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Chalcone Oxides—Potent Selective Inhibitors of Cytosolic Epoxide Hydrolase[1]

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The systematic screening of over 150 compounds for inhibitory activity on mammalian cytosolic epoxide hydrolase led to identification of chalcone oxide (*trans*-1-benzoyl-2-phenyloxirane) as an optimal inhibitory structure. Important structural features for inhibition include two hydrophobic moieties preferably orientating in a *trans* manner from an electrophilic center such as an activated olefin or epoxide, with the epoxide giving maximal activity. Synthesis of chalcone oxide derivatives bearing a single *p*-substituent on either phenyl ring has led to very potent inhibitors of the enzyme, the best being 4-phenylchalcone oxide (50% inhibition at 6.4×10^{-8} M). Multiple factorial analysis on the inhibition data for the two series of chalcone oxides prepared (phenyl or benzoyl substituted) revealed both the essentialness of hydrophobic interactions and the apparent nonequivalence of the two hydrophobic sites involved in the inhibitory process. Steric factors were considerably less crucial while electronic effects were unimportant in the compounds examined. The chalcone oxides were either inactive or only weak inhibitors of the other major epoxide-metabolizing enzymes in mouse liver, cytosolic glutathione *S*-transferase, and microsomal epoxide hydrolase. The nature of the inhibition of cytosolic epoxide hydrolase by chalcone oxides was further investigated through steady-state kinetic analysis and the use of amino acid modifiers. Chalcone oxides give a slowly reversible mixed-noncompetitive inhibition. They may interact covalently with a cysteine residue possibly essential to the catalytic action of cytosolic epoxide hydrolase, and may indeed be alternative substrates with very low turnover. The cytosolic and microsomal epoxide hydrolases can be clearly distinguished by these inhibitors, further indicating different catalytic mechanisms.

All organisms studied to date possess degradatory enzymes to contend with the hormonal (1) or deleterious effects (2-4) of reactive heterocycles known as epoxides. The two major pathways of epoxide metabolism are catalyzed by epoxide hydrolases (EC 3.3.2.3) and glutathione *S*-transferases (EC 2.5.1.18) (5) leading to the considerably more polar diols and GSH conjugates, respectively. Thus, these enzymes are thought to serve a detoxification role by generating readily excretable products from the more lipophilic epoxide precursors.

In mammals there appear to be at least two distinct forms of epoxide hydrolases, a low-molecular-weight form located largely, but not exclusively, in the membranes of the cell and a second,

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higher-molecular-weight form located largely in the cytosol and the mitochondrial lumen. Although a preponderance of information is now available on membrane-bound epoxide hydrolases from eucaryotic organisms (2-4, 6, 7), relatively little is known about the more recently characterized mammalian cytosolic enzyme (8) that readily hydrates some epoxides refractory to the microsomal epoxide hydrolase. The ability of the cytosolic epoxide hydrolase to hydrate α-phenyl *trans*-1,2-disubstituted epoxides, which are not or only slowly converted by the microsomal enzyme, is a useful criterion to distinguish the microsomal and cytosolic enzymes within hepatic tissues of the species examined (8-10). The apparently tight interaction of *trans*-1,2-disubstituted oxiranes with cytosolic epoxide hydrolase could prove useful in designing specific inhibitors of the enzyme. Effective inhibitors of microsomal epoxide hydrolase (1, 11-13) such as 1,2-epoxy-3,3,3-trichloropropane are known and a variety of glutathione *S*-transferase inhibitors have been noted (5), however, only inhibition with certain inorganic cations and competitive substrate inhibition by juvenoid epoxides have been observed for the cytosolic epoxide hydrolase (8, 14, 15). Clearly, specific inhibitors for each epoxide-metabolizing enzyme would be highly advantageous for clarifying the relative importance of these enzymes to epoxide degradation in mammalian tissue.

Chalcone oxides (Table I) whether biosynthetically generated in plants (16) or chemically prepared (17, 18) are predominantly of the thermodynamically stable *trans* geometry. The parent compound is an *in vitro* stimulator of rat microsomal epoxide hydrolase (19, 20). Cytosolic epoxide hydrolase has to date exhibited properties markedly dissimilar (indeed almost an antithesis) to its microsomal counterpart, and to this extent the chalcone oxide behaves similarly. This report will describe first the structure-activity optimization and then consider the mode of action of chalcone oxides as potent, selective inhibitors of the cytosolic epoxide hydrolase.

MATERIALS AND METHODS

Chemicals. The 4'-hydroxy, 4- and 4'-methoxy, and 4-nitro substituted chalcones were from Fairfield, Blythewood, South Carolina and chalcone, *trans*-stilbene oxide, 4'-nitro- and 4-hydroxychalcones, 2'-bromo-6-nitroacetophenone, and 1,2-epoxy-3,3,3-trichloropropane were obtained from Aldrich. All acetophenones and benzaldehydes required for chalcone synthesis were from Aldrich except for *p*-bromoacetophenone (MCB, Cincinnati, Ohio). 1-(4'-Ethylphenoxy)-6,7-epoxy-3,7-dimethyl-*trans*-2-octene was a gift of Stauffer Chemical Company, Mountain View, California, and 1-(4'-bromophenoxy)-6,7-epoxy-3,7-dimethyloctane was synthesized previously (15). All other reagents and buffer salts used here were of highest purity commercially available.

Synthesis of both the radiolabeled epoxides and standard diols for the epoxide hydrolase assays for *trans*-β-ethylstyrene oxide and *cis*-stilbene oxide were as described (10, 21). The cold epoxide substrates were prepared from *trans*-1-phenyl-1-butene (Chemical Samples Co., Columbus, Ohio) and *cis*-stilbene (Aldrich) respectively with *m*-chloroperbenzoic acid, and *meso*-stilbene glycol was from Tridom/Fluka. Synthesis of a glutathione-conjugate of *trans*-β-ethylstyrene oxide was achieved by titrating aqueous GSH (0.5 M) to pH 9.5 with 1 N sodium hydroxide, adding an equimolar amount of the epoxide in ethanol (final ethanol concentration, 25%, v/v) and stirring at room temperature in the dark for 20 h. The pH was then adjusted to 3.5 with 6 N hydrochloric acid, the mixture extracted under N_2 with an equal volume of ethyl acetate, and the water phase remaining after precipitate removal freeze-dried to give mostly conjugate (ninhydrin positive; uv absorbing at 254; R_f 0.27 on silica gel: *n*-propanol/2 N NH_4OH, 3/1) and some unreacted glutathione (R_f 0.05).

Synthesis of chalcone oxides and their derivatives. Purity and identity of the various chalcones, chalcone oxides and their 2-mercaptoethanol adducts synthesized here were determined by melting points, NMR spectroscopy (Varian EM-390), TLC[4] (silica gel F-254, EM reagents) using toluene/ethyl acetate (10/1), hexane/ether (10/1), and toluene/methanol (9/1) with visualization by 254-nm quenching, 340-nm fluorescence, and a 4-(*p*-nitrobenzyl) pyridine spray reagent for epoxides (22), and in some cases elemental analysis. All compounds were satisfactory pure (>95%) except for 4-acetamidochalcone oxide, which gave a low elemental analysis (C 68.73, H 4.68; expect 72.59, 5.38).

[4] Abbreviations used: DFP, diisopropyl fluorophosphate; *I*, ionic strength, mp, melting point; *MR*, molar refractivity; pI_{50}, $-\log I_{50}$; TLC, thin-layer chromatography.

The substituted chalcones (cf. Tables I and II) were prepared by condensation of the appropriate *p*-substituted acetophenone (4′ series) or *p*-substituted benzaldehyde (4 series) with benzaldehyde and acetophenone, respectively, in alkaline ethanol (23). Thus, 30 mmol of each of the appropriate acetophenone and benzaldehyde in 45 ml absolute ethanol were stirred vigorously in the dark for 45 min at room temperature with 3 ml aqueous sodium hydroxide (2 N). Following neutralization with hydrochloric acid and addition of water, precipitated solids (ice temperature) were collected, washed with water, and recrystallized from absolute ethanol.

Chalcone oxides (Table II) were obtained from the respective chalcones by treatment with alkaline hydrogen peroxide (24). The chalcone (10 mmol) in 80 ml methanol after addition of a trace of base was treated all at once with 5.2 ml of 30% (v/v) hydrogen peroxide and rapidly stirred while 6 ml of sodium hydroxide (1 N) was added over a 0.5-h period, maintaining the reaction near room temperature with an ice bath if required. In some cases acetone (up to 40 ml) was added to effect solution, but this resulted in side reactions for 4′-nitrochalcone. The mixture was then further stirred at room temperature in the dark for 3 h, water added at ice temperature, and precipitated solids washed with water and recrystallized from absolute ethanol or in some cases an ethanol/benzene mixture. The 16 chalcone oxides synthesized here (Table II) were exclusively *trans*-1,2-oxiranes (17, 18, 25) by NMR ($CDCl_3$): δ 4.06 ± 0.03 (H_a), 4.26 ± 0.04 (H_b), $J_{a,b}$ = 1.96 ± 0.07 ($\bar{x}$ ± SD, and used hereafter). *trans*-Dibenzoylethylene oxide was prepared similarly from the olefin to give white needles (mp 125°C, ethanol).

