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Specific rearrangement reactions of acetylated lysine containing peptide b_n ($n = 4–7$) ion series

A. Emin Atik,[a] Oscar Hernandez,[b] Philippe Maître[b]* and Talat Yalcin[a]*

Characterization of ε-*N*-acetylated lysine containing peptides, one of the most prominent post-translational modifications of proteins, is an important goal for tandem mass spectrometry experiments. A systematic study for the fragmentation reactions of *b* ions derived from ε-*N*-acetyllysine containing model octapeptides (K_{Ac}YAGFLVG and YAK_{Ac}GFLVG) has been examined in detail. Collision-induced dissociation (CID) mass spectra of b_n ($n = 4–7$) fragments of ε-*N*-acetylated lysine containing peptides are compared with those of *N*-terminal acetylated and doubly acetylated (both ε-*N* and *N*-terminal) peptides, as well as acetyl-free peptides. Both direct and nondirect fragments are observed for acetyl-free and singly acetylated (ε-*N* or *N*-terminal) peptides. In the case of ε-*N*-acetylated lysine containing peptides, however, specific fragment ions (*m/z* 309, 456, 569 and 668) are observed in CID mass spectra of b_n ($n = 4–7$) ions. The CID mass spectra of these four ions are shown to be identical to those of selected protonated *C*-terminal amidated peptides. On this basis, a new type of rearrangement chemistry is proposed to account for the formation of these fragment ions, which are specific for ε-*N*-acetylated lysine containing peptides. Consistent with the observation of nondirect fragments, it is proposed that the *b* ions undergo head-to-tail macrocyclization followed by ring opening. The proposed reaction pathway assumes that b_n ($n = 4–7$) of ε-*N*-acetylated lysine containing peptides has a tendency to place the K_{Ac} residue at the *C*-terminal position after macrocyclization/reopening mechanism. Then, following the loss of CO, it is proposed that the marker ions are the result of the loss of an acetyllysine imine as a neutral fragment. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: peptide fragmentation; lysine acetylation; macrocyclization of *b* ions

Introduction

Tandem mass spectrometry (MS/MS) has become an invaluable tool particularly in peptide sequencing and protein identification.[1] The primary structure of peptides can be obtained by collision-induced dissociation (CID)-based MS/MS experiments.[2,3] Under low-energy CID conditions, consecutive series of *N*-terminal *b* and *a* ions and/or *C*-terminal *y* ions have been predominantly generated via cleavage of the amide bonds along the peptide backbone.[4,5] In the last two decades, tremendous efforts have been made in order to understand the structures, formation mechanisms and fragmentation reactions of gas-phase *b* ions.[6–10] Extensive MS/MS studies[6,7] have provided evidence for the formation of protonated five-membered oxazolone ring at the *C*-terminal end of the peptide in the case of small b_n ($n = 2–4$) ions. The protonated oxazolone structure has been confirmed by several infrared multiple photon dissociation spectroscopy combined with theoretical studies[10–13] as well as gas-phase H/D exchange experiment.[14] Recently, numerous experimental and theoretical studies have revealed that middle-sized b_n ($n = 5, 6, 7 \ldots$) ions undergo intramolecular head-to-tail cyclization to form a protonated cyclic peptide intermediate.[15–25] Upon proton transfer (PT), this cyclic structure subsequently reopens at different amide bonds to form a variety of *C*-terminal oxazolone *b* isomers with a permuted sequence, which may result in erroneous assignment of fragment ions for database searches.

The lysine acetylation is one of the most prominent post-translational modification found in proteins and plays vital roles in regulation of protein activity and gene expression.[26–28] Therefore, a large number of gas-phase fragmentation reaction studies have been carried out for acetylated lysine containing peptides in order to find the localization of the acetylation in the peptide sequence.[29–32]

Previous studies have shown that the *m/z* 143 and 126 fragments can be accepted as marker ions for peptides containing ε-*N*-acetylated lysine residues via gas-phase fragmentation.[29–31] These ions correspond to an ε-*N*-acetylated lysine immonium ion and a loss of ammonia from the acetylated lysine immonium ion. Trelle and Jensen[31] have proved that the *m/z* 126 ion has higher specificity and sensitivity over the ion at *m/z* 143 ion for the screening of acetylated lysine residue containing peptides. The authors also emphasized that the intensities of these diagnostic marker ions are more significant when the ε-*N*-acetylated lysine (K_{ac}) residue is located at the *N*-terminal as compared with internal positions in peptides. Recently, Fu *et al.*[32] also discussed the fragmentation of protonated peptides containing internal lysine and side chain acetylated lysine residues using a triple quadrupole instrument. In their study, it was stated that the acetylation of the ε-amine group of lysine residue enhances the cleavage of K_{Ac}-Gly amide bond and allow forming b_n ions with higher abundances in the corresponding MS/MS spectra.

