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Inhibition of Epoxide Hydrolases and Glutathione S-Transferases by 2-, 3-, and 4-Substituted Derivatives of 4'-Phenylchalcone and Its Oxide[1]

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4'-Phenylchalcones, chalcone oxides, and related compounds were synthesized and tested as inhibitors of cytosolic epoxide hydrolase, microsomal epoxide hydrolase, and glutathione *S*-transferases from mouse and rat liver. Several compounds were more potent inhibitors of the cytosolic epoxide hydrolase than the parent 4'-phenylchalcone oxide while large substituents in the 4- and especially the 2-position caused a reduction in inhibition. The chalcone oxides showed selectivity as inhibitors of the cytosolic epoxide hydrolase acting on *trans*-stilbene oxide, while chalcones were inhibitors of cytosolic glutathione *S*-transferase acting on *cis*-stilbene oxide. Data are consistent with the hypothesis that much of the inhibition of the glutathione *S*-transferase is caused by the glutathione conjugate of the chalcone.

The extremes of reactivity demonstrated by the epoxide functionality explain, in part, its interest to biochemists. Some epoxides are so stable that they are potential environmental contaminants and may bioaccumulate in the food chain. Typical of such an environmentally persistent epoxide is heptachlorepoxide. At the other extreme of reactivity, some epoxides are but hypothetical reaction intermediates which have yet to be isolated such as the epoxide of aflatoxin B_1. Between these extremes of reactivity lie the vast number of natural epoxides, important industrial and biosynthetic intermediates, and potent chemical mediators (1).

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Epoxides are most notorious for their ability to alkylate biological nucleophiles. For epoxides to present such toxic risks, they must be of the appropriate reactivity and possess the physical properties which will bring them into contact with their biological target. Since epoxides are dietary constituents as well as intermediates in the conversion of olefinic and aromatic xenobiotics to hydrophilic products, an understanding of their metabolism is essential. They appear to be metabolized by two major epoxide hydrolases (EC 3.3.2.3) and a family of glutathione *S*-transferases (EC 2.5.2.18) (1-7), but important to understanding epoxide metabolism is the development of potent, selective inhibitors for the enzymes involved. Such inhibitors will facilitate an evaluation of the role of epoxide metabolism *in vitro* and *in vivo* and may lead to compounds of therapeutic or agricultural significance. Such inhibitors also may act as probes to investigate the mechanism and catalytic site of the enzymes involved.

To this end we followed the observation that some chalcones could inhibit gluta-

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thione *S*-transferase activity and that some chalcone oxides were inhibitors of the cytosolic epoxide hydrolase activity (8–11). However, these compounds are not highly active *in vivo*. Thus, we have undertaken a synthetic program to optimize inhibitory potency. In this manuscript we report the relative inhibitory potency of a variety of chalcones and chalcone oxides on epoxide-metabolizing enzymes. Most of the compounds are derivatives of 4′-phenylchalcone. We also report initial observations on the mechanism by which chalcone derivatives inhibit glutathione *S*-transferases.

MATERIALS AND METHODS

Analytical TLC[5] was performed on 0.20-mm precoated silica gel $60F_{254}$ plastic sheets (EM reagents), and compounds were detected by uv light (254 nm) 0.5% palladium chloride in diluted hydrochloric acid ($PdCl_2$ reagent) and/or iodine vapor (12). ir Spectra were determined with a Beckman Model 4240 spectrophotometer. Important diagnostic peaks are reported. ^1H NMR spectra were measured with a Varian EM-390 90-MHz NMR spectrometer or a Bruker WM 300 using tetramethylsilane as an internal standard. MS spectra were obtained on a V.G.ZAB-2F with a VG 11-250 data system.

Chemicals. The chemical structures and designations of compounds used in the present study are shown in Fig. 1, and their designations are described in the legend. Olefin **H,** 4′-phenylchalcone, was prepared by Claisen–Schmidt reaction of 4-acetylbiphenyl with benzaldehyde. To a mixture of 4.0 g (19.9 mmol) of 4-acetylbiphenyl (97%) and 2.3 g (22.0 mmol) of benzaldehyde in 50 ml of ethanol was added dropwise with stirring 13.1 ml of 3 N sodium hydroxide solution at room temperature over 5 min; then stirring was continued for 3 h. The mixture was acidified with 3 N hydrochloric acid, and the precipitate was collected by filtration and then recrystallized from ethanol to obtain 3.7 g of olefin **H** in a 65.2% yield based on 4-acetylbiphenyl (mp 159.5–160). Anal. Calcd for $C_{21}H_{16}O$: C, 88.70; H, 5.67. Found: C, 88.27; H, 5.53. A summary of IR and NMR spectral data is shown in Table I.

