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Substrate Enantioselectivity in the Rabbit Liver Microsomal Epoxide Hydrolase Catalyzed Hydrolysis of trans and cis 1-Phenylpropene Oxides. A Comparison with Styrene Oxide

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Abstract: A preferential consumption of the (1S,2S) enantiomer of (±)-*trans*-1-phenylpropene oxide (**3**) and of the (1R,2S) enantiomer of *cis*-1-phenylpropene oxide (**5**) is observed during the rabbit liver mEH catalyzed hydrolysis of these epoxides. This preference is, respectively, much lower and much higher than that found for the consumption of the (R) enantiomer in the hydrolysis of (±)-styrene oxide. These results are rationalized in terms of the K_M and V_{max} of the respective reactions.

INTRODUCTION

The microsomal epoxide hydrolase (mEH)[1] is an important enzyme effecting the detoxification of the often cytotoxic, mutagenic and carcinogenic epoxides formed in the cytochrome P-450 promoted oxidation of xenobiotics containing olefinic and aromatic functions in mammals. In spite of its very broad structural selectivity, this enzyme often exhibits a remarkable substrate enantioselectivity,[2] leading to an easy hydrolysis of one enantiomer of a racemic epoxide and to the accumulation of its antipode. This enantioselection can have important toxicological consequences,[3] because the biological activity of epoxides can be markedly dependent on their stereochemistry.[4] A knowledge of the enantioselecting ability of the mEH and of its dependence on substrate structural changes can therefore be very useful to understand the toxicological effects associated to the metabolism of certain xenobiotics. Furthermore, the substrate enantioselectivity of mEH catalyzed hydrolyses can be exploited, in principle, for a small scale kinetic resolution of racemic epoxides[5] and for the obtainment of chiral 1,2-diols in fair to high ee.[2] A knowledge of the stereochemical requirements of the active site of the enzyme, that can be obtained by comparative kinetic studies of the hydrolysis of simple stereoisomeric epoxides,[6] can help in predicting the structural features of substrates that can be successfully subjected to this kinetic resolution.

As a further contribution to this field, we are now reporting on the results of an investigation of the enantioselectivity of the enzymatic hydrolysis of racemic styrene oxide (**1**), *trans*-1-phenylpropene oxide (**3**) and *cis*-1-phenylpropene oxide (**5**).The determination of the kinetic parameters for the single enantiomers of these three substrates has provided a rationalization of the observed enantioselectivities, and has allowed us to obtain further information about the topology of the active site of mEH.

RESULTS AND DISCUSSION

The ability of both enantiomers of *cis*- and *trans*-1-phenylpropene oxide to be substrates for the rabbit mEH was preliminarily investigated in comparison with the two enantiomers of styrene oxide, a typical mEH substrate,[7] by measuring the saturation velocities for their hydrolysis with the same lot of liver microsomal preparation. Incubations were carried out at 37 °C and pH 7.4 for 15-30 min, and were stopped by extraction of the products. Only 1-phenylethane-1,2-diol (**2**), *erythro*-1-phenylpropane-1,2-diol (**4**) and *threo*-1-phenylpropane-1,2-diol (**6**) were detected by HPLC as the hydrolysis products of epoxides **1**, **3** and **5**, respectively. This showed that, as expected, both diastereoisomeric 1-phenylpropene oxides were opened in an anti stereospecific way. The rates of diol production were linear both with the time and with the amount of microsomal protein, and were independent of the epoxide concentrations, indicating substrate saturation. The saturation rates, expressed in nmol/min per mg protein, were the following: (R)-**1**, 7; (S)-**1**, 20; (1R,2R)-**3**, 6; (1S,2S)-**3**, 12; (1R,2S)-**5**, 2; (1S,2R)-**5**, 5. These results established that under enzyme saturation conditions the single enantiomers of each epoxide are hydrolyzed at different rates, the enantiomer having an (S) configuration at the phenyl substituted carbon always reacting 2-3 times as fast as its antipode. Furthermore, these data show that the presence of a trans methyl group has little effect on the V_{max} with respect to styrene oxide, while a cis methyl group reduces by about four times the hydrolysis rates of both enantiomers.

