The application of pyrolytic methylation and esterification to the mass spectrometric sequencing of peptides

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THE APPLICATION OF PYROLYTIC METHYLATION
AND ESTERIFICATION TO THE MASS SPECTROMETRIC
SEQUENCING OF PEPTIDES

A THESIS

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by

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This Thesis is dedicated to my wife

Dorothy

and to my children

David and Karen-Anne
SUMMARY

A requisite for successful biochemical applications of mass spectrometric sequencing of peptides is the development of a suitable micromethod for transforming the peptide into a volatile derivative. We have now developed a new mass spectrometric sequencing procedure which offers advantages in speed of analysis and convenience over previously described methods.

The new procedure depends upon the conversion of an N-protected peptide trimethylphenylammonium salt into a volatile peptide methyl ester using the solid probe heater of a mass spectrometer as a reactor. Under these conditions the residues of Tyr, Asp and Glu are completely methylated in the side chain, Trp and His are partially methylated whilst Gln and Asn peptides are stable under the derivatisation conditions. Apart from these desirable side reactions, the mass spectra of the volatile derivatives obtained by the new pyrolytic esterification procedure are essentially identical to those obtained by conventional methods.

A number of reagents were investigated as to their suitability for N-protection of peptide trimethylphenylammonium salts, and the resultant condensates were examined by low resolution e.i. mass spectrometry. Acetylacetonyl derivatives were found to be most suitable for this purpose, as their e.i. mass spectra were characterised by intense "amine" fragment ions, such as \( \text{CH}_3\text{CO CH = C(CH}_3 \text{)} + \text{NH = CHR} \), which could be used to identify the
N-terminal amino acid residue. In addition, these ions were also useful as starting points in the search for the other sequence ions. Peptides containing between 2 to 7 amino acid residues could be sequenced by the new method, but attempts to sequence longer peptides were rarely completely successful.

To cope with the larger peptides, a combined enzymatic and mass spectrometric procedure was developed. The method makes use of the enzyme Dipeptidylaminopeptidase-I which is known to hydrolyse a polypeptide from the N-terminus to give a mixture of dipeptides. Such a dipeptide mixture could be derivatised with trimethylphenylammonium hydroxide and acetylacetone, and following pyrolytic esterification within the mass spectrometer the component dipeptides could be identified without separation by c.i. (helium/H$_2$O) mass spectrometry. To reduce the derivatisation time, ethylacetoacetate was used in place of acetylacetone, while identification of the component dipeptides was aided by the use of dimethyltrideuteromethylphenylammonium hydroxide instead of trimethylphenylammonium hydroxide, to produce artificial isotope clusters. With this reagent every methyl group that is transferred to the peptide has an isotope ratio of 2:1 at 3 amu apart, and the number of methyl groups acquired by the N-terminal amino acid residues and the molecular ions can be readily established. The N-terminal residues and the protonated molecular ions could be readily matched from their isotope ratios in a dipeptide mixture. However, the identification of a dipeptide mixture in itself is not sufficient for the elucidation of the sequence of the original polypeptide.
In order to do this, it is necessary to apply the enzyme to another sample of the polypeptide from which the N-terminal amino acid has been removed. From this hydrolysis a second set of dipeptides are formed which correspond to the odd cleavage points of the polypeptide. Following identification of the two mixtures as their ethoxycarbonylpropenyl dipeptide methyl ester derivatives by c.i. (helium/H$_2$O) mass spectrometry the correct sequence of the polypeptide could be deduced. A selection of oligopeptides (4-8 residues) were sequenced by this procedure. It is believed that polypeptides of up to 25 residues should be amenable for sequencing by this route.
TABLE OF CONTENTS

Summary

Introduction

I. Protein Structure

II. Primary Structure Determination
   A. General
   B. N-Terminal Methods
   C. C-Terminal Methods
   D. Sequential Degradation
   E. Mass Spectrometric Methods

III. Aim of the Present Work

Discussion

A. Esterification of Acetyl Amino Acids
B. Permethylation of Acetyl Amino Acids
C. Pyrolytic Esterification of Acetyl Peptides
D. Esterification of Acyl and Sulphonyl Protected Peptides
E. Aldehydes and β-Diketones as Protecting Groups
F. The Mass Spectrometric Identification of Dipeptide Mixtures Obtained from Dipeptidylaminopeptidase-I Hydrolysates

Experimental

General

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B. Permethylation of Acetyl Amino Acids
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Bibliography

Acknowledgements
INTRODUCTION
I. PROTEIN STRUCTURE

The nature and basic functions of living organisms depend on proteins. No known form of life exists which does not rely on proteins. The proteins are the first recognizable and distinctive expressions of genetic information.

The chemical nature and the structure of proteins enables them to act as: the regulators (hormones) of physiological relationships; the oxygen carries; the participators in muscle contraction; the antibodies concerned with immunological defence mechanisms; or the catalysts of the cell (enzymes) which control the rate of all biological reactions. Other proteins may act as structural extracellular elements, for example as hair, wool and the collagen of connective tissue.

Because of the many functions and processes in which proteins are involved, they constitute as much as three-quarters of the dry substance of animal tissue. A major objective of protein chemistry is to explain the physiological functions of these molecules in terms of their structure.

The proteins have molecular weights ranging from approximately 5,000 to many millions. Each is a macromolecule and the repeating units are amino acids, of which twenty are commonly found in proteins. The amino acids that have been isolated are α-amino acids, that is, the carboxyl and amino groups are attached to the same carbon atom. Their general formula is,
where \( R^- \), called the amino acid side chain or residue represents a variety of structures. These side chains include, among others, both cationic and anionic groups, aromatic and aliphatic hydroxyls, amides, thiols, heterocycles, as well as strongly hydrophobic aliphatic and aromatic side chains. The usual classification of amino acids depends on the number of acidic and basic groups that are present. Thus, the neutral amino acids contain one amino group and one carboxyl group. The acidic and basic amino acids are characterised by an excess of either carboxyl groups or basic functions in their side chain.

The main mode of linkage between the amino acids in proteins is by a peptide bond (Diag.-1) which couples the \( \alpha \)-carboxyl group of one amino acid residue to the \( \alpha \)-amino group of another residue with the loss of one mole of water per residue.
If the resulting peptide is made up of less than ten amino acids, it is called an oligopeptide. Peptides containing more than ten amino acid residues are known as polypeptides. Individual proteins may consist of one or more polypeptide chains, each chain containing from approximately twenty to several hundred amino acid residues. The order in which the amino acids are joined by peptide bonds is termed the amino acid sequence or referred to as the primary structure.

The peptide bond has one hydrogen atom associated with it and being very weakly acidic, ionizes only under special conditions. It does form hydrogen bonds readily (Diag.-2), a property which is one of the factors responsible for the three dimensional structure of proteins.

\[ R-C-H \quad H-C-R \]
\[ C=O \quad H-N \quad C=O \]

**DIAG 2**

The hydrogen bonding between the hydrogen of the peptide amide and carbonyl groups within one polypeptide chain gives rise to folding or twisting of the primary structure, and this is called the secondary structure of a protein. The aggregation of individual structured units by hydrogen bonds, hydrophobic contacts, electrostatic interactions or covalent disulphide bonds has been called the tertiary structure. The general term "chain conformation"
has been used to combine the terms secondary and tertiary structure as the distinction between these two terms is not always clear. The quarternary structure describes the aggregation of several peptide chains to form a defined molecule.

II. PRIMARY STRUCTURE DETERMINATION

A. General

As the quarternary structure, and the resultant biological function of proteins are products of the primary structure, the amino acid sequence determination becomes a prerequisite to the complete understanding of the molecules.

As a preliminary to determining the amino acid sequence of a protein, it is necessary to establish its amino acid composition. Various methods are available for the analysis of protein hydrolysates but the main quantitative method used involves ion-exchange chromatography. For many proteins, the minimal molecular weight calculated from amino acid analyses is much smaller than the molecular weight obtained by physical methods. In these cases the proteins consist of a number of subunits which may or may not be identical. Thus, before attempting to determine the amino acid sequence of a protein, it is essential to determine the minimal physical unit, as well as the minimal chemical unit. If subunits do exist or are held together by non covalent forces, then they may be dissociated by treatment with reagents such as, urea or guanidine. In general, the smaller proteins of molecular weight
up to 30,000 are likely to consist of a single unit that cannot be dissociated, whereas many of the larger ones, for example, haemoglobin and glutamic acid dehydrogenase, consist of subunits which can be dissociated by such methods.

If protein chains are linked by disulphide bonds such as in the insulin molecule, these are usually cleaved to facilitate subsequent enzymic hydrolysis of the protein chain. This cleavage of disulphide bonds, resulting in conversion to free chains, can be accomplished by oxidation with performic acid, or reduction followed by alkylation with iodoacetic acid, its amide or bromoethylamine.

The usual approach to the sequencing of a single polypeptide chain, whose molecular weight and amino acid composition are known has been to:

1. partially hydrolyse the polypeptide,
2. isolate the resulting peptides,
3. determine the amino acid sequence of the peptides, and
4. deduce the complete primary structure from the sequence of peptides of overlapping structures.

Two general methods of partial hydrolysis of peptides and proteins are used:

a. partial acidic hydrolysis, and
b. hydrolysis catalysed by proteolytic enzymes.

A partial cleavage of a polypeptide or protein can be obtained
by limiting the time of acid hydrolysis, lowering the temperature or working with dilute acid. These procedures yield a mixture of free amino acids and a large variety of small peptides. The technique is useful with smaller peptides but is seldom employed with larger peptides or proteins, because the yield of peptides is low, hydrolysis is essentially random, and larger peptides are rarely obtained in significant yields.

Proteolytic enzymes in comparison catalyse only a limited cleavage, producing relatively large fragments in good yields. Each proteolytic enzyme will hydrolyse only certain types of peptide bonds. For example, (Diag.-3), trypsin catalyses the hydrolysis of -CONH- bonds in which the carboxyl group of lysine or arginine residues participate. Thus, from a knowledge of the lysine and arginine content of a given protein or polypeptide, the number of bonds susceptible to trypsin may be calculated.

\[
\text{Phe-Val-Arg-Ala-Leu-Gly-Lys-Asp-Ser}
\]

\[
\text{Trypsin} \quad \text{Trypsin}
\]

\[
\text{Diag.-3}
\]

Other proteinases, such as, chymotrypsin, pepsin and papain hydrolyse proteins or peptides at other specific loci in the peptide chain. For the complete sequence determination of a protein, at least two different forms of enzymatic hydrolysis must be used in order to deduce the structure by overlapping sequences.
A hypothetical case illustrating this approach is as follows. If a peptide obtained from a tryptic digest with the sequence Val-Leu-Trp-Ala-Met-Arg is treated with chymotrypsin the specificity of chymotrypsin is such that hydrolysis will occur at the Trp residue, liberating the peptides Val-Leu-Trp. and Ala-Met-Arg. The positioning of these two peptides relative to each other is already established by the enzyme specificities of trypsin and chymotrypsin, the peptide containing Trp being amino-terminal and the one containing Arg being carboxy-terminal. Further sequence work on these peptides can now be carried out, following separation and purification of the peptides by standard electrophoretic and chromatographic techniques. Early sequencing techniques were based on the use of specific reagents to label N-terminal or C-terminal residues.

Numerous reagents have been used for this end group determination, which still plays an important role in the elucidation of the primary structure, particularly for the estimation of the number of chains present in a protein.

B. N-Terminal Methods

Although a number of reagents have been used to form derivatives with the alpha-amino end of peptides, the reagent 1-fluoro-2, 4-dinitrobenzene first introduced by Sanger⁴ has been one of the most widely used. This reagent readily reacts in mildly alkaline solution, and following the removal of the excess reagent the peptide is subject to acid hydrolysis. The resulting Dinitrophenyl
(Dnp) amino acids are then identified by paper chromatography, thin layer chromatography, on polyamide thin layer sheets, or mass spectrometry.

An improvement on Sanger's procedure, using 2-chloro-3, 5-dinitropyridine instead of fluorodinitrobenzene can improve the yields of N-terminal amino acid following acid hydrolysis of the N-protected peptide. The pyridine nitrogen facilitates cleavage of the first peptide bond and shorter hydrolysis times can be employed, thereby reducing one of the principle causes of poor end group recovery in the earlier method.

A more sensitive modification of the above is the use of 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride). When this is coupled with the alpha-amino group of a protein or peptide a dansyl (Dns) derivative is formed which on acid hydrolysis yields a fluorescent Dns-amino acid. These derivatives are commonly used for sequencing experiments and are usually detected after chromatography, on polyamide sheets, although mass spectrometry has also been used for identification.

As these methods result in destruction of all the peptide bonds and loss of sequence information, other procedures are desirable which are not subject to this limitation.

The Edman method caters for this need, and can be used for the N-terminal analysis of peptides by conversion of the N-terminal amino acid to a phenylthiohydantoin derivative (Pth) whilst leaving the remainder of the peptide intact.
FIGURE I. EDMAN REACTION FOR STEPWISE DEGRADATION OF PROTEIN OR PEPTIDE CHAIN FROM THE N-TERMINUS

A. \[
\text{C}_6\text{H}_5\text{N} = \text{C} = \text{S} + \text{H}_2\text{N} - \text{CHR} - \text{CO} - \text{NH} - \text{CHR} - \text{CO} \rightarrow \text{C}_6\text{H}_5\text{NH} - \text{NH} - \text{CHR} - \text{CO} - \text{NH} - \text{CHR} - \text{CO}
\]

B. \[
\text{C}_6\text{H}_5\text{NH} - \text{NH} - \text{CHR} - \text{CO} - \text{NH} - \text{CHR} - \text{CO} \rightarrow \text{C}_6\text{H}_5\text{NH} - \text{NH} - \text{CHR} - \text{CO} - \text{NH} - \text{CHR} - \text{CO} + \text{H}^+
\]

C. \[
\text{C}_6\text{H}_5\text{NH} - \text{NH} - \text{CHR} - \text{COOH} + \text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_5\text{NH} - \text{NH} - \text{CHR} - \text{COOH} + \text{H}^+ + \text{H}_2\text{O}
\]

II

III
The first reaction (A) in the Edman procedure (Fig. - I) is based on coupling the free alpha-amino group with phenylisothiocyanate to form the phenylthiocarbamyl (PTC) derivative of the peptide (I). This reaction is referred to as the coupling reaction.

The next step (B) involves the cleavage of the PTC-peptide at the peptide bond nearest to the PTC substituent. This requires a strongly acid medium and results in the formation of a 2-anilino-5-thiazolinone derivative (II) and a peptide with one less amino acid than the original. This is referred to as the cleavage reaction. The shortened peptide has a free alpha-amino group, and may therefore be subjected to a new cycle of the degradation.

The thiazolinone cleaved off is a derivative of the N-terminal amino acid, but because of instability of these derivatives, they are not identified, but are converted to the isomeric and stable Pth derivative namely the 3-phenyl-2-thiohydantoin (III). This conversion reaction (C) takes place in an aqueous acid medium and consists of two reactions, i.e. the hydrolysis of the thiazolinone to the PTC-amino acid (C₁) and cyclization of the latter to the corresponding Pth amino acid (C₂).

The N-terminal amino acid can now be identified as its Pth derivative. The Pth amino acids are with few exceptions, Ser, Thr and Cys Cys, chemically stable. The hydroxy and mercapto compounds show, to a varying degree, a tendency for beta-elimination. This tendency is stronger in Pth-Ser than Pth-Thr and the formation of a highly reactive methylene group at C-5 of the thiohydantoin
ring leads to further reactions, e.g. polymerisation. These side reactions hamper identification of these derivatives, while in Pth-Cys Cys and Pth-Cys this tendency for beta-elimination is so strong that the derivatives are not useful for identification purposes. However, the S-alkylated Pth derivatives of Cys are more stable, as is also Pth-cysteic acid.

The Pth derivatives of Asn and Gln are unstable to the extent that the amide group is readily hydrolysed by the acidic conditions leading to their formation. In sequencing, these Pth-amino acids are found to be contaminated by the corresponding acids. Pth-Trp is also susceptible to the action of strong acids, presumably due to decomposition of the indole nucleus, but identification can still be made.

The Pth-amino acids may be identified by chromatography, and for this purpose paper chromatography\textsuperscript{14}, thin layer chromatography\textsuperscript{16} and gas liquid chromatography\textsuperscript{17}, systems have been proposed. Mass spectrometry either by electron impact\textsuperscript{18,19} or chemical ionisation\textsuperscript{20} has also been used for this purpose, and can be done without isolation of the Pth-amino acid\textsuperscript{21}.

An alternative approach to the sequence analysis of peptides from the amino terminal end, involves the use of the enzyme leucine aminopeptidase. This enzyme only acts on a free terminal α-amino group. The aminopeptidase acts most rapidly on a terminal leucine residue but it also liberates the other N-terminal amino acids commonly found in proteins except if a Pro residue is the penultimate
amino acid in the sequence. Leucine aminopeptidase acts in a sequential manner, and at any given time the concentration of the first N-terminal amino acid liberated will be greater than the concentration of the second amino acid liberated etc. By examining the relative rates of release of amino acid during the enzyme hydrolysis, the N-terminal amino acid can be identified. However, this method cannot be used to identify more than several amino acid residues at the amino-terminal end of a peptide.

C. C-Terminal Methods

The carboxyl-terminal amino acid identification can provide useful information for the primary structure determination of proteins and peptides. Several methods are available for the identification of the C-terminal residue, however, each method is subject to certain limitations.

The hydrazinolysis\textsuperscript{22} of peptides containing a free alpha-carboxyl group can be used for the identification of the C-terminal amino acid. The method involves treatment of the protein or peptide with hydrazine under anhydrous conditions, which converts all amino acid residues to amino acid hydrazides with exception of the carboxyl terminal residue which remains as the free amino acid. The hydrazides are precipitated as Schiff bases by the reaction with benzaIdehyde, while the C-terminal amino acid, which remains in solution, can be separated and identified by chromatography. However, the loss of the C-terminal Asn or Gln as $\gamma$-hydrazides has been reported\textsuperscript{23,24} and this imposes a severe limitation to the usefulness of the procedure.
The C-terminal residue in polypeptides can also be identified by a cyclisation to the corresponding oxazalone with acetic anhydride (Scheme-1).

\[
\begin{align*}
R-C-NH-CH-COCH & \xrightarrow{Ac_2O} R-C=N \\
\end{align*}
\]

Scheme-1

The hydrogen on the α-carbon atom in the oxazalone can be replaced with tritium by treatment with \(^3\)H\(_2\)O and pyridine. Hydrolysis produces a mixture of amino acids which can be separated by paper chromatography and the labelled C-terminal amino acid can be identified. The method can be used for the identification of all C-terminal amino acids present in proteins, with the exception of Pro which does not react under these conditions.

Another chemical approach to the C-terminal analysis of peptides, which does not result in the complete destruction of all the peptide bonds is based on the reaction of peptides with ammonium thiocyanate and acetic anhydride which forms the phenylthiohydantoins at the C-terminus. The thiohydantoin is cleaved from the peptide by treatment with acetohydroxamate, and a new C-terminal residue is exposed. The possibility of extending this procedure to the sequential degradation of peptides from the C-terminus has been re-investigated. A number of peptides have been degraded, and up to six residues have been identified. The method is limited in that carboxyl-terminal Asp and Pro are not
removed. Unfortunately, the step wise degradation becomes increasingly
difficult to interpret because the terminal residue is not removed
completely during each cycle.

An enzymatic method based on the use of carboxypeptidase has
also found application for the C-terminal analysis of peptides.
Carboxypeptidase-A liberates only those residues that contain a
free «carboxyl group, with the exception of Lys, Arg or Pro
residues, which may be incompletely hydrolysed or not liberated
at all, although another enzyme carboxypeptidase-B can be used
for C-terminal Arg or Lys peptides. The carboxyl terminal amino
acid can be identified by examining the rate of liberation of
successive residues. This procedure has been used successfully
to determine part of the amino acid sequence, but is also limited
to the determination of only a few C-terminal amino acid residues.

D. Sequential Degradation

The N-terminal step wise degradation first employed by Edman14
(Fig.-I) has been the most successful approach to peptide and protein
sequencing. Because the peptide is sequentially degraded, one amino
acid residue at a time, great control can be exercised over this
sequencing procedure, and sample sizes in the range of 0.1 - 0.5
micromoles are commonly used.

The Pth derivatives of the Edman degradation, are readily
identified, but other approaches aimed at increased sensitivity
have been used for the identification of the newly exposed N-terminal
amino acid, resulting from this procedure.
For example, the Dns-Edman technique\textsuperscript{27} has been widely used for determination of the amino acid sequence of peptides and proteins with high sensitivity. In this procedure a Dns estimation of the N-terminal amino acid residue is carried out after each successive stage of the Edman degradation. The Dns-amino acids can then be identified in the picomole range by thin layer chromatography on polyamide layer sheets\textsuperscript{28}.

The most outstanding achievement in protein sequencing was the development of an automated method, introduced by Edman and Begg\textsuperscript{29} known as the protein sequenator. In this instrument the reaction vessel is a spinning glass cup housed in a bell-jar. Reagents and solvents are supplied to the spinning cup and are spread by centrifugal force into a thin film on the inner surface of the cup where protein or peptide had previously been deposited. Solvents are added in excess and rise over the protein film, reach an annular groove and are removed through a Teflon tube.

The protein sequenator has overcome many problems associated with the Edman procedure, resulting in average conversions of approximately 92-94\% at each step of the degradation. The high efficiency of this procedure has allowed for the determination of up to sixty successive residues of a protein.

The major application of the sequenator has been in the analysis of whole proteins or large polypeptide fragments, the study of subunit structure\textsuperscript{30}, the determination of purity or heterogeneity and to study the sequence of proteins in mixtures\textsuperscript{31}. 
However, when the Edman degradation is applied to short chain peptides, up to 30 residues, the extractive losses become a major problem and the yield no longer approaches 92-94% at each stage of the degradation. Because of these problems, which are associated with the loss of material from the cup during solvent extractions, the amount of polypeptide required often increases in inverse proportion to chain length. Thus, sequencing can be done with as little as 25 nanomole of protein, whereas 3.6 micromole is required for the sequencing of a hexapeptide from collagen.

The problem of the Edman procedure associated with short chain peptides has focused attention on alternative approaches.

The 'Solid-Phase Edman Degradation' has been applied specifically for the sequence determination of small peptides up to thirty residues. By this method peptides are chemically bonded to a solid support and are then degraded sequentially in the solid-phase sequencer.

For example, the N-terminal residue of the peptide is protected by reaction with t-butyloxycarbonyl azide to form the t-butyloxycarbonyl (t-Boc) peptide. This N-protected peptide is treated with carbonyldiimidazole under stringent anhydrous conditions, and is then attached to an amino polystyrene resin. The excess resin amino groups are blocked with methylisothiocyanate and the t-Boc protecting group is removed with trifluoroacetic acid, after which the normal Edman degradation scheme is followed.

