Mechanistic Studies of Inactivation of Glutathione *S*-transferase Pi Isozyme by a Haloenol Lactone Derivative

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Abstract: Cancer chemotherapy often fails due to acquired drug resistance. One of the most critical biochemical changes observed in drug-resistant tumor cells is over-expression of glutathione *S*-transferase Pi isozyme (GSTP1). Glutathione *S*-transferase inhibitors have been used as potentiating agents of chemotherapeutic drugs. Earlier we reported haloenol lactone **1** as a site-directed GSTP1 inactivator. We proposed that enzymatic hydrolysis of the haloenol lactone may be the initial step of GSTP1 chemical modification, resulting in the inactivation of the enzyme. Enzyme inactivation is initiated through addition of Cys-47 to the lactone ring, which is opened in the process to form an α-bromoketone adduct. The acidity of Cys-47 confers good leaving group properties, and rapid hydrolysis occurs to generate an α-bromoketoacid intermediate. The reaction may proceed *via* alkylation of the transient thioester to form a six-membered ring episulfonium ion intermediate which would be yet more reactive toward hydrolysis, with either process leading to the observed mass increase of 230 Da.

To probe the importance of the bromine of the lactone in GST inactivation, we designed and synthesized compound **2**. Unlike lactone **1**, lactone **2** did not show time-dependent inhibitory effect on GSTP1. Incubation of compounds **1** and **2** with excess of *N*-acetyl cysteine produced the corresponding di-*N*-acetyl cysteine conjugate and mono-*N*-acetyl cysteine conjugate, respectively. To probe the role of Cys-47 in the inactivation of GSTP1 by compound **1**, we prepared mutant C47A GSTP1. The mutant GSTP1 still showed good activity toward CDNB, but it lost susceptibility to the inactivation by compound **1**. In addition, LC-MS/MS technique allowed us to identify the modified Cys-47 after the enzyme was exposed to compound **1**.

INTRODUCTION

Drug resistance to chemotherapy is often an obstacle in the effectiveness of cancer treatment (*1*). Multidrug resistance is a complicated biochemical process, and many drug resistant tumors express high levels of glutathione *S*-transferases (GSTs) (*2*). GSTs are a family of enzymes catalyzing detoxification of electrophilic xenobiotics. Cytosolic GSTs are encoded by six distantly related gene families designated as classes: Alpha, Pi, Mu, Theta, Kappa, and Sigma based on their chemical properties, immuno-cross-reactivity, and amino acid sequence homology (*3*). GST isozymes constitute 3-8% of cytosolic proteins in mammalian liver.

Correlations between expression of specific GST isozymes and resistance to chemotherapeutic drugs including adriamycin, chlorambucil, and other alkylating agents are well established (*4-7*). Malignancies including carcinoma of breast, colon, lung, kidney, and ovary demonstrate elevated levels of the Pi class GST, hGSTP1 (*8-12*). Measurement of hGSTP1 levels within tumors has been proposed as a diagnostic indicator of the progression of cancer with potential clinical value in treating certain malignancies (*13-15*).

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Successful cancer therapy is based on the toxicity of cancer drugs being greater in tumors than in other tissues, and such selectivity may be enhanced by inhibiting their detoxification in tumor cells. The diuretic drug ethacrynic acid and its GSH conjugate are competitive inhibitors of GSTs (*16*), and ethacrynic acid enhances cytotoxicity of several alkylating agents in cultured drug-resistant tumor cells (*17, 18*). Selective inhibition of GST isoenzymes overexpressed in tumor cells, offers the prospect of lower effective doses of chemotherapeutic agents and has stimulated interest in development of specific and potent GST inhibitors (*19, 20*).

Although the catalytic mechanism of Pi class GST is still a subject of study, spectroscopic studies revealed that the cosubstrate GSH exists as a thiolate anion in the active site of the enzyme (*21*). In addition, sulfhydryl groups of human Pi class GST are sensitive to alkylating agents such as iodoacetamide (*22*). The Pi class GSTs from mammalian species have three highly conserved cysteine residues (Cys-14, Cys-47, and Cys-169), and a fourth cysteine residue (Cys-101) is conserved in all studied mammalian species but mouse (*23*). Site-directed mutagenesis studies demonstrated that none of these residues is essential for catalysis. The sulfhydryl group of Cys-47 is unusually acidic and has a pK_a of 4.2 (*24*). On the basis of these observations and studies describing mechanism-based inactivation of α-chymotrypsin by haloenol lactone derivatives (*25*), we postulated that either the thiolate anion of GSH or the reactive sulfhydryl of

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Cys-47 would be capable of activating haloenol lactone derivatives and would produce enzyme inactivation through formation of either a GSH-haloketone conjugate or a protein–haloketone adduct.

