Preparation of enantiopure epoxides by biocatalytic kinetic resolution

Promotor
Dr. ir. J.A.M. de Bont
Hoogleraar in de Industriële Microbiologie

Promotiecommissie
Dr. D.J. Leak, Imperial College of Science, Technology and Medicine
Prof. dr. H.E. Schoemaker, Universiteit van Amsterdam
Prof. dr. E.J.R. Sudhölter, Wageningen Universiteit
Prof. dr. ir. J. Tramper, Wageningen Universiteit

Preparation of enantiopure epoxides by biocatalytic kinetic resolution

Carel A.G.M. Weijers

PROEFSCHRIFT

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op woensdag 21 november 2001
des namiddags te vier uur in de Aula

ISBN: 90-5808-480-9

1. Enantioselectieve ringopening van epoxiden is een bijzondere vorm van ontspanning. *dit proefschrift*

2. Voor de productie van enantiomeerzuivere 2-methyl-3-alkyl-gesubstitueerde epoxiden is biokatalytische kinetische resolutie (nog steeds) de meest geschikte methode. *dit proefschrift*

3. Bij toepassing van een epoxide hydrolase in een proces op grote schaal is *in-situ* verwijdering van het gevormde diol de meest cruciale parameter. *dit proefschrift*

4. In de vergelijking van productiviteit van het gist epoxidehydrolase en de Jacobsen katalysator is het resultaat in hoge mate bepaald door de keuze van bijbehorende substraten. *Chemistry & Biology 1998, 5, 73-79*

5. De consumptie van doorbakken vlees zou zeker aan personen met een verhoogde dan wel verlaagde epoxidehydrolase activiteit ontraden moeten worden.
Cancer Research 2001, 61, 2381-2385
Cancer Epidemiology, Biomarkers & Prevention 2001, 10, 875-882

6. Als modelorganisme voor de selectie van verbindingen met antischimmel en antitumor activiteit is *Saccharomyces cerevisiae* geen goede keuze vanwege het beperkte detoxificeringsvermogen van deze gist.
Clinical Microbiology Reviews 1999, 12, 583-611

7. Indien men een biokatalytisch kinetisch resolutie proces als 'Green Chemistry' wil typeren zou men er ook naar kunnen streven om de geïntegreerde synthese van substraten volgens overeenkomstige richtlijnen uit te voeren.
Tetrahedron 2001, 57, 695-701
Green Chemistry: designing chemistry for the environment, ACS Symposium Series 626, 1998, pp 1-17

8. DNA Copyright zou een effectief voorbehoedsmiddel zijn.
BBC News, August 15, 2001

9. Wachtlijsten in de zorgsector zijn het gevolg van de toegenomen vraag naar een betere 'kwaliteit van leven' voor het personeel.

10. Het laten opgroeien van kinderen op een 'Vinexlocatie' kan gezien worden als een hedendaagse vorm van Spartaanse opvoeding.

Stellingen behorende bij het proefschrift: Preparation of enantiopure epoxides by biocatalytic kinetic resolution. Carel A.G.M. Weijers.
Wageningen, 21 november 2001.

CONTENTS

1

Introduction

Introduction

1.1 Enantiopure epoxides as building blocks

1.1.1 Molecular chirality

Objects that cannot be superimposed on their mirror image are called *chiral*, derived from the Greek word *chiros* meaning 'handed'. The term *chirality* is therefore based on the familiar analogy of the morphological mirror-image relation between the left and right hand. The concepts of chirality can be extended from macroscopic objects like hands, screws and spiral staircases to molecules. A molecule is chiral if it contains a *chirality center*, which is usually a tetrahedral carbon atom attached to four different atoms or groups of atoms. The four atoms or groups can be arranged in different ways corresponding to different *stereoisomers*. The precise arrangement of substituents at a chirality center is termed *absolute configuration*.

Stereoisomers that are related as nonsuperimposable mirror images are called *enantiomers*. The nomenclature of enantiomers is based on their absolute configurations and generally assigned by the Sequence Rule notation of the 'Cahn-Ingold-Prelog system'[1]. In this way, molecules are assigned the (*R*)-configuration (descending priority of substituents in clockwise direction) or (*S*)-configuration (anticlockwise direction). Mixtures containing equal quantities of enantiomers are called *racemic* mixtures or *racemates*. Abiotically produced chemical compounds are generally racemic. In sharp contrast to this, the chemistry of living organisms is *homochiral*, being based almost exclusively on L-amino acids (or (*S*)) and D-polysaccharide monomers (or (*R*)). The current biosphere is thus commonly characterized as being composed of building blocks with biomolecular homochirality. The term 'homochirality' is synonym with 'enantiopurity' but the latter is more commonly used to describe a certain compound in particular.

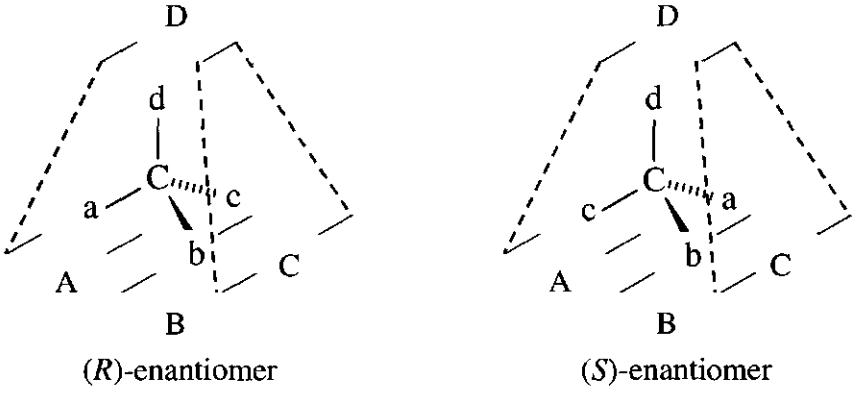
Many physical properties like density, melting point and boiling point are identical for enantiomers. There is one difference in physical properties: enantiomers can be distinguished because they rotate plane-polarized light (PPL) in opposite directions. Enantiomers that rotate PPL to the right are indicated by a (+) before the name; those that rotate PPL to the left are indicated by a (−). The direction and amount of rotation of an enantiopure compound, determined under standard conditions, is termed *specific rotation* ($[\alpha]$) and is a typical physical property of that compound. The chemical properties of enantiomers are identical in a symmetric environment, but they are distinct in a chiral environment. This difference is of crucial importance in many reactions in living cells.

The importance of 'handedness' in Nature is such that scientists have long been wondered about its origin. Theories for the origin of homochirality were at first distinguished in two main categories, biotic and abiotic[2,3]. The biotic or selection theories suggest that life arose in a racemic environment and that homochirality as such developed later.

Nowadays it is generally accepted that homochirality did not have a biogenic origin[4-6].

Homochirality is now assumed to be a prerequisite for the enantiopure monomers to form the folded structures of a protein (for example the α-helix) essential for the catalytic function of enzymes, and the double helix of DNA, essential for replication. In view of this, abiotic theories suggest that primitive biomolecules required an initial enantiomeric excess. Such an enantiomeric imbalance could have occurred by chance or in a determinate way. Abiotic theories have been discussed recently in literature[4-9]. Chance or random mechanisms include competing autocatalysis, asymmetric adsorption and spontaneous crystallization of conglomerates. Alternatively, determinate mechanisms suggest that some chiral physical interaction acting on racemates would have caused an initial very small enantiomeric excess that subsequently could be amplified. Examples of this are photochemistry with circularly polarized light and magnetochiral photochemistry. Despite the many theories, the mystery of the origin of the homochirality of life still has not been solved.

At a molecular level, biological systems are homochiral environments composed of biopolymers from chiral precursors as amino acids and carbohydrates. Many biochemical processes involve an interaction with these homochiral biomolecules. Examples are interactions with enzymes and receptor systems for hormones, sensory recognition and drug metabolism. It can thus be understood that biological receptors almost always show a stereochemical preference for one of a pair of enantiomers. In order to rationalize these observed differences in biological activity, a 3-point fit model has been proposed[10,11]. Such a model is schematically depicted in Scheme 1.



Scheme 1. Enantiomeric interactions of a chiral ligand with protein sites A, B, C and D of a biological receptor molecule.

In interactions between chiral ligands and receptor molecules, the geometry of the active site on the biological receptor is the determinant, rather than the geometry of the ligand. In Scheme 1, the receptor is considered as a fixed conformation and thus showing maximal interaction with the (*R*)-enantiomer of the chiral ligand (combination Aa+Bb+Cc+Dd). For the (*S*)-enantiomer only two interactions are possible. Due to the difference in interaction and subsequent biological activity, the (*R*)-enantiomer is termed in this case as the *eutomer* and the (*S*)-enantiomer as distomer[10]. The terminology of *eutomer* and *distomer* is only related to a higher or lower activity, respectively, regardless of the absolute configuration of the ligand enantiomers.

Very recently, a more general model for enantiomeric receptor interactions has been reported[12]. This new model is called a 'four-point location' model in which a protein might provide two sites (D' and D") in either of two locations for interaction with site D: D' would bind one enantiomer and D" the other one. In this model, it is not necessary for there to be four binding sites, but, for example, three interaction sites and a fourth direction also makes discrimination of enantiomers possible.

1.1.2 Enantiopure bioactive compounds

In biological systems, the transfer of information is largely controlled by interaction of specific substrates with particular sites on enzymes, receptors and carrier molecules[13]. On the molecular level, selective interaction with specific substrates is based upon complementary chemistry with a high degree of homochirality as described before. Endogenous and exogenous substrates that activate biological activity by their interaction are called *bioactive compounds*. Since the late 1980s, it has been understood and generally accepted that exogenous bioactive compounds like agrochemicals and pharmaceuticals can be more effectively exploited by using their single eutomer rather than the racemate. Apart from low or high activity, also the presence or absence of adverse side effects, and desired or antagonistic effects has been observed additionally[13-16]. In view of their differential molecular configuration and consequent biological activity, stereoisomers have to be regarded as essentially different compounds[13]. Bioactive compounds are therefore now preferably produced as single isomers.

In the case of racemic synthetic agrochemicals, differential biological activity of stereoisomers can be expressed by different degrees of intrinsic activity and by different selectivity between the target and all other species with which the product comes into contact[17,18]. For these compounds most concern has been directed towards the environmental consequences of application of the less active distomer which poses an additional loading on the environment. Differing biological activities, synthesis and analysis of isomers of various synthetic agrochemicals have been reported[19,20].

The structure-activity relationship of stereoisomers from pharmaceuticals has been similarly well recognized to have a major influence on their pharmacological response[16].

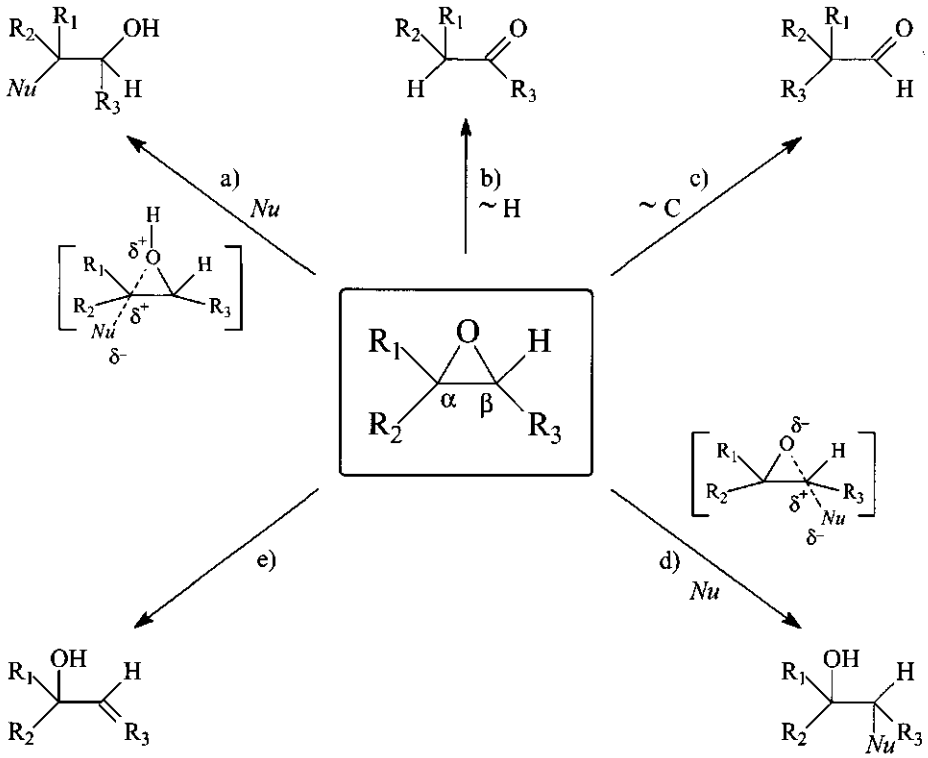
Nowadays, pharmaceutical companies are aware that, where appropriate, new pharmaceuticals should be enantiopure to avoid the possibility of adverse side effects due to an undesirable stereoisomer. During the last decade, the portion of synthetic pharmaceuticals introduced as single isomers has grown very rapidly. This growth was made possible by (*i*) a strategic conversion to the development of single isomers as initiated by the publication of the 'Stereoisomer Drug Policy' of the US Food and Drug Administration (FDA)[21,22] in 1992 and (*ii*) recent advances in the synthesis of enantiopure compounds[23]. For the production of enantiopure agrochemical and pharmaceutical intermediates, an increasingly prominent position has been occupied by *biocatalytic processes*, using enzymes and microbial cells as catalysts[24-29].

1.1.3 Enantiopure epoxides

A great number of bioactive compounds can be effectively synthesized from enantiopure epoxides used as chiral building blocks. *Epoxides*, or *oxiranes*, are three-membered cyclic ethers that are highly reactive due to their considerable bond angle strain. Products of ring-opening reactions have normal tetrahedral bond angles and are not strained (Scheme 2).

In organic synthesis, ring-opening reactions by addition of nucleophilic reagents to epoxides has been widely used to form 1,2-disubstituted products[30]. In this way, nucleophiles containing oxygen, nitrogen, sulfur and halides can be added to the target molecule. As a consequence of their high reactivity, epoxides must be handled carefully and regarded as hazardous genotoxic compounds which are able to alkylate nucleophilic centers of DNA[31,32] .

Nucleophilic ring-opening can occur at either neutral, basic, or acid conditions, but acids generally accelerate ring-opening. Unsymmetrical epoxides give different products under acid-catalyzed and base-catalyzed or neutral conditions, as shown in Scheme 2. Normally, backside attack of the nucleophile on an epoxide carbon occurs, resulting in inversion of configuration at this center. Under base-catalyzed and neutral conditions, attack predominantly takes place at the sterically less hindered C_β carbon atom in a typical S_N2-type reaction. The mechanism under acid-catalyzed conditions is dictated by the stability of the protonated transition state, which favors nucleophilic attack at the more substituted C_α carbon. Although these are typical S_N1 conditions, the actual acid-catalyzed mechanism is somewhere between S_N1 and S_N2. The influence of S_N2 is contributed by the stereochemistry of the reaction: the nucleophile approaches from the side opposite of the leaving oxygen. Because such reactions have features of both the S_N1 mechanism (regiochemistry) and the S_N2 mechanism (stereochemistry), they are termed 'borderline S_N2' reactions.

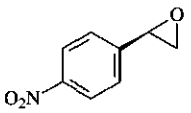
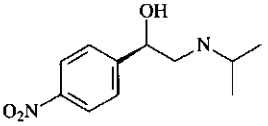
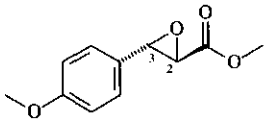
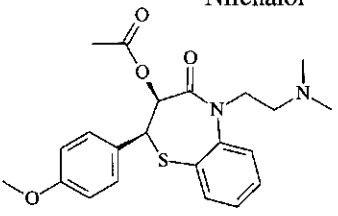
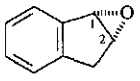
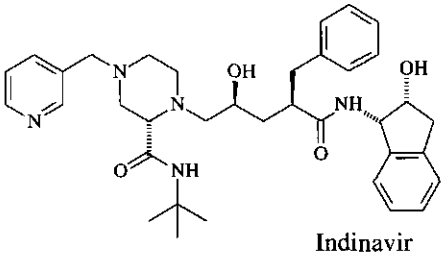


Scheme 2. Ring-opening of epoxides under acid-catalyzed (a, b, c; attack at C_α) and base-catalyzed conditions (d, e; attack at C_β). Under acid-catalyzed conditions, epoxides are protonated and opened to a carbocation-like intermediate. The positively charged intermediate can be converted to (a) a 1,2-disubstituted product by addition of a nucleophile following a 'borderline S_N2' mechanism, to (b) a ketone by an internal hydride-shift, or to (c) an aldehyde by an internal carbon-shift. Most important base-catalyzed reactions are (d) nucleophilic ring-opening by a pure S_N2 reaction and (e) hydrogen abstraction from C_β adjacent R_3 and subsequent epoxide opening to form allylic alcohols.

Attack on C_α or C_β is not strictly defined by the reaction conditions but depends as well on the balance of steric and electronic factors arising from both substituent and nucleophile. For example, alkyl substituents favor attack at C_β whereas conjugating substituents tend to stabilize carbocations and thus favor attack at C_α. The chemistry and chemocatalytical ring-opening of epoxides has been reviewed in literature[30-35].

Enantiopure epoxides can be used to introduce one or two adjacent chirality centers in a target molecule or, in some specific cases, directly as compound with intrinsic biological activity. Biologically important enantiopure natural epoxides are for example Juvenile insect hormones[36] and the antitumor agent Epothilone A[37]. Table 1 shows 'classical' examples of enantiopure synthetic epoxides used as key intermediates in the synthesis of specific pharmaceuticals.

Table 1. Synthetic epoxides prepared by kinetic resolution and used as enantiopure pharmaceutical intermediates.

Epoxide	product	pharmacological activity
 (*R*)-**1**	 Nifénalol	β-adrenergic blocker[38]
 (2*R*,3*S*)-**2**	 Diltiazem	Ca^{2+} channel blocker[39]
 (1*S*,2*R*)-**3**	 Indinavir	HIV protease inhibitor[40]

The desired biological activity of the pharmaceutical products given in Table 1 is present only in the eutomers having the configuration as shown. Preparation of the enantiopure epoxides **1**, **2**, and **3** has been demonstrated by biocatalytic kinetic resolution processes (see paragraph 1.2). For this, optimized methods using bacterial lipases for the large-scale preparation of epoxide-**2** and fungal epoxide hydrolases for preparation of epoxide-**1** and **3** respectively, were applied successfully[41-43].

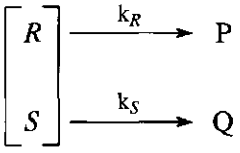
1.2 Kinetic resolution of epoxides

1.2.1 Principles of kinetic resolution

The process for separation of enantiomers forming a racemic compound is defined as *resolution*. In fact, two types of resolution can be distinguished, depending on the composition of the racemic starting material.

Spontaneous resolution is defined as a process in which the two enantiomers of a conglomerate are equally separated[44]. A *conglomerate* is a 1:1 mechanical mixture of crystals, each crystal consisting of pure enantiomers. It is estimated that about 10% of the chiral organic solids form conglomerates[45]. In conglomerate systems, the racemate is more soluble that the constituent enantiomers. During crystallization of conglomerates, separation of the crystals, consisting of the single enantiomers, occurs spontaneously.

Kinetic resolution is an enantioselective reaction in which one of the enantiomer constituents of a racemic mixture is more readily transformed into a product, or adsorbed to a matrix, than the other[46].



Kinetic resolution by substrate transformation occurs if $k_R \neq k_S$ and the reaction is terminated between 0% and 100% conversion of the racemate (*R,S*). Kinetic resolution essentially requires the partial transformation of a racemic mixture. This is in contrast with spontaneous resolution, which usually involves complete transformation of a racemic mixture.

Kinetic resolution is basically linked to kinetics and can be applied to both chemocatalytic as well as biocatalytic reactions. In the case of kinetic resolution of racemic epoxides, the reaction can be described as an irreversible conversion of a single substrate (*R* or *S*) into a single product (*P* or *Q*). Therefore, the quantitative analysis of biocatalytic kinetic resolutions as developed by Chen *et al.*[47] can be applied to enzymatic epoxide ring-opening reactions.

The (biochemical) stereoselectivity factor *E*, or *enantiomeric ratio E*, is by definition an intrinsic property of the biocatalyst. The value of *E* depends on the ratio of the enzyme specificity constants *V* (V_{max}, maximal velocity) and *K* (K_m, Michaelis-Menten constant) of the (*R*)- and (*S*)-enantiomers, as shown in equation [1]:

$$E = \frac{V_R K_R}{V_S K_S} \qquad [1]$$

The enantiomeric composition of a racemate during the course of resolution is represented by its e.e. value (*enantiomeric excess*). In case of preferential conversion of a (*S*)-enantiomer, the enrichment in (*R*)-enantiomer is determined by equation [2]:

$$\text{e.e.} = \frac{([R] - [S])}{([R] + [S])} \qquad [2]$$

For the determination of E, several different methods are available, dependent on the combination of monitored parameters[48]. In irreversible enantioselective ring-opening reactions of epoxides, equations [3], [4] and [5] are appropriate methods for the determination of E. Equation [3] represents the e.e. of substrate versus extent of conversion C, and equation [4] the e.e. of product versus extent of conversion C.

$$E = \frac{\ln[(1- C)(1- \text{e.e.}_s)]}{\ln[(1- C)(1+ \text{e.e.}_s)]} \qquad [3]$$

$$E = \frac{\ln[(1- C)(1+ \text{e.e.}_p)]}{\ln[(1- C)(1- \text{e.e.}_p)]} \qquad [4]$$

In equation [5], R_0 and S_0 are the initial concentrations of the individual enantiomers at t=0. The slope of the plotted curve, obtained by data from equation [5], represents the E value.

$$E = \frac{\ln ([R_0] / [R])}{\ln ([S_0] / [S])} \qquad [5]$$

In Figure 1, the evolution of e.e. (substrate) as a function of conversion, for various values of E, is visualized. These graphic representations are very helpful to estimate the amount of enantiopure remaining substrate that can be obtained. The curves represented in Figure 1 show that the maximal feasible yield is 50%, in kinetic resolutions. It is also obvious that reactions with E=100 are almost as effective as those are with $E = \infty$. In general, reactions with significant enantioselectivities show values of E>20.

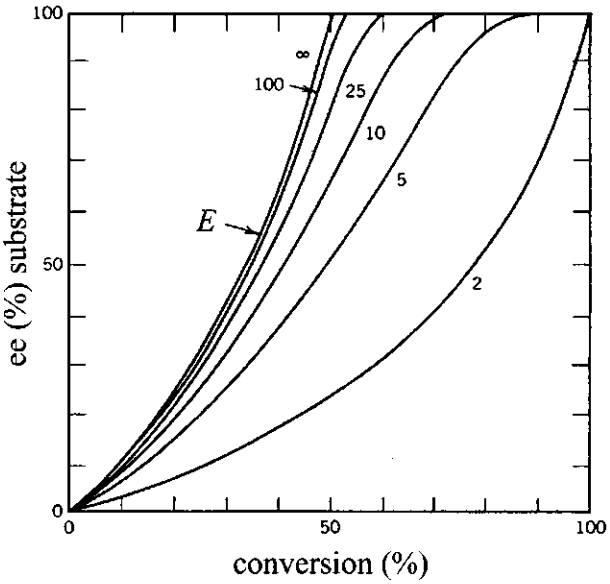
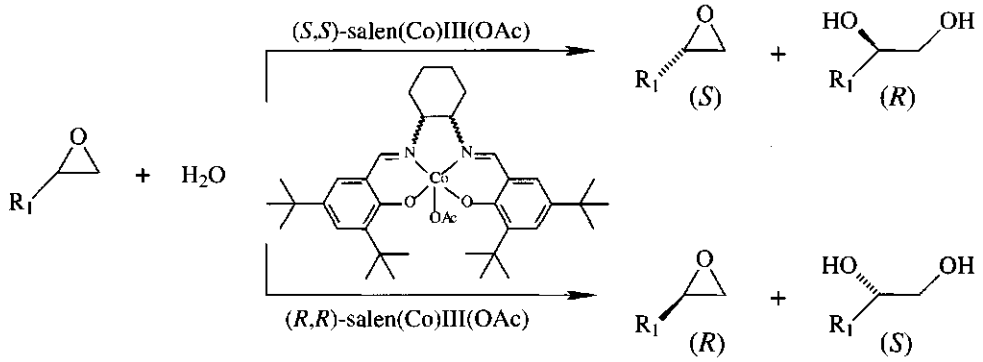


Figure 1. Effect of the e.e. of remaining substrate as function of the conversion, for various enantiomeric ratios *E*[46].

1.2.2 Chemocatalytic and biocatalytic kinetic resolution of epoxides

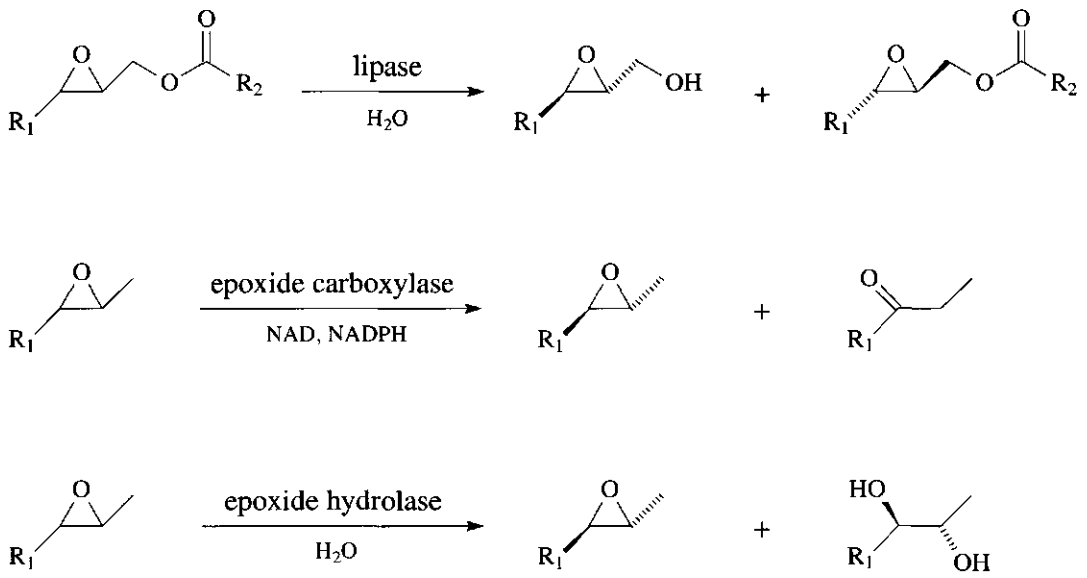
The synthesis of enantiopure epoxides by chemocatalytic and biocatalytic methods has been studied extensively during the last decade. Well developed are chemocatalytic epoxidation reactions for the preparation of enantiopure epoxides from allylic alcohols and from conjugated alkenes[49]. However, due to the absence of methods for preparation of simple alkyl epoxides also alternative procedure have been explored. From these investigations, a most promising method has appeared to be chemocatalytic hydrolytic kinetic resolution. The method has shown to be successful in the preparation of several enantiopure aliphatic 1,2-epoxides and 1,2-diols (Scheme 3)[50]. For this, synthetic chiral catalysts have been prepared with their structure mimicking the enzyme active-site geometry. By use of the synthetic Jacobsen's (*R,R*)- and (*S,S*)-salen(Co)III(OAc) catalysts, both enantiopure (*S*)- and respectively (*R*)-1,2-epoxides were prepared from C_3 and from C_{10} to C_{20} racemic epoxides[51]. Recently, the range of substrates has been extended by epoxides bearing substituents with varying steric demand and composition. The method however is still restricted to the resolution of monosubstituted epoxides[35].



Scheme 3. Chemocatalytic hydrolytic kinetic resolution of various aliphatic 1,2-epoxides.

Alternatively, biocatalytic production methods for enantiopure epoxides have been developed simultaneously. Biocatalytic production methods have been reviewed in literature[52-56]. However, mainly due to the high toxicity of epoxides towards the biocatalysts, several biocatalytic methods were hampered by product inhibition and therefore are difficult to scale-up.

Most effectively were the methods based on the use of enzymes that were able to enantioselectively degrade epoxides. Known epoxide-converting enzymes are epoxide hydrolases and epoxide carboxylases. And, also lipases have been used for the kinetic resolution of glycidyl esters. In Scheme 4, general reactions of biocatalytic epoxide resolutions are shown.



Scheme 4. Biocatalytical kinetic resolution of epoxides.

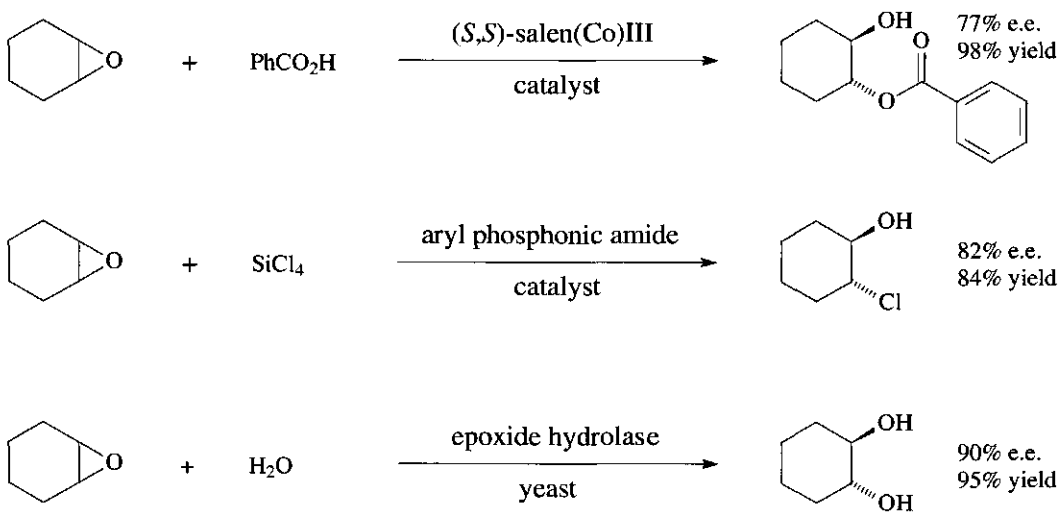
Lipase-catalyzed resolutions are generally easy to scale-up because the enzyme is not deactivated by reaction with the epoxide moiety. The reaction was successful in the preparation of enantiopure epoxy alcohols[57] and enantiopure glycidyl esters[58,59]. But lipase-catalyzed resolutions, being based on hydrolysis of the ester side-chain, are however limited by the scope of suitable epoxide substrates.

Resolution of epoxides by direct asymmetric ring-opening are catalyzed by epoxide carboxylase and epoxide hydrolase enzymes (Scheme 4). All epoxide-bearing compounds can thus be regarded as potential substrates for these enzymes. Consequently, these two enzymes can be regarded as attractive tools in biocatalysis.

1.2.3 Asymmetric ring-opening of meso *epoxides*

Compounds that have two or more equivalent chirality centers, but are nevertheless achiral, are called *meso* compounds. *Meso* compounds can be converted to enantiopure products in a theoretical 100% chemical yield[60]. The possible high yield makes this process of *desymmetrization* in certain cases even more advantageous than kinetic resolution.

Unfortunately, desymmetrization of *meso* epoxides is limited to some specific examples of symmetric *cis*-disubstituted and tetrasubstituted molecules. Asymmetric ring-opening of *meso* epoxides is possible by use of synthetic catalysts[35,61,62] and epoxide hydrolase enzymes[63]. In Scheme 5, asymmetric nucleophilic ring-opening reactions of *meso* 1,2-epoxycyclohexane are shown.



Scheme 5. Formation of enantiopure (1*R*,2*R*)-disubstituted cyclohexanes by asymmetric ring-opening of *meso* 1,2-epoxycyclohexane.

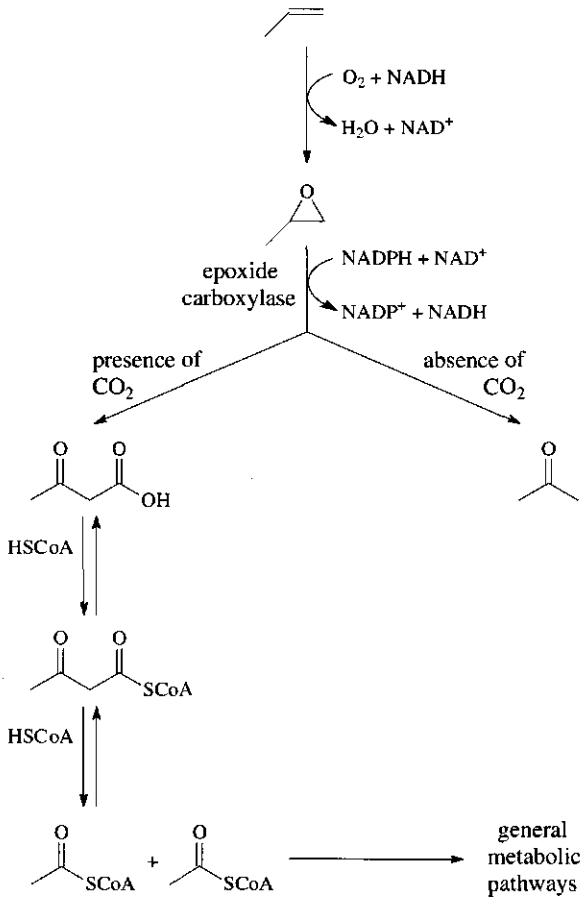
1.3 Bacterial epoxide carboxylase

1.3.1 Biological function

Biologically produced epoxides can either occur as end products of biosynthetic routes or as intermediates in the metabolism of alkenes and aromatic compounds. In most metabolic routes, epoxides are converted by ring-opening to less reactive and consequently less harmful compounds by detoxifying enzymes like epoxide hydrolases and glutathione *S*-transferases.

However, in microbial metabolism, some aliphatic epoxides play a completely different role. In alkene-utilizing bacteria, epoxides are generated by mono-oxygenases and subsequently further metabolized by known biochemical pathways. The bacteria involved play an important role in the global carbon cycle by using various short-chain alkenes (C_2 to C_7) as sole source of carbon and energy[64,65]. Their contribution to the reduction of gaseous alkenes in the atmosphere is of importance because the global emission of these compounds has grown to more than 200 million tons produced annually from biogenic and anthropogenic sources[65,66].

Bacterial metabolism of 1,2-epoxypropane has been studied in detail for two propene-utilizing bacteria, *Xanthobacter* strain Py2 and *Rhodococcus rhodochrous* strain B276 (formerly referred to as *Nocardia corallina* B276). The interest in epoxide metabolism in *Xanthobacter* Py2 has been initiated by the study on the highly enantioselective conversion of aliphatic 2,3-epoxides by this organism[67,68]. Kinetic resolution of short-chain 2,3-epoxides was successful with cells of *Xanthobacter* Py2 since disubstituted epoxides were no intermediates in 1-alkene metabolism and therefore only partially degraded. In a subsequent study[69], the *in vitro* isomerization of epoxides to ketones has been described for various aliphatic 1,2- and (2*S*)-epoxides by cell extracts of *Xanthobacter* Py2. Under physiological conditions, however, ketones were not further metabolized by propene-grown *Xanthobacter* cells. The enzymes required for ketone metabolism are repressed during growth on propene and are expressed only upon addition of acetone or 2-propanol to the growth medium[70]. Follow-up studies by other research groups concentrated on epoxide metabolism under physiological conditions. Eventually, these studies showed that intermediate epoxides in alkene metabolism were carboxylated with CO_2 as a cosubstrate to produce β-keto acids[71]. The key enzyme in this route is an *epoxide carboxylase* which catalyzes the formation of β-keto acids or ketones by addition of CO_2 or H^+ to the ring-opening product of 1,2-epoxypropane, respectively. In the absence of CO_2, epoxides were found to be stoichiometrically converted to ketones, confirming preceding investigations[72]. The summarized pathway of propene metabolism in *Xanthobacter* Py2 is shown in Scheme 6.



Scheme 6. Role of the epoxide carboxylase system[71] in propene metabolism by *Xanthobacter* Py2.

1.3.2 Enzyme characteristics

Enzymatic isomerization of epoxides to ketones initially was shown to be dependent on both NAD+ and a reducing dithiol compound and was sensitive to inhibition by *N*-ethylmaleimide (NEM)[69]. These findings strongly indicated that oxidation and reduction of reaction intermediates would play a central role in this reaction. And, in addition, from the sensitivity of the enzyme to inhibition by *N*-ethylmaleimide (NEM) involvement of enzyme thiol groups were concluded[69].

The first progress in refinement of this puzzling reaction was made by Chion and Leak[73]. They found that synthetic dithiol compounds could be replaced by the physiological reductant NADPH, and also that in the reaction at least two proteins should be involved. NADPH-dependency and further fractionation of the enzyme system has been

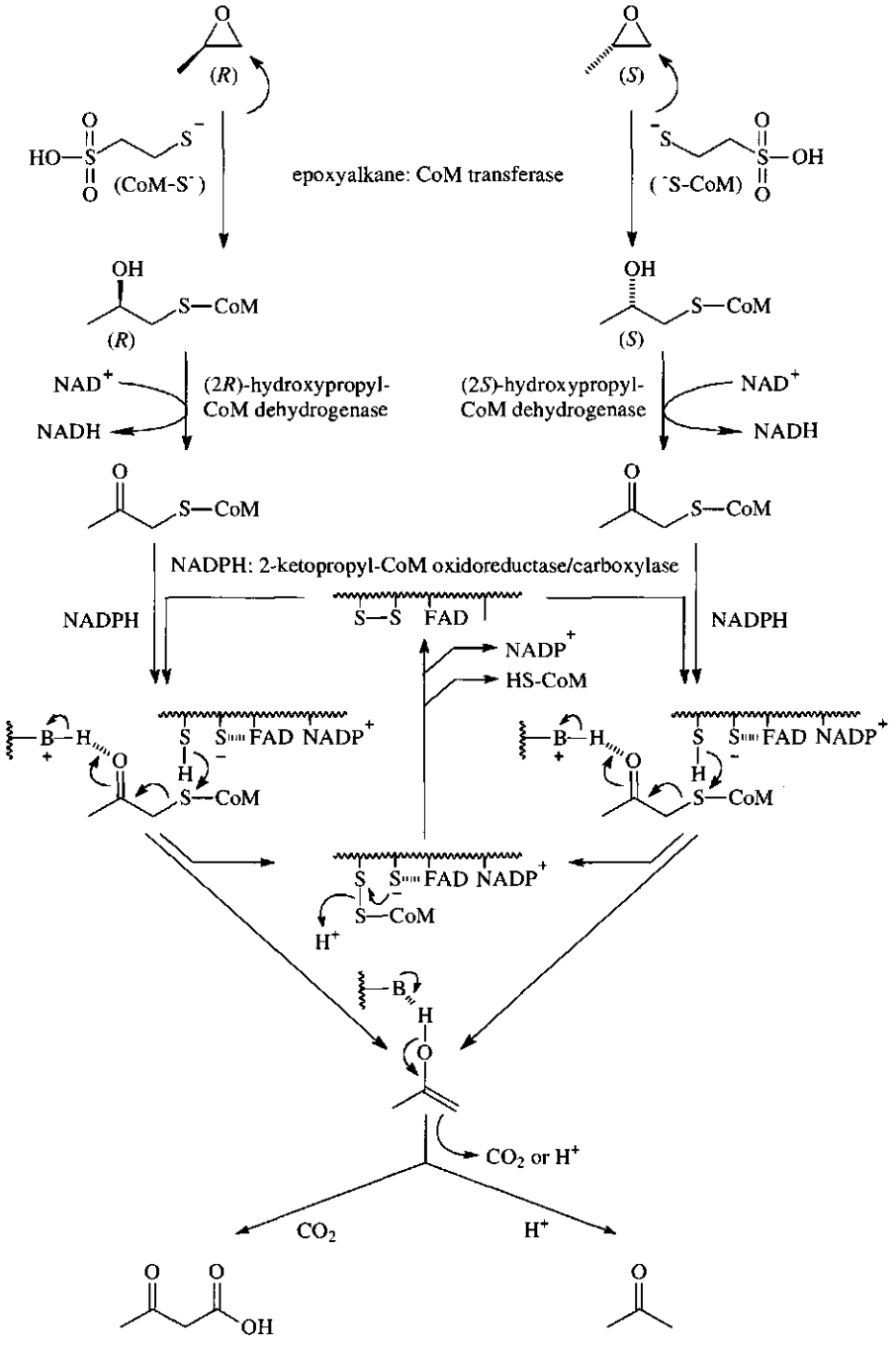
described by Swaving *et al.*[74]. The NADPH-dependent enzyme was subsequently purified and characterized as a member of the NADPH-dependent FAD-containing disulfide oxidoreductases as reported by Westphal *et al.*[75]. Very recently, the catalytic activities of this enzyme have been studied in detail by Clark *et al.*[76]. The authors reported that this enzyme is involved in the third step of the epoxide metabolism, catalyzing the NADPH-dependent reductive cleavage and carboxylation of a β-ketothioether to form a β-keto acid and coenzyme M (see next paragraph). In the absence of CO_2, a proton can serve as an alternative electrophile for ketone production. Therefore, the enzyme is renamed as NADPH: 2-ketopropyl-CoM oxidoreductase/carboxylase. The mini-metabolic pathway of epoxide to β-keto acid or ketone, respectively, is called the *epoxide carboxylase system.*

Ring-opening of the epoxide was initially proposed by nucleophilic attack of an enzyme thiol on the less substituted carbon to form a covalently bound β-hydroxythioether intermediate[69]. Recently, Allen *et al.* have shown in their investigations that the physiological nucleophile for this reaction is CoM (coenzyme M, 2-mercaptoethanesulfonic acid)[77]. The ring-opening by conjugation of the deprotonated nucleophile CoM-S^- has thus been described as an epoxyalkane: CoM transferase reaction. CoM biosynthesis is coordinately regulated with the expression of the alkene mono-oxygenase and enzymes of the epoxide carboxylase system. The lack of CoM in glucose-, propane-, 2-propanol- and acetone-grown cells shows the highly specialized role for CoM in metabolism of alkenes and epoxides. The latter observations recently have been found in cells of *Xanthobacter* Py2 and of *Rhodococcus rhodochrous* B276[78].