* *Correspondence to: Talat Yalcin, Department of Chemistry, Izmir Institute of Technology, Urla-Izmir 35430, Turkey. E-mail: talatyalcin@iyte.edu.tr*

* *Correspondence to: Philippe Maître, Laboratoire de Chimie Physique, Université Paris Sud, UMR8000 CNRS, Faculté des Sciences, Bât. 349, 91405 Orsay Cedex, France. E-mail: philippe.maitre@u-psud.fr*

a *Department of Chemistry, Faculty of Science, Izmir Institute of Technology, 35430, Urla-Izmir, Turkey*

b *Laboratoire de Chimie Physique, Université Paris Sud, UMR8000 CNRS, Faculté des Sciences, Bât. 349, 91405 Orsay Cedex, France*

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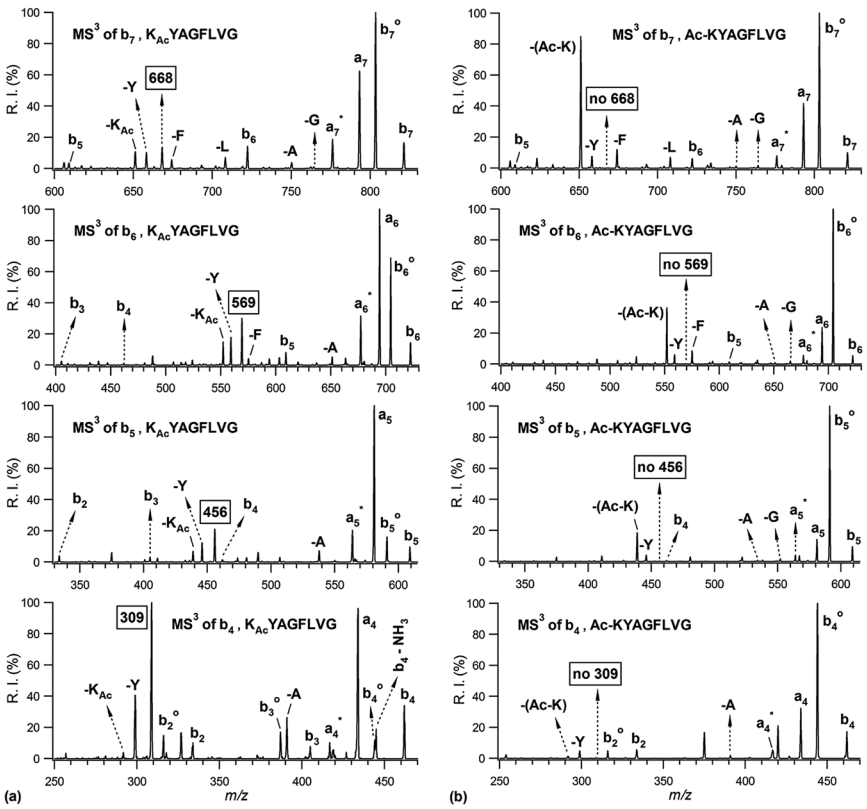


Figure 1. Comparison of the MS^3 mass spectra of b_7, b_6, b_5 *and* b_4 ions originated from protonated (a) K_{Ac}YAGFLVG and (b) Ac-KYAGFLVG.

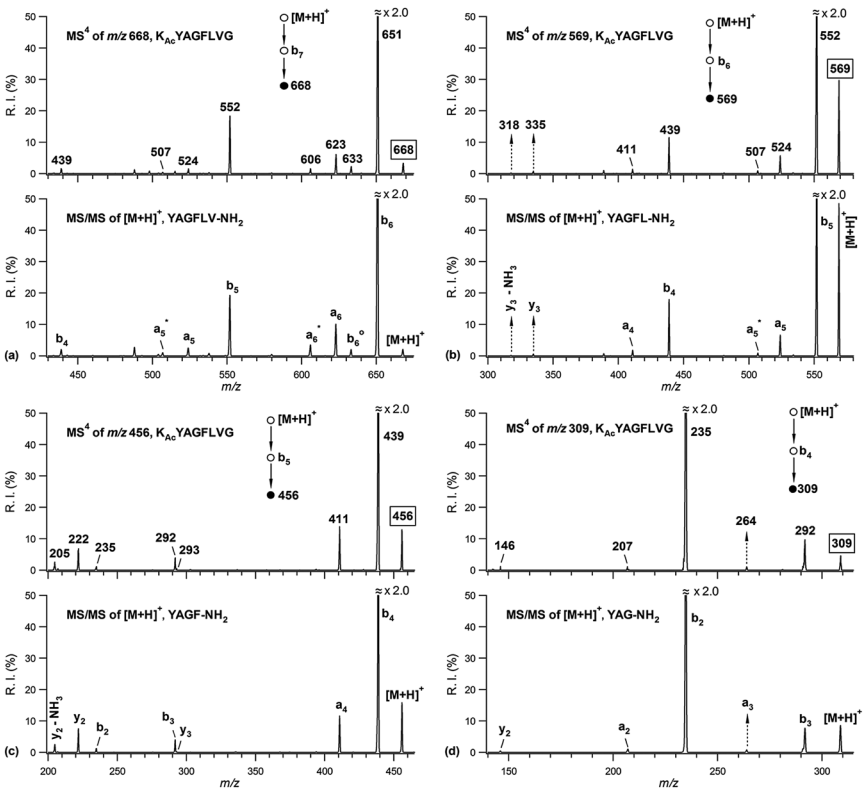


Figure 2. Comparison of (a) MS^4 mass spectrum of *m/z* 668 ion from b_7 ion of protonated K_{Ac}YAGFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated YAGFLV-NH_2, (b) MS^4 mass spectrum of *m/z* 569 ion from b_6 ion of protonated K_{Ac}YAGFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated YAGFL-NH_2, (c) MS^4 mass spectrum of *m/z* 456 ion from b_5 ion of protonated K_{Ac}YAGFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated YAGF-NH_2 and (d) MS^4 mass spectrum of *m/z* 309 ion from b_4 ion of protonated K_{Ac}YAGFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated YAG-NH_2.

