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A STUDY OF SOME NEW AND USEFUL N-TERMINAL GROUPS IN MASS SPECTROMETRY OF PEPTIDES

THE USE OF 3-HYDROXYALKANOYL AND UNSATURATED ACYL GROUPS[a]

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Abstract—Using model peptides ranging from tri- to dodecapeptides, the utility of several new N-protecting groups, i.e., 3-hydroxyalkanoyl and Δ^2-acyl groups including Oct(OH), Dec(OH), Dod(OH), Myr(OH), Pal(OH), Ste(OH), Δ^2-Dec, Cro, and Cin, for mass spectrometric sequence analysis were examined. Among these, the Dec(OH)- and Δ^2-Dec peptide derivatives were found to be superior to the hitherto reported Ac or Dec derivatives, since they gave comparable or sometimes better mass spectra. Particularly the Dec(OH) derivatives yielded two series of sequence peaks (as doublets of 18 mass units apart) ascribable to partial dehydration of the protecting group, the fact which makes the recognition of sequence determining peaks much easier. The Cro and Cin derivatives also gave mass spectra comparable to those of the corresponding Ac or Dec ones, except in a few cases where (Pro-Pro-Gly)$_{2-4}$ were model peptides. Permethylation technique can also be effectively applied to the all new acyl derivatives for the mass spectrometric sequence studies.

Mass spectrometry has been effectively applied to structure analysis problems of protein and peptide chemistry, particularly as a valuable method for confirming or determining amino acid sequences in synthetic and natural peptides.[1] Free peptides are unsuitable for mass spectrometry owing to their very low volatility and tendency to undergo irregular fragmentation. Therefore their termini are usually modified with appropriate protecting groups. The C-terminus can be left free, but N-protection is practically essential in order to convert peptides into sufficiently volatile derivatives and to mark their N-terminal amino acids allowing easy recognition of the ions bearing sequence informations. Since the nature of N-protecting groups of peptides has a significant effect on the volatility of peptide sample, the choice of the N-protecting group is of much importance in mass spectrometric work of peptide.

Among various N-protecting groups for the purpose of amino acid sequence determination, Ac and its higher homologs such as Dec or stearoyl have been the most frequently employed ones because of the ease of derivative formation and the favourable volatility of the resulting derivatives. However, these protecting groups have the following disadvantages. First, some oligopeptides undergo the partial cleavages of the peptide bonds during N-acetylation,[2] the fact which sometimes might mislead the structural assignments of the peptides. Second, for the acetylated peptide derivatives there are certain troubles in distinguishing the structurally significant N-terminal fragment ions from non-sequence ions which occur abundantly in low mass regions of the spectra. On the other hand, peptide derivatives acylated with long chain fatty acids are of relatively low volatility and therefore the abundance of their sequence ions are usually lesser than those of the corresponding Ac derivatives. Further it was claimed that some longer chain N-acyl peptides such as Dec derivatives are apt to be split off a portion of the acyl groups under electron impact, thus complicating the spectra.[3]

In the course of studies on the structures of 3-hydroxyacyl group-containing peptidolipids,[4,5] our attention has been drawn to the fact that they yielded excellent mass spectra suitable for peptide sequencing; most of the sequence ions were clearly recognized as twin peaks separated by 18 mass units making interpretation of the spectra much simple.

These observations have prompted us to investigate a series of 3-hydroxyalkanoyl groups as a

[a] Amino acid symbols denote the L-configuration unless otherwise stated. The abbreviated designations of amino acids, peptides and their derivatives in this paper are those from *Biochemistry* 5, 2585 (1966); 6, 362 (1967). The following abbreviations are used: Ac = acetyl; Dec = decanoyl; Δ^2-Dec = 2-decenoyl; Cro = crotonyl; Cin = cinnamoyl; Oct(OH) = 3 - hydroxyoctanoyl; Dec(OH) = 3 - hydroxydecanoyl; Dod(OH) = 3 - hydroxydodecanoyl; Myr(OH) = 3 - hydroxytetradecanoyl; Pal(OH) = 3 - hydroxyhexadecanoyl; Ste(OH) = 3 - hydroxyoctadecanoyl; pMZ = *p* - methoxybenzyloxycarbonyl.

promising N-protecting group for the mass spectrometry of peptides. Furthermore, since the occurrence of the above 18 mass units lower satellite peaks indicates undoubtedly the dehydration of the 3 - hydroxyalkanoyl peptide to the corresponding 2-alkenoyl peptides which gave rise to their own series of sequence ions, unsaturated acyl groups also looked promising. Therefore we have also carried out investigations on the usefulness of several N-unsaturated acyl groups including Δ^2-Dec, Cro, and Cin, for the peptide sequence analysis.

For comparing the contribution of the N-protecting group to the sequence analysis we have chosen acyl derivatives of tri- to dodecapeptide methyl esters (**1–6**), which were prepared by the conventional or Merrifield's procedure. As a measure of comparing relative merits of the protecting groups, the mass number and relative abundance of the sequence ions resulting from the normal fragmentations of the peptide and ester bonds were determined.

R-Leu-Gly-Ser-D-Val-Thr-Leu-OMe
1c, g-k

R-Leu-Gly-Ser-OMe
2a-f

R-(Pro-Pro-Gly)$_2$-OMe
3a-f

R-(Pro-Pro-Gly)$_3$-OMe
4a-f

R-(Pro-Pro-Gly)$_4$-OMe
5a-e

R-Val-D-Leu-Ala-Val-Leu-D-Ser-Leu-D-Ser-Ile-OMe
6a-f

a: R = Ac; **b**: R = Dec; **c**: R = Dec(OH); **d**: R = Δ^2-Dec; **e**: R = Cro; **f**: R = Cin; **g**: R = Oct(OH); **h**: R = Dod(OH); **i**: R = Myr(OH); **j**: R = Pal(OH); **k**: R = Ste(OH).