The NAD-dependent oxidation in epoxide metabolism was proposed to be a hydride abstraction of the β-hydroxythioether intermediate to form a β-ketothioether[69]. It is now more precisely described that the β-hydroxythioether is not covalently bound to an enzyme-thiol but coupled to CoM. The NAD-dependent enzyme is therefore named 2-hydroxypropyl-CoM dehydrogenase[76]. Very interestingly, there are two specific dehydrogenases involved: one for each hydroxyalkyl enantiomer formed by ring-opening of the respective epoxide enantiomer[79]. The enzymes of the epoxide carboxylase system of *Xanthobacter* Py2 are summarized in Table 2.

Table 2. Enzymes of the epoxide carboxylase system of *Xanthobacter* Py2

Enzyme	cofactor / coenzyme
Epoxyalkane: CoM transferase	HS-CoM, Zn
(2*R*)-hydroxypropyl-CoM dehydrogenase	NAD^+
(2*S*)-hydroxypropyl-CoM dehydrogenase	NAD^+
NADPH: 2-ketopropyl-CoM oxidoreductase/carboxylase	FAD, $NADPH^+$ H^+, CO2



Scheme 7. Proposed mechanism[76] of carboxylation/isomerization of (*R*)- and (*S*)-1,2-epoxypropane by the epoxide carboxylase system of *Xanthobacter* Py2.

1.3.3 Catalytic mechanism

The results obtained by the various research groups suggested that the epoxide carboxylase system should be regarded as a multiprotein enzyme complex. Actually, recent insights in the reaction mechanism[75,76] justifies the term mini-metabolic pathway with distinct intermediates as shown in Scheme 7.

The proposed pathway of carboxylation/isomerization of 1,2-epoxypropane starts with the conjugation of CoM as catalyzed by an epoxyalkane: CoM transferase. Actually, this is a base-catalyzed S_N2 ring-opening with the deprotonated CoM-S- acting as the nucleophile. In this way, (*R*)- and (*S*)-2-hydroxypropyl-CoM are formed from the corresponding epoxide enantiomers. In the second step, the individual 2-hydroxypropyl enantiomers are oxidized by specific NAD-dependent dehydrogenases to form the common achiral intermediate 2-ketopropyl-CoM. The terminal enzyme is an NADPH: 2-ketopropyl-CoM oxidoreductase/carboxylase which catalyzes the reductive cleavage of 2-ketopropyl-CoM to form the enol of acetone. As shown in Scheme 7, this reaction proceeds by binding of NADPH to the oxidized enzyme. Subsequently, NADPH reduces FAD, which reduces the redox-active disulfide. The protonated thiol opens the 2-ketopropyl thioether by nucleophilic attack, thereby forming a CoM/interchange mixed disulfide and the enol of acetone. The final step is completed by base-catalyzed deprotonation of the enol and electrophilic addition of either a proton or CO_2 to generate acetone or acetoacetate, respectively. Concomitantly, the redox-active thiol of the terminal enzyme is reoxidized, the CoM/interchange mixed disulfide is reduced and CoM and $NADP^+$ are released.

The puzzling epoxide degradation pathway in *Xantobacter* Py2 has been studied thoroughly in the case of 1,2-epoxypropane. The question still remains to what extend these highly specialized enzymes are also involved in the enantioselective isomerization of 2,3-epoxides, being substrates which are no intermediates in 1-alkene metabolism.

1.4 Yeast epoxide hydrolase

1.4.1 Biological function

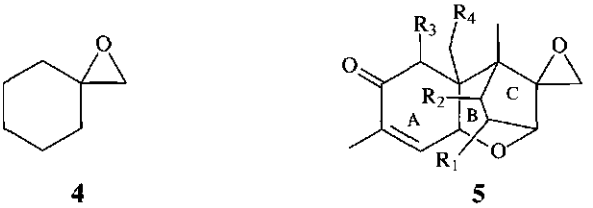
Epoxides with intrinsic biological activity in many cases are toxic to living cells[34]. This toxicity can be displayed by both acute cytotoxicity and by long-term mutagenic effects. In many eukaryotic organisms, the first line of biochemical defense against these exogenous epoxides is the detoxifying enzyme epoxide hydrolase. Epoxide hydrolases have been found in all types of living organisms, including mammals, insects, plants and microorganisms, and are thus ubiquitous in Nature. In mammals, these enzymes are also involved in the detoxification of endogenous produced arene oxides, which are formed *in vivo* by oxidation of aromatic hydrocarbons. A main role for biochemical detoxification of epoxides has been assigned to the mammalian microsomal epoxide hydrolase (MmEH)[80-82].

Epoxide hydrolases (EC 3.3.2.3) catalyze the addition of water to epoxides resulting in the formation of vicinal diols. Because this reaction in many cases proceeds with a high degree of enantioselectivity are these enzymes of interest as biocatalysts for kinetic resolution. The biocatalytical potential of epoxide hydrolases from various microbial sources has been reviewed recently[63,83-85].

Among the sources of microbial epoxide hydrolases, yeasts are particularly of interest as catalysts since these unicellular organisms are (*i*) easy to cultivate, especially on a large scale and (*ii*) are relatively insensitive to mechanical shear damage which facilitates whole-cell biotransformations on a large scale. Yeast epoxide hydrolase activity with moderate to high enantioselectivity was found to be restricted to specific strains of basidiomycetes genera, which include *Xanthophyllomyces*, *Trichosporon*, *Rhodosporidium* and *Rhodotorula*[86-88]. The substrate scope and catalytic activity of the epoxide hydrolase of *Rhodotorula glutinis* has been reviewed in comparison with other epoxide hydrolases[63].

The biological function of epoxide hydrolases in yeasts, and almost all other microbial sources, is still unknown. As an exception, the very specific bacterial limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* DCL 14 was found to be involved in the limonene metabolism of this microorganism[89]. Nevertheless, it is likely to consider that yeast epoxide hydrolases (YEHs) play a role in the defense mechanisms against cytotoxic compounds. The key characteristic of detoxifying enzymes is their loose restriction in substrate recognition in order to be able to react with xenobiotics of variable structure[90]. YEH shares this common feature as it can accept structurally diverse molecules as substrates thus being actually only functional group specific[63]. Final proof for the biological role of YEH will be given by the identification of the physiological substrate(s).

Possibly, natural YEH substrates belong to the epoxide-bearing mycotoxins, the trichothecenes. These fungal metabolites are a large group of tricyclic sesquiterpenes produced by strains of *Fusarium*, *Trichoderma*, *Trichothecium* and *Myrothecium*, showing potent antifungal and cytostatic activity[91]. About more than 45 compounds have been classified in this group. The toxic effects of these compounds appear to result from their ability to inhibit protein synthesis. For the biological activity of trichothecenes, the 12,13-epoxide moiety is generally assumed to be essential and derivatives lacking this structural feature are inactive[91]. The structure of the trichothecene skeleton is represented by compound **5** in Scheme 8.



4 **5**

Scheme 8. Structures of 1-oxaspiro[2.5]octane **4** and the trichothecene skeleton **5**.

The inhibitory effect of trichothecenes on yeast growth and metabolism has been investigated[92-94]. These studies showed an interspecies differential trichothecene-sensitivity, which indicates the existence of a biochemical detoxification mechanism in yeasts. As a possible detoxification mechanism, enzymatic reduction of the 12,13-epoxide group has been suggested[94]. Similarly, epoxide reduction to a diene has been proposed for some gastrointestinal bacteria to be the explanation of their trichothecene-resistance[95]. However, there is not much support for this hypothesis in literature. Epoxide reductases have not been observed in any other type of microorganism and a related enzyme present in mammalian cells, was assigned no role in epoxide detoxification as it accepts no trichothecene T-2 toxin or other structurally divers xenobiotic epoxide[96]. Hence it will be more likely, due to their characteristics and distribution, that epoxide hydrolases will be involved in such a detoxification mechanism.

A preliminary study has been set up recently to test whether substrates containing the spiro attachment of an epoxide group, like the epoxypyran ring B of trichothecenes (Scheme 8), are accessible substrates for the YEH of *Rhodotorula glutinis*[97]. Interestingly, the YEH showed a very high activity for the tested carbocyclic trichothecene analogue 1-oxaspiro[2.5]octane (epoxide **4**). Oxaspiro epoxycompounds, and the structurally related trichothecenes, may thus be regarded as potentially accessible substrates for YEH.

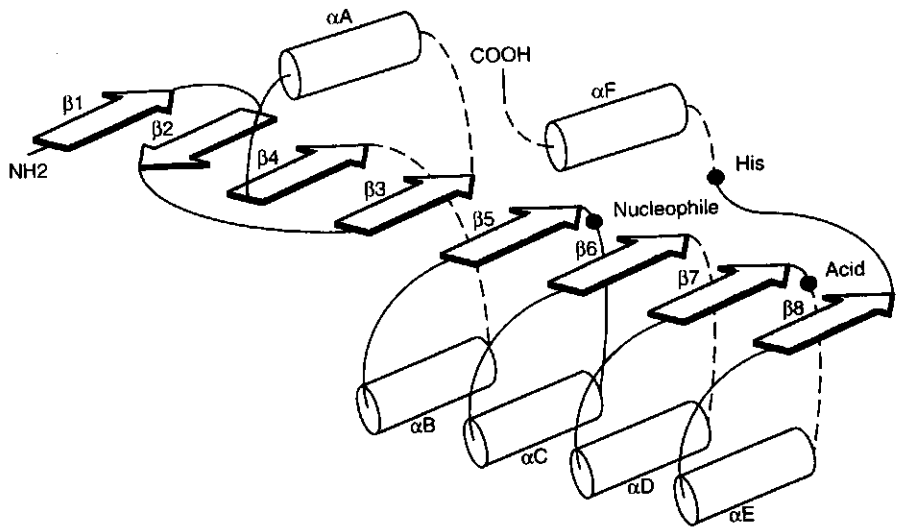
The cytotoxic activity of synthetic oxaspiro trichothecene analogues has been investigated. Independent studies, using cells of *Salmonella* and mice, respectively, showed that epoxide **4** was more biologically active than synthetic oxaspirooctanes containing the pyran structure[98,99]. A lower general cytotoxicity was, however, to be expected from the less polar epoxide **4**. The differential biological activity therefore indicated that the pyran oxygen was not essential for maximal activity. Other results from the study of Fullerton *et al.*[99] were (*i*) an observed complete inactivation of the biological activity after hydrolysis of the trichothecene 12,13-epoxide followed by a molecular rearrangement to the apotrichothecene structure, and (*ii*) a significant influence of the oxaspiro stereochemistry in biological activity. The influence of stereochemistry is in accordance with the generally observed phenomenon of chiral recognition in the action of many other bioactive compounds.

All these findings clearly support a possible role for enzymatic epoxide hydrolysis in microbial mycotoxin detoxification. For elucidation of the biological role of YEH and other microbial epoxide hydrolases, more detailed investigations will be necessary for the identification of the natural epoxide substrates.

1.4.2 *Enzyme characteristics*

Epoxide hydrolases comprise a group of functionally related enzymes that catalyze the cofactor-independent hydrolysis of epoxides to their corresponding diols. In spite of their broad range of accepted substrates, epoxide hydrolases show a narrow specificity to their cosubstrate: only water can be used as a cosubstrate.

Epoxide hydrolases have been divided into two classes based on homology and properties: soluble epoxide hydrolases (sEH) and membrane-associated microsomal epoxide hydrolases (mEH). Most epoxide hydrolases belong to the *α/β hydrolase fold* family of enzymes[100]. This family is an evolutionary divergent group of enzymes that function with a catalytic triad consisting of (*i*) a nucleophile (Ser, Cys or Asp) for formation of an alkyl-enzyme intermediate, (*ii*) a general base (always His) that initiates hydrolysis of the intermediate, and (*iii*) a charge relay residue (Asp or Glu) to activate and assist His by hydrolysis of the intermediate. The catalytic triads of all members are arranged in the same manner by the sequence: nucleophile, charge relay acid, general base. The 'canonical' α/β hydrolase fold consists of a mostly parallel eight-stranded polypeptide β-sheet surrounded on both sides by polypeptide α-helices (Scheme 9). The parallel β-strands 3 to 8 are connected by α-helices, which pack on either side of the central β-sheet. The nucleophile is located at the 'nucleophile elbow' (between strand β5 and the connecting α-helix), which positions the nucleophile such that it is easily accessible for the substrate on one side and for the hydrolytic water molecule on the other side. Characteristic enantio- and regioselectivities for the different enzymes can be explained by the specific shape and composition of the respective active sites.



Scheme 9. Schematic diagram of the 'canonical' α/β hydrolase fold[100].

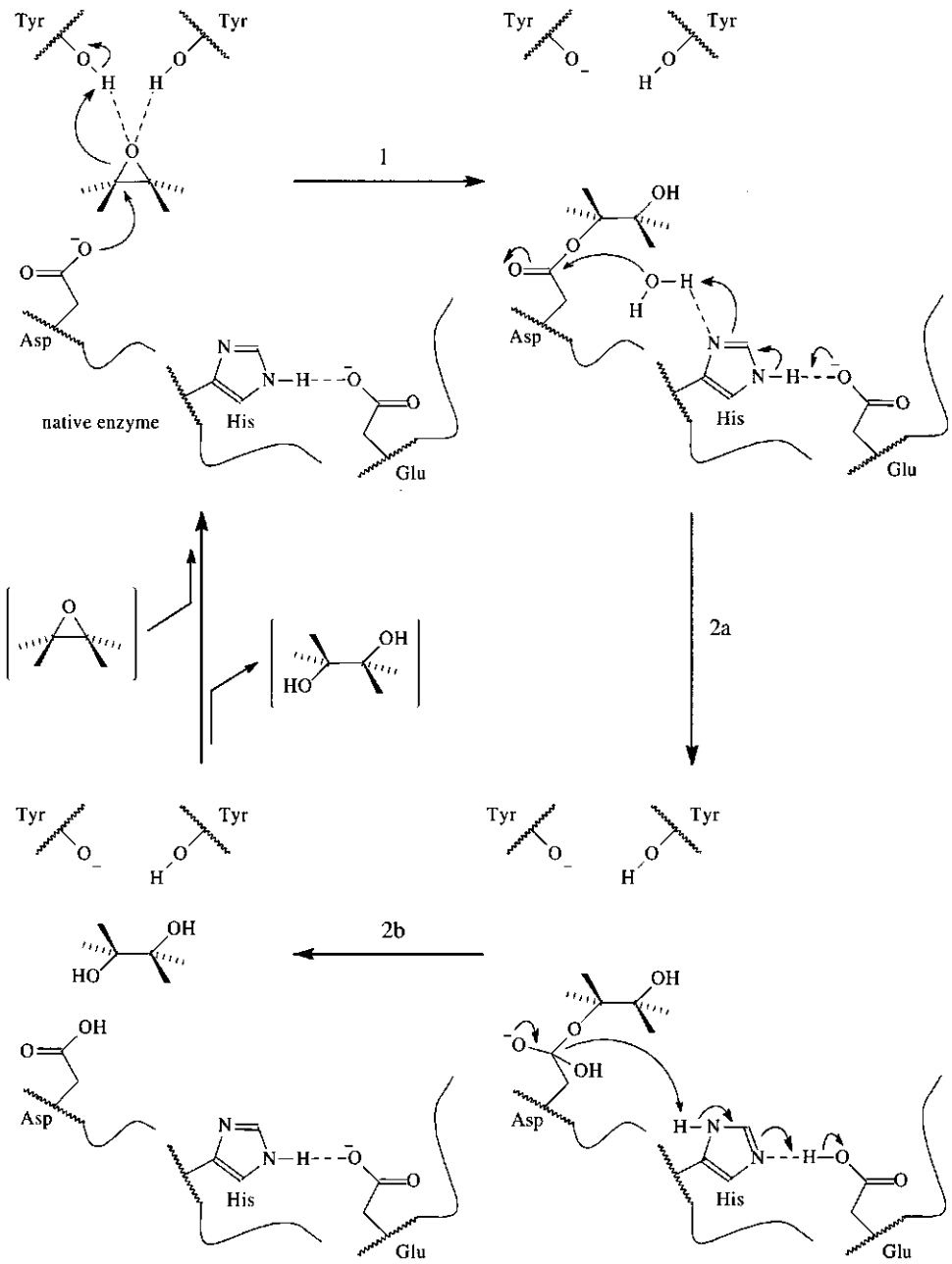
In all epoxide hydrolases, a common role of acidic tyrosine residues in the active site is proposed. Support by Tyr is suggested for substrate activation by protonation and substrate binding by hydrogen bond donation. Sequence alignments have shown that a catalytic Tyr residue is present in all sequenced microbial and eukaryotic epoxide hydrolases, and a second Tyr is at least partly conserved[101-104]. Recent studies indicate that epoxide hydrolases in general need two Tyr residues for catalytic activity and high substrate affinity[102,104].

The yeast epoxide hydrolase (YEH) of *Rhodotorula glutinis* has been characterized very recently by Visser *et al.* to be a member of the α/β hydrolase fold family[105]. For this, the epoxide hydrolase-encoding gene was isolated and the encoded polypeptide characterized. A molecular mass of 46 kDa was calculated for the YEH. The catalytic triad (nucleophile, charge relay acid, base) was proposed to consist of Asp190, Glu359 and His385. Corresponding Tyr residues for substrate binding and activation are highly conserved as well in YEH[106]. Highest amino acid sequence similarities were found for two mammalian microsomal epoxide hydrolases: human MmEH and rat MmEH. Particularly the Glu residue as charge relay function in contrast to Asp residues, is unique for YEH and MmEH when compared to other well-characterized enzymes including bacterial sEH, mammalian MsEH and fungal AnEH[102-104]. These results are in accordance with previously reported common features of YEH and MmEH, like similar localization in the cell membrane[107] and corresponding substrate specificities[63]. The recent findings indicate that both the eukaryotic MmEH and YEH share a common ancestry and proposed role in biochemical detoxification.

1.4.3 Catalytic mechanism

Most epoxide hydrolases are members of the α/β hydrolase fold enzymes which implies that their catalytic mechanism proceeds in two steps *via* an alkyl-enzyme intermediate. The commonly accepted two-step mechanism has been first elucidated for the mammalian enzyme MmEH[108] and independently confirmed for the mammalian MsEH[109]. The first step in this mechanism, epoxide ring-opening, is assumed to be a base-catalyzed S_N2 reaction[110]. Exceptional acid-catalyzed ring-opening has however been reported for the non-α/β hydrolase fold limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* DCL14, indicating the existence of alternative mechanisms[111].

Common features of the mechanism[112] of α/β hydrolase fold epoxide hydrolases are: (*i*) backside nucleophilic attack with inversion of configuration at the attacked epoxide carbon, (*ii*) distinction of two different transition states, one formed by epoxide ring-opening and the second during hydrolysis of the alkyl-enzyme intermediate, and (*iii*) substrate binding and subsequent activation by two tyrosine residues. Based on the generally accepted mechanism and the recent characterization of the epoxide hydrolase from *Rhodotorula glutinis*[105], a catalytic mechanism for YEH is proposed (Scheme 10).



Scheme 10. Proposed catalytic mechanism for epoxide hydrolysis by α/β hydrolase fold epoxide hydrolases. A catalytic triad of Asp190, Glu359 and His385 is proposed for the yeast epoxide hydrolase of *Rhodotorula glutinis*. The two-step mechanism proceeds via nucleophilic ring-opening of the epoxide (step 1) and subsequent hydrolysis of the alkyl-enzyme intermediate (step 2a and 2b).

In the YEH reaction mechanism, epoxide hydrolysis is initiated by positioning of the substrate in the active site by hydrogen bonding of two tyrosine residues. Subsequent substrate protonation by one of the tyrosine residues promotes the ring-opening in concert by attack of the nucleophile Asp190 (step 1). The so formed alkyl-enzyme (ester) intermediate is subsequently hydrolyzed by a water molecule, activated by the general base His385. The basicity of His is enhanced by the negatively charged carboxylate side-chain of Glu359. Nucleophilic attack by the deprotonated water molecule to the carbonyl of the alkyl-enzyme intermediate results in the formation of a tetrahedral oxyanion intermediate (step 2a). The carbonyl oxygen of this second intermediate is negatively charged ('oxyanion') and stabilized by at least two hydrogen bonds from amide nitrogens of residues forming the 'oxyanion hole' (not shown in Scheme 10). The tetrahedral intermediate collapses upon protonation of the aspartate oxygen by His, resulting in release of the diol from the broken ester bond (step 2b). Proton transfer from the resultant Asp carboxylic acid to one of the Tyr phenolate anions completes the reaction. Eventually, the diol is released from the active site and the enzyme is ready for another catalytic cycle.

Final evidence for the detailed information on the residues involved the YEH catalytic mechanism eventually will be obtained from subsequent high-resolution crystal structures of this epoxide hydrolase.

References

1. Cahn, R.S.; Ingold, C.K.; Prelog, V. Specification of molecular chirality. *Angew. Chem. Int. Ed.* **1966**, *5*, 385-415.
2. Mason, S.F. Origins of biomolecular handedness. *Nature* **1984**, *311*, 19-23.
3. Bonner, W.A. Origins of chiral homogeneity in Nature. *In*: Eliel, E.L.; Wilen, S.H. (eds), *Topics in Stereochemistry*, Wiley Interscience, New York, **1988**, *18*, 1-96.
4. Feringa, B.L.; van Delden, R.A. Absolute asymmetric synthesis: the origin, control, and amplification of chirality. *Angew. Chem. Int. Ed.* **1999**, *38*, 3418-3438.
5. Meierhenrich, U.; Thiemann, W.H.-P.; Rosenbauer, H. Molecular parity violation via comets? *Chirality* **1999**, *11*, 575-582.
6. Bonner, W.A. Parity violation and the evolution of biomolecular homochirality. *Chirality*, **2000**, *12*, 114-126.
7. Buschmann, H.; Thede, R.; Heller, D. New developments in the origins of the homochirality of biologically relevant molecules. *Angew. Chem. Int. Ed.* **2000**, *39*, 4033-4036.
8. Avalos, M.; Babiano, R.; Cintas, P.; Jiménez, J.L.; Palacios, J.C. From parity to chirality: chemical implications revisited. *Tetrahedron Asymmetry* **2000**, *11*, 2845-2874.
9. Rikken, G.L.J.A.; Raupach, E. Enantioselective magnetochiral photochemistry. *Nature* **2000**, *405*, 932-935.
10. Lehmann, P.A.F.; Rodrigues de Miranda, J.F.; Ariëns, E.J. Stereoselectivity and affinity in molecular pharmacology. *Progress in Drug Research* **1976**, *20*, 101-142.

11. Crossley, R. The relevance of chirality to the study of biological activity. *Tetrahedron* **1992**, *48*, 8155-8178.
12. Mesecar, A.D.; Koshland, D.E. A new model for protein stereospecificity. *Nature* **2000**, *403*, 614-615.
13. Ariëns, E.J.; Wuis, E.W.; Veringa, E.J. Stereoselectivity of bioactive xenobiotics. *Biochemical Pharmacology* **1988**, *37*, 9-18.
14. Lien, E.J. Chirality and drug targeting: pros and cons. *J. Drug Targeting* **1995**, *2*, 527-532.
15 Gibaldi, M. Stereoselective and isozyme-selective drug interactions. *Chirality* **1993**, *5*, 407-413.
16. Hutt, A.J.; Tan, S.C. Drug chirality and its clinical significance. *Drugs* **1996**, *52*, 1-12.
17. Williams, A. Opportunities for chiral agrochemicals. *Pesticide Science* **1996**, *46*, 3-9.
18. Williams, A. The role of chirality in the agrochemical industry. *Phytoparasitica* **2000**, *28*, 1-4.
19. Kurihara, N.; Miyamoto, J.; Paulson, G.D.; Zeeh, B.; Skidmore, M.W.; Hollingworth, R.M.; Kuiper, H.A. Chirality in synthetic agrochemicals: bioactivity and safety consideration. *Pure Appl. Chem.* **1997**, *69*, 1335-1348.
20. Hirohara, H.; Nishizawa, M. Biochemical synthesis of several chiral insectiside intermediates and mechanisms of action of relevant enzymes. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1-9.
21. US Food and Drug Administration. FDA's policy statement for the development of new stereoisomeric drugs. *Chirality* **1992**, *4*, 338-340.
22. Strong, M. FDA policy and regulation of stereoisomers: paradigm shift and the future of safer, more effective drugs. *Food and Drug Law Journal* **1999**, *54*, 463-487.
23. Crosby, J. *In*: Collins, A.N.; Sheldrake, G.N.; Crosby, J. (eds), *Chirality in Industry II*, Wiley, Chichester, **1997**, 1-10.
24. Margolin, A.L. Enzymes in the synthesis of chiral drugs. *Enzyme Microb. Technol.* **1993**, *15*, 266-280.
25. McCoy, M. Biocatalysis grows for drug synthesis. *Chem. Eng. News* **1999**, *77*, 10-14.
26. Rozzell, J.D. Commercial scale biocatalysis: myths and realities. *Bioorg. Med. Chem.* **1999**, *7*, 2253-2261.
27. Schulze, B.; Wubbolts, M.G. Biocatalysis for industrial production of fine chemicals. *Curr. Opin. Biotechnol.* **1999**, *10*, 609-615.
28. Patel, R.N. *In*: Patel, R.N. (ed.), *Stereoselective Biocatalysis*, Dekker, New York, **2000**, 87-130.
29. Seelbach, K. *In*: Liese, A.; Seelbach, K.; Wandrey, C. (eds), *Industrial Biotransformations*, Wiley, Weinheim, **2000**, 3-27.
30. Smith, J.G. Synthetically useful reactions of epoxides. *Synthesis 1983*, 629-656.
31. Kirkovsky, L.; Lermontov, A.; Zavorin, S.I.; Sukhozhenko, I.I.; Zavelsky, V.I.; Thier, R.; Bolt, H.M. Hydrolysis of genotoxic methyl-substituted oxiranes: experimental kinetic and semiempirical studies. *Environ. Toxicol. Chem.* **1998**, *17*, 2141-2147.
32. Pauwels, W.; Veulemans, H. Comparison of ethylene, propylene and styrene 7,8-oxide in vitro adduct formation on N-terminal valine in human haemoglobin and on N-7-guanine in human DNA. *Mutation Research* **1998**, *418*, 21-33.
33. Buchanan, J.G.; Sable, H.Z. Stereoselective epoxide cleavages. *In*: Thyagarajan, B.S. (ed.), *Selective Organic Transformations*, Wiley, New York, **1972**, *2*, 2-95.
34. Lewars, E.G. Oxiranes and Oxirenes. *In*: Katritzky, A.R.; Rees, C.W. (eds), *Comprehensive Heterocyclic Chemistry*, Pergamon, Oxford, **1984**, *7*, 96-119.
35. Jacobsen, E.N. Asymmetric catalysis of epoxide ring-opening reactions. *Acc. Chem. Res.* **2000**, *33*, 421-431.

36. Linderman R.J.; Roe, R.M.; Harris, S.V.; Thompson, D.M. Inhibition of insect juvenile hormone epoxide hydrolase: asymmetric synthesis and assy of glycidol-ester and epoxy-ester inhibitors of *Trichoplusia ni* epoxide hydrolase. Insect *Biochem. Molec. Biol.* **2000**, *30*, 767-774.
37. Ojima, I.; Chakravarty, S.; Inoue, T.; Lin, S.; He, L.; Band Horwitz, S.; Kuduk, S.D.; Danishefsky, S.J. A common pharmacophore for cytotoxic natural products that stabilize microtubules. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4256-4261.
38. Almirante, L.; Murmann, W. Relationship between configuration and adrenergic β-receptor blocking activity of optical isomers of 1-(4-nitrophenyl)-2-isopropylaminoethanol (INPEA). *J. Med. Chem.* **1966**, *9*, 650-653.
39. Kraus, R.L.; Hering, S.; Grabner, M.; Ostler, D.; Striessnig, J. Molecular mechanism of diltiazem interaction with L-type Ca^{2+} channels. *J. Biol. Chem.* **1998**, *273*, 27205-27212.
40. Lin, J.H. Role of pharmacokinetics in the discovery and development of indinavir. *Advanced Drug Delivery Reviews* **1999**, *39*, 33-49.
41. Shibatani T.; Omori, K.; Akatsuka, H.; Kawai, E.; Matsumae, H. Enzymatic resolution of diltiazem intermediate by *Serratia marcescens* lipase: molecular mechanism of lipase secretion and its industrial application. *J. Mol. Catal. B* **2000**, *10*, 141-149.
42. Pedragosa-Moreau, S.; Morisseau, C.; Baratti, J.; Zylber, J.; Archelas, A.; Furstoss, R. Microbiological Transformations 37: An enantioconvergent synthesis of the β-blocker (*R*)-Nifénalol® using a combined chemoenzymatic approach. *Tetrahedron* **1997**, *53*, 9707-9714.
43. Zhang, J.; Reddy, J.; Roberge, C.; Senanayake, C.; Greasham, R.; Chartrain, M. Chiral bio-resolution of racemic indene oxide by fungal epoxide hydrolases. *J. Ferment. Bioeng.* **1995**, *80*, 244-246.
44. Collet, A.; Brienne, M.-J.; Jacques, J. Optical resolution by direct crystallization of enantiomer mixtures. *Chem. Rev.* **1980**, *80*, 215-230.
45. Wolff, A.; van Asperen, V.; Straathof, A.J.J.; Heijnen, J.J. Potential of enzymatic kinetic resolution using solid substrates suspension: improved yield, productivity, substrate concentration, and recovery. *Biotechnol. Prog.* **1999**, *15*, 216-227.
46. Kagan, H.B.; Fiaud, J.C. Kinetic Resolution. *In*: Eliel, E.L.; Wilen, S.H. (eds), *Topics in Stereochemistry*, Wiley Interscience, New York, **1988**, *18*, 249-331.
47. Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C.J. Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.* **1982**, *104*, 7294-7299.
48. Straathof, A.J.J.; Jongejan, J.A. The enantiomeric ratio: origin, determination and prediction. *Enzyme Microb. Technol.* **1997**, *21*, 559-571.
49. Finney, N.S. Enantioselective epoxide hydrolysis: catalysis involving microbes, mammals and metals. *Chem. Biol.* **1998**, *5*, 73-79.
50. Tokunaga, M.; Larrow, J.F.; Kakiuchi, F.; Jacobsen, E.N. Asymmetric catalysis with water: efficient kinetic resolution of terminal epoxides by means of catalytic hydrolysis. *Science* **1997**, *277*, 936-938.
51. Savle, P.S.; Lamoreaux, M.J.; Berry, J.F.; Gandour, R.D. A convenient resolution of long-chain alkyl epoxides with Jacobsen's salen(Co)III(OAc) catalysts. *Tetrahedron: Asymmetry* **1998**, *9*, 1843-1846.
52. Weijers, C.A.G.M.; de Haan, A.; de Bont, J.A.M. Microbial production and metabolism of epoxides. *Microbiol. Sciences* **1988**, *5*, 156-159.
53. Leak, D.J.; Aikens, P.J.; Mahmoudian, M.S. The microbial production of epoxides. *TIBTECH* **1992**, *10*, 256-261.

54. Bont de, J.A.M. Bioformation of optically pure epoxides. *Tetrahedron: Asymmetry* **1993**, *4*, 1331-1340.
55. Besse, P.; Veschambre, H. Chemical and biological synthesis of chiral epoxides. *Tetrahedron* **1994**, *50*, 8885-8927.
56. Archelas, A.; Furstoss, R. Biocatalytic approaches for the synthesis of enantiopure epoxides. *Topics in Curr. Chem.* **1999**, *200*, 159-191.
57. Ladner, W.E.; Whitesides, G.M. Lipase-catalyzed hydrolysis as a route to esters of chiral epoxy alcohols. *J. Am. Chem. Soc.* **1984**, *106*, 7251-7252.
58. Wu, D.R.; Cramer, S.M.; Belfort, G. Kinetic resolution of racemic glycidyl butyrate using a multiphase membrane enzyme reactor: experiments and model verification. *Biotechnol. Bioeng.* **1993**, *41*, 979-990.
59. Matsumae, H.; Furui, M.; Shibatani, T. Lipase-catalyzed asymmetric hydrolysis of 3-phenylglycidic acid ester, the key intermediate in the synthesis of diltiazem hydrochloride. J. Ferment. Bioeng. **1993**, *75*, 93-98.
60. Schoffers, E.; Golebiowski, A.; Johnson, C.R. Enantioselective synthesis through enzymatic asymmetrization. *Tetrahedron* **1996**, *52*, 3769-3826.
61. Hodgson, D.M.; Gibbs, A.R.; Lee, G.P. Enantioselective desymmetrisation of achiral epoxides. *Tetrahedron* **1996**, *52*, 14361-14384.
62. Brunel, J.M.; Legrand, O.; Reymond, S.; Buono, G. Beneficial effect of *ortho*-methoxy groups in the asymmetric ring-opening of *meso* epoxides with silicon tetrachloride catalyzed by chiral *ortho*-methoxyphenyldiazaphosphonamide Lewis Bases. *Angew. Chem. Int. Ed.* **2000**, *39*, 2554-2557.
63. Weijers, C.A.G.M.; de Bont, J.A.M. Epoxide hydrolases from yeasts and other sources: versatile tools in biocatalysis. *J. Mol. Catal. B* **1999**, *6*, 199-214.
64. van Ginkel, C.G.; Welten, H.G.J.; de Bont J.A.M. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 334-337.
65. Hartmans, S.; de Bont, J.A.M.; Harder, W. Microbial metabolism of short-chain unsaturated hydrocarbons. *FEMS Microbiol. Rev.* **1989**, *63*, 235-264.
66. Cleveland, C.C.; Yavitt, J.B. Microbial consumption of atmospheric isoprene in a temperate forest soil. *Appl. Environ. Microbiol.* **1998**, *64*, 172-177.
67. Weijers, C.A.G.M.; de Haan, A.; de Bont, J.A.M. Werkwijze voor het winnen van optisch zuivere (2*R*)-vormen van al dan niet gesubstitueerde 2,3-epoxyalkanen. Patent NL 8700468A.
68. Weijers, C.A.G.M.; de Haan, A.; de Bont, J.A.M. Chiral resolution of 2,3-epoxyalkanes by *Xanthobacter* Py2. *Appl. Microbiol. Biotechnol.* **1988**, *27*, 337-340.
69. Weijers, C.A.G.M.; Jongejan, H.; Franssen, M.C.R.; de Groot, Ae. Dithiol- and NAD-dependent degradation of epoxyalkanes by *Xanthobacter* Py2. *Appl. Microbiol. Biotechnol.* **1995**, *42*, 775-781.
70. Sluis, M.K.; Small, F.J.; Allen, J.R.; Ensign, S.A. Involvement of an ATP-dependent carboxylase in a CO2-dependent pathway of acetone metabolism by *Xanthobacter* strain Py2. *J. Bacteriol.* **1996**, *178*, 4020-4026.
71. Ensign, S.A.; Small, F.J.; Allen, J.R.; Sluis, M.K. New roles for CO_2 in the microbial metabolism of aliphatic epoxides and ketones. *Arch. Microbiol.* **1998**, *169*, 179-187.
72. Allen, J.R.; Ensign, S.A. Carboxylation of epoxides to ß-keto acids in cell extracts of *Xanthobacter* strain Py2. *J. Bacteriol.* **1996**, *178*, 1469-1472.
73. Chion, C.K.; Leak, D.J. Purification and characterization of two components of epoxypropane isomerase/carboxylase from *Xanthobacter* Py2. *Biochem. J.* **1996**, *319*, 499-506.

