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Stereoselectivity and Substrate Specificity in the Kinetic Resolution of Methyl-Substituted 1-Oxaspiro[2.5]octanes by *Rhodotorula glutinis* Epoxide Hydrolase

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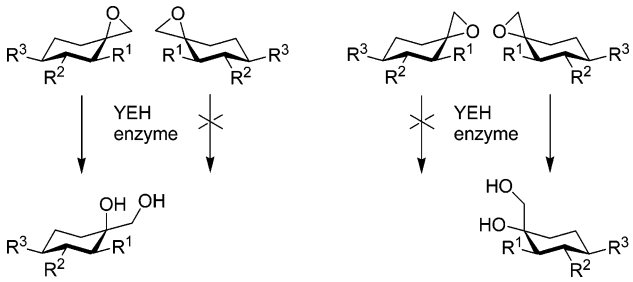

The kinetic resolution of a range of methyl-substituted 1-oxaspiro[2.5]octanes by yeast epoxide hydrolase (YEH) from *Rhodotorula glutinis* has been investigated. The structural determinants of substrate specificity and stereoselectivity of YEH toward these substrates appeared to be the configuration of the epoxide ring and the substitution pattern of the cyclohexane ring. For all compounds tested, O-axial epoxides were hydrolyzed faster than the corresponding O-equatorial compounds. In concern of the ring substituents, YEH preferred methyl groups on the *Re* side of the ring. Placement of substituents close to the spiroepoxide carbon decreased the reaction rate but increased enantioselectivity. YEH-catalyzed kinetic resolutions of 4-methyl 1-oxaspiro[2.5]octane epimers were most enantioselective ($E > 100$).

Introduction

1-Oxaspiro[2.*n*]alkanes are compounds in which an epoxide moiety is joined to an alicyclic fragment by a common carbon atom. The spiro attachment makes this type of epoxides easy accessible and thus highly sensitive for nucleophilic ring opening. This will result in an enhanced reactivity when compared to their epoxycycloalkane counterparts.[1] In nature, this high reactivity is translated to the high toxicity of spiroepoxide-containing mycotoxins. Typical examples thereof are trichothecenes and fumagillin-related toxins.[2,3]

Preparation of synthetic spiroepoxides has focused to a great extent on compounds mimicking the biological activity of mycotoxins. Many studies were initiated on the preparation of effective 1-oxaspiro[2.5]octane derivatives since the first observation that spiroepoxides such as fumagillin show significant antitumor activity. Several fumagillin analogues were synthesized which possess angiogenesis inhibiting activity (Figure 1).[4]

Apart from their potential value as end products with biological activity, 1-oxaspiro[2.5]octanes can serve as chiral starting materials in asymmetric synthesis. As an

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(1) Kas'yan, L. I.; Kas'yan, A. O.; Tarabara, I. N. *Russ. J. Org. Chem.* **2001**, *37*, 1361−1404.

(2) (a) Bennet, J. W.; Klich, M. *Clin. Microbiol. Rev.* **2003**, *16*, 497−516. (b) Amagata, T.; Rath, C.; Rigot, J. F.; Tarlov, N.; Tenney, K.; Valeriote, F. A.; Crews, P. *J. Med. Chem.* **2003**, *46*, 4342−4350. (c) Eriksen, G. S.; Pettersson, H.; Lundh, T. *Food Chem. Toxicol.* **2004**, *42*, 619−624.

(3) (a) Todd Lowther, W.; McMillen, D. A.; Orville, A. M.; Matthews, B. W. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12153−12157. (b) Halász, J.; Podányi, B.; Vasvári-Debreczy, L.; Szabó, A.; Hajdú, F.; Böcskei, Z.; Hegedús-Vajda, J.; Gyórbíró, A.; Hermecz, I. *Tetrahedron* **2000**, *56*, 10081−10085. (c) Zhou, G.; Tsai, C. W.; Liu, J. O. *J. Med. Chem.* **2003**, *46*, 3452−3454.

(4) (a) Griffith, E. C.; Su, Z.; Niwayama, S.; Ramsay, C. A.; Chang, Y.-H.; Liu, J. O. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15183−15188. (b) Baldwin, J. E.; Bulger, P. G.; Marquez, R. *Tetrahedron* **2002**, *58*, 5441−5452. (c) Vosburg, D. A.; Weiler, S.; Sorensen, E. J. *Chirality* **2003**, *15*, 156−166. (d) Pyun, H.-J.; Fardis, M.; Tario, J.; Yang, C. Y.; Ruckman, J.; Henninger, D.; Jin, H.; Kim, C. U. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 91−94. (e) Rodeschini, V.; Boiteau, J.-G.; Van de Weghe, P.; Tarnus, C.; Eustache, J. *J. Org. Chem.* **2004**, *69*, 357−373.

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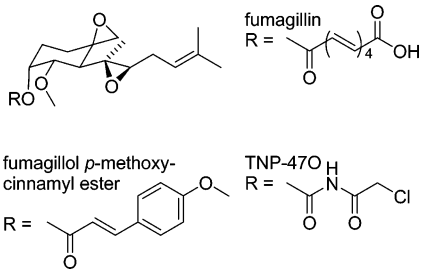


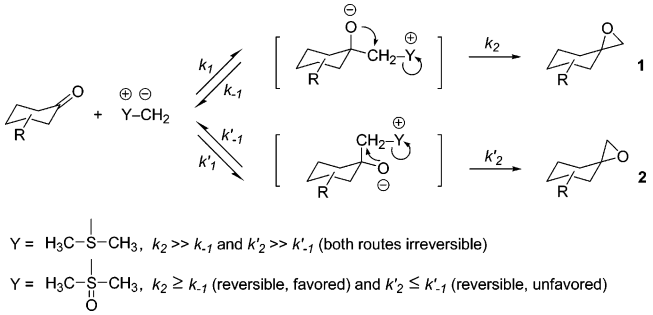
FIGURE 1. Antiangiogenic agents based on the fumagillin structure.

