hypothesis that merostabilization of the biradicaloid intermediate predisposes these salts to utilize this mechanism rather than the concerted one.

Acknowledgment. We thank the National Science Foundation and Conicit (Venezuela) for support of this work. We also thank Raymond A. Firestone for a letter in which he made helpful comments about our previous work.

Registry No. 4 (R = C_6H_5), 68001-26-3; 4 (R = methyl), 68001-32-1; 4 (R = benzyl), 82026-92-4; 4 (R = cyclopropyl), 68001-36-5; 4 (R = *m*-ClC_6H_4), 82026-94-6; 4 (R = *p*-$MeOC_6H_4$), 41745-69-1; 4 (R = 1-naphthyl),, 68001-38-7; 4 (R = styryl), 82043-96-7; **9a**, 82026-95-7; **9c**, 68001-17-2; **9d**, 10425-52-2; **9e**, 13226-09-0; **9g**, 76583-58-9; **9i**, 82026-96-8; **11** (R = C_6H_5), 76456-91-2; **11** (R = *p*-$MeOC_6H_4$), 82026-98-0; **12** (R = R' = H), 20958-78-5; **12** (R = *n*-Bu; R' = H), 82026-99-1; **13** (R = R' = CO_2Me), 76456-96-7; **13** (R = C_6H_5; R' = CO_2Et), 76456-98-9; **13** (R = R' = CO_2H), 76456-97-8; **13** (R = R' = H), 82027-00-7; **13** (R = H; R' = $CONH_2$), 66858-02-4; **13** (R = C_6H_5; R' = CO_2H), 82027-01-8; **13** (R = C_6H_5; R' = H), 82027-02-9; **13** (R = C_6H_5; R' = $CONH_2$), 82027-03-0; **14**, 82027-05-2; **15** (R = R' = CO_2Me), 82027-06-3; **15** (R = C_6H_5; R' = CO_2Et), 82027-07-4; ethylene, 74-85-1; acetylene, 74-86-2; *cis*-stilbene, 645-49-8; 1-phenylpropene, 637-50-3; 1-hexyne, 693-02-7; dimethyl acetylenedicarboxylate, 762-42-5; ethyl phenylpropiolate, 2216-94-6; 2-benzoyl-1-cyano-1,2-dihydrophthalazine, 13925-27-4; acrylonitrile, 107-13-1; cinnamonitrile, 1885-38-7; ethyl acrylate, 140-88-5; styrene, 100-42-5; diethyl maleate, 141-05-9; 1-hexene, 592-41-6; cyclohexene, 110-83-8; *trans*-stilbene, 103-30-0; phenylacetylene, 536-74-3; diphenylacetylene, 501-65-5.

Stereoselectivity in the Epoxide Hydrolase Catalyzed Hydrolysis of the Stereoisomeric 3-*tert*-Butyl-1,2-epoxycyclohexanes. Further Evidence for the Topology of the Enzyme Active Site[1]

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(±)-*cis*-3-*tert*-Butyl-1,2-epoxycyclohexane is converted by rabbit liver microsomal epoxide hydrolase exclusively into the diaxial diol. The 1*S*,2*R*,3*S* enantiomer reacts at a much faster rate to yield the 1*R*,2*R*,3*S* diol, which is isolated at least 96% optically pure in the first stages of the reaction, up to almost 50% conversion. (±)-*trans*-3-*tert*-Butyl-1,2-epoxycyclohexane is a poorer substrate than the cis isomer: only the 1*S*,2*R*,3*R* epoxide undergoes slow enzymatic hydrolysis to produce exclusively optically pure 1*R*,2*R*,3*R* diol, the product of diequatorial opening, in contrast with the acid-catalyzed hydrolysis of the same epoxide that yields both the diequatorial and the diaxial diols. The absolute configurations of the diols and epoxides were established by chiroptical methods on appropriate derivatives. The enantiomeric excesses in the diols were determined with chiral shift reagents. The present results confirm previous hypotheses on the topology of the hydrolase active site, involving a large hydrophobic pocket situated in such a way as to accommodate bulky substituents to the right of the oxirane ring in the ES complex. They also are consistent with and supplement previous evidence on a general-base catalysis in the enzymatic reaction mechanism.

The microsomal epoxide hydrolase (EC 3.3.2.3) is an important enzyme involved in both the detoxification[2,3] and the further metabolic activation[4,5] of epoxides and arene oxides arising by oxidation of olefinic and aromatic substrates by the cytochrome P-450 containing monooxygenases. In spite of its low substrate specificity, the hydrolase exhibits a remarkable regioselectivity[6-10] and in several cases appears to be able to discriminate between substrate enantiomers.[11-13] The latter feature is exceedingly important in view of the well-known influence of stereochemical factors on biological activity. In particular, marked effects of absolute and relative configuration on the mutagenic and carcinogenic activity of stereoisomeric epoxides, dihydrodiols, and diol epoxides formed in the metabolism of polycyclic aromatic hydrocarbons have been reported.[14]

Substituted epoxycyclohexane rings are present in a variety of naturally occurring or metabolically formed products and represent suitable models on which the stereoselectivity of the epoxide hydrolase can be simply investigated by virtue of the rather advanced understanding of steric, electronic, and conformational factors involved in their ring-opening reactions[15] and of the facility of conformational and configurational determinations with

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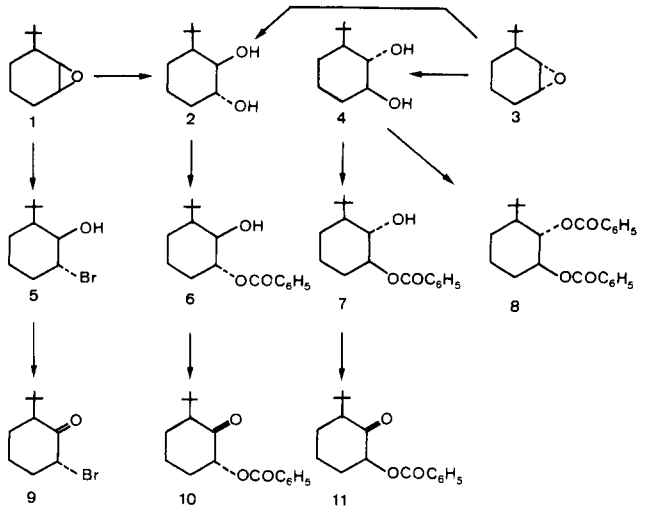
0022-3263/82/1947-3105$01.25/0

Scheme I



these compounds. Important information on the topology of and geometric constraints in the active site of the epoxide hydrolase can be gained in this way.[12]

In a previous paper[12] we examined the influence exerted on the stereoselectivity of the enzymatic hydrolysis by the presence on the epoxycyclohexane ring of a remote *tert*-butyl group, providing conformational rigidity but unable to exert a direct steric effect on the reaction center. For the present investigation we chose the enantiomeric couples of the diastereoisomeric *cis*- and *trans*-3-*tert*-butyl-1,2-epoxycyclohexanes (**1** and **3**), in which the proximity of the bulky anchoring group to the epoxide ring was expected to cause a shielding effect on protonation of the oxirane oxygen in the cis form and on nucleophilic attack at C(2) in the trans form.

