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JMS Letters

Dear Sir,

Tandem Mass Spectrometry of Geranylgeranylcysteine

In recent years there has been a revival of interest in the mass spectrometric analysis of polyisoprenoids. de Hoffmann and co-workers[1–3] have studied both phosphorylated and glycosylated polyisoprenoids using fast atom bombardment (FAB) and desorption chemical ionization (DCI) with low-energy tandem mass spectrometry. In this laboratory,[4] investigations have been made on free polyisoprenols and polyisoprenol sulphate and phosphate esters using impact (EI), FAB and electrospray (ES) high-energy tandem mass spectrometry.

The discovery by Farnsworth *et al.*[5] and Rilling *et al.*[6] that polyisoprenoids are covalently linked to cellular proteins constituting a new type of post-translational modification has stimulated considerable interest in such compounds. Initial findings indicated that short-chain polyisoprenoids such as farnesol and geranylgeraniol were bound to proteins,[7] but evidence now exists that other mevalonate-derived compounds,[8] including long-chain polyisoprenoids, are linked covalently to proteins.[9] Prenylated proteins are often divided into two classes on the basis of their *C*-terminal sequence. In both classes the polyisoprenol is bound to a cysteine residue via a thioether linkage. The first group has the *C*-terminal sequence $CX_1X_2X_3$, where C is the single-letter code for cysteine. When X_1 and X_2 are aliphatic amino acids and X_3 is a polar amino acid such as methionine or serine, the protein is farnesylated. If X_3 is a non-polar amino acid such as leucine or phenylalanine, the protein will be geranylgeranylated. In a second group of prenylated proteins, the *C*-terminal sequence may be XXCC, XCXC, CCXX or CCXX, in which case the cysteine(s) are modified by geranylgeraniol. Association of polyisoprenoids with proteins through linkages other than thioether bonds have also been reported.[10]

In previous mass spectrometric studies of prenylated proteins,[8,9] polyisoprenoids were released from proteins by treatment with methyl iodide and were subsequently analysed by EI mass spectrometry. An alternative approach is to digest the prenylated protein enzymatically with a non-specific protease mixture and collect the chromatographic fraction containing radiolabelled prenylated cysteine. This fraction can subsequently be analysed by ES mass spectrometry. The purpose of the present study was to investigate the ES mass spectrometry of a model cysteinyl polyisoprenoid.

Electrospray spectra were recorded on a hybrid magnetic sector–orthogonal acceleration time-of-flight (OATOF) tandem mass spectrometer[11] equipped with a focal plane array detector (FPD) located after the magnet (AutoSpec OATOFFPD, Micromass, Manchester, UK). Synthetic geranylgeranylcysteine (GG-Cys) was obtained from Larodan Fine Chemicals (Malmö, Sweden) and polyethylene glycol (PEG) from Sigma (St Louis, MO. USA). Solvents were of HPLC grade and doubly distilled before use. Samples were introduced by loop injection or continuous infusion in a chloroform–methanol–water solvent (3:3:1) containing 1%

