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GAS-LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF DEUTERIUM-CONTAINING AMINO ACIDS AS THEIR TRIMETHYLSILYL DERIVATIVES

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SUMMARY

The gas-liquid chromatography and mass spectrometry of specifically labeled deuterium-containing amino acids as their trimethylsilyl derivatives has been investigated. In many cases the retention time of the deuterated compound was measurably shorter than that of its protium analog. The small retention time differences, to a first approximation, were proportional to the number of deuterium atoms. Under our experimental conditions separation of mixtures (bifurcated peak) was observed within only two pairs of compounds—leucine and leucine-D_{10}, and isoleucine and isoleucine-D_{10}. Fractionation of the protium and deuterium forms within a single gas-liquid chromatography peak has been demonstrated by combined gas-liquid chromatography-multiscan mass spectrometry. Comparison of the mass spectra of normal amino acid trimethylsilyl derivatives and their deuterium-containing analogs has facilitated the elucidation of the mass spectral fragmentation patterns observed for these compounds.

INTRODUCTION

We have recently reported on the gas-liquid chromatography-mass spectrometry (GLC-MS) of the trimethylsilyl (TMSi) derivatives of carbon-13 enriched (15% randomly labeled) amino acids[1,**]. The presence of the isotopic labels facilitated the assignment of structure to mass spectral fragmentation products. While no evidence was found for the GLC separation or fractionation of the normal and corresponding carbon-13 enriched amino acids, there are reports in the literature on GLC separation of a variety of deuterated compounds from their protium analogs. The methyl esters of perdeutero fatty acids have been shown to possess shorter retention times than the corresponding normal esters[4], and isotope fractionation of protium and deuterium

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** BAKER *et al.*[2] and TEETER[3] have reported on the MS of TMSi derivatives of several amino acids.

forms of sugars (as TMSi ethers) during GLC has also been reported[5,6]. More recently, WALLER *et al.*[7] observed the GLC separation of TMSi and TMSi-D_9 derivatives of α-D-glucose prepared from normal and deuterated reagents, respectively; for a penta-ether, this results in a molecular weight difference of 45 amu, but the deuterium-containing derivative exhibits the shorter retention time. Other workers have also observed the effect of isotope substitution upon GLC behavior (see refs. 1 and 4–7). The availability of a considerable number of isotopically pure deuterated amino acids (obtained from algae grown in deuterium oxide (99%) by hydrolysis[8] and separated by ion-exchange chromatography[9]) has enabled us to investigate the effect of deuterium content upon the GLC behavior of the TMSi derivatives of these important natural products. Further, mass spectrometry of these specifically labeled compounds has allowed an instructive comparison with our earlier comments[1]. We now wish to present the results of our study on the GLC and MS of deuterated amino acids.

EXPERIMENTAL

GLC retention behavior studies were carried out with a Barber-Colman Model 5000 instrument (hydrogen flame ionization detection). The column was a 12 ft. × 4 mm glass W-tube; 2% F-60 (DC-560) coated over 1.5% SE-30 on 80–100 mesh acid-washed and silanized[10] Gas-Chrom P; 20 p.s.i. Four different temperatures were employed for the isothermal separations because of the widely different retention times of the amino acid derivatives: 90° (alanine, glycine), 105° (valine, proline, leucine, isoleucine, serine, threonine), 150° (methionine, aspartic acid, phenylalanine, glutamic acid), 180° (tyrosine, lysine); the amino acids are listed in order of increasing retention time. Unless indicated otherwise, all carbon-bound hydrogen is replaced by deuterium in the deuterated amino acids. Partial exchange during the isolation procedure resulted in incomplete deuteration with aspartic and glutamic acids[9]. Methionine-α,β-D_3 was prepared synthetically by MARKLEY[11]* following the method of MARTIN AND MORLINO[12]. The TMSi derivatives were prepared as in ref. 1.

The LKB Model 9000 instrument, fitted with a 6 ft. × 3.5 mm I.D. spiral glass column packed with 3% OV-1 on acid-washed and silanized[10] Gas-Chrom P, was employed for combined GLC–MS experiments. Spectra were obtained with the previously reported instrument conditions[1].

RESULTS AND DISCUSSION

The retention times of the deuterium-containing amino acid TMSi derivatives are less than those of their protium analogs. To a first approximation, the retention time difference was proportional to the number of deuterium atoms. For example, the separation factor (retention time normal acid/retention time deuterium-containing acid) for the pair glycine and glycine-D_2 is < 1.01, and that for phenylalanine and phenylalanine-D_8 is 1.02 (see Table I). In addition to a measurable difference in retention times between the members of most pairs of amino acid derivatives when chromatographed individually, in a number of cases GLC of a mixture of the two compounds resulted in a peak broadening effect. This is reflected by a peak width at half-height for the mixed peak measurably larger than that for either component run

* We are grateful to Dr. MARKLEY for this sample.

