

Improving Mass Spectrometric Sequencing of Arginine-containing Peptides by Derivatization with Acetylacetone

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Modification of arginine residues in bradykinin, [1–5]-bradykinin, splenopentin and two synthetic pentapeptides with acetylacetone (pentane-2,4-dione) significantly increases the relative abundance of sequence-specific fragment ions produced by matrix-assisted laser desorption/ionization (MALDI). The fragmentation efficiency as measured by post-source decay in a reflectron time-of-flight mass spectrometer increases by a factor of 2–3.5. Peptide bonds adjacent to modified residues are more susceptible to cleavage than in the non-derivatized peptide ions. The increased lability of these bonds gives rise to more complete sequence information. In addition, the relative abundances of sequence-specific fragment ions are enhanced. This strategy makes it possible to obtain valuable structural information from arginine-containing peptides that otherwise do not fragment well. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

The introduction of post-source decay (PSD)¹ for acquiring metastable ion (MI) and collision-induced dissociation (CID) mass spectra of peptide $[M + H]^+$ ions formed by matrix-assisted laser desorption/ionization (MALDI)² greatly expands the utility of mass spectrometry. Peptide fragmentation patterns obtained by MALDI/TOF/PSD are very similar to spectra obtained by liquid secondary ion mass spectrometry (LSIMS) using a tandem sector instrument.³ The former has the advantage, however, of much higher sensitivity. MALDI/TOF/PSD requires 10–1000 times less material than LSIMS tandem mass spectrometry (MS/MS) and can tolerate higher salt concentrations.³

Although the intrinsic basicity of peptides facilitates production of gas-phase $[M + H]^+$ ions, the presence of basic amino acid residues (e.g. arginine, lysine, histidine and ornithine) increases the abundance of $[M + H]^+$ ions in the mass spectrum.⁴ The basic groups are easily protonated but this can have deleterious effects for structural characterization by MS/MS.⁵ For example, protonation of the basic groups results in charge localization and the localized charge

results in suppression of random cleavage of backbone bonds and enhancement of amino acid specific fragmentation reactions. During the development of MS/MS for the structural characterization of peptides, a great deal of attention has been directed toward arginine-containing peptides.^{6–8} Arginine is the most basic amino acid; its gas-phase proton affinity is higher than that of the next basic amino acid residue, histidine, based on studies using the kinetic method,^{9–11} or lysine, based on data obtained by the bracketing method.^{12,13} The frequency of occurrence of arginine in proteins is 5.7%, which is higher than the average (5.0%) and much higher than that of histidine (2.2%).¹⁴ Trypsin, which cleaves the amide bond of proteins after arginine or lysine, is the most commonly used enzyme for protein digestion to yield peptide fragments for mass spectrometric sequence analysis, hence C-terminal arginine containing peptides are frequently encountered in mass spectrometric experiments.¹⁵

The presence of arginine at the C- or N-terminus of a peptide molecule has a direct impact on the abundance of C- or N-terminal fragment ions, respectively.¹⁶ Peptides containing an arginine in their sequence readily undergo rearrangement during the fragmentation process. For example, $[M + H]^+$ ions rearrange to yield $[b_{n-1} + H_2O]^+$ ions and the mechanism for formation of $[b_{n-1} + H_2O]^+$ fragment ions was deduced from ¹⁸O-labeling studies on bradykinin.¹⁷ The nomenclature used to identify the fragment ions throughout this paper is that proposed by Roepstorff and Fohlman^{18a} and modified by Biemann.^{18b} For internal fragments we use the two letter nomenclature $(b, y)_n$, where subscripts *l* and *m* are sites of cleavage and *n* is the number of residues in the fragment. This mechanism involves

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proton transfer from the C-terminal carboxyl group to the carboxyl oxygen of the $n - 1$ residue and formation of a heterocyclic intermediate followed by loss of CO and $\text{NH}=\text{CH}-\text{R}_n$. Glish and co-workers¹⁹ recently proposed another mechanism for the same process that is consistent with the isotopic labeling data. Gross and co-workers^{20,21} also showed that $[\text{M} + \text{Li}]^+$ ions undergo rearrangement reactions to form $[\text{b}_{n-1} + \text{Li} + \text{OH}]$ ions.

Although low-energy CID is commonly used for peptide sequencing, Boyd and co-workers²² noted that multiply protonated arginine-containing peptides show a low degree of structurally informative fragmentation. Low-energy CID was also used to study the energetics of peptide fragmentation. Wysocki and co-workers⁵ showed that the fragmentation of arginine-containing peptides requires higher energy than lysine-containing peptides, which in turn requires higher energy than peptides that do not contain basic residues. Their results are consistent with results reported by Glish and co-workers.¹⁹ They demonstrated that arginine-containing peptides experience higher energy losses in the formation of fragment ions involving a dissociation adjacent to an arginine residue. Glish and co-workers proposed that the additional energy required for dissociation of bradykinin was due to intramolecular interactions between the guanidino group of the arginine side chain and the carbonyl oxygen of the $n - 1$ residue. That is, the gas-phase ionic species adopts structural conformations to delocalize the charge site.^{19,23}

More recently, dissociation of arginine-containing peptides was studied by 'blackbody infrared radiative dissociation (BIRD).^{23,24} Bradykinin showed a lack of fragmentation in these experiments. The rationalization for this involves the formation of a salt-bridge structure with the peptide backbone folded around the charged guanidino and carboxyl groups.²³ A similar structure was proposed by Bowers and co-workers²⁵ for the bradykinin $[\text{M} + \text{H}]^+$, $[\text{M} + \text{Na}]^+$ and $[\text{M} - \text{H} + 2\text{Na}]^+$ ions using ion mobility spectrometry and molecular mechanics/dynamics calculations. The presence of a strong intramolecular hydrogen bond between the guanidino groups of the N-terminal and the C-terminal arginine residues was proposed based on MALDI/TOF/PSD fragmentation patterns.²⁶

Gaskell and co-workers⁸ further illustrated the influence of charge-carrying residues on the fragmentation of peptide ions by balancing the positive charge localized on arginine side-chains with the negative charge (formation of a salt bridge) on cysteic acid side-chains; the latter were created by oxidation of the cysteine mercapto groups. The model arginine-containing peptides that also contain cysteic acid residues fragmented more efficiently and produced a higher number of the sequence-specific fragment ions.

This research was aimed at enhancing the fragmentation of arginine-containing peptides in metastable ion (MI) decomposition by derivatizing the guanidino groups with acetylacetone (pentane-2,4-dione). Derivatization can also be used to enhance the utility of CID for peptide sequencing; however, it is not clear whether structural features that enhance CID spectra have similar effects on PSD MI spectra. The trend in the derivatization for high-energy CID is to put the posi-

tively charged group at the N-terminus, thereby favoring the formation of the N-terminal fragment ions.^{27,28} Recently, a similar approach was used in MALDI/PSD.²⁹ Conversely, our scheme is designed to derivatize the guanidino groups, thereby decreasing the basicity of the arginine residues and reducing intramolecular interactions. Gaskell and co-workers¹⁷ previously used derivatized peptides to study mechanisms of rearrangement reactions that yield $[\text{b}_{n-1} + \text{H}_2\text{O}]$ fragment ions. Derivatization with acetylacetone was previously utilized in electron ionization (EI) mass spectrometry to make arginine-containing peptides accessible for analysis.^{30,31} A similar derivatization using cyclohexane-1,2-dione has also been suggested for fast atom bombardment (FAB) mass spectrometry.³² In this work we evaluated the fragmentation efficiency of derivatized peptides and compared it with that for non-derivatized peptides. This study covers peptides with an arginine residue at the N-terminus, the C-terminus and in the middle of the sequence.

EXPERIMENTAL

Materials

Bradykinin, [1-5]-bradykinin, splenopentin and acetylacetone were purchased from Sigma Chemical (St Louis, MO, USA). α -Cyano-4-hydroxycinnamic acid was purchased from Aldrich Chemical (Milwaukee, WI, USA). All the above materials were used without further purification. Doubly deionized water was used to prepare all aqueous solutions.

