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Determination of Amino Acid Sequences in Oligopeptides by Mass Spectrometry. IV. Synthetic *N*-Acyl Oligopeptide Methyl Esters*

E. Bricas,† J. van Heijenoort,† M. Barber,‡
W. A. Wolstenholme,‡ B. C. Das,§ and E. Lederer§

ABSTRACT: A series of *N*-acylated di-, tri, tetra-, and octapeptide methyl esters has been prepared, the *N*-acyl groups used being acetyl, decanoyl, stearoyl, and an equimolecular mixture of heptadecanoyl and octadecanoyl. In all cases, a good molecular ion peak as well as intense peaks corresponding to the cleavage of the peptide bonds were obtained.

The basic fragmentation pattern of the peptide derivative is the same regardless of the *N*-acyl group, but the determination of amino acid sequences is easier with the *N*-stearoyl peptide esters and even more so with the peptide esters acylated with an equimolecular mixture of heptadecanoic and octadecanoic acids.

In the first paper of this series the structure of fortuitine, a natural eicosanoyl nonapeptide methyl ester of molecular weight 1359, was determined by mass spectrometry (Barber *et al.*, 1965a). In the second paper the structure proposed for peptidolipin NA, a natural macrocyclic *N*-acyl heptapeptide of molecular weight 963, was confirmed (Barber *et al.*, 1965b). The third paper describes the determination by mass spectrometry of the structure of a natural peptidolipid from *Mycobacterium johnei*. This compound is an *N*-acyl pentapeptide methyl ester of molecular weight 945 (Lanéelle *et al.*, 1965). In each of these three compounds, the principal fragmentation observed was that of the peptide bond, thus allowing an unambiguous determination of the sequence of amino acids in these peptide derivatives.

The principal difference with respect to previous mass spectrometric studies of oligopeptide derivatives (Stenhagen, 1961; Heyns and Grützmacher, 1963a,b; Weygand *et al.*, 1963) was the presence in these compounds of a long-chain N-terminal acyl group. We have, therefore, undertaken a study of a series of synthetic long-chain *N*-acyl di-, tri-, tetra-, and octapeptide methyl esters.

For comparing the effect of the length of the *N*-acyl group we have prepared *N*-acetyl, -decanoyl, -stearoyl, and mixed -heptadecanoyl and -stearoyl tetrapeptide methyl esters.

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† From the Institut de Biochimie, Faculté des Sciences Paris-Orsay, Orsay, France.
‡ From the Scientific Apparatus Department, Associated Electrical Industries, Manchester, England.
§ From the Institut de Chimie des Substances Naturelles, Centre National de la Réchèrche Scientifique, Gif-sur-Yvette, France.

Experimental Section

Preparation of N-Acyl Oligopeptide Methyl Esters. The *N*-acyl oligopeptide methyl esters were obtained by acylation of the corresponding peptide methyl esters. Their homogeneity was confirmed by elemental analysis, by determination of physical constants (melting point

TABLE 1: Physical Constants and Elemental Analyses of Peptide Derivatives.

