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Preparation of (*S*)-2-, 3-, and 4-chlorostyrene oxides with the epoxide hydrolase from *Sphingomonas* sp. HXN-200

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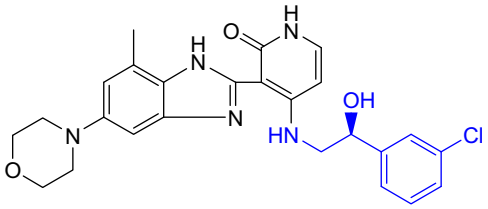
Abstract—The epoxide hydrolase from *Sphingomonas* sp. HXN-200 catalyzed the enantioselective hydrolysis of racemic 2-, 3-, and 4-chlorostyrene oxides **1**–**3** to form the corresponding (*R*)-diols and gave the (*S*)-epoxides **1**–**3** in high ee. The reactions were examined with frozen/thawed cells as well as cell-free extracts of *Sphingomonas* sp. HXN-200 as catalysts in an aqueous, and a two-liquid phase system, respectively. Biotransformation in the two-liquid phase system containing *n*-hexane as an organic phase showed a higher enantioselectivity than that in the single aqueous phase, due to the reduced non-enzymatic hydrolysis. Hydrolysis of 60 mM 2-chlorostyrene oxide **1** gave 31.3% of (*S*)-2-chlorostyrene oxide **1** in 98.8% ee with an enantioselectivity factor (*E*) of 12; hydrolysis of 100 mM 4-chlorostyrene oxide **3** afforded 30.8% of (*S*)-4-chlorostyrene oxide **3** with 98.6% ee with an *E*-value of 11. The best results were obtained with the hydrolysis of 3-chlorostyrene oxide **2**: biotransformation with 100 mM substrate gave 44.0% of (*S*)-3-chlorostyrene oxide **2** in 99.0% ee with an *E*-value of 41; such enantioselectivity is much higher than that of any other known epoxide hydrolases for this reaction; preparative biotransformation demonstrated the efficient synthesis of (*S*)-3-chlorostyrene oxide **2**, an intermediate for the preparation of an IGF-1R kinase inhibitor BMS-536924, with 99.1% ee and 41% isolated yield.

1. Introduction

The production of enantiopure compounds has become more and more important because of the increasing needs of manufacturing chiral drugs as single stereoisomers. On the other hand, environmental concern from governments and the public requires green processes for chemical production. Due to its high enantioselectivity and non-toxicity, enzyme catalysis has become an ideal tool for enantioselective and green syntheses. For instance, epoxide hydrolases are useful catalysts for the production of enantiopure epoxides which are useful synthons for pharmaceutical intermediates via hydrolytic kinetic resolution: enantioselective epoxide hydrolase catalyzes the hydrolysis of the two enantiomers of a chiral epoxide at different rates, leading to the recovery of the less reactive enantiomer in high ee.[1] Epoxide hydrolases are co-factor independent, relatively stable, and widely spread in Nature. Microbial epoxide hydrolases are of particular interests for organic syntheses due to their wide substrate spectrum, easy availability, and high activity.[1]

We are currently interested in developing the efficient synthesis of (*S*)-2-, 3-, and 4-chlorostyrene oxides by using epoxide hydrolase. (*S*)-2-, 3-, and 4-chlorostyrene oxides **1**–**3** are useful intermediates in the preparation of antiviral agents EMI39.3, EMI40.1, and EMI37.1, respectively.[2] In addition, (*S*)-3-chlorostyrene oxide **2** is an useful intermediate for preparing an IGF-1R kinase inhibitor BMS-536924 (Scheme 1).[3] Many epoxide hydrolases have been



BMS-536924

Scheme 1.

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examined for the syntheses of these epoxides.[4–7] While the hydrolysis of racemic 4-chlorostyrene oxide **3** with the epoxide hydrolase from *Aspergillus niger* demonstrated good enantioselectivity with an enantioselectivity factor (*E*)[8] of up to 100,[6,7] the enantioselectivities for the hydrolysis of racemic 2- and 3-chlorostyrene oxides **1**–**2** have so far been unsatisfactory: the highest *E*-values reported are 9.5 and 15, respectively,[4] and both were achieved with the epoxide hydrolase from *Agrobacterium radiobacter*. Previously, we discovered a novel bacterial epoxide hydrolase from *Sphingomonas* sp. HXN-200 that catalyzed the hydrolysis of several alicyclic and aromatic chiral epoxides as well as meso-epoxides with good to excellent enantioselectivity.[9–11] Recently, we have explored the use of this epoxide hydrolase for the enantioselective hydrolysis of racemic 2-, 3-, and 4-chlorostyrene oxides **1**–**3** to achieve higher enantioselectivity. Herein, we report our results on these enzymatic transformations and the preparation of the useful and valuable (*S*)-epoxides **1**–**3**.

2. Result and discussion

2.1. Preparation of biocatalysts and substrates

The cells of *Sphingomonas* sp. HXN-200 were grown in 2 L E2 medium in a bioreactor with *n*-octane vapor as a carbon source.[9] Cells were harvested at the late exponential phase (OD_{450} of 30) and stored at −80 °C. The enzymatic hydrolysis of 2-, 3-, and 4-chlorostyrene oxides (±)-**1**–**3** was performed either with the frozen/thawed cells or with cell-free extracts of *Sphingomonas* sp. HXN-200.

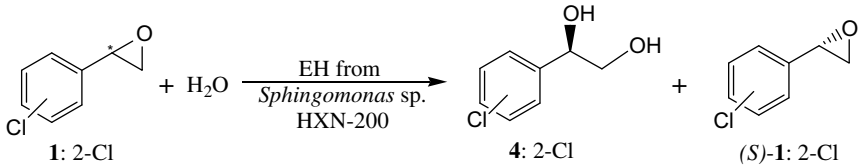
All substrates used were prepared in high purity. 2-Chlorostyrene oxide (±)-**1** was synthesized by the oxidation of 2-chlorostyrene with *m*-CPBA in chloroform at 0 °C.[12] Purification by flash chromatography on a silica gel column afforded (±)-**1** in 98.5% purity and 59% yield. Similarly, 3-chlorostyrene oxide (±)-**2** was prepared from 3-chlorostyrene in 98.2% purity and 36% yield. 4-Chlorostyrene oxide (±)-**3** in 97% purity was commercially available and it was further purified by flash chromatography on a silica gel column to 99.9% purity in 71% yield.

