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Fast Atom Bombardment Mass Spectrometry of Carbobenzyloxy-protected Amino Acids and Peptides†

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The positive fast atom bombardment (FAB) mass spectra of 15 *N*-carbobenzyloxy derivatives of α-amino acids are presented together with those of some synthetic peptides containing other widely employed protecting groups. The data obtained allow a fragmentation pattern to be established for the *N*-carbobenzyloxy moiety and to obtain detailed structural information on the main fragment ions. A study of the ion current ratio vs. time pattern shows that important fragments derive from the parent protonated molecule through FAB-induced condensed-phase reactions.

INTRODUCTION

The *N*-carbobenzyloxy (*N*-Cbz) protecting group is largely employed in the liquid-phase synthesis of peptides due to its stability to mild acidic conditions and easy cleavage by hydrogenolysis. In the course of our investigation on synthetic peptides, we noticed the peculiar mass spectrometric behaviour of the *N*-Cbz moiety in protected amino acids and peptides under fast atom bombardment (FAB).

Whereas a wealth of information is available on the fragmentation behaviour of unprotected peptides under FAB conditions,[1,2] mass spectrometric work on synthetic protected peptides has been greatly overlooked. The fragmentation pattern of *t*-butyloxycarbonyl (Boc) protected amino acids was reported by Sedgwick and co-workers,[3] and Heerma has recently made a comparison between the sequence information in the spectra of underivatized and Boc-protected peptides.[4] In a recent paper concerning the monitoring of deprotection reactions in synthetic peptides by FAB-MS[5] no discussion was presented on the fragmentation behaviour of the protected peptides examined.

As it could be of interest to have an overall picture of the behaviour of the protecting groups in peptides under fast atom bombardment, the present paper deals with the condensed and gas-phase ion chemistry of *N*-Cbz protected amino acids and some peptides.

EXPERIMENTAL

The *N*-carbobenzyloxy derivatives of glycine **1**, alanine **2**, serine **3**, proline **4**, valine **5**, threonine **6**, leucine **7**, asparagine **8**, aspartic acid **9**, glutamic acid **10**, histidine **11**, phenylalanine **12**, arginine **13**, tryptophan **14** and *Nα,Nδ,Nω*-tri-carbobenzyloxy-arginine **15** were purchased from commercial sources or synthesized according to standard literature methods, and were not especially purified. Samples of the free amino acids and/or their hydrochlorides were obtained from various commercial sources and were of synthetic grade. *N*-Cbz-1-^{13}C-L-leucine **16** was prepared from 1-^{13}C-L-leucine (Amity, 90 at%). *N*-Cbz-phenethylamine **17** was prepared by the action of benzylchloroformate on phenethylamine. *N*-Benzylphenylalanine **18** was synthesized in a one-step procedure involving sodium cyanoborohydride reduction of the *in situ* generated benzeneimine of phenylalanine.[6] DL-*N*-*o*-toluidyl-phenylalanine **19** was synthesized as before from phenylpyruvic acid and *o*-toluidine. Protected peptides **20** to **24** were supplied by Prof. Chillemi of this Department.

All FAB mass spectra were recorded on a VG Analytical 7070EQ-HF instrument, equipped with its own standard FAB source. The ion gun was supplied with xenon gas; the beam was at 8 keV energy and 20 mA intensity. The spectra were run from glycerol or thioglycerol solutions of the compounds, placed on standard copper tips. Both '1st FFR' *B/E* and quadrupole daughter ion spectra were scanned manually under the operating conditions specified by the manufacturer; the latter experiment is referred to as MS/MS. Collisional activation was accomplished with helium gas at a pressure so as to reduce the intensity of the parent ion to 1/3.

The irradiation experiments were carried out by bombarding a sample of compound **12** (3.4 mg cm^{-3}) in glycerol (10^{-2} cm^3) for 30 min. with the sputtering primary beams. Spectra were taken at time intervals, occasionally replacing the matrix as it became dry. At the end of the irradiation, glycerol was finally evaporated off the probe, the resulting sample transferred to a glass vial and reacted with BSTFA. The reaction product was dissolved in CH_2Cl_2 (5×10^{-2} cm^3) and examined for the presence of Phe and *N*-benzyl-Phe by GC/MS (SE-54 25 m × 0.25 mm BPFS; He 0.6 psi; inj.

