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AN INVESTIGATION OF THE OLIGOPEPTIDE FRAGMENTATION  
MECHANISMS BY CURIE-POINT PYROLYSIS TANDEM MASS  
SPECTROMETRY

by

Wenhui Zhang

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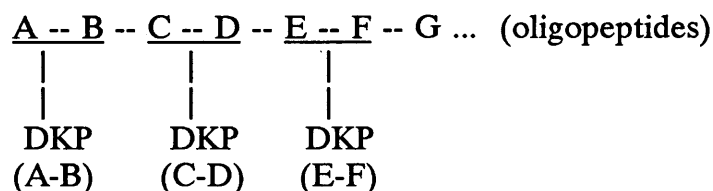
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## ABSTRACT

The mechanisms of fragmentations of oligopeptides (ranging from three to sixteen amino acid residues) upon Curie-point pyrolysis mass spectrometry have been studied. The results point to the formation of diketopiperazine (DKP) or cyclic dipeptide as the major mode of decomposition. The measured pyrolysis mass spectra show that the formation of DKPs occurs via unique consecutive cyclizations which are indicated as:



\* G = an amino acid in odd-numbered oligopeptides

Cleavage of the DKPs involves rearrangement or cleavage of the side groups before breakup of the ring. In contrast to most previous studies, the intensities of these fragment ions that yield sequence information, are much higher than the intensity of the molecular ion. Backbone and side-group cleavage of the oligopeptides also occurred, whereas, no evidence has been found for a N-terminal cleavage in the oligopeptides with more than three amino acids.

T-4289

The free C-terminus eliminated from odd-numbered oligopeptide was observed which would serve as well as to identify the sequence.

## TABLE OF CONTENTS

	<u>Page</u>
ABSTRACT .....	iii
LIST OF FIGURES.....	vii
LIST OF TABLES .....	xii
ACKNOWLEDGEMENTS .....	xiii
INTRODUCTION .....	1
PREVIOUS WORK .....	5
Diketopiperazine Formation .....	5
Peptide Sequencing .....	11
EXPERIMENTAL .....	14
RESULTS AND DISCUSSION .....	18
Tripeptides .....	18
Phenylalanyl-Leucyl-Methionine .....	18
Methionyl-Leucyl-Phenylalanine .....	37
Some other tripeptides .....	47
Tetrapeptides .....	54
Alanyl-Phenylalanyl-Leucyl-Methionine .....	54
Alanyl-Methionyl-Leucyl-Phenylalanine .....	65
Pentapeptides .....	71
Alanyl-Phenylalanyl-Leucyl-Methionyl-Tyrosine .....	71
Alanyl-Tyrosyl-Leucyl-Methionyl-Phenylalanine .....	74
Hexapeptides .....	79
Other oligopeptides .....	85
CONCLUSIONS .....	89
REFERENCES CITED .....	91

T-4289

APPENDIX I ..... 96

## LIST OF FIGURES

	<u>Page</u>
Figure 1. Schematic of a triple quadrupole mass spectrometer modified for analysis of Curie-point pyrolysis products . . . . .	2
Figure 2. Fragmentation processes of the DKPs . . . . .	7
Figure 3. Schematic illustration of the formation of 2,5-diketopiperazines (DKP) by the desorption laser beam . . . . .	9
Figure 4. Permethylation of a heptapeptide derivative . . . . .	12
Figure 5. Pyrolysis-mass spectrum of phenylalanyl-leucyl-methionine . . . . .	19
Figure 6. Pyrolysis-mass spectrum of phenylalanyl-leucine . . . . .	20
Figure 7. Thermal fragmentation pathway of phenylalanyl-leucyl-methionine . . . . .	22
Figure 8. Pyrolysis-mass spectrum of daughter ions of m/z 260 of the DKP of phenylalanyl-leucine . . . . .	23
Figure 9. Pyrolysis-mass spectrum of daughter ions of m/z 204 of the DKP of PHE-LEU . . . . .	24
Figure 10. Pyrolysis-mass spectrum of daughter ions of m/z 169 of the DKP of PHE-LEU . . . . .	25
Figure 11. Pyrolysis-mass spectrum of daughter ions of m/z 141 of the DKP of PHE-LEU . . . . .	26
Figure 12. Pyrolysis-mass spectrum of daughter ions of m/z 120 of the DKP of PHE-LEU . . . . .	27

Figure 13.	Pyrolysis-mass spectrum of daughter ions of m/z 113 of the DKP of PHE-LEU . . . . .	28
Figure 14.	Pyrolysis-mass spectrum of daughter ions of m/z 103 of the DKP of PHE-LEU . . . . .	29
Figure 15.	General fragmentation pathway 1 of the DKP of phenylalanyl-leucine and leucyl-phenylalanine . . . . .	30
Figure 16.	General fragmentation pathway 2 of the DKP of phenylalanyl-leucine and leucyl-phenylalanine . . . . .	31
Figure 17.	Pyrolysis-mass spectrum of methionine . . . . .	32
Figure 18.	Fragmentation pathway of N-terminal cleavage for phenylalanyl-leucyl-methionine . . . . .	34
Figure 19.	Pyrolysis-mass spectrum of parent ions of m/z 120 of phenylalanyl-leucyl-methionine . . . . .	35
Figure 20.	Pyrolysis-mass spectrum of daughter ions of m/z 409 of phenylalanyl-leucyl-methionine . . . . .	36
Figure 21.	Fragmentation pathway 1 for the DKP of methionyl-leucine . . .	38
Figure 22.	Fragmentation pathway 2 for the DKP of methionyl-leucine . . .	39
Figure 23.	Pyrolysis-mass spectrum of methionyl-leucyl-phenylalanine . . . .	40
Figure 24.	Pyrolysis-mass spectrum of daughter ions of m/z 244 of the DKP of methionyl-leucine and leucyl-methionine . . . . .	41
Figure 25.	Pyrolysis-mass spectrum of daughter ions of m/z 183 of the DKP of MET-LEU and LEU-MET . . . . .	42
Figure 26.	Pyrolysis-mass spectrum of daughter ions of m/z 170 of the DKP MET-LEU and LEU-MET . . . . .	43

Figure 27.	Pyrolysis-mass spectrum of daughter ions of m/z 155 of the DKP MET-LEU and LEU-MET . . . . .	44
Figure 28.	Pyrolysis-mass spectrum of daughter ions of m/z 113 of the DKP MET-LEU and LEU-MET . . . . .	45
Figure 29.	Pyrolysis-mass spectrum of phenylalanine . . . . .	46
Figure 30.	Pyrolysis-mass spectrum of daughter ions of m/z 409 of methionyl-leucine-phenylalanine . . . . .	48
Figure 31.	Fragmentation pathway of N-terminal cleavage for methionyl-leucyl-phenylalanine . . . . .	49
Figure 32.	Pyrolysis-mass spectrum of phenylalanyl-methionyl-leucine . . . . .	50
Figure 33.	Pyrolysis-mass spectrum of tyrosyl-tyrosyl-phenylalanine . . . . .	51
Figure 34.	Pyrolysis-mass spectrum of leucyl-leucyl-leucine . . . . .	52
Figure 35.	Pyrolysis-mass spectrum of alanyl-phenylalanyl-leucyl-methionine . . . . .	55
Figure 36.	Fragmentation pathway of the DKP consecutive cyclization of alanyl-phenylalanyl-leucyl-methionine . . . . .	56
Figure 37.	Fragmentation pathway for the DKP of alanyl-phenylalanine . . . . .	57
Figure 38.	Pyrolysis-mass spectrum of daughter ions of m/z 218 of the DKP of alanyl-phenylalanine . . . . .	58
Figure 39.	Pyrolysis-mass spectrum of daughter ions of m/z 194 of the DKP of ALA-PHE . . . . .	59
Figure 40.	Pyrolysis-mass spectrum of daughter ions of m/z 166 of the DKP of ALA-PHE . . . . .	60

Figure 41.	Pyrolysis-mass spectrum of daughter ions of m/z 127 of the DKP of ALA-PHE . . . . .	61
Figure 42.	Pyrolysis-mass spectrum of parent ions of m/z 91 of alanyl-phenylalanyl-leucyl-methionine . . . . .	62
Figure 43.	Pyrolysis-mass spectrum of parent ions of m/z 127 of alanyl-phenylalanyl-leucyl-methionine . . . . .	63
Figure 44.	Pyrolysis-mass spectrum of alanyl-methionyl-leucyl-phenylalanine . . . . .	66
Figure 45.	Pyrolysis-mass spectrum of daughter ions of m/z 202 of the DKP of alanyl-methionine . . . . .	67
Figure 46.	Pyrolysis-mass spectrum of daughter ions of m/z 187 of the DKP of ALA-MET . . . . .	68
Figure 47.	Pyrolysis-mass spectrum of daughter ions of m/z 128 of the DKP of ALA-MET . . . . .	69
Figure 48.	Fragmentation pathway for the DKP of ALA-MET . . . . .	70
Figure 49.	Pyrolysis-mass spectrum of alanyl-phenylalanyl-leucyl-methionyl-tyrosine . . . . .	72
Figure 50.	Pyrolysis-mass spectrum of tyrosine . . . . .	73
Figure 51.	Fragmentation pathway of the DKP consecutive cyclization of alanyl-tyrosyl-leucyl-methionyl-phenylalanine . . . . .	75
Figure 52.	Pyrolysis-mass spectrum of alanyl-tyrosyl-leucyl-methionyl-phenylalanine . . . . .	76
Figure 53.	Pyrolysis-mass spectrum of daughter ions of m/z 234 of the DKP of alanyl-tyrosine . . . . .	77
Figure 54.	Pyrolysis-mass spectrum of ALA-TYR-LEU-MET-PHE-PHE . . . . .	81

Figure 55.	Pyrolysis-mass spectrum of ALA-PHE-LEU-MET-TYR-PHE . .	82
Figure 56.	Pyrolysis-mass spectrum of PHE-ALA-PHE-LEU-MET-TYR . .	83
Figure 57.	Pyrolysis-mass spectrum of TYR-TYR-TYR-TYR-TYR-TYR . . .	84
Figure 58.	Pyrolysis-mass spectrum of R-G-L-I-V-F-H-T-S . . . . .	86
Figure 59.	Pyrolysis-mass spectrum of A-N-E-R-A-D-L-I-A-Y-L-K-Q-A- T-K . . . . .	87
Figure 60.	Pyrolysis-mass spectrum of N-P-N-A-N-P-N-A-N-P-N-A-N-P-N-A	88

**LIST OF TABLES**

	<u>Page</u>
Table 1. Major peaks in the mass spectra of several hexapeptides . . . . .	80

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## INTRODUCTION

The complexity and non-volatility of most naturally occurring organic substances make structure analysis difficult. However, various thermal methods have been developed and applied to analyze solid or non-volatile compounds, such as proteins (1), nucleic acids (2), polysaccharides (3) and plant materials (4) including biopolymers (5). The techniques which have been developed fall into two basic approaches. One, involves mass spectral ionization methodologies, such as fast atom bombardment mass spectrometry, secondary ion mass spectrometry, laser desorption, field ionization and field desorption. The other approach is based on actual thermal degradation to produce smaller or modified volatile substances which permit analysis by gas chromatography (GC), mass spectrometry (MS), or the combination of GC-MS.

Studies have shown that Curie-point pyrolysis (6) in conjunction with mass spectrometry (Py-MS) and gas chromatography (Py-GC) has great potential for characterization and differentiation of complex bio-organic samples. In addition, Curie-point pyrolysis with GC or tandem MS allows for the investigation of the thermal fragmentation mechanisms of organic compounds. Figure 1 shows a schematic diagram of a typical Curie-point tandem Py-MS system. Curie-point pyrolysis is undertaken on microgram amounts of sample deposited onto the

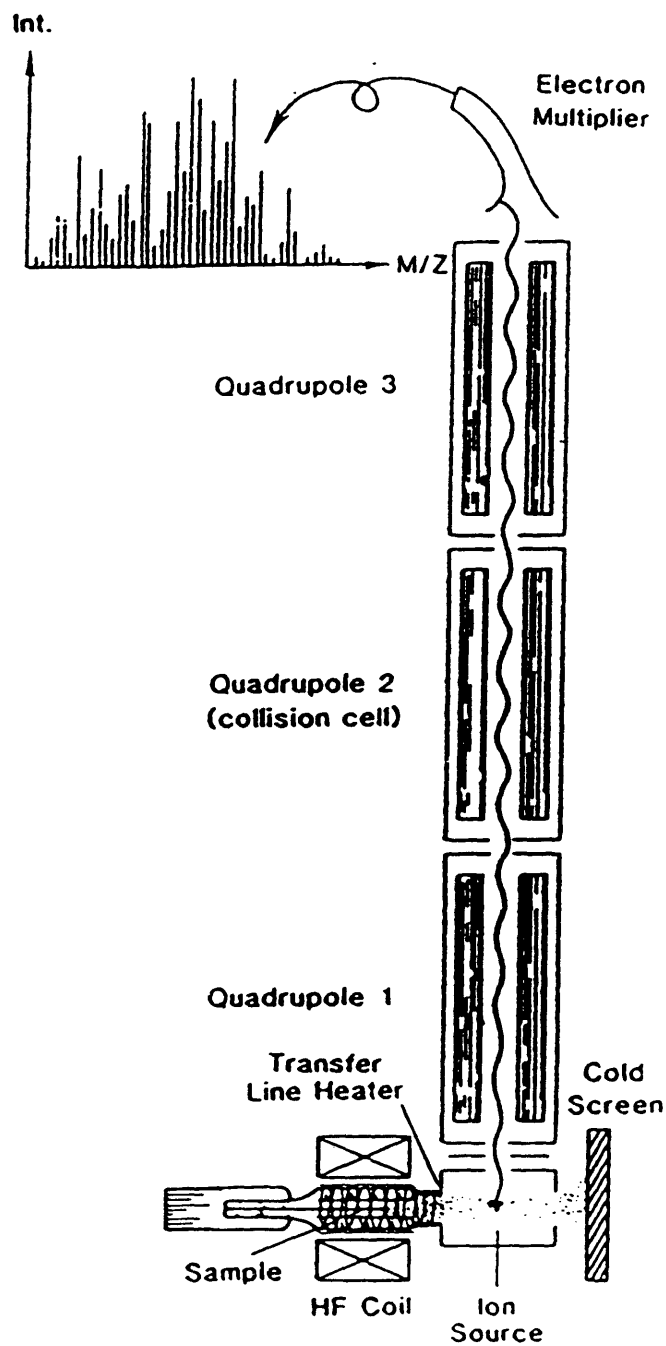


Figure 1. Schematic of a triple quadrupole mass spectrometer modified for analysis of Curie-point pyrolysis products

surface of a ferromagnetic wire. This is held within a Pyrex tube attached to a probe. The sample is inserted into the center of an induction coil connected to a high-frequency power supply, which heats the ferromagnetic wire to its Curie-point temperature (7). At the Curie-point, the wire becomes paramagnetic and reaches an equilibrium temperature. The molecular beam formed from the pyrolysates is passed through a high-energy beam of electrons generated within the ion source. Collisions with this beam result in the expulsion of an electron to form positive ions. Further fragmentations may occur if sufficient internal energy is present.

For proteins, Py-MS or Py-GC methods have been limited to small volatile peptides which could be heated to the temperatures required to vaporize a sufficient amount of the sample to obtain usable spectra or chromatograms (8). Since higher molecular weight peptides have low vapor pressure, they had not been directly analyzed until recently by mass spectrometry (5). Many researchers have derivatized peptides to increase volatility, but this leads to different MS fragmentation pathways from those for the underivatized peptides. However, most of the studies on peptide derivatives have been concerned with just sequence information from backbone cleavages of peptides.

A major part of this research effort has been focused on the investigation of the effects of polar and non-polar side groups on the thermal fragmentation mechanisms of oligopeptides occurring in Curie-point pyrolysis tandem mass spectrometry. It is crucial to understand under what conditions thermal decomposition occurs and what are the resulting products in order to control this process for structural analysis and identification. An update on the performance level of tandem Py-MS, a discussion of the variety of approaches used to sequence peptides, and interpretation of the pyrolysis mass spectra of underivatized linear oligopeptides are presented.

## PREVIOUS WORK

### Diketopiperazine Formation

Although there is considerable literature on mass spectral electron ionization reactions of peptides, only limited work has been done on the formation of 2,5-diketopiperazine or cyclo-dipeptide (DKP) which is an important aspect of using electron ionization MS for potential protein sequence analysis. From previous studies (6,9,10,11,12,13), dipeptide cleavage and dipeptide cyclization have been proposed. Thermally volatilized dipeptides dehydrate to form substituted DKPs. Svec and Junk (12) reported DKP formation for a group of dipeptides which were sublimed at 120-160°C in the mass spectrometer. The observed dipeptide spectra were shown to be the summation spectra of the dipeptides and the DKP. They stated that cleavage of the DKP normally proceeds through the retention of the amino acid residue side groups. The possible loss of a residue side group before ring cleavage is unlikely from energy and steric considerations. Others (14,15) have applied different techniques not involving direct mass spectrometry to dipeptide analysis and found the same cyclization pathway. However, Bowers *et al.* (16) did not observe the DKPs upon ultraviolet laser radiation and Fourier transform mass spectrometry, neither did Robertson (17) or Bradley (18). The apparent differences are due to drastic variations in the methodologies and other

experimental conditions such as pyrolysis temperature, sample size, type of column, flow rate, and temperature variations.

Fragmentation processes of DKPs have been thoroughly discussed by Svec and Junk (12). Diketopiperazine of Gly-Gly and Ala-Ala were investigated and only the hydrogen migration reactions (1)-(2) was found, Figure 2. Ratcliff *et al.* (11) pyrolysed a selected group of DKPs of valine, alanine and glycine and analyzed the results by GC-MS. With the possible exception of the glycine DKP, all of the other DKPs were shown to be decomposed mainly through cleavage pathway A followed by a dehydrogenation reaction, Figure 2, reaction (3).

Direct cleavage of dipeptides can also occur. Bowers *et al.*(16) detected the peaks corresponding to the cleavage of the  $\alpha$ -C-C bond in dipeptides with transfer of a hydrogen and charge retention on the N-terminus, Figure 3. Further, Biemann (19) and Niederwieser (20) have noted that EI always results in cleavage of the peptide linkage on either side of the carbonyl group.

Noguerola (6) described the formation and characterization of pyrolysis products from twelve dipeptides and of their DKPs using Curie-point tandem Py-MS. Two levels of general electron ionization and thermal fragmentations for dipeptides were established. The first level involves direct dehydration of dipeptide to form DKP which occurred simultaneously with cleavage of the dipeptide. On the second level, three pathways for the fragmentation of the side

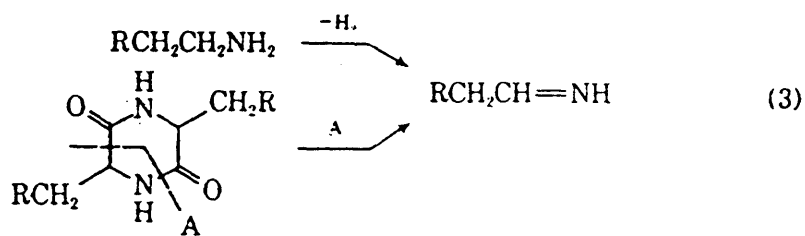
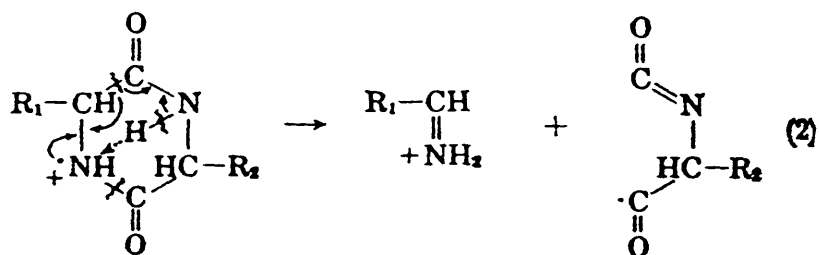
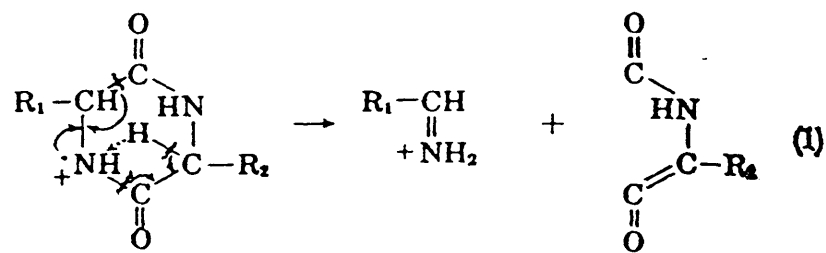


Figure 2. Fragmentation processes of the DKPs

groups were postulated. These reactions were simple cleavage of a side group to form a stable product; cleavage of a side group with migration or rearrangement, especially by a six-membered rearrangement if there is a  $\gamma$ -hydrogen relative to a carbonyl group; or no cleavage of a side group if it is aliphatic with three or fewer carbons.

Li and Lubman have also investigated pyrolysis of peptides using laser desorption mass spectrometry (9). Based on their R2PI/PMI (R2PI= resonant two-photon ionization; MPI= multiphoton ionization) mass spectra, they did observe DKPs in the laser-induced thermal decomposition process for oligopeptides and their derivatives whose N-terminal group was not blocked. TYR-GLY-GLY and TYR-GLY-GLY-PHE-MET produced intact molecular ions and their thermally decomposed species were present. In addition, an elimination of one glycine moiety in the tripeptide and GLY-PHE-MET released from the cyclization of the N-terminal pair of amino acid residues were also seen. The suggested mechanism of formation of DKPs is shown in Figure 3 which can be suitable for several cases as follows: 1) X can be a hydroxy group where DKP is formed by a water loss, as the case in dipeptides. 2) X can be an amino acid or peptide, as the case of oligopeptides, the displacement of the amino acid or peptide forms the cyclic dipeptide.

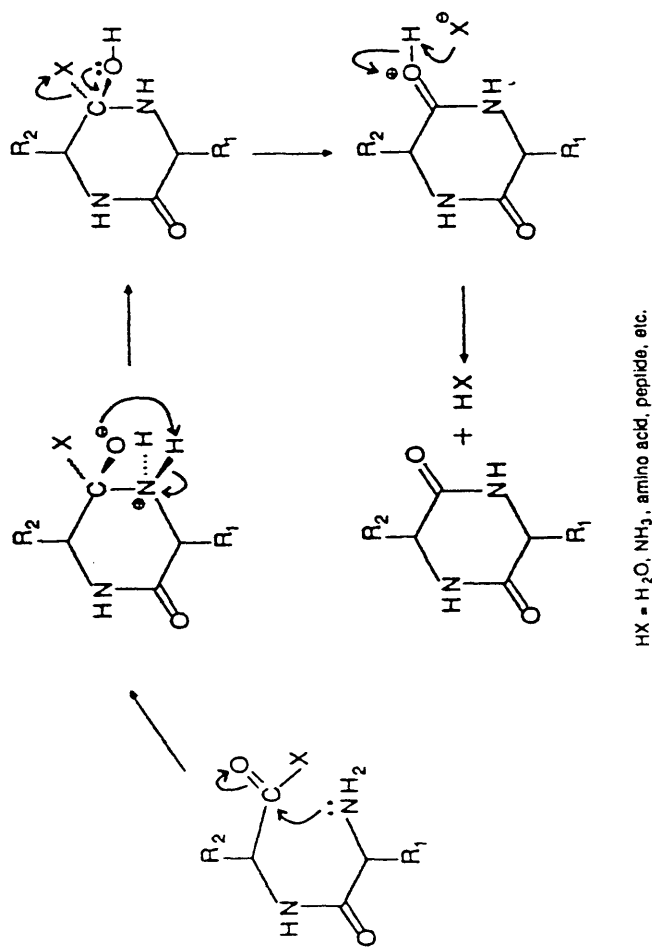


Figure 3. Schematic illustration of the formation of 2,5-diketopiperazines (DKP) by the desorption laser beam

### Peptide Sequencing

Following the work of Sanger (21) in determining the primary structure of insulin, the most important contribution to amino acid sequence analysis was made by Edman, who introduced (22-24) the use of phenylisothiocyanate (PITC) for sequential degradation of one amino acid at a time from the amino terminus of a polypeptide chain. Up to the present time, the methodology has been modified and new techniques developed at an unexpected pace in order to obtain sequence information on smaller and smaller amounts of sample (25-28). Of all the techniques, mass spectrometry has received the most attention. A number of researchers have sequenced peptides using fast atom bombardment (FAB) mass spectrometry. This method enables the study of underivatized peptides with a small sample size, as well as the study of larger peptides. Dudley (29) and Barber and Bordolin (30) reported that the molecular weight of underivatized peptides in the range 300-2000 daltons has been routinely determined. Therefore, peptides containing 4 to 30 amino acid residues are conveniently studied with FAB. Using FAB for amino acid sequencing, Buko *et al.* (31) stated there are always two pathways for decomposition of the protonated molecular ion into fragment ions that are the basis for sequence information, both of which result from peptide bond fission. The first is peptide bond cleavage with charge retention on the amino terminal fragment and with loss of the carboxyl fragment as a neutral

species, forming a series of acylium ions. The second, is the reverse of the first, with charge retention on the carboxyl fragment, producing a series of ammonium ions. A variety of publications (32-35) can be consulted for reproductions of actual spectra and for further discussion of their interpretation.

Recent publications have presented other applications of desorption techniques on peptides, such as laser desorption (36), field desorption (FD) (37), and plasma desorption mass spectrometry (38) or the combination of these methodologies with FAB and Edman degradation procedure (39). The fragmentation patterns discussed in these studies were almost the same as that previously described for FAB. The use of methylated-acetylated derivatives of polypeptides, which renders them volatile enough to be desorbed by simple heating of the direct inlet probe, has been involved in the structural analysis of numerous of peptides. Bieman (40) and Krutzsch (41) successfully did a sequence analysis on the permethylated peptide derivatives by using methyl iodide as a catalyst, Figure 4. In an extended investigation, workers (42) pyrolysed some synthetic tetradecapeptides and pentadecapeptides, which yielded mass spectra which showed sequence peaks up to and including the first 11 or 12 amino acid residues. It was apparently proved that all these derivatives had appreciable volatility after methylation, as well as the fact that the intensity of successive sequence ions decreases and at some points these ions may represent such a small

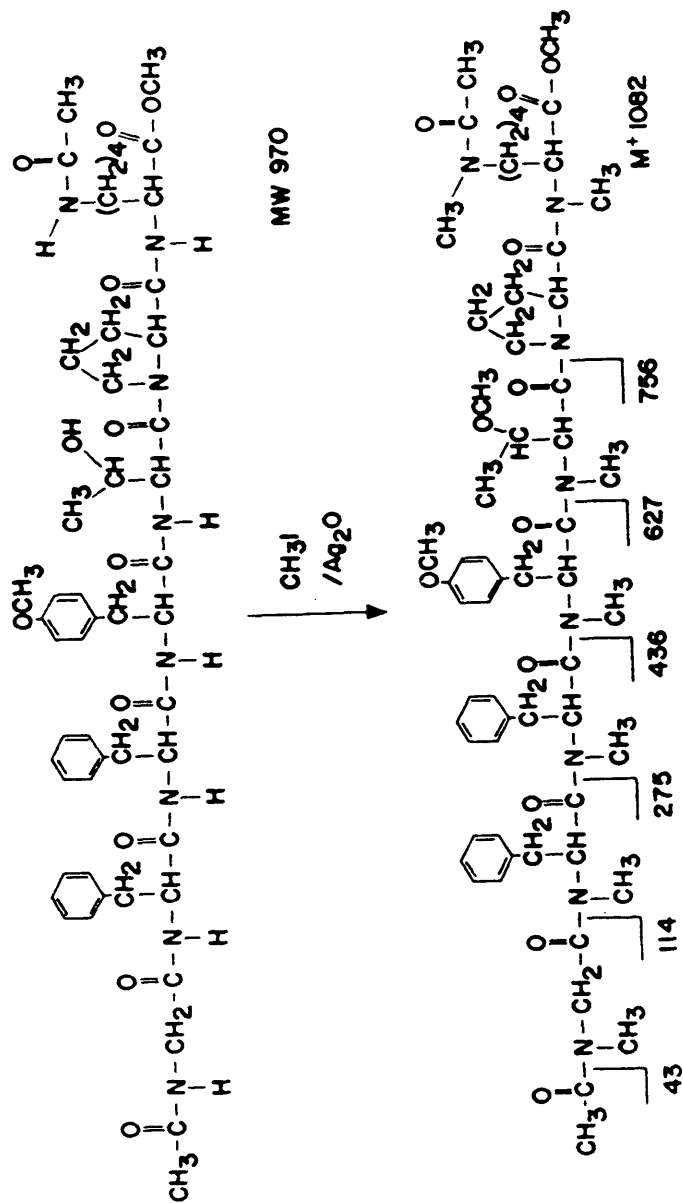


Figure 4. Permethylation of a heptapeptide derivative

sequence ions decreases and at some points these ions may represent such a small part of the total ion current that they are no longer detectable above the background noise.

