Peptide Sequence Scrambling Through Cyclization of b_5 Ions

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The CID mass spectra of the MH^+ ions and the b_5 ions derived therefrom have been determined for the hexapeptides YAAAAA, AYAAAA, AAYAAA, AAAYAA, and AAAAYA. The CID mass spectra for the b_5 ions derived from the five isomers are essentially identical and show abundant ion signals for nonsequence b ions. This result is consistent with cyclization of the b_5 ions to a cyclic pentapeptide before fragmentation; this cyclic peptide can open at various positions, leading to losses of amino acid residues that are not characteristic of the original amino acid sequence. These nonsequence b ions are also observed in the fragmentation of the MH^+ ions and increase substantially in importance with increasing collision energy. A comparison of the fragmentation of AAAYAA and Ac-AAAYAA indicates that N-acetylation eliminates the cyclization of b_5 ions and, thus, eliminates the nonsequence ions in the CID mass spectra of both b_5 and MH^+ ions. (J Am Soc Mass Spectrom 2008, 19, 1776–1780) © 2008 American Society for Mass Spectrometry

Collision-induced dissociation (CID) [1, 2] of protonated peptides, produced by soft ionization techniques, has become a widely used method for deriving sequence information for peptides and proteins [3–5]. Under low-energy CID conditions protonated peptides most often fragment by amide bond cleavage to produce N-terminal b ions and/or C-terminal y ions [6, 7]; indeed, it is the sequence of b_n and y_n ions that provides sequence information. Although it has been clearly established [8, 9] that the y ions are protonated amino acids (y_1) or protonated truncated peptides (y_n), the structure(s) of the b ions presents a much more complicated picture. Although it was originally proposed [6, 7] that b ions had an acylium ion structure, extensive studies [10–15] of b_2 and b_3 ions have shown that, in many cases, cyclization has occurred to form a protonated five-membered oxazolone ring. When there is a strong nucleophile in the side-chain alternative, cyclization reactions involving this nucleophile may occur [16–19].

More recently, several groups [20–23] have presented evidence that b_5 ions (and larger b ions) form fully cyclic structures; ab initio calculations [21] indicate that the pathway to these cyclic structures involves attack of the N-terminal amine function on the C-terminal oxazolone, which is formed in the initial fragmentation process. Elegant infrared (IR) photodissociation studies coupled with theoretical calculations have provided direct evidence for the oxazolone structure for the b_4 ion derived from Leu-enkephalin but also showed evidence for some proportion of the fully cyclic structure [24, 25]. The pathway [21] for formation of the cyclic structure for the b_5 case is shown in Scheme **1**. As shown in the scheme, the cyclic peptide may reopen at various positions to form a variety of oxazolones, thus achieving scrambling of the initial amino acid sequence. Further fragmentation of these rearranged oxazolones will lead to "nonsequence" fragment ions that have the potential to lead to confusion when sequencing an unknown peptide. Although such "nonsequence" ions have been observed in a number of the recent studies [20–23], their significance in the CID spectra of protonated peptides has not been clearly established and further studies seemed desirable. Accordingly, we have made a detailed study of the fragmentation of a series of protonated hexapeptides containing five alanine residues and one tyrosine residue with the position of the tyrosine residue varied. As will be shown in the following text, all b_5 ions show identical CID mass spectra irrespective of the original position of the tyrosine residue, indicative of cyclization to a common structure. Further, the CID spectra of the MH^+ ions show substantial signals for "nonsequence" ions originating by further fragmentation of the rearranged oxazolones formed by ring opening of the cyclic b_5 ion. It is also shown that this cyclization and concomitant sequence scrambling can be avoided by acetylation of the N-terminal amine function.