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Table 1. Main ions in the spectra of compounds 1–15

	Compound	MH^+	*a*	*b*	*c*	*d*	*e*	*f*
1	Cbz-Gly	210	120	76	102	59	164	166
2	Cbz-Ala	224	134	90	116	73	182	180
3	Cbz-Ser	240	150	106	132	89	194	196
4	Cbz-Pro	250	160	116	142	99	204	206
5	Cbz-Val	252	162	118	144	101	206	208
6	Cbz-Thr	254	164	120	146	103	208	210
7	Cbz-Leu	266	176	132	–	–	220	222
8	Cbz-Asn	267	177	133	159	116	221	223
9	Cbz-Asp	268	178	134	160	117	222	224
10	Cbz-Glu	282	192	148	–	131	236	238
11	Cbz-His	290	200	156	182	139	244	246
12	Cbz-Phe	300	210	166	192	149	254	256
13	Cbz-α-Arg	309	219	175	201	158	263	265
14	Cbz-Trp	339	249	–	–	188	293	295
15	$(Cbz)_3$-Arg[a]	577	–	443	469	–	531	533

[a] Other ions present: *m/z* 399, 355, 353, 335, 265, 221, 219, 201 (see text).

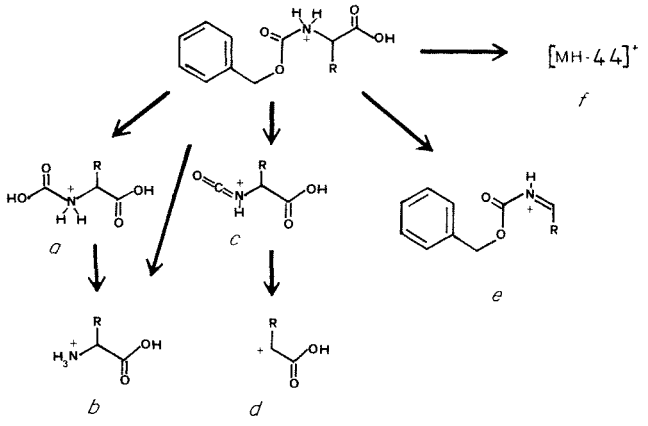
temp. 280 °C; oven temp. from 100 to 270 °C at 5 °C min^{-1}; EI 70 eV).

RESULTS AND DISCUSSION

The positive mass spectra of *N*-carbobenzyloxy amino acids **1–15** are reported in Table 1. The spectrum of each compound contains ions related to the fragmentation of the side chain, which nevertheless have been omitted. As the extent of cationization is greatly dependent on the amount of alkaline cations in the solution, cationized species are not reported, although $[M + Na]^+$ and $[M + K]^+$ ions were clearly visible in the spectra of all compounds. Moreover, in the case of the derivatives of aspartic and glutamic acid, the $[M + Na]^+$ ion is particularly intense and a weak, yet clearly distinguishable sodiated *A*-type (*vide infra*) fragment is also present, owing to the chelating aptitude of the three carboxyl groups.

The main ions of compounds **17**, **18** and **19** are collected in Table 2, whereas the most important ions in the spectra of the synthetic protected peptides **20–24** are reported in Table 3.

Prominent fragmentation pathways are depicted in Scheme 1. Three main decomposition processes arise from the protonated molecule: the loss of 90 u (a formal benzylcarbene) yields an *a*-type ion to which the structure of a carbamic acid is assigned. This is in excellent



Scheme 1

Table 2. Main ions in the spectra of compounds 17, 18 and 19

17 $PhCH_2OCONH(CH_2)_2Ph$
256 (MH^+, 27.6), 214 (8.2), 212 (11.2), 122 (49.4), 120 (7.6), 105 (38.0, 91 (100.0)

18 $PhCH_2NHCH(CH_2Ph)COOH$
256 (MH^+, 5.1), 215 (1.6), 201 (4.8), 181 (2.1), 149 (3.4), 120 (3.0), 115 (3.8), 105 (4.2), 91 (100.0)

19 $o\text{-}CH_3C_6H_4NHCH(CH_2Ph)COOH$
256 (MH^+, 9.5), 237 (3.9), 235 (7.8), 215 (5.8), 207 (15.4), 164 (7.3), 149 (8.8), 108 (12.5), 105 (98.0), 91 (100.0)

