ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 242, No. 1, October, pp. 11–15, 1985

Inhibition of Epoxide Metabolism by α,β-Epoxyketones and Isosteric Analogs[1]

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Received April 23, 1985, and in revised form June 13, 1985

Chalcone oxides and several isosteric compounds have been prepared to examine the importance of the α,β-epoxyketone moiety in the inhibition of the hydrolysis of [^{3}H]-*trans*-stilbene oxide to its *meso*-diol by mouse liver cytosolic epoxide hydrolase (cEH). Inhibition of microsomal EH and glutathione *S*-transferase were also examined. For cEH, replacement of the carbonyl by methylidene reduces inhibitor potency by a factor of 44, while replacement of the epoxide ring with a cyclopropyl ring reduces inhibition by a factor of 450. A 2′-hydroxyl also reduces cEH inhibition by 100 times. These observations are consistent with a model of the active site in which the carbonyl is hyhydrogen-bonded to an acidic site presumed to be involved in initiating epoxide hydrolysis. The chalcone oxides thus bind tightly but are not readily turned over as substrates.

The chalcone oxides constitute an important class of tight-binding inhibitors of cytosolic epoxide hydrolase (cEH)[4] which show slow association kinetics (1). The mechanism of action proposed for these analogs involves hydrophobic binding of the 4- and 4′-aryl groups into different hydrophobic pockets flanking the active site and interaction of the epoxyketone moiety with the hydrolytic site (1). Stereochemical, kinetic, and isotopic labeling studies support a simple nucleophilic opening in which the enzyme directs a hydroxide anion equivalent to the backside of the least-hindered epoxide carbon [summarized in Ref. (2)]. We have proposed that the hydrolytic site possesses an electrophilic site to initiate epoxide-opening from one face, with a basic site on the opposite face to generate the hydroxyl anion equivalent required to complete the hydrolytic opening. The synchronous, or at least nonionic, nature of this conversion receives support from the failure of cyclopropyl oxiranes to inactivate the enzyme irreversibly through a suicide-type cyclopropylcarbinyl-homoallylic rearrangement (2). We felt that the tight binding was due to the α,β-epoxyketone moiety of the chalcone oxides. A series of chalcone oxide analogs was thus prepared to examine the importance of the epoxide ring in determining inhibitory potency. We now demonstrate that reduction of the Lewis-basicity of the electron pairs of the carbonyl or replacement of the carbonyl or the epoxide with a methylene

[1] We gratefully acknowledge the Herman Frasch Foundation for grants to G.D.P. and B.D.H. The latter also thanks the NIH (ES-02710-04) for support.

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[3] NIEHS Research Career Development Awardee (ES-00107).

[4] Abbreviations used: cEH, cytosolic epoxide hydrolase; mEH, microsomal epoxide hydrolase; GST, glutathione *S*-transferase; CSO, *cis*-stilbene oxide; TSO, *trans*-stilbene oxide; DMSO, dimethyl sulfoxide.

0003-9861/85 $3.00

(CH_2) unit drastically reduces the inhibitory potency of isosteric analogs of the chalcone oxides. In contrast, replacement of aryl rings with aliphatic chains has little effect if the α,β-epoxyketone moiety is retained.

EXPERIMENTAL PROCEDURES

Chemicals. The substituted chalcone oxides **A–G** were prepared previously (1, 3) (Fig. 1). The *cis*- and *trans*-dypnone oxides, **I** and **J**, were gifts of Dr. C. A. Kingsbury (University of Nebraska). All other inhibitors were prepared as outlined below. Complete synthetic and spectral details, including ^1H NMR, ^{13}C NMR, ir, and MS, are presented in the M.Sc. Thesis of J.-W. Kuo.

2'-Hydroxychalcone oxide, ***H*** (4). The parent chalcone was obtained from the base-catalyzed condensation of benzaldehyde with 2-hydroxyacetophenone; yield, 60% recrystallized (C_2H_5OH), mp 83.5–85.0°C. Epoxidation with three equivalents of *m*-chloroperbenzoic acid in $CHCl_3$ (60°C, 24 h) gave, after usual extractive isolation and SiO_2 chromatography (5% ethyl acetate-benzene), a 45% yield of **H.** A recrystallized (benzene) sample had mp 71.0–72.5°C [lit. mp unreported, Ref. (4)].

