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The role of lysine ε-amine group on the macrocyclization of *b* ions

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ABSTRACT

A study was carried out to examine if the amine (—NH_2) group located on the side chains of lysine (K), glutamine (Q), or asparagine (N) residue has any effect on the macrocyclization of *b* ions even though the N-terminals of the peptides were acetylated. The work utilized the model peptides Ac-KYAGFLVG, Ac-QYAGFLV-NH_2, and Ac-NYAGFLV-NH_2. The CID mass spectra of b_7 ions originated from these three peptides exhibited that the macrocyclization still occurred for the lysine containing peptide in spite of the N-terminal of the peptide was acetylated, but was failed to be observed for glutamine and asparagine containing peptides. These current results reveal that the lysine side chain ε-amine group has been involved in the macrocyclization of the peptide *b* ions for the N-terminal acetylated peptides and consequently, non-direct sequence *b* ions were observed in the CID mass spectra. However, due to the amide group on the side chains of the glutamine and asparagine residues, the nucleophilicity of their groups greatly reduced; therefore the scrambling *b* ions were not detected in their b_7 ion CID mass spectra. In addition, the effect of the lysine position was also studied for series of six isomeric octapeptides such as, Ac-KYAGFLVG, Ac-YKAGFLVG, Ac-YAKGFLVG, Ac-YAGKFLVG, Ac-YAGFKLVG and Ac-YAGFLKVG in order to examine the relationship between the intensities of non-direct sequence *b* ions and the lysine position in the octapeptide series. The results clearly demonstrated that the most abundant non-direct sequence *b* ions were observed for the first position of lysine residue in the N-terminal acetylated octapeptide, however, when the lysine residue gets closer to the C-terminal position the relative intensities of the scrambled *b* ions were greatly decreased.

1. Introduction

Tandem mass spectrometry (MS/MS) with collision-induced dissociation (CID) has become widely used in peptide sequence analysis [1] after the introduction of soft ionization techniques, namely ESI and MALDI [2–4]. It is quite obvious that the correct assignment of an amino acid sequence from each MS/MS spectrum of peptides or proteins is a crucial step in mass spectrometry based proteomics. Under low-energy CID conditions, protonated peptides primarily undergo amide bond cleavage to form a series of informative N-terminal *b* and *a* ions and/or C-terminal *y* ions which reflect the sequence of peptide [5,6]. It has been well defined that *y* ions are protonated truncated peptides or protonated C-terminal amino acids [7,8]. By contrast, most interest has been devoted to the structures and fragmentation mechanism of *b* ions [9–13] in which further fragmentation results in lower *b* ions as well as *a* ions under low CID conditions. In early studies, an acylium ion structure was proposed for *b* ion structure [5,6]. However, Harrison and co-workers reported that the five-membered oxazolone ring structure is the most stable for b_n ions (n = 2–4) [9,10]. Afterwards, the oxazolone structure of b_n (n = 2–4) has been confirmed by different groups using spectroscopic and theoretical [14–16] and gas-phase H/D exchange studies [17]. As well, a diketopiperazine structure [18] and a mixed of diketopiperazine and oxazolone structure [19] have been also proposed as the structure of the b_2 ions. A review paper by Paizs and Shuai [20] summarized the fragmentation reaction mechanisms of peptides.

In early studies, formal internal amino acid residue eliminations have been reported that are due to the intramolecular rearrangement reactions, which make the interpretation of peptide MS/MS spectra more difficult [21,22]. Recently, there has been strong evidence of larger b_n (n = 5, 6, 7, ...) ions to form a protonated macrocyclic structure [23–26]. This macrocyclic intermediate can undergo a ring opening process at different amide bonds due to the mobile proton on the ring and forms various isomeric linear C-terminal oxazolones. As a result, non-direct sequence fragment ions have been observed which lead to uncertainty in the sequencing of unknown peptides through MS/MS experiments [25]. Harrison and co-workers proposed that macrocyclization is initiated by a nucleophilic attack of the free N-terminal amine (α-amine) group on the carbonyl carbon of the C-terminal linear oxazolone structure [23,25]. The infrared multiple photon dissociation (IRMPD)

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spectroscopy, DFT calculations and ion mobility-mass spectrometry (IM-MS) studies also confirmed the macrocyclic *b* ion structure [27–29].