Mass spectra of 3-hydroxyalkanoyl (C_8–C_{18}) derivatives of hexapeptide methyl esters (**1c, g-k**)

In order to compare the effect of the chain length of N - 3 - hydroxyalkanoyl groups, the mass spectra of a homologous series of 3 - hydroxylalkanoyl derivatives of hexapeptide methyl ester (**1**)[5a] were examined.

The spectrum of **1g** is drawn out in full as a representative example (Fig 1) and the principal sequence peaks in the spectra of other 3 - hydroxyalkanoyl hexapeptide methyl esters are summarized in Table 1. In the mass spectra of this series of peptides the molecular ions were usually very weak and sometimes absent, instead, M-H_2O or M-2H_2O ions were clearly observed. Neither acyl peptide showed (M-OCH_3) ion, whereas ions due to the N-terminal acylleucine fragment R–NH–CH $[(CH_2)_2CH(CH_3)_2]$–C$\equiv$O$^+$ were the most abundant ions in every case. In general, peaks corresponding to the simple cleavage of the peptide bonds dominated the spectra, and most of them were accompanied by the dehydration peaks, thus highly facilitating interpretation. Also observed in the most compounds were peaks at M-30 and/or M-44, which are due to loss of the side chains from serine and threonine, respectively. Aldimine fragment ions due to fission of the C–CO bonds of the amino acid residues were of low intensity in all cases, except several peaks in the lower mass regions, i.e., *m/e* 228, 372, and 471 peaks (Fig 1).

By comparing the mass spectra we can conclude that the basic fragmentation patterns of the peptide derivatives are practically the same regardless of the chain-length of the N-protecting groups.

Comparison of the utility of 3 - hydroxydecanoyl-, 2-decenoyl-, crotonyl-, and cinnamoyl - groups

Acyl derivatives of tripeptide methyl esters (**2a-f**)

Fig 2 shows the low-resolution mass spectra of Dec(OH)- (**2c**), Δ^2-Dec- (**2d**), Cro- (**2e**), and Cin - Leu - Gly - Ser - OMe (**2f**), as well as Ac- (**2a**) and Dec - Leu - Gly - Ser - OMe (**2b**), the latters being used as reference standards. All of them were sufficiently volatile allowing to give good mass spectra in source temperature below 200°. There was no significant difference in the principal fragmentation patterns (so-called amino acid fragmentation) between them; the spectra displayed abundant ions resulting from single cleavage of the peptide bonds (for example, *m/e* 301, and 244 in **2f**), and, in analogy to the spectra of compounds **1** described above, cleavage of the C-terminal ester bond occurred very little, while the spectra contained prominent

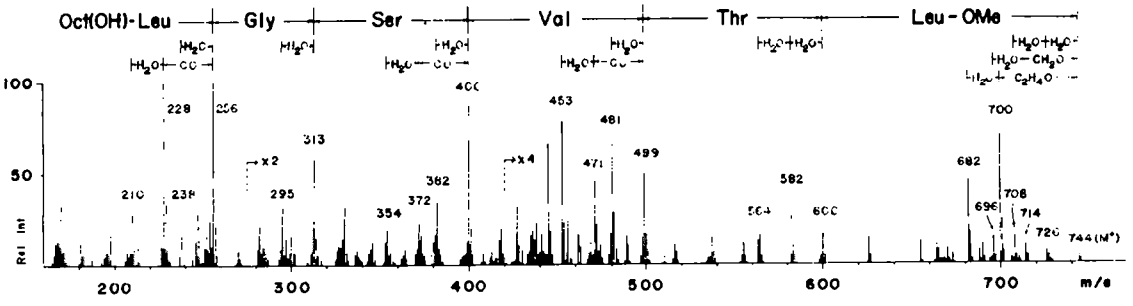

Fig 1. Mass spectrum of Oct(OH)-Leu-Gly-Ser-D-Val-Thr-Leu-OMe (**1g**); source temperature 170°.

Table 1. Mass spectra of 3-hydroxyacylhexapeptide methyl esters (**1c, h–k**)

No.	Compound R	Temp.		Sequence peaks;[a] R-Leu ↓ Gly ↓ Ser ↓ D-Val ↓ Thr ↓ Leu-OMe	Mass (Intensity)[c] [M-CH_2O]	[M-C_2H_4O]
1c	Dec(OH)	190°	A	284 (100) 341 (18) 428 (12) 527 (2) — 772 (0·2)	742 (1)	728 (4)
			B	266 (14) — 410 (6) 509 (5) 610 (1) 754 (1) 736[b] (2)	724 (1)	710 (6)
1h	Dod(OH)	190°	A	312 (100) 369 (15) 456 (19) 555 (5) 656 (6) —	—	756 (5)
			B	294 (22) — 438 (20) 537 (11) 638 (4) 782 (3) 764[b] (6)	752 (1)	738 (11)
1i	Myr(OH)	190°	A	340 (100) 397 (22) 484 (6) 583 (16) — —	—	—
			B	322 (25) — 466 (6) 565 (14) 666 (3) 810 (1) 792[b] (10)	780 (4)	766 (12)
1j	Pal(OH)	210°	A	368 (100) 425 (10) 512 (4) 611 (3) 712 (1) —	—	812 (4)
			B	— — 494 (4) 593 (4) 694 (1) 838 (2) 820[b] (2)	808 (2)	794 (8)
1k	Ste(OH)	200°	A	396 (100) 453 (14) 540 (5) — — —	—	—
			B	378 (75) 435 (54) 522 (9) 621 (6) 722 (1) — 848[b] (3)	—	822 (5)

[a] For convenience these refer to molecular and acyl carbonium ions (A) due to the normal fragmentation of amino acid type; B, [A-H_2O].
[b] [M-2H_2O].
[c] Percent relative abundance.

