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STRUCTURAL AND SEQUENCING STUDIES ON PEPTIDES, PROTEINS, AND GLYCOPEPTIDE ANTIBIOTICS BY MASS SPECTROMETRY

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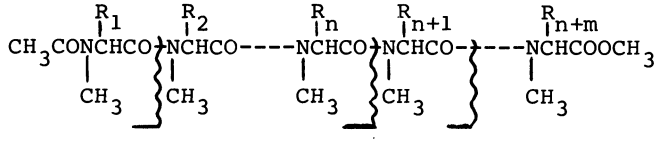
Abstract - Mass spectrometry is now established as a useful method for establishing the sequence of small peptides (2-10 residues), following their derivatisation (by N-acetylation and permethylation) to increase volatility. The strength of the method lies in its ability to deal with simple mixtures of peptides and to identify unusual amino acids. The weakness of the method (relative to some wet-chemical approaches to sequencing) lies in the current inability of the method to sequence larger peptides, and the relatively low sensitivity when working with peptides near the molecular weight limit of the method. This last shortcoming is due to our present inability to volatilise and efficiently produce high mass ions from the larger peptides, the inherent sensitivity of mass spectrometry being in contrast extremely high. The use of deuterated reagents in degradation and derivatisation reactions provides a powerful method for structure elucidation of glycopeptide antibiotics containing unusual amino acids.

INTRODUCTION

The work to be described in this paper falls naturally into two parts. The first deals with the sequence determination of peptides derived from proteins, and the second with structural studies on some glycopeptide antibiotics. Both of these areas of research require appropriate ionisation methods and chemical derivatisation techniques, and the current approaches to these problems will therefore be described first. Computer based approaches to peptide sequencing by mass spectrometry will be covered in another part of the symposium.

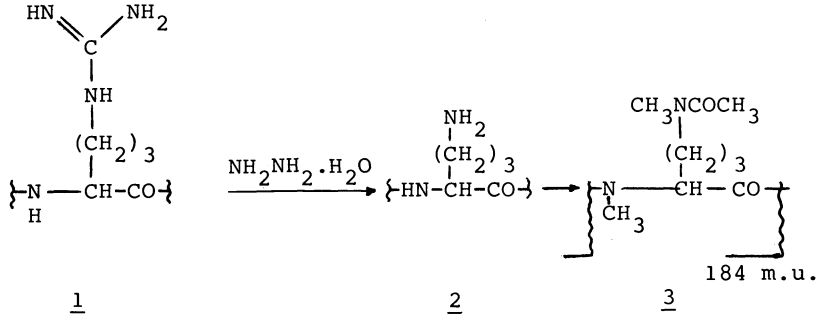
CHEMICAL DERIVATISATION METHODS

The most widely used method of derivatisation for the sequence determination of protein-derived peptides is that of N-acetylation (acetic anhydride/methanol) (Ref. 1) followed by permethylation ($^-CH_2SOCH_3$ in dimethyl sulphoxide, and subsequent treatment with a large excess of methyl iodide) (Ref. 2&3). Each derivatised amino acid has a characteristic mass (with the exception of the isomeric pair leucine and isoleucine). Cleavage of the derivatised peptide at the peptide bonds (as shown below), following ionisation upon electron impact, occurs such that the charge is retained by the N-terminal fragment. Ions formed in this way are known as sequence ions. The mass difference between sequence ions formed by cleaving the n^{th} and $(n+1)^{th}$ peptide bonds identifies the $(n+1)^{th}$ amino acid from the N-terminus. The N-terminal amino acid is recognised by the presence of an ion corresponding to its characteristic mass plus 43 m.u. (CH_3CO).



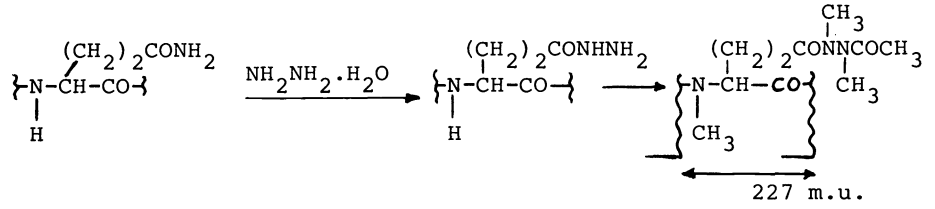
The C-terminal residue can often be recognised by the presence of molecular ion ($M^{+\cdot}$) and M^{+}-15 peaks occurring 31 (OCH_3) and 16(O) m.u. above the sequence ion. The method is currently limited to peptides containing a maximum of about 10 amino acids.