Experimental

All experimental work was carried out using an electrospray/quadrupole/time-of-flight (QqToF) mass spectrometer (Ultima III, Waters/Micromass, Manchester, UK). MS^2 experiments were carried out in the usual fashion for MH^+ ions by mass-selecting the ions of

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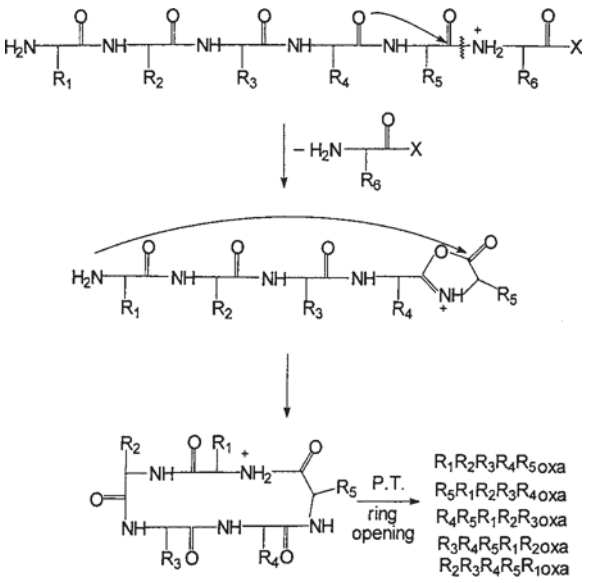

Scheme 1

interest with the quadrupole mass analyzer Q with CID in the quadrupole collision cell q and mass analysis of the ionic products with the time-of-flight analyzer. In the MS^3 experiments, CID in the interface region produced fragment ions with those of interest being mass-selected by the quadrupole mass analyzer for fragmentation and analysis in the usual way. The cone voltage in the interface region was varied to achieve the best yield of the fragment ion of interest; typically a cone voltage of 50 V was found to be optimum. By varying the collision energy in the quadrupole cell, breakdown graphs—expressing, in a qualitative way, the relative energy dependencies of the fragmentation reactions—were obtained under multiple collision conditions.

Ionization was by electrospray with the sample dissolved in 1:1 CH_3OH:1% aqueous formic acid and introduced into the source at a flow rate of 10 μL min^{-1}. Nitrogen was used as nebulizing and drying gas, whereas argon was used as collision gas in the quadrupole collision cell. There is no provision for measuring the collision gas pressure; with the collision gas flowing the pressure gauge for the quadrupole region read approximately 5×10^{-5} mbar.

The peptide AAAYAA was obtained from Bachem Biosciences (King of Prussia, PA, USA); all other peptides were obtained from Celtek Peptides (Nashville, TN, USA). All samples were used as received.

Results and Discussion

The fragmentation reactions of protonated hexaalanine have been studied in detail previously [26]. A major fragmentation of the MH^+ ion involves loss of neutral alanine to form the b_5 ion, which fragments further, in part, by sequential losses of alanine residues to form the lower b ions. With all residues the same it is not possible to determine whether any skeletal rearrangement has occurred during this sequential fragmentation. Consequently, we have used the tyrosine residue as a marker to probe for sequence scrambling in the fragmentation of b_5 ions. Figure 1 shows the CID mass spectra obtained at 14 eV collision energy for the b_5 ions formed from protonated YAAAAA, AYAAAA, AAYAAA, AAAYAA, and AAAAYA by loss of neutral alanine. As can be seen, the CID mass spectra of the five b_5 ions are essentially identical. These results are not consistent with formation of an oxazolone at the C-terminus on loss of alanine and fragmentation from this oxazolone structure. Specifically, the strong signal for loss of the tyrosine residue (Y) in the first four cases as well as the loss of the alanine residue (A) from b_5 in the last case indicate a much more complex behavior. The