In addition, the stable structure of the *m/z* 129 ion has been reported as a protonated α-amino-ε-caprolactam in the dissociation reactions of lysine containing small peptides.[33] Moreover, Kish and Wesdemiotis[34] investigated the selective cleavage of the amide bonds at the *C*-terminal position of protonated and metalated peptides containing internal lysine residues. Payne and co-workers[35] also reported the alteration of peptide fragmentation chemistry by acetylating the *N*-terminal amino group of the peptide.

In the present study, we investigated the fragmentation reactions of b_n ($n = 4$–7) ions originated from ε-*N*-acetylated lysine containing octapeptide (K_{Ac}YAGFLVG). For the purpose of comparison, the CID mass spectra of *b* ion series produced from Ac-KYAGFLVG (acetylation of *N*-terminal), Ac-K_{Ac}YAGFLVG (acetylation of both ε-*N* and *N*-terminal) and KYAGFLV-NH_2 (no acetylation) are also recorded. In addition, the positional effect of acetylated lysine residue is also studied for the fragmentation of *b* ion series.

Experimental

All model peptides (either *C*-terminal amidated or free acid, purity >95%) were purchased from GL Biochem Ltd. (Shanghai, China) and used without any purification. The appropriate amount of peptides were dissolved in 1 : 1 (v/v) mixture of high-performance liquid chromatography-grade methanol (MeOH) and deionized water to make up stock solutions with a concentration of 10^{-3} or 10^{-4} M. All the stock solutions were stored at −20 °C.

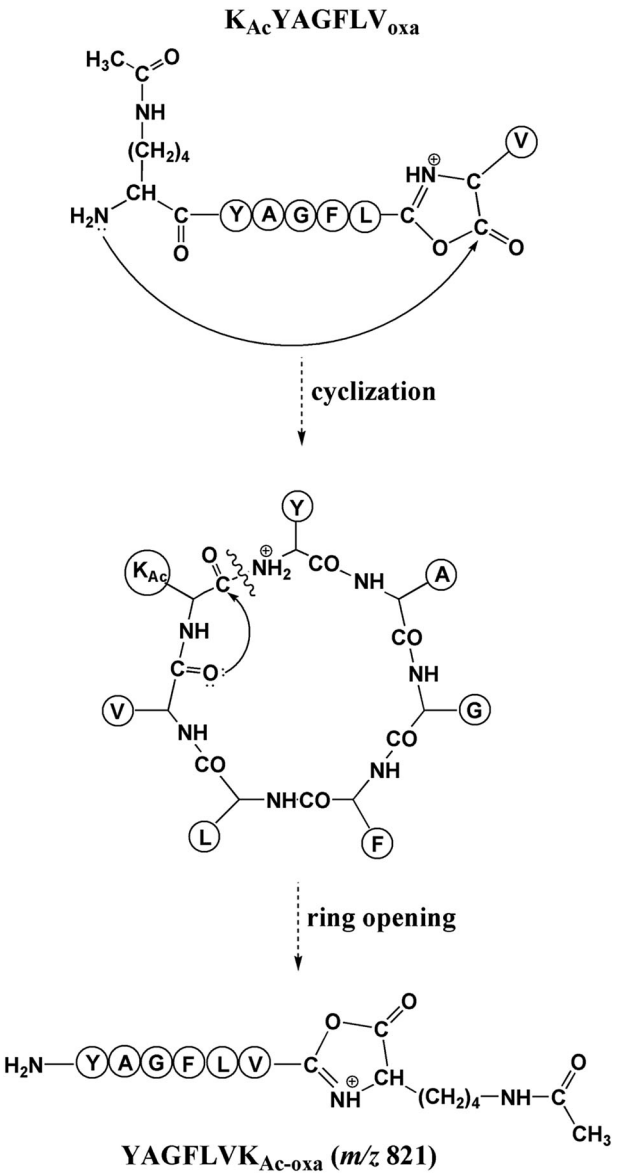
The low-energy MS/MS (MS^n) experiments were performed with an LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray ionization source. The stock peptide solutions were diluted to a 100 μM in 50 : 50 (v/v) methanol/water solvent containing 1% formic acid and infused directly into a mass spectrometer with an incorporated syringe pump at a flow rate of 5 μl min^{-1}. The instrument was calibrated with a Calmix solution composed of caffeine, MRFA and Ultramark 1621 prior to experiments. The ion optics was optimized in order to provide maximum precursor ion transmission into the trap. The experimental conditions were the same as previously described in detail.[25] Briefly, the spray voltage was set at +5.0 kV, and the heated capillary temperature was maintained at 275 °C. Nitrogen was used as the sheath, sweep and auxiliary gas with a flow rate of 10, 1 and 1 (all arbitrary units), respectively. An isolation width (*m/z*) of 1.2–2.0 Da was used for each MS^n acquisitions, and an activation (*q*) of 0.250 with a 30-ms activation time was applied at each CID stage. The normalized collision energy was varied from 20% to 28% (arbitrary units) for the dissociation of selected precursor ion. The MS/MS spectra were recorded in the *m/z* scan range of 150–920 in the positive-ion mode, and at least 400 scans were averaged. Helium was used as the collision gas for CID and also as a damping gas in the collision cell. Data acquisition was performed using XCALIBUR™ (version 2.0) software data system (Thermo Fisher Scientific).

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 to construct the breakdown graph. The experimental parameters were the same as previously described.[25] Briefly, the ion spray voltage was set at +5.5 kV. Peptide solution with a concentration of 10 μM was introduced via an infusion pump at a flow rate of 5 μl min^{-1}. The MS/MS experiment was carried out in enhanced product ion scan mode. The collision energy was varied from 10 to 60 eV in increments of 5 eV, and 40 cycles were averaged with a scan rate of 1000 Da/s. All data were processed using Igor Pro Software package (WaveMetrics, Lake Oswego, OR) for presentation of experimental data.

