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PAPER

Catalytic activity of halohydrin dehalogenases towards spiroepoxides†

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A novel activity of halohydrin dehalogenases towards spiroepoxides has been found. The enzyme from *Arthrobacter* sp. (HheA) catalysed highly regioselective azidolysis of spiroepoxides containing 5, 6 and 7-membered cycloalkane rings, while the enzyme from *Agrobacterium radiobacter* (HheC), besides high regioselectivity, also displayed moderate to high enantioselectivity (*E* up to >200) that can be applied for the kinetic resolution of chiral spiroepoxides. The orientations of spiroepoxides in the active site of halohydrin dehalogenases were studied by quantum-chemical calculations and docking simulations. Analyses of the complexes obtained revealed the origins of diastereoselectivity and enantioselectivity of the investigated biotransformations.

Introduction

The ability of enzymes to catalyse the ring-opening of epoxides has been intensively studied over the past years.[1] Epoxide hydrolases are popular catalysts for the enantioselective hydrolysis of racemic epoxides,[2] while halohydrin dehalogenases can accept several anionic nucleophiles, such as halides, azide, cyanide, cyanate and nitrite, affording a broad range of 1,2-difunctionalised organic compounds.[3] Substrates for halohydrin dehalogenases are terminal mono- and disubstituted epoxides, while activity with non-terminal substrates has not been observed until now.[4] In this paper, we describe a newly discovered activity of halohydrin dehalogenases for the nucleophilic ring-opening of spiroepoxides. Spiroepoxides are compounds with a three-membered oxygen-containing heterocycle joined to an alicyclic fragment by a common carbon.[5] This scaffold can be found in many natural products,[6] as well as synthetic bioactive compounds.[7]

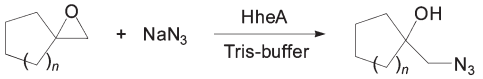
To date, several examples of epoxide hydrolase catalysed hydrolysis of spiroepoxides have been reported.[8,9] As far as racemic substrates are concerned, some kinetic resolutions were successful and afforded enantiopure products.[8,9] Here, for the first time, azidolysis of spiroepoxides catalysed by halohydrin dehalogenases is described. The importance of enantioselective azidolysis of epoxides stems from the facile conversion of the 1,2-azido alcohol products into valuable, highly enantiomerically enriched 1,2-amino alcohols in just one step. In order to investigate the nature of the interactions of spiroepoxides with the enzymes and to predict required structural changes for obtaining better selectivity, quantum-chemical calculations and docking simulations were performed as well.

Results and discussion

Regioselective azidolysis of spiroepoxides catalysed by HheA

Studying the various substrates of halohydrin dehalogenase HheA (an enzyme from *Arthrobacter sp*. AD2) we noticed very rapid conversion of spiroepoxide **1b** to azido alcohol **2b** (Scheme 1). The observed initial enzyme activity (9.8 μmol min^{-1} mg^{-1}) was several fold higher compared to the reaction of monosubstituted aliphatic epoxides, *e.g.* 1,2-epoxybutane.[10] To expand a substrate scope of HheA we have chosen to evaluate spiroepoxides containing 5- and 7-membered cycloalkane ring (**1a** and **1c**) as well (Scheme 1).

HheA-catalysed transformations of epoxides **1a–1c** were carried out in Tris-SO_4 buffer (pH 7.0) containing 0.5% DMSO to increase the solubility of the substrate, epoxide (50 mM) and



1a: *n*=1
1b: *n*=2
1c: *n*=3

2a-2c

Scheme 1 Azidolysis of spiroepoxides **1a–1c** catalysed by HheA.

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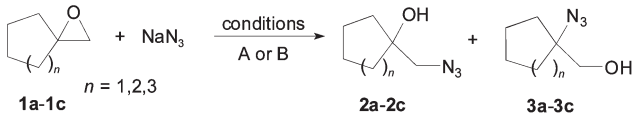
Table 1 HheA-catalysed (A) and non-catalysed (B) ring-opening of epoxides **1a–1c**



Substrate	Conditions[a]	*t*/h	Conv. [b]/ %	Yield / %	Ratio (**2** : **3**)
1a[e]	A	1.5	>99	75[c]	98 : 2
	B	20	100	28[d]	59 : 41
1b	A	2	>99	95[c]	100 : 0
	B	20	77	61[d]	97 : 3
1c	A	3	>99	96[c]	99 : 1
	B	20	38	26[d]	57 : 43

[a] Conditions A: Epoxide (1.0 mmol, 50 mM), NaN_3 (2.0 eq), 0.5 mg purified HheA in Tris-SO_4 buffer (20 mL, 0.5 M, pH 7.0). Conditions B:[12] Epoxide (1.0 mmol, 0.5 M), NaN_3 (5.0 eq) in 2 mL water at 30 °C, pH 9.5. [b] Determined by GC. [c] Yield of **2**. [d] Total yield **2** + **3**. [e] Unstable compound in water.

NaN_3 (100 mM). Reactions were performed with purified enzyme at room temperature and monitored by GC analysis. In the control experiment performed in the absence of the enzyme the non-catalysed formation of azido alcohols was found to be slow. The stability of the substrates was also checked under the experimental conditions. Significant chemical hydrolysis has been observed only in the case of substrate **1a** containing 5-membered ring (*ca*. 15% per hour). All reactions proceeded with the high enzyme activity and almost complete regioselectivity. At 1.0 mmol scale quantitative conversions of epoxides were achieved after 1.5–3 h and azido alcohols **2a–2c** were isolated in high yields (75–96%, Table 1, conditions A). It was observed that the size of the ring had minor influence on the enzyme activity. Somewhat lower isolated yield of **2a** can be explained with the above mentioned chemical instability of the starting epoxide **1a**.

A common method for the synthesis of 1,2-azido alcohols is the ring-opening reaction of epoxides employing NaN_3.[11] Azidolysis of epoxides is typically performed in aqueous medium and requires relatively long reaction times (12–48 h).[12] It was found that under acidic conditions these reactions can be strongly accelerated, although with compromised regioselectivity.[12] To evaluate our results of the enzyme-catalysed azidolysis, we have performed chemical azidolysis of **1a–1c** following the protocol of Fringuelli *et al*.[12] The results are summarized in Table 1 (conditions B). Comparing the HheA-catalysed (A) and non-catalysed (B) azidolysis it can be concluded that the biotransformation proceeds with the much higher rate and almost complete regioselectivity. In the case of substrates **1a** and **1c**, a minor azido alcohol regioisomer **3a** (2%) and **3c** (1%) appear as a result of slow non-catalysed reaction. The high β-regioselectivity of the enzymatic reaction is remarkable and stands in contrast to the majority of ring-opening reactions involving spiroepoxides.

