Enantioselective biocatalytic conversions of epoxides
Lutje Spelberg, Jeffrey Harald

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Chapter 1

Introduction
Importance of chirality

Epoxides are versatile building blocks in synthetic organic chemistry. As a result of the ring strain, their susceptibility towards nucleophiles, oxidizing and reducing agents makes them versatile intermediates for the preparation of various complex molecules. An epoxide ring can be opened by a nucleophile yielding the corresponding substituted alcohol. Generally, a base-catalysed ring opening will take place at the least hindered carbon atom whereas an acid-catalysed ring opening occurs at the more substituted carbon atom. Their high reactivity and directable regioselectivity makes epoxides valuable starting compounds for the preparation of biologically active compounds.

Many biologically active compounds such as drugs are chiral, which means that they can exist in two forms called enantiomers, which are non-superimposable mirror images of each other. Since many drugs are chiral compounds and interact with a chiral receptor in the body, only one of the enantiomers has the optimal therapeutic action. To avoid side effects of the unwanted enantiomer, government regulations have, over the last 15 years, increasingly demanded the use of drugs containing only the biologically active enantiomer. One example of a chiral drug in which both enantiomers have a different therapeutic effect is Darvon and Novrad (Figure 1). Novrad is an anti-cough agent and Darvon a painkiller.

![Figure 1](image-url) Enantiomers of a drug that have a different therapeutic effect.

In Figure 2, some examples of biologically active compounds that can be prepared from epoxides are shown. Halohydrins can be used as direct precursors for epoxides. As the complexity of synthetic target molecules increases, more selective and mild methods for preparing and converting epoxides and halohydrins are needed. Various methods are known for obtaining a single enantiomer of a chemical compound. They can be divided in three general preparation strategies: asymmetric synthesis, use of the chiral pool, and separation of the enantiomers present in a racemic mixture. Asymmetric synthesis is the preparation of an enantiomerically pure compound, starting from a compound containing a prochiral reaction center. The chirality can be introduced by using a chiral auxiliary, by internal asymmetric
induction from a nearby chiral center in the molecule, or by external asymmetric induction using a chiral catalyst. The last method is the commercially most attractive since only a catalytic amount of a chemical or biochemical catalyst is needed to obtain the optically active compound.

![Figure 2](image)

**Figure 2**  Examples of biologically active compounds prepared from optically pure epoxides and halohydrins.

The chiral pool consists of the optically active products present in nature, such as amino acids, organic acids, sugars, terpenes, and complex carbohydrates. An example of using the chiral pool is the anticancer drug Taxol, which can be obtained from the Pacific Yew tree *Taxus brevifolia*. A total synthesis of Taxol is impractical due to the complexity of the molecule. Semi-synthetic Taxol can be obtained by using the intermediate Baccatin III which is more abundant in nature, and converting it with the use of synthetic chemistry.

The third strategy is the separation of a racemic mixture of enantiomers. One such a technique is preferential crystallisation, in which a supersaturated solution is seeded with a crystal of one enantiomer causing selective crystallisation of that enantiomer. A second technique involves the conversion of enantiomers into diastereomers, followed again by a crystallisation. A third technique is kinetic resolution, which is based on the differences in reaction rate of the enantiomers in a racemic mixture, which can be achieved by using a chiral catalyst. If one enantiomer has been entirely converted, the reaction is stopped and the remaining enantiomer is obtained in optically pure form. A drawback to this method is that the maximum yield is only 50% of the total amount of the starting compound. Kinetic resolutions have been described using chemo-catalysts and biocatalysts.
Chapter 1

Enzymes

Fermentation has been a process that is known for thousands of years and is used for brewing beer, baking of bread and the production of sauerkraut, yoghurt and wine. The chemical changes that occur during the fermentation are the result of the activity of enzymes. Over the past decades, enzymes have become increasingly recognized as attractive catalysts in numerous processes and their use is still increasing. The major markets for enzymes are technical enzymes (used in detergents and fuels), food enzymes (used in bread, beer and wine) and feed enzymes (used in animal feed). Only a few percent of the total enzyme market consists of enzymes for applications in the fine chemicals industry, such as the manufacturing of pharmaceuticals, agrochemicals, and flavors. The market for such enzymes is expected to grow 7 to 10% annually. The demand for single-enantiomer compounds, particularly in the strongly regulated pharmaceutical industry, has increased the demand for enzymes for fine chemical applications. Since these biocatalysts can make products with high enantioselectivity and regioselectivity, drug manufacturers integrate enzyme-catalysed reaction steps in the early stages of the development of synthetic routes for drugs. Some single enantiomer compounds made by enantioselective biocatalysis are shown in Table 1.