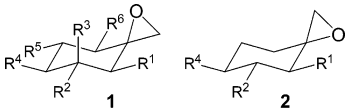
example, preparation of enantiopure 4,4,5,8-tetramethyl-1-oxaspiro[2.5]octane as a key intermediate for highly active fragrances has been reported.[5]

In most synthetic approaches, enantiopure spiroepoxides are prepared by sulfur ylide epoxidation of the corresponding cyclic ketones. Initially, the desired stereochemistry was simply defined by the starting carbonyl compound, but subsequent development of chiral sulfur ylide catalysts has rendered this method into a catalytic process for asymmetric epoxidation.[6] Nevertheless, the novel methodology is still limited in terms of substrate scope and has been applied only recently for the epoxidation of a symmetric cyclic ketone in the preparation of enantiopure 2-phenyl-1-oxaspiro[2.5]octane.[7]

The alternative approach to obtain enantiopure epoxides is kinetic resolution of racemic epoxides by stereoselective ring opening.[8,9] Although this method has clearly proven its practical utility, it has been very rarely tested for the resolution of spiroepoxides.[1] Stereoselective hydrolytic ring opening of a spiroepoxide has been initially demonstrated in the enzymatic hydrolysis of the (3*R*)- and (3*S*)-epimers of 3-spiro[5α-androstane-3,2′-oxirane]-17β-ol by whole cells of *Mycobacterium aurum*.[10] In two other studies, enzymatic hydrolytic kinetic resolution of 1-oxaspiro[2.5]octanes has been described. Mammalian microsomal epoxide hydrolase (mEH) has been used in the kinetic resolution of several methyl-substituted 1-oxaspiro[2.5]octanes.[11] Bellucci and co-workers showed in their study that spiroepoxides in general were excellent substrates for the mammalian enzyme and that enantioselectivity was high in case of the presence of a geminal dimethyl group on the cyclohexane ring. More recently, epoxide hydrolase of *Rhodotorula glutinis*, which was overexpressed in recombinant *Escherichia coli* BL21(DE3)(pEph1), was used for the enantioselective hydrolysis of racemic 2-isocyano-1-oxaspiro[2.5]octane.[12]

SCHEME 1. Sulfur Ylide Epoxidation of Substituted Cyclohexanones





1a: $R^1 = CH_3$, R^2–R^6 = H
1b: $R^2 = CH_3$, R^1, R^3–R^6 = H
1c: $R^4 = CH_3$, R^1–R^3, R^5, R^6 = H
1d: R^1, $R^6 = CH_3$, R^2–R^5 = H
1e: R^2, R^3, $R^5 = CH_3$, R^1, R^4, R^6 = H

2a: $R^1 = CH_3$, R^2, R^4 = H
2b: $R^2 = CH_3$, R^1, R^4 = H
2c: $R^4 = CH_3$, R^1, R^2 = H

In the present study, yeast epoxide hydrolase (YEH) containing cells of *Rhodotorula glutinis* ATCC 201718 were tested for kinetic resolution of a range of methyl-substituted 1-oxaspiro[2.5]octanes (see structure block). Discrimination between epimeric structures **1** and **2** was investigated, as well as effects of methyl substituents on varying positions of the cyclohexane ring, to gain insight into the structural requirements of spiroepoxides to serve as substrates for YEH. In this way, our study aims to extend the scope of enzymatic kinetic resolution of epoxides to the structurally and chemically divergent spiroepoxides.

Results and Discussion

Preparation of Racemic Substrates.

Racemic 1-oxaspiro[2.5]octanes are easy accessible by sulfur ylide epoxidation of the corresponding cyclohexanones (Scheme 1).[13] Dimethyl sulfonium ylide was used for the preparation of spiroepoxides **2a**–**2c**, which were obtained as mixtures of O-axial and O-equatorial epimers. Our results, as summarized in Table 1, are in agreement with findings reported in the literature.[13,14]

The stereochemical outcomes of sulfur ylide epoxidations are known to be controlled by the extent of reversibility in the formation of intermediate betaines: high reversibility leads to high stereoselectivity.[6,15] Epoxida-

(5) (a) Chapuis, C.; Brauchli, R. *Helv. Chim. Acta* **1993**, *76*, 2070–2088. (b) Liebens, A.; Laufer, W.; Hölderich, W. *Catal. Lett.* **1999**, *60*, 71–75.

(6) (a) Aggarwal, V. K.; Richardson, J. *Chem. Commun.* **2003**, 2644–2651. (b) Aggarwal, V. K.; Winn, C. L. *Acc. Chem. Res.* **2004**, *37*, 611–620.

(7) Aggarwal, V. K.; Bae, I.; Lee, H.-Y.; Richardson, J.; Williams, D. T. *Angew. Chem., Int. Ed.* **2003**, *42*, 3274–3278.

(8) (a) Jacobsen, E. N. *Acc. Chem. Res.* **2000**, *33*, 421–431. (b) Schaus, S. E.; Brandes, B. D.; Larrow, J. F.; Tokunaga, M.; Hansen, K. B.; Gould, A. E.; Furrow, M. E.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2002**, *124*, 1307–1315. (c) Nielsen, L. P. C.; Stevenson, C. P.; Blackmond, D. G.; Jacobsen, E. N. *J. Am. Chem. Soc.* **2004**, *126*, 1360–1362.

(9) (a) Weijers, C. A. G. M.; de Bont, J. A. M. *J. Mol. Catal., B* **1999**, *6*, 199–214. (b) Orru, R. V. A.; Faber, K. *Curr. Opin. Chem. Biol.* **1999**, *3*, 16–21. (c) Archelas, A.; Furstoss, R. *Curr. Opin. Chem. Biol.* **2001**, *5*, 112–119. (d) Smit, M. S. *TIBTECH* **2004**, *22*, 123–129.

(10) Escoffier, B.; Prome, J.-C. *Bioorg. Chem.* **1989**, *17*, 53–63.

(11) Bellucci, G.; Chiappe, C.; Ingrosso, G.; Rosini, C. *Tetrahedron: Asymmetry* **1995**, *6*, 1911–1918.

(12) Visser, H.; de Oliveira Villela Filho, M.; Liese, A.; Weijers, C. A. G. M.; Verdoes, J. *Biocatal. Biotransform.* **2003**, *21*, 33–40.

(13) (a) Corey, E. J.; Chaykovsky, M. *J. Am. Chem. Soc.* **1965**, *87*, 1353–1364. (b) Gololobov, Y. G.; Nesmeyanov, A. N.; Lysenko, V. P.; Boldeskul, I. E. *Tetrahedron* **1987**, *43*, 2609–2651.

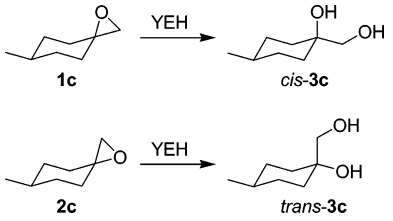
(14) (a) Toda, F.; Imai, N. *J. Chem. Soc., Perkin Trans. 1* **1994**, 2673–2674. (b) Migneco, L. M.; Vecchi, E. *Gazz. Chim. Ital.* **1997**, *127*, 19–24.

(15) Johnson, C. R.; Schroeck, C. W.; Shanklin, J. R. *J. Am. Chem. Soc.* **1973**, *95*, 7424–7431.