2-Mercaptoethanol adducts [ring-substituted 1,3-diphenyl-3-(2-hydroxyethylthio)propan-1-ones] of the chalcones were synthesized essentially as follows for the parent chalcone. To chalcone (5 mmol) in 10 ml benzene were added 2-mercaptoethanol (10 mmol) and 2 drops of triethylamine and the reaction was stirred under nitrogen at room temperature for 24 h. Recrystallization of the crude residue from ether/petroleum ether gave white crystals, mp 73°C (lit. mp 68°C, 26). NMR (d_6-acetone): benzylic H 4.64 (1 H, t).

Assays for epoxide-metabolizing enzymes. Cytosolic epoxide hydrolase with *trans*-2-[2-^{3}H]ethyl-1-phenyloxirane (*trans*-β-ethylstyrene oxide, 47 Ci/mol) as substrate and microsomal epoxide hydrolase with *cis*-1,2-[1-^{3}H]diphenyloxirane (*cis*-stilbene oxide, 64 Ci/mol) were both routinely measured by the respective radiometric partition assays (10, 21). Inhibitors were incorporated into the assay procedures as follows. Mouse hepatic cytosol (15 μg protein; final volume 50 μl) in pH 7.2 sodium phosphate (I = 0.2) buffer containing 0.1 mM EDTA (buffer A) was preincubated 1 min at 37°C. The inhibitor in 1 μl ethanol was then added followed 15 s later by the substrate solution (final concentration, 5×10^{-4} M; 20,000 cpm) in 1 μl ethanol unless indicated otherwise for specific experiments. Incubations after 10 min were terminated with 100 μl isooctane (2,2,4-trimethylpentane) as before (10) and an aliquot of the aqueous phase (20 μl) with 92% of the diol residue was removed for liquid scintillation counting in OCS (Amersham Corporation)/Triton X-100 (3/1). Counting efficiency was 36% for ^{3}H in Omni-vials (Wheaton). Similarly, inhibitors were incubated with washed mouse liver microsomes (44 μg protein) in 0.1 M sodium pyrophosphate (pH 8.5) buffer containing 0.1 mM EDTA (buffer B) and analyzed as above for the epoxide hydration of *cis*-stilbene oxide. Cytosolic GSH *S*-epoxy transferase acting on *trans*-β-ethylstyrene oxide was measured by a TLC assay (10) with inhibitors included in the incubation as follows. Mouse liver cytosol (16 μg protein) in a buffer A (40 μl final volume) was incubated 1 min at 37°C and 1 μl of the inhibitor in ethanol was added. After 1 min incubation the reaction was initiated with 10 μl of freshly prepared glutathione in buffer A (26 mM), then substrate as above and subsequently terminated with 25 μl of tetrahydrofuran after 5 min of incubation at 37°C. Reactions were kept on ice prior to TLC on Whatman LK5DF silica gel plates (10). Thus, the loading zone of the plate was scraped and analyzed by liquid scintillation counting as above. The GSH-dependent conjugate(s) produced by mouse liver cytosol cochromatographed with a chemically synthesized standard.

Radiometric assays of the epoxide-metabolizing enzymes incorporating inhibitors were run in triplicate with percentage inhibition calculated relative to product formation above background (boiled enzyme, always less than 3% of control) in ethanol controls. For determination of the concentration required for 50 or 90% inhibition of the enzyme (I_{50} and I_{90}, respectively), duplicate incubations at five different concentrations of the inhibitor (to give 5–95% inhibition) were used and the inhibition constants obtained from a linear fit of data on a log dosage-probit plot.

The kinetics of the inhibition of cytosolic epoxide hydrolase by chalcone oxides was determined, in part, by a continuous spectrophotometric assay[5] based on the decrease in absorbance of *trans*-stilbene oxide at 229 nm upon its hydration to the hypochromic diol ($\Delta\epsilon$ = 18,500 M^{-1} cm^{-1}). Mouse hepatic cytosol (272 μg) in 1.96 ml buffer A was preincubated at 37°C for 5 min in the sample and reference beams of a Varian Carey 219 spectrophotometer. The inhibitor (20 μl) in ethanol was added to both sides followed by ethanol (20 μl) to the reference cuvette, and the initial velocity then measured after addition of

[5] L. Hasegawa and B. D. Hammock, unpublished data.

20 μl of *trans*-stilbene oxide (final concentration, $0.56\text{–}5 \times 10^{-5}$ M) in ethanol to the sample cuvette. There was no detectable nonenzymatic (sonicated boiled cytosol) conversion of the epoxide.

Enzyme preparation. Eleven-week-old male Swiss-Webster mice (Simonsen Laboratories, Gilroy, Calif.) were killed by a blow to the head, decapitated, and their livers placed in buffer A. The gall bladder was excised intact and then both the liver perfused and a 20% (w/v) homogenate prepared with buffer A, using a smooth glass Potter–Elvehjem homogenizer with a motor-driven Teflon pestle. Cytosolic and microsomal fractions were obtained as before (9). The microsomal fraction was washed in buffer B (27), and then resuspended to 35 mg protein/ml in buffer B containing 50% glycerol and 0.01% (w/v) butylated hydroxytoluene. All enzymes preparations were either kept on ice up to 1 week or stored at −70°C until required. Full epoxide-metabolizing capability was maintained under these conditions even after storage of frozen microsomal and cytosolic fractions for up to 2 and 10 months, respectively. Protein was assayed by the method of Lowry *et al.* (28) with bovine serum albumin as standard.

Multiple regression analysis. All substituent constants and the nomenclature used here were from the extensive compilation of Hansch (29), except for the E_s value of the acetamido group which was estimated to be −2. Only the most widely accepted values were used. Multiple regression analysis of the inhibitor potency of the 4- and 4′-substituted chalcone oxides on cytosolic epoxide hydrolase was obtained using a Minitab II program (Statistics Dept., Penn State University, State College, Pa.), and combinations of up to four free-energy parameters were investigated. The *F* test was used to determine the confidence of adding an extra parameter to a regression equation.

RESULTS

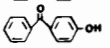
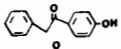
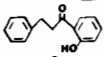
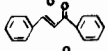
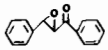
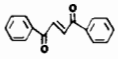
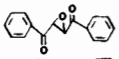
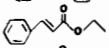
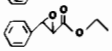
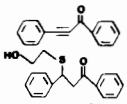
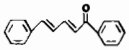
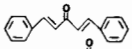
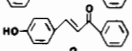
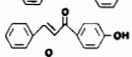
Identification of Chalcone Oxide as an Optimal Inhibitory Structure for Cytosolic Epoxide Hydrolase

Numerous organic compounds (over 150) were screened for their inhibitory potency on the cytosolic epoxide hydrolase of mouse liver, and a sample of essential structural features for inhibition is presented in Table I. An electrophilic center in the molecule was important for maximal inhibitory potency, and in all cases good inhibitors possessed a hydrophobic region on both sides of the electrophilic moiety (Table I, cf. **N**, **G**). Orientation of the two hydrophobic regions away from each other (i.e., *trans* instead of aligned or *cis* to) markedly improved inhibition [cf. **K**, **L**; (15)]. Fortuitously, the *trans* isomer is the thermodynamically stable geometric form of the 3-phenyl-2-propen-1-one moiety (16–18) thus making available many compounds to study. In addition, the olefin appears more inhibitory than the corresponding hydrocarbon (cf. **W**, **V**; **T**, **D**). With the diphenyl ketone structure, a hydrocarbon spacer was disadvantageous (cf. **A–D**); similarly, increasing olefinic conjugation in the chalcone structure (**F**, **Q**, and **R**) had no significant effect on activity; however, further ketonic conjugation although ineffective for benzophenone (cf. **A**, **E**) markedly increased the inhibitory potency of the chalcone (**H**). Most dramatic in increasing inhibition activity was epoxidation of the unsaturated center to give a more reactive alkylating agent (cf. **G**, **F**; **K**, **J**; **N**, **M**) with the exception again of *trans*-1,2-dibenzoylethylene oxide (**I** and **H**). Chalcone oxide was a potent inhibitor of the enzyme. No modification tested in the basic chalcone structure proved to be more advantageous (e.g., **O**, **P**) although ring hydroxylation always increased inhibition (**A**, **B**; **F**, **S**, and **T**). Unfortunately, preparation of phenolic chalcone oxides, that is molecules possessing both features of a good inhibitor, are difficult due to their propensity to self-polymerize. Interestingly, the mercaptoethanol adducts (structure **P**) of chalcone, 4-hydroxy- and 4′-hydroxychalcones (**F**, **S**, and **T**, respectively) had the same inhibitory potency ($I = 1.8\text{–}2.0 \times 10^{-4}$ M) indicating that an essential feature for inhibition by the later two compounds in addition to the epoxide functionality, possibly *trans* geometry, had been removed.