No.	Compound[a]	Recrystn Solvent[b]	R_F[c]	Mp, °C	Rotation Value $[\alpha]_D$ (deg)	Rotation Value c, Solvent[b]	Formula	C (%) Calcd	C (%) Found	H (%) Calcd	H (%) Found	N (%) Calcd	N (%) Found
I	H-Gly-L-Leu-OCH_3, HCl	A	0.84	169.5	−34.8	0.9, J	$C_9H_{19}ClN_2O_3$	45.28	45.10	8.02	8.02	11.74	11.74
II	*N*-Stearoyl-Gly-L-Leu-OCH_3	B, C	0.56	78	+4.6	1, G	$C_{27}H_{52}N_2O_4$	69.19	69.18	11.18	11.60	5.98	6.06
III	*N*-Z-L-Val-Gly-L-Leu-OCH_3	B	0.3	134.5–135	−26.1	1.5, J	$C_{22}H_{33}N_3O_6$	60.67	60.36	7.64	7.68	9.65	9.66
IV	*N*-Acetyl-L-Val-Gly-L-Leu-OCH_3	B, D	0.4	172–173	−22.4	1, J	$C_{16}H_{24}N_3O_5$	55.95	55.39	8.51	8.41	12.24	12.32
V	*N*-Stearoyl-L-Val-Gly-L-Leu-OCH_3	E	0.3	140	−2.3	1, G	$C_{32}H_{61}N_3O_5$	67.68	67.46	10.83	10.80	7.40	7.47
VI	*N*-Z-L-Ala-L-Val-Gly-L-Leu-OCH_3	F	0.14	197–199	−47.8	0.7, J	$C_{25}H_{38}N_4O_7$	59.27	58.99	7.56	7.52	11.06	11.16
VII	*N*-Acetyl-L-Ala-L-Val-Gly-L-Leu-OCH_3	E	0.2	229	−78.1	0.8, J	$C_{19}H_{34}N_4O_6$	55.05	55.01	8.27	8.24	13.52	13.45
VIII	*N*-Decanoyl-L-Ala-L-Val-Gly-L-Leu-OCH_3	B, E	0.4	186–187	−8.7	1, G	$C_{27}H_{50}N_4O_6$	61 57	61.57	9.57	9.64	10.64	10.59
IX	*N*-Stearoyl-L-Ala-L-Val-Gly-L-Leu-OCH_3	C, G	0.2	187–188			$C_{35}H_{66}N_4O_6$	65.79	65.69	10.41	10.56	8.77	8.84
X	*N*-Heptadecanoyl- and *N*-octadecanoyl-L-Ala-L-Val-Gly-L-Leu-OCH_3	C, G	0.2	185–187			$C_{34}H_{64}N_4O_6$ $C_{35}H_{66}N_4O_6$	65.57	65.46	10.37	10.22	8.87	9.29
XI	*N*-Z-L-Ala-L-Val-Gly-Leu-L-Ala-L-Val-Gly-L-Leu-OCH_3	H	0.5	280–281 dec			$C_{41}H_{66}N_8O_{11}$	58.11	57.79	7.85	7.83	13.26	13.19
XII	*N*-Heptadecanoyl- + *N*-octadecanoyl-L-Ala-L-Val-Gly-Leu-L-Ala-L-Val-Gly-L-Leu-OCH_3	I	0.6	283–284 dec			$C_{50}H_{92}N_8O_{10}$ $C_{51}H_{94}N_8O_{10}$	62.46	62.45	9.66	9.90	11.48	11.36

[a] Z = benzyloxycarbonyl. [b] Solvents used for recrystallization and $[\alpha]_D$ measurements: A, methanol–ether; B, ethyl acetate–petroleum ether (bp 40–65°); C, absolute ethanol; D, acetonitrile–benzene; E, ethyl acetate; F, 95% ethanol; G, chloroform; H, DMF–methanol; I, chloroform–methanol; J, methanol. [c] Paper chromatography of run I in 1-butanol–pyridine–acetic acid–water (30:20:6:24). Silica gel thin layer chromatography (detection with morin) (Schellenberg, 1962) of II, V, and IX run in chloroform–methanol (96:4); VIII and XII run in chloroform–methanol (92:8); III and XI run in chloroform–acetone (80:20); IV and VII run in chloroform–acetone (50:50).

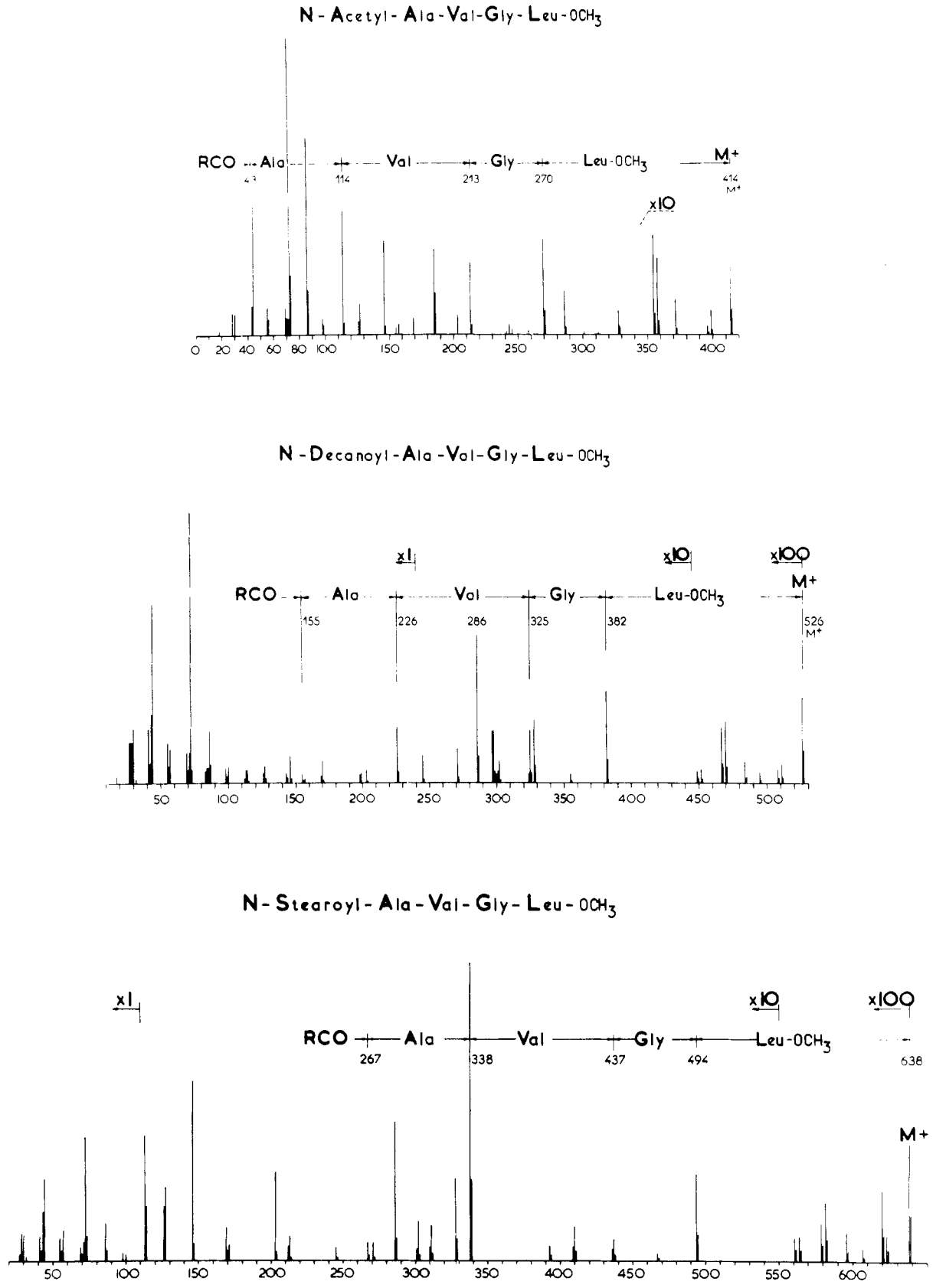


FIGURE 1: Line drawings of the mass spectra of *N*-acetyl (top), *N*-decanoyl- (middle), and *N*-stearoylalanylvalylglycylleucine (bottom) methyl esters.

and rotation), and by silica gel thin layer chromatography (see Table I).

Preparation of Methyl Esters. In general, oligopeptides are easily esterified by treating the free peptide with methanolic hydrogen chloride at 25° for a few hours (Leggett-Bailey, 1962). This technique was used for the preparation of the dipeptide methyl ester hydrochloride (I).

The tri-, tetra-, and octapeptide methyl ester hydrochlorides were prepared by catalytic hydrogenolysis (5% palladized carbon) of the corresponding *N*-benzyloxycarbonyl peptide methyl esters. The *N*-benzyloxycarbonyl tri- and tetrapeptide methyl esters (III and VI) had been obtained in excellent yields by coupling the corresponding *N*-benzyloxycarbonyl amino acid *p*-nitrophenyl ester with the di- or tripeptide methyl ester. The *N*-benzyloxycarbonyl octapeptide methyl ester (XI) had been prepared according to the mixed anhydride method by coupling the *N*-benzyloxycarbonyl derivative of the tetrapeptide Ala-Val-Gly-Leu with the methyl ester of the same tetrapeptide.

