Enzymology:

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J. Biol. Chem. 1996, 271:20421-20425.
doi: 10.1074/jbc.271.34.20421

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 34, Issue of August 23, pp. 20421–20425, 1996
Printed in U.S.A.

Haloenol Lactone Is a New Isozyme-selective and Active Site-directed Inactivator of Glutathione *S*-Transferase*

(Received for publication, May 31, 1996, and in revised form, June 28, 1996)

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A haloenol lactone derivative has been synthesized and found to be an isozyme-selective and active site-directed inactivator of glutathione *S*-transferase (GST). Preincubation of the haloenol lactone (100 μM) with murine Alpha, Mu, or Pi GST isozyme (1.0 μM) at pH 6.5, 37 °C resulted in time-dependent loss of enzyme activity with highly selective inhibition of the Pi isozyme ($t_{1/2}$, ~ 2 min). In a separate experiment, a 10-fold excess of the lactone was incubated with GST-Pi isozyme at 37 °C for 3 h, followed by dialysis against Nanopure water. GST activity lost upon incubation with the lactone could not be restored by exhaustive dialysis, and only 8% of enzyme activity for the modified GST remained relative to the control that was treated identically except the lactone was omitted from the incubation. Both control and modified GST were characterized using electrospray ionization mass spectrometry. No native GST (23,478 Da) was observed in the spectrum of modified GST. Instead, protein incubated with the lactone exhibited an increase in molecular mass of 230 Da relative to control GST. The lactone (100 μM) was incubated with GST Pi isozyme (1.0 μM) in the presence of the competitive inhibitor *S*-hexylglutathione (10 μM), which suppressed time-dependent inhibition of GST by the lactone. The results suggest that this haloenol lactone is an irreversible and active site-directed inhibitor of GST that appears to inhibit the enzyme through two consecutive steps of nucleophilic attack.

Glutathione *S*-transferases (GSTs)[1] are a family of enzymes that play a critical role in protection of cells from carcinogenic and cytotoxic xenobiotics by catalyzing the addition of GSH to electrophiles or by donating reducing equivalents to organic hydroperoxides. Four principal classes of cytosolic GST isozymes have been isolated from various eukaryotes and named Alpha, Pi, Mu, and Theta based upon biochemical and immunological properties, amino acid sequences, and x-ray crystal structures (1–3). GSTs exhibit broad and overlapping substrate specificities and can also bind various nonspecific ligands (4). The catalytic mechanisms of GSTs are not fully understood but have been the subject of intense investigation (5, 6). It has been reported that the sulfhydryl group(s) of GST are reactive to certain electrophilic and oxidizing reagents (7–11), although site-directed mutagenesis studies showed that these sulfhydryl groups are not required for GST catalysis (12–14). These enzymes are also responsible for metabolism (deactivation) of many drugs used in the treatment of cancer (15), and it has become evident that overexpression of GST isozymes (particularly the Pi isozyme) plays a significant role in acquired drug resistance of tumor cells (16, 17). In this study, we report a haloenol lactone derivative, compound **1**, as a new isozyme-selective and potent active site-directed inactivator of GST. This haloenol lactone derivative can potentially be used as a synergetic agent for cancer chemotherapy, particularly for the treatment of GST overexpression-mediated drug-resistant cancers.

MATERIALS AND METHODS

Synthesis—Compound **1,** 3-cinnamyl-5(*E*)-bromomethylidenetetrahydro-2-furanone, was synthesized as shown in Scheme I. Specific details of the synthetic procedures used to prepare compound **1** and other analogs will be described in a separate study. Briefly, ethyl malonate was sequentially alkylated with cinnamyl chloride and propargyl bromide. The resulting alkylated malonic ester was hydrolyzed in aqueous NaOH and decarboxylated to generate a pentynoic acid derivative (**2**). Haloenol lactone **1** was prepared through the halolactonization (18) of the pentynoic acid (**2**) using *N*-bromosuccinimide. Purity of compound **1** was estimated to be about 95% based upon analysis by FAB-MS and HPLC. ^{1}H-NMR ($CDCl_3$): 7.2–7.4 (5H, m), 6.52 (1H, *d*, J = 15.9 Hz), 6.11 (1H, *tt*, J = 7.2, 15.6 Hz), 6.02 (1H, s), 2.9–3.1 (2H, m), 2.5–2.8 (3H, m). FAB MS: m/z 293, 295 $[M+H]^+$. High resolution FAB MS: m/z 293.0169 $[M+H]^+$; calculated for $C_{14}H_{14}O_2{}^{79}Br$, m/z 293.0177.

