

Formaldehyde adducts of glutathione

Structure elucidation by two-dimensional n.m.r. spectroscopy and fast-atom-bombardment tandem mass spectrometry

Stephen NAYLOR,* Ralph P. MASON,* Jeremy K. M. SANDERS,*† Dudley H. WILLIAMS* and Gloriano MONETI†

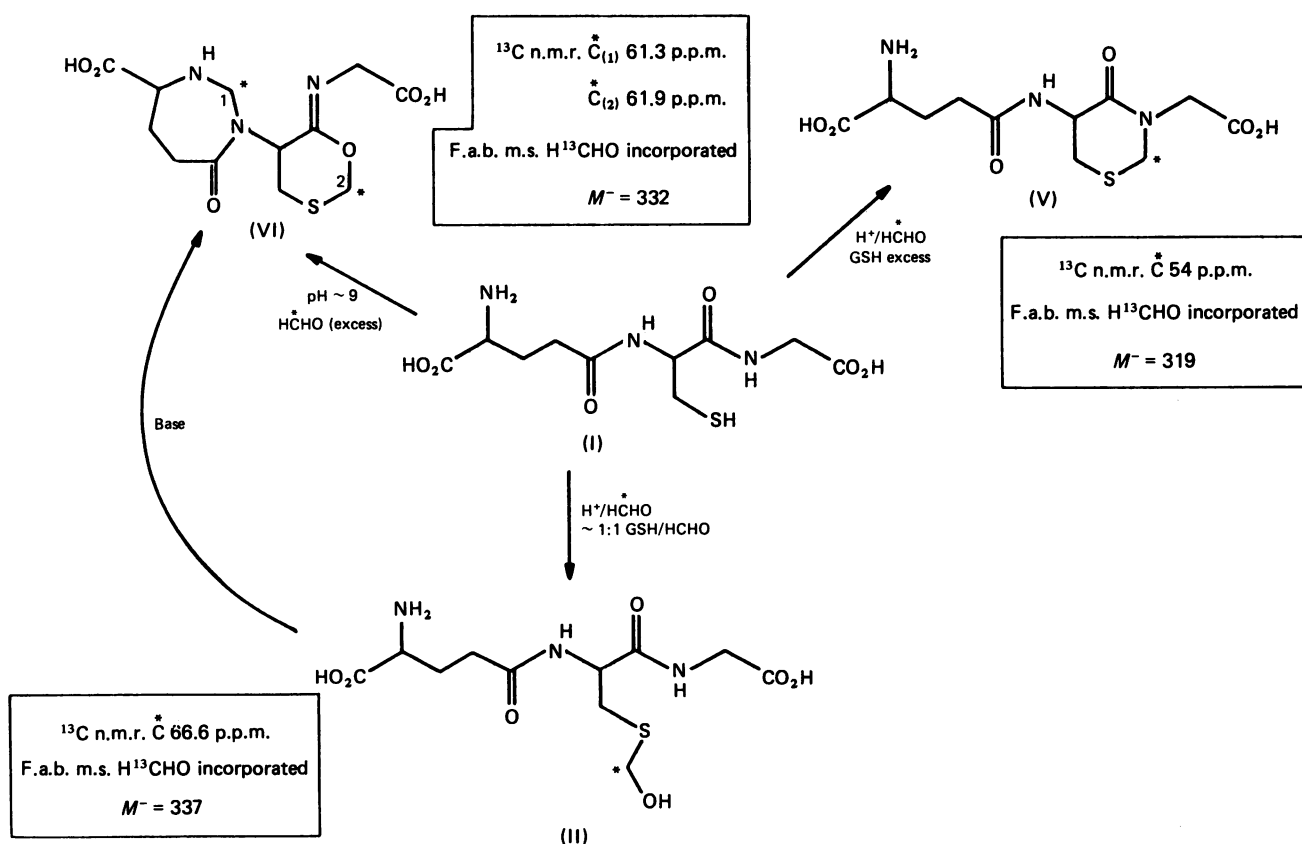
*University of Cambridge Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K., and †Mass Spectrometry Centre, Department of Pharmacology, Viale Morgagni 65, University of Florence, 50134 Florence, Italy

Aqueous mixtures of formaldehyde and glutathione react to form a variety of cyclized adducts in addition to *S*-hydroxymethylglutathione. The adducts are in labile equilibrium with each other and are not readily separated. The structures of two of the other major adducts were determined by concerted application of ^{13}C - ^1H two-dimensional chemical-shift correlation, fast-atom-bombardment mass spectrometry and tandem mass spectrometry to the adduct mixtures in aqueous solution.