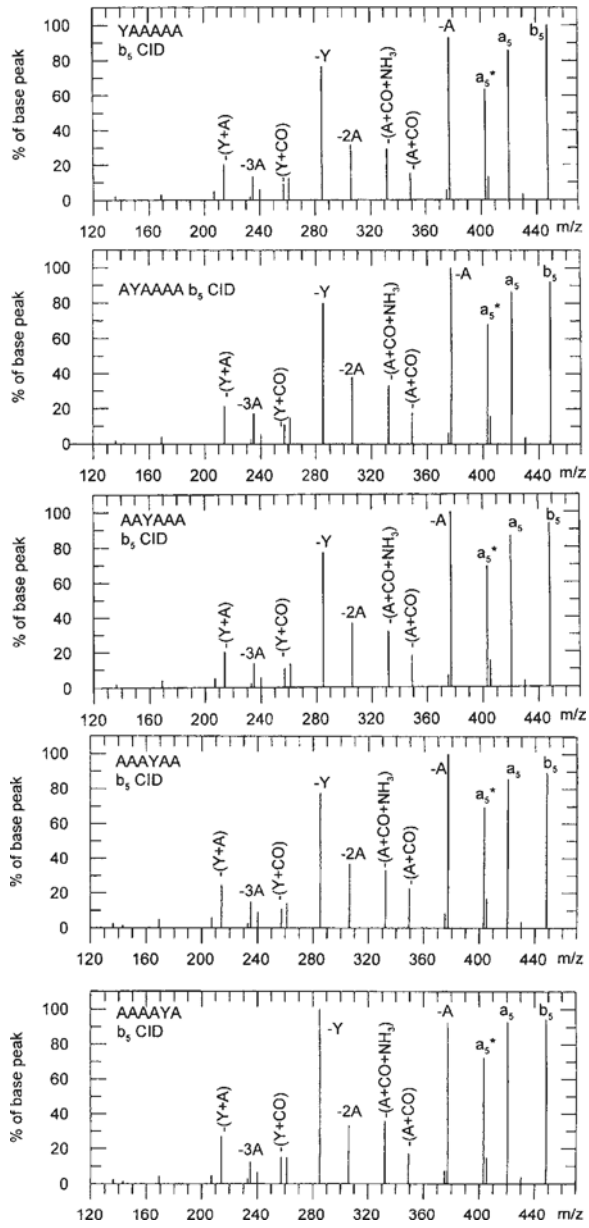

Figure 1. Comparison of CID mass spectra of b_5 ions at 14 eV collision energy.

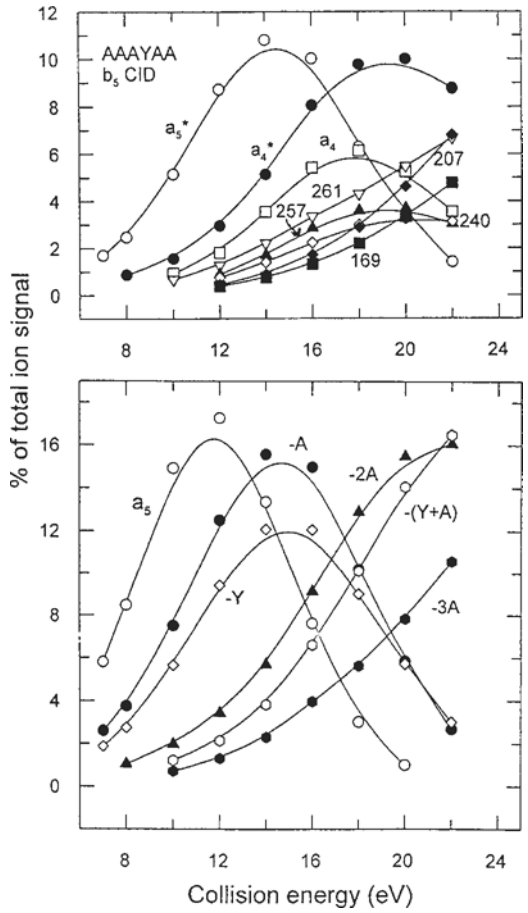

Figure 2. Breakdown graph for the b_5 ion derived from AAAYAA. b_5 ion signal not shown.

results are consistent with the mechanism outlined in Scheme **1**, in which a protonated cyclic pentapeptide is formed, which, upon activation, opens to form a variety of oxazolones, one of which results in the tyrosine residue at the C-terminus, thus leading to loss of the tyrosine residue upon fragmentation. This cyclization mechanism means that the fragment ions identified as -A, −2A, and −3A can have more than one amino acid sequence. Computational studies by Paizs (B. Paizs, private communication) on the fragmentation of protonated cyclo-(-YAGFL) indicates that cleavage of the Y—A amide bond and subsequent loss of the Y residue is the least energy-demanding fragmentation route; the same appears to be the case in the present system.

The breakdown graphs for the b_5 ions were essentially identical. As an example, Figure 2 shows the complex breakdown graph for the b_5 ion derived from protonated AAAYAA. Besides the b ions formed by loss of the tyrosine (Y) and alanine (A) residues from b_5, followed by further fragmentation to lower-mass b ions, there are abundant a and a* (a-NH_3) ions, particularly a_5, a_5*, a_4, and a_4*. (Although these ions are so labeled, the actual amino acid sequence in each ion is uncertain.) The minor ions labeled with the *m/z* ratios 261 to 169 correspond to loss of CO or CO + NH_3 from the various b ions; for example, the signal at *m/z* 169 corresponds to loss of CO + NH_3 from the -(Y + A) ion (*m/z* 214).

The question of greatest interest is how much the cyclization of the b_5 ion results in confusion in interpreting the CID mass spectra of the MH^+ ions. Figure 3 presents the CID mass spectra of the five MH^+ ions recorded at 18 eV collision energy. In each case the b ions (b_5, b_4, b_3) that correspond, nominally, to sequence ions are identified as such, whereas the nonsequence b ions are identified in parentheses [i.e., (b_5-Y), (b_5-2A), etc.]. In general the nominal sequence ions are more abundant but the nonsequence b ions are of sufficient abundance to cause some uncertainty if a complete

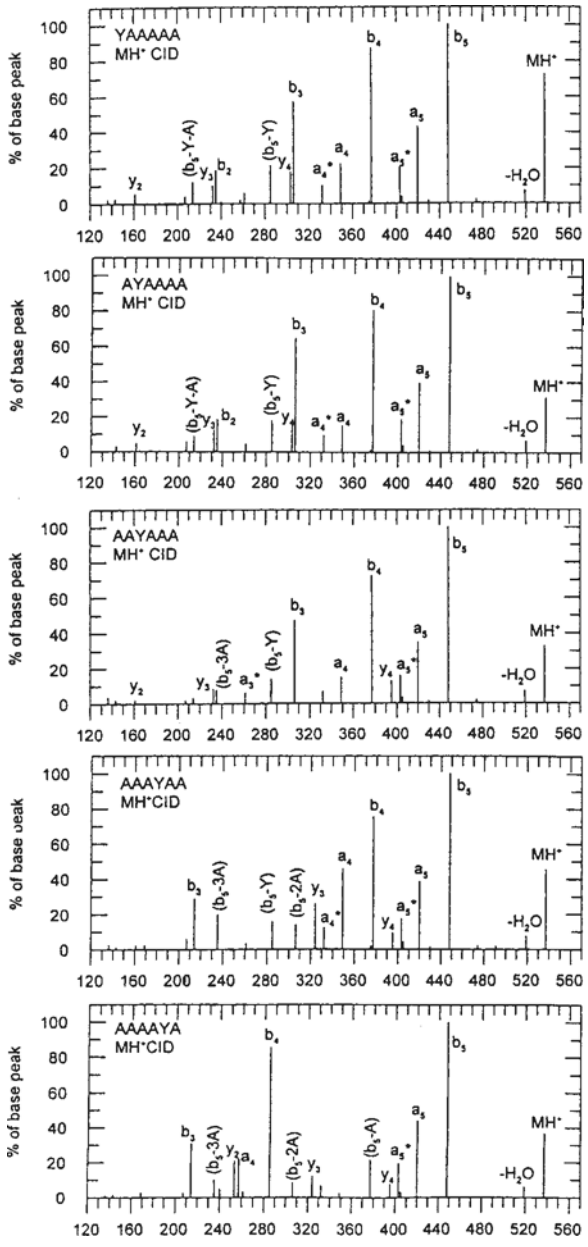

Figure 3. Comparison of CID mass spectra of MH^+ ions at 18 eV collision energy.

unknown was being studied. This is particularly true for protonated AAAYAA, where the nonsequence b ions (b_5-2A), (b_5-Y), and (b_5-3A) are nearly as abundant as the nominal b_3 sequence ion. The breakdown graph for protonated AAAYAA is presented in Figure 4, from which it can be seen that, at high collision energies, these nonsequence b ions and their further fragmentation products become major ions in the CID mass spectrum. Clearly, at 24 eV collision energy it would be difficult to establish the amino acid sequence of this relatively simple peptide from the CID mass spectrum obtained. Similar results were obtained for the MH^+ ions of the other peptides since the lower-mass nonsequence b ions originate by further fragmentation of the primary b_5 ion product and this fragmentation increases in importance with increasing collision energy.