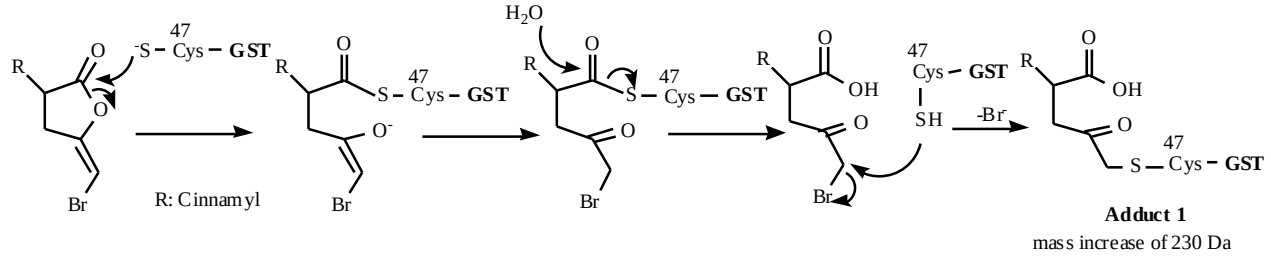
In early studies, we demonstrated that a novel haloenol lactone (3-cinnamyl-5(*E*)-bromoethylidenetetrahydro-2-furanone, **1**) is a competitive and time-dependent inhibitor of murine mGSTA3, mGSTP1, and mGSTM3, displaying greatest reactivity toward mGSTP1 (*26*). Time-dependent inhibition studies indicated that 50% inactivation of mGSTP1 was achieved in less than 3 min. In contrast, mGSTM3 and mGSTA3 were less susceptible to the haloenol lactone (*26*). Electrospray ionization (ESI) mass spectra obtained for mGSTP1 incubated with 5 molar equivalence of the haloenol lactone indicated that inactivation was due to a covalent modification, which produced an increase in molecular weight of 230 Da.

The objectives of the present study were to further investigate the mechanism by which haloenol lactone **1** inactivates hGSTP1 and to determine the role of Cys-47 in the inactivation of the enzyme by lactone **1**.

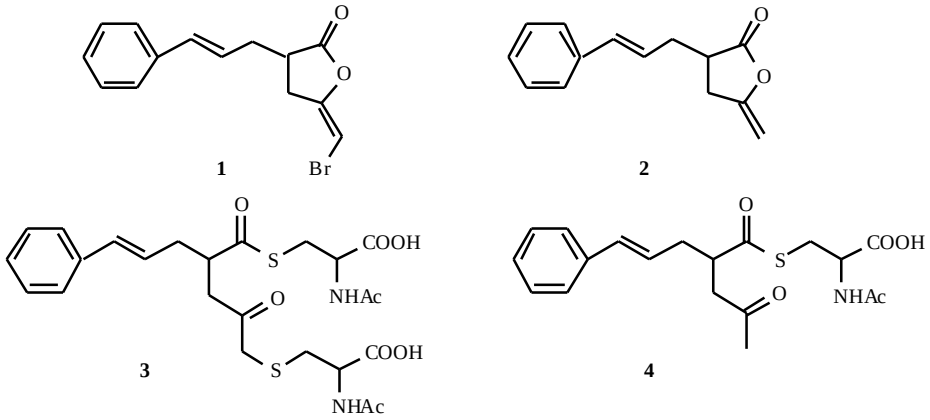
RESULTS AND DISCUSSION

The investigation of mechanism by which haloenol lactone derivatives inactivate glutathione *S*-transferase Pi (GSTP1) isozyme is the key focus in this study. We proposed that enzymatic hydrolysis of the haloenol lactone may be the initial step of GSTP1 chemical modification, resulting in the inactivation of the enzyme. Mass spectrometric analysis of proteolytic fragments from haloenol lactone-modified GSTP1 indicated that the haloenol lactone is covalently attached to the protein (*26*). In addition, kinetic studies employing recombinant hGSTP1 with replacement of cysteine by serine at Cys-47 and Cys-101 demonstrated that rapid inactivation occurs only when residue 47 is cysteine (*27*). As shown in Scheme **1**, it is likely that enzyme inactivation is initiated through addition of Cys-47 to the lactone ring, which is opened in the process to form an α-bromoketone adduct. The acidity of Cys-47 confers good leaving group properties, and rapid hydrolysis occurs to generate an α-bromoketoacid intermediate. The reaction may proceed *via* alkylation of the transient thioester to form a six-membered ring episulfonium ion intermediate, which would be yet more reactive toward hydrolysis, with either process leading to the observed mass increase of 230 Da (*26*).

To study the role of the bromine in the inactivation of hGSTP1 by compound **1**, we designed and synthesized compound **2** (Scheme **2**) as a chemical probe. The rationale for the design was based on the hypothesis that the bromide acts as a leaving group in the inactivation of hGSTP1 by haloenol lactone **1**. Parallel studies were performed to test time-dependent inhibitory effects of compounds **1** and **2** on hGSTP1. Recombinant hGSTP1 was incubated with compound **1** or **2** (250 μM) in phosphate buffer (pH 7.4) at 30 °C. As expected, a time-dependent GST activity loss was observed after exposure to compound **1**, while compound **2** did not produce time-dependent inhibitory effect on hGSTP1 (Figure **1**). It may be explained that the lack of bromine at α carbon prevents the development of an α-bromoketone as an electrophilic species.



Scheme 1.



Scheme 2.

