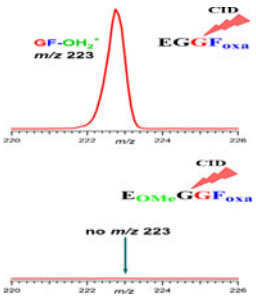
J. Am. Soc. Mass Spectrom. (2013) 24:1543–1554
DOI: 10.1007/s13361-013-0694-x

RESEARCH ARTICLE

Protonated Dipeptide Losses from b_5 and b_4 Ions of Side Chain Hydroxyl Group Containing Pentapeptides

A. Emin Atik, Talat Yalcin

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



Abstract. In this study, C-terminal protonated dipeptide eliminations were reported for both b_5 and b_4 ions of side chain hydroxyl group (–OH) containing pentapeptides. The study utilized the model C-terminal amidated pentapeptides having sequences of XGGFL and AXVYI, where X represents serine (S), threonine (T), glutamic acid (E), aspartic acid (D), or tyrosine (Y) residue. Upon low-energy collision-induced dissociation (CID) of XGGFL (where X=S, T, E, D, and Y) model peptide series, the ions at *m/z* 279 and 223 were observed as common fragments in all b_5 and b_4 ion (except b_4 ion of YGGFL) mass spectra, respectively. By contrast, peptides, namely S_{Me}GGFL-NH_2 and E_{OMe}GGFL-NH_2, did not show either the ion at *m/z* 279 or the ion at *m/z* 223. It is shown that the side chain hydroxyl group is required for the possible mechanism to take place that furnishes the protonated dipeptide loss from b_5 and b_4 ions. In addition, the ions at *m/z* 295 and 281 were detected as common fragments in all b_5 and b_4 ion (except b_4 ion of AYVYI) mass spectra, respectively, for AXVYI model peptide series. The MS^4 experiments exhibited that the fragment ions at *m/z* 279, 223, 295, and 281 entirely reflect the same fragmentation behavior of $[M+H]^+$ ion generated from commercial dipeptides FL-OH, GF-OH, YI-OH, and VY-OH. These novel eliminations reported here for b_5 and b_4 ions can be useful in assigning the correct and reliable peptide sequences for high-throughput proteomic studies.

Key words: Dipeptide loss, Peptide fragmentation, Water migration, Macrocyclization

Received: 22 February 2013/Revised: 6 June 2013/Accepted: 9 June 2013/Published online: 31 July 2013

Introduction

Tandem mass spectrometry (MS/MS) with collision-induced dissociation (CID) has become an invaluable technique in peptide sequencing and protein identification with the introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [1–7]. Upon low-energy CID, the amide bonds along the peptide backbone have been cleaved. During this cleavage, a series of complementary sequence-informative *b* and *a* and/or *y* fragment ions have been produced as a result of charge retention on the N- or C-terminal, respectively [8, 9]. Knowledge of the fragmentation routes of these ions has enabled developing database search algorithms and bioinformatics tools, which are used to deduce the peptide sequence for high throughput proteomic studies [10, 11]. In the last two decades, considerable effort has been devoted for understanding the structures and dissociation mechanisms of the gas-phase *b* ions [12–16]. In early studies, the acylium ion structure was adopted for *b* ions [8, 9]. However, extensive studies [14, 15] have shown that the most stable structure for the small b_n ions (n=2–4) is protonated five-member oxazolone ring at the C-terminal of the peptide. Moreover, the theoretical calculations and gas-phase H/D exchange studies have supported that the b_2 ion has an oxazolone structure [16–18]. Additionally, Polfer et al. [19, 20] have presented infrared multi-photon dissociation (IRMPD) experiments as well as density functional theory (DFT) calculations to support the oxazolone structure for b_4 ions originated from Leu-enkephalin.

The formation of $b_n + H_2O$ ions (where n represent the total number of residues in the peptides) through fragmentation of a variety of protonated peptides in the gas phase have been reported over the years [21–26]. Gaskell and co-workers [21] have reported for the first time the rearranged $[b_{n-1} + H_2O]$ product ions through metastable decomposition of $[M + H]^+$ ions obtained from N-terminal basic amino acid containing peptides. Additionally, Ballard and Gaskell used the ^{18}O isotopic exchange experiments to demonstrate the rearrangement reactions can generate $[b_n + H_2{}^{18}O]$ product

Electronic supplementary material The online version of this article (doi:10.1007/s13361-013-0694-x) contains supplementary material, which is available to authorized users.

Correspondence to: Talat Yalcin; *e-mail:* talatyalcin@iyte.edu.tr

ion containing labeled oxygen in the MS/MS spectra of protonated or cationized peptides [22]. On the other hand, Fang et al. [23] showed that the formation of novel rearranged product ions designated as [b_{n-1} + H_2O] for serine- or threonine-containing bradykinin and its synthetic derivatives in MALDI-TOF post-source decay (PSD) mass spectra. The authors demonstrated that the hydroxyl group was transferred to the neighboring residue's carbonyl group, which led to a loss of the C-terminal end of the peptide [23]. Similarly, Hiserodt and co-workers highlighted that [b_1+ H_2O] product ion was formed via rearrangement reaction for the dipeptides containing lysine, arginine, and histidine residues located at the N-terminal position [24]. Farrugia and O'Hair [25] reported the importance of salt-bridge (SB) intermediate formation that yields [b_n + H_2O] product ion by a charge-directed mechanism in mass spectra of protonated arginine containing dipeptides. In addition to this, Gonzalez et al. [26] emphasized the effect of the position of arginine residue within a series of synthetic tetra- to heptapeptides for the formation of [b_{n-1} + H_2O] product ion through C-terminal rearrangement reaction.

In this work, the gas-phase fragmentation behaviors of b_5 and b_4 ions originated from XGGFL-NH_2 and AXVYI-NH_2, where X is either serine (S), threonine (T), glutamic acid (E), aspartic acid (D), or tyrosine (Y) residue, are reported. The common feature of these five amino acid residues is that they contain a hydroxyl group (-OH) on their side chains.

Experimental

The synthetic C-terminal amidated model pentapeptides, such as SGGFL-NH_2, S_{Me}GGFL-NH_2 (methylation on the side chain of serine), TGGFL-NH_2, EGGFL-NH_2, E_{OMe}GGFL-NH_2 (methylation on the side chain of glutamic acid), DGGFL-NH_2, YGGFL-NH_2, ASVYI-NH_2, ATVYI-NH_2, AEVYI-NH_2, ADVYI-NH_2, and AYVYI-NH_2 and free acid forms of dipeptides, such as FL-OH, LF-OH, GF-OH, FG-OH, YI-OH, IY-OH, VY-OH, and YV-OH were obtained from GL Biochem Ltd. (Shanghai, China) and used without further purification. One to two mg of each solid peptide was dissolved in 1:1 (vol/vol) mixture of HPLC-grade methanol and deionized water to make stock solutions with a concentration of 10^{-3} or 10^{-4} M.

All tandem mass spectrometry (MS^n) experiments were conducted on a LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The stock peptide samples were diluted to a 100 pmol μL^{-1} in 50:50:1 (vol/vol/vol) MeOH/H_2O/HCOOH and introduced via infusion at a flow rate of 5 $\mu L\ min^{-1}$ with an incorporated syringe pump. Before the experiments, the instrument was calibrated with a Calmix solution (company's calibration mixture) containing caffeine, MRFA, and Ultramark 1621. The ion optics (multipoles, lens voltages, etc.) were optimized in order to obtain maximal precursor ion transmission into the trap. The experimental conditions were the similar as those described previously [27]. Briefly, the scan range was from *m/z* 150 to 700 in the positive-ion mode for all MS^n stages, and at least 400 scans were averaged in profile mode. Spray voltage was set at +5.0 kV, and the heated capillary (desolvation) temperature was maintained at 275 °C. Nitrogen gas was used as a sheath, a sweep, and an auxiliary gas during the experiments, whereas helium was introduced into the system for both as the collision gas and as a damping gas. The capillary voltage was kept at 20 V. The mass isolation window (*m/z*) was varied between 1.6 and 2.2 for MS^n acquisitions in order to isolate a single and the most abundant precursor ion. The activation time was set to 30 ms at each CID stage using an activation (*q*) of 0.250 and the normalized collision energy was varied from 18 % to 28 % for the dissociation of selected precursor ion. Data acquisition was carried out with Xcalibur (ver. 2.0) software data system.

