Cyclization Reaction of Peptide Fragment Ions during Multistage Collisionally Activated Decomposition: An Inducement to Lose Internal Amino-Acid Residues

Chenxi Jia, Wei Qi, and Zhimin He

Chemical Engineering Research Center, School of Chemical Engineering and Technology, Tianjin University, Tianjin, People's Republic of China

During characterization of some peptides (linear precursors of the cyclic peptides showing potential to be anticancer drugs) in an ion trap, it was noted that many internal amino acid residues could be lost from singly charged b ions. The phenomenon was not obvious at the first stage of collisionally activated decomposition (CAD), but was apparent at multiple stages of CAD. The unique fragmentation consisting of multiple steps is induced by a cyclization reaction of b ions, the mechanism of which has been probed by experiments of N-acetylation, MS^n, rearranged-ion design, and activation-time adjustment. The fragmentation of synthetic cyclic peptides demonstrates that a cyclic peptide intermediate (CPI) formed by b ion cyclization exhibits the same fragmentation pattern as a protonated cyclic peptide. Although no rules for the cyclization reaction were discerned in the experiments of peptide modification, the fragmentations of a number of b ions indicate that the "Pro and Asn/Gln effects" can influence ring openings of CPIs. In addition, large-scale losses of internal residues from different positions of a-type ions have been observed when pure helium was used as collision gas. The fragmentation is initiated by a cyclization reaction forming an a-type ion CPI. This CPI with a fixed-charge structure cannot be influenced by the "Pro effect", causing a selective ring opening at the amide bond Pro-Xxx rather than Xxx-Pro. With the knowledge of the unique fragmentations leading to internal residue losses, the misidentification of fragments and sequences of peptides may be avoided. (J Am Soc Mass Spectrom 2007, 18, 663–678) © 2007 American Society for Mass Spectrometry

Tandem mass spectrometry (MS/MS) plays a key role in the characterization of the primary structures of proteins and peptides. In the last two decades, innovations in MS instrumentation and techniques fundamentally drove the development of proteomics [1, 2]. So far, two approaches have been developed for protein identification [3]: top-down [4] and bottom-up [5, 6] sequencings. For the top-down sequencing, the protein sample without enzymatic digestion is transferred into the gas phase intact, which has the potential for 100% sequence coverage of the protein and improved detection of post-translational modifications [4]. The bottom-up sequencing requires MS/MS analysis of the proteolytic fragments and database searching using algorithms such as Mascot and Sequest [5, 6]. In addition, structural elucidations of natural or synthetic peptides, including linear peptides [7, 8], cyclic peptides [9, 10], and peptide analogues [11] also rely on MS/MS analysis. Undoubtedly, all of the applications described above are based on understanding the dissociation mechanisms of peptides, which have been well summarized in recent reviews [12–16]. However, conventional knowledge of fragmentation pathways and rules cannot reasonably explain many anomalous fragmentations. For example, some groups have reported that certain peptides show enhanced cleavage at special amino acid residues to produce incomplete sequence information [15]. Hence, the study on fragment-ion intensity relationship was of further concern and specially reviewed by Paizs and Suhai [16]. Also, another class of anomalous fragmentations introduced in this paper should receive attention.

For peptide sequencing, the ideal fragmentation pattern of peptides is that the amino acid residues are sequentially lost from the C/N-terminus. However, if the residues are lost from the interior of the peptide chain, the peptide sequence will be easily misidentified. Referring to related literatures [17–23], we summarize this class of anomalous fragmentations accompanied by losses of internal amino acid residues into three steps (Scheme **1**). (1) A cyclic peptide intermediate (CPI) is formed through a nucleophilic attack by the electron-rich group on the electron-poor group of the peptide

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Address reprint requests to Dr. W. Qi, Chemical Engineering Research Center, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China. E-mail: qiwei@tju.edu.cn

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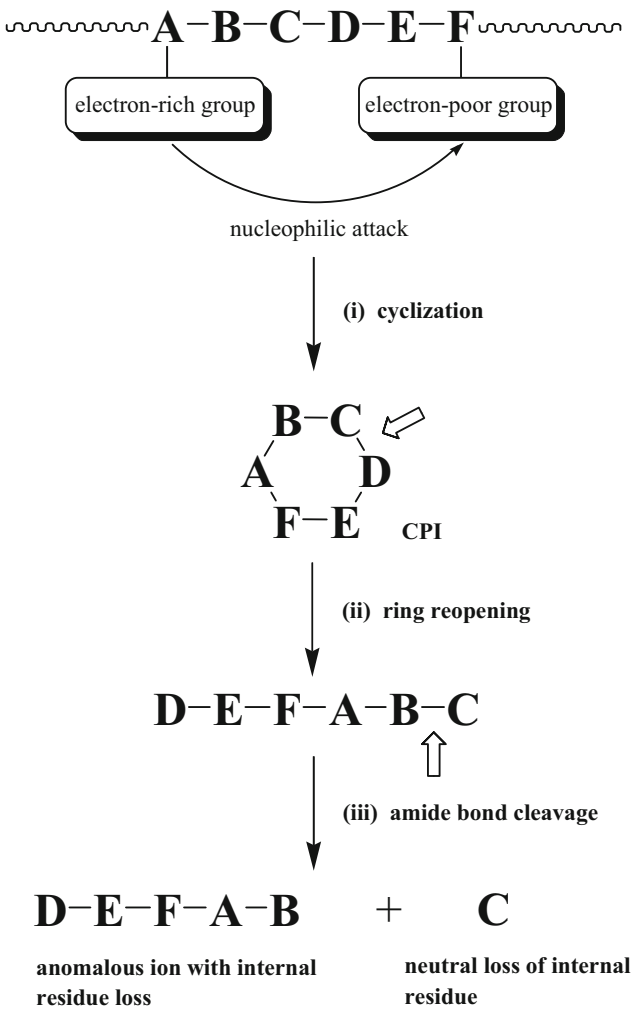


Scheme 1

chain. (2) The CPI ring reopens at other sites, producing linear fragment ions where internal residues are relocated at the C/N-termini. (3) The linear fragment ions further dissociate via conventional fragmentation pathways, forming daughter ions with neutral losses of internal residues from the parent ion. Obviously, the cyclization reaction is critical for inducing this class of fragmentations. The related literature is briefly described as follows.

Tang et al. [17, 18] have reported a head to side-chain cyclization of b^{2+} ions leading to internal residue losses, which proceeds via electrophilic attack by the protonated ω-amino group of Lys (or Orn) in $[M + H]^{2+}$ on the backbone amide carbonyl groups at/near the C-terminus. The analogous behavior of Lys was also observed by Fuchs and Budzikiewicz [21] in MS/MS analysis of pyoverdins. Subsequently, Yagüe et al. [22] described a head-to-tail cyclization of b^{2+} ions, arising from a nucleophilic attack by the N-terminal nitrogen over the carbonyl carbon of one peptide bond. The investigations described above focused on doubly charged ions. For singly charged ions, Vachet et al. [19] suggested that the a-type ion can undergo a head-to-tail cyclization reaction accompanied by an NH_3 loss from N-terminus, which was also proposed by Harrison and Young [24]. A similar dissociation pattern was observed by Craig and Taylor [20] during characterization of plicatamide 1. Recently, in the negative ion spectra of citropin 1.1 and analogues, Brinkworth et al. [23] found that the loss of an internal Val residue was induced by a head-to-tail cyclization between the $CONH^-$ group of the C-terminus and the carbonyl group of the Ser-Val amide bond. Coincidentally, when our manuscript was being prepared, Harrison et al. [25] published a communication, which showed that b ions could be cyclized by attack of the N-terminal amino group on the carbonyl carbon of C-terminal oxazolone ring. These findings described above suggest that the cyclization reactions leading to internal residue losses frequently occur during peptide fragmentations and need to be further studied.

In our previous work [26], using multistep MS/MS in an ion trap we sequenced some cyclic peptides and observed some anomalous ions with internal residue losses as reported by Ngoka and Gross [10] as well as Stefanowicz [27]. In the present work, we devote our attention to investigating the unique fragmentation of b ions from linear peptides. At the first stage of CAD, this unique fragmentation of singly charged b ions is difficult to be observed as reported by Yagüe et al. [22]. However, under multistage CAD almost all of the investigated b ions (containing three to seven residues) notably display the unique fragmentation pattern, which is quite intensive. This paper focuses on studying the cyclization mechanisms of b ions, which are probed by the experiments including N-acetylation, MS^n, rearranged-ion design, activation-time adjustment, synthetic cyclic peptide fragmentation and peptide modification. Particular attention is paid on discussing the relationship between the fragmentation patterns of the CPI produced by b ion cyclization and the protonated cyclic peptide produced by electrospray ionization (ESI). We attempt to introduce the fragmentation mechanism of cyclic peptides into CPIs and to find some valuable predictive rules, e.g., the "Pro and Asn/Gln effects" [26, 28–30]. Vachet and Glish [19] have reported that residues can be lost from the second positions of C-termini of a-type ions when a mixture of helium and 5% xenon was used as collision gas in an ion trap. Following their research, we observed this phenomenon using 100% helium as collision gas. In addition, it was found that not only the internal residues at the second position of C-terminus [19] but also those at other positions could be eliminated. Moreover, another interesting phenomenon is that the amide bond Pro-Xxx rather than Xxx-Pro in CPIs formed by a-type ion cyclization exhibits enhanced cleavage, and a tentative explanation has been proposed in this research.

Experimental

Materials and Reagents

Wang-resin, Fmoc-amino acids, and coupling reagents for peptide synthesis were purchased from GL Biochem

(Shanghai, China). All solvents for peptide synthesis were commercial analytical grade and were redistilled before use. HPLC-grade formic acid and acetonitrile were supplied by Merck (Darmstadt, Germany).

