Stereochemical preference of yeast epoxide hydrolase for the O-*axial* C3 epimers of 1-oxaspiro[2.5]octanes†

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Received 27th June 2007, Accepted 5th July 2007
First published as an Advance Article on the web 21st August 2007
DOI: 10.1039/b709742e

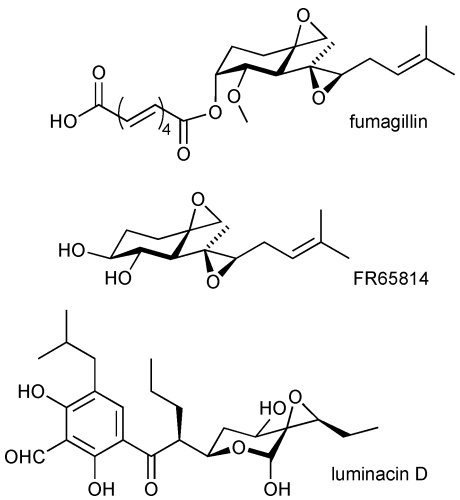
The 1-oxaspiro[2.5]octane moiety is a common motif in many biologically active spiroepoxide compounds. Stereochemistry plays an important role in the action of these spiroepoxides, since the O-*axial* C3 epimers are predominantly responsible for biological activity. In view of this, the reactivity of the yeast epoxide hydrolase (YEH) from *Rhodotorula glutinis* towards both O-*axial* and O-*equatorial* C3 epimers of various 1-oxaspiro[2.5]octanes was investigated. O-*axial* C3 Epimers were hydrolyzed faster than the O-*equatorial* C3 epimers. The stereochemical preference was greatly dependent on the type of substitution on the cyclohexane ring. The preference of YEH for O-*axial* C3 epimers, found throughout this study, illustrates the effectiveness of YEH in enzymatic detoxification of spiroepoxides.

Introduction

Many natural products and synthetic drugs are biologically active through the covalent modification of proteins.[1,2] The biologically active compounds possess reactive groups which are able to selectively label and inactivate specific proteins. A prominent example of such reactive groups is the electrophilic spiroepoxide moiety which is present in various fungal toxins and synthetic derivatives thereof. Spiroepoxides in most cases are highly reactive due to the spiro attachment of the epoxide which makes this group easy accessible and thus sensitive for nucleophilic ring opening.[3] Common spiroepoxide motifs in fungal toxins are the 1-oxaspiro[2.5]octane and corresponding spiro-epoxypyranose functionalities which are present in fumagillin-related toxins and in the group of luminacins, respectively (Scheme 1).[4–6]

Luminacins as well as fumagillin-related toxins show, besides their antibiotic activities, a remarkable inhibition of endothelial cell growth and subsequently selectively block angiogenesis (formation of new blood vessels).[7] Inhibition of tumor-induced angiogenesis has a great therapeutic potential for the treatment of a variety of cancers. The spiroepoxide-containing fungal toxins have therefore inspired synthetic chemists to prepare numerous derivatives of fumagillin.[8]

For the fumagillin molecule, it was found that two stable conformers of the cyclohexane ring may exist. In the dominant form of fumagillin (and related fungal toxins), the cyclohexane ring adopts a conformation in which the position of the spiroepoxide oxygen is (*pseudo*) *axial*. It is also this conformation which is to be necessary for biological activity, since the opposite conformer is by far less active.[4] Similar stereoselectivity had been observed in experiments using a synthetic thioureido derivative of fumagillin, showing only weak anti-angiogenic activity due to an opposite chair conformation of the cyclohexane ring (and *equatorial* position of the spiroepoxide oxygen).[9]



Scheme 1 Spiroepoxide-containing fungal toxins.

The question arises whether Nature's defence mechanism is active against spiroepoxide containing compounds and what role stereochemistry plays in such detoxification reactions. In order to study this, epoxide hydrolase should be the enzyme to focus on. Enzymatic hydrolysis of 1-oxaspiro[2.5]octane compounds has been reported for epoxide hydrolases from *Mycobacterium aurum*, mammalian rabbit liver, fungal strains of *Ulocladium* and *Zopfiella*, and the enzyme from *Rhodotorula glutinis* which was over expressed in recombinant *E. coli*.[10] More recently, we used the yeast epoxide hydrolase (YEH) in wild-type cells of *Rhodotorula glutinis* ATCC 201718 for the kinetic resolution of a range of methyl substituted 1-oxaspiro[2.5]octanes.[11] In that study, we found a preference of the YEH enzyme for *Re* substituted and for O-*axial* spiroepoxide substrates. The observed epimeric preference of YEH could however be influenced by equilibria of C3-epimeric conformers of the conformationally inhomogeneous mono methyl 1-oxaspiro[2.5]octanes which were used as substrates.

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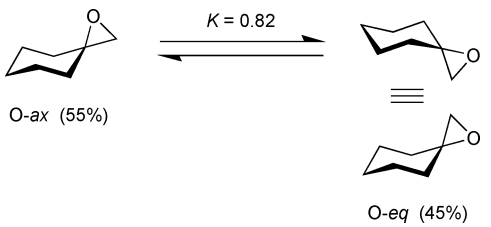
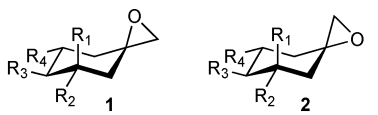
† Electronic supplementary information (ESI) available: NMR spectra of spiroepoxides and bromohydrins, and conditions for GC analysis. See DOI: 10.1039/b709742e

1-Oxaspiro[2.5]octane is a conformationally mobile compound which is rapidly interconverting between two different chair conformations (Scheme 2).[12] A small enthalpy difference of 0.13 kcal mol^{-1} in favor for the O-*axial* epimer has been reported, which corresponds to a 55 : 45 O-*axial*–O-*equatorial* ratio at room temperature.[13]



Scheme 2 Conformational equilibrium of 1-oxaspiro[2.5]octane.[13]

Conformational equilibria can thus influence the stereoselectivities and substrate specificities of epoxide hydrolases in the conversion of C3 epimeric 1-oxaspiro[2.5]octanes. In the present study, we therefore prepared conformationally homogeneous C3 epimers of 1-oxaspiro[2.5]octanes with different substitution patterns, and tested these compounds as substrates for YEH (Scheme 3).



