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INHIBITION OF CYTOSOLIC EPOXIDE HYDROLASE BY *trans*-3-PHENYLGLYCIDOLS

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Abstract—The inhibition of murine cytosolic epoxide hydrolase has been studied with both racemic and enantiomerically pure *trans*-3-phenylglycidols. These compounds are the first enantioselective, slow binding inhibitors of cytosolic epoxide hydrolase. The (2*S*,3*S*)-3-phenylglycidol enantiomer was always a better inhibitor than the (2*R*,3*R*)-enantiomer. When the I_{50} values of (2*S*,3*S*)- and (2*R*,3*R*)-3-(4-nitrophenyl)glycidol were compared, the (2*S*,3*S*)-enantiomer was at least a 750-fold better inhibitor (I_{50} = 1.6 μM) than the (2*R*,3*R*)-enantiomer (I_{50} = 1200 μM), and it was the most potent inhibitor tested in the 3-phenylglycidol series. If the hydroxyl group of the glycidol was masked or converted to another functionality, the potency of the inhibitor decreased and the (2*S*,3*S*)-enantiomer was not necessarily the better inhibitor. In addition, *trans*-3-phenylglycidols demonstrated slow binding inhibition of cytosolic epoxide hydrolase. Inhibitors without a hydroxyl group, or with a blocked hydroxyl group, were not slow binding inhibitors. These results suggested that the hydroxyl group was important in both enantioselectivity and time dependence of inhibition of cytosolic epoxide hydrolase by *trans*-3-phenylglycidols. The hydration pattern of (2*S*,3*S*)- and (2*R*,3*R*)-2,3-epoxy-3-(4-nitrophenyl)glycidol by cytosolic epoxide hydrolase also differed. When incorporation of [^{18}O] from water catalyzed by cytosolic epoxide hydrolase was measured, the (2*S*,3*S*)-enantiomer gave 12% incorporation into the benzylic carbon and the (2*R*,3*R*)-enantiometer gave 40% incorporation into the benzylic carbon. Finally, *trans*-3-phenylglycidols were found to be poor inhibitors of microsomal epoxide hydrolase.

Cytosolic epoxide hydrolase (CEH§, EC 3.3.2.3) is one of several known epoxide hydrolases. This group of enzymes is thought to protect the cell against cytotoxic and genotoxic effects of epoxide-containing compounds [1, 2]. The cell is exposed to epoxide-containing compounds (epoxides) by ingestion of epoxides present in the environment, spontaneous oxidation of membrane lipids, catabolism of xenobiotics to epoxides, and epoxidation of endogenous metabolites [2–4]. Although the *in vivo* substrates of CEH are not known, it is thought that CEH may be important in hydrolysis of epoxides formed during oxidation of fatty acids [5, 6].

The basic physical properties of CEH from various species are well known [7–9]. However, the structure and primary sequence of CEH are not known. In addition, little is known about the reaction mechanism or active site of CEH. However, general acid catalysis is not thought to be involved in the rate-limiting step [10]. Chalone oxides have been shown to be potent selective inhibitors of CEH and their inhibitory activity correlates with hydrophobicity [11].

To probe the active site of CEH, we studied the interaction of *trans*-3-phenylglycidols with CEH. Chalcone oxides, the most potent known inhibitors of CEH, are *trans*-epoxides. Reduction of chalcone oxides with sodium borohydride yields the corresponding glycidol (Fig. 1A). In addition, the epoxidation method of Sharpless [12] can be used to synthesize enantiomerically pure *trans*-glycidols. Finally, the glycidol hydroxyl group is also easily derivatized (Fig. 1, B and C). This makes a large number of different chiral *trans*-epoxides accessible. We have examined the effect of chirality and the presence of hydroxyl group on the inhibition of CEH with *trans*-3-phenylglycidols and their derivatives.

MATERIALS AND METHODS

Reagents

Chiral glycidols, *trans*-stilbene oxide (TSO), chalcone, [^{18}O]H_2O (normalized, 97 atom% [^{18}O]), and synthetic intermediates were purchased from the Aldrich Chemical Co. (Milwaukee, WI). ACS liquid scintillation fluid was purchased from Amersham (Arlington Heights, IL). [^{3}H]TSO, [^{3}H]*cis*-stilbene oxide (CSO), and CSO were synthesized according to Hammock *et al.* [13]. 4-Nitrochalcone oxide (4-NCO), 4-fluorochalcone oxide (4-FCO), and chalcone oxide (CO) were synthesized as described [11]. Diisopropyl fluorophosphate (DFP) and bovine

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§ Abbreviations: CEH, cytosolic epoxide hydrolase; TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; DFP, diisopropyl fluorophosphate; BSA, bovine serum albumin; BCA, bicinchoninic acid; 4-NCO, 4-nitrochalcone oxide; 4-FCO, 4-fluorochalcone oxide; CO, chalcone oxide; MEH, microsomal epoxide hydrolase; GC, gas chromatography; MS, mass spectrometry; [I], inhibitor concentration; TMS, trimethylsilyl-; EI, electron impact ionization; and CI, chemical ionization.

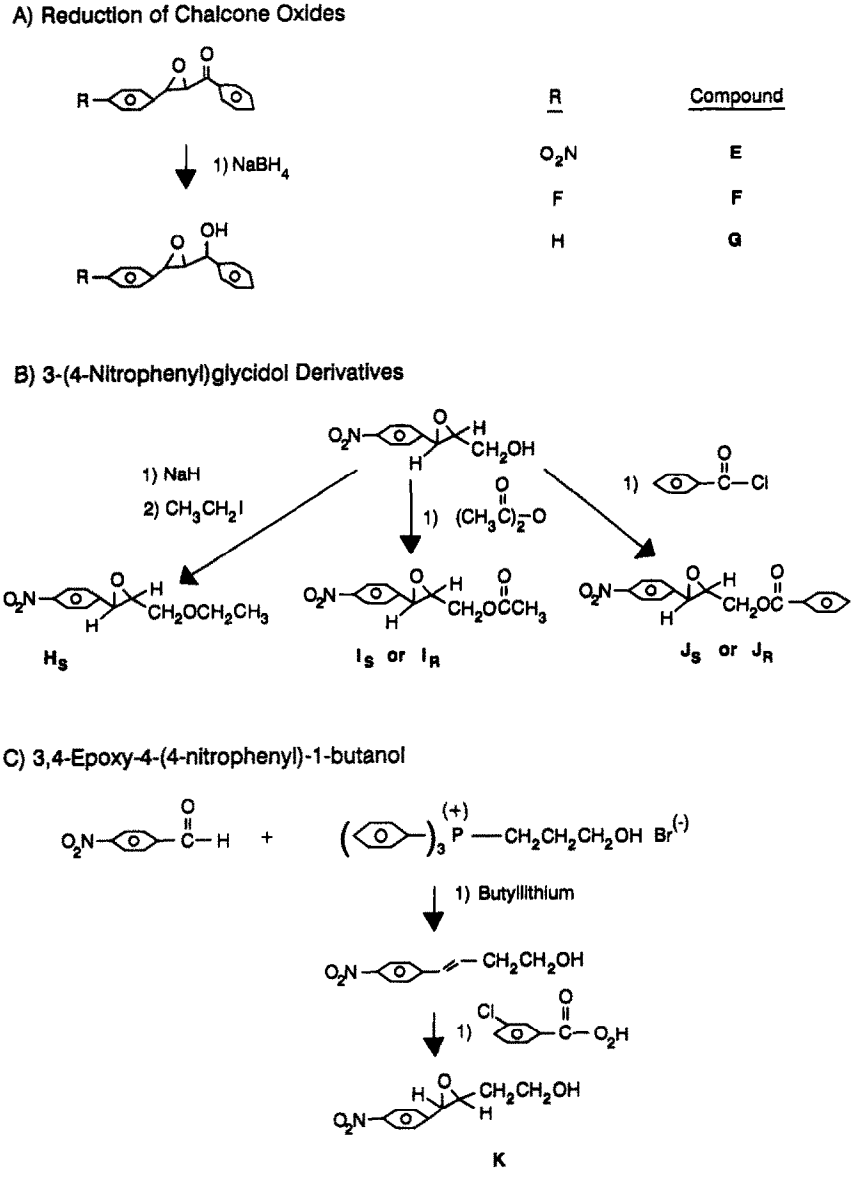

Fig. 1. Synthesis of inhibitors. (A) Reduction of chalcone oxides to phenylglycidols. (B) Derivatization of 3-(4-nitrophenyl)glycidols. (C) Synthesis of 3,4-epoxy-4-(4-nitrophenyl)-1-butanol.

serum albumin, grade V (BSA) were purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) reagent and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide were purchased from the Pierce Chemical Co. (Rockford, IL). Buffer salts were purchased from Fisher Scientific (Pittsburgh, PA). HPLC solvents were obtained from Fisher, Aldrich, or the Baker Chemical Co. (Phillipsburg, NJ). HPLC water was purified with a Milli-Q water purification system (MilliPore, Bedford, MA). All water for enzyme purification and assay was deionized and glass distilled. All other reagents were of the highest quality commercially available.

Inhibitor synthesis

The structures and physical properties of all compounds are listed in Tables 1 and 2. Letters refer to the compound structures shown in Table 1. Subscripts (*R* or *S*) indicate the *R,R*- or *S,S*-enantiomer. Racemic compounds lack subscripts. All values for the optical rotation, $[\alpha]_D^{17}$, were for $c = 1.0$ in CH_2Cl_2 and were carried out at 17°. Melting points were uncorrected. The ^{1}H-NMR spectra were obtained using a Varian EM-390 (Varian Associates, Palo Alto, CA) spectrometer using tetramethyl silane as the internal standard. All samples were dissolved in $CDCl_3$. All final products were checked for purity using thin-layer chromatography and two solvent systems of different polarity.

2,3-*Epoxy*-3-(4-*nitrophenyl*)-1-*phenyl*-1-*propanol*[E]. To a cooled (ice bath) suspension of 0.3 g of sodium borohydride in 20 mL of ethanol, 1.5 g of 4-NCO was added. After stirring for 12 hr at room temperature, the mixture was poured into ice water

Table 1. Inhibition of cytosolic epoxide hydrolase by 3-phenylglycidols and their derivatives*

Compound†	Structure R	σ_p	π	Structure R_1	I_{50} (μM) *R,R*	*S,S*	Racemic
A_R/A_S	H	0	0	CH_3	1100 ± 23	2400 ± 46	—
B_R/B_S	H	0	0	CH_2OH	430 ± 19	370 ± 49	—
C_R/C_S	Br	0.26	0.88	CH_2OH	46 ± 1.6	7.2 ± 0.16	—
D_R/D_S	NO_2	0.81	(−)0.27	CH_2OH	1200 ± 25	1.6 ± 0.55	5.0 ± 1.3
E	NO_2	0.81	(−)0.27	$CH(OH)C_6H_5$	—	—	1.7 ± 0.70
F	F	0.15	0.13	$CH(OH)C_6H_5$	—	—	32.2 ± 6.41
G	H	0	0	$CH(OH)C_6H_5$	—	—	28.7 ± 3.12
H_S	NO_2	0.81	(−)0.27	$CH_2OC_2H_5$	—	160 ± 14	—
I_R/I_S	NO_2	0.81	(−)0.27	$CH_2OC(O)CH_3$	69 ± 4.9	12 ± 0.63	—
J_R/J_S	NO_2	0.81	(−)0.27	$CH_2OC(O)C_6H_5$	24 ± 1.0	39 ± 2.9	—
K	NO_2	0.81	(−)0.27	CH_2CH_2OH	—	—	12 ± 0.42

* Inhibitors were preincubated with CEH for 5 min on ice, 5 min at 37°, and assayed for 15 min with 50 μM TSO. Inhibitors not checked for enantiomeric purity, — = not tested. Under the same conditions, the I_{50} of 4-FCO was 26.1 ± 4.58 μM and the I_{50} of CO was 54.8 ± 9.20. Inhibition assays were run in quadruplicate at least three times, and values are shown as means ± SD.

† Subscript *R* indicates the *R,R*-enantiomer and subscript *S* indicates the *S,S*-enantiomer. No subscript indicates the racemic mixture.

and extracted with ether. The ether extract was washed with brine and dried over sodium sulfate. The extract was then concentrated and the residue purified by flash chromatography on silica gel using a hexane–ethyl acetate (3:1, v/v) mobile phase. Concentration of the eluate yielded 1.0 g (66.2%) of *E*.

2,3-*Epoxy*-3-(4-*fluorophenyl*)-1-*phenyl*-1-*propanol*[F]. 2,3-Epoxy-3-(4-fluorophenyl)-1-phenyl-1-propanol was prepared by the same method as *E* starting with 0.75 g of 4-FCO. The product was purified by preparative TLC on silica gel plates with a hexane–ethyl acetate (10:1, v/v) solvent system. The yield was 0.48 g (63.5%) of *F*.

2,3-*Epoxy*-1,3-*diphenyl*-1-*propanol* [G]. 2,3-Epoxy-1,3-diphenyl-1-propanol was prepared by the same method as *E* starting from 1.25 g of CO. The product was purified by flash chromatography using a hexane–ethyl acetate (3:1, v/v) mobile phase. Concentration of the eluate gave 0.69 g (54.6%) of *G*.

1-*Ethoxy*-(2S,3S)-2,3-*epoxy*-3-(4-*nitrophenyl*)-*propane* [H_S]. To a suspension of 0.1 g sodium hydride [60% (w/w) in oil] in 10 mL dimethylformamide at 0–5°, 0.5 g (2*S*,3*S*)-3-(4-nitrophenyl) glycidol was added. After stirring for 1 hr at room temperature, 0.4 g iodoethane was added dropwise to the mixture. The mixture was stirred for 3 hr at room temperature and then poured into water. The product was extracted with ether and the ether layer washed with brine and dried over sodium sulfate. After removal of the ether, the residue was purified by flash chromatography on silica gel using a hexane–ethyl acetate (3:1, v/v) mobile phase. Concentration under reduced pressure and recrystallization from hexane yielded 0.15 g H_S (26.2%).

1-*Acetoxy*-2,3-*epoxy*-3-(4-*nitrophenyl*)*propane* [I_S, (2S,3S)] and [I_R, (2R,3R)]. Acetic anhydride (0.2 mL) was added to a solution of 0.2 g of (2*S*,3*S*)-3-(4-nitrophenyl)glycidol, in 5 mL of pyridine at room temperature. After stirring for 4 hr, the mixture was poured into ice water and the precipitate was collected by filtration. The crude product was recrystallized from ethanol to yield 0.20 g (82.3%) of I_S.

The (2*R*,3*R*)-enantiomer, I_R, was prepared in the same manner starting from (2*R*,3*R*)-3-(4-nitrophenyl)glycidol.

1-*Benzoyloxy*-2,3-*epoxy*-3-(4-*nitrophenyl*)*propane* [J_S, (2S, 3S)] *and* [J_R, (2R,3R)]. 1 - Benzoyloxy - (2*S*, 3*S*) - 2, 3 - epoxy - 3 - (4 - nitrophenyl) was prepared in the same manner as I_S using benzoyl chloride instead of acetic anhydride. The yield was 78.5% recrystallized from ethanol.

The (2*R*,3*R*)-enantiomer, J_R, was prepared in the same manner and gave a yield of 86.7%.

