# Substrate Enantioselection in the Microsomal Epoxide Hydrolase Catalyzed Hydrolysis of Monosubstituted Oxiranes. Effects of Branching of Alkyl Chains

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The hydrolysis of racemic 1,2-epoxyhexane (**1a**), 1,2-epoxydecane (**1b**), 4,4-dimethyl-1,2-epoxypentane (**1c**), and 3,3-dimethyl-1,2-epoxybutane (**1d**) catalyzed by rabbit liver microsomal epoxide hydrolase (MEH) has been investigated in order to further clarify the enantioselecting ability of this enzyme. Both enantiomers of all four epoxides were good substrates. The alkyl chain showed, however, a remarkable effect on the enantiomeric excess of the diols **2** produced during the course of the hydrolysis under enzyme saturation conditions. Linear-chain epoxides gave diols with very low enantiomeric excesses even at very low conversions, whereas branched-chain epoxides gave enantiomerically enriched diols. Furthermore, the ee obtained with the latter epoxides increased with increasing ratio of substrate to microsomal protein. *R* configurations were demonstrated for the excess enantiomers of diols **2c** and **2d** by CD and NMR measurements of their dibenzoate esters. In the case of **1c** nucleophilic attack by water was proved using $^{18}OH_2$ followed by MS analysis of the produced diol to occur ≥95% at the unsubstituted oxirane carbon. A markedly biphasic profile was found for the time course of the hydrolysis of (±)-**1d**. A mechanistic explanation for the observed substrate enantioselection is proposed.

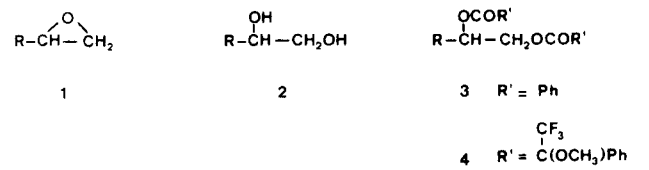
On account of the remarkable influence of chirality on the biological activity of molecules, chiral recognition, a typical feature of many enzyme reactions, is expected to play an important role in the enzymatic detoxification of xenobiotics. Compounds containing arene and alkene functions are known[1] to be biotransformed in mammalians by the action of Cytochrome P-450 dependent monooxygenases to epoxides, which, in turn, are converted to more easily excreted trans diols by a water addition catalyzed by epoxide hydrolases.[2] Owing to their ability to bind to cellular macromoles, the most electrophilically reactive of these epoxides can give rise to toxic, mutagenic, and carcinogenic effects[3] to extents that can be markedly dependent on the stereochemistry.[4] In this respect, the capacity of epoxide hydrolases to modulate, in conjunction with the other important epoxide conjugating enzymes glutathione-*S*-transferases,[5] the cellular concentrations of different enantiomers of epoxides by effecting an enantioselective hydrolysis can be of crucial importance.

Systematic investigations of substrate enantioselection in the enzymatic hydrolysis of racemic epoxides have so far been devoted to the reaction of microsomal epoxide hydrolase (MEH) with aryl-substituted epoxides[6-8] and arene oxides[9] and with epoxides having the oxirane ring fused to substituted cyclohexane and tetrahydropyran rings,[10-16] for which regiospecific water attack associated with remarkable kinetic resolution has been observed. Very little information is instead available concerning the MEH-catalyzed hydrolysis of simple racemic aliphatic epoxides. A recent report[17] claims that substrate enantioselection is a general feature also for these reactions, and that "*S* configurated enantiomers are preferentially metabolized in the biotransformations of aliphatic oxiranes by epoxide hydrolases". However, only the qualitative statement that "the *S* enantiomer reacts more rapidly than the *R* antipode" has been reported for monoalkyl-substituted oxiranes.[17]

With the aim at clarifying this point and at giving a further contribution to the understanding of the factors determining the enantioselectivity of the MEH-catalyzed hydrolyses, we undertook an investigation using simple 1,2-epoxyalkanes differing by the length and the branching of the alkyl chain.

## Results

Two linear 1,2-epoxyalkanes of different chain length, 1,2-epoxyhexane (**1a**) and 1,2-epoxydecane (**1b**), and two branched epoxy derivatives differing for the distance of the branching from the oxirane ring, 4,4-dimethyl-1,2-epoxypentane (**1c**) and 3,3-dimethyl-1,2-epoxybutane (**1d**), were chosen for this investigation of the MEH-catalyzed hydrolysis.