The cyclization mechanism of Scheme **1** involves attack of the N-terminal amine on the C-terminal oxazolone formed initially and presumably requires a free amine group. To test this hypothesis the N-acetylated peptide Ac-AAAYAA was studied. The breakdown graph for the b_5 ion (actually N-Ac-b_5) formed by loss of neutral alanine from MH^+ is shown in Figure 5. Compared to the breakdown graph of Figure 2 a much simpler result is observed. Not only is there an absence of nonsequence b ions, there is also an absence of a_n* ion signals and much reduced ion signals for a_n ions. In a similar fashion, the breakdown graph for protonated Ac-AAAYAA (Figure 6) is also much simplified compared to that for protonated AAAYAA (Figure 4). Nonsequence b ion signals are absent as are, for the most part, ion signals corresponding to a_n and a_n* ions. The series of b ions provides clear sequence information. The lack of sequence scrambling in the N-acetylated peptide clearly illustrates the role of the

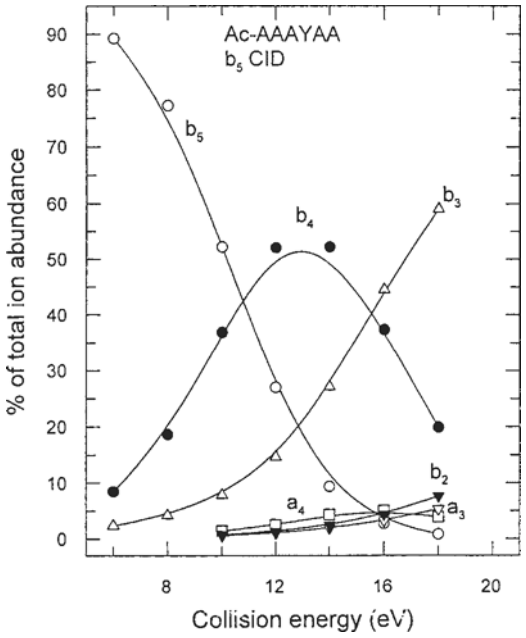

Figure 5. Breakdown graph for b_5 ion derived from Ac-AAAYAA.

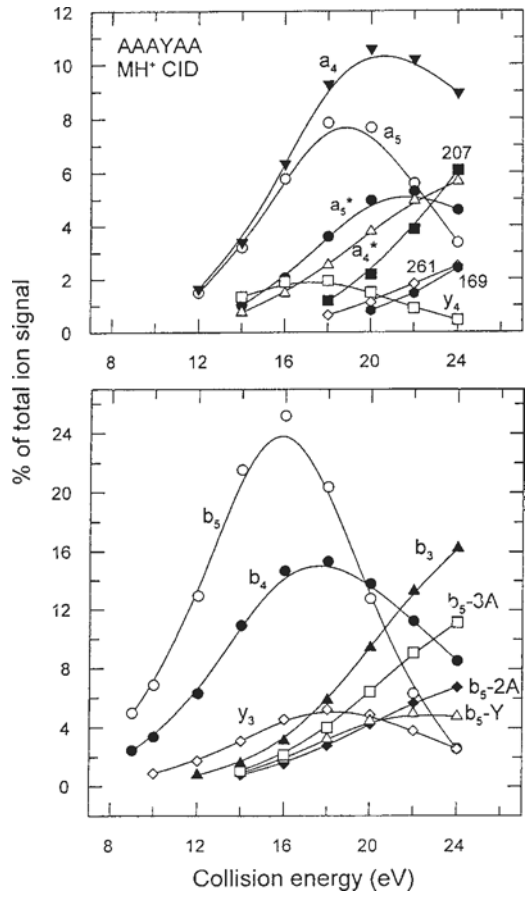

Figure 4. Breakdown graph for protonated AAAYAA. MH^+ ion signal not shown.

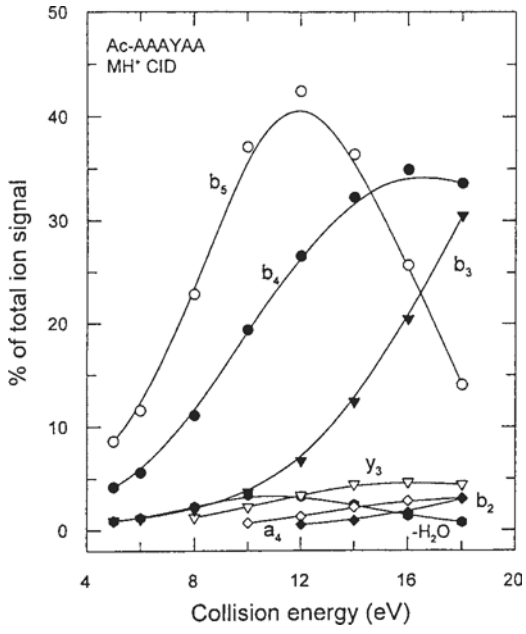

Figure 6. Breakdown graph for protonated Ac-AAAYAA. MH^+ ion signal not shown.

free N-terminal amino group in the cyclization and subsequent scrambling process.