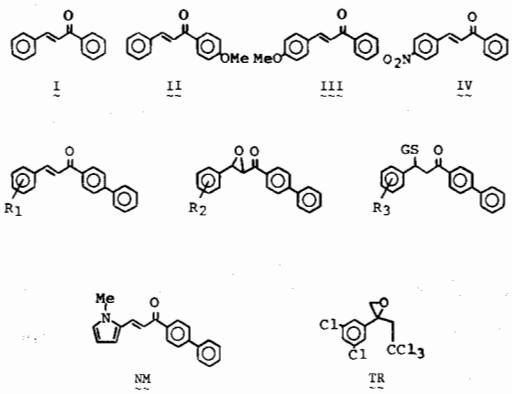


FIG. 1. Chemical structures and designations of compounds used in the present study. Roman numerals identify the first three compounds in the figure. The R groups on the remaining compounds are indicated by the code shown below with a number indicating the position of the substituent on the phenyl ring and an abbreviation for the substituent on the chalcone nucleus (left). The same system followed by E is used for epoxides of chalcones (center) while GS indicates glutathione conjugates (right). **I,** chalcone; **II,** 4′-methoxychalone; **III,** 4-methoxychalcone; **IV,** 4-nitrochalcone; **H,** R_1 = H; **2F,** R_1 = *o*-F; **3F,** R_1 = *m*-F; **4F,** R_1 = *p*-F; **2C,** R_1 = *o*-Cl; **2B,** R_1 = *o*-Br; **3B,** R_1 = *m*-Br; **4B,** R_1 = *p*-Br; **2MO,** R_1 = *o*-CH_3O; **3MO,** R_1 = *m*-CH_3O; **4MO,** R_1 = *p*-CH_3O; **2M,** R_1 = *o*-CH_3; **2T,** R_1 = *o*-CF_3; **3T,** R_1 = *m*-CF_3; **4T,** R_1 = *p*-CF_3; **3OH,** R_1 = *m*-OH; **4Ph,** R_1 = *p*-C_6H_5; **HE,** R_2 = H; **2FE,** R_2 = *o*-F; **3FE,** R_2 = *m*-F; **4FE,** R_2 = *p*-F; **2BE,** R_2 = *o*-Br; **3BE,** R_2 = *m*-Br; **4BE,** R_2 = *p*-Br; **2MOE,** R_2 = *o*-CH_3O; **3MOE,** R_2 = *m*-CH_3O; **4MOE,** R_2 = *p*-CH_3O; **2TE,** R_2 = *o*-CF_3; **3TE,** R_2 = *m*-CF_3; **4TE,** R_2 = *p*-CF_3; **GS-3B,** R_3 = *m*-Br; **GS-4B,** R_3 = *p*-Br; **TR,** tridiphane.

Epoxide **HE,** 4′-phenylchalcone oxide, was prepared by epoxidation of olefin **H** with hydrogen peroxide. To a mixture of 1.0 g (3.5 mmol) of olefin **H** and 1.1 ml of 5 N sodium hydroxide solution in 30 ml of ethanol was added dropwise with stirring 5 ml of hydrogen peroxide (30%) at room temperature over 2.5 h. After the mixture was filtered *in vacuo*, the precipitate was recrystallized from ethanol:benzene (4:1) to obtain 562 mg of epoxide **HE** in a 52.8% yield based on olefin **H** (mp 144.5–145). Anal. Calcd for $C_{21}H_{16}O_2$: C, 83.97; H, 5.37. Found: C, 78.23; H, 4.80. A summary of IR and NMR spectral data is shown in Table II.

Other olefinic and epoxide containing compounds except compounds **I** to **IV** as shown in Fig. 1 also were synthesized in the same manner described above with

[5] Abbreviations used: TLC, thin-layer chromatography; uv, ultraviolet; PMR, proton magnetic resonance; MS, mass spectrophotometer; IR, infrared red; GSH, reduced glutathione; THF, tetrahydrofuran; DW, distilled water; R_f, relative mobility; MHz, megahertz; FAB, fast atom bombardment; cEH, cytosolic epoxide hydrolase; cGST, cytosolic glutathione S-transferase; mEH, microsomal epoxide hydrolase; TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; DEM, diethylmaleate; ppm, parts per million.

TABLE I

IR AND NMR SPECTRAL DATA OF SELECTED CHALCONES

Compound	IR[a] (cm^{-1})	NMR (δ)
H	1664, 1607, 1594	7.21–7.93 (m, 14H), 807 (d, 2H)[b]
2F	1664, 1608, 1597, 1485	—
2B	1664, 1607, 1598	7.21–8.34 (m)[c]
2MO	1657, 1603, 1583, 1485	3.88 (S, 3H, CH_3O), 6.83–8.27 (m, 15H)[b]
2T	1670, 1611, 1599, 1489	—
3F	1664, 1613, 1601, 1579	—
3B	1664, 1609, 1596	7.29–8.42 (m)[c]
3MO	1660, 1605, 1597, 1496	3.82 (S, 3H, CH_3O), 6.83–7.93 (m, 13H), 8.08 (d, 2H)[b]
3T	1660, 1605, 1592	—
4F	1659, 1608, 1594, 1586, 1509	—
4B	1663, 1611, 1602	7.35–8.38 (m)[c]
4MO	1661, 1599, 1511	3.81 (S, 3H, CH_3O), 6.93 (d, 2H), 7.22–7.97 (m, 11H), 808 (d, 2H)[b]
4T	1657, 1608, 1596	—

[a] The spectra were determined on nujol mulls.

[b] The spectra were obtained in chloroform-d solution.

