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Enantioselective biocatalytic conversions of epoxides
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Chapter 2

Enantioselectivity of a recombinant epoxide hydrolase from *Agrobacterium radiobacter*
Chapter 2

Introduction

Enantiomerically pure epoxides are important building blocks for the production of a wide range of pharmaceutical products. Especially during the past decade much effort has been devoted to the development of biocatalytic methods for the production of these compounds.\(^1\) Epoxide hydrolases are cofactor-independent enzymes that hydrolyze epoxides to diols. If these enzymes display enantioselectivity, they can be used as chiral catalysts for the production of enantiomerically pure epoxides or diols.\(^2\) The enzymes from mammalian sources have been studied extensively because of their involvement in the metabolism of toxic xenobiotics.\(^3\) Styrene oxide, for example, is hydrolyzed to the less toxic product phenylethanediol. The potential of mammalian epoxide hydrolases as chiral catalysts on a preparative scale is limited due to the low availability of these enzymes.

Several epoxide hydrolases from microbial sources have been discovered recently.\(^4\) High enantioselectivity was obtained with the hydrolysis of (substituted) styrene oxides using fungal cells,\(^5\) and with a broad variety of aryl and aliphatic epoxides with the yeast *Rhodotorula glutinis*.\(^6\) During the last few years enantioselective bacterial epoxide hydrolases have been detected in *Rhodococcus sp.*, \(^7\) *Nocardia sp.*, \(^8\) *Corynebacterium sp.* \(^9\) and some other genera.\(^10\) The availability of the bacterial enzymes is considerably higher compared to that of microsomal epoxide hydrolase. However, unlike for the mammalian enzyme, very little biochemical information is available for the above mentioned epoxide hydrolases from microbial sources. The genes have not been cloned and their structure and mechanism is unknown.

Recently we have characterized a bacterial epoxide hydrolase from *Agrobacterium radiobacter* AD1.\(^11\) The bacterium was initially isolated for environmental reasons because of its ability to degrade epichlorohydrin. Such xenobiotic-degrading enzymes must have high turnover numbers, since they are involved in the assimilation of a primary substrate for growth. The epoxide hydrolase gene was cloned and brought to expression in *E. coli* and the mechanism was characterized.\(^12\) The epoxide ring is opened by a nucleophilic attack of an aspartic acid residue yielding a covalent ester intermediate. Subsequently this intermediate is hydrolyzed and the diol is released. This mechanism is similar to what has been proposed for mammalian epoxide hydrolase.\(^13\) In a recombinant *E. coli* strain containing the epoxide hydrolase gene, up to 40% of the total cellular protein content consisted of the epoxide hydrolase, allowing the isolation of up to 200 mg of pure enzyme from a one liter culture.

In this paper we describe the biocatalytic resolution of a number of chiral epoxides by the epoxide hydrolase from *A. radiobacter* AD1. Since this recombinant epoxide hydrolase showed good enantioselectivity towards several substrates and can be produced on multi-gram scale, it has the potential to be used as an industrial biocatalyst for the production of enantiomerically pure epoxides.
Results and discussion

The epoxide hydrolase gene from *A. radiobacter* AD1 was cloned and brought to overexpression in *E. coli* as described previously. The recombinant enzyme was produced by fermentation and lyophilized partially purified enzyme was prepared. The epoxides 1 - 7 were subjected to enzymatic hydrolysis by the epoxide hydrolase. The results are shown in Table 1. With all the kinetic resolutions an enantiomeric excess (e.e.) of the remaining epoxide of higher than 99% e.e. was reached. The yields of the remaining optically pure epoxides varied between 27% and 36%. A factor contributing to the lower yield was some instability of the epoxides in the reaction medium, which is caused by chemical hydrolysis yielding the racemic diol.

The remaining enantiomerically pure epoxides 1 - 6 were all of the (S)-configuration, indicating that the substituents do not influence the positioning of the substrate in the active site of the enzyme. In the case of epoxide 3 the absolute configuration was determined by comparison to a previously reported optical rotation, although a contradictory optical rotation was also reported in literature. The assignment coincided with the fact that with the kinetic resolutions of all the styrene oxide derivatives the remaining enantiomer was of (S)-configuration. The absolute configuration of epoxide 4 was determined by the product of the kinetic resolution, para-chloro-1-phenyl-1,2-ethanediol. Direct determination of the epoxide was not possible because no literature value was found. For the optical rotation the para-chloro-1-phenyl-1,2-ethanediol was reduced to 1-phenyl-1,2-ethanediol which was analysed by chiral HPLC using a chiraldex OB column. The phenyl glycidyl ether 7 yielded the (R)-enantiomer which has the same relative configuration at the chiral carbon atom as the styrene oxide derivatives. The opposite stereochemical designation is due to a change in priority.

