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Note

Gas chromatography–mass spectrometry of permethylated peptides and their reduced and trimethylsilylated derivatives

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Peptide derivatives suitable for mass spectrometry (MS) can be divided into two groups: (a) those which are introduced directly into the ion source of the mass spectrometer; and (b) those which are introduced via the gas chromatographic (GC) inlet of the mass spectrometer. Peptide derivatives of group (a), particularly those obtained by N-acetylation and permethylation[1,2], are suitable for the analysis of single peptides or of very simple peptide mixtures, since the method of vaporization into the mass spectrometer affords only limited separation of the derivatives present[3–5]. On the other hand, derivatives (b) can be analyzed by the direct combination of GC and MS[6–16] which is ideal for the analysis of very complex mixtures. Some 50 oligopeptides have been identified by a single GC–MS experiment of a polypeptide hydrolysate[17]; even hydrolysates of entire enzymes have been analyzed successfully[18].

However, the volatility requirement of GC is a limitation to the GC–MS sequencing methods; thus it is often difficult to analyze derivatives larger than those of hexapeptides (containing amino acids of medium polarity). On the other hand, the permethylated peptide derivatives (a) have been successfully prepared and analyzed from deca- to dodecapeptides[19].

The complete characterization of peptide mixtures, particularly of those obtained by limited hydrolysis of polypeptides or entire enzyme molecules[18,20], requires the identification of both the small and the large oligopeptides present. One obvious approach would be to divide the sample into two parts and to prepare the permethylated peptide derivatives (a) from one part for the analysis of large oligopeptides, and to prepare the derivatives (b) from the other part for the identification of the smaller oligopeptides present.

In this paper an approach is described which uses smaller amounts of sample and requires less time than the one outlined above: new peptide derivatives have been prepared from permethylated peptides by reduction with $LiAl^2H_4$ and O-trimethylsilylation. These derivatives are of sufficient volatility to be efficiently identified in complex mixtures by a GC–MS system. Thus, after a mixture of permethylated peptides has been analyzed for larger oligopeptides, the remainder of the sample can now be reduced and trimethylsilylated to increase further the volatility of the peptide derivatives for the analysis of the smaller oligopeptides present. Furthermore, the new derivatives are particularly suitable for the GC–MS analysis of glutamine- and asparagine-containing peptides, which hitherto caused difficulties with other types of peptide derivatives suitable for GC–MS.

EXPERIMENTAL

Mixtures of peptides containing between 0.1 and 1 μmole of di- to pentapeptides (Serva, Heidelberg, G.F.R.; Sigma, St. Louis, Mo., U.S.A.; Cyclo Chem., Los Angeles, Calif., U.S.A.; and P-L Biochemicals, Milwaukee, Wisc., U.S.A.) were acetylated or trifluoroacetylated with the respective anhydrides; after the excess of reagents had been removed, permethylation was affected by use of a ten-fold excess of methyl iodide and methylsulphinylmethanide carbanion, according to the procedure of Leclercq and Desiderio[21]. After 3 min (*cf.* ref. 22) the reaction was terminated by the addition of 1 ml of water, and the products were extracted three times with 1 ml of chloroform. The chloroform layer was washed three times with 1 ml of water and dried in a stream of nitrogen. The residue was dissolved in 100 μl of chloroform and an aliquot portion (2–10%) was injected into the gas chromatograph (F-22, Perkin-Elmer, Überlingen, G.F.R.) equipped with a flame ionization detector; the temperature of the oven was raised at 12.5 or 15°/min from 60 to 330° (or 350° if Dexsil was used as stationary phase). Glass columns (0.25 in.) were used and were packed with 2% Dexsil 300 GC on Chromosorb W AW DMCS (80–100 mesh, 50 cm and 1 m), 3% SE-30 on Chromosorb W HP (100–120 mesh, 1 m) or 4% OV-17 on Chromosorb W HP (100–120 mesh, 2 m).

