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GAS CHROMATOGRAPHY OF SOME DIPEPTIDE DERIVATIVES

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SUMMARY

The gas chromatographic behaviour of acetyl-, acetyl permethylated-, trifluoroacetyl- and pentafluoropropionyldipeptide methyl esters has been studied to assess their potential usefulness in the dipeptidyl aminopeptidase I technique of peptide sequencing using gas chromatography–mass spectrometry (GC–MS). None of these derivatives gives volatile products with all dipeptides and hence these procedures do not provide the basis for a satisfactory GC–MS sequencing method.

INTRODUCTION

The possibility of reducing the problem of peptide sequencing to that of characterizing completely a mixture of dipeptides using the specificity of the enzyme dipeptidyl aminopeptidase I (cathepsin C) has been discussed by a number of authors[1–5]. The feasibility of the technique has been demonstrated in test situations using ion exchange and paper chromatography[4], high-voltage paper electrophoresis[2] and gas chromatography–mass spectrometry (GC–MS) of trifluoroacetyl[5] or pentafluoropropionyl derivatives[6]. If, however, the technique is to be of general application, it is important that a complete solution to the problem of separation and identification of all the possible 400 dipeptides should be found. The only systematic attempt at a completely general solution to this problem has been by Callahan *et al.*[7], who devised a combination of paper and column chromatography which, they claim, is capable of separating and identifying any dipeptide mixture. The main disadvantages of this technique are that it is time consuming, and identification of any dipeptide ultimately rests on elution times or R_F values, which, in the case of chromatographically similar species, may lead to erroneous assignments.

MS appears to offer an ideal solution to this problem because it is fast, sensitive and potentially capable of giving an unequivocal answer. Three strategies can be envisaged in which mass spectrometry could be used:

(1) Separation of the dipeptide mixture into its components followed by derivatization and characterization of each derivative by mass spectrometry. This involves using the mass spectrometer merely to identify the dipeptide and leaves the problem of fractionation of the dipeptides as a separate unrelated problem.

(2) A second possibility, frequently mentioned but little exploited, is to obtain

mass spectra on the unseparated mixture of dipeptide derivatives and use a computer to analyse the results. This could ultimately be the solution, but the technique obviously needs a lot of development time directed to it. It is worth noting that where mixture analysis of peptides has been used, it has involved careful programming of the probe temperature and a study in the variation of the mass spectra with time. The technique is thus really a rather crude form of GC using the computer largely to resolve overlapping peaks.

(3) A further possibility exists in the derivatization of the peptide mixture, separation of this and subsequent MS identification of each component.

The obvious choice of separative technique is GC since this can be coupled directly to the mass spectrometer affording a saving in time, handling and sensitivity. Suitably volatile derivatives which have been suggested are trifluoroacetyl methyl esters[5], pentafluoropropionyl methyl esters[6] and acetyl permethylated methyl esters[8]. The use of trifluoroacetyl and pentafluoropropionyl methyl esters has been proposed as a general solution to the problem and examples of their use in peptide sequencing, using cathepsin C, have been given[5,6]. However, these examples were on *known* sequences and the universality of the technique was not established.

This paper is concerned with the GC behaviour of these derivatives of a representative collection of dipeptides. The simple acetyl methyl esters which are intermediates on the way to the permethylated derivatives were also examined. The MS data on these compounds will be reported elsewhere.

MATERIALS AND METHODS

The dipeptides were purchased either from Cyclo Chemical Corporation (Los Angeles, Calif., U.S.A.) or Calbiochem (Los Angeles, Calif., U.S.A.): Val–His, Trp–Trp and Lys–Gly were obtained from Calbiochem, Yeda (Rehovoth, Israel) and Mann Labs. (New York, N.Y., U.S.A.), respectively.

Trifluoroacetic anhydride was a product of Fluka (Buchs, Switzerland) and pentafluoropropionic anhydride was obtained from Pierce (Rockford, Ill., U.S.A.). The *n*-paraffin mixtures for retention indices and the Supelcoport, 80–100 mesh, came from Supelco (Bellefonte, Pa., U.S.A.), whilst Dexsil 300 GC is a product of Analabs (North Haven, Conn., U.S.A.). Sodium hydride was obtained from BDH (Poole, Great Britain) as a 60% dispersion in oil.