Identification of chalcone oxide as a model inhibitory structure for cytosolic epoxide hydrolase allowed the directed synthesis, based on free-energy parameters (29), of analogs for structure-activity optimization. Two series of compounds, the 4- and 4′-substituted chalcone oxides, were prepared (Table II) by methods that gave strictly *trans* epoxides. Markedly potent inhibitors (I_{50}'s into the 10^{-8} M range)

TABLE I

APPROACH TO AN OPTIMAL INHIBITORY STRUCTURE FOR CYTOSOLIC EPOXIDE HYDROLYASE

Compound	Structure	Source[a]	I_{50} (M) or percentage inhibition at 5×10^{-4} M[b]
A Benzophenone		1	39 ± 3
B *p*-Hyxdroxybenzophenone		2	2.8×10^{-4}
C 4-Hydroxyphenylbenzylketone		3	1.0×10^{-3}
D *o*-Hydroxy-β-phenylpropriophenone		1	17 ± 2[c]
E Benzil		4	30 ± 2
F Chalcone		1	39 ± 2[d]
G Chalcone oxide		5	8.2×10^{-6}
H *trans*-1,2-Dibenzoylethylene		1	9.0×10^{-5}
I *trans*-1,2-Dibenzoylethylene oxide		5	22 ± 5
J *trans*-Stilbene		1	6 ± 7
K *trans*-Stilbene oxide		1	9.2×10^{-5}
L *cis*-Stilbene oxide		5	17 ± 3
M Ethyl cinnamate		1	36 ± 1
N Ethyl glycidate		6	2.2×10^{-4}
O Benzoylphenylacetylene		7	31 ± 2
P 1,3-Diphenyl-3-(2-hydroxyethylthio)- propan-1-one		5	2.0×10^{-4}
Q 5-Phenyl-2,4-pentadienophenone		1	22 ± 2
R Dibenzalacetone		1	34 ± 5
S 4-Hydroxybenzylideneacetophenone		1	4.4×10^{-5}
T 4′-Hydroxychalcone		8	1.3×10^{-4}
U 9-Fluorenone		1	22 ± 2
V Flavanone		1	33 ± 3
W Flavone		1	1.7×10^{-4}

[a] Sources: 1, Aldrich; 2, Dow; 3, ICN/KNK; 4, MC & B; 5, synthesized in this study; 6, Pfaltz & Bauer; 7, gift from Zoecon; 8, Fairfield.

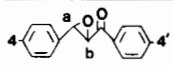
[b] Concentration (M) giving 50% inhibition (I_{50}) of mouse liver cytosolic epoxide hydrolase was determined by the partition assay as described under Materials and Methods. Otherwise, inhibition at inhibitor equal to substrate concentration of 5×10^{-4} M reported (mean ± SD for at least $n = 3$). Control activity was 58 ± 4 nmol/min-mg protein ($n = 9$).

[c] At 1.0×10^{-3} M.

[d] 4-Phenylchalcone gives 15 ± 4%.

TABLE II

PHYSICAL PROPERTIES AND INHIBITOR POTENCY OF THE CHALCONE OXIDES

 Chalcone oxide	Melting point			Inhibition (M) of cytosolic epoxide hydrolase[a]	
	Experimental	Literature	(ref.)	I_{50}	I_{90}
4-Substituted					
Phenyl	132	132	(52)	$6.4 \pm 1.2 \times 10^{-8}$	$6.0 \pm 0.6 \times 10^{-7}$
Isopropyl	75	76	(53)	$4.6 \pm 0.3 \times 10^{-7}$	$4.0 \pm 1.1 \times 10^{-6}$
Bromo	89.5	90	(17)	$9.0 \pm 1.4 \times 10^{-7}$	$8.5 \pm 3.5 \times 10^{-6}$
Methyl	76	76	(17)	$1.0 \pm 0.1 \times 10^{-6}$	$9.4 \pm 0.1 \times 10^{-6}$
Methoxy	84	85	(17)	$1.5 \pm 0.1 \times 10^{-6}$	$1.6 \pm 0.8 \times 10^{-5}$
Fluoro	89	89	(17)	$2.1 \pm 0.1 \times 10^{-6}$	$2.1 \pm 0.3 \times 10^{-5}$
Nitro	148	150	(24)	$3.9 \pm 0.1 \times 10^{-6}$	$4.0 \pm 0.4 \times 10^{-5}$
Acetamido	156	NC[b]	—	$8.8 \pm 0.8 \times 10^{-6}$	$8.6 \pm 1.3 \times 10^{-5}$
Hydrogen	88	89	(17)	$8.2 \pm 0.2 \times 10^{-6}$	$7.6 \pm 0.4 \times 10^{-5}$
4'-Substituted					
Phenyl	135	136	(52)	$7.6 \pm 0.1 \times 10^{-7}$	$6.6 \pm 1.3 \times 10^{-6}$
Methyl	85	85	(52)	$2.6 \pm 0.6 \times 10^{-6}$	$3.0 \pm 0.1 \times 10^{-5}$
Bromo	126	125	(54)	$2.8 \pm 0.1 \times 10^{-6}$	$2.9 \pm 0.2 \times 10^{-5}$
Nitro	101.5	103	(25)	$4.7 \pm 0.1 \times 10^{-6}$	$5.0 \pm 0.4 \times 10^{-5}$
Methoxy	77	79	(18)	$5.9 \pm 1.0 \times 10^{-6}$	$6.4 \pm 0.1 \times 10^{-5}$
Fluoro	85	83	(18)	$8.1 \pm 1.8 \times 10^{-6}$	$7.6 \pm 0.4 \times 10^{-5}$
Acetamido	155	NC	—	$1.8 \pm 0.5 \times 10^{-5}$	$1.7 \pm 0.1 \times 10^{-4}$

[a] The concentration (M) of inhibitor causing a 50% decrease (I_{50}) or a 90% decrease (I_{90}) in *trans*-β-ethylstyrene oxide (5×10^{-4} M final concentration) hydrolase activity in mouse liver cytosol was determined as in methods; $\bar{x} \pm$ SD for two experiments.

[b] New compound.

of the cytosolic epoxide hydrolase were noted in the two series of compounds (Table II), particularly for an enzyme in such a crude state of purity (100,000*g* supernatant). Clearly, the 4-substituted compounds are more potent than the 4'-substituted analogs as inhibitors of the cytosolic epoxide hydrolase, and in all cases the chalcone was a much poorer inhibitor ($I_{50} > 10^{-4}$ M or solubility, unpublished data) than the corresponding epoxide.

The probit lines for inhibition were remarkably parallel for all the chalcone oxides studies (slopes: 1.31 ± 0.05 probits/log concentration; range 1.23–1.38, $n = 16$), indicating that all compounds were interacting with a common catalytic site. Lack of antagonistic or synergistic activity for binary combinations of 4-isopropyl, 4-bromo, 4-nitro, and 4'-methoxychalcone oxides on cytosolic epoxide hydrolase of mouse liver (i.e., additive inhibition only, data not shown) again supports interaction with a common binding site.