Enzyme Purification—The three major mouse liver GSTs were purified according to established methods (19). Briefly, mice were killed by cervical dislocation, and livers were removed and rinsed with ice-cold 10 mM Tris buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol (buffer A). The tissues were homogenized in buffer A (1.0 g of tissue/2 ml of buffer). Centrifugation and all subsequent purification steps were carried out at 4 °C. The homogenate was centrifuged at 10,000 × *g* for 30 min. The supernatant fraction was then collected and centrifuged at 100,000 × *g* for 80 min. Supernatant was next passed through a Sephadex G-25 column (100 × 2.5 cm), which had been pre-equilibrated in 25 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol (buffer B). Column eluants were monitored at 280 nm using an Isco-UA5 detector. The eluant from the G-25 column containing GST activity was applied to an affinity column (11 × 2.5 cm, GSH-linked agarose, Sigma) at a flow rate of 0.64 ml/min. The first two GST isozymes were eluted using a linear gradient (0–75 mM) of GSH in buffer B (250 × 250 ml). The third peak containing activity toward CDNB was eluted by using a linear gradient of increasing pH (7.4–9.0) in buffer B, which was 75 mM in GSH (150 × 150 ml). The three main peaks of activity were recovered and pooled separately. Each pool was concentrated to 25–30 ml in an Amicon ultrafiltration cell (Amicon, Inc., Beverly, MA) with a PM 10 membrane and dialyzed against 4 liters of Nanopure water. Pooled proteins were analyzed by ESI-MS to determine purity and subunit molecular weight. Dialyzed concentrates were lyophilized and stored at −80 °C.

Kinetics of Enzyme Inhibition—A solution of glutathione *S*-transferase (1.0 μg/μl) was prepared in a 0.1 M potassium phosphate buffer (pH

* This work was supported by the University of California, Davis NIEHS Superfund Basic Research Program (Grant 2P42 ES04699–08) and the University of California, Davis NIEHS Center for Environmental Health Science (Grant 1P30 ES05707). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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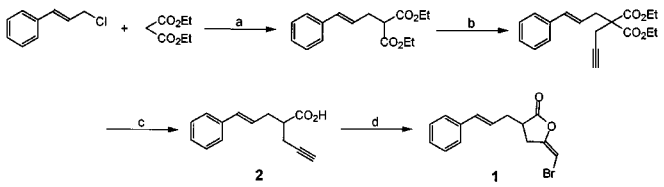
[1] The abbreviations used are: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment ionization mass spectrometry; HPLC, high performance liquid chromatography.



SCHEME I. *a*, NaOH/EtOH for 1.5 h; *b*, propargyl bromide/NaOEt/EtOH for 1 h; *c*, 1 N NaOH at 80 °C for 1 h or at 140 °C for 2 h; *d*, *N*-bromosuccinimide/$KHCO_3$/Bu_4NOH/CH_2Cl_2/H_2O for 0.5 h.

6.5). To 350 μl of 0 °C 0.1 M potassium phosphate buffer (pH 6.5) was sequentially added 10 μl of the reconstituted GST solution and 5 μl of a solution containing the haloenol lactone or *N*-ethylmaleimide in ethanol. In some experiments 1.0 mM *S*-hexylglutathione dissolved in a 0.1 M potassium phosphate buffer (pH 6.5) was added in the incubation solution. Aliquots (50 μl) were withdrawn for enzyme activity assay, and the remaining solution was immediately transferred to a water bath for incubation at 37 °C. Aliquots were withdrawn at 1, 3, 5, and 8 min, and GST activity was determined using a spectrophotometric assay described below.

Enzyme Assays—Glutathione *S*-transferase activity was measured using GSH and CDNB as substrates according to the method of Habig *et al.* (20). The activity of the enzyme was determined in a 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM GSH and 1 mM CDNB. The rate of product formation was monitored by measuring the change in absorbance at 340 nm using a Shimadzu PC-2101 uv-visible spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Enzyme activities were calculated after correction for nonenzymatic reaction. Specific activities are based on protein concentrations as determined using Bio-Rad protein spectrophotometric assay kits.