More recently, the effects of histidine [30] and arginine residue [31] on the macrocyclization of b_5 ions were investigated. It was documented that sequence scrambling was observed for histidine [30] containing peptides when the histidine residue gets closer to the C-terminal position; conversely no scrambling ions were observed in the b_5 ion CID mass spectra originating from arginine containing peptides [31]. Moreover, we have reported a systematic study to examine the effects of acidic amino acids (either glutamic or aspartic acid) as well as the position of the acidic group on the macrocyclization of b_7 and b_8 ions [32]. It was clearly shown that the side chain of acidic residue(s) did not prevent the sequence scrambling of *b* ions. The effects of the nucleophilic side chain of amino acid residues, either glutamic acid (E), aspartic acid (D), glutamine (Q), asparagine (N), and lysine (K) on the selective opening of macrocyclic b_5 ion of the YAXFLG peptides were studied by Van Stipdonk et al. [33]. The effect of hepta-, octa-, and nonapeptides on the macrocyclization of *b* ions has been studied by the same group [34]. Additionally, Chen and co-workers presented a detailed study on oligoglycine b_2–b_8 ions to determine the influence of the peptide size on the cyclization [35]. Furthermore, Harrison reported a study on the cyclization of b_9 ions [36]. It was shown that the product ion mass spectra of b_9 ions obtained from YA_9, A_4YA_5, and A_8YA (where A is alanine and Y is tyrosine residue) are very similar to each other, which could be used as evidence of the macrocyclization of larger *b* ions. In spite of all these, Siu et al. reported that the presence of non-direct sequence *b* ions does not affect correct peptide and protein identifications via MS/MS spectra [37].

In early studies, Tang et al. reported that some lysine and ornithyl side chains may also trigger the formation of a cyclic structure [38,39]. It was also postulated that protonated α-amino-ε-caprolactam was formed via involvement of a lysine side chain ε-amine group in lysine derivatives of dipeptides and tripeptides [40]. As it was proposed by Harrison et al. [23] the free N-terminal amine group is required for the formation of the macrocyclic *b* ion structure in the gas-phase. It was shown that cyclization process is completely blocked by N-terminal acetylation [22,24,26,36]. More recently, O'Conner and coworkers [41] suggested that the macrocyclization of *b* ions may take place between the lysine side chain ε-amine group and the C-terminal oxazolone carbonyl group. However, there is no systematic study on the side chain amine group assisted macrocyclization (can be defined as "side-to-tail cyclization") of *b* ions. In this work, we examined whether side-to-tail cyclization can take place for lysine, glutamine, and asparagine containing peptides in which these residues are located at the N-terminal position of the N-terminal acetylated peptides. The common feature of these three amino acid residues is that they contain an amine group on their side chains that might play a crucial role in the side-to-tail cyclization of the *b* ions. In addition, the positional effect of the lysine residue in the octapeptides was also studied for the side chain assisted macrocyclization process.

2. Experimental

All model peptides (either C-terminal amidated or free acid) were purchased from GL Biochem Ltd. (Shanghai, China) and were used as received. Approximately 2 mg of each of the solid peptide samples was dissolved to a concentration of 10^{-3} or 10^{-4} M in HPLC-grade methanol (MeOH).

Two types of mass spectrometers were used throughout the study, namely the LTQ XL linear ion-trap (Thermo Finnigan, San Jose, CA) and a hybrid triple quadrupole/linear ion trap instrument (4000 Q-TRAP, Applied Biosystems/MDS Sciex, Concord, Canada).

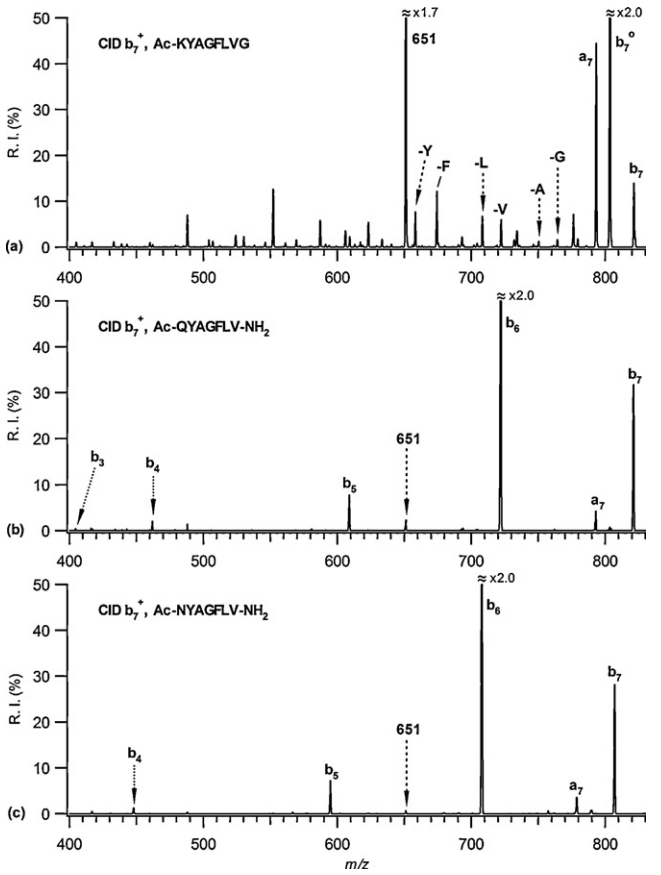


Fig. 1. Comparison of the MS^3 mass spectra of b_7 ions of protonated Ac-KYAGFLVG, Ac-QYAGFLV-NH_2, and Ac-NYAGFLV-NH_2.

