Journal of Chromatography, 87 (1973) 499–512

CHROM. 6984

GAS–LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF AMINO ACID THIOHYDANTOINS AND THEIR USE IN PROTEIN SEQUENCING

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(Received August 1st, 1973)

SUMMARY

The method of Stark for the sequential degradation of peptides from the carboxyl end involves the formation of peptidyl thiohydantoins. The suitability of amino acid thiohydantoins for gas–liquid chromatography was investigated and retention times of their trimethylsilyl derivatives on two stationary phases are reported. All derivatives except arginine gave satisfactory peaks and identification was possible with either OV-17 or Dexsil 300 GC stationary phases. Some derivatives gave two peaks. The mass spectra are given for sixteen thiohydantoins and twelve trimethylsilyl derivatives.

INTRODUCTION

There are well established methods for determining the primary structure of a protein by labelling the N-terminal amino acid[1,2]. The method of sequential degradation for determining the primary structure which was introduced by Edman[3] in 1949 was developed for routine use[4,5]. This involved the use of phenyl isothiocyanate reagent with subsequent cleavage and identification of the phenylthiohydantoin derivative formed by the N-terminal amino acid. The use of gas–liquid chromatography (GLC) for identification of this derivative was reported[6,7]. Waterfield and Haber[8] substituted methyl isothiocyanate for phenyl isothiocyanate and the methylthiohydantoin amino acid derivatives were identified by GLC[7–9].

Limited success has been obtained with carboxyl end group methods. Only the C-terminal amino acid was identified by hydrazinolysis[10] and tritium-labelling[11,12]. The method for sequential analysis using carboxypeptidases presented many problems[13–15]. Considerable progress was achieved when Stark[16] re-investigated the method originally proposed by Schlack and Kumpf[17] for the formation of C-terminal peptidylthiohydantoins. By specific cleavage the C-terminal amino acid was removed as its thiohydantoin derivative and was identified by thin-layer chromatography[16,18,19]. Our investigations reported here show that the separation and identification by GLC is possible. Mass spectrometry (MS) was used to confirm the identity of the GLC

peaks. The method of Stark has been developed for use with GLC for sequence analysis of proteins and these studies will be published.

EXPERIMENTAL

Materials

A Pye Series 104 gas chromatograph, Model 24, fitted with dual-flame ionization detectors (Pye-Unicam, Cambridge, Great Britain) was used with a Speedomax W 1 mV strip chart recorder (Leeds & Northrup, Birmingham, Great Britain). Nitrogen (99.9% "white spot"; British Oxygen Co., London) was used as carrier gas. Glass columns (3.25 m × 2.5 mm I.D.) were packed with HP Chromosorb W coated with stationary phase in a flat-bottomed dish as previously described[20]. Amino acids and chemicals were obtained from BDH Chemicals (Poole, Great Britain) except L-isoleucine (*allo*-free) (Calbiochem, London, Great Britain), L-arginine HCl, L-phenylalanine and N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma, St. Louis, Mo., U.S.A.), L-lysine mono-HCl and L-tryptophan (Koch-Light, Colnbrook, Great Britain), ammonium thiocyanate and potassium thiocyanate (Fison's Scientific Apparatus, Loughborough, Great Britain), Silicone OV-17 (Phase Separations, Queensferry, Great Britain) and Dexsil 300 GC and HP Chromosorb W 80–100 mesh (Field Instruments, Richmond, Great Britain).

Crystalline thiohydantoins of alanine, arginine, asparagine, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, dehydrothreonine, tryptophan, tyrosine and valine and a non-crystalline dehydroserine thiohydantoin were prepared as described[18]. Crystalline thiohydantoins of aspartic acid and 1-acetylserine and a non-crystalline proline thiohydantoin were prepared by the method of Yamashita[19], using KCNS instead of NaCNS.

Trimethylsilylation

About 1 mg of the thiohydantoin was silylated by the addition of 500 μl of a mixture of pyridine–bis(trimethylsilyl)trifluoroacetamide (1:1) and reacted at 50° for 10 min in a 2.5-ml tube with ground glass stopper. Aliquots of 1 μl were injected directly onto the GLC column.

Mass spectrometry

Mass spectra were measured on an AEI MS-30 mass spectrometer with a 70-eV ionizing beam (AEI, Manchester, Great Britain). The amino acid thiohydantoins were injected by using a direct insertion probe. The silylated thiohydantoins were injected onto a GLC column (3.25 m × 2.5 mm I.D.) packed with HP Chromosorb W 80–100 mesh coated with 5% (w/w) Silicone OV-17 coupled to the mass spectrometer. The reagent gas was at about 1 mm pressure and the source temperature at 250°.

RESULTS AND DISCUSSION

Amino acid phenyl- and methylthiohydantoins were gas chromatographed with silicone stationary phases[6–9]. Because the amino acid thiohydantoins are more polar satisfactory peaks could not be obtained with these phases. Fig. 1 shows the

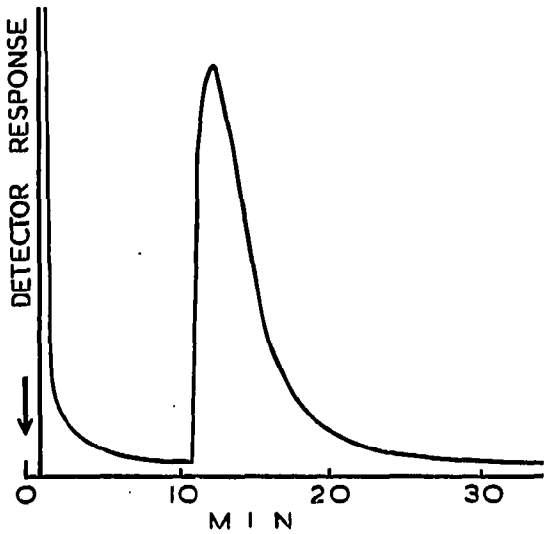


Fig. 1. Gas–liquid chromatogram of alanine thiohydantoin. Gas chromatograph, Pye Series 104, Model 24, dual FID. Glass column, 3.25 m × 2.5 mm I.D. packed with HP Chromosorb W 80–100 mesh coated with OV-17 (5% w/w). Oven temperature, 200°. Nitrogen carrier gas flow-rate, 30 ml/min. Attenuation, 1×10^{-9} A f.s.d. Arrow indicates time of injection of 1 μl sample.