TABLE I

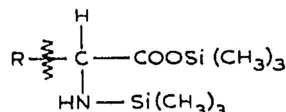
EFFECT OF DEUTERIUM CONTENT UPON GLC BEHAVIOR OF AMINO ACIDS AS TMSi DERIVATIVES

Compound	*Separation factor*	*Peak width (cm) at half height*[a]	
		Individually	*Mixture*
Gly	< 1.01	0.28	0.29
Gly-D_2		0.27	
Ala	1.02	0.25	0.30
Ala-D_4		0.23	
Val	1.02	0.22	0.31
Val-D_8		0.22	
Phe	1.02	0.24	0.32
Phe-D_8		0.25	
Leu	1.04	0.29	0.59[b]
Leu-D_{10}		0.28	
Ile	1.04	0.31	0.68[b]
Ile-D_{10}		0.30	

[a] These values are ± 0.01 cm.
[b] Partial separation is observed on the chromatogram.

separately (see Table I), and can also be discerned in the more favorable instances by simple visual inspection. For example, the difference in GLC retention behavior between the TMSi derivatives of valine and valine-D_8 is reflected by a separation factor of 1.02, but the peak resulting from chromatography of a mixture shows only a slight broadening, and no indication of splitting. In only two instances (leucine and leucine-D_{10}, and isoleucine and isoleucine-D_{10}) was separation (peak bifurcation) clearly visible (see Fig. 1). Those compounds which differ the greatest in deuterium content exhibit the greatest separation. The greater volatility of the labeled compounds is in complete agreement with the observations of other workers[4–7].

In our previous paper[1] we described four pathways of fragmentation which amino acid TMSi derivatives undergo during MS, and suggested that they are (a) loss of a methyl group from the molecular ion M (M-15)*; (b) loss of methyl and CO with retention of the silyloxy group (M-43); (c) loss of carbotrimethylsilyloxy (M-117)*; (d) loss of the amino acid side chain group R, *i.e.*,



to yield a fragment of m/e 218 (previously reported by PIERCE[13]). Other signals at m/e 73, $[Si(CH_3)_3]^+$, and 147, $[(CH_3)_2Si{=}O{-}Si(CH_3)_3]^+$, are commonly observed with O-TMSi compounds. Concerning fragmentation (a) there can be little doubt with those amino acids which do not possess a side chain methyl group (*e.g.*, proline, phenylalanine) that the eliminated methyl comes from a TMSi group. For methyl group-containing amino acids such as valine and leucine, however, the methyl could

* Also observed by BAKER *et al.*[2].

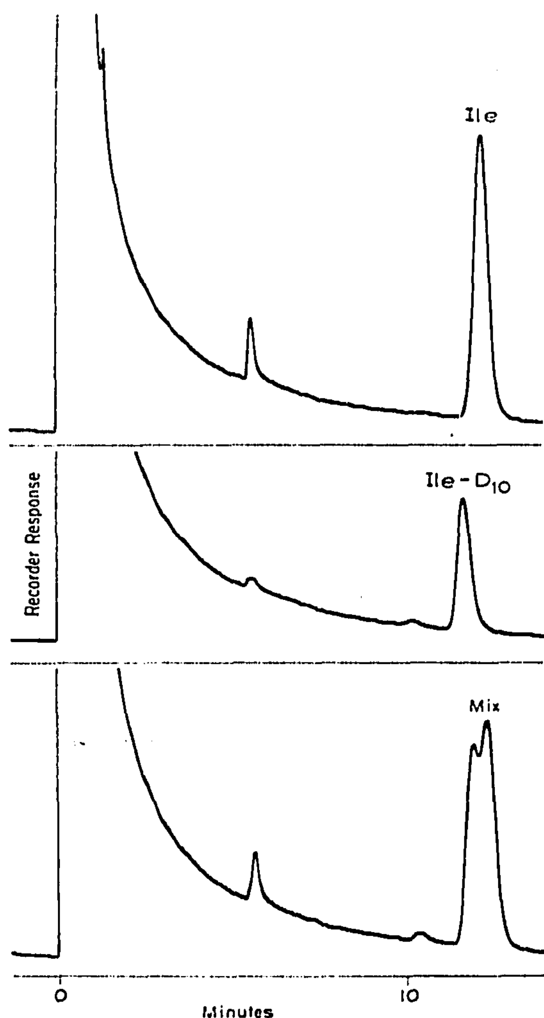


Fig. 1. GLC analysis of the TMSi derivatives of isoleucine (top panel), isoleucine-D_{10} (middle panel) and a mixture of these two compounds (bottom panel).