The procedure has several disadvantages. The most serious problem
is that the β and γ carboxyl groups of Asp and Glu have to be selectively blocked to prevent their becoming attached to the resin along with the C-terminal carboxyl. In the case of Asp, the degradation fails to proceed past this residue. It has been suggested that the β-carboxyl group, after activation by carbodiimide, reacts with neighbouring amide nitrogen to form an imide, thus blocking further degradation\textsuperscript{36}. If a peptide contains Glu, this remains attached to the resin, and no derivative can be identified, thus creating a gap in the peptide sequence. Peptides with a Lys ε-amino group, such as many tryptic peptides, can be cross linked to aminopolystyrene or aminopropyl-glass by p-phenyldiisothiocyanate. Arg containing peptides can be converted by hydrazinolysis into peptides which can then be attached like Lys by p-phenyldiisothiocyanate. However, this procedure can lead to rupture of the peptide bonds. The Asp and Glu problem originally encountered in the 'Solid-Phase' sequencing method has been largely overcome by the use of a water soluble carbodiimide. The rationale of this procedure is that the t-Boc peptide is treated with N-ethyl, N\textsuperscript{1}-(3-dimethylaminopropyl)-carbodiimide to specifically activate the \textgreek{N}-terminal carboxyl group by conversion to an oxazolinone\textsuperscript{37}. Under these conditions side chain carboxyl groups from Asp or Glu initially form the O-acyl urea derivative at the side chain carboxyl group. This N-protected peptide can now be coupled to the amino-polystyrene resin, and the usual reaction scheme can be followed.
E. Mass Spectrometric Methods

Because of problems associated with the sequencing of small peptides, mass spectrometric methods were developed to cater for this special need.

The rationale that has been used can best be illustrated in the following scheme.

When a peptide is exposed to Electron Ionization (e.i.) the ionized peptide undergoes a process of fragmentation, and if sufficient energy is supplied cleavage occurs preferentially but not exclusively at the peptide bonds. Fragmentation occurs on both sides of the carbonyl function (Diag.-4) resulting in two types of ions, amine fragments \((A_n^+)\) and amino acyl fragments \((B_n^+)\), which can be readily identified by characteristic masses determined by the side chain substituents \((R)\).

\[
X - \text{NH} - \text{CHR} - \text{CO} \ldots \text{NH} - \text{CHR} - \text{CO} \quad \text{OZ}
\]

\[
\begin{align*}
A_1 & \quad J \quad \text{(A)} \\
B_1 & \quad J \quad \text{(B)} \\
A_n & \quad J \quad \text{(A)} \\
B_n & \quad J \quad \text{(B)} \\
\end{align*}
\]

Diag.-4

The total peptide can often be reconstructed by noting the various fragments (Diag.-5) starting from the N-terminal end and identifying each residue by consecutive mass difference along the entire sequence.

These fragment ions are commonly observed in the e.i. mass spectra of peptide derivatives, but because of the high energy associated with this form of ionization other processes of fragmentation, such as side chain cleavages and rearrangements also take place.
Expected Fragmentation Pattern

<table>
<thead>
<tr>
<th>X</th>
<th>X^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_1</td>
<td>X - NH - CHR^+</td>
</tr>
<tr>
<td>B_1</td>
<td>X - NH - CHR - CO^+</td>
</tr>
<tr>
<td>A_n</td>
<td>X - NH - CHR - CO ............... NH - CHR^+</td>
</tr>
<tr>
<td>B_n</td>
<td>X - NH - CHR - CO ............... NH - CHR - CO^+</td>
</tr>
<tr>
<td>M</td>
<td>X - NH - CHR - CO ............... NH - CHR - COOZ^+</td>
</tr>
</tbody>
</table>

Diag.-5

For example, the mass spectra of Val containing peptides frequently exhibit ions resulting from elimination of the side chain as an olefin or as a radical (C\textsubscript{3}H\textsubscript{7}'). A similar cleavage is observed for Leu containing peptides with a loss of isobutylene or C\textsubscript{4}H\textsubscript{9}'. Part of the side chain can also be lost as C\textsubscript{3}H\textsubscript{7}' or propylene. The fragmentation of peptides containing residues of the hydroxyamino acids (Ser, Thr and Hyp) are accompanied by dehydration of the side chain. Ser and Thr residues are further characterised by the loss of the whole side chain, with hydrogen atom migration to the charged fragment. Peptides with residues of Asp, Asn, Glu and Gln under mass spectrometric conditions are characterised by conversion of the side chain carboxyloxy group or the carboxyamide group into a ketene (loss of 31 or 17 amu respectively). In addition, the carboxyloxy or carboxyamide group of these residues can be expelled completely by homolytic fission of the C-C bond, or with concomitant hydrogen atom migration.
The amino acid side chain of these residues can also be partially or entirely cleaved. The side chains of peptides containing residues of aromatic amino acids (Phe and Tyr) and heterocyclic amino acids (His and Trp) can be eliminated as the cation $\text{ArCH}_2^+$. These ions are usually intense and can be used as additional evidence as to the presence of these amino acids in the peptide. Alternatively, the side chain may be eliminated as the radical $\text{ArCH}_2^*$, which can be accompanied by hydrogen atom migration to the charged fragment in Trp and Tyr peptides. Met residues on electron impact frequently lose their entire side chain and this sometimes occurs with concomitant migration of the hydrogen atom to the charged fragment. Simultaneous fissions of the $\text{C}_\beta - \text{C}_\gamma$ and $\text{C} - \text{S}$ bonds in the Met side chain have also been observed, but the corresponding peaks usually are of low intensity. The mass spectra of Cys and Cys Cys peptides are frequently recorded on their S-carboxymethyl (CmCys, hereafter abbreviated CmC) derivatives, as the sequence assignment to free Cys or Cys Cys peptides is often difficult. The modified side chain may be cleaved completely, or alternatively partial elimination of the carbomethoxy group or methyl mercaptoacetate is often observed. Lys peptides in which the ε-amino group is protected, undergoes C-C and C-N bond cleavage in the side chain. The mass spectrometric fragmentation of Arg containing peptides is usually complex and for this reason, Arg residues are chemically modified before a mass spectrum is recorded.

Despite these side reactions which have been established using model peptides, it is often possible to deduce the correct amino acid sequence from a peptide's mass spectrum.
The ionization and fragmentation of peptides depends on their prior volatilisation, but as peptides are internal salts (one amino group for every COOH group), they have a very low vapour pressure and they often decompose when heated to temperatures which are required for vapourisation. It is, therefore, necessary to convert the peptides to more volatile derivatives which must retain all the original structural features. Furthermore, it is desirable that the derivatisation procedure employed is simple and quantitative.

The first attempt to apply mass spectrometry to peptide sequencing was made by Biemann\(^{38,39,40,41}\). He reduced acetyl peptide esters with lithium aluminium hydride (or deuteride) to form volatile polyamino alcohols (Scheme-2).

\[
\begin{align*}
R & \quad R' \quad O \\
\text{CH}_3\text{CONH} - \text{CH} - \text{CO} - (\text{NH} - \text{CHR} - \text{CO})_n - \text{NH} - \text{CH} - \text{C} - \text{OCH}_3 \\
\text{LiAlH}_4 & \\
\text{C}_2\text{H}_5 - \text{NH} - \text{CH} - \text{CH}_2 - (\text{NH} - \text{CH} - \text{CH}_2)_n - \text{NH} - \text{CH} - \text{CH}_2 - \text{OH}
\end{align*}
\]

Scheme-2

These derivatives fragmented under electron impact mainly by C-C bond splitting (Diag. -6) and this process resulted in a series of ions corresponding to a stepwise loss of amino acid residues from the C-terminal end, thus enabling the sequence of the original peptide to be deduced. However, the reduction of small quantities
of the larger acetyl peptide esters with good yields, was difficult. Biemann therefore suggested an alternative procedure which comprised the following steps:

1. non specific hydrolysis of the compound to a mixture of smaller peptides;
2. acetylation and esterification of the shorter peptides to convert them into compounds soluble in organic solvents;
3. reduction of the mixture of the N-acyl peptide esters with LiAlH₄, and
4. gas chromatographic separation of the resulting polyamino-alcohol mixture followed by mass spectrometric analysis of the separate fractions.

However, it was necessary to modify the hydroxyl groups present in the peptide (Ser, Thr) or in a Glu and Asp containing reduced peptide, into chlorides by treatment with thionyl chloride prior to LiAlH₄ reduction. Unfortunately, the intermediate amino-chlorides are
unstable and liable to undergo further reactions, which complicate the interpretation of the resulting mass spectra. His and Arg containing peptides were even more troublesome. Because of these problems and the complexity of the chemical treatment, this method has had only limited use for the sequencing of peptides.

In the period 1958-1961, Anderson and Stenhagen showed that methyl esters of trifluoroacetyl peptides were sufficiently volatile for mass spectral analysis, and that the spectra could be used to determine the primary structure of the peptide. A little later, Weygand confirmed that the mass spectra of N-trifluoroacetyl-peptide esters exhibited ions (CF₃CO-NH-CHR)⁺ and (H₂N-CHR-COOCH₃)⁺, and that these were useful for amino acid sequencing. Similar ions were also found in the mass spectra of acetyl-peptides and their esters and in acetylacefonyl peptide esters. Numerous acyl protecting groups such as decanoyl, ethoxycarbonyl and acetyl have been used. These N-protecting groups often increase volatility or yield higher intensity sequence ions. Recently, a number of Schiff-base protecting groups have been investigated as potential sequencing reagents. The 4-dimethylaminonaphthylidene derivatives have proven to be the most useful in terms of volatility, and the tendency to maximize cleavage into N-terminal fragments. They also yield intense molecular ions and the mass spectra can be readily interpreted. Modification of the carboxyl group usually involves esterification and methyl, ethyl or butyl esters are usually employed. The esterification procedure has been carried
out using diazomethane, methanolic HCL or thionyl chloride-methanol. These three esterification methods suffer from a number of disadvantages. For example, diazomethane treatment of free or unmodified peptides forms non-volatile quaternary ammonium salts, as the free amino group also reacts with the reagent. The other procedures lead to degradation of Trp containing residues, loss of ammonia from Gln and Asn and in addition, non-specific hydrolysis occurs if traces of water are present in the reaction media.

Despite these disadvantages, these techniques of N- and C-terminal modifications have been applied successfully to a number of peptides and volatile peptide derivatives have been formed. However, the larger oligopeptide derivatives are still not sufficiently volatile to give satisfactory mass spectra, and thus primary structure determination by this route is still difficult.

It was observed in 1963 that the naturally occurring nonapeptide derivative fortuitine was sufficiently volatile, without chemical modification, to give an acceptable mass spectrum. This enhanced volatility was considered to be due to the reduced hydrogen bonding in the peptide, as it contained three tertiary amide bonds. It was also shown that the tetrapeptide methyl ester H-Ile-Pro-Sar-MeVal-OMe containing no -CONH- groups gave a mass spectrum exhibiting a molecular ion even without acylation of the terminal amino-group.

These observations suggested that, if a procedure leading to methylation of the -CONH- groups of oligopeptide derivatives could be found, the resulting modified peptide might be more volatile and thus suitable for the determination of the amino acid sequence.
by mass spectrometry.

This resulted in the development of permethylation techniques, which were based on a procedure for carbohydrates described by Kuhn et al.\textsuperscript{,60}. The N-methylation procedure consisted of treating an N-acyl peptide methyl ester in dimethylformamide with an excess of methyl iodide in the presence of silver oxide (Scheme-3). In order to achieve complete reaction with this technique, it was necessary to stir the mixture for 3 days at room temperature or 4 hours at 50°C\textsuperscript{61}.

![Scheme-3](image)

The permethylation reaction was improved by Hakamori\textsuperscript{62} who used the methylsulphinyl carbanion catalysed reaction in dimethylsulphoxide for permethylation of glycolipids and polysaccharides (Scheme-4). This technique was rapidly extended to the permethylation of peptides\textsuperscript{63,64}, and reaction times could be reduced to one hour at room temperature\textsuperscript{62}. 
The permethylation reaction has been used extensively for the sequencing of peptides by mass spectrometry. There are, however, some inherent disadvantages associated with this technique. These are:

1. the amide bond of Asn and Gln is converted to the expected dimethylamide, but some dehydration to a nitrile is possible;
2. the Lys residue is also methylated at the ε-N-acetyl group, but at a slower rate and the product contains a significant amount of a lower homologue, which may make interpretation of the mass spectrum difficult;
3. Ser and Thr are O- and N- permethylated, but the mass spectra show partial (or occasionally complete) loss of methanol from all fragments containing one of these residues;
4. Met and His form involatile salts which are unsuitable for mass spectrometry;
5. Arg, Cys, Cys and Cys also form nonvolatile salts and must be modified chemically before permethylation.

Variations in the application of the Hakamori reaction, such as the use of different reagent concentrations and reaction times\textsuperscript{65,66,67} has overcome some of these problems. However, additional chemical manipulation is still required to cater for some peptides.

The elucidation of the primary structure of a peptide from its mass spectrum, depends upon the identification of the amine \((A_n)^+\) and/or the amino acyl \((B_n)^+\) fragment ions. Because side chain losses, rearrangements and other fragmentation processes also occur in the mass spectrometer, the peptide may not exhibit all the ions corresponding to these cleavages\textsuperscript{68,69,70}, but techniques designed specifically to simplify interpretation have been developed.

Lederer suggested recording the mass spectra of two derivatives for each peptide, prepared with a mixed reagent (e.g., CH\textsubscript{3}CO + CD\textsubscript{3}CO), so that all ions arising from the fragmentation of the peptide would appear as regular doublets (e.g., 3 amu apart). McLafferty\textsuperscript{71} suggested acyl groups containing a bromine or chlorine atom for the same purpose, so that fragments bearing these atoms will yield characteristic groups of peaks due to the specific isotopic composition of these elements.

High resolution mass spectrometry can be used for the interpretation of mass spectra. Exact mass measurement allows for the calculation of the empirical formulae of all the fragment ions, and has resulted in the automated determination of the amino acid sequence in peptides\textsuperscript{72,73,74,75}. Programs utilising this information have used amine \((A_n)^+\) and amino acyl
(B)_{n}^{+} fragment ions for identification, but even so an unambiguous answer is not always obtained. However, most mass spectra can be interpreted at low resolving power and it is only necessary to use high resolution mass spectrometry in doubtful cases.

It is clear that the sequencing of peptides by mass spectrometry is limited by: the volatility of the peptide derivative; the type and number of constituent amino acids; the thermal stability; and the type of mass spectrometer used. Obviously, the chemical methods utilised to overcome some of these problems are not entirely satisfactory, as they have required time consuming multistep manipulations and have been shown to produce undesirable side reactions with a number of amino acid residues.

III. AIM OF THE PRESENT WORK

In view of these problems it was considered desirable to develop a one step procedure for the derivatization of peptides.

As current esterification procedures are not entirely satisfactory for all amino acids it was considered necessary that a non destructive esterification procedure be developed. In addition it was felt that a reagent should be selected that would react with the N-terminal amino group of all the common amino acids under the conditions required for esterification so that volatile peptide derivatives could be prepared in one operation.

A literature search had shown that the pyrolytic conversion of tetraalkylammonium salts to alkyl esters might be a useful reaction suitable for the esterification of peptides under pyrolytic conditions. The general approach that has been used for
Pyrolytic esterification, has involved reaction of a carboxyl group with a quaternary ammonium hydroxide to form a salt (Scheme-5). This quaternary ammonium salt is thermally decomposed to form an ester and a tertiary amine biproduct (Scheme-6).

\[
R\text{-COOH} + R_4^+N\text{OH} \rightarrow R\text{-COONR}_4^- + H_2O
\]

Scheme-5

to form an ester and a tertiary amine biproduct (Scheme-6).

\[
R\text{-COONR}_4^- \rightarrow R\text{-COOCH}_3^- + R_3^N
\]

Scheme-6

This technique of pyrolytic esterification or alkylation has been used successfully for fatty acids, purines and pyrimidines, barbituates, phenolic alkaloids and phenolic acids.

These pyrolytic conversion reactions have been done in solution at reflux or in an injection port of a gas chromatograph at 250°C.

The aim of the present work was to prepare volatile peptide derivatives suitable for low resolution mass spectrometry by a simple and non-destructive one-step chemical operation. In particular, it was decided to exploit the pyrolytic route for alkylation and esterification and to develop a suitable N-protecting group compatible with this approach.
DISCUSSION
A. ESTERIFICATION OF ACETYL AMINO ACIDS

The first objective was to establish a peptide esterification procedure that would reduce sample manipulation and avoid side reactions, such as the hydrolysis of Gln, Asn and the decomposition of Trp encountered with an acid catalysed esterification. In particular the concept of converting an N-protected peptide into its ester derivative by thermal decomposition of its quaternary ammonium salt was to be investigated. The ultimate aim was to carry out such a reaction in a mass spectrometer using the solid probe heater as a heat source and utilising the high vacuum system to drive the pyrolytic esterification reaction to completion.

This idea was first tested on simpler but structurally related compounds. For this purpose N-acetyl amino acids were chosen as suitable models, as their alkyl ester derivatives were known to yield simple mass spectral fragmentation patterns. Since a large proportion of the published work on the sequencing of peptides by mass spectrometry had utilised methyl esters for carboxyl protection, these early esterification experiments were concentrated on the formation of methyl esters.

Previous workers had established that fatty acids could be converted to their methyl esters via pyrolytic decomposition of either their tetramethylammonium or trimethylphenylammonium salts. These salts had been prepared by dissolving the acids in tetramethylammonium hydroxide (TMAH) or trimethylphenylammonium...
hydroxide (TMPAH) respectively. The literature also suggested that TMPAH would be a better methylating agent than TMAH as dimethylaniline is a better leaving group than trimethylamine. The experiments of Brochmann-Hanssen and Oke\textsuperscript{79} on the methylation of barbiturates, supported this view. These workers found that the gas-chromatographic pyrolytic methylation of tetramethylammonium salts of barbituric acids gave several products which were shown to be due to incomplete methylation of the barbituric acids, but that the pyrolysis of the trimethylphenylammonium salts of the same barbiturates yielded only the completely methylated product.

In an effort to test this hypothesis, a selection of N-acetyl amino acids were converted to their methyl esters by pyrolytic decomposition of their tetramethylammonium and trimethylphenylammonium salts respectively, in the solid probe heater of a mass spectrometer. In these initial pyrolytic esterification experiments, stoichiometric quantities of quaternary ammonium hydroxide were used (one equivalent of base per -COOH group).

In a typical experiment the acetyl amino acid was dissolved in the equivalent amount of methanolic TMAH or TMPAH, which resulted in the formation of the tetramethylammonium salt (TMA) or the trimethylphenylammonium salt (TMPA) respectively (Scheme - 7). These acetyl amino acid salts were then introduced

\[
\text{CH}_3\text{CONHCHR}COOH + \text{(CH}_3\text{)}_4\text{NOH} \quad \text{--\textgreater} \quad \text{CH}_3\text{CONHCHR}COON\text{(CH}_3\text{)}_4 + \text{H}_2\text{O}
\]

\[
\text{CH}_3\text{CONHCHR}COOH + \text{PhN(CH}_3\text{)}_3\text{OH} \quad \text{--\textgreater} \quad \text{CH}_3\text{CONHCHR}COOPhN\text{(CH}_3\text{)}_3 + \text{H}_2\text{O}
\]

\text{\textbf{Scheme - 7}}
into the mass spectrometer via the solid probe inlet and the esterification was induced by slowly increasing the temperature of the solid probe heater. The onset of the reaction was readily detected by an increase in sample pressure, which was due principally to the liberation of the volatile biproducts i.e., trimethylamine or dimethylaniline (Scheme - 8). After most of these compounds had been pumped off the solid probe

\[
\begin{align*}
\text{CH}_3\text{CONHCHRCON(CH}_3)_4 & \xrightarrow{\text{HEAT}} \text{CH}_3\text{CONHCHRCOOCH}_3 + (\text{CH}_3)_3\text{N} \\
\text{CH}_3\text{CONHCHRCON(CH}_3)_3 & \xrightarrow{\text{HEAT}} \text{CH}_3\text{CONHCHRCOOCH}_3 + \text{PhN(CH}_3)_2
\end{align*}
\]

Scheme - 8

temperature was increased until the acetyl amino acid methyl ester became sufficiently volatile to give a mass spectrum.

The most suitable temperature for esterification of TMA salts was 110-130°C, and for TMPA salts it was 80-110°C. Whilst lower temperatures could be used to initiate these conversions, longer reaction times (in excess of thirty minutes) were required to achieve complete esterification. Pyrolysis temperatures in excess of 150°C could also be used, but under these conditions the amine biproduct and the acetylamino acid ester were volatilised concurrently. This resulted in a large increase in sample pressure.
Table 1. Relative Intensities\(^2\) of Selected Ions from the Mass Spectra of Acetyl Amino Acid Esters.

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2. All ions are calculated as a percentage of the base peak, this calculation excluding those ions from TMA or TMAH.
3. Cleavage M-R represents complete or partial side chain loss.
and in some cases pressures of $2 \times 10^{-5}$ torr were recorded. In practice it was found to be more desirable to remove most of the amine biproduct prior to recording a mass spectrum of the acetylamino acid methyl ester. Under these conditions (80-130°C) some 4-8 minutes were required for decomposition of the TMA salt and 3-5 minutes for the TMPA salts respectively.

The characteristic amine $(A_n)^+$ and amino acyl $(B_n)^+$ ions in the e.i. mass spectra of the acetylamino acid methyl esters prepared by pyrolytic methylation procedures (method B-TMPAH, method C-TMAH) were compared with those of authentic acetylamino acid methyl esters which were prepared by published methods (Method-A) (Table-1). The mass spectra of the derivatives of Gly, Ala, Val, Leu, Thr, Phe, Pro, Asp, Glu, Met and Lys were essentially identical for all three esterification methods. A minor variation in the spectra of the derivatives of the thermally labile amino acids, Ser, Gln and Asn was noted. For example, the mass spectrum of acetyl Ser methyl ester when prepared from its TMA salt resulted in more intense ions at $m/e = M - 18$ due to dehydration, and at $m/e = 84 = M - (\text{COOCH}_3 + \text{H}_2\text{O})$ than if prepared by the other methods. Also, the molecular ion was less intense and overall the spectrum was more consistent with the derivative, methyl 2-N-acetylaminoacrylate, the dehydrated form of acetyl Ser methyl ester. The mass spectra of N-acetyl Gln and N-acetyl Asn methyl esters prepared by the reference method of esterification or by thermal decomposition of the TMPA salt were essentially identical.
The reference method used diazomethane for esterification, instead of the more commonly used thionyl chloride–methanol reagent, as acidic esterification methods completely hydrolyse amides to the corresponding acids. The most pronounced side chain loss in the mass spectrometer was due to the liberation of ammonia at m/e = M-17. This loss was more pronounced for the TMA esterified derivatives and once again, the molecular ion was less intense.