Peptide Synthesis and Chemical Modifications

The linear peptides were prepared manually using Wang-resin as solid support following the Fmoc methodology. The obtained linear peptides were cyclized in solution to yield the cyclic peptides. The details were described in our previous reports [26, 31]. Acetylation of the free amine moiety at the N-terminus of peptide was achieved by adding 50% (vol/vol) acetic anhydride in pyridine to the peptide-resin following a final deprotection step (before the final cleavage from resin).

Mass Spectrometry Conditions

Experiments were performed with a Finnigan LCQ ion-trap mass spectrometer (San Jose, CA) equipped with an ESI source. The spray needle was set at a potential of 5.0 kV and a nitrogen sheath gas flow of 20 (arbitrary units) was used to stabilize the spray. The capillary temperature was maintained at 200 °C. The tube-lens offset was 20 V and the electron multiplier voltage was −800 V. Helium gas was introduced into the ion trap to improve the trapping efficiency of the analyte ions introduced into the ion trap. The background helium gas also served as the collision gas during the CAD event. A 50 pmol/μl solution of peptide in 50:50 (vol/vol) acetonitrile/H_2O containing 1% formic acid was infused into the mass spectrometer at a flow-rate of 2 μl/min by syringe pump. Isolation widths of 1.0 *m/z* and activation times of 30 ms were used at each stage of CAD; 400 scans were averaged. Spectra were acquired in the centroid mode.

Nomenclature for Labeling the Fragment Ions

The nomenclature for labeling the conventional fragment ions of peptides was developed by Roepstorff and Fohlmann [32] and Biemann [33]. An extension [18, 22] of this nomenclature is used to label the anomalous ions with internal residue losses. The general formula is "x_n–AA. . .," where "x_n" is the designation for the ion such as "a_n" and "b_n" and "AA. . .", are the one letter codes of lost amino acid sequence. In addition, "*" and "0" superscripts, respectively, denote neutral loss of one ammonia molecular and one water molecule; "E" superscript denotes N-acetylation fragment ion. Because all ions in this study are singly charged, for simplicity no special symbol is used to label charges.

Results and Discussion

Deduction for Cyclization Reaction of Singly Charged b-Type Ions

Finding of the unique fragmentation. Initially, we were interested in sequencing cyclic peptides with antitumor activities and in studying the fragmentation mechanisms using multistep CAD in an ion trap [26]. Sequencing cyclic peptides usually depends on detection of b ions [10]. However, some CAD spectra of b ions present the rearranged fragment ions with losses of internal amino acid residues [10, 26, 27]. In this work, we further study the b ions arising from the linear precursors of these cyclic peptides to verify whether it is a common phenomenon. In the first step, the ESI-produced $[M + H]^+$ of the linear peptides were subjected to the first stage of CAD to produce MS^2 spectra. No anomalous ions were observed obviously (Figure S1 in Supplementary Material which can be found in the electronic version of this article). In the second step, the b ions were selected from the obtained MS^2 spectra and subjected to the second stage of CAD to produce MS^3 spectra. The obtained data indicate that almost all of the investigated b ions exhibit the unique fragmentation pattern with internal residue losses (see the second column in Table 1). Figure 1a, b, and c illustrate the CAD spectra (MS^3) of the b_6, b_5, and b_4 ions from peptide PLIFSPI (a precursor of Stylopeptide 1 [34], respectively. Figure 2a, b, and c give the CAD spectra (MS^3) of the b_6, b_5 and b_4 ions from peptide PFNSLAI (a precursor of Stylostatin 1 [35], respectively.

Deduction for cyclization. The MS^3 spectrum of b_6 $PLIFSP^+$ (Figure 1a) is taken as a representative case to introduce the deduction process. According to conventional fragmentation rules, the b_6 ion dissociates mainly via $b_n \rightarrow b_{n-1}$ and $b_n \rightarrow a_n$ pathways to form lower b- and a-type ions [16, 36, 37]. The ions b_5, b_4, b_3, and b_2 are observed clearly in the spectrum. On the basis of classical sequencing methods [1, 3, 16], the sequences of these b ions are determined as $PLIFS^+$, $PLIF^+$, PLI^+, and PL^+, respectively. Accordingly, the linear parent ion b_6 are identified as $PLIFSP^+$. However, some anomalous ions formed via other fragmentation pathways reveal sequential elimination of residues from the interior of the parent ion b_6. For example, b_6–S at *m/z* 568.7 differs from b_6 at *m/z* 655.8 by 87.1 Da, the mass of a Ser residue. The mass differences among b_6–S (*m/z* 568.7), b_6–FS (*m/z* 421.7), and b_6–IFS (*m/z* 308.6) are 147.0 and 113.1 Da, which respectively, correspond with the mass of Phe and Ile residues. Furthermore, the anomalous ions display the feature of b-type ions because sometimes there are a-type ions formed by a CO loss near them (the evidence shown at the end of the Exploring Predictive Rules section). Therefore, the sequences of b_6–S, b_6–FS, and b_6–IFS are deduced as $PPLIF^+$, $PPLI^+$, and PPL^+, respectively. The sequence of their linear

Table 1. b-Type ions undergoing cyclization reaction in this investigation

Peptide	Parent b ions undergoing cyclization reaction		CPI formed by cyclization reaction	Preferential ring-opening amide bond of CPI[a]	
PLIFSPI[b]	b_6	$PLIFSP^+$	$cyclo(PLIFSP + H)^+$	Ser-Pro	Pro-Pro
	b_5	$PLIFS^+$	$cyclo(PLIFS + H)^+$	Ser-Pro	Leu-Ile
	b_4	$PLIF^+$	$cyclo(PLIF + H)^+$	Phe-Pro	Leu-Ile
PFNSLAI[b]	b_6	$PFNSLA^+$	$cyclo(PFNSLA + H)^+$	Asn-Ser	Ala-Pro
	b_5	$PFNSL^+$	$cyclo(PFNSL + H)^+$	Asn-Ser	Leu-Pro
	b_4	$PFNS^+$	$cyclo(PFNS + H)^+$	Asn-Ser	Ser-Pro
	b_3	PFN^+	$cyclo(PFN + H)^+$	—[c]	—[c]
IFSPIP[b]	b_6	$IFSPIP^+$	$cyclo(IFSPIP + H)^+$	Ser-Pro	Ile-Pro
	b_5	$IFSPI^+$	$cyclo(IFSPI + H)^+$	Ser-Pro	Ile-Ile
	b_4	$IFSP^+$	$cyclo(IFSP + H)^+$	Ser-Pro	Pro-Ile
LPVNPFV[b]	b_6	$LPVNPF^+$	$cyclo(LPVNPF + H)^+$	Asn-Pro	Leu-Pro
	b_5	$LPVNP^+$	$cyclo(LPVNP + H)^+$	Asn-Pro	Leu-Pro
	b_4	$LPVN^+$	$cyclo(LPVN + H)^+$	Asn-Leu	Val-Asn
VPVNPFV[b]	b_6	$VPVNPF^+$	$cyclo(VPVNPF + H)^+$	Asn-Pro	Val-Asn
	b_5	$VPVNP^+$	$cyclo(VPVNP + H)^+$	Asn-Pro	Val-Asn
	b_4	$VPVN^+$	$cyclo(VPVN + H)^+$	Asn-Val	Val-Asn
FPQPFPFI[b]	b_7	$FPQPFPF^+$	$cyclo(FPQPFPF + H)^+$	Gln-Pro	Phe-Pro
	b_6	$FPQPFP^+$	$cyclo(FPQPFP + H)^+$	Gln-Pro	Phe-Pro
	b_5	$FPQPF^+$	$cyclo(FPQPF + H)^+$	Gln-Pro	Phe-Phe
	b_4	$FPQP^+$	$cyclo(FPQP + H)^+$	Gln-Pro	Phe-Pro
	b_3	FPQ^+	$cyclo(FPQ + H)^+$	—[c]	—[c]
APFNSL	b_5	$APFNS^+$	$cyclo(APFNS + H)^+$	Ala-Pro	Pro-Phe
	b_4	$APFN^+$	$cyclo(APFN + H)^+$	Ala-Pro	Asn-Ala
LPPFI	b_4	$LPPF^+$	$cyclo(LPPF + H)^+$	Leu-Pro	Pro-Pro
	b_3	LPP^+	$cyclo(LPP + H)^+$	—[c]	—[c]
FSPLII	b_5	$FSPLI^+$	$cyclo(FSPLI + H)^+$	Ser-Pro	Leu-Ile
	b_4	$FSPL^+$	$cyclo(FSPL + H)^+$	Ser-Pro	Phe-Ser
FPLII	b_4	$FPLI^+$	$cyclo(FPLI + H)^+$	Phe-Pro	Leu-Ile
PVNPF	b_4	$PVNP^+$	$cyclo(PVNP + H)^+$	Pro-Pro	Asn-Pro

[a]When the CPI dissociates via more than two ring-opening pathways, the main two are listed.
[b]Linear precursors of the cyclic peptides showing potential to be anticancer drugs [34, 35, 43].
[c]In the CAD spectra (MS^3) of the b_3 ions, the anomalous ions are lowly abundant. Due to containing three residues, the cyclization of the b_3 ions is possibly incomplete. So the preferential ring-opening amide bonds cannot be determined.

parent ion b_6 can be inferred as $PPLIFS^+$. Another set of ions showing sequential elimination of internal residues are b_6–L, b_6–PL, b_6–PPL, and b_6–SPPL. Their sequences can be deduced as $IFSPP^+$, $IFSP^+$, IFS^+, and IF^+. The sequence of their linear parent ion b_6 is inferred as $IFSPPL^+$. To sum up, there are three deduced sequences of the parent ion b_6, i.e., $PLIFSP^+$, $PPLIFS^+$, and $IFSPPL^+$.

