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Nitrite-mediated hydrolysis of epoxides catalyzed by halohydrin dehalogenase from *Agrobacterium radiobacter* AD1: a new tool for the kinetic resolution of epoxides

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Abstract—Halohydrin dehalogenase obtained from *Agrobacterium radiobacter* AD1, has been tested for the nitrite-mediated ring opening of epoxides. This reaction mainly leads to the formation of unstable hydroxynitrite ester intermediates, which can be further hydrolyzed to the corresponding diols. This conversion proceeds with high enantioselectivity and high regioselectivity towards styrene oxide derivatives. It has been concluded that halohydrin dehalogenase can serve as an attractive alternative to epoxide hydrolases in the preparation of enantiopure epoxides by kinetic resolution.

1. Introduction

Epoxides are valuable intermediates in the synthesis of various pharmaceutical intermediates. Due to the strain on the oxirane ring, epoxides can easily be ring-opened by various nucleophiles, affording a broad range of β-substituted alcohols. There is an increasing need for enantiopure biologically active compounds, and as a result much effort has been put into the development of stereoselective processes for the preparation of enantiopure epoxides. Methods that have been explored over the past two decades are stereoselective synthesis from achiral alkenes and enantioselective conversion of epoxides.[1,2] Biocatalytic approaches have also proven to be very promising.[3,4]

The halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 (HheC)[5] is involved in the bacterial degradation of halohydrins, such as 1,3-dichloro-2-propanol. This enzyme catalyzes the intramolecular displacement of a halogen substituent by a vicinal hydroxyl group to form an epoxide and halide ions.[6] This reaction occurs in a highly enantioselective fashion with aromatic halohydrins such as 1-(*para*-nitrophenyl)-2-chloroethanol. Furthermore, the enzyme can catalyze not only the ring closure of the halohydrin but also the reverse reaction, that is, the nucleophilic ring opening of an epoxide.[6] Using *para*-nitrostyrene oxide (*p*-NSO), it was observed that anions, such as azide, cyanide or nitrite, could be accepted as alternative nucleophiles in the ring-opening reaction.[7,8] HheC-catalyzed ring opening of various styrene oxides with azide resulted in highly enantioselective and regioselective production of azidoalcohols.[9] Ring opening of an epoxide by the cyanide ion has also been shown using a halohydrin dehalogenase isolated from *Corynebacterium* sp. strain N-1074.[10]

To the best of our knowledge, enzyme-catalyzed ring opening of an epoxide by NO_2^- has not been explicitly described in the literature so far, although chemical ring opening of an epoxide by nitrite has been reported.[11] Since nitrite is an ambident nucleophile that can attack through its nitrogen or oxygen, four types of adducts can theoretically be expected from the reaction of nitrite with an epoxide. As shown in Figure 1, the

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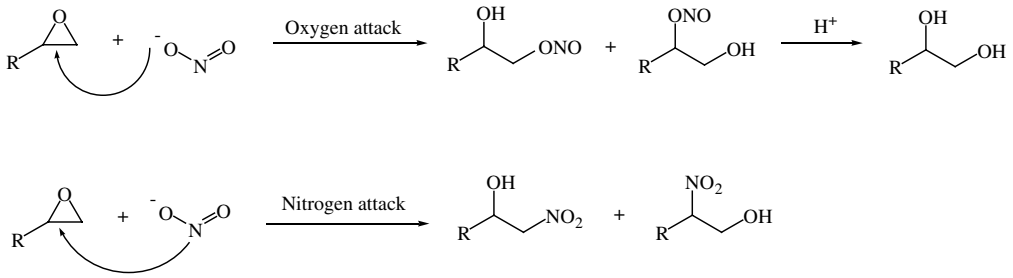

Figure 1. Possible reactions between an epoxide and nitrite ion. In theory a total of four different adducts, plus their enantiomers, can be formed, depending on α/β and N/O selectivity of the nucleophilic attack. When attack occurs through the oxygen, nitrite esters are formed, which are spontaneously hydrolyzed to diols.

formation of products depends on two types of regioselectivity: oxygen or nitrogen attack, and α– or β-selectivity of the attack on the epoxide ring. When the attack occurs through the nitrogen, 1,2-nitroalcohols are formed, which are stable compounds in neutral aqueous media. When the reaction proceeds via an oxygen attack, unstable nitrite esters are formed, which can be readily hydrolyzed to their corresponding alcohols.

Herein we report for the first time an enzyme-catalyzed ring opening of epoxides by nitrite. We show that HheC catalyzes the enantioselective and β-regioselective ring opening of *para*-nitrostyrene oxide with $NaNO_2$ via an oxygen attack leading to the corresponding hydroxy-nitrite ester. Due to its inherent unstable character, this compound hydrolyzes to give its parent diol.[12–14] Therefore, HheC in combination with nitrite acts as a highly enantioselective epoxide hydrolase.

