# Identification of a *S*-Hexahydro-1*H*-azepine-1-carbonyl Adduct Produced by Molinate on Rat Hemoglobin $\beta_2$ and $\beta_3$ Chains in Vivo

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Molinate is a thiocarbamate herbicide used in the rice industry for over 25 years, and regulatory reports have shown that administration of molinate results in reproductive toxicity in male rats. Previous in vitro studies indicate that molinate undergoes oxidative metabolism, forming reactive electrophilic intermediates capable of undergoing nucleophilic addition by protein nucleophiles. On the basis of in vitro studies, carbamylation of an active site serine residue in Hydrolase A has been proposed to be the mechanism responsible for the observed testicular toxicity. The experiments presented here utilize hemoglobin to characterize covalent protein modifications produced in vivo by molinate. Rats were dosed intraperitoneally with molinate as a function of exposure duration. Examination of globin from molinate-treated rats by HPLC demonstrated a new peak in the isolated samples and, when collected and analyzed using MALDI-TOF MS, revealed a 126 Da increase in mass relative to the native $\beta_3$ chain. Digestion of the globin using Glu-C and analysis by MALDI-TOF MS revealed two modified peptide fragments at *m*/*z* 2743 and 4985 consistent with a 126 Da increase to peptide fragments [122−146] and [102−146] in the unmodified $\beta_2$ and $\beta_3$ chains of globin. Using selected reaction monitoring LC/MS/MS, *S*-hexahydro-1*H*-azepine-1-carbonyl cysteine (HHAC-Cys) was identified in the globin hydrolysates isolated from the molinate-treated rats, but not in the control samples, and the quantity of adduct exhibited a cumulative dose response. These experiments demonstrate the ability of molinate to covalently modify proteins in vivo in a dose dependent manner. For hemoglobin this modification was a carbamylation at Cys-125 similar to the modification produced by disulfiram and *N*,*N*-diethyldithiocarbamate. The ability of molinate to covalently modify cysteine residues provides a potential mechanism to account for enzyme inhibition following molinate exposure and suggests that enzymes with cysteine residues in their active site may be inhibited by molinate.

## Introduction

Molinate (Ordram) is a widely used thiocarbamate herbicide that is particularly important in the rice industry (*1*). Previously, molinate administration was shown to cause reproductive impairment in male rats marked by a disruption of spermatogenesis and a reduction in fertility characterized by a testicular lesion seen after a single dose (≥200 mg/kg) of molinate. Molinate has been shown to undergo metabolic bioactivation via oxidation to form the reactive metabolites molinate sulfoxide and molinate sulfone (*2*). Both of these electrophilic species are capable of forming glutathione conjugates and can be further metabolized into the mercapturates and excreted in the urine (*2*). Previous studies have supported the sulfoxidation of molinate as the bioactivation pathway responsible for the testicular toxicity observed following molinate administration (*3*). Inhibition of Hydrolase A, a carboxylesterase required for the production of testosterone in the rat, is the proposed mechanism of testicular toxicity. Evidence supporting this mechanism was derived from observations that molinate inhibits esterase activity in Leydig cells following administration to rats and that molinate, molinate sulfoxide, and molinate sulfone demonstrate increasing potencies for inhibition of liver and testis nonspecific esterase in vitro (*4*). Upon the basis of the specificity of radiolabeled molinate binding and the molecular weight of the modified protein, it was proposed that molinate was binding to Hydrolase A through carbamylation of an active site serine.

Disulfiram, a dithiocarbamate, carbamylates proteins in vivo, presumably through generation of an *S*-methyl *N*,*N*-diethylthiocarbamate sulfoxide or sulfone metabolite (*5*). Consequently, administration of disulfiram to rats results in high levels of carbamylation on hemoglobin that is selective for Cys-125 located on the $\beta_2$ and $\beta_3$ globin chains. Similarly, in vivo carbamylation of the active site cysteine residue of mitochondrial aldehyde dehydrogenase (ALDH)[1] by disulfiram has been observed and proposed to be responsible for inhibition of this enzyme (*6*). Because disulfiram and molinate are both metabolized to thiocarbamate sulfoxides and sulfones, molinate may produce similar protein modifications to those observed for disulfiram. This suggests that hemoglobin may be a good surrogate for characterizing protein modifications produced by molinate in vivo as well.

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The investigation presented here was performed to extend previous in vitro studies through determining if molinate exposure results in covalent protein modifications in vivo and to identify the adducts generated and the amino acid residues modified. To accomplish this, rats were exposed to molinate as a function of dose administered in 4 and 11 day durations and hemoglobin isolated and analyzed using mass spectrometry and HPLC. Characterization of the protein modifications produced by molinate will aid in evaluating potential biological effects of molinate in addition to testicular toxicity, and may provide a basis for the development of biomarkers of exposure or effect.

## Materials and Methods

**Chemicals**. Sigma Chemical Co. (St. Louis, MO) was the source for corn oil and Endoproteinase Glu-C (from *Staphylococcus aureus* strain V8). J. T. Baker (Phillipsburg, NJ) was the source for poly(ethylene glycol) 200 (PEG). The Alzet osmotic minipumps (2 mL capacity, 4 week delivery) were obtained from Durect Corporation (Cupertino, CA). Unless otherwise specified, additional chemicals were also obtained from commercial sources.

**Animals**. This study was performed in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee. For animal exposures, male Sprague Dawley rats, 16 weeks old and 370−450 g, (Harlan, Sprague Dawley, Indianapolis, IN) were used. Rats were housed in a room on a 12 h diurnal light cycle and given rodent chow and water ad libitum.

**Molinate Exposures**. Prior to molinate administration, male rats ($n = 4$) were anesthetized and 0.5 mL of whole blood was collected in a heparinized syringe from the tail vein and globin isolation performed. A once daily dose of 100 mg/kg molinate in corn oil (320 $\mu$L of molinate added to 1.78 mL corn oil) was then given by intraperitoneal injection for a period of 4 days. On day 5, the rats were anesthetized and blood was drawn again for globin isolation.

For the second exposure experiment, four week Alzet osmotic minipumps delivering 2.5 $\mu$L/h of molinate in PEG 200 (389 mg/mL) for an effective dose of 1 mmol/kg/day (187 mg/kg/day) were surgically implanted in the abdomen of three male rats. Whole blood (0.5 mL) was collected and globin isolation performed on day 11 following implantation.