Table 3. Main ions in the spectra of protected peptides 20–24

20 Cbz-Ser-Phe-OtBu $C_{23}H_{30}N_2O_5$ MW 442
443, 387, 371, 369, 343, 309, 297, 279, 256, 253, 233, 222, 210, 207, 165, 150

21 Cbz-Tyr(OBz)-Ser-Phe-OH $C_{35}H_{35}N_3O_6$ MW 695
696, 652, 640, 624, 622, 606, 596, 562, 550, 532, 514, 506, 488, 481, 475, 458, 431, 403, 385, 373, 360, 343, 341, 324, 316, 313, 309, 307, 279, 256, 253, 237, 226, 222, 217, 215, 201, 197, 136, 120

22 Cbz-Lys(Boc)-Tyr-Ser-Phe-OH $C_{44}H_{59}N_5O_{11}$ MW 833
834, 818, 784, 778, 734, 700, 678, 662, 660, 644, 634, 632, 626, 600, 588, 572, 570, 557, 544, 530, 526, 513, 495, 485, 472, 443 426, 416, 409, 408, 398, 379, 351, 343, 336, 324, 309, 308, 307, 292, 290, 280, 273, 264, 263, 253, 245, 235, 226, 166, 136, 120

23 Cbz-Gln-Lys(Boc)-Tyr-Ser-Phe-OH $C_{49}H_{67}N_7O_{12}$ MW 961
962, 946, 912, 906, 862, 828, 806, 790, 788, 773, 772, 734, 728, 716, 700, 698, 678, 672, 641, 613, 600, 598, 571, 554, 544, 536, 526, 472, 464, 420, 416, 408, 391, 375, 363, 343, 330, 320, 318, 309, 308, 301, 280, 273, 263, 257, 253, 251, 245, 240, 235, 229, 226, 222, 190, 166, 136, 129, 120

24 Cbz-Glu(O*t*Bu)-Gln-Lys(Boc)-Tyr-Ser-Phe-OH $C_{58}H_{82}N_8O_{16}$ MW 1146
1148, 1131, 1091, 1047, 1013, 991, 975, 958, 957, 935, 919, 917, 901, 861, 857, 828, 826, 806, 801, 798, 783, 770, 756, 739, 728, 727, 711, 700, 683, 672, 655, 650, 639, 636, 611, 600, 598, 593, 576, 549, 544, 537, 525, 520, 504, 492, 472, 465, 459, 448, 420, 416, 409, 403, 392, 376, 364, 348, 320, 281, 258, 253, 237, 166, 136, 129, 120

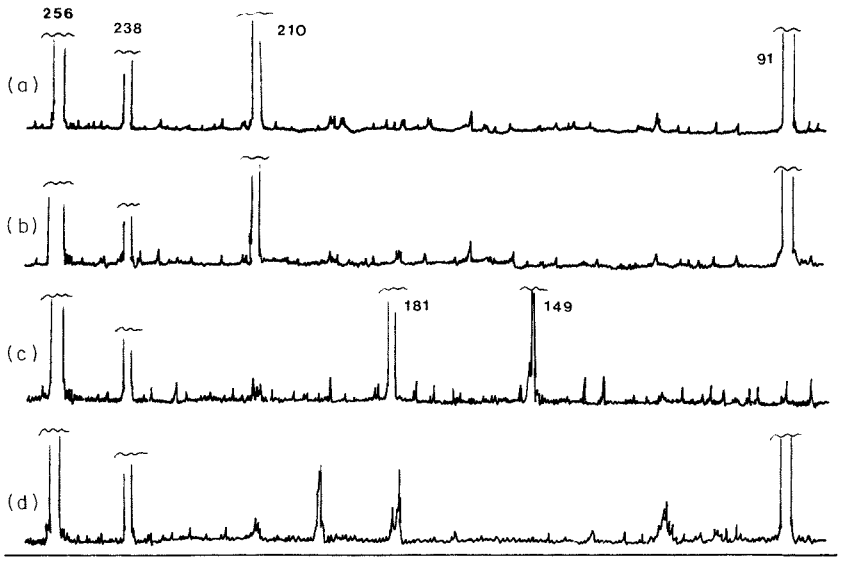


Figure 1. CAD MS/MS spectra of *m/z* 246 from compounds **12** (a), **18** (b) **19** (c) and **17** (d).

agreement with its gas-phase reactivity, in that the CAD *B/E* spectrum shows that the *a*-type ion yields a *b*-type one by loss of 44 u, and that the *B/E* spectrum of the latter is superimposable on the one of the corresponding free amino acid. Such behaviour parallels that of *t*-butyloxycarbonyl amino acids and is in accordance with the well-known solution reactivity of both protecting groups.

