Enantioselective biocatalytic conversions of epoxides
Lutje Spelberg, Jeffrey Harald

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Chapter 6

Highly enantioselective and regioselective biocatalytic azidolysis of aromatic epoxides
Chapter 6

Introduction

Chiral non-racemic 1,2-azidoalcohols are precursors for a wide variety of 1,2-aminoalcohols as well as useful intermediates in carbohydrate chemistry. A common method for the synthesis of an 1,2-azidoalcohol is ring-opening of the corresponding epoxide whereby, if the epoxide is unsymmetrical, azidolysis occurs via a bimolecular reaction at the least substituted carbon atom. Aryl epoxides such as styrene oxide form an exception, because the phenyl group stabilizes the formation of a positive charge at the benzylic carbon atom (Cα) in the transition state, favoring bimolecular attack at this position. Variation of the metal counter-ion of azide or addition of a catalytic amount of an organometallic reagent increases the rate of azidolysis, but noteworthy change of regioselectivity towards the less substituted β-carbon atom is not observed. A substantial increase in the β-selectivity of the azidolysis of styrene oxide can be obtained with LiN3/HMPA (94% β-attack) and with LiN3 in the presence of β-cyclodextrin. In the latter case a partial kinetic resolution leads to 1-phenyl-2-azido-ethanol, enantiomerically enriched to a low extent. Enantioselective azidolysis of meso-epoxides and various terminal epoxides using chiral salen complexes has been described by the group of Jacobsen, but the regioselectivity of ring-opening of styrene oxide is obscured by decomposition of the products.

Results and discussion

Recently we described the use of the halohydrin dehalogenase from Agrobacterium radiobacter AD1 to obtain optically active epoxides and halohydrins such as (S)-2,3-dichloro-1-propanol (E>100) and (S)-2-chloro-1-phenylethanol (E=73) by means of kinetic resolution of halohydrins. Here we show that azide can be used as a nucleophile in the reverse reaction. Furthermore, this enzyme catalysed azidolysis is both highly enantioselective and β-regioselective towards (substituted) styrene oxides.

Table 1  Equilibrium constants of the reaction of various sodium salts with (R)-1a by the halohydrin dehalogenase from Agrobacterium radiobacter AD1.

<table>
<thead>
<tr>
<th>sodium salt</th>
<th>Keq (mM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaBr</td>
<td>480</td>
</tr>
<tr>
<td>NaCl</td>
<td>40</td>
</tr>
<tr>
<td>NaF</td>
<td>no reaction</td>
</tr>
<tr>
<td>NaN3</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

a) The exact equilibrium constant was too low to be determined using the chromogenic substrate.
Highly enantioselective and regioselective biocatalytic azidolysis

\[ K_{eq} = \frac{[NaX] \times ([R]-1a)}{([R]-alcohol)} \]

Using the chromogenic substrate para-nitrostyrene oxide (\(R\))-1a we determined the equilibrium constant (\(K_{eq}\)) of the reversible reaction catalysed by the halohydrin dehalogenase with a variety of halides and nucleophiles. The nucleophilic ring opening of para-nitrostyrene oxide leads to a decrease in the extinction coefficient at 310 nm, which made it possible to monitor the enzymatic ring opening online by the decrease of absorbance. Using chiral HPLC, it was established that the enzymatic ring opening is highly regioselective towards the \(\beta\)-carbon atom. With chloride and bromide the equilibrium is predominantly on the side of the epoxide (Table 1). This limits a practical application of this reaction since a large excess of halide would be necessary to favor the formation of the halohydrin over the epoxide. With azide as nucleophile, the equilibrium lies on the side of the product of ring-opening, although the exact equilibrium constant was too small to be determined accurately using the chromogenic substrate. This implies that a total enzymatic conversion of the epoxide to the azido alcohol is possible with only a small excess of sodium azide.