Table 1  Examples of single enantiomer compounds produced on an industrial scale by enantioselective biocatalysts.

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<thead>
<tr>
<th>Chemical structure</th>
<th>Production details</th>
<th>Enzyme</th>
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<td><img src="image" alt="Aspartame Structure" /></td>
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<td>aminopeptidase</td>
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<tr>
<td><img src="image" alt="Intermediate in Diltiazem Structure" /></td>
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<td>lipase</td>
</tr>
<tr>
<td><img src="image" alt="Intermediate Structure" /></td>
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<td>lipase</td>
</tr>
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<td>additive in nutrition, 99.9% e.e., 300 ton yr(^{-1})</td>
<td>carnitine dehydratase</td>
</tr>
</tbody>
</table>
Introduction

The use of biocatalysts in organic synthesis can have some advantages and disadvantages. An accredited benefit is the high selectivity, such as chemoselectivity, enantioselectivity, and regioselectivity. These aspects make it possible to perform reactions that are synthetically unfeasible. The high regioselectivity avoids the use of complicated protection and deprotection steps used in chemical synthesis. Enzymes are easy to dispose of and can be used in water as a solvent making them environmentally friendly. A disadvantage of enzymes may be their poor operational stability that often requests narrower operation parameters than conventional chemical methods.

To make biocatalysis economically competitive with existing technologies, molecular biology is often used to increase the production scale and improve the catalytic properties of the enzymes. Cloning and overexpressing the genes in suitable hosts has drastically increased the production scale of the enzymes. Genetic engineering can adapt enzymes to unnatural reaction conditions or modify them in such a way that they can perform other reactions than they were designed to catalyse. Important goals are expanded substrate range, higher (enantio-)selectivity and improved stability of the enzyme at elevated temperatures and extreme pH-values.

Microorganisms involved in degradation pathways as a source for biocatalysts

A variety of man-made synthetic compounds is released in the natural environment. Halogenated compounds such as 1,2-dichloroethane, chloroform, dichloromethane, and epichlorohydrin have a widespread use in the industry as solvents, intermediates in chemical synthesis, insecticides, or degreasing agents. Some halogenated compounds can also be generated by plants (for example chloromethane by seaweeds), fungi, or mammals. Since many of these compounds are relatively new to the environment, they are poorly biodegradable. Partial degradation leads to the formation of highly toxic intermediate products such as epoxides and halo-carbonyl compounds. Since epoxides are highly reactive, they can react with a large variety of biomolecules such as proteins and DNA. These characteristics make many halogenated compounds highly toxic to organisms and therefore a fast degradation by microorganisms in the environment and in biotreatment systems is required.

Various organisms have been isolated that can use halogenated hydrocarbons as the sole source of carbon and energy. To understand the mechanism of dehalogenation and the evolution of the catabolic routes in several organisms, the involved enzymes have been studied extensively using microbial and genetic techniques. The organism Xanthobacter autotrophicus GJ10 is able to totally degrade 1,2-dichloroethane and Agrobacterium radiobacter AD1 can convert the toxic epichlorohydrin to glycerol (Figure 3.).
Three different types of dehalogenases are involved in these degradation routes: a haloalkane dehalogenase, a haloacid dehalogenase and a haloalcohol dehalogenase. All three enzymes have been studied extensively to determine their involvement in the degradation of halogenated compounds. Besides the use of these enzymes and organisms for bioremediation purposes, they can potentially also be used as biocatalysts for synthetic purposes. One of the dehalogenating enzymes that has been characterized extensively is the haloalcohol dehalogenase. As early as in the 1970s a process for the production of propylene oxide was developed using a haloalcohol dehalogenase as a part of a multi-enzyme process. During the last decade, haloalcohol dehalogenases have been considered for use in the biocatalytic synthesis of optically pure aliphatic epoxides and halohydrins.

Applications of organisms that contain haloalkane dehalogenase have been directed towards bioremediation and wastewater treatment. Recently, the stereospecificity of two different haloalkane dehalogenases was investigated. The results showed that a variety of chiral aliphatic halocarbons can be hydrolysed, but the enantioselectivity is low. These examples illustrate that microorganisms that degrade halogenated aliphatic compounds are a potential source of enzymes for (enantioselective) biocatalysis.
Optically active epoxides have been synthesized by a variety of biotransformation reactions (Figure 4). They can be prepared by direct epoxidation of alkenes using monooxygenases or peroxidases, by enantioselective hydrolysis using epoxide hydrolases, or by enantioselective nucleophilic ring opening using a haloalcohol dehalogenase or lipase. Optically active halohydrins, which are direct precursors of epoxides, have been prepared using haloalcohol dehalogenases, haloperoxidases, lipases or by microbial reduction of haloacetophenones.