If sequence information is to be obtained from peptides containing His, Met and carboxymethyl-Cys, then "short" permethylation conditions must be used (Ref. 4), whereby the permethylation reaction is quenched with water after 1 minute. It is believed that in the case of the sulphur-containing amino acids, the short permethylation reaction avoids the formation of polar sulphonium salts. Peptides which show a positive stain for arginine (1) are first subject to mild hydrazinolysis (treatment with hydrazine hydrate at 75°C for 15 min.) (Ref. 5). This treatment causes cleavage of the guanidine group of arginine, without significant cleavage of amide bonds in the peptide. The resulting ornithine residue (2) is then modified by acetylation and permethylation in the normal manner (2→3), and after derivatisation is characterised by an in-chain mass of 184 m.u.



1 2 3

It should be noted that if the peptide (or mixture of peptides), treated in the above manner, contains asparagine or glutamine, then the amide side-chain of such residues will be modified as follows during derivatisation e.g. for glutamine, (Ref 6):



The ease with which this variation was uncovered by mass spectrometry (Ref. 6) attests to the power of the method in determining the presence of novel amino acids in peptides, as will be illustrated subsequently.

When ambiguities arise in the interpretation of the mass spectrum, they can often be resolved by the use of perdeuteroacetic anhydride and/or trideuteromethyl iodide in place of the normal reagents. Thus, the mass increment associated with derivatised lysine is 198 m.u.; however, if sequencing is being carried out from the N-terminus in the normal way (Ref. 7), then the interposition of a mass increment of 71, 85, 113, or 127 m.u., would also be consistent with the sequences Gly Leu, Ala Val, Val Ala, or Leu Gly. These possibilities can obviously be differentiated from a Lys sequence ion by the use of perdeuteroacetic anhydride in derivatisation, which causes the mass shift 198→201 in the case of lysine only.

IONISATION METHODS

Electron impact mass spectrometry at low pressure (ca. 10^{-6} mm. Hg) is still the most widely used method, but negative and positive ion chemical ionisation (NICI and PICI) and field desorption (FD) mass spectrometry are becoming of increasing importance.

A major technical breakthrough, which appears to have relevance to protein sequencing by mass spectrometry, is one which allows simultaneous recording of positive ion and negative ion chemical ionisation (PICI and NICI) mass spectra (Ref. 8). In general, negative ion mass spectrometry has hitherto not been popular in the area of structure elucidation, because of poor sensitivity. Conventional electron impact sources produce high energy electrons, and the probability of resonance capture by a sample molecule A to give a molecular anion-radical is extremely low:-

$$A + e \longrightarrow A^{-\cdot}$$

This process requires electrons with very low translational energies. It has now been realised (Ref. 8) that when a mass spectrometer operates under chemical ionisation conditions, a large population of electrons having near thermal energies is produced. Such electrons are produced because, in chemical ionisation, a reagent gas (e.g. methane) at a pressure of ca. 1 Torr is ionised by high energy electrons (100-500 eV), and each ionisation event will reduce the electron energy on average by ca. 16 eV. The electrons can also lose translational energy by collisions with neutral gas molecules, until eventually a near-thermal population is produced. Negative ions are then produced with such efficiency that, in favourable cases, a 10^2-10^3 increase in negative ion sample current over its positive ion counterpart can be achieved (Ref. 8).

It has been shown that PICI and NICI spectra can be simultaneously recorded by using a quadrupole mass spectrometer. This has been achieved by pulsing the polarity of the ion source potential (± 1-10 volts) and focusing-lens potential (± 10-30 volts) at a rate of 10 kHz (Fig. 1). Thus, "packets" of positive and negative ions are ejected from the ion source in rapid succession. Both positive and negative ions are analysed with equal facility by the quadrupole field, and leave the analyser at the same point (although simultaneous analysis of positive and negative ions is of course impossible in a magnetic sector instrument). The positive and negative ion beams are separately recorded by two electron multipliers, placed side by side, with the potentials on the first dynodes being 1200 to 2000 volts and opposite in sign (Fig. 1). Consequently, positive and negative ions are deflected in opposite directions to their respective multipliers and simultaneous PICI and NICI spectra conveniently recorded.