conceivably be lost from either a TMSi group or the amino acid moiety. Mass spectrometry of the carbon-13 enriched amino acids proved to be very helpful with this problem, and simple calculations based on the carbon-13 content of the fragments (reflected in the (S+1) isotope peak) indicated that a TMSi group is the source of the expelled methyl group[1]. Deuterium labeling has been employed extensively in MS to deduce fragmentation modes and the structure of fragment ions, as has been exemplified by the recent reports of CAPELLA AND ZORZUT[14] and DIEKMAN *et al.*[15]. That our conclusion concerning the source of the lost methyl group was correct can be seen in Table II, where one observes (M-15), rather than (M-18), fragments for the TMSi derivatives of the methyl group-containing deuterated amino acids. Within each pair of these compounds the difference in m/e values for the fragment ions resulting from loss of a methyl group corresponds exactly to the difference in molecular weight between the two derivatives. Loss of methyl and CO (b), and loss of carbotrimethylsilyloxy (c), are seen not to involve deuterium-containing portions of the amino acids. Finally, the identity of the 218 fragment, observed for many of the amino acids, is also con-

TABLE II

MASS SPECTRAL FRAGMENTS FOR NORMAL AND DEUTERATED AMINO ACIDS AS TMSi DERIVATIVES

Amino acid	*M*[a] *or M-15*[b]	*M-43*[c]	*M-117*[d]	*M-R*[e]
Gly	204	176	102	—
Gly-D_2	206	178	104	—
Ala	218	190	116	218[f]
Ala-D_4	222	194	120	219
Pro	244	216	142	—
Pro-D_7	251	223	149	—
Val	246	218[f]	144	218[f]
Val-D_8	254	226	152	219
Leu, Ile	260	232	158	218
Leu-D_{10}, Ile-D_{10}	270	242	168	219
Met	293*, 278	250	176	218
Met-D_3	296*, 281	253	179	219
Phe	294	266	192	218
Phe-D_8	302	274	200	219
Asp	349*, 334	306	232	218
Asp-$D_{1,2,3}$	350*, 335	307	233	219
	351*, 336	308	234	
	352*, 337	309	235	
Tyr	382	354	280	218
Tyr-D_7	389	361	287	219

[a] M indicated by asterisk.
[b] Loss of methyl.
[c] Loss of methyl + CO.
[d] Loss of carbotrimethylsilyloxy.
[e] Loss of amino acid side chain.
[f] See p. 410.

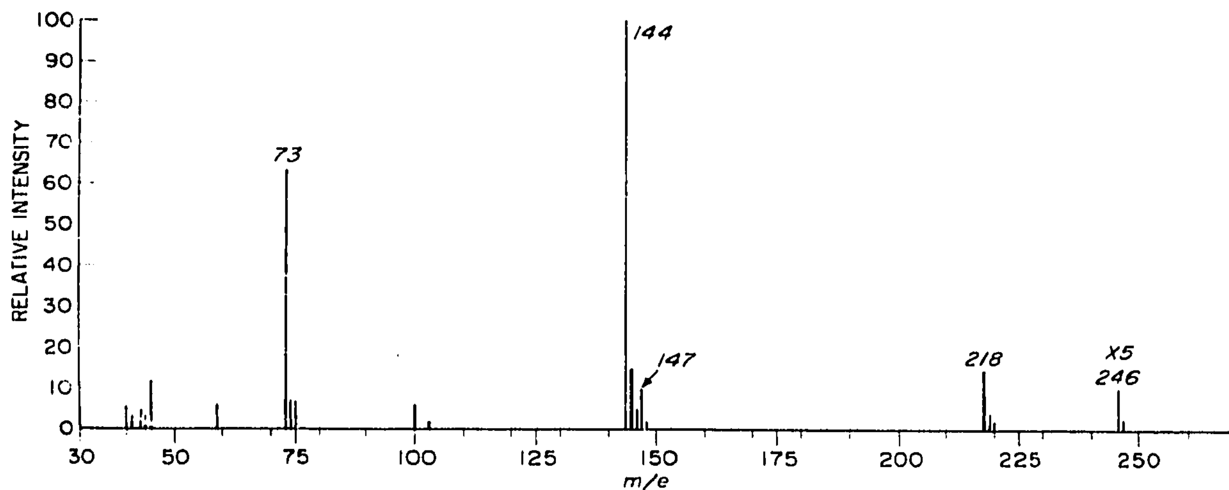


Fig. 2. Mass spectrum of the TMSi derivative of valine.

