MASS SPECTROMETRY OF NATURAL AND SYNTHETIC PEPTIDE DERIVATIVES

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INTRODUCTION

Preliminary studies on the use of mass spectrometry for the determination of amino acid sequences in peptides have been published by Biemann et al.[1, 2] who reduced the peptide bonds and the terminal carboxyl by $LiAlH_4$ or $LiAlD_4$ to obtain polyamino-alcohols or the corresponding polyamines.

For the purpose of obtaining sufficiently volatile derivatives, all later authors have used N-acyl derivatives of oligopeptide methyl (or ethyl, or t-butyl) esters. Thus, Stenhagen[3] and Weygand et al.[4] have obtained mass spectra of N-trifluoroacetyl methyl esters of oligopeptides up to pentapeptides and Manusadzhyan et al.[5] have used N-acetyl oligopeptide ethyl esters. With a direct inlet system, Heyns and Grützmacher[6,7] have studied N-acetyl peptides and have obtained a fairly satisfactory sequential splitting, the highest peptide measured being a pentapeptide derivative of mass 539.

Detailed studies of the fragmentation of peptides and their derivatives have been performed by Weygand et al.[8, 9] and Shemyakin et al.[10], the latter having especially studied depsipeptides and cyclic peptides[11, 11a].

The usefulness of mass spectrometry for the determination of the sequence of amino acid residues was clearly recognized in 1964, due to structural studies on natural peptidolipids[12].

Since then mass spectrometry has become a very helpful tool for correcting[12, 13], or confirming[14] the structure of oligopeptide derivatives established by classical analytical methods; the most interesting application of mass spectrometry in this field is, however, *the determination of the sequence of amino acids in oligopeptides* and their derivatives. The minute quantities (10–100 μg) needed for obtaining a mass spectrum and the rapidity of the measurements as well as the possibilities of automation compete favourably with other well established methods.
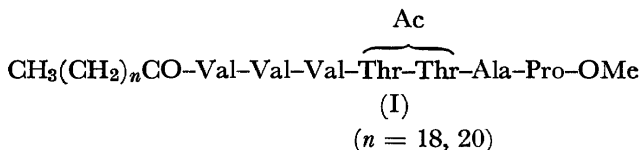
We shall not go into details of fragmentation mechanisms, our principal aim being to try to show how the "normal" splitting of the peptide bond can be used for sequence determination. For recent reviews see references 15–19.
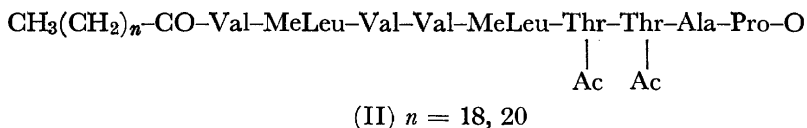
NATURAL PEPTIDOLIPIDS

The story of fortuitine

Mycobacterium fortuitum produces a peptidolipid (m.p. 199–202°, $[\alpha]_D = -72°$) which was discovered in 1963 by E. Vilkas[20] and named fortuitine.

It was shown to be essentially an *N*-acyl-oligopeptide methyl ester and the preliminary structure (I) was proposed[21]:

$$Ac$$

$$CH_3(CH_2)_nCO-Val-Val-Val-\overset{\frown}{Thr-Thr}-Ala-Pro-OMe$$

$$(I)$$

$$(n = 18, 20)$$

A closer examination of the results of quantitative determinations of the amino acids of a total hydrolysate of fortuitine showed, however, that a nitrogenous portion of the molecule was not yet accounted for.

Just at that moment we learnt that Dr. Michael Barber, at the A.E.I. works at Manchester, was looking for high molecular weight compounds for testing the performance of his MS9 mass spectrometer. We sent him a few mg of fortuitine and received in reply a detailed letter giving the identification of the "unknown constituent" as two molecules of *N*-methyl leucine and the complete structure of fortuitine (II) which is thus an *N*-acyl-nonapeptide methyl ester with two acetylated threonine residues[12].

$$CH_3(CH_2)_n-CO-Val-MeLeu-Val-Val-MeLeu-Thr-Thr-Ala-Pro-O$$

$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad | \quad\ |$$

$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad Ac \quad Ac$$

$$(II)\ n = 18, 20$$

The mass spectrum (*Figure 1*) of fortuitine shows two parent peaks at 1331 and 1359, due to its being a mixture of two homologues containing a C_{20} and C_{22} fatty acid respectively. All ions containing the lipid moiety will therefore give sets of peaks 28 mass units apart, thus facilitating interpretation.

The parent peaks, at 1331 and 1359, each lose $2 \times CH_3COOH = 120$ to give peaks at 1211 and 1239. This shows the presence of two acetyl groups: both threonine molecules are *O*-acetylated. Two small peaks at 1083 and 1111 are apparently due to the loss of —Pro—OMe (128 m.u.). A prominent set of peaks occurs at 1011 and 1039. This is 72 m.u. less than 1083 and 1111, due to the loss of Ala + H (migration of one H atom). The next set of peaks occurs at 928 and 956. This loss of 83 m.u. from 1011 and 1039 corresponds to the loss of one molecule of anhydrothreonine, again after migration of one H atom. This gives the sequence R–Thr–Ala–Pro–OMe.

The further interpretation of *Figure 1* gives clearly the whole structure of fortuitine as (II), corresponding to $C_{70}H_{125}N_9O_{15} = 1331$ and $C_{72}H_{129}N_9O_{15} = 1359$ respectively. The above interpretation was confirmed by mass measurements of the peaks at m/e 521, 648, 719, 846 and 928.

This work showed for the first time that mass spectrometry could be very useful for sequence determination of oligopeptide derivatives of unknown structure and that the possible mass range for such work was at least as high as 1400, and that even nonapeptide derivatives could be tackled. Contrary to previous experiments in this field, the principal fragmentations observed were those of the peptide bond, thus facilitating sequence determination.
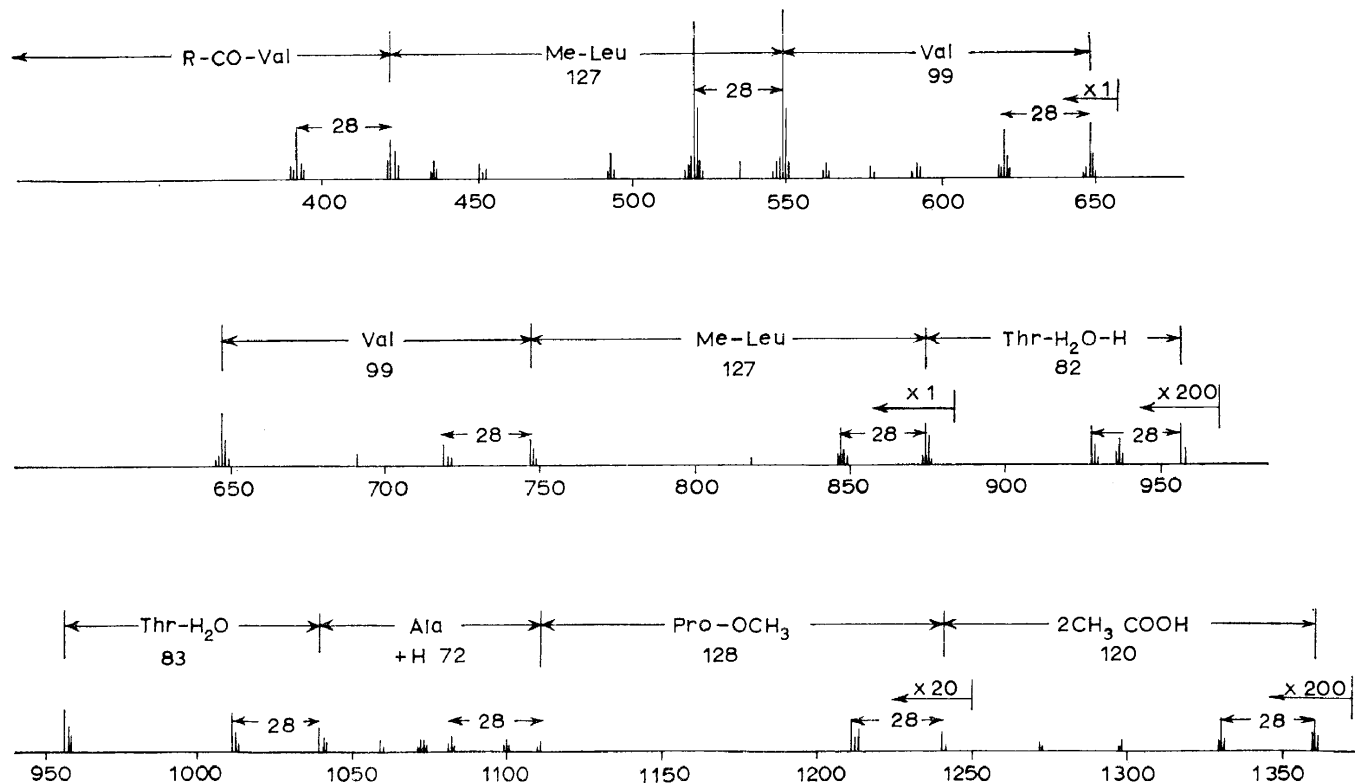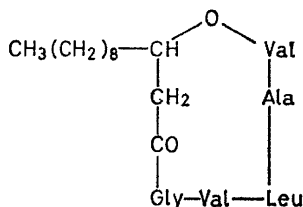
Figure 1. Mass spectrum of fortuitine (Barber *et al.*[12])

Encouraged by this success, we examined a few other natural peptidolipid compounds by mass spectrometry.

