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Article

Gas Phase Fragmentation Behavior of Proline in Macrocyclic b_7 Ions

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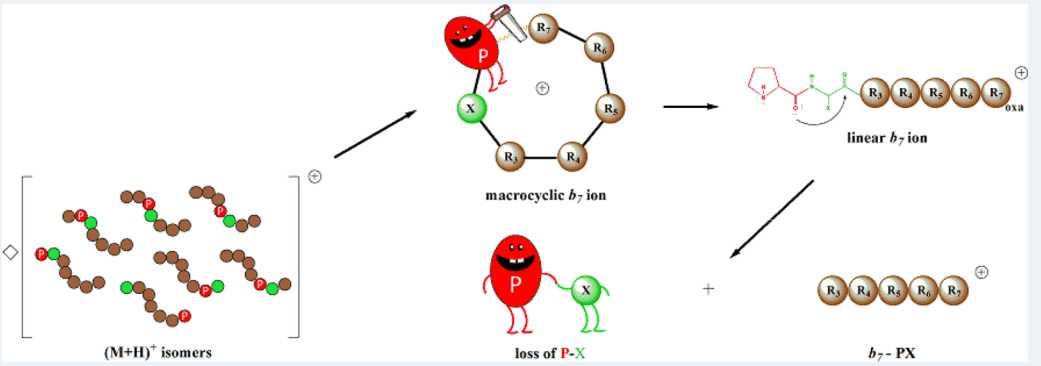

ABSTRACT: The fragmentation characteristics of b_7 ions produced from proline-containing heptapeptides have been studied in detail. The study has utilized the following C-terminally amidated model peptides: PA_6, APA_5, A_2PA_4, A_3PA_3, A_4PA_2, A_5PA, A_6P, PYAGFLV, PAGFLVY, PGFLVYA, PFLVYAG, PLVYAGF, PVYAGFL, YPAGFLV, YAPGFLV, YAGPFLV, YAGFPLV, YAGFLPV, YAGFLVP, PYAFLVG, PVLFYAG, A_2PXA_3, and A_2XPA_3 (where X = C, D, F, G, L, V, and Y, respectively). The results have shown that b_7 ions undergo head-to-tail cyclization and form a macrocyclic structure. Under the collision-induced dissociation (CID) condition, it generates nondirect sequence ions regardless of the position of the proline and the neighboring amino acid residues. This study highlights the unusual and unique fragmentation behavior of proline-containing heptapeptides. Following the head-to-tail cyclization, the ring opens up and places the proline residue in the N-terminal position while forming a regular oxazolone form of b_2 ions for all peptide series. Then, the fragmentation reaction pathway is followed by the elimination of proline with its C-terminal neighbor residue as an oxazolone (e.g., PX_{oxa}) for all proline-containing peptide series.

KEYWORDS: *proline, peptide fragmentation, scrambling of sequence, nondirect sequence ions, macrocyclization, ESI MS/MS mass spectrometry*

INTRODUCTION

Due to its imino group being held in a stiff confirmation and lowering the structural flexibility of the polypeptide chain, proline amino acid plays a significant role in the stability of proteins.[1] Therefore, it is not surprising that 99.8% of human proteins analyzed contain proline. In comparison, certain proteins have up to 40% proline residues,[2] and correct identification of proline-containing peptides by mass spectrometric methods is important for proteomic studies.

In the last two decades, tandem mass spectrometry (MS^n) with collision-induced dissociation (CID) has become an indispensable analytical technique used for protein/peptide sequencing[3,4] with the emergence of soft ionization methods: electrospray ionization (ESI)[5,6] and matrix-assisted laser desorption/ionization (MALDI).[7,8] Low-energy CID conditions lead to cleavages at the peptide backbone and mainly produce sequence-informative *a*, *b*, and *y* ions[9,10] through charge-directed reactions (mobile proton model).[11] Theoretical *m*/*z* data of enzymatic peptides from known proteins are matched to those obtained for product ions to identify the protein. Mascot[12] and SEQUEST[13] are popular matching algorithms that utilize simple and primitive peptide fragmentation chemistry. This limitation, in turn, may result in erroneous assignments in protein identification.