In addition to these specific cleavages, the mass spectra of the pyrolytically esterified derivatives had other distinguishing characteristics. The most prominent feature was the presence of ions due to the biproduct amine (m/e 58, 59 trimethylamine or m/e 120, 121 dimethylaniline) in the spectra of the acetyl amino acid esters. These biproducts could be selectively removed before a mass spectrum was recorded for the ester derivative, simply by maintaining the solid probe at a sufficiently low temperature to prevent volatilisation of the acetyl amino acid ester. This technique was not generally implemented as the presence of the amine spectrum did not interfere with the interpretation of the mass spectrum of the acetyl amino acid ester, and only lengthened the analysis time.

The pyrolytic esterification procedure also resulted in partial methylation of the N-terminal acetamido group. Satellite ions of low abundance (<3%) were observed 14 amu higher in mass than the expected fragment and molecular ions, for most of the amino acid derivatives investigated. These ions were attributed to N-methylation of the acetamido group, the methylation being facilitated by the
electron withdrawing effect of the acetyl group. N-acetyl Pro did not undergo methylation, because it has a tertiary N-acetyl amide bond with no hydrogen for replacement.

The mass spectra of conventionally and pyrolytically esterified acetyl amino acids contain in addition to a molecular ion (M)\(^+\) an ion at m/e = M + I. The intensity of this ion was less than 30% of the molecular ion for the conventionally esterified derivatives, with the exception of the mass spectra of Ser, Thr, Asn and Gln which showed ions at m/e = M + I of greater intensity than the (M)\(^+\) ions. As expected\(^8\) the intensity of the ion at m/e = M + I was shown to be pressure dependent, thus at pressures of 8 \(\times\) 10\(^{-6}\) to 2 \(\times\) 10\(^{-5}\) torr, when the amine biproduct was present in large concentration, the ion at m/e = M + I was of greater abundance than the molecular ion. If the amine biproduct was removed prior to volatilisation of the acetyl amino acid ester, the (M + I)\(^+\) peak was less intense than the molecular ion (M)\(^+\).

These results demonstrated that a pyrolytic esterification reaction in a mass spectrometer was a viable alternative to conventional esterification procedures. Since it was observed that the thermal decomposition of TMPA salts of the thermally labile amino acids, Ser, Gln and Asn was superior to that of the respective IMA salts, TMPAH was selected as the reagent of choice for all further esterification reactions.
B. PERMETHYLATION OF ACETYL AMINO ACIDS

The volatility of peptide derivatives can be increased by N-methylation of the amide bonds\textsuperscript{61,62}, and it was thought likely that the pyrolytic methylation procedure with excess TMPAH may lead to the permethylation of peptide bonds. The acetyl amino acids were also suitable models for this study as the degree of methylation of the acetamido group could be used as a guide for the likely excess of TMPAH required for amide bond methylation. In addition, side reactions could be more easily recognised and tabulated. The literature suggested that an excess of TMPAH ranging from 1-10 equivalents had been used in some cases to achieve complete methylation of the functional groups of barbiturates\textsuperscript{78,79} phenolic alkaloids\textsuperscript{79} and pyrimidines\textsuperscript{77} and for this reason these early experiments were carried out using a substantial excess of TMPAH. The results showed that 4 equivalents of TMPAH (for each COOH group) were required for complete permethylation of Gly, Ala, Val, Leu, Met, Asp and Glu. The mass spectra for these derivatives had ions 14 amu higher in mass than the reference compounds. In addition, ions of low abundance (<2\%) 14 amu higher in mass than these were obtained on the derivative of Gly. N-acetyl Pro with a tertiary acetamido bond, also exhibited ions 14 amu higher in mass than expected. The increase in molecular weight for both derivatives was attributed to the presence of some C-methylation byproducts in the sample.
Similar observations have been reported by other investigators when using conventional permethylation methods.

The amino acids Ser, Thr, Gln and Asn could also be completely N-methylated under these conditions. However, the mass spectra of these derivatives were quite complicated due to the presence of partially methylated side chain functional groups and the presence of ions corresponding to dehydration, loss of ammonia and other rearrangements. Because of the difficulties of interpreting the mass spectra of some of these permethylated acetyl amino acids, a large excess of TMPAH was not considered suitable for the permethylation of peptides. During these permethylation studies, it was observed that 1.2 equivalents of TMPAH per equivalent of -COOH group provided a useful base excess for pyrolytic methylation studies, since it aided dissolution of the acids and resulted in a minimum of side reactions. Utilising these reaction conditions, methylation of the acetamido group did not exceed 10% and undesirable side chain methylation of Ser, Thr, Gln, Asn, Gly and Pro was avoided. For this reason, future pyrolytic esterification procedures utilised these reaction conditions.

C. PYROLYTIC ESTERIFICATION OF ACETYL PEPTIDES

A number of N-acetyl peptides were esterified by pyrolytic methylation using the same reaction conditions as before. N-acetyl peptides were chosen for this initial study because they have been used extensively for mass spectrometric sequencing, and it has been shown that peptides acylated with low molecular weight aliphatic
Table 2. Relative Intensities\(^a\) of Selected Ions for N-Acetyl Peptide Methyl Esters

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<th>Peptide</th>
<th>Procedure</th>
<th>A₁</th>
<th>B₁</th>
<th>A₂</th>
<th>B₂</th>
<th>A₃</th>
<th>B₃</th>
<th>H⁺-R₁</th>
<th>H⁺-R₂</th>
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<td>233</td>
<td>120</td>
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(a) Sequence ion abundances < 10% are in bold, 10-30% in italic, > 30% in ordinary type.
(b) P = Pyrolytic Methylation; R = Reference
(c) The *- and 0*-amino groups are both acetylated.
(d) The sequence ions shown correspond to fragmentation of [(M/2) + 1].
(e) Side chain -leavage R may be complete or partial loss of the side chain.
acids (from acetic to decanoic) are most suitable for mass spectrometry.\textsuperscript{85,86,87}

The pyrolytic esterification of acetyl peptides by thermal decomposition of their TMPA salts required solid probe temperatures of 80-110°C and a reaction time of less than five minutes. Not all of the resulting acetyl peptide esters were sufficiently volatile at this low temperature and some of these required a solid probe temperature of up to 250°C before a mass spectrum could be obtained. For example, the derivative of a neutral dipeptide such as Ala-Leu was sufficiently volatile at a 120°C for mass spectral analysis whilst the derivative of Phe-Phe required a temperature of 210°C before a satisfactory mass spectrum could be obtained.

Table-2 gives the e.i. mass spectral data for a selection of acetyl peptide methyl esters esterified in this manner. The e.i. mass spectra of these compounds were consistent with previously published fragmentation mechanisms\textsuperscript{88} and prominent ions corresponding to amine (\(A_n\))\(^+\) and amino acyl (\(B_n\))\(^+\) cleavages were common (Diag.-7), allowing the amino acid sequence of the starting peptide to be deduced.

\[
\begin{array}{c}
\text{CH}_3\text{CONH - CH} \quad \text{CO} \quad \text{(NH - CH - CO)}_n \quad \text{NH - CH} \quad \text{CO}_1\text{OCH}_3 \\
\text{R} \quad \quad \quad \text{R} \\
\text{A}_1 \quad \text{B}_1 \\
\text{A}_n \quad \text{B}_n \\
\end{array}
\]

\textbf{Diag.-7}

A small selection of acetyl peptides were esterified by standard methods\textsuperscript{82} and their mass spectra were compared with the mass spectra obtained on the corresponding pyrolytically esterified derivatives.
Fig. 2. Acetyl Leu-Val Methyl Ester.
The results (Table-2) were with minor exceptions essentially the same. In particular, the presence of ions at m/e 120, 121 due to dimethylaniline were a characteristic feature of the pyrolytically esterified derivatives, but this did not impair identification of the acetyl peptide ester, as can be seen for the derivative of Leu-Val, (Fig-2). The other feature of the spectra of the pyrolytically esterified derivative was the presence of satellite ions 14 amu higher in mass than the molecular ion. These satellite ions were attributed to permethylation of the acetamido group, but there was no evidence of permethylation of the other amide bonds. In these experiments 1.2 equivalents of TMPAH per equivalent of carboxyl group was used, and under these conditions, both the alpha and the side chain carboxyl groups were completely esterified. At the same time, the side chains of Tyr, Trp and His were also partially methylated. This was deduced from the presence of satellite ions 14 and 28 amu higher in mass than the molecular ion in the mass spectra of peptides containing these residues. These ions were attributed to partial N-methylation of the acetamido group and partial methylation of the side chain. In fact, ions 14 amu higher in mass than the molecular ion were also detected in the mass spectra of His and Trp peptide esters prepared by conventional techniques. These ions have been shown to arise by thermal intermolecular N-methylation of the imidazole or indole rings\textsuperscript{89,90}, the C-terminal carbomethoxy group being the main source of methyl groups (Scheme-9). It is probable that this type of process accounts for some of the methylation
observed for His and Trp residues, although the percentage methylation of the conventionally esterified acetyl peptides with these residues was less than 0.1% for the Trp peptides and less than 1% for the His peptides. However, the pyrolytically esterified derivatives contained more of the methylation product than could be attributed to intermolecular N-methylation. In addition the phenolic group of Tyr residues was also susceptible to pyrolytic methylation with 18-22% methylated product being observed.

As these residues possessed obvious methylation potential, the excess of TMPAH used was increased to a 20% excess per residue, i.e., 1.2 equivalents of TMPAH per equivalent of His, Trp or Tyr, so that methylation of the side chains of these residues could be enhanced. Under these conditions the phenolic group of Tyr residues was completely methylated, as shown by the 14 amu increase in molecular weight of these peptides, together with an intense ion at m/e 121
due to cleavage of the aromatic side chain as $^{+} \text{CH}_2\text{-C}_6\text{H}_4\text{-OCH}_3$. The use of less TMPAH gave incomplete methylation of this group and molecular and sequence ions separated by 14 amu were obtained, in addition to intense ions at m/e 107 and 121.

His and Trp residues in contrast to Tyr were only incompletely methylated using a 1.2 molar excess of TMPAH per residue. The mass spectra of peptides with these residues had molecular ions and some sequence ions separated by 14 amu. In addition both residues had intense ions separated by 14 amu corresponding to cleavage of the side chain as ArCH$_2^+$ (His, m/e 81 and 95; Trp, m/e 130 and 144). These ions were usually of equal intensity suggesting that approximately 50% conversion to the N-methyl derivative was possible under these conditions. Complete methylation of His and Trp residues was possible, if 4 equivalents of TMPAH was used for derivatisation. However, this was not desirable under e.i. conditions, as a considerable increase in sample pressure was obtained from excess TMPAH within the mass spectrometer necessitating long residence times (twenty to thirty minutes) before the excess dimethylaniline could be removed. In addition, the large excess of TMPAH induced partial methylation of all the amide bonds in the molecule and completely methylated the N-terminal acetamido group. However, the presence of these ions made the interpretation of the mass spectrum very much more difficult. For this reason, the TMPAH excess was restricted to 1.2 equivalents for every equivalent of His
### TABLE - 3

Summary of Reaction Conditions and Products of Pyrolytic Esterification.

<table>
<thead>
<tr>
<th>Peptide Residue</th>
<th>Functional Group Modified</th>
<th>TMPAH (equivalents)</th>
<th>Product Obtained</th>
<th>Yield</th>
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<td><img src="image" alt="Chemical Structure" /></td>
<td>1.2</td>
<td><img src="image" alt="Chemical Structure" /></td>
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<td><img src="image" alt="Chemical Structure" /></td>
<td>1.2</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>≈ 50%</td>
</tr>
</tbody>
</table>
or Trp residue present in the peptide. Although incomplete methylation (approx. 50%) of both His and Trp residues is likely under these conditions, this partial methylation can be used to advantage to confirm the presence of these residues in an unknown peptide.

In summary, the reaction conditions found to be most suited for the pyrolytic methylation of acetyl peptides was the use of 1.2 equivalents of TMPAH per equivalent of carboxyl group, Tyr, His, and Trp residue (Table-3). Solid probe temperatures of 80-110°C were found most suitable, with esterification being achieved in less than five minutes.

Finally, it was decided to demonstrate the general applicability of the pyrolytic esterification reaction to the derivatisation of other N-acylated peptides, and a small selection of some of the commonly used N-acyl protected peptides were derivatised under these conditions.

D. ESTERIFICATION OF ACYL AND SULPHONYL PROTECTED PEPTIDES

Val-Ile-Ala was acylated with a number of reagents and the resulting acyl peptide was esterified by pyrolytic methylation. The mass spectra of the resulting derivatives (Table-4) were compared with published reference spectra^{51,52,53}. The results showed that the only difference in mass spectra was the presence of satellite ions 14 amu higher in mass due to partial N-methylation of an acetamido or
Table 4. Relative Intensities$^1$ of Selected Ions for the Derivatives of Valylisoleucylalanine

\[
\begin{array}{cccccccc}
\text{Amino Protecting} & & & & & & & \\
\text{Group} & \text{A}_{1} & \text{B}_{1} & \text{A}_{2} & \text{B}_{2} & \text{A}_{3} & \text{B}_{3} & \text{M} & \text{Source of data}\$^2$
\hline
\text{Dansyl} & m/e 305 & 333 & 410 & 446 & 489 & 517 & 548 & a \\
 & INT. 30 & <1 & 5 & 2 & - & 24 & - & \\
 & INT. 52 & 0.9 & 6 & 11 & - & 15 & - & \\
\text{Acetyl} & m/e 114 & 142 & 227 & 255 & 298 & 326 & 357 & b \\
 & INT. 100 & 36 & 26 & 12 & 0.3 & 0.5 & 0.3 & c \\
 & INT. 100 & 29 & 23 & 9 & - & 0.4 & - & b \\
\text{Tritfluoroacetyl} & m/e 168 & 196 & 281 & 309 & 352 & 380 & 411 & c \\
 & INT. 51 & - & 100 & 7 & 1.5 & 0.8 & 0.1 & b \\
 & INT. 56 & - & 100 & 3 & 2 & - & 0.3 & b \\
\text{Formyl} & m/e 176 & 204 & 289 & 317 & 360 & 388 & 419 & b \\
 & INT. 100 & 10 & 89 & 19 & 1.4 & 1.3 & 0.4 & c \\
 & INT. 100 & 8 & 60 & 13 & 2.0 & 0.8 & 0.4 & c \\
\text{Benzoyl} & m/e 156 & 184 & 269 & 297 & 340 & 368 & 399 & a \\
 & INT. 39 & 27 & 5 & 2 & 0.04 & 0.2 & 0.1 & c \\
 & INT. 51 & 23 & 3 & 2 & - & 0.4 & 0.3 & b \\
\text{Pivaloyl} & m/e 156 & 184 & 269 & 297 & 340 & 368 & 399 & d \\
 & INT. 100 & 9 & 12 & 2 & 1 & 0.1 & 0.1 & b \\
 & INT. 100 & 7 & 9 & 3 & 2 & 0.3 & 0.1 & b \\
\end{array}
\]

1. All intensities are calculated as a percentage of the base peak after background subtraction.

2. (a) From an Hitachi Perkin-Elmer RMJ-7 mass spectrometer,$^{53}$ (b) From an AEI QUAD 300-D mass spectrometer using pyrolytic esterification,$^{51}$ (c) From an AEI MS-9 mass spectrometer,$^{51}$ (d) From an AEI QUAD 300-D mass spectrometer using conventional esterification.$^{51}$
sulphonamido group for the pyrolytically esterified derivatives. These methylation peaks did not significantly hamper the identification of the relevant sequence ions. The mass spectral results suggested that the pyrolytic esterification reaction could be potentially useful for the esterification and the subsequent sequencing of most volatile N-acyl or sulphonyl protected peptides.

However, it was clear, that the potential value of the pyrolytic reaction would be greatly enhanced if N-protection could be achieved during salt formation. In this way the chemical manipulation of the peptides could be considerably reduced and a one step derivatisation might become feasible. Of the acyl protecting groups already used for the sequencing of peptides, the trifluoroacetyl function seemed most suitable for this purpose, as the trifluoroacetylation of amino groups with methyltrifluoroacetate can be effected in weakly alkaline solution. This condensation reaction was applied to the derivatisation of the TMPA salts (Method II) and the products of this reaction were run on thin-layer chromatography against trifluoroacetylated peptides prepared by the conventional trifluoroacetylation method (Ref. Method). In all cases, the Rf of TFA-peptides by the two methods were found to be identical (Table-5). The condensation of methyltrifluoroacetate with peptide TMPA salts required either refluxing for fifteen minutes or seven hours at room temperature. Under these conditions the side chain amino group of Lys is also trifluoroacetylated. In order to check the
Table 6. Relative intensities of selected ions in the mass spectra of TFA-peptide methyl esters prepared by different methods

<table>
<thead>
<tr>
<th>Peptide</th>
<th>A₁</th>
<th>A₁</th>
<th>A₂</th>
<th>A₂</th>
<th>A₃</th>
<th>A₃</th>
<th>M⁻</th>
<th>M⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Trp</td>
<td>126</td>
<td>126</td>
<td>126</td>
<td>154</td>
<td>154</td>
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<td>326</td>
</tr>
<tr>
<td>Asp-Phe</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>168</td>
<td>168</td>
<td>168</td>
<td>287</td>
<td>287</td>
</tr>
<tr>
<td>Ile-Ala</td>
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<td>253</td>
</tr>
<tr>
<td>Pro-Val</td>
<td>166</td>
<td>166</td>
<td>166</td>
<td>194</td>
<td>194</td>
<td>194</td>
<td>265</td>
<td>265</td>
</tr>
<tr>
<td>Hyp-Gly</td>
<td>102</td>
<td>102</td>
<td>102</td>
<td>210</td>
<td>210</td>
<td>210</td>
<td>239</td>
<td>239</td>
</tr>
<tr>
<td>Ser-Val</td>
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<tr>
<td>Thr-Gly</td>
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<td>227</td>
</tr>
<tr>
<td>Asp-Phe</td>
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<td>226</td>
<td>226</td>
<td>226</td>
<td>345</td>
<td>345</td>
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<tr>
<td>Lys-Val</td>
<td>293</td>
<td>293</td>
<td>293</td>
<td>321</td>
<td>321</td>
<td>321</td>
<td>372</td>
<td>372</td>
</tr>
<tr>
<td>Ala-Tyr</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>168</td>
<td>168</td>
<td>168</td>
<td>303</td>
<td>303</td>
</tr>
</tbody>
</table>

(a) All intensities are calculated as a percentage of the base peak above m/z 60 after a background subtraction.
(b) M = Reference method of trifluoroacetylation and esterification; 1 = Reference method of trifluoroacetylation and pyrolytic esterification;
   1 = Trifluoroacetylation with methyl trifluoroacetate, and esterification by pyrolysis.
(c) Approx. 10% methyl ester of the indole side chain of this residue has occurred during pyrolytic esterification.
(d) MS were recorded on an SEI QUAD 3000 mass spectrometer.
Table 5. Thin-layer chromatography of TFA-peptides

Plates were developed with butanol-acetone-acetic acid-ammonia-water (18:6:4:1:8).

<table>
<thead>
<tr>
<th>Peptide group</th>
<th>Rp peptide Ref. compd Method II</th>
<th>Peptide group</th>
<th>Rp peptide Ref. compd Method II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Leu</td>
<td>0.45 0.72 0.72\textsuperscript{A}</td>
<td>Met-Ser</td>
<td>0.47 0.75 0.75</td>
</tr>
<tr>
<td>Val-Leu</td>
<td>0.25 0.73 0.73\textsuperscript{A}</td>
<td>His-Leu</td>
<td>0.37 0.81 0.81</td>
</tr>
<tr>
<td>Gly-Trp</td>
<td>0.37 0.81 0.81</td>
<td>Lys-Val</td>
<td>0.33 0.70 0.70</td>
</tr>
<tr>
<td>Ile-Ala</td>
<td>0.33 0.73 0.73</td>
<td>Asp-Phe</td>
<td>0.28 0.73 0.73</td>
</tr>
<tr>
<td>Ser-Val</td>
<td>0.43 0.80 0.80</td>
<td>Gln-Gly</td>
<td>0.31 0.72 0.72</td>
</tr>
<tr>
<td>Hyp-Gly</td>
<td>0.43 0.73 0.73</td>
<td>Pro-Phe</td>
<td>0.37 0.81 0.81</td>
</tr>
</tbody>
</table>

\textsuperscript{A} The trifluoroacetylation of these peptides was followed by thin-layer chromatography over a period of 8 hr. Reaction was complete after 15 min. at reflux or 7 hr. at room temperature.

Stability of the N-TFA group under pyrolytic conditions, the TFA-peptides trifluoroacetylated by the reference method were divided into two portions and half of the derivative was esterified by conventional methods\textsuperscript{45,59} (Ref. Method) and the other half was esterified by the pyrolytic technique (Method I). The mass spectra of these TFA-peptide esters were then compared with those prepared by the methyltrifluoroacetate-pyrolysis method (Method II). The results (Table-6) showed that the mass spectra of TFA-peptide methyl esters by these three methods were almost identical. Again, the only differences in these spectra were the presence of low abundant ions due to partial methylation of the trifluoroacetamido group, and the side chain methylation of peptides containing Tyr, His and Trp residues.
Table 7. Relative Intensities\(^a\) of Sequence Ions in the Mass Spectra\(^d\) of TFA - Peptide Methyl Esters\(^b\)

\[
\text{C}_6\text{H}_5\text{CO} - \text{NH} + \text{CH} + \text{CO} + \text{NH} - \text{CH} + \text{CO} + \text{NH} - \text{CH} + \text{CO} + \text{OCH}_3
\]

<table>
<thead>
<tr>
<th>Peptide</th>
<th>A(_1)</th>
<th>B(_1)</th>
<th>A(_2)</th>
<th>B(_2)</th>
<th>A(_3)</th>
<th>B(_3)</th>
<th>m/e</th>
<th>Base Peak</th>
<th>S Methyl</th>
</tr>
</thead>
<tbody>
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<td>Ala-Yal</td>
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<td>168</td>
<td>219</td>
<td>298</td>
<td>298</td>
<td>267</td>
<td>160</td>
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<td>6</td>
</tr>
<tr>
<td></td>
<td>INT</td>
<td>100</td>
<td>5</td>
<td>50</td>
<td>50</td>
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<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>Ala-Leu</td>
<td>140</td>
<td>168</td>
<td>219</td>
<td>298</td>
<td>298</td>
<td>267</td>
<td>160</td>
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<td>5</td>
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<tr>
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</tr>
<tr>
<td>Val-Yal</td>
<td>166</td>
<td>196</td>
<td>267</td>
<td>326</td>
<td>326</td>
<td>395</td>
<td>160</td>
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<td>4</td>
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<td>40</td>
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<td>0.3</td>
<td></td>
<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>Pro-Phe</td>
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<td>194</td>
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<td>395</td>
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<td>20</td>
<td>4</td>
<td>4</td>
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<td>5</td>
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<tr>
<td>Glu-Tyr</td>
<td>212</td>
<td>240</td>
<td>360</td>
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</tr>
<tr>
<td>Met-Phen Gly</td>
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<td>228</td>
<td>347</td>
<td>406</td>
<td>406</td>
<td>432</td>
<td>160</td>
<td>432</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>INT</td>
<td>20</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td></td>
<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>Glu-Gly</td>
<td>197</td>
<td>223</td>
<td>254</td>
<td>282</td>
<td>282</td>
<td>313</td>
<td>160</td>
<td>313</td>
<td>6</td>
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<tr>
<td></td>
<td>INT</td>
<td>16</td>
<td>-</td>
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<td>0.8</td>
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<tr>
<td>Trp(^\text{6})-Glu</td>
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<td>297</td>
<td>402</td>
<td>430</td>
<td>430</td>
<td>471</td>
<td>160</td>
<td>471</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>INT</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td></td>
<td>100</td>
<td>0.2</td>
</tr>
<tr>
<td>His(^\text{5})-His(^\text{5})</td>
<td>220</td>
<td>244</td>
<td>371</td>
<td>399</td>
<td>399</td>
<td>430</td>
<td>95</td>
<td>430</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>INT</td>
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<td>20</td>
<td>0.4</td>
<td>0.4</td>
<td>-</td>
<td></td>
<td>100</td>
<td>0.4</td>
</tr>
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<td>Ala-Ala-Ala</td>
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<td>211</td>
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<td>259</td>
<td>282</td>
<td>100</td>
<td>282</td>
<td>6</td>
</tr>
<tr>
<td></td>
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<td>60</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td></td>
<td>100</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\(^a\) All intensities are calculated as a percentage of the base peak above m/e 80 after a background subtraction.

