Acrolein Mercapturates: Synthesis, Characterization, and Assessment of Their Role in the Bladder Toxicity of Cyclophosphamide

Kumar Ramu,*,† Lucy H. Fraiser,†,‡ Blain Mamiya,§ Tamer Ahmed,† and James P. Kehrer[†]

Division of Pharmacology and Toxicology and Division of Medicinal Chemistry, College of Pharmacy, University of Texas at Austin, Austin, Texas 78712-1074

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Acrolein is the metabolite of cyclophosphamide (CP) believed to be involved in the bladder toxicity associated with this anticancer drug. The mechanism by which this extremely reactive intermediate is delivered to the bladder is not known. Glutathione (GSH) readily conjugates with acrolein, and the acrolein mercapturate S-(3-hydroxypropyl)-N-acetylcysteine (3-hydroxy-PrMCA) has been found in the urine of animals and man given CP. The objectives of this study were to prepare and characterize synthetic standards of the GSH acrolein adduct (3oxopropyl)glutathione (3-oxoPrGSH), the acrolein mercapturates S-(3-oxopropyl)-N-acetylcysteine (3-oxoPrMCA) and 3-hydroxyPrMCA, and the S-oxidation product of 3-oxoPrMCA (3oxoPrMCA S-oxide). In addition, the release of acrolein from, and the bladder toxicity of, these conjugates was determined. 3-OxoPrGSH and 3-oxoPrMCA were prepared with a 99% yield by condensing acrolein with GSH and N-acetylcysteine, respectively. 3-HydroxyPrMCA was prepared with a 63% yield by refluxing 3-chloropropanol and N-acetylcysteine in a basic medium. Oxidation of 3-oxoPrMCA with H_2O_2 was used to prepare 3-oxoPrMCA S-oxide. By decreasing the reaction time to 1 h, and adjusting the ratio of 3-oxoPrMCA to H_2O_2 , the yield of 3-oxoPrMCA S-oxide was increased to 96%. The anhydrous aldehyde, 3-oxoPrMCA, afforded characteristic aldehydic proton resonances (¹H NMR) in deuterated dimethyl sulfoxide. New resonances were observed in deuterated water, indicating a 75% hydration of the aldehyde to the corresponding geminal diol. This phenomenon was enhanced with 3-oxoPrMCA S-oxide where $\sim 100\%$ hydration of the aldehyde to the corresponding geminal diol was observed. When incubated at 25 °C in 100 mM potassium phosphate buffer containing 1 M KCl, pH 8.0, 3-oxoPrMCA released $\sim 6\%$ and 3-oxoPrMCA S-oxide released $\sim 16-18\%$ of the theoretical maximum yield of acrolein after 30 min, as indicated by an increase in absorbance at 210 nm and confirmed by trapping this aldehyde as a semicarbazone. There was less than a 2% yield of acrolein from 3-hydroxyPrMCA or 3-oxoPrGSH under similar conditions. At pH 7.4 the release of acrolein from 3-oxoPrMCA and 3-oxoPrMCA S-oxide was decreased by 50%. An assay where aldehydes are reacted with *m*-aminophenol in acid media produced fluorescence consistent with 72%, 46%, 23%, and 1% yields of acrolein from 3-oxoPrMCA S-oxide, 3-oxoPrMCA, 3-oxoPrGSH, and 3-hydroxyPrMCA, respectively. These yields were unaffected by incubation in buffer for up to 2 h. Acrolein, 3-oxoPrMCA S-oxide, 3-oxoPrMCA and 3-oxoPrGSH, but not 3-hydroxyPrMCA, damaged the bladder dose-dependently when instilled intravesically in mice at concentrations of 10-20 mM. Potency was acrolein > 3-oxoPrMCA S-oxide > 3-oxoPrMCA > 3-oxoPrGSH. These data support the possibility that a mercapturic acid may be involved in the bladder toxicity of CP.

Introduction

Cyclophosphamide $(CP)^1$ (1), an oxazaphosphorine alkylating agent, is a prodrug used orally in over 200000 patients in the United States each year as an antitumor

agent and an immunosuppressant (1). A dose-limiting bladder toxicity (hemorrhagic cystitis) occurs in most patients receiving CP (2). Acrolein (2), which is formed via a β -elimination reaction following an initial 4-hydroxylation of CP (Figure 1), has been implicated as the metabolite responsible for bladder damage (3). The mechanism by which this extremely reactive intermediate is delivered to the bladder following bioactivation of CP in the liver and/or other tissues is not known.

Acrolein, an α . β -unsaturated aldehyde, is highly electrophilic and will react directly with cellular constituents, primarily at or near its site of formation (4). With thiols that are nucleophilic, acrolein can react at C-3 via a 1,4addition or at C-1 via a 1,2-addition. Michael addition of the thiol to the β carbon of the α,β -unsaturated aldehyde is kinetically favored over the nucleophilic

^{*} Address correspondence and reprint requests to this author at the Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin, TX 78712-1074. (512) 471-5188 (Office); (512) 471-5002 (FAX); Internet: kumar@mail.utexas.edu.

Division of Pharmacology and Toxicology.

[‡] Present address: Texas Natural Resources Conservation Commission, P.O. Box 13087, Austin, TX. [§] Division of Medicinal Chemistry.

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 ¹ Abbreviations: CP, cyclophosphamide; GSH, glutathione; 3-oxo-PrGSH, (3-oxopropyl)glutathione; 3-oxoPrMCA, S-(3-oxopropyl)-N-acetylcysteine; 3-hydroxyPrMCA, S-(3-hydroxypropyl)-N-acetylcysteine; 3-oxoPrMCA S-oxide, S-(3-oxopropyl)-N-acetylcysteine S-oxide.



(4) 3-oxopropyl MCA S-oxide

Figure 1. Postulated bioactivation mechanism of CP leading to mercapturic acid metabolites. The GSH-acrolein conjugate is hypothesized to undergo cleavage to a cysteine derivative followed by acetylation to give the mercapturic acid, 3-oxopropylMCA, or its reduced congener 3-hydroxypropylMCA. S-Oxidation of 3-oxopropylMCA by monooxygenases results in formation of the corresponding S-oxide, which, together with the parent mercapturate, may be excreted in the urine. These compounds can then undergo a nonenzymatic β -elimination reaction, generating acrolein that, along with the parent mercapturates, may damage the bladder.

addition to the aldehydic carbonyl. This reaction results in a product formed by addition of the thiol across the activated double bond (5).