The organism *A. radiobacter* AD1, previously referred to as a *Pseudomonas* species, was isolated from freshwater sediment that was polluted with chlorinated hydrocarbons. *A. radiobacter* AD1 can utilize 1,3-dichloro-2-propanol or epichlorohydrin as sole source of carbon and energy. Investigation of conversion of epichlorohydrin to glycerol revealed that two enzymes are involved in the biodegradation route. A haloalcohol dehalogenase catalyses the ring closure of haloalcohols to yield epoxides. The second enzyme, an epoxide hydrolase, catalyses the hydrolysis of epoxides to vicinal diols. Both enzymes are active with various chiral substrates, and it was the goal of the work described in this thesis to investigate the potential of the use of these two enzymes as biocatalysts for the preparation of optically pure compounds. In the next paragraphs the structure, mechanism, function and biocatalytic properties of both types of enzymes will be discussed.
Occurrence of epoxide hydrolases

Epoxide hydrolases catalyse the hydrolysis of an epoxide to yield a vicinal diol (Figure 5). The occurrence of these enzymes is widespread. Epoxide hydrolase activity has been found in bacteria, yeasts, fungi, insects, plants and mammals.

![Figure 5](image-url) Hydrolysis of an epoxide to a vicinal diol.

Five different types of mammalian epoxide hydrolases have been identified: microsomal epoxide hydrolase (mEH), soluble epoxide hydrolase (sEH), cholesterol epoxide hydrolase, leukotriene A₄ epoxide hydrolase, and hepoxilin hydrolase. The most extensively studied mammalian epoxide hydrolases are the membrane bound mEH and the soluble sEH. The latter is mainly found in the cytosol of liver and kidney cells. The primary function of mEH is the detoxification of xenobiotic epoxides, which are formed by cytochrome P-450-catalysed oxidation of aliphatic and aromatic compounds. The soluble epoxide hydrolase (sEH) has a different substrate specificity and is involved in both the detoxification of epoxides and the biosynthesis of endogenous compounds.

Epoxide hydrolases in insects are involved in the hydrolysis of pheromones. In plants, these enzymes are hypothesized to aid in the biosynthesis of cutin, a polymer that plays an important role in the defense against pathogens. Until recently, epoxide hydrolases were mainly studied for their biological role and catalytic mechanism. Only in a few studies, the enantioselectivity of mEH and sEH towards the hydrolysis of aromatic and aliphatic epoxides was investigated. The discovery of (enantioselective) microbial epoxide hydrolases in *Rhodococcus* sp. and *Aspergillus niger* prompted an increased interest in biocatalytic applications.

Routes to optically pure epoxides and diols by hydrolysis of epoxides

Several types of chiral recognition by epoxide hydrolase can lead to an optically active epoxide or diol. In Figure 6 three reaction types are shown. A kinetic resolution, in which only one of the enantiomers is hydrolysed, yields the remaining enantiomer of the epoxide in an optically pure form (Figure 6A). If the ring opening of the preferred enantiomer is completely regioselective towards the α- or β-carbon atom of the epoxide, the produced diol is obtained in optically pure form. If one enantiomer is attacked solely at the α-carbon and the other enantiomer solely at the β-carbon atom, a complete conversion will lead to an
Introduction

optically pure diol in 100% yield (Figure 6B). This type of reaction is called enantioconvergent. In most cases the enantioselectivity and regioselectivity will not be absolute, resulting in a combined effect of the two reaction types described above. The asymmetric hydrolysis of a *meso* compound like cyclopentene oxide can theoretically result in an optically pure trans-diol with a yield of 100% (Figure 6C). In this case, the optical purity of the product (e.e.) is defined by the ratio of the rates of formation of the enantiomers of the product (product enantioselectivity) and is constant throughout the conversion.

![Figure 6](image-url)  
*Figure 6*  
Routes to optically pure epoxides and diols by epoxide hydrolase catalysed conversions. (A), kinetic resolution, (B), enantioconvergent conversion and (C), asymmetric hydrolysis of a *meso* compound. Indicated yields are maximum values.

**Enantioselectivity of mammalian epoxide hydrolases**

The enantioselectivity of ring opening using mammalian epoxide hydrolases (mEH and sEH) has been investigated since 1970. Belluci *et al.* studied the enantioselective hydrolysis of a variety of cycloalkane oxides and epoxides of heterocycles. Both mEH and sEH catalysed the hydrolysis of the *meso* compounds cycloalkane oxides (C₅ - C₈) to optically active diols. The highest optical purity was achieved with the mEH-catalysed hydrolysis of cyclopentene oxide, which yielded the (1R,2R)-diol in 90% e.e. An opposite regioselectivity towards both enantiomers was observed in the hydrolysis of 3,4-
epoxytetrahydrofuran. This enantioconvergent process yielded (-)-(3R,4R)-3,4-dihydroxytetrahydrofuran in 96% e.e.