**Globin Isolation**. A total of 0.5 mL of whole blood was collected in a heparinized syringe at each bleeding time and then centrifuged at 3000*g* for 5 min to separate the plasma from the red cells. A 5 mM phosphate-buffered saline solution (pH 7.4) containing 150 mM NaCl was added in equal volume to the red cells, resuspended, and centrifuged at 3000*g*. The supernatant and buffy coat were discarded and the washing procedure repeated twice more. The washed red cells were then lysed with a 2× volume of 5 mM phosphate buffer (pH 7.4) and centrifuged at 20000*g* for 25 min. The hemolysate (supernatant) was removed and mixed with 100 mL of 1 M ascorbic acid and added dropwise to 10 mL of cold 2.5% oxalic acid in acetone and allowed to precipitate for 15 min. The mixture was then centrifuged at 12000*g* for 10 min, the supernatant aspirated, and the globin washed by adding 5 mL of acetone, resuspending with a metal spatula, and centrifuging at 12000*g* for 10 min. The supernatant was aspirated and the globin pellet dried under a stream of nitrogen and then stored at −78 °C.

[1] Abbreviations: ACN, acetonitrile; ALDH, aldehyde dehydrogenase; *m*-CPBA, meta-chloroperoxybenzoic acid; CID, collision-induced dissociation; COS, carbonyl sulfide; DSF, disulfiram; ESI, electrospray ionization; HHAC-Cys, *S*-hexahydro-1*H*-azepine-1-carbonyl cysteine; MeOH, methanol; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PIP-Cys, *S*-piperidine-1-ylcarbonyl cysteine; SA, sinapinic acid; SRM, selected reaction monitoring; TFA, trifluoroacetic acid.

**Chemical Synthesis**. **Molinate (*S*-Ethyl-hexahydro-1*H*-azepine-1-carbothioate) (1)**. Hexamethyleneimine (1.13 mL, 10 mmol), ethanol (10 mL), and 10 N NaOH (1 mL) were taken up in a 25 mL two-neck flask and fitted with a Dewar condenser containing dry ice and 2-propanol. The flask was stirred and cooled in a dry ice/2-propanol bath (−70 °C), and carbonyl sulfide (COS) (1 mL) was condensed into the flask. The second neck was sealed, and the temperature of the bath increased to 0−10 °C and maintained in that temperature range while keeping the Dewar condenser cold while COS evaporated and condensed into the reaction. After 4 h, the condenser was removed, and iodoethane (0.88 mL, 11 mmol) was added with stirring for 2 h, after which the EtOH was removed, and the residue distributed between the water (10 mL) and chloroform (20 mL) layers. The organic layer was separated and dried, and the solvent was removed using a rotary evaporator. $^1$H NMR ($CDCl_3$) $\delta$ 1.27 (t, 3H, $CH_3$), 1.51 (m, 4H, $CH_2$), 1.70 (m, 4H, $CH_2$), 2.88 (q, 2H, $CH_2CH_3$), 3.41 (t, 2H, ring $CH_2$−N), 3.52 (t, 2H, ring $CH_2$−N). $^{13}$C NMR ($CDCl_3$) $\delta$ 15.8 ($CH_3$), 24.9 ($CH_2CH_3$), 27.4 and 27.6 ($CH_2$), 28.3 and 28.8 ($CH_2$), 47.6 and 48.0 ($CH_2$N), 168.2 (C═O).

***S*-Ethyl-hexahydro-1*H*-azepine-1-carbothioate sulfone (2)**. A solution of molinate (0.94 g, 5 mmol) in dichloromethane (10 mL) was cooled in an ice bath, and *m*-chloroperoxybenzoic acid (2.70 g, 11 mmol) in the same solvent (25 mL) was added with stirring. The reaction was stirred at 0 °C for 4 h, the solid was filtered and the filtrate was washed with saturated $NaHCO_3$ (2 × 25 mL), and then dried and concentrated using a rotary evaporator. The product was purified using column chromatography (hexane; 9:1 hexane/ethyl acetate; 5:1 hexane/ethyl acetate) and fractions containing the product giving a GC peak at 10.5 min were combined and the solvent removed under reduced pressure yielding a colorless liquid. $^1$H NMR ($CDCl_3$) $\delta$ 1.36 (t, 3H, $CH_3$), 1.57 (m, 4H, ring $CH_2$), 1.78 (m, 4H, ring $CH_2$), 3.30 (q, 2H, $CH_2CH_3$), 3.49 (t, 2H, $CH_2$−N), 3.84 (t, 2H, $CH_2$−N). $^{13}$C NMR ($CDCl_3$) $\delta$ 6.7 ($CH_3$), 26.0, 26.3, 27.3, 28.8 (ring $CH_2$), 46.1 ($CH_2CH_3$), 46.8 and 48.8 ($CH_2$−N), 160.6 (C═O).

***S*-(Hexahydro-1*H*-azepine-1-carbonyl)cysteine (HHAC-Cys) (3)** was prepared according to previously published procedures with slight modifications (*5*, *7*). Briefly, the pH of a solution of L-cysteine (0.52 g, 4 mmol) in water was adjusted to 8 with 1 N NaOH and stirred with molinate sulfone (0.44 g, 2 mmol) in methanol for 24 h. After removing the ACN, purification was achieved by HPLC by injecting the reaction mixture onto a C18 PRP-1 column (Hamilton, 70 $\mu$m, 7.0 × 305 mm, 100 Å). The desired product was eluted at a flow rate of 2.0 mL/min using a linear gradient of 10−90% B over 10 min and then maintained at 90% B for 9 min before returning to initial conditions. Solvent A contained 5 mM formic acid in water, and solvent B contained 5 mM formic acid in acetonitrile. The separation was monitored at 214 nm, and fractions containing the sulfone were combined and lyophilized leaving a white solid. $^1$H NMR ($CDCl_3$) $\delta$ 1.48 (m, 4H, $CH_2$), 1.65 (m, 4H, $CH_2$), 3.26 (q, 2H, $CH_2$), 3.64 (m, 4H, $CH_2$), 3.96 (q, 1H, CH). $^{13}$C NMR ($CDCl_3$) $\delta$ 26.4, 26.8, 27.36 and 27.07 (ring $CH_2$), 30.7 (S−$CH_2$), 48.4 and 48.7 (ring $CH_2$), 55.2 (CH−$NH_2$), 169.2 (C═O), 172.6 (N−C═O). ESI-MS *m/z* 247 $(M + H)^+$.