Result and Discussion

The Formation of m/z 279 Fragment Ion in the b_5 Ion Mass Spectra of XGGFL-NH_2 (where X Is S, T, E, D, or Y)

The CID mass spectra of the b_5 ions obtained from SGGFL-NH_2, TGGFL-NH_2, EGGFL-NH_2, DGGFL-NH_2, and YGGFL-NH_2 peptides were studied separately via low-energy CID-MS^3 ($[M + H]^+ \rightarrow b_5$) consecutive experiments. The peptide sequences were confirmed by MS/MS spectrum of their $[M + H]^+$ ion, as shown in Figure S1 of the Electronic Supplementary Material of this article. It is worth mentioning that the fragment ion at *m/z* 279 appeared in all b_5 ion mass spectra as a common peak. Table 1 lists the relative intensities of the *m/z* 279 fragment ions originated from each b_5 ion.

The CID mass spectrum of the b_5 ion derived from SGGFL-NH_2 ($SGGFL_{oxa}$, *m/z* 462) is shown in Figure 1a. The *m/z* 279 fragment ion was detected with a relative intensity of 7.23 % to the base peak of b_5-H_2O (denoted as b_5^o). The MS^4 ($[M + H]^+ \rightarrow b_5 \rightarrow 279$) consecutive experiments were conducted for the *m/z* 279 fragment ion in order to elucidate its gas-phase fragmentation pattern as well as the

Table 1. Relative Intensities of *m/z* 279 and 223 Fragments in the b_5 and b_4 ions CID Mass Spectra of XGGFL-NH_2 (Where X Is S, T, E, D, or Y) Model Peptides, Respectively

Peptide	Relative intensity (%)	
	m/z 279	*m/z* 223
SGGFL-NH_2	7.23	2.51
TGGFL-NH_2	10.3	0.440
EGGFL-NH_2	0.586	100
DGGFL-NH_2	0.266	0.852
YGGFL-NH_2	0.00659	ND

ND=not detected

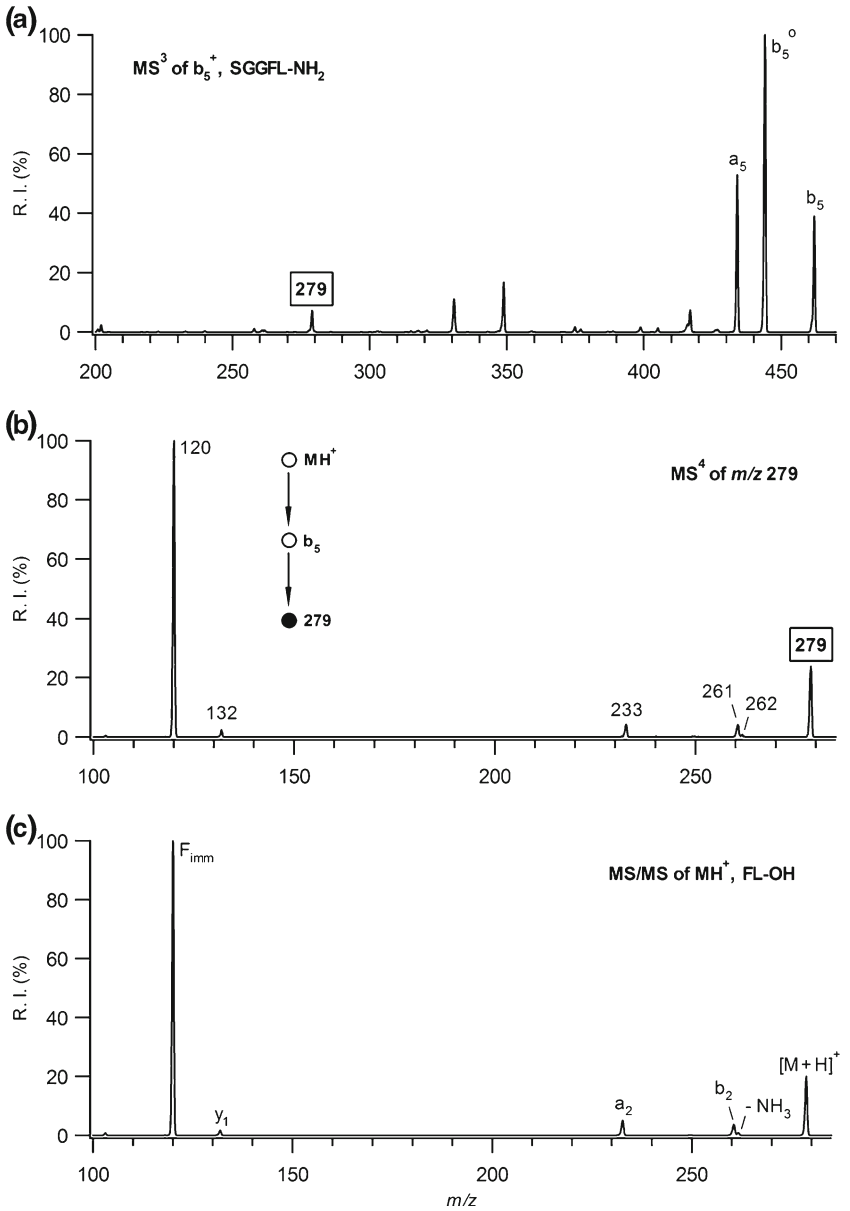


Figure 1. **(a)** MS^3 mass spectrum of b_5 ion from protonated SGGFL-NH_2, **(b)** MS^4 mass spectrum of m/z 279 ion from b_5 ion of protonated SGGFL-NH_2, **(c)** MS^2 mass spectrum of $[M + H]^+$ ion from protonated FL-OH

structure through CID. The CID-MS^4 spectrum (see Figure 1b) is dominated by the product ion at m/z 120 with weak intensities of the ions at m/z 132, 233, 261, and 262. The strong m/z 120 product ion is an evidence for the presence of phenylalanine (phenylalanine immonium) within the sequence of m/z 279 ion [28] and the ion at m/z 132 could be accepted as an evidence of protonated leucine. Therefore, it could be concluded that phenylalanine (F) and leucine (L) amino acid residues are dissociated together in the form of protonated dipeptide from b_5 ion. The source of the water molecule is thought to be the side chain of serine residue. In order to confirm our proposal, the MS/MS spectra of $[M + H]^+$ ion produced from FL-OH and LF-OH dipeptides (both of them have the same molecular mass) were recorded and compared with the MS^4 spectrum of the m/z 279 fragment ion. It is clearly shown that (Figure 1b and c) the MS/MS spectrum of $[M + H]^+$ ion of FL-OH yields entirely the same fragment ions with the same relative intensities to that originated from the m/z 279 fragment ion derived from b_5 ion of SGGFL-NH_2. In contrast, the fragmentation behavior of LF-OH is totally different from that of m/z 279 ion. The product ion mass spectrum of $[M + H]^+$ ion of LF-OH is shown in Electronic Supplementary Material Figure S2. Therefore, the sequence of m/z 279 fragment ion was verified as a protonated FL-OH dipeptide because of the completely identical fragmentation behaviors under the same CID conditions. Additionally, the product ions at m/z 132, 233, and 261 correspond to the y_1, a_2, and

