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Regioselectivity of *Rhodococcus* NCIMB 11216 Epoxide Hydrolase: Applicability of *E*-Values for Description of Enantioselectivity Depends on Substrate Structure

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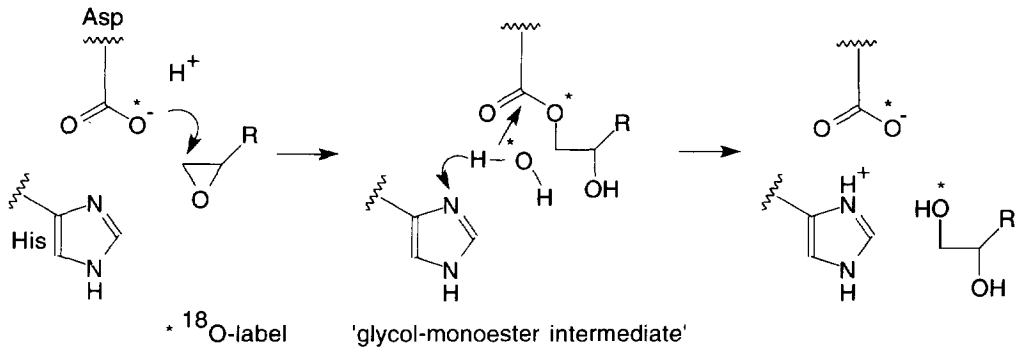
Abstract: The regioselectivity of purified epoxide hydrolase from *Rhodococcus* NCIMB 11216 was investigated by hydrolyzing a series of structurally different epoxides **1a-5a** in ^{18}O-labelled water followed by GC/MS analysis of the 1,2-diols formed **1b-5b**. The enzyme introduced a single ^{18}O-atom in a *trans*-specific fashion with a varying degree of regioselectivity depending on the substitution pattern of the substrate. With an aliphatic *mono*- **1a** and 2,2-disubstituted oxirane **3a** the attack occurred exclusively at the less hindered C-atom and complete retention of configuration was retained. On the other hand, the regioselectivity was low with a 2,3-di- **4a** and a trisubstituted epoxide **5a**, and with a substrate bearing a benzylic oxirane atom **2a**. As a consequence, the 'Enantiomeric Ratio' (E) can be applied to describe the selectivity of kinetic resolutions for some, but not for all types of substrates.

Introduction

Epoxide hydrolases have emerged as powerful biocatalysts for the synthesis of enantiomerically pure epoxides and 1,2-diols[1]. Although enzymes from mammalian sources such as liver tissue have been thoroughly investigated during studies on the detoxification of xenobiotics[2-4], their application in biotransformations on a preparative scale is impeded by the limited supply of enzyme[5,6]. This drawback has recently been circumvented by the discovery of epoxide hydrolases from microbial sources such as fungi[7,8] and bacteria[9-11] which allows an almost unlimited production of biocatalyst.

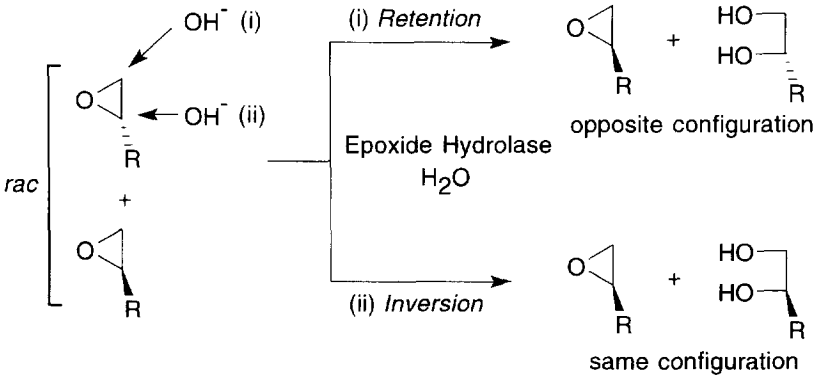
The mechanism of epoxide hydrolases, which neither require any prostetic group nor metal ions, was long debated, and it was formerly assumed, that it constitutes a nucleophilic opening of the oxirane ring by a hydroxide ion, which in turn is supplied by the aid of a histidine[12]. It was only recently, that convincing evidence was provided (at least for microsomal epoxide hydrolase from liver tissue) showing that the reaction occurs via a covalent glycol-monoester-enzyme intermediate[13,14] (Scheme 1). Thus, the epoxide is opened by a nucleophilic attack of an aspartate residue by forming a *mono*-ester of the corresponding 1,2-diol. The latter species - which resembles an 'inverted' acyl-enzyme intermediate found in serine-hydrolase mechanism - is subsequently hydrolyzed by a hydroxide ion which is provided from water by proton abstraction via a histidine, with concomitant release of the diol.

Scheme 1: Proposed mechanism of epoxide hydrolases.



As a consequence, the epoxide is opened in a *trans*-specific fashion[9,15,16] with one oxygen from water being incorporated into the diol[17,18]. These facts have important consequences on the stereochemical outcome of kinetic resolutions of racemic epoxides. In contrast to reactions performed with commonly used hydrolytic enzymes such as esterases, lipases and proteases where the configuration of the stereogenic centre remains the same during the course of the biocatalytic reaction, enzymatic epoxide-hydrolysis may proceed via two different pathways[8] (Scheme 2). (i) Attack of the (formal) hydroxide ion at the more accessible carbon atom effects *retention* of configuration, whereas (ii) attack on the sterically more shielded stereogenic centre causes *inversion*. The existence of both pathways has been proven with different enzyme systems[8,17,19].

Scheme 2: Stereochemical pathways of enzymatic epoxide hydrolysis.



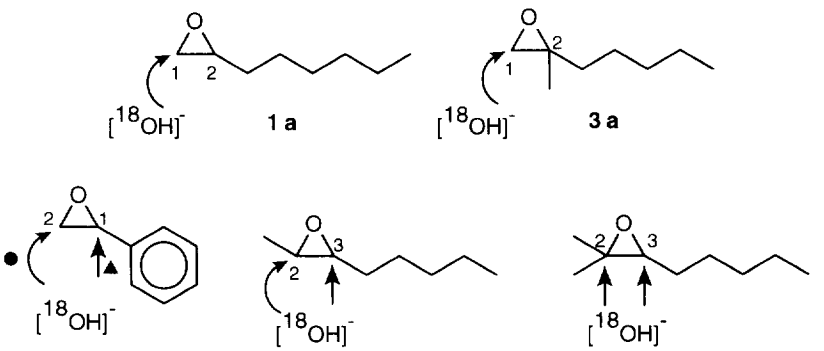
The above mentioned facts cause a general problem associated with the description of the selectivity of the reaction. The selectivity of biocatalytic kinetic resolutions of enantiomers is most conveniently described by the (dimensionless) Enantiomeric Ratio (E)[20-22]. The latter remains constant throughout the reaction and is only determined by the 'environment' of the system, such as the enzyme, the substrate structure, the solvent system, pH and temperature. E-Values have been used most commonly with ester-hydrolysis and -synthesis catalyzed

by lipases, esterases and proteases, because the preconditions - such as (i) irreversibility of reaction, (ii) absence of inhibition phenomena and (iii) the existence of a single stereochemical pathway with respect to the configuration of the stereogenic centre - can be met. It is obvious that E-values can only be applied to epoxide-hydrolase catalyzed reactions, where the regioselectivity of the [OH]-incorporation is absolute. In principle, the calculation of E-values from the enantiomeric excess of substrate and product (e.e.$_S$, e.e.$_P$) and/or conversion (c) is applicable only to reactions displaying a single stereochemical pathway, *i.e. either* retention *or* inversion of configuration, but not to those, where retention *and* inversion occurs during the course of the reaction[23].
The excellent enantiospecificities obtained from our recently published study on the kinetic resolution of epoxides[11] led us to investigate the regioselectivity of the purified epoxide hydrolase from *Rhodococcus* NCIMB 11216[24] in order to determine whether E-values (calculated from e.e.$_S$, e.e.$_P$ and/or c) can be applied for the description of the selectivity[23].

Results and Discussion

The regioselectivity of enzymatic epoxide hydrolysis catalyzed by epoxide hydrolases can be accurately determined via ^{18}O-labelling experiments. This may either be performed by employing an ^{18}O-labelled epoxide[17,25,26] or (more commonly) by carrying out the enzymatic reaction in ^{18}O-labelled aqueous media, followed by GC/MS with electron impact (EI) and chemical ionization (CI). According to their molecular or pseudomolecular ions, the diols obtained from epoxides **1a-5a** through enzymatic hydrolysis in $^{18}OH_2$ (95-98% ^{18}O) were *mono*-labelled (>97%). The position of the label can be determined with high accuracy by analysis of the diol fragmentation pattern provided that the diol splits during EI between the carbon atoms bearing the hydroxyl groups.

Scheme 3: Regioselectivity of *Rhodococcus* NCIMB 11216 epoxide hydrolase with substrates **1a-5a**.



Hydrolysis of both aliphatic *mono*- and 2,2-disubstituted epoxides **1a**, **3a** by *Rhodococcus* NCIMB 11216 epoxide hydrolase gave octane-1,2-diol **1b** and 2-methylheptane-1,2-diol **3b**, respectively, both exclusively labelled at C-1 (>97%), identified by using the fragment ion m/z 115 for both compounds. No significant enhancement of m/z 117 (being the corresponding fragment ion of the putative regioisomeric C-2 addition

product) was observed. In other words, nucleophilic attack occurred at the less hindered (unsubstituted) oxirane atom with retention of configuration at the stereogenic centre. These results are in line with observations reported from *Corynebacterium* epoxide hydrolases[27] and mammalian liver microsomes[28-30].

Surprisingly, the ^{18}O-incorporation pattern was different for the monosubstituted substrate bearing a benzylic carbon atom **2a**. In this case, ^{18}O was incorporated at both oxirane carbon atoms at a ratio of C-1/C-2 of 64:36, calculated from the relative intensities of the fragment ions m/z 107 and 109, respectively. In the absence of enzyme, however, a significant spontaneous (non-enzymatic) hydrolysis of substrate **2a** was observed under the conditions used with a regioselectivity of C-1/C-2 ~95:5 in favor of the benzylic position. The formal 'shift' of the regioselectivity observed in the enzymatic experiment shows the enzyme´s preference for the less hindered C-2 rather than C-1. These results are in line with those obtained by hydrolysis of **2a** using epoxide hydrolases from mammalian hepatic microsomes[17] and the fungus *Aspergillus niger*[18].

A similar pattern - *i.e.* incomplete regioselectivity with concomitant ^{18}O-incorporation at both carbon atoms - was observed with the sterically more demanding *trans*-2,3-disubstituted epoxide **4a**. The ratio of the ^{18}O-incorporation, calculated from the relative intensities of the fragment ions m/z 101 and 103, respectively, was C-2/C-3 67:33. The results for the trisubstituted substrate **5a** are similar (C-2/C-3 44:56, fragment ions m/z 59 and 61). A similar low regioselectivity with structurally related 2,3-di- and trisubstituted oxiranes has been observed with the mammalian cytosolic enzyme[17]. In contrast, enzymes from other sources - such as insects[31], mammalian hepatic microsomes[25], and the fungi *Aspergillus niger*[26] and *Helminthosporium sativum*[32] - exhibited high specificities for the sterically less hindered oxirane carbon atom on trisubstituted oxiranes. It is noteworthy that epoxide **4a** was hydrolyzed in a pure *trans*-specific fashion yielding *erythro*-2-methyloctane-2,3-diol **4b**. No trace of the *threo*-isomer was detected[16].

In a recently published study it was suggested[18] that there is good evidence for the existence of two types of epoxide hydrolases acting via different mechanisms, *i.e.* (i) 'pure' S_N by $[OH]^-$ with complete retention of configuration at the stereogenic centre, and (ii) S_N with concomitant aid of general acid-catalysis in catalytic assistance of the dispersal of the electron density on the oxirane oxygen thus destabilizing the epoxide. It is obvious, that both mechanisms may lead to different regioselectivities depending on the substitutional pattern of the substrate. Regioselectivities typical for enzymes following pathway (i) have been reported from *Aspergillus niger*[18,26], *Helminthosporium sativum*[32], *Corynebacterium*[27], insects[31] and from mammalian hepatic microsomes[17,25,28-30]. Pathway (ii) is less common and has been associated with *Beauveria bassiana*[8,18] and mammalian cytosolic epoxide hydrolase[17]. Exact proof for the existence of these two distinctly different epoxide-hydrolase mechanisms will become available once the 3-D structure of these enzymes has been elucidated.

Summary: By means of ^{18}O-labelling experiments we could show that the regioselectivity of *Rhodococus* NCIMB 11216 epoxide hydrolase is absolute for the less substituted oxirane carbon atom when aliphatic *mono*- and 2,2-disubstituted oxiranes are employed as substrates. As a consequence, for these substrates the Enantiomeric Ratio can be used for the description of the selectivity. On the other hand, the regioselectivity was low with 2,3-di- and trisubstituted epoxides, and with those bearing a benzylic oxirane atom. In these cases E-values cannot be calculated from $e.e._S$, $e.e._P$ and/or c.

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Experimental
General: EI (70 eV) or CI (*iso*-butane) GC/MS data were recorded on a Shimadzu Profile mass spectrometer. GC-analyses were performed on a Shimadzu GC 14A using a Hewlett-Packard column (HP-5MS, 30m). Program: Injection port 250°C; 60°C, hold 2 min., 10°/min. to 180°C, 20°/min. to 255°C. ^{13}C-NMR spectra (200 MHz) were recorded on a Jeol Gemini 200 in $CDCl_3$ solution. Chemical shifts are reported in δ (ppm) from TMS as internal standard. Column chromatography was performed on silica gel Merck 60 (40-63μm). ^{18}O-Labeled water (95-98% ^{18}O) was purchased from Promochem.
Synthesis of Substrates and Reference Materials: 1,2-Epoxyoctane (±)-**1a** (Merck), styrene oxide (±)-**2a** (Merck), 1,2-octanediol (±)-**1b** (Aldrich) and 1-phenyl-1,2-ethanediol (±)-**2b** (Aldrich) are commercially available. 2-Methyl-1,2-epoxyheptane (±)-**3a** and 2-methyl-1,2-heptanediol (±)-**3b** were prepared according to ref. 11. *Trans*-2-methyl-3-*n*-pentyloxirane (±)-**4a** was obtained by epoxidation of *trans*-2-octene (3g, 26.7mmol) using *m*-chloroperbenzoic acid (50%, 11.06g, 32mmol) and K_2CO_3 (3.7g, 26.7mmol) in CH_2Cl_2 (50ml) . Due to the high volatility of *trans*-2-octene the flask containing the solvent was cooled to 0°C prior to addition of the alkene. After *m*-CPBA has been added, the the mixture was left at room temperature overnight. The solids were filtered, the organic layer was extracted several times with aqu. $Na_2S_2O_5$ and $NaHCO_3$ and dried (Na_2SO_4). The solvent was removed in vacuo (closed system) and the product was purified by bulb-to-bulb distillation (b.p. 105°C, 30mm) yielding 2.24g (66%) of highly volatile (±)-**4a**. ^{13}C-NMR: δ = 13.9 (CH_3), 17.6 (CH_2), 22.5 (CH_3), 25.6 (CH_2), 31.6 (CH_2), 31.9 (CH_2), 54.5 (oxirane-C), 59.7 (oxirane-C). *Erythro*-2,3-octanediol **4b** was obtained by base catalyzed hydrolysis of (±)-**4a** (100mg, 0.8mmol) in aqu. NaOH (10%)/THF 8:2 at reflux. After 24h the solution was saturated with NaCl and extracted with ethyl acetate. Column chromatography (petrol ether/ethyl acetate 5:1) gave 95mg (81%) of (±)-**4b**. ^{13}C-NMR: δ = 14.0 (CH_3), 16.7 (CH_2), 22.6 (CH_3), 25.7 (CH_2), 31.8 (CH_2), 31.9 (CH_2), 70.5 (C-OH), 75.0 (C-OH). 