***S*-Methyl-1-piperidinecarbothioate (4)**. To piperidine (20 mmol, 1.98 mL) in ethanol (20 mL) was added 10 N NaOH (2 mL) in a 25 mL two-neck flask and fitted with a Dewar condenser containing dry ice and 2-propanol. The flask was stirred and cooled in a dry ice/2-propanol bath (−70 °C) and COS (2 mL) was condensed into the flask. The second neck was sealed, and the temperature of the bath raised to 10 °C and maintained at that temperature while keeping the Dewar condenser cold while COS evaporated and condensed into the reaction. After 4 h, the condenser was removed and iodomethane (1.4 mL, 22 mmol) was added with stirring for 2 h after which the EtOH was removed, and the residue distributed between water (10 mL) and chloroform (20 mL) layers. The organic layer was separated, dried and evaporated. $^1$H NMR ($CDCl_3$) $\delta$ 1.53 (m, 6H), 2.25 (s, 3H, $CH_3$), 3.41 (m, 4H, $CH_2$−N). $^{13}$C NMR

($CDCl_3$) $\delta$ 13.2 ($CH_3$), 24.8 ($NCH_2CH_2$), 26.0 ($CH_2$), 46.6 ($NCH_2$), 167.6 (C═O).

***S*-Methyl-1-piperidinecarbothioate sulfone (5)**. A solution of *S*-methyl-1-piperidinecarbothioate (0.71 g, 4.4 mmol) in dichloromethane (10 mL) was cooled in an ice bath and *m*-CPBA (2.4 g) in the same solvent (20 mL) was stirred overnight at room temperature. The solid was filtered off and the filtrate was washed with $NaHCO_3$. The organic layer was dried and evaporated and purified by column chromatography (hexane; 9:1 hexane/ethyl acetate; 5:1 hexane/ethyl acetate; 1:1 hexane/ethyl acetate). Fractions containing the product giving a GC peak at 9.2 min were combined and evaporated. $^1$H NMR ($CDCl_3$) $\delta$ 1.67 (m, 6H, ring $CH_2$), 3.1 (s, 3H, methyl), 3.33 (m, 2H, $CH_2$−N), 3.65 (m, 2H, $CH_2$−N). $^{13}$C NMR ($CDCl_3$) $\delta$ 23.9, 25.3, 25.9, 39.6 ($CH_3$), 45.7 and 46.2 ($CH_2$−N), 159.6 (C═O).

***S*-(Piperidine-1-ylcarbonyl)cysteine (PIP-Cys) (6)**. The pH of a solution of L-cysteine (0.52 g, 4 mmol) in water was adjusted to 8 with 1 N NaOH and stirred with *S*-methyl-1-piperidinecarbothioate sulfone (0.38 g, 2 mmol) in MeOH (20 mL) for 24 h. After removing the MeOH, purification was accomplished by HPLC by injecting the reaction mixture onto a PRP-1 column (Hamilton, 70 $\mu$m, 7.0 × 305 mm, 100 Å). The desired product was eluted at a flow rate of 2.0 mL/min using a linear gradient of 10 to 90% B over 20 min using the same solvent system for (3). The eluant was monitored at 214 nm and fractions containing the product were lyophilized, leaving a white solid. $^1$H NMR ($D_2O$) $\delta$ 1.54 (m, 6H, ring $CH_2$), 3.29 (dd, 2H, S−C*H*$_2$), 3.50 (m, 4H, ring $CH_2$), 3.93 (q, 1H, C*H*-$NH_2$). $^{13}$C NMR ($D_2O$) $\delta$ 24.0, 25.5, 31.0, 45.8, 47. 9, 55.3, 167.8 (NC═O), 172.9 (C═O).

**Chromatography**. Analysis of intact globin samples was performed on a Waters 2690 HPLC system. Globin (1.8−2.5 mg) from nonexposed and molinate-exposed rats was dissolved in 0.1% TFA (750 $\mu$L). The absorbance was measured at 280 nm and diluted as necessary to produce a final absorbance of 1. Fifty microliters was injected onto a C4 RP column (4.6 mm × 150 mm, 5 $\mu$m, 300 Å, Phenomenex, Torrance, CA). Globin samples were eluted at a flow rate of 1 mL/min with a linear gradient from initial conditions of 56% A [solvent A = ACN/$H_2O$/TFA (20:80:0.1%)] and 44% B [solvent B = ACN/$H_2O$/TFA (60:40:0.08%)] to 30% A and 70% B over 30 min while monitoring at 214 nm.

Globin α- and $\beta$-chains were separated using HPLC by dissolving globin collected from nonexposed and molinate-exposed rats in solvent A to a final concentration of 10 mg/mL and loading 50−100 $\mu$L onto a C4 Waters Delta Pak column (15 $\mu$m, 3.9 × 300 mm, 300 Å). Protein fractions were eluted at a flow rate of 2 mL/min with a linear gradient from initial conditions of 56% A [solvent A = ACN/$H_2O$/TFA (20:80:0.1%)] and 44% B [solvent B = ACN/$H_2O$/TFA (60:40:0.08%] to 40% A and 60% B over 30 min while monitoring at 214 nm. Protein fractions were collected, and ACN was removed and lyophilized before analysis with MALDI-TOF MS.

Separations and collection of fractions were performed on a Shimadzu LC-10ADvp connected to a SPD-10A detector. The software used in the analysis of the data was HP Chemstation.

**Enzymatic Digestion**. All enzymatic digestions on globin preparations and purified $\beta$-chain fractions were performed in solution by adding 5 $\mu$L of globin (1 mg/mL) or purified $\beta$-chains (0.3−0.5 mg/mL) to 10 $\mu$L 100 mM $NH_4HCO_3$ buffer (pH 6.5) and 1.5 $\mu$g Glu-C. Samples were digested for 2 h at 37 °C. Following digestion, 2−4 $\mu$L of the digest was added to an equivalent amount of a saturated sinapinic acid (SA) matrix solution and applied to a MALDI probe using the dried droplet method.

**Acid Hydrolysis**. For nano-ESI MS experiments, approximately 1 mg of globin was added to a hydrolysis tube, placed in a hydrolysis chamber containing 200 $\mu$L of 6 N HCl, flushed with argon gas, and hydrolyzed for 18 h at 110 °C. Following completion of hydrolysis, samples were reconstituted in 100 $\mu$L of MeOH/$H_2O$/formic acid mixture (50:49:1%) and filtered using a Costar Spin-X centrifuge spin filter (0.2 $\mu$m pore size). Samples were stored at −20 °C until analysis.

Samples for LC/MS/MS analysis were prepared in a similar manner using 10 mg of protein and reconstituting the hydrolyzed sample in 100 $\mu$L ACN/$H_2O$/formic acid (10:90:0.02%).