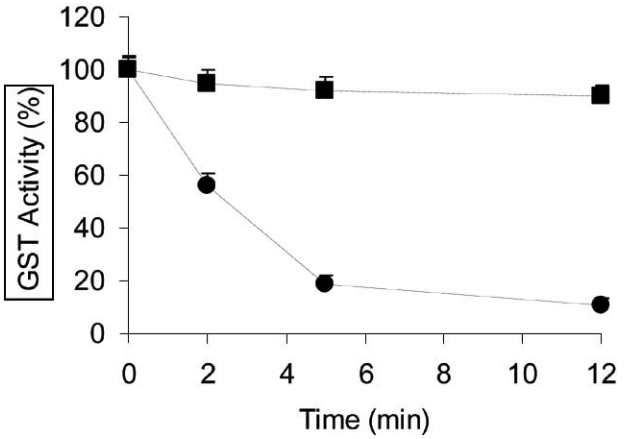


Fig. (1). Time-dependent inhibition of hGSTP1-1 by compound **1** and **2**. Recombinant hGSTP1 (3.0 mg protein contents/mL) was incubated in phosphate buffer (pH 7.4) with compound **2** (■, 250 μM) or compound **1** (●, 250 μM) at 30°C. The GST activity was determined periodically by Habig method [32].

In an attempt to test the two sequential steps of nucleophilic attack proposed in Scheme **1**, we incubated compound **1** with excess of *N*-acetyl cysteine as a nucleophilic trapping agent. The resulting mixture was analyzed by LC-MS/MS. After 1 hour of incubation at 30 °C, most of the compound **1** was found to be depleted by *N*-acetyl cysteine. A polar product was detected by LC-MS/MS with molecular ion of 539 $[M+H]^+$ m/z (Figure **2a**), which fits the molecular weight of compound **3** (538 Da). Like compound **1**, compound **2** was also found to be depleted by *N*-acetyl cysteine. However, incubation of compound **2** with excess of *N*-acetyl cysteine gave a mono-*N*-acetyl cysteine substituted adduct, compound **4**. The LC-MS/MS analysis showed that the molecular mass (378 Da *m/z*) of the resulting product from the incubation, matched the molecular weight of compound **4** (377 Da) (Figure **2b**). The mechanism for the formation of the di-*N*-acetyl cysteine substituted adduct as well as formation of the mono-*N*-acetyl cysteine substituted adduct are proposed in Scheme **3**. The lactone ring is opened through nucleophilic attack by the sulfur of *N*-acetyl cysteine to generate a thioester. The resulting enolate is tautomerized to the corresponding α-bromoketone, followed by nucleophilic replacement of bromide by the sulfur of the second *N*-acetyl cysteine to form a thioether compound through S_N2 reaction. However, only a thioester was formed after compound **2** was exposed to excess of *N*-acetyl cysteine because of lacking in substituted bromine at α-carbon of the resulting ketone as a potential leaving group. The studies above, using model compounds, support the proposed mechanism by which haloenol lactone **1** inhibits hGSTP1, and two sequential steps of nucleophilic attack may be involved in the inactivation of GSTP1 by compound **1** as shown in Scheme **1**.

Identification of the amino acid modified by haloenol lactone **1** is one of key steps in investigating the mechanism of the enzyme inactivation. GSTP1 purified by affinity chromatography was incubated with haloenol lactone **1**, followed by dialysis against water to remove small molecules. The modified protein was digested with endo-Lys

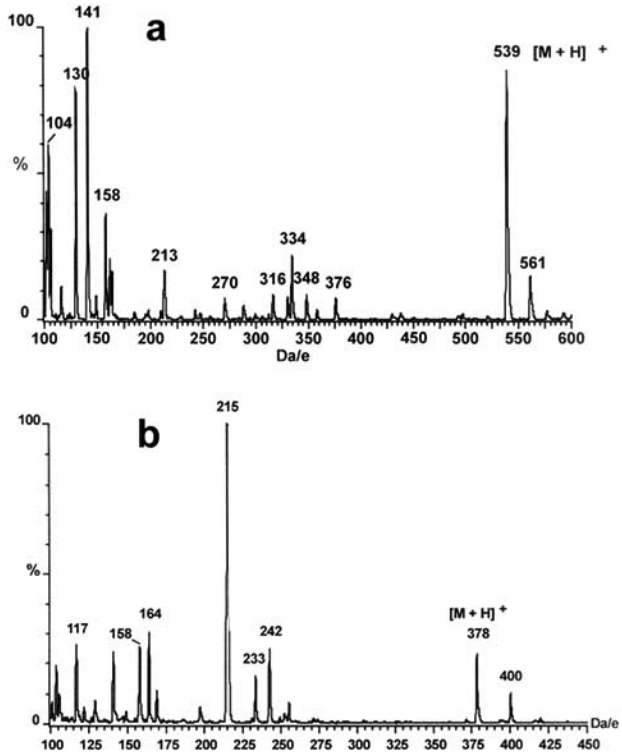
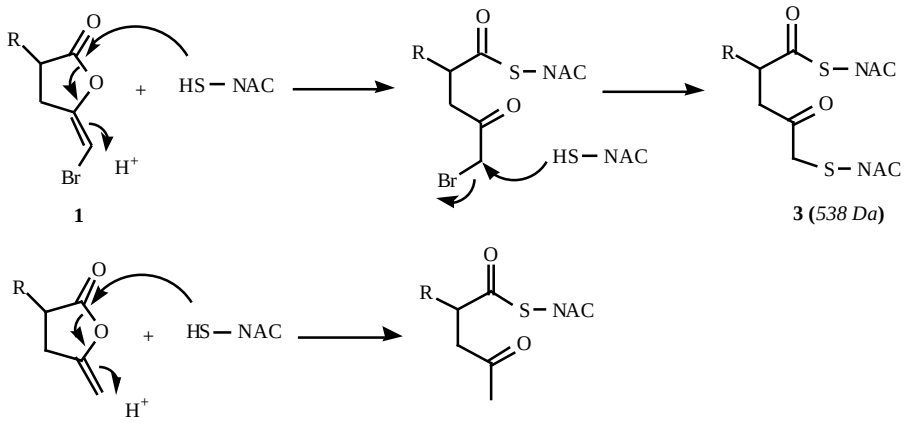