While *y* ions were established to be truncated peptides,[14,15] acylium ion was suggested as a structure of *b* ion by early publications.[9,10] In contrast, it was Boyd's group first proposing the formation of the cyclic structure of doubly protonated *b* ions.[16,17] In addition, Yalcin et al.[18] revealed that cyclization occurs via nucleophilic attack of adjacent carbonyl oxygen on the N-terminus to generate a five-membered oxazolone ring structure of *b* ions. This mechanism was also verified by the infrared multiple photon dissociation (IRMPD) techni-

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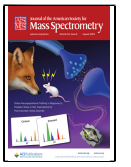

ques[19−23] and deuterium labeling experiments.[24] Diketopiperazine was then alternatively proposed as the structure of b_2 ions by Wesdemiotis and co-workers.[15,25] In contrast, Wysocki et al.[26] reported that the structure of b_2 ions produced from the dipeptide of HA is a mixture of oxazolone and diketopiperazine. It was also shown that the cyclic structure of b_n ion (n = 2 or 4) is peptide-side-chain-dependent.[27−34] However, b_n ions ($n \geq 5$) formed a macrocyclic structure via head-to-tail cyclization. This macrocyclic b_n ion randomly breaks apart and forms "*nondirect sequence ions*" and "*direct sequence ions*" upon CID conditions[35−38] CID condition.

Proline has the highest proton affinity among amino acid residues without basic functional groups.[39] Many studies[3,40−46] demonstrated proline-directed fragmentations that produce very prominent y ions due to the cleavage of N-terminal to proline residue (proline effect). The position of proline was also found to have a direct role at the cleavage site of protonated peptides[47] and deprotonated ones, too.[48] Eckart's group published the first experimental and theoretical results of an alternative gas-phase structure for the b_2 ion (immonium ion type of b_2) produced from GP.[49] Wysocki's laboratory suggested a diketopiperazine structure for b_2 ion obtained from VP b_2 ion;[50,51] however, then they observed predominant oxazolone structures of b_2 ion structure for PPG tripeptide[52] and concluded that the neighbor residue has an influence on the b_2 ion structure because Martens et al.[53] found earlier that the diketopiperazine pathway is almost 100% preferred for the b_2 ion of PPP.

Here we investigate the gas phase fragmentation behavior of a proline-containing heptapeptide series in the gas phase. We observe a unique fragmentation pathway eliminating proline with its C-terminal neighbor residue as a neutral oxazolone (PX_{oxa}). MS/MS results show the formation of proline-containing macrocyclic b_7 ions, followed by ring opening (linearization), rearrangement of the proline residue at the N-terminal position, and then the elimination of proline together with its C-terminal neighbor residue as a neutral oxazolone. To our knowledge, such elimination of oxazolone is the first experimental observation for the proline-containing heptapeptide series.

EXPERIMENTAL SECTION

Materials. All synthetic model peptides were obtained from GL Biochem and used as received with no further purification. HPLC-grade methanol and formic acid were supplied by Merck (Darmstadt, Germany). The water was ultrapure grade (Arium 611 UV, Sartorius AG, Goettingen, Germany). Stock solutions of peptides were prepared by dissolving solid material in a 1:1 (v/v) mixture of methanol and water to a concentration of 10^{-2} M. Peptide samples at micromolar concentration level were prepared by diluting stock solutions with 1:1 methanol:water containing 1% formic acid. All of the model peptides are C-terminally amidated except for YIHPFHL-OH.

Mass Spectrometry. All low-energy CID experiments were conducted on an LTQ XL linear ion trap mass spectrometer (Thermo Scientific, NJ, USA), equipped with an electrospray ionization source. Peptide samples were infused into an electrospray source at a flow rate of 5 μL/min using an incorporated syringe pump.

The linear ion trap MS analyses were performed under the following conditions: All of the instrumental parameter settings were optimized to get maximum precursor ion transmission to the ion trap mass analyzer using the autotune routine within the LTQ Tune program. Ion spray voltage was kept at 5.00 kV, and nitrogen was used as a sheath gas (10 au), auxiliary gas (1 au), and sweep gas (1 au) for spray stabilization. Ion-trapping efficiency and collisional cooling were improved by using helium as a bath gas that was also used as a collision (target) gas for CID analysis. The capillary temperature was held constant at 275 °C. Multistage mass (MS^n) analysis was carried out using an isolation width of 2.1 m/z, an activation time of 30 ms, and a time of acquisition of 0.30 min. The normalized collision energy was maintained between 23% and 27% for all CID experiments.