**Mass Spectrometry**. MALDI-TOF MS analysis of intact proteins and digests were performed on a Voyager Elite (PerSeptive Biosystems, Inc.) time-of-flight mass spectrometer. The ion acceleration was 25 kV, the grid voltage 95% for intact proteins and 93% for peptides, and the guide wire voltage 0.1%. Mass spectra were acquired as the sum of ions generated by irradiation of the sample with 100−300 laser pulses and processed using GRAMS/386 software (Galactica Industries Corp., Salem, NH). A saturated solution of sinapinic acid (SA) in ACN/$H_2O$/TFA (50:49:0.1 v/v/v) was used as the matrix. For analysis of intact proteins, 2 $\mu$L of a globin mixture (1 mg/mL) was added to 2 $\mu$L of the SA matrix and applied to the plate by the dried-droplet technique and air-dried. Myoglobin was used as an internal standard with an *m*/*z* of 16952.5. For analysis of the peptide mixtures following enzymatic digestion of intact or purified protein fractions, 2−4 $\mu$L of the mixture was added to an equivalent volume of the SA matrix and applied to the sample plate in a similar manner. The mass of two known peptide fragments derived from the $\beta_3$ chain, [27−43] and [27−90] with *m*/*z* of 2098.5 and 7047.1, respectively, were used as internal calibration standards (*5*).

Tandem mass spectrometry was performed on a Waters 2690 HPLC system coupled with a Finnigan TSQ 7000 triple-quadrupole ESI/MS (Finnigan, San Jose, CA) equipped with a nano-ESI ion source. Ten microliters of the globin acid hydrolysate was loaded into the nanospray capillary (Picotips, New Objectives, Cambridge, MA). The heated capillary was operated at 20 V and 100 °C, tube lens voltage set to 80 V and the needle voltage 1000 V. Collision-induced dissociation (CID) occurred in Q2 with argon (2.5 mT) as the carrier gas with collision energy varied between 5 and 30 eV.

Liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis of HHAC-Cys with selected reaction monitoring (SRM) was performed using a Waters 2690 HPLC system coupled with a Finnigan TSQ 7000 triple-quadrupole ESI/MS (Finnigan, San Jose, CA). A PE Biosystems (Foster City, CA) 785A Programmable Absorbance UV detector was used and set to monitor at 214 nm. The electrospray voltage was maintained at 4 kV and the capillary temperature held at 200 °C. Collision-induced dissociation (CID) occurred in Q2 at a collision energy of 20 eV with the collision gas (argon) at 2.5 mT. Solvent A contained 5 mM formic acid in water and solvent B contained 5 mM formic acid in acetronitrile. LC separations were performed on a C18 PRP-1 column (3 $\mu$m, 2.1 × 100 mm, 100 Å, Hamilton, Reno, NV). Separations were achieved using initial conditions of 10% B (90% A) held for 10 min followed by a linear gradient to 90% B (10% A) over 5 min and held for 6 min before returning to initial conditions. Equilibration with 10% B (90% A) was allowed for 13 min and total run time was 35 min. Twenty-five microliters of the sample treated with 5 $\mu$L of the internal standard (80 ng/$\mu$L) was injected and the eluant from 2−9 min directed into the mass spectrometer. SRM experiments used to detect HHAC-Cys monitored the *m*/*z* 247→126 transition and the *m*/*z* 233→112 transition monitored for PIP−Cys, the internal standard. The scan width for the daughter ions was 1 amu and total cycle time was 2 s. The ratios of the resulting peak areas were used to calculate the amount of HHAC-Cys in the acid hydrolysates by reference to a calibration curve. A calibration curve was generated by dissolving varying amounts of HHAC-Cys in solvent A and adding a fixed amount of PIP−Cys so each 25 $\mu$L injection would contain 0−1000 ng of HHAC-Cys and 100 ng of PIP−Cys. The ratio of the area of the analyte to the internal standard was plotted against the concentration of HHAC-Cys in the sample to generate a standard curve ($r^2$ = 0.992, $y = 0.005142x + 0.228125$).

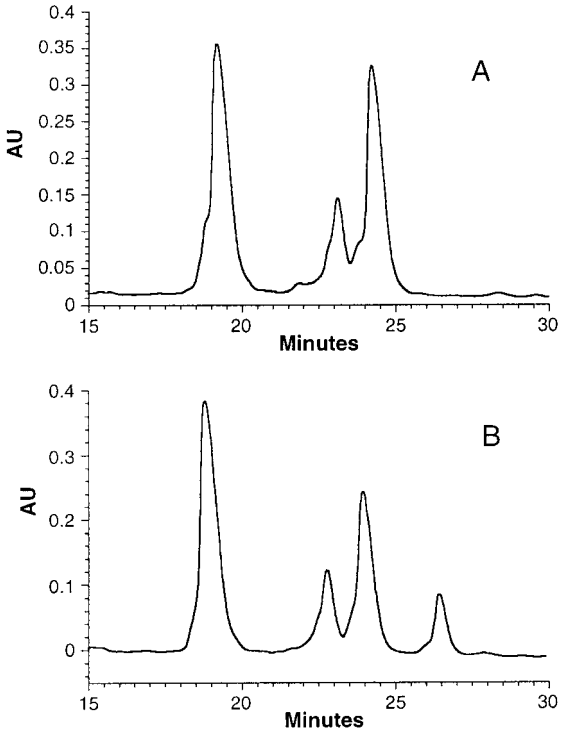



**Figure 1.** HPLC analysis of rat globin isolated by precipation followed by separation using a C4 reversed-phase column and detection by UV absorption at 214 nm. (A) Representative chromatogram of nonexposed globin showing a combined α-peak eluting between 18 and 20 min and two peaks resulting from the unresolved four native *β*-chains eluting between 22 and 25 min. (B) Globin preparations isolated from molinate exposed animals produced an additional late eluting peak (26−27 min).

## Results

**Intact Globin Analyis by MALDI-TOF MS**. The HPLC chromatograms obtained from the globins of molinate-exposed and nonexposed rats are shown in Figure 1. HPLC analysis of both the nontreated and molinate-treated globin samples produced a single peak eluting between 18 and 20 min corresponding to the two native α-chains, and two peaks eluting between 22 and 25 min corresponding to the four native *β*-chains. The globin isolated from molinate-exposed rats contained an additional late eluting peak between 26 and 27 min that was not seen in the controls. MALDI-TOF MS analysis of the control globin samples did not resolve individual peaks for each globin chain but appeared to resolve the two α-chains at *m*/*z* 15 150 and 15 197 with a single peak for the *β*-chains at *m*/*z* 15 849 (Figure 2A). Analysis of the globin isolated from molinate-exposed rats also produced peaks at *m*/*z* 15 150, 15 197, and 15 849 with an additional signal at *m*/*z* 15 975 not seen in the control (Figure 2B). Isolation of the late eluting HPLC peak between 26 and 27 min present in molinate-exposed samples followed by MALDI-TOF MS demonstrated it to have an *m*/*z* of 15 975 (Figure 2C). MALDI-TOF MS analysis of the fraction eluting between 22 and 25 min produced a signal at *m*/*z* 15 990 in samples from exposed rats (Figure 2D) but not from control samples (data not shown). Myoglobin was used as an internal standard (*m*/*z* 16 952.5) and SA matrix adducts 200 or 225 Da larger in mass than the protein signals were also observed.