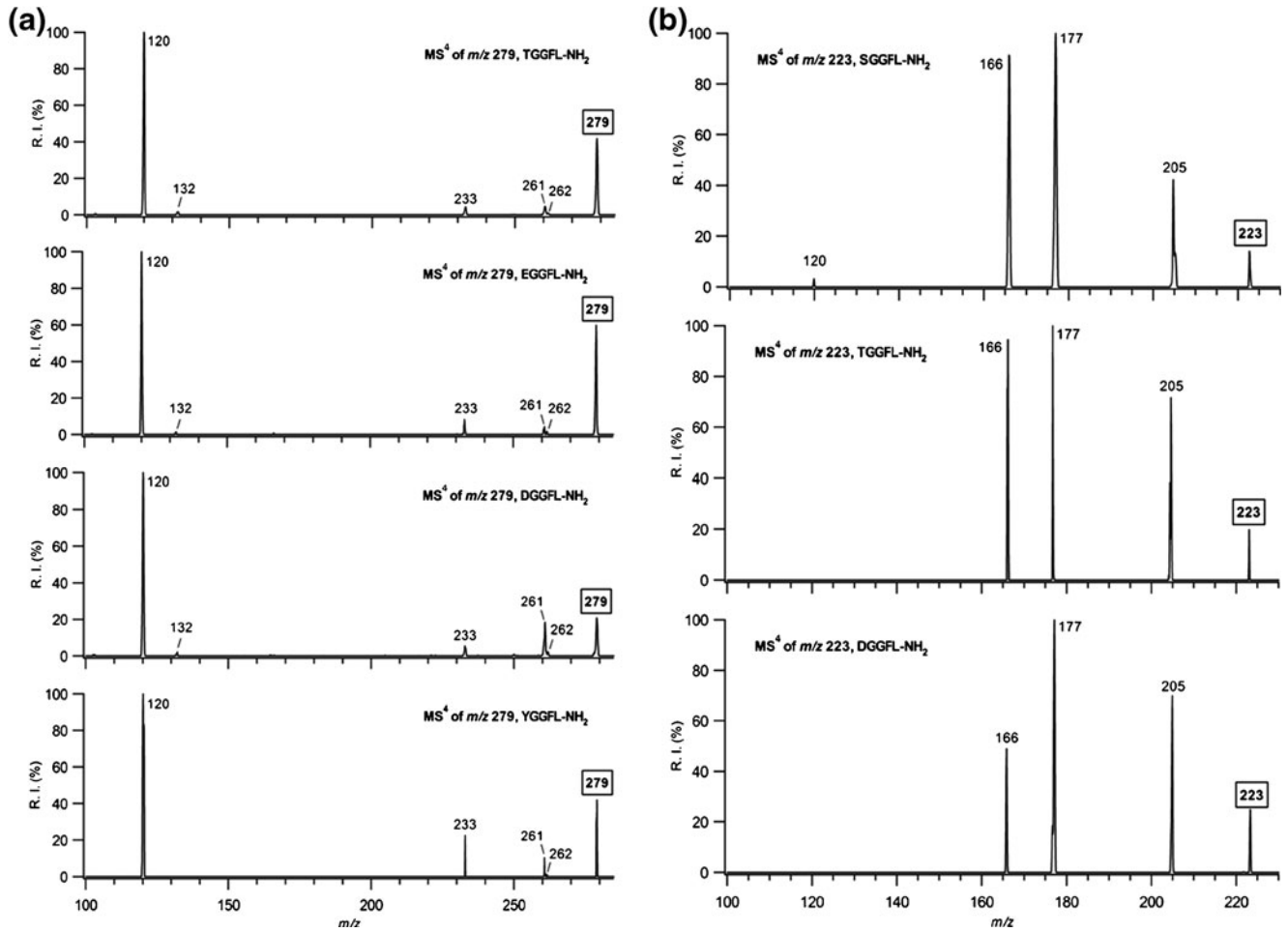


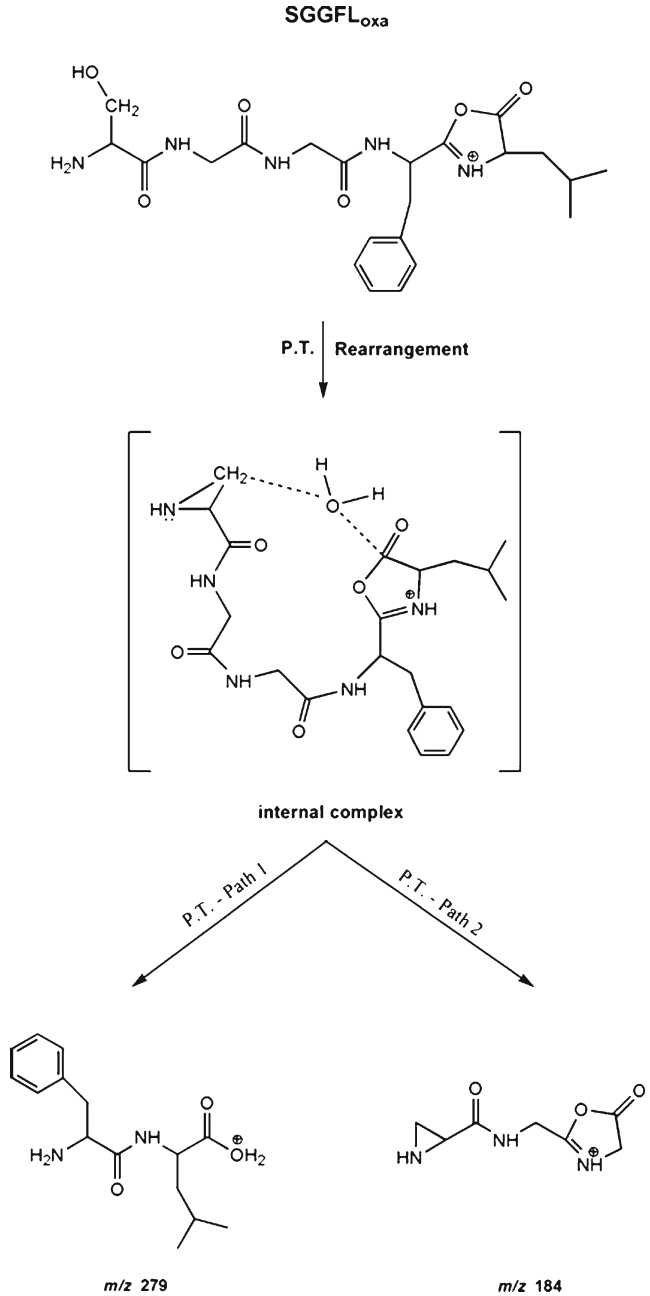
Figure 2. **(a)** Comparison of the MS^4 mass spectra of *m/z* 279 fragment ions ($MH^+ \rightarrow b_5 \rightarrow 279$) originated from protonated TGGFL-NH_2, EGGFL-NH_2, DGGFL-NH_2, and YGGFL-NH_2, respectively. **(b)** Comparison of the MS^4 mass spectra of *m/z* 223 fragment ions ($MH^+ \rightarrow b_4 \rightarrow 223$) originated from protonated SGGFL-NH_2, TGGFL-NH_2, and DGGFL-NH_2, respectively

b_2, respectively, whereas the ion at *m/z* 262 was assigned as ammonia loss (–NH_3) from protonated FL-OH.

Each *m/z* 279 fragment ion was selected from b_5 ions of TGGFL-NH_2, EGGFL-NH_2, DGGFL-NH_2, and YGGFL-NH_2 peptides individually and allowed to dissociate under the same experimental conditions in an ion trap mass spectrometer. As depicted in Figure 2a, the similar fragmentation patterns were obtained in all *m/z* 279 ion's mass spectra which are also the same as the MS/MS spectrum of protonated FL-OH. However, the only difference in the leucine-enkephalin (YGGFL-NH_2) peptide is that the *m/z* 132 ion was not detected in the MS^4 spectrum of *m/z* 279 fragment ion. These results clearly demonstrate that the sequence of *m/z* 279 ions originated from each protonated b_5 ions of XGGFL-NH_2 (where X is S, T, E, D, or Y) are protonated FL-OH.

The water losses from the side chain of hydroxyl group containing amino acid residues have been extensively investigated by several groups [29–36]. In line with these works, a possible mechanism for the formation of *m/z* 279 fragment ion from protonated b_5 ion of SGGFL-NH_2 is shown in Scheme 1. After proton transfer (P.T.) and rearrangement reaction, a water molecule can migrate to the C-terminal oxazolone ring, giving rise to form an intermediate internal complex. The complex was then dissociated to yield protonated FL-OH (*m/z* 279) in the gas phase. Note that the internal complex can follow two competing dissociation paths: either charge retention on the N- or C-terminal resulting in the formation of *m/z* 184 or *m/z* 279 fragment ions. The same mechanism is also acceptable for the formation of the *m/z* 279 fragment ion from the b_5 ions of XGGFL-NH_2 (where X is T, E, or D) peptides. It should be mentioned that the b_5 ion of YGGFL-NH_2 solely follows pathway 1 after dissociation of internal complex. Additionally, the possible fragmentation channel has very low contribution to the formation of *m/z* 279 fragment ion in the dissociation of the b_5 ion of YGGFL-NH_2 (see Table 1). It is known that phenolic hydrogen is not acidic enough and that is why leu-enkaphalin did not show strong protonated dipeptide elimination compare to others [30].