Identification of Hydrolysis Product of Haloenol Lactone 1—To 100 ml of 0.1 M potassium phosphate buffer (pH 8) was added 70 mg of compound **1** dissolved in 10 ml of ethanol, followed by incubation in a water bath shaker at 37 °C for 36 h. The mixture was extracted with chloroform, and the aqueous layer was acidified to pH 5 with dilute HCl. The hydrolytic product was extracted with chloroform and purified by a preparative thin layer chromatography plate developed with 50% ethyl acetate in hexane with 2% acetic acid. The structure of the purified product was elucidated by ^{1}H NMR and mass spectrometry.

Electrospray Ionization Mass Spectrometry—Lyophilized proteins were dissolved in acetonitrile/water (50:50) to give a final concentration of 1 μg/μl (40 pmol/μl). Intact proteins were analyzed on a VG/Fisons Quattro-BQ triple quadrupole mass spectrometer (VG Biotech, Altrincham, UK) using acetonitrile/water (50:50) + 0.05% formic acid as the mobile phase. Proteins were analyzed by direct flow injection. Spectra were obtained in positive ion mode using a capillary voltage of +3.5 kV. The cone voltage was set at 50 V. Spectra were scanned over the range of 600-1400 *m/z* at 20 s/scan and summed using the MCA acquisition mode in the Fisons Masslynx software.

RESULTS AND DISCUSSION

N-Ethylmaleimide, ethacrynic acid, iodoacetamide, 5,5′-dithiobis(2-nitrobenzoate), and 1-chloro-2,4-dinitrobenzene have been reported to inactivate glutathione *S*-transferases by chemical modification of reactive sulfhydryl groups of the enzyme (7–9, 21, 22). Dibromodihydroethacrynic acid, an α-bromoketone derivative, has demonstrated higher potency but less isozyme selectivity of GST inactivation than ethacrynic acid (10). We designed and synthesized haloenol lactone **1**, an α-bromoketone precursor, as an inactivator of GST. This haloenol lactone inactivator was designed to take advantage of the presence of multiple potentially reactive nucleophiles present in the active site region in GST-Pi. The reactivity of the acidic cysteine residue (Cys-47) has been documented (21, 22), and similarities to the reactivities of cysteine proteases have been noted (23). The sulfhydryl group of enzyme-bound GSH could serve as an activating nucleophile as it has also been shown to be acidic (24), with the neighboring Tyr-7 residue serving as a general base. Recent crystallographic studies revealed the presence of water molecules near the GSH binding site (6). Furthermore, the research of Caccuri and co-workers (25) suggested that product release can be the rate-limiting step in

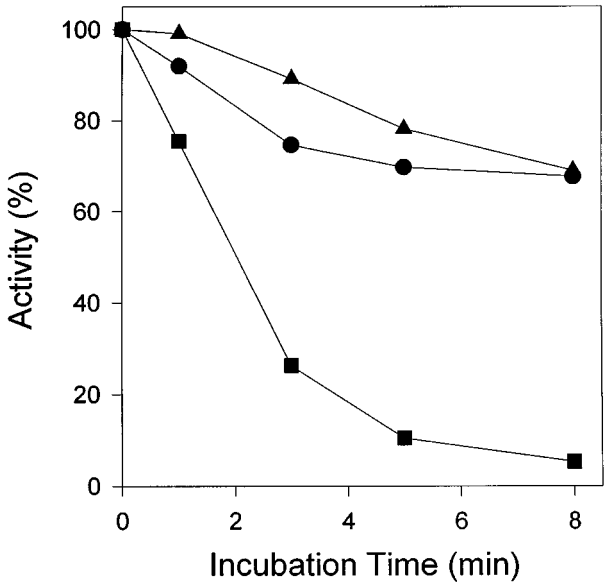


FIG. 1. **Time-dependent inhibition of GSTs by haloenol lactone 1.** Purified mouse GST (1.0 μM) was incubated with haloenol lactone **1** (100 μM in 0.1 M potassium phosphate buffer (pH 6.5) at 37 °C. The GST activity was determined periodically by the Habig method (20). ●, Alpha isozyme; ▲, Mu isozyme; ■, Pi isozyme.

GSH conjugation catalyzed by GST-Pi. The combination of these factors suggested that several nucleophiles near the active site of GST-Pi could facilitate the opening of the lactone ring, leading to formation of an α-bromoketone intermediate capable of irreversible modification of the protein with isozyme selectivity based upon the reactivity of residues near the active site.