A recent development in mass spectrometry, electrospray/ion spray ionization mass spectrometry (ESMS), has greatly extended the application of mass spectrometry to a wide range of important biological macromolecules. ESMS has been used by a number of researchers (43-45) for its accurate determination of the molecular weight of proteins and peptides in the range of 24,000 to 26,000 daltons. The accuracy is better than 0.02%. Several workers (46-48) have reported on the coupling of ESMS with a triple quadrupole mass spectrometer for structural characterization of constituents of the peptide.

C-terminal oligopeptide sequencing was established back in 1926 when the thiocyanate degradation was introduced by Schlack and Kumpf. Arguments (49) for the importance of C-terminal sequencing always include the following: 1) Information especially suited to the specification of nucleotide probes. 2) Access to N-terminally blocked proteins. Hunt *et al.* (50) successfully claimed C-terminal fragments arise mainly in peptides containing aromatic or heterocyclic amino acids in the mass spectra generated by electron impact. On the other hand, C-terminal fragments are common in Schiff base derivatives of peptides and are significant factors in interpreting such spectra.

## EXPERIMENTAL

The pyrolysis tandem MS data were obtained with an Extrel Model EL-400 triple quadrupole mass spectrometry, fitted with a Fisher Model 0310 (1 KW) rf-generator to supply power to a Curie-point pyrolysis coil. A schematic of this instrument was shown in Figure 1. The transfer line was heated to 325°C and the ion source to 250°C. 70 eV spectra were generated from a 510°C pyrolysis under high vacuum. The three scan modes: full scan, parent ion scans, and daughter ion scans, were obtained. Full scan spectra were generated by scanning quadrupole 1 (Q1) and setting quadrupole 2 (Q2) and quadrupole 3 (Q3) to pass all ions. In parent ion mode, Q1 was scanned while Q3 was set to a selected daughter, and in daughter ion mode, Q1 was set to a selected parent while Q3 was scanned. Argon was used as the collision gas for the daughter ion and parent ion scans.

The pyrolytic Curie-point procedure used was based on the direct application of underviatized oligopeptide samples onto the wires. To obtain known amounts of the peptides on the wires, approximately 5 mg of the peptides was weighed out and suspended in 1 ml methanol. For each sample, 15-20 ul of sample was applied to a 510°C (610°C and 358°C as options) wire and the methanol was evaporated under a stream of hot air. Triplicate Py-MS spectra were made for each oligopeptide in the mass range of  $m/z$  35 to  $m/z$  500, as well as daughter-ion

and parent-ion scans.

All oligopeptides used in the study were synthesized by Dr. Craig Miles, Biochemistry Department, Colorado State University (Fort Collins, CO). These included:

Tripeptides:

Phenylalanyl-leucyl-methionine (PHE-LEU-MET)

Methionyl-leucyl-phenylalanine (MET-LEU-PHE)

Phenylalanyl-methionyl-leucine (PHE-MET-LEU)

Tyrosyl-tyrosyl-phenylalanine (TYR-TYR-PHE)

Leucyl-leucyl-leucine (LEU-LEU-LEU)

Tetrapeptides:

Alanyl-phenylalanyl-leucyl-methionine (ALA-PHE-LEU-MET)

Alanyl-methionyl-leucyl-phenylalanine (ALA-MET-LEU-PHE)

Pentapeptides:

Alanyl-phenylalanyl-leucyl-methionyl-tyrosine

(ALA-PHE-LEU-MET-TYR)

Alanyl-tyrosyl-leucyl-methionyl-phenylalanine

(ALA-TYR-LEU-MET-PHE)

Hexapeptides:

Alanyl-tyrosyl-leucyl-methionyl-phenylalanyl-phenylalanine

(ALA-TYR-LEU-MET-PHE-PHE)

Alanyl-phenylalanyl-leucyl-methionyl-tyrosyl-phenylalanine

(ALA-PHE-LEU-MET-TYR-PHE)

Phenylalanyl-alanyl-phenylalanyl-leucyl-methionyl-tyrosine

(PHE-ALA-PHE-LEU-MET-TYR)

Tyrosyl-tyrosyl-tyrosyl-tyrosyl-tyrosyl-tyrosine

(TYR-TYR-TYR-TYR-TYR-TYR)

Other oligopeptides:

Arginyl-glycyl-leucyl-isoleucyl-valyl-phenylalanyl-histidyl-threonyl-serine

(R-G-L-I-V-F-H-T-S)

Alanyl-asparagyl-glutamic acid-arginyl-alanyl-aspartic acid-leucyl-isoleucyl-alanyl-tyrosyl-leucyl-lysyl-glutamyl-alanyl-threonyl-lysine

(A-N-E-R-A-D-L-I-A-Y-L-K-Q-A-T-K)

Asparagyl-prolyl-asparagyl-alanyl-asparagyl-prolyl-asparagyl-alanyl-asparagyl-prolyl-asparagyl-alanyl-asparagyl-prolyl-asparagyl-alanyl

(N-P-N-A-N-P-N-A-N-P-N-A-N-P-N-A)

The derivatized oligopeptides were obtained from Dr. Paul Fennessery, University of Colorado Medical Center. They included:

Seryl-phenylalanyl-phenylalanyl-arginine (TFA)

(SER-PHE-PHE-ARG) (TFA)

Phenylalanyl-seryl-phenylalanyl-phenylalanyl-arginine (TFA)

(PHE-SER-PHE-PHE-ARG) (TFA)

Glycyl-phenylalanyl-seryl-phenylalanyl-arginine (TFA)

(GLY-PHE-SER-PHE-ARG) (TFA)

## RESULTS AND DISCUSSION

Noguerola (6) investigated the formation of DKPs from dipeptides using the system described in Figure 1. The present study extends the dipeptide investigation to determine if oligopeptides decompose by similar mechanisms or if new reactions occur.

### Tripeptides

#### Phenylalanyl-Leucyl-Methionine

Noguerola (6) found that PHE-LEU thermally decomposes to produce a series of peaks:  $m/z$  260, 204, 169, 141, 113, 103, 91, 85. Many of these peaks correlate with the DKP and its fragments. The tripeptide PHE-LEU-MET is composed of the dipeptide PHE-LEU plus a methionine C-terminus. The mass spectrum of PHE-LEU-MET is shown in Figure 5. Much of this spectrum of PHE-LEU-MET is an additive spectrum of the dipeptide PHE-LEU spectrum (Figure 6) plus the spectrum of the single amino acid methionine. The common appearance of the same series of peaks corresponding to DKP of PHE-LEU in both di- and tri-peptides suggests that the thermal and EI decomposition pathways are quite similar.

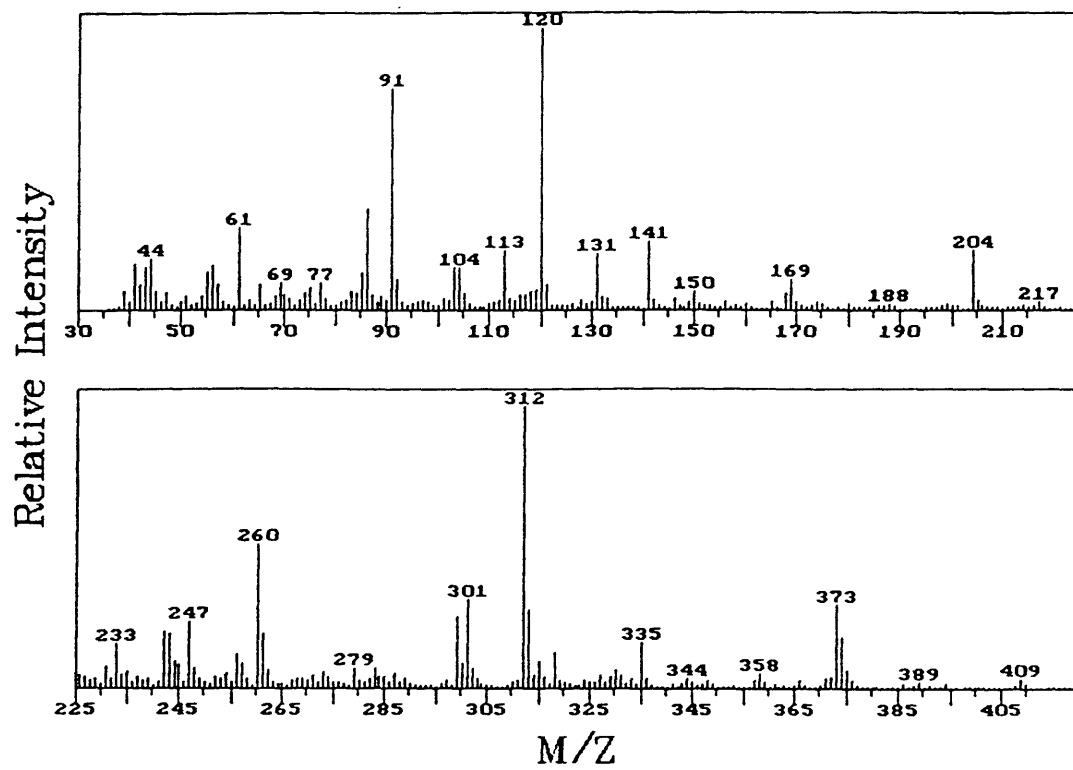


Figure 5. Pyrolysis-mass spectrum of phenylalanyl-leucyl-methionine

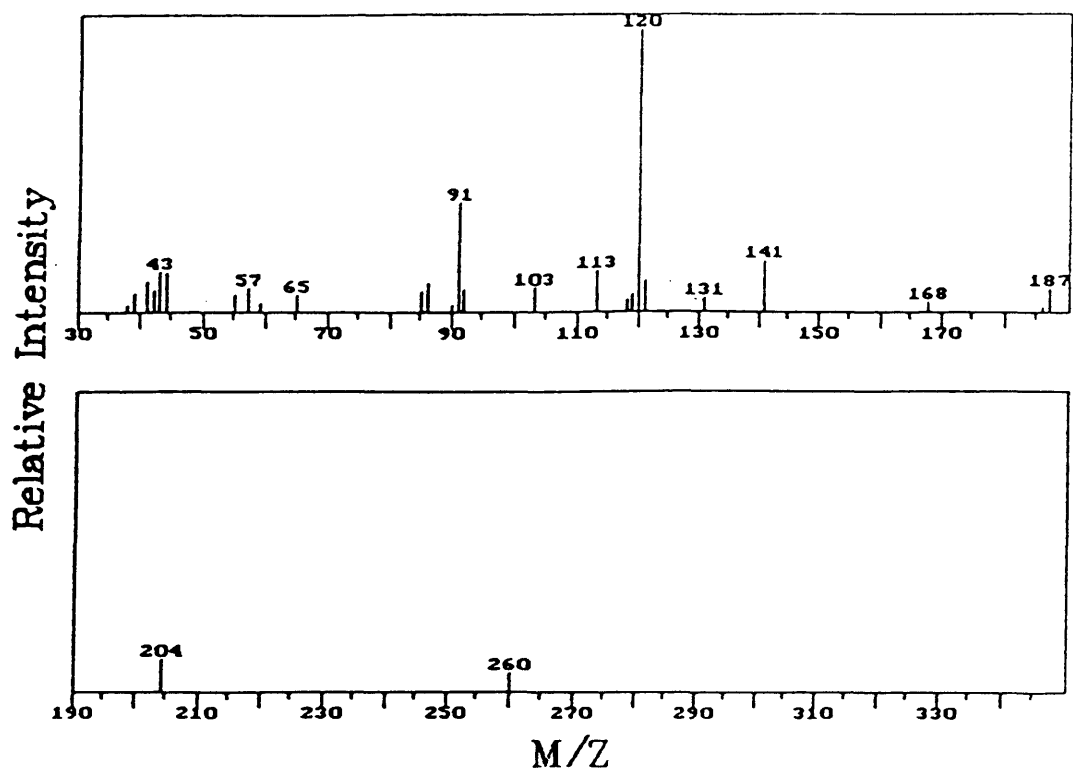


Figure 6. Pyrolysis-mass spectrum of phenylalanyl-leucine

The DKP formation occurs by the electron pair of the N-terminus attacking the carbonyl of the amino acid. However, the mechanism of the formation of DKP from di- and tri-peptides cannot be identical. This is because the DKP in the dipeptide is produced by the elimination of water, while in the tripeptide cyclization is followed by the elimination of the terminal amino acid, Figure 7.

The peak at  $m/z$  260 known to correspond to the DKP of PHE-LEU is supported to be a DKP by the daughter ion spectra of all pertinent peaks in PHE-LEU-MET in Figures 8-14. The peaks at  $m/z$  204, 169, 141, 113, 103, 91, 85 can be attributed to the fragmentation of the DKP. The peaks at  $m/z$  204 and 169 are due to the cleavage of benzyl or isobutene groups from the DKP. The other peaks at  $m/z$  113, 103, 91 and 85 are due to further fragmentation of the diketopiperazine skeleton, which is consistent with Noguerola' observation, Figures 15 and 16.

In the formation of the DKP of PHE-LEU from the tripeptide PHE-LEU-MET, the C-terminal amino acid residue was eliminated. For identification, the full spectrum of PHE-LEU-MET was compared with the EI mass spectrum of pure methionine, Figure 17. Most of the peaks present in the methionine spectrum, such as  $m/z$  150, 131, 61, were observed in the PHE-LEU-MET spectrum with significant intensity. This formation of the free C-terminal amino

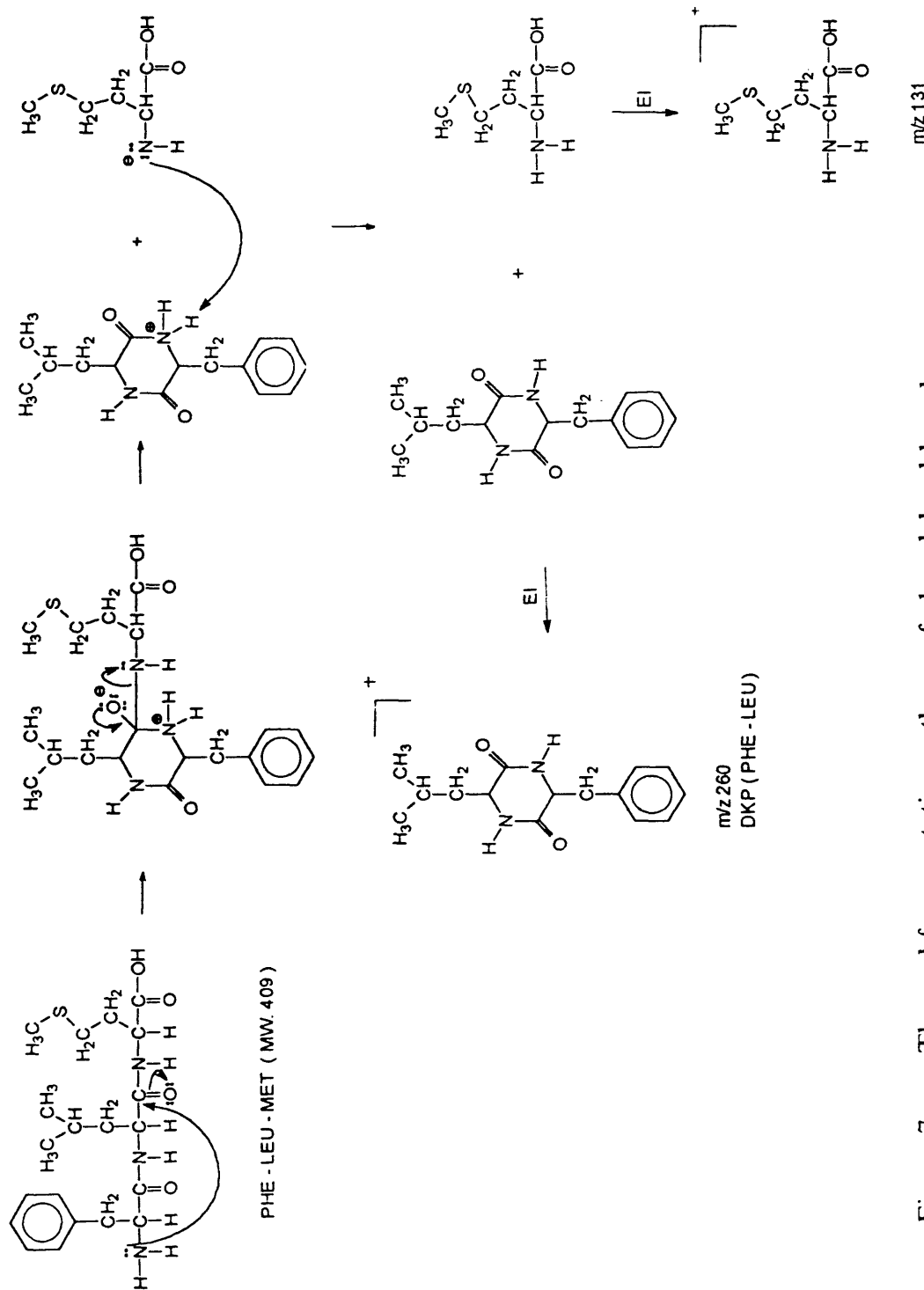


Figure 7. Thermal fragmentation pathway of phenylalanyl-leucyl-methionine

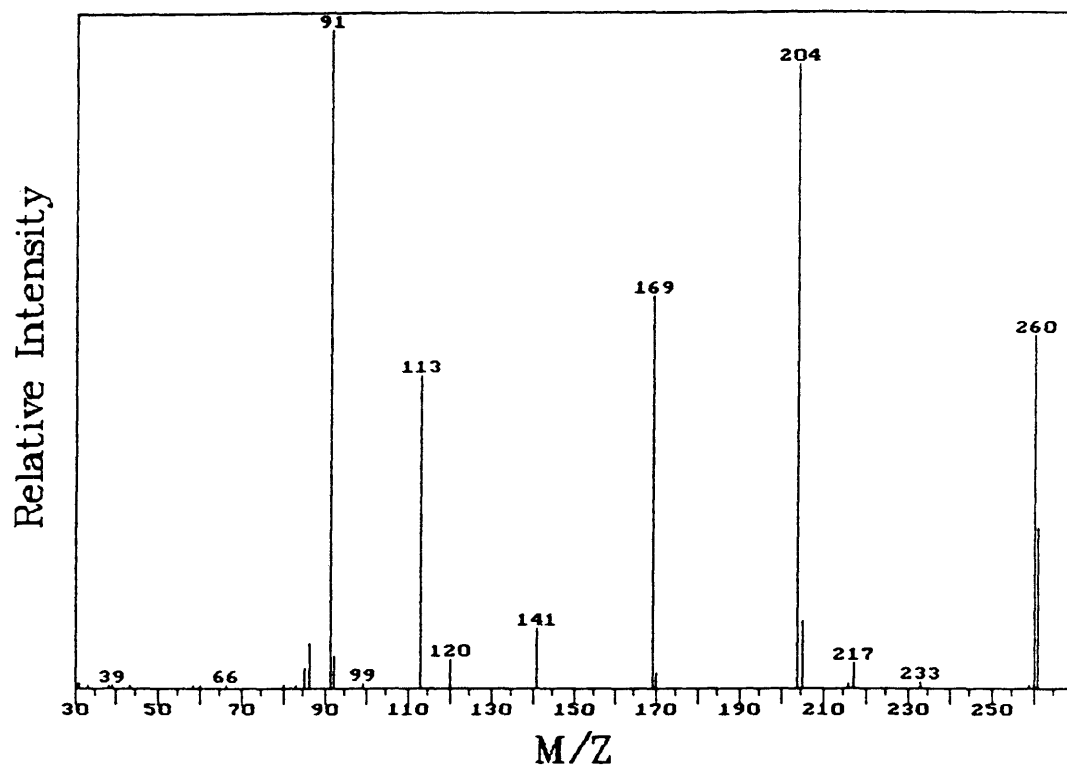


Figure 8. Pyrolysis-mass spectrum of daughter ions of m/z 260 of the DKP phenylalanyl-leucine

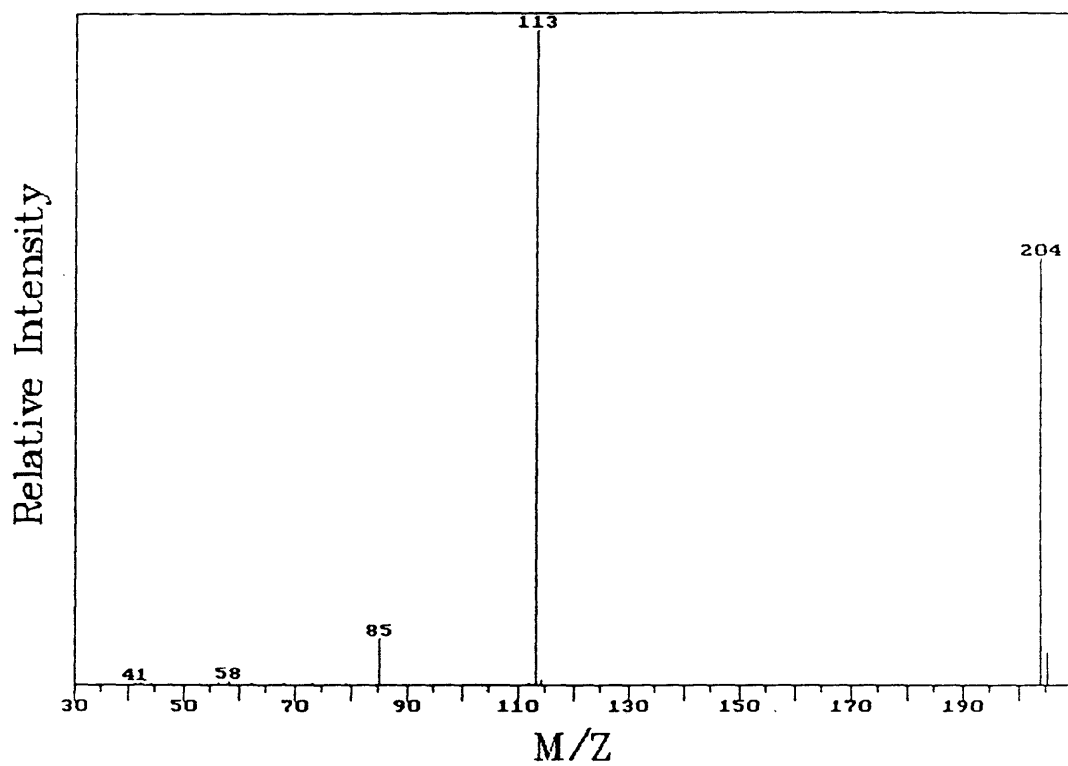


Figure 9. Pyrolysis-mass spectrum of daughter ions of  $m/z$  204 of the DKP of PHE-LEU

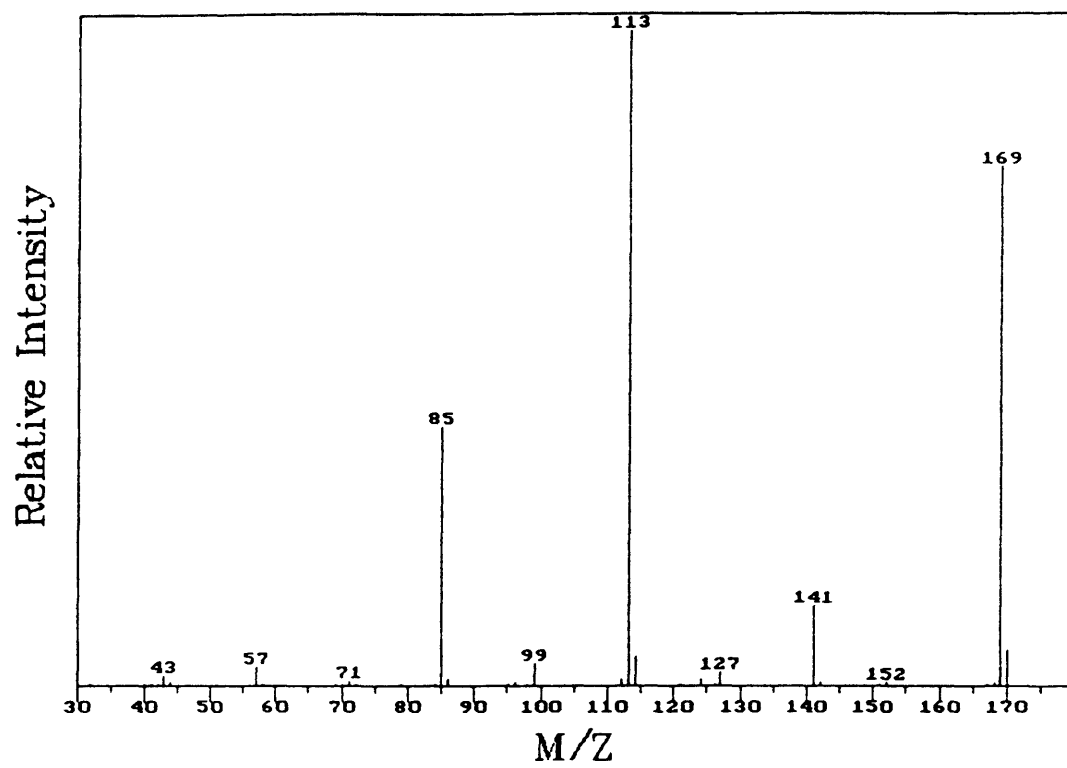


Figure 10. Pyrolysis-mass spectrum of daughter ions of m/z 169 of the DKP of PHE-LEU

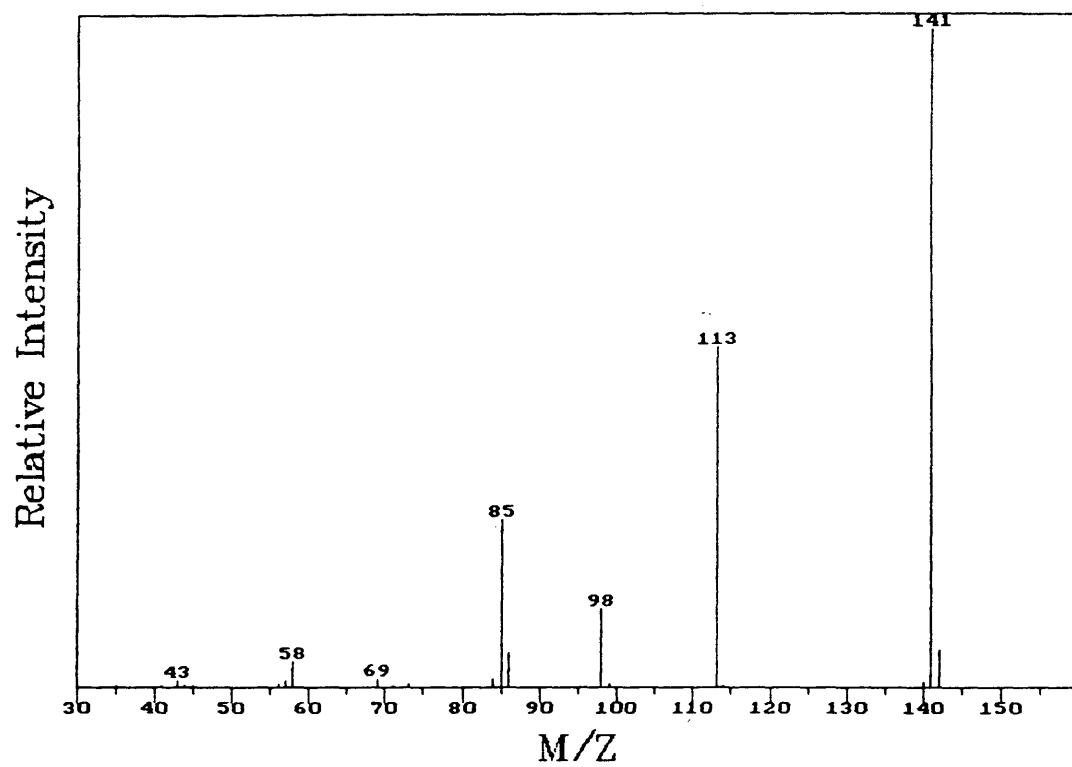


Figure 11. Pyrolysis-mass spectrum of daughter ions of m/z 141 of the DKP of PHE-LEU

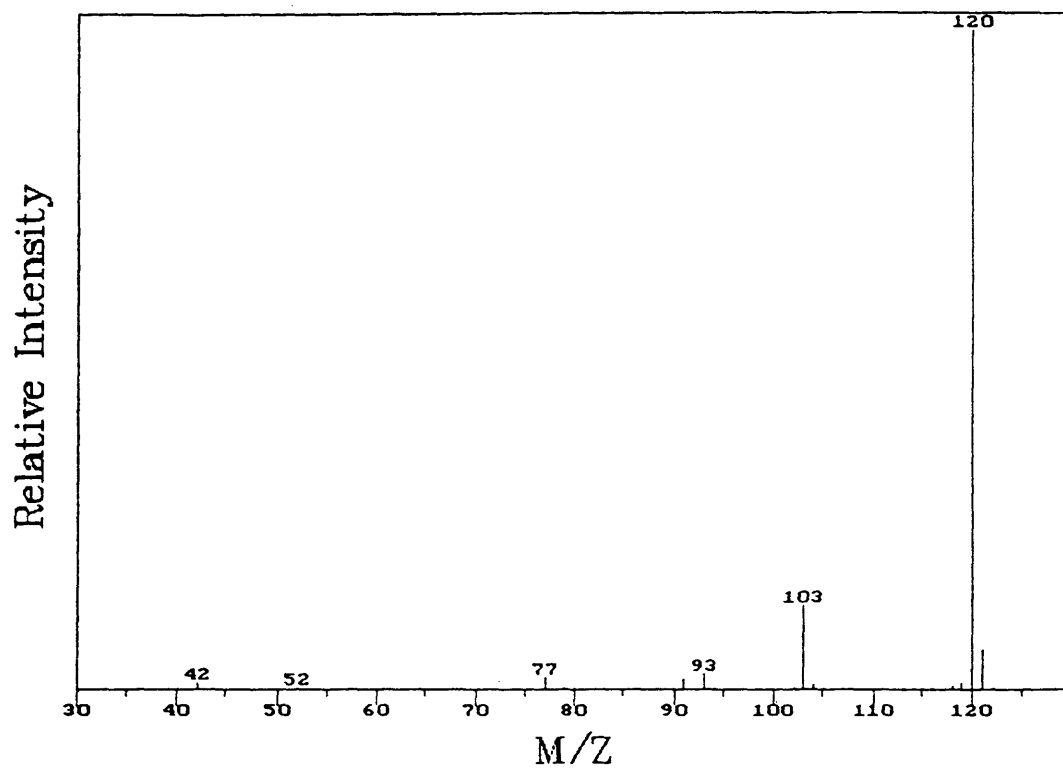


Figure 12. Pyrolysis-mass spectrum of daughter ions of m/z 120 of the DKP of PHE-LEU

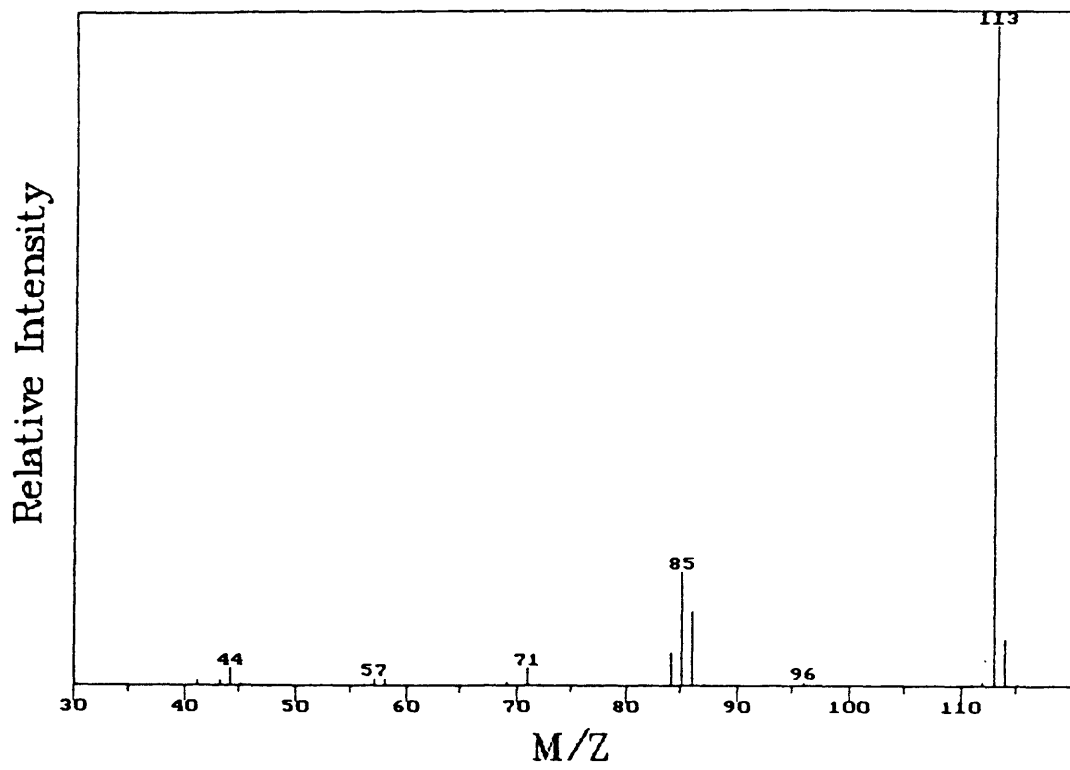


Figure 13. Pyrolysis-mass spectrum of daughter ions of m/z 113 of the DKP of PHE-LEU

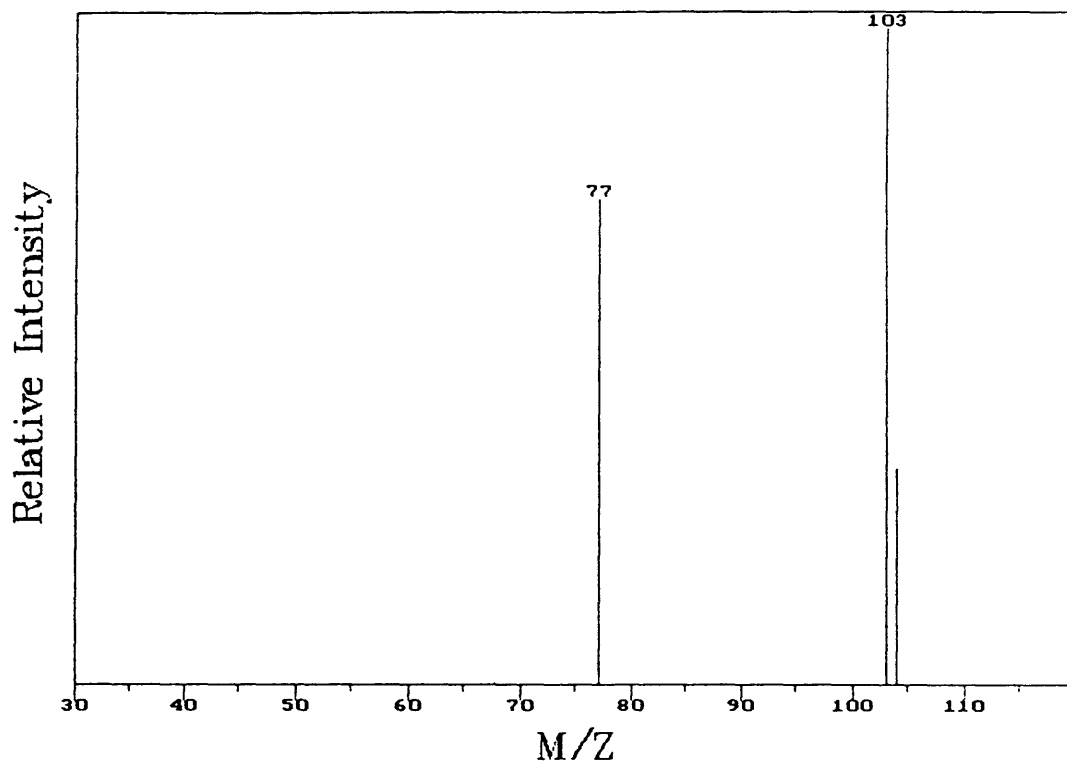


Figure 14. Pyrolysis-mass spectrum of daughter ions of  $m/z$  103 of the DKP of PHE-LEU



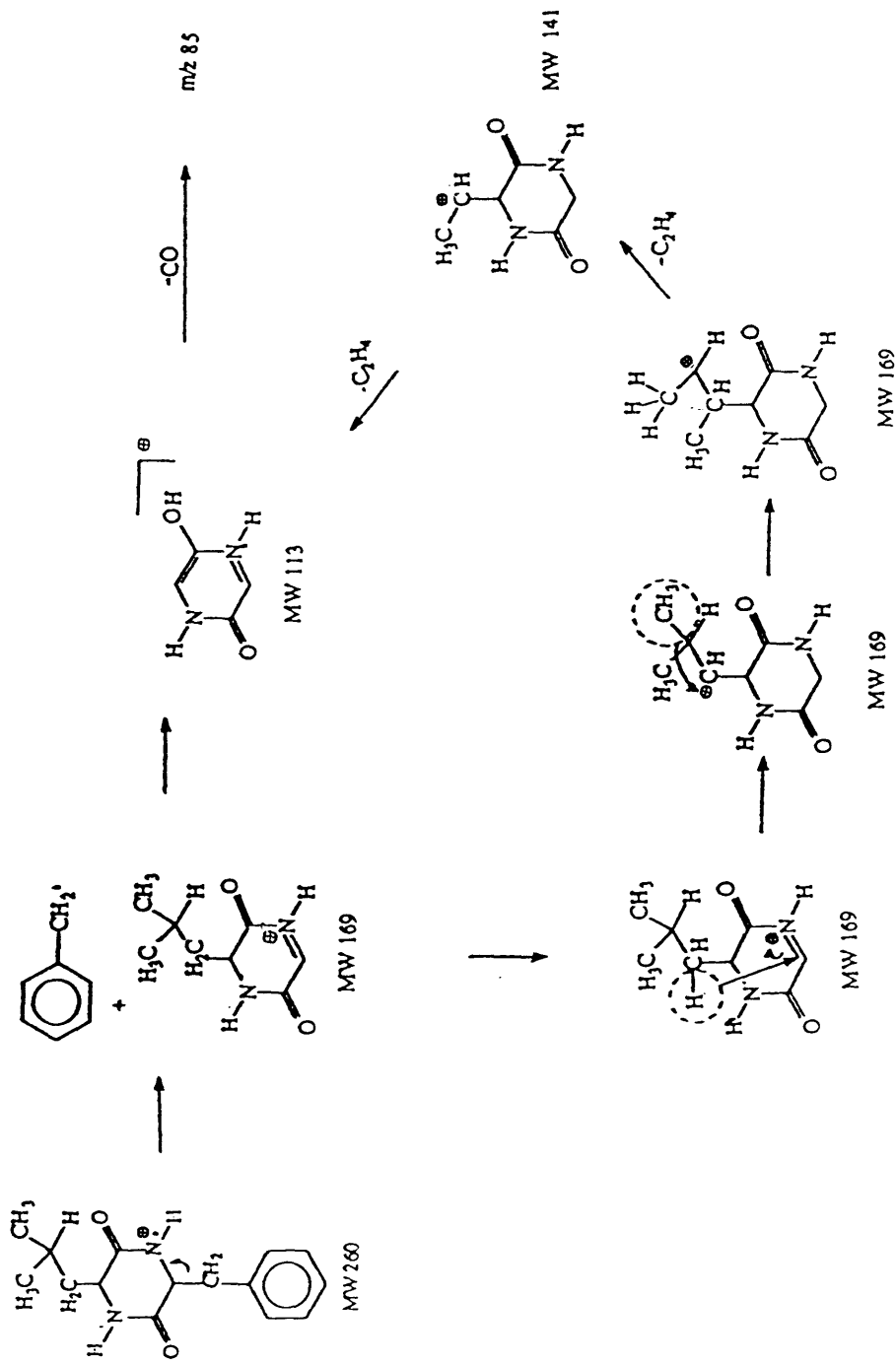


Figure 16. General fragmentation pathway 2 of the DKP of phenylalanyl-leucine and leucyl-phenylalanine

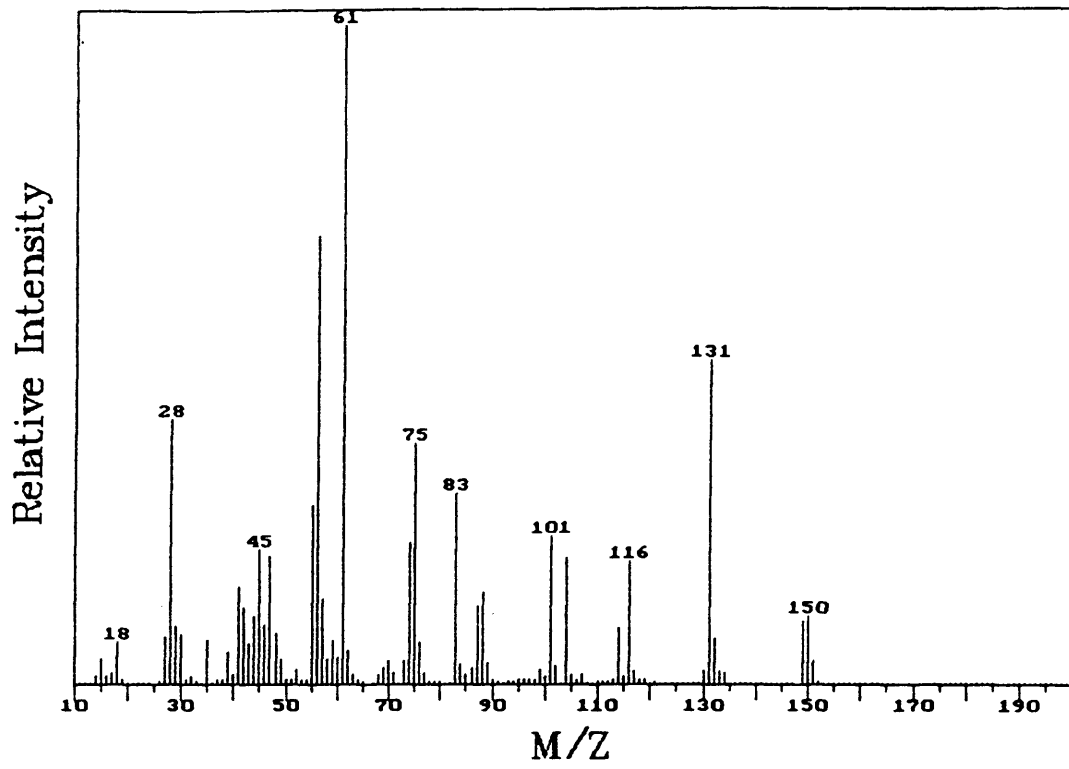
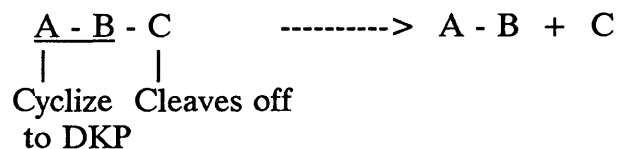


Figure 17. Pyrolysis-mass spectrum of methionine

acid provides additional evidence that the thermal decomposition pattern of the tripeptide PHE-LEU-MET follows



The full spectrum of PHE-LEU-MET also shows a weak molecular ion peak at  $m/z$  409. The peaks at  $m/z$  373, 335, 318, 294 can be attributed by daughter ion experiments to the fragmentation of the side groups directly from the tripeptide backbone when electron ionization occurs. In addition, we postulate that the base peak ( $m/z$  120) in PHE-LEU-MET spectrum is due to the N-terminal cleavage from the tripeptide molecular ion, Figure 18. Inspection of the daughter and parent ion spectra provides strong evidence for this hypothesis, which is supported by (a) molecular ion  $m/z$  409, was present in the parent ion spectrum of  $m/z$  120, Figure 19; and (b) the daughter ion spectrum of  $m/z$  409 with a prominent  $m/z$  120 peak, Figure 20. These results show that the molecular ion is a precursor to  $m/z$  120.

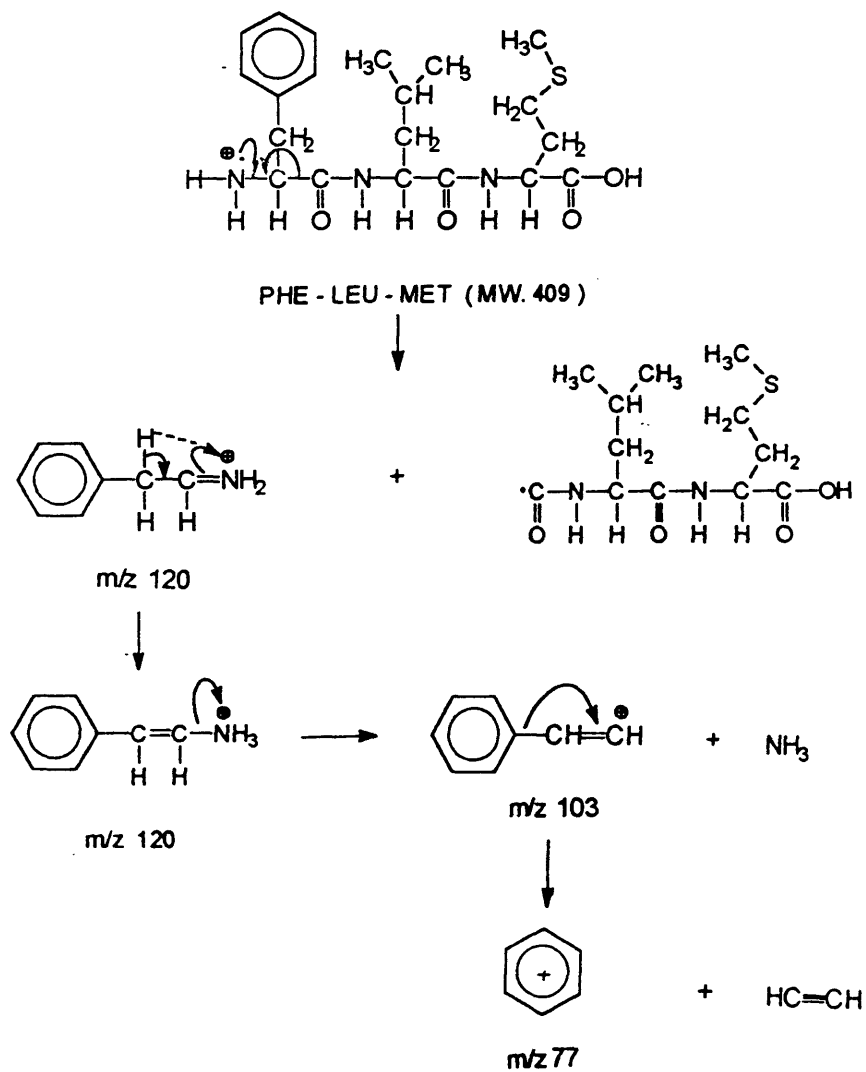


Figure 18. Fragmentation pathway of N-terminal cleavage for phenylalanyl-leucyl-methionine

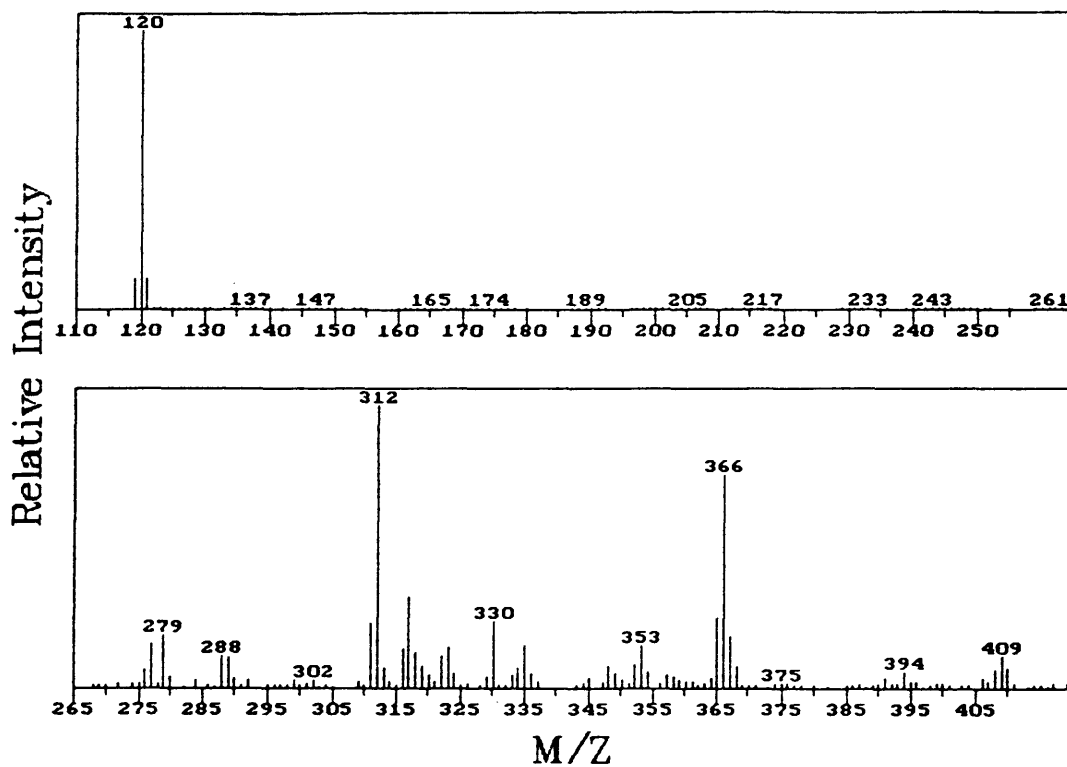


Figure 19. Pyrolysis-mass spectrum of parent ions of  $m/z$  120 of phenylalanyl-leucyl-methionine

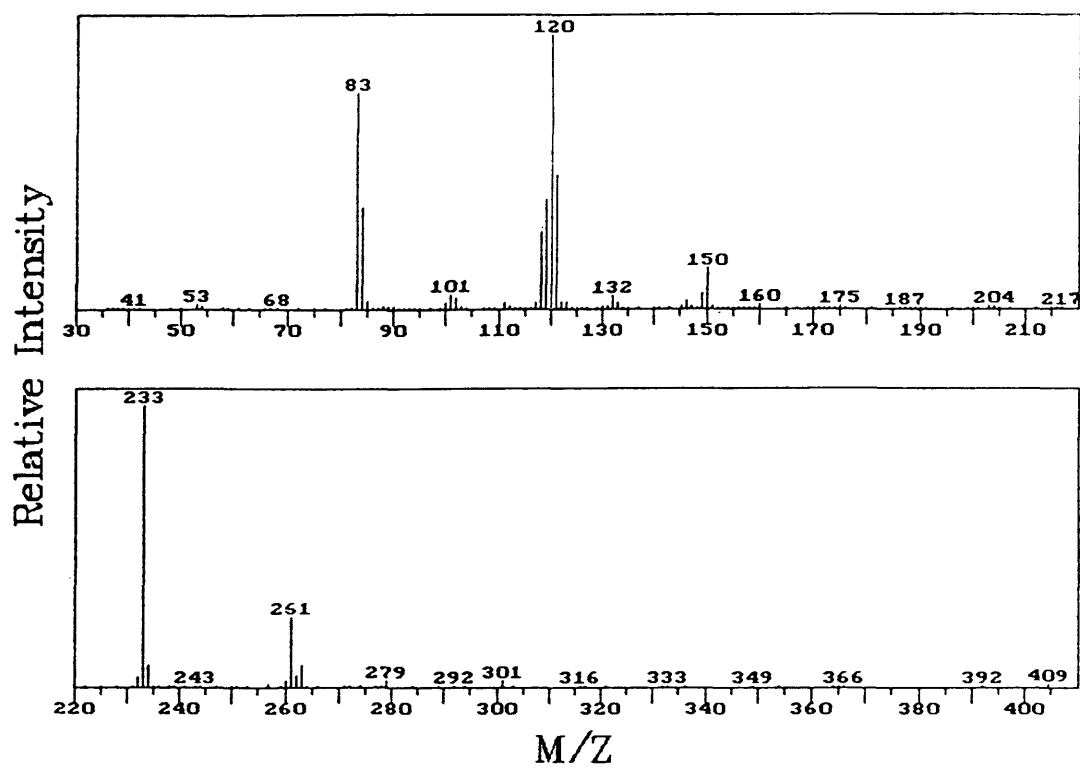


Figure 20. Pyrolysis-mass spectrum of daughter ions of  $m/z$  409 of phenylalanyl-leucyl-methionine

### Methionyl-Leucyl-Phenylalanine

Noguerola (6) also investigated the dipeptide MET-LEU and interpreted a series of peaks at  $m/z$  244, 183, 170, 155, 127, 113, 85, as resulting from the thermal decomposition of the dipeptide MET-LEU to a DKP and its related fragments. He noted two pathways involved in the cleavage of the DKP of MET-LEU. One was the initial loss of ethenyl methyl sulfide, which was followed by the loss of isobutene, both through an EI induced six-membered  $\gamma$ -hydrogen rearrangement, Figure 21. Another was the radical cleavage of methyl methylene sulfide followed by two successive ethylene losses, Figure 22.