Multiple Regression Analysis of Chalcone Oxides as Inhibitors of Epoxide Hydrolase

Numerous free-energy parameters [σ_p, σ_p^0, σ_p^-, σ_p^+, π, *MR*, E_s; cf. Ref. (29)] were considered for their role in improvement of the inhibitory potency of the chalcone oxide structure (Table II) via the addition of 4 and 4' substituents separately. For both the 4 and 4' series of chalcone oxides, highest correlations by single-parameter regression analysis for inhibition of the cytosolic epoxide hydrolase was obtained employing the hydrophobic parameter π (Fig. 1), with inhibition always increasing with hydrophobicity. Although electronic factors did not appear important ($r < 0.25$

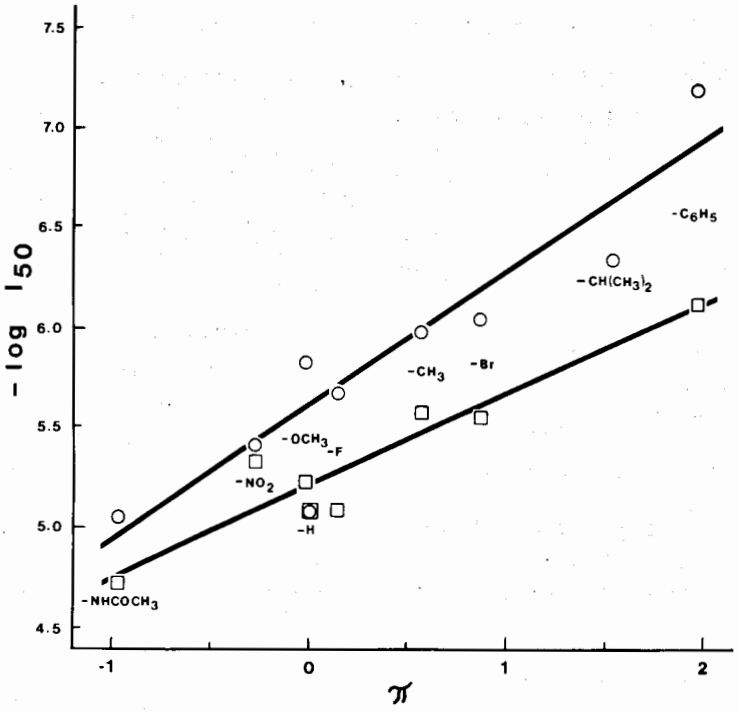


FIG. 1. Regression plots for the inhibitory potency of 4- or 4'-substituted chalcone oxides relative to the Hansch π parameter. Inhibitor concentration (M) resulting in a 50% decrease in mouse liver cytosolic epoxide hydrolase was determined as in methods for each 4-substituted (○) and 4'-substituted (□) chalcone oxide and is plotted on a negative log scale relative to the hydrophobic parameter π (29). The resulting regression lines are described more fully in the text.

for all σ terms), significant correlations were obtained with the steric terms E_s and MR ($r = 0.57$–0.68) and thus were examined further by multiple regression analysis.

Significant improvement ($p < 0.10$, F test) of the regression equation [1] for the 4-chalcone oxides was not obtained by addition of any parameter or appropriate square of the parameter listed above or their combination thereof up to four parameters per equation.

$$pI_{50} = 0.675\pi + 5.563,$$

$$r = 0.926, \quad s = 0.268, \quad n = 9. \quad [1]$$

There is some indication that molar refractivity, a constant denoting both steric bulk and polarizability (29, 30), might be important.

$$pI_{50} = 0.885\pi - 0.671\pi^2 + 0.0458MR + 0.0025MR^2 + 5.284$$

$$r = 0.980, \quad s = 0.189. \quad [2]$$

However, Eq. [2] does not give a more significant correlation than [1], [$F(3,4) = 3.39$; $p > 0.10$], perhaps a reflection of the small number (nine) of compounds analyzed.

In contrast to the 4-chalcone oxides, significant improvement ($p < 0.05$) to the regression Eq. [3],

$$pI_{50} = 0.455\pi + 5.212,$$

$$r = 0.947, \quad s = 0.145, \quad n = 8, \quad [3]$$

for 4'-chalcone oxides was realized with the addition of both the steric term E_s and a square π term [$F(2,4) = 7.57$] indicating that increasing bulk of the 4'-substituent improves binding of the chalcone oxide to the enzyme.

$$pI_{50} = 0.529\pi - 0.168\pi^2 - 0.175E_s + 5.060,$$
$$r = 0.990, \quad s = 0.0811. \qquad [4]$$

The addition of a fourth parameter to Eq. [4] only resulted in poorer correlations.

The substituent set chosen for structure-activity optimization in the two series of chalcone oxides possess free-energy parameters which are strictly noncolinear (π relative to *MR*, $r^2 = 0.29$; π to E_s, $r^2 = 0.18$) and aids in the assignment of hydrophobic and steric contributions to optimal inhibition of cytosolic epoxide hydrolase. However, the orthogonality of the *MR* and E_s set of constants ($r^2 = 0.74$) does not allow a clear discrimination of the two sets in this study.

Examination of Table II clearly demonstrates that the substituent order of potency in the two series of chalcone oxides is different. An explanation for this discrepancy is that two separate hydrophobic binding sites are being "titrated" for by these *trans*-oxiranes; one by the more potent *p*-substituted phenyl series, and the other by the *p*-substituted benzoyl series. Although hydrophobicity is the predominant factor in directing the binding of the *p*-substituted chalcone oxides to the catalytic site of the enzyme, multiple regression analysis does indicate divergent roles of other substituent terms (steric, polarizability, etc.) in the expression of inhibition for the two series of compounds.

Effect of Chalcone Oxides on Other Epoxide-Metabolizing Enzymes

A comparison of the effects chalcone oxides and a few other epoxides had on the activity of the three major epoxide-metabolizing enzymes of mouse liver was made (Table III) to verify the selectivity of the compounds. All the chalcone oxides studied were poor inhibitors for both the microsomal epoxide hydrolase and cytosolic glutathione *S*-epoxide transferase with I_{50} values greater than 4×10^{-4} M. The low solubility of some of the chalcone oxides within the assay mixture (e.g., 4-

TABLE III

INHIBITORY POTENCY OF VARIOUS EPOXIDES ON THE MAJOR EPOXIDE-METABOLIZING ENZYMES OF MOUSE LIVER[a]

Compound	I_{50} or highest percentage inhibition noted (M)		
	Epoxide hydrolase		Glutathione *S*-transferase
	Microsomal	Cytosolic	
Chalcone oxide	6.8×10^{-4}	8.2×10^{-6}	6.6×10^{-4}
4'-Acetamidochalcone oxide	4.7×10^{-4}	1.8×10^{-5}	4.5×10^{-4}
4-Phenylchalcone oxide	18 (2×10^{-4})	6.4×10^{-8}	26[b] (5×10^{-5})
3,3,3-Trichloropropene oxide	2.0×10^{-6}	1.6×10^{-4}[c]	0 (5×10^{-4})
1-(4'-Bromophenoxy)-6,7-epoxy-3,7-dimethyloctane	0 (1×10^{-5})	2.0×10^{-6}	—

[a] Mouse hepatic enzymes were prepared and assayed with and without (ethanol control) inhibitors as under Materials and Methods at the final substrate concentration of 5×10^{-4} M. Control activities (nmol diol formed/min-mg protein) with the indicated substrate were: Microsomal epoxide hydrolase, 7.8 (*cis*-stilbene oxide); cytosolic epoxide hydrolase, 61 (*trans*-β-ethylstyrene oxide); and cytosolic glutathione *S*-transferase, 66 (*trans*-β-ethylstyrene oxide). Data represent the average of two experiments for each enzyme activity.

[b] No further increase in inhibition was observed up to 2×10^{-4} M (apparent concentration).

[c] Identical results were obtained using either the partition or TLC (10) assays.

phenyl for cytosolic glutathione transferase, Table III) made I_{50} values unattainable, and distinct structure-activity relationships were not observed for the chalcone oxides toward these other epoxide-metabolizing enzymes. The 4'-acetamido-, 4-bromo, and 4'-methoxychalcone oxides were the best inhibitors for the microsomal enzyme (I_{50}'s = 4.7–5.8 × 10^{-4} M) in the series, while glutathione transferase inhibition was highest with the 4'-acetamido-, 4'-nitro- (I_{50} = 5.2 × 10^{-4} M), and unsubstituted chalcone oxides. In general, the chalcone oxides were weak inhibitors of the microsomal epoxide hydrolase and glutathione transferase, and interestingly the poorest inhibitor of the cytosolic epoxide hydrolase appeared to be the most potent for the other epoxide-metabolizing enzymes.

