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Note

Use of *tert.*-butyldimethylsilyl derivatives for gas chromatographic–mass spectrometric analysis of dipeptides

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Gas chromatographic–mass spectrometric (GC–MS) methods have been reported for the analysis and identification of dipeptides [1]. The dipeptides are often converted to trimethylsilyl (TMS) derivatives which are amenable to GC analysis and furnish mass spectra from which the N-terminal amino acid can be identified and the molecular weight established. Krutzsch and co-workers have reported the electron-impact (EI) mass spectra of about 200 dipeptides [2] and also the chemical-ionisation (CI) mass spectra of over 40 dipeptides [3]. In view of the weak M − 15 fragment seen in the EI spectra, they recommend the use of CI to determine the molecular mass of low concentrations of dipeptides. However, the TMS derivatives are not stable for more than a few hours and are highly sensitive to traces of water and active sites on used GC columns. The vigorous conditions (heating to 140°C for 10 min) required for their preparation can lead to cyclisation or modification of certain amino acid residues.

We now report the use of N-methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) to prepare *tert.*-butyldimethylsilyl (TBDMS) derivatives of several dipeptides and the EI and CI mass spectra of the TBDMS derivatives. These derivatives can be prepared under much milder conditions than TMS derivatives [4], removing the complications such as cyclisation when applied to dipeptides. The TBDMS derivatives are stable in heptane solution, chromatograph well and give more informative mass spectra in both the EI and CI mode than the corresponding TMS derivatives.

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EXPERIMENTAL

Materials

Amino acids, dipeptides, dichloromethane and pyridine were obtained from Sigma (Poole, U.K.). All amino acids and dipeptides were the L form. *n*-Heptane (Analar grade) was obtained from BDH (Poole, U.K.). Pyridine was dried by storage over potassium hydroxide pellets. MTBSTFA and MTBSTFA containing 1% *tert.*-butyldimethylchlorosilane (TBDMCS) as catalyst were obtained from Phase Separations (Clwyd, U.K.).

Gas chromatography–mass spectrometry

GC–MS was carried out on a Finnigan Mat 1020 GC–MS system, fitted with a 20 m×0.3 mm I.D. OV-1 WCOT chemically bonded fused-silica column (Pierce U.K., Cambridge, U.K.) programmed from 75 to 250°C at 15°C/min after an initial time of 1 min. Injection was in the splitless mode with an injector temperature of 280°C and a splitless injection time of 30 s. The carrier gas was helium at a pressure of 40 kPa. The mass spectrometer was operated either in the EI mode with an ionisation energy of 70 eV or in the CI mode using methane as the reagent gas at a source pressure of 0.34 Torr.

Sample preparation and derivatisation

Solutions (10 m*M*) of the dipeptides Gly-Gly, Leu-Leu, Leu-Gly, Gly-Leu, Ala-Leu, Leu-Ala and Ala-Gln in distilled water were prepared. Solutions of the mixed dipeptides and of the mixed dipeptides with an admixture of fourteen amino acids commonly found in biological materials, both containing *S*-methyl-L-cysteine as an internal standard, were also prepared at 10 m*M*.

Aliquots (10 μl) of these solutions were freeze-dried in polypropylene Eppendorf vials (Sarstedt, F.R.G.) and the residue was azeotroped with 2×250 μl dichloromethane under a gentle flow of nitrogen. Pyridine (10 μl) was added to the dried sample in the Eppendorf vial and after vortex-mixing, 10 μl MTBSTFA were added and the vial was tightly capped. The mixture was heated at 60°C for 90 min, cooled, and the reagents were evaporated under a stream of nitrogen at room temperature. The sample was dissolved in 100 μl of heptane and 1 μl was used for injection into the GC–MS system.

In some experiments the reaction time was varied from 30 to 120 min while the temperature was varied from 50 to 80°C. The reaction was also carried out using MTBSTFA containing 1% TBDMCS for comparison with the above.

To examine the application of the method to physiological fluids, 200 μl rat plasma were enriched with either Ala-Gly, Gly-Gly or Leu-Gly. These were deproteinised with 200 μl methanol–12 *M* hydrochloric acid (4:1) [5] and after centrifugation the supernatant was dried under a gentle stream of nitrogen at room temperature. Subsequent derivatisation was carried out as above, using 100 μl of both pyridine and MTBSTFA, and the product taken up in 250 μl of heptane.