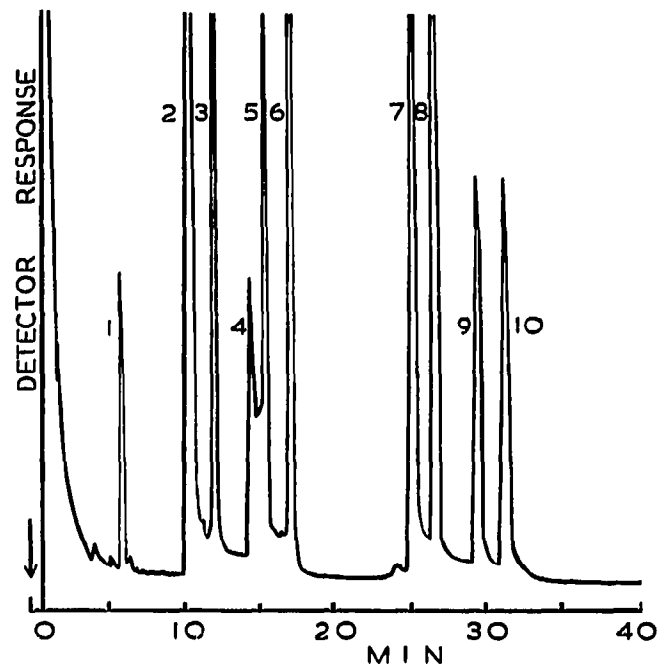
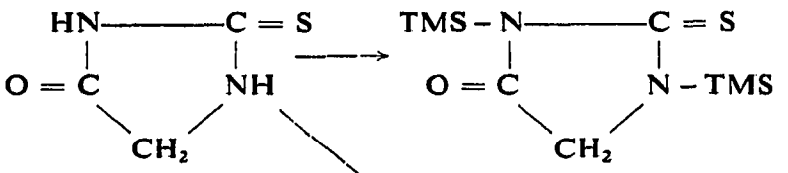


Fig. 2. Gas–liquidchromatogram of TMS-thiohydantoins of amino acids. Gas chromatograph, Pye Series 104, Model 24, dual FID. Glass column, 3.25 m × 2.5 mm I.D. packed with HP Chromosorb W 80–100 mesh coated with Dexsil 300 GC (1% w/w). Temperature programme, 130° × 2°/min to 230°. 1 = Bibenzyl; 2 = glycine; 3 = valine; 4 = threonine; 5 = threonine; 6 = glycine; 7 = aspartic acid; 8 = methionine; 9 = glutamic acid; 10 = phenylalanine.

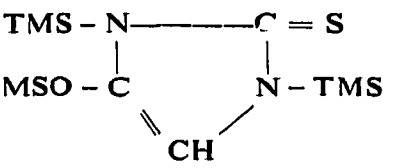
GLC tracing for alanine thiohydantoin chromatographed on a column with OV-17 stationary phase. This was typical of our attempts to chromatograph these compounds. They showed low plate efficiencies and peak tailing. Attempts to use GLC for the identification of thiohydantoins were reported to be unsuccessful[18].

The trimethylsilyl(TMS)thiohydantoin derivatives of amino acids gave sharp, symmetrical peaks and since they are usually more volatile compounds, a lower oven temperature was possible. In Fig. 2 seven amino acid TMS-thiohydantoins were chromatographed with Dexsil 300 stationary phase. Some of these amino acid thiohydantoins gave two GLC peaks after trimethylsilylation. Analysis of the peaks shown in Fig. 2 by GLC–MS (to be discussed later) confirmed that peak No. 2 was glycine N,N-bis(TMS)thiohydantoin and peak No. 6 was glycine N,N',O-tris(TMS)thiohydantoin. These were derived as follows:



Glycine thiohydantoin

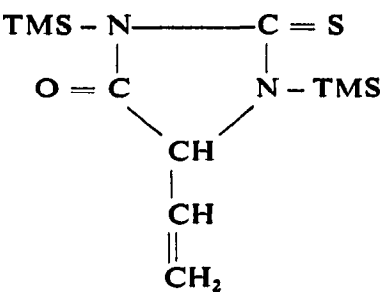
Glycine N,N',O-bis(TMS)thiohydantoin



Glycine N,N',-tris(TMS)thiohydantoin

It was shown[21] that the solvent used for trimethylsilylation of amino acids affected the number of peaks obtained. We tried solvents such as methylene chloride, chloroform, dichloroethane, hexane, heptane and dioxane, but these were discarded because of the insolubility of the thiohydantoins. Using dioxane as solvent only the GLC peak for glycine N,N'-bis(TMS)thiohydantoin was obtained. All other polar solvents tried gave two peaks with glycine. Dimethylformamide showed no advantage over pyridine.

After trimethylsilylation the serine and threonine thiohydantoins each gave two peaks on GLC, as shown for threonine in Fig. 2. By GLC–MS peak No. 4 was shown to be the di-TMS derivative as shown:



Dehydrothreonine N,N',-bis(TMS)thiohydantoin

Peak No. 5 corresponded to the mono-TMS derivative, but it was not established on which nitrogen of the thiohydantoin ring the TMS group was attached. We offer no formal proof that the serine and threonine thiohydantoins prepared by the methods quoted[18,19] were in the dehydro form as claimed by these authors. Dehydration could have occurred here as a result of the high temperature of the inlet chamber in the mass spectrometer.