The free thiol group on the tripeptide glutathione (GSH) is nucleophilic and reacts with acrolein through a Michael addition forming the acrolein-GSH adduct (3-oxoPrGSH) (5) following incubation of isolated rat hepatocytes with CP (6). Although 3-oxoPrGSH has not been identified *in vivo*, this route of biotransformation is widely believed to be involved in the formation of the mercapturic acid S-(3-hydroxypropyl)-N-acetylcysteine (3-hydroxyPrMCA) (6) which is found in urine after administration of CP (7-9) or acrolein (10) to rats (Figure 1).

Although the formation of glutathione conjugates is normally considered to be a detoxification pathway, *in vitro* and *in vivo* studies (11) suggest that glutathione conjugates can undergo several types of bioactivation reactions (12). In vitro, 3-oxoPrGSH is only minimally toxic to cultured cells (13) unless incubated for prolonged times (unpublished data). In vivo, 3-oxoPrGSH is nephrotoxic in rats, and its toxicity is blocked by acivicin, a γ -glutamyl transpeptidase inhibitor (14). This suggests that the conjugate requires processing through the mercapturate pathway before eliciting toxicity and may explain the nephrotoxicity occasionally seen with acrolein (6, 15). Other studies have shown that cysteine adducts of acrolein, and other α,β -unsaturated aldehydes, can slowly release the precursor aldehydes and may, therefore, serve as transport forms for cytotoxic aldehydes (5). Similarly, the decomposition of acrolein mercapturic acids has been postulated to regenerate acrolein (16). This latter study demonstrated the greater release of acrolein from S-(3-oxopropyl)-N-acetyl-L-cysteine S-oxide (3-oxo-PrMCA S-oxide) (4) than from S-(3-oxopropyl)-N-acetyl-L-cysteine (3-oxoPrMCA) (3), although both compounds were cytotoxic to freshly isolated renal proximal tubular cells and LLC-PK1 cells. This indicated that either acrolein could be released from 3-oxoPrMCA in the culture system (perhaps following S-oxidation) or that both compounds may be toxic without additional biotransformation or breakdown to acrolein.

In order to perform more detailed studies on the toxicity of 3-oxoPrMCA, 3-hydroxyPrMCA, 3-oxoPrMCA S-oxide, and 3-oxoPrGSH, it was necessary to synthesize

quantities of each of these compounds. Although methods have been published to accomplish this goal (14, 16), we found that previously reported NMR spectra did not reveal some interesting properties of these compounds. Further, by modifying these prior procedures, we were able to significantly increase our yields of products. The current study reports on the details of these synthetic procedures and provides an in-depth NMR and mass spectroscopic characterization of each compound as well as their ability to release acrolein and damage the bladder. (2,4-Dinitrophenyl)hydrazine (2,4-DNPH) derivatives are also described that may be useful for demonstrating the presence of these mercapturates in biological systems.

Experimental Procedures

Drugs and Chemicals. Acrolein, 30% w/w hydrogen peroxide, glutathione, hydroxylamine hydrochloride, semicarbazide, 2,4-DNPH, deuterated water (D₂O) (internal standard as 0.75%3-(trimethylsilyl)propionic acid-2,2,3,3-d₄, sodium salt) and deuterated dimethyl sulfoxide (internal standard as 1% tetramethylsilane) as NMR solvents, and potassium phosphate (monobasic and dibasic) were obtained from Sigma Chemical Co. (St. Louis, MO). *m*-Aminophenol was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). A mixture of ketamine and xylazine for anesthesia was obtained from the Animal Resources Center at the University of Texas at Austin. All other chemicals used were of reagent grade.

Animals. Male ICR mice, weighing between 30 and 45 g, were supplied by Harlan Sprague Dawley and housed at the Animal Resource Center at the University of Texas at Austin. The animals were maintained on a 12 h light/dark cycle and were provided with food and water *ad libitum*.

Chromatography. Thin-layer chromatographic (TLC) analyses of the synthesized conjugates were carried out on precoated aluminum backed (Art. 5554, DC-Alufoilen Kieselgel) silica gel 60 F254 plates (0.2 mm, E. Merck). TLC plates were developed in ethyl acetate/methanol/acetic acid (49.5:49.5:1) and visualized either in an iodine chamber or with *p*-anisaldehyde-sulfuric acid spray reagent, followed by heat. The p-anisaldehydesulfuric acid spray reagent (200 mL) was prepared by adding in the following order: p-anisaldehyde 1 mL, glacial acetic acid 20 mL, methanol 169 mL, and concentrated sulfuric acid 10 mL. TLC analyses of the *m*-aminophenol derivatives were performed on silica gel 60 precoated glass plates without a fluorescent indicator (E. Merck). Plates were developed by the ascending technique at room temperature in butanol/acetic acid/water (60: 15:25). Spots were visualized for fluorescence by irradiating them with long wavelength ultraviolet light.

Column chromatography was conducted on standard flash columns using silica gel 60 Art. 9385 (40-60 mm, E. Merck). Melting points were determined in open capillary tubes with a Thomas-Hoover capillary melting point apparatus and were uncorrected.

Reversed phase high performance liquid chromatography of DNPH derivatives was done using a C18 column (Whatman Partisil, 25 cm \times 4 mm, ODS-3, 5 μ m) and a mobile phase of 25–90% acetonitrile in 0.1 M ammonium acetate (linear gradient over 30 min). Detection was at 355 nm.