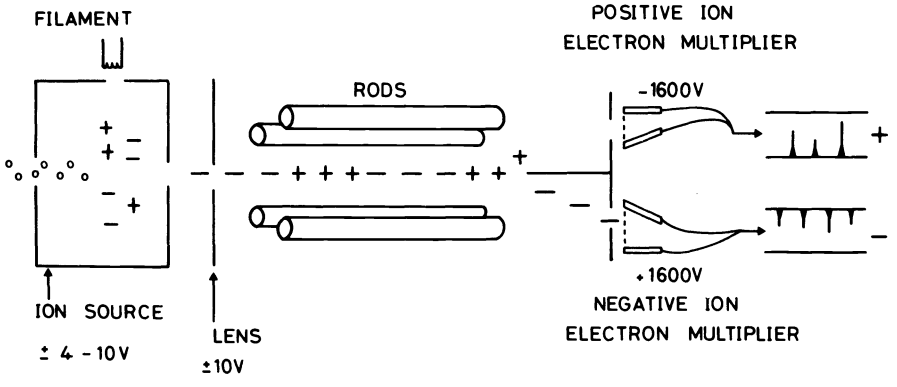


Fig. 1. Schematic illustration of a quadrupole mass spectrometer modified for simultaneous recording of positive and negative ion spectra (ref. 8).

Although the possible advantages of such techniques in peptide sequencing have not yet been fully explored, the experiments performed so far (Ref. 8) look encouraging. In Fig. 2 the simultaneously recorded PICI and NICI spectra of N-acetylated permethylated MetGlyMetMet are reproduced (Ref. 8).

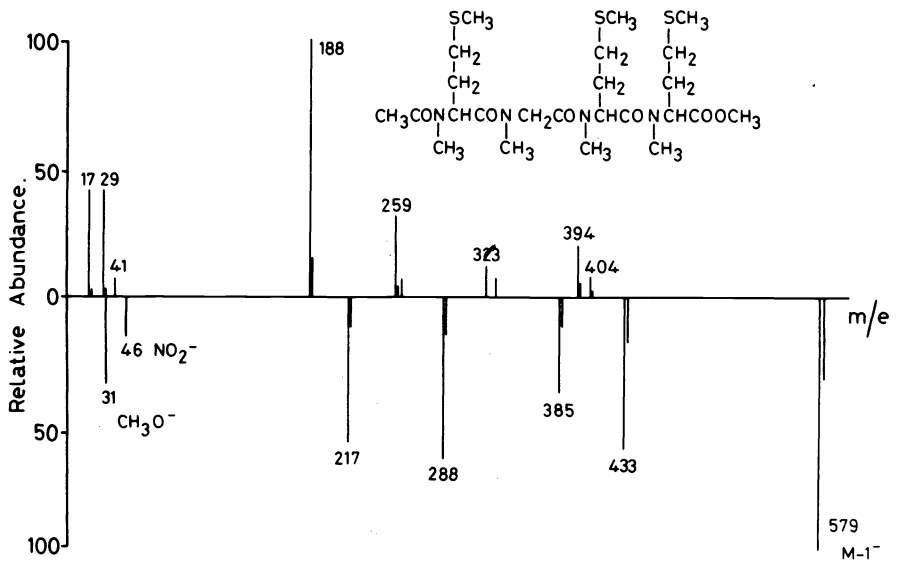
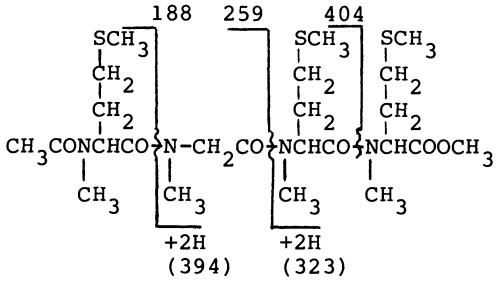


Fig. 2. (a) PICI and (b) Bronsted base NICI spectra of the N-aceylated permethylated peptide MetGlyMetMet (Ref. 8).

The PICI spectrum is of the form expected from earlier work (Refs.9 & 10), showing both N-terminal sequence ions (m/e 188, 259 and 404) and C-terminal sequence ions (m/e 323 and 394).

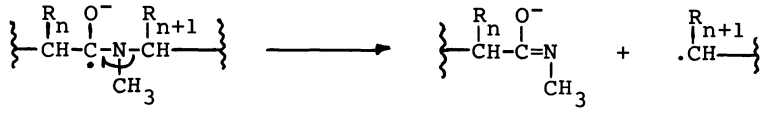


However, a protonated molecular ion of this peptide (m.wt. 580) is not obtained in the PICI spectrum (which was generated using CH_4 as reagent gas). In contrast, the NICI spectrum contains an abundant M-1 ion (Fig. 2). The generation of such an ion was promoted by doping the methane chemical ionisation gas with a small amount (<0.1%) of methyl nitrite (Ref. 8). Methyl nitrite forms the strong base CH_3O^- by a dissociative electron capture reaction (Ref. 11), which can then produce M-1 ions of characteristic molecular weight, by proton abstraction from sample molecules AH.

$$CH_3ONO + e \longrightarrow CH_3O^- + NO$$

$$A + CH_3O^- \longrightarrow A^- + CH_3OH$$

Moreover, if the population of thermal electrons is not totally depleted by methyl nitrite (achieved by maintaining it at appropriately low concentration), then electron capture by the peptide produces a molecular anion-radical which dissociates into the enolate anion of an amide and a radical.