Peptide synthesis

[5-9]-Bradykinin and the pentapeptide Lys-Glu-Arg-Val-Tyr were synthesized on a Model 431A automatic peptide synthesizer (Applied Biosystems, Foster City, CA, USA) utilizing standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry³³ on Wang resin (0.25 mmol scale). After completion of the synthesis, the peptide-resin was washed with dichloromethane and methanol, dried under vacuum and then cleaved with a mixture of trifluoroacetic acid (95%), thioanisole (2.5%) and water (2.5%) for 4 h. Crude peptide was precipitated by an eightfold volume excess of cold diethyl ether and centrifuged until it formed a pellet on the bottom of the tube. The supernatant was poured out and the precipitated peptide dried under vacuum over sodium hydroxide, dissolved in water-dimethyl sulfoxide (2:1), purified using reversed-phase C_{18} high-performance liquid chromatography (HPLC) and lyophilized. Homogeneity of the material was established by a combination of reversed-phase HPLC and MALDI/MS.

Modification of peptides with acetylacetone

This reaction was carried out by adopting the method published by Gilbert and O'Leary.³⁴ The peptide (5-9

μmol) was dissolved in 1 ml of 0.1 M sodium carbonate–hydrogencarbonate buffer (pH 9.0) and then 50–90 μl (100-fold molar excess) of acetylacetone were slowly added. The reaction mixture was stirred for 3 days, monitoring the progress of the reaction by HPLC. The crude product was lyophilized, dissolved in 1 ml of water and purified by HPLC. In the case of peptides containing lysine, a 200-fold molar excess of acetylacetone was used. These peptides were treated with a solution of 50 mg (100-fold molar excess) of hydroxylamine hydrochloride in 0.35 ml of water for 15 min, before injection into the HPLC system, in order to remove the acetylacetone moiety from the lysine side-chain.

HPLC

All separations were carried out using solutions consisting of 0.2% trifluoroacetic acid in acetonitrile–water (5:95) (solvent A) and 0.2% trifluoroacetic acid in water–acetonitrile (5:95) (solvent B). The absorbance was monitored at 226 nm.

Preparative HPLC was carried out to purify synthetic pentapeptides. A Waters Model 600 HPLC system (Millipore, Milford, MA, USA) equipped with a variable-wavelength monitor (Knauer, Berlin, Germany) at a flow rate of 10 ml min⁻¹ was used for this purpose. A Waters RCM extended column with two 25 mm $\times$ 10 (m) Delta-Pac C₁₈ cartridges was used. [5–9]-Bradykinin was eluted with linear gradient from 5% to 35% solvent B over 40 min and the pentapeptide Lys–Glu–Arg–Val–Tyr was eluted with a linear gradient from 0% to 30% solvent B over 30 min.

Semi-preparative HPLC of the modified peptides was performed with a system consisting of a Model 410 BIO LC pump and a Model LC-95 UV/VIS detector (Perkin-Elmer, Norwalk, CT, USA) at flow rate of 1.5 ml min⁻¹ or on the Waters HPLC system described above at a flow rate of 2.0 ml min⁻¹. A Waters RCM extended column with two 8 mm $\times$ 10 (m) Radial-Pac C₁₈ cartridges was used for all modified peptide separations. Products of bradykinin modification were eluted with a non-linear gradient (slower at the beginning of the program) from 18% to 40% solvent B over 40 min. In order to resolve them completely, a second consecutive separation was carried out using the same program. The modified synthetic pentapeptide Lys–Glu–Arg–Val–Tyr was eluted with the same gradient as described for the preparative HPLC of this peptide. For all other modified peptides the linear gradient used for preparative HPLC of [5–9]-bradykinin was utilized.

Sample preparation for mass spectrometry

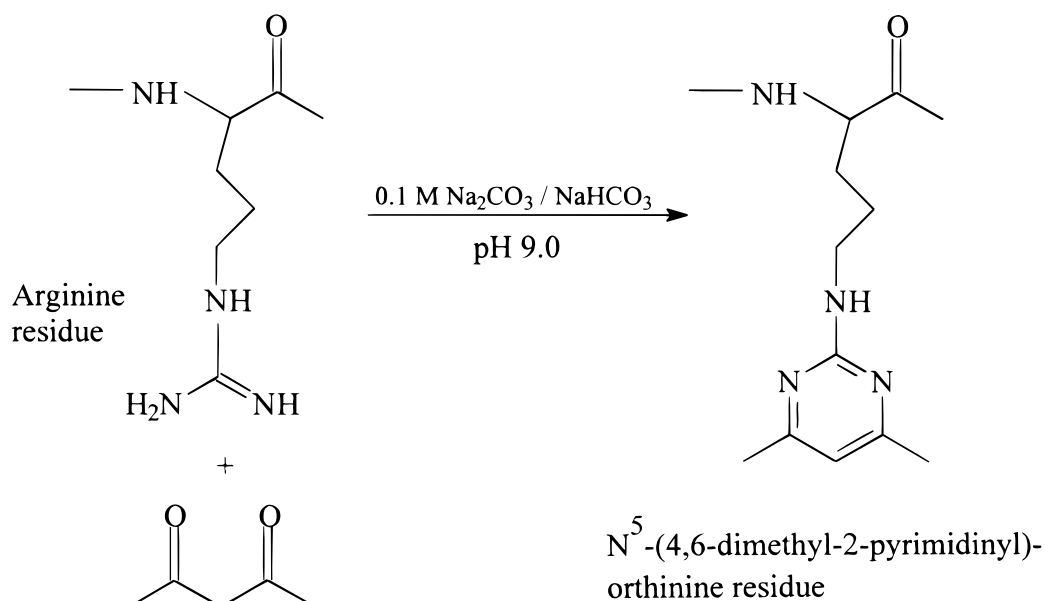
A 2 mg ml⁻¹ aqueous solution of each peptide was mixed with a 7.5 mg ml⁻¹ methanol solution of α -cyano-4-hydroxycinnamic acid in ratio of 1:2 (v/v) and 3 μl of the final solution were deposited on a stainless-steel sample plate similar to that described by Martin and co-workers.³⁵ The deposited droplet was allowed to evaporate to dryness. The amount of peptide placed on the sample plate corresponds to 2–3 nmol.

Mass spectrometry

Mass spectra were acquired on a Voyager Elite XL MALDI reflectron time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with 337 nm pulsed nitrogen laser previously described by Vestal *et al.*³⁶ Reflector and post-source decay (PSD) modes of the instrument were utilized. The measured m/z values are monoisotopic for both precursor ions and fragment ions. All the peptides were initially analyzed in the reflector mode with external calibration using the protonated angiotensin I peak (1296.685) and the $[2M + H]^+$ (379.093) or $[M + H]^+$ (190.050) matrix peak. Fragmentation information was obtained in the PSD mode. Protonated peptide ions generated by the laser pulse were accelerated with 25.0 kV. Ion acceleration was fine-tuned with a 14.0 kV voltage on the grid in the source. Accelerated ions drifted in the field-free 3.3 m flight tube and reached the timed ion selector pre-set to the $[M + H]^+$ mass of the analyte. The wire ion guide was biased at –12.5 V. Fragmentation occurred in the flight tube but since fragmented and precursor ions of one peptide have the same initial velocity, they reach the timed ion selector at the same time. Hence only one dissociating peptide species at a time was analyzed. Peptide ions which passed the timed ion selector entered the reflector where parts of the fragment population were reflected by different voltages (PSD mirror ratio settings) and focused on the detector. Eight to ten spectra with different PSD mirror ratio settings were taken and then 'stitched' together using the PerSeptive Biosystems version of GRAMS 386 software for a PC to produce one combined PSD spectrum. Each acquired spectrum was averaged from 100 laser shots with a laser power in the range 27–40 mJ cm⁻².

RESULTS

The influence of arginine residues on the dissociation of protonated peptide ions was investigated by derivatizing the guanidino group with acetylacetone yielding the *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine (Pyo) residue (Scheme 1). Figure 1 contains MI spectra of bradykinin $[M + H]^+$ ions [Fig. 1(A)], formed by MALDI using α -cyano-4-hydroxycinnamic acid as a matrix, and three derivatized bradykinins: [Pyo 1]-bradykinin [Fig. 1(B)], [Pyo 9]-bradykinin [Fig. 1(C)] and the dimodified [Pyo 1, Pyo 9]-bradykinin [Fig. 1(D)]. MI spectra of the pentapeptides [1–5]-bradykinin (Arg–Pro–Pro–Gly–Phe) [Fig. 2(A)] and splenopentin (Arg–Lys–Glu–Val–Tyr) [Fig. 3(A)] which contain *N*-terminal arginine residues are compared with MI spectra of the Pyo derivatives (Figs 2(B) and 3(B), respectively). Figure 4 contains MI spectra of a synthetic [5–9]-bradykinin (Phe–Ser–Pro–Phe–Arg) with arginine at the *C*-terminus and its Pyo derivative. A peptide containing arginine in the middle of the sequence was synthesized (Lys–Glu–Arg–Val–Tyr) and the MI spectra for this peptide and its derivative (Lys–Glu–Pyo–Val–Tyr) are shown in Fig. 5.