When isoleucine thiohydantoin was trimethylsilylated, two GLC peaks of similar size were obtained, with little retention time difference, as shown in Fig. 3. Both peaks gave the same mass spectrum. This is consistent with the view that during the preparation of the thiohydantoin by the method described[18], when L-isoleucine in acetic acid was heated in the presence of a molar excess of acetic anhydride, racemization occurred at the α-carbon atom leading to the formation of a mixture of L-isoleucine and D-*allo*-isoleucine[22,23]. The reaction mechanism for this racemization was confirmed[24,25].

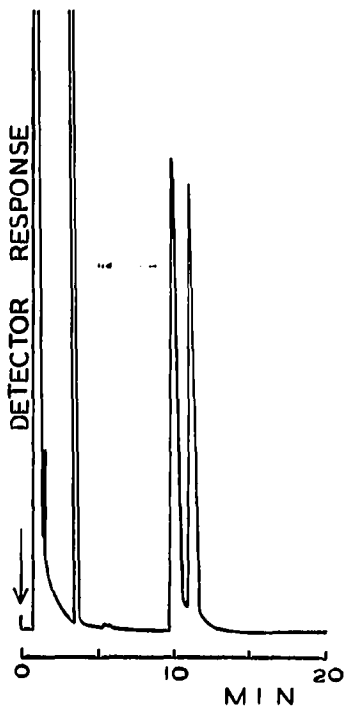


Fig. 3. Gas–liquid chromatogram of TMS-thiohydantoin of isoleucine. Instrument and conditions, as in Fig. 1, except oven temperature, 190°.

Because the protein amino acids led to the formation of TMS-thiohydantoins with a wide range of volatility it was necessary to use stationary phases with good stability at high temperatures. No stationary phase was found to separate all the protein amino acid derivatives. Both OV-17 and Dexsil 300 GC gave some good separations. Table I presents the retention times of 19 TMS amino acid thiohydantoins relative to those of bibenzyl and pyrene, which were used as internal standards. It was

TABLE I

RETENTION TIMES OF TMS AMINO ACID THIOHYDANTOINS RELATIVE TO BIBENZYL OR PYRENE TAKEN AS 1.0 ON TWO STATIONARY PHASES

The retention times for bibenzyl and pyrene (in minutes) are given in brackets for each temperature. Columns and conditions, as in Figs. 1 and 2.

Compound	*Dexsil 300 GC 1% w/w*	*OV-17 5% w/w*
	120°	*160°*
Alanine	1.95	1.26
N-Acetylcysteine	2.87	1.71
Glycine	2.49	1.57
Glycine	7.16	2.54
Isoleucine	4.35	2.07
Isoleucine	5.03	2.24
Leucine	4.54	2.15
Proline	1.04	1.05
Serine	1.94	1.25
Serine	2.45	1.75
Dehydrothreonine	4.34	2.53
Dehydrothreonine	5.11	3.54
Valine	3.07	1.58
Bibenzyl	(10.50)	(11.22)
	190°	*190°*
Asparagine	0.72	0.40
Aspartic acid	0.56	0.40
Glutamic acid	0.75	0.55
Methionine	0.64	0.50
Phenylalanine	0.94	0.81
Pyrene	(9.00)	(58.00)
	210°	*260°*
Histidine	2.26	1.43
N-Acetyllysine	3.04	1.28
Tyrosine	2.16	1.28
Tryptophan	—	4.48
Pyrene	(6.10)	(6.40)
	240°	
Tryptophan	5.37	
Pyrene	(3.00)	

possible to identify the thiohydantoin derivatives by GLC on one or other of these two columns. The characteristic two peaks obtained with glycine, isoleucine, serine and threonine also aided in their identification. Threonine methylthiohydantoin also gave two peaks[8].

Pisano and Bronzert[6] tried to analyse arginine phenylthiohydantoin by GLC but they were unsuccessful presumably because of the high polarity of the guanidino group. It was reported[26] that TMS-arginine decomposed on GLC columns with SE-30 stationary phase. Waterfield and Haber[8] were able to chromatograph arginine methylthiohydantoin only after prior reaction of the guanidino group with cyclohexanedione followed by trimethylsilylation. Gehrke and Leimer reacted arginine at 135° for 4 h

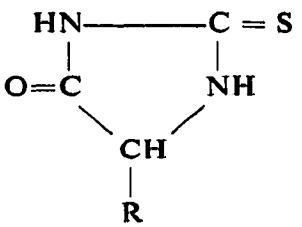
(ref. 21) or at 150° for 2.5 h (ref. 27) to obtain a fully trimethylsilylated derivative for GLC. In our work, arginine thiohydantoin was trimethylsilylated for 4 h at 135° in sealed tubes, but no peaks were found by GLC. Weygand and Obermeier[28] reported that a mass spectrum of arginine *p*-bromophenylthiohydantoin could be obtained only by starting with a very large amount of material. Because of its low volatility it was not possible to identify unequivocally this derivative from its mass spectrum. They claimed that it could be identified by converting it into the more volatile N^{δ}-4,6-dimethyl-2-pyrimidinylornithine *p*-bromophenylthiohydantoin.