Just as Noguerola found for the dipeptides, the tripeptide MET-LEU-PHE similarly undergoes many of the same reactions, Figure 23 shows the pyrolysis mass spectrum of MET-LEU-PHE. Again, as we discussed above, the common appearance of the same series of peaks in both the dipeptide MET-LEU and the tripeptide MET-LEU-PHE spectra, together with the daughter ion spectra of these peaks from the tripeptide, Figures 24-28, support the mechanism for the formation and fragmentation of the DKP of MET-LEU.

As noted above about identification of the methionine C-terminus from PHE-LEU-MET, similarly, the determination of the C-terminal amino acid in MET-LEU-PHE is based on the comparison of tripeptide spectrum with the phenylalanine amino acid spectrum, Figure 29. The series of peaks at  $m/z$  147,

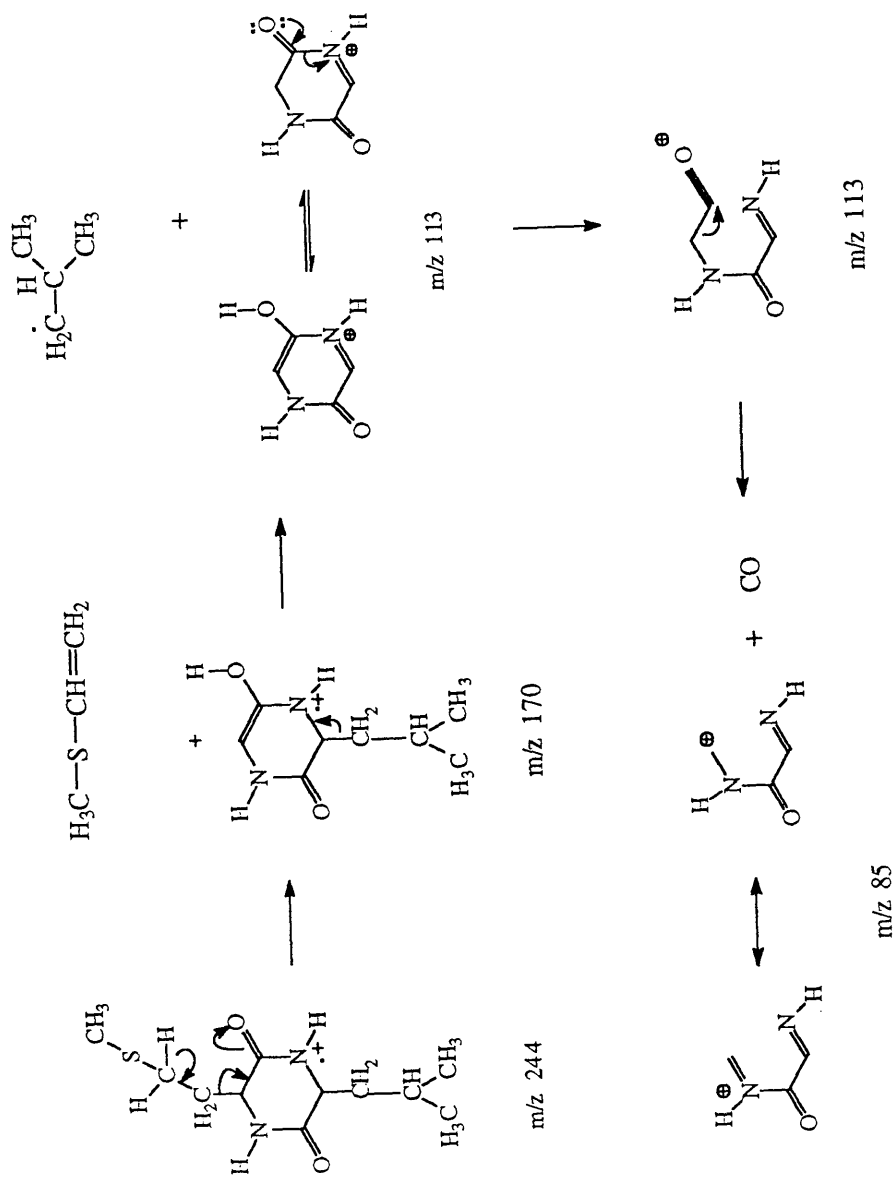


Figure 21. Fragmentation pathway 1 for the DKP of methionyl-leucine

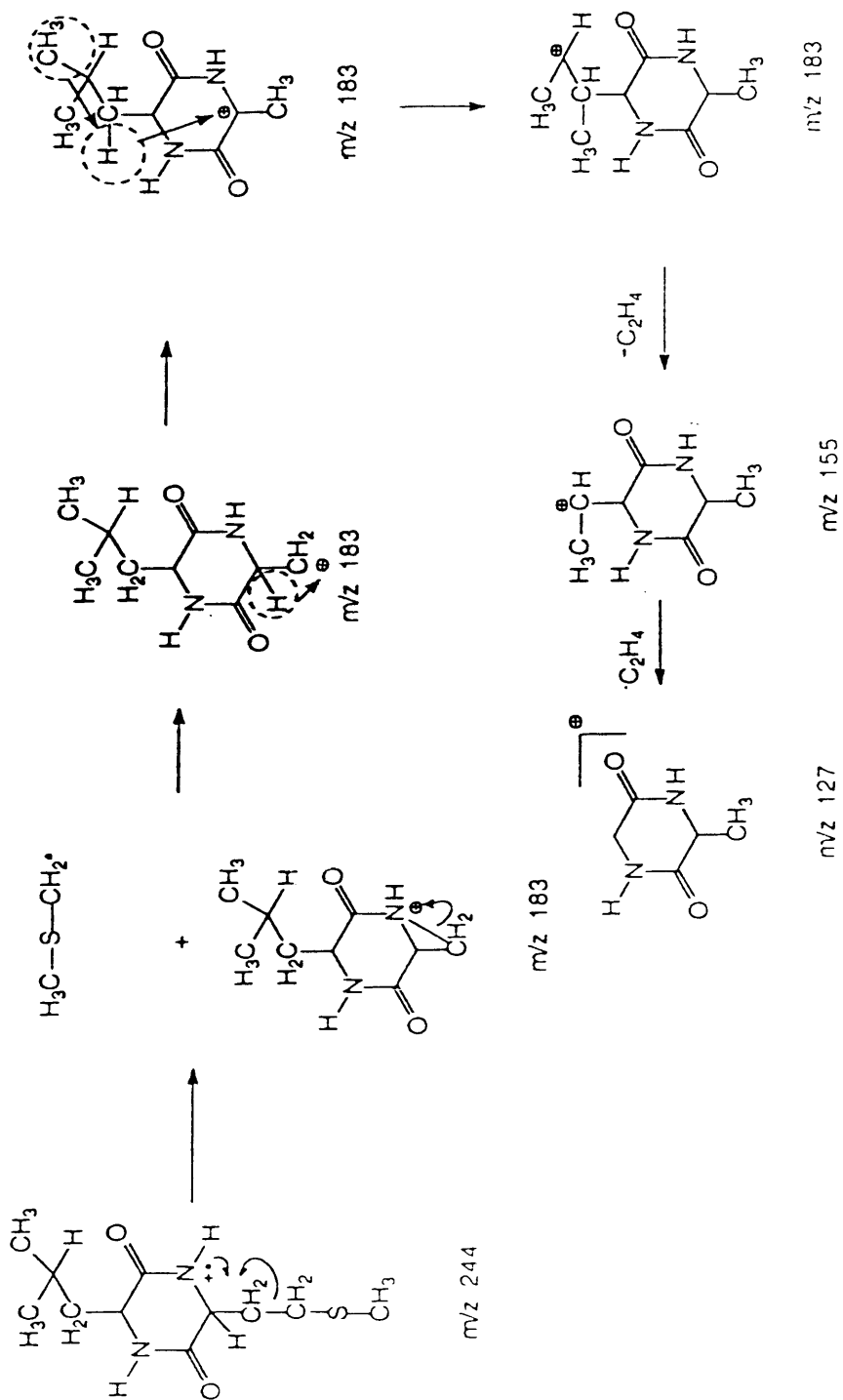


Figure 22. Fragmentation pathway 2 for the DKP of methionyl-leucine

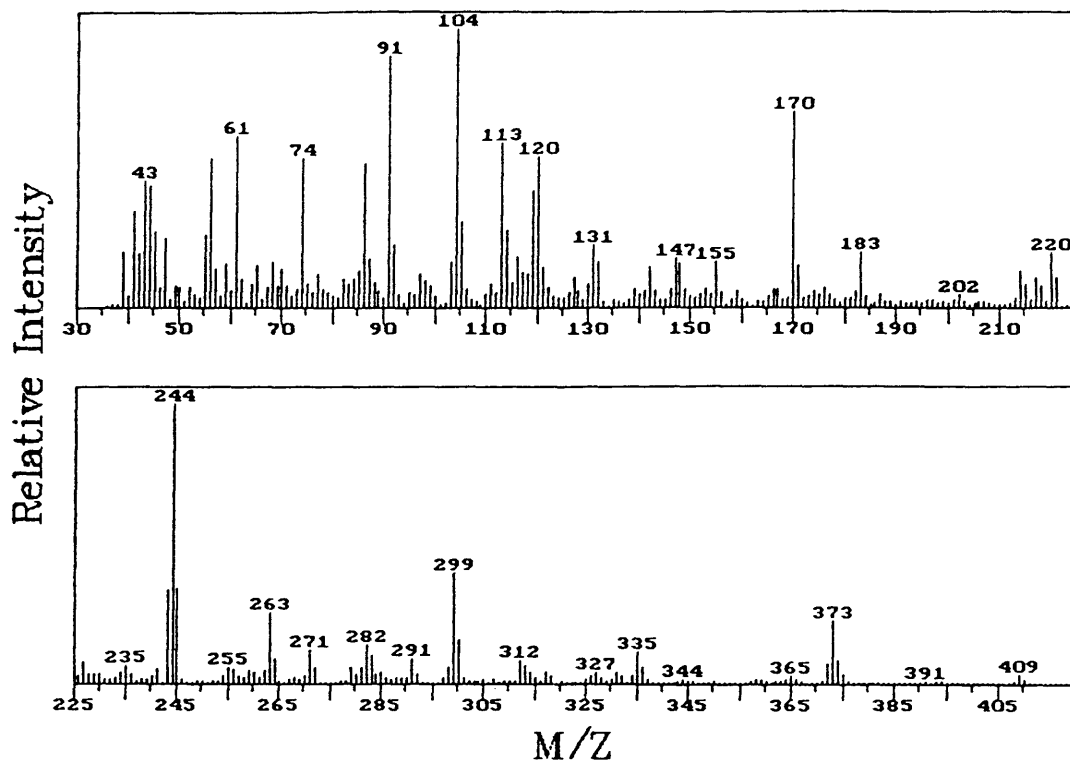


Figure 23. Pyrolysis-mass spectrum of methionyl-leucyl-phenylalanine

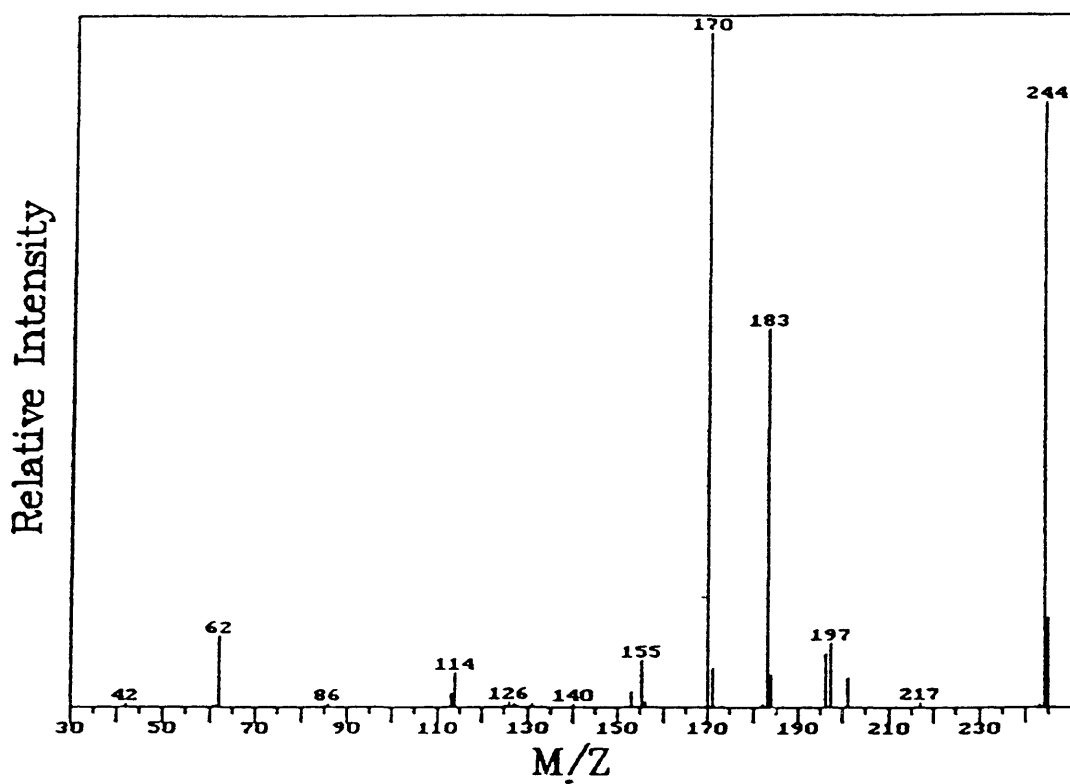


Figure 24. Pyrolysis-mass spectrum of daughter ions of m/z 244 of the DKP of methionyl-leucine and leucyl-methionine

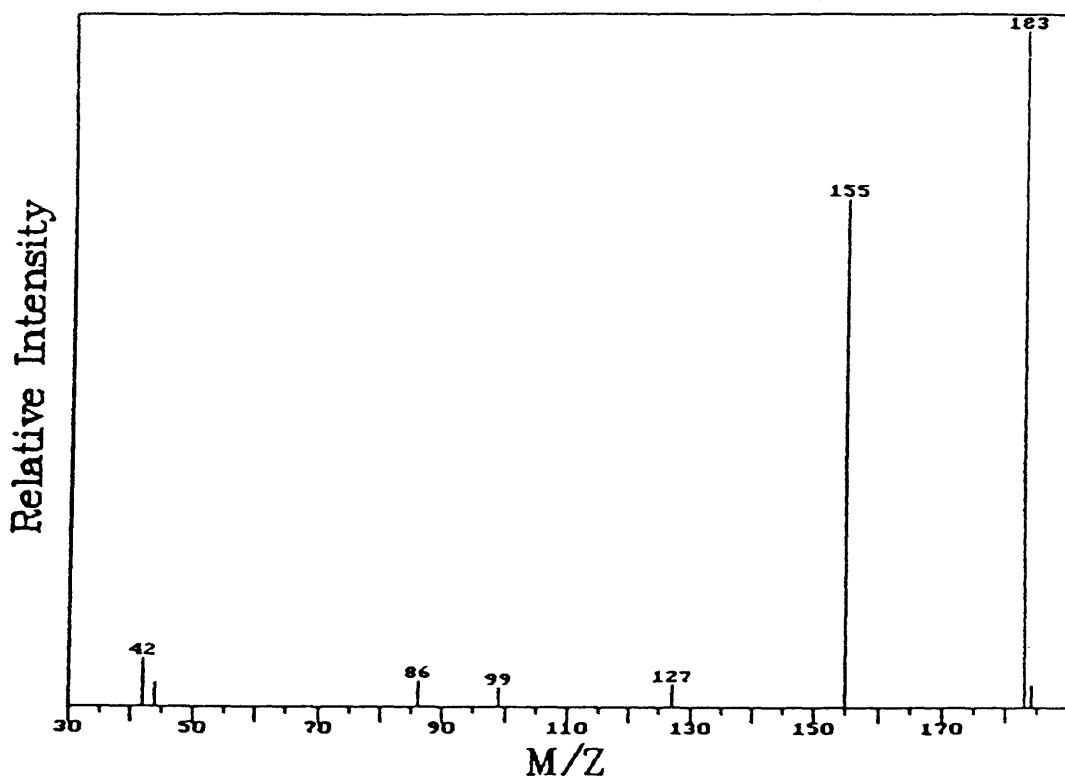


Figure 25. Pyrolysis-mass spectrum of daughter ions of m/z 183 of the DKP of MET-LEU and LEU-MET

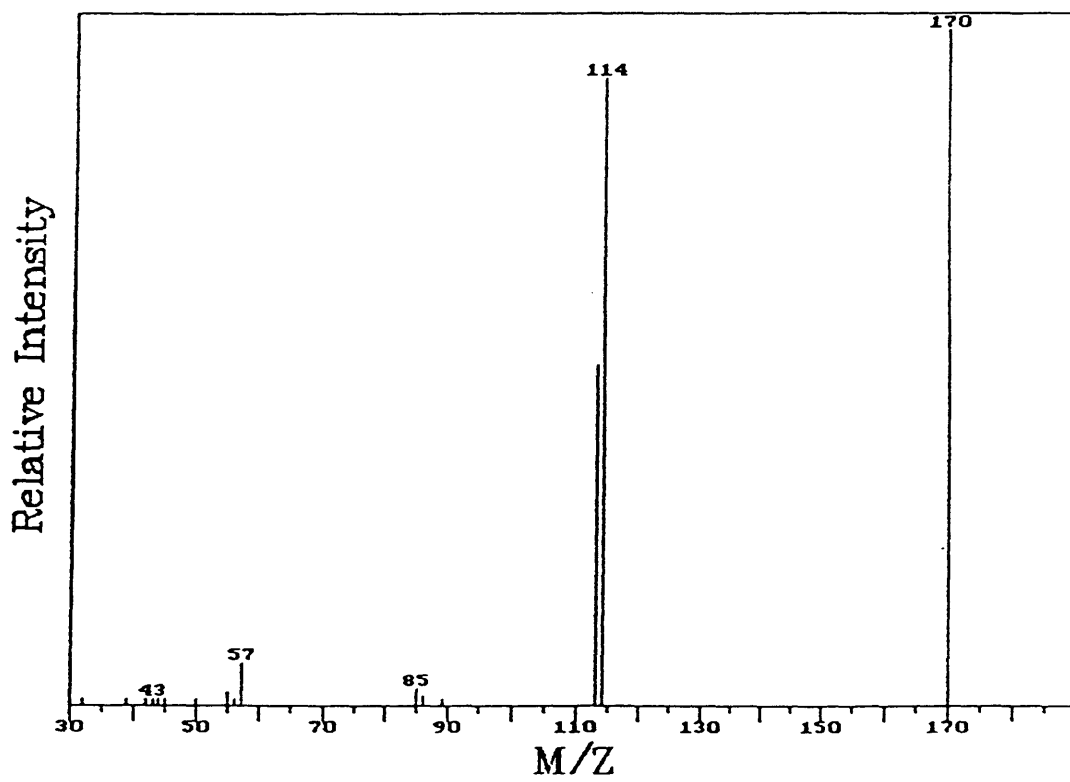


Figure 26. Pyrolysis-mass spectrum of daughter ions of m/z 170 of the DKP Met-Leu and Leu-Met

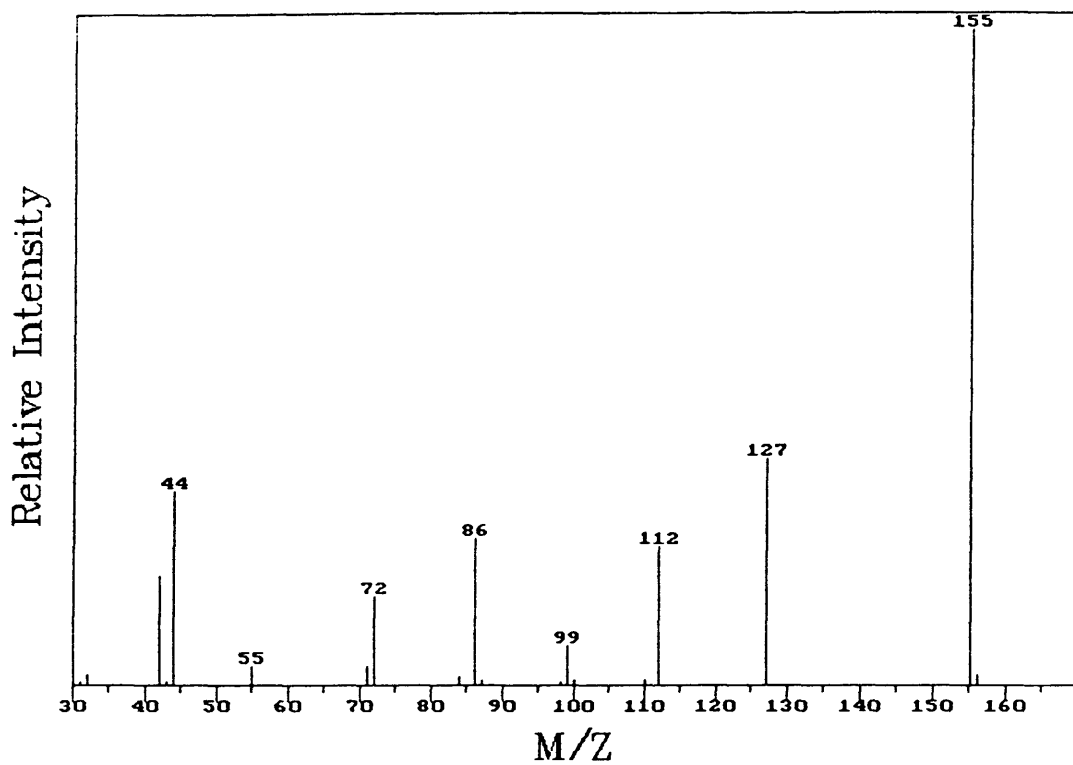


Figure 27. Pyrolysis-mass spectrum of daughter ions of m/z 155 of the DKP Met-Leu and Leu-Met

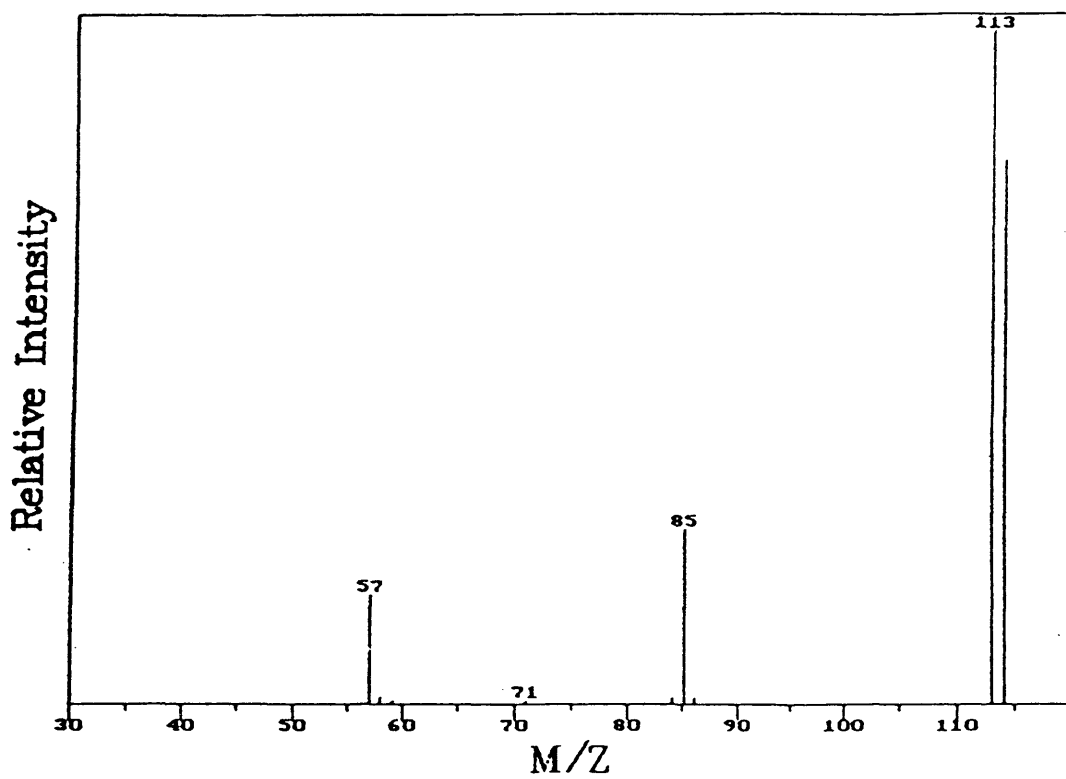


Figure 28. Pyrolysis-mass spectrum of daughter ions of m/z 113 of the DKP Met-Leu and Leu-Met

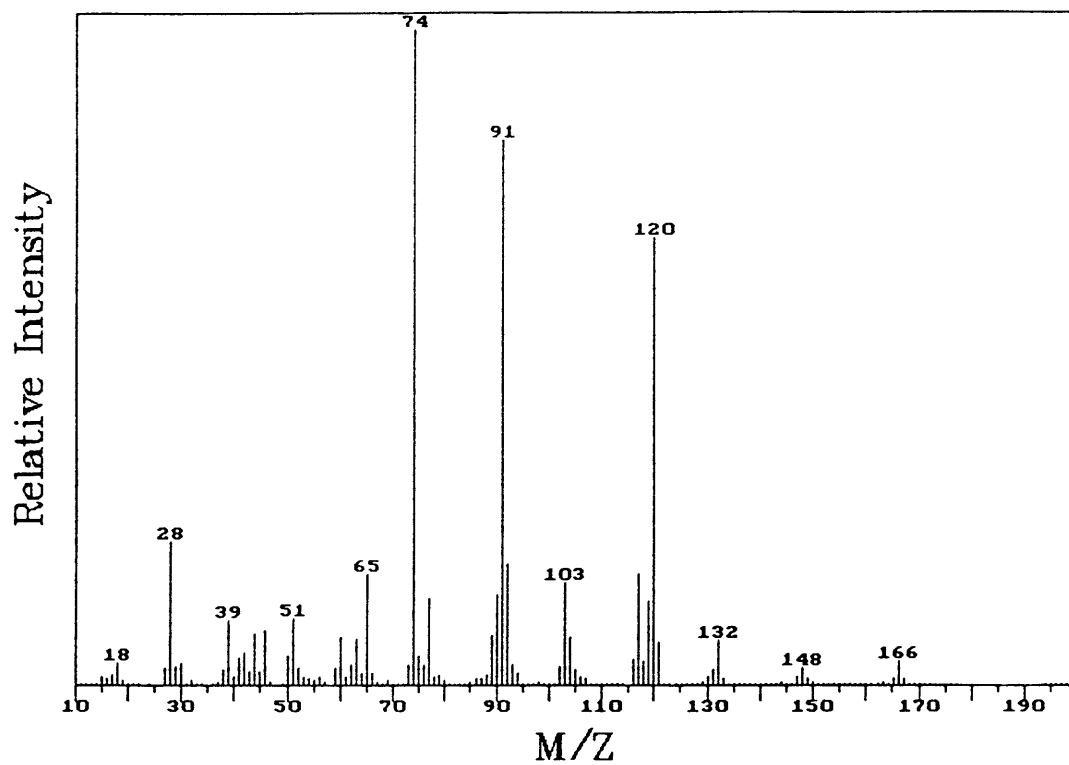


Figure 29. Pyrolysis-mass spectrum of phenylalanine

120, 91 that appear in both spectra serve as finger-printing peaks for determining the loss of phenylalanine C-terminus in the tripeptide during DKP formation.