3,4 - *Epoxy* - 4 - (4 - *nitrophenyl*) - 1 - *butanol* [K]. A solution of *n*-butyllithium in hexane (2.5 M, 22 mL) was added to an ice cooled, stirred suspension of 11 g of 3-hydroxypropyl triphenylphosphonium bromide in 50 mL of tetrahydrofuran at 2–5°. After stirring for 30 min at room temperature and cooling to −30°, a solution of 4-nitrobenzaldehyde in 10 mL of tetrahydrofuran was added dropwise to the mixture. The mixture was stirred for 3 hr at −30° to −20°, poured into ice water, and neutralized with 5% HCl. The product was extracted with ether and the extract washed with brine, dried over sodium sulfate, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel with a hexane–ethyl acetate mobile phase (2:1, v/v). Concentration of the eluate under reduced pressure gave 1.5 g (29.4%) of crude 4-(4-nitrophenyl)but-3-en-1-ol.

Table 2. Properties of synthesized inhibitors*

Compound	Optical rotation ($[\alpha]_D^{17}$, degrees)	Melting point (°)	Elemental analysis (%)	^{1}H-NMR (δ)
E		ND	Found: ND Calculated: C, 66.42; H, 4.80; N, 5.17 $C_{15}H_{13}NO_4$	2.5–2.7 (1H, dd, J_a = 3Hz, J_b = 6Hz/D_2O exchangeable); 3.2–3.4 (1H, m); 4.08 (0.5H, d, J = 2Hz); 4.20 (0.5H, d, J = 2Hz); 4.78 (0.5H, t, J =6Hz); 5.00 (0.5H, t, J = 3Hz; 7.2–7.4 (7H, m); 8.22 (2H, d, J = 9Hz)
F		Oil	Found: ND Calculated: C, 73.70; H, 5.36; N, 7.78 $C_{15}H_{13}FO_2$	2.60–2.95 (1H, m, D_2O exchangeable); 3.23 (1H, d, J = 3Hz); 4.10 (1H, s); 4.93 (1H, d, J = 3Hz); 6.95–7.03 (2H, m); 7.33–7.49 (7H, m)
G		Oil	Found: ND Calculated: C, 79.56; H, 6.24 $C_{15}H_{14}O_2$	2.52–2.37 (1H, m, D_2O exchangeable); 3.33 (1H, d, J = 3Hz); 4.18 (1H, s); 5.01 (1H, d, J = 3Hz); 7.33–7.49 (10H, m)
H_S	−46	73–74	Found: C, 59.13; H, 5.89; N, 6.29 Calculated: C, 59.19; H, 5.83; N, 6.28 $C_{11}H_{13}NO_4$	1.25 (3H, t, J = 7Hz); 3.1–3.3 (1H, m); 3.4–3.8 (4H, m); 3.92 (1H, d, J = 2Hz); 7.46 (2H, d, J = 9Hz); 8.25 (2H, d, J = 9Hz)
I_S	−62	122–124	Found: C, 55.75; H, 4.70; N, 5.99 Calculated: C, 55.70; H, 4.64; N, 5.91 $C_{11}H_{11}NO_5$	2.10 (3H, s); 3.1–3.3 (1H, m); 3.85 (1H, d, J = 2Hz); 4.08 (1H, dd, J_a = 5Hz, J_b = 12Hz); 4.45 (1H, dd, J_a = 3Hz, J_b = 12Hz); 7.36 (2H, d, J = 9Hz); 8.13 (2H, d, J = 9Hz)
I_R	+62	122–123	Found: C, 55.68; H, 4.67; N, 5.98 Calculated: C, 55.70; H, 4.64; N, 5.91 $C_{11}H_{11}NO_5$	See compound I_S
J_S	−38	139–141	Found: C, 64.20; H, 4.41; N, 4.74 Calculated: C, 64.21; H, 4.35; N, 4.68 $C_{16}H_{13}NO_5$	3.2–3.5 (1H, m); 3.96 (1H, d, J = 2Hz); 4.36 (1H, dd, J_a = 5Hz, J_b = 12Hz); 4.72 (1H, dd, J_a = 3Hz, J_b = 12Hz); 7.2–7.6 (5H, m); 7.9–8.3 (4H, m)
J_R	+42	140–141	Found: C, 64.18; H, 4.40; N, 4.76 Calculated: C, 64.21; H, 4.35; N, 4.68 $C_{16}H_{13}NO_5$	See compound J_S
K		84–85	Found: C, 57.49; H, 5.36; N, 6.73 Calculated: C, 57.42; H, 5.26; N, 6.70 $C_{10}H_{11}NO_4$	1.7–1.9 (1H, m, D_2O exchangeable); 1.8–2.3 (2H, m); 3.1–3.3 (1H, m); 3.8–4.1 (3H, m); 7.48 (2H, d, J = 9Hz); 8.26 (2H, d, J = 9Hz)

* Optical rotation was determined at c = 1.0 in CH_2Cl_2 at 17°. Melting points were uncorrected. All compounds were *trans*-. ND = not determined.

To a cooled (ice bath) solution of 0.6 g of 4-(4-nitrophenyl)but-3-en-1-ol in 10 mL of dichloromethane, 0.9 g of 85% *m*-chloroperbenzoic acid was added. After stirring for 3 hr at room temperature, the mixture was washed with 5% $NaHCO_3$ and then brine. Next, the solvent was dried over Na_2SO_4, the solvent was removed, and the residue recrystallized from diisopropyl ether to yield 0.50 g (76.9%) of *K*.

Enzyme purification and assay

CEH was prepared, from the livers of Swiss Webster mice that had been fed clofibrate, and assayed as described [5, 14]. DFP (0.1 mM final concentration) was added to the homogenization buffer to avoid proteolytic degradation of CEH during purification. The mice (Charles River, Cambridge, MA) were fed a diet of ground Purina Rodent Chow (Purina Mills, Inc., St. Louis, MO) with 5% corn oil and 0.5% clofibrate (Ayerst, New York, NY), housed in stainless steel cages, at constant temperature and humidity with a 12-hr light/dark cycle, and given free access to water and food. The concentration of purified CEH was determined by a modified BCA assay [14]. Purified enzyme was stored at 4° and used within 2 weeks.

Microsomes were prepared and used as a source of microsomal epoxide hydrolase (MEH). In brief, the microsomes were pelleted (130,000 *g* at 4° for 1 hr), resuspended in 0.1 M Tris buffer (pH 9.0), and pelleted again under the same conditions. The final pellet was resuspended in the same Tris buffer and stored at −80°. MEH activity was measured using the CSO partition assay [5]. Both CEH and MEH were assayed in buffer containing 0.1 mg/mL BSA with 50 μM TSO and 50 μM CSO, respectively.

Enzyme inhibition

Purified CEH was diluted to 1.8 μg/mL in buffer containing 0.10 mg/mL BSA to give an activity that was in the linear range of the assay. Aliquots of 0.50 mL of diluted enzyme were put in borosilicate test tubes that had been placed in ice. Five microliters of solvent or 100× stock inhibitor, prepared in acetone or acetone/ethanol (1:1, v/v), was added, the solution was mixed, and the tubes were incubated on ice for 5 min. Four aliquots of 0.10 mL were removed from each tube, placed in separate test tubes prewarmed to 37°, and incubated at 37° for 5 min with shaking. Substrate was added and the assay allowed to proceed for 15 min. Assays were performed at least two separate times in quadruplicate. The I_{50} was determined by plotting percent inhibition vs log [I]. Inhibitor concentrations were chosen to give three points above and below 50% CEH inhibition for each individual inhibitor. The concentration of inhibitor used was not limited by solubility.

Inhibition of MEH followed the same protocol and used a 1/20 dilution of microsomes in 100 mM, pH 9.0 Tris buffer. The assay, using CSO as the substrate, was carried out two times in duplicate.

Determination of the time dependence of inhibition was carried out by preequilibrating 0.10 mL of diluted CEH (0.90 μg/mL) at 37°, adding 1 μL of stock inhibitor (at a concentration chosen to give a maximum of 45–80% CEH inhibition), and adding substrate after the appropriate time interval. CEH activity was determined after preincubation with inhibitor for 0, 15, 30, 60, 90, 120, 300, and 600 sec. Assays were done in quadruplicate and carried out two separate times.

GC–MS

Hydrolysis of 40 nmol (2*R*,3*R*)- and (2*S*,3*S*)-3-(4-nitrophenyl)glycidol was carried out in 0.10 mL of unbuffered $[^{18}O]H_2O$ for 1.5 hr at 37° with 1.2 μg of CEH, with 0.50% (v/v) $HClO_4$, or for 4 hr in 0.10 N NaOH. The mixtures were then extracted three times with ether, washed with 5% $NaHCO_3$, dried over Na_2SO_4, and the ether extracts pooled and evaporated. The concentrated extracts were then derivatized with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide by dissolving the dried extracts in 50 μL of acetonitrile, adding 50 μL of *N*-methyl-*N*-trimethylsilyl trifluoroacetamide, and incubating for 2 hr at 60°. The silylated extract was injected without further treatment onto a Hewlett–Packard 5890 GC (Hewlett–Packard, Palo Alto, CA) using a DB-5 capillary column (J+W Scientific, Folsom, CA) with a programmed temperature gradient (125°–220° at 5°/min). The GC was coupled to a VG TRIO-2 mass spectrometer (VG Instruments, Altrincham, England) and the data were collected and processed with a VG11-250 data system. The samples were analyzed using both electron impact (EI) and chemical ionization (CI) with isobutane.

HPLC

HPLC of *R*-(α)-methoxyphenylacetic acid esters of (2*R*,3*R*)- [D_R] and (2*S*,3*S*)-3-(4-nitrophenyl)glycidol [D_S] were carried out on a Supelcosil LC-(R)-Urea column (Supelco, Bellefonte, PA). The esters were synthesized as follows. Dicyclohexylcarbodiimide (0.4 g) was added to a cooled (ice bath) solution of 0.4 g D_S, 0.32 g (*R*)-(α)-methoxyphenylacetic acid and 40 mg dimethylaminopyridine in 10 mL dichloromethane. The mixture was stirred for 30 min at 0–5° and then at room temperature for 3 hr. The precipitate was removed by filtration and the filtrate washed with 5% HCl, 5% $NaHCO_3$, and brine, and dried over sodium sulfate. The solvent was removed and the residue purified by flash chromatography on silica gel with a mobile phase of hexane–ethyl acetate (2:1, v/v). After concentration of the eluate the yield was 0.69 g. The crude product was not further purified in order to avoid enrichment of either diastereomer. The ester of (2*R*,3*R*)-2,3-epoxy-3-(4-nitrophenyl)glycidol [D_R] was prepared in the same fashion. The ^{1}H-NMR spectrum of both products was consistent with the pattern predicted based on their structure.

A Perkin–Elmer Bio410 solvent delivery system was used (Perkin–Elmer, Norwalk, CT) for HPLC. It was equipped with a Perkin–Elmer 235 diode array detector and a Rheodyne 7125 injection port with a 250-μL sample loop (Rheodyne, Cotati, CA). The detector output was recorded with a Hewlett-Packard 4490 recording integrator (Hewlett-Packard, Palo Alto, CA) and a Perkin–Elmer GP-100 recorder. The solvent system used was aqueous 7.5% acetonitrile, 5.0% 1-propanol, 2.5% tetrahydrofuran, and two drops pyridine/L. The flow rate

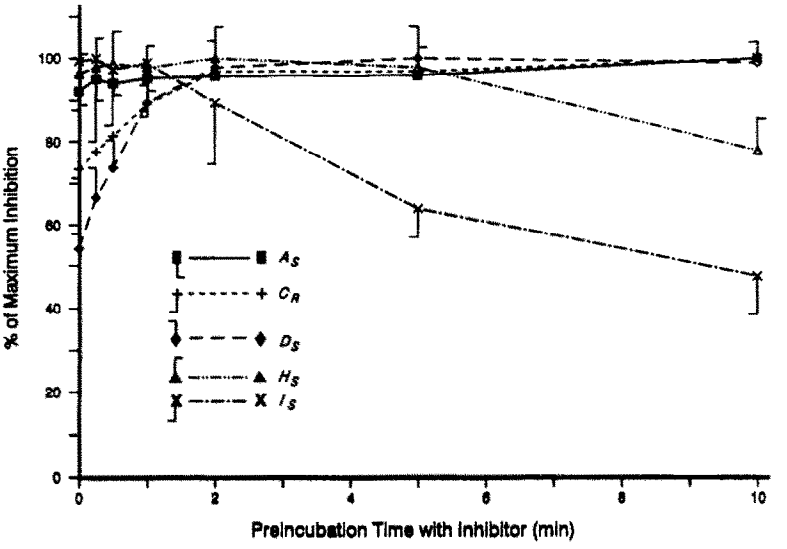

Fig. 2. Time dependence of cytosolic epoxide hydrolase inhibition by *trans*-3-phenylglycidols and their derivatives. CEH was preincubated with inhibitor at 37° for the time indicated and then assayed with TSO in quadruplicate. Data are expressed as the ratio of the percent inhibition observed at any given time to the maximum observed percent inhibition and is the mean of two determinations with standard deviation shown. The maximum observed percent inhibition for each inhibitor was: A_S 70 ± 0.62, C_R 78 ± 1.2, D_S 76 ± 2.1, H_S 79 ± 1.3, and I_S 46 ± 0.92.

was 0.2mL/min. The compounds were detected by monitoring absorbance at 255 nm. Between 1 and 2 nmol of each compound was injected, separately or together. Each of the synthetic precursors was also chromatographed. The retention times of the (*R*)-(α)-methoxyphenylacetic acid esters of D_S and D_R were 33.2 and 41.8 min, respectively.

RESULTS

The I_{50} values of (2*S*,3*S*)-3-phenylglycidols [B_S, C_S, D_S] were found to be lower than those of the corresponding (2*R*,3*R*)-enantiomer [B_R, C_R, D_R] (Table 1). The relative degree of inhibition of the paired enantiomers varied from 1.2-fold, for *trans*-3-phenylglycidol [B_R/B_S], to 750-fold, for *trans*-3-(4-nitrophenyl)glycidol [D_R/D_S]. The best enantiomer tested in the *trans*-3-phenylglycidol series was (2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)glycidol [D_S]. D_S was equally effective as an inhibitor of purified and crude CEH.* When the enantiomers of *trans*-1,2-epoxy-1-phenylpropane [A_R, A_S] (which lacks the hydroxyl group) were tested, they were found to be poor inhibitors of CEH and the (1*R*,2*R*)-enantiomer [A_R] was a 2.2-fold better inhibitor than the (1*S*,2*S*)-enantiomer [A_S].

R-(α)-Methoxyphenylacetic acid esters of both (2*R*,3*R*)- [D_R] and (2*S*,3*S*)- [D_S] 3-(4-nitrophenyl) glycidol were made in order to test the enantiomeric purity of the respective enantiomers as purchased and used. The esterified enantiomers were separated by HPLC and the percent contamination of D_R by D_S was estimated. D_R was found to contain approximately 0.5 ± 0.02% (N = 2) of D_S (data not shown). This observation indicated that inhibition of CEH by D_R may have been entirely due to contamination by D_S.

When CEH was inhibited with several 3-(4-nitrophenyl)glycidol derivatives and analogs which lacked a free hydroxyl group, there was no consistent pattern in the I_{50} values for *R*,*R*- and *S*,*S*-enantiomers (Table 1). Also, every member of this series of compounds had higher I_{50} values than the parent glycidol. The I_{50} of the (2*S*,3*S*)-enantiomer was higher than the I_{50} for the corresponding (*R*,*R*)-enantiomer for both 1,2-epoxy-1-phenylpropane [A_R/A_S] (1100 μM/2400 μM), as noted above, and 1-benzoyloxy-2,3-epoxy-3-(4-nitrophenyl)propane [J_R/J_S] (24 μM/39 μM). The I_{50} of the (2*S*,3*S*)-enantiomer was lower for only 1-acetoxy-2,3-epoxy-3-(4-nitrophenyl)propane [I_R/I_S] (69 μM/12 μM). (2*S*,3*S*)-2,3-Epoxy-1-ethoxy-3-(4-nitrophenyl)propane [H_S] was the sole enantiomer tested.

The only inhibitor tested with a lower I_{50} than racemic 3-(4-nitrophenyl)glycidol [*D*] was racemic 2,3-epoxy-3-(4-nitrophenyl)-1-phenyl-1-propanol [*E*]. The I_{50} was 1.7 ± 0.70 μM as compared to 5.0 ± 1.3 μM for racemic 3-(4-nitrophenyl)glycidol. 2,3-Epoxy-3-(4-fluorophenyl)-1-phenyl-1-propanol [*F*] had an I_{50} of 32 μM and 2,3-epoxy-1,3-diphenyl-1-propanol [*G*] has an I_{50} of 29 μM. The I_{50} values for the corresponding chalcone oxides were 26 μM for 4-FCO and 55 μM for CO. 3,4-Epoxy-4-(4-nitrophenyl)-1-butanol [*K*] was found to have a slightly higher I_{50} than racemic 3-(4-nitrophenyl)glycidol. Addition of a methylene group

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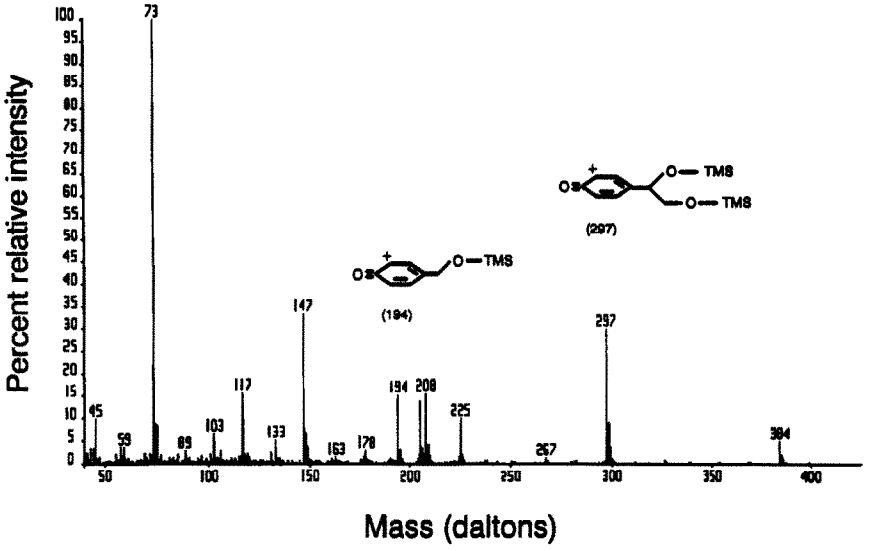


Fig. 3. Mass spectrum of 1,2,3-trihydroxy-3-(4-nitrophenyl)propane formed by hydrolysis of (2*R*,3*R*)-2,3-epoxy-3-(4-nitrophenyl)glycidol by cytosolic epoxide hydrolase and ionized by electron impact with structures of key ions used for [^{18}O] incorporation studies shown.

between the epoxide ring and the hydroxyl group raised the I_{50} to 12 ± 0.42 μM.

The time course of CEH inhibition by (2*R*,3*R*)-3-(4-bromophenyl)glycidol [C_R] and (2*S*,3*S*)-3-(4-nitrophenyl)glycidol [D_S] showed increasing inhibition over the first 2 min of preincubation with inhibitor (Fig. 2). CEH inhibition by C_R and D_S increased from 59 ± 1.34% and 41 ± 1.06%, with no preincubation with inhibitor, to 78 ± 1.2% and 75.6 ± 2.11%, with a 2-min preincubation, respectively. (1*S*,2*S*)-1,2-Epoxy-1-phenyl-propane [A_S] showed no change in the degree of CEH inhibition as the length of preincubation increased (66.3 ± 3.07% inhibition with a 0-min preincubation and 70 ± 0.624% inhibition with a 10-min preincubation). CEH inhibition by 1-ethoxy-(2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)propane [H_S] decreased from 79 ± 1.27% to 63 ± 4.18%, and 1-acetoxy-(2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)propane [I_S] inhibition decreased from 46 ± 0.919% to 22 ± 2.19%, as the length of preincubation with inhibitor increased from 0 to 10 min.

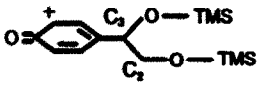
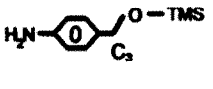
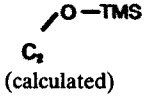
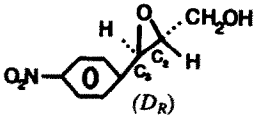
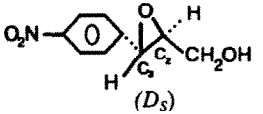
The MS spectrum of (2*R*,3*R*)-3-(4-nitrophenyl)glycidol [D_R], after hydrolysis by CEH in [^{18}O]-H_2O, trimethylsilyl- (TMS) derivatization, and EI ionization, is shown in Fig. 3. The ratio of [^{18}O] to [^{16}O] incorporation from H_2O was calculated for two ions. The first, mass = 297 for [^{16}O] (or 299 for [^{18}O]), was produced by EI and contained both C_2, C_3, and their TMS-derivatized hydroxyl groups. The second, mass = 194 for [^{16}O] (or 196 for [^{18}O]), was produced by CI and contained only C_3 and its TMS-derivatized hydroxyl group (Fig. 3). This ion was not seen reliably in the EI spectrum so the ratio was confirmed based on the appearance of the ion in the CI spectrum (Table 3). An aromatic nitro- group gives loss of NO during EI and reduction to an amino group during CI. The mass to charge ratio of these ions is the same. Therefore, the differences in fragmentation pattern and ion structure between EI (Fig. 3) and CI (Table 3) did not influence data analysis. Hydrolysis of *trans*-3-(4-nitrophenyl)-glycidol in [^{18}O]H_2O with CEH gave 40% incorporation into C_3, the benzylic position, and 60% incorporation into C_2 with the (2*R*,3*R*)-enantiomer [D_R] (Table 3). The (2*S*,3*S*)-enantiomer [D_S] gave 12% incorporation into C_3 and 88% incorporation into the C_2 position. Hydrolysis under acid conditions gave 15 and 6.3% [^{18}O] incorporation into C_3 and 95 and 94% [^{18}O] incorporation into C_2 for the (2*R*,3*R*)- [D_R] and (2*S*,3*S*)- [D_S] enantiomers. Hydrolysis under basic conditions gave 46 and 51% incorporation of [^{18}O] into C_3 and 54 and 49% [^{18}O] incorporation into C_2 for the (2*R*,3*R*)- [D_R] and (2*S*,3*S*)- [D_S] enantiomers. Since the hydrolysis of the enantiomers should be identical with either $HClO_4$ or NaOH, this variability gives an indication of the accuracy of the method. The pattern of incorporation under the different conditions of hydrolysis is shown in Fig. 4.

trans-3-Phenylglycidol [B_R, B_S] and *trans*-3-(4-nitrophenyl)glycidol [D_R, D_S] were found to be much less effective as inhibitors of MEH (Table 4). While I_{50} values were not determined, the degree of inhibition at the concentrations tested was much lower than for CEH. This was also found to be true with 1-benzoyloxy-2,3-epoxy-3-(4-nitrophenyl)-propane [J_R, J_S]. However, 1,2-epoxy-1-phenylpropane [A_R, A_S] was more effective as an inhibitor of MEH than of CEH. The percent inhibition of MEH by *S*,*S*-enantiomers [A_S, D_S, J_S] was consistently higher than by the corresponding *R*,*R*-enantiomers [A_R, D_R, J_R]. Under the same conditions the I_{50} of cyclohexene oxide was 2.22 ± 0.323 μM.

