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RESEARCH ARTICLE

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Gas-phase fragmentation reactions of a_7 ions containing a glutamine residue

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Abstract

The gas-phase fragmentation reactions of the a_7 ions derived from glutamine (Q) containing model heptapeptides have been studied in detail with low-energy collision-induced dissociation (CID) tandem mass spectrometry (MS/MS). Specifically, the positional effect of the Q residue has been investigated on the fragmentation reactions of a_7 ions. The study involves two sets of permuted isomers of the Q containing model heptapeptides. The first set contains the QAAAAAA sequence, and the second set involves of QYAGFLV sequence, where the position of the Q residue is changed from N- to C-terminal gradually for both peptide series. An intense loss of ammonia from the *a*7 ions followed by internal amino acid eliminations strongly supports forming the imine-amides structure via cyclization/rearrangement reaction for all studied a_7 ions. This is in agreement with the pioneering study reported by Bythell et al. (2010, 10.1021/ja101556g). A novel rearrangement reaction is detected upon fragmentation of imine-amide structure, which yields a protonated C-terminal amidated hexapeptide excluding the Q residue. A possible fragmentation mechanism was proposed to form the protonated C-terminal amidated hexapeptide, assisted via nucleophilic attack of the side chain amide nitrogen of the Q residue on its N-protonated imine carbon atom of the rearranged imine-amide structure.

Highlights

- The gas-phase fragmentation reactions of a_7 ions obtained from protonated model peptides containing glutamine residue were studied by ESI-MS/MS.
- A rearranged imine-amide structure is the predominant even for a_7 ions.
- Novel rearrangement reaction is observed which forms a protonated C-terminal amidated hexapeptide excluding Q residue upon fragmentation of the imine-amide structure.

KEYWORDS
a_7 ion, CID, glutamine, imine-amide structure, peptide fragmentation, tandem mass spectrometry

Abbreviations: CID, collision-induced dissociation; MS/MS, tandem mass spectrometry; Q, glutamine.

1 | INTRODUCTION

Understanding the gas-phase fragmentation reaction mechanisms of ions produced from peptides is necessary to provide correct amino acid sequences for high-throughput proteomic studies.[1] Upon low-energy collision-induced dissociation (CID), the fragmentation occurs along with protonated amide bonds via a charge-directed pathway. As a consequence of backbone cleavage, sequence informative N-terminal *b*- and C-terminal *y*- type ions[2] are formed. These fragment ions are used to derive the primary peptide structure by using database search algorithms and bioinformatics tools.[3–5] In the last two decades, most of the attention was focused on the elucidation of gas-phase structures and the reactivities of *b* ions.[6–12] Earlier reports[6,7] demonstrated that a protonated five-membered oxazolone ring at the C-terminal end of the peptide is the most stable structure for many small *b* ions, and this structure has been confirmed by numerous density-functional theories (DFT) and infrared multiphoton dissociation (IRMPD) spectroscopies.[11,13–16] For larger *b*n ($n \geq 5$) ions, a protonated macrocyclic structure has been proposed through an intramolecular head-to-tail cyclization reaction that re-opens at different amide bonds to form permuted *b* ion isomers.[17–21] IRMPD studies have provided strong evidence for forming macrocyclic structures in the gas phase.[22–24] Previous studies have been conducted to investigate the influences of different parameters (i.e., peptide length, amino acid type, and side-chain functionality) on the formation of macrocyclic structures.[23,25–33]

Recently, much more interest has been devoted to the gas-phase structures and fragmentation reactions of peptide ions.[34–36] a_n ions are formed either via CO loss (−28 Da) from their corresponding b_n ions[6,7] or directly from protonated peptides.[37,38] The structure of the a_n ions was initially proposed as a linear peptide with an imine-protonated (—HN+═CHR) at the C-terminal end.[37–39] However, recent extensive studies have shown that *a*n ($n = 2–5$) ions can adopt acyclic and/or a linear rearranged imine-amide structure(s) prior to fragmentation.[13,35,36,40–48] For instance, IRMPD and DFT results have revealed that the a_2 ions of GGG (triglycine) adopt a cyclic N1-protonated 4-imidazolidinone structure in the gas phase in a lower energy state than its linear imine-protonated isomer.[34,40,42] Moreover, Good et al.[44] provided statistical analysis data for many high-energy CID mass spectra, which supports the cyclic nature of the a_2 ions. On the contrary, most of the a_3 ions are not observed in the CID mass spectra of protonated peptides. This aspect was explained by facile fragmentation into the a_3^* (a_3—NH_3) or the b_2 ions.[49,50] However, Bythell et al.[34] highlighted that a_3 ions derived from protonated GGGG (tetraglycine) are stable with a seven-membered ring structure formed via nucleophilic attack to carbonyl oxygen of the N-terminal glycine residue on the C-terminal imine carbon atom.

Additionally, detailed analysis of IRMPD spectroscopy and DFT calculations have shown that the rearranged linear imine-amide structure is energetically the most favorable form for the majority of a_4 ions.[34,35,43,51] The intermediate macrocyclic isomer is formed first via nucleophilic attack of the N-terminal amino group on the carbon atom of C-terminal imine. After a proton transfer, the cyclic structure is dissociated to form a series of rearranged linear isomers with N-terminal imine and a C-terminal amide functionality. This structure quickly eliminates ammonia (—NH_3) from its C-terminal end to generate its corresponding a^* ion.[52–54] Moreover, Yalcin et al.[7] and Harrison and Young[55] have reported that the a_n ions with C-terminal amine groups can dissociate to form next-lower b_n ions (viz., b_{n-1}). More recently, Harrison[36] has examined the fragmentation reactions of a_n ($n = 4–8$) ion-containing alanine residues solely and named the fragmentation reactions of ions as either "amide pathway" or "imine pathway."

The possible effects in the presence of proline (P) and methionine (M) residue and their position on the fragmentation reactions of ions have been studied in detail by Harrison.[47,48] Here, we examine the potential effects of glutamine (Q) residue and its position within the permuted model heptapeptides on the fragmentation reactions of the a_7 ions.

2 | EXPERIMENTAL

2.1 | Sample preparation

All C-terminal amidated synthetic model peptides (QAAAAAA-NH_2, AQAAAAA-NH_2, AAQAAAA-NH_2, AAAQAAA-NH_2, AAAAQAA-NH_2, AAAAAQA-NH_2, AAAAAAQ-NH_2, QYAGFLV-NH_2, YQAGFLV-NH_2, YAQGFLV-NH_2, YAGQFLV-NH_2, YAGFQLV-NH_2, YAGFLQV-NH_2, YAGFLVQ-NH_2, AAAAAA-NH_2, AAAAA-NH_2, AAAA-NH_2, YAGFLV-NH_2, AGFLVY-NH_2, GFLVYA-NH_2, FLVYAG-NH_2, LVYAGF-NH_2, and VYAGFL-NH_2) were purchased from GL Biochem Ltd. (Shanghai, China) and used as received. Approximately 2 mg of each peptide was dissolved in a mixture of HPLC-grade methanol (MeOH) and ultra-pure water (1:1, v/v) to make up stock solutions with a concentration of 10^{-3} M. The resulting peptide solutions were stored at −20°C until use.

2.2 | Mass spectrometry

The low-energy tandem mass spectrometry (MS/MS) experiments were conducted on an LTQ XL linear ion-trap (Thermo Finnigan, San Jose, CA) equipped with an ESI source. Peptide solutions of 100 μM were prepared in 50:50:1 (v/v/v) MeOH/H2O/HCOOH and were infused directly into the mass spectrometer at a flow rate of 5 μl min^{-1} using an incorporated syringe pump. Before experiments, the instrument was calibrated with a Calmix solution containing caffeine, MRFA, and Ultramark 1,621. The ESI needle was held at +5 kV throughout the analysis, and the capillary temperature was set to 300°C. Nitrogen was used as the sheath, sweep, and auxiliary gas with a flow rate of 10, 1, and

1 (all arbitrary units), respectively, while helium was introduced as a collision gas into the trap to perform CID. The normalized collision energy was set at between 18–26% (arbitrary units) to dissociate selected precursor ions. The isolation width was set between 1.0–1.8 Da with an activation (q) of 0.250 and a 30 ms activation time at each CID stage. All MS/MS spectra were acquired in the positive ion profile mode, and at least 400 scans were averaged for the best representation. Data acquisition was performed using the Xcalibur™ (version 2.0) software data system (Thermo Fisher Scientific). The collected data were further analyzed with the Igor Pro software package (WaveMetrics, Lake Oswego, OR).

