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Cyclopropyl Oxiranes: Reversible Inhibitors of Cytosolic and Microsomal Epoxide Hydrolases[1]

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A series of aryl- and alkyl-substituted cyclopropyl oxiranes were synthesized as potential suicide inhibitors of mouse liver epoxide hydrolase (EH). The inhibitory potency of each compound and its corresponding alkene precursor was determined with mouse liver EHs using [^{3}H]-*cis*-stilbene oxide as substrate for microsomal EH (mEH) and for glutathione-*S*-transferase, and using [^{3}H]-*trans*-stilbene oxide for cytosolic EH (cEH). The cyclopropyl oxiranes all showed low (26–60% at 5×10^{-4} M) inhibition of glutathione transferase and moderate inhibition ($I_{50} = 5 \times 10^{-4}$ to 6×10^{-6} M) for cEH and mEH. *cis*-Phenylcyclopropyl oxirane had an I_{50} for mEH near that for a commonly used inhibitor, 1,1,1-trichloropropene oxide. Inhibition appeared competitive and reversible, and the cyclopropyl oxiranes appeared to function as alternate substrates. Absence of irreversible inhibition is evidence against a strongly electrophilic epoxide-opening mechanism involving a cyclopropyl carbinyl–homoallyl cation rearrangement. Instead, a concerted mechanism is favored, in which electrophilic opening and hydroxide attack occur in a concerted fashion.

Epoxide hydrolases (EHs)[4] (EC 3.3.2.3) and glutathione-*S*-transferases (EC 2.5.1.18) represent two well-studied enzymatic pathways for epoxide catabolism (1, 2). EHs convert epoxides into polar diols while glutathione-*S*-transferases transform them into the corresponding hydroxy cysteinyl adducts. These so-called detoxication processes generally result in the conversion of hydrophobic endogenous or foreign compounds to a more hydrophilic derivative which can be conjugated and/or excreted. In some instances, the epoxide hydration can lead to a more toxic metabolite. For this reason, there is a continuing need to examine the mechanism of the EHs and the selective inhibition of these enzymes by substrate analogs, mechanism-based inhibitors, and active site-directed reagents.

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[4] Abbreviations used: EH, epoxide hydrolase; cEH, cytosolic EH; mEH, microsomal EH; TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; THF, tetrahydrofuran; MCPBA, *m*-chloroperbenzoic acid; TMS, tetramethylsilane; HRMS, high-resolution mass spectroscopy; DMSO, dimethyl sulfoxide; TCPO, trichloromethyl oxirane; PCO, phenylcyclopropyl oxirane; PPCO, *trans*-β-(2′-phenyl)-cyclopropylstyrene oxide; TESO, *trans*-β-ethyl styrene oxide; PHCO, 1-(2′-phenyl)-cyclopropyl-1,2-epoxyheptane.

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In this study, we describe the preparation and inhibitory potency of a series of cyclopropyl-substituted oxiranes. These were designed as potential active site-directed, mechanism-based inhibitors which would probe for significant electrophilic character during the opening of the epoxide ring. That is, if significant electropositive character developed adjacent to the cyclopropane, a rearrangement of the cyclopropylcarbinyl cation to a homoallyl cation might occur. The reactive homoallylic form of this cation would be a potent alkylating agent in which the positive center would be several Angstroms removed from the normal site of hydroxyl attack.

There appear to be multiple forms of epoxide hydrolases (3-5); we will discuss only two forms of murine hepatic EH. The cytosolic form (cEH) occurs largely in the 100,000*g* supernatant of a subcellular homogenate and is defined in this paper as the form which optimally hydrates *trans*-stilbene oxide (TSO) at or near a neutral pH. The microsomal form (mEH) resides largely in the 100,000*g* pellet and optimally hydrates *cis*-stilbene oxide (CSO) at a more basic pH.

Most of the known inhibitors for each form seem to act as alternate substrates (Fig. 1). For the mEH the most widely used inhibitors include 1,2-epoxycyclohexane and 1,1,1-trichloropropene oxide (trichloromethyl oxirane) (6). Substituted chalcone oxides are potent inhibitors of the cEH, as are sulfhydryl reagents (7). A variety of epoxidized organic compounds are also competitive inhibitors (8) and alternative substrates (9). With both enzymes, the lipophilic nature of the inhibitor seems important for activity. In the case of the cytosolic enzyme, the most potent inhibitors have lipophilic groups substituted on both carbons of the epoxide.

The main characteristic which mEH and cEH share is the apparent mechanism of oxirane opening. Stereochemical, kinetic, and ^{18}O-labeling studies support a simple nucleophilic opening, in which the enzyme directs a hydroxide ion equivalent to the backside of the least hindered oxirane carbon (10, 11). Involvement of a histidine imidazole as a general base catalyst has been suggested for mEH (12). For the more "neutral" cEH, a push-pull mechanism has been postulated in which general base activates the water while a general acid weakens the *anti*-periplanar C-O bond by interaction with the oxygen electrons (7, 13).

The cyclopropyl oxiranes were thus designed to probe the electronic details of epoxide ring opening by cEH (Fig. 2). If the mechanism involves a concerted process of electrophilic activation-nucleophilic addition, the cyclopropyl oxiranes would at best be reversible competitive

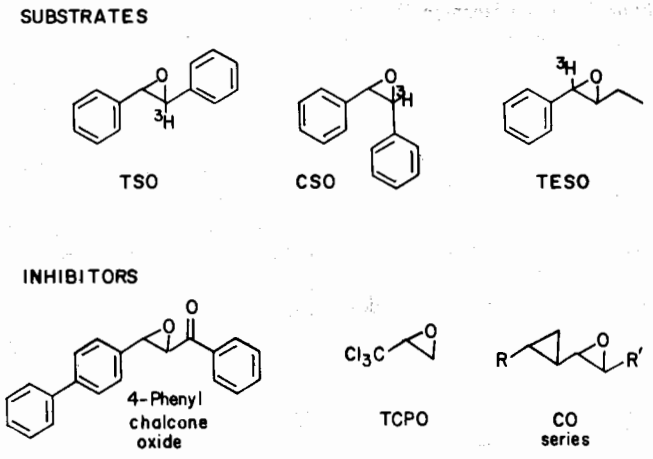


FIG. 1. Epoxide hydrolase substrates and inhibitors.