The molar responses and retention times of both glutamic acid and glutamine phenyl- and methylthiohydantoins were reported[6,8]. Using *p*-bromophenyl-[28] and methylthiohydantoin[29] derivatives MS distinction was possible between asparagine and aspartic acid and between glutamine and glutamic acid. Table I shows that we were able to separate asparagine and aspartic acid thiohydantoin derivatives with Dexsil 300 GC but not with OV-17 stationary phase. We have attempted to prepare glutamine thiohydantoin, but trimethylsilylation always resulted in a GLC peak having the same retention time of TMS glutamic acid thiohydantoin. MS showed them to be identical. Suzuki *et al.*[30] reported that they were unable to prepare glutamine hydantoin.

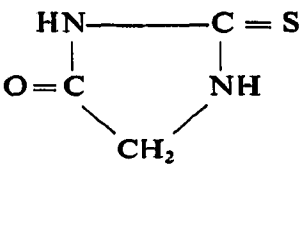
Mass spectrometry

The mass spectra of 7 thiohydantoin derivatives of amino acids were reported[31]. We now present the mass spectra of 16 of these derivatives and 12 derivatives obtained after trimethylsilylation under the conditions given under Experimental. There were only minor differences between our spectra and those published[31].

The mass spectra of the thiohydantoins (I) are given in Fig. 4. The fragmentation patterns show similarities and their study may be rationalized in terms of loss



(I)



(II)

of the substituent group R, followed by fragmentation of the ring, or by fragmentation of the ring with R still attached. The stability of the thiohydantoin ring (II) was demonstrated by its presence (M = 116) in the spectra of several of these derivatives. This ion with *m/e* 116 arose by a rearrangement process and the necessary condition for this to take place seemed to be the presence of a hydrogen on the carbon atom β to the ring. This would explain why this ion was not present in the spectrum of alanine but was found in those of valine, isoleucine, leucine, N-acetylcysteine, methionine and N-acetyllysine. Its absence from the spectrum of glutamic acid was possibly due to the proximity of the carboxyl group, which somehow prevented the rearrangement process.

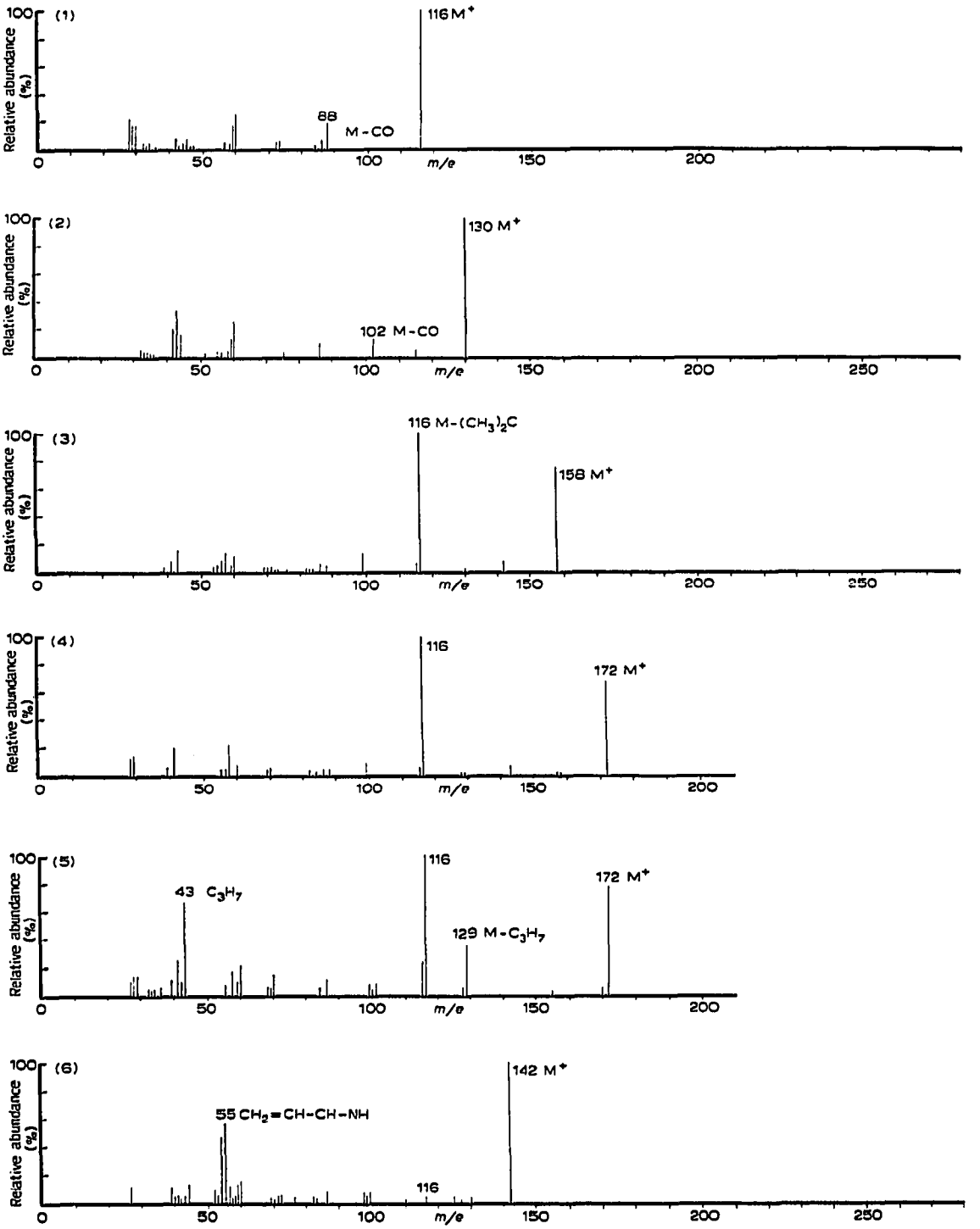
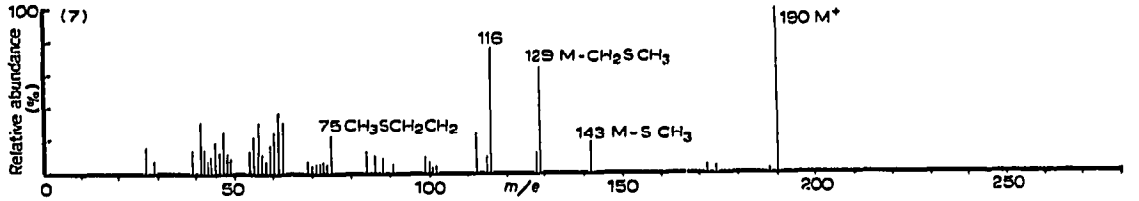
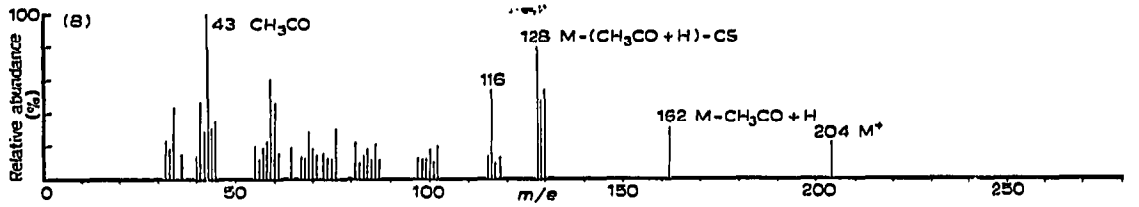
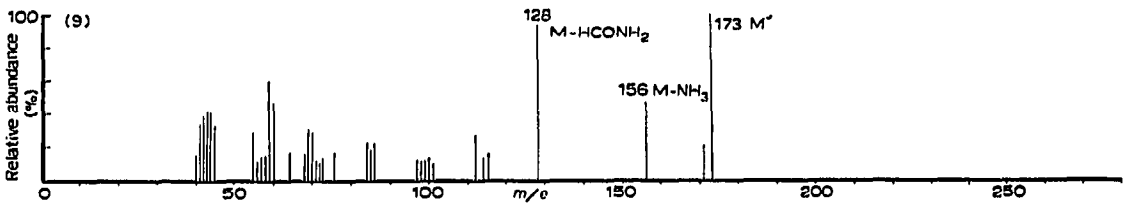
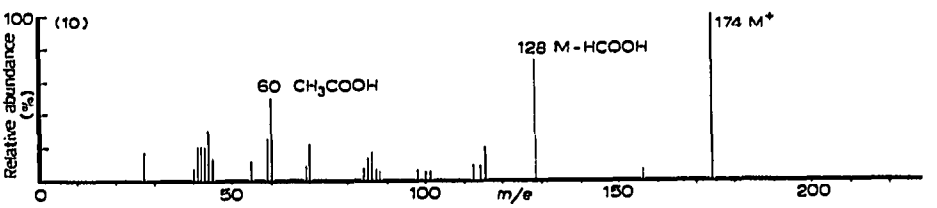