The gas chromatograph was a Packard-Becker Model 409 with temperature programmer and flame ionization detectors. Standard conditions were used throughout, *viz.*: Carrier gas (nitrogen) flow-rate 30 ml/min; glass column 2 m × 2 mm I.D. packed with 3% Dexsil 300 GC on Supelcoport; injection port temperature 200°; detector temperature 250°; 1-μl samples in methanol were injected on to the column at an initial oven temperature of 100° for 1 min and then programmed from 100–350° at 8°/min. After the retention time had been determined in this way, a second sample, mixed with the appropriate *n*-paraffin mixture, was injected using the same temperature program and the retention index thus determined.

EXPERIMENTAL

Conversion of the dipeptides to their methyl ester hydrochlorides was effected

by reaction with 1 *N* HCl in methanol at room temperature (20°) for 2 h followed by evaporation to dryness on a rotary evaporator.

Acetyl-, trifluoroacetyl- and pentafluoropropionyldipeptide methyl esters were prepared from the ester hydrochlorides by reaction with the appropriate acid anhydride for 1 h at room temperature and removal of excess of reagent on a rotary evaporator.

The permethylation of the acetyldipeptide esters was carried out as follows. About 200 mg of sodium hydride dispersion were weighed into a tared, stoppered centrifuge tube and washed three times with light petroleum (b.p. 40–60°), decanting the washing at each stage. The oil-free sodium hydride was then taken to dryness on a rotary evaporator and the tube re-weighed to give the clean dry weight of sodium hydride. Dry dimethyl sulphoxide was added in the proportion of 1 ml for every 24 mg sodium hydride, the centrifuge tube was stoppered with a punctured plastic cap and heated at 70° on a water-bath until effervescence ceased. The solution was then centrifuged (ca. 3000 *g*) and allowed to cool to room temperature. The acetyldipeptide methyl ester (1–2 mg) was treated with 250 μl of the supernatant dimethyl sulphoxide–sodium hydride reagent and 100 μl methyl iodide for 5 min at room temperature. Approximately 2 ml of chloroform were then added to the mixture followed by 7–8 ml of water. After thorough mixing and settling, the aqueous layer was discarded and the chloroform layer washed a further three times, with water, and dried over anhydrous sodium sulphate. The product was isolated by evaporation of the filtered chloroform solution in a stream of nitrogen.

RESULTS

Table I gives the retention indices of the acetyl-, trifluoroacetyl- and pentafluoropropionyldipeptide methyl esters studied. The results with the acetyl permethylated derivatives are referred to in the discussion.

DISCUSSION

If one compares the values for corresponding acetyl- and trifluoroacetyldipeptide methyl esters, it is apparent that the trifluoroacetyl derivatives are uniformly more volatile than the acetyl derivatives. For the 37 dipeptides for which data are presented the mean difference in retention index between corresponding trifluoroacetyl- and acetyldipeptide methyl esters is 273 points.

The pentafluoropropionyl derivatives are uniformly more volatile than the corresponding trifluoroacetyl compounds, but the difference in this case is much smaller and amounts only to an average of 36 points in retention index. (This average is based on values for 30 dipeptides and neglects the values for Ile–Thr and CmCys–Asn where the differences seem anomalously large although mass spectrometry shows these samples to be the expected, pure derivatives.)