2. Results and discussion

2.1. Epoxide ring opening with nitrite

In order to examine the acceptance of nitrite as a nucleophile for the ring opening of epoxides by halohydrin dehalogenase from *A. radiobacter*, *para*-nitrostyrene oxide (*p*-NSO) was chosen as the model substrate. This epoxide is known from previous studies to be accepted as a substrate by the enzyme and its relative stability towards chemical hydrolysis makes it an attractive compound for testing enzyme-catalyzed conversion.

For these experiments, *p*-NSO (1.5 mM) was incubated with nitrite (10 mM) and HheC (35 μM) in a buffered solution (Tris–SO_4 pH = 7.5) and its conversion followed using chiral HPLC. As reported in Figure 2, (*R*)-*p*-NSO was converted preferentially, with an initial activity of the enzyme of 0.2 μmol min^{-1} mg^{-1} (0.2 U). The remaining (*S*)-epoxide was obtained with an ee of 99% and a calculated yield of 48% (maximum theoretical yield is 50%).

Conversion of *p*-NSO was accompanied by the formation of a mixture of products as judged by the appearance of several unidentified peaks in the HPLC elution profile. Among these products, one was formed in a larger amount (t_R = 24.5 min) when compared to the other

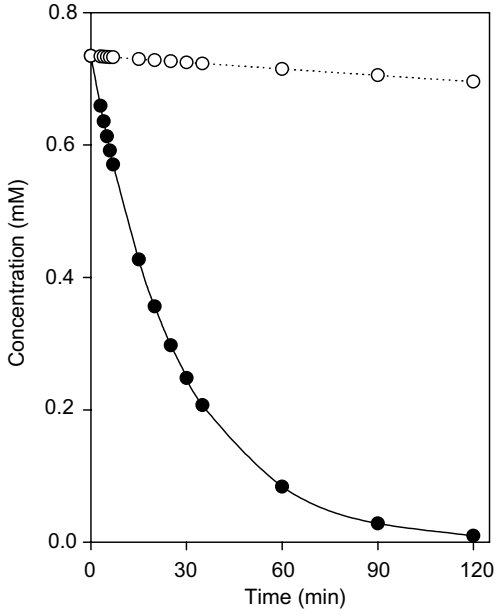

Figure 2. Kinetic resolution of *para*-nitro-styrene oxide (*p*-NSO) catalyzed by HheC and nitrite. Symbols: (●): (*R*)-*p*-NSO, (○): (*S*)-*p*-NSO.

compounds, suggesting that the enzyme might display some regioselectivity. One other product (t_R = 11.9 min) transiently accumulated before it slowly disappeared (Fig. 3), indicating that the nitrite might attack through the oxygen yielding a nitrite ester, which is expected to be unstable.[12–14] Furthermore, two other peaks were observed (t_R = 34.3 min and t_R = 46.5 min), corresponding to stable products.

2.2. Oxygen versus nitrogen regioselectivity

To identify the formed products and investigate the oxygen and nitrogen selectivity of the nitrite attack, the expected products 2-hydroxy-2-(*para*-nitrophenyl)-nitroethane (Fig. 4, **d**), 2-hydroxy-2-(*para*-nitrophenyl)-ethylnitrite ester (Fig. 4, **b**) and *para*-nitrophenyl-ethane-1,2-diol (Fig. 4, **c**) were chemically synthesized and used as reference compounds.

From comparison of the retention times of the synthesized reference compounds on chiral HPLC with those

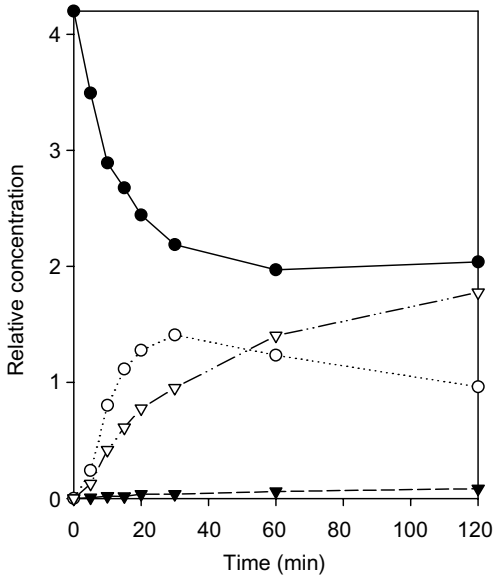

Figure 3. Product formation during the conversion of *p*-NSO by HheC and nitrite. Symbols: (●): *p*-NSO, (○): nitrite ester (t_R = 11.9 min), (▽): (*R*)-2-(*para*-nitrophenyl)-1,2-ethanediol (t_R = 24.5 min.), (▼): (*S*)-2-(*para*-nitrophenyl)-1,2-ethanediol (t_R = 30.4 min).