74. Swaving, J.; de Bont, J.A.M.; Westphal, A.; de Kok, A. A novel type of pyridine nucleotide-disulfide oxidoreductase is essential for NAD^+- and NADPH-dependent degradation of epoxyalkanes by *Xanthobacter* strain Py2. *J. Bacteriol.* **1996**, *178*, 6644-6646.
75. Westphal, A.; Swaving, J.; Jacobs, L.; de Kok, A. Purification and characterization of a flavoprotein involved in the degradation of epoxyalkanes by *Xanthobacter* Py2. *Eur. J. Biochem.* **1998**, *257*, 160-168.
76. Clark, D.D.; Allen, J.R.; Ensign, S.A. Characterization of five catalytic activities associated with the NADPH: 2-ketopropyl-coenzyme M [2-(2-ketopropylthio) ethanesulfonate] oxidoreductase/carboxylase of the *Xanthobacter* strain Py2 epoxide carboxylase system. *Biochemistry* **2000**, *39*, 1294-1304.
77. Allen, J.R.; Clark, D.D.; Krum, J.G.; Ensign, S.A. A role for coenzyme M (2-mercaptoethanesulfonic acid) in a bacterial pathway of aliphatic epoxide carboxylation. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 8432-8437.
78. Krum, J.G.; Ensign, S.A. Heterologous expression of bacterial epoxyalkane: coenzyme M transferase and inducible coenzyme M biosynthesis in *Xanthobacter* strain Py2 and *Rhodococcus rhodochrous* B276. *J. Bacteriol.* **2000**, *182*, 2629-2634.
79. Allen. J.R.; Ensign, S.A. Two short-chain dehydrogenases confer stereoselectivity for enantiomers of epoxypropane in the multiprotein epoxide carboxylating systems of *Xanthobacter* strain Py2 and *Nocardia corallina* B276. *Biochemistry* **1999**, *38*, 247-256.
80. Seidegard, J.; DePierre, J.W. Microsomal epoxide hydrolase: properties, regulation and function. *Biochim. Biophys. Acta* **1983**, *695*, 251-270.
81. Fretland, A.J.; Omiecinski, C.J. Epoxide hydrolases: biochemistry and molecular biology. *Chem. Biol. Interact.* **2000**, *129*, 41-59.
82. Omiecinski, C.J.; Hassett, C.; Hosagrahara, V. Epoxide hydrolase: polymorphism and role in toxicology. *Toxicol. Lett.* **2000**, *112-113*, 365-370.
83. Archer, I.V.J. Epoxide hydrolases as asymmetric catalysts. *Tetrahedron* **1997**, *53*, 15617-15662.
84. Archelas, A.; Furstoss, R. Epoxide hydrolases: new tools for the synthesis of fine organic chemicals. *TIBTECH* **1998**, *16*, 108-116.
85. Kroutil, W.; Faber, K. Stereoselective syntheses using microbial epoxide hydrolases. *In*: Patel, R.N. (ed.), *Stereoselective Biocatalysis*, Dekker, New York, **2000**, 205-237.
86. Visser, H.; de Bont, J.A.M.; Verdoes, J.C. Isolation and characterization of the epoxide hydrolase-encoding gene from *Xanthophyllomyces dendrohous*. *Appl. Environ. Microbiol.* **1999**, *65*, 5459-5463.
87. Botes, A.L.; Weijers, C.A.G.M.; Botes, P.J.; van Dyk, M.S. Enantioselectivities of yeast epoxide hydrolases for 1,2-epoxides. *Tetrahedron: Asymmetry* **1999**, *10*, 3327-3336.
88. Weijers, C.A.G.M. Enantioselective hydrolysis of aryl, alicyclic and aliphatic epoxides by *Rhodotorula glutinis*. *Tetrahedron: Asymmetry* **1997**, *8*, 639-647.
89. Werf van der, M.J.; Overkamp, K.M.; de Bont, J.A.M. Limonene-1,2-epoxide hydrolase from *Rhodococcus erythropolis* DCL14 belongs to a novel class of epoxide hydrolases. *J. Bacteriol.* **1998**, *180*, 5052-5057.
90. Armstrong, R.N. Enzyme-catalyzed detoxification reactions: mechanisms and stereochemistry. *CRC Crit. Rev. Biochem.* **1987**, *22*, 39-88.
91. Ueno, Y. Mode of action of trichothecenes. *Ann. Nutr. Aliment.* **1977**, *31*, 885-900.
92. Sukroongreung, S.; Schappert, K.T.; Khachatourians, G.G. Survey of sensitivity of twelve yeast genera toward T-2 toxin. *Appl. Environ. Microbiol.* **1984**, *48*, 416-419.

93. Boeira, L.S.; Bryce, J.H.; Stewart, G.G.; Flannigan, B. Inhibitory effect of *Fusarium* mycotoxins on growth of brewing yeasts. 2. Deoxynivalenol and nivalenol. *J. Inst. Brew.* **1999**, *105*, 376-381.
94. Boeira, L.S.; Bryce, J.H.; Stewart, G.G.; Flannigan, B. The effect of combinations of *Fusarium* mycotoxins (deoxynivalenol, zearalenone and fumonisin B1) on growth of brewing yeasts. *J. Appl. Microbiol.* **2000**, *88*, 388-403.
95. Liptay-Reuter, I.; Dose, K.; Guenthner, T.; Wörner, W.; Oesch, F. Vitamin K epoxide reductase activity in the metabolism of epoxides. *Biochem. Pharmacol.* **1985**, *34*, 2617-2620.
96. King, R.R.; McQueen, R.E.; Levesque, D.; Greenhalgh, R. Transformation of deoxynivalenol (vomitoxin) by rumen microorganisms. *J. Agr. Food Chem.* **1984**, *32*, 1181-1183.
97. Weijers, C.A.G.M. Unpublished results.
98. Sinsheimer, J.E.; Chakraborty, P.K.; Messerly, E.A.; Gaddamidi, V. Mutagenicity of oxaspiro compounds with *Salmonella*. *Mutat. Res.* **1989**, *224*, 171-175.
99. Fullerton, D.S.; Chen, C.M.; Hall, I.H. Trichothecene analogues. 1. 1,5-Dioxaspiro[2.5]octanes. *J. Med. Chem.* **1976**, *19*, 1391-1395.
100. Ollis, D.L.; Cheah, E.; Cygler, M.; Dijkstra, B.; Frolow, F.; Franken, M.; Harel, M.; Remington, S.J.; Silman, L.; Schrag, J.; Sussman, J.L.; Verschueren, K.H.G., Goldman, A. The α/β hydrolase fold. *Protein Eng.* **1992**, *5*, 197-211.
101. Nardini, M.; Ridder, I.S.; Rozeboom, H.J.; Kalk, K.H.; Rink, R.; Janssen, D.B.; Dijkstra, B.W. The X-ray structure of epoxide hydrolase from *Agrobacterium radiobacter* AD1. *J. Biol. Chem.* **1999**, *274*, 14579-14586.
102. Rink, R.; Kingma, J.; Lutje Spelberg, J.H.; Janssen, D.B. Tyrosine residues serve as proton donor in the catalytic mechanism of epoxide hydrolase from *Agrobacterium radiobacter*. *Biochemistry* **2000**, *39*, 5600-5613.
103. Yamada, T.; Morisseau, C.; Maxwell, J.E.; Agririadi, M.A.; Christianson, D.W.; Hammock, B.D. Biochemical evidence for the involvement of tyrosine in epoxide activation during the catalytic cycle of epoxide hydrolase. *J. Biol. Chem.* **2000**, *275*, 23082-23088.
104. Zou, J.; Hallberg, B.M.; Bergfors, T.; Oesch, F.; Arand, M.; Mowbray, S.L.; Jones, T.A. Structure of *Aspergillus niger* epoxide hydrolase at 1.8 Å resolution: implications for the structure and function of the mammalian microsomal class of epoxide hydrolases. *Structure* **2000**, *8*, 111-122.
105. Visser, H.; Vreugdenhil, S.; de Bont, J.A.M.; Verdoes, J. Cloning and characterization of an epoxide hydrolase-encoding gene from *Rhodotorula glutinis*. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 415-419.
106. Visser, H. Personal communication.
107. Kronenburg, N.A.E.; Mutter, M.; Visser, H.; de Bont, J.A.M.; Weijers, C.A.G.M. Purification of an epoxide hydrolase from *Rhodotorula glutinis*. *Biotechnol. Lett.* **1999**, *21*, 519-524.
108. Lacourciere, G.M.; Armstrong, R.N. The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate. *J. Am. Chem. Soc.* **1993**, *115*, 10466-10467.
109. Borhan, B.; Jones, A.D.; Pinot, F.; Gran, D.F.; Kurth, M.J.; Hammock, B.D. Mechanism of soluble epoxide hydrolase. *J. Biol. Chem.* **1995**, *270*, 26923-26930.
110. Armstrong, R.N. Kinetic and chemical mechanism of epoxide hydrolase. *Drug Metab. Rev.* **1999**, *31*, 71-86.
111. Werf van der, M.J.; de Bont, J.A.M.; Swarts, H.J. Acid-catalyzed enzymatic hydrolysis of 1-methylcyclohexene oxide. *Tetrahedron: Asymmetry* **1999**, *10*, 4225-4230.
112. Armstrong, R.N.; Cassidy, C.S. New structural and chemical insight into the catalytic mechanism of epoxide hydrolases. *Drug Metab. Rev.* **2000**, *32*, 327-338.

1.5 Outline of this thesis

This thesis describes the biocatalytic kinetic resolution of epoxides by enantioselective ring-opening. For this study, the catalytic activity of two different enzymes has been explored: the bacterial epoxide carboxylase of *Xanthobacter* Py2 and the yeast epoxide hydrolase of *Rhodotorula glutinis*.

In *Chapter 2*, the first example of kinetic resolution of epoxides by enzymatic ring-opening is presented. (2*R*)-Enantiomers of short-chain aliphatic disubstituted epoxides were obtained by selective conversion of the respective (2*S*)-enantiomers using cells of *Xanthobacter* Py2.

Epoxide conversion by *Xanthobacter* Py2 is described in more detail in *Chapter 3*. Ketones were identified as reaction products of epoxides. The reaction was found to be dependent on NAD^+ and an unknown reducing cofactor, which could be replaced by synthetic dithiol compounds. Involvement of catalytic thiol residues was indicated by sensitivity for the thiol blocking reagent *N*-ethylmaleimide. Based on these findings, a putative catalytic mechanism is proposed.

In *Chapter 4*, the biocatalytical potential of yeast epoxide hydrolases (YEHs) is explored. Epoxide hydrolysis by cells of *Rhodotorula glutinis* was examined and the scope of substrates presented. Enantioselective hydrolysis of *trans*-1-phenyl-1,2-epoxypropane is described in more detail.

In *Chapter 5*, YEH-catalyzed hydrolysis of a homologous range of monosubstituted aliphatic epoxides is described. Reaction rates as well as enantioselectivities were found to be strongly influenced by the substrate chain length. Resolution of 1,2-epoxyhexane showed the best combination of enantioselectivity and activity.

In *Chapter 6*, two methods for YEH-catalyzed preparative-scale kinetic resolution of 1,2-epoxyhexane are presented. Critical parameters in scaling-up were determined to be the limited water-solubility of the epoxide substrate, and toxicity of both the epoxide and formed diol. An efficient process to overcome these problems was developed using a two-phase hollow fiber membrane bioreactor. The process configuration was operated in a batch-wise mode and subsequently modified for continuous extractive kinetic resolution.

A *discussion* on the work presented in this thesis is given in *Chapter 7*, followed by a *Summary* in English and in Dutch.

2

Chiral resolution of 2,3-epoxyalkanes by *Xanthobacter* Py2

Abstract

With propene-grown cells of Xanthobacter *Py2 it was possible to resolve racemic mixtures of 2,3-expoxyalkanes. Only* 2S*-forms were metabolized bij this organism, resulting in pure* 2R*-2,3-expoxylkanes. Chiral resolution was obtained with* trans*-2,3-epoxybutane,* trans*-2,3-epoxypentane and* cis*-2,3-epoxypentane.* Xanthobacter *Py2 was however not able to discriminate between the enantiomeric forms of 1,2-epoxyalkanes, resulting in the complete degradation of both enantiomers of 1,2-epoxyalkanes.*

C.A.G.M. Weijers, A. de Haan, and J.A.M. de Bont

Applied Microbiology and Biotechnology **1988**, *27*, 337-340

Introduction

Epoxides are valuable starting materials in organic synthesis and they are used on a large scale in chemical industry as precursors of many different compounds. Epoxides are of even greater interest when available in enantiopure form because they then can serve as synthons in the preparation of other more valuable optically active compounds. Synthesis of chiral epoxides by chemical methods has been studied extensively[1], but apart from the asymmetric epoxidation of allylic alcohols[2] and apart from the formation of some other chiral epoxides as for instance 1,2-epoxypropane[3] no general procedure is available for the selective formation of the respective enantiomeric forms of epoxides. Consequently, many attempts have been made in recent years to produce chiral epoxides from alkenes by biological methods. Bacteria have been described that are able to produce chiral epoxyalkanes from gaseous alkenes[4,5], from higher alkenes[6] and from more complex alkenes as for instance arylallyl ethers[7]. Prospects and limitations of such systems that all depend on the action of mono-oxygenases are presently studied by several research groups[8,9].

However, another biological method to obtain enantiopure epoxides may be available by using enzymes that degrade epoxides stereoselectively. Such method would involve the complete degradation of one enantiomer while the other would not be affected. Although resolution of epoxides in this manner involves the destruction of half of the amount of epoxide, it nevertheless may be an economically feasible method in view of the difference in price between the racemic and optically pure epoxides. Enantioselective hydrolysis has previously been observed for microsomal epoxide hydrolase[10], and the conversion of epoxides into *trans*-diols by this enzyme has recently been reviewed by Berti[11] from the point of view of the relations between substrate structure and the regio- and stereoselectivity of the enzyme.

Recently we have screened several epoxide-degrading bacteria for their ability to degrade epoxyalkanes enantioselectively and the effect of these strains on racemic mixtures of both 1,2-epoxyalkanes and 2,3-epoxyalkanes has been investigated. In the present paper we report on the results obtained with *Xanthobacter* Py2 for the selective degradation of epoxyalkanes. The organism was originally isolated on propene[12] and it also grows on other 1-alkenes (ethene, 1-butene) as well as on 1,2-epoxyalkanes (1,2-epoxypropane, 1,2-epoxybutane)[13]. It contains an alkene mono-oxygenase that oxidizes 1-alkenes to (*R*)-1,2-epoxyalkanes[5]. It is now shown that the organism contains an enzyme involved in the further metabolism if epoxyalkanes that is able to discriminate between the enantiomeric forms of 2,3-epoxyalkanes.

Table 1. Degradation of epoxyalkanes by propene-grown *Xanthobacter* Py2

Substrate	Degradation rate*
1,2-Epoxypropane	35
1,2-Epoxybutane	32
Cis-2,3-epoxybutane	10
Trans-2,3-epoxybutane	6
Cis-2,3-epoxypentane	9
Trans-2,3-epoxypentane	4
1,2-Epoxyhexane	15

* Rates are expressed in nmol per minute per mg protein

Results and discussion

Epoxyalkane utilization by Xanthobacter Py2

Xanthobacter Py2, when growing on an alkene, in an at present unknown type of reaction further degrades epoxyalkanes to eventually cell biomass and carbon dioxide[14]. Washed cell suspensions of propene-grown cells metabolize several epoxyalkanes and the initial rates of degradation of various racemic epoxyalkane mixtures are given in Table 1. From these results it appears that an epoxide-degrading enzyme with a broad substrate specificity is present in propene-grown *Xanthobacter* Py2 cells, although it can not be excluded that more than one enzyme system is involved in the metabolism of the various epoxyalkanes.

Stereoselectivity of epoxyalkane degradation

The enantioselectivity of the epoxide-degrading enzyme in *Xanthobacter* Py2 was tested by incubating washed propene-grown cells with racemic mixtures of 1,2-epoxypropane and *trans*-2,3-epoxybutane respectively. Enantiomeric resolution of the epoxyalkanes was followed by taking samples from head space and analysis by complexation gas chromatography[15]. No clear preferential utilization of one of the configurations of 1,2-epoxypropane was observed; both isomers were fully degraded (Figure 1a). Interestingly, a complete different situation was met with *trans*-2,3-epoxybutane. In this case only the (2*S*,3*S*)-isomer was degraded while the (2*R*,3*R*)-isomer was not metabolized at all (Figure 1b).

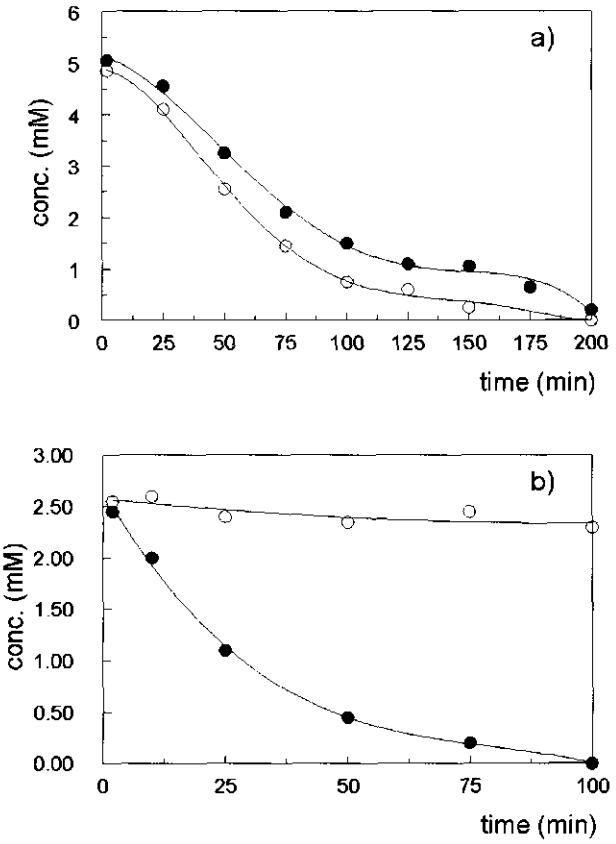


Figure 1.Degradation of epoxyalkanes by propene-grown *Xanthobacter* Py2. (**a**) Simultaneous degradation of (*R*)-1,2-epoxypropane (○) and (*S*)-1,2-epoxypropane (●); (**b**) degradation of *trans*-(2*S*,3*S*)-epoxybutane (●), *trans*-(2*R*,3*R*)-epoxybutane (○) was not degraded.

Furthermore, no effect of the (2*R,3R*)-isomer on the degradation of the (2*S,3S*)-isomer was observed since an extra addition of racemic *trans*-2,3-epoxybutane, after resolution of the initial mixture, did not affect the rate of degradation of the (2*S*,3*S*)-isomer (Figure 2). In another experiment was the initial concentration of the racemic *trans*-2,3-epoxybutane raised to 100 mM and under that condition was also a complete resolution of the mixture obtained. In this way it was possible to obtain in a very simple way the pure *trans*-(2*R*,3*R*)-epoxybutane whereas the chemical method for the preparation of this chiral compound from (2*S*,3*S*)-tartaric acid involves eight different steps[16].

Cis-2,3-epoxybutane is not a chiral compound and it was degraded to completion by washed-cell suspensions of propene-grown *Xanthobacter* Py2.

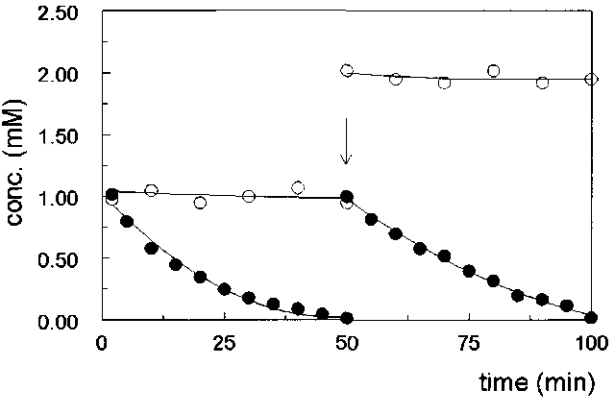


Figure 2. Effect of *trans*-(2*R*,3*R*)-epoxybutane (○) on the degradation of *trans*-(2*S*,3*S*)-epoxybutane (●) by *Xanthobacter* Py2.

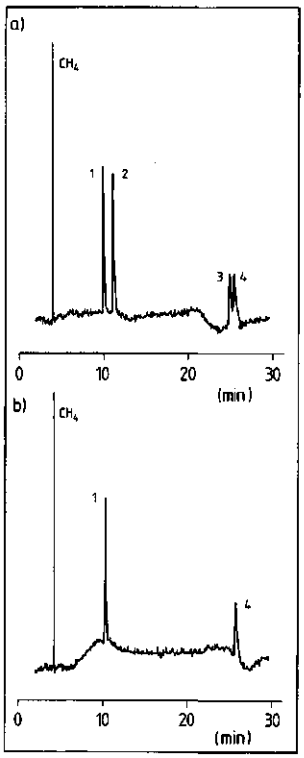
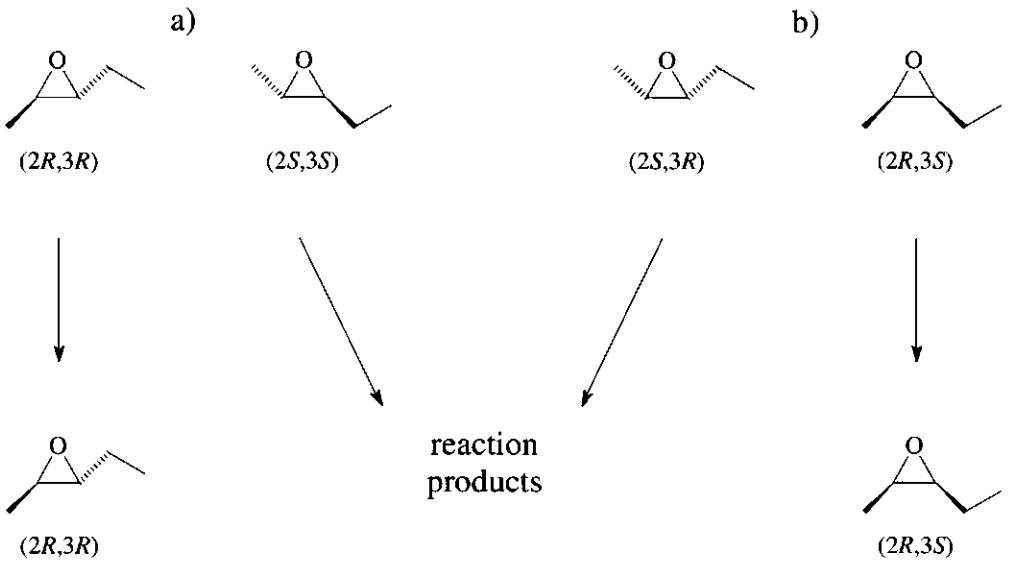


Figure 3. Chromatograms of 2,3-epoxypentanes as obtained by complexation gas chromatography. *Trans*-(2*R*,3*R*)-epoxypentane (1), *trans*-(2*S*,3*S*)-epoxypentane (2), *cis*-(2*S*,3*R*)-epoxypntane (3) and *cis*-(2*R*,3*S*)-epoxypentane (4); (**a**) initial mixture, (**b**) mixture remaining after treatment with *Xanthobacter* Py2.

Investigations were further extended by preparing racemic 2,3-epoxypentanes from both *cis*-2-pentene and from *trans*-2-pentene and the effect of *Xanthobacter* Py2 cells on the resulting four epoxyalkanes was recorded. The racemic mixtures of both the *trans* and the *cis* form were given in separate experiments to the washed cells. Both mixtures were resolved and the remaining enantiomers were *trans*-(2*R*,3*R*)-epoxypentane and *cis*-(2*R*,3*S*)-epoxypentane respectively. In another experiment were both racemic mixtures given simultaneously to the cells again resulting in the degradation of the (2*S*)-forms and in an accumulation of the (2*R*)-forms (Figure 3).



Scheme 1. Racemic mixtures of (**a**) *trans*-2,3-epoxypentane, (**b**) *cis*-2,3-epoxypentane and the remaining enantiomers after resolution by *Xanthobacter* Py2.

Conclusions

From the results presented it is concluded that *Xanthobacter* Py2 cells are able to discriminate between the enantiometric forms of 2,3-epoxyalkanes (Scheme 1) whereas both enantiomers of 1,2-epoxyalkanes are fully degraded. At present it remains uncertain whether only one enzyme is involved in these epoxide-degrading reactions, but it nevertheless is obvious that raising *Xanthobacter* Py2 it is possible to resolve racemic mixtures of 2,3-epoxyalkanes. This stereoselective degradation of epoxyalkanes appears to be a very elegant way to obtain an epoxyalkane in an enantiomerically pure form and we

therefore at present are studying the epoxide-degrading enzyme of *Xanthobacter* Py2 involved in the resolution of 2,3-epoxyalkanes and we also are testing other organisms for the resolution of 1,2-epoxyalkanes.

Experimental section

Organism and culture conditions

Xanthobacter Py2 has been described previously by van Ginkel and de Bont[14]. The organism was cultivated continuousley in mineral medium in a 3-litre fermentor (with 2 litre working volume) at 30°C, with a dilution rate of 0.02 h^{-1}. The pH of the culture was maintained at 7.0. As carbon source propene was supplied as a 100 ml/min 4% in air mixture. The cells were harvested by centrifugation at 16,000*g*, washed twice with 50mM potassium phosphate buffer pH 7.0 and stored at -20°C.

Chemicals

Gaseous alkenes were obtained from Hoek Loos, Schiedam (NL). Other alkenes, 1,2-epoxyalkanes, *trans*-2,3-epoxybutane and *cis*-2,3-epoxybutane and *cis*-2,3-epoxybutane were from Aldrich Chemie N.V., Brussels. *Trans*-2,3-epoxypentane and *cis*-2,3-epoxypentane were synthesized by oxidation of the corresponding 2-pentenes with *m*-chloroperoxybenzoie acid[17].

Analysis of epoxyalkanes

The characterization of the synthesized epoxypentanes was carried out by NMR spectroscopy and mass spectroscopy. ^{1}H-NMR spectra were recorded on a Varian EM-390 90 Mhz spectrometer and mass spectra on a Vacuum Generators Ltd. Micromass 7070F Mass-spectrometer.

A complexation GLC method[15] was used to determine the enantiomeric composition of the various epoxyalkanes. Analyses were carried out on a Packard model 438 gas chromatograph. As carrier gas N_2 was used and the oven temperature was 50°C. A glass capillary column was used: Length 25 m, diameter 0.25 mm, coated with 'Chirametal-27-*R*-3-1-13'. This column was obtained from CC & CC, P.O. Box 14, D-7402 Kirchentellinsfurt, FRG. Peak areas were determined with a Shimadzu model Chromatopac C-R3A integrator.

Degradation of epoxyalkanes by Xanthobacter *Py2*

Epoxyalkane degradation by *Xanthobacter* Py2 was tested in 27 cm^3 screw-cap bottles sealed with rubber septa. Bottles contained 4ml 50mM potassium phosphate buffer with the appropiate epoxyalkanes and 0.05 ml methane serving as internal standard. The bottles were placed into an shaking waterbath (30°C, 3 Hz) and the reaction was started by injecting 1 ml washed cell-suspension (25 mg protein). Periodically 100µl samples of the gas phase were taken and analyzed by complexation GLC. Concentrations of epoxyalkanes were derived from calibration curves using heat killed cells. Epoxyalkane concentrations are expressed in mM in the water-phase assuming the quantity of epoxyalkane in the gas-phase is negligible in comparison with the quantity epoxyalkane in the water-phase.

Acknowledgements

We are grateful to Dr. B.J.M. Jansen (Dept. of Organic Chemistry, WU) for his advice in the synthesis of the epoxypentanes and to Dr. M.A. Posthumus (Dept. of Organic Chemistry, WU) for performing the mass spectrum analyses.

References

1 Scott, J.W. *In*: Morrison J.D., Scott J.W. (eds) *Asymmetric Synthesis*. Academic Press, Orlando, **1984**, *vol. 4*, p 5.

2 Katsuki, T.; Sharpless, K.B. The First Practical Method for Asymmetric Epoxidation. *J. Am. Chem. Soc.* **1980**, *102*, 5974-5976.

3 Golding, B.T. Synthesis of optically active propylene oxides. *In*: Porter R, Clark S (eds) *Enzymes in organic synthesis*, Pitman, London, **1985**, pp 165-169.

4 Furuhashi, K.; Taoka, A.; Uchida S.; Karube, I.; Suzuki, S. Production of 1,2-Epoxyalkanes from 1-Alkenes by *Nocardia corallina* B276. *Eur. J. Appl. Microbiol. Biotechnol.* **1981**, *12*, 39-45.

5 Habets-Crützen, A.Q.H.; Carlier, S.J.N.; Bont de, J.A.M.; Wistuba, D.; Schurig, H.; Hartmans, S.; Tramper, J. Stereospecific formation of 1,2-epoxypropane, 1,2-epoxybutane and 1-chloro-2,3-epoxypropane by alkene-utilizing bacteria. *Enzyme Microbiol. Technol.* **1985**, *7*, 17-21.

6 Smet de, M.J.; Kingma, J.; Wynberg, H.; Witholt, B. *Pseudomonas oleovorans* as a tool in bioconversion characteristics in different two-phase systems. *Enzyme Microbiol. Technol.* **1983**, *5*, 352-360.

7 Johnstone, S.L.; Phillips, G.T.; Robertson, B.W.; Watts, P.D.; Bertola, M.A.; Koger, H.S.; Marx, A.F. Stereoselective synthesis of *S*-(-)-ß-blockers via microbially produced epoxide intermediates. *In*: Laane C, Tramper J, Lilly MD (eds). *Biocatalysis in Organic Media* Elsevier, Amsterdam, **1986**, pp 387-392.

8 Tramper, J.; Brink, L.E.S.; Hamstra, R.S.; Bont de, J.A.M.; Habets-Crützen, A.Q.H.; Ginkel van, G. Production of (chiral) epoxyalkanes in second-generation bioreactors. *Proceeding of Third European Congress on Biotechnology* München, **1984**, *II*, pp 269-276.

9 Drozd, J.W.; Balley, M.L. Biotransformation. *European Patent Application* **1984**, 0,099,609.

10 Seidegard, J.; DePierre, J.W. Microsomal Epoxide Hydrolase properties, regulation and function. *Biochim. Biophys. Acta* **1983**, *695*, 251-270.

11 Berti, G. Enantio- and diastereoselectivity of microsomal epoxide hydrolase: Potential applications to the preparation of non-racemic epoxides and diols. *In*: Schneider MP (ed) *Enzymes as Catalysts in Organic Synthesis*. Reidel D Publishing Company, Dordrecht, **1986**, pp 349-354.

12 Habets-Crützen, A.Q.H.; Brink, L.E.S.; Ginkel van, C.G.; Bont de, J.A.M.; Tramper, J. Production os epoxides from gaseous alkenes by resting-cell suspensions and immobilized cells of alkene utilizing bacteria. *Appl. Microbiol. Biothechnol.* **1984**, *20*, 245-250.

13 Ginkel van, C.G.; Welten, H.G.J.; Bont de, J.A.M. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 334-337.

14 Ginkel van, C.G.; Bont de, J.A.M. Isolation and characterization of alkene-utilizing *Xanthobacter* spp. *Arch. Microbiol.* **1986**, *145*, 403-407.

15 Schurig, V.; Bürkle, W. Extending the Scope of Enantiomer Resolution by Complexation Gas Chromatography. *J. Am. Chem. Soc.* **1982**, *104*, 7573-7580.

16 Schurig, V.; Koppenhöfer, B.; Bürkle, W. Preparation of Configurationally Pure *trans*-(2*S*,3*S*)-2,3-Epoxybutane. *J. Org. Chem.* **1980**, *45*, 538-541.

17 Swern, D.; Billen, G.N.; Scanlan, J.T. Hydroxylation and epoxidation of some 1-olefins with per-acids. *J. Am. Chem. Soc.* **1946**, *68*, 1504-1507.

3

Dithiol- and NAD-dependent degradation of epoxyalkanes by *Xanthobacter* Py2

Abstract

A broad range of epoxyalkanes was converted into the corresponding ketones by cell extracts of Xanthobacter *Py2. Both 1,2-and 2,3-epoxyalkanes were degraded and in addition, the degradation of 2,3-epoxyalkanes in all cases was highly enantioselective.*

Conversion of a deuterium-labelled substrate indicated that the ketone product was probably formed indirectly via a hydroxy intermediate.

Degradation of epoxyalkanes by Xanthobacter *Py2 was dependent on both NAD and another, not yet identified cofactor which was present in the low molecular weight fraction (LMF) of propene-grown cells. It is proposed that the LMF was involved in a reductive reaction step since it could be replaced by dithiothreitol (DTT) and various other dithiol compounds. Epoxyalkane-degrading activity was inhibited by the sulfhydryl blocking reagent* N*-ethylmaleimide (NEM). Inhibition by NEM and stimulation by LMF, DTT and other dithiols was only effective in the simultaneous presence of NAD.*

C.A.G.M. Weijers, H. Jongejan, M.C.R. Franssen, Ae. de Groot and J.A.M. de Bont

Applied Microbiology and Biotechnology **1995**, *42*, 775-781

Introduction

Studies on epoxide metabolism have focused on the biotransformation of arene oxides by mammalian systems and fungi and only limited information is available on the bacterial metabolism of epoxides. In bacterial metabolism, epoxides are not frequently observed as intermediates, but nevertheless, different types of reactions involved in microbial epoxide degradation are known[1]. Metabolism of epoxides in microorganisms has been studied mainly because these compounds are very useful starting materials in organic synthesis. Epoxides may be obtained in enantiopure form from racemic mixtures if they are metabolized enantioselectively. Such epoxides can be used in subsequent chemical synthesis of valuable bioactive compounds. Enantioselective degradation and various other biological methods for production of enantiopure epoxides have recently been reviewed[2,3].

Examples of well-studied enzymes in bacterial epoxide metabolism are a styrene oxide isomerase from a *Xanthobacter* sp. with very narrow substrate specificity[4] and epoxide hydrolases from different *Pseudomonas* spp. catalyzing the hydrolysis of several epoxides[5,6]. These enzymes were not enantioselective. Epoxide hydrolase activity has further been observed in the 1,2-epoxypropane-utilizing *Nocardia* A60[7] and also in crude enzyme preparations of a *Rhodococcus* sp.. The latter enzyme showed enantioselectivity in the hydrolysis of branched methyl-alkyl epoxides but not in the case of straight-chain terminal epoxides[8]. Fungal hydrolysis of an epoxide has been described for *Aspergillus niger* and *Beauveria sulfurescens*. These two strains show opposite enantioselectivity in the degradation of racemic styrene oxide[9]. Bacterial hydrolytic degradation of cyclic epoxides has recently been observed in a *Corynebacterium* sp.[10].

A specific group of epoxides, epoxyalkanes, are known as intermediates in the bacterial metabolism of aliphatic alkenes. Very little is known, however, about the enzyme systems that degrade these epoxyalkanes. In a study with ethene-grown *Mycobacterium* E20 it was proposed that epoxyethane was converted directly into acetyl-CoA in the presence of NAD, CoA and an unknown cofactor[11]. The alkene-utilizing *Xanthobacter* Py2 grows on short chain 1-alkenes as well as on the corresponding epoxyalkanes[12]. Propene-grown cells of *Xanthobacter* Py2 degrade both enantiomers of 1,2-epoxyalkanes tested but from 2,3-epoxyalkanes only the (2*S*)-forms are degraded, resulting in enantiopure (2*R*)-epoxyalkanes[13].

In the present study, the enzymatic degradation of 1,2- and 2,3-epoxyalkanes in *Xanthobacter* Py2 has been investigated in more detail.

Results

Epoxyalkane degradation by crude cell extracts

Crude cell extracts of propene-grown *Xanthobacter* Py2 showed the same degradation pattern as the corresponding whole cells: degradation of both enantiomers of 1,2-epoxyalkanes and highly enantioselective degradation of only the (2*S*)-forms of 2,3-epoxyalkanes. The degradation rates for 1,2-epoxyalkanes were about ten times higher than for (2*S*)-epoxyalkanes. Therefore, most experiments were performed with 1,2-epoxybutane as substrate while enantioselectivity was verified with *trans*-2,3-epoxybutane as substrate.

Both the pH and the temperature optimum were determined to optimize assay conditions. Maximum activity in the extract was at pH 8.2 and at a temperature of 38°C. The extract was stable for 30 minutes at temperatures of up to 50°C and between pH values of 6.5 to 8.5. Stability of the enzyme activity was further enhanced by adding glycerol (10% v/v). Purification of the enzyme using various chromatographic techniques was not successful. A reason for this was probably the loss of an essential non-protein component as will be discussed later in this report.

Cofactor requirements were studied in crude cell extracts under standard assay conditions. NAD (2 mM) and NADP (2 mM) raised the initial degradation rates of 1,2-epoxybutane from 4 to 12 and 5 nmoles per minute per milligram protein respectively.

Identification of the initial products

Crude cell extracts of *Xanthobacter* Py2 with additional NAD converted all 1,2- and 2,3-epoxyalkanes tested into ketone compounds. From 1,2-epoxyalkanes the corresponding 2-ketones were formed stoichiometrically and (2*S*)-epoxyalkanes were similarly converted into 3-ketones (Table 1). A deuterium labelled substrate was used to elucidate whether the ketone was formed via a rearrangement reaction, or whether a mechanism with a hydroxy intermediate was involved in the epoxyalkane degradation. *Cis*-2,3-dideutero-2,3-epoxypentane was converted into 2-deutero-3-pentanone as determined by GC/MS analysis (Figure1). The two major peaks in the mass spectrum at m/e 57 and m/e 58 show the mass fragments without and with one deuterium label at the keto-neighbouring C-atom respectively. Obviously one of the two deuterium atoms of the epoxyalkane was lost in the reaction. This result indicates that formation of the ketone was not accompanied by rearrangement of the deuterium atoms. In additional experiments it has been found that, apart from epoxides, ketones were only formed from the corresponding alcohols by cell extracts, and not from other possible intermediates like vicinal diols or α-hydroxy ketones. However, attempts to isolate any intermediate alcohol were unsuccessful. Subsequently, 2-methyl-1,2-epoxypropane and 2,3-dimethyl-2,3-

epoxybutane were synthesized in order to enzymatically accumulate the corresponding tertiary alcohols which would not be oxidized to a ketone. Unfortunately, these epoxides were not converted at all.

Table 1. Conversion of various epoxyalkanes by crude cell extract of *Xanthobacter* Py2.

Substrate	Detected product
1,2-Epoxypropane	Acetone
1,2-Epoxybutane	Butanone
Trans-2,3-epoxybutane	Butanone
Cis-2,3-epoxybutane	Butanone
1,2-Epoxypentane	2-Pentanone
Trans-2*S*,3*S*-epoxypentane	3-Pentanone
Cis-2*S*,3*R*-epoxypentane	3-Pentanone
1,2-Epoxyhexane	2-Hexanone
1,2-Epoxyethylbenzene (styrene oxide)	Acetophenone

Conversions were performed at pH 8.2 and at a temperature of 38°C with 5 ml crude cell extract (100 mg protein). The concentrations of the compounds added were glycerol (10% v/v), NAD (2 mM) and epoxyalkane (3 mM). The composition of the reaction products was determined by GC/MS analysis.

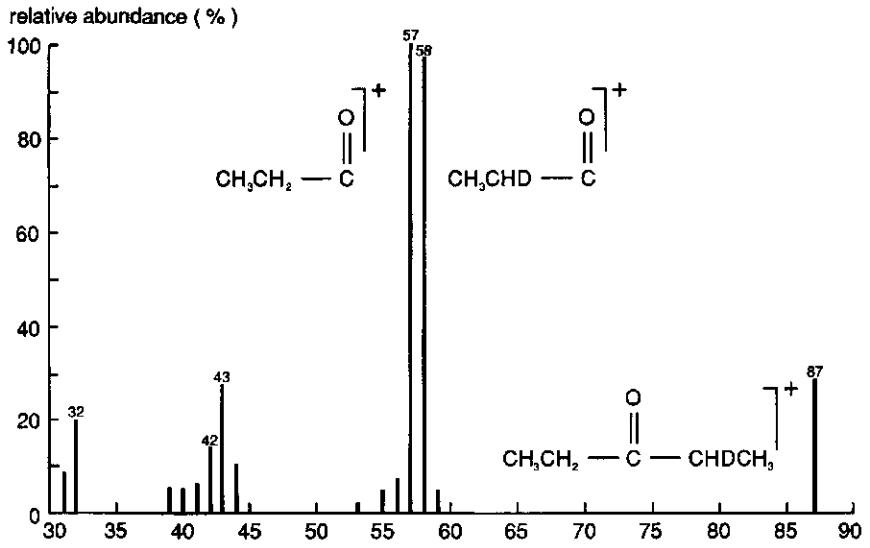


Figure 1. Relative abundances of mass fragments in the spectrum of 2-deutero-3-pentanone, formed by conversion of *cis*-2,3-dideutero-2,3-epoxypentane by crude cell extract of *Xanthobacter* Py2.

