Novel Fragmentation Pathway for CID of $(b_n - 1 + Cat)^+$ Ions from Model, Metal Cationized Peptides

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We report a new fragmentation pathway for the CID of $(b_3 - 1 + Cat)^+$ product ions derived from the model peptide AXAG, where X = β-alanine, γ-aminobutyric acid, ε-amino-n-caproic acid, or 4-aminomethylbenzoic acid. By changing the amino acid to the C-terminal side of the amino acid X, and incorporating ^{15}N and ^{13}C labeled residues at the same position, we conclude that the dissociation pathway most likely leads to a metal cationized nitrile. With respect to the various amino acids at position X, the putative nitrile product becomes more prominent, relative to the conventional $(a_3 - 1 + Cat)^+$ species, in the order β-alanine < γ-aminobutyric acid < ε-aminocaproic acid < 4-aminomethylbenzoic acid. The pathway is not observed for peptides with α-amino acids at position X. The product ion is observed most prominently during the CID of Li^+ and Na^+ cationized peptides, only to a small extent for Ag^+ cationized peptides, and not at all from protonated analogues. (J Am Soc Mass Spectrom 2005, 16, 1305–1310) © 2005 American Society for Mass Spectrometry

In an attempt to enhance the understanding of how cation and sequence influence peptide fragmentation, we recently investigated the dissociation of model N-acetylated tetrapeptides with general sequence AcFGGX that featured C-termini designed to allow transfer of the −OH required to generate the $(b_3 + 17 + Cat)^+$ product ion, but not necessarily as the most favored pathway [1]. The amino acid placed at position X either required a larger cyclic intermediate than the five-member ring presumably formed with α-amino acids (β-alanine, γ-aminobutyric acid, and ε-amino-*n*-caproic acid to generate 6-, 7-, or 9-member rings, respectively) or prohibited cyclization because of the inclusion of a rigid ring (*para*- and *meta*-aminobenzoic acid). For Ag^+, Li^+ and Na^+ cationized AcFGGX, formation of $(b_3 + 17 + Cat)^+$ was suppressed when the amino acids requiring the adoption of larger ring intermediates were used, while amino acids that prohibit cyclization eliminated the reaction pathway completely—an observation in accord with proposed mechanisms for the formation of this important sequence ion [2–8].

During subsequent experiments involving peptides containing similar "alternative" amino acids, we observed an unusual fragmentation pathway when the $(b_3 - 1 + Li)^+$ product ion derived from the synthetic peptide A(βA)AG was subjected to collision-induced dissociation (CID). The pathway resulted in a neutral loss one mass unit (u) greater than the residue mass of the amino acid that composed the presumed oxazolinone ring [9–15] of the $(b_3 - 1 + Li)^+$ species, and thus could not be attributed to the formation of the $(b_2 - 1 + Li)^+$ ion. We describe here experiments involving a group of tetrapeptides of general sequence AXAG, where X = β-alanine, γ-aminobutyric acid, ε-amino-*n*-caproic acid, and 4-aminomethylbenzoic acid, and analogous peptides with ^{15}N and ^{13}C labels in specific positions that were designed to probe the unusual reaction pathway. This specific set of experiments, focused on the fragmentation of the $(b_n - 1 + Cat)^+$ ions (as opposed to their formation), showed that the reaction pathway most likely generates a metal-cationized nitrile product.

Experimental

All peptides used in this study were generated by conventional solid-phase synthesis methods [16] using 9-fluorenylmethoxycarbonyl (Fmoc)-glycine loaded Wang resin (Sigma Chemical, St. Louis MO) and a custom-built, multiple reaction vessel peptide synthesis apparatus. Glycine-^{15}N, glycine-1-^{13}C, γ-aminobutyric acid (**γAbu**), and Fmoc-chloride were purchased from Sigma Chemical and used to generate Fmoc-glycine^{15}N (**G-^{15}N**), -glycine-1-^{13}C (**G-1-^{13}C**) and -γAbu for incorporation into the model peptides. Fmoc protected glycine, alanine, valine, β-alanine (**βA**), ε-amino-n-caproic acid (**εCap**), and 4-aminomethylbenzoic acid (**4AMBz**) were purchased from Sigma and used as received.