[c] The spectra were measured in a mixture of DMSO-d_6 and acetone-d_6.

purification by repeated crystallization and/or flash chromatography. IR, NMR, and MS spectral data are shown in Tables I–III (see also Refs. (8-11) and included references for related syntheses). All compounds were judged pure by the absence of signals for starting materials on IR or NMR, sharp melting points, and the appearance of only one spot on TLC in at least two systems.

Glutathione conjugates of olefins **3B** and **4B** (**GS-3B** and **GS-4B**) were prepared by reaction of the corresponding olefin with reduced glutathione in borate buffer under inert atmosphere. To a mixture of 1.16 g (3.19 mmol) of olefin **4B** and 1.07 g (3.48 mmol) of reduced glutathione (GSH) in 95 ml of tetrahydrofuran (THF) was added dropwise with stirring 95 ml

TABLE II

IR AND NMR SPECTRAL DATA OF CHALCONE OXIDES

Compound	IR[a] (cm^{-1})	NMR[b] (δ)
HE	1662, 1605	4.06 (d, 1H), 4.26 (d, 1H), 7.19–7.80 (m, 12H), 8.06 (d, 2H)[b]
2FE	1682, 1608, 1590, 1495	4.27 (d, 1H), 4.32 (d, 1H), 6.88–7.84 (m, 11H), 8.09 (d, 2H)[b]
2BE	1683, 1603	4.21 (d, 1H), 4.72 (d, 1H), 7.20–7.97 (m, 11H), 8.17 (d, 2H)[c]
2MOE	1680, 1602, 1590, 1499	3.78 (S, 3H, CH_3O), 4.14 (d, 1H), 4.34 (d, 1H), 6.78–7.07, 7.13–7.80 (m, 11H), 8.08 (d, 2H)
2TE		4.14 (d, 1H), 4.42 (d, 1H), 7.16–7.86 (m, 11H), 8.03 (d, 2H)
3FE	1687, 1605, 1595, 1497	4.06 (d, 1H), 4.21 (d, 1H), 6.87–7.78 (m, 11H), 8.05 (d, 2H)
3BE	1689, 1607, 1487	4.16 (d, 1H), 4.87 (d, 1H), 7.26–7.96 (m, 11H), 8.13 (d, 2H)[c]
3MOE	1687, 1607, 1587, 1499	3.79 (S, 3H, CH_3O), 4.04 (d, 1H), 4.24 (d, 1H), 6.75–7.05, 7.18–7.78 (m, 11H), 8.05 (d, 2H)
3TE		4.13 (d, 1H), 4.24 (d, 1H), 7.16–7.86 (m, 11H), 8.04 (d, 2H)
4FE	1663, 1606, 1514	4.04 (d, 1H), 4.21 (d, 1H), 6.93–7.80 (m, 11H), 8.05 (d, 2H)
4BE	1657, 1603	4.13 (d, 1H), 4.80 (d, 1H), 7.28–8.01 (m, 11H), 8.09 (d, 2H)[c]
4MOE	1662, 1603, 1512	3.79 (S, 3H, CH_3O), 4.01 (d, 1H), 4.25 (d, 1H), 6.88 (d, 2H), 7.18–7.78 (m, 9H), 8.06 (d, 2H)
4TE		4.13 (d, 1H), 4.22 (d, 1H), 7.16–7.83 (m, 11H), 8.04 (d, 2H)

[a] The spectra were determined on nujol mulls.

[b] The spectra of epoxides **2BE**, **3BE**, and **4BE** were obtained in DMSO-d_6 solution while the spectra of other epoxides were measured in chloroform-d solution.

TABLE III

LOW AND HIGH RESOLUTION MASS SPECTRAL (LRMS AND HRMS) CHALCONES AND CHALCONE OXIDES

Compound	LRMS	HRMS Calcd. for		HRMS Found
2B	362, 364, (M^+, 6%, 6%), 361, 363 (M-1, 4%, 7%), 283 (M-Br, 100%), 152 (22%)	$C_{21}H_{15}O^{79}Br$ $C_{21}H_{15}O^{81}Br$	(362.0306) (364.0286)	362.0304 364.0286
2MO	314 (M^+, 2%), 313 (M-1, 1%), 284 (31%), 283 (M-CH_3O, 100%), 152 (30%)	$C_{22}H_{18}O_2$	(314.1307)	314.1291
3MO	314 (M^+, 70%), 313 (m-1, 80%), 283 (M-CH_3O, 41%), 152 (30%)	$C_{22}H_{18}O_2$	(314.1307)	314.1285
4MO	314 (M^+, 62%), 313 (M-1, 75%), 299 (16%), 181 (17%), 152 (100%)	$C_{22}H_{18}O_2$	(314.1307)	314.1289
2BE	378, 380 (M^+, 1%, 0.7%), 377, 379 (M-1, 0.6%, 0.8%), 283 (M-Br-16, 37%), 181 (100%), 153 (23%), 152 (97%)	$C_{21}H_{15}O_2^{79}Br$ $C_{21}H_{15}O_2^{81}Br$	(378.0255) (380.0235)	378.0254 380.0225
2MOE	330 (M^+, 7%), 283 (M-CH_3O-16, 59%), 181 (100%), 153 (24%), 152 (75%)	$C_{22}H_{18}O_3$	(330.1256)	330.1278
3MOE	330 (M^+, 12%), 314 (M-16, 14%), 313 (M-16-1, 17%), 283 (18%), 181 (100%), 153 (25%), 153 (25%), 152 (92%)	$C_{22}H_{18}O_3$	(330.1256)	330.1243
4MOE	330 (M^+, 10%), 314 (M-16, 6%), 313 (M-16-1, 6%), 181 (100%), 152 (81%), 121 (22%)	$C_{22}H_{18}O_3$	(330.1256)	330.1255