LMF-dependent epoxyalkane degradation

Dialyzed extracts of *Xanthobacter* Py2 were completely inactive in degrading epoxyalkanes, also in the presence of NAD. The activity was also lost when various chromatographic separation techniques were used for enzyme purification. Epoxyalkane-degrading activity could be restored by addition of a low molecular weight fraction (LMF), prepared by ultrafiltration from the corresponding crude cell extract. Addition of the LMF was only effective in the simultaneous presence of NAD. The relationship between the amount of LMF added to dialyzed extract and the specific activity restored is shown in Figure 2. The active form of LMF was only present in propene-grown cells, but not in glucose-grown cells. Attempts to purify the active compound from the LMF by gel filtration, ion-exchange chromatography or by solvent extraction were not successful. The molecular mass of the compound was lower than 3 kDa as indicated by ultrafiltration experiments. The compound is not a protein since it was not affected by proteolytic enzymes or heat (15 minutes at 110°C) and since it was stable for at least 30 minutes at pH-values between 2 and 11.

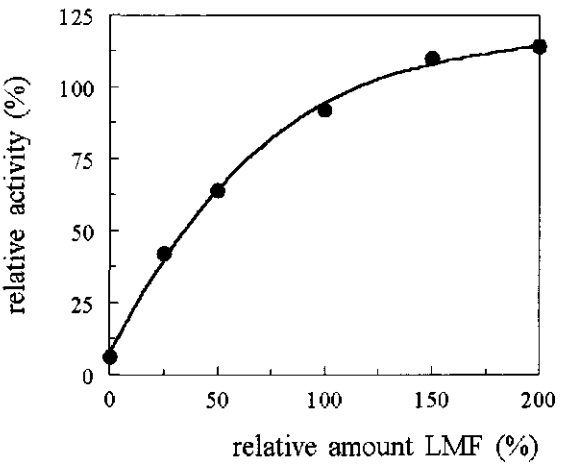


Figure 2. Recovery of the epoxyalkane degrading activity in dialyzed extract of *Xanthobacter* Py2 by addition of increasing amounts of the low molecular weight fraction (LMF). The relative amount of 100% LMF corresponds with the LMF concentration of the original crude cell extract. The specific activity of the original crude cell extract, taken as 100%, was 9.4 nmoles epoxyalkane per minute per milligram protein.

DTT-dependent epoxyalkane degradation

The finding that NAD is essential for epoxyalkane degradation indicates that an oxidative step is involved in this reaction. On the other hand, the oxidation states of an epoxyalkane and a ketone are identical and therefore a reductive counterpart is necessary. Possibly, the LMF has a role in this respect and consequently it was tested if some reducing reagents might replace the LMF. Addition of ascorbate and cysteine resulted in no or only in partly restored activity of dialyzed extracts. However, dithiothreitol (DTT) in the simultaneous presence of NAD restored activity. The stimulating effect of DTT was observed not only with 1,2-epoxybutane but also with *trans*-2,3-epoxybutane as substrate. The degradation of *trans*-2,3-epoxybutane was completely enantioselective excluding a non-enzymic conversion of the compound. The effect of different concentrations of DTT on enzyme activity was tested in analogy with the concentration-dependent LMF-effect. In Figure 3 it is shown that the stimulating effect of DTT was also concentration-dependent. A final concentration of 5 mM was chosen as optimal DTT concentration for further experiments.

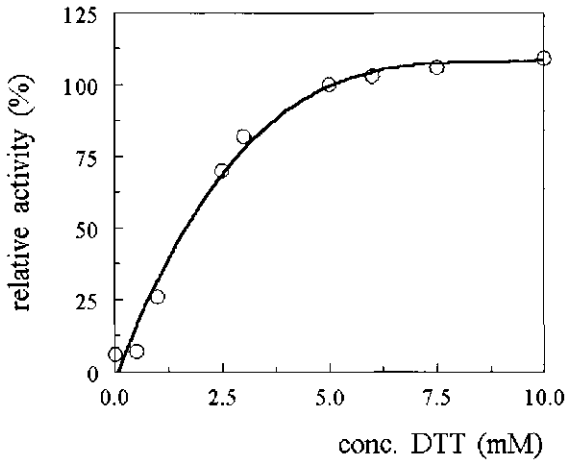


Figure 3. Effect of various concentrations dithiothreitol (DTT) on the epoxyalkane degrading activity of dialyzed extract of *Xanthobacter* Py2. The specific activity with addition of DTT to a final concentration of 5 mM, taken as 100%, was 13.8 nmoles epoxyalkane per minute per milligram protein.

Inhibition experiments with the sulfhydryl blocking reagent *N*-ethylmaleimide (NEM) were undertaken to investigate the effect of DTT. Inhibition with 1 mM NEM was only effective in the presence of NAD and the order of additions of NEM, DTT or NAD was important: NEM inhibition was maximal when NEM was added to the dialyzed extract

prior to either NAD or DTT. The order of addition of NAD and DTT also had an effect: maximal activity required NAD as the first binding component. The effects of DTT, NAD and NEM on the activity of dialyzed extract are summarized in Table 2.

Table 2. Effect of DTT, NAD, and NEM on the rate of degradation of 1,2-epoxybutane by dialyzed extract of *Xanthobacter* Py2

Compound added	Relative activity (%)[a]
None	6
NAD	7
NADH	10
DTT	22
NAD + DTT	100
DTT + NAD	84
NEM + DTT	20
NAD + DTT + NEM	92
NAD + NEM + DTT	65
DTT + NAD + NEM	40
DTT + NEM + NAD	16
NEM + NAD + DTT	21
NEM + DTT + NAD	12
DTT, heat-denaturated extract	3

[a] The maximum specific activity, taken as 100%, was 16.2 nmoles epoxyalkane per minute per milligram protein. The concentrations of the compounds added were NAD (2 mM), NADH (2 mM), DTT (5 mM) and NEM (1 mM). Rates were determined by adding 1,2-epoxybutane (3 mM) to extracts and subsequently the compounds were added in the order indicated and at intervals of 10 minutes.

Other thiol compounds serving as cofactor

Apart from DTT, various other synthetic and biological monothiol and dithiol compounds were tested for their ability to serve as a cofactor for epoxyalkane degradation. For this, these compounds were added to dialyzed extract with additional NAD and the initial rates for degradation of 1,2-epoxybutane were determined. In Table 3 these activities are compared with the activity achieved with DTT as cofactor. In the cases where significant activity was observed, enantioselectivity of the reaction was checked using *trans*-2,3-epoxybutane as substrate. In this way nonenzymatic reactions due to thiols was excluded.

In general, it was found that monothiol compounds were inactive as cofactor while most dithiols were as active as DTT.

Table 3. The effect of various monothiol and dithiol compounds on the rate of degradation of 1,2-epoxybutane by dialyzed extract of *Xanthobacter* Py2

Thiol compound	Relative activity (%)[a]
None	8
1-Propanethiol	16
2-Propanethiol	13
1-Butanethiol	14
Glutathione (red)	17
L-Cysteine	34
1,2-Ethanedithiol	112
1,3-Propanedithiol	102
2,3-Butanedithiol	12
1,4-Butanedithiol	94
1,5-Pentanedithiol	38
Lipoic acid (red)	10
Dithioerythritol	109
Dithiothreitol	100

[a] The specific activity with addition of dithiothreitol, taken as 100%, was 12.4 nmoles epoxyalkane per minute per milligram protein. The concentrations of the compounds added were NAD (2 mM) and thiol compounds (5 mM). Rates were determined by adding 1,2-epoxybutane (3 mM) to extracts and subsequently NAD was added prior to the thiol compounds.

Discussion

Cofactor requirement

In the present study it is shown that epoxyalkane degradation by *Xanthobacter* Py2 is dependent on both NAD and another cofactor which is present in the LMF of propene-grown cells. The requirement of a LMF as additional cofactor in epoxyalkane metabolism has been observed previously in *Mycobacterium* E20[11]. The composition of the active components of the LMF in either case is still unknown. The synthetic thiol compound dithiothreitol (DTT) restored the activity of dialyzed extracts and showed a similar concentration-dependent pattern as observed for the LMF (Figures 2,3). The involvement of DTT in the bioconversion of an epoxide has been described previously for the extensively studied mammalian enzyme vitamin K epoxide reductase. Whitlon *et al.*[14] reported that among various biological reducing agents tested, DTT was the most effective cofactor in vitro for this specific enzyme. The mammalian enzyme and our *Xanthobacter* enzyme furthermore have similar substrate specificities for thiols since they are both stimulated by the dithiol compounds whereas synthetic monothiols exhib-

ited no cofactor activity[15]. Vitamin K epoxide reductase, however, differs from the enzyme of *Xanthobacter* Py2 in that it is not NAD-dependent. At this moment the physiological cofactor for vitamin K epoxide reductase is still unknown.

From the data in Table 3 it can be concluded that the only apparent structural requirement for cofactor activity is the presence of two terminal thiol groups. Nevertheless, 2,3-butanedithiol was inactive which probably is due to the terminal methyl groups present, thus preventing the formation of the sterically favorable cyclic disulfide after oxidation[16]. Therefore, reduction reactions with 2,3-butanedithiol and also with monothiol compounds result in much lower steady-state concentrations of reduced forms of the interacting enzyme. Of the natural occuring thiol compounds tested only cysteine showed some cofactor activity while glutathione and lipoic acid did not. The data presented for the dithiol-dependent activities do not distinguish between direct action of the dithiol as a primary reductant or its ability to reduce some other compound which is then acting as the in vitro reducing agent.

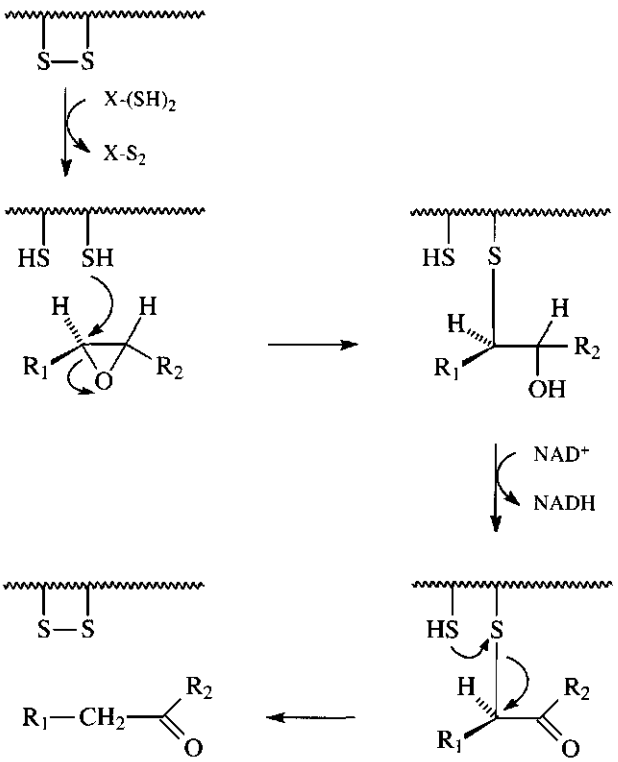
Stimulation by dithiol compounds suggests that the reduction of one or more critical disulfide bonds is required for enzyme activity. The involvement of sulfhydryl groups in the active center of the epoxyalkane degrading enzyme of *Xanthobacter* Py2 was confirmed using the sulfhydryl blocking reagent *N*-ethylmaleimide (NEM). Inhibition with 1 mM NEM was maximal when it was added to the dialyzed extract prior to NAD or DTT (Table 2). The effect observed by the order of addition of NAD and NEM, and also of NAD and DTT, indicates that sulfhydryl groups might participate in binding of NAD. Alkylation of sulfhydryl groups by NEM could not be reversed by the addition of NAD. Protection against sulfhydryl group inhibitors by first adding NAD has previously been established for several other enzymic reactions[17]. More recently, this NAD-effect has been observed in the reduction of formaldehyde by the NAD-linked factor-dependent formaldehyde dehydrogenase from Amycolatopsis methanolica. This enzyme and its unidentified cofactor have been described[18]. The properties of their unknown cofactor are reminiscing of the cofactor from *Xanthobacter* Py2 because it was found to be a heat-stable, low-molecular-mass compound which inactive form could be reactivated by reduction with DTT.

Proposed enzyme mechanism

Formation of ketones in the degradation of epoxyalkanes was studied in detail using cis-2,3-dideutero-2,3-epoxypentane. Cell extracts of *Xanthobacter* Py2 converted this compound to 2-deutero-3-pentanone (Figure 1), indicating no rearrangement of the deuterium atoms had taken place. A one step conversion would have been accompanied with an internal 1,2-shift, resulting in 2,2-dideutero-3-pentanone. A mechanism consisting of more than one reaction step will probably proceed via the intermediate 2,3-dideutero-3-pentanol which will be oxidized to 2-deutero-3-pentanone. Oxidation of 2,3-dideutero-3-pentanol by crude cell extract also resulted in the 2-deutero-3-pentanone product, thus

supporting the proposed mechanism. Other possible intermediates can be excluded because it has been observed in related experiments that formation of ketones did not occur from vicinal diols or from the corresponding α-hydroxy ketones. Under standard assay conditions, ketones were only formed from the corresponding secondary alcohols by cell extracts of *Xanthobacter* Py2.

Enzymic reduction of an epoxide to an alcohol has only been described for very few and very specific reactions. The first example was given by Steckbeck *et al.*[19] who reported that incubation of (24*R*)-24,25-oxidolanosterol with standard S_{10} rat liver homogenate had resulted in the formation of (24*R*)-hydroxycholesterol. A hydroxy intermediate was further observed in the very specific formation of hydroxyvitamin K by warfarin-resistant rat liver microsomes, whereas it was not found in those from normal rats[20]. This hydroxy compound has also been demonstrated as an intermediate in a chemical model study for the mechanism of vitamin K epoxide reductase[21]. However, activity of the latter enzyme is restricted to the reduction of vitamin K epoxide to the corresponding vitamin K. Other epoxides of different size, structure and polarity, including styrene oxide, were not converted by the mammalian enzyme[22].



Scheme 1. Proposed enzyme mechanism in the degradation of epoxyalkanes to ketones by *Xanthobacter* Py2. R_1= H, CH_3; R_2= CH_3, C_2H_5, C_3H_7, C_4H_9, C_6H_5. The unknown reducing cofactor is represented by X.

Scheme 1 shows a proposed enzyme mechanism in the degradation of epoxyalkanes to ketones by *Xanthobacter* Py2. The degradation of epoxyalkanes in four steps is based on the conversion of the deuterium labelled substrate and on the requirement of both the reductive and oxidative cofactors. The proposed covalent bond between the enzyme and substrate is also based on the inability to isolate any intermediate alcohol in the degradation reaction. Elucidation of the mechanism may be possible by purifying the epoxyalkane-degrading enzyme. However, many attempts to purify the enzyme while using several techniques (results not reported) were without success. Consequently, we subsequently will investigate the genetics of epoxyalkane degradation. Furthermore, it would be extremely interesting to identify the cofactor involved in epoxyalkane-degradation in *Xanthobacter* Py2 since a similar and presently unknown factor is involved in a NAD-linked formaldehyde dehydrogenase[18] and in vitamin K epoxide reduction[15].

Experimental section

Organism and culture conditions

The isolation and characterization of *Xanthobacter* Py2 has been described previously by van Ginkel and de Bont[23]. During the present study the organism was continuously cultivated in mineral medium in a 2-litre fermentor (with 1-litre working volume) at 30°C, with a dilution rate of 0.025 h^{-1}. The pH of the culture was maintained at 7.0. As carbon source propene was supplied as a 100 ml min^{-1} 1.25% in air mixture. The cells were harvested by centrifugation at 16,000 *g*, washed twice with 50 mM potassium phosphate buffer pH 7.0, concentrated, and stored at -20°C.

Preparation of cell extracts

Crude cell extracts were obtained after ultrasonic disintegration of concentrated *Xanthobacter* Py2 cells followed by removal of cell debris and unbroken cells by centrifugation at 30,000 *g* for 20 min at 4°C. Subsequently, glycerol was added to the crude cell extract to a final concentration of 10% (v/v) in order to stabilize enzyme activity. Dialyzed extracts were prepared by dialysing crude cell extracts overnight at 4°C against 50 mM potassium phosphate buffer pH 8.0 supplemented with 10% (v/v) glycerol.

Preparation of the low molecular weight fraction (LMF)

The low molecular weight fraction (LMF) was prepared by ultrafiltration of crude cell extracts without additional glycerol. Ultrafiltration was performed in Centriprep-10 con-

centrators (Amicon Ltd., Rotterdam, The Netherlands) by stepwise centrifugation at 3,000 *g* for 20 min each, at room temperature. The collected colorless, clear filtrate was designated as the LMF. The LMF could be stored at -20°C for at least six months without any significant loss of activity.

Chemicals

2,3-Dimethyl-2,3-epoxybutane and 2-methyl-1,2-epoxypropane were synthesized by direct oxidation of the corresponding alkenes with *m*-chloroperbenzoic acid (MCPBA)[24]. Other epoxides, alkenes, unnatural thiol compounds and Lindlar-catalyst were purchased from Aldrich (Belgium). Glutathione, L-cysteine and lipoic acid were from Sigma (Belgium). *N*-ethylmaleimide (NEM), MCPBA and lithium aluminium hydride ($LiAlH_4$) were obtained from Janssen-Chimica (Belgium) and all biochemicals were from Boehringer Mannheim (Germany).

Synthesis of the deuterium labelled compounds

Cis-2,3-dideutero-2,3-epoxypentane was obtained by direct oxidation of *cis*-2,3-dideutero-2-pentene with MCPBA[24]. For the synthesis of *cis*-2,3-dideutero-2-pentene 680 mg 2-pentyne was dissolved in 10 ml pentane and subsequently 68 mg Lindlar-catalyst and one drop of quinoline were added. Hydrogenation of the reaction mixture was performed with 220 ml of deuteriumgas at room temperature[25]. Eventually, the catalyst was filtered off and the filtrate was used for synthesis of *cis*-2,3-dideutero-2,3-epoxypentane. For this, 10 ml diethylether and 320 mg of sodium bicarbonate were added to the filtrate, cooled to 5°C and the reaction was started by addition of 1,01 eq. of MCPBA. The reaction mixture was stirred for 15 min, warmed to room temperature and stirred for another three hours. Subsequently, the mixture was washed with sodium thiosulphate and sodium bicarbonate solution and dried. The solvent was evaporated via Spaltrohr distillation and the epoxide was further purified by preparative GC. The yield was 350 mg (40% from 2-pentyne).

2,3-Dideutero-3-pentanol was obtained by $LiAlH_4$ reduction of *cis*-2,3-dideutero-2,3-epoxypentane and further purified by preparative HPLC (column: Silica Nucleosil ET 250, with hexane:t-butylmethylether = 85:15 as eluent) and preparative GC (2 m* 3/8" column with 30% superox 0.6 on chromosorb WAW 45/60 Mesh at 45°C). ^{1}H-NMR spectra and mass spectra of the synthesized compounds were in accordance with the proposed structures.

Analytical methods

Epoxyalkane- and ketone-concentrations were determined by headspace analysis on a Carlo Erba model 4200 gas chromatograph fitted with a capillary Poraplot Q column (Chrompack, The Netherlands). As carrier gas N_2 was used and the oven temperature was 170°C. The complexation gas-liquid chromatography (GLC) method of Schurig and Bürkle[26] was used to determine the enantiomeric composition of the epoxyalkanes, as described in one of our previous studies[13]. The characterization of the synthesized methyl-substituted epoxyalkanes and all deuterium labelled compounds was carried out by NMR spectroscopy and GC/MS analysis. ^{1}H-NMR spectra were recorded on a Varian EM-390 90 MHz spectrometer and mass spectra on a Hewlett-Packard 5970 mass selective detector coupled to an HP 5890 gas chromatograph.

The protein content of cell extracts was determined by the method of Bradford[27], using bovine serum albumin as a standard.

Degradation of epoxyalkanes by cell extracts

Epoxyalkane-degradation by crude cell extracts of *Xanthobacter* Py2 was tested in 40 ml screw-cap bottles sealed with rubber septa. In standard assays, the bottles contained 4 ml crude cell extract (100 mg protein), 1 ml potassium phosphate buffer pH 8.2 supplemented with 10% (v/v) glycerol and NAD in a final concentration of 2 mM. Subsequently, the bottles were placed into a shaking waterbath of 38°C and the reaction was started by addition of the appropriate epoxyalkane to a final concentration of 3 mM. Periodically 100 µl headspace samples were taken and analyzed by gas chromatography in order to determine the initial degradation rates. Complexation GLC was performed in experiments where enantioselective degradations were studied. Concentrations of epoxyalkanes were derived from calibration curves using heat-denaturated cell extracts. In experiments with dialyzed extracts, the LMF, dithiothreitol (DTT) and other thiol compounds were added after 10 minutes equilibrium of the complete reaction mixture with epoxyalkane.

Accumulation of reaction products was followed by periodically analyzing headspace samples from the reaction mixture in analogy with the analysis of epoxyalkanes. Eventually, the composition of the reaction products was determined by GC/MS analysis.

Acknowledgements

This study was supported by a grant from the European Community (BIOT-CT91-0269). Cooperation and discussions through this EC-project with Dr. D.J. Leak and Prof.Dr. M.M.R. da Fonseca are greatly appreciated. Thanks are also due to Prof.Dr. C. Syldatk for his contribution to this work.

References

1. Weijers, C.A.G.M.; Haan de, A.; Bont de, J.A.M. Microbial production and metabolism of epoxides. *Microbiological Sciences* **1988**, *5*, 156-159.
2. Bont de, J.A.M. Bioformation of optically pure epoxides. *Tetrahedron: Asymmetry* **1993**, *4*, 1331-1340.
3. Leak, D.J.; Aikens, P.J.; Seyed-Mahmoudian, M. The microbial production of epoxides. *TIBTECH* **1992**, *10*, 256-261.
4. Hartmans, S.; Smits, J.P.; Werf van der, M.J.; Volkering, F.; Bont de, J.A.M. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Appl. Environm. Microbiol.* **1989**, *55*, 2850-2855.
5. Kasai, N.; Tsujimura, K; Unoura, K.; Suzuki, T. Degradation of 2,3-dichloro-1-propanol by a *Pseudomonas* sp. *Agric. Biol. Chem.* **1990**, *54*, 3185-3190.
6. Jacobs, M.H.J.; Wijngaard van den, A.J.; Pentenga, M.; Janssen, D.B. Characterization of the epoxide hydrolase from an epichlorohydrin-degrading *Pseudomonas* sp. *Eur. J. Biochem.* **1991**, *202*, 1217-1222.
7. Bont de, J.A.M.; Dijken van, J.P.; Ginkel van, C.G. The metabolism of 1,2-propanediol by the propylene oxide utilizing bacterium *Nocardia* A60. *Biochim. Biophys. Acta* **1982**, *714*, 465-470.
8. Hechtberger, P.; Wirnsberger, G.; Mischitz, M.; Klempier, N.; Faber, K. Asymmetric hydrolysis of epoxides using an immobilized enzyme preparation from *Rhodococcus* sp. *Tetrahedron: Asymmetry* **1993**, *4*, 1161-1164.
9. Pedragosa-Moreau, S.; Archelas, A.; Furstoss, R. Enantiocomplementary epoxide hydrolyses as a preparative access to both enantiomers of styrene oxide. *J. Org. Chem.* **1993**, *58*, 5533-5536.
10. Carter, S.F.; Leak, D.J. The isolation and genetic construction of cyclic epoxide degrading bacteria. *In*: *Abstracts European Symposium on Biocatalysis*, P-50, **1993**, Graz, Austria.
11. Bont de, J.A.M.; Harder, W. Metabolism of ethylene by *Mycobacterium* E20. *FEMS Microbiol. Lett.* **1978**, *3*, 89-93.
12. Ginkel van, C.G.; Welten, H.G.J.; Bont de, J.A.M. Epoxidation of alkenes by alkene-grown *Xanthobacter* spp. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 334-337.
13. Weijers, C.A.G.M.; Haan de, A.; Bont de, J.A.M. Chiral resolution of 2,3-epoxyalkanes by *Xanthobacter* Py2. *Appl. Microbiol. Biotechnol.* **1988**, *27*, 337-340.

14. Whitlon, D.S.; Sadowski, J.A.; Suttie, J.W. Mechanism of coumarin action: significance of vitamin K epoxide reductase inhibition. *Biochemistry* **1978**, *17*, 1371-1377.
15. Lee, J.J.; Fasco, M.J. Metabolism of vitamin K and vitamin K 2,3-epoxide via interaction with a common disulfide. *Biochemistry* **1984**, *23*, 2246-2252.
16. Cleland, W.W. Dithiothreitol, a new protective reagent for SH groups. *Biochemistry* **1964**, *3*, 480-482.
17. Torchinskii, Yu.M. The role of SH groups in enzymes. *In*: Torchinskii YuM (ed) *Sulfhydryl and Disulfide Groups of Proteins*. Consultants Bureau, London, **1974**, pp 185-198.
18. Ophem van, P.W.; Beeumen van, J.; Duine, J.A. NAD-linked, factor-dependent formaldehyde dehydrogenase or trimeric, zinc-containing, long-chain alcohol dehydrogenase from *Amycolatopsis methanolica*. *Eur. J. Biochem.* **1992**, *206*, 511-518.
19. Steckbeck, S.R.; Nelson, J.A.; Spencer, T.A. Enzymic reduction of an epoxide to an alcohol. *J. Am. Chem. Soc.* **1982**, *104*, 893-895.
20. Fasco, M.J.; Preusch, P.C.; Hildebrandt, E.; Suttie, J.W. Formation of hydroxyvitamin K by vitamin K epoxide reductase of warfarin-resistant rats. *J. Biol. Chem.* **1983**, *258*, 4372-4380.
21. Silverman, R.B. Chemical model studies for the mechanism of vitamin K epoxide reductase. *J. Am. Chem. Soc.* **1981**, *103*, 5939-5941.
22. Liptay-Reuter, I.; Dose, K.; Guenthner, T.; Wörner, W.; Oesch, F. Vitamin K epoxide reductase activity in the metabolism of epoxides. *Biochem. Pharmacol.* **1985**, *34*, 2617-262018.
23. Ginkel van, C.G.; Bont de, J.A.M. Isolation and characterization of alkene-utilizing *Xanthobacter* spp. *Arch. Microbiol.* **1986**, *145*, 403-407.
24. Pasto, D.J.; Cumbo, C.C. An improved method for the preparation of volatile epoxides. *J. Org. Chem.* **1965**, *30*, 1271-1272.
25. Dobson, N.A.; Eglinton, G.; Krishnamurti, M.; Raphael, R.A.; Willis, R.G. Selective catalytic hydrogenation of acetylenes. *Tetrahedron* **1961**, *16*, 16-24.
26. Schurig, V.; Bürkle, W. Extending the scope of enantiomer resolution by complexation gas chromatography. *J. Am. Chem. Soc.* **1982**, *104*, 7573-7580.
27. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.

4

Enantioselective hydrolysis of aryl, alicyclic and aliphatic epoxides by *Rhodotorula glutinis*

Abstract

Enantioselective epoxide hydrolysis by yeasts has been demonstrated for the hydrolysis of several aryl, alicyclic and aliphatic epoxides by a strain of Rhodotorula glutinis. *High enantioselectivity was obtained in the hydrolysis of disubstituted aryl and aliphatic epoxides whereas selectivity towards terminal epoxides in all cases was lower. Enantiopure vicinal diols were formed from several disubstituted epoxides and also from* meso *epoxides. Kinetic resolution of* trans-*1-phenyl-1,2-epoxypropane was studied in more detail.*

C.A.G.M. Weijers

Tetrahedron: Asymmetry **1997**, *8*, 639-647

Introduction

Enantiomerically pure epoxides are important chiral building blocks in organic synthesis and can be used as key intermediates in the synthesis of more complex enantiopure bioactive compounds. Therefore, a great interest exists in the development of methods for the synthesis of enantiopure epoxides. Various chemical and biological production methods have been reviewed[1,2]. Among biological production routes, enantioselective bioconversion might be a very promising method because epoxides with very high enantiomeric purities can be obtained and the enzymes involved seem to react with a homologous range of epoxides[3,4,5]. However, only limited information is available on the use of microbial enzymes in enantioselective bioconversion. Nevertheless, different types of reactions involved in microbial epoxide conversion are known[6]. Enantioselectivity in microbial epoxide conversion has been detected in the case of conversion of 1,2-epoxyalkanes by a *Nocardia* sp[7]. This organism and also a *Xanthobacter* sp contains an NAD-dependent enzyme which is able to resolve enantiomerically pure (2*R*)-epoxyalkanes from racemic 2,3-epoxyalkanes[8].

Hydrolytic enzymes[9] may be more promising as enantioselective biocatalysts, because they are cofactor independent and because the epoxide enantiomer degraded may be recovered as enantiomerically pure diol. Enantioselectivity of epoxide hydrolases of mammalian origin (MmEH and MsEH) has been studied extensively with special attention to the microsomal enzyme[5,10]. However, biocatalysts from microbial sources might be more applicable on an industrial scale and therefore studies on microbial enantioselective epoxide hydrolysis have been set up recently. An enantioselective epoxide hydrolase from a bacterial source has been observed in a *Rhodococcus* sp which showed selectivity in the hydrolysis of 2,2-disubstituted epoxides[11]. Deracemization of epoxides using bacterial epoxide hydrolases has subsequently been reported for the hydrolysis of *cis*-2,3-epoxyheptane by a *Nocardia* sp[12] and of 1-methyl-1,2-epoxycyclohexane by a *Corynebacterium* sp[13]. Fungal epoxide hydrolysis has been described for *Aspergillus niger* and *Beauvaria sulfurescens*. These two strains showed different enantio- and regioselectivities in the hydrolysis of styrene oxide[14] and several other substituted styrene oxide derivatives[15,16]. Based on hypothetical active site models it was proposed that the two fungal enzymes operate with different mechanisms for oxirane ring-opening. Other examples of fungal epoxide hydrolase activities have been reported for the enantioselective hydrolysis of indene oxide by *Diplodia gossipina*[17] and of aliphatic epoxides by *Ulocladium atrum* and *Zopfiella karachiensis*[18].

Recently, we have investigated the presence of enantioselective epoxide hydrolases in yeasts. These organisms are most promising because they are easy to cultivate which would make the production of epoxide hydrolases on a large scale more feasible. Enantioselective hydrolysis of various aryl, alicyclic and aliphatic epoxides by a strain of the yeast *Rhodotorula glutinis* has been observed.

Results and Discussion

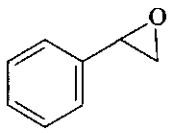
Scope of epoxide hydrolysis

Screening for epoxide hydrolase activity in yeasts was performed with styrene oxide and *trans*-1-phenyl-1,2-epoxypropane as potential substrates. These two aryl epoxides are known to be typical substrates for the microsomal (MmEH), and soluble (MsEH) mammalian epoxide hydrolase respectively[19,20]. We tested both substrates not knowing which type of enzyme was to be expected in yeasts. A number of yeast strains was tested for epoxide hydrolase activity and *Rhodotorula glutinis* strain CIMW 147 was eventually selected for further studies. *Rhodotorula glutinis* is able to hydrolyse both aryl epoxides tested, with relatively high activity and, in addition, the organism is very easy to cultivate. When grown on various simple carbon sources, the yeast possessed in all cases epoxide hydrolase activity which facilitates the production of large amounts of this biocatalyst. In the present study, *Rhodotorula glutinis* was routinely grown in a chemostat culture on a mineral medium supplemented with glucose as carbon source.

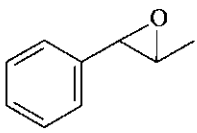
Substrate specificity and enantioselectivity of the yeast epoxide hydrolase was investigated by incubating epoxide (±)-**1** to (±)-**15** with resting cell suspensions of *Rhodotorula glutinis* (Scheme 1). The reactions were monitored by periodically taking samples and subsequent analysis by GLC using enantioselective columns.

In the hydrolysis of racemic epoxide **1** to **15**, the reaction was terminated when the residual epoxide reached an e.e. of more than 98%. Then the reaction time, yield and configuration of the epoxide and e.e. and configuration of the formed diol were determined. Reactions with *meso* epoxides were terminated when more than 98% conversion was reached. Initial hydrolysis rates, absolute configurations and yields of the residual epoxides **1** to **15** and e.e. values and absolute configurations of the formed diols **1a** to **15a** are summarized in Table 1. Absolute configurations of the diols were determined *via* prepared, or commercially available, compounds by co-injection on chiral GLC and, in specific cases, by comparison of the specific rotation values.

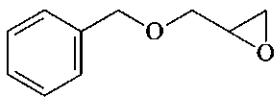
From the results of Table 1 it is concluded that the epoxide hydrolase from *Rhodotorula glutinis* has a broad substrate specificity because all tested epoxides, with the exception of epoxide **10**, are accepted by this enzyme. Nevertheless, among the accepted substrates there are significant differences in reaction rate and enantioselectivity, resulting in chiral epoxides and diols with different yield and e.e. values respectively. Terminal epoxides were, as expected, hydrolyzed with relatively low regioselectivity to (*R*) diols with retention of configuration at the more hindered carbon atom.



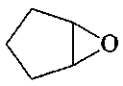
1



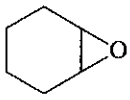
2



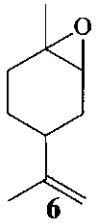
3



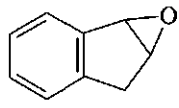
4



5

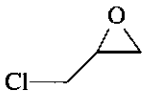


6

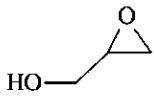


7

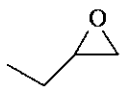
8



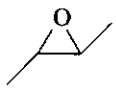
9



10

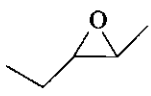


11

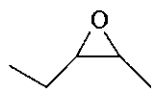


12

13



14



15

Scheme 1. Epoxides used as substrates for epoxide hydrolysis by *Rhodotorula glutinis*.

In the case of disubstituted epoxides the reaction proceeded with *inversion* of configuration at the more hindered carbon atom. Enantioselectivity in the second case was to such an extend that very high values of enantiomeric purity of the formed diol and of the yield of the residual epoxide were obtained.

Table 1. Hydrolysis of various epoxides by *Rhodotorula glutinis*

Epoxide (residual substrate)						Diol product		
	reaction rate[a]	e.e.	abs. conf.	yield	reaction time		e.e.	abs. conf.
1	6.5	> 98 %	(*S*)	18%	0.8	**1a**	48%	(*R*)
2	0.91	> 98 %	(1*R*,2*R*)	45%	1.3	**2a**	> 98%	(1*R*,2*S*)
3	12.7	> 98 %	(*R*)	14%	0.2	**3a**	33%	(*S*)
4	0.29	—	*meso*	< 2%	11.0	**4a**	> 98%	(1*R*,2*R*)
5	2.1	—	*meso*	< 2%	1.4	**5a**	90%	(1*R*,2*R*)
(-)-**6**	1.2	> 98%	(1*S*,2*R*,4*S*)	48%	1.2	(-)-**6a**	> 98%	(1*R*,2*R*,4*S*)
(+)-**6**	0.64	> 98%	(1*S*,2*R*,4*R*)	28%	3.2	(+)-**6a**	30%	(1*R*,2*R*,4*R*)
7	11.4[b]	> 98%	(1*R*,2*S*)	22%	0.2	**7a**	54%	n.d.
8	0.50	> 98%	(*S*)	15%	3.0	**8a**	30%	(*R*)
9	2.0	> 98%	(*R*)	10%	1.0	**9a**	22%	(*S*)
10	—	no epoxide hydrolysis		—	—	**10a**	—	—
11	1.4	> 98%	(*S*)	16%	0.8	**11a**	25%	(*R*)
12	0.07	> 98%	(2*R*,3*R*)	47%	18.3	**12a**	—[c]	*meso*
13	0.52	—	*meso*	< 2%	13.0	**13a**	90%	(2*R*,3*R*)
14	0.21	> 98%	(2*R*,3*R*)	48%	3.4	**14a**	54%	(2*S*,3*R*)
15	1.2	> 98%	(2*R*,3*S*)	48%	0.6	**15a**	> 98%	(2*R*,3*R*)

a) Initial rate of epoxide hydrolysis in nmol/min, mg dw

b) Under the present reaction conditions there was also significant chemical hydrolysis of epoxide **7**

c) > 98 % *cis* diol

n.d. not determined

Hydrolysis of 10 mM epoxide in 20 ml reaction mixture with glucose-grown cells of *Rhodotorula glutinis* (dry weights ranging from 1.0 to 1.5 g).

Experiments for epoxide hydrolysis were routinely performed under aerobic conditions resulting in the data presented in Table 1. Under these conditions, however, it was observed that in the hydrolysis of some of the short-chain aliphatic epoxides **8** to **15** small amounts of the formed diols were subsequently oxidized to the corresponding α-hydroxy

keto compounds. Fortunately, it was found that partial oxidation of vicinal diols by one or more oxidoreductases from *Rhodotorula glutinis* could be minimized under anaerobic conditions. No oxidation of the more complex diols **1a** to **7a** was detected in either reaction condition.

The aryl epoxides **1**, **2**, and **3** appeared to be very good substrates for the epoxide hydrolase of *Rhodotorula glutinis* with maximum activity for benzyl glycidyl ether **3**. From the reaction rates determined for the various epoxides it can be concluded that the epoxide hydrolase has a preference for phenyl group containing substrates. Relatively high activity combined with high enantioselectivity was found for the hydrolysis of the *trans*-methyl substituted styrene oxide derivative **2**. Furthermore, biocatalytic preparation of this compound will be of interest because chemical synthesis using recently developed selective catalysts[21,22] is still less satisfactory. For these reasons kinetic resolution of epoxide **2** was eventually selected to be studied in more detail.

Alicyclic epoxides **4** to **7** were all hydrolyzed to (1*R*,2*R*) diols with relatively high reaction rates. The hydrolyses of the *meso* epoxides **4** and **5** are of special interest because in these cases enantiopure *trans* diols can be obtained possibly in 100% theoretical yield. In this context, our results obtained for diol (1*R*,2*R*)-**4a** (>98% e.e. and 98% yield) and diol (1*R*,2*R*)-**5a** (90% e.e. and 93% yield) are rather encouraging. For the hydrolysis of substituted alicyclic epoxides, the (4*S*)-(-) and (4*R*)-(+) isomers of limonene oxide **6** were selected. It was observed that the position of the sidechain at the stereogenic C4 carbon atom is of great influence on the activity and enantioselectivity. Very high selectivity was found when the alkylsubstituent was in the (4*S*) configuration, leading to the single trans-(1*R*,2*R*,4*S*)-**6a** diol and 48% yield of epoxide (1*S*,2*R*,4*S*)-**6**. With the sidechain of epoxide **6** in the (4*R*) configuration a much lower activity and selectivity were obtained, resulting in a low yield for the residual epoxide. The diol formed was in this case a mixture of 65% (1*R*,2*R*,4*R*)-**6a** and 35% (1*S*,2*S*,4*R*)-**6a**. A similar effect of the orientation of the sidechain has been observed in the hydrolysis of limonene oxide by rabbit MmEH[23]. The mammalian enzyme, however, showed a preference for the two other enantiomers of (-)- and (+)-limonene oxide. Indene oxide **7** was chosen as another example of a substituted alicyclic epoxide. Enantiomerically pure indene oxide is of commercial interest because it is a precursor to a side chain of a specific HIV protease inhibitor. In our experiments for biocatalytic resolution of epoxide **7** we observed, however, that the reaction was strongly hampered by the chemical instability of this compound. As a result, the yield of (1*R*,2*S*)-**7** as well as the e.e. of diol-**7a** were unsatisfactory. At the moment, experiments for enhancement the stability of indene oxide by use of organic solvents are in progress.