Results

The acid-catalyzed hydration of *cis*- and *trans*-3-*tert*-butyl-1,2-epoxycyclohexane (**1** and **3**) in water was first examined, and the previous results[16] were confirmed. The *cis*-epoxide **1** was attacked only at C(1) to give the diaxial diol **2**, whereas a 53:47 mixture of the same diaxial and diequatorial diol **4**, respectively arising by attack at C(2) and at C(1), was obtained from the *trans*-epoxide **3** (Scheme I).

The hydrolysis of (±)-**1** and (±)-**3** in the presence of rabbit liver microsomes in phosphate buffer at pH 7.4 was then examined. In order to ensure a meaningful comparison of the results, most of the incubations were carried out under similar conditions, at substrate concentrations certainly far in excess of the presumed K_m.[17] Incubations were stopped at different times, the products extracted into ethyl acetate, and the yields determined by GLC. Only the diaxial diol **2** and only the diequatorial diastereoisomer **4** were respectively found as the products of the enzymatic hydrolysis of epoxides **1** and **3**, although the GLC method could detect less than 1% of the former in a sample of the latter diol and vice versa. The product diol and the unreacted epoxide were then separated and subjected to measurement of optical rotations. The results are shown in Tables I and II.

Although both epoxides **1** and **3** appeared to be much worse substrates with respect to the previously investigated 4-*tert*-butyl-substituted isomers, probably owing to the higher steric crowding around the epoxide ring, the cis

(16) Freppel, C., Thèse de Doctorat, University of Montréal, 1971.
(17) Values of K_m ranging between ~2 μM and 0.7 mM have been reported for largely different substrates (see ref 14 and 18).

Table I. Conversion of (±)-*cis*-3-*tert*-Butyl-1,2-epoxycyclohexane (**1**) into *t*-3-*tert*-Butylcyclohexane-*r*-1,*t*-2-diol (**2**)

incubation time, h	recovd epoxide 1 yield, %	recovd epoxide 1 $[\alpha]^{20}_D$, deg	recovd diol 2 yield, %	recovd diol 2 $[\alpha]^{20}_D$, deg
1	74	−2.9	11	+35.8
2	52	−10.3	27	+35.0
4	40	−16.2	40	+35.0
6	31	−20.5	60	+20.9
11[a]	25	−20.6	53	+26.5

[a] Incubation carried out with two-thirds of the standard amount (see Experimental Section) of a less active lot of microsomes.

Table II. Conversion of (±)-*trans*-3-*tert*-Butyl-1,2-epoxycyclohexane (**3**) into *c*-3-*tert*-Butylcyclohexane-*r*-1,*t*-2-diol (**4**)

incubation time, h	pH	recovd epoxide 3 yield, %	recovd epoxide 3 $[\alpha]^{20}_D$, deg	recovd diol 4 yield, %	recovd diol 4 $[\alpha]^{20}_D$, deg
10[a]	7.4[b]	54	−1.0	15	+0.8
12	9.0[c]	55	−1.5	27	+0.9
20	7.4[b]	28	−2.2	40	+1.0
20	9.0[c]	36	−2.3	41	+1.2
30	9.0[c]	30	−2.1	40	+1.1

[a] Incubation carried out with half of the standard amount (see Experimental Section) of microsomal preparation. [b] Phosphate buffer. [c] Tris–HCl buffer.

compound **1** was hydrolyzed much faster than the trans one **3**, and also when the latter was incubated in a Tris–HCl buffer at pH 9.0.

Whereas fairly high optical rotations were measured both for the diaxial diol **2** obtained by partial hydrolysis of (±)-**1** and for the unreacted *cis*-epoxide, the diequatorial diol **4** formed from (±)-**3** as well as the recovered *trans*-epoxide exhibited extremely low optical activity, independently of the extent of hydrolysis, pointing possibly to a very low enantioselectivity in the hydrolase-promoted hydrolysis of (±)-**3**.

The optical rotation of **2** obtained by partial hydrolysis of (±)-**1** remained nearly unchanged until about half of the substrate was used up, while the rotation of the recovered *cis*-epoxide **1** increased steadily. On the other hand, beyond 50% conversion the rotation of **2** decreased, while that of recovered **1** reached a maximum constant value. These facts strongly pointed to a practically enantiospecific reaction in which one enantiomer of **1** underwent enzymatic hydrolysis at a much higher rate, so that it was completely transformed before the other, slow-reacting enantiomer was significantly attacked. In this way nearly optically pure diaxial diol was expected to be formed during the first half of the reaction, and the optically pure *cis*-epoxide should be recovered in the second half.

In order to test this interpretation, we checked the enantiomeric excess (ee) of (+)-**2** obtained at different incubation times with a chiral lanthanide shift reagent. Figure 1 shows the high- and medium-field part of the NMR spectra of (±)-**2** and of (+)-**2** ($[\alpha]^{25}_D$ +35.8°) in the presence of Eu(facam)$_3$. Whereas two *tert*-butyl singlets and four multiplets of equal area, due to the equatorial protons α to the hydroxyl groups, were observed for the racemic compound, indicating a chiral discrimination between the enantiomers of (±)-**2**, only a *tert*-butyl and two α-proton signals were found for (+)-**2** obtained after incubation times of up to 4 h. This confirmed that the dextrorotatory diaxial diol formed in the first half of the

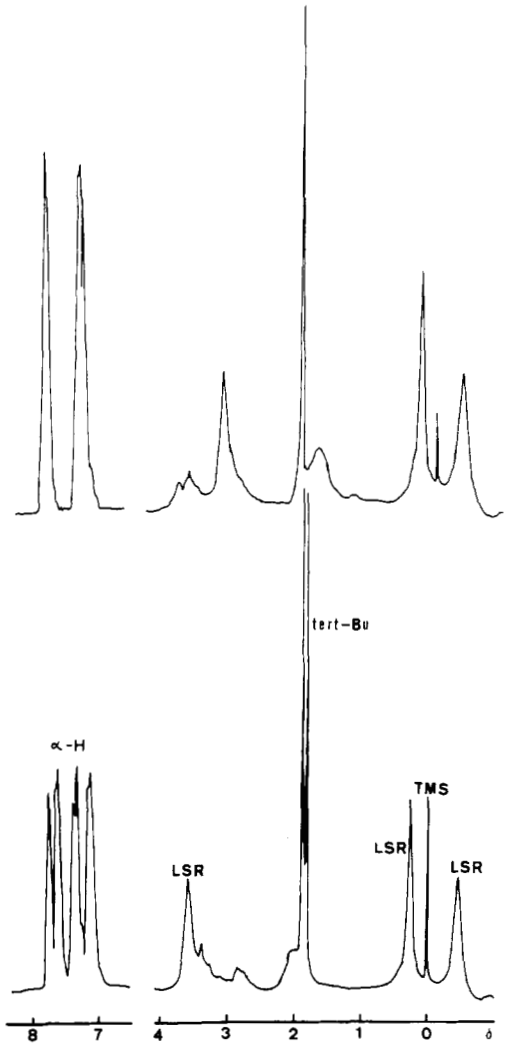


Figure 1. *tert*-Butyl protons signals and the signals of the protons α to the hydroxyl groups in the NMR spectra of diol (±)-2 and of (+)-2 in $CDCl_3$ in the presence of $Eu(facam)_3$.

