leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl] (MeByt[1]-CsA, 5). The title compound was prepared in 39% yield from undecapeptide **36c** according to the procedure for **3**: R_f 0.42 (40% acetone/hexane); $[\alpha]^{rt}_D$ −229.1° (*c* 0.50, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 0.78–1.11 [m, 42 H, CH_3-C(4^1), CH_3-C(3^2), 2 CH_3-C(4^4), 2 CH_3-C(3^5), 2 CH_3-C(4^6), 2 CH_3-C(4^9), 2 CH_3-C(4^{10}), 2 CH_3-C(3^{11})], 1.18–2.62 [m, 19 H, HC(4^1), 2 H-C(5^1), 2 H-C(3^2), 2 H-C(3^4), H-C(4^4), H-C(3^5), 2 H-C(3^6), H-C(4^6), 2 H-C(3^9), H-C(4^9), 2 H-C(3^{10}), H-C(4^{10}), H-C(3^{11})], 1.26 [d, *J* = 7.0 Hz, 3 H, CH_3-C(2^8)], 1.36 [d, *J* = 7.0 Hz, 3 H, CH_3-C(2^7)], 1.76 [m, 3 H, CH_3-C(7^1)], 2.70 (s, 6 H, CH_3-N^{11}, CH_3-N^{10}), 3.06 (s, 3 H, CH_3-N^4), 3.16 (s, 3 H, CH_3-N^9), 3.18 [d, *J* = 11.5 Hz, 1 H, H-C(2^3)], 3.26 (s, 3 H, CH_3-N^6), 3.38 (s, 3 H, CH_3-N^3), 3.49 (s, 3 H, CH_3-N^1), 3.85 [m, 1 H, H-C(3^1)], 4.48 [m, 1 H, H-C(2^7)], 4.68 [m, 2 H, H-C(2^3), H-C(2^5)], 4.82 [m, 1 H, H-C(2^8)], 4.98 [m, 1 H, H-C(2^2)], 5.10 [m, 2 H, H-C(2^6), H-C(2^{10})], 5.20 [d, *J* = 10.0 Hz, 1 H, H-C(2^{11})], 5.30 [dd, *J* = 4.0, 10 Hz, 1 H, H-C(2^4)], 5.36 [d, *J* = 8.0 Hz, 1 H, H-C(2^1)], 5.70 [dd, *J* = 3.5, 10 Hz, 1 H, H-C(2^9)], 7.26 (d, *J* = 8.0 Hz, 1 H, H-N^8), 7.43 (d, *J* = 8.0 Hz, 1 H, H-N^5), 7.76 (d, *J* = 7.0 Hz, 1 H, H-N^7), 8.18 (d, *J* = 7.5 Hz, 1 H, H-N^2). MS: Exact mass calcd for $C_{62}H_{110}N_{11}O_{12}$ (M^+ + 1), 1200.8308; found (HR-FAB), 1200.8335.

Acknowledgment. We gratefully acknowledge support from the National Institutes of Health (AR 32007) and from Merck Sharp and Dohme. High-resolution mass spectra were performed by the Midwest Center for Mass Spectrometry, an NSF regional instrumentation facility (CHE 8620177). We thank the Departments of Chemistry and Biochemistry (NMR FAM) for the use of the 500-MHz NMR spectrometers.

Registry No. **1a**, 59865-13-3; **1b**, 59865-15-5; **3**, 114865-22-4; **4**, 114891-20-2; **5**, 124562-06-7; **6**, 124562-07-8; **7**, 108536-03-4; **8**, 920-08-1; **9**, 124648-21-1; (±)-**9**, 124562-32-9; **9** [(+)-ephedrine salt], 124562-33-0; **11**, 104324-14-3; **12**, 928-92-7; **13**, 25166-87-4; **14**, 5927-18-4; **15**, 25184-12-7; **15** (2*E*-isomer), 25172-05-8; **16**, 124562-08-9; **17**, 121741-83-1; **18**, 124562-09-0; **19**, 121741-84-2; **20**, 108536-06-7; **21**, 124562-10-3; **22**, 26817-34-5; **23**, 124562-11-4; **24a**, 124562-12-5; **24b**, 124562-22-7; **25**, 124562-13-6; **26**, 124562-14-7; **27**, 124562-15-8; **28a**, 124562-16-9; **28b**, 124562-23-8; *cis*-**28c**, 124562-24-9; *trans*-**28c**, 124562-25-0; **29a**, 124581-07-3; **29b**, 124562-26-1; **30a**, 124562-17-0; **30b**, 124581-08-4; **31**, 124562-18-1; **32**, 81135-30-0; **33a**, 124562-19-2; **33b**, 124562-27-2; **33c**, 124562-28-3; **34a**, 124562-20-5; **34b**, 124562-29-4; **34c**, 124562-30-7; **35**, 115141-96-3; **36a**, 124562-21-6; **36b**, 124562-31-8; **36c**, 124581-09-5; (±)-$CH_3CH(OH)CH{=}CH_2$, 6118-14-5; Me_2CHCHO, 78-84-2; MeNCO, 624-83-9; Ph_3CCl, 76-83-5; Me_2CO, 67-64-1; (+)-ephedrine, 321-98-2.