To some extent, the deduction method and the sequencing result described above are very similar to those for a cyclic peptide sequencing [9, 10, 26, 27]. If Figure 1a is a MS^2 spectrum of $[M + H]^+$ of a cyclic peptide, we may determine the sequence of the cyclic peptide as cyclo(PLIFSP). But it is indeed the MS^3 spectrum of b_6 $PLIFSP^+$ from the linear peptide PLIFSPI. Therefore, it can be assumed that b_6 $PLIFSP^+$ is cyclized to form a CPI $cyclo(PLIFSP + H)^+$ and the CPI reopens at different sites to produce the fragment ions presented in Figure 1a. In addition, from other MS^3 spectra of b ions in Figures 1 and 2 (and the b ions listed in Table 1), we also observe large-scale anomalous ions and draw the same conclusion that these b ions as parent ions have undergone cyclization reactions.

Proposed Mechanism for Cyclization Reaction of b-Type Ions

The MS^3 experiment of b_4 ($PLIF^+$, *m/z* 471.6) from peptide PLIFSPI (Figure 1c) is taken as a representative case to discuss the unique fragmentation mechanism. In the MS^3 spectrum, not only the normal ions b_3 (*m/z* 324.4) and b_2 (*m/z* 211.3) exhibiting a sequential loss of residues from C-terminus are observed, but also the anomalous ions b_4–L (*m/z* 358.5) and b_4–PL (*m/z* 261.4) displaying a sequential loss of residues from interior are detected.

$MH^+ \rightarrow CPI \rightarrow b_n^{rea}$ pathway. A proposed mechanism is shown in Scheme **2**. At the first stage of CAD, the normal ion b_4 can be formed via the conventional b_x–y_z pathway [13, 16]. The protonation of the nitrogen of the amide bond Phe–Ser lowers the bond stability as well as induces the neighboring carbonyl oxygen to attack the carbon of the amide bond, leading to cleavage of the amide bond to form b_4 as an oxazolone derivative and a neutral counterpart (Pathway 1) [16, 38]. The b_4 ion further dissociates to form b_3 and b_2 via conventional

$b_n \rightarrow b_{n-1}$ pathways (Pathway II) [16, 36]. At the first stage of CAD, if the N-terminal nitrogen attacks the carbon of the amide bond Phe–Ser, a b_4 CPI will be produced by a head-to-tail cyclization reaction (Pathway III). The fragmentation of the CPI is identical to that of a protonated cyclic peptide, involving proton retransfer, ring opening, and subsequent $b_n \rightarrow b_{n-1}$ fragmentation [16, 26, 39]. At the second stage of CAD, if the mobile proton locates on the nitrogen of the amide bond Leu-Ile of the CPI, the neighboring carbonyl oxygen will attack the carbon of the amide bond, leading to ring reopening to form the rearranged b_4 with the Leu residue relocated at the C-terminus (Pathway IV). Subsequently, the anomalous ions b_4–L and b_4–PL are generated via conventional $b_n \rightarrow b_{n-1}$ pathways (Pathways V and VI). The series of fragmentations leading to anomalous ions with internal residue losses is tentatively named as $MH^+ \rightarrow CPI \rightarrow b_n^{rea}$ pathway.

$b_n \rightarrow CPI \rightarrow b_n^{rea}$ pathway. The b_4 CPI can also be formed via other pathways. The linear b_4 ion may be cyclized by attack of the N-terminal nitrogen on the carbonyl carbon of oxazolone ring to form the CPI (Pathway VII in Scheme 2) [25]. The CPI further dissociates to produce the anomalous b ions (Pathways IV, V, and VI). The series of unique fragmentations is tentatively named as $b_n \rightarrow CPI \rightarrow b_n^{rea}$ pathway.

The question that remains is the origination of the normal b ions, i.e., b_3 (*m/z* 324.4) and b_2 (*m/z* 211.3) in Figure 1c. Are they really formed via b_x–y_z (Pathway I) and subsequent $b_n \rightarrow b_{n-1}$ pathways (Pathway II)? The answer is that other pathways are possible. As stated above, the CPI reopens at an amide bond whose nitrogen is protonated. At the second stage of CAD, if the CPI reopens at the amide bond Phe–Pro, b_4 will be reproduced via a $CPI \rightarrow b_n^{rea}$ pathway (Pathway XIII) and be further fragmented to produce b_3 and b_2 (Pathway II). In some sense, the ions b_3 and b_2 in Figure 1c may also be reassigned as b_4–F and b_4–IF, respectively. Thus, the real origination of these normal b ions needs to be determined (see the Fragmentation of Cyclic Peptide section).

It is worth noting that the $MH^+ \rightarrow CPI$ pathway proposed in this investigation has relationship with the diketopiperazine–y_{N-n} pathway which has been studied by some groups [13, 16, 40, 41]. These two pathways (Scheme 3) have the same nucleophile-electrophile mechanism and product structures. When the proton is attached to the produced cyclic peptide to form a CPI ion, it is a $MH^+ \rightarrow CPI$ pathway. On the other hand, when the proton is attached to the produced C-terminal fragment to form a y-type ion, it is a diketopiperazine–y_{N-n} pathway. The alternation of these two fragmentation pathways is induced by a proton transfer between these two product fragments: cyclic peptide and C-terminal fragment. Possibly, these two pathways occur together and transform each other.

The cyclization phenomenon of doubly charged b ions has been found by Yague et al. [22] and a similar mechanism has been proposed for the formation of cyclic b ions and amino acid rearrangement. Following their research, we try to introduce the fragmentation mechanism of cyclic peptides into CPIs and to find some fragmentation rules. Moreover, it should be noted here that Harrison, Paizs et al. [25] published a communication about cyclization of singly charged b ions. Their experimental and computational results provide great insight into our proposed mechanism.

To support our proposed mechanisms, four points should be verified by experiments. (1) The assumption that the cyclization reaction requires a free N-terminus will be examined by peptide N-acetylation; (2) the existence of cyclization reaction and CPI will be verified by MS^4 and rearranged-ion design; (3) the hypothesis that CPIs have the same fragmentation pattern as protonated cyclic peptides will be tested by fragmentation of synthetic cyclic peptides; (4) the factors affecting the cyclization reaction and the CPI fragmentation will be determined by performing activation-time adjustment and peptide modification as well as by generalizing the fragmentation rules of a number of b ions.

Evidence to Support the Proposed Mechanism for b-Type Ions

Peptide N-acetylation. To examine whether the cyclization requires a free N-terminus, N-acetylation experiments were performed. Peptides were acetylated in solution and directly subjected to ESI, but the sample cannot be ionized effectively. To obtain efficient ionization, peptide IFSPIPL (another precursor of Stylopeptide 1) [34] was acetylated on resin and then cleaved from resin to yield pure N-acetylated peptide. Because the Ser side chains were still protected by *t*-butyl groups during acetylation, there was no acetylation to the Ser residues.

Figure 3a and b illustrate a comparison of MS^3 spectra between b_5 ($IFSPI^+$, *m/z* 558.7) from peptide IFSPIPL and b_5^E (CH_3CO-$IFSPI^+$, *m/z* 600.7) from N-acetylated peptide CH_3CO-IFSPIPL. The anomalous ions are presented in the MS^3 spectrum of b_5 (Figure 3a). In contrast, when the N-terminus is blocked by an acetyl (+42 Da), these anomalous ions are absent in the MS^3 spectrum of b_5^E (Figure 3b). The same phenomenon can also be found by comparing the MS^3 spectra of b_4 ($IFSP^+$, *m/z* 445.5) with that of b_4^E (CH_3CO-$IFSP^+$, *m/z* 487.5) ions (Figure 3c, d). These experimental results indicate that the N-acetylation hinder the N-terminal nitrogen from attacking the carbonyl carbon of the C-terminus or one amide bond. Thus, the $MH^+ \rightarrow CPI \rightarrow b_n^{rea}$ or $b_n \rightarrow CPI \rightarrow b_n^{rea}$ pathway no longer takes place, leading to disappearance of anomalous ions.

In addition, in the MS^2 spectrum of peptide IFSPIPL, the y and b ions are observed with relatively higher intensities (Figure S2A), whereas in that of N-acetylated peptide CH_3CO-IFSPIPL, the y and b^E ions is detected with relatively lower intensities (Figure S2B). The pos-