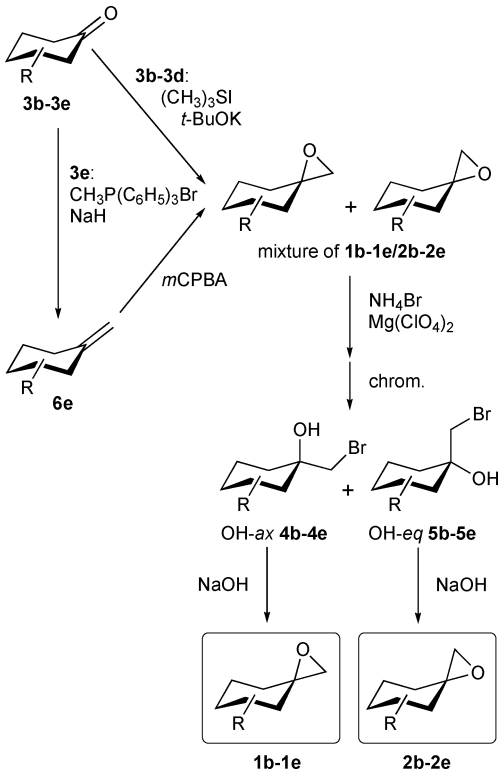
1	2
1a: R_1–R_4 = H	**2a**: R_1–R_4 = H
1b: R_3 = CH_3	**2b**: R_3 = CH_3
R_1, R_2, R_4 = H	R_1, R_2, R_4 = H
1c: R_3 = *t*-Bu	**2c**: R_3 = *t*-Bu
R_1, R_2, R_4 = H	R_1, R_2, R_4 = H
1d: R_2, R_4 = CH_3	**2d**: R_2, R_4 = CH_3
R_1, R_3 = H	R_1, R_3 = H
1e: R_1, R_2, R_4 = CH_3	**2e**: R_1, R_2, R_4 = CH_3
R_3 = H	R_3 = H

Scheme 3 1-Oxaspiro[2.5]octanes used as YEH substrates in this study.

Results and discussion

Synthesis of spiroepoxide epimers

Epimeric mixtures of 1-oxaspiro[2.5]octanes **1**–**2a–d** were prepared by sulfur ylide epoxidation with the dimethyl sulfonium ylide of the corresponding cyclohexanones as described before.[10] Epimers of **1a**–**2a** were indistinguishable under the experimental conditions and therefore the **1a**–**2a** mixture was used as the YEH substrate. For separation of the C3 epimers of substrates **1**–**2b–e**, we increased their difference in polarity *via* conversion to the corresponding β-bromohydrins (Scheme 4). For this, magnesium perchlorate-catalyzed ring opening with ammonium bromide of



Scheme 4 Synthesis of spiroepoxide C3 epimers.

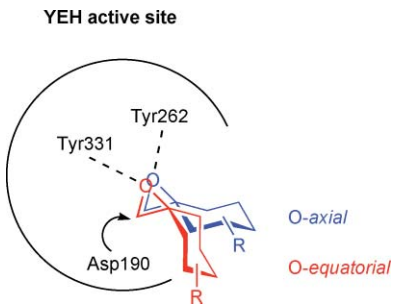
the spiroepoxides was performed.[14] The β-bromohydrins were subsequently purified by column chromatography and isolated as pure epimers. Spiroepoxide epimers were eventually obtained by base-catalyzed ring closure of the individual β-bromohydrins using concentrated NaOH.[15]

Preparation of C3 epimeric mixtures of **1e**–**2e** appeared unsuccessful by sulfur ylide epoxidation using dimethyl sulfonium ylide. Due to increased steric effects, the O-*axial* C3 epimer **1e** was formed exclusively in all experiments.[16] We therefore prepared the mixture **1e**–**2e** in an alternative way, starting from the conversion of 3,3,5-trimethylcyclohexanone to 3,3,5-trimethyl-1-methylenecyclohexane by a Wittig reaction.[17] Subsequent epoxidation by using *m*CPBA gave the corresponding spiroepoxides as an 1 : 2 mixture of **1e**–**2e**. Purification and isolation of the pure C3 epimers **1e** and **2e** took place as described for spiroepoxides **1**–**2b–d** (Scheme 4)

Substrate binding in the YEH active site

The YEH of *Rhodotorula glutinis* has been characterized as an α/β hydrolase fold enzyme, being a member of the microsomal EH superfamily.[18] Substrate docking in the active site of α/β hydrolase fold epoxide hydrolases is known to be initiated by hydrogen bonding with two conserved Tyr residues.[19] Epoxide substrates are thereby positioned and activated for nucleophilic attack by a catalytic Asp residue. A two-step mechanism is generally accepted for this group of α/β hydrolase fold enzymes.[20] For the YEH of *Rhodotorula glutinis*, the catalytic triad has been determined as Asp190 (nucleophile), Glu359 (charge relay acidic residue) and His385 (general base).[21] Proton donation to the epoxide oxygen is performed by Tyr262 and Tyr331, respectively.

Based on this information, orientations of spiroepoxide C3 epimers in the YEH active site are proposed and shown in Scheme 5. In the depicted substrate bindings, the epoxide moieties are similarly oriented for most optimum binding with the two Tyr residues, resulting in a different spatial orientation of the connected cyclohexane rings. Consistent regio- and enantioselectivities of YEH in the hydrolysis of racemic 1-oxaspiro[2.5]octanes have been explained by the proposed binding conformations and were described in a previous study.[11]



Scheme 5 Proposed binding of spiroepoxide C3 epimers in the active site of the YEH enzyme.

Mono-substituted spiroepoxide substrates

Enzymatic hydrolysis of spiroepoxides was studied with YEH-containing cells of wild-type *Rhodotorula glutinis* ATCC 201718. In this way, the membrane associated YEH enzyme was still present in its physiological surroundings for optimum performance. For substrates **1b** and **2b**, a preference for the O-*axial* C3 epimer **1b** had already been observed in the hydrolysis of a 4 : 6 epimeric mixture in a previous study.[11] In the present study, equal initial concentrations of epimers **1b** and **2b** were used in separate reactions, in order to exclude a previously observed sequential reaction profile due to mutual competition of epimers. Determination of the individual initial reaction rates is more accurate in this way. The time courses of the YEH-catalyzed hydrolysis of individual C3 epimers of **1b** and of **2b** are shown in Fig. 1.