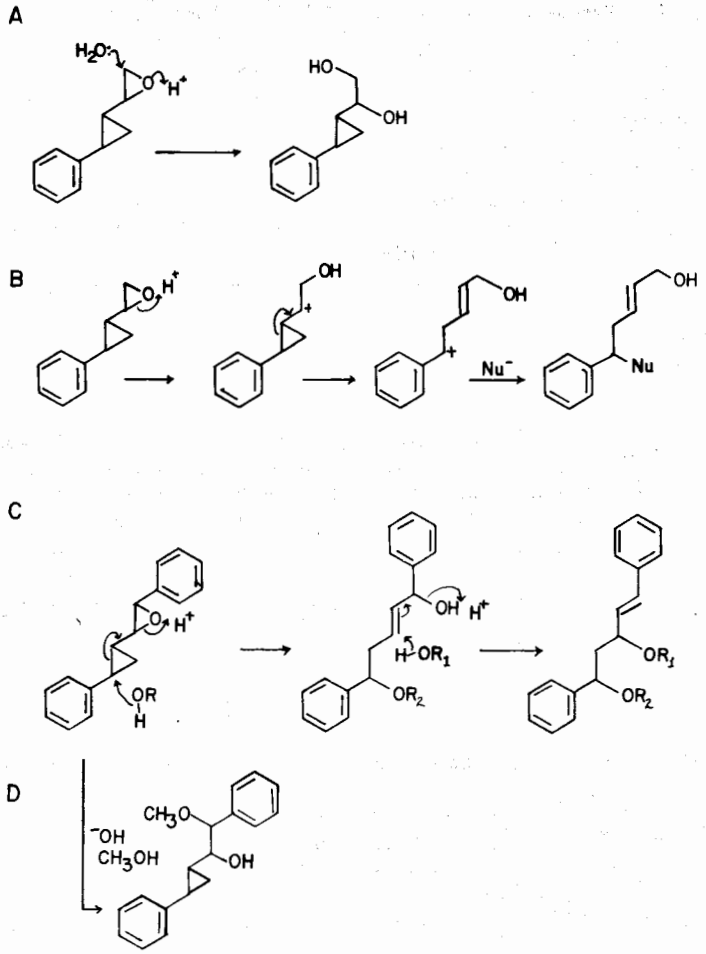


FIG. 2. (A) Nucleophilic hydrolysis, (B) postulated suicide-inactivation by cyclopropyl oxiranes during nonconcerted epoxide hydrolysis, (C) acid-catalyzed hydrolytic opening of PPCO, and (D) base-catalyzed opening of PPCO.

inhibitors (substrate analogs). If substantial cationic character developed on the carbon α to the cyclopropane, then an irreversible alkylating agent could be unmasked during the catalytic hydration even if the overall mechanism appeared to be a simple nucleophilic opening.

EXPERIMENTAL PROCEDURES

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All reactions and distillations were performed under nitrogen. Tetrahydrofuran (THF) was distilled from benzophenone sodium ketyl. Methylene chloride was distilled from phosphorus pentoxide and stored over sodium hydroxide pellets. Methanol was distilled from calcium hydride and dried over 3-Å molecular sieves. Toluene was azeotropically distilled and stored over 3-Å molecular sieves. Dimethyl sulfoxide (DMSO) was distilled from barium oxide and stored over molecular sieves. 1-Bromohexane and 1-bromodecane were distilled before use. Hexane, ethyl acetate, and anhydrous diethyl ether were Fisher HPLC grade and were used without further purification. Acetonitrile was stored over calcium hydride. Potassium fluoride was activated by heating at 100°C (1 mm Hg) for 1 h and *m*-chloroperbenzoic acid (MCPBA) was purified by washing with a pH 7.4 KH_2PO_4-Na_2HPO_4 buffer.

^1H NMR spectra were recorded at 80 MHz on a Varian HFT-80 spectrometer or at 300 MHz on an NT-300 instrument. ^{13}C NMR spectra were recorded

at 20 MHz on a Varian CFT-20 or at 75 MHz on the NT-300 with off-resonance proton decoupling. All NMR samples were prepared in $CDCl_3$ and shifts reported in ppm relative to δ(TMS) = 0 ppm. The following abbreviations are used for peak multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Infrared spectra were obtained from a Perkin-Elmer 727 instrument. Only important diagnostic peaks are reported.

Thin-layer chromatography was performed using MN Polygram Sil G/UV 254 (4 × 8 cm). TLC plates were visualized with uv light and ethanolic-vanillin-sulfuric acid reagent. Flash chromatography was performed on Merck silica gel G (400-230 mesh) under nitrogen pressure. Analytical gas-liquid chromatography (GLC) was performed on a Varian model 3700 Chromatograph equipped with a flame ionization detector and helium carrier gas using (a) DX-4 fused silica, 30 m × 0.25 mm, (b) DB-5 fused silica, 30 m × 0.25 mm, or (c) DB-1701 fused silica, 30 m × 0.25 mm. Preparative GLC was performed on a Varian Aerograph Model 920 using a 3/8 in. × 15 ft 20% Carbowax 20M column.

Melting points were taken in capillary tubes by using a Thomas-Hoover melting point apparatus and are uncorrected.

Synthesis of substrates. Synthetic and spectral details (Fig. 3) are presented only for the alkenes and epoxides. Preparations of other intermediates may be found elsewhere (14).

β-Cyclopropylstyrene (**5a**). Benzyltriphenylphosphonium bromide (0.74 g, 1.7 mmol) was dissolved in THF and treated with 0.73 ml of a 2.2 M solution of *n*-butyllithium-hexane. Cyclopropylcarboxaldehyde **3** (0.12 g, 1.7 mmol) in ether was added dropwise after the red ylide solution was refluxed for 0.5 h. The resulting mixture was allowed to stir and reflux for 24 h. Solvent was removed *in vacuo;* the residue was extracted into 1:1 ether-hexane; the organic layer was washed with water, dried ($MgSO_4$), and concentrated; and the residue was evaporatively distilled to give 0.145 g (59%) of the alkene **5a** (96% pure by GLC; *cis:trans*, 1:1.4) (R_f = 0.43, 2% ethyl acetate-hexane). Further purification by preparative GLC allowed the separation of the *cis/trans* isomers. ^{1}H NMR, δ7.23 (m, aromatic), *trans:* 6.48 (d, 15.8 Hz, H-1), 5.65 (dd, J_1 = 9.1, J_2 = 15.8 Hz, H-2); *cis:* 6.40 (d, 11.6 Hz, H-1), 5.08 (dd, J_1 = 9.9, J_2 = 11.6 Hz, H-2); 1.00 – 0.85 (m, H-3, 4); ^{13}C NMR, (mixture) δ136.83, 134.91, 128.75 – 125.66 (aromatic and olefinic), 14.58 (C-3), 8.10, 7.29 (C-4, 5). High-resolution mass spectrum (HRMS); Calcd for $C_{11}H_{12}$: 144.0919. Found: 144.0929.