of 100 mM sodium tetraborate (Borax, Sigma) buffer (pH 9.2) at room temperature over 45 min, and stirring was continued at room temperature for 190 min. After filtration of the precipitate, most THF was removed *in vacuo* at 35°C, and the remaining solvent was removed by lyophilization. The residue was triturated and washed repeatedly with a total of 100 ml of benzene and then extracted several times with a total of 40 ml of methanol. The methanol extract was mixed with distilled water (DW) to prepare a DW:methanol (3:1) mixture. A part of the mixture (each ca. 20 ml) was adsorbed on and then eluted from a SEP-PAC C_{18} cartridge (Waters Associates, Inc.), which had been previously washed with 30 ml of methanol, 30 ml of DW, and 15 ml of DW:methanol (3:1). DW:methanol (30 ml; 3:1) and DW:methanol (40 ml; 1:1) solutions were further passed through the cartridge, and 30 fractions were collected and analyzed by TLC. The eluate containing only a product with an R_f value of 0.44 (1-butanol:acetic acid:water, 3:1:1) was collected and the solvents were removed as described above to obtain a white solid **GS-4B** in a low yield. Solid **GS-4B** was detected as essentially one spot on TLC with the above solvent system using uv, iodine vapor, and $PdCl_2$ reagent. In this solvent system the starting chalcone had an R_f of 0.81, and the reduced and oxidized glutathiones were near the origin. An unequivocal NMR spectrum was not obtained because of the poor solubility of **GS-4B** in a variety of solvents including acetone, methanol, water and acetic acid. Furthermore, solid **GS-4B** decomposed in dimethyl sulfoxide and trifluoroacetic acid at room temperature. However, the 300-MHz NMR spectra obtained in either acidic DMSO or methanol clearly demonstrated the peptidic and aromatic signals of the proposed structure with appropriate integration. The mass

spectrum of **GS-4B** was obtained as a FAB spectrum using glycerol. MS (*m/z*): 714, 716 (M_1^+ of disodium salt form), 692, 694 (M_2^+ of monosodium salt form), 670, 672 (M_3^+ of free acid form), 636 (M_1 - Br + 1), 614 (M_2 - Br + 1), 592 (M_1 - Br + 1), 413, 391, 369, 363, 365 (M_1, M_2, M_3 - GS). Solid **GS-3B** was also synthesized and identified in the same manner described above (13–15).

4-Methoxy-, 4-nitro-, and 4′-methoxychalcones (**III, IV,** and **II**) and reduced glutathione were purchased from Fairfield Chemical, Inc., and from Sigma, respectively. Chalcone (**I**) and other synthetic starting materials were supplied by Aldrich Chemical, Inc. Tridiphane (TR, Fig. 1) was generously provided by Dr. Paul Zorner (formerly of Dow Chemical Co., Walnut Creek, CA).

Assays for cGST, cEH, or mEH of mouse and rat livers. Enzymes were prepared as described previously (16–18) from livers of male Swiss-Webster mice (25–30 g) or male Sprague-Dawley rats (250–300 g) maintained on Purina Rodent Chow. Cytosolic fractions were used for the determination of cytosolic epoxide hydrolase (cEH) and cytosolic glutathione *S*-transferase (cGST) activity; washed microsomal fractions were used for the determination of microsomal epoxide hydrolase (mEH) activity. The cytosol and microsomes were frozen in aliquots immediately after preparation at −80°C.

Protein and enzyme assays. Protein levels were estimated using a previously described modification (18) of the method of Bradford (19). Data were collected and analyzed using an Atari 400 microcomputer interfaced with a Gilford Manual EIA reader.

Two substrates were used to monitor EH and cGST activity. *trans*-Stilbene oxide (TSO) is rapidly hydrated by cEH (pH 7.4), and *cis*-stilbene oxide (CSO) is hydrolyzed by mEH (pH 9.0), while in the presence of GSH and cGST, CSO is conjugated (pH 7.4 in buffer with 5 mM GSH) (1, 9, 20). All assays were partition assays which monitored radiolabeled diol formed or radiolabeled glutathione conjugate formed (in the case of cGST) from 50,000 dpm of the appropriate substrate. TSO (5×10^{-5} M to monitor cEH activity), and CSO (5×10^{-5} M to monitor mEH activity), were extracted with isooctane to terminate the reaction. The diol which remained in the aqueous layer was analyzed by liquid scintillation counting. Chromatographic analysis indicated that the diol accounted for >98% of the radioactivity in the aqueous phase. Assays for cEH in rat cytosol involved depletion of GSH by a 10-min preincubation with diethylmaleate (DEM, 0.5 mM) before addition of TSO since GSH levels are high enough in rat cytosol to interfere with cEH values (21). This procedure gives the same results as the removal of GSH by dialysis or gel permeation chromatography. For monitoring cGST activity, CSO (5×10^{-5} M) was extracted with *n*-hexanol to terminate the reaction. Hexanol extracted both the diol and epoxide from the radiolabeled conjugate which remained in the aqueous phase (1, 9, 20, 21). At least three replicates were run for each reported point, and each experiment was repeated at least twice. Each assay was linearly dependent on time for the assay periods used in this study.