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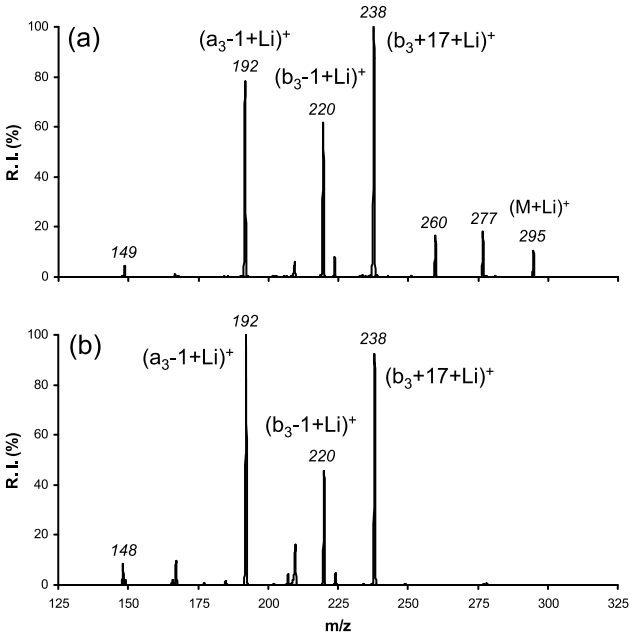


Figure 1. CID (MS/MS) spectra of Li^+ cationized tetrapeptides: (**a**) AAAG; (**b**) A(βA)AG.

Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies.

Metal nitrate salts (Li^+, Na^+ and Ag^+) were purchased from Aldrich Chemical (St. Louis, MO) and used as received. Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (vol:vol) mixture of HPLC grade MeOH (Aldrich Chemical) and deionized H_2O to produce final concentrations of 10^{-5}–10^{-4} M. Equimolar metal nitrate solutions were prepared in deionized H_2O.

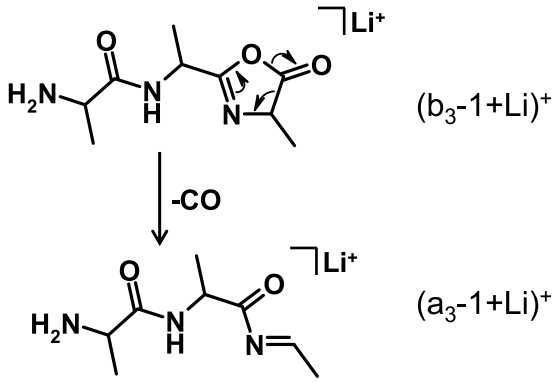
ESI mass spectra were collected using a ThermoFinnigan LCQ-Deca ion-trap mass spectrometer (San Jose, CA). Mixtures (1:1 by volume) of metal nitrate and peptide, prepared by mixing 0.25 mL of the respective stock solutions, were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 3–5 μl/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octopole voltage offsets, etc.) were optimized for maximum $(M + Cat)^+$ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N_2 sheath gas flow at 25 units (arbitrary for the Finnigan systems, corresponding to approximately 0.375 L/min) and the capillary (desolvation) temperature at 200 ° C. The ion trap analyzer was operated at a pressure of $\sim 1.5 \times 10^{-5}$ Torr. Helium gas, admitted directly into the ion trap, was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The multiple-stage CID studies were performed as follows. The alkali metal cationized peptides were isolated for the initial CID stage (MS/MS) using an isolation width of 1.5 mass to charge (*m/z*) units. The Ag^+ cationized peptides were isolated using a width of 6 *m/z* units, with the isolation mass centered between the ^{107}Ag and ^{109}Ag isotopic peaks. The activation amplitude, which defines the amplitude of the RF energy applied to the end cap electrodes in the CID experiment, was set between 20 and 35% (chosen empirically, representing approximately 0.66 → 1.54 V, 0-p, laboratory frame) and the activation Q (as labeled by ThermoFinnigan, used to adjust the q_z value for the precursor ion) was set at 0.30. The activation time employed at each CID stage was 30 ms.