The terminal aliphatic epoxides **8**, **9**, and **11** were found to be poor substrates for the yeast epoxide hydrolase. Activity and enantioselectivity for these compounds was relatively low and was slightly increased by increasing length of the side chain. Glycidol **10** was not hydrolyzed at all by the yeast epoxide hydrolase, nor at lower concentrations of 5 and 1 mM.

Activity for disubstituted 2,3-epoxides, represented by substrates **12** to **15**, was low in the case of *trans* substituted side chains and higher when the side chains were *cis* situated. Enantioselectivity was very high for the epoxides **12**, **14** and **15** resulting in residual (2*R*) epoxides with nearly the maximal feasible yields of 50%. Hydrolysis of the *meso* epoxide **13** was somewhat unsatisfactory concerning the relatively low yield of 78% of diol product **13a**. This was most probably caused by some subsequent oxidation of the diol since significant amounts of 3-hydroxy-2-butanone were detected in the final reaction mixture.

Kinetic resolution of epoxide-2

A detailed study of the resolution of epoxide (1*R*,2*R*)-**2** was performed by monitoring the course of the e.e. values of (1*R*,2*R*)-**2** in relation to the proceeding conversion in the hydrolysis of (±)-**2**. In Figure 1 it is shown that a complete kinetic resolution of (1*R*,2*R*)-**2** (e.e. >98%) is achieved at a conversion of 55% which is a rather encouraging result for a resolution process. During the initial phase of the reaction enantiomerically pure (1*R*,2*S*)-**2a** was found to be the product of the hydrolysis of (±)-**2** and was even detected slightly above 50% conversion, indicating that this diol was also formed in some very small amounts from (1*R*,2*R*)-**2**. From Figure 1 it is also obvious that at conversion values of 60% or more the decrease of e.e.'s seems to be dramatic. However, it must be considered that resolution of the initial reaction mixture takes place in about one hour whereas the final part of the reaction is still not completed after more than 40 hours. In this period, chemical hydrolysis of epoxides may be significant. Formation of other diol enantiomers was investigated by monitoring the chemical hydrolysis in the final part of the reaction. For this, the yeast cells were separated from the reaction mixture after complete hydrolysis of (1*S*,2*S*)-**2** after which the incubation of the reaction mixture was continued without cells. In Figure 1 it is shown that the loss of enantiomeric purity of (1*R*,2*S*)-**2a** in the reaction mixture is mainly caused by formation of the other diol enantiomers by chemical hydrolysis of the residual (1*R*,2*R*)-**2**.

Enantioselective hydrolysis of *trans*-1-phenyl-1,2-epoxypropane has been studied before with epoxide hydrolases from other sources. In a study on the substrate enantioselectivity of the rabbit microsomal epoxide hydrolase (MmEH) only a low selectivity in favor of epoxide (1*S*,2*S*)-**2** has been observed, resulting in epoxide (1*R*,2*R*)-**2** and diol (1*S*,2*R*)-**2a** both with low enantiopurity and low yield[20]. Better results were obtained with an epoxide hydrolase from the fungus *Beauvaria sulfurescens*. It has been reported that with this organism resolution of (±)-**2** is possible resulting in epoxide (1*R*,2*R*)-**2** (e.e. 98%, yield 30%) and diol (1*R*,2*S*)-**2a** (e.e. 90%, yield 38%)[15]. The results from the present study

indicate the yeast *Rhodotorula glutinis* performs even better than the fungus.

Epoxide hydrolases, in some cases showing high enantioselectivity, have been reported from mammalian sources[4,5,9], plants[24] and from fungal[15,17,18] and bacterial[11,12,13] microorganisms. The pool of sources of these useful biocatalytic enzymes has now been extended with the yeast *Rhodotorula glutinis*. By further research on the yeast enzyme and other epoxide hydrolases a better selection for a specific enzyme, based on characteristics of both enzyme as well as of source organism, will be made possible.

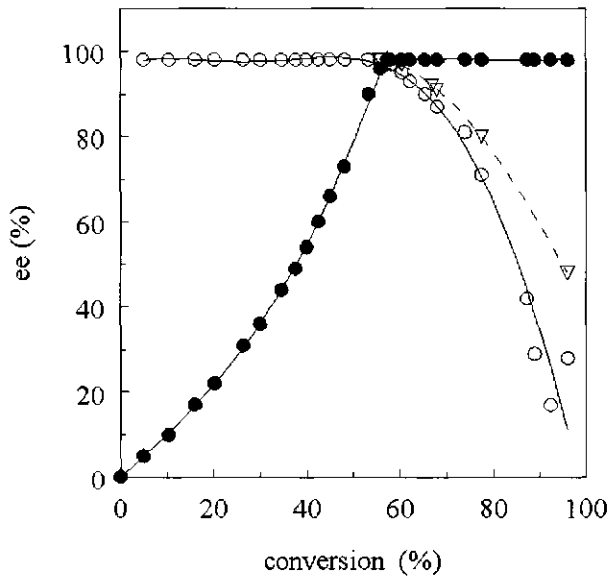


Figure 1. Resolution of 10 mM *trans*-1-phenyl-1,2-epoxypropane by *Rhodotorula glutinis* (2.3 g weight of dry cells in 50 ml). (●) Epoxide (1*R*,2*R*)-**2**; (○) diol (1*R*,2*S*)-**2a**; (▽) diol (1*R*,2*S*)-**2a** by chemical hydrolysis of residual (1*R*,2*R*)-**2** (dotted line).

Conclusions

Enantioselective epoxide hydrolysis by yeasts has been first demonstrated for the hydrolysis of several aryl, alicyclic and aliphatic epoxides by cells of *Rhodotorula glutinis*. By use of this biocatalyst enantiomerically pure forms of disubstituted aryl and aliphatic epoxides as well as the corresponding diols can be prepared. Diol formation was also promising in the hydrolysis of (-)-limonene oxide and of alicyclic *meso* epoxides. At the moment, experiments for enhancement of the hydrolase activity and isolation and characterization of the epoxide hydrolase from *Rhodotorula glutinis* are in progress.

Experimental Section

General

Gas chromatography (GLC) was performed on Chrompack CP9000 and Hewlett-Packard 6890 gaschromatographs equipped with FID detectors and using N_2 as carrier gas. Determination of the enantiomeric excesses was performed by GLC using fused silica cyclodextrin capillary columns (30 m length, 0.25 mm ID and 0.25 μm film thickness). For epoxide **1**, **2**, **6** and **7** an α-DEX 120 column (Supelco Inc.) was used at an oven-temperature of 85°C, 90°C, 80°C and 110°C respectively. Enantiomer analysis of epoxide **3** and **10** was done at a β-DEX 120 column (Supelco Inc.) at oventemperatures of 110°C and 60°C. Complexation gas chromatography[25,3] with a Chirametal-27-*R*-3-1-13 column was used at an oven temperature of 55°C to determine the enantiomeric composition of epoxide **8**, **9** and **11** to **15**. Chiral GLC analysis for diol **3a** and **7a** was performed on a β-DEX 120 column at an oven temperature of 145°C after derivatization into their acetonides. Enantiomer analysis of the other diol compounds was carried out by direct analysis on a β-DEX 120 column at oven temperatures ranging from 70°C to 145°C. Quantification of epoxides and diols was by GLC analysis using the respective capillary columns and conditions as for determination of e.e.'s. The concentrations of epoxides and diols were derived from calibration curves. Mass spectra of diol compounds were recorded on a Hewlett-Packard 5970 mass selective detector coupled to an HP 5890 gas chromatograph and were in accordance with the proposed structures. Optical rotation values were measured on a Perkin-Elmer 241 polarimeter at 589 nm.

Epoxides 1 to 15

The commercially available racemic and *meso* substrates cyclopentene oxide **4**, cyclohexene oxide **5**, *cis/trans*-(4*S*)-limonene oxide **6**, (±)-1,2-epoxybutane **11**, (±)-*trans*-2,3-epoxybutane **12** and *cis*-2,3-epoxybutane **13** were all obtained from Aldrich Chemie. Several of the other epoxides were available in enantiomeric pure form as well: (1*R*,2*R*)- and (1*S*,2*S*)-*trans*-1-Phenyl-1,2-epoxypropane **2** (Aldrich), (*S*)- and (*R*)-Benzyl glycidyl ether **3** (Fluka), (1*R*,2*S*,4*R*)- and (1*S*,2*R*,4*R*)-limonene oxide **6** (Fluka), (*R*)- and (*S*)-1,2-epoxypropane **8** (Fluka), (*S*)- and (*R*)-epichlorohydrin **9** (Fluka) and (*R*)- and (*S*)-glycidol **10** (Aldrich).

(±)-Indene oxide **7** was synthesized by cyclization of the indene bromohydrin[26]. To 75 ml 13N KOH solution 12.5 g *trans*-2-bromo-1-indanol (Aldrich) was added. After stirring for 1 hour at roomtemperature, the reaction mixture was extracted three times with ethyl acetate. The organic extracts were combined, washed with water and dried over $MgSO_4$. Evaporation under reduced pressure yielded 7.2 g of a white solid of epoxide **7**.

The structure of epoxide **7** was characterized by GC/MS analysis.

(±)-*trans*-2,3-Epoxypentane **14** and (±)-*cis*-2,3-epoxypentane **15** were prepared by direct epoxidation of the corresponding *trans*- and *cis*-2-pentene using *m*-chloroperoxybenzoic acid (MCPBA) as described in our previous studies[3,8].

*Reference compounds **1a** to **15a***

For identification and determination of the absolute configurations and yields of the formed diols from epoxides **1** to **15**, the corresponding reference diol compounds were purchased or prepared. Commercially available were the enantiomerically pure forms of diol **1a** (Fluka), **3a** (Fluka), **4a** (Fluka), **5a** (Fluka), **8a** (Fluka), (*R*)-**9a** (Fluka), **10a** (Aldrich), **11a** (Aldrich), **12a** (Aldrich) and **13a** (Fluka).

A reference compound of enantiopure (1*R*,2*S*)-**2a** was prepared according to the reported method of microbial asymmetric reduction of 1-phenyl-1,2-propanedione with *Saccharomyces cerevisiae*[27]. By using this method with *Saccharomyces cerevisiae* CBS 1394 (from Centraalbureau voor Schimmelcultures, Yeast Division, The Netherlands), 38 mg (1*R*,2*S*)-**2a** was obtained from 70 mg 1-phenyl-1,2-propanedione (Fluka Chemie). The structure of the reference compound (1*R*,2*S*)-**2a** was confirmed by GC/MS analysis and the enantiomeric excess and specific optical rotation were determined as 90% e.e. and $[\alpha]^{24}_{D}$ = - 36.3 (c = 1.9, $CHCl_3$) respectively. Chiral GLC analysis with an β-DEX 120 column at 145°C resulted in a major peak at Rt = 33.8 min (95%) and a minor peak at Rt = 30.8 min (5%).

trans-8-*p*-Menthene-1,2-diols **6a** were prepared by hydrolysis of the corresponding epoxides[28]. For this, a solution of 150 mg epoxide in 10 ml of 0.1 M $HClO_4$ in 1:9 (v/v) dioxane-water was stirred for 5 minutes at room temperature. After addition of 2 ml water, the diols were extracted twice with ethyl acetate. The ethyl acetate extracts were combined, washed with 2 ml of water, and dried over $MgSO_4$. Removal of the solvent yielded about 90% diol compound. In this way, (1*R*,2*R*,4*R*)-**6a** was prepared from (1*R*,2*S*,4*R*)-**6** and (1*S*,2*S*,4*R*)-**6a** was the minor hydration product of (1*S*,2*R*,4*R*)-**6** respectively. Diol (1*R*,2*R*,4*S*)-**6a** was prepared from (1*R*,2*S*,4*S*)-**6**.

(±)-*trans*-Indan-1,2-diol **7a** was prepared by a reported method[29] from indene oxide **7**. A solution of 1.0 g epoxide **7** in 100 ml 2N KOH was heated to 102°C for 4 hours. Subsequently, the reaction mixture was saturated with NaCl, extracted with ethyl acetate, dried over $MgSO_4$ and evaporated under reduced pressure yielding 0.43 g of compound **7a**.

Acid-catalyzed epoxide hydrolysis was used in the synthesis of *erythro* (±)-2,3-pentanediol **14a** from *trans* **14** and *threo* (±)-2,3-pentanediol **15a** from *cis* **15** respectively. After derivatization of the diols into their acetonides, determination of the absolute configurations was performed by complexation gas chromatography[30] using a Ni(II)-*bis*-(3-heptafluorobutyryl-(1*R*,2*S*)-pinan-4-onate column at 75°C. The obtained peak elution

orders were used for correlation to those obtained after direct analysis on a β-DEX 120 column at 75°C.

Derivatization of diols into their acetonides

Samples of 1.0 ml of diol-containing reaction mixture or 10 mM reference solution, were saturated with NaCl and subsequently extracted with an equal amount of 2,2-dimethoxypropane. To 0.5 ml of the organic phase, 200 mg Amberlite IR-120 H^+ (BDH, UK) was added and the mixture was stirred for an hour at room temperature. After neutralization with approximately 200 mg $NaHCO_3$, the mixture was extracted with 1.0 ml ethyl acetate and dried over $MgSO_4$. Analysis of the prepared acetonides was by chiral GLC.

Absolute configuration of diol ***2a***

Absolute configurations were determined of the diols formed from hydrolysis of 0.50 mmol of epoxide (±)-**2**, (1*R*,2*R*)-**2** and (1*S*,2*S*)-**2** by *Rhodotorula glutinis* cells. Experiments for epoxide hydrolysis were carried out as described in this paper. By monitoring the duplicate reaction bottle, the moment for terminating the reaction of each epoxide was determined. The reactions were stopped by removal the yeast cells by centrifugation (20,000 *g*, 10 minutes). The supernatants were saturated with NaCl and subsequently extracted twice with an equal volume of ethyl acetate containing decane as an internal standard. GLC analysis was performed for determination the yields and e.e. values of the formed diols.

The combined organic layers from the respective reaction mixtures were dried over $MgSO_4$ and evaporated under reduced pressure to give each an oily residu. For measurement of the specific optical rotation values the diols were redissolved in $CHCl_3$. Data of chiral GLC analysis (Rt's on ß-DEX 120 column at 145°C) and specific optical rotation values are:

diol **2a** from (±)-**2**: Rt = 33.8 min; $[\alpha]^{24}_D$ = - 31.6 (c = 1.2, $CHCl_3$)

diol **2a** from (1*R*,2*R*)-**2**: Rt = 30.8 and 32.7 min; $[\alpha]^{24}_D$ = + 8.8 (c = 0.16, $CHCl_3$)

diol **2a** from (1*S*,2*S*)-**2**: Rt = 33.8 min; $[\alpha]^{24}_D$ = - 32.8 (c = 0.6, $CHCl_3$).

Determination of the absolute configurations was by comparison of the results with data from literature[27,31] and from the prepared reference compound of (1*R*,2*S*)-**2a**.

Growth conditions Rhodotorula glutinis

The yeast *Rhodotorula glutinis* strain CIMW 147 was obtained from our own laboratory culture collection. A mineral medium supplemented with 0.2% (w/v) yeast extract and

1% (w/v) glucose was used for cultivation. In the present study, *R. glutinis* was routinely grown in a chemostat culture under aerobic conditions in a 2-l fermentor (with 1-l working volume) at 30°C, with a dilution rate of 0.15 h^{-1}. The pH of the culture was maintained at 6.0. The cells were harvested by centrifugation at 16,000 *g*, washed twice with 50 mM potassium phosphate buffer pH 7.5, concentrated, and stored at -20°C.

Epoxide hydrolysis by Rhodotorula glutinis

Hydrolysis of epoxides was performed in 100 ml screw-capped bottles sealed with rubber septa. The bottles contained 10 ml concentrated washed cell suspension of *Rhodotorula glutinis* (1.0 to 1.5 g dry weight) and 50 mM potassium phosphate buffer pH 7.5 to a total volume of 20 ml. The bottles were placed into a shaking waterbath at 35°C and the reaction was started by addition of 0.20 mmol epoxide. The course of the epoxide hydrolysis was followed by periodically taking samples from the reaction mixture, centrifuging for 4 minutes at 15.000 *g*, extracting 0.5 ml supernatants with 1.0 ml ethyl acetate and analysis by chiral GLC. Diols were analyzed similarly, however, extraction with ethyl acetate was after saturation of the supernatants with NaCl. Chiral GLC of epoxide **8**, **9**, **11** and **12** to **15** was performed by analysis of headspace samples taken from the reaction mixture[3]. Initial reaction rates were determined from the epoxide disappearance and correlated to the dry weight of the used yeast suspension. In general, reactions were terminated when the residual epoxides reached e.e.'s of more than 98%.

The detailed kinetic resolution of (±)-**2** was performed in 250 ml screw-capped bottles with 25 ml concentrated washed cells, 0.50 mmol epoxide and phosphate buffer to a total volume of 50 ml. Chemical hydrolysis of residual epoxide from (±)-**2** was tested by removal of the yeast cells by centrifugation (20,000 *g*, 10 minutes) from the reaction mixture after complete hydrolysis of (1*S*,2*S*)-**2**. Subsequently, the obtained clear supernatant was further incubated at 35°C. Chemical hydrolysis was determined by monitoring the diol formation and epoxide conversion in the supernatant without cells. Eventually, four enantiomers of diol **2a** were detected in the final part of the reaction. There was no significant difference in the relative ratio of the four enantiomers in the final reaction mixtures with and without cells (4:9:21:66, ratio of peaks with ascending elution order by chiral GLC). A detailed course of the kinetic resolution of (±)-**2** is shown in Figure 1.

Acknowledgements

The author is indebted to Prof. Dr. Ir. J.A.M. de Bont for critically reading the manuscript. Thanks are also due to Mr. M. de Wit (Div. of Industrial Microbiology, WU) and Mr. H.J. Swarts (Dept. of Organic Chemistry, WU) for advice and assistance in the use of various analytical techniques and synthesis of reference compounds.

References

1. Bont de, J.A.M. Bioformation of optically pure epoxides. *Tetrahedron: Asymmetry* **1993**, *4*, 1331-1340.
2. Besse, P.; Veschambre, H. Chemical and biological synthesis of chiral epoxides. *Tetrahedron* **1994**, *50*, 8885-8927.
3. Weijers, C.A.G.M.; Haan de, A.; Bont de, J.A.M. Chiral resolution of 2,3-epoxyalkanes by *Xanthobacter* Py2. *Appl. Microbiol. Biotechnol.* **1988**, *27*, 337-340.
4. Bellucci, G.; Chiappe, C.; Ingrosso, C.; Rosini, C. Kinetic resolution by epoxide hydrolase catalyzed hydrolysis of racemic methyl substituted methylenecyclohexene oxides. *Tetrahedron: Asymmetry* **1995**, *6*, 1911-1918.
5. Bellucci, G.; Chiappe, C.; Cordoni, A. Enantioconvergent transformation of racemic cis-ß-alkyl substituted styrene oxides to (*R,R*) threo diols by microsomal epoxide hydrolase catalysed hydrolysis. *Tetrahedron: Asymmetry* **1996**, *7*, 197-202.
6. Weijers, C.A.G.M.; Haan de, A.; Bont de, J.A.M. Microbial production and metabolism of epoxides. *Microbiological Sciences* **1988**, *5*, 156-159.
7. Weijers, C.A.G.M.; Bont de, J.A.M. Enantioselective degradation of 1,2-epoxyalkanes by *Nocardia* H8. *Enzyme Microb. Technol.* **1991**, *13*, 306-308.
8. Weijers, C.A.G.M.; Jongejan, H.; Franssen, M.C.R.; Groot de, Ae.; Bont de, J.A.M. Dithiol- and NAD-dependent degradation of epoxyalkanes by *Xanthobacter* Py2. *Appl. Microb. Biotechnol.* **1995**, *42*, 775-781.
9. Arand, M.; Grant, D.F.; Beetham, J.K.; Friedberg, T.; Oesch, F.; Hammock, B.D. Sequence similarity of mammalian epoxide hydrolases to the bacterial haloalkane dehalogenase and other related proteins. Implication for the potential catalytic mechanism of enzymatic epoxide hydrolysis. *FEBS Letters* **1994**, *338*, 251-256.
10. Bellucci, G.; Chiappe, C.; Conti, L.; Marioni, F.; Pierini, G. Substrate enantioselection in the microsomal epoxide hydrolase catalyzed hydrolysis of monosubstituted oxiranes. Effects of branching of alkyl chains. *J. Org. Chem.* **1989**, *54*, 5978-5983.
11. Mischitz, M.; Kroutil, W.; Wandel, U.; Faber, K. Asymmetric microbial hydrolysis of epoxides. *Tetrahedron: Asymmetry* **1995**, *6*, 1261-1272.
12. Kroutil, W.; Mischitz, M.; Plachota, P.; Faber, K. Deracemization of (±)-*cis*-2,3-epoxyheptane via enantioconvergent biocatalytic hydrolysis using *Nocardia* EH1 epoxide hydrolase. *Tetrahedron Letters* **1996**, *37*, 8379-8382.
13 Archer, I.V.J.; Leak, D.; Widdowson, D.A. Chemoenzymic resolution and deracemisation of (±)-1-methyl-1,2-epoxycyclohexane: the synthesis of (1*S*,2*S*)-1-methylcyclohexane-1,2-diol. *Tetrahedron Letters* **1996**, *37*, 8819-8822.
14. Pedragosa-Moreau, S.; Archelas, A.; Furstoss, R. Microbiological transformations XXIX. Enantioselective hydrolysis of epoxides using microorganisms: a mechanistic study. *Bioorg. Med. Chem.* **1994**, *2*, 609-616.
15. Pedragosa-Moreau, S.; Archelas, A.; Furstoss, R. Microbiological transformations 32. Use of epoxide hydrolase mediated biohydrolysis as a way to enantiopure epoxides and vicinal diols: application to substituted styrene oxide derivatives. *Tetrahedron* **1996**, *52*, 4593-4606.

16. Pedragosa-Moreau, S.; Morisseau, C.; Zylber, J.; Archelas, A.; Baratti, J.; Furstoss, R. Microbiological transformations 33. Fungal epoxide hydrolases applied to the synthesis of enantiopure *para*-substituted styrene oxides. A mechanistic approach. *J. Org. Chem.* **1996**, *61*, 7402-7407.
17. Zhang, J.; Reddy, J.; Roberge, C.; Senanayake, C.; Greasham, R.; Chartrain, M. Chiral bioresolution of racemic indene oxide by fungal epoxide hydrolases. *J. Ferm. Bioeng.* **1995**, *80*, 244-246.
18. Grogan, G.; Roberts, S.M.; Willetts, A.J. Novel aliphatic epoxide hydrolase activities from dematiaceous fungi. *FEMS Microbiol. Lett.* **1996**, *141*, 239-243.
19. Ota, K.; Hammock, B.D. Cytosolic and microsomal epoxide hydrolases: differential properties in mammalian liver. *Science* **1980**, *207*, 1479-1480.
20. Bellucci, G.; Chiappe, C.; Gordoni, A.; Marioni, F. Substrate enantioselectivity in the rabbit liver microsomal epoxide hydrolase catalyzed hydrolysis of *trans* and *cis* 1-phenylpropene oxides. A comparison with styrene oxide. *Tetrahedron: Asymmetry* **1993**, *4*, 1153-1160.
21. Zhang, W.; Loebach, J.L.; Wilson, S.R.; Jacobsen, E.N. Enantioselective epoxidation of unfunctionalized olefins catalyzed by (salen)manganese complexes. *J. Am. Chem. Soc.* **1990**, *112*, 2801-2803.
22. Konishi, K.; Oda, K.; Nishida, K.; Aida, T.; Inoue, S. Asymmetric epoxidation of olefins catalyzed by manganese complexes of chiral "strapped" porphyrins with diastereotopic faces. A novel strategy for stereochemical modeling of the active site of cytochrome P-450. *J. Am. Chem. Soc.* **1992**, *114*, 1313-1317.
23. Bellucci, G.; Berti, G.; Ingrosso, G.; Mastrorilli, E. Stereoselectivity in the epoxide hydrase catalysed hydrolysis of the stereoisomeric 4-tert-butyl-1,2-epoxycyclohexanes. *J. Org. Chem.* **1980**, *45*, 299-303.
24. Blee, E.; Schuber, F. Stereocontrolled hydrolysis of the linoleic acid monoepoxide regioisomers catalyzed by soybean epoxide hydrolase. *Eur. J. Biochem.* **1995**, *230*, 229-234.
25. Schurig, V.; Bürkle, W. Extending the scope of enantiomer resolution by complexation gas chromatography. *J. Am. Chem. Soc.* **1982**, *104*, 7573-7580.
26. Gagis, A.; Fusco, A; Benedict, J.T. A stereochemical study of the ring opening of indene oxide by benzoic acid. *J. Org. Chem.* **1972**, *37*, 3181-3183.
27. Takeshita, M.; Sato, T. Synthesis of optically active 1-phenyl-1,2-propanediol by use of baker's yeast. *Chem. Pharm. Bull.* **1989**, *37*, 1085-1086.
28. Royals, E.E.; Leffingwell, J.C. Reactions of limonene-1,2-oxides. I The stereospecific reactions of the (+)-*cis*- and (+)-*trans*-limonene-1,2-oxides. *J. Org. Chem.* **1966**, *31*, 1937-1944.
29. Balsamo, A.; Berti, G.; Crotti, P.; Ferretti, M.; Macchia, B.; Macchia, F. The "anomalous" steric course of ring opening reactions of indene oxide. A reexamination. *J. Org. Chem.* **1974**, *39*, 2596-2598.
30. Schurig, V.; Wistuba, D. Analytical enantiomer separation of aliphatic diols and acetals by complexation gas chromatography. *Tetrahedron Letters* **1984**, *25*, 5633-5636.
31. Takeshita, M.; Yaguchi, R.; Akutsu, N. Enzymatic preparation of chiral 1-phenylglycidols and 1-phenyl-1,2-propanediols. *Tetrahedron: Asymmetry* **1992**, *3*, 1369-1372.

5

Enantioselective hydrolysis of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*

Abstract

Epoxide hydrolase catalysed resolution of aliphatic terminal epoxides has been demonstrated for the hydrolysis of a homologous range of unbranched 1,2-epoxyalkanes by the yeast Rhodotorula glutinis. *Both enantioselectivity and reaction rate were strongly influenced by the chain length of the epoxide used. Enantioselectivity showed an optimum in the hydrolysis of 1,2-epoxyhexane* (E = *84). Resolution of (±)-1,2-epoxyhexane resulted in* (S)*-1,2-epoxyhexane (e.e. > 98%, yield = 48%) and* (R)*-1,2-hexanediol (e.e. = 83%, yield = 47%).*

C.A.G.M. Weijers, A.L. Botes, M.S. van Dyk and J.A.M. de Bont

Tetrahedron: Asymmetry **1998**, *9*, 467-473

Introduction

A great interest exists in the development of methods for the synthesis of enantiomerically pure epoxides, because they are important chiral building blocks in the preparation of more complex enantiopure bioactive compounds. Various chemical and biological production methods have been reviewed[1,2]. In biological production methods, special attention has been given to terminal aliphatic epoxides because of their relatively high chemical stability in water-containing reaction mixtures. Furthermore, the absence of reactive side groups other than the oxirane ring, in most cases excludes undesired side-reactions while using crude enzyme preparations or whole cell biocatalysts.

Most studies on the biological production of enantiopure aliphatic epoxides involved direct epoxidation of alkenes by mono-oxygenase containing bacterial cells[3,4,5]. In all cases, aliphatic 1,2-epoxides were produced either as a racemic mixture or with the *(R)* configuration in excess. The method, however, was problematic due to product toxicity[6]. Resolution of several aliphatic 1,2-epoxides has been observed in a previous study for non-hydrolytic epoxide conversions by *Nocardia* H8[7]. However, because of insufficient enantioselectivities, the yields of the obtained enantiopure *(S)* epoxides were low.

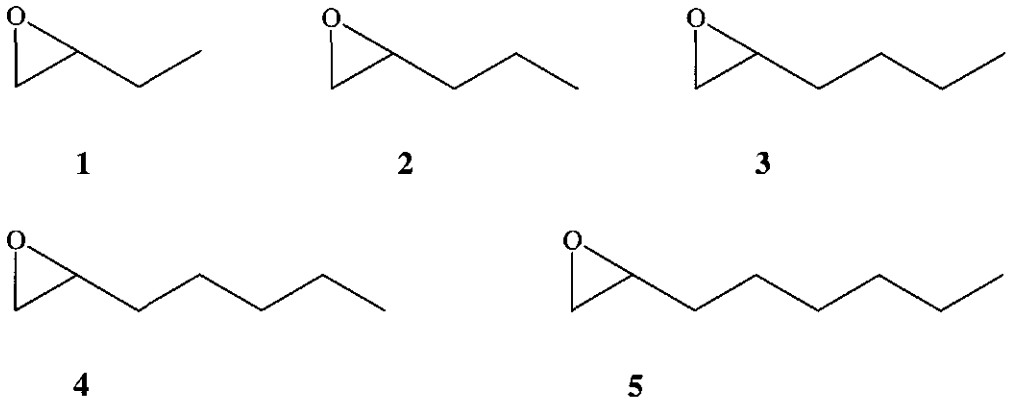
Epoxide hydrolase catalyzed hydrolysis of aliphatic 1,2-epoxides has been studied with biocatalysts from different sources. Mammalian microsomal epoxide hydrolase (MmEH) was used for the hydrolysis of 1,2-epoxyhexane and 1,2-epoxydecane[8]. However, very low enantioselectivities were observed and the formed diols were of low enantiomeric purity, even at very low conversions. Fungal epoxide hydrolase activities towards aliphatic 1,2-epoxides has been reported for the hydrolysis of C_6 to C_{14} 1,2-epoxyalkanes by crude cell extracts from *Ulocladium atrum* and *Zopfiella karachiensis*[9]. Activities were maximal for C_8 and C_{10} epoxides but, enantioselectivities were not given. Bacterial epoxide hydrolase activities were tested in a study for the resolution of 1,2-epoxyoctane with cell suspensions of the genera *Rhodococcus*, *Nocardia* and *Mycobacterium*[10]. From this screening it was concluded that the enantioselectivities of all biocatalysts tested were very low for the hydrolysis of this substrate ($E < 6$) .

Recently, we have investigated the presence of enantioselective epoxide hydrolases in yeasts. Enantioselective hydrolysis of various aryl, alicyclic and aliphatic epoxides by a strain of the yeast *Rhodotorula glutinis* has been demonstrated[11]. We have now extended our study by investigating substrate specificities and enantioselectivities for a homologous range of C_4 to C_8 aliphatic 1,2-epoxides.

Results and Discussion

Substrate specificity and enantioselectivity of the yeast epoxide hydrolase towards aliphatic 1,2-epoxides was investigated by incubating epoxides (±)-**1** to (±)-**5** with rest-

ing cell suspensions of glucose-grown *Rhodotorula glutinis* (Scheme 1). The reactions were monitored by periodic sampling and followed by GLC analysis using enantioselective columns. The present study was initiated by optimizing the reaction conditions for epoxide **1**. In comparison with experiments performed in our previous study[11], we have now raised the substrate concentration to a final concentration of 20 mM and lowered the biocatalyst concentration to maximal 0.5 grams dry weight per 10 ml reaction mixture. Under these conditions the reaction rate as well as the enantioselectivity for epoxide **1** were improved (Table 1). The optimized reaction conditions were used in subsequent experiments for the hydrolysis of epoxides (±)-**2** to (±)-**5**.



Scheme 1: Aliphatic 1,2-epoxides used as substrates for hydrolysis by *Rhodotorula glutinis*

In the hydrolysis of racemic epoxides **1** to **5**, the reaction was terminated when the residual epoxide reached an e.e. of more than 98%. The reaction time, yield of the epoxide and e.e. and yield of the formed diol were then determined. Initial hydrolysis rates, absolute configurations, e.e. values and yields of the residual epoxides and of the formed diols are summarized in Table 1. Absolute configurations of the epoxides and diols were determined by co-injection of commercially available reference compounds on chiral GLC and by comparison of the specific rotation values with data from the literature.

From the results summarized in Table 1 it is evident that the epoxide hydrolase from *Rhodotorula glutinis* has a preference for substrates with a chain length of six carbon atoms and more. The reaction rates for epoxides **3**, **4** and **5** are very much higher than those for epoxide **1** and **2**. The high enantioselectivity observed for hydrolysis of epoxide (±)-**4** and, in particular, for epoxide (±)-**3** is remarkable. Resolution of unbranched aliphatic terminal epoxides with moderate or high selectivities has not been reported for epoxide hydrolases from other sources.

Table 1. Hydrolysis of linear-chain aliphatic 1,2-epoxides by *Rhodotorula glutinis*

Epoxide (residual substrate)						Diol product			
	reaction rate[a]	e.e.	abs. conf.	yield	reaction time (h)		e.e.	abs. conf.	yield
1	2.3	> 98%	(*S*)	21%	3.0	**1a**	29%	(*R*)	78%
2	7.2	> 98%	(*S*)	40%	0.7	**2a**	66%	(*R*)	54%
3	50.8	> 98%	(*S*)	48%	0.4	**3a**	83%	(*R*)	47%
4	106.1	> 98%	(*S*)	44%	0.3	**4a**	73%	(*R*)	52%
5	85.2	> 98%	(*S*)	38%	0.3	**5a**	55%	(*R*)	60%

a) Initial rate of epoxide hydrolysis in nmol/min, mg dw.
Hydrolysis of 20 mM epoxide in 10 ml reaction mixture with glucose-grown cells of *Rhodotorula glutinis* (dry weights ranging from 0.1 to 0.5 g).

All tested epoxides were hydrolyzed to (*R*) diols with retention of configuration at the more hindered carbon atom. The enantiomeric purities of the diols, obtained after complete resolution of the corresponding epoxides, were in all cases low. Enantiopure diols could only be obtained from the corresponding epoxides at low conversions.

For determination of the enantiomeric ratio *E*, ln [Ro]/[R] versus ln [So]/[S] was plotted. The slope of this curve represents the *E* value. The concentrations of the (*R*) enantiomer at times 0 and t are represented by [Ro] and [R], while [So] and [S] are the concentrations of the (*S*) enantiomer at times 0 and t, respectively. Concentrations at times t were determined from samples taken at two minute intervals, during the course of the reaction. This method for determining *E* has previously been used in a study on the hydrolysis of *p*-nitrostyrene oxide by an epoxide hydrolase preparation from *Aspergillus niger*[12]. The effect of the substrate chain length on the enantiomeric ratio *E* and on the initial reaction rate in the resolution of epoxides (±)-**1** to (±)-**5** is shown in Figure 1a and b, respectively. In other revelant studies on the hydrolysis of aliphatic terminal epoxides, it was found that enantioselectivities were only significantly higher when substrates with branched alkyl-chains were used[8,9,10].

In order to investigate the effect of increased substrate concentration on the biocatalyst, we raised the concentration of epoxide (±)-**3**. In this experiment 500 mg epoxide (±)-**3** was added to 1200 mg (dry weight) cells of *Rhodotorula glutinis* in a total volume of 10 ml. Such a substrate concentration, calculated to be theoretically 500 mM, exceeds the maximal solubility of epoxide-**3**. Under these conditions, substantial amounts of epoxide will be separated from the aqueous phase during the reaction. Chemical hydrolysis of the epoxide will be minimized and larger quantities of substrate can thus be resolved. This high epoxide concentration had no significant adverse effect on the biocatalyst since

there was only about 10% decrease in reaction rate (r = 45 nmol/min,mg dw), compared with the rate for 20 mM epoxide (±)-**3**. The use of high substrate concentrations will greatly facilitate the application of this method on a preparative scale.

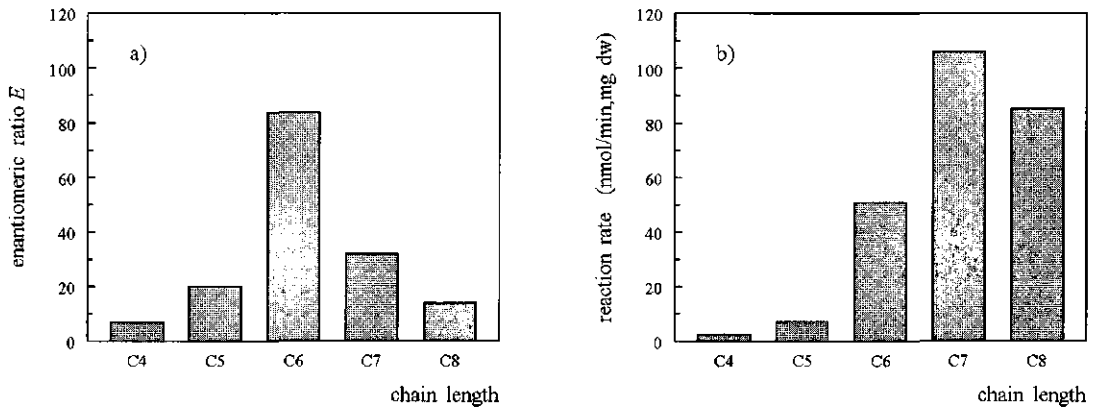


Figure 1. Effect of the substrate chain length on the enantioselectivity *E* (a) and reaction rate (b) in the hydrolysis of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*.

Conclusions

Epoxide hydrolysis with moderate to high enantioselectivities has, for the first time, been demonstrated for the resolution of a homologous range of unbranched aliphatic 1,2-epoxides by cells of the yeast *Rhodotorula glutinis*. (*R*) Epoxides were preferentially hydrolyzed to (*R*) diols with *retention* of configuration at the more hindered carbon atom. Reaction rates as well as enantioselectivities were strongly influenced by the chain length of the epoxide used. High enantioselectivity (*E* = 84) in combination with relatively high activity was observed in the resolution of (±)-1,2-epoxyhexane.

Experimental Section

General

Gas chromatography (GLC) was performed on Chrompack CP9000 and Hewlett-Packard 6890 gaschromatographs equipped with FID detectors and using N_2 as carrier gas. Determination of the enantiomeric excesses was performed by GLC using fused silica cyclodextrin capillary columns (30 m length, 0.25 mm ID and 0.25 μm film thickness). For

epoxides **1** and **2**, a ß-DEX 225 column (Supelco Inc.) was used at oven temperatures of 50°C and 55°C, respectively. Enantiomer analysis of epoxides **3**, **4** and **5** was done on a β-DEX 120 column (Supelco Inc.) at oven temperatures of 45°C, 45°C and 55°C respectively. Chiral GLC analysis for diols **1a**, **2a**, **3a**, **4a** and **5a** was performed on a β-DEX 120 column at oven temperatures of 90°C, 100°C, 125°C, 125°C and 130°C. Concentrations of epoxides and diols were derived from calibration curves using heat-killed cells of *Rhodotorula glutinis*. Optical rotation values were measured on a Perkin-Elmer 241 polarimeter at 589 nm. ^{1}H-NMR spectra were recorded on a Brucker 300MHz spectrometer.

*Epoxides **1** to **5***

The commercially available racemic substrates (±)-1,2-epoxybutane **1**, (±)-1,2-epoxypentane **2**, (±)-1,2-epoxyhexane **3**, and (±)-1,2-epoxyoctane **5** were all obtained from Aldrich Chemie. Epoxide **5** was available from Fluka in enantiomeric pure (*R*) and (*S*) form as well.