![Figure 1](image-url)  
*Figure 1*  Epoxides used as substrates by the epoxide hydrolase from *A. radiobacter* AD1.
The enantioselective hydrolysis of racemic styrene oxide was followed in time (Figure 2). After the start of the reaction, the (R)-enantiomer of styrene oxide was hydrolyzed to (R)-phenylethanol, indicating that the attack was preferentially at the terminal carbon atom. During the first 40% of the total conversion, the e.e. of the (R)-1-phenyl-1,2-ethanediol was 73%. When the (R)-enantiomer was completely hydrolysed (e.e. > 99%) the e.e. of the diol had decreased to 49%. Similar results were obtained with the hydrolysis of epoxide 7. Because of the moderate enantioselectivity, the e.e. of the remaining (R)-diol was 38% at the
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timepoint at which the e.e. of the (S)-enantiomer of epoxide 7 was higher than 99%. These low enantiomeric purities make the application of this epoxide hydrolase less interesting for preparing enantiomerically pure diols.

After conversion of the (R)-enantiomer, the (S)-enantiomer of the styrene oxide started to be hydrolyzed too, and at a much faster rate than the (R)-enantiomer. A possible explanation for this kinetic behavior is that the (R)-enantiomer has a higher affinity for the active site, thereby inhibiting the binding of the (S)-enantiomer. Once 90% of the (R)-enantiomer has been hydrolyzed, the (S)-enantiomer reacts with a higher rate due to faster chemical reaction steps at the enzyme active site. A similar kinetic behavior has been observed previously with styrene oxide, para-nitrostyrene oxide and tert-butyloxirane by microsomal epoxide hydrolases. To further investigate these results, the conversion of both enantiomers was studied separately. The specific activity of the (R)-enantiomer of styrene oxide was 1.82 µmol.min\(^{-1}\).mg\(^{-1}\) and for the (S)-enantiomer 5.14 µmol.min\(^{-1}\).mg\(^{-1}\). Both activities were measured for 25% pure enzyme. Initial experiments to determine the K\(_m\) value failed because the value was below the detection limit of 50 µM for both enantiomers.

Epoxides 2 - 7 showed a more classical kinetic resolution. As an example, the enantioselective hydrolysis of epoxide 6 is shown in Figure 2b. During the hydrolysis of the faster reacting (R)-enantiomer, the hydrolysis of the (S)-enantiomer was inhibited. After more than 90% of the (R)-enantiomer was hydrolyzed, the (S)-enantiomer was hydrolyzed at a lower rate. This is contrary to the hydrolysis rate of the (S)-enantiomer of epoxide 1. The same kinetic resolution pattern was observed for the epoxides 2 - 5 and 7.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Hydrolysis of epoxides 1 - 7 by the cloned epoxide hydrolase from A. radiobacter AD1.</th>
</tr>
</thead>
<tbody>
<tr>
<td>epoxide</td>
<td>% e.e.</td>
</tr>
<tr>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>2(^e)</td>
<td>&gt;99</td>
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<tr>
<td>3</td>
<td>&gt;99</td>
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<tr>
<td>4(^e)</td>
<td>&gt;99</td>
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<td>5(^e)</td>
<td>&gt;99</td>
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<tr>
<td>6(^e)</td>
<td>&gt;99</td>
</tr>
<tr>
<td>7</td>
<td>&gt;99</td>
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</table>

\(^{a}\) determined by an additional injection of optically pure epoxide. \(^{b}\) determined by optical rotation. \(^{c}\) deduced from a previously described elution order on chiral GLC. \(^{d}\) determined by reduction of the corresponding diol to 1-phenyl-1,2-ethanediol. \(^{e}\) in buffer containing 10% DMSO.
The enantioselectivity of the reaction can be described by using the enantiomeric ratio \( E \) which is defined by equation (1), where \( V_{\text{max}} \) and \( K_m \) represent the Michaelis-Menten parameters.