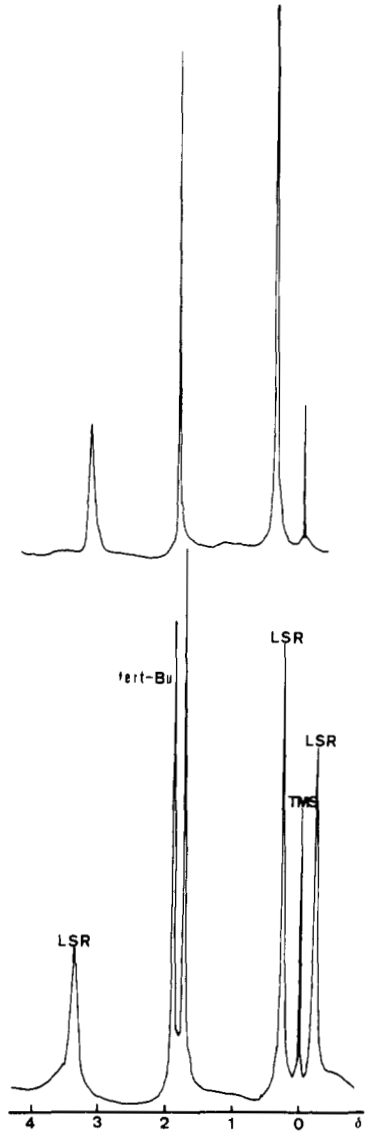


Figure 2. *tert*-Butyl protons signals in the NMR spectra of diol (±)-4 and of (+)-4 in $CDCl_3$ in the presence of $Eu(facam)_3$.

hydrolysis reaction of (±)-1 actually had an ee certainly higher than 96%.

When the ee of the diequatorial diol 4 obtained from the enzymatic hydrolysis of (±)-3 for up to 20 h (both at pH 7.4 and at pH 9.0) was checked in the same way, the signals for the protons α to the hydroxyl groups were not clearly resolved, but only one *tert*-butyl signal was observed, although two singlets of equal area were clearly apparent in the spectrum of (±)-4 in the presence of $Eu(facam)_3$ (Figure 2). This showed that, in spite of the very low optical rotations observed both in the product diol 4 and in the recovered *trans*-epoxide 3, the former had an ee of at least 96%, independent of the incubation time. Furthermore, the formation of diol 4 stopped after 20 h, the extent of hydrolysis of (±)-3 not exceeding 50% even after a much longer incubation time. All these facts showed that also the enzymatic hydrolysis of the *trans*-epoxide was an entirely enantiospecific process: whereas one enantiomer of this compound was a substrate, the other one was not attacked at an appreciable rate.

The absolute configurations of the products of the partial enzyme hydrolysis of both (±)-1 and (±)-3 were determined by chiroptical methods after transformation into suitable compounds as shown in Scheme I. All reactions had been previously carried out with the racemic compounds.

Preliminary attempts at selective acetylation of the diaxial diol 2 at C(1) with acetic anhydride failed because the two hydroxyl groups exhibited not very different reaction rates with this reagent. Selective esterification was easily accomplished with pivaloyl chloride in pyridine, the large *tert*-butyl group hindering the approach of the bulky reactant at the adjacent axial hydroxyl, thus allowing exclusive attack at the C(1) hydroxyl group. However, a preliminary NMR investigation showed an extensive incursion of flexible conformations of the six-membered ring[19] that made the use of this pivaloyloxy derivative for application of the octant rule to the determination of absolute configuration[20] unadvisable.

Selective esterification at C(1) was also obtained with benzoyl chloride, and the resulting monobenzoate 6 was shown by NMR to have the expected chair conformation with axial hydroxy and benzoyloxy groups. Oxidation of 6 with Jones reagent[21] gave a 6-*tert*-butyl-substituted 2-(benzoyloxy)cyclohexanone. The NMR spectrum of the latter compound, however, was not consistent with the

(18) Belvedere, G.; Pachecka, J.; Cantoni, L.; Mussini, E.; Salmona, M. *J. Chromatogr.* **1976**, *118*, 387. Bentley, P.; Oesch, F.; Tsugita, A. *FEBS Lett.* **1975**, *59*, 296.
(19) Results to be submitted for publication.
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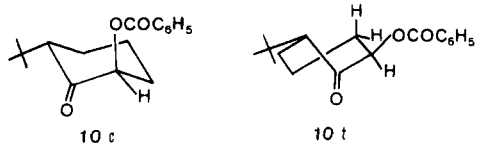
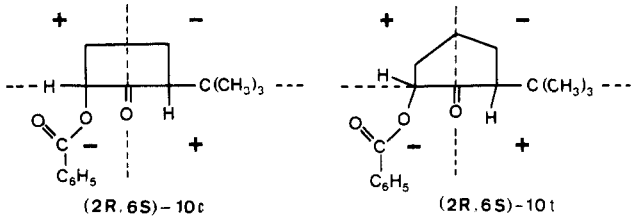
(21) Djerassi, C.; Engle, R. R.; Bowers, A. *J. Org. Chem.* **1956**, *21*, 1547.

expected trans configuration and chair conformation with an equatorial *tert*-butyl and an axial benzoyloxy group (**10c**), since the proton α to the latter substituent gave a three-line pattern (X part of an ABX sistem) with $J_{AX} + J_{BX} = 12$ Hz, a value definitely too high for one equatorial–equatorial and one equatorial–axial vicinal coupling.[22]



The possibility of an epimerization of **10** to the more stable cis isomer **11** during the oxidation of **6** was ruled out by preparing the latter compound by selective monobenzoylation of the diequatorial diol **4** followed by oxidation. The resulting product turned out to be different from the oxidation product of **6**, its NMR spectrum showing for the proton α to the benzoyloxy group a multiplet 20 Hz wide, in agreement with the relative configuration shown in **11** and the expected conformation with both substituents equatorial.

The abnormally large width of the signal due to the α-proton in **10** was therefore considered as evidence for the contribution of a twist conformation of type **10t**, in which the bulky *tert*-butyl and benzoyloxy groups are both in the more favorable, respectively, pseudoequatorial and isoclinal positions,[23] and the diedral angles between the proton at C(2) and the cis and trans protons at C(3) should be respectively ca. 40° and ca. 160°,[24] thus accounting for the observed increase in coupling constants. A nonchair conformation of this type was found for 2β-acetoxycholestan-3-one[25] and was attributed to the 1,3-diaxial interaction with the C(19)-methyl group. In the present case the unfavorable axial orientation of the benzoyloxy group in **10c** may increase the inherent tendency expected for 2-*tert*-butylcyclohexanone to adopt nonchair conformations owing to nonbonded interactions between *tert*-butyl and carbonyl groups,[26] and this could again introduce a complication in the application of the octant rule to **10**. However, an examination of the octant projections shows



that a Cotton effect of the same sign is to be expected for both **10c** and **10t**. In fact, in both conformations the *tert*-butyl group should lie on or near the horizontal nodal plane, whereas the benzoyloxy group is placed in the same negative octant. The latter is therefore expected to determine the sign of the Cotton effect, even if in **10t** the contributions of C(3) and C(5) do not cancel out exactly, as in the chair conformation **10c**, and the hydrogen at C(2) is slightly bent into the upper positive octant.