Trichloropropene oxide, a potent inhibitor of microsomal epoxide hydrolase (2), was only weakly inhibitory toward the cytoslic epoxide hydrolase (Table III) and had no effect on glutathione *S*-transferase probably because of its chemical reactivity with the substrate glutathione (3). A juvenoid epoxide known to be a good inhibitor of the cytosolic juvenoid epoxide hydrolase (15), 1-(4'-bromophenoxy)-6,7-epoxy-3,7-dimethyloctane, was also a strong inhibitor of *trans*-β-ethylstyrene

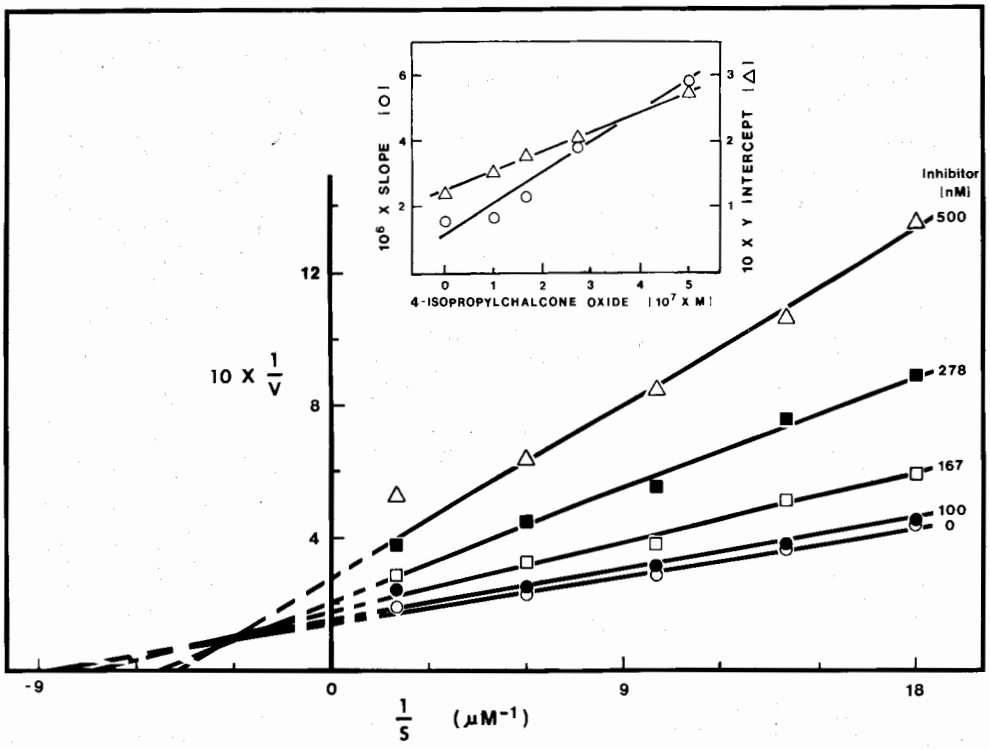


FIG. 2. Kinetic analysis for the inhibition of mouse liver cytosolic epoxide hydrolase by 4-isopropylchalcone oxide. Epoxide hydrolase activity was measured spectrophotometrically with the substrate *trans*-stilbene oxide. Lineweaver-Burk plots in the absence (K_m = 1.1 × 10^{-5} M; V_{max} = 6.9 nmol/min-mg protein) or presence of 4-isopropylchalcone oxide (ranging from 1 to 5 × 10^{-7} M) where each initial velocity (V) in nanomoles diol formed/min-mg protein (mean of three experiments of at least duplicate incubations) measured at five different substrate concentrations from 0.56 to 5 × 10^{-5} M are presented. A replot (inset) of the slopes (○) and y intercepts (△) of the corresponding regression lines relative to the inhibitor concentration was used to extract the additional dissociation constants described in the text. Protein concentration was 0.136 mg/ml incubation mix. At the highest apparent substrate concentration (5 × 10^{-5} M), some retardation of rates was noted, and the data were not included in calculations of kinetic constants.

oxide hydrolase, and again implicates a single cytosolic epoxide hydrolase with wide substrate specificity which is further supported by gel filtration studies (14, 22).

Nature of the Inhibition

To facilitate kinetic analysis of the inhibition of cytosolic epoxide hydrolase by chalcone oxides, a continuous assay based on the substrate *trans*-stilbene oxide[5] rather than a point assay with *trans*-β-ethylstyrene oxide was resorted to. With the former assay an I_{50} for 4-isopropylchalcone oxide of 2.7×10^{-7} M was obtained which is comparable to that from the radiometric assay (Table II, 4.6×10^{-7} M), and thus demonstrates the utility of the method.

Lineweaver-Burk plots for the inhibition of a representative chalcone oxide on cytosolic epoxide hydrolase (Fig. 2) suggests a noncompetitive (mixed-inhibition type) interaction of the inhibitor with the active site (i.e., site distinct from substrate binding site). Replotting (31) of both the slopes and y intercepts versus inhibitor concentration (Fig. 2) allows extraction of the dissociation constants for the enzyme-inhibitor [E · I] complex (K_1, 1.7×10^{-7} M) and the inhibitor from the enzyme-inhibitor-substrate [E · I · S] complex (K_2, 5.3

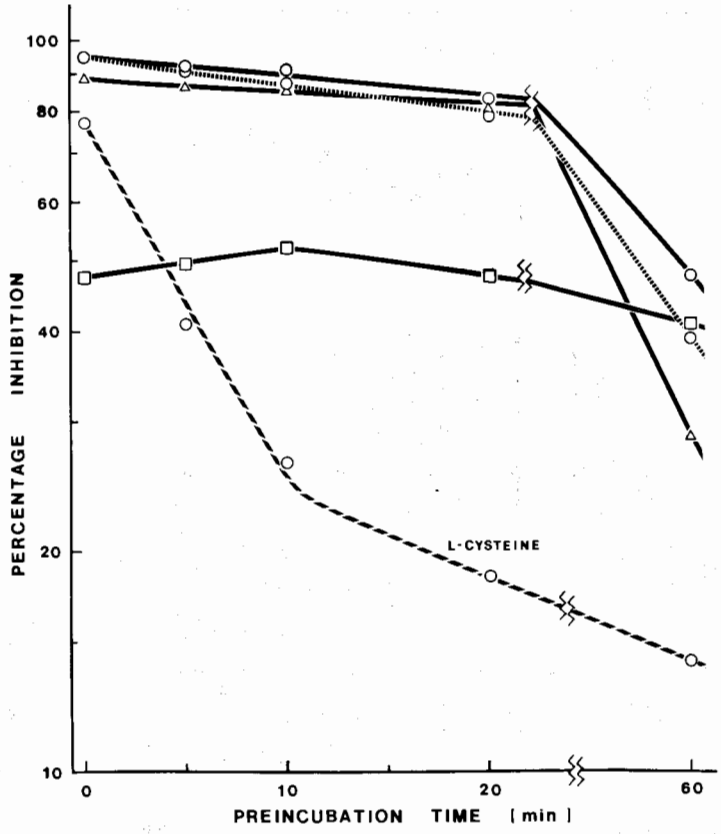


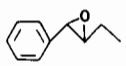
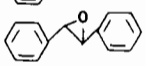
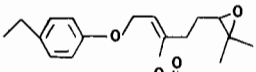
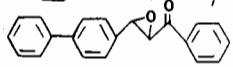
FIG. 3. Effect of enzyme and inhibitor preincubation on the inhibitory potency of chalcone oxides for cytosolic epoxide hydrolase: protective action of L-cysteine. Mouse liver cytosol (15 μg protein) was preincubated for various times (min) at 37°C with either 4-phenyl [(○), final concentration 8.2×10^{-7} M], 4-isopropyl [(△), 4.0×10^{-6}], or 4'-acetamido [(□), 2×10^{-5}] chalcone oxides in phosphate (——), Tris (· · ·), or phosphate containing 5 mM L-cysteine (- - -) buffers (I = 0.2, pH 7.2, 0.1 mM EDTA). Following preincubation, epoxide hydrolase activity was then measured in triplicate with *trans*-β-ethylstyrene oxide as described under Materials and Methods. Percentage inhibition (log scale) relative to identically preincubated controls lacking the chalcone oxide is presented.

$\times 10^{-7}$ M). Further analysis (31) gives the dissociation constant for the substrate from the ternary complex (K_3, 3.4×10^{-5} M) since the apparent K_m (1.1×10^{-5} M) is known. Whereupon K_1 and K_m are, respectively, threefold less than K_2 and K_3, ternary complex formation [E·I·S] will occur much less often than binary complex formation, [E·I] or [E·S], demonstrating that binding sites for S and I are not mutually exclusive. However, characterizing the extent of overlap of the binding and inhibitor sites for cytosolic epoxide hydrolase will require additional kinetic analysis on a purer enzyme. Indeed, the potency of the chalcone oxide inhibition ($I_{50} < 10^{-7}$ M) in such a crude enzyme preparation is more indicative of a tight binding inhibition (inhibitor ≃ enzyme concentration) where steady-state kinetic analysis does not adequately express the interaction of the enzyme and inhibitor (32). Data presented below (Fig. 3) provide support for a reversible covalent-type interaction.