(1)
116 M+
88
M-CO
(2)
130 M+
102 M-CO
(3)
116 M-(CH3)2C
158 M+
(4)
116
172 M+
(5)
116
43 C3H7
172 M+
129 M-C3H7
(6)
142 M+
55 CH2=CH-CH-NH
116
Relative abundance (%)
m/e

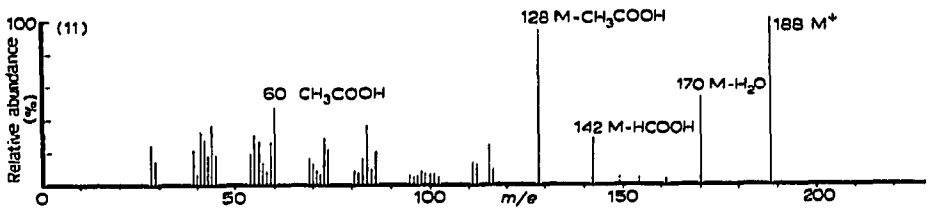
Fig. 4.

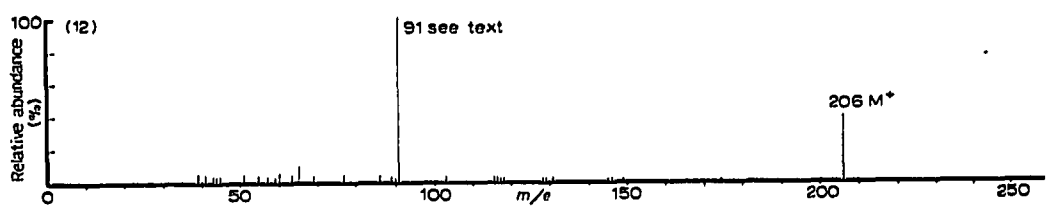












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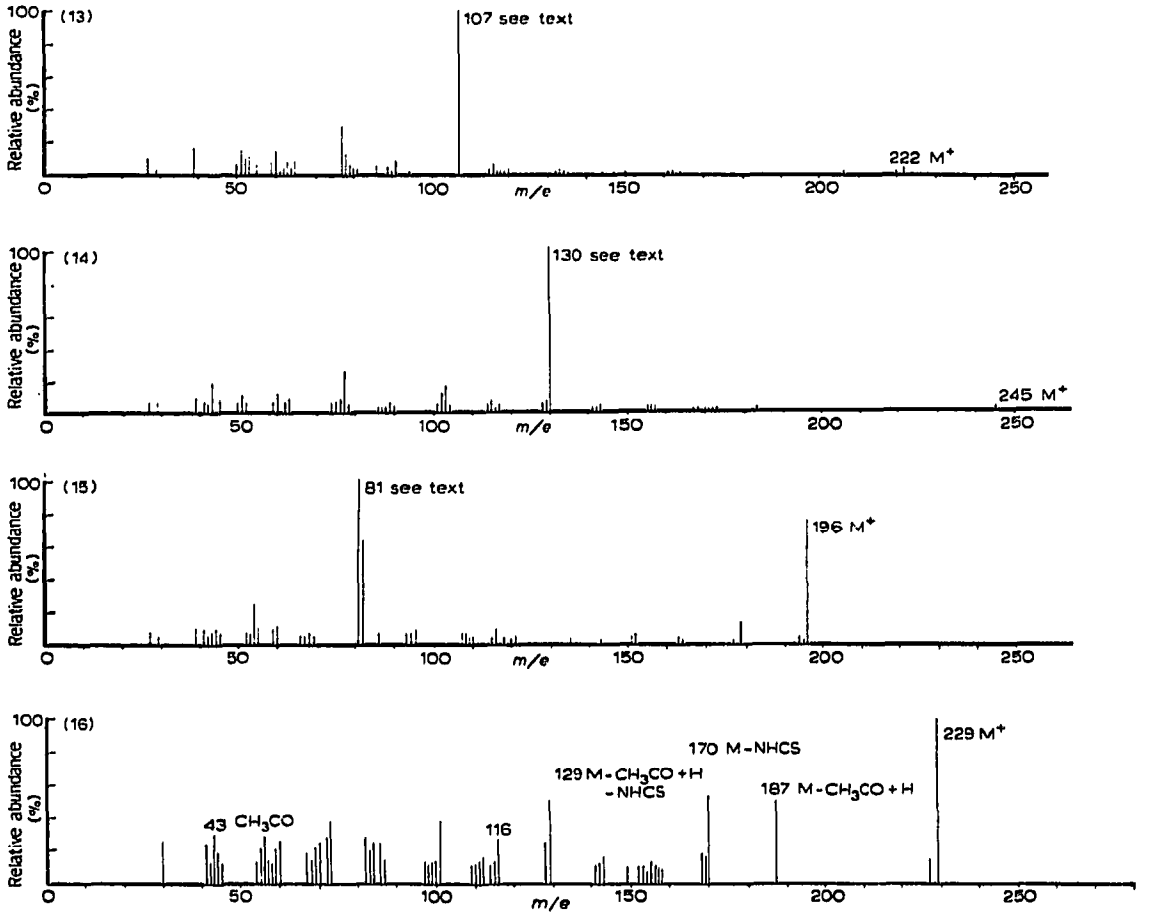

Fig. 4. Mass spectra of 16 amino acid thiohydantoins. 1 = Glycine thiohydantoin; 2 = alanine thiohydantoin; 3 = valine thiohydantoin; 4 = isoleucine thiohydantoin; 5 = leucine thiohydantoin; 6 = dehydrothreonine thiohydantoin; 7 = methionine thiohydantoin; 8 = N-acetylcysteine thiohydantoin; 9 = asparagine thiohydantoin; 10 = aspartic acid thiohydantoin; 11 = glutamic acid thiohydantoin; 12 = phenylalanine thiohydantoin; 13 = tyrosine thiohydantoin; 14 = tryptophan thiohydantoin; 15 = histidine thiohydantoin; 16 = N-acetyllysine thiohydantoin.

In each case the molecular ion was easily recognisable, and this showed a relative abundance greater than 67% of the base peak, except for phenylalanine, tyrosine and tryptophan. With these three compounds loss of the stable aromatic ring led to the much decreased relative abundance of the molecular ion. The ion corresponding to the substituent R was always present. When R was aromatic this became the base peak and its relative abundance may be attributed to the process of "ring expansion" which occurred as described for trifluoroacetylated aromatic amino acid esters[32]. In Fig. 4 the base peaks for phenylalanine, tyrosine, tryptophan and histidine were derived from the aromatic rings by "ring expansion" to give *m/e* ratios of 91, 107, 130 and 81, respectively.