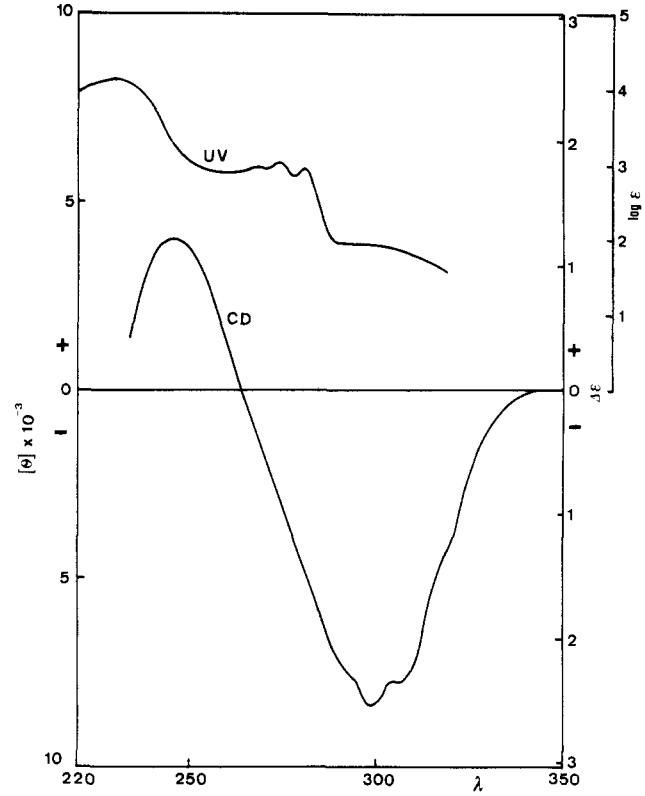


Figure 3. UV and CD spectra of (−)-*trans*-2-(benzoyloxy)-6-*tert*-butylcyclohexanone (**10**) in cyclohexane.

The UV and CD spectra of (−)-**10** ($[\alpha]^{20}_D$ −92.8°) obtained from (+)-**2** ($[\alpha]^{20}_D$ +35.0°) are shown in Figure 3. A large negative Cotton effect was associated with the n $\rightarrow \pi^*$ transition of the asymmetrically perturbed carbonyl group around 300 nm. A negative Cotton effect, $a = -33$, corresponding to a $[\theta]$ of −2700, has been reported for (*S*)-(−)-2-*tert*-butylcyclohexanone[27] and ascribed to the fact that the substituent is not exactly in the nodal plane but is slightly tilted in a negative octant. The much higher negative value found for (−)-**10** ($[\theta]_{298}$ −8400) shows that the ester group lies in the other negative octant, pointing to a 2*R*,6*S* absolute configuration for the levorotatory enantiomer.[28] The parent dextrorotatory diaxial diol obtained by partial enzymatic hydrolysis of (±)-**1** was therefore (1*R*,2*R*,3*S*)-**2**, and the levorotatory unreacted epoxide had the 1*R*,2*S*,3*R* configuration.

The latter conclusion was further checked by transforming the recovered epoxide (−)-**1** into (+)-**9** by ring opening with hydrogen bromide followed by oxidation with Jones reagent. It had been shown[29] that the first step occurs with more than 90% inversion at C(1) and that the second one does not involve epimerization.[30] The CD

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(23) Kellie, G. M.; Riddel, G. *Top. Stereochem.* **1974**, *8*, 225.
(24) These values can be calculated on the basis of the ring torsion angles reported for one of the two possible twist forms of cyclohexanone: Bucourt, R.; *Top. Stereochem.* **1974**, *8*, 159.
(25) Williamson, K. L.; Johnson, W. S. *J. Am. Chem. Soc.* **1961**, *83*, 4623.
(26) Allinger, N. L.; Blatter, H. M.; Freiberg, L. A.; Karkowsky, F. M. *J. Am. Chem. Soc.* **1966**, *88*, 2999.
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(28) The application of the simple octant rule in this case could be misleading if a $\pi \rightarrow \pi^*$ benzoate transition could interact with the carbonyl n $\rightarrow \pi^*$ maximum to give an exciton-split CD curve. Chiral interactions of a benzoate with conjugated enones, esters, and lactones have been reported (Koreeda, M.; Harada, N.; Nakanishi, K. *J. Am. Chem. Soc.* **1974**, *95*, 266), but they always involve the benzoate intramolecular charge-transfer transition at 230 nm, which is located far from the 300-nm carbonyl transition. No Cotton effects have been observed for the 195- and the 280-nm benzoate transitions (Harada, N.; Ohashi, Mo.; Nakanishi, K. *Ibid.* **1968**, *90*, 7349. Harada, N.; Nakanishi, K. *Ibid.* **1968**, *90*, 7351) which therefore should not interfere with the carbonyl Cotton effect of **10**.
(29) Bellucci, G.; Berti, G.; Ferretti, M.; Ingrosso, G.; Mastrorilli, E. *J. Org. Chem.* **1978**, *43*, 422.
(30) Bellucci, G.; Ingrosso, G.; Mastrorilli, E. *Tetrahedron* **1978**, *34*, 387.

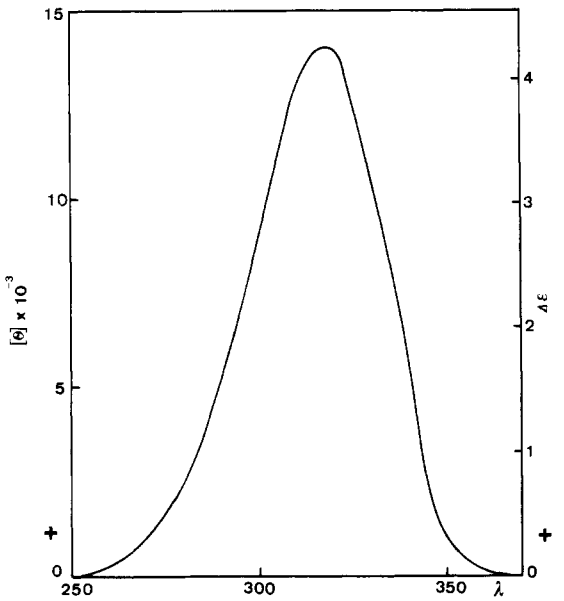


Figure 4. CD spectrum of (+)-*trans*-2-bromo-6-*tert*-butylcyclohexanone (**9**) in cyclohexane.

spectrum of (+)-**9** ($[\alpha]^{25}_D$ +125.0°) obtained from (−)-**1** ($[\alpha]^{20}_D$ −20.5°) showed a large positive Cotton effect (Figure 4). Since this compound had been shown[30] to adopt a chair conformation with an equatorial *tert*-butyl group and an axial bromine atom, the observed Cotten effect unequivocally indicated[20] for the dextrorotatory bromo ketone a 2*S*,6*R* configuration, thus confirming the assigned 1*R*,2*S*,3*R* configuration for the starting levorotatory epoxide.

Finally, the absolute configuration of the diequatorial diol **4** obtained by enzymatic hydrolysis of the *trans*-epoxide (±)-**3** has been deduced from the split n → π* Cotten effects of its dibenzoate **8**, ($[\alpha]^{24}_D$ −89.6°; Figure 5). According to the dibenzoate chirality rule[31] the negative sign of the first CD Cotton effect at 237 nm indicated a negative chirality or a 1*R*,2*R*,3*R* absolute configuration for (−)-**8** and therefore for the slightly dextrorotatory parent diol **4**. Since the enzymatic hydrolysis of (±)-**3** occurred with inversion at C(1), the slightly levorotatory unreacted epoxide recovered from the half-hydrolysis had the 1*R*,2*S*,3*S* configuration.