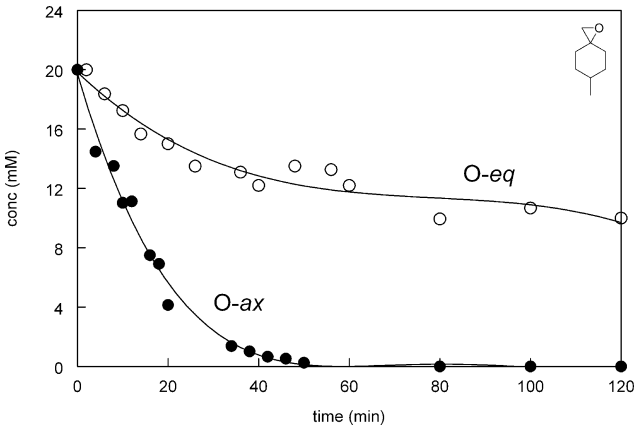
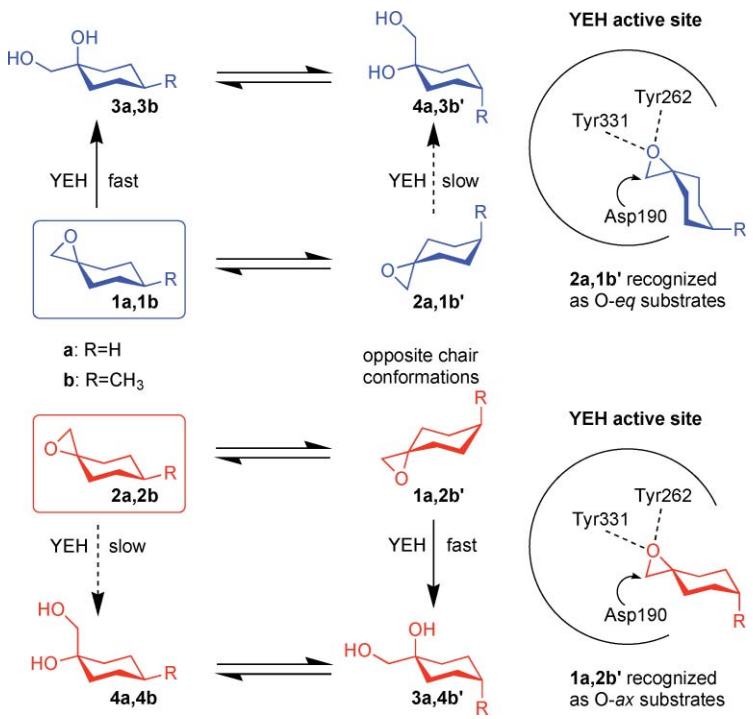


Fig. 1 Time courses of biohydrolysis of 200 μmol O-*axial* **1b** (●) and of 200 μmol O-*equatorial* **2b** (○) by YEH-containing cells of *Rhodotorula glutinis* (103.9 mg dw and 196.7 mg dw, respectively).

A clear, but nevertheless not absolute, preference for O-*axial* substrate **1b** can be concluded from the results shown in Fig. 1. However, mono methyl substituted substrates like **1b** and **2b** are conformationally mobile compounds which will interconvert to their opposite chair conformers (Scheme 6). For equilibria in aqueous solution at room temperature between **1b** and **1b**′ and between **2b** and **2b**′, free energy differences $-\Delta G$ were reported as



Scheme 6 Proposed contribution of conformational equilibria to the enzymatic hydrolysis of spiroepoxides **1a**, **2a**, **1b** and **2b**.

1.84 and 1.55 kcal mol^{-1} respectively.[22] These values correspond with opposite conformer populations of 4% for **1b′** and 7% for **2b′**. In Scheme 6, a possible contribution of conformational equilibria in the YEH-catalyzed hydrolysis of substrates **1b** and **2b** is schematically depicted.

The phenomenon can play a role in the biohydrolysis of the non-preferred O-*equatorial* spiroepoxide **2b**, and will be of less significance in the hydrolysis of **1b**. Enzymatic hydrolysis of **2b** can proceed in a direct way, and/or indirectly *via* its opposite conformer **2b′** which in that case can be recognized by YEH as an O-*axial* substrate. However, if YEH would only be able to hydrolyze exclusively O-*axial* substrates like **1b** and **2b′**, a much lower (overall) reaction rate for **2b** should be observed and consequently a higher stereoselectivity. This is based on the given free energy differences for equilibria of **1b**–**1b′** and **2b**–**2b′**, and expected comparable reaction rates for the structurally corresponding compounds **1b** and **2b′**. Only a possible small contribution by hydrolysis of the opposite chair conformer **2b′** to the overall reaction rate of O-*equatorial* substrate **2b** can thus be concluded from our experiments. Analysis of the formed diols will not assist to unravel to what extend **2b** and **2b′** are converted by YEH, due to the equilibrium between diols **4b** and **4b′**. We therefore continued our study with the biohydrolysis of conformational homogeneous substrates **1c** and **2c**.

Determination of the intrinsic stereochemical preference of YEH for hydrolysis of substrates **1c** and **2c** was not hampered by interconversion of these epimers to their opposite chair conformers, due to the bulky *tert*-butyl substituent present.[23] Interestingly, all experiments demonstrated a nearly absolute preference for the O-*axial* C3 epimer **1c**. Even in cases of raising the amount of biocatalyst and simultaneously lowering the substrate concentration there was almost no conversion of **2c**. Due to increased steric effects of the *tert*-butyl substituent, and blocked interconversion of **2c** to its opposite conformer, YEH appeared not to be able to hydrolyze this O-*equatorial* spiroepoxide in a direct nor indirect way.

YEH-catalyzed hydrolyzes of all individual epimeric substrates were followed in detail by direct headspace analysis and the initial reaction rates could be determined accurately in this way. Initial reaction rates for the biohydrolysis of all substrates tested in this study are summarized in Fig. 2.

From the results shown in Fig. 2 it is clear that increase of ring substitution caused lowering of activities for the substrates tested. The specific activity for the non-substituted compound **1a**–**2a** is high, due to (*i*) smallest steric effects by substrate docking in the tunnel-shaped active site cavity of YEH, and, to a lesser extend, (*ii*) rapid conversion of O-*equatorial* **2a** to the more preferred O-*axial* **1a** (Schemes 2 and 6).

Methyl substitution at C6 causes only a small decrease in specific activity for substrate **1b**. For O-*equatorial* epimer **2b**, the activity is significantly lower but still moderate. Increased steric effects are caused by *tert*-butyl substitution at C6, resulting in a further decrease of specific activity for O-*axial* substrate **1c**, and moreover, an almost absolute preference for this epimer.

Di- and trimethyl-substituted spiroepoxides

Spiroepoxide substrates with different substitution patterns were accordingly prepared and tested. *cis*-3,5-Disubstituted cyclohexane compounds are generally accepted as conformationally homogeneous.[24] For the present study, the conformationally homogeneous epimers **1d** and **2d** of *cis*-5,7-dimethyl-1-oxaspiro[2.5]octane should be the ideal substrates: no interconversion of conformers and no extreme bulky substituents reducing the activity too much. An absolute preference of YEH for O-*axial* **1d** was therefore expected. However, substrates **1d** as well as **2d** were found to be hydrolyzed by the enzyme (Fig. 3).

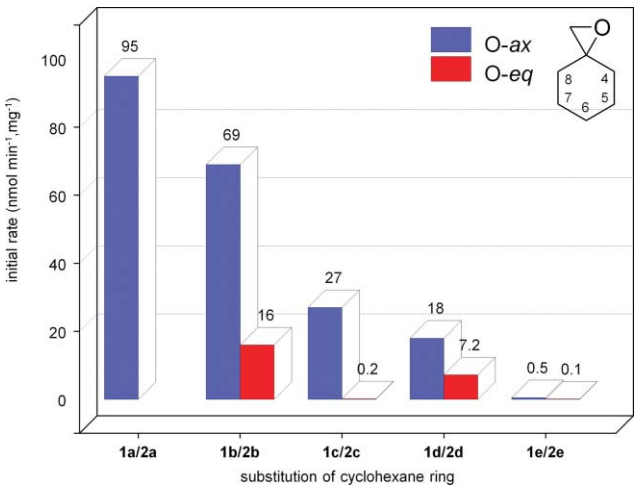


Fig. 2 Effect of ring substitution on the activity of YEH-containing cells of *Rhodotorula glutinis* for the individual C3 epimers of 1-oxaspiro[2.5]octanes.