Inhibition of TSO and CSO hydrolysis by chalcones and chalcone oxide. To inhibit cEH, mEH, or cGST activities, the enzyme solutions were preincubated for 10 min, unless specified otherwise, before addition of substrate. DEM was added to rat cytosol and preincubated for 10 min before inhibitor was added, and then preincubated another 10 min before substrate was added.

Potential inhibitors were ranked according to their potency by screening them at concentrations ranging from 1×10^{-4} to 1×10^{-9} M. For determination of the concentration required for 50% inhibition of the enzyme, triplicate incubations at four or five different concentrations of the inhibitor were used. The experiments to determine I_{50} were repeated at least two times and the same trends were observed in each case. I_{50} was calculated using Finney's probit analysis (22) on an Apple II Plus computer. Confidence limits of 5% for the I_{50}'s reported ranged from 0.94 to 5.5% of the reported I_{50}'s with an average of 2.6%.

*Time-dependent reaction of olefins **3B** and **4B** with glutathione in phosphate buffer.* To 50 μl of 5 mM GSH in pH 7.4 phosphate buffer was added 1 μl of 1.5×10^{-5} M olefin **3B** (or 2.5×10^{-3} M olefin **4B**) in acetone and each mixture was reacted at 37°C for varying times (5 to 120 min) followed by the addition of 50 μl of 0.1% (liver wet wt eq/vol) cEH and 5 mM GSH in phosphate buffer. After the preincubation for 10 min, 1 μl of CSO as substrate was added and the incubation was carried out for 10 min.

RESULTS AND DISCUSSION

In this study the effect on inhibitory activity of introducing fluoro, bromo, methoxy, and trifluoromethyl groups into the 2-, 3-, and 4-positions of the parent compound 4′-phenylchalcone (**H**, Fig. 1) was evaluated in hopes of obtaining more potent and selective materials. The microsomal and cytosolic epoxide hydrolases as well as cytosolic glutathione transferase were used as target enzymes. In addition, 2-chloro-, 2-methyl-, 3-hydroxy-, and 4-phenyl-4′-phenylchalcones (**2C, 2M, 3OH,** and **4Ph**) and **NM** were synthesized. The inhibitory potency of these compounds was tested along with that of chalcone (**I**), 4-methoxy-, 4-nitro-, and 4′-methoxychalcones (**III, IV,** and **II**) on the cytosolic glu-

tathione *S*-transferase and microsomal and cytosolic epoxide hydrolase activities. The potency of these compounds as inhibitors was compared to that of tridiphane (a known inhibitor of some plant GSTs).

Syntheses. Structural assignments for the chalcones and their oxides were based principally on the IR and NMR spectra (Tables I and II) with mass spectral data acquired on selected compounds (Table III). Diagnostic IR signals for the chalcones included the weak absorption of the α,β-unsaturated ketone which was shifted towards lower wavenumbers (ca. 20–30 cm^{-1}) compared with that of the nonconjugated ketones. The NMR signals at 4 to 5 ppm were diagnostic for the protons on the oxirane ring. These signals appeared as doublets with coupling constants of less than 2.0 Hz. All compounds were judged to be pure based on spectral and chromatographic evidence as well as sharp melting points. When the chalcones were reacted with GSH, one main product was obtained which both absorbed uv (indicative of aromatic systems) and yielded a colored spot with the $PdCl_2$ reagent (indicative of sulfur) on TLC. The isolated product gave one spot on TLC with 1-butanol:acetic acid:water (3:1:1). However, using compound **GS-4B** as an example, the mass spectrum gave three clear doublet molecular ion peaks with the differences of 2 mass units at *m/z* 670, 672, 692, 694, and 714, 716. The difference between each of these doublets was 22 mass units. These data suggested that this product was a mixture of the free acid, monosodium salt and disodium salt of the glutathione conjugate. As evidence to support this argument, the mass spectrum also gave three peaks at *m/z* 592, 614, and 636. These peaks could be rationalized as the fragment ions M_1, M_2, and M_3-Br. These data clearly indicated that the compound was a Michael addition product of glutathione and the corresponding chalcone as shown in Fig. 1. However, the NMR spectra did not support unequivocal assignment of the location of the thioether, although benzylic attack would be expected.