RESULTS AND DISCUSSION

Fragmentation Reactions of b_7 Ions from Isomers of PA_6. MS/MS spectra of b_7 (524 m/z) ions produced from protonated heptapeptides (containing six alanine (A) residues and a single proline (P) residue) are given in Figure 1. Nearly identical fragmentation patterns are obtained for all of the isomers. In addition to direct sequence ions of H_2O loss (506

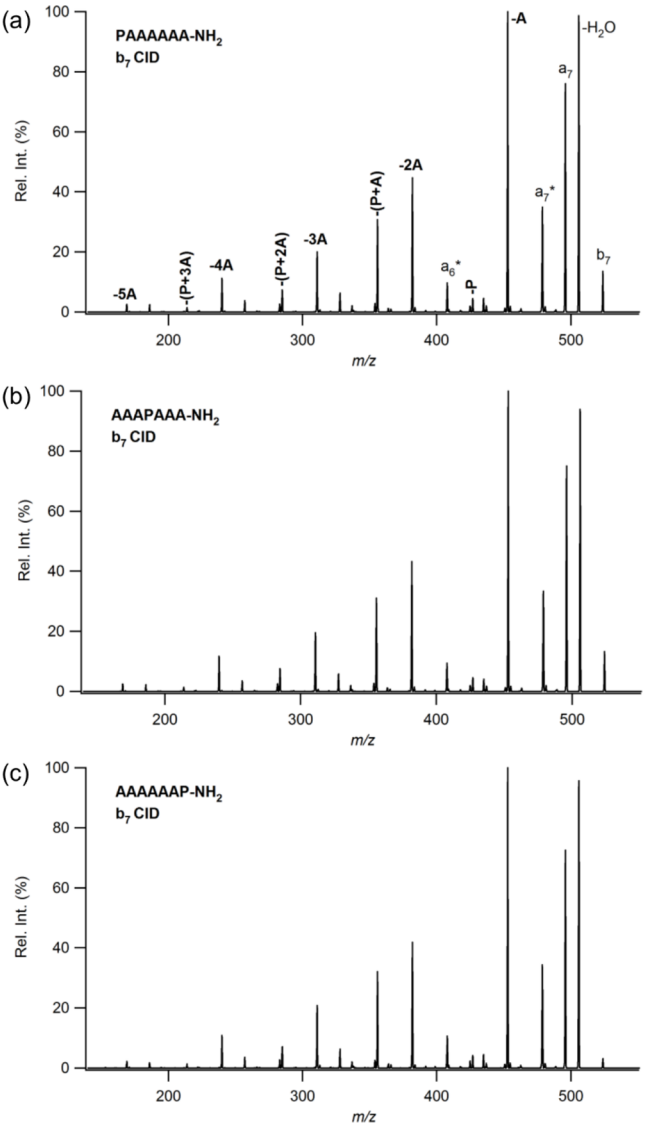

Figure 1. MS/MS spectra of b_7 ions derived from protonated (a) PAAAAAA-NH_2, (b) AAAPAAA-NH_2, and (c) AAAAAAP-NH_2.

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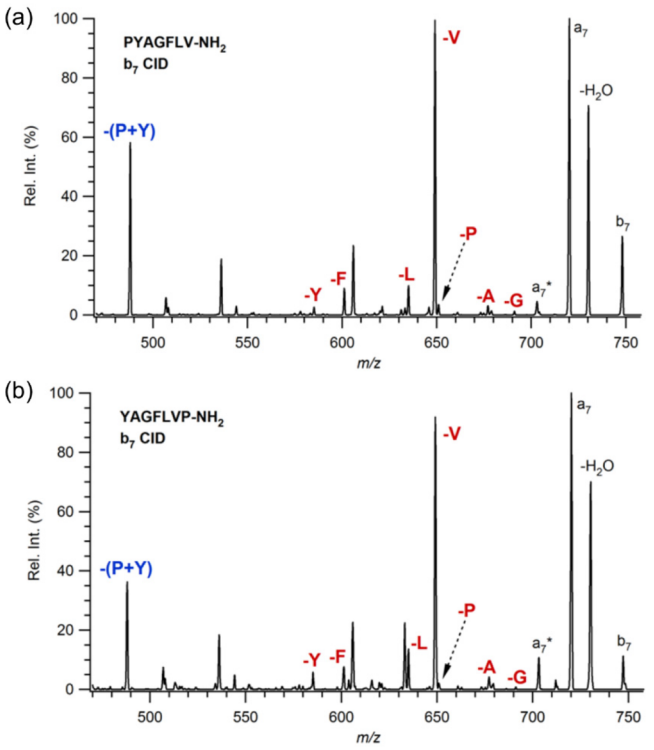