For the mouse liver cytosolic epoxide hydrolase (Table IV), inhibition potency seems to be directly related to the affinity of the compound for the catalytic site of the enzyme (low I_{50}, low K_m), and the best inhibitors have low turnover rates (low V). However, the similarity in the V/K_m [proportional to the specificity constant k_{cat}/K_m, Ref. (31)] for *trans*-β-ethylstyrene oxide, *trans*-stilbene oxide, and the juvenoid epoxide R 20458 may indicate that cytosolic epoxide hydrolase is nonspecific, and will accommodate many substrates, but that turnover is more dependent on other rate-limiting factors. Indeed, the chalcone oxides, although extremely potent inhibitors for the enzyme, are reversible inhibitors and display a slow decrease in inhibition with time of exposure to the enzyme (Fig. 3), and disappear only very slowly from the reaction mixture when analyzed by TLC. The rate of decay in inhibitor potency with 4-phenylchalcone oxide was similar in both an anionic (phosphate, $t_{1/2}$ ≃ 62 min) and cationic (Tris, $t_{1/2}$ ≃ 49 min) buffer suggesting that ring opening of the epoxide by a chemical reaction with the buffer was not responsible for the inhibition decrease. A similar decay was noted with 4-isopropylchalcone oxide ($t_{1/2}$ ≃ 41 min, Fig. 3); however, a chalcone oxide such as the 4′-acetamido derivative which requires a much higher concentration to

TABLE IV

COMPARISON OF VARIOUS EPOXIDES AS INHIBITORS AND SUBSTRATES OF THE CYTOSOLIC EPOXIDE HYDROLASE[a]

Compound	I_{50}[b] (M)	V[c]	K_m (M)	V/K_m[d]
	5.8×10^{-4}	65[e]	7.2×10^{-5}[e]	15
	9.2×10^{-5}	6.9	1.1×10^{-5}	10
	1.2×10^{-5}	2.4[f]	2.0×10^{-6}[f]	20
	6.4×10^{-8}	—	—	—

[a] Apparent kinetic constants presented for hepatic cytosolic epoxide hydrolase of male Swiss-Webster mice.

[b] For *trans*-β-ethylstyrene oxide as substrate.

[c] V in units of nmol diol min^{-1} mg $protein^{-1}$.

[d] V/K_m in units of 1 s^{-1} kg $protein^{-1}$.

[e] From Ref. (10).

[f] From Ref. (15).

TABLE V

EFFECT OF VARIOUS THIOL GROUP MODIFIERS ON CYTOSOLIC EPOXIDE HYDROLASE ACTIVITY[a]

Sulfhydryl reagent	Percentage inhibition[b] 0.1 mM	1 mM
More polar		
3-Bromo-1,1,1-trifluoropropane	0	—
Iodacetamide	7	6
Diethyl maleate	4	3
N-Ethyl maleimide	14	33
More nonpolar		
Styrene oxide	14	49
5,5'-Dithiobis(2-nitrobenzoic acid)	22	55
2'-Bromo-6-nitroacetophenone	37	78
1,2-Epoxy-3,3,3-trichloropropane	42	92
Sodium *p*-hydroxymercuriphenylsulfonate	98	—
Sodium *p*-chloromercuriphenylsulfonate	97	—

[a] Inhibition measured at a substrate concentration of 0.5 mM. The amino group modifier sodium picryl sulfonate had little effect (3%, 0.1 mM; 27%, 1 mM).

[b] Mean of three to nine determinations.

attain the same degree of inhibition has a considerably lower decay rate ($t_{1/2} \simeq 240$ min). One possible explanation of these results is that the chalcone oxide is slowly being converted by the hydrolase to a noninhibitory product. Attempts to isolate a degradation product of the reaction with 4-phenylchalcone oxide (long-term batch incubations) have to date failed, but the expected α-keto-1,2-diol of the reaction is not a chemically known derivative of the chalcone oxides and predictably would be very unstable.

The decrease in inhibition potency of 4-phenylchalcone oxide with time of preincubation with the enzyme in the absence of substrate ($t_{1/2} \simeq 62$ min) could be stimulated 10-fold by the inclusion of the thiol L-cysteine (Fig. 3, $t_{1/2} \simeq 6.2$ min). The role cysteine might play in the catalytic mechanism of the cytosolic epoxide hydrolase was further investigated with a selection of thiol-modifying reagents (Table V). The more nonpolar (i.e., hydrophobic binding) thiol group modifiers (e.g., phenylmercurials) were the strongest inhibitors out of the series of compounds. Also, the modifier styrene oxide, a widely studied substrate of microsomal epoxide hydrolase (2), was mildly inhibitory. Amino group selective reagents such as sodium picrylsulfonate had little effect. This is strong evidence that the chalcone oxides are interacting with an essential cysteine residue in the active site of cytosolic epoxide hydrolase.

Cytosolic epoxide hydrolase activity was very sensitive to the solvent used to dispense the substrate into the incubation mixture when substrates with relatively high K_m's were used (Table VI). Introduction of more nonpolar water-miscible solvents to the assay would result in marked inhibition of the enzyme activity, with the effect being more pronounced with decrease in the dielectric constant of the incubation mix.

DISCUSSION

Chalcone oxides possess all the structural features that appear optimal for inhibitory activity on cytosolic epoxide hydrolase, i.e., two aromatic hydrophobic substituents orienting in a *trans* manner from a reactive electrophilic center. The epoxide function is crucial to the marked activity of this chalcone derivative. However, various olefins such as the chalcones (Table I, **F**, **S**, **T**) and the *trans*-cinnamate ester (Table I, **M**) had substantial inhibitory activity and could be metabolically epoxidized to the more inhibitory structure. Indeed, hydroxylated chalcones are important if not key intermediates in biosynthetic pathways leading to the flavonoids (16, 33) and thus have wide occurrence in plants along with the ubiquitous *trans*-cinnamates. The chalcone oxide has been suggested as the possible reactive form of the chalcone in flavonoid biosynthesis (16). Natural and synthetic chalcones have a wide spectrum of biological activity including antihelmintic (34) and molluscacidal (35) properties, and are for example potent inhibitors of 3,4-dihydroxyphenylalanine decarboxylase (36). Flavone in this study (Table I, **W**) was a good inhibitor of cytosolic epoxide hydrolase; numerous other toxicologically important activities have been associated with flavones and their derivatives in mammals (35, 37). Certainly, there is need

TABLE VI

SOLVENT EFFECTS OF CYTOSOLIC EPOXIDE HYDROLASE

Solvent	10 × Concentration (M) in incubation[a]	Percentage inhibition[b]	Inhibitory potency[c]	ϵ[d]
Methanol	4.75	7	1.0	32.6
Ethanol	3.28[e]	13	2.5	24.3
Dimethyl sulfoxide	2.71	18	4.2	49
Acetonitrile	3.63	39	7.0	36.2
Acetone	2.62	32	7.6	20.7
Dimethylformamide	2.47	32	8.2	36.7
Isopropanol	2.50	58	15	18.3
Dioxane	2.26	56	16	2.21
Tetrahydrofuran	2.37	66	18	7.58
Ethyl acetate	1.94[f]	53	18	6.02

[a] Concentration resulting from adding 1 μl solvent to standard incubation mix of 51 μl.

[b] Mouse liver cytosolic epoxide hydrolase was measured in Tris–HCl (I = 0.2, pH 7.4) buffer using the partition assay; and inhibition relative to the ethanol control (1 μl in 51 μl final volume) for solvent introduction is presented.

[c] (Percentage inhibition ÷ 10) ÷ log C, where C = concentration of solvent (dM).

[d] Dielectric constants from National Bureau of Standards; values at 25°C.

[e] Dose not include ethanol present in standard incubation mix.

[f] Assuming miscibility at this solvent level.

to examine the consequences of nutritional flavonoids and their precursors on cytosolic epoxide hydrolase in mammals. Interestingly, ethyl glycidate (Table I, **N**) and its 3-methyl derivative are major components of synthetic strawberry and apple flavors, and can occur at up to 470 ppm in chewing gum (38).

Improvements to the inhibitory activity of the basic chalcone oxide structure were made by addition of 4- and 4′-substituents of increasing hydrophobic character and bulk. Hydrophobicity was the predominate factor in the two series of compounds screened here in conferring inhibitory potency on cytosolic epoxide hydrolase. Based on similar multiple factor analyses, hydrophobic bonding was also found important for substrate and inhibitor interactions with other toxicologically important enzymes including cytochrome *P*-450 (39, 40) and cholinesterases (41). Directed synthesis in this study led to 4-phenylchalcone oxide which is the most potent inhibitor of any epoxide-metabolizing enzyme noted to date. Furthermore, the benzoyl and phenyl portions of these *trans*-epoxides are apparently interacting with two distinct binding sites on the cytosolic epoxide hydrolase since both the regression equation and substituent order of potency differ for the two series of compounds. Multiple regression analysis also revealed additional factors (steric and polarizability) which appear important in optimal inhibitory interaction with cytosolic epoxide hydrolase. Electronic factors were unimportant. Nevertheless, additional chalcone oxides incorporating a wider diversity of substituents together with combinations of substituents must be examined before a fuller expression of this binding interaction can be ascertained and the above trends verified. Ultimately, a purified enzyme will be required to divorce alternative binding sites from the analysis. Further directed synthesis toward optimization of the chalcone oxide structure should provide both an extremely potent *in vitro* and perhaps an effective *in vivo* inhibitor for cytosolic epoxide hydrolase.