Syntheses. (a) 3-OxoPrMCA (3). Acrolein (MW 56) (2) (1.68 g, 2.0 mL, 30 mmol) in 3-fold molar excess was added to a stirring suspension of N-acetyl-L-cysteine (MW 163) (1.63 g, 10 mmol) in 5 mL of water. This mixture was stirred overnight under nitrogen, at room temperature. A single spot corresponding to the product ($R_f = 0.48$) was seen by TLC, indicating total consumption of N-acetyl-L-cysteine ($R_f = 0.58$) in the reaction. Water and excess acrolein from the reaction were removed under reduced pressure at 40 °C, and the product was placed under high vacuum overnight to give 2.17 g of 3-oxoPrMCA (3) (MW 219) (9.9 mmol, 99% yield), C_8H_{13}NO_4S, viscous yellow liquid. EI mass spectrometry showed a quasimolecular ion (M - H⁺) at m/z 218 (Figure 2), R_f 0.48, ¹H NMR (Table 1, Figure 3), ¹³C



Figure 2. Scanning EI mass spectra (mass intensity vs m/z) of the acrolein mercapturates.

NMR (Table 2). Exact mass calculated for (MH⁺) $C_8H_{14}NO_4S$ 220.0643; found 220.0642. The melting point of the 2,4-DNPH derivative was 94–97 °C.

(b) 3-OxoPrMCA S-Oxide (4). To a stirring solution of 3-oxoPrMCA (3) (MW 219) (660 mg, 3.01 mmol) in 5 mL of water was added a 1.2-fold molar excess of 30% H₂O₂ (MW 34) (409 mg, 409 μ L, 3.61 mmol). This mixture was stirred for 1 h in a water bath at room temperature. Total consumption of 3-oxo-PrMCA was seen in the reaction as determined by TLC, where a single spot corresponding to the product ($R_f = 0.25$) was seen. Water from the reaction was removed under reduced pressure at 40 °C, and the product was placed under high vacuum

overnight to give 680 mg of 3-oxoPrMCA S-oxide (4) (MW 235) (2.9 mmol, 96% yield), $C_8H_{13}NO_5S$, highly hygroscopic white solid. EI mass spectrometry showed a quasimolecular ion (M – H⁺) at m/z 234 (Figure 2), R_f 0.25, ¹H NMR (Table 1), ¹³C NMR (Table 2). Exact mass calculated for (MH⁺) $C_8H_{14}NO_5S$ 236.0593; found 236.0583. The melting point of the 2,4-DNPH derivative was 80–84 °C.

(c) 3-OxopropylGSH (5). To a stirring solution of glutathione (MW 307) (614 mg, 2.0 mmol) in 5 mL of water was added a 1.5-fold molar excess of acrolein (MW 56) (2) (168 mg, 200 μ L, 3.0 mmol). This mixture was stirred for 1 h under nitrogen in a water bath at room temperature. Water from the reaction was removed under reduced pressure at 40 °C, and the product was placed under high vacuum overnight to give 720 mg of 3-oxopropylGSH (5) (MW 363) (1.98 mmol, 99% yield), C₁₃H₂₁N₃O₇S, white solid, decomposes at 215 °C. EI mass spectrometry showed a quasimolecular ion (M – H⁺) at *m/z* 362 (Figure 2), R_f 0.10, ¹H NMR (Table 1), ¹³C NMR (Table 2). Exact mass calculated for (MH⁺) C₁₃H₂₂N₃O₇S 364.1978; found 364.2012.

(d) 3-HvdroxvPrMCA (6). The procedure of Onkenhout et al. (17) as modified by Hazelton Laboratories (Madison, WI; personal communication) was used. To a stirring suspension of N-acetyl-L-cysteine (MW 163) (4.9 g, 30 mmol) in methanol (100 mL) was added a NaOH solution (50% w/w, 4.8 g, 60 mmol). 3-Chloropropanol (MW 94.5) (2.9 g, 30 mmol) was then added, and the mixture was refluxed under nitrogen for 18 h. The resulting solution was concentrated under reduced pressure, and acetone (100 mL) was added. The solution was made acidic using concentrated HCl. Water from the reaction was removed under reduced pressure at 40 °C and the product placed under high vacuum overnight. The product was then flash chromatographed on a silica gel column using methanol/chloroform/acetic acid (10:85:5). The column fractions having the product were pooled (analyzed by TLC and visualized by iodine vapors) to give 4.2 g of 3-hydroxyPrMCA (6) (MW 221) (19 mmol, 63% yield), C₈H₁₅NO₄S, viscous colorless liquid. EI mass spectrometry showed a quasimolecular ion (M^+) at m/z 221 (Figure 2), R_f 0.60, ¹H NMR (Table 1), ¹³C NMR (Table 2). Exact mass calculated for (MH^+) C₈H₁₆NO₄S 222.0799; found 222.0811.

(e) 2,4-DNPH Derivatives. A 2,4-DNPH solution was prepared by adding 2 mL of concentrated sulfuric acid to 0.4 g of 2,4-DNPH in a 25 mL Erlenmeyer flask. Water (3 mL) was then added dropwise with stirring until solution was complete. To this warm solution was added 10 mL of 95% ethanol. 2,4-DNPH derivatives were prepared at room temperature by adding approximately 0.1 g of the mercapturate aldehyde (3 or 4) dissolved in 1 mL of 95% ethanol. Crystallization of the (2,4dinitrophenyl)hydrazone occurred within 5-10 min. The precipitate was filtered and recrystallized using a mixture of ethyl acetate and 95% ethanol. The derivatives were dried under vacuum and their melting points determined.

(f) Deuterated Potassium Phosphate (K_2DPO_4 and KD_2PO_4) Solutions. To 17.4 mg (100 mM, pH 8.3) of K_2HPO_4 in a 2 mL vial was added 1 mL of D_2O , and the solution was evaporated to dryness. This was repeated twice to ensure exchange of the hydrogens by deuterium. The deuterated residue was then dissolved in 1 mL of D_2O . Similarly, a solution of 13.6 mg (100 mM, pH 4.5) of KH₂PO₄ in 1 mL of D_2O was prepared. Approximately 0.5 mL of either solution was used to run the NMR of 3-oxoPrMCA (3).

Acrolein Release Determinations. The incubation system consisted of a solution (100 nmol/mL in 100 mM potassium phosphate buffer containing 0.1 M KCl, pH 8.0) of each of the conjugates (3-oxoPrGSH, 3-oxoPrMCA, 3-hydroxyPrMCA, 3-oxo-PrMCA S-oxide) as well as acrolein and GSH, in 25 mL tightly sealed Erlenmeyer flasks (total volume 22 mL). The reaction mixture was incubated at 37 °C in a water bath, and 4 mL aliquots were taken at time intervals of 0, 15, 30, 60, and 120 min. Separate 4 mL aliquots were used, and a standard curve was generated with authentic acrolein, for each of the methods.

(a) Absorbance at 210 nm. The change in absorbance at 210 nm was measured on a Beckman DU-62 spectrophotometer. This procedure was also done in a separate experiment with 3-oxoPrMCA and 3-oxoPrMCA S-oxide at pH 7.4.

(b) Fluorescence. MAP reagent (250 mg of *m*-aminophenol and 300 mg of hydroxylamine hydrochloride in 25 mL of 1 N HCl) was prepared and stored in an amber colored bottle. One milliliter of MAP reagent and 1 mL of 5 N HCl were added to a 4 mL aliquot of the incubation mixture. This solution was vortexed and heated for 10 min in a boiling water bath. It was then allowed to cool, and the fluorescence was recorded at excitation and emission wavelengths of 355 and 510 nm, respectively (18).

(c) Semicarbazone Formation. The pH of a 4 mL aliquot of the incubation mixture was adjusted to 4 with 5 N HCl. Semicarbazide (600 nmol) was then added. This mixture was incubated for 30 min at 37 °C in a water bath and semicarbazone formation quantified by measuring the increase in absorbance at 257 nm. The loss of acrolein was also monitored by measuring the decrease in absorbance at 210 nm.

Instrumental Analysis. Electron impact (EI) mass spectra were recorded on a TSQ Finnigan-MAT-70 mass spectrometer. Accurate mass determinations were performed on a Fisons (VG analytical) VG ZAB-2E mass (FAB mass spectra) spectrometer. A Bruker AM-250 NMR spectrometer was employed for ¹H NMR, ¹³C NMR, and DEPT (distortionless enhancement through polarization transfer experiment) spectra (operating at 250 MHz for proton and 62.5 MHz for carbon). The spectra were recorded at 25 °C in either deuterated water (D₂O) or deuterated dimethyl sulfoxide (DMSO).

Bladder Toxicity Studies. The various acrolein conjugates were administered as single intravesicle injections. Mice were anesthetized subcutaneously prior to injection with 0.1 mL of a cocktail of ketamine and xylazine. Intravesical instillation of the acrolein adducts (10, 15, and 20 mM solutions; 0.2 mL/ mouse) dissolved in 100 mM potassium phosphate buffer, pH 7.4, containing 0.9% NaCl, was carried out by means of a simple needle puncture of the urinary bladder following a skin incision. The bladder was emptied of any urine before instilling the compounds. Wounds were closed with clips. Controls received an equal volume of isotonic phosphate buffer.

Mice were euthanized 24 h after treatment by cervical dislocation. Bladders were removed, expressed, trimmed of extraparenchymal tissues, and rinsed with cold 50 mM Tris-HCl containing 0.1 mM EDTA (pH 7.6). The bladders were homogenized for 30 s with a Tekmar Tissuemizer in 3 mL of 50 mM Tris-HCl. The resulting crude homogenate was filtered through four layers of gauze, made to a volume of 4 mL/bladder with the same buffer, and centrifuged at 1800g for 5 min. The supernatant was filtered through a 5 μ m syringe filter and the absorbance at 415 nm measured (19). Blood enriched aliquots of bladder homogenates used to construct calibration curves were treated in an identical manner.

Statistical Analysis. Data are expressed as the mean \pm SE and were analyzed using the unpaired Students *t* test where appropriate. Multiple group data were analyzed by one-way analysis of variance. Post hoc analyses were carried out using the Student-Newman-Keuls test. A *p* value of less than 0.05 was considered significant.

Results

Synthesis and Characterization of 3-OxoPrMCA. The procedure used for the synthesis of 3-oxoPrMCA (3) was modified from Hashmi *et al.* (16). This original procedure used a 1:1 molar ratio of acrolein (2) to *N*-acetylcysteine and required purification by flash chromatography. Doing the reaction under nitrogen and using a 3:1 molar ratio of acrolein to *N*-acetylcysteine gave a 98% yield of 3-oxoPrMCA (3) of mass 219. This compound was characterized by ¹H (Table 1) and ¹³C (Table 2) NMR in DMSO as solvent. Using DMSO as solvent allows visualization of the amino proton which would be otherwise exchanged in D₂O. On the basis of TLC and NMR results, no further purification seemed essential.

	3		4 ^b	6	5	N-Ac-cysteine ^c	2 ^c
proton no.	DMSO	D_2O	D_2O	D ₂ O	D_2O	DMSO	D_2O
1 (HC=O) 1' [CH(OH) _{2]}	9.60 s	9.50 s 4.97 t (5.6)	5.05 t, 5.10 t	(CH ₂) 3.46 t	9.55 br s 5.05 t	(SH) 2.32 t	9.38 s
$2\;(CH_2)$	2.70* m	1.72 q (5.6, 7.4)	1.97 q, 1.97 q	1.62 quint	1.76 q		6.41 dd
$3\;(CH_2)$	2.70* m	2.50 t (7.4)	2.8 - 2.9	2.47 t	2.55 t		6.47 dd
5 (CH ₂)	2.89 dd	2.94 dd (4.8, 14.0)	3 05-3 35	2.86 dd	2.97 dd	2.8 br m	6.61 dd
	2.70* m	2.79 dd (7.8, 14.0)	0.00 0.00	2.66 dd	2.80 dd		
6 (CH)	4.40 dd	4.40 dd (4.8, 7.8)	4.65 dd, 5.25 dd	4.42 dd	4.46 br dd	4.38 dd	
7 (NH) 9 (CH ₃) 10 (CH ₂) 11 (CH) 15 (CH ₂)	8.25 d 1.85 s	exchanged 1.90 s	exchanged 1.88 s, 1.91 s	$\begin{array}{c} exchanged \\ (CH_2) \ 1.97 \ s \end{array}$	exchanged 2.05 dd 2.44 br dt 3.74 t 3.87 s	8.16 d 1.87 s	
diol OH		2.7 s					

a (') indicates protons that showed substantial changes in chemical shift for the geminal diol form. (*) indicates protons with the same chemical shift. ^b Chemical shifts for both diastereomers are listed. ^c Numbering of protons corresponds to their positions in the mercapturates.

Table 2. ¹³C NMR Chemical Shift Assignments of the Mercapturic Acids of Acrolein^a

	3		4^{b}	6	5	N-Ac-cysteine ^c	2 ^c
carbon no.	DMSO	D_2O	D_2O	D_2O	$\overline{D_2O}$	DMSO	D_2O
1 (HC=O) 1' [CH(OH) ₂₁	202.0	206.6 90.4	90.6. 99.7	$(CH_2) 60.9$	206.8 90.5		199.7
$2 (CH_2)$ 2' (CH ₂)	42.8	$43.5 \\ 37.5$	47.6, 47.4	33.3	43.8 37.5		138.2
$3(CH_2)$ 3'(CH ₂)	24.1	$24.8 \\ 27.8$	24.6, 26.5	29.0	$27.4 \\ 29.0$		142.5
5 (CH ₂) 6 (CH)	$32.9 \\ 51.9$	33.5 53.3	$31.4^{'}$ 53.8, 49.1	32.0 53.5	33.7 53.8	$25.9 \\ 54.6$	
8 (C=O) 9 (CH ₃)	169.6^{*} 22.3	$\begin{array}{c} 174.8\\ 22.5\end{array}$	174.8, 174.9 22.5, 21.1	174.3^{*} 22.5	174.6^{*} (CH ₂) 26.8	169.9^{*} 22.6	
10 (C=O) 11 (CH) 12 (C=O) 13 (C=O)	172.1*	174.8	174.5, 175.2	174.6*	(CH_2) 32.0 54.6 175.5* 174.4*	171.9*	
$\begin{array}{c} 15 \; (CH_2) \\ 16 \; (C=0) \end{array}$					$42.5 \\ 173.4^*$		

a (') indicates carbons that showed substantial changes in chemical shift for the geminal diol form. (*) indicates assignments within the column that are interchangeable. ^b Chemical shifts for both diastereomers are listed. ^c Numbering of protons corresponds to their positions in the mercapturates.

Synthesis and Characterization of 3-OxoPrMCA S-Oxide. An earlier procedure (16) for the synthesis of 3-oxoPrMCA S-oxide (4) used a 1:1 molar ratio of 3-oxo-PrMCA (3) to H_2O_2 and a reaction time of 24 h. The reported yield of this method was only 10%. When we repeated this procedure, a single compound (as indicated by TLC) with a mass of m/z 235 corresponding to that of 3-oxoPrMCA S-oxide was isolated. However, the negligible intensity or absence of an aldehydic proton in the ¹H NMR of the product, using DMSO as solvent, led us to believe that the aldehyde had been oxidized to the diacid (which has the same molecular weight). Thus, it seemed possible that the major product of this procedure contained very little or none of the S-oxide.

To more directly compare our results to earlier work (16), ¹H and ¹³C NMR spectra of 3-oxoPrMCA were acquired in D₂O as solvent. ¹H NMR showed extra resonances at $\delta \sim 5.0$ while ¹³C NMR showed eleven (three more than there should be) carbons with a characteristic methine carbon around 90.0 ppm. Although decomposition of 3-oxoPrMCA could explain this finding, the ¹H and ¹³C NMR spectra in DMSO as solvent

indicated that this was something unique to the NMR in D₂O as solvent (Figure 3) because the anhydrous aldehyde 3-oxoPrMCA afforded characteristic aldehydic proton resonances at $\delta \sim 9.5$ in DMSO and ¹³C NMR showed only eight carbons.

Aldehydes are easily hydrated to their geminal diol in water (20). This can be seen distinctly with glyoxals such as methylglyoxal (21, 22). The glyoxals afford characteristic aldehydic proton resonances $\delta \sim 9.5$ in CDCl₃ as solvent (21), but in D₂O as solvent no aldehyde resonances are detected. Instead, new resonances are observed at δ 5.1–5.2 (21) while the corresponding carbon moves upfield to around 90 ppm (22) from 200 ppm. This indicates a virtually complete hydration of the aldehyde to the corresponding geminal diol [CH(OH)₂].

This same hydration phenomenon was seen with 3-oxoPrMCA in D_2O as solvent. The aldehyde resonance was still seen, but a new resonance was observed at $\delta \sim 5.0$ (H-1') while the corresponding carbon (C-1') moved upfield to around 90.0 ppm. This implied that the 3-oxoPrMCA solution consisted of a mixture of two forms of the compound. Integrating the protons (equating the



Figure 3. ¹H NMR of 3-oxopropyl mercapturic acid in DMSO and D₂O.



Figure 4. Equilibrium between 3-oxopropylMCA (aldehyde) and its geminal diol in aqueous solution.

observed aldehydic peak to one proton) indicated that a 75% hydration of the aldehyde to the corresponding diol had occurred (Figure 4) within minutes. In the case of the geminal diol, one of the methylene carbons C-2 moved upfield (C-2') by around 6.0 ppm and C-3 moved downfield (C-3') by 3.0 ppm, while the carbons C-5, C-6, C-8, C-9, and C-10 remained unchanged. The corresponding protons H-2 and H-3 did not show substantial changes in chemical shift.

Except for being able to differentiate the acid and amide carbonyls in the ¹³C NMR spectra of 3-oxoPrMCA (3) using the monobasic (KD₂PO₄, pH 4.5) and dibasic (K₂DPO₄, pH 8.3) phosphate solutions in D₂O as solvent, no change was observed with respect to the ratio of the two forms of 3-oxoPrMCA even after 48 h. There was also negligible decomposition (<5%) of 3-oxoPrMCA in the dibasic (K₂DPO₄) phosphate solution after 48 h, indicating the stability of the geminal diol under these conditions.