Scheme 1

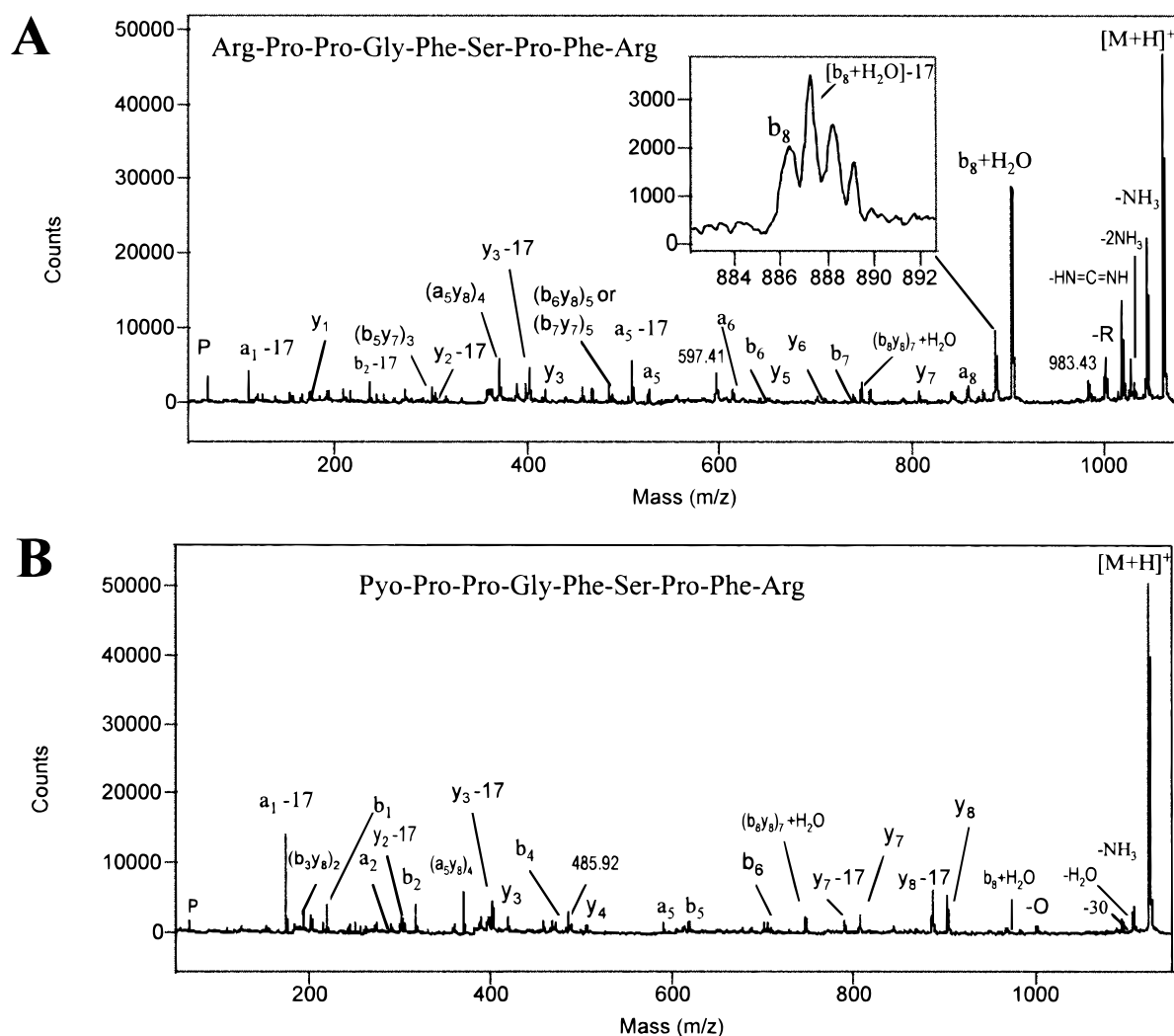


Figure 1. Metastable ion MALDI/PSD spectra of (A) bradykinin, (B) [Pyo 1]-bradykinin, (C) [Pyo 9]-bradykinin and (D) [Pyo 1, Pyo 9]-bradykinin.

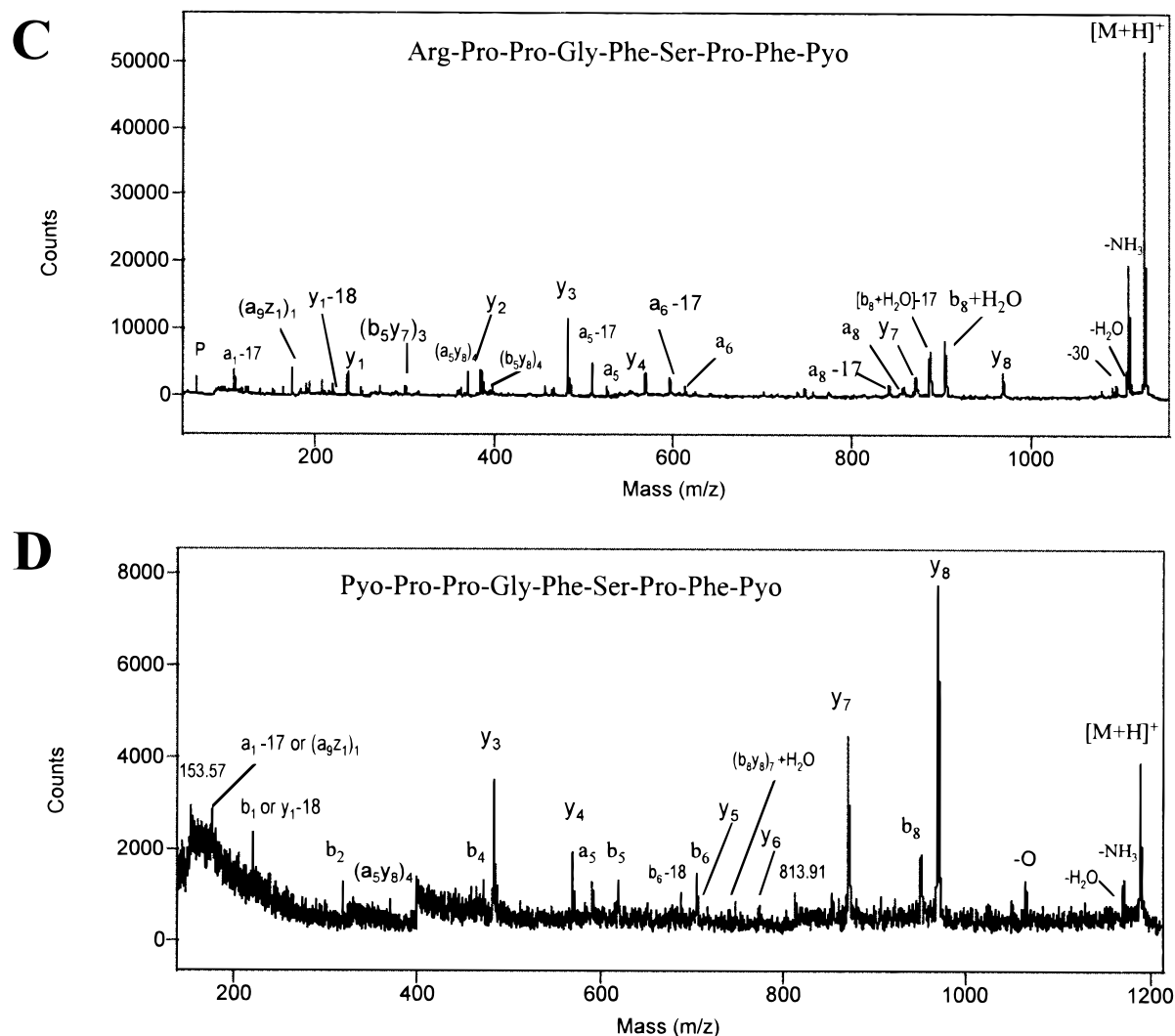


Figure 1. (Continued).