Figure 2. MS/MS spectra of b_7 ions derived from protonated (a) PYAGFLV-NH_2 and (b) YAGFLVP-NH_2.

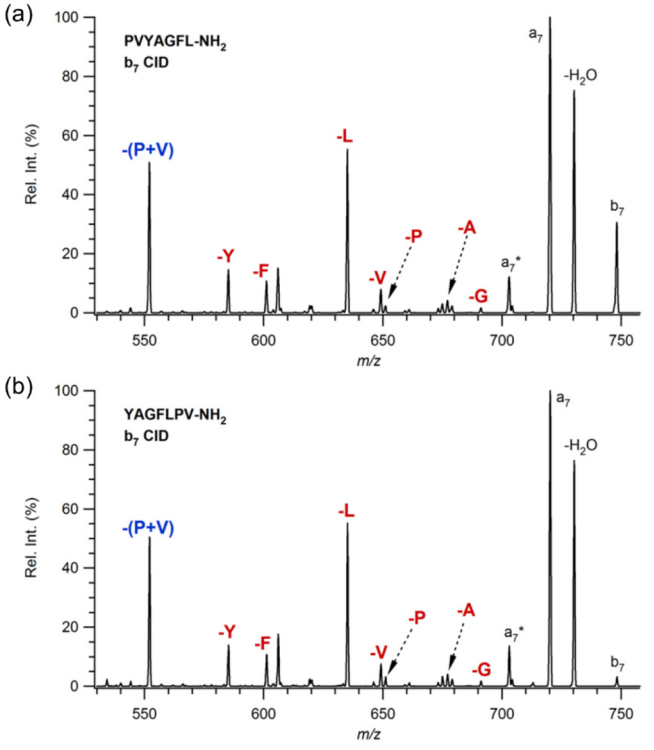

Figure 3. MS/MS spectra of b_7 ions derived from protonated (a) PVYAGFL-NH_2 and (b) YAGFLPV-NH_2.

m/z), a_7 (496 m/z), and a_7* (479 m/z), we also observe nondirect sequence ions as eliminations of A (453 m/z), 2A (382 m/z), 3A (311 m/z), 4A (240 m/z), 5A (169 m/z), P (427 m/z), P+A (356 m/z), P+2A (285 m/z), and P+3A (214 m/z). MS/MS spectra of b_7 ions of APAAAAA-NH_2, AAPAAAA-NH_2, AAAAPAA-NH_2, and AAAAAPA-NH_2 isomers also produce the same product ions and fragmentation patterns (Figure S1). The obtained results are in good agreement with the work of Harrison,[54] showing identical MS/MS spectra of b_5 ions derived from PAAAA, AAPAA, and AAAAP series. Internal eliminations and similarity of dissociation patterns are the direct evidence of a head-to-tail macrocyclization reaction before reopening at various amide bond positions regardless of the original sequence. These findings clearly show that the position of proline does not influence the course of fragmentation for the alanine-containing heptapeptide series.