Glutathione *S*-transferase Alpha, Pi, and Mu isozymes were isolated from mouse liver with specific activity (toward CDNB) of 64, 74, and 244 μmol·min^{-1}·mg^{-1}, respectively, and no impurity determined by ESI-MS was observed in each protein sample. Small amounts of enzyme-bound GSH may have been present based upon separate experiments. The inactivation of GST by compound **1** was initially studied by testing its time-dependent inhibitory effect upon GSTs. An excess of lactone **1** (100 μM) was incubated with each isozyme (1.0 μM) in phosphate buffer (pH 6.5) at 37 °C. As shown in Fig. 1, compound **1** demonstrated a selective inhibitory effect on Pi GST isozyme, and only 4% of enzyme activity remained after 8 min with a $t_{1/2}$ of inactivation of about 2 min. For comparison, *N*-ethylmaleimide (100 μM) as a GST inactivator was incubated with these three GST isozymes (1.0 μM) in phosphate buffer (pH 6.5) at 37 °C. Similar to compound **1**, 3% of GST-Pi activity remained in 8 min, and isozymes Alpha and Mu were slightly inactivated by *N*-ethylmaleimide. To confirm the irreversible enzyme inhibition, a 10-fold excess of lactone **1** was incubated with GST-Pi isozyme at 37 °C for 3 h, followed by dialysis against six changes of Nanopure water to facilitate mass spectrometric analysis. The dialyzed protein sample was dried by lyophilization. The recovered protein was redissolved in Nanopure water, and specific enzyme activity was determined by measuring the protein content and GST activity. The GST activity lost upon incubation with compound **1** could not be restored by exhaustive dialysis, and only 8% of enzyme activity for the GST modified by compound **1** remained relative to the control, which was treated identically except for the absence of compound **1**. Dialysis did not cause appreciable reduction in GST activity, as greater than 95% of the catalytic activity was recovered after dialysis for the control GST. This finding points to irreversible covalent modification and inhibition of GST Pi by compound **1**.

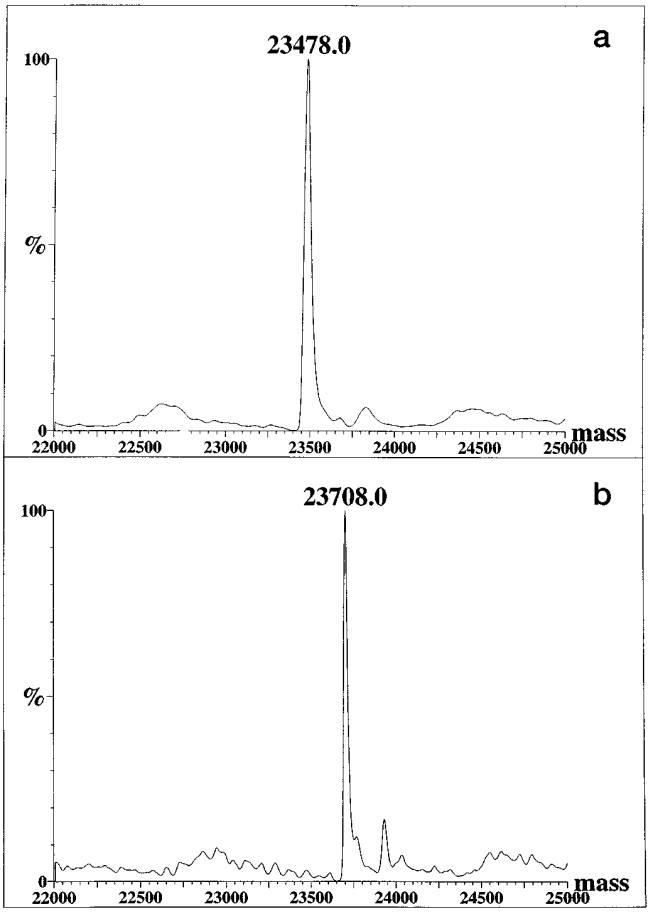
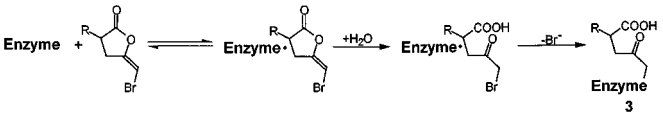


FIG. 2. **Electrospray ionization MaxEnt™ transformed mass spectra of native GST-Pi isozyme (*a*) and modified GST-Pi isozyme (*b*).** GST-Pi isozyme was incubated with vehicle or a 10-fold excess of lactone **1** at 37 °C for 3 h, followed by dialysis against six changes of Nanopure water at 4 °C. Both were then lyophilized, redissolved, and analyzed by electrospray ionization mass spectrometry.



