J. Pesticide Sci. **19**, 53–58 (1994)

Original Article

Glutathione Conjugates as the Activated Form of Chalcones for Glutathione *S*-Transferase Inhibition

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(Received June 12, 1993; Accepted September 1, 1993)

Chloro-substituted 4′-phenylchalcones inhibited glutathione *S*-transferase (GST) ***in vitro***. They first reacted with the reduced glutathione (GSH) to give the GSH conjugates of the chalcones, which actually inhibited GST. The chemical structure of the conjugates was inferred from the ultraviolet spectral change during conjugation and established by synthesis as the GSH addition product on the β-position of the α, β-unsaturated ketone moiety of the chalcone. Among the GSH conjugates of 2-, 3- and 4-chloro- and 2,3-, 2,4-, 2,6- and 3,4-dichloro-4′-phenylchalcones, 2-chloro-compound was remarkable in GST inhibition, but others are also active. The overall inhibition of GST by chalcones depends mostly on the rate of GSH conjugation which is affected by the position and number of chloro-substituents. The GSH conjugation of 4-chloro-compound was, if any, very slow and gave lower GST inhibition, although its GSH conjugate gave inhibition comparable to others. The mode of GST inhibition seemed incompetitive as shown with the GSH conjugate of 2-chloro-4′-phenylchalcone, which had the K_i value comparable to that of tridiphane.

INTRODUCTION

The glutathione *S*-transferase (EC 2.5.1.18) (GST) is one of the important enzymes in xenobiotic metabolism. They catalyze the conjugation of a variety of electrophilic and hydrophobic compounds with the reduced glutathione (GSH) to the more hydrophilic forms. Such detoxication is an important mechanism for the pest resistance to pesticides and for selective toxicity. The inhibitors of GST may serve as a probe to elucidate such mechanisms, which provides ideas for designing pesticides of appropriate properties. During the search for the inhibitors of mouse epoxide hydrolases, one of the authors (T. Miyamoto) found incidentally that certain chalcones inhibited mouse GST.[1)]

In this paper, mechanism of GST inhibition by chalcones is investigated.

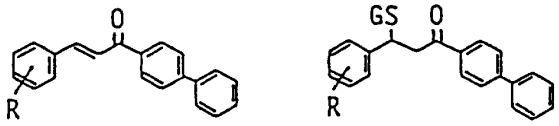
MATERIALS AND METHODS

Analytical TLC was performed on 0.2 mm precoated Silica gel $60F_{254}$ plastic sheets (Merck), and compounds were detected by UV light (254 nm) and 0.5% palladium chloride in diluted hydrochloric acid ($PdCl_2$ reagent). ^{13}C-NMR spectra were measured in deuterochloroform or deuterium oxide solution using tetrahydrofuran (THF) as an internal standard with a JEOL JNM-EX90A NMR spectrometer. MS spectra were obtained by DI system on a Shimadzu GCMS-QP1000 gas chromatograph-mass spectrometer. UV spectra were determined with a Hitachi U-3200 spectrophotometer.

1. Chemicals

Chalcones and their GSH conjugates as shown in Table 1 were synthesized in our laboratory. Chalcones were prepared by

Table 1 Inhibitory activity of monochloro- and dichloro-substituted 4′-phenylchalcones and their GSH conjugates against GST from ICR mouse liver.



R	I_{50} (M)	
	Chalcone	GSH conjugate
2-Cl	7.1×10^{-8}	6.2×10^{-8}
3-Cl	3.2×10^{-7}	4.3×10^{-7}
4-Cl	4.0×10^{-6}	6.6×10^{-7}
2,3-diCl	2.5×10^{-7}	1.7×10^{-7}
2,4-diCl	2.4×10^{-7}	3.9×10^{-7}
2,6-diCl	8.6×10^{-8}	3.6×10^{-7}
3,4-diCl	1.0×10^{-6}	—

These data were obtained when 4′-phenylchalcone had an I_{50} of $2.3\text{–}3.6\times10^{-7}$ M against the GST.

Claisen-Schmidt reaction of 4-acetylbiphenyl with the appropriate benzaldehydes.[1] To a mixture of 4-acetylbiphenyl (98%, 4.0 g, 20.0 mmol) and 2-chlorobenzaldehyde (3.1 g, 22.1 mmol) in ethanol (50 ml) was added dropwise with stirring 3 N sodium hydroxide solution (13.1 ml) at room temperature over 5 min, and then the stirring was continued for 3 hr. The mixture was acidified with 3 N hydrochloric acid, and the precipitate was collected by filtration and recrystallized from ethanol to obtain 3.6 g of 2-chloro-4′-phenylchalcone in a 56.5% yield based on 4-acetylbiphenyl.

