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FOCUS: MS/MS PEPTIDE IDENTIFICATION: RESEARCH ARTICLE

Fragmentation Reactions of b_5 and a_5 Ions Containing Proline—The Structures of a_5 Ions

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Abstract

A detailed study has been made of the b_5 and a_5 ions derived from the amides H-Ala-Ala-Ala-Ala-Pro-NH_2, H-Ala-Ala-Ala-Pro-Ala-NH_2, and H-Ala-Ala-Pro-Ala-Ala-NH_2. From quasi-MS^3 experiments it is shown that the product ion mass spectra of the three b_5 ions are essentially identical, indicating macrocyclization/reopening to produce a common mixture of intermediates prior to fragmentation. This is in agreement with numerous recent studies of sequence scrambling in b ions. By contrast, the product ion mass spectra for the a_5 ions show substantial differences, indicating significant differences in the mixture of structures undergoing fragmentation for these three species. The results are interpreted in terms of a mixture of classical substituted iminium ions as well as protonated C-terminal amides formed by cyclization/rearrangement as reported recently for a_4 ions (Bythell, Maître , Paizs, *J . Am. Chem. Soc.* **2010,** *132*, 14761–14779). Novel fragment ions observed upon fragmentation of the a_5 ions are protonated H-Pro-NH_2 and H-Pro-Ala-NH_2 which arise by fragmentation of the amides. The observation of these products provides strong experimental evidence for the cyclization/rearrangement reaction to form amides and shows that it also applies to a_5 ions.

Key words: b_5 ion structures, a_5 Ion structures, Tandem mass spectrometry

Introduction

Peptide sequence information in proteomics is achieved by tandem mass spectrometry (MS/MS) of singly- or multiply-protonated peptides [1–3]. These studies frequently involve collision-induced dissociation (CID) to produce fragment ions. In favorable cases, fragmentation occurs by cleavage of amide bonds to give a series of y and/or b ions that contain, respectively, the C-terminus and N-terminus residues [4]. Additionally, a ions, often arising by loss of CO from b ions [5], provide further confirming sequence information.

Although it has been established [6, 7] that y ions are protonated amino acids (y_1) or protonated truncated peptides (y_n), the structure(s) and fragmentation reactions of b ions present a much more complex picture [8]. Although it was originally proposed [9, 10] that b ions were acylium ions, extensive MS/MS and theoretical studies of small b ions [5, 11–15] have presented strong evidence that in many cases, cyclization has occurred to form a protonated oxazolone ring at the C-terminus. More recently, a number of infrared multiphoton dissociation (IRMPD) studies [16–20] have provided direct evidence for the protonated oxazolone structure for smaller b ions. Such cyclic b ions are expected to fragment by loss of CO and by sequential loss of amino acid residues to give lower mass sequence-specific b ions [4, 8].

In early studies, Boyd and co-workers [21, 22] reported observation of non-direct sequence ions in the fragmentation of doubly-protonated b ions containing lysyl or ornithyl residues, which were interpreted in terms of cyclization/reopening reactions prior to fragmentation. In the past few years, there have been a large number of experimental and theoretical studies [23–35] (primarily of b_5 and larger b ions), which have shown that these b ions, initially formed with an oxazolone ring at their C-terminus, frequently undergo head-to-tail cyclization to form a macrocyclic isomer. This macrocyclic isomer may reopen at different amide bonds leading to a variety of oxazolones, many of which fragment to give non-direct sequence ions, i.e., those not anticipated from the original sequence of the peptide [24, 27]. It might be noted that ion mobility data indicate, in