(1) Jerina, D. M.; Daly, J. W. *Science* **1974**, *185*, 573.
(2) Oesch, F. *Enzymatic Basis of Detoxication*; Jacoby, W. B., Ed.; Academic: New York, 1980; pp 277-290. Seidegard, J.; DePierre, J. W. *Biochim. Biophys. Acta* **1983**, *698*, 251. Meijer, J.; DePierre, J. W. *Chem.-Biol. Interact.* **1988**, *64*, 207.
(3) Ehremberg, L.; Hussain, S. *Mutation Res.* **1981**, *86*, 1.
(4) Levin, W.; Buening, M. K.; Wood, A. W.; Chang, R. L.; Kedzierski, B.; Thakker, D. R.; Boyd, D. R.; Gadaginameth, G. S.; Armstrong, R. N.; Yagi, H.; Karle, T. J.; Slaga, T. J.; Jerina, D. M.; Conney, A. H. *J. Biol. Chem.* **1980**, *255*, 9067.
(5) Mannervik, B. *Adv. Enzymol.* **1985**, *57*, 357.
(6) Watabe, T.; Ozawa, W.; Yoshikawa, K. *Biochem. Pharmacol.* **1981**, *30*, 1695.
(7) Westkaemper, R. B.; Hanzlik, R. P. *Arch. Biochem. Biophys.* **1981**, *208*, 195.
(8) (a) Watabe, T.; Akamatsu, K. *Biochim. Biophys. Acta* **1972**, *279*, 297. (b) Dansette, P. M.; Makedonska, V. B.; Jerina, D. M. *Arch. Biochem. Biophys.* **1978**, *187*, 290.
(9) Boyd, D. R.; Jerina, D. M. *The Chemistry of Heterocyclic Compounds. Small Ring Heterocycles*; Hassner, A., Ed.; Wiley: New York, Part 3, Chapter II, pp 197-282.
(10) Bellucci, G.; Berti, G.; Ingrosso, G.; Mastrorilli, E. *J. Org. Chem.* **1980**, *45*, 299.
(11) Bellucci, G.; Berti, G.; Bianchini, R.; Cetera, P.; Mastrorilli, E. *J. Org. Chem.* **1982**, *47*, 3105.
(12) Bellucci, G.; Berti, G.; Ferretti, M.; Mastrorilli, E.; Silvestri, L. *J. Org. Chem.* **1985**, *50*, 1471.
(13) Bellucci, G.; Ferretti, M.; Lippi, A.; Marioni, F. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2715.
(14) Bellucci, G.; Berti, G.; Catelani, G.; Mastrorilli, E. *J. Org. Chem.* **1981**, *46*, 5148.
(15) Catelani, G.; Mastrorilli, E. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2717.
(16) Barili, P. L.; Berti, G.; Catelani, G.; Colonna, F.; Mastrorilli, E. *J. Org. Chem.* **1987**, *52*, 2886.
(17) Wistuba, D.; Schurig, V. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1032.

0022-3263/89/1954-5978$01.50/0 

**Table I. Optical Rotations, Enantiomeric Ratios, and Absolute Configurations of Diols 2 Obtained by MEH-Catalyzed Hydration of Epoxides (±)-1**

| | | | incubation conditions | | | | product | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| run | epoxide | μmol/mg of protein | volume, mL | microsomal proteins, mg | time, min | diol | yield, % | enantiomeric ratio[a] | $[\alpha]^{25}_D$, deg | absolute config |
| 1 | **1a** | 1.8 | 26 | 365 | 30 | **2a** | 50 | 54:46 | | |
| 2 | **1a** | 2.3 | 53 | 676 | 5 | **2a** | 14 | 56:44 | | |
| 3 | **1a** | 1.8 | 20 | 270 | 30 | **2a** | 16 | 62:38 | +2.0 | *R*[b] |
| 4 | **1b** | 4.7 | 21 | 310 | 10 | **2b** | 14 | 58:42 | | |
| 5 | **1b** | 10.5 | 12 | 160 | 25 | **2b** | 9 | 58:42 | | |
| 6 | **1c** | 4.3 | 10 | 200 | 25 | **2c** | 10 | 70:30 | | |
| 7 | **1c** | 4.3 | 10 | 200 | 70 | **2c** | 28 | 55:45 | | |
| 8 | **1c** | 18.0 | 10 | 100 | 40 | **2c** | 13 | 86:14 | | |
| 9 | **1c** | 18.0 | 10 | 100 | 90 | **2c** | 28 | 86:14 | +16.0 | *R*[c] |
| 10 | **1d** | 2.2 | 21 | 310 | 15 | **2d** | 14 | 75:25 | | |
| 11 | **1d** | 2.4 | 27 | 338 | 30 | **2d** | 28 | 58:42 | | |
| 12 | **1d** | 2.8 | 27 | 387 | 15 | **2d** | 11 | 87:13 | | |
| 13 | **1d** | 4.5 | 21 | 310 | 25 | **2d** | 11 | 96:4 | −20.5 | *R*[c] |
| 14 | **1d** | 10.0 | 10 | 250 | 30 | **2d** | 15 | 97:3 | | |
| 15 | **1d** | 10.0 | 10 | 250 | 90 | **2d** | 38 | 96:4 | | |
| 16 | **1d** | 10.0 | 10 | 250 | 130 | **2d** | 58 | 88:12 | | |
| 17 | **1d** | 10.0 | 10 | 250 | 170 | **2d** | 78 | 63:37 | | |

[a] Determined by HPLC analysis of the diastereoisomeric bis(MTPA) esters. [b] Based on literature reports (ref 18). [c] Determined by the CD and $^1$H NMR spectra of the dibenzoate (see text).

The ability of these epoxides to be substrates for MEH was preliminarily checked with reference to a typical good substrate, styrene oxide. Incubations were carried out at 37 °C and pH 7.4, with 3–8 mM epoxides and 1–2 mg of protein/mL for 5–10 min, and stopped by addition of methanol. The amounts of formed diols were determined by GLC by direct injection of the supernatant after addition of an appropriate standard, *trans*-cyclohexane-1,2-diol, for diols **2a–d**, and hexadecan-1-ol for styrene diol. Under the incubation conditions initial rates of diol production were linear both with the time and with the amount of microsomal protein, but were independent of the concentration of the epoxide, consistent with substrate saturation. Although the individual rate values exhibited fluctuations using different microsomal preparations, the relative rates with respect to styrene oxide hydrolysis were constant at the following values: **1a**, 1.0 ± 0.1; **1b**, 0.8 ± 0.1; **1c**, 0.4 ± 0.05; **1d**, 0.35 ± 0.05. Similar relative values were also obtained working at 25 mM epoxide and 12 mg of protein/mL. Although these experiments could not give true kinetic parameters, since each employed epoxide consisted of two competing substrates, they show that all examined compounds are good substrates for MEH and that saturating conditions had been used throughout this investigation.

In order to obtain amounts of diols suitable for isolation and determination of their enantiomeric excesses and absolute configurations, preparative incubations were carried out with larger quantities of microsomal proteins and substrate amounts high enough to ensure saturation. Several microsome lots of different activity were used. The formed diols were first quantified by GLC, after stopping the reactions by addition of methanol. The supernatants were then evaporated, and the residues were chromatographed over a silica gel column to isolate the diols which, in typical experiments, were subjected to the measurement of the optical rotation. The amount of substrate and of microsomal protein and the incubation times were varied in order to change the percent hydrolysis. The results are reported in Table I.

No definitely measurable optical rotation was found for diol **2b**. A small positive rotation was measured for **2a** in run 3, and large optical rotations were found in runs 9 and 13 for the branched diols, the value being again positive for **2c** and negative for **2d**.

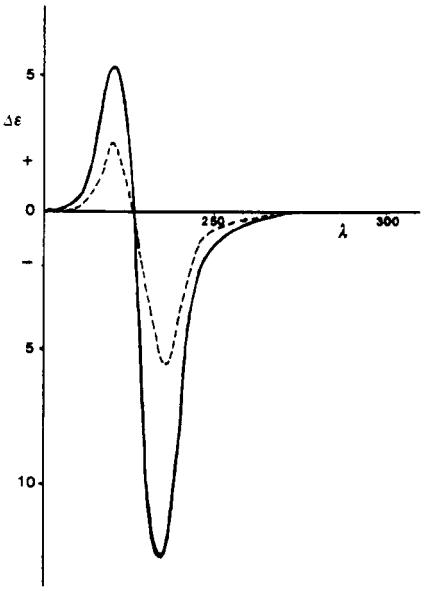



**Figure 1.** CD spectra of dibenzoates **3c** (- -) and **3d** (–) in acetonitrile.

The relations between optical rotations and absolute configurations, (+)-(*R*)-**2a**[18] and (–)-(*R*)-**2d**,[19] were known for these diols. This established *R* configurations for the excess enantiomers of both diols obtained in the enzymatic hydrolyses of (±)-**1a** and (±)-**1d**. No information was available concerning the absolute configuration of diol (+)-**2c**. This was therefore sought through a combination of the exciton coupling method and NMR analysis. The same approach was applied to check the *R* configuration of (–)-**2d**. Samples of **2c**, $[\alpha]^{25}_D$ +16.0°, and of **2d**, $[\alpha]^{25}_D$ −20.5°, were converted into their dibenzoates **3c** and **3d**, in order to apply the reliable dibenzoate chirality, rule.[20] The CD spectra in acetonitrile at 25 °C of **3c** and **3d** so

(18) (a) Levene, A.; Haller, H. L. *J. Biol. Chem.* **1928**, *79*. (b) Mulzer, J.; Augermann, A. *Tetrahedron Lett.* **1983**, 2843.
(19) (a) Guetté, J.-P.; Spassky, N. *Bull. Soc. Chim.* **1972**, 4217. (b) Ko, K. Y.; Frazee, W. J.; Eliel, E. L. *Tetrahedron* **1984**, *40*, 1333.
(20) Harada, N.; Nakanishi, K. *Circular Dichroic Spectroscopy. Exciton Coupling in Organic Stereochemistry*; Oxford University: London, 1983.

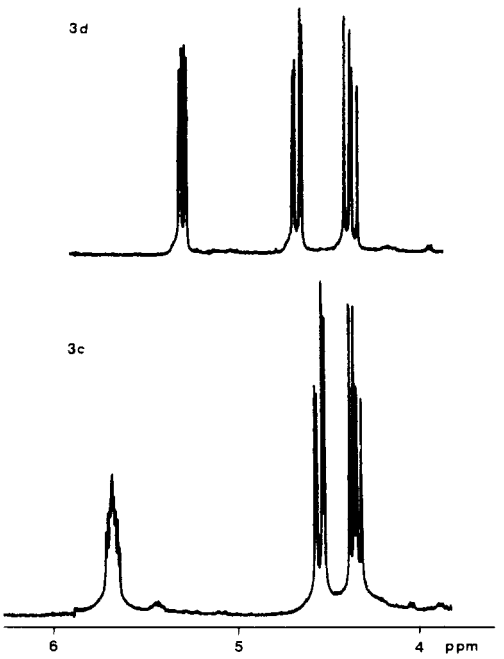
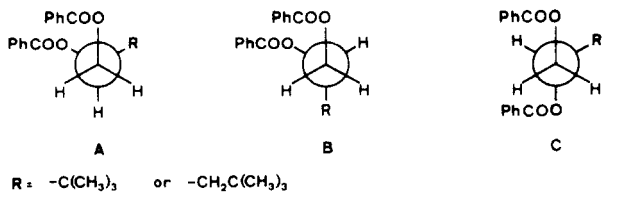



**Figure 2.** Medium-field part of the 300-MHz NMR spectra of dibenzoates **3c** and **3d** in $CD_3CN$.

obtained are shown in Figure 1. Both spectra exhibited the expected exciton splittings, with negative Cotton effects around 235 nm and positive ones around 220 nm, due to conformers of **3c** and **3d** having gauche dibenzoate groups with a left-handed screwness. Either conformer A of (*S*)-**3** or conformer B of (*R*)-**3** were consistent with these CD spectra. A choice between these two alternatives was made on the basis of coupling constant measurements.

The medium-field part of the 300-MHz NMR spectrum of dibenzoate **3d** (Figure 2) shows an AMX system, for the two diastereotopic protons at C(1) and the proton at C(2), with a geminal coupling of 12 Hz and vicinal couplings of 9 and 2.7 Hz. A very similar line pattern, with values of the geminal and vicinal couplings practically identical with those measured for **3d**, is observed for the diastereotopic protons at C(1) in the spectrum of **3c** (Figure 2). In this case, however, the signal of the C(2) proton is a complex multiplet due to further couplings to the diastereotopic methylene protons at C(3). The measured $^3J$ values exclude conformers of type A, having all gauche hydrogens, and are consistent with conformers of type B and/or C. However, conformer C (or its antipode) cannot contribute, because of the anti orientation of the two benzoyloxy groups, to the split Cotton effect, which must therefore be due to B. These considerations establish an *R* configuration for both dibenzoates **3c** and **3d** and consequently for their parent diols (+)-**2c** and (−)-**2d**.