The regiochemistry of the hydrolysis of the (R) and (S) enantiomers of styrene oxide by mEH had been investigated using rat microsomes.[8] A >98% regiospecific attack at C(2) was found in both cases.[8] The same regiospecificity was found in this work by glc analysis of the diacetates of diols obtained from the single enantiomers of styrene oxide on a chiral column. The same method was employed in order to establish the regiochemistry of the ring opening of each enantiomer of *cis*- and *trans*-1-phenylpropene oxide. Only one peak, corresponding respectively to the diacetate of (1R,2S)-**4** or (1R,2R)-**6**, was found for the hydrolysis product of (1R,2R)-**3** and (1R,2S)-**5**. Two peaks in a 88:12 ratio, corresponding to the diacetates of (1S,2R)-**4** and (1R,2S)-**4**, were observed for the product arising from (1S,2S)-**3**, and two peaks in a similar 90:10 ratio, corresponding to the esters of (1S,2S)-**6** and (1R,2R)-**6**, were found in the case of (1S,2R)-**5**. Thus, while the ring opening of (1R,2R)-**3** and (1R,2S)-**5** occurred by a >98% regiospecific attack at C(2), the opening of (1S,2S)-**3** and (1S,2R)-**5** involved an only 88-90% regioselective attack at the same C(2), a 12-10% opening occurring by an unusual attack[9] at the more hindered phenyl substituted oxirane carbon (Scheme). An 89:11 ratio for attack at C(2) and C(1) had been determined by mass spectrometry for the hydrolysis of ^{18}O enriched racemic epoxide **5** by rabbit liver mEH.[10]

The substrate enantioselectivities of the mEH catalyzed hydrolyses were investigated by incubating 5 mM (±)-**1** and (±)-**5** and 5 or 40 mM (±)-**3** with a microsomal preparation containing 5 mg/ml of protein. Incubations were stopped at different times and the formed diols and the residual epoxides were quantified by HPLC analysis of the extracts. The ee's of epoxides **1**, **3** and **5** were then obtained directly, and those of diols **2**, **4** and **6** after acetylation, by glc using the chiral column. The results are reported in Table 1.