Table 1. Fragmentation efficiency of derivatized and non-derivatized peptides in MALDI/PSD

No.	Peptide ^a	Sequence ^b	[M + H] ⁺ ^c	Fragmentation efficiency ^d
1	Bradykinin	RPPGFSPFR	1060.54	4.1
2	[Pyo 1]-bradykinin	OPPGFSPFR	1124.59	2.5
3	[Pyo 9]-bradykinin	RPPGFSPFO	1124.61	2.7
4	[Pyo 1, Pyo 9]-bradykinin	OPPGFSPFO	1188.65	14.6
5	[1-5]-Bradykinin	RPPGF	573.29	2.3
6	[Pyo 1]-[1-5]-bradykinin	OPPGF	637.33	7.0
7	Splenopentin	RKEVY	694.34	2.6
8	[Pyo 1]-splenopentin	OKEVY	758.43	7.5
9	[5-9]-Bradykinin	FSPFR	653.39	3.3
10	[Pyo 9]-[5-9]-bradykinin	FSPFO	717.40	3.5
11		KERVY	694.40	1.8
12		KEOVY	758.40	3.3

^a Pyo represents the *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue.^b Standard single letter code used. O represents the *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue.^c The measured mass is monoisotopic.^d Fragmentation efficiency is calculated as the sum of the product ion absolute abundances divided by the [M + H]⁺ ion absolute abundance and is averaged from two sets of experimental data.

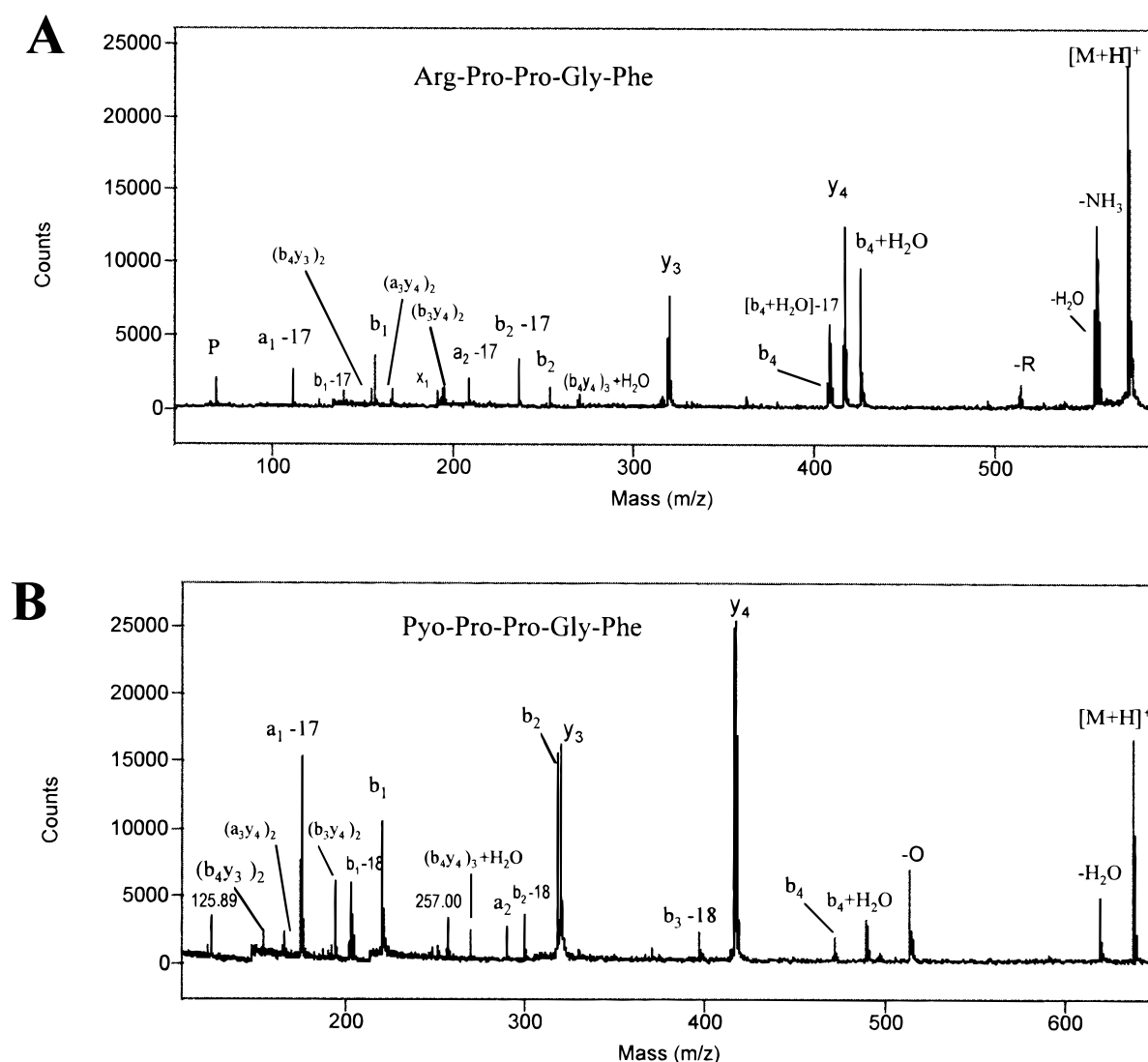


Figure 2. Metastable ion MALDI/PSD spectra of (A) [1-5]-bradykinin and (B) [Pyo 1]-[1-5]-bradykinin.

Fragmentation of the derivatized and non-derivatized peptides are compared on the basis of fragmentation efficiency (Table 1). The latter is calculated as the sum of product ion absolute abundances divided by the absolute abundance of $[M + H]^+$ ion signal. To explain trends in fragmentation efficiency and to compare *N*-terminal and *C*-terminal fragmentation, we summarize the relative abundances of *b* and *y* fragments in Tables 2 and 3. Table 2 contains data for bradykinin, [Pyo 1]-bradykinin, [Pyo 9]-bradykinin and [Pyo 1, Pyo 9]-bradykinin. Table 3 summarizes the relative abundances of *b* and *y* fragments for the derivatized and non-derivatized pentapeptides. The reason for using

relative abundance as opposed to absolute abundance is the difference in ionization threshold of different samples and, thus, slightly different laser power used to ionize them.

Signals corresponding to the losses of small neutral molecules are decreased in number and abundance for derivatized peptides as compared with non-derivatized peptides. Table 4 compares the abundances of ions formed by loss of small neutrals (neutral loss factor) calculated as the sum of the absolute abundances of their ion signals divided by the $[M + H]^+$ ion absolute abundance. Values for this factor and fragmentation efficiency values and relative abundances of *b*-type and

Table 2. Relative abundance of *b*- and *y*-type fragments for bradykinin and its derivatization products^a

Peptide ^b	b_1/y_6	b_2/y_7	b_3/y_6	b_4/y_6	b_5/y_4	b_6/y_3	b_7/y_2	b_8/y_1
Bradykinin	—/—	—/4	—/3	—/4	—/—	3/6	4/—	11/3
[Pyo 1]-bradykinin	7/19	10/12	—/—	4/—	7/7	6/7	—/—	—/—
[Pyo 9]-bradykinin	—/8	—/7	—/—	—/—	—/8	—/24	—/9	—/7
[Pyo 1, Pyo 9]-bradykinin	36/100	22/64	—/15	17/14	20/38	20/61	—/—	24/—

^a Expressed as a percentage of the most abundant signal in the spectrum and averaged from two sets of experimental data.

^b Pyo represents the *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue.

