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Comparing Mass Spectrometric Characteristics of Peptides and Peptoids—2†

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The high-energy collision-induced dissociation (CID) spectra of the $[M + H]^+$ and $[M - H]^-$ ions of substance P fragment 6–11, the retropeptide and the corresponding peptoid and retropeptoid were compared. The CID spectra of the $[M + H]^+$ ion of the corresponding (retro)peptide and (retro)peptoid exhibit both B- and Y″-type sequence ions at identical *m*/*z*-values. The differences in the relative abundances of these sequence ions, however, can be related to structural characteristics of the compounds. The fragmentation behaviour of the *N*-substituted immonium ions differs from that of the immonium ions derived from common amino acids by showing a preferential loss of a $CH_2{=}NH$ imine molecule. This dominant fragmentation reaction is suppressed in the CID spectrum of the *N*-substituted glutamine immonium ion in favour of a less energy demanding cyclization reaction involving the loss of NH_3. The CID spectra of the $[M - H]^-$ ions of both peptides and peptoids show C-type ions, which appeared to be more abundant in the spectra of the peptides than in those of the peptoids. *C*-Terminal ″Z- and Y-type ions are characteristic of the peptides, whereas the peptoid spectra show relatively abundant *N*-terminal ″B-type ions. Both $[M + H]^+$ and $[M - H]^-$ ion CID spectra show loss of amino acid-specific side-chains, which occurs as radical loss in the case of peptides and as molecular loss in peptoids. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

The application of peptides in drug design is limited owing to their biological instability. The development of biologically modified peptides or peptidomimetics possessing enhanced metabolic stability is a goal of many pharmaceutical companies.[1] Peptoids are an interesting class of peptidomimetics that differ from peptides in that the amino acid-specific side-chains are located on the nitrogen atom rather than on the α-carbon atom.[2] Particularly interesting is the retropeptoid mimic of a peptide, with the reverse order of *N*-substituted glycine residues. The relative positions of side-chains and carbonyl groups in the retropeptoid are identical with those in a peptide and therefore these mimics might exhibit a similar biological activity as the peptide.[1]
It is obvious that a reliable structural analysis of synthetic peptoids is of great importance. Mass spectrometric identification and structural analysis of isobaric compounds require distinguishable spectrometric characteristics and preferably the occurrence of recognizable sequence ions. Recognition of peptoids and metabolic products containing structural elements resulting from administered peptoids in biological samples will probably become an important issue in the near future.

The positive ion mass spectra of peptoids exhibit a great resemblance to those of the corresponding peptides. Both low-[3] and high-energy[4] collision-induced dissociation (CID) spectra revealed that peptoids also fragment at the amide bonds to yield B- and Y″-type ions.

In Part 1[4] we compared the positive ion CID spectra of acetylated Leu-enkephalin amide peptide and the corresponding peptoid and retropeptoid which are constitutional isomers of each other. We now present the CID spectra of another set of isomers, consisting of a substance P fragment 6–11, as the *N*-acetylated peptide and retropeptide together with the corresponding peptoid and retropeptoid. Significant biological activity of substance P fragment 6–11 and also the corresponding peptoid has been observed.[5]

The aim of this work was to explore further the differences in the fragmentation behaviour of corresponding peptides and peptoids. Although it is known that the product ion spectrum of a protonated peptide molecule shows a rational pattern from which the amino acid

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sequence and additional structural features can be derived, it was decided to include the CID spectra of the deprotonated molecules in order to investigate whether additional and possibly more pronounced differences between peptides and peptoids could be traced.

EXPERIMENTAL

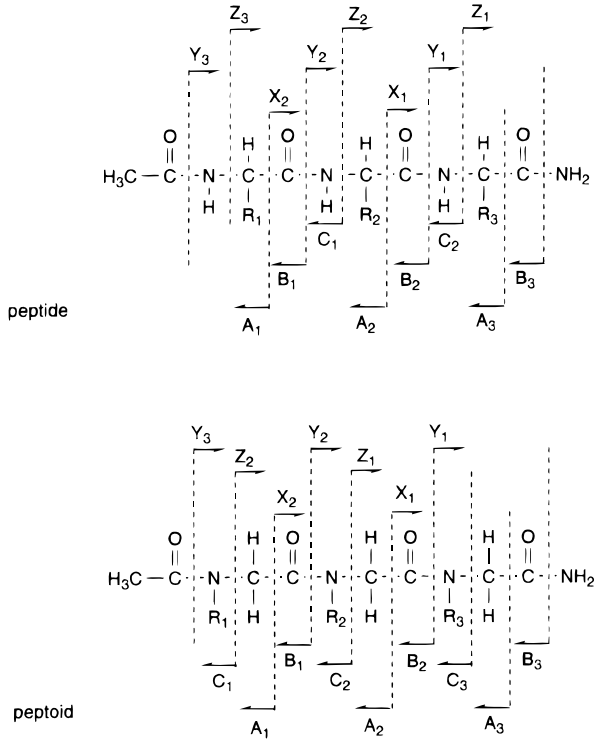
Fast atom bombardment (FAB) mass spectra were acquired using a Jeol JMS SX/SX 102A four-sector mass spectrometer, operated at 10 kV accelerating voltage and equipped with a Jeol FAB gun set at 5 mA emission current and producing a beam of 6 keV Xe atoms. Full mass spectra were recorded over about 15 s for the *m/z* 10–1000 range by averaging 5–10 scans. The data were acquired and processed with an HP-9000 data system using Jeol Complement software. The samples were dissolved in water or deuterium oxide and approximately 1 μl of the solution, containing about 5 μg of the compound, was mixed with a microdroplet of glycerol or trideuteroglycerol on the FAB probe. Normal mass spectra were obtained by scanning MS-1, while CID product ion spectra of selected precursor ions were acquired by scanning MS-2 in the linked scan at constant *B/E* mode using a collision cell in the third field-free region of the instrument with air as the collision gas. The pressure of the collision gas was adjusted to obtain a 50% intensity reduction of the main beam.