Scheme

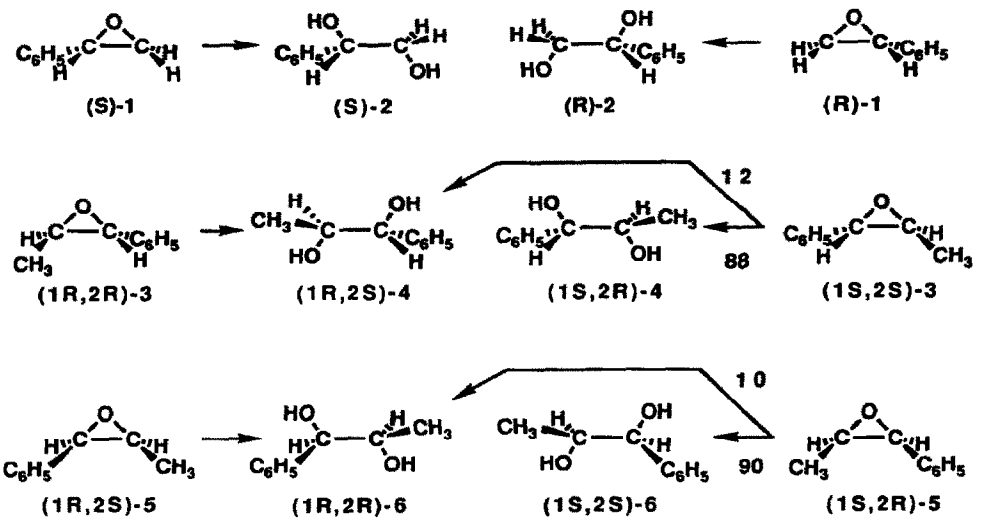


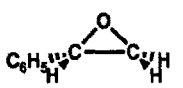
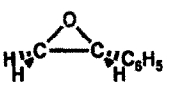
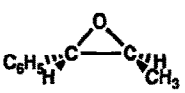
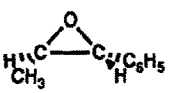
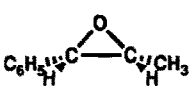
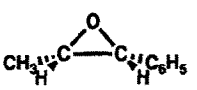
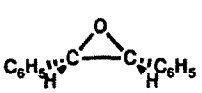
Table 1. Enantiomeric excesses (ee) in the rabbit liver mEH catalyzed hydrolysis of racemic epoxides **1**, **3** and **5**.

Epoxide	Unreacted %	e e	Abs. Conf.	Diol	Yield %	e e	Abs. Conf.
1[a]	86	20	S	**2**	12	>98	R
	71	30	S		26	76	R
	60	46	S		37	68	R
	42	76	S		56	45	R
	22	>98	S		77	25	R
3[a]	88	6	1R,2R	**4**	10	23	1S,2R
	64	14	1R,2R		33	10	1S,2R
	55	24	1R,2R		44	8	1S,2R
	42	30	1R,2R		56	6	1S,2R
3[b]	89	4	1R,2R	**4**	9	6	1S,2R
	66	6	1R,2R		32	4	1S,2R
5[a]	84	20	1S,2R	**6**	15	>98	1R,2R
	70	45	1S,2R		28	>98	1R,2R
	50	>98	1S,2R		50	>98	1R,2R
	25	>98	1S,2R		74	38	1R,2R

[a] Reactions carried out at 5 x 10^{-3} M substrate concentration with microsomal preparations containing 5 mg/ml of protein.
[b] Reactions carried out at 4 x 10^{-2} M substrate concentration.

The data of Table 1 show for (±)-styrene oxide (**1**) a remarkable preference for the hydrolysis of the (R) enantiomer, leading to (R)-**2**, in spite of the fact that this enantiomer was hydrolyzed at a V_{max} lower than that of its (S) antipode. This behaviour is analogous to that found for the hydrolysis of (±)-**1** by rat liver microsomes.[8] The introduction of a methyl group β and trans to the phenyl ring, while not producing relevant changes in the V_{max} of the two enantiomers, caused a dramatic drop in the substrate enantioselectivity. Furthermore, the residual enantioselectivity favoured the hydrolysis of the (1S,2S) enantiomer, having the higher V_{max}. This enantioselectivity was practically suppressed at the higher substrate concentration, i.e. under enzyme saturation conditions (see infra). On the other hand, a *cis*-β-methyl substituent, which induced a larger decrease in the V_{max} of both enantiomers, increased the substrate selection in favour of the (1R,2S) enantiomer of **5**, having the lower V_{max}, so much that at half reaction both the (1S,2R) epoxide and the (1R,2R) diol were obtained in a practically enantiomerically pure form, a complete kinetic resolution being thus easily achieved. It is noteworthy that of the two enantiomers of (±)-*cis*-2-ethyl-3-methyloxirane only the (2R,3S) one, having the same configuration as (1R,2S)-**5**, has been reported[11] to be hydrolyzed by the rat mEH, while both enantiomers of the (±) trans oxirane react, although with some preference for the (2S,3S) form corresponding to (1S,2S)-**3**. Thus, epoxides differing for the presence of a phenyl group in place of an ethyl group on the oxirane ring are hydrolyzed with similar substrate enantioselectivities.

