ADVANTAGES AND LIMITATIONS OF THE MASS SPECTROMETRIC SEQUENCE DETERMINATION OF PERMETHYLATED OLIGOPEPTIDE DERIVATIVES *

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Summary. - The mass spectrometric sequence determination of O,N-permethylated oligopeptide derivatives can be extremely facile because their spectra consist principally of peaks arising from CO-NCH_3 cleavage. In addition, the increased volatility resulting after permethylation has permitted even compounds as large as pentadecapeptide derivatives to be analyzed by this method.

The previous two publications in this series have described N-permethylation as a convenient method to increase the volatility of oligopeptide derivatives (Das et al.,1967, 1968). As a direct consequence, the application of mass spectrometry to the determination of amino acid sequences can be extended to higher oligopeptides which have insufficient volatility before methylation. We have continued work on the permethylation technique with several purposes in mind : a) to investigate the behavior of peptides which contain more complex amino acids, b) to examine the upper size limit of permethylated oligopeptides which can be vaporized in the mass spectrometer, and c) to develop improved experimental conditions applicable to submilligram quantities. Some significant and partly unsuspected results of these investigations are presented here.

Simplified Mass Spectra. The limited volatility of peptide derivatives has frequently been regarded as the major difficulty in the determination of amino acid sequences by mass spectrometry. However, even if a

* Part XII in the series "Determination of amino acid sequences in oligopeptides by mass spectrometry ; part XI, Das et al., 1968.

spectrum can be readily obtained, its interpretation can sometimes be difficult. The mass spectral peaks most useful for determining the amino acid sequence of a peptide result from cleavage at each CO-NH bond, with charge retention on the acyl fragment ; but it should be emphasized that fragmentations of amino acid side chains and N-terminal acyl groups also commonly occur, leading to very intense peaks which can dominate a spectrum. Consequently, interpretation can be troublesome as well as time-consuming. As a solution to this problem Barber et al. (1966), Biemann et al. (1966) and Senn et al. (1966) have proposed computer-aided interpretation of high resolution mass spectra. However, this elegant method is not available to numerous laboratories, due to the difficulty of obtaining high resolution mass spectrometers and computer facilities.

In the course of our research on a large number of N-permethylated peptides, it became apparent that mass spectral fragmentation was in all cases extremely simplified as a result of the methylation. The spectra consist almost exclusively of the "sequence-determining" peaks which result from $CO-NCH_3$ cleavage, the intensities of successive peaks decreasing in a regular manner toward higher mass. As a consequence, the amino acid sequence can be determined within a few minutes simply by measuring the mass differences between the major peaks of the spectrum. No computer analysis or mass measurements at high resolution are necessary, and in fact would contribute no useful information except in the case of new amino acids.

This important result, which makes the N-permethylation procedure so attractive for oligopeptide sequence determination, can be demonstrated by a comparison of Figures 1 and 2. The first is a mass spectrum of the octapeptide derivative 1*. Despite the fact that only simple amino acid residues are present, some initial difficulty in interpretation of this spectrum would be experienced, especially if the compound were of unknown structure. Replacement of the N-benzyloxycarbonyl group of 1 by an N-acyl function eliminates the interfering fragmentations caused by the former, but some of the "sequence-determining" peaks are even more difficult to recognize (Bricas et al., 1965).

* Spectra were obtained with an A.E.I. model MS9 mass spectrometer.

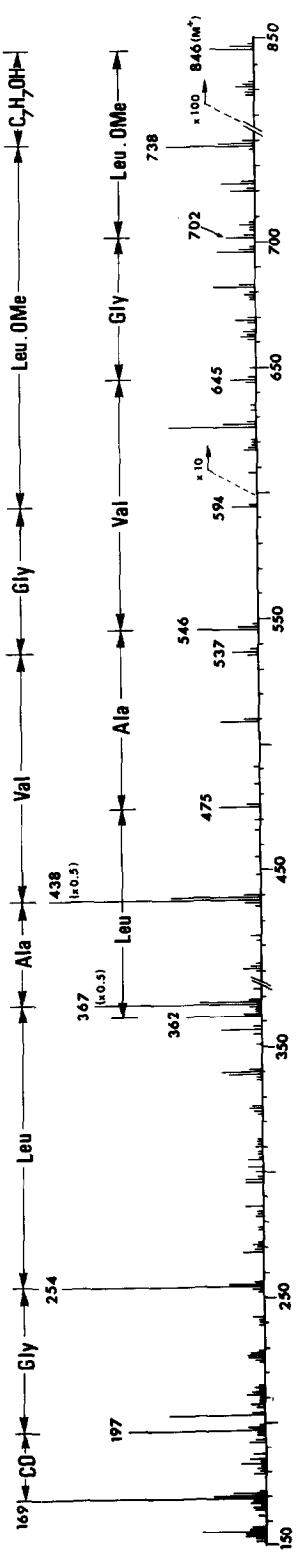

Figure 1. Mass spectrum of Z.Ala.Val.Gly.Leu.Ala.Val.Gly.Leu.OMe (1)

