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Kinetic Resolution by Epoxide Hydrolase Catalyzed Hydrolysis of Racemic Methyl Substituted Methylenecyclohexene Oxides

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Abstract: A complete kinetic resolution of 2,2-dimethylmethylenecyclohexene oxide and a partial resolution of the *cis* and *trans*-2-methylmethylenecyclohexene oxides, but not of their 3-methyl substituted isomers, have been achieved by partial hydrolysis to the corresponding diols using the microsomal epoxide hydrolase. No substrate enantioselectivity was in found for the hydrolysis of all these substrates by the cytosolic epoxide hydrolase.

INTRODUCTION

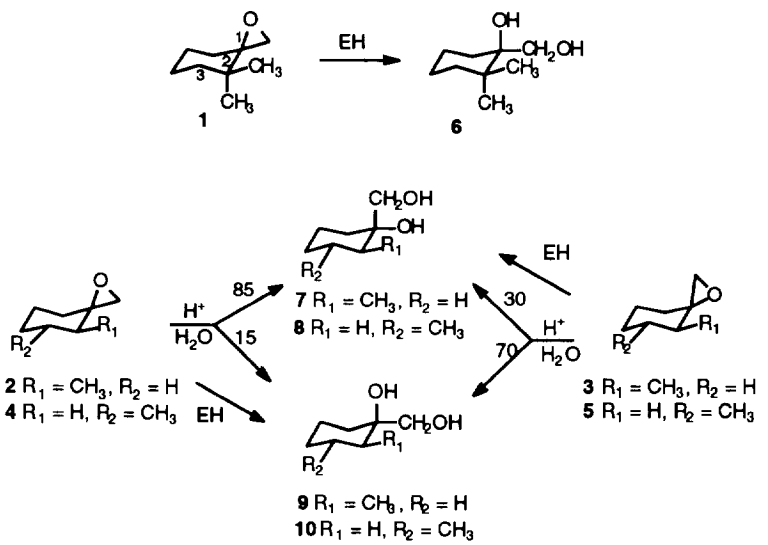
The microsomal and cytosolic epoxide hydrolases (mEH and cEH) are important enzymes[1,2] catalyzing a trans addition of water to a large variety of the frequently mutagenic and carcinogenic epoxides[3] formed in the Cytochrome P-450 catalyzed biooxidation of xenobiotics containing olefinic and aromatic functionalities.[4] Single turnover experiments performed in $H_2{}^{18}O$ have demonstrated[5] that the basic catalytic mechanism of mEH involves a diol monoester intermediate, which is subsequently hydrolyzed to diol with the assistance of an histidine residue. Stereochemical investigations have also shown that the cytosolic enzyme exhibits a substrate and product enantioselectivity of the same type as that shown by mEH with racemic monosubstituted aliphatic epoxides[6] as well as with meso epoxides,[7] an indication for similar mechanisms and steric requirements of the active sites of the two enzymes. On the other hand, cEH has recently been found to exhibit a markedly different regio- and enantioselectivity with phenyl substituted oxiranes.[8] This has suggested[8a] that an oxirane protonation, favouring the opening at a benzylic carbon, may be more important for cEH than for mEH.

In order to have further information on the factors affecting the regio- and stereochemical course of the mEH and cEH catalyzed hydrolysis of racemic oxiranes and to check the possibility of using these processes for small scale kinetic resolutions, the reactions of both enzymes with the five racemic epoxides **1-5**, deriving from methylenecyclohexanes, have been investigated and compared with the previously examined ones of the analogous acyclic 2-methyl, 2-alkyl substituted oxiranes.

RESULTS AND DISCUSSION

The ability of the differently substituted racemic exocyclic oxiranes **1-5** to be substrates for the rabbit liver mEH and cEH was preliminarily checked. Incubations were carried out at 37 °C and pH 7.4 at several substrate and protein concentrations and stopped by extraction with ethyl acetate. The organic phases were analyzed by GLC before and after derivatization and addition of an appropriate standard. At variance with the $HClO_4$ catalyzed oxirane ring opening, which led to mixtures of diols **7** and **9** from epoxides **2** and **3**, and to **8** and **10** from **4** and **5** (Scheme 1), only diols **9** and **7** were respectively obtained from epoxides **2** and **3**, and diols **10** and **8** were respectively formed from **4** and **5**, both with mEH and with cEH. Diols **7-10** were identified by correlation with the corresponding 1-methylcyclohexanol derivatives, as reported.[9]