(±)-1,2-Epoxyheptane **4** was synthesized by direct epoxidation of the corresponding 1-heptene using *m*-chloroperoxybenzoic acid (M-CPBA) in dichloromethane at 0°C. The structure of epoxide **4** was characterized by GC/MS analysis m/z 85(12, M^+-CHO), 71(100, $CH_3(CH_2)_4^+$), 55(43, $CH_3(CH_2)_2^+$). ^{1}H-NMR of (±)-**4** in $CDCl_3$: δ_H 0.91 (3H, t, J=7 Hz, CH_3), 1.28-1.68 (8H, m, -$(CH_2)_4$-), 2.48 (1H, dd, J=5.1 and 2.8 Hz,CH_2(O)CH-), 2.76 (1H, dd, J=5.1 and 4Hz, CH_2(O)CH), 2.90-2.95 (1H, m, CH_2(O)CH-).

*Reference compounds **1a** to **5a***

For identification and determination of the yields of the formed diols from epoxides **1** to **5**, the corresponding racemic reference diol compounds **1a**, **2a**, **3a** and **5a** were purchased from Aldrich Chemie. Acid catalyzed hydrolysis was used to prepare (±)-1,2-heptanediol **4a** from the corresponding epoxide (±)-**4**. GC/MS analysis m/z 101(27, M^+-CH_2OH), 83(77, M^+-(CH_2OH+H_2O)), 55(100, $CH_3(CH_2)_2^+$). ^{1}H-NMR of (±)-**4a** in acetone-d_6: d_H 0.89 (3H, t, J=7 Hz, CH_3), 1.18-1.42 (6H, m, -$(CH_2)_3$-), 1.42-1.60 (2H, m, -CH_2CH(OH)-), 2.04-2.08 (1H, m, -CH(OH)-), 3.30-3.53 (2H, m, -CH_2OH).

Growth conditions for Rhodotorula glutinis

The yeast *Rhodotorula glutinis* strain CIMW 147 was from our own laboratory culture collection. A mineral medium supplemented with 0.2% (w/v) yeast extract and 1% (w/v) glucose was used for cultivation. *Rhodotorula glutinis* was routinely grown in a chemostat culture under aerobic conditions in a 2-l fermentor (with 1-l working volume)

at 30°C, with a dilution rate of 0.15 h^{-1}. The pH of the culture was maintained at 6.0. The cells were harvested by centrifugation at 16,000 *g*, washed twice with 50 mM potassium phosphate buffer pH 7.5, concentrated, and stored at -20°C.

Epoxide hydrolysis by Rhodotorula glutinis

Hydrolysis of epoxides was routinely performed in 100 ml screw-capped bottles sealed with rubber septa. The bottles contained 1 to 5 ml concentrated washed cell suspension of *Rhodotorula glutinis* (0.1 to 0.5 g dry weight) and 50 mM potassium phosphate buffer pH 7.5 to a total volume of 10 ml. The bottles were placed into a shaking waterbath at 35°C and the reaction was started by addition of 0.20 mmol epoxide. The course of the epoxide hydrolysis was followed by periodic taking headspace sampling followed by analysis with chiral GLC. Initial reaction rates were determined from the epoxide disappearance and correlated to the dry weight of the used yeast suspension. In general, reactions were terminated when the residual epoxides reached e.e.'s of more than 98%. Subsequently, diols were extracted with ethyl acetate from NaCl saturated supernatants, obtained after centrifugation of the reaction mixture. Analysis of the diols was by chiral GLC.

*Absolute configuration of epoxides **1** to **5***

Absolute configurations were determined of the residual epoxides obtained after hydrolysis of 300 mg of the appropriate epoxide by 800 mg (dry weight) of *Rhodotorula glutinis* cells as described in this paper. The moment for terminating the reactions was determined by monitoring headspace samples with chiral GLC. The reactions were stopped by removal of the yeast cells by centrifugation (20,000*g*, 10 minutes, 4°C). Subsequently, the supernatants were extracted twice with an equal volume of cold pentane. The combined organic layers were dried over $MgSO_4$ and concentrated by evaporation at 40°C under atmospheric pressure. Because of the high volality of most epoxides, concentration was not further continued and measurement of the specific optical rotation values was performed in the concentrated pentane fraction. Chiral GLC was used for determination of e.e.'s and concentrations were derived from calibration curves.

Data of chiral GLC analysis and specific optical rotation values of the residual epoxides are:

epoxide (*S*)-**1**: $[\alpha]^{24}_D$ = - 15.5 (c = 0.20, pentane; e.e. = 95%)
[Lit.[13]: (*R*)-**1**: $[\alpha]^{16}_D$ = + 12.4 (c = 5.98, dioxane; e.e. > 98%)]
epoxide (*S*)-**2**: $[\alpha]^{24}_D$ = - 16.8 (c = 0.28, pentane; e.e. > 98%)

epoxide (*S*)-**3**: $[\alpha]^{24}_D$ = - 18.7 (c = 0.93, pentane; e.e. > 98%)
[Lit.[4]: (*R*)-**3**: $[\alpha]^{25}_D$ = + 12.1 (neat; e.e. = 66%)]
epoxide (*S*)-**4**: $[\alpha]^{24}_D$ = - 12.3 (c = 0.31, pentane; e.e. = 78%)
[Lit.[4]: (*R*)-**4**: $[\alpha]^{25}_D$ = + 15.1 (neat; e.e. = 94%)].

Determination of the absolute configurations was by comparison of our results with the data reported in the literature[13,4]. Absolute configuration of the resolved residual epoxide (*S*)-**5** was determined by co-injection on chiral GLC with the enantiopure reference compounds (*R*)-**5** and (*S*)-**5** from Fluka.

*Absolute configuration of diols **1a** to **5a***

Absolute configurations were determined of the diols formed from duplicate hydrolysis of 300 mg of the appropriate epoxide by 800 mg (dry weight) of *Rhodotorula glutinis* cells as described. Experiments were carried out in duplicate as described in this paper for resolution of the epoxides. However, in case of the diols, the reactions were already terminated at low conversions in order to obtain diols with higher enantiomeric purities. The residual epoxides are removed from the reaction mixture by extraction with pentane as described. The remaining aqueous reaction mixtures were subsequently saturated with NaCl and extracted twice with an equal amount of ethyl acetate. The combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure to give an oily residu of diols **1a** to **4a**, and a white solid in the case of diol **5a**. For measurement of the specific optical rotation values and enantiomeric purities, the diols were redissolved in methanol. Chiral GLC analysis was performed for determination of the e.e. values. Data of chiral GLC analysis and specific optical rotation values of the formed diols are:

diol (*R*)-**1a**: $[\alpha]^{24}_D$ = + 3.6 (c = 3.5, methanol; e.e. = 36%)
[Lit.[14]: (*S*)-**1a**: $[\alpha]^{20}_D$ = - 8.6 (c = 1.0, methanol; e.e. > 99%)]
diol (*R*)-**2a**: $[\alpha]^{24}_D$ = + 9.9 (c = 5.4, methanol; e.e. = 67%)
[Lit.[14]: (*S*)-**2a**: $[\alpha]^{20}_D$ = - 17.3 (c = 1.0, methanol; e.e. > 99%)]
diol (*R*)-**3a**: $[\alpha]^{24}_D$ = + 13.5 (c = 5.8, methanol; e.e. = 91%)
[Lit.[14]: (*S*)-**3a**: $[\alpha]^{20}_D$ = - 16.4 (c = 1.0, methanol; e.e. > 99%)]
diol (*R*)-**4a**: $[\alpha]^{24}_D$ = + 14.4 (c = 2.4, methanol; e.e. = 93%)
[Lit.[14]: (*S*)-**4a**: $[\alpha]^{20}_D$ = - 15.4 (c = 1.0, methanol; e.e. > 99%)]
diol (*R*)-**5a**: $[\alpha]^{24}_D$ = + 12.8 (c = 0.95, methanol; e.e. = 80%)
[Lit.[14]: (*S*)-**5a**: $[\alpha]^{20}_D$ = - 13.6 (c = 1.0, methanol; e.e. > 99%)]

Determination of the absolute configurations was by comparison of our results with the data reported in the literature[14].

Acknowledgements

Thanks are due to Mr. M. de Wit (Div. of Industrial Microbiology, WU, NL), Mr. H.J. Swarts (Dept. of Organic Chemistry, WU, NL) and Prof. J.A. Steenkamp (Dept. of Organic Chemistry, UOFS, SA) for advice and assistance in the use of various analytical techniques. Financial support from the South African Foundation for Research Development and SASOL is also gratefully acknowledged.

References

1. Bont de, J.A.M. Bioformation of optically pure epoxides. *Tetrahedron: Asymmetry* **1993**, *4*, 1331-1340.
2. Besse, P.; Veschambre, H. Chemical and biological synthesis of chiral epoxides. *Tetrahedron* **1994**, *50*, 8885-8927.
3. May, S.W. Enzymatic epoxidation reactions. *Enzyme Microb. Technol.* **1979**, *1*, 15-22.
4. Furuhashi, K. A fermentation process for the production of optically active epoxides. *Chemical Economy and Engineering Review* **1986**, *18*, 21-26.
5. Weijers, C.A.G.M.; Ginkel van, C.G.; Bont de, J.A.M. Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane- and alkene-utilizing bacteria. *Enzyme Microb. Technol.* **1988**, *10*, 214-218.
6. Habets-Crützen, A.Q.H.; Bont de, J.A.M. Inactivation of alkene oxidation by epoxides in alkene- and alkane-grown bacteria. *Appl. Microbiol. Biotechnol.* **1985**, *22*, 428-433.
7. Weijers, C.A.G.M.; Bont de, J.A.M. Enantioselective degradation of 1,2-epoxyalkanes by *Nocardia* H8. *Enzyme Microb. Technol.* **1991**, *13*, 306-308.
8. Bellucci, G.; Chiappe, C.; Conti, L.; Marioni, F.; Pierini, G. Substrate enantioselection in the microsomal epoxide hydrolase catalyzed hydrolysis of monosubstituted oxiranes. Effects of branching of alkyl chains. *J. Org. Chem.* **1989**, *54*, 5978-5983.
9. Grogan, G.; Roberts, S.M.; Willetts, A.J. Novel aliphatic epoxide hydrolase activities from dematiaceous fungi. *FEMS Microbiol. Lett.* **1996**, *141*, 239-243.
10. Osprian, I.; Kroutil,W.; Mischitz, M.; Faber, K. Biocatalytic resolution of 2-methyl-2-(aryl)alkyloxiranes using novel bacterial epoxide hydrolases. *Tetrahedron: Asymmetry* **1997**, *8*, 65-71.
11. Weijers, C.A.G.M. Enantioselective hydrolysis of aryl, alicyclic and aliphatic epoxides by *Rhodotorula glutinis*. *Tetrahedron: Asymmetry* **1997**, *8*, 639-647.
12. Morisseau, C.; Nellaiah, H.; Archelas, A.; Furstoss, R.; Baratti, J.C. Asymmetric hydrolysis of racemic *para*-nitrostyrene oxide using an epoxide hydrolase preparation from *Aspergillus niger*. *Enzyme Microb. Technol.* **1997**, *20*, 446-452.
13. Coke, J.L.; Shue, R.S. Nucleophilic ring opening of optically pure (*R*)-(+)-1,2-epoxybutane synthesis of new (*R*)-2-butanol derivatives. *J. Org. Chem.* **1973**, *38*, 2210-2211.
14. Hasegawa, J.; Ogura, M.; Tsuda, S.; Maemoto, S.; Kutsuki, H.; Ohashi, T. High-yield production of optically active 1,2-diols from the corresponding racemates by microbial stereoinversion. *Agric. Biol. Chem.* **1990**, *54*, 1819-1827.

6

Preparative-scale kinetic resolution of 1,2-epoxyhexane by *Rhodotorula glutinis*

Abstract

Preparative-scale kinetic resolution of epoxides was investigated with 1,2-epoxyhexane as a model substrate and cells of the yeast Rhodotorula glutinis *as biocatalyst with epoxide hydrolase (EH) activity. Critical parameters observed in scaling-up this process were the limited water-solubility and high cytotoxicity of the epoxide substrate. Therefore, organic solvents were tested for application in a two-phase reaction medium. From the relationship between log* P *and EH activity, long-chain aliphatic alkanes were determined as suitable biocompatible organic solvents. Dodecane was eventually selected for further applications. Scale-up experiments showed that product inhibition by the formed diol additionally hampered prolongation of the kinetic resolution process.*

A two-phase hollow fiber membrane bioreactor in cascade configuration was successfully used to (i) *minimize toxicity of the solvent and epoxide and* (ii) *to remove inhibitory amounts of formed diol. By this method, preparative-scale resolution of 22 g 1,2-epoxyhexane resulted in 6.5 g enantiopure* (S)*-epoxide, obtained as a concentrated 0.9 M solution in the organic solvent containing feed reservoir.*

A process for continuous extractive kinetic resolution was performed using a modified configuration of the two-phase cascade membrane bioreactor. By this method, long-term operation was possible to produce 13.4 g enantiopure (S)*-1,2-epoxyhexane in the effluent solvent phase. The process was operated for 12 days and allowed production of enantiopure epoxide without the need for complete resolution of the racemic substrate in the feed reservoir.*

The main part of this chapter has been published in:
W.J. Choi, C.Y. Choi, J.A.M. de Bont, C.A.G.M. Weijers
Applied Microbiology and Biotechnology **1999**, *53*, 7-11
and
W.J. Choi, C.Y. Choi, J.A.M. de Bont, C.A.G.M. Weijers
Applied Microbiology and Biotechnology **2000**, *54*, 641-646

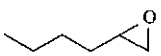
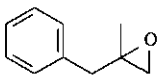
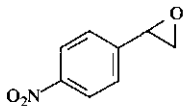
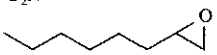
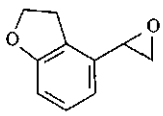
Introduction

The biocatalytical potential of epoxide hydrolases (EHs) from microbial sources, either used as enzyme preparations or whole cells, has been regarded as to be superior to that of their counterparts from mammalian cells[1-3]. Microbial EHs have shown to be easily accessible tools for application in organic synthesis because large-scale production of these biocatalysts is relatively easy. However, despite of this, their use in industrial-scale production of enantiopure epoxides is still not widespread. Scaling-up has most probably been hampered by experimental limitations such as inhibition of catalytic activity at high substrate concentrations. This inhibition phenomenon has already been observed in one of the early studies on the hydrolysis of styrene oxide by fungal cells[4].

Single-phase systems

Scaling-up EH-catalyzed resolution of epoxides in aqueous buffer media has been reported for cells of *Nocardia* EH1[5], crude enzyme from *Aspergillus niger*[6] and cells of *Rhodosporidium toruloides*[7] and *Rhodotorula glutinis* strains[8,3] which are summarized in Table 1.

Table 1. Reported EH-catalyzed preparative-scale kinetic resolution in aqueous media

Substrate	substrate conc.	reaction medium	EH source	ref.
	100 mM	buffer	yeast	3.
	270 mM	buffer	bacterial	5.
	330 mM	buffer/DMSO	fungal	6.
	500 mM	buffer	yeast	7.
	62 mM	buffer	yeast	8.

The initial substrate concentrations as given in Table 1 in most cases will exceed the maximum solubility of the respective compounds in aqueous buffer. Under these conditions, substantial amounts of epoxide will not be dissolved in the aqueous phase but will be highly dispersed without forming a separate layer. In this way, relatively large amounts of epoxide can be resolved with high efficiency. However, when enzymes or cells are exposed to substrate concentrations from millimolar range up to 1 M or more, severe substrate inhibition will certainly affect the action of the biocatalyst as a general rule[9]. Moreover, in case of whole-cell biocatalysis, acute cytotoxicity of the epoxide substrate will occur due to membrane damage and subsequent misfunctioning of membrane associated enzymes[10]. In most of the studies referred to in Table 1, inhibition of epoxide hydrolysis at high substrate concentrations had been observed yet. In some cases, this inhibition could be effectively compensated by using higher amounts of biocatalyst, indicating that only the ratio of substrate to cells/enzyme was of importance[4,8]. For other sources of EH, substrate inhibition was independent to the biocatalyst concentration, and was possibly due to the intrinsic toxicity of epoxides[4,5]. The high toxicity of epoxides will certainly limit scaling-up possibilities.

For scaling-up in single-phase media, special attention has to be given to the decreased solubility in water when more complex epoxides are used as substrates. Hydrolysis of large amounts of these kinds of epoxides will be hampered by mass-transfer limitations. Addition of water-miscible cosolvents will then be necessary to reduce the problem without causing a large decrease in volumetric productivity. It has been stated that the use of cosolvent containing single-phase systems is not desirable in biotransformations with toxic substrates. This is based on the assumptions that these systems will enhance the toxicity towards the biocatalyst by (*i*) the basic cytotoxicity of both the cosolvent and the substrate and (*ii*) the speed of action[11]. For water-miscible cosolvents in a single-phase system, direct access to the cell membrane or enzyme itself is easily possible so that any toxic effects will appear rapidly. Apart from direct physical damage to the membrane and associated enzymes, additional protein denaturation will occur by unfolding. Application of cosolvents has been investigated in the resolution of *para*-nitrostyrene oxide by EH from *Aspergillus niger*[6]. In this study, the use of a cosolvent was essential because of the low solubility of the substrate in aqueous buffer. All cosolvents tested were inhibitory towards the activity and stability of the EH. Best results were obtained with dimethylsulfoxide (DMSO) but, however, the activity of the EH was still inhibited for 84% by addition of this compound to a final low concentration of 0.2% (v/v).

It can be concluded that single-phase systems are not suitable reaction media for kinetic resolution of epoxides on a preparative scale.

Two-phase systems

Further increase of substrate concentrations in aqueous media will be of interest for scaling-up, because with many epoxides being organic liquids, a simple two-phase system will be obtained with the concentrated epoxide serving as both organic phase and substrate reservoir. In these systems, the substrate transfer rate depends on the interfacial area between the two phases, and the aqueous substrate levels are determined by the water-solubility only.

Application of such a two-phase system has been reported for the preparative-scale resolution of *para*-bromo-α-methyl styrene oxide by *Aspergillus niger* LCP 521[12]. The epoxide used in this study was poorly soluble in water and a concentration of 770 mM therefore formed a separate organic layer. The method has been successfully scaled-up for the complete resolution of 6 g epoxide substrate (380 mM) using crude fungal EH preparation.

Nevertheless, such a simple two-phase system, with the epoxide itself serving as organic phase, will not be generally applicable for scaling-up kinetic resolutions of all kinds of epoxide substrates. In particular, epoxides which are relatively well soluble in water will cause severe inhibition of the biocatalyst due to exposure to high substrate concentrations. For these substrates a better-controlled dosage towards the biocatalyst can be obtained by the use of an epoxide containing organic solvent serving as second phase. And, in addition, for epoxides which are solids in concentrated form this would be the only possibility, apart from the less promising application of cosolvents as alternative.

In the present study, we will focus on scaling-up epoxide hydrolysis in two-phase systems using cells of *Rhodotorula glutinis*. The EH present in this yeast was found to be a membrane associated protein and it was therefore assumed that the enzyme will perform best and be stabilized under the most optimal conditions in the intra-cellular environment[13].

In this view, the effect of solvents on catalytic activity of yeast cells is of importance. Whole-cell yeast biotransformations in two-phase systems have not been described extensively. Studies that have been reported include (*i*) effects on cell structure[14] (*ii*) toxicity of *n*-alkanes and their derivatives[15] (*iii*) effects of interphase mixing[16] and (*iiii*) effects on catalytic activity and enantioselectivity[17,18]. From these investigations it can be concluded that for optimization of novel biocatalytic two-phase systems an empirical screening of organic solvents will be necessary. In particular biocompatibility, based on low or acceptable toxicity towards the biocatalyst, is a restrictive criterion as it is an absolute condition, whereas other desirable solvent characteristics are more relative conditions.

Classification of solvents in terms of biocompatibility is generally based on parameters that are all related to the polarity of the solvent[19,20]. The most widely used selection cri-

terion for solvent biocompatibility has been studied first by Laane *et al.*[21]. They demonstrated the correlation between biocatalytic activity and log *P*. The log *P* is the logarithm of the partition coefficient that is arbitrary determined in a standard 1-octanol-water two-phase system. The partition coefficient *P* is defined as

$$P = \frac{[\text{compound}]_{\text{octanol}}}{[\text{compound}]_{\text{water}}}$$

Log *P* has conventionally been used in the pharmaceutical and medicinal chemistry fields as a part of drug activity studies[22]. As a general rule, biocatalytic activity is low in polar solvents with a log $P < 2$ and the activity will be retrieved in apolar solvents having a log $P > 4$[23]. Cell activity as function of log *P* values for different homologous series of solvents has been studied by Vermuë *et al.*[24]. The results of this study indicates that biocompatibility of solvents can not be determined by their log *P* alone but is related to their concentration used and chemical structure as well. For membrane associated enzymes catalytic activity was found to be well correlated with the concentration of solvent in the cell membranes[25]. Loss of activity was supposed to be mainly caused by alterations of membrane fluidity, and to some lower extend by lipid phase separation, direct protein-solvent interactions and membrane permeabilization.

The use of organic solvents for biocatalytic resolution of epoxides has several potential advantages including (*i*) increased substrate concentrations for high volumetric productivity (*ii*) protection of cells against inhibitory amounts of epoxide and (*iii*) prevention of chemical hydrolysis of the epoxide substrate at longer reaction times. The main disadvantage that can be expected is a dramatic decrease in reaction rates due to additional solvent toxicity[11].

Membrane bioreactors

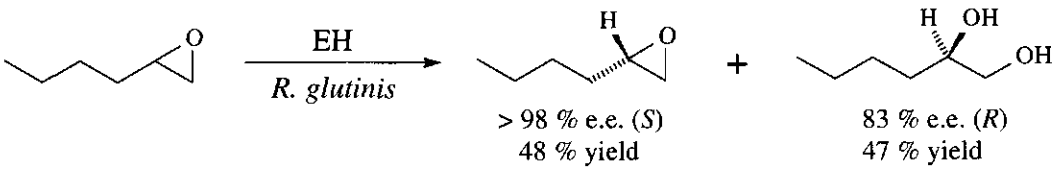
Suitable organic solvents can be selected for scaling-up EH-catalyzed resolution of epoxides. However, for long-term operational stability of the biocatalyst, a more effective protection to the solvent and other reactants will be needed. This can be achieved by the use of a membrane bioreactor with a microporous membrane serving as a barrier between the used toxic compounds and the biocatalyst. Apart from enhanced activity and extended operational stability of the biocatalyst, this methodology has several other advantages, such as (*i*) prevention of the formation of emulsions and subsequent loss of solvent and substrate, (*ii*) possibility to easily remove reaction products for increased conversions and (*iii*) modular design which allows simple scale-up. Operating principles, mathematics and applications of hollow-fiber membrane reactors have been reviewed recently[26].

Applications of two-phase membrane bioreactors in whole-cell biocatalysis have been reported for bioconversions in general[27-29] and for one example of kinetic resolution in

particular[30]. Enzymatic resolutions using membrane bioreactors have been described in a few cases more[31-33]. Resolution of epoxides by use of membrane bioreactors has not been reported before in either case.

Present study

In the present study, scaling-up epoxide resolution was investigated with 1,2-epoxyhexane as a model substrate and cells of the yeast *Rhodotorula glutinis* were used as biocatalyst with yeast epoxide hydrolase activity (YEH)(Scheme 1). A membrane reactor consisting of hollow-fibers with a large surface area was used as bioreactor for the kinetic resolution process. The membrane bioreactor was used in a batchwise operation mode and subsequently in a modified configuration for continuous extraction of the resolved enantiopure epoxide.



Scheme 1. YEH-catalyzed kinetic resolution of (±)-1,2-epoxyhexane[3]

Results and Discussion

Solvent selection

Solvent selection for aided resolution of 1,2-epoxyhexane was performed in a reaction medium consisting of aqueous buffer with cells of *Rhodotorula glutinis* and the appropriate organic solvent in a concentration of 30% (v/v). Water-miscible cosolvents like acetonitrile, methyl sulfoxide and 1,4-dioxane were initially tested but all of them showed almost complete inhibition of the YEH activity and were therefore excluded from further investigation.

Water-immiscible solvents were found to be less inhibitory. But, nevertheless, minimum inhibition still was about 75% when tested under the standard assay conditions. The disappointing result can be partly explained by low biocompatibility of the tested solvents but a greater part of inhibition was most probably caused by a limiting amount of epoxide available in the aqueous phase due to solvent extraction. In this context, kinetic constants for the expression of whole-cell epoxide hydrolysis are of importance. From data obtained by epoxide hydrolysis using similar experimental conditions, we

determined the maximum diol production rate V^*_{max} as 48 ± 4 nmol/min,mg dw and a K^*_M of 14 ± 2 mM for (*R*)-1,2-epoxyhexane. Both extrinsic parameters were calculated from a "Eadie-Hofstee" plot. From the K^*_M value it can be understood that an epoxide concentration of 20 mM in the presence of extracting organic solvent can generate very low substrate concentrations in the aqueous phase which become rate limiting. We therefore raised the epoxide concentration up to 100 mM and screened a range of water-immiscible solvents under the improved conditions. A concentration of 100 mM was obtained by addition of 2 mmol epoxide. This amount of epoxide could be resolved to completion within 180 minutes by 0.4 g cells of *Rhodotorula glutinis*. Partition coefficients for 1,2-epoxyhexane in the tested solvents were in the range of 64 to 34, resulting in epoxide concentrations from 5 to 9 mM in the aqueous phase by addition of 2 mmol substrate.

Table 2. Epoxide hydrolase activity of *Rhodotorula glutinis* in aqueous/organic two-phase reaction media

Solvent [a]	log *P*	reaction rate[b]	yield diol[c]	e.e. (*R*)-diol[c]
Aqueous buffer	-	43	47 %	86 %
Glycerol	-	33	43 %	87 %
Ethyl acetate	0.68	0.1	0.13 %	n.d.
Diethyl ether	0.85	3.9	12 %	92 %
Dipropyl ether	1.9	14	26 %	94 %
Pentane	3.0	20	33 %	92 %
Hexane	3.5	20	38 %	90 %
Octane	4.5	24	38 %	91 %
Decane	5.6	28	41 %	91 %
Dodecane	6.6	30	49 %	92 %
Tetradecane	7.6	31	48 %	92 %
Hexadecane	8.8	36	52 %	91 %
1,2-Epoxyhexane[a]	1.9	38	2.2 %	95 %

Hydrolysis of 100 mM 1,2-epoxyhexane in 20 ml reaction mixture with cells of *Rhodotorula glutinis* (dry weight 0.4 g).

a) Reaction mixture containing 30 % (v/v) solvent or epoxide as an organic phase.

b) Initial rate of diol production in nmol/min, mg dw at 35 °C, reaction started after 60 min pre-incubation with the appropriate solvent.

c) Concentration of 1,2-hexanediol and e.e. of (*R*)-1,2-hexanediol after 3 h of incubation.

n.d. Not determined

In order to minimize possible variations, caused by differences in time-dependent solvent sensitivity, a 60 min pre-incubation period was included in the experiment. During pre-incubation, cells were exposed to the appropriate solvent without epoxide substrate. Under the present experimental conditions a more accurate determination of the EH activity was found by analysis of the formed diol rather than by analysis of the residual epoxide. The results are compiled in Table 2.

Both reaction rate as well as degree of conversion was found to be dependent on the polarity of the solvent used. The effect of organic solvents on the initial reaction rates as shown in Table 2 is in accordance with the effect observed in the hydrolysis of 20 mM epoxide substrate. However, inhibition by the tested non-polar solvents is much less severe than in case of the experiment using the lower initial substrate concentration.

Glycerol has been included in the experiment for observation of possible effects caused by decrease of the aqueous volume and subsequent increase of the cell concentration. In the control situation using glycerol, only a small inhibition of the reaction rate was observed due to the reduced amount of water. Glycerol itself was assumed not to show any intrinsic toxicity because this polyalcohol has been widely used for stabilization of the catalytic activity of enzymes[34] and cells[35]. In fact, glycerol is able to maintain both the hydrophobic interactions essential for the native structure of the enzyme and the water shell around the protein molecule[36].

The relationships between retention of activity and log *P* and between enantiomeric ratio *E* and log *P* are shown in Figure 1. From these results, biocompatible solvents can be selected for the desirable activity and/or enantioselectivity. The relationship between activity retention and log *P* is somewhat divergent from the generally observed sigmoidal patterns. In the present case, there are only extreme log *P* values indicating the range of solvents being completely inhibitory or supporting complete retention of activity. The results show that an increase of V_{max} for the substrate hydrolysis is correlated to increasing log *P* values of the solvents used. This relationship is assumed to be equal for both the (*R*)- and (*S*)-enantiomers individually. The overall small effect of enantioselectivity (Figure 1b) can thus be explained by the ratio of $V_{max}{}^{R}$ / $V_{max}{}^{S}$ remaining almost constant. Corresponding effects of solvents have been reported for other kind of hydrolytic enzymes[37,38].

Apart from biocompatibility, also other solvent characteristics are of importance. At first, limitations of substrate transfer must be avoided, so therefore, suitable solvents should have low or moderate partition coefficients for the substrate. And, secondly, suitable solvents should have a good selectivity in relation to partition of substrate and product. In case of selecting a solvent for the present study, a good selectivity would imply a moderate partition for the substrate and a very low one for the product. Such good selectivity greatly facilitates the separation of epoxide and diol as basically will be obtained by separation of the organic and aqueous phase. In view of these requirements,

and based on other more practical criteria[22], dodecane was eventually selected from the biocompatible solvents. In dodecane, the partition coefficient for 1,2-epoxyhexane was determined to be 36. 1,2-Hexanediol showed no solubility in dodecane and hence the partition coefficient was accordingly determined as 0.02. From these data, the separation factor of dodecane for extraction of 1,2-epoxyhexane was calculated as 1800.

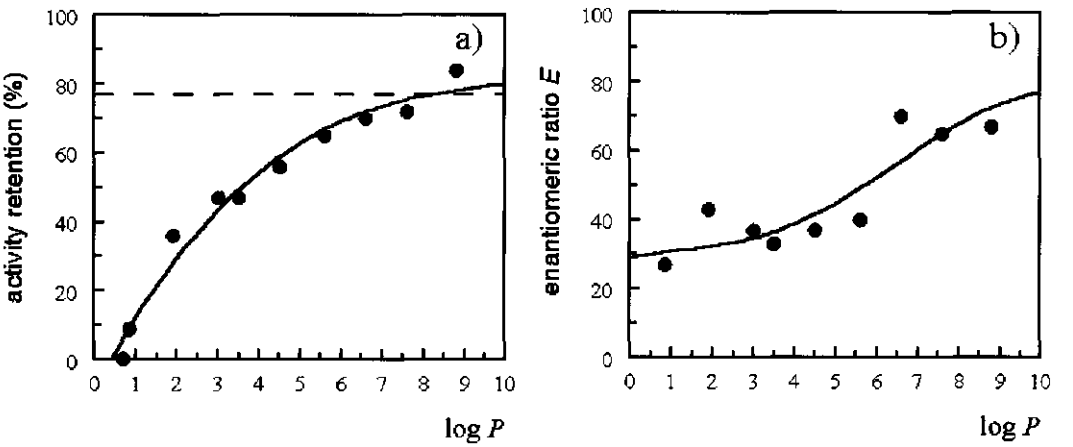


Figure 1. Relationship between activity retention (a) and log *P* and between enantiomeric ratio *E*(c,p) (b) and log *P*, in the hydrolysis of 1,2-epoxyhexane by cells of *Rhodotorula glutinis* when exposed to various organic solvents. The activity retention in case of addition of glycerol in the control experiment is represented by the dotted line in figure (a).

Diol inhibition

Hydrolysis of 2 mmol 1,2-epoxyhexane proceeded to complete resolution of the racemic epoxide within the duration of the experiments. However, in case of epoxide hydrolysis in the two-phase reaction medium where 1,2-epoxyhexane (50 mmol) served for both substrate and organic phase, a very low conversion of only 2 % was obtained. The YEH activity was most probably inhibited by the high concentration of 30% (v/v) and intrinsic toxicity of the epoxide. But, however, loss of activity could also have been partly caused by product inhibition. The latter possibility was supported by the observation that in the course of this reaction, diol production leveled off when the formed diol reached concentrations of 50 mM or more. The effect of product inhibition was examined by comparison of YEH activity in the presence and absence of 50 mM 1,2-hexanediol. In this experiment, a similar amount of 50 mmol of 1,2-epoxyhexane was used but now dissolved in an equal volume of dodecane. Analysis of the aqueous phase resulted in an

epoxide concentration of about 55 mM under these conditions. The biocompatible solvent was expected to be able to protect the yeast cells to the epoxide but not to the diol. The courses of these reactions are shown in Figure 2.

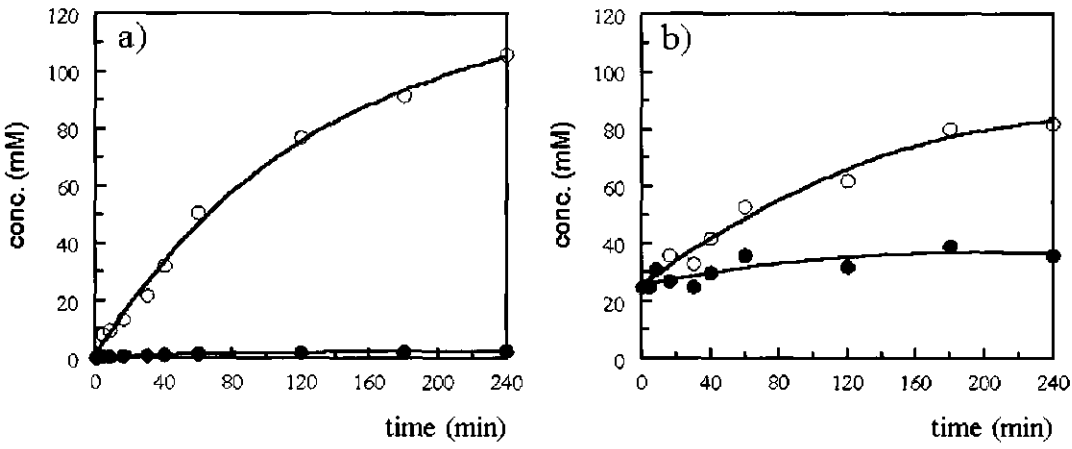


Figure 2. Product inhibition in the hydrolysis of 50 mmol 1,2-epoxyhexane by cells of *Rhodotorula glutinis* (0.5 g dw) in a dodecane containing two-phase system. Course of production of (*R*)-1,2-hexanediol (●) and (*S*)-1,2-hexanediol (○) (a) and in the presence of 50 mM (±)-1,2-hexanediol (b).

YEH activity could be prolonged by addition of dodecane, but, however, resolution of the epoxide nevertheless remained incomplete due to product inhibition. This phenomenon was observed more clearly in the presence of 50 mM 1,2-hexanediol. Inhibition of the initial reaction rate was determined to be 37% and, after terminating the experiment after 20 h of incubation, a decrease of 74% in the yield of formed diol was calculated. In a corresponding reference experiment, dodecane was replaced by glycerol. Inhibition of reaction rate and decrease in yield of formed diol were 36% and 72% respectively in this case. The inhibitory effect of increasing diol concentrations was tested in more detail. Inhibition of YEH activity was found to be directly correlated to the diol concentration. Diol concentrations from 100 to 200 mM resulted in inhibition of reaction rates of 60 to 90% respectively.

Resolution of 1,2-epoxyhexane using a two-phase membrane bioreactor

A cascade hollow-fiber membrane bioreactor was used for preparative-scale resolution of 1,2-epoxyhexane. The bioreactor is schematically depicted in Scheme 2 (Experimental Section).

Dodecane was used as organic solvent in the substrate feed reservoir due to its characteristics as discussed before. Especially the low substrate partition in dodecane was of interest for application in a membrane bioreactor as was indicated by theoretical data from literature[31]. Both hydrophilic as well as hydrophobic membranes were tested for the process. It was observed that hydrophobic membranes supported transfer of the non-polar dodecane through the pores of the membrane surface where the solvent became in direct contact with the cells. We therefore selected a hollow-fiber membrane module consisting of hydrophilic cellulose membranes. In the present bioreactor, yeast cells are not able to pass the membrane pores and remain in the shell side without direct contact with the organic phase. Aqueous solutions however, are permitted to pass the membrane barrier towards the lumen side filled with the hydrophobic organic solvent. Cells of *Rhodotorula glutinis* were used as biocatalyst.

The bioreactor was operated by recirculating racemic 1,2-epoxyhexane dissolved in dodecane through the fiber lumen side with transfer of epoxide to the yeast cells in aqueous buffer in the shell side. In the aqueous phase, 1,2-epoxyhexane was hydrolyzed by the biocatalyst. Subsequently, the aqueous phase was passed to the shell side of the second membrane unit where formed inhibitory high concentrations of diol were lowered by buffer recirculating through the lumen side.

To strip off diol from the cell suspension, water-immiscible organic solvents with high extraction capacities for diols were tested. However, tested suitable solvents, like long-chain aliphatic alcohols, in all cases extracted the partially resolved epoxide as well. In this way, complete resolution of epoxide was strongly hampered. We therefore applied phosphate buffer in the second membrane unit for dilution of the formed inhibitory diol in the cell suspensions. Since addition of buffer direct to the cell suspension would result in a substantial decrease of cell concentration, a second membrane unit was applied in a cascade configuration.

In Table 3, epoxide and diol concentrations in dodecane, cell suspension and diol extracting buffer during the kinetic resolution process are shown. From the data presented, it can be concluded that the large amount of highly concentrated epoxide was successfully hydrolyzed and that the formed diol maintained below the previously observed inhibitory concentrations. A disadvantage of the cascade membrane configuration is loss of epoxide by undesired extraction in the diol extraction buffer.

Table 3. Concentrations of 1,2-epoxyhexane and 1,2-hexanediol in solvent, yeast cell suspension and diol extracting buffer, during epoxide resolution in a cascade two-phase membrane bioreactor

Time (h)	epoxide, dodecane (mM)	epoxide, cells (mM)	epoxide, extr. buffer (mM)	diol, cells (mM)	diol, extr. buffer (mM)
- 3[a]	3000	0	0	0	0
0[a]	2025	28	28	0	0
0.3	1903	33	28	14	2
1	1778	31	24	18	10
3	1412	20	17	32	28
7	1120	17	11	41	42
10	913	14	11	47	50
14	867	13	10	52	53

[a]) Pre-run period

In Figure 3, the course of epoxide conversion and diol production is shown. Kinetic resolution of 226 mmol 1,2-epoxyhexane by cells of *Rhodotorula glutinis* (7 g dw) was completed within 14 hours. Enantiopure epoxide was obtained as 65 mmol (6.5 g) (*S*)-1,2-epoxyhexane (e.e. > 98%) in 30 % yield in the dodecane containing organic phase. No 1,2-hexanediol was detected in the dodecane phase. A decrease in yield of enantiopure epoxide was mainly caused by the loss of, racemic as well as enantiopure, epoxide in the diol extraction buffer. Secondly, a small decrease in enantioselectivity during the final course of the reaction also influenced the yield of (*S*)-epoxide and e.e. of formed (*R*)-diol. 1,2-Hexanediol accumulated up to 126 mmol in the diol extraction buffer. The initial rate of YEH activity was 43 nmol/min mg dw.

The results show that application of a two-phase cascade membrane bioreactor allows kinetic resolution of large amounts of epoxide in high concentrations without significant product inhibition. In addition, formed diols can be easily separated from the epoxides and, in specific cases, obtained in enantiopure form as well. The method can be optimized for resolution of other epoxides and/or diol production. Results will even be better by using epoxides that are less soluble in water because then loss of substrate in the diol extraction buffer will decrease. In conclusion, the method is useful for large-scale resolution of epoxides and particularly when chemically unstable and/or water-insoluble epoxides are used as substrates.