3 | RESULTS AND DISCUSSIONS

3.1 | Fragmentation reactions of the a_7 ions derived from model heptapeptides containing six alanine residues and one glutamine residue

In the first part of the study, C-terminal amidated model heptapeptides containing six alanine (A) residues and one glutamine (Q) residue (QAAAAAA-NH_2, AQAAAAA-NH_2, AAQAAAA-NH_2, AAAQAAA-NH_2, AAAAQAA-NH_2, AAAAAQA-NH_2, and AAAAAAQ-NH_2) have been used to examine the position of the Q residue and also effects of the Q residue on the fragmentation reactions of the a_7

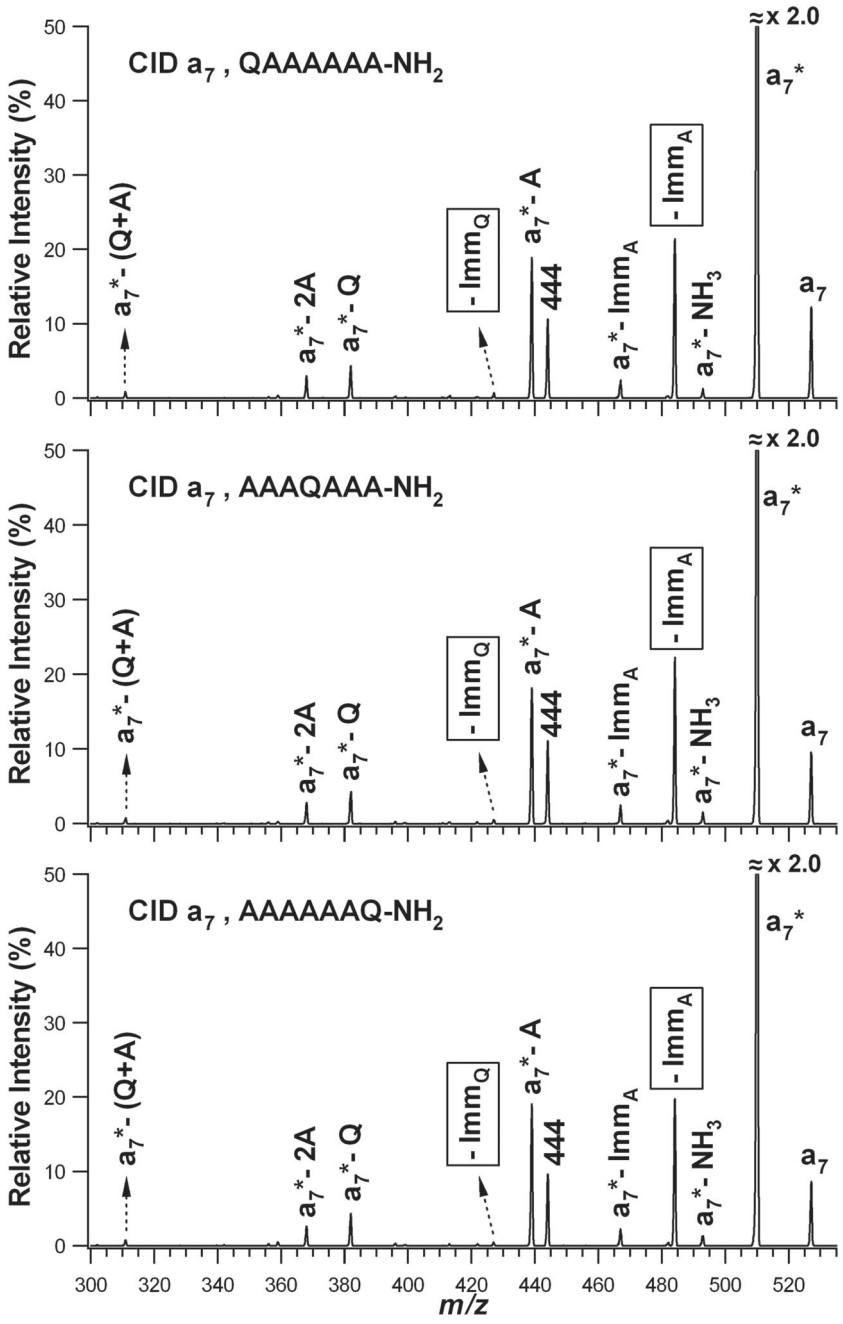


FIGURE 1 Comparison of the CID mass spectra of the a_7 ions (m/z 527) from QAAAAAA-NH_2, AAAQAAA-NH_2, and AAAAAAQ-NH_2

ions. The model peptides were designed as a C-terminal amide form rather than as free acid to obtain the b_7 ion and its corresponding a_7 ion with high intensity. The a_7 ions were isolated [MS4 ([M + H]$^+ \rightarrow b_7 \rightarrow a_7$)] and allowed to dissociate under low-energy CID. For simplicity, only the comparison of mass spectra for the a_7 ions from QAAAAAA-NH_2, AAAQAAA-NH_2, and AAAAAAQ-NH_2 is