NMR Studies of the Conjugation of Mechlorethamine with Glutathione

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Many cancer cells are resistant to chemotherapeutic treatment with mechlorethamine and other alkylating agents. These drug-resistant cells often show an increase in the intracellular concentration of glutathione and an increase in the activity of glutathione-*S*-transferase when compared to the sensitive cells. Both of these components are thought to be involved with inactivation of the drug either through conjugation with glutathione or by hydrolysis. NMR spectroscopy was used to monitor the nonenzymatic conjugation of mechlorethamine with glutathione. Several intermediates along the pathway to the doubly glutathione substituted mustard, including both mustard–aziridinium adducts, can be observed. The assignment of the ^1H NMR spectrum of these adducts are presented. At 30 °C, pH 7.0, no hydrolyzed mustard was detectable. With the use of ^{13}C-labeled mustard, the conjugation reaction can be shown to proceed through an aziridinium intermediate rather than by direct nucleophilic substitution.

The nitrogen mustards are a class of bifunctional alkylating agents commonly used in cancer chemotherapy. These agents are believed to exert their cytotoxic effect through the alkylation of cellular components.[1] Alkylation is thought to occur via the formation of a reactive mustard aziridinium intermediate. Cancer cells displaying resistance to these alkylating agents often have an increased intracellular concentration of glutathione (GSH) and a higher activity of the enzyme(s) glutathione-*S*-transferase (GST).[2] The role these species play in conferring the drug resistance of these cells is not clear. However, it has been proposed that the drug may be inactivated by hydrolysis or by conjugation with GSH.[3,4] It is not known whether inactivation must be catalyzed by GST or will occur spontaneously under physiological conditions.

In this paper, we report the NMR characterization of the inactive derivative of the nitrogen mustard mechlorethamine which is formed by the conjugation with GSH. The formation of this bisthioether can be monitored dynamically by ^1H NMR spectroscopy. Several intermediates in the reaction of GSH with mechlorethamine can be observed, including the stepwise formation of the two aziridinium derivatives and the mono-GSH-substituted adduct.

Mechlorethamine specifically labeled with the ^{13}C nucleus was used to prove that the formation of the conjugated adduct proceeds through the aziridinium intermediate and not through direct nucleophilic substitution of the chlorine atom with the thiolate of GSH.

This report presents the NMR characterization of the inactive analogues of this alkylating agent and forms a basis for the study of the enzyme-catalyzed reactions in vitro and in vivo.

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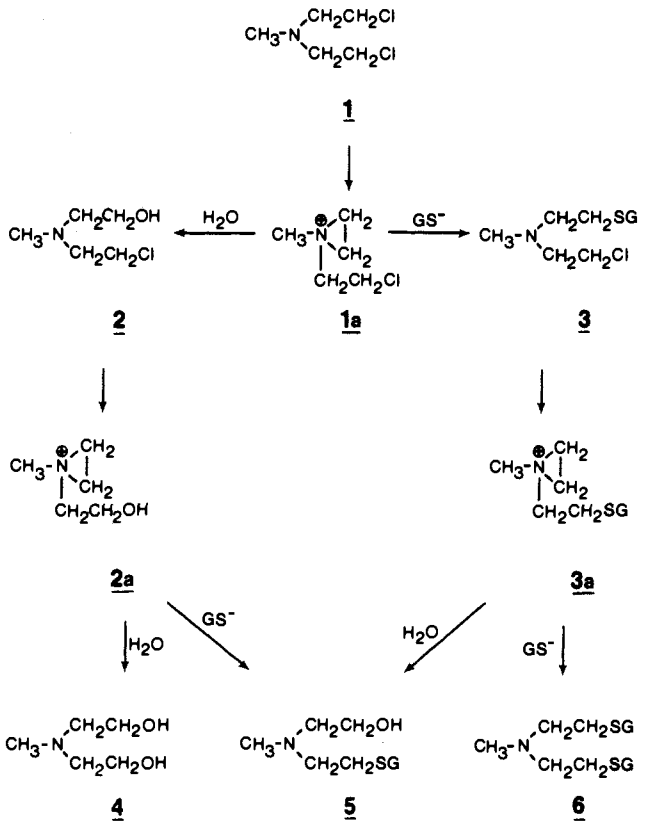
Scheme I



Results and Discussion

A solution of mechlorethamine (1) at pH 7.0 spontaneously forms a reactive aziridinium derivative (**1a**). This highly electrophilic species may be hydrolyzed or react with GSH, resulting in formation of the hydroxyethyl derivative **2** or the GSH conjugate of the mustard **3**, respectively (Scheme I). This process may then be repeated to produce the presumed inactive *N*-methyldiethanolamine (**4**), bisthioether derivative **6**, or the unsymmetrically substituted species **5**.

The *N*-methyldiethanolamine species (**4**) has been characterized previously.[5] Under the conditions of our experiment, the methyl resonance of **4** appears at 3.11 ppm, the α-CH_2 protons (the α-position is adjacent to the mustard nitrogen) at 3.52 ppm, and the β-CH_2 protons at 4.08 ppm. The assignments of the methylene protons was confirmed by observation of the long-range ^1H–^{13}C heteronuclear coupling between the carbon resonance of the methyl group and the methylene group proton resonance at 3.52 ppm (data not shown).