The base peak at  $m/z$  104 results from the cleavage of the N-terminal methionine residue. This is supported by the following: the presence of a strong  $m/z$  104 peak in the daughter ion spectrum of molecular ion ( $m/z$  409), Figure 30, and the absence of  $m/z$  104 in the daughter ion spectrum of the DKP of MET-LEU ( $m/z$  244), Figure 24. These strongly serve to confirm our understanding that the molecular ion is a main precursor of the base peak rather than DKP, Figure 31.

#### Some other tripeptides

Py-MS spectra of several other tripeptides have been examined, including: PHE-MET-LEU, TYR-TYR-PHE and LEU-LEU-LEU, Figures 32-34. The peaks in these spectra can be explained based on previous results and provides evidence that their fragmentation mechanisms are similar to that for PHE-LEU-MET and MET-LEU-PHE decomposition.

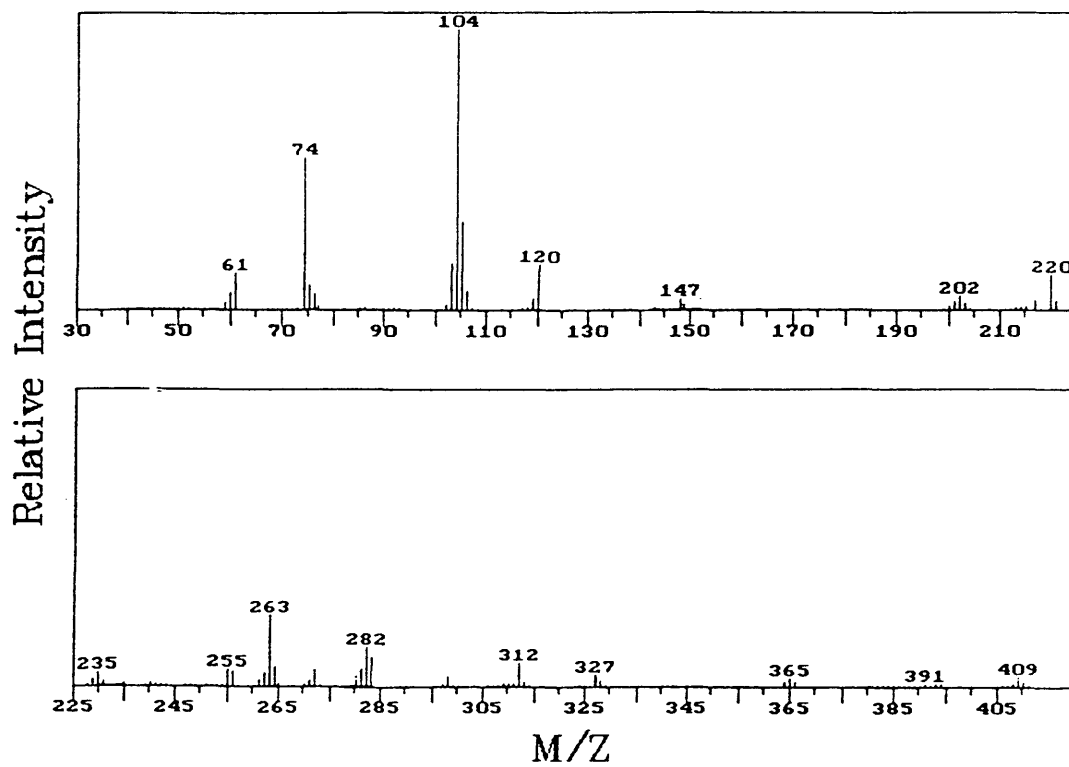


Figure 30. Pyrolysis-mass spectrum of daughter ions of  $m/z$  409 of methionyl-leucine-phenylalanine

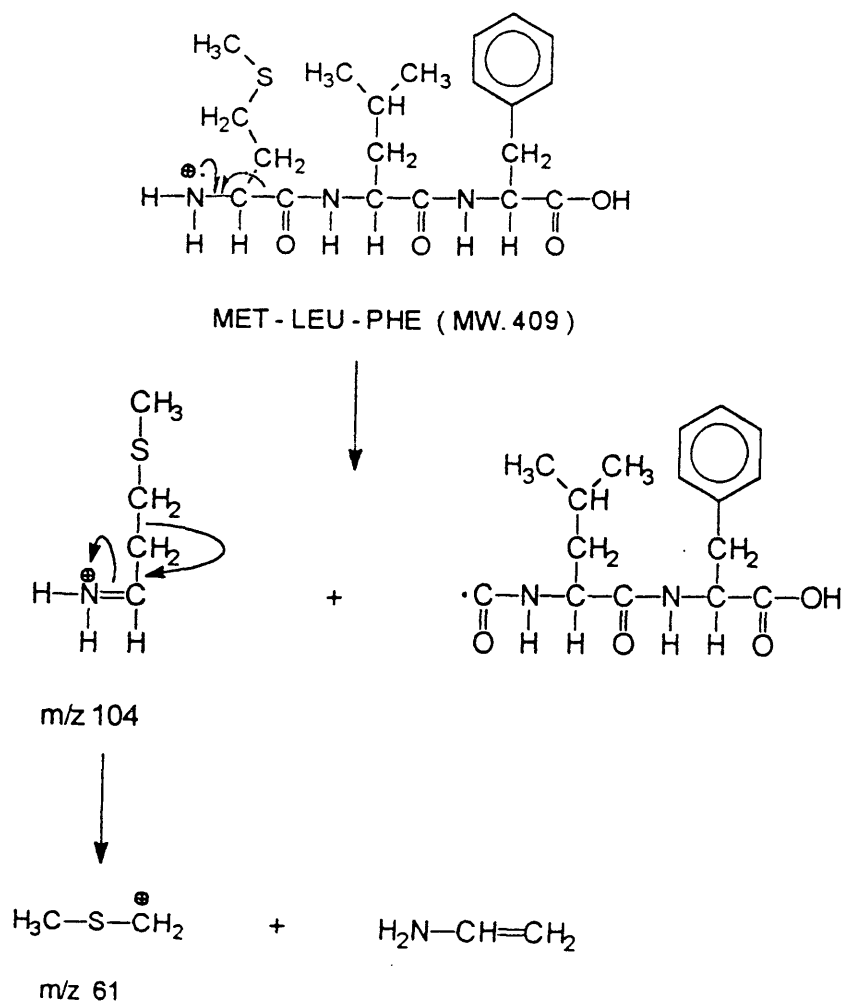


Figure 31. Fragmentation pathway of N-terminal cleavage for methionyl-leucyl-phenylalanine

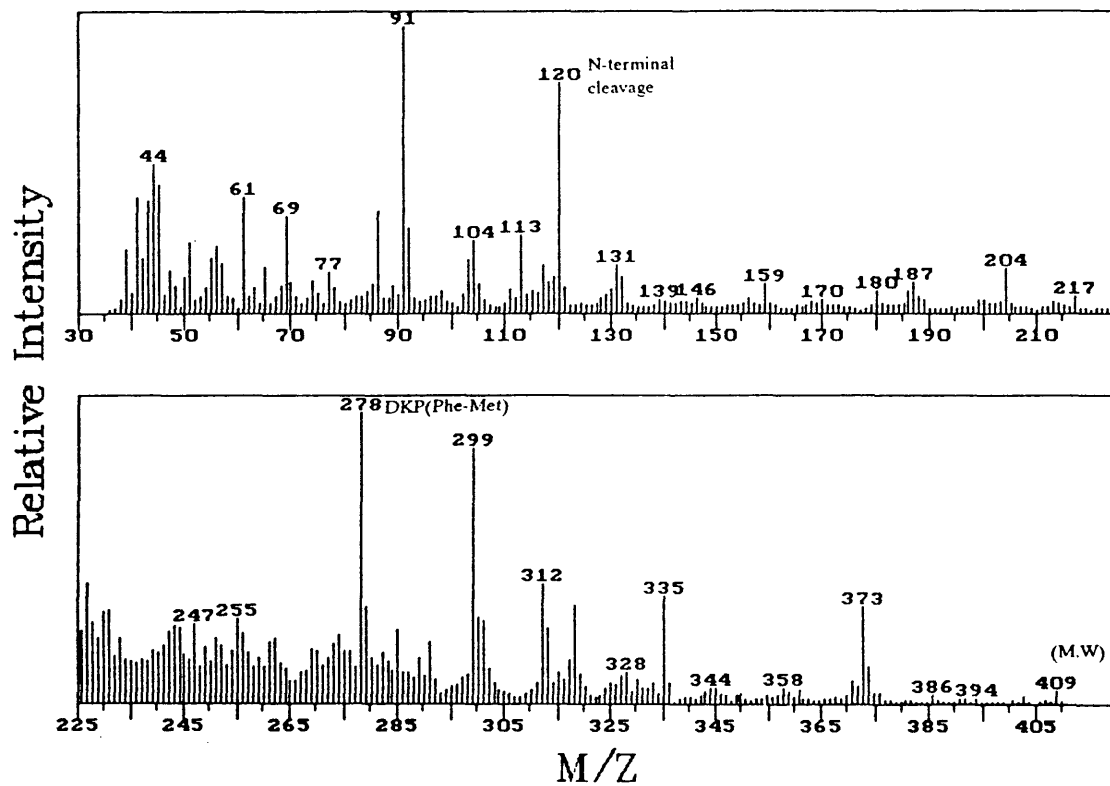


Figure 32. Pyrolysis-mass spectrum of phenylalanyl-methionyl-leucine

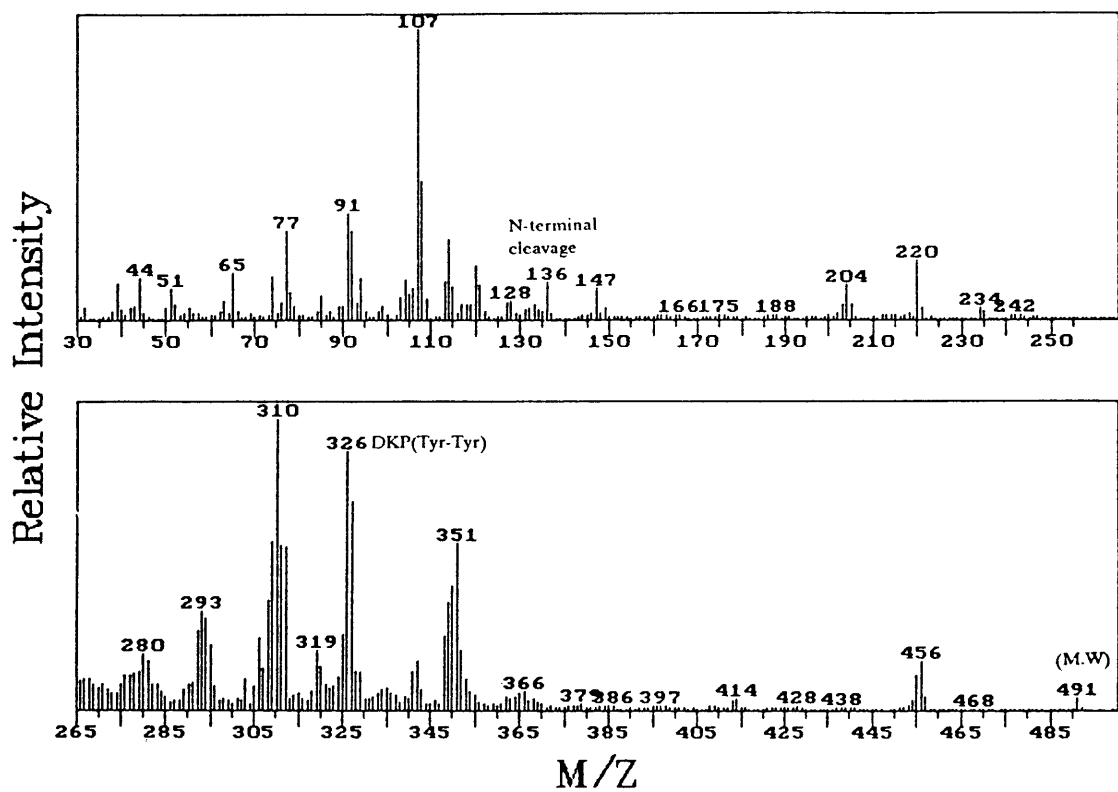


Figure 33. Pyrolysis-mass spectrum of tyrosyl-tyrosyl-phenylalanine

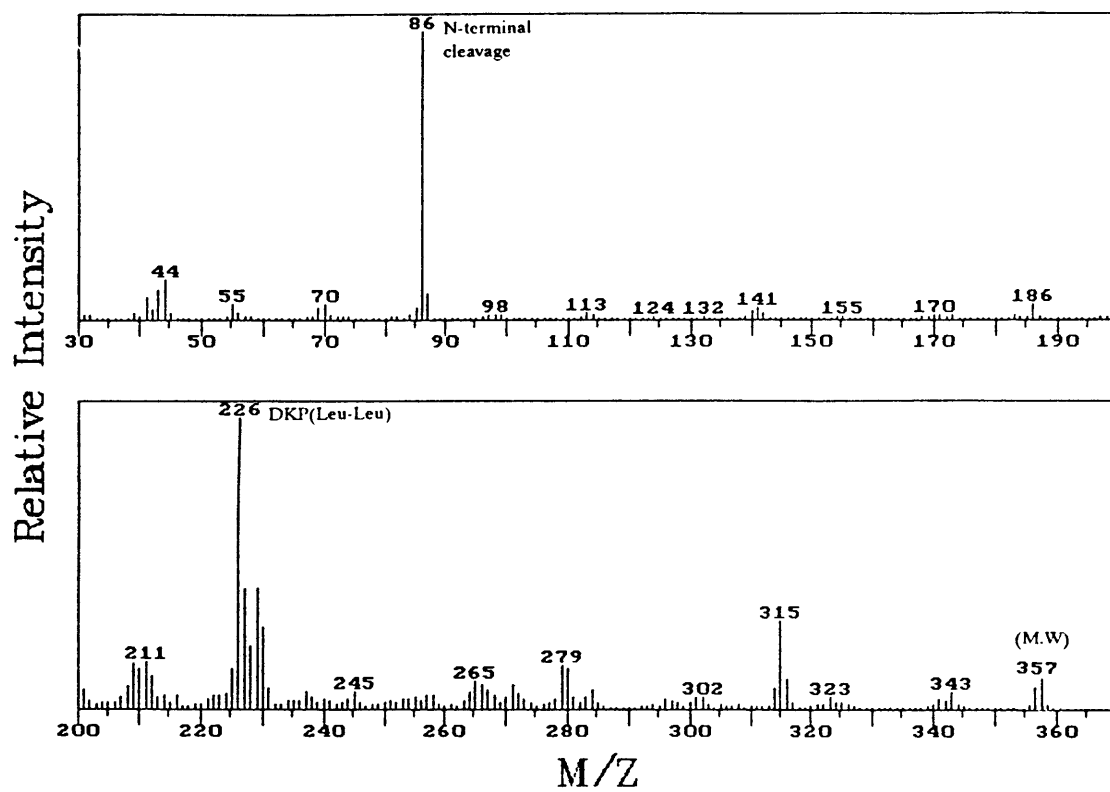
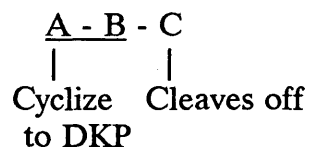


Figure 34. Pyrolysis-mass spectrum of leucyl-leucyl-leucine

So far, based on the thermal and EI fragmentation pattern:



Where A, B, and C are amino acids

a sequence analysis of tripeptides can be done successfully with the aid of three sets of commonly appearing peaks. One set of peaks corresponds to the tripeptide molecular ion and its fragmentation to yield the prominent iminium ion peak. The second set is due to the DKP of the N-terminal pair of amino acid residues, and its fragmentation. The third set is due to the C-terminal amino acid residue, which is eliminated in the cyclization process of the tripeptide to form DKP. The first set of peaks corresponding to the N-terminal cleavage gives the identity of the N-terminal amino acid residue of the tripeptide. The set of peaks observed from the DKP reveals the appearance of the first two sets of amino acid residues including the N-terminus. The third set is used to identify the C-terminal amino acid residue of the tripeptide. The sequence of the tripeptide can be correctly determined by analyzing these three sets of peaks.

### Tetrapeptides

#### Alanyl-Phenylalanyl-Leucyl-Methionine

Inspection of the complete pyrolysis-mass spectra of ALA-PHE-LEU-MET (Figure 35) and tripeptides PHE-LEU-MET and MET-LEU-PHE reveals that some of the fragmentations observed are different. The appearance of two series of peaks at  $m/z$  244, 183, 170, 155, 127, 113, 85 and  $m/z$  218, 194, 166, 127, 99, 91, 71, provides important information for the fragmentation pathway of ALA-PHE-LEU-MET, Figure 36. The first series of peaks can be assigned to the DKP of MET-LEU or LEU-MET and its fragments. Noguerola studied the dipeptides MET-LEU and LEU-MET, and he assumed that the two dipeptides underwent identical fragmentation pathways. The second series of peaks can be correlated with the DKP of ALA-PHE and its fragments, Figure 37.

The daughter ion spectra of DKP of ALA-PHE ( $m/z$  218), Figures 38-41, together with the parent ion spectra of  $m/z$  91, 127, Figures 42 and 43, can be used to understand the fragmentation mechanism of the DKP of ALA-PHE. The parent ion spectrum of  $m/z$  91 has a prominent peak at  $m/z$  218. This indicates the direct loss of an ionic benzyl from the phenylalanine residue. The spectrum of parent ions of  $m/z$  127 exhibits an intense peak at  $m/z$  218 as well as a smaller peak at  $m/z$  244, accompanied by other peaks at  $m/z$  183, 170, 155, which are known to be the fragments of the DKP of LEU-MET. The parent ion spectrum

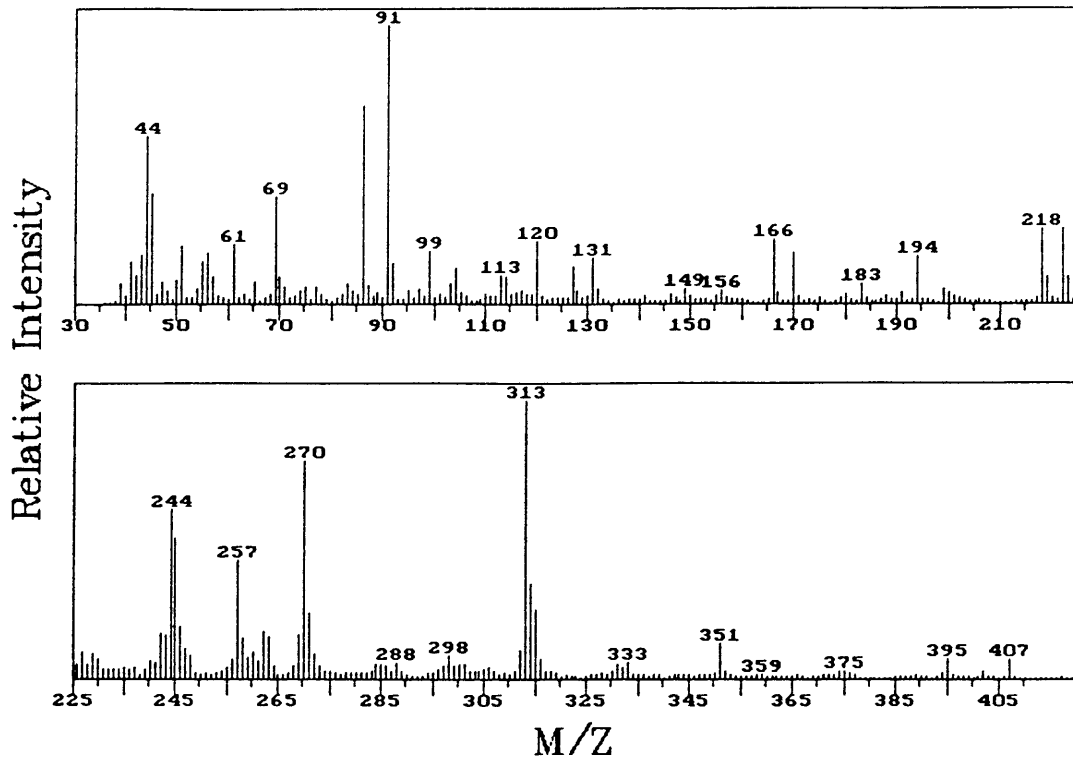


Figure 35. Pyrolysis-mass spectrum of alanyl-phenylalanyl-leucyl-methionine

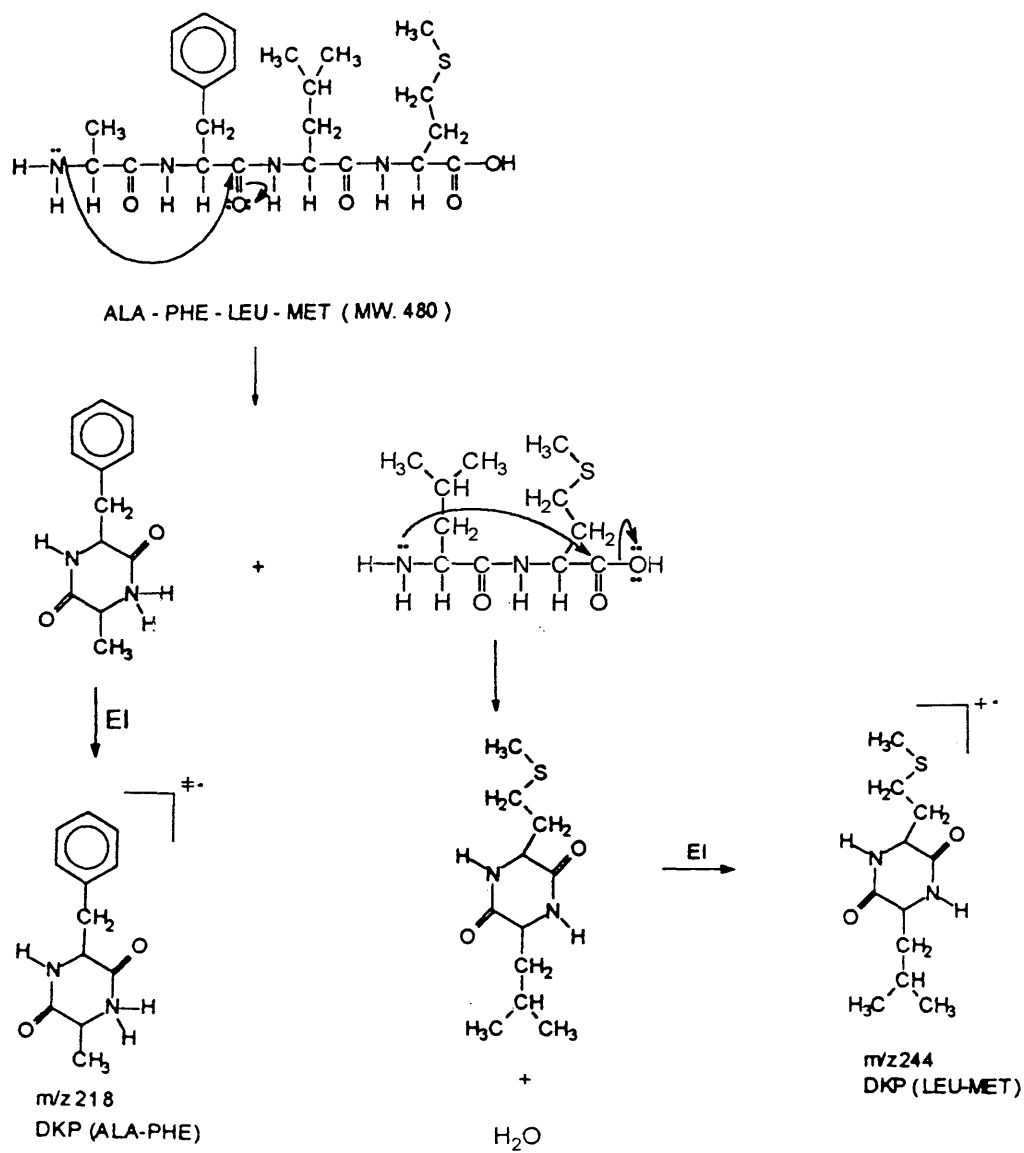


Figure 36. Fragmentation pathway of the DKP consecutive cyclization of alanyl-phenylalanyl-leucyl-methionine

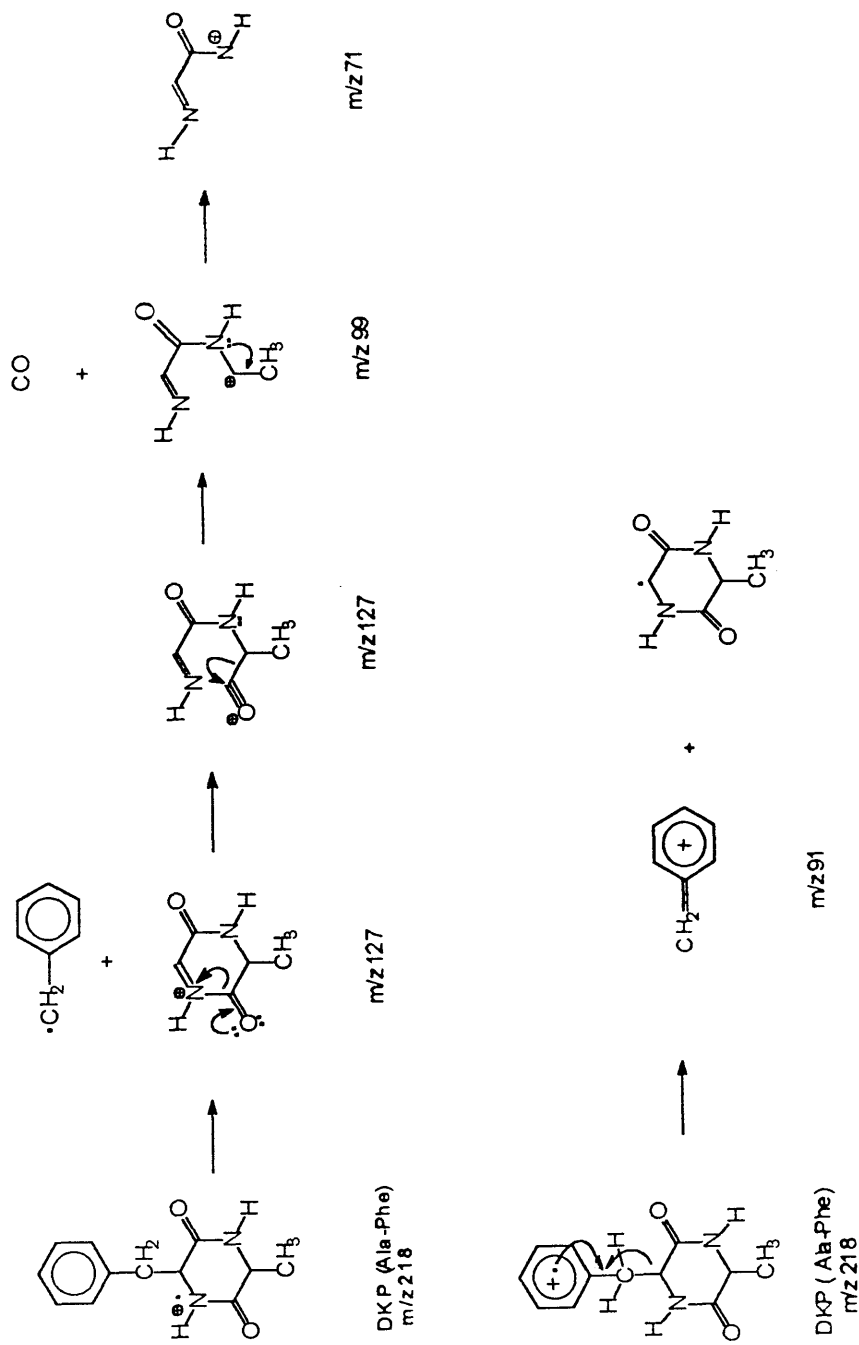


Figure 37. Fragmentation pathway for the DKP of alanyl-phenylalanine

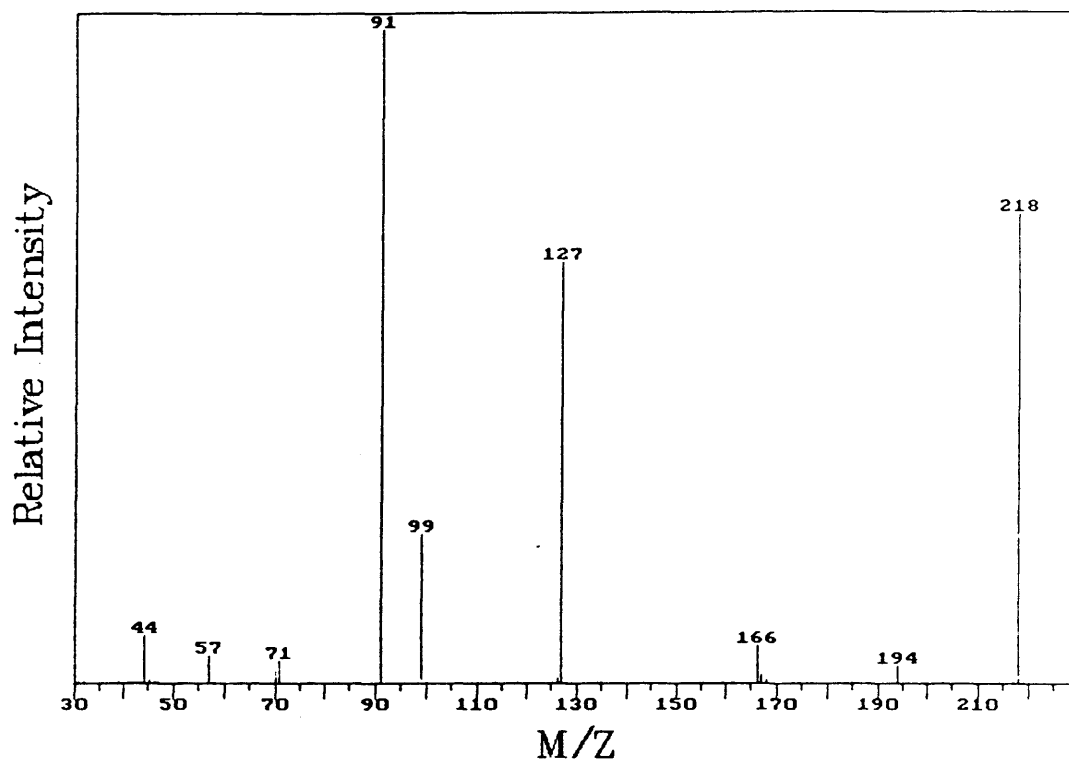


Figure 38. Pyrolysis-mass spectrum of daughter ions of m/z 218 of the DKP of alanyl-phenylalanine

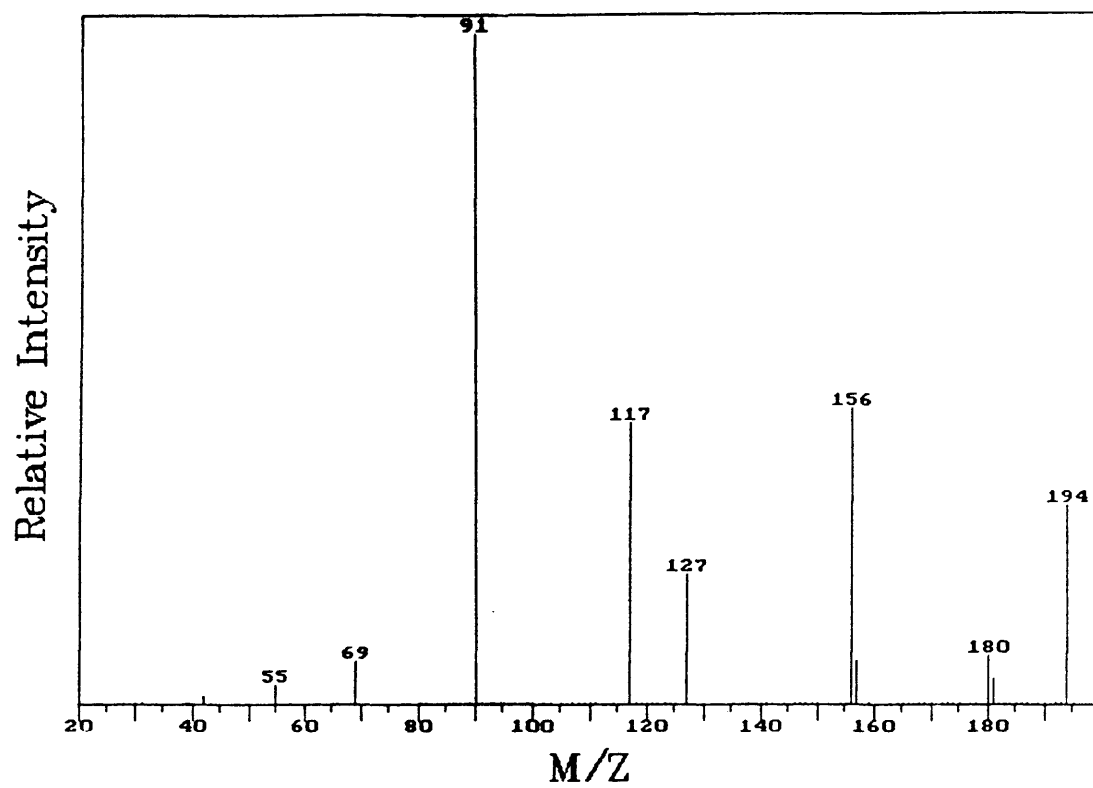


Figure 39. Pyrolysis-mass spectrum of daughter ions of m/z 194 of the DKP of ALA-PHE

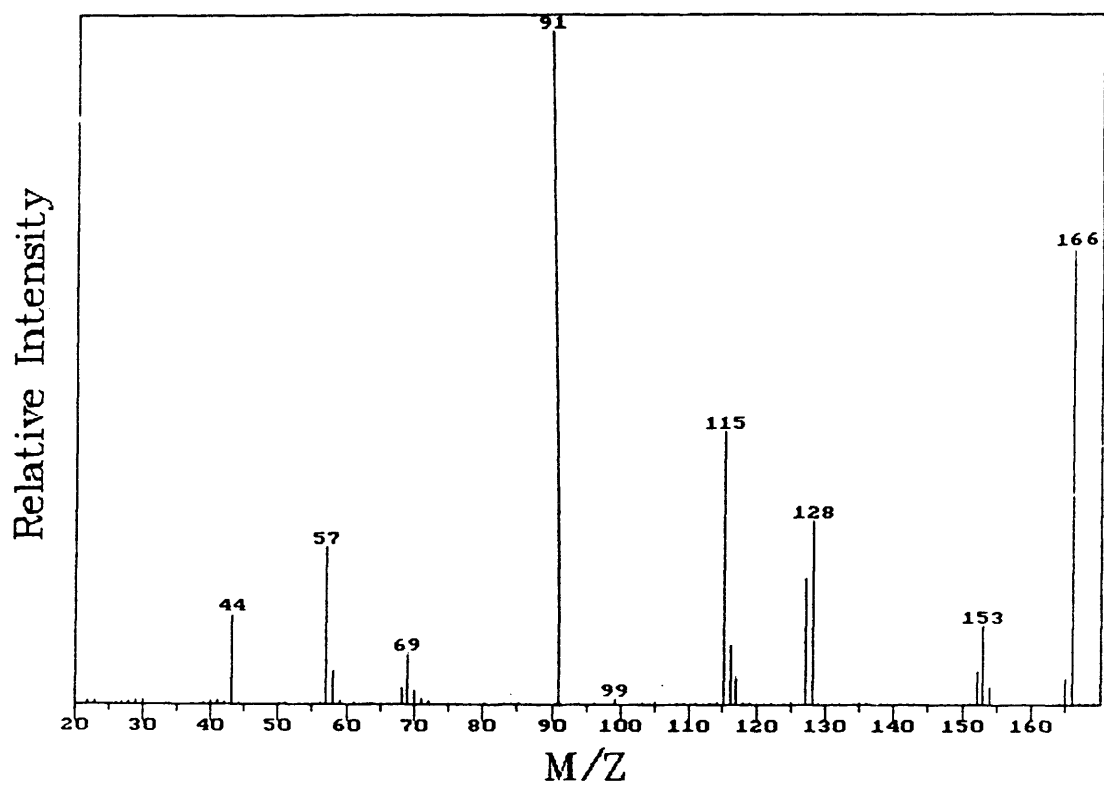


Figure 40. Pyrolysis-mass spectrum of daughter ions of m/z 166 of the DKP of ALA-PHE

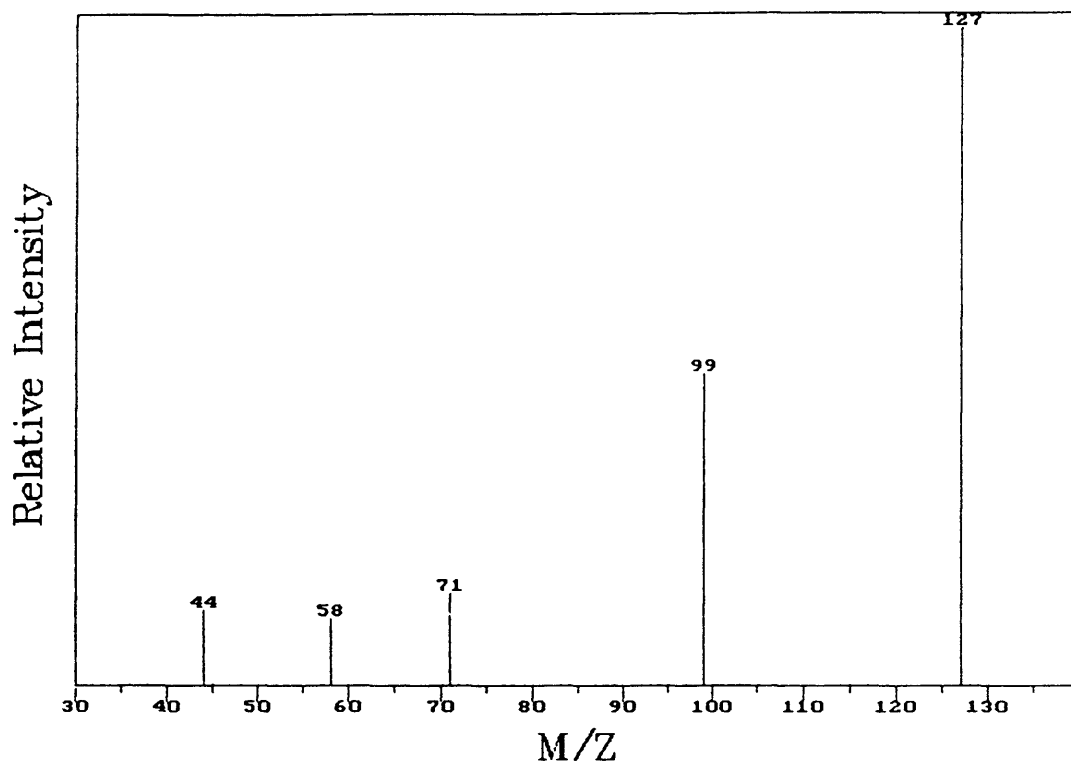


Figure 41. Pyrolysis-mass spectrum of daughter ions of m/z 127 of the DKP of ALA-PHE

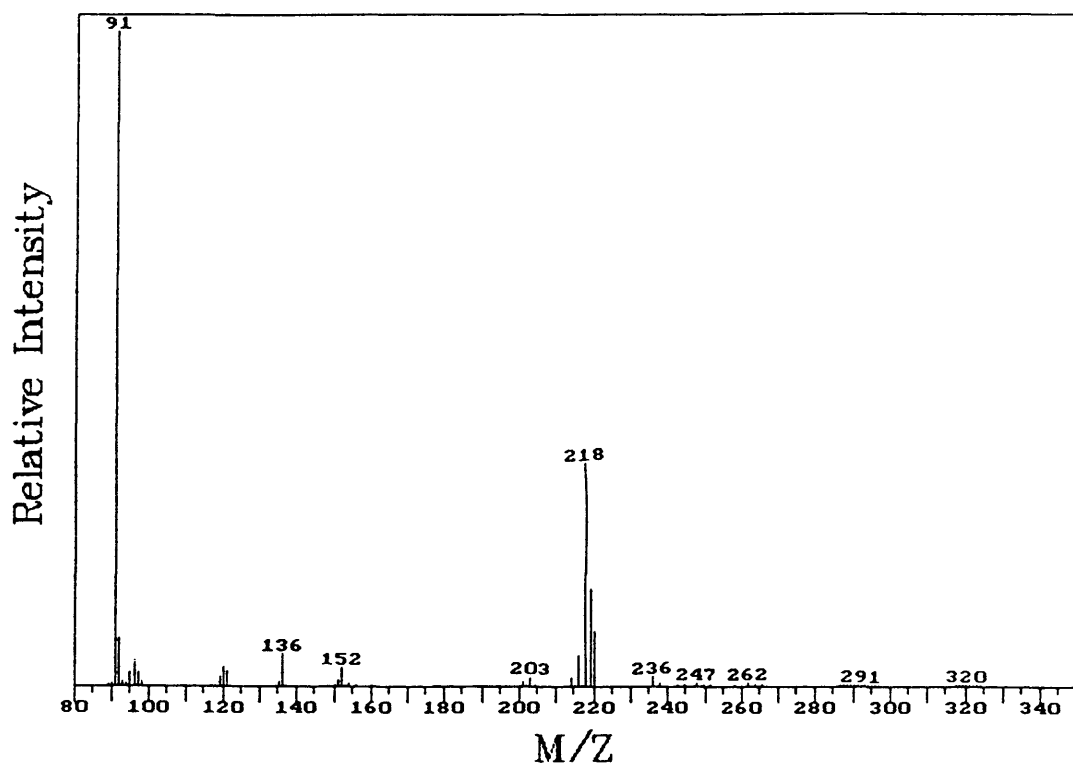


Figure 42. Pyrolysis-mass spectrum of parent ions of m/z 91 of alanyl-phenylalanyl-leucyl-methionine

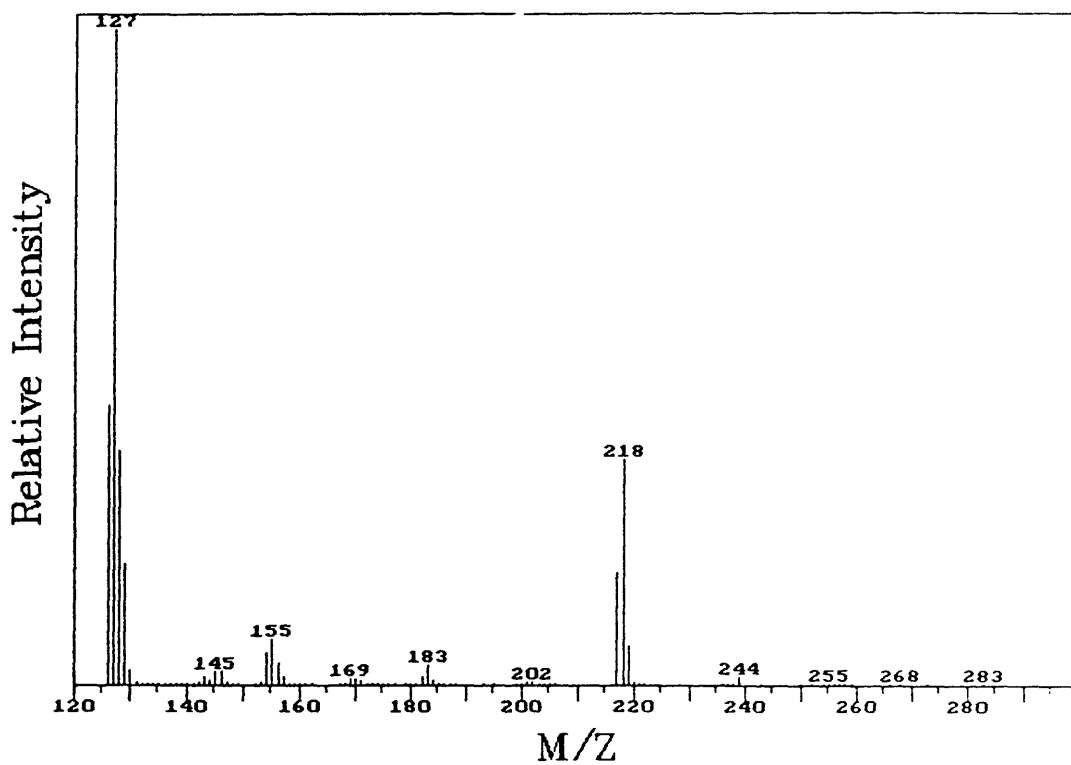


Figure 43. Pyrolysis-mass spectrum of parent ions of m/z 127 of alanyl-phenylalanyl-leucyl-methionine

of  $m/z$  127 shows the DKP of ALA-PHE to be a significant source of this peak, relative to the DKP of LEU-MET. The absence of the peak at  $m/z$  203 in the daughter ion spectrum of  $m/z$  218 (Figure 38), which corresponding to a loss of a radical methyl group from the DKP of ALA-PHE confirms Noguerola's hypothesis (6), that a methyl group from the alanine residue is always retained on the DKP.

The ALA-PHE-LEU-MET pyrolysis-mass spectrum yields an intensive peak at  $m/z$  44. It is not certain whether this peak comes from the N-terminal cleavage of alanine residue or from  $\text{CO}_2$ . Several attempts were made to obtain the molecular ion peak ( $m/z$  480), assuming that this peak is the most important peak in the spectrum. No  $m/z$  480 was observed. The obvious remedy in such cases is to run the spectrum at maximum sensitivity and to use a larger sample. However, the molecular ion peak did not appear. Probably, the tetrapeptides molecular ion could not be detected because tetrapeptides are not of sufficient volatility to be analyzed directly without extensive thermal degradation. Without the appearance of the molecular ion peak, i.e, a precursor of N-terminal cleavage, the peak at  $m/z$  44 is most likely caused by  $\text{CO}_2$  rather than N-terminal cleavage. Due to the limitation of the instrument, the N-terminal cleavage was not confirmed with the increasing of the molecular weight of the oligopeptides. No C-terminal amino acid from ALA-PHE-LEU-MET was detected, either.

### Alanyl-Methionyl-Leucyl-Phenylalanine

ALA-MET-LEU-PHE also exhibits a strong tendency to cyclize to form the DKPs of ALA-MET and LEU-PHE as ALA-PHE-LEU-MET did. The unique products in the ALA-MET-LEU-PHE spectrum, Figure 44, can be attributed to the change in the constituent amino acid sequence.

The series of peaks for the DKP of LEU-PHE,  $m/z$  260, 204, 169, 141, 113, 103, 91, 85 are analogous to those of PHE-LEU as discussed above. The fragmentation pattern of the DKP of ALA-MET ( $m/z$  202), supported by the daughter ions spectra in Figures 45-47, is the electron ionization that is mostly due to the cleavage of side groups by six-membered EI-induced rearrangement. For instance, a peak at  $m/z$  128 corresponds to the elimination of the ethenyl methyl sulfide group, 74u. Another peak at  $m/z$  187 is identified as the loss of a methyl radical from the alanine residue in the DKP of ALA-MET, Figure 48. It is not readily apparent why the loss of methyl radical from the DKP of ALA-MET is more energetically favored than a loss from the DKP of ALA-PHE as discussed in tetrapeptide ALA-PHE-LEU-MET. Similarly, neither N-terminal cleavage or C-terminal amino acid in ALA-MET-LEU-PHE was detected.

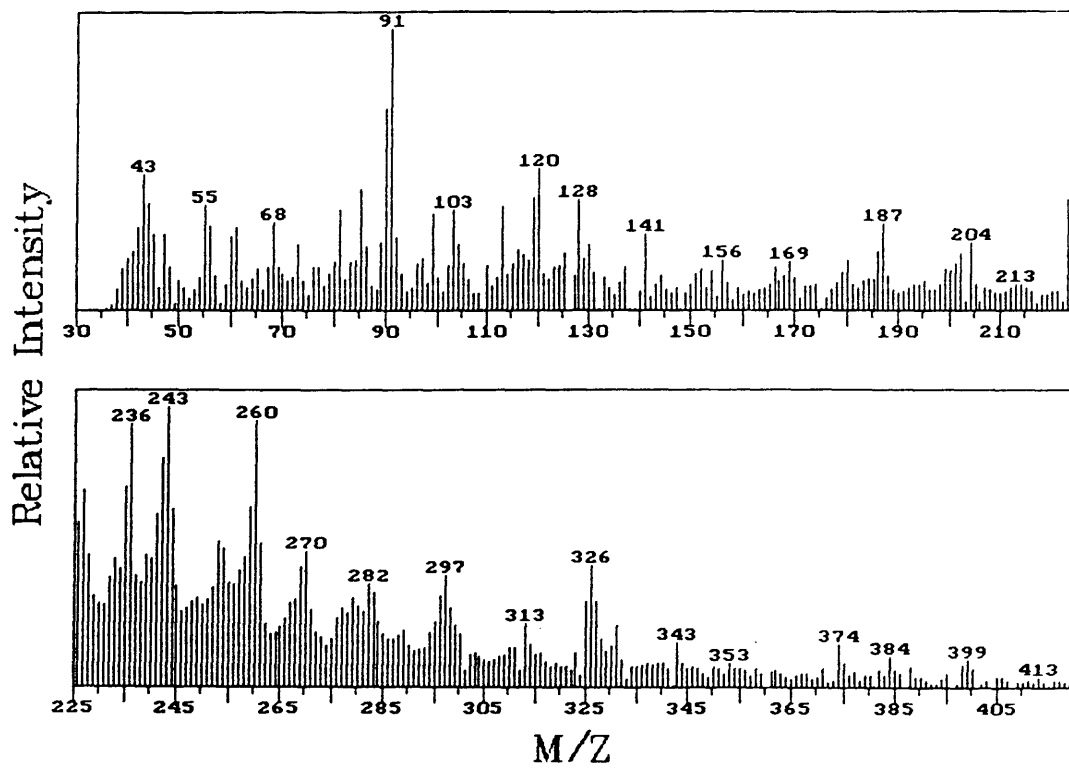


Figure 44. Pyrolysis-mass spectrum of alanyl-methionyl-leucyl-phenylalanine

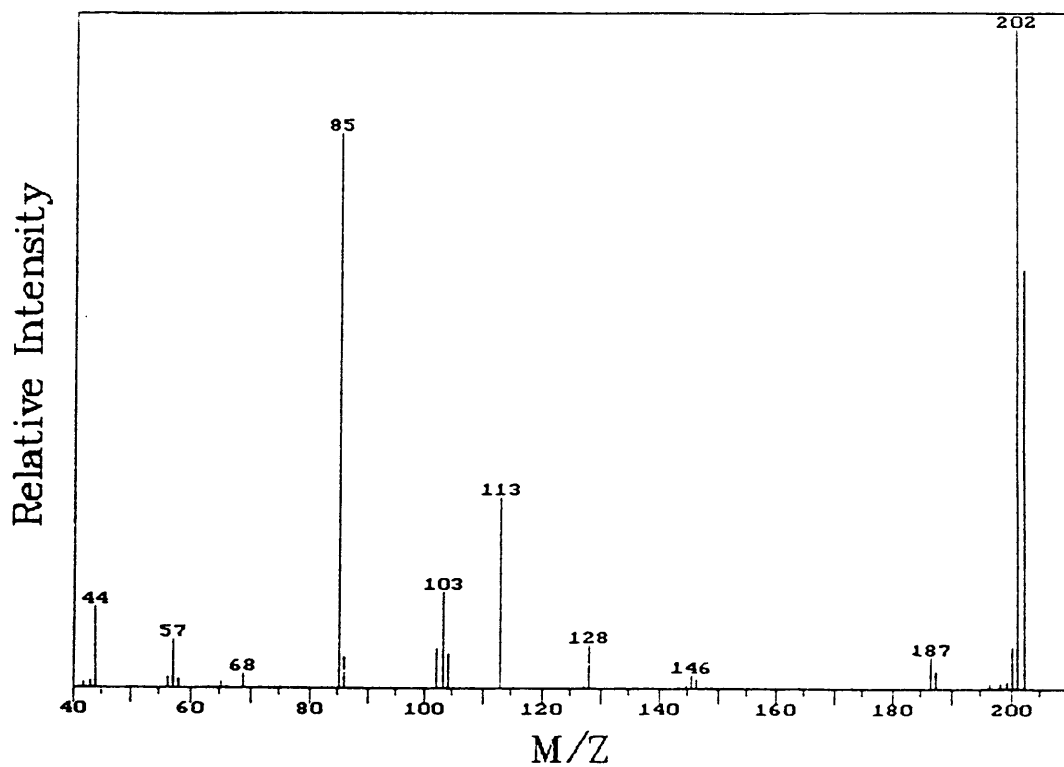


Figure 45. Pyrolysis-mass spectrum of daughter ions of m/z 202 of the DKP of alanyl-methionine