UV λ_{max}^{MeOH} nm (ϵ): 234 (6800), 318 (24,300).

^{13}C-NMR $\delta_{THF}^{CDCl_3}$ ppm: 124.0, 126.7, 127.3, 127.7, 128.4, 128.6, 129.7, 130.6, 132.7, 134.9, 136.1, 139.2, 139.6, 145.0, 188.6. MS *m/z*: 318,320 (M^+), 283 (M^+-Cl), 241,243 ($M^+-C_6H_5$), 207 ($M^+-C_6H_4Cl$).

The properties of other chalcones were close to those of 2-chloro-4′-phenylchalcone.

Glutathione conjugates of the chalcones were prepared by reaction of the corresponding chalcone with GSH in borate buffer under inert atmosphere.[1] To a mixture of 2-chloro-4′-phenylchalcone (1.02 g, 3.20 mmol) and GSH (1.07 g, 3.50 mmol) in THF (95 ml) was added dropwise with stirring 100 mM sodium tetraborate buffer (pH 9.2, 95 ml) at room temperature over 45 min, and the 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 aqueous solvent was removed by lyophilization. The residue was triturated, washed repeatedly with benzene, and then extracted several times with methanol. The methanol extract was mixed with distilled water (DW) to prepare a DW–methanol mixed solution (3: 1). A part of the mixture (each *ca.* 20 ml) was adsorbed on a SEP-PAC C_{18} cartridge (Waters Associates Inc.) and then eluted from the cartridge, which had been previously washed with methanol, DW and DW–methanol (3: 1) mixed solvent. DW–methanol mixed solvent was further passed through the cartridge (3: 1, 30 ml; 1: 1, 40 ml) and 30 fractions were collected and analyzed by TLC. The eluates containing only a product with an *Rf* value of near 0.5 (butanol–acetic acid–DW, 3: 1: 1) were combined and the solvent was removed to obtain 52.9 mg of a white solid in a low yield. The solid was detected as one spot on the TLC by UV absorption and $PdCl_2$ coloration.

UV λ_{max}^{MeOH} nm (ϵ): 286 (17,500).

^{13}C-NMR $\delta_{THF}^{D_2O}$ ppm: 26.4, 31.7, 41.0, 43.1, 43.9, 53.2, 54.2, 126.7, 127.4, 128.4, 128.7, 128.9, 129.5, 133.0, 133.2, 135.0, 138.6, 139.0, 145.5, 170.7, 173.2, 174.2, 197.0.

Other GSH conjugates also were synthesized and identified in the same manner described above.

2. *Enzyme Preparation*

The cytosolic GST was prepared from the liver of male ICR mouse (7 weeks, 25–30 g) to obtain in 10% cytosol solution of phosphate buffer (pH 7.4) in the usual way. The cytosol was frozen immediately at −80°C in aliquots after the preparation.

The enzyme activity is expressed by nmol of GSH conjugate of 1,2-dichloro-4-nitrobenzene (DCNB) per min in protein mg. The total protein content was measured by the method of Laury.

3. *Inhibitory Test against GST by Chalcones and their GSH Conjugates*[2]

Cytosol solution (0.2%, 1.45 ml) in 1/15 M

sodium phosphate buffer (pH 7.4) containing 5 mM GSH was preincubated at 37°C for 1 min and to this was added 25 μl of the chalcone in acetone or the GSH conjugate in methanol. For control only 25 μl of acetone or methanol was added instead of the chalcone or the conjugate solution. After 10 min 25 μl of DCNB in acetone was added and incubated for 10 min before termination of the reaction by addition of 3 ml of chloroform and vigorous vortexing. Following centrifugation ($1500 \times g$, 10 min), the aqueous phase was taken to UV cell and the absorbance was measured at 345 nm. The difference in absorbance between reaction mixtures with and without the inhibitor corresponds to the inhibitory activity against GST by the inhibitor. Triplicate incubations at 4 or 5 different concentrations of the inhibitor were run to determine the concentration required for 50% inhibition of the GST (I_{50}) from a linear fit of data on a log dosage-probit plot.