2,2-Dimethyl-3-pentyloxirane (±)-**5a** was prepared by addition of diphenylsulfonium isopropylid to *n*-hexanal[33,34]: To a mixture of diphenyl sulfide (4.69g, 25.2mmol) and ethyl iodide (19.5g, 125mmol), $AgBF_4$ (4.96g, 25.2mmol) was added portionwise over a period of 30 min. The solution was stirred at room temperature for 12h under light protection. The yellow precipitate (AgI) was removed by filtration and washed with ether, the volatiles were evaporated and the residual oil was recrystallized from 1-butanol yielding ethyl diphenyl sulfonium tetrafluoroborate as white crystals (6.09g, 20.2mmol, mp = 72° C). To a mixture of diisopropyl amine (1.5ml, 20.6mmol) in dimethoxyethane abs. (5ml) and CH_2Cl_2 abs. (0.6ml, 9.9mmol), *n*-butyl lithium (6.1ml, 11mmol) was added at -70°C under N_2. After 15 min a solution of ethyl diphenyl sulfonium tetrafluoroborate (3g, 9.9mmol) in dimethoxyethane abs. (20ml) was added dropwise followed by stirring at -60°C for 30min. Then methyl iodide (1.48g, 10.4mmol) was added and the temperature was maintained for 2h between -50°C and -60°C, while a white precipitate was formed. To this mixture a freshly prepared solution of LDA (10.9mmol, prepared as above) was added slowly and stirring was continued at -70°C for one hour while the colour changed to orange. Finally, *n*-hexanal (0.99g, 9.9mmol) was added and the solution was stirred for 1h at -70°C and 2h at -70°C to -50°C. The reaction was quenched by addition of water (200ml). After phase separation the aqueous layer was extracted several times with petrol ether. The combined organic phases were dried (Na_2SO_4), evaporated and the product was purified by column chromatography (petrol ether/methyl acetate 20:1), followed by bulb-to-bulb distillation (bp 120° C, 30mm) yielding 0.32g (23%) of (±)-**5a**. ^{13}C-NMR: δ = 14.0 (CH_3), 18.7 (CH_2), 22.6 (CH_3), 24.9 (CH_3), 26.2 (CH_2), 28.8 (CH_2), 31.7 (CH_2), 58.1 (oxirane-C), 64.5 (oxirane-C). 2-Methyl-2,3-octanediol (±)-**5b** was obtained by acid catalysed hydrolysis of (±)-**5a** (50mg, 0.35mmol) in THF/water (10:1, 5ml) and one drop of H_2SO_4 (2M), yielding 45mg (80%) of (±)-**5b**. ^{13}C-NMR: δ = 14.1 (CH_3), 22.7 (CH_2), 23.1 (CH_3), 26.5 (CH_2), 26.5 (CH_2), 31.7 (CH_2), 31.9 (CH_2), 73.2 (CH-OH), 78.6 (C-OH).
Protein Purification: The epoxide hydrolase from Rhodococcus NCIMB 11216 was purified to homogeneity according to ref. 24. Enzyme samples from the final purification step in Tris buffer were lyophilized (0.5ml volume, 50mM Tris, pH 7.5) in 1ml vials.

Biocatalytic Transformations: Freeze dried stock samples containing an equal amount of purified epoxide hydrolase (50µg) were redissolved in $^{18}OH_2$ (120µl) in 1ml vials. Substrates were added by means of a GLC-syringe: (±)-**1a**: 2µl (110mM), (±)-**2a**: 1µl (75mM), (±)-**3a**: 2µl (110mM), (±)-**4a**: 1µl (50mM), (±)-**5a**: 1µl (50mM). The vials were shaken for 15h at 30°C. The reaction was stopped by saturation with NaCl and the diols formed were extracted with two portions of ethyl acetate (200µl each). The organic phase containing the ^{18}O-labeled diols were subjected to GC/MS analysis as described above. To determine any ^{18}O-incorporation into (±)-**2a** due to spontaneous (non-enzymatic) hydrolysis, (±)-**2a** (1µl) was incubated in $^{18}OH_2$ buffer solution without enzyme. Non-enzymatic hydrolysis was absent with the other substrates.

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