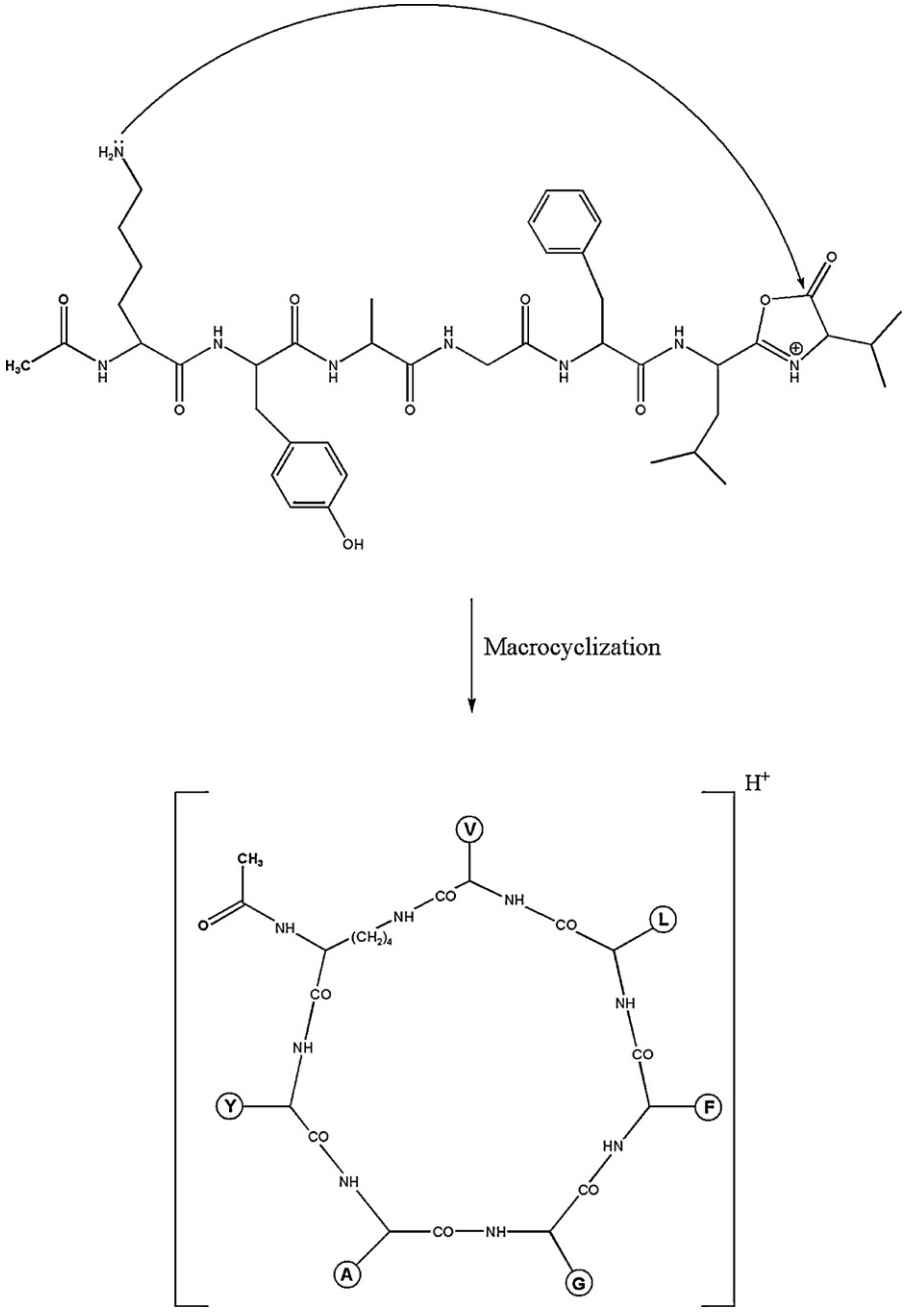
The LTQ XL mass spectrometer system is equipped with an ESI source and was operated in the positive mode. The experimental conditions were the same as previously described in detail [32]. Briefly, 100 pmol μL^{-1} of each peptide solution was diluted in 50:50:1 (v/v/v) MeOH/H_2O/HCOOH and was introduced into the ion source with an incorporated syringe pump at a flow rate of 5 μL min^{-1}. Other experimental parameters are as follows. The isolation width (*m*/*z*) for precursor ions was set at between 0.8 and 2.4 for MS^n stages and at least 400 scans were averaged. The normalized collision energy was varied between 20% and 28% (arbitrary units) where helium was used as the collision gas for CID and as a damping gas.