These anions constitute useful sequence ions in the NICI spectrum, and occur at higher mass by 29 n.u. relative to their positive ion counterparts (owing to the incremental NCH_3 group). Such ions are evident in Fig. 2 at m/e 288 and 433, and support the sequence assignments Met-Gly and Met-Gly-Met, respectively. The absence of the Met sequence ion at m/e 217 has been rationalised (ref. 8) in terms of the higher energy requirement for a primary radical loss (glycine being the next residue.

Since NICI spectra may be sufficiently "clean" to render a manual "counting calibration" of the spectrum impossible or inconvenient, it appeared desirable to obtain NICI spectra in a magnetic sector instrument either with (i) a data system or (ii) a double-beam instrument. In the latter approach (Ref. 12), electron-capture by perfluoro compounds may be so efficient that electron impact operation in one beam at pressures only slightly higher than normal ($\sim 10^{-5}$ mm Hg) can be used to provide a reference spectrum. Thus, perfluorotributylamine affords, under these conditions the main negative ion (electron impact) peaks shown in Fig. 3. The second source is then operated under NICI conditions; for example, the N-acetyl S-2,4-dinitrophenyl derivative of the methyl ester of cysteine yields the NICI spectrum reproduced in Fig. 4 (Ref. 12).

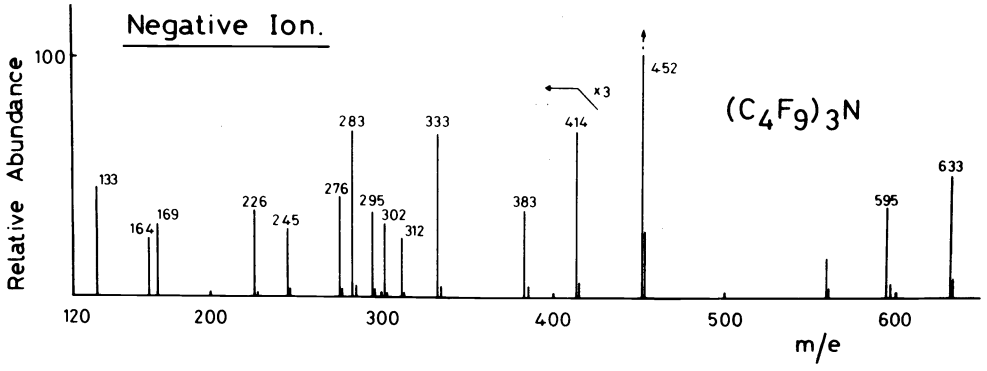


Fig. 3. Negative ion electron impact spectrum of perfluorotributylamine recorded at a source pressure of ca. 10^{-5} mm Hg.

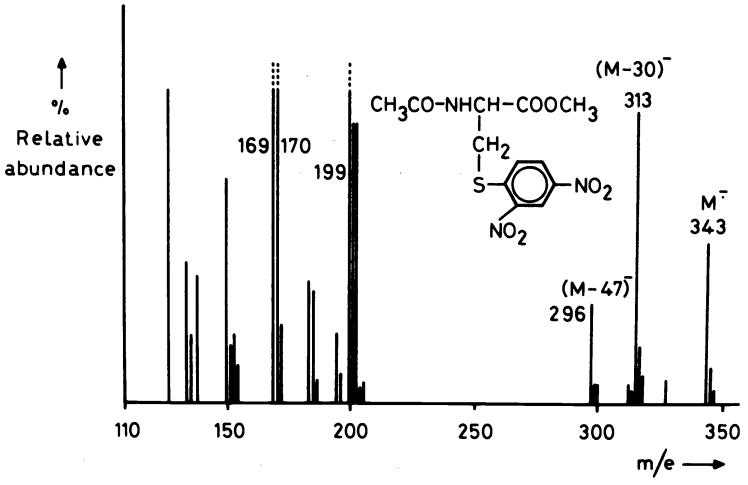


Fig. 4. NICI spectrum of the N-acetyl S-2,4-dinitrophenyl derivative of cysteine (Ref. 12).

SEQUENCE DETERMINATION OF PEPTIDE AND PROTEINS

A strategy for the sequence determination of proteins by mass spectrometry has been described previously (Ref. 3), and this strategy has been successfully applied to determine some 80% of the sequence of ribitol dehydrogenase (Ref. 6), (m.wt. ≃ 27,000

for each of four identical subunits). However, the sequences determined are largely those of tri- to nona-peptides, with an average size of about a hexa-peptide. Consequently, many overlaps would be required to give a complete sequence and to date, insufficient have been forthcoming. Thus, the largest sequence created by overlaps was no more than 19 amino acids units long (Ref. 6). Hence mass spectrometry is not suited at its present stage of development to complete sequence determination, but rather to rapid screening to give partial structure information. In this area, it is therefore best used in conjunction with dansyl-Edman (Ref. 13), and sequenator methods (Refs. 14&15). In the dansyl-Edman method, the differentiation of Asp and Asn, or Glu and Gln (these pairs having the difference -COOH vs $-CONH_2$ in their side-chains) can be tedious, since production of the dansyl amino-acid from the N-terminus by hydrolysis also causes the conversion $CONH_2 \rightarrow COOH$ in the amino-acid side-chains. Therefore, additional electrophoresis experiments on peptides are necessary to differentiate the acidic and neutral side-chains. Mass spectrometry obviates such tedious experiments since the masses of the derivatised side-chains are characteristically different.

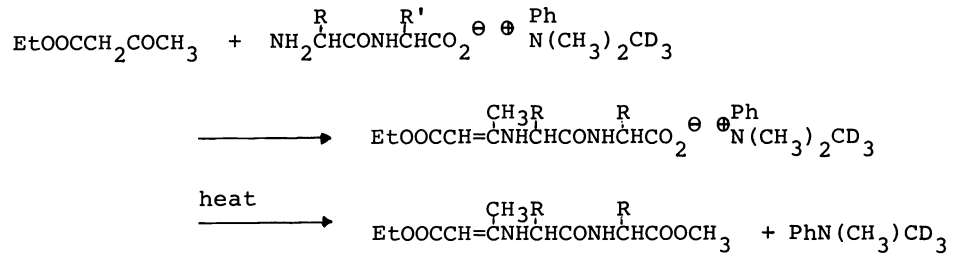
The above general conclusions are supported by current mass spectrometric sequencing studies (Ref. 16) on a manganese-containing superoxide dismutase form B. stearothermophilus (Ref. 17) (two identical sub-units each of 185-190 amino acids). The superoxide dismutase enzymes are ubiquitous among oxygen metabolising organisms, and are responsible for the dismutation of superoxide radicals into peroxide and molecular oxygen.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

A pepsin digest of the enzyme, followed by gel filtration on Sephadex (to give fractionation of the peptide products according to molecular weight), gave fractions which were sensibly pooled and examined by paper electrophoresis. Simple mixtures of small peptides (di- to deca-peptides) were washed from the paper, derivatised, and examined in the mass spectrometer, using a temperature gradient in the source to separate partially peptides of differing volatility. The one pepsin digest gave sequence information of ca 50 amino acids, the average chain length corresponding approximately to tetra-peptides (Ref.16).

A strategy for obtaining sequence information for a polypeptide digested by dipeptidylaminopeptidase I (DAP-I) has been reported (Ref. 18). This strategy builds extensively on some previous work in this area (Refs. 19-21). DAP-I cleaves polypeptides from the N-terminus at alternate amide bonds, yielding a mixture of dipeptides. If in a second experiment, the N-terminal amino acid of the polypeptide is removed (by the Edman procedure), a further DAP-I digest leads to a new set of dipeptides. The identification of all the dipeptides in the two mixtures will in principle permit the sequence determination of the polypeptide, although repetition of a specific amino acid residue can cause difficulties.

In the most recent work (Ref. 18), polypeptide digests with DAP-I were carried out at the 0.1 μmol level, and the resulting mixture of dipeptides derivatised by reacting the N-terminal amino group with ethyl acetoacetate. The reaction mixture for this step also contains dimethyltrideuteromethyl anilinium hydroxide which methylates carboxylic acid groups when the sample is heated on a direct insertion probe inside the mass spectrometer. Apart from the reaction shown below, the methylated peptides will, at any one site, obviously incorporate CD_3 groups to the extent of approximately 33%.



Although complete details of this derivatisation procedure will not be discussed here, it is noteworthy that the side-chain functional groups of Tyr, Asp, Glu and carboxymethylcysteine (CmCys) are methylated, while Trp and His are incompletely methylated in the ring.

Chemical Ionisation mass spectra were obtained using He gas at 0.8-1.0 Torr, and showed intense N-terminal fragment ions and, in general, protonated molecular ions of similar intensity (e.g. Fig. 5).