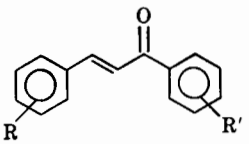
Inhibition of microsomal epoxide hydrolase. Both the chalcones and chalcone oxides tested were exceptionally weak inhibitors of murine microsomal epoxide hydrolase activity acting on CSO (Table IV). With the possible exception of **2TE, 4BE,** and **4MOE,** the chalcone oxides were slightly more potent inhibitors than the corresponding olefins. Ganu and Alworth (23) noted a stimulation of microsomal epoxide hydrolase from rat liver by some chalcone oxides. As previously reported with other substrates (8), no or only very slight (<10%) stimulation was seen in this study. It should be noted that these compounds are quite lipophilic and might have caused stimulation if they could have been tested at higher concentrations or with other substrates (24). Thus, both the chalcone and chalcone oxides can be used as selective inhibitors of other epoxide metabolizing systems without fear of inhibiting the mEH.

Inhibition of cytosolic epoxide hydrolase. The chalcones tested caused but slight inhibition of the cytosolic EH at the highest concentrations examined. If there is a trend, it is that the less hydrophobic compounds **I** and **II** caused the greatest cEH inhibition. Interestingly, 4′-methoxychalcone (**II**) was a better inhibitor than the corresponding 4-compound (**III**) (Table IV). In contrast the corresponding epoxides of many of these chalcones were potent inhibitors of cEH (Table V) (8).

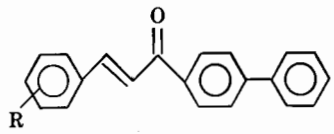
Mullin and Hammock (8) reported that 4- and 4′-substituents of increasing hydrophobic character and bulk improved the inhibitory activity of chalcone oxide on cEH. For instance, 4-phenyl, 4-isopropyl, and 4′-phenylchalcone oxides were potent inhibitors of cEH acting on *trans*-β-ethylstyrene oxide. In this study, introduction of bromo-, fluoro-, methoxy-, and trifluoromethyl groups into the 2-, 3-, and 4-positions on the benzene ring of 4′-phenylchalcone oxide caused no dramatic change in the inhibition of cEH activity. From the limited series of compounds available, it was not possible to draw clear conclusions regarding linear free energy parameters associated with inhibitory potency. However, substituents in the 2-position larger than F clearly destroy inhibitory potency, presumably for steric reasons. The inhibitory activity of **HE** allows the current se-

TABLE IV

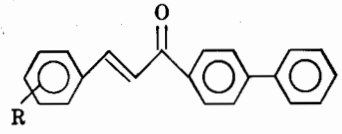
INHIBITORY ACTIVITY OF VARIOUS CHALCONES ON THREE EPOXIDE-METABOLIZING SYSTEMS FROM MOUSE LIVER



Compound designation	R	R′	I_{50} or highest percentage inhibition noted (mM)[a] Glutathione S-transferase[b]	Epoxide hydrolase[b] Cytosolic	Epoxide hydrolase[b] Microsomal
Chalcone (I)	H	H	1.8×10^{-5}	43 (0.1)	23 (0.1)
II	H	4′-CH_3O	52 (1.0×10^{-4})	43 (0.1)	18 (0.1)
III	4-CH_3O	H	8.7×10^{-6}	28 (0.1)	17 (0.1)
IV	4-NO_2	H	44 (1.0×10^{-4})	10 (0.1)	3 (0.1)



	R			
4′-Phenylchalcone (H)	H	3.0×10^{-6}	27 (0.2)	0 (0.1)
2F	2-F	6.5×10^{-7}	25 (0.2)	6 (0.1)
2C	2-Cl	5.0×10^{-7}	18 (0.1)	12 (0.1)
2B	2-Br	2.5×10^{-7}	27 (0.2)	7 (0.1)
2MO	2-CH_3O	55 (1.0×10^{-4})	32 (0.2)	21 (0.1)
2M	2-CH_3	56 (1.0×10^{-4})	13 (0.1)	11 (0.1)
2T	2-CF_3	9.5×10^{-7}	14 (0.1)	17 (0.1)
3F	3-F	1.0×10^{-6}	23 (0.2)	8 (0.1)
3B	3-Br	6.1×10^{-7}	23 (0.2)	7 (0.1)
3MO	3-CH_3O	7.6×10^{-7}	29 (0.2)	14 (0.1)



	R			
3T	3-CF_3	1.9×10^{-6}	11 (0.1)	15 (0.1)
3OH	3-OH	1.9×10^{-6}	6 (0.1)	14 (0.1)
4F	4-F	53 (1.0×10^{-4})	15 (0.2)	0 (0.1)
4B[c]	4-Br	66 (1.0×10^{-4})	14 (0.2)	5 (0.1)
4MO	4-CH_3O	51 (1.0×10^{-4})	29 (0.2)	7 (0.1)
4T	4-CF_3	28 (1.0×10^{-4})	1 (0.1)	2 (0.1)
4Ph[c]	4-C_6H_5	27 (1.0×10^{-4})	17 (0.1)	0 (0.1)
N-CH_3	NA	32 (1.0×10^{-4})	18 (0.1)	21 (0.1)

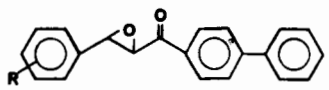
[a] Data are from triplicate assays performed on at least two separate occasions at a minimum of five different concentrations for active compounds. I_{50} data were calculated using the regression program of Lieberman (22).