**Glu-C Digests of Globin**. Globin samples from exposed and nonexposed rats were digested in solution with

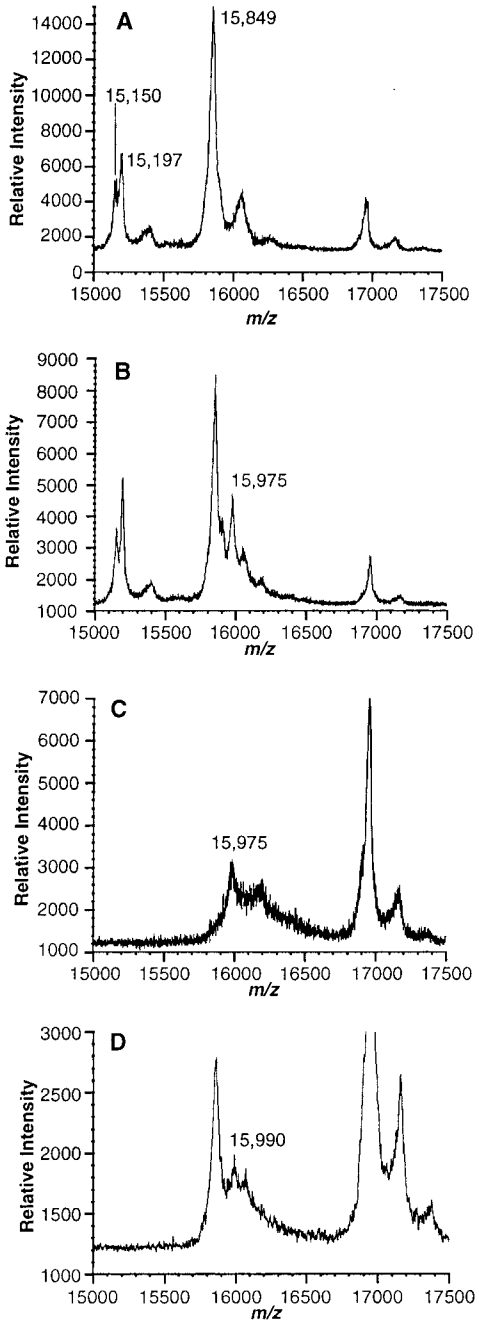



**Figure 2.** MALDI-TOF MS spectra of (A) control globin showing broad signals at *m*/*z* 15 150, 15 197, and 15 849 corresponding to the two native α- and four native *β*-chains, respectively. (B) Spectra of globin isolated from molinate-treated rats contained a signal at *m*/*z* 15 975 additional to those observed in the nontreated globins. (C) A spectrum produced by the late eluting HPLC fraction (26−27 min) isolated from exposed globin preparations demonstrating an *m*/*z* of 15 975 consistent with the addition of a 126 Da *S*-hexahydro-1*H*-azepine-1-carbonyl adduct to the $\beta_3$ chain. (D) In the fraction eluting between 22 and 25 min isolated from molinate exposed globins a signal at *m*/*z* 15 990 consistent with the addition of a 126 Da *S*-hexahydro-1*H*-azepine-1-carbonyl adduct to the $\beta_2$ chain could be detected. Myoglobin was used as an internal standard (*m*/*z* 16 952.2) and SA matrix adducts can been seen as signals 200 or 225 Da larger than the parent protein signals described above.

Glu-C for 2 h and analyzed using MALDI-TOF MS. The peptide fragments originating from the $\beta_3$ chain of globin could then be assigned (Figure 3). Two signals from the peptide fragments with an *m*/*z* at 2743 and 4985, corre-