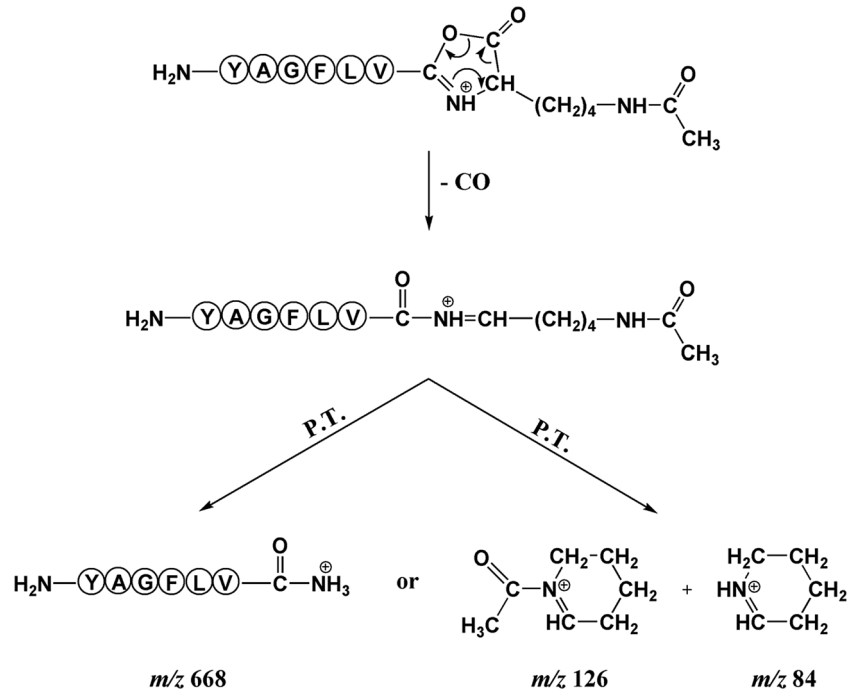
Results and discussions

Fragmentation reactions of b_n ($n = 4$–7) ions originated from K_{Ac}YAGFLVG and Ac-KYAGFLVG

The low-energy CID mass spectra of b_7, b_6, b_5 and b_4 ions were recorded separately for the lysine side chain acetylated; K_{Ac}YAGFLVG, octapeptide and their CID mass spectra are shown in Fig. 1(a). As labeled Fig. 1(a), both direct and nondirect sequence ions were observed in the tandem mass spectra of b_n ($n = 4$–7) ions. The formation of nondirect sequence ions can be explained by head-to-tail cyclization and subsequent ring opening of *b* ions, which forms permuted linear *b* isomers.[15,17] In addition to these product ions,



Scheme 1. The proposed macrocyclization reaction mechanism for b_7 ion of K_{Ac}YAGFLVG.



Scheme 2. The proposed fragmentation pathways for the formation of protonated YAGFLV-NH_2 (*m/z* 668) and/or protonated *m/z* 126 and 84 from permuted b_7 isomer, $YAGFLVK_{Ac\text{-}oxa}$.