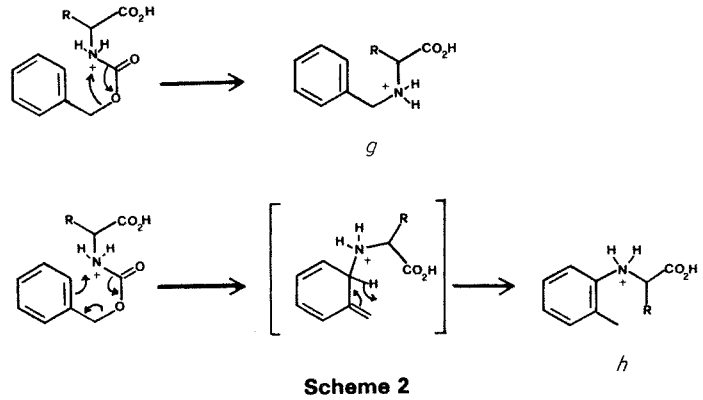
The spectra of many *N*-Cbz-protected amino acids show the loss of 108 u (formally benzyl alcohol) to generate *c*-type ions to which the structure of a protonated isocyanide can be assigned, on the basis of the loss of 43 u, which leads to *d*-type ions (Ion *d* is depicted as an α-oxo carbenium ion, however any other compatible structure can be accepted.)

A minor fragmentation pathway to *e*-type ions through a formal loss of formic acid from the protonated molecule yields a very weak iminium ion, in agreement to a similar process observed in the spectra of *N*-Boc amino acids. *f*-Type fragment ions due to the loss of 44 u (i.e. loss of CO_2) are very abundant in the spectra of all protected amino acids examined, and are always accompanied by the appropriate intense metastable ions. These fragments can be originated either from reductive decarboxylation at C(1) or, in a more reasonable way, from the elimination of CO_2 from the benzylurethane carboxyl group.

The evidence that the last hypothesis is the correct one is supplied by the spectrum of Cbz-1-^{13}C-L-leucine **16**, in which *a*-, *b*-, *c*-, *d*- and *f*-type ions retain the ^{13}C label, whereas the *e*-type ion loses the carboxylic carbon atom. Additional support is given by the analysis of the spectrum of protonated *N*-carbobenzyloxy-phenethylamine **17**, which would result by 'wrong carbon' loss from **12**. Fragment ions (Table 2) and collisionally activated dissociation (CAD) MS/MS daughter ion spectrum (Fig. 1) clearly rule out the structure of protonated **17** for ion *f* derived from **12**. The detailed structure of the product ion arising from CO_2 loss from the benzylurethane moiety can be represented by either of the two isomeric structures *g* and *h* formed along the pathways shown in Scheme 2.

Taking as an example the *N*-carbobenzyloxy-phenylalanine **12**, *N*-benzylphenylalanine **18** and *N*-(*o*-tolyl)-phenylalanine **19**, corresponding to the two possible structures *g* and *h* were synthesized. The spectra were recorded (Table 2) and the CAD *B/E* and MS/MS daughter ion spectra of their protonated molecules compared with those of the ion at *m/z* 256 in the spectrum of **12** (Fig. 1).