The recombinant halohydrin dehalogenase from Agrobacterium radiobacter AD1 catalysed the highly enantioselective and regioselective azidolysis of substituted styrene oxides (Table 2). The enzyme converted para-nitrostyrene oxide 1a to the corresponding azido alcohol 1b with high regioselectivity and enantioselectivity. The remaining (\(S\))-1a was obtained with an e.e.>99% and the azido alcohol (\(R\))-1b was formed with an e.e. of 96%. The corresponding E-value was calculated to be higher than 200 either from the e.e.’s of the epoxide and the azido alcohol or by using conversion and e.e. of the epoxide.\(^{10}\) The initial activity with (\(R\))-1a at a substrate concentration of 1 mM was 180 mU.mg\(^{-1}\). Even at initial substrate concentrations higher than 1 mM the curve showed first order kinetics, indicating a considerably higher \(K_m\)-value of the substrate. The initial activity of (\(S\))-1a was too low to be measured (<1 mU.mg\(^{-1}\)). The epoxides 2a and 3a were also converted with high enantioselectivity leading to optically pure epoxides (e.e.>99%) and the almost optically pure (98% e.e.) azido alcohols 2b and 3b. A complication is chemical azidolysis leading mainly to the azido alcohols 2c and 3c. The intrinsic E-value (Table 2, ee.a/ee.p) of the enzymatic resolutions based on the e.e.’s of a and b was higher than 200, but the apparent E-value (conv./ee.), which takes the unwanted chemical conversion into account, was considerably lower.

The high \(\beta\)-regioselectivity of the enzyme catalysed reaction is striking and is, as mentioned, opposite to the observed selectivity in the non-catalysed azide ion ring opening. For the kinetic resolution of 2a, \(\beta\)-attack took place to the extent of 89% at 55% conversion compared to chemical azidolysis, which involves only 3% reaction at the \(\beta\)-position. During the enzymatic reaction the apparent selectivity percentage is continuously lowered due to the chemical side-reaction. When the reaction is performed with an excess of enzyme, the initial
β-regioselectivity for all substrates was higher than 98% indicating an almost absolute opposite regioselectivity compared to the chemical reaction.

Would the chemical azidolysis leading to product c derail a practical application of this halohydrin dehalogenase as a biocatalyst for large scale conversions? To determine the optimal conditions for a large scale synthesis, the influence of increasing azide concentrations on the conversion of 1a to 1b was investigated by monitoring the initial activities (concentration 1a, 250 µM) online at 310 nm. The apparent \( K_{m} \)-value for azide was determined to be 0.2 mM, from which we may expect that above a concentration of approximately 0.5 mM the enzymatic activity will become independent of the azide concentration. If in a kinetic resolution, azide is present in a higher concentration, only an increase in the disadvantageous chemical azidolysis will be observed. To circumvent the problem of excessive volumes owing to the low solubility (3 mM) of 1a we added the substrate (0.47 gr) as a second solid phase to 60 ml buffer (MOPS, pH = 7.0) containing 29 mg of purified enzyme. The amount of substrate was 7.8 gr/l, which is 17-fold the solubility. Sodium azide (0.6 molar equivalent) was slowly added over 24 h keeping the azide concentration around 0.5 to 1 mM.

Table 2  
*Kinetic resolution of epoxides 1a, 2a and 3a by the recombinant halohydrin dehalogenase from Agrobacterium radiobacter AD1.*

<table>
<thead>
<tr>
<th>epoxide</th>
<th>conv.a</th>
<th>e.e. a</th>
<th>e.e. b</th>
<th>E-value</th>
<th>β-attackb</th>
<th>init.act. c</th>
<th>k_{az} d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>ee_{ee}, ee_{p}</td>
<td>conv., ee_{s}</td>
<td>%</td>
<td>mU.mg(^{-1})</td>
</tr>
<tr>
<td><strong>1a</strong></td>
<td>51</td>
<td>&gt;99 (S)</td>
<td>96 (R)</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>98 (37)</td>
<td>180</td>
</tr>
<tr>
<td><strong>2a</strong></td>
<td>55</td>
<td>&gt;99 (S)</td>
<td>98 (R)</td>
<td>&gt;200</td>
<td>51</td>
<td>89 (3)</td>
<td>640</td>
</tr>
<tr>
<td><strong>3a</strong></td>
<td>64</td>
<td>&gt;99 (S)</td>
<td>98 (R)</td>
<td>&gt;200</td>
<td>15</td>
<td>79 (2)</td>
<td>190</td>
</tr>
</tbody>
</table>