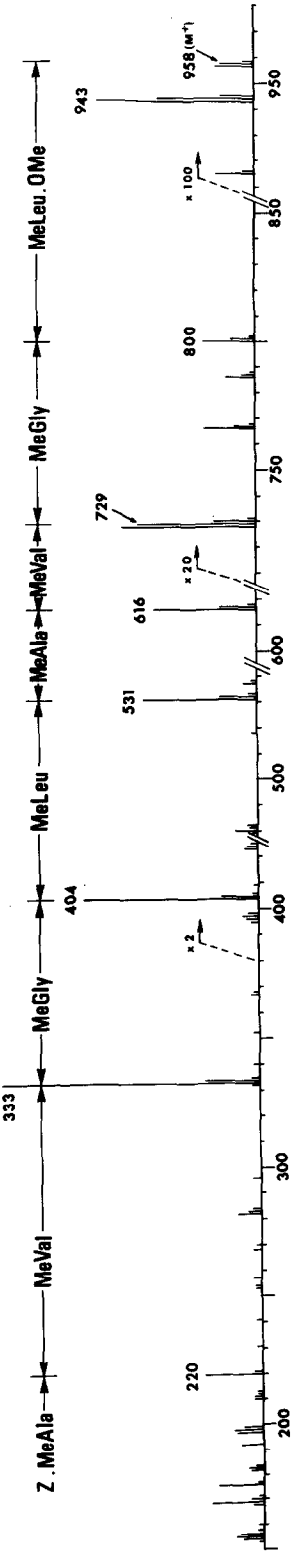

Figure 2. Mass spectrum of Z.MeAla.MeVal.MeGly.MeLeu.MeAla.MeVal.MeGly.MeLeu.OMe

A striking change occurs in the mass spectrum of the same peptide after N-methylation (Figure 2). The fragmentations involving the benzyloxycarbonyl group and losses of carbon monoxide and alkyl substituents, which had occurred before methylation, are no longer observed. The major peaks of the spectrum clearly delineate the amino acid sequence without the need of a detailed interpretation. Thus, because of the resultant simplification of mass spectral fragmentation (observed regardless of the type of N-terminal protecting group), N-permethylation can even be advantageous for the sequence determination of simple peptides where volatility poses no problem.

Larger Oligopeptides. To investigate the applicability of the permethylation technique to larger, less-volatile peptides, the decapeptide derivative 2 was considered. After methylation, a mass spectrum (Figure 3) was obtained which showed not only the expected molecular weight of 1580, but also the entire amino acid sequence. A few "non-sequence" peaks (e.g. m/e 1155 and 1297) occur in this spectrum, the origins of which are as yet unknown; however, they are not a serious handicap to the sequence determination.

The success with this decapeptide derivative, which contained four trifunctional amino acid residues (two each of lysine and tyrosine) prompted us to try even larger oligopeptides, and in fact, sufficient volatility resulted after methylation of some tetradeca- and pentadecapeptide derivatives. For example :

a) The pentadecapeptide derivative 3 from the naturally occurring antibiotic stendomycin (Bodanszky et al., 1968b and references therein) gave a mass spectrum after permethylation which enabled us to establish correctly the sequence of the first ten amino acid residues without prior knowledge of the sequence which had been chemically determined by Bodanszky et al. (1968a).

b) Methylation of the tetradecapeptide 4, a synthetic peptide related to the bee-venom constituent melittin (Lübke and Schröder, 1967) produced a volatile derivative, the spectrum of which consisted exclusively of "sequence-determining" peaks up to and including the first eleven amino acid residues.

c) The pentadecapeptide derivatives gramicidin A (5) and B (6) (Sarges and Witkop, 1965) gave volatile derivatives after methylation, the