SCHEME II

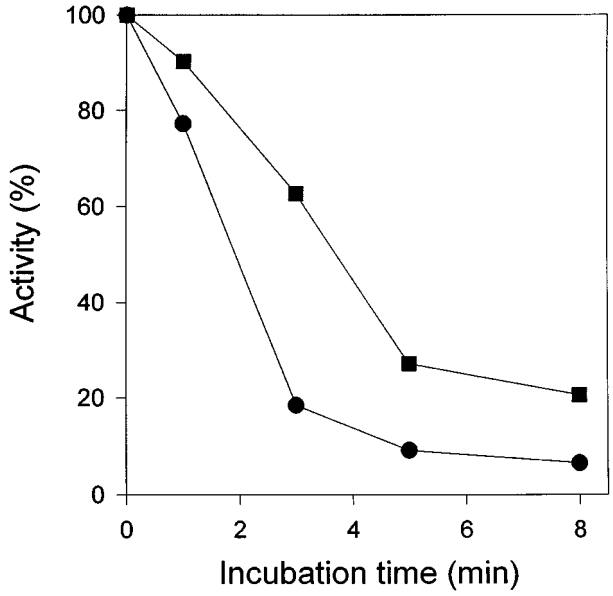


FIG. 3. **Inactivation of GST Pi isozyme by haloenol lactone 1.** The GST-Pi (1.0 μM) was incubated with haloenol lactone **1** (100 μM) in the absence of *S*-hexylglutathione (●) or in the presence of *S*-hexylglutathione (■, 10 μM) in 0.1 M potassium phosphate buffer (pH 6.5) at 37 °C. The GST activity was monitored periodically using the Habig method (20).

To confirm the covalent modification of GST, both native and modified enzymes were analyzed using electrospray ionization mass spectrometry (ESI-MS). Native GST-Pi (23,478 Da) was not detectable in the ESI mass spectrum of GST-Pi incubated with a 10-fold excess of compound **1** (Fig. 2). Separate experiments indicated that approximately 3% of unmodified GST could have been detected if present. It is not clear why 8% of GST activity remained after incubation with compound **1**, because no evidence was found to suggest the presence of other GST isozymes as contaminants. Though it is conceivable that small amounts of native GST might not be visible in the mass spectrum, residual activity may be attributable to a minor amount of covalent modification occurring at a residue distant from the active site. Such modifications would not be distinguishable from active site modifications in the ESI mass spectra. An alternative possibility is that the modification does not completely eliminate catalytic activity. A mass addition of 230 Da relative to the molecular weight of the control GST-Pi isozyme was observed after incubation with the inhibitor. This mass addition matched the molecular mass of GST inhibitor adduct, compound **3** (Scheme II). These mass spectra demonstrated a loss of bromine from compound **1** and provide strong evidence of nucleophilic attack by a nucleophile of the enzyme and displacement of the bromine substituent at C_α (Scheme II). To probe whether inactivation involved the enzyme active site, compound **1** (100 μM) was incubated with GST-Pi isozyme (1.0 μM) in the presence of *S*-hexylglutathione (10 μM), a known competitive inhibitor of GST. As shown in Fig. 3, this concentration of *S*-hexylglutathione caused a 2-fold reduction in the rate of time-dependent inhibition of GST by compound **1**. In other experiments, the presence of 1.0 mM *S*-hexylglutathione completely suppressed the time-dependent inhibition of GST-Pi by compound **1** (data not shown). This critical finding suggested that compound **1** inhibits the enzyme by chemical modification at or near the active site rather than random modification at other sites. In a parallel study, *S*-hexylglutathione (10 μM) was unable to protect the GST-Pi (1.0 μM) from inactivation by *N*-ethylmaleimide (100 μM) (not shown), implying that *N*-ethylmaleimide inactivated GST by modification of the protein at sites other than the active site, thus documenting less selectivity of modification.