Results and Discussion

Figure 1 shows the CID (MS/MS stage) spectra generated from Li^+ cationized AAAG (Figure 1a) and A(βA)AG (Figure 1b). For both peptides, CID generated the $(b_3 + 17 + Li)^+$, $(b_3 - 1 + Li)^+$, and $(a_3 - 1 + Li)^+$ ions at *m/z* 238, 220, and 192, respectively. Peaks at *m/z* 277 and 260, corresponding to the elimination of H_2O (18 mass units, u) and the combination of H_2O and NH_3 (35 u) were generated by CID of AAAG but not of the analogue containing βA. Recent investigations in our laboratory suggest that the reaction to eliminate H_2O and combination of H_2O and NH_3 are initiated by nucleophilic attack by the N-terminal amide carbonyl oxygen atom upon the carbon atom of the adjacent amide group. The absence of the two reaction products for the peptide containing βA adjacent to the N-terminus is consistent with the cyclization and intra-molecular attack involving a kinetically and entropically less favored 6-member ring and with our earlier study of the influence of amino acids on the generation of $(b_n + 17 + Cat)^+$ products [1].

The $(b_3 - 1 + Li)^+$ ion generated from AAAG and A(βA)AG is presumably a lithium-cationized oxazolinone [11]. Often, the most prominent dissociation pathway for $(b_n)^+/(b_n - 1 + Cat)^+$ species is the elimination of CO to produce the $(a_n - 1 + Cat)^+$ ion (Scheme 1) [9, 10, 14, 17, 18]. Figure 2 shows the MS^3 spectra for the dissociation of the $(b_3 - 1 + Li)^+$ species (*m/z* 220)



Scheme 1. Suggested mechanism for formation of $(a_3 - 1 + Cat)^+$ from $(b_3 - 1 + Cat)^+$ from Li^+ cationized AAAG.

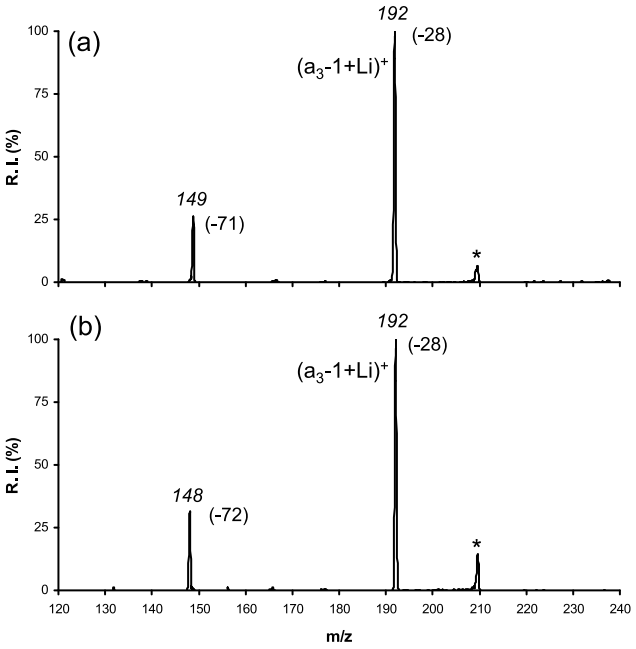


Figure 2. Product ion spectra from CID of $(b_3 - 1 + Li)^+$ derived from dissociation (MS^2 stage) of (**a**) AAAG and (**b**) A(βA)AG. Asterisks mark H_2O adducts to product ions. Values in parenthesis represent neutral losses.

derived from Li^+ cationized AAAG (Figure 2a) and A(βA)AG (Figure 2b). Fragmentation pathways for CID of $(b_3 - 1 + Li)^+$ derived from AAAG included formation of $(a_3 - 1 + Li)^+$, *m/z* 192, by loss of CO and $(b_2 - 1 + Li)^+$, *m/z* 149, by elimination of 71 u. Fragmentation pathways observed for CID of the $(b_3 - 1 + Li)^+$ species derived from A(βA)AG included formation of $(a_3 - 1 + Li)^+$ and the elimination of 72 u to generate a product ion at *m/z* 148.

The 72 u eliminated from the $(b_3 - 1 + Cat)^+$ product derived from A(βA)AG is 1 u greater than the residue mass of the alanine residue adjacent to the C-terminus of the original peptide and suggested an alternative reaction pathway for decomposition of the Li^+ cationized oxazolinone. Figure 3 compares the spectra generated following the CID of $(b_3 - 1 + Li)^+$ initially generated from A(βA)AG (Figure 4a), A(γAbu)AG (Figure 4b), A(εCap)AG (Figure 4c), and A(4AMBz)AG (Figure 4d). For this series of peptides, the tendency to eliminate 72 u from the $(b_3 - 1 + Li)^+$ species increased substantially, relative to the more conventional elimination of CO (to furnish the a type ion), as the size of the amino acid adjacent to the N-terminus increases and was highest for the amino acid containing the aromatic amino acid.