Fig. (2). Electrospray ionization mass spectra of *N*-acetyl cysteine adducts derived from compound **1** (**a**) and compound **2** (**b**). Compound **1** or **2** was incubated with 5-fold molar excess of *N*-acetyl cysteine in 0.1 M potassium phosphate buffer (pH 7.4) at 30°C for 1 hr. The resulting samples were analyzed by LC-MS/MS.

C protease, and the resulting peptides were separated on HPLC and analyzed by mass spectrometry; peptides of interest were sequenced by MS/MS. The same protocol was followed for the endo-Lys C digest of native hGSTP1. Mass spectrometry detected peptide *ASCLYGQLPK* (residues 45-54) in the digest of the native protein. The calculated MH_2^{2+} for this peptide is 540.27 and the experimentally determined MH_2^{2+} is 540.28. However, MH_2^{2+} detected by mass spectrometry for the peptide containing residues 45-54 in the digest of the haloenol lactone **1** modified protein is 655.32, which matches the calculated MH_2^{2+} (655.33) for the peptide modified with a haloenol lactone **1** [540.28 + (230.09/2) for the doublet charged ion]. The mass addition exactly matches that of intact enzyme after exposure to lactone **1** (*26, 28*). Figure **3a** shows the MS/MS spectrum for the native peptide *ASCLYGQLPK* and Figure **3b** shows the MS/MS spectrum for the haloenol lactone **1** modified peptide *ASCLYGQLPK*. Ions corresponding to fragments y_1-y_3, y_5-y_8, b_2, b_3, and a_4 detected in the MS/MS spectrum of the native peptide confirmed the sequence, ASCLYGQLPK. For the modified peptide, the same ions corresponding to fragments y_1-y_3, y_5-y_7 and b_2 were also detected in its MS/MS spectrum, which confirmed the sequence *AS***X***LYGQLPK*, except for residue Cys-47. This demonstrates that the modification did not occur on any residues *AS* and *LYGQLPK*. Comparison of the MS/MS spectrum of the modified peptide (Figure **3a**) with that of the native peptide (Figure **3b**) revealed a few new ions with m/z values of 306.1, 401.2, 429.2, 474.2, and 492.2



R: Cinnamyl; NAC: *N*-acetyl cystein

Scheme 3.

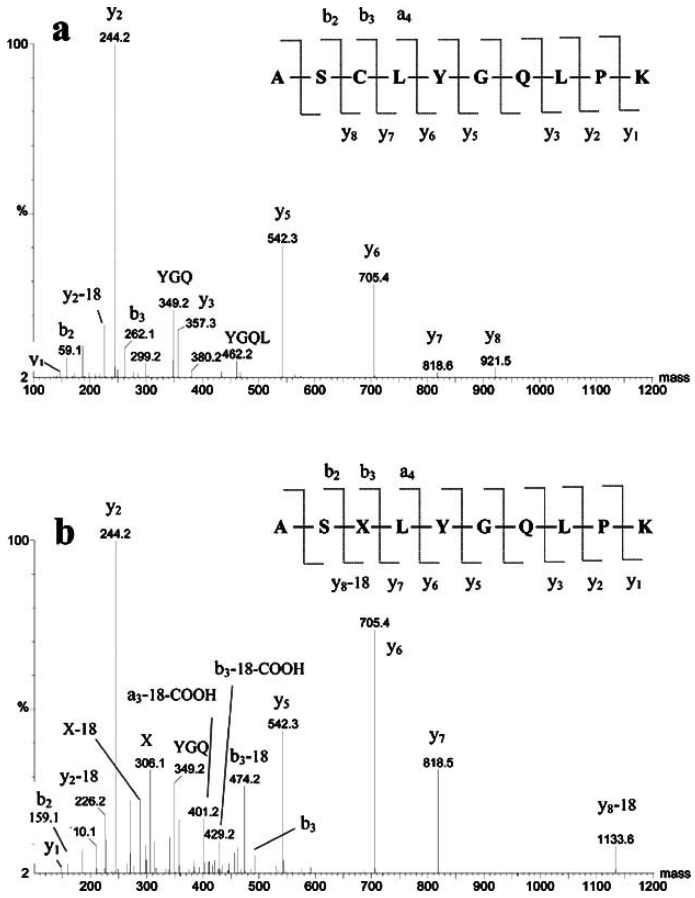


Fig. (3). MaxEnt3 deconvoluted MS/MS spectra for the peptide containing residues 45-54; **a**, native peptide; **b**, modified peptide by haloenol lactone **1**.