Regioselectivity of the non-enzyme ring opening reaction

To understand the different regioselectivity ratio in the azidolysis of tested spiroepoxides **1a–1c** without an enzyme, the epoxide ring-opening was modelled using reactants and one molecule of water. The energy barriers, optimized reactant and transition state structures for the attack on α (TSα) and β (TSβ) epoxide carbon atoms are presented in Table 2 and in the ESI Fig. S1–S3.† Reactions proceeding *via* transition states TSβ for all three compounds may be considered as a very simple simulation of the biocatalysed reaction as well, where the water molecule mimics catalytic triad Tyr hydroxyl group. While the structures of TSβ are very similar, Gibbs energies of activation for TSα are dependent on unfavourable steric interactions between the cycloalkane ring and the approaching nucleophile (Table 2). The calculated barriers for the attack of azide at Cα and Cβ atom of **1a** and **1b** were in a very good agreement with the experimentally determined regioselectivity (Table 1), whereas in the case of **1c** the calculated differences did not meet completely the observed regioselectivity (probably due to the greater flexibility of the 7-membered ring).

Table 2 Non-catalysed ring-opening of epoxides **1a–1c**: calculated standard Gibbs energies of activation (B3LYP/6-311++g(d,p) in H_2O, ε = 78.3553) for the transition states of α and β azidolysis

Comp.	Transition state	$\Delta^{\ddagger}G°$/ kcal mol^{-1}	$\Delta\Delta^{\ddagger}G°$/ kcal mol^{-1}	Ratio (**2** : **3**)
1a	TSα	31.65	0.33	64 : 36
	TSβ	31.32		
1b	TSα	35.11	4.40	100 : 0
	TSβ	30.71		
1c	TSα	33.26	1.08	86 : 14
	TSβ	32.18		

ONIOM calculations of HheA-spiroepoxide and azide complexes

To comprehend the high regioselectivity of HheA-catalysed azidolysis and define the important interactions within the active site, a hybrid approach, combining quantum chemical (DFT) and semiempirical (PM3MM) calculations within the ONIOM scheme was chosen. A model of the enzyme active site was built by selecting the most important amino acids (26 amino acids) with several atoms' positions fixed, and placing the substrate **1a–1c** and azide ion inside of the model site. The results are presented in Fig. 1.

Overlay of the obtained geometries showed that the interactions with the amino acids of the active site, are pretty much alike for all compounds. The azide ion is bound in the halide binding site where it forms one very strong hydrogen bond with a water molecule and several weak H-bonds with the backbone amides in the surroundings. The epoxide ring is positioned in such a way that the oxygen atom forms two strong hydrogen bonds with catalytic Ser134 and Tyr147. The atoms of the hydrophobic aliphatic rings are in close contacts with the residues of Asn87, Ser135, Leu141, Asn144, Tyr147 (aromatic ring) and Tyr185. The optimal position of the oxygen atom of the substrate as well as the spiro substituent enforced the position of the Cβ atom as the closest to the azide nucleophile, thus resulting in a high regioselectivity of the attack. The observed differences in the enzyme activity of **1a–1c** (Table 1) can be related to the slight increase in distance of the Cβ atom from the nucleophile (from 2.64 for **1a** to 2.84 Å for **1c**).

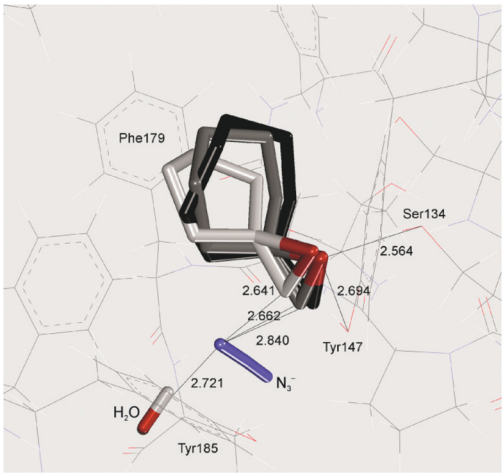
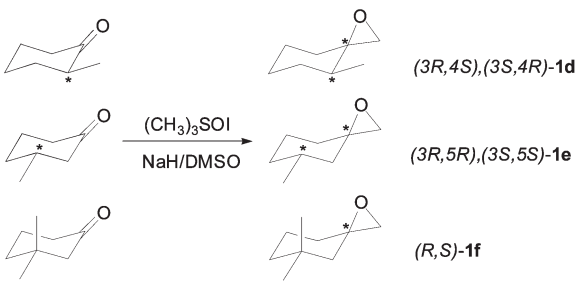


Fig. 1 Overlay of **1a** (white), **1b** (grey), **1c** (black) and the azide ion in the HheA active site model represented by 26 selected amino acids obtained by ONIOM calculations. Interatomic distances are given in Å and hydrogen atoms are omitted for clarity.

Enantioselective azidolysis of chiral spiroepoxides catalysed by HheA and HheC

Investigations were further extended towards chiral spiroepoxides. Spiroepoxide substrates with different substitution patterns were prepared. All substrates **1a–1f** were prepared from the corresponding cyclohexanones by using trimethylsulfoxonium iodide in 30–80% yield.[13] Although epoxide formations were quantitative, a considerable loss of the material during isolation of **1a–1c** occurred due to the low boiling points of the products. The stereochemistry of sulfoxonium ylide epoxidation of cyclohexanones are known to be controlled in such a manner that racemic 1-oxaspiro[2.5]octanes **1d–1f** were formed almost exclusively as *O-axial* epimers (>97%, Scheme 2).[9]



Scheme 2 Synthesis of racemic 1-oxaspiro[2.5]octanes **1d–1f**.

Racemic methyl substituted 1-oxaspiro[2,5]octanes **1d–1f**, were tested in kinetic resolution catalysed by HheA. Enantioselectivities were low (E = 1–3, Table 3) which was not surprising knowing the low enantiodiscrimination of this enzyme.[4] Apparently, both enantiomers of **1d–1f** can be accommodated in the HheA active site with less difference in spatial requirements. Conversion of the substrate **1e** was the fastest and the reaction was slightly more selective compared to the other two chiral substrates. The slow conversion of **1d** and **1f** can be assumed to be due to the steric reasons, see Docking studies (*vide infra*). The highest activity among all substrates was observed with non-substituted **1b**.

The absolute configuration has been ascertained by comparing the chiral GC traces of the remaining substrate to standards of known configuration. For substrates **1d** and **1e**, a slight preference for the (3*S*)-enantiomer (spiro C-atom) was observed, opposite to the **1f** where inverted preference in favour of (*R*)-enantiomer is shown.

It was shown previously that HheC (an enzyme from *Agrobacterium radiobacter* AD1) is a very powerful and versatile biocatalyst offering high activity and enantioselectivity.[14] According to the known high enantioselectivity of the HheC towards 2,2-disubstituted epoxides, *e.g.* 2-methyl-1,2-epoxybutane[15] low activity of HheC towards cyclic 2,2-disubstituted epoxides was therefore expected. It has been shown that (*R*)-enantiomer of 2-methyl-1,2-epoxybutane fits rather tightly into the active site pocket with the methyl group pointing into a small bulge bordered by the amino acids Trp139, Asn176 and Thr134.[15] A bulkier group at this position would lead to steric conflict with those amino acids and thus the (*S*)-enantiomer binds with great difficulties in a productive manner, resulting in a high enantioselectivity of the process. To our great surprise HheC not only catalysed azidolysis of spiroepoxides **1b** and **1e** but reaction proceeded with the high rate, even higher than the one catalysed by HheA (Table 3). Azidolyses were observed with **1d** and **1f** as well, but both reacted much slower (*ca.* 40 times slower than **1e**).

Table 3 Results of halohydrin dehalogenase catalysed azidolysis of spiroepoxides **1b**, **1d–1f**[a]

Comp.	Enzyme	Initial activity[d]	*t*/h	Conv.[e]/%	ee_s/%	ee_p/%	Config.[f]	*E*
1b	HheA[b]	9.8	3	60	—	—	—	—
	HheC[b]	13.9	3	63	—	—	—	—
1d	HheA[c]	0.2	23	25	1	3	3*S*,4*R*	1
	HheC[c]	0.4	23	31	39	87	3*S*,4*R*	21
1e	HheA[b]	4.8	3	39	27	42	3*S*,5*S*	3
	HheC[b]	20.4	1	39	58	90	3*R*,5*R*	34
1f	HheA[c]	0.5	23	57	16	12	*R*	1.5
	HheC[c]	0.5	22	39	63	>99	*R*	>200

[a] Conditions: Epoxide (5.0 mM), NaN_3 (1 eq), purified enzyme in Tris-SO_4 buffer (20 mL, 50 mM, pH 7.0). [b] 56 μg enzyme. [c] 150 μg enzyme. [d] μmol min^{-1} mg^{-1}. [e] The conversion ratio could be determined on the basis of the two enantiomeric excesses [$c = ee_s/(ee_s + ee_p)$].[34] [f] Faster reacting epoxide enantiomer.

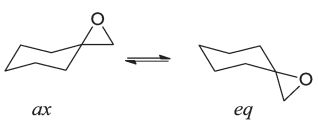
For all tested spiroepoxides HheC showed much higher enantioselectivity (E = 21–200), compared to HheA (E = 1–3). For 4-methyl substituted spiroepoxide **1d** moderate enantioselectivity (E = 21) and an enantioselective preference in favour of (3*S*,4*R*)-**1d** enantiomer has been observed. This result was not in agreement with previous studies which pointed out that HheC is (*R*)-selective (absolute configuration at oxirane ring) for most substrates.[4,15] Geometry of the spiro substrate and conformational flexibility of the 6-membered ring play a crucial role in this inverted stereopreference as described further in the molecular modelling and docking study. As mentioned earlier HheC catalysed kinetic resolution of **1e** with the highest rate, but also with moderate enantioselectivity (E = 34) in favor of the (3*R*,5*R*)-**1e** enantiomer. The same enantiopreference was found in **1f** where an additional *axial* methyl substituent was introduced on the cyclohexane ring. This compound was resolved with excellent selectivity (E > 200), although with significantly reduced specific activity (Table 3). Small changes in the substrate structure and the enzyme itself had showed a remarkable influence on both activity and enantioselectivity.

Docking studies of 1-oxaspiro[2,5]octane compounds 1b and 1d–1f in the active site of HheA and HheC

To explain enantioselectivities observed in the halohydrin dehalogenase catalysed azidolysis of racemic spiroepoxides **1d–1f**, molecular docking simulations were performed (AutoDock 4.2 suite of programs).[16] A molecular-level interpretation of the experimentally obtained results is based on the X-ray crystal structure of halohydrin dehalogenase HheA (PDB code: 1ZMO)[17] and HheC (PDB code: 1ZMT).[18] In the 1-oxaspiro [2,5]octane compounds the cyclohexane ring can adopt a conformation in which the position of the epoxide oxygen is pseudo-*axial* (*O-ax*) or pseudo-*equatorial* (*O-eq*) (Scheme 3). A rapid interconversion between the two chair conformers of the cyclohexane ring occur and the position of equilibria is influenced by the substitution pattern.[8]

Conformational analyses were performed for all substrates and results are presented in Table 4.

DFT calculations for all compounds confirmed that *O-ax* conformer prevails. **1e** showed the greatest *O-ax* preference due to the unfavourable 1,3-diaxial interactions of the epoxide methylene group and 5-methyl substituent. Therefore, for the flexible ligand docking both chair conformers of all tested compounds were used with the exception of **1e** where only largely predominate *O-ax* conformer was studied.



Scheme 3 Two conformers of the cyclohexane ring: oxygen atom in the pseudo-*axial* (**1b**$_{O\text{-}ax}$) and pseudo-*equatorial* (**1b**$_{O\text{-}eq}$) position.

Table 4 Conformational analyses of cyclohexane spiroepoxides **1b**, **1d–1f** (calculated at B3LYP/6-311++g(d,p) level)

Comp.	$\Delta_r G_{eq}$–$\Delta_r G_{ax}$/kcal mol^{-1}	Ratio (*O-ax* : *O-eq*)
1b	0.22	59 : 41
1d	0.59	73 : 27
1e	2.53	99 : 1
1f	0.25	60 : 40

Overlay of geometries obtained by docking studies for **1b** and enantiomers of **1d–1f** in the active site of HheA are presented in Fig. 2. Generally, there are two main ways in which an epoxide oxygen atom can efficiently bind in the active site: I) productive binding: making two hydrogen bonds with the hydroxyl groups of Ser134 and Tyr147 (two amino acids of the catalytic triad) and II) non-productive binding: forming two hydrogen bonds with the side chain amides of Asn87 and Asn144, as shown for conformers of **1b** (Fig. 2A). Analysis of the geometries obtained for **1d** enantiomers showed productive binding for both conformers of (3*S*)-**1d** (*O-eq* being in a somewhat better position for a nucleophilic attack), as well as (3*R*)-**1d**$_{O\text{-}ax}$ (Fig. 2B). In the case of **1e** both enantiomers can bind productively but optimal positioning of the methyl group resulted in the closer distance of the Cβ atom of the (3*S*)-enantiomer to the azide, which is in agreement with the observed enantiopreference (Fig. 2C).

Due to the bigger size (additional methyl group), conformers of (*S*)- and (*R*)-**1f** bind differently than those of **1d**. (*R*)-**1f**$_{O\text{-}eq}$ makes strong H-bonds with the amino acids of the catalytic triad, but Cβ atom is shifted away from the optimal position for the reaction to occur. In a complex with (*S*)-**1f**$_{O\text{-}eq}$, the epoxide ring is positioned toward Asn87 and Asn144 (Fig. 2D). The docking studies showed that the topology of the HheA active site permits the binding of *axial* and *equatorial* conformers of both enantiomers of the investigated compounds, resulting in low *E*-values.

HheC is known to possess a smaller active site compared to HheA[17] therefore different binding modes were expected. Overlay of geometries obtained by docking studies for conformers of **1b** and enantiomers of **1d–1f** in the active site of HheC are presented in Fig. 3. In the active site of HheC, asparagine side chain amides are missing compared to HheA, thus the best position for binding the epoxide ring is the one stabilized with catalytic Ser132 and Tyr145 (Fig. 3A). Additional non-productive binding is possible through single H-bond stabilization with hydroxyl group of Tyr187 found for the *O-ax* conformer of (3*R*)-**1d**$_{O\text{-}ax}$ (Fig. S9†). As in HheA complexes, *O-ax* confomer of **1b** is productively and *O-eq* non-productively bound. Although epoxide oxygen atom is stabilized through H-bonds with the Ser132 and Tyr145, the optimized position of cyclohexane ring is resulting with Cβ shifting from the azide ion. Analysis of the geometries for **1d** enantiomers showed that (3*S*)-**1d**$_{O\text{-}ax}$ binds better then (3*R*)-**1d**$_{O\text{-}eq}$ which is in agreement with the experimentally determined unexpected stereopreference towards (3*S*)-enantiomer (Fig. 3B). On the other hand, (3*S*)-enantiomers of **1e** and **1f** cannot be easily productively bound resulting in a greater enantiomeric ratio of that reactions towards (3*R*)-enantiomer. In the case of (3*R*)-**1e**$_{O\text{-}ax}$, the atoms of the epoxide and cyclohexane ring are completely overlapped with those of **1b**$_{O\text{-}ax}$ (Fig. 3C) and the activity of HheC is even higher toward this substrate than to non-substituted **1b** due to the additional favorable interactions of the 5-methyl substituent within the active site. (*R*)-**1f**$_{O\text{-}ax}$ fits tightly into the active site and although the most sterically demanding substrate occupies

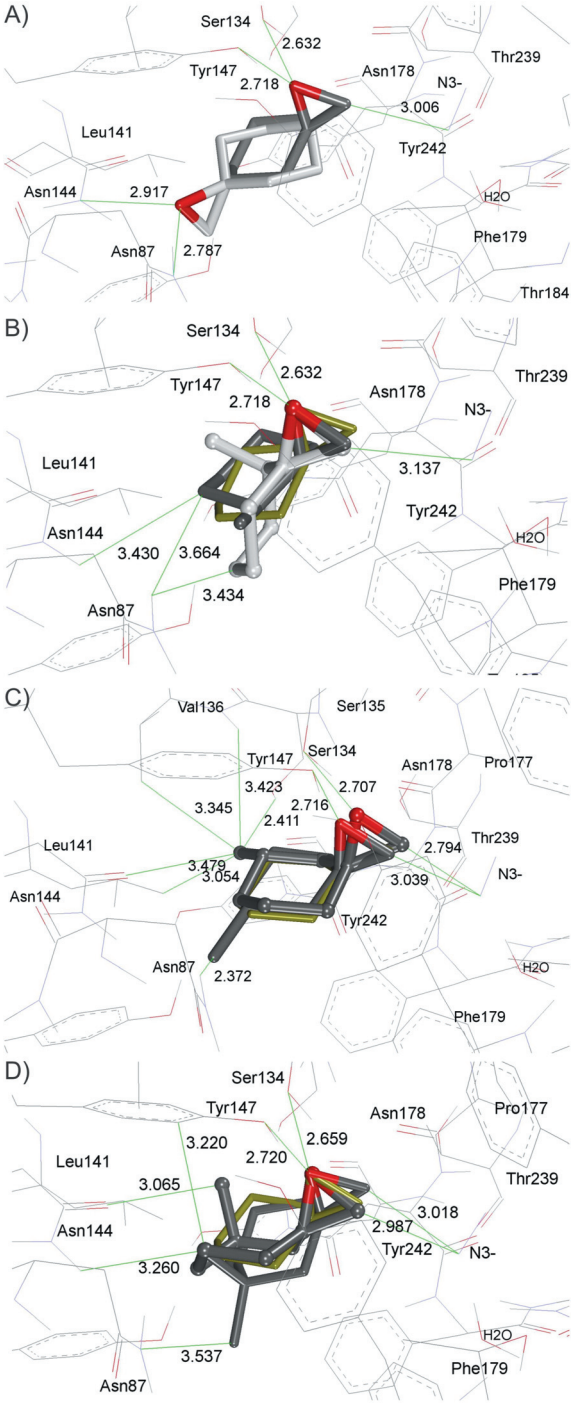


Fig. 2 Epoxide-HheA complexes derived from docking studies. The HheA active site is represented by some structurally important amino acids. Only polar hydrogen atoms are presented. Interatomic distances are given in Å. (**A**) overlay of **1b**$_{O\text{-}ax}$ (grey) and **1b**$_{O\text{-}eq}$ (white); (**B**) overlay of **1b**$_{O\text{-}ax}$ (dark yellow), (3*R*,4*S*)-**1d**$_{O\text{-}ax}$ (grey) and (3*S*,4*R*)-**1d**$_{O\text{-}eq}$ (white); (**C**) overlay of **1b**$_{O\text{-}ax}$ (dark yellow), (3*R*,5*R*)-**1e**$_{O\text{-}ax}$ (stick model) and (3*S*,5*S*)-**1e**$_{O\text{-}ax}$ (ball and stick model); (**D**) overlay of **1b**$_{O\text{-}ax}$ (dark yellow), (*R*)-**1f**$_{O\text{-}ax}$ (stick model) and (*S*)-**1f**$_{O\text{-}ax}$ (ball and stick model).