In order to emphasize the involvement of side chain hydroxyl group for the possible mechanism, we used S_{Me}GGFL-NH_2 pentapeptide in which the side chain of serine residue was



Scheme 1. The possible mechanism for the formation of either *m/z* 279 or 184 fragment ions from the b_5 ion of SGGFL-NH_2

methylated. It is clear that the *m/z* 279 fragment ion was not detected in its b_5 ion CID mass spectrum, which is the direct evidence for migration of hydroxyl group from the side chain of serine residue to the C-terminal oxazolone ring (Electronic Supplementary Material Figure S3).

On the other hand, it was reported that the b_5 ions (or even larger) undergo head-to-tail cyclization to form a macrocyclic intermediate [37, 38]. This macrocyclic structure may reopen at different amide bonds to form various linear *b* isomers. In the current study, the b_5 ions derived from XGGFL-NH_2 (where X is S, T, E, D, Y) also form macrocyclic structures, which then generate internal amino acid eliminations from each b_5 ion, as Electronic Supplementary Material Figure S4 illustrates. After reopening of macrocyclic structures, LXGGF$_{oxa}$ b_5 isomer may have been formed. The protonated GF dipeptide losses (*m/z* 223) are expected to be formed in the dissociation of LXGGF$_{oxa}$ isomer using similar fragmentation mechanism. The *m/z* 223 fragment ion was detected in the dissociation of permuted LSGGF$_{oxa}$, LTGGF$_{oxa}$, and LEGGF$_{oxa}$ b_5 isomers (with very low relative intensities), which show nearly the same fragmentation pattern of $[M+H]^+$ ion produced from GF-OH (spectra not shown).

The Formation of m/z 223 Fragment Ion in the b_4 Ion Mass Spectra of XGGFL-NH_2 (where X Is S, T, E, D, or Y)

In addition to the b_5 ions, the b_4 ions from the SGGFL-NH_2, TGGFL-NH_2, EGGFL-NH_2, DGGFL-NH_2, and YGGFL-NH_2 peptides were studied individually by means of low-energy CID-MS^3 consecutive experiments. In this case, the ion at *m/z* 223 was observed in all b_4 ion mass spectra except the YGGFL-NH_2 (leucine-enkephalin). This fragment ion was not detected in the b_4 ion mass spectrum derived from YGGFL-NH_2 because of the same reason as was explained previously. The relative intensities of the *m/z* 223 fragment ions are summarized in Table 1. The CID mass spectrum of b_4 ion (EGGF$_{oxa}$, *m/z* 391) that originated from EGGFL-NH_2 is shown in Figure 3a. It is clear that the *m/z* 223 fragment ion is the most abundant peak in the mass spectrum. In order to identify the gas-phase structure of this fragment ion, the MS^4 ($[M + H]^+ \rightarrow b_4 \rightarrow 223$), consecutive experiments were probed in an ion trap mass spectrometer. The CID mass spectrum of *m/z* 223 fragment ion contains the product ions at *m/z* 166, 177, and 205 as the major peaks with a weak intensity of *m/z* 120 (Figure 3b). The ions at *m/z* 166 and 120 correspond to the masses of protonated phenylalanine residue and its immonium ion, respectively. Hence, we can assume that glycine (G) and phenylalanine (F) residues are dissociated together as a protonated dipeptide form from the b_4 ion. To verify the sequence of protonated *m/z* 223 ion, the $[M + H]^+$ ion MS/MS spectra of GF-OH and FG-OH dipeptides were recorded and the resultant MS/MS spectra were compared with the MS^4 spectrum of the *m/z* 223 fragment ion. The two spectra in Figure 3b and c clearly exhibit that the sequence of *m/z* 223 fragment ion that originated from the b_4 ion of EGGFL-NH_2 is identical to the MS/MS spectrum of protonated GF-OH. The products at *m/z* 120, 166, 177, and 205 refer to the phenylalanine immonium F_{imm}, y_1, a_2, and b_2 ions, respectively. In contrast, the fragmentation behavior of FG-OH is totally different from that of *m/z* 223 ion (shown in Electronic Supplementary Material Figure S2).

The *m/z* 223 fragment ions that originated from the b_4 ions of the SGGFL-NH_2, TGGFL-NH_2, and DGGFL-

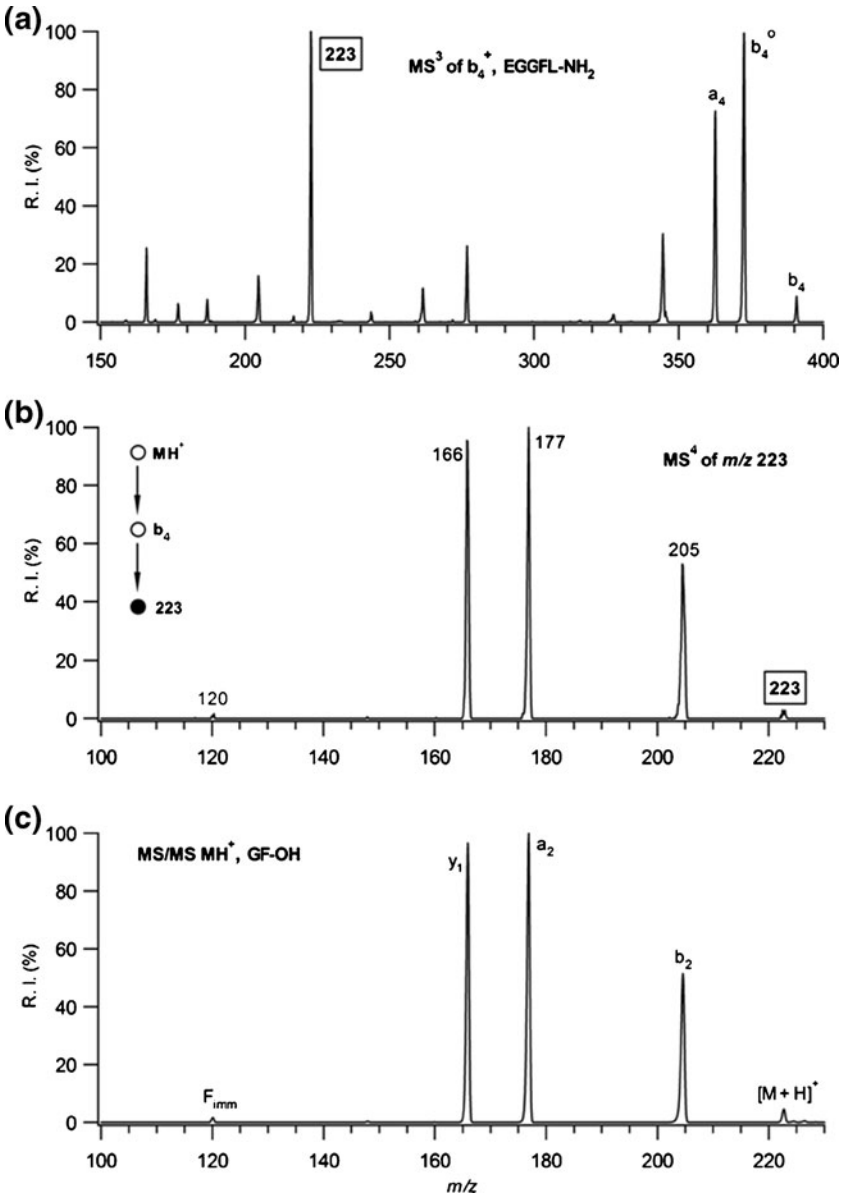
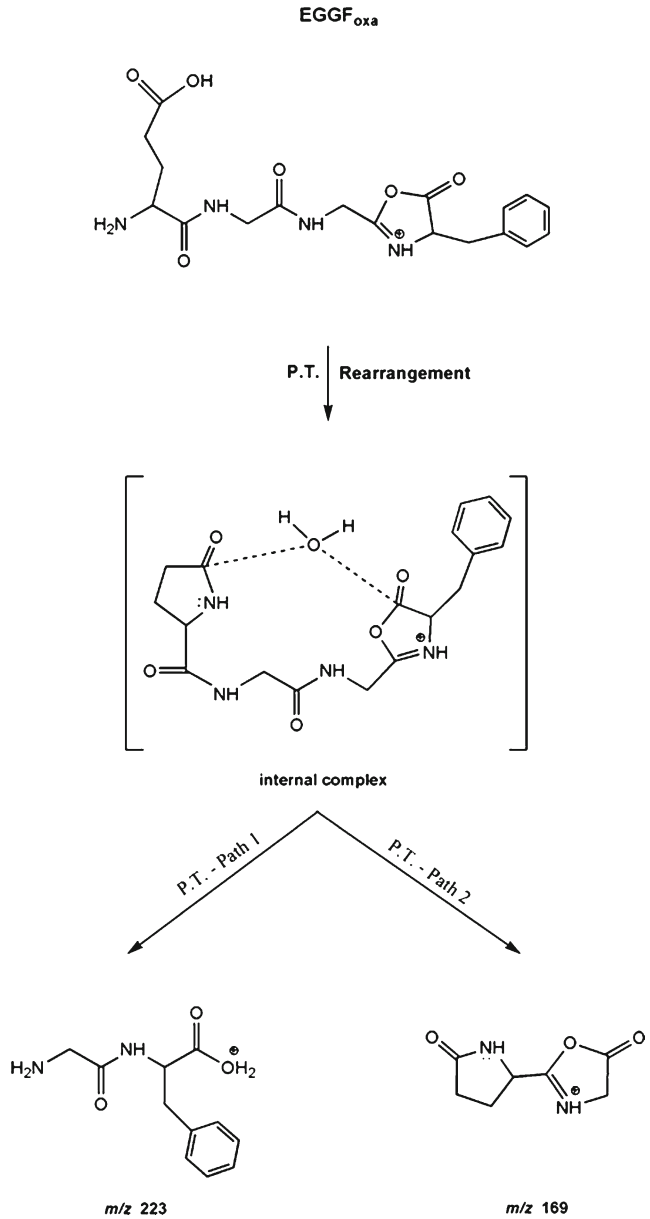