of the products, which were formed during the enzymatic conversion of *p*-NSO, it was concluded that the main product of the reaction was diol **c** (Fig. 4), of which one enantiomer (t_R = 24.5 min) was formed in a larger amount than the other (t_R = 30.4 min). The unstable intermediate at t_R = 11.9 min was identified as nitrite ester **b** (Fig. 4), which was expected to be formed from an oxygen attack by the nitrite ion. The peaks at t_R = 34.3 min and 46.5 min corresponded to the enantiomers of the adduct expected from the nitrogen attack of the nitrite on the epoxide, namely 2-hydroxy-2-(*para*-nitrophenyl)-nitroethane **d** (Fig. 4), of which one enantiomer was formed in excess. These results show that *p*-NSO could be attacked both by the oxygen and by the nitrogen atom of nitrite ion. When the attack occurred through the oxygen, an unstable nitrite ester **b** was formed, which was then slowly hydrolyzed to yield its corresponding diol **c**.

In order to achieve a quantitative analysis of the oxygen/nitrogen regioselectivity, the reaction of *p*-NSO with nitrite catalyzed by HheC was also followed using on-line ^{1}H NMR spectroscopy. The formation of products was monitored by recording, at regular intervals, the spectra of the incubation mixture containing HheC and nitrite in an NMR tube containing a near-saturated solution of *p*-NSO in a D_2O buffer at pD = 7.5 (reading 7.1 on a pH meter). The results of the integration of the aromatic proton signals were directly correlated to concentrations. The identity of detected products was again established by comparing the signals of the aromatic protons with those of the synthesized reference compounds. For this, the ^{1}H NMR spectra of the substrate and the reference compounds were recorded in the same deuterated buffer that was used for the enzymatic reaction, showing again that diol **c** was the main product that nitrite ester **b** was formed as an unstable

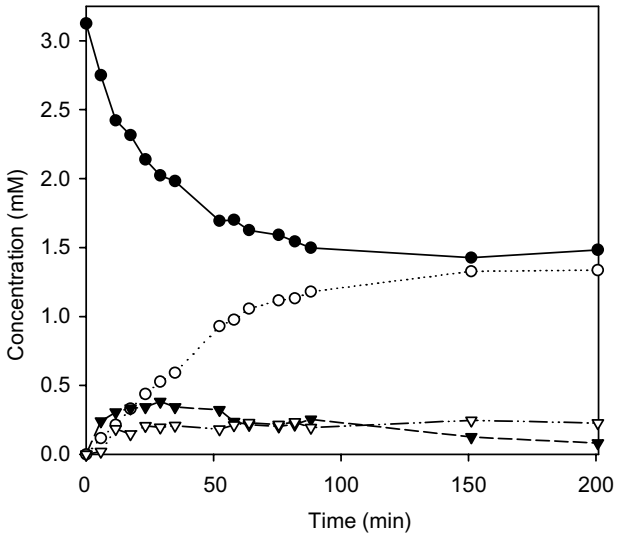

Figure 5. On-line NMR monitoring of ring opening of *p*-NSO by nitrite catalyzed by HheC. Symbols: (●): *p*-NSO; (○): main product 2-(*para*-nitrophenyl)-1,2-ethanediol **c** (Fig. 4); (▼): unstable 2-hydroxy-2-(*para*-nitrophenyl)-ethylnitrite ester **b** (Fig. 4); (▽): 2-hydroxy-2-(*para*-nitrophenyl)-nitroethane **d** (Fig. 4) as a side product.

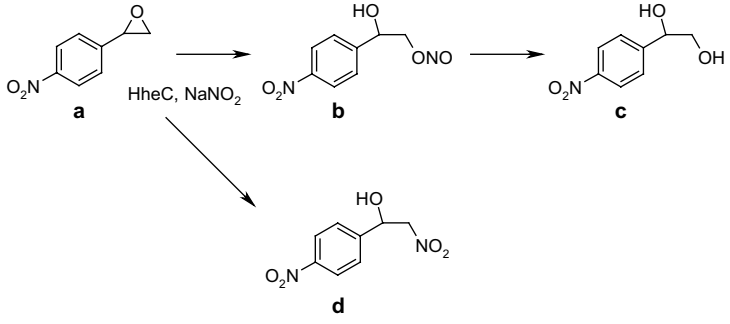

Figure 4. Nitrite-mediated ring opening of *p*-NSO catalyzed by HheC. Substrate **a** is mainly converted to 2-hydroxy-2-(*para*-nitrophenyl)-ethylnitrite ester **b**, due to the attack of the oxygen of nitrite on the β-position of the epoxide ring. This unstable intermediate is then spontaneously hydrolyzed while the nitrite is recovered and the corresponding 2-(*para*-nitrophenyl)-1,2-ethanediol **c** formed. 2-Hydroxy-2-(*para*-nitrophenyl)-nitroethane **d** resulting from the attack of nitrite on the β-position of the epoxide through its nitrogen atom is formed in low amounts.