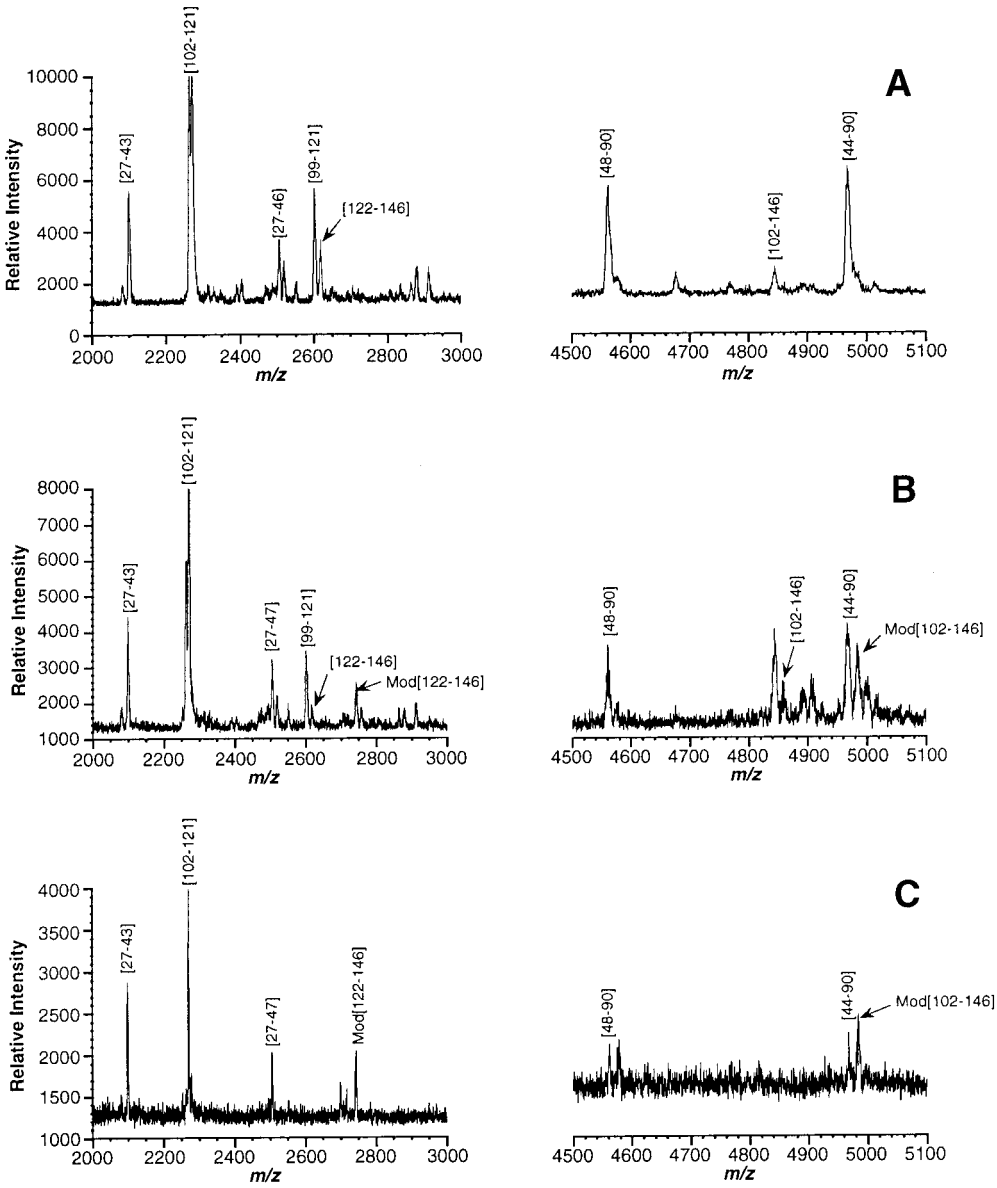



**Figure 3.** MALDI-TOF MS spectra of Glu-C digests in the regions of *m*/*z* 2000−3000 and 4500−5100. (A) Spectra of globin isolated from a nonexposed rat showing the predicted peptide fragments. (B) Spectra of globin isolated from molinate-treated rats produced two additional peptide fragments at *m*/*z* 2743 and 4985, corresponding to the addition of 126 Da to the peptides [122−146] and [102−146], respectively. (C) Spectra of the isolated HPLC fraction eluting between 26 and 27 min containing the modified $\beta_3$ chain. Both modified fragments observed in panel B are present although the signal at *m*/*z* 2617 corresponding to the native peptide [122−146] is not present. For all spectra, peptides at [27−43] (*m*/*z* 2098.5) and [27−90] (*m*/*z* 7047.1 not shown) were used for internal calibration.

sponding to the addition of 126 Da to the peptides [122−146] and [102−146], respectively, were observed in the digest of the molinate-exposed rats (Figure 3B) but were not present in the nontreated globin (Figure 3A). Fractions obtained from the peak eluting between 26 and 27 min were digested and while no signal at *m*/*z* 2617 corresponding to the native [122−146] peptide was observed, the two signals at *m*/*z* 2743 and 4985 appeared, corresponding to the modified fragments (Figure 3C).

**Nano-ESI-MS of Acid Hydrolyzed Globins**. A CID of the parent ion of the synthetic authentic HHAC-Cys at *m*/*z* 247 produced fragments ions at *m*/*z* 230, 201, 160, 126, 98, 83, and 55 (Figure 4A). The ion at *m*/*z* 126 can be attributed to the loss of the carbonyl hexamethyleneimine moiety from the parent compound. Although the control hydrolysate produced several fragments from the parent of *m*/*z* 247 using identical CID conditions, there were no ions detected at *m*/*z* 247, 230, 201, 126, 98, 83, or 55, characteristic of the HHAC-Cys adduct (Figure 4B). Acid hydrolysates of globin from molinate-treated rats contained the same fragment ions observed for the synthetic HHAC-Cys standard in addition to those observed for the control samples (Figure 4C).

**Analysis of Globins using LC/MS/MS**. Acid hydrolysates of nonexposed and molinate-exposed globin were analyzed for the HHAC-Cys adduct using selected reaction monitoring (SRM). The retention times of synthetic HHAC-Cys and the internal standard, PIP-Cys, were 6.5 and 4 min, respectively, when chromatographed on a C18 PRP-1 column (Figure 5A). Control hydrolysates did not contain a peak for the HHAC-Cys adduct at 6.5 min, although the internal standard could be seen (Figure 5B). Molinate-treated globin hydrolysates did, however, contain a peak having the same retention time as the

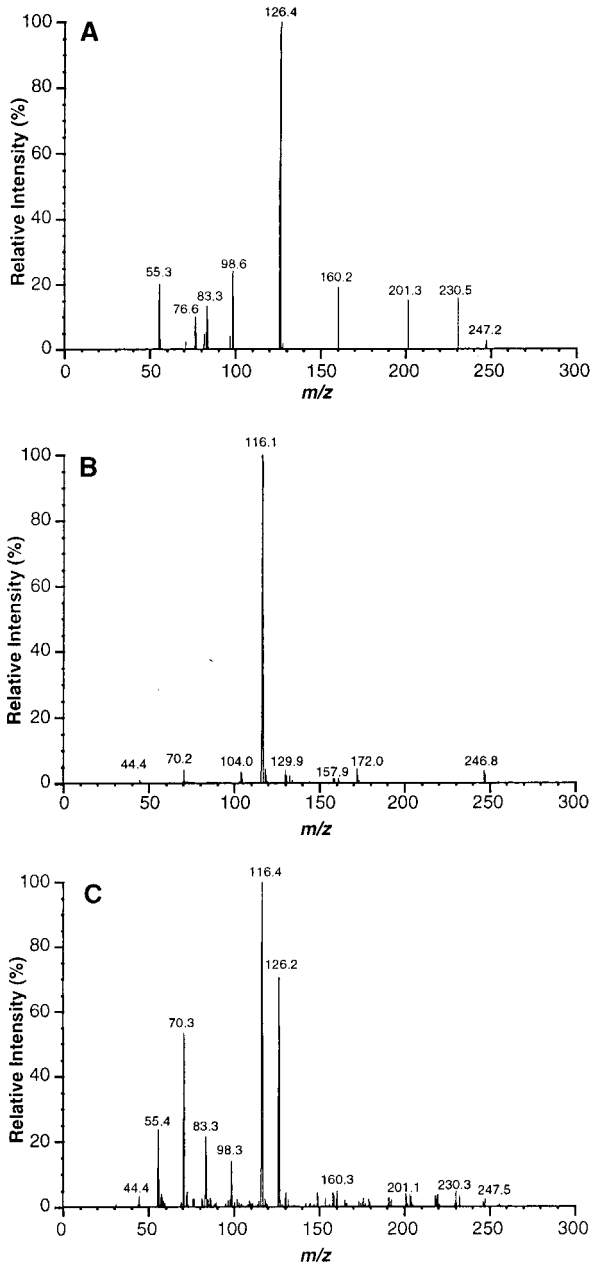



**Figure 4.** Product ions obtained from CID of the [$MH^+$] ion *m*/*z* 247 of *S*-hexahydro-1*H*-azepine-1-carbonyl cysteine. (A) A spectrum of the synthetic standard *S*-hexahydro-1*H*-azepine-1-carbonyl cysteine (HHAC-Cys) showing characteristic fragments at *m*/*z* 230, 201, 160, 126, 98, 83, and 55. (B) A spectrum obtained from control hydrolysate showing one major fragment at *m*/*z* 116. (C) A spectrum obtained from molinate-exposed hydrolysates showing a combination of the fragments present in the synthetic standard and control hydrolysate. The ion at *m*/*z* 126 is attributed to loss of the *S*-hexahydro-1*H*-azepine-1-carbonyl adduct and is observed in the standard and exposed rats (A and C) but not in the control (B).

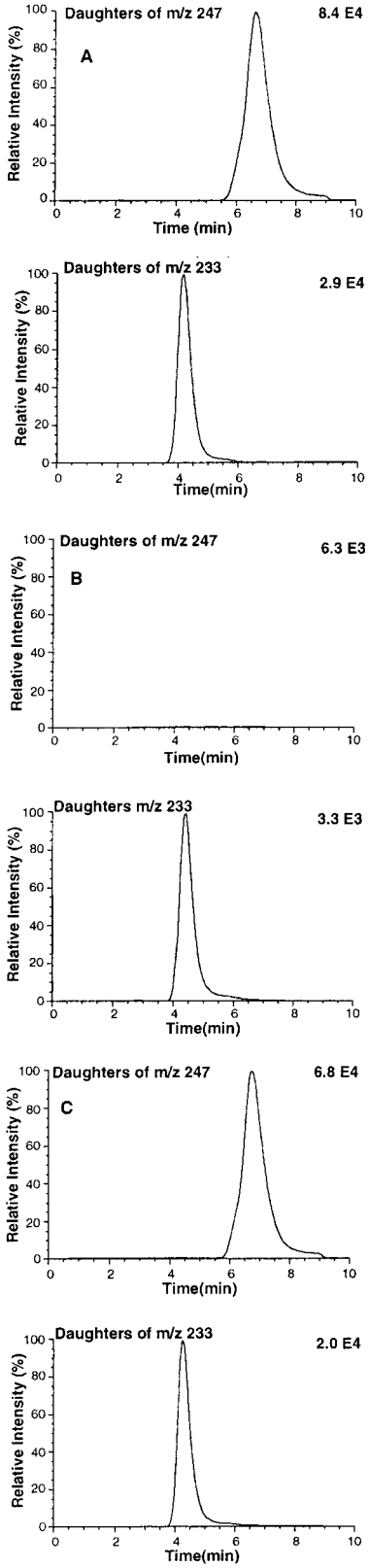



**Figure 5.** LC/MS/MS with SRM of globin hydrolysates. The internal standard, *S*-(piperidine-1-ylcarbonyl) cysteine (PIP-Cys *m*/*z* 233), is shown in the lower trace of each chromatogram with a retention time of 4 min. The presence of HHAC-Cys, with a retention time of 6.5 min, is shown in the upper trace of each chromatogram for the synthetic standard (A) and the molinate-exposed globin (C) while HHAC-Cys is not detected in the control (B).

synthetic standard (Figure 5C). The quantity of adduct detected in the samples is shown as a function of the cumulative exposure (Figure 6). Estimates of the amounts of adduct were calculated from the calibration curve generated from known quantities of the authentic standard. Analysis performed on 10 mg samples demonstrated that rats dosed with molinate for 4 days at 100 mg/kg/day (400 mg/kg total dose) corresponded to 720 ± 99 ng/10 mg of protein of the adduct while 1233 ± 41 ng/10 mg of protein adduct was detected in the rats dosed for 11 days at 187 mg/kg/day (2000 mg/kg total dose).

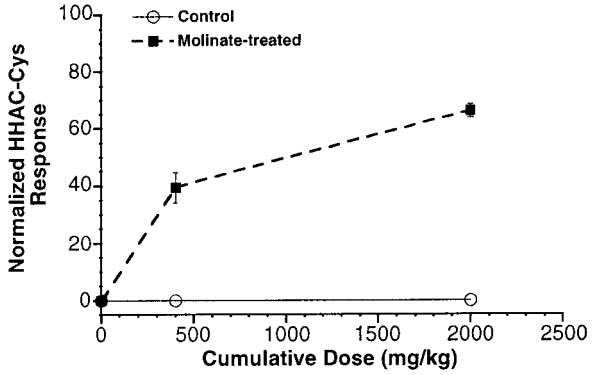
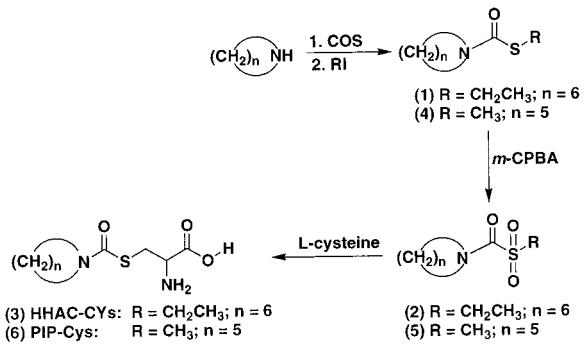



**Figure 6.** HHAC-Cys formation as a function of cumulative dose (mg/kg/day × days of exposure) for globin samples obtained from control (○) and molinate-treated (■) rats. Dose−response curves relate to the quantity of HHAC-Cys detected for control, 4 day (400 mg/kg total dose), and 11 day (2000 mg/kg total dose) exposures. Normalized values for HHAC-Cys responses were obtained by dividing the mass spectrometric response for HHAC-Cys by the internal standard PIP−Cys and the amount of protein hydrolyzed multiplied by 100 to give a dimensionless number. Error bars represent standard errors ($n = 3$).