Compounds

The acetylated substance P-amide fragment 6–11 Ac-QFFGLM-NH_2 (**1**), retropeptide (**3**), peptoid (**2**) and retropeptoid (**4**) were synthesized on an Applied Biosystems 433A peptide synthesizer using Fmoc-protected *N*-substituted glycine derivatives,[6] as will be described in due course.[7] The deuterium-labelled retropeptide Ac-MLGFFQ-NH_2-d_{10} (**3d**) and the peptoid Ac-nQnFnFGnLnM-NH_2-d_5 (**2d**) were obtained by dissolving ~50 μg of the peptoid in a mixture of 10 μl of D_2O and 2 μl of tetradeuteroacetic acid (D_2O, 99.75%; CD_3COOD, >98%; Merck, Darmstadt, Germany). Trideuteroglycerol was obtained by repeatedly exchanging the hydroxyl hydrogen atoms with deuterium from D_2O and distilling the glycerol after three treatments.

RESULTS AND DISCUSSION

The proposed nomenclature for assigning sequence ions in peptoids[4] is very similar to that generally used for peptides[8,9] so that B-, Y-, A- and X-type ions are observed at the same *m/z* value. The C- and Z-type ions of peptoids, however differ by a CH_2CONH group (57 u less and more, respectively) from the corresponding ions in the peptide (see Scheme 1), which means that corresponding C- and Z-type ions in peptides an peptoids are not isobaric. In order to indicate peptoid



Scheme 1. Nomenclature of peptoid backbone sequence ions compared with those of peptides (Refs 4 and 8).

amino acid residues, the prefix N in the three-letter code or n in the one-letter code is used.

Positive ion spectra

We have studied the fragmentation behaviour of substance P fragment 6–11 (**1**) and its three constitutional isomers **2**, **3** and **4**. The CID spectra of the *m/z* 783 $[M + H]^+$ ion of the peptide and the peptoid are given in Fig. 1(a) and (b), respectively. From these spectra it is clear that both compounds yield predominantly B-type ions. The complete series of B ions, including B_1 since we are dealing with *N*-acetylated compounds,[10] is present and allows a full sequence determination. The main difference is the relative abundance of the Y_2'' and Y_5'' ions in their spectra. The Y_5'' ion is even the most abundant product ion in the CID spectrum of the peptoid. The phenomenon of increased Y″ ion abundances has also been observed in the CID spectrum of the pentapeptide Leu-enkephalin,[4] where the Y_2''/B_3 intensity ratio is 0.13 in the case of the peptide while this ratio is found to be 1.9 for the corresponding peptoid.

The increased intensity of Y″-type ions in the case of peptoids compared with corresponding peptides could be caused by the enhanced proton affinity of the N-atom of the amide bond which is substituted by the characteristic amino acid side-chain and thus acts as a more basic secondary amine group than the primary amine group in common peptides.[11]

The CID spectra of **3** and **4** also contain mainly B- and Y″-type ions [Fig. 2(a) and (b)]. Notable differences in the spectra are the occurrence of a peak at *m/z* 708 in the spectrum of the retropeptide and at *m/z* 709 in the spectrum of the retropeptoid. The *m/z* 708 ion orig-