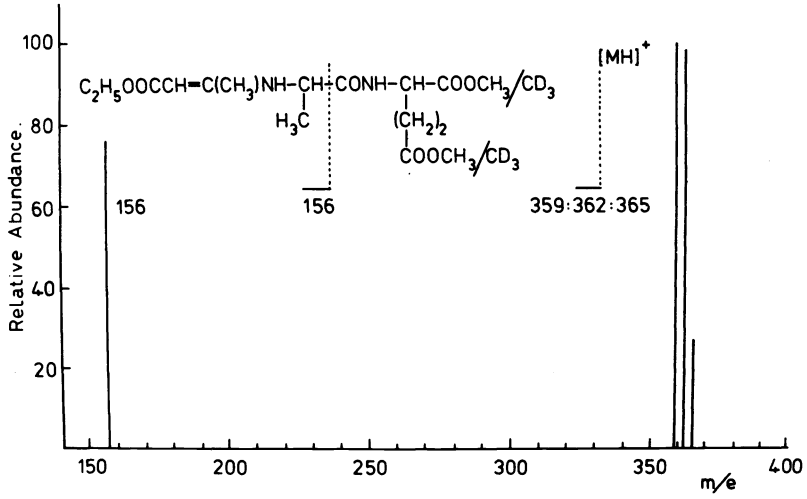
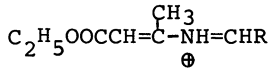


Fig. 5. The C.I. (helium) mass spectrum of ethoxycarbonyl-propenyl-AlaGlu dimethyl ester (partially deuterated) obtained by pyrolytic esterification (Ref. 18).

Note that the N-terminal fragment ions do not correspond to the acylium ions which are dominant in electron impact spectra of N-methylated peptides, but rather to imminium ions 4.



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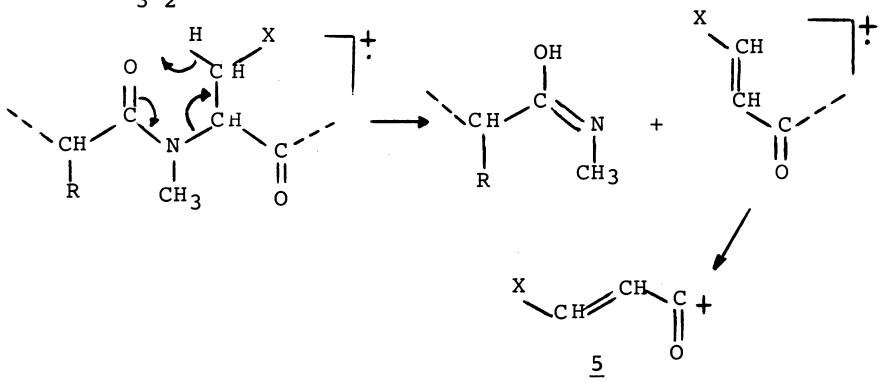
The spectrum reproduced in Fig. 5 clearly establishes dimethylation of the C-terminal residue from the 4:4:1 intensity of the $M+H^+$ ions at m/e 359, 362 and 365. The $M+H^+$ ions are obtained owing to the presence of a proton donor in the source. Thus, water present in the helium gives rise to the proton donor H_3O^+.

Since the spectra are so simple, it is possible to deal directly with the mixtures of dipeptides. The two sets of identified peptides (from the initial DAP-1 digest and the second DAP-I digest following Edman degradation) are matched in such a way that every C-terminal amino acid of one digest corresponds to an N-terminal amino acid in the other digest, and the sequence of the polypeptide can be deduced. The overall strategy has been carefully worked out, and used to sequence a number of small polypeptides (3-8 residues) (Ref. 18). Although the approach is elegant, a problem seems to be that, since