\(^b\) TFA - Peptide Methyl Esters were prepared by the methyliododfluoroacetate/trifluoromethanesulfonic acid condensation and pyrolytic esterification. (Method II.)

\(^c\) Sequence ions are shown for the N-methyl derivative.

\(^d\) MS were recorded on an Elmer 3000 mass spectrometer.
Since the sequencing of unknown peptides by mass spectrometry relies on the presence of 'sequence-determining ions' [amine \( (A_n)^+ \) and amino acyl \( (B_n)^+ \)], the relative abundances of these ions in the mass spectra of a series of TFA-peptide esters prepared by the new method (Method II) have been tabulated (Table-7). The partial methylation (<10%) of the trifluoroacetamido group during pyrolytic methylation is undesirable, as it interferes with the interpretation of some of the peptides' mass spectra. As such, some attempts have been made to reduce the degree of permethylation. This was most conveniently done by the addition of a small amount of cation exchange resin (Zerolit 236 Permutit) until the pH of the reaction mixture was reduced to approximately 8. Care has to be taken in this step as if too much TMPAH is removed, the yield of ester following pyrolytic esterification is drastically reduced. This procedure significantly reduced the amount of permethylation of the trifluoroacetamido group, but it was not possible to completely eliminate the process.

It is clear that the elimination of permethylation is desirable if the sequencing of peptides is to be attempted by this method, and for this reason other N-protecting groups such as aldehyde and \( \beta \)-diketone adducts were investigated. These condensates do not have an acidic NH-group and thus the tendency for permethylation seemed less likely.
Table 8. Relative Intensities of Selected Ions for N-protected Valylisoleucylalanine Methyl Esters

<table>
<thead>
<tr>
<th>Amino Protecting Group</th>
<th>A₁</th>
<th>A₂</th>
<th>A₃</th>
<th>B₁</th>
<th>B₂</th>
<th>B₃</th>
<th>p#</th>
<th>O</th>
<th>H</th>
<th>Ref. 1</th>
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<tr>
<td>Acetylacetonyl</td>
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<td>182</td>
<td>267</td>
<td>295</td>
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<td>497</td>
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<td>2</td>
<td>181</td>
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<td>Benzoylacetonyl</td>
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<td>357</td>
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<td>Ethoxycarbonylpropenyl</td>
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<td>3</td>
<td>183</td>
</tr>
<tr>
<td>N-methylindane</td>
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<td>4-Methlybenzylidene</td>
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<tr>
<td>4-Dimethylaminobenzylidene</td>
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<td>316</td>
<td>344</td>
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<td>415</td>
<td>148</td>
<td>2</td>
<td>3</td>
<td>183</td>
</tr>
<tr>
<td>4-Dimethylamino-</td>
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<td>257</td>
<td>342</td>
<td>370</td>
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<td>306</td>
<td>336</td>
<td>379</td>
<td>407</td>
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<td>2</td>
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<td>183</td>
</tr>
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<td>4-Naphthylmethyline</td>
<td>210</td>
<td>238</td>
<td>323</td>
<td>351</td>
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<td>427</td>
<td>127</td>
<td>2</td>
<td>3</td>
<td>183</td>
</tr>
<tr>
<td>4-Dimethylaminonaphthylmethyline</td>
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<td>366</td>
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<td>465</td>
<td>197</td>
<td>2</td>
<td>3</td>
<td>183</td>
</tr>
</tbody>
</table>

1. Source of reference spectra, a(36), b - peptides were esterified and N-protected by standard methods(79, 96), c(155).
2. These ref. spectra were recorded as their ethyl ester derivatives.
3. The ions shown are for the isotope of greatest abundance.
4. Cleavage P may be complete or partial loss of the protecting group.
5. All intensities are calculated as a percentage of the base peak above m/z 80 after background subtraction.
E. ALDEHYDES AND β-DIKETONES AS PROTECTING GROUPS

The reaction of aldehydes and β-diketones with peptides in alkaline media results in formation of Schiff base and enamine protected peptides[93,94]. As these derivatives have no acidic hydrogen for replacement, methylation in the amino terminal position is unlikely. Aldehydes and β-diketones can be coupled with peptide TMPA salts in slightly basic conditions. This condensation makes one step derivatisation possible and chemical manipulations can be kept to a minimum allowing as little as 50-100 nmoles to be handled satisfactorily.

In order to evaluate the more common aldehyde and β-diketone condensates under pyrolytic methylation conditions, a series of protected Val-Ile-Ala TMPA salts were prepared and the mass spectra of their enamine and Schiff's base derivatives were compared with those obtained by conventional derivatisation procedures. The results showed that the molecular (M)^+ and sequence ions (A_n^+, B_n^+) of the N-protected Val-Ile-Ala-OMe listed in Table-8 were essentially identical to the published reference spectra[51,52,53].

The aldehyde or β-diketone condensation of peptide TMPA salts could be prepared in a one step reaction using molecular sieves (type-3A) as water scavengers (Scheme-10). In this manner the mass spectrometric sequencing of peptides was possible without purification or isolation of the derivatives. The selection of suitable reagents for this purpose was based on the published work of Day et.al.[53] on the mass spectrometric sequencing of Schiff base and enamine condensates
Table 9. Relative abundance of selected ions of Neopentylidene peptide derivatives

Sequence ion abundances < 10% are in bold, 10–50% in *italic* and > 50% in ordinary type. The base peak is indicated by *.

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{CH=N} \quad \text{CHR} \quad \text{CO} \quad \text{NH} \quad \text{CHR} \quad \text{CO} \quad \text{NH} \quad \text{CHR} \quad \text{CO} \quad \text{OCH}_3 \\
\text{H}_2\text{C} & \quad \text{CH} = \text{N} \quad \text{CHR} \quad \text{CO} \quad \text{NH} \quad \text{CHR} \quad \text{CO} \quad \text{NH} \quad \text{CHR} \quad \text{CO} \quad \text{OCH}_3
\end{align*}
\]

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<sup>a</sup> Base peaks:

\[
\begin{align*}
\text{CH}_2 & \quad \text{OCH}_3 \\
\text{CH}_2 & \quad \text{OCH}_3 \\
\text{CH}_2 & \quad \text{OCH}_3 \\
\text{CH} & \quad \text{CH}_2 \text{CCN} \\
\text{CH} & \quad \text{NH}_3
\end{align*}
\]

<sup>b</sup> Sequence ions represented are for the uncondensed proline peptide.

<sup>c</sup> Methylation of the phenolic group on tyrosine has taken place.
of peptide esters.

Of the aldehydes tested, pivaldehyde was found to be the most suitable for our pyrolytic methylation studies. The low boiling point of the aldehyde (b.p. 77-78°C) allowed the selective removal of any excess reagent present in the ion source, and the electron impact mass spectra of the resultant neopentylidene peptide esters contained sufficient information to allow sequence assignment in each instance (Table-9).

The use of analogous aromatic Schiff base peptide condensates were not quite as suitable as pivaldehyde. Selective removal of excess aldehyde such as 4-dimethylaminobenzaldehyde in the mass spectrometer was quite time consuming requiring in excess of twenty minutes before pyrolytic methylation could be attempted. The peptide condensation with aromatic aldehydes took some six hours, whilst pivaldehyde condensation took about fifteen minutes. In
Table 10. Relative abundance of selected ions for Benzyldiene peptide derivatives

Sequence ion abundances < 10% are in bold, 10-50% in italic and > 50% in ordinary type. The base peak is indicated by *

\[ X = N^+CH(\text{CH}_3)CO\text{NH}^+CH(\text{CH}_3)CO\text{NH}^+CH(\text{CH}_3)CO\text{OCH}_3 \]

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| X = \( p \)-Dimethylaminobenzylidene

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* Base peaks:

![Base peaks](image-url)

\(^a\) These residues have undergone complete methylation of the phenolic group or the indole or imidazole nitrogen. \(^b\) The ions shown are in the dehydro form. \(^c\) The ions shown have undergone the loss of NH\(_3\). \(^d\) The ions shown have undergone the loss of CH\(_3\)OH. \(^e\) All selected ions represent the mass of the lower m/e for the Cl isotopic ratio.
addition, the e.i. spectra of aromatic Schiff base peptide condensates (Table-10) contained prominent ions (P)^+ and (Q)^+ due to cleavage of N^\alpha - C^\alpha bonds of the first and second amino acid residue (Diag.-7).

These ions, not being sequence determining ions (A^+_n, B^+_n) made interpretation of the mass spectrum more difficult. Cleavage P is only occasionally observed with the neopentylidene derivative, although ions corresponding to the loss of part of the protecting group are encountered (Table-9). Ions corresponding to cleavage Q are rarely observed and are usually of low relative abundance. The use of aldehydes for N-protection of peptides is not applicable to Pro or Hyp and we have found the mass spectra of the Arg condensates difficult to interpret. The use of β-diketones for N-protection of peptides overcomes this limitation, as the condensation can be effected with all peptides regardless of their amino acid composition.

The pyrolytic esterification of acetylacetonyl, benzoylacetonyl and α,α,α'-trifluoroacetylacetonyl peptide TMPA salts yielded prominent ions in the e.i. spectra due to cleavage of the amine
Table II. Relative abundance of selected ions for Enamine peptide derivatives

Sequence ion abundances < 10% are in bold, 10-50% in italic and > 50% in ordinary type. The base peak is indicated by *

X—\text{CHR}—CO—\text{NH}—\text{CHR}—CO—\text{NH}—\text{CHR}—\text{CO}—\text{OCH}_3

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X = Acetylacetonyl

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</table>

A Cleavage P may be complete or partial loss of the protecting group. B Base peaks:

61.

C These residues have undergone complete methylation of the phenolic group or the indole or imidazole nitrogen. D Complete dehydration has occurred and sequence ions are in the dehydro form. E The ions indicated have undergone the loss of C₆H₅COCH-C(CH₃)NH₃. F All ions shown have undergone the loss of CH₂OH.
These ions are distinctive N-terminal tags and can be used as an unambiguous starting point in the search for the other sequence ions ($B_1^+, A_2^+, B_2^+, \ldots A_n^+, B_n^+$). However, certain difficulties have been encountered in the application and use of some of these protecting groups.

The e.i. mass spectra of the benzoylacetylonyI derivatives (Table-II) was complicated by a prominent cleavage of part of the marker group as $(PhCO)^+$. This ion was often the base peak and appreciably reduced the intensity of the sequence ions of the peptide. In addition, the removal of excess benzoylaceton required some twenty to thirty minutes prior to volatilisation of the ester derivative. However, useful e.i. mass spectra were obtained from the trifluoroacetylacetonyl peptide methyl esters (Table-12). Here some solubility problems were encountered when Ser peptide TMPA salts were derivatised with trifluoroacetylacetone, although in some cases this could be overcome by a gradual addition of the reagent over a period of time.

Of the compounds investigated, acetylacetone was found to be the most useful reagent for N-protection and subsequent pyrolytic
Table 12. Relative Intensities\textsuperscript{b} of Selected Ions for Trifluoroacetylation Derivatives

\[ \text{CH}_3 \quad A_1 \quad B_1 \quad A_2 \quad B_2 \quad M \]
\[ \text{CF}_3\text{COCH} = \text{C} - \text{NH} - \text{CHR}_1 \quad \text{CO}_1 \quad \text{NH} - \text{CHR}_2 \quad \text{CO}_2 \quad \text{OCH}_3 \]

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\textsuperscript{a}. Sequence ions are shown in the N-Methyl form as 60\% Methylation is observed.

\textsuperscript{b}. Sequence ion abundances: \(< 10\% \) are in bold, 10-50\% in italic, \(> 50\% \) in ordinary type.
methylation. The condensation was readily effected with all peptides regardless of the amino acid composition and Arg and Lys were the only two amino acids to react with a second molecule of acetylacetone. Lys forms a di-Schiff's base, while the guanidine side chain of Arg is converted to the much less polar δ-N-(2-pyrimidinyl) ornithine system\(^{95,96}\). Acetylacetone has been used for the N-protection and subsequent sequencing of over 120 peptides. In all these cases condensation and pyrolytic methylation could be induced readily. The only biproduct of the reaction is as yet an unidentified self condensation product of acetylacetone with prominent ions at m/e 225 and 326. Since excess acetylacetone and the biproduct are rapidly removed at 100°C, the contaminent is of no great consequence. The e.i. mass spectra of a selection of acetylacetonyl peptide methyl esters (Table-11) shows very little cleavage of the protecting group, the only ion of note is at m/e 43 (CH\(_3\)CO\(^+\))\(^+\) but this is usually of low relative abundance.

The sequencing of acetylacetonyl peptide esters by e.i. mass spectrometry results in intense amine fragment ions (A\(_1\))\(^+\) which can be used as a starting point in the search for the other sequence determining ions. This ion falls at unique m/e values for all amino acids, with the exception of Leu, Ile and Hyp (Table-13). It is frequently the most intense ion in the spectrum, except for N-terminal Tyr, Trp, Lys and Arg which are generally of 10-20% relative abundance. Lys and Arg are further
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characterised by intense ions at m/e = A_1 - 99 corresponding to cleavage of the C-N bond of the protected peptide. This cleavage is between the N^α - C^α bond for the Arg derivative, and the N^ε - C^ε bond for Lys peptides. This ion is very characteristic for these residues, (for example, Fig.-3 of Lys-Val), and can be used for the identification of the N-terminal amino acid residue. For some peptides, partial loss of the side chain is observed with N-terminal Met, Ser, Thr, Asp and Glu. Some larger Ser and Thr peptides tend to dehydrate at higher probe temperatures, whilst Gln and Asn may lose NH_3, thus making recognition of these residues in the N-terminal position more difficult. His, Trp, Phe and Tyr peptides show some elimination of the side chain as ArCH_2^+ or ArCH_2^*, but these cleavages can be used to confirm the presence of these residues. Cys Cys derivatives undergo S-S bond rupture accompanied by hydrogen transfer.

Bacon et.al. have utilised acetylacetone ethyl ester derivatives for the sequencing of peptides of 2-10 residues, but have found the volatility to be affected by amino acid composition and peptide chain length. We have obtained similar results with acetylacetonyl peptides utilising the pyrolysis procedure for esterification. The derivative of Ala-Leu-Ala-Val-Gly-Ala-Phe gave a readily interpretable mass spectrum with the necessary sequence ions (Fig.-4), but the derivative of glucagon (residues 21-29) Phe-Val-Gln-Trp-Leu-Met-Asn-Thr of similar size but with a number
Fig. 3. Acetylaceptonyl Lys-Val Methyl Ester.
of polyfunctional amino acids, had low volatility and gave low abundant ions, allowing identification of only the first four residues. Although this approach to peptide sequencing is rapid and allows identification of all residues (with the exception of Leu/Ile), it is of course limited by the volatility of peptide derivatives. To overcome this limitation of volatility, a combination of a mass spectrometric and an enzymatic approach was sought. By a suitable enzyme degradation of a polypeptide it was hoped to generate a mixture of smaller peptides. Peptide mixtures have been successfully identified by mass spectrometry and we hoped to apply our derivatisation procedure to identify the component peptides in these mixtures.

F. THE MASS SPECTROMETRIC IDENTIFICATION OF DIPEPTIDE MIXTURES OBTAINED FROM DIPEPTIDYLAMINOPePTIDASE-I HYDROLYSATES

It has been shown previously that the enzyme Dipeptidylaminopeptidase-I (DAP-I), also known as Cathepsin C hydrolyses a polypeptide from the N-terminal end to give a mixture of dipeptides. The potential of DAP-I for the sequencing of polypeptides has already been demonstrated on polypeptide hormones such as, β-corticotropin, angiotensin II, glucagon, secretin, and the A and B chains of oxidised bovine insulin. The published approach for the sequencing of a polypeptide by this route involves the treatment of a polypeptide with the enzyme to give a mixture of dipeptides. This is then followed by the separation and identification of all the dipeptides in the mixture. In a second experiment the N-terminal
Fig. 1. Aromatic Aromatic - Ala - Leu - Ala - Val - Gly - Ala - Ph - Methyl Ester.
amino acid residue of the polypeptide is removed by the Edman procedure or alternatively an amino acid is coupled to the N-terminal residue of the polypeptide. A further DAP-I digest of the modified peptide then leads to a new set of dipeptides. As the N-terminal amino acids of all the dipeptides obtained from the original polypeptide become C-terminal amino acids in the modified dipeptide mixture, the identification of the dipeptides in the two mixtures can be used to reconstruct the amino acid sequence of the polypeptide. The sequence determination is made very much more difficult by the repetition of a specific amino acid residue or peptide unit in the polypeptide. In addition, the action of the enzyme is limited by its inability to remove dipeptides with an N-terminal Arg or Lys and its inability to cleave the bond on either side of Pro residues. The presence of an amino-terminal Lys or Arg residue can be avoided by using only Trypsin generated peptides as substrates. With these substrates Pro residues still constitute an obstruction, but this can sometimes aid in the interpretation of the dipeptide mixture. Since the Pro residue provides a complete stop, the remaining polypeptide containing Pro in the second or third position can be degraded further by the Edman reaction to provide a fresh start for the enzyme and a new set of dipeptides. Despite these limitations, the method has been shown to be of value for the sequencing of tryptic oligopeptides.

The experimental approach taken by other investigations has been to separate the dipeptides from one another by either liquid chromatography or electrophoresis and then to identify each
of the dipeptides by comparing their Rf values with that of a set of dipeptide standards. An alternative method has made use of dansylation of the mixture followed by separation of the dansyl dipeptides\textsuperscript{107}. Here the identification of the N-terminal amino acid of each purified dipeptide derivative could be made by hydrolysis and subsequent separation of the dansyl amino acids by thin layer chromatography. The C-terminal amino acid of each dipeptide could also be identified if the above hydrolysate was dansylated and chromatographed in the same way. The dipeptide mixtures have also been derivatised and separated by gas chromatography\textsuperscript{108,109} and the components have been identified by mass spectrometry. Unfortunately, many of the 441 possible dipeptides, derivable from the 21 protein amino acids, cannot be made sufficiently volatile for gas chromatography and thus a complete sequence determination by this method is rarely possible\textsuperscript{110}. To fully utilise the potential of the DAP-I digestion for peptide sequencing, an unequivocal identification of the component dipeptides of a DAP-I digest without prior separation of the mixture is desirable.

The e.i. mass spectra of acetylacetonyl dipeptide methyl ester derivatives can be used for this purpose. These mass spectra almost always contain the amine fragment ion \((A_1)^+\), and thus the N-terminal amino acids of the component dipeptides from a DAP-I digest can be identified. Similarly, the C-terminal amino acid residues can be determined from the difference in molecular weight between the \((M)^+\) and the \((A_1)^+\) fragment. Unfortunately, the e.i. spectra of these
Table 14. The c.i. (isobutane) mass spectra of ACA-dipeptide methyl esters

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<td>359</td>
<td>100</td>
</tr>
<tr>
<td>ACA-Gly-Leu-OMe</td>
<td>285</td>
<td>100</td>
</tr>
<tr>
<td>ACA-Gly-Tyr-OMe</td>
<td>349$^b$</td>
<td>100</td>
</tr>
<tr>
<td>ACA-Glu-Glu-OMe</td>
<td>401$^b,c$</td>
<td>83</td>
</tr>
<tr>
<td>ACA-Trp-Trp-OMe</td>
<td>515$^b,c$</td>
<td>20</td>
</tr>
<tr>
<td>ACA-Arg-Glu-OMe</td>
<td>487$^b$</td>
<td>91</td>
</tr>
<tr>
<td>ACA-Hyp-Glu-OMe</td>
<td>285</td>
<td>100</td>
</tr>
<tr>
<td>ACA-His-Leu-OMe</td>
<td>365</td>
<td>50</td>
</tr>
<tr>
<td>ACA-Lys-Val-OMe</td>
<td>424</td>
<td>100</td>
</tr>
<tr>
<td>ACA-Asp-Phe-OMe</td>
<td>391$^c$</td>
<td>23</td>
</tr>
<tr>
<td>ACA-Met-Glu-OMe</td>
<td>389$^b$</td>
<td>100</td>
</tr>
<tr>
<td>ACA-Thr-Met-OMe</td>
<td>347</td>
<td>-</td>
</tr>
<tr>
<td>ACA-Ser-Val-OMe</td>
<td>301</td>
<td>100</td>
</tr>
<tr>
<td>ACA-Gln-Gly-OMe</td>
<td>300</td>
<td>77</td>
</tr>
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<td>ACA-Trp-Leu-OMe</td>
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<td>27</td>
</tr>
<tr>
<td>ACA-Asp-Val-OMe</td>
<td>345$^c$</td>
<td>47</td>
</tr>
</tbody>
</table>

$^a$All intensities are quoted as a percentage of the total ion current summed above m/e 130. All ions containing $^{13}$C are combined with the corresponding $^{12}$C ions for the purposes of this calculation. Intensities of less than 1% are not reported.

$^b$Methylation of the side chain of the C-terminal amino acid has occurred.

$^c$Methylation of the side chain of the N-terminal amino acid has occurred.

$^d$A side chain hydroxyl group has been lost as water.
compounds show low intensity molecular ions and an unequivocal identification of the component dipeptide \((M)^+\) ions in a mixture is difficult. This problem has been overcome by the use of chemical ionisation (c.i.) mass spectrometry, a technique first demonstrated by Munson and Field in 1966. The exact mechanism of the ionisation and the nature of the product ion depends upon the reactants and the conditions used, but in all cases the \((MH)^+\) ions are of greater intensity in c.i. mass spectrometry than the \((M)^+\) ion is in e.i. mass spectrometry. For this reason, the c.i. (isobutane) mass spectra of acetylacetonyl methyl esters were recorded and it was shown that under these conditions all the dipeptide derivatives gave large protonated molecular ions (Table-14). The only other ions noted in some cases, were those formed by the loss of water or methanol from the protonated molecular ion. The loss of water was important in the c.i. mass spectra of Ser and Thr containing peptides, while the loss of methanol appeared to be more facile in the cases of Asp containing peptides than with the corresponding Glu peptides. The terminal carbomethoxy groups of the acetylacetonyl dipeptide methyl esters were stable under these conditions.

However, the c.i. (isobutane) mass spectra by themselves were not sufficient for the identification of acetylacetonyl-dipeptide methyl esters, because they only show the protonated molecular ion of the dipeptide. On the other hand, the e.i. mass spectra taken alone were also inadequate, because the low intensity molecular ions were difficult to identify and only the N-terminal amino acids
Fig. 5 and 6. C.I. and e.i. mass spectra of a mixture of Acetylacetonyl dipeptide methyl esters.

Fig. 5 CI (isobutane) mass spectra of a mixture of ACA-dipeptide methyl esters taken at various probe temperatures. (*) Ion formed by loss of MeOH from the [MH]+ ion. ** Ion formed by the methylation of the neutral molecule.

Fig. 6 E.I. mass spectra of a mixture of ACA-dipeptide methyl esters taken at various probe temperatures. (*) Ion formed by the loss of MeOH from the [MH]+ ion. ** Ion formed by the methylation of the neutral molecule.
of the dipeptide mixture could be positively identified from the
intense N-terminal fragment ions \((A_1)^+\). For example, the c.i.
(isobutane) mass spectra of the acetylacetonyl methyl ester
derivatives of a synthetic dipeptide mixture (Fig.-5) gave the
following ions at m/e: 327, Val-Leu \((MH)^+\); 391 Asp-Phe \((MH)^+\);
359, Asn-Phe \((MH)^+ - (CH_3OH); 365, His-Leu \((MH)^+; 379, (N-Me)
His-Leu \((MH)^+; 511, Lys-Trp \((MH)^+. The e.i. mass spectra of the
same mixture (Fig.-6) contained the \((A_1)^+ fragment ions of the
dipeptides, and these were found to be at m/e: 154, Val \((A_1)^+;
184, Asp \((A_1)^+; 192, His \((A_1)^+; 206, (N-Me) His \((A_1)^+;
166, Lys \((A_1 - 99)^+. These N-terminal amino acid residues were
matched with the \(MH^+\) ions from Fig.-5, by making use of the relative
volatility of the dipeptide components. For example, the most
volatile compound present, the derivative from Val-Leu had a
protonated molecular ion at m/e 327 and a \((A_1)^+ fragment ion
at m/e 154. The least volatile component, the derivative of
Lys-Trp provided the last protonated molecular ion and N-terminal
fragment to appear at m/e 511 and 166 respectively.

This procedure had obvious application to the identification
of dipeptide mixtures from DAP-I digests, but since the condensation
of the peptide-TMPA salts with acetylacetone was slow, requiring three
to six hours, it was more convenient to exploit the faster
condensation reaction of ethylacetoacetate with amino groups\(^{112}\), for
N-terminal protection. This condensation was effected in thirty
minutes at 70°C and with two exceptions (Lys, Arg) all dipeptide
salts reacted only with one equivalent of ethylacetoacetate.

(Scheme-11). Lys and Arg dipeptides reacted with a second equivalent of ethylacetoacetate to form an ε-ethoxycarbonylpropenyl derivative I, or a δ-N-3-methyl-5-hydroxy-2-pyrimidyl ornithine derivative II, respectively\(^ {113}\) (Diag.-9). The non volatile N-protected dipeptide salts were introduced into a mass spectrometer via a direct insertion probe. At temperatures in the range of 80-110°C dimethylaniline was evolved with concomitant formation of the ethoxycarbonylpropenyl dipeptide methyl ester (Scheme-12).

As expected, the side chain functional groups of Tyr, Asp and Glu were completely methylated, whilst Trp and His were incompletely
methylated in the ring. Carboxymethylcysteine (CmC) residues, the

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{C}_2\text{H}_5\text{OCCH} = \text{CNHCHRCONHCHRCOOPhN(CH}_3)_3 + \text{HEAT} & \quad \text{C}_2\text{H}_5\text{OCCH} = \text{CNHCHRCONHCHRCOOCCH}_3 + \text{PhN(CH}_3)_2
\end{align*}
\]

(Scheme - 12)

product of the reduction and carboxymethylation of proteins and the Arg derivative II were also completely methylated by this process.

To further simplify the handling procedure and to reduce sample requirements, the use of only one mass spectrometer operation was desirable. For this purpose, the c.i. mass spectrometry of these compounds with different reagent gases such as hydrogen, helium, argon, methane and isobutane was investigated.

It was hoped that \((\text{MH})^+\) ions and N-terminal fragments of roughly equal intensity would be obtained with a suitable reagent gas. As expected, isobutane produced predominantly \((\text{MH})^+\) ions and very little fragmentation. Methane was more energetic, but the appearance of ions 26 amu and 40 amu higher in mass than the protonated molecular ion, was an undesirable feature of this reagent gas, as it could lead to difficulties in the interpretation. The c.i. fragmentation by hydrogen and the spectra produced by argon were very similar, with intense N-terminal fragment ions and protonated molecular ions being obtained in both cases. The spectra obtained with helium (Industrial grade), at pressures of between 0.8-1.00 torr gave the most desirable fragmentation characteristics for these peptide derivatives, as these spectra had intense N-terminal fragment ions
Table 15. The c.i. (helium) mass spectra of Ethoxycarbonylpropenyl-dipeptide Methyl Esters.

\[
\begin{align*}
\text{CH}_3 \\
C_2H_5OOCCH & = C - NH - CH \quad \text{CO} - NH - CH - \text{COOCH}_3 \\
& \quad | R_1 | R_2 |
\end{align*}
\]

<table>
<thead>
<tr>
<th>Peptide</th>
<th>([A^+\text{]}) Ion</th>
<th>([\text{MH}^+\text{]}) Ion</th>
<th>Other Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/e Intensity</td>
<td>m/e Intensity</td>
<td>m/e Intensity</td>
</tr>
<tr>
<td>Gly-Ala</td>
<td>142 100</td>
<td>273 83</td>
<td></td>
</tr>
<tr>
<td>Ala-Hyp</td>
<td>156 94</td>
<td>329 100</td>
<td>310 3</td>
</tr>
<tr>
<td>Val-Pho</td>
<td>184 62</td>
<td>391 100</td>
<td>345 5</td>
</tr>
<tr>
<td>Leu-Met</td>
<td>198 100</td>
<td>389 50</td>
<td></td>
</tr>
<tr>
<td>Ile-Ala</td>
<td>198 100</td>
<td>329 75</td>
<td>283 13</td>
</tr>
<tr>
<td>Pro-Val</td>
<td>182 100</td>
<td>341 93</td>
<td></td>
</tr>
<tr>
<td>Hyp-Gly</td>
<td>198 47</td>
<td>315 100</td>
<td>180 15</td>
</tr>
<tr>
<td>OMeC-Asn</td>
<td>260 11</td>
<td>434 94</td>
<td>417 100</td>
</tr>
<tr>
<td>TrpC-Arg (^d)</td>
<td>262 22</td>
<td>558 66</td>
<td>252 6.0</td>
</tr>
<tr>
<td>ArgC-Leu (^e)</td>
<td>192 100</td>
<td>494 60</td>
<td>508 18</td>
</tr>
<tr>
<td>Phe-Pho</td>
<td>232 65</td>
<td>439 100</td>
<td></td>
</tr>
<tr>
<td>HisC-Ala (^d)</td>
<td>236 38</td>
<td>367 100</td>
<td>222 50</td>
</tr>
<tr>
<td>Ser-Ala</td>
<td>172 45</td>
<td>303 50</td>
<td>154 25</td>
</tr>
<tr>
<td>Thr-Lys</td>
<td>186 75</td>
<td>486 60</td>
<td>168 100</td>
</tr>
<tr>
<td>Lys-Ala</td>
<td>196 100</td>
<td>456 74</td>
<td>325 32</td>
</tr>
<tr>
<td>Met-Asp (^d)</td>
<td>216 100</td>
<td>405 83</td>
<td></td>
</tr>
<tr>
<td>Gin-Trp (^d)</td>
<td>213 100</td>
<td>473 60</td>
<td>459 50</td>
</tr>
<tr>
<td>Asn-Thr</td>
<td>199 100</td>
<td>360 88</td>
<td>343 25</td>
</tr>
<tr>
<td>TrpC-Met</td>
<td>285 58</td>
<td>476 96</td>
<td>271 38</td>
</tr>
<tr>
<td>AspC-Phe</td>
<td>214 100</td>
<td>421 90</td>
<td></td>
</tr>
<tr>
<td>GluC-Hyp</td>
<td>228 100</td>
<td>401 22</td>
<td>383 4</td>
</tr>
</tbody>
</table>

(a) Intensities are quoted as a percentage of the base peak (100%) calculated for ions greater than m/e 140. All ions containing \( ^{13}C \) are combined with the corresponding \( ^{12}C \) ions for the purposes of this calculation. Intensities of less than 1% are not reported.

(b) All m/e values shown are for the most abundant isotope for each of the elements present.

(c) Methylatlon of the side chain of the N-terminal amino acid has occurred.

(d) Methylatlon of the side chain of the C-terminal amino acid has occurred.

(e) The Ions indicated are at \([A^+\text{]} -129\).
and a protonated molecular ion of almost equal intensity (Table-15).

It is probable that the formation of \((\text{MH})^+\) ions was caused by a proton donor such as \(\text{H}_3\text{O}^+\) (from water present in the helium as an impurity) or the anilinium ion (from the dimethylaniline present in the source). The spectra (hereafter referred to as c.l.) almost always contained an intense ion \((A_1)^+\) (Diag.-10) which allowed identification of the N-terminal amino acid of the dipeptide.

\[
\begin{align*}
\text{CH}_3 \\
\bigg| \\
\text{C}_2\text{H}_5\text{OOCCH} = \text{C} - \text{NH} = \text{CHR} \\
(A_1)^+
\end{align*}
\]

Diag.-10

These ions had unique m/e values for all amino acid residues, with the exception of Leu, Ile and Hyp. In the case of N-terminal Tyr, Trp, CmC, Lys and Arg, the intensity of \((A_1)^+\) is usually 10-20% of the molecular ion intensity, but the presence of an N-terminal Lys or Arg can be confirmed by intense ions at \((A_1 - 129)^+\) \((A_1 - (\text{NH}_2\text{C}(\text{CH}_3)\text{CHCOOC}_2\text{H}_5))\). Ions resulting from other processes were used to confirm the presence of certain amino acids. These were, the loss of water from Ser, Thr and Hyp, the loss of methanol from Ser, Glu and Asp, the loss of ethanol from Thr, the loss of ammonia from Gln and Asn, and normal amino acid side chain cleavages. In addition, the loss of ethanol from the N-protecting group and the presence of low intensity Quasimolecular ions due to the addition of dimethylaniline were also observed.

In an effort to simplify the matching of N-terminal ions with \((\text{MH})^+\) ions in the mixture, dimethyltrideuteromethylphenylammonium
Fig. 7 c.i. (Helium) Mass Spectrum of Ethoxycarbonylpropenyl Ala-Glu Dimethyl ester obtained by Pyrolytic Esterification.
hydroxide (DTMPAH) (Diag.-II) was used in place of TMPAH. In this way pyrolytic esterification yielded labelled protonated molecular ions and the residues of Trp, His, Tyr, Arg, CmC, Asp and Glu were labelled in their side chains. In this case, every methyl group that is subsequently transferred from this reagent to the dipeptide is 66% CH₃ and 33% CD₃. Thus, any ion which contains one methyl will appear as a (2:1) doublet separated by 3 amu and any ion which contains two methyl groups will appear a (4:4:1) triplet spaced 3 amu apart and any ion which contains 3 methyl groups will appear as an (8:12:6:1) quadruplet spaced 3 amu apart. If the (A₁)⁺ ion in the mass spectrum of the same dipeptide appears as a (2:1) doublet, then one of these polyfunctional amino acids must be in the N-terminal position. If, however, the (A₁)⁺ ion is a singlet, then both of the additional methyl groups are associated with the C-terminal amino acid (Fig.-7). The c.i. (helium) mass spectra of ethoxycarbonylpropenyl dipeptide methyl esters were quite simple and thus it was possible to deal with mixtures of dipeptides without a prior separation step. All that was normally required was to derivatize the sample with ethylacetoacetate and DTMPAH and to record the c.i. (helium) mass spectrum of the mixture at different probe temperatures.

The application of the DAP-1 technique to polypeptide sequencing required a second hydrolysis on the des N-terminal amino acid polypeptide. In these experiments the Edman procedure with methyl
TABLE 16. Computer Generated Listing of the Protonated Molecular Ions and the N-terminal Amino Acid Residues of the Ethoxycarbonylpropenyl methyl Ester Derivatives of the Possible Dipeptides.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Dipeptide</th>
<th>Molecular Ions</th>
<th>N-terminal Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Val</td>
<td>Ac-Val</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Lys</td>
<td>Ac-Lys</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Arg</td>
<td>Ac-Arg</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Asp</td>
<td>Ac-Asp</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Glu</td>
<td>Ac-Glu</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-His</td>
<td>Ac-His</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Pro</td>
<td>Ac-Pro</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Leu</td>
<td>Ac-Leu</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Ser</td>
<td>Ac-Ser</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Thr</td>
<td>Ac-Thr</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Ile</td>
<td>Ac-Ile</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Met</td>
<td>Ac-Met</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Phe</td>
<td>Ac-Phe</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Tyr</td>
<td>Ac-Tyr</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Trp</td>
<td>Ac-Trp</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Val</td>
<td>Ac-Val</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Lys</td>
<td>Ac-Lys</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Arg</td>
<td>Ac-Arg</td>
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<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Asp</td>
<td>Ac-Asp</td>
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<td>Phe-CMC</td>
</tr>
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<td>Ac-Glu</td>
<td>Ac-Glu</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
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<td>Ac-His</td>
<td>233 262</td>
<td>Phe-CMC</td>
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<tr>
<td>Ac-Pro</td>
<td>Ac-Pro</td>
<td>233 262</td>
<td>Phe-CMC</td>
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<td>Ac-Leu</td>
<td>Ac-Leu</td>
<td>233 262</td>
<td>Phe-CMC</td>
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<td>Ac-Ser</td>
<td>233 262</td>
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<td>Ac-Thr</td>
<td>233 262</td>
<td>Phe-CMC</td>
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<td>Ac-Ile</td>
<td>Ac-Ile</td>
<td>233 262</td>
<td>Phe-CMC</td>
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<td>Ac-Met</td>
<td>Ac-Met</td>
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<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Phe</td>
<td>Ac-Phe</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Tyr</td>
<td>Ac-Tyr</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
<tr>
<td>Ac-Trp</td>
<td>Ac-Trp</td>
<td>233 262</td>
<td>Phe-CMC</td>
</tr>
</tbody>
</table>

+Trp, His, Tyr, Arg are shown only for the N-methyl derivative.
Isothiocyanate was used to form the modified peptide and the N-terminal amino acid was identified as the methyl thiohydantoin by c.i. (isobutane) mass spectrometry.

The enzyme and the buffer salts used in the hydrolysis of the polypeptide interfered with the derivatization and subsequent mass spectrometric identification. A centrifugation through an Amicon CF50A Centriflo membrane followed by a purification through a short ion exchange column (Dowex 50W, H+) reduced this interference. The recovery of model peptides after this purification step was in excess of 95% for the neutral, 85% for the basic and 75% for the acidic dipeptides. DAP-I digests have been carried out on as little as 20 nmole of polypeptide, but as some dipeptides such as CmC-Asn yield only low intensity \((A_1)^+\) fragment ions (10-20%), it was found more desirable to use at least 0.1 µmole. It was found most convenient to do both DAP-I hydrolyses in parallel. After derivatisation and mass spectrometry the original polypeptide sequence could be reconstructed from the knowledge of the identity of the Edman derivative and the dipeptides in the two mixtures. This was most conveniently done with a computer generated listing of the \((MH)^+\) ions and the N-terminal amino acid residues of the possible dipeptides (Table-16) using the following Scheme:

**Step 1.** Record the ions and their isotope ratios in the region 259 to 630 amu of the two mass spectra and use Table-16 to make a listing of all the possible dipeptides which have matching \((MH)^+\) ions.
Table 17. Characteristic Ions for the Identification of N-Terminlal and C-Terminal Amino Acids.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>m/e</th>
<th>Origin of Fragment</th>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>m/e</th>
<th>m/e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>142</td>
<td></td>
<td>202</td>
<td>2:1</td>
<td>187</td>
<td></td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>156</td>
<td></td>
<td>216</td>
<td>2:1</td>
<td>201</td>
<td></td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>184</td>
<td></td>
<td>244</td>
<td>2:1</td>
<td>229</td>
<td></td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>196</td>
<td></td>
<td>258</td>
<td>2:1</td>
<td>243</td>
<td></td>
<td>187</td>
<td></td>
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<tr>
<td>Ile</td>
<td>198</td>
<td></td>
<td>258</td>
<td>2:1</td>
<td>243</td>
<td></td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>Hyp</td>
<td>198</td>
<td></td>
<td>258</td>
<td>2:1</td>
<td>243</td>
<td></td>
<td>187</td>
<td></td>
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<td>Ser</td>
<td>172</td>
<td></td>
<td>232</td>
<td>2:1</td>
<td>217</td>
<td></td>
<td>161</td>
<td></td>
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<tr>
<td>Thr</td>
<td>186</td>
<td></td>
<td>246</td>
<td>2:1</td>
<td>231</td>
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<td>175</td>
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<td>214</td>
<td>2:1</td>
<td>274</td>
<td>4:4:1</td>
<td>259</td>
<td></td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>228</td>
<td>2:1</td>
<td>280</td>
<td>4:4:1</td>
<td>273</td>
<td></td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>Asn</td>
<td>199</td>
<td>182</td>
<td>259</td>
<td>2:1</td>
<td>244</td>
<td></td>
<td>188</td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>213</td>
<td>196</td>
<td>273</td>
<td>2:1</td>
<td>258</td>
<td></td>
<td>202</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>232</td>
<td></td>
<td>292</td>
<td>2:1</td>
<td>277</td>
<td></td>
<td>222</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>262</td>
<td>2:1</td>
<td>322</td>
<td>4:4:1</td>
<td>307</td>
<td></td>
<td>237</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>285</td>
<td>2:1</td>
<td>271</td>
<td>(unmethylated)</td>
<td>345</td>
<td>4:4:1</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>236</td>
<td>2:1</td>
<td>222</td>
<td>(unmethylated)</td>
<td>296</td>
<td>4:4:1</td>
<td>281</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>196</td>
<td>325</td>
<td>385</td>
<td>2:1</td>
<td>370</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>192</td>
<td>2:1</td>
<td>381</td>
<td>4:4:1</td>
<td>366</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>188</td>
<td></td>
<td>248</td>
<td>2:1</td>
<td>233</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmC</td>
<td>260</td>
<td>154</td>
<td>320</td>
<td>4:4:1</td>
<td>305</td>
<td></td>
<td>236</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>182</td>
<td></td>
<td>242</td>
<td>2:1</td>
<td>227</td>
<td></td>
<td>171</td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>216</td>
<td>199</td>
<td>276</td>
<td>2:1</td>
<td>261</td>
<td></td>
<td>205</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>142</td>
<td>266</td>
<td>2:1</td>
<td>205</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Step 2. Identify the N-terminal residues in the two mixtures by locating all the \((A_1)^+\) ions in the region 142 to 322 amu in the two spectra. They will have even mass numbers except if N-terminal Asn, Gln, Lys, Arg and Trp are present. The \((A_1)^+\) ions normally appear as single peaks or as (2:1) doublets separated by 3 amu if N-terminal Asp, Glu, His, Trp, Arg, Tyr or CmC are present. All dipeptides in the list generated in Step 1 which do not contain an N-terminal amino acid in the list generated here, should be excluded as possibilities.

Step 3. An attempt can now be made to match \((A_1)^+\) with \((MH)^+\) ions on the basis of isotope ratios. In addition the amino acid composition of the polypeptide should now be used to firm up the list of dipeptides in the mixtures. If this is not available, the relative volatility of the components of the dipeptide mixtures can sometimes be used to associate \((A_1)^+\) and \((MH)^+\) ions for the most volatile or involatile component.

Step 4. Identify the C-terminal amino acid from its \((MH)^+\) ion in one of the mass spectra of the two dipeptide mixtures (Table-17). It will be located in the range 202 to 381 amu. Identify the N-terminal amino acid of the polypeptide from the c.i. (isobutane) mass spectrum of the Edman derivative (Table-17).

Step 5. Further reduce the number of possible dipeptides in the mixtures by matching the two peptide lists and reconstruct the polypeptide sequence by overlapping the dipeptides in the two mixtures in such a way that every C-terminal amino acid in every
dipeptide in the first mixture corresponds to an N-terminal amino acid in every dipeptide in the second mixture.

The above technique has been used to sequence the following peptides and peptide amides: Ser-Gly-Ala-Gly-Ala-Gly; Asp-Glu-Leu-Thr-Lys; Glu-Leu-Thr-Lys-ε-methylthiocarbamyl; Ala-Ala-Ala-Ala; Met-Phe-Gly; Phe-Val-Gln-Trp-Leu-Met-Asn-Thr; Phe-Gly-Gly-Phe; Met-Gly-Met-Met; Ala-Phe-Gly; Val-Leu-Ser; Gly-Leu-Tyr; Phe-Phe-Gly-Leu-Met.NH₂; Gly-Leu-Met.NH₂; Leu-Met.NH₂; Val-Phe.NH₂; Trp-Met-Asp-Phe.NH₂; Trp-Met-Gly-Asp-Phe.NH₂.

The identifying ions for the dipeptide mixtures from each peptide and its des N-terminal peptide are tabulated in the experimental (Section-F). However, the following examples have been selected to demonstrate the method and to highlight a number of problems which have been encountered.

Example I describes the application of the method to the sequencing of an octapeptide. In this case the dipeptide mixture derived from the DAP-I hydrolysis of polypeptide-I yielded the following ions (Fig.-8) in the region 259 to 630 amu which were matched with possible (MH)⁺ ions.