In order to construct the breakdown graph, a hybrid triple quadrupole/linear ion trap instrument (4000 Q-TRAP, Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a turbo ion spray source was used as described previously [32]. Briefly, the ion spray voltage was +5.5 kV and MS/MS experiment was carried out in enhanced product ion (EPI) scan mode. The collision energy was varied from 18 to 48 eV in increments of 2 eV, and 50 cycles were averaged.

3. Results and discussion

3.1. Effect of lysine, glutamine, and asparagine side chain amine groups on the side-to-tail cyclization of b_7 ions

The CID mass spectra of b_7 ions derived from Ac-KYAGFLVG, Ac-QYAGFLV-NH_2, and Ac-NYAGFLV-NH_2 are shown in Fig. 1. It is clearly shown that non-direct sequence *b* ions are still observed for the Ac-KYAGFLVG peptide even though the α-amine group of the model peptide is acetylated (Fig. 1a). However, it was

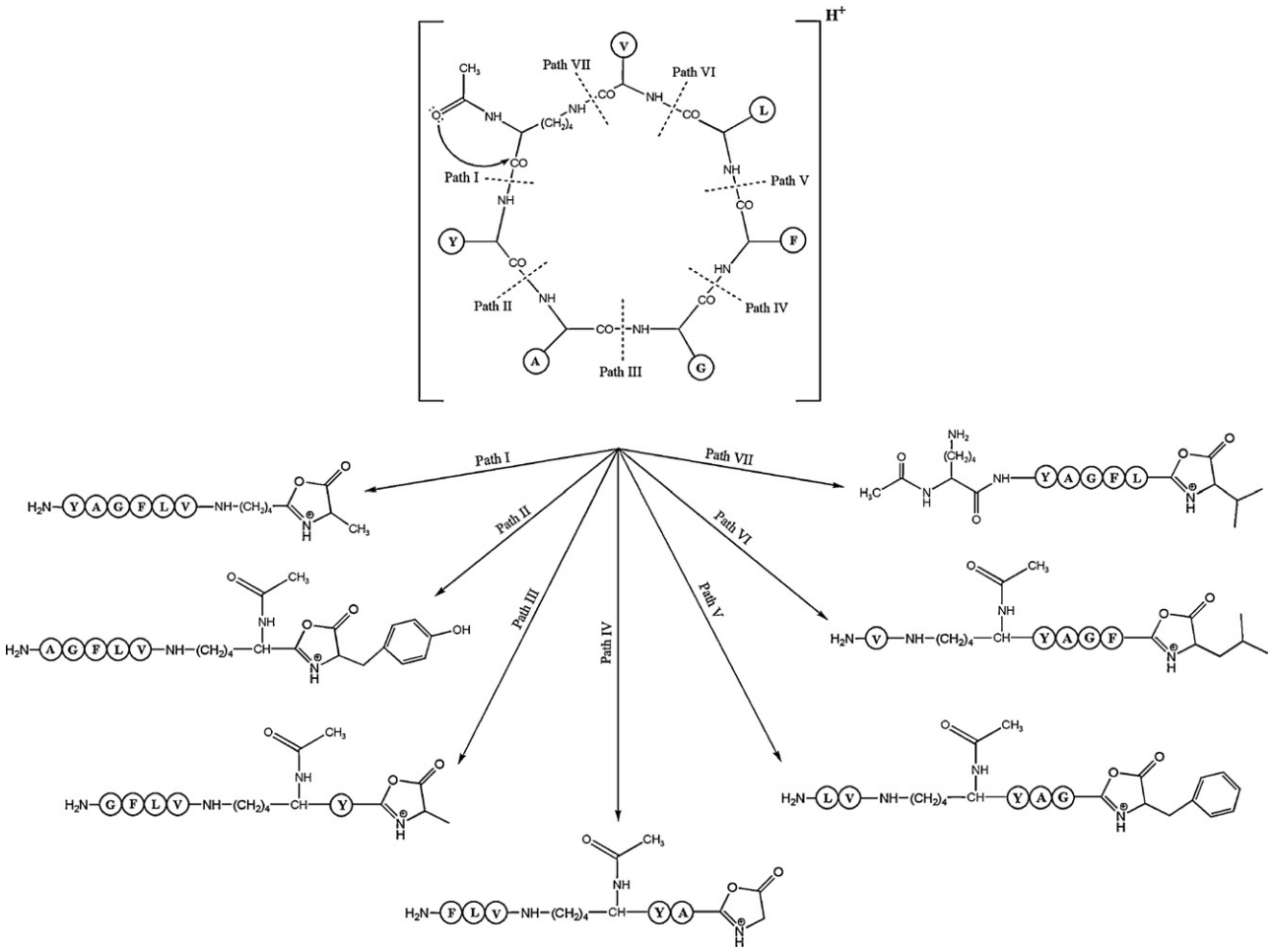


Scheme 1. The proposed macrocyclization reaction mechanism for b_7 ion of Ac-KYAGFLVG.