[b] The rates of enzymatic reactions in solvent controls (no inhibitor added) were 29.7, 3.98, and 2.05 nmol/min/mg protein for glutathions-S-transferase and cytosolic and microsomal epoxide hydrolase, respectively.

[c] Chalcones 4B and 4Ph were not dissolved completely in acetone at a concentration of 1×10^{-4} M.

TABLE V

INHIBITORY ACTIVITY OF VARIOUS EPOXIDES ON THE MAJOR EPOXIDE-METABOLIZING ENZYMES OF MOUSE LIVER



Compound Designation	R	I_{50} or highest percentage inhibition noted at 0.1 mM[a]		
		Glutathione *S*-transferase[b]	Epoxide hydrolase[b]	
			Cytosolic	Microsomal
4'-Phenylchalcone oxide (**HE**)	H	65	7.2×10^{-7}	5
2FE	2-F	63	2.5×10^{-6}	19
2BE	2-Br	44	26	11
2MOE	2-CH_3O	54	24	35
2TE	2-CF_3	24	24	14
3FE	3-F	62	1.0×10^{-6}	13
3BE	3-Br	55	1.4×10^{-6}	16
3MOE	3-CH_3O	54	5.0×10^{-7}	19
3TE	3-CF_3	43	2.5×10^{-6}	19
4FE	4-F	64	6.6×10^{-7}	4
4BE	4-Br	61	4.7×10^{-6}	2
4MOE	4-CH_3O	49	4.3×10^{-7}	5
4TE	4-CF_3	56	7.5×10^{-6}	8
TR	NA	42	—	—

[a] Data are from triplicate assays performed on at least two separate occasions at a minimum of five different concentrations for active compounds. I_{50} data were calculated using the regression program of Lieberman (22).

[b] The rates of enzymatic reactions in solvent controls (no inhibitor added) were 29.7, 3.98, and 2.05 nmol/min/mg protein for glutathione *S*-transferase, cytosolic epoxide hydrolase, and microsomal epoxide hydrolase, respectively.

ries of compounds shown in Table V to be compared with literature values. It is important to remember that *trans*-stilbene oxide rather than *trans*-β-ethylstyrene oxide was used as a substrate in this study, and that I_{50} values can be greatly influenced by the K_m of the substrate used.

Inhibition of glutathione transferase. As earlier reported (8, 9), chalcone oxides are very weak inhibitors of cGST with CSO as a substrate. The only result of note was the very low inhibition caused by the compound with a trifluoromethyl group in the two position (Table V). These data suggest that chalcone oxides compete poorly with CSO as a substrate for cGST, that glutathione conjugates of the chalcone oxides tested form slowly, and/or that the resulting conjugate is unstable or a poor inhibitor of the cGST isozymes analyzed in this study.

An exciting aspect of the study was the strong inhibition of cGST by a variety of the chalcones tested (Table IV). When compared to chalcone (**I**), compound **II** with a methoxy in the 4'-position showed weak inhibition while the same substituent in the 4-position (**III**) increased activity as did the 4'-phenyl substitutent **H**. In contrast, a nitro group in the 4'-position (**IV**) significantly reduced biological activity. Thus, the role of the 4-substituent seems to be different from that of the 4'-substituent for cGST inhibition and may correlate with the electrophilicity of the beta carbon of the conjugated enone.

Based on the high inhibitory activity of 4'-phenylchalcone (**H**), the effects of 2-, 3-,

TABLE VI

INHIBITORY ACTIVITY OF VARIOUS CHALCONES FOR CYTOSOLIC GLUTATHIONE *S*-TRANSFERASE ACTIVITY OF RAT LIVER

Compound designation	R (see Table V)	Highest percentage I_{50} or inhibition noted (mM)[a]
H	H	9.0×10^{-6}
2B	2-Br	8.9×10^{-7}
2MO	2-CH_3O	57 (0.1)
2T	2-CF_3	6.8×10^{-6}
3B	3-Br	7.5×10^{-7}
3MO	3-CH_3O	1.4×10^{-6}
3T	3-CF_3	1.3×10^{-6}
4B	4-Br	36 (0.067)
4MO	4-CH_3O	62 (0.1)
4T	4-CF_3	36 (0.1)

[a] These data were obtained under conditions when chalcone **H** for mouse cGST had an I_{50} of 4.0×10^{-6} M. The rate of enzymatic reactions in solvent controls (no inhibitor added) was 89.5 nmol/min/mg protein for glutathione *S*-transferase.

and 4-substituents on cGST inhibition were investigated using **H** as the parent molecule. All 4'-phenylchalcones with a substituent in the 4-position were weak inhibitors of cGST, regardless of the substituent's electronegativity. These data suggest a maximum size of the substituted chalcone for good inhibitory activity. However, the steric contribution of the 4-fluoro substituent in **4F** would be slight, indicating that the above hypothesis may be simplistic.