1-Cyclopropyl-1-heptene (**5b**) was prepared in the same manner as **5a**, using hexytriphenylphosphonium bromide. The crude material was evaporatively distilled and purified on a flash column (2% ethyl acetate-hexane) to give a 37% yield of the alkene **5b.** The *cis/trans* isomers could not be separated by prep GLC or silver nitrate-silica flash chromatography. ^{1}H NMR, *trans:* δ5.52 (m, H-2), 4.97 (m, H-1); *cis:* 5.35 (m, H-2), 4.72 (m, H-1); 2.15 (m, H-3), 1.95 (m, H-4), 1.55 (m, H-1'), 1.31 – 0.70 (m, H-5-7), 0.33 (H-2'). HRMS: Calcd for $C_{10}H_{18}$: 138.1378. Found: 138.1393.

1-Cyclopropyl-1-undecene (**5c**) was prepared as described for **5a** using decyltriphenylphosphonium bromide. Evaporative distillation and chromatography (2% ethyl acetate-hexane) gave the alkene **5c** in 53% yield. Further purification by preparative GLC and silver nitrate-silica chromatography proved unsuccessful in separating the *cis/trans* isomers (R_f = 0.71, 24% ethyl acetate-hexane). ^{1}H NMR, δ5.37 – 4.70 (m, H-1, 2), 2.00 (m, H-3), 1.41 (m, H-4), 1.08

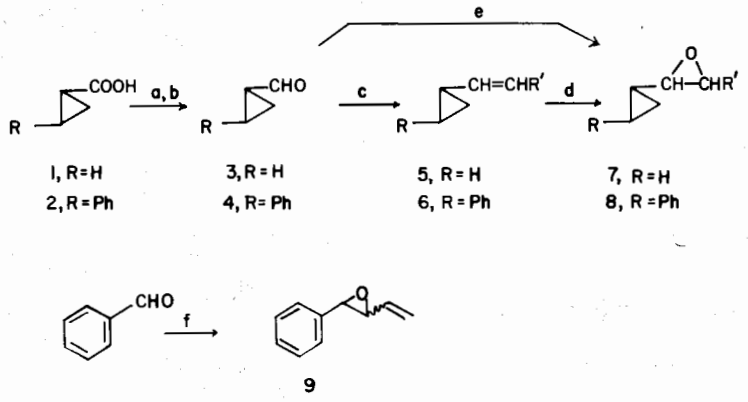


FIG. 3. Synthesis of cyclopropyloxiranes. Reagents: (a) $LiAlH_4$, THF; (b) PCC, CH_2Cl_2, 20°C; (c) $RCH_2\overset{+}{P}Ph_3\bar{I}$, nBuLi, THF, 0°C; (d) MCPBA-KF, CH_2Cl_2 or MCPBA, CH_2Cl_2-aqueous $NaHCO_3$ (two phase); (e) for R' = H: $(CH_3)_3\overset{+}{S}O\bar{I}$, I^-, $Na^+\bar{C}H_2SOCH_3$, DMSO; (f) $CH_2{=}CH{-}CH_2\overset{+}{S}Ph_2\bar{B}F_4$, THF, *t*BuLi.

– 0.68 (m, H-5–11, 1′), 0.42 – 0.05 (m, H-2′, 3′); ^{13}C NMR, δ133.85, 128.52 (C-1, 2), 32.02 – 22.78 (C-3–11), 14.19 (C-1′), 9.66, 6.78 (C-2′, 3′). HRMS: Calc'd for $C_{14}H_{26}$: 194.2048. Found: 194.2041.

β-(2′-Phenyl)-cyclopropylstyrene (**6a**). Aldehyde **4** was employed in the Wittig reaction giving 0.871 g (51%) of **6a** after purification by flash chromatography (R_f = 0.65, 20% ethyl acetate–hexane). ^{1}H NMR, δ7.20 (m, aromatic), 6.48, 6.00 (m, H-1, 2), 2.20 (m, H-3), 1.45 (m, H-4); ^{13}C NMR, δ132.98, 128.74 – 125.82 (C-1, 2 and aromatic), 27.45, 25.84 (C-3, 4), 17.17 (C-5). HRMS: Calc'd for $C_{17}H_{16}$: 220.1236. Found: 220.1244.

1-(2′-Phenyl)-cyclopropyl-1-heptene (**6b**) was prepared analogously. Flash chromatography gave a 44% yield of the alkene **6b** (R_f = 0.74, 24% ethyl acetate–hexane). *Cis/trans* isomers were separated by preparative GLC. ^{1}H NMR, δ7.22, 7.04 (m, aromatic), *trans:* 5.52 (m, H-2), 5.14 (dd, J_1 = 15.1, J_2 = 8.3 Hz, H-1); *cis:* 5.39 (dt, J_1 = 10.8, J_2 = 7.3 Hz, H-2), 4.93 (dd, J_1 = 7.9, J_2 = 10.5 Hz, H-1); 1.95 – 0.91 (m, H-3–7, 1′-3′); ^{13}C NMR, δ144.32, 133.83 (C-1, 2), 131.10 – 127.17 (aromatic), 33.17 – 26.62 (C-3–7), 24.29, 18.86 (C-1′, 2′) 15.74 (C-3′). HRMS: Calc'd for $C_{16}H_{22}$: 214.1722. Found: 214.1722.

β-Cyclopropylstyrene oxide (**7a**). The *cis* isomer of alkene **5a** (0.028 g, 0.19 mmol) was added to suspension of 0.10 g (0.58 mmol) of MCPBA and 0.034 g (0.58 mmol) of activated KF in CH_2Cl_2 at 0°C (15). The mixture was allowed to stir overnight at room temperature and then filtered. Potassium fluoride (0.034 g) was added to the solution and it was stirred for 0.5 h. Upon filtration, the solvent was evaporated and the colorless oil was evaporatively distilled, yielding 0.015 g (48%) of the *cis*-epoxide **7a**. The *trans* isomer (0.017 g) was treated in the same way to give 0.015 g (79%) of the *trans*-epoxide **7a** (R_f = 0.51, 22% ethyl acetate–hexane). ^{1}H NMR, δ7.35 (m, aromatic), *cis:* 4.10 (d, J = 4.2 Hz, H-1), 2.61 (m, H-2); *trans:* 3.69 (d, J = 2.1 Hz, H-1), 2.74 (dd, J_1 = 5.5, J_2 = 2.1 Hz, H-2); 1.25 (m, H-4), 0.87, 0.45 (m, H-5, 6); ^{13}C NMR, δ131.89 – 125.53 (aromatic), 64.88, 63.76 (C-1, 2), 11.60 (C-4), 7.51 (C-5), 2.10 (C-6). HRMS: Calc'd for $C_{11}H_{12}O$: 160.0896. Found: 160.0892.