4. Absorption Spectra by Repeated Scanning of the Reaction Mixture of a Chalcone with GSH in the Cytosolic Solution

Cytosol solution (0.2%, 1.18 ml) containing 5 mM GSH was preincubated at 37°C for 1 min in the absorption cell on UV spectrophotometer, and 20 μl of a chalcone in an appropriate concentration in methanol was reacted with GSH in the cell to measure the spectrum between 240 nm and 390 nm after 0, 20, 40, 60 and 80 min by repeated scanning.

5. Inhibitory Kinetics of GST[2]

Cytosol solution (1.04%, 0.96 ml) containing 5.21 mM GSH was preincubated at 37°C for 1 min, and to this was added 40 μl of the methanol solution of a GSH conjugate (an appropriate concentration) and DCNB (3.75, 5, 6.25, 8.75, 12.5, 18.75 or 25 mM). After starting, the rate of DCNB conjugation of GSH was measured every 0.3 min from 0.3 through 3.3 min by the change in the absorbance at 345 nm according to the rate assay system with the U-3200 spectrophotometer. For control the GSH conjugate as inhibitor was not added in the above methanol solution. Also, 40 μl of the methanol solution of a GSH conjugate and GSH (8.75, 12.5, 16.25, 25, 50 or 125 mM) was added to 0.96 ml of 1.04% cytosol solution containing 1.04 mM DCNB to measure the rate of conjugation in the same manner described above.

To recognize the inhibitory activity of 2-chloro-4′-phenylchalcone, the Lineweaver-Burk plots in the presence of 3 different concentrations of the GSH conjugate as the activated form were obtained, which were replotted into the Dixon plots to determine the K_i value from the intersection. The K_i was compared with that for GST inhibition of tridiphane.

RESULTS AND DISCUSSION

1. Synthesis of GSH Conjugates

The starting chalcones had *Rf* values near 0.8, and GSH and its dimer $(GS)_2$ were at the origin on TLC with butanol–acetic acid–DW (3 : 1 : 1) as the developing solvent. A spot obtained from GSH conjugation in methanol extract, which had an *Rf* value near 0.5, could be detected by treatment with $PdCl_2$ reagent and by absorption in UV (254 nm). The product corresponding to this spot was isolated from the extract through Sep-Pak cartrige. In our previous report,[1] the GSH conjugate of 2-bromo-4′-phenylchalcone was identified but without its unequivocal PMR spectrum because of the poor solubility of the product in acetone, methanol, dioxane, DW and acetic acid, and because of the decomposition of the product in dimethyl sulfoxide and trifluoroacetic acid at room temperature. In this paper, ^{13}C-NMR spectrum of the product formed from 2-chloro-4′-phenylchalcone was obtained in DW–THF mixed solvent (*ca.* 1 : 1), which indicated the disappearance of two methine carbon signals (δ 124.0, 130.6 ppm) of α,β-unsaturated carbonyl bond and the appearance of new –CHSG– methine and $-CH_2C(O)-$ methylene carbon signals (δ 43.1, 43.9 ppm). Therefore, the product was identified as the GSH conjugate of 2-chloro-4′-phenylchalcone. The product showed the same behaviour on the TLC as the GSH conjugates of 2- and 3-bromo-4′-phenylchalcones. Also, the above reaction was monitored by the change of the UV spectrum. To 50 ml of a THF solution of 2-, 3- or 4-chloro-4′-phenylchalcone and GSH (each 1×10^{-4} M) was added dropwise with

stirring 50 ml of 100 mM sodium tetraborate buffer (pH 9.2) at room temperature to measure the UV spectrum between 240 nm and 390 nm with the elapse of time.

Other conjugates synthesized were also identified in the same manner as described above.

2. *Inhibitory Activity of Chalcones and their GSH Conjugates against GST from Mouse Liver*

Based on the highly inhibitory activity of 4′-phenylchalcone *in vitro*, the effect of monochloro- and dichloro-substitutions at the benzene ring on the GST inhibition was investigated. As shown in Table 1, all 4′-phenylchalcones tested were strongly inhibitory against the GST except 4-chloro- and 3,4-dichloro-4′-phenylchalcones. The chloro-substitution at 2- and 2,6-positions gave higher inhibition. Thus, the inhibitory activity was affected by the position and number of chloro-substituents.

