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Determination of the enantiomeric purity of synthetic peptides by gas chromatography–mass spectrometry

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ABSTRACT

Hydrolysis using ^2H-labelled HCl and H_2O, derivatization of free amino acids as N,O,S-trifluoroacetyl isobutyl esters, separation by gas chromatography on a chiral stationary phase and detection by mass spectrometry in selected-ion monitoring mode have been used in order to determine the enantiomeric purity of several synthetic peptides. Chromatographic separation has been optimized for proline, whose two enantiomers are difficult to resolve under standard conditions. Electron impact and methane chemical ionization mass spectra and chromatographic resolution of unnatural amino acids, such as 3-(1-naphthyl)-alanine and *p*-chlorophenylalanine, are reported. For both natural and unnatural amino acids selected-ion monitoring of the different fragmentation peaks has been carried out. The results are interpreted from the point of view of whether or not the fragments contain a hydrogen atom on the α-carbon, and a comparison between electron impact and methane chemical ionization has been carried out. The main advantage of the latter method is that a quasimolecular ion can be observed for all the amino acids studied.

INTRODUCTION

Racemization of amino acid residues in a synthetic peptide is one of the most important side-reactions in peptide synthesis. It can take place at several points in the synthesis: at the coupling of the residues, at the deprotection step, or during the cleavage of the peptide from the resin [1]. Conventional methods of determination of the enantiomeric purity involve acid hydrolysis of the peptide, derivatization of the free amino acids and separation of the enantiomers using a suitable system [2–4]. None of these methods takes into consideration the racemization produced in the acid hydrolysis step. Several laboratories have proposed hydrolysis of the peptide in ^2HCl–^2H_2O followed by esterification, perfluoroacylation and analysis by capillary gas chromatography (GC)–mass spectrometry (MS), using a chiral stationary phase, of the individual amino acids. Under these conditions, the inversion of the chiral centre of the amino acid takes place by enolization of the carbonyl group giving a planar species. In the absence of non-deuterated hydroniun ions, the new enantiomer formed will be labelled with deuterium in the α-position and these molecules will weigh one unit more. Detec-

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tion by MS provides a way of distinguishing normal amino acids from labelled amino acids.

Hartmut *et al.* [5] have used this deuterium-labelling method, derivatizing the amino acids as N,O,S-pentafluoropropionyl isopropyl esters and analysing them by capillary GC with chemical ionization (CI) MS, using the chiral stationary phase Chirasil-Val. Kusumoto *et al.* [6] and simultaneously Liardon *et al.* [7] employed N,O,S-trifluoroacetyl isopropyl esters of the amino acids on the same stationary phase, but electron impact (EI) was used as the ionization method. In this case some amino acids, such as Trp, could not be analysed because there is no detectable ion containing a hydrogen atom on the α-carbon. Liardon *et al.* [7] also proposed a set of equations for the exact calculation of D-isomer content and the hydrolysis-induced racemization from the experimental values obtained from the integration of the selected-ion monitoring chromatograms.

Here we report the application of the deuterium-labelling method to several amino acids, derivatized as N,O,S-trifluoroacetyl isobutyl esters, that are of particular interest. First, we studied the separation of proline, an amino acid whose enantiomers are usually poorly resolved compared with other amino acids [7,8]. In the search for new peptides with improved stability against proteolysis for therapeutic use, a common trend is the substitution of naturally occurring amino acids by unnatural amino acids. We have also studied the EI and methane CI mass spectra and separation conditions of racemic amino acids 3-(1-naphthyl)-alanine and *p*-chlorophenylalanine. Finally, a comparison between EI and methane CI has been carried out, using the equations proposed by Liardon *et al.* in both EI and CI modes.

