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Enantioselectivity of a recombinant epoxide hydrolase from *Agrobacterium radiobacter*

Jeffrey H. Lutje Spelberg,[a] Rick Rink,[a] Richard M. Kellogg[b] and Dick B. Janssen[a,*]

[a]*Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands*

[b]*Department of Organic and Molecular Inorganic Chemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands*

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Abstract

The recombinant epoxide hydrolase from *Agrobacterium radiobacter* AD1 was used to obtain enantiomerically pure epoxides by means of a kinetic resolution. Epoxides such as styrene oxide and various derivatives thereof and phenyl glycidyl ether were obtained in high enantiomeric excess and in reasonable yield. The enantioselectivity (E-value) of the resolution was calculated from progress curves for styrene oxide (E=16.2) and *para*-chlorostyrene oxide (E=32.2). © 1998 Elsevier Science Ltd. All rights reserved.

1. 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

* Corresponding author. Tel.: 31-50-363-4208; fax: 31-50-363-4165; e-mail: d.b.janssen@chem.rug.nl

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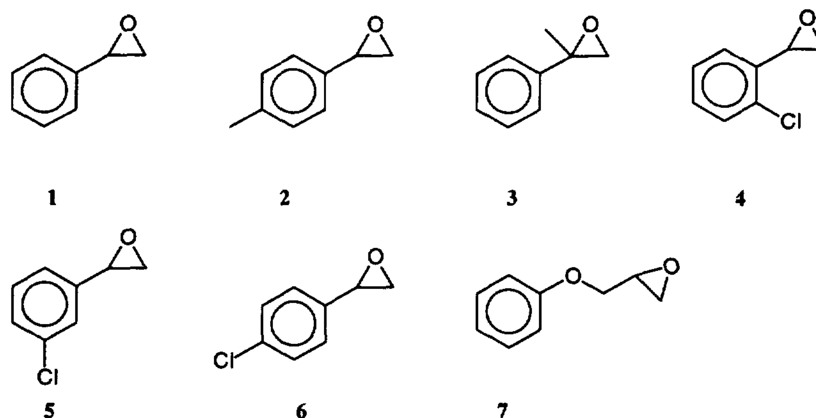

Fig. 1. Epoxides used as substrates by the epoxide hydrolase from *A. radiobacter* AD1

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 enzymes, 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. This intermediate is subsequently hydrolyzed and the diol is released. This mechanism is similar to that which 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 a multi-gram scale, it has the potential to be used as an industrial biocatalyst for the production of enantiomerically pure epoxides.

2. Results and discussion

The epoxide hydrolase gene from *A. radiobacter* AD1 was cloned and brought to overexpression in *E. coli* as described previously.[12] The recombinant enzyme was produced by fermentation and lyophilized partially purified enzyme was prepared. The epoxides **1–7** (Fig. 1) were subjected to enzymatic hydrolysis by the epoxide hydrolase. The results are shown in Table 1. With all the kinetic resolutions an enantiomeric excess (*ee*) of the remaining epoxide of higher then 99% was reached. The yields of the remaining enantiomerically 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

Table 1
Hydrolysis of epoxides **1–7** by the cloned epoxide hydrolase from *A. radiobacter* AD1

epoxide	% e.e	yield (%)	abs. conf.
1	99	33	(S)[a]
2[e]	>99	36	(S)[b]
3	>99	27	(S)[b]
4[e]	>99	35	(S)[d]
5[e]	>99	27	(S)[a]
6[e]	>99	34	(S)[c]
7	>99	28	(R)[a]

[a]Determined by an additional injection of enantiomerically pure epoxide.
[b]Determined by specific rotation.[24]
[c]Deduced from a previously described elution order on chiral GLC.[25]
[d]Determined by reduction of the corresponding diol to 1-phenyl-1,2-ethanediol.[21]
[e]In buffer containing 10% DMSO.

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 with a previously reported optical rotation, although a contradictory optical rotation was also reported in literature.[14] The assignment coincided with the fact that with the kinetic resolutions of all the styrene oxide derivatives the remaining enantiomer was of the (*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. Therefore, the *para*-chloro-1-phenyl-1,2-ethanediol was reduced to 1-phenyl-1,2-ethanediol which was analysed by chiral HPLC using a Chiralcell OB column. The phenyl glycidyl ether **7** yielded the (*R*)-enantiomer which has the same relative configuration at the stereogenic carbon atom as the styrene oxide derivatives. The opposite stereochemical designation is due to a change in priority.

The enantioselective hydrolysis of racemic styrene oxide was followed with time (Fig. 2). After the start of the reaction, the (*R*)-enantiomer of styrene oxide was hydrolyzed to (*R*)-phenylethanediol, indicating that the attack was preferentially at the terminal carbon atom. During the first 40% of the total conversion, the *ee* of the (*R*)-1-phenyl-1,2-ethanediol was 73%. When the (*R*)-enantiomer was completely hydrolysed (*ee*>99%) the *ee* of the diol had decreased to 49%. Similar results were obtained with the hydrolysis of epoxide **7**. Because of the moderate enantioselectivity, the *ee* of the remaining (*R*)-diol was 38% at the timepoint at which the *ee* of the (*S*)-enantiomer of epoxide **7** was higher then 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

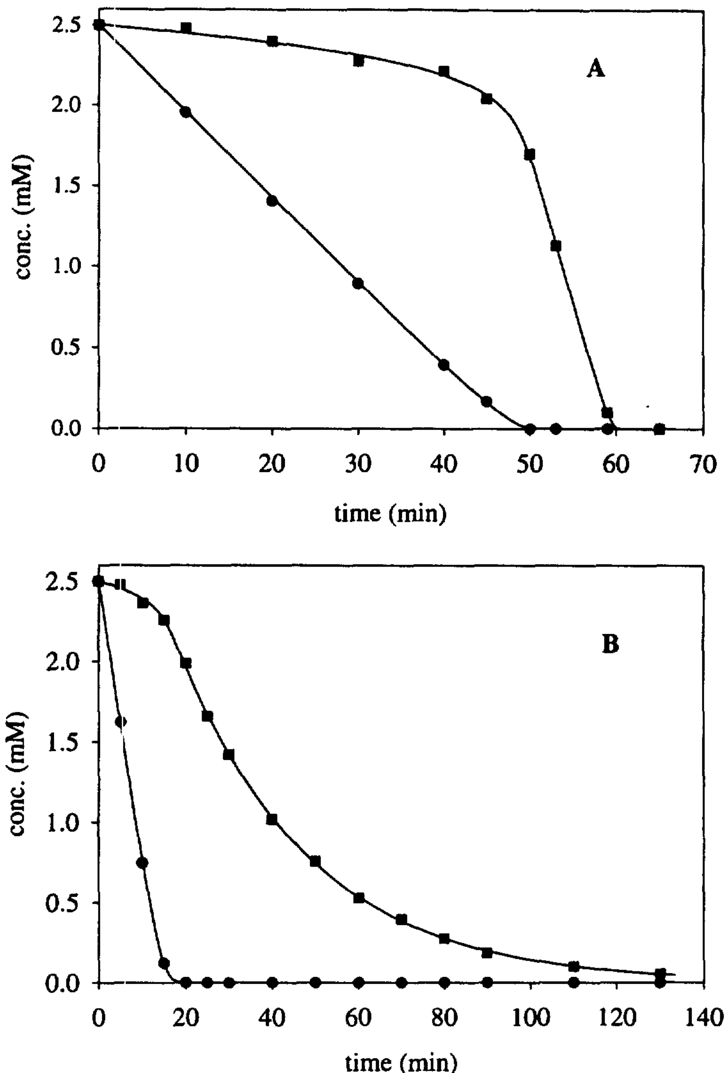

Fig. 2. Kinetic resolution of **A**; styrene oxide ((●), (*R*)-enantiomer; (■), (*S*)-enantiomer) and **B**; *para*-chlorostyrene oxide ((●), (*R*)-enantiomer, (■), (*S*)-enantiomer)

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,[15] *para*-nitrostyrene oxide[16] and *tert*-butyloxirane[17] by microsomal epoxide hydrolases. To investigate these results further, 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 Fig. 2b. During the hydrolysis of the faster reacting (*R*)-enantiomer, the hydrolysis of the (*S*)-enantiomer was inhibited. After more then 90% of the (*R*)-enantiomer was hydrolyzed, the (*S*)-enantiomer was hydrolyzed at a lower rate. This is in contrast 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**.

