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Short communication

Halohydrin dehalogenase-catalysed synthesis of fluorinated aromatic chiral building blocks

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ABSTRACT

Kinetic resolution of a series of fluorinated styrene oxide derivatives was studied using halohydrin dehalogenase. A mutant HheC-W249P catalysed nucleophilic ring-opening with azide and cyanide ions with excellent enantioselectivity (*E*-values up to >200), which gives access to various enantiopure β-substituted alcohols and epoxides. It was found that the enzyme tolerates substrates in concentrations over 50 mM. However, different side reactions were observed at elevated concentrations and with prolonged reaction time. The biocatalytic azidolysis and cyanolysis of racemic 4-trifluoromethylstyrene oxide were performed on preparative scale, affording (*R*)-2-azido-1-(4-trifluoromethyl-phenyl)-ethanol in 38% yield and 97% *ee*, and (*S*)-3-hydroxy-3-(4-trifluoromethyl-phenyl)-propionitrile in 30% yield and 98% *ee*.

1. Introduction

Enantiomerically pure epoxides and their ring-opening products are frequently used building blocks for the synthesis of pharmaceuticals. Different resolution methods have been described including application of organometallic catalysts [1] and biocatalysts [2]. Among them, biocatalytic transformations employing halohydrin dehalogenase (HHDH) are very promising due to its capability to catalyse ring-opening reactions with several nucleophiles [3,4]. The usefulness of HHDHs in organic synthesis has been demonstrated by various preparative scale experiments [5,6] and an industrial application [7]. HHDHs are cofactor-independent enzymes and can be expressed in recombinant form in *E. coli* up to 60% of the total cellular protein content, allowing the isolation of up to 160 mg of pure enzyme from 1 L culture [8]. Easy preparation, stability and high selectivity of already discovered enzymes make HHDHs attractive catalysts for laboratory scale synthesis. However, to establish the role as an industrial biocatalyst, protein engineering is necessary in order to increase catalytic performances, expand their substrate range and provide access to both enantiomers of desired products [9].

Catalytic activity of HHDHs toward several styrene oxide derivatives has been reported in combination with azide [10–14], nitrite [15], cyanide [16] and cyanate ions [17,18] as nucleophiles. Since the fluoroaryl moiety is an attractive pharmacophore [19,20], we were interested in exploring the possibility of employing HHDH in the resolution of fluorine-substituted styrene oxides with the aim of synthesising the corresponding β-azido alcohol and β-hydroxy nitriles in enantiopure form. The growing interest in fluorinated organics makes the development of synthetic procedures leading to such compounds highly desirable. This task is not simple due to the difficulties of the incorporation of the fluorine atom into organic molecules. One of the approaches to the synthesis of fluorinated organics implies development of fluorination methods using various fluorinating agents [21]. The main drawback of this approach is often a high price and/or toxicity of the fluorinating agent. Another approach consists of synthetic modifications of simple commercially available fluorine containing compounds and a design of new intermediates [22]. Such building blocks must possess both C-F moiety and a functional group, which can be used for the incorporation of C-F fragment into a target molecule. In addition, such compounds can be modified in enantioselective fashion leading to optically active intermediates, for example alcohols, amines, amino alcohols and epoxides. In this study, we report enantioselective ring-opening reaction of

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epoxides **1a**-**1e** (Scheme 1) in the presence of azide and cyanide ions catalysed by variant HheC-W249P [23].

2. Experimental

2.1. Kinetic resolution experiments – general procedure

To 2.0 mL of Tris-SO_4 buffer (50 mM, pH 7.5) at room temperature, 125 μL of a stock solution of substrate in DMSO was added (5 μmol, final concentration 2 mM) followed by the addition of NaN_3 or NaCN (125 μL, 7.5 μmol). Reactions were initiated by the addition of 250 μL of cell-free extract of enzyme in TEMG buffer (10 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, pH 7.5). Reaction progress was followed by periodically taking samples (0.5 mL) from the reaction mixture. Samples were extracted with MTBE (1.0 mL) containing mesitylene as internal standard, dried over anhydrous Na_2SO_4, and analyzed by GC on HP-5 column to determine the conversion and regioisomeric ratio. In parallel, control reactions were performed by following the substrate consumption and product formation in the absence of enzyme. The enantiomeric excess (*ee*) of the remaining epoxides and products was determined by chiral GC and HPLC, respectively, under conditions described in Supplementary data.