Scheme 1



The diol production was always linear with time and with the microsomal or cytosolic protein amount, and was independent of the substrate concentration, showing that saturating conditions had been used. The average rates measured using the microsomal preparation were: **1**, 70; **2**, 45; **3**, 60; **4**, 50; **5**, 60 nmol/(mg protein x min). Those found with the cytosolic preparation were **1**, 10; **2**, 13; **3**,15; **4**, 10; **5**, 10 nmol/(mg protein x min). Although these experiments could not give true kinetic parameters, since each employed epoxide consisted of two competing enantiomeric substrates, they show that all examined compounds were excellent substrates for mEH and fairly good ones for cEH.

In order to determine the substrate and product enantioselectivity, incubations of epoxides **1-5**, carried out under saturation conditions, were stopped at different times and the residual epoxides and the formed diols were analyzed by GLC using a chiral column. The ees of epoxides **1-5** were obtained directly, those of diols **6-10** after acetylation or trifluoroacetylation. The results are reported in Table 1.

Table 1. Enantiomeric excesses and absolute configurations of epoxides and diols obtained by partial mEH and cEH catalyzed hydrolysis of racemic substartes **1-5**.

Epoxide	Enzyme	Unreacted Epoxide %	ee[a] %	Abs. Conf.	Formed Diol	Yield %	ee[a] %	Abs. Conf.
1	M[b]	75	50	S	**6**	23	>95	R
1	M[b]	50	>95	S	**6**	48	>95	R
1	M[b]	25	>95	S	**6**	75	33	R
2	M[b]	80	25	1S,2R	**9**	18	95	1R,2S
2	M[b]	58	56	1S,2R	**9**	40	46	1R,2S
2	M[b]	20	80	1S,2R	**9**	81	20	1R,2S
3	M[b]	78	20	1S,2S	**7**	20	90	1R,2R
3	M[b]	50	40	1S,2S	**7**	50	40	1R,2R
3	M[b]	20	60	1S,2S	**7**	80	15	1R,2R
4	M[b]	78	<5		**10**	20	<5	
4	M[b]	50	<5		**10**	47	<5	
5	M[b]	70	<5		**8**	28	<5	
5	M[b]	50	<5		**8**	48	<5	
1	C[c]	50	<5		**6**	47	<5	
2	C[c]	52	<5		**9**	50	<5	
3	C[c]	50	<5		**7**	50	<5	
4	C[c]	48	<5		**10**	51	<5	
5	C[c]	50	<5		**8**	48	<5	

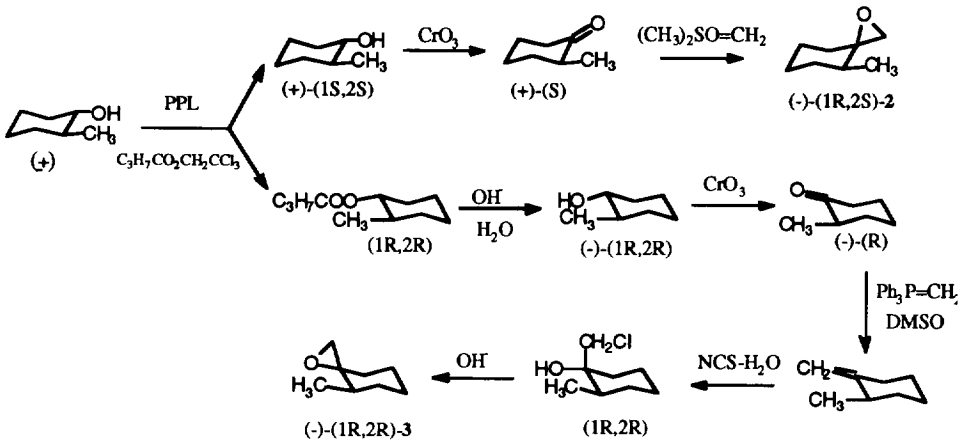
[a]Average error of three determinations: ±2%. At the same percent hydrolysis the ees obtained with different rabbit liver microsomal preparations were reproducible within ±2%.

[b]Reactions carried out at 20 mM substrate concentration with microsomal preparations containing 5 mg of protein/ml.

[c]Reactions carried out at 20 mM substrate concentration with cytosolic preparations containing 15 mg of protein/ml.