Chalcone oxides were only weak inhibitors of the other major epoxide-metabolizing enzymes in mouse liver (Table III) with the more polar compounds (e.g., 4′-acetamido) being the most effective for

microsomal epoxide hydrolase and cytosolic glutathione S-epoxide transferase. However, this trend may be due, in part, to water solubility. Conversely, trichloropropene oxide, a strong inhibitor of microsomal epoxide hydrolase (11), was only moderately inhibitory for the cytosolic enzyme and inactive on the transferase. Indeed, for a juvenoid epoxide substrate with high binding affinity for the cytosolic enzyme, trichloropropene oxide is essentially noninhibitory (8). The specificity and potency of these inhibitors should thus aid in defining the functional role of each epoxide-metabolizing enzyme in the tissue sources where they concur.

Chalcone oxide has been reported as a strong *in vitro* stimulator of microsomal epoxide hydrolase (19, 20) of rat liver using styrene oxide as substrate and the substrate solvent acetonitrile. However, only weak inhibition was noted for any of the *p*-substituted chalcone oxides studied here including the parent epoxide (Table III). Perhaps the contradicting observations are the result of the differing assay conditions used here (mouse liver, *cis*-stilbene oxide, ethanol).

Inhibitory potency for cytosolic epoxide hydrolase generally increases with increasing lipophilic character of the substituted oxirane [Tables II and IV; Ref. (15)] with order of potency being trisubstituted > *trans*-1,2-disubstituted > *cis*-disubstituted oxiranes [cf. (15)]. The opposite trend is reported for microsomal epoxide hydrolase (11). Synthesis of tri- or tetra-substituted analogs of the chalcone epoxides may provide even more potent compounds than those developed here. Furthermore, substrates with high initial hydration rates tend to be poorer inhibitors of the cytosolic epoxide hydrolase [this study, (15)], however this is not necessarily the case with the microsomal enzyme (11).

Clearly, substrate binding differences are apparent between the cytosolic and microsomal epoxide hydrolases with hydrophobic binding being less critical for the efficacy of the active site of microsomal epoxide hydrolase (6, 7); however, optimal hydration by cytosolic epoxide hydrolase often requires hydrophobic interaction between the enzyme and substrate on both sides of the epoxide moiety [Table IV, Ref. (15)] with *trans*-β-propylstyrene oxide being the best substrate known to date.[5] Another indication of the critical nature of hydrophobic binding in the efficacy of enzymatic hydration is the marked susceptibility of the enzymatic hydration of t-β-ethylstyrene oxide to inhibition by organic solvents (Table VI) of decreasing polarity presumably due to disruption of hydrophobic interactions between the enzyme and substrate. In contrast, many of the same solvents either have no effect or increase microsomal epoxide hydrolase activity (7, 20).

A steady-state kinetic analysis for the inhibition of cytosolic epoxide hydrolase by chalcone oxides suggests a noncompetitive (mixed)-type interaction whereupon considerable overlap of the substrate and inhibitor binding sites is occurring. Thus, species where both the substrate and inhibitor are enzyme bound will be infrequent. However, the potency of some of the chalcone oxides particularly within a crude enzyme preparation where many competing nonspecific interactions might occur, is more consistent with a tight-binding or covalent model for the inhibition (32). Establishing the inherent kinetics of this interaction will require a pure enzyme. Nevertheless, this inhibition is reversible (Fig. 3), and together with the apparent nonspecificity of cytosolic epoxide hydrolase further implies that chalcone oxides may be alternative substrates with extremely low turnover; hence be site-specific reagents [affinity label, cf. (42)].

Amino acid modifiers (42), particularly hydrophobic thiol reagents such as the phenylmercurials, were strong inhibitors of the cytosolic epoxide hydrolase, although the amino group modifiers sodium picrylsulfonate and 2′-bromo-6-nitroacetophenone and also the serine modifier DFP (14) were less potent or without effect. The converse has been observed for the microsomal epoxide hydrolase (43) where an essential histidine has been implicated at the active site. Thus, a cysteine

residue may be necessary for the catalytic action of cytosolic epoxide hydrolase. The strong inhibition observed with copper salts (14), potent complexers of inorganic sulfide, is consistent with this model. In fact the inhibition of the cytosolic epoxide hydrolase by a variety of inorganic cations correlates well with the solubility product constants of their sulfide complexes. Moreover, ring-opening reactions of epoxides preferentially occur with attack of "soft" nucleophiles (44) such as thiols and amines, and with the chalcone oxides a rapid benzylic attack results to give the corresponding 2-hydroxy adduct (45, 46). Due to this alkylating ability, epoxides such as styrene oxide (cf. Table V) are useful as active-site-directed cysteine and methionine modifiers (42, 47). This inhibition explains the lack of styrene oxide metabolism by the cytosolic epoxide hydrolase at high substrate concentrations (9), although it is rapidly hydrated at low substrate concentrations. Chalcones, similarly, react with soft bases (26, 48) as do other biologically active α,β-unsaturated ketones (49) to give Michael adducts. Sulfur anions are more reactive than amino groups in Michael additions, and the reactions are both thermally and chemically reversible (26, 49). In addition, electron-donating substituents in the benzoyl ring stabilize the interaction of soft bases with the chalcone oxide structure (45, 46). Appropriately, 4-hydroxychalcone (Table I, **S**) was at least 10-fold more inhibitory than chalcone, and this potency was decreased 5-fold through formation of the Michael adduct with 2-mercaptoethanol. Indeed, a high concentration of L-cysteine (Fig. 3) markedly protected cytosolic epoxide hydrolase from the effect of even the strongest inhibitor of the enzyme. Seemingly, chalcone oxides together with their olefin precursors are interacting covalently, but reversibly, with an essential cysteine residue at or near the active site of cytosolic epoxide hydrolase.

In summary, mammalian cytosolic epoxide hydrolase is inherently mechanistically distinct form the corresponding microsomal enzyme (8, 50). Some plausible structural features of the active site of the cytosolic enzyme are depicted in Fig. 4. Although the mechanism of hydration by both enzymes apparently proceeds by a "backside" nucleophilic addition of water at the less hindered oxirane carbon to give the *trans*-diol (2, 3, 6, 8, 50), the cytosolic enzyme has two distinct hydrophobic binding sites [cf. (15)], a wide substrate specificity with less steric requirements (8, 9, 15), and a catalytically essential cysteine which could be an effective general-base catalyst at the neutral pH optimum (9, 10) of the enzyme. In contrast, microsomal epoxide hydrolase features a more sterically restrictive active site with less essential hydrophobic interactions and a histidine serving as the general-base catalyst (6, 7, 43). These data further support the hypothesis that the microsomal and cytosolic epoxide hydrolases in normal mouse liver are distinctly different enzymes. Moreover, the cytosolic and microsomal epoxide hydrolases of mammalian liver appear immunologically distinct (51).

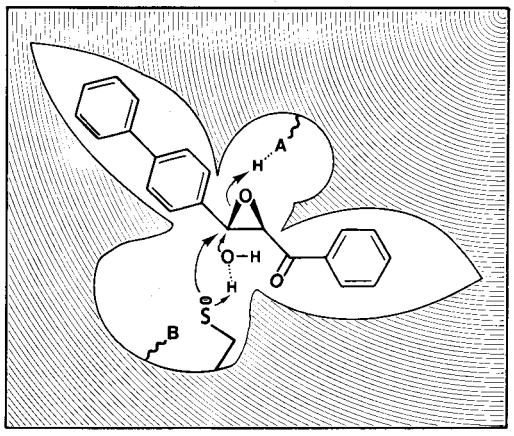


FIG. 4. Proposed active-site topography for the interaction of chalcone oxide inhibitors with the cytosolic epoxide hydrolase. The representative inhibitor or possible substrate 4-phenylchalcone oxide is depicted interacting with the two hydrophobic regions extending from the active core of the enzyme. An essential cysteine in its thiolate form and a possible specific acid catalyst **A** and general base catalyst **B** are also included. Additional studies are needed to fully elucidate the topography of the active site since the hydrolase rapidly turns over some, but not all *cis*-disubstituted epoxides, while having a greater affinity for *trans*-substituted epoxides. See text for further details.

