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Catalytic Promiscuity of Halohydrin Dehalogenase and its Application in Enantioselective Epoxide Ring Opening

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The catalytic promiscuity of some enzymes is thought to play an important role in the evolution of new catalytic activities, and can be used to apply enzymes in unnatural reactions of synthetic importance.[1] However, unnatural enzyme-catalyzed reactions are often slow and most documented cases concern hydrolytic conversions instead of synthetic reactions. The enantioselectivity of promiscuous enzymes also may be low. Here, we report that an unusual dehalogenase that has recently been explored structurally and mechanistically[2] can accept at least nine different anionic nucleophiles in enantioselective epoxide ring-opening reactions that are catalyzed by this enzyme. This enables the preparation of a broad range of highly enantioenriched β-substituted alcohols and epoxides by kinetic resolution.

Halohydrin dehalogenase (HheC) from the epichlorohydrin degrading bacterium *Agrobacterium radiobacter* AD1 catalyzes the dehalogenation of 1,3-dichloropropanol and 1-chloropropane-2,3-diol to produce an epoxide and HCl. Recently solved X-ray structures of HheC with various ligands revealed that the active site consists of a binding site for the epoxide and a spacious halide-binding pocket, and provided insight into the catalytic mechanism and cause of enantioselectivity of the enzyme.[2] The dehalogenase also catalyzes the reverse reaction: ring opening of epoxides.[3] Nucleophilic epoxide ring opening is a powerful way to produce β-functionalized alcohols, which can be applied in the synthesis of pharmaceuticals and biologically active compounds.[4] Considering that such compounds are often required as pure enantiomers, enantio- and regioselective epoxide ring-opening reactions have been studied intensively, for example with the use of chiral metal catalysts.[5] So far, few nucleophiles have been used for epoxide ring opening, apart from water, halides, azide, and cyanide.[6]

We explored the catalytic versatility of HheC in epoxide ring opening by determining the products that are formed upon incubation of the enzyme with a series of different nucleophiles and 1,2-epoxybutane (5 mM) in buffer. Conversion was observed with Br^-, Cl^-, I^-, CN^-, NO_2^-, N_3^-, OCN^-, SCN^-, and $HCOO^-$. No reaction (epoxide conversion less than 0.05 μmol min^{-1} mg^{-1}) occurred with nonanionic nucleophiles (primary alcohols and amines), and with the sulfur-containing compounds H_2S, SO_3^{2-}, SO_4^{2-}, and $S_2O_5^{2-}$. Acetic acid, chloro-, bromo-, and iodoacetic acid, 2-chloropropionic acid, malonic acid, PO_4^{2-}, BO_3^{3-}, PO_4^{2-}, CO_3^{2-}, H_2O_2, ClO_4^-, and F^- were also not accepted. A low conversion rate (0.1 μmol min^{-1} mg^{-1}) was obtained with NO_3^-. This indicates that the enzyme can accept a range of monovalent anions with a linear shape.

In order to determine the rate of 1,2-epoxybutane conversion and monitor product formation, epoxybutane was incubated in a buffered solution at pH 7.5 with each accepted nucleophile and HheC. The rate of epoxybutane conversion was very much dependent on the nucleophile used, but with calculated k_{cat} values of 2–100 s^{-1} for most substrates the enzyme is sufficiently active for preparative application (Table 1). Azide gave the highest reaction rates. For the other nucleophiles, the initial activities were lower even when their concentrations were saturating, but the values still fall in the range of synthetically useful enzymatic activities. For product identification, all expected products and their regioisomers were chemically synthesized according to literature procedures, and were subse-

Table 1. Catalytic activity of halohydrin dehalogenase HheC in the nucleophilic ring opening of epoxybutane.