The absolute configurations of the two enantiomers of the unreacted epoxides **2** and **3** were determined by comparison of their GLC retention times with those of optically active samples of (-)-(1R,2S)-**2** and (-)-(1R,2R)-**3**. These epoxides were prepared, respectively, from (+)-(S)-2-methylcyclohexanone (40% ee), and (-)-(R)-2-methylcyclohexanone (95% ee), obtained by oxidation of (1S,2S) and (1R,2R)-*trans*-2-methylcyclohexanol arising from a kinetic resolution of the racemic alcohol by transesterification with 2,2,2-trichloroethanol butyrate using Porcine Pancreatic Lipase.[10] The (S)-ketone was directly transformed into (1R,2S)-**2** by reaction with dimethyloxosulfonium methylide, using the Corey procedure.[11] The (R)-ketone was instead converted to 2-methylmethylenecyclohexane by the Corey modification of the Wittig reaction,[12] followed by N-chlorosuccinimide-water addition and dehydrochlorination[13] to (-)-(1R,2R)-**3** (Scheme 2).

Scheme 2



The absolute stereochemistry of epoxide (+)-**1**, recovered from partial mEH catalyzed hydrolysis of the racemic substrate, was established by CD measurements. The CD spectra of optically active oxiranes have been studied quite extensively in the last years, and a chirality (quadrant) rule has been formulated,[14] which correlates the absolute configuration of the epoxide with the sign of the lowest energy transition (a Rydberg n(O)→ 3s excitation peaked at about 175 nm) of the oxirane chromophore. The four quadrants around the chromophore as well as the signs of the CD contributions are shown in Figure 1 for epoxide **1**. A sample of (+)-**1** (hexane) showed in this solvent a positive CD down to 180 nm:

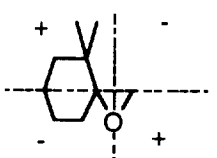


Figure 1. Quadrant projection of epoxide (S)-**1**

this is certainly the long-wavelength tail of the n(O) → 3s Cotton effect at 175 nm. The C(1) atom becomes a stereogenic centre owing to the double methyl substitution at C(2). In order to have a positive CD these substituents must be located in the upper left quadrant as reported in the Figure. This corresponds to an (S) absolute configuration at C(1).

The absolute configurations of the diols reported in Table 1 were derived from those of their parent epoxides, the relative configurations being known.

All mEH and cEH reactions involved retention of configuration at the chiral oxirane carbon. Since with both enzymes water attack has always been found to occur in an anti stereospecific way, a syn ring opening at the disubstituted oxirane carbon can reasonably be excluded and the opening must occur at the unsubstituted carbon. This is consistent with all previous findings for mEH catalyzed reactions of non-symmetrically substituted oxiranes, in which water attack always occurs at the less substituted or less hindered carbon.[8,15,16] On the other hand, the stereochemical outcome observed for the presently reported cEH

reactions indicates that the requisite of charge stabilization in the transition state for ring opening, suggested by the course of the cEH catalyzed opening of phenyl substituted oxiranes,[8a] is overbalanced by the steric effects present at the quaternary carbon.

The data of Table 1 show that no appreciable enantioselection occurs in the cEH catalyzed hydrolysis of epoxides **1**-**5**. The position and the number of substituents on the cyclohexane ring affects instead the enantioselectivity of the mEH catalyzed hydrolyses. In fact, whereas no enantioselection was found in the mEH catalyzed reactions of epoxides **4** and **5**, bearing a methyl group at the cyclohexane C-3, the same substituent at C-2 determined a significant substrate enantioselection in favour of (1R,2S)-**2** and (1R,2R)-**3**. On the other hand, the presence of two methyl groups at C-2 led to a complete enantioselection, with a >95% ee of both (S)-**1** and (R)-**6** obtained at 50% conversion. A typical biphasic profile, analogous to those previously obtained for other racemic substrates undergoing a kinetic resolution,[15,17,18] was found for the hydrolysis of (±)-**1** (Figure 2), showing that diol (R)-**6** was produced from (R)-**1** in the first half of the reaction, at a rate lower than that at which its S-enantiomer was formed from (S)-**1** during the subsequent course of the hydrolysis. This is consistent with the (R) enantiomer of the substrate fitting better than the (S) one into the mEH active site, the former acting as a competitive inhibitor for the hydrolysis of the latter.