Optical rotations of diols **2a** and **2d** have been reported in the literature.[18,19] However, an evaluation of the optical purity of these diols obtained in the enzymatic hydrolysis of epoxides **1a** and **1d** by measurements of this type could give very inaccurate results, owing to the hygroscopic nature of the diols and to fluctuations reported for the maximum optical rotations.[19b] A HPLC technique was

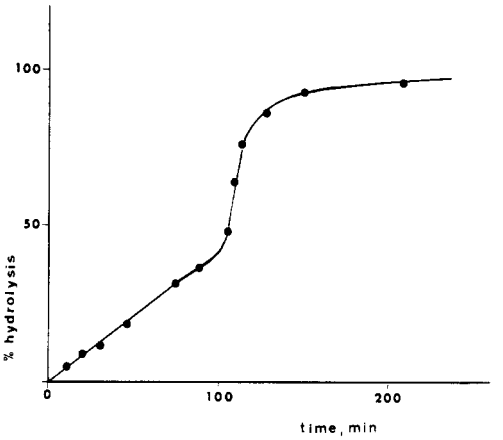



**Figure 3.** Time course of the MEH-catalyzed hydrolysis of (±)-**1d**.

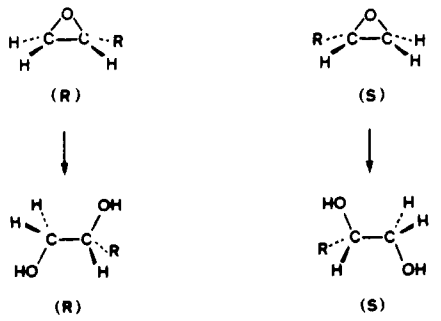
then employed after transformation of the diols to their diastereoisomeric bis(MTPA) esters (**4a–d**) by reaction with (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPACl). Preliminary experiments carried out with the racemic diols showed in all cases a 1:1 ratio of the two peaks, showing that no kinetic resolution was occurring during the esterification. The enantiomeric ratios determined in this way for diols **2a–d** obtained in the different runs are reported in Table I.

The data of Table I show a remarkable effect of the alkyl chain on the course of the hydrolysis of the different epoxides. Linear-chain epoxides gave diols with very low enantiomeric excesses, independent of the length of the alkyl chain, whereas branched-chain epoxides yielded enantiomerically enriched diols. Furthermore, at comparable conversions, the ee obtained with the latter epoxides increased with increasing ratio of substrate to the microsomal protein. The results obtained with **1d**, which was more extensively investigated, show that at the lowest examined ratio the ee was 50% at 14% conversion and decreased sharply with the progression of hydrolysis (runs 10–11). Using much higher substrate amounts, up to exceeding the solubility limit of the epoxide, (−)-(*R*)-**2d** with more than 92% ee was found up to 38% hydrolysis of (±)-**1d** (runs 14–15) and relevant amounts of the (+)-*S* enantiomer of **2d** started appearing only after 50% hydrolysis (runs 16–17). A similar trend was observed for the hydrolysis of (±)-**1c**, where a 40% ee of (+)-(*R*)-**2c** was found at the lower substrate to microsomal protein ratio and 10% conversion (run 6) and a maximum 76% ee was obtained at the highest ratio and 13% conversion (run 8).

The progress of the diol formation was followed by GLC during the entire course of the hydrolysis of (±)-**1d**, which exhibited the highest substrate enantioselectivity. The reaction was carried out with 30 μmol/mL of **1d** and 6 mg of protein/mL, that is under surely saturating conditions and a substrate to protein ratio comparable to that of run 13 of Table I. The resulting curve (Figure 3) exhibited a clear biphasic shape, typical of substrate enantioselective reactions, with a ca. 5-fold increase in slope after 50% hydrolysis. These results, together with the enantioselectivity and absolute configuration data of Table I, show that diol (−)-(*R*)-**2d** was produced in the first half of the reaction at a rate that was only about one-fifth of that at which its (+)-*S* enantiomer was formed during the subsequent course of the hydrolysis.

The regioselectivity of the enzymatic hydrolysis was investigated in the case of **1c** using $^{18}O$-labeled water. To this purpose, a rabbit liver microsomal preparation, suspended in Tris·HCl buffer, pH 7.4, was lyophilized and

**Scheme I**




resuspended in 50% $^{18}OH_2$. This preparation retained almost entirely the original epoxide hydrolase activity and was employed to hydrolyze (±)-**1c**. Diol **2c** obtained at 85% conversion was analyzed by GLC–mass spectrometry. No molecular peak was observed at 70 eV. A very intense base peak appeared at $m/z$ 57 due to a *tert*-butyl ion. The second most abundant peaks in the spectrum of the labeled diol, $m/z$ 117 and 119, were due to the loss of a methyl from the neopentyl group of the molecular ion. They had equal abundances, thus allowing us to evaluate an overall 50% content of $^{18}O$ in the product. The amount of $^{18}O$ present at C(2) was then obtained from the relative abundances of the peaks at $m/z$ 101 and 103 derived from the loss of the terminal hydroxymethyl group. A ≥95% incorporation of $^{18}O$ at C(1) was thus established.