Peptidolipid of *M. johnei*

A peptidolipid analogous to fortuitine had been isolated from *M. johnei* by Lanéelle and Asselineau[22]; a preliminary structure R–CO–(Phe)$_2$,Ala, Ala–OMe had been considered. Mass spectrometry showed, however, quite unequivocally, that two molecules of isoleucine had escaped the Moore and Stein analysis (isoleucyl-isoleucine being very difficult to hydrolyse) and that the correct structure of this compound is (III)[13]. Here again the most prominent peaks corresponded to the splitting of the peptide bond.

$$CH_3(CH_2)_n\text{–CO–Phe–ILe–ILe–Phe–Ala–OCH}_3$$

(III) $n = 14, 16, 18, 20$

The peptidolipin NA group

Peptidolipin NA, m.p. 232–233°, $[\alpha]_D = + 42°$, isolated from *Nocardia asteroides*, is a peptidolipid for which Guinand and Michel[23, 24] have proposed structure (IV) corresponding to the molecular formula $C_{50}H_{89}N_7O_{11}$ (mol. weight 963).


```
                   H              1
CH3—(CH2)n—C —CH2—CO→(X)→L - Val   2
           |                 ↓
           O·                D - Ala  3
           |                 ↓
           CO                L - Pro  4
           |  (L-Thr)        ↓
           HC—NH←(Y)←—D-allo-ILe  5
           |      6
         7H COH
           |
           CH3
```

Peptidolipin NA (IV)	X = L – Thr Y = L – Ala n = 16
Val^6-peptidolipin NA (V)	X = L – Thr Y = L – Val n = 16, 17, 18
α-AB^1-peptidolipin NA (VI)	X = L – α-AB Y = L – Ala n = 16, 17, 18

In this case, mass spectrometry fully confirmed this structure; one of the more prominent fragmentations observed is the elimination of CO_2 from the lactone ring, thus leading to an open chain acyl-oligopeptide ion quite analogous to the cases discussed above; from then on, the sequential splitting of peptide bonds could be recognized[14].

The principal compound, peptidolipin NA is accompanied by two or three analogues, in which one amino acid of the macrocyclic lactone is replaced by another. The structure determination of these compounds by mass spectrometry was particularly easy, based on the experience obtained with the parent compound. It was thus established that one of the "com-

panions" is a Val[6]-peptidolipin NA (V)[25], another is an α-aminobutyryl[1]-peptidolipin NA (VI)[26].

Isariin

In Manchester, the structure of isariin (VII), a metabolite of the fungus *Isaria cretacea* was determined, in an analogous way[27] (see also Kiryushkin *et al.*[28]). In all this work high resolution mass measurements were very useful for confirming the identification of various peaks. Biemann *et al.*[29] have described the analysis of isariin by computer interpretation of high resolution mass spectra.†

$$CH_3(CH_2)_8\text{---}CH \underset{\overset{|}{CH_2}}{\overset{O}{\diagup}} Val$$

(VII)

SYNTHETIC PEPTIDES

In most of the above mentioned natural peptidolipids, the principal fragmentation observed was that of the peptide bond, thus allowing an unambiguous determination of the sequence of amino acids in these peptide derivatives. The principal difference with respect to previous mass spectrometric studies of oligopeptide derivatives was the presence in these compounds of a *long-chain N-terminal acyl group* (II–VII).

It was therefore thought necessary to undertake a study of the fragmentation of a series of synthetic long-chain *N*-acyl oligopeptide methyl esters. For comparing the effect of the length of the *N*-acyl group, *N*-acetyl,*N*-decanoyl and *N*-stearoyl Ala–Val–Gly–Leu methyl esters were prepared[31]‡. All three acyl derivatives gave good molecular ions.

Measurements of the intensities of the peaks in the spectra of the three compounds showed that there is no difference in the basic fragmentation. Despite this, the spectrum of the stearoyl derivative is clearly simpler in that the peaks corresponding to cleavage of the peptide bonds are more easily recognized. The principal advantage of the long-chain acyl group is the shifting of the structurally significant peaks to a higher mass region, e.g. in the case of stearoyl peptides, all peaks below m/e 267 $(CH_3(CH_2)_{16}\text{–CO})^+$ can be disregarded for the purpose of sequence determination.

Mixed acyl derivatives

In the case of fortuitine[12] (II) and also of the *M. Johnei* peptidolipid (III)[13] the presence of homologues in the acyl chain made interpretation easier because all acyl-containing ions gave a group of peaks corresponding to the

† See also the paper of Kingston *et al.*[30] on the structure of ostreogrycin A.
‡ The Russian authors[31a] have also used the decanoyl group for *N*-acylation.

homologue pattern, Thus, it was thought to be an advantage to try mixed acyl group.

After considering the use of a mixture of heptadecanoyl and octadecanoyl groups we proposed the use of a mixture of normal and ω-trideuterated acyl groups ($CH_3CO-+ CD_3CO-$ or decanoyl- $+CD_3(CH_2)_8CO-$); all significant peaks still containing the acyl radical thus give doublets with a difference of 3 m.u.[32].

N-METHYLATED PEPTIDE DERIVATIVES

In studying the mass spectrometric behaviour of higher synthetic N-acyl oligopeptide methyl esters quite unexpected difficulties were, however, encountered. Whereas fortuitine (II) with its nine amino acids and heavy acyl groups had given an excellent spectrum up to m/e 1359, several derivatives of synthetic hepta- or octapeptides were apparently not volatile enough to give useful mass spectra. The explanation of this was found by J. Van Heijenoort[33], who suggested that an important factor concerning the low volatility of peptide derivatives might be hydrogen bonding due to the presence of –CO–NH–groups. An argument in favour of this hypothesis was that the tetrapeptide methyl ester H–Ile–Pro–Sar–MeVal–OMe containing no peptide hydrogen (i.e. absence of –CO–NH–) gave a mass spectrum exhibiting a molecular ion peak even without acylation of the terminal amino group. Also, the satisfactory volatility of the nonapeptide derivative fortuitine (II) could be explained by the presence of three tertiary amide bonds (two –MeLeu– and one –Pro–). These observations suggested that, if a procedure leading to permethylation of the –CO–NH– groupings of oligopeptide derivatives could be found, the resulting modified peptide might be more volatile and particularly suitable for the determination of the amino acid sequence by mass spectrometry†.

The methylation of the peptide bond had not yet been described and was considered to be a very difficult enterprise (personal communications of several peptide specialists). We were thus all the more pleased to find that the well known methylation procedure of Kuhn et al.[34] using silver oxide and methyl iodide in dimethylformamide gave complete N-methylation of peptide derivatives in a few hours.[35] In the same operation the C-terminal carboxy group and OH groups of threonine, serine and tyrosine are also methylated.

In our first paper on "N-methylation of N-acyl oligopeptides"[35] the usefulness of the procedure for obtaining more volatile peptide derivatives was shown by the fact that the heptapeptide derivative (VIII) was not volatile enough to give a mass spectrum, but after complete N-methylation, (IX) gave a satisfactory mass spectrum which exhibited a molecular ion peak at m/e 1082. This molecular weight indicates that the seven–CONH–groups as well as the threonine hydroxyl have been methylated by the methyl iodide–silver oxide treatment. The mass spectral fragmentation pattern (*Figure 2*) is also consistent with the amino acid sequence of this synthetic heptapeptide derivative (IX) and shows that the reaction –CONH → –CO–NMe–proceeds quantitatively to give a completely methylated product.

† Other reactions, such as trimethylsilylation had been tried, but without success (J. Van Heijenoort, unpublished experiments).
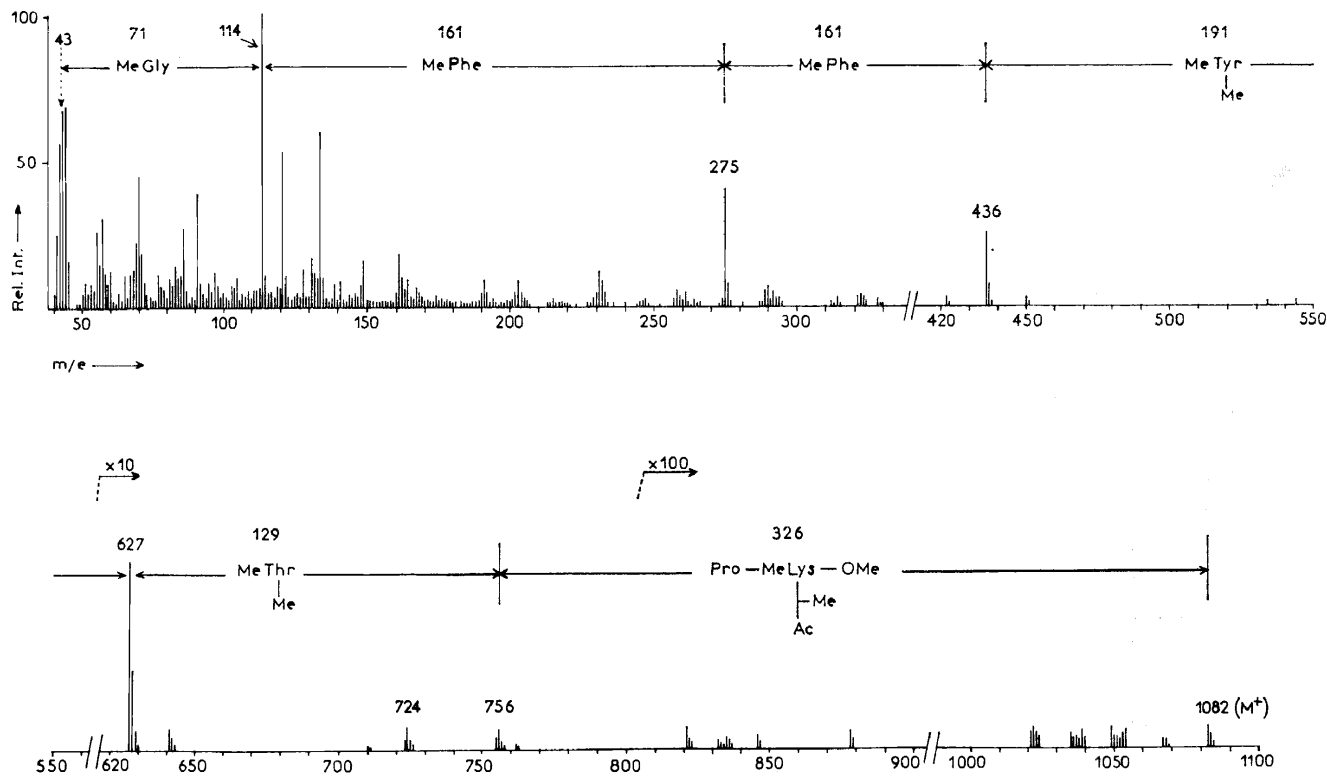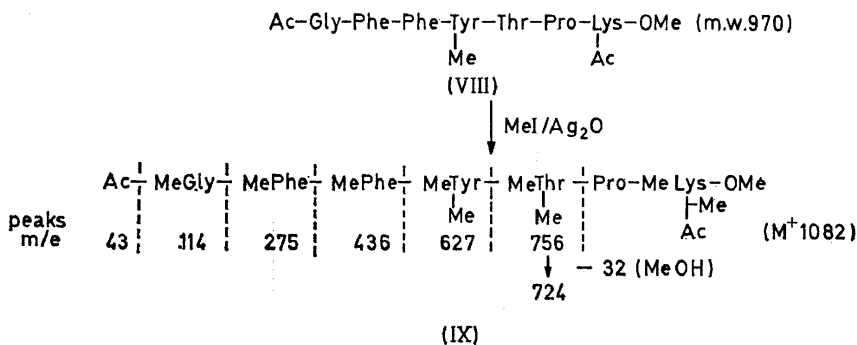
Figure 2. Mass spectrum of the methylated heptapeptide derivative (IX) (Das *et al.*[35])

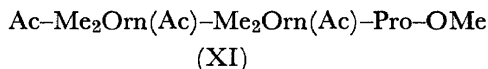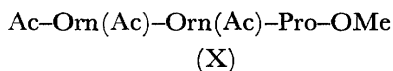Ac–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–OMe (m.w.970)
           | |
           Me   Ac

(VIII)

$\downarrow$ MeI/Ag$_2$O

Ac—MeGly—MePhe—MePhe—MeTyr—MeThr—Pro–Me Lys–OMe

| | | | | | Me | Me | |–Me |
| peaks m/e | 43 | 114 | 275 | 436 | 627 | 756 | Ac | (M$^+$1082) |

$\downarrow$ − 32 (MeOH)
724

(IX)

The hitherto unknown *permethylated peptide derivatives* have thus become easily accessible; a detailed study of their chemical and, especially, biological properties might well be rewarding.