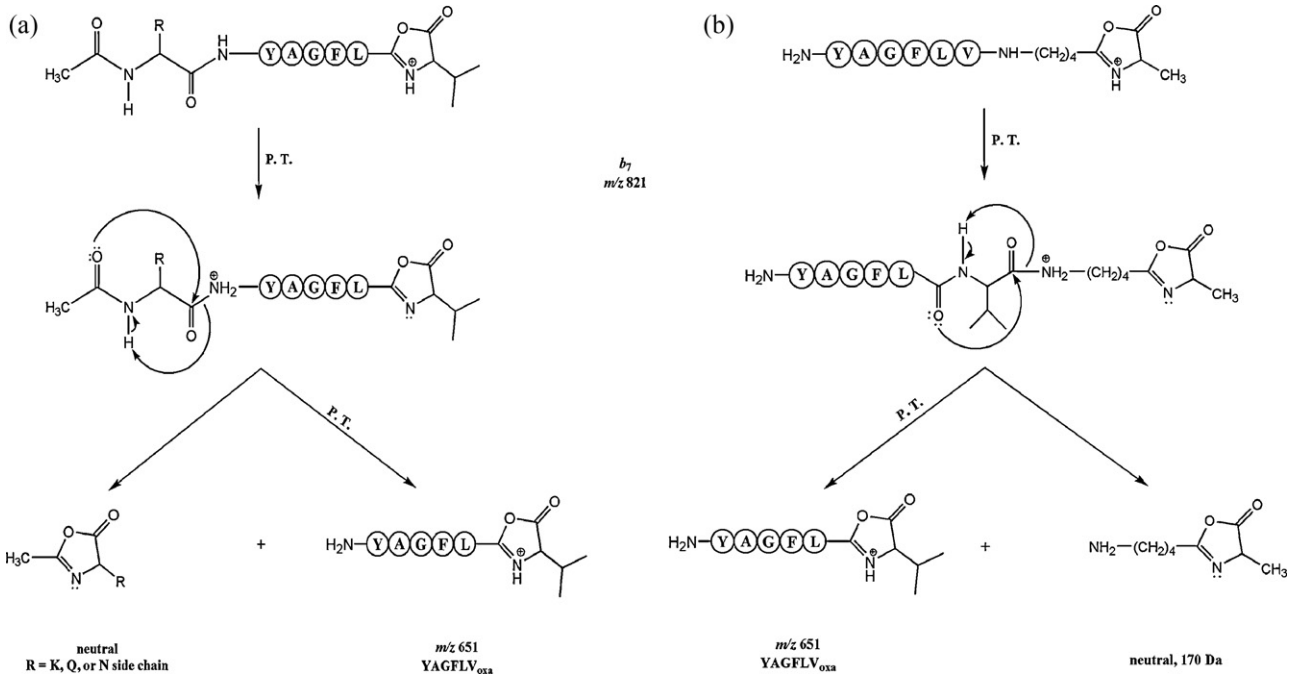
reported that N-acetylation blocks the cyclization reactions and eliminates the non-direct sequence *b* ions in the peptide CID mass spectra [36]. These non-direct sequence ions can be accepted as a major evidence of the macrocyclization of b_7 ion in the gas-phase and the macrocyclization reaction can be explained by a nucleophilic attack of the lysine side chain ε-amine group to the carbonyl carbon of valine oxazolone, side-to-tail cyclization, as shown in Scheme 1. Afterwards, this intermediate macrocyclic structure undergoes seven different ring opening pathways at various amide bonds to yield isomeric linear *b* oxazolones (Scheme 2). The product ions at *m*/*z* 764, 750, 708, 674, and 658 correspond to the non-direct sequence *b* ions which is the elimination of G (−57 Da), A (−71 Da), L (−113 Da), F (−147 Da), and Y (−163 Da), respectively. Here, it should be mentioned that the valine loss (−99 Da) represents the direct sequence b_6 ion (*m*/*z* 722) from the b_7 ion.

By contrast, non-direct sequence *b* ions or any evidence for the macrocyclization could not be observed in the b_7 ion CID mass spectra for Ac-QYAGFLV-NH_2 and Ac-NYAGFLV-NH_2 peptides, in which only direct sequence *b* ions (b_6, b_5, b_4, . . .) were detected in their b_7 ion CID mass spectra (Fig. 1b and c). This aspect can be explained by having amide group on the side chains of glutamine and asparagine residues which makes the nucleophilic reactivity of their amine group less compared to the lysine side chain ε-amine group.