The enantioselectivity of the reaction can be described by using the enantiomeric ratio[18] which is defined by Eq. 1, where V_{max} and K_m represent the Michaelis–Menten parameters.

$$E = \frac{(V_{max}^S)/(K_m^S)}{(V_{max}^R)/K_m^R)} \quad (1)$$

The conversion of both enantiomers with time can be described by competitive Michaelis–Menten kinetics,[19,20] using Eqs 2 and 3 which also account for chemical hydrolysis.

$$\frac{dS}{dt} = -\frac{V_{max}^S S}{S + \left(\frac{R}{K_m^R} + 1\right) K_m^S} - k_c S \quad (2)$$

$$\frac{dR}{dt} = -\frac{V_{max}^R R}{R + \left(\frac{S}{K_m^S} + 1\right) K_m^R} - k_c R \quad (3)$$

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 8.63×10^{-6} s^{-1} and for epoxide **6** 6.2×10^{-6} s^{-1}. To estimate the kinetic parameters, Eqs 2 and 3 were fitted by numerical integration to the data shown in Fig. 2, and a good fit was obtained. With the obtained kinetic parameters, the *E*-value was calculated using Eq. 1. For epoxide **1** a value of 16.2 and for epoxide **6** a value of 32.2 was calculated.

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 calculated for the V_{max} of the (*R*)-enantiomer. In the case of styrene oxide unique solutions were obtained for the values of V_{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 K_m value for each enantiomer. The estimated K_m of the (*S*)-enantiomer is lower than 50 μM and that of the (*R*)-enantiomer lower than 1 μM, 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_{max} of the (*S*)-enantiomer compared to that of the (*R*)-enantiomer.

According to the method described by Chen et al. (Eq. 4), the enantiomeric ratio is derived from the degree of conversion (*c*) and the enantiomeric excess of the remaining enantiomer of the substrate (ee_s). Because this method is time independent it does not take into account the influence of the chemical hydrolysis.

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \quad (4)$$

Using the data points from each curve (Fig. 2), the enantiomeric ratios determined with Eq. 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 *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 feasible. 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.

3. Experimental

3.1. 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 phenylethanediol was determined by HPLC using a Chiralcell 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.

3.2. 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 *E. coli* as described previously.[12] Briefly, plasmid DNA was transformed by electroporation to competent *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. 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-SO_4 buffer, lyophilized and stored at 4°C. Prior to use the lyophilized enzyme preparation was suspended in Tris-SO_4 buffer (pH 9.0, 50 mM). From a 1 liter culture, 300 to 400 mg of lyophilized partially purified enzyme could be prepared with a purity of 25 to 40%.

3.3. 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 the case of the epoxides **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 diethyl ether 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 specifc rotation of the remaining enantiomers, the reaction was terminated at the point where an *ee* of >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 **4**, the water phase was extracted with hexane, saturated with NaCl and extracted twice with ethyl acetate. 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.[21] 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 ethyl acetate, dried ($MgSO_4$) and the 1-phenyl-1,2-ethanediol was found to be the (*R*)-enantiomer as determined by HPLC using a Chiralcell 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 specific rotations are as follows: (**1**) col. I: t_r (*S*)=12.4 min and t_r (*R*)=14.8 min at 110°C; (**2**) (*S*) $[\alpha]^{25}_D$ +19.5 (c 1.97, $CHCl_3$),[24] col. II: t_r (*R*)=52.8 min and t_r (*S*)=54.4 min at 90°C; (**3**) $[\alpha]^{25}_D$ −7.8 (c 3.84, $CHCl_3$),[24] col. I: t_r (*S*)=27.9 min and t_r (*R*)=30.3 min at 90°C; (**4**) $[\alpha]^{25}_D$ +32.2 (c 1.19, $CHCl_3$), col. I: t_r (*R*)=15.6 min and t_r (*S*)=20.6 min at 120°C; (**5**) col. I: t_r (*S*)=20.2 min and t_r (*R*)=31.1 min at 120°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 coinjection 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 Chiralcell 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).

3.4. 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.[22] 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: ^{1}H NMR δ: 2.46 (s, 3H), 2.90 (dd, 1H), 3.25 (dd, 1H), 7.3 (m, 4H); ^{13}C 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: ^{1}H NMR δ: 1.62 (s, 3H), 2.65 (d, 1H), 2.85 (d, 1H), 7.2 (m, 5H); ^{13}C 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: ^{1}H NMR δ: 2.63 (dd, 1H), 3.19 (dd, 1H), 4.21 (dd, 1H), 7.20–7.38 (m, 4H); ^{13}C NMR δ: 49.8 (C-2), 50.5 (C-1), 125.5, 126.9, 128.8, 135.4 (C-Ar).

6: ^{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-2), 51.5 (C-1), 126.7, 128.6, 133.8, 136.1 (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.[23]

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