EXPERIMENTAL

Reagents

^{2}HCl was from Aldrich (Steinheim, F.R.G.), 20% solution in ^2H_2O, 99%. Ethylmercaptan was obtained from Fluka (Buchs, Switzerland). Isobutanol was from Scharlau (Barcelona, Spain). HCl was obtained from Union Carbide (Livingston, U.K.). Acetonitrile was from Merck (Darmstadt, F.R.G.), HPLC grade. Trifluoroacetic anhydride was obtained from Koch-Light (Colnbrook, U.K.). Standards of racemic amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). Methane was from Linde (Unterschleissheim, F.R.G.).

Hydrolysis and derivatization

About 2–5 mg of peptide, 0.5 ml of 6 *M* ^{2}HCl and a drop of ethylmercaptan were placed in a screw-cap culture tube reduced to 2 cm in length with a blow-pipe. Hydrolysis took place for 18–20 h at 110°C under argon. The mixture of free amino acids was treated with 400 μl of isobutanol–3 *M* HCl at 110°C for 1 h. Isobutanol was evaporated at 100°C under an argon stream, and 100 μl of acetonitrile and 70 μl of trifluoroacetic anhydride were added. The mixture was reacted

for 10 min at 110°C and was evaporated to dryness under an argon stream at room temperature. Samples were diluted in 10–30 μl of acetonitrile, and 1–2 μl of this solution were injected in the GC–MS system.

Gas chromatography–mass spectrometry

General conditions for the GC separation of amino acid enantiomers and their quantitative determination by MS were as follows, using a Hewlett-Packard 5988 A system, high mass option. (Hewlett-Packard, Palo Alto, CA, U.S.A.), controlled by an MS Chemstation HP 59970, revision 3.1.1 software: fused-silica capillary column (50 m × 0.25 mm I.D.) coated with Chirasil-Val III (Applied Science Labs., State College, P.A., U.S.A.); carrier gas (helium) flow-rate, 3.2 ml/min; inlet split ratio, 30:1; injector temperature, 250°C; transfer line temperature, 200°C. Three temperature programmes were used. Programme A: initial temperature, 60°C; final temperature, 200°C; rate, 4°C/min; final time, 20 min; total time, 55 min. Programme B: several isothermal conditions (120, 130, 140 and 150°C) were used; after all the amino acids were eluted, the oven temperature was raised to 200°C in order to clean the column. Programme C: initial temperature, 60°C; final temperature I, 150°C; rate I, 2°C/min; final temperature II, 200°C; rate II, 4°C/min; final time, 40 min; total time, 97.5 min.

In EI mode, the ion source was at 250°C and the pressure was *ca.* $2–3 \cdot 10^{-6}$ Torr. Scans were taken in 40–500-dalton range, with a threshold of 50 counts. The number of analogue-to-digital measurements per datum point was 2, corresponding to 1.86 scan/s. For quantitative determinations, the mass spectrometer was run in selected-ion monitoring mode with a dwell time of 100 ms. Peak surface areas were measured by post-run integration.

CI was carried out with methane as reagent gas at 150°C, and the pressure in the ion source was 1–2 Torr. Scans were taken from 100 to 600 dalton with a threshold of 50 counts, the number of analogue-to-digital measurements per datum point was 4, corresponding to 0.86 scans/s. For quantitative determinations, the mass spectrometer was run in selected-ion monitoring mode with a dwell time of 100 ms. Peak surface areas were again measured by post-run integration.

RESULTS AND DISCUSSION

Determination of the enantiomeric purity of proline is of interest because this amino acid is placed in the C-terminal position when the risk of racemization is high (segment coupling, basic treatments, etc.) owing to its low tendency to racemize. It is often simply assumed that racemization does not take place with this residue, but is interesting to be able to determine exactly the degree of this side-reaction.