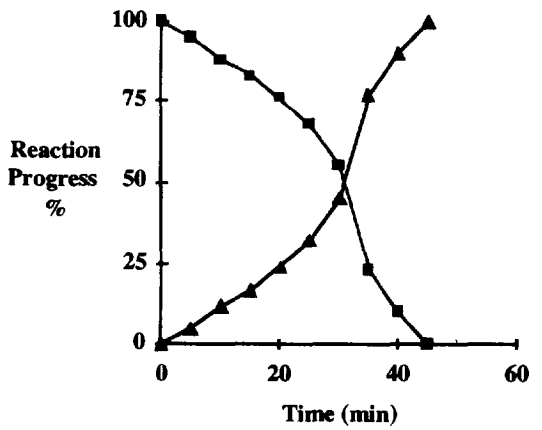


Figure 2. Time course of the mEH-catalyzed hydrolysis of (±)-**1**. Residual (+)-**1** (_), formed (-)-**6** ().

These results are consistent with those reported for racemic aliphatic monosubstituted oxiranes,[15b] pointing to the formation of a more stable complex of the enzyme with the (R)-enantiomers of epoxides having a bulky branched group at one oxirane carbon. They are, however, at variance with the lack of enantioselectivity found in the mEH catalyzed hydrolysis of 2-methyl-2-*tert*-butyloxirane,[19] an acyclic analogue of **1**. A different insertion of the cyclic and acyclic oxirane in the enzyme active site, for which two hydrophobic pockets of different shape and size, situated at the right and left back side of the epoxide binding site, have been postulated,[8b] may be responsible for the observed difference in enantioselection.

Finally, it must be remarked that, in agreement with the generally lower enantioselectivity observed with cEH with respect to mEH, no enantioselection has been found in all the presently reported reactions carried out with the cytosolic enzyme. This once again indicates that the microsomal enzyme is more useful

for small scale kinetic resolutions of epoxides, and that it may became of much greater utility when the enzyme produced by biotechnologies will become available in large amounts.

EXPERIMENTAL SECTION

Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The ^1H and ^{13}C NMR spectra were registered in $CDCl_3$ with a Bruker AC 200 instrument using TMS as the internal reference. CD spectra were registered with a JASCO J 600 spectropolarimeter. GLC analyses were carried out with a C. Erba HRGC 5300 instrument equipped with a 10 m Chiraldex G-TA (ASTEC) column, evaporator and detector 240 °C, helium flow 1 ml/min, at the following temperatures :**1**, **2**, **4**, 75 °C; **3**, 70 °C, **6-9**, as diacetyl derivatives, 104 °C, **10**, as bis(trifluoromethyl)derivative, 84 °C.

Materials

Epoxides 1-5. Epoxide (±)-**1** and the diastereoisomerically pure (GLC) epoxides (±)-**2** and (±)-**4** were respectively prepared in > 80% yield from commercial 2,2-dimethylcyclohexanone, 2-methylcyclohexanone and 3-methylcyclohexanone using dimethyloxosulfonium methylide, according the Corey's procedure,[11] followed by column chromatography over silica gel.

2-Methylmethylenecyclohexane and 3-methylmethylenecyclohexane were synthesized from the corresponding cyclohexanones using Corey's procedure.[12] They were converted to the diastereoisomerically pure (±)-**3** and (±)-**5** in > 80% yield by reaction with N-chlorosuccinimide in 7:3 THF-water,[13] followed by dehydrochlorination of the formed chlorohydrins by titration with NaOH (0.1 N) in 2-propanol-water (3:2), and purification by column chromatography.