Formation of the aziridinium can be followed in a solution of buffer containing the mustard alone and is characterized by shifts in all of the proton resonances. The methyl group resonance shifts from 2.53 ppm in mechlorethamine to 3.18 ppm in the aziridinium intermediate. The aziridinium ring protons are identified from their AA'BB' multiplet pattern centered at 3.30 ppm expected for the ring methylenes of the aziridinium group.[5] A small four-bond coupling between the aziridinium methylene protons and the methyl group is detectable as a cross-peak in a homonuclear ^1H–^1H chemical shift correlated (COSY) spectrum (data not shown). The α-CH_2 and β-CH_2 protons of the chloroethyl group of the aziridinium are located at 3.64 and 4.07 ppm, respectively. The position of the

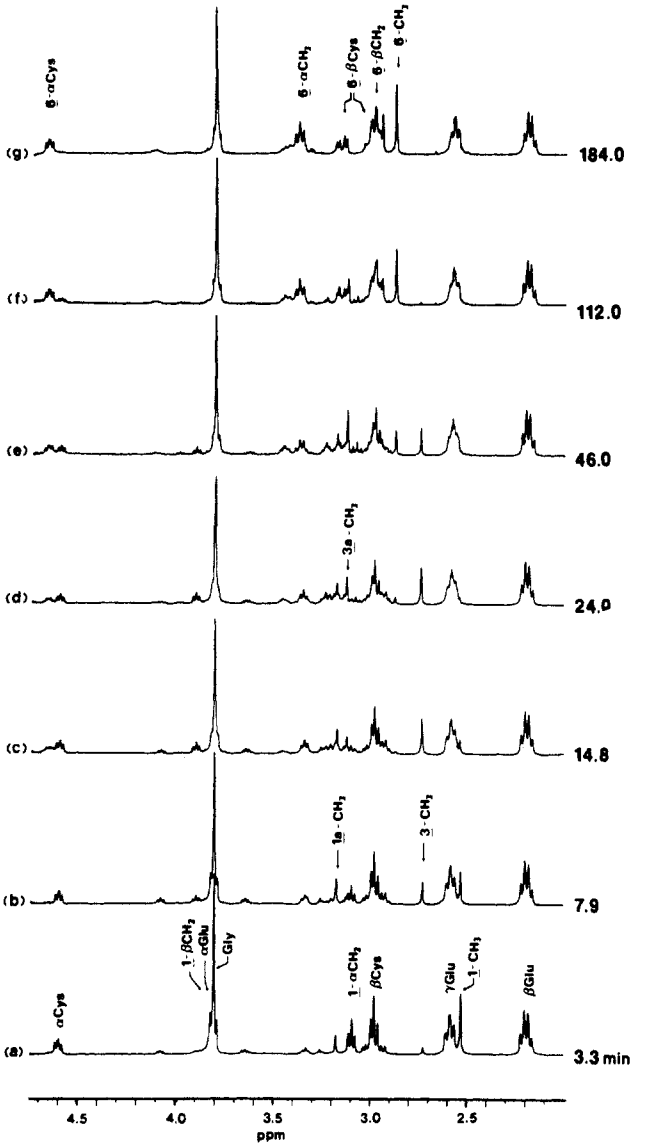


Figure 1. ^1H NMR spectra of the reaction of 4 mM GSH with 2 mM mechlorethamine at 30 °C in 0.06 M phosphate, D_2O, pH 7.0. The GSH resonances are identified by their amino acid residues. The resonances of the mechlorethamine and derivatives are identified by using the numbering system presented in Scheme I. Each spectrum is the result of 64 scans acquired in 1.3 min with the acquisition starting at the times indicated.

methyl and methylene resonances of the aziridinium closely corresponds to those observed by previous investigators.[5,6]

Mechlorethamine is converted rapidly into the aziridinium intermediate with the data closely fitting a first-order rate equation. This is consistent with the results of previous investigators who observed first order kinetics for the formation of the aziridinium with other 2-haloethylamines.[6] At 30 °C in 0.05 M phosphate buffer, pH 7.0, the half-life of mechlorethamine is 5.52 ± 0.44 min (k = 0.126 ± 0.010 min^{-1}). Under these conditions, the aziridinium reacts extensively with the phosphate buffer (see below).

The conjugation of mechlorethamine and GSH involves several transient species (i.e. **1a**, **3**, and **3a**), which can be observed by ^1H NMR spectroscopy. The ^1H NMR spectra of a reaction mixture containing GSH and mechlorethamine at pH 7 are shown in Figure 1. The resonance

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Table I. ^{1}H NMR Assignments of Mechlorethamine and Inactive Derivatives

compd	CH_3	CH_2CH_2Cl		CH_2CH_2X		aziridinium ring protons
		α-CH_2	β-CH_2	α-CH_2	β-CH_2	
1	2.53	3.09	3.80			
1a	3.19	3.64	4.07			3.30
3 (X = SG)	2.73	3.33	3.92	3.24	2.92	
3a	3.12					
4 (X = OH)	3.11			3.52	4.08	
6 (X = SG)	2.91			3.41	3.00	

assignments for the nonexchangeable protons of GSH given in Figure 1(a) agree with previous results.[7] Approximately 2 min after addition of mechlorethamine, the spectrum in Figure 1(a) was acquired. The singlet resonance of the methyl group of mechlorethamine is observed at 2.53 ppm. The α-methylene resonance is detectable at 3.09 ppm and the β-methylene resonance is obscured by the GSH glycine and α-glutamate resonances at 3.80 ppm. These positions are indistinguishable from that in phosphate buffer in the absence of GSH. Likewise, the aziridinium methyl resonance is observed at 3.19 ppm as are the ring methylene multiplet centered at 3.30 ppm and the chloroethyl group methylenes at 3.64 and 4.07 ppm. The rate of breakdown of mechlorethamine in the presence of GSH is similar to that in phosphate buffer alone; a half-life of 5.44 ± 0.37 min (k = 0.127 ± 0.009 min^{-1}) at 30 °C, pH 7.0. The aziridinium methyl resonance at 3.19 ppm is present only at lower concentration and reaches maximum intensity after 9 min and then rapidly disappears.

Another singlet methyl resonance is observable at 2.73 ppm and increases in intensity for the first 20 min of the experiment followed by a decrease until it is no longer detectable after 3 h. This can be assigned to the methyl group of the monosubstituted thioether derivative **3**. The methyl resonance of **3** can be correlated to the methylene resonances from the relative integrated intensity of these peaks. A COSY[8] experiment was performed at 15 °C (where the conjugation reaction is much slower) to identify the coupled methylene resonances of the intermediates. By following the temperature dependence of these resonances back to 30 °C, the methylene group at 3.33 ppm can be assigned to the α-CH_2 of the chloroethyl side chain of **3**. The multiplet at 3.92 ppm coupled to this is the β-CH_2 resonance. The only other coupled resonances of similar integrated intensity are located at 3.24 and 2.92 ppm. This is consistent with assignment of these resonances to the α- and β-methylene of the GSH-substituted side chain of **3**. The position of the resonance at 2.92 ppm is typical of methylene groups in thioether linkages (see below). The α-C*H* resonance of cysteine shifts from 4.61 ppm in free GSH to 4.68 ppm in conjugated GSH. A comparison of the integrated intensity of the α-C*H* resonance of Cys at 4.68 ppm to the α-CH_2 resonance of the chloroethyl moiety at 3.33 ppm confirms the structure of **3** as the monosubstituted mechlorethamine.

After 24 min (Figure 1(d)), another singlet methyl resonance appears at 2.91 ppm and steadily increases in intensity for the remainder of the reaction. This can be assigned to the methyl group of the bisthioether **6**. The resonance of the α-CH_2 group of **6** is located at 3.41 ppm, and the β-CH_2 resonance appears at 3.00 ppm and partially overlaps with one of the β-protons of the cysteine resonance of GSH. The scalar coupling network in the GSH and mustard component of **6** are determined from a COSY experiment. With the exception of the cysteine protons, the resonances of GSH are unchanged. The cysteine α-C*H* shifts upon conjugation as described previously and is coupled to the inequivalent β-CH_2 protons at 3.17 and 2.94 ppm. An NOE between the methyl resonance at 2.91 ppm and the methylene resonance at 3.41 ppm suggests that the latter can be assigned to the α-CH_2 group. Further proof for this assignment is provided by heteronuclear ^{1}H–^{13}C NMR experiments (vide supra).

Definitive proof of the assignment of the proton resonances and of the structure of the bisthioether derivative must rely upon observation of long-range scalar couplings. Long-range ^{1}H–^{1}H correlation experiments did not detect scalar coupling between the methyl group of **6** to either of the methylene resonances. Due to the overlap of the resonances, a long-range scalar coupling between the β-CH_2 resonances of the cysteine of GSH and the mechlorethamine β-CH_2 was not observable. Detection of scalar coupling between the cysteine and mechlorethamine resonances provides unambiguous evidence for the thioether linkage between the cysteine of GSH and the mustard. By use of a long-range ^{13}C–^{1}H heteronuclear correlation experiment (COLOC),[9] definitive structural determination and proton assignments can be made.

Single-bond ^{1}H–^{13}C heteronuclear spectroscopy[10] was used to correlate the proton and carbon resonances. Any ambiguity in the assignment of the mustard methylene groups to the α- or β-position can be removed by a three-bond ^{1}H–^{13}C heteronuclear COLOC experiment (Figure 2). A long-range correlation between the methyl carbon resonance at 40.4 ppm and the methylene group at 3.33 ppm is observable as a cross-peak in this spectrum. Conversely, the methylene carbon resonance of the α-CH_2 of the mustard at 54.9 ppm is correlated with the methyl proton resonance at 2.91 ppm. Proof that the conjugate is formed is provided by detection of a correlation between the carbon resonance of the β-CH_2 at 26.0 ppm to the cysteine β-protons. Observation of this long-range scalar coupling provides unambiguous proof that a thioether linkage has formed between mechlorethamine and GSH. Previous investigators observed shifts in the ^{1}H and ^{13}C NMR spectrum of GSH consistent with formation of a GSH conjugate.[11-17] Therefore definitive proof of the