The enantioselectivity of the hydrolysis of substituted styrene oxides by sEH and mEH is usually low to moderate. An exception is the mEH-catalysed hydrolysis of cis-β-substituted styrene oxides. Rabbit liver mEH catalysed the hydrolysis of cis-β-methyl styrene oxide and cis-β-ethyl styrene oxide with very high enantioselectivity, yielding nearly optically pure (1S,2R)-epoxides and (1R,2R)-diols. With the exception of cis-β-methylstyrene oxide, cis-β-substituted styrene oxides were enantioconvergently hydrolysed. The same trend can be observed with aliphatic epoxides as mostly cis-β-substituted aliphatic epoxides are hydrolysed by mEH with high enantioselectivity. A high enantioselectivity was observed for the hydrolysis of 2,2-dimethylmethylene cyclohexene oxide.

Although mammalian epoxide hydrolases show high enantioselectivity towards specific substrates, a large-scale application has not been described. The low availability of the enzymes prevents the production of optically active epoxides or diols on multigram scale. Epoxide hydrolases from microbial sources such as fungi and bacteria can easily be obtained in larger amounts and therefore have a much higher potential to be used for the production of optically pure compounds.

Enantioselectivity of microbial epoxide hydrolases

Although the presence of epoxide hydrolase activity has been described for many microorganisms, the enantioselectivity of the conversions has only been investigated recently. Faber et al. discovered that a crude enzyme preparation from a Rhodococcus sp. (NOVO SP 409), which was previously used for the hydrolysis of nitriles, showed low to moderate enantioselectivity towards a variety of aliphatic epoxides. This initiated a comprehensive screening for enantioselective epoxide hydrolases in bacteria. The highest enantioselectivity was observed with aliphatic 2,2-disubstituted epoxides using a Rhodococcus sp. (NCIMB 11216) and Nocardia sp. Kinetic resolutions of 2,2-disubstituted epoxides bearing various functional oxygen groups were studied using 11 bacterial strains. A correct choice of ether functionality and biocatalyst resulted in various substituted optically pure epoxides and diols. Functionalised epoxides were used in the total synthesis, including an epoxide hydrolase catalysed kinetic resolution, of (S)-frontalin and (R)-mevalonolactone.

The combination of a kinetic resolution using Nocardia EH1 cells and a sequential acid-catalysed hydrolysis step yielded one enantiomer of a diol as sole product (Figure 7A). The enzyme-catalysed ring opening of the epoxide occurred at the terminal carbon atom and the subsequent chemical hydrolysis of the remaining epoxide at the non-terminal carbon atom, yielding the (S)-diols in >90% e.e. and >90% yield. A chemoenzymatic process was developed by Archer et al. (Figure 7C). Racemic 1-methyl-1,2-epoxycyclohexane was converted to the (1S,2S)-1-methylcyclohexane-1,2-diol in a one-pot combination of a
Corynebacterium epoxide hydrolase and acid-catalysed hydrolysis. A very extensive screening by researchers from Daicel Chemical Industries showed enantioselective epoxide hydrolase activity in more than 50 different microorganisms.\(^\text{58}\)

![Chemical structures](image)

\textbf{Figure 7} Examples of enantioconvergent hydrolysis of racemic epoxides yielding optically pure diols. (A) and (C), chemoenzymatic sequential conversion and (B), simultaneous conversion with two organisms.

Furstoss et al. studied the hydrolysis of aromatic epoxides by fungal epoxide hydrolases. Styrene oxide was hydrolysed by \textit{Aspergillus niger} LCP 521 with an opposite enantiopreference (\((R)\)-selective) and regioselectivity compared to conversion catalysed by \textit{Beauveria sulfurescens} ATCC 7159 (\((S)\)-selective).\(^\text{28}\) The combined use of both organisms yielded \((R)\)-phenylethanol in 92% yield and 83% e.e. (Figure 7B). The hydrolysis of a range of \textit{para}-substituted styrene oxides suggested different mechanisms for both enzymes.\(^\text{59}\) With the same range of epoxides a shift in regioselectivity was observed going from electron-donating to electron-withdrawing substituents using a crude extract from \textit{Syncephalastrum racemosum}.\(^\text{60}\) A kinetic study revealed that the mechanism could be described, in contrast to that of the \textit{A. niger} enzyme, as a concerted process implying a general acid activation. Recent results with \textit{A. niger} showed that this enzyme could be used to obtain optically pure \((S)\)-2,3- and 4-pyridyloxiranes.\(^\text{61}\)

Enantioselective epoxide hydrolase activity has also been detected in various yeast strains.\(^\text{62,63}\) The yeast \textit{Rhodotorula glutinis} showed a remarkably wide substrate range in the
enantioselective hydrolysis of aliphatic and aromatic epoxides. More recent screenings showed that enantioselective epoxide hydrolase activity is relatively common in various bacteria, fungi and yeasts. 