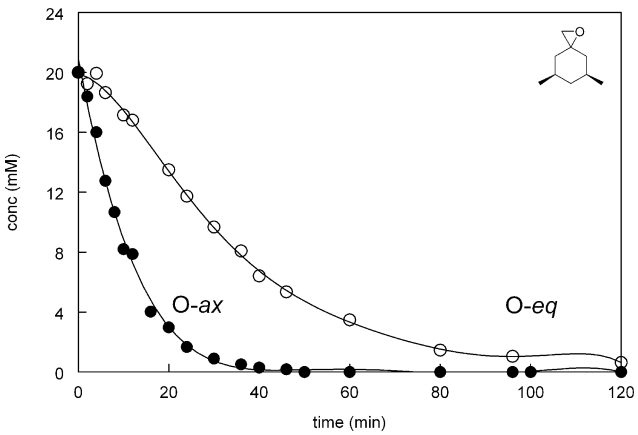


Fig. 3 Time courses of hydrolysis of 200 μmol O-*axial* **1d** (●) and of 200 μmol O-*equatorial* **2d** (○) by YEH-containing cells of *Rhodotorula glutinis* (602.8 mg dw and 583.2 mg dw, respectively).

Although the O-*axial* C3 epimer **1d** was still hydrolyzed much more rapidly, there was only moderate stereoselectivity observed in this case. Apparently, substrates **1d** and **2d** can be accommodated in the YEH active site with less difference in spatial requirements.

The influence of *axial* substituents on the cyclohexane ring was studied by YEH-catalyzed hydrolysis of spiroepoxides **1e** and **2e**. Each single C3 epimer of **1e**–**2e** can be regarded as a conformationally homogeneous compound but still consisting of a pair of enantiomers. Stereochemical preferences of YEH will therefore include enantiomeric discrimination as well. For substrate **1e**, a preference for the (3*S*,7*R*)-**1e** enantiomer had already been described in a previous study.[11] Time courses of

the presently investigated enzymatic hydrolysis reactions of the individual C3 epimers **1e** and **2e** are presented in Fig. 4.

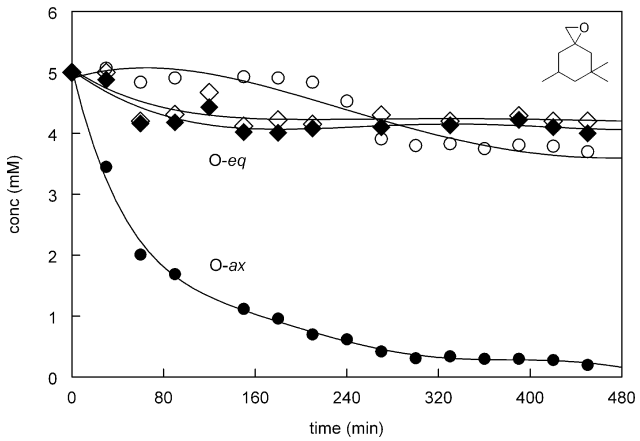


Fig. 4 Time courses of hydrolysis of 50 µmol O-*axial* **1e** and of 50 µmol O-*equatorial* **2e** by YEH-containing cells of *Rhodotorula glutinis* (550.7 mg dw and 561.6 mg dw, respectively). The four enantiomers are represented as: (3*S*,7*R*)-**1e** (●), (3*R*,7*S*)-**1e** (○), (3*R*,7*R*)-**2e** (♦) and (3*S*,7*S*)-**2e** (◇).

A high preference for the O-*axial* substrate **1e** can be concluded from results presented in Fig. 4. Moreover, an enantioselective hydrolysis in favor of the (3*S*,7*R*)-**1e** enantiomer is shown. When compared to the activity for substrate **1d**, we found a much lower activity for **1e**, caused by the *axial* methyl substituent on the cyclohexane ring (Scheme 7). The steric effect apparently is so considerable that it even generated a change in the general enantioselectivity as observed for other spiroepoxide substrates.[11] No suitable accommodation of O-*equatorial* **2e** seemed possible due to hindrance of the *axial* methyl substituent.

Comparison of the spatial orientations of the tested substrates is shown in Scheme 7. For catalytic activity, the orientation and substitution pattern of O-*axial* substrate **1b** can be regarded as being most optimal. Deviations in either substrate substitution or orientation will cause a decrease in catalytic activity by YEH. Spiroepoxides **2c** and **2e** were not hydrolyzed by the enzyme, so apparently their spatial requirements exceed the tunnel-shaped substrate binding area. Also the acceptance of both the *cis*-5,7-disubstituted C3 epimers **1d** and **2d** but nevertheless clear preference for **1d**, can accordingly be understood due to their relatively smaller 'steric volume' than **2c** and **2e**.

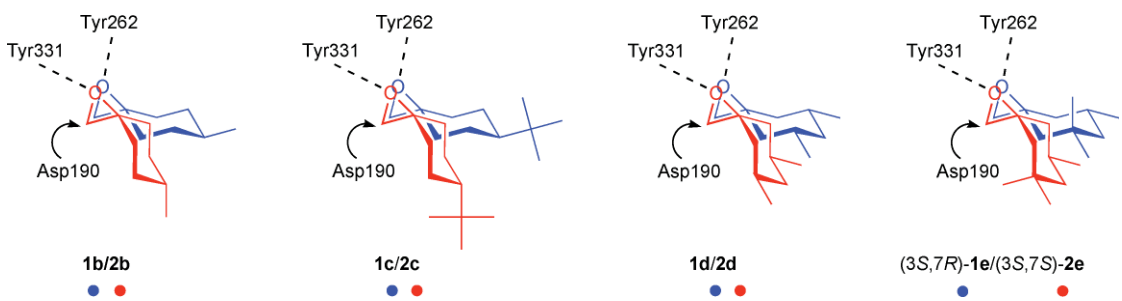
Conclusions

Most epoxide hydrolases belong to the α/β hydrolase fold family of enzymes and are ubiquitous in Nature. For studying spiroepoxide hydrolysis, the yeast epoxide hydrolase (YEH) is an appropriate representative of the α/β hydrolase fold epoxide hydrolases. We used YEH-containing cells of *Rhodotorula glutinis* ATCC 201718 to investigate the degree of stereochemical recognition in the hydrolysis of spiroepoxide substrates.