**Scheme 1. Synthesis of HHAC-Cys and PIP-Cys**

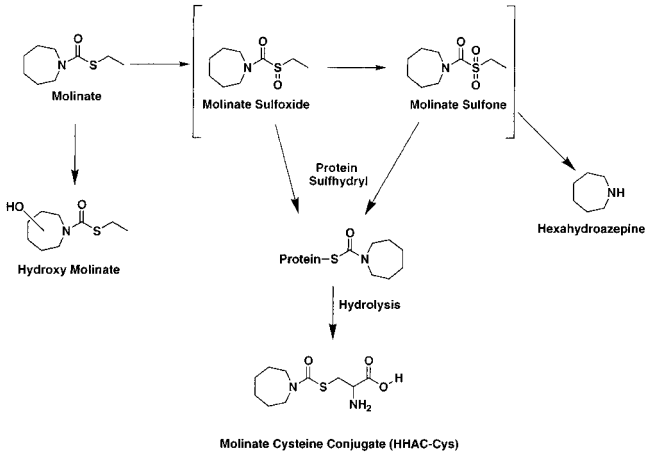



## Discussion

HPLC chromatograms of globin samples isolated from molinate-exposed rats contained a late eluting peak additional to the native α- and $\beta$-chains. Isolation and MALDI-TOF MS analysis of the late eluting peak revealed a mass corresponding to the $\beta_3$ chain plus 126 Da, consistent with the formation of a *S*-hexahydro-1*H*-azepine-1-carbonyl adduct. MALDI-TOF MS also revealed a small signal at *m*/*z* 15 990, indicative of a similarly modified $\beta_2$ chain. Glu-C has been shown to digest $\beta$-chains with minimal digestion of the α-chains, alleviating the need for HPLC separation of the two subgroups of chains prior to digestion (*8*, *9*). Using Glu-C enzymatic digestion followed by MALDI-TOF MS analysis of the digests, the 126 Da increase was localized to two peptides of $\beta_2$ and $\beta_3$, [122−146] and the partial digestion product [102−146], both containing a reactive cysteine at residue 125. To confirm the structure of the proposed covalent modification at Cys-125, hydrolysates from the molinate-treated globins were examined by ESI-MS and found to produce fragments identical to those produced from the HHAC-Cys synthetic standard, whereas no analogous fragments were observed in the control hydrolysates. LC/MS/MS analysis using SRM to detect the HHAC-Cys adduct following hydrolysis indicated that the quantity of the HHAC-Cys adduct displayed a cumulative dose−response over the duration of molinate exposure with adducts detected as early as 4 days. These results are consistent with molinate being bioactivated to a sulfoxide or sulfone intermediate that undergoes nucleophilic addition by Cys-125 of globin to yield an acid stable carbamoyl adduct (Scheme 2). Additional metabolic pathways have been reported for molinate that are not expected to result in protein modifications. Oxidative metabolism of molinate to produce ring-hydroxylated metabolites that are typically excreted unchanged or as glucuronide conjugates are generated, and hydrolysis of the sulfone to liberate hexahydroazepine may also occur (*2*, *10*). The molinate sulfoxide and sulfone can also form glutathione conjugates, which, after further metabolism are excreted in the urine as mercapturic acids (*2*).

Previous in vitro studies using microsomal preparations have provided evidence that covalent binding of radiolabeled molinate and its metabolites occurs preferentially on a single carboxylesterase in the liver and testis (*3*). The relative potencies for carboxylesterase inhibition observed for the sulfoxide and sulfone derivatives of molinate were consistent with the inhibition occurring through the generation of an *S*-hexahydro-1*H*-azepine-1-carbonyl adduct (*1*). The further observation that prior incubation with phenylmethanesulfonyl fluoride (PMSF) prevented molinate binding was interpreted to support the covalent modification occurring at the active site (*3*). In contrast, the data derived from the present in vivo study examining globin digests demonstrates the formation of the analogous adduct on Cys-125 in the absence of serine modification. Most likely this discrepancy arises due to the high reactivity of these two specific residues on their respective proteins (*11*, *12*).

**Scheme 2. Proposed Metabolic Pathway for *S*-Hexahydro-1*H*-azepine-1-carbonyl Adduct Formation**



Although, there is some uncertainty regarding the exact site of the protein modification in the microsomal preparation in that the structure and location of the modification was not determined and PMSF has been reported to also modify cysteine residues (*13*). Regardless, the current data is consistent with the ability of molinate to potentially alter protein function through the modification of cysteine residues in addition to the serine based modification proposed previously.

The metabolism of DSF has been characterized and, similar to molinate, is thought to be bioactivated through a series of intermediates to form *S*-methyl *N*,*N*-diethylthiocarbamate sulfoxide (DETC-MeSO), which is thought to be the active metabolite of DSF and ultimate inhibitor of ALDH (*14*−*17*). Recently DSF has been shown to covalently modify ALDH in vivo at the cysteine active site through carbamylation, increasing the mass of the enzyme by 100 Da (*6*). Disulfiram has also been shown to covalently modify rat hemoglobin in vivo, generating a stable *N*,*N*-diethylthiocarbamate adduct through carbamylation of Cys-125 on the $\beta_2$ and $\beta_3$ chains (*5*). It is interesting to note that, in addition to generating analogous covalent modifications on globin, molinate has also been shown to inhibit ALDH, to increase levels of acetaldehyde, and to produce a disulfiram-like ethanol reaction in rats challenged with ethanol (*18*). The results presented here for globin suggest that inhibition of ALDH by molinate may also occur through carbamylation of an active site cysteine by the sulfoxide metabolite. The ability of molinate to undergo bioactivation to form reactive metabolites which can carbamylate proteins in vivo, similar to DSF, suggests that other biological effects associated with DSF may also be relevant to molinate. In particular, administration of DSF has been shown to be hepatotoxic and produce a peripheral neuropathy that targets Schwann cells, resulting in demyelination (*19*). Although the literature regarding the neurotoxicity associated with molinate is limited, there have been reports revealing an increased incidence of nerve degeneration, Schwann cell hyperplasia, and increased frequency of eosinophilic bodies in the spinal cord and medulla (*20*). If protein carbamylation is a contributing mechanism in the neurotoxicity of disulfiram then molinate may also be neurotoxic through a similar mechanism and may display a greater potency given the fact that fewer bioactivation steps are required to generate the molinate sulfoxide or sulfone.

## Conclusion

Molinate covalently modifies rat hemoglobin in vivo in a cumulative dose dependent manner. Characterization of the covalent modification demonstrated a *S*-hexahydro-1*H*-azepine-1-carbonyl adduct on the Cys-125 residue of the $\beta_2$ and $\beta_3$ chains consistent with bioactivation of molinate to a sulfoxide or sulfone intermediate. The globin modifications produced by molinate are analogous to those produced by disulfiram, suggesting that molinate may also covalently modify other proteins that are modified by disulfiram and share biological effects that have been demonstrated by disulfiram. Because similar metabolism to sulfoxide and sulfone metabolites is available to other thiocarbamate herbicides, the results obtained for molinate may also extend to other compounds within this class and measurement of cysteine carbamylation may provide a biomarker for assessing exposure to thiocarbamate herbicides. However, since human hemoglobin lacks the highly reactive Cys-125 exclusive to rat hemoglobin, further experiments will be required to determine the reactivity of molinate and its metabolites toward human hemoglobin.

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