**Epoxide hydrolase from A. radiobacter AD1**

The organism *A. radiobacter* AD1 is able to grow on epichlorohydrin as sole carbon source. The first step of the degradation route is the hydrolysis of epichlorohydrin to 3-chloro-1,2-propanediol catalysed by an epoxide hydrolase (Figure 3). The enzyme was purified and characterized by Jacobs *et al.* The epoxide hydrolase is a soluble, cofactor-independent, monomeric protein with a molecular mass of 35 kDa. The highest activity of the enzyme was obtained at pH 8.4 – 9.0 at 50°C. An initial investigation in the substrate specificity showed that the enzyme can hydrolyse terminal aliphatic and aromatic epoxides such as epibromohydrin, epoxyoctane and styrene oxide but not cyclohexene oxide. The low activity towards glycidol suggests the presence of a second epoxide hydrolase in *A. radiobacter* AD1, since the formed glycidol is further converted to glycerol. Originally, it was concluded that the enzyme was not enantioselective. This conclusion was drawn from the fact that all tested epoxides were completely hydrolysed. The likelihood of a sequential conversion of both enantiomers was not considered and the decrease of both enantiomers was not monitored separately. The epoxide hydrolase gene was cloned and brought to overexpression in *Escherichia coli* by Rink *et al.* This was at that moment the first cloned bacterial epoxide hydrolase gene. The enzyme was overexpressed to up to 40% of the total cellular protein content and could easily be purified in a two-step purification procedure. Sequence similarity with haloalkane dehalogenases indicated that the enzyme belongs to the α/β-hydrolase fold family. This family includes, besides epoxide hydrolases and haloalkane dehalogenases, also lipases, proteases, and esterases. Based on the conserved features of the α/β-hydrolase fold, a catalytic triad was proposed. Site-directed mutations showed that the Asp107, His275, and Asp246 form the catalytic triad in the active site. 

**Structure and catalytic mechanism of the epoxide hydrolase**

Recently, Nardini *et al.* determined the X-ray structure of the epoxide hydrolase from *A. radiobacter* AD1 at 2.1 Å resolution. The structure confirmed the proposed α/β-hydrolase fold topology of the enzyme and the presence of a catalytic triad in the active site. The main domain consists of a central parallel β-sheet flanked by 6 α-helices. The second domain or cap domain lies on top of the main domain and consists of 5 α-helices. The active site is located between the main domain (which also contains the catalytic triad) and the cap
domain. Two tyrosine residues (Tyr152 and Tyr215) present in the cap domain are positioned close to Asp107 in such a way that they can be involved in catalysis by activating the substrate or by donating a proton to the leaving group. Sequence alignment studies showed that the two tyrosines are conserved in all known soluble epoxide hydrolases from microorganisms, mammals and plants.⁷⁰

**Figure 8**  
*Reaction mechanism of the epoxide hydrolase from A. radiobacter AD1. (A) alkylation reaction and (B) hydrolysis of covalent intermediate.*

The catalytic mechanism shown in Figure 8 is based on information obtained from the structure and mutagenesis studies. The mechanism consists of two steps, an alkylation step resulting in the formation of a covalent intermediate (Figure 8A) and a subsequent hydrolysis reaction (Figure 8B). The oxygen atom in the epoxide is bound in the active site between Tyr152 and Tyr215. The epoxide is opened by a nucleophilic attack from Asp107, resulting in the formation of the covalent intermediate. In the second step, a water molecule, activated
by proton abstraction by His275, attacks the ester group of the covalent intermediate and the
diol is released. Mutating the histidine residue, resulting in the trapping of the substrate-
enzyme, proved the existence of a covalent intermediate.  

### Occurrence of haloalcohol dehalogenases

Haloalcohol dehalogenases catalyse the intramolecular nucleophilic displacement of a
halogen to yield an epoxide, a proton and the inorganic halide, as well as the reverse reaction.
Halohydrin dehalogenase, halohydrin epoxidase or hydrogen-halide lyase are alternative
names for the haloalcohol dehalogenase. The occurrence of this class of enzymes is limited to
microbial sources.  