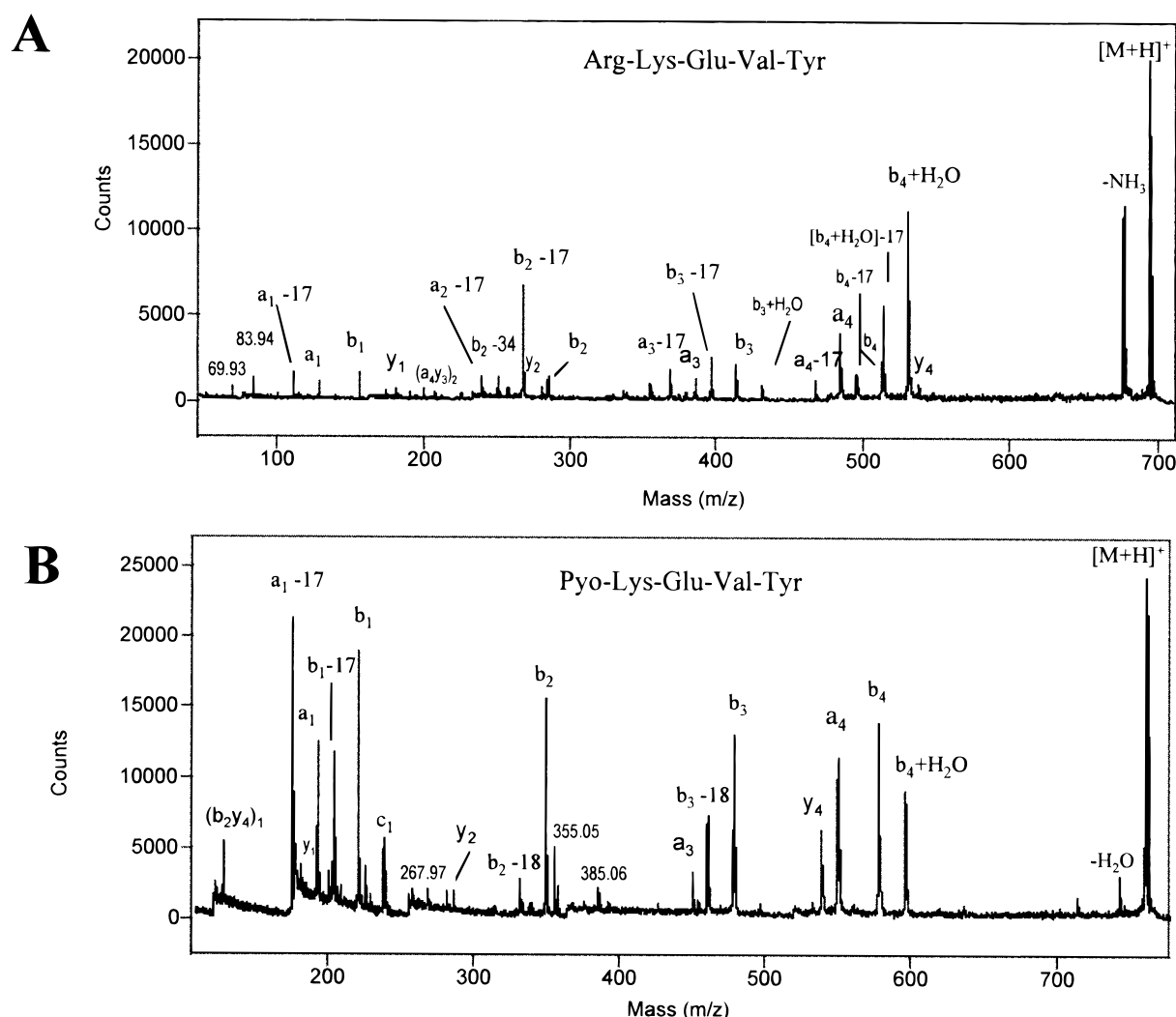


Figure 3. Metastable ion MALDI/PSD spectra of (A) splenopentin and (B) [Pyo 1]-splenopentin.

y-type fragment ions were averaged from two sets of experimental data.

DISCUSSION

Derivatization of the arginine residues of a peptide influences the total MALDI yields for $[M + H]^+$ ions

and also the fragmentation reactions of the $[M + H]^+$ ions. The MALDI ion yields for the derivatized peptides are probably reduced because the overall basicity of the molecule is lower;^{37,38} in particular, the basicity of the guanidino group is reduced. The increased fragmentation efficiency of the derivatized peptides in comparison with the non-derivatized peptides is illustrated by comparing the types of fragment ions and their relative

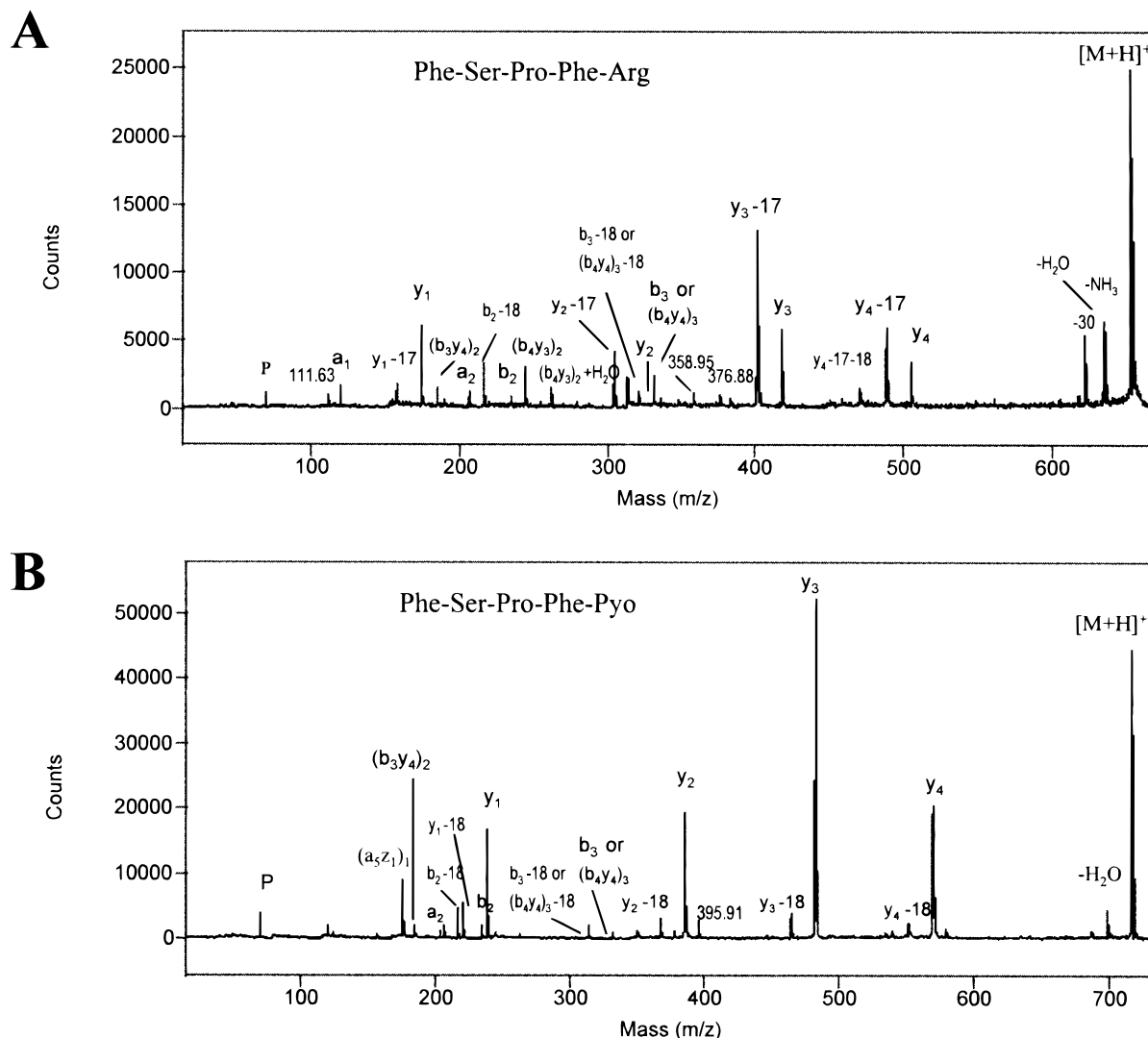
Table 3. Relative abundance of b- and y-type fragments for derivatized and non-derivatized pentapeptides^a

No. ^b	Peptide sequence ^c	b ₁ /y ₄	b ₂ /y ₃	b ₃ /y ₂	b ₄ /y ₁
5	Arg-Pro-Pro-Gly-Phe	12/39	4/22	—/—	6/—
6	Pyo-Pro-Pro-Gly-Phe	44/100	59/63	—/—	10/—
7	Arg-Lys-Glu-Val-Tyr	7/4	5/—	9/3	8/3
8	Pyo-Lys-Glu-Val-Tyr	78/26	64/—	54/8	57/15
9	Phe-Ser-Pro-Phe-Arg	—/12	5/23	9/5	—/24
10	Phe-Ser-Pro-Phe-Pyo	—/43	5/97	2/34	—/36
11	Lys-Glu-Arg-Val-Tyr	8/3	2/13	21/2	2/—
12	Lys-Glu-Pyo-Val-Tyr	6/9	3/26	43/—	18/—

^a Expressed as a percentage of the most abundant signal in the spectrum and averaged from two sets of experimental data.