The *N*-methylation method also allows the easy *preparation of monomethyl derivatives* of primary amines and in particular of amino acids, by the sequence R–NH$_2$ → R–NH–Ac → RN(Me)Ac → RNHMe, which is less tedious than other methods.

Another advantage of the use of permethylated peptide derivatives for sequence determination was noticed by Thomas *et al.*[36]; the *fragmentation pattern* of the permethylated derivatives *is much simpler* than that of the corresponding non-methylated derivatives (the spectra consisting almost exclusively of the "sequence determining" peaks resulting from –CO–NCH$_3$ cleavage); this can be seen by comparing the spectra of *Figures 3 and 4.*

The simplification of the spectra obtained by permethylation is certainly in part at least due to the decrease of pyrolytic reactions as a result of the lower temperature used.

As an example for the enhancement of volatility obtained by permethylation let us quote the case of peptide (X) (mol. wt. 483), which before methylation could be volatilized in the mass spectrometer at 210°, whereas the permethylated product (XI) (mol. wt. 539) gave a spectrum at a source-sample temperature of 150°.

Ac–Orn(Ac)–Orn(Ac)–Pro–OMe

(X)

Ac–Me$_2$Orn(Ac)–Me$_2$Orn(Ac)–Pro–OMe

(XI)

The permethylation technique will prove especially valuable in combination with the interesting (but also most expensive) computer techniques described by Biemann *et al.*[29], Senn *et al.*[37], and Barber *et al.*[38], because it allows the analysis of much larger peptides than those described by the above authors. Moreover, the computer could easily give useful information with impure peptide preparations, where the usual technique is of little help.

How to recognize natural *N*-methylamino acids

Previously, it has not been possible to detect and locate with certainty the presence of an *N*-methylamino acid residue in a peptide by mass spectro-
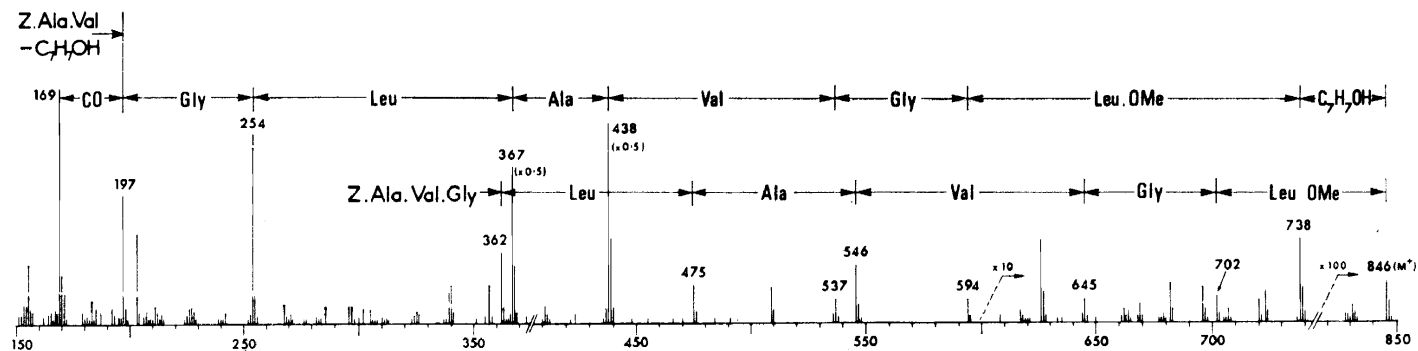
Figure 3. Mass spectrum of the octapeptide derivative Z–Ala–Val–Gly–Leu–Ala–Val–Gly– Leu–OMe (Thomas *et al.*[36])
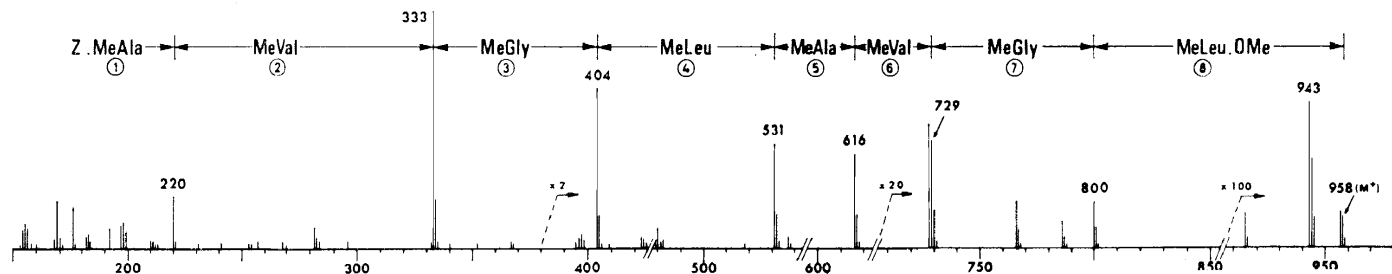

Figure 4. Mass spectrum of the octapeptide derivative of *Figure 3*, after permethylation (Thomas *et al.*[36])

metry because the mass number as well as the elemental composition of a given amino-acid and its N-methylated lower homologue (for example leucine and N-methylvaline) are the same. This problem can, however, be solved by the use of the mass spectrometric shift technique.

A comparative study of the mass spectra of an N-acyl-oligopeptide methyl ester and its permethylated derivative provides information on the presence as well as the location of originally existing N-methylamino-acid residues in oligopeptide derivatives[39]. The increase in molecular weight after complete N-methylation indicates the number of methyl groups introduced in the molecule and thus the number of peptide –CONH– groups (together with –OH groups, if present) in the oligopeptide derivative before methylation. Each amino acid residue which has gained a methyl group reveals itself by an increase of 14 mass units recognizable from the peaks arising by peptide bond cleavage. An amino acid residue with its peptide nitrogen originally methylated or fully substituted shows no change in mass number after methylation (unless it contained other methylatable functional groups), and thus its position can be determined.

This has been illustrated with fortuitine (II) as model compound†.

This procedure cannot, however, be used for oligopeptide derivatives which do not give mass spectra before methylation, for example, the heptapeptide derivative (VIII)[35]. In such cases, the N-acyl peptide methyl ester may be treated with a mixture of trideuterio-methyl iodide and silver oxide in dimethylformamide to obtain the corresponding perdeuteriomethylated product. Comparison of the mass spectra of the permethylated and the perdeuteriomethylated derivatives would indicate a shift of 3 mass units (CH_3 versus CD_3) for each N-deuteriomethylated amino acid residue. Such a shift would not be observed if the amino acid residue did not originally contain any methylatable hydrogen.

This approach was illustrated by studying the mass spectrum of perdeuteriomethylfortuitine[39] and has been used for the study of the structure of stendomycine (see below).

Some comments on methylation techniques

The permethylation of peptide derivatives is presently carried out in our laboratory by using silver oxide washed successively with water, methanol and ether, then dried under vacuum and stored over P_2O_5. By heating 0·3 to 20 mg of an acyl peptide derivative with 50 mg silver oxide in 0·3 ml dimethylformamide in presence of 0·2 ml methyl iodide in a screw cap vial to 50° for 4 hours, complete O- and N-methylation is obtained[36].

It is interesting to note that if moist silver oxide is used, only O-methylation is observed; thus, Guinand and Michel[24] have obtained the O-permethylated derivative of peptidolipic acid (XII) (mol. wt 1037) by using moist silver oxide, whereas the same compound gives the O,N-permethylated derivative (mol. wt 1121) when methylated with dry silver oxide[36].

$$\text{RCO–Thr–Val–Ala–Pro–aIle–Ala–Thr–OH} \qquad \text{(XII)}$$

† E. Vilkas had, independently, also performed the permethylation of fortuitine (unpublished experiments).

Agarwal *et al.*[40] have also recently described permethylation of peptide derivatives using silver oxide; these authors have observed cleavage of the peptide bonds, especially next to glutamic acid or methionine, which could be due to methanolysis.

Other methylation techniques can also be considered; thus, Vilkas[41] has recently described the permethylation of a peptidoglycolipid (a mycoside C_b from *M.butyricum*, see below) using the method first described by Hakomori[42] for methylating a glycolipid; silver oxide is replaced by the methylsulphinyl carbanion of Corey and Chaykovsky[43] which is generated by dissolving NaH in dimethylsulphoxide. Complete *O*- and *N*-methylation was obtained in 12 hours at room temperature.

The "troublesome" amino acids

Before the development of the permethylation technique it had been shown that peptides containing several trifunctional amino acids could be analyzed by mass spectrometry, provided that the dicarboxylic amino acids (Asp, Glu) were esterified on their free carboxyl group, that tyrosine was present as its *O*-methyl ether, that lysine was ε-acylated; derivatives of cystine and of histidine had given mass spectra[32] so that only arginine was left as the most "troublesome" one. Then Shemyakin *et al.*[44] showed that arginine peptides can be condensed with a β-diketone (e.g. acetylacetone) to give pyrimidine derivatives which give good mass spectra (see also Vetter–Diechtl *et al.*[45]). Shemyakin *et al.*[44] further reported that a treatment of arginine peptides with hydrazine produces the corresponding ornithine derivatives.

After introduction of the permethylation technique new problems arose with some of the tri-functional amino acids; let us consider these briefly and define the solutions which can be adopted.

Free amino groups present either at the *N*-terminal end of the peptide chain, or as the ω-function of lysine or ornithine are usually acylated to ensure volatility of oligopeptide derivatives to be analyzed by mass spectrometry[32]; methylation of free amino groups would, of course, lead to quaternary methyl iodide salts with low volatility. Therefore acylation must precede methylation.

Thomas *et al.*[46] proposed acetylation of the peptide with methanolic acetic anhydride which leads only to *N*-acetyl derivatives leaving OH groups free; these are then methylated in the subsequent permethylation procedure.
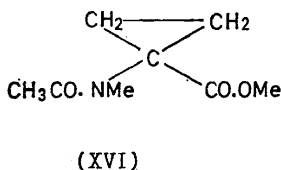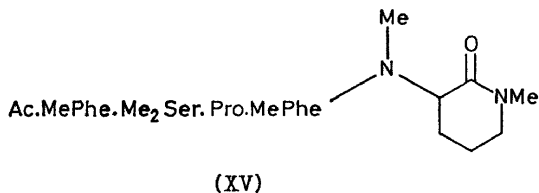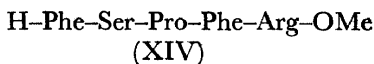
Arginine. When arginine peptides are acylated and methylated by the usual procedure, chloroform-soluble products of sufficient volatility can be obtained, but the mass spectra show sequence peaks only up to but not including the arginine residue. After treatment with hydrazine, following the procedure of Shemyakin *et al.*[44], the corresponding ornithine peptides are obtained which can then be permethylated.

Thomas *et al.*[46] have recently described experimental details for arginine peptides consisting of (i) treatment with hydrazine, (ii) acetylation, (iii) permethylation of the corresponding ornithine peptides. The tripeptide deriva-

<center>H–Arg–Arg–Pro–OBu^t</center>
<center>(XIII)</center>

<center>499</center>

tive (XIII) containing two arginine residues thus gave the tetramethyl-triacetyl-diornithine methyl ester XI (*Figure 5*). When arginine methyl ester is at the *C*-terminal (peptides obtained from proteins by tryptic hydrolysis frequently contain *C*-terminal arginine), the lactam of ornithine is obtained; thus, the pentapeptide methyl ester (XIV) gave the permethyl-ated lactam (XV) (*Figure 6*).
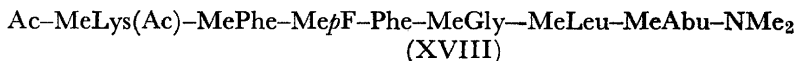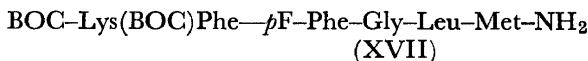
Histidine derivatives give a quaternary base after permethylation, but in the mass spectrometer CH_3I is lost thermally, so that mass spectra can be obtained (D. W. Thomas, unpublished results).

H–Phe–Ser–Pro–Phe–Arg–OMe
(XIV)

Ac.MePhe.Me$_2$ Ser. Pro.MePhe

(XV)

(XVI)

Methionine peptides show a peculiar behaviour after permethylation. Agarwal *et al.*[40] have found that methylation of acetyl methionine gave a volatile crystalline product $C_8H_{13}NO_3$ which they have shown to be the cyclopropyl derivative (XVI). Thomas *et al.*[46] have not observed this reaction. Instead, methionine derivatives seem to give on methylation sulphonium derivatives.

The spectra obtained with such permethylated methionine-peptides show only peptide cleavage peaks up to, but not including the methionine residue (in analogy with the situation observed with methylated arginine peptides, see above).

A solution to the problem of methionine can be found by treating methionine peptides (for instance XVII) with Raney nickel, thus producing an α-aminobutyric acid (Abu) residue from each methionine residue (see XVIII). Experimental details have been given by Thomas *et al.*[46]. The treatment with Raney nickel would transform cystin and cysteine residues in peptides to alanine residues. In order to recognise newly formed alanine residues we are investigating the use of deuterated Raney nickel.
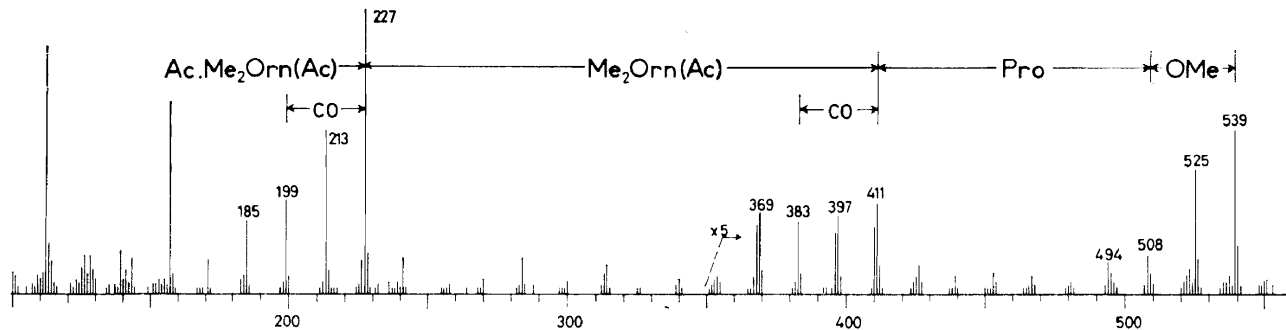
BOC–Lys(BOC)Phe—*p*F–Phe–Gly–Leu–Met–NH$_2$
(XVII)

Ac–MeLys(Ac)–MePhe–Me*p*F–Phe–MeGly—MeLeu–MeAbu–NMe$_2$
(XVIII)

Figure 5. Mass spectrum of the product obtained after treatment of H–Arg–Arg–Pro–OBu with hydrazine, then methanolic acetic anhydride, trifluoroacetic acid and methyl iodide-silver oxide (Thomas *et al.*[46])
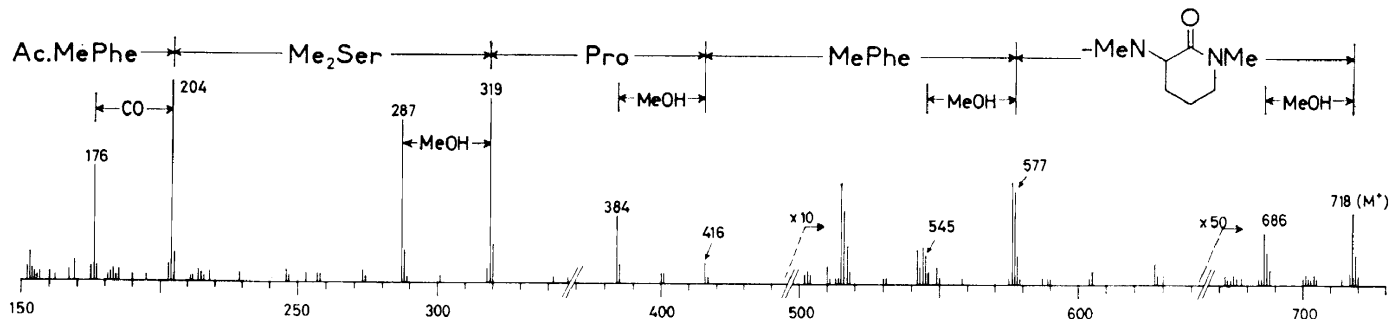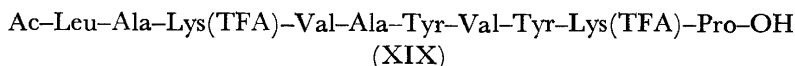

Figure 6. Mass spectrum of the product obtained after treatment of H–Phe–Ser–Pro–Phe–Arg–OMe with hydrazine, then methanolic acetic anhydride and methyl iodide–silver oxide (Thomas *et al.*[46])

Aspartic and glutamic acid peptides. Peptides containing aspartic and glutamic acid have been repeatedly analyzed by mass spectrometry [15, 16, 31, 32]; the free carboxyl group is usually esterified by methanol or t-butanol. The per-methylation technique introduces, however, an unexpected difficulty with aspartic residues†, as also noted by Agarwal *et al.*[40] who have ascribed this to *C*-methylation as a side reaction.

D. W. Thomas did also observe some difficulties with Asp-peptides after permethylation; this problem is currently under investigation.

Upper limits for mass spectrometry of peptide derivatives

The upper limit for mass spectrometry is of course an important question, which interests all biochemists performing sequence determination of peptides. The situation can be summed up as follows[36];without permethylation, *N*-acyl oligopeptide methyl esters can be "tackled" up to hepta-or octapeptides, depending on the amino acid residues they contain. After permethylation, this limit is increased allowing in the most favourable cases sequence determination of the first twelve *N*-terminal amino acid residues. Thus the *decapeptide* derivative (XIX) gave, after permethylation, a mass spectrum (*Figure 7*) showing the expected molecular ion at m/e 1580‡

Ac–Leu–Ala–Lys(TFA)–Val–Ala–Tyr–Val–Tyr–Lys(TFA)–Pro–OH

(XIX)

and also all specific cleavages giving the entire amino acid sequence; only a few "non-sequence" peaks occur, mainly in the molecular weight region and do not represent a serious handicap for a correct sequence deter-mination. Even tetradecapeptide derivatives such as stendomycic acid (see below), or the pentadecapeptide derivatives gramicidin A and B (see below) are volatile enough, after permethylation, to give mass spectra, but the sequence of only ten to twelve *N*-terminal amino acid residues can be deduced. This is apparently due to the fact that the intensity of each suc-cessive sequence peak is lower than the preceding (frequently 10% or less) so that the ions beyond 10 to 12 residues become a very small percentage of the total ionization and are thus below the sensitivity limits of the now available mass spectrometers.

With the peptide derivatives studied up to now in our laboratory, the upper mass limit for the detection of significant peaks seem to be at m/e 1600–1700. The ions of other categories of organic compounds are apparently more stable in the mass spectrometer and give mass spectra up to approxi-mately m/e 2000. A peracetylated 6,6′diester of trehalose studied by Adam *et al.*[47] gave an M-60 peak at m/e 1910. This seems to be the "highest mass spectrum" described so far for a purely organic compound.§

It is clear, that instrumental improvements will allow further advances in this field. I shall now consider some structural problems concerning *natural* peptide derivatives.