TABLE 1. Preparation of Racemic Spiroepoxide Substrates by Sulfonium Ylide Epoxidation of Methyl-Substituted Cyclohexanones

prepared spiroepoxide substrate	diMe-sulfonium ylide		diMe-oxo-sulfonium ylide	
	yield **1** + **2**	ratio **1**:**2**	yield **1** + **2**	ratio **1**:**2**
1a, **2a**	77%	80:20	81%	97:3
1b, **2b**	69%	49:51	84%	97:3
1c, **2c**	72%	40:60	83%	91:9
1d			80%	95:5
1e			85%	99:1

SCHEME 2. YEH-Catalyzed Hydrolysis of 1c and 2c



tion by dimethylsulfonium ylide is more or less irreversible due to the low stability of the sulfonium ylide. As a consequence, reactions with substituted cyclohexanones will initially generate a mixture of betaine intermediates, which are irreversibly converted to their respective spiroepoxides. Increased steric hindrance around the ketone carbonyl changed the preference of the direction of methylene addition from axial toward equatorial, thus reducing the amount of O-equatorial epimers in **2a** compared to **2b** and **2c**. Dimethyl sulfonium ylide was not used for epoxidation of di- and trisubstituted cyclohexanones because the O-equatorial epimers of **1d** and **1e** were expected to be obtained in even less amounts, due to enhanced steric effects.[14]

Epoxidation with the sulfoxide-stabilized dimethyloxosulfonium ylide facilitated the reversion of unfavored betaines to their more stable starting materials. Diastereoselectivity was thereby enhanced to such an extent that spiroepoxides **1a**−**1e** were obtained almost exclusively as O-axial epimers.

Configurational assignments of the epimers **1** and **2** were performed using NMR analysis. Particularly ^{13}C chemical shift values for epimeric 1-oxaspiro[2.5]octanes are reported to be very consistent.[16] Characteristic deshielding by an equatorial epoxide oxygen results in higher values of ^{13}C chemical shifts for the cyclohexane ring carbons C3−C8 of O-equatorial epimers. ^{1}H and ^{13}C NMR spectra of the synthesized epimers **1** and **2** are given in the Supporting Information.

Stereoselective Hydrolysis of Epimeric Spiroepoxides 1c and 2c.

In preliminary experiments, spiroepoxide **1c** was tested for bioconversion by YEH-containing cells of *Rhodotorula glutinis* ATCC 201718. We noticed a very rapid hydrolysis of **1c** and a concomitant formation of the diol *cis*-**3c** (Scheme 2). Apparently, there is a facile enzymatic

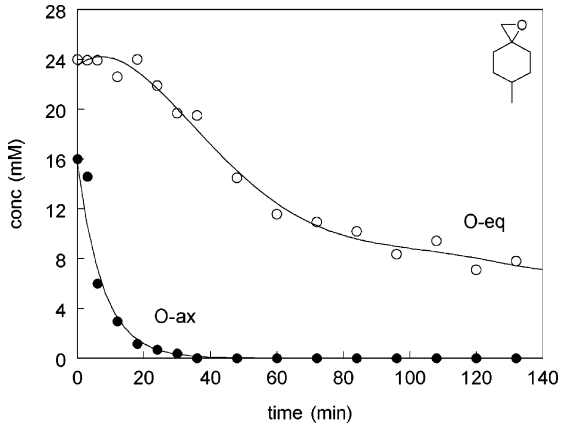


FIGURE 2. Stereoselective hydrolysis of 6-methyl-1-oxaspiro-[2.5]octane (400 μmol) by YEH-containing cells of *Rhodotorula glutinis* (132 mg dw). The substrate was prepared as a 4:6 mixture of O-axial epimer **1c** (●) and O-equatorial epimer **2c** (○).

nucleophilic attack on the easily accessible exocyclic epoxide carbon. The observed reaction rate (v_i = 85 nmol/min, mg dw) was by far above values determined for YEH-catalyzed hydrolysis of any other alicyclic epoxide.[17] Further investigation on biohydrolysis of epimeric mixture **1c/ 2c** showed however a much lower activity for spiroepoxide **2c** (Figure 2).

In solution, compounds **1c** and **2c** occur in two different chair conformations, which are in equilibrium. Equatorial methyl conformations are reported to predominate for both epimers (**1c**, 96% eq Me; **2c**, 93% eq Me, respectively).[18] Stereochemical discrimination by YEH will thus primarily concentrate on the configuration of the epoxide moiety. From the results shown in Figure 2, we can conclude that the YEH enzyme has a clear preference for substrate **1c**, having the epoxide in the O-axial position.

Docking of substrates in the enzyme active site has been studied in detail for various α/β hydrolase fold epoxide hydrolases (EHs).[19] It is known that initial binding of the substrate occurs by hydrogen bonding of the epoxide oxygen to two Tyr residues from the covering lid. Further modulation of the position of the epoxide carbon takes place by hydrophobic interactions with other residues in the active-site cavity. Since the X-ray structure of YEH is still under investigation, substrate docking in the YEH active site has not been studied in much detail yet. It is likely that binding of substrates by YEH is comparable with that of other eukaryotic EHs. The reason is that YEH has been characterized as an α/β hydrolase fold enzyme, and the Eph1 gene sequence revealed high similarity to mammalian mEHs and other eukaryotic EHs, including the two conserved Tyr residues.[9,20] YEH is assumed to be present as the single EH

(16) (a) Bottin-Strzalko, T.; Roux-Schmitt, M. C. *Org. Magn. Reson.* **1980**, *14*, 145−146. (b) Davis, R.; Kluge, A. F.; Maddox, M. L.; Sparacino, M. L. *J. Org. Chem.* **1983**, *48*, 255−259. (c) Bellucci, G.; Chiappe, C.; Lo Moro, G. *J. Org. Chem.* **1995**, *60*, 6214−6217. (d) Vedejs, E.; Dent, W. H.; Kendall, J. T.; Oliver, P. A. *J. Am. Chem. Soc.* **1996**, *118*, 3556−3567.

(17) Weijers, C. A. G. M. *Tetrahedron: Asymmetry* **1997**, *8*, 639−647.

(18) Uebel, J. J. *Tetrahedron Lett.* **1967**, *47*, 4751−4754.

(19) (a) Schiøtt, B.; Bruice, T. C. *J. Am. Chem. Soc.* **2002**, *124*, 14558−14570. (b) Arand, M.; Cronin, A.; Oesch, F.; Mowbray, S. L.; Jones, T. A. *Drug Metab. Rev.* **2003**, *35*, 365−383.

(20) (a) Visser, H.; Vreugdenhil, S.; de Bont, J. A. M.; Verdoes, J. C. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 415−419. (b) Visser, H.; Weijers, C. A. G. M.; van Ooyen, A. J. J.; Verdoes, J. C. *Biotechnol. Lett.* **2002**, *24*, 1687−1694. (c) Visser, H. Ph.D. Thesis, Wageningen University, The Netherlands, 2002.

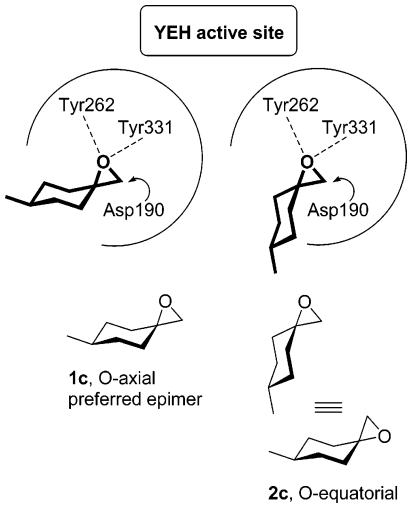


FIGURE 3. Schematic presentation of proposed bindings of substrates **1c** and **2c** in the YEH active site. Orientation of the epoxide moiety of **1c** and **2c** represents initial noncovalent binding of the preferred O-axial epimer **1c** and is based on key interactions with Asp190, Tyr262, and Tyr331 residues. O-Equatorial epimer **2c** is oriented with epoxide oxygen upward for most optimum binding to the two tyrosines, just like in **1c**. Residues of *Rhodotorula glutinis* YEH have been determined in a previous study.[20]

enzyme in *Rhodotorula glutinis* ATCC 201718 since there is only one EH-encoding gene found in this yeast.[20c]

Proposed binding of epimers **1c** and **2c** in the YEH active site is schematically depicted in Figure 3. The proposed binding indicates that the two epimers will require a different spatial orientation in the active site, from which the stereochemical discrimination by YEH can be understood.

Diastereoselective and Enantioselective Hydrolysis of Spiroepoxides 1b and 2b.

To investigate the diastereoselectivity and enantioselectivity of YEH toward 1-oxaspiro[2.5]octanes, we decided to study to the nonsymmetrical 5-methyl-substituted substrates **1b** and **2b**. YEH-catalyzed hydrolyses of racemic **1b** and the epimeric mixture **1b/2b** were investigated. In the latter reaction, the O-axial epimer **1b** was preferably hydrolyzed, thus confirming the results obtained with mixture **1c/ 2c**. Furthermore, significant enantioselectivity was found in the hydrolysis of individual racemic substrates **1b** and **2b**, respectively (Figure 4). This enabled the isolation of the enantiopure epoxides (3*S*,5*S*)-**1b** and (3*S*,5*R*)-**2b** by sequential kinetic resolution of the epimeric mixture **1b/2b**, showing the potential of YEH for the resolution of these compounds.

Diastereoselective and Enantioselective Hydrolysis of Spiroepoxides 1a and 2a.

Increased diastereoselectivity and enantioselectivity, when compared to hydrolysis of **1b** and **2b**, were observed in kinetic resolutions of the more sterically hindered 4-methyl-1-oxaspiro[2.5]octanes **1a** and **2a**. Discrimination between **1a** and **2a** was very high, with preferential hydrolysis of the O-axial substrate **1a**. Enantioselectivity toward enantiomers of the individual epimers **1a** and **2a** was in both cases nearly absolute ($E > 100$). Only the (3*R*,4*S*)-isomer of **1a** and (3*R*,4*R*)-isomer of **2a** were accepted by the YEH enzyme, whereas the other enantiomers remained unaffected in resolution experiments (see Figure S1 Supporting Information).

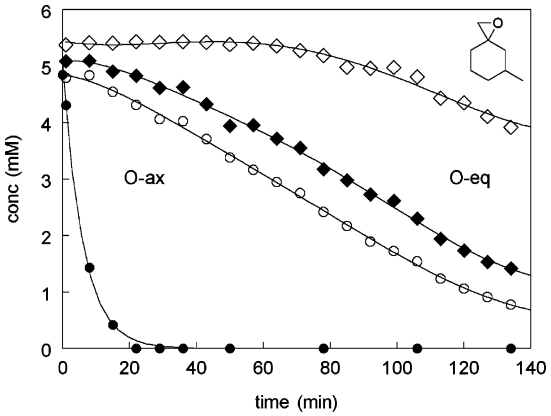


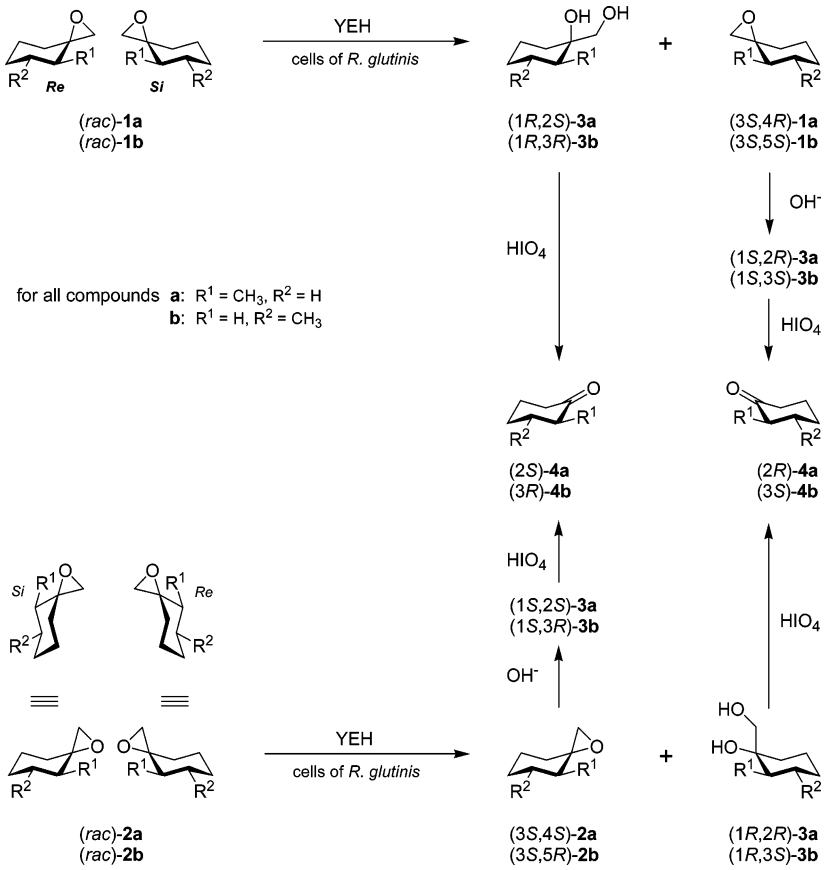
FIGURE 4. Time course of the kinetic resolution of spiroepoxides **1b/2b** (200 μmol) by YEH-containing cells of *Rhodotorula glutinis* (106 mg dw). The solution contained an epimeric 1:1 mixture of **1b** (O-ax) and **2b** (O-eq), consisting of (3*R*,5*R*)-**1b** (●), (3*S*,5*S*)-**1b** (○), (3*R*,5*S*)-**2b** (◆), and (3*S*,5*R*)-**2b** (◇).

The kinetic resolutions of substrates **1a** and **2a** and **1b** and **2b** are presented in Scheme 3. Substrates **2a** and **2b** are depicted in their proposed binding conformation with the oxygen atoms pointing upward, according to the orientation of **2c** in Figure 3. In Scheme 3, it is shown that enantiomers of **1a**, **2a**, **1b**, and **2b** with methyl substituents on the *Re* side of the cyclohexane ring were preferably hydrolyzed by YEH of *Rhodotorula glutinis*. Our results were most striking since it had been reported that the mammalian mEH did not show a significant discrimination between the O-axial and O-equatorial epimers of substrates **1a** and **2a** and **1b** and **2b**.[11] In addition, enantioselectivities of mEH were only moderate in hydrolysis of the individual substrates. Because of the close similarity of YEH and mammalian mEH, no such large differences in substrate specificity were expected.[20] A possible explanation for the much better stereoselective performance of YEH might be a difference in protein conformation, caused by the difference in environment where both membrane associated enzymes were situated during the biocatalysis experiments. In the present study, YEH was still embedded in its most optimal surroundings, the parent yeast cell, whereas the mammalian mEH has been used as an isolated enzyme. However, preferential conversion of O-axial spiroepoxide epimers is not restricted to the yeast enzyme, since an O-axial epimeric preference has been described for the hydrolysis of steroidal spiroepoxides by cells of *Mycobacterium aurum*.[10]

The effect of methyl substituents on varying positions of the cyclohexane ring of substrates **1a**−**1c** and **2a**−**2c** on the activity of YEH is summarized in Figure 5. The results demonstrate that the effect of the substitution pattern of the cyclohexane ring on the YEH activity is similar for both the O-axial and O-equatorial spiroepoxides. The substrate specificity, as presented in Figure 5, clearly shows that YEH has a preference for (i) spiroepoxides with substituents remote from the epoxide moiety and (ii) spiroepoxide isomers bearing substituents on the *Re* side of the cyclohexane ring.

The determination of the absolute configurations of residual spiroepoxides and formed diols was performed

SCHEME 3. YEH-Catalyzed Kinetic Resolution of 1a, 2a, 1b, and 2b and Conversion of Products to the Corresponding Cyclohexanones for Stereochemical Assignment



as follows: (i) the relative configuration of the quaternary carbon was assigned directly by ^{13}C NMR spectroscopy or by enantioselective gas chromatography (GC) by co-injection with a prepared reference compound with known configuration; (ii) the configuration of the methyl-substituted carbons was determined by oxidation to the corresponding cyclohexanones, followed by analysis of their optical rotation. Specific optical rotations of enantiopure methyl-substituted cyclohexanones were available as reference data.[23a,25,27]

More specifically, residual spiroepoxides and formed diols were isolated from the final reaction mixture by selective extraction, with pentane and ethyl acetate, respectively. Sodium metaperiodate-mediated oxidative cleavage was used for the subsequent conversion of diols to their respective cyclohexanones.[21] Base-catalyzed hydrolysis to the corresponding diols preceded metaperiodate oxidation in case of residual spiroepoxides (Scheme 3). The results of the kinetic resolutions are summarized in Table 2.

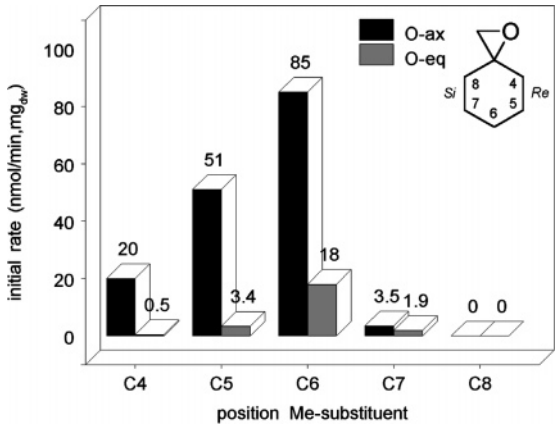


FIGURE 5. Substrate specificity in YEH-catalyzed kinetic resolution of monomethyl-substituted 1-oxaspiro[2.5]octanes.

Di- and Trimethyl-Substituted Spiroepoxides 1d and 1e.

Investigations were further extended toward substrates bearing methyl substituents on the *Si* side of the cyclohexane ring. Dimethyl-substituted spiroepoxide **1d** was not converted by YEH-containing cells. This result is in accordance with the observation that compounds bearing a methyl substituent adjacent to the spirocarbon on the *Si* side of the cyclohexane ring were not hydrolyzed

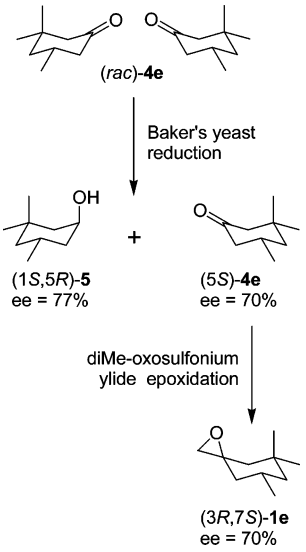
(21) (a) Zhong Y.-L.; Shing, T. K. M. *J. Org. Chem.* **1997**, *62*, 2622−2624. (b) Doderer, K.; Lutz-Wahl, S.; Hauer, B.; Schmid, R. D. *Anal. Biochem.* **2003**, *321*, 131−134. (c) Mateo, C.; Archelas, A.; Furstoss, R. *Anal. Biochem.* **2003**, *314*, 135−141.
(22) (a) Prelog, V. *Pure Appl. Chem.* **1964**, *9*, 119−130. (b) Tanikaga, R.; Obata, Y.; Kawamoto, K.-I. *Tetrahedron: Asymmetry* **1997**, *8*, 3101−3106.
(23) (a) Allinger, N. L.; Riew, C. K. *J. Org. Chem.* **1975**, *40*, 1316−1321. (b) Laruelle, C.; Lepant, M. U.S. Patent, 1992, WO 92/22529.
(24) Morisseau, C.; Nellaiah, H.; Archelas, A.; Furstoss, R.; Baratti, J. C. *Enzyme Microb. Technol.* **1997**, *20*, 446−452.
(25) Meyers, A. I.; Williams, D. R.; Erickson, G. W.; White, S.; Druelinger, M. *J. Am. Chem. Soc.* **1981**, *103*, 3081−3087.
(26) Park, J.; Pedersen, S. *Tetrahedron* **1992**, *48*, 2069−2080.
(27) Thurkauf, A.; Hillery, P.; Jacobson, A. E.; Rice, K. C. *J. Org. Chem.* **1987**, *52*, 5466−5467.