for the modified peptide. The m/z values of these new ions actually fit very well to fragment ions having a haloenol lactone **1** group on residue Cys-47: e.g., *m/z* 306.1 is the imminium ion of the modified Cys (calculated m/z = 306.12); *m/z* 401.2 is the fragment a_3-(H_2O + COOH) [*m/z* = 401.19]; *m/z* 429.2 is the fragment b_3-(H_2O + COOH) [*m/z* = 429.18]; m/z 474.2 is b_3-H_2O (*m/z* = 474.17); and *m/z* 492.2 is b_3 (m/z = 492.18). Thus, the MS/MS spectrum demonstrates that the cysteine residue (Cys-47) is modified with haloenol lactone **1**. Furthermore, the absence of the y_8 (*m/z* 921.5) and b_3 (*m/z* 262.1) ions, along with the presence of y_7 (*m/z* 818.5) and b_2 (*m/z* 159.1) ions, provides the solid evidence for the modification at Cys-47.

To confirm the importance of Cys-47 in the inactivation of hGSTP1 by compound **1**, we prepared C47A mutant enzyme. The cDNA of the mutant hGSTP1 was confirmed by DNA sequencing. Time-dependent inhibition studies of recombinant hGSTP1 (wild-type) and C47A mutant by compound **1** were performed at concentration of 170 μM in phosphate buffer (pH 7.4) at 30 °C. As expected, haloenol lactone **1** caused rapid inhibition of the wild type enzyme (Figure **4**). However, lactone **1** showed no inhibitory effect at all on the mutant enzyme (Figure **4**). Replacement of Cys-47 with Ala made hGSTP1 completely resistant to haloenol lactone **1**, indicating the importance of Cys-47 in the enzyme inactivation by the lactone. Our earlier studies showed that mutant C47S showed partial susceptibility to haloenol lactone **1** (*27*). We proposed that Ser-47, serving as the nucleophile like Cys-47, is capable of opening the lactone ring, but this reaction is slower than that of Cys-47 due to differences in nucleophilicities of hydroxyl and sulfhydryl. Also, it is unlikely for Ser to form an intermediate analogous to the episulfonium ion. The observed inertness of C47A mutant to compound **1** strongly supports our hypothesized mechanism of hGSTP1 inactivation by lactone **1**. Ala lacks the nucleophilic functional group responsible for opening the lactone ring. That may explain why lactone **1** shows slower inactivation of C47S than the wild type, while it shows no inhibitory effect at all on C47A.

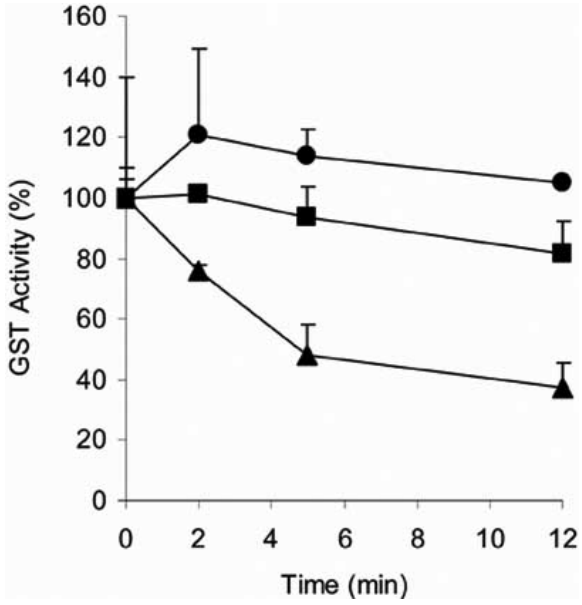


Fig. (4). Time-dependent inhibition of wild-type hGSTP1 and C47A mutant hGSTP1 by compound **1**. Recombinant wild type hGSTP1 (3.0 mg protein contents/mL) was incubated in phosphate buffer (pH 7.4) with vehicle (■) or compound **1** (▲, 170 μM) at 30 °C. C47A mutant hGSTP1-1 (3.0 mg protein contents/mL) was incubated in phosphate buffer (pH 7.4) with compound **1** (●, 170 μM) at 30°C. The GST activity was determined periodically by Habig method [32].

In summary, lactone **1** is a site-directed inactivator of GSTP1, and the enzyme participates in the formation of an α-bromoketone derivative. The resulting electrophilic species modifies Cys-47, and the protein modification causes enzyme inactivation. Although Cys-47 is not required for GST activity, modification of the sulfhydryl group of Cys-47 is critical for GST inactivation by lactone **1**.