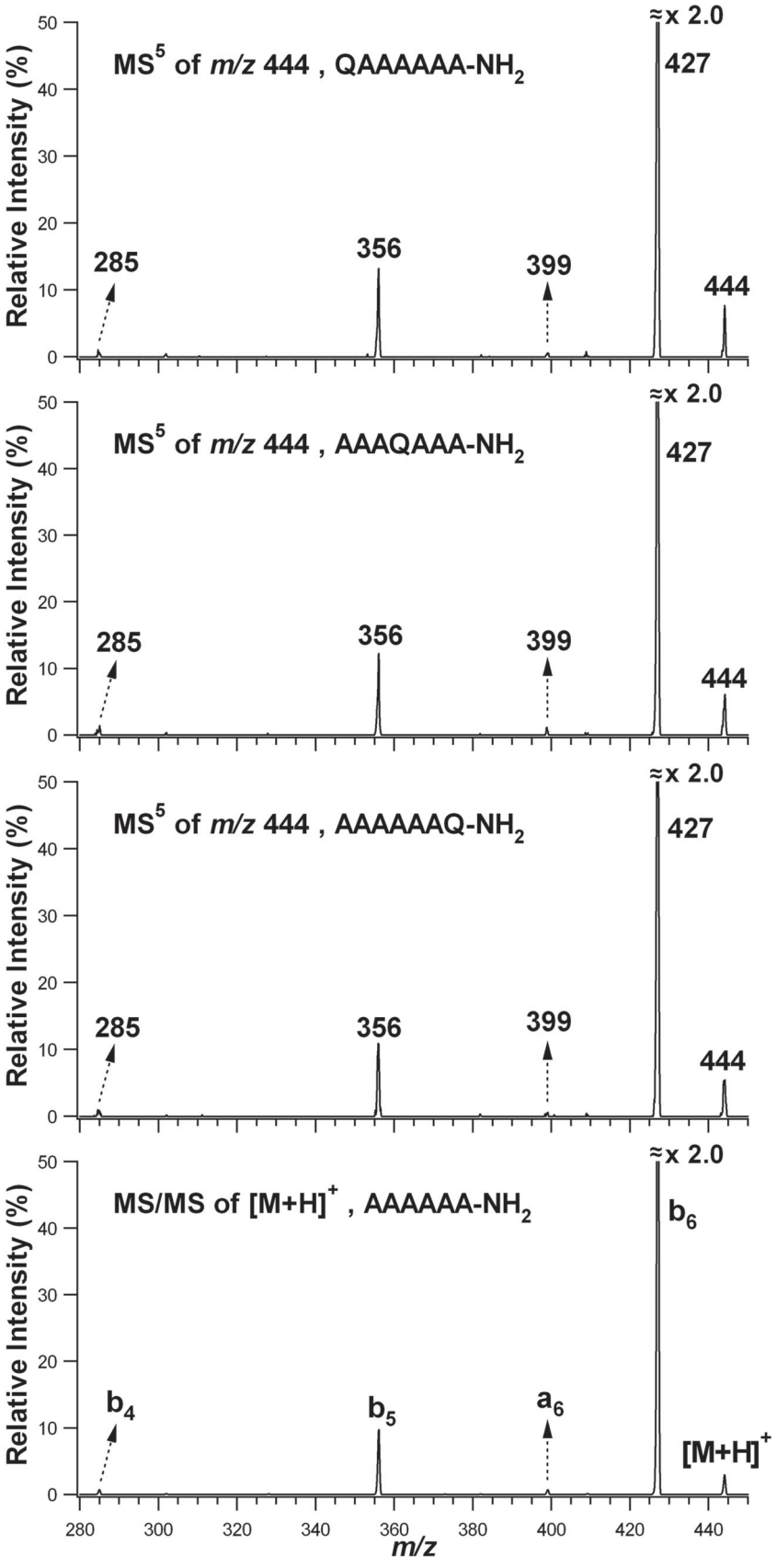
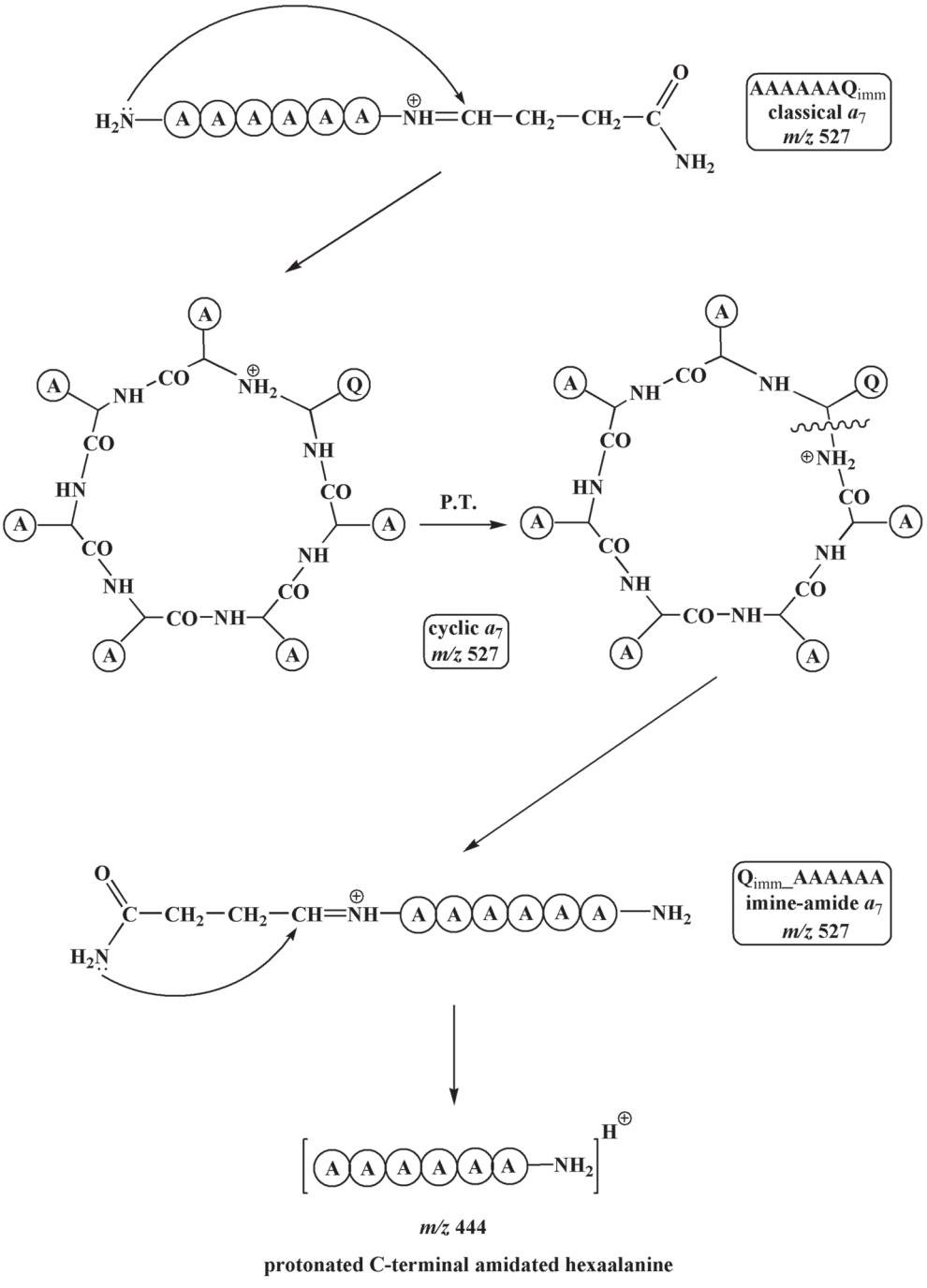


FIGURE 2 Comparison of the CID-MS^5 mass spectra of *m/z* 444 ions from QAAAAAA-NH_2, AAAQAAA-NH_2, and AAAAAAQ-NH_2 with MS/MS spectrum of [M + H]$^+$ ion (*m/z* 444) from AAAAAA-NH_2

shown in Figure 1 (the CID mass spectra of the a_7 ions obtained from seven isomeric heptapeptides are illustrated in the supporting information Figure S1). As displayed in Figure 1, the CID mass spectra of the a_7 ions (m/z 527) obtained from these three peptides are virtually identical and have the same fragment ions with the same relative intensities. Additionally, the CID mass spectra for the a_7 ions derived from seven isomeric heptapeptides containing the Q residue are indistinguishable (see Figure S1). This behavior can be explained by the full macrocyclization of their initial precursor b_7 ions (m/z 555) regardless of the Q position. CID-MS^3 mass spectra of the b_7 ions produced from QAAAAAA-NH_2, AAAQAAA-NH_2, and AAAAAAQ-NH_2 were also acquired, and their comparative spectra are shown in Figure S2. The CID mass spectra are completely identical, regardless of the original sequence. This finding provides evidence for macrocyclization followed by subsequent ring openings to generate the same mixture of protonated oxazolones. It has been noted here that the relative intensity arising from the loss of the Q residue from the b_7 ion (b_7—Q, m/z 427) is the most dominant peak in all three mass spectra. This observation clearly shows that the b_7 ions tend to place the Q residue at the C-terminal position of the protonated oxazolones before the fragmentation.

As illustrated in Figure 1, the a_7 ion (m/z 527) predominantly fragments to the a_7* ion (m/z 510) through an ammonia loss (−17 Da) which appears to be the most abundant fragment among other ions. It



SCHEME 1 The proposed mechanism for the formation of m/z 444 fragment from imine-amide a_7 ions derived from six alanines and one glutamine containing model heptapeptides

has been previously reported that an intense ammonia loss strongly indicates the formation of an imine-amide isomer in the gas phase.[34,43] The authors provided that an imine-amide structure for the a_4 ion is energetically more favorable than its classical linear N-protonated imine isomer by DFT calculation.[34,43] In line with the proposed fragmentation mechanism for the a_4 ion, the a_7 ion with an imine-amide structure eliminates ammonia from its C-terminal end to form the a_7* ion, which is followed by sequential amino acid losses (a_7*—A (*m/z* 439), a_7*—2A (*m/z* 368), a_7*—Q (*m/z* 382), and a_7*—(Q + A) (*m/z* 311)). Detection of these fragment ions demonstrates that the a_7 ions mainly adopt an imine-amide structure under the applied CID condition.