To determine whether or not the fragmentation reaction leading to the loss of 72 u involved elimination of the amino acid residue that composed the oxazolinone ring (i.e., the residue originally adjacent to the C-terminus), the $(b_3 - 1 + Li)^+$ species derived from Li^+ cationized A(εCap)VG, A(εCap)GG, A(εCap)(G-1-^{13}C)G, and A(εCap)(G-^{15}N)G were subjected to CID. As shown in Figure 4a and b, the neutral losses to form the peak at *m/z* 148 shifted to 100 for the peptide containing V and to 58 for the peptides containing G adjacent to the C-terminus. The elimination of 100 and 58 is 1 u greater than the residue masses of V and G, respectively, confirming the elimination of a portion of the presumed oxazolinone ring. The neutral losses observed shifted to 29 (^{13}CO), 45, and 59 when the peptide with G-1-^{13}C was investigated (Figure 4c). For the peptide containing the G-^{15}N (Figure 4d) the dominant product *ion* shifted by 1 u to *m/z* 149, indicating retention of the isotope label.

A dissociation reaction in which the net neutral loss was 1 u greater than the residue mass of the amino acid that composed the oxazolinone ring, along with the retention of the ^{15}N label by the product ion, suggested the formation of a nitrile as depicted in Scheme **2a**. While the composition and structure of the neutral species is not revealed by the CID experiment, it is depicted in Scheme **2a** as an α-lactone resulting from a direct ring-opening reaction. Formation of α-lactones has been proposed to explain certain dissociation products for Ag^+-cationized phenylalanine [19, 20]. An alternative reaction pathway might involve another intramolecular nucleophilic attack upon the oxazolinone by the carbonyl group to the N-terminal side of the ring. However, we reason that such cyclization reactions might be less favored for the larger amino acids (i.e., because of kinetic and entropic factors associated

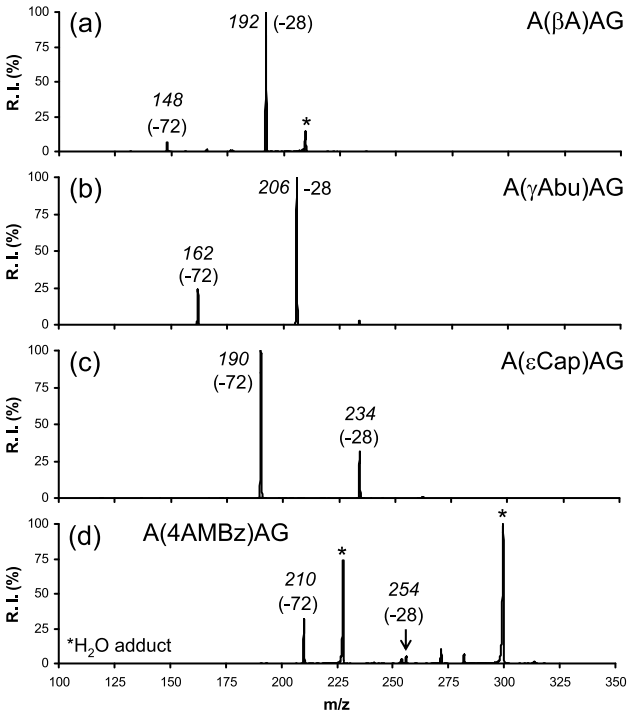


Figure 3. Comparison of the product ion spectra generated from CID (MS^3 stage) from $(b_3 - 1 + Li)^+$ sequence ions derived from the dissociation (MS^2 stage) of (**a**) A(βA)AG, (**b**) A(γAbu)AG, (**c**) A(εCap)AG, and (**d**) A(4AMBz)AG. Asterisks mark H_2O adducts to product ions. Values in parenthesis represent neutral losses.