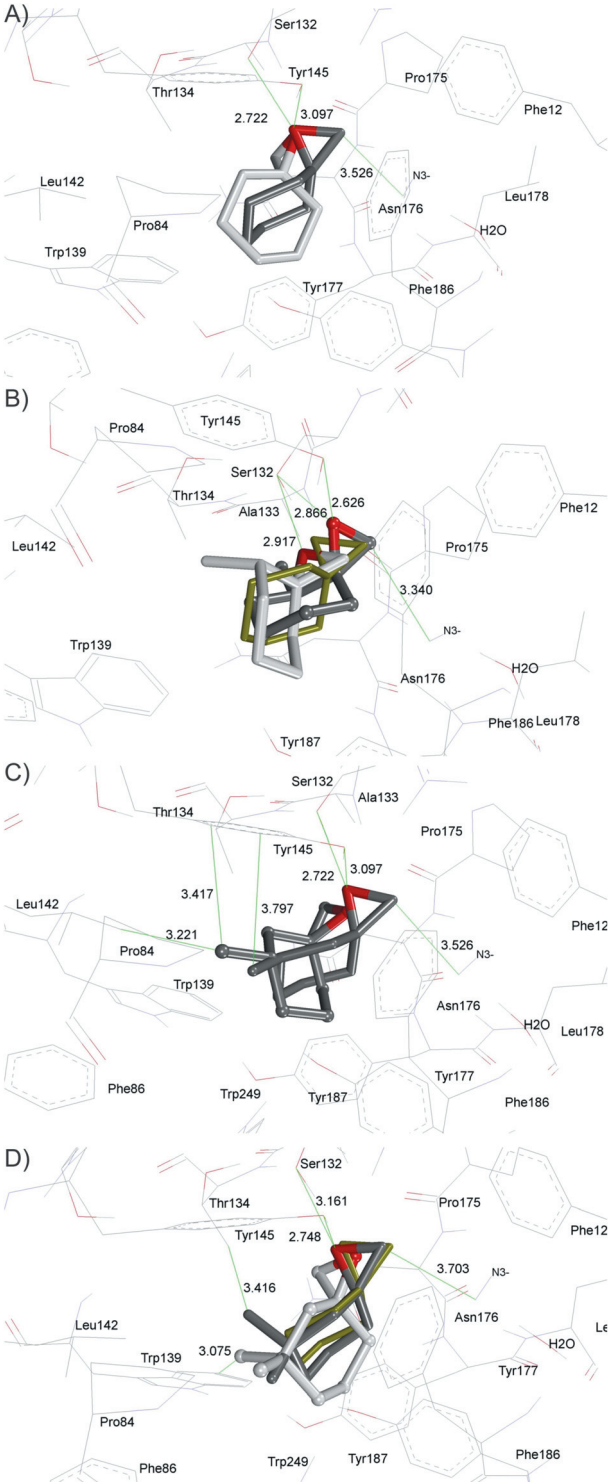


Fig. 3 Epoxide-HheC complexes derived from docking studies. The HheC active site is represented by some structurally important amino acids. Only polar hydrogen atoms are presented. Interatomic distances are given in Å. (**A**) overlay of **1b**$_{O\text{-}ax}$ (grey) and **1b**$_{O\text{-}eq}$ (white); (**B**) overlay of **1b**$_{O\text{-}ax}$ (dark yellow), (3*R*,4*S*)-**1d**$_{O\text{-}eq}$ (white) and (3*S*,4*R*)-**1d**$_{O\text{-}ax}$ (grey); (**C**) overlay of (3*R*,5*R*)-**1e**$_{O\text{-}ax}$ (stick model), (3*S*,5*S*)-**1e**$_{O\text{-}ax}$ (ball and stick model) and **1b**$_{O\text{-}ax}$ (dark yellow, not seen because of the complete overlap with (3*R*,5*R*)-**1e**$_{O\text{-}ax}$); (**D**) overlay of **1b**$_{O\text{-}ax}$ (dark yellow), (*R*)-**1f**$_{O\text{-}ax}$ (grey), and (*S*)-**1f**$_{O\text{-}eq}$ (white).

analogous space as $\mathbf{1b}_{O\text{-}ax}$. On the other hand, the optimal positioning of the methyl substituents orient the epoxide ring of (*S*)-$\mathbf{1f}_{O\text{-}eq}$ to a wrong position and thus prevents the enzymatic azidolysis reaction. Only axial conformers of all tested spiroepoxides can bind productively in the HheC active site, which results in a higher enantioselectivity of the reactions compared to the HheA catalyses.

Conclusions

The results presented in this paper demonstrate that halohydrin dehalogenase catalysed reactions are simple, convenient and highly regioselective for preparing variety of β-substituted *tert*-azido alcohols (achiral or chiral) starting from spiroepoxides. Biocatalysts are environmental friendly, inexpensive and can be easily produced by fermentation. The HheC was confirmed to be more enantioselective enzyme among halohydrin dehalogenases that can be applied also for the kinetic resolution of racemic substrates. With the molecular modelling and docking studies the conformational flexibility and binding of spiroepoxides were explored and the preferable interactions within the enzymes active sites determined giving the better insight into the regioselectivity and enantioselectivity of both enzymes. To the best of our knowledge, this is the first study of the enzyme catalysed azidolysis of spiroepoxides.

Experimental

General methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 300 (^{1}H 300 or 600 MHz and ^{13}C 75 or 150 MHz) spectrometer in $CDCl_3$. Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constants are given in Hz. IR spectra were recorded as thin films on NaCl plates on a Perkin-Elmer Spectrum Two FT-IR and the peaks reported in cm^{-1}. Elemental analyses were carried out on a Perkin-Elmer Series II 2400 CHNS/O analyser. Enzymatic reactions were monitored by gas chromatography (GC) using β-DEX 225 column. GC analyses was performed on a Hewlett Packard HP 5890 Series II gas chromatograph equipped with FID detector (set at 300 °C) and a split injector (set at 250 °C), using N_2 as a carrier gas. Determination of enantiomeric excesses (ee's) was performed by GC using different chiral columns: β-DEX 225 (30 m × 0.25 mm × 0.25 μm, Supelco), Chiraldex G-TA (30 m × 0.25 mm × 0.12 μm, Supelco), Lipodex E (25 m × 0.25 mm × 0.25 μm, Machery–Nagel) and CP Chirasil-DEX CB (25 m × 0.25 mm × 0.25 μm, Varian). Column chromatography was done using silica gel (Fluka, 0.063–0.2 mm). TLC was performed on 0.25 mm silica gel 60-F plates (Merck). Spots were visualised after dipping the TLC plate in a mixture of phosphomolybdic acid (25 g): cerium(IV)sulphate (7.5 g): sulphuric acid (25 mL): water (495 mL) and subsequent heating. Kugelrohr distillation was performed using glass oven B-585 instrument, Büchi.

Materials

The commercially available, cyclohexanone and cycloheptanone were supplied by Merck. Cyclopentanone, 2-methylcyclohexanone, 3-methylcyclohexanone, (*R*)-3-methylcyclohexanone, 3,3-dimethylcyclohexanone (assay 90%), trimethylsulfoxonium iodide, absolute DMSO, arabinose, ampicillin sodium salt, β-mercaptoethanol, sorbitol, and EDTA were purchased from Sigma-Aldrich. Bacto-tryptone, yeast extract, and bacto-agar were purchased from Difco. Complete Protease Inhibitor Cocktail Tablets were supplied by Roche, while glycerol was obtained from Kemika.

Production and purification of the enzyme

Enzymes were prepared according to the modified literature procedure.[19] In brief, two constructs of the plasmid pBAD containing the gene *hheA* from *Arthrobacter species* AD2, and the gene *hheC* from *Agrobacterium radiobacter* AD1, were used for protein expression of respective halohydrin dehalogenases. Each of the constructs was transformed into *E. coli*, strain MC1061, and grown on Luria broth (LB) media supplemented with 50 μg mL^{-1} ampicillin, at 37 °C, over night. Several colonies of respective constructs were inoculated into 50 mL of Terrific broth media (TB) supplemented with 50 μg mL^{-1} ampicillin and grown over night at 30 °C with shaking at 250 rpm. The initial culture was then added into 0.5 L of modified TB media (containing double phosphate buffer concentration and 0.5 M sorbitol). To induce protein expression, arabinose was added to final concentration of 0.02%, and the culture was incubated for 72 h at 30 °C. Bacterial cells were pelleted by centrifugation at 5000 g, at 4 °C, for 15 min, and stored at −20 °C until further protein purification.