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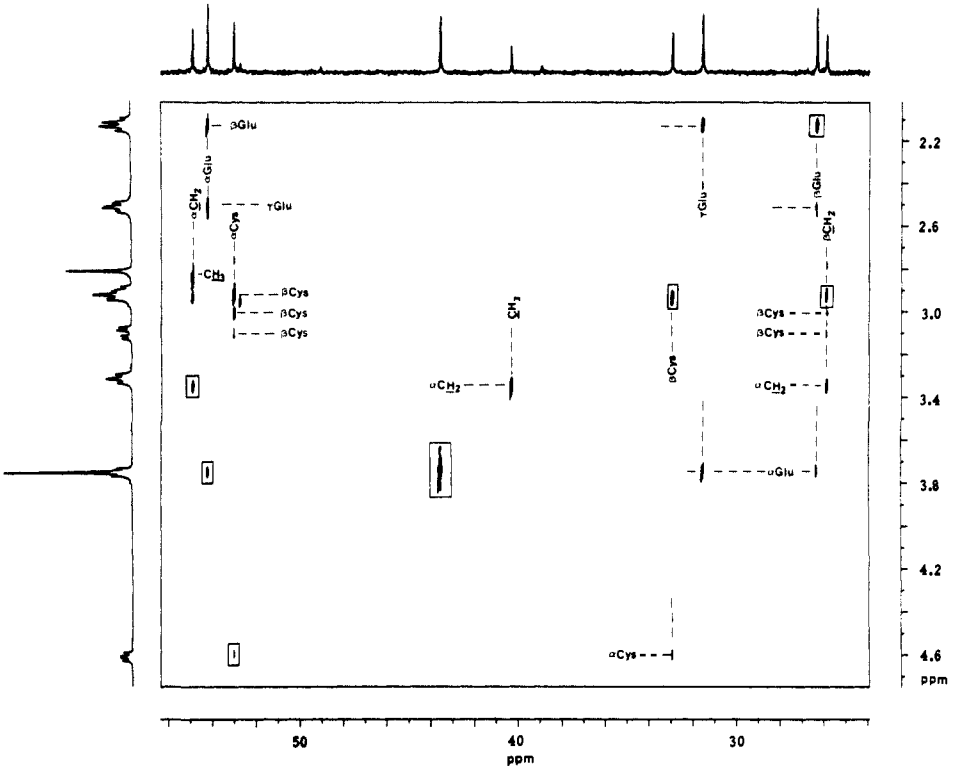

Figure 2. Long-range $^{13}C-^{1}H$ heteronuclear correlated (COLOC) spectrum of bis(GSH)-mustard **6**. Cross-peaks enclosed by rectangles are one-bond $^{13}C-^{1}H$ correlations which also appear in a one-bond correlation map. The β-Cys protons appear as three peaks due to the effect of decoupling in the f_1 dimension.

structure of these conjugates required additional purification and chemical analysis.[11-17]

In addition to resonances assignable to **1**, **1a**, **3**, and **6**, another transient singlet resonance is observable in Figure 1(d) at 3.12 ppm, which increases in intensity and finally disappears at the end of the experiment. Although detailed assignment of the resonances of this species is difficult due to overlap of resonances, the position, intensity, and transient behavior suggest that this peak as the methyl resonance of the second aziridinium derivative (**3a**). The chemical shifts of the mustard resonances and derivatives are presented in Table I.

The rate of change in the concentration of species **1**, **1a**, **3**, **3a**, and **6** over the time course of the conjugation reaction is illustrated in Figure 3. The relative concentration of each was determined from the intensity of the methyl resonances and normalized. Quantitation of the second aziridinium derivative **3a** is difficult due to the overlap of the methyl resonance of this derivative with one of the β-Cys protons which increases in intensity during the course of the experiment. When the GSH concentration is increased, the steady-state levels of the aziridinium intermediates are much lower and **3a** disappears much more rapidly.

Close inspection of the spectrum in Figure 1(g) reveals the presence of resonances in addition to those assigned to GSH conjugates or intermediates. Broad resonances are centered at 4.09 and 3.44 ppm, and a sharp singlet is observable at 2.95 ppm. All of these resonances are absent from the spectrum of a reaction mixture prepared without phosphate buffer. For example, the ^{1}H NMR spectrum shown along the vertical axis in Figure 2 was obtained with the same concentrations of GSH and mustard as that shown in Figure 1; however, the pH of the solution was