Figure 3. **(a)** MS^3 mass spectrum of b_4 ion from protonated EGGFL-NH_2, **(b)** MS^4 mass spectrum of m/z 223 ion from b_4 ion of protonated EGGFL-NH_2, **(c)** MS^2 mass spectrum of $[M+H]^+$ ion from protonated GF-OH

NH_2 peptides were also studied under the same fragmentation conditions. Similar fragmentation patterns (the same peaks with similar intensities) were obtained (shown in Figure 2b) and were also comparable to the dissociation products of the protonated GF-OH. However, the relatively low intensity of m/z 120 product ion is not detected in the m/z 223 ion's MS^4 mass spectra of TGGFL-NH_2 and DGGFL-NH_2.

The glutamic acid and aspartic acid residues have been known to lose water through dissociation reactions because of the presence of a hydroxyl group on their side chains [27, 29, 30, 36]. In line with these reference works, a possible mechanism for the formation of m/z 223 fragment ion from the b_4 ion of EGGFL-NH_2 is shown in Scheme 2. In this case, two competitive dissociation pathways of internal complex are only valid for the b_4 ion of the EGGFL-NH_2 peptide. (The other b_4 ions, i.e., $SGGF_{oxa}$, $TGGF_{oxa}$, and $DGGF_{oxa}$, may not follow the second pathway through the fragmentation of internal complex).

To gain further evidence of side chain hydroxyl group migration to the C-terminal oxazolone ring, we used side chain methylated glutamic acid containing pentapeptide, E_{OMe}GGFL-NH_2. The CID mass spectrum of b_5 ion clearly shows that the m/z 223 fragment ion has not been observed



Scheme 2. The possible mechanism for the formation of either *m/z* 223 or 169 fragment ions from the b_4 ion of EGGFL-NH_2

after replacing side chain acidic hydrogen with methyl group (Electronic Supplementary Material Figure S5).

The Formation of m/z 295 Fragment Ion in the b_5 Ion Mass Spectra of AXVYI-NH_2 (where X Is S, T, E, D, or Y)

The b_5 ions that originated from ASVYI-NH_2, ATVYI-NH_2, AEVYI-NH_2, ADVYI-NH_2, and AYVYI-NH_2 were isolated via MS^3 ($[M + H]^+ \rightarrow b_5$) experiments individually and allowed to dissociate under low-energy CID conditions. The peptide sequences were validated by MS/MS spectrum of their $[M + H]^+$ ion, as shown in Electronic Supplementary Material Figure S6. It has been demonstrated that the fragment ion at *m/z* 295 was observed in all b_5 ion mass spectra as a common peak, and the relative intensities of the *m/z* 295 fragment ions are tabulated in Table 2. Figure 4a shows the CID mass spectrum of the b_5 ion (ASVYI$_{oxa}$, *m/z* 534) obtained from ASVYI-NH_2 and the relative intensity of *m/z* 295 fragment ion is 2.04 % compared with the base peak of b_5^o. To elucidate its gas-phase structure, the detailed MS^4 ($[M + H]^+ \rightarrow b_5 \rightarrow 295$) consecutive experiments were performed. As depicted in Figure 4b, the MS^4 spectrum contains the product ions at *m/z* 132, 136, 249, 277, and 278 in which the highest relative abundance is observed for the *m/z* 136. It is well known that the *m/z* 136 represents the tyrosine immonium ion's mass and it provides evidence for the presence of tyrosine residue in the sequence of *m/z* 295. Additionally, the ion at *m/z* 132 can be accepted as evidence of protonated isoleucine. As mentioned above in the fragmentation reactions of the XGGFL-NH_2 peptide series, it could be concluded that tyrosine (Y) and isoleucine (I) amino acid residues are dissociated together in the form of protonated dipeptide from b_5 ion. Again, the source of water comes from the side chain of serine residue. To confirm the sequence of protonated fragment ion, the $[M + H]^+$ ions of YI-OH and IY-OH dipeptides were further examined by CID MS/MS experiments. The resulting $[M + H]^+$ product ion mass spectrum of YI-OH and the MS^4 spectrum of the *m/z* 295 fragment ion show entirely the same fragmentation behavior (shown in Figure 4b and c) via CID. The mass spectra provide evidence that these ions are structurally identical in the gas phase. The products at *m/z* 132, 136, 249, 277, and 278 were assigned to y_1, Y_{imm}, a_2, b_2, and $[M + H - NH_3]^+$ ions, respectively. The fragmentation behavior of IY-OH is totally different from that of *m/z* 295 ion (shown in Electronic Supplementary Material Figure S2).

The MS^4 consecutive experiments were also carried out separately for each *m/z* 295 fragment ion derived from the b_5 ions of ATVYI-NH_2, AEVYI-NH_2, ADVYI-NH_2, and AYVYI-NH_2. As Figure 5a displays, the obtained fragmenta-

Table 2. Relative Intensities of *m/z* 295 and 281 Fragments in the b_5 and b_4 Ions CID Mass Spectra of AXVYI-NH_2 (Where X Is S, T, E, D, or Y) Model Peptides, Respectively

Peptide	Relative intensity (%)	
	m/z 295	*m/z* 281
ASVYI-NH_2	2.04	0.818
ATVYI-NH_2	0.173	1.11
AEVYI-NH_2	0.0795	0.234
ADVYI-NH_2	0.268	0.0658
AYVYI-NH_2	0.0227	ND

ND=not detected.