2.2. Hydrolysis of (±)-2-, 3-, and 4-chlorostyrene oxides 1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in aqueous buffer

For the initial test, hydrolysis of (±)-2-, 3-, and 4-chlorostyrene oxides**1**–**3** (Scheme 2) was examined at a concentration of 5.0 mM with frozen/thawed cells of *Sphingomonas* sp. HXN-200 (8 g cdw/L; cdw: cell dry weight) in 50 mM Tris–HCl buffer (pH 7.5) at 25 °C on a 10 mL-scale, respectively. Samples were taken at different time points and centrifuged to remove the cells. The supernatant was extracted with an equal volume of *n*-hexane, and the resulting samples were analyzed on normal phase HPLC with a chiral column (Diacel™ AS-H, 250 × 4.6 mm) for quantifying the ee and concentration of the epoxides. The retention times for (*R*)-**1** and (*S*)-**1** are 16.9 min and 18.3 min, respectively, with *n*-hexane as an eluent at 0.6 mL/min. The retention times for (*R*)-**2**, (*S*)-**2**, (*R*)-**3**, and (*S*)-**3** are 9.4 min, 10.0 min, 10.8 min, and 12.8 min, respectively, with 90% *n*-hexane/10% isopropanol as an eluent at 0.6 mL/min. The configuration of the remaining epoxide **2** was established as (*S*) by comparing its retention time with those of standard (*R*)-**2** and (±)-**2** in the chiral HPLC chromatograms. On the other hand, the configurations of hydrolysis products **1** and **3** were deduced to be both (*S*) by comparing the $[\alpha]_D$ values of the isolated epoxides with the reported values for (*R*)- and (*S*)-**1** and **3**, respectively. The product concentrations were also established by reversed phase HPLC analysis of the samples prepared from the same aliquots taken from the reaction mixtures, and the results were the same as those established by the chiral HPLC. The biotransformation results were demonstrated by plotting the concentrations of (*R*)- and (*S*)-enantiomers in the reaction mixtures versus time. As shown in Figure 1, the (*R*)-epoxides were hydrolyzed faster than (*S*)-epoxides for all the three cases, giving rise to the recovery of the remaining (*S*)-enantiomers in high ee. The hydrolysis of (±)-3-chlorostyrene oxide **2** was the fastest among three transformations: an initial catalytic activity reached 6.1 U/g cdw (U = μmol/min); nearly all (*R*)-**2** were hydrolyzed into the corresponding diol after 1 h and 37.8% of (*S*)-**2** remained with 97.2% ee. This corresponds to an *E*-value of 15. The hydrolysis of (±)-2-chlorostyrene oxide **1** was relatively slow, with an initial activity of 2.9 U/g cdw. Nevertheless, 33.2% of (*S*)-**1** was obtained in 87.8% ee after 4 h of reaction, corresponding to an *E*-value of 7. The hydrolysis of (±)-**3** was the slowest with an activity of 1.1 U/g cdw, while it was also much less enantioselective: (*S*)-**3** was obtained in only 15.9% with 87.3% ee after 4 h of reaction, corresponding to an *E*-value of 3.

2.3. Hydrolysis of (±)-2-, 3-, and 4-chlorostyrene oxides 1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in a two-phase system

It is known that styrene oxide undergoes non-enzymatic hydrolysis in an aqueous buffer.[9] To check the non-enzymatic hydrolysis of (±)-2-, 3-, and 4-chlorostyrene oxides



1: 2-Cl **2**: 3-Cl **3**: 4-Cl — **4**: 2-Cl **5**: 3-Cl **6**: 4-Cl — (*S*)-**1**: 2-Cl (*S*)-**2**: 3-Cl (*S*)-**3**: 4-Cl

Scheme 2.

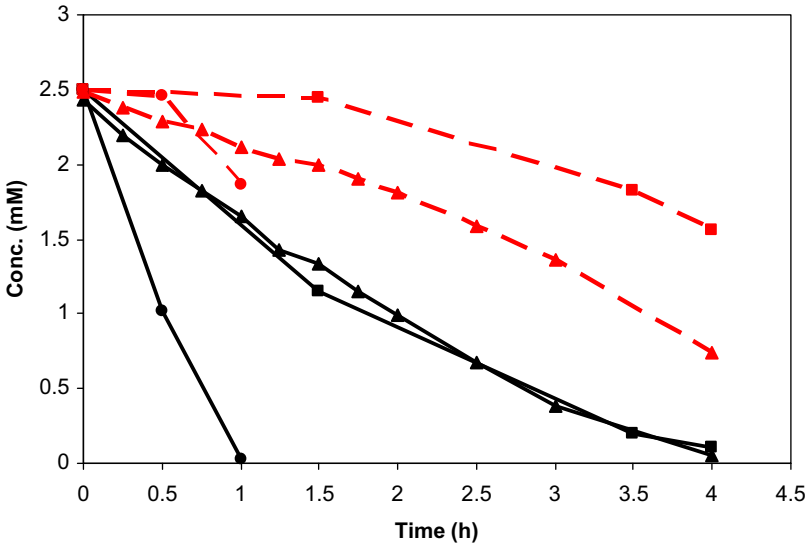


Figure 1. Enantioselective hydrolysis of 5.0 mM (±)-**1**–**3** with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in 10 mL 50 mM Tris–HCl buffer (pH 7.5). ■: (*R*)-**1**. ■: (*S*)-**1**. ●: (*R*)-**2**. ●: (*S*)-**2**. ▲: (*R*)-**3**. ▲: (*S*)-**3**.