Table 2. K_M and V_{max} values for the rabbit liver mEH catalyzed hydrolysis of epoxides **1**, **3**, **5** and **7**.[a]

Epoxide	K_M	V_{max}	Epoxide	K_M	V_{max}
 (S)-**1**	$2.2\pm0.2 \times 10^{-4}$	22±3	 (R)-**1**	$8.4\pm0.8 \times 10^{-5}$	6.9±1.0
 (1S,2S)-**3**	$3.2\pm0.3 \times 10^{-3}$	13±2	 (1R,2R)-**3**	$4.7\pm0.5 \times 10^{-3}$	5.2±0.8
 (1S,2R)-**5**	$1.38\pm0.2 \times 10^{-3}$	5.8±0.9	 (1R,2S)-**5**	$2.03\pm0.2 \times 10^{-5}$	1.8±0.3
			 **7**	$5.3\pm0.4 \times 10^{-5}$ [b]	13±0.2[b]

[a] K_M are expressed in M, V_{max} in nmol/min x mg of protein.

[b] Taken from ref. 12.

In order to rationalize the different substrate enantioselectivities found for the mEH catalyzed hydrolyses of (±)-**1**, (±)-**3** and (±)-**5**, a knowledge of the K_M values for the single enantiomers of each epoxide was needed. The initial hydrolysis rates were measured as a function of the substrate concentration in the following concentration ranges: 8×10^{-2} - 3.6×10^{-3} mM for (R)-**1** and (S)-**1**, 1 - 0.1 mM for (1R,2R)-**3** and (1S,2S)-**3**, 0.8 - 6×10^{-2} mM for (1S,2R)-**5**, and 8×10^{-2} - 3.6×10^{-3} mM for (1R,2S)-**5**. The K_M and V_{max} values, obtained from the respective Lineweaver-Burk plots, are reported in Table 2, that also includes the values previously determined[12] for the mEH catalyzed hydrolysis of *cis*-stilbene oxide.

The data of Table 2 show that the ratio of K_M for the (S) and (R) enantiomers of styrene oxide is 2.6. Although smaller, this ratio is in the same direction of that (5.3) found[8] for the rat liver mEH catalyzed hydrolysis of **1**, and indicates that also with the rabbit enzyme the kinetic resolution observed for (±)-**1** is due to a partial competitive inhibition by the (R) enantiomer, forming a more stable enzyme-substrate complex, on the hydrolysis of the (S) antipode. The K_M of both (1S,2S)-**3** and (1R,2R)-**3** increases with respect to (S)-**1** and (R)-**1**, and their ratio is nearer to unit. No competitive inhibition of one enantiomer on the other is possible in this case, and no relevant kinetic resolution is observed, even if minor differences are found under non-saturating and saturating conditions. The K_M ratio increases instead to 68 for (1S,2R)-**5** and (1R,2S)-**5**. The latter exerts therefore a very strong competitive inhibition on the hydrolysis of the former enantiomer, which starts to be hydrolyzed only when practically all (1R,2S)-**5** has been consumed.

The data also show that, with the exception of (1R,2R)-**3**, all forms having a phenyl group on the right back side of the epoxide ring, when the substrate is viewed with the oxygen on the topside, have similar K_M values around 10^{-5} M. This is in agreement with the previously proposed topology of the mEH enzyme active site,[6,13,14] in which the presence of a hydrophobic pocket situated to the right back side and able to accommodate the phenyl group should allow a preferential binding of the forms having an (R) configuration at the benzylic carbon. Furthermore, the fact that (1R,2S)-**5** and **7** have K_M values comparable to that of (R)-**1** shows that the presence of either a methyl or a phenyl group on the left back side of the epoxide ring does not affect the accommodation of the substrate in the active site. This suggests that a large area, able to accommodate these groups but providing only a low contribution to the binding, should be present on the left back side of the oxygen binding site. This is consistent with the higher K_M values of (S)-**1**, (1S,2R)-**5** and (1S,2S)-**3**, in which the phenyl group is lacking on the right back and present on the left back side. Finally, the higher K_M of (1R,2R)-**3** relative to (1R,2S)-**5**, (R)-**1** and **7** indicates that a substituent on the left front side exerts an unfavourable interaction with the corresponding area of the active site. This conclusion is in agreement with the inference based on the kinetic parameters for the mEH catalyzed hydration of epoxides derived from benz[*a*]anthracene.[14]