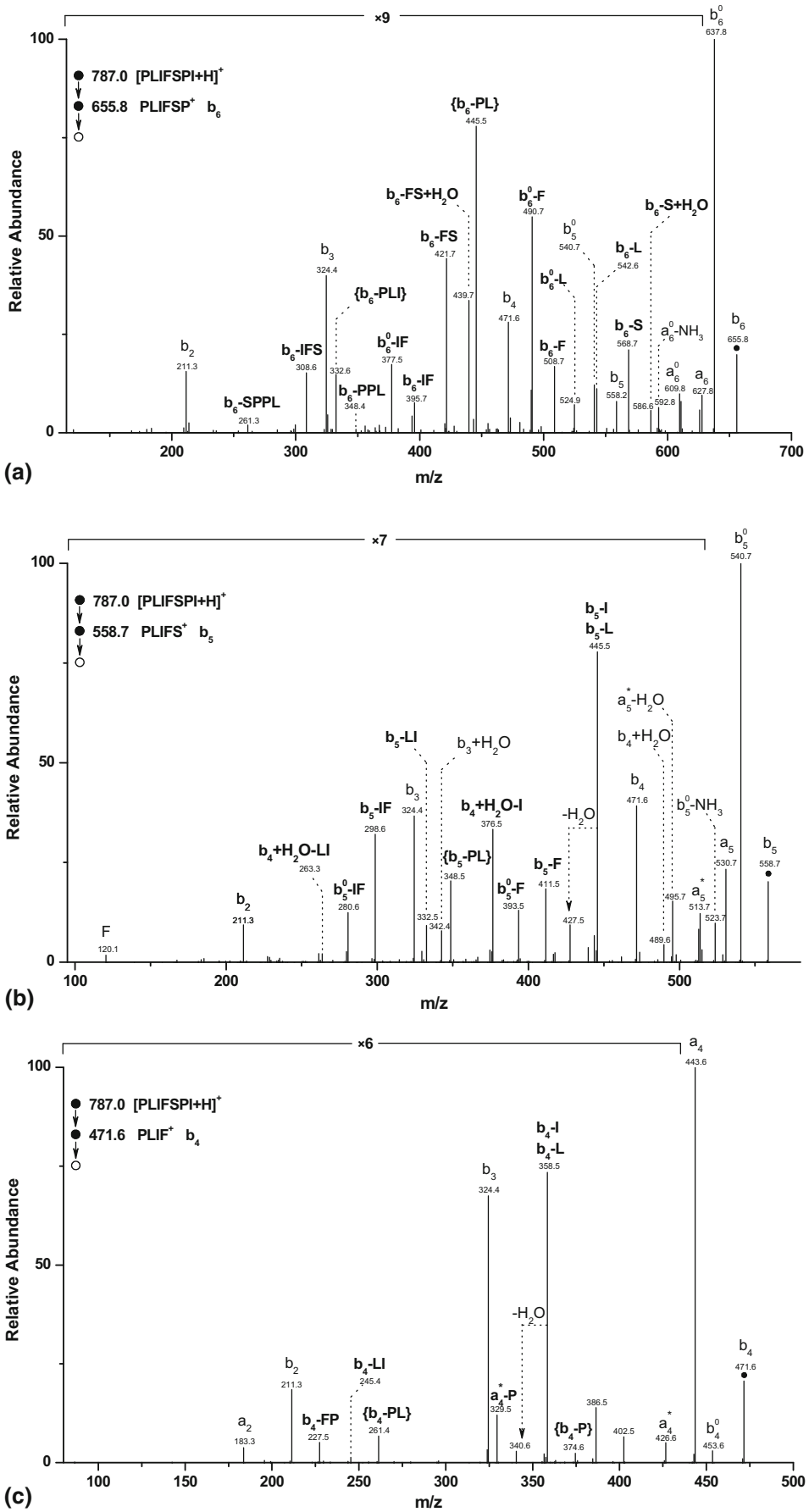


Figure 1. CAD spectra (MS^3) of the (**a**) b_6, (**b**) b_5, and (**c**) b_4 ions from peptide PLIFSPI. In this and the following figures, all of the anomalous ions with internal residue losses are labeled in bold font; the ions which may also be formed via the a_1–y_{n-1} pathway [16] are labeled in curly brackets {}. The details of the nomenclature are interpreted in the Experimental section.

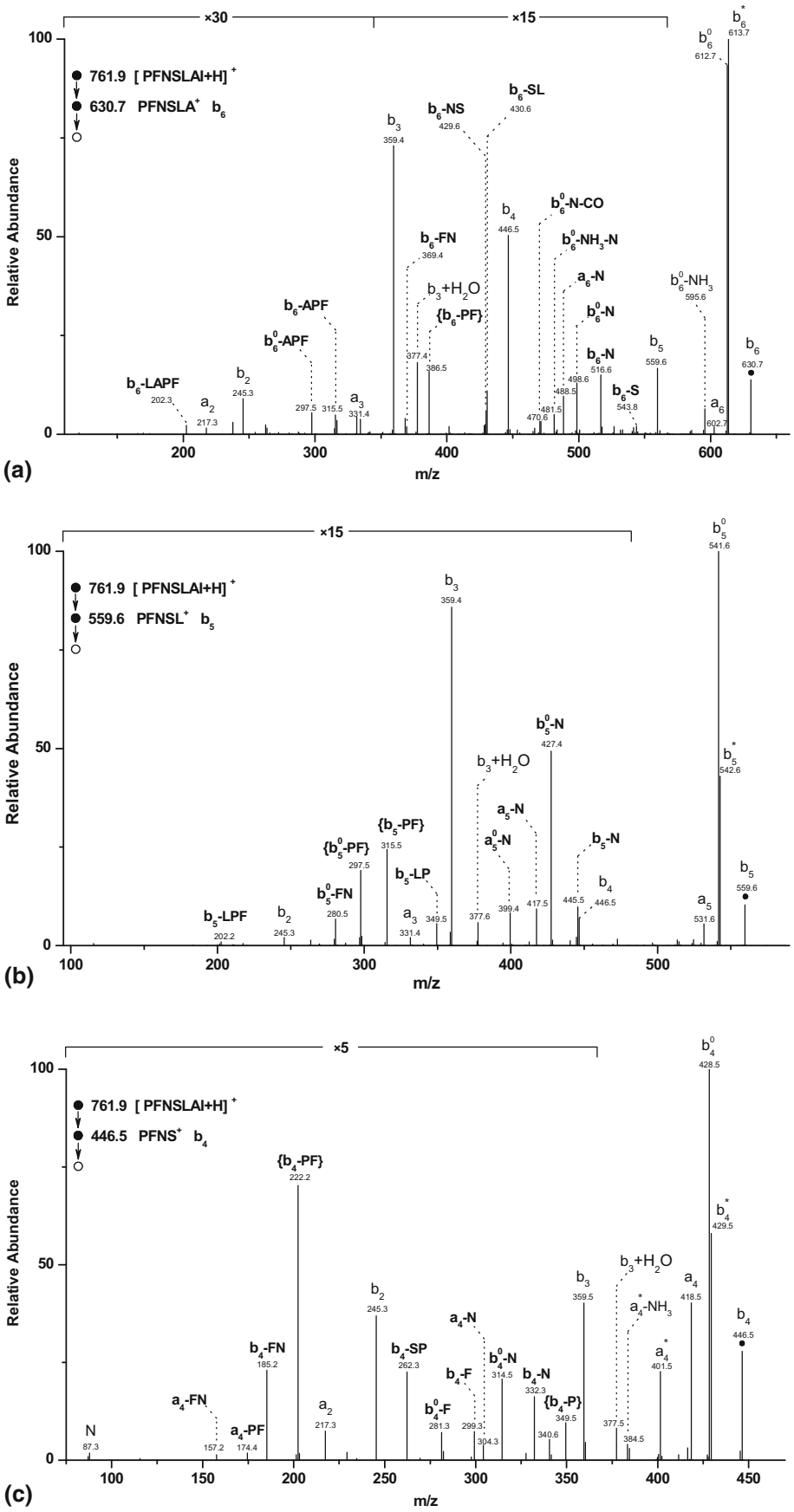
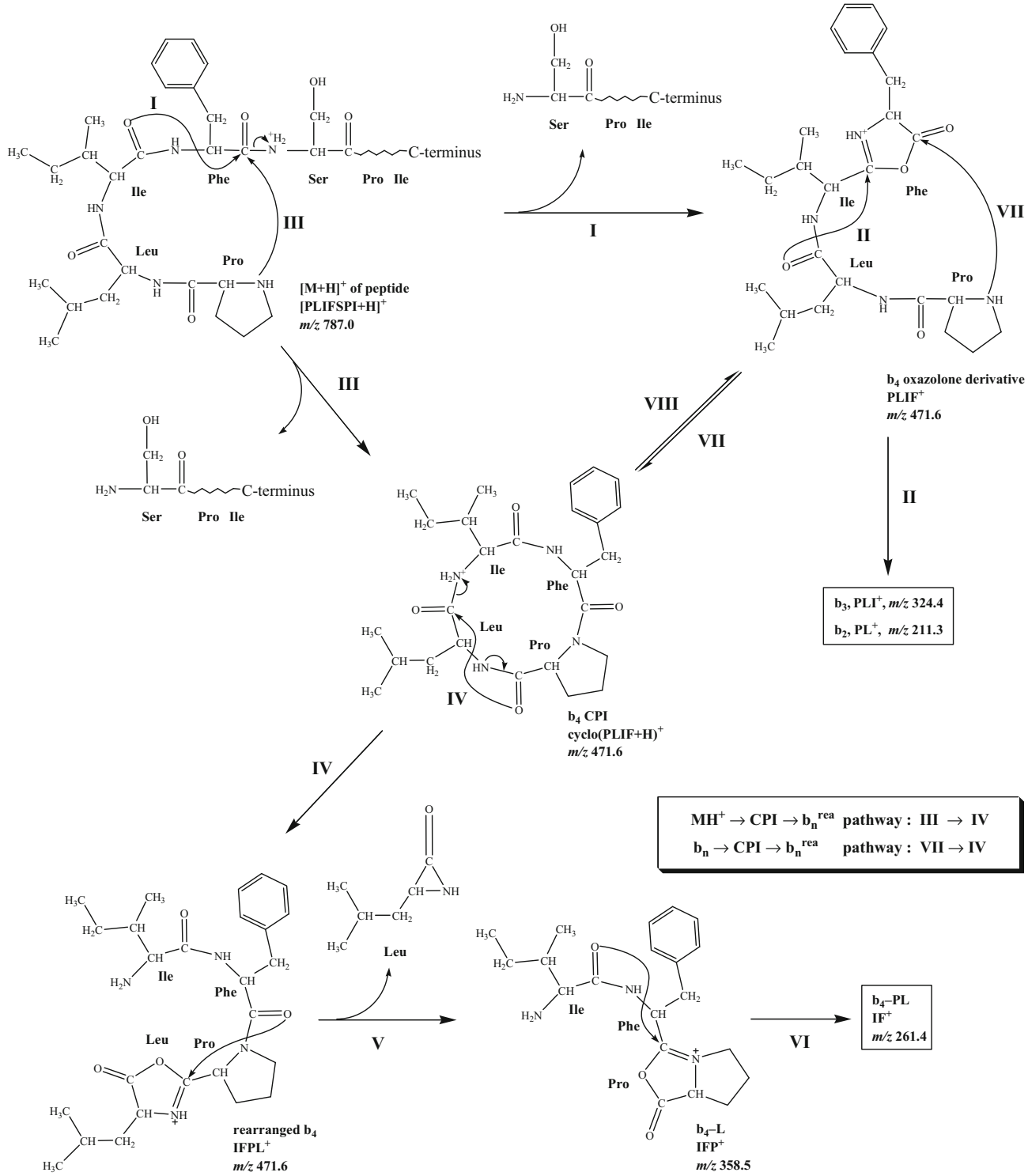


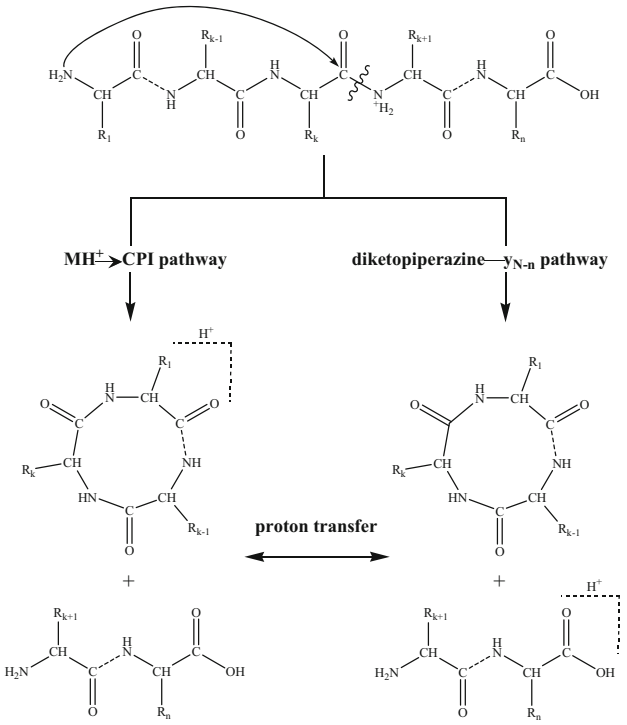
Figure 2. CAD spectra (MS^3) of the (**a**) b_6, (**b**) b_5, and (**c**) b_4 ions from peptide PFNSLAI.



Scheme 2

sible reason is that when the N-terminus is exposed, b_x–y_z, diketopiperazine–y_{N-n} and $MH^+ \rightarrow$CPI pathways are available for producing the y and b ions. However, as the N-terminus is acetylated, the fragmentations via $MH^+ \rightarrow$CPI and diketopiperazine–y_{N-n} pathways are ceased, resulting in the decrease of the produced y and b^E ions.

MS^4 experiment. MS^4 experiments were carried out to examine whether the sequences of these anomalous ions are identical with our expectation. b_5–S (*m/z* 471.6) and b_5–FS (*m/z* 411.5) in Figure 3a were subjected to the next stage of CAD to produce MS^4 spectra, respectively. In the MS^4 spectrum of b_5–S (Figure S3A), b_5–FS (*m/z* 324.4, 15%) and b_5–IFS (*m/z* 211.3, 3%) are observed; in that of



Scheme 3

b_5–F (Figure S3B), b_5–IF (*m/z* 298.4, 57%) is observed. Moreover, the spectra illustrate that b_5–S and b_5–F, which have lost internal residues from the parent ion b_5, undergo cyclization again to produce anomalous ions with internal residue losses. Further, the MS^4 experiment was performed on b_5^0 at *m/z* 540.7 (Figure 3a). From the obtained MS^4 spectrum (Figure S3C), fragment ions with losses of internal residues and one molecular water, e.g., b_5^0–F (*m/z* 393.5, 22%) and b_5^0–IF (*m/z* 280.6, 38%) are seen, revealing that the parent ion b_5^0 has undergone cyclization reaction. This also indicates that in Figure 3a the anomalous ions with loss of one water molecule, such as b_5^0–F and b_5^0–IF, derive from fragmentation of b_5^0 CPI.

Rearranged-ion design. If the cyclization reaction and the CPI exist during fragmentation, two b ions with different amino acid sequences, which can be cyclized to form the same CPI, may produce identical fragments and CAD spectra. For instance, $PLIFS^+$ and $FSPLI^+$ can both be cyclized to generate CPI cyclo(FSPLI + H)$^+$, and the CPI is able to further dissociate to produce the identical CAD spectra. Thus, peptides FSPLII and FPLII were designed and synthesized. Figure 4a shows the MS^3 spectrum of b_5 ($FSPLI^+$, *m/z* 558.7) from peptide FSPLII, which is designed for comparison with that of the b_5 ($PLIFS^+$, *m/z* 558.7) from peptide PLIFSPI shown in Figure 1b. Surprisingly, the two spectra have the identical fragment ions and even the corresponding relative intensities. Similarly, the MS^3 spectrum of b_4 ($FPLI^+$, *m/z* 471.6) from peptide FPLII (Figure 4b) is identical to that of b_4 ($PLIF^+$, *m/z* 471.6) from peptide PLIFSPI (Figure 1c). These experimental results are consistent with our hypothesis. Interestingly, the MS^3 spectrum of $IFSPI^+$ (Figure 3a) is almost identical to those of $PLIFS^+$ (Figure 1b) and $FSPLI^+$ (Figure 4a). The reason is that $IFSPI^+$ can be cyclized to form cyclo(IFSP*I*+H)$^+$ and $PLIFS^+$/$FSPLI^+$ can be similarly cyclized to form cyclo(IFSP*L*+H)$^+$. Note that the fifth residue of the former CPI is Ile, and that of the latter is Leu. The two CPIs are able to dissociate via similar fragmentation pathways to produce the identical CAD spectra because the isomeric residues Ile and Leu have similar features. The evidences described above strongly prove our assumption about cyclization reaction.

Activation-time adjustment. It has been reported that the similar cyclization reactions of other fragment ions rely on time scales of collisional activation [19, 20, 22]. For experiments performed at the first stage of CAD (MS^2), it was easy to adjust the activation time. However, our experiments were carried out using two stages of CAD (MS^3), so the cyclization reaction of b ions may occur at each stage of CAD, leading to two stages of activation times requiring adjustment. As activation time was reduced, relative intensities of some anomalous ions decreased. However, when the activation time at each stage was less than 3 ms, many desired ions could not be detected due to low signals. Thus, it was difficult to measure the critical value of activation time required for cyclization. On the contrary, we used long activation times (30 to 8000 ms) to study the effect of activation time on the fragment-ion intensity relationship of b_5 from PLIFSPI. No fundamental change was found in comparison between the spectra obtained using activation times of 30 (Figure 1b) and 8000 ms (Figure S4). Figure S5 shows the ratios of the fragment-ion intensities versus the activation time, which reflects the competitive relationship between the normal $b_n \rightarrow b_{n-1}$ pathway and the anomalous CPI$\rightarrow b_n^{rea} \rightarrow b_{n-1}^{rea}$ pathway. b_5–L&I and b_5–F arise from fragmentation of CPI via $MH^+ \rightarrow CPI \rightarrow b_5^{rea} \rightarrow b_4^{rea}$ and $b_5 \rightarrow CPI \rightarrow b_5^{rea} \rightarrow b_4^{rea}$; b_4 derives from fragmentation of the linear b_5 ion via $b_5 \rightarrow b_4$ and from fragmentation of the cyclic b_5 CPI ion via CPI$\rightarrow b_5 \rightarrow b_4$. As the activation time rises, the (b_5–L&I)/b_4 and (b_5–F)/b_4 ratios both increase, indicating that the long time activation inside an ion trap benefits the occurrence of cyclization reaction. However, the increments of the ratios are not large, revealing that the cyclization reaction and the CPI fragmentation can be mainly accomplished in a normal activation time of 30 ms at each stage of CAD.

Fragmentation of synthetic cyclic peptide. To explore whether the CPI produced by b ion cyclization has the same fragmentation pattern as protonated cyclic peptide produced by ESI, the cyclic peptide cyclo(PFNSLA) was synthesized and analyzed by ESI-MS/MS to produce a MS^2 spectrum (Figure S6), which was used for comparison with the MS^3 spectrum of b_6 $PFNSLA^+$ from peptide PFNSLA (Figure 2a). (Cyclo[PFNSLA] has

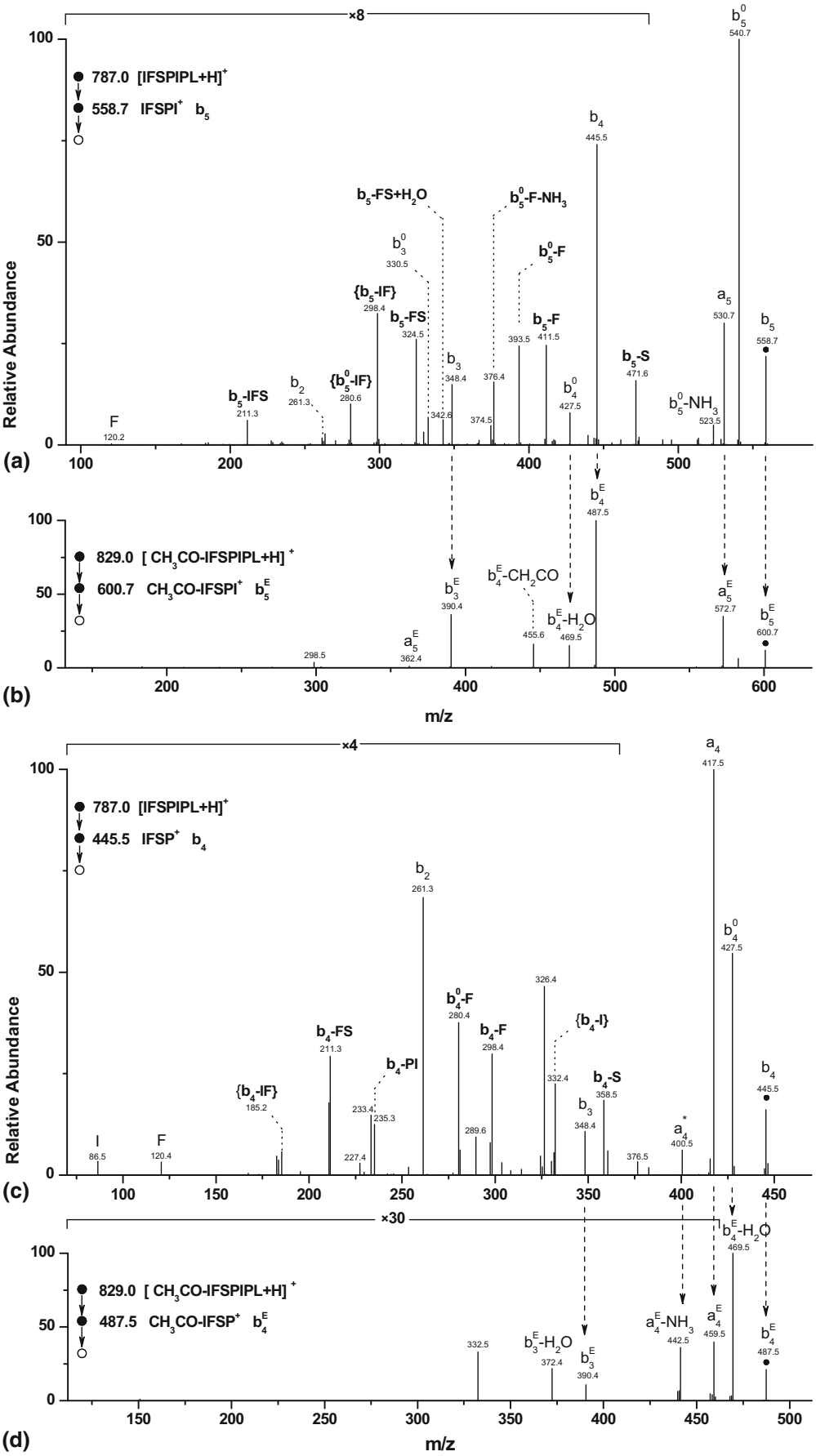


Figure 3. CAD spectra (MS^3) of (**a**) the b_5 ion from peptide IFSPIPL, (**b**) the b_5^E ion from N-acetylated peptide CH_3CO-IFSPIPL, (**c**) the b_4 ion from peptide IFSPIPL, and (**d**) the b_4^E ion from N-acetylated peptide CH_3CO-IFSPIPL.

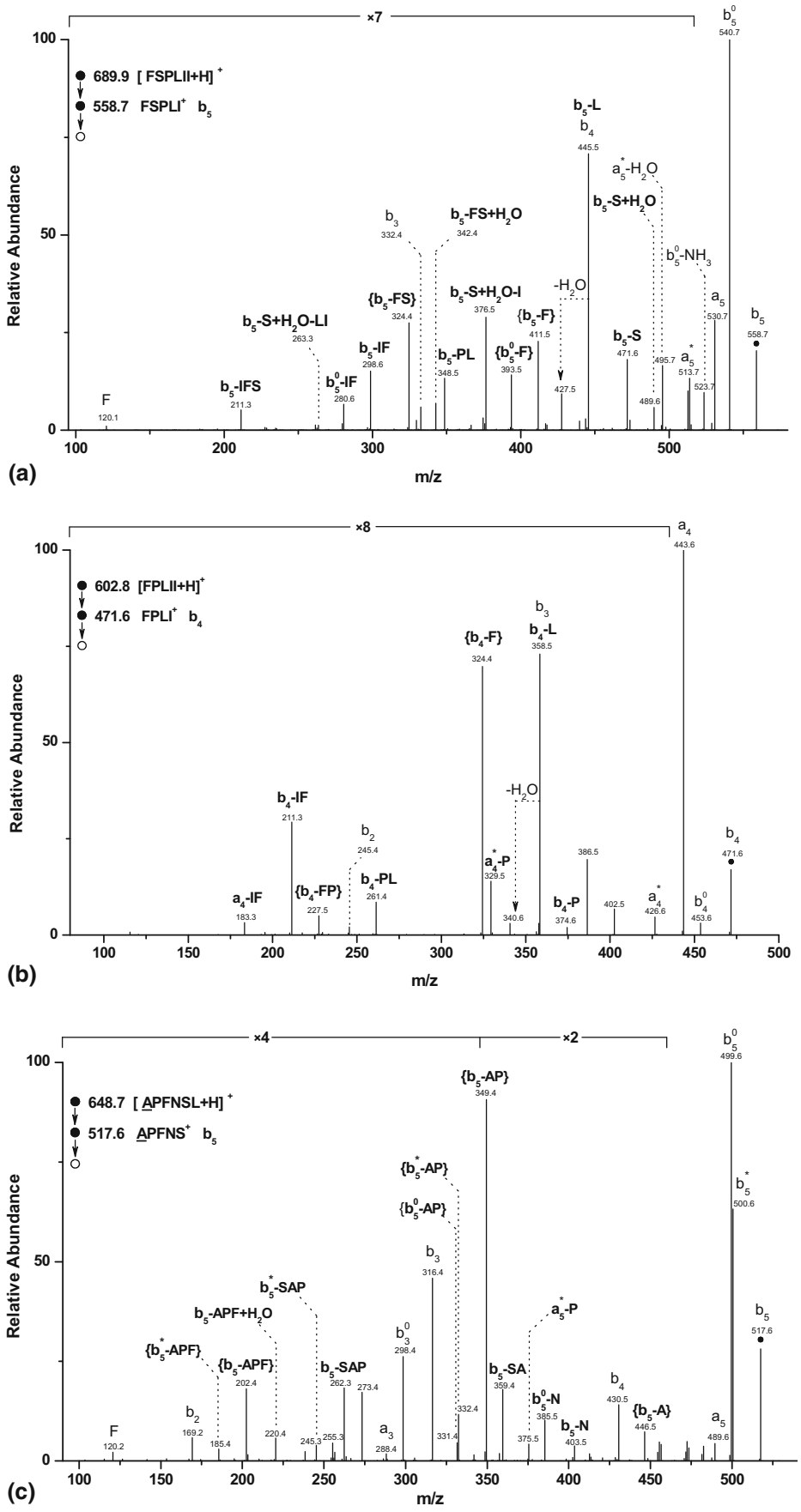


Figure 4. CAD spectra (MS^3) of (**a**) the b_5 ion from peptide FSPLII, (**b**) the b_4 ion from peptide FPLII, and (**c**) the b_5 ion from peptide *A*PFNSL.

been sequenced by multistep MS/MS in our previous work [26].) As we expected, these two spectra have the same fragment ions and relative intensities, strongly demonstrating that CPIs have the same dissociation feature as protonated cyclic peptides. This experimental result also clarifies the fact that the normal b ions, such as b_3 (*m/z* 359.4) and b_2 (*m/z* 245.3) in Figure 2a, mostly originate from CPI fragmentation. Moreover, Harrison et al. [25] reported that the b_5 ion from peptide LFGAY gave a breakdown graph, which was very similar to that of protonated cyclo(LFGAY). This finding also confirms the assumption proposed above.

Exploring Predictive Rules for the Unique Fragmentation of b-Type Ions

Because the $MH^+ \rightarrow CPI \rightarrow b_n^{rea}$ or $b_n \rightarrow CPI \rightarrow b_n^{rea}$ pathway includes the processes of cyclization and ring opening, the predictive rules involve two aspects, i.e., predictability of cyclization reaction and predictability of CPI ring opening.

Predictive rules for cyclization reaction. All of the six parent b ions in Figures 1 and 2, in which have undergone cyclization reaction, have Pro residues located at N-termini. The Pro residue with a high proton affinity [28] is easy to initiate a nucleophilic attack on the C-terminus or one amide bond. Do the N-terminal Pro residues contribute to the high-frequency of cyclization reactions? To answer this question, we performed peptide modification. The b ion *A*PFNS$^+$ was designed to compare its CAD spectrum with that of PFNS$^+$ in Figure 2c. An Ala residue is added to the N-terminus of PFNS$^+$ to block the Pro residue without breaking the C-terminal conformation in gas phase. In Figure 4c many anomalous ions are presented, demonstrating that *A*PFNS$^+$ have also suffered from cyclization. This means that the N-terminal Pro has no obvious relationship with the occurrence of cyclization reactions.

Other investigations [19,22] have demonstrated that displacing internal residues cannot prevent a- and b^{2+}-type ions from cyclizing. Nevertheless, for cyclic peptide synthesis in solution or solid phase, the yield of cyclization strongly relies on choice of cyclization sites, i.e., the two terminal residues of linear peptides [42]. This means that the two terminal residues possibly affect b ion cyclizations. Moreover, in Figure 1a, b, and c, b_6 (PLIFSP$^+$), b_5 (PLIFS$^+$), and b_4 (PLIF$^+$) as parent ions all undergo cyclization reactions, revealing that the change of the C-terminal residues cannot influence cyclization reactions. Also, the cyclizations of the b ions from peptides PFNSLAI (Figure 2) and IFSPIPL (Figure 3) all support this point. As a result, the most possible factor to affect cyclization reactions is the characteristics of N-terminal residues. To examine whether N-terminal residues control the cyclization process in gas phase, two peptides *L*PVNPFV and *V*PVNPFV (precursors of Axinastatin 3 and 1 [43]) were synthesized. Figure 5a and b show the MS^3 spectra of b_6 (*L*PVNPF$^+$, *m/z* 668.8) from peptide *L*PVNPFV and b_6 (*V*PVNPF$^+$, *m/z* 654.8) from peptide *V*PVNPFV, respectively. Apparently, the two b ions have undergone cyclization reactions. Furthermore, the two b_6 ions display similar fragmentation patterns. For instance, b_6–PV at *m/z* 472.6 in panel A corresponds with b_6–PV at *m/z* 458.5 in panel B; b_6–VN at *m/z* 455.6 in panel A corresponds with b_6–VN at *m/z* 441.6 in panel B. Similarly, the phenomenon has also been found in the MS^3 spectra of the b_5 ions from these two peptides (Figure 5c, d). Overall, no evidence reveals that the cyclization reaction is predictable. In other words, the unique fragmentation reaction is very intensive.

Predictive rules for CPI ring opening. To some extent, the CPI ring opening may be predicted. As discussed above, the CPIs and the protonated cyclic peptides have the same fragmentation pattern. It has been reported that fragmentations of protonated cyclic peptides containing Pro, Asn, Gln, Asn, and Glu always display selective ring-opening behaviors [10, 26, 44, 45]. For example, the amide bonds Xxx-Pro [28] and Asn/Gln-Xxx [16, 29] show enhanced cleavage. Similarly, the fragmentations of CPIs also display the selective ring-opening behaviors. Table 1 lists all of the investigated b ions, the formed CPIs and the preferred ring-opening amide bonds, which clearly exhibits the "Pro and Asn/Gln effects" acting on CPIs. As a result, if we consider CPIs as protonated cyclic peptides, many of the fragmentations will be predictable.

Finally, two questions remain to be addressed. First, the b_6–S + H_2O ion at *m/z* 586.6 and the b_6–FS + H_2O ion at *m/z* 439.7 are presented in Figure 1a. Since the parent b_6 ion PLIFSP$^+$ contain a Ser residue, this type of ions belongs to b_{n-1} + H_2O [46, 47] or b_{n-1} + H + OH [48] ions. These ions can also be found in other CAD spectra of serine-containing ions. Second, in Figure 5a it is observed that b_6–N at *m/z* 554.7 is accompanied by a_6–N at *m/z* 526.7. This phenomenon that b-type ions are accompanied by a-type ions can also be observed from other spectra (Figure 5b, c, d). This proves that the anomalous ions have the features of b-type ions as assumed above.

Cyclization Reaction of Singly Charged a-Type Ions

Vachet et al. [19] described that the cyclization reaction of the singly charged a-type ion accompanied by an NH_3 loss can induce an internal residue loss from the second position of C-terminus. This unique fragmentation was observed when a mixture of helium and 5% xenon was used as collision gas in an ion trap. In our CAD experiments using pure helium as collision gas, the same phenomenon has also been found. In addition, we noted that the internal residues can be eliminated from different positions.

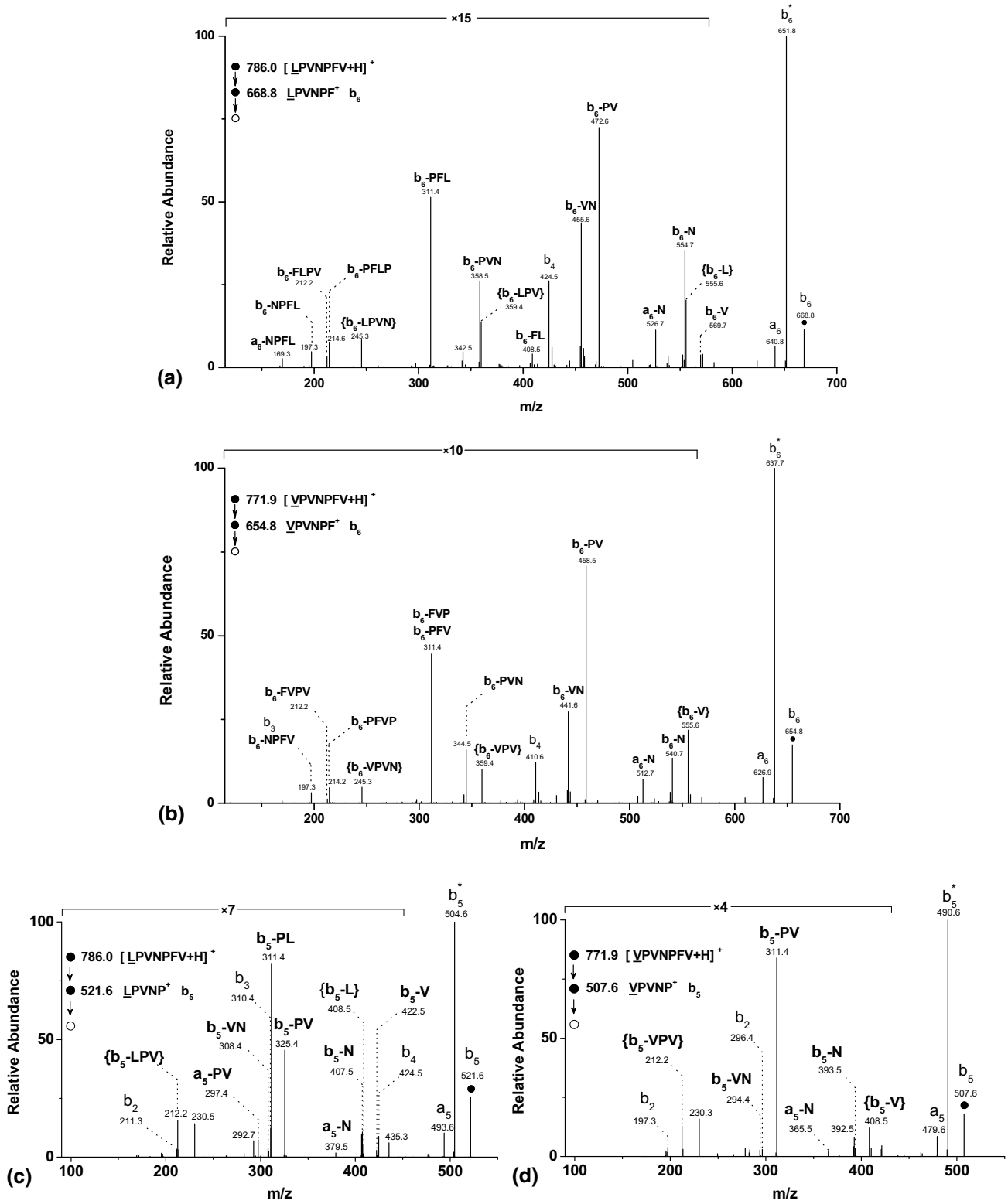


Figure 5. CAD spectra (MS^3) of the b_6 ions from peptides, (**a**) *L*PVNPFV and (**b**) *V*PVNPFV; CAD spectra (MS^3) of the b_5 ions from peptides, (**c**) *L*PVNPFV and (**d**) *V*PVNPFV.

Experimental results. In the MS^3 spectrum of a_5 IFSPI$^+$–CO from peptide IFSPIPLI (Figure 6), the anomalous ions a_5*−P at *m/z* 416.5 and a_5*−SP at *m/z* 329.5 are presented. In the MS^4 experiment on a_5*–P (Figure S7A), a_5*–SP (100%) was detected. The results indicate that the Pro residue is lost from the second position of C-terminus of IFS*P*I$^+$–CO. Similarly, a_5*–F at *m/z* 366.6 and a_5*–IF at *m/z* 253.5 are presented in Figure 6; in a_5*–IF

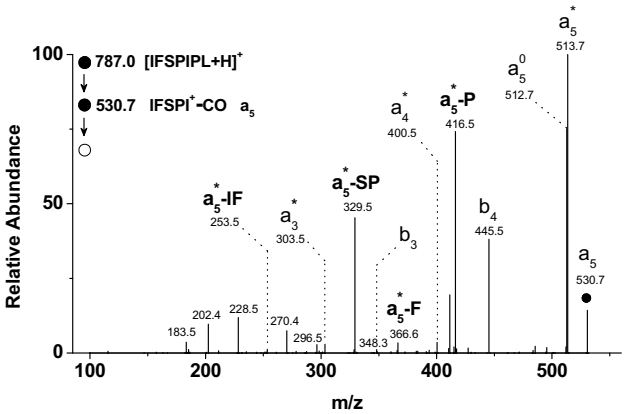


Figure 6. CAD spectrum (MS^3) of the a_5 ion from peptide IFSPIPL.

(100%) was observed in the MS^4 experiment on a_5*–F (Figure S7B). The results demonstrate that the Phe residue is lost from the fourth position of the C-terminus of I*F*SPI$^+$–CO. When peptide IFSPIPL is N-acetylated, the anomalous fragmentation no longer occurs (Figure S8), indicating that the cyclization also needs a free N-terminus. Table 2 lists the parent a-type ions and the observed anomalous ions in this study. Interestingly, the acquired spectra are always dominated by a_n*–P ions, indicating that Pro residues are preferred to lose from interior of parent ion. In other words, the amide bond Pro-Xxx in CPIs formed by a-type ion cyclization is preferred to cleave, which contrasts with the conventional concept that Xxx-Pro is easy to cleave [28].