We have studied the racemization in two synthetic peptides rich in proline that correspond to the N-therminal repeat sequence of maize glutelin-2 (Fig. 1). The first one is Boc-Val-His-Leu-Pro-Pro-Pro-OH (I). This peptide was cleaved from

Boc-L-Val-L-His-L-Leu-L-Pro-L-Pro-L-Pro-OH (I)

H-(L-Val-L-His-L-Leu-L-Pro-L-Pro-L-Pro)$_2$-OH (II)

H-D-β-Nal-L-Cys-L-Tyr-D-Trp-L-Lys-L-Val-L-Cys-L-Thr-NH$_2$ (III)

Fig. 1. Synthetic peptides used in this study.

the resin by treatment with dioxane–methanol–4 *M* NaOH (30:9:1) for 3 min and dioxane–2-dimethylaminoethanol (DMAE)–4 *M* NaOH (30:9:1) for 15 min [9,10]. Even though these are short treatment times, the use of base implies, at least in principle, some risk of racemization, and it is necessary to determine the amount (if any) at this point, in the C-terminal proline residue. The second peptide was H-(Val-His-Leu-Pro-Pro-Pro)$_2$-OH (II), and was obtained by solid-phase segment coupling of one unit of the peptide I on a peptide-resin that contained another unit of I [11]. It is interesting to analyse the inversion level of proline, because the risk of racemization of the C-terminal residue of the peptide incorporated in the resin is relatively high, owing to the long times used in the coupling step [12]. In order to resolve the two enantiomers of proline, we have prepared a standard with racemic mixtures of alanine and proline, and we have assayed different chromatographic conditions, obtaining the best results with isothermic profiles. We have determined a resolution factor for every couple of enantiomers to be $R=(2\Delta t_{L-D})/(W_L+W_D)$,where Δt_{L-D} is the retention time difference between the two enantiomer peaks, and $W_{L(D)}$ is the widt at half-height. According to this definition, two symmetrical peaks can be considered to be completely resolved when the resolution factor is greater than 3 [7]. We have also defined t_m as the time corresponding to the average of the retention times of the two enantiomers of an amino acid. Table I shows the values of R and t_m obtained for alanine and proline at different temperatures.

TABLE I

VALUES OF R AND t_m FOR ALANINE AND PROLINE AT DIFFERENT TEMPERATURES

$R=(2\Delta t_{L\ D})/(W_L+W_D)$, where $\Delta t_{L\ D}$ is the retention time difference between two enantiomer peaks and $W_{L(D)}$ is the width at half-height. $t_m=(t_L+t_D)/2$.

Temperature (°C)	Alanine		Proline	
	R	t_m (min)	R	t_m (min)
110	26.6	12.0	3.6	42.4
120	13.2	9.0	3.2	28.3
130	6.9	7.1	2.7	19.4
140	6.1	6.0	2.3	14.4
150	4.1	5.3	2.1	11.1