intermediate, and that nitroalcohol **d** was obtained as a minor side product (Fig. 5). From the NMR data obtained during conversion of *p*-NSO, it could be calculated that the total yield of diol **c** and nitroalcohol **d** formed from the conversion of (*R*)-*p*-NSO were 40% and 10%, respectively. These results established that HheC could catalyze the nucleophilic ring opening of *p*-NSO by nitrite both via oxygen and nitrogen attack. However, the conversion of (*R*)-*p*-NSO occurred with a regioselectivity of 80% in favour of the oxygen attack.

2.3. α/β-Regioselectivity

The α- or β-regioselectivity of the attack on the oxirane ring determines the structure of the formed products and their absolute configuration. To establish the epoxide-regioselectivity of the O-attack, it was necessary to isolate the unstable hydroxynitrite ester formed during the enzymatic conversion of (*R*)-*p*-NSO, and to determine whether this intermediate carried a primary or secondary hydroxyl function. This was achieved by letting *p*-NSO react with HheC and nitrite at a low temperature in order to reduce the rate of hydrolysis of the hydroxynitrite ester adduct. The intermediate was then isolated by extraction with ether and crystallization from dichloromethane and its ^{1}H NMR spectrum compared to that of the chemically synthesized compound, thus confirming its structure. The analysis of the ^{1}H NMR spectrum recorded in dry DMSO showed the presence of a doublet at $\delta = 5.72$. This signal disappeared when deuterated water was added to the medium, indicating that it corresponded to the hydroxyl proton. The fact that the hydroxyl proton appeared as a doublet shows that it is coupled with only one adjacent proton. This can only be the case when the hydroxyl group is carried by a secondary carbon. Consequently, the molecule contains a secondary hydroxyl function, which implies that the *O*-nitrosyl function is at the β-position. This means that the oxygen attack of the nitrite occurred on the β-position of the epoxide. The ee of the obtained *para*-nitrophenylethane-1,2-diol, as determined by chiral HPLC, was 91%, which would indicate a β-regioselectivity of the oxygen attack of 95.5%. However, this value does not account for the spontaneous chemical hydrolysis of *pNSO*. Therefore, the purely enzyme-catalyzed ring opening of *p*-NSO is expected to happen with a regioselectivity higher than 95.5%.

The regioselectivity of the nitrogen attack could be assessed from the on-line NMR analysis and the chiral HPLC analysis of incubation of racemic *p*-NSO with nitrite and HheC. Using these methods, it was found that only 2-(*para*-nitrophenyl)-2-hydroxynitroethane **d** was produced. This suggests that the nitrogen attack occurred with high β-regioselectivity (>99%), but also with high enantioselectivity since this compound was formed with an ee higher than 98%.

In conclusion, both oxygen and nitrogen attacks were highly β-selective, implying that the epoxide was converted with retention of configuration. (*R*)-*p*-NSO was ring-opened through attack of the nitrite on the β-position, thus yielding the (*R*)-nitrite ester. Since the hydrolysis of nitrite esters is known to occur with retention of configuration[14] it can be concluded that the absolute configuration of the formed *para*-nitrophenylethane-1,2-diol **c** is (*R*). As the product of nitrogen attack of nitrite on the β-position of (*R*)-*p*-NSO, the formed 2-hydroxy-2-(*para*-nitrophenyl)-nitroethane was also of (*R*)-configuration.

2.4. HheC and nitrite as a novel tool for the enantioselective and regioselective hydrolysis of epoxides

The high enantioselectivity and high β-regioselectivity of the ring opening of *p*-NSO by nitrite catalyzed by HheC makes the enzyme a potentially attractive tool for the kinetic resolution of epoxides and the synthesis of optically active diols. As described above and summarized in Table 1, HheC and nitrite catalyzed the kinetic resolution of a racemic mixture of *p*-NSO in an efficient way, allowing the recovery of (*S*)-epoxide in a yield of 48% and an ee of 99% while *para*-nitrophenyl-1,2-ethanediol **c** was formed with an ee of 91%. Thus, the system acts as an epoxide hydrolase.