\( a)\) Substrate concentration 2 mM in Tris-SO\(_4\) (50 mM, pH = 7.3, 3 mM mercaptoethanol, 22°C); NaN\(_3\) concentration 1.3 mM. \( b)\) Value between parentheses is the % β-attack of the chemical conversion. \( c)\) Initial activity of (R)-enantiomer in kinetic resolution, 1 mU equals 1 nmol.min\(^{-1}\). \( d)\) Bimolecular reaction constant of chemical azidolysis of the epoxide.
After work-up, the yields and e.e.'s of the compounds present in the mixture were determined using chiral HPLC. The mixture consisted of (S)-1a in 46% yield (98% e.e.) and (R)-1b in 47% yield (97% e.e.). Compound 1c, the result of the non-enzymatic reaction, formed a total of 4% of the reaction mixture and the product of chemical hydrolysis of the epoxide, para-nitrophenylethanediol is present in 3% yield.

In conclusion, we describe here for the first time a highly enantioselective (E>200) and β-regioselective azidolysis of (substituted) styrene oxides using the halohydrin dehalogenase from *Agrobacterium radiobacter* AD1. This selectivity appears to be limited to the enzyme from the above mentioned organism. We have also examined two distinct halohydrin dehalogenases from other organisms in this investigation. The recombinant halohydrin dehalogenases from *Mycobacterium* sp. GP1 and *Arthrobacter* sp. AD2 also catalysed the azidolysis of 1a but the E-value with both enzymes was lower than 5.

This method provides at low azide concentration a suitable synthesis of an optically pure aromatic β-azido alcohol under the very mild conditions of room temperature, aqueous medium and neutral pH. Although a and b could be obtained in a very high e.e., an intrinsic limitation of a kinetic resolution is the maximum yield of 50% for both remaining substrate and formed product. It gives direct access to optically active 2-amino-1-arylethanol, which are building blocks for a wide range of pharmaceutical products, via the inexpensive and easily accessible racemic epoxides. Recently, the gene encoding the halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 has been cloned and the enzyme has been brought to overexpression. This makes the enzyme available in multi-gram quantities and thus an application on an industrially interesting scale may become feasible.

**Experimental**

**General**

The enantiomeric excesses (e.e.) and/or the yields of 1a, 1b and 1c and para-nitrophenylethanediol were determined by HPLC using a Chiralpak AS column from Daicel. The enantiomeric excesses (e.e.) and/or yields of 2a, 2b, 2c, 3a, 3b, and 3c were determined by chiral GC using a Chiralsil Dex CB column from Chrompack. Substrate depletion curves of epoxide 1a were recorded on a Perkin Elmer Lambda BIO 40 spectrophotometer provided with a temperature-controlled cell holder. NMR-spectra were recorded in CDCl$_3$.

**Production of halohydrin dehalogenase**

A gene library of *A. radiobacter* AD1 was constructed in the cosmid vector pLAFR3. After in vitro packaging, the library was transduced to *E. coli* HB101. Transconjugants were screened for dehalogenase activity with 1,3-dichloro-2-propanol. The halohydrin dehalogenase gene, designated HheC, was sequenced and subsequently amplified by PCR and cloned behind the T7 promoter of the expression vector pGEF$^+$, yielding pGEFhheC.
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The halohydrin dehalogenase gene was overexpressed up to 30% of soluble protein by introduction of pGEFhheC in *E. coli* BL21(DE3). For the described kinetic resolutions purified enzyme was used. Plasmid DNA was transformed by electroporation to competent *E. coli* BL21 (DE3) cells, which were then plated out on LB medium containing tetracycline and incubated overnight at 30 °C. A preculture was started by inoculating 100 ml of LB medium containing tetracycline with the transformants from a plate to an initial OD$_{600}$ of 0.1. The culture was incubated at 30 °C until an OD$_{600}$ of 1-2 was reached, diluted in 1 l of LB medium containing tetracycline and 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 Resource Q column. The overexpression and production of the halohydrin dehalogenases from *Mycobacterium* sp. GP1 and *Arthrobacter* sp. AD2 will be published.