^b Peptides have the same numbers as in Table 1.

^c Pyo represents the N⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue.



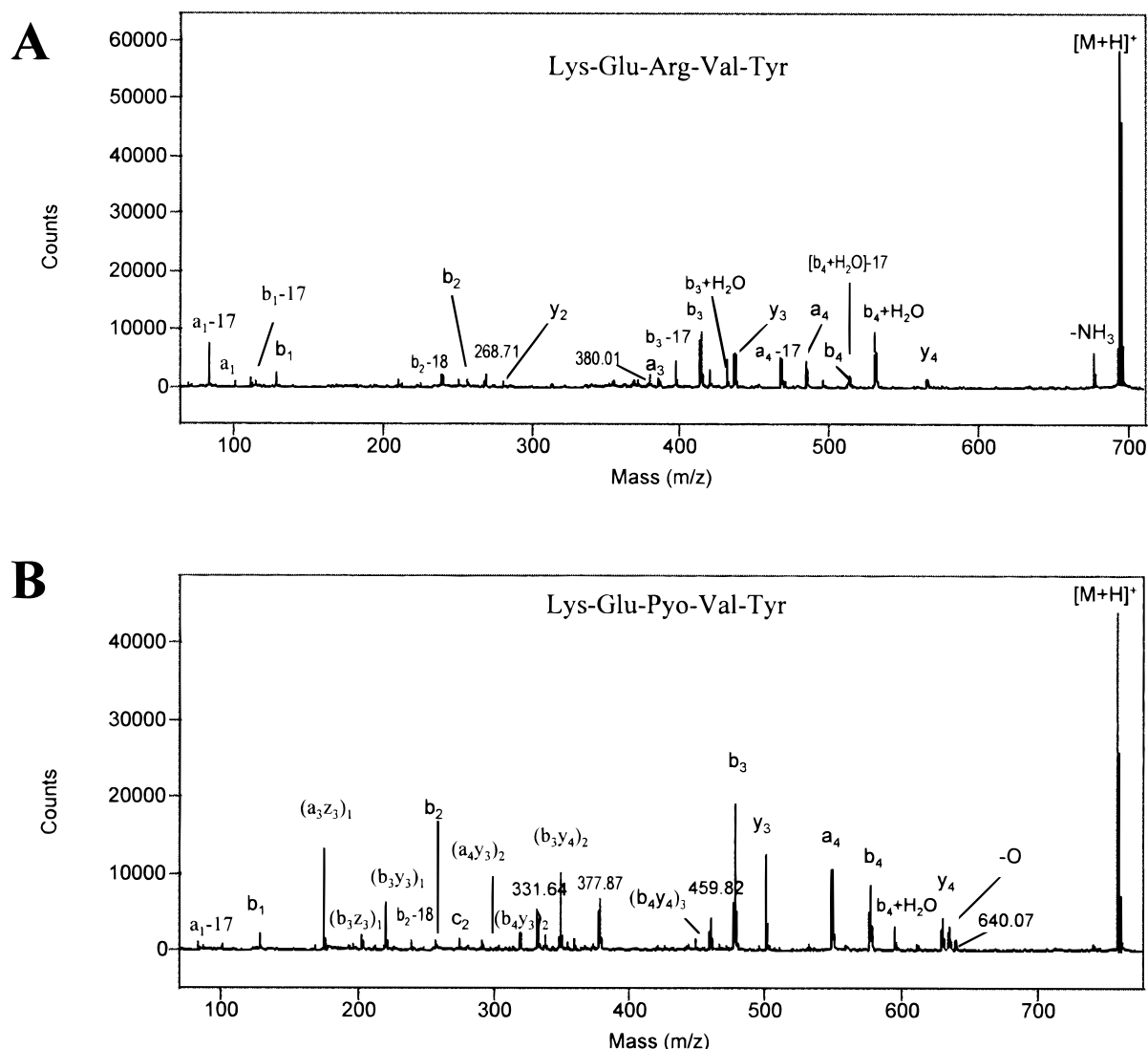


Figure 5. Metastable ion MALDI/PSD spectra of (A) Lys-Glu-Arg-Val-Tyr and (B) Lys-Glu-Pyo-Val-Tyr.

backbone.²⁶ When the basicity of the peptide is decreased by converting both arginines into *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithines, the basic amide groups compete for the ionizing proton and the population of protonated molecules becomes heterogeneous with respect to proton location and therefore charge-directed fragmentation plays a more important role.

Another possible explanation for the increase in fragmentation efficiency for [Pyo 1, Pyo 9]-bradykinin relative to bradykinin is related to the secondary structure of gas-phase peptide ions, particularly the formation of hydrogen bonds and their influence on fragmentation patterns. Our proposal is that activated bradykinin $[M + H]^+$ ions with sufficient internal energy to dissociate as metastable ions have a proton bridge between guanidino groups of the *N*-terminal and the *C*-terminal arginine residues and a hydrogen bond between the serine hydroxyl group and the *C*-terminal arginine guanidino group.²⁶ The secondary structure as a result of the proton bridge between the *N*-terminal and the *C*-terminal arginine residues and intramolecular hydrogen bonds decrease the number of fragmentation pathways.

The $[M + H]^+$ ions of [1–5]-bradykinin and spleenopentin both have *N*-terminal arginine residues and

exhibit a trend in fragmentation efficiency similar to that observed for bradykinin. The Pyo derivatives show a three times higher fragmentation efficiency than the non-derivatized peptides. The [5–9]-bradykinin compound has a *C*-terminal arginine residue and the fragmentation efficiency of the Pyo derivative is also higher than that of the non-derivatized peptide. Derivatization of the arginine of Lys-Glu-Arg-Val-Tyr increases the fragmentation efficiency by a factor of two, which is smaller than the increase in fragmentation efficiency for peptides containing derivatized *N*-terminal arginine (see Table 1).

The MI spectra of bradykinin and [Pyo 1, Pyo 9]-bradykinin $[M + H]^+$ ions are different [Fig. 1(A) and (D)]. The MI spectrum of [Pyo 1, Pyo 9]-bradykinin $[M + H]^+$ ions [Fig. 1(D)] has an almost complete series of *b* and *y* fragment ions (only the *b*₃, *b*₇ and *y*₁, *y*₂ fragment ions¹⁸ are missing) as compared with the spectrum of bradykinin $[M + H]^+$ ions [Fig. 1(A)] which contains only the *b*₆, *b*₇, *b*₈, *y*₁, *y*₃, *y*₅, *y*₆ and *y*₇ fragment ions. Also, there are several *a*-type fragment ions in the MI spectrum of bradykinin $[M + H]^+$ ions. The relative abundances of the *b* and *y* fragment ions are 2–16 times higher for [Pyo 1, Pyo 9]-bradykinin

Table 4. Neutral loss factor for derivatized and non-derivatized peptides^a

No.	Peptide ^b	Neutral loss ^c factor
1	Bradykinin	1.5
2	[Pyo 1]-bradykinin	0.4
3	[Pyo 9]-bradykinin	0.5
4	[Pyo 1, Pyo 9]-bradykinin	0.5
5	[1-5]-Bradykinin	1.0
6	[Pyo 1]-[1-5]-bradykinin	0.3
7	Splenopentin	0.4
8	[Pyo 1]-splenopentin	0.1
9	[5-9]-Bradykinin	0.5
10	[Pyo 9]-[5-9]-bradykinin	0.1
11	Lys-Glu-Arg-Val-Tyr	0.1
12	Lys-Glu-Pyo-Val-Tyr	— ^d

^a This factor is calculated as the sum of the absolute abundances of signals that correspond to small neutral molecule losses divided by $[M + H]^+$ ion absolute abundance and is averaged from two sets of experimental data.

^b Pyo represents the N^5 -(4,6-dimethyl-2-pyrimidinyl) ornithine residue.

^c Small neutral molecule losses from $[M + H]^+$ ion most often include loss of NH_3 and loss of H_2O . In the case of non-derivatized peptides, loss of 30 u (loss of CH_2O from the serine side-chain) and loss of $NH=C(NH_2)_2$ from the arginine side-chain are sometimes observed.

^d Small neutral molecule losses from $[M + H]^+$ ion are not detected.