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>473</td>
<td>(4:4:1)</td>
<td>Gln-Trp, Trp-Gln, His-Tyr, Tyr-His</td>
</tr>
<tr>
<td>459</td>
<td>(2:1)</td>
<td>Asn-Trp, Trp-Asn</td>
</tr>
<tr>
<td>456</td>
<td>(4:4:1)</td>
<td>Ala-Lys, Lys-Ala</td>
</tr>
<tr>
<td>442</td>
<td>(2:1)</td>
<td>Pro-Trp, Trp-Pro, Gly-Lys, Lys-Gly</td>
</tr>
<tr>
<td>391</td>
<td>(2:1)</td>
<td>Phe-Val, Val-Phe, Cys-Glu, Glu-Cys, Ala-CmC, CmC-Ala</td>
</tr>
</tbody>
</table>
Possible Dipeptides

Leu-Met, Met-Leu, Thr-Glu, Glu-Thr, Pro-Phe, Phe-Pro, Ile-Met, Met-Ile, Hyp-Met, Met-Hyp, Asn-Asp, Asp-Asn

Asn-Thr, Thr-Asn, Ser-Gln, Gln-Ser

Pro-Thr, Val-Val, Thr-Pro

no match

A search for ions in the region 142-322 amu produced the following (A₁)⁺ ions at m/e:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>389</td>
<td>(2:1)</td>
<td>Leu-Met, Met-Leu, Thr-Glu, Glu-Thr, Pro-Phe, Phe-Pro, Ile-Met, Met-Ile, Hyp-Met, Met-Hyp, Asn-Asp, Asp-Asn</td>
</tr>
<tr>
<td>360</td>
<td>(2:1)</td>
<td>Asn-Thr, Thr-Asn, Ser-Gln, Gln-Ser</td>
</tr>
<tr>
<td>343</td>
<td>(2:1)</td>
<td>Pro-Thr, Val-Val, Thr-Pro</td>
</tr>
<tr>
<td>342</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
</tbody>
</table>

Since Asn and Gln have been identified, the ions at m/e 182 and 196 could also be due to Asn-(NH₃) and Gln-(NH₃) respectively. After matching ions and isotope ratios in the two lists a large number of the dipeptide possibilities could be eliminated. For example, the isotope ratio (4:4:1) of the ions at m/e 473 was not compatible with the expected isotope ratio (8:12:6:1) for the (MH)⁺ ions of His-Tyr, Tyr-His, the ratio of the ion at m/e 456 (4:4:1) did not fit the assignment of Ala-Lys and Lys-Ala which should be (2:1) and the isotope ratio of (2:1) at m/e 459, 442, 391 and 389 were not compatible with that expected for the (MH)⁺ ion of Asn-Trp, Trp-Asn, Pro-Trp, Trp-Pro, Cys-Glu, Glu-Cys, Ala-CmC, CmC-Ala, Thr-Glu, Glu-Thr, Asn-Asp, Asp-Asn which should be (4:4:1). Finally, the absence of an (A₁)⁺ ion for N-terminal Trp, Gly, Met, Thr, Ser and Val was used to eliminate Trp-Gln, Gly-Lys, Met-Leu, Val-Phe, Thr-Asn, Ser-Gln,
FIG. 9 POLYPEPTIDE-1 DIPEPTIDE MIXTURE-2
Thr-Pro and Val-Val so that the list of dipeptides could be
reduced to the following possibilities at m/e:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>473</td>
<td>Gln-Trp</td>
</tr>
<tr>
<td>459</td>
<td>no match</td>
</tr>
<tr>
<td>456</td>
<td>no match</td>
</tr>
<tr>
<td>442</td>
<td>Lys-Gly</td>
</tr>
<tr>
<td>391</td>
<td>Phe-Val</td>
</tr>
<tr>
<td>389</td>
<td>Leu-Met, Pro-Phe, Hyp-Met, Ile-Met, Phe-Pro</td>
</tr>
<tr>
<td>360</td>
<td>Asn-Thr, Gln-Ser</td>
</tr>
<tr>
<td>343</td>
<td>Pro-Thr</td>
</tr>
<tr>
<td>342</td>
<td>no match</td>
</tr>
</tbody>
</table>

On the basis of the amino acid analysis of the polypeptide Phe (1.0),
Val (0.97), Glx (1.01), Trp (not determined), Leu (1.03), Met (1.00),
Asx (1.00) and Thr (1.00) the Pro, Lys, Hyp, Ile and Ser in the
above list could be eliminated and the dipeptide possibilities in
mixture I could be reduced to: m/e 473 Gln-Trp; 391 Phe-Val;
389 Leu-Met; 360 Asn-Thr and the ions for which no match could be
found previously could be identified as m/e 459 (2:1) Gln-Trp
(unmethylated; 456 (4:4:1) Gln-Trp-(NH₃); 442 (2:1) Gln-Trp
(unmethylated-NH₃); 343 (2:1) Asn-Thr-(NH₃); 342 (2:1) Asn-Thr-(H₂O).

In a similar fashion the dipeptide mixture generated from the
modified polypeptide yielded the following dipeptide possibilities
and N-terminal residues. (Fig.-9)

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>458</td>
<td>(4:4:1)</td>
<td>Trp-Leu, Trp-Ile, Trp-Hyp, Leu-Trp, Ile-Trp, Hyp-Trp</td>
</tr>
<tr>
<td>444</td>
<td>(2:1)</td>
<td>Trp-Val, Val-Trp</td>
</tr>
<tr>
<td>m/e</td>
<td>Isotope Ratio</td>
<td>Possible Dipeptides</td>
</tr>
<tr>
<td>-----</td>
<td>---------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>390</td>
<td>(2:1)</td>
<td>Met-Asn, Asn-Met</td>
</tr>
<tr>
<td>373</td>
<td>(2:1)</td>
<td>Asn-Asn, Val-Asp, Pro-Met, Asp-Val, Met-Pro</td>
</tr>
<tr>
<td>355</td>
<td>(2:1)</td>
<td>Pro-Leu, Pro-Ile, Pro-Hyp, Leu-Pro, Ile-Pro, Hyp-Pro</td>
</tr>
<tr>
<td>168</td>
<td>(Singlet)</td>
<td>no match</td>
</tr>
<tr>
<td>184</td>
<td>&quot;</td>
<td>Val</td>
</tr>
<tr>
<td>186</td>
<td>&quot;</td>
<td>Thr</td>
</tr>
<tr>
<td>216</td>
<td>&quot;</td>
<td>Met</td>
</tr>
<tr>
<td>228</td>
<td>(2:1)</td>
<td>Glu</td>
</tr>
<tr>
<td>246</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>271</td>
<td>(Singlet)</td>
<td>no match</td>
</tr>
<tr>
<td>285</td>
<td>(2:1)</td>
<td>Trp</td>
</tr>
</tbody>
</table>

After matching the ions and the isotope ratios in the two lists, the isotope ratio of (2:1) at m/e 444 and 373, were not compatible with the expected isotope ratio (4:4:1) of Trp-Val, Val-Trp, Asp-Val, Val-Asp and these dipeptides could be eliminated. The absence of \((A_1)^+\) ions for Leu, Ile, Hyp, Asn, Pro, Gln was used to remove Leu-Trp, Ile-Trp, Hyp-Trp, Asn-Met, Asn-Asn, Pro-Met, Leu-Asn, Hyp-Asn, Ile-Asn, Gln-Val, Asn-Leu, Asn-Hyp, Asn-Ile, Pro-Leu, Pro-Ile, Pro-Hyp, Leu-Pro, Ile-Pro and Hyp-Pro from the list. On the basis of the amino acid analysis Trp-Ile, Trp-Hyp could also be eliminated and the list could be reduced to: m/e 444 Trp-Leu (unmethylated); 373 Met-Asn-(NH₃) and 355 Val-Gln-(NH₃).

Finally, the C-terminal amino acid in mixture 2 could be identified as Thr from its \((MH)^+\) ion at m/e 246 (2:1), and ions
at m/e 228 Thr-(H₂O) and 186 [(A₁)⁺, Thr] and the N-terminal amino acid was shown to be Phe from the c.i. (isobutane) mass spectrum of the Edman derivative (MH)⁺ of m/e 222.

From the above information the dipeptides in the two mixtures could be overlapped;

Mixture 1. Phe-Val, Gln-Trp, Leu-Met, Asn-Thr
Mixture 2. Val-Gln, Trp-Leu, Met-Asn, Thr

and the polypeptide sequence could be identified as:

Phe-Val-Gln-Trp-Leu-Met-Asn-Thr.

Example 2 illustrates the application of the procedure to the sequencing of a polypeptide which contains a repeated dipeptide unit. Because the method relies only on the identification of the (A₁)⁺ and the (MH)⁺ ions of the dipeptide mixtures, a replication of a peptide sequence in the polypeptide is difficult to detect; unless some degree of quantitative measurement is attempted. In some cases this problem can be overcome if an amino acid analysis of the polypeptide is available. For example, the mass spectrum (Fig.-10) of the DAP-I digest of Ser-Gly-Ala-Gly-Ala-Gly yielded the following dipeptide possibilities and N-terminal residues.

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Dipeptide Possibilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>289</td>
<td>(2:1)</td>
<td>Gly-Ser, Ser-Gly</td>
</tr>
<tr>
<td>273</td>
<td>(2:1)</td>
<td>Gly-Ala, Ala-Gly</td>
</tr>
<tr>
<td>243</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>227</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>172</td>
<td>(Singlet)</td>
<td>Ser</td>
</tr>
<tr>
<td>156</td>
<td>(Singlet)</td>
<td>Ala</td>
</tr>
</tbody>
</table>
After matching the ions, Gly-Ser (m/e 289) and Gly-Ala (m/e 273) could be eliminated. The list of possible dipeptides was thus reduced to:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>289</td>
<td>Ser-Gly</td>
</tr>
<tr>
<td>273</td>
<td>Ala-Gly</td>
</tr>
</tbody>
</table>

and the ions for which no match could be found were identified as Ser-Gly-(C$_2$H$_5$OH), m/e 243 and Ala-Gly-(C$_2$H$_5$OH), m/e 227.

On the basis of the amino acid analysis of the peptide Ser (0.96) Gly (3.01) and Ala (2.03), the presence of a second Ala-Gly unit in mixture I was indicated. The ions generated in mixture 2 (Fig.-II) yielded the following possibilities:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>273</td>
<td>(2:1)</td>
<td>Gly-Ala, Ala-Gly</td>
</tr>
<tr>
<td>227</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>202</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>142</td>
<td>(Singlet)</td>
<td>Gly</td>
</tr>
</tbody>
</table>

After matching of these ions Ala-Gly (m/e 273) could be eliminated and the dipeptide present was identified as Gly-Ala (m/e 273).

On the basis of the amino acid analysis of the original polypeptide two Gly-Ala residues are present in mixture 2 and the ion at m/e 227 was identified as Gly-Ala-(C$_2$H$_5$OH). The C-terminal amino acid in mixture 2 was shown to be Gly m/e 202 (2:1) and the N-terminal amino acid was identified as Ser [(MH)$^+$ m/e 161] from the mass spectrum of the Edman derivative.

From this information the dipeptides in the two mixtures could
FIG. 12 POLYPEPTIDE-3 DIPEPTIDE MIXTURE-1
be overlapped:

Mixture 1. Ser-Gly, Ala-Gly, Ala-Gly

Mixture 2. Gly-Ala, Gly-Ala, Gly

and the sequence was identified as:
Ser-Gly-Ala-Gly-Ala-Gly.

Example 3 highlights an obvious practical problem which arises from the application of the procedure to the sequencing of tryptic peptides, since the use of the Edman degradation may partially block the ε-amino group of a C-terminal Lys residue. Our experiments indicated that the DAP-I hydrolysate of the modified peptide may contain both the Lys dipeptide and its ε-methylthiocarbamyl Lys (ε-MTC-Lys) dipeptide which can be identified from its mass spectrum.

The DAP-I hydrolysate of such a polypeptide (Fig.-12) yielded the following (MH)$^+$ and ($A_1$)$^+$ ions at:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>417</td>
<td>(8:12:6:1)</td>
<td>Asp-Glu, Glu-Asp, Pro-CmC, CmC-Pro</td>
</tr>
<tr>
<td>385</td>
<td>(2:1)</td>
<td>Pro-Glu, Glu-Pro</td>
</tr>
<tr>
<td>359</td>
<td>(2:1)</td>
<td>Thr-Leu, Thr-Ile, Thr-Hyp, Ala-Glu, Leu-Thr, Ile-Hyp, Hyp-Thr, Glu-Ala</td>
</tr>
<tr>
<td>341</td>
<td>(2:1)</td>
<td>Pro-Val, Val-Pro</td>
</tr>
<tr>
<td>325</td>
<td>(Singlet)</td>
<td>Lys</td>
</tr>
<tr>
<td>256</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>214</td>
<td>(2:1)</td>
<td>Asp</td>
</tr>
<tr>
<td>198</td>
<td>(Singlet)</td>
<td>Leu, Ile, Hyp</td>
</tr>
<tr>
<td>196</td>
<td>(Singlet)</td>
<td>Lys-(129)</td>
</tr>
</tbody>
</table>

After matching the ions and the isotope ratios in the two lists, the isotope ratio of (8:12:6:1) at m/e 417 was not compatible with the expected isotope ratio (4:4:1) of Pro-CmC, CmC-Pro and
FIG. 13 POLYPEPTIDE-3 DIPEPTIDE MIXTURE-2

RELATIVE ABUNDANCE

Thr[Al]⁺ - (H₂O)

Thr[Al]⁺

Glu[Al]⁺
the ratio of (2:1) at m/e 385 and 359 were not in agreement with the expected ratio of (4:4:1) for Pro-Glu, Glu-Pro, Ala-Glu, Glu-Ala. The absence of \((A_1)^+\) ions for Glu, Thr, Pro and Val was used to eliminate Glu-Asp, Thr-Leu, Thr-Ile, Thr-Hyp, Pro-Val and Val-Pro. The list could now be reduced to:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>417</td>
<td>Asp-Glu</td>
</tr>
<tr>
<td>385</td>
<td>no match</td>
</tr>
<tr>
<td>359</td>
<td>Leu-Thr, Ile-Thr, Hyp-Thr</td>
</tr>
<tr>
<td>341</td>
<td>no match</td>
</tr>
</tbody>
</table>

The ions for which no match could be found were identified as:

m/e 385 Lys and 341 Leu-Thr-(H\(_2\)O), Ile-Thr-(H\(_2\)O), Hyp-Thr-(H\(_2\)O).

On the basis of the amino acid analysis of the polypeptide, Asx(1.00), Glx(1.01), Leu(1.01), Lys(1.03) and Thr(1.00) the list of possibilities in mixture I could be reduced to Asp-Glu (m/e 417), Leu-Thr (m/e 359) and Lys (m/e 385).

The dipeptide mixture generated from the modified polypeptide (Fig.-13) gave the following dipeptide and \((A_1)^+\) possibilities:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>486</td>
<td>(2:1)</td>
<td>Thr-Lys, Lys-Thr</td>
</tr>
<tr>
<td>468</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>442</td>
<td>(2:1)</td>
<td>Gly-Lys, Lys-Gly, Pro-Trp, Trp-Pro</td>
</tr>
<tr>
<td>423</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>401</td>
<td>(4:4:1)</td>
<td>Leu-Glu, Ile-Glu, Hyp-Glu, Gln-Gln, Glu-Leu, Glu-Ile, Glu-Hyp</td>
</tr>
<tr>
<td>228</td>
<td>(2:1)</td>
<td>Glu</td>
</tr>
<tr>
<td>186</td>
<td>(Singlet)</td>
<td>Thr</td>
</tr>
<tr>
<td>168</td>
<td>(Singlet)</td>
<td>no match</td>
</tr>
</tbody>
</table>
After matching the isotope ratios of the two sets of ions the (2:1) ratio at m/e 442 was not compatible with Pro-Trp, Trp-Pro (4:4:1) and the (4:4:1) ratio at m/e 401 was not in agreement with the expected (2:1) ratio for Gln-Gln. The absence of \((A_1)^+\) ions for Lys, Gly, Leu, Ile, Hyp could be used to remove Lys-Thr, Gly-Lys, Lys-Gly, Leu-Gln, Ile-Glu and Hyp-Glu from the list of possible dipeptides. On the basis of the amino acid analysis of the polypeptide the lists of dipeptides in mixture 2 could be reduced to Thr-Lys and Glu-Leu and the ions for which no match could be found were identified as: m/e 468, Thr-Lys-(H2O); 442, Thr-Lys-ε-MTC; 423, Thr-Lys-ε-MTC-(H2O) and 168, Thr \([(A_1)^+ - (H_2O)]\). The dipeptide components of the two mixtures were overlapped on the basis of the c.i. (isobutane) spectra of the Edman derivative Asp \([(MH)^+ \text{ at m/e } 189]\) and the identification of the C-terminal amino acid as Lys \([(MH)^+ \text{ at m/e } 385 (2:1)]\) in mixture 1 and the sequence of the polypeptide was shown to be: Asp-Glu-Leu-Thr-Lys. The ε-MTC group of Lys in the modified peptide, can be conveniently removed with aqueous hydrazine\(^{119}\). The mass spectra of the deblocked polypeptide was also sequenced by the above procedure. Similar results were obtained, with the exception that the ions at m/e 442 and 423 were absent.

Example 4 describes the application of the technique to the sequencing of polypeptide amides. Here the DAP-I degradation proceeded normally and all alternative peptide bonds were hydrolysed. Peptide amides with an even number of residues yielded the expected dipeptide mixture,
whilst peptide amides with an odd number of residues yielded dipeptides and the C-terminal amino acid amide. The pyrolytic derivatisation procedure converted the amino acid amides to the ethoxycarbonylpropenyl amide derivatives which were identified from their c.i. (helium) mass spectra. In some cases a small amount of the original polypeptide amide was hydrolysed to the free peptide during the work up of the Edman degradation.

The DAP-1 digest of such a polypeptide amide (Fig.-14) yielded the following \( (\text{MH})^+ \) and \( (\text{A}_1)^+ \) ions:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>476</td>
<td>(4:4:1)</td>
<td>Met-Trp, Trp-Met</td>
</tr>
<tr>
<td>462</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>331</td>
<td>(4:4:1)</td>
<td>Gly-Asp, Asp-Gly, Ser-Val, Val-Ser</td>
</tr>
<tr>
<td>285</td>
<td>(2:1)</td>
<td>Trp</td>
</tr>
<tr>
<td>277</td>
<td>(Singlet)</td>
<td>no match</td>
</tr>
<tr>
<td>271</td>
<td>(Singlet)</td>
<td>no match</td>
</tr>
<tr>
<td>232</td>
<td>(Singlet)</td>
<td>Phe</td>
</tr>
<tr>
<td>144</td>
<td>(2:1)</td>
<td>no match</td>
</tr>
<tr>
<td>142</td>
<td>(Singlet)</td>
<td>Gly</td>
</tr>
</tbody>
</table>

After matching ions and isotope ratios in the two lists, the isotope ratio of (4:4:1) at m/e 331 was not compatible with the expected ratio of (2:1) for Val-Ser and Ser-Val, and the absence of an \( (\text{A}_1)^+ \) ion for Met was used to eliminate Met-Trp. The list could now be reduced to Trp-Met (m/e 476) and Gly-Asp (m/e 331) and the ions for which no match could be found were identified as Trp-Met (m/e 462 unmethylated), Phe.NH\(_2\) (m/e 277), Trp(MH\(^+\)) (unmethylated) (m/e 271), m/e 144 (Diag.-12).
The DAP-I digest on the modified peptide, gave rise to the following \((\text{MH})^+\) and \((A_1)^+\) ions (Fig.-15) at:

<table>
<thead>
<tr>
<th>m/e</th>
<th>Isotope Ratio</th>
<th>Possible Dipeptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>421</td>
<td>(4:4:1)</td>
<td>Val-Tyr, Tyr-Val, Asp-Phe, Phe-Asp, Thr-CmC, CmC-Thr</td>
</tr>
<tr>
<td>333</td>
<td>(2:1)</td>
<td>Met-Gly, Gly-Met, Ser-Thr, Thr-Ser</td>
</tr>
<tr>
<td>216</td>
<td>(Singlet)</td>
<td>Met</td>
</tr>
<tr>
<td>214</td>
<td>(2:1)</td>
<td>Asp</td>
</tr>
</tbody>
</table>

After matching the ions and the isotope ratios in the above list, the dipeptides Val-Tyr, Tyr-Val, Thr-CmC, CmC-Thr, Gly-Met, Ser-Thr and Thr-Ser could be eliminated on the basis of the absence of \((A_1)^+\) ions for Val, Tyr, Thr, CmC, Gly and Ser. The list of possible dipeptides in mixture 2 was thus reduced to Asp-Phe (m/e 421) and Met-Gly (m/e 333).

After identification of the N-terminal residue as Trp \((\text{MH})^+\ m/e 260\), from its Edman derivative, the two dipeptide mixtures could be overlapped:

Mixture 1. Trp-Met, Gly-Asp, Phe.NH₂

Mixture 2. Met-Gly, Asp-Phe

to give the reconstructed sequence of the polypeptide:

Trp-Met-Gly-Asp-Phe.NH₂
This method of sequencing which relies upon identification of the c.i. (helium) mass spectra of the ethoxycarbonylpropenyl methyl ester derivatives of dipeptide mixtures from DAP-I hydrolysates without separation of the component dipeptides has been applied successfully to the identification of up to an octapeptide. Although larger peptides have not yet been sequenced by this procedure, the method is potentially useful for the sequencing of peptides up to 25 residues. But, beyond this size the possibility of deriving a unique sequence decreases markedly. However, if tryptic peptides are used for DAP-I digests, then polypeptides greater than 25 residues are seldom obtained.
GENERAL

Mass Spectrometry

Low resolution e.i. mass spectra were recorded on an E.A.I. QUAD 300D mass spectrometer using the solid probe inlet system. Operating conditions for the mass spectrometer were: ion source temperature 220°C, ionising current 50 µamps and ionising voltage 70 eV. The solid probe temperature was varied from ambient to 300°C.

Chemical ionisation mass spectra were recorded on an A.E.I. MS-902 mass spectrometer or on a Dupont 21-491B mass spectrometer, both modified for operation in the c.i. mode. All c.i. mass spectra were measured at a source temperature of 200°C and the probe temperature was varied from ambient to 300°C.

Peptides

All peptides were obtained from Schwarz/Mann Research Laboratories (Orangeburg, New York); Bachem (Marina Del Ray, Cal.); Cyclo Chemical Corp., (Los Angeles, Cal.) and Research Plus Laboratories (Denville, New Jersey).

Reaction Vessels

All peptide derivatisations were carried out in reactor vials (1ml capacity) fitted with teflon lined caps (Regis, Illinois).