EXPERIMENTAL SECTION

Synthesis

Solvents and reagents were purchased from commercial sources and were used without further purification except as noted below. Melting points were uncorrected on a Büchi-510. Thin-layer chromatography was carried out on glass precoated with silica gel. ^{1}H-NMR spectra were determined on a Bruker AMX-400 spectrometer and referenced to Me_4Si. Mass spectra were recorded on a MAT-95 spectrometer. All air-sensitive experiments were carried out under nitrogen with freshly distilled dried solvents. Ethanol was distilled after reaction with sodium. Chloroform and methylene chloride were distilled from CaH_2. The petroleum ether used in column chromatography had a boiling point range of 60-90 ^{o}C.

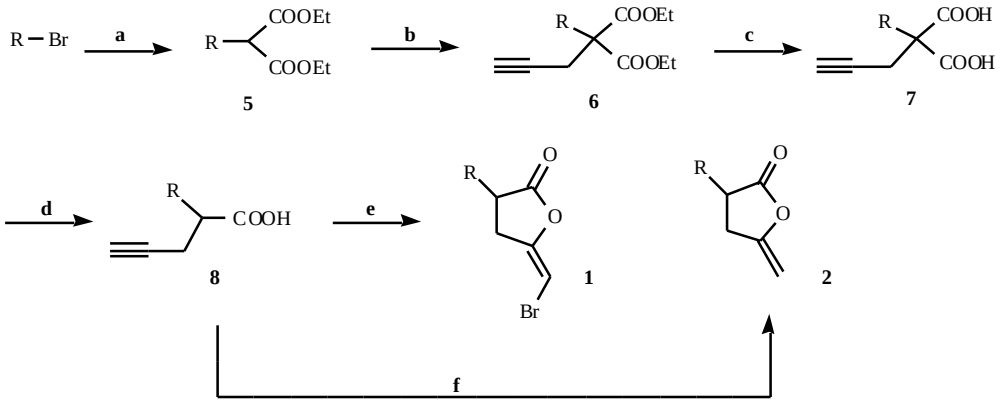
Both compounds **1** and **2** were synthesized from intermediate **8** as shown in Scheme **4**. Briefly, ethyl malonate was sequentially alkylated with cinnamyl bromide and propargyl bromide. The resulting alkylated malonic ester was hydrolyzed in aqueous NaOH and decarboxylated to generate a pentynoic acid derivative (**7**). Compound **1** was prepared through the halolactonization (*29*) of the pentynoic acid (**7**) using *N*-bromosuccinimide, and compound **2** prepared by the lactonization (*29*) of **7** by use of mercury trifluoroacetate.

2-Cinnamyl-malonic Acid Diethyl Ester (5)

To a solution of EtONa (750 mg of Na dissolved in 25 ml of EtOH) was sequentially added 4.8 g (30 mmol) of diethyl malonate and a solution of 5.88 g (30 mmol) of cinnamyl bromide in 25 ml of EtOH in dropwise. The resulting mixture was stirred overnight at room temperature. The solvent was removed by rotary evaporation, followed by addition of 20 ml of water. The crude mixture was extracted with chloroform, and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated in vacuum. Flash chromatography (petroleum ether/ ethyl acetate, 100:1) over silica gel afforded the pure **5** (2.2 g, 26.7% in yield): ^{1}H NMR ($CDCl_3$) δ 7.33-7.20 (m, 5H), 6.48 (d, J=15.74Hz, 1H), 6.16 (dt, J_1=7.32Hz, J_2=15.74Hz, 1H), 4.21 (q, J=7.32Hz, 4H), 3.50 (dt, J_1=1.1Hz, J_2=7.50Hz, 1H), 2.80 (dd, J_1=7.32Hz, J_2=7.50Hz, 2H), 1.27 (t, J=7.32Hz, 6H); EIMS *m/z* 276 (M^+), 129 (100).

2-Cinnamyl-2-propargyl-malonic Acid Diethyl Ester (6)

Sodium (334 mg, 14.5 mmol) was dissolved in 40 ml of absolute EtOH. To the EtONa solution, 2.7 g (9.6 mmol) of compound **5** was added, followed by addition of 1.3 g (10 mmol) of propargyl bromide in 20 ml of absolute EtOH. The resulting mixture was stirred at 40 ^{o}C overnight. The solvent and remaining propargyl bromide were removed by evaporation in vacuum, followed by addition of 20 ml of H_2O. The resulting aqueous was extracted with chloroform, and the combined organic layers were dried over anhydrous



a: EtONa, EtOH, diethyl malonate; **b**: EtONa, EtOH, propargyl bromide;
c: EtOH, 2N NaOH, reflux, 3h; **d**: 140 ^{o}C, 3h; **e**: NBS, $KHCO_3$, Bu_4NOH, CH_2Cl_2, H_2O, 30 min;
f: $Hg(OAc)_2$, CH_2Cl_2, 2h.
R: Cinnamyl

Scheme 4.

Na_2SO_4 and concentrated in vacuum to provide 2.6 g (87.2%) of **6** as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.34-7.22 (m, 5H), 6.52 (d, *J*=15.75 Hz, 1H), 6.04 (dt, J_1=7.69Hz, J_2=15.75 Hz, 1H), 4.23 (q, *J*=7.32Hz, 4H), 2.97 (dd, J_1=1.1Hz, J_2=7.69 Hz, 2H), 2.84 (d, *J*=2.57Hz, 2H), 2.06 (t, *J*=2.57 Hz, 1H), 1.26 (t, *J*=7.32 Hz, 6H).