[a]	Nucleophile	Concentration [mM]	Activity[b] [s^{-1}]	Conversion [%]	ee_p [%]	*E* value[c]	Regioselectivity [% terminal]
1	N_3^-	10	160	30	90	28	99
2	NO_2^-	10	18	26	96	28	97
3	CN^-	10	2.5	30	90	28	99
4	Cl^-	100	1.8	45	8	–[d]	89
5	Br^-	100	0.93	45	6	–[d]	86
6	I^-	100	3.2	42	11	–[d]	96
7	OCN^-	100	1.9	27	90	24	>99
8	SCN^-	100	1.9	27	n.d.[e]	24	>99
9	$HCOO^-$	100	0.61	32	90	31	94

[a] Product structures are shown in Scheme 1. [b] Initial activities (k_{cat}) per tetramer measured with *rac*-epoxybutane [5 mM]. [c] *E* values were calculated from the enantiomeric excess of the substrate (ee_s) and conversion according to the formula $E = [\ln[(1-c)(1-ee_s)]/[(1-c)(1+ee_s)]$. The faster reacting enantiomer with all nucleophiles was the *R* epoxide. [d] *E* values could not be determined from conversion and *ee* due to the reversibility of the reaction. [e] Not determined.

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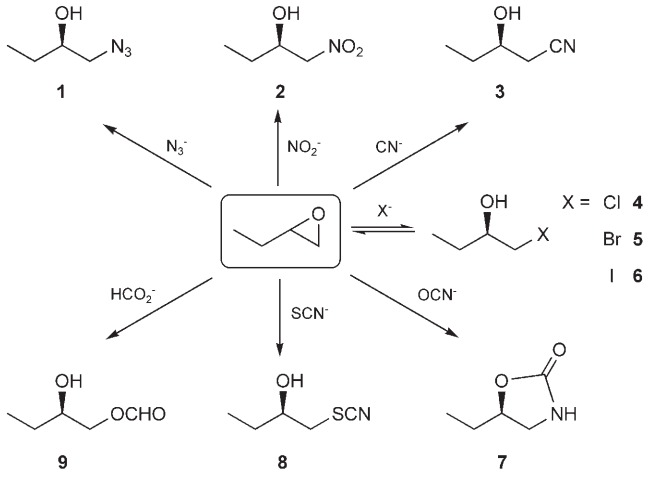
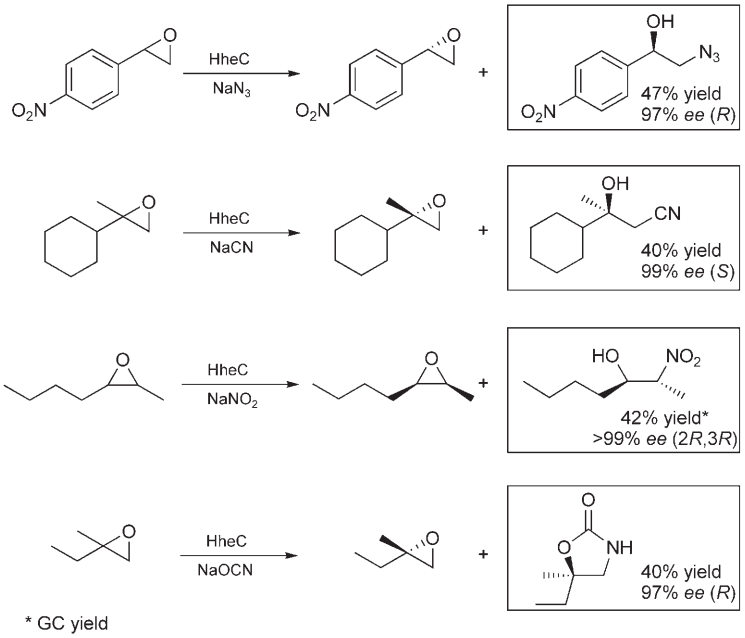
quently used as standards in GC analysis. In the enzyme-catalyzed reaction, all nucleophiles attacked predominantly at the less substituted carbon of 1,2-epoxybutane (terminal position), and in all cases there was a strong enantiopreference for the *R* epoxide; this resulted in the formation of enantioenriched 1-substituted butan-2-ols with retained *R* configuration (Scheme 1).



Scheme 1. Conversions catalyzed by the halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 (HheC).

Ring opening of epoxybutane with azide yielded the expected (*R*)-1-azidobutan-2-ol (**1**). Besides epoxybutane, several styrene oxide derivatives were azidolyzed by HheC with high enantioselectivity and almost complete terminal regioselectivity (Scheme 2); this is contrary to what is observed in noncatalyzed azidolysis of epoxides that bear an aromatic substituent, and in which the benzylic position is attacked.[3b,7]



Scheme 2. Examples of highly enantioselective epoxide ring-opening reactions catalyzed by halohydrin dehalogenase.

Nitrite is an ambident nucleophile that could perform nucleophilic attack on the epoxide ring either through its nitrogen or oxygen atoms. The HheC-catalyzed ring opening of epoxybutane in the presence of nitrite resulted in a mixture of nitrite ester, which is formed by oxygen attack and undergoes hydrolysis to butane-1,2-diol (46%), and 1-nitrobutan-2-ol (54%), which is formed by nitrogen attack. Ring opening of styrene oxide derivatives proceeds mainly by oxygen attack to give 1,2-diols via unstable nitrite esters; this causes HheC to act as an alternative epoxide hydrolase.[8] The result with the aliphatic epoxide shows that HheC can also be used for the production of optically pure nitroalcohols, which are attractive inermediates for preparing optically active aminoalcohols. The highly enantio- and regioselective conversion that was found with 2,3-epoxyheptane made it possible to obtain the nitro compound in high yield (Scheme 2); this also indicates that the substrate range of the enzyme is not restricted to terminal epoxides.

Ring opening with cyanide yielded enantioenriched (*R*)-1-cyanobutan-2-ol (**3**); this confirms that HheC is capable of enantioselective carbon–carbon bond formation. Despite lower reaction rates with cyanide than with either azide or nitrite, some epoxides, especially aliphatics, could be converted efficiently with good to excellent enantioselectivities and yield (Table 1, Scheme 2).[9] Epoxide ring opening with cyanide was observed earlier with a different dehalogenase.[10] One of the possible applications of halohydrin dehalogenation and cyanide-mediated epoxide ring opening is in the synthesis of the side chain of the cholesterol-lowering agent atorvastatin. For this process HheC was optimized by directed evolution.[11]

Ring-opening reactions of epoxybutane with Cl^-, Br^-, and I^- led to equilibrium mixtures with equilibrium constants of 0.007, 0.0025, and 0.01 mM^{-1}, respectively. This means that the formation of epoxides from halohydrins was favored over halide-mediated epoxide ring opening. Since racemic halohydrins are easily available and in view of the equilibrium constants it is more practical to use HheC for ring closure reactions of halohydrins than for halide-mediated epoxide ring opening. This was recently explored in tandem reactions.[12] Of the halides, fluoride showed no reactivity.

Ring opening of 1,2-epoxybutane with cyanate yielded (*R*)-5-ethyl-oxazolidin-2-one (**7**) as the sole product. Both nitrogen and oxygen attack would lead to the same product since organic cyanates rapidly undergo isomerization to form the more stable isocyanates,[13] which subsequently cyclize to form the corresponding oxazolidinones. Since no intermediates were found, the cyclization step must occur at a high rate. This reaction can yield a route to enantiopure oxazolidinones, which are convenient precursors for the production of amino alcohols and amino acids, and are used as chiral auxiliaries in aldol condensations. Highly enantioenriched (*R*)-5-methyl-5-ethyloxazolidinone was isolated in good yield (Scheme 2). Oxazolidinones have also attracted interest for their antibacterial activity, particularly

toward multidrug resistant Gram positive bacteria, as is the case for the commercialized antibiotic linezolid.[14]

With thiocyanate and epoxybutane, the sole product of the enzymatic reaction was (*R*)-1-isothiocyanatobutan-2-ol (**8**); this shows that attack took place via the sulfur atom. The product of a nitrogen attack, which would cyclize to form 5-ethyl-oxazolidine-2-thione, was not detected; this confirms that thiocyanate attacked exclusively through the sulfur. As expected,[15] compound **8** slowly rearranged nonenzymatically to 2-ethylthiirane. Formation of 2-ethylthiirane was accompanied by release of OCN^-, which further reacted with **8** to give unidentified side products. Thiocyanatohydrins and thiiranes can both be used in the preparation of fragrance compounds.[16]

Upon reaction of 1,2-epoxybutane with formate both regioisomers of hydroxybutyl formic acid ester were formed, and subsequently hydrolyzed to 1,2-butanediol. Determination of the *ee* and absolute configuration of the formed diol indicated that formate mainly attacked the α (terminal) carbon of the epoxide ring and that the other regioisomer was formed by transesterification.