Modifying the procedure for the synthesis of 4 by decreasing the reaction time to 1 h and using a 1.2-fold molar excess of H_2O_2 to 3-oxoPrMCA (3) gave a 96% yield of 3-oxoPrMCA S-oxide (4). Again, no further purification seemed essential. Two aldehydic peaks were seen in the ¹H NMR spectrum of the product in DMSO that disappeared within a half hour of making up the solution. In contrast, ~100% hydration of the aldehyde occurred almost instantaneously in D_2O . The conversion of the aldehyde to the geminal diol in D_2O could be predicted from the NMR spectra. Two sets of peaks for the geminal diol [H-1', CH(OH)₂] and the methine H-6 protons indicated the formation of both diastereomers of the *S*-oxide in a 50:50 mixture as in the case of L-methionine *S*-oxide (23). In water, the solution consisted of a mixture of the *d*,*l* and *l*,*l* diastereomers of the 3-oxoPrMCA *S*-oxide, both in their geminal diol forms.

The sulfoxide 4 showed 100% hydration of the aldehyde to the geminal diol while the sulfide 3 showed only 75% hydration. The precipitation of the hydrazone derivative occurred at a much slower rate for the S-oxide than for the sulfide. This slow reaction can be attributed to the equilibrium between the aldehyde and the geminal diol which, in case of the sulfoxide, was more toward the geminal diol than the aldehyde. A strong inductive effect in the case of the sulfoxide (octahedral) 4 on the aldehydic carbon could cause a more favorable and complete hydration of the aldehyde to the geminal diol. The weaker inductive effect of the sulfide (tetrahedral), in turn, is less favorable for the formation of the diol and helps partially stabilize the aldehyde (3).

2,4-DNPH Derivatives. Aldehydes form hydrazone derivatives in the presence of hydrazines. The 2,4-DNPH derivatives of the aldehyde mercapturates **3** and **4** were prepared. Their melting points (see Experimental Procedures) were found to be different from that of the 2,4-DNPH derivative of acrolein (mp 137-139 °C). Spectral analyses of all three 2,4-DNPH derivatives showed absorbance maxima around 355 nm. The retention times of these highly colored derivatives in an HPLC system (see Experimental Procedures) were 10.1, 23.0, and 24.8 min for oxoPrMCA, acrolein, and oxoPrMCA S-oxide, respectively.



Figure 5. Acrolein generation from 100 nmol/mL (3-hydroxypropyl)-N-acetylcysteine (3-hydroxyPrMCA); (3-oxopropyl)-Nacetylcysteine (3-oxoPrMCA); (3-oxopropyl)glutathione (3-oxopropylGSH); and (3-oxopropyl)-N-acetylcysteine S-oxide (3oxoPrMCA S-oxide) as a function of time. Experiments were conducted at 37 °C and a pH of 8.0 (solid lines) or 7.4 (dotted lines). Acrolein formation was determined by measuring the change in absorbance at 210 nm. Data are expressed as mean nmol of acrolein/mL \pm SE (n = 3).

Synthesis and Characterization of 3-OxoPrGSH and HydroxyPrMCA. Using a modified procedure from Horvath *et al.* (14), 3-oxoPrGSH (5) was synthesized from glutathione with a 1.5-fold molar excess of acrolein (2). The yield with this procedure was 99%. NMR analyses revealed that the formation of the geminal diol product was also seen in this case. HydroxyPrMCA (6) was synthesized using a modified method (17) starting from 3-chloropropanol instead of 1,3-dibromopropane. This procedure was somewhat more complex than those used for the other mercapturates, and some purification by flash chromatography on a silica gel column was required. The final product was obtained in a 63% yield as a viscous colorless liquid.

Stability of Acrolein Conjugates. Three approaches were used to assess the release of acrolein. Measuring the change in absorbance at 210 nm, measuring acrolein semicarbazone formation at 257 nm, and evaluating the fluorescent response using the *m*-aminophenol assay were the methods used.

The kinetics of the generation of acrolein from 3-oxo-PrMCA, 3-hydroxyPrMCA, 3-oxoPrMCA S-oxide, and 3-oxopropylGSH were measured over 120 min in potassium phosphate buffer, pH 8.0. As assessed by the change in absorbance at 210 nm, small amounts of acrolein were released from 3-oxoPrMCA after 30 min (6%) while 3-oxoPrMCA S-oxide showed a much greater release of acrolein (16%) (Figure 5). Release of acrolein from hydroxyPrMCA and 3-oxopropylGSH was less than 2%. The release of acrolein from all conjugates was maximal by 30 min. Except for 3-oxoPrMCA S-oxide which showed a decrease, there were no further changes in absorbance with any conjugate up to 2 h. When examined in potassium phosphate buffer at pH 7.4, the release of acrolein from 3-oxoPrMCA or 3-oxoPrMCA was only about half as great as that measured at pH 8.0 (Figure 5).



Figure 6. Percentage of acrolein remaining following incubation of a solution containing 100 nmol/mL in 100 mM potassium phosphate buffer containing 0.1 M KCl, pH 8.0. Acrolein content was assessed by measuring absorbance at 210 nm, semicarbazone formation, and fluorescence following reaction with maminophenol.

When authentic acrolein was incubated under identical conditions, there was a linear decrease in absorbance at 210 nm with time, indicating a loss of 55% of the initial concentration after 2 h (Figure 6). Assessing acrolein content with a fluorescence assay gave readings that remained relatively constant until 60 min while formation of the semicarbazone derivative decreased at a slower rate than absorbance at 210 nm (Figure 6). Decreases in acrolein content are likely the result of polymerization (24). Differences between the various assays may be due to differing abilities to react with the polymerized material. Polymerization might explain the absence of a continual increase in acrolein with time when the different conjugates were incubated in buffer (Figure 5).

Because an increase in absorbance at 210 nm is not definitive evidence of acrolein generation, acrolein release from the various conjugates was confirmed by measuring acrolein semicarbazone formation at 257 nm (Figure 7) and the concomitant loss of absorbance at 210 nm (data not shown). Both parameters correlated with the presence of acrolein in these assays.

The reaction between m-aminophenol and acrolein, substituted acrolein, or compounds that can generate them under acid conditions results in the formation of the fluorescent compound 7-hydroxyquinoline (18). Various other aldehydes will also react with m-aminophenol, producing compounds with less intense fluorescence than acrolein (18). Despite the lack of absolute specificity, this assay has been commonly used to detect acrolein in biologic systems.