In order to confirm the involvement of the lysine side chain ε-amine group in the side-to-tail cyclization of *b* ion, we purchased Ac-K(Ac)YAGFLVG peptide where both of the α-amine of the peptide and ε-amine group of lysine residue have been acetylated. The CID mass spectrum of b_7 ion obtained from doubly acetylated peptide contains only direct sequence *b* ions, as Fig. 2 illustrates. This spectrum clearly shows evidence of the lysine ε-amine group involvement in the side chain assisted macrocyclization of *b* ion



Scheme 2. Seven possible ring opening pathways of macrocyclic b_7 ion of Ac-KYAGFLVG.



Scheme 3. (a) The proposed fragmentation pathways leading to the neutral Ac-X (X = K, Q, or N) and protonated $YAGFLV_{oxa}$ from Ac-KYAGFLVG, Ac-QYAGFLV-NH_2, and Ac-NYAGFLV-NH_2, respectively. (b) The proposed fragmentation pathway for the formation of neutral Ac-K and protonated $YAGFLV_{oxa}$ from YAGFLV-Ac-K_{oxa} b_7 isomer.

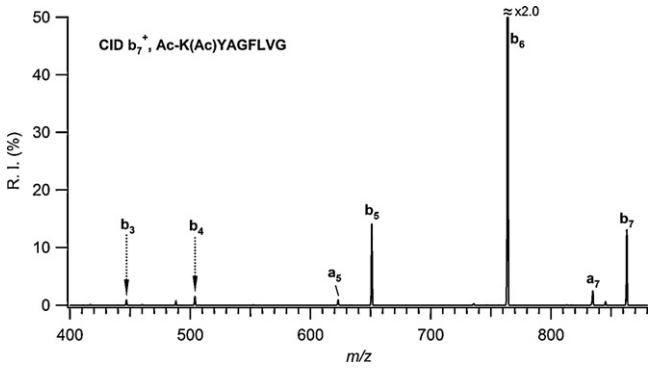


Fig. 2. The MS^3 mass spectrum of b_7 ion from protonated Ac-K(Ac)YAGFLVG.

(The peptide sequence was validated by MS/MS spectrum of its MH^+, as shown in Fig. S1 of Supplemental Information).

3.2. Formation and structure of m/z 651 in the b_7 ion CID mass spectra of N-terminal acetylated lysine, glutamine, and asparagine containing peptides

The peak at m/z 651 is observed as a common fragment ion in all b_7 ions of Ac-KYAGFLVG, Ac-QYAGFLV-NH_2, and Ac-NYAGFLV-NH_2 peptides, as labeled in Fig. 1. The gas-phase structure of m/z 651 ion was probed via MS^4 ($MH^+ \rightarrow b_7 \rightarrow 651$) consecutive experiments for these three peptides individually. It was shown that the fragmentation patterns of the m/z 651 ions derived from N-terminal acetylated lysine, glutamine, and asparagine containing peptides contain entirely the same product ions in their CID mass spectra (Fig. 3). We proposed that that N-terminal acetylated lysine, glutamine, or asparagine is cleaved as a neutral from b_7 ions, leaving the $YAGFLV_{oxa}$ as a b-type protonated oxazolone structure in the gas-phase (Scheme 3a). To support this proposed fragmentation mechanism, the model peptide YAGFLV-NH_2 was also purchased and its b_6 ion (m/z 651) CID mass spectrum was compared with the other m/z 651 ion's mass spectra, as shown in Fig. 3. It is clear that the m/z 651 ions obtained from b_7 ions of N-terminal acetylated lysine, glutamine, and asparagine containing peptides and the b_6 ion obtained from YAGFLV-NH_2 have the same fragmentation pattern along with the same fragment ion intensities.