\[
E = \frac{V_{\text{max}}^S}{V_{\text{max}}^R} \frac{K_m^S}{K_m^R}
\] (1)

The conversion of both enantiomers in time can be described by competitive Michaelis-Menten kinetics, \(^{21,22}\) using equations (2) and (3) which also account for chemical hydrolysis.

\[
\frac{dS}{dt} = -\frac{V_{\text{max}}^S \cdot S}{S + \left( \frac{R}{K_m^R} + 1 \right) \cdot K_m^S} - k_c \cdot S
\] (2)

\[
\frac{dR}{dt} = -\frac{V_{\text{max}}^R \cdot R}{R + \left( \frac{S}{K_m^S} + 1 \right) \cdot K_m^R} - k_c \cdot R
\] (3)

In these equations \( R \) represents the concentration of the \((R)\)-enantiomer, \( S \) the concentration of the \((S)\)-enantiomer and \( k_c \) the first order chemical hydrolysis rate constant. The constant \( k_c \) was determined under the same conditions as the enzymatic conversion, but in the absence of the enzyme. The \( k_c \) for epoxide 1 was found to be \( 8.63 \times 10^{-6} \text{ sec}^{-1} \) and for epoxide 6 \( 6.2 \times 10^{-6} \text{ sec}^{-1} \). To estimate the kinetic parameters, the equations (2) and (3) were fitted by numerical integration to the data shown in Figure 2, and a good fit was obtained. With the obtained kinetic parameters, the E-value was calculated using equation (1). For epoxide 1 a value of 16.2 and for epoxide 6 a value of 32.2 was obtained.

This method could not be used to obtain unique solutions for all of the kinetic parameters of epoxides 1 and 6. For epoxide 6 a unique solution could only be obtained for the \( V_{\text{max}} \) of the \((R)\)-enantiomer. In the case of styrene oxide unique solutions were obtained for the values of \( V_{\text{max}} \) and for the ratio between the \( K_m \) of the \((S)\)-enantiomer and that of the \((R)\)-enantiomer (57.9). The resulting E-value was independent of the absolute \( K_m \) value for each enantiomer. The estimated \( K_m \) of the \((S)\)-enantiomer is lower than 50 \( \mu \text{M} \) and of the \((R)\)-enantiomer lower than 1 \( \mu \text{M} \), but to obtain the exact data a more sensitive analysis method is needed. These results show that the progress of the kinetic resolution of styrene oxide, with a fast conversion of the remaining \((S)\)-enantiomer after the depletion of the \((R)\)-enantiomer, can be explained by the higher \( K_m \) and \( V_{\text{max}} \) of the \((S)\)-enantiomer compared to that of the \((R)\)-enantiomer.
According to the method described by Chen et al. (equation 4), the enantiomeric ratio is derived from the degree of conversion (c) and the enantiomeric excess of the remaining enantiomer of the substrate (ee). Because this method is time-independent it does not take into account the influence of the chemical hydrolysis.

\[ E = \frac{\ln[(1 - c)(1 - ee)]}{\ln[(1 - c)(1 + ee)]} \]  

Using the datapoints from each curve (Figure 2), the enantiomeric ratios determined with equation (4) were 13.8 for epoxide 1 and 28.2 for epoxide 6, which is significantly lower than when a correction was made for chemical hydrolysis.

The above results demonstrate that the cloned epoxide hydrolase from \textit{A. radiobacter} AD1, which can be produced in large amounts, is enantioselective for a number of epoxides. This makes the production of enantiomerically pure epoxides on an industrial scale applicable. Since the mechanism of this epoxide hydrolase has been elucidated and the active site is known, it becomes possible to construct mutants of the enzyme with altered substrate specificity and enantioselectivity. Activities towards the creation of such (improved) enzymes are currently underway in our laboratory.

**Experimental**

**General**

The enantiomeric excess (%ee) and the yield of the epoxides was determined with a Hewlett-Packard 5890 gas chromatograph equipped with a FID-detector, using a Chiraldex G-TA capillary column (col I) or a CP-cyclodextrin-b-2,3,6-M-19 capillary column (col II), both of 50 m length and 0.25 mm inside diameter. The absolute configuration of the phenylethanolamine was determined by HPLC using a Chiralcel OB column. Optical rotations were determined on a Perkin-Elmer 241 MC polarimeter at 589 nm. NMR-spectra were recorded in CDCl$_3$ using TMS as an internal standard. Purification of the remaining epoxides was performed by flash chromatography using silica 60 H.