N-Acylation. The *N*-acetyl peptide methyl esters were prepared by treating the peptide methyl esters with acetic anhydride in pyridine or in a mixture of ethyl acetate and a saturated solution of sodium bicarbonate. The *N*-decanoyl, *N*-octadecanoyl, and mixed *N*-heptadecanoyl and *N*-octadecanoyl peptide methyl esters were prepared by the standard mixed anhydride method involving the condensation of a peptide methyl ester with the mixed anhydride of ethyl carbonate (Wieland and Bernhard, 1951; Boissonnas, 1951; Vaughan, 1951) and of the corresponding decanoic, octadecanoic, or equimolecular mixture of hepta- and octadecanoic acids. The purity of the decanoic, heptadecanoic, and octadecanoic acids had been checked by gas chromatographic analysis of their methyl esters.

The following example describes the stearoylation of a tetrapeptide methyl ester. A solution of 1 mmole of stearic acid and 1 mmole of triethylamine in 10 ml of tetrahydrofuran was cooled to −5° and 1 mmole of ethyl chlorocarbonate was added. After 20 min a solution of 1 mmole of L-alanyl-L-valylglycyl-L-leucine methyl ester hydrochloride and 0.14 ml of triethylamine in 20 ml of tetrahydrofuran was added and the mixture was stirred vigorously for 2 hr during which time it was allowed to warm to room temperature. After concentration to dryness *in vacuo* the residue was dissolved in chloroform.[1] The solution was washed successively with 1 N HCl and water; the product then precipitates in a high state of purity; over-all yield, 76%.

Mass Spectrometry. The instrument used for this work was the A.E.I. MS9 double-focusing mass spectrometer. The compounds (approximately 0.1 mg) were mounted on the end of a direct insertion probe which was then admitted to the ion chamber through a vacuum lock. The sample was evaporated by heating the ion chamber. Low-resolution spectra were recorded for each compound, and, in the case of the decanoyl derivative, accurate mass measurements were made of several of the peaks in the spectrum in order to determine unambiguously their atomic compositions.

[1] The corresponding decanoyl tetrapeptide ester is readily soluble in ethyl acetate and the washings are carried out in this solvent.

Results

In the three cases of natural peptidolipids reported earlier (Barber *et al.*, 1965a,b; Lanéelle *et al.*, 1965) the amino acid sequence could easily be determined, since peaks due to simple cleavage of the peptide bonds were clearly recognized. In the synthetic long-chain acyl derivatives we therefore anticipated that peaks due to this fragmentation would also be more prominent than in the acetyl peptides studied by other authors (Stenhagen, 1961; Heyns and Grützmacher, 1963a,b; Weygand *et al.*, 1963).

Acyl Derivatives of Di- and Tripeptide Methyl Esters. *N*-Stearoyl-Gly-Leu-OCH_3 and *N*-acetyl- and *N*-stearoyl-Val-Gly-Leu-OCH_3 were prepared (Table I). All of these give good molecular ion peaks as well as intense peaks corresponding to the cleavage of the peptide bonds.

Acyl Derivatives of Tetrapeptide Methyl Esters. Figure 1 shows the low-resolution spectra of *N*-acetyl-, *N*-decanoyl-, and *N*-stearoyl-L-alanyl-L-valylglycyl-L-leucine methyl ester. By comparing these mass spectra we can conclude that there is no important difference in the basic fragmentation patterns of the acetyl and higher acyl derivatives. For example, the acetyl compound also shows intense peaks resulting from simple cleavage of the peptide bonds, *i.e.*, *m/e* 270, 213, and 114. Other peaks which are present in the spectra of all of these derivatives are *m/e* 328 and 286. The mass measurements on the decanoyl derivative (see Table II) show these to correspond to parts of the peptide chain and not to contain the acyl group; hence they occur at the same mass in each compound.

TABLE II: Results of Accurate Mass Measurements of *N*-Decanoyl-L-Ala-L-Val-Gly-L-Leu-OMe.

m/e	Measured Mass	Assigned Formula	Difference from Calcd Mass (millimass units)
526	526.3754	$C_{27}H_{50}N_4O_6$	−2.4
470	470.3117	$C_{23}H_{42}N_4O_6$	−1.3
382	382.2712	$C_{20}H_{36}N_3O_4$	−0.6
328	328.1876	$C_{15}H_{26}N_3O_5$	−0.2
325	325.2490	$C_{18}H_{33}N_2O_3$	+0.1
286	286.1892	$C_{14}H_{26}N_2O_4$	0
226	226.1806	$C_{13}H_{24}NO_2$	+0.1
155	155.1437	$C_{10}H_{19}O$	−0.1

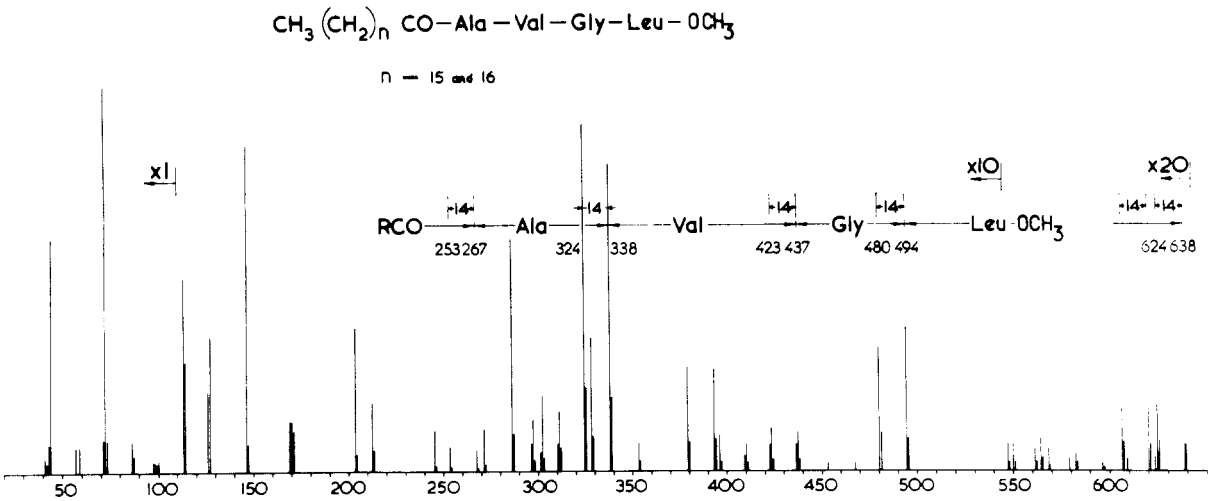
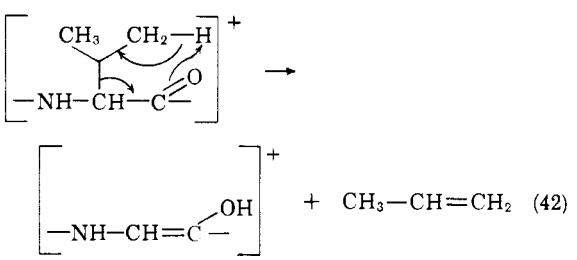


FIGURE 2: Line drawing of the mass spectrum of *N*-heptadecanoyl- + *N*-octadecanoylalanylvalylglycylleucine methyl ester.

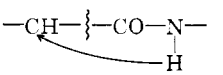
All three acyl derivatives gave good molecular ions and peaks at M − 15, M − 18, and M − 59 (M − $COOCH_3$). Also observed in each case were peaks at M − 42 and M − 56. These are due to loss of the valine and leucine side chains presumably *via* a McLafferty-type rearrangement (McLafferty, 1959) and would appear to be characteristic of the presence of these amino acids in the peptide chains; see Scheme I for a valyl peptide.

SCHEME I



Measurements of the intensities of the peaks in the spectra of the three compounds also confirm that there is no difference in the basic fragmentation. Despite this, the spectrum of the *stearoyl* derivative is clearly simpler in that the peaks corresponding to cleavage of the peptide bonds are more easily recognized. The principal advantage of the long-chain acyl group seems to be the shifting of the structurally significant peaks to a higher mass region; for example, in the case of stearoyl peptides, all peaks below *m/e* 267 $[CH_3(CH_2)_{16}CO]^+$ can be disregarded for the purpose of sequence determination, whereas the spectrum between *m/e* 267 (R–CO) and M (638) consists of groups of peaks corresponding to the amino acid sequence. The evidence from the mass measurements indicates that negligible fragmentation of the acyl chain occurs, so that these peaks are all due to the complete acyl group plus a part of the peptide chain remaining after simple cleavage of one of the peptide bonds

Other peaks in this region occur at 28 and 27 mass units below these. The former correspond to the further loss of CO (confirmed in some cases by the presence of metastable peaks), whereas the latter seem to be due to a different fragmentation, *i.e.*,



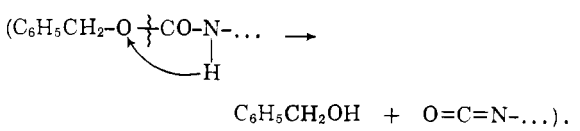
which occurs to a lesser extent than the simple cleavage.

In the case of fortuitine (Barber *et al.*, 1965a) and also of the *M. johnei* peptidolipid (Lanéelle *et al.*, 1965), the presence of homologs in the acyl chain made interpretation even easier because all acyl-containing ions gave a group of peaks corresponding to the homolog pattern. For example, for fortuitine, pairs of peaks 28 mass units apart were observed. Thus, we thought it would be an advantage to use a mixed acyl group. Since pairs of peaks 28 mass units apart can in some cases be due to the loss of CO from the heavier ion, we thought it would be better to use two acyl groups differing by CH_2 only. We have, therefore, prepared *mixed acyl derivatives* using an equimolecular mixture of heptadecanoic and octadecanoic acids. All acyl-containing ions in the spectra of such compounds give pairs of peaks 14 mass units apart, thus facilitating interpretation. In the spectrum of the stearoyl tetrapeptide methyl ester, peaks at *m/e* 328, 302, and 286 could thus be shown not to contain the acyl group. Figure 2 shows the mass spectrum of the tetrapeptide methyl

ester acylated with an equimolecular mixture of heptadecanoic and octadecanoic acids.

Acyl Derivatives of an Octapeptide Methyl Ester. The mixed *N*-heptadecanoyl- + *N*-octadecanoyl-Ala-Val-Gly-Leu-Ala-Val-Gly-Leu methyl ester gave molecular ion peaks at *m/e* 964 and 978; the peaks corresponding to the fragmentation of the peptide bonds are indicated in Figure 3.

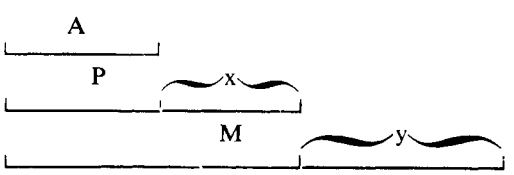
The spectrum of the nonacylated octapeptide methyl ester did not show peaks corresponding to the cleavage of the peptide bonds and was not, therefore, suitable for sequence determinations. The benzyloxycarbonyl derivative of the same octapeptide methyl ester gave a spectrum showing a more prominent fragmentation of the peptide bonds which starts after loss of benzyl alcohol (*m/e* 108) from the molecular ion (M^+ 846).



$$(C_6H_5CH_2\text{-}O\text{-}CO\text{-}N\text{-}\ldots \rightarrow C_6H_5CH_2OH + O{=}C{=}N\text{-}\ldots).$$

Conclusion

In general, for an *N*-acyl peptide methyl ester of mass M with an acyl group of mass A and a peptide ester moiety of mass P, peaks in the spectrum up to *m/e* A will not be useful for sequence determination. If the peptide moiety has greater mass than the acyl chain, between masses A and P (region x) two types of peaks will occur, *i.e.*, those useful for sequence determination and those due to parts of the peptide chain only. From the mass of the peptide chain, P, up to the molecular ion (M) the peaks contain the complete acyl group as described above and make sequence determination easy (region y).



In theory, the ideal acyl chain would be one having the same or greater mass than the peptide itself, though in practice this could introduce volatility problems, and in the compounds studied so far it has been relatively easy to distinguish the structurally significant peaks where acyl groups somewhat lower in molecular weight than the peptide were present. A good alternative which affords considerable advantage is the use of a mixed acyl derivative as described above.

In the previously described natural peptidolipids (Barber *et al.*, 1965a,b; Lanéelle *et al.*, 1965) and in the *N*-acyl oligopeptide methyl esters prepared for this work, only monoaminomonocarboxylic acids and one hydroxylamino acid, threonine, were present. The mass spectrometry of peptide derivatives containing other trifunctional amino acids and other acyl groups such as benzyloxycarbonyl, etc., is under investigation.

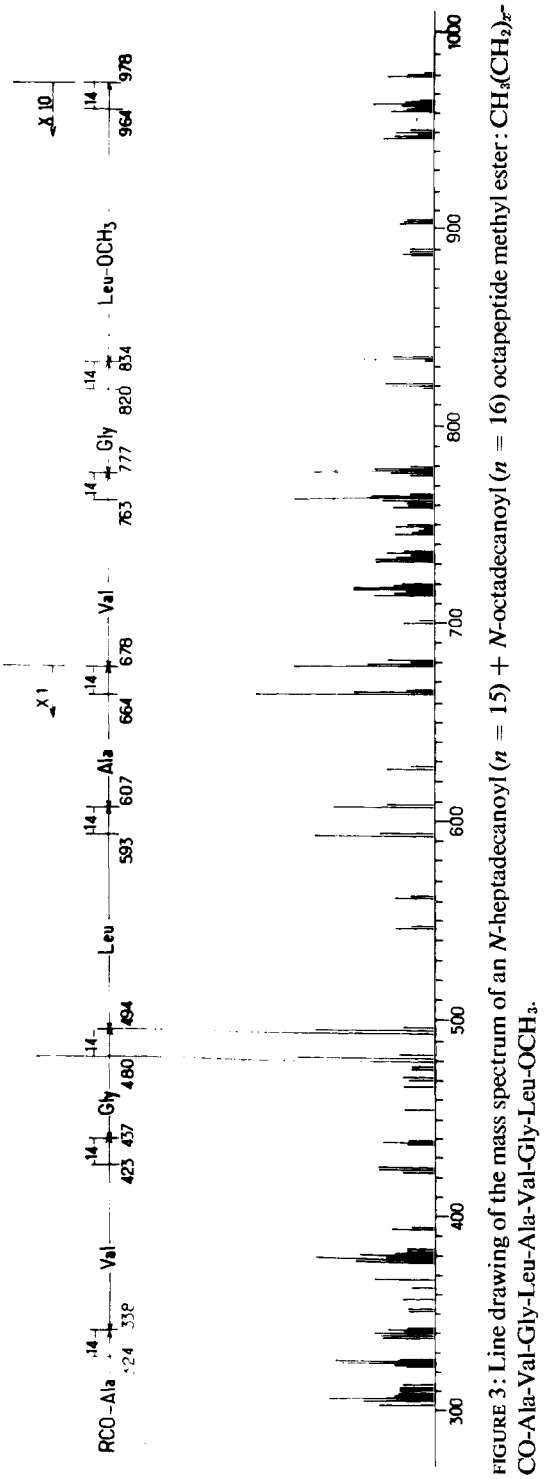
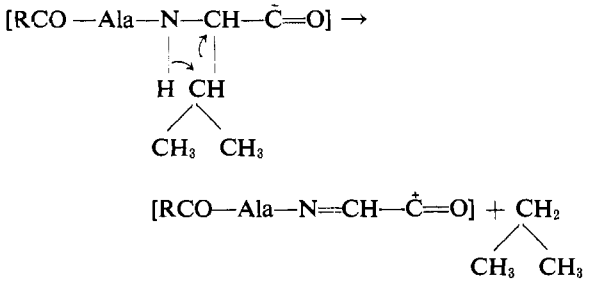


FIGURE 3: Line drawing of the mass spectrum of an *N*-heptadecanoyl ($n = 15$) + *N*-octadecanoyl ($n = 16$) octapeptide methyl ester: $CH_3(CH_2)_n$-CO-Ala-Val-Gly-Leu-Ala-Val-Gly-Leu-OCH_3.

Addendum

(August 31, 1965). One of the referees has drawn our attention to the presence of peaks 14 units apart, at *m/e* 379 and 393 (Figures 2 and 3), which do not correspond to cleavage of a peptide bond and which could cause difficulties in sequence determination. We have now made mass measurements of these peaks;

they show that these peaks correspond to a loss of C_3H_8 (= 44) from the C-terminal valine residue of the ions at *m/e* 423 and 437. This can be explained by the loss of the valine side chain, as follows



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