Molecular Sieves

Molecular sieve type 3A (1/16" pellets) was manufactured by the Linde Air Products Co., and was distributed by BDH Chemicals Ltd.
Esterification Reagents

Preparation of Anhydrous Methanol

A dry one-litre round-bottom flask was fitted with a double surface condenser and drying tube. Clean dry magnesium turnings (2.5g) and iodine (0.25g) were added to the flask followed by 30ml of methanol. The mixture was warmed until the iodine had disappeared and most of the magnesium was converted to the methylate. More methanol (450ml) was then added and the mixture was refluxed for thirty minutes. The methanol was then distilled off and stored over 3A molecular sieve.

Preparation of Thionyl Chloride-Methanol Reagent

Anhydrous methanol (9ml) was cooled in a dry ice/acetone bath and freshly distilled thionyl chloride (1ml) was added dropwise. The reagent was used within fifteen minutes of preparation.

Preparation of Diazomethane Reagent

p-ToluenesulphonylmethylNitrosamide (2.14g) (Fluka A.G. Buchs S.G.) was dissolved in ether (30ml) and the solution was cooled in an ice bath. Potassium hydroxide (0.4g) in ethanol (10ml) was then added. After five minutes the ethereal solution of diazomethane was distilled from a water bath. The reagent was stored in a brown bottle at 0°C.

Preparation of Tetramethylammonium Hydroxide (TMAH)

A mixture of tetramethylammonium bromide (E. Merck Darmstadt) (1.54g, 10 mmole) and silver oxide (1.8g, 14.4 meq.) was stirred in anhydrous methanol (40ml) for two hours. The silver bromide was
filtered off, and the base concentrations of the filtrate was determined by titration with standard sulphuric acid (0.1N) to a phenolphthalein end point. The solution was diluted to a strength of 0.1 mmole/ml (≈ 60ml) and it was stored under refrigeration. Under these conditions the solution could be kept indefinitely.

Preparation of Trimethylphenylammonium Hydroxide (TMPAH)

Trimethylphenylammonium Bromide (Fluka A.G. Buchs S.G.) (2.16g, 10 mmole) and silver oxide (1.8g, 14.4 meq.) was stirred in anhydrous methanol (30ml) for two hours. The solution was filtered, and the hydroxide ion concentration of the filtrate was determined by titration with standard sulphuric acid (0.1N) to a phenolphthalein end point. The solution was diluted with anhydrous methanol to a concentration of 0.1 mmole/ml (≈ 70ml) and stored under refrigeration at 0°C. Under these conditions the solution was indefinitely stable.

A. ESTERIFICATION OF ACETYL AMINO ACIDS

Acetylation of Amino Acids

The amino acid (10 μ mole) was dissolved in water (1ml) and acetic anhydride (1ml) (E. Merck A.G. Darmstadt) was added. The solution was agitated for ten minutes at room temperature and then for five minutes at 30°C. The excess solvent was then removed in vacuo and the residue was redissolved in ethanolic ammonia (4:1) (0.5ml). The product was checked for unreacted amino acid by paper chromatography on Whatman No.2 paper, using butanol, methanol
ammonia (4:1:5) as developer and ninhydrin (0.5% in butanol) reagent was used to detect the unreacted amino acid. The acetyl amino acid was detected as a yellow spot on a blue background by spraying the paper with bromocresol green spray (E. Merck A.G. Darmstadt). If unreacted amino acid was detected by this method, the acetylation was repeated until a homogenous product was obtained. Finally, the excess ethanolic ammonia was removed on the rotary evaporator and the product was dried over silica-gel.

Preparation of Acetyl Amino Acid Methyl Esters

The acetyl amino acids Gly, Ala, Val, Leu, Phe, Met, Lys, Pro, Glu, Asp, Ser and Thr were esterified by heating the amino acids (1 µ mole) with the thionyl chloride-methanol reagent (0.5ml) under reflux for thirty minutes or until all the amino acid had dissolved. After removal of the solvent and the excess reagent in vacuo, the residue was redissolved in methanol (0.1ml) and an aliquot (20µl) was dried under vacuum in a glass capillary tube. The dried sample was then examined by mass spectrometry and the reference mass spectra were recorded.

The acetyl amino acids Asn and Gln (1 µ mole) were esterified by dissolution of the acetyl amino acid in methanol (0.1ml) and diazomethane (0.5ml) was added dropwise over a twenty minute period or until a persistent yellow colour was obtained. The excess solvent was then evaporated and the residue was redissolved in methanol (0.1ml). An aliquot (20µl) was then dried under vacuum in a glass capillary tube and the sample was examined by mass
spectrometry. The results are shown in Table-1.

**Esterification of Acetyl Amino Acids by Pyrolysis**

The dry N-acetyl amino acid (10 μ mole) was dissolved in TMAH (0.1N) or in TMPAH (0.1N) (100 μl of this reagent was added per COOH group). An aliquot (2 μl) was transferred to a glass capillary tube and the solvent was evaporated under vacuum. The glass capillary was transferred to the solid probe inlet of the mass spectrometer and the trimethylamine was removed at a solid probe temperature of 60°C. This process could be monitored on the oscilloscope by following the disappearance of ions at m/e 58, 59. Similarly, the ions at m/e 120, 121 could be used to monitor the concentration of dimethylaniline. The temperature was then slowly increased until the mass spectrum of the acetyl amino acid methyl ester appeared on the oscilloscope. When the e.i. spectra of the acetyl amino acid ester reached a constant intensity, a chart record was taken. The pyrolytic esterification was also carried out at solid probe temperatures of 80, 90, 110, 130, 150, 170, 200 and 230°C. The mass spectra of acetyl amino acid esters prepared by pyrolytic esterification of TMPA salts at 80-100°C and of TMA salts at 110-130°C are shown in Table-1.

**B. PERMETHYLATION OF ACETYL AMINO ACIDS**

**Preparation of Permethylated Acetyl Amino Acids by Pyrolytic Methylation**

The dry N-acetyl amino acid (0.2 μ mole) was dissolved in TMPAH (0.1N) and the solution was evaporated under reduced pressure
In a glass capillary tube. The sample was inserted into the mass spectrometer, and at a solid probe temperature of 100°C the sample was pyrolytically methylated. The experiment was repeated using TMPAH volumes of 2.4 µl (0.22 µ moles), 3 µl (0.3 µ moles), 4 µl (0.4 µ moles), 6 µl (0.6 µ moles) and 8 µl (0.8 µ moles).

C. PYROLYTIC ESTERIFICATION OF ACETYL PEPTIDES

Preparation of N-Acetyl Peptides

The peptide (1 µ mole) was shaken with a mixture of acetic anhydride (1ml) and water (1ml) at room temperature for two hours. The solution was then evaporated under vacuum and the residue was then redissolved in water (1ml). The purity of the product was checked by paper chromatography on Whatman No.2 paper using butanol: methanol:ammonia (4:1:5) as developer. Untreated peptide was detected with ninhydrin and acetyl peptides were detected with bromocresol green spray reagent, (acetyl peptides stained as a yellow spot against a blue background). In cases of incomplete acetylation the acetylation procedure was repeated until a homogenous product was obtained. The solution was then re-evaporated to dryness under reduced pressure and the sample was dried under vacuum over KOH pellets and silica-gel. The following peptides Gly-Ile, Ala-Leu, Val-Tyr-Val, Val-Gly-Gly, Leu-Gly-Phe, Pro-Val, Hyp-Gly, Phe-Val, His-Leu, Lys-Val, Ser-Val, Ser-Ala, Thr-Met, Asp-Phe, Gln-Gly, Met-Phe-Gly, Phe-Tyr, Trp-Lys, Ile-Ala, Ala-Tyr, Gly-Trp, Ala-Asn, Glu-Ala, (Cys-Val)2, His-Val, Gly-Pro, Ala-Val, Val-Leu, Leu-His, Pro-Phe, His-Ala, Trp-Leu and Trp-Ala were
Esterification of Acetyl Peptides

The acetyl peptide (1 μ mole) was refluxed with dry thionyl chloride-methanol reagent (0.5ml) for thirty minutes. The excess reagent and solvents were removed in vacuo and the acetyl peptide ester was then dissolved in methanol (1ml) and the solvent was re-evaporated. This was repeated twice more and then the residue was redissolved in methanol (0.1ml). An aliquot (20 μl) was then transferred to a glass capillary tube and the solvent was removed in vacuo. The dried residue was used for mass spectral analysis and a reference spectrum was recorded. The following peptides: Gly-Pro, Ala-Val, Val-Leu, Leu-His, Pro-Phe, His-Ala, Trp-Leu and Trp-Ala were esterified in this way and the sequence ions present in their e.i. mass spectra are shown in Table-2.

Pyrolytic Esterification of Acetyl Peptides

The dry N-acetyl peptide (0.2 μ mole) was dissolved in TMPAH [(0.1N) using 2.4 μl per -COOH group] and the solution was evaporated to dryness under reduced pressure. The sample was transferred to the solid probe inlet of the mass spectrometer and excess solvent was removed in the forepump region. The sample was then introduced into the high vacuum region and the temperature was raised to 100°C and it was maintained at 100°C for 3-5 minutes or until the evolution of dimethylaniline had subsided. The solid probe temperature was then slowly increased until the mass spectrum of the acetyl peptide ester appeared on the oscilloscope display. An e.i. mass spectrum was recorded when the spectrum had reached a
constant intensity. The sequence ions of acetyl peptide esters prepared by this procedure are listed in Table-2.

**Pyrolytic Esterification of Acetyl Peptides with residues of His, Trp and Tyr**

The dry N-acetyl peptide 0.2 μ mole was dissolved in TMPAH [(0.1N) 2.4 μl were added per -COOH group and for every His, Trp and Tyr residue in the peptide], and the solution was evaporated to an oil under reduced pressure in a glass capillary tube. The dry residue was transferred to the mass spectrometer and pyrolytic esterification was induced by the foregoing procedure. The peptides: Val-Tyr-Val, His-Leu, Phe-Tyr, Gly-Trp, Leu-His, His-Ala and Trp-Leu were derivatised in this manner. In a similar fashion the experiment was repeated using 4, 6 or 8 μl of TMPAH per -COOH group and for every His, Trp and Tyr residue in the peptide.

**D. ESTERIFICATION OF ACYL AND SULPHONYL PROTECTED PEPTIDES**

**Acetylation Procedure**

Val-Ile-Ala was acetylated by the procedure in Section C.

**Trifluoroacetylation Procedure**

Val-Ile-Ala (1 μ mole) was dissolved in trifluoroacetic acid (0.5ml) and trifluoroacetic anhydride (0.5ml) was added. The solution was heated for ten minutes to 50°C and the reagents were then removed under reduced pressure. The derivative was dried over KOH pellets and silica-gel.

**Formylation Procedure**

Val-Ile-Ala (1 μ mole) was dissolved in formic acid (90%) (1ml) and warmed to 50°C. Acetic Anhydride (0.2ml) was then
added dropwise over a period of ten minutes. The excess solvent and reagent were removed under reduced pressure, and the derivative was dried over KOH pellets and silica-gel.

**Benzoylation and Pivaloylation Procedure**

Val-Ile-Ala (1 µ mole) was dissolved in aqueous NaHCO₃ (0.1M) (0.5ml) and benzoyl or pivaloyl chloride (Fluka A.G. Buchs S.G.) (0.1ml) was added. The solution was shaken for two hours at room temperature and it was then concentrated under reduced pressure. The residue was acidified to pH 3 and the acylated peptide was isolated by extraction with ethylacetate (3 x 0.5ml). The ethylacetate fractions were combined, dried with solid MgSO₄ (anhyd.), filtered and the filtrate was evaporated to dryness. The residue of benzoyl or pivaloyl Val-Ile-Ala was dried over silica-gel.

**Dansylation Procedure**

Val-Ile-Ala (1 µ mole) was dissolved in triethylamine (0.1N) (20 µl) and a solution of 1-dimethylaminonaphthalene 5-sulphonyl chloride (Dansyl Chloride) (Fluka A.G. Buchs S.G.) in acetone (20 µl, 0.5%) was added. The solution was warmed to 40°C and it was maintained at this temperature for one hour. The excess reagent was removed by extraction with toluene (1ml) and this was repeated three times. The aqueous solution was acidified to pH 2 and the Dns-Val-Ile-Ala was extracted with ethylacetate (3 x 0.5ml). The ethylacetate fractions were combined, dried with MgSO₄ (anhyd.) and the filtrate was evaporated to dryness. The residue was dried over silica-gel.
Pyrolytic Esterification of Acyl and Sulphonyl Protected Val-Ile-Ala

The N-protected Val-Ile-Ala (1 μ mole) was dissolved in TMPAH (0.1N) (12 μl) and an aliquot (2.4 μl) was transferred to a glass capillary and the solution was evaporated to dryness under reduced pressure. The residue was transferred to the mass spectrometer, and pyrolytic esterification was induced by heating the probe to 100°C for 3-5 minutes. The probe temperature was slowly increased until a mass spectrum of the N-protected Val-Ile-Ala methyl ester was obtained. The sequence ions obtained from the e.i. mass spectra of these derivatives are shown in Table-4.

Preparation of TFA-Peptide Methyl Esters

Trifluoroacetylation of Peptides: Reference Method\textsuperscript{124}

The peptide (1 μ mole) was dissolved in trifluoroacetic acid (0.5ml) and trifluoroacetic anhydride (0.5ml) was added. The solution was heated at 40°C for twenty minutes and the excess reagent was removed in vacuo. The residue was redissolved in methanol (50 μl), and an aliquot 5 μl was spotted on a t.l.c. plate for chromatography. The excess solvent was removed under reduced pressure and the residue dried over KOH pellets and silica-gel.

Methyltrifluoroacetate Condensation with Peptide TMPA salts

Establishment of Reaction Conditions

The peptide (1 μ mole) was dissolved in TMPAH (0.1N) (12 μl), methanol (2ml) and methyltrifluoroacetate (10μl) was added. The solution was divided into two portions, the first (1ml) portion
being heated under reflux for thirty minutes with aliquots (10 µl) being taken at five minute intervals, these were spotted immediately on t.l.c. plates (Merck. Kieselgel. G.). The second portion was allowed to stand at room temperature (~ 18°C) with aliquots (10 µl) being taken half hourly for two hours, and then hourly for six hours. These samples were also spotted on t.l.c. plates. Chromatography was performed in butanol:acetone:acetic acid:ammonia:H2O (18:6:4:1:8). The TFA-peptides and unmodified peptides were detected by spraying the plate with Morin\textsuperscript{127} (3, 5, 7, 2', 4' - pentahydroxyflavone) (B.D.H.) (0.05% in methanol) and heating at 100°C for two minutes. The TFA-peptides fluoresced yellow under U.V. light. The unreacted peptides and TFA-peptides were further detected by spraying the plate with the sodium hypochloride (10-15% available chlorine). After thirty minutes at room temperature the plates were heated at 100°C for five minutes and they were then sprayed with o-tolidine\textsuperscript{128}. The TFA-peptides and unreacted peptides stained as blue spots. This procedure was used on Ala-Leu and Ile-Ala. The results are discussed in Section D (discussion). The following method was developed from these results.

**Trifluoroacetylation of Peptides: Methyltrifluoroacetate Condensation (Method II)**

The peptide (1 µ mole) was dissolved in TMPAH (0.1N) (12 µl), methanol (2ml) and methyltrifluoroacetate (10 µl) was added, and the solution was heated under reflux for fifteen minutes. The excess solvent was then removed under reduced pressure, and the
residue was redissolved in methanol (50 μl). An aliquot (5 μl) was spotted immediately on a t.l.c. plate for chromatography. A further aliquot (20 μl) was transferred to a glass capillary tube and evaporated under vacuum to an oil. The oily residue was now suitable for pyrolytic esterification.

**Comparison of TFA Peptide Derivatives prepared by the Reference Method and Method II**

The peptides Ala-Leu, Val-Leu, Gly-Trp, Ile-Ala, Ser-Val, Hyp-Gly, Met-Ser, His-Leu, Lys-Val, Asp-Phe, Gln-Gly, Pro-Phe were trifluoroacetylated by both methods and the derivatives were chromatographed in butanol:acetone:acetic acid:ammonia: H₂O (18:6:4:1:8) on Kieselgel-G t.l.c. plates (E. Merck, Darmstadt). The Rf values of the TFA-peptides prepared by these methods are summarised in Table-5.

**Comparison of the Mass Spectra of TFA-Peptide Methyl Esters prepared by Different Methods**

**Reference Method**

The TFA-peptide (1 μ mole) (prepared by the Ref. Method) was dissolved in methanol (1ml) and a solution of diazomethane in ether was added until a yellow colour persisted. The solution was allowed to stand at room temperature for thirty minutes, at which time the excess solvents and reagents were removed by evaporation with a stream of dry N₂. E.i. mass spectra were recorded on a 0.2 μ mole sample and the sequence ions obtained in this way are listed in Table-6.
Method I

The TFA-peptide (1 μ mole) (prepared by Ref. Method) was redissolved in TMPAH (12 μl) (0.1N) and an aliquot (2.4 μl) was transferred to a glass capillary and this was evaporated to dryness under vacuum. The capillary was transferred to the mass spectrometer and pyrolytic esterification was initiated. An e.i. mass spectrum was then recorded on the TFA-peptide methyl ester (c.f. Table-6).

Method II

The TFA-peptide TMPA salt, prepared by Method II with methyltrifluoroacetate, was esterified directly by pyrolytic esterification within the mass spectrometer at 100°C. The e.i. mass spectral results for the TFA-peptide methyl esters are recorded in Table-6 and 7.

Removal of Excess TMPAH Reagent by Ion Exchange

Procedure for the Preparation of the Ion Exchange Resin

The cation exchange resin Zerolit 236 Permutit (Brookevale, N.S.W.) (H+ form) (10g) was washed with deionised distilled water (100ml), and then with methanol (100ml). The resin was dried by vacuum filtration and it was then resuspended in methanol (50ml). The mixture was allowed to stand at room temperature for four hours and the excess methanol was then removed by vacuum filtration. The resin was washed with anhydrous methanol (3 x 20 ml) under vacuum filtration. The dry resin was then resuspended in anhydrous methanol (30ml).
Procedure for the Removal of Excess TMPAH from a Reaction Mixture

The peptides Ala-Val and Val-Leu (1 μ mole) were trifluoroacetylated by the reference method and excess TMPAH (0.1N) (20 μl) was added. Zeolit 236 was added in 0.1ml portions to the TFA-peptide TMPA salt and the pH was checked with Merck Universal Indicator paper after each addition, until the pH was reduced to 8. An aliquot (4 μl) was then dried under vacuum, and it was pyrolytically esterified at 100°C in the mass spectrometer. A mass spectrum of the ester was recorded.

E. ALDEHYDES AND β-DIKETONES AS PROTECTING GROUPS

Reagents

Ethylacetoacetate, pivaldehyde, 4-methoxybenzaldehyde, 4-dimethylaminobenzaldehyde, 4-dimethylaminocinnamaldehyde, 4-chlorobenzaldehyde were obtained from Fluka A.G. (Buchs S.G.). Benzoylaceton and benzaldehyde were obtained from B.D.H. Chemicals (Poole, England). 1-Naphthaldehyde was obtained from Pfaltz and Bauer, Inc. (Flushing, N.Y.) and 4-dimethylamino-1-naphthaldehyde was synthesised. All reagents were used as obtained commercially, except for benzaldehyde, which was washed with Na₂CO₃ solution (10%) dried over Na₂CO₃ (anhyd.), and distilled at reduced pressure (8mm Hg) from a little zinc dust. The fraction boiling at 57-59°C (8mm) was collected.

Preparation of 4-Dimethylamino-1-Naphthaldehyde

Hexamine (4.5g) and glacial acetic acid (4.5ml) were heated on
a boiling water bath, while a mixture of dimethyl-α-naphthylamine (4.0g) and glacial acetic acid (8.0ml) were added dropwise during one hour. Heating was continued for a further two hours, after which the hot mixture was poured into 0.5N HCl (60ml) and the solution was left overnight. The thick brown oil which separated was extracted with ether (3 x 10ml) and this was washed with water (3 x 10ml) and the extract was dried over MgSO₄ (anhyd.). After evaporation of the ether, the thick oil was distilled under reduced pressure (10mm) and the distillate boiling between 190° and 195°C was collected (yield 1.4g - 32%).

Preparation of Acetyacetonyl Peptide Methyl Esters

The peptide (1 μ mole) was dissolved in TMPAH[0.1N], 12 μl were added per COOH group, and for every His, Trp and Tyr residue in the peptide] methanol 20 μl was added. Dissolution of the peptide was aided by ultrasonication for 5-10 minutes. Acetylacetone (5 μl) and a molecular sieve (Type-3A) were then added, and the solution was allowed to stand overnight at room temperature. An aliquot (7 μl) was evaporated to dryness in a glass capillary tube and pyrolytic esterification was induced within the mass spectrometer at 100°C. When the sample pressure, due to dimethylaniline, had subsided (3-5 minutes), the probe temperature was increased and a mass spectrum of the acetylacetonyl peptide methyl ester was recorded. The characteristic sequence ions for a selection of some of these peptide derivatives are shown in Table-8 and II.
Preparation of Benzoylacetonyl Peptide Methyl Esters

The peptide (1 μ mole) was dissolved in TMPAH [(0.1N) 12 μl were added per COOH group and for every His, Trp and Tyr residue in the peptide] and methanol (20 μl) was added. Dissolution of the peptide was aided by ultrasonic treatment for ten minutes. Benzoylacetone (5mg) and a molecular sieve (Type 3A) were added and the solution was allowed to stand overnight. An aliquot (7 μl) was evaporated to dryness in a glass capillary tube, and pyrolytic esterification was induced within the mass spectrometer at 100°C. This required 3-5 minutes before the intensity of the ions, due to dimethylaniline (m/e 120, 121), had subsided. However, it was necessary to maintain this temperature (in excess of twenty minutes) until the excess benzoylacetone was selectively pumped from the sample. When the sample pressure, due to excess reagents, had dropped, the probe temperature was increased until the benzoylacetonyl peptide ester became sufficiently volatile to record a mass spectrum. Sequence ions from the e.i. spectra are recorded in Table-8 and 11.

Preparation of Trifluoroacetylacetonyl Peptide Methyl Esters

These derivatives were prepared by the same method used for the preparation of the acetylacetonyl derivatives, except that trifluoroacetylacetone was used in place of acetylacetone. The sequence ions from the e.i. mass spectra are recorded in Table-12.
Preparation of Ethoxycarbonylpropenyl Val-Ile-Ala Methyl Ester

Val-Ile-Ala (1 μ mole) was dissolved in TMPAH (0.1N) (12 μl) and methanol (20 μl) was added. Ethylacetoacetate (5 μl) and a molecular sieve (Type 3A) were then added. The solution was heated at 70°C for fifteen minutes (in a sand bath), followed by cooling to room temperature. An aliquot (8 μl) was then evaporated to dryness in a glass capillary tube and the residue was pyrolytically esterified using the same conditions as described for the acetylacetonyl derivatives. The sequence ions from the e.i. mass spectra are recorded in Table-8.