The above inventory of the nucleophile promiscuity of the halohydrin dehalogenase HheC indicates that the enzyme accepts a range of halides and small negatively charged nucleophiles that are often referred to as pseudohalides (Scheme 1). The size and the shape of a nucleophile determine its acceptance by HheC, which is in agreement with the presence of a well-defined occluded anion-binding site observed in the X-ray structure of the enzyme. The accepted, more voluminous nucleophiles, such as the triatomic anions nitrite, azide, cyanate, and thiocyanates, have the same linear or slightly bent shapes. Although formate deviates from this common shape it is accepted by HheC; this shows that the enzyme can accommodate an additional hydrogen, but not larger atoms, such as the extra oxygen of a carbonate. These features also explain why anions such as phosphate, borate, and perchlorate were not accepted. Besides the shape and size of the nucleophile, the negative charge appears to be essential for activity of HheC. In view of this, it is not surprising that small species, such as hydrogen sulfide or water, displayed no activity. Structural inspection also suggests that the nucleophiles attack at the terminal carbon of the epoxybutane—it is closer to the nucleophile-binding site than the carbon atom that bears the more bulky *R* substiuent.[2] Nevertheless, we have identified 2,3-epoxyheptane as a substrate; this indicates that activity is not restricted to terminal epoxides.

The conversions described here show that halohydrin dehalogenase has the unique capacity to form carbon–halogen, carbon–oxygen, carbon–sulfur, and carbon–carbon bonds. The products formed during epoxide ring opening are of high *ee* and can be obtained in good yield (Scheme 2); therefore the enzyme could find wide application in the preparation of enantiopure β-functionalized alcohols from or via epoxides. Azido-, cyano-, and nitroalcohols can be used as precursors for the preparation of aminoalcohols, which are highly versatile starting materials for the synthesis of biologically active molecules. Isothiocyanates, thiiranes, and oxazolidinones find applications in agrochemicals, pharmaceuticals, and polymer chemistry. The remarkable catalytic promiscuity of halohydrin dehalogenase illustrates the potential of bacteria that degrade halogenated environmental pollutants as a source of useful industrial enzymes.

Experimental Section

Halohydrin dehalogenase from *Agrobacterium radiobacter* AD1 (HheC, GenBank accession no. AF397296) was isolated from a recombinant *E. coli* as described previously.[3b] Screening for nucleophiles for HheC was carried out by the addition of purified enzyme (10 μM final concentration) to a mixture of racemic 1,2-epoxybutane (25 mM) and the nucleophile (25 mM) in Tris·SO_4 buffer (2 mL, 100 mM, pH 7.5, containing 0.5% (*v*/*v*) DMSO to facilitate solubilization of the epoxide). After 2 h the reaction mixture was extracted with diethyl ether containing 1-chlorohexane as the internal standard. Samples were dried over $MgSO_4$ and analyzed by GC. To determine initial rates, enantioselectivities, and the nature of the formed products, reactions were performed with epoxybutane (5 mM) in Tris·SO_4 (20 mL, 100 mM, pH 7.5) containing an appropriate concentration of the sodium salt of an anionic nucleophile (10–100 mM). After addition of enzyme, samples were collected at regular time intervals, extracted with diethyl ether, dried with $MgSO_4$, and analyzed by chiral GC. GC conditions and analytical details for products are given in the Supporting Information.

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Keywords: biocatalysis · enantioselectivity · epoxides · halohydrin dehalogenase · nucleophiles

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