TABLE 2. YEH-Catalyzed Kinetic Resolution of Methyl-Substituted 1-Oxaspiro[2.5]octanes

		residual spiroepoxide				diol product[a]		
substrate	*E* value	ee[b]	yield[b]	abs. conf.	$[\alpha]^{20}_D$	ee[b]	abs. conf.	$[\alpha]^{20}_D$
(*rac*)-**1a**	>100	>98%	49%	(3*S*,4*R*)-**1a**	+6.2 (*c* 1.1, hexane)	91%	(1*R*,2*S*)-**3a**	−6.5 (*c* 1.1, $CHCl_3$)
(*rac*)-**2a**	>100	76%	11%	(3*S*,4*S*)-**2a**	nd[c]	nd	(1*R*,2*R*)-**3a**	nd
(*rac*)-**1b**	28	>98%	42%	(3*S*,5*S*)-**1b**	+39.1 (*c* 1.2, hexane)	62%	(1*R*,3*R*)-**3b**	−1.2 (*c* 1.1, $CHCl_3$)
(*rac*)-**2b**	4	70%	19%	(3*S*,5*R*)-**2 b**	+24.0 (*c* 0.1, hexane)	nd	(1*R*,3*S*)-**3b**	nd
1c/2c[e]		>98%[d]	48%	*trans*-**2c**		74%[d]	*cis*-**3c**	
1d				no reaction			no reaction	
(*rac*)-**1e**	12	>98%	25%	(3*R*,7*S*)-**1e**	*f*	48%	(1*S*,5*R*)-**3e**	*g*

[a] 1-Hydroxy-*x*-methyl-cyclohexanemethanol. [b] ee and yield of single epimer. [c] Not determined. [d] Value of de. [e] Mixture of epimers **1c** (cis) and **2c** (trans). [f] Specific rotation reference compound (3*R*,7*S*)-**1e** (ee = 70%) $[\alpha]^{20}_D = +5.2$. (*c* 0.7, hexane). [g] Specific rotation diol product (1*S*,5*R*)-**3e** (ee = 73%) $[\alpha]^{20}_D = -2.8$. (*c* 0.6, $CHCl_3$).

SCHEME 4. Preparation of (3*R*,7*S*)-1e by Baker's Yeast Mediated Reduction



(enantiomers (3*S*,4*R*)-**1a** and (3*S*,4*S*)-**2a**, respectively, Figure 5).

Acceptance of substituents on the carbon atom β to the spirocarbon on the *Si* side was expected to be less tight, considering the lower enantioselectivity observed in the hydrolysis of **1b** and **2b** (Figure 4). Trimethyl-substituted spiroepoxide **1e** was used to study the effect of substitution on this β-position. Although the reaction rate has dropped dramatically (v_i = 0.52 nmol/min, mg dw) compared to the rate for **1b** (Figure 5, C5), it was still possible to perform a complete kinetic resolution of substrate **1e** with cells of *Rhodotorula glutinis* (see Supporting Information).

Determination of the absolute configurations of residual spiroepoxide **1e** and diol **3e** was initially set up according to the procedure shown in Scheme 3. However, due to the greatly increased hydrophobic character of diol **3e**, selective extraction of residual spiroepoxide **1e** was hampered by co-extraction of amounts of diol **3e**. We therefore turned to an alternative approach for determination of the absolute configuration by preparing an enantiomerically enriched spiroepoxide reference compound **1e**. This was achieved by yeast-mediated enantioselective reduction of ketone (*rac*)-**4e**, followed by sulfur ylide epoxidation to give the desired epoxide (Scheme 4).

For enantioselective reduction of ketone (*rac*)-**4e**, baker's yeasts from several commercial sources were screened and a highly enantioselective baker's yeast preparation (Fermipan Red) was selected for this reaction. Baker's yeast reduction of (*rac*)-**4e** proceeded according to Prelog's rule (*Re* side hydride attack on the ketone carbonyl), resulting in predominant formation of *trans*-(1*S*,5*R*)-**5**.[22] Alcohol (1*S*,5*R*)-**5** and residual ketone (5*S*)-**4e** were isolated from the reaction mixture and subsequently purified by column chromatography. Determination of the absolute configurations of (1*S*,5*R*)-**5** and (5*S*)-**4e** was done by determination of the specific rotations and comparison with reference data.[23] Epoxidation of (5*S*)-**4e** by dimethyloxosulfonium ylide yielded reference compound (3*R*,7*S*)-**1e**, enantiomeric excess (ee) = 70%; $[\alpha]^{20}_D = +5.2$ (*c* 0.7, hexane).

By comparative GC analysis, we determined the configuration of the residual spiroepoxide **1e**, obtained by YEH-catalyzed resolution, as (3*R*,7*S*)-**1e**. Consequently, the enantiomer bearing the geminal dimethyl group on the *Si* side of the cyclohexane ring was preferably hydrolyzed by YEH. This result was not in accordance with the observed preferences for other tested spiroepoxides, but it has to be considered that substrate **1e** in any case was a poor substrate for YEH. The axial methyl of the geminal dimethyl group will possibly hamper optimal orientation of either enantiomer of **1e** in the YEH active site. For a better understanding of this observation, more detailed information about the crystal structure of the YEH enzyme will be necessary.

Conclusions

Kinetic resolution of 1-oxaspiro[2.5]octanes by YEH-containing cells of *Rhodotorula glutinis* ATCC 201718 has been demonstrated. Several methyl-substituted spiroepoxides have been prepared in enantiopure form by this methodology. Substrate recognition of spiroepoxides by YEH was found to be primarily dependent on the configuration of the epoxide moiety: O-axial spiroepoxides were preferably hydrolyzed.

Methyl substituents on varying positions of the cyclohexane ring were found to have a large effect on both activity and enantioselectivity of substrate hydrolysis. Placement of substituents closer to the epoxide moiety resulted in a decrease of reaction rate and an increase of enantioselectivity. Substitution on the *Re* side of the cyclohexane ring was strongly preferred above *Si* side substitution. Preferential conversion of *Re* substituted substrates actually defined the enantiomeric discrimination in spiroepoxide hydrolysis.

Throughout this study, O-axial-preferred spiroepoxide hydrolysis has been consistently observed in YEH-

catalyzed conversions. It is hence assumed that epimeric discrimination is an intrinsic feature of the YEH enzyme. Although O-equatorial epimers were gradually converted as well, it cannot be fully excluded that some apparent activity for O-equatorial epimers was (partly) due to hydrolysis of their O-axial conformers, concomitantly present in the reaction mixture by conformational equilibria. The latter phenomenon could in this way possibly mask an even more definite intrinsic preference of YEH for O-axial epimers. We have therefore continued our study with investigations on the behavior of conformationally locked spiroepoxide substrates in YEH-catalyzed reactions. The complete results of the subsequent studies will be presented elsewhere. Initial results of these studies confirm the preferential hydrolysis of O-axial spiroepoxide epimers.

Experimental Section

General Procedure for the Synthesis of 1, Exemplified by O-ax 4-Methyl-1-oxaspiro[2.5]octane (1a).