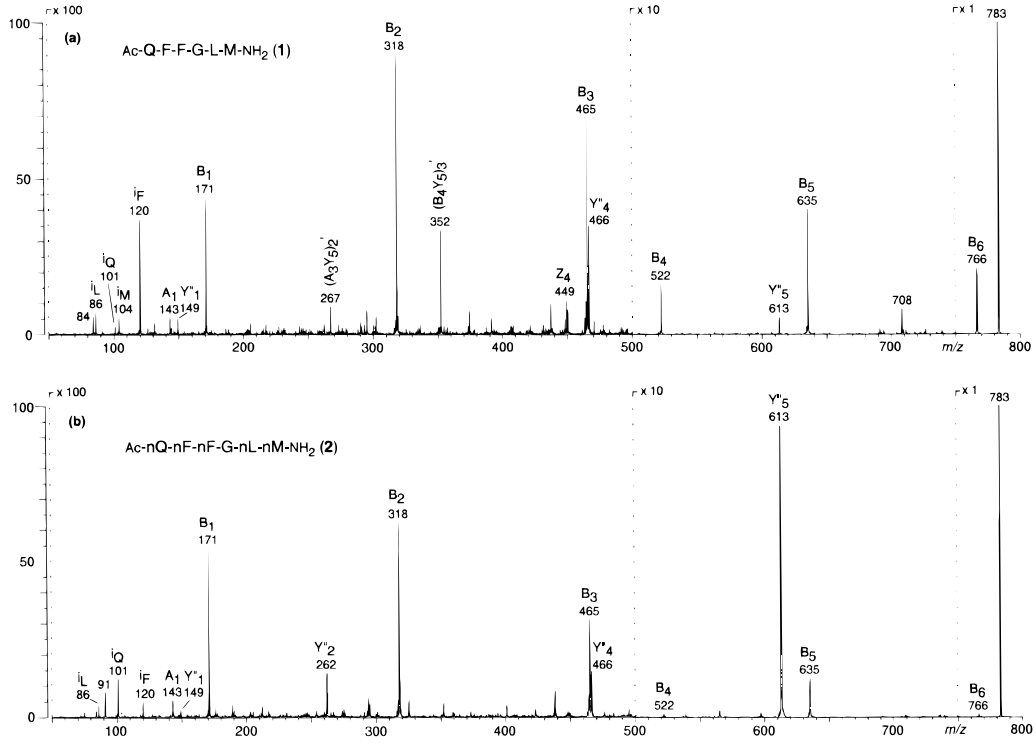


Figure 1. CID spectra of the *m/z* 783 $[M + H]^+$ ions of (a) Ac-QFFGLM-NH_2 (**1**) and (b) Ac-nQnFnFGnLnM-NH_2 (**2**).

inates from loss of the methionine side-chain ($CH_3SCH_2CH_2^{\cdot}$) thus resulting in the generation of an odd-electron ion [see Figs 1(a) and 2(a) and Scheme 2]. Similar product ions, due to the losses of the Q and L side-chain radicals, can be observed in the spectra of **1** and **3** at *m/z* 711 and 726, respectively.

The formation of *m/z* 709 [Fig. 2(b)], however, requires the elimination of a 74 u neutral and occurs if the methionine side-chain is attached to the N-atom by loss of a $CH_3SCH{=}CH_2$ molecule and by simultaneous transfer of a hydrogen atom from the side-chain towards the nitrogen atom (see Scheme 2). The position of the nM residue in the peptoid chain determines the extent of this elimination process. The *m/z* 709 ion is very weak if the nM residue is at the *C*-terminal position but shows an increased abundance when the

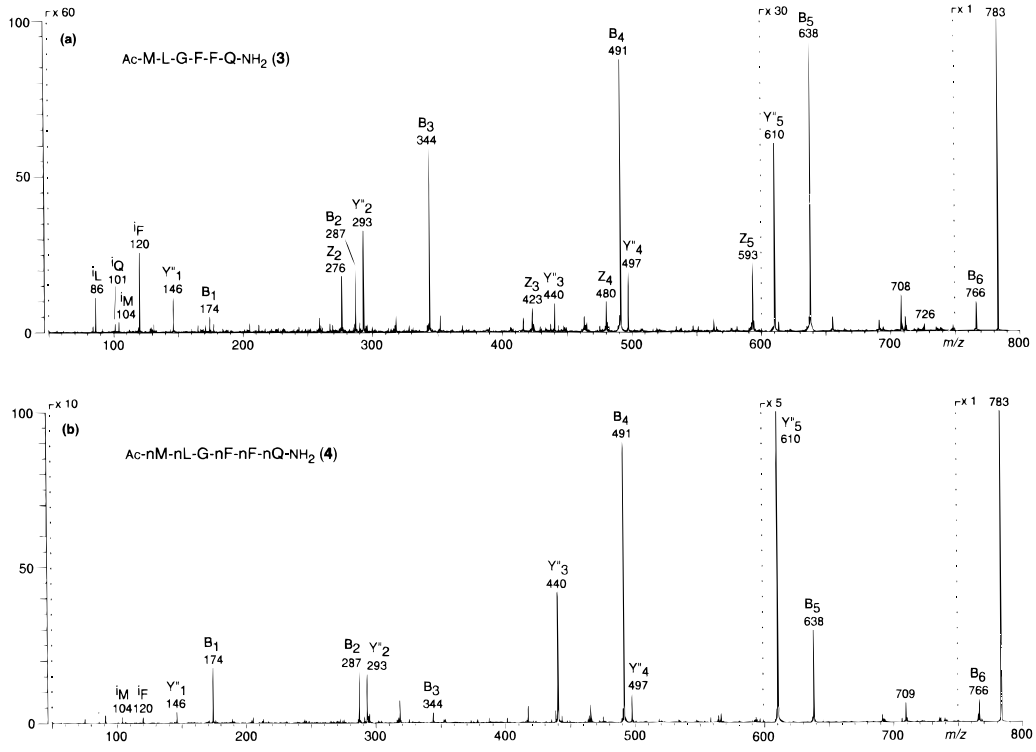
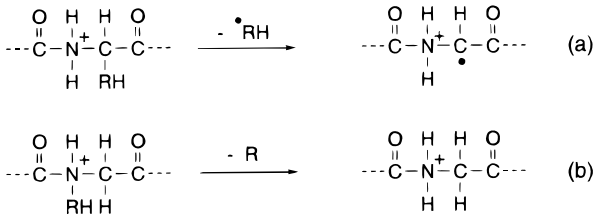


Figure 2. CID spectra of the *m/z* 783 $[M + H]^+$ ions of (a) Ac-MLGFFQ-NH_2 (**3**) and (b) Ac-nMnLGnFnFnQ-NH_2 (**4**).