1-Cyclopropyl-1,2-epoxyheptane (**7b**). To a suspension of 0.17 g (1.0 mmol) of MCPBA and 0.11 g (1.3 mmol) of $NaHCO_3$ in $CHCl_3$ at 0°C was added 0.14 g (1.0 mmol) of the alkene **5b.** After allowing the mixture to stir overnight, it was washed with water, extracted into ether–hexane, washed with saturated aqueous $NaHSO_3$, washed with saturated aqueous $NaHCO_3$, dried ($MgSO_4$), concentrated, and purified by flash chromatography (2% ether, 2% triethylamine–hexane) to give 0.047 g (30%) of the epoxide **7b** (R_f = 0.38, 20% ethyl acetate–hexane). Attempted separation of isomers by preparative GLC proved unsuccessful. ^{1}H NMR, δ2.95 (m, H-1), 2.39 (m, H-2), 1.58 (m, H-3), 1.60 – 0.42 (m, H-4–7, 1′-3′); ^{13}C NMR, δ61.00 (C-1), 57.88 (C-2), 32.00 – 25.70 (C-3–7), 22.66 (C-1′), 14.10, 8.08 (C-2′, 3′). HRMS: Calc'd for $C_{10}H_{18}O$: 154.1375. Found: 154.1366.

1-Cyclopropyl-1,2-epoxyundecane (**7c**). The alkene **5c** was epoxidized following the same method described for **7b.** Evaporative distillation of the crude mixture gave a 57% yield of the *cis-* and *trans*-epoxides **7c,** which could not be separated by preparative GLC. ^{1}H NMR, δ2.96 (m, H-1), 2.50 (m, H-2), 1.59 (m, H-3), 1.37 – 0.41 (m, H-4–11, 1′-3′); ^{13}C NMR, δ61.05 (C-1), 57.93 (C-2), 31.99 – 26.59 (C-3–11), 22.76 (C-1′), 14.18, 8.11 (C-2′, 3′). HRMS: Calc'd for $C_{14}H_{26}O$: 210.1940. Found: 210.1962.

β-(2′-Phenyl)-cyclopropylstyrene oxide (**8a**). The alkene **6a** was epoxidized following exactly the method described for **7b.** Flash chromatography under the same mildly basic conditions gave a 40% yield of the oxirane **8a.** *trans/cis* isomers were separated during this step, affording a 3:1 ratio, respectively [R_f (*trans*) = 0.52, R_f (*cis*) = 0.43, 20% ethyl acetate–hexane]. ^{1}H NMR, δ7.15 (m, aromatic); *cis:* 4.16 (d, 4 Hz, H-1), 2.98 (m, H-2); *trans:* 3.75 (d, 2.4 Hz, H-1), 2.98 (m, H-2); 2.05 (m, H-4), 1.40 – 0.92 (m, H-5, 6); ^{13}C NMR, δ128.50 – 125.60 (aromatic), 63.33 (C-2), 58.24 (C-1), 23.47 (C-4), 20.53 (C-5), 12.19 (C-6). HRMS: Calc'd for $C_{17}H_{16}O$: 236.1207. Found: 236.1204.

1-(2′-Phenyl)-cyclopropyl-1,2-epoxyheptane (**8b**). The *cis* and *trans* alkene isomers of **6b** were oxidized in the manner of **7a.** Yields of 24 and 19% were obtained, respectively, after flash chromatography (R_f = 0.60, 24% ethyl acetate–hexane). ^{1}H NMR of mixture, δ7.03 (m, aromatic), 2.68 (m, H-1), 2.16 (m, H-2), 1.73 – 0.63 (m, H-3–7, 1′-3′); ^{13}C NMR, δ130.00 – 127.46 (aromatic), 61.05 (C-1), 59.57 (C-2), 33.28 – 22.39 (C-3–7), 22.29, 21.87 (C-1′, 2′), 15.62 (C-3′). HRMS: Calc'd for $C_{16}H_{22}O$: 230.1648. Found: 230.1659.

1-(2′-Phenyl)-cyclopropyl-1,2-epoxyethane (**8c**). According to the method of Clark and Goldsmith (16), 0.055 g (1.37 mmol) of sodium hydride (Aldrich, 60% mineral oil dispersion) was placed in a dry flask, washed three times with dry hexane, and pumped until free of solvent. Then, 0.30 g (1.37 mmol) of trimethylsulfoxonium iodide (Aldrich) was placed in the flask and 2 ml of dry DMSO was added dropwise with stirring. The mixture turned white and was allowed to stir for 15 min until hydrogen gas evolution ceased. The carboxaldehyde (0.2 g, 1.37 mmol) **4** in DMSO was added over 1 h at room temperature and the mixture was allowed to stir an additional 1.5 h at 50°C. The suspension was cooled, poured onto ice–water, and extracted into ether. Evaporation of solvent and purification by flash chromatography (2% ether, 2% triethylamine–hexane) afforded 0.065 g (30%) of the epoxide **8c** (R_f = 0.60, 22% ethyl acetate–hexane). ^{1}H NMR, δ7.20 (m, aromatic), 2.78 (m, H-2), 2.43 (m, H-1), 1.82 (m, H-4), 1.07 (m, H-5), 0.92 (m, H-6); ^{13}C NMR, δ128.46 – 125.91 (aromatic), 53.17 (C-2), 46.69 (C-1), 23.14, 20.40 (C-4, 5), 19.91 (C-6).

β-Vinylstyrene oxide (**9**). To 299 mg (0.95 mmol) of allylphenylsulfonium tetrafluoroborate was added 15 ml of dry THF under nitrogen. The slurry was stirred at −78° for 30 min, and then 0.42 ml (0.97 mmol) of 2.3 M *t*-BuLi in pentane was added. After the solution had stirred for 15 min, a solution of 100 mg (0.94 mmol) benzaldehyde in 1 ml of THF was added. The mixture was stirred 15 min at −78°C, warmed to room temperature, worked up as above, and purified by preparative GLC to give 17 mg (12%) of *cis*-β-vinylstyrene oxide and 10 mg (7%) of *trans* isomer (the crude product contained 4:1 ratio of *cis*:*trans* isomer). *cis* isomer, ^{1}H NMR ($CDCl_3$) δ7.42 (s, 5H, aromatic), 5.78 − 5.26 (m, 3H, vinylic), 4.25 (d, 1H, J = 4.38 Hz, H-1′), 3.76 (dd, 1H, J = 4.38 and 8.25 Hz, H-2′); *trans* isomer: ^{1}H NMR ($CDCl_3$) δ7.34 (s, 5H, aromatic), 6.01 − 5.28 (m, 3H, vinylic), 3.81 (d, 1H, J = 2.50 Hz, H-1′), 3.42 (dd, 1H, J = 2.50 and 6.50 Hz, H-2′).