1–**3**, a cell-suspension (8 g cdw/L) in 50 mM Tris–HCl buffer (pH 7.5) was boiled for 40 min and then used for the incubation of 10 mM (±)-**1**–**3** at 25 °C for 24 h. Analysis of samples prepared at different time points revealed an average non-enzymatic hydrolysis rates of 1.3%, 1.1%, and 2.0% per h for (±)-**1**–**3**, respectively.

The non-enzymatic hydrolysis of epoxides could be effectively avoided by the use of a two-liquid phase system containing an organic solvent.[9] Thus, the kinetic resolutions of the three epoxides were examined in a two-liquid phase system containing *n*-hexane and an aqueous buffer at a 1:1 (v/v) ratio at 25 °C on a 20 mL scale. Here again the initial concentration of the epoxides (±)-**1**–**3** was 5.0 mM and the cell density of *Sphingomonas* sp. HXN-200 in the aqueous buffer (50 mM Tris–HCl, pH 7.5) was 8 g cdw/L. As shown in Figure 2, all three reactions became much slower in the two-phase system than in the single aqueous buffer. The initial activity was reduced from 2.9 to 0.6 U/g cdw, 6.1 to 2.6 U/g cdw, and 1.1 to 0.7 U/g cdw, respectively. Nevertheless, all the enantioselectivities were improved. The *E*-value was increased from 7 to 10 for (±)-**1**, from 15 to 18 for (±)-**2**, and from 3 to 15 for (±)-**3**. The (*S*)-epoxides **1**–**3** were obtained in 30.0% with 98.0% ee, 38.4% yield with 98.0% ee, and 37.2% with 97.5% ee, respectively. Moreover, the use of the two-liquid phase system for the kinetic resolution of these epoxides has other obvious advantages: high concentration of the epoxide substrates can be used due to their high solubility in *n*-hexane and the enantiopure epoxides can be easily separated from the diol products that were present only in the aqueous phase.

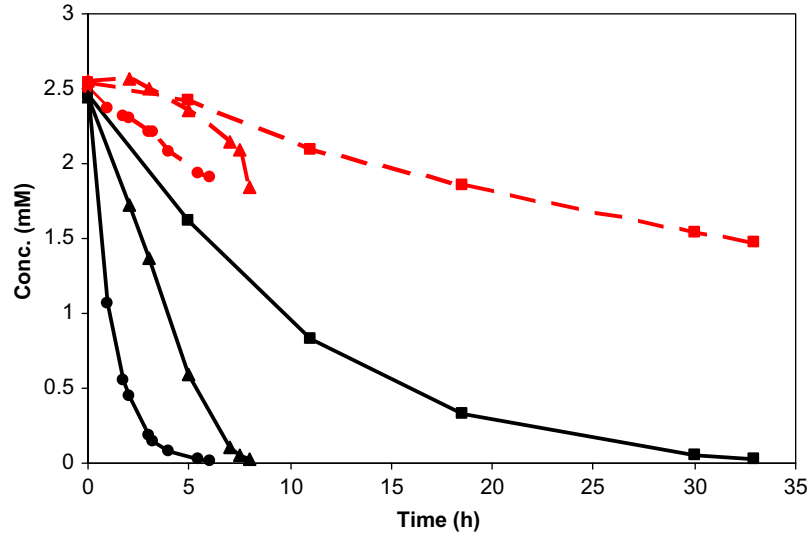


Figure 2. Enantioselective hydrolysis of 5.0 mM (±)-**1**–**3** with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in two-phase system containing 10 mL 50 mM Tris–HCl buffer (pH 7.5) and 10 mL *n*-hexane. ■: (*R*)-**1**. ■: (*S*)-**1**. ●: (*R*)-**2**. ●: (*S*)-**2**. ▲: (*R*)-**3**. ▲: (*S*)-**3**.

To investigate the stereochemistry of product **5**, the enzymatic hydrolyses of 10 mM (*S*)-**2** and 10 mM (*R*)-**2** were performed, respectively, in a mixture of 10 mL *n*-hexane and 10 mL aqueous buffer at a cell density of 16 g/L. Samples were taken at different time points; diol product **5** existed in the aqueous phase and was extracted into chloroform; and the organic phase was analyzed by HPLC on an OD-H column with *n*-hexane/ isopropanol (95:5) as an eluent. Under such analytic conditions, (*R*)-**5** and (*S*)-**5** were successfully separated with retention times of 18.0 min and 21.4 min, respectively. The assignment of the peaks of (*R*)- and (*S*)-**5** was based on the comparison with the reported separation on the same column.[13] Hydrolysis of (*S*)-**2** with the epoxide hydrolase for 1 h gave the corresponding diol (*S*)- and (*R*)-**5** in 92.3:7.7, while the hydrolysis of (*R*)-**2** for 1 h afforded (*S*)- and (*R*)-**5** in 3.1:96.9. In both the cases, the major enzymatic hydrolysis resulted in the retention of the configuration at the stereogenic center, suggesting ring opening at the terminal position. On the other hand, chemical hydrolysis of (*R*)-**2** with acid gave rise to (*S*)-**5**, indicating opening of the epoxide at the benzyl position. Further investigations were carried out on the enzymatic hydrolysis of 20 mM of (±)-**2**, (±)-**1**, and (±)-**3** in the same two-phase system at the same cell density, respectively. Similar to diol **5**, diols **4** and **6** were analyzed by HPLC on an OD–H column with *n*-hexane/isopropanol (95:5) as an eluent. The retention times were 14.8 min for (*R*)-**4**, 19.8 min for (*S*)-**4**, 19.5 min for (*R*)-**6**, and 21.9 min for (*S*)-**6**. While the peaks for (*R*)- and (*S*)- **4** were assigned by comparison with the reported data,[13] the peaks for (*R*)- and (*S*)-**6** were deduced from the similar separation of (±)-**4** and (±)-**5** on the same chiral column with the same eluent. In the enzymatic hydrolysis of (±)-**1**–**3**, all (*R*)-epoxides were preferentially hydrolyzed to give the corresponding (*R*)-diols. Such enantioselectivities are the same as those for the hydrolysis of styrene oxide with the same epoxide hydrolase.[9] In general, the hydrolysis of racemic styrene oxide and chlorostyrene oxides with the epoxide hydrolase of *Sphingomonas* sp. HXN-200 gave rise to the formation of the corresponding (*R*)-diols leaving the less reactive (*S*)-epoxides in high ee.