$[M + H]^+$ ions in comparison with bradykinin $[M + H]^+$ ions (see Table 2). Note also that the $[M + H]^+$ ion is the most abundant ion in the bradykinin spectrum, but fragment ions are more abundant (y_8) or approximately of the same abundance (y_3 , y_7) as $[M + H]^+$ ion in the spectrum of [Pyo 1, Pyo 9]-bradykinin. Interestingly, more internal fragment ions are observed in the bradykinin $[M + H]^+$ ion spectrum than in the [Pyo 1, Pyo 9]-bradykinin $[M + H]^+$ ion spectrum. In contrast to the MI spectrum of [Pyo 1, Pyo 9]-bradykinin, the $[M + H]^+$ ion is the strongest signal in the MI spectra of [Pyo 1]-bradykinin and [Pyo 9]-bradykinin [Fig. 1(B) and (C)].

The abundance of C-terminal fragment ions (y_1 , y_2 , y_3 , y_4 , y_7 , y_8) relative to N-terminal fragment ions (a_5 , a_6 , a_8) in the MI spectrum of [Pyo 9]-bradykinin [Fig. 1(C)] is surprising because more N-terminal fragment ions are expected based on the position of arginine residue.¹⁶ The relative abundances of the same b and y fragment ions in [Pyo 1]-bradykinin and [Pyo 9]-bradykinin spectra are higher than those in the bradykinin spectrum. However, the increase in abundance is not as substantial as in the case of [Pyo 1, Pyo 9]-bradykinin (see Table 2).

The same b- and y-type fragment ions as observed in the MI spectrum of [1-5]-bradykinin are present in the spectrum of [Pyo 1]-[1-5]-bradykinin but in higher abundance [Fig. 2(A) and (B)]. The relative abundance of y-type fragments (y_3 , y_4) increases by a factor of three for the derivatized peptide in comparison with the non-derivatized peptide, whereas b-type fragment ions (b_1 , b_2 , b_4) increase in abundance by a factor of 1.7 for b_4 , 3.7 for b_1 and 15 for b_2 (Table 3).

Trends similar to those for [1-5]-bradykinin and [Pyo 1]-[1-5]-bradykinin were observed for splenopentin and [Pyo 1]-splenopentin. These peptides have complete series of b-type ions and almost complete y-type series (y_3 is missing) [Fig. 3(A) and (B)]. The relative abundance of b and y fragment ions increased 3–13-fold in the spectrum of [Pyo 1]-splenopentin in comparison with splenopentin (Table 3).

The MI spectrum of [5-9]-bradykinin is dominated by C-terminal fragment ions [Fig. 4(A)]. In fact, we observe a complete series of y-type ions as well as y – 17-type ions which are isobaric with z-type ions. The y – 17 ion series can be derived by a loss of ammonia from y-type ions or, alternatively, by loss of ammonia from the C-terminal arginine side-chain followed by y-type cleavage. Fragment ions of the y – 17-type are more abundant than the corresponding y-type ions with the exception of $y_1 - 17$ and y_1 . The high abundance of y – 17-type fragment ions is unusual for MI spectra.

The C-terminal fragment ion pattern of [Pyo 9]-[5-9]-bradykinin is different from that of [5-9]-bradykinin. In the former, abundant and complete y-type fragment ion series is observed with the y_3 ion signal of approximately the same abundance as the $[M + H]^+$ ion signal [Fig. 4(B)]. These y fragment ions are 1.5–6.8 times more abundant than the same fragment ions in the spectrum of the non-derivatized peptide (Table 3). However, the y – 17 ion series is not observed because loss of NH_3 from the side-chain of the C-terminal Pyo residue is not possible. Instead, a complete y – 18 ion series of very low abundance ions that involves water loss from y fragment ions is observed.

The MI spectra of Lys-Glu-Arg-Val-Tyr and Lys-Glu-Pyo-Val-Tyr are dominated by N-terminal fragments [Fig. 5(A) and (B)]. Both spectra contain a complete series of b-type fragment ions. In addition, the spectrum of Lys-Glu-Arg-Val-Tyr has a nearly complete set of a-type fragment ions; only a_2 is missing. In addition to a complete series of b-type fragment ions, the a_4 and c_2 N-terminal fragment ions are present in the Lys-Glu-Pyo-Val-Tyr spectrum. Note that the relative abundance of b_1 and b_2 fragment ions in the MI spectrum of the derivatized peptide is approximately the same as that in the non-derivatized peptide spectrum, but the relative abundance of b_3 and b_4 increases dramatically (see Table 3). The reason for this effect is that b_1 and b_2 have the same structure in both peptides, whereas b_3 and b_4 contain the Pyo residue in the case of the derivatized peptide or Arg in the case of the non-derivatized peptide. Three y-type fragment ions (y_2 , y_3 , y_4) are present in the Lys-Glu-Arg-Val-Tyr spectrum and only two (y_3 , y_4) in the Lys-Glu-Pyo-Val-Tyr spectrum. However, these two y-type fragment ions are 2–3 times more abundant than the same fragments in the spectrum of the non-derivatized peptide.

A characteristic feature of Lys-Glu-Pyo-Val-Tyr MI spectrum is the abundance of internal fragment ions, e.g. ($a_4 y_3$)₂, ($b_4 y_3$)₂, ($b_3 y_4$)₂ and ($b_4 y_4$)₃. Three internal fragment ions containing only Pyo residue are also observed, e.g. ($a_3 z_3$)₁, ($b_3 z_3$)₁ and ($b_3 y_3$)₁. This suggests a dramatically increased susceptibility for bond cleavages around the N^5 -(4,6-dimethyl-2-pyrimidinyl)ornithine residue as compared with a peptide having an

arginine residue in the same position. Another observation that illustrates this point is the presence of abundant $y_8 - 17$ and y_8 fragment ions in addition to b_1 and $a_1 - 17$ in the MI spectrum of [Pyo 1]-bradykinin [Fig. 1(B)]. In the case of [Pyo 9]-bradykinin, the presence of a_8 , $a_8 - 17$, $[b_8 + H_2O]$, $[b_8 + H_2O] - 17$ and y_1 fragment ions also supports the increased susceptibility hypothesis [Fig. 1(C)].

Small neutral molecule loss ions in spectra of derivatized and non-derivatized peptides

Ions that are formed by losses of small neutral molecules from $[M + H]^+$ ions are common in MI spectra of arginine-containing peptides. Although losses of small neutral molecules do not provide sequence information, such ions are useful diagnostics of the fragmentation process.²³ Small neutral molecule losses observed most often include loss of ammonia ($[M + H]^+ - 17$) and loss of water ($[M + H]^+ - 18$). In addition, some peptide $[M + H]^+$ ions which contain serine residues lose formaldehyde ($[M + H]^+ - 30$; abbreviated to -30 in the spectra). A similar fragmentation process yielding $[M - H]^- - 30$ fragment ions was previously observed in the negative-ion mode using CID.⁴¹ Loss of guanidine ($[M + H]^+ - 59$; abbreviated to $-R$ in the spectra) from the arginine side-chain is observed in the spectra of $[M + H]^+$ ions of bradykinin and [1-5]-bradykinin. We also observed an ion at m/z 1000.38 that corresponds to $[M + H]^+ - 60$ but only for the $[M + H]^+$ ion spectrum of bradykinin; however, even in the bradykinin spectrum the $[M + H]^+ - 59$ fragment ion is more abundant than the $[M + H]^+ - 60$ fragment ion. Because this observation contradicts previously reported fragmentation spectra of bradykinin $[M + H]^+$ ions,⁴² we are investigating this reaction channel further.

Bradykinin has the highest neutral loss factor among the 12 peptides studied (see Table 4). This is consistent with previous data suggesting that the small neutral molecule loss (especially loss of NH_3) is the lowest energy dissociation process for bradykinin.¹⁹ The major neutral losses observed for bradykinin $[M + H]^+$ ions are the four ions described in the previous paragraph and also the loss of two ammonia molecules ($[M + H]^+ - 34$) and loss of $HN=C=NH$ ($[M + H]^+ - 42$) [Fig. 1(A)]. Peaks corresponding to loss of water and loss of formaldehyde (loss of 30 u) are not labeled in the spectrum owing to lack of space. The neutral loss factor for the three derivatized bradykinins decreases by a factor of three. Note that six neutral losses are observed in the MI spectrum of bradykinin $[M + H]^+$ ions as compared with only three (loss of NH_3 , loss of H_2O and loss of CH_2O) for [Pyo 1]-bradykinin and [Pyo 9]-bradykinin and only loss of NH_3 and H_2O is observed for [Pyo 1, Pyo 9]-bradykinin. The signal for NH_3 loss in the MI spectrum of [Pyo 9]-bradykinin is more intense (relative abundance 44%) than that for [Pyo 1]-bradykinin (15%) but less intense than that for bradykinin (50%) [Fig. 1(A), (B) and (C)]. This observation could be explained by assuming that NH_3 loss occurred more readily from the *N*-terminal arginine guanidino group than from the *C*-terminal arginine or α -amino group.