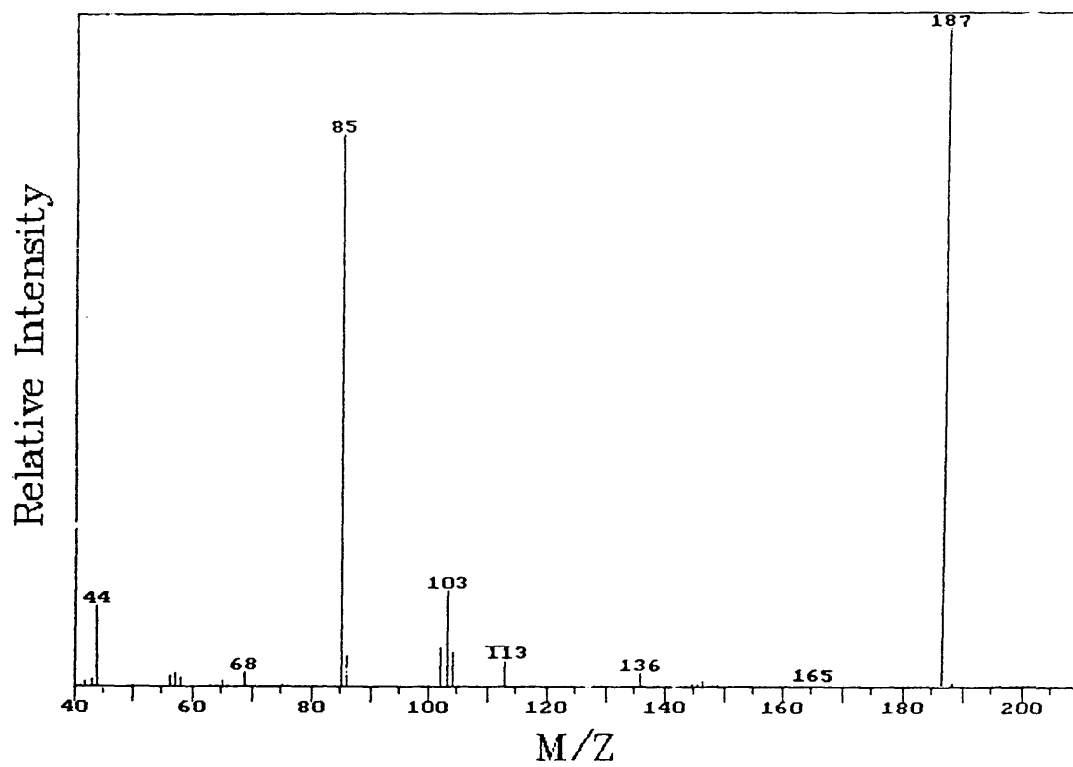


Figure 46. Pyrolysis-mass spectrum of daughter ions of m/z 187 of the DKP of ALA-MET

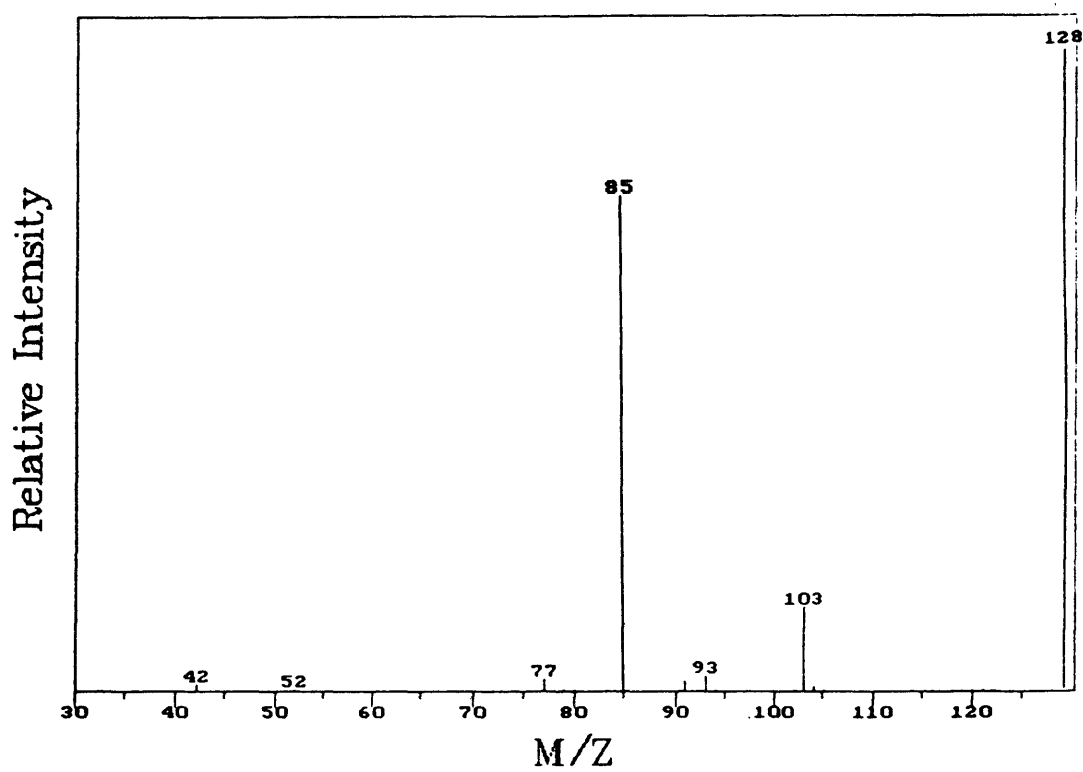


Figure 47. Pyrolysis-mass spectrum of daughter ions of  $m/z$  128 of the DKP of ALA-MET

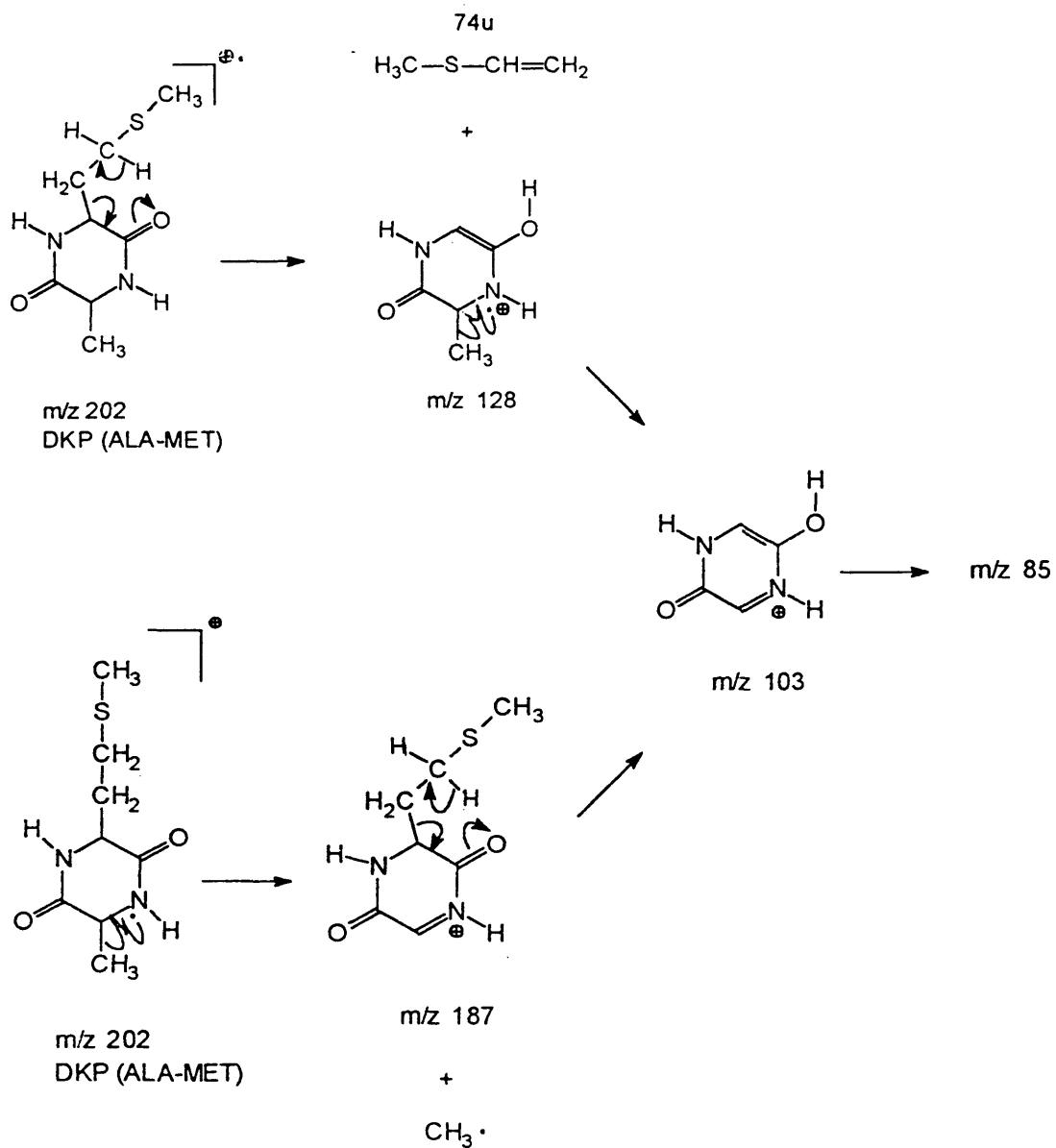


Figure 48. Fragmentation pathway for the DKP of ALA-MET

### Pentapeptides

#### Alanyl-Phenylalanyl-Leucyl-Methionyl-Tyrosine

A comparison of the pyrolysis mass spectrum of ALA-PHE-LEU-MET-TYR, Figure 49, with the corresponding spectrum of ALA-PHE-LEU-MET, Figure 35, shows them to be similar with only a few significant differences. One difference is the strong change in the relative intensities of  $m/z$  91 and  $m/z$  107. The base peak for ALA-PHE-LEU-MET,  $m/z$  91, is lower in ALA-PHE-LEU-MET-TYR, while the base peak for ALA-PHE-LEU-MET-TYR,  $m/z$  107, is very weak in ALA-PHE-LEU-MET. The other difference is the significant presence of  $m/z$  182, 136, 77 in the ALA-PHE-LEU-MET-TYR spectrum. These peaks correspond to the pure tyrosine amino acid fragments, Figure 50. Hence, the major differences between ALA-PHE-LEU-MET and ALA-PHE-LEU-MET-TYR can be attributed to the C-terminal amino acid tyrosine in the pentapeptides.

The peaks in the spectrum are consistent with the already discussed cyclization pathway, i.e., consecutive cyclization in tetrapeptides. In the case of the pentapeptides, the consecutive cyclization results in the formation of two DKPs with a free C-terminal amino acid. The series of peaks at  $m/z$  218, 194, 166, 127 and  $m/z$  244, 183, 170, 155, 127 are the DKP of ALA-PHE and the DKP of LEU-MET, respectively. The daughter ion experiments verify our interpretation

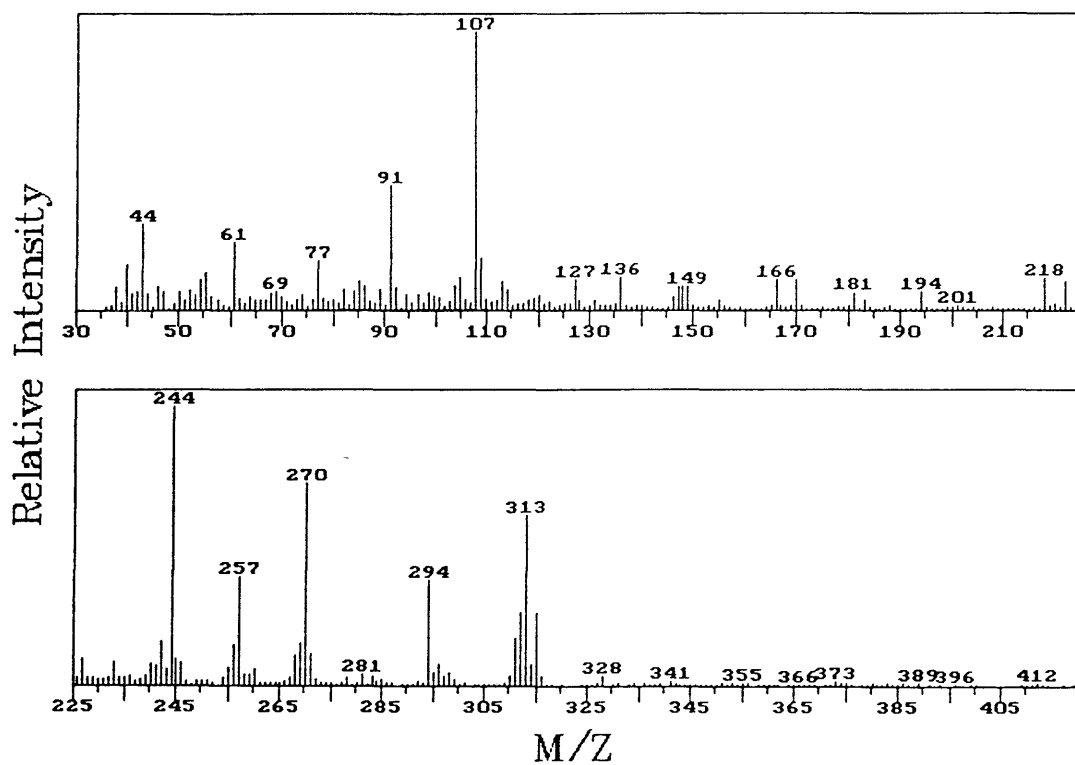


Figure 49. Pyrolysis-mass spectrum of alanyl-phenylalanyl-leucyl-methionyl-tyrosine

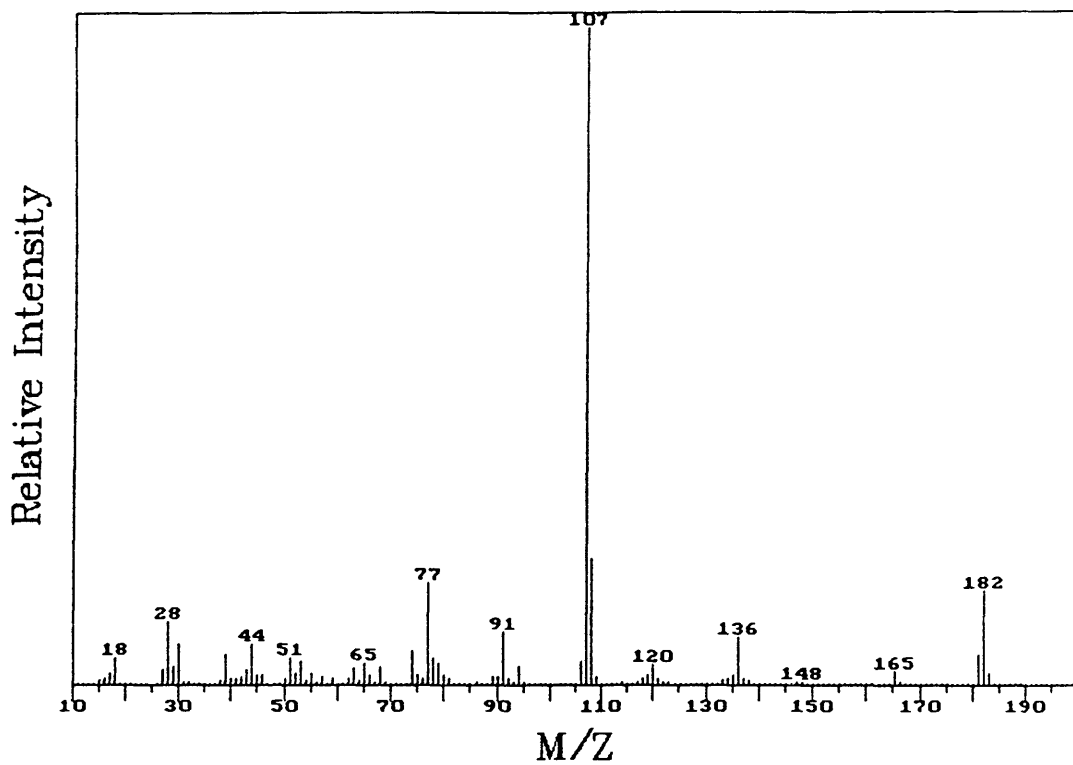


Figure 50. Pyrolysis-mass spectrum of tyrosine

of the fragmentation of the DKP of ALA-PHE. These daughter ion spectra are shown in the Section of the tetrapeptide of ALA-PHE-LEU-MET.

#### Alanyl-Tyrosyl-Leucyl-Methionyl-Phenylalanine

From the previously reported studies, it has already been clearly established that the even-numbered oligopeptides usually undergo the consecutive cyclization which form a number of pairs of DKPs. The odd-numbered oligopeptides undergoes the same cyclization plus the elimination of an amino acid from their C-terminus.

For ALA-TYR-LEU-MET-PHE, the formation of the DKP of ALA-TYR,  $m/z$  234, is accompanied by both the cleavage of a radical 4-methyl-phenol group from the tyrosine residue which leads to an ion of  $m/z$  128, and the formation of an ionic methyl phenol group which produces  $m/z$  107, Figure 51. The full mass spectrum, Figure 52, and the daughter ion spectrum of  $m/z$  234, Figure 53, strongly support this interpretation. A series of peaks for the DKP of LEU-MET at  $m/z$  244, 183, 170, 155, 127, 113 also stands out in the full mass-spectrum (Figure 52).

The peaks at  $m/z$  91 and  $m/z$  147 are significant for identifying the phenylalanine C-terminus which is cleaved from ALA-TYR-LEU-MET-PHE.

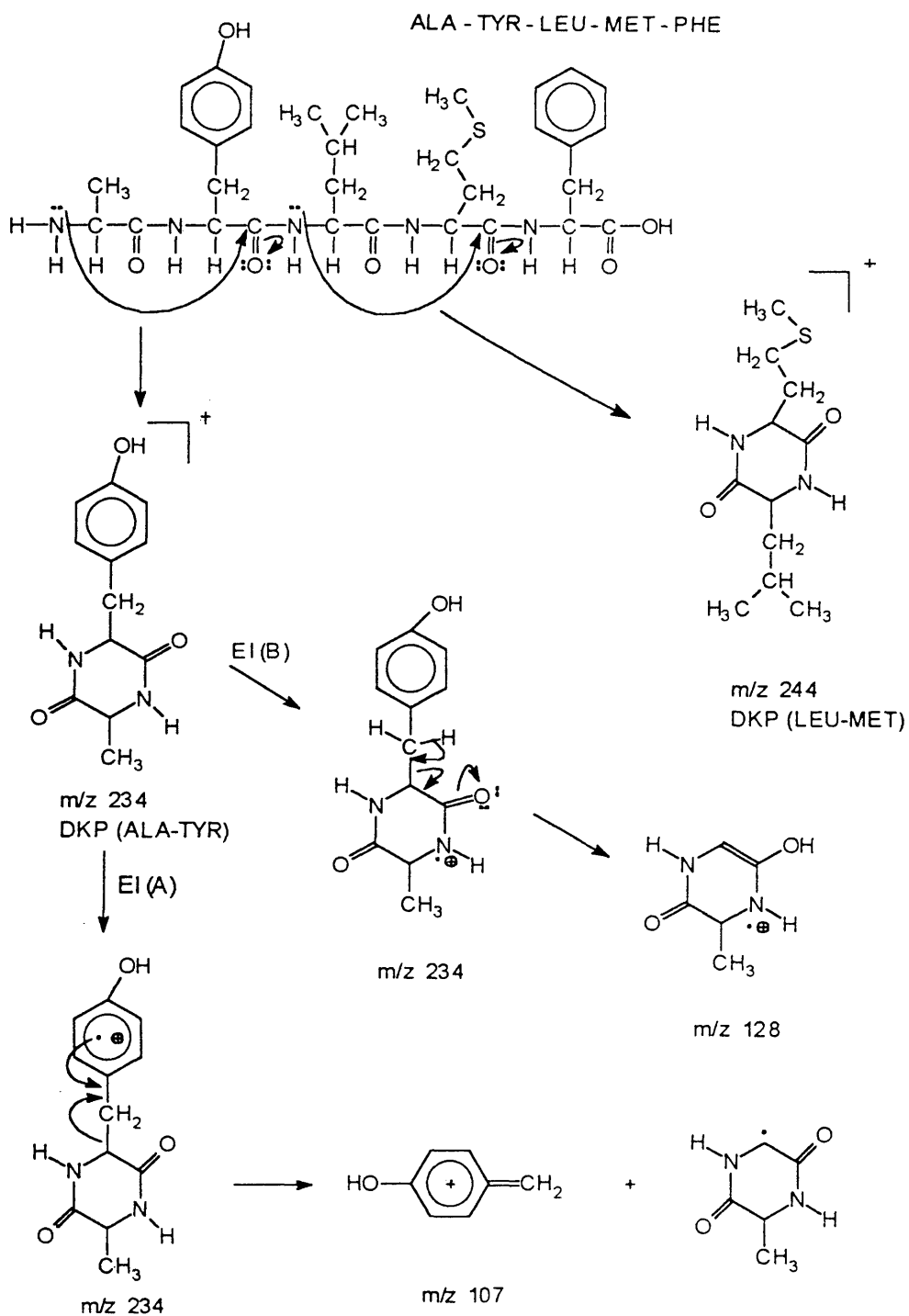


Figure 51. Fragmentation pathway of the DKP consecutive cyclization of alanyl-tyrosyl-leucyl-methionyl-phenylalanine

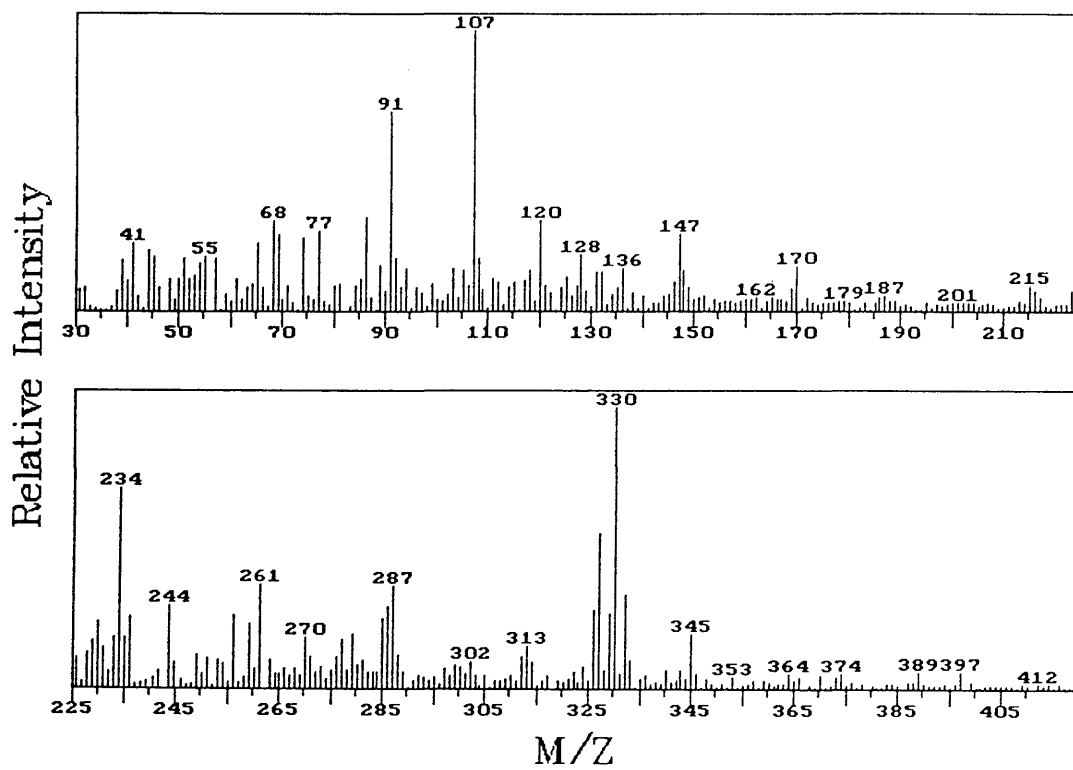


Figure 52. Pyrolysis-mass spectrum of alanyl-tyrosyl-leucyl-methionyl-phenylalanine

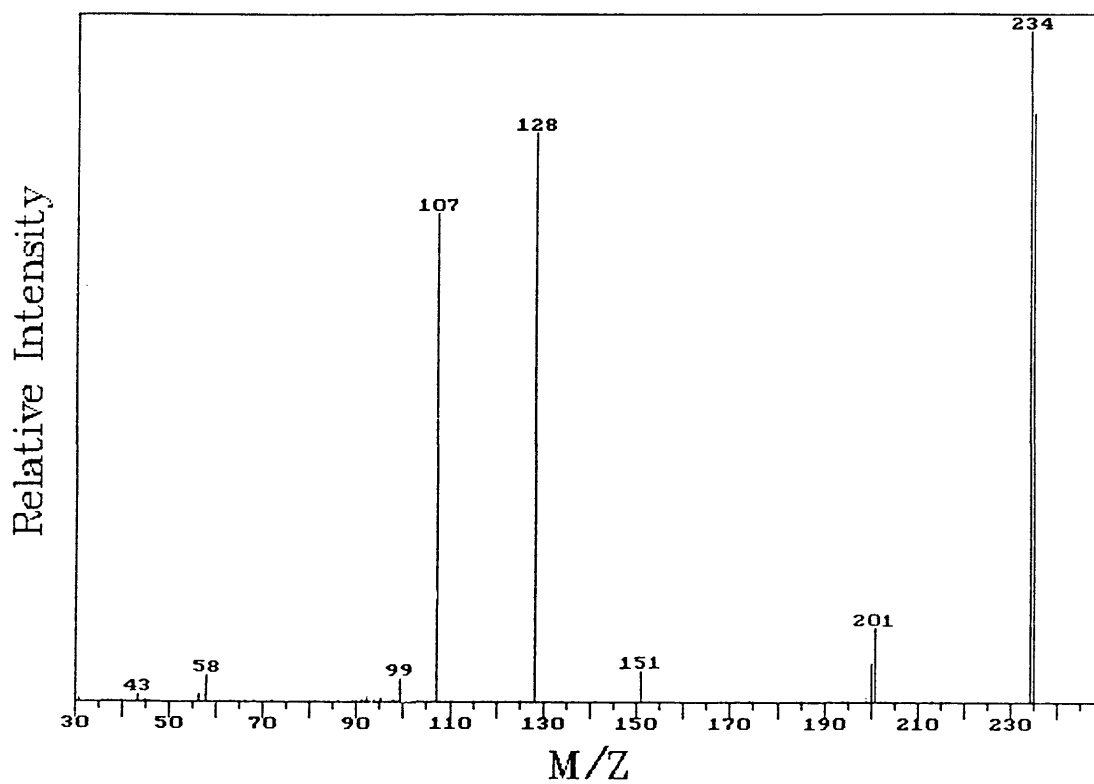
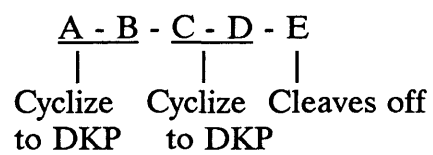


Figure 53. Pyrolysis-mass spectrum of daughter ions of  $m/z$  234 of the DKP of alanyl-tyrosine

No observation on the formation of any other DKPs implies that the fragmentation pattern of pentapeptides:



is the only thermal decomposition pathway.

### Hexapeptides

The hexapeptides should thermally fragment by consecutive cyclization to produce three DKP pairs, A - B - C - D - E - F. ALA-TYR-LEU-MET-PHE-PHE, ALA-PHE-LEU-MET-TYR-PHE, PHE-ALA-PHE-LEU-MET-TYR and TYR-TYR-TYR-TYR-TYR-TYR have been analyzed to test this hypothesis. Table 1 summarizes the major peaks corresponding to the related DKPs. The Py-MS spectra are shown in Figure 54-57.

In some cases, pyrolysis at a particular peptide bond produces mass spectral peaks with low intensities, making a sequence assignment at this point rather difficult. At times, these possible difficulties can be overcome by merely applying more sample and rerunning the mass spectrum. Lowering the Curie-point temperature from 510°C to 358°C seems to enhance the peak intensities of the DKPs in the mass spectra of the oligopeptides. The mass spectra of these oligopeptides are also much cleaner at 358°C. This could be attributed to other thermal degradation reactions of the oligopeptides that occurs at 510°C, which are suppressed to larger extent at lower temperature.