In order to purify Hhe proteins, the pellet was resuspended in TEMG buffer (10 mM Tris-SO_4, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, pH 7.5) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets), 10 mL g^{-1}. Following sonication (10 × 10 s, Labsonic M), extract was centrifuged (10 000 g, 1 h, 4 °C). Supernatant was filtered through filter (0.45 μm, Millipore) and subjected to a HiPrep Q FF 16/10 – Sepharose anion exchange column (GE Healthcare, Sweden) connected to the $ÄKTA_{FPLC}$ system (GE Healthcare, Sweden). The elution of the Hhe proteins was carried out with gradient of 0–0.45 $(NH_4)_2SO_4$ in TEMG, at flow rate of 1–3 mL min^{-1}. The collected fractions were screened by SDS-PAGE. Fractions containing purified protein of interest were pooled, concentrated by using Centriprep YM-10 (Millipore, Ireland), and protein concentration was identified according to Bradford.[20] The enzymes were stored at −20 °C until further use.

Synthesis of substrates and reference compounds

General procedure for the synthesis of epoxides. Epoxides **1a–1f** and (*3R*)-**1e** were prepared according to a literature procedure.[13] DMSO (20 mL) was added dropwise to a mixture of trimethylsulfoxonium iodide (5.1 g, 23.1 mmol) and sodium hydride (a 60% disp. in mineral oil; 0.94 g, 23.1 mmol) cooled to 0 °C, under argon. After stirring for 30 min at room temperature, a solution of the corresponding ketone (17.8 mmol) in DMSO (10 mL) was added dropwise. Reaction mixture was stirred for 20 h. Water was added (20 mL) and the mixture extracted with diethyl ether (3 × 30 mL). The combined organic layers were washed with water (2 × 30 mL) and brine (20 mL),

dried over Na_2SO_4, filtered and evaporated under reduced pressure.

*1-Oxaspiro[2.4]heptane (**1a**).* Compound **1a** was prepared from cyclopentanone (1.5 g, 17.8 mmol), according to general procedure, and obtained as a colorless liquid after Kugelrohr distillation (70 °C, 23 mmHg). Yield: 0.52 g, 30%; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.60–1.74 (4H, m), 1.77–1.95 (4H, m), 2.83 (2H, s); δ_C (150 MHz; $CDCl_3$; Me_4Si) 25.2, 32.4, 52.1, 65.2; ν_{max}(film)/cm^{-1} 3032, 2961, 2872, 1485, 1438, 1389, 1208, 947, 907; El. an. calcd for $C_6H_{10}O$: C, 73.4; H, 10.3. Found: C, 73.55; H, 10.6%.

*1-Oxaspiro[2.5]octane (**1b**)*[21] Compound **1b** was prepared from cyclohexanone (1.75 g, 17.8 mmol), according to the general procedure. The crude product was first purified by column chromatography (SiO_2; pentane–diethyl ether, 85 : 15) followed by Kugelrohr distillation (65 °C, 2 mmHg). Epoxide **1b** was obtained as a colorless liquid. Yield: 0.84 g, 42%; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.48–1.59 (8H, m), 1.69–1.81 (2H, m), 2.58 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 24.9, 25.2, 33.6, 53.3, 54.4. ν_{max}(film)/cm^{-1} 3033, 2933, 2858, 1495, 1484, 1448, 1249, 911, 821.

*1-Oxaspiro[2.6]nonane (**1c**)*[21] Compound **1c** was prepared from cycloheptanone (1.99 g, 17.8 mmol), according to general procedure. The crude product was first purified by column chromatography (SiO_2; pentane–diethyl ether, 85 : 15) followed by Kugelrohr distillation (75 °C, 2 mmHg). Epoxide **1c** was obtained as a colorless liquid. Yield: 1.36 g, 61%; δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46–1.78 (12H, m), 2.59 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 24.6, 28.9, 35.9, 55.6, 60.5. ν_{max}(film)/cm^{-1} 3029, 2928, 2856, 1455, 1264, 944, 886.

*(3R,4S),(3S,4R)-4-Methyl-1-oxaspiro[2,5]octane(**1d**)*[9] Racemic compound **1d** was prepared from *rac*-2-methylcyclohexanone (1.0 g, 8.9 mmol), according to general procedure. The crude product was first purified by column chromatography (SiO_2; *n*-hexane–ethyl acetate, 9 : 1). Epoxide **1d** was obtained as a colorless liquid. Yield: 0.95 g, 85%; δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.83 (3H, d, J = 7.0 Hz), 1.39–1.76 (9H, m), 2.48 (1H, d, J = 5.0 Hz), 2.75 (1H, d, J = 5.0 Hz); δ_C (75 MHz; $CDCl_3$; Me_4Si) 14.3, 24.0, 24.6, 32.6, 32.9, 34.5, 52.5, 60.9. ν_{max}(film)/cm^{-1} 3042, 2930, 2855, 1447, 1369, 1232, 958, 828.

*(3R,5R),(3S,5S)-5-Methyl-1-oxaspiro[2,5]octane (**1e**)*[9] Racemic compound **1e** was prepared from *rac*-3-methylcyclohexanone (1.0 g, 8.9 mmol), according to general procedure. Epoxide was obtained as a colorless liquid. Yield: 0.90 g, 80%; δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.92 (3H, d, J = 6.5 Hz), 0.95–1.03 (1H, m), 1.20–1.29 (2H, m), 1.48 (1H, dd, J_1 = 13.5 Hz, J_2 = 11.5 Hz), 1.59–1.81 (5H, m), 2.60 (1H, d, J = 4.7 Hz), 2.62 (1H, d, J = 4.7 Hz); δ_C (75 MHz; $CDCl_3$; Me_4Si) 22.2, 23.4, 30.4, 32.6, 33.7, 41.5, 53.9, 58.4. ν_{max}(film)/cm^{-1} 3032, 2977, 2868, 1491, 1457, 1289, 914, 866.

*(3R,5R)-5-Methyl-1-oxaspiro[2,5]octane ((3R,5R)-**1e**).* Reference compound (3*R*,5*R*)-**1e** was prepared in 80% yield (0.90 g), starting from (*R*)-3-methylcyclohexanone (1.0 g, 8.9 mmol). δ_H (600 MHz; $CDCl_3$; Me_4Si) 0.92 (3H, d, J = 6.5 Hz), 0.93–1.00 (1H, m), 1.22–1.28 (2H, m), 1.48 (1H, dd, J_1 = 13.5 Hz, J_2 = 11.5 Hz), 1.62–1.67 (1H, m), 1.72–1.82 (4H, m), 2.59 (1H, d, J = 4.5 Hz), 2.61 (1H, d, J = 4.5 Hz); δ_C (150 MHz; $CDCl_3$; Me_4Si) 21.7, 22.9, 29.9, 32.1, 33.3, 41.0, 53.4, 57.9; El. an. calcd for $C_8H_{14}O$: C, 76.1; H, 11.2. Found: C, 76.0; H, 11.2%.