Discussion

As underlined in the introduction, the investigation of the substrate reactivity as a function of its structure and configuration is a valid tool to get information about the topology of the active site of an enzyme. The approach followed in this and the previous papers,[12,32] consisting in the contextual determination of the absolute configuration and of the ee of both the formed diol and unreacted epoxide as a function of the progress of the hydrolysis promoted by the microsomal epoxide hydrolase of enantiomeric couples of diastereoisomeric substrates at concentrations far in excess of the presumed K_m, has the advantage of providing a simple, even if only qualitative or semiquantitative,[33] estimate of the effect of absolute and relative configuration of the epoxide on the hydrolysis rates without performing individual rate measurements on enantiomerically pure substrates, which are often rather difficult to obtain.

The correlation established in this investigation between the *cis*- and *trans*-3-*tert*-butyl-1,2-epoxycyclohexanes and

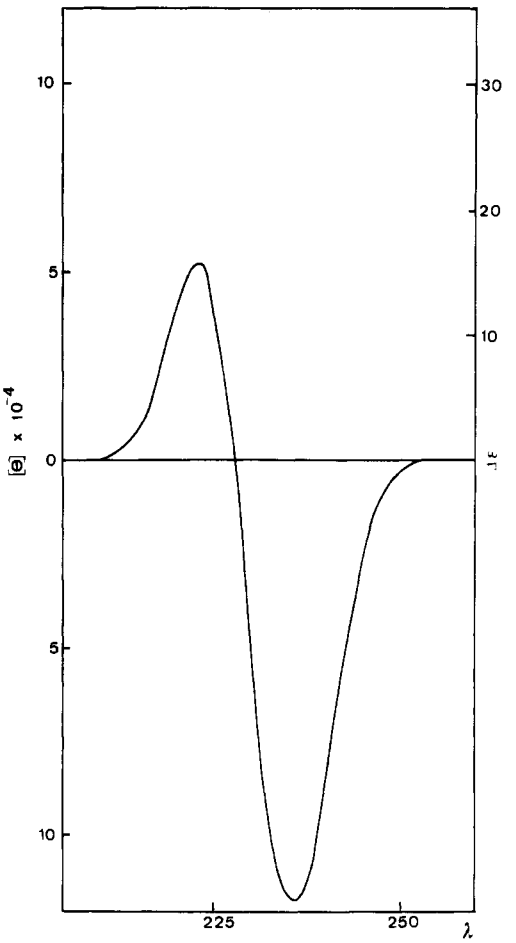
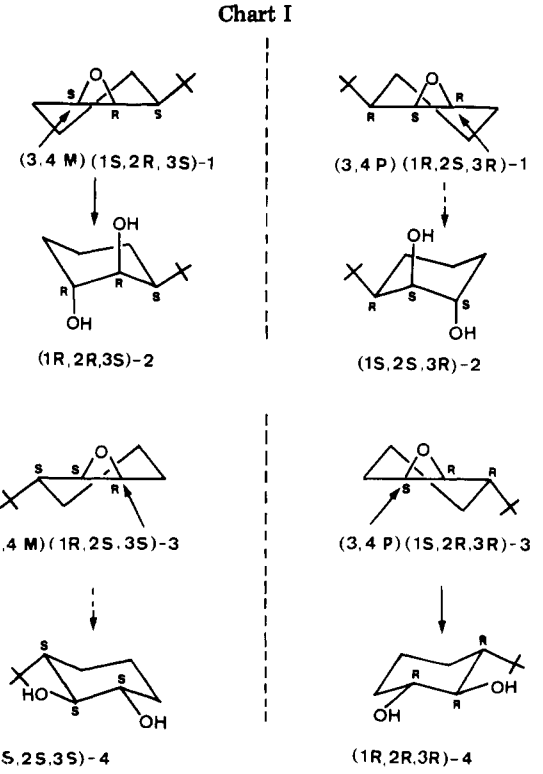


Figure 5. CD spectrum of (−)-*c*-3-*tert*-butyl-cyclohexane-*r*-1,*t*-2-diol dibenzoate (**8**) in ethanol.

Chart I



(31) Harada, N.; Nakanishi, K. *J. Am. Chem. Soc.* **1969**, *91*, 3989. Harada, N.; Nakanishi, K. *Acc. Chem. Res.* **1972**, *5*, 257.
(32) Bellucci, G.; Berti, G.; Catelani, G.; Mastrorilli, E. *J. Org. Chem.* **1981**, *46*, 5148.
(33) Competitive inhibition between substrate enantiomers can substantially affect rate values obtained with racemic substrates (see ref 14 and 35).

their hydrolysis products is shown in Chart I, where the helicities[34] of the monoplanar conformations of the six-membered rings are also indicated for each stereoisomeric epoxide, and the full and dashed arrows denote, respectively, the faster and slower reacting enantiomer in each couple.

The enzymatic hydrolysis of both racemic substrates was enantiospecific and regiospecific, only one enantiomer being practically attacked in the presence of the other and the nucleophilic attack occurring only at C(1). Whereas for (±)-1 this enantiospecificity could be due to a competitive inhibition[35] of the (+)-(1*S*,2*R*,3*S*) enantiomer on the hydrolysis of the (−)-(1*R*,2*S*,3*R*) one, because the latter was also hydrolyzed after the first had been consumed, this was not the case with the *trans*-epoxide **3**, whose 1*R*,2*S*,3*S* enantiomer did not seem to be attacked at all by the epoxide hydrolase. Apparently, this enantiomer cannot be accommodated in the enzyme active site, or its accommodation does not meet the geometric requirements for attack by water.

Previous work on the epoxide hydrolase catalyzed hydrolysis of epoxycyclohexanes[12] and 3,4-epoxytetrahydropyran[32] had led to the individuation of two factors affecting the enantioselectivity of these reactions, the ring helicity and the disposition of substituents with respect to the oxirane ring. Chairlike transition states deriving from antiparallel attack by water on the epoxides having the six-membered ring in a monoplanar conformation of 3,4 *M* helicity (or in the equivalent 1,2 *M* conformation in the case of 3,4-epoxytetrahydropyran) appeared to be preferentially stabilized by the enzyme. A higher degree of stabilization, resulting in a predominant contribution to the observed enantioselectivity, was, however, provided by the presence of a lipophilic substituent placed to the right of the epoxy oxygen if the substrate in the active site of the enzyme is viewed with the oxygen on the topside as shown in Chart I. The latter observation suggested a corresponding geometry of the active site in which the presence of a hydrophobic pocket situated to the right of the epoxide ring and able to accommodate the substituent should allow the preferential binding of the proper enantiomer of the substrate in an orientation suitable for the nucleophilic attack by water. This conclusion was later substantiated by Armstrong et al., who rationalized on the basis of the same interpretation the enantioselectivity of the microsomal epoxyde hydrolase toward three K-region arene oxides.[36]

The enantiospecificity observed for the enzymatic hydrolysis of the present substrates is fully consistent with the above working hypothesis. In fact, by far the fastest reacting stereoisomer is the 1*S*,2*R*,3*S* cis isomer **1**, in which the six-membered ring has the favorable 3,4 *M* helicity and the 3-substituent is situated to the right of the epoxide ring. On the other hand, neither enantiomer of the trans isomer **3** meets the above requirements, and only the 1*S*.2*R*,3*R* one, having the proper disposition of the *tert*-butyl group but the wrong (3,4 *P*) helicity of the six-membered ring, is hydrolyzed, although at a much reduced rate.

The observed difference in rates of enzymatic hydrolysis of **1** and **3** can further be related to the regiospecificity of these reactions. Whereas for the cis epoxide **1** the exclusive attack at C(1) is in agreement with the usual high preference for antiparallel attack in the hydrolysis of epoxycyclohexane derivatives,[15] in the case of the trans substrate **3**, opening at C(1) involves a parallel attack to give a diequatorial diol through a conformationally unfavorable boatlike transition state, and this must cause a reduction in the rate of enzyme hydrolysis, if nucleophilic attack is the rate-limiting step, as usually assumed for nonenzymatic ring opening reactions.[37] An approximatly 20:1 ratio has been found, for instance, between the rates of attack at C(1) of epoxides **1** and **3** in the hydrogen chloride addition to these substrates.[37]

Watabe et al. have recently interpreted[38] the higher rate of the hydrolysis catalyzed by hepatic microsomes of estratetraenol 16α,17α-epoxide with respect to the 16β,17β isomer as the result of a steric hindrance effect of the angular 18β-methyl group of the latter substrate on the interaction of the epoxy oxygen with a dissociating hydrogen of the epoxide hydrolase that was considered as the crucial requirement for ring opening by water. The present results, while not excluding simultaneous acid and base catalysis as recently proposed,[35] are inconsistent with rate determination by proton transfer from the enzyme to the epoxy oxygen,[39] since it would lead to the wrong prediction of a higher hydrolysis rate for the *trans*- (**3**) than for the *cis*-epoxide (**1**). Both the reduced rate and the complete C(1) regiospecificity observed in the hydrolysis of **3** are rather consistent with a general-base mechanism, in which steric effects during nucleophilic attack are expected to play a determinant role.

Diequatorial products in amounts ranging between 50% and 70% have been found in acid-catalyzed ring-opening reactions of the *trans*-epoxide **3**.[29,40] The observed trend in formation of these products as a function of the reagent, HBr > HCl > HCOOH and H_2O, confirms that the steric shielding of antiparallel nucleophilic attack at C(2) by the bulky substituent is responsible for this unusual steric course. The exclusive parallel attack at C(1) in the enzyme hydrolysis, as compared with the about 1:1 ratio of attack at C(1) and C(2) in the acid-catalyzed hydration, seems to indicate a large increase in the steric demand of the attacking nucleophile in the former with respect to the latter reaction.

The chemical modification of a histidine residue essential for the catalytic activity of epoxide hydrolase[41] and the observed dependence of the catalytic rate constant for the hydration of phenantrene 9,10-oxide on an ionization with an apparent pK_a of 6.7 have suggested that an imidazole moiety is involved as a general base assisting in the removal of a proton from the attacking water molecule at the active site.[13] The increase in the bulk of water due to hydrogen bonding to the general base may be resonsible for the suppression of attack at C(2) of **3**, syn to the adjacent *tert*-butyl group, thus determining the C(1) regiospecificity and resulting in a consequent reduction in rate of this reaction.

In conclusion, the results of the present investigation on the hydrolysis of *cis*- and *trans*-3-*tert*-butyl-1,2-epoxycyclohexanes fully confirm the previously suggested topology for the reaction site of the microsomal epoxide hydrolase. The proposed model allows one to rationalize

(34) Cahn, R. S.; Ingold, C.; Prelog, V. *Angew. Chem., Int. Ed. Engl.* **1966**, *5*, 385. See also ref 12, footnote 9.
(35) Westkaemper, R. B.; Hanzlik, R. P. *Arch. Biochem. Biophys.* **1981**, *208*, 195.
(36) Armstrong, R. N.; Kedzierski, B.; Levin, W.; Jerina, D. M. *J. Biol. Chem.* **1981**, *256*, 4726.
(37) Bellucci, G.; Berti, G.; Ingrosso, G.; Vatteroni, A.; Conti, G.; Ambrosetti, R. *J. Chem. Soc., Perkin Trans. 2* **1978**, 627.
(38) Watabe, T.; Ichihara, S.; Sawahata, T. *J. Biol. Chem.* **1979**, *254*.
(39) Previous results by Japanese workers with 2,3-epoxy steroids (Watabe, T.; Kiyonaga, K.; Akamatsu, K.; Hara, S. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 1252) are also in contrast with this assumption.
(40) Richer, J. C.; Freppel. C. *Can. J. Chem.* **1970**, *48*, 145.
(41) DuBois, G. C.; Apella, E.; Levin, W.; Lu, A. Y. H.; Jerina, D. M. *J. Biol. Chem.* **1978**, *253*, 2932.

the observed kinetic discrimination between enantiomers of substrates having the oxirane ring fused to a six-membered ring, as in simple epoxycyclohexane derivatives and in epoxides derived from polycyclic aromatic hydrocarbons, and appears to be of sufficiently general validity to be used successfully for predictions of the relative rates of enzyme hydrolysis of enantiomeric and diastereoisomeric substrates of the above type.

These results also rule out a simple general-acid mechanism in which the rate of enzymatic hydrolysis is substantially determined by the ease of protonation of the epoxy oxygen by a dissociating hydrogen of the hydrolase and are instead consistent with an important role of general-base catalysis of nuclephilic attack by water at an oxirane carbon. More direct evidence for a mechanism of the latter type has been reported by us in a communication[42] submitted concurrently with the present paper.

Experimental Section

Melting points were determined on a Kofler block and are uncorrected. GLC analyses were carried out with a C. Erba Fractovap, Model GV, and a Perkin-Elmer Model F_{11} instrument. Optical rotations were measured, unless otherways specified, in ethyl acetate (c = 2–4) with a Perkin-Elmer Model 241 photoelectric polarimeter. CD spectra were obtained on a Cary 61 and a Jobin Yvon Mark III spectropolarimeter thermostated at 20 °C. UV spectra were measured with a Perkin-Elmer Model 575 spectrophotometer. NMR spectra were taken from $CDCl_3$ solutions with JEOL C 60 HL and Varian CFT 20 spectrometers with Me_4Si as an internal standard. Petroleum ether was the fraction of boiling range 40–60 °C. $MgSO_4$ was always used as the drying agent. Preparative TLC were carried out on 20 × 20 cm silica gel plates (Merck, PSC Fertigplatten Kieselgel 60 F_{254}, 2 mm thick).

Racemic Substrates and Products. *cis*- and *trans*-3-*tert*-butyl-1,2-epoxycyclohexanes (**1** and **3**) were prepared as previously reported.[29] A 1:1 mixture of *t*-3-*tert*-butylcyclohexane-*r*-1,*t*-2-diol (**2**) and *c*-3-*tert*-butylcyclohexan-*r*-1,*t*-2-diol (**4**) was obtained[16] by refluxing with 0.35 M sulfuric acid for 24 h the 1:9 mixture of *cis*- and *trans*-epoxides formed in the epoxidation of 3-*tert*-butylcyclohexene with peroxybenzoic acid.[29] Chromatography over a silica gel column allowed separation of pure **4** [mp 53–54 °C (lit.[40] mp 54 °C)] and pure **2** [mp 98 °C (lit.[40] mp 98 °C)] eluted in succession with 80:20 petroleum ether–ethyl ether, without previous transformation of **4** into the acetonide as described in the reported separation procedure.[16,40]

Acid-Catalyzed Hydrolysis of 1 and 3. A sample (35 mg) of the epoxide **1** or **3** was stirred in 0.1 N $HClO_4$ (2 mL) at 37 °C for 20 h and then extracted with ethyl acetate, and the extract was subjected to GLC under the conditions used for the analysis of the enzyme incubations products (see below). Only diol **2** was detected in the reaction mixture from **1** and a 53:47 mixture of diols **2** and **4** in that from **3**.

(±)-*t*-2-(Benzoyloxy)-*c*-6-*tert*-butyl-*r*-1-cyclohexanol (6). A sample of the racemic diol **2** (52 mg, 0.30 mmol) was refluxed for 7 h with 0.1 mL (0.86 mmol) of benzoyl chloride in 5 mL of dry benzene and 0.5 mL of pyridine. After being allowed to stand overnight at room temperature, the mixture was poured into cold diluted aqueous HCl and worked up as usual. The crude product was subjected to preparative TLC and developed with chloroform to give 57 mg of pure (±)-**6**: mp 82 °C (from petroleum ether); NMR δ 1.00 (s, $C(CH_3)_3$, 9 H), 4.32 (m, $W_{1/2}$ = 7.5 Hz, CHOH, 1 H), 5.22 (m, $W_{1/2}$ = 7.5 Hz, CH–OCO, 1 H), ~7.6 and ~8.2 (2 m, 5 aromatic H).

Anal. Calcd for $C_{17}H_{24}O_3$: C, 73.88; H, 8.75. Found: C, 73.82; H, 8.86.

(±)-*t*-2-(Benzoyloxy)-*t*-6-*tert*-butyl-*r*-1-cyclohexanol (7). The racemic diol **4** (500 mg, 2.9 mmol) and benzoyl chloride (0.34 mL, 2.9 mmol) were reacted in dry pyridine (2 mL) at room temperature for 2 days and then poured onto cold diluted aqueous HCl. The usual workup and preparative TLC developed with chloroform yielded the pure monoester (±)-**7**: 400 mg; mp 66–67 °C (from petroleum ether); NMR δ 1.05 (s, $C(CH_3)_3$, 9 H), 3.70 (t with further splitting, $W_{1/2}$ = 21 Hz, CHOH, 1 H), 5.00 (br m, CH–OCO, 1 H), ~7.6 and ~8.2 (2 m, 5 aromatic H).

Anal. Calcd for $C_{17}H_{24}O_3$: C, 73.88; H, 8.75. Found: C, 73.50; H, 8.80.

(±)-*trans*-2-(Benzoyloxy)-6-*tert*-butylcyclohexanone (10). The monoester (±)-**6** (100 mg) was oxidized with Jones reagent[21] (0.3 mL) in acetone (2 mL) at room temperature. The excess of reagent was reduced with 2-propanol and the mixture worked up as usual to give 72 mg of (±)-**10**: mp 62–63 °C (from petroleum ether); NMR δ 1.03 (s, $C(CH_3)_3$, 9 H), 5.20 (distorted t, splittings 5.7 and 6.3 Hz, CH–OCO, 1 H), 7.5 and 8.1 (2 m, 5 aromatic protons).

Anal. Calcd for $C_{17}H_{22}O_3$: C, 74.42; H, 8.08. Found: C, 74.40; H, 8.15.

(±)-*cis*-2-(Benzoyloxy)-6-*tert*-butylcyclohexanone (11). The treatment of monoester (±)-**7** (200 mg) in 2 mL of acetone with Jones reagent[21] (0.5 mL) under the conditions used for **6** gave (±)-**11**: 130 mg; mp 63–65 °C (from petroleum ether); NMR δ 1.05 (s, $C(CH_3)_3$, 9 H), 5.50 (m, $W_{1/2}$ = 20 Hz, CH–OCO, 1 H), ~7.8 and ~8.4 (2 m, 5 aromatic H).

Anal. Calcd for $C_{17}H_{22}O_3$: C, 74.42; H, 8.08. Found: C, 74.40; H, 8.15.

(±)-*c*-3-*tert*-Butylcyclohexane-*r*-1,*t*-2-diol Dibenzoate (8). The racemic diol **4** (133 mg, 0.77 mmol) and benzoyl chloride (1 mL, 8.6 mmol) were reacted in 5 mL of dry pyridine under the conditions used for the preparation of monester **7**. The diester (±)-**8** (134 mg) was separated from the crude reaction mixture, containing a large amount of benzoic anhydride, by TLC developed with 9:1 petroleum ether–ethyl ether. After crystallization from petroleum ether (±)-**8** had the following: mp 105–107 °C; NMR δ 1.00 (s, $C(CH_3)_3$, 9 H), ~5.3 (br m, CH–O–CO, 1 H), 5.70 (t, J = 9.7 Hz, CH–O–CO, 1 H), ~7.6 and 8.15 (2 m, 10 aromatic H).

Anal. Calcd for $C_{24}H_{28}O_4$: C, 75.76; H, 7.41. Found: C, 76.10; H, 7.26.

Microsomal Preparations. Liver microsomes were prepared from phenobarbital-treated male New Zealand white rabbits as previously described,[12] suspended in 0.01 M phosphate buffer (pH 7.4) or in Tris–HCl buffer (pH 9.0) to a final protein concentration of a ca. 16 mg/mL, and stored at −40 °C.

Incubations. Unless otherways specified, the incubations were carried out with the following standard procedure: the microsomal preparation (15 mL) was added to the epoxide **1** or **3** (130 to 150 mg) in 10 mL of 0.01 M phosphate buffer (pH 7.4) and 3 mL of ethanol, and the mixture was incubated with shaking at 37 °C. The incubations of **3** at pH 9.0 were carried out by adding a solution of ~130 mg of the substrate in 0.5 mL of acetonitrile to 10 mL of the microsomal preparation. After the times reported in Tables I and II, the incubations were terminated by cooling at −40 °C, the proteins were precipitated by addition of saturated aqueous barium hydroxide followed by shaking, and the resulting mixtures were directly extracted five times with 10 mL each of ethyl acetate and shaking for 5 min. The extracts were dried and combined in a volumetric flask (50 mL), biphenyl (1 mg) was added as a standard to 1 mL of this solution, and the amounts of recovered epoxide and diol were determined by GLC (2-m glass column, 2.5-mm i.d., packed with 10% Carbowax 20M on silanized Chromosorb W, 80–100 mesh; low isotherm 100 °C, high isotherm 195 °C, temperature increment 4.5 °C/min, evaporator and detector 210 °C, nitrogen flow 30 mL/min) by using a calibration curve obtained with the pure reference compounds. The relative retention times were as follows: (1) biphenyl, 1; **1**, 0.26; **2**, 1.85; (2) biphenyl 1; **3**, 0.30; **4**, 1.26. Only the diaxial diol **2** and only the diequatorial diol **4** were respectively detected in the incubation mixtures of the *cis*- and *trans*-epoxides **1** and **3**. The average total recovery of epoxide and diol was around 80% starting from the cis epoxide, and around 75% from the *trans*-epoxide. Higher recovery was obtained from Tris–HCl than from phosphate buffer with the latter substrate. Blank experiments carried out with each epoxide and boiled microsomes and with each pure diol and active microsomes showed that no spontaneous hydrolysis occurred even at the longest incubation times and that racemic diols were recovered from incubations in 80–90% yields.