Acidic hydrolysis of PPCO (**8a**). To a solution of *trans*-β-(2′-phenyl)-cyclopropylstyrene oxide (138 mg, 0.58 mmol) in 2 ml of dioxane was added 2 ml of 0.1 N aqueous perchloric acid solution. The mixture was stirred for 24 h and extracted 3× with chloroform, and the extracts were washed (saturated $NaHCO_3$, brine), dried ($MgSO_4$), and chromatographed on deactivated silica gel (5% water) by elution with 10 and 20% ethyl acetate–hexane. The major product (91 mg, 62% yield) was the mixture of *erythro*- and *threo*-1,5-diphenyl-4-pentene-1,3-diol (1.1:1 ratio by GLC analysis after derivatization to diacetate). The structural assignments for the alcohols are based on the methanolysis products described below. ^{1}H NMR (300 MHz): δ6.59 (d, 1 H, J = 15.8 Hz, H-5), 6.27 (dd, 0.5 H, J = 15.8 and 6.1 Hz, *threo* H-4), 6.20 (dd, 0.5 H, J = 15.8 and 6.4 Hz, *erythro* H-4), 5.05 (dd, 0.5 H, J = 8.2 and 3.2 Hz, *threo* H-1), 4.97 (dd, 0.5 H, J = 9.8 and 3.0 Hz, *erythro* H-1), 4.55 (m, 1 H, H-3), 2.12 − 1.87 (m, 2H, H-2). ^{13}C NMR: *threo*; δ144.06 − 125.53 (aromatic carbons and C-4 and C-5), 71.23, 69.84 (C-1 and C-3), 44.53 (C-2), *erythro*; δ143.99 − 125.60 (aromatic carbons and C-4 and C-5), 74.21, 72.79 (C-1 and C-3), 45.23 (C-2); IR (thin film): 3265, 1590, 1480, 1440, 1060, 1040, 1000, 960, 900 cm^{-1}; LRMS (solid): 254 (M^+, 0.1) 107 (29, C-1, 2 fission), 105 (59), 104 (100).

The structure of the alcohols was further confirmed by examining the acidic methanolysis of PPCO (**8a**). To a solution of PPCO (**8a**) (47.3 mg, 0.20 mmol) in 3.5 ml of dry methanol was added 4.8 mg (0.025 mmol) of *p*-toluenesulfonic acid monohydrate. The mixture was stirred for 2 h, quenched with 24 mg of solid $NaHCO_3$ and partitioned in ether–water. The combined organic layers were washed with brine, dried ($MgSO_4$), concentrated, and purified by column chromatography (SiO_2) using 2.5% ethyl acetate–hexane as the eluent.

The first fraction afforded 20.4 mg (57%) of *threo*-3,5-dimethoxy-1,5-diphenyl-1-pentene (91% pure by GLC; rt, 6.45 min on DB-5, 200° + 5°C/min); ^{1}H NMR (300 MHz, $CDCl_3$): δ7.4 − 7.2 (m, 10H, aromatic), 6.56 (d, 1H, J = 15.9 Hz, H-1), 6.04 (dd, 1H, J = 15.9 and 8.0 Hz, H-2), 4.38 (dd, 1H, J = 9.3 and 4.4 Hz, H-5), 3.96 (ddd, 1 H, J = 8.0 and 4.2 and 8.9 Hz, H-3), 1.99 (ddd, 1H, J = 4.4 and 9.3 and −14.2 Hz, H-4); ^{13}C NMR ($CDCl_3$): δ142.18, 136.53, 128.52, 128.39, 127.63, 127.49, 126.55, 126.40 (aromatic carbons and C-1 and C-2), 79.83, 78.82 (C-3 and C-5), 56.70, 56.46 (-OMe), 44.96 (C-4).

The second fraction gave a mixture (11.7 mg, 21% yield) of a 1:2 ratio (by GLC) of 1,5-dimethoxy-1,5-diphenyl-2-pentene and *erythro*-3,5-dimethoxy-1,5-diphenyl-1-pentene. The latter had rt 6.13 min on DB-5; ^{1}H NMR (300 MHz, $CDCl_3$), δ6.49 (d, ^{1}H, J = 16.0 Hz, H-5), 6.05 (dd, 1H, J = 16.0 and 8.1 Hz, H-4), 4.27 (dd, 1H, J = 6.1 and 7.1 Hz, H-5), 3.64 (dd, 1H, J = 7.43 and 6.39, H-3), 2.30 (ddd, 1H, J = 7.1, 7.4 and −14.01 Hz, H-4), 1.77 (dd, 1H, J = 6.1, 6.4 and −14.1 Hz, H-4).

Attempted basic hydrolysis of PPCO (**8a**). To a solution of PPCO (50 mg, 0.21 mmol) in THF and methanol (0.5 ml and 0.3 ml) was added 0.1 ml of 40% aqueous potassium hydroxide solution. The mixture was stirred for 1 week at room temperature. The solvents were removed under reduced pressure and the residue was extracted with chloroform, washed with brine, and dried ($MgSO_4$). After removal of the solvent, column chromatography on a silica gel column (prewashed with 10% Et_3N in hexane) using 5% EtOAC–2% Et_3N in hexane as eluent gave unreacted starting material (33.8 mg, 68%) and methoxyhydrins (14%), consisting of 2.0 mg (3.5% of *erythro*- and 5.4 mg (10%) *threo*-2-methoxy-1-(2′-phenylcyclopropyl)-benzeneethanol. None of the desired diol could be obtained under any of the conditions employed.

Assays for epoxide-metabolizing enzymes. The cytosolic and microsomal fractions from male Swiss-Webster mice were prepared as described by Hammock and Ota (17), except that the 100,000*g* pellet was resuspended in 125 mM KCl, 50 mM Tris, pH 7.4, and centrifuged an additional time to obtain washed microsomes. The cytosol was diluted to 0.2% (w/v) of the original net weight of the liver with sodium phosphate buffer (76 mM, pH 7.4) and the microsomes were diluted to 1% (w/v) with Tris-HCl (100 mM, pH 9.0).

Cytosolic EH was assayed using [^{3}H]-*trans*-stilbene oxide (69 mCi/mmol, 5×10^{-5} M) as substrate, and microsomal EH and cytosolic GSH *S*-transferase were assayed using [^{3}H]-*cis*-stilbene oxide (69 mCi/mmol, 5×10^{-5} M) determined by partition assays (18). Inhibition of the hydration of these substrates was monitored as follows. Mouse liver cytosol (final volume, 100 μl) in 76 mM sodium phosphate buffer, pH 7.4, was preincubated at 37°C for 1 min, after which 1 μl of the inhibitor in ethanol or acetone was added. This addition was followed 10 min later

by the addition of 1 μl of substrate in ethanol. After 10 min, the reaction was terminated with 200 μl of dodecane and vigorous vortexing. Following centrifugation (1000*g*, 3 min), 50 μl of the aqueous phase was then removed for liquid scintillation counting.