To explore further the potential use of HheC with nitrite in enantioselective epoxide ring-opening reactions, some other epoxides were tested. In these experiments, besides wild-type HheC also as a mutant enzyme in which tryptophan 249 is replaced by a phenylalanine, HheC–W249F, was used. This HheC–W249F catalyzed the ring closure of 2-bromo-1-(*para*-nitrophenyl)-ethanol with higher enantioselectivity and higher activity than the wild-type enzyme.[15] The wild-type enzyme (WT) and the W249F mutant were both tested for their ability to resolve several aromatic epoxides **1**–**6** (Fig. 6). Substrates (2–5 mM) were incubated with enzyme (WT: 35 μM, W249F: 10 μM) and nitrite (10 mM) and conversion of the epoxides monitored using chiral HPLC or chiral GC. To compare the enantioselectivities of the two systems towards these substrates, the enantioselectivity ratio (*E*) was calculated. The *E*-values, which give the ratio between the V_{max}/K_m values for the separate enantiomers, were obtained by fitting the experimental data with competitive Michaelis-Menten kinetics, using equations described before.[16] For these calculations, the effect of the chemical hydrolysis was neglected. The results of the kinetic resolution experiments are reported in Table 1.

A kinetic resolution is usually regarded as practically useful when the *E*-value is higher than 50.[17] The wild-type enzyme displayed good to excellent enantioselectivity towards three aromatic epoxides **1**, **2** and **3**, providing an attractive system for kinetic resolution. Remarkably, when the W249F mutant of HheC was used, the *E*-values obtained were dramatically increased in most cases, as illustrated by the example of *para*-methylstyrene oxide, for which the enantioselectivity ratio increased from 5 to 193 (Fig. 7). Also for other aromatic epoxides, very high *E*-values were obtained, indicating that nitrite-mediated epoxide ring opening by the mutant halohydrin dehalogenase proceeds with excellent enantioselectivity. Only in the case of epoxide **3** was the enantioselectivity displayed by the mutant

Table 1. Kinetic resolution of substrates **1**–**6** using wild-type and W249F mutant halohydrin dehalogenase from *A. radiobacter* AD1

	WT		W249F	
	E[a]	Initial activity (μmol min^{-1} mg^{-1})	E[a]	Initial activity (μmol min^{-1} mg^{-1})
1	200 (*S*)	0.20	>200 (*S*)	0.30
2	55 (*S*)	0.30	100 (*S*)	0.75
3	45 (*R*)	7.0	28 (*R*)	7.5
4	15 (*S*)	0.30	40 (*S*)	0.70
5	12 (*S*)	0.14	90 (*S*)	0.20
6	5 (*S*)	1.90	193 (*S*)	1.70

[a] The absolute configuration of the remaining epoxide is indicated between brackets.

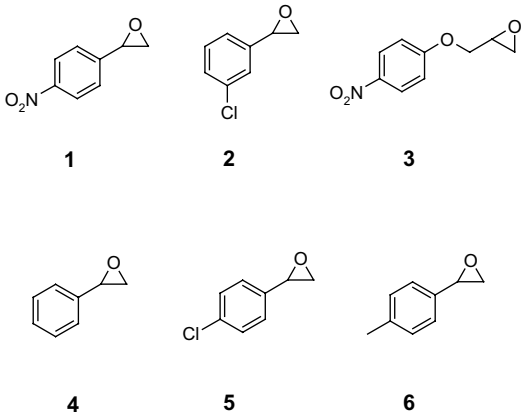

Figure 6. Substrates used in the kinetic resolution by HheC (WT and W249F) and nitrite.

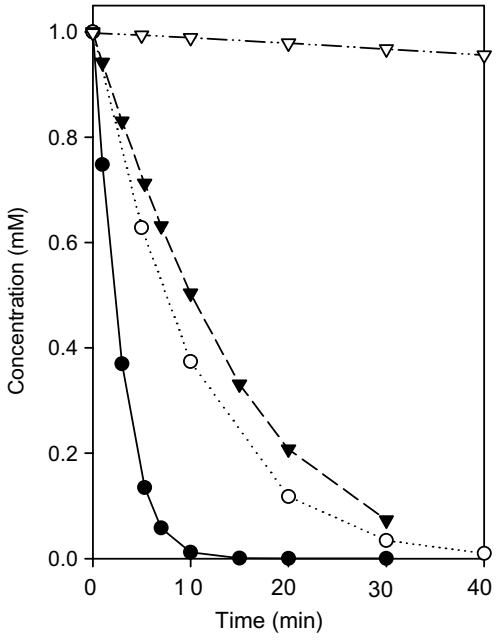

Figure 7. Kinetic resolution of *para*-methyl styrene oxide catalyzed by wild-type HheC, and mutant W249F in presence of nitrite. Symbols: filled symbols: HheC wild-type (35 μM); open symbols: HheC–W249F (10 μM); (▼, ▽): (*S*)-enantiomer; (●, ○): (*R*)-enantiomer.

HheC ($E = 28$) lower when compared to the wild-type enzyme ($E = 45$).