The molecular mass of the protein-inactivator adduct documents the displacement of bromine and covalent addition of one water molecule. Two mechanisms can be invoked to explain this behavior. Addition of a side chain nucleophile to the lactone ring would effect ring opening and formation of the α-bromoketone moiety, allowing a second nucleophile to displace bromide. Subsequent hydrolysis of the initial attachment may occur during dialysis. An alternative explanation proposes active site hydrolytic opening of the lactone ring of compound **1** followed by displacement of bromide by a reactive side chain such as Cys-47. This latter mechanism would suggest esterase activity for the GST-Pi active site. Spontaneous nonenzymatic hydrolysis of the lactone is slow, as determined by recovery of 94 and 87% of compound **1** (determined by HPLC) after 10 min of incubation in phosphate buffer at pH 6.5 and 7.4 at 37 °C (conditions otherwise identical to incubation of compound **1** with enzyme), respectively. These kinetic observations suggest the microenvironment of the enzyme-inhibitor complex accel-

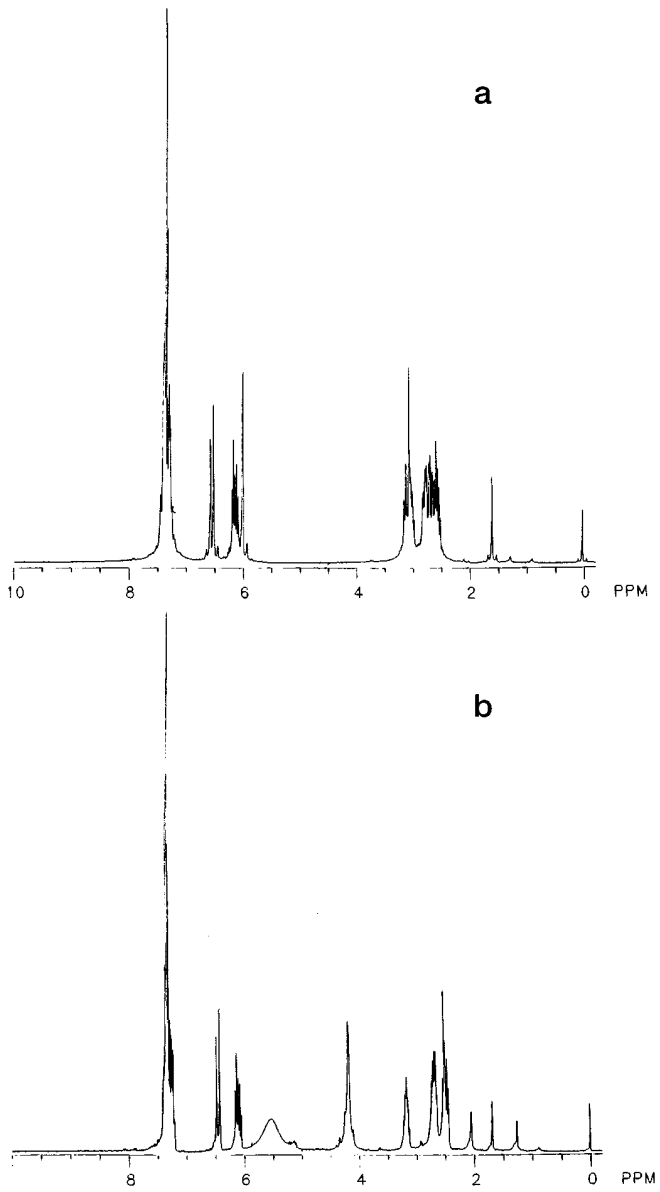


FIG. 4. **Proton NMR spectra ($CDCl_3$ as solvent) of haloenol lactone 1 (*a*) and its hydrolytic product (*b*).**

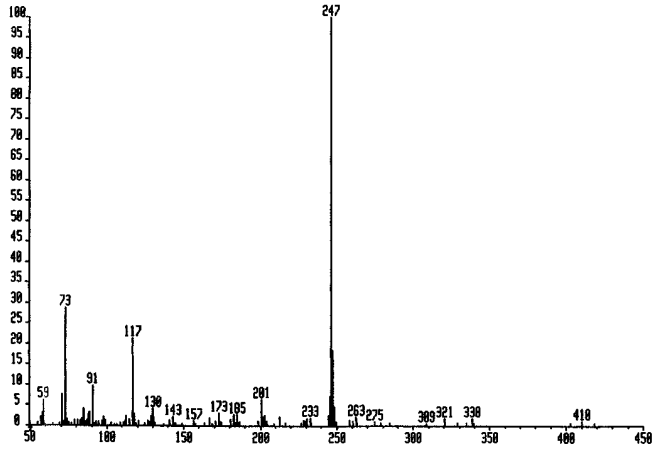
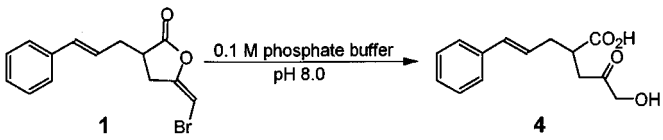