The assay for the mEH was carried out in 100 mM Tris-HCl, pH 9.0, and that for cytosolic GSH *S*-transferase in 76 mM sodium phosphate, pH 7.4, in the presence of 5 mM GSH. Extraction with 200 μl of 1-hexanol was used to separate CSO from its GSH conjugate (18).

Assays of the epoxide-metabolizing enzymes in the presence of inhibitors were run in triplicate, and the percentage inhibition was calculated relative to product formation above background in solvent controls. To determine the concentration required for 50% inhibition of the enzyme (I_{50}), triplicate incubations at four or five different concentrations of the inhibitor were used. The I_{50} values were obtained from a linear fit of data on a log dosage-probit plot. At least three of the data points were on the linear portion of the curve and bracketed the I_{50}. Compounds with similar I_{50}'s were compared in the same run.

The effect of preincubation with the enzyme on inhibitor potency was studied as follows. A mixture of 2 ml of enzyme and 20 μl inhibitor in ethanol was preincubated at 37°C. At various time points, duplicate 100-μl aliquots were removed and pipetted into tubes each containing 1 μl of substrate (5×10^{-5} M). After vortexing, the tubes were incubated for 10 min at 37°C and the reaction was terminated as described above. Inhibition at time zero was evaluated by adding 100 μl of enzyme to a tube containing 1 μl of inhibitor and 1 μl of substrate. Percentage inhibition was determined relative to activity in control incubations containing enzyme and ethanol.

To determine the reversibility of inhibition, ethanol or an I_{80} concentration of the inhibitor was incubated with diluted cytosol for 15 min at 37°C, and the mixtures were dialyzed against 800 ml of 76 mM sodium phosphate, pH 7.4, at 4°C. At different time intervals (0–28 h), aliquots from control and inhibited samples were taken and assayed for activity with TSO. Buffer was changed at 2, 6, 10, and 22 h after dialysis. Dialysis bags containing control cytosol in the same container were monitored for activity to ensure that the process of dialysis did not greatly reduce enzyme activity and that the control cytosol was not inhibited by compounds in the buffer.

RESULTS AND DISCUSSION

Cyclopropyl oxiranes were synthesized via a Wittig reaction followed by epoxidation with MCPBA with the exception of monosubstituted oxirane **8c**, which was prepared by a sulfur ylide route to avoid handling a volatile precursor olefin. Epoxidation using KF–MCPBA complex by the method of Camps *et al.* (15) worked most efficiently. Although ^{1}H NMR and TLC showed that the reactions went to completion, yields of the recovered, purified oxiranes were low due to the inherent acid lability of these compounds. The purification of cyclopropyloxiranes **7a** through **8b** was accomplished by flash chromatography. Silica gel was pretreated with a solution of 10% triethylamine, 2% ether–hexane and then equilibrated with 2% triethylamine, 2% ether–hexane. Recoveries as low as 2% were obtained when flash chromatography was performed in the absence of triethylamine. The pH of the silica gel should not be below 6.4 (measured on 10 g of SiO_2 in 100 ml H_2O), since acid-catalyzed cleavage may occur in the absence of triethylamine.

The *cis* and *trans* isomers of epoxide **8a** were recovered by careful chromatography using the method outlined above. The remaining epoxides could not be separated in that manner, nor were they separable by preparative GLC using an 8-m column of 20% Carbowax 20M. None of the alkene isomers could be separated by 20% silver nitrate–silica flash chromatography with 2% ethyl acetate–hexane or pure hexane elution. The cyclopropane moiety may interact with the silver ions so as to obscure any separation on the basis of alkene geometry. The isomers of two alkenes, **5a** and **6b**, were isolated by preparative GLC. In contrast, the aliphatic alkenes **5b** and **5c** could not be resolved readily into the pure isomers. The presence of an aromatic ring facilitated separation of geometrical isomers, and the presence of two aromatic rings (as in **8a**) made the separation virtually effortless.

A manifestation of thermal instability appeared during analytical GLC. TLC and NMR-homogeneous epoxide *cis*-**7a** was injected onto DX-4 fused silica with a programmed range of 90–108°C. With the injector temperature set at over 100°C, four major peaks were observed. With lower injector temperatures and a shorter packed column only a single major peak was observed. This behavior was found to a lesser degree in some of the other ep-

oxides and is consistent with the overall reactivity and instability of these epoxides.

Under the conditions employed, the uninhibited microsomal fraction hydrated 5.6 nmol CSO min^{-1} mg $protein^{-1}$, the cytosolic fraction hydrated 5.7 nmol TSO min^{-1} mg $protein^{-1}$ or 61 nmol TESO min^{-1} mg $protein^{-1}$, and the cytosolic fraction led to production of 40 nmol min^{-1} mg $protein^{-1}$ of the conjugate of CSO in the presence of 5 mM reduced glutathione. Under these conditions, trichloromethyl oxirane (TCPO) had an I_{50} of 4×10^{-6} M with mEH and 4-phenyl chalcone oxide had an I_{50} of 6×10^{-7} M with cEH. The latter compound had an I_{50} of 6.4×10^{-8} M for cEH when TESO was used as substrate (7).

In examining Table I, a number of correlations may be drawn. The alkenes, which lack the electrophilic epoxide, are all poor inhibitors at the limits of their solubilities. The epoxides show cEH and mEH inhibition with a range of I_{50}'s from $>5 \times 10^{-4}$ down to 6×10^{-6}; they show also only modest glutathione *S*-transferase inhibitory potency, with 26 to 63% inhibition at 5×10^{-4}.

In most cases, differences can be noted between the values for cEH and mEH for each epoxide. The compounds for which there exist the least difference between mEH and cEH forms are the disubstituted epoxide **8b** (including *cis* and *trans* forms) and the monosubstituted epoxide **8c.** In comparing epoxides **8b** and **8c,** where one lacks the hexyl substituent, there is little difference in inhibitory activity with respect to each other and with respect to the different EH forms. Stereochemistry does not seem to be a factor for these substrates.

If the nature of the substituent on the cyclopropyl moiety is held constant, and the substitution at C-2 is varied, then the presence of the aromatic ring in epoxide **8a** gives less potent inhibition for mEH, but better inhibition for cEH. The other substituents on oxiranes **8b** and **8c,** pentyl and hydrogen, respectively, offer approximately constant values overall. In comparing compounds **7a** (aryl), **7b** (pentyl), and **7c** (nonyl), the strongest inhibitor is the nonyl substituted oxirane **7c** for mEH and for cEH.

By varying the group on the cyclopropyl moiety, and keeping the 2-epoxide substituent constant, there are only modest differences for the mEH form between hydrogen-substituted **7a** and aryl-substituted **8a** and between pentyl-substituted **7b** and aryl-substituted **8b.** However, for cEH, aryl substitution on the cyclopropyl moiety (compounds **8a** and **8b**) appears to increase inhibitory potency.