The stereochemistry of epoxides **2-5** was established through conversion into the corresponding methyl carbinols by reduction with lithium aluminium hydride.[13] **1**, oil: ^1H NMR δ 0.88 (s, 3H, CH_3); 0.92 (s, 3H, CH_3); 1.30-1.70 (m, 8H, cyclohexane CH_2); 2.44 (d, 1H, J = 4.6 Hz, CH_2O); 2.80 (d, 1H, J = 4.6 Hz, CH_2O).^{13}C NMR δ 22.51 (CH_3); 23.85 (C5); 25.39 (C3); 25.64 (CH_3); 31.90 (C4); 39.62 (C6); 51.71 (CH_2O) . **2**, oil: ^1H NMR δ 0.83 (d, 3H, J = 6.7 Hz, CH_3); 1.40-1.70 (m, 9H, cyclohexane CH); 2.48 (d, 1H, J = 4.6 Hz, CH_2O); 2.75 (d, 1H, J = 4.6 Hz, CH_2O). ^{13}C NMR δ 15.66 (CH_3); 24.51 (C5); 26.17 (C3); 25.29 (C4); 33.22 (C6); 35.22 (C2); 53.22 (CH_2O),61.5 (C1). **3**, oil: ^1H NMR δ 0.82 (d, 3H, J = 6.7 Hz, CH_3); 1.20-1.80 (m, 9H, cyclohexane CH); 2.44 (d, 1H, J = 4.5 Hz, CH_2O); 2.69 (d, 1H, J = 4.5 Hz, CH_2O). ^{13}C NMR δ 15.66 (CH_3); 24.63 (C5); 26.27 (C4); 34.09 (C3 and C6); 36.33 (C2); 51.34 (CH_2O); 62.54 (C1). **4**, oil: ^1H NMR δ 0.91 (d, 3H, J = 6.4 Hz, CH_3); 1.20-1.80 (m, 9H, cyclohexane CH); 2.61 (s, 2H, CH_2O). ^{13}C NMR δ 22.85 (CH_3); 24.02 (C5); 31.10 (C3); 33.24 (C4); 34.40 (C6); 48.10 (C2); 54.58 (CH_2O). **5**, oil: ^1H NMR δ 0.96 (d, 3H, J = 6.4 Hz, CH_3); 1.20-1.90 (m, 9H, cyclohexane CH); 2.58 (s, 2H, CH_2O). ^{13}C NMR δ 22.80 (CH_3); 25.35 (C5); 33.80 (C3); 34.04 (C4); 34.59 (C6); 42.90 (C2); 55.91 (CH_2O).

(+)-(S)-2-Methylcyclohexanone (40% ee) and (-)-(R)-2-methylcyclohexanone (95% ee) were obtained by oxidation with the Jones reagent of (1S,2S)- and (1R,2R)-*trans*-2-methylcyclohexanol, prepared by kinetic resolution of the (±)-alcohol *via* transesterification using 2,2,2-trichloroethyl butyrate and Porcine Pancreatic Lipase (Type II) in anhydrous hexane, as reported.[10] The same reaction sequences respectively employed for the preparation of the racemic epoxides **2** and **3** lead to (-)-(1R,2S)-**2** ($[\alpha]_D$= - 1 (hexane), 40% ee) from (+)-(S)-2-methylcyclohexanone and to (-)-(1R,2R)-**3** ($[\alpha]_D$ = -15 (hexane), 95% ee) from (-)-(R)-2-methylcyclohexanone.