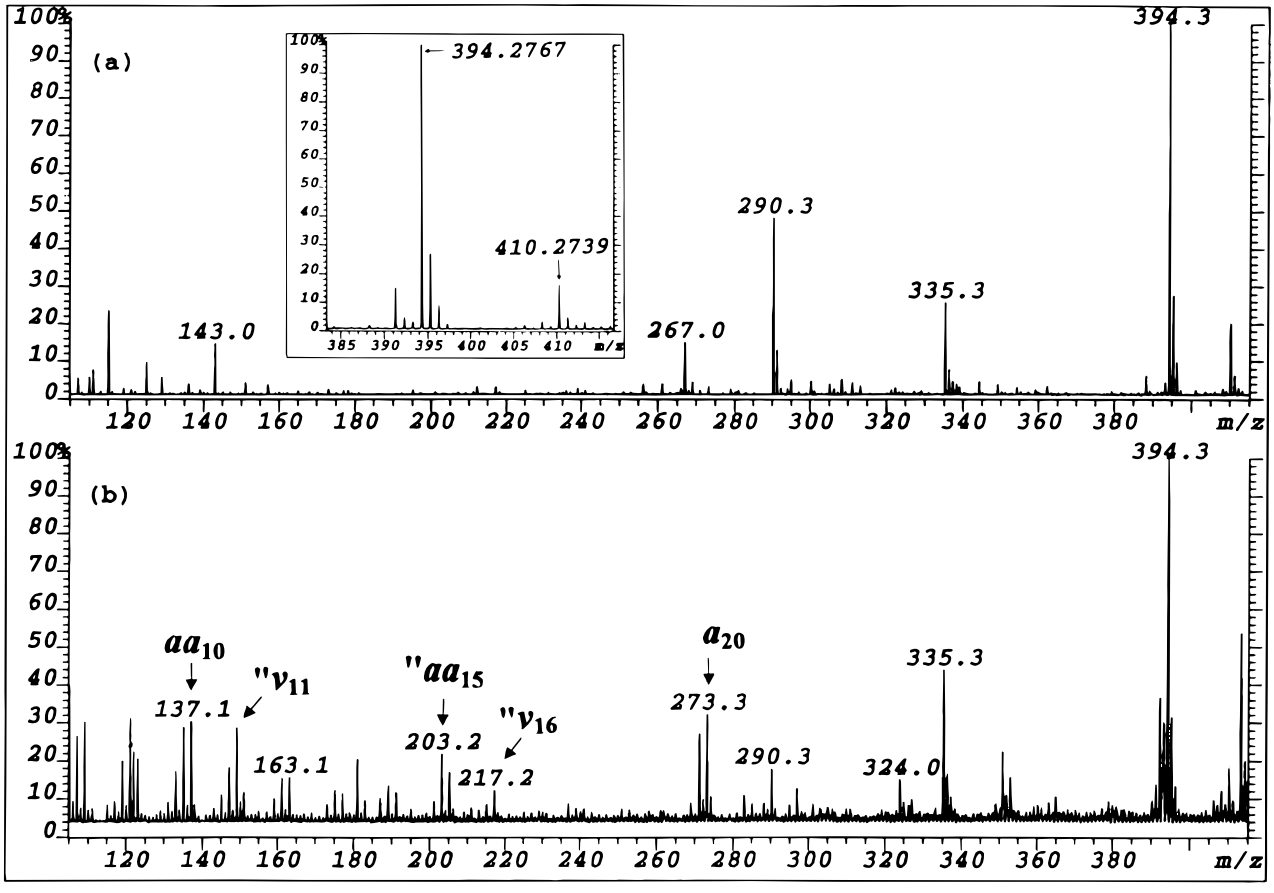

Figure 1. Electrospray mass spectra of synthetic GG-Cys with the electrospray interface (a) optimized on the ion of *m/z* 394 and (b) set at a high cone to skimmer voltage differential. Both spectra were recorded from 25 ng of sample injected. The inset in (a) is the high-resolution accurate mass spectrum covering the $[M + H]^+$ ion region.