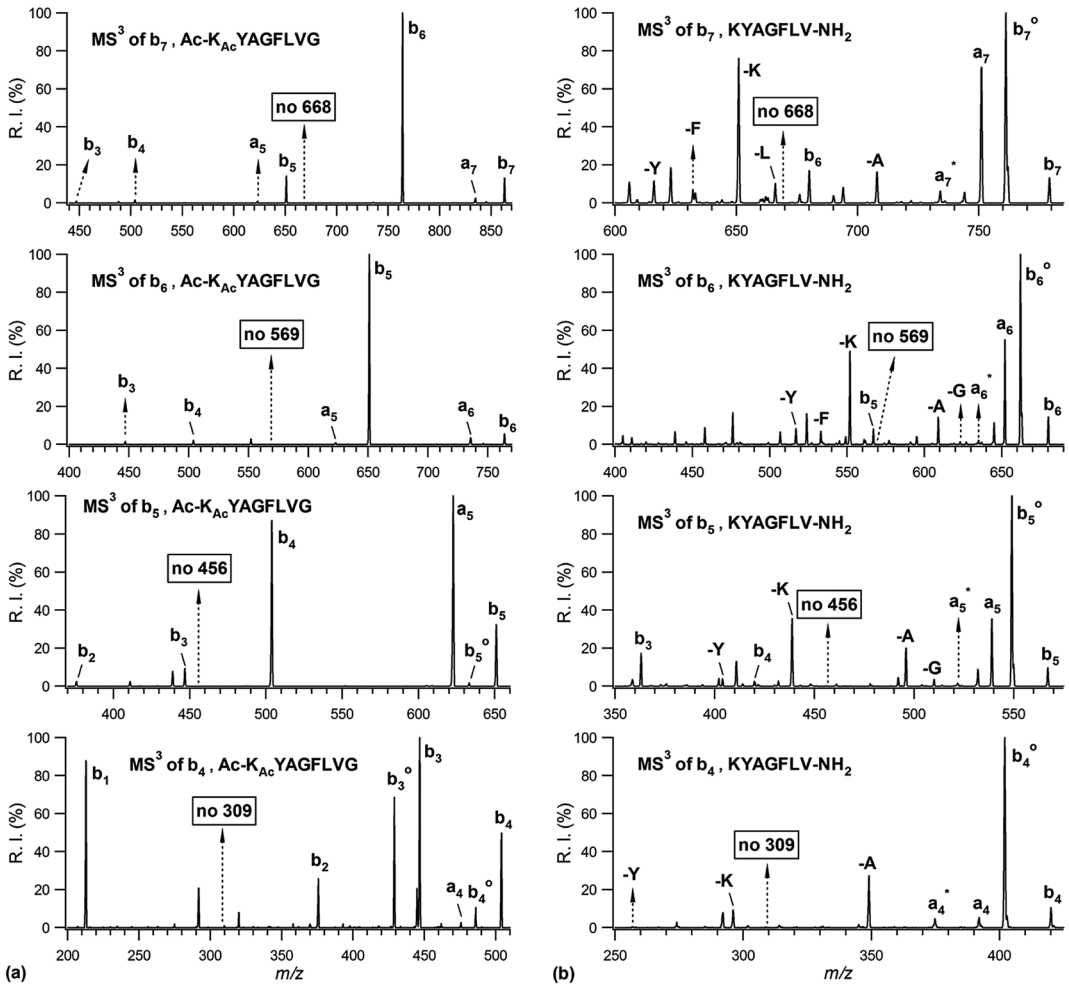


Figure 3. Comparison of the MS^3 mass spectra of b_7, b_6, b_5 *and* b_4 ions originated from protonated (a) Ac-K_{Ac}YAGFLVG and (b) KYAGFLV-NH_2.

the fragments ions at *m/z* 668, 569, 456 and 309 were also observed in the dissociation of b_7, b_6, b_5 and b_4 ions mass spectra, respectively, which do not reflect any direct and nondirect sequence ions. In contrast, as can be seen in Fig. 1(b), these *m/z* 668, 569, 456 and 309 fragments ions are not detected in the CID mass spectra of the *N*-terminal acetylated Ac-KYAGFLVG peptide.

In order to obtain more insight into the structure of the *m/z* 668 ion, its CID mass spectrum has been recorded (Fig. 2(a)). The fragmentation of *m/z* 668 generates *m/z* 651, 623 and 552 as the major product ions and also generates *m/z* 633, 606, 524, 507, 488 and 439 as the minor ions. The *m/z* 651 ion was observed as the most abundant fragment ion in the CID-MS^4 mass spectrum, and the further isolation (CID-MS^5) of the *m/z* 651 ion have resulted the same CID mass spectra of b_6 ions derived from permuted isomers of YAGFLV-NH_2, whose CID mass spectra have been reported from our group previously.[36,37] The CID mass spectrum of *m/z* 668 fragment from b_7 ion of protonated K_{Ac}YAGFLVG and YAGFLV-NH_2 peptides is given in Fig. 2(a). Comparison of these two CID mass spectra reveals that they have the same fragmentation pathway, which suggests that they have the same peptide sequence. In addition to that, the MS/MS spectrum of $[M+H]^+$ ion produced from YAGFLV-NH_2, AGFLVY-NH_2, GFLVYA-NH_2, FLVYAG-NH_2, LVYAGF-NH_2 and VYAGFL-NH_2 was also recorded (spectra not shown). It was found that only the CID spectrum of protonated YAGFLV-NH_2 shows entirely identical fragment ions to that of the *m/z* 668 from b_7 ion of K_{Ac}YAGFLVG.

The proposed mechanism for the formation of *m/z* 668 fragment ion is depicted in Schemes 1 and 2. Briefly, it is proposed that the b_7 ion of K_{Ac}YAGFLVG could undergo macrocyclization and ring-opening process to form permuted *b* isomer, YAGFLV$K_{Ac\text{-}oxa}$, in which acetylated lysine residue is relocated at the *C*-terminal position (Scheme 1). This new *b* oxazolone isomer eliminates CO (−28 u) to form its corresponding a_7 ion with an iminium structure (Scheme 2). As proposed for the formation of *m/z* 143 and 126 markers ions of ε-*N*-acetylated lysine, nucleophilic attack of the acetylated ε-*N* on the iminium carbon could lead to a cyclic iminium. Subsequent PT could lead to two dissociation pathways; either generation of protonated *C*-terminal amidated hexapeptide YAGFLV-NH_2 (*m/z* 668) or protonated $C_7H_{12}NO$ (*m/z* 126), which in turn could fragment into $C_5H_{10}N$ (*m/z* 84). The breakdown graph of b_7 ion of K_{Ac}YAGFLVG peptide reveals that the branching ratio depends on the applied collison energy (Fig. S1). It is evident that the relative ion intensity of b_7 ion decreases sharply as the collision energy increases. On the other hand, the formation of a_7 (*m/z* 793) and *m/z* 668 fragment ions is favored below 30 eV, whereas their corresponding intensities start to decrease above 30 eV. Conversely, the relative intensities of *m/z* 126 and 84 fragments increase as the collision energy is increased from 30 to 60 eV.

To support proposed mechanism, we further analyzed YAGFLVK_{Ac}-NH_2 heptapeptide where the ε-*N*-acetylated lysine is originally positioned at the *C*-terminal end. The obtained CID-MS^3 mass spectrum of b_7 ion is identical compared with the b_7 ion of K_{Ac}YAGFLVG (Fig. S2). The mass spectra similarities strongly support our proposed mechanism in which the ε-*N*-acetylated lysine is relocated to the *C*-terminal position after macrocyclization/reopening mechanism of b_7 ion.