Diols 6-10. (±)-1-hydroxymethyl-2,2-dimethylcyclohexanol (**6**), was obtained by $HClO_4$ (0.05 M) promoted hydrolysis of (±)-**1** in 7:3: THF-water for 10 h. Racemic 1-hydroxymethyl-*trans*-2-methylcyclohexanol (**7**) and 1-hydroxymethyl-*cis*-2-methylcyclohexanol (**9**) were likewise obtained in ratios of 85:15 and 30:70, respectively, from epoxides (±)-**2** and (±)-**3**, and 1-hydroxymethyl-*cis*-3-methylcyclohexanol (**8**) and 1-hydroxymethyl-*trans*-3-methylcyclohexanol (**10**) were likewise obtained in ratios of 85:15 and 30:70 by hydrolysis of epoxides (±)-**4** and (±)-**5**. The relative stereochemistry of diols **7-10** was established by conversion to the corresponding methyl carbinols using the reported[9] two-step procedure: the primary alcoholic group was selectively tosylated, and the tosylate group was removed by reduction with lithium aluminium hydride. **6**, oil: ^1H NMR δ 0.92 (s, 3H, CH_3); 0.97 (s, 3H, CH_3); 1.30-1.80 (m, 8H, cyclohexane CH_2); 3.47 (d, 1H, J = 10.8 Hz, CH_2OH); 3.72 (d, 1H, J = 10.8 Hz, CH_2OH). ^{13}C NMR δ 22.14 (CH_3); 22.48 (C5); 24.05 (C3); 25.00 (CH_3); 30.78 (C4); 38.02 (C6); 66.00 (CH_2OH). **7**, oil: ^1H NMR δ 0.93 (d, 3H, J = 6.8 Hz, CH_3); 1.50-1.80 (m, 9H, cyclohexane CH); 3.53 (s, 2H, CH_2OH). ^{13}C NMR δ 15.74 (CH_3); 23.19 (C5); 23.95 (C4); 31.03 (C3); 33.05 (C6); 38.92 (C2); 65.47 (CH_2OH); 75.07 (C1). **8**, oil: ^1H NMR δ 0.91 (d, 3H, J = 6.4 Hz, CH_3); 1.20-1.90 (m, 9H, cyclohexane CH); 3.59 (s, 2H, CH_2OH). ^{13}C NMR δ 15.76 (CH_3); 22.10 (C5); 25.68 (C4); 31.07 (C3); 34.55 (C6); 36.90 (C2); 69.56 (CH_2OH); 74.00 (C1). **9**, oil: ^1H NMR δ 0.91 (d, 3H, J = 6.6 Hz, CH_3); 1.20-1.80 (m, 9H, cyclohexane CH); 3.40 (d, 1H, J = 10.8 Hz, CH_2OH); 3.64 (d, 1H, J = 10.8 Hz, CH_2OH). ^{13}C NMR δ 23.14 (CH_3); 23.60 (C5); 30.56 (C3); 34.80 (C4); 35.30 (C6); 44.46 (C2); 66.73 (CH_2OH); 74.00 (C1). **10**, oil: ^1H NMR δ 0.89 (d, 3H, J = 6.2 Hz, CH_3); 1.20-1.80 (m, 9H, cyclohexane CH); 3.40 (s, 2H, CH_2OH). ^{13}C NMR δ 23.24 (CH_3); 21.77 (C5); 28.09 (C3); 38.70 (C4); 35.50 (C6); 43.00 (C2); 72.52 (CH_2OH); 72.90 (C1).

Rates of mEH and cEH catalyzed hydrolysis of epoxides 1-5. Aliquotes (20 µl) of ethanolic stock solutions of (±)-**1**, or (±)-**2**, or (±)-**3**, or (±)-**4**, or (±)-**5** were added to 2 ml of diluted microsomal or cytosolic preparations[8b] containing 2.5 or 5 mg of protein/ml, to obtain a 10 or 20 mM final substrate concentration, and the mixtures were incubated with shaking at 37 °C. After 10 and 20 min the reactions were stopped by extraction with ethyl acetate containing a proper amount of cyclohexanol as a standard. The extracts were analyzed by GLC for the quantification of the unreacted epoxides and the formed diols. Only diol **6** from **1**, **7** from **3**, **8** from **5**, **9** from **2** and **10** from **4** was always formed. The diol formation was linear with time and protein concentration, and was independent of the subtrate concentration, indicating substrate saturation. The average saturation rates obtained for the various substrates with the microsomal and the cytosolic preparations are reported in the Results and Discussion Section.

Enantiomeric excesses and absolute configurations. Aliquotes (20 µl) of 2 M ethanolic stock solutions of (±)-**1**, or (±)-**2**, or (±)-**3**, or (±)-**4**, or (±)-**5** were added to 2 ml of microsomal or cytosolic preparations containing, respectively, 5 and 15 mg of protein/ml and the mixtures were incubated with shaking at 37 °C. At prefixed times the reactions were stopped by extraction with ethyl acetate containing a proper amount of cyclohexanol as a standard, and the extracts were analyzed using the chiral column for the determination of the ee of the epoxides and, after derivatization with acetic anhydride or trifluoroacetic anhydride, of the diols. At least three determinations were made at each time. The average results are reported

in Table 1. The absolute configurations of the two enantiomers of the unreacted epoxides **2** and **3** were directly determined by comparison of their GLC retention times with those of (-)-(1R,2S)-**2** and (-)-(1R,2R)-**3**.

In order to determine the absolute configuration of the excess enantiomer of **1** from CD measurements, 60 mg of the racemic epoxide **1** were added to 25 ml of the microsomal preparation containing 5 mg of protein/ml. After 30 min the reaction mixtures was extracted first with hexane, and then with ethyl acetate. The hexane extract, dried and evaporated, gave 35 mg of **1**, $[\alpha]_D$ = 18.8 (c = 0.02, hexane), 66% ee, while 28 mg of diol **6** $[\alpha]_D$ = - 7.9 (c = 0.02, $CHCl_3$), >95 % ee, were obtained from the ethyl acetate extract.

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