3-OxoPrMCA S-oxide, 3-oxoPrMCA, and 3-oxoPrGSH gave strong fluorescent responses in the m-aminophenol assay while 3-hydroxyPrMCA yielded no fluorescent material (Table 3). The determination of excitation and emission maxima verified that 7-hydroxyquinoline was present. There was, however, no time-dependent increase in the production of fluorescent material (Table 3). The possibility that the fluorescence assay itself was generating acrolein was examined by reacting acrolein, 3-oxoPrMCA, and 3-oxoPrMCA S-oxide with m-aminophenol and subjecting the resultant fluorescent mate-



Figure 7. Acrolein generation from 100 nmol/mL (3-hydroxypropyl)-*N*-acetylcysteine (3-hydroxyPrMCA); (3-oxopropyl)-*N*acetylcysteine (3-oxoPrMCA); (3-oxopropyl)glutathione (3-oxopropylGSH); and (3-oxopropyl)-*N*-acetylcysteine *S*-oxide (3oxoPrMCA *S*-oxide) as a function of time. Experiments were conducted at 37 °C and a pH of 8.0. Acrolein formation was determined by measuring semicarbazone formation by the absorbance at 257 nm following reaction with semicarbazide. Data are expressed as mean nmol of acrolein/mL \pm SE (n = 3).

 Table 3. Fluorescence following the Reaction of Mercapturic Acid Analogs with *m*-Aminophenol^a

time ^b (min)	3-hydroxy- PrMCA	3-oxo- PrMCA	3-oxo- PrGSH	3-Oxo-PrMCA S-oxide
0	0.8 ± 0.4	45.5 ± 1.3	23.4 ± 0.3	72.0 ± 1.6
15	0.6 ± 0.4	46.8 ± 1.4	24.6 ± 0.5	75.3 ± 2.3
30	0.8 ± 0.4	47.1 ± 1.6	25.0 ± 0.4	73.4 ± 1.9
60	0.9 ± 0.4	48.1 ± 1.5	25.5 ± 1.5	70.5 ± 1.8
120	0.8 ± 0.4	35.3 ± 1.3	23.4 ± 0.3	65.6 ± 1.7

^a Fluorescence was determined with excitation at 355 nm and emission at 510 nm. A standard curve was generated using authentic acrolein. Data are expressed as the mean percentage yields of acrolein \pm SE assuming the potential for 100% yields. ^b Incubations were done in 100 mM KPO₄ and 0.1 M KCl, pH 8.0.

rial to thin-layer chromatography. The results showed that most of the fluorescent material formed from 3-oxo-PrMCA or its S-oxide had R_f values identical to that formed with acrolein (0.39).

Toxicity Studies. There was a significant dosedependent increase in bladder hemoglobin levels when acrolein, 3-oxoPrMCA S-oxide, 3-oxoPrMCA, and 3-oxo-PrGSH were instilled into the bladder (Figure 8). The highest concentration of 3-oxoPrMCA S-oxide (20 mM) produced essentially the same degree of damage as 15 mM acrolein. HydroxyPrMCA failed to produce bladder injury when instilled intravesically up to 20 mM. The potency of the various conjugates in terms of bladder injury was as follows: acrolein > 3-oxoPrMCA S-oxide > 3-oxoPrMCA > 3-oxoPrGSH. This correlated with the release of acrolein (Figures 5 and 7), and even better with the ability to react with *m*-aminophenol (Table 3). The pHs of 20 mM solutions of 3-oxoPrMCA and 3-oxoPrMCA S-oxide in unbuffered saline were 2.55 and 2.46, respectively. Since bladder installations were done in phosphatebuffered saline, it is unlikely that acidic damage was a factor in the measured injury.



Figure 8. Bladder damage 24 h following intravesical instillation of (3-hydroxypropyl)-*N*-acetylcysteine (3-hydroxyPrMCA); (3-oxopropyl)-*N*-acetylcysteine (3-oxoPrMCA); (3-oxopropyl)glutathione (3-oxoPrMCA S-oxide) or acrolein. Compounds were dissolved in phosphate-buffered saline (pH 7.4). Data represent the mean μ L of blood/bladder ± SE minus controls (n = 3). Bladder blood content of controls = 0.015 ± 0.001 μ L. (*) Significantly different from controls treated with buffer alone (p < 0.05).

Discussion

The protective effects against CP-induced bladder injury that are achieved with cysteine, acetylcysteine, or mesna (2-mercaptoethanesulfonate) are consistent with the presence in urine of an electrophilic compound(s) from CP metabolite(s) (1). Acrolein is by far the strongest electrophile and shows the highest reactivity of the α . β unsaturated aldehydes (25). It has been convincingly demonstrated that only oxazophosphorine derivatives capable of generating acrolein are able to cause bladder toxicity (3). However, although acrolein is reportedly present in human urine following treatment with CP(26), it remains unclear how this aldehyde, which arises from metabolites formed mainly in the liver, is delivered to the bladder. Circulating 4-hydroxyCP, secreted into the urine and then broken down to acrolein and phosphoramide mustard, is one possibility (1). It has been suggested that 3.5% of an administered dose of CP is excreted in this form (27). However, the identification of urinary 4-hydroxyCP in these studies relied on the m-aminophenol fluorescence assay that cannot differentiate among compounds capable of releasing acrolein, and more recent data suggest only 0.5% of a CP dose is excreted as the 4-hydroxy metabolite (28). Thus, other transport forms may be involved. For example, cysteine adducts of acrolein and other α,β -unsaturated aldehydes can slowly release the precursor aldehydes and may serve as transport forms for cytotoxic aldehydes (5, 11, 29). In addition, the decomposition of mercapturic acid adducts has been postulated to regenerate acrolein (7).

We previously reported that acrolein measurable by the fluorescence assay rapidly disappeared when authentic acrolein was added to urine of untreated mice (which contains large amounts of protein under normal conditions) (19). Recovery of acrolein from tissue homogenates has also been found to be quite low (30), probably due to covalent binding. In contrast, acrolein equivalents mea-

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surable in the urine of mice treated with CP were stable for several hours (19). This was not due to a saturation of acrolein binding sites since virtually all acrolein added to urine from CP-treated mice disappeared within 30 min. These results indicate that acrolein measured in urine samples is not present in the free form.