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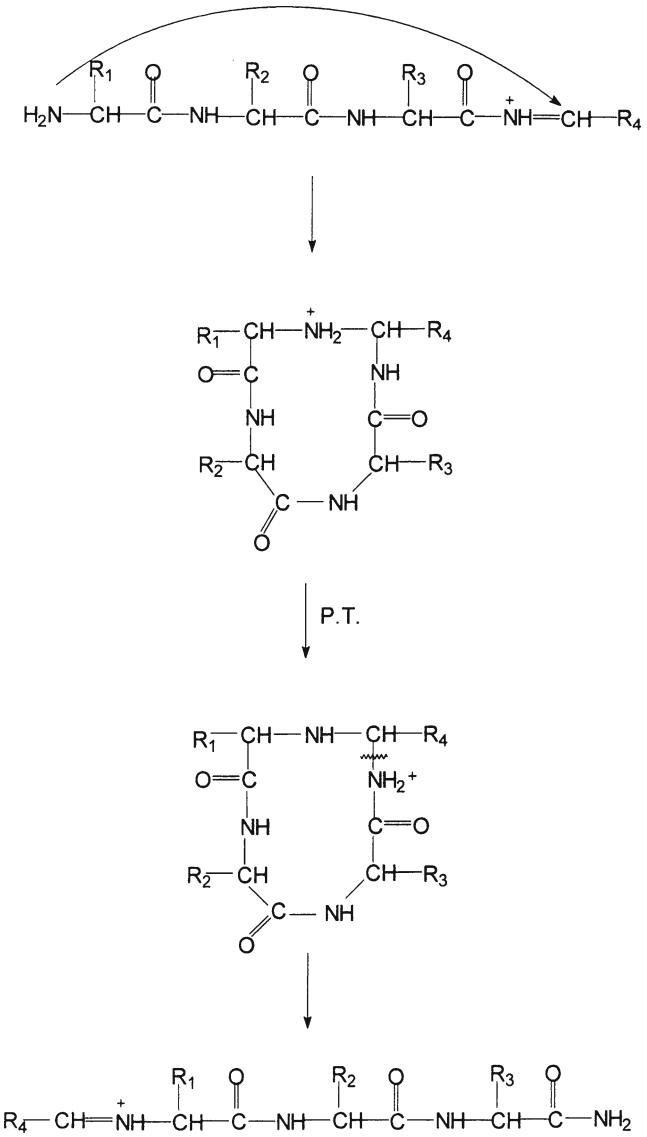
many cases, that b ions have a mixture of structures [36–38]. The effect of this possible sequence scrambling in sequencing proteins is not yet clear; two recent studies [39, 40] have indicated that such scrambling has a limited effect on sequencing, while a further publication [41] suggests a greater effect in some cases.

The a_n ions frequently observed [42–44] in product ion mass spectra of peptides carry useful information as to the residues present and also their sequence, although they sometimes are not considered in peptide sequencing programs. Such a_n ions may be formed directly from the protonated species [45, 46] or by loss of CO from the corresponding b_n ion [4, 5, 11]. Indeed, the observation of an a_n ion 28 mass units lower than the supposed b_n ion helps confirm the assignment as a b ion.

As initially formed a_n ions are immonium (iminium) ion derivatives, although, as with b_n ions, there is ample opportunity for rearrangement, including cyclization. The simplest iminium ions, R-CH=NH_2^+, may be formed from any residue present in the peptide and serve to identify the amino acid residues present [42, 43]. Theoretical calculations and infrared multiphoton dissociation (IRMPD) studies [47–49] have shown that a_2 ions have undergone cyclization to from a cyclic protonated 4-imidazolidone. By contrast, a_3 ions are rarely observed with any intensity [44, 50]; this has been attributed to the ready fragmentation to form b_2 ions as well as a_3* (a_3-NH_3) ions [50].

However, a_4 ions are commonly observed. In early studies, Glish and co-workers [51] observed that the a_4 ion derived from leucine enkephalin, nominally an iminium ion of sequence YGGF, fragmented by loss of NH_3 to form the a_4* ion, which fragmented further by elimination of a glycine residue. Studies of related a_4 ions showed that the residue lost from a_4* involved the third residue, for example the proline residue from the a_4* ion derived from YGPFL. They proposed a concerted cyclization reaction involving loss of the N-terminal amino group as NH_3 and reopening of the cyclic structure to expose the third residue at the C-terminus of the rearranged a_4* ion. Bythell et al. [52] have studied in detail the fragmentation of the a_4 ion derived from FGGFL, nominally the FGGF iminium ion. Using ^{15}N labeling they showed that the ammonia lost in formation of the a_4* ion originated almost equally from the two phenylalanine (F) residues. They proposed prior sequence rearrangement in the b_4 precursor to the a_4 ion, loss of the N-terminal amine as ammonia and rearrangement similar to that proposed by Glish and co-workers but involving a proton bound intermediate complex rather than the concerted mechanism involving cyclization as proposed by Glish. Quite recently, Bythell et al. [49] have shown from theoretical calculations and IRMPD studies of the a_4 ions derived from pentaglycine and penta-alanine that rearrangement of the a_4 ions occurred as shown in a generalized way in Scheme 1. (Throughout, "P.T." is used in the schemes as an abbreviation for "proton transfer.") This rearrangement results in an amide function at the C-terminus of the