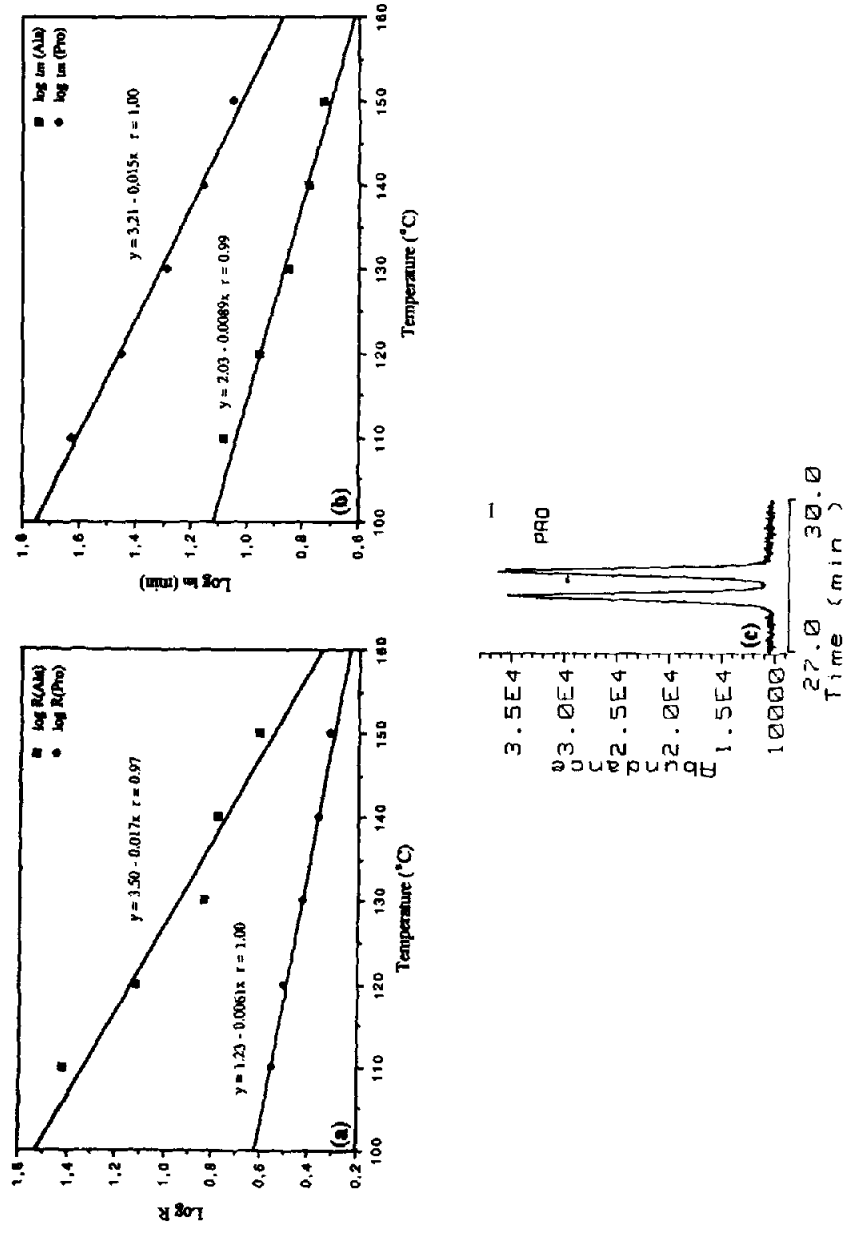

Fig. 2. (a) Graph of log R and *versus* temperature for alanine and proline. (b) Graph of log t_m *versus* temperature for alanine and proline. (c) Chromatographic profile of proline at 120°C.

We have also plotted log R and log t_m *versus* temperature, obtaining linear relationships. Fig. 2 shows these graphs, and also the chromatographic profile for proline at 120°C that is the best compromise between resolution and retention time.

Calculation of the D-enantiomer content in peptides I and II has been carried out using the equations reported by Liardon *et al.* [7]. Selected-ion monitoring was performed for $[F+1]^+$, $[F]^+$ and $[F-1]^+$ of proline ($m/z = 165$, 166 and 167 dalton), valine ($m/z = 167$, 168 and 169 dalton) and leucine ($m/z = 139$, 140 and 141 dalton), and after integration of the chromatographic profiles, the experimental values were introduced in the equations shown in Table II. The wrong enantiomer content (I) was calculated using a personal computer.

Table III shows the racemization level of the residues for both peptides I and II. All D-isomer contents are low, and no racemization took place in the treatments carried out on the peptides. Because there is more than one proline residue in all cases studied, is not possible to determine exactly the racemization level of any one proline in particular. We have estimated the highest racemization level of

TABLE II

EQUATIONS USED FOR THE CALCULATION OF THE INITIAL WRONG ENANTIOMER CONTENT

$[F+1]_D$, $[F]_D$, $[F+1]_L$, $[F]_L$ and $[F-1]_L$ are experimental values; X is the contribution (%) due to the racemization during hydrolysis; Y is that the due to the D-isomer present in the sample before hydrolysis; Z and T are factors relating the formation probability of fragment ion F + 1 or F − 1 to that of F; I is the initial wrong enantiomer content (%).

$$\frac{[F+1]_D}{[F]_L} 100 = X + YZ + \frac{XY}{100 - XT} \quad (1)$$

$$\frac{[F]_D}{[F]_L} 100 = XT + Y \quad (2)$$

$$\frac{[F+1]_L}{[F]_L} 100 = 100Z + X - YZT + \frac{XY}{100 - XT} \quad (3)$$

$$\frac{[F-1]_L}{[F]_L} 100 = 100T - XT^2 + \frac{XY}{100 - XT} \quad (4)$$

$$I = 100 \frac{Y + \dfrac{2XY}{100 - XT}}{100 + Y + X(2 - T) + \dfrac{XY(2 - T)}{100 - XT}} \quad (5)$$

TABLE III

RACEMIZATION VALUES FOR PEPTIDES I AND II UNDER DIFFERENT TREATMENTS USED IN SOLID-PHASE PEPTIDE SYNTHESIS

Sample	Initial wrong enantiomer content (*I*)		
	Val	Leu	Pro
Boc-VHLPPP-OH-(I) from dioxane–methanol–4 *M* NaOH (30:9:1)	0.19	0.07	0.10 (0.30)[a]
Boc-VHLPPP-OH (I) from dioxane–DMAE–4 *M* NaOH (30:9:1)	0.21	0.24	0.22 (0.66)[a]
H-(VHLPPP)$_2$-OH (II) from segment coupling	–	–	0.12 (0.72)[b]

[a] Highest level of racemization in C-terminal proline.

[b] Highest level of racemization of activated proline in the coupling.

a specific residue by supposing that the other proline residues were not racemized. Values in parenthesis in Table III show these highest values of inversion in the worst cases.

Other amino acids of special interest that have been studied are 3-(1-naphthyl)alanine (β-Nal) and *p*-chlorophenylalanine (Cl-Phe), two unnatural amino acids. EI mass spectra of these two compounds follow the typical fragmentation patterns previously described for the aromatic amino acids [9,10]. Fig. 3 shows this pattern, and the ions and intensities are tabulated for each amino acid in Table IV.

β-Nal shows small peak intensities except for the ion VIII, which corresponds to a ring expansion. For this reason, the method described cannot be applied in this case, because the α-carbon does not carry a hydrogen atom. The other peaks are too small to be of use in the analysis. (Although ion VII shows an intensity of 33%, when hydrolysis is carried out with ^{2}HCl polydeuteration of the aromatic ring results. The intensity is decreased because a cluster peak is produced and, even though there is a hydrogen on the α-carbon, it is too weak to be useful.) Polydeuteration phenomena will be discussed later. Cl-Phe shows an ion (III) of reasonable intensity and, if polydeuteration is not extensive, the correction can be applied. Methane CI was carried out on β-Nal and Cl-Phe and mass spectra are shown in Fig. 4. For those two amino acid derivatives, the base peak corresponds to the $[M+H]^+$ quasimolecular ion. Using this ionization technique it is possible to analyse amino acids such as Trp and β-Nal satisfactorily, which is not possible in the EI mode.

Liardon *et al.* [7] reported that some amino acids, such as Asp, Glu, Tyr and Trp, underwent deuterium-labelling at other positions in the molecule. If the exchange is complete, it is necessary to detect only those new ions with masses increased by a convenient number of mass units. If the exchange is not complete,