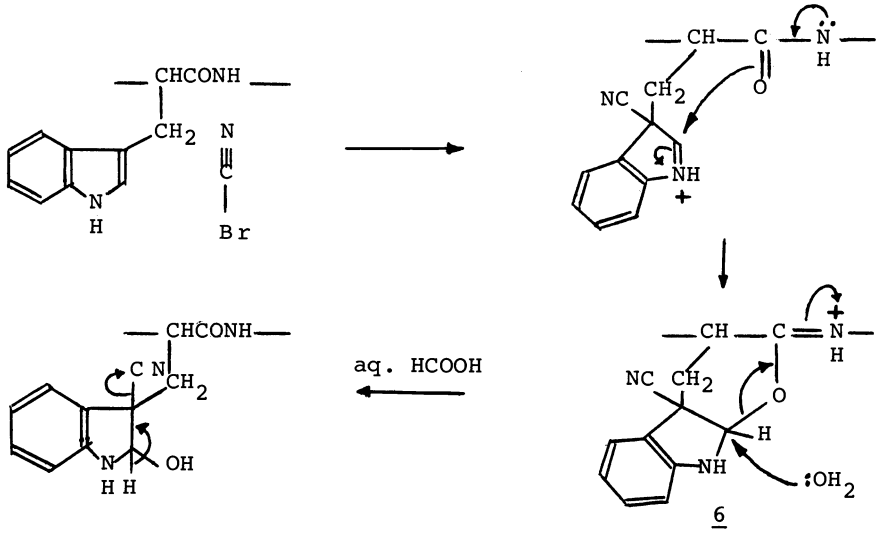
very small fragments are generated, a large amount of reconstructional logic is necessary to establish the sequence of even an octapeptide.

As indicated earlier, the strength of mass spectrometry in this area lies in identifying novel amino acids, and in analysing mixtures. For example, mass spectrometry was used in the recognition of ten γ-carboxyglutamic acid residues in the N-terminal region of prothrombin, (Ref. 22 and 23) and in identifying the enkephalins (the brain's natural opiates) as a mixture of the two penta-peptides TyrGlyGlyPheMet and TyrGlyGlyPheLeu (Ref. 24). A further example is found (Ref. 25) in the identification of an oxidised tryptophan residue in a cyanogen bromide digest of an azurin (specifically, a small respiratory copper protein from the bacterium Pseudomonasfluorescens biotype G). After derivatisation, one fraction from a Sephadex column exhibited electron-impact sequence ions (N-terminal) at m/e 126, 370, 483, 610 and 739. The relatively high abundance of the m/e 126 ion suggested that this ion was characteristic of the presence of in-chain Asn, from which m/e 126 (5, X=$CON(CH_3)_2$) is produced as shown below.



5

This type of in-chain cleavage also occurs for other amino acids which contain β-hydrogen atoms which are activated towards transfer (see above), (Ref. 6), and the ions 5 so produced then serve in lieu of N-terminal ions as a starting point to deduce sequence information. However, in the case cited above, the mass difference between m/e 126 and 370 (244 m.u.) did not correspond to a mass difference associated with one of the usual amino acids. These peaks were not shifted on deuteroacetylation but after permethylation with perdeuteromethyl iodide occurred at m/e 132 and 385. Hence, three methylatable sites must be accommodated in the mass interval m/e 126→370, and the data appear to be consistent only with occurrence of hydroxytryptophan as the residue following Asn. The sequence is therefore AsnHOTrpValLeuThr. The previously unreported oxidation of a Trp residue in the course of the cyanogen bromide digest could occur as follows (the digest being carried out in aqueous formic acid).



6

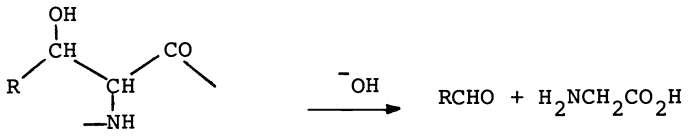
Evidence to support the intermediacy of 6 is found from the presence of the peptide ValLeuThrSerAlaAlaAsnHomoSer in the same cyanogen bromide digest (Ref. 25). Thus in the protein, the sequence .... AsnTrpValLeuThrSer.... has been cleaved at the N-terminal side of Val, notwithstanding the reported (Ref. 26) specificity of CNBr for cleaving only at methionine linkages. The observed cleavage is readily understandable in terms of a hydrolytic split of the imminium linkage in the proposed intermediate 6.

The sequencing of permethylated peptides by mass spectrometry after their separation by GLC has recently been described (Ref. 27). Permethylated peptides were eluted from a 0.3 m glass column of 2mm internal diameter, using helium as a carrier gas at 330°C. Columns packed with OV-1 (a simple dimethylsilicone) permitted the transmittance and subsequent sequencing of a number of tetra- and penta-peptides (after acylation and permethylation), present at the 15-30 nmol level in the source after injection of quantities in the 100-150 nmol range.

STRUCTURAL STUDIES ON GLYCOPEPTIDE ANTIBIOTICS

This section will deal briefly with some aspects of work in our own group on the structures of the antibiotics vancomycin, ristocetin A and ristomycin A. Although these antibiotics are available in quantity, structural studies on them have made slow progress, largely because they contain unusual amino acids which are linked together in polycyclic structures (Refs. 28-30). Their structure elucidation therefore presents an interesting challenge to current methods, especially since some aspects of the molecular basis for their physiological action are known (Refs. 31,32).