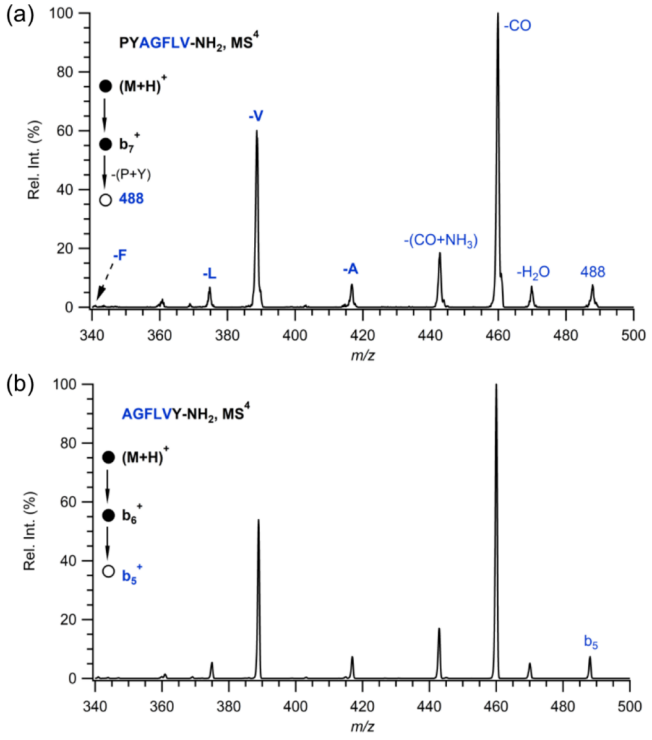

Figure 4. Comparison of MS^4 spectra of (a) P−Y residue elimination from b_7 of protonated PYAGFLV-NH_2 and (b) b_5 of protonated AGFLVY-NH_2.

Fragmentation Reactions of b_7 Ions from Isomers of PYAGFLV. The MS/MS spectra of b_7 ions produced from PYAGFLV-NH_2 and its other isomers are compared to determine the influence of the neighbor residue and the position of proline on the fragmentation pathway. Figure 2 shows the MS/MS spectra of b_7 ions produced from PYAGFLV-NH_2 (a) and YAGFLVP-NH_2 (b) in the high mass range. Similar MS/MS spectra are observed for all isomeric heptapeptide series. The most abundant ions in the mass spectra were H_2O loss at m/z 730, a_7 ions at m/z 720, and b_7-V at m/z 649. In addition, internal single residue eliminations such as b_7-X ions (where X is P, Y, A, G, F, or L) corresponding to m/z 651, 585, 677, 691, 601, and 635, respectively, are observed. What is noticeable is abundant proline, P, and tyrosine, Y, residue elimination, together from the b_7 ion for both isomers. The single P elimination from b_7 is not a preferred route; instead, the loss of P and Y residues is observed as the favored pathway. Similar behavior is also observed for the isomeric pair of PVYAGFL and YAGFLVP, where P and valine, V, residue loss is abundant in the MS/MS

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Table 1. Comparison of Peptide Fragments to Prove the Identity of the P−X Residue Elimination[a]

Series A	Series B	m/z matched[a]
b_7 -PY from PYAGFLV	b_5 of AGFLVY	488 m/z
b_7 -PA from PAGFLVY	b_5 of GFLVYA	580 m/z
b_7 -PG from PGFLVYA	b_5 of FLVYAG	594 m/z
b_7 -PF from PFLVYAG	b_5 of LVYAGF	504 m/z
b_7 -PL from PLVYAGF	b_5 of VYAGFL	538 m/z
b_7 -PV from PVYAGFL	b_5 of YAGFLV	552 m/z

[a]The term "m/z matched" corresponds to the mass of the common colored sequence of compared peptides in each series.