In a similar way, the *m/z* 569, 456 and 309 ions were isolated from b_6, b_5 and b_4 ions, respectively, and allowed to dissociate under the same experimental conditions in order to derive their gas-phase structures. The resultant mass spectra are given in Fig. 2(b)–(d). The mass spectra entirely show the same fragmentation behavior (same peaks with the identical relative intensities) to that obtained for the dissociation of $[M+H]^+$ ions of commercial *C*-terminal amidated YAGFL-NH_2, YAGF-NH_2 and YAG-NH_2 peptide. As a result, formation of *m/z* 569, 456 and 309 fragments could be rationalized using a reaction mechanism similar to that proposed for *m/z* 668 in Schemes 1 and 2.

These results indicate that the *m/z* 668, 569, 456 and 309 fragment ions are specific for side chain acetylated lysine (K_{Ac}) containing peptides. They are not observed for *N*-terminal acetylated peptide isomer. However, as reported in our early study,[36] nondirect sequence ions have been detected with different relative intensities in the dissociation of each b_7, b_6, b_5 and b_4 ions CID spectra produced from Ac-KYAGFLVG. When the *N*-terminal of the lysine containing peptide was acetylated, the observation of nondirect fragment ions suggested that lysine ε-amine group could attack to the carbonyl carbon of the oxazolone ring (side-to-tail cyclization) to form macrocyclic structure of *b* ion.[36]

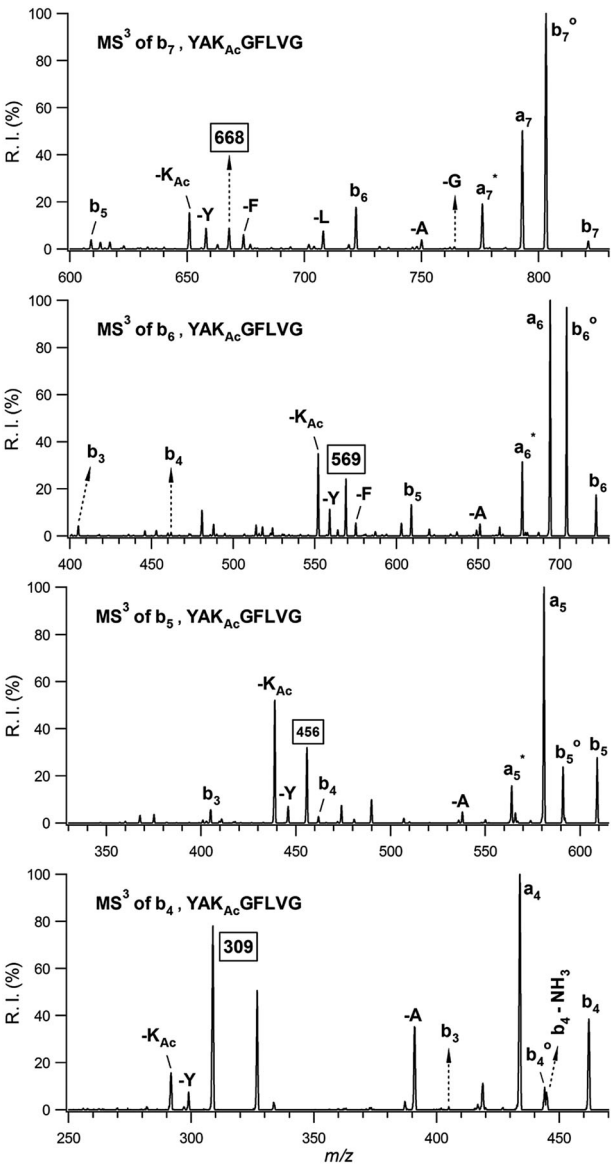


Figure 4. Comparison of the MS^3 mass spectra of b_7, b_6, b_5 and b_4 ions originated from protonated $YAK_{Ac}GFLVG$.

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Fragmentation reactions of b_n ($n = 4–7$) ions originated from Ac-K_{Ac}YAGFLVG and KYAGFLV-NH_2

Doubly acetylated peptide, Ac-K_{Ac}YAGFLVG, in which both of the α-amine of the peptide and ε-amine of lysine residue have been acetylated, was also investigated. As shown in Fig. 3(a), the mentioned characteristic fragment ions were not detected in the corresponding b_n ($n = 4–7$) CID mass spectra. It is apparent that the CID mass spectra of *b* ion series contain only direct sequence *b* ions together with some of their corresponding α ions. Conversely, while both direct and nondirect peptide fragments are observed for unmodified peptide sequence, KYAGFLV-NH_2, the CID mass spectra of the b_n ($n = 4–7$) do not show the presence of the *m/z* 668, 569, 456 and 309 fragment ions (Fig. 3(b)).

In summary, the *m/z* 668, 569, 456 and 309 fragment ions are not detected in the dissociation ion mass spectra of b_n ($n = 4–7$) ions derived from Ac-KYAGFLVG, Ac-K_{Ac}YAGFLVG or KYAGFLV-NH_2 peptide sequence. These results support our proposed mechanism and, in particular, the involvement of free *N*-terminal amine and ε-*N*-acetylated lysine for the formation of characteristic *m/z* 668, 569, 456 and 309 fragment ions.