INTRODUCTION

Glutathione (GSH) (I) has an important detoxifying role in cells, and is also a cofactor for many dehydrogenases (Koivusalo *et al.*, 1982). Two of us have confirmed that it is implicated in formaldehyde detoxification in *Escherichia coli* by demonstrating the intra-

cellular formation and enzymic consumption of the hemithioacetal adduct *S*-hydroxymethylglutathione (GSCH_2OH) (II) (Mason *et al.*, 1986); this is probably the true substrate for formaldehyde dehydrogenase. In the course of that work we noticed that mixtures of GSH and [^{13}C]formaldehyde slowly reacted to give several new ^{13}C -n.m.r. resonances in addition to those of formalde-



Scheme 1. Structures and spectroscopic properties of adducts formed between GSH and formaldehyde

Abbreviations used: c.a.d., collision activated dissociation; f.a.b., fast atom bombardment.

† To whom correspondence should be addressed.

hyde and GSCH_2OH . We now report on the structures of two of these new products, which are cyclic 1:1 and 1:2 adducts in labile equilibrium with each other. The strategy used in this work has been to use n.m.r. spectroscopy to define the chemical environment of the formaldehyde-derived carbon atoms, f.a.b. m.s. to determine the molecular mass of the adducts (Barber *et al.*, 1981*a,b*) and fragmentation patterns in a c.a.d. tandem mass spectrum to locate the added carbon atoms (for reviews see McLafferty, 1983; Yost & Fetterolf, 1983). A summary of the adduct structures, and of some of their salient properties, is given in Scheme 1.

These new adducts are formed under non-physiological conditions and presumably have no biological significance in themselves. However, we believe that the use of f.a.b. and tandem m.s. to determine the structures of modified peptides in intractable aqueous mixtures is new. Most importantly, we demonstrate that, by careful control of conditions, it is possible to reproduce the delicate equilibria of aqueous chemistry within the largely non-aqueous environment of the f.a.b.-m.s. matrix.

RESULTS

^{13}C -n.m.r. spectroscopy

We had previously reported that $[^{13}\text{C}]$ formaldehyde and GSH (I) react rapidly with each other in aqueous solution to give $\text{GS}^{13}\text{CH}_2\text{OH}$ (II) (Mason *et al.*, 1986). The formaldehyde-derived carbon atom in the adduct resonates at 66.6 p.p.m., whereas formaldehyde itself appears at 83.2 p.p.m. Solutions of the adduct at pH 5 were indefinitely stable. However, if the pH was raised to 6 or above, new resonances gradually appeared at the expense of GSCH_2OH (II); the relative intensities of these new signals depended in a complex manner on pH and on the absolute and relative concentrations of the reactants. Two signals at 61.9 and 61.3 p.p.m. always appeared simultaneously, the former being slightly more intense; these were ultimately assigned to the bis-adduct (VI). Re-acidification to pH 1 gave slow reversion of these new signals to that of GSCH_2OH . When GSH was in excess at slightly acid pH, additional signals at 44 p.p.m. (and, to a lesser extent, 54 p.p.m.) also grew in intensity. Under these conditions, no natural-abundance GSH-derived carbon atoms were observed in our spectra. Fig. 1 shows a typical spectrum. Addition of unlabelled formaldehyde to a solution containing $\text{GS}^{13}\text{CH}_2\text{OH}$, 61.3 and 61.9 p.p.m. resonances, led to virtually instant exchange of the GSCH_2OH signal, followed by slower (approx. 1 h) exchange of the 61.3 p.p.m. peak, and very slow (overnight) exchange of the 61.9 p.p.m. peak.

^1H -coupled ^{13}C -n.m.r. spectra revealed that all the formaldehyde-derived carbon atoms were still CH_2 groups, and allowed measurement of their $^1J_{\text{CH}}$; two-dimensional ^1H - ^{13}C chemical-shift correlation (Sanders & Hunter, 1987) revealed the shifts of the directly attached protons for most of the adducts (Table 1). The complexity of the reaction mixtures rendered both one- and two-dimensional ^1H -n.m.r. spectra too complicated to unravel. The large non-equivalence of the two formaldehyde-derived protons in all the adducts other than GSCH_2OH is a strong indication that they are incorporated into rings, and the large J_{CH} of the 61.3 and 61.9 p.p.m. signals is indicative of NCH_2N , OCH_2S or

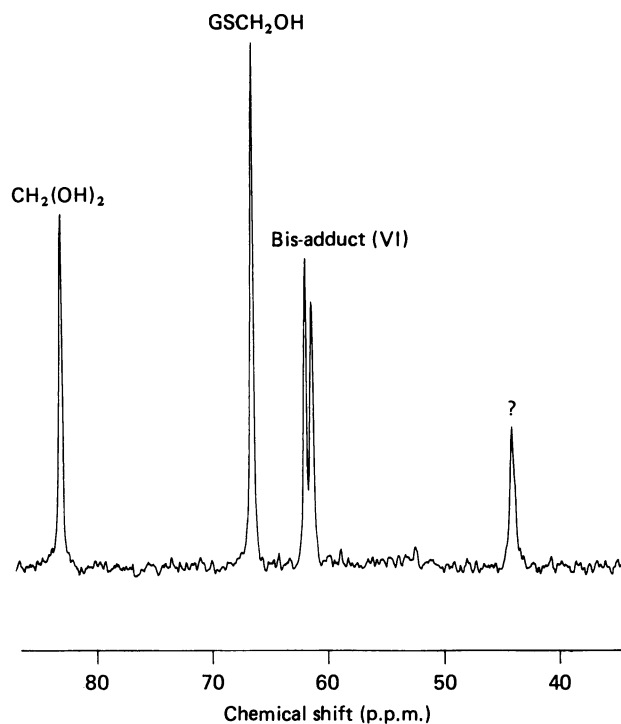


Fig. 1. 100.6 MHz ^{13}C -n.m.r. spectrum of a reaction mixture containing equimolar GSH and $[^{13}\text{C}]$ formaldehyde at pH 6

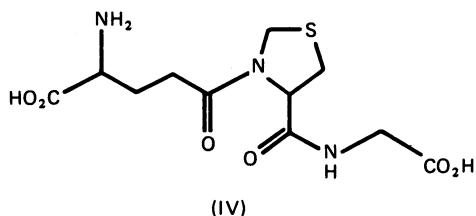
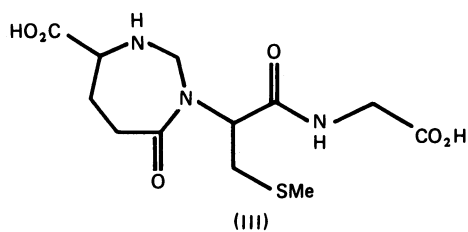
Table 1. N.m.r. properties of the formaldehyde-derived groups in GSH adducts

^{13}C shift (p.p.m.)	$^1J_{\text{CH}}$ (Hz \pm 1)	^1H shifts (p.p.m.)
66.6	159	4.70, 4.72
61.9	156	5.0, 4.3
61.3	156	4.0, 3.0
54.0	152	—*
44.0	152	—*

* Experiment not carried out.

NCH_2S groups. The large shifts of the protons attached to the 61.9 p.p.m. signal are strongly indicative of attachment to oxygen. The ^{13}C shift and coupling of the 54 p.p.m. signal are consistent with attachment to less electronegative groups such as NCH_2S . However, although this information is indicative, it is inadequate for structure assignment, given the plethora of functional groups available for reaction in GSH. Attempts to carry out long-range ^1H - ^{13}C correlation and COLOC experiments (Sanders & Hunter, 1987) failed to yield useful spectra.

In an attempt to discover which functional groups of GSH were involved in the observed chemistry, we also looked at the reaction of *S*-methylglutathione (GSMe) and several amino acids with formaldehyde. Above pH 9, $[^{13}\text{C}]$ formaldehyde and GSMe rapidly gave an adduct showing a resonance at 70 p.p.m.; by analogy with previous work (Tome & Nault, 1981) this may be assigned to the *N*-terminal aminomethylol (NCH_2OH)



adduct. Below pH 8.5, the only observed reaction was slow growth of a signal at 61.3 p.p.m., but, significantly, none at 61.9 p.p.m. In view of this simplicity, we assigned the natural-abundance ^{13}C spectrum of GSMe by two-dimensional ^1H - ^{13}C correlation, and repeated the reaction with formaldehyde labelled only with 10% ^{13}C . On adduct formation, the Glu α -carbon atom signal shifted from 58 to 67 p.p.m., while that of the Glu β -carbon atom moved from 28 to 27 p.p.m.; there were no other significant shifts. The conclusion to be drawn from these observations is that the 61.3 p.p.m. signal in the GSMe adduct (and, by implication, the GSH adduct) arises from the seven-membered ring adduct shown (III).

Further evidence that the *N*-terminal nitrogen atom and the sulphur atom were the most reactive sites of hydroxymethylation came from attempting to make *NS*-diacetylglutathione react with [^{13}C]formaldehyde. Under neither acidic nor basic conditions were adducts detected by using ^{13}C n.m.r. and f.a.b. m.s.

Reaction of [^{13}C]formaldehyde with *N*-acetylcysteine gave only the expected SCH_2OH adduct resonating at 66.6 p.p.m., whereas reaction with cysteine itself also gave a 54 p.p.m. signal. The latter shift is characteristic of CONHCH_2S groups (Tome & Naulet, 1981), and implies formation of a cyclic adduct. In the cysteine adduct this is necessarily a five-membered ring (IV), but in the corresponding GSH adduct a six-membered ring (V) is also possible. M.s. confirms the six-membered ring structure (V) (see below).

In model studies, no products exhibiting a 44 p.p.m. ^{13}C resonance were obtained, and the structure of that adduct remains unknown.

Conditions for f.a.b. m.s.

In order to simulate, in an f.a.b.-m.s. matrix, conditions under which adducts are normally formed by reaction between GSH and formaldehyde, both acidic and basic matrices were required. Saito & Kato (1984) used dimethyl sulphoxide glycerol as a matrix to monitor the formation *in situ* of GSH-arylnitroso adducts, but we observed only a limited increase in ion abundance when this combination was used (relative to the signal observed with aq. glycerol alone). For the determination of both positive-ion and negative-ion f.a.b. mass spectra, under acidic conditions, a matrix consisting of thioglycerol/aq.

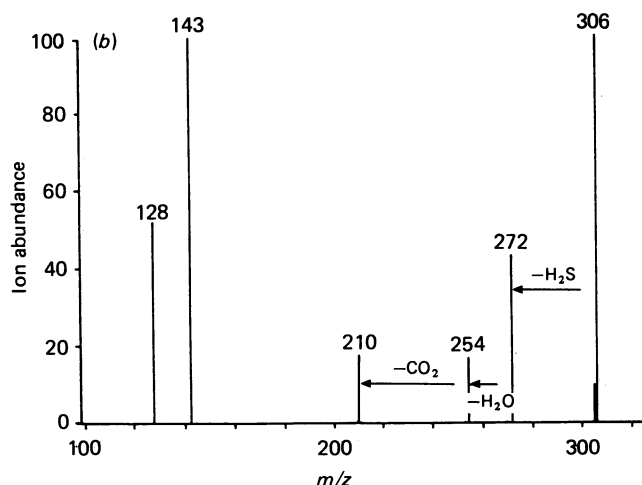
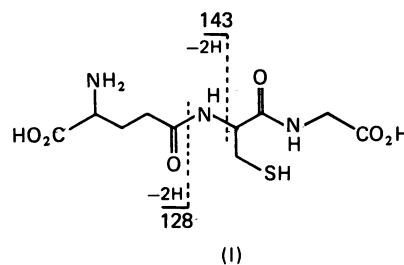
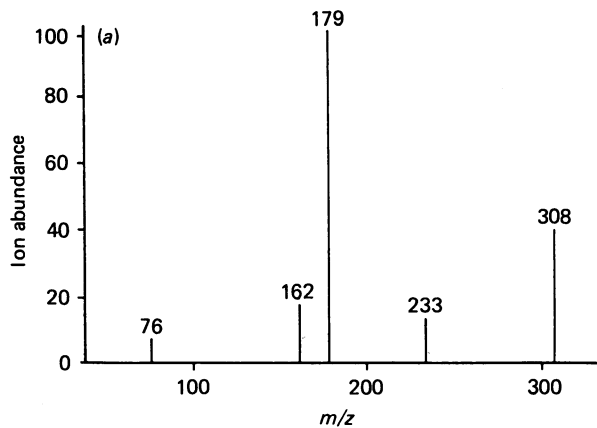
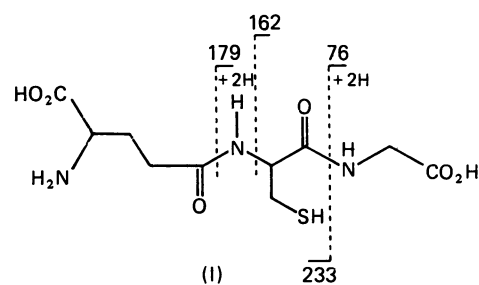


Fig. 2. Positive-ion (a) and negative-ion (b) c.a.d. mass spectra of GSH (I)

5% (v/v) glycerol/15-crown-5 (10:10:1, by vol.) was used. The presence of 15-crown-5 caused a dramatic increase in the abundance of the adduct ions of interest relative to peaks due to the matrix. This observation is similar to an effect observed by De Pauw *et al.* (1984) when using various sulphonic acids as a means of

Table 2. Adduct ions observed in f.a.b. m.s.

The values given correspond to $[M-H]^-$ ions obtained in negative-ion spectra. The values in parentheses are the $[MH]^+$ ions observed in positive-ion spectra.

Reactant	<i>m/z</i> values
H ¹² CHO	318 (320), 336 (338)
H ¹³ CHO	319 (321), 337 (339)
² H ¹² C ² HO	320 338

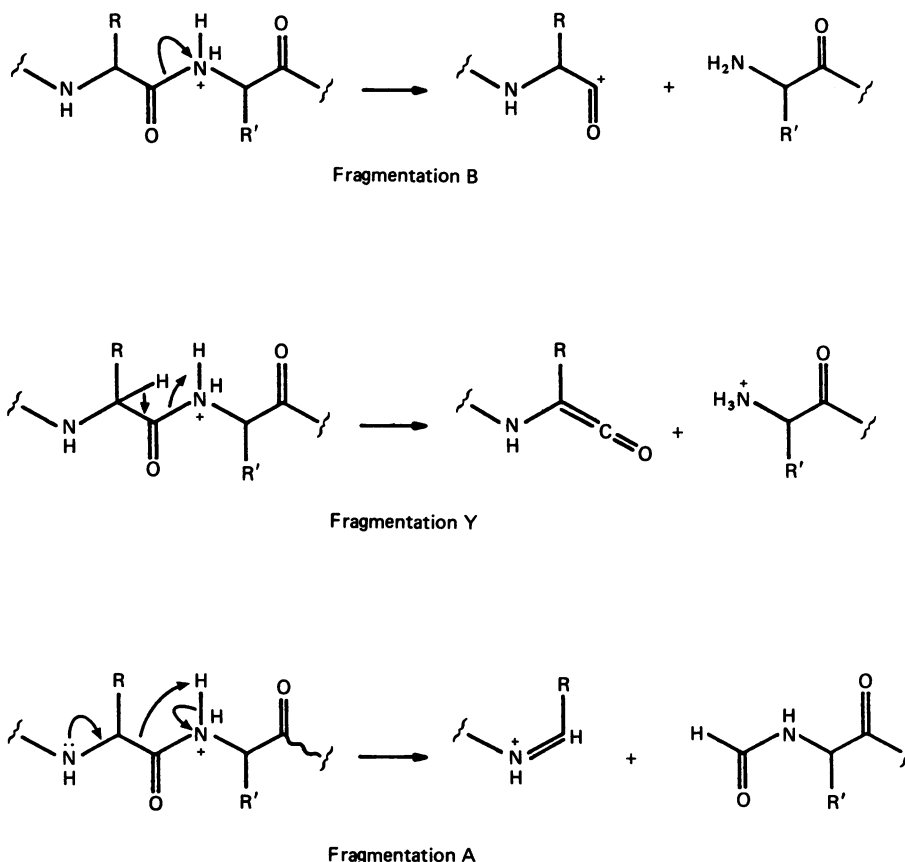
suppressing matrix relative to sample signals. The beneficial effects of addition of 15-crown-5 to glycerol have been independently noted by Fujii *et al.* (1985). However, increasing the concentration of 15-crown-5 relative to thioglycerol/aq. glycerol can lead to complete suppression of peptide signal. For example, a thioglycerol/aq. glycerol/15-crown-5 (1:1:1, by vol.) matrix containing GSH completely suppressed the molecular ion (M^-) at *m/z* 306 of the peptide for 12 min. This is consistent with the hydrophobic crown ether excluding peptide from the surface region of the matrix. Only after the 15-crown-5 has been sputtered away does a signal corresponding to M^- of GSH appear. The basic matrix was generated by adding approx. 25% (v/v) of triethylamine to this mixture. NaOH could not be satisfactorily used as base owing to the formation of a wide variety of cationized species from the matrix.

Results from f.a.b. m.s.

F.a.b. m.s. has aided the structural studies of the adducts of formaldehyde and GSH in three ways: first, by confirming the nature of the reactive functionalities of GSH towards formaldehyde; secondly, by determination of the molecular masses of these adducts; thirdly, by the use of c.a.d. in a tandem mass spectrometer to indicate the sites of adduct formation and ring formation. These aspects are discussed separately in this section.

Reactive sites of GSH. *NS*-Diacetylglutathione was treated with [¹³C]formaldehyde in the matrix under both acidic and basic conditions. In neither case were molecular ions due to any [¹³C]formaldehyde addition or condensation products observed. These experiments indicate the amino group of the γ -glutamic acid residue, and the thiol group of cysteine residue, as the sites of reactivity for adduct formation in GSH itself.

Molecular masses of the adducts. Initially ¹³C-n.m.r. samples containing a predominant adduct were subjected to f.a.b. m.s. in order to ascertain the molecular mass of that adduct. Subsequently, adducts were formed directly in the f.a.b.-m.s. matrix on the probe tip and their f.a.b. mass spectra were obtained. In acid conditions with a variety of labelled formaldehydes, two different adducts were formed (see Table 2). The molecular masses of the two adducts were 335 and 317 Da (incorporating [¹²C]-formaldehyde). The increase in molecular mass of the adducts from GSH corresponds to addition of formal-



Scheme 2. Major fragmentation pathways observed for peptides subjected to positive-ion f.a.b. m.s.

The nomenclature of Roepstorff & Fohlmann (1984) is used to describe fragment type.

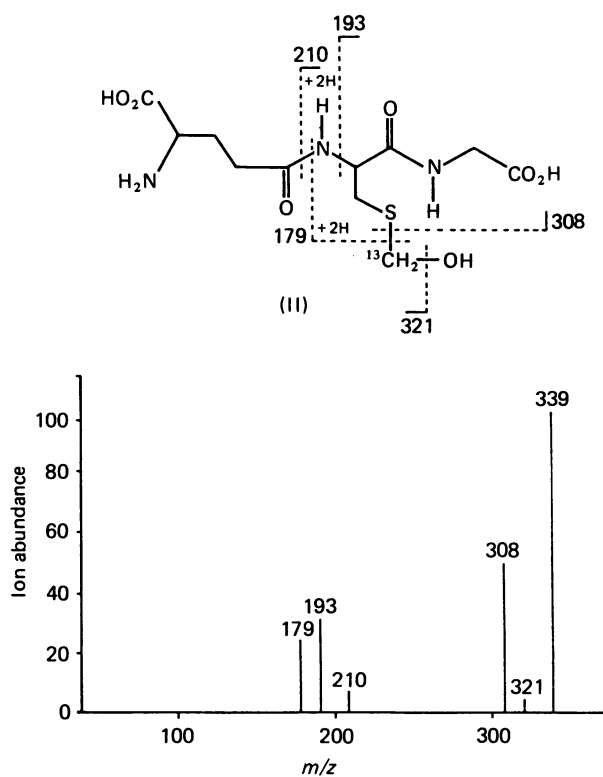


Fig. 3. Positive-ion c.a.d. mass spectrum of GSCH₂OH (II)

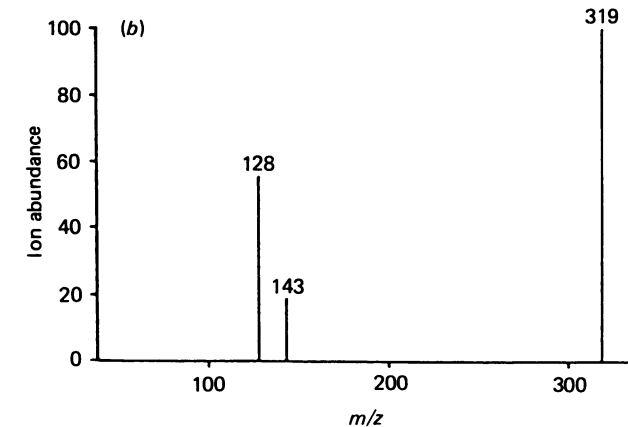
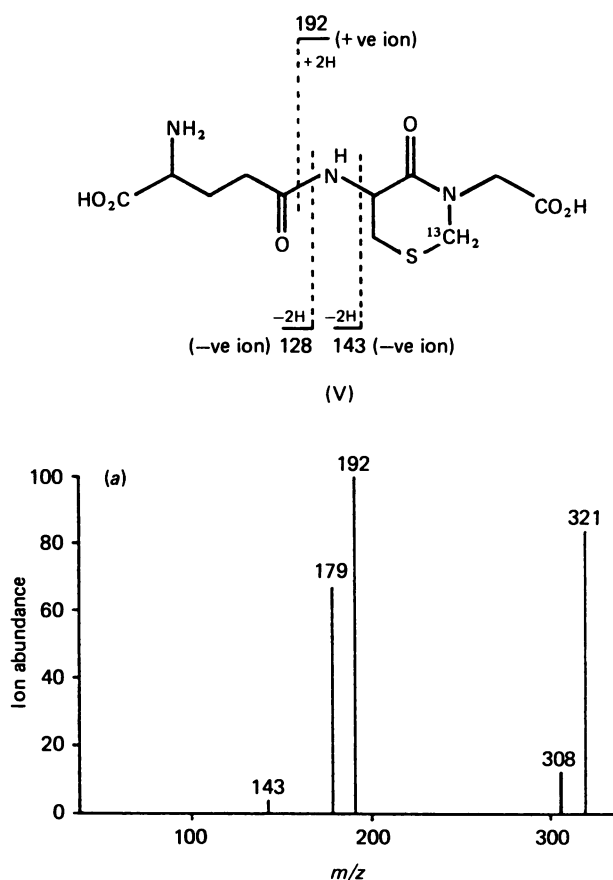


Fig. 4. Positive-ion (a) and negative-ion (b) mass spectra of the single-ring adduct (V) of formaldehyde and GSH

dehyde and addition of formaldehyde with associated loss of water respectively. These observations are consistent with the formation of compounds (II) and (V) (see Scheme 1).

Further experiments were carried out in which both GSH (I) and GSCH₂OH (II) were treated with an excess of formaldehyde in the matrix under basic conditions (addition of triethylamine). Under these conditions, a new molecular ion $[M-H]^-$ appeared in negative-ion spectra at m/z 330. This peak was shifted to m/z 332 when [¹³C]formaldehyde replaced unlabelled formaldehyde, and to m/z 334 when [²H₂]formaldehyde replaced unlabelled formaldehyde. It therefore corresponds to formation of an adduct containing two CH₂ groups from formaldehyde, but the reactions are associated with the loss of two water molecules. These data therefore provide supporting evidence for the formation of a bicyclic adduct; and, in conjunction with the ¹³C-n.m.r. evidence, indicate the formation of compound (VI) (Scheme 1). It is noteworthy that the formation of this adduct as deduced from f.a.b. m.s. occurs under the same conditions as those leading to signals at 61.9 and 61.3 p.p.m. in the ¹³C-n.m.r. spectrum.

Use of c.a.d. tandem m.s. A characteristic feature of f.a.b. m.s. is the normally pronounced abundance of MH^+ or $[M-H]^-$ ions relative to fragment ions. Thus, in order to extract structural information from f.a.b.-m.s. spectra, it is sometimes advantageous to break up the MH^+ or $[M-H]^-$ ions via c.a.d., and then to separate the resulting fragment ions. If this strategy is applied to obtain structural information from a mixture of compounds, it is necessary to separate the various ions characteristic of the molecular masses of the components

in a first mass spectrometer, and then to bring about c.a.d. of a selected ion before separating the resultant fragments in a second mass spectrometer. This technique is known as c.a.d. tandem m.s., and it has been employed to derive structural information from the present mixture of adducts. The fragmentation of peptides in c.a.d. tandem m.s. has been investigated and reviewed previously (Eckart *et al.*, 1985; Lippstreu-Fisher & Gross, 1985; Biemann & Martin, 1987), and the major frag-

mentations of linear peptides are outlined in Scheme 2. The nomenclature of Roepstorff & Fohlman (1984) is used to describe particular fragmentation processes.

To obtain information on the c.a.d. of the GSH backbone, initial studies were carried out on the parent peptide. The c.a.d. f.a.b. mass spectra of GSH were obtained in the positive-ion (Fig. 2a) and negative-ion (Fig. 2b) modes. The origins of the fragments are indicated in the Figures.

The interpretation of the positive-ion spectrum was supported by comparing the spectrum with the corresponding spectrum (Fig. 3) of $\text{GS}^{13}\text{CH}_2\text{OH}$. Thus in the latter spectrum the ions observed at m/z 162 and 179 in Fig. 2(a), should appear at m/z 193 and 210 if the ^{13}C formaldehyde was added at the sulphur rather than the nitrogen atom; these peaks are observed, and exemplifies how c.a.d. tandem m.s. can be used to determine the location of the added formaldehyde.

The positive-ion c.a.d. f.a.b. mass spectrum of the adduct (from reaction with ^{13}C formaldehyde) previously deduced to have the monocyclic structure (V) was then obtained (Fig. 4a). The most abundant fragment ion occurs at m/z 192. The mass of this ion is in accord with the cleavage shown in the Figure, which was also observed in GSH and GSCH_2OH . Thus the $^{13}\text{CH}_2$ unit, which has been incorporated via addition followed by water loss, must be attached at a site other than the γ -glutamic acid residue, in accord with the assigned structure. It is clear from the spectrum that in the aqueous matrix a small amount of the adduct has produced GSH (m/z 308); fragmentation of this hydrolysis product will produce m/z 179, as in the case of GSH itself (Fig. 2a).

The negative-ion c.a.d. f.a.b. mass spectrum of the same adduct is reproduced in Fig. 4(b). The only fragment ions of significant abundance occur at m/z 128 and 143. Linked scan experiments (carried out on the $^2\text{H}_2$ -formaldehyde adduct) showed that both m/z 128 and 143 arise directly from the parent ion at m/z 320. Additionally, the spectra of the adducts with either $^2\text{H}_2$ -formaldehyde or ^{13}C formaldehyde afforded only m/z 128 and 143, establishing that these fragments do not retain the methylene group incorporated from formaldehyde. This observation is consistent with their origins as shown in Fig. 4(b), which is analogous to that proposed in Fig. 2(b). These findings are not only in accord with the proposed structure (V), but also exclude the alternative structure (IV). In this latter structure the formation of m/z 143 is precluded by the ring of which the cysteine residue $\alpha\text{-CH-N}$ bond is a part.

The positive-ion and negative-ion c.a.d. studies on the bisformaldehyde adduct (VI) gave complementary evidence on the existence of the seven-membered and six-membered rings respectively. The negative-ion c.a.d. mass spectrum displayed prominent fragment ions (incorporation of $^2\text{H}_2$ formaldehyde) corresponding to loss of CO_2 , $\text{C}^2\text{H}_2\text{S}$ and $\text{CO}_2 + \text{C}^2\text{H}_2\text{S}$. This ready loss of $-\text{SX}-$ in negative-ion c.a.d. mass spectra was also observed in GSH, GSMe , GSCH_2OH and the single-ring adduct (V).

In the positive-ion c.a.d. mass spectrum of compound (VI) three fragment ions were observed. The spectrum was analogous to the positive-ion c.a.d. mass spectrum of the seven-membered ring GSMe derivative (III) insofar as the same neutral losses from MH^+ were observed. These observations are consistent with the presence of

the same structural feature, i.e. a seven-membered ring, in both compounds (III) and (VI).

CONCLUSION

The interaction of GSH with formaldehyde was explored by using a combination of ^{13}C n.m.r., f.a.b. m.s. and tandem f.a.b. m.s.-m.s. Two new adducts of GSH with formaldehyde were identified and their structures determined. More importantly, we have shown that this approach is capable of determining structures in mixtures that are intractable by virtue of the labile equilibria that are occurring: the adducts cannot be easily isolated because they are continuously interconverting.

EXPERIMENTAL

F.a.b. m.s.

Matrices. All matrices were vacuum-distilled before use. Glycerol, thioglycerol and 15-crown-5 were purchased from Sigma Chemical Co.

Mass spectra. All f.a.b. mass spectra were recorded on one of the following instruments.

(a) Kratos MS50 operating at 8 keV. The instrument was equipped with a standard Kratos FAB source and an Ion Tech gun. The energy of the primary atom beam was 8 keV at an ion current of 40 μA . Xenon was used as the source of high-energy atoms in the primary beam. Spectra were obtained with a magnet scan rate in the range 30–100 s per decade with a magnet of 10000 Da mass range at 8 keV. The source pressure was typically 1.3 mPa (10^{-5} Torr).

Samples were deposited on an acid-etched stainless-steel probe tip, and a measured amount (normally 2 μl) of matrix was delivered to the probe with a Hamilton syringe. The sample and matrix were thoroughly mixed, then subjected to f.a.b. m.s.

(b) F.a.b. m.s.-m.s. data were obtained on a VG7070EQ mass spectrometer. The instrument had a standard VG f.a.b. source (1.3 mPa) and an Ion Tech gun (xenon beam operated at 8 keV). Spectra were obtained with a magnet scan rate in the range 5–30 s per decade with a magnet of 2000 Da mass range at 6 keV. Typical collision-cell conditions were 20–30 eV collision energy at a pressure of 0.13 mPa with argon as the collision gas. The mass range of the second quadrupole was 1200 Da. The data were processed by a Digital PDP8/A computer system.

N.m.r.

Solutions (10–50 mM) of labelled formaldehyde were prepared as described previously (Mason *et al.*, 1986), and were added to solutions of GSH; the pH was adjusted with NaOH. The 100.6 MHz ^{13}C spectra were acquired at ambient temperatures on a Bruker WH 400 instrument. In all 50–500 transients were collected into 8K data points across 29000 Hz, with 50° pulses and a 1 s relaxation delay between pulses.

R.P.M. thanks Unilever Research and the Science and Engineering Research Council for a C.A.S.E. studentship, and S.N. thanks Churchill College and the Science and Engineering Research Council for financial support. G.M. was the recipient

of a C.N.R.-N.A.T.O. Senior Fellowship. J.K.M.S. and D.H.W. are grateful to the Science and Engineering Council for the provision of n.m.r. and m.s. instrumentation.

REFERENCES

- Barber, M., Bordoli, R. S., Sedgwick, R. D. & Tyler, A. N. (1981*a*) *J. Chem. Soc. Chem. Commun.* 325-327
- Barber, M., Bordoli, R. S., Sedgwick, R. D. & Tyler, A. N. (1981*b*) *Nature (London)* **293**, 270-275
- Biemann, K. & Martin, S. A. (1987) *Mass Spectrom. Rev.* **6**, 1-76
- De Pauw, E., Pelzer, G., Dao Viet, D. & Marien, J. (1984) *Biochem. Biophys. Res. Commun.* **123**, 27-32
- Eckart, K., Schwarz, H., Tomer, K. B. & Gross, M. L. (1985) *J. Am. Chem. Soc.* **107**, 6765-6769
- Fujii, I., Isobe, R. & Kanematsu, K. (1985) *J. Chem. Soc. Chem. Commun.* 405-406
- Koivusalo, M., Koivula, T. & Votila, L. (1982) *Enzymology of Carbonyl Compounds: Aldehyde Dehydrogenases and Aldo/Keto Reductases*, pp. 155-168, A. R. Liss, New York
- Lippstreu-Fisher, D. L. & Gross, M. L. (1985) *Anal. Chem.* **57**, 1174-1180
- Mason, R. P., Sanders, J. K. M., Crawford, A. & Hunter, B. K. (1986) *Biochemistry* **25**, 4504-4507
- McLafferty, F. W. (ed.) (1983) *Tandem Mass Spectrometry*, John Wiley and Sons, New York
- Roepstorff, P. & Fohlman, J. (1984) *Biomed. Mass Spectrom.* **11**, 601
- Saito, K. & Kato, R. (1984) *Biochem. Biophys. Res. Commun.* **124**, 1-5
- Sanders, J. K. M. & Hunter, B. K. (1987) *Modern NMR Spectroscopy*, pp. 155-159, Oxford University Press, Oxford and New York
- Tome, D. & Naulet, N. (1981) *Int. J. Pept. Protein Res.* **17**, 501-507
- Yost, R. A. & Fetterolf, D. D. (1983) *Mass Spectrom. Rev.* **2**, 1-45

Received 14 May 1987/12 August 1987; accepted 25 September 1987