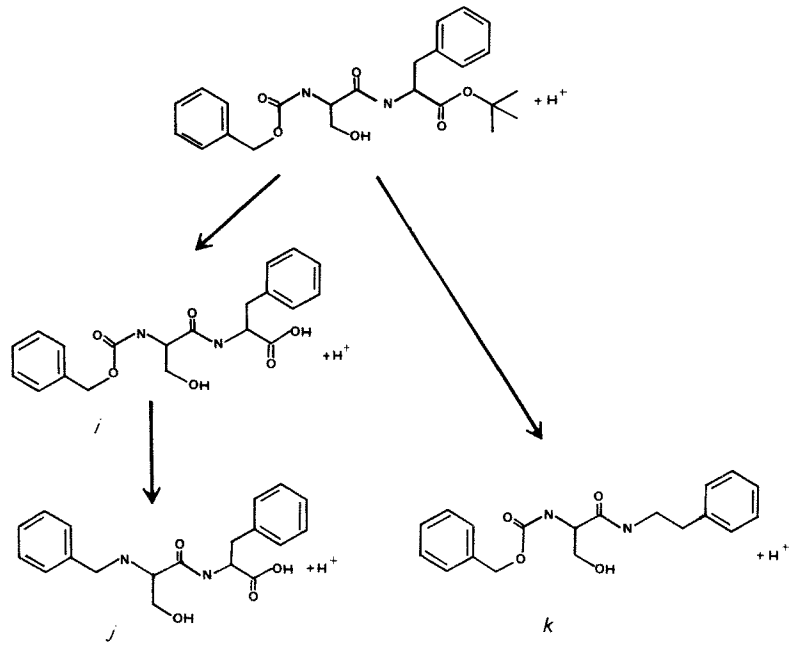
The comparison points at protonated **18** as the most likely candidate to represent the *f*-type ion. In particular, the ion at *m/z* 149 in the MS/MS spectrum of protonated **19** is due to the loss of *o*-toluidine, and the



g

h

Scheme 2



Scheme 3

analogous loss of benzylamine is not observed in the spectrum of protonated **18**. Moreover, the absence of *m/z* 91 in protonated *N*-*o*-tolyl-phenylalanine shows that no benzyl cation comes from the amino acid side chain. It is therefore proposed that the *f*-type ions of all compounds possess a protonated *N*-benzyl amino acid structure.

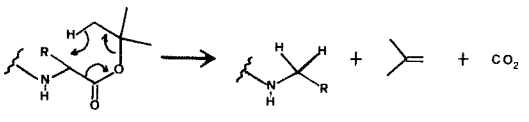
This behaviour is also in agreement with a sensible analysis of the spectrum of the protected dipeptide **20** (Table 3, Scheme 3). In this compound, the protonated molecule at *m/z* 443 suffers a well-known isobutene loss from the *t*-butyl ester group to yield the ion at *m/z* 387 (*i*), which in turn yields the ion at *m/z* 343 through the loss of CO_2. In principle, this ion may have either the structure of a *N*-benzyl-seryl-phenylalanine (*j*) or of a *N*-carbobenzyloxy-seryl-phenethylamine (*k*) as in Scheme 3. Its *B/E* spectrum contains ions at 325, 297 and 150, which account for the accepted fragmentation pattern of the benzylpeptide *j*.

Other much weaker ions are also present at *m/z* 299, 253 and 235, which cannot be explained by the fragmentation of *j*, but would account for the presence of an intact carbobenzyloxy group (*f*-, *a*- and *c*-type ions, respectively). A careful inspection of the *B/E* spectrum of *m/z* 443 shows a weak unimolecular loss of 100 u, which can be interpreted by the extrusion of COO-*t*Bu with H-rearrangement as depicted in Scheme 4. It is therefore apparent that the resulting isomeric structure *k*, although scarcely abundant, accounts for the above mentioned ions at *m/z* 299, 253 and 235. Although these data cannot rule out completely the formation of ion *k* either from *i* or through a double metastable $MH \rightarrow j$ transition,† the main fragmentation of protonated **20** is the loss of the benzylurethane carboxyl group, whereas the loss of the terminal carboxyl group either from MH^+ or from *i* is a minor process. A similar behaviour is shared by all other *N*-terminal carbobenzyloxy-protected peptides **21**, **22**, **23** and **24**, (Table 3) which show in their spectra some of the ions corresponding to the fission of the backbone of a *N*-benzyl peptide. This process tends, however, to be of lesser importance as the protected peptides grow in molecular weight and as more easily cleavable protecting groups are present.

The behaviour of *Nα,Nδ,Nω*-tri-Cbz-arginine **15**, which shows a complex spectrum originating from the superimposition of the fragmentation pathways of each of the three carbobenzyloxy groups present, deserves some comment. The protonated molecule at *m/z* 577 is accompanied by ions at *m/z* 399 and 265, representing the successive losses of one whole carbobenzyloxy moiety. Each of the three above ions is associated with its *c*- (469, 335 and 201), *e*- (531, 353, and 219) and *f*-type (533, 355 and 221) fragments, while *a*-type ions are absent. The fragmentation processes on each of the three groups seem virtually independent of one another, in that 'mixed fragmentation' ions are present.

