

Unusual Steric Course of the Epoxide Hydrolase Catalyzed Hydrolysis of (\pm)-3,4-Epoxytetrahydropyran. A Case of Complete Stereoconvergence

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The steric course of the hydrolysis of racemic 3,4-epoxytetrahydropyran promoted by rabbit liver microsomal epoxide hydrolase has been investigated. Both enantiomers of the epoxide give (-)-(*R,R*)-*trans*-tetrahydropyran-3,4-diol at practically equal rates, the optical purity being at least 96% at any stage of conversion. These results can be justified by assuming that both enantiomers react in the 1,2-*M* conformation, nucleophilic attack by water taking place exclusively at position 3 of the 3*S*,4*R* enantiomer and at position 4 of the 3*R*,4*S* one, in contrast with the nonenzymic ring opening in which a high preference for attack at C(4) has been found.

The microsomal epoxide hydrolase, an important enzyme involved in the metabolism of xenobiotic compounds and in the detoxification of carcinogenic epoxides and arene oxides,¹ exhibits a low substrate specificity but often a high regio- and stereoselectivity.² Cyclohexene oxide, a meso compound actually consisting of a 50/50 mixture of two rapidly equilibrating chiral conformers that can be designated as 3,4-*P* and 3,4-*M* according to their helicities,² is hydrolyzed by the enzyme with an 85:15 preference for attack by water at the *S* carbon (Scheme I) to give a 70% enantiomeric excess (ee) of (1*R*,2*R*)-cyclohexane-1,2-diol.³ If opening of the oxirane ring takes place, as usual, in the diaxial (antiparallel) fashion (Fürst-Plattner rule), the preferential conformation of the epoxide in the enzyme active site must be the 3,4-*M* one.² This was further substantiated by an investigation of the enzyme-catalyzed hydrolysis of analogous rigid epoxides, *cis*- and *trans*-4-*tert*-butyl-² and -3-*tert*-butyl-1,2-epoxycyclohexanes,⁴ which showed that the enantiomers in which the ring has the 3,4-*M* helicity are hydrolyzed faster, unless predominant steric factors oppose this course.

In order to bring further support to the above hypothesis we investigated the hydrolysis promoted by rabbit liver microsomes of (\pm)-3,4-epoxytetrahydropyran (**3**), a substrate very similar in its steric requirements to **1** but in which the presence of the heteroatom in the six-membered ring causes nonequivalence of the oxirane carbons and enantiomerism. The hydrolysis of **3** was relatively slow as in the case of epoxide **1** and gave exclusively (Table I) the *trans*-diol **4** which was isolated at time intervals and found to be levorotatory. Its hygroscopic nature caused an uncertainty in the determination of its specific rotation and optical purity. Two very different data on this compound are reported in the literature, although the same synthetic procedure was followed in each case: $[\alpha]_D -44.9^\circ$ (H_2O),⁵ $[\alpha]^{20}_D -29.6^\circ$ (H_2O).⁶ It was therefore quantitatively converted into the corresponding diacetate with acetic anhydride in pyridine. The specific rotation of the pure (>99% by GLC) diacetate had a constant value ($[\alpha]^{22}_D -38.0 \pm 0.3^\circ$ in $CHCl_3$; $-32.8 \pm 0.3^\circ$ in EtOH) that was independent of the percent conversion of epoxide into glycol. Whereas the value in $CHCl_3$ was coincident with the reported specific rotation (-38°)⁶ for the optically pure

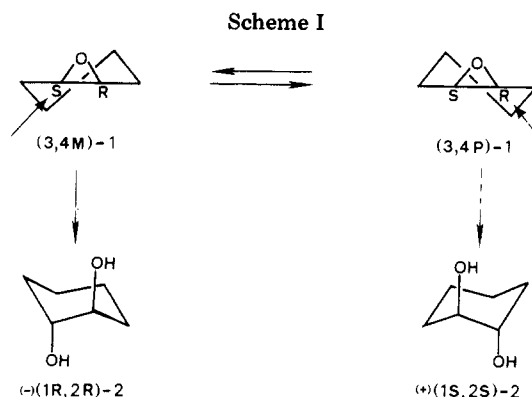


Table I. Epoxide Hydrolase Catalyzed Hydrolysis of (\pm)-3,4-Epoxytetrahydropyran

incubn time, ° h	% hydrolysis	$[\alpha]^{22}_D$ of 4 (<i>c</i> 1.5, H_2O), deg	$[\alpha]^{22}_D$ of 4 diacetate (<i>c</i> 1.4, $CHCl_3$), deg
2	32	-38.6	
4	58	-38.4	-37.8
6	84	-33.5	-38.3
15	100	-35.0	-38.0

^a See text. ^b Lit.⁶ $[\alpha]^{20}_D -38^\circ$ ($CHCl_3$). ^c The ee of **4** diacetate was >96% in every case.

compound prepared from natural D-xylose, the value in EtOH corresponded to only 86% optical purity if related to the value (-38.3°) reported by a different literature source.⁵ We therefore checked the enantiomeric excess of our product with the chiral lanthanide shift reagent Eu(facam)₃. In the absence of the shift reagent the protons of the two acetyl groups give a single signal at δ 2.02 ppm; when Eu(facam)₃ is added to the racemate, this signal gives rise to four singlets of equal area (figure 1), indicating a different LIS for the two acetyl groups and a chiral discrimination between the enantiomers. The spectrum of the diacetate of the enzymic hydrolysis product in the presence of Eu(facam)₃ showed only two of the four signals (Figure 1) thus confirming an ee of at least 96%. The correlation with D-xylose⁶ further indicated a 3*R*,4*R* configuration.

The enzymic hydrolysis of the two enantiomers of **3** proceeds at very similar rates; this was shown by the lack of a significant change in rate after 50% hydrolysis and by the fact that the epoxide recovered after partial conversion showed no appreciable optical rotation. When it was subjected to acid-catalyzed hydrolysis, the recovered diol **4** had less than 1% optical purity. One can assume that, because of the previously observed preference for ring opening at C(4),⁷ the diol obtained in the chemical hy-

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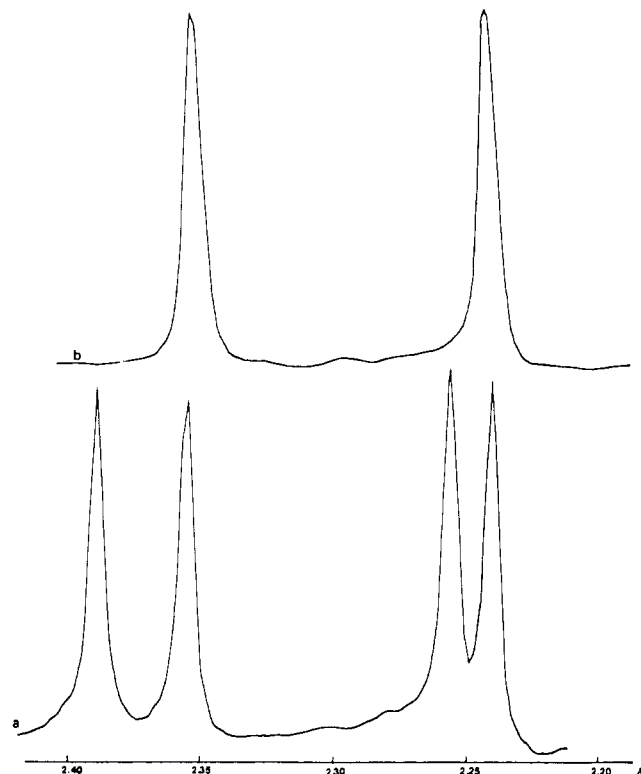
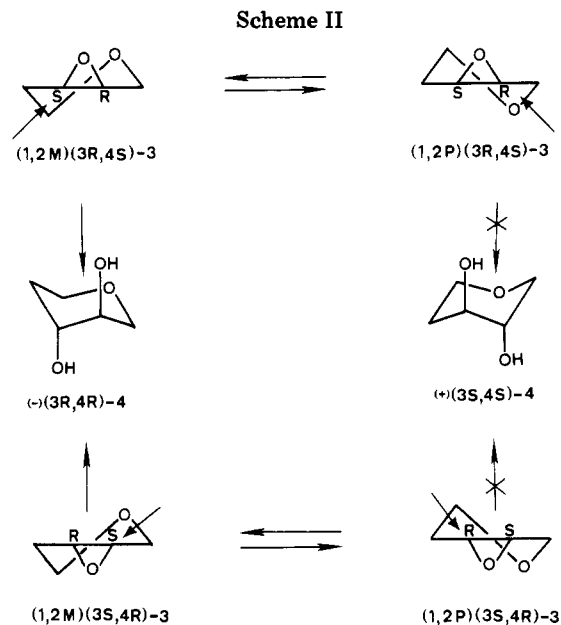


Figure 1. Acetyl proton signals in the NMR spectra of ca. 0.05 M (\pm)-3 diacetate (a) and (-)-3 diacetate, $[\alpha]_D^{25} -38.0^\circ$ (b), in the presence of ca. 0.025 M Eu(facam)₃.

drolysis should have an optical purity about 80% of that of the starting epoxide. Since all enzymic reactions were run on a preparative scale, at substrate concentrations (ca. 0.1 M) well above the K_m of the enzyme, the comparison of the rates for the two enantiomers of 3 is certainly meaningful.

In order to explain the observed steric course, one must assume that *the epoxide rings of the enantiomers of 3 are opened with total but opposite regioselectivity*. The situation is analyzed in Scheme II. Each of the enantiomers of 3 exists in two nonequivalent half-chair conformations. If one assumes, as appears very likely, that both enantiomers are opened in the diaxial fashion,⁸ again, as in the case of 1, a high preference for the 1,2-*M* conformations⁹ in the enzyme active site is exhibited by both, although this involves nucleophilic attack by H₂O at C(4) of the 3*R*,4*S* enantiomer and at C(3) of the 3*S*,4*R* enantiomer. These two positions exhibit a rather different reactivity in nonenzymic reactions, owing to the unfavorable inductive effect of the tetrahydropyran ring oxygen on the breaking of the nearer oxirane C–O bond. A 9:1 preference of attack at C(4) has been observed in the reactions of 3 with hydrogen halides and LiAlH₄⁷ and a 67:33 one in its reaction with Me₂NH.¹⁰ Apparently the enzymic reaction is insensitive to this inductive effect but highly sensitive to the ring helicity. However, the observed ratio of at least 98:2 of the enantiomers of 4 implies a difference of at least 2.4 kcal/mol between the ΔG^\ddagger of the two ap-



proximately equienergetic transition states giving (-)-4 and the one of lower energy between those giving (+)-4. This is hard to explain on the basis of helicity alone if one considers that in the case of cyclohexene oxide $\Delta\Delta G^\ddagger$ is only about 1 kcal/mol.² Some, as yet undefined, kind of interaction of the oxygen in the six-membered ring with the active site must probably enter into the picture.

The major and novel finding of this study is that both enantiomers of substrate 3 react at practically equal rates (no substrate enantiodiscrimination) but exhibit completely opposite regioselectivity in the oxirane ring opening (complete product stereoselectivity). Whereas there exist in the literature reports on each of these two features found separately for different substrates, as far as we were able to establish this is the first case in which both features are present in such a complete way for a single substrate. One must also keep in mind that only relatively few of the literature data were concerned with preparative enzymic hydrolyses involving separation and characterization of the products. Furthermore, in most cases where the optical purities of the product diols were ascertained, this was done on runs in which conversion of epoxide into diol was not complete; if one uses a racemic substrate in which the enantiomers react at different rates, one obtains optical purities of products and recovered substrate that change considerably with the percent conversion. These facts may explain some contrasting reports on the steric course of hydrolase catalyzed hydrolysis of certain substrates (see below). From the presently available picture one can only generalize the fact that the steric course is rather unpredictable, and whereas some rationalization is possible in a narrow range of structurally similar substrates such as the substituted cyclohexene oxides,^{2,4} in other cases such as the polycyclic arene oxides widely different types of behavior have been found. For instance, in the case of benzo[*a*]pyrene 7,8-oxide attack by water takes place with high regioselectivity and almost exclusively at position 8 of both enantiomers, and moderate (72% ee)¹¹ or low (8% ee)¹² product stereoselectivities have been reported. On the other hand, benzo[*a*]pyrene 4,5-oxide was reported to undergo preferential attack at the *S* carbon (position 4 in

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(8) The hypothesis that one of the enantiomers of 3 is opened in the diaxial and the other in the diequatorial fashion is made very unlikely by their identical rates of reaction, which contrast with the very different energy demands of the two processes.

(9) Because of the difference in numbering, the 1,2-*M* conformations of 3 correspond to the 3,4-*M* one of 1.

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one enantiomer and position 5 in the other) of both enantiomers with a fairly high product stereoselectivity (78% ee),¹² a behavior similar to (even if less pronounced than) that of **3**; however, there is a large difference in the rate of hydration of the (+) and (-) enantiomers.¹³ Another report¹¹ on the same substrate, however, claims an ee of only 42% and exclusive attack at C(4) of both enantiomers.

Another case that can in part be related to the hydrolysis of **3** is that of the substituted *cis*-stilbene oxides, where a very high preferences for attack by water at the *S* carbon of either enantiomer of each substrate was observed,¹⁴ independently of the presence of an electron-donating or electron-withdrawing substituent on one of the benzene rings; this proves, as in the case of **3**, that the regioselectivity of hydrolase catalysis, in contrast with that of acidic catalysis, is rather insensitive to electronic effects. On the other hand, differently from what was observed with **3**, relative rates of attack on the two enantiomers ranged from about 3 (*p*-methyl derivative) to about 80 (*p*-nitro derivative).¹⁴

The situation is obviously still rather confused: no single stereochemical scheme can yet account for the behavior of hydrolase catalysis on different substrates. The low substrate specificity, the existence of more than one type of enzyme in the microsomal fractions of the same or different species,¹⁵ and that of a very different hydrolase in the cytosolic fraction¹⁶ can contribute to the existing confusion. It can be hoped, however, that actively progressing studies on the structure of these hydrolases¹⁷ and a rigorous characterization of the hydrolysis products of a wide range of epoxides in preparative scale experiments will soon allow one to clarify completely the mechanism and structural and steric requirements of these important enzymes.

Experimental Section

GLC analyses were carried out with a C. Erba Fractovap Model GV instrument equipped with a 2-m glass column (2.5 mm i.d.) packed with 10% Carbowax 20M on 80–100-mesh silanized Chromosorb. Optical rotations were measured in water (*c* 1.5) for the diol **4** and in chloroform for its diacetate (*c* 1.5) with a Perkin-Elmer Model 141 photoelectric polarimeter. NMR spectra of the diacetate of **4** in the presence of the chiral LSR were taken from CDCl₃ solutions of ca. 0.05 M diacetate and ca. 0.025 M

Eu(facam)₃ with a Varian CFT 20 spectrometer with Me₄Si as an internal standard.

Starting and Reference Materials. (±)-3,4-Epoxytetrahydropyran (**3**) was prepared as previously described.⁷ (±)-*trans*-Tetrahydropyran-3,4-diol (**4**)¹⁸ was obtained by hydrolysis of (±)-**3** with 0.35 M aqueous H₂SO₄ for 20 h at room temperature, followed by neutralization with solid BaCO₃, evaporation of the aqueous solution, extraction of the residue with methanol, evaporation, and purification by Kugelrohr distillation [bath temperature 120 °C (1 mmHg)]. The conversion of (±)-**4** to the diacetate was carried out with excess Ac₂O in pyridine at room temperature for 24 h, followed by the usual workup and purification by Kugelrohr distillation [bath temperature 120 °C (1 mmHg)].

Preparation of Liver Microsomes and Incubation of (±)-3. Livers of male New Zealand white rabbits (2.5–3 kg) pretreated with sodium phenobarbital for 3 days (35 mg/kg/day) by intraperitoneal injection were homogenized in 0.01 M phosphate buffer (pH 7.4) containing KCl (1.15% w/v). The homogenate was centrifuged at 10 000g for 30 min, and the resultant supernatant was further centrifuged at 105 000g for 2 h. The microsomal pellets were resuspended in the same buffer to a final protein concentration of ca. 15 mg/mL and stored at -40 °C.

(±)-3,4-Epoxytetrahydropyran (150 mg) in 1.5 mL of EtOH was added to 6 mL of the microsomal preparation and 8 mL of 0.01 M phosphate buffer containing 1.15% (w/v) KCl (pH 7.4), and the mixture was shaken at 37 °C. At the given times (Table I) the mixture was cooled at 0 °C and centrifuged (8000g), and a small sample of the aqueous solution was analyzed by GLC (low isotherm 90 °C, high isotherm 195 °C, temperature increment 4.5 °C/min, evaporator and detector 220 °C, nitrogen flow 30 mL/min) by using a calibration curve obtained with synthetic mixtures of pure (±)-epoxide and (±)-diol in order to get the percent conversion of **3** into **4**. Unreacted **3** was extracted with petroleum ether (bp 30–50 °C, 4 × 20 mL) with shaking, centrifugation, and freezing. After evaporation of the combined organic extracts, the residue, consisting of pure **3** (GLC), exhibited a zero optical rotation. This product was subjected to acid-catalyzed hydrolysis under the above conditions. The pure (GLC) diol **4** so obtained had [α]_D 0 ± 0.5°. The diol **4** was then extracted with ethyl acetate from the residual aqueous incubation mixture after evaporation in vacuo and was purified by Kugelrohr distillation. After measurement of the optical rotation (Table I), the diol was converted into its diacetate, which was purified by Kugelrohr distillation and subjected to measurement of the optical rotation (Table I). The enantiomeric excess of a sample of diacetate so obtained from each incubation was also checked by NMR in the presence of Eu(facam)₃.

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Registry No. (±)-**3**, 78870-52-7; (±)-*trans*-**4**, 79026-39-4; (-)-(3*R*,4*R*)-**4**, 79026-40-7; (-)-(3*R*,4*R*)-**4** diacetate, 78870-53-8.

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