2.2. Preparative-scale azidolysis

To a solution of epoxide **1b** (250 mg, 1.33 mmol, 50 mM final concentration) in the mixture of DMSO (1.3 mL) and Tris-SO_4 buffer (20 mL, 0.5 M, pH 7.0), NaN_3 was added (86 mg, 1.33 mmol) followed by addition of HheC-W249P (*ca* 20 mg in 5.3 mL buffer). The reaction was carried out at room temperature on a magnetic stirrer at 1000 rpm. After 7 h of incubation the reaction mixture was extracted with ethyl acetate (3 × 25 mL). The combined organic extracts were dried with Na_2SO_4 and solvent was removed by rotary evaporation. Column chromatography (hexane/ethyl acetate 9:1) yielded pure (*R*)-**2b** and (*S*)-**1b**.

(*R*)-2-Azido-1-(4-trifluoromethyl-phenyl)-ethanol ((*R*)-**2b**) was obtained in 35% yield (117 mg), 97% *ee*, $[\alpha]^{23}_D$ -70.3 (c = 1.18 in $CHCl_3$). The remaining (*S*)-2-(4-(trifluoromethoxy)phenyl)oxirane ((*S*)-**1b**) was obtained in 35% yield (88 mg), 87% *ee*, $[\alpha]^{23}_D$ + 15.7 (c = 1.46 in $CHCl_3$). The NMR data were identical with synthesized racemic reference compounds.

2.3. Preparative-scale cyanolysis

To a solution of epoxide **1b** (100 mg, 0.532 mmol, 20 mM final concentration) in the mixture of DMSO (1.3 mL) and Tris-SO_4 buffer (14.7 mL, 0.5 M, pH 7.0), NaCN was added (26 mg, 0.532 mmol) followed by addition of HheC-W249P (*ca* 40 mg in 10.6 mL buffer). The reaction was carried out at room temperature on a magnetic stirrer at 1000 rpm. After 15 h of incubation the reaction mixture was extracted with ethyl acetate (3 × 25 mL). The combined organic extracts were dried with Na_2SO_4 and solvent removed by rotary evaporation. Column chromatography of the residue (hexane/ethyl acetate 7:3) yielded pure (*S*)-**4b** and (*S*)-**1b**.

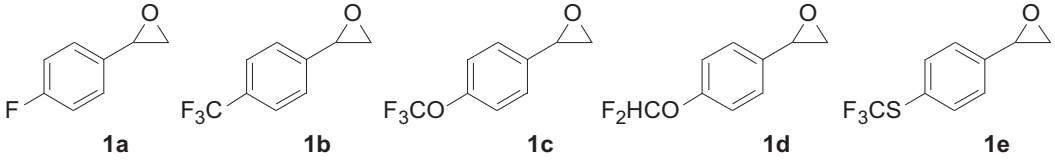
(*S*)-3-Hydroxy-3-(4-trifluoromethyl-phenyl)-propionitrile ((*S*)-**4b**) was obtained in 30% yield (35 mg), 98% *ee*, $[\alpha]^{23}_D$ -45.3 (c = 0.54 in $CHCl_3$). The remaining (*S*)-2-(4-(trifluoromethoxy)phenyl)-oxirane ((*S*)-**1b**) was obtained in 33% yield (33 mg), 61% *ee*, $[\alpha]^{23}_D$ + 11.2 (c = 1.11 in $CHCl_3$). The NMR data were identical with synthesized racemic reference compounds.

3. Results and discussion

3.1. Kinetic resolution of 1b catalysed by HheC and HheC-W249P mutant

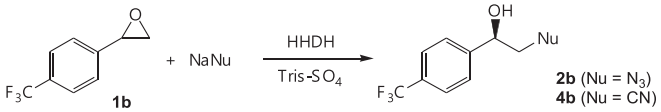
Among the wild-type HHDHs, the most commonly applied in biocatalysis is the enzyme from *Agrobacterium radiobacter* AD1 (HheC), due to its remarkable stereoselectivity and broad nucleophile scope. HheC was shown to catalyse azidolysis of styrene oxide, *p*-Cl- and *p*-NO_2-styrene oxide with excellent enantioselectivity ($E > 200$) [10]. Since in this study we chose to focus on styrene oxide derivatives with fluorine bearing groups, HheC was considered as a catalyst of choice.

Initially, the wild-type (WT) enzyme and the mutant W249P were tested in the ring-opening reaction of **1b** in the presence of azide and cyanide ions (Table 1). Both reactions catalysed by HheC proceeded with very high enantioselectivity, however azidolysis reaction was faster compared to cyanolysis, as observed in previous studies [4,24] (Table 1,



Scheme 1. Racemic epoxides **1a**-**1e** used as substrates.