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† In some cases permethylation of glutamic acid peptides leads to secondary products (see aso Agarwal *et al.*[40]); glutamine and asparagine behave normally.
‡ The highest molecular ion obtained so far for a peptide derivative.
§ Fales[48] obtained a mass peak at m/e 3628 for the compound $C_{72}H_{24}O_8F_{128}N_4P_4$.
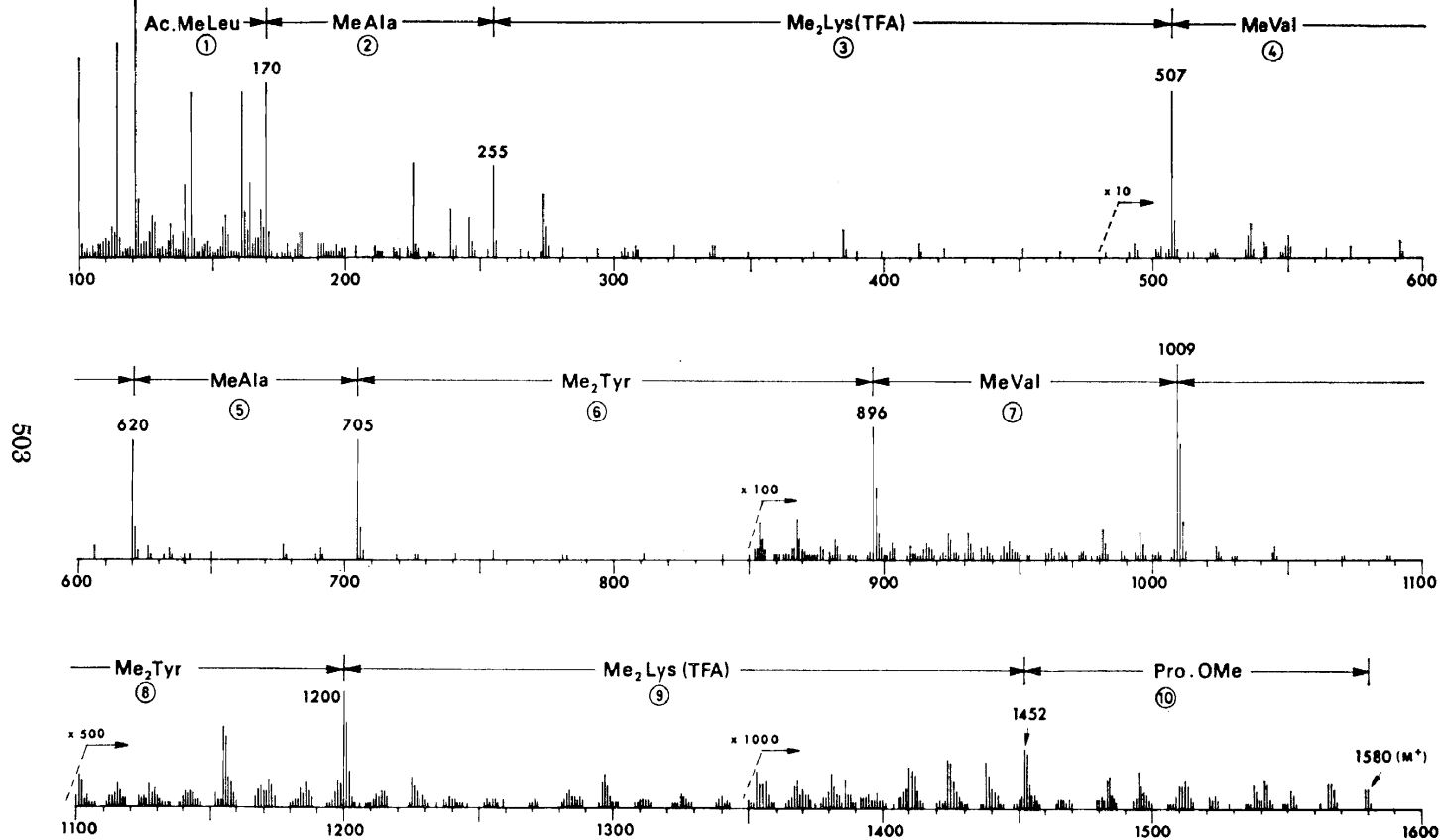
Figure 7. Mass spectrum of the permethylated decapeptide derivative Ac–Leu–Ala–Lys(TFA)–Val–Ala–Tyr–Val–Tyr–Lys(TFA)–Pro–OMe (Thomas *et al.*[36])

The mycoside C group

The mycosides, discovered by Smith and Randall[49-52] have been defined as "type-specific glycolipids of mycobacterial origin"[53]. They all contain typical 6-deoxyhexoses[52-54]. I shall only discuss the mycoside C compounds, which also contain amino acids; they are thus peptidoglycolipids. Initially they were thought to be restricted to strains of *M. avium*[50-54], but they have since been found also in *M. marianum*[55], *M. butyricum*[56] and *M. scrofulaceum*[57].

Total acid hydrolysis of all mycoside C compounds gives three categories of components: (i) a mixture of long chain fatty acids and several molecules of acetic acid; (ii) at least two 6-deoxyhexoses; (iii) three different amino acids, all of the D-series: D-phenylalanine, D-*allo*threonine and D-alanine[58] as well as one amino alcohol, such as alaninol[59, 60].

The complexity of the mycoside C fractions, the large number of their components and the lack of criteria of purity of the starting material, are the reasons for the slow advances and some occasional errors made in elucidating their structures during the last 10 years. It is mostly due to mass spectrometry that precise structural formulae could be established quite recently for several representatives of the mycoside C group.

In a first stage[61, 62], a partial formula of an acyl-tripeptide RCO–Phe–*allo*–Thr–Ala emerged, where the detailed structure of RCO– a long chain, unsaturated and oxygenated acyl group could not be determined, because it usually decomposes during the strong acid hydrolysis necessary for splitting the RCO–Phe amide linkage; it is, however, relatively easy to obtain, by partial acid hydrolysis of all C-mycosides, the amide of phenylalanine, the methyl ester of which (RCO–PheOMe) gives a good mass spectrum[56], confirming the structure of the acyl radical deduced by mass spectrometry of the acyl methyl ester.

The sequence RCO–Phe–*allo*–Thr–Ala, first elaborated chemically by standard methods was then confirmed by mass spectrometry[56].

The presence of a fourth nitrogenous constituent had been noticed already four years ago by E. Vilkas and then by M. Barber (A.E.I., Manchester), when he ran the first mass spectrum of a mycoside C; the search for this fourth "amino acid" was rather frustrating, due to the unsuspected inhomogeneity of the fractions studied; finally, from a small fraction of the mycoside C of *M.butyricum* a "new amino acid" could be isolated and identified as *N*-methyl *O*-methyl L-serine[63]. This, however, is not a major constituent and up to now no pure molecular species has been isolated, containing the hypothetical sequence RCO–Phe–*allo*–Thr–Ala–N–MeSer(OMe)–.

During a study of the constituents of an atypical photochromogenic strain No. 1217, Lanéelle *et al.*[64] isolated a mycoside C and Lanéelle[60] succeeded in identifying the mysterious fourth nitrogenous constituent as *alaninol*, which had partly escaped notice due to its volatility. This leads to the sequence RCO–Phe–*allo*–Thr–Ala–alaninol, which is in agreement with the mass spectrum[65].
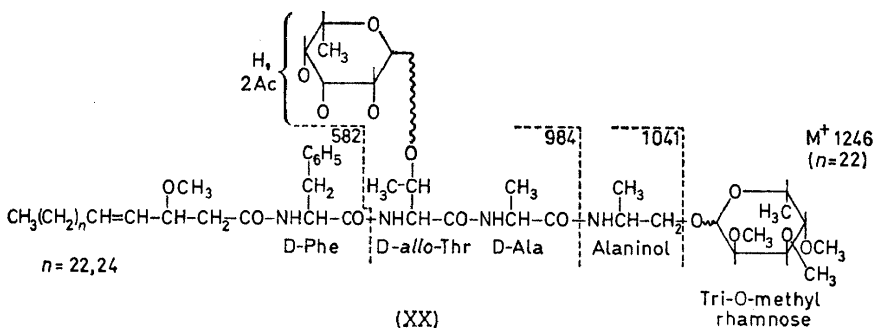
We have now to determine the position of the deoxy-sugar molecules. All mycoside C compounds described until now contain one molecule of 6-deoxytalose, which is usually di-or tri-acetylated and one molar proportion of partly or entirely *O*-methylated rhamnoses[54].

The ease with which the 6-deoxytalose is split off in alkaline medium was first interpreted as a proof of an ester linkage of a C-terminal carboxyl group with the C–1 hydroxyl group of this sugar[62]. Then came the discovery[66, 67] that glycosides of serine and threonine, such as exist in glycoproteins easily suffer a β-elimination reaction with liberation of the sugar molecule and formation of dehydroalanine from serine and dehydro α-amino butyric acid from threonine. This lead to a revision of the location of the 6-deoxytalose in the mycoside C compounds, as it was found that here too, β-elimination takes place with liberation of the sugar and destruction of *allo*-threonine[56].

It now seems that in all mycoside C compounds the talose derivative is in glycosidic linkage on the OH group of one molecule of *allo*-threonine as also shown by mass spectrometry. This leaves only the rhamnose derivative to be located; chemical degradation and mass spectrometry show unambiguously that it is linked to the OH group of the terminal alaninol molecule[56, 65].

The structure of the long chain fatty acids which form the RCO–group of these mycosides seems to vary from strain to strain; their chemistry has been largely defined by mass spectrometry of their amide with phenylalanine, as already mentioned above. They all represent new structural types in fatty acid chemistry (see *Table 1*).

We thus arrive at formula (XXa) showing the general structure of the C-mycosides of *M.butyricum*, of the atypical strain No. 1217 and of *M.scrofulaceum*. The case of *M.avium* will be discussed below.

(XX)

It is noteworthy that several mycoside C compounds (for instance XX, a mycoside C_b from *M. butyricum*) give good partial mass spectra; permethylation has, however, definite advantages and can be performed either by the method of Kuhn *et al.*[34] as used by Das *et al.*[35, 36] or by the method of Hakomori[42], as shown by Vilkas[41].

The mycoside C compounds of M. avium and M. marianum. The structure RCO–Phe–*allo*–Thr–Ala–alaninol seems to represent the "smallest" and the most frequent one for a mycoside C. A reexamination of fractions of mycoside C described previously as containing a pentapeptide[62] –Phe–(*allo*–Thr–Ala)$_2$–, or even a heptapeptide[55] –Phe–(*allo*–Thr–Ala)$_3$– has shown that a pentapeptide as quoted above does exist and that it is linked to a terminal amino alcohol molecule. Mass spectrometry of these more complex

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compounds was not successful, but after partial acid hydrolysis and acetylation a good mass spectrum of a 2-O-acetyl 3,4-di-O-methyl rhamnoside of N-acetyl alaninol was obtained, thus suggesting a structure analogous to (XX), but having the pentapeptide sequence Phe–(allo–Thr–Ala)$_2$ instead of the tripeptide sequence Phe–allo–Thr–Ala[68].
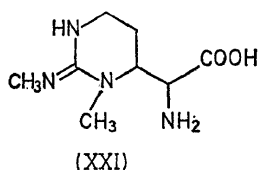
Table 1. Mycoside C compounds of various strains

R–C–Phe–alloThr–Ala–NH–CH–CH$_2$O–Rhamnose
(with O double bond on C, Diacetyl 6-deoxy-talose and CH$_3$ substituents)
(XXa)

3,4-di-O-methyl   *M.avium, M.scrofulaceum*

2,3,4-tri-O-methyl   *M.*No. 1217, *M. butyricum*

	RCOOH	STRAIN	REFERENCES
$C_{28}H_{56}O_3$	$CH_3(CH_2)_{24}CH–CH_2–COOH$ with OH	Atypical No. 1217	Lanéelle[60]
$C_{29}H_{56}O_3$	$CH_3(CH_2)_{22}CH=CH–CH–CH_2–COOH$ with OCH_3	*M.avium, M. butyricum*	Vilkas et al.[56]
$C_{32-35}H_{62-68}O_3$	$CH_3C_nH_{(2n-2)}CH–COOH$ with OCH_3, $n = 28, 29, 30, 31$	*M.scrofulaceum*	Vilkas et al.[57]

Stendomycin

The antifungal antibiotic stendomycin was isolated by Thomson and Hughes[69] from *Streptomyces antimycoticus*. Bodanszky et al.[70] showed that it is a cyclic acyl tetradecapeptide lactone resembling peptidolipin NA (IV) and that it is a mixture of homologous compounds.

After acid hydrolysis they identified isomyristic acid and its lower homologues, as well as 14 moles of amino acids: Ala$_1$ Gly$_1$, Ser$_1$, Pro$_1$, Val$_3$, allo–Ile$_2$, allo–Thr$_2$, one mole of N-methylthreonine, one mole of "dehydrobutyrine" [= dehydro-α-aminobutyric acid = Δ-Abu[71]] and one mole of a basic compound ("B") shown to be (XXI[72]).

This was the only information available to us, when we received from Dr. Bodanszky a few mg of "stendomycic acid", the "open form" obtained by mild alkaline hydrolysis of the antibiotic-lactone.

(XXI)

Permethylation of one mg gave a product of sufficient volatility for mass spectral analysis, a rather satisfactory result, considering the presence of four hydroxyamino acids in addition to a basic component expected to form a quaternary amine derivative of low voltability.

Several unusual problems arose, not normally encountered with simple amino acids, involving the determination of the location of the proline, threonine, N-methylthreonine, and "dehydrobutyrine" residues known to be present. All ions containing permethylated threonine usually lose methanol, giving ions containing N-methyldehydro-a-aminobutyric residues instead. The mass of these is the same as that of proline (97 m.u.) thus it is impossible to distinguish between each of the residues listed above without employing other techniques.

Use of CD_3I for the permethylation reaction (performed again on 1 mg) allowed the division of these four residues into two groups: the masses of proline and (N–CH_3 threonine–CD_3OH) remained unchanged (97 m.u.), whereas the N–CD_3 group introduced in the threonine and Δ-Abu residues were detected by the shift of their masses to 100. The mass spectrum of the deuteriomethylated product is shown in *Figure 8*.

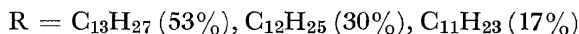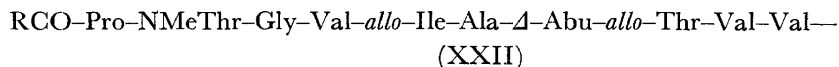The sequence RCO–Pro–MeThr– is preferred over the inverse sequence RCO–MeThr–Pro, because the latter would be expected to give rise to a minor but nevertheless discernible mass spectral fragment, which contains the threonine methoxyl group :RCO–CH_3Thr– (m/e 343 for R = $C_{11}H_{23}$).

$$\overset{|}{O}CD_3$$

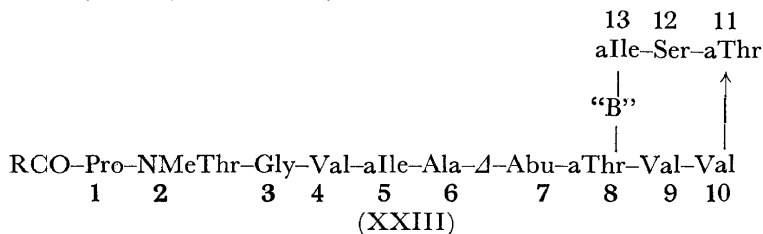This was not observed and thus the assignment of a proline to position 1 is preferred although not proven.

Positions 7 and 8 were defined by catalytic hydrogenation of permethylated stendomycic acid; the dihydroderivative (Abu) thus obtained showed an increment of two mass units at the 7 position (*Figure 9*).

The partial structure (XXII) can thus be written for stendomycic acid:

RCO–Pro–NMeThr–Gly–Val–*allo*–Ile–Ala–Δ–Abu–*allo*–Thr–Val–Val—

(XXII)

R = $C_{13}H_{27}$ (53%), $C_{12}H_{25}$ (30%), $C_{11}H_{23}$ (17%)

Sequence-determining peaks in the mass spectra beyond the tenth amino acid could not be detected above the noise and background level[73].