The rate for the enzymatic hydrolysis of 10 mM (*R*)-**2** was found to be 2.1 times faster than that for the hydrolysis of 10 mM (*S*)-**2**, whereas the *E*-value for the hydrolysis of 20 mM (±)-**2** reached 16. This indicates that the K_m of (*R*)-**2** is at least 7 times smaller than that of (*S*)-**2**. A similar phenomenon was also found for the hydrolysis of styrene oxide with the same epoxide hydrolase.[9]

Different from non-enzymatic hydrolysis, the enzymatic openings of styrene epoxide and chlorostyrene epoxides happened at the terminal position but not at the chemically more active benzylic position. This selectivity is possibly caused by the shape of the enzyme catalytic pocket and substrate binding, but not by the chemical reactivity of different positions. Therefore, the electron-withdrawing and donating effect of the substituents on the benzene ring of the epoxides may not have any significant influence on the enzymatic hydrolysis. Instead, the size, hydrophobicity, and binding ability of the substituents on the benzene ring could play more important roles in the enzymatic hydrolysis. While styrene oxides with alkyl, Br, or OMe substitution might be appropriate substrates for our epoxide hydrolase, hydrophilic substitutions such as NO_2, CN, OH, and NH_2 could create problems in the binding of the corresponding substrates in the enzyme catalytic pocket.

It was recently reported that the phase ratio may influence the enantioselectivity of an epoxide hydrolase in a two-phase system.[14] To investigate this effect, hydrolyses of (±)-**2** (5 mM based on aqueous volume) were carried out in a two-phase system containing hexane and an aqueous buffer at a cell density of 8 g/L in 5:10, 10:10, and 20:10 mL, respectively. Analysis of the conversion and ee of the epoxide at different times revealed no differences in the activity or enantioselectivity ($E = 16$–17) among the three different phase ratios.

Other organic solvent such as *n*-octane, 1-octanol, and *n*-dodecane was compared with *n*-hexane in the two-phase system at a ratio of 1:1 for the hydrolysis of 5 mM (±)-**2**. While the use of 1-octanol as organic phase resulted in low conversion and low *E*-value, the use of *n*-octane and *n*-dodecane as the organic phase gave the same conversion and *E*-value as those with the two-phase system containing *n*-hexane and the aqueous buffer.

2.4. Hydrolysis of (±)-2-, 3-, and 4-chlorostyrene oxides 1–3 with cell-free extracts of *Sphingomonas* sp. HXN-200 in a two-phase system containing aqueous buffer/*n*-hexane (1:1)

To further explore such enzymatic hydrolyses, cell-free extracts (CFE) were used as catalysts since they might give higher enantioselectivity and less side reactions. The CFE was prepared by the treatment of a cell suspension (18 g cdw/L) in 50 mM Tris–HCl buffer (pH 7.5) in a homogenizer at 30 psi and subsequent removal of cell debris through centrifugation at 25,200*g* for 0.5 h. All the operations were carried out at 4 °C to avoid the possible deactivation of enzyme. The resulting CFE was found to contain 7.0 g protein/L by Bradford assay,[15] and it was used for the hydrolysis of epoxides by mixing with equal volume (10 mL:10 mL) of *n*-hexane containing 5.0 mM (±)-**1**–**3**. As shown in Figure 3, the use of CFE for the hydrolysis of (±)-**1**–**3** in the two-phase system did not increase the enantioselectivity in comparison with the use of frozen/thawed cells in the same system. We obtained *E*-values of 8, 17, and 10 for the hydrolysis of (±)-**1**–**3**, respectively. The initial hydrolysis activities of (±)-**1**–**3** were 0.34, 4.8, and 3.0 U/g protein, respectively. Thus, no obvious advantages were achieved with the CFE as biocatalysts.

2.5. Optimization of enzymatic hydrolysis of (±)-2-, 3-, and 4-chlorostyrene oxides 1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in a two-phase system containing aqueous buffer/*n*-hexane (1:1)

Since the use of frozen/thawed cells in a two-liquid phase system was proven to be the best for the enantioselective hydrolysis of epoxides **1**–**3**, such a system was further explored for the production of (*S*)-epoxides in high

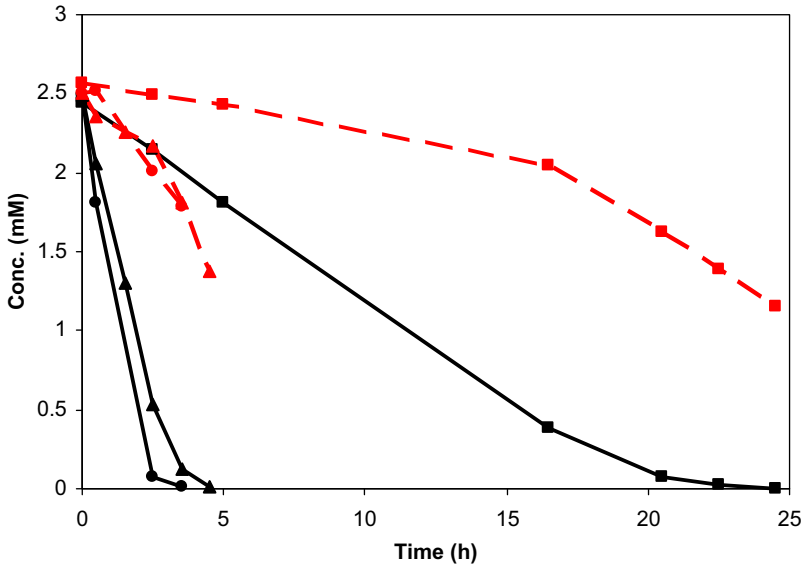


Figure 3. Enantioselective hydrolysis of 5.0 mM (±)-**1**–**3** with CFE of *Sphingomonas* sp. HXN-200 in two-phase system containing 10 mL 50 mM Tris–HCl buffer (pH 7.5) and 10 mL *n*-hexane. ■: (*R*)-**1**. ■: (*S*)-**1**. ●: (*R*)-**2**. ●: (*S*)-**2**. ▲: (*R*)-**3**. ▲: (*S*)-**3**.