Preparation of Neopentylidene Peptide Methyl Esters

The peptide (1 μ mole) was dissolved in TMPAH [(0.1N) and 12 μl were added per COOH group and for every His, Trp or Tyr residue in the peptide methanol (20 μl) was added. Dissolution of the peptide was aided by ultrasonic treatment for ten minutes. Pivaldehyde (5 μl) and a molecular sieve (Type 3A) were then added. The solution was allowed to stand at room temperature for fifteen minutes, and an aliquot (8 μl) was evaporated to dryness in a glass capillary tube under vacuum. The capillary was heated to 100°C within the mass spectrometer, and after the esterification period (3-5 minutes) the probe temperature could be immediately increased to the volatilisation temperature of the neopentylidene peptide methyl ester. The sequence ions from the e.i. mass spectra are recorded in Table-4.
Preparation of Arylidene Peptide Methyl Ester Derivatives

The peptide (1 μ mole) was dissolved in TMPAH [(0.1N) 12 μl were added for every COOH group, His, Trp and Tyr residue in the peptide] and methanol (20 μl) was added. One of the following aromatic aldehydes, benaldehyde (2 μl), salicylaldehyde (2 μl), 4-dimethylaminobenzaldehyde (2mg), 4-dimethylaminocinnamaldehyde (2mg), 4-chlorobenzaldehyde (2 μl), 1-naphthaldehyde (2 μl) or 4-dimethylamino-1-naphthaldehyde (2 μl) was added with a molecular sieve (Type 3A) to the solution. The solution was then allowed to stand at room temperature for six hours (or overnight). An aliquot (8 μl) was transferred to a glass capillary tube, and evaporated to dryness under vacuum. Following pyrolytic esterification at 100°C, the probe temperature was maintained until excess reagents were selectively pumped from the sample, (this required 20-30 minutes). The e.i. mass spectra for these derivatives are recorded in Table-8 and 10.

Preparation of Schiff Base and Enamine Peptide Methyl Esters:

Reference Compounds

Val-Ile-Ala, (1 μ mole) was dissolved in thionyl chloride-methanol reagent and refluxed for thirty minutes, excluding moisture with a calcium chloride drying tube. The excess solvent and reagent was removed under vacuum, and the residue was redissolved in methanol (20 μl) and triethylamine (5 μl). One of the reagents benzoylacetone (2 μl), ethylacetoacetate (2 μl), or 4-chlorobenzaldehyde (2 μl) was then added, together with a
molecular sieve (Type 3A). Condensation times were as described previously. Following condensation, an aliquot (6 μl) was then evaporated to dryness in a glass capillary tube under vacuum, and admitted to the mass spectrometer. The excess reagents were selectively removed at 100°C, and a reference mass spectrum was recorded. The e.i. mass spectra for the respective derivatives are recorded in Table-8.

F. THE MASS SPECTROMETRIC IDENTIFICATION OF DIPEPTIDE MIXTURES

OBTAINED FROM DIPEPTIDYLAMINOPEPTIDASE-I HYDROLYSATES

Preparation of Acetylaceptonyl Methyl Ester Derivatives of Dipeptide Mixtures

The peptides Val-Leu (0.2 μ mole), Asp-Phe (0.2 μ mole), His-Leu (0.2 μ mole) and Lys-Trp (0.2 μ mole) were weighed into a reaction vial. TMPAH (0.1N) was then added slowly, with gentle agitation, until the peptide mixture was dissolved. (This required 18 μl.) Methanol (10 μl), acetylaceptone (5 μl) and a molecular sieve (Type 3A) were added and the mixture was allowed to stand overnight at room temperature. After the reaction period the molecular sieve was withdrawn from the solution with a spatula and rinsed off into the reaction solution with 10 μl of methanol. The solution containing the peptide mixture was then transferred to two glass capillary tubes and evaporated to dryness under reduced pressure.

E.I. Mass Spectrometry of the Acetylaceptonyl Dipeptide Mixture TMPA salt

A capillary containing 0.1 μ moles of the dipeptide mixture was transferred to an E.A.I. QUAD 300D mass spectrometer and pyrolytic
esterification was induced at 80°C. A background spectrum was recorded after subsidence of the sample pressure due to the dimethylaniline, and the probe temperature was slowly increased with the mass spectrum being monitored on a scanning oscilloscope. Spectra were recorded at 20°C intervals between m/e 100 and 280. The results obtained are shown in Fig.-6, with the background spectrum having been subtracted.

C.i. (isobutane) Mass Spectrometry of the Acetyacetonyl Dipeptide Mixture TMPA Salt

A capillary containing 0.1 μ moles of the derivatised dipeptide mixture was transferred to an A.E.I. MS-902 mass spectrometer and c.i. (isobutane) mass spectra were recorded at pressures of 0.8-1.00 Torr. Pyrolytic esterification was induced at 80°C and a background spectrum was recorded. The probe temperature was then slowly increased and spectra were recorded at 20°C intervals between m/e 220 and 600. The mass spectra of the mixture is shown in Fig.-5, with the background spectrum having been subtracted. The mass spectral results for two other mixtures derivatised and identified by the foregoing procedure are listed below. The results are documented as:

m/e (Intensity) Fragment ion^+

Mixture II

Ala-Leu: m/e 126 (100) (A₁)⁺; 299 (100)(MH)⁺
Pro-Val: m/e 152 (93) (A₁)⁺; 311 (90)(MH)⁺
Thr-Leu: m/e 156 (45) (A₁)⁺; 138 (18)(A₁)⁺ - H₂O; 111 (A₁)⁺ - C₂H₅OH
329 (40) (MH)$^+$; 310 (33) (MH)$^+$ – H$_2$O

Val-Glu: m/e 154 (90) (A$_1$)$^+$; 357 (60) (MH)$^+$

Mixture III

Glu-Arg: m/e 198 (53) (A$_1$)$^+$; 478 (43) (MH)$^+$

Leu-Met: m/e 168 (100) (A$_1$)$^+$; 359 (80) (MH)$^+$

Ala-Ala: m/e 126 (90) (A$_1$)$^+$; 257 (100) (MH)$^+$

Investigation of Suitable C.I. Reagent Gases to Induce Peptide Fragmentation

Reagent gases used were: isobutane (Instrument Grade, 99.5 mole %) (Matheson): methane (C.P. Grade, 99.0 mole %) (Matheson): hydrogen (High Purity Grade, 99.995%) (C.I.G.): argon (High Purity Grade, 99.995%, moisture < 12 v.p.m.): helium (Industrial, 99.99%, moisture < 25 v.p.m.). Mass spectra were recorded at pressures of 0.8-1.00 Torr for all gases. The results are tabulated below for the ratio of (A$_1$)$^+/(MH)^+$ obtained on acetylacetonyl peptide methyl esters using the different reagent gases:

- Acetylacetonyl Val-Val methyl ester: isobutane (.06), methane (.54), hydrogen (1.10), argon (1.30), helium (1.75).
- Acetylacetonyl Trp-Ala methyl ester: isobutane (0), methane (.05), hydrogen (.16), argon (.18), helium (.24).
- Acetylacetonyl Pro-Val methyl ester: isobutane (.15), methane (.90), hydrogen (1.30), argon (1.53), helium (2.10).
- Acetylacetonyl Tyr-Gly methyl ester: isobutane (0), methane (.14), hydrogen (.16), argon (.13), helium (.19).

Preparation of Ethoxycarbonylpropenyl Dipeptide Methyl Esters

Reagents
TMPAH (0.1N) was prepared by the general procedure, but ethanol was used as a solvent in place of methanol.

**Procedure**

The peptide (0.2 μ moles) was dissolved in TMPAH [(0.1N) 2.4 μl were added for every COOH group, Tyr, His or Trp residue in the peptide] and methanol 20 μl was added. Ethylacetoacetate (3 μl) and a molecular sieve (Type 3A) were then added. The solution was heated at 70°C for thirty minutes (in a sand bath), cooled, and then evaporated to dryness in a glass capillary tube. The pyrolytic esterification was induced at 100°C in the solid probe inlet of a Dupont 21-491 B mass spectrometer. Helium was used as the reagent gas at pressures of 0.8-1.00 Torr, and mass spectra of the ethoxycarbonylpropenyl dipeptide methyl esters were recorded when they were of constant intensity. The mass spectral results are recorded in Table-15.

**Edman Degradation**

**Reagents**

Pyridine, (B.D.H. Ltd., Poole, England) was refluxed over solid KOH for three hours and then distilled. B.p. 115° to 116°C.

Benzene, (one litre, Practical Grade) was stirred over conc. H₂SO₄ (50 ml) with several changes of acid and then washed with distilled water. It was shaken in a mechanical shaker overnight with an aqueous KMnO₄ solution (30g/l) (100ml). The KMnO₄ was removed by washing with distilled water and the preparation dried over KOH. The benzene was distilled, the fraction boiling 80° to 81°C was collected.
Ethylacetate, (Practical Grade) was stirred with aqueous Na$_2$CO$_3$ (50 g/l) and then with a saturated solution of CaCl$_2$ in water. The organic phase was separated, shaken with solid KMnO$_4$ (1 g/l) in a mechanical shaker overnight, and then washed with distilled water until colourless. It was dried, first over anhyd. CaCl$_2$ (200 g/l) for twenty four hours and then for the same period over anhyd. CaSO$_4$ (200 g/l) from which it was distilled, B.p. 77°C.

Trifluoroacetic Acid, (Fluka A.G.) was refluxed with solid CrO$_3$ until there was no colour change. The acid was distilled and again refluxed with CrO$_3$. The acid was again distilled and dried over anhyd. CaSO$_4$, distilled and the fraction boiling at 72 to 73°C collected.

Ethylene chloride, (purum grade, Fluka A.G.) was stirred twice with conc. H$_2$SO$_4$, then washed in succession with distilled water, dilute Na$_2$CO$_3$ solution and distilled water. It was then shaken in a mechanical shaker overnight with an aqueous solution of KMnO$_4$ (30 g/l). The KMnO$_4$ was removed by repeated washings with distilled water, and the preparation was then dried over anhyd. CaSO$_4$, and finally distilled. B.p. 83°C to 84°C.

Methylisothiocyanate, (Fluka A.G.) was used as obtained commercially.

Reaction Buffer$^{106}$; N-ethylmorpholine (Pfaltz and Bauer, Flushing, N.Y.) (3.3 ml), glacial acetic acid (0.5 ml) and water (46.7 ml) were mixed and used immediately.

Procedure$^{131,132}$

The peptide (0.1 to 0.3 μ mole) was dissolved in 500 μl of
pyridine. Buffer (100 µl), water (100 µl) and methylisothiocyanate (5 mg) were added. The tube was flushed with N₂, stoppered and left at 40°C for one hour. The solution was then extracted with benzene (3 x 2.5 ml), and the aqueous phase dried with a stream of N₂. The residue was washed with ethylacetate (3 x 0.5 ml) and re-evaporated to dryness. Trifluoroacetic acid (0.4 ml) was added, and the reaction allowed to proceed at 40°C for fifteen minutes. The excess trifluoroacetic acid was evaporated with N₂ and the methylthiocarbamyl amino acid was extracted with ethylene chloride (3 ml). The peptide residue was washed with 0.5 ml of ethylene chloride, and the precipitate dried with a stream of N₂. This peptide was now sufficiently pure for further degradation. The ethylene chloride phase was brought to dryness with N₂ and the residue was suspended in 0.3 ml of 1N. HCL. The tube was heated to 80°C for ten minutes, after which time the reaction mixture was cooled, and then extracted with ethylacetate (3 x 0.5 ml). The ethylacetate fractions were combined and dried with N₂, redissolved in ethylacetate (10 µl), and then transferred to a glass capillary tube and evaporated to dryness under reduced pressure. C.i. (isobutane) mass spectra of these methylthiohydantoin derivatives were recorded at pressures of between 0.8 to 1.00 Torr using the Dupont 491 B mass spectrometer. Edman degradations were performed on all peptides sequenced using DAP-I (c.f. below).

Removal of ε-Methylthiocarbamyl Group of C-Terminal Lys

The peptide (0.1 µmole) was dissolved in aqueous hydrazine (2ml, 50%) and refluxed for one hour. The excess reagent was
removed under reduced pressure and the residue was re-evaporated with three (1ml) water washes. The deblocked peptide was further dried with a stream of N₂.

Digestion of Peptides with DAP-I

Reagents

Dipeptidylaminopeptidase I was obtained as a 500 unit vial from Schwarz/Mann (Orangeburg, N.Y.) with a specific activity of 18.1 units/mg of protein. (Unit Definition: 1 unit equals the amount of enzyme which catalyses the formation of 1 µmole of β-naphthylamine per minute at 37° at pH 6 from Gly-(L)-Phe-β-naphthylamine). The freeze-dried enzyme was reconstituted in 1% NaCl-0.5mM EDTA (20 ml) to provide a working solution of 25 units per ml. This solution was stored at 0°C on ice.

Procedure

The peptide (0.1 µmole) was dissolved in pyridine (4%) (40 µl) and diluted with water (80 µl). Acetic acid (0.5%) (40 µl), HCl (0.1M) (32 µl), EDTA (0.1M) (1 µl) and mercaptoethanol (0.375M) (8 µl) were added with mixing. The pH of this solution was checked and if not pH 5.0, was adjusted with 4% pyridine or 0.5% acetic acid. The solution was preincubated at 37° for five minutes, and hydrolysis of the peptide was initiated by adding 40 µl, (1 unit) of enzyme to the reaction mixture. The reaction mixture was flushed with N₂, sealed with waxed film and gently shaken for four hours at 37°C. The reaction was terminated after
this period by centrifugation through an Amicon CF50A centriflo
membrane (Scientific and Research Equipment Co. Pty. Ltd., Pennant
Hills) for ten minutes at 1,000 r.p.m.

Removal of Buffer Salts from a DAP-I Digest

Reagents

Dowex 50W x 8 [(H\(^{+}\) form) (200-400 mesh)] resin (100g) (Biorad,
Richmond, Cal.) was washed with HCL (IN) 500 ml, distilled water
until neutral, ammonia (2N) 500 ml, distilled water until neutral,
and the process repeated, with the resin being washed, finally,
with HCL (IN) 500 ml and then with distilled water until neutral.
The resin was dried by vacuum filtration and stored in a semi
moist state.

Procedure

The filtrate from the DAP-I digest was acidified with HCL
(IN) to pH 2 (17 µl) and applied at the rate of 0.2 ml/minute
to a Dowex 50W x 8 (H\(^{+}\)), ion exchange column (400 mg resin bed).
The resin was washed with distilled water until neutral (4 ml)
at 1ml/minute and the peptide mixture eluted with ammonia
(2N) (5ml). The elute was evaporated to a small volume (0.5 ml)
under reduced pressure and transferred to a reactor vial, where
it was evaporated to dryness with N\(_2\). The dipeptide mixture was
now suitable for derivatisation.

Dipeptide Recovery from Dowex 50W x 8 Ion Exchange Resin

The peptide Lys-Val (0.5 µ mole) was dissolved in the DAP-I
digest buffer, acidified to pH 2 with HCl (IN) and the solution
ion exchanged (c.f. foregoing procedure). To the peptide
recovered from the ion exchange resin was added aminooctanoic acid 0.5 μ mole (Internal standard). A calibration curve was also prepared using 0.25, 0.50, 0.75 and 1.00 μ moles of the peptide together with 0.50 μ mole of an internal standard aminooctanoic acid in each vial. All vials were evaporated to dryness with nitrogen and the residue was dissolved in thionyl chloride-methanol reagent (1 ml). The solution was refluxed for thirty minutes, evaporated to dryness with N₂, and the residue re-dissolved in methanol (50 μl), triethylamine (2 μl), pivaldehyde (10 μl) and a stick of molecular sieve was added. The reaction was allowed to stand for thirty minutes at room temperature, and submitted immediately to gas chromatography. Gas chromatography was performed on a Packard model 419 gas chromatograph, fitted with a stainless steel column packed with 5% silicone OV-17 on DMCS-treated chromosorb W (100 mesh), with helium as the carrier gas at a flow rate of 20 ml/minute. The chromatograph oven was programmed at 6°C/minute beginning at 140°C. The recovery from the ion exchange resin as calculated from a standard curve was 85% for Lys-Val. In a similar fashion, the recovery from ion exchange of Glu-Ala (81%), Glu-Glu (75%), Ala-Ala (99%) and Val-Val (95%) was determined by gas chromatography.

Derivatisation of Dipeptide Mixtures from DAP-I Hydrolysates

Reagents

Preparation of Dimethyltrideuteromethylphenylammonium Hydroxide (DTMPAH)

Dimethylaniline (Unilab, Ajax Chemical Sydney) (10 ml) and
trideuteromethyl iodide (Fluka A.G. Buchs S.G.) (5 ml) were dissolved in ethylacetate (20 ml) and the resulting solution was kept at 0°C overnight. The insoluble dimethyltrideuteromethylphenylammonium iodide was filtered and recrystallised from ethanol/ethylacetate. Yield (71%). The iodide (2.63g) was redissolved in ethanol (20ml) and silver oxide (2g) was added. After two hours stirring at room temperature, the solution was filtered and the concentration of the reagent was adjusted to 0.1 mm/ml, after standardisation with sulphuric acid (0.1N) to a phenolphthalein end point. The reagent was stored over molecular sieves (Type 3A) at 0°C.

Procedure

The dry dipeptide mixture (0.1 μ mole) was dissolved in an ethanolic solution of DTMPAH (0.1N) (5-10 μl) until just dissolved (pH 9). Dry ethanol (20 μl), ethylacetate (5 μl) and a stick of molecular sieve (Type 3A) were then added. The reaction mixture was heated at 70°C in a screw cap reactor vial for thirty minutes. The molecular sieve was partly removed, washed with ethanol (10 μl) and removed. The solution was transferred to a glass capillary and the solvent evaporated under vacuum. The capillary was then admitted to the mass spectrometer, Dupont 21-491B, using a heatable direct insertion probe.

Pyrolytic esterification was induced by heating the probe to 100°C and maintaining this temperature for 3-5 minutes. The biproduct dimethyltrideuteromethylaniline was evolved as observed
by the presence of ions at m/e 120-125. The probe temperature was then slowly increased and c.i. (helium) mass spectra were recorded at 20°C intervals in the region 240-630 amu.

The following peptides and peptide amides were hydrolysed with DAP-I, the buffer salts removed by ion exchange, and the dipeptide mixtures identified as their ethoxycarbonylpropenyl dipeptide methyl esters. All ions shown are for the most abundant isotope and are listed as: m/e (intensity), identity;

Ala-Ala-Ala-Ala (0.1 μ mole): m/e 287 (80), Ala-Ala (MH)^+;
241 (2), Ala-Ala (MH)^+ - C₂H₅OH; 156 (100) Ala (A₁)^+.

Met-Phe-Gly (0.1 μ mole): m/e 423 (100), Met-Phe (MH)^+;
216 (60) Met (A₁)^+; 202 (80) Gly (MH)^+; 142 (30) Gly (A₁)^+.

Phe-Gly-Gly-Phe (0.1 μ mole): m/e 349 (100), Phe-Gly (MH)^+;
Gly-Phe (MH)^+; 232 (60), Phe (A₁)^+;

Des(Phe), Phe-Gly-Gly-Phe (0.1 μ mole): m/e 259 (100), Gly-Gly (MH)^+;
213 (6), Gly-Gly (MH)^+ - (C₂H₅OH); 292 (80), Phe (MH)^+;
232 (60), Phe (A₁)^+; 142 (40), Gly (A₁)^+.

Val-Leu-Ser (0.1 μ mole): m/e 357 (80), Val-Leu (MH)^+; 311 (8),
Val-Leu (MH)^+ - C₂H₅OH; 232 (60), Ser (MH)^+; 184 (100), Val (A₁)^+;
172 (50), Ser (A₁)^+; 154 (11), Ser (A₁)^+ - H₂O; 213 (10), Ser (MH)^+ - H₂O.

Gly-Leu-Tyr (0.1 μ mole): m/e 315 (100), Gly-Leu (MH)^+; 322 (60), Tyr (MH)^+;
262 (15), Tyr (A₁)^+; 142 (70), Gly (A₁)^+.

Phe-Phe-Gly-Leu-Met.NH₂ (0.2 μ mole): m/e 439 (100), Phe-Phe (MH)^+;
315 (24), Gly-Leu (MH)^+; 260 (36), Met.NH₂ (36); 232 (32), Phe (A₁)^+;
216 (17), Met (A₁)^+; 186 (20), Phe (A₁)^+ - C₂H₅OH; 142 (18), Gly (A₁)^+. 
Des(Phe), Phe-Phe-Gly-Leu-Met.NH₂ (0.2 μ mole): m/e 349 (90),
  Phe-Gly (MH)⁺; 389 (80), Leu-Met (MH)⁺; 232 (100), Phe (A₁)⁺;
  198 (100), Leu (A₁)⁺.

Gly-Leu-Met.NH₂ (0.1 μ mole): m/e 315 (100), Gly-Leu (MH)⁺;
  279 (18), (MH)⁺ - C₂H₅OH; 261 (70), Met.NH₂ (MH)⁺; 216 (90),
  Met (A₁)⁺; 142 (80), Gly (A₁)⁺.

Leu-Met. NH₂ (0.1 μ mole): m/e 389 (80), Leu-Met (MH)⁺; 374 (30),
  Leu-Met.NH₂ (MH)⁺; 357 (8), Leu-Met.NH₂ (MH)⁺ - NH₃; 198 (100),
  Leu (A₁)⁺.

Val-Phe.NH₂ (0.1 μ mole): m/e 391 (100), Val-Phe (MH)⁺; 345 (3)
  Val-Phe (MH)⁺ - C₂H₅OH; 184 (40), Val (A₁)⁺.

Trp-Met-Asp-Phe.NH₂ (0.1 μ mole): m/e 476 (60), Trp (NCH₃) - Met (MH)⁺;
  462 (45), Trp-Met (MH)⁺; 421 (100), Asp-Phe (MH)⁺; 399 (8),
  Asp-Phe (MH)⁺ - CH₃OH; 285 (8), Trp (NCH₃) (A₁)⁺; 271 (6), Trp (A₁)⁺;
  214 (11), Asp (A₁)⁺, 182 (15), Asp (A₁)⁺ - CH₃OH.

Des(Trp), Trp-Met-Asp-Phe.NH₂ (0.2 μ mole): m/e 405 (42), Met-Asp (MH)⁺
  292 (86), Phe (MH)⁺; 277 (30), Phe.NH₂ (MH)⁺; 232 (100), Phe (A₁)⁺;
  216 (80), Met (A₁)⁺; 186 (30), Phe (A₁)⁺ - C₂H₅OH.
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