The reactivity of 2-, 3- or 4-chloro-4′-phenylchalcone with GSH was examined spectroscopically. The chalcones in methanol showed the λ_{max} at 313–318 nm because of the α,β-unsaturated ketone system having a long conjugation with aryl groups. However, for example, the λ_{max} of 4-chloro-4′-phenylchalcone in phosphate buffer (pH 7.4) shifted about 15–20 nm to shorter wavelength, and the λ_{max} by the $n\rightarrow\pi^*$ transitions of the carbonyl group remarkably depended on solvent polarity. On the other hand, their GSH conjugates (described below) in methanol showed the λ_{max} at 285–289 nm. These chalcones were reacted with GSH in the 0.2% cytosol solution and the absorption spectrum was directly measured with the elapse of time. The λ_{max} of 2-chloro-4′-phenylchalcone when reacted with GSH shifted to shorter wavelength and became weaker. The spectra by repeated scanning are shown in Fig. 1 (a). In the reaction of 3-chloro-4′-phenylchalcone with GSH, the λ_{max} shifted more rapidly to shorter wavelength and became still weaker as compared with the case of 2-chloro-4′-phenylchalcone (Fig. 1 (b)). It was suggested that both chalcones reacted with GSH with different reaction rate by GSH addition to the α,β-unsaturated system to

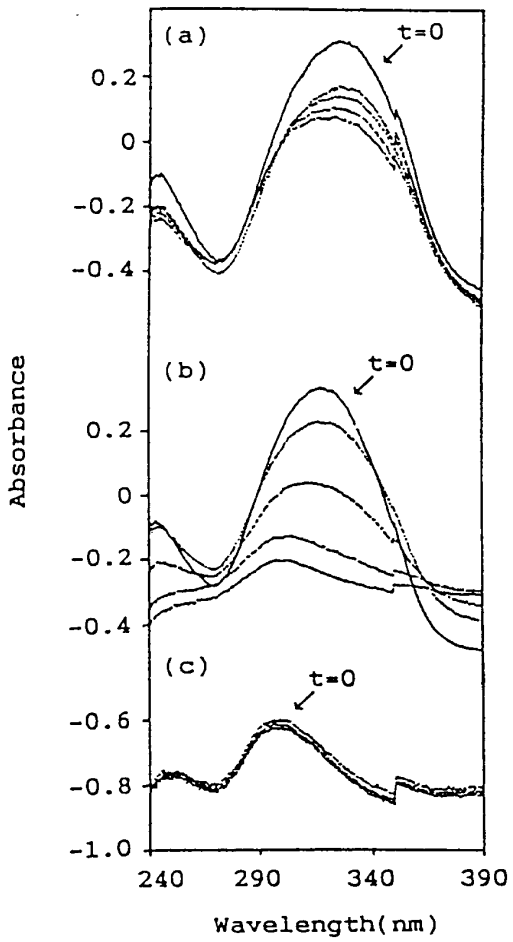


Fig. 1 Absorption spectra of the reaction mixture of a chalcone with GSH in the cytosolic solution by repeated scanning.

(a), (b) and (c) showed the reaction spectra of 2-, 3- and 4-chloro-4′-phenylchalcones with GSH (each 5×10^{-5} M), respectively (at 37°C, after 0, 20, 40, 60 and 80 min[t]). The cytosol were 0.2% phosphate buffer solution prepared from ICR mouse liver.

form the GSH conjugate possessing the two spectroscopically separated conjugated systems. On the other hand, in the reaction of 4-chloro-4′-phenylchalcone with GSH (Fig. 1 (c)), there was almost no change in spectrum even after 80 min, indicating that the GSH addition reaction, if any, was very slow.

The fact that 2-chloro- and 3-chloro-compounds were faster in conjugation but stronger in GST inhibition than 4-chloro-compound suggests the possibility that the GSH conjugate was the activated form of chalcones for inhibition of GST. Therefore, the GSH conjugates of chalcones were synthesized and ex-

amined for the GST inhibition and their spectroscopical change similar to the above enzymatic reaction. As shown in Table 1, 2-chloro-compound was distinguished (I_{50} 6.2×10^{-8} M) but all the compounds tested were more or less highly inhibitory (I_{50} $1.7–6.6\times10^{-7}$ M). The production of the GSH conjugate in the cytosolic solution was not directly confirmed by TLC *etc.*, but changes of the repeated UV spectra obtained by the reaction of not only 2- and 3-chloro-compounds but also 4-chloro-compound with GSH in THF-borate buffer showed the same tendency as those by the enzymatic reaction as described above. The overall results support the idea that chalcones first reacted with GSH in cytosolic solution to give their GSH conjugates, which as an activated form inhibited GST, and that the inhibition by chalcones depends on both the rate of GSH conjugation and the intrinsic inhibitory activity of the conjugates but the former seems more important.