Throughout this study, we found a consistent preference for O-*axial* spiroepoxides by YEH. We described the proposed orientations of spiroepoxides in the YEH active site and took into account that O-*equatorial* C3 epimers of conformationally mobile spiroepoxides could possibly be hydrolyzed *via* their O-*axial* opposite chair conformers. For spiroepoxide substrate 6-methyl 1-oxaspiro[2.5]octane, we looked at this phenomenon in more detail. We found a still moderate activity for the O-*equatorial* C3 epimer which was predominantly caused by the moderate stereoselectivity of the enzyme and to a much lesser extend by hydrolysis of the O-*axial* opposite chair conformer. This makes contribution of the latter phenomenon to the hydrolysis of conformationally mobile substrates quite unlikely.

Conformationally homogeneous C3 epimers were prepared and used as substrates to determine the intrinsic stereoselectivity of the YEH enzyme. Substrate binding after possible interconversion of conformers will be excluded in this way. We found an almost absolute preference for the O-*axial* C3 epimers of *tert*-butyl and trimethyl substituted spiroepoxides. High, but no absolute stereoselectivity was observed in the biohydrolysis of *cis*-5,7-dimethyl substituted substrates. The latter substitution pattern apparently caused a less distinctive orientation of C3 epimers in the YEH active site.

Nature has developed the constitutively present epoxide hydrolases for enzymatic detoxification. Based on our experiments, it is likely that the natural substrate for YEH is an O-*axial* spiroepoxide, possibly a fungal toxin of the fumagillin type. Since the mammalian microsomal epoxide hydrolase shows a very close sequence similarity to YEH,[21,25] the present study supplies useful information for further development of drug design for the treatment of tumor-induced angiogenesis based on the



Scheme 7 Proposed accommodations for catalytic activity of substituted spiroepoxides in the YEH active site. Steric differences caused by substrate docking of the spiroepoxide C3 epimers are shown in comparison to the most optimal spatial orientation of O-*axial* substrate **1b**. For spiroepoxides **1e** and **2e**, only the accepted enantiomer (3*S*,7*R*)-**1e** and the correspondingly substituted (in proposed binding conformation) C3 epimer (3*S*,7*S*)-**2e** are shown.

1-oxaspiro[2.5]octane scaffold, and subsequent metabolic studies of these compounds.

Experimental

General experimental procedures

Analyses of the epimers of all used spiroepoxides and the enantiomers of **1e** and **2e**, ketones, bromohydrins and diols were performed by gas chromatography (GC) on a β-DEX 120 fused silica cyclodextrin capillary column, with either H_2 or N_2 as carrier gas. Concentrations of spiroepoxides and diols were derived from calibration curves in the presence of heat-killed cells of *Rhodotorula glutinis* in order to correct for possible adsorption of compounds to the cells and any spontaneous hydrolysis.

Molecular formula assignment was performed by HRMS analysis. Combustion elemental analysis appeared not to be appropriate for molecular formula assignment of the volatile spiroepoxides used in this study. HRMS data were consistent and in agreement with values calculated for the expected structures.

All reagents were obtained from commercial sources and used without further purification. The yeast *Rhodotorula glutinis* ATCC 201718 was originally obtained from the culture collection of the Dept. of Industrial Microbiology WU, and previously referred to as *Rhodotorula glutinis* CIMW 147.

1-Oxaspiro[2.5]octane (1a–2a)

Spiroepoxide substrate **1a**–**2a** was prepared as follows. To a clear yellow solution of trimethylsulfonium iodide (16.6 g, 81 mmol) in 150 mL dry DMSO was added 5.9 g (50 mmol) cyclohexanone with stirring. The mixture was brought under N_2 atmosphere and a solution of potassium *tert*-butoxide (8.5 g, 75 mmol) in 100 mL dry DMSO was slowly added. The resulting solution was stirred at room temperature for 16 hours under N_2. The reaction was quenched by addition of water (300 mL), and extracted with diethyl ether (3 × 100 mL). Combined organic layers were washed with water (100 mL), dried over Na_2SO_4, and concentrated under reduced pressure.

1-Oxaspiro[2.5]octane was obtained in an indistinguishable mixture of **1a**–**2a** (4.5 g, 89%) as a colorless liquid. δ_H (300 MHz, $CDCl_3$) 1.44–1.68 (8 H, m), 1.66–1.68 (2 H, m), 2.52 (2 H, s); δ_C (75 MHz, $CDCl_3$) 24.8 (2 × CH_2), 25.2 (CH_2), 33.6 (2 × CH_2), 54.4 (CH_2), 58.9 (C); *m/z* (EI) 112.0882 (M^+, 46%, $C_7H_{12}O$ requires 112.0888), 41 (52%), 54 (64), 67 (88), 84 (100), 97 (59), 111 (73).

O-*ax* 6-Methyl-1-oxaspiro[2.5]octane (1b). General synthetic procedure for stereochemically pure spiroepoxide substrates

Spiroepoxide substrates **1b** and **2b** were prepared *via* base-catalyzed ring closure of their corresponding β-bromohydrins, obtained by conversion of a synthesized spiroepoxide mixture **1b**–**2b** and subsequent purification by column chromatography.

For synthesis of spiroepoxide mixture **1b**–**2b**, trimethylsulfonium iodide (31.0 g, 151 mmol) was dissolved in 150 mL dry DMSO and subsequently 10.1 g (90 mmol) 4-methylcyclohexanone was added with stirring at room temperature. The mixture was brought under N_2 atmosphere and a solution of potassium *tert*-butoxide (15.8 g, 158 mmol) in 100 mL dry DMSO was slowly added. The resulting solution was stirred at room temperature for 16 hours under N_2. The reaction was quenched by addition of water (300 mL), and extracted with diethyl ether (3 × 100 mL). Combined organic layers were washed with water (100 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The resulting spiroepoxides were obtained in 90% yield as a colorless liquid (10.2 g) in an epimeric mixture of **1b** (40%)–**2b** (60%).

For conversion to their corresponding β-bromohydrins, 10.2 g (81 mmol) spiroepoxide mixture **1b**–**2b** was dissolved in 75 mL acetonitrile. To this solution, 26.8 g (120 mmol) $Mg(ClO_4)_2$ and 11.7 g (120 mmol) NH_4Br were added. The mixture was stirred at 65 °C under reflux for 28 hours. After cooling to room temperature, the reaction was quenched by addition of 75 mL water and the product extracted with diethyl ether (3 × 100 mL). Combined organic layers were washed with water (100 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The β-bromohydrins of 1-bromomethyl-4-methyl-1-cyclohexanol were obtained in 78% yield as a yellow oil (8.0 g) in a mixture of OH-*ax* **4b** (40%)–OH-*eq* **5b** (60%).

Purification of the individual β-bromohydrin epimers was by careful chromatography on silica gel with petroleum ether (bp 40–60 °C)–diethyl ether (2 : 1) pooling only the very pure fractions, followed by concentration under reduced pressure. In this way, stereochemically pure OH-ax **4b** was isolated as a yellow oil: de >98%; yield 19% (1.5 g). δ_H (300 MHz, $CDCl_3$) 0.91 (3 H, d), 1.27–1.38 (5 H, m), 1.53 (2 H, d), 1.77 (2 H, d), 1.91 (1 H, s, broad), 3.41 (2 H, s); δ_C (75 MHz, $CDCl_3$) 21.9 (CH_3), 30.0 (2 × CH_2), 32.2 (CH), 35.2 (2 × CH_2), 47.4 (CH_2), 69.2 (C).