Scheme 1. Rearrangement of a_4 ions

rearranged a_4 ion, which is ideally suited to eliminate NH_3 to form an a_4* ion with a C-terminal oxazolone which can fragment by elimination of what was the third amino acid residue in the original iminium ion. This sequence of fragmentation reactions is consistent with the observations of Glish and co-workers [51]. It appears that the ^{15}N labeling results [52] also are consistent with this reaction pathway if we allow for appropriate sequence rearrangement [24] in the precursor b_4 ion.

In the present work, we have studied the structures and fragmentation reactions of b_5 and a_5 ions containing four Ala residues and one Pro residue. These were derived from the amides AAAAP-NH_2, AAAPA-NH_2, and AAPAA-NH_2. Not surprisingly, the product ion mass spectra for the b_5 ions are essentially identical indicating cyclization to a common structure prior to fragmentation. On the other hand, the product ion mass spectra for the a_5 ions show significant

differences including formation of products, which are most readily explained by formation, in part, of a C-terminal amide function prior to fragmentation, consistent with the final rearrangement product of Scheme 1 but applied to a_5 ions.

Experimental

All experimental work was carried out using an electrospray/quadrupole/time-of-flight (QqTOF) mass spectrometer (QStarXL; SCIEX, Concord, Canada). MS^2 experiments were carried out in the usual manner by selecting the ions of interest with the mass analyzer Q followed by CID in the quadrupole collision cell q and mass analysis of the ionic products with the time-of-flight analyzer. In the quasi-MS^3 studies of fragment ions, CID in the interface region produced fragment ions with those of interest being selected by the quadrupole mass analyzer Q for fragmentation and analysis in the usual manner. The cone voltage in the interface region was varied to achieve the best yield of the fragment ions of interest. The product ion mass spectra were independent of the cone voltage employed.

Ionization was by electrospray with the sample, at micromolar concentrations dissolved in 1:1 CH_3OH:1% aqueous formic acid, introduced into the source at a flow rate of 10 μL min^{-1}. Nitrogen was used as nebulizing gas and drying gas and as collision gas in the quadrupole cell q.

The compounds studied were obtained from Celtek Peptides (Nashville, TN, USA); they showed no impurities in their mass spectra and were used as received.

Results and Discussion

The ions studied were derived from the amides A-A-A-A-P-NH_2, A-A-A-P-A-NH_2, and A-A-P-A-A-NH_2. The b_5 ions arise by loss of NH_3 from the protonated species while the a_5 ions arise by loss of NH_3 and CO from the protonated species. ^{15}N labeling experiments on F-A-G-F-L-NH_2 have shown [27] that the NH_3 lost contains the amide nitrogen. The yield of b_5 and a_5 ions from A-A-A-A-P-NH_2 was rather low but sufficient to obtain reliable product ion mass spectra.

Figure 1 shows the product ion mass spectra at 22 eV collision energy for the three b_5 ions (*m/z* 382) studied. The spectra are essentially identical, indicating cyclization to a common macrocyclic intermediate which reopens to the same mixture of protonated oxazolone or mixture of oxazolones [24, 27] prior to fragmentation. Apart from the loss of CO the spectra are dominated by sequential loss of three Ala residues with only very minor loss of the Pro residue. This indicates that the cyclization/reopening reaction leads primarily to the Pro residue near the N-terminus of the rearranged protonated oxazolones. A similar result, although less clear-cut, was observed [34] in the fragmentation of b_5 ions containing four Ala residues and one His residue. In that study [34], computations showed that opening the macrocyclic species to form oxazolones with His near the C-terminus had higher energy barriers than the energy barriers to form oxazolones with the His residue near the N-terminus. Presumably the same also applies in the Pro case. Two unexpected products are observed at *m/z* 115 and 186; their origin will be discussed below with respect to the fragmentation of the a_5 ions.

Figure 2 presents the product ion mass spectra for the three a_5 ions studied. In contrast to the results for the b_5 ions, the spectra are not identical; although the same product ions are formed, the relative intensities show substantial differences in the three spectra. The product ions of *m/z* 115 and 186 are much more intense than they were in the b_5 ion spectra indicating that they originate by fragmentation of the a_5 ions. The *m/z* 186 ion is quite abundant in the product ion mass spectrum of the MH^+ ion of A-A-A-P-A-NH_2 but is not seen in the product ion mass spectrum of the MH^+ ion of A-A-P-A-A-NH_2 (spectra not shown), whereas it is the base peak in the product ion spectrum of the a_5 ion derived from AAPAA-NH_2. Table 1 compares the product ion mass spectrum of *m/z* 186 derived from the latter compound with the product ion mass spectra for the MH^+ ions of A-P-NH_2 and P-A-NH_2; all spectra were obtained at 14 eV collision energy. The two amides A-P-NH_2 and P-A-NH_2 are readily distinguishable from the CID mass spectra of the MH^+ ions with the former showing the y_1 ion (protonated prolinamide) as the base peak and the latter showing the proline iminium ion (Im_P) at much greater relative intensity. The spectrum obtained for the *m/z* 186 ion derived from A-A-P-A-A-NH_2 is similar to that obtained for protonated P-A-NH_2, indicating that the major part of the *m/z* 186 signal corresponds to protonated P-A-NH_2. However, the relatively weak signal at *m/z* 115 indicates that there is some population (probably small) of protonated A-P-NH_2. Comparison of the product ion mass spectrum of the *m/z* 115 ion derived from protonated A-A-A-P-A-NH_2 with the product ion mass spectrum of the y_1 ion derived from protonated A-P-NH_2 clearly showed that the former was protonated prolinamide. The pathways to these amides will be discussed in the following.

Accepting that a_n ions are initially formed, at least in part, by loss of CO from the corresponding (cyclic) b_n ions and, initially, have an iminium ion functionality at the C-terminus, there are five initial a_5 ions that may be formed. This results from sequence scrambling in the b_5 ions, which may put the Pro residue in any position. These structures are illustrated in Scheme 2, which also includes the structures that would be formed by rearrangement as outlined in Scheme 1. Structure 1 has the proline residue at the N-terminus. It most likely fragments from the original structure by loss of $CH_3CH{=}NH$ followed by sequential loss of Ala residues. It appears that rearrangement as per Scheme 1 does not occur. Because of the secondary nature of the Pro nitrogen, such a rearrangement would lead to the charge on the Pro nitrogen and no mobile proton.

Initial Structure 2 has the proline residue in the second position; this initial structure can fragment in the same fashion as initial Structure 1. Alternatively, the initial

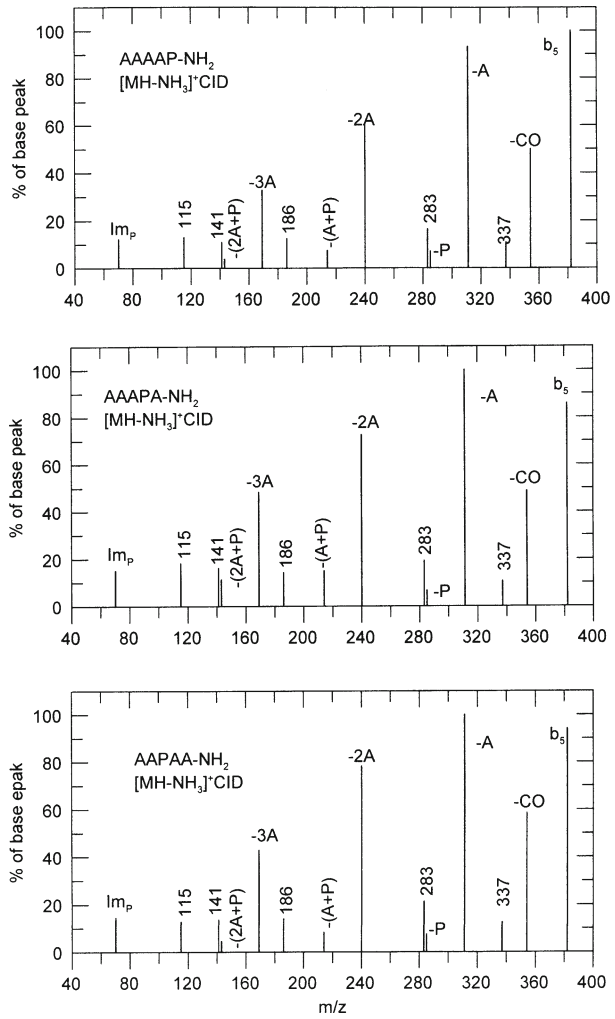


Figure 1. Product ion mass spectra for b_5 ions at 22 eV collision energy

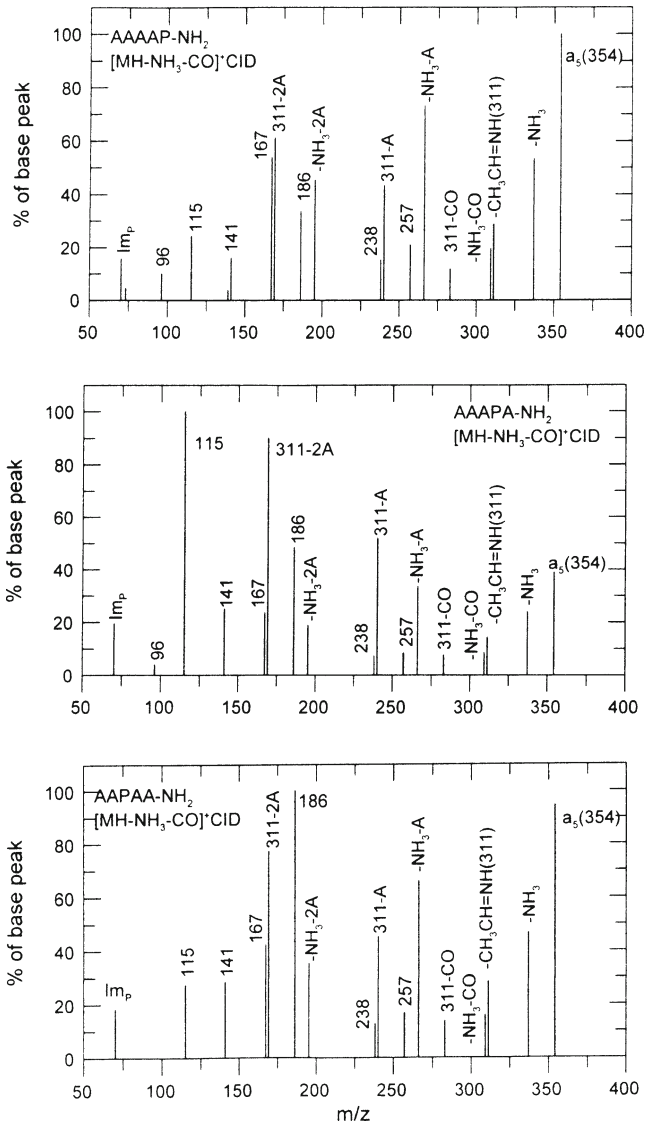


Figure 2. Product ion mass spectra for a_5 ions at 22 eV collision energy

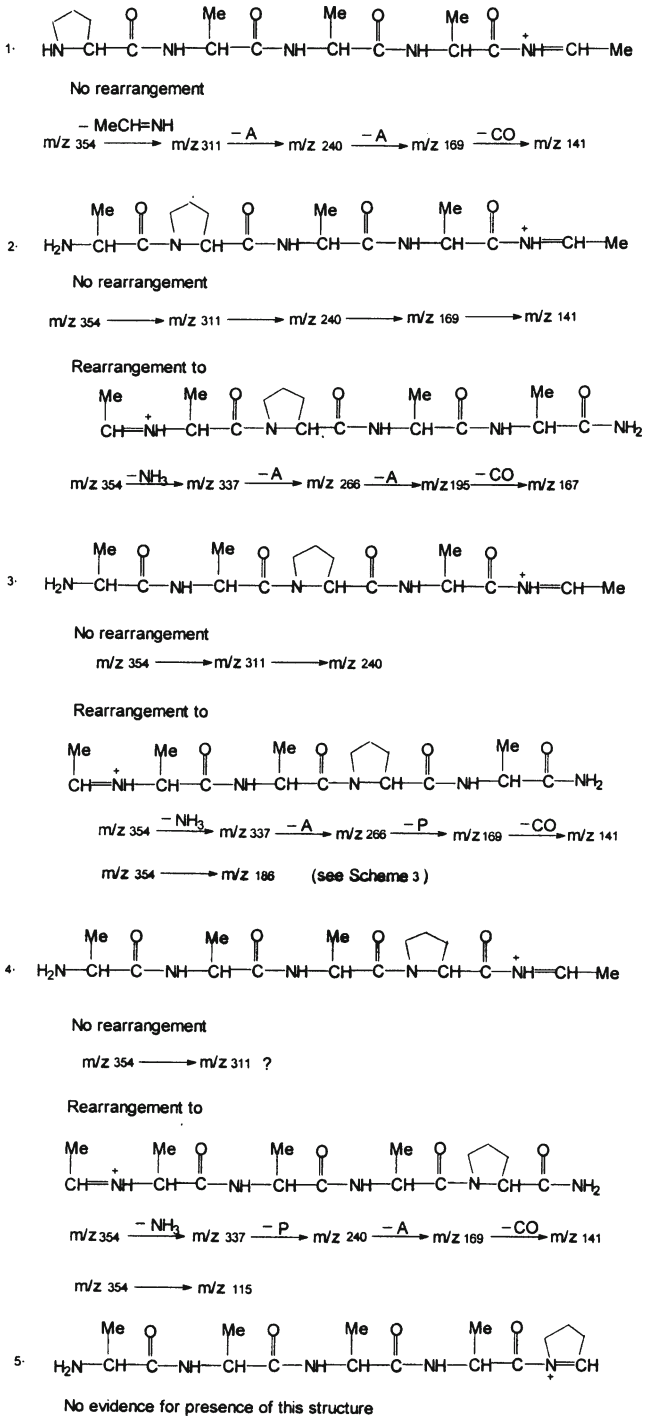
structure can rearrange by the pathway outlined in Scheme 1 to give the amide structure shown. With migration of the proton to the C-terminal amide, sequential loss of NH_3 and Ala residues can occur as shown. Fragmentation of this rearranged structure clearly is the pathway that leads to the *m/z* 195 ($-NH_3$-2A) product observed in the three a_5 product ion mass spectra (Figure 2).

Initial Structure 3 may fragment directly by loss $CH_3CH=NH$ and an Ala residue but there is no signal for subsequent loss of the Pro residue from the *m/z* 240 product. It is likely that the preferred fragmentation pathway is by rearrangement to form the amide shown. Again, with migration of the proton, the sequence involving loss of NH_3 followed by sequential loss of first an Ala residue followed by loss of a Pro residue can occur. This reaction sequence is supported by the breakdown graph for the a_5* ion (*m/z* 337, a_5-NH_3) derived from A-A-P-A-A-NH_2 shown in Figure 3. In addition to loss of CO, loss of an Ala residue to give *m/z* 266, followed by loss of a Pro residue to give *m/z* 169 clearly provides the main fragmentation pathway.

Table 1. Product Ion Mass Spectra of Ions of *m/z* 186 (Intensities as % of Base Peak)

	Source		
m/z(Ion)[a]	$APNH_2.H^+$	$PANH_2.H^+$	*m/z* 186[b]
186(MH^+)	83.5	100	100
169(b_2)	67.7	64.6	69.0
141(a_2)	41.9	45.1	45.9
115(y_1)[c]	100	_	6.0
70(Im_P)	16.4	68.2	74.4
44(Im_A)	6.5	_	_

[a]Applies to first two entries.
[b]Derived from protonated $AAPAA-NH_2$.
[c]Applies only to first entry.



Scheme 2. Structures and fragmentation of a_5 ions

Alternatively, for the a_5 ion, transfer of the proton to the basic nitrogen of the Pro residue leads to the fragmentation pathway of Scheme 3 resulting in formation of protonated H-Pro-Ala-NH_2 (*m/z* 186). The most favorable site of the proton in the protonated amide is not known; for convenience it has been placed on the Pro nitrogen.

Initial Structure 4 may lose $CH_3CH{=}NH$ to give *m/z* 311 but there is no ion signal supporting further fragmentation along this pathway. It appears that rearrangement along the

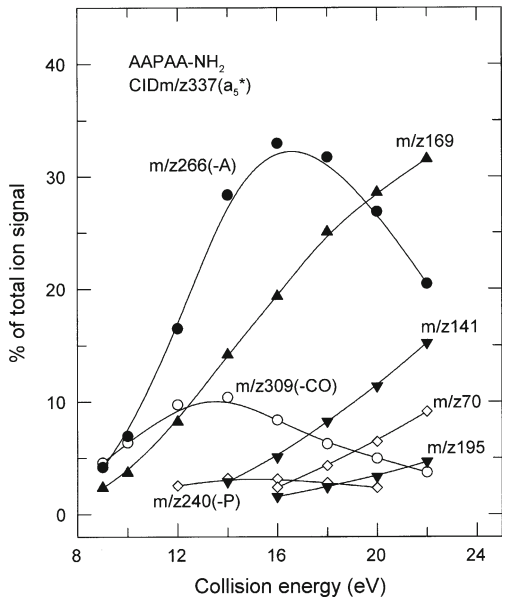
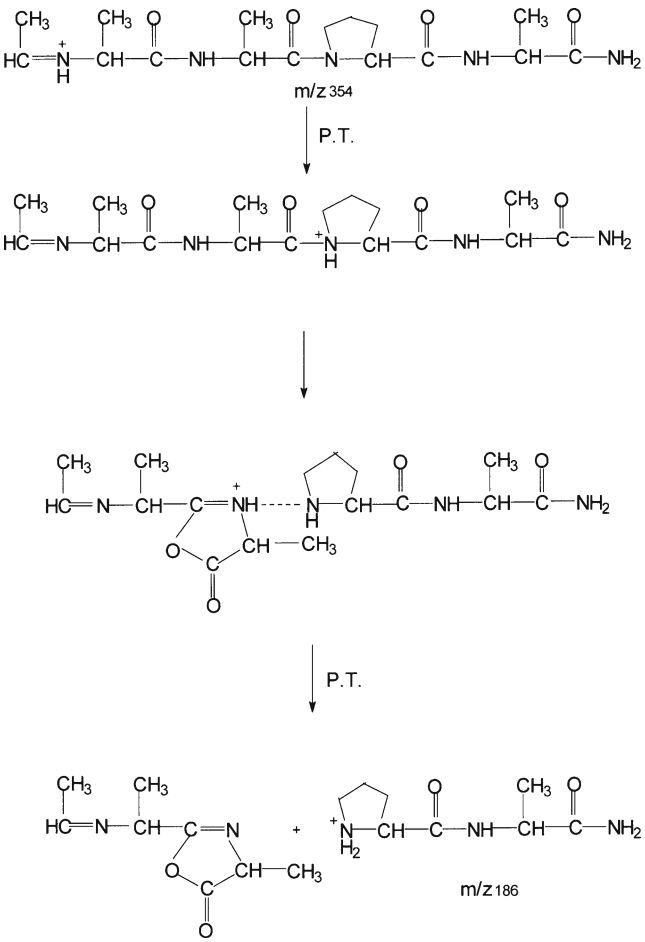


Figure 3. Breakdown graph for a_5* ion derived from H-Ala-Ala-Pro-Ala-Ala-NH_2



Scheme 3. Pathway to protonated Pro-Ala-NH_2

lines of Scheme 1 occurs to give the amide structure shown. Clearly, this amide structure can fragment by proton transfer to the Pro residue followed by fragmentation along the lines of Scheme 3 only giving protonated prolinamide (*m/z* 115) as the final product. Figure 4 shows the breakdown graph for the a_5* (*m/z* 337) derived from A-A-A-P-A-NH_2. In agreement with the rearranged amide structure, there is significant loss of the Pro residue to give *m/z* 240 with further fragmentation by loss of an Ala residue to give *m/z* 169. However, there obviously is a second structure for the a_5* ion studied which fragments initially by loss of an Ala residue; this becomes the major fragmentation pathway at higher collision energies. The amide form of Structure 4 is the likely origin of the low yield of protonated H-Ala-Pro-NH_2, which was discussed above. Proton transfer to the nitrogen of the Ala residue nearest the C-terminus and further reaction along the pathway indicated in Scheme 3 can occur, but clearly is less favorable than proton transfer to the Pro residue because of the greater basicity of the latter nitrogen.