concentration, high ee, and high yield. The hydrolyses were examined under different conditions, such as high substrate concentrations [20–60 mM for (±)-**1**; 40–120 mM for (±)-**2**; 20–120 mM for (±)-**3**] and high cell densities (8–32 g cdw/L) and the results are summarized in Table 1. Hydrolysis of 60 mM epoxide **1** at a cell density of 16 g cdw/L gave 31.3% of (*S*)-**1** in 98.8% ee with an *E*-value of 12. Hydrolysis of 100 mM substrate **3** at a cell density of 32 g cdw/L afforded 30.8% (*S*)-**3** in 98.6% ee with an *E*-value of 11. The highest enantioselectivity was observed in the hydrolysis of 100 mM epoxide **2** at a cell density of 16 g cdw/L giving 44.0% of (*S*)-**2** in 99.0% ee. This corresponds to an *E* of 41, demonstrating much higher enantioselectivity of the epoxide hydrolase from *Sphingomonas* sp. HXN-200 than any other known epoxide hydrolases for such reaction. It is also interesting to observe that the enantioselectivity for (±)-**2** is better at higher substrate concentrations, providing the possibility of preparing (*S*)-**2** in high concentration. High substrate concentration in the two-phase system might result in a concentration of (*R*)-**2** in the aqueous buffer higher than its K_m even at the late stage of the resolution. This could efficiently compete

Table 1. Enantioselective hydrolysis of (±)-**1**–**3** with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in a two-phase system containing 10 mL 50 mM Tris–HCl buffer (pH 7.5) and 10 mL *n*-hexane

Subs.	Concentration[a] (mM)	Cell density (g cdw/L)[a]	Activity (U/g cdw)[b]	Time (h)	Product	ee (%)	Yield (%)	*E*
(±)-**1**	20	16	0.44	44	(*S*)-**1**	99.3	25.5	9
(±)-**1**	40	16	0.32	129	(*S*)-**1**	98.1	29.1	9
(±)-**1**	60	16	0.27	194	(*S*)-**1**	98.8	31.3	12
(±)-**1**	20	24	0.35	32	(*S*)-**1**	98.9	27.5	10
(±)-**1**	40	24	0.31	77	(*S*)-**1**	98.3	31.4	11
(±)-**1**	60	24	0.29	116	(*S*)-**1**	98.6	29.2	10
(±)-**1**	40	32	0.27	52	(*S*)-**1**	97.5	32.1	11
(±)-**1**	60	32	0.28	98	(*S*)-**1**	99.0	30.6	12
(±)-**2**	60	16	1.74	27	(*S*)-**2**	99.8	39.3	30
(±)-**2**	80	16	2.06	27	(*S*)-**2**	98.2	42.7	29
(±)-**2**	100	16	1.97	48	(*S*)-**2**	99.0	44.0	41
(±)-**2**	120	16	1.97	67	(*S*)-**2**	98.7	43.8	37
(±)-**3**	20	8	1.25	33	(*S*)-**3**	97.4	30.9	10
(±)-**3**	40	8	0.87	68	(*S*)-**3**	98.9	31.4	12
(±)-**3**	60	8	0.87	134	(*S*)-**3**	95.7	30.0	8
(±)-**3**	60	16	1.30	47	(*S*)-**3**	97.7	30.8	10
(±)-**3**	80	16	1.67	56	(*S*)-**3**	94.4	32.7	9
(±)-**3**	60	24	1.33	31	(*S*)-**3**	96.6	33.9	11
(±)-**3**	80	24	1.89	51	(*S*)-**3**	97.6	30.9	10
(±)-**3**	100	24	1.16	68	(*S*)-**3**	98.5	29.5	10
(±)-**3**	120	24	1.04	87	(*S*)-**3**	97.4	25.5	7
(±)-**3**	100	32	1.37	47	(*S*)-**3**	98.6	30.8	11

[a] Based on the volume of buffer.
[b] Average activity in the first 30 min.

with the binding and conversion of (*S*)-**2**, thus increasing the enantioselectivity.

2.6. Preparation of (*S*)-2-, 3-, and 4-chlorostyrene oxides 1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in a two-phase system containing aqueous buffer/*n*-hexane (1:1)

To demonstrate the application potential of these transformations, biohydrolyses were performed in 100 mL oftwo-phase system. The results were shown in Figure 4. Hydrolysis of 0.305 g (±)-**1** in 50 mL *n*-hexane (39.4 mM) and 50 mL buffer containing frozen/thawed cells (32 g cdw/L) for 75 h followed by workup and flash chromatography gave 0.109 g (*S*)-**1** in 98.5% ee with an $[\alpha]_D^{20} = +58.2$ (*c* 0.20, $CHCl_3$) {lit.:[16] +61.5 (*c* 1.62, $CHCl_3$)}. The yield obtained was 37% after the deduction of the volume taken for sampling during the biotransformation.

Similarly, hydrolysis of 0.656 g (±)-**2** in 50 mL *n*-hexane (84.8 mM) and 50 mL buffer was carried out at a cell density of 16 g cdw/L for 100 h. Workup and flash chromatography resulted in 0.254 g (*S*)-**2** in 99.1% ee {$[\alpha]_D^{20} = +11.5$ (*c* 1.61; $CHCl_3$); lit.:[16] −10.9, (*c* 1.80, $CHCl_3$) for (*R*)-**2**} with a yield of 41% after deduction of the sampling.

Finally, hydrolysis of 0.618 g (±)-**3** in 50 mL *n*-hexane (79.9 mM) and 50 mL buffer at a cell density of 32 g cdw/L was performed for 61 h. After workup and purification by chromatography, 0.117 g of (*S*)-**3** was isolated in 99.1% ee $[\alpha]_D^{20} = +24.5$ (*c* 1.37, $CHCl_3$); {lit.:[16] +24.9 (*c* 1.50, $CHCl_3$)}, corresponding to a yield of 20% after the deduction of analytic samples taken.

Kinetic resolutions of terminal epoxides with chiral (salen)-Co^{III} complexes are well known.[17] Hydrolysis of (±)-**1**–**3** with an (*S*,*S*)-(salen)Co^{III} complex (0.8–1.5 mol %; 3.5–7.0 wt %) afforded (*S*)-**1**–**3** in >99% ee with an isolated yield of 38%, 40%, and 38%, respectively.[17] Compared with these metal-catalyzed syntheses, our enzymatic preparations of (*S*)-**1** and (*S*)-**2** gave the same product ee and isolated yields. In view of the non-toxicity, easy availability, and low cost of whole cell catalysts, our methods provide green and economical syntheses of these (*S*)-epoxides.

3. Conclusion

Epoxide hydrolase of *Sphingomonas* sp. HXN-200 catalyses the enantioselective hydrolysis of racemic 2-, 3-, and 4-chlorostyrene oxides **1**–**3** forming the corresponding (*R*)-diols and giving (*S*)-epoxides **1**–**3** in high ee, with an *E* of 12, 41, and 11, respectively. This epoxide hydrolase is much more enantioselective than any other known epoxide hydrolase for the hydrolysis of 3-chlorostyrene oxide **2**. It also has better enantioselectivity than the best known epoxide hydrolase for the hydrolysis of 2-chlorostyrene oxide **1**. The best catalytic system for the hydrolysis of (±)-**1**–**3** is the two-phase system containing *n*-hexane and aqueous buffer with frozen/thawed cells of *Sphingomonas* sp. HXN-200 as catalysts. This system allows for the use of high substrate concentration, the reduction of non-enzymatic hydrolysis, and the easy recovery of the epoxide by phase separation. Enzymatic syntheses of (*S*)-**1**–**3** in high ee and high concentration have been successfully demonstrated. The best result is the preparation of (*S*)-3-chlorostyrene oxide **2** in 99.1% ee with 41% yield by the hydrolysis of 100 mM (±)-**2**, which provides a green and efficient synthesis of (*S*)-**2**, an intermediate for the preparation of an IGF-1R kinase inhibitor BMS-536924.

4. Experiment

4.1. Analytical method

Concentrations and ee of **1**, **2**, and **3** were determined with a Shimadzu™ Prominence HPLC on a Diacel™ AS-H chiral column (250 × 4.6 mM, 5 μm) at 25 °C. Detection:

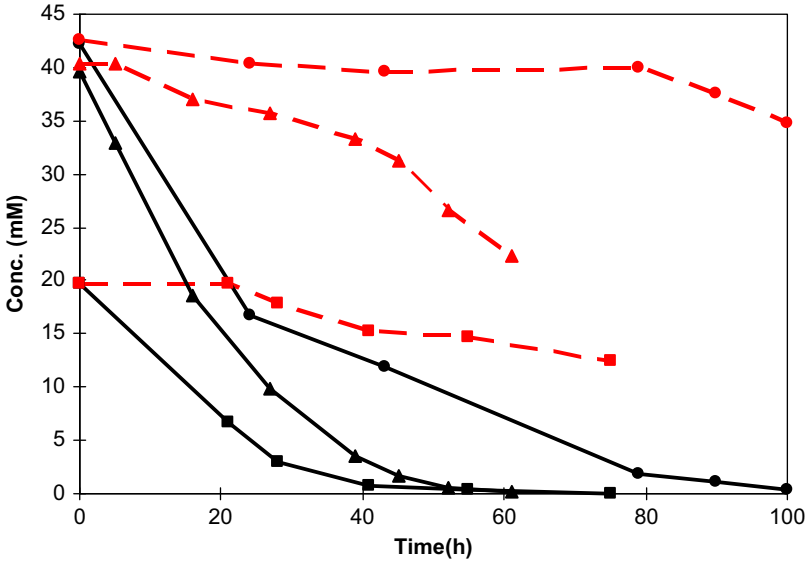


Figure 4. Enzymatic preparation of (*S*)-**1**–**3** by enantioselective hydrolysis of (±)-**1**–**3** with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in two-phase system containing 50 mL 50 mM Tris–HCl buffer (pH 7.5) and 50 mL *n*-hexane. ■: (*R*)-**1**. ■: (*S*)-**1**. ●: (*R*)-**2**. ●: (*S*)-**2**. ▲: (*R*)-**3**. ▲: (*S*)-**3**.

210 nm; eluent: *n*-hexane for the analysis of **1**, *n*-hexane/IPA (90:10) for the analysis of **2** and **3**; flow rate: 0.6 mL/min; internal standard: (*R*)-styrene oxide for quantifying **1**, benzyl alcohol for quantifying **2** and **3**; retention times: 16.9 min for (*R*)-**1**, 18.3 min for (*S*)-**1**, 31.8 min for (*R*)-styrene oxide; 9.4 min for (*R*)-**2**, 10.0 min for (*S*)-**2**, 11.8 min for benzyl alcohol; 10.8 min for (*R*)-**3**, 11.8 min for benzyl alcohol, and 12.8 min for (*S*)-**3**. The ee values of **4**, **5**, **6** were determined with the same HPLC on a Diacel™ OD-H chiral column (250 × 4.6 mM, 5 μm) at 25 °C. Detection: 210 nm; eluent: *n*-hexane/IPA (95:5); flow rate: 0.9 mL/min; retention times: 14.8 min for (*R*)-**4**, 19.8 min for (*S*)-**4**; 18.0 min for (*R*)-**5**, 21.4 min for (*S*)-**5**; 19.5 min for (*R*)-**6**, and 21.9 min for (*S*)-**6**. The concentrations of (±)-**1**–**3** in aqueous samples were determined with a Shimadzu™ class-VP HPLC system on a Hypersil BDS-C18 (5 μm, 4 × 125 mM) at 25 °C. Detection: 210 nm; eluent: KH_2PO_4 buffer (10 mM, pH 7.0)/CAN (70/30); flow rate: 1.0 mL/min; internal standard: benzyl alcohol; retention time: 15.2 min for **1**; 14.2 min for **2**; 13.8 min for **3**, and 2.2 min for benzyl alcohol. Optical rotations were determined in chloroform at 20 °C with a Jasco™ spectropolarimeter.