1-Phenyl-3-(2-naphthyl)-2,3-epoxy-1-propanone, ***K*** (5). The parent chalcone analog, 1-phenyl-3-(2-naphthyl)-2-propen-1-one, was prepared from 2-naphthaldehyde and acetophenone to give, after recrystallization, an 81% yield of yellow crystals, mp 155–156.5°C [lit. mp, 154°C; Ref. (6)]. The epoxide was prepared using aqueous methanolic H_2O_2 with NaOH and benzyltrimethylammonium hydroxide as a catalyst. The crude epoxide (96%) was recrystallized from ethanol and then from pentane to give white crystals, mp 82°C [lit. mp, 80°C; Ref. (5)].

1-(2-Naphthyl)-3-phenyl-2,3-epoxy-1-propanone, ***L.*** The parent enone (2 mmol), mp 104–105°C, was dissolved in 40 ml of CH_3OH and 20 ml of acetone and stirred (20°C, 3 h) with 1 ml of 30% H_2O_2 and a few drops of 1 N NaOH. The epoxyketone was precipitated

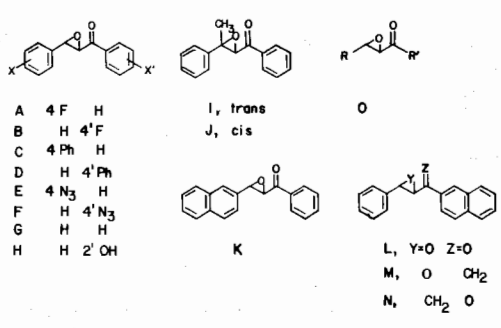

FIG. 1. Inhibitors tested with epoxide-metabolizing enzymes.

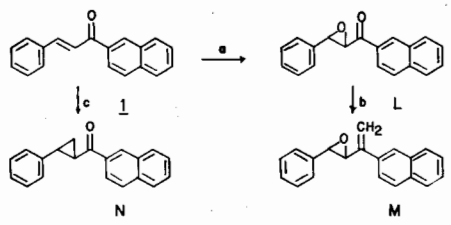

FIG. 2. Synthesis of α,β-epoxyketones and isosteres: (a) H_2O_2, ^-OH; (b) $Ph_3P{=}CH_2$; (c) $(CH_3)_2S(O){=}CH_2$.

with water, filtered, and recrystallized (C_2H_5OH) to give 74% of epoxide, **L;** mp 120–121°C [lit. mp, 124°C; Ref. (5)].

3-(2-Naphthyl)-1-phenyl-1,2-epoxy-3-butene, ***M.*** To a solution of 170 mg (0.477 mmol) of methyl triphenylphosphonium bromide and 2 mg of triphenylmethane (carbanion indicator) in 4 ml of DMSO was added dropwise a 1.0 M solution of potassium dimsylate in DMSO at 20°C. To the yellow solution was added 57 mg (0.208 mmol) of epoxyketone (**L**) as dry powder, and the red solution was stirred at 50°C for 3 h. The mixture was cooled, poured into H_2O and extracted with hexane (3 × 20 ml). The combined organics were washed (saturated NaCl), dried ($MgSO_4$), and evaporated to give 62 mg of a yellowish material. Flash chromatography on silica gel with 4% ethyl acetate in hexane gave 11.5 mg of the epoxide product (20%) which was 97% homogeneous by GC. ^1H NMR, δ3.78 (br s, H-1,2), 5.56, 5.60 (s,s, H-4); LRMS, *m/z* 272 (19%, M^+), 165 (100%, M^+-phenyl-CH_2O); HRMS, Calcd. for $C_{20}H_{16}O$, 272.1201. Found, 272.1196.