On the contrary, a minor population of the a_7 ions adopt a classical linear C-terminal imine-protonated isomer, and this structure leads to the loss of A immonium (—Imm_A, *m/z* 484) and Q immonium (—Imm_Q, *m/z* 427), as illustrated in Figure 1. The relative intensity of the former ion is much greater than the latter, which ultimately supports the migration of the Imm_Q to the N-terminal of the a_7 ion via cyclization to generate a rearranged imine-amide structure. Additionally, a second ammonia loss from the a_7 ion (a_7*—NH_3, *m/z* 493) was also observed with minor intensity, which might be due to the loss of the side chain of the Q residue.[1]

In addition to these eliminations, *m/z* 444 fragment ions were detected in three CID mass spectra of the a_7 ions, which do not match any common fragment masses (see Figure 1). CID-MS5 ($[M + H]^+ \rightarrow b_7 \rightarrow a_7 \rightarrow m/z$ 444) consecutive experiments were conducted to elucidate the gas-phase structure and its fragmentation pattern. The ions dominate the CID mass spectrum at *m/z* 427 and

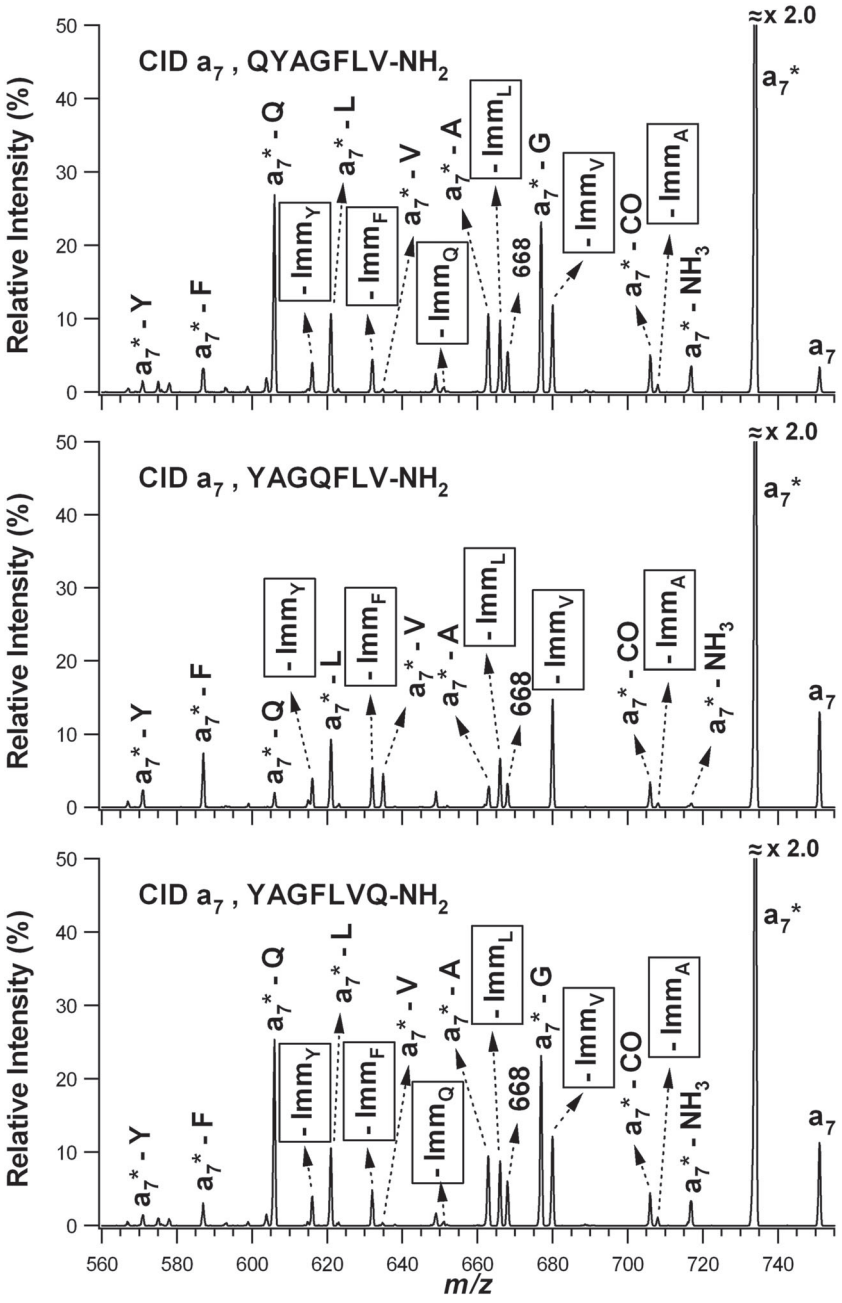


FIGURE 3 Comparison of the CID mass spectra of the a_7 ions (*m/z* 751) from QYAGFLV-NH_2, YAGQFLV-NH_2, and YAGFLVQ-NH_2

356 along with weak intensities of *m/z* 399 and 285 ions shown in Figure 2. Based on our knowledge of peptide fragmentation chemistry, we assume that these fragment ions represent the fragmentation chemistry of a protonated C-terminal amidated hexaalanine. Therefore, a commercial AAAAAA-NH_2 was purchased, and the MS/MS spectrum of its molecular ion was recorded. The CID mass spectra of *m/z* 444 ions obtained from the a_7 ions are entirely identical to the MS/MS spectrum of protonated AAAAAA-NH_2. As depicted in Scheme 1, a possible mechanism for forming a protonated C-terminal amidated hexaalanine was proposed. Briefly, the b_7 ion undergoes macrocyclization/ring-opening reactions to locate the Q residue at the C-terminal position. The a_7 ion with Q_{imm} group at the C-terminal end is formed (a classical a_7 ion) with subsequent CO loss (−28 Da). Then, a nucleophilic attack of the N-terminal nitrogen atom on the carbon atom of the protonated C-terminal imine group forms a cyclic a_7 ion. This cyclic structure can re-open after proton transfer (P.T.) to generate a rearranged linear a_7 isomer with an imine group at the N-terminal and amide group at the C-terminal (imine-amide structure). Finally, a nucleophilic attack of the side chain amide nitrogen of the Q residue on its N-protonated imine carbon atom leads to forming a protonated AAAAAA-NH_2 (see Scheme 1).

Moreover, the a_6 ions derived from QA_6, AQA_5, A_2QA_4, A_3QA_3, A_4QA_2, and A_5QA (all C-terminal amidated) peptides are entirely identical (see Figure S3). As proposed for the a_7 ions, the *a6* ions also