The above partial sequence for stendomycic acid was then communicated to Dr. Bodanszky, who informed us that it was identical with the one established independently by his group by classical chemical methods leading to formula (XXIII) for stendomycin[72].

```
                              13   12    11
                            aIle–Ser–aThr
                               |        ↑
                             "B"        |
                               |        |
RCO–Pro–NMeThr–Gly–Val–aIle–Ala–Δ–Abu–aThr–Val–Val
  1    2      3    4    5    6    7     8    9   10
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(XXIII)

More recently the tetrapeptide sequence *allo*–Thr–Ser–*allo*–Ile–"B"– obtained by partial hydrolysis was also submitted to mass spectrometry after
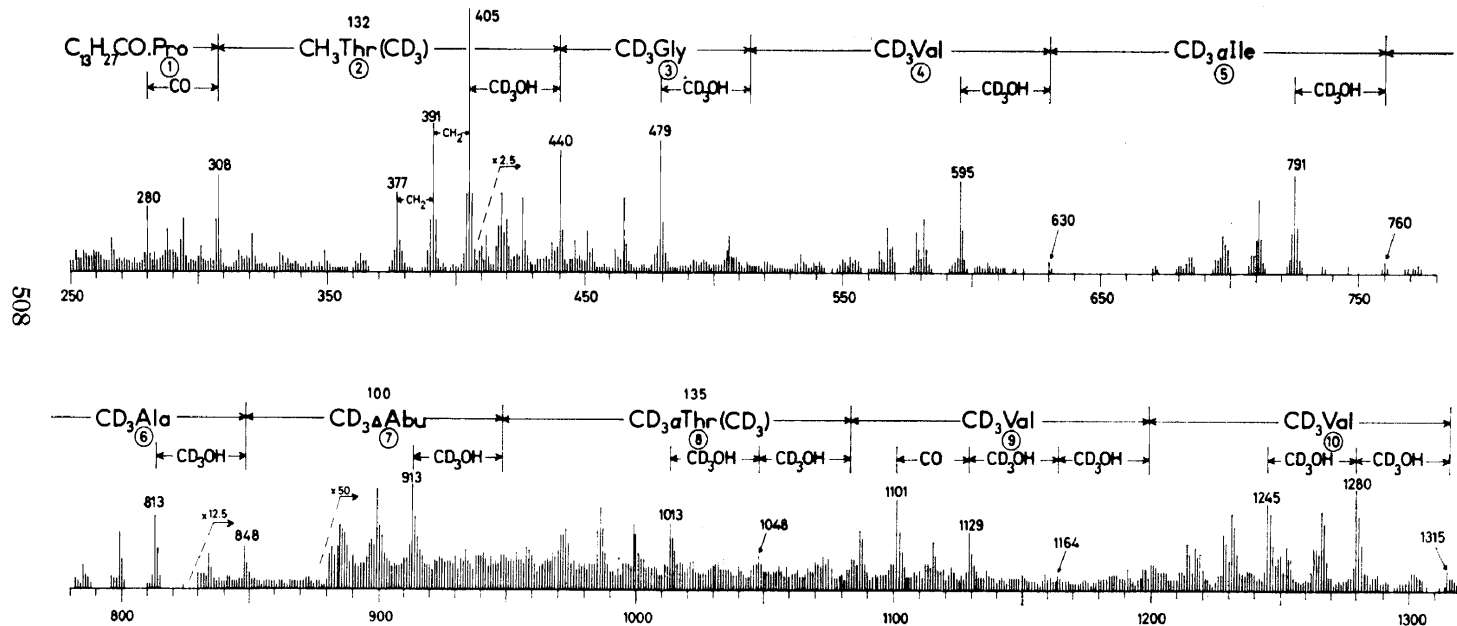
507

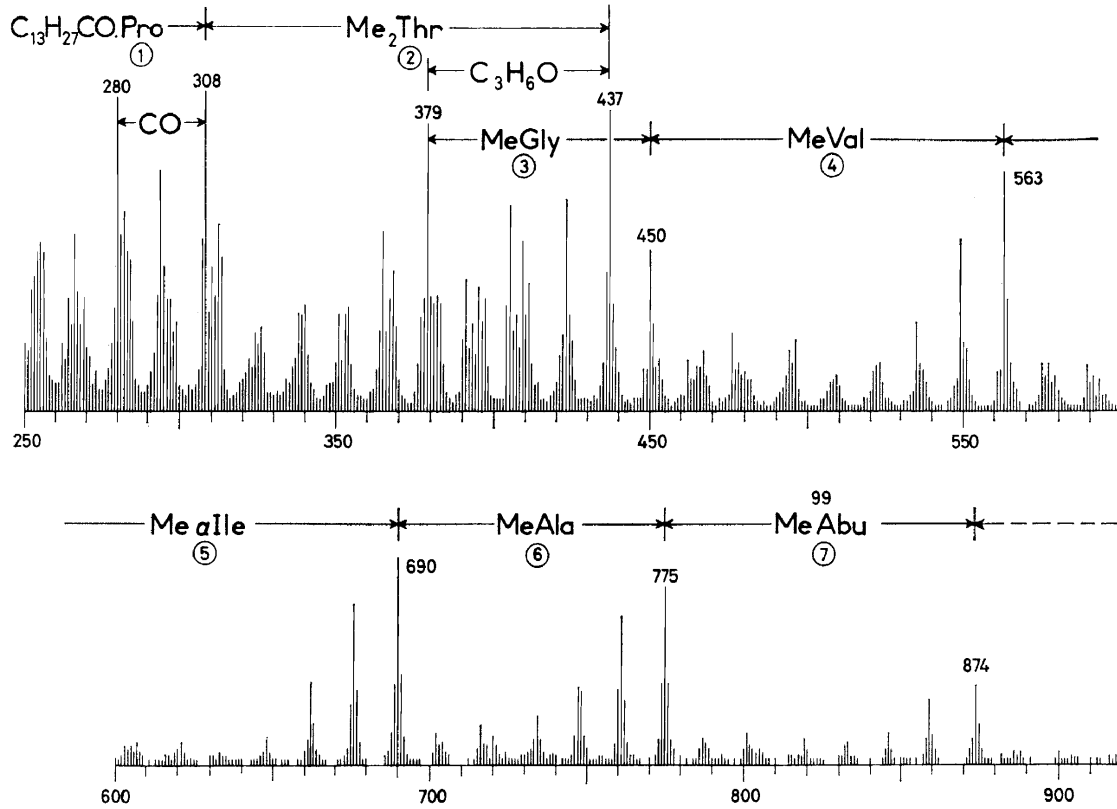*Figure 8.* Mass spectrum of perdeuteriomethylated stendomycic acid (Thomas *et al.*[73])

Figure 9. Mass spectrum of permethylated dihydrostendomycic acid (Thomas *et al.*[73])

acylation and permethylation. The result was in agreement with the proposed structure.

The gramicidins

The gramicidins A, B and C are polypeptide antibiotics produced by *Bacillus brevis*, first described by Hotchkiss and Dubos[74]. Sarges and Witkop[75, 76] have shown that they are N-formyl pentadecapeptides linked to a C-terminal ethanolamine residue; the complete structures as determined by these authors are shown in *Table 2*.

The synthesis of gramicidin A has been described by Sarges and Witkop[77]; the synthetic compound was found to be identical in all respects with the natural one.

Due to the courtesy of Dr. Witkop we have received generous samples of the natural gramicidins A and B. Mass spectrometry of these compounds showed some sequence peaks up to the seventh N-terminal residue (W. A. Wolstenholme, unpublished experiments). After permethylation, however, the sequence could be recognized *up to and including the twelfth residue (Figures 10 and 11)*.

On closer examination of these spectra, an unexpected result was discovered. The tryptophan residue in position 9 of both gramicidins A and B did *not* give the theoretical residue weight, but 30 m.u. more, indicating the presence of a methoxyl group in the permethylated compound. An experiment of perdeuteriomethylation with CD_3I showed that the "supplementary OMe group" was apparently due to the methylation of an OH group in Trp[9]†.

Table 2. Structures of the gramicidins, A, B and C (R. Sarges and B. Witkop[76])

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Valine-gramicidin A :
HCO–*Val*–Gly–Ala–leu–Ala–val–Val–val–Trp–leu–*Trp*–leu–Trp–leu–Trp–NHCH₂CH₂OH

Isoleucine-gramicidin A :
  –*Ileu*–   –*Trp*–

Valine gramicidin B :
  –*Val*–   –*Phe*–

Isoleucine gramicidin B :
  –*Ileu*–   –*Phe*–

Valine-gramicidin C :
  –*Val*–   –*Tyr*–

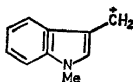*Isoleucine–gramicidin C :*
  –*Ileu*–   –*Tyr*–

NB: Val = L–Val; val = D–Val; etc.

Further examination of the mass spectrum showed that the "mysterious Trp[9]" residue was not hydroxylated on the indole nucleus‡, nor on the β-carbon because the mass spectrum shows no ion at m/e 174, which is expected to be a prominent one for a tryptophan methoxylated in these positions§.

† Trp gives on methylation (Me₂)Trp, a hydroxy Trp would give MeO(Me)₂Trp.
‡ An OH on the indole nucleus was also ruled out by Dr. Witkop who found no u.v. shift in alkaline medium (private communication).
§ *N*-methyl tryptophane gives a strong ion at m/e 144 corresponding to

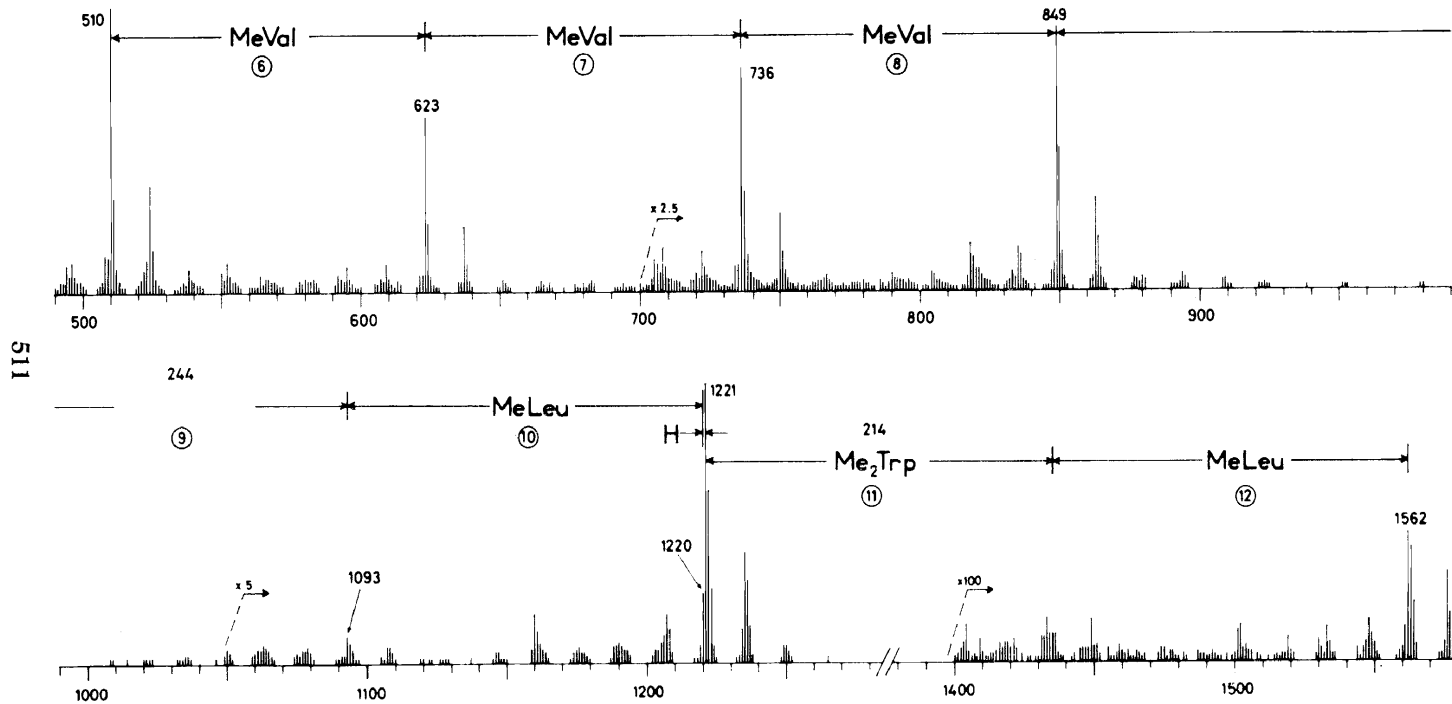Any methoxy tryptophane derivative having an ⸰OCH₃ on this part of the molecule would give an ion at m/e 174.

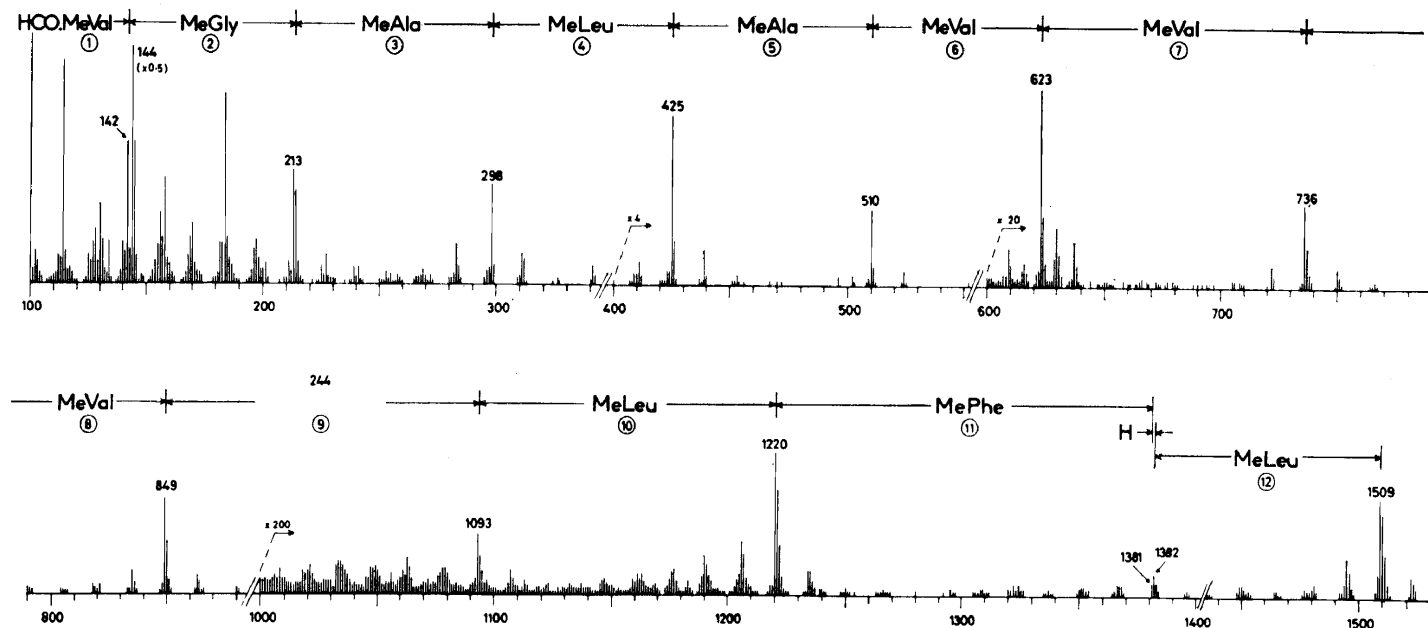*Figure 10.* Mass spectrum of permethylated natural gramicidin A (Thomas *et al.*[79])

Figure 11. Mass spectrum of permethylated gramicidin B (Thomas *et al.*[79])

Obviously there are two explanations for these results: (i) Trp[9] in the gramicidins A and B is in reality a 2-hydroxytryptophane (a very unusual and labile structure indeed), or (ii) the $MeO(Me)_2Trp^9$ is an artefact obtained by the methylation procedure.

Dr. Thomas had checked that N-acetyl tryptophane itself gives, after methylation the expected product; also methylation of Ac–Gly–Trp–OMe gave Ac–MeGly–Me$_2$Trp–OMe without a trace of a M + 30 peak. Moreover the Trp in position 11, in the same molecule of gramicidin A and having the same adjacent amino acids, also behaved normally. Thus an artefact seemed very improbable. A dehydrotryptophane such as found recently by Sheehan *et al.*[78] in the antibiotic telomycin was also excluded because the gramicidin A and B do not have the corresponding u.v. absorption at 339 nm.

The easiest way to settle the question was to study the mass spectrum of permethylated *synthetic* gramicidin A prepared by Sarges and Witkop[77]. This could be done only a few days before this lecture. Again an "abnormal Trp[9]" peak was found, thus indicating the occurrence of an abnormal reaction of this residue or of an unusual fragmentation mechanism. This problem will have to be investigated in more detail. In particular mass measurement of the peak at m/e 1221 (*Figure 10*) would be very desirable, but is difficult to perform in this high mass region.