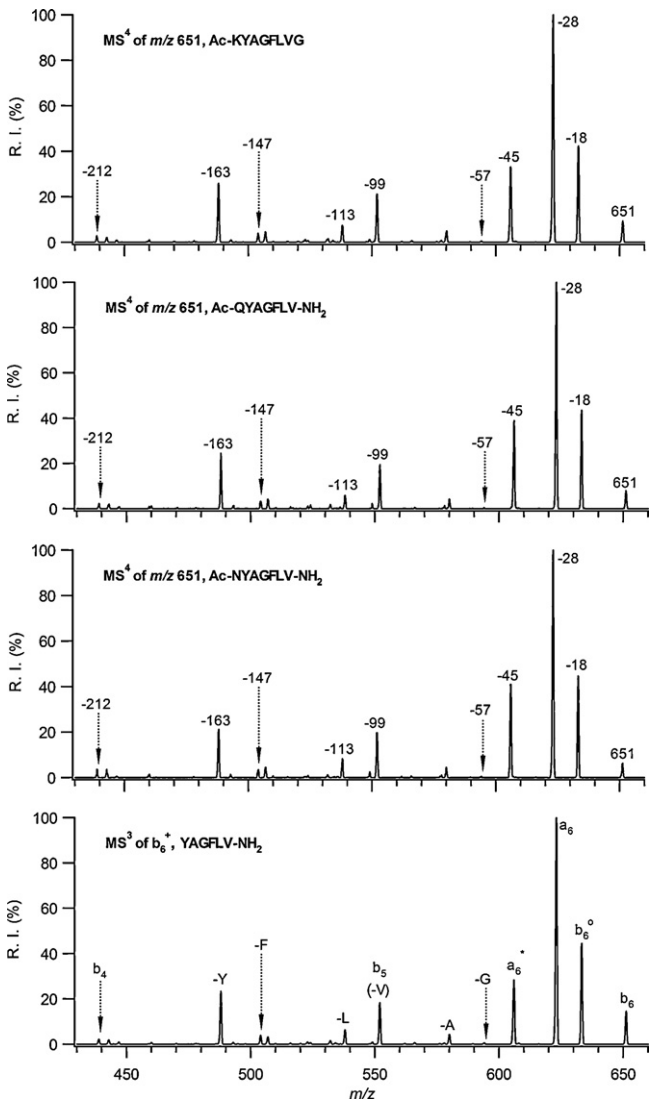


Fig. 3. Comparison of the MS^4 ($MH^+ \rightarrow b_7 \rightarrow 651$) mass spectra of m/z 651 ions originated from protonated Ac-KYAGFLVG, Ac-QYAGFLV-NH_2, Ac-NYAGFLV-NH_2 with MS^3 ($MH^+ \rightarrow b_6$) mass spectrum of b_6 ion derived from YAGFLV-NH_2.

3.3. Positional effect of lysine residue on the side-to-tail cyclization of b_7 ions in the N-terminal acetylated octapeptides

In the second part of the study, we performed a detailed analysis for six N-terminal acetylated isomeric octapeptides in order to examine the positional effect of the lysine residue for the side chain assisted macrocyclization of b ions. This set of model peptides comprised of Ac-KYAGFLVG, Ac-YKAGFLVG, Ac-YAKGFLVG, Ac-YAGKFLVG, Ac-YAGFKLVG and Ac-YAGFLKVG where the lysine was positioned at the N-terminal position 1 through 6 in the model octapeptides. (The MS/MS spectra of MH^+ derived from each peptide were shown in Fig. S2 of Supplemental Information for the confirmation of peptide sequence). The MS^3 CID mass spectra of b_7 ions derived from each six octapeptides are shown in Fig. 4a–f. It is clear from the spectra that the relative intensities of the non-direct sequence b ions have greatly decreased (below to the 1%) for the isomeric peptides where the lysine residue gets closer to the C-terminal position. We can explain this dramatic reduce in intensities of the non-direct sequence b ions as having a bulky group near to the lysine residue whose ε-amine group can rationalize the side-to-tail cyclization. However, further studies are necessary to figure out this behavior.

For the peptide Ac-KYAGFLVG, we proposed that there are two fragmentation routes leading to a neutral Ac-K loss from the b_7 ion: either the direct cleavage of peptide bond between Ac-K and $YAGFLV_{oxa}$ (as already described in Scheme 3a) or the YAGFLV-Ac-K_{oxa} was formed as a result of sequence scrambling chemistry via path I, shown in Scheme 2, and is followed by peptide bond cleavage between the valine carbonyl group and the lysine ε-amine group at the C-terminal (Scheme 3b). On the other hand, the spectrum also contains the ion at m/z 552 arising from loss of valine residue (-99 Da) from $YAGFLV_{oxa}$ which forms $YAGFL_{oxa}$. The structure of this fragment ion was also supported by comparing the b_5 ion spectrum obtained from a commercial YAGFLV-NH_2 peptide (spectrum not shown).

For the octapeptides where the lysine residue is located at the N-terminal position 2 or any other internal position, we proposed and reported the detailed mechanisms for the formation of each of the labeled fragments in the b_7 ion CID mass spectra and can be found in Supplementary Materials of this article.