DISCUSSION

The key findings of this study were that (2*S*,3*S*)-3-phenylglycidols [B_S, C_S, D_S] gave better inhibition

Table 3. Hydrolysis of 3-(4-nitrophenyl)glycidol in $[^{18}O]H_2O$*

Reagent	Substrate structure	Fragment ratio ($[^{18}O]/[^{16}O]$) 		 (calculated)	% $[^{18}O]$ incorporation C_2	% $[^{18}O]$ incorporation C_3	Ratio % incorporation C_2/C_3
			2R,3R				
CEH		9.50	7.65	11.4	60	40	1.5
NaOH		3.50	3.22	3.78	54	46	1.2
$HClO_4$	(D_R)	62.0	18.6	105	85	15	5.7
			2S,3S				
CEH		58.6	13.7	104	88	12	7.3
NaOH		3.40	3.50	3.30	49	51	0.96
$HClO_4$	(D_S)	128	16.2	240	94	6.3	15

* Hydrolysis of 40 nmol in 0.1 mL $[^{18}O]H_2O$ for 1.5 hr at 37° with 1.2 μg CEH, 0.5% $HClO_4$, or 0.1 N NaOH. Fragment containing C_2 and C_3 was from EI and fragment containing C_2 was from CI. A two-way factorial analysis of variance (ANOVA) was used to analyze the unreplicated data with the assumption that there was no significant interaction between hydrolysis conditions and the chirality of the substrate. Incorporation of $[^{18}O]$ into substrate catalyzed by CEH was found to differ significantly with $P = 0.21$.

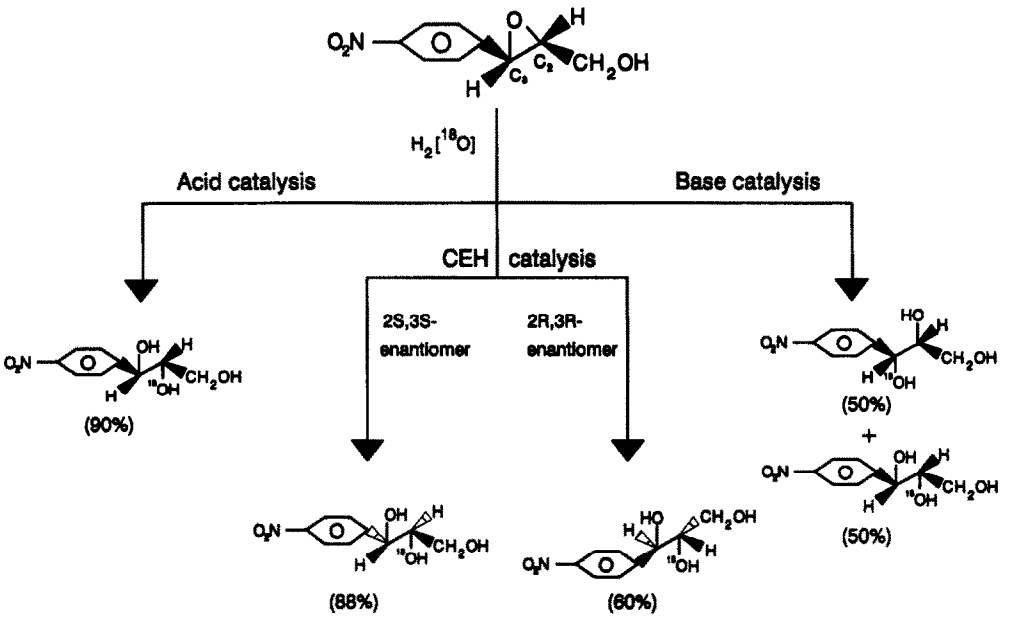


Fig. 4. Pattern of [^{18}O] incorporation from water into (2*R*,3*R*)-2,3-epoxy-3-(4-nitrophenyl)glycidol produced by hydrolysis in 0.50% (v/v) $HClO_4$, 0.10 N NaOH, or cytosolic epoxide hydrolase. Stereochemistry of products assumes *trans*- opening of the epoxide ring and inversion of configuration. The predominant product (and percent observed) is illustrated.

Table 4. Inhibition of microsomal epoxide hydrolase*

Compound†	Inhibitor R	Inhibitor R_1	Concentration (μM)	% Inhibition *R,R*	% Inhibition *S,S*
A_R/A_S	H	CH_3	800	88	89
			200	23	75
B_R/B_S	H	CH_2OH	640	0.0	0.0
			64	0.0	0.0
D_R/D_S	NO_2	CH_2OH	200	8.2	17
			20	1.9	10
J_R/J_S	NO_2	$CH_2OC(O)C_6H_5$	100	9.0	11
			10	0.0	0.0

* Microsomes were incubated with inhibitor for 5 min on ice, 5 min at 37°, and assayed for 15 min with CSO. Data are the mean of two determinations. The mean rate of CSO hydrolysis by controls was 0.520 nmol/min/mg microsomes. The I_{50} of cyclohexane oxide under the same conditions was 2.22 ± 0.323 μM.

† Subscript *R* indicates *R,R*-enantiomer and subscript *S* indicates *S,S*-enantiomer.

of CEH than the corresponding (2*R*,3*R*)-enantiomers [B_R, C_R, D_R]. This is the first demonstration of enantioselective inhibition of CEH. The (*R,R*)- and (*S,S*)-enantiomers of 4-fluorochalcone oxide have been tested as inhibitors of CEH and no difference was found.* Also, (2*S*,3*S*)-3-(4-nitrophenyl)glycidol [D_S] and (2*R*,3*R*)-3-(4-bromophenyl)glycidol [C_R] were slow binding inhibitors of CEH. Inhibitor potency, the preference for the (2*S*,3*S*)-enantiomer, and slow binding inhibition depended on the presence of a free hydroxyl group. When the hydroxyl group was removed or blocked, inhibitor potency decreased and the (*S,S*)-enantiomer was not always the best inhibitor. None of the inhibitors lacking a free hydroxyl group showed time dependence of inhibition

* Personal communication, Roger Wixtron. Cited with permission.

[A_S, H_S, I_S]. Finally, moving the hydroxyl group one carbon further away from the epoxide ring decreased the potency of 3,4-epoxy-4-(4-nitrophenyl)-1-butanol [K] 2.4-fold relative to 3-(4-nitrophenyl)glycidol [D].

Inhibition of MEH by *trans*-3-phenylglycidols was also tested. The interaction of these inhibitors with MEH was clearly different than that with CEH. *trans*-3-Phenylglycidols were poor inhibitors of MEH but good inhibitors of CEH. The (*S*,*S*)-enantiomers [A_S, D_S, J_S] were somewhat better inhibitors of MEH than the corresponding (*R*,*R*)-enantiomers [A_R, D_R, J_R] which was consistent with the results for CEH. However, (1*S*,2*S*)-1,2-epoxy-1-phenylpropane [A_S] was a moderately good inhibitor of MEH and a very poor inhibitor of CEH. Finally, the (1*S*,2*S*)-enantiomer [A_S] of 1,2-epoxy-1-phenylpropane was a better inhibitor of MEH than the (1*R*,2*R*)-enantiomer [A_R]. This was the opposite of the respective inhibitor's potency with CEH and a further demonstration of differences between CEH and MEH.

Of the glycidol enantiomers tested against CEH, (2*S*,3*S*)-3-(4-nitrophenyl)glycidol [D_S], was found to be the best inhibitor of CEH and to have the largest difference in potency between the enantiomers (Table 1). It is likely that the true difference in I_{50} values between D_R and D_S was even larger. As noted, it was found that D_R was contaminated by approximately 0.5% D_S. At this level of contamination, the concentration of D_S present in 1200 μM D_R would have been 6 μM. Since the I_{50} of D_S was found to be 1.6 μM, D_R may be essentially inactive as an inhibitor of CEH. Unfortunately, we were unable to obtain enough sufficiently pure D_R to test this possibility.

The relative ability of the *R*,*R*- and *S*,*S*-enantiomers to inhibit CEH was dependent on the nature of the 4-phenyl substituent, the chirality of the inhibitor, and the presence or absence of a free hydroxyl group (Table 1). Neither 3-phenylglycidol [B_R/B_S] nor 3-(4-bromophenyl)-glycidol [C_R/C_S] demonstrated the same degree of enantiomeric selectivity as 3-(4-nitrophenyl)glycidol [D_R/D_S]. This may be due to one or more of several factors. The nitro- group is the bulkiest of the 4-phenyl substituents and purely geometric considerations may have been overriding in the determination of relative inhibitory potencies of the enantiomers. However, based on a limited number of substrates, there appears to be a contribution of both the hydrophobicity, π, and the Hammet sigma coefficient, σ_p [15, 16]. The potency of the (2*R*,3*R*)-enantiomers [B_R, C_R, D_R] appeared to correlate with the hydrophobicity of the 4-phenyl substituent. The potency of the (2*S*,3*S*)-enantiomers [B_S, C_S, D_S] appeared to correlate with the σ_p value of the 4-phenyl substituent (Table 1).

In prior studies, the ability of chalcone oxides to inhibit CEH was found to correlate only with the hydrophobicity of the 4- or 4'-phenyl substituent [11, 17]. This indicates that CEH has a hydrophobic binding site. Chalcone oxides are more hydrophobic than their corresponding alcohols. This leads to the prediction that chalcone oxides should be better CEH inhibitors than their corresponding alcohols. However, when assayed under the same conditions, 4-FCO had the same potency as its corresponding glycidol, 2,3-epoxy-3-(4-fluorophenyl)-1-phenyl-1-propanol [F], and CO was a less potent CEH inhibitor than its coresponding glycidol, 2,3-epoxy-1,3-diphenyl-1-propanol [G]. Mullin and Hammock [11] found that the I_{50} of 4-FCO was 1.9-fold lower than that of 4-NCO. In the 1,3-diphenylglycidol series, the I_{50} of the 4-nitro derivative, [E], is 19-fold lower than that of the 4-fluoro derivative, [F] (Table 1). Thus, the inhibitor potency of these two compounds was reversed between the chalcone oxide series and the 1,3-diphenylglycidol series. The same reversal in potency was also seen in the (2*S*,3*S*)-phenylglycidol series (NO_2 > Br). Finally, the I_{50} of racemic 2,3-epoxy-3-(4-fluorophenyl)-1-phenyl-1-propanol [F] was only 3-fold lower than that of racemic 3-(4-nitrophenyl)glycidol, indicating a small additional contribution to binding by the 1-phenyl group. These data indicate that for (2*S*,3*S*)-3-phenylglycidols [B_S, C_S, D_S] and 1,3-diphenylglycidols [E, F, G], unlike chalcone oxides, hydrophobicity of the 4-phenyl substituent is not the most important factor in determining inhibitor potency.

While it was not possible to separate the relative contributions of the 4-phenyl substituent and the hydroxyl group to inhibitor potency with this set of inhibitors, it is clear that the hydroxyl group plays an important role. When 1,2-epoxy-1-phenylpropane enantiomers [A_R, A_S] were compared to 3-phenylglycidol enantiomers [B_R, B_S] it was clear that removal of the hydroxyl group resulted in both decreased inhibitor potency and a reversal in the potency of the enantiomers. Any modification to 3-(4-nitrophenyl)glycidol resulting in removal [1-ethoxy-(2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)propane, H_S] or blocking [1-acetoxy-2,3-epoxy-3-(4-nitrophenyl)propane, I_R and I_S, or 1-benzoyloxy-2,3-epoxy-3-(4-nitrophenyl)propane, J_R and J_S] of the hydroxyl group also led to a decrease in potency of the (2*S*,3*S*)-enantiomer and either decreased, or abolished, the higher potency of the (2*S*,3*S*)-enantiomer. Addition of a methylene group between the hydroxyl group and the epoxide ring [3,4-epoxy-4-(4-nitrophenyl)-1-butanol, [K]] resulted in a 2.4-fold increase in the I_{50}. It is probable that this alteration diminished, but did not abolish, any contribution of the hydroxyl group to inhibitor potency.

More importantly, it was shown that the presence of the hydroxyl group resulted in time dependence of CEH inhibition (Fig. 2). Both (2*S*,3*S*)-3-(4-nitrophenyl)glycidol [D_S] and (2*R*,3*R*)-3-(4-bromophenyl)glycidol [C_R] showed increasing CEH inhibition with up to a 2-min preincubation with the enzyme. (2*R*,3*R*)-3-(4-Nitrophenyl)glycidol [D_R] was not tested due to the likelihood that its observed inhibition was due to contamination by the (2*S*,3*S*)-enantiomer [D_S]. 1-Ethoxy-(2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)propane [H_S] and 1-acetoxy-(2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)propane [I_S], in which the hydroxyl group is "masked" and (1*S*,2*S*)-1,2-epoxy-1-phenylpropane [A_S], which lacks a hydroxyl group, showed no time dependence of CEH inhibition. Thus, the hydroxyl group appears to be primarily responsible for the observed slow binding kinetics.