Scheme 2. Amino acid side-chain loss in (a) peptides and (b) peptoids.

residue is at the *N*-terminus, most probably as the result of a preferential charge localization at this site. Peptides containing *N*-substituted tyrosine show loss of a C_7H_6O molecule in a similar hydrogen rearrangement process.[4]

Immonium ions

The immonium ions of common and *N*-substituted amino acid residues in the spectra of peptides and peptoids can be used for identification and discrimination purposes.[12–16] These immonium ions, which are abundant in the normal FAB mass spectra of peptides and peptoids and thus can be selected for tandem mass spectrometric (MS/MS) analysis, reveal differences of which the abundant loss of a CH_2=NH imine molecule has been reported to be characteristic in the CID spectra of nL-, nF- and nY-immonium ions.[4]

In Fig. 3(a)–(e), pairs of CID spectra of the α-amino acid Q- and M-immonium ions and their *N*-substituted analogues are shown together with the ε-ND_2-labelled immonium ion of nQ. The distinction between M and nM is obviously facile because of the expected abundant CH_2=NH loss from the nM-immonium ion [Fig. 3(c) and (d)]. The difference between the CID spectra of the Q- and nQ-immonium ions is less clear at first sight. Both spectra [Fig. 3(a) and (b)] are dominated by the loss of an NH_3 molecule while the loss of CH_2=NH from nQ, yielding a product ion at *m/z* 72, is relatively weak. The NH_3 loss from the immonium ion of nQ occurs presumably in an energetically favourable cycli-

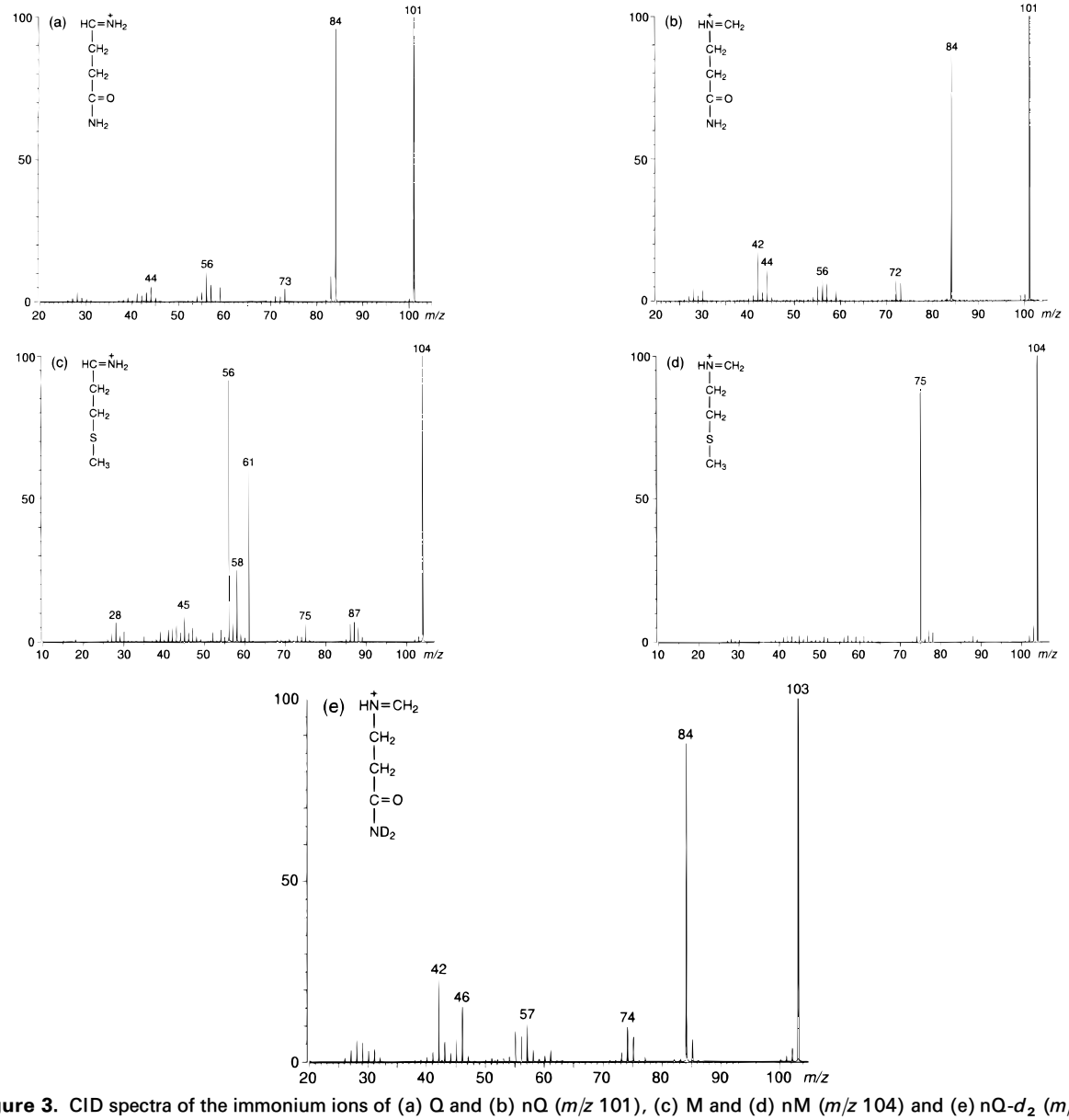


Figure 3. CID spectra of the immonium ions of (a) Q and (b) nQ (*m/z* 101), (c) M and (d) nM (*m/z* 104) and (e) nQ-d_2 (*m/z* 103).

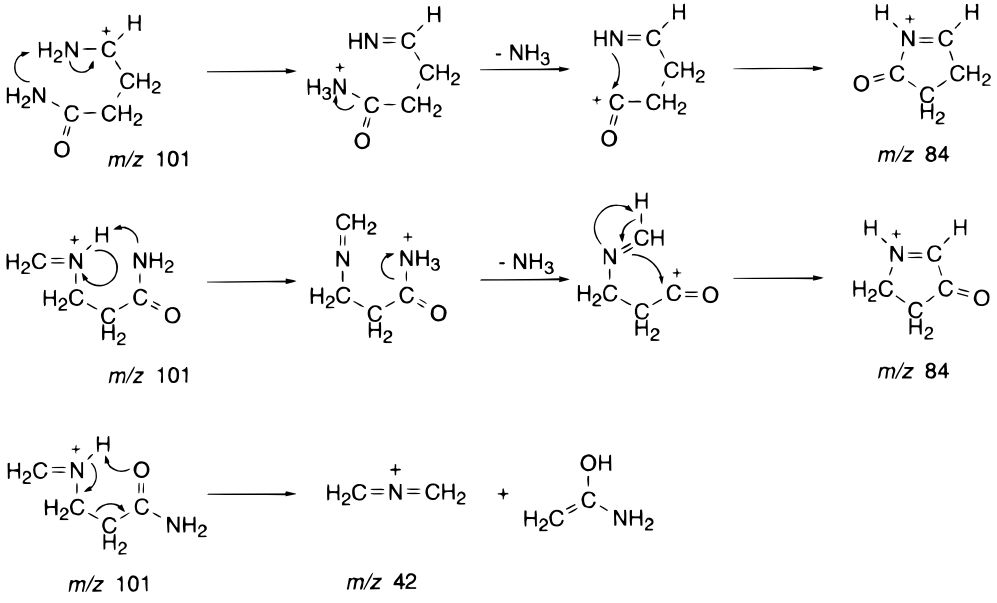
zation process (see Scheme 3) similarly to the NH_3 loss from protonated glutamine[17] and involves the exclusive participation of the ε-amino group, which is apparent from the non-shifted *m/z* 84 peak in the *m/z* 103 immonium ion spectrum [Fig. 3(e)] of the ε-ND_2-labelled analogue. This nQ-d_2 precursor ion was selected from the mass spectrum of **2**d. Another difference between the *m/z* 101 spectra of Q and nQ is the formation of an *m/z* 42 ion [$CH_2{=}N^+{=}CH_2$] which is more facile in the case of nQ (Scheme 3).

Finally, it should be noted that isomeric peptides and peptoids can be easily distinguished based on their number of exchangeable hydrogen atoms from the NH_2, NHCO, NH_2CO, COOH and OH groups. In the case of our model compounds, peptides **1** and **3** have ten exchangeable hydrogens whereas peptoids **2** and **4** have only five exchangeable hydrogen atoms which exhibits, in the case of complete deuteration and using trideuteroglycerol as a matrix, a molecular ion mass shift from *m/z* 783 $[M + H]^+$ to *m/z* 794 $[M + D]^+$ for peptides but only to *m/z* 789 $[M + D]^+$ for peptoids; similarly, in the negative ion mass spectra $[M - H]^-$ ions at *m/z* 781 shift to $[M - D]^-$ ions at *m/z* 790 and 785 for peptides and peptoids, respectively.

Negative ion spectra

The $[M - H]^-$ CID spectra of peptide and peptoid couples are presented in Figs 4 and 5. The eliminated proton in the $[M - H]^-$ ion is assumed to originate from the *C*-terminal amidic nitrogen, thus resulting in the formation of pseudo-molecular ions with an $RCONH^-$ structure. Waugh and Bowie[18] reported three different types of fragmentation reactions for collisionally activated peptide $[M - H]^-$ ions, viz. (i) backbone cleavages, (ii) side-chain cleavages which are independent of the position of the amino acid residue and (iii) fragmentation reactions which are characteristic of particular residues at *N*- and *C*-terminal positions. It should be noted that these observations were made using $[M - H]^-$ precursor ions from common underivatized di- and tripeptides,[18] and were later extended to tetra-[19] and pentapeptides[20] with *C*-terminal COOH and/or $CONH_2$ groups. Backbone cleavages in both peptides of the carboxylic acid and amidated type can yield *N*-terminal fragments with an elemental composition of ″A-, ″B- and C-type ions, while the *C*-terminal fragments are found as ″X-, Y- and ″Z-type ions.[21] Bradford *et al.*,[19] who used a different nomenclature for backbone sequence ions (α^- for Y- and β^- for ″B-type ions), observed mainly ″B- and Y-type ions as typical backbone sequence ions, while in the spectra of our *N*-acetylated peptides **1** and **3** the C- and Y-type ions prevail [Figs 4(a) and 5(a)]. These data are in good agreement with recent observations in the matrix-assisted laser desportion/ionization time-of-flight (MALDI-TOF) anion post-source decay mass spectra of substance P.[22] C-type ions in the $[M - H]^-$ CID spectra of peptides [... $NHCH(R_n)CONH^-$], or peptoids [... $N(R_{n-1})CH_2CON(R_n)^-$] can be formed in a direct bond-cleavage process by elimination of a cyclic neutral molecule, viz. a diketopiperazine. Their proposed formation is rationalized in Scheme 4 and agrees fully with the *m/z* values obtained from the CID spectra of the $[M - D]^-$ ions of **3**d and **2**d. The C-type ions appeared to be more abundant in the peptide than in the peptoid spectra.

The negative ion spectra show more pronounced differences between peptides and peptoids than the positive ion spectra. In contrast to the spectra of **1** and **3**, the CID spectrum of the $[M - H]^-$ ions of **2** [Fig. 4(b)] exhibits intense ″B-type ions. In peptides, these ″B_n ions are depicted as [... $NHC(R_n){=}C{=}O - H]^-$ ions, formed in a fragmentation reaction of the $[M - H]^-$ ion complex, with the one hydrogen deficiency at a methine carbon atom and the second one at an undetermined position.[19] The strong preference for ″B-type ion formation in the $[M - H]^-$ CID spectra of peptoids might reflect a specific stability of the resulting ion which can be rationalized as an ion with a resonance stabilized [... $N(R_n)C{\equiv}C - O^-$] structure. From the negative ion mass spectrum of **2**d it could be derived that the $[M - D]^-$ ion is composed of ~70% of a d_4



Scheme 3. Proposed NH_3 loss from Q- and nQ-immonium ions and formation of *m/z* 42 from nQ-immonium ions.

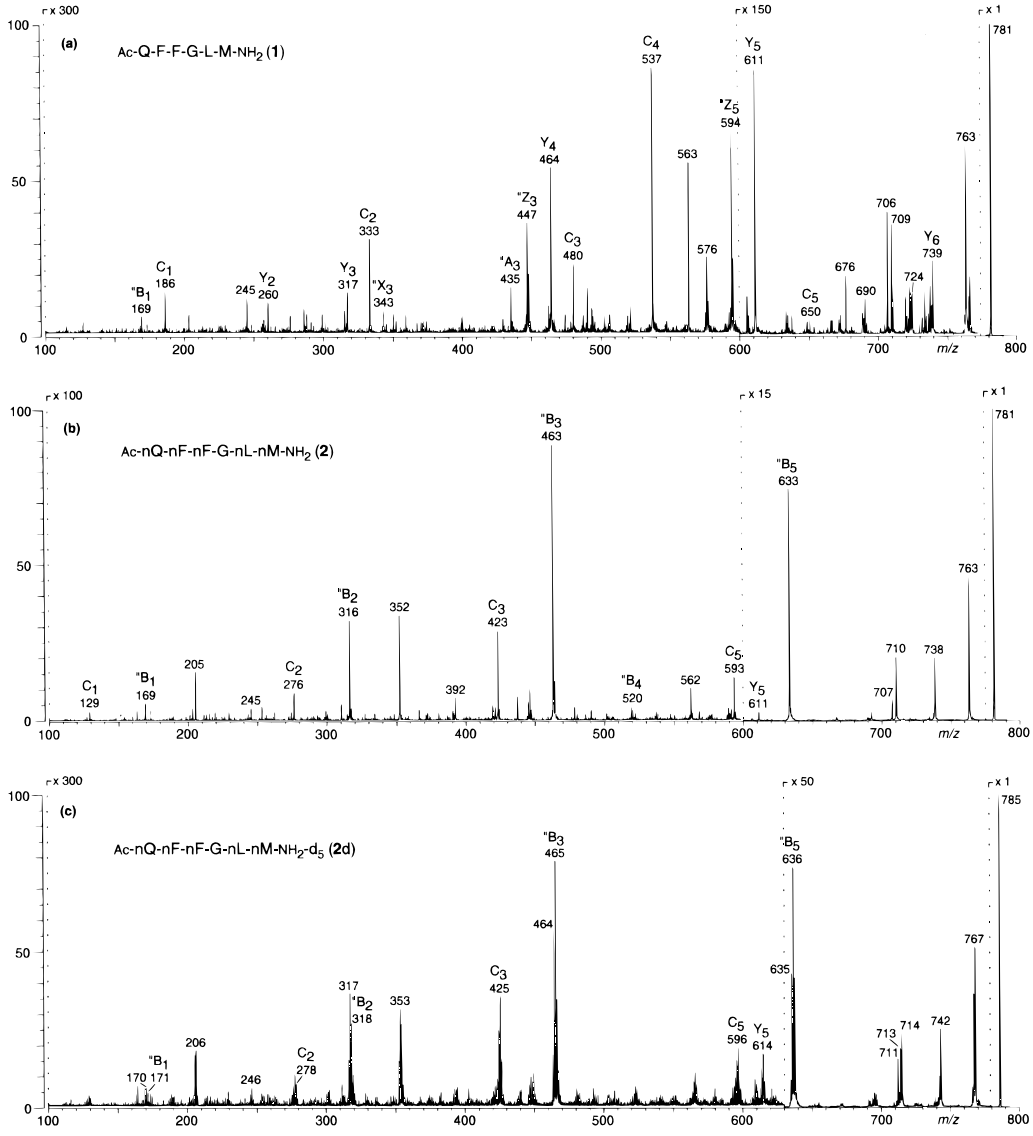


Figure 4. CID spectra of the *m/z* 781 $[M-H]^-$ ions of (a) Ac-QFFGLM-NH_2 (**1**) and (b) Ac-nQnFnFGnLnM-NH_2 (**2**) and the *m/z* 785 $[M-D]^-$ ion of (c) Ac-nQnFnFGnLnM-NH_2-d_5 (**2d**).

ion and about 30% of the d_3 species mainly combined with one ^{13}C, ^{15}N or ^{33}S isotope. The $''B_n$ product ions in the *m/z* 785 $[M-D]^-$ CID spectrum **2d** are now observed as ion clusters [Fig. 4(c)]. The *m/z* 633 $''B_5$ ion of **2** shifts to an ion cluster in **2d** of which *m/z* 636 is the most prominent peak. As the formation of a $''B_5$ ion in **2** (loss of 148 u) requires the loss of the complete *C*-terminal amide $[C_5H_{12}N_2SO]$, the *m/z* 636 ion in **2d** must be mainly formed by loss of $[C_5H_{11}D_2SO]$, which corresponds to full retention of the original d_3 label in the resulting $''B_5$ ion. The *m/z* 635 ion of **2d** can contain only two deuterium atoms, which means that one of the original deuterium labels has been transferred to the *C*-terminal residue and will be lost in the fragmentation of the $[M-D]^-$ ion; the *m/z* 637 ion of **2d** represents mainly the $''B_5$ ion containing the original d_3 label combined with one of the previously mentioned isotopes.

A remarkable phenomenon is the occurrence of a peak at *m/z* 763 corresponding to the loss of an H_2O molecule from all $[M-H]^-$ ions, in contrast to the loss of an NH_3 molecule from the $[M+H]^+$ ions, which is considered to be diagnostic of amidated compounds (Figs 1 and 2). The water loss in the negative ion spectra most probably must be preceded by an enolization of a keto function in the molecular ion, since no hydroxylic groups are present in our model compounds.

Radical side-chain loss from the peptide $[M-H]^-$ ions has been noticed to occur from F, M, Q and L residues, yielding radical anions at *m/z* 690, 706, 709 and 724, respectively.[18,19,23] Losses of the molecules CH_3SH[19,24] (*m/z* 733) and CH_3SCH_3[19] (*m/z* 719) are also clearly visible in the spectra of the peptides [Figs 4(a) and 5(a)] and are characteristic for the presence of M independent of its position in the peptide chain. It should be noted that Bradford *et al.*[19] reported that radical side-chain loss from L and F was observed in the spectra of their dipeptides but not in those of their tetrapeptides, whereas in our hexapeptide spectra the resulting product ions are obviously present. The $[M-H]^-$ ion spectra of the peptoids [Figs 4(b) and 5(b)] show peaks at *m/z* 707 (from **2** only) and *m/z* 710 corresponding to the elimination of C_3H_6S and

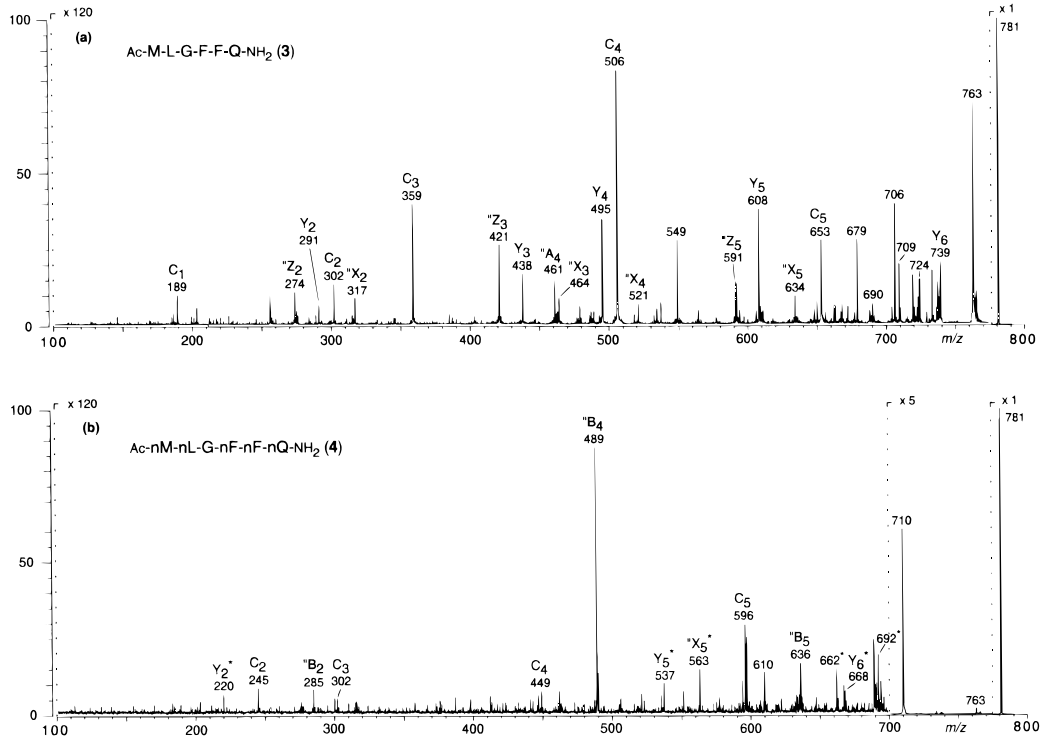
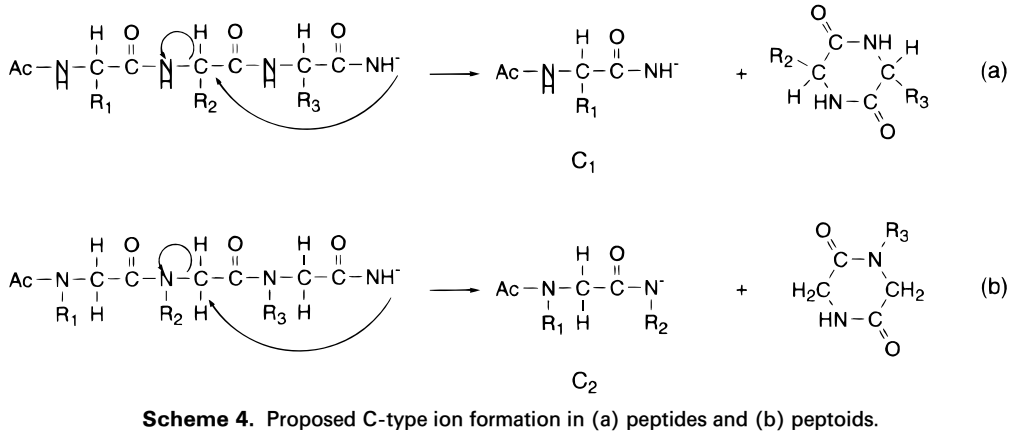