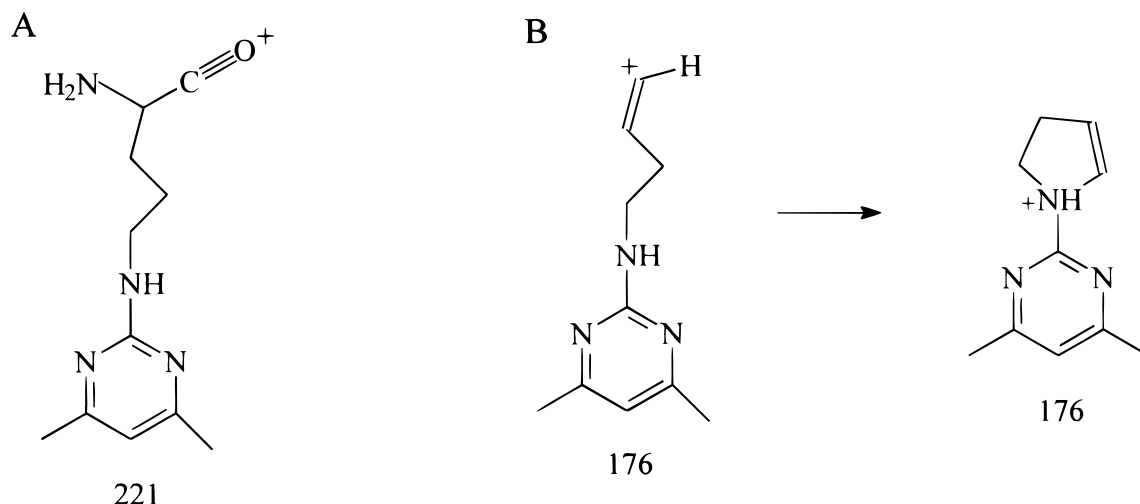
For those peptides with an *N*-terminal arginine residue, the neutral loss factor also decreases 3–4-fold after derivatization (Table 4). For [1-5]-bradykinin and [Pyo 1]-[1-5]-bradykinin we observe a decrease in the number of ions corresponding to the loss of small neutral molecules from three for the non-derivatized peptide (loss of NH_3 , loss of H_2O and loss of $NH=C(NH_2)_2$) to one for the derivatized peptide (loss of H_2O). The same trend is observed for the $[M + H]^+$ ions of Phe-Ser-Pro-Phe-Arg and Phe-Ser-Pro-Phe-Pyo. The number of ions corresponding to small neutral molecule losses decreases from three in the spectrum of Phe-Ser-Pro-Phe-Arg (loss of NH_3 , loss of H_2O and loss of CH_2O) to one in the spectrum of Phe-Ser-Pro-Phe-Pyo (loss of H_2O). The neutral loss factor decreases by a factor of 5 from 0.5 to 0.1.

For Lys-Glu-Arg-Val-Tyr, which has arginine in the middle of the sequence, the only fragment ion formed by loss of a small neutral molecule corresponds to NH_3 loss. Interestingly, small neutral molecule losses are not detected in the MI spectrum of Lys-Glu-Pyo-Val-Tyr.

Rearrangement ion $[b_{n-1} + H_2O]$ vs. $[b_{n-1} + H_2O] - 17$ and b_{n-1}

The rearrangement ion $[b_{n-1} + H_2O]$ was studied by Gaskell and co-workers¹⁷ using ^{18}O -labeling techniques. Based on their study, we assign the major component of the m/z 904.40 peak in the MI spectrum of bradykinin $[M + H]^+$ ion to $[b_8 + H_2O]$ (y_8 has the same mass). Conversely, the dominant fragment ion at m/z 968.52 in the spectrum of [Pyo 1, Pyo 9]-bradykinin $[M + H]^+$ ions could be assigned as y_8 or $[b_8 + H_2O]$ because the ratio of y_8 to $[b_8 + H_2O]$ was found to be 6:1.¹⁷

The relative abundance of the $[b_{n-1} + H_2O]$ ion decreases in the MI spectra of derivatized peptides in comparison with the non-derivatized peptides. This observation is consistent with Gaskell and co-workers' results.¹⁷ We observe the $[b_{n-1} + H_2O] - 17$ fragment ion which is formed by NH_3 loss from the $[b_{n-1} + H_2O]$ ion or, alternatively, by NH_3 loss from $[M + H]^+$ ion followed by rearrangement to form $[b_{n-1} + H_2O]$ ions. The rearrangement ion $[b_{n-1} + H_2O] - 17$ has not been observed before, to the best of our knowledge. The important trend is that the relative abundance of the $[b_{n-1} + H_2O] - 17$ ion is higher for the non-derivatized peptides than is the abundance of b_{n-1} ion which is just 1 Da lower in mass. The trend is observed for bradykinin [see the inset in Fig. 1(A)], [1-5]-bradykinin, splenopentin, and Lys-Glu-Arg-Val-Tyr. In the case of partially derivatized [Pyo 9]-bradykinin the b_8 ion is not detected but the $[b_8 + H_2O] - 17$ ion is present in the spectrum. On the other hand, the $[b_{n-1} + H_2O] - 17$ ion is not detected in the spectrum of derivatized peptides such as [Pyo 1]-[1-5]-bradykinin and [Pyo 1]-splenopentin, but the $[b_{n-1} + H_2O]$ fragment ion is present. Based on these facts, it seems reasonable to assume that the loss of NH_3 which transforms $[b_{n-1} + H_2O]$ into $[b_{n-1} + H_2O] - 17$ probably occurs from the arginine residue. In the case of [5-9]-bradykinin, which contains the *C*-terminal arginine, the rearrangement ion $[b_{n-1} + H_2O]$ is not detected, which is consistent with the



Scheme 2

data for Ala-Ser-Val-Phe-Arg published by Gonzalez *et al.*⁷

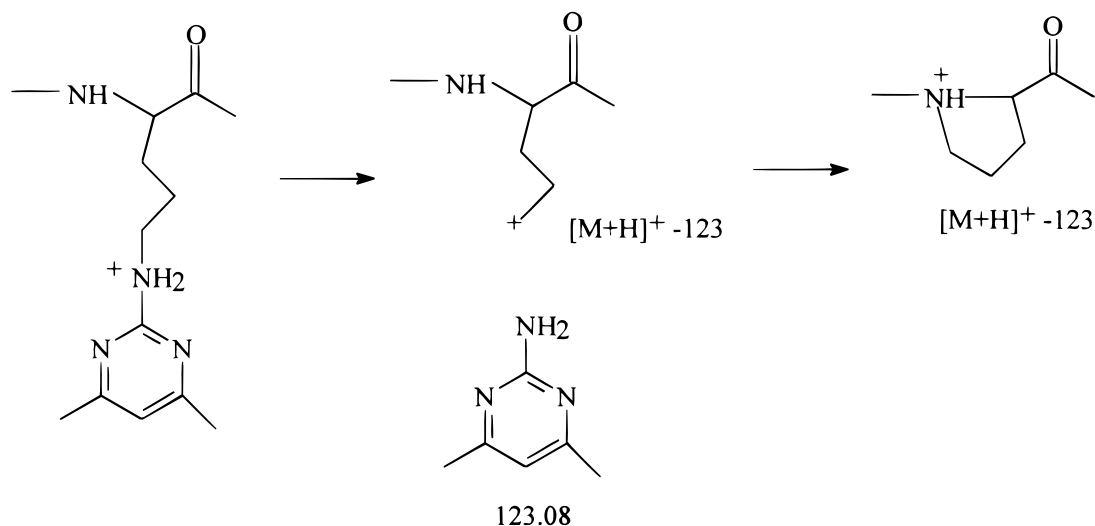
Specific fragmentation of *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue

In the MALDