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The anomalous course of the microsomal transformation of the *exo*-2,3-epoxides of norbornene and norbornadiene. The possible involvement of a general acid activation during the enzymatic hydrolysis of these oxides

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Abstract

The enzymatic hydrolysis of exo-2,3-epoxy-norbornane (**1**) with a crude rabbit liver microsomal preparation occurred with a rearrangement and gave selectively (2*R*,7*S*)-bicyclo[2.2.1]heptane-2,7-diol (**3**), enantiomeric excess (ee) $30 \pm 2\%$. The analogous exo-2,3-epoxy-5-norbornene (**2**) gave, under the same conditions, exclusively endo-6-hydroxymethylbicyclo-[3.1.0]hex-2-ene (**4**), arising from the microsomal catalyzed reduction of the first formed endo-6-formylbicyclo-[3.1.0]hex-2-ene (**5**). A mechanistic explanation for the observed products is proposed.

Keywords: Microsomal biotransformation; mEH; Reaction mechanism

1. Introduction

Chiral epoxides and vicinal diols are very important for organic synthesis and a number of synthetic methodologies and reagents have been developed to prepare enantiomerically pure molecules [1,2]. Although a high enantiomeric purity has often been achieved, during the last years intense research has been devoted to the development of biocatalytic methods for their preparation. Among several types of enzymes that have been employed epoxide hydrolases (EH) are particularly attractive [3], being able to catalyze the hydrolysis of a broad variety of epoxides, without the need of co-factors, affording the corresponding vicinal diols. Furthermore, stereochemical investigations have demonstrated that these enzymes often exhibit a remarkable substrate and/or product enantioselectivity towards racemic epoxides and product enantioselectivity with *meso* epoxides [3]. Owing to their involvement in the metabolism of xenobiotics, these enzymes have been extensively studied in mammals up to the recent years [4,5]. However, a survey of recent literature shows that a number of EH have been purified from other sources, such as bacteria [6], yeasts [7], fungi [8], plants [9] and insects [10].

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Two main EH forms, a microsomal, mEH, and a soluble one, sEH (or cEH), both catalysing the anti addition of water to the oxirane ring and having broad and complementary substrate selectivities, are known [3]. The catalytic mechanism with most substrates involves two discrete chemical steps, at least for murine mEH. The first one is the nucleophilic attack by a carboxylate oxygen of an aspartate residue, generally on the least hindered carbon atom of the epoxide, to give a covalent enzyme-substrate ester intermediate. The second step is the hydrolysis of the ester intermediate by a water molecule and involves a general base and a charge relay residue which assists in the deprotonation of the attacking water molecule and an oxyanion hole to stabilize the tetrahedral intermediate [11–15]. The possibility that the oxirane ring cleavage involves an activation of the epoxide in the transition state, by protonation or hydrogen bonding before or during the nucleophilic attack of the carboxylate anion, has also been repeatedly suggested for soluble EHs, on the basis of the regioselectivity observed for the opening of aryl-substituted epoxides [16], of kinetic studies on the hydrolysis of *para*-substituted aryl oxiranes [8,17], and more recently, of crystallographic data [18,19] and steady-state kinetics of the mutant enzymes [18,20].

With the aim of giving a further contribution to the understanding of the factors determining the enantioselectivity of mammalian EH catalyzed hydrolyses, and to obtain further data about the possibility to apply this method to prepare optically pure diols, we undertook an investigation on *meso* epoxy derivatives of bridged rings, and in particular *exo*-2,3-epoxynorbornane (**1**) and *exo*-2,3-epoxy-5-norbornene (**2**).

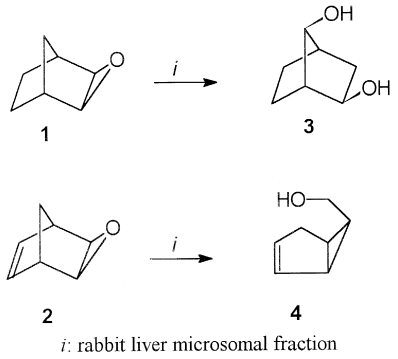
2. Experimental

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The ^{1}H and ^{13}C NMR spectra were registered in $CDCl_3$ with a Bruker AC 200 instrument using TMS as the internal reference. The enantiomeric excess (ee) of the diol **3**, after conversion into the corresponding diacetyl derivative, and alcohol **4** were determined by GLC analysis using a Carlo Erba HRGC 5300 instrument equipped with a 20-m Chiraldex G-TA (ASTEC) column, evaporator and detector 245°C, helium flow 1 ml/min, at the following temperatures: **3**, as diacetyl derivative, 80°C for 20 min, at 6°C/min, and 135°C for 10 min; **4**, 70°C. The yields of recovered **4** were determined by GLC analysis, under the same conditions used for the determination of the ee, by adding cyclohexanol as an internal standard.

2.1. Materials

2,3-Epoxynorbornane (**1**) and norbornadiene were commercial products and were used without further purification. *Endo*-6-Formylbicyclo[3.1.0]hex-2-ene (**5**) was prepared by reaction of commercial norbornadiene with *m*-chloroperbenzoic acid, as previously reported [21]. (±)-Bicyclo[2.2.1]heptane-2,7-diol (**3**) was prepared by acid catalyzed hydrolysis of **1**, as previously reported and was purified by column chromatography [22]. 6-Hydroxymethylbicyclo[3.1.0]-hex-2-ene (**4**) was prepared by reduction of **5**, as previously reported [23].

2,3-Epoxy-5-norbornene (**3**). ANHYDROUS KF (2.10 g, 36 mmol, obtained by heating at 120°C and 0.1 mm Hg for 2 h) was added to a dichloromethane solution (180 ml) of 70% *m*-chloroperbenzoic acid (4.41 g, 18 mmol), previously dried over Sikkon and $MgSO_4$, and the mixture was stirred at room temperature for 30 min. A solution of commercial norbornene (660 mg, 7.2 mmol) in dry CH_2Cl_2 (10 ml) was added and the mixture was stirred for 24 h at room temperature. The insoluble complexes were then filtered off, and the solvent was removed under reduced pressure to give (95% yield) a 95:5 mixture of epoxide **2** and aldehyde **5**, which were identified on the basis of the



Scheme 1. Rabbit liver microsomal catalyzed transformation of epoxides **1** and **2**.

NMR spectra [21]. Epoxide **2**: ^{13}C NMR; δ 40.4 (CH_2); 43.30 (*C*H); 59.5 (CHO): 140.7 (CH═).

2.2. Enzymatic biotransformations. Product isolation

Epoxides **1** and **2**, or aldehyde **5**, as neat liquids (15–65 mg, 0.14–0.6 mmol) were added to 25 ml of the microsomal preparation [24], containing 3–4 mg of protein/ml, and the reaction mixtures were incubated with shaking at 37°C for the time necessary to obtain the appropriate conversion (24 h). In order to achieve a practically complete biotransformation of epoxides a fresh microsomal preparation (5 ml, containing 20 mg of protein/ml) was added after 8 h. The incubation mixtures were then stopped by extraction with ethyl acetate (3×20 ml). The organic phases were diluted to an exactly known volume (50 ml) by dilution with ethyl acetate and a proper amount of the standard was added to an aliquot (5 ml) of these extracts in order to verify the conversion and/or the ee by GLC. The remaining part of the organic phases were evaporated in vacuo and the residues were chromatographed on silica gel columns (70% AcOEt/hexane) to give **3** and **4**, respectively. **3**, $[\alpha]_D = +3.8$ ($c = 1$, MeOH); ee 30% ($[\alpha]_D = +10.5$, (MeOH); ee 81%, [21]). The incubations of **2** were carried out also in the presence of NADH and NADPH (10 mM). Blank experiments were carried out under identical conditions but using microsomal preparation deactivated by boiling.

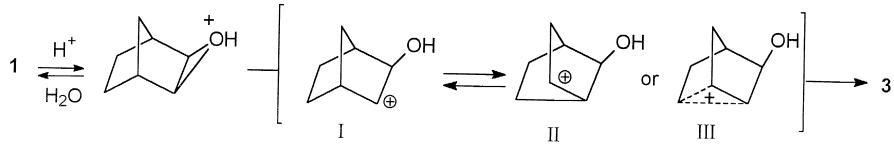
3. Results and discussion

The *meso* epoxides *exo*-2,3-epoxynorbornane (**1**) and *exo*-2,3-epoxy-5-norbornene (**2**), the latter prepared by epoxidation of norbornadiene with MCPBA-KF in anhydrous CH_2Cl_2, were submitted to biohydrolysis with a crude rabbit liver microsomal preparation (Scheme 1) [24]. Incubations were carried out at 37°C and pH 7.4 or 8.7 with 5–24 mM epoxides and 3–4 mg/ml of protein. The reactions were monitored by periodic sampling and followed by GLC analysis. In order to achieve a complete biotransformation of the epoxides incubations were protracted for 24 h by adding a fresh microsomal preparation after 8 h. It is known [3] that *meso* alicyclic epoxides generally are only slowly hydrolyzed by EHs, and this was the case for our compounds. The formed products, extracted with ethyl acetate, were analyzed by NMR and GLC and purified by column chromatography (Table 1). Diol **3** and alcohol **4** were identified, on the basis of their NMR spectra, as the sole products arising by the microsomal transformation of **1** and **2**, respectively. No trace of the corresponding vicinal

Table 1
Product distribution obtained by rabbit liver microsomal catalyzed hydration of epoxides **1** and **2**

Substrate			pH	Product				
	μmol	Conc. (mM)		Yield (μmol)	%	ee	Abs. conf.	
1	150	6	7.4	**3**	105[a]	70	20	(2*R*,7*S*)
1	150	6	8.7	**3**	110[a]	73	20	(2*R*,7*S*)
1	150	6	8.7[b]	**3**	112[a]	75	30	(2*R*,7*S*)
2	125	5	7.4	**4**	62.5[c]	50	5	n.d
2	500	20	7.4	**4**	60[c]	12	7	n.d.
2	600	24	8.7	**4**	300[c]	50	7	n.d.
2	600	24	8.7[b]	**4**	450[c]	75	8	n.d.

[a]After column chromatography.
[b]Microsomal preparation from Phenobarbital pre-treated animals.
[c]Determined by GLC on the crude reaction mixtures.



Scheme 2. Acid catalyzed formation of **3** from **1**.

trans-diols was observed in the crude reaction mixtures analyzed by NMR and GLC.

A very small positive optical rotation was measured for **4**, whereas a larger rotation was found for **3**. Anyway, the ee of diol **3** and alcohol **4** were determined by GLC analysis on a chiral column. No significant enantioselection ($< 10\%$) was found in the formation of **4** and a moderate product enantioselectivity was found ($30 \pm 2\%$ ee) in the case of **3**. On the basis of literature data [25], the excess enantiomer of the latter compound has the (2*R*,7*S*) absolute configuration. Finally, blank experiments carried out with a deactivated microsomal preparation showed that the formation of both products involved at least one enzymatic step. Epoxide **1** was indeed stable under the reaction conditions and was recovered unchanged after 24 h, whereas compound **2** gave a complex product mixture containing only a small amount ($<$ 10%) of *endo*-6-formylbicyclo[3.1.0]-hex-2-en, the precursor of **4** (see below).

The formation of diol **3**, instead of the expected *trans*-2,3-dihydroxynorbornane, by mEH catalyzed hydrolysis of **1** is surely an unexpected result. The rearranged diol **3** and its epimer on C-7 are indeed the main products arising from the acid catalyzed hydrolysis of **1** Scheme 2 [22]. These compounds are formed by the nucleophilic attack on the cation II, produced through a Wagner–Meerwein rearrangement, or on its "non-classical" relative III. Independent of the nature of the intermediate, classical or non-classical, the formation of **3** involves, however, a positively charged intermediate. Since it is known [26] that epoxide **1** is able to give *trans*-2,3-adducts by nucleophilic oxirane ring opening in the absence of acid, the formation of the optically active diol **3**, as the sole product by enzymatic hydrolysis of **1**, strongly suggests the involvement of an electrophilic activation during the mEH catalyzed oxirane ring opening.

Furthermore, it is noteworthy that the intermediacy of III may easily explain the formation of **3** as the sole product in the enzymatic reaction. In III, both the 1 and 2 positions may be attacked by a nucleophile. However, electronic and steric factors arising from the interaction of this intermediate with the enzyme active site may favour the attack on 2, from the *exo* direction, with respect to the attack on 1 (Fig. 1).

Finally, as far as the formation of **4** from **2** is concerned, it is noteworthy that the different oxidation state of this compound suggests the involvement of a reducing system. The possibility that a reductase could be able to give, under the reaction conditions (absence of co-factors), alcohol **4** from the corresponding aldehyde, was therefore checked by incubating **5**, obtained by reaction of norbornadiene with *m*-chloroperbenzoic acid, with the same microsomal preparation under conditions identical to those employed to prepare **4**. In agreement with this hypothesis, **5** was converted into **4** in high yield ($> 80\%$). Furthermore, it was verified that the yield of **4** from **2** was not affected by addition of NADH or NADPH, showing that the amount of co-factors present in the crude preparation was sufficient to convert the first formed carbonyl compound **5** into **4**. Finally, the formation rate of **4**

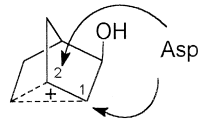
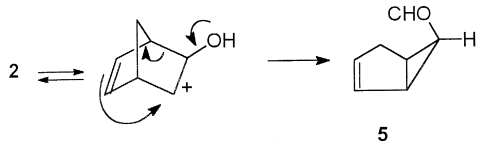


Fig. 1.



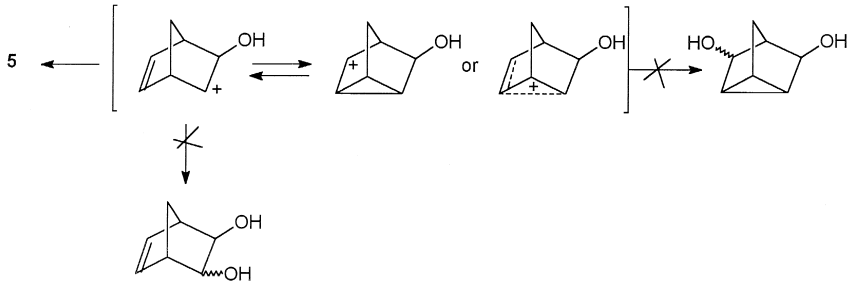
Scheme 3.

was independent of the epoxide concentration while it was affected by the activity of the microsomal preparation (Table 1). When the incubations of **2** were carried out at pH 8.7, using microsomal preparation obtained from Phenobarbital pre-treated rabbits, higher yields of **4** (ca. 75%) were obtained.

On the basis of these data, taking into account the blank experiment, using deactivated microsomal preparations, it is possible suppose that the formation of **5** implies an enzymatic process. Furthermore, also in this case the biotransformation of **2** into the aldehyde **5** should occur through the initial activation of the oxirane oxygen by protonation, followed by oxirane ring opening and skeletal rearrangement. No distinction can be made, however, between a mechanism that involves an intermediate in the carbonyl forming reaction and a concerted mechanism in which the protonated epoxide yields aldehyde **5** in a single step (Scheme 3).

Finally, although the multienzymatic nature of the microsomal preparation does not allow to exclude the possible involvement of an "epoxide isomerase" in this process; however, considering (1) the structural analogy between compounds **2** and **1**, (2) the influence of pH; 8.7 is indeed the optimal pH for mEH, (3) the induction effect; Phenobarbital is a typical inductor of mEH, it is highly probable that the formation of **5** may occur in the mEH active site. In this case, the presence of a second double bond may favour the concerted rearrangement to carbonyl compound **5**, or if an intermediate is involved, may render the nucleophilic attack by aspartate, on the classical or "non-classical" carbocation intermediate, uncompetitive (Scheme 4).

In conclusion, the present results, and in particular the data related to the hydrolysis of **1**, strongly suggest the involvement of an oxirane activation during the microsomal epoxide hydrolase catalyzed hydrolysis. Although the possibility of an acid co-catalysis during the EH catalyzed hydrolysis has been often stressed for the soluble enzyme, this possibility has been generally ruled out for the microsomal form. On the basis of Hammett plots obtained from styrene and *cis*-stilbene oxide derivatives [27], it has been concluded that the rate determining step was a nucleophilic attack not involving a free carbocation. Furthermore, the formation of *trans*-2,3-epoxycyclohexanol from *trans*-3-bromo-1,2-epoxycyclohexane was interpreted [28] as an evidence that the nucleophilic attack occurred at the oxirane ring without apparent electrophilic protonation at the epoxide. These results, however, do not exclude the possibility that, at least with specific substrates, an activation of the oxygen during or before the nucleophilic step may occur. It is noteworthy that from crystallographic data for EH from *A. radiobacter* AD1 [18], supported [20] by steady-state kinetics of the mutated enzyme, and more recently on the basis of the crystal structure of



Scheme 4.

recombinant murine sEH [19], it has been suggested that at least a Tyr residue may act as proton donors in the reaction mechanism of these EHs. Furthermore, since Tyr^{215} is conserved within the EHs, despite of the fact that the similarities among various EHs are often less that 20%, a highly probable activating role of this residue even in other EHs, not excluding the mammalian one, has been proposed [18,20].

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