2-Cinnamyl-2-propargyl-malonic Acid (7)

To a solution of 2.60 g (8.3 mmol) of **6**, in 20 ml of EtOH, was added 20 ml of 2N NaOH. The resulting mixture was heated at reflux for 3 hours. The ethanol was removed by evaporation, and the remaining aqueous was washed with chloroform. The washed aqueous was acidified to pH 2 with cold HCl and extracted with chloroform. The extracts were pooled, dried over anhydrous Na_2SO_4 and concentrated to provide 2.08 g of **7** in 97.4% yield as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 9.77 (broad s, 2H), 7.35-7.21 (m, 5H), 6.59 (d, *J*=15.74 Hz, 1H), 6.06 (dt, J_1=7.69 Hz, J_2=15.74 Hz, 1H), 3.00 (d, *J*=7.69 Hz, 2H), 2.88 (d, *J*=2.56 Hz, 2H), 2.08 (t, *J*=2.56Hz, 1H).

2-Cinnamyl-4-pentynoic Acid (8)

Compound **7** (2.00 g, 7.8 mmol) was heated at 140 ^{o}C for 3 hours and the resulting oil was chromatographed (petroleum ether/ ethyl acetate/ TFA, 30:1:0.1%) to provide 0.96 g of **8** in 57.9% yield as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 10.50 (broad s, 1H), 7.35-7.21 (m, 5H), 6.51 (d, *J*=15.75 Hz, 1H), 6. (dt, J_1=7.69 Hz, J_2=15.75 Hz, 1H), 2.80 (m, 1H), 2.72-2.51 (m, 4H), 2.05 (t, *J*=2.56 Hz, 1H).

3 - Cinnamyl - 5(*E*) - bromomethylidenetetrahydro - 2 - furanone (1)

To a solution of **8** (0.428 g, 2 mmol) in 25 ml of CH_2Cl_2 was sequentially added 356 mg (2 mmol) of *N*-bromosuccinimide, 200 mg (2 mmol) of $KHCO_3$, and 0.5 ml of Bu_4NOH (40 % in water). After stirring vigorously at room temperature for 30 min, the mixture was diluted with 10 ml of CH_2Cl_2. The CH_2Cl_2 phase was washed with 5% $Na_2S_2O_3$ and H_2O, dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography (petroleum ether/ ethyl acetate, 100:1) to afford 0.36 g of **1** in 61.4% yield as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 7.38-7.24 (m, 5H), 6.52 (d, *J*=15.74Hz, 1H), 6.12 (dt, J_1=7.32 Hz, J_2=15.74Hz, 1H), 5.99 (t, *J*=2.38 Hz, 1H), 3.12-2.98 (m, 2H), 2.80-2.74 (m, 1H), 2.68-2.62 (m, 1H), 2.58-2.50 (m, 1H); EIMS m/z 294 (M^++2), 292 (M^+), 149 (100); HREIMS calcd. for $C_{14}H_{13}BrO_2$ 292.098, found 292.0091.

3-Cinnamylmethylidenetetrahydro-2-furanone (2)

To a solution of **8** (0.428 g, 2 mmol) in 25 ml of CH_2Cl_2 was added 85.2 mg (0.3 mmol) of mercury trifluoroacetate. After stirring at room temperature for 2 h, the reaction mixture was diluted with 25 ml of CH_2Cl_2. The CH_2Cl_2 phase was washed with 5% $Na_2S_2O_3$ and H_2O, dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography (petroleum ether/ ethyl acetate, 100:1) to afford **2** in 79.2% yield as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.21-7.39 (m, 5 H), 6.51 (d, *J* = 15.9 Hz, 1 H), 6.11 (dt, J_1 = 7.2 Hz, J_2 = 15.6 Hz, 1 H), 4.75 (m, 1 H), 4.32 (m, 1 H), 3.91 (dd, J_1 = 7.1 Hz, J_2 = 8.2 Hz, 1 H), 2.74-3.45 (m, 4 H).; EIMS m/z 294 (M^++2), 292 (M^+), 149 (100); HREIMS calcd. for $C_{14}H_{14}O_2$ 214.2640, found 214.2497.

Expression of Wild Type hGSTP1

Human GSTP1-1 complete sequence was amplified by PCR from a gift vector pcDNA3.1-GSTP1 from Dr. David Eaton, University of Washington, using the PCR primers 5'-GGCCTCTAGACTCTCGAGTCACTGTTTCCCGTTGCCATTGATGG-3', 5'-GGCCTCGAGTCTAGATCACTGTTTCCCGTTGCCATTGATGG-3', followed by being ligated into a pGEM(R)-T Easy Vector (Fisher Scientific, Pittsburgh, PA). The resulting pGEM-GSTP1 plasmid was digested by HindIII and XhoI to get a GSTP1 fragment. The fragment was then inserted into a pET21a(+) vector (Novagen, San Diego, CA) by Hind III and Xho I for E. coli expression. A

Rosetta-gami™ B(DE3) competent cell (Novagen, San Diego, CA) was used for expression of wild type hGSTP1-1 in E. coli (*30*).