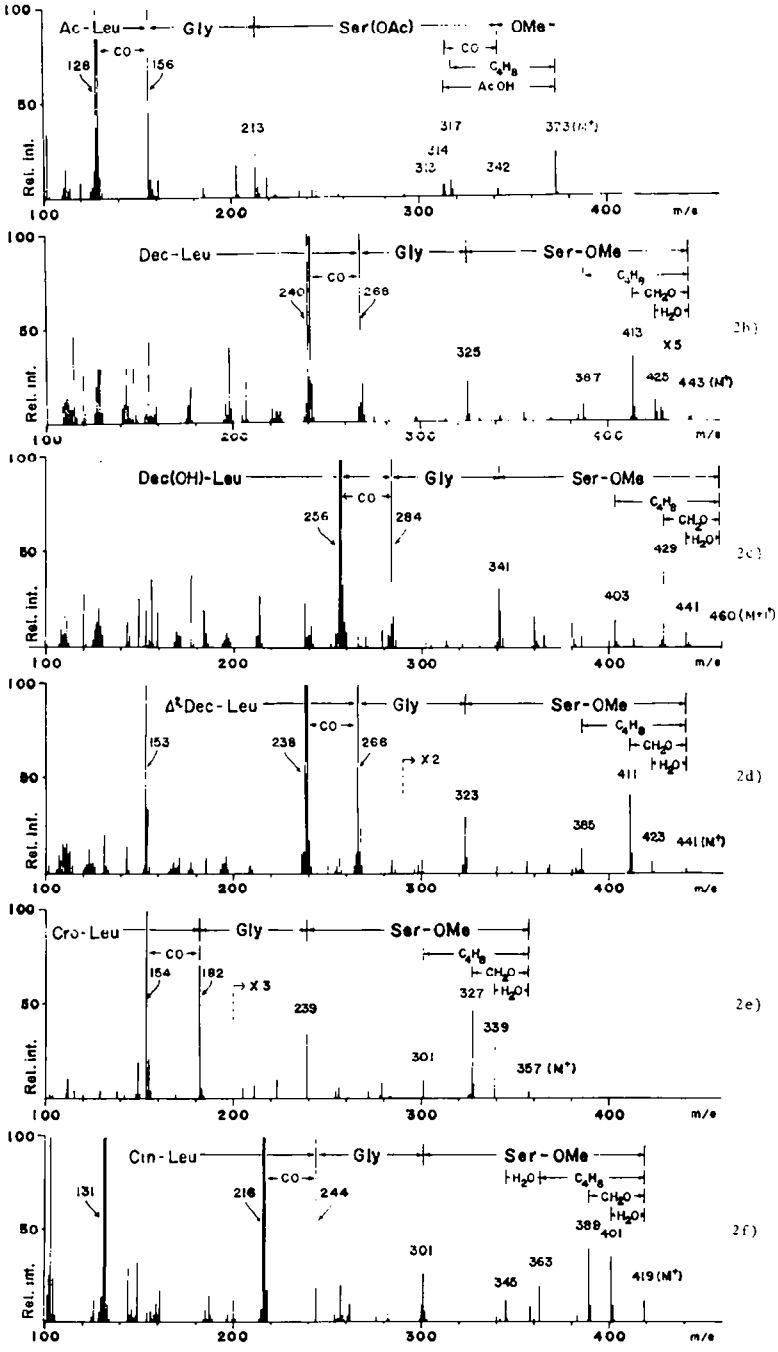

Fig 2. Mass spectra of acyl - Leu - Gly - Ser - OMe (**2a–f**); source temperature 140°(**2a**); 180°(**2b**); 160°(**2c**); 160°(**2d**); 180°(**2e**); 170°(**2f**).

M-18 (or M-60 in **2a**), M-30 and M-56 ions which can be rationalized as side-chain cleavage of H_2O (AcOH in **2a**), CH_2O, and C_4H_8 from the molecular ions, respectively.

Also in each case, intense aldimine peaks corresponding to the splitting of the C–CO bond of the N-terminal leucine were observed, each of them being employed as the base peak. The accurate mass measurements on compounds **2c–f**, as shown in Table 2, determined unambigously the atomic

Table 2. Results of accurate mass measurements of R-Leu-Gly-Ser-OMe (2c–f)

Compound No.	R	m/e	Measured mass	Assigned formula	Difference from calcd. mass (mmU)
2c	Dec(OH)	460	460·304	$C_{22}H_{42}O_7N_3$	+2
		441	441·284	$C_{22}H_{39}O_6N_3$	+1
		429	429·286	$C_{21}H_{39}O_6N_3$	+3
		423	423·271	$C_{22}H_{37}O_5N_3$	−2
		403	403·230	$C_{18}H_{33}O_7N_3$	−2
		385	385·220	$C_{18}H_{31}O_6N_3$	−1
		341	341·242	$C_{18}H_{33}O_4N_2$	−2
		284	284·221	$C_{16}H_{30}O_3N$	−1
		266	266·214	$C_{16}H_{28}O_2N$	+2
		256	256·221	$C_{15}H_{30}O_2N$	−6
		238	238·217	$C_{15}H_{28}O\ N$	0
2d	Δ^2-Dec	441	441·286	$C_{22}H_{39}O_6N_3$	+3
		423	423·273	$C_{22}H_{37}O_5N_3$	0
		411	411·269	$C_{21}H_{37}O_5N_3$	−4
		385	385·222	$C_{18}H_{31}O_6N_3$	+1
		367	367·214	$C_{18}H_{29}O_5N_3$	+3
		323	323·235	$C_{18}H_{31}O_3N_2$	+2
		266	266·211	$C_{16}H_{28}O_2N$	−1
		238	238·221	$C_{15}H_{28}O\ N$	+4
		153	153·125	$C_{10}H_{17}O$	−3
2e	Cro	357	357·191	$C_{16}H_{27}O_6N_3$	+1
		339	339·178	$C_{16}H_{25}O_5N_3$	−1
		327	327·178	$C_{15}H_{25}O_5N_3$	−1
		301	301·123	$C_{12}H_{19}O_6N_3$	−4
		239	239·139	$C_{12}H_{19}O_3N_2$	−1
		182	182·115	$C_{10}H_{16}O_2N$	−3
		154	154·124	$C_9\ H_{16}O\ N$	+1
2f	Cin	419	419·202	$C_{21}H_{29}O_6N_3$	−4
		401	401·197	$C_{21}H_{27}O_5N_3$	+2
		389	389·197	$C_{20}H_{25}O_5N_3$	+2
		363	363·142	$C_{17}H_{21}O_6N_3$	−1
		345	345·135	$C_{17}H_{19}O_5N_3$	+3
		301	301·151	$C_{17}H_{21}O_3N_2$	−4
		244	244·132	$C_{15}H_{18}O_2N$	−2
		216	216·136	$C_{14}H_{18}O\ N$	−3

compositions of the important peaks in each spectrum.

Acyl derivatives of sequential tripeptide methyl esters (**3a–f**, **4a–f**, and **5a–e**) *and nonapeptide methyl ester* (**6a–f**). The most important considerations in the choice of a protecting group for use in mass spectrometric analysis of an unknown peptide are whether (i) the protecting group can be effectively introduced to a small amount of the sample, since oligopeptides from protein hydrolysates are usually isolated in microgram quantities only, and (ii) the resulting peptide derivative can yield a reliable spectrum with high abundance of sequence ions. From these analytical standpoints, further examinations of the relative merits of the new protecting groups have been undertaken using 1–2 μmoles of larger model peptides.

As large peptides near the upper limit in peptide sequencing by mass spectrometry, we have used a series of collagen model sequential tripeptides of defined molecular weight, (Pro-Pro-Gly)$_n$ (n = 2–4),* and a nonapeptide, Val - D - Leu - Ala - Val - Leu - D - Ser - Leu - D - Ser - Ile. The use of the different sizes of the collagen model peptides seemed particularly advantageous for the reason that their amino acid ratios are identical irrespective to the molecular sizes, therefore facilitating discussion of the comparative merits of the protecting groups.

Table 3 shows the relative intensities of amino acid fragment ions of the acyl derivatives of the sequential tripeptide methyl ester (**3**), and the spectrum of Δ^2-Dec derivative (**3d**) is illustrated in Fig 3 as a typical representative. Dec(OH)-(**3c**), Δ^2-Dec-(**3d**), and Cro derivatives (**3e**) gave good molecular ion peaks together with the complete series of

*These compounds were kindly supplied by Dr. S. Sakakibara of Protein Research Foundation (Osaka) [S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai and K. Kikuchi, *Bull. Chem. Soc. Japan* **41**, 1273 (1968)].

Table 3. Mass spectra of R-(Pro-Pro-Gly)$_2$-OMe (3a–c, e, f)

No.	Compound R	Temp.		Sequence peaks; Mass (Intensity)[b] R↓ Pro ↓	Pro ↓	Gly ↓	Pro ↓	Pro ↓	Gly ↓	OMe
3a	Ac	170°	A[a]	140	237	294	391	488	545	576
				(100)	(42)	(100)	(22)	(1)	(2)	(25)
3b	Dec	200°	A	252	349	406	503	600	657	688
				(100)	(8)	(20)	(10)	(1)	(1)	(12)
3c	Dec(OH)	230°	A	268	—	422	519	616	673	704
				(70)		(18)	(5)	(2)	(1)	(13)
			B	250	347	—	501	—	655	686
				(100)	(9)		(2)		(2)	(5)
3e	Cro	210°	A	166	263	320	417	514	—	602
				(100)	(10)	(13)	(3)	(1)		(4)
3f	Cin	150°	A	228	324	—	—	—	—	—
				(100)	(65)					

[a] A, Amino acid fragments; B, [A-H_2O].
[b] Percent relative abundance.

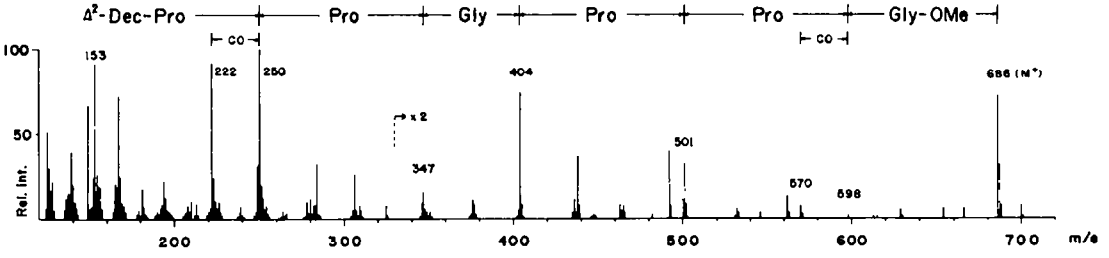

Fig 3. Mass spectrum of Δ^2-Dec-(Pro-Pro-Gly)$_2$-OMe (3d); source temperature 200°.

abundant sequence ions, except one peak [m/e 571 due to (M-OCH_3) in 3e]; the intensities of these peaks were nearly comparable to those of the corresponding ions in the reference peptide (3b). Cin derivative (3f), in contrast, yielded only sequence peaks corresponding to the acylium fragments up to the second residue from the N-terminus.

Principal sequence ions of the acyl methyl esters of collagen model nonapeptide (4) and dodecapeptide (5) are summarized in Tables 4 and 5, respectively.

Owing to their probable low volatility, all derivatives gave neither molecular ion nor ions bearing sequence information beyond the sixth (in 5) or seventh (in 4) residue from the N-terminus, however, simple visual inspection of the spectra provided an instructive knowledge of the relative merits of the acyl groups; the Dec(OH)-(4c, 5c) and Δ^2-Dec derivatives (4d, 5d) of each of the collagen model nona- and dodecapeptide gave obviously much more excellent spectra than the corresponding Cro- and Cin derivatives in terms of the number and intensity of the sequence ions.

Comparisons of the spectra of Dec(OH) - (3c, 4c, 5c) and Δ^2-Dec derivatives (3d, 4d, 5d) with those of the corresponding Ac-(3a, 4a, 5a) or Dec derivatives (3b, 4b, 5b) indicated that there is no great difference in fundamental fragmentation. Despite this, the former two derivatives seemed to be preferable from the point of view of sequence determination in the following senses; the sequence information ions were more easily recognized and no appreciable decrease in the incidence of the sequence ions were caused with increase of molecular sizes of the peptides. On the contrary, this is not the case for the Ac- or Dec derivatives, in which a decreasing tendency in the number of the sequence ions was obvious with increase of the chain length of the peptides.

A similar observation on the usefulness of the Dec(OH)- and Δ^2-Dec groups was also obtained after applying the permethylation technique[6] which increases the volatility of peptides and simplifies the mass spectra. Permethylated 4c and 4d (spectra not shown) exhibited, together with a molecular ion (at m/e 1011 in 4c) or M + 14* peak (at m/e 993 in 4d), a complete series of sequence peaks for the respective peptides, whereas in permethylated 4a, 4b, and 4e, the former peak was lacking, sequence

*The peak is due to C-methylation at the glycine residues.[6b]

Table 4. Mass spectra of R-(Pro-Pro-Gly)$_3$-OMe (**4a–f**)

No.	Compound R	Temp.		Sequence peaks; Mass (Intensity)[b] R-Pro ↓	Pro ↓	Gly ↓	Pro ↓	Pro ↓	Gly ↓	Pro ↓
4a	Ac	240°	A[a]	140	237	294	391	488	545	642
				(100)	(22)	(37)	(7)	(0·5)	(0·3)	(0·3)
4b	Dec	130°	A	252	349	—	503	600	—	—
				(100)	(7)		(5)	(0·5)		
4c	Dec(OH)	240°	A	268	365	422	519	616	673	770
				(72)	(6)	(12)	(7)	(1)	(2)	(0·5)
			B	250	347	404	501	598	655	752
				(100)	(7)	(13)	(6)	(1)	(3)	(1)
4d	Δ^2-Dec	270°	A	250	347	404	501	598	655	—
				(100)	(8)	(9)	(4)	(1)	(1)	
4e	Cro	250°	A	166	—	—	—	—	—	—
4f	Cin	200°	A	228	—	—	—	—	—	—

[a] A, Amino acid fragments; B, [A-H_2O].
[b] Percent relative abundance.

Table 5. Mass spectra of R-(Pro-Pro-Gly)$_4$-OMe (**5a–e**)

No.	Compound R	Temp.		Sequence peaks; Mass (Intensity)[b] R-Pro ↓	Pro ↓	Gly ↓	Pro ↓	Pro ↓	Gly ↓
5a	Ac	240°	A[a]	140	237	294	391	488	—
				(100)	(6)	(2)	(3)	(0·2)	
5b	Dec	200°	A	252	348	406	502	599	—
				(100)	(22)	(9)	(22)	(1)	
5c	Dec(OH)	240°	A	268	365	422	519	616	673
				(21)	(5)	(3)	(3)	(1)	(0·5)
			B	250	347	404	501	—	655
				(100)	(7)	(15)	(5)		(1)
5d	Δ^2-Dec	240°	A	250	347	404	501	598	655
				(100)	(13)	(19)	(37)	(2)	(1)
5e	Cro	250°	A	166	—	—	—	—	—

[a] A, Amino acid fragments; B, [A-H_2O].
[b] Percent relative abundance.

ions corresponding to at most six or seven amino acid residues from the N-termini being recognized, and in permethylated **4f** only the peaks for the first three residues were observed. A similar tendency was also observed with the spectra (not shown) of permethylated derivatives of **5**; the permethylated **5c** provided fairly profitable combination in number and abundance of sequence ions permitting to assign the sequence of the first nine amino acids from the N-terminus, while in the spectra of the permethylated **5a** and **5b** only the first six and seven residues were assigned, respectively. Neither the Cro derivatives (**4e**, **5e**) of the collagen model peptides yielded satisfactory spectrum even after permethylation, unfortunately.

For the nonapeptide Val - D - Leu - Ala - Val - Leu - D - Ser - Leu - D - Ser - Ile, the most satisfactory spectrum was obtained with Δ^2-Dec derivative (**6d**). It gave a complete set of abundant peaks arising from the rupture of the peptide bonds, as shown in Fig 4.

Neither spectra of the other five acyl derivatives (**6a–c, e, f**) could provide any sequence information beyond the fifth amino acid. Permethylation of **6a–f**, however, gave sufficiently volatile products to allow assignment of the complete sequences in every derivative, except the Ac- and Dec ones (**6a, b**), the spectra of which are missing the molecular ions and/or the sequence ions of the last residue (Table 6).

DISCUSSION

Using small model peptides (tri- to tetrapeptides), the relative merits of different acyl groups for use in mass spectrometric amino acid sequence determination were investigated by several workers,[2b,7] who have concluded that N-acylation by low-molecular-weight aliphatic acid are most suitable