3. *Mode of GST Inhibition by the GSH Conjugates of Chalcones*

The K_ms for GSH and DCNB in the absence of inhibitor was 697 μM and 1.23 mM, respectively, and GSH had higher affinity ($1/K_m$) to GST than DCNB. *In vitro* GSH conjugation of DCNB was then conducted in the presence of the GSH conjugates of chalcones to examine the mode of inhibition. The Lineweaver-Burk plots at 1×10^{-6} M of the conjugate of 2-, 3- or 4-chloro-4′-phenylchalcone are shown in Fig. 2. At a fixed concentration of GSH (Fig. 2 (a)) and DCNB (Fig. 2 (b)), these conjugates inhibited the GST in an incompetitive fashion with respect to DCNB and GSH, respectively. The inhibitory activity was in the order of 2-, 4- and 3-chloro-compounds. To determine a K_i value of the conjugate of 2-chloro-4′-phenylchalcone, the above reaction was again performed at 3 different concentrations of the inhibitor. From the Dixon plots as shown in Fig. 3, a K_i value of 0.190 μM was estimated.

It is generally understood that the GSH conjugation of xenobiotics is a detoxication reaction. However, by GSH-GST system, thiocyanate compounds release hydrogen cyanide which is responsible for their toxicity.[8] Also, a few instances have been known for GST inhibition by the GSH conjugates. *S*-Hexyl-

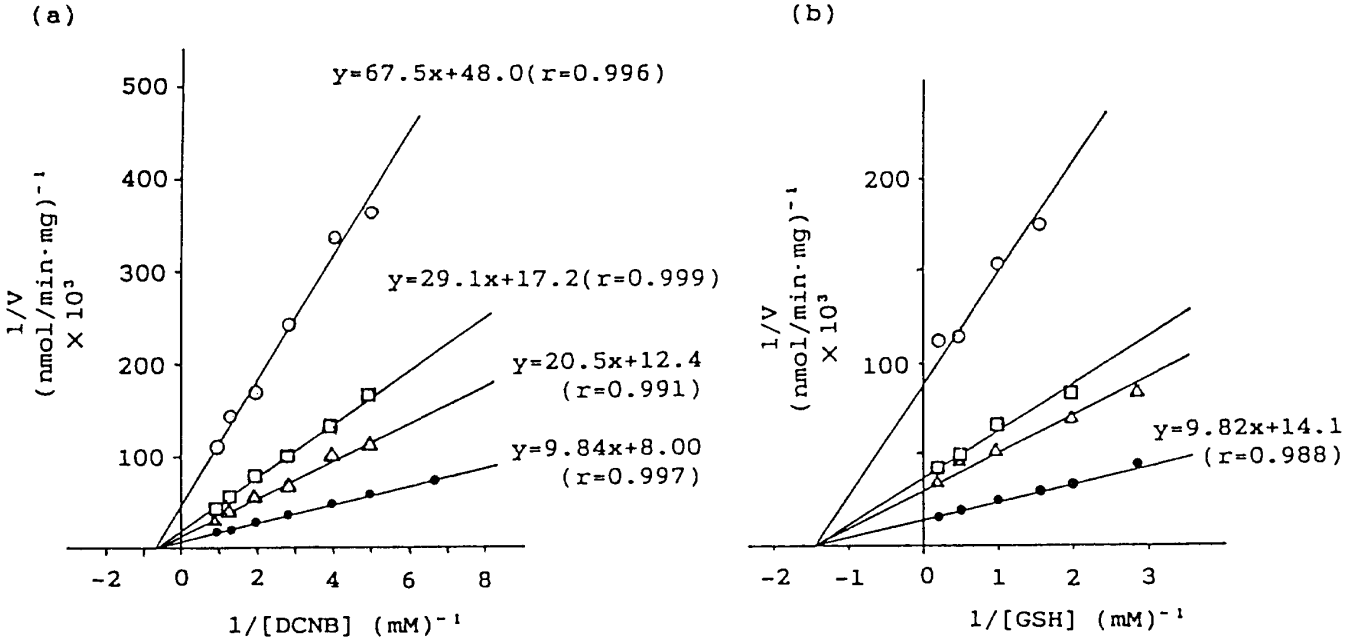


Fig. 2 Lineweaver-Burk plots showing incompetitive inhibition of DCNB conjugation of GSH by the GSH conjugates of 2-, 3- and 4-chloro-4′-phenylchalcones with respect to DCNB (a, [GSH]: 5 mM) and GSH (b, [DCNB]: 1 mM).