## Discussion

In agreement with the general base-catalyzed mechanism proposed for epoxide hydration by MEH,[21] this reaction is known to proceed through water attack at the least substituted or least hindered oxirane carbons. This has been shown for aryl and *n*-alkyl monosubstituted[22,23] and for unsymmetrically *cis*-1,2-disubstituted oxiranes,[24] using $^{18}OH_2$ and unlabeled substrates or [$^{18}O$]epoxides and ordinary buffers. The ≥95% $^{18}O$ incorporation at C(1) observed in this work for the MEH-catalyzed hydrolysis of (±)-**1c** with $^{18}OH_2$ shows that, as expected, also with branched-chain monosubstituted oxiranes opening occurs in an essentially completely stereospecific way, both enantiomers being attacked at the unsubstituted oxirane carbon. Thus, the hydrolysis of each enantiomer occurs with retention of configuration (Scheme I), and the formation of an optically active diol by incomplete hydrolysis can only be the result of a kinetic resolution operated by the enzyme. This is consistent with the dependence of the ee on the progress of the hydrolysis and with the markedly biphasic shape of the curve shown in Figure 3 for the hydration of (±)-**1d**.

The hydrolysis of racemic styrene oxide[6] and its *p*-nitro derivative[7] by rat liver MEH was likewise shown to exhibit a similar, although less marked, biphasic profile, the *R* diol being preferentially formed in the first part and the *S* enantiomer arising at a faster rate mostly in the second part of the reaction. In these cases, the observed substrate enantioselection was shown by $K_m$ and $V_S$ measurements of both enantiomers of the epoxides to be due to an inhibitory effect of the *R* enantiomers on the hydrolysis of the *S* ones because of the higher affinity of the former for the MEH active site.

Unfortunately, the values of $K_m$ for the two enantiomers of the aliphatic epoxides **1** are not directly accessible at present, because of the difficulty in finding an analytical tool sensitive enough to quantify diols **2** produced in incubations carried out at nonsaturating, very low epoxide concentrations. However, even in the absence of $K_m$ data for **1d**, the similar biphasic profiles found for the hydrolysis of this racemic epoxide and for styrene oxide strongly imply the same mechanistic explanation, which, by analogy, likely holds also for the enantioselection observed in the reaction of (±)-**1c**. Under the employed saturating conditions, epoxide (*R*)-**1d** is preferentially hydrolyzed to diol (−)-(*R*)-**2d** when using the racemic mixture as the substrate, in spite of the fact that diol (+)-(*S*)-**2d** is produced from epoxide (*S*)-**1d** at a much higher $V_S$. This is consistent with the *R* enantiomer of the epoxide fitting better than the *S* one into the MEH active site to give a more stable enzyme–substrate complex (lower $K_m$) that releases the product more slowly (lower $V_S$), as in the case of styrene oxide.

These conclusions meet the expectation based on the previously proposed[10-13,25,26] hypothesis on the topology of the active site of MEH. According to this hypothesis, a hydrophobic pocket should be situated to the right back side of the epoxide oxygen-binding site in such a way as to allow a better binding of the *R* enantiomers of epoxides **1** oriented with the oxygen upward, as in Scheme I. Branched, bulky alkyl groups, like a *tert*-butyl or a neopentyl, appear to be suitable for this binding. The reasons for the very low or insignificant substrate enantioselection found instead for the hydrolysis of epoxides having long linear chains, like a *n*-butyl, or a *n*-octyl, are not clear at present.

The present results are inconsistent with the report, mentioned in the introduction, of a preferential hydration of *S* enantiomers of racemic methyl-, ethyl-, and vinyloxirane by MEH.[17] We remark, however, that all these compounds have very small alkyl chains which may contribute little to substrate binding and that, anyway, the significance of this inconsistency cannot be evaluated until quantitative ee data as a function of the hydrolysis progress can be reported for these compounds.

Finally, the increase in enantioselection observed with increasing ratio of substrate to microsomal protein is noteworthy. This effect is very small or insignificant with unbranched substrates, having an intrinsically low tendency to undergo enantioselective hydration, but is marked with branched epoxides. It occurs with substrate amounts exceeding by far not only enzyme saturation, but also the solubility in the microsomal preparation. Although we do not have a conclusive explanation for this unprecedented result, we can speculate that, when present in a large excess, more substrate may accumulate in or around the microsomes and alter the environment of the enzyme, which is localized in the endoplasmic reticulum membrane, producing some modification of the enzyme itself, that may increase its enantioselecting ability. We want to stress that this effect, whatever its origin may be, can be very useful for preparative purposes. Since in the presence of a large amount of substrate all the *R* enantiomer of epoxide (±)-**1d** is hydrated before the reaction of the *S* enantiomer starts,

(21) Bellucci, G.; Berti, G.; Ferretti, M.; Marioni, F.; Re, F. *Biochem. Biophys. Res. Commun.* **1981**, *103*, 838.
(22) Jerina, D. M.; Ziffer, H.; Daly, J. W. *J. Am. Chem. Soc.* **1970**, *92*, 1056.
(23) Hanzlik, R. P.; Edelman, M.; Michaely, W. J.; Scott, G. *J. Am. Chem. Soc.* **1976**, *98*, 1952.
(24) Hanzlik, R. P.; Heideman, S.; Smith, D. *Biochem. Biophys. Res. Commun.* **1978**, *82*, 310.
(25) Armstrong, R. N.; Kedzierski, B.; Levin, W.; Jerina, D. M. *J. Biol. Chem.* **1981**, *256*, 4726.
(26) Sayer, J. M.; Yagi, H.; van Bladeren, P. J.; Levin, W.; Jerina, D. M. *J. Biol. Chem.* **1985**, *260*, 1630.

a nearly complete kinetic resolution of this, and probably also of other substrates, can be simply achieved by working with the appropriate amount and stopping the incubation at half conversion.

## Experimental Section

$^1H$ NMR spectra were recorded at 300 MHz. CD spectra were recorded with a JASCO J 500 C spectropolarimeter. Mass spectra were obtained at 70 eV. HPLC analyses were carried out with a UV detector and a normal-phase column, 15 cm, 5 μm, Hypersil (HPLC), in the following conditions: (a) 98:2 hexane/ethyl acetate, 1.5 mL/min, λ 260 nm; (b) 99.7:0.3 hexane/acetone, 1.5 mL/min, λ 240 nm. GLC analyses were carried out with a 2-m glass column (2.5 mm i.d.) packed with 10% NPGS on 80–100-mesh sylanized Chromosorb: column 170 °C, nitrogen flow 40 mL/min.

**Materials.** Commercial (±)-1,2-epoxyhexane (**1a**) (Aldrich-Chemie, >99%), (±)-1,2-epoxydecane (**1b**) (Aldrich-Chemie, >98%), (±)-4,4-dimethyl-1,2-epoxypentane (**1c**) (Fluka AG, >98%), (±)-3,3-dimethyl-1,2-epoxybutane (**1d**) (Aldrich-Chemie, >98%), (±)-1,2-hexanediol (**2a**) (EGA-Chemie, >98%), (±)-1,2-decanediol (**2b**) (Aldrich-Chemie, >98%), (±)-styrene oxide (Aldrich-Chemie, 96–98%), (±)-1-phenyl-1,2-ethanediol (Merck, 98%) were distilled before use. (±)-3,3-Dimethyl-1,2-butanediol (**2d**) (Aldrich-Chemie, >85%) was purified by distillation with a Vigreux column, bp 200–201 °C. (±)-4,4-Dimethyl-1,2-epoxypentane (**1c**) was obtained by epoxidation of 4,4-dimethyl-1-pentene with *m*-chloroperoxybenzoic acid in $CH_2Cl_2$ for 24 h, followed by distillation, bp 123–125 °C [lit.[27] bp 126–128 °C (744 mm)]. (±)-4,4-Dimethyl-1,2-pentanediol (**2c**) was prepared by hydrolysis of **1c** with 0.05 N $HClO_4$ in 60:40 THF/$H_2O$ and purified by chromatography on a silica gel column (Kieselgel 150–230 mesh, ASTM, Merck) using 3:2 hexane/ethyl acetate as the eluant. Benzoyl chloride (Aldrich-Chemie >99%) was distilled before use. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride, (MTPACl), bp 54 °C (1 mm), was prepared from the (*R*)-(+)-acid and thionyl chloride.[28] Solvents were reagent grade.

**Microsomal Preparations.** Liver microsomes were obtained from male New Zealand white rabbits (2.5–3 kg) using the previously reported procedure[29] and were suspended in 50 mM Tris·HCl buffer (pH 7.4) to a final protein concentration ranging between 10 and 25 mg/mL. These microsomal preparations were directly used or stored at −40 °C for no longer than 1 month. A preparation containing 22 mg of protein/mL was subjected to lyophilization. A sample of this lyophilized preparation was resuspended in water to obtain the same protein concentration and tested for its EH activity in comparison with the initial fresh preparation using styrene oxide at 0.025 M concentration. Comparable activities were found.

**Enzymatic Hydrolyses. A. Kinetic Measurements.** Samples of 20 μL of an ethanolic stock solution (0.15 or 0.4 M) of (±)-**1a–d** and styrene oxide were respectively added to 1 mL of microsomal preparation previously diluted to a final protein concentration of 1 or 2 mg/mL, thermostated at 37 °C. The mixtures were incubated at 37 °C for 5 and 10 min. The reactions were stopped by addition of 1 mL of methanol containing 50 μg of the appropriate internal standard, *trans*-cyclohexane-1,2-diol for **2a–d** and hexadecan-1-ol for styrene diol, and analyzed by GLC. Each experiment was carried out in triplicate. The rates of formation of diols **2a–d** (expressed in nmol/min per mg of protein) were compared with that of styrene diol, and the relative rates are given in the text.

**B. Time Course of Hydrolysis of (±)-1d.** Aliquots (20 μL) of 1.5 M ethanolic solution of (±)-**1d** were added to 1-mL samples of microsomal preparation previously diluted to a final concentration of 6 mg/mL and thermostated at 37 °C. The mixtures were incubated at 37 °C for prefixed times (10–210 min), stopped by addition of methanol (1–3 mL) containing the appropriate amount of *trans*-cyclohexane-1,2-diol as internal standard, and analyzed by GLC. Each experiment was carried out in duplicate. The amount of diol **2d** formed at each time was used to obtain the kinetic curve reported in Figure 3.

**C. Product Isolation.** Epoxides (±)-**1a–d** were added as ethanolic solutions (0.5 mL) or neat liquids to the microsomal preparation, and the mixtures were incubated at 37 °C with shaking for the times reported in Table I. The reactions were then stopped by addition of methanol, and the mixtures were centrifuged at 5000 rpm. The precipitate was resuspended in methanol and further centrifuged. This procedure was repeated twice. The combined supernatants were reduced to an exactly known volume by evaporation in vacuo, a proper amount of an ethanolic stock solution of *trans*-cyclohexane-1,2-diol was added to an aliquot of this supernatant, and the amount of diol was determined by GLC. The yields of diols obtained in the different runs are reported in Table I. The remaining part of the methanolic supernatants was evaporated in vacuo, and the residue was chromatographed on a silica gel column using 3:2 hexane/ethyl acetate as the eluant and monitoring the eluate by GLC. The fractions containing the diol were combined and evaporated. The diols so obtained had IR and NMR spectra identical with those of synthetic samples of (±)-**2**. In typical experiments they were subjected to the measurement of the optical rotation.

When the experiment of run 15 of Table I was carried out on a duplicate scale (0.5 g of (±)-**1d** and 20 mL of microsomal preparation containing 25 mg of protein/mL), 0.2 g of (−)-**2d**, 92% ee, were isolated.

