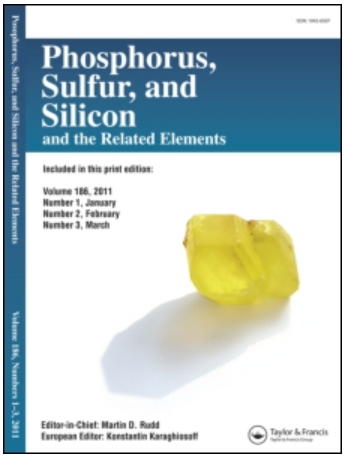

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Synthesis, Characterization and Mass Spectrometric Analysis of Cysteine and Valine Adducts of Sulphur Mustard

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SYNTHESIS, CHARACTERIZATION AND MASS SPECTROMETRIC ANALYSIS OF CYSTEINE AND VALINE ADDUCTS OF SULPHUR MUSTARD

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The synthetic standards for cysteine and valine adducts of sulphur mustard have been synthesized using 2-(2-chloroethylthio) ethanol (half-mustard). These adducts have been recognized as biomarkers for exposure to sulphur mustard. The adducts have been fully characterized using ^{1}H and ^{13}C NMR spectroscopy and mass spectrometric techniques.

Keywords: Biomarker; cysteine; GC/MS; GC/NCI-MS; half mustard; sulphur mustard; valine

INTRODUCTION

Sulphur mustard (SM), 2,2-bis(2-chloroethyl) sulphide, is a carcinogenic compound by virtue of its ability to alkylate deoxyribonucleic acid (DNA) and proteins.[1] It has been allegedly used as a chemical warfare agent in various conflicts.[2,3] A lot of interest has been generated in the recent past for the retrospective determination of sulphur mustard in various biological matrices.

Sulphur mustard reacts with nucleophiles such as water, tripeptide glutathione, and various amino acid residues present in proteins and DNA in human system under physiological conditions.[1] The metabolites derived from an initial reaction with water and glutathione are excreted in urine; adducts with hemoglobin and albumin are present in blood and adducts with DNA are formed in various tissues, blood, and urine.[1]

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Attempts have been made to prepare synthetic standards for both DNA and protein adducts of SM and to develop analytical techniques for their detection and analysis. Recently, we have reported a simplified methodology for the synthesis of N7-guanine-SM, adduct and its detection by high performance liquid chromatography (HPLC), and liquid chromatography mass spectrometry (LC/MS) techniques.[4]

The protein adducts of SM are much more stable and abundant as compared to the DNA-SM adduct. The two most reactive sites in the amino acid residue of the protein available for sulphur mustard to react are the SH group of cysteine and the free NH_2 on valine. However, human hemoglobin possesses only three cysteine residues, one of which is not accessible for alkylation. Valine is the N-terminal amino acid on both α- and β-chains of hemoglobin. The synthetic standards of cysteine-SM and valine-SM adducts are required for an unequivocal determination of these adducts in the real biological samples. Methods for the preparation of these adducts have been reported in the literature but in many instances these procedures give products that are not only impure but inadequately described and characterized.[5] This article describes the synthesis of these two adducts with high purity giving complete data about their preparation in the laboratory and their spectral characterisation.

RESULTS AND DISCUSSION

The fact that sulphur mustard reacts readily with sulphur containing nucleophiles suggested that the –SH group of cysteine is the primary site of alkylation in a readily isolable protein, such as hemoglobin in blood. Hydrolysis of such an alklylated protein to individual amino acids should result in the liberation of mono-cysteine adduct which may serve as a useful biomarker for retrospective detection of SM in bio-samples.