For subsequent conversion of the β-bromohydrin towards the corresponding spiroepoxide, 1.5 g (7.0 mmol) OH-*ax* **4b** was dissolved in 50 mL of a water–isopropanol (2 : 3) mixture. The solution was stirred at room temperature and 1.5 mL 0.1 M NaOH solution (1 eq) was added dropwise. After stirring for 1 hour, the formed spiroepoxide was extracted with pentane (3 × 50 mL). The combined organic layers were washed with water (3 × 50 mL), dried over Na_2SO_4 and concentrated under reduced pressure.

Spiroepoxide O-*ax* **1b** was obtained as a colorless liquid: de >98%; overall yield 8% (0.8 g) from the starting ketone. δ_H (300 MHz, $CDCl_3$) 0.88 (3 H, d), 1.16–1.31 (4 H, m), 1.39 (1 H, m), 1.60–1.63 (2 H, m), 1.74–1.83 (2 H, m), 2.55 (2 H, s); δ_C (75 MHz, $CDCl_3$) 21.9 (CH_3), 31.2 (CH), 32.2 (2 × CH_2), 32.5 (2 × CH_2), 53.7 (CH_2), 57.9 (C); *m/z* (EI) 126.1043 (M^+, 3%, $C_8H_{14}O$ requires 126.1045), 43 (11%), 54 (11), 55 (16), 67 (6), 81 (15), 84 (100), 97 (4), 111 (21).

O-*eq* 6-Methyl-1-oxaspiro[2.5]octane (2b)

Spiroepoxide substrate **2b** was prepared from its corresponding β-bromohydrin OH-*eq* **5b**, which was isolated by the procedure as described for OH-*ax* **4b**. Stereochemically pure OH-*eq* **5b** was accordingly isolated as a yellow oil: de >98%; yield 16% (1.3 g). δ_H (200 MHz, $CDCl_3$) 0.89 (3 H, d), 1.44–1.56 (3 H, m), 1.62–1.72 (4 H, m), 1.81–1.89 (2 H, m), 2.19 (1 H, s, broad), 3.59 (2 H, s); δ_C (50 MHz, $CDCl_3$) 21.0 (CH_3), 31.2 (2 × CH_2), 31.3 (CH), 35.3 (2 × CH_2), 43.9 (CH_2), 70.3 (C).

For conversion of the β-bromohydrin towards the corresponding spiroepoxide, 1.3 g (6.4 mmol) OH-*eq* **5b** was submitted to the base-catalyzed ring closure reaction as described.

Spiroepoxide O-*eq* **2b** was accordingly obtained as a colorless liquid: de >98%; overall yield 7% (0.8 g). δ_H (300 MHz, $CDCl_3$) 0.88 (3 H, d), 1.03–1.16 (2 H, m), 1.20–1.25 (2 H, m), 1.34–1.50 (1 H, m), 1.73–1.82 (4 H, m), 2.50 (2 H, s); δ_C (75 MHz, $CDCl_3$) 21.5 (CH_3), 31.4 (CH), 33.1 (2 × CH_2), 33.8 (2 × CH_2), 54.7 (CH_2), 59.3 (C); *m/z* (EI) 126.1044 (M^+, 4%, $C_8H_{14}O$ requires 126.1045), 43 (14%), 54 (16), 55 (21), 81 (20), 84 (100), 97 (7), 111 (26).

O-*ax* 6-*tert*-Butyl-1-oxaspiro[2.5]octane (1c)

Substrate **1c** was prepared according to the procedure described for compound **1b**. The starting spiroepoxides **1c**–**2c** were synthesized by epoxidation of 4-*tert*-butylcyclohexanone (13.9 g, 90 mmol) using trimethylsulfonium iodide (31.0 g) and obtained in 87% yield as a colorless liquid (13.5 g), in an epimeric mixture of **1c** (38%)–**2c** (62%).

From mixture **1c**–**2c** (13.5 g, 80 mmol), the corresponding β-bromohydrins were obtained in 89% yield as a yellow oil, as a mixture of OH-*ax* **4c** (42%)–OH-*eq* **5c** (58%). Purification by chromatography on silica gel with petroleum ether (bp 40–60 °C)–diethyl ether (2 : 1) resulted in stereochemically pure OH-*ax* **4c** as a yellow oil: de = 97%; yield 13% (1.8 g). δ_H (300 MHz, $CDCl_3$) 0.88 (9 H, s), 0.92–0.98 (1 H, m), 1.26–1.44 (4 H, m), 1.62–1.65 (2 H, m), 1.83–1.86 (2 H, m), 3.42 (2 H, s, broad); δ_C (75 MHz, $CDCl_3$) 22.4 (2 × CH_2), 27.5 (3 × CH_3), 32.3 (C), 35.8 (2 × CH_2), 47.5 (CH_2), 47.7 (CH), 69.2 (C).

Spiroepoxide O-*ax* **1c** was eventually formed by ring closure of OH-*ax* **4c** and obtained as a colorless liquid: de = 97%; overall yield 10% (1.1 g). δ_H (300 MHz, $CDCl_3$) 0.87 (9 H, s), 0.94–1.08 (1 H, m), 1.09–1.41 (4 H, m), 1.75–1.89 (4 H, m), 2.61 (2 H, s); δ_C (75 MHz, $CDCl_3$) 25.2 (2 × CH_2), 28.0 (3 × CH_3), 32.9 (C), 33.8 (2 × CH_2), 47.6 (CH), 54.2 (CH_2), 58.7 (C); *m/z* (EI) 168.1516 (M^+, 4%, $C_{11}H_{20}O$ requires 168.1514), 41 (30%), 57 (86), 79 (24), 81 (26), 84 (100), 95 (13), 111 (40), 112 (23), 153 (56).

O-*eq* 6-*tert*-Butyl-1-oxaspiro[2.5]octane (2c)

Substrate **2c** was accordingly prepared by ring closure from its corresponding β-bromohydrin OH-*eq* **5c**. For this, stereochemically pure OH-*eq* **5c** has been isolated as a yellow oil: de >98%; yield 9% (1.2 g). δ_H (300 MHz, $CDCl_3$) 0.86 (9 H, s), 0.93–1.10 (2 H, m), 1.20–1.30 (1 H, m), 1.54–1.61 (2 H, m), 1.73–1.76 (2 H, m), 2.00 (2 H, d), 2.27 (1 H, s, broad), 3.64 (2 H, s, broad); δ_C (75 MHz, $CDCl_3$) 24.3 (2 × CH_2), 27.5 (3 × CH_3), 32.1 (C), 36.6 (2 × CH_2), 43.3 (CH_2), 47.3 (CH), 70.4 (C).