CONCLUSIONS

The results of this investigation show that both cis and trans 1,2-disubstituted epoxides are hydrolyzed by the microsomal epoxide hydrolase to diols in an anti stereospecific and regiospecific or highly regioselective way. Furthermore, while a moderate or low substrate enantioselectivity is found with racemic trans epoxides, the mEH catalyzed hydrolysis of cis epoxides occurs with a very high enantioselectivity, due to the particular topology of the enzyme active site. This feature can presently be exploited only for small scale kinetic

resolutions, but may become of much greater utility when EH enzymes produced by biotechnologies will be easily available in large amounts.

EXPERIMENTAL

HPLC analyses were performed with a Waters 660E instrument equipped with a 990 diode array detector allowing to obtain the UV-vis spectra of each eluted compound, and with a Nitrile S5 column, using hexane/2-propanol (95:5) as the eluent at a flow rate of 1 ml/min. The yields of diols were evaluated by using calibration curves obtained for each compound monitoring at 220 nm. Glc analyses were carried out with a C. Erba HRGC 5300 instrument equipped with a 15 m Chiraldex G-TA (ASTEC) column, evaporator and detector 245 °C, nitrogen flow 1 ml/min, at the following temperatures (k'_1 and α for the two enantiomers are reported in parentheses): 95 °C for **1** (4.7, 1.2), **3** (6.8, 1.3) and **5** (6.5, 1.6); 120 °C for the diacetates of **2** (23, 1.2), **4** (20, 1.06) and **6** (19, 1.05).

Materials

Commercial *trans*-1-phenylpropene (Aldrich, 99%), (±)-styrene oxide (Aldrich, 97%), (R)-(+)-styrene oxide (Aldrich, 98%), (S)-(-)-styrene oxide (Aldrich, 98%), (1R,2R)-(+)-*trans*-1-phenylpropene oxide (Aldrich, 98%) and (1S,2S)-(-)-*trans*-1-phenylpropene oxide (Aldrich, 98%) were used after distillation. Commercial (R)-(-) and (S)-(+)-1-phenyl-1,2-ethanediol (Fluka, >98%) were used as reference compounds for the hydrolysis of styrene oxides. *cis*-1-Phenylpropene was prepared from 1-phenyl-1-propyne (Aldrich, 99%) as previously reported.[15] (±)-*trans*-1-Phenylpropene oxide and (±)-*cis*-1-phenylpropene oxide were obtained by epoxidation of the corresponding olefins with *m*-chloroperoxybenzoic acid in dichloromethane for 24 h, followed by distillation. Optically pure (1R,2S)-(-)-*cis*-1-phenylpropene oxide and (1S,2R)-(+)-*cis*-1-phenylpropene oxide were respectively prepared from commercial (1R,2R)-(-)- and (1S,2S)-(+)-ψ-ephedrine (Sigma), as reported.[16] (±)-*erythro*-1-Phenyl-1,2-propanediol and (±)-*threo*-1-phenyl-1,2-propanediol were respectively obtained by hydrolysis of epoxides (±)-**3** and (±)-**5** with 0.05 N $HClO_4$ in 60:40 THF/water, followed by crystallization from chloroform.[16]

Microsomal and cytosolic preparations.

Livers of New Zealand white rabbits (2.5- 3 kg) were removed and homogenized in four volumes of 0.05 M Tris-HCl buffer (pH 7.4) containing KCl (1.15% w/v), and the resulting suspensions were centrifuged at 9,000*g* for 30 min. The supernatant was further centrifuged at 125,000*g* for 1.5 h, and the microsomal pellets were resuspended in the same buffer to a final protein concentration of 16-25 mg/ml. This microsomal preparation was used directly or stored at -80 °C.

Enzymatic hydrolyses.