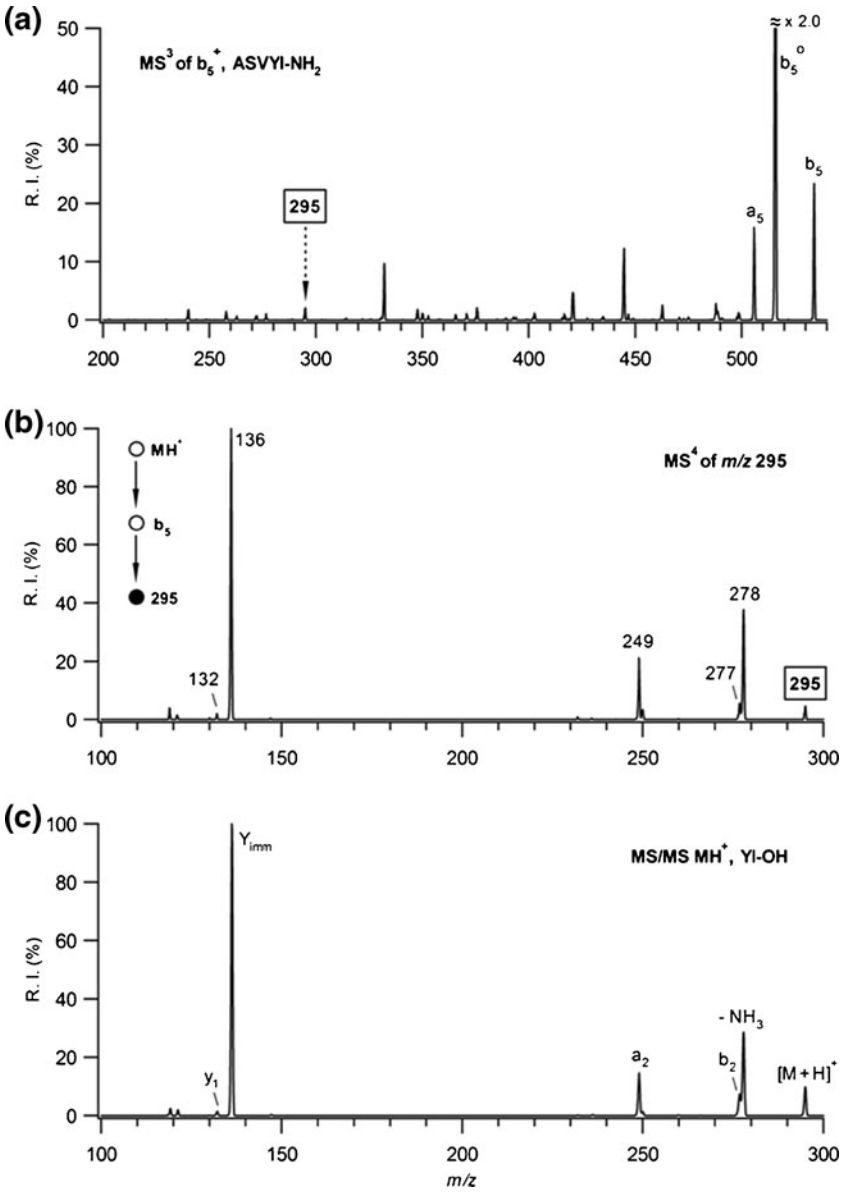


Figure 4. **(a)** MS^3 mass spectrum of b_5 ion from protonated ASVYI-NH_2, **(b)** MS^4 mass spectrum of m/z 295 ion from b_5 ion of protonated ASVYI-NH_2, **(c)** MS^2 mass spectrum of $[M + H]^+$ ion from protonated YI-OH

tion patterns are remarkably alike compared with the fragmentation products of YI-OH. However, the only difference is the absence of the m/z 277 ion in the MS^4 spectrum of the m/z 295 fragment ion derived from AYVYI-NH_2. As a consequence of identical fragmentation behaviors, it was confirmed that the sequence of the m/z 295 fragment ions obtained from b_5 ions of AXVYI-NH_2 (where X is S, T, E, D, or Y) have a sequence of protonated dipeptide, YI-OH.

The possible fragmentation mechanism for the formation of the m/z 295 fragment ion is similar to Scheme 1, and the two competitive dissociation pathways are valid for all b_5 ions after dissociation of internal complex (scheme not shown).

In addition, the b_5 ions that originated from AXVYI-NH_2 undergo macrocyclization reaction followed by ring opening at various positions to form linear oxazolones, which generate internal amino acid losses in the product ion mass spectra (see Electronic Supplementary Material Figure S7). Similar to the mechanism for original sequence, the m/z 203 fragment ions (IA-OH) were detected with relatively low intensities in the dissociation of permuted $SVYIA_{oxa}$ and $EVYIA_{oxa}$ isomers. The fragmentation patterns of m/z 203 ions and MS/MS spectrum of $[M + H]^+$ ion form IA-OH dipeptide are similar (spectra not shown).

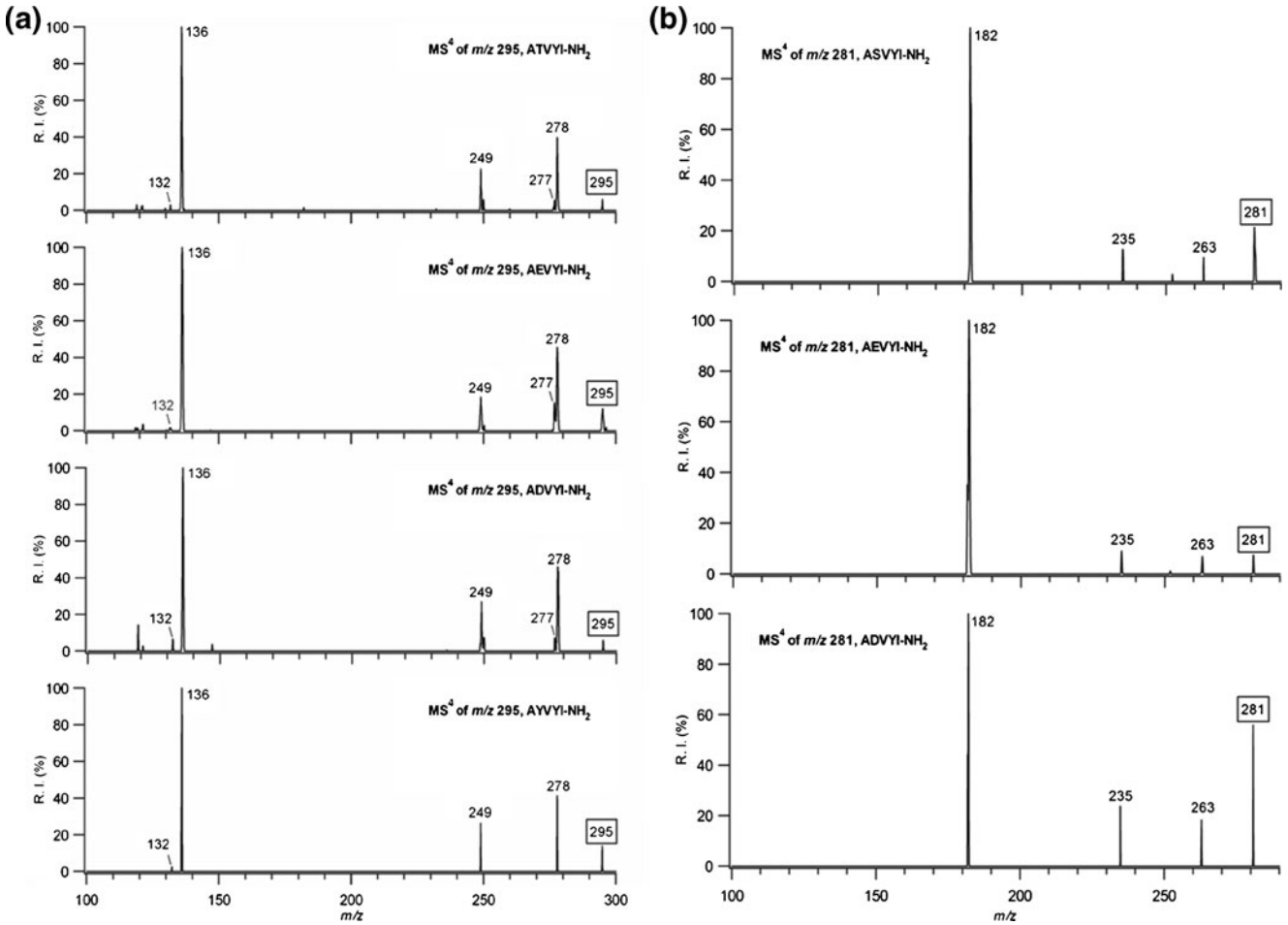


Figure 5. **(a)** Comparison of the MS^4 mass spectra of m/z 295 fragment ions ($MH^+ \rightarrow b_5 \rightarrow 295$) originated from protonated ATVYI-NH_2, AEVYI-NH_2, ADVYI-NH_2, and AYVYI-NH_2, respectively. **(b)** Comparison of the MS^4 mass spectra of m/z 281 fragment ions ($MH^+ \rightarrow b_4 \rightarrow 281$) originated from protonated ASVYI-NH_2, AEVYI-NH_2, and ADVYI-NH_2, respectively

The Formation of m/z 281 Fragment Ion in the b_4 Ion Mass Spectra of AXVYI-NH_2 (where X Is S, T, E, D, or Y)