![Figure 9](image)

**Figure 9**  Enzyme-catalysed interconversion of a haloalcohol to an epoxide.

Castro and Bartinicki reported the first enzyme-catalysed conversion of a haloalcohol
to an epoxide.  A *Flavobacterium sp.* was isolated from soil samples because of its ability to
degrade 2,3-dibromo-1-propanol. The first step of the degradation route is the conversion of
2,3-dibromo-1-propanol to epibromohydrin catalysed by a haloalcohol dehalogenase. Further
degradation of epibromohydrin to glycerol occurred by the same degradation route as
described above for *A. radiobacter* AD1. The substrate range of the haloalcohol dehalogenase
was investigated with partially purified enzyme. The reverse reaction, the ring opening of
epoxides by a halide, was also observed. The ring opening of epibromohydrin by a chloride
ion and the subsequent ring closure of 1-bromo-3-chloro-2-propanol yielded epichlorohydrin.
This process, in which both steps are catalysed by the haloalcohol dehalogenase, is known as
transhalogenation.

Besides the above described organisms, haloalcohol dehalogenase activity has been
found in *Arthrobacter* sp.,  *Pseudomonas* sp.,  *Alcaligenes* sp.,  *Corynebacterium*
sp. and very recently also in *Rhizobium* sp. In *Corynebacterium* sp. strain N-1074 two
distinctly different haloalcohol dehalogenases (HheA and HheB) were identified and the
corresponding genes were cloned. A haloalcohol dehalogenase from the 3-chloro-1,2-
propanediol utilizing bacterium *Arthrobacter* strain AD2 was purified and characterized.
Recently, the genes encoding haloalcohol dehalogenases from *A. radiobacter* AD1,
*Arthrobacter* strain AD2 and *Mycobacterium* sp. strain GP1 have been cloned and expressed
in *E. coli*.  

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Together with HheA and HheB from Corynebacterium sp., a total of five sequences encoding haloalcohol dehalogenases are presently known (Table 2). The enzyme from Arthrobacter strain AD2 is highly similar to HheA (97.1% sequence identity) while that of Mycobacterium sp. strain GP1 is highly similar to HheB (98.4% sequence identity). The relationships of the associated enzyme pairs were confirmed by their similar substrate range. The low sequence identities show that the enzyme from A. radiobacter AD1 is different from the other four enzymes. Sequence homology and structure predictions indicate that haloalcohol dehalogenases are structurally similar to proteins belonging to the family of short-chain dehydrogenases and reductases (SDR). Although their sequence identity (15% to 25%) is rather low, β-strands and α-helices were predicted at the same position as those in the SDR proteins.

### Table 2  Sequence identities (%) between haloalcohol dehalogenases.

<table>
<thead>
<tr>
<th></th>
<th>HheA</th>
<th>AD2</th>
<th>HheB</th>
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### Routes to optically pure compounds using haloalcohol dehalogenase

Enantioselective conversions of haloalcohols and epoxides catalysed by haloalcohol dehalogenase can be used to obtain optically pure epoxides and alcohols. If a chiral racemic haloalcohol is transformed with high enantioselectivity to the epoxide, the reaction will stop at 50% conversion and produced epoxide and non-reacted haloalcohol substrate can be obtained in high optical purity and 50% yield (Figure 10A). By alkaline treatment an isolated optically pure haloalcohol can be converted in an optically pure epoxide. This gives the possibility to obtain both enantiomers of an epoxide optically pure with the use of one enzymatic and one chemical reaction step. The disadvantage of the 50% yield maximum can be overcome by using prochiral substrates, such as 1,3-dihalo-2-propanol (Figure 10B).

Since the enzyme also catalyses the reverse reaction, addition of a nucleophile to an epoxide can be used to produce alcohols. A highly enantioselective ring opening by an anionic nucleophile, can yield the remaining epoxide and the formed alcohol in optically pure form (Figure 10C).
Enantioselectivity of haloalcohol dehalogenases

A *Pseudomonas sp.* and an *Alcaligenes sp.* enantioselectively degraded 2,3-dichloro-1-propanol.\(^{73,74}\) In both cases, the product of the ring closure could not be isolated. This was due to the presence of an epoxide hydrolase, which hydrolysed the formed epichlorohydrin to 3-chloro-1,2-propanediol. The ring closure reaction was slower than the hydrolysis of the epoxides resulting in a lack of accumulation of the epoxide. The enantiopreference of both organisms is opposite to each other. This allowed, by the appropriate choice of organism, both enantiomers of 2,3-dichloro-1-propanol to be obtained optically pure (e.e. > 99%).