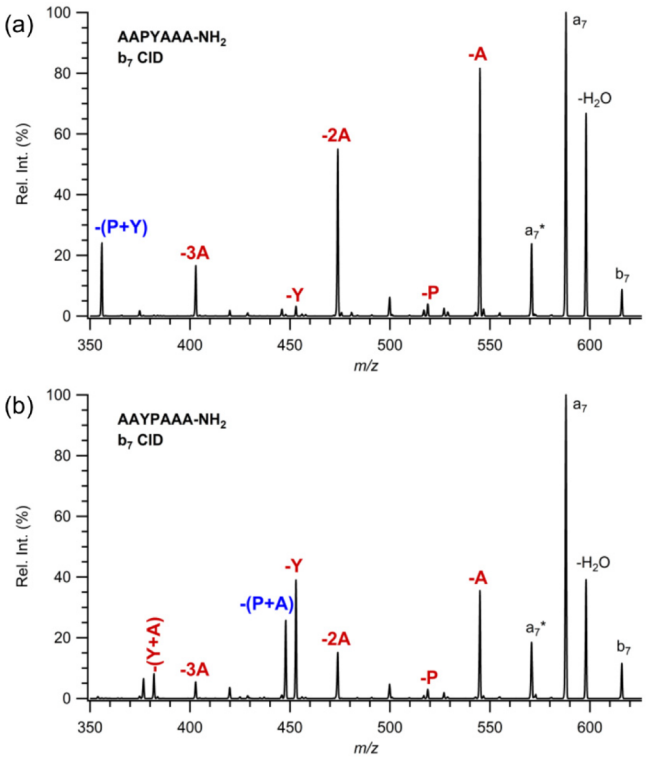

Figure 5. Comparison of MS/MS spectra of b_7 from protonated (a) AAPYAAA-NH_2 and (b) AAYPAAA-NH_2.

spectra. At the same time, single eliminations of each residue are also observed and are shown in Figure 3a and b.

Further experiments are carried out for the isomeric pairs of PLVYAGF/YAGFPLV, PAGFLVY/YPAGFLV, PGFLVYA/YAPGFLV, and PFLVYAG/YAGPFLV. The MS/MS spectra of b_7 ions produced from these isomeric peptide series show a similar fragmentation pathway, and always b_7-PX residue elimination is observed for each isomer (where X is L, A, G, and F, and it is a C-terminally connected residue to P). The results are shown in Figures S2−S5. Because each pair has the same ring sequence when macro-cycled, it can be concluded that the original position of the proline does not affect the fragment ion distribution and course of fragmentation.

Contrary to abundant PY and PV residue eliminations, a minor proline-phenylalanine (PF) elimination is observed from b_7 ions produced from the PFLVYAG/YAGPFLV peptide pair (Figure S5). Since the glycine residue stays N-terminal to the proline residue when PFLVYAG and YAGPFLV are macro-cycled, it is conceivable to consider that the glycine residue is the reason for the low abundance of PF elimination. The MS/MS spectra of b_7 ions produced from PYAFLVG and PVLFYAG show similar behavior where the elimination of PY and PV residues from b_7 ions is very low (Figure S6). Thus, it can be concluded that mobile protons would not prefer to be retained at the G−P amide bond due to glycine's low proton affinity. Thus, the intense proline-X residue elimination from b_7 ions significantly decreases when the glycine is N-terminal to proline. The glycine effect is well-known in the peptide fragmentation mechanism.[38,43,55]

The MS^4 experiments are performed for the b_7-PY residue (nominally b_5) and b_5 ion produced from AGFLVY to confirm the remaining amino acid sequence after the PY residue loss from the b_7 ion produced from PYAGFLV. The MS/MS spectra of the remaining peptide after PX residue elimination from the b_7 ion are shown in Figure 4. The MS/MS spectra of b_7-PY ions produced from PYAGFLV and the MS/MS spectra of b_5 ions produced from AGFLVY are precisely the same as expected. Similarly, other fragment pairs in Table 1 also produce the same pattern (Figures S7−S11). The PX residue elimination observed from b_7 ions is a unique fragmentation behavior of heptapeptides containing a proline residue. The MS/MS spectra of all isomer pairs show similar fragmentation pathways such as the elimination of H_2O, CO, and CO+NH_3. In addition, single residue eliminations indicate the macro-cyclization/reopening pathway for the remaining pentapeptide.

The behavior of proline residues is further studied using model peptides YAGHFLV and YAGKFLV, where P is replaced with other amino acids such as histidine (H) and lysine (K). The CID of b_7 of YAGHFLV does not result in the loss of H and its C-terminal residue F, while b_7 of YAGKFLV shows a minor K+F elimination with approximately 3% intensity (Figure S12a and b). Additionally, 26% K+G elimination is observed from the b_7 ions of the same peptides, further confirming the specificity of our findings to proline residues.

To determine if the behavior of proline residues is consistent in the presence of a more basic residue, we examined another peptide model, YIHPFHL-OH (Figure S12c). We observe abundant elimination of P+F from b_7, but unexpectedly, P+H elimination was also observed, most likely due to the higher basicity of the H residue. Nonetheless, this observation does not contradict our finding that P and its C-terminal neighbor residue are neutrally eliminated from b_7 ions.