Table 1
Ring-opening of *rac*-**1b** in the presence of sodium azide or sodium cyanide catalysed by HHDH.[a]



Entry	HHDH	NaNu	t (h)	Conversion (%)[b]	ee_s (%)[c]	ee_p (%)[d]	*E*-value
1	HheC	NaN_3	2	45 (39)	63 (*S*)	>99 (*R*)	>200
2	HheC	NaCN	3	34 (22)	27 (*S*)	98 (*S*)	>100
3	HheC-W249P	NaN_3	2	53 (50)	>99 (*S*)	>99 (*R*)	>200
4	HheC-W249P	NaCN	3	43 (31)	45 (*S*)	>99 (*S*)	>200

[a] *Conditions*: **1b** (2 mM), NaN_3 or NaCN (3 mM), 250 μL cell-free extract HHDH, Tris-SO_4 buffer (4 mL, 0.5 M, pH 7.0), 5% DMSO, total volume 5 mL.
[b] Apparent conversion determined by GC (value in parentheses is conversion catalysed by enzyme = intrinsic).
[c] Determined by GC.
[d] Determined by HPLC.

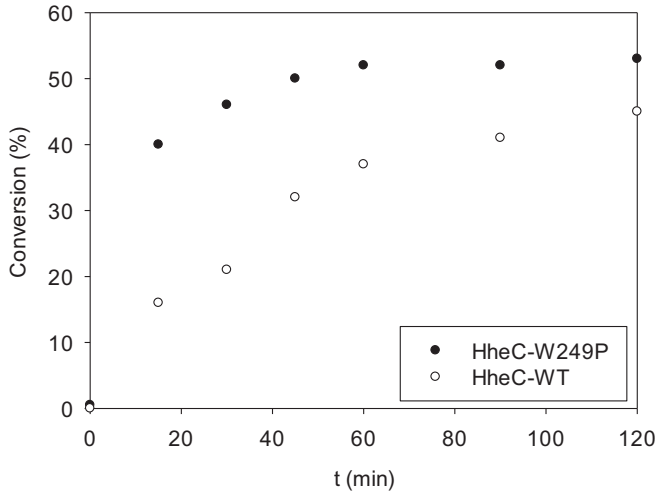


Fig. 1. Progress curves of biocatalytic azidolysis of **1b** catalysed by WT and mutant HheC.

Entries 1 and 2).

Residue Trp249 in HheC is known for its importance regarding the catalytic activity and enantioselectivity. This residue is positioned in the loop that forms the halide-binding site; therefore, its replacement destabilises the site, leading to faster halide release and a higher reaction rate [23,25]. The HheC-W249P mutant showed significantly higher activity in the kinetic resolution of **1b** (Table 1, Entries 3 and 4). While both enzymes catalyse the formation of azido alcohol (*R*)-**2b** with >99% *ee*, activity of W249P is considerably higher. 40% Conversion of *rac*-**1b** was achieved already after 15 min, while 50% conversion after 1 h, resulting in complete kinetic resolution of substrate (Fig. 1). Thus, (*R*)-enantiomer was transformed to azido alcohol ((*R*)-**2b**, >99% *ee*), while (*S*)-enantiomer remained as unreacted epoxide ((*S*)-**1b**, >99% *ee*). The stereoselectivity of the cyanolysis reaction is unchanged compared to azidolysis; however, inversion of the absolute configuration in the corresponding β-hydroxy nitrile **4b** appears due to a different substituent priority according to the Cahn–Ingold-Prelog convention. Due to superior catalytic properties, W249P was selected for further biocatalytic studies.