The wild-type HheC and the W249F mutant were also compared in the ring opening of *p*-NSO **1** with regard to the regioselectivities, that is, nitrogen versus oxygen attack and the preference for attack on the α or β carbon of the epoxide ring. For this, the conversion of *p*-NSO catalyzed by the mutant HheC was monitored using chiral HPLC. Diol **c** was formed with an overall yield of 40% while the remainder was converted to nitro alcohol **d**, which is similar to the result obtained with the conversion catalyzed by wild-type HheC. This indicates that both enzymes displayed the same oxygen versus nitrogen regioselectivity. However, the 80% ee of the formed diol **c** was significantly lower when HheC–W249F was used as compared to wild-type HheC (91%) catalyzed conversion. Since the mutant enzyme displayed a higher enantioselectivity for the conversion of *p*-NSO, the lower ee of formed diol **c** cannot be due to the higher conversion of (*S*)-*p*-NSO and concomitant formation of (*S*)-diol **c**, but rather to an increase of the α-attack of (*R*)-*p*-NSO occurring with inversion of configuration and subsequent formation of (*S*)-diol **c**.

To the best of our knowledge, the enzyme-catalyzed ring opening of epoxides by nitrite has not been described for any other enzyme. Our study shows that halohydrin dehalogenase from *A. radiobacter* accepts nitrite as a nucleophile in the ring opening of various epoxides. We have shown that the ambident nitrite ion attacks the epoxide not on the electronically favoured benzylic position but on the sterically less obstructed position, mainly through its oxygen, yielding an unstable nitrite ester intermediate. This nitrite ester adduct is then spontaneously hydrolyzed in such a way that the final product of the reaction is a diol (Fig. 4).

This reaction can be compared to chemical reactions involving nitrite. The first is the nucleophilic substitution of a halogen from haloalkanes by nitrite.[18] In this non-catalyzed conversion, nitrite also behaves as an ambident nucleophile and two types of attack can be observed, yielding a mixture of two possible products of which the predominant one is formed by replacing the halogen by a nitrogen, that is, yielding nitroalkanes. Epoxides can also be ring-opened by nitrite in the presence of $MgSO_4$.[11] In this reaction, nitrite attacks exclusively through the nitrogen. Thus, the N versus O regioselectivity of the enzyme-catalyzed reaction is very different from the chemical conversion, since the HheC-catalyzed ring opening of epoxides **1** to **6** mainly proceeds via oxygen attack. Consequently, the unstable nitrite ester is the most important intermediate. Over the course of the spontaneous hydrolysis of this intermediate, the nitrite ion is regenerated in such a way that it is not consumed in the overall reaction. This means that the nitrite acts more as an assisting catalyst in the overall conversion of epoxides, than as a co-substrate.

The HheC-catalyzed ring opening of *p*-NSO not only occurred with good oxygen regioselectivity but also with good β-regioselectivity and good enantioselectivity. This

made it possible to perform the kinetic resolutions of epoxides, and to produce the corresponding diols with good enantiomeric excess (>91%). Apparently, HheC and nitrite can serve as alternatives to epoxide hydrolases for the kinetic resolution of epoxides. Similar to what was found for most epoxide hydrolases,[19] HheC converts preferentially the (*R*)-enantiomer of the various styrene oxide derivatives. In the case of epoxide **3**, the (*S*)-enantiomer was preferentially hydrolyzed, but this is due to a change in substituent priority rather than to an opposite enantiopreference.

Our study showed that racemic terminal aromatic epoxides were resolved in a satisfactory manner using the wild-type enzyme. Much higher enantioselectivities were observed with the W249F mutant. This HheC and nitrite displayed a significantly higher enantioselectivity than the epoxide hydrolase, that was obtained from the same microorganism (*A. radiobacter* AD1). The *E*-value of the kinetic resolution of epoxides **2** ($E = 103$) and **3** ($E = 45$), which both can be used as intermediates in the synthesis of biologically active molecules, are among the highest values reported so far as compared to microbial epoxide hydrolases.[16,19] The observed increase in enantioselectivity of the W249F mutant enzyme with most of the tested epoxides was the result of both a decrease in the conversion of the non-preferred (*S*)-enantiomer and an increase in the initial activity towards the preferred (*R*)-enantiomer. Thus, the mutant is improved both with regard to its enantioselectivity as well as concerning its activity.

The HheC-catalyzed ring opening of epoxides by azide was reported earlier.[8] The enantioselectivity of this reaction was found to be higher than what was observed here with nitrite. In the HheC-catalyzed azidolysis of styrene oxide, the *E*-value was higher than 200, while for the ring opening of the same epoxide by nitrite, the *E*-value was 15. This suggests that the enantiodiscrimination not only depends on the binding of the organic substrate but is also significantly influenced by the nature of the nucleophile used.