The reactions were carried out at 37°C in 1% cytosol solution prepared from ICR mouse liver. ○, □ and △: the GSH conjugates of 2-, 4- and 3-chloro-4′-phenylchalcones, respectively (each 1×10^{-6} M), ●: inhibitor free.

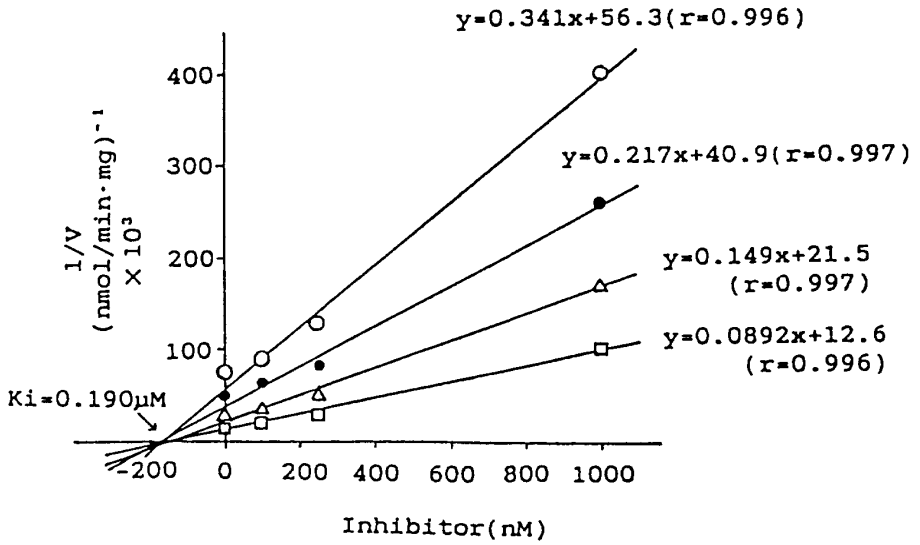


Fig. 3 Dixon plot showing incompetitive inhibition of DCNB conjugation of GSH by the GSH conjugate of 2-chloro-4′-phenylchalcone with respect to DCNB (○: 0.15 mM, ●: 0.25 mM, △: 0.5 mM and □: 1 mM).

glutathione inhibited the conjugation between DCNB and GSH in mouse liver cytosol in a competitive fashion with respect to DCNB and GSH, respectively (unpublished). Its inhibitory activity was about 1/50 of that of the GSH conjugate of 3-chloro-4′-phenylchalcone, and extremely weak as compared to that of 2-chloro-4′-phenylchalcone. *S*-(2-Hydroxybenzyl)glutathione inhibited the isolated GST of equine liver, which catalyzed the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and GSH, in a competitive fashion with respect to GSH, and its K_i was 11.0 μM.[4] Tridiphane is known as the inhibitor of some plant GST and has been shown to be a promising herbicide synergist in field studies.[1] Its K_i values have been reported as ranging from hundreds nM to several μM.[5,6] The fact that the GSH conjugate of 2-chloro-4′-phenylchalcone was comparable in inhibitory activity to tridiphane opens the possibility that certain chalcones are useful in the areas where the GSH-GST system is involved.

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要　　約

カルコンによるグルタチオン *S*-トランスフェラーゼ阻害の活性本体としてのグルタチオン抱合体

宮本　徹，山本　出

塩素置換 4′-phenylchalcone 類は *in vitro* で還元型グルタチオン (GSH) と反応して GSH 抱合体を生成し，これがグルタチオン *S*-トランスフェラーゼ (GST) を阻害した．抱合体の化学構造は，酵素反応液の紫外線吸収スペクトルの経時的変化から推測し，カルコンの α，β-不飽和ケトン部位の β 位への GSH 付加物を合成，決定した．2-, 3-, 4-chloro- また 2,3-, 2,4-, 2,6-, 3,4-dichloro-4′-phenylchalcone の GSH 抱合体の中で，2-chloro 体の GST 阻害が著しかったが，その他の抱合体も阻害活性を示した．カルコンによる GST 阻害は塩素の数と置換位置により異なり，GSH による抱合速度に大きく支配されていた．4-Chloro 体は，その抱合体自体の阻害活性は他の抱合体と同程度であるにもかかわらず，GSH 抱合がきわめて遅いため GST 阻害が弱かった．2-Chloro 体の抱合体による GST 阻害は非拮抗的であり，tridiphane と同程度の K_i 値を示した．