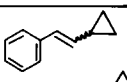
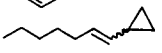
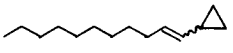
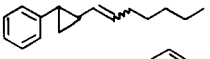
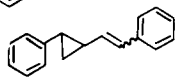
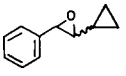
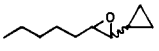
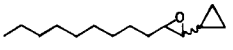
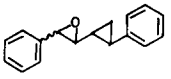
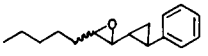
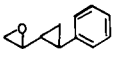
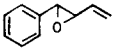
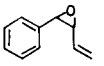
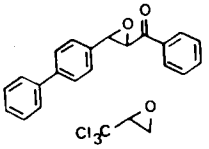
Differences between *cis* and *trans* epoxides are also apparent for some pairs. The most dramatic example is seen in aryl-substituted **7a.** The *cis* isomer of PCO (**7a**) is a 100-fold better inhibitor than the *trans* isomer for mEH, while the *trans*-PCO is two times better for cEH inhibition. Similarly, *cis*-PPCO (**8a**) has a lower I_{50} for mEH than *trans*-PPCO and a slightly higher I_{50} for cEH. The differences for *cis*- and *trans*-PHCO (**8c**) and the vinyl oxiranes **9** are not clear cut. The general trend of *trans*-1,2-disubstituted oxiranes showing higher inhibitory activity for cEH and the *cis* isomers showing higher activity for mEH is best seen in the 1,2-diaryl-substituted cases.

The two isomeric β-vinyl styrene oxides **9,** which are unsaturated analogs of TESO, were prepared by condensation of the allyldiphenylsulfonium ylid with benzaldehyde and separation by chromatography. These substrates could also act as irreversible inhibitors if an allylic cation resulted from electrophilic epoxide opening. The high I_{50} values for these substrates do not support such a mode of inhibition, and these appear to be simple competitive inhibitors for the active site.

The data in Fig. 4 indicate that the inhibition of the cytosolic epoxide hydrolase by *trans*-PPCO and the inhibition of the microsomal epoxide hydrolase by *trans*-PCO are not time dependent on the time scale of the assay. One would expect that inhibition of the enzymes by a suicide mechanism such as that shown in Fig. 2 would occur in a time-dependent process. In this case, an inactivation rate much faster than substrate turnover would be required for time-dependent inactivation

TABLE I

INHIBITORY POTENCY OF VARIOUS EPOXIDES ON EPOXIDE-METABOLIZING ENZYMES OF MOUSE LIVER

Compound	R	R′	Code	Structure	Epoxide hydrolase		Glutathione S-transferase
					Microsomal	Cytosolic	
5a	H	Ph	PCE		15% (5×10^{-4})	23% (5×10^{-4})	—
5b	H	C_5H_{11}	HCE		—	—	—
5c	H	C_9H_{19}	DCE		—	—	—
6a	Ph	Ph	PPCE		14 (1×10^{-4})	18 (1×10^{-4})	—
6b	Ph	C_5H_{11}	PHCE		30 (2×10^{-4})	—	—
7a	H	Ph	PCO		2.0×10^{-5}	2.9×10^{-4}	34 (5×10^{-4})
trans-**7a**	H	Ph			41 (5×10^{-4})	2.5×10^{-4}	26 (2×10^{-4})
cis-**7a**	H	Ph			6.6×10^{-6}	5×10^{-4}	30 (2×10^{-4})
7b	H	C_5H_{11}	HCO		1.7×10^{-5}	30 (5×10^{-4})	30 (5×10^{-4})
7c	H	C_9H_{19}	DCO		7.5×10^{-6}	9×10^{-5}	—
8a	Ph	Ph	PPCO		4×10^{-4}	3.3×10^{-5}	36 (5×10^{-4})
trans-**8a**	Ph	Ph			5×10^{-4}	2.2×10^{-5}	52 (5×10^{-4})
cis-**8a**	Ph	Ph			1.4×10^{-5}	1.7×10^{-4}	60 (5×10^{-4})
8b	Ph	C_5H_{11}	PHCO		2.8×10^{-5}	8.6×10^{-5}	63 (5×10^{-4})
trans-**8b**	Ph	C_5H_{11}			1.6×10^{-4}	1.5×10^{-4}	51 (5×10^{-4})
cis-**8b**	Ph	C_5H_{11}			2.0×10^{-5}	6.0×10^{-5}	63 (5×10^{-4})
8c	Ph	H	FCO		2.9×10^{-5}	8.0×10^{-5}	48 (5×10^{-4})
trans-**9**	—	Ph			30 (5×10^{-4})	5.0×10^{-4}	35 (5×10^{-4})
cis-**9**	—	Ph			1.4×10^{-4}	1.2×10^{-4}	25 (5×10^{-4})
4-Phenyl chalcone oxide					—	6×10^{-7}	—
TCPO					4×10^{-6}	—	—

Note. Microsomal EH and cytosolic glutathione transferase were assayed with *cis*-stilbene oxide (CSO) (69 mCi/mmole, 5×10^{-5} M) and cytosolic EH with *trans*-stilbene oxide (TSO) (69 mCi/mmole, 5×10^{-5} M).

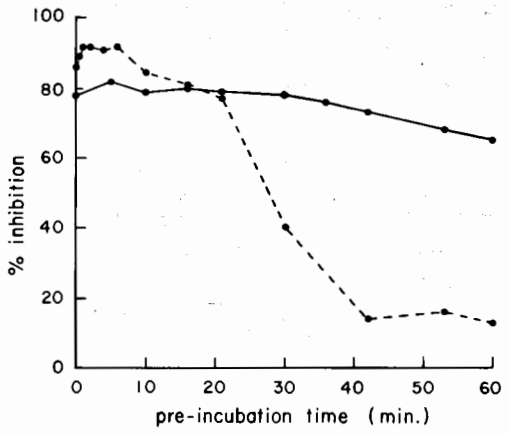


FIG. 4. Time course of inhibition of cEH by *trans*-PPCO (**8a**) (- - -) and of mEH by *trans*-PCO (**7a**) (—). Enzyme and inhibitor (final concentration, 5×10^{-5} M) were preincubated at 37°C. At the time indicated, duplicate 100-μl aliquots were removed and added to tubes containing 1 μl substrate in ethanol. Enzyme activity was assayed as described in text and expressed as the average of two replicates. Inhibition is expressed relative to activity of the ethanol-treated control enzyme at each time point.

to be occurring. Moreover, a covalent intermediate would not be expected to rapidly reactivate as shown in the dialysis experiment (Fig. 5). It is also clear from this figure that the inhibition decreases with time, especially in the case of cEH. These data support the hypothesis that the compounds are acting as alternate substrates. This decrease cannot be explained by the nonenzymatic reaction of PPCO and PCO with water or other nucleophiles, since they are stable for over 40 h over a range from pH 3 to pH 11 in aqueous THF.