Positional effect of acetylated lysine residue for the formation of novel fragment ions

In the perspective of probing the effect of the position of the ε-*N*-acetylated lysine, the YAK_{Ac}GFLVG octapeptide was also studied. The CID mass spectra of b_n ($n = 4–7$) ions derived from protonated YAK_{Ac}GFLVG octapeptide are given in Fig. 4. Each mass spectrum contains both direct and nondirect sequence *b* ions. In addition, the *m/z* 668, 569, 456 and 309 fragment ions were also observed for this octapeptide sequence. In contrast, the CID-MS^3 spectra of the b_n ($n = 4–7$) ions of protonated Ac-YAKGFLVG (*N*-terminal acetylated) and Ac-YAK_{Ac}GFLVG (doubly acetylated) octapeptides do not display these *m/z* 668, 569, 456 and 309 characteristic fragment ions (Fig. S3). The CID-MS^4 spectrum of each of these fragment ions were collected in the same CID conditions. As can be seen in Fig. 5 (a), the MS^4 spectrum of *m/z* 668 fragment ion is dominated by the product ions at *m/z* 651, 580 and 417, and ions with weaker intensities at *m/z* 633, 623, 552, 463, 389 and 351 can also be observed. The CID mass spectrum of *m/z* 668 ion originated from the b_7 ion of YAK_{Ac}GFLVG is identical to the MS/MS spectrum of protonated GFLVYA-NH_2 (Fig. 5(a)). Hence, it can be concluded that these two

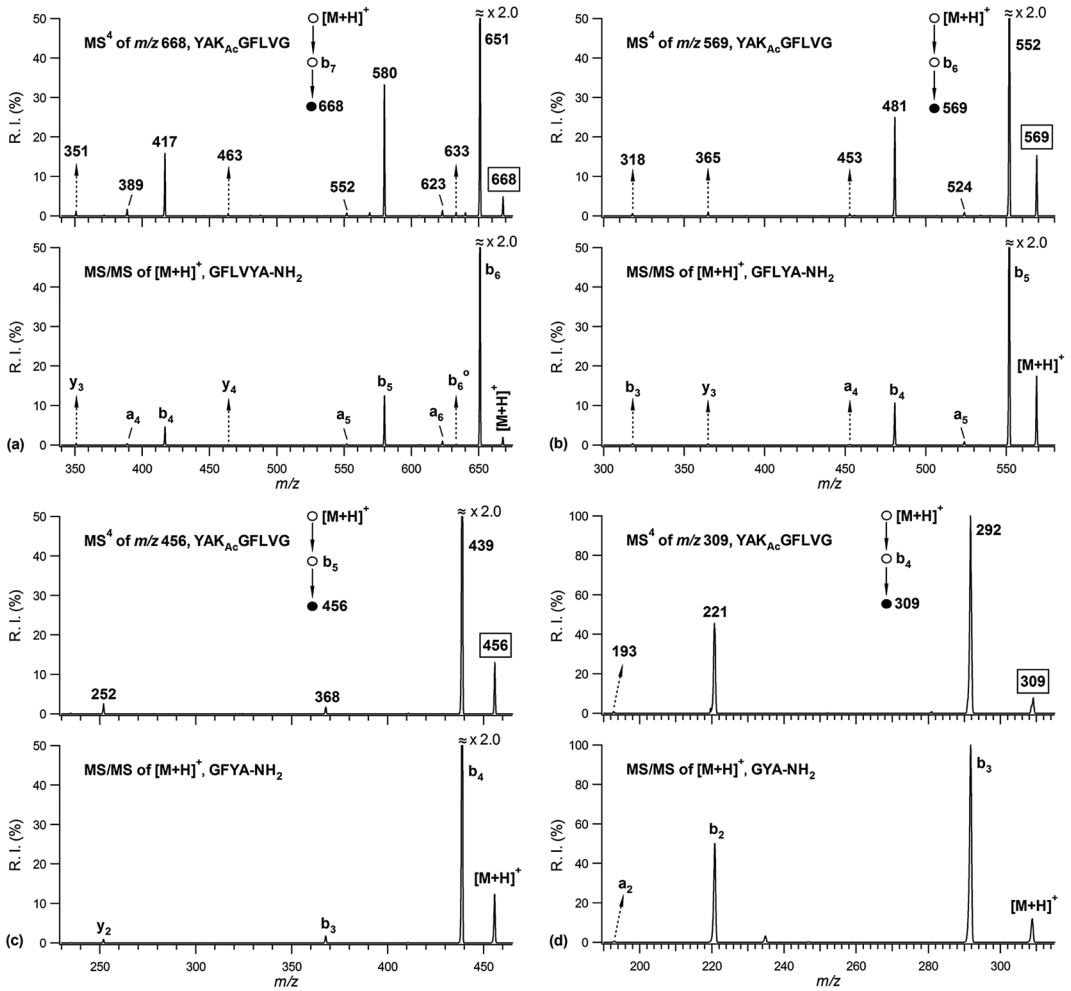

Figure 5. Comparison of (a) MS^4 mass spectrum of *m/z* 668 ion from b_7 ion of protonated YAK_{Ac}GFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated GFLVYA-NH_2, (b) MS^4 mass spectrum of *m/z* 569 ion from b_6 ion of protonated YAK_{Ac}GFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated GFLYA-NH_2, (c) MS^4 mass spectrum of *m/z* 456 ion from b_5 ion of protonated YAK_{Ac}GFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated GFYA-NH_2 and (d) MS^4 mass spectrum of *m/z* 309 ion from b_4 ion of protonated YAK_{Ac}GFLVG and MS/MS mass spectrum of $[M+H]^+$ ion from protonated GYA-NH_2.

ions are structurally identical because they are having the same dissociation pattern in the gas phase.