FIG. 5. **Negative mode fast atom bombardment mass spectrum of hydrolytic product of haloenol lactone 1.**



SCHEME III

erates opening of the lactone ring, resulting in formation of the active α-bromoketone intermediate.

To determine the role of the product formed from spontaneous hydrolysis in the inactivation of the enzyme, compound **1** was hydrolyzed by incubation in pH 8.0 phosphate buffer at 37 °C. After 36 h of hydrolysis, a polar product along with the starting material was found by HPLC. The resulting product was isolated by thin layer chromatography and identified by proton NMR and mass spectrometry. The proton NMR spectrum of the hydrolytic product (Fig. 4*b*) showed that the vinyl proton at 6.01 ppm observed in the proton NMR spectrum of compound **1** (Fig. 4*a*) disappeared, and two protons at 4.17 ppm were observed instead, indicating that the enol lactone ring had been hydrolyzed and a tautomeric keto intermediate had been produced. The negative mode FAB mass spectrum of the product showed a base peak at *m/z* 247 corresponding to $[M\text{-}H]^-$ rather than *m/z* 309 anticipated for the α-bromoketone derivative (Fig. 5). The molecular mass of 248 Da is consistent with the α-hydroxyketone compound **4** (see Scheme III for the structure). The observation of compound **4** as the hydrolytic product of compound **1** indicates that the bromo group at the α-carbon of the hydrolytic product was replaced by a hydroxy group. As expected, this α-hydroxyketone derivative (**4**) showed no inhibitory effect on GSTs (data not shown). All efforts to isolate the initial α-bromoketone intermediate were unsuccessful, as only the starting lactone or the α-hydroxyketone was observed, and no additional peaks were observed in HPLC chromatograms to suggest accumulation of the α-bromoketone intermediate. Though we are unable to completely rule out spontaneous nonenzymatic hydrolysis as a source of low levels of a transient α-bromoketone intermediate, our results indicate the resulting α-bromoketone intermediate is more reactive toward water than the haloenol lactone and suggest ring opening of the haloenol lactone occurs after formation of the enzyme-inhibitor complex. The initial α-bromoketone intermediate further modifies the protein via alkylation of nearby nucleophilic residues. There are two suggested mechanisms by which the enol lactone gets hydrolyzed. 1) The nucleophile involved in ring opening is not an amino acid side chain but instead is a water molecule bound near the active site; or (2) ring opening is effected by a nucleophilic side chain, and the resulting adduct undergoes rapid hydrolysis in a manner analogous to the mechanism of catalysis of soluble epoxide hydrolase (26). Either mechanism implies GST-Pi possesses esterase-like activity. Based upon these findings, we propose that enzyme-mediated hydrolysis of the lactone generates the reactive α-haloketone intermediate that forms a covalent adduct with GST-Pi. The inertness of the lactone toward nonenzymatic hydrolysis coupled with the evidence of hydrolysis and bromide displacement provided by the ESI mass spectrum of the protein-inhibitor adduct indicates that compound **1** inhibits GST by a different mechanism than simple one-step alkylation of the sulfhydryl group of Cys-47 typical of electrophilic reagents such as *N*-ethylmaleimide. It appears that GST-Pi inactivation by compound **1** involves two consecutive steps, *i.e.* hydrolytic ring opening and nucleophilic displacement (Scheme II), and such features of the mechanism may confer greater selectivity for irreversible inactivation of GST-Pi by compound **1** and related analogs.

In summary, haloenol lactone **1** was found to be a potent and selective inactivator of mouse GST-Pi isozyme. Chemical mod-

ification of the protein by compound **1** takes place at the active site of the enzyme, and the bromine of the haloenol lactone is lost during inactivation of GST-Pi. The process of enzyme inactivation is believed to involve two consecutive steps including hydrolysis and nucleophilic displacement. Further mechanistic studies of enzyme inactivation by compound **1** and related analogs are under way.

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