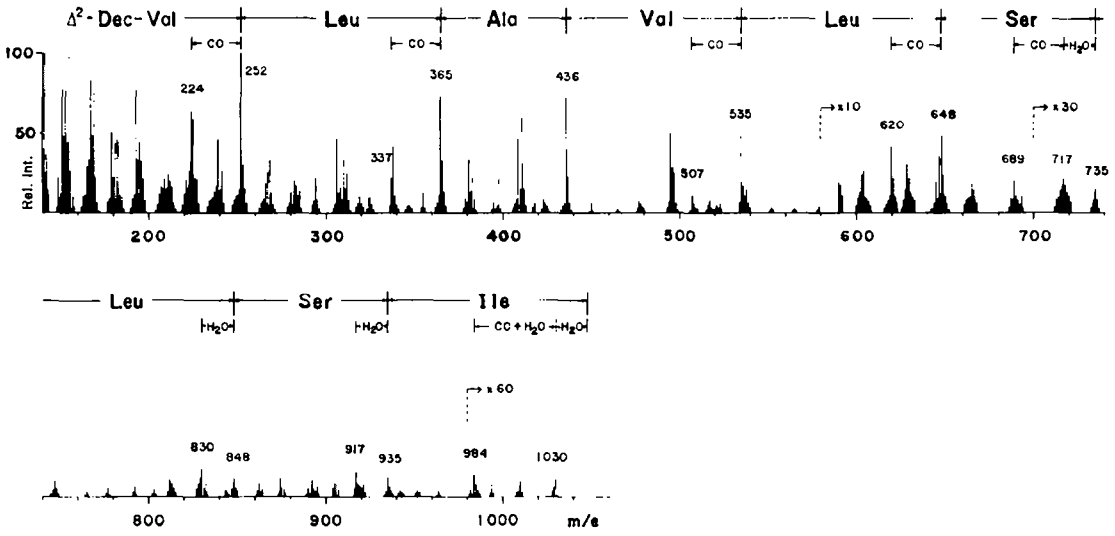

Fig 4. Mass spectrum of Δ²-Dec-Val-D-Leu-Ala-Val-Leu-D-Ser-Leu-D-Ser-Ile-OMe (**6d**); source temperature 230°.

for the purpose. However, there are very few studies on the use of unsaturated acyl-containing N-terminal tag for the sequence analysis of peptides except a few peculiar instances.[8,9]

The results in this paper obtained from the mass spectra of widely different sizes of model peptides including tri- to dodecapeptides show that the Dec-(OH)- and Δ²-Dec derivatives, throughout the model peptides used, gave the spectra comparable to or sometimes more excellent than those of the corresponding Ac derivatives. On the other hand, many of the Dec derivatives of peptides afforded, together with their own series of sequence ions, some acylium type ions (e.g., *m/e* 140, 294, and 391 peaks in each of **3b**, **4b**, and **5b**, or *m/e* 156, 283, 368, and 481 peaks in **6b**); these peaks are likely due to the normal fragmentation of N-Ac peptide ester resulted from a McLafferty rearrangement of the Dec group.[3] This cleavage of the N-blocking group might be the principal reason for lower incidences of sequence ions in the mass spectra of some of the Dec derivatives (i.e., **4b** and permethylated **4b** and **5b**). In the spectra of the Dec(OH) derivatives, however, the most of sequence ions could easily be recognized as doublets of 18 mass units apart, which indicates dehydration of the Dec(OH) peptides to the corresponding Δ²-Dec peptides. This feature does not complicate the analysis, but rather make easier sequence determination. Such an advantage of permitting easy recognition of sequence peaks appeared analogous to those mentioned for mixed N-acyl- or isotopic acyl-containing peptide derivatives.[3,10]

Furthermore both the Dec(OH)- and Δ²-Dec groups possessed also the same advantage as demonstrated with peptide esters acylated with long chain acyl groups.[10] The shifting of the structurally significant peaks to a higher mass region has allowed to recognize all acyl-containing sequence ions in the spectra much easy; e.g., in the case of Δ²-Dec peptides, all peaks below *m/e* 155 $[CH_3(CH_2)_6CH{=}CH{-}CO]^+$ can be disregarded for the purpose of sequence determination. Cro- and Cin-(Pro - Pro - Gly)$_n$ (n = 3 or 4) did not yield good spectra, but the Cro- and Cin esters of nonapeptide (**6e**, **f**) showed reliable results, utilities of which were approximately comparable to the Ac- and Dec-esters (**6a**, **b**); the fact which suggests that the utility of these types of N-protection should depend largely on the nature of parent peptide to be examined.

Application of the permethylation technique to the model peptides resulted in significant increase in the volatility of the all peptide derivatives examined except for the Cro derivatives of the collagen model peptides, thus leading to mass spectra containing sufficient information to allow sequence assignment. This result is in contrast to an observation that sequencing was actually impossible after permethylation in peptide derivatives containing enamino - ketone function at the N-terminus.[8]

To test the practical use of the Dec(OH) N-protection method, we have then applied this method to microgram scale samples derived from natural sources, viscosic acid dimethyl ester [Dec(OH) - Leu - D - Glu(γ - OMe) - D - *a*Thr - D - Val - Leu - D - Ser - Leu - D - Ser - Ile - OMe][4,5b] and a collagenolytic peptide (Pro - Lys - Ile - Pro - Pro) of potentiator B, one of the bradykinin potentiating peptides from the venom of *Agkistrodon halys blomhoffii*.[11]

Each of these gave sufficient mass spectrum to

Table 6. Mass spectra of permethylated derivatives of R-Val-D-Leu-Ala-Val-Leu-D-Ser-Leu-D-Ser-Ile-OMe (**6a–f**)

No.	Compound R	Temp.		Sequence peaks; Mass (Intensity)[c] R-MeVal ↓ MeLeu ↓ MeAla ↓ MeVal ↓ MeLeu ↓ Me_2Ser ↓ MeLeu ↓ Me_2Ser ↓ MeIle ↓ OMe									
6a	Ac	230°	A[a]	156	283	368	481	606	723	818[b]	965	—	1091[b]
				(100)	(47)	(40)	(25)	(9)	(2)	(2)	(1)		(0·5)
6b	Dec	250°	A	268	395	480	593	720	835	930	1077	—	—
				(100)	(17)	(11)	(8)	(4)	(1)	(1)	(0·5)		
6c	Dec(OH)	220°	A	298	425	510	623	750	865	992	1107	1234	1265
				(100)	(70)	(45)	(30)	(12)	(3)	(4)	(4)	(1)	(1)
			B	266	393	478	591	718	833	960	1075	1202	1233
				(65)	(17)	(17)	(12)	(6)	(4)	(3)	(2)	(2)	(1)
6d	Δ^2-Dec	190°	A	266	393	478	591	718	833	928[b]	1075	1202	1233
				(100)	(43)	(37)	(31)	(13)	(5)	(4)	(4)	(1)	(1)
6e	Cro	200°	A	182	309	394	507	634	749	844[b]	991	1086[b]	1149
				(100)	(53)	(48)	(43)	(24)	(5)	(5)	(3)	(1)	(1)
6f	Cin	160°	A	244	371	456	569	696	811	906[b]	1053	1148[b]	1211
				(100)	(67)	(33)	(22)	(10)	(1)	(2)	(1)	(0·2)	(0·4)

[a] A, Amino acid fragments; B, [A-MeOH].
[b] [A-MeOH].
[c] Percent relative abundance.

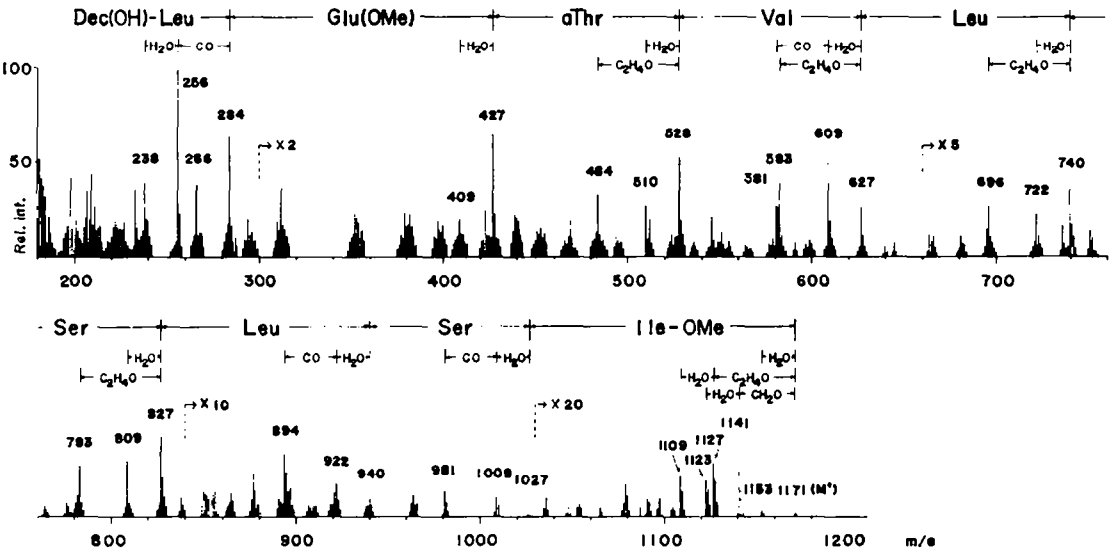

Fig 5. Mass spectrum of viscosic acid dimethyl ester; source temperature 200°.

allow confirming smoothly the respective total sequences. Viscosic acid dimethyl ester exhibited molecular ion at *m/e* 1171 as well as a series of peaks due to the fragmentation of the peptide bonds (Fig 5). Since the fragmentation is usually accompanied by dehydration from the hydroxyacyl and/or any of the hydroxyamino acid residues, the spectrum contained two groups of sequence ions; the normal amino acid type fragment ions at *m/e* 284, 427, 528, 627, 740, 827, 940, and 1171(M^+) and a series of 18 mass units lower satellite peaks at *m/e* 266, 409, 510, 609, 722, 809, 922, 1009, and 1153. In addition, observed were M-30 and M-44 ions, which are characterized by loss of the side chains from the serine and allo-threonine residues *via* a McLafferty-type rearrangement, respectively.[1]

As has also been described in the previous paper,[12] it was concluded that the use of the Dec(OH) group is highly recommended in the sequencing of a small amount of peptides of natural origin.

EXPERIMENTAL*

Mass spectra were determined using Nippon Denshi Model JMS-01SG mass spectrometer with the direct sample inlet system operating at 75 eV: the heating temp varied between 140° and 270°. Accurate mass measurements were performed with the same spectrometer using perfluorokerosene as the reference compound. Specific rotations were measured with a Jasco DIP-SL polarimeter. The amino acid composition of the acid hydrolysate was determined with a Hitachi Model KLA-2. Thin layer chromatography was carried out on Kieselgel G (Merck) with the solvent system of $CHCl_3$:MeOH:AcOH (95:5:3, v/v, R_f). Ninhydrin-HBr combination reagent was used for the detection.

*All m.p.s were uncorrected.

N-Acyltripeptide methyl esters (**2a–f**). (i) N - Acyloxysuccinimides: The N - hydroxysuccinimide esters of decanoic and 3 - hydroxydecanoic acid were prepared according to the method described in a previous paper.[13] Using essentially the similar method the following esters were prepared from the corresponding acids and N - hydroxysuccinimide. 2 - Decenoic acid N - hydroxysuccinimide ester: m.p. 65–65·5°. (Found: C, 62·85; H, 7·74; N, 5·26. Calcd for $C_{14}H_{21}O_4N$: C, 62·90; H, 7·92; N, 5·24%). Crotonic acid N - hydroxysuccinimide ester: m.p. 81–83°. (Found: C, 52·77; H, 4·90; N, 7·73. Calcd for $C_8H_9O_4N$: C, 52·46; H, 4·95; N, 7·65%). Cinnamic acid N - hydroxysuccinimide ester: m.p. 179–180°. (Found: C, 63·92; H, 4·45; N, 5·66. Calcd for $C_{13}H_{11}O_4N$: C, 63·67; H, 4·52; N, 5·71%). (ii) H - Leu - Gly - Ser - OMe . HCl: Z - Leu - Gly - Ser - OMe[14] (4·4 mmole) was hydrogenated over 10% Pd-C in MeOH (10 ml) containing 4N HCl (1·1 ml) for 2 h. After filtration the filtrate was evaporated *in vacuo* to yield crystalline H - Leu - Gly - Ser - OMe . HCl (84%). (iii) N - Acyl - Leu - Gly - Ser - OMe (**2a–f**): H - Leu - Gly - Ser - OMe . HCl (0·3 mmole) was treated with a mixture of AcOH–Ac_2O (1:1, v/v) (2 ml) and Et_3N (0·04 ml) at room temperature overnight. The reaction mixture was evaporated *in vacuo* and the residual oil was dissolved in AcOEt (10 ml). The solution was washed with H_2O and dried over $MgSO_4$. Evaporation of the solvent gave an oily residue, which was triturated with light petroleum to give a solid mass. Reprecipitation from AcOEt–light petroleum gave the Ac derivative **2a** in 60% yield: m.p. 117–119°, $[\alpha]_D^{25} - 1°$ (c = 1, EtOH); $R_f = 0·53$. (Found: C, 51·51; H, 7·10; N, 11·23. Calcd for $C_{16}H_{27}O_7N_3$: C, 51·46; H, 7·29; N, 11·25%). The other acyl derivatives (**2b–f**) were prepared from the corresponding N - acyloxysuccinimides and H - Leu - Gly - Ser - OMe . HCl according to the following general procedure: A soln containing 0·3 mmole each of the components and Et_3N (0·04 ml) in DMF (0·5 ml) and AcOEt (1 ml) was stirred at room temp for 16 h and diluted with AcOEt (5 ml). The mixture was washed successively with 1N HCl, H_2O, 1N $NaHCO_3$ and H_2O, dried over $MgSO_4$ and evaporated *in vacuo* to give a product. Recrystallization from