**Synthesis of substrates and reference compounds**

Racemic *para*-nitrostyrene oxide 1a. To a cooled solution of ω-bromo-*para*-nitroacetophenone (5.0 g, 20 mmol) in MeOH (50 ml), sodium borohydride was added (1.0 g, 26 mmol) and stirred for 3 h. Water (50 ml) was added and the mixture was extracted with diethyl ether. After separating, the organic phase was washed with brine, dried with MgSO$_4$ and the solvent was removed by a rotary evaporator yielding 4.1 gram of an orange solid. To 1.0 gram of the solid dissolved in diethyl ether 15 ml of an aqueous solution (1 M) of KOH was added. The mixture was refluxed for 15 min, cooled, diluted with sulfuric acid (20 ml, 1 M) and extracted with diethyl ether. After separation, the organic layer was dried with MgSO$_4$, and removed by a rotary evaporator. Recrystallisation in ethanol yielded 0.76 gram of 1a. $^1$H NMR, δ: 2.72 (dd, 1H, $J = 2.6$ Hz and 5.5 Hz); 3.17 (dd, 1H, $J = 4.0$ Hz and 5.5 Hz); 3.91 (dd, 1H, $J = 2.6$ Hz and 4.0 Hz); 7.40 (d, 2H$_{ar}$, $J = 8.8$ Hz); 8.16 (d, 2H$_{ar}$, $J = 8.8$ Hz) $^{13}$C NMR δ: 48.9 (C-1); 49.2 (C-2); 121.3; 123.7; 142.7; 145.3 (C$_{ar}$).

Racemic *para*-chlorostyrene oxide 2a. To a solution of *para*-chlorobenzaldehyde (2.8 gram, 19.9 mmol) in CH$_2$Cl$_2$ (20 ml) trimethylsulphonium 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$_2$Cl$_2$. 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$_2$Cl$_2$ was removed on a rotary evaporator. The *para*-chlorostyrene oxide was obtained by flash chromatography on silica 60 H. $^1$H NMR, δ: 2.88 (dd, 1H), 3.27 (dd, 1H), 3.96 (dd, 1H), 7.32-7.46 (m, 4H), $^{13}$C-NMR δ: 51.0 (C-1); 126.7, 128.6, 133.8, 136.1 (C$_{ar}$).

Racemic azido alcohols 1b and 1c. To a 400 mM solution of sodium azide in water (300 ml), 200 mg of racemic 1a was added and the mixture was stirred for 15 h at room temperature. The water phase was extracted four times with diethyl ether. After separating,
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the organic phase was dried with MgSO$_4$ and removed by a rotary evaporator yielding an orange oil that consisted of 1b and 1c in a 37:63 ratio. Flash chromatography on silica 60 H using heptane/ethyl acetate (ratio 7:3) yielded pure 1b and 1c.

2-azido-1-(para-nitro-phenyl)-ethanol 1b: $^1$H NMR, $\delta$: 2.49 (d, 1H, OH, $J = 3.7$ Hz); 3.45 (m, 2H); 4.95 (m, 1H); 7.51 (d, 2H$_{ar}$, $J = 8.8$ Hz); 8.17 (d, 2H$_{ar}$, $J = 8.8$ Hz). $^{13}$C NMR $\delta$: 55.3 (C-1); 70.0 (C-2); 121.3; 124.3; 145.1; 145.3 (C$_{ar}$).

2-azido-2-(para-nitro-phenyl)-ethanol 1c: $^1$H NMR, $\delta$: 1.99 (t, 1H, OH, $J = 5.5$ Hz); 3.75 (m, 2H); 4.74 (dd, 1H, $J = 4.4$ Hz and 7.3 Hz); 7.49 (d, 2H$_{ar}$, $J = 8.8$ Hz); 8.21 (d, 2H$_{ar}$, $J = 8.8$ Hz). $^{13}$C NMR $\delta$: 63.9 (C-1); 64.2 (C-2); 121.5; 125.6; 141.2; 145.3 (C$_{ar}$).