Determination of K_M and V_{max}

Aliquots (20 µl) of ethanolic stock solutions of epoxides (+)-**1**, (-)-**1**, (+)-**3**, (-)-**3**, (+)-**5** and (-)-**5** of appropriate concentrations were added to 3 ml of diluted microsomal preparation containing 2-6 mg protein/ml, preincubated ad 37 °C for 5 min. The final substrate concentrations in the incubation mixtures are reported in the text. After shaking at 37 °C for 5, or 10, or 20 min, the reactions were stopped by addition of ethyl acetate (3 ml) and the mixtures were centrifuged at 5000 rpm. The precipitates were resuspended in ethyl acetate (3 ml)

and further centrifuged. The combined supernatants were reduced to an exactly known volume and the produced diols **2**, or **4**, or **6** were quantified by HPLC analysis. Initial rates of hydrolysis of each epoxide were calculated at each substrate concentration from the amounts of diols formed. Each experiment was carried out in triplicate and the average data were used to obtain Lineweaver-Burk plots from the slope and intercept of which the K_M and V_{max} reported in Table 2 were calculated.

The V_{max} for the hydrolysis of the pure enantiomers of all epoxides were also independently measured under enzyme saturation conditions as follows. Aliquots (20 μl) of a 0.75 to 3 M stock solutions of epoxides in ethanol were added to 2 ml of diluted microsomal preparation containing 1.25, 2.5, or 5 mg protein/ml. Incubations were protracted for 15 and 30 min, and stopped by addition of ethyl acetate (2 ml) followed by centrifugation, and the produced diols were quantified as described above. All experiments were carried out in duplicate. In all cases the diol formation was linear with the time and the protein concentration, and independent from the substrate concentration. Blank experiments carried out under identical conditions but using boiled microsomal preparations showed that no spontaneous hydrolysis occurred under the employed conditions. Average saturation velocities for the enzymatic hydrolyses are quoted in the text.

Determination of the regioselectivity

Enantiomerically pure (+)-**1** and (-)-**1** (0.025 mmol) or (+)-**3**, (-)-**3**, (+)-**5** and (-)-**5** (0.75 mmol) were added to a microsomal preparation (5 ml) containing 5 mg protein/ml, and the mixtures were incubated at 37 °C for 30 min in the case of **1** and for 3 h in all other cases. The reactions were then stopped by extraction of the unreacted epoxides with hexane followed by extraction of the formed diols with ethyl acetate. The absolute configurations of the isolated diols **4** and **6** were checked by comparing their optical rotations with those reported for (+)-(1S,2R)-**4**, (-)-(1R,2S)-**4**, (+)-(1S,2S)-**6** and (-)-(1R,2R)-**6**.[17] The diols were then trasformed into their diacetyl derivatives by treatment with acetic anhydride in pyridine and subjected to glc analysis using the chiral column for the determination of the enantiomer ratios, from which the regioselectivities reported in the Scheme were obtained.

Determination of the enantiomeric excesses

Aliquots (20 μl) of ethanolic stock solutions of (±)-**1** (0.75 M) or of (±)-**3** (0.75 or 6 M), or of (±)-**5** (0.75 M) were added to 3 ml of microsomal preparation containing 5 mg protein/ml and the mixtures were incubated at 37 °C. The final substrate concentration was 5 mM for (±)-**1** and (±)-**5** and 5 or 40 mM for (±)-**3**. At prefixed times (5 to 420 min) the reactions were extracted with ethyl acetate (3 x 3 ml) containing a proper amount of *p*-methoxybenzaldehyde as internal standard for glc analysis. The extracts were analyzed by HPLC for the quantification of the formed diols, and by glc for the quantification of the residue epoxides and for the determination of their enantiomeric excesses. The absolute configurations of the two enantiomers of the unreacted epoxides were directly determined by comparison of their retention times with those of authentical samples of (+)-**1**, (-)-**1**, (+)-**3**, (-)-**3**, (+)-**5** and (-)-**5**. The extracts were then evaporated, and acetic anhydride in pyridine was added to the residues in order to transform the diols into their diacetyl derivatives which were subjected to glc analysis for the determination of the ee. Each experiment was carried out in triplicate. The average results are reported in Table 1.

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