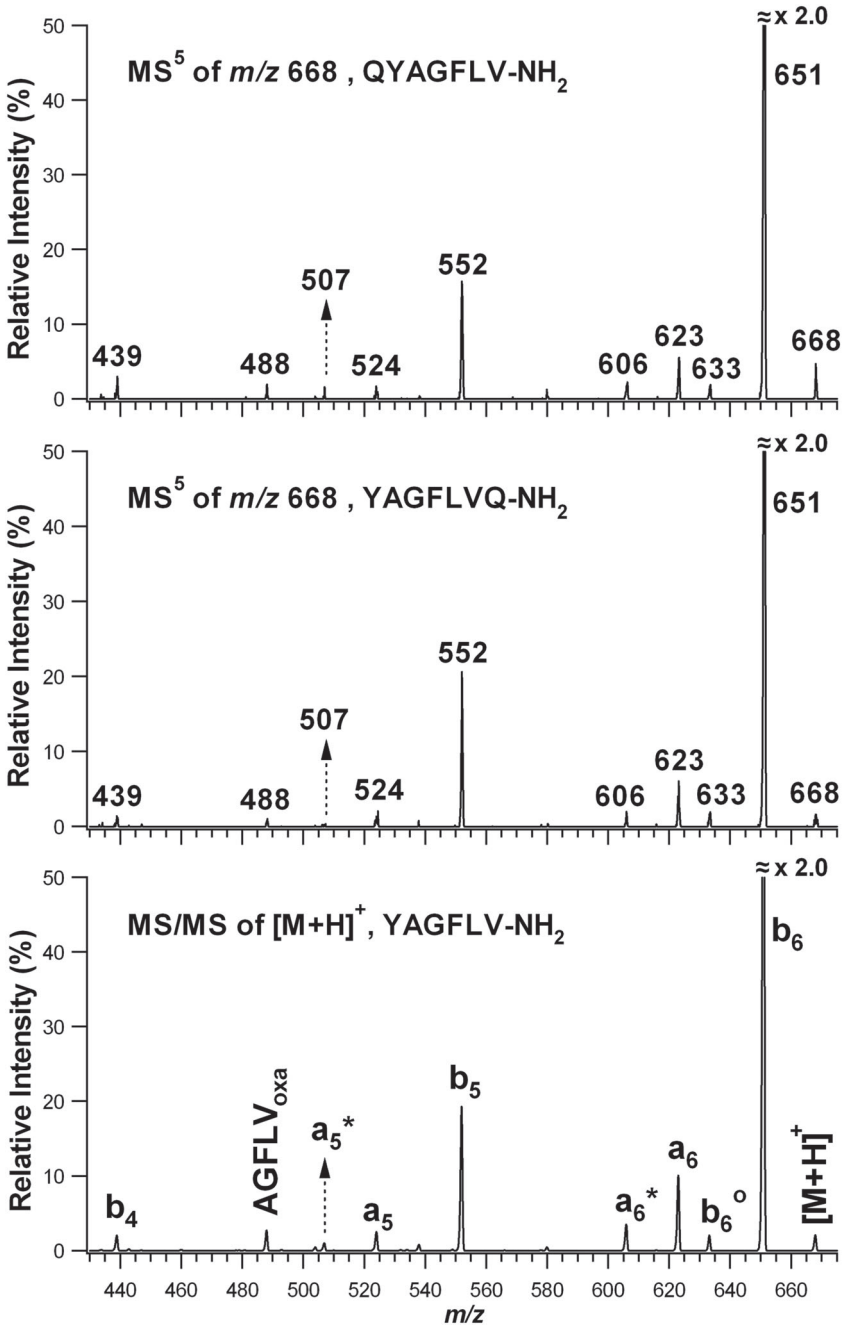
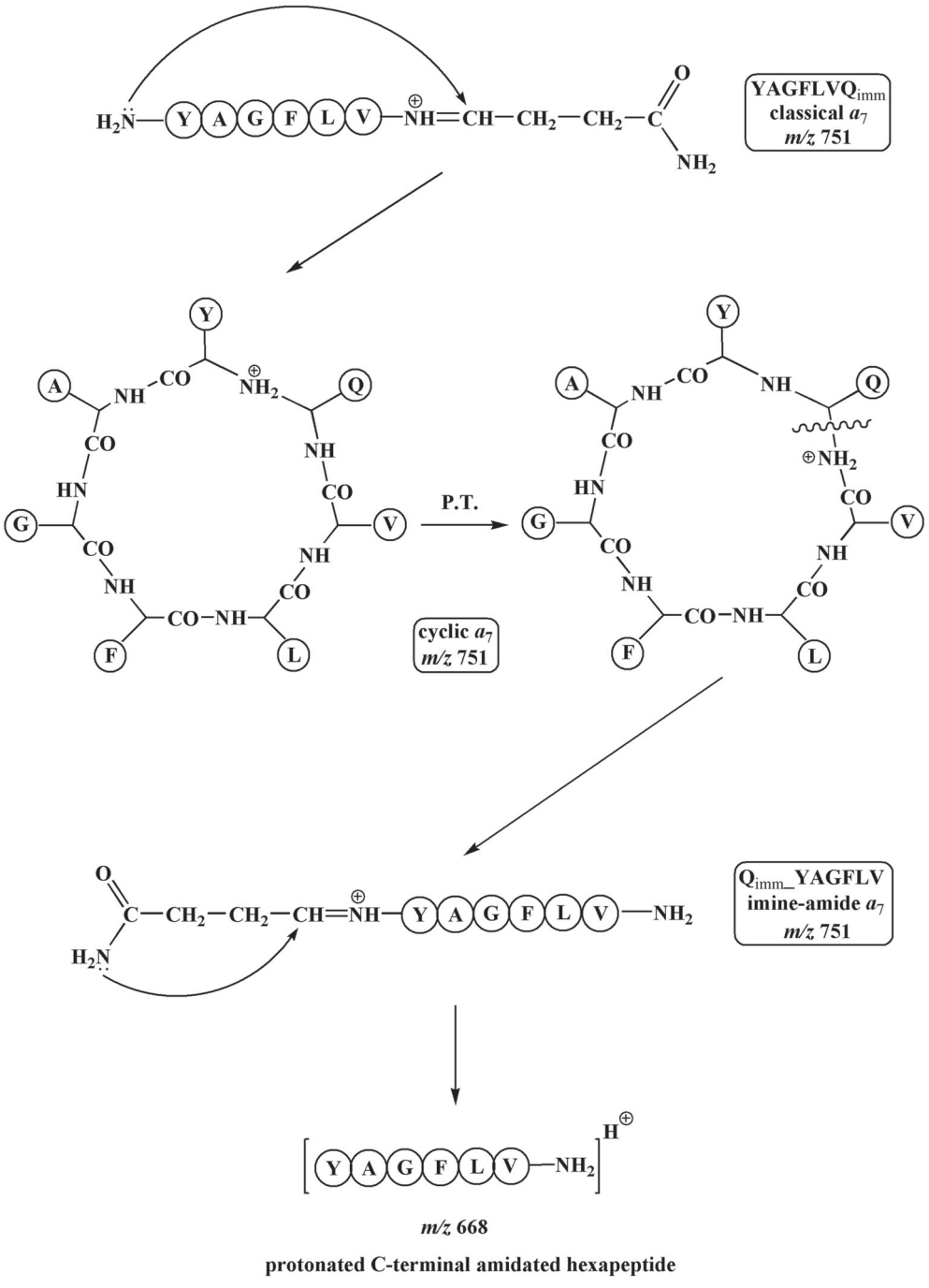


FIGURE 4 Comparison of the CID-MS^5 mass spectra of *m/z* 668 ions from QYAGFLV-NH_2, YAGFLVQ-NH_2 with MS/MS mass spectrum of $[M + H]^+$ ion (*m/z* 668) from YAGFLV-NH_2

adopt a linear rearranged imine-amide structure in the gas phase, which leads to the formation of the a_6* ion as a base peak followed by sequential amino acid losses. *m/z* 373 was detected in the CID mass spectra corresponding to the protonated C-terminal amidated pentaalanine (AAAAA-NH_2) (MS/MS data not shown). Additionally, the CID mass spectra of the a_5 ions obtained from QA_6, AQA_5, A_2QA_4, A_3QA_3, and A_4QA_2 (all C-terminal amidated) peptides are virtually identical (see Figure S4). The mass spectra contain *m/z* 302 fragment ions, reflecting the protonated C-terminal amidated tetraalanine (AAAA-N_{H2}) (MS/MS data not shown). The formation of the *m/z* 373 and 302 fragment ions from CID mass spectra of the a_6 and a_5, respectively, can be rationalized using a similar mechanism proposed for *m/z* 444. Overall, these results provide strong evidence for the formation of an imine-amide structure for most of the a_7, a_6, and *a*5 ions for six A residues and one Q residue containing model peptides.

To better understand the possible effects of different peptide sequences on the novel fragmentation reaction (i.e., formation of a C-terminal amidated hexapeptide excluding the Q residue), YAGFLV peptide series were used, and the obtained results were discussed in the following section.



SCHEME 2 The proposed mechanism for the formation of *m/z* 668 fragment from imine-amide a_7 ions derived from QYAGFLV-NH_2 and YAGFLVQ-NH_2 heptapeptides

3.2 | Fragmentation reactions of the a_7 ions derived from glutamine containing YAGFLV model heptapeptide series

In the second part of the study, YAGFLV peptide series (Y, Tyrosine; A, Alanine; G, Glycine; F, Phenylalanine; L, Leucine; and V, Valine) containing the Q residue at seven different positions have been used. The study focused on seven isomeric heptapeptides, namely, QYAGFLV-NH_2, YQAGFLV-NH_2, YAQGFLV-NH_2, YAGQFLV-NH_2, YAGFQLV-NH_2, YAGFLQV-NH_2, and YAGFLVQ-NH_2. The peptides were designed with C-terminal amide functionality to obtain the b_7 and a_7 ions with the highest intensity. For each peptide, a_7 ions (m/z 751) were selected individually through MS4 ($[M + H]^+ \rightarrow b_7 \rightarrow a_7$) consecutive stages and allowed to dissociate under low-energy CID. For comparison, CID mass spectra of the a_7 ions generated from QYAGFLV-NH_2, YAGQFLV-NH_2, and YAGFLVQ-NH_2 are shown in Figure 3. All three mass spectra show an intense loss of NH3 (−17 Da) to produce the a_7* ion (m/z 734) followed by internal amino acid losses. These eliminations provide a strong indication for the formation of imine-amide structures for the studied a_7 ions.

The CID mass spectra of the a_7 ions derived from QYAGFLV-NH_2 and YAGFLVQ-NH_2 entirely reflect the same fragmentation behavior, which demonstrates that the generated a_7 ions adopt the same gas-phase structures. As Figure 3 illustrates, internal amino acid losses from the a_7* ions were detected, such as a_7*—G (m/z 677), a_7*—A (m/z 663), a_7*—V (m/z 635), a_7*—L (m/z 621), a_7*—Q (m/z 606), a_7*—F (m/z 587), and a_7*—Y (m/z 571). Additionally, C-terminal immonium ion losses from the a_7 ion were also observed, such as —Imm_A (m/z 708), —Imm_V (m/z 680), —Imm_L (m/z 666), —Imm_Q (m/z 651), —Imm_F (m/z 632), and —Imm_Y (m/z 616). These eliminations (i.e., a_7*—X and a_7—Imm_X) provide evidence that a rearranged imine-amide and a classical C-terminal imine isomer co-exist while the former structure is the major isomer based on the formation of an abundant a_7* ion. However, it should be noted here that the relative percentage population for each isomer cannot be predicted based on the collected ion-trap data.

Moreover, m/z 668 fragment was detected in the CID mass spectra of the a_7 ions derived from QYAGFLV-NH_2 and YAGFLVQ-NH_2. In line with the first set of peptide series, we assumed that this fragment might have the structure of a C-terminal amidated hexapeptide excluding the Q residue. In order to verify its gas-phase structure, m/z 668 fragment was further selected via MS^5 ($[M + H]^+ \rightarrow b_7 \rightarrow a_7 \rightarrow m/z$ 668) consecutive stages from each peptide, and the obtained mass spectra were compared with MS/MS spectra of the $[M + H]^+$ ion of YAGFLV-NH_2. Figure 4 clearly showed that the MS^5-CID spectra of m/z 668 ions are identical to the MS/MS spectrum of commercial YAGFLV-NH_2 peptide (the same fragment ions as well as very similar relative intensities). Due to the inconsistency with Scheme 1, a possible mechanism was proposed to rationalize the formation of a protonated C-terminal amidated hexapeptide (see Scheme 2). The b_7 ions of QYAGFLV-NH_2 and YAGFLVQ-NH_2 undergo macrocyclization/ring-opening reactions to generate YAGFLVQ_{oxa} isomers where the Q residue is positioned at the C-terminal end. This b_7 isomer can quickly eliminate CO (−28 Da) to form its corresponding a_7 ion with Q_{imm} at the C-terminal

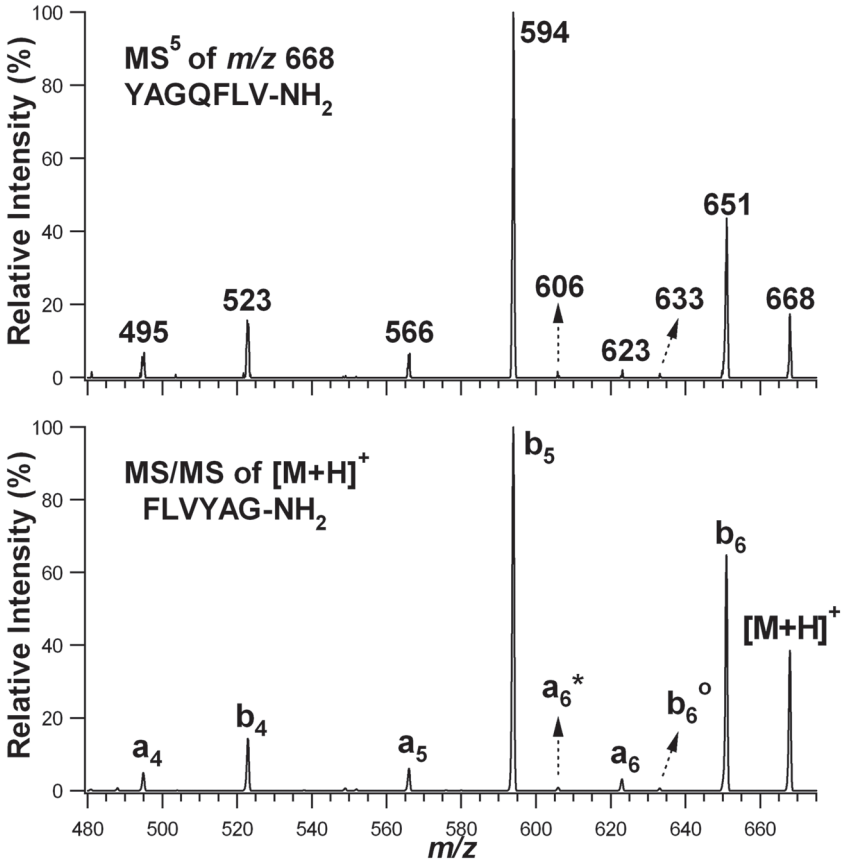


FIGURE 5 Comparison of the CID-MS^5 mass spectrum of m/z 668 ion from YAGQFLV-NH_2 with MS/MS mass spectrum of $[M + H]^+$ ion (m/z 668) from FLVYAG-NH_2

end. Because of cyclization followed by ring-opening chemistry of the a_7 ion, Q_{imm} moiety is transferred to the N-terminal of the peptide to generate a rearranged linear imine-amide isomer. Finally, amide nitrogen of the Q residue attacked its N-protonated imine carbon atom to form a protonated YAGFLV-NH_2 (Scheme 2) peptide ion.

In the case of the a_7 ion obtained from YAGQFLV-NH_2 (see the middle panel of Figure 3), *m/z* 668 fragments were also detected in addition to the internal amino acid losses from the a_7* ion (−Y, −F, −Q, −L, −V, and −A) and C-terminal immonium ion losses from the a_7 ion (−Y, −F, −L, −V, and −A). These losses demonstrate that imine-amide isomers and classical C-terminal imine isomers co-exist under the applied CID conditions. The MS^5-CID mass spectrum of *m/z* 668 was compared with the MS/MS spectrum commercial FLVYAG-NH_2 peptide (see Figure 5). Both spectra are virtually identical, which provides evidence for the formation of a protonated hexapeptide excluding the Q residue via fragmentation of imine-amide a_7 isomer. In a similar fragmentation mechanism proposed in Scheme 2, a sequence scrambling of the b_7 ion results in the formation of the FLVYAGQ_{oxa} isomer, which then loses —CO to generate its corresponding a_7 ion. Afterward, Q_{imm} is positioned to the N-terminal of the a_7 ion via cyclization/re-opening chemistry to form imine-amide isomers. Following the nucleophilic attack of the amide nitrogen of the Q residue to the imine carbon atom, a protonated FLVYAG-NH_2 peptide molecular ion is formed.