Hexapeptides	Diketopiperazine (DKP) (u)	Major Peaks (m/z)
Ala-Tyr-Leu-Met-Phe-Phe	Ala-Tyr (234u)	128, 107
	Leu-Met (244u)	183, 170, 155
	Phe-Phe (294u)	203, 112
Ala-Phe-Leu-Met-Tyr-Phe	Ala-Phe (218u)	127, 99, 91, 77
	Leu-Met (244u)	183, 170, 155
	Tyr-Phe (310u)	293, 204, 187, 91
Phe-Ala-Phe-Leu-Met-Tyr	Phe-Ala (218u)	128, 107, 91
	Phe-Leu (260u)	204, 187, 169, 141
	Met-Tyr (294u)	203, 188, 107
Tyr-Tyr-Tyr-Tyr-Tyr-Tyr	Tyr-Tyr (326u)	310, 220, 203, 107

Table 1. Major peaks in the mass spectra of several hexapeptides

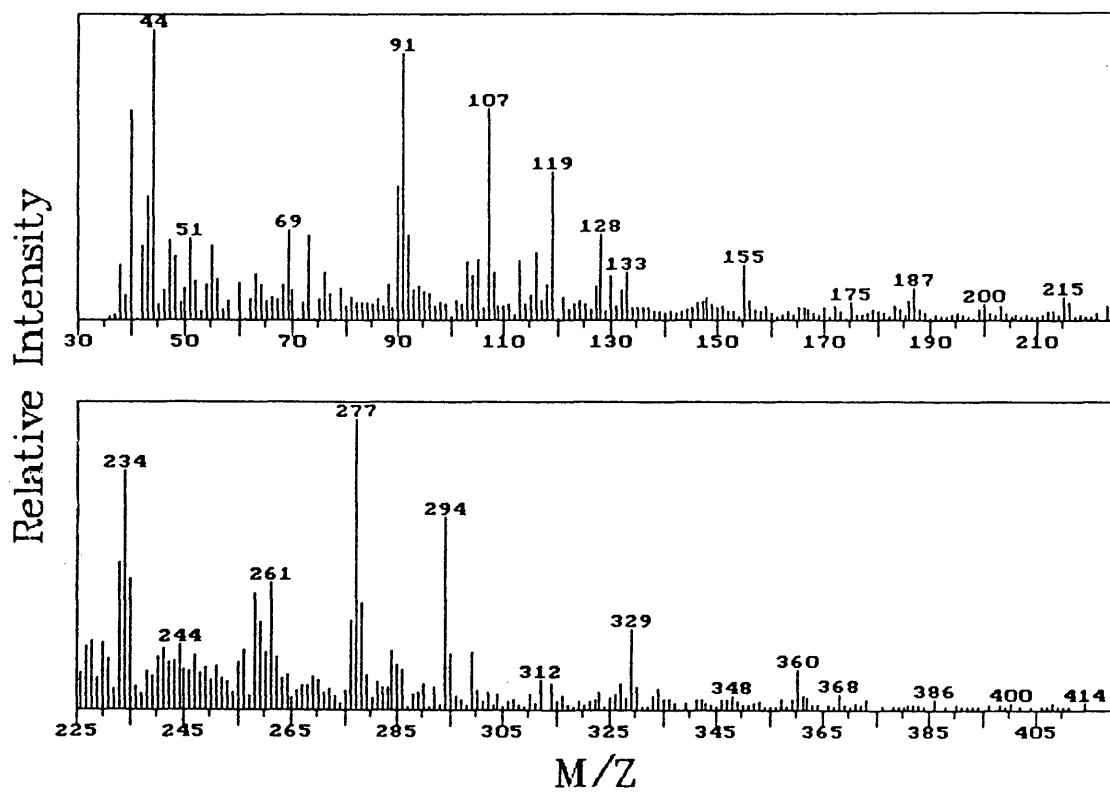


Figure 54. Pyrolysis-mass spectrum of ALA-TYR-LEU-MET-PHE-PHE

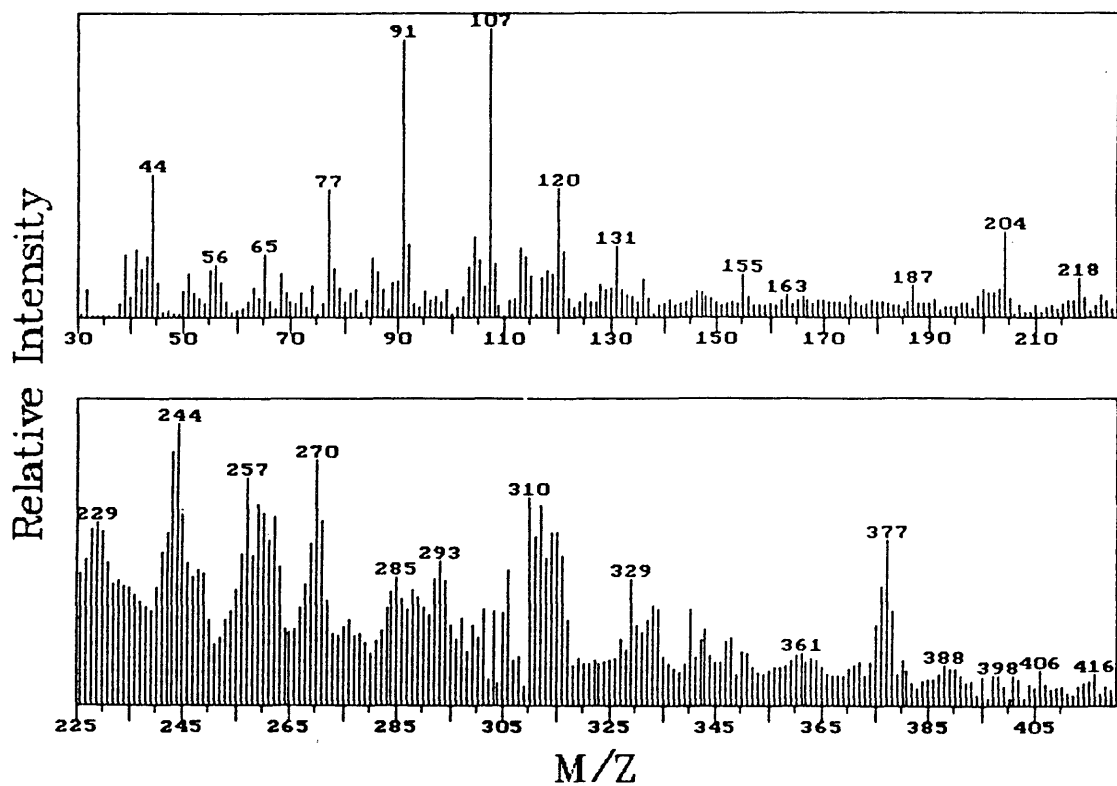


Figure 55. Pyrolysis-mass spectrum of ALA-PHE-LEU-MET-TYR-PHE

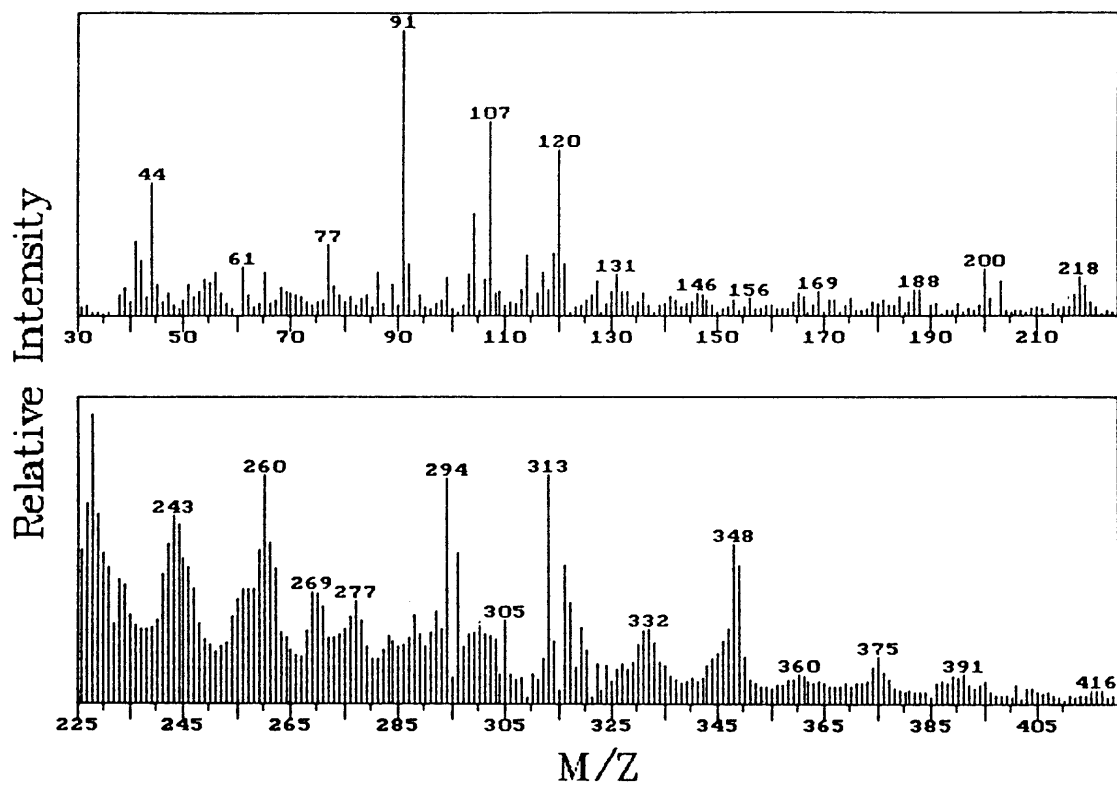


Figure 56. Pyrolysis-mass spectrum of PHE-ALA-PHE-LEU-MET-TYR

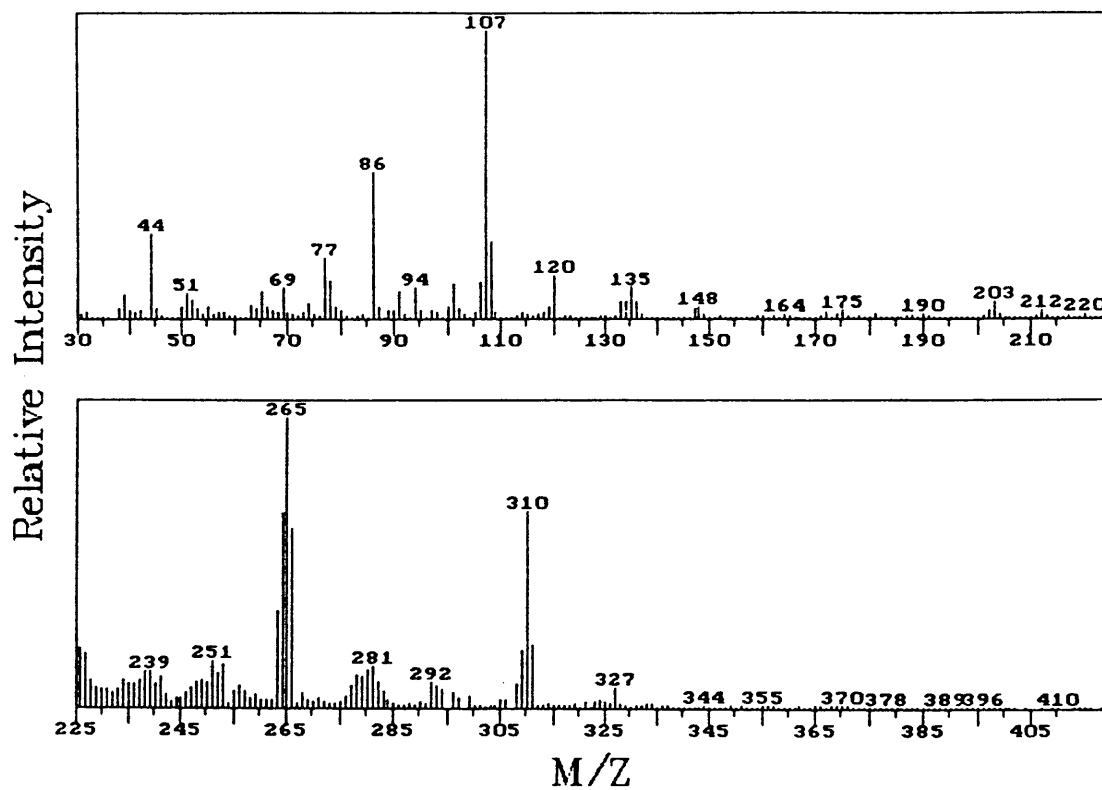


Figure 57. Pyrolysis-mass spectrum of TYR-TYR-TYR-TYR-TYR-TYR

### Other Oligopeptides

The oligopeptides including R-G-L-I-V-F-H-T-S, A-N-E-R-A-D-L-I-A-Y-L-K-Q-A-T-K and N-P-N-A-N-P-N-A-N-P-N-A-N-P-N-A have been examined. No peaks due to the formation of DKPs were observed. The mass-spectra of these oligopeptides are shown in Figure 58-60.

Inspection of the structure of these oligopeptides reveals that big side groups can be one of the important factors to hinder the consecutive cyclization. For example, the amino acid arginine (R) has a long side group. In the oligopeptides where arginine becomes one of the amino acid residues, instead of the formation of DKPs, this side group reaction might occur first. The other amino acid residues, such as histidine (H) and proline (P), have cyclic side groups. These are also considered to hinder the formation of DKPs. The investigation of larger peptides should be the emphasis of a future study.

A series of derivatized oligopeptides have also been examined. All of these peptides were derived from the trifluoroacetyl (TFA) on the N-terminus. As a result, the electron pair on the N-terminus was strongly delocalized which resulted in less possibility of attacking the carbonyl of the amino acid to form a DKP. No peaks corresponding to any DKPs were observed in the spectra which helped confirm that a pair of undelocalized electrons on the N-terminus of the oligopeptides is the basic requirement of the formation of a DKP.

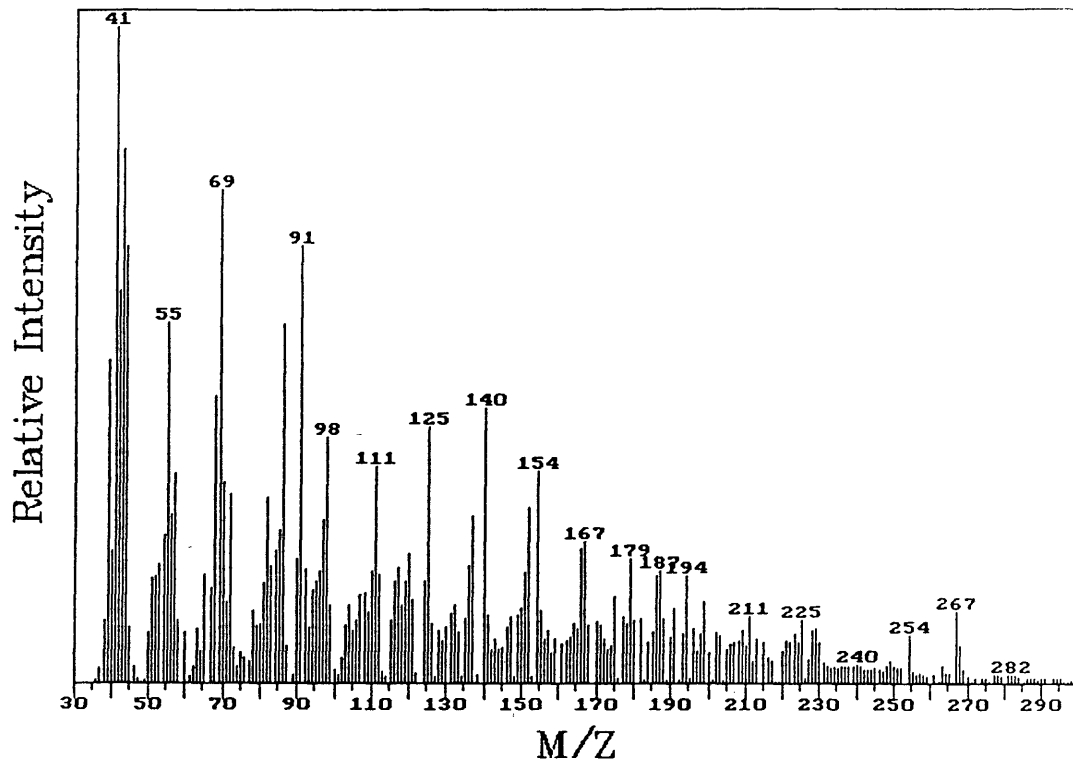


Figure 58. Pyrolysis-mass spectrum of R-G-L-I-V-F-H-T-S

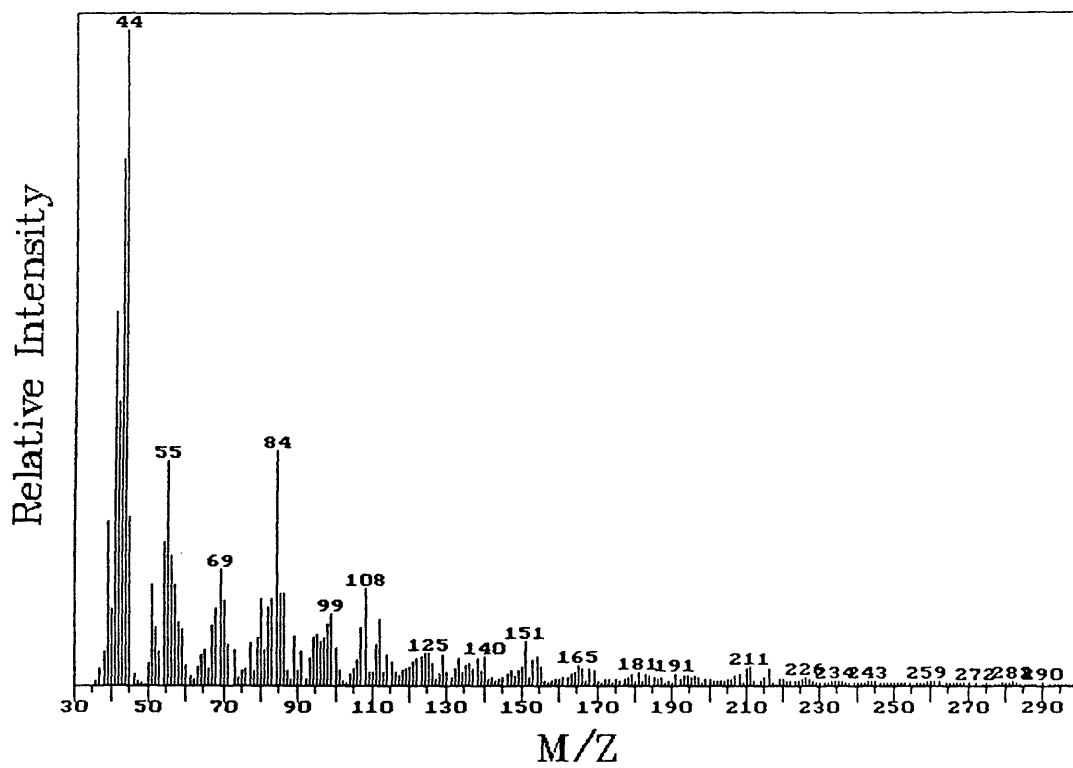


Figure 59. Pyrolysis-mass spectrum of A-N-E-R-A-D-L-I-A-Y-L-K-Q-A-T-K

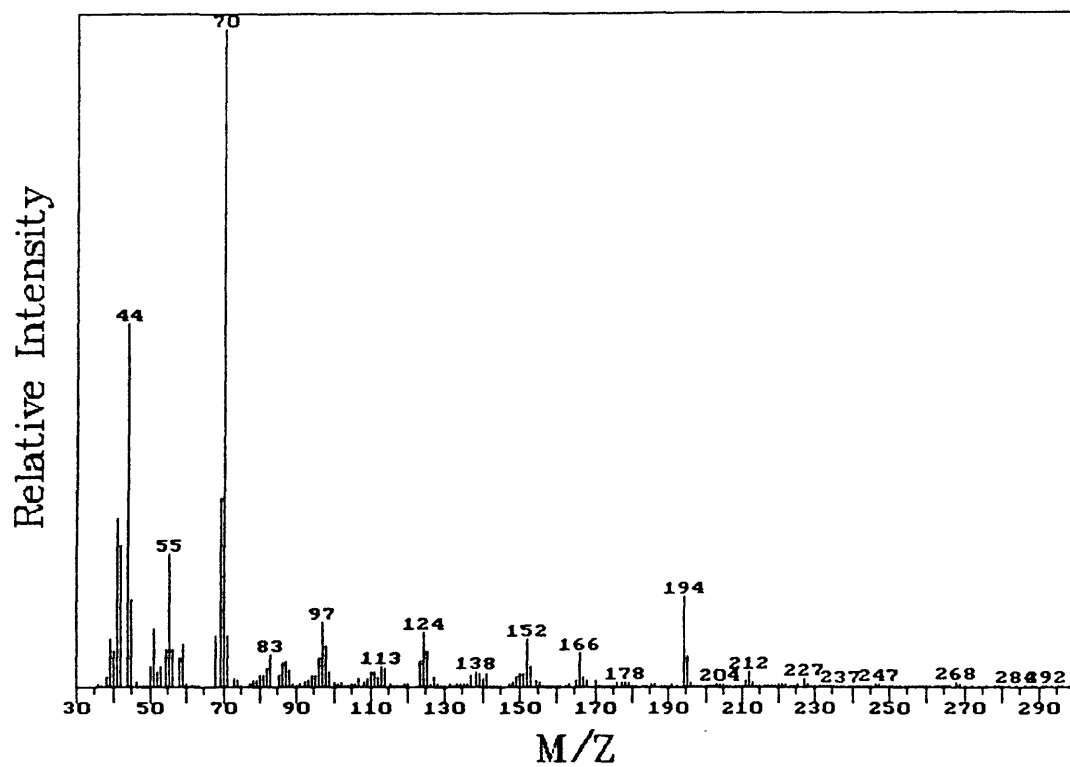


Figure 60. Pyrolysis-mass spectrum of N-P-N-A-N-P-N-A-N-P-N-A-N-P-N-A

## CONCLUSIONS

As is evident from the proceeding discussions, three levels of general electron ionization and thermal fragmentation pathways of underivatized linear oligopeptides can be clearly established. First of all, cyclization which form 2,5-diketopiperazines (DKPs) takes place readily under pyrolytic conditions. Although the formation of DKPs has been reported by others (6,52,53,54), the occurrence was limited exclusively on the two amino acids residing on the N-terminal end of the oligopeptides, while, the rest of the amino acid moieties were eliminated. Our experiments have revealed that the formation of DKPs could also happen as the continuous depolymerization of the oligopeptides. As a result, the DKP molecular ion together with its fragmentation ions would strongly serve to identify the peptide moieties when present in complex systems.

The second decomposition mode involves N-terminal cleavage. However, with the increase of the molecular weight of the oligopeptides, the N-terminal cleavage does not detract significantly from most of the mass spectra with the exception of the di- and tri-peptides. This observation makes it possible for the sequence analysis in the di- and tri-peptides. Evidence from the product ion spectra of di- and tri-peptides was provided to support our understanding that the molecular ion of these peptides is the precursor to the N-terminal cleavage. In

this regard, N-terminal cleavage is a result of electron ionization. Finally, C-terminal elimination from odd-numbered oligopeptides to produce a free amino acid was observed in peptides containing three and five amino acids.

In summary, Curie-point tandem pyrolysis mass spectrometry is a valuable and unique tool for oligopeptide composition analysis. However, a particular MS method may be employed to solve only a particular sequencing problem, the combination of several MS methods, such as FAB, FD, ESMS and GC-MS should be able to solve various aspects of a peptide or protein structure determination.

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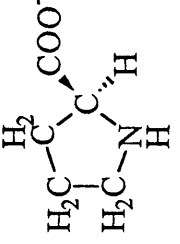
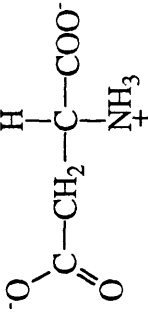
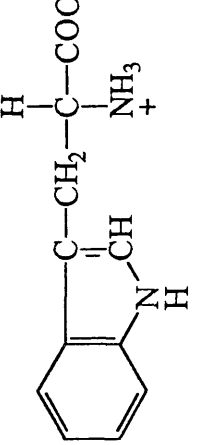


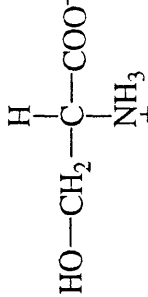
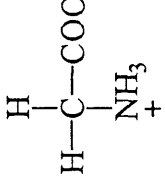
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**Appendix I**

Amino acids with nonpolar side groups. The three-letter and one-letter symbols and the molecular weights are also given.

Alanine Ala A MW 89	$\begin{array}{c} \text{H} \\   \\ \text{CH}_3-\text{C}-\text{COO}^- \\   \\ \text{NH}_3^+ \end{array}$	Phenylalanine Phe F MW 165	$\begin{array}{c} \text{H} \\   \\ \text{C}_6\text{H}_5-\text{CH}_2-\text{C}-\text{COO}^- \\   \\ \text{NH}_3^+ \end{array}$
Valine Val V MW 117	$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{C}-\text{CH}-\text{C}-\text{COO}^- \\   \quad   \\ \text{H}_3\text{C} \quad \text{NH}_3^+ \end{array}$	Tyrosine Tyr Y MW 181	$\begin{array}{c} \text{H} \\   \\ \text{HO}-\text{C}_6\text{H}_4-\text{CH}_2-\text{C}-\text{COO}^- \\   \\ \text{NH}_3^+ \end{array}$
Leucine Leu L MW 131	$\begin{array}{c} \text{H} \\   \\ \text{H}_3\text{C}-\text{CH}-\text{CH}_2-\text{C}-\text{COO}^- \\   \quad   \\ \text{H}_3\text{C} \quad \text{NH}_3^+ \end{array}$	Asparagine Asn N MW 132	$\begin{array}{c} \text{H} \\   \\ \text{H}_2\text{N}-\text{C}(=\text{O})-\text{CH}_2-\text{C}-\text{COO}^- \\   \\ \text{NH}_3^+ \end{array}$
Isoleucine Ile I MW 131	$\begin{array}{c} \text{H} \\   \\ \text{CH}_3-\text{CH}_2-\text{CH}-\text{C}-\text{COO}^- \\   \quad   \\ \text{CH}_3 \quad \text{NH}_3^+ \end{array}$	Glutamine Gln Q MW 146	$\begin{array}{c} \text{H} \\   \\ \text{H}_2\text{N}-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-\text{C}-\text{COO}^- \\   \\ \text{NH}_3^+ \end{array}$

<p>Proline Pro P MW 115</p> 	<p>Aspartic acid Asp D MW 133</p> 
<p>Tryptophan Trp W MW 204</p> 	<p>Glutamic acid Glu E MW 147</p> 
<p>Methionine Met M MW 149</p> 	<p>Serine Ser S MW 105</p> 
<p>Glycine Gly G MW 75</p> 	<p>Threonine Thr T MW 119</p> 