TABLE I

RELATIVE MOLAR RESPONSE OF DIPEPTIDE TBDMS DERIVATIVES WITH REACTION TIME

Temperature, 60 °C; internal standard, *S*-methyl-L-cysteine; relative molar response of 1.

Dipeptide	Relative molar response			
	30 min	60 min	90 min	120 min
Leu-Leu	0.57	0.65	0.86	0.86
Gly-Gly	0.34	0.38	0.44	0.38
Leu-Ala	0.50	0.55	0.69	0.59
Ala-Leu	1.01	0.98	1.21	1.21
Leu-Gly	0.61	0.67	0.76	0.64
Ala-Gln	0.054	0.076	0.120	0.080

RESULTS AND DISCUSSION

The derivatisation procedure is similar to that used by Biermann et al. [6] for amino acids. The optimum conditions of 60 °C and 90 min achieve the maximum response for all the dipeptides relative to the methylcysteine internal standard, and further heating results in the loss of some dipeptide derivatives. Table I shows the molar response for each dipeptide relative to the internal standard when the reaction is carried out at 60 °C for various times. The low response for Gly-Gly and Ala-Gln was also noted when a flame-ionisation detector was used. The presence of 1% TBDMCS as catalyst in the MTBSTFA made no difference to the response of any of the dipeptides. The samples were stable at room temperature in heptane for at least 24 h. The GC column was also used for several other analyses including acidic mixtures, and no decomposition of the TBDMS derivatives was observed. This was a welcome finding as we had previously found TMS derivatives to be very sensitive to column contamination. The chromatograms obtained from the reaction in polypropylene Eppendorf vials compare favourably with those obtained using glass Reacti-Vials, with no extra peaks observed.

Fig. 1. shows the reconstructed ion current chromatogram for the dipeptide and amino acid mixture and Fig. 2 shows that of the dipeptide mixture alone. Of the dipeptides studied, all show chromatographic separation, except Leu-Gly and Gly-Leu. A number of isomeric dipeptides have been separated as TMS derivatives [7], but Leu-Gly and Gly-Leu were not included in this study. All the isomeric dipeptide TBDMS derivatives are readily distinguished by their mass spectra. The dipeptides separate from all the amino acids studied, although the retention times of the later running amino acids overlap the earlier running dipeptides.

In the plasma extracts, the dipeptide derivatives were also well separated from derivatives of the other plasma constituents (amino acids, keto acids and urea), and 25 μM concentrations of the dipeptides could be detected. This is a simple but highly effective method of sample preparation from plasma, requiring noth-

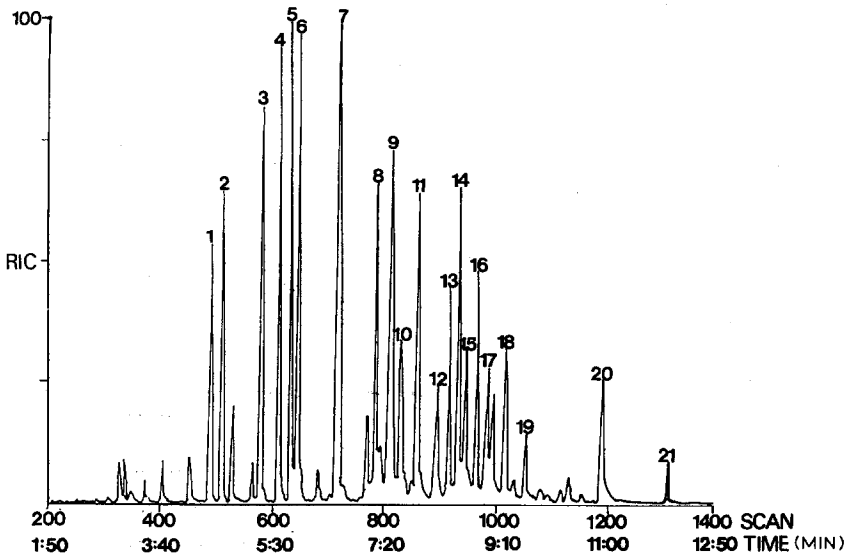

Fig. 1. Relative ion current (RIC) trace for dipeptide and amino acid mixture. EI mass spectra scanned from 100 to 600 a.m.u. over 0.5 s. Peaks: 1=alanine; 2=glycine; 3=valine; 4=leucine; 5=isoleucine; 6=proline; 7=methylcysteine; 8=methionine; 9=serine; 10=threonine; 11=phenylalanine; 12=glycylglycine; 13=aspartate; 14=alanylleucine; 15=leucylalanine; 16=leucylglycine and glycylleucine; 17=glutamate; 18=leucylleucine; 19=lysine; 20=tyrosine; 21=alanylglutamine.