(42) Bellucci, G.; Berti, G.; Ferretti, M.; Marioni, F.; Re, F. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 838.

The extracts were evaporated in vacuo(<40 °C), and the residues were subjected to preparative TLC developed with 9:1 petroleum ether–ethyl ether and made visible by spraying the strip with sulfuric acid. Only the epoxides migrated under these conditions. The recovered diols were also further purified by complete sublimation at 0.5 mm. The optical rotations of the pure (GLC) epoxides and diols obtained by stopping the incubations at different times were then measured (Tables I and II). No optical activity was detected in the products recovered from control experiments in which the racemic diols were incubated with the microsomes under the conditions employed for the incubations of the respective parent epoxides.

Enantiomeric Composition of Products. The enantiomeric excesses of the diols **2** and **4** obtained from the enzyme-promoted hydrolysis of epoxides **1** and **3** were determined by the NMR spectra taken with a Varian CFT 20 spectrometer [$CDCl_3$ solutions of ca. 0.02 M diol and Eu(facam)$_3$] on the basis of the areas of the differently shifted singlets due to the *tert*-butyl groups of the two diastereoisomeric complexes [$\Delta\delta$ = 3.6 Hz for (±)-**2**, $\Delta\delta$ = 12.9 Hz for (±)-**4**]. With this method a 74% ee was evaluated for a sample of **2** with $[\alpha]^{20}_D$ +26.5°, giving a maximum optical rotation of $[\alpha]^{20}_D$ +35.8° for the diaxial diol. Similar although less accurate results were obtained for the ee of diol **2** on the basis of the multiplets at δ 7.03, 7.25, 7.53, and 7.65, due to the protons α to the hydroxyl groups of the two diastereoisomeric complexes with the chiral LSR. Only one *tert*-butyl singlet and two multiplets at δ 7.03 and 7.53 were detected with samples of **2** having $[\alpha]^{20}_D$ +35.0–35.8°.

Only one *tert*-butyl singlet was observed for all samples of diol **4** ($[\alpha]^{20}_D$ + 1.0 ± 0.2°) obtained by enzyme hydrolysis of (±)-**3** below 50% conversion.

Absolute Configurations of Products. (1*R*,2*R*,6*S*)-(−)-*t*-2-(Benzoyloxy)-*c*-6-*tert*-butyl-*r*-1-cyclohexanol (6). The treatment of a sample of **2** (35 mg, $[\alpha]^{20}_D$ +35.0°) with benzoyl chloride as reported for (±)-**2** gave 40 mg of the pure monoester **6**: $[\alpha]^{20}_D$ −29.3°, $[\alpha]^{20}_{546}$ −33.7°, $[\alpha]^{20}_{436}$ −61.6°, $[\alpha]^{20}_{365}$ −103.8° (*c* 4.6).

(2*R*,6*S*)-(−)-*trans*-2-(Benzoyloxy)-6-*tert*-butylcyclohexanone (10). Oxidation of 35 mg of (−)-**6** ($[\alpha]^{20}_D$ −29.3°) with Jones reagent as reported for (±)-**6** yielded 27 mg of (−)-**10** with IR and NMR spectra identical with those of (±)-**10**: mp 63–65 °C; $[\alpha]^{20}_D$ −92.9°, $[\alpha]^{20}_{546}$ −109.2°, $[\alpha]^{20}_{436}$ −237.0°, $[\alpha]^{20}_{365}$ −566.5°; UV (cyclohexane) λ_{max} 274 nm (ϵ 1060), 300 (80); CD (*c* 1.1 × 10^{-3} M, cyclohexane) $[\theta]_{260}$ 0, $[\theta]_{298}$ 8400, $[\theta]_{340}$ 0.

(2*S*,6*R*)-(+)-*trans*-2-Bromo-6-*tert*-butylcyclohexanone (9). A solution of (−)-**1** (50 mg, $[\alpha]^{20}_D$ −20.5°) in chloroform (10 mL) was saturated with dry HBr at room temperature and then washed with water, 10% aqueous Na_2CO_3, and water. GLC analysis of the crude product (1.5-m glass column, 2.5-mm i.d., packed with 2% neopentyl glycol succinate on silanized Chromosorb W, 80–100 mesh; column 95 °C, evaporator 160 °C, nitrogen flow rate 30 mL/min) showed the presence of about 5% of *t*-2-bromo-*c*-3-*tert*-butyl-*r*-1-cyclohexanol besides bromohydrin **5**.[29] Oxidation of this product with Jones reagent yielded (+)-**9** as an oil, identified by comparison of its IR and UV spectra and GLC retention time with those of a sample of (±)-**9**:[30] $[\alpha]^{20}_D$ +125.0°, $[\alpha]^{20}_{546}$ +160°, $[\alpha]^{20}_{436}$ +400.0°, $[\alpha]^{20}_{365}$ +1390° (*c* 0.2, cyclohexane); UV (cyclohexane) λ_{max} 307 nm (ϵ 120); CD (*c* 7.5 × 10^{-3} M, cyclohexane) $[\theta]_{250}$ 0, $[\theta]_{318}$ 14.080, $[\theta]_{380}$ 0.

(1*R*,2*R*,3*R*)-(−)-*c*-3-*tert*-Butylcyclohexane-*r*-1,*t*-2-diol Dibenzoate (8). A sample of the diol **4** (70 mg, $[\alpha]^{20}_D$ +1.0°) obtained by enzymatic hydrolysis of (+)-**2** was reacted with a tenfold excess of benzoyl chloride in 2.5 mL of pyridine for 2 days at room temperature. Dilution with cold aqueous HCl, the usual workup, and purification by TLC as described for (+)-**9** gave the pure dibenzoate [mp 98–100 °C (from petroleum ether)] with an NMR spectrum identical with that of (±)-**8**: $[\alpha]^{24}_D$ −89.6°, $[\alpha]^{24}_{546}$ −107.4°, $[\alpha]^{24}_{436}$ −197.7°, $[\alpha]^{24}_{365}$ −347.4° (*c* 0.87, AcOEt); CD (*c* 2.20 × 10^{-5} M, EtOH) $[\theta]_{255}$ 0, $[\theta]_{236}$ −117 000, $[\theta]_{228}$ 0, $[\theta]_{223}$ 52 500, $[\theta]_{210}$ 0.

Anal. Calcd for $C_{24}H_{28}O_4$: C, 75.76; H, 7.41. Found: C, 76.07; H, 7.51.

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Registry No. (±)-**1**, 81800-94-4; (−)-**1**, 81800-95-5; (±)-**2**, 81800-96-6; (+)-**2**, 81800-97-7; (±)-**3**, 81800-98-8; (−)-**3**, 81800-99-9; (±)-**4**, 81801-00-5; (+)-**4**, 81801-01-6; **5**, 38512-64-0; (±)-**6**, 81769-10-0; (−)-**6**, 81801-02-7; (±)-**7**, 81801-03-8; (±)-**8**, 81769-11-1; (−)-**8**, 81801-04-9; (+)-**9**, 81801-05-0; (±)-**10**, 81769-12-2; (−)-**10**, 81801-06-1; (±)-**11**, 81769-13-3; *t*-3-bromo-*c*-3-*tert*-butyl-*r*-1-cyclohexanol, 81801-07-2.