The 12 derivatives shown in Fig. 5 contained up to three trimethylsilyl groups

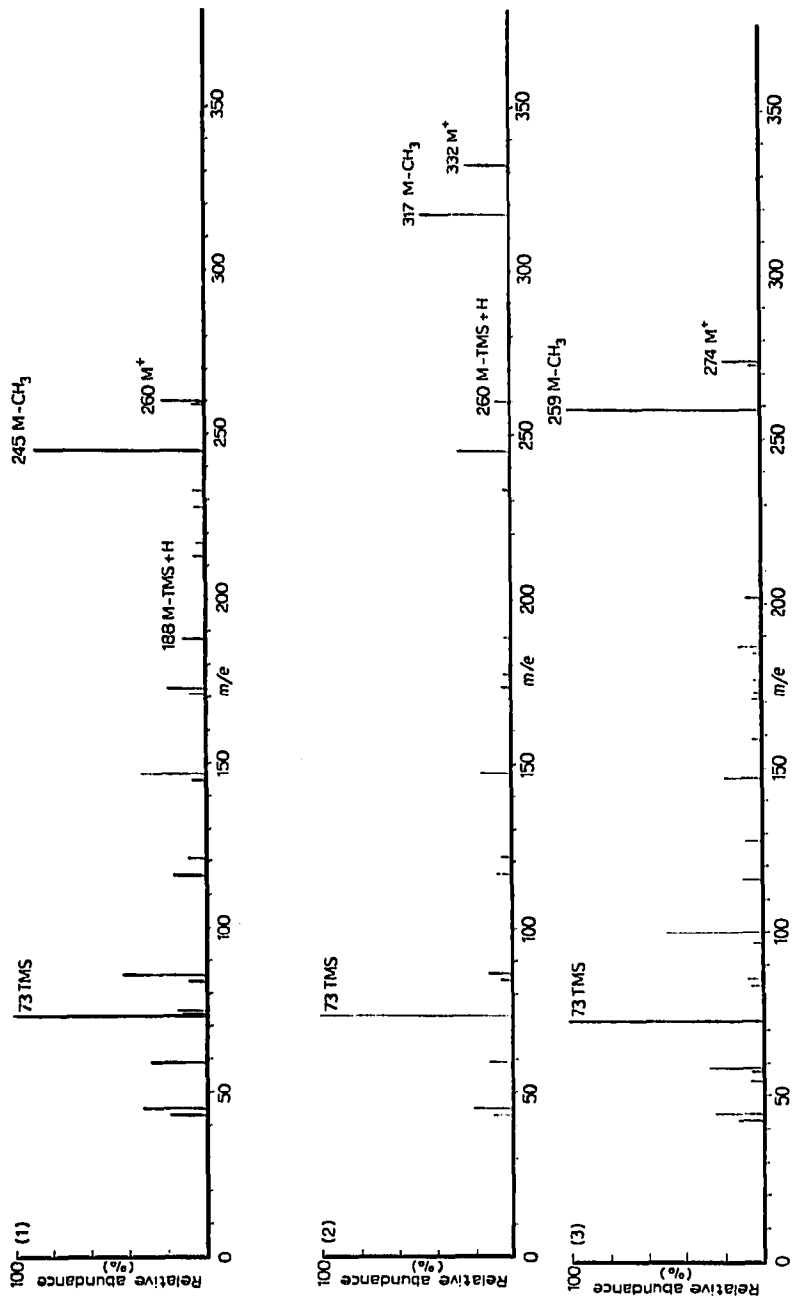

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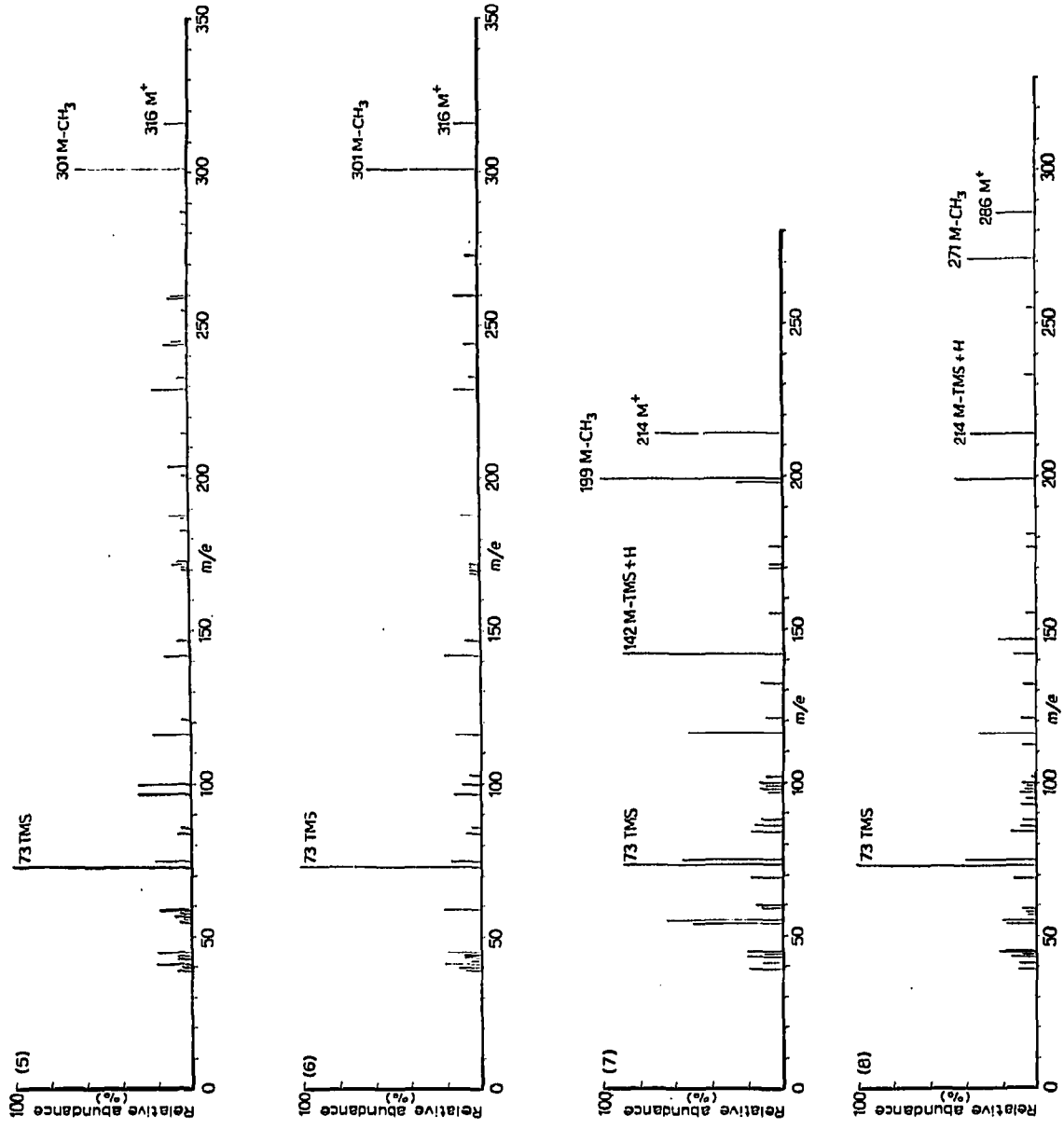
Fig. 5.