Similar to the b_5 ions, the m/z 281 fragment ion was generated in all the b_4 ion mass spectra of ASVYI-NH_2, ATVYI-NH_2, AEVYI-NH_2, and ADVYI-NH_2 pentapeptides with different relative intensities (Table 2). Figure 6a displays the CID mass spectrum of the b_4 ion ($ATVY_{oxa}$, m/z 435) originated from the ATVYI-NH_2 pentapeptide. In the mass spectrum, the m/z 281 fragment ion was detected with a relative intensity of 1.11 % compared with the base peak of b_5^o. The MS^4 ($[M + H]^+ \rightarrow b_4 \rightarrow 281$) consecutive experiments were performed for this fragment ion to obtain its gas-phase structure under low-energy CID conditions. As Figure 6b illustrates, the CID-MS^4 spectrum contains an abundant product ion at m/z 182, whereas the m/z 136, 235, and 263 product ions have minor intensities in the mass spectrum. The ions at m/z 182 and 136 reflect the masses of protonated tyrosine residue and immonium ion, respectively. Hence, we can assume that valine (V) and tyrosine (Y) residues are dissociated together as a protonated dipeptide form from the b_4 ion. To verify this assumption, the VY-OH and YV-OH dipeptides were used, and their $[M + H]^+$ ion was selected separately and allowed to fragment in the gas phase. The MS/MS spectrum of $[M + H]^+$ ion of VY-OH was identical to that obtained from the m/z 281 ion (Figure 6b and c). The comparison of these two mass spectra proves that the m/z 281 fragment ion has a sequence of VY-OH. The product ions at m/z 136, 182, 235, and 263 represent the Y_{imm}, y_1, a_2, and b_2 ions, respectively. By contrast, the product ion mass spectrum of YV-OH was totally different than the ion at m/z 281 and is shown in Electronic Supplementary Material Figure S2.

Each m/z 281 fragment ion obtained from the b_4 ions of ASVYI-NH_2, AEVYI-NH_2, and ADVYI-NH_2 was isolated individually and subjected to CID for further fragmentation.

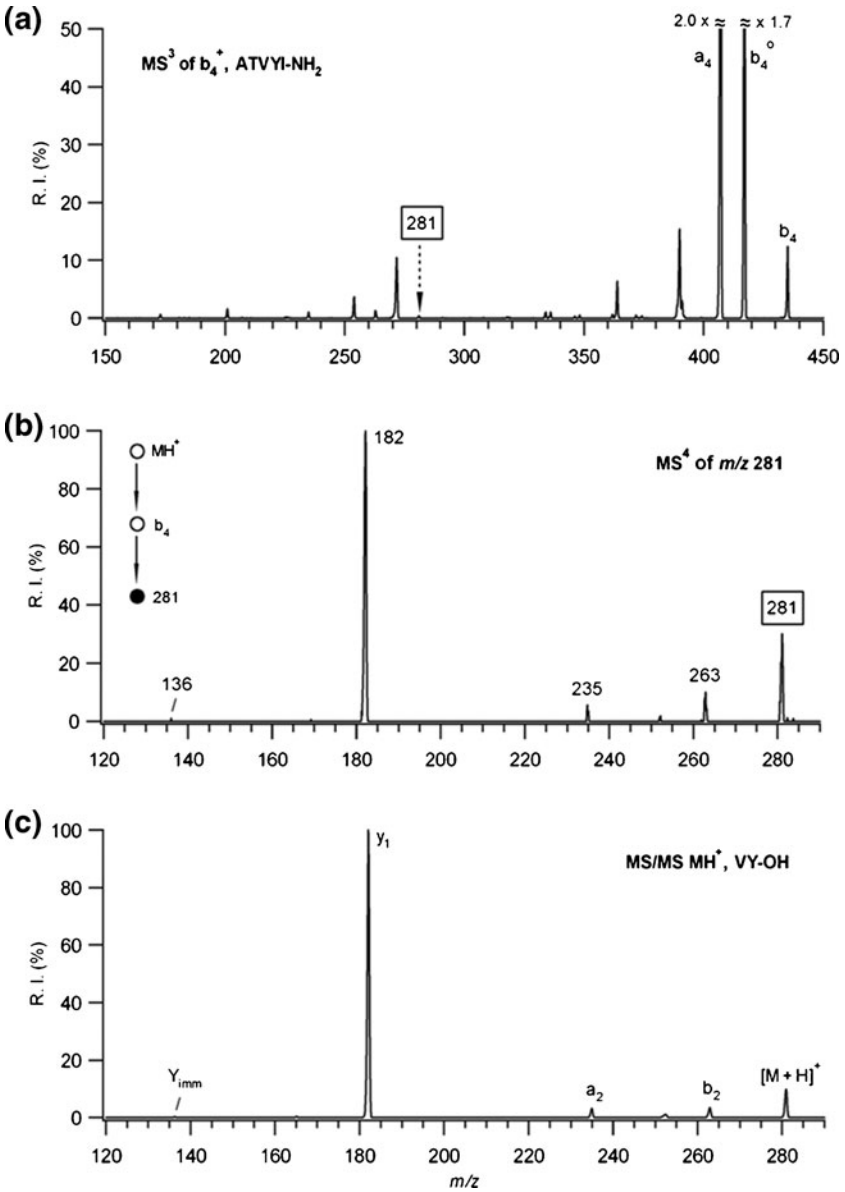


Figure 6. **(a)** MS^3 mass spectrum of b_4 ion from protonated ATVYI-NH_2, **(b)** MS^4 mass spectrum of m/z 281 ion from b_4 ion of protonated ATVYI-NH_2, **(c)** MS^2 mass spectrum of $[M+H]^+$ ion from protonated VY-OH

The results obtained from each MS^4 spectrum indicate that the sequence of the m/z 281 fragment ion is a protonated VY-OH (shown in Figure 5b). However, the relatively low intensity of Y_{imm} ion was not detected in all three mass spectra. Moreover, the m/z 281 fragment ion could not be observed in the b_4 ion mass spectrum of AYVYI-NH_2 peptide.

The possible mechanism for the formation of the m/z 281 fragment ion is similar to the other mechanisms described above. Additionally, only b_4 ions of $AEVY_{oxa}$ and $ADVY_{oxa}$ follow two competitive dissociation pathways after the dissociation of the internal complex (scheme not shown).

Conclusion

In the present study, the C-terminal protonated dipeptide losses are reported in the dissociation process of the b_5 and b_4 ions of pentapeptides containing side chain hydroxyl group residues. The protonated FL-OH (m/z 279) and GF-OH (m/z 223) dipeptide eliminations have been generated with different relative intensities in the dissociations of all the b_5 and b_4 ions (except the b_4 ion of YGGFL-NH_2), respectively, for the XGGFL-NH_2 (where X is S, T, E, D, or Y) model peptide series. Similarly, the protonated YI-OH (m/z 295) and VY-OH (m/z 281) dipeptide losses have been detected in the dissociations of all the b_5 and b_4 ions (except

the b_4 ion derived from AYVYI-NH_2), respectively, derived from the AXVYI-NH_2 (where X is S, T, E, D, or Y) model peptides. The sequences of these fragment ions were verified by comparing the MS/MS fragmentation behavior of the $[M+H]^+$ ions of commercial dipeptides, namely FL-OH, GF-OH, YI-OH, and VY-OH, via low-energy CID. It should be noted that the *m/z* 223 and *m/z* 281 fragment ions are not detected in the b_4 ion mass spectra of YGGFL-NH_2 and AYVYI-NH_2, respectively. Similarly, the relative intensities of *m/z* 279 and 295 fragment ions are remarkably low (below 0.03 %) in the dissociation of the b_5 ions of YGGFL-NH_2 and AYVYI-NH_2, respectively. The reason could be explained as the phenolic hydrogen behavior. It is known that phenolic hydrogen is not acidic enough and that is why tyrosine-containing peptides did not show strong protonated dipeptide elimination compared with others.

These eliminations clearly demonstrate that a novel rearrangement reaction takes place through gas-phase fragmentation of the b_5 and b_4 ions from hydroxyl side chain containing pentapeptides.

Acknowledgments

The authors acknowledge support for this study by the Scientific and Technological Research Council of Turkey, TUBITAK, under project no. 109T430. The State Planning Organization, DPT, is gratefully acknowledged for its financial support of the Biological Mass Spectrometry and Proteomics Facility. A.E.A. also thanks to the Ph.D. scholarship of TUBITAK during the studies.