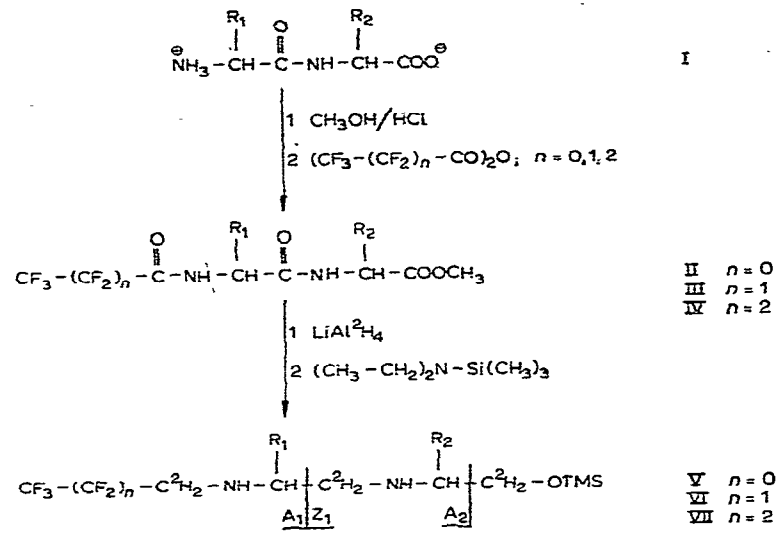
The same aliquot portion was then injected into the GC–MS computer system consisting of a Perkin-Elmer F-22 gas chromatograph, a MS-30 mass spectrometer (AEI Scientific Co.) and a DS-50 data system (AEI). The GC column was coupled by a 0.25-in. Swagelok union (drilled to 0.25 in.) to a 0.25-in. glass tube leading to the membrane separator of the mass spectrometer. Mass spectra were scanned continuously (every 7 sec) and recorded by the data system; subsequently, selected ion records (mass chromatograms)[23] were generated and used for the identification of the minor components of the mixture.

The remainder of the sample was transferred with chloroform into a 5-ml glass bulb, and the solvent was evaporated by use of a stream of nitrogen followed by drying with a vacuum pump (5 min). The sample was then reduced by 2 ml of a 1 *N* solution of $LiAl^2H_4$ in dimethoxyethane according to a previously published procedure[13]. The O-trimethylsilylated products (2–10% of a benzene solution) were finally analyzed by GC and GC–MS under the conditions described above for the permethylated peptides.

RESULTS AND DISCUSSION

The methods of preparation and structures of the peptide derivatives II–IV[7,8,10,14] and V–VII[6,9,12,13,15–18], which have previously been used successfully for the sequence analysis of polypeptides by GC–MS, are shown in Fig. 1. The derivatives II–IV are easily and quickly prepared, while the derivatives V–VII require a more involved transformation procedure, but possess excellent GC as well as MS properties[13,15–17].

The N-acetylated and permethylated derivatives VIII (Fig. 2) are of lower volatility (compare the retention indices of the derivatives V and VIII of a dipeptide in Table I) and therefore must be introduced by the direct probe inlet of the mass spectrometer; only permethylated derivatives of small peptides have been analyzed

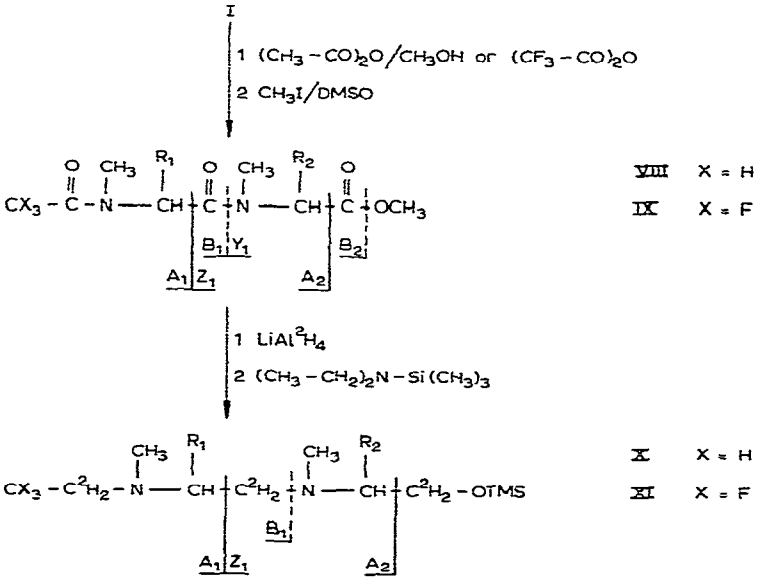


I

II n = 0
III n = 1
IV n = 2

V n = 0
VI n = 1
VII n = 2

Fig. 1. Scheme of the preparation and structures of peptide derivatives II–VII. TMS = Trimethyl silyl.



VIII X = H
IX X = F

X X = H
XI X = F

Fig. 2. Scheme of the preparation and structures of peptide derivatives VIII–XI. DMSO = Dimethyl sulphoxide.

by GC[11,24]. Recently I have successfully analyzed the permethylated derivatives of even tetra- and pentapeptides by GC: for example, the derivative VIII of Phe-Asp-Ala-Ser-Val emerged on a 50-cm column of Dexsil at 350° with a retention index of 4520.

TABLE I

RETENTION INDICES ON DEXSIL 300 GC, AND SEQUENCE IONS RELATIVE TO THE MOST INTENSE SEQUENCE ION (= 100), OF Phe–Phe DERIVATIVES

Peptide derivative	*Retention index*	*Sequence ions*								
		A_1	B_1	A_2	B_2	Z_1	Y_1	$M-91$	$M-15$	M
V	2391	31		29		100		29	4.0	
VIII	2939	100	39	0.3	0.5	3.6	3.5*	2.7		0.4
IX	2780	100	27**	7.0	3.9***	16	78	2.5		2.75
X	2566	100		0.62		10.5		0.82	0.46	
XI	2419	8.0		11		100		3.8	0.80	

* Y_1-H, 4.4.
** B_1+H, 35.
*** As for B_2+H.

However, the volatility of these derivatives must still be significantly increased if they are to be used for the analysis of complex mixtures of oligopeptides. In the first attempt, N-trifluoroacetylated peptides II, rather than N-acetylated peptides, were permethylated in order to yield the derivatives IX. The latter are more volatile and also exhibit mass spectra with intensity-balanced sequence ions (Table I): while the sequence ions of the N-terminal amino acids appeared as very intense peaks in the mass spectra of both derivatives VIII and IX of Phe-Phe (Table I), the other sequence ions, such as those indicating the carboxyl terminal residue (Z_1, Y_1) and the amino terminal dipeptide (A_2, B_2), gave much more pronounced peaks in the mass spectra of the derivatives IX than in those of VIII. Unfortunately, GC revealed that side products had been formed in addition to the desired derivatives IX. Optimum derivatization procedures leading to single reaction products must be developed before the derivatives IX can be used for the analysis of complex mixtures.

It was then investigated whether the volatility of the permethylated peptides could be increased by reduction with $LiAl^2H_4$ and subsequent O-trimethylsilylation, analogous to the preparation of the derivatives V–VII from II–IV (see Fig. 1). The resulting derivatives X and XI (Fig. 2) have much increased volatility. The difference in retention index between the derivatives VIII and X and between IX and XI is *ca.* 400 units (see Table I). The volatility of the derivatives XI approaches that of the most volatile derivatives V–VII. The mass spectra of the derivatives XI also exhibited pronounced sequence ions: the ions indicating the amino terminal amino acid (A_1) and dipeptide (A_2), as well as the carboxyl terminal amino acid (the Z_1 ion is the base peak in the mass spectra of the dipeptides), were all prominent and easily recognised. However, due to the present difficulties with multiple derivatives (see above), the derivatives of type X were usually employed.