3. Conclusion

In conclusion, we have characterized herein the first example of a nitrite-mediated ring opening of an epoxide by an enzyme, which to the best of our knowledge, is also the first example of an enzyme that accepts nitrite as a nucleophile. Nitrite attacks the epoxide through its oxygen in such a way that the main product was an unstable nitrite ester intermediate, which then spontaneously hydrolyzed to the corresponding diol. This reaction occurred with good oxygen regioselectivity on the terminal position of the oxirane ring, and with good to excellent enantioselectivity when using an improved mutant halohydrin dehalogenase. This makes it possible to apply halohydrin dehalogenase and nitrite to the kinetic resolution of various racemic epoxides.

4. Experimental

4.1. General

Wild type and mutant W249F halohydrin dehalogenases were produced and purified as described before.[15] Enantiomeric excess and yields of epoxides and products were determined using chiral HPLC and/or chiral gas chromatography. Liquid chromatography was done with a Jasco HPLC system equipped with chiral columns [Chiralcel OD (col I), AS (col II) and AD (col III)], a Jasco diode array detector and a Chrompack single wavelength detector. For gas chromatography the following chiral columns were used: Chiraldex GT-A (col IV, 50 m, Astec), β-dex 120 (col V, 30 m, Supelco). NMR experiments were performed using a 300 MHz Varian NMR spectrometer. Spectra were recorded in $CDCl_3$ and DMSO, or in phosphate–D_2O buffer. Epoxides **2**, **5** and **6** were gifts from Enzis. Epoxides **3** and **4** were purchased, respectively, from Aldrich and Acros Chemicals, and used as received.

4.2. Synthesis of substrate and reference compounds

4.2.1. Racemic *para*-nitro-styrene oxide a. To a cooled solution of commercial (Fluka) ω-bromo-*para*-nitroacetophenone (5.0 g, 20 mmol) in MeOH (30 mL), a solution of sodium borohydride (1.0 g, 26 mmol in 20 mL MeOH) was slowly added and the mixture stirred overnight. Water (50 mL) was added and the mixture extracted with diethyl ether. After separation, the organic phase was washed with water, dried over $MgSO_4$ and the solvent removed by a rotary evaporator yielding an orange solid. To 1 g of this solid dissolved in diethyl ether, 15 mL of an aqueous solution of NaOH (1 M) were added. The mixture was refluxed for 20 min, cooled, diluted with sulfuric acid (20 mL, 1 M) and extracted with diethyl ether. After separation, the organic phase was dried over $MgSO_4$ and the solvent removed by rotary evaporator. Recrystallization in ethanol yielded 0.68 g of **1a**. ^{1}H NMR in $CDCl_3$: $\delta = 2.7$, dd, 1H; $\delta = 3.1$, dd, 1H; $\delta = 3.9$, dd, 1H; $\delta = 7.4$, d, 2H; $\delta = 8.2$, d, 2H.

4.2.2. 2-(*para*-Nitrophenyl)-2-hydroxyethylnitrite b

4.2.2.1. Enzymatic synthesis. *p*-NSO **1** (100 mg, 0.6 mmol) and sodium nitrite (50 mM) were incubated in Tris–SO_4 buffer (100 mL, 100 mM, pH = 8) with HheC at 5 °C overnight. The mixture was extracted twice with ether. After separation, the organic phase was dried over $MgSO_4$ and the solvent removed under vacuum at room temperature, yielding a yellow solid. This yellow solid was dissolved in dichloromethane. Crystals of pure **b** were obtained by slowly adding heptane to the solution. ^{1}H NMR in DMSO: $\delta = 3.50$, m, 1H; $\delta = 5.62$, dd, 1H; $\delta = 5.72$, d, 1H, (OH); $\delta = 5.82$, t, 1H; $\delta = 7.51$, d, 2H; $\delta = 8.16$, d, 2H.

4.2.2.2. Chemical synthesis. To a solution of 2-bromo-1-(*para*-nitrophenyl)-ethanol (13 mmol, 3 g) in DMSO (100 mL), sodium nitrite was added (26 mmol, 1.7 g). The solution was stirred overnight, after which 100 mL of a strong buffer (500 mM Tris–SO_4,

pH = 8.5) was added to the mixture, which was subsequently extracted twice with ether. The organic phase was dried over $MgSO_4$ and the solvent removed by a rotary evaporator yielding an orange oil. This oil was loaded on a silica column (60 Å) and eluted using a mixture of heptane and ethyl acetate (7:3). Fractions were analyzed using chiral HPLC.

4.2.3. Racemic 2-hydroxy-2-(*para*-nitrophenyl)-nitroethane d. To a solution of *para*-nitro benzaldehyde (0.5 g, 3.3 mmol) and nitromethane (2 g, 33 mmol) in ethanol (50 mL), potassium *tert*-butoxide (2 mg) was added. The mixture was stirred for 4 h at room temperature and monitored by TLC (eluent: heptane, ethyl acetate 7:3). After complete conversion, water (50 mL) was added and the mixture extracted twice with ether. After separation, the organic phase was washed with water and dried over $MgSO_4$. The solvent was then removed by a rotary evaporator yielding 0.6 g of an orange oil. Recrystallization in ethanol yielded 0.4 g of crystals of pure **d**. ^{1}H NMR in DMSO: δ = 3.29, m, 1H; δ = 3.60, dd, 1H; δ = 4.08, t, 1H; δ = 5.07, d, 1H (OH); δ = 6.38, d, 2H; δ = 6.88, d, 2H.