**Determination of Enantiomeric Excesses.** Samples of diols **2** (5–30 mg), obtained by enzymatic hydrolysis of epoxides **1**, were dissolved in pyridine (0.5–2 mL) and treated with a triple molar excess of (*S*)-(+)-MTPACl. After 24 h at room temperature, the mixtures were diluted with 10% HCl and extracted with ethyl acetate. The extracts were washed with saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated in vacuo, and the residues were analyzed by HPLC (conditions a for **4a**, **4b**, and **4d**; conditions b for **4c**). Two peaks, corresponding to the diastereoisomeric bis(MTPA) esters **4**, were detected with the following retention times: **4a**, 8.3 and 9.3 min; **4b**, 6.6 and 7.4 min; **4c**, 7.8 and 9.0 min; **4d** 7.9 and 8.5 min. The ratios of these peaks are reported in Table I. When racemic **2** were used as starting material, the two diastereoisomeric bis(MTPA) esters were present in 1:1 ratios.

**Determination of the Absolute Configuration of (+)-2c and (−)-2d.** Benzoyl chloride (71 mg, 0.5 mmol) was added to a solution of 20 mg (0.17 mmol) of (−)-**2d**, $[\alpha]^{25}_D$ −20.5°, in pyridine (0.5 mL). After 3 days at room temperature, the reaction mixture was diluted with $H_2O$ and extracted with ethyl acetate. The organic phase was washed with 10% aqueous HCl and saturated $NaHCO_3$, dried ($MgSO_4$), and evaporated in vacuo. The crude residue was chromatographed on a silica gel column using 9:1 hexane/ethyl acetate as eluant to give pure **3d**. $^1H$ NMR ($CD_3CN$): δ 1.10 (s, 9 H, $(CH_3)_3C$), 4.40 (d of d, 1 H, $CH_2O$, $J$ = 12 and 9 Hz), 4.72 (d of d, 1 H, $CH_2O$, $J$ = 12 and 2.7 Hz), 5.30 (d of d, 1 H, CHO, $J$ = 9 and 2.7 Hz), 7.3–8.1 (10 aromatic H). UV ($CH_3CN$): $\lambda_{max}$ 229 nm (ε 19000). CD ($CH_3CN$): $\Delta\epsilon_{235}$ = −12.6, $\Delta\epsilon_{220}$ = +5.33.

Diol (+)-**2c**, $[\alpha]^{25}_D$ +16.0°, was treated with benzoyl chloride, and the product, **3c**, was purified as described for **3d**. $^1H$ NMR ($CD_3CN$): δ 1.0 (s, 9 H, $(CH_3)_3C$), 1.65 (d of d, 1 H, $CH_2$, $J$ = 16 and 3 Hz), 1.85 (d of d, 1 H, $CH_2$, $J$ = 16 and 9 Hz), 4.33 (d of d, 1 H, $CH_2O$, $J$ = 12 and 9 Hz), 4.52 (d of d, 1 H, $CH_2O$, $J$ = 12 and 3 Hz), 5.62 (m, 1 H, CHO), 7.4–8.2 (10 aromatic H). UV ($CH_3CN$): $\lambda_{max}$ 229 nm (ε 23000). CD ($CH_3CN$): $\Delta\epsilon_{236}$ = −5.59, $\Delta\epsilon_{221}$ = +2.48.

**Determination of the Regioselectivity of the Enzymatic Hydrolysis of 1c.** Epoxide **1c** (26 mg) was added to a microsomal preparation obtained by resuspending a sample of the lyophilized preparation in $^{18}OH_2$ (1 mL) to obtain the original protein concentration (22 mg/mL), and the mixture was incubated at 37 °C for 17 h. The reaction was then stopped by addition of methanol (3 mL), and the mixture was worked up in the usual way. GLC analysis showed an 85% yield of diol **2c**. The diol, isolated in the usual way, was subjected to GC/MS analysis: $m/z$ (relative abundance) 119 (66), 117 (67), 103 (<1), 101 (44), 81 (17), 57 (100), 41 (33). The spectrum of unlabeled **2c** showed no peaks at $m/z$ 119 and 103.

(27) Hurst, S. J.; Bruce, J. M. *J. Chem. Soc.* **1963**, 1321.
(28) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543.
(29) Bellucci, G.; Berti, G.; Chiappe, C.; Lippi, A.; Marioni, F. *J. Med. Chem.* **1987**, *30*, 768.

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**Registry No.** **1a**, 122922-40-1; **1b**, 67210-45-1; **1c**, 123052-35-7; **1d**, 62137-90-0; **2g**, 84994-66-1; (+)-**2c**, 123052-36-8; **2d**, 31612-63-2; (±)-**2c**, 123122-61-2; MeH, 9048-63-9.

# Notes

## Reactivity of *N,N*-Dialkylamide Enolate Ions. Arylation of 1-Methyl-2-pyrrolidinone Enolate Ions by the $S_{RN}1$ Mechanism

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The radical nucleophilic substitution or $S_{RN}1$ mechanism is well-known.[3] The main steps of the propagation cycle are outlined in Scheme I.