In an attempt to synthesize the cysteine adduct of sulphur mustard, we reacted sulphur mustard with cysteine but it resulted in the formation of bis-adduct rather than mono-adduct,[6] primarily due to the presence of two active electron deficient sites in SM which are susceptible to be attacked by :SH nucleophile through the intermediate three-membered cyclic sulphonium species. In order to prepare the mono-adduct, therefore, we reacted half-mustard, 2-(2-chloroethylthio) ethanol with cysteine as per the method reported in the literature.[5] However, the method reported[7,8] for the synthesis of half-mustard gave a mixture of products in poor yields. Half mustard was then prepared by making slight modification to the reported method. We used sodium methoxide instead of sodium hydride which gave quite pure

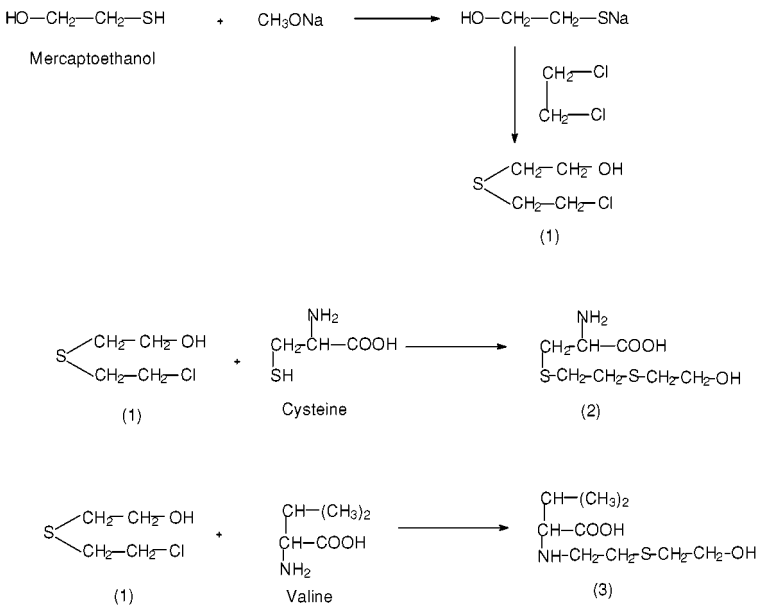


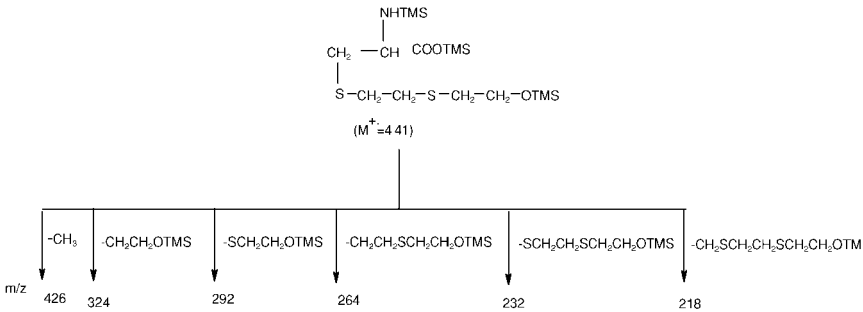
FIGURE 1 Scheme for the synthesis of (1) half-mustard, (2) cysteine adduct, and (3) valine adduct.

half mustard in excellent yields (85%). The resulting half mustard was stored in ether which was used without any further purification. Furthermore, the reaction was completed in 6–8 h, against the reported method where the reaction mixture was kept in refrigerator for 5 days. The cysteine-SM adduct was then prepared according to the method described earlier[5] but with improved yields. The scheme for the synthesis of half mustard (**1**) and its subsequent reaction with cysteine is shown in Figure 1.

The cysteine adduct (**2**) was characterized using ^{1}H and ^{13}C NMR and mass spectral data. The NMR data matched fully with the reported values[6] (Table I). The adduct was analyzed by gas chromatography mass spectrometry (GC/MS) after derivatization with bis(trimethylsilyl) trifluoroacetamide (BSTFA). The mass spectrum of the silylated adduct recorded under electron ionisation (EI) mode showed characteristic fragmentation pattern (Scheme 1) but lacked the molecular ion information. In order to confirm the molecular weight of the derivatized adduct, we recorded the positive chemical ionisation (PCI) spectrum, which gave an abundant pseudomolecular ion at m/z 442. The cysteine adduct also has been analyzed by the earlier reported negative chemical ionisation (NCI) GC/MS after converting the adduct

TABLE I NMR and Mass Spectral Data for Cysteine-SM and Valine-SM Adducts

Compound	NMR and mass spectral data
Cysteine-SM adduct	^{1}H NMR (d_6-DMSO) δ (ppm): 2.59 (2H, t, S$\underline{CH_2}CH_2OH$), 2.70 (4H, broad s, S$\underline{CH_2CH_2}$S), 2.83 and 3.00 (each 1H of S$\underline{CH_2}$CH, each dd, AB part of ABX system), 3.55 (2H, t, $\underline{CH_2}$OH), 4.2 (1H, $NH_2\underline{CH}CH_2$ dd, X part of ABX system).
	^{13}C NMR (d_6-DMSO) δ (ppm): 32.30, 32.39, 33.62, 34.66 ($\underline{CH_2}$ S$\underline{CH_2CH_2}$S $\underline{CH_2}$), 54.54 ($SCH_2\underline{CH}NH_2$), 62.19 (CH_2OH), 169.61 (COOH).
	Mass (Silylated adduct) (EI): m/z 218 (M-$CH_2SCH_2CH_2SCH_2$-CH_2OTMS, 100%), 232 (M-$SCH_2CH_2SCH_2CH_2$OTMS, 9%), 264 (M-$CH_2CH_2SCH_2CH_2$OTMS, 6%), 292 (M- SCH_2CH_2OTMS, 20%), 324 (M- CH_2CH_2OTMS, 15%).
	Mass (Silylated adduct) (CI, Isobutane): m/z 442 $(M+H)^+$
Valine-SM adduct	^{1}H NMR (D_2O) δ (ppm): 0.84 (3H, d, CH_3), 0.89 (3H, d, CH_3), 2.15 (1H, m, $\underline{CH}(CH_3)_2$), 2.71 (t, 2H, S$\underline{CH_2}CH_2OH$), 2.84 (2H, m, $NHCH_2\underline{CH_2}$S), 3.19 (2H, m, $NHCH_2$), 3.55 (d, 1H, NHCH), 3.60 (2H, t, CH_2OH)
	^{13}C NMR (D_2O) δ (ppm):18.11, 18.84 (2 × CH_3) 27.94 (S$\underline{CH_2}$ CH_2NH) 30.03 ($\underline{CH}(CH_3)_2$), 33.96 ($HOCH_2\underline{CH_2}$S), 47.36 ($HNCH_2$), 61.01 (CH_2OH), 69.07 (CHNH), 170.12 (COOH).
	PFTH derivative of valine adduct:
	^{19}F NMR ($CDCl_3$) δ (ppm): Aromatic Fluorines 146.5 [2F, d, Ortho], 157.2 [1F, t, Para], 162.8 [2F, t, Meta].
	Mass (EI): m/z 428 ($M^{+\cdot}$, 40%), 410 (M-H_2O, 11%), 383 (M-CH_2CH_2OH, 100%), 351 (M-SCH_2CH_2OH, 67%).
	Mass (CI, Methane): m/z 429 $(M+H)^+$
	HFBA derivative of valine adduct:
	Mass (NCI, Methane): m/z 624 ($M^{-\cdot}$), 604 (M-HF), 584 (M-2HF), 564 (100%, M-3HF), 544 (M-4HF), 524 (M-5HF), 504 (M-6HF), 427 (M-COC_3F_7)
	Mass (PCI, Methane): m/z 625 $(M+H)^+$

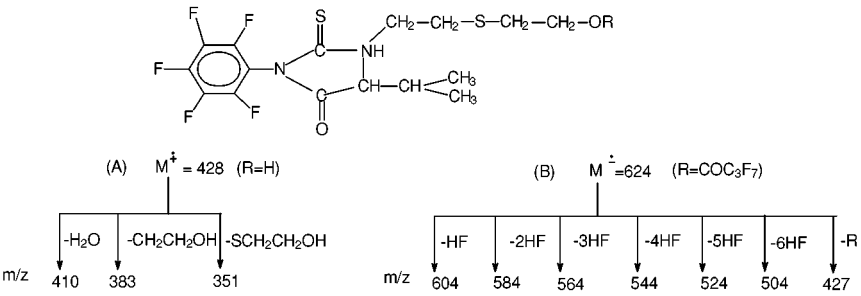


SCHEME 1 Fragmentation pattern for the cysteine-SM adduct (silylated) under electron impact (El) conditions.

into an oxazolidinone using 1,3-dichlorotetrafluoroacetone followed by derivatization with heptafluorobutyric anhydride (HFBA). It gave $M^{-\cdot}$ ion at m/z 601 and M^{-}-HF—CO_2 at m/z 537.[9]

In a similar manner valine-SM adduct (**3**) was synthesized by reacting half-mustard with valine in aqueous solution. The method developed by us is much simpler than the reported method[10] which involved a multistep process culminating in a very poor yield (23%) of the adduct. Our method resulted in an excellent yield (70%) of the adduct. The scheme for the synthesis of valine-SM adduct is shown in Figure 1.

The valine-SM adduct also was characterized on the basis of ^{1}H and ^{13}C NMR and mass spectral data. The NMR data matched well with reported values[10] (Table I). The mass spectrometric analysis was performed using GC/MS after converting the adduct into hydantoin by reacting with pentafluorophenyl isothiocyanate. The pentafluorophenyl thiohydantoin (PFTH) derivative of the adduct thus obtained gave molecular ion peak at m/z 428 under EI conditions (Table I). The EI mass spectrum showed characteristic fragmentation pattern (Scheme 2). The product was further confirmed by PCI-GC/MS which gave the pseudomolecular ion peak at m/z 429. However, the chromatographic properties of this derivative were very poor in both EI and CI modes. We therefore, derivatized it further with HFBA, and subjected it to NCI-GC/MS analysis which gave $M^{-\cdot}$ at m/z 624 and an abundant ion at m/z 564 (M-3HF). The other characteristic fragment ions formed are shown in Scheme 2. The PCI-GC/MS of the derivatized adduct gave $(M+H)^{+}$ ion at m/z 625 confirming thereby the formation of adduct. NCI-GC/MS was, however, the most sensitive method for the detection and analysis of the valine-SM adduct in derivatized form. The detection limit achieved was 1 picogram in full scan mode and 1 femtogram in selected ion monitoring (SIM) mode.



SCHEME 2 Fragmentation pattern for the PFTH-valine adduct (A) in EI mode (B) in NCl mode.

CONCLUSIONS

Simple and convenient methods for the synthesis of cysteine and valine adducts of sulphur mustard have been worked out. In the process an improved process for the synthesis of half mustard also has been developed which not only gives half mustard in excellently pure form in high yields, but also avoids the cumbersome distillation of this highly toxic chemical. The GC/MS analysis methods developed for the analysis of synthetic standards of the protein adducts will be quite useful for retrospective detection and analysis of these adducts isolated from real biological samples obtained from the patients allegedly exposed to SM.

EXPERIMENTAL

(*Caution:* 2,2-bis(2-chloroethyl) sulphide (sulphur mustard; SM) is a potent vesicant and carcinogenic and should be handled only by suitably qualified and protected individuals using a well-ventilated fume cupboard. Half mustard also has pronounced vesicant properties and should be handled accordingly.)

General Procedure

All the solvents used in this study were of HPLC grade. Cysteine and valine were procured from Sigma Chemical Co. while all derivatizing agents were obtained from Lancaster. NMR spectra were recorded on a Varian 400 MHz instrument. Chemical shifts (δ) are reported in ppm downfield from TMS. Mass spectra were obtained using a Finnigan Mat TSQ 7000 triple quadruple mass spectrometer. The electron ionisation (EI) spectra were recorded at 70 eV at a source temperature of 180°C; chemical ionisation (CI) spectra were recorded using methane or isobutane as the reagent gas at a source pressure of 4000 mTorr.