An early observation in structural studies on vancomycin was that basic hydrolysis of vancomycin gave ca. 2 molar equivalents of glycine, although this substance was not produced in significant amounts from acid hydrolyses (Ref. 33). We therefore considered the possibility that the glycine might arise from two β-substituted serine units (7) via a base-catalysed cleavage reminiscent of the retro-aldol reaction.



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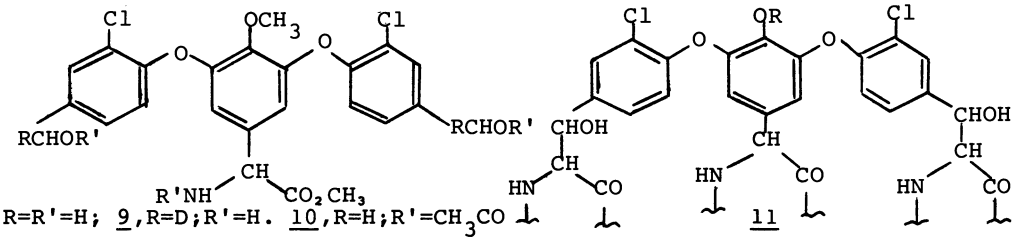
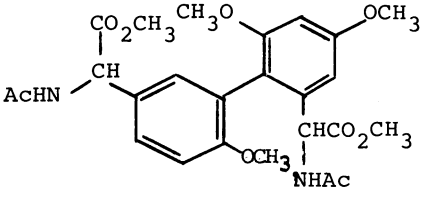
The putative aldehyde groups could not be characterised in the products of simple basic hydrolysis although this was not too surprising in view of the severe reaction conditions necessary to bring about hydrolysis. Basic hydrolyses were therefore carried out in the presence of sodium borohydride, to reduce in situ any carbonyl groups produced via fragmentation. When aglucovancomycin methylated with diazomethane, was hydrolysed in the presence of sodium borohydride, 8 was obtained after treatment with methanolic hydrogen chloride; 9 was obtained in the presence of sodium borodeuteride, as shown by appropriate mass shifts in the mass spectrum. The presence of three acylable sites in 8 was established by acetylation to give 10 (mass increment 3 x 42).

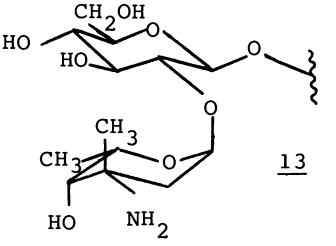


8,R=R'=H; 9,R=D;R'=H. 10,R=H;R'=CH_3CO 11

The presence of one ester group in 8 was shown when esterification experiments were carried out with CH_3OH/CD_3OH 1:1, the molecular ion abundances indicating the presence of $-COOCH_3$ and $-COOCD_3$ groups in the ratio 1:1. These experiments (Ref. 28) exemplify how simple derivatisation techniques and the use of isotope labels give information about the functionalities in, and origins of, degradation products. They are applicable to quantities in the 10μg to 1 mg range. In the present case they indicate the presence of the unit 11 in vancomycin (Ref. 28), the nature of the aromatic skeleton having been established in earlier work (Ref. 34).

Related experiments (Ref. 28) led, following acetylation and esterification of hydrolytic products, to characterisation of the amino acid derivative 12. Other than in this group of antibiotics and latex (Ref. 35), the occurrence of phenylglycines in nature is very rare. Preliminary experiments indicate, however, that the analogous fragment is also present in ristocetin A (Ref. 36).





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Proton magnetic resonance studies (Ref. 37) at 270 MHz indicate that chemical degradation experiments have now accounted for all the skeleton of vancomycin. The antibiotic is constituted from 11 (R = the disaccharide 13), the free triphenol corresponding to 12 (incorporated through amide bonds which utilise the underivatised amino acid residues of the phenylglycines), aspartic acid, and N-terminal N-methylleucine. On this basis the formula of penta-O-methyl-N-acetylaglucovancomycin should be $C_{60}H_{64}N_8O_{18}Cl_2$ (m.wt. 1254 for the species containing two ^{35}Cl isotopes). Electron impact mass spectrometry does not give a molecular ion for this compound, but a field desorption spectrum affords an abundant ion at m/e 1277. When the compound is loaded on to the wire in methanol saturated with potassium chloride the ion shifts by 16 m.u. to higher mass (m/e 1293). This results indicates that the ions in the two spectra correspond to MNa^+ and MK^+, respectively (Ref. 38). Thus the field desorption spectrum confirms the molecular formula of the antibiotic which had been derived from chemical degradation experiments.

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