In the spectrum of the synthetic gramicidin A, the sequence of 14 residues (up to m/e 1903) could be recognised.

Mass spectrometry as a tool for checking the purity of synthetic peptides

In the course of our work we have received, through the kindness of several colleagues, a large number of synthetic peptide derivatives. Some of these were examined before, some after permethylation; most of them gave the expected mass spectra. In several cases, however, the mass spectra either showed the presence of several impurities, or were entirely incompatible with the theoretical structures.

Sometimes it was evident that one or the other of the protecting groups had not been removed, or that a coupling reaction had not succeeded. Two special cases might be described in more detail†.

1. A hexapeptide related to eledoisin: H–Ala–Phe–Ile–Gly–Leu–Met–NH$_2$ prepared by Schröder *et al.*[80] was acetylated and then permethylated. The mass spectrum of the resulting product was in agreement with the expected N-acetyl permethylated hexapeptide derivative, with sequence peaks visible up to but not including methionine (see above). The spectrum showed, however, clearly the presence of another compound containing an additional oxygen atom in the N-terminal residue, or its protecting group (confirmed by a mass measurement of m/e 144) (*Table 3*). Furthermore, methylation of the initial peptide without prior acylation yielded a spectrum of this "oxygen homologue" in pure form.

It was concluded that part of the original peptide fraction must already be acylated and contains a methoxycarbonyl protecting group(CH_3OCO– Ala). This quite unexpected result was very puzzling for Dr. Schröder and his colleagues, but could finally be explained after analysis of the batch of

† Unpublished experiments by B. C. Das, S. D. Géro and D. W. Thomas.
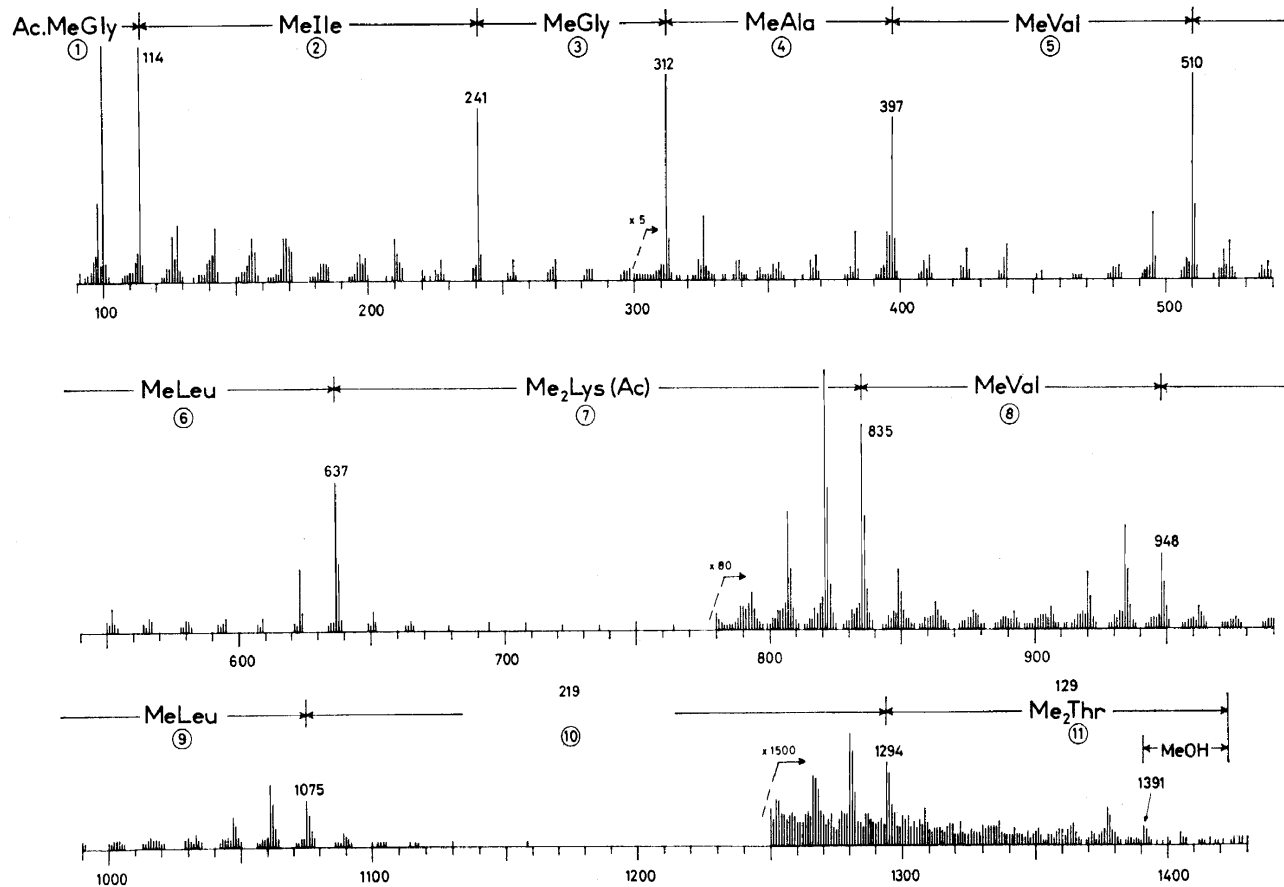
Figure 12. Mass spectrum of a synthetic tetradecapeptide derivative

t-butoxy-carbonyl chloride used for the synthesis of the hexapeptide; it was found that it contained a certain amount of methoxycarbonyl chloride, which had thus been incorporated into the peptide and then escaped "deprotection".

Table 3. Detection of an unexpected impurity in a synthetic peptide: Ala–Phe–Ile–Gly–Leu–Met–NH₂

(The mass spectrum of the acetylated and permethylated peptide shows two series of peaks with a mass difference of 16 m.u.)

		128	289	446	487	614	
Theoretical structure	CH₃CO-MeAla	MePhe	MeIle	MeGly	MeLeu	MeMet–NMe₂
		144	305	432	503	630	
"Impurity"	CH₃OCO-MeAla	—	— —	—	—	—

Mass measurement of the peak at m/e 144. Calculated for $C_6H_{10}NO_3$ = 144·0661. Observed: 144·0665

2. A tetradecapeptide corresponding to the partial sequence 1-14 of the bee venom melittin H–Gly–Ile–Gly–Ala–Val–Leu–Lys–Val–Leu–Thr–Thr–Gly–Leu–ProOH, also received from Dr. Schröder[81], gave after acetylation and permethylation a volatile derivative, the mass spectrum of which showed the sequence of eleven N-terminal amino acid residues, consistent with the expected structure with one exception (*Figure 12*). The methylated amino acid residue at position 10 had a mass of 219 and contained only one methyl group introduced by methylation (mass 222 after methylation with CD₃I): the expected dimethyl threonine should have a mass of 129 (or 97 after loss of MeOH), as observed for residue 11. The most probable explanation for this discrepancy is that Thr at position 10 is present as a benzoate. (Unfortunately, mass measurement was not possible for this minor peak at such a high mass). A re-examination of the conditions of synthesis did not give any clue as to the possible origin of the supposed benzoyl group (E. Schröder, private communication)†.

CONCLUSION

We hope to have shown in this lecture that mass spectrometry promises to become more and more useful for the sequence determination of natural and synthetic peptide derivatives, especially as a result of the permethylation technique. Further improvements in methodology and instrumentation will certainly extend the range and precision of this method.

† I am particularly grateful to Dr. E. Schröder for his permission to mention these two cases in this lecture.

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Note added in proof

More recently, it has been found that the permethylation technique of Hakomori-Vilkas[41, 42], as well as the use of NaH + CH_3I in dimethylfor-mamide, as described by J. Coggins and L. Benoiton (*Abstracts of the American Chemical Society*, BIOL. 18, Sept. 1968) allows the preparation of permethylated aspartic and glutamic acid containing peptides, without formation of artefacts; the gramicidins could also be permethylated without oxidation of the Trp[9] residue mentioned above (D. W. Thomas, *Biochem. Biophys. Res. Comm.* **33**, 483, 1968). The use of the two above-mentioned techniques has lead to the elucidation of the structure of the peptide antibiotic esperin (D. W. Thomas and K. Ito. *Tetrahedron*, in the press).