Spiroepoxide **2c** was obtained as a colorless liquid: de >98%; overall yield 7% (0.5 g). δ_H (300 MHz, $CDCl_3$) 0.87 (9 H, s), 1.02–1.33 (5 H, m), 1.81–1.91 (4 H, m), 2.57 (2 H, s); δ_C (75 MHz, $CDCl_3$) 26.6 (2 × CH_2), 27.6 (3 × CH_3), 32.3 (C), 33.9 (2 × CH_2), 47.2 (CH), 55.0 (CH_2), 60.0 (C); *m/z* (EI) 168.1516 (M^+, 2%, $C_{11}H_{20}O$ requires 168.1514), 41 (36%), 57 (100), 79 (21), 81 (21), 84 (92), 95 (13), 111 (37), 112 (18), 153 (45).

cis-3,5-Dimethylcyclohexanone (3d)

Ketone **3d** was used as the starting compound for the synthesis of substrates **1d** and **2d**. Ketone **3d** was prepared by stereoselective hydrogenation of 3,5-dimethylcyclohex-2-enone.[26] From preliminary experiments, the sterically hindered isopropanol was selected as the appropriate solvent for stereoselective hydrogenation, since the use of methanol and ethanol resulted in undesired byproducts.

For stereoselective hydrogenation, 5.0 g (40 mmol) 3,5-dimethylcyclohex-2-enone was dissolved in 50 mL isopropanol and 0.3 g Pd/C 5% was added. Hydrogenation was conducted at 2 atm for 1 hour at room temperature using a Parr apparatus.

The reaction mixture was filtered through Celite and the filtrate diluted with 50 mL water. The ketone was extracted with pentane (3 × 50 mL). The combined organic layers were washed with water (3× 50 mL), dried over Na_2SO_4 and concentrated under reduced pressure.

Ketone **3d** was obtained as a yellow oil: de >99%; yield 93% (4.7 g), containing trace amounts of solvent. δ_H (300 MHz, $CDCl_3$) 0.94 (6 H, d), 1.75–1.89 (4 H, m), 2.24 (4H, d); δ_C (75 MHz, $CDCl_3$) 22.1 (2 × CH_3), 33.0 (2 × CH), 42.5 (CH_2), 49.1 (2 × CH_2), 211.0 (C). The reaction was repeated several times to obtain sufficient amounts of starting ketone.

O-*ax* 5,7-Dimethyl-1-oxaspiro[2.5]octane (1d)

Substrate **1d** was prepared according to the procedure described for compound **1b**. The starting spiroepoxides **1d**–**2d** were synthesized by epoxidation of the prepared *cis*-3,5-dimethylcyclohexanone **3d** (19.0 g, 151 mmol) using trimethylsulfonium iodide (55.2 g). Spiroepoxides **1d**–**2d** were obtained in 90% yield as a colorless liquid (19.0 g), as an epimeric mixture of **1d** (67%)–**2d** (33%).

From mixture **1d**–**2d** (19.0 g, 140 mmol), the corresponding β-bromohydrins were obtained in 93% yield as a yellow oil, as a mixture of OH-*ax* **4d** (70%)–OH-*eq* **5d** (30%). Purification by chromatography on silica gel with petroleum ether (bp 40–60 °C)–diethyl ether (2 : 1) resulted in stereochemically pure OH-*ax* **4d** as a yellow oil: de = 93%; yield 15% (2.9 g). δ_H (300 MHz, $CDCl_3$) 0.44 (2 H, q), 0.84 (6 H, d), 1.57–1.83 (6 H, m), 1.87 (1 H, s, broad), 3.34 (2 H, s); δ_C (75 MHz, $CDCl_3$) 22.1 (2 × CH_3), 27.8 (2 × CH), 43.4 (CH_2), 43.5 (2 × CH_2), 47.3 (CH_2), 70.9 (C).

Spiroepoxide O-*ax* **1d** was formed by ring closure of OH-*ax* **4d** and obtained as a colorless liquid: de = 93%; overall yield 10% (0.6 g). δ_H (300 MHz, $CDCl_3$) 0.54 (2 H, q), 0.85 (6 H, d), 1.14 (2 H, t, broad), 1.37 (2 H, t), 1.64–1.79 (2 H, m), 2.54 (2 H, s); δ_C (75 MHz, $CDCl_3$) 22.2 (2 × CH_3), 30.1 (2 × CH), 41.0 (2 ×CH_2), 43.0 (CH_2), 53.5 (CH_2), 58.6 (C); *m/z* (EI) 139.1123 (M^+, 2%, $C_9H_{15}O$ requires 139.1127), 41 (10%), 55 (13), 67 (10), 68 (8), 69 (10), 95 (13), 98 (11), 125 (100), 139 (4).

O-*eq* 5,7-Dimethyl-1-oxaspiro[2.5]octane (2d)

Substrate **2d** was accordingly prepared by ring closure from its corresponding β-bromohydrin OH-*eq* **5d**. For this, stereochemically pure OH-*eq* **5d** has been isolated as a yellow oil: de = 90%; yield 12% (2.3 g). δ_H (300 MHz, $CDCl_3$) 0.48 (1 H, q), 0.86 (6 H, d), 1.07 (1 H, t), 1.28–1.41 (2 H, m), 1.58–1.63 (2 H, m), 1.84 (2 H, d, broad), 2.11 (1 H, s, broad), 3.55 (2 H, s); δ_C (75 MHz, $CDCl_3$) 22.2 (2 × CH_3), 29.7 (2 × CH), 43.3 (CH_2), 44.3 (CH_2), 44.6 (2 × CH_2), 71.3 (C).

Spiroepoxide **2d** was obtained as a colorless liquid: de = 90%; overall yield 12% (0.4 g). δ_H (300 MHz, $CDCl_3$) 0.58 (2 H, q), 0.88 (6 H, d), 1.14–1.19 (2 H, m), 1.38 (2 H, t), 1.50–1.64 (2 H, m), 2.51 (2 H, s); δ_C (75 MHz, $CDCl_3$) 22.2 (2 × CH_3), 31.8 (2 × CH), 41.9

(2 × CH_2), 43.0 (CH_2), 55.0 (CH_2), 59.2 (C); *m/z* (EI) 139.1125 ($M^+ - 1$, 4%, $C_9H_{15}O$ requires 139.1127), 41 (13%), 55 (14), 67 (11), 68 (10), 69 (10), 83 (10), 95 (15), 98 (13), 125 (100).