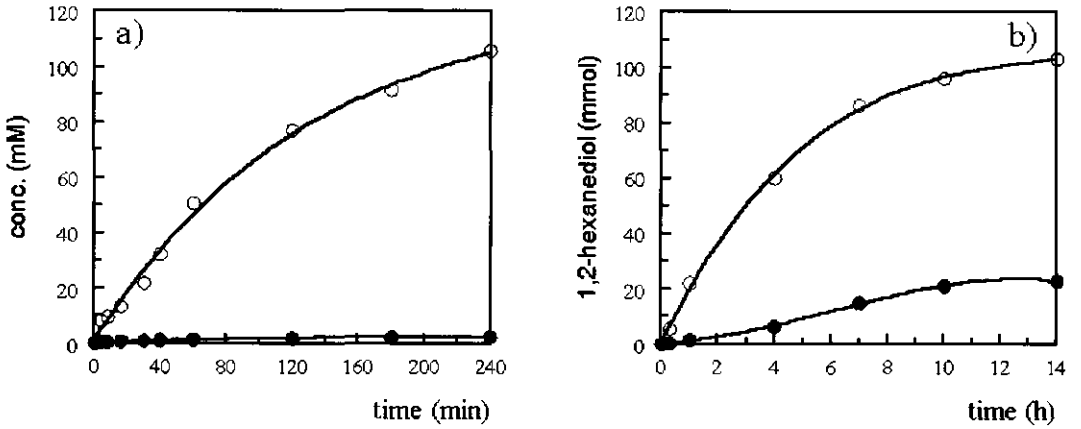


Figure 3. Enantioselective hydrolysis of 226 mmol (22 g) 1,2-epoxyhexane by cells of *Rhodotorula glutinis* (7 g dw) in a cascade two-phase membrane bioreactor. Epoxide and diol during the pre-run are not shown. Course of epoxide hydrolysis (a) showing the total amounts of (*R*)-1,2-epoxyhexane (○) and (*S*)-1,2-epoxyhexane (●) in dodecane (75 ml). Course of diol production (b) showing the total amounts of (*S*)-1,2-hexanediol (○) and (*R*)-1,2-hexanediol (●) in diol extraction buffer (2.4 l).

Continuous production of enantiopure 1,2-epoxyhexane in a two-phase membrane bioreactor

Application of a two-phase membrane bioreactor for batch-wise kinetic resolution of epoxides has shown to be a method with satisfactory results. In order to investigate the feasibility of using a membrane bioreactor for continuous production of enantiopure epoxides, we modified the previously used configuration of the equipment. The new process was designed for continuous extraction of the resolved epoxide during the course of kinetic resolution. Enantioselective hydrolysis of 1,2-epoxyhexane by cells of *Rhodotorula glutinis* was studied in a dodecane containing bioreactor consisting of microporous hydrophilic hollow-fiber membrane modules. The bioreactor for continuous extractive kinetic resolution is schematically depicted in Scheme 3 (Experimental Section).

A critical parameter in continuous biocatalytical processes is the long-term operational stability of the biocatalyst. Apart from the intrinsic stability characteristics of the YEH enzyme, inactivation in two-phase membrane bioreactors can additionally be attributed to cytotoxicity of the reactants and mechanical shear stress caused by pumping. We therefore evaluated the stability of YEH activity in the cell suspension while mimicking experimental conditions in the membrane reactor. Because it has been reported that EH stability could be enhanced by lowering the temperature[39], we operated the bioreactor at

two different temperatures, 35°C and 25°C. Based on the inactivation patterns, half-lives for the two different temperatures of 35°C and 25°C were determined as 2 and 5 days, respectively. The EH activity at 25°C was 85% of the maximum value obtained at 35°C, while the half-life was 2.5 times longer at 25°C. Based on the improvement in enzyme stability, a reaction temperature of 25°C was chosen for the continuous process.

The bioreactor was operated by circulating racemic 1,2-epoxyhexane dissolved in dodecane from the feed reservoir through the fiber lumen side of the first membrane module. Amounts of epoxide are transferred to the aqueous phase in the shell side for subsequent enantioselective hydrolysis of the (*R*) enantiomer by the yeast cells. The aqueous cell suspension with residual (*S*)-epoxide was passed to the shell side of the second membrane unit. Continuous extraction of the (*S*)-epoxide was performed in this unit by a dodecane flow passing through the lumen side. Eventually, inhibitory formed 1,2-hexanediol was stripped off continuously in the third membrane unit by effluent-extracting aqueous buffer. The process for continuous extracting kinetic resolution was started with 3 mol (300 g) (±)-1,2-epoxyhexane as substrate. The time courses of extraction of (*S*)-epoxide and (*R*)-diol are shown in Figure 4.

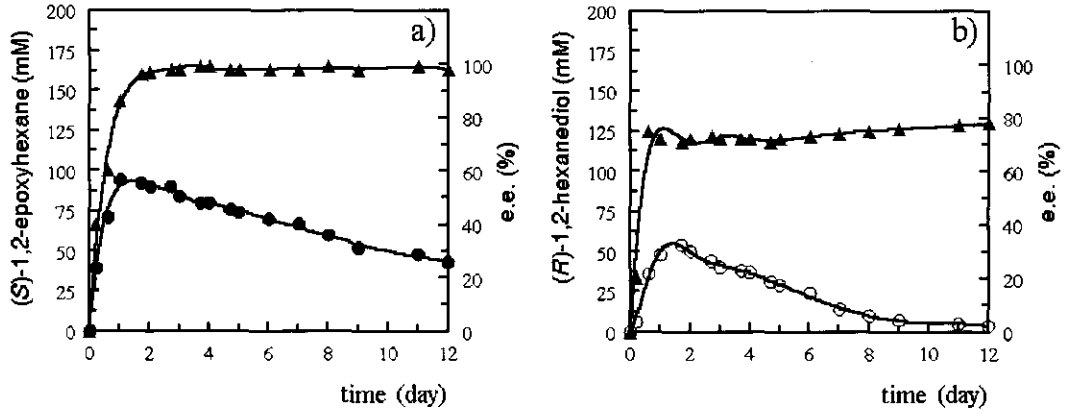


Figure 4. Continuous extractive kinetic resolution of 3 mol 1,2-epoxyhexane by cells of *Rhodotorula glutinis* (9 g dw) in a two-phase membrane bioreactor. Time course of continuous extraction in effluent dodecane (a) showing concentration (●) and e.e. (▲) of residual (*S*)-epoxide. Course of continuous extraction in aqueous buffer (b) showing concentration (○) and e.e. (▲) of the produced (*R*)-diol.

In Figure 4, the time course of extracted epoxide shows a low enantiopurity of the residual (*S*)-epoxide during the early stage of operation. This phenomenon indicates that the initial amounts of epoxide transported to the yeast cells exceeded the amount that could be hydrolyzed by the biocatalyst. Unreacted racemic epoxide thereby remained together with the resolved enantiopure (*S*)-epoxide in the aqueous cell suspension. Partially resolved epoxide with low enantiopurity was subsequently extracted by dodecane in the

second membrane unit. After several recirculations, the aqueous cell suspension contained residual (*S*)-epoxide in excess, due to the resolution process. The amount of (*S*)-epoxide decreased the transfer of racemic epoxide from the substrate reservoir to the aqueous cell suspension. After 2 days of operation, the transfer of racemic substrate was in equilibrium with the amount of (*R*)-epoxide that could be hydrolyzed completely by the yeast cells. Consequently, in the remaining resolution process, epoxide extracted from the aqueous phase in the second membrane unit consisted merely of the enantiopure residual (*S*)-epoxide.

These observations indicate that optimization of the resolution process is highly dependent on the interfacial mass transfer of the epoxide and the amount of biocatalyst. In a given two-phase membrane bioreactor, the mass transfer is influenced by the substrate concentration in the feed solution and by the recirculation flow rates of both phases. However, optimization of the flow rates is strongly limited by the characteristics of the membrane. Careful consideration has to be taken to control the flow rates on both sides of the membrane in such a way that the generated transmembrane pressures do not exceed the 'breakthrough' pressure. The flow rates of the dodecane phases were therefore optimized to the minimum values that did not cause phase-mixing.

The course of production of enantiopure (*S*)-epoxide, obtained in the extracting dodecane phase, is shown in Figure 5. The volumetric productivity gradually decreased during the operation, due to loss of activity of the yeast cells. The initial specific activity, determined from the first 2 days interval, was 22 nmol/min, mg dw at 25°C. After 12 days of continuous operation an amount 134 mmol (13.4 g) enantiopure (*S*)-epoxide was obtained. An amount of 140 g of racemic epoxide was still present in the substrate reservoir indicating that an excess amount of epoxide was supplied at the beginning under these conditions. The overall yield of enantiopure (*S*)-1,2-epoxyhexane (e.e. >98%) was determined as 24% whereas the yield of epoxide in extracting dodecane was only 30% of this value. The low yield was caused by significant loss of epoxide by the continuous flow of extracting aqueous buffer in the third membrane unit.

Enhancement of the productivity can be achieved by changing the epoxide extracting solvent used in the second membrane unit into a solvent with a much higher extracting capacity for the specific epoxide. The amount of epoxide transported to the third membrane unit will then be substantially lowered. Consequently, a minimal loss of epoxide by the diol-extracting buffer will be achieved. However, use of higher extracting, more polar solvents may cause (*i*) inactivation of the biocatalyst due to higher cytotoxicity and (*ii*) lower chemical purity of the resolved epoxide due to undesired co-extraction of amounts of diol. A higher yield of residual epoxide will also be obtained in case of resolution of less water-soluble epoxides due to similar reduced loss of epoxide in diol extraction buffer. Further optimization will be necessary for enhanced utilization of this new approach to kinetic resolutions.

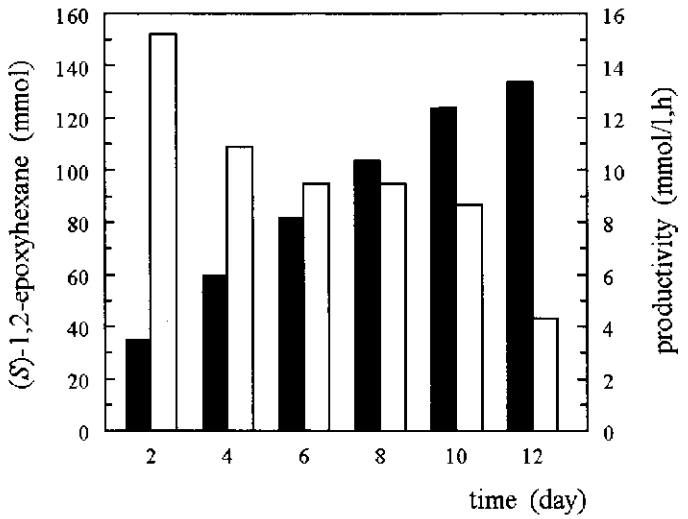


Figure 5. Production of enantiopure epoxide by continuous extractive kinetic resolution in a two-phase membrane bioreactor. Cumulative production of enantiopure (*S*)-1,2-epoxyhexane (■) in extracting effluent dodecane and course of the productivity (□) for this epoxide during the process.

In the present study we have developed a method to produce enantiopure epoxides in an effluent extracting solvent during the course of a kinetic resolution process. This method allows the continuous production of enantiopure epoxide without the need for complete resolution of the racemic epoxide in the substrate reservoir. In this way, the continuous process is potentially applicable to EH-catalyzed resolutions in which the biocatalyst shows a high activity but relatively low enantioselectivity towards a specific substrate.

Conclusions

Biocatalytic kinetic resolution of epoxides has been demonstrated in solvent containing two-phase reaction media. Long-chain aliphatic alkanes were found suitable biocompatible organic solvents for epoxide hydrolase containing cells of *Rhodotorula glutinis*. Scaling-up of this reaction was hampered by inhibition due to substrate toxicity as well by product inhibition of formed diol.

Preparative-scale resolution of highly concentrated epoxide was successfully performed in a two-phase membrane bioreactor. A cascade configuration of two hollow-fiber membrane modules was used to (*i*) minimize toxicity of the solvent and epoxide and (*ii*) to remove inhibitory amounts of diol. After complete resolution, enantiopure epoxide was obtained in the organic solvent containing feed reservoir.

In a modified process design, the membrane bioreactor was used for continuous extractive kinetic resolution. Under these conditions, enantiopure epoxide was obtained in the effluent solvent phase during long-term operation. The process configuration allowed continuous production of enantiopure epoxide without the need for complete resolution of the racemic substrate in the feed reservoir. Further optimization of this process will be necessary for improvement of the volumetric productivity.

Experimental Section

General

Gas chromatography (GLC) was performed on a Hewlett-Packard 6890 gaschromatograph equipped with a FID detector and using N_2 as carrier gas. Determination of the enantiomeric excesses was performed by chiral GLC using a fused silica cyclodextrin capillary ß-DEX 120 column (30 m length, 0.25 mm ID and 0.25 mm film thickness, Supelco Inc.) as described previously[40]. Racemic 1,2-epoxyhexane, (*R*)-1,2-epoxyhexane, 1,2-hexanediol, organic solvents and medium components were purchased from Sigma-Aldrich Chemie BV, The Netherlands.

Concentrations of epoxide and diol were derived from calibration curves using heat-killed cells of *Rhodotorula glutinis*. For determination of diol concentrations in two-phase reaction media, individual calibration curves with the corresponding amounts of appropriate organic solvent and heat-killed cells were prepared.

Biocatalyst

The yeast *Rhodotorula glutinis* ATCC 201718 was originally obtained from our own laboratory culture collection and previously referred to as strain CIMW 147. The yeast strain was cultivated for 48 h in a mineral medium supplemented with 2% (m/v) glucose and 0.2% (m/v) yeast extract, at 30°C in a shaking incubator. Cells were harvested by centrifugation at 16,000 *g*, washed with 100 mM potassium phosphate buffer pH 8.0, concentrated and stored - 20°C for future experiments.

Solvent selection

Hydrolysis of epoxide was performed in 100 ml screw-capped bottles sealed with rubber septa. The bottles contained 3 ml concentrated cell suspension of *Rhodotorula glutinis* (0.4 g dw), 6 ml appropriate organic solvent, and 50 mM potassium phosphate buffer pH 8.0 to a total volume of 20 ml. The bottles were placed into a shaking waterbath at 35°C and the cells were pre-incubated with the organic solvent for 60 minutes. Under these

conditions, the solvent was dispersed in the reaction mixture but formed a separate layer when shaking was terminated. Epoxide hydrolysis was started by addition of 2.0 mmol 1,2-epoxyhexane. The course of the reaction was followed for 3 h, by periodic taking samples from the cells containing aqueous buffer phase. Subsequently, formed 1,2-hexanediol was extracted with ethyl acetate from NaCl saturated supernatants, obtained after centrifugation of samples from the aqueous cell suspension. Analysis of the diol was by chiral GLC. Initial reaction rates were determined from diol production and correlated to the dry weight of the used yeast suspension. Partition coefficients for 1,2-epoxyhexane were determined under similar experimental conditions.

Diol inhibition

Epoxide hydrolysis was performed in 100 ml screw-capped bottles sealed with rubber septa. The bottles contained 3 ml concentrated cell suspension of *Rhodotorula glutinis* (0.5 g dw), 6 ml dodecane and 5 ml potassium phosphate buffer pH 8.0. Duplicate reference experiments were performed by replacing dodecane by glycerol. Product inhibition was studied in bottles by addition of 1,2-hexanediol to a final concentration of 50 mM. The bottles were placed into a shaking waterbath at 35°C for pre-incubation during 60 minutes. Epoxide hydrolysis was started by addition of 50 mmol (6 ml) 1,2-epoxyhexane. The course of the reaction was followed for 4 h, by periodic sampling and subsequent analysis of produced 1,2-hexanediol in the aqueous cell suspension. Total yield formed diol was determined after 20 h of incubation. Concentrations of diol were derived from calibration curves using heat-killed cells of *Rhodotorula glutinis* under similar experimental conditions.

Effects of increasing concentrations of diol were studied under standard assay conditions[40] in the presence of 30 mM up to 400 mM 1,2-hexanediol.

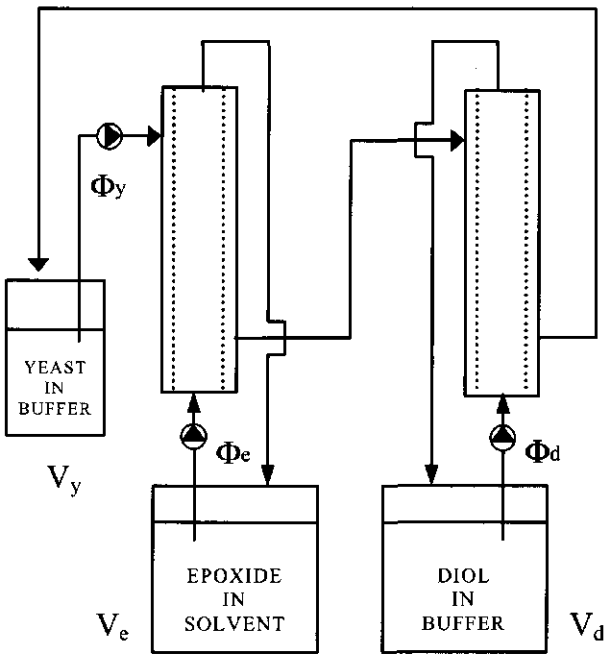
Membrane bioreactor for batchwise operation

Two identical hollow-fiber membrane units were used in cascade for epoxide hydrolysis and subsequent diol extraction. The hollow-fiber module consisted of a microporous hydrophilic ultrafiltration membrane (diameter 200 mm, thickness 6.5 μm, 6500 membranes/module). The ultrafiltration membrane was made of Hemophan, regenerated cellulose, with a diffusive surface area of 1.05 m^2 and housing material of acrylonitrile-butadiene-styrene copolymers. Bio Nephross Allegro membrane modules were purchased from COBE Nephross, The Netherlands.

Epoxide hydrolysis was performed by cells of *Rhodotorula glutinis* (7 g dw) in 100 mM potassium phosphate buffer pH 8.0 (V_y, 160 ml), placed in a waterbath at 35°C. The cell suspension was recirculated through the shell sides of the two membrane units by a peristaltic pump (Φ_y, 60 ml/min). Substrate, (±)-1,2-epoxyhexane dissolved in dodecane

(V_e, 75 ml), was recirculated through the lumen side of the first membrane unit in a counter current direction (Φ_e, 30 ml/min). For diol extraction, 100 mM potassium phosphate buffer pH 8.0 (V_d, 2.4 l) was recirculated through the lumen side of the second membrane unit (Φ_d, 60 ml/min).

The membrane bioreactor was operated for 3 h without cells to obtain the partition equilibrium of the epoxide substrate between the organic and aqueous phases. Chemically inert Viton (Cole-Palmer, USA) was used for connecting tubing. The membrane bioreactor could be reused several times without significant damage.

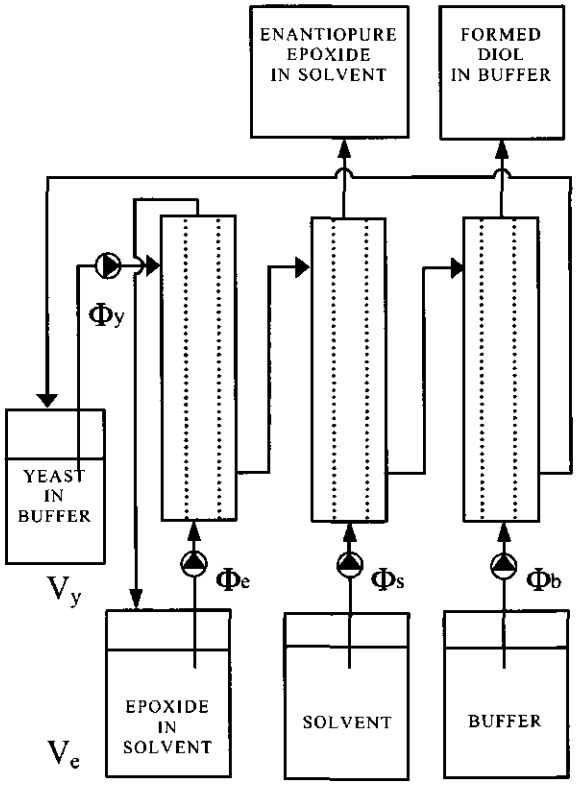


Scheme 2. Schematic diagram of a two-phase cascade membrane bioreactor for batch-wise epoxide resolution.

Samples of the reaction mixture were withdrawn separately from the organic solvent phase, cell suspension and diol extraction buffer at periodic intervals. Samples (100 μl) from the solvent phase were diluted ten times with ethyl acetate and analyzed directly by chiral GLC. Aqueous samples (300 μl) were mixed with an equal volume of ethyl acetate, centrifuged at 16,000 *g*, and the remaining organic supernatants analyzed by chiral GLC. The membrane bioreactor is schematically depicted in Scheme 2.

Membrane bioreactor for continuous operation

Three identical hollow-fiber membrane units were used in cascade for epoxide hydrolyis and subsequent extracting of residual epoxide and formed diol. The hollow-fiber modules used were identical as the ones applied in the batch-wise operation of the bioreactor.



Scheme 3. Schematic diagram of a two-phase cascade membrane bioreactor for continuous extractive epoxide resolution.

Epoxide hydrolyis was performed by cells of *Rhodotorula glutinis* (9 g dw) in 100 mM potassium phosphate buffer pH 8.0 (V_y, 400 ml), placed in a waterbath at 25°C. The cell suspension was recirculated through the shell sides of the three membrane modules by a peristaltic pump (Φ_y, 30 ml/min). Substrate, a solution of 1 M (±)-1,2-epoxyhexane dissolved in dodecane (V_e, 3 l), was recirculated through the lumen side of the first membrane unit in a counter current direction (Φ_e, 2 ml/min). In the second membrane unit, epoxide was continuously extracted from the cell suspension in the shell side by dode-

cane passing through the lumen side ((Φ_s, 10 ml/h). For diol extraction, 100 mM potassium phosphate buffer pH 8.0 was passed through the lumen side of the third membrane unit (Φ_d, 100 ml/h).

Sampling and subsequent analysis by GLC were in analogy as described for the batch-wise operation of the bioreactor. The membrane bioreactor for continuous operation is schematically depicted in Scheme 3.

References

1. Archelas, A. Fungal epoxide hydrolases: new tools for the synthesis of enantiopure epoxides and diols. *J. Mol. Catal. B: Enzymatic*. **1998**, *5*, 79-85.
2. Orru, R.V.A.; Archelas, A.; Furstoss, R.; Faber, K. Epoxide hydrolases and their synthetic applications. *Adv. Biochem. Eng.* **1999**, *63*, 145-167.
3. Weijers, C.A.G.M.; Bont de, J.A.M. Epoxide hydrolases from yeasts and other sources: versatile tools in biocatalysis *J. Mol. Catal. B: Enzymatic.* **1999**, *6*, 199-214
4. Pedragosa-Moreau, S.; Archelas, A.; Furstoss, R. Microbiological Transformations. 28. Enantiocomplementary epoxide hydrolyses as a preparative access to both enantiomers of styrene oxide. *J. Org. Chem.* **1993**, *58*, 5533-5536.
5. Orru, R.V.A.; Osprian, I.; Kroutil, W.; Faber, K. An efficient large-scale synthesis of (*R*)-(-)-mevalonolactone using simple biological and chemical catalysts. *Synthesis* **1998**, 1259-1263.
6. Morisseau, C.; Nellaiah, H.; Archelas, A.; Furstoss, R.; Baratti, J.C. Asymmetric hydrolysis of racemic *para*-nitrostyrene oxide using an epoxide hydrolase preparation from *Aspergillus niger*. *Enzyme Microb. Technol.* **1997**, *20*, 446-452.
7. Botes, A.L.; Weijers, C.A.G.M.; van Dyk, M.S. Biocatalytic resolution of 1,2-epoxyoctane using resting cells of different yeast strains with novel epoxide hydrolase activities. *Biotechnol. Lett.* **1998**, *20*, 421-426.
8. Goswami, A.; Totleben, M.J.; Singh, A.K.; Patel, R.N. Stereospecific enzymatic hydrolysis of racemic epoxide: a process for making chiral epoxide. *Tetrahedron Asymmetry* **1999**, *10*, 3167-3175.
9. Kühl, P.W. Biocatalysts operating at high substrate concentrations. In: Tramper, J.; Vermüe, M.H.; Beeftink, H.H; von Stockar, U. (eds), *Biocatalysis in non-conventional media*, Elsevier Science Publishers BV, Amsterdam, **1992**, 245-252.
10. Sikkema, J.; de Bont, J.A.M.; Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Reviews*, **1995**, *59*, 201-222.
11. Salter, G.J.; Kell, D.B. Solvent selection for whole cell biotransformations in organic media. *Crit. Rev. Biotechnol.*, **1995**, *15*, 139-177.
12. Cleij, M.; Archelas, A.; Furtstoss, R. Microbiological transformations. Part 42: A two-liquid-phase preparative scale process for an epoxide hydrolase catalysed resolution of *para*-bromo-α-methyl styrene oxide. Occurence of a susprising enantioselectivity enhancement. *Tetrahedron: Asymmetry* **1998**, *9*, 1839-1842.
13. Kronenburg, N.A.E.; Mutter, M.; Visser, H.; Bont de, J.A.M.; Weijers, C.A.G.M. Purification of an epoxide hydrolase from *Rhodotorula glutinis*. *Biotechnol. Lett.* **1999**, *21*, 519-524.

14. Nikolova, P.; Ward, O.P. Whole cell yeast biotransformations in two-phase systems: Effect of solvent on product formation and cell structure. *J. Ind. Microbiol.* **1992**, *10*, 169-177.
15. Gill, C.O.; Ratledge, C. Toxicity of *n*-alkanes, *n*-alk-1-enes, *n*-alkan-1-ols and *n*-alkyl-1-bromides towards yeasts. *J. Gen. Microbiol.* **1972**, *72*, 165-172.
16. Bar, R. Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. *J. Chem. Tech. Biotechnol.* **1988**, *43*, 49-62.
17. Omata, T.; Iwamoto, N.; Kimura, T.; Tanaka, A.; Fukui, S. Stereoselective hydrolysis of *dl*-menthyl succinate by gel-entrapped *Rhodotorula minuta* var. *texensis* cells in organic solvent. *Eur. J. Appl. Microbiol. Biotechnol.* **1981**, *11*, 199-204.
18. Nikolova, P.; Ward, O.P. Whole cell biocatalysts in nonconventional media. *J. Ind. Microbiol.* **1993**, *12*, 76-86.
19. León, R.; Fernandes, P.; Pinheiro, H.M.; Cabral, J.M.S. Whole-cell biocatalysis in organic media. *Enzyme Microb. Technol.* **1998**, *23*, 483-500.
20. Brink, L.E.S.; Tramper, J. Optimization of organic solvent in multiphase biocatalysis. *Biotechnol. Bioeng.* **1985**, *27*, 1258-1269.
21. Laane, C.; Boeren, S.; Vos, K. On optimizing organic solvents in multi-liquid-phase biocatalysis. *Trends Biotechnol.* **1985**, *3*, 251-252.
22. Bruce, L.J.; Daugulis, A.J. Solvent selection strategies for extractive biocatalysis. *Biotechnol. Prog.* **1991**, *7*, 116-124.
23. Laane, C.; Boeren, S.; Vos, K.; Veeger, C. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **1987**, *30*, 81-87.
24. Vermüe, M.; Sikkema, J.; Verheul, A.; Bakker, R.; Tramper, J. Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. *Biotechnol. Bioeng.* **1993**, *42*, 747-758.
25. Osborne, S.J.; Leaver, J.; Turner, M.K.; Dunnill, P. Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.* **1990**, *12*, 281-291.
26. Gabelman, A.; Hwang, S.T. Hollow fiber membrane contactors. *J. Membrane Sci.* **1999**, *159*, 61-106.
27. Van Sonsbeek, H.M.; Beeftink, H.H.; Tramper, J. Two-liquid-phase bioreactors. *Enzyme Microb. Technol.* **1993**, *15*, 722-729.
28. Prazeres, D.M.F.; Cabral, J.M.S. Enzymatic membrane bioreactors and their applications. *Enzyme Microb. Technol.* **1994**, *16*, 738-750.
29. Molinari, F.; Aragozzini, F.; Cabral, J.M.S.; Prazeres, D.M.F. Continuous production of isovaleraldehyde through extractive bioconversion in a hollow-fiber membrane bioreactor. *Enzyme Microb. Technol.* **1997**, *20*, 604-611.
30. Westgate, S.; Vaidya, A.M.; Bell, G.; Halling, P.J. High specific activity of whole cells in an aqueous-organic two-phase membrane bioreactor. *Enzyme Microb. Technol.* **1998**, *22*, 575-577.
31. Wu, D.R.; Belfort, G.; Cramer, S.M. Enzymatic resolution with a multiphase membrane bioreactor: A theoretical analysis. *Ind. Eng. Chem. Res.* **1990**, *29*, 1612-1621.
32. Gutman, A.L.; Meyer, E.; Kalerin, E.; Polyak, F.; Sterling, J. Enzymatic resolution of racemic amines in a continuous reactor in organic solvents. *Biotechnol. Bioeng.* **1992**, *40*, 760-767.
33. Sousa, H.A.; Crespo, J.G.; Afonso, C.A.M. Asymmetric hydrolysis of a *meso*-diester using pig liver esterase immobilised in hollow fiber ultrafiltration membrane. *Tetrahedron: Asymmetry* **2000**, *11*, 929-934.

34. Arroyo, M.; Torres-Guzmán, R.; de la Mata, I.; Pilar Castillón, M.; Acebal, C. Activation and stabilization of penicillin V acylase from *Streptomyces lavendulae* in the presence of glycerol and glycols. *Biotechnol. Prog.* **2000**, *16*, 368-371.
35. D'Cunha, G.B.; Satyanarayan, V.; Madhusudanan Nair, P. Stabilization of phenylalanine ammonia lyase containing *Rhodotorula glutinis* cells for the continuous synthesis of L-phenylalanine methyl ester. *Enzyme Microb. Technol.* **1996**, *19*, 421-427.
36. Gekko, K.; Timasheff, S.N. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry*, **1981**, *20*, 4667-4676.
37. Secundo, F.; Riva, S.; Carrea, G. Effects of medium and of reaction conditions on the enantioselectivity of lipases in organic solvents and possible rationales. *Tetrahedron: Asymmetry* **1992**, *3*, 267-280.
38. Wolff, A.; Straathof, A.J.J.; Jongejan, J.A.; Heijnen, J.J. Solvent induced change of enzyme enantioselectivity: rule or exception? *Biocatal. Biotransform.* **1997**, *15*, 175-184.
39. Nellaiah, H.; Morisseau, C.; Archelas, A.; Furtstoss, R.; Baratti, J.C. Enantioselective hydrolysis of *p*-nitrostyrene oxide by an epoxide hydrolase preparation from *Aspergillus niger*. *Biotechnol. Bioeng.* **1996**, *49*, 70-77.
40. Weijers, C.A.G.M.; Botes, A.L.; van Dyk, M.S.; de Bont, J.A.M. Enantioselective hydrolysis of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*. *Tetrahedron: Asymmetry* **1998**, *9*, 467-473.

7

Discussion

Enantiopure epoxides are versatile building blocks in fine chemistry. For their preparation, chemical and biotechnological production methods have been developed during the past decade. In the early start of the present study, no specific synthetic catalysts were yet available and enzymatic production of enantiopure epoxides had been described for mono-oxygenase reactions only. Mono-oxygenase-catalyzed epoxidations were, however, difficult to scale-up due to product inhibition by the toxic epoxides. A more promising alternative was expected from enzymatic kinetic resolution of racemic epoxides. Microorganisms, being easily accessible sources of enzymes, were therefore screened for the desired catalytic activity.

Enantioselective epoxide conversion has been first observed in microbial strains that produce epoxides as intermediates: alkene-utilizing bacteria. Suitable enzymatic activities (with values of $E > 20$) were found in strains of *Mycobacterium*, *Nocardia* and *Xanthobacter*. Based on its catalytic activities and growth characteristics, *Xanthobacter* Py2 was selected for this study.

Improved catalytic properties were subsequently found in epoxide hydrolase-containing yeasts and the epoxide hydrolase activity of *Rhodotorula glutinis* has been explored. Characteristics of both the cell structure and the yeast epoxide hydrolase allowed preparative-scale epoxide resolution by whole cells of this yeast in aqueous/organic two-phase media.

Bacterial epoxide carboxylase

Epoxide carboxylase activity was initially studied in cells of *Xanthobacter* Py2 for the kinetic resolution of 2,3-disubstituted aliphatic epoxides. (2*R*)-Enantiomers of short-chain aliphatic epoxides were resolved from racemic mixtures with very high enantioselectivities ($E > 100$). And, in addition, substrate concentrations of 100 mM or more were found not to be inhibiting the biocatalyst. Both these observations were interesting and epoxide conversion was studied in more detail in a subsequent study.

Epoxide substrates were converted to ketones *via* a hydroxy intermediate. The enzymatic isomerization was dependent on NAD^+ and a reducing cofactor, which could be replaced by dithiothreitol (DTT) or other synthetic dithiol compounds. The proposed putative enzyme mechanism suggested four steps for the epoxide isomerization reaction. The multi-step conversion and particularly the dependence on both an oxidizing and reducing cofactor, was judged to be a major disadvantage for further development of the epoxide carboxylase reaction. Consequently, application of this reaction in a kinetic resolution process would be feasible only in case of using a whole-cell system and not by using the isolated enzyme(s).

Follow-up studies by various other research groups concentrated on the metabolism of the physiological substrate 1,2-epoxypropane. In these studies, the enzymatic steps involved in 1,2-epoxypropane metabolism were eventually elucidated. The enzymes

involved in this mini-metabolic pathway are called the *epoxide carboxylase system*[1]. Also the physiological nucleophile and reducing cofactor were identified as 2-mercaptoethanesulfonic acid (CoM) and NADPH, respectively. It will now be of interest to investigate if the enzymes of the epoxide carboxylase system are similarly involved in the isomerization of non-physiological 2,3-disubstituted epoxides. Subsequent characterization of the enzyme responsible for the high enantioselectivity in this reaction can provide further insight into the structure requirements of chiral catalysts for epoxide resolution.

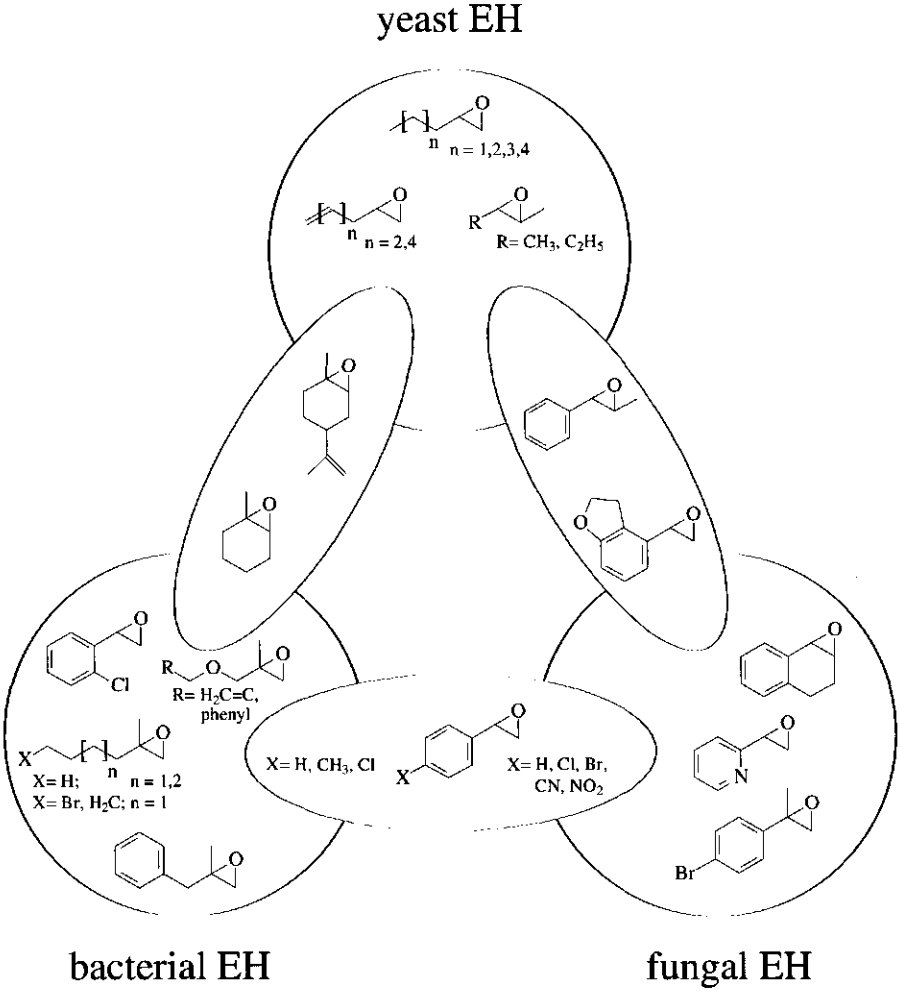
Yeast epoxide hydrolase

Initiated by the growing interest in enantiopure compounds, studies on the screening of alternative biocatalysts for kinetic resolution of epoxides were set up. For this, cofactor-independent epoxide hydrolases from microbial origin were tested and their substrate scopes mapped out. Initially, the biocatalytical potential of fungal and bacterial epoxide hydrolases was investigated by several other research groups. The enzymes from fungal and bacterial origin share a common catalytic activity but nevertheless are divergent with respect to substrate scope and operational characteristics. Properties of both enzyme sources could be possibly found combined in yeasts, since (*i*) these eukaryotic organisms may contain enzymes with fungal and mammalian-resembling substrate specificities and (*ii*) their unicellular appearance would be advantageous in cultivation and in whole-cell biocatalysis.

Yeast epoxide hydrolase (YEH) activity has been first demonstrated in our study on the hydrolysis of various epoxides by *Rhodotorula glutinis* ATCC 201718. The yeast enzyme showed a partially complementary substrate specificity in comparison with other microbial epoxide hydrolases (Scheme 1)[2-10]. Asymmetric hydrolysis of *meso* epoxides has been demonstrated as well and was interestingly restricted to the yeast enzyme. The substrate scopes, presented in Scheme 1, show that the structural requirements for YEH are less than for bacterial and fungal enzymes. Monosubstituted aliphatic epoxides, 2,3-disubstituted aliphatic and aryl epoxides, and trisubstituted alicyclic epoxides were all hydrolyzed by YEH with significant enantioselectivities ($E > 20$).

Interesting and typical substrates for YEH are the monosubstituted aliphatic epoxides. Enantiomeric discrimination was expected to be difficult for these highly flexible and rather 'slim' molecules. Therefore, kinetic resolution of a homologous range of aliphatic 1,2-epoxides by *Rhodotorula glutinis* was studied in more detail. Activities as well as enantioselectivities were found to be strongly influenced by the chain length of the substrate used. Similar patterns were observed in a subsequent study using these substrates and their unsaturated corresponding epoxyalkenes for YEH-catalyzed hydrolysis by *Rhodotorula glutinis* and related basidiomycetes yeast strains[8]. Best results, in case of *Rhodotorula glutinis* ATCC 201718, were obtained in the resolution of 1,2-epoxyhexa-

ne and its unsaturated structure analog 1,2-epoxyhexene. To date, no other epoxide hydrolases have been found suitable for the kinetic resolution of monosubstituted aliphatic epoxides. An alternative method for kinetic resolution of corresponding substrates is by using the synthetic Jacobsen's salen(Co)III(OAc) catalysts[11].