Acetyl permethylated methyl esters were prepared from all the dipeptides using the above procedure but were found to give complex gas chromatograms with at least two major peaks in most instances. Since this behaviour pattern occurs with some glycine-containing dipeptides, it is not merely racemization which is involved and attempts to explain the patterns in terms of diketopiperazine formation or hy-

TABLE I

RETENTION INDICES OF ACETYL-, TRIFLUOROACETYL- AND PENTAFLUOROPROPIONYLDIPEPTIDE METHYL ESTERS (ALL L-L COMPOUNDS)

Peptide	*Derivative*		
	Acetyl	*Trifluoroacetyl*	*Pentafluoropropionyl*
Ile-Ala*	1905	1648	1926
Ile-Gly*	1943	1699	1671
Ile-Val*	1994	1729	1709
Ile-Ile*	2073	1800	1775
Ile-Leu*	2059	1800	1770
Ile-Phe*	2472	2161	2090
Ile-Pro*	2124	1890	1851
Ile-Met*	2362	2081	2046
Ile-Ser*	2155	1876	—
Ile-Thr*	2126	1934	1633
Ile-Asp*	2189	1909	1876
Ile-Glu*	2312	2027	1995
Ile-Tyr*	—	2440	2400
Ile-Asn*	—	1814	1928
Ile-Trp*	—	2635	2534
Ile-Gln*	—	2101	—
Ile-Lys*	—	2330	2239
Ala-Leu**	1910	1664	1639
Gly-Leu**	1980	1748	1715
Val-Leu**	1978	1730	1705
Leu-Leu**	2046	1800	1750
Phe-Leu**	2450	2147	2169
Pro-Leu**	2164	1909	1880
Met-Leu***	2344	2054	2000
Thr-Leu**	2134	1748	—
Tyr-Leu**	—	2444	2400
Asp-Leu**	2135	1850	1817
Trp-Leu**	—	2531	2418
His-Leu**	—	2497	2456
Lys-Leu**	—	2309	—
Val-Ile**	1991	1735	1713
Val-Val*	1910	1655	—
Gly-Phe§	2392	2103	2073
Gly-Asp§§	2111	1826	1808
Pro-Phe§§§	2631	2333	2288
Phe-Phe§§§	2906	2560	2504
CmCys-Asn*	—	2353	2118

* Supplied by Calbiochem.
** Supplied by Cyclo Chemicals.
*** Supplied by Bachem, Los Angeles, Calif., U.S.A.
§ Supplied by Dr. D. E. Rivett of these laboratories.
§§ Supplied by Yeda Chemicals.
§§§ Supplied by Dr. F. H. C. Stewart of these laboratories.
* Prepared by carboxymethylation of cysteinylasparagine[9].

drolysis of the dipeptides give no further insight into the unexpected behaviour. Since claims have been made that these derivatives are suitable for GC analysis[8], we have included in the experimental section full details of our permethylation technique. This

is based on that of Morris and co-workers[10,11] and in our hands has been consistently successful with larger peptides (four or more residues) but quite unreliable with di- and tripeptides. The large number of variants of the permethylation technique which have been published may perhaps indicate that a universally applicable procedure has so far not been achieved. Because of the complexity of the GC pattern and because also even the most volatile acetyl permethylated dipeptide combination was not significantly more volatile than the corresponding acetyl derivative, it was judged that these derivatives were quite useless for our purpose and they have not been further studied.

However, if GC–MS is to be a viable technique for analysing dipeptide mixtures, it is essential that it works satisfactorily with every possible dipeptide and in our experience none of the dipeptide derivatives we have studied meets this criterion. Some dipeptides which failed to give volatile derivatives in a specific instance are noted in the table, but there are other peptides which have been tried which have not given any volatile derivative. These include Val–His, Gly–Asn[9], Trp–Trp, Lys–Gly. No arginine-containing peptides were studied but they would certainly require additional modification of the guanido group in order to give a suitably volatile derivative.

We should also like to mention one observation that we have made on more than one occasion and that suggests some reservations about the technique in general. This is that a dipeptide derivative may fail completely to emerge from the column on the first attempt at gas chromatography and yet a subsequent sample may give a satisfactory peak. Obviously this is unimportant in a situation involving routine analysis for a small number of components, but in the application we envisage one could encounter the "once only" occurrence of one of more than 400 compounds and possibly fail completely to detect it.

Our general conclusion from this study is therefore that none of the dipeptide derivatives we have studied is suitable for the complete GC characterization of an unknown mixture of dipeptides.

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