*(R,S)-5,5-Dimethyl-1-oxaspiro[2,5]octane (**1f**).* Racemic compound *rac*-**1f** was prepared from 3,3-dimethylcyclohexanone (4.98 g of 90% purity, 35.6 mmol), according to general procedure. Epoxide was obtained as a colorless liquid. Yield: 3.7 g, 74%; δ_H (600 MHz; $CDCl_3$; Me_4Si) 0.94 (3H, s), 1.00 (3H, s), 1.31–1.33 (3H, m), 1.41 (1H, d, J = 13.5 Hz), 1.46–1.53 (2H, m), 1.58–1.64 (1H, m), 1.72–1.78 (1H, m), 2.54 (1H, d, J = 5.0 Hz), 2.58 (1H, d, J = 5.0 Hz); δ_C (150 MHz; $CDCl_3$; Me_4Si) 20.9, 28.4, 29.5, 32.7, 33.1, 38.3, 45.9, 53.8, 57.6; ν_{max}(film)/cm^{-1} 3033, 2947, 2868, 1457, 1365, 1268, 917, 818, 798; El. an. calcd for $C_9H_{16}O$: C, 77.1; H, 11.5. Found: C, 77.3; H, 11.4%.

General procedure for the synthesis of β-azido alcohols. Azido alcohols were prepared according to a literature procedure starting from 1.0 mmol of epoxide.[12] By following the protocol at pH 9.5 a mixture of regioisomers **2a**/**3a** and **2c**/**3c** as well as single **2b**, **2d**, **2e** and **2f** were obtained. At pH 4.2 both regioisomers **2b**/**3b** were obtained. Column chromatography (SiO_2; *n*-hexane–ethyl acetate, 9 : 1) furnished the pure azido alcohols.

*1-(Azidomethyl)cyclopentan-1-ol (**2a**).* δ_H (600 MHz; $CDCl_3$; Me_4Si) 1.60–1.70 (6H, m), 1.80–1.85 (2H, m), 1.86 (1H, s), 3.38 (2H, s); δ_C (150 MHz; $CDCl_3$; Me_4Si) 23.5, 37.4, 60.0, 81.6; El. an. calcd for $C_6H_{11}N_3O$: C, 51.1; H, 7.9; N 29.8. Found: C, 49.9; H, 7.85; N, 29.95%.

*1-Azido-1-hydroxymethylcyclopentane (**3a**)*[22] δ_H (600 MHz; $CDCl_3$; Me_4Si) 1.68–1.72 (4H, m), 1.77–1.84 (5H, m), 3.58 (2H, s); δ_C (150 MHz; $CDCl_3$; Me_4Si) 24.2, 33.9, 68.4, 73.9.

*1-(Azidomethyl)cyclohexan-1-ol (**2b**).* δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.46–1.62 (10H, m), 1.71 (1H, s), 3.25 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 21.7, 25.6, 35.0, 61.5, 71.6; El. an. calcd for $C_7H_{13}N_3O$: C, 54.2; H, 8.4; N 27.1. Found: C, 54.4; H, 8.5; N, 27.0%.

*1-Azido-1-hydroxymethylcyclohexane (**3b**).* δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.29–1.82 (11H, m), 3.55 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 21.8, 25.4, 31.1, 64.7, 69.3; El. an. calcd for $C_7H_{13}N_3O$: C, 54.2; H, 8.4; N 27.1. Found: C, 54.35; H, 8.3; N, 27.2%.

*1-(Azidomethyl)cycloheptan-1-ol (**2c**)*[23] δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.42–1.74 (12H, m), 1.74 (1H, s), 3.26 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 22.2, 29.7, 38.5, 61.7, 75.6.

*1-Azido-1-hydroxymethylcycloheptane (**3c**).* δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.45–1.85 (12H, m), 3.50 (2H, d, J = 6 Hz); δ_C (150 MHz; $CDCl_3$; Me_4Si) 22.5, 29.8, 34.4, 68.5, 69.0; El. an. calcd for $C_8H_{15}N_3O$: C, 56.8; H, 8.9; N 24.8. Found: C, 56.7; H, 9.0; N, 24.85%.

*1-Azidomethyl-2-methylcyclohexan-1-ol (**2d**).* δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.90 (3H, d, J = 6.5 Hz), 1.21–1.77 (9H, m), 3.21 (1H, d, J = 12.0 Hz), 3.40 (1H, d, J = 12.0 Hz); δ_C (75 MHz; $CDCl_3$; Me_4Si) 21.7, 22.9, 29.9, 32.1, 33.2, 41.0, 53.4; El. an. calcd for $C_8H_{15}N_3O$: C, 56.8; H, 8.9; N 24.8. Found: C, 56.6; H, 8.85; N, 24.9%.

*1-Azidomethyl-3-methylcyclohexan-1-ol (**2e**).* δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.91 (3H, d, J = 6.0 Hz), 1.16–1.27 (1H, m), 1.59–1.81 (8H, m), 3.23 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 21.0, 22.4, 27.4, 34.2, 34.5, 43.2, 62.9, 72.1; El. an. calcd for $C_8H_{15}N_3O$: C, 56.8; H, 8.9; N 24.8. Found: C, 56.95; H, 9.0; N, 24.7%.

*1-Azidomethyl-3,3-dimethylcyclohexan-1-ol (**2f**).* δ_H (600 MHz; $CDCl_3$; Me_4Si) 0.92 (3H, s), 1.10 (3H, s), 1.13–1.26 (3H, m), 1.44–1.52 (3H, m), 1.63 (1H, s), 1.64–1.67 (1H, m), 1.75–1.82 (1H, m), 3.19 (1H, d, J = 12.0 Hz), 3.21 (1H, d, J = 12.0 Hz); δ_C(150 MHz; $CDCl_3$; Me_4Si) 18.0, 27.1, 30.5, 33.6, 34.9, 39.0, 46.6, 63.5, 72.6; El. an. calcd for $C_9H_{17}N_3O$: C, 59.0; H, 9.4; N 22.9. Found: C, 58.9; H, 9.3; N, 22.9%.

Regioselectivity of the ring opening of epoxides 1a–1c with sodium azide in water. Epoxide (0.5 mmol) was added to 1 mL of water containing sodium azide (2.5 mmol) at room temperature. After 20 h reaction mixtures were extracted with CH_2Cl_2, extracts were dried and analyzed by GC under conditions described in ESI.†

Determination of absolute configuration. Absolute configurations were assigned by chiral GC analysis using reference compounds. In the case of epoxide **1d** assignment was based on previously reported data.[9] (3*R*,5*R*)-**1e** was prepared from optically pure (*R*)-3-methylcyclohexanone (Aldrich). The enantiomerically enriched epoxide (*R*)-**1f** (97% ee) was prepared by (*R*,*R*)-(salen)CrCl catalysed ring opening with $TMSN_3$ according to Label and Jacobsen.[24]

Calculation of enantioselectivity. *E*-values were calculated from ee_p and ee_s according to formula $E = \ln[(1 - ee_s)/(1 + ee_s/ee_p)]/\ln[(1 + ee_s)/(1 + ee_s/ee_p)]$.[14]

General procedure for the enzymatic ring-opening of epoxides on analytical scale

To 20 mL of Tris-SO_4 buffer (50 mM, pH 7.0) at room temperature, 100 μL of a stock solution of substrate in DMSO was added (final concentration 5.0 mM), followed by addition of a stock solution in water of NaN_3 (1.0 mL, final concentration 5.0 mM). Reactions were initiated by addition of enzyme HheA or HheC (56–150 μg) in TEMG buffer. The progress of the reaction was followed by periodically taking samples (0.5 mL) from reaction mixture. Samples were extracted with MTBE (1.0 mL) containing chlorobenzene as internal standard and analysed by GC on a β-DEX 225 column. Non-enzymatic ring-opening reaction was determined by monitoring product formation in the absence of enzyme.