**Enzyme preparation**

For the described enzymatic resolutions a partially purified enzyme was used. Initially the epoxide hydrolase gene was cloned by means of polymerase chain reaction and brought to expression in \textit{E. coli} as described previously. Briefly, plasmid DNA was transformed by electroporation to competent \textit{E. coli} BL21 (DE3) cells, which were then plated out on LB plates containing ampicillin and incubated overnight at 30 °C. A preculture was started by inoculating 100 ml of LB containing ampicillin with the transformants from a plate to a starting OD$_{600}$ of 0.1. The culture was incubated at 30 °C until an OD$_{600}$ of 1-2 was reached,
and then diluted in 1 liter of LB medium containing ampicillin, and the culture was incubated overnight at 20 °C. The cells were subsequently centrifuged, washed and resuspended. A crude extract was prepared by ultrasonic disruption and centrifugation of the cells. This was followed by a purification step with a DEAE-cellulose anion exchange column. Finally, the partially purified enzyme was dialyzed against a Tris-\(\text{SO}_4\) buffer, lyophilized and stored at 4°C. Prior to use the lyophilized enzyme preparation was suspended in Tris-\(\text{SO}_4\) buffer (pH 9.0, 50 mM). From a 1 liter culture, 300 to 400 mg lyophilized partially purified enzyme could be prepared with a purity of 25 to 40%.

**Kinetic resolution with epoxide hydrolase**

A reaction vessel containing 20 to 100 ml Tris buffer (50 mM, pH 9) was incubated in a shaking waterbath at 30 °C. In case of the epoxides \(\text{2, 4, 5 and 6}\) the buffer contained 10 % DMSO. The epoxide was added to a final concentration of 5 mM. The reaction was started by the addition of the enzyme solution to a final concentration of 25 to 125 µg/ml. The reaction was monitored by periodically taking samples from the reaction mixture. The samples were extracted with diethylether containing mesitylene as an internal standard. Prior to analysis by chiral GLC, the samples were dried on a short column containing MgSO\(_4\).

For the determination of the optical rotation of the remaining enantiomers, the reaction was terminated at the point where an e.e. >99% was reached. The water phase was extracted twice with hexane. The combined organic phases were dried (MgSO\(_4\)) and evaporated. The residue was further purified by flash chromatography using hexane/ether as eluent. In the case of epoxide \(\text{4}\), the water phase was extracted with hexane, saturated with NaCl and extracted twice with ethylacetate. The combined organic phases were dried (MgSO\(_4\)) and evaporated.

The obtained para-chloro-1-phenyl-1,2-ethanediol was converted to 1-phenyl-1,2-ethanediol, by reduction with sodium metal in ethanol.\(^{23}\) For this, the para-chloro-1-phenyl-1,2-ethanediol (50 mg) was dissolved in absolute ethanol (3 ml) and refluxed under argon for 30 min. Sodium (150 mg) was added in 25 mg portions to the stirring solution. After 2.5 hours the suspension was evaporated to near dryness and aqueous HCl was added. The aqueous phase was extracted with ethylacetate, dried (MgSO\(_4\)) and the 1-phenyl-1,2-ethanediol was found to be the \((R)\)-enantiomer as determined by HPLC using a Chiralcel OB column. According to the enzyme mechanism, by which the \((R)\)-enantiomer of the epoxide is hydrolysed with retention of configuration, it was concluded that with the kinetic resolution the remaining epoxide was of the \((S)\)-configuration.

The data from the chiral GLC and the obtained optical rotations are as follows:

1. \(t_c(S) = 12.4 \text{ min and } t_c(R) = 14.8 \text{ min at 110 } ^\circ \text{C};\) \(\alpha\) \(\text{D}^{25}\) +19.5(c 1.97, CHCl\(_3\))^18, col II: \(t_c(R) = 52.8 \text{ min and } t_c(S) = 54.4 \text{ min at 90 } ^\circ \text{C};\)
2. \(t_c(S) = 27.9 \text{ min and } t_c(R) = 30.3 \text{ min at 90 } ^\circ \text{C};\)
3. \(t_c(S) = 20.2 \text{ min and } t_c(R) = 31.1 \text{ min at 120 } ^\circ \text{C};\)
4. \(t_c(S) = 20.2 \text{ min and } t_c(R) = 31.1 \text{ min at 120 } ^\circ \text{C};\)
5. \(t_c(S) = 20.2 \text{ min and } t_c(R) = 31.1 \text{ min at 120 } ^\circ \text{C};\)
6. \(t_c(S) = 20.2 \text{ min and } t_c(R) = 31.1 \text{ min at 120 } ^\circ \text{C};\)
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°C; 6, col I t_r(S) = 40.7 min and t_r(R) = 43.0 min at 100 °C; 7, col I t_r(S) = 83.3 min and t_r(R) = 84.5 min at 95 °C.

The absolute configuration of the enantiomers of phenylethanediol was determined by HPLC by co-injection of both enantiomers on a Chiralcell OB column using hexane/2-propanol as the eluent (ratio 9:1, 0.5 ml/min, retention times 14.7 min and 17.9 min). The formation of 3-phenoxy-1,2-propanediol during the hydrolysis of epoxide 7 was monitored by HPLC on a Chiralcel OD column using hexane/2-propanol as the eluent (ratio 9:1, 1.0 ml/min, t_r(R) = 17.2 min and t_r(S) = 35.9 min).

Synthesis of epoxides

The epoxides 1 and 7 were bought from Aldrich and epoxide 5 was a gift from DSM. The epoxides 2, 3, 4 and 6 were synthesized from their corresponding aldehydes and ketones with trimethylsulfonium methylsulfate. The synthesis of para-chlorostyrene oxide is a typical procedure for the epoxidation of aldehydes and ketones. To a solution of para-chlorobenzaldehyde (2.8 gram, 19.9 mmol) in CH_2Cl_2 (20 ml) trimethylsulfonium methylsulphate was added (4.4 gram, 23.3 mmol). Aqueous NaOH (50 %, 10 ml) was added and the reaction mixture was stirred overnight. Water was added and the organic phase was separated. The water phase was extracted twice with CH_2Cl_2. The combined organic phase was washed twice with 20 ml portions of water, subsequently shaken for 20 minutes with a saturated solution of sodium metabisulphite and finally washed twice with 20 ml portions of water. The organic phase was dried and the CH_2Cl_2 was removed on a rotary evaporator. The para-chlorostyrene oxide was obtained by bulb-to-bulb distillation or by flash chromatography on silica 60 H. In the case of epoxide 3 the reaction was performed at the same conditions with the exception that the reaction mixture was heated to reflux.

2: 1H NMR, δ: 2.46 (s, 3H), 2.90 (dd, 1H), 3.25 (dd, 1H), 7.3 (m, 4H), 13C-NMR δ: 20.9 (CH_3), 50.9 (C-2), 52.2 (C-1), 125.4, 128.9, 134.4, 137.8 (C-Ar)
3: 1H NMR, δ: 1.62 (s,3H), 2.65 (d, 1H), 2.85 (d, 1H), 7.2 (m, 5H), 13C-NMR δ: 21.6 (CH_3), 56.5 (C-1), 56.8 (C-2), 125.2, 127.4, 128.2, 141.1 (C-Ar)
4: 1H NMR, δ: 2.63 (dd, 1H), 3.19 (dd, 1H), 4.21 (dd, 1H), 7.20-7.38 (m, 4H), 13C-NMR δ: 49.8 (C-2), 50.5 (C-1), 125.5, 126.9, 128.8, 135.4 (C-Ar)
6: 1H NMR, δ: 2.88 (dd, 1H), 3.27 (dd, 1H), 3.96 (dd, 1H), 7.32-7.46 (m, 4H), 13C-NMR δ: 51.0 (C-2), 51.5 (C-1), 126.7, 128.6, 133.8, 136.1 (C-Ar)
3: 1H NMR, δ: 2.46 (s, 3H), 2.90 (dd, 1H), 3.25 (dd, 1H), 7.3 (m, 4H), 13C-NMR δ: 20.9 (CH_3), 50.9 (C-2), 52.2 (C-1), 125.4, 128.9, 134.4, 137.8 (C-Ar)

The reference compounds (S)-1 and (R)-5 were bought from Aldrich and (R)-7 was synthesized from (S)-glycidol and phenol, according to a literature procedure. 25

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