In line with the proposed mechanism given in Schemes 1 and 2, the GFLVYAK$_{Ac\text{-}oxa}$ isomer may have been formed via macrocyclization and ring-opening process of b_7 ion of the original sequence. Then, this isomer can dissociate to yield the protonated GFLVYA-NH$_2$ (*m/z* 668) in the gas phase. In the same vein, the fragment ions at *m/z* 569, 456 and 309 were isolated from b_6, b_5 and b_4 ion, respectively, and subjected to CID for further fragmentation. The fragmentation behaviors of these ions are virtually identical to the fragmentation products of $[M+H]^+$ ions obtained from GFLYA-NH$_2$, GFYA-NH$_2$ and GYA-NH$_2$ (Fig. 5(b)–(d)). The proposed mechanism leading to the formation of these ions involves the cyclization and subsequent ring-opening of b_6, b_5 and b_4 ions separately, in which acetylated lysine residue was position at the *C*-terminal in each case.

In addition, it seems that the fragmentation chemistry depicted in Scheme 2 also occurs with the b_3 ion of protonated YAK$_{Ac}$GFLVG peptide. Indeed, as illustrated in Fig. 6, the mass spectrum of this b_3 ion contains a fragment ion at *m/z* 252. In order to challenge this hypothesis, the CID-MS^4 mass spectrum of the *m/z* 252 ion is compared with the CID mass spectrum of protonated YA-NH$_2$ dipeptide (see last panel of Fig. 6). The *C*-terminal amidated dipeptide sequence was verified as protonated $[M+H]^+$ ion of commercial YA-NH$_2$. Interestingly, the CID mass spectrum of b_3 ion originated from K$_{Ac}$YAGFLVG did not show any ion signal that corresponds to rearranged protonated fragment ion, YA-NH$_2$ (Fig. S4).

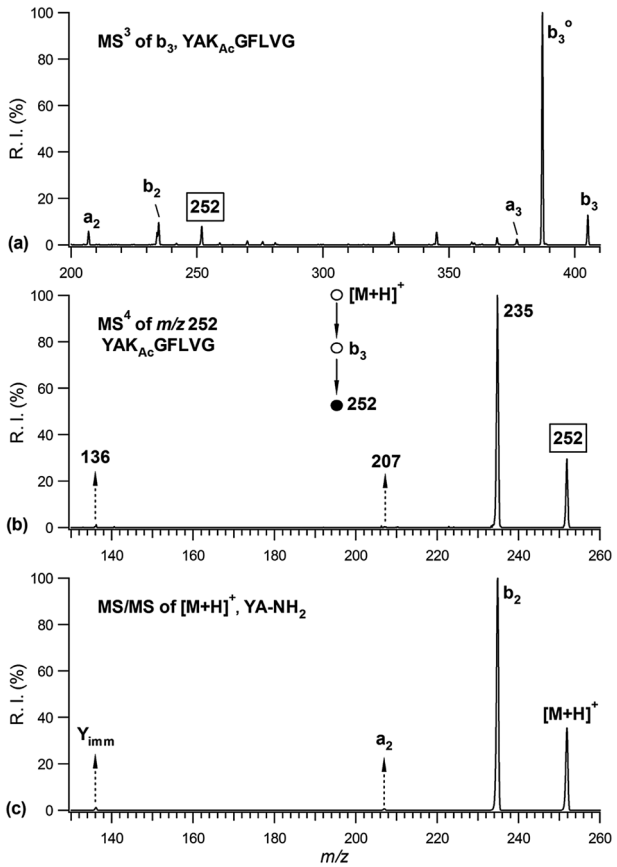

Figure 6. (a) MS^3 mass spectrum of b_3 ion from protonated YAK$_{Ac}$GFLVG, (b) MS^4 mass spectrum of *m/z* 252 ion from b_3 ion of protonated YAK$_{Ac}$GFLVG and (c) MS/MS mass spectrum of $[M+H]^+$ ion from protonated YA-NH$_2$.

Conclusion

Specific rearrangement chemistry has been evidenced in the case of ε-*N*-acetylated lysine containing peptides, which could be useful for the characterization of one of the most prominent post-translational modification of proteins. Marker ions of this specific rearrangement have been identified in the MS^3 low-energy CID mass spectra series of b_n ($n=4$–7) ions of ε-*N*-acetylated lysine peptide case, K$_{Ac}$YAGFLVG. These ions appear for the ε-*N*-acetylated lysine peptide, but not for the corresponding *N*-terminal acetylated peptide, doubly acetylated peptide or non-acetylated peptide. Similar conclusions could be drawn for the YAK$_{Ac}$GFLVG model peptide, i.e. when changing the position of the lysine.

More insights into the mechanism of the specific rearrangement of the ε-*N*-acetylated lysine containing peptides could be drawn based on the one-to-one similarity of the MS^4-CID spectra of the marker ions and the CID mass spectra of selected *C*-terminal amidated peptides. The one-to-one similarity between the CID spectra of the two sets of CID mass spectra strongly suggests that each marker ion is the result of a rearrangement chemistry starting with the formation of a macrocyclic isomer resulting from the head-to-tail macrocyclization of the corresponding b_n ($n=4$–7) ion of the ε-*N*-acetylated lysine containing peptide. Following PT, the marker ions could be formed following the ring opening at the amide bond on the lysine *C*-terminal side, leading to an oxazolone structure with the K$_{Ac}$ at the *C*-terminus. Following the loss of the CO leading to the corresponding *a* ion, the marker ion is proposed to be the result of the loss of the acetyllysine imine.

Acknowledgements

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