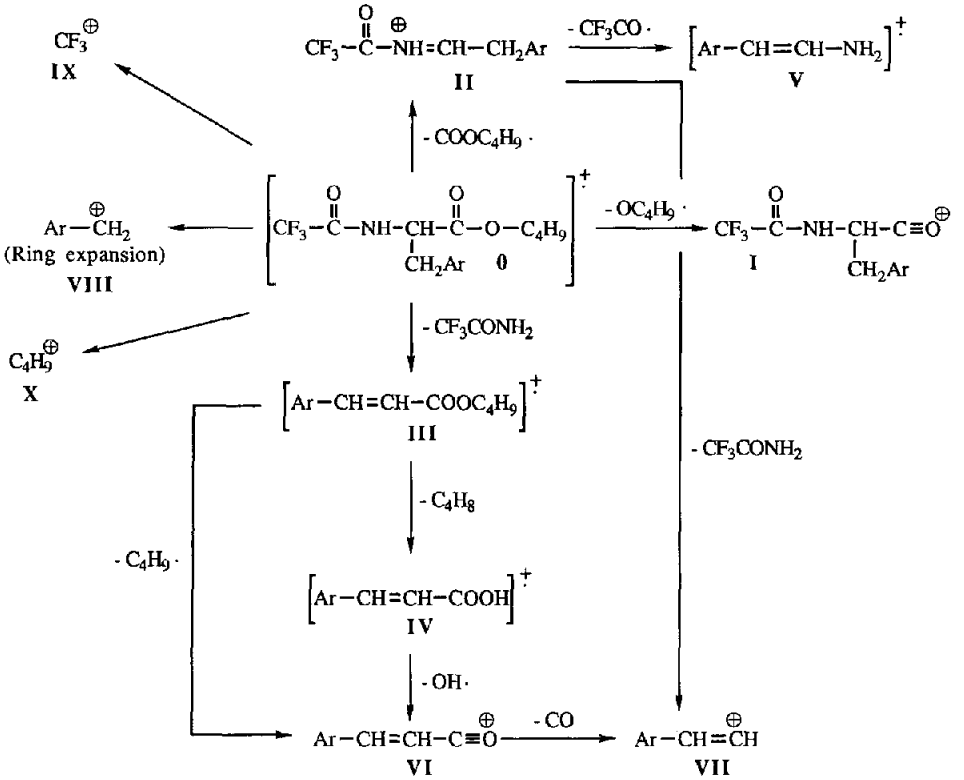

Fig. 3. Fragmentation pattern of trifluoroacetyl isobutyl esters of β-Nal and Cl-Phe.

TABLE IV

TABULATION OF THE EI SPECTRA FOR THE TRIFLUOROACETYL ISOBUTYL ESTERS OF β-Nal AND Cl-Phe

Percentage abundances in parentheses refer to the ion with highest intensity. (ND) means peaks expected from the typical fragmentation pattern of aromatic amino acids but with less than 0.1% of the intensity and therefore not detected.

Ion	β-Nal	Cl-Phe	Ion	β-Nal	Cl-Phe
0	367 (4)	351-3 (ND)	VI	181 (1)	165-7 (20)
I	249 (1)	278–80 (ND)	VII	153 (3)	137–9 (0.9)
II	266 (ND)	250–2 (0.9)	VIII	141 (100)	125–7 (100)
III	254 (8)	238–40 (16)	IX	69 (35)	69 (18)
IV	198 (5)	184–2 (80)	X	57 (36)	57 (19)
V	169 (4)	153–5 (13)			