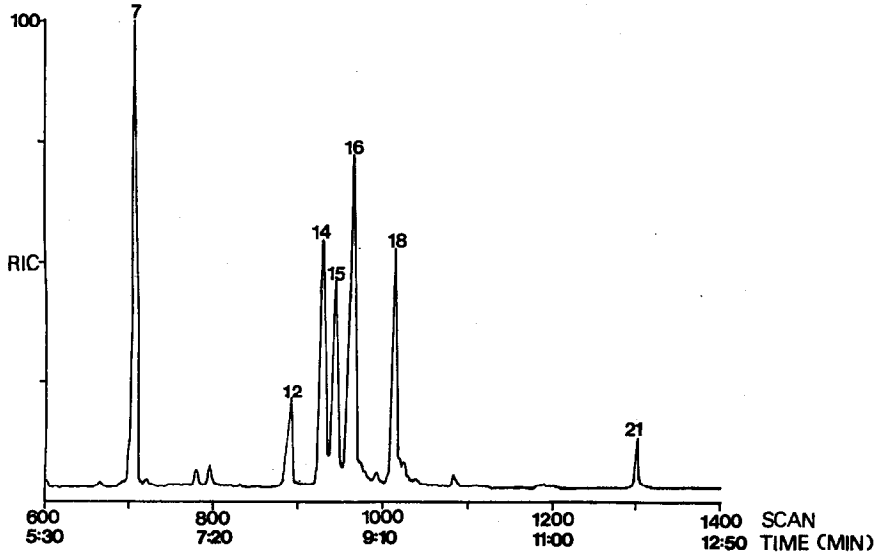

Fig. 2. Relative ion current (RIC) trace for dipeptide mixture. Conditions and peak identification as in Fig. 1.

ing other than protein precipitation, and it can be applied to physiological studies of dipeptide transport.

TABLE II

CI AND EI MASS SPECTRAL FRAGMENTATION OF DIPEPTIDE TBDMS DERIVATIVES

Dipeptide	M^+	M−15	M−57	M−85	M−159	M−302	M+1	M+29	M+41	R+143	R+103
Leu-Leu											
m/z	472	457	415	387	313	170	473	501	513	200	160
EI(%)			8			4				100	18
CI(%)		46	50				100	15	5	39	
Gly-Gly											
m/z	360	345	303	274	201	58	361	389	401	144	104
EI(%)		2	45	2						20	100
CI(%)		30	40				100	15	6	3	
Leu-Ala											
m/z	430	415	373	345	271	128	431	459	471	200	160
EI(%)			8			5				100	23
CI(%)		40	47				100	14	5	36	
Ala-Leu											
m/z	430	415	373	345	271	128	431	459	471	158	118
EI(%)			18			7				100	42
CI(%)		41	43				100	16	6	25	
Gly-Leu											
m/z	416	401	359	331	257	114	417	445	457	144	104
EI(%)			15							28	100
CI(%)		36	44	2			100	18	6	4	
Leu-Gly											
m/z	416	401	359	331	257	114	417	445	457	200	160
EI(%)			16			1				100	31
CI(%)		38	44				100	15	7	25	2
Ala-Gln											
m/z	559	544	502	474	400	257	560	588	600	158	118
EI(%)			4							100	18
CI(%)		62	53	3	3	2	96	16	4	58	

Mass spectra

The significant ions in the EI and CI mass spectra of a number of dipeptides are shown in Table II and the EI mass spectra of Ala-Leu and Leu-Ala and their constituent amino acids are shown in Fig. 3A–D. The mass spectra confirm that these are all di-TBDMS derivatives except for Ala-Gln which is a tri-TBDMS derivative. Unlike the TMS derivatives of N-glycyl dipeptides there is no cyclisation to a diketopiperazine.