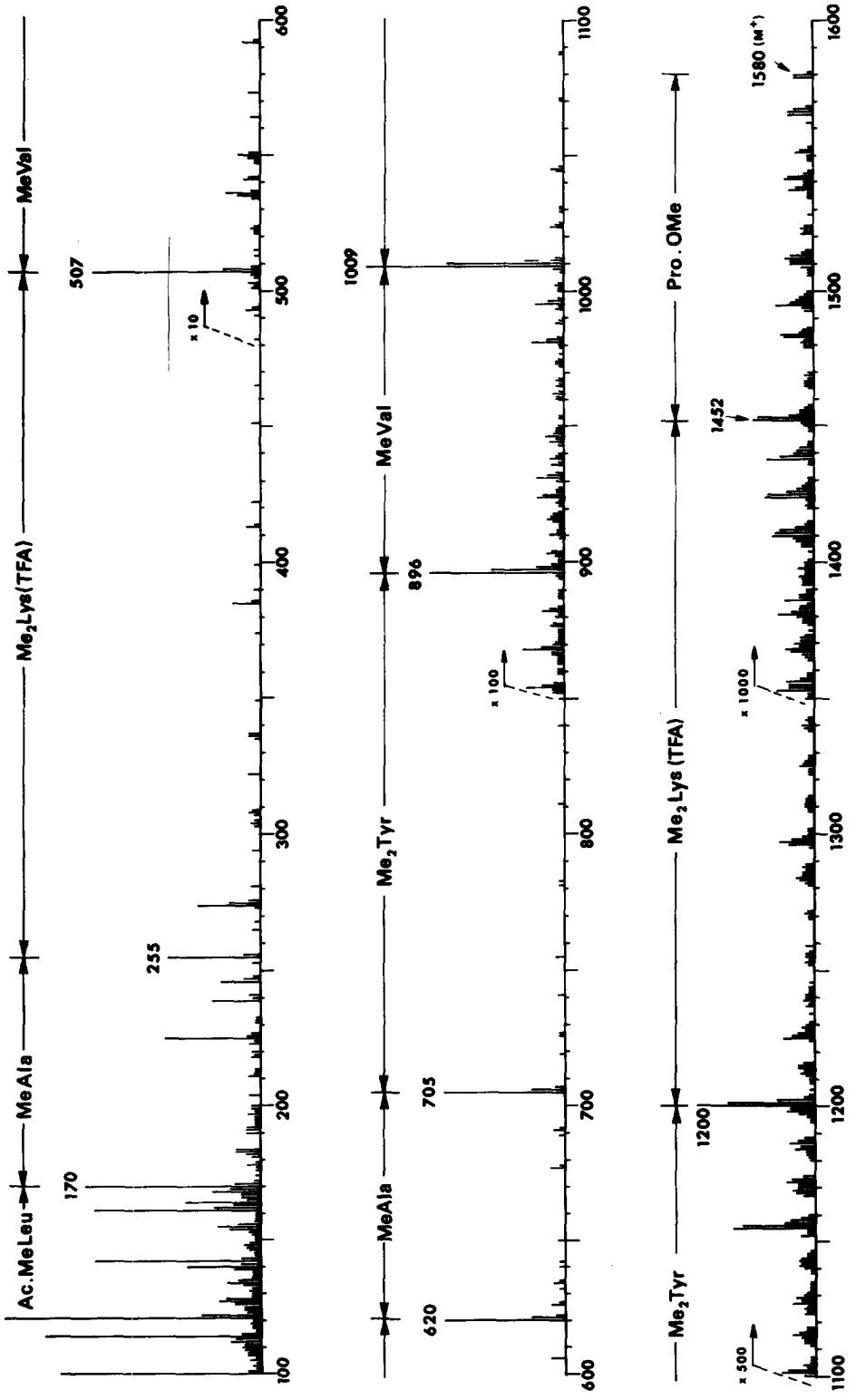

Figure 3. Mass spectrum of Ac.MeLeu.MeAla.Me_2Lys(TFA).MeVal.MeAla.Me_2Tyr.-MeVal.Me_2Tyr.Me_2Lys(TFA).Pro.OMe

mass spectra of which showed sequence peaks for the first twelve residues.

More detailed results of these examples, including structural implications, will be reported separately. It is surprising that these large methylated peptide derivatives have appreciable volatility, especially considering the presence of a significant number of trifunctional and aromatic amino acid residues. On the other hand, a new and initially unexpected difficulty is now encountered: no mass spectral peaks have been detected beyond twelve residues. Because the intensity of each successive sequence peak in the mass spectrum of a peptide is lower than the preceding (frequently ten per cent or less), the ions beyond a certain point (ten to twelve residues) become a very small percentage of the total ionization and are below the sensitivity of the mass spectrometers now available.

Discussion of the methylation technique. The permethylation method as previously described was inconvenient because of its three-day time requirement (Das et al., 1967). We have now shortened the time to four hours by heating the reaction mixture at fifty degrees in a sealed tube (a screw-cap vial ; 0.3-20 mg peptide, 50 mg silver oxide, 0.3 ml dimethylformamide, 0.2 ml methyl iodide). The product is isolated as previously described, and since the reaction is nearly quantitative, no final purification is necessary. This permethylation technique is therefore applicable to the microgram quantities frequently encountered in studies on naturally occurring peptides and their hydrolysis products. For example the mass spectrum of Figure 2 was obtained after methylation of 0.3 mg of the peptide derivative 1. The lower limit appears to be governed by the requirements of the mass spectrometer ; much compound is lost by thermal decomposition at the high temperatures (up to 300°) required for vaporization, thus a relatively large sample size of 50 to 100 micrograms is necessary to produce sufficient intensity of the very weak high-mass peaks.