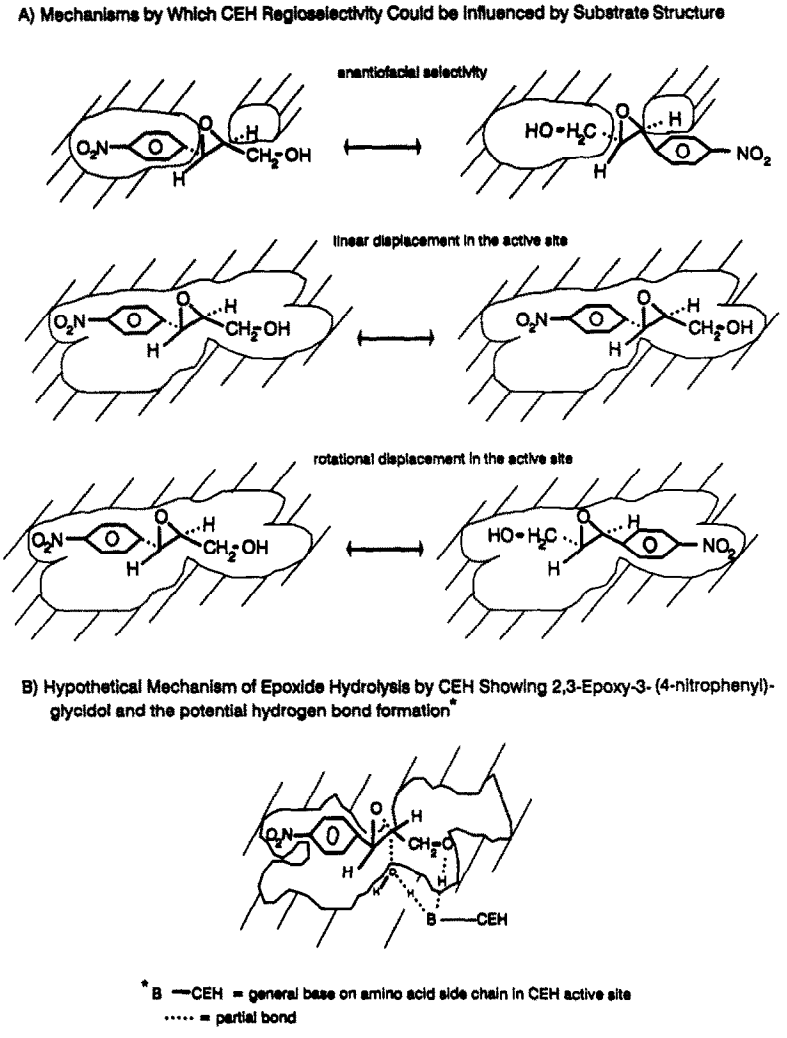


Fig. 5. Hypothetical models of the active site of cytosolic epoxide hydrolase. (A) Mechanisms of interaction between the active site and substrates which could explain the observed difference in the regioselectivity of $[^{18}O]H_2O$ addition to (2*R*,3*R*)- and (2*S*,3*S*)-2,3-epoxy-3-(4-nitrophenyl)glycidol. These include differences in enantiofacial selectivity, linear displacement of the substrate in the active site. (B) A model of the mechanism of epoxide hydrolysis by cytosolic epoxide hydrolase consistent with a general base mechanism of catalysis. This representation shows 4-nitrophenyl glycidol in the active site and the hypothesized hydrogen bond between glycidols and cytosolic epoxide hydrolase.

Slow binding kinetics of inhibition has been postulated to be due to either slow release of the inhibitor from its binding site or a two-step binding process in which the second step is slow [18–20]. A slow step during inhibitor binding might be due to conformational changes in either enzyme or substrate [18] or trapping of a water molecule during the initial binding step [21, 22]. From the data presented, it is not possible to determine which of these mechanisms might be correct. However, preliminary evidence indicates that CEH is easily and rapidly reactivated following D_S inhibition by passage through a short G-25 Sephadex column. A slow step during inhibitor binding, perhaps rearrangement to form a hydrogen bond between the glycidol hydroxyl group and CEH or release of a water molecule bound in the active site, could explain the observed time-dependent inhibition.

The results of $[^{18}O]$ incorporation from H_2O also showed that CEH behaves differently toward (2*R*,3*R*)- $[D_R]$ and (2*S*,3*S*)- $[D_S]$ 2,3-epoxy-(4-nitrophenyl)glycidol. The (2*R*,3*R*)- and (2*S*,3*S*)-enantiomers showed essentially equal incorporation into C_2 and C_3 when hydrolysis was carried out in 0.10 N NaOH and predominant incorporation into C_2 when hydrolysis was carried out in 0.50% $HClO_4$ (Table 3 and Fig. 4). As expected, the two enantiomers showed the same pattern of $[^{18}O]$ incorporation from H_2O when hydrolysis was carried out in either NaOH or $HClO_4$. However, CEH-catalyzed hydrolysis of the (2*R*,3*R*)-enantiomer gave nearly equal $[^{18}O]$ incorporation into C_2 and C_3,

resembling hydrolysis in 0.10 N NaOH, but CEH-catalyzed hydrolysis of the (2*S*,3*S*)-enantiomer gave incorporation of [^{18}O] largely into C_2, resembling hydrolysis in 0.50% $HClO_4$. As was confirmed by [^{18}O] incorporation, studies carried out in model systems suggest that hydrolysis in NaOH should occur approximately equally at C_2 and C_3 and hydrolysis in $HClO_4$ should occur nearly exclusively at C_2 [23, 24].

These results suggest that the differences in the hydrolysis patterns of (2*R*,3*R*)- and (2*S*,3*S*)-2,3-epoxy-(4-nitrophenyl)glycidol [D_R and D_S] by CEH were due to differences in their binding to the CEH active site. If the inhibitors were bound tightly by CEH in the same orientation, it would be expected that incorporation of [^{18}O] from water during CEH-catalyzed hydrolysis would be the same for both enantiomers. This was not the case.

It has been shown that CEH preferentially attacks the *S*-carbon of *cis*-epoxides [25]. Depending on the substrate, a 33–82% enantiomeric excess of the *R*,*R*-diol was produced. Under the same conditions, MEH produced a 57–95% enantiomeric excess of the *R*,*R*-diol. This is consistent with earlier studies employing MEH which showed that, with 1,2-disubstituted *cis*-epoxides, MEH preferentially attacks at the *S*-chiral center [25–28]. In the case of monosubstituted epoxides, MEH attacks the unsubstituted carbon [29]. These studies demonstrated that epoxide hydrolase preferentially, but not exclusively, attacked a specific carbon atom on the epoxide ring and that the preferred position of attack depended on substrate structure.

The results of [^{18}O] incorporation from water during CEH-catalyzed hydrolysis of (2*R*,3*R*)- [D_R] and (2*S*,3*S*)- [D_S] 2,3-epoxy-3-(4-nitrophenyl)glycidol were similar. Incorporation of [^{18}O] occurred in either carbon atom of the epoxide ring with both enantiomers. A possible explanation for the observed [^{18}O] incorporation patterns is that the (2*R*,3*R*)-enantiomer [D_R] had no strongly preferred binding orientation and bound nearly equally well in more then one orientation. Therefore, either C_2 or C_3 could be attacked by CEH during ring opening. However, if the (2*S*,3*S*)-enantiomer [D_S] did have a preferred binding orientation, one carbon would be preferentially attacked. It is not possible from the available data to tell whether the observed effect of chirality on [^{18}O] incorporation from water was due to alteration of enantiofacial selectivity in binding, linear displacememt in the binding site, or rotational displacement in the binding site (Fig. 5A).

The results presented here clearly indicate that CEH interacts differently with *R*,*R*- and *S*,*S*-epoxides. They also suggested that the hydroxyl group, possibly by formation of a hydrogen bond, was important in the mechanism of glycidol inhibition. In Fig. 5B, a model for the interaction of 3-(4-nitrophenyl)glycidol with the active site of CEH is shown. This model assumes an asymmetric binding site and general base catalysis of the hydrolysis reaction. These assumptions are based on the results of [^{18}O] addition to the two enantiomers reported here, which indicated that the (2*S*,3*S*)-enantiomer bound CEH differently than the (2*R*,3*R*)-enantiomer, the apparent importance of the hydroxyl group to both inhibitor potency and time-dependent inhibition, *trans*- addition of the attacking water molecule [24, 30], and the work of Prestwich *et al.* [10], which indicated that the rate-limiting step of CEH-catalyzed hydrolysis of epoxides did not involve general acid hydrolysis.

Several predictions can be made using this model. Glycidol enantiomers and, certainly, all derivatives/analogs lacking a hydrogen bonding interaction should be classical competitive inhibitors. Time-dependent inhibition by (2*S*,3*S*)-3-phenylglycidols may be due to hydrogen bonding of the glycidol hydroxyl group to the proposed general base in the CEH active site. If the proposed active site configuration is correct (Fig. 5B), it should be possible to design an active site directed irreversible inhibitor of CEH by placing an electrophile approximately in the position occupied by the hydroxyl hydrogen of (2*S*,3*S*)-3-(4-nitrophenyl)glycidol. Finally, it should be possible to use a series of chiral epoxides with two to four substituents on the epoxide ring, test their ability to inhibit CEH, and begin to determine the configuration of the CEH binding pocket.

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