4.2.4. Racemic *para*-nitrophenylethane-1,2-diol c. To a solution of epoxide **1** (200 mg, 1.2 mmol) in 10 mL of acetonitrile, 10 mL of 1 M H_2SO_4 was added. The mixture was then stirred overnight. The solution was then neutralized by the addition of 5 mL of a solution of 1 M NaOH and extracted with ether. After separation, the organic phase was dried over $MgSO_4$ and the solvent removed by rotary evaporator yielding 130 mg of a white powder. This powder was dissolved in warm ethyl acetate after which the slow addition of cold hexane yielded pure crystals of **c**. ^{1}H NMR in DMSO: δ = 3.50, m, 1H; δ = 4.35, m, 1H; δ = 4.79, t, 1H; δ = 5.34, m, 1H (OH); δ = 5.70, d, 1H (OH); δ = 7.55, d, 2H; δ = 8.12, d, 2H.

4.3. Kinetic resolution of epoxides

4.3.1. NMR monitoring of kinetic resolution of 1. Seven hundred microlitres of a near-saturated solution of *p*-NSO **1** (3 mM) and sodium nitrite (10 mM) in phosphate–D_2O buffer (pD = 7.1) were introduced in an NMR tube. After locking and shimming, 10 μL HheC solution to a final concentration of 0.27 mg mL^{-1} was added and the spectra automatically recorded at regular intervals. Signals corresponding to aromatic protons of substrate and products were sufficiently distinct from each other to allow correct integration. The integration results were directly correlated to absolute concentrations, using the first point as a calibration point. Chemical shifts of aromatic protons used for the analysis by ^{1}H NMR: *p*-NSO **a** (Fig. 4): δ = 7.56, d; 2-(*para*-nitrophenyl)-2-hydroxyethylnitrite **b** (Fig. 4): δ = 7.67, d; 2-(*para*-nitrophenyl)-1,2-ethanediol **c** (Fig. 4): δ = 7.63, d; 2-(*para*-nitrophenyl)-2-hydroxynitroethane **d** (Fig. 4): δ = 7.68, d.

4.3.2. Chiral chromatography monitoring of kinetic resolution of epoxide 1–6. To 20 mL of Tris–SO_4 buffer (100 mM, pH = 7.5), containing either epoxide **1** (3 mM), **2** (2 mM), **3** (5 mM), **4** (2 mM), **5** (2 mM) or **6** (2 mM) and 2% DMSO to facilitate the solubilization of the substrate, purified enzyme was added to a final concentration of (10 or 35 μM). Samples of 1 mL were taken at regular times, extracted with 1.5 mL of diethyl ether or hexane, containing an internal standard. Samples were dried over $MgSO_4$ prior to analysis chiral HPLC or GLC.

Conditions and retention times were as follows:

Epoxide **1**, col. I (heptane/isopropanol 90:10), (*R*)-**1** t_R = 9.0 min, (*S*)-**1** t_R = 14.0 min; 2-(*para*-nitrophenyl)-1,2-ethanediol **c**, 2-hydroxy-2-(*para*-nitrophenyl)-ethylnitrite ester **b** and 2-hydroxy-2-(*para*-nitrophenyl)-nitroethane **d**: col. II, (heptane/isopropanol 90:10), respectively: (*R*)-**c** t_R = 24.5; (*S*)-**c** t_R = 30.4 min; (*R*, *S*)-**b** t_R = 11.9 min; (*R*)-**d** t_R = 34.3 min; (*S*)-**d** t_R = 46.5 min.

Epoxide **2**, col. IV (temperature program: 7 min at 120 °C followed by an increase by 5 °C/min to 150 °C): (*R*)-**2** t_R = 11.5 min; (*S*)-**2** t_R = 13.5 min.

Epoxide **3**, col. III (heptane/isopropanol 95:5): (*S*)-**3** t_R = 20.5 min; (*R*)-**3** t_R = 22.1 min.

Epoxide **4**, col. V (110 °C): (*R*)-**4** t_R = 12.4 min, (*S*)-**4** t_R = 14.8 min.

Epoxide **5**, col. V (100 °C): (*R*)-**5** t_R = 35.0 min, (*S*)-**5** t_R = 38.5 min.

Epoxide **6**, col. I (heptane/isopropanol 99:1): (*R*)-**6** t_R = 7.1 min, (*S*)-**6** t_R = 8.3 min.

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