Expression of hGSTP1 Mutant (C47A)

A QuikChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used for mutation of hGSTP1-1 at 47 residue from cysteine to alanine in plasmid pET21a(+)-hGSTP1. Briefly, a PCR was performed using pfuTurbo DNA polymerase for replication of both plasmid strands from plasmid pET21a(+)-GSTP1. PCR primers 5'-GCTCACTCAAAGCCTCCGCTCTATACGGGCAGCTCC C-3' and 5'-GGGAGCTGCCCGTATAGAGCGGAGGCTT TGAGTGAGC-3' were used for hGSTP1 mutation. Following temperature cycling, the product was treated with Dpn I which is specific for methylated and hemimethylated DNA for digestion of the parental DNA template, to select mutation-containing synthesized DNA. The resulting mutation-containing plasmid was transformed to a DH5a host for the production of mutant-containing plasmid. The clones containing the desired mutant were identified by DNA sequencing. A Rosetta-gami™ B(DE3) competent cells (Novagen, San Diego, CA) was also used for expression of hGSTP1 mutant (C47A) in E. coli (*31*).

Enzyme Preparation

Cell pellet was homogenized in 0.1 M potassium phosphate buffer (pH 7.4) by sonication. The homogenate was centrifuged at 5000g for 20 min at 4°C. The supernatant fraction was then collected and centrifuged at 14,000g for 30 min 4°C. The resulting supernatant was used freshly for enzyme activity and kinetic studies.

Kinetics of Enzyme Inhibition

To 300 μL of 0 ºC cell supernatant solution containing 3.0 mg/mL protein content was added 5 μL of stock solution containing compound **1** or **2**. Aliquots were withdrawn for enzyme activity determination and the remaining solution was immediately placed in a water bath for incubation at 30 ºC. Aliquots were withdrawn at 0, 2, 5, and 12 min, and GST activity was determined using spectrophotometric assays described below.

Enzyme Assays

Glutathione *S*-transferase activity was measured using GSH and CDNB as substrates according to the method of Habig (*32*). The activity of the enzyme was determined in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1.0 mM GSH and 1.0 mM CDNB. The rate of product formation was monitored by measuring the change in absorbance at 340 nm using a 96-well microplate reader (VersaMax, Molecular Devices Co. Sunnyvale, CA). Enzyme activities were calculated after correction for non-enzymatic reaction.

GST Modification

To 5.0 mg of pure recombinant human GSTP1 in 25 ml of potassium phosphate buffer (pH 7.4) was added **1** (1.0 mg in 1.0 mL of absolute ethanol) or **2** (0.1 mg in 1.0 mL of ethanol). The resulting mixtures were incubated at 30°C for 2 hours and dialyzed against distilled water for mass spectrometry analysis.

Digestion of GST and Analysis of the Digests by LC-MS/MS

Protein samples of about 5 μg were digested 5% (w/w) of endoproteinase Lys-C (Wako Pure Chemical Industries, Ltd.) in 0.1 M Tris-HCl, 1 mM EDTA, pH 8.0, at room temperature for 20 h. The digest then was adjusted with urea to 4M and with DTT to 20 mM. After one hour at room temperature the digest was analyzed on an LC-MS system composed of a reversed-phase HPLC (2695 Alliance Separations Module), a dual wavelength UV detector, and a LCT mass spectrometer (Waters Corp., Milford, MA). A 1.0-mm x 25-cm YMC C18 column (Waters AA12S052501 WT) was used with a 70-min gradient (0-70% acetonitrile) in 0.03% TFA at a flow rate of 0.07 mL/min. MS spectra were collected in m/z range 400-2000, with sampling every 1 sec, with 0.1 sec separation between consecutive spectra. Peaks from peptide maps were identified using MassLynx software version 4.0.

Sequencing of GST Peptides

Peptides of interest were sequenced by LC-MS/MS experiments on a LC-Q-TOF mass spectrometer system, equipped with a Z-Spray electrospray source (Model API US, Waters Corp., Milford, MA). LC Experimental conditions were the same as described above. MS/MS spectra were acquired using the data dependent acquisition function (DDA). MS/MS spectra were collected in the m/z range 50-1500, with sampling every 0.3 sec, 0.05 sec separation between consecutive spectra. MS/MS spectra were analyzed using Mass Lynx software version 4.0 with MaxEnt3 and BioLynx options.

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ABBREVIATIONS

Ala	=	Alanine
CDNB	=	1-chloro-2,4-dinitrobenzene
Cys	=	Cysteine
EDTA	=	Ethylenediamine tetraacetic acid
ESI-MS	=	Electrospray ionization mass spectrometry
hGSTP1	=	Human glutathione S-transferase pi
GSH	=	Glutathione
mGSTA3	=	Mouse GST alpha
mGSTM3	=	Mouse GST mu
mGSTP1	=	Mouse GST pi
Ser	=	Serine
NAC	=	*N*-acetyl cysteine

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