Scheme 1. Complementary and overlapping substrate specificities of microbial epoxide hydrolases. Substrates are selected when hydrolyzed with significant enantioselectivities (values of $E > 20$). *Meso* substrates are not included. Bacterial EH comprises epoxide hydrolases from the genera of *Rhodococcus*, *Nocardia*, *Streptomyces* and *Agrobacterium*, fungal EH those from *Aspergillus* and *Beauveria* and yeast EH those from *Rhodotorula* and *Rhodosporidium*.

The present study shows that YEH can accept structurally diverse molecules as substrates, which is a common feature of detoxifying enzymes in general. In this view it will be of interest to identify the physiological substrate(s) of YEH in order to (*i*) determine whether its biological function is involved in detoxification and (*ii*) to exploit its substrate scope even more effectively.

Preparative-scale YEH-catalyzed resolution

Scaling-up kinetic resolution of epoxides was studied by using cells of *Rhodotorula glutinis* for the resolution of 1,2-epoxyhexane, which was used as a model substrate. Substrate concentrations up to 100 mM could be effectively hydrolyzed in aqueous media. Under these conditions, substantial amounts of epoxide will however not be dissolved in the aqueous phase but will be highly dispersed without forming a separate layer. Scaling-up by further increase of substrate amounts seemed very attractive, because a simple two-phase system would be obtained with the excess epoxide serving as both organic phase and substrate reservoir. However, under these conditions the YEH-catalyzed reaction was severely inhibited due to (*i*) substrate toxicity and (*ii*) product inhibition by the formed diol.

In order to reduce substrate toxicity, biocompatible organic solvents were tested for application in an aqueous/organic two-phase reaction medium. Dodecane was selected to serve as substrate reservoir, showing no significant loss of catalytic productivity of the yeast cells (Figure 1, B_1). However, dodecane (and other biocompatible solvents) gave no protection to the formed diol and consequently further scaling-up was hampered. In this respect, a distinction can be made between the actual amount of epoxide recovered in the process by extraction (*effective* catalytic productivity) and the amount of epoxide resolved by the biocatalyst (*overall* catalytic productivity). Catalytic productivities of kinetic resolution of 1,2-epoxyhexane under various reaction conditions are shown in Figure 1.

An aqueous/organic two-phase membrane bioreactor in cascade configuration was applied for separate recirculation of the yeast cells, the organic solvent with concentrated epoxide, and formed diol in aqueous buffer. In this way, the diol concentration was kept below the critical concentration of 50 mM. Enantiopure (*S*)-epoxide was thus obtained as a concentrated 0.9 M solution in dodecane. The overall catalytic productivity was significantly enhanced in this process (Figure 1, B_3). The effective catalytic productivity was, however, slightly lower by some undesired loss of epoxide in the recirculating diol extracting buffer.

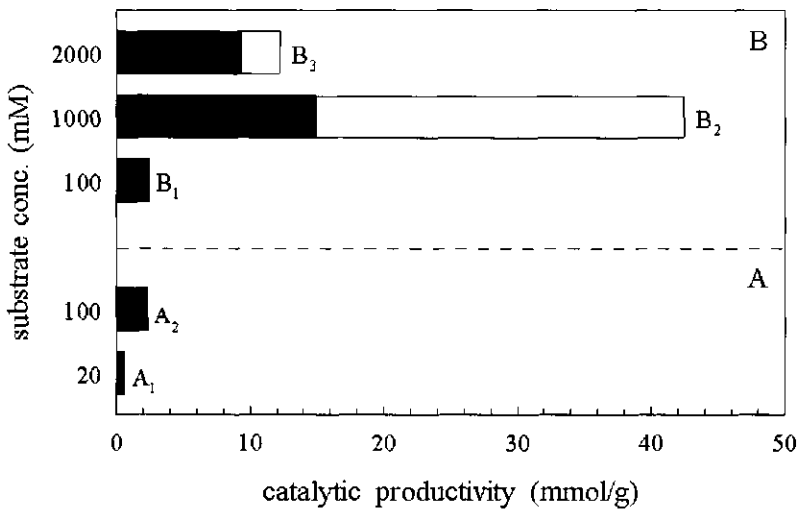


Figure 1. Overall (□) and effective (■) catalytic productivities in YEH-catalyzed kinetic resolutions of 1,2-epoxyhexane. Methods used are: kinetic resolution in aqueous buffer (A_1, A_2), in aqueous/dodecane two-phase medium (B_1), and by using a two-phase cascade membrane bioreactor in a batchwise operation mode (B_3) and in continuous extraction mode (B_2). Catalytic productivities are expressed as mmol (*S*)-1,2-epoxyhexane (e.e. > 98%) resolved from racemic substrate, per g dry weight of biocatalyst used. The overall catalytic productivity is based on the total amount of epoxide resolved by the biocatalyst; the effective catalytic productivity on the amount eventually recovered in the process. Amounts of biocatalyst were 0.18 g (A_1), 0.4 g (A_2), 0.4 g (B_1), 9 g (B_2) and 7 g (B_3) cells of *Rhodotorula glutinis*.

Almost similar strategies for scaling-up kinetic resolution of epoxides have been followed by other research groups in subsequent studies: either by using an aqueous/organic two-phase system without membrane[12], or a membrane bioreactor without organic solvent[13].

Further enhancement of the catalytic productivity was achieved by using the membrane bioreactor in a modified configuration for continuous extractive kinetic resolution. The modified configuration allowed continuous production of enantiopure epoxide without the need for complete resolution of the racemic substrate in the feed reservoir.

After 12 days of operation, an amount of 134 mmol (13.4 g) enantiopure (*S*)-epoxide (e.e. > 98%) was obtained by incomplete resolution of 3 mol substrate. A low yield of only 35% of the total resolved enantiopure (*S*)-epoxide (379 mmol), very much reduced the increase in overall catalytic productivity (Figure 1, B_2). The result was mainly caused by incomplete extraction of the enantiopure (*S*)-epoxide in the second membrane unit, followed by subsequent loss of the epoxide in the diol extraction buffer.

Optimization can possibly be achieved by using a solvent with a higher extracting capacity for the epoxide passing through the second membrane unit. However, significant amounts of diol may than be extracted as well, resulting in more complicated product isolation. In order to completely utilize the overall high catalytic productivity, a refined screening of solvents for selective epoxide extraction will then be necessary. Alternatively, better results will also be obtained in case of resolution of less water-soluble epoxides due to similar improved extraction characteristics.

YEH enzyme vs. whole cells

Crude enzyme preparations of YEH can be obtained from cells of *Rhodotorula glutinis* in a yield of 90% of the total activity. Further purification will however result in a low yield of only 3% of this membrane-associated enzyme[14]. In organic solvent-containing reaction media, it can be advantageous to use enzymes in stead of whole cells because enzymes require smaller amounts of water to maintain their catalytic activity. The activity of crude YEH enzyme was tested in a dodecane-containing reaction mixture. From this, the minimal amount of water to maintain activity was determined as 1% (v/v) of the total reaction volume (10% moisture content related to the amount of protein). The use of the YEH enzyme on a large-scale has however been hampered by the limited amounts of enzyme that can be isolated from *Rhodotorula glutinis*.

YEH-containing cells of *Rhodotorula glutinis*, on the other hand, are easily available in large quantities and have attractive intrinsic biocatalyst characteristics like (*i*) an unicellular appearance and a relatively low sensitivity towards (*ii*) mechanical shear damage and (*iii*) toxicity of organic solvents. These characteristics, the good availability, and the absence of undesired side-reactions or product degradation, make the use of whole cells more advantageous to study preparative-scale reactions. By using cells of *Rhodotorula glutinis*, the feasibility of preparative-scale YEH-catalyzed epoxide resolution has been demonstrated and the critical parameters for scaling-up determined.

Further application of YEH

In the course of the present study, related studies were set up in our laboratory to characterize the YEH enzyme and its encoding gene. As a result from these studies, the YEH enzyme of *Rhodotorula glutinis* has been purified[14] and its encoding gene cloned and characterized[15]. Subsequently, the YEH has been overexpressed in *Escherichia coli*[16].

The productivities shown in Table 1 indicate, that both the YEH and the synthetic Jacobsen's catalyst intrinsically can be equally effective. The use of the synthetic catalyst has already been demonstrated in a preparative-scale resolution. But the YEH, either as isolated enzyme or overexpresed in *E. coli*, has been tested only on a smaller scale.

Table 1. Overall catalytic productivities in batch-wise kinetic resolutions of 1,2-epoxyhexane

Catalyst	amount of resolved (*S*)-1,2-epoxyhexane	yield	productivity (mmol/g)	ref.
YEH / cells of *Rhodotorula glutinis*	90 mmol	40 %	12	present study
YEH / cells of recombinant *E. coli* BL21(DE3)	4.10^{-3} mmol	40 %	133	16
YEH / enzyme of *Rhodotorula glutinis*	4.10^{-3} mmol	40 %	333	14
Jacobsen's catalyst / (*S*,*S*)-salen(Co)III(OAc)	460 mmol	46 %	164	11

A major challenge for application of the improved catalytic productivity of YEH will be the scaling-up from bench-scale reactions to a large-scale production process. For this, application of either (*i*) the isolated YEH enzyme or (*ii*) cells from a host, containing overexpressed YEH, should be further investigated. In the case of whole-cell application, expression of YEH in a more suitable host will probably be necessary. Cells of recombinant *E. coli* don't have the favorable biocatalyst characteristics like the wild-type *Rhodotorula glutinis*, and are thus much less applicable for whole-cell epoxide resolutions on a large-scale. However, the price to be paid for replacement of *E. coli* by another host, might be a lower expression of YEH. Alternatively, *E. coli* can be effectively used for the production of high amounts of YEH enzyme, for further development of a process with isolated enzyme.

In conclusion, the catalytic properties of YEH make this biocatalyst the ultimate candidate for application in a large-scale process for the preparation of a number of enantiopure epoxides.

References

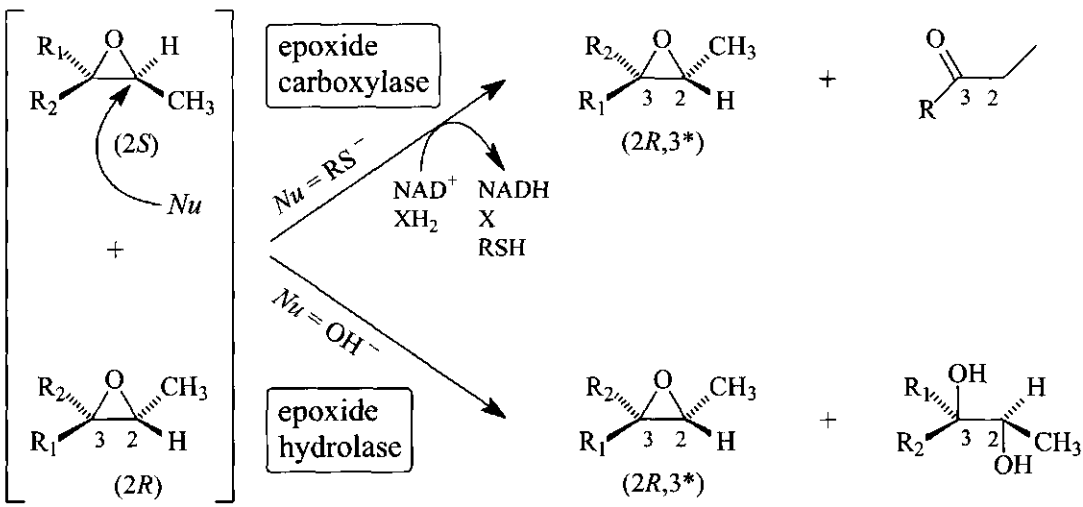
1. Clark, D.D.; Allen, J.R.; Ensign, S.A. Characterization of five catalytic activities associated with the NADPH: 2-ketopropyl-coenzyme M[2-(2-ketopropylthio)ethanesulfonate] oxidoreductase/carboxylase of the *Xanthobacter* strain Py2 epoxide carboxylase system. *Biochemistry* **2000**, *39*, 1294-1304.
2. Werf van der, M.J.; de Bont, J.A.M.; Swarts, H.J. Acid-catalyzed enzymatic hydrolysis of 1-methylcyclohexene oxide. *Tetrahedron: Asymmetry* **1999**, *10*, 4225-4230.
3. Kroutil, W.; Faber, K. Stereoselective syntheses using microbial epoxide hydrolases. *In*: Patel, R.N. (ed.), *Stereoselective Biocatalysis*, Dekker, New York, **2000**, 205-237.

4. Steinreiber, A.; Osprian, I.; Mayer, S.F.; Orru, R.V.A.; Faber, K. Enantioselective hydrolysis of functionalized 2,2-disubstituted oxiranes with bacterial epoxide hydrolases. *Eur. J. Org. Chem.* **2000**, 3703-3711.
5. Genzel, Y.; Archelas, A.; Broxterman, Q.B.; Schulze, B.; Furstoss, R. Microbiological transformations. Part 46: preparation of enantiopure (S)-2-pyridyloxirane via epoxide hydrolase-catalysed kinetic resolution. *Tetrahedron: Asymmetry* **2000**, *11*, 3041-3044.
6. Choi, W.J. Biocatalytic production of enantiopure epoxides using epoxide hydrolase. Dissertation Seoul National University **2001**, pp 25-33.
7. Goswami, A.; Totleben, M.J.; Singh, A.K.; Patel, R.N. Stereospecific enzymatic hydrolysis of racemic epoxide: a process for making chiral epoxide. *Tetrahedron: Asymmetry* **1999**, *10*, 3167-3175.
8. Botes, A.L.; Weijers, C.A.G.M.; Botes, P.J.; van Dyk, M.S. Enantioselectivities of yeast epoxide hydrolases for 1,2-epoxides. *Tetrahedron: Asymmetry* **1999**, *10*, 3327-3336.
9. Weijers, C.A.G.M.; de Bont, J.A.M. Epoxide hydrolases from yeasts and other sources: versatile tools in biocatalysis. *J. Mol. Catal. B* **1999**, *6*, 199-214.
10. Zocher, F.; Enzelberger, M.M.; Bornscheuer, U.T.; Hauer, B.; Wohlleben, W.; Schmid, R.D. Epoxide hydrolase activity of *Streptomyces* strains. *J. Biotechnol.* **2000**, *77*, 287-292.
11. Jacobsen, E.N.. Asymmetric catalysis of epoxide ring-opening reactions. *Acc. Chem. Res.* **2000**, *33*, 421-431.
12. Baldascini, H.; Ganzeveld, K.J.; Janssen, D.B.; Beenackers, A.A.C.M. Effect of mass transfer limitations on the enzymatic kinetic resolution of epoxides in a two-liquid-phase system. *Biotechnol. Bioeng.* **2001**, *73*, 44-54.
13. Krieg, H.M.; Breytenbach, J.C.; Keizer, K. Resolution of 1,2-epoxyoctane by enantioselective catalytic hydrolysis in a membrane bioreactor. *J. Membrane Sci.* **2000**, *180*, 69-80.
14. Kronenburg, N.A.E.; Mutter, M.; Visser, H.; de Bont, J.A.M.; Weijers, C.A.G.M. Purification of an epoxide hydrolase from *Rhodotorula glutinis*. *Biotechnol. Lett.* **1999**, *21*, 519-524.
15. Visser, H.; Vreugdenhil, S.; de Bont, J.A.M.; Verdoes, J.C. Cloning and characterization of an epoxide hydrolase-encoding gene from *Rhodotorula glutinis*. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 415-419.
16. Visser, H.; de Oliveira Villela Filho, M.; Liese, A.; Weijers, C.A.G.M.; Verdoes, J.C. Construction and characterization of a genetically engineered *Escherichia coli* strain for the epoxide hydrolase-catalyzed kinetic resolution of epoxides. Submitted for publication.

Summary

Molecular chirality is of great importance for many processes in biological systems. Examples are interactions with enzymes and receptor systems for hormones, sensory recognition and drug metabolism. Activation of biological activity when initiated by interaction with bioactive compounds is highly based on complementary stereochemistry. Synthetic bioactive compounds, like agrochemicals and pharmaceuticals, are therefore now preferably produced as single enantiomers.

Various bioactive compounds can be effectively prepared from enantiopure epoxides. These chiral building blocks can be used to introduce one or two adjacent chirality centers in a target molecule. In the present study, enzymatic kinetic resolution *via* direct epoxide ring-opening has been studied for the preparation of enantiopure epoxides. For this method, the biocatalytic activities of a bacterial epoxide carboxylase and a yeast epoxide hydrolase have been explored (Scheme 1).



Scheme 1. Kinetic resolution of *cis*- (R_1= H, R_2= alkyl) and *trans*- (R_1= alkyl, R_2= H) 2,3-disubstituted epoxides by enzymatic nucleophilic ring-opening. XH_2 represents NADPH or a reducing dithiol compound. (Ketone: R= alkyl).

Bacterial epoxide carboxylase

In alkene-utilizing bacteria, epoxides are generated by mono-oxygenases and subsequently further degraded. The epoxide-degrading enzyme system has been recently identified as an epoxide carboxylase. However, in the absence of CO_2, the reaction catalyzed is actually an isomerization of the epoxide. The enzyme can therefore also be regarded as an epoxide isomerase.

Epoxide carboxylase/isomerase from *Xanthobacter* Py2 was found enantioselective in the conversion of 2,3-disubstituted aliphatic epoxides (Scheme 1). Only (2*S*)-enantiomers were converted by propene-grown cells of *Xanthobacter* Py2 and (2*R*)-enantiomers were thus resolved from a racemic mixture with almost maximal feasible yield (*Chapter 2*). Aliphatic 1,2-epoxides, being intermediates in 1-alkene metabolism, were converted without remarkable enantioselectivity.

In the subsequent study, epoxide bioconversion was studied in more detail (*Chapter 3*). Epoxide substrates were found to be converted to ketones *via* a hydroxy intermediate. The enzymatic reaction was dependent on NAD^+ and a reducing cofactor, which could be replaced by synthetic dithiol compounds. Based on these findings, a four-step reaction mechanism was proposed starting from nucleophilic ring-opening of the epoxide. Follow-up studies by various other research groups concentrated on the metabolism of the physiological substrate 1,2-epoxypropane. By these studies, the enzymatic steps involved in 1,2-epoxypropane metabolism were further elucidated.

Yeast epoxide hydrolase

Cofactor-independent microbial epoxide hydrolases are generally regarded as attractive biocatalytical tools. Epoxide hydrolase catalyzed ring-opening of epoxides can be exploited for the production of enantiopure epoxides and vicinal diols (Scheme 1). The biocatalytical potential of microbial epoxide hydrolases has been first recognized in studies using enzymes from fungal and bacterial origin. Epoxide hydrolase activities in yeasts have been subsequently explored.

Yeast epoxide hydrolase (YEH) activity has been demonstrated for the hydrolysis of various structurally divergent epoxides by *Rhodotorula glutinis* ATCC 201718 (*Chapter 4*). Very high enantioselectivities were determined in the hydrolysis of 2,3-disubstituted aryl and aliphatic epoxides (Scheme 1). Asymmetric hydrolysis of *meso* epoxides has been demonstrated and interestingly this property has been restricted to yeasts in particular.

Typical other substrates for the yeast enzyme are monosubstituted aliphatic epoxides. Enantiomeric discrimination was expected to be difficult for these highly flexible and rather 'slim' molecules. Therefore, kinetic resolution of a homologous range of aliphatic 1,2-epoxides by *Rhodotorula glutinis* was studied in more detail (*Chapter 5*). Activities as well as enantioselectivities were found to be strongly influenced by the chain length of the substrate used. Best results were obtained in the resolution of 1,2-epoxyhexane.

Preparative-scale YEH-catalyzed resolution

Preparative-scale kinetic resolutions were investigated with 1,2-epoxyhexane as a model substrate and cells of *Rhodotorula glutinis* as biocatalyst (*Chapter 6*). Scaling-up was hampered by inhibition due to substrate toxicity, and to an even higher extend, by product inhibition of the formed diol. A critical inhibitory diol concentration was determined as 50 mM for 1,2-hexanediol. For protection against high epoxide concentrations, aqueous/organic two-phase reaction media were tested. Long-chain aliphatic alkanes were suitable biocompatible solvents and dodecane was selected for further applications. However, dodecane and other biocompatible solvents gave no protection towards the diol.

Preparative-scale resolution of 1,2-epoxyhexane (22 g) was performed successfully in an aqueous/organic two-phase membrane bioreactor. A cascade configuration of two hollow-fiber membrane modules was used (*i*) to separate the biocatalyst from the organic solvent containing feed solution with concentrated epoxide (2 M) and (*ii*) to remove inhibitory amounts of diol.

In a modified process design, the membrane bioreactor was used for continuous extractive kinetic resolution of 1,2-epoxyhexane (1 M in dodecane). Under these conditions, enantiopure (*S*)-epoxide (13 g) was obtained in the effluent solvent phase. The process allowed long-term continuous production of enantiopure epoxide without the need for complete resolution of the racemic substrate in the feed reservoir. Optimization of this process will however be necessary for improvement of the productivity.

Samenvatting

Epoxiden zijn verbindingen die door hun cyclische structuur een hoge chemische reactiviteit bezitten. De meest bekende reactie van epoxiden is de nucleofiele substitutie, waarbij na ringopening een 1,2-additie product gevormd wordt. Op deze manier kunnen diverse substituenten als nucleofiel in het reactieproduct gebracht worden. In enantiomeer zuivere vorm kunnen epoxiden gebruikt worden voor de synthese van specifieke biologisch actieve stoffen. Bij interacties tussen receptoren en biologisch actieve stoffen speelt de enantiomere zuiverheid van biologisch actieve stoffen vaak een grote rol voor het genereren van de gewenste activiteit.

Een methode om enantiomeren in zuivere vorm te verkrijgen is de kinetische resolutie van racemische mengsels. In het geval van enantiomeer zuivere epoxiden, is nucleofiele ringopening met een selectieve chemische- of enzymatische katalysator, hiervoor een geschikte reactie. Enzymatische opening van epoxiden kan op eenvoudige wijze uitgevoerd worden onder de milde omstandigheden zoals die plaatsvinden in biologische systemen.

Het onderzoek, zoals beschreven in dit proefschrift, was gericht op de bestudering van de kinetische resolutie van epoxiden via ringopening met behulp van microbiële enzymen. De methode werd bestudeerd met een bacterieel epoxidecarboxylase en een gist epoxidehydrolase.

Bacterieel epoxidecarboxylase

Biologisch gevormde epoxiden ontstaan in de meeste gevallen door mono-oxygenase gekatalyseerde epoxidaties van alkenen of aromaten. Gezien de hoge toxiciteit van epoxiden hebben epoxide producerende organismen eveneens systemen om de epoxiden via ringopening onschadelijk te maken. In alkeen gebruikende bacteriën worden epoxiden evenwel gevormd als intermediairen in het alkeen metabolisme. Het bacterieel alkeen metabolisme is uitgebreid bestudeerd aan *Xanthobacter* Py2. Groeisubstraten voor *Xanthobacter* Py2 waren bekend als (C_2 tot C_6) 1-alkenen en overeenkomstige 1,2-epoxiden werden dan ook volledig omgezet.

Conversie van methylgesubstitueerde 2,3-epoxiden door *Xanthobacter* Py2 ging daarentegen gepaard met een hoge enantioselectiviteit ($E > 100$). Van racemische substraten werden enkel de (2*S*)-enantiomeren omgezet zodat (2*R*)-enantiomeren in zuivere vorm verkregen werden (*Hoofdstuk 2*).

Epoxide conversie werd vervolgens bestudeerd in celextracten van *Xanthobacter* Py2 (*Hoofdstuk 3*). De reactie bleek afhankelijk te zijn van zowel NAD^+ als een reducerende cofactor, welke vervangen kon worden door dithiol verbindingen. Ketonen werden geïdentificeerd als producten van de reactie met 1,2- en (2*S*)-epoxiden. Aan de hand van de resultaten werd een reactiemechanisme opgesteld. Ringopening van het epoxide en NAD^+-afhankelijke oxidatie van een ß-hydroxythioether intermediair, vormden de centrale reacties in dit mechanisme. Het fysiologische mechanisme voor de omzetting van

1,2-epoxypropaan is vervolgens door andere onderzoeksgroepen verder in detail bestudeerd (zie *Hoofdstuk 1, 1.1.3.*). De laatste stap van dit mechanisme geeft aan dat in aan- of afwezigheid van CO_2, respectievelijk een ß-ketocarbonzuur of een keton als reactieproduct kan ontstaan. Het betrokken enzymsysteem is overeenkomstig omschreven als een epoxidecarboxylase.

Gist epoxidehydrolase

Het huidige onderzoek werd voortgezet met de bestudering van een epoxidehydrolase uit gist. Epoxidehydrolasen katalyseren de additie van water aan epoxiden en maken op deze wijze deel uit van het detoxificerende enzymsysteem van vele organismen.

Epoxidehydrolase activiteit in gist werd beschreven voor *Rhodotorula glutinis* ATCC 201718 (*Hoofdstuk 4*). Een opvallende eigenschap van dit epoxidehydrolase was de grote variatie in molecuulstructuren die als substraat geaccepteerd werden. Daarnaast was asymmetrische hydrolyse van *meso* substraten nog niet waargenomen bij andere, reeds bekende bacteriële en schimmel epoxidehydrolasen. Hoge enantioselectiviteiten ($E > 100$) werden gevonden in de hydrolyse van methylgesubstitueerde aryl- en alifatische 2,3-epoxiden.

Hydrolyse van een reeks alifatische 1,2-epoxiden werd vervolgens in detail bestudeerd (*Hoofdstuk 5*). Enzymatische kinetische resolutie met voldoende enantioselectiviteit was voor deze substraten nog niet mogelijk gebleken. Het ontbreken van specifieke substituenten en de grote bewegingsmogelijkheid van deze groep van substraten zou een selectieve binding van de enantiomeren bemoeilijken. Deze verwachting werd bij epoxide hydrolyse door *Rhodotorula glutinis* echter maar ten dele waargenomen. De resultaten waren afhankelijk van het gebruikte substraat: de ketenlengte van het epoxide bleek een grote invloed te hebben op de activiteit en enantioselectiviteit van het enzym. Goede resultaten werden verkregen bij de resolutie van 1,2-epoxyhexaan.

Opschaling van de epoxidehydrolase reactie

Opschaling van de kinetische resolutie werd bestudeerd met 1,2-epoxyhexaan als modelsubstraat en *Rhodotorula glutinis* als biokatalysator (*Hoofdstuk 6*). Kritische parameters in de opschaling bleken remming van de reactie door toxiciteit van het epoxide en daarnaast door hoge concentraties diol. Remming door 1,2-hexaandiol werd waargenomen bij concentraties van 50 mM of hoger.

Scheiding tussen gistcellen en hoge concentraties epoxide is mogelijk door toepassing van een reactiemedium met een gescheiden tweede fase, bestaande uit een organisch oplosmiddel met daarin opgelost substraat. Oplosmiddelen die zelf weinig remming van de epoxidehydrolase reactie vertoonden, werden gevonden in de groep van apolaire alifatische alkanen. Hiervan is dodecaan geselecteerd voor verdere toepassingen.

Gebruik van hollevezel membranen bleken effectief voor een proces met gescheiden circulatie van gistcellen en buffer met diol. Op deze manier werd de concentratie van het gevormde diol voldoende laag gehouden. Minimaal contact tussen gistcellen en epoxide was mogelijk door gescheiden circulatie van celsuspensie en geconcentreerd 1,2-epoxyhexaan in dodecaan. Epoxide resolutie werd daarom uitgevoerd door recirculatie van gistcellen door twee membraan modulen: de eerste module voor hydrolyse van het epoxide en de tweede module voor verwijdering van het gevormde diol. Met de methode werd 6,5 g (*S*)-1,2-epoxyhexaan verkregen, als een 0,9 M oplossing in dodecaan, na volledige resolutie van 22 g substraat.

Kinetische resolutie met continue extractie werd uitgevoerd door toepassing van drie membraan modules en modificatie van het proces. Hierbij werd (*S*)-1,2-epoxyhexaan, verkregen na resolutie van het substraat, direct geëxtraheerd in dodecaan en kwam daardoor continu en apart beschikbaar. Op deze manier kon 13 g enantiomeer zuiver epoxide worden verkregen zonder volledige kinetische resolutie van het substraat in het voorraad reservoir. Optimalisering van de continue extractie zal nodig zijn om de epoxidehydrolase activiteit meer efficiënt te kunnen benutten.

Nawoord

Een proefschrift is als bekroning van een onderzoek gewoonlijk als een van de doelstellingen in een onderzoeksproject opgenomen. Bij het huidige onderzoek was er aanvankelijk nog geen doel om een proefschrift te schrijven. Het hier beschreven onderwerp leverde dan ook louter het plezier om in grote vrijheid met het onderzoek bezig te zijn en aan de hieraan verbonden 'randactiviteiten' te kunnen deelnemen.

Bij de afsluiting van het onderzoek werd door mijn promotor Jan de Bont het voorstel gedaan om de resultaten in een proefschrift samen te vatten. Jan, voor deze mogelijkheid om te kunnen promoveren ben ik je dan ook zeer dankbaar. Ook wil ik je bedanken voor de nimmer aflatende belangstelling en waardering voor het epoxide-onderzoek tijdens de gehele periode. Het was vooral daardoor aangenaam om te werken in de bijzondere werkomgeving van Industriële Microbiologie. Naast de aanpak van mijn eigen onderwerp heb ik ook veel van je geleerd over de organisatie van onderzoek en onderwijs in het algemeen. Ondanks de veranderde situatie was het overleg tijdens het schrijven van het boekje gelukkig nog steeds als vanouds.

Lof ook voor de mensen van Organische Chemie en Proceskunde. Jullie steun in het afgelopen jaar maakte het mede mogelijk dat ik het onderzoek op positieve wijze heb kunnen afronden. Met name Maurice Franssen en Ernst Sudhölter wil ik bedanken voor hun inzet om bij Organische Chemie te kunnen komen en mij de ruimte te geven om het proefschrift af te ronden. Zonder hun steun zou ik niet aan het boekje zijn begonnen. Maurice, daarnaast wil ik je bedanken voor je hulp bij 'mechanistische chemie' en voor de bijdrage die je samen met Hugo Jongejan hebt geleverd aan het onderzoek zoals beschreven in hoofdstuk 3.

Het grootste deel van het werk is van dichtbij meegemaakt door mijn ex-kamergenoot Martin de Wit. Martin, jouw steun en manier van relativeren heeft mij vaak geholpen om de zaken op het goede spoor te houden. Ook de directe hulp bij het gebruik van apparatuur en PC's is voor mij altijd belangrijk geweest om het onderzoek te kunnen uitvoeren. Bedankt voor je collegialiteit.

Een woord van dank eveneens aan Jan Verdoes. Jan, in de periode dat ik aan dit boekje werkte was jij degene die nog direct in de buurt was om mijn vragen en twijfels aan te horen. Jouw vermogen om onder alle omstandigheden gewoon door te werken was voor mij (en vele andere IM-ers) een sterke stimulans om zelf ook door te gaan. Bedankt voor je steun en voor het overnemen van diverse taken in die periode.

Een stapeltje onderzoeksresultaten is nog geen proefschrift. Om van de tekst en plaatjes uiteindelijk een proefschrift te maken is het werk van Karin Scheele essentieel geweest. Karin, dankzij jouw ervaring en inzet is er nu een boekje met een uniforme layout. Ook voor de vele adviezen en begeleiding tijdens het eindtraject ben ik je heel dankbaar.

Door een aantal studenten is tijdens hun afstudeervak een bijdrage aan het onderzoek geleverd. Met betrekking tot het eerste onderwerp wil ik daarvoor bedanken: Jan Dirk Jansma, Nicolette Klijn, Imke Leenen, Bart Fraaye, Maureen Cox, Marco Fraaye en in

het bijzonder André de Haan die voor een belangrijk deel aan de start van het onderzoek heeft bijgedragen. Het epoxidehydrolase onderzoek heeft vorm gekregen dankzij de bijdrage van Eyke van den Ban, Bert Beekman, Anneke van der Zwet, Ilse van Nijhuis, Herman van Herk en Jeroen Rouwkema.

Een sterke impuls voor het onderzoek was ook de samenwerking met binnenlandse en buitenlandse collega's. Hierbij is de hulp van Henk Swarts vooral terug te vinden in de hoofdstukken 4 en 5. Henk, bedankt dat je altijd wel tijd wist vrij te maken om me te helpen bij de synthese en analyse van nieuwe verbindingen. Op een heel ander terrein was de samenwerking met Hans Visser. Hans, bedankt dat je me wilde inwijden (of althans geprobeerd hebt) in de moleculaire biologie van het gist epoxidehydrolase. Ik ben blij dat jouw onderzoek uiteindelijk de goede kant op is gegaan en nu ook met een boekje afgerond kan worden.

I am grateful to Christoph Syldatk, David Leak and Manuela da Fonseca for their contribution to the study on the epoxide isomerization. Dear David, I enjoyed and learned a lot from the discussions with you during participation in the EC-project.

Very successful was the collaboration with Martie van Dyk and Adri Botes from the University of the Orange Free State in South Africa. Dear Martie and Adri, I very much enjoyed the time I spent in Bloemfontein. Our work has initiated the use of aliphatic 1,2-epoxides as substrates for the yeast epoxide hydrolase and our first joint paper is included as chapter 5 in this thesis.

Chapter 6 is based on the work done together with Won Jae Choi from the Republic of South Korea. Dear Won Jae, from the day of your arrival it was a pleasure to work with you during your stay here. It was remarkable how you rapidly adapted to our life and actually felt like a Dutchman. From all the work you have done we were able to publish only a small part of it. The results are however of great importance for the application of the yeast epoxide hydrolase.

Lieve Elly, Julia en Paul, een deel van het boekje is geschreven in de tijd die jullie hebben ingeleverd ten koste van andere activiteiten. Bedankt dat jullie me een jaartje 'vrijaf' hebben gegeven om aan het proefschrift te kunnen werken.

Carel

Curriculum vitae

Carel Weijers werd op 17 mei 1957 geboren te Nijmegen. Aan het Canisiuscollege-Mater Dei in diezelfde plaats werd in 1974 het HAVO diploma behaald. Vervolgens werd de opleiding voor laboratorium medewerker gevolgd aan de HMLS te Oss, richting HBO-B biochemie. Na afsluiting hiervan werd hij in 1977 aangesteld in dienst van de Stichting voor Biofysica bij het Laboratorium voor Chemische Cytologie aan de Katholieke Universiteit Nijmegen. In 1980 werd de overstap gemaakt naar de toenmalige Landbouwhogeschool te Wageningen. Het dienstverband werd gestart bij het Laboratorium voor Microbiologie, sectie Technische Microbiologie.

Vanaf de oprichting in 1987 was hij in dienst bij de sectie Industriële Microbiologie aan de Landbouwuniversiteit Wageningen. Bij de sectie Industriële Microbiologie verrichtte hij onderzoek binnen het thema 'biotransformaties', daarnaast was hij werkzaam als financieel beheerder en verzorgde hij de organisatie en de begeleiding van het praktische onderwijs. Het onderzoek richtte zich op microbiële omzettingen voor de productie van epoxiden. Een groot deel van het onderzoek is beschreven in dit proefschrift. De loopbaan werd vanaf januari 2001 voortgezet bij het Laboratorium voor Organische Chemie aan de Wageningen Universiteit.

List of publications

C.A.G.M. Weijers, C.G. van Ginkel and J.A.M. de Bont.
Enantiomeric composition of lower epoxyalkanes produced by methane-, alkane- and alkene-utilizing bacteria.
Enzyme and Microbial Technology, **1988**, *10*, 214 - 218.

C.A.G.M. Weijers, A. de Haan and J.A.M. de Bont.
Microbial production and metabolism of epoxides.
Microbiological Sciences, **1988**, *5*, 156 - 159.

C.A.G.M. Weijers, A. de Haan and J.A.M. de Bont.
Chiral resolution of 2,3-epoxyalkanes by *Xanthobacter* Py2.
Applied Microbiology and Biotechnology, **1988**, *27*, 337 - 340.

C.A.G.M. Weijers and J.A.M. de Bont.
Enantioselective degradation of 1,2-epoxyalkanes by *Nocardia* H8.
Enzyme and Microbial Technology, **1991**, *13*, 306 - 308.

C.A.G.M. Weijers, M.J.J. Litjens and J.A.M. de Bont.
Synthesis of optically pure 1,2-epoxypropane by microbial asymmetric reduction of chloroacetone.
Applied Microbiology and Biotechnology, **1992**, *38*, 297 - 300.

J. Swaving, C.A.G.M. Weijers, C.K. Chan Kwo Chion, D.J. Leak, A.J.J. van Ooyen and J.A.M. de Bont.
Initial characterization of the enzyme and cloning of genes involved in the enantioselective epoxyalkane degradation by *Xanthobacter* Py2.
Biocatalysis, **1994**, *10*, 227 - 232.

C.A.G.M. Weijers, H. Jongejan, M.C.R. Franssen, Ae. de Groot and J.A.M. de Bont.
Dithiol- and NAD-dependent degradation of epoxyalkanes by *Xanthobacter* Py2.
Applied Microbiology and Biotechnology, **1995**, *42*, 775 - 781.

J. Swaving, C.A.G.M. Weijers, A.J.J. van Ooyen and J.A.M. de Bont.
Complementation of *Xanthobacter* Py2 mutants defective in epoxyalkane degradation, and expression and nucleotide sequence of the complementing DNA fragment.
Microbiology, **1995**, *141*, 477 - 484.

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

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

C.A.G.M. Weijers, A.L. Botes, M.S. van Dyk and J.A.M. de Bont.
Enantioselective hydrolysis of unbranched aliphatic 1,2-epoxides by *Rhodotorula glutinis*.
Tetrahedron: Asymmetry, **1998**, *9*, 467 - 473.

N.A.E.Kronenburg, M. Mutter, H. Visser, J.A.M. de Bont and C.A.G.M. Weijers.
Purification of an epoxide hydrolase from *Rhodotorula glutinis*.
Biotechnology Letters, **1999**, *21*, 519 - 524.

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

C.A.G.M. Weijers and J.A.M. de Bont.
Epoxide hydrolases from yeasts and other sources: versatile tools in biocatalysis.
Journal of Molecular Catalysis B: Enzymatic, **1999**, *6*, 199 - 214.

W.J. Choi, C.Y. Choi, J.A.M. de Bont and C.A.G.M. Weijers.
Resolution of 1,2-epoxyhexane by *Rhodotorula glutinis* using a two-phase membrane bioreactor.
Applied Microbiology and Biotechnology, **1999**, *53*, 7 - 11.

W.J. Choi, C.Y. Choi, J.A.M. de Bont and C.A.G.M. Weijers.
Continuous production of enantiopure 1,2-epoxyhexane by yeast epoxide hydrolase in a two-phase membrane bioreactor.
Applied Microbiology and Biotechnology, **2000**, *54*, 641-646.

H. Visser, M. de Oliveira Villela Filho, A. Liese, C.A.G.M. Weijers and J.C. Verdoes.
Construction and characterization of a genetically engineered *Escherichia coli* strain for the epoxide hydrolase-catalyzed kinetic resolution of epoxides.
Submitted for publication.

H. Visser, C.A.G.M. Weijers, A.J.J. van Ooyen and J.C. Verdoes.
Cloning, characterization and heterologous expression of epoxide hydrolase-encoding cDNA sequences from yeasts belonging to the genera *Rhodotorula* and *Rhodosporidium*.
Manuscript in preparation.

B.W.T. Gruijters, A. van Veldhuizen, C.A.G.M. Weijers and J.B.P.A. Wijnberg.
Total synthesis and bioactivity of some naturally occurring pterulones.
Submitted for publication.

Cover: Boudewijn van Veen / Carel Weijers
Lay-out: *B*-Point, Karin Scheele
Druk: FEBOdruk, Enschede