All the spectra contain clear information about both the N-terminal amino acid and the molecular mass of the dipeptide. This information may be extracted from either the EI or CI spectrum. The CI spectra give the clearest indication of the molecular mass with the base peak at M+1, confirmatory adduct ions at M+29 and M+41 and fragment ions at M−15 and M−57. The M−57 ion in the EI spectrum is normally 10% or more of the base peak and a confirmatory M−15 peak may be detected. The dominant ions in the EI spectrum originate from the N-terminal amino acid. The base peak normally results from cleavage of CH–CO bond to the N-terminal acid (Fig. 4), as observed in the TMS derivatives. This

fragment is also found in the TBDMS derivatives of the corresponding amino acid, but at a lower intensity. A further peak is observed at an *m/z* 40 less than this fragment, usually 20–50% of the base peak except in the N-glycyl dipeptides where this fragment is the base peak. No similar peak is noted in dipeptide TMS derivatives and it is not significant in the amino acid TBDMS derivatives. This fragment may arise from cleavage of the central NH–CH bond followed by loss of *tert.*-butyl and a cyanide radical (Fig. 4). The absence of an intense *m/z* 147

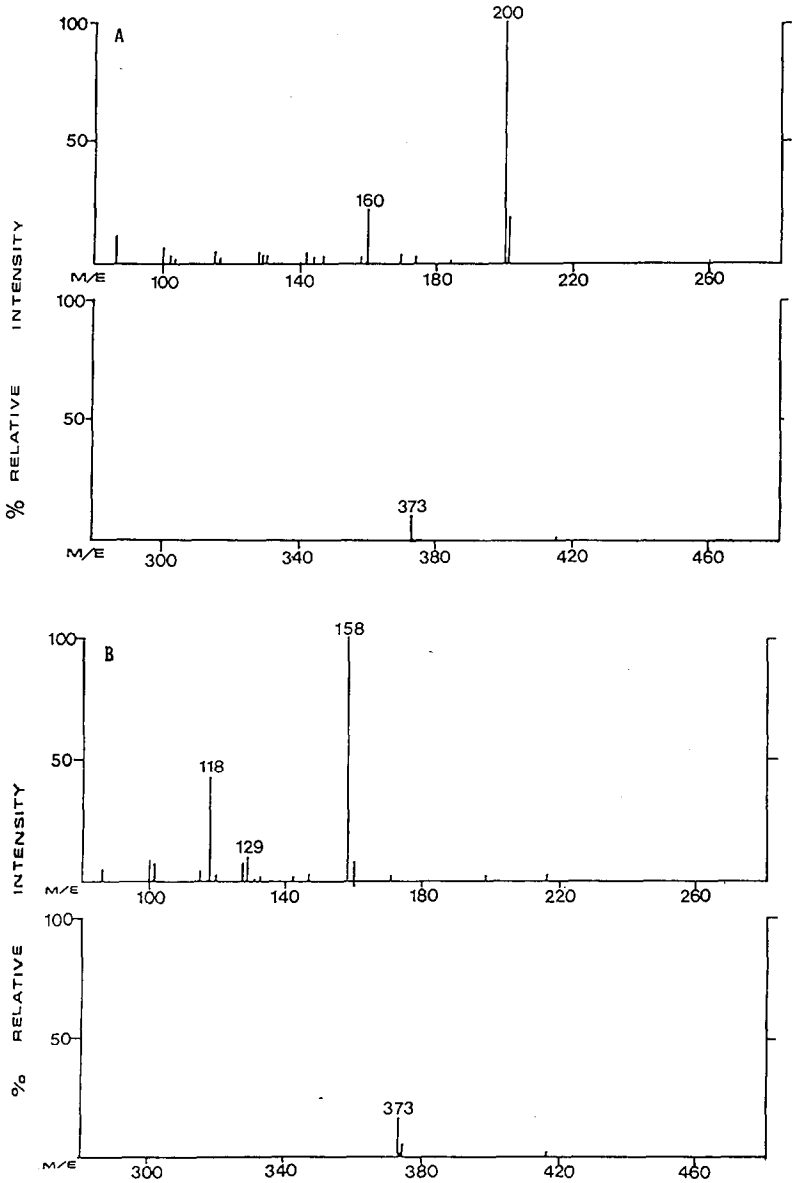

Fig. 3.

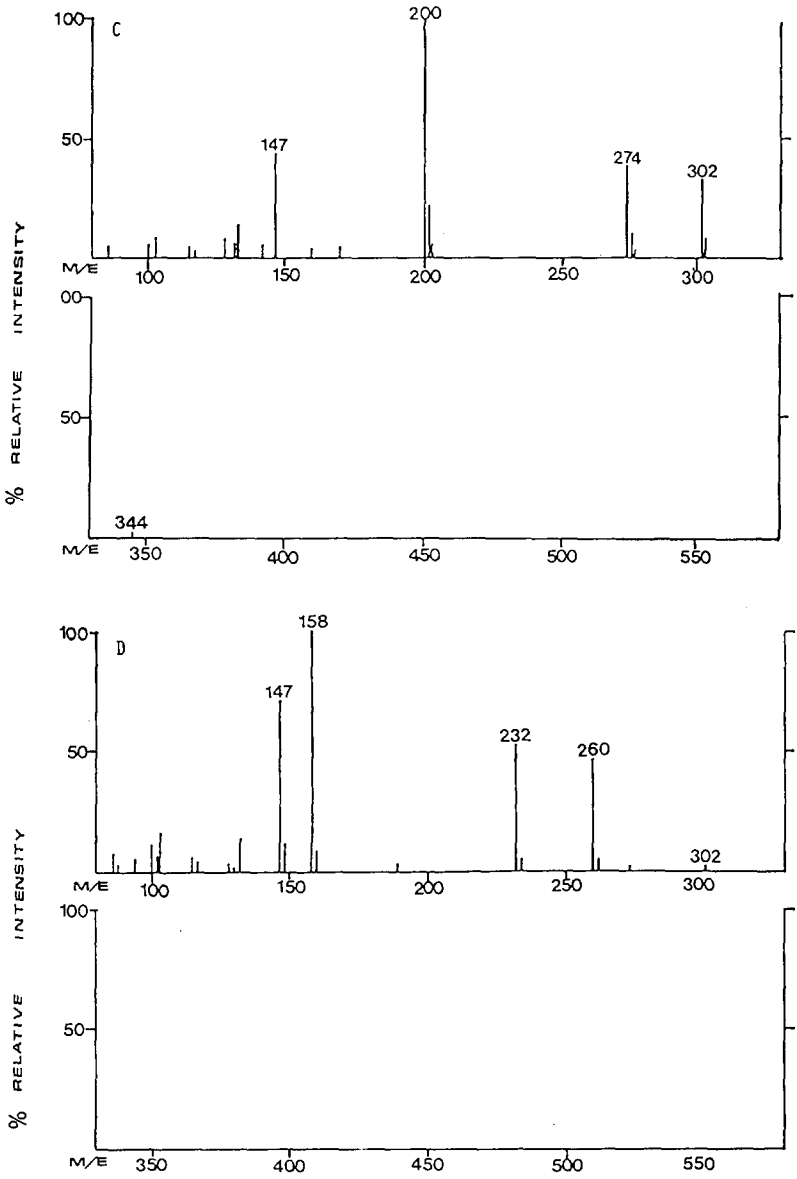

Fig. 3. Electron impact mass spectra of leucylalanine (A), alanylleucine (B), leucine (C) and alanine (D).

ion and the distinctive pair of ions separated by *m/z* 40 clearly distinguish the spectra of amino acid and dipeptide TBDMS derivatives.

CONCLUSION

TBDMS derivatives are a useful new method for GC–MS analysis of dipep-

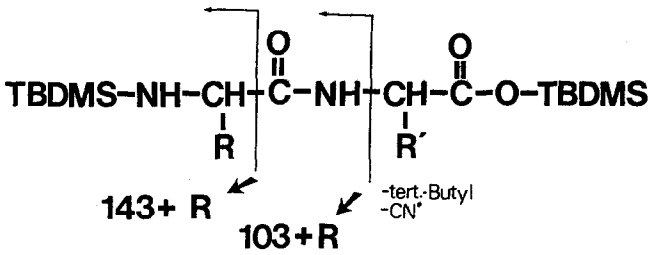

Fig. 4. Schematic representation of the cleavage of TBDMS derivatives of peptides.

tides. They are formed under mild conditions by the reaction of the dipeptide with MTBSTFA in pyridine. Once formed, the derivatives are stable in heptane solution and are not sensitive to active sites on the GC column. Their mass spectra give definitive information about the N-terminal amino acid and the molecular mass of the dipeptide in either the EI or CI mode. Concentrations of 25 μM of dipeptides in plasma can be detected after deproteinising the plasma with acidic methanol and derivatisation.

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