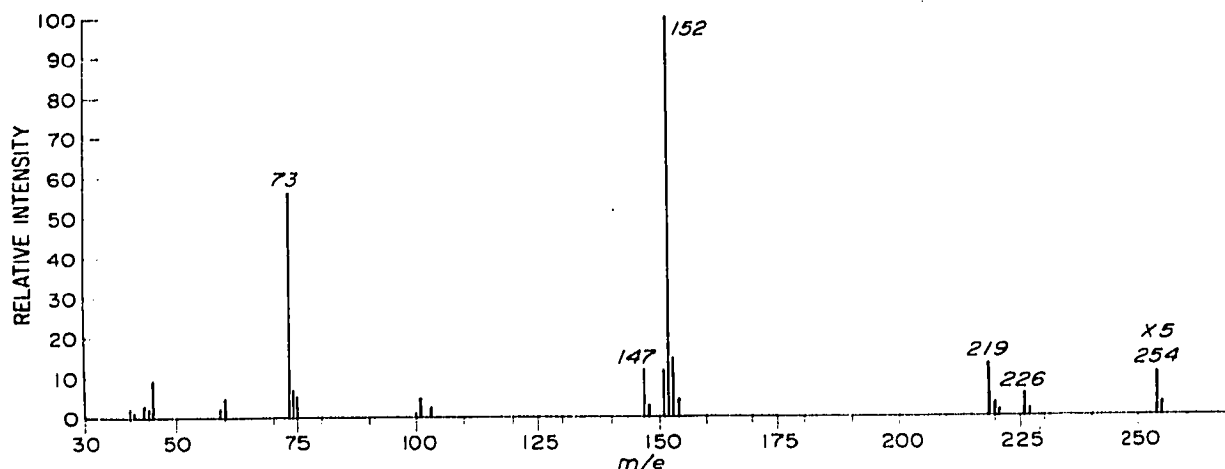


Fig. 3. Mass spectrum of the TMSi derivative of valine-D_8.

firmed, since it is replaced by a fragment 1 amu greater for the deuterated analogs (see Table II).

The mass spectrum of the TMSi derivative of valine is presented in Fig. 2, and contains fragments as described above, *e.g.*, (M-15) (*m/e* 246), and (M-COOSi$(CH_3)_3$) (*m/e* 144). A signal is also noted, as expected, at *m/e* 218. This fragment could arise either by loss of methyl and CO (b), or by loss of the isopropyl side chain (d); both result in loss of 43 amu. Data obtained from the carbon-13 enriched amino acid led to the suggestion that the main pathway was the latter[1]. As is clear from the mass spectrum of the TMSi derivative of valine-D_8 (Fig. 3), this is indeed the main pathway, for there is a strong signal at *m/e* 219 (M-50). The alternative pathway, loss of methyl and CO, is not insignificant, however, for a fragment of *m/e* 226 (M-43) is readily apparent. It was also deduced from work with carbon-13 enriched alanine that its *m/e* 218 fragment is formed by loss of methyl from a TMSi group (a), and not the amino acid methyl (d)[1]. Examination of the mass spectrum of the TMSi derivative of alanine-D_4 has shown this to be essentially correct; however, in addition to the expected *m/e* 222 signal, one also observes a much less intense (1/7) signal at *m/e* 219, indicating a minor contribution of pathway (d).

A number of methods are frequently employed to determine peak homogeneity in GLC, *viz.* increase in column efficiency (*e.g.*, use of a longer column)[5], use of both non-selective and selective stationary phases[10,16], and exposure of the sample to derivative formation conditions[10]. The homogeneity of a peak may also be examined by taking a mass spectrum, either in direct combination with a GLC column[10,17–19] or by conventional means following collection[20,21]. If the peak is multi-component in nature, and if the components exhibit significantly different mass spectra, the non-homogeneity will be apparent from visual inspection of the composite spectrum. If the co-eluted compounds undergo partial fractionation but not separation, collection of several fractions during elution of the peak followed by MS of each "cut" may indicate the multi-component nature of the peak[20]. The peak may be scanned successively during combined GLC–MS, and the spectra will be seen to change with time across the peak. With this latter approach, one is using MS as a highly selective detection system, and, in a sense, the combination of techniques possesses greater