**Scheme I**

$$(RX)^{\bullet-} \rightarrow R^{\bullet} + X^- \quad (1)$$

$$R^{\bullet} + Nu^- \rightarrow (RNu)^{\bullet-} \quad (2)$$

$$(RNu)^{\bullet-} + RX \rightarrow RNu + (RX)^{\bullet-} \quad (3)$$

$$RX + Nu^- \rightarrow RNu + X^- \quad (4)$$

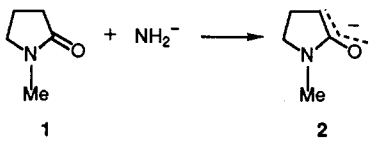
Summation of these steps 1–3 leads to eq 4, which is a nucleophilic substitution, but with radical and radical anions as intermediates. When no spontaneous formation of the radical anion of the substrate takes place to initiate the reaction, it can be catalyzed with solvated electrons in liquid ammonia[4] or with electrons from an electrode,[5] but the most common initiation step is photostimulation.[3,6]

Many aromatic substrates react by this mechanism with different types of nucleophiles, such as the carbanions derived from ketone,[3,7] ester,[3,8] and nitrile enolates ions.[3,9]

We have reported that *N,N*-dialkylamide enolate ions are suitable nucleophiles to react with haloarenes by this mechanism. For instance, the photostimulated reaction in liquid ammonia of several *N,N*-dialkylacetamide enolate ion with haloarenes, such as halobenzenes (chloro, bromo, and iodo), 1-chloronaphthalene, and 9-bromophenanthrene gave good yields of substitution products, together with a low amount of the disubstitution products.[10]

It is known that α,α-diaryl-*N,N*-dialkylamide derivatives constitute an important class of herbicides, such as *N,N*-dimethyldiphenylacetamide (Diphenamid).[11] Therefore we are using this mechanism because it offers the possibility to synthesize different α,α-diaryl-*N,N*-dialkylamide compounds, in order to test their biological activity. We have already synthesized several of these substrates by the $S_{RN}1$ mechanism to study their biological activity.[12] We now attempt to extend this study to *N*-alkyl lactams.

It was reported that the 1-methyl-2-pyrrolidinone enolate ion **2** formed by the acid–base reaction of 1-methyl-2-pyrrolidinone **1** with sodium amide in liquid ammonia gave no reaction with bromobenzene (eq 5 and 6).[13] There




(5)

**1** **2**

$$PhBr + \mathbf{2} \xrightarrow{dark} \text{no reaction} \quad (6)$$

were other attempts to form the enolate ion **2** using different bases, such as $NaNH_2$–KH or $NaNH_2$–$KOC(CH_3)_3$; however neither photostimulation nor solvated electrons were used to stimulate the reactions.[13] In general, there are few examples of thermal or spontaneous $S_{RN}1$ reactions, and most of the carbanions derived from enolate ions need solvated electrons or photostimulation to occur in liquid ammonia, and they react slowly, or fail to react in the dark.[3] Therefore, we decided to reinvestigate the reaction of 1-methyl-2-pyrrolidinone enolate ion **2** with

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(3) For reviews see: (a) Kornblum, N. *The Chemistry of Functional Groups*; Patai, S., Rappoport, Z., Eds.; Wiley: Chichester, UK, 1982; Supplement F, Chapter 10. (b) Rossi, R. A.; de Rossi, R. H. *Aromatic Substitution by the $S_{RN}1$ Mechanism*; ACS Monograph Series, No. 178; American Chemical Society: Washington, DC, 1983. (c) Norris, R. K. *The Chemistry of Functional Groups*; Patai, S., Rappoport, Z., Eds.; Wiley: Chichester, UK, 1983; Supplement D, Chapter 16.
(4) (a) Kim, J. K.; Bunnett, J. F. *J. Am. Chem. Soc.* **1970**, *92*, 7463, 7464. (b) Rossi, R. A.; Bunnett, J. F. *J. Am. Chem. Soc.* **1974**, *96*, 112.
(5) Saveant, J. M. *Acc. Chem. Res.* **1980**, *13*, 323, and references therein.
(6) Rossi, R. A.; Bunnett, J. F. *J. Org. Chem.* **1973**, *38*, 1407.
(7) (a) Bunnett, J. F.; Sundberg, J. E. *Chem. Soc. Bull.* **1975**, *23*, 2620. (b) Komin, A. P.; Wolfe, J. F. *J. Org. Chem.* **1977**, *42*, 2481. (c) Oostveen, E. A.; van der Plas, H. C. *Recl. Trav. Chim. Pays-Bas* **1979**, *98*, 441.
(8) Semmelhack, M. F.; Bargar, T. *J. Org. Chem.* **1977**, *42*, 1481; *J. Am. Chem. Soc.* **1980**, *102*, 7765.
(9) (a) Bunnett, J. F.; Gloor, B. F. *J. Org. Chem.* **1973**, *38*, 4156. (b) Rossi, R. A.; de Rossi, R. H.; López, A. F. *J. Org. Chem.* **1976**, *41*, 3371. (c) Rossi, R. A.; de Rossi, R. H.; Pierini, A. B. *J. Org. Chem.* **1979**, *44*, 2662. (d) Alonso, R. A.; Austin, E.; Rossi, R. A. *J. Org. Chem.* **1988**, *53*, 6065.
(10) Rossi, R. A.; Alonso, R. A. *J. Org. Chem.* **1980**, *45*, 1239. Also see: Wolfe, J. F.; Sleevi, M. C.; Goehring, R. R. *J. Am. Chem. Soc.* **1980**, *102*, 3646.
(11) (a) Caswell, R. L., Ed. *Pesticide Handbook*; Entomological Society of America: College Park, MD, 1980. (b) Ashton, F. M.; Crafts, S. A. *Mode of Action of Herbicides*; Wiley-Interscience: New York, 1981. (c) Audus, L. J. *Biochemicals and Physiology of Herbicides*; Academic Press: New York, 1967. (d) Worthing, C. R. *The Pesticide Manual, A World Compendium*; The British Corp.: Worthing, 1980.
(12) Palacios, S. M.; Rodriguez, C. H.; Rossi, R. A., unpublished results.
(13) Stewart, J. D.; Fields, S. C.; Kochhar, S. K.; Pinnick, H. W. *J. Org. Chem.* **1987**, *52*, 2110.

0022-3263/89/1954-5983$01.50/0 