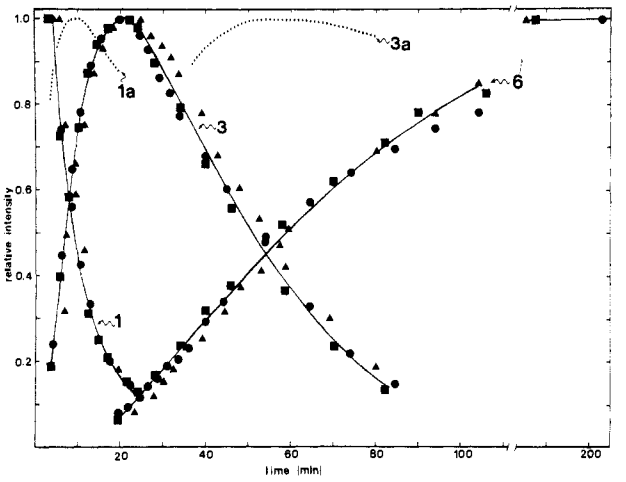

Figure 3. Time course for reaction of mechlorethamine and the production of intermediates and GSH conjugates. The concentration of each species was determined from the intensity of the methyl proton resonance. The intensities were normalized to the highest intensity found for that resonance. The time course of the reaction for three separate determination is presented as different symbols. The time course for the production and breakdown of the aziridinium intermediates are shown as dotted lines. The overlap of the aziridinium resonances with other resonances, especially for **3a**, resulted in relative intensities which do not approach zero.

maintained at 7.0 through the course of the reaction by the addition of 0.1 M NaOH. After completion of the reaction, the ^{1}H NMR spectrum shown in Figure 2 was obtained. Only resonances due to the bithioether **6** are observed. In phosphate buffer, the intensity of the resonances at 4.09, 3.44, and 2.95 ppm increase with an increase in the buffer concentration. Selective proton decoupling of the ^{31}P NMR spectrum indicates that the resonances at 4.09 ppm are coupled to the phosphorous resonance and

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Scheme II

$$BrCH_2^{13}CO_2Et \xrightarrow{CH_3NH_2\cdot HCl} CH_3N(CH_2^{13}CO_2Et)_2 \xrightarrow{LiAlH_4}$$

7 **8**

$$CH_3N(CH_2^{13}CH_2OH)_2 \xrightarrow{SOCl_2} CH_3N(CH_2^{13}CH_2Cl)_2$$

9 **10**

proves the presence of the phosphate conjugate of mechlorethamine. The reaction of mechlorethamine with inorganic phosphate has been observed by other investigators.[18] In the ^{31}P NMR spectrum, the resonance of the phosphate derivative occurs 2.47 ppm downfield of the inorganic phosphate resonance. Doubling the initial concentration of GSH reduces the final intensity of the resonances at 4.09, 3.44, and 2.95 ppm. The ^{31}P NMR resonance of the phosphate derivative was present after 1 week at room temperature and after the addition of a second aliquot of GSH, indicating that the phosphate derivative is a stable species. In order to minimize the contribution of this competing reaction, the spectra in Figure 1 were acquired in a minimum concentration of phosphate buffer needed to control the pH. The pH of the reaction mixture in Figure 1 decreased by 0.06 unit, resulting in slight shifts (0.015 ppm) in some of the resonances.

The position of the GSH and mustard resonances are influenced by the ionic strength of the medium. The methyl resonance of 1 mM mechlorethamine with 2 mM GSH in 0.1, 0.2, and 0.4 M sodium phosphate pH 7.0 shifts to 2.54, 2.56, and 2.59 ppm, respectively. The resonances of GSH shift in the opposite direction. For example, the position of the glycine CH_2 resonances in the same buffers shift 0.05 ppm upfield over this range of ionic strength.

The proposed mechanism for the alkylation of cellular components by nitrogen mustards is thought to proceed through an aziridinium intermediate by an S_N1 type reaction.[1] With GSH or other reactive nucleophiles, direct S_N2 type substitution is also possible. By use of ^{13}C NMR, the intermediacy of the aziridinium in the conjugation reaction can be demonstrated easily. Mechlorethamine labeled in the β-carbon (**10**) was synthesized from methylamine hydrochloride and ethyl bromo[1-^{13}C]acetate (Scheme II). The carbon spectrum of **10** is shown in Figure 4(a). Upon completion of the conjugation reaction with GSH, the resulting ^{13}C NMR spectrum is shown in Figure 4(b). For a comparison, the natural-abundance ^{13}C spectrum of **6** is shown in Figure 4(c). The ^{13}C NMR spectrum of **6** in Figure 4(c) was assigned from one-bond ^{13}C–^1H heteronuclear correlation experiments and multiple-bond correlation experiments (e.g. Figure 2). From the spectra shown in Figure 4, it is obvious that the ^{13}C label has been scrambled equally to both the α- and β-carbon positions of the bisthioether conjugate. This is consistent with the S_N1 type mechanism where the GSH can add with equal facility at either carbon of the aziridinium intermediate. An S_N2 type reaction would result in retention of the carbon label at the β-position. The similar intensity of the carbon resonances indicates a completely random addition of GSH to either carbon of the aziridinium moiety.