Preparation of the silver oxide which is used for the permethylation reaction requires some discussion. Freshly prepared silver oxide is not necessary and in fact a commercially available product is completely satisfactory (J. van Heijenoort, personal communication). However, certain authors have been unable to obtain N-permethylated products, probably due to the use of silver oxide which has been washed with water but not dried

(M. Guinand, personal communication). For example, peptidolipic acid (7) gave only an O-permethylated derivative of molecular weight 1037 (Guinand and Michel, 1966). In our procedure, the silver oxide is washed successively with water, methanol and ether, then dried under vacuum and stored over phosphorous pentoxide. With this dry reagent we have repeated the methylation of 7 and obtained the N,O-permethyl derivative (MW 1121).

The importance of the silver oxide can be illustrated by another case. Agarwal et al. (1968)* have recently applied a similar permethylation procedure to peptides, and have pointed out that cleavage of the peptide amide bonds may occur, especially when glutamic acid or methionine are present. However, these authors use silver oxide containing methanol, and their results are interpreted as a base-catalysed methanolysis. In the absence of methanol or water, no solvolysis would be expected to occur. In fact, we have investigated a number of glutamic acid peptides, including one (8) very similar to those studied by Agarwal et al., and in no case has evidence for peptide cleavage been obtained.

Scope and Limitations. The permethylation reaction has now been successfully extended to peptides containing a large variety of more complex amino acids. A few examples are indicated by structures 9 - 13. Glutamic acid, phenylalanine, tyrosine, and tryptophane become permethylated (including the indole N-H of tryptophane) and mass spectral cleavage occurs principally at the $CO-NCH_3$ bonds. Threonine and serine also are O-, N-permethylated, but the mass spectra show partial (or occasionally complete) loss of methanol from all fragments containing one of these residues. During methylation, the amide of asparagine and glutamine is converted to the expected dimethylamide, but occasionally dehydration has been observed (probably occurring during prior acetylation of the peptide) to give a nitrile. Lysine and ornithine residues are also N-methylated at the ω-N-acetyl group, but at a slower rate, and the product usually contains a significant amount of a lower homolog. In fact, if a t-butoxycarbonyl protecting group is present, no methylation at all occurs at this nitrogen.

All the abnormalities mentioned above, however, are readily recog-

* We thank Professor Kenner for communicating a preprint to us.

nized in the mass spectra and give no problems in interpretation. More unusual results are obtained if the peptide contains histidine, arginine, methionine, or aspartic acid residues; some possible solutions which exist for such peptides will be discussed in subsequent communications.

1 Z.Ala.Val.Gly.Leu.Ala.Val.Gly.Leu.OMe

2 Ac.Leu.Ala.Lys(TFA).Val.Ala.Tyr.Val.Tyr.Lys(TFA).Pro.OH

3* RCO.Pro.NMeThr.Gly.Val.aIle.Ala.ΔBut.aThr.-
-Val.Val.aThr.Ser.aIle."B".OH

4 Ac.Gly.Ile.Gly.Ala.Val.Leu.Lys(Ac).Val.Leu.Thr.Thr.Gly.Leu.Pro.OH

5 HCO.Val(Ile).Gly.Ala.Leu.Ala.Val.Val.Val.-
-Trp.Leu.Trp.Leu.Trp.Leu.Trp.$NHCH_2CH_2OH$

6 HCO.Val(Ile).Gly.Ala.Leu.Ala.Val.Val.Val.-
-Trp.Leu.Phe.Leu.Trp.Leu.Trp.$NHCH_2CH_2OH$

7 RCO.Thr.Val.Ala.Pro.aIle.Ala.Thr.OH

8 Ac.Glu(OBu^t).Glu(OBu^t).Glu(OBu^t).Glu(OBu^t).Glu(OBu^t).Ala.Tyr.Gly.OMe

9 Ac.Orn(Ac).Orn(Ac).Pro.OMe

10 Z.Lys(BOC).Ile.Val.Gly.OBu^t

11 Ac.Asp(NH_2).Ala.Phe.Ile.Gly.Leu.OH

12 BOC.Glu(OBz).Ala.Glu(NH_2).Ala.Lys(TFA).Leu.Asp(NH_2)Ile.OH

13 Z.Ala.Glu(NH_2).Ala.Phe.Pro.Leu.Glu.Phe.OH

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* For the structure of ΔBut and "B", see Bodanszky et al. (1968 a).

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