3.2.1 | YQAGFLV-NH_2 and YAQGFLV-NH_2

The MS^4-CID mass spectra of the a_7 ions originated from YQAGFLV-NH_2 and YAQGFLV-NH_2 are presented in Figure 6A,B, respectively. As expected, both a_7*—X (where X is Y, F, Q, L, V, or A) and a_7—Imm_X (where X is Y, F, L, V, or A) eliminations were observed with different relative intensities. Specifically, observation of the a_7*—X losses strongly supports the formation of an imine-amide structure where

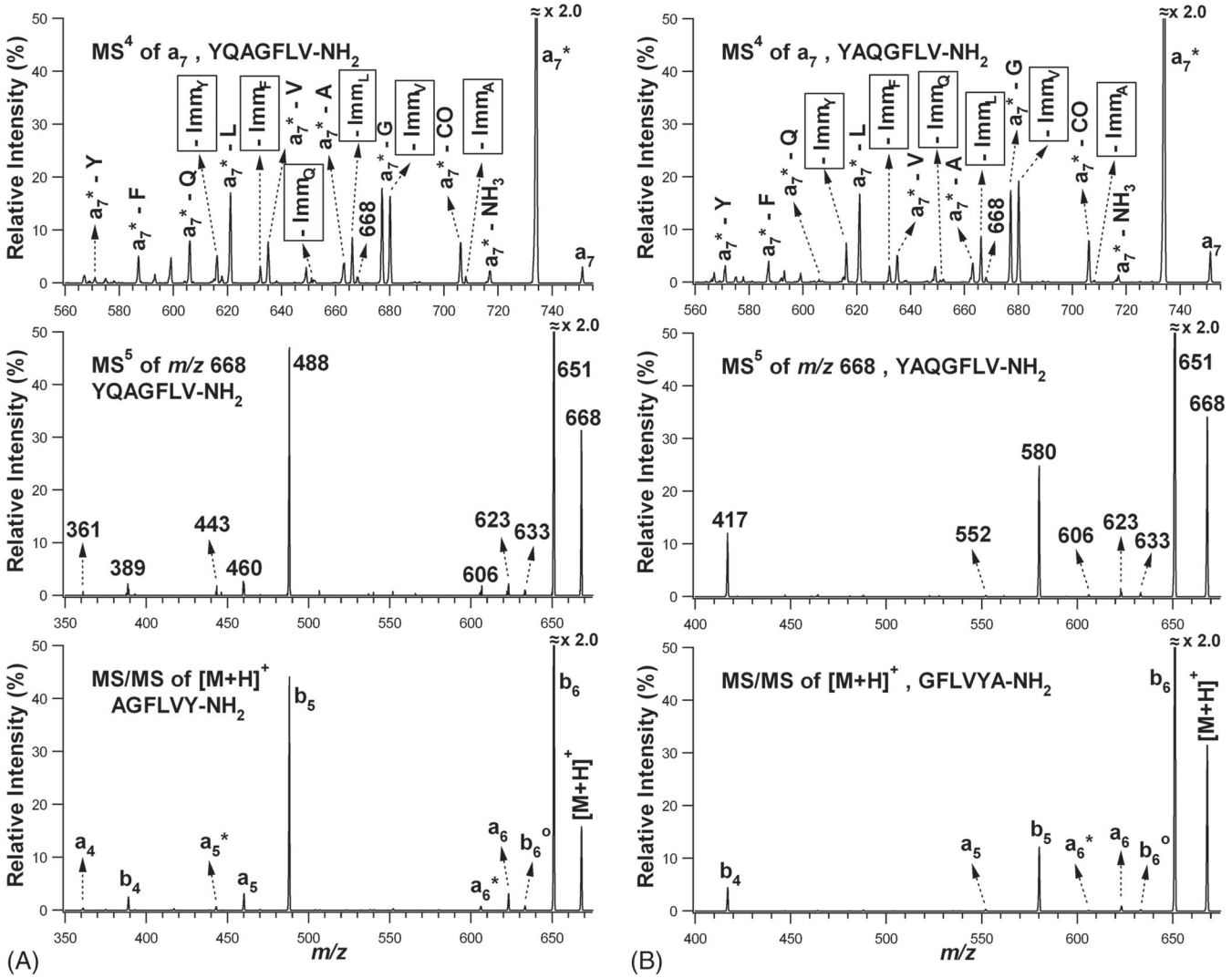


FIGURE 6 (A) MS^4 mass spectrum of a_7 ion from protonated YQAGFLV-NH_2, MS^5 mass spectrum of *m/z* 668 ion from a_7 ion of protonated YQAGFLV-NH_2, MS/MS spectrum of $[M + H]^+$ ion from protonated AGFLVY-NH_2 and (B) MS^4 mass spectrum of a_7 ion from protonated YAQGFLV-NH_2, MS^5 mass spectrum of *m/z* 668 ion from a_7 ion of protonated YAQGFLV-NH_2, and MS/MS spectrum of $[M + H]^+$ ion from protonated GFLVYA-NH_2

the detection of a_7—Imm_X losses indicates the existence of a classical C-terminal imine isomer as well.

Moreover, *m/z* 668 fragment ions were detected in the a_7 ion's mass spectra. MS^5-CID mass spectra of these fragment ions were acquired individually from each peptide (see the middle panel of Figure 6A,B) and compared with MS/MS spectra of $[M + H]^+$ ions AGFLVY-NH_2 and GFLVYA-NH_2, respectively. The middle and last panel of Figure 6A compares the CID-MS^5 mass spectrum of *m/z* 668 fragment ion from the a_7 ion of YQAGFLV-NH_2 with the MS/MS spectrum of $[M + H]^+$ ion from a commercial AGFLVY-NH_2 hexapeptide. The one-to-one similarity between the two mass spectra strongly suggests that they adopt the same structure in the gas phase.

Similarly, the CID-MS^5 mass spectrum of the *m/z* 668 fragment ion from the a_7 ion of YAQGFLV-NH_2 and the MS/MS spectrum of $[M + H]^+$ ion from a commercial GFLVYA-NH_2 hexapeptide is compared in the middle and last panel of Figure 6B. Comparing these two mass spectra reveals that *the m/z* 668 fragment has a protonated GFLVYA-NH_2 molecular ion gas-phase structure.

In conclusion, the obtained results demonstrate that an imine-amide structure is predominant for the a_7 ions derived from YQAGFLV-NH_2, and YAQGFLV-NH_2 heptapeptide populations have a classical C-terminal imine structure as well. The former structure yields a protonated C-terminal amidated hexapeptide, excluding the Q residue during CID fragmentation. The possible mechanism for forming a protonated C-terminal amidated hexapeptide could be explained as illustrated in Scheme 2.

3.2.2 | YAGFQLV-NH_2 and YAGFLQV-NH_2

The MS^4-CID mass spectra of the a_7 ions generated from YAGFQLV-NH_2 and YAGFLQV-NH_2 were shown in Figure 7A,B, respectively.