Fig. 3 shows the GC separation of some peptide derivatives X which emerged as well-shaped and symmetrical peaks. However, small amounts of side products were also present, which were eluted shortly after the desired derivatives and were often not completely separated from them. As the mass spectral data reveal, these side products are formed by over-methylation and racemization of the peptides

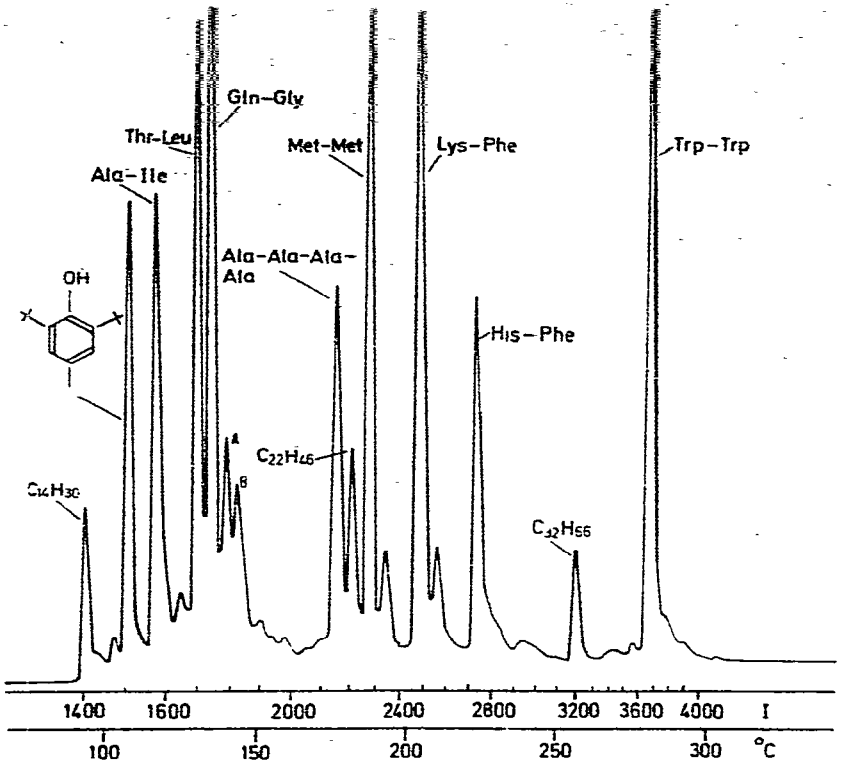
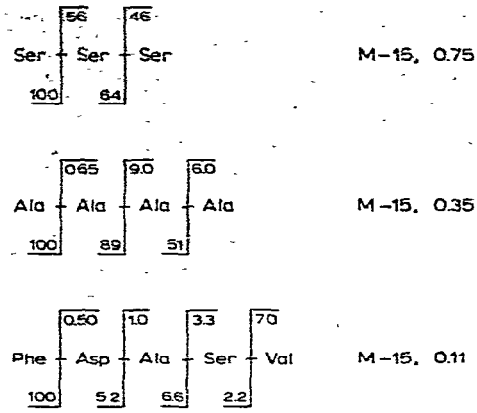


Fig. 3. Gas chromatogram (obtained on a 50-cm Dexsil column) of peptide derivatives obtained by N-acetylation, permethylation, $LiAl^2H_4$ reduction and O-trimethylsilylation of a peptide mixture. A, B = side products formed by C-methylation on the glycine and glutamine residue of Gln-Gly.

at the α-carbon atoms of the amino acid residues. The degree of this C-methylation depends on the nature of the amino acids present and was usually much less than 5% of the desired product. The main components are shown "off-scale" in Fig. 3 so as to accentuate the presence of these side products. Amino acids such as glutamine, asparagine, the corresponding acid analogs and glycine were particularly susceptible to C-methylation: for example, the peaks A and B in Fig. 1 correspond to Gln-Gly derivatives, which had been C-methylated on the glycine and glutamine residues, respectively.