The absence of a_n* ions in the spectrum of the N-acetyl peptide is not surprising in light of the mechanism of formation of a* ions proposed by van Stipdonk and colleagues [27], which involves migration of the $RCH{=}NH_2^+$ group from the C-terminus of the a ion to the N-terminal amine; this presumably also requires a free amine group. More surprising is the much reduced intensity of the ion signals for a_n ions, observed on N-acetylation of the peptide. The reasons for this are not clear but, obviously, if a_n ions are not formed in any abundance, a_n* ions will not be observed.

Conclusions

We initially undertook studies [21] of the fragmentation of larger b ions in anticipation that such MS^3 studies would provide additional information in the sequencing of peptides. Unexpectedly, such is not the case but, rather, the cyclization and subsequent sequence scrambling that are observed [20–23] complicate sequence determination. As the present work has shown, when a major fragmentation pathway of the MH^+ ion involves formation of a b_5 (or larger b) ion, nonsequence ions are likely to be observed with an increasing importance as the collision energy is increased. The present work also indicates that N-acetylation of the peptide eliminates this sequence scrambling and apparently MS^n studies of the b ions formed from the N-acetylated peptide will prove useful in sequence determination.

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References

1. McLafferty, F. W., editor. *Tandem Mass Spectrometry*; Wiley: New York, 1983.
2. Busch, K. L.; Glish, G. L.; McLuckey, S. A. *Mass Spectrometry/Mass Spectrometry: Techniques and Applications of Tandem Mass Spectrometry*; VCH: New York, 1988.
3. Larsen, M. R.; Roepstorff, P. Mass Spectrometric Identification of Proteins and Characterization of their Post-translational Modifications in Proteome Analysis. *Fresenius J. Anal. Chem.* **2000,** *366,* 677–690.
4. Aebersold, R.; Goodlett, D. R. Mass Spectrometry in Proteomics. *Chem. Rev.* **2001,** *101,* 269–295.
5. Medzihradszky, K. F. Peptide Sequence Analysis. *Methods Enzymol.* **2005,** *402,* 209–244.
6. Roepstorff, P.; Fohlmann, J. Proposal for a Common Nomenclature for Sequence Ions in Mass Spectra of Peptides. *Biomed. Mass Spectrom.* **1984,** *11,* 601.
7. Biemann, K. Contributions of Mass Spectrometry to Peptide and Protein Structure. *Biomed. Environ. Mass Spectrom.* **1988,** *16,* 99–111.
8. Mueller, D. R.; Eckersley, M.; Richter, W. Hydrogen Transfer Reactions in the Formation of "Y + 2" Sequence Ions from Protonated Peptides. *Org. Mass Spectrom.* **1988,** *23,* 217–222.
9. Cordero, M. M.; Houser, J. J.; Wesdemiotis, C. The Neutral Products Formed During Backbone Fragmentation of Protonated Peptides in Tandem Mass Spectrometry. *Anal. Chem.* **1993,** *65,* 1594–1601.
10. Yalcin, T.; Khouw, C.; Csizmadia, I. G.; Peterson, M. R.; Harrison, A. G. Why Are b Ions Stable Species in Peptide Mass Spectra? *J. Am. Soc. Mass Spectrom.* **1995,** *6,* 1165–1174.
11. Yalcin, T.; Csizmadia, I. G.; Peterson, M. R.; Harrison, A. G. The Structures and Fragmentation of B_n ($n \geq 3$) Ions in Peptide Mass Spectra. *J. Am. Soc. Mass Spectrom.* **1996,** *7,* 233–242.