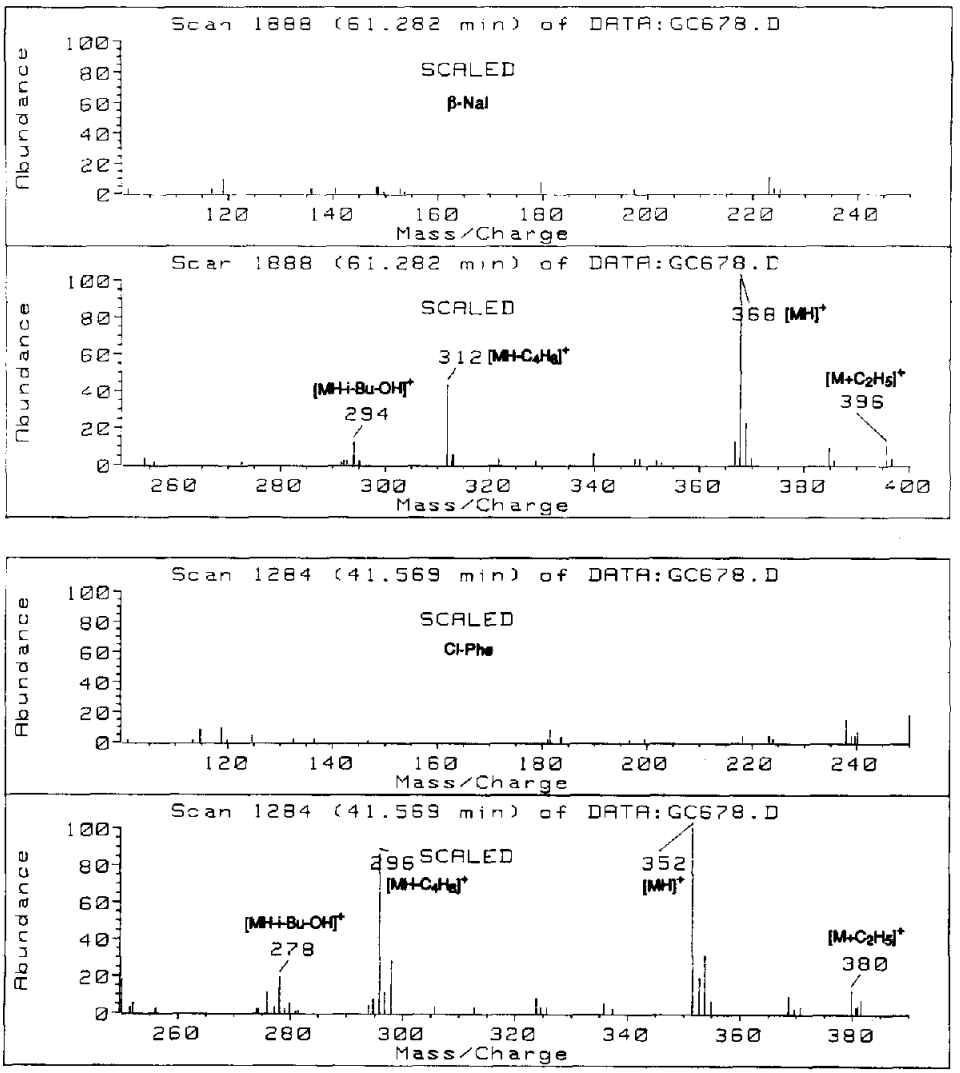

Fig. 4. Methane CI spectra of trifluoroacetyl isobutyl esters of β-Nal and Cl-Phe.

detection of initial mass is carried out and the factors Z and T in the equations in the Table II take this phenomenon into account. β-Nal and Cl-Phe also undergoes this process. Fig. 5a shows the molecular ion region of the mass spectrum of (D)β-Nal in methane CI mode after hydrolysis with ^{2}HCl; it is possible to observe the increase of four units of mass as a consequence of the labelling of four positions on the naphthalene ring. Fig. 5b corresponds to the mass spectrum of (L)β-Nal, produced as a result of inversion during the hydrolysis process. It shows one more unit of mass as a consequence of additional labelling at the α-position.

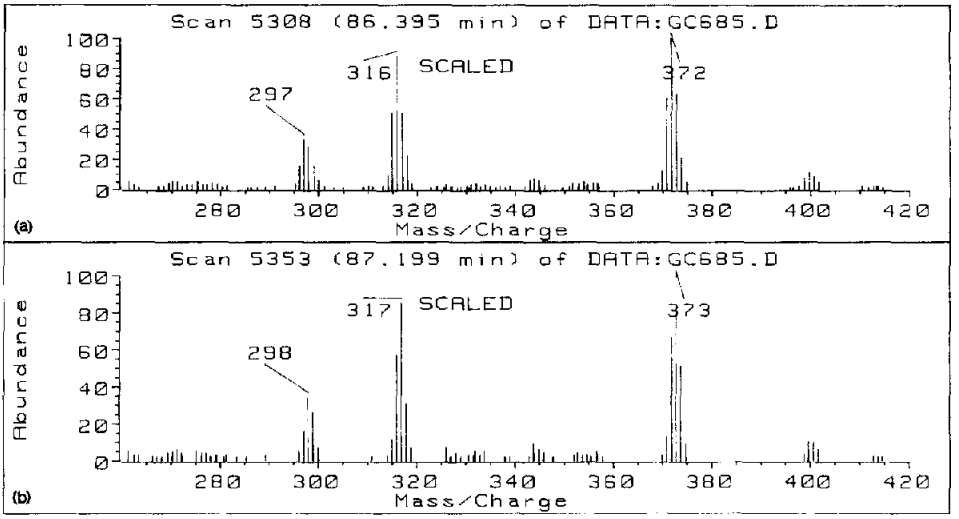

Fig. 5. (a) Methane CI spectrum of trifluoroacetyl isobutyl ester of (D)β-Nal after hydrolysis with ^{2}HCl without inversion. (b) Methane CI spectrum of trifluoroacetyl isobutyl ester of (L)β-Nal with inversion as a result of the hydrolysis with ^{2}HCl (in this case the true isomer was D).

In order to test the application of this method to unnatural amino acids, and to make a comparison between EI and CI modes, we studied the octapeptide H-D-β-Nal-L-Cys-L-Tyr-D-Trp-L-Lys-L-Val-L-Cys-L-Thr-NH_2 (III). This is an analogue of somatostatin (SRIF), which has potential therapeutic use in the treatment of certain human small cell lung carcinomas [15]. Table V shows the results obtained by the two methods, as well as the characteristic ions employed in each case and the retention time of the L-enantiomer.

All analysis were carried out a minimum of twice to give the average values and deviations listed in the Table. Agreement between the CI and EI methods is good, except for the cases of Trp and β-Nal where values for the EI mode are higher because it is not possible to apply the correction method in this mode. These results show the importance of racemization during the hydrolysis, especially in the case of some residues such as β-Nal.

In summary, we have increased the resolution of the two enantiomers of proline, and shown by the deuterium-labelling method that proline does not racemize under either the basic conditions used to cleave the peptide-resin bond or the segment-coupling conditions. EI and methane CI of the unnatural amino acids studied, such as β-Nal and Cl-Phe, follow the general fragmentation pattern of the aromatic amino acids, and their enantiomers show good chromatographic separation although the EI spectrum of β-Nal does not give useful peaks. Methane CI give intense quasimolecular ions for all amino acids studied. Application of the deuterium-labelling method in EI and methane CI gives comparable results for all the amino acids studied, except for Trp and β-Nal. The importance

TABLE V

COMPARISON BETWEEN EI AND CI MODES FOR PEPTIDE III

t_R is the retention time of the L-isomer, and I is the initial wrong enantiomer content.

Amino acid	t_R (min)	Ionization Mode	Mass (dalton)	I (%)
Val	29.7	EI	168	0.33 ± 0.01
		CI	270	0.30 ± 0.01
Thr	30.0	EI	153	0.04 ± 0.03
		CI	368	0.02 ± 0.01
Cys[a]	47.1	EI	268	1.13 ± 0.06
		CI	370	0.98 ± 0.03
Tyr	59.7	EI	262	0.18 ± 0.02
		CI	432	0.20 ± 0.06
Lys	70.1	EI	180	0.49 ± 0.06
		CI	395	0.31 ± 0.03
Trp	77.9	EI[b]	227–8–9	1.55 ± 0.10
		CI	455	0.16 ± 0.04
β-Nal	85.6	EI[b]	143–4–5	26.0 ± 3.00
		CI	372	1.42 ± 0.30

[a] Since there are two Cys residues, the highest value will be double that indicated in the table.

[b] In these cases it was not possible to substract the racemization due to the hydrolysis, thus the three ions indicated in the table were used and the final value was an average of the results obtained for each ion.

of the correction due to deuterium labelling is shown, especially in the case of β-Nal, the racemization of which was much less, showing the importance of racemization during the hydrolysis step. Deuterium exchange at other positions of some amino acids increases the complexity of this method, but the mathematical treatment is powerful enough to cope with this extra factor.

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