O-*ax* 5,5,7-Trimethyl-1-oxaspiro[2.5]octane (1e)

For this, 4.0 g (100 mmol) of a 60% suspension of sodium hydride in mineral oil was added to 200 mL dry DMSO, under N_2 atmosphere. The mixture was heated to 65 °C for 2 h and subsequently cooled to room temperature. To this solution, 36.5 g (100 mmol) methyl-triphenylphosphonium bromide was added stepwise and stirred for 1 h at room temperature. The starting ketone, 10.0 g (70 mmol) 3,3,5-trimethylcyclohexanone **3e**, was added dropwise and the reaction was performed at 50 °C for 18 h. The reaction was quenched by addition of 250 mL water, resulting in the formation of a white precipitate which was filtered off. The filtrate was extracted with pentane (3 × 200 mL). The combined pentane layers were washed with water (3 × 200 mL), dried over Na_2SO_4 and concentrated under reduced pressure. 3,3,5-Trimethyl-1-methylenecyclohexane **6e** was obtained in 64% yield as a colorless liquid (6.2 g). δ_H (300 MHz, $CDCl_3$) 0.84 (6 H, s), 0.89 (3 H, d), 1.25 (2 H, d), 1.52–1.56 (2 H, m), 2.15–2.19 (2 H, m), 4.49 (1 H, s), 4.58 (1 H, s); δ_C (75 MHz, $CDCl_3$) 22.3 (CH_3), 22.6 (CH_3), 25.0 (CH_3), 25.0 (CH), 29.7 (CH_2), 32.6 (C), 43.5 (CH_2), 48.3 (CH_2), 108.1 (CH_2), 147.7 (C). Synthesis of alkene **6e** was performed in duplicate and the products combined.

For subsequent epoxidation, 22.6 g (75 mmol) *m*CPBA was added stepwise to a 0.3 M solution of $NaHCO_3$ in water at 0 °C. The mixture was stirred vigorously, 9.7 g (70 mmol) of alkene **6e** was added stepwise and stirring continued for 3 h, while slowly warming up to room temperature. Formed epoxides were extracted with diethyl ether (3 × 200 mL). The combined organic layers were washed with 10% NaOH solution (3 × 200 mL), saturated NaCl solution (200 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The resulting spiroepoxides were obtained in 68% yield as a light yellow liquid (7.3 g) in an epimeric mixture of **1e** (34%)–**2e** (66%).

The corresponding β-bromohydrins were prepared as described and obtained in 22% yield as a yellow oil (2.5 g) in a mixture of OH-*ax* **4e** (75%)–OH-*eq* **5e** (25%). The epimeric ratio has changed significantly, due to the favored formation of the energetically more stable OH-*ax* β-bromohydrin. Purification by chromatography on silica gel with petroleum ether (bp 40–60 °C)–diethyl ether (2 : 1) resulted in stereochemically pure OH-*ax* **4e** as a yellow oil: de >98%; yield 16% (0.4 g). δ_H (300 MHz, $CDCl_3$) 0.72–1.00 (2 H, m), 0.91 (3 H, d), 0.92 (3 H, s), 1.11 (3 H, s), 1.12 (1 H, d), 1.38–1.46 (1 H, m), 1.56 (1 H, dt), 1.73–1.86 (2 H, m), 1.92–2.08 (1 H, m), 3.39 (2 H, s); δ_C (75 MHz, $CDCl_3$) 22.2 (CH_3), 24.4 (CH_3), 27.0 (CH_3), 31.5 (C), 34.1 (CH), 44.1 (CH_2), 46.7 (CH_2), 48.3 (CH_2), 48.6 (CH_2), 71.6 (C).

Spiroepoxide O-*ax* **1e** was formed by ring closure and obtained as a colorless liquid: de >98%; overall yield 1% (0.12 g) from the starting ketone. δ_H (300 MHz, $CDCl_3$) 0.84 (3 H, d), 0.86 (3 H, s), 0.92 (1 H, dt), 0.98 (3 H, s), 1.11–1.14 (1 H, m), 1.17 (1 H, d), 1.32 (1 H, d), 1.37–1.43 (1 H, m), 1.61 (1 H, d), 1.84–1.95 (1 H, m), 2.42 (2 H, dd); δ_C (75 MHz, $CDCl_3$) 22.3 (CH_3), 26.1 (2 × CH_3), 32.3 (C), 32.9 (CH), 41.3 (CH_2), 44.7 (CH_2), 47.7 (CH_2), 51.1 (CH_2), 57.6 (C); *m/z* (EI) 154.1358 (M^+, 1%, $C_{10}H_{18}O$ requires 154.1358), 41 (14%), 55 (13), 67 (11), 69 (9), 83 (13), 98 (6), 109 (7), 139 (100), 153 (1).

O-*eq* 5,5,7-Trimethyl-1-oxaspiro[2.5]octane (2e)

Substrate **2e** was prepared by ring closure from its corresponding β-bromohydrin OH-*eq* **5e**. For this, stereochemically pure OH-*eq* **5e** has been isolated as a yellow oil: de >98%; yield 8% (0.2 g).

Spiroepoxide **2e** was obtained as a colorless liquid: de >98%; overall yield <1% (0.09 g) from starting ketone. The compound is not pure, which hampers the interpretation of the ^1H NMR spectrum. Attempts for further purification failed because of the small quantity and the similar polarity of the impurities. δ_C (75 MHz, $CDCl_3$) 22.3 (CH_3), 25.8 (CH_3), 28.1 (CH_3), 32.6 (CH), 33.3 (C), 42.1 (CH_2), 46.0 (CH_2), 47.7 (CH_2), 55.8 (CH_2), 57.8 (C); *m/z* (EI) 154.1351 (M^+, 1%, $C_{10}H_{18}O$ requires 154.1358), 41 (13%), 55 (13), 67 (11), 69 (10), 83 (13), 98 (6), 109 (6), 139 (100), 153 (1).

General procedure for biohydrolysis by YEH-containing *Rhodotorula glutinis* cells

Cells of *Rhodotorula glutinis* ATCC 201718 were cultivated for 48 h in a mineral medium supplemented with 2% glucose and 0.2% yeast extract, at 30 °C in a shaking incubator. The yeast cells were harvested by centrifugation at 10000*g*, washed with 50 mM potassium phosphate buffer pH 8.0, concentrated, and stored at −20 °C for future experiments.

Hydrolysis of spiroepoxides was routinely performed in 100 mL screw-capped bottles sealed with rubber septa. The bottles contained cells of *Rhodotorula glutinis* (0.1–1.0 g dw) and 50 mM potassium phosphate buffer pH 8.0 to a total volume of 10 mL. The bottles were placed into a shaking water bath at 35 °C and the reaction was started by addition of 0.20 mmol of the appropriate (neat) substrate. The course of the reaction was followed by monitoring headspace samples with enantioselective GC. Initial reaction rates were determined from substrate disappearance, from data obtained by duplicate experiments.

Subsequently, yeast cells were removed from the reaction mixture by centrifugation. Residual spiroepoxides and formed diols were isolated from the supernatants by selective extraction with pentane and ethyl acetate, respectively.

Acknowledgements

The authors are indebted to Dr M. A. Posthumus for performing mass spectral analyses, to Mr B. van Lagen for recording the NMR spectra and to Mr E. J. C. van der Klift for support in the use of other analytical techniques. We also thank Ms K. Span and Ms C. Grotenhuis for skilful technical assistance.

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