12. Nold, M. J.; Wesdemiotis, C.; Yalcin, T.; Harrison, A. G. Amide Bond Dissociation in Protonated Peptides. Structures of the N-terminal Ionic and Neutral Fragments. *Int. J. Mass Spectrom. Ion Process.* **1997,** *164,* 137–153.
13. Paizs, B.; Lendvay, G.; Vékey, K.; Suhai, S. Formation of b_2^+ Ions from Protonated Peptides. An Ab Initio Study. *Rapid Commun. Mass Spectrom.* **1999,** *13,* 525–533.
14. Harrison, A. G.; Csizmadia, I. G.; Tang, T. H. Structures and Fragmentation of b_2 Ions in Peptide Mass Spectra. *J. Am. Soc. Mass Spectrom.* **2000,** *11,* 427–436.
15. Rodriquez, C. F.; Shoeib, T.; Chu, I. K.; Siu, K. W. M.; Hopkinson, A. C. Comparison Between Protonation, Lithiation and Argentination of 5-Oxazolones. A Study of a Key Intermediate in Gas-Phase Peptide Sequencing. *J. Phys. Chem. A* **2000,** *104,* 5335–5342.
16. Yalcin, T.; Harrison, A. G. Ion Chemistry of Protonated Lysine Derivatives. *J. Mass Spectrom.* **1996,** *31,* 1237–1243.
17. Tu, Y. P.; Harrison, A. G. The b_1 Ion Derived from Methionine Is a Stable Species. *Rapid Commun. Mass Spectrom.* **1998,** *12,* 849–851.
18. Farrugia, J. M.; Taverner, T.; O'Hair, R. A. J. Side-Chain Involvement in the Fragmentation Reactions of Protonated Methyl Esters of Histidine and Its Peptides. *Int. J. Mass Spectrom.* **2001,** *209,* 99–112.
19. Farrugia, J. M.; O'Hair, R. A. J.; Reid, G. A. Do All b_2 Ions Have Oxazolone Structures? Multistage Mass Spectrometry and Ab Initio Studies on Protonated N-Acyl Amino Acid Methyl Ester Model Systems. *Int. J. Mass Spectrom.* **2001**, *210–211,* 71–87.
20. Yague, J.; Paradela, A.; Ramos, M.; Ogueta, S.; Marina, A.; Barabona, F.; de Castro, J. A.; Vazquez, J. Peptide Rearrangement During Ion Trap Fragmentation: Added Complexity to MS/MS Spectra. *Anal. Chem.* **2003,** *75,* 1524–1535.
21. Harrison, A. G.; Young, A. B.; Bleiholder, C.; Suhai, S.; Paizs, B. Scrambling of Sequence Information in Collision-Induced Dissociation of Peptides. *J. Am. Chem. Soc.* **2006,** *128,* 10364–10365.
22. Jia, C.; Qi, W.; He, Z. Cyclization Reactions of Peptide Fragment Ions During Multistage Collisionally Activated Decomposition: An Inducement to Lose Internal Amino Acid Residues. *J. Am. Soc. Mass Spectrom.* **2007,** *18,* 663–678.
23. Mouls, L.; Aubagnac, J. L.; Martinez, J.; Enjalbal, C. Low Energy Peptide Fragmentations in an ESI-Q-Tof Type Mass Spectrometer. *J. Proteome Res.* **2007,** *6,* 1378–1391.
24. Polfer, N. C.; Oomens, J.; Suhai, S.; Paizs, B. Spectroscopic and Theoretical Evidence for Oxazolone Ring Formation in Collision-Induced Dissociation of Peptides. *J. Am. Chem. Soc.* **2005,** *127,* 17154–17155.
25. Polfer, N. C.; Oomens, J.; Suhai, S.; Paizs, B. Infrared Spectroscopy and Theoretical Studies on Gas-Phase Protonated Leu-enkephalin and Its Fragments: Direct Experimental Evidence for the Mobile Proton. *J. Am. Chem. Soc.* **2007,** *129,* 5887–5897.
26. Harrison, A. G.; Young, A. B. Fragmentation of Protonated Oligoalanines: Amide Bond Cleavage and Beyond. *J. Am. Soc. Mass Spectrom.* **2004,** *15,* 1810–1819.
27. Cooper, T.; Talaty, E.; Grove, J.; Van Stipdonk, M.; Suhai, S.; Paizs, B. Isotope Labelling and Theoretical Study of the Formation of a_3* Ions from Protonated Tetraglycine. *J. Am. Soc. Mass Spectrom.* **2006,** *17,* 1654–1664.