4.2. Materials

4-Chlorostyrene oxide (±)-**3** (96%), 2-chlorostyrene (97%), and 3-chlorostyrene (98%) were purchased from Aldrich. (*R*)-Chlorostyrene oxide **2** (98%), (*R*)-styrene oxide (98%), and benzyl alcohol (>97%) were obtained from Fluka. *m*-Chloroperoxybenzoic acid (70%) was purchased from Sigma.

2-Chlorostyrene oxides (±)-**1** (98.5%) and 3-chlorostyrene oxide (±)-**2** (98.2%) were prepared from 2-chlorostyrene and 3-chlorostyrene, respectively.[12] 4-Chlorostyrene oxide (±)-**3** (99.9%) was purified from (±)-**3** (96%) by flash chromatography.

Cells of *Sphingomonas* sp. HXN-200 were grown in E2-medium with *n*-octane as a carbon source in a 2 L fermentor according to the published procedure,[9] harvested at late exponential phase, and stored at −80 °C. The frozen cells were thawed shortly before the use for biotransformation.

4.3. Chemical hydrolysis of (*R*)-3-chlorostyrene oxide 2

To a solution of 0.61 g of (*R*)-3-chlorostyrene oxide **2** in THF (25 mL) and water (5 mL) were added 2 drops of concentrated sulfate acid (98%) and the mixture was stirred for 24 h. The solution was neutralized by adding saturated $NaHCO_3$ and the product was extracted into ether (3 × 30 mL). The organic layer was separated, dried over $MgSO_4$, and the solvent was removed by evaporation. Flash chromatography on a silica gel column (*n*-hexane/ethyl acetate = 1:1, $R_f = 0.33$) afforded 0.30 g of (*S*)-1-(3-chlorophenyl)-1,2-ethanediol **5** with an $[\alpha]_D^{20} = +22.3$ (*c* 0.93; EtOH) [lit.:[13] −22.5, (*c* 1.1, EtOH) for (*R*)-**5**].

4.4. Non-enzymatic hydrolysis of 2-, 3-, and 4-chlorostyrene oxides (±)-1–3 in aqueous buffer

Frozen/thawed cells of *Sphingomonas* sp. HXN-200 in 50 mM Tris–HCl buffer (pH 7.5) were boiled for 40 min and diluted to a cell density of 8 g cdw/L. To 10 mL of this suspension was added (±)-**1**, **2** or **3** to 10 mM and the mixture was incubated at 300 rpm, 25 °C, for 24 h. 300 μL aliquots were taken out at different time points for HPLC analysis. Analytic samples were prepared by centrifugation and dilution of 100 μL supernatant with 100 μL MeOH containing 5.0 mM (*R*)-styrene oxide or benzyl alcohol as the internal standard. The concentrations of the remaining epoxides **1**, **2**, or **3** were analyzed by HPLC on a Hypersil BDS-C18 column. Average non-enzymatic hydrolysis rates of 1.3%, 1.1%, and 2.0% per h were obtained for (±)-**1**–**3**, respectively.

4.5. General procedure for enzymatic hydrolysis of 2-, 3-, and 4-chlorostyrene oxides (±)-1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in aqueous buffer

To a 10 mL suspension of frozen/thawed cells (8 g cdw/L) of *Sphingomonas* sp. HXN-200 in 50 mM Tris–HCl buffer (pH 7.5) was added (±)-**1**, **2**, or **3** to a concentration of 5.0 mM. The mixture was incubated at 300 rpm and 25 °C. Then, 300 μL aliquots were taken out at different time points for HPLC analysis. Cells were removed by centrifugation and the supernatants were divided into two portions. Then a 100 μL supernatant was mixed with 100 μL MeOH containing 2.0 mM (*R*)-styrene oxide or benzyl alcohol as the internal standard for the analysis of product concentration by a HPLC with a Hypersil BDS-C18 column; 100 μL supernatant was extracted with equal volume of *n*-hexane containing 5.0 mM (*R*)-styrene oxide or benzyl alcohol as the internal standard, and the organic phase was separated, and dried over $NaSO_4$ for determining the ee and concentration of the remaining epoxide by a chiral HPLC. The results are shown in Figure 1.

4.6. General procedure for the enzymatic hydrolysis of 2-, 3-, and 4-chlorostyrene oxides (±)-1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in a two-phase system containing aqueous buffer/*n*-hexane (1:1)

To a 10 mL suspension of frozen/thawed cells of *Sphingomonas* sp. HXN-200 (8–32 g cdw/L) in 50 mM Tris–HCl buffer (pH 7.5) was added 10 mL *n*-hexane containing 5.0–120 mM of (±)-**1**, **2**, or **3**, and the mixture was incubated at 300 rpm and 25 °C. Then, 500 μL samples were taken out at different time points for analysis. After centrifugation, 100 μL organic phase was separated, and diluted with equal volume of *n*-hexane containing 2.0 mM (*R*)-styrene oxide or benzyl alcohol as the internal standard, and the ee and concentration of the epoxide were quantified by HPLC with a chiral column. The results are given in Figure 2 and Table 1.