In conclusion, studies with chalcone oxides, active-site-directed inhibitors of cytosolic epoxide hydrolase, have contributed substantially to "mapping" the catalytic site of this epoxide scavenging enzyme and should aid in elucidating the functional role of the enzyme in living systems.

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REFERENCES

1. HAMMOCK, B. D., AND QUISTAD, G. B. (1981) *in* Progress in Pesticide Biochemistry (Hutson, D. H., and Roberts, T. R., eds.), Vol. 1, pp. 1–83, Wiley, New York.
2. GUENTHNER, T. M., AND OESCH, F. (1981) *in* Polycyclic Hydrocarbons and Cancer (Gelboin, H. V., and Ts'o, P. O. P., eds.), Vol. 3, pp. 183–212, Academic Press, New York.
3. LU, A. Y. H., AND MIWA, G. T. (1980) *Annu. Rev. Pharmacol. Toxicol.* **20,** 513–531.
4. BROOKS, G. T. (1977) *Gen. Pharmacol.* **8,** 221–226.
5. CHASSEAUD, L. F. (1979) *Adv. Cancer Res.* **29,** 175–274.
6. ARMSTRONG, R. N., LEVIN, W., AND JERINA, D. M. (1980) *J. Biol. Chem.* **255,** 4698–4705.
7. MULLIN, C. A., AND WILKINSON, C. F. (1980) *Pest. Biochem. Physiol.* **14,** 192–207.
8. HAMMOCK, B. D., GILL, S. S., MUMBY, S. M., AND OTA, K. (1980) *in* Molecular Basis of Environmental Toxicity (Bhatnagar, R. S., ed.), pp. 229–272, Ann Arbor Science Publ., Ann Arbor, Mich.
9. OTA, K., AND HAMMOCK, B. D. (1980) *Science* **207,** 1479–1481.
10. MULLIN, C. A., AND HAMMOCK, B. D. (1980) *Anal. Biochem.* **106,** 476–485.
11. OESCH, F., KAUBISCH, N., JERINA, D. M., AND DALY, J. W. (1971) *Biochemistry* **10,** 4858–4866.
12. SLADE, M., BROOKS, G. T., HETNARSKI, H. K., AND WILKINSON, C. F. (1975) *Pest. Biochem. Physiol.* **5,** 35–46.
13. HANZLIK, R. P., AND WALSH, J. S. A. (1980) *Arch. Biochem. Biophys.* **204,** 255–263.
14. GILL, S. S., AND HAMMOCK, B. D. (1980) *Biochem. Pharmacol.* **29,** 389–395.
15. MUMBY, S. M., AND HAMMOCK, B. D. (1979) *Pest. Biochem. Physiol.* **11,** 275–284.
16. WONG, E. (1976) *in* Chemistry and Biochemistry of Plant Pigments (Goodwin, T. W., ed.), pp. 464–526, Academic Press, New York.
17. TURNER, A. B., LUTZ, R. E., MCFARLANE, N. S., AND BOYKIN, D. W., JR. (1971) *J. Org. Chem.* **36,** 1107–1112.
18. WEBER, F. G., AND LIEPERT, H. (1980) *Z. Chem.* **20,** 22–23.
19. GANU, V. S., AND ALWORTH, W. L. (1978) *Biochemistry* **17,** 2876–2881.
20. SEIDEGÅRD, J., AND DEPIERRE, J. W. (1980) *Eur. J. Biochem.* **112,** 643–648.
21. GILL, S. S., AND HAMMOCK, B. D. (1981) *Biochem. Pharmacol.*, **30,** 2111–2120.
22. EL-TANTAWY, M. A., AND HAMMOCK, B. D. (1980) *Mutat. Res.* **79,** 59–71.
23. DAVEY, W., AND GWILT, J. R. (1957) *J. Chem. Soc.*, 1008–1014.
24. WEITZ, E., AND SCHEFFER, A. (1921) *Chem. Ber.* **54,** 2327–2344.
25. VERESHCHAGIN, A. N., VUL'FSON, S. G., DONSKOVA, A. I., AND SAVIN, V. I. (1974) *Dokl. Akad. Nauk. SSSR* **215,** 339–342.
26. ALLEN, C. F. H., FOURNIER, J. O., AND HUMPHLETT, W. J. (1964) *Canad. J. Chem.* **42,** 2616–2620.
27. MULLIN, C. A., AND WILKINSON, C. F. (1980) *Insect Biochem.* **10,** 681–691.
28. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
29. HANSCH, C., AND LEO, A. (1979) Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley, New York.
30. HANSCH, C., SMITH, R. N., ROCKOFF, A., CALEF, D. F., JOW, P. Y. C., AND FUKUNAGA, J. Y. (1977) *Arch. Biochem. Biophys.* **183,** 383–392.
31. ROBERTS, D. V. (1977) Enzyme Kinetics, pp. 48–82, Cambridge Univ. Press, Cambridge/London.
32. WILLIAMS, J. W., AND MORRISON, J. F. (1979) *in* Methods in Enzymology (Purich, D. L., ed.), Vol. 63, pp. 437–467, Academic Press, New York.
33. SÜTFELD, R., AND WIERMANN, R. (1980) *Arch. Biochem. Biophys.* **201,** 64–72.
34. LALIBERTÉ, R., CAMPBELL, D., AND BRUDERLEIN, F. (1967) *Canad. J. Pharmac. Sci.* **2,** 37–43.
35. MABRY, T. J., AND ULUBELEN, A. (1980) *J. Agr. Food Chem.* **28,** 188–196.
36. HARTMAN, W. J., AKAWIE, R. I., AND CLARK, W. G. (1955) *J. Biol. Chem.* **216,** 507–529.
37. BUENING, M. K., CHANG, R. L., HUANG, M. -T.,

FORTNER, J. G., WOOD, A. W., AND CONNEY, A. H. (1981) *Cancer Res.* **41,** 67–72.

38. FURIA, T. E., AND BELLANCA, N. (1971) Feraroli's Handbook of Flavor Ingredients, pp. 531, 395, Chemical Rubber Co., Cleveland, Ohio.
39. WILKINSON, C. F., HETNARSKI, H. K., CANTWELL, G. P., AND DI CARLO, F. J. (1974) *Biochem. Pharmacol.* **23,** 2377–2386.
40. BLAKE, R. C., II, AND COON, M. J. (1980) *J. Biol. Chem.* **255,** 4100–4111.
41. WUSTNER, D. A., SMITH, C., AND FUKUTO, T. R. (1978) *Pest. Biochem. Physiol.* **9,** 281–292.
42. GLAZER, A. N. (1976) *in* The Proteins (Neurath, H., and Hill, R. L., eds.), Vol. 2, pp. 1–103, Academic Press, New York.
43. DUBOIS, G. C., APPELLA, E., LEVIN, W., LU, A. Y. H., AND JERINA, D. M. (1978) *J. Biol. Chem.* **253,** 2932–2939.
44. KAYSER, M. M., AND MORAND, P. (1980) *Canad. J. Chem.* **58,** 302–306.
45. SEMENOVA, S. N., EL SADANI, S. K., KARAVAN, V. S., AND TEMNIKOVA, T. I. (1978) *Zh. Org. Khim.* **14,** 1268–1273.
46. CHANDA, B. K., KAR, J. N., BEHERA, G. B., ROUT, M. K. (1971) *J. Indian Chem. Soc.* **48,** 867–871.
47. KLINMAN, J. P., WELSH, K. M., AND HOGUE-ANGELETTI, R. (1977) *Biochemistry* **16,** 5521–5527.
48. MENGER, F. M., AND SMITH, J. H. (1969) *J. Amer. Chem. Soc.* **91,** 4211–4216.
49. KUPCHAN, S. M., GIACOBBE, T. J., KRULL, I. S., THOMAS, A. M., EAKIN, M. A., AND FESSLER, D. C. (1970) *J. Org. Chem.* **35,** 3539–3543.
50. HAMMOCK, B. D., RATCLIFF, M., AND SCHOOLEY, D. A. (1980) *Life Sci.* **27,** 1635–1641.
51. GUENTHNER, T. M., HAMMOCK, B. D., VOGEL, U., AND OESCH, F. (1981) *J. Biol. Chem.* **256,** 3163–3166.
52. KOROTKOV, S. A., ORLOV, V. D., AND LAVRUSHIN, V. F. (1972) *Visn. Kharkiv. Univ. Khim.* **84,** 72–75.
53. BODFORSS, S. (1916) *Chem. Ber.* **49,** 2795–2813.
54. SIPOS, GY., SCHÖBEL, GY., AND BALÁSPIRI, L. (1970) *J. Chem. Soc. C,* 1154–1156.