Although the mass spectra of the dipeptide derivatives of type X are dominated by the A_2 ion of the amino terminal residue (particularly if the residue is aromatic), the less intense A_2, Z_1 and M — 15 ions usually yield peaks of sufficient intensity for the nature of the carboxyl terminal residue to be determined (see the mass spectra in Fig. 4). In addition, the retention indices of the derivatives can be used for confirmation of the structure. The mass spectra of larger peptide derivatives exhibit a more balanced spectrum of A ions (containing the amino terminal) as shown in the following examples:



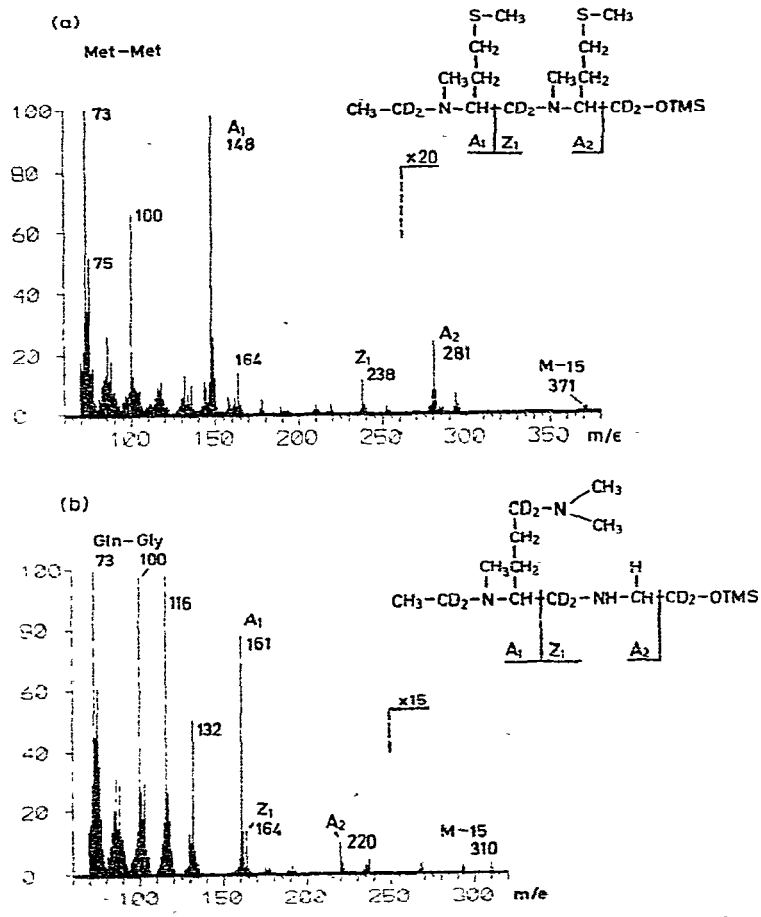


Fig. 4. Mass spectra of two GC fractions obtained by GC–MS of a mixture of peptide derivatives of type X (see Fig. 3 for the gas chromatogram).

In addition, the ions containing the carboxyl terminal groups are readily discernable, although of low abundance. Selected ion records and retention indices[25] can be used for the efficient identification of these derivatives, since both the sequence ions and the GC behaviour can readily be predicted for all of the peptides.

Glutamine- and asparagine-containing peptides are derivatized by the previously published procedure[13] with rather low yields, presumably because primary amine groups are formed upon reduction with $LiAl^2H_4$, and the resulting products are not easily extracted and analyzed by GC. By use of the derivatization procedure described in this paper, the side chains of Gln and Asn may be dimethylated, leading to products with excellent GC properties (Figs. 3 and 4); the yields obtained were 80–90% as compared to the yields of "unproblematic" peptides[26,27].

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