Tests for hydrolytic stability of epoxides

Typically, reactions were performed analogous to the procedure described for the enzymatic ring opening of epoxides without adding enzyme and nucleophile. To 21 mL of Tris-SO_4 buffer (50 mM, pH 7.0) at room temperature, 100 μL of a stock solution of substrate in DMSO was added (final concentration 5.0 mM). Concentration of epoxide was followed by periodically taking samples (0.5 mL) from reaction mixture. Samples were extracted with MTBE (1.0 mL) containing chlorobenzene as internal standard and analysed by GC on a β-DEX 225 column.

Enzymatic preparation of β-azido alcohols 2a–2c. The epoxide (1.0 mmol, 50 mM) was dissolved in a 19.4 mL of Tris-SO_4 buffer (0.5 M, pH 7.0) followed by addition of 130 mg of NaN_3 (2.0 mmol, 100 mM) and purified HheA (0.5 mg in 0.6 mL TMEG buffer). The reaction was performed at room temperature, followed by GC and stopped at >99% conversion. The reaction mixture was saturated with NaCl and extracted with CH_2Cl_2. The combined organic extracts were dried on filtered and evaporated. The organic phase was dried over Na_2SO_4. The crude product was purified by column chromatography (SiO_2; *n*-hexane–ethyl acetate, 9 : 1).

*1-(Azidomethyl)cyclopentan-1-ol (**2a**).* Obtained as colorless liquid (107 mg, 75%) after column chromatography. The NMR data were identical to **2a** prepared by standard chemical procedure. δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.59–1.72 (6H, m), 1.82–1.86 (2H, m), 3.39 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 24.0, 37.9, 60.5, 82.1; ν_{max}(film)/cm^{-1} 3401, 2961, 2101, 1284; El. an. calcd for $C_6H_{11}N_3O$: C, 51.1; H, 7.9; N 29.8. Found: C, 50.0; H, 7.85; N, 29.9%.

*1-(Azidomethyl)cyclohexan-1-ol (**2b**).* Obtained as colorless liquid (147 mg, 95%) after column chromatography. The NMR data were identical to **2b** prepared by standard chemical procedure. δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.24–1.73 (11H, m), 3.26 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 21.7, 25.6, 35.0, 61.5, 71.6; ν_{max}(film)/cm^{-1} 3422, 2934, 2859, 2103, 1447, 1286; El. an. calcd for $C_7H_{13}N_3O$: C, 54.2; H, 8.4; N 27.1. Found: C, 54.3; H, 8.4; N, 27.0%.

*1-(Azidomethyl)cycloheptan-1-ol (**2c**).* Obtained as colorless liquid (163 mg, 96%) after column chromatography. The NMR data were identical to **2c** prepared by standard chemical procedure. δ_H (300 MHz; $CDCl_3$; Me_4Si) 1.38–1.73 (13H, m), 3.25 (2H, s); δ_C (75 MHz; $CDCl_3$; Me_4Si) 22.2, 29.7, 38.5, 61.7, 75.6. ν_{max}(film)/cm^{-1} 3428, 2927, 2856, 2102, 1461, 1280; El. an. calcd for $C_8H_{15}N_3O$: C, 56.8; H, 8.9; N 24.8. Found: C, 56.9; H, 8.95; N, 24.7%.

Quantum chemical calculations

Optimization of reactants, transition states and products were performed at the B3LYP/6-311++g(d,p) level of the theory[25,26] using the Gaussian 09 program package.[27] For all optimized structures harmonic frequencies were calculated to insure that obtained geometries correspond to the local minimum (or maximum) on the potential energy surface. The Gibbs energies were calculated at T = 298.15 K and p = 1 atm (Table S6†). Solvation effects were incorporated in the calculations using the reformulation of polarizable continuum model (PCM)[28,29] known as integral equation formalism (IEFPCM) of Tomasi and co-workers.[30–33] Contents of obtained products were estimated from the Boltzmann distribution using the differences in Gibbs energies of activation. For a hybrid approach, combination of quantum chemical (B3LYP/6-31+g(d)) and semiempirical (PM3MM) calculations within the ONIOM[34] scheme was chosen. A model for enzyme placed in the low layer was made of 26 amino acids surrounding the active site. The residues included in the model were Phe12, Asp80, Arg84, Asn87, Arg88, Thr133, Ser134, Ser135, Val136, Gly137, Leu141, Tyr143, Asn144, Tyr147, Arg151, Gly176, Pro177, Asn178, Phe179, Phe180, Asn182, Thr184, Tyr185, Phe186, Thr239, Tyr242 and two molecules of water. To ensure the consistency of the model, several atoms positions were kept fixed to their

crystallographic position during the optimizations. In high layer an azide, ligand and one water molecule were placed.

Flexible ligand docking

Docking studies were performed by using the AutoDock 4.2.2 suite of programs.[16] AutoDock requires a precalculated electrostatic grid map for each atom type present in the substrate molecule. These electrostatic maps were calculated using the AutoGrid part of the suite with 0.2 Å spacing between grid points and the centre of the grid was placed in the γ-oxygen atom of catalytic Ser200. Dimensions of the active site box were set to 20 Å × 20 Å × 20 Å thus ensuring appropriate size of the ligand-accessible space. Consistencies of electrostatic maps were ascertained by checking maximum and minimum values of van der Waals energies and electrostatic potentials for each calculated grid map.

Flexible ligand docking for all compounds were carried out using the Lamarckian genetic algorithm and all parameters were the same for each docking run. We used initially a population of 5000 random individuals, a maximum number of 2×10^7 energy evaluations, a maximum number of generations of 1×10^6, elitism value of 1, mutation rate of 0.02 and crossover rate of 0.08. For the local search, the pseudo-Solis and Wets method was used with maximum of 10 000 iterations per local search, the probability of performing a local search on an individual in the same population was 0.06, the maximum number of consecutive successes or failures before changing the rho was 4 in both cases, size of local search space to sample was 1.0 and the lower bound on rho was 0.01. To ensure the validity of results, the docking procedure for each substrate consisted of 100 independent docking runs. The resulting positions were clustered according to r.m.s. criterion of 0.5 Å. Obtained structures were analyzed visually and evaluated based on their interactions with the amino acids within the active site of the enzyme.

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