As shown in Fig. 5, the majority of the enzyme activity is recovered when cytosol previously inhibited with PPCO is dialyzed. These data also suggest that the cyclopropyl epoxides are acting as alternate substrates since no covalent modification of the enzyme has taken place.

The inhibitor *trans*-**8a** (PPCO) was subjected to pH extremes to determine its chemical stability to nonenzymatic hydrolytic stability. In the range pH 4–10 essentially no reaction occurs in a 10^{-2} M solution of *trans*-PPCO during several hours at room temperature. Using forcing acidic conditions, a reaction product containing the rearranged enediol moiety was isolated and characterized spectroscopically as the *erythro*- and *threo*-1,3-diols (Fig. 2C, $R_1 = R_2 = H$). These structures were proven by acidic methanolysis to the enedimethoxy compound (Fig. 2C, $R_1 = R_2 = CH_3$). This product was assayed for inhibitory potency and found to be 20-fold less active (I_{50} for cEH $= 4 \times 10^{-4}$ M). Forcing basic conditions [5:5:1 (v/v) THF: MeOH:40% KOH] failed to produce any of the cyclopropane-containing unrearranged diol. Only the methoxyhydrins (Fig. 2D) were isolated but not assayed.

All previous studies of epoxide hydrolase (6–13) support a classical S_N2 mechanism, i.e., nucleophilic attack of activated water (e.g., hydroxide ion) at the least substituted carbon with inversion of stereochemistry. However, none of the experiments reported to date rule out the possibility of electrophilic activation prior to or during the nucleophilic attack. Indeed, the inhibitory potency of the chalcone oxides (7) strongly implicates a general acid which assists epoxide cleavage from the opposite face of the molecule to the hydroxide attack. Moreover, unpublished work from our laboratories stresses the importance of the carbonyl of the α,β-epoxy ketones in hydrogen bonding to such a general acid as the major interaction leading to inhibition.

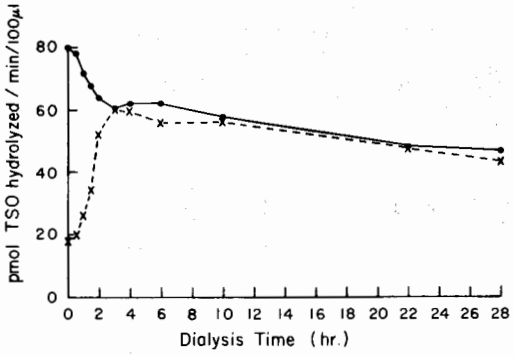


FIG. 5. Reactivation of inhibited cEH following dialysis against phosphate buffer (76 mM, pH 7.4): (●), cEH activity in control sample; (×), cEH activity in *trans*-PPCO-inhibited sample (*trans*-**8a**). Each point represents the average of two measurements.

The cyclopropyl oxiranes were designed as suicide substrates (19) to probe for electropositive character at the epoxide carbon adjacent to the cyclopropyl ring. Such a cyclopropylmethyl cation is expected to be on an energy surface consisting of delocalized or extremely rapidly interconverting cyclopropylmethyl, cyclobutyl, and homoallylic carbocationic species (20, 21). Many useful reactions have emerged in which the preferred trapping mode by a nucleophilic species, such as a halide, leads to the homoallylic halide (22). This cationic rearrangement has not previously been exploited in designing mechanism-based inhibitors. However, unpublished results from our labs have shown irreversible inhibition of sterol dealkylation in insects using a cyclopropyl oxiranyl steroid; the approach is therefore a valid one (G. D. Prestwich and M. Angelastro, unpublished results). In addition, a related rearrangement, namely the rapid ring-opening of a cyclopropylcarbinyl radical, was used to probe for hydrogen atom transfer by NAD(H) in alcohol dehydrogenase action on α-hydroxyalkyl cyclopropanes as substrates (23). In this case, the extremely rapid (10^8 s^{-1}) ring opening of cyclopropylmethyl radicals would have provided ring-opened products in the event of hydrogen atom vs hydride abstraction by NAD^+. No ring opening was observed in either hydrogenase or dehydrogenase directions, ruling out radical intermediates.

No irreversible inhibition was observed. Several explanations are possible: (a) complete absence of electrophilic contribution to the hydrolysis, (b) electrophilic weakening of the epoxide C–O bond concomitant with nucleophilic attack of water from the opposite face, (c) electrophilic opening of the epoxide followed by rapid capture of the potential alkylating site by water in the absence of reactive nucleophiles at the active site, or (d) failure of the enzyme to hydrolyze the substrate. Hypothesis (a) is unrealistic in terms of what is known of the cEH hydrolytic mechanism, as discussed above. Hypothesis (c) cannot be ruled out, since we know that a water molecule is present at the active site in the hydrolytic mechanism. However, there is also excellent evidence for the presence of catalytically important histidine (mEH active site) and cysteine (cEH active site) residues. One would expect competitive trapping of a reactive alkylating species by the available nucleophiles at the active site, and inactivation when a catalytically active residue was the nucleophile involved. If a cyclopropyl cation were involved, the distribution of the positive charge throughout the delocalized cyclopropylcarbinyl–cyclobutyl–homoallylic cation energy surface would necessarily place strongly electrophilic carbons in a larger effective area of the active site; the probability of intercepting one of the nucleophilic residues would thus be expected to be increased.

With respect to hypothesis (d), we have noted above that PPCO is very stable in the presence of acidic and basic media; therefore, no spontaneous nonenzymatic hydrolysis occurs during the preincubation period. However, since we can observe (Fig. 4) a time-dependent decrease in the inhibition of cEH by PPCO, we conclude that this inhibitor is a substrate for the enzyme and (as also demonstrated) that the hydrolytic products are noninhibitory for cEH. Note that in Fig. 4 the time-dependence for loss of inhibitory activity of PPCO for cEH is much slower than the corresponding effect of PCO on mEH, consistent with the generally poor turnover rates of *trans*-epoxides by mEH relative to the cEH enzyme.

Hypothesis (b), therefore, is the most consistent with our results. The cyclopropyl oxiranes appear to function as alternative substrates, i.e., as reversible inhibitors of [^{3}H]TSO hydrolysis. They are apparently hydrolyzed without significant damage to the enzyme active site (Fig. 5). Further evidence for the electrophilic site of cEH will be described in due course (G. D. Prestwich, J.-W. Kuo, B. D. Hammock, and D. Loury, unpublished results).

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