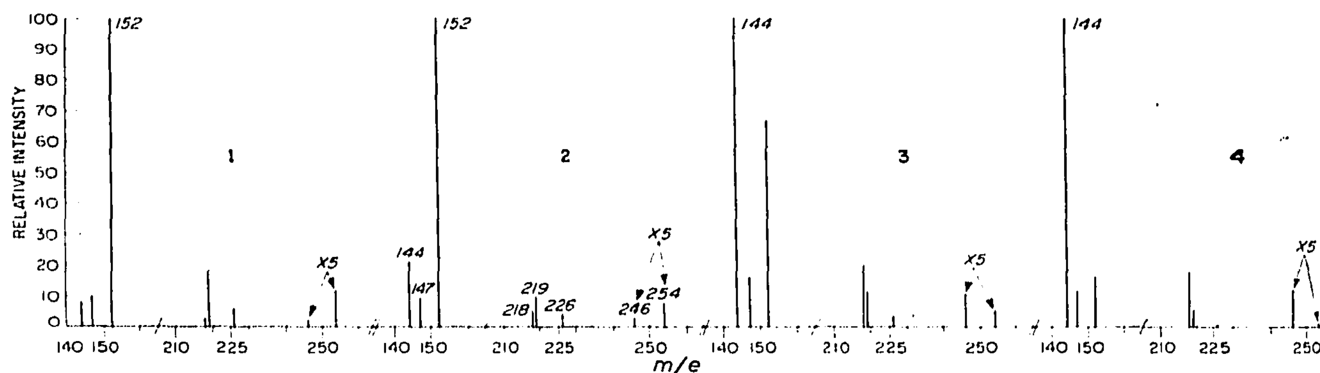


Fig. 4. Partial mass spectra (separated by double slash marks) obtained for four successive scans across the GLC peak resulting from chromatography of a mixture of the TMSi derivatives of valine-D_8 and valine. Spectra 1 and 2 were produced by scanning the ascending portion of the GLC peak, and spectra 3 and 4 result from scans on the descending side of the peak. The *m/e* values for all the signals are given in spectrum 2—152, 219, 226 and 254 arise from valine-D_8; 144, 218 and 246 are the corresponding signals for valine.

GLC resolution than the same column equipped with a normal mass detector (*e.g.*, hydrogen flame detector). SWEELEY *et al.*[6] were able to demonstrate isotope fractionation of the TMSi derivatives of α-D-glucose and α-D-glucose-D_7 on a short (2 m) GLC column by this means. We have utilized this multiple scanning MS approach to detect unresolved normal and deuterated amino acids in GLC effluents (Fig. 4). The symmetrical peak resulting from the chromatography of an approximately 1:1 mixture of valine and valine-D_8 (TMSi derivatives) was subjected to four successive scans, two (1 and 2) on the ascending side, and two (3 and 4) on the descending side. The partial (*m/e* 140–260) mass spectra from the four scans are presented in Fig. 4. Panel 1 (scan 1) corresponds favorably to the mass spectrum of valine-D_8 (Fig. 3), with only relatively small signals at *m/e* 144, 218 and 246, indicating that the fraction of the peak scan 1 represents is mainly valine-D_8. The proportion of normal valine is seen to have increased by the time of scan 2. The composition of the peak at scan 3 has changed radically—the signals for the valine fragments are of greater intensity than the corresponding signals from valine-D_8 (*m/e* 152, 219 and 226, and 254), and the spectrum resulting from scan 4 is essentially that of the TMSi derivative of valine (Fig. 2). The number of deuterium atoms necessary for the occurrence of such GLC fractionation is not large; using the multi-scan GLC–MS technique we have demonstrated this phenomenon within pairs of amino acids which exhibit separation factors of 1.01 or less (*e.g.*, glycine and glycine-D_2).

The use of deuterium-labeled drugs as an aid in the mass spectrometric identification of metabolites has been discussed by BOYD AND BULLOCK[22]; the recognition of fragment ions arising from drug-related compounds is facilitated by the presence of the elevated isotope peaks. MCCLOSKEY *et al.*[23] have discussed the value of deuterium-labeled trimethylsilyl derivatives (*i.e.*, TMSi-D_9) in the structure determination of unknown molecules. Combined GLC–MS techniques are finding increasing use in drug metabolism studies[24–31], and the possibility of encountering the GLC isotope fractionation of deuterium-labeled and non-labeled drug or metabolite is very real. Indeed, it could serve as an additional means for distinguishing drug-related substances.

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