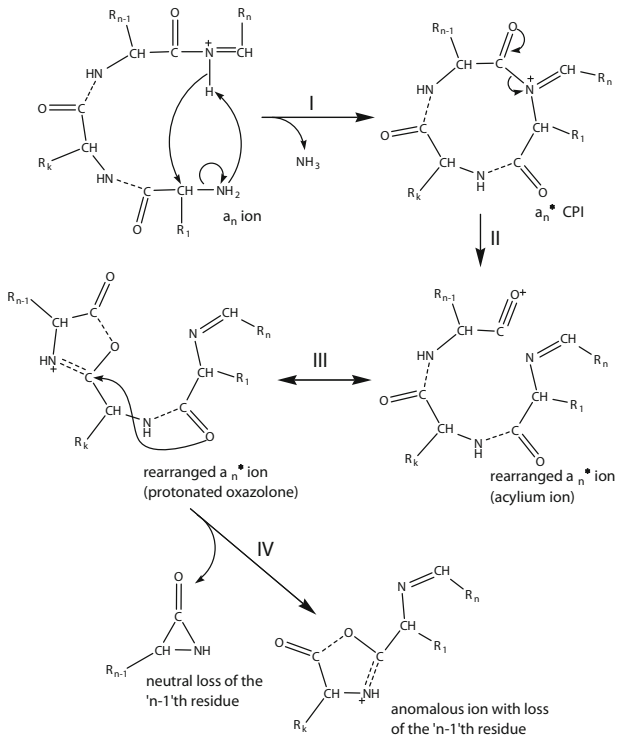
Proposed mechanism. A proposed mechanism of the unique fragmentation is shown in Scheme **4**. The a_n ion can be cyclized to form a CPI accompanied by an NH_3 loss (Pathway I) [19, 24]. The CPI reopens to produce the rearranged a_n* ion (acylium ion or protonated oxazolone) where the second residue (the 'n-1'th residue) of the C-terminus of the parent ion relocate at the C-terminus of the new ion (Pathways II and III). The rearranged a_n* ion with the feature of b-type ions further dissociates via $b_n \rightarrow b_{n-1}$ pathways to generate the anomalous ion with loss of the second residue (the 'n-1'th residue) of C-terminus (Pathway IV). In our experiment, the anomalous ions with losses of residues at other positions have been found as depicted above, indicating that the CPI is able to reopen at other amide bonds (Pathway I in Scheme **5**). The 'k'th residue can be removed by further dissociation of the rearranged a_n^A ion (Pathway III).

It should be noted that the ring opening of a protonated cyclic peptide is initiated by a proton transfer, whereas the a-type ion CPI is a fixed-charge structure without any mobile protons. Therefore, the "Pro effect" cannot influence CPI ring openings, causing Xxx-Pro no longer to be the preferred cleavage site. Why do amide bonds Pro-Xxx in the CPIs exhibit enhanced cleavage?

Table 2. a-Type ions undergoing cyclization reaction in this investigation

Peptide	Parent a-type ions undergoing cyclization reaction[a]		Anomalous fragment ions with internal amino-acid residue losses		Preferred ring-opening amide bond of CPI[b]
IFSPIPL	a_5	IFS**P**I$^+$–CO	a_5*–P (*m/z* 416.5, 74%)	a_5*–SP (*m/z* 329.5, 45%)	Pro-Ile
			a_5*–F (*m/z* 366.6, 3%)	a_5*–IF (*m/z* 253.5, 1%)	
LPVNPFV	a_6	LPVN<u>**P**</u>F$^+$–CO	a_6*–P (*m/z* 526.8, 100%)	a_6*–NP (*m/z* 412.7, 12%)	Pro-Phe
	a_5	L**P**VN**P**$^+$–CO	a_5*–P (*m/z* 379.3, 100%)		—[c]
	a_4	L**P**VN$^+$–CO	a_4*–P (*m/z* 282.2, 12%)	a_4*–LP (*m/z* 169.4, 2%)	Pro-Val
VPVNPFV	a_6	V**P**VN<u>**P**</u>F$^+$–CO	a_6*–P (*m/z* 512.6, 100%)	a_6*–NP (*m/z* 398.7, 22%)	Pro-Phe
			a_6*–VNP (*m/z* 299.6, 4%)		
	a_5	V**P**VN**P**$^+$–CO	a_5*–P (*m/z* 365.5, 64%)		—[c]
	a_4	V**P**VN$^+$–CO	a_4*–P (*m/z* 268.3, 7%)	a_4*–VP (*m/z* 169.4, 5%)	Pro-Val
FPQPFPFI	a_7	FPQPF**P**F$^+$–CO	a_7*–P (*m/z* 719.9, 100%)	a_7*–FP (*m/z* 572.9, 44%)	Pro-Phe
			a_7*–PFP (*m/z* 475.8, 59%)		
	a_5	F**P**QPF$^+$–CO	a_5*–P (*m/z* 475.7, 100%)	a_5*–FP (*m/z* 328.5, 42%)	Pro-Gln
PFNSLAI	a_6	**P**F<u>NS</u>LA$^+$–CO	a_6*–P (*m/z* 488.6, 44%)	a_6*–L (*m/z* 472.6, 19%)	Pro-Phe
			a_6*–S (*m/z* 498.6, 17%)	a_6*–NS (*m/z* 384.7, 14%)	
	a_5	**P**F<u>NS</u>L$^+$–CO	a_5*–P (*m/z* 417.5, 15%)	a_5*–S (*m/z* 427.6, 9%)	Pro-Phe
			a_5*–NS (*m/z* 313.5, 9%)		
PLIFSPI	a_5	**P**LIFS$^+$–CO	a_5*–P (*m/z* 416.8, 11%)	a_5*–L (*m/z* 400.6, 2%)	Pro-Leu
			a_5*–PL (*m/z* 303.6, 5%)		
	a_4	P**LI**F$^+$–CO	a_4*–P (*m/z* 346.5, 1%)	a_4*–L/I (*m/z* 313.4, 11%)	—[c]
FSPLII	a_5	FS**P**LI$^+$–CO	a_5*–P (*m/z* 416.6, 8%)	a_5*–SP (*m/z* 329.5, 5%)	Pro-Leu
			a_5*–F (*m/z* 366.6, 2%)		
	a_4	FS**P**L$^+$–CO	a_4*–P (*m/z* 303.3, 100%)		Pro-Leu
LPPFI	a_4	L**PP**F$^+$–CO	a_4*–P (*m/z* 313.4, 100%)		Pro-Pro/Phe

[a]The internal residues which are preferred to lose are labeled in bold font. The two residues Asn-Xxx are underlined, indicating that the residue at the right position of Asn is easy to lose.
[b]When the CPI dissociates via more than one ring-opening pathway, the preferred one is listed.
[c]The preferred ring-opening amide bond cannot be determined.



Scheme 4

A tentative explanation is given as follows. The ring-opening pathway of the CPI is reversible (Pathways I and II in Scheme **5**). In the process of fragmentation, if the rearranged a_n* ion never dissociate timely to produce anomalous ions with internal residue losses (Pathway III), the reversible reaction will occur to reform the CPI (Pathway II). The a_n* acylium ion can transform to the protonated oxazolone ion. The latter structure has a mobile proton, so it can be affected by the "Pro effect". Therefore, if a Pro residue is relocated at the C-terminus of the rearranged a_n* ion, the amide bond Xxx-Pro will be preferred to cleave via the $b_n \rightarrow b_{n-1}$ pathway to form an a_n*−P ion (Pathway III), driving the reversible reaction toward right orientation (Pathway I). On the basis of the "pathways in competition" model [16], the ring-opening pathways of the CPI are competitive. As a result, the amide bond Pro-Xxx in CPI is preferred to dissociate. In a word, the "Pro effect" cannot act on ring opening of CPI, but can act on dissociation of the rearranged a_n* ion formed by ring opening of CPI. Consequently, it can be concluded that the enhanced cleavage of a_n* ion at Xxx-Pro results in the preferred ring opening of CPI at Pro-Xxx. Additionally, another interesting finding can further support this explanation. From Table 2 it is found that the residue at the right position of Asn is easy to lose, revealing that the right amide bond of Asn-Xxx-Xxx in CPI is preferential to cleave. The phenomenon also indicates that the "Asn effect" cannot act on ring opening of CPI, but can act on dissociation of the rearranged a_n* ion.

Conclusions

In this investigation, it was found that internal amino acid residues could be lost from singly charged b- and a-type ions during multistage CAD. Our results indicate that the unique fragmentations are induced by cyclization reactions.

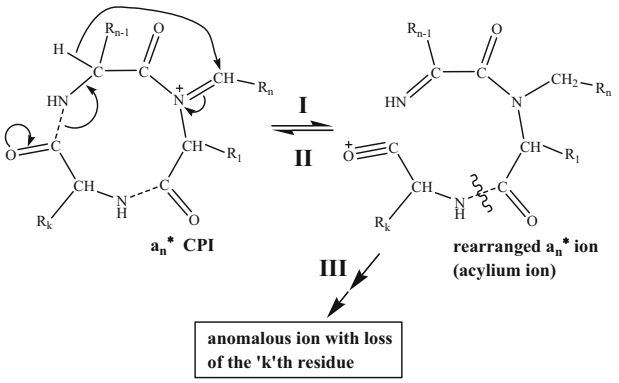
b-Type Ions

The cyclization of b ion is initiated by a nucleophilic attack of the N-terminal nitrogen on the carbonyl carbon at C-terminus or at one amide bond to produce a CPI. Subsequently, the CPI is reopened at another position and an internal residue is relocated at the C-terminus. The ring-opened structure further dissociates via $b_n \rightarrow b_{n-1}$ pathways to produce the anomalous ions with internal residue losses. This reaction can be mainly completed in activation time of 30 ms. The CPI produced by b ion cyclization displays the same fragmentation pattern as the protonated cyclic peptide produced by ESI, and shows selective ring-opening behaviors owing to the "Pro and Asn/Gln effects".

a-Type Ions

The a-type ion can be cyclized to form a CPI accompanied by an NH_3 loss. The CPI may then reopen at different positions to generate rearranged a_n* ions with the feature of b-type ions. The rearranged a_n* ions further dissociate via $b_n \rightarrow b_{n-1}$ pathways to form anomalous ions with internal residue losses. The CPI is a fixed-charge structure, whereas the rearranged a_n* ion has a mobile proton. Therefore, the "Pro and Asn effects" cannot act on the former but can act on the latter.

Overall, we studied a limited number of synthetic peptides, so we cannot yet conclude that the cyclizations of b- and a-type ions are very general phenomena for other peptides, such as tryptic and natural peptides. If the phenomena receive further attention, the new cyclization reactions of other ions will possibly be discovered and the misidentification of fragments and sequences of peptides will be avoided.



Scheme 5

Acknowledgments

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