There is no substantial evidence for formation of Structure 5. All a_5 product ion mass spectra show a low intensity ion signal at *m/z* 257, which could correspond to loss of the Pro residue from the initial iminium ion structure. However, protonated A-A-P-NH_2 also has an *m/z* ratio of 257 and, indeed, is isobaric with a_5-P. It is more likely that the signal observed corresponds to this protonated amide. It might also be noted that the *m/z* 169 ion, presumably formed from unrearranged ions of Structures 2 and 3, has a different structure than the *m/z* 169 ions formed from the rearranged ions of Structures 3 and 4; however, again the elemental composition is identical for the two ions.

The product ion mass spectra obtained for the a_5 ions clearly show that there are a variety of structures for the ions

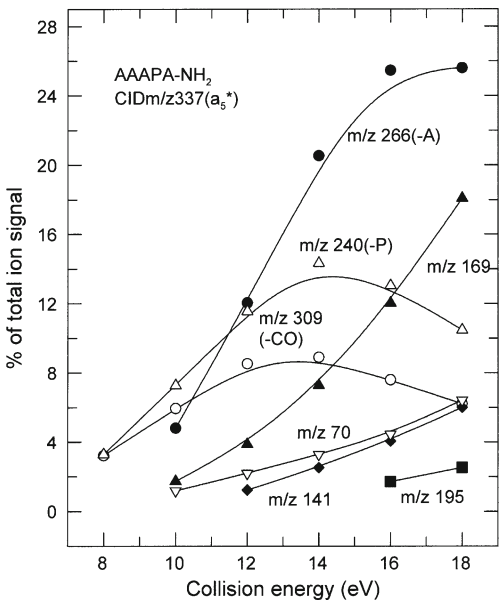


Figure 4. Breakdown graph for a_5* ion derived from H-Ala-Ala-Ala-Pro-Ala-NH_2

subjected to collisional activation. Because of this multitude of possible structures, it is not possible to identify quantitatively the contribution of each. The structures are summarized in Scheme 2. In part, this variety of structures arises from sequence scrambling [24, 27] in the b_5 ions, which serve as precursors for the a_5 ions. While some of the product ions observed can be rationalized in terms of fragmentation of the "classical" substituted iminium ions, many of the product ions observed clearly result from fragmentation of protonated amide structures formed by the pathway analogous to Scheme 1. The most striking evidence for such structures, apart from the loss of NH_3, is the formation of protonated prolinamide (*m/z* 115) and protonated H-Pro-Ala-NH_2 (*m/z* 186). The results, thus, serve to support the mechanism of cyclization/rearrangement proposed by Paizs and co-workers [49] for a_4 ions and shows that this rearrangement to amide structures also applies to a_5 ions.

Conclusions

Not surprisingly, the present work shows that b_5 ions containing one proline residue undergo sequence scrambling [24, 27] prior to fragmentation, thus giving the same product ion mass spectra irrespective of the original position of the Pro residue. The chief contribution of the present work relates to the structures and fragmentation reactions of the a_5 ions. Although the a_5 ions are formed initially with a substituted iminium ion structure, clear evidence is presented that prior to fragmentation, there is substantial rearrangement to an amide structure. This amide structure is formed by the cyclization/rearrangement pathway recently elucidated by Paizs and co-workers [49] as applicable to a_4 ions (Scheme 1) and shown here to also be applicable to a_5 ions.

A second interesting feature is that the product ion mass spectra for the a_5 ions differ markedly in terms of the relative fragment ion intensities. A major pathway to a ions is thought to be by loss of CO from the corresponding b ion. Since the results in the present study indicate common mixture of structures for the b_5 ions derived from the three precursors, one might have expected a common mixture of structures for the a_5 ions. It is not clear whether the different behavior of the a_5 and b_5 ions in the present study reflect a direct pathway from MH^+ to a_5 or whether, at the internal energies necessary for formation of a_5 ions from b_5 ions, the lifetime of the b_5 ions is sufficiently short so that full sequence scrambling does not occur.

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