Figure 5. CID spectra of the *m/z* 781 $[M - H]^-$ ions of (a) Ac-MLGFFQ-NH_2 (**3**) and (b) Ac-nMnLGnFnFnQ-NH_2 (**4**).

C_3H_5NO molecules from M and Q side-chains respectively, accompanied by hydrogen transfer to the vacancy at the nitrogen atom. The *m/z* 707 peak shifts completely to *m/z* 711 in the *m/z* 785 $[M - D]^-$ CID spectrum of **2d**, again indicating the loss of C_3H_6S, while the *m/z* 710 peak shifts to two approximately equally intense peaks at *m/z* 714 and 713, corresponding to the losses of C_3H_5NO and C_3H_5DNO, which means that an amidic hydrogen is preferably transferred to the nitrogen atom [Fig. 4(c)]. Furthermore, it should be noted that the methionine side-chain loss is most abundant in the peptides, whereas glutamine side-chain loss prevails in the peptoids. The *m/z* 738 ion **2** has its isotopic analogue at *m/z* 742 in **2d** and therefore must result from $C_3H_7^{\bullet}$ loss from the isobutyl chain and not from loss of O=C=N—H, the isoelectronic equivalent of CO_2 which is commonly observed in peptides with a carboxylic acid terminus.[19] The loss of the C_3H_5NO molecule is extremely dominant in the retropeptoid Ac-nMnLGnFnFnQ-NH_2 with the nQ residue at the *C*-terminus where the negative charge is presumed to be localized. This predominant generation of *m/z* 710 ions in the $[M - H]^-$ CID spectrum of the retropeptoid [Fig. 5(b)] obstructs the formation of other sequence ions in reasonable abundances, so that sequence analysis using this spectrum CID spectrum is seriously hampered. The resulting *m/z* 710 ion acts as a new $[M - H]^-$ precursor ion of a peptoid with an Ac-nMnLGnFnFG-NH_2 structure, producing a variety of product ions marked with an asterisk in the spectrum in Fig. 5(b). In the peptoid Ac-nQnFnFGnLnM-NH_2 with the nQ residue at the *N*-terminus, the elimination of C_3H_5NO molecules can also occur from various ″B- and C-type ions resulting in the formation of product ions at *m/z* 562 (″B_5 − 71), 392 (″B_3 − 71), 352 (C_3 − 71), 245 (″B_2 − 71) and 205 (C_2 − 71), similarly as the C_7H_6O elimination proceeds from many sequence ions in the positive ion spectrum of Leu-enkephalin.[4] These fragment ions are diagnostic for the position of the nQ residue in the peptoid.



Scheme 4. Proposed C-type ion formation in (a) peptides and (b) peptoids.

CONCLUSIONS

It can be stated that the $[M + H]^+$ CID spectra of the investigated hexapeptides and peptoids exhibit characteristic B- and Y″-type ions, which allow full sequence analysis. The relative abundances of Y″-type ions in peptoids are higher than those of the corresponding ions in peptides. The identity of *N*-substituted glycine residues can be derived from the CID spectra of their immonium ions. Peptoids tend to lose their *N*-substituted side-chain as a molecule accompanied by hydrogen rearrangement, while peptides can lose the complete side-chain as a radical. The CID spectra of the $[M - H]^-$ ions are more complex because of the occurrence of various side-chain fragmentation reactions. In contrast to previously reported high-energy CID spectra of peptides with a carboxylic acid or amide terminus showing abundant ″B-type ions, the formation of C-type ions appears to be favoured in the peptides investigated in this study. Side-chain loss in peptoids will nearly always occur as loss of molecule, while the magnitude of this elimination reaction is strongly dependent on the position of the specific residue in the peptoid chain. Such a preferential fragmentation reaction channel can reduce the occurrence of backbone fragmentation to such an extent that a reliable sequence analysis will become very difficult or even impossible. Just as for peptides, positive ion CID spectra of peptoids appear to be more suitable for structure determination, although the negative ion CID spectra of both peptides and peptoids are also very characteristic and certainly can provide complementary information.

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