2-Naphthalenyl-(trans-phenylcycloprop-1-yl) methanone, ***N.*** A solution of dimethyloxosulfonium methylide in DMSO (7) was prepared from 12.4 mmol of NaH, 12.4 mmol of trimethylsulfoxonium iodide, and 15 ml of DMSO, cooled to 0°C, and treated with 9.2 mmol of enone **1** in 15 ml of DMSO (Fig. 2). The reaction was stirred for 16 h at 20°C, poured into H_2O, and extracted three times with ether, and the combined organic layers were washed (H_2O, saturated NaCl), dried ($MgSO_4$), and evaporated. The residue was recrystallized from absolute alcohol to yield 2.20 g (88%) cyclopropanated product, mp 98–99°C [lit. mp, 99°C; Ref. (7)]. IR (KBr) 1640 cm^{-1} (C=O); ^1H NMR, δ1.61, 1.99 (m, cyclopropyl CH_2), 2.79, 3.07 (m, cyclopropyl CH, sum of coupling constants = 20 Hz); ^{13}C NMR, δ19.41, 29.37, 30.32 (cyclopropyl), 140.51 (carbonyl); LRMS, *m/z* 272 (74%, M^+); 127 (100%, M^+-naphthyl); HRMS, Calcd. for $C_{20}H_{16}O$, 272.1203. Found, 272.1197.

Mixture of 9-oxo-10,11-epoxyoctadecanoic acid and 10-oxo-8,9-epoxyoctadecanoic acid, ***O.*** The methyl esters of the (*E*)-9-oxo-10-octadecenoic acid and (*E*)-10-oxo-8-octadecenoic acid mixture were prepared by photooxygenation of oleic acid (8) and were provided as a gift by Dr. D. J. Eickhoff (Proctor and Gamble) and

E. D. Mihelich (Eli Lilly Co.). The esters were dissolved in CH_3OH and epoxidized with basic hydroperoxide in the usual fashion (0°C, 7 h). Extraction (ether) and chromatography (5% ethyl acetate-hexane, SiO_2), gave 36% of the α,β-epoxy keto esters. Hydrolysis with one equivalent of LiOH in CH_3OH:H_2O (2:1) for 17 h at 20°C gave, after acidification (H_3PO_4), extraction (ether), and chromatography (20% ethyl acetate-hexane, SiO_2), 60% of pure keto acids **O**: ^1H NMR ($CDCl_3$), δ10.8 (s, CO_2H), 3.18 (d, J = 1.7 Hz, H_α), 3.03 (br s, H_β), 2.34 (br t, $-CH_2CO-$).

Assays for epoxide-metabolizing enzymes. The cytosolic and microsomal fractions from male Swiss-Webster mice were prepared as described by Hammock and Ota (9), except that the 100,000g pellet was resuspended in 125 mM KCl, 50 mM Tris (pH 7.4), and centrifuged an additional 30 min 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 (TSO) while the microsomal and GSH *S*-transferases were measured with [^{3}H]-*cis*-stilbene oxide (CSO) using partition assays described in detail elsewhere (10–12). CSO and TSO were used at 5×10^{-5} M and GSH at 5×10^{-3} M. The inhibitor was preincubated with enzyme at 37°C for 10 min following its addition in 1 μl of acetone or ethanol. The assay was initiated by addition of labeled substrate. Triplicate incubations at four or five different concentrations of the inhibitor were used and I_{50} values were obtained from a linear fit of data on a log dosage–probit plot.

RESULTS AND DISCUSSION

Compounds tested as inhibitors of epoxide metabolism are shown in Fig. 1. Chalcones were prepared by Claisen-Schmidt condensation of substituted aromatic aldehydes with the aryl methyl ketones, and the *trans*-α,β-epoxyketones were obtained by conjugate addition of hydroperoxide anion to the (*E*)-enones. The methylidene isostere (**M**) of epoxyketone **L** was prepared by Wittig olefination of **L** with methylene triphenylphosphorane in dimethyl sulfoxide (Fig. 2). The *trans*-1,2-disubstituted cyclopropyl isostere **N** was prepared by controlled addition of dimethylsulfoxonium methylide in DMSO (7).