The same procedure was used for the azidolysis of epoxides 2 and 3. The product mixture was identified by NMR and GC. The product ratio of the two regio-isomers b and c were close to identical to previous reports.$^{13}$

Optically pure (R) and (S) para-nitrostyrene oxide 1a were obtained from racemic 1a by preparative HPLC using an analytical Chiralpak AS column with hexane/isopropanol (95:5) as eluent (1 ml/min). Yields and retention times of the individual enantiomers: (R)-1a, yield 39.2 mg, retention time 17.2 min, e.e.>99%; (S)-1a, yield 37.0 mg, retention time 25.3 min.

Enzymatic conversions

Typical kinetic resolution experiment: to 20 ml of Tris-SO$_4$ buffer (50 mM, pH = 7.3, 30 °C) containing 2 mM of the epoxide and 1.3 mM of NaN$_3$, purified enzyme was added to a concentration of 8 µM. The reaction was monitored by periodically taking 1 ml samples and extracting them with 1.5 ml of diethyl ether containing an internal standard. The organic phase was analysed by chiral HPLC and GC. The bimolecular reaction constants ($k_{az}$) of chemical azidolysis of the epoxides were determined from the slope of a $k_{observed}$ vs [NaN$_3$] plot.

Large scale conversion: To 60 ml of MOPS buffer (50mM, pH = 7.0), 0.47 gram (3.2 mmol) of racemic 1a was added and the suspension was stirred for 60 min. After addition of 29 mg of the enzyme, a prepared stock solution of 0.6 molar equivalents sodium azide in 5 ml MOPS buffer was slowly added over a period of 24 hours. The reaction was stopped and the suspension was extracted three times with diethyl ether. After separation, the organic phase was dried with MgSO$_4$, and removed by a rotary evaporator yielding an orange oil. This mixture was redissolved in diethyl ether and the composition and e.e. of the products were determined by chiral HPLC. The yields given in the text are calculated yields. Flash chromatography on silica 60 H using heptane/ethylacetate (ratio 7:3) yielded pure epoxide and azido alcohols. The NMR data were identical with the synthesized racemic compounds.

Absolute configurations and enantioselectivity

The absolute configurations of the azido alcohols were established by chemical azidolysis of the optically pure epoxide to the corresponding azido alcohols. The products
and side product were analysed by chiral HPLC. Retention times eluent hexane/isopropanol (92:8): (R)-1a, 10.8 min; (S)-1a, 15.9 min; (R)-1b, 41.1 min; (S)-1b, 26.2 min; (R)-1c, 18.7 min; (S)-1c, 22.6 min; para-nitrophenylethanediol 30.9 and 35.6 min. The enantiomeric excess (e.e.) and yields of 2a, 2b, 3a, and 3b were determined by chiral GC using a Chiralsil Dex CB column from Chrompack. The enantioselectivity of the kinetic resolutions was calculated using Formula (1) (eeₐ is the e.e. of the substrate a and eeₚ that of azido alcohol b and in Formula (2), c equals the conversion of substrate a).\(^{10,14}\)

\[
E = \frac{\ln[(1 - \text{ee}ₚ) / (1 + \text{ee}ₚ / \text{ee}ₐ)]}{\ln[(1 + \text{ee}ₚ) / (1 + \text{ee}ₚ / \text{ee}ₐ)]}
\]  

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

The outcome of the above formulas will be the same if no non-enzymatic side reaction occurs. In the case of the kinetic resolutions described in this article, a chemical side reaction to product c occurs. This will result in an overestimated enzymatic conversion and thus an erroneous E-value (apparent E-value) when formula (2) is used. The use of formula (1) is insensitive to the chemical conversion and results in the intrinsic E-value of the enzyme catalysed kinetic resolution. The regioselectivity of formation of 1b versus 1c was calculated using Formula 3.

\[
\beta - \text{regioselectivity} = \frac{\beta_{\text{attack}}}{\beta_{\text{attack}} + \alpha_{\text{attack}}}
\]

References

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