4.7. General procedure for enzymatic hydrolysis of 2-, 3-, and 4-chlorostyrene oxides (±)-1–3 with cell-free extracts of *Sphingomonas* sp. HXN-200 in a two-phase system containing aqueous buffer/*n*-hexane (1:1)

Frozen cells of *Sphingomonas* sp. HXN-200 were thawed and then suspended in 50 mM Tris–HCl buffer (pH 7.5) to a cell density of 18 g/L. The cells were disrupted with a homogenizer (Constant Cell Disruption System™) at 30 psi. Centrifugation at 25,200*g* for 0.5 h in a Hitachi high-speed refrigerated centrifuge followed by the removal of cell debris gave the cell-free extracts. All the operations were carried out at 4 °C to avoid enzyme deactivation. The protein concentration was determined to be 7.0 g protein/L with Bradford method.[15]

Then, 10 mL of freshly prepared CFE (7.0 g protein/L) was mixed with an equal volume of *n*-hexane containing 5.0 mM (±)-**1**, **2**, or **3**, and the mixture was incubated at 300 rpm and 25 °C. At different time points, 500 μL sample was taken out and centrifuged for phase separation, 100 μL of organic phase was diluted with equal volume of *n*-hexane containing 2.0 mM benzyl alcohol as the internal standard, and the sample was analyzed by chiral HPLC to determine the ee and concentration of the epoxide. The results are shown in Figure 3.

4.8. Enzymatic preparation of (*S*)-1–3 by the hydrolysis of 2-, 3-, and 4-chlorostyrene oxides (±)-1–3 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 in a two-phase system containing aqueous buffer/*n*-hexane (1:1)

4.8.1. (*S*)-3-Chlorostyrene oxide 2. A suspension of frozen/thawed cells (16 g cdw/L) of *Sphingomonas* sp. HXN-200 in 50 mL 50 mM Tris–HCl buffer (pH 7.5) was mixed with an equal volume of *n*-hexane containing 3-chlorostyrene oxide (±)-**2** (0.656 g, 4.24 mMol, 84.8 mM) in a 250 mL shaking flask. The mixture was incubated at 300 rpm and 25 °C, and 300 μL samples of liquid were taken at 0, 24, 43, 79, 90, and 100 h, respectively, to follow the reaction. Samples were prepared by centrifugation, separation, dilution of 20 μL organic layer into 1.580 mL 1 mM benzyl alcohol (internal standard), and analyzed by chiral HPLC to determine the ee and the concentration of the remaining epoxide **2**. After 100 h, the ee of epoxide **2** exceeded 98%, and the reaction was stopped. The mixture was centrifuged at 10,000 rpm for 30 min at 4 °C, and the organic phase was collected. The aqueous phase was extracted with *n*-hexane (2 × 50 mL), and all organic phase was combined. After drying over $MgSO_4$, the solvent was removed by evaporation. The crude product was then purified by flash chromatography on a silica gel column with *n*-hexane ($R_f = 0.31$). 0.254 g (*S*)-**2** was obtained in 38.7% yield and 99.1% ee, with an $[\alpha]_D^{20} = +11.5$ (*c* 1.61, $CHCl_3$) {lit.:[16] −10.9, (*c* 1.80, $CHCl_3$) for (*R*)-**2**}. Deducing the volume of sampling during the biotransformation gave a corrected yield of 41%.

4.8.2. (*S*)-2-Chlorostyrene oxide 1. In a procedure similar to the synthesis of (*S*)-**2**, 50 mL *n*-hexane containing (±)-**1** (0.305 g, 1.97 mMol, 39.4 mM) and 50 mL 50 mM Tris–HCl buffer (pH 7.5) with a cell density of 32 g cdw/L was mixed and shaken at 300 rpm and 25 °C. For following the reaction, 300 μL liquid samples were taken at 0, 21, 28, 41, 55, and 75 h, respectively, and analytical samples were prepared by centrifugation, separation, and dilution of 20 μL organic layer with 1.580 mL 1 mM (*R*)-styrene oxide as an internal standard. The ee and concentration of the remaining epoxide **1** were quantified by chiral HPLC. The reaction was stopped at 75 h with an ee of (*S*)-**1** >98%. The product was recovered according to the same procedure described above for (*S*)-**2**. Purification by flash chromatography on a silica gel column with *n*-hexane ($R_f = 0.46$) gave 0.109 g (*S*)-**1** in 35.7% yield and 98.5% ee with an $[\alpha]_D^{20} = +58.2$ (*c* 0.20, $CHCl_3$) {lit.:[16] +61.5, (*c* 1.62, $CHCl_3$)}. The yield was corrected as 37% after deducing the sampling volume during the biotransformation.

4.8.3. (*S*)-4-Chlorostyrene oxide 3. In a procedure similar to the synthesis of (*S*)-**2**, a mixture of 50 mL *n*-hexane containing (±)-**3** (0.618 g, 4.00 mMol, 79.9 mM) and 50 mL 50 mM Tris–HCl buffer (pH 7.5) with cell density of 24 g cdw/L was shaken at 300 rpm and 25 °C. To follow the bioconversion, 300 μL samples were taken at 0, 5, 16, 27, 39, 45, 52, and 61 h, respectively. Samples for chiral HPLC analysis were prepared by centrifugation and separation of organic layer followed by diluting 20 μL organic layer with 0.620 mL 1.032 mM benzyl alcohol as the internal standard. The reaction was stopped after 61 h when ee of (*S*)-**3** reached >98%, and the product was recovered according to the same procedure described above for (*S*)-**2**. After workup and flash chromatography on a silica gel column with *n*-hexane ($R_f = 0.29$), 0.117 g of (*S*)-**3** was obtained in 18.9% yield and 99.1% ee {$[\alpha]_D^{20} = +24.5$ (*c* 1.37, $CHCl_3$); lit.:[16] +24.9, (*c* 1.50, $CHCl_3$)}. The corrected yield was 20% after the deduction of analytic samples taken during the biotransformation.

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