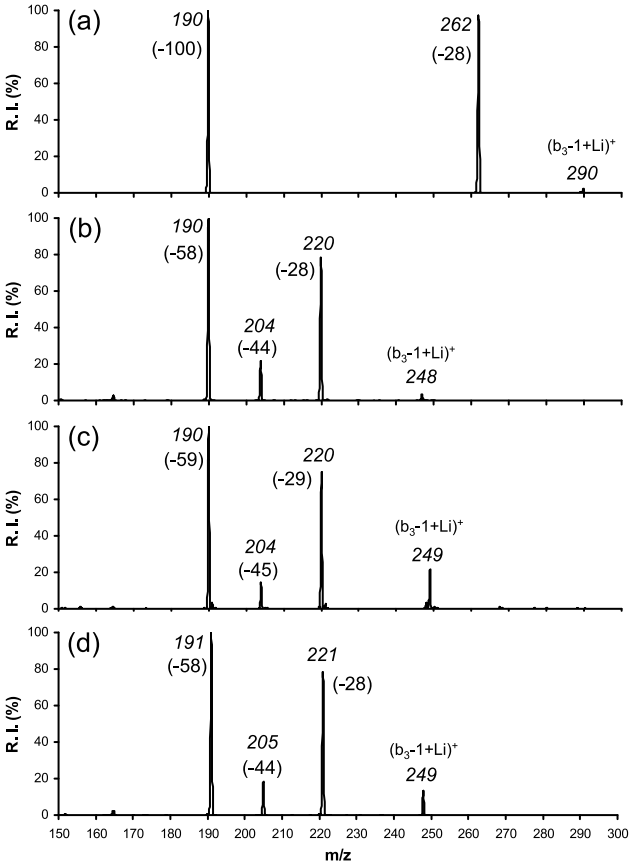
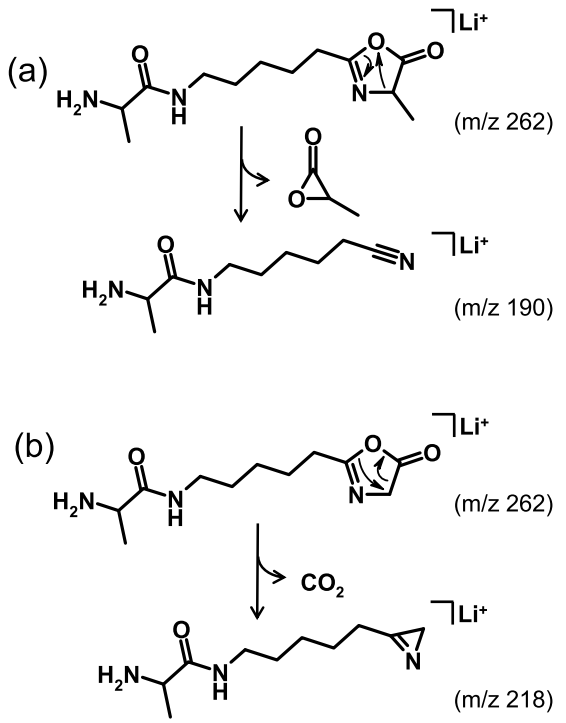


Figure 4. Comparison of the product ion spectra generated from CID (MS^3 stage) from $(b_3 - 1 + Li)^+$ sequence ions derived from the dissociation (MS^2 stage) of (**a**) A(εCap)VG, (**b**) A(εCap)GG, (**c**) A(εCap)(G − 1 − ^{13}C)G, and (**d**) A(εCap)(G − ^{15}N)G. Values in parenthesis represent neutral losses.

with the larger cyclic intermediates, as demonstrated in our earlier study [1]) such as γAbu and εCap, and impossible for 4AMBz because of the rigid aromatic ring.

In the spectra shown in Figure 4b and c, the fragmentation pathway involving the elimination of 44 and 45 u is attributed to the loss CO_2 and $^{13}CO_2$, respectively, from the oxazolinone ring. Here the identity of the product ion is less clear, but may be a substituted 2*H*-azirine as shown in Scheme **2b**. The elimination of 44 u from the $(b_3 - 1 + Cat)^+$ species was only observed for the peptides AXGG, suggesting that the relative stability of neutral α-lactones, particularly with respect to the 2*H*-azirine structure, may play a role in the tendency to produce the nitrile product. 2*H*-azirines are more stable than the anti-aromatic 1*H*-azirines, and even the unsubstituted version of the former has been isolated at low temperatures [21], but not the unsubstituted α-lactone.