3.2. Kinetic resolution of epoxides 1a-1e in the presence of NaN_3 catalysed by HheC-W249P

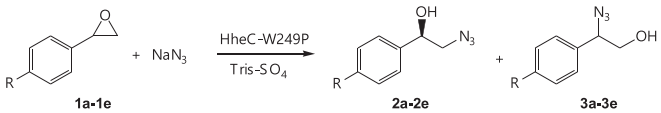
Substrates used in this study were prepared from the corresponding substituted benzaldehydes by using trimethylsulfoxonium iodide in 54–68% yield after purification [26], except commercially available **1a**. HheC-W249P-catalysed transformations of epoxides **1a**–**1e** (2 mM) with 1.5 equivalent of NaN_3 were carried out in Tris-SO_4 buffer containing 5% DMSO for the solubility improvement. Reactions were performed with cell-free extract at room temperature and monitored by GC analysis. Conversions determined by GC were always found higher compared to calculated conversions (given in parentheses; calculated according to the formula $c = ee_s / (ee_s + ee_p)$). The difference observed in the enzyme-catalysed conversion of substrate (intrinsic) and conversion measured by GC (apparent) is mainly the result of hydrolytic instability of the epoxide. Spontaneous hydrolysis of substrates becomes significant when the rate of biocatalytic reaction is low. Spontaneous azidolysis leading to *rac*-**2** was found to be negligible under the conditions used, reflecting in very high product *ee* (97% *ee* for **2a** and > 99% *ee* for **2b**-**2e**, Table 2). To minor extent, spontaneous chemical reaction occurs at Cα leading to *rac*-**3**, which was observed in control reaction performed without enzyme. However, enzyme-catalysed azidolysis is β-regioselective toward tested substrates (96–100% of β-attack). All kinetic resolutions occurred with high enantioselectivity toward the (*R*)-enantiomer, yielding almost enantiopure products while leaving the (*S*)-enantiomer of the epoxide behind. *E*-value was calculated from the enantiomeric purity of substrate and product, as well as the percentage of enzymatic conversion (intrinsic conversion) as already mentioned, while absolute configurations were determined by chiral GC analysis using the chemically prepared reference compounds of known configuration; for more information see Supplementary data.

This method is not applicable to *ortho*-substituted styrene oxides due to very low enzyme activity, while *meta*-substituted derivatives could be converted to products with high enantioselectivity (e.g. $E = 128$ in the case of *m*-F and $E > 200$ with *m*-CF_3-styrene oxide; unpublished data).

3.3. Kinetic resolution of epoxides 1a-1e in the presence of NaCN catalysed by HheC-W249P

To further explore the potential of HheC-W249P for producing β-hydroxy nitriles, we investigated the reaction of epoxides **1a**-**1e** with cyanide ion as a nucleophile. As illustrated in Table 3, HheC-W249P showed activity with entire series of epoxides generating β-hydroxy nitriles in completely regioselective fashion and with high enantioselectivity ($E > 100$). However, the reaction rate strongly depends on the nucleophile, and lower rate was observed with cyanide compared to azide ion. Control experiments, without enzyme, revealed negligible spontaneous cyanolysis reaction leading to *rac*-**4**, but again significant spontaneous substrate hydrolysis.

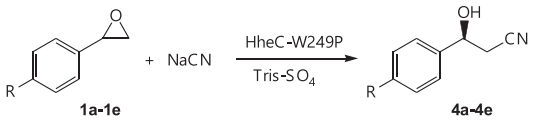
Table 2
Conversion of epoxides **1a**-**1e** with sodium azide catalysed by HheC-W249P.[a]



Entry	Epoxide	R	Conversion (%)[b]	*ee***1** (%)[c]	*ee***2** (%)[d]	β-regioselectivity[e]	*E*-value
1	**1a**	F	55 (37)	56 (*S*)	97 (*R*)	82 (100)	>100
2	**1b**	CF_3	53 (50)	>99 (*S*)	>99 (*R*)	97 (99)	>200
3	**1c**	OCF_3	60 (50)	>99 (*S*)	>99 (*R*)	93 (97)	>200
4	**1d**	$OCHF_2$	52 (50)	>99 (*S*)	>99 (*R*)	81 (96)	>200
5	**1e**	SCF_3	53 (49)	96 (*S*)	>99 (*R*)	97 (100)	>200

[a] *Conditions*:**1a-1e** (2 mM), NaN_3 (3 mM), 250 μL HheC-W249P, Tris-SO_4 buffer (4 mL, 0.5 M, pH 7.0), 5% DMSO, total volume 5 mL, reaction time 2 h.
[b] Apparent conversion determined by GC (value in parentheses is conversion catalysed by enzyme = intrinsic).
[c] Determined by GC.
[d] Determined by HPLC.
[e] Apparent regioselectivity determined by GC (intrinsic value is in parentheses).