References

1. Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., Whitehouse, C.M.: Electrospray ionization mass spectrometry of large molecules. *Science* **246**, 64–71 (1989)
2. Karas, M., Hillenkamp, F.: Laser desorption ionization of proteins with molecular masses exceeding 10,000 Da. *Anal. Chem.* **60**, 2299–2301 (1988)
3. Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y., Yoshida, T., Matsuo, T.: Protein and polymer analyses up to *m/z* 100,000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2**, 151–153 (1988)
4. Hunt, D.F., Bone, W.M., Shabanowitz, J., Rhodes, J., Ballard, J.M.: Sequence-analysis of oligopeptides by secondary ion/collision activated dissociation mass-spectrometry. *Anal. Chem.* **53**, 1704–1706 (1981)
5. Hunt, D.F., Yates, J.R.I.I.I., Shabanonowitz, J., Winston, S., Hauer, C.R.: Protein sequencing by tandem mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6233–6237 (1986)
6. Aebersold, R., Goodlett, D.R.: Mass spectrometry in proteomics. *Chem. Rev.* **101**, 269–295 (2001)
7. Medzihradszky, K.F.: Peptide sequence analysis. *Methods Enzymol.* **402**, 209–244 (2005)
8. Roepstorff, P., Fohlmann, J.: Proposals for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* **11**, 601–601 (1984)
9. Biemann, K.: Contributions of mass spectrometry to peptide and protein structure. *Biomed. Environ. Mass Spectrom.* **16**, 99–111 (1988)
10. Eng, J., McCormack, A., Yates III, J.R.: An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976–989 (1994)
11. Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S.: Probability based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551–3567 (1999)
12. Arnott, D., Kottmeir, D., Yates, N., Shabanowitz, J., Hunt, D.F.: *Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics.* Chicago, IL, June (1994).
13. Cordero, M.M., Houser, J.J., Wesdemiotis, C.: The neutral products formed during backbone fragmentation of protonated peptides in tandem mass spectrometry. *Anal. Chem.* **65**, 1594–1601 (1993)
14. Yalcin, T., Khouw, C., Csizmadia, I.G., Peterson, M.B., Harrison, A.G.: Why are *B* ions stable species in peptide spectra? *J. Am. Soc. Mass Spectrom.* **6**, 1165–1174 (1995)
15. Yalcin, T., Csizmadia, I.G., Peterson, M.R., Harrison, A.G.: The structures and fragmentation of B_n ($n \geq 3$) ions in peptide spectra. *J. Am. Soc. Mass Spectrom.* **7**, 233–242 (1996)
16. Paizs, B., Lendvay, G., Vekey, K., Suhai, S.: Formation of b_2^+ ions from protonated peptides: an ab initio study. *Rapid Commun. Mass Spectrom.* **13**, 525–533 (1999)
17. Rodriquez, C.F., Shoeib, T., Chu, I.K., Siu, K.W.M., Hopkinson, A.C.: Comparison between protonation, lithiation and argentination of 5-oxazolones: a study of a key intermediate in gas-phase peptide sequencing. *J. Phys. Chem. A* **104**, *5335–5342 (2000)*
18. Bythell, B.J., Somogyi, A., Paizs, B.: What is the structure of b_2 ions generated from doubly protonated tryptic peptides? *J. Am. Soc. Mass Spectrom.* **20**, 618–624 (2009)
19. Polfer, N.C., Oomens, J., Suhai, S., Paizs, B.: Spectroscopic and theoretical evidence for oxazolone ring formation in collision-induced dissociation of peptides. *J. Am. Chem. Soc.* **127**, 17154–17155 (2005)
20. Polfer, N.C., Oomens, J., Suhai, S., Paizs, B.: Infrared spectroscopy and theoretical studies on gas-phase protonated leu-enkephalin and its fragments: direct experimental evidence for the mobile proton. *J. Am. Chem. Soc.* **129**, 5887–5897 (2007)
21. Thorne, G.C., Ballard, K.D., Gaskell, S.J.: Metastable decomposition of peptide $[M+H]^+$ ions via rearrangement involving loss of the C-terminal amino acid residue. *J. Am. Soc. Mass Spectrom.* **1**, 249–257 (1990)
22. Ballard, K.D., Gaskell, S.J.: Intramolecular [^{18}O] isotopic exchange in the gas phase observed during the tandem mass spectrometric analysis of peptides. *J. Am. Chem. Soc.* **114**, 64–71 (1992)
23. Fang, S., Takao, T., Satomi, Y., Mo, W., Shimonishi, Y.: Novel rearranged ions observed for protonated peptides via metastable decomposition in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Am. Soc. Mass Spectrom.* **11**, 345–351 (2000)
24. Hiserodt, R.D., Brown, S.M., Swijter, D.F.H., Hawkins, N., Mussinan, C.J.: A study of $b_1 + H_2O$ and b_1-ions in the product ion spectra of dipeptides containing N-terminal basic amino acid residues. *J. Am. Soc. Mass Spectrom.* **18**, 1414–1422 (2007)
25. Farrugia, J.M., O'Hair, R.A.J.: Involvement of salt bridges in a novel gas phase rearrangement of protonated arginine-containing dipeptides which precedes fragmentation. *Int. J. Mass Spectrom.* **222**, 229–242 (2003)
26. Gonzalez, J., Besada, V., Garay, H., Reyes, O., Padron, G., Tambara, Y., Takao, T., Shimonishi, Y.: Effect of the position of a basic amino acid on the C-terminal rearrangement of protonated peptides upon collision-induced dissociation. *J. Mass Spectrom.* **31**, 150–158 (1996)
27. Atik, A.E., Yalcin, T.: A systematic study of acidic peptides for *b*-type sequence scrambling. *J. Am. Soc. Mass Spectrom.* **22**, 38–48 (2011)
28. Ambihapathy, K., Yalcin, T., Leung, H., Harrison, A.G.: Pathways to immonium ions in the fragmentation of protonated peptides. *J. Mass Spectrom.* **32**, 209–215 (1997)
29. Ballard, K.D., Gaskell, S.J.: Dehydration of peptide $[M+H]^+$ ions in the gas phase. *J. Am. Soc. Mass Spectrom.* **4**, 477–481 (1993)
30. Harrison, A.G., Yalcin, T.: Proton mobility in protonated amino acids and peptides. *Int. J. Mass Spectrom. Ion Process* **165/166**, 339–347 (1997)
31. O'Hair, R.A.J., Reid, G.E.: Does side chain water loss from protonated threonine yield N-protonated dehydroamino-2-butyric acid? *Rapid Commun. Mass Spectrom.* **12**, 999–1002 (1999)
32. Reid, G.E., Simpson, R.J., O'Hair, R.A.J.: Leaving group and gas phase neighboring group effects in the side chain losses from protonated serine and its derivatives. *J. Am. Soc. Mass Spectrom.* **11**, 1047–1060 (2000)
33. Serafin, S.V., Zhang, K., Aurelio, L., Hughes, A.B., Morton, T.H.: Decomposition of protonated threonine, its stereoisomers, and its homologues in the gas phase: evidence for internal backside displacement. *Org. Lett.* **6**, 1561–1564 (2004)

34. Neta, P., Pu, Q.-L., Yang, X., Stein, S.E.: Consecutive neutral losses of H_2O and C_2H_4O from N-terminal Thr-Thr and Thr-Ser in collision-induced dissociation of protonated peptides. position dependent water loss from single Thr or Ser. *Int. J. Mass Spectrom.* **267**, 295–301 (2007)
35. Harrison, A.G.: Pathways for water loss from doubly protonated peptides containing serine or threonine. *J. Am. Soc. Mass Spectrom.* **23**, 116–123 (2012)
36. Paizs, B., Suhai, S.: Fragmentation pathways of protonated peptides. *Mass Spectrom. Rev.* **24**, 508–548 (2005)
37. Harrison, A.G.: Peptide sequence scrambling through cyclization of b_5 ions. *J. Am. Soc. Mass Spectrom.* **19**, 1776–1780 (2008)
38. Bleiholder, C., Osburn, S., Williams, T.D., Suhai, S., Van Stipdonk, M., Harrison, A.G., Paizs, B.: Sequence-scrambling pathways of protonated peptides. *J. Am. Chem. Soc.* **130**, 17774–17789 (2008)