A similar strategy was used to prove the existence of an aziridinium intermediate in the reaction of a deuterated phosphoramide mustard with ethanethiol[19] and ^{13}C-labeled

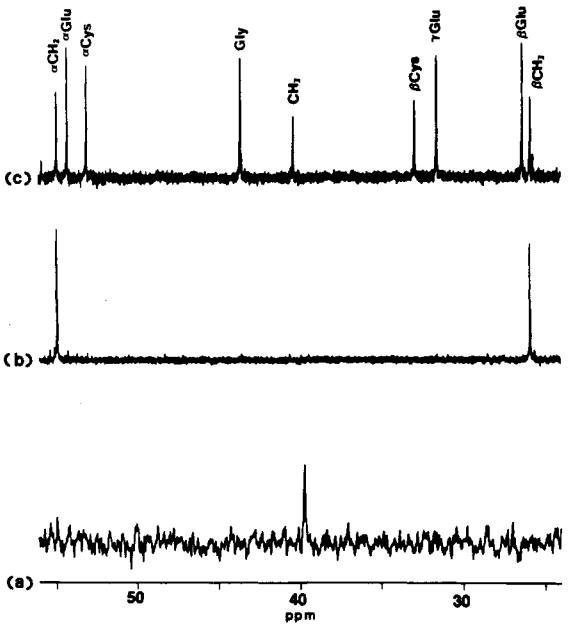

Figure 4. ^{13}C NMR spectra of (a) 2 mM β-^{13}C-mechlorethamine (**10**) in 0.06 M phosphate, D_2O, pH 7.0, 30 °C. (b) After conjugation with 4 mM GSH. (c) Natural-abundance ^{13}C NMR spectrum of the protonated carbons of the bisthioether conjugate **6**.

mechlorethamine with other nucleophiles.[5]

In addition to the spectra shown in Figure 4(a) and 4(b), the time course for the reaction of ^{13}C-labeled mustard with GSH can be followed by ^{13}C NMR. The rate of reaction and distribution of label in the final product was unchanged by the addition of 0.1 M chloride ion. The rate of aziridinium formation, as monitored by ^1H NMR spectroscopy, is unaffected by the addition of 0.1 M chloride. This suggests that **1a** does not react with chloride ion to reversibly form **1**, leading to label scrambling by an S_N2 type mechanism.[5]

The use of labeled drug greatly simplifies the identification of components in the reaction mixture. For example, the COLOC experiment presented in Figure 2 was obtained from the natural abundance ^{13}C nuclei present in the bis(GSH) conjugate. This required a high concentration of the conjugate and a 12-h experiment. An increase in sensitivity is possible by use of ^1H detected heteronuclear multiple-bond spectroscopy[20] or by use of the ^{13}C-enriched sample. In the latter case, a COLOC experiment can be performed within 1 h.

In all of the reaction mixtures containing GSH, there was no evidence for the production of hydroxylated species **4** or unsymmetrically substituted adduct **5**.

Although NMR assignments of conjugates of GSH have been presented,[11-17] identification relied upon purification and other methods of chemical analysis. The NMR evidence presented in these previous communications identified shifts in resonances consistent with conjugation of GSH by a thioether linkage. Engle et al.[21] determined the kinetics of the reaction of phosphoramide mustard with 2-mercaptoethanol using ^{31}P NMR and provided ^1H NMR evidence in support of the identity of the bis-alkylated product. This paper presents NMR data for the unambiguous determination of the formation of the bisthioether through the use of ^1H and ^{13}C NMR spectroscopy. The

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results show that the position of the methyl resonance is distinct for each of the metabolites. The determination of the structure and the characterization of the ^1H NMR spectrum of the GSH derivatives of mechlorethamine is the first step in the analysis of the inactivation of nitrogen mustards in vitro and in vivo. NMR has the capability to monitor the formation of inactive metabolites of the drug and in some cases detect the formation and breakdown of transient species. Future studies will determine the effect of added glutathione-*S*-transferase on the kinetics and mechanism of the inactivation. In addition, NMR studies are currently underway to monitor metabolism of these drugs in the whole cell.

Experimental Section

Mechlorethamine (2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine), deuterium oxide, and glutathione were purchased from Aldrich Chemical Co.

^1H NMR spectroscopy was performed at 360 MHz on a Bruker AM360 WB spectrometer. Temperature was maintained to within ±1 °C with the Bruker Variable Temperature Unit. ^1H NMR spectra were referenced to external 3-(trimethylsilyl)propionic-*2,2,3,3,-d*$_4$ acid sodium salt (TSP) (Merck) contained within a capillary tube. ^{13}C NMR spectra are referenced to external dioxane at 66.5 ppm. Two-dimensional chemical shift correlation spectroscopy (COSY),[8] one-bond ^1H–^{13}C heteronuclear chemical shift correlation spectroscopy,[10] and three-bond ^1H–^{13}C heteronuclear correlation spectroscopy (COLOC)[9] with f_1 decoupling were performed in the absolute value mode.

Glutathione and buffers were lyophilized several times from 99.6% D_2O before final dissolution of D_2O. The pH values given in D_2O are not corrected for the isotope effect.

For the time-course experiments in the NMR spectrometer, a solution of GSH in buffer was added directly to a preweighed sample of mechlorethamine in a 5-mm NMR tube and immediately placed in the NMR spectrometer. Typically, data acquisition began 3–4 min after mustard and GSH were mixed.

The half-life for the formation of the aziridinium intermediate from mechlorethamine was determined assuming a first-order process.[6] The values given are the mean plus or minus one standard deviation of at least five experiments.