The prochiral compound 1,3-dichloro-2-propanol can be converted to \((R)-3\)-chloro-1,2-propanediol by *Corynebacterium* sp. strain N-1074. The diol was obtained in 97.3\% yield with a moderate e.e. of 83.8\%.\(^{80}\) Since two haloalcohol dehalogenases (HheA and HheB) are present in this organism, the enantioselective properties of the recombinant enzymes were investigated separately. Although both HheA and HheB catalysed the ring closure of 1,3-dichloro-2-propanol, only HheB yielded an optically active epoxide. The optical purity of the formed epichlorohydrin continuously decreased during the reaction. This effect could be explained by the enzyme-catalysed racemisation of epichlorohydrin via the reverse reaction.\(^{81}\)

Hardman *et al.* observed the same racemisation with a haloalcohol dehalogenase obtained from *Arthrobacter erithii* H10a.\(^{82}\) The prochiral 1,3-dichloro-2-propanol was initially converted to \((R)-epichlorohydrin (89\% e.e., 61\% yield), but the e.e. decreased upon prolonged incubation. When the conversion was performed in the presence of an excess of
KBr, (R)-epichlorohydrin was obtained with a high enantiomeric excess (>95% e.e.) but in an unsatisfactory yield of 14.5%. The higher optical purity was caused by the reverse reaction, the ring opening of epichlorohydrin by Br⁻. In this reaction, the less formed (S)-enantiomer was the preferred substrate, resulting in a sequential kinetic resolution and therefore an increase in the e.e. of (R)-epichlorohydrin. Two different types of haloalcohol converting enzymes were detected in *Alcaligenes* sp. DS-S-7G and *Enterobacter* sp. The cofactor dependent dehalogenating enzymes obtained from *Alcaligenes* sp. DS-S-7G converted the haloalcohol to hydroxyacetone, acetic acid and formic acid. Bacterial cells from the *Enterobacter* species dechlorinated 4-chloro-3-hydroxybutyrate to give 3-hydroxy-γ-butyrolactone. Since these enzymes do not catalyse the conversion of haloalcohols to epoxides, the enantioselectivity of the reactions will not be discussed here.

**Haloalcohol dehalogenase from *A. radiobacter* AD1**

The gene encoding the haloalcohol dehalogenase from *A. radiobacter* AD1 was brought to overexpression in *E. coli* making the enzyme available in sufficient quantities for biocatalytic applications. The enzyme has a predicted molecular mass of 28 kD and is a tetrameric protein consisting of four identical subunits. The protein could be obtained in pure form by a simple purification procedure. The highest activity of the enzyme was obtained between pH 8.0 – 9.0. A variety of chlorohydrins, bromohydrins, but not fluorohydrins were converted to the corresponding epoxides. Generally, bromohydrins were converted at a higher rate than chlorohydrins. The haloalcohol dehalogenase also catalysed the conversion of chloroacetone to hydroxyacetone. The mechanism of this conversion is still unclear.

A catalytic mechanism was proposed based on the high sequence similarity to SDR proteins (as discussed above) and site-directed mutagenesis studies. In the first step Tyr145 abstracts a proton from the haloalcohol (Figure 11). This process is facilitated by Arg149, lowering the pKₐ of the tyrosine. The subsequent attack of the substrate oxygen on the neighboring halogen-substituted carbon atom causes the release of the halide and the formation of the epoxide.

![Proposed catalytic mechanism of the haloalcohol dehalogenase.](image-url)
Chapter 1

IOP catalysis

The research described in this thesis is the result of an IOP-catalysis (IKA 94007a) project titled “Biocatalytic synthesis of chiral epoxides and diols” from the Dutch Ministry of Economic Affairs. The IOP promotes research in a number of promising fields in catalysis in order to improve the competitive position of industry in the Netherlands. By providing universities with funding for research projects, which are aimed specifically at meeting the needs of the industry, novel catalytic routes for the production of fine chemicals can be developed. Since epoxides and alcohols are building blocks for a variety of fine chemicals, several Dutch industries were participating in this project. The project also aimed to work in close cooperation with an IOP-catalysis project (IKA 94007b) carried out at the Division of Industrial Microbiology at the University of Wageningen.

Scope and outline of the thesis

As summarized in the previous paragraphs, epoxide hydrolases and haloalcohol dehalogenases can be valuable tools for obtaining optically pure epoxides and alcohols. During the last years, several of these enzymes have been brought to overexpression, making them available in quantities that make practical applications in (organic) chemistry possible. In this thesis the biocatalytic potential of epoxide hydrolase and haloalcohol dehalogenase from *A. radiobacter* AD1 are explored. At the start of the research, only the epoxide hydrolase gene was brought to overexpression. Initial investigations in the enantioselectivity of this enzyme suggested that the enzyme is not enantioselective. The goal of the research therefore was to investigate the enantioselectivity of the epoxide hydrolase from *A. radiobacter* AD1 along with a range of epoxide hydrolases from other organisms. The haloalcohol dehalogenase from the same organism had not been investigated for its properties as an enantioselective biocatalyst, and the description of the substrate range of other haloalcohol dehalogenases was limited to the conversion of small aliphatic epoxides. During this study the enantioselective properties of the epoxide hydrolase and haloalcohol dehalogenase were studied with the goal to obtain new tools for the production of optically pure precursors of biologically active compounds.