To a clear-yellow solution of trimethylsulfoxonium iodide (7.75 g, 35 mmol) in 75 mL of dry DMSO was added 2.52 g (22.5 mmol) of 2-methylcyclohexanone with stirring. The mixture was brought under a N_2 atmosphere, and a solution of potassium *tert*-butoxide (3.95 g, 35 mmol) in 50 mL dry DMSO was slowly added. The resulting solution was stirred at room temperature for 16 h under N_2. The reaction was quenched by addition of water (150 mL) and extracted with diethyl ether (3 × 50 mL). Combined organic layers were washed with water (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. Bulb-to-bulb distillation gave (*rac*)-**1a** (2.04 g, 81%) as a colorless liquid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80 (3H, d), 1.39−1.77 (9H, m), 2.46 (1H, d), 2.73 (1H, d). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.3 (CH_3), 24.0 (CH_2), 24.6 (CH_2), 32.6 (CH_2), 32.9 (CH_2), 34.5 (CH), 52.6 (CH_2), 60.9 (C). MS *m/z* 126 $[M]^+$; HRMS calcd for $C_8H_{14}O$ 126.1045, found 126.1042.

General Procedure for the Synthesis of 2, Exemplified by O-eq 4-Methyl-1-oxaspiro[2.5]octane (2a).

Substrate **2a** was prepared from 2-methylcyclohexanone (2.52 g) according to the method for compound **1a**, using trimethylsulfonium iodide (7.75 g, 38 mmol) for epoxidation. Compound **2a** was obtained as a mixture of **1a/2a** (80:20) in 77% yield (1.94 g) as a colorless liquid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.70 (3H, d, CH_3 1a/2a), 1.22−1.72 (9H, m, 1a/2a), 2.33 (1H, d, CH_2 of C2 2a), 2.35 (1H, d, CH_2 of C2 1a), 2.57 (1H, d, CH_2 of C2 2a), 2.63 (1H, d, CH_2 of C2 1a). ^{13}C NMR (50 MHz, $CDCl_3$): δ 14.1 (CH_3 1a), 14.7 (CH_3 2a), 23.7 (CH_2 2a), 23.9 (CH_2 1a), 24.5 (CH_2 1a), 25.3 (CH_2 2a), 32.4 (CH_2 1a), 32.7 (CH_2 1a), 33.1 (2*CH_2 2a), 34.3 (CH 1a), 35.4 (CH 2a), 50.3 (CH_2 2a), 52.3 (CH_2 1a), 60.6 (C 1a), 65.6 (C 2a). MS *m/z* 126 $[M]^+$; HRMS calcd for $C_8H_{14}O$ 126.1045, found 126.1045.

General Procedure for YEH-Catalyzed Kinetic Resolution with cells of *Rhodotorula glutinis*.

Rhodotorula glutinis ATCC 201718 was cultivated for 48 h in a mineral medium supplemented with 2% glucose and 0.2% yeast extract, at 30 °C in a shaking incubator. Cells were harvested by centrifugation at 10 000 *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. In general, reactions were terminated when the residual spiroepoxides reached ee > 98%. Subsequently, yeast cells were removed from the reaction mixture by centrifugation. Residual spiroepoxides in formed diols were isolated from the supernatants by selective extraction with pentane and ethyl acetate, respectively.

General Procedure for the Determination of the Enantiomeric Ratio (*E* value).

For determination of the enantiomeric ratio *E*, $\ln[\text{fast}]_0/[\text{fast}]$ vs $\ln[\text{slow}]_0/[\text{slow}]$ was plotted. The slope of this curve represents the *E* value.[24] Concentrations of the fast-reacting enantiomer at times 0 and *t* are represented by $[\text{fast}]_0$ and [fast], while $[\text{slow}]_0$ and [slow] are the concentrations of the slow-reacting enantiomer at times 0 and *t*, respectively.

General Procedure for the Determination of the Absolute Configuration of Residual Spiroepoxides and Formed Diols.

Kinetic resolutions were scaled-up for conversion of 0.5 g of substrate. For hydrolysis of substrates **1a**, **1b**, and **2a**−**2c**, 0.3−1.3 g (dw) of cells of *Rhodotorula glutinis* were used and incubations were continued for complete resolution within 6 h. Resolution of substrate **1e** (0.5 g) was performed by 2.0 g (dw) of yeast cells and incubation for 24 h. The reactions were monitored by analysis with enantioselective GC. Residual spiroepoxides and formed diols were isolated by selective extraction, dried, and concentrated. Determination of the absolute configuration of residual spiroepoxides and formed diols was as follows: (i) the relative configuration of the quaternary carbon was assigned directly by ^{13}C NMR spectrometry or by enantioselective GC by co-injection with a prepared reference compound with known configuration; (ii) configuration of the methyl-substituted carbons was determined by subsequent oxidative cleavage with periodate to the corresponding cyclohexanones, followed by analysis of their optical rotation. Specific optical rotations of enantiopure methyl-substituted cyclohexanones were available as reference data.[23a,25,27]

Oxidative cleavage of diols to cyclohexanones was performed by adding 0.10 g of diol to a stirred solution of 0.15 g of $NaIO_4$ in 5 mL of water. The mixture was stirred at room temperature for 1 h and extracted with pentane (3 × 5 mL). The combined organic layers were dried and concentrated under reduced pressure. The resulting cyclohexanones were redissolved in appropriate solvents for the measurement of ee by enantioselective GC and specific optical rotation.

The residual spiroepoxides were hydrolyzed to their corresponding diols, preceding oxidative cleavage. For base-catalyzed hydrolysis, 0.10 g of spiroepoxide was added to a solution of 10 mL of 1 M NaOH and stirred at room temperature for 24 h. The mixture was neutralized with H_3PO_4, saturated with NaCl, and extracted with ethyl acetate. The combined organic layers were dried and the solvent removed under reduced pressure. Acid-catalyzed hydrolysis was accordingly performed in 1 N H_3PO_4 solution instead of NaOH. The obtained diols were subsequently used for reaction with $NaIO_4$.

(3*S*,4*R*)-4-Methyl-1-oxaspiro[2.5]octane (1a) and (1*R*,2*S*)-1-Hydroxy-2-methyl-cyclohexanemethanol (3a) by YEH-Catalyzed Resolution.

Residual spiroepoxide (3*S*,4*R*)-**1a** was obtained in 49% yield as a colorless liquid: ee > 98%; $[\alpha]^{20}_D$ +6.2 (*c* 1.1, hexane). Base-catalyzed hydrolysis, followed by oxidative cleavage of enantiopure (3*S*,4*R*)-**1a**, yielded ketone (2*R*)-**4a** as a colorless liquid: ee = 91%; $[\alpha]^{20}_D$ −21.5 (*c* 0.4, methanol). ^{1}H and ^{13}C spectra of (2*R*)-**4a** matched the spectra of commercially available (*rac*)-**4a**. The absolute configuration of (2*R*)-**4a** was determined by comparison of the specific optical rotation with literature data (2*S*)-**4a**: $[\alpha]^{23}_D$ +12.2 (*c* 4.0, methanol); ee = 87%. The absolute configuration of (3*S*,4*R*)-**1a** was derived from the configuration of (2*R*)-**4a** and the O-axial configuration of (*rac*)-**1a** (Scheme 3).