Table 3
Conversion of epoxides **1a**-**1e** with sodium cyanide catalysed by HheC-W249P.[a]



1a-1e **4a-4e**

Entry	Epoxide	R	Conversion (%)[b]	*ee***1** (%)[c]	*ee***4** (%)[d]	β-regioselectivity[e]	*E*-value
1	**1a**	F	54 (47)	86 (*S*)	97 (*S*)	100	>100
2	**1b**	CF_3	37 (27)	37 (*S*)	>99 (*S*)	100	>200
3	**1c**	OCF_3	43 (33)	48 (*S*)	97 (*S*)	100	>100
4	**1d**	$OCHF_2$	43 (36)	56 (*S*)	>99 (*S*)	100	>200
5	**1e**	SCF_3	22 (19)	23 (*S*)	98 (*S*)	100	>100

[a] *Conditions*: **1a-1e** (2 mM), NaN_3 (3 mM), 250 μL HheC-W249P, Tris-SO_4 buffer (4 mL, 0.5 M, pH 7.0), 5% DMSO, total volume 5 mL, reaction time 2 h.
[b] Apparent conversion determined by GC (value in parentheses is conversion catalysed by enzyme = intrinsic).
[c] Determined by GC.
[d] Determined by HPLC.
[e] Apparent regioselectivity determined by GC.

3.4. Preparative scale reaction

Tris-SO_4 buffer containing 5% DMSO allows substrates to be dissolved in 2 mM concentration, which was considered too low for preparative transformation. Therefore, by increasing substrate concentration and decreasing the biocatalyst loading, we tried to enhance the reaction and perform it on a preparative scale. It was found that enzyme tolerates substrate **1b** in concentration over 50 mM. However, at elevated concentrations and with the prolonged reaction time, optical purity and product yields are affected by chemical side reactions (see Table S2 in the Supplementary data). Particularly, chemical azidolysis that takes place at Cβ reduces the product *ee*, while reaction at Cα increases the amount of α-regioisomer (**3b**). Besides, substrate spontaneously hydrolyses giving the corresponding diol, which becomes significant under these conditions over a period of several hours.

Thus, preparative-scale experiments using 250 mg of *rac*-**1b** were performed with 50 mM substrate concentration (9.4 g/L) and 1 equivalent of NaN_3, by using cell-free extract containing approximately 20 mg (8 wt%) of HheC-W249P. To avoid the reduction of *ee*, the reaction was conducted for 7 h and terminated at *ca* 45% conversion, allowing isolation of (*R*)-**2b** in 38% yield and 97% *ee* and (*S*)-**1b** in 35% yield and 87% *ee*. Enzymatic reaction was very fast in the beginning but slowed down significantly once conversion of 40% was reached (see Fig. S1 in the Supplementary data). In order to achieve complete conversion of (*R*)-**1b** and to obtain (*S*)-**1b** with >99% *ee*, the reaction time should be prolonged.

Similarly, a reaction with NaCN was performed. However, a greater amount of enzyme (40 wt%), lower substrate concentration (20 mM) and a longer reaction time (15 h) were required to achieve comparable conversion and to obtain (*S*)-**4b** in 30% yield and 98% *ee*. With a prolonged reaction time, a slight reduction of product *ee* was observed due to chemical reaction of epoxide with NaCN, while product resulting from the Cα-attack was not detected. Practical limitations to scale-up may be encountered due to high catalyst loading required for cyanolysis reaction, probably caused by enzyme deactivation in the presence of cyanide. Detailed kinetic analyses will be performed in the future to optimise both reactions on preparative scale.

4. Conclusions

In conclusion, enantioselective ring-opening of five fluorine-substituted styrene oxide derivatives was performed by using HHDH. HheC-W249P possesses high enantioselectivity toward all substrates with *para*-substitution pattern, preferentially converting (*R*)-enantiomer, and high regioselectivity, attacking at Cβ-position (opposite to chemical reaction). Under elevated concentrations substrates were sensitive to spontaneous hydrolysis; moreover, the spontaneous reaction between the epoxide and nucleophile occurred. In order to minimize the rate of chemical side-reactions and perform preparative scale transformations, higher concentrations of enzyme and lower concentrations of substrates were required. Azidolysis of epoxide **1b** at 50 mM substrate concentration gave the corresponding azido alcohol (*R*)-**2b** in 38% yield and 97% *ee* and remaining epoxide (*S*)-**1b** in 30% yield and 87% *ee*, respectively. Due to lower enzymatic activity in cyanolysis reaction, very high catalyst loadings were required to obtain (*S*)-**4b** in 30% yield and 98% *ee*. Nevertheless, preparative scale transformations require further improvement in order to minimize the effect of chemical side-reactions and increase productivity. This can be facilitated by a detailed kinetic analysis of all reactions involved in the system. Thus, further optimization will be done by employing enzyme reaction engineering.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.catcom.2021.106285.

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