2-(2-Chloroethylthio) Ethanol [Half-Mustard] (1)

Sodium methoxide, prepared by adding 5.8 g (0.25 mmol) of sodium in small proportions to 150 mL dry methanol, was added to a stirred solution of 2-mercaprtoethanol (18 mL, 19.8 g, 0.25 mmol). Stirring was continued for an additional 30 min when 1,2 dichloroethane (150 mL) was added. The mixture was allowed to stand at 5°C (refrigerator) for 8 h. The precipitated sodium chloride was filtered off, the solvent removed, and the residue washed with water. The half mustard thus obtained was kept in a known volume of ether and stored over anhydrous

magnesium sulphate at −20°C until required. An aliquot of ethereal solution was evaporated to dryness for spectroscopic analysis. The compound was identified by NMR and MS and the purity was checked by GC/MS.

S-(2-Hydroxyethylthioethyl)-L-cysteine (2)

Cysteine hydrochloride (783 mg, 0.005 mmol) was dissolved in water (3 mL) at room temperature. Nitrogen was bubbled through the solution and 1 ml of 10 N sodium hydroxide was added to make the mixture alkaline. Half mustard (1.35 g, 0.015 mmol) in 30 mL of ether was added in small portions during about 90 min and sufficient 10 N sodium hydroxide solution was added to maintain the mixture alkaline. When the reaction was complete, the solution was shaken with dichloromethane (3 × 5 mL). The aqueous solution was adjusted to pH 5.5 by addition of concentrated hydrochloric acid. After cooling in the refrigerator overnight, the precipitate, which formed, was filtered off and washed with 8-10 mL ethyl alcohol. The dry powder was mixed thoroughly with 1 mL concentrated hydrochloric acid and dissolved in 10 mL of boiling absolute ethanol. After filtering, 50 mL of ether was added and the solution refrigerated to yield a white precipitate, Yield 1.01 g (90%); m.p. 128°C (lit. m.p. 128.5–129°C).[5]

N-(2-Hydroxyethylthioethyl)-L-valine (3)

A solution of L-valine (0.5 g, 0.0042 mmol) was prepared in 4 mL of water and 0.2 mL of 10 N sodium hydroxide by warming at 50–70°C. Ether (15 mL) containing half mustard (1.2 g, 0.084 mmol) was added in small portions. The reaction flask was kept warm on the hot plate and nitrogen was bubbled through the reaction mixture to accelerate the evaporation of ether. The pH of the reaction mixture was maintained at 10 by adding 10 N sodium hydroxide solution. When the reaction was complete, the aqueous solution was shaken with 3 × 5 mL portions of dichloromethane. The pH was adjusted to 2.7 by addition of concentrated hydrochloric acid (0.4 mL) and the solution was shaken twice with 5 mL portions of ether. The water was evaporated under vacuum and the residual solid was washed twice with 10 mL of isopropyl alcohol. Extraction of the solid with 70–80 mL of boiling isopropyl alcohol and subsequent cooling gave a white precipitate, Yield 0.35 g (70%); m.p. 208–211°C (dec.) (lit. m.p. 209–212°C dec.).[5]

TMS Derivatization of Cysteine-SM Adduct

Dried cysteine-SM adduct (1 mg) was heated with N, O bis (trimethylsilyl)trifluoro acetamide (BSTFA) (250 μL) for 4 h at 60°C to yield the TMS derivative of the Cysteine-SM adduct.

Oxazolidinone Formation of Cysteine-SM Adduct

The cysteine-SM adduct (1 mg) was derivatized with 1,3-dichlorotetrafluoroacetone (DCTFA) (20 μL) followed by acylation with heptafluorobutyric anhydride (HFBA) (20 μL) at 40°C. The organic layer was extracted with sodium carbonate (1 mL, 1M), HCl (1 mL, 1M), and water (1 mL) respectively. The organic layer was evaporated under a nitrogen stream, dissolved in n-heptane (2.5 mL) and HFBA (125 μL) and heated at 70°C for 5 min after which the samples was ready for analysis.

PFTH Derivatization of Valine-SM Adduct

Valine-SM adduct (10 mg) was dissolved in a mixture of aqueous potassium bicarbonate (5 ml, 0.5 M) and 1-propanol (2.5 mL). Pentafluorophenyl isothiocynate (PFPITC) (25 μL) was added and the mixture was heated for 2 h at 45°C. The PFPTH derivative was extracted with n-heptane (2 $\times$ 10 mL), concentrated to dryness, dissolved in toluene, and analyzed.

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