Examination of Table I provides several insights into the nature of the enzyme active sites of the three enzymes. First, none of the chalcone oxides or isoteric analogs show high inhibitory potency for mEH. Only the epoxy keto fatty acid mixture and the more hydrophobic chalcone oxides approach micromolar I_{50} values.

Second, none of the chalcone oxides or isosteres are potent inhibitors of GST. In contrast, the enones (precursors to the α,β-epoxyketones) strongly inhibited GST with I_{50}'s of 5.4×10^{-6} and 8.5×10^{-7} M for the 1-naphthyl-3-phenyl and 1-phenyl-3-naphthyl propenones, respectively, and 8×10^{-6} M for the oxooctadecenoic acids. This observation is expected since enones add RSH compounds rapidly even in the absence of enzyme catalysis, but this inhibition is not due simply to depletion of glutathione (10). Indeed, it appears that the GSH-chalcone adducts are the inhibitory species for the transferase reaction (1, 10).

Third, for the biphenyl and naphthyl chalcone oxide systems, both of which have hydrophobic biaryl moieties, the I_{50} values decrease in both the 4 and 4′ series (compare **A** with **K** and **B** with **L**). The planar naphthyl ring in the 4 position gives somewhat better inhibition than in the 4′ position.

Fourth, substitution of methyl for hydrogen on the C-3 position of the oxirane ring of chalcone oxide does not substantially alter inhibitory potency for cEH (compare **G** and **I**). However, altering the geometry of the aryl-containing moieties from *trans* to *cis* on the oxirane ring causes a dramatic 500-fold reduction in inhibition (compare **I** and **J**), which mirrors the CSO/TSO dichotomy as mEH/cEH assay substrates.

Fifth, replacement of the aryl groups with aliphatic chains causes a modest reduction in activity. Retention of inhibitory potency in **O** indicates that it is not essential to have aryl groups adjacent to the epoxide or carbonyl functions.

Sixth, the presence of a 2′-hydroxy function reduces inhibition of cEH over 100-fold. Since the carbonyl oxygen is hydrogen bonded to the 2′-OH, its electron pairs are not available to interact with the active site electrophile; its inhibitory potency is thus reduced.

Finally, the substitution of methylene for oxygen in the oxirane or carbonyl groups causes a dramatic decrease in in-

TABLE I

INHIBITORY POTENCIES [I_{50} (M) OR HIGHEST PERCENTAGE INHIBITION NOTED] FOR CHALCONE OXIDES AND ANALOGS[a]

Trivial name and code	cEH[b]	mEH[c]	cGST[d]
4-Fluoro chalcone oxide, **A**	1.5×10^{-6}	—	—
4′-Fluoro chalcone oxide, **B**	3.5×10^{-6}	—	—
4-Phenyl chalcone oxide, **C**	6.0×10^{-7}	—	—
4′-Phenyl chalcone oxide, **D**	7.0×10^{-7}	—	—
4-Azido chalcone oxide, **E**	1.2×10^{-6}	1.3×10^{-4}	3.2×10^{-5}
4′-Azido chalcone oxide, **F**	4.8×10^{-6}	2.5×10^{-4}	2.5×10^{-4}
Chalcone oxide, **G**	4.0×10^{-6}	2.0×10^{-4}	1.3×10^{-4}
2′-Hydroxychalcone oxide, **H**	$43(5.0 \times 10^{-4})$	$38(5.0 \times 10^{-4})$	1.4×10^{-5}
trans-Dypnone oxide, **I**	3.1×10^{-6}	1.3×10^{-4}	$35(5.0 \times 10^{-4})$
cis-Dypnone oxide, **J**	$35(5.0 \times 10^{-4})$	3.7×10^{-4}	$39(5.0 \times 10^{-4})$
Naphthalenyloxiranyl phenyl methanone, **K**	2.4×10^{-7}	2.1×10^{-5}	8.0×10^{-6}
Phenyloxiranyl naphthyl methanone, **L**	1.2×10^{-6}	9.0×10^{-5}	8.0×10^{-6}
Methylidene analog, **M**	5.3×10^{-5}	3.1×10^{-4}	1.8×10^{-4}
Cyclopropyl analog, **N**	$46(5.0 \times 10^{-4})$	6.0×10^{-5}	$19(5.0 \times 10^{-4})$
α-β-Epoxy oxo-octadecanoic acids, **O**	1.6×10^{-5}	1.0×10^{-5}	6.0×10^{-5}

[a] Under the conditions used for the enzyme assays, 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 $protein^{-1}$, and the cytosolic fraction led to the production of 40 nmol min^{-1} mg $protein^{-1}$ of the glutathione conjugate of CSO. For comparison with other assays (1, 2, 9, 10), 3,3,3-trichloropropane-1,2-oxide (TCPO) had an I_{50} of 4×10^{-6} M with mEH. For cEH, 4-phenylchalcone oxide, **C**, had an I_{50} value of 6.4×10^{-8} M with [^{3}H]*trans*-β-ethylstyrene oxide as substrate but a value of 6.0×10^{-7} M with [^{3}H]TSO as substrate.

[b] Cytosolic epoxide hydrolase (cEH) was measured using [^{3}H]-*trans*-stilbene oxide, 5×10^{-5} M, 37°C, 10 min, pH 7.4.

[c] Microsomal epoxide hydrolase (mEH) was measured using [^{3}H]-*cis*-stilbene oxide, 5×10^{-5} M, 37°C, 10 min, pH 9.0.

[d] Cytosolic glutathione *S*-transferase (cGST) was measured using [^{3}H]-*cis*-stilbene oxide, 5×10^{-5} M, with 5 mM glutathione, 37°C, 10 min, pH 7.4.

hibitory potency. The ene-epoxide **M** shows 44-fold lower inhibition of cEH relative to epoxyketone **L**, and the cyclopropylketone **N** is 450-fold less potent than **L**. These two analogs provide the most important information, in that they embody major electronic differences but relatively minor steric differences in the inhibitory epoxyketone moiety.

These observations lead us to a simple model (Fig. 3) for the cEH catalytic site. Two hydrophobic binding regions are spatially arranged to accept *trans*-oriented hydrophobic moieties and in some cases *cis*-moieties capable of free rotation. Interaction of the oxiranyl oxygen lone-pair electrons with an electrophile (general or specific acid) at the active site activates the epoxide for ring opening, while an essentially synchronous delivery of water, activated by enzymic deprotonation, occurs

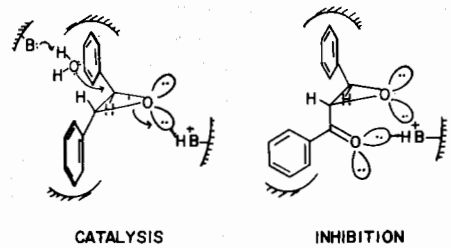

FIG. 3. Modes of binding for hydrolysis (TSO binding) and inhibition (chalcone oxide binding) for cytosolic epoxide hydrolase.

in an *anti*-periplanar fashion. The lack of irreversible inhibition by the cyclopropyl oxiranes provided some evidence to argue against a purely electrophilic ring-opening mechanism (2). However, the inhibitory nature of the α,β-epoxyketone moiety is best rationalized by invoking electrophilic initiation in the course of epoxide opening. The lone-pair electrons of the carbonyl oxygen are in sp^2-like orbitals in the plane containing the carbonyl and oxirane carbon and oxygen atoms, and are thus stronger Lewis bases than the oxiranyl oxygen lone pairs which are more sp^3-like in hybridization. Because the epoxyketones can apparently bind to the hydrolytic site electrophile through hydrogen bonding to the carbonyl group, the activation of the oxirane ring to nucleophilic attack is substantially reduced. Hydration of substrates and the inhibitor is thereby reduced. When the methylidene group is substituted for the carbonyl, the electrostatic interaction is no longer possible, although the material is still a substrate analog. When the epoxide oxygen is removed, the carbonyl is insufficient to allow tight binding of the hydrophobic cyclopropyl to the electrophilic region of the active site.

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