AcOEt–light petroleum afforded almost theoretical yields of **2b–g**. **2b**: m.p. 108–110°, $[\alpha]_D^{27}+6°$ (c = 1, EtOH); R_f = 0·57. (Found: C, 59·49; H, 9·18; H, 9·63. Calcd for $C_{22}H_{41}O_6N_3$: C, 59·57; H, 9·32; N, 9·47%). **2c**: m.p. 117–123°, $[\alpha]_D^{27}+5°$ (c = 1, EtOH); R_f = 0·44. (Found: C, 57·28; H, 9·02; N, 9·31. Calcd for $C_{22}H_{41}O_7N_3$: C, 57·49; H, 8·99; N, 9·14%). **2d**: m.p. 132–132·5°, $[\alpha]_D^{23}+5·5°$ (c = 1, EtOH); R_f = 0·58. (Found: C, 59·40; H, 8·97; N, 9·56. Calcd for $C_{22}H_{39}O_6N_3$: C, 59·84; H, 8·90; N, 9·52%). **2e**: amorphous powder, $[\alpha]_D^{29}-8°$ (c = 1, EtOH); R_f = 0·55. (Found: C, 51·56; H, 7·73; N, 11·14. Calcd for $C_{16}H_{27}O_6N_3 . H_2O$: C, 51·19; H, 7·79; N, 11·19%). **2f**: m.p. 190·5–192·5, $[\alpha]_D^{22}+24°$ (c = 1, EtOH); R_f = 0·60. (Found: C, 60·18; H, 6·89; N, 10·01. Calcd for $C_{21}H_{30}O_6N_3$: C, 60·13; H, 6·97; N, 10·02%).

H - Val - D - Leu - Ala - Val - Leu - D - Ser - Leu - D - Ser - Ile - OH. (i) Z - Val - D - Leu - Ala - Val - $NHNH_2$: The hydrazide was prepared by treatment of Z - Val - D - Leu - Ala - Val - OMe[15] (1·6 g) with $NH_2NH_2 . H_2O$ (3 g) in MeOH (40 ml) at room temperature, yield 1·1 g (65%); m.p. 251–252°, $[\alpha]_D^{20}+10°$ (c = 1, 0·1N HCl-DMF). (Found: C, 59·28; H, 8·03; N, 15·34. Calcd for $C_{27}H_{44}O_6N_6$: C, 59·10; H, 8·08; N, 15·32%). (ii) Z - Val - D - Leu - Ala - Val - Leu - D - Ser - Leu - D - Ser - Ile - OBzl: After elimination of the pMZ group from pMZ - Leu - D - Ser - Leu - D - Ser - Ile - OBzl[5b] (790 mg) by an exposure (0°, 30 min) to trifluoroacetic acid (3 ml) in the presence of anisole (0·5 ml), the resulting pentapeptide ester was coupled with Z - Val - D - Leu - Ala - Val - $NHNH_2$ (550 mg) by the usual azide method. The product was isolated by column chromatography on silica gel using $CHCl_3$–MeOH–AcOH (95:5:3, v/v) as a solvent, and finally crystallized from MeOH–$CHCl_3$–Et_2O. Yield 213 mg (34%); m.p. 195–197°, $[\alpha]_D^{18}-18°$ (c = 1, MeOH). (Found: C, 60·90; H, 7·97; N, 10·98. Calcd for $C_{58}H_{91}O_{14}N_9$: C, 61·19; H, 8·00; N, 11·07%). (iii) H - Val - D - Leu - Ala - Val - Leu - D - Ser - Leu - D - Ser - Ile - OH: The protected nonapeptide (114 mg) was hydrogenated over 10% Pd-C in MeOH (20 ml) containing 4N HCl in dioxane (0·05 ml) for 7 h. After filtration and evaporation of the solvent, the residue obtained was crystallized from MeOH–Et_2O to afford 74 mg (80%) of the free nonapeptide: m.p. 209–211°, $[\alpha]_D^{18}-27°$ (c = 1, MeOH); R_f = 0·30. Amino acid ratios in an acid hydrolysate (105°, 24 h in 6N HCl): Ser, 1·71; Ala, 0·92; Val, 2·35; Leu, 3·44; Ile, 1·00. (Found: C, 52·64; H, 8·24; N, 12·39. Calcd for $C_{41}H_{79}O_{12}N_9 . 4H_2O$: C, 52·37; H, 8·89; N, 12·78%).

N-Acylation and esterification of free peptides in a small scale (the preparation of compounds **3**, **4**, **5**, and **6**). Peptide (0·5–2 mg) was acetylated in Ac_2O–AcOH (1:1, v/v) at room temp by the method of Agarwal *et al.*[16] Other N-acylation were carried out according to the method previously described.[13] The resulting N-acylpeptides were esterified by treating with an excess of a solution of CH_2N_2 in Et_2O or CH_2Cl_2 (2 ml) or with 0·1N methanolic HCl at 20° for 12 h.

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