Organic Synthesis. Methylamine hydrochloride, triethylamine, lithium aluminum hydride (1.0 M in tetrahydrofuran), and ethanol were purchased from Aldrich Chemical Co., and the ethyl bromo[1-^{13}C]acetate was purchased from Cambridge Isotope Laboratories and were used without further purification.

^1H NMR spectra of the intermediates and products of organic synthesis were obtained on an IBM/Bruker NR80A spectrometer operating at 80 MHz and were referenced to internal tetramethylsilane in $CDCl_3$ or TSP in D_2O.

The IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR or a Perkin-Elmer 399B spectrometer.

The tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl, the benzene was distilled from calcium hydride, and the thionyl chloride was distilled through a Vigreux column before use.

[1,1′-^{13}C]-*N*-Carboxymethyl-*N*-methylglycine Diethyl Ester (8). A slurry of methylamine hydrochloride (604 mg, 8.9 mmol) in ethanol (3 mL) containing triethylamine (3.7 mL, 26.8 mmol) was cooled to 0 °C and treated with a solution of bromo[1-^{13}C]acetate (3 g, 18 mmol) in ethanol (3 mL) via syringe over 10 min, producing a white opaque mixture. The ice bath was warmed slowly to ambient temperature and the mixture was stirred overnight. The mixture was filtered through a Celite pad and concentrated under reduced pressure. The residue was placed in a separatory funnel containing diethyl ether (50 mL) and aqueous 10% sodium hydroxide (20 mL). The layers were separated, and the aqueous layer was washed with diethyl ether (2 × 25 mL). The organic layers were combined, dried ($MgSO_4$), and concentrated in vacuo to give 1.7 g of a clear, pale yellow liquid. Purification on silica gel (Kieselgel 60, 15 g), eluting with hexane/diethyl ether (3:1) gave 1.06 g (59%) of **8**, as a clear, pale yellow liquid. ^1H NMR (δ, $CDCl_3$): 1.28 (6 H, t, J = 1.7 Hz), 2.54 (3 H, s), 3.49 (4 H, d, $J(^{13}C–^1H)$ = 5.2 Hz), 4.19 (4 H, dq, $J(^1H–^1H)$ = 7.1 Hz, $J(^{13}C–^1H)$ = 3.0 Hz). IR (cm^{-1}, neat): 1737.

[2,2′-^{13}C]-2-Hydroxy-*N*-(2-hydroxyethyl)-*N*-methylethanamine (9). A solution of **8** (1.05 g, 5.1 mmol) in THF (5 mL) was added over 15 min via syringe to a solution of lithium aluminum hydride (1.0 M in THF, 24 mL, 24 mmol) at 0 °C to give a clear, colorless solution. The reaction mixture was warmed slowly to ambient temperature and stirred overnight. The reaction mixture was cooled to 0 °C and quenched by slow addition of a saturated aqueous solution of sodium sulfate over 1 h. Methylene chloride was added along with solid anhydrous sodium sulfate, and the slurry was stirred for 30 min. The mixture was filtered through a pad of Celite, dried ($MgSO_4$), and concentrated in vacuo to give 311 mg of **9** as a clear, colorless liquid. The filter cake was placed in a Soxhlet extractor and refluxed with THF for 12 h. The THF was dried ($MgSO_4$) and concentrated in vacuo to give an additional 395 mg of **9** as a clear, yellow liquid. ^1H NMR (δ, $CDCl_3$): 2.32 (3 H, s), 2.65 (4 H, t), 3.71 (4 H, dt, $J(^1H–^1H)$ = 5.3 Hz; $J(^{13}C–^1H)$ = 150 Hz). IR (cm^{-1}) 3360, 1100. The two fractions of **9** were combined and used without purification in the next step.

[2,2′-^{13}C]-2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine Hydrochloride (β-^{13}C-Mechlorethamine Hydrochloride) (10). A solution of crude **9** (705 mg, 5.83 mmol) in benzene (2.6 mL) was added via syringe over 10 min to a solution of thionyl chloride (1.16 mL, 15.9 mmol) in benzene (2.6 mL) at room temperature. After the addition was complete, the reaction was heated to 55–60 °C for 2 h to produce a clear brown solution. The reaction mixture was cooled to ambient temperature and concentrated at reduced pressure. Several milliliters of ethanol were added, and this mixture was concentrated under reduced pressure to give a brown semisolid. Crystallization from acetone produced 564 mg (58% from **9**) of **10** as a tan solid: mp 111–112 °C (lit.[22] mp 110 °C). ^1H NMR (δ, D_2O): 3.0 (3 H, s), 3.6 (4 H, m), 4.9 (4 H, m).

Acknowledgment. This work was supported by a grant from the Johns Hopkins University Institutional Grant Committee (M.P.G.) and Grant No. CA44703 from the National Institutes of Health (M.C.). We thank Drs. Ian J. McLennan and John Hilton for helpful discussions.

Registry No. 1, 51-75-2; **1a**, 36375-20-9; **3**, 124605-73-8; **3a**, 124605-77-2; **4**, 105-59-9; **6**, 124605-74-9; **7**, 61203-71-2; **8**, 124605-75-0; **9**, 111036-14-7; **10**, 124605-76-1; GSH, 70-18-8; $MeNH_2$·HCl, 593-51-1.

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