Mercapturates are, almost without exception, metabolized to S-oxides (30), and mercapturic acid S-oxides of several compounds are excreted in trace amounts into the urine of animals (32-35). This is normally a detoxication pathway, and S-oxide-dependent bioactivation has only recently been described (16, 36, 37). Interestingly, propylthiouracil, an oxidase inhibitor, reduces urinary excretion of acrolein-like material (38) and toxicity following administration of CP to rats (39). Although the thiol moiety on this compound could react with acrolein, thereby explaining this protection, prevention of Soxidation of the mercapturic acids may also be a factor.

The reactivity of acrolein has made its detection in biologic samples difficult. The assay most often used to quantitate acrolein measures the fluorescence that is produced when a variety of compounds, most of them aldehydes, are heated with an acid solution of *m*aminophenol (7). While acrolein produces more intense fluorescence than other compounds (18), the assay is not specific for this aldehyde. Conjugates in which the aldehyde moiety remains intact should theoretically test positive in the assay. In the case of α,β -unsaturated aldehydes such as acrolein, nucleophilic attack by thiols at C-3 is preferred, leaving the aldehyde group undisturbed which could react in the fluorescence assay.

Acrolein equivalents were detected by fluorescence spectroscopy in the 3-oxoPrMCA, 3-oxoPrMCA S-oxide, and 3-oxoPrGSH incubates at time zero. Increasing the incubation period did not affect the level detected. Mass spectrometry data suggest that these compounds are subject to thermal decomposition. The harsh conditions (100 °C and pH <1.0) used in the reaction with maminophenol may, therefore, have caused their breakdown. This possibility is supported by data in a paper describing an HPLC assay for acrolein in urine following derivatization with m-aminophenol (26). Interestingly, the authors reported that the reaction yield was very small unless ferrous sulfate was added as a catalyst. This may be because of binding to urine proteins or, in the case of CP, the need to release acrolein from a precursor. Alternately, since these mercapturic acids contain an aldehyde moiety, it is possible that the intact molecule tests positive in the assay, albeit at a much lower fluorescence intensity. This latter possibility is supported in part by the mobility of the fluorescent material derived from 3-oxoPrMCA or its S-oxide on TLC. However, the presence of fluorescent material with R_f values identical to that obtained with authentic acrolein, together with determination of acrolein semicarbazone formation, supports the formation of acrolein from precursor material.

CP is not a potent hepatic or renal toxin even though its metabolism to acrolein occurs predominantly in the liver (and possibly in the kidney). A hypothetical pathway by which toxic species may be formed and reach the bladder after treatment with CP is illustrated in Figure 1. Following initial oxidation of CP, mainly by the mixedfunction oxidase system in the liver, acrolein is released by a nonenzymatic process. This aldehyde is then conjugated with GSH, and once formed, the conjugate may be further transformed by the mercapturate pathway to 3-oxoPrMCA. In general, aldehydic conjugates are rapidly reduced (personal communication from Dr. Melanie M. C. G. Peters). With 3-oxoPrGSH and/or 3-oxoPrMCA this would yield the nontoxic 3-hydroxy-PrMCA. However, it is also possible that the aldehyde moiety is oxidized (40). 3-OxoPrMCA or its oxidized product could be involved in the toxicity of CP (40) perhaps by releasing a reactive species following an S-oxidation step which generates an even less stable compound (16).

An alternate toxic mechanism is that the aldehydic mercapturic acids themselves are reactive. The aldehydic moiety of 3-oxoPrMCA is capable of reacting directly with cysteine or amino groups of proteins (13) and may explain the cytotoxicity associated with this compound (16). This concept is supported by the current findings that instillation of compounds with a free aldehyde group directly into the urinary bladders of mice resulted in a hemorrhagic response similar to that observed following systemic administration of CP (19) and intravesical instillation of acrolein. This is true even though the amount of acrolein released at neutral or acidic pH is relatively small and would not be expected to be sufficient to explain the observed damage. Calculations have suggested that bladder toxic CP metabolites are present at concentrations up to 2 mM (27). Although direct instillation of potential metabolites is a poor model of the in vivo situation because of severe kinetic deficiences, thereby requiring extremely high doses, the data presented here do demonstrate the potential for mercapturic acid-induced bladder damage.

Nephrotoxicity associated with the mercapturic acids of numerous compounds has been reported (41-44). While the mechanism(s) of mercapturate toxicity have not been elucidated, various renal enzyme systems involved in mercapturic acid biosynthesis and catabolism as well as regional physiologic transport factors have been implicated. Redox cycling with concomitant generation of reactive oxygen species has been demonstrated for several mercapturic acids (45) and may be involved in the observed toxicities. A similar mechanism could be operative in the bladder toxicity associated with intravesical instillation of these compounds although the bladder is generally considered to be a reducing environment.

In previous work, we reported that a 2-h exposure to 3-oxoPrMCA or acrolein significantly inhibited the growth of A549 cells while CP, 3-oxoPrGSH, and 3-hydroxyPrMCA were nontoxic (13). The dehydration of the geminal diol to the aldehyde and the subsequent shift in the equilibrium dictates the release of acrolein from the mercapturate aldehydes by the retro-Michael mechanism. In biological culture media, the mercapturate aldehydes would be expected to exist predominantly as their geminal diols. However, it is not known if the equilibrium between the aldehyde and the geminal diol forms would be shifted in vivo or if the geminal diols by themselves are toxic. Aldehydes form hydrazone derivatives in the presence of hydrazines while the geminal diols do not. Thus, the formation of the hydrazone derivative provides evidence of the aldehydic product in aqueous media, but does not eliminate a contributing effect of the geminal diol to toxicity.

Because of their role in facilitating the excretion of reactive metabolites, the biosynthesis of mercapturic acids has been considered as a detoxication pathway. However, the excretion of acrolein adducts capable of regenerating the parent aldehyde, or that are directly reactive, may provide a mechanism to explain the selective bladder toxicity of CP, particularly if the presence of other CP metabolites catalyzes decomposition or reactivity. The presence of 3-hydroxyPrMCA in urine after administration of CP to humans (7) and rats (7-9)demonstrates that this type of metabolism is occurring. Although it remains unclear whether more reactive acrolein mercapturic acids are present in the urine of CPtreated animals, the current data support a possible role for these compounds in bladder damage.

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