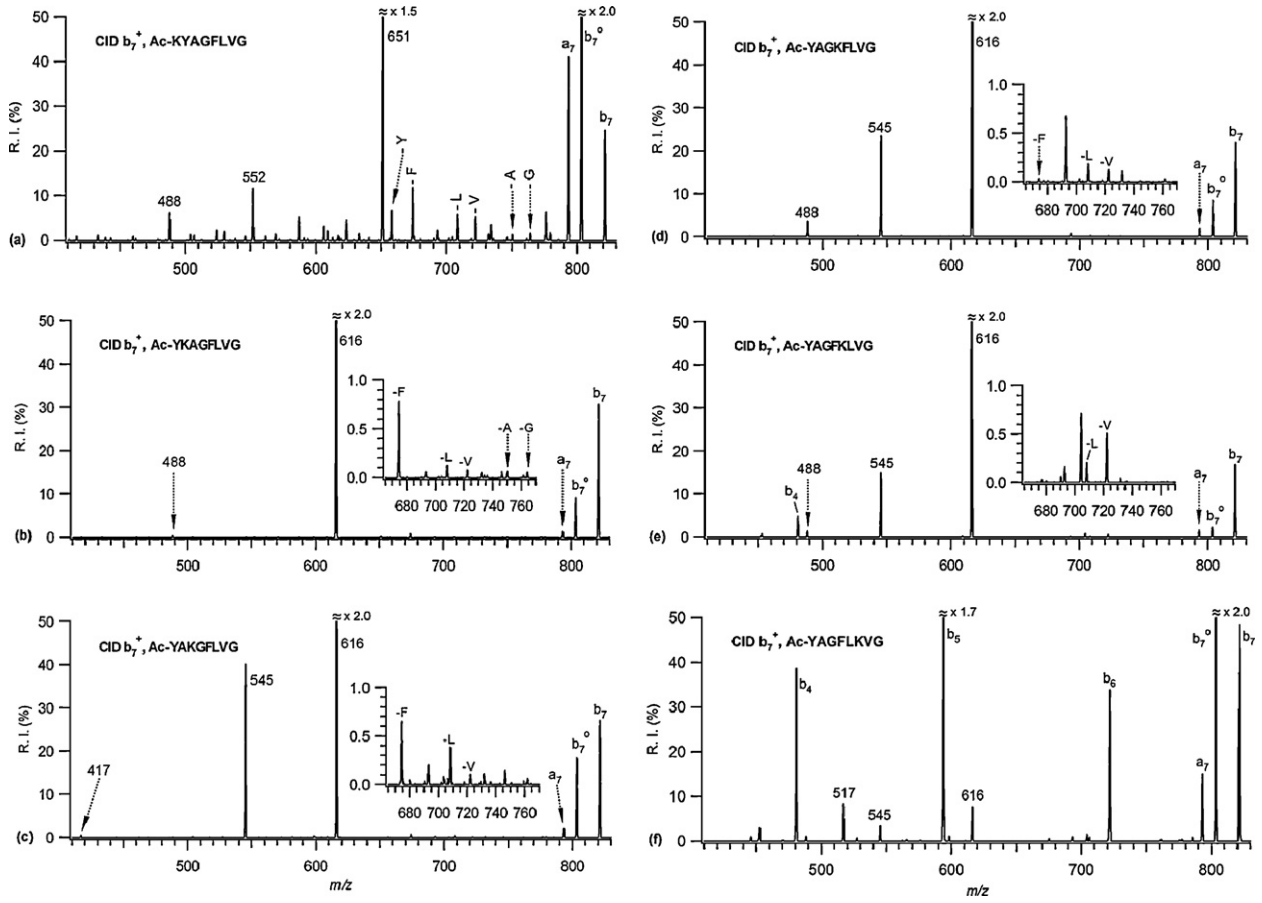


Fig. 4. The MS^3 mass spectra comparison of the of b_7 ions from protonated (a) Ac-KYAGFLVG, (b) Ac-YKAGFLVG, (c) Ac-YAKGFLVG, (d) Ac-YAGKFLVG, (e) Ac-YAGFKLVG and (f) Ac-YAGFLKVG.

4. Conclusion

In the first part of the study, we examined the effects of the side chain amine group in the N-terminal acetylated lysine, glutamine or asparagine containing octapeptides for the side chain assisted macrocyclization of *b* ions. The obtained results indicated that the ε-amine group of a lysine residue induced macrocyclization of *b* ion in the gas-phase even though the N-terminal of the peptides was acetylated. However, the non-direct sequence *b* ions were not observed for N-terminal acetylated glutamine and asparagine containing peptides due to the amide groups on the side chains of these residues which reduce the nucleophilicity of the amide groups.

In the second part of the work, our attention was focused on the varying positions of lysine residues in the N-terminal acetylated octapeptides in order to investigate whether the side chain assisted macrocyclization of *b* ions was affected by their position. The MS^3 experiments have shown that the intensities of non-direct sequence *b* ions were drastically reduced as the lysine residue was shifted into the second and any other internal positions. It was thought that the extent of macrocyclic structure formation is affected by bulky group near to the lysine residue. Nonetheless, further studies are needed using other lysine containing model isomeric peptides to clarify this behavior. It was clearly shown that the lysine position is an important factor for the complete side-to-tail cyclization of b_7 ions derived from N-terminal acetylated octapeptides.

The current results reported here explain that macrocyclization of *b* ions can be accomplished between ε-amine group of lysine residue and the C-terminal oxazolone when the lysine residue is positioned at the N-terminal position 1 in the N-terminal acetylated octapeptide and the position of the lysine residue has a crucial importance in order to obtain significant scrambling ions as a consequence of the cyclization process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.12.008.

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