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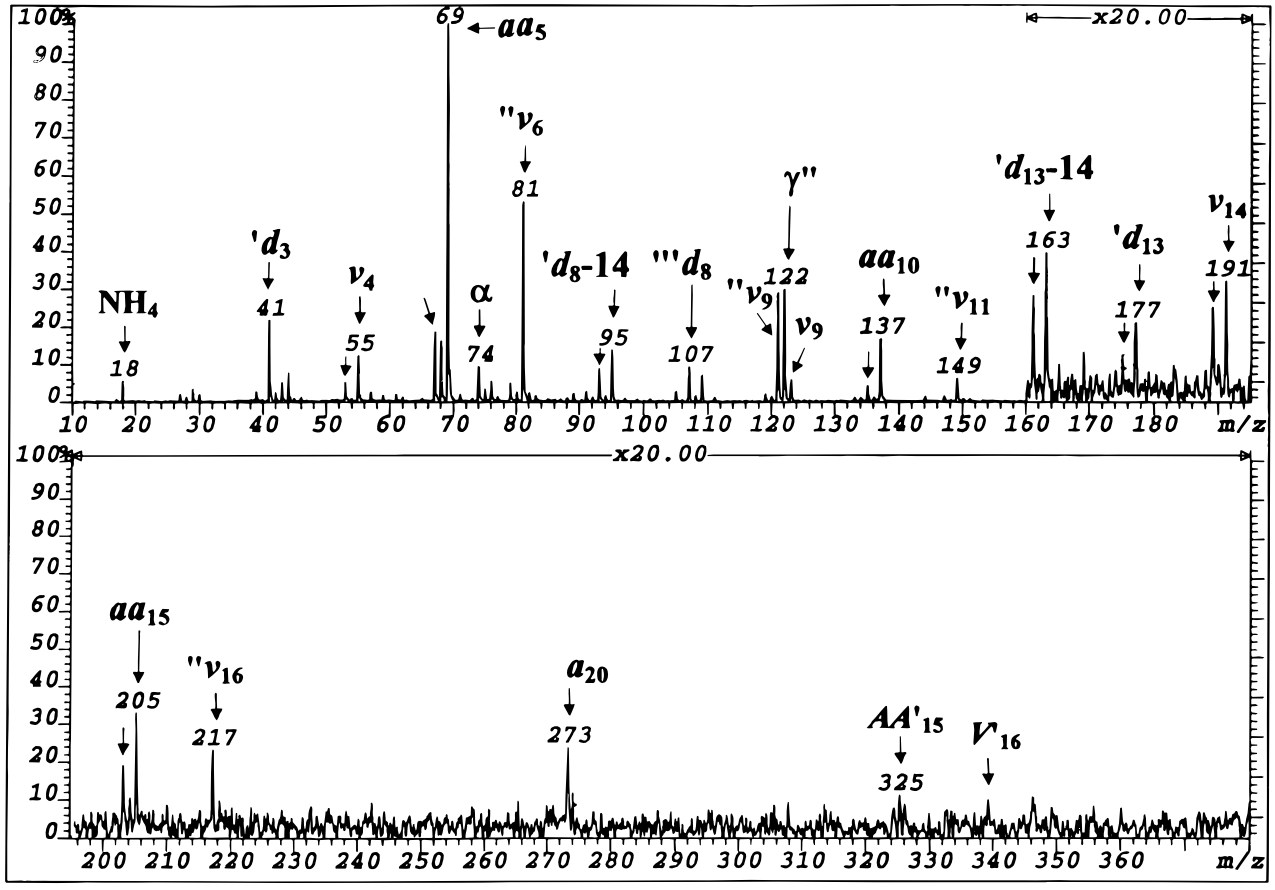


Figure 2. CID spectrum of GG-Cys $[M + H]^+$ ions at *m/z* 394.3 (160 ng infused). E_{lab} 400 eV, collision gas Xe, 75% precursor ion beam attenuation, spectrum recorded on the OATOF.

acetic acid. Electrospray low-resolution (<1500, 5% valley) mass spectra were recorded as magnet scans over the *m/z* range 1000–100 at a rate of 15 s per decade. The electrospray interface conditions were optimized for transmission of the GG-Cys $[M + H]^+$ ion at *m/z* 394.3. High-resolution (>4500, 5% valley) accurate mass spectra were recorded as voltage scans. Scan rates were 15 s per scan. Sample injections were bracketed between calibrant injections (PEG 200–1500). Collision-induced dissociation (CID) spectra were recorded either as linked scans at constant *B/E* on the focal plane array detector or using the orthogonal acceleration time-of-flight mass analyser.

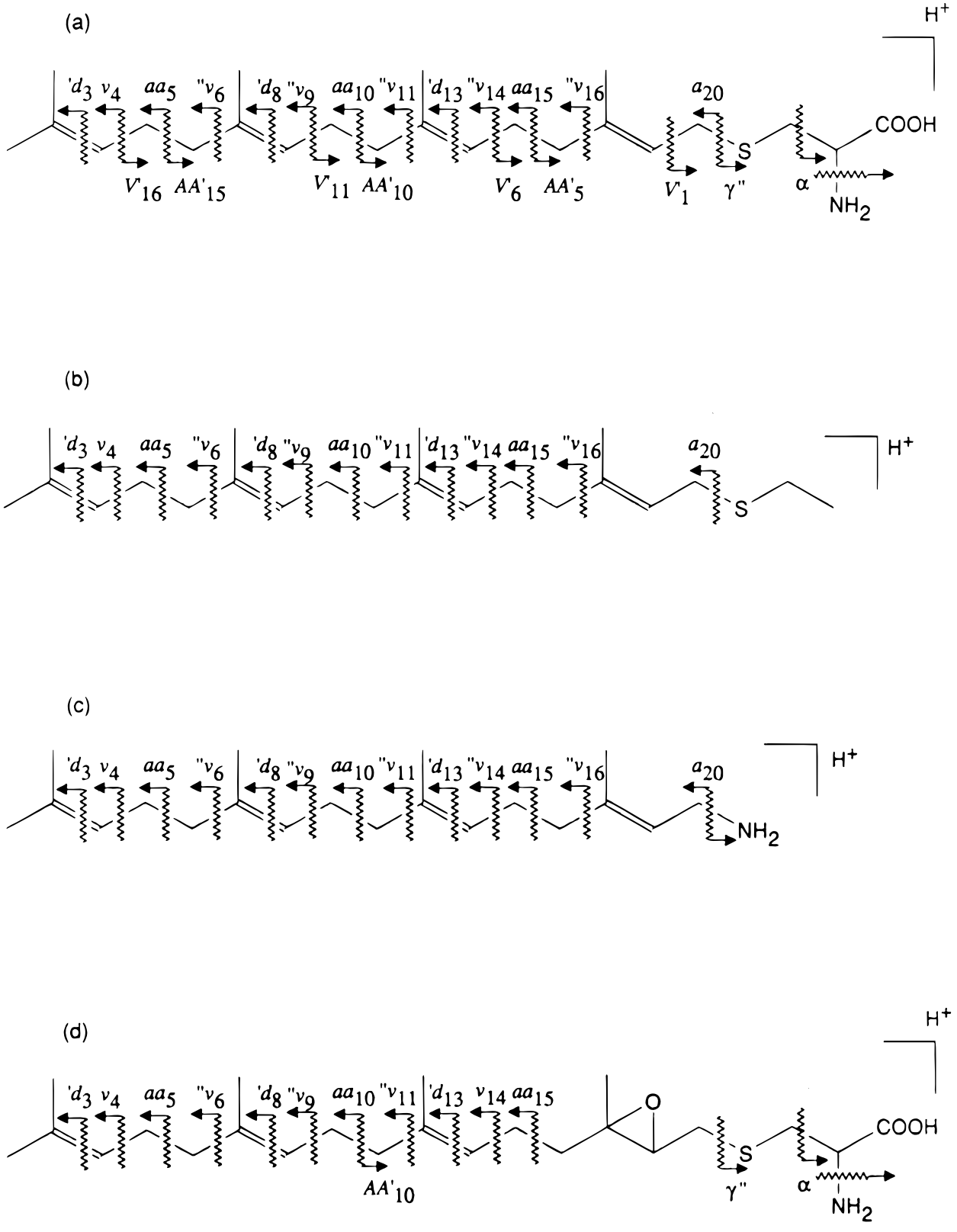
The low-resolution ES mass spectrum of a synthetic sample GG-Cys is shown in Fig. 1(a). The ES interface was optimized on the $[M + H]^+$ ion at *m/z* 394.3. The spectrum is dominated by the ion at *m/z* 394.3. At higher mass an ion is observed at *m/z* 410.3, probably corresponding to an impurity such as an oxidized form of GG-Cys. At lower mass other abundant ions of unknown origin are present at *m/z* 335.3 and 290.3. These molecular masses are consistent with the formulae $[H(C_5H_8)_4SCH_2CH_3 + H]^+$ and $[H(C_5H_8)_4NH_2 + H]^+$. By increasing the cone to skimmer voltage differential within the electrospray interface it is possible to induce collisional fragmentation reactions between the accelerated precursor ions and residual atmosphere. The low-resolution ES 'cone voltage' (CV) fragmentation spectrum of the synthetic GG-Cys sample is shown in Fig. 1(b). In both spectra shown in Fig. 1 impurity ions at *m/z* 410.3, 335.3 and 290.3 are observed; however, abundant fragments at *m/z* 273.3, 271.2, 205.2, 203.2, 149.1, 137.1, 135.1 and 121.1 characteristic of the isoprenoid nature of their precursor ion (see below) are only present in the ESCV spectrum [Fig. 1(b)]. As the synthetic sample of GG-Cys was impure, it is not possible to assign the above fragment ions to a given parent. Although no attempt was made to determine the detection limit for GG-Cys, for a 1 ng injection of impure sample the signal-to-noise ratio for the $[M + H]^+$ ion was in excess of 10:1.

High-resolution accurate mass spectra were recorded to confirm the chemical composition of the GG-Cys $[M + H]^+$ ion at *m/z* 394.3 and determine the chemical compositions of the ions at *m/z* 410.3, 335.3 and 290.3. The high-resolution mass spectrum covering the $[M + H]^+$ ion region of GG-Cys is shown in the inset to Fig. 1(a). From accurate mass measurements the mass of this ion was determined to be 394.2767, which corresponds to within 3 ppm of the theoretical mass of $H(C_5H_8)_4SCH_2CH(NH_3)COOH$, i.e. [GG-Cys + H]. From the accurate mass measurements made on ions at *m/z* 410.3, 335.3 and 290.3, their probable chemical formulae were determined and are given in Table 1.

To investigate further the structures of the above ions, CID spectra were recorded at laboratory frame collision energies of 4 keV (as *B/E* linked scans) and 400 eV (on the OATOF) with argon or xenon as the respective collision gases. The CID

Table 1. Accurate mass measurements made on selected ions in the ES mass spectrum of synthetic GG-Cys

Formula	Theoretical mass (Da)	Measured mass (Da)	Error (ppm)
$C_{23}H_{40}O_3NS$	410.2729	410.2739	3
$C_{23}H_{40}O_2NS$	394.2780	394.2767	3
$C_{22}H_{39}S$	335.2772	335.2784	4
$C_{20}H_{36}N$	290.2848	290.2856	3



Scheme 1. Fragmentation of (a) [GG-Cys + H]$^+$ *m/z* 394.3, (b) precursor ion of *m/z* 335.3, (c) precursor ion of *m/z* 290.3 and (d) precursor ion of *m/z* 410.3.

spectrum of GG-Cys $[M + H]^+$ ions of *m/z* 394.3 recorded at a collision energy of 400 eV with xenon as the collision gas is shown in Fig. 2. The amount of sample injected for recording this spectrum was 160 ng, although spectra showing identical fragment ions were obtained from 40 ng of sample. The 4 keV CID spectrum of GG-Cys $[M + H]^+$ ions is described in Table 2. The 400 eV and 4 kV CID spectra are similar, which is not surprising as the centre of mass collision energies (E_{cm}) are calculated to be 100 and 369 eV at the respective 400 eV and 4 keV laboratory frame collision energies (E_{lab}), and both of these values of E_{cm} are within the high-energy CID regime. The spectrum in Fig. 2 is interpreted in Scheme 1(a) and Table 2. The nomenclature used here is a modified form of that described earlier[12] and the basic rules are as follows:

Table 2. Fragmentation of geranylgeranyl-containing ions

Fragment ion and theoretical mass (Da)	Relative abundance of product ions (%)[a,b] CV fragments[c]	CID of *m/z* 410.3	CID of *m/z* 394.3	CID of *m/z* 335.3	CID of *m/z* 290.3
V'_{16}	—	—	1	—	—
339.22			*12*	—	
AA''_{15}	—	—	—	—	—
326.22			*9*	—	
AA'_{15}	—	—	1	—	—
325.21			*13*	—	
a_{20}	29	—	1	5	5
273.26			*61*	*100*	
V'_{11}	25	—	—	—	—
271.16			*13*	—	
AA''_{10}	—	3[d]	—	—	—
258.15			*12*	—	
AA'_{10}		4[e]	—	—	—
257.15			*11*	—	
$''v_{16}$	9	—	1	—	1
217.20			*6*	*20*	
aa_{15}	14	3	2	4	3
205.20			*16*	*16*	
$''aa_{15}$	19	—	1	—	—
203.18			*11*	*30*	
V_6'	3	—	—	—	—
203.10			*11*	—	
v_{14}	8	2	2	1	1
191.18			*9*	*30*	
AA''_5	—	—	—	—	—
190.09			*19*	—	
$''v_{14}$	10	—	1	1.9	1
189.16			*26*	*17*	
AA_5'	4	—	—	—	—
189.08			*26*	—	
$'d_{13}$	1	3	1	4	3
177.16			*13*	*16*	
$'''d_{13}$	1	3	1	2	3
175.15			—	*13*	
$'d_{13}-14$	12	3	2	5	4
163.15			*33*	*25*	
$'''d_{13}-14$	12	3	1	2	2
161.13			*19*	*13*	
$''v_{11}$	26	7	6	11	7
149.13			*96*	*42*	
aa_{10}	28	57	17	22	16
137.13			*31*	*58*	
$''aa_{10}$	14	7	5	4	5
135.12			*46*	*49*	
V_1'	1	—	—	—	—
135.04			*46*	—	
v_9	17	5	6	8	5
123.12			*42*	*25*	
γ''	19	5	30	—	—
122.03			*100*	—	
$''v_9$	28	12	29	17	12
121.10			*52*	*73*	
$'d_8$	27	4	8	10	9
109.10			*33*	*20*	
$'''d_8$	24	9	9	14	12
107.09			*8*	*39*	
$'d_8-14$	25	11	14	20	16
95.09			*27*	*25*	
$'''d_8-14$		7	10	4	13
93.07			*40*	*25*	
$''v_6$		48	53	54	66
81.07			*87*	*86*	
α		7	9	—	—
74.02			*11*	—	
aa_5		100	100	100	100
69.07			*81*	*54*	
$'aa_5$		16	11	15	20
68.06			*13*	*30*	
$''aa_5$		19	13	20	23
67.05			*21*	*30*	
v_4		15	12	13	11
55.05					
$''v_4$		5	6	8	11
53.04					
$'d_3$		20	22	29	45
41.04					
$'''d_3$		—	3	4	6
39.02					
$'d_3-14$		—	2	3	5
27.02					
NH_4		7	6	—	20
18.03					

[a] Relative abundances of product ions in 400 eV CID spectra are shown in roman type. Spectra recorded in the *m/z* range 500–1.
[b] Relative abundances of product ions in the 4 keV CID spectra are shown in *italics*. Spectra recorded in the *m/z* range 500–60.
[c] Cone voltage fragment ions. Spectrum recorded in the *m/z* range 1000–100 at a resolution of 4500.
[d] Fragment ion appears at *m/z* 274.2.
[e] Fragment ion appears at *m/z* 273.2.

1. The capital letters *AA* and *V* are used to describe the nature of the bond broken with protonation formally of the cysteine amino group. When an allyl–allyl bond is broken, this is represented by *AA*, and a vinyl bond by *V*. In situations where charge does not reside on the cysteine amino group and fragmentation proceeds from the α-end of the lipid, lower case letters are used. When a double bond is broken, this is represented by *d*, a vinyl bond by *v*, an allyl bond by *a* and an allyl–allyl bond by *aa*.
2. A subscript to the right of a letter written in *italics* indicates the number of carbon atoms remaining in the lipid portion of the ion, e.g. a_{20}.
3. For a molecule M, the protonated molecule $[M + H]^+$ is represented as *M′*, where the prime to the right of the capital letter indicates that the ion has one hydrogen more than the molecular ion M^+. Alternatively, a double prime to the left of the letter representing a fragmentation process indicates that fragmentation has resulted in a fragment ion, e.g. $''v_n$, being deficient in two hydrogens, as compared with a fragment ion formed by a homolytic fragmentation at the same point in the molecular ion M^+. A prime to the right indicates the addition of H to the fragment.

In an attempt to determine the structures of the unknown compounds giving ions at *m/z* 410.3, 335.3 and 290.3, 400 eV CID spectra of these precursor ions were recorded. The fragment ions observed are listed in Table 2 and their origin are proposed in Scheme 1. The 400 eV CID spectra of precursor ions of *m/z* 410.3, 394.3, 335.3 and 290.3 are all extremely similar, showing almost identical fragment ions in the *m/z* range 27–273. The presence of ions at *m/z* 122 and 74 is characteristic of the cysteine residue and they are only observed in the CID spectra of precursor ions at *m/z* 410.3 and 394.3, while ions at *m/z* 18 corresponding to NH_4^+ ions are not observed from the precursor ion of *m/z* 335.3 The accurate mass of the precursor ion of *m/z* 335.3 corresponds to the

formula $[H(C_5H_8)_4SCH_2CH_3 + H]$ while the 400 eV CID spectrum indicates the presence of an $H(C_5H_8)_4$ group and the absence an amino group and the cysteine residue. The accurate mass of the precursor ion of m/z 290.3 is consistent with the formula $[H(C_5H_8)_4NH_2 + H]$. Again, the 400 eV CID spectrum indicates the presence of the $H(C_5H_8)_4$ group and the absence of the cysteine residue; however, as discussed above, the appearance of fragment ions at m/z 18 indicates that the precursor ion is an amine. The 400 eV CID spectrum of the precursor ion of m/z 410.3 is more difficult to interpret. Accurate mass measurements indicate that this ion has the formula $[H(C_5H_8)_4SCH_2CH(CO_2H)NH_2 + OH]^+$, and the CID spectrum suggests that oxidation of the double bond proximal to the cysteine residue has occurred. There is, in fact, a remarkable degree of homology between each of the 400 eV CID spectra and the ESCV fragmentation spectrum. In each CID spectrum a repeating series of aa, $''aa$, v, $''v$, $'d$, $'''d$, $'d - 14$, $'''d - 14$ and $''v$ fragment ions is observed, confirming the isoprenoid nature of the precursor ions. The above fragment ions are not formed by formal charge remote mechanisms, as is implicit by the absence of the basic amino group in the fragment ions.

There is excellent agreement between the theoretical and measured fragment ion masses for data obtained on the OATOF and only in the case of peaks of low intensity (<2% RA) was the error in excess of 0.05 Da. This degree of mass accuracy allows the confident assignment of the above fragment ions. In each of the 400 eV CID spectra the aa_5 (m/z 69.1) fragment ions were the most abundant. This is not unexpected as these ions correspond in mass to $[C_5H_8 + H]^+$ fragments which may originate from the ω-terminal isoprenoid moiety, or alternatively may be internal fragment ions.

CID spectra of the precursor ions of m/z 394.3 and 335.3 were also recorded at 4 keV E_{lab}. The 4 keV CID spectrum of GG-Cys ions of m/z 394.3 (Table 2) is more complex than that recorded at 400 eV (Fig. 2). At the higher collision energy charge-remote fragmentations becomes more important and short series of V' and AA' fragment ions are observed (Table 2). The presence of this second fragment ion series complicates the 4 keV spectrum, and in a number of cases ions from both series have the same nominal mass. Unfortunately, neither the resolution nor mass accuracy in the linked scan product ion spectra were sufficiently high to allow the composition of such fragment ions to be determined. As a result of this, studies at 4 keV E_{lab} were not pursued further.

In the present investigation, the aim was to investigate the ES mass spectrometry of a cysteinyl polyisoprenoid. As our synthetic sample was impure we also had the opportunity to use ES high-resolution accurate mass and tandem mass spectrometry in the structural determination of unknown polyisoprenoids. The results of this investigation are promising, and indicate that ES mass spectrometry should be ideal for the analysis of cysteinyl polyisoprenoids obtained from protein digests.

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Yours,

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