(5)
73 TMS
301 M-CH3
316 M+
(6)
73 TMS
301 M-CH3
316 M+
(7)
73 TMS
142 M-TMS+H
199 M-CH3
214 M+
(8)
73 TMS
214 M-TMS+H
271 M-CH3
286 M+
Relative abundance
(%)
m/e

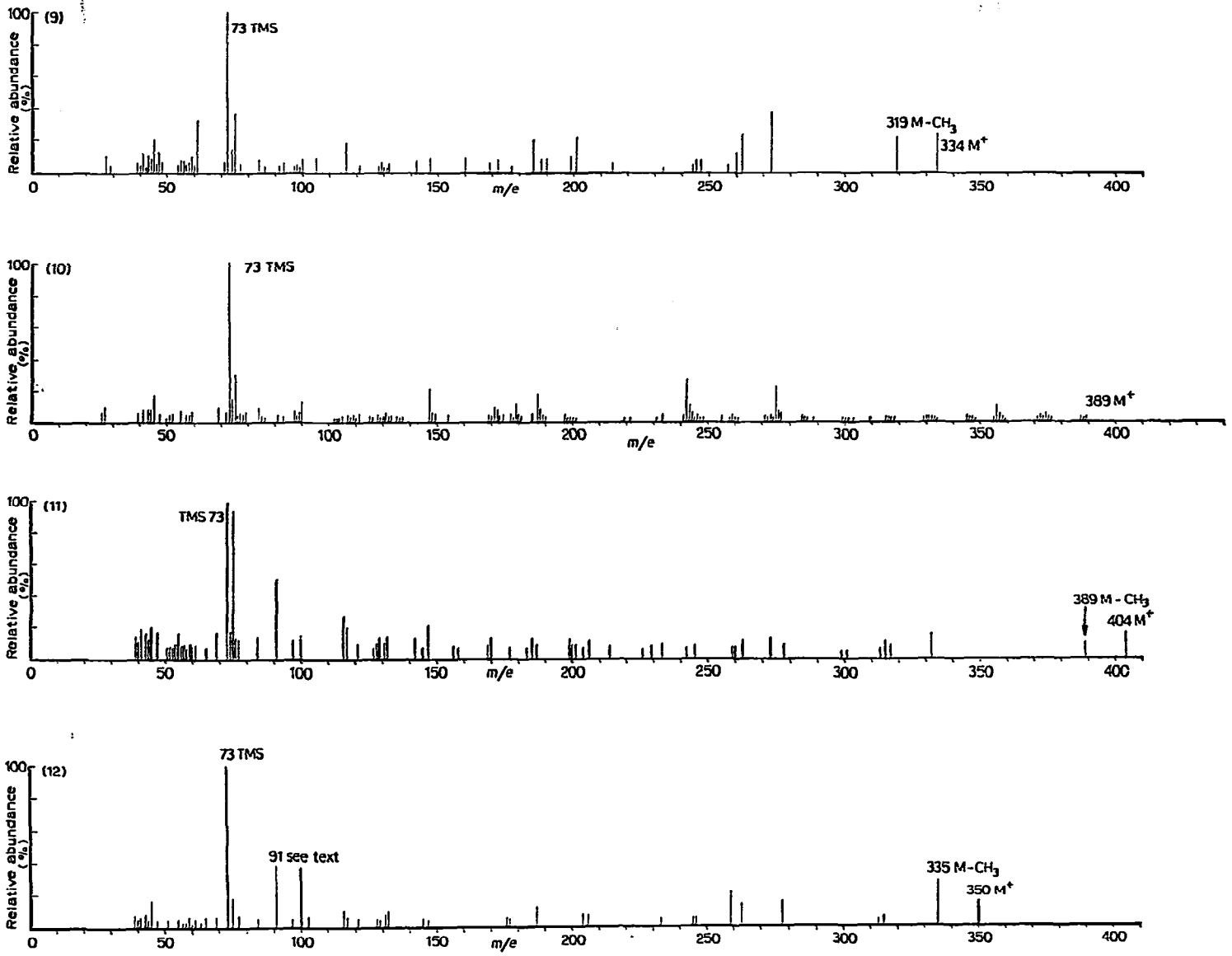


Fig. 5. Mass spectra of 12 TMS derivatives of amino acid thiohydantoins. 1 = Glycine bis-TMS thiohydantoin; 2 = glycine tris-TMS thiohydantoin; 3 = alanine bis-TMS thiohydantoin; 4 = valine bis-TMS thiohydantoin; 5 = isoleucine bis-TMS thiohydantoin; 6 = leucine bis-TMS thiohydantoin; 7 = dehydrothreonine mono-TMS thiohydantoin; 8 = dehydrothreonine bis-TMS thiohydantoin; 9 = methionine bis-TMS thiohydantoin; 10 = asparagine tris-TMS thiohydantoin; 11 = glutamic acid tris-TMS thiohydantoin; 12 = phenylalanine bis-TMS thiohydantoin.

but the mode of fragmentation was similar in each case. The molecular ion was easily recognisable, except for that of asparagine. A peak was always found with *m/e* ratio corresponding to loss of a methyl group from the molecular ion ($M^+ - 15$). Other peaks were derived by the loss of a TMS group and the migration of a proton from this to the thiohydantoin ring. This process was then repeated to give eventually the ion of *m/e* 116 which corresponded to the ring (II). The fragmentation pattern became complicated with the TMS-containing fragments, the elemental compositions of which were not further investigated. The peak with *m/e* 73 was due to the TMS group and this was the base peak with most samples.

ACKNOWLEDGEMENTS

A. D. wishes to thank the Medical Research Council, Science Research Council and Wellcome Trust for financial support. We are grateful to Professors H. R. V. Arnstein and A. J. B. Robertson for their interest and encouragement and to Professor V. Gold (Department of Chemistry, King's College) for use of the mass spectrometer.

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