In the normal spectrum of all Cbz-amino acids the only metastable transition observed corresponds to the gas-phase formation of ion *f* from the protonated molecule. On the other hand, metastable and CAD daughter ion analyses reveal that only the *f*-type fragment derives from the protonated molecule and that the *a* to *b* transition occurs only under collisional activation. As *a*- and *b*-type fragments are necessarily formed from the precursor protonated molecule, it is a matter of question whether any hydrogenolysis could occur by radical processes in the condensed phase under FAB conditions,[7,8] paralleling the well-known deblocking reaction of the carbobenzyloxy group.

In order to achieve information on this particular aspect, irradiation experiments were performed on a



Scheme 4

† We thank a referee for this suggestion.

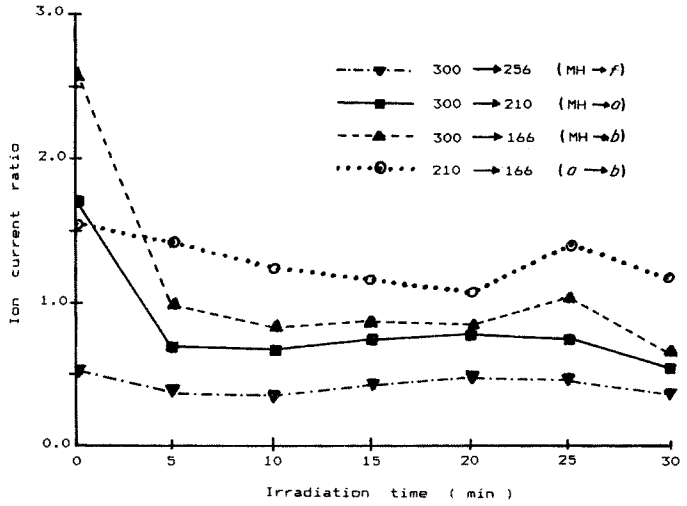


Figure 2. Plot of selected ion current ratios vs. irradiation time for Cbz-phenylalanine **12**.

sample of compound **12**, as reported in the Experimental section. Thioglycerol was not employed as the matrix because it is known to act as a radical scavenger.

The ratios of the ion currents 300/256 (MH → *f*) and 300/210 (MH → *a*) are plotted against irradiation time[9] (Fig. 2). As the 300 → 256 transition is accompanied by a metastable ion, it is implicitly assumed that this guarantees the essentially gas-phase nature of the process. It is also assumed that the internal energy distribution in the protonated molecule is not a function of the irradiation time, and therefore the ratio of the 300/256 ion current should be, and effectively is, approximately constant in the time. The 300/210 ratio, instead, is decreased by a factor of two to three within the first ten minutes, and reaches a long-lasting plateau. An analogous situation is shared by the 300/166 ratio (MH → *b*).

As no trace of either *N*-benzyl or free amino acid is detected in the matrix after irradiation, it can be therefore suggested that the reactions MH → *a* and MH → *b* take place in the energized condensed phase, possibly triggered by radical production under irradiation, with subsequent sputtering of ions *a* and *b* into the gas phase. Moreover, if we consider the *m/z* 210 to *m/z* 166 ratio, we note a slow decrease indicating that the *b*-type ions are also formed from *a*-type ions, either in the condensed phase and/or in the dense, high-pressure region inside the source.[10]

This hydrogenolytic behaviour is similar to that obtained while analyzing ring-halogenated pyrilium salts.[11] FAB-induced reactions were observed in the spectra of a broad range of compounds,[8] including protected nucleotides[12] and silatranes.[9]

Finally, the higher peptides protected with different groups show that the Boc group is lost before the *t*-butyl ester and it does not show the intermediate loss of isobutene.[3] The loss of the carbobenzyloxy moiety tends to be suppressed as ions are often observed which belong to the fragmentation pattern of the backbone in a terminal Cbz-protected peptide.

Another peculiar behaviour is the usual absence of the *a*-type ion with respect to the *b*-type one. A further similarity in the mass spectrometric behaviour vs. solution chemistry of the protecting groups is supplied by the *O*-benzyl-tyrosine residue in compound **21**. No cleavage of the benzyl ether occurs even in the low mass ions coming from the most energized precursor, in agreement with the stronger conditions required for the cleavage of this group.

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