Diol (1*R*,2*S*)-**3a** was obtained in 44% yield as a yellow oil: ee = 91%; $[\alpha]^{20}_D$ −6.5 (*c* 1.1, $CHCl_3$). ^1H NMR (200 MHz, $CDCl_3$): δ 0.86 (3H, d), 1.20−1.64 (8H, m), 1.72−1.79 (1H, m), 2.11 (2H, OH, broad), 3.35 (1H, d), 3.60 (1H, d). ^{13}C NMR (50 MHz, $CDCl_3$): δ 15.1 (CH_3), 21.5 (CH_2), 25.1 (CH_2), 30.5 (CH_2), 33.9 (CH_2), 36.3 (CH), 69.0 (CH_2), 73.2 (C). Comparison of ^{13}C NMR values with data from literature revealed that the relative stereochemistry of diol (1*R*,2*S*)-**3a** was cis.[26] Oxidative cleavage of (1*R*,2*S*)-**3a** yielded ketone (2*S*)-**4a** as a colorless liquid: ee = 83%; $[\alpha]^{20}_D$ +19.2 (*c* 0.73, methanol). The absolute configuration of (2*S*)-**4a** was determined by comparison of the specific optical rotation with literature data (2*S*)-**4a**: $[\alpha]^{25}_D$ +12.2 (*c* 4.0, methanol); ee = 87%.[25] The absolute configuration of (1*R*,2*S*)-**3a** was derived from the configuration of (2*S*)-**4a** and the assigned cis stereochemistry of (1*R*,2*S*)-**3a** (Scheme 3).

***trans*-6-Methyl-1-oxaspiro[2.5]octane (2c) and *cis*-1-Hydroxy-4-methyl-cyclohexanemethanol (3c) by YEH-Catalyzed Resolution.**

Spiroepoxide *trans*-**2c** was prepared by resolution of substrate **2c** and obtained as a colorless liquid: diasteromeric excess (de) > 98%; yield 48% (0.24 g). Purification was done by flash chromatography on silica gel with petroleum ether (bp 40−60 °C)/diethyl ether (4:1), followed by concentration under reduced pressure. ^1H NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, d), 1.03−1.26 (4H, m), 1.42 (1H, m), 1.71−1.82 (4H, m), 2.51 (2H, s). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.6 (CH_3), 31.5 (CH), 33.2 (2*CH_2), 33.9 (2*CH_2), 54.8 (CH_2), 59.5 (C).

Diol *cis*-**3c** was prepared by a duplicate experiment and isolated after termination of the reaction at lower conversion of substrate **2c**. Diol *cis*-**3c** was obtained as a yellow solid: de > 98%; yield 28% (0.14 g). Purification was done by flash chromatography on silica gel with petroleum ether (bp 40−60 °C)/diethyl ether (4:1), followed by removal of the solvent under reduced pressure. ^1H NMR (200 MHz, $CDCl_3$): δ 0.90 (3H, d), 1.16−1.36 (5H, m), 1.42−1.72 (4H, m), 2.27 (2H, OH, broad), 3.38 (2H, s). ^{13}C NMR (50 MHz, $CDCl_3$): δ 22.3 (CH_3), 29.0 (2*CH_2), 32.4 (CH), 33.5 (2*CH_2), 71.1 (C), 71.7 (CH_2). The stereochemistry of biohydrolysis product diol *cis*-**3c** was determined by enantioselective GC analysis by co injection with a reference compound *cis*-**3c**, prepared by known base-catalyzed hydrolysis of *cis*-**1c**.

Reference Compound (3*R*,7*S*)-5,5,7-Trimethyl-1-oxaspiro[2.5]octane (1e), Prepared by Baker's Yeast Mediated Reduction of 3,3,5-Trimethylcyclohexanone.

For reduction of ketone **4e**, 300 g of lyophilized baker's yeast (Fermipan Red) was suspended in 1 L of phosphate buffer pH 7.0. The reaction was started by addition of (*rac*)-**4e** (2.2 g), and incubation was continued for 2 days in an orbital shaker at 35 °C. Yeast cells were discarded from the reaction mixture by centrifugation (10 000 *g*, 10 min, 4 °C), and the supernatants extracted with diethyl ether. The combined organic layers were dried and the solvent removed under reduced pressure. Alcohol **5** and residual ketone **4e** were obtained as a mixture. Purification was done by flash chromatography on silica gel with petroleum ether (bp 40−60 °C)/diethyl ether (10:1), followed by concentration under reduced pressure.

Residual ketone (5*S*)-**4e** was isolated in 36% yield (0.8 g) as a colorless liquid: ee = 70%; $[\alpha]^{20}_D$ +20.8 (*c* 0.25, $CHCl_3$). ^1H and ^{13}C NMR spectra of (5*S*)-**4e** matched the spectra of commercially available (*rac*)-**4e**. The absolute configuration of (5*S*)-**4e** was determined by comparison of the specific optical rotation with literature data (5*S*)-**4e**: $[\alpha]^{24}_D$ +20.3 (*c* 1, $CHCl_3$); ee = 75%.[23a]

Alcohol (1*S*,5*R*)-**5** was isolated in 10% yield (0.2 g) as a yellow oil: ee = 77%; $[\alpha]^{20}_D$ −9.2 (*c* 1.1, $CHCl_3$). The absolute configuration of *trans*-(1*S*,5*R*)-**5** was determined by comparison of the specific optical rotation with literature data of *trans*-(1*S*,5*R*)-**5**: $[\alpha]^{24}_D$ −15.5 (*c* 1, $CHCl_3$).[23b]

Reference compound (3*R*,7*S*)-**1e** was prepared by epoxidation of 0.3 g of ketone (5*S*)-**4e** with dimethyloxosulfonium ylide, according to the method for **1a**. Compound (3*R*,7*S*)-**1e** was obtained in 81% yield (0.24 g) as a colorless liquid: ee = 70%; $[\alpha]^{20}_D$ = +5.2 (*c* 0.7, hexane). ^1H and ^{13}C NMR spectra of (3*R*,7*S*)-**1e** matched the spectra of substrate (*rac*)-**1e**.

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Supporting Information Available: Graphs of YEH-catalyzed kinetic resolution of substrates **1a**, **2a**, and **1e**. General experimental procedures, descriptions of the synthesis, and characterization of substrates **1b**−**1e**, **2b**, and **2c**. Product characterization data of compounds obtained by YEH-catalyzed kinetic resolutions of **1b**, **2a**, **2b**, and **1e**. ^1H and ^{13}C NMR spectra of substrates and reaction products, mass spectroscopy peak listings of substrates, and conditions of GC analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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