Based on the observations described above, it appears that the $(b_3 - 1 + Li)+$ ion from the AXAG series is capable of fragmenting by several competing pathways: (1) loss of the residue mass of α-alanine to form the $(b_2 - 1 + Cat)^+$ species, (2) elimination of CO to produce $(a_2 - 1 + Li)^+$, (3) ejection of an α-lactone to generate a lithiated nitrile, and (4) elimination of CO_2 to leave a lithium cationized 2*H*-azirine. Formation of the $(b_2 - 1 + Li)^+$ and $(a_2 - 1 + Li)^+$ correspond to well-documented reaction pathways as recorded here for Li^+ cationized AAAG. The absence of the nitrile and azirine products in the CID spectrum of $(b_3 - 1 + Li)^+$ derived from AAAG suggests a special stabilization of the $(a_3 - 1 + Li)^+$ product; stabilization that may be lacking in the same product derived from the peptides with the amino acids such as βA, γAbu, and εCap at position X. To account for the differences in the competition between imine type, $(a_n - 1 + Cat)^+$, and nitrile or azirine products, we suggest that the $(a_3 - 1 + Li)^+$ species derived from AAAG may be stabilized by the pseudo 5-membered hydrogen bonded structure, **a**, shown in Figure 5. Moreover, the traditional imine-like structure may be in equilibrium with the entropically favored 5-membered ring structure, **a**′, resulting from a Michael-type addition. In structure **a**′ both nitrogen atoms of the oxazolidinone ring can participate in amide-type resonance, a feature lacking in structure **a** because the lone pair of the imine nitrogen would occupy an sp^2 orbital to accommodate conjugation with the carbonyl group. Even this conjugation in **a** of the imine-carbonyl type is less resonance stabilized than the traditional olefin-carbonyl type because of the electronegativity of the nitrogen atom, thus promoting formation of **a**′. The presence of the imine nitrogen, on the other hand, would facilitate the initial Michael addition (species **b** in Figure 5). In the case of Structure **a**,



Scheme 2.. Formation of (**a**) nitrile from CID of $(b_3 - 1 + Li)^+$ derived from A(eCap)AG and (**b**) 2*H*-azirine product from CID of $(b_2 - 1 + Li)^+$ derived from A(εCap)GG.

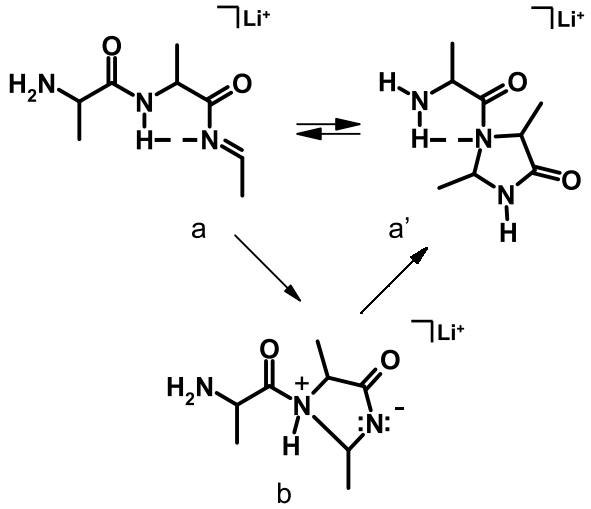


Figure 5. Potential structures for $(a_3 - 1 + Cat)^+$ species derived from Li^+ cationized AAAG.

potentially formed from AXAG (with X representing amino acids other than α-alanine), not only would intra-molecular hydrogen bonding become progressively more difficult as the pseudo-ring enlarges from 5 to 6 to 7 or 9 members (as for X = βA, γAbu, or εCap) or becomes impossible with 4AMBz, but formation of structure **a**′ would be kinetically less favorable. Reduced probability for formation of structure **a**′ might then allow pathways leading to the nitrile or azirine to become more competitive. Another factor may be progressively increased stability of the nitrile product in proceeding from A to βA, γAbu, and to εCap. This is reminiscent of the increase in proton affinity with chain length of a homologous series of nitriles reported by Cao° and° Holmes° [22],° and° the° trends° in° gas-phase acidity of alcohols first reported by Brauman and Blair [23,°24].°In°part,°the°influence°of°an°alkyl°group°on°the acidity of gas phase alcohols has been attributed to increasing polarizability with chain length and partial delocalization of charge. In addition to the inability of 4AMBz to compete with formation of the oxazolidinone,°**a**′,°in°Figure°5°on°account°of°its°rigid°structure, conjugation of the nitrile with the aromatic ring would certainly favor its formation in comparison with the other aliphatic nitriles.

We also examined the influence of the cation on the tendency°to°generate°the°putative°nitrile°product.°Figure°6°shows°the°spectra°generated°from°the°CID°of A(εCap)AG°when°cationized°with°proton°(Figure°6a), Li^+ (Figure°6b),°Na^+,°(Figure°6c),°and°Ag^+ (Figure°6d). CID of the $(b_3)^+$ ion, derived from the protonated version of the peptide, caused the elimination of 89 u but not the loss of 28 u to form $(a_3)^+$ or 72 u to generate the nitrile product. The mechanism behind the elimination of 89 u from the protonated peptide is yet to be resolved. The loss of 72 u to make the nitrile product was the dominant pathway for the CID of both the Li^+ and Na^+ cationized versions of the species, but only minor for the CID of the Ag^+ cationized analog.

In summary, we have identified a novel reaction pathway for the CID of $(b_3 - 1 + Cat)^+$ product ions. The pathway is not observed when the amino acid adjacent to the ring structure of the oxazolinone is an α-amino acid, but increases in prominence when β- or γ-amino acids are substituted in the same position. For tetrapeptides in which the "alternative" amino acids are incorporated, this reaction pathway is not observed when the residues are positioned either at the N-terminus or adjacent to the C-terminus (spectra not shown). The dissociation pathway is also observed for the CID of $(b_4 - 1 + Cat)^+$ derived from model pentapeptides with sequence AAXAG (with X = βA and εCap, spectra not shown), suggesting that the reaction is not unique to the fragmentation of $(b_3 - 1 + Cat)^+$. By examining the respective neutral losses from a series of peptides, and the retention of a specific ^{15}N label by the product ion, we conclude that the reaction most likely leads to formation of a metal cationized nitrile. The reaction pathway is most prominent for CID of the Li^+ and Na^+ cationized versions of $(b_3 - 1 + Cat)^+$ species. The reason for the pronounced "metal effect" is not clear and requires a significant computational investigation. Preliminary ab initio modeling of likely precursor and transition state structures, including possible important hydrogen-bonded conformations, is currently underway.

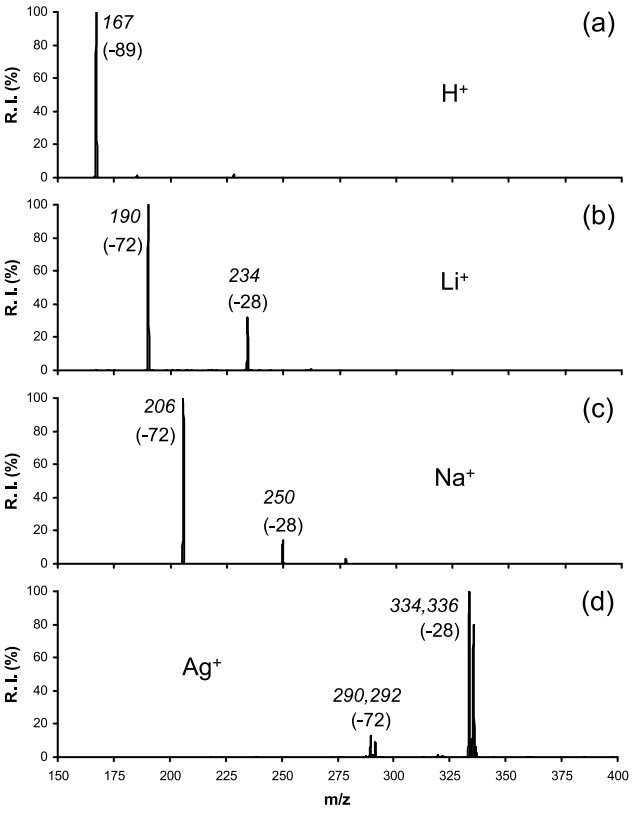


Figure 6. Comparison of the product ion spectra generated from CID (MS^3 stage) from $(b_3 - 1 + Li)^+$ sequence ions derived from protonated and metal cationized A(εCap)AG: (**a**) protonated, (**b**) Li^+ cationized, (**c**) Na^+ cationized, and (**d**) Ag^+ cationized. Values in parenthesis represent neutral losses.

Acknowledgments

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