All of the compounds with a substituent in the 3-position (**3F, 3B, 3MO, 3T, 3OH**) were good inhibitors of cGST and essentially equipotent. Compounds with electronegative substituents in the 2-position were also good inhibitors (**2T, 2F, 2C, 2B**), while sterically similar molecules lacking electronegative properties were weak inhibitors (**2MO, 2M**). This general trend is expected for compounds acting as general Michael acceptors, but additional compounds should be tested to distinguish clearly among the physicochemical parameters involved. Several of these compounds were tested under the same conditions as inhibitors of cGST from rat liver

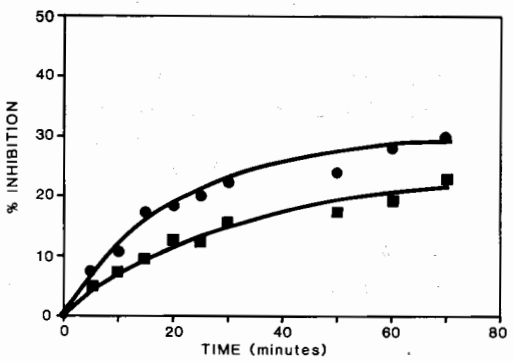


FIG. 2. Increase in inhibition of cGST following incubation of chalcone **3B** (■) (1.5×10^{-7} M) or **4B** (●) (2.5×10^{-6} M) with GSH (5×10^{-8} M) prior to addition of cytosol, substrate, and GSH. The data are the average of three replicates for each point. The experiment was performed on five separate occasions with similar trends. The SD is less than 6% of the reported values.

(Table VI). Similar trends were observed with the compounds (notably **2T**) being less active in rat than in mouse cytosol.

Time dependence of inhibition. As shown in Fig. 3, inhibition of cGST by **3B** or **4B**

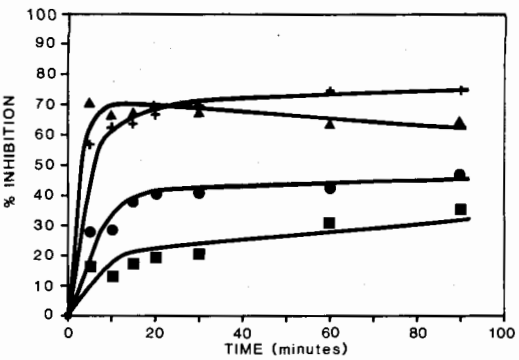


FIG. 3. Time-dependent inhibition of cGST by purified glutathione conjugates of two chalcones. Lines (●) and (▲) are for **GS-4B** at 1×10^{-6} and 1×10^{-5} M, respectively. Lines (■) and (+) are for **GS-3B** at 2×10^{-7} and 2×10^{-6} M, respectively. Points represent an average of three replications each with the experiment replicated three times for **GS-4B** on separate occasions with similar results. No further change in inhibition was observed out to 120 min. The SD is less than 7% of the reported values.

increases with the time of preincubation with GSH, while preincubation with cytosol lacking GSH did not result in a time-dependent increase in inhibition. These data suggest that the glutathione conjugate of the chalcone may be the ultimate inhibitor of the enzyme. Thus, glutathione conjugates were synthesized for a good inhibitor (**3B**) and a poor inhibitor (**4B**) as shown in Fig. 1. These conjugates, **GS-3B** and **GS-4B,** had I_{50}'s of 4.3×10^{-7} and 2.5×10^{-6} M, respectively (Fig. 2). The semilog plots of inhibition versus concentration of **GS-3B** and **GS-4B** are parallel with each other and with those of the other chalcones tested such as **3B,** providing some evidence for a similar mechanism of inhibition. **GS-3B** was a slightly more potent inhibitor than the corresponding olefin, while **GS-4B** was far more potent than its olefinic precursor. These data strongly suggest that the conjugate makes a major contribution to the inhibitory activity observed for weak and possibly strong inhibitors as well. These data further indicate that the I_{50} values reported in Tables IV and VI reflect the inhibitory potency of the compound shown, the potency of the corresponding conjugate as well as the speed of formation of the conjugate by chemical and/or enzymatic mechanisms.

The data in Fig. 3 show that inhibition of cGST by glutathione conjugates occurs rapidly compared to inhibition by the corresponding chalcone (Fig. 2). However, a degree of time dependence occurs even with the conjugates, indicating that these compounds may not be simple competitive inhibitors.

Field studies have shown tridiphane (**TR,** Fig. 1) to be a promising herbicide synergist. It presumably acts as its glutathione conjugate by inhibiting glutathione transferase activity in target plants (25, 26). In this study using CSO as a substrate, tridiphane was a poor inhibitor of cGST (Table V). Thus by comparative studies of epoxide hydrolases and glutathione transferases in target and nontarget species, it may be possible to develop other classes of compounds as pesticides or pesticide synergists.

A variety of chalcone oxides and chalcones have now been shown to be potent inhibitors of the cytosolic epoxide hydrolase and the cGST isozyme(s) metabolizing CSO *in vitro* and in isolated hepatocytes (data not shown). These potent, selective inhibitors will prove to be useful probes for defining routes of epoxide metabolism in the above systems and as active site probes and photo affinity labels for the enzyme. It is hoped that further investigations will lead to compounds which are useful *in vivo* as well. Such probes would be valuable in the study of xenobiotic metabolism and may be of therapeutic value since a variety of biosynthetic intermediates and chemical mediators contain an epoxide functionality.

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