In Chapter 2 the recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1 was used to obtain enantiomerically pure epoxides by means of kinetic resolution. Epoxides such as styrene oxide and various derivatives thereof and phenyl glycidyl ether were obtained in high optical purity and in reasonable yield. The enantioselectivity (E-value) of the resolution was calculated from progress curves for styrene oxide (E = 16) and para-chlorostyrene oxide (E = 32).

In Chapter 3 the enantioselectivity of the conversions using substituted styrene oxides is described in more detail. Based on the X-ray structure, it was hypothesized that two
tyrosine residues in the active site are involved in substrate binding.\textsuperscript{88,89} The enantioselectivity and regioselectivity of the wild type enzyme and a tyrosine mutant towards a range of styrene oxides containing electron donating and electron-withdrawing substituents were investigated. Mutation of either one of the tyrosine mutants resulted in an enzyme with an increased enantioselectivity towards aromatic substrates. Besides the increase in enantioselectivity, the relatively small decrease in the conversion rate of the preferred enantiomer and the strong decrease in activity of the remaining enantiomer makes the mutant enzyme a very suitable biocatalyst for the preparation of optically pure epoxides and diols.\textsuperscript{90}

In Chapter 4 the recombinant haloalcohol dehalogenase from \textit{A. radiobacter} AD1 was used to obtain a variety of optically pure haloalcohols by means of a kinetic resolution. Because of the reversibility of the enzyme action, an excess epoxide hydrolase was added to draw the kinetic resolutions to completion, creating a tandem enzyme reaction. The combined use of the recombinant haloalcohol dehalogenase and of epoxide hydrolase resulted in a novel biocatalytic method to produce optically active aromatic haloalcohols, epoxides and diols.\textsuperscript{91}

In Chapter 5 a spectrophotometric assay for haloalcohol dehalogenases based on the difference between the absorbance of \textit{para}-nitro-2-bromo-1-phenylethanol and \textit{para}-nitrostyrene oxide is described. The enantioselectivity of ring-closure reactions catalysed by three distinctly different haloalcohol dehalogenases could be predicted from the shape of the progress curves. Evaluation of ring-opening reactions catalysed by the haloalcohol dehalogenase from \textit{A. radiobacter} AD1 established that, in addition to Cl\textsuperscript{−} and Br\textsuperscript{−} also N\textsubscript{3}\textsuperscript{−}, CN\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} are accepted as nucleophiles in the ring opening of \textit{para}-nitrostyrene oxide.\textsuperscript{92}

In Chapter 6 the ring opening of substituted styrene oxides by N\textsubscript{3}\textsuperscript{−} is described. The reactions occurred in a highly enantioselective manner. The high regioselectivity (>95% β-selective) of the enzyme-catalysed reaction is opposite to the observed selectivity in the non-catalysed ring opening by N\textsubscript{3}\textsuperscript{−}. A controlled feeding of azide and the addition of the epoxide as a second phase could suppress the formation of side products due to chemical side reactions. The reaction was scaled up to 0.5 gram without loss of enantioselectivity or regioselectivity.\textsuperscript{93}

In Chapter 7 the ring opening of epichlorohydrin and epibromohydrin by N\textsubscript{3}\textsuperscript{−} using the haloalcohol dehalogenase from \textit{A. radiobacter} AD1 was investigated. The enzyme catalysed, besides the ring opening by azide, also the racemisation of the remaining enantiomer and the enantioselective ring closure of the product. Optimisation of the reaction conditions resulted in 1-azido-3-bromo-2-propanol in 84% yield and 94% e.e. Due to an \textit{in situ} sequential kinetic resolution by ring closure of the azido-alcohol, which is catalysed by the same enzyme, the product can be obtained optically pure (e.e. > 99%) with an acceptable 7% loss in yield.

In conclusion, an overview of the research, concluding remarks and opportunities for future research are presented in Chapter 8.
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85 The industrial researchers involved in this project were Dr. M. Lubben (DSM), Dr. B. Schulze (DSM), Dr.ir. A.G.G. Kieboom (DSM), Dr. J. Frankena (Solvay Duphar), Ir. G.F.H. Kramer (Unilever).

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