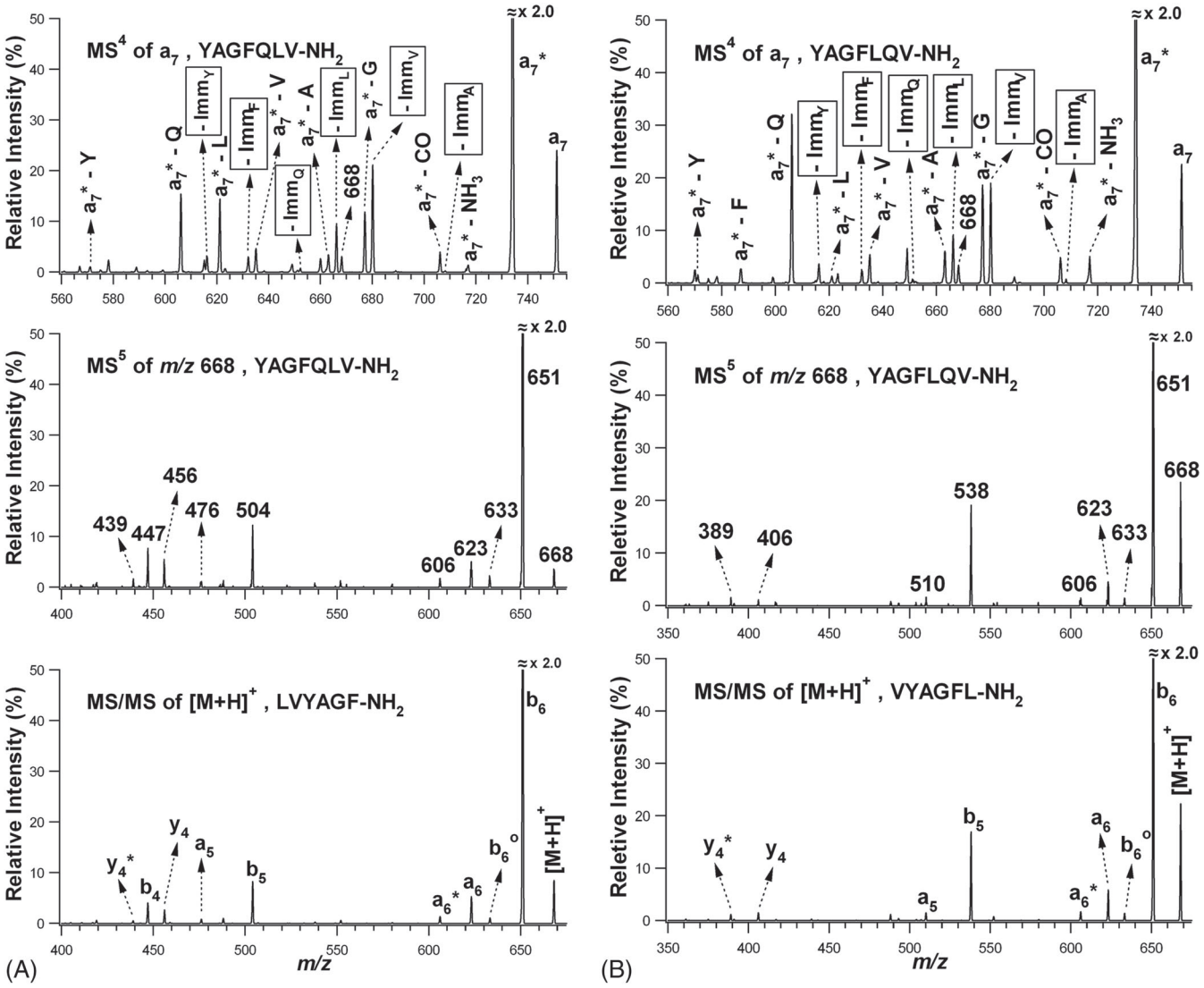


FIGURE 7 (A) MS^4 mass spectrum of a_7 ion from protonated YAGFQLV-NH_2, MS^5 mass spectrum of *m/z* 668 ion from a_7 ion of protonated YAGFQLV-NH_2, MS/MS spectrum of $[M + H]^+$ ion from protonated LVYAGF-NH_2 and (B) MS^4 mass spectrum of a_7 ion from protonated YAGFLQV-NH_2, MS^5 mass spectrum of *m/z* 668 ion from a_7 ion of protonated YAGFLQV-NH_2, and MS/MS spectrum of $[M + H]^+$ ion from protonated VYAGFL-NH_2

The observation of the a_7*—X (where X is Y, F, Q, L, V, A, or G) and a_7—ImmX (where X is Y, F, Q, L, V, or A) losses provide evidence for the existence of an imine-amide isomer and a C-terminal imine isomer for a_7 ions, respectively.

Additionally, the CID mass spectrum of the a_7 ion from YAGFQLV-NH_2 includes of *m/z* 668 fragment ion. The MS^5-CID consecutive stages were conducted, and the obtained mass spectrum was compared with the MS/MS spectra of $[M + H]^+$ ions of the commercial LVYAGF-NH_2 hexapeptide (see Figure 7A) to probe its gas-phase structure. Based on their having identical CID mass spectra, it can be accepted that the *m/z* 668 fragment has the structure of a protonated C-terminal amidated LVYAGF-NH_2 peptide ion.

Similarly, *m/z* 668 fragment was detected in the CID mass spectra of the a_7 ion derived from YAGFLQV-NH_2. This fragment was further selected and allowed to fragment in the gas phase. The obtained CID-MS^5 mass spectrum of the *m/z* 668 fragment ion is identical to the MS/MS spectrum of $[M + H]^+$ ions of commercial VYAGFL-NH_2 (see Figure 7B). Therefore, *m/z* 668 fragment has the structure of a protonated C-terminal amidated VYAGFL-NH_2 peptide ion.

In summary, the resultant mass spectra have demonstrated that the possible structures of a_7 ions derived from YAGFQLV-NH_2 and YAGFLQV-NH_2 are imine-amide and C-terminal imine at the same time under applied CID conditions.

4 | CONCLUSION

In the present work, the gas-phase fragmentation reactions of the a_7 ions derived from permuted isomers of the Q-containing model peptides have been investigated via multi-stage mass spectrometry experiments. The obtained results demonstrated that the a_7 ions undergo cyclization/rearrangement reactions to form rearranged imine-amide isomers in line with the previous reports.[34,36,43,47,48] In addition to these isomers, a minor population of the classical C-terminal imine structures was also observed based on detection of ithe immonium ion losses. During the fragmentation of the imine-amide isomers, a novel rearrangement reaction was observed, which yields a protonated C-terminal amidated hexapeptide excluding the Q residue. For instance, AAAAAA-NH_2 (*m/z* 444) was detected upon fragmentation of the a_7 ions for the first set of peptides. The one-to-one similarity of the MS^5-CID spectra of the *m/z* 444 ions with MS/MS spectra of the $[M + H]^+$ ions for commercial AAAAAA-NH_2 strongly supports the formation of the imine-amide structure. The possible fragmentation mechanism is initiated via nucleophilic attack of the side chain amide nitrogen of the Q residue on its N-protonated imine carbon atom of imine-amide structure.

On the other hand, CID mass spectra of the a_7 ions derived from permuted isomers of the Q containing YAGFLV peptide series involves of the a_7*—X and a_7—Imm_X (where X is Y, A, G, F, L, V, or Q) ions with different relative intensities. Observations of these fragment ions have provided strong evidence that rearranged imine-amides and classical C-terminal imine isomers co-exist under applied CID conditions while the former isomer is the primary structure for all the a_7 ions studied. Upon fragmentation of the imine-amide isomers, *m/z* 668 ions are observed, which have the structure of C-terminal hexapeptides without the Q residue. For example, CID mass spectra of the a_7 ion from QYAGFLV-NH_2 generate *m/z* 668 fragments whose structures are verified as protonated YAGFLV-NH_2 using multi-stage tandem mass spectrometry experiments.

The formation of the imine-amide isomer is more prominent for the AAAAAA peptide series compared to the YAGFLV peptide series. This aspect could be explained by hindering cyclization/re-opening chemistry for the a_7 ions from the YAGFLV peptide set due to the bulky nature of the comprising amino acid residues. Therefore, we detected *m/z* 444 fragment ions (for AAAAAA peptide series) in higher abundance than the *m/z* 668 fragments (YAGFLV peptide series), which are formed by the fragmentation imine-amide structure.

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CONFLICT OF INTEREST

There is no conflict of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

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