Fragmentation Reactions of b_7 Ions from AAPXAAA and AAXPAAA (Where X Is C, D, F, G, L, V, and Y). A different set of model peptides, such as AAPXAAA and AAXPAAA, where X is C, D, F, G, L, V, and Y residues, is investigated to gather information about PX or XP residue elimination from b_7 ions. The MS/MS spectra of b_7 ions

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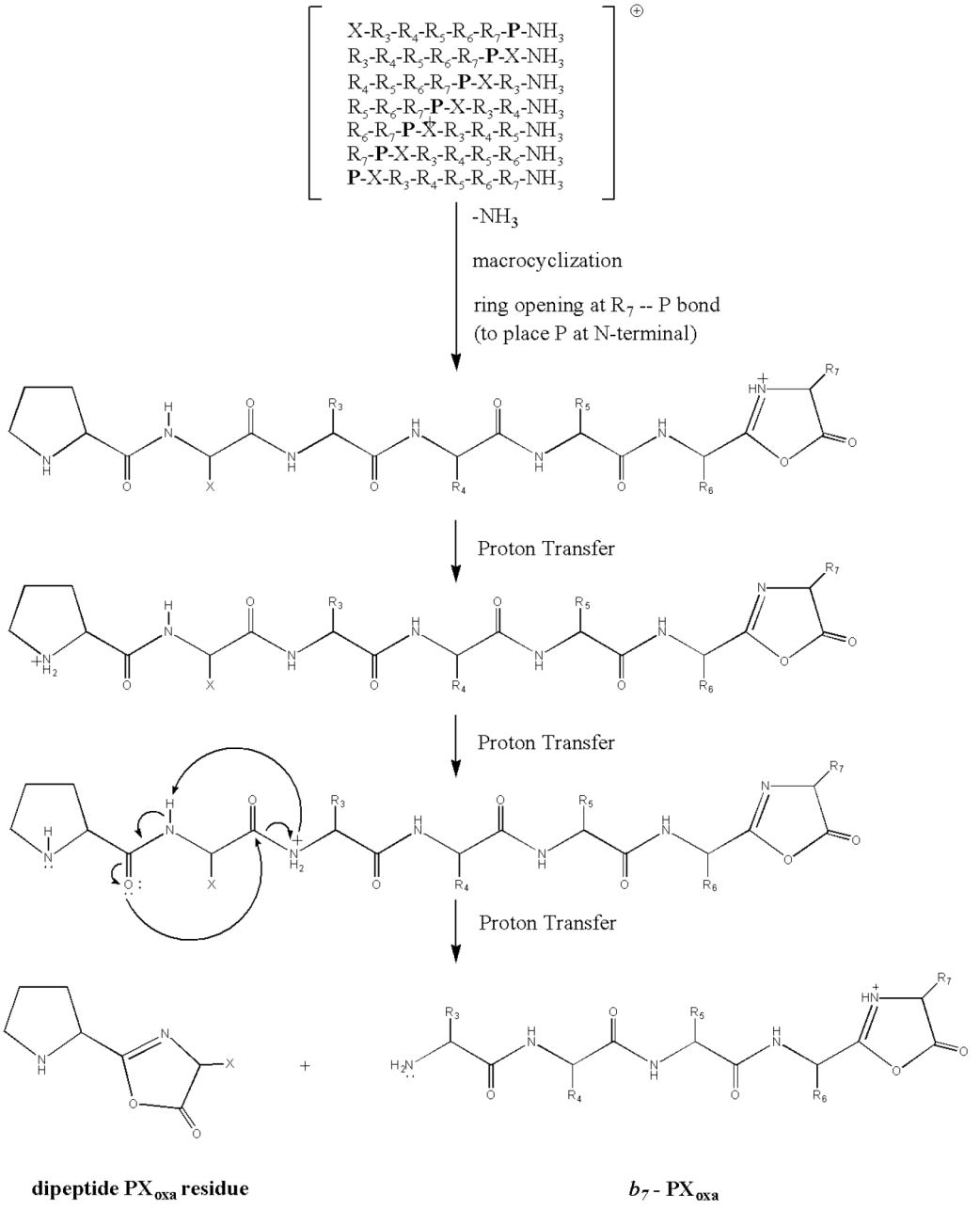
Scheme 1. Proposed Reaction Mechanism for Neutral Dipeptide PX Elimination



produced from AAPYAAA and AAYPAAA are shown in Figure 5a and b. As expected, an abundant elimination of the PY residue is observed from b_7 ions produced from AAPYAAA. By contrast, PA residue elimination is abundant in the MS/MS spectra of b_7 ions produced from AAYPAAA. This result is consistent with our findings suggesting that proline and its adjacent C-terminal residue are lost as a neutral PX residue regardless of their position in the peptide backbone. This behavior is well-supported by increased Y elimination from b_7 when Y is N-terminal to the P residue (Figure 5b). The same fragmentation pathway is observed for other model peptides where X is C, D, F, G, L, and V (Figures S13−S18). Abundant single elimination of amino acid residues N-terminal to proline can be explained by a propensity of proline to stay at the N-terminus after macrocyclization and reopening reaction. And then, the related residue can be located at the C-terminus to be eliminated as a single residue (direct sequence pathway). However, this behavior is not favored when the X amino acid residue is replaced by P. In this case, negligible PP residue elimination is seen (~0.6%) in b_7 spectra of PPAAAAA, AAPPAAA, and AAAAAPP peptide series (Figure S19). This behavior is most likely due to the proline's unique rigid cyclic structure, where the N-terminal proline's carbonyl group cannot effectively attack the carbonyl group of the neighboring proline for the formation and elimination of neutral oxazolone. The unique structure of PP and its enhanced basicity may be attributed to basic functional groups, such as amino groups, which can readily accept protons. PP is a cyclic dipeptide that contains two proline residues, which are known to have unique structural characteristics that distinguish them from those of other amino acids. For example, proline contains a rigid cyclic structure due to its side chain being connected to the backbone nitrogen atom, which can influence the overall conformation of a protein or peptide. Additionally, the presence of multiple

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nitrogen atoms in proline residues can increase the basicity of the molecule, enhancing its propensity to accept protons. This combination of unique structural features and enhanced basicity may contribute to the distinct behavior of PP in various chemical and biological contexts.[46,56]

These results have shown that preferential rearrangement occurs in the peptide sequence during the ring opening of the macrocyclic b_7 structure so that the proline is always located at the N-terminus, no matter its original position in the peptide backbone. The mobile proton is first transferred to the proline pyrrolidine side chain group and then to the second amide bond. This initiates the cleavage of the second amide bond to form a neutral oxazolone dipeptide residue, which explains the PX elimination from the b_7 ion (Scheme 1).

CONCLUSIONS

The current article mainly examines the fragmentation behavior of a series of different model heptapeptides containing a proline residue. The b_7 ions produced from all model isomers of PAAAAAA, PYAGFLV, and AAPXAAA undergo head-to-tail macrocyclization/reopening reactions. Then, a unique fragmentation reaction pathway is observed: neutral proline and its C-terminal residue elimination from the N-terminus of all proline-containing peptides. These abundant dipeptide eliminations from b_7 ions produced from all model heptapeptides suggest that proline-containing b_7 ions tend to place the proline residue in the N-terminal position during the ring opening of the macrocyclic structure which is then followed by elimination of proline plus its adjacent C-terminal residue as neutral b_2 oxazolone (e.g., PX_{oxa}). Overall, the obtained results are particularly important, highlighting the unique behavior of proline-containing heptapeptide isomers. This observation can help in the development of new search algorithms to prevent false positives in protein identification.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.3c00049.

Figure S1, CID spectra of b_7 ions from other isomers of the PAAAAAA-NH_2 series; Figures S2−S6, CID spectra of b_7 ions from PLVYAGF/YAGFPLV, PAGFLVY/YPAGFLV, PGFLVYA/YAPGFLV, PFLVYAG/YAGPFLV, and PYAFLVG/PVLFYAG; Figures S7−S11, comparison of MS^4 spectra of PX_{oxa} eliminations; Figure S12, CID spectra of b_7 ions from protonated (a) YAGHFLV-NH_2, (b) YAGKFLV-NH_2, and (c) YIHPFHL-OH; Figures S13−S18, comparison of CID spectra of b_7 ions from (a) AAPXAAA-NH_2 and (b) AAXPAAA-NH_2; Figure S19, CID spectra of b_7 ions from (a) PPAAAAA-NH_2, (b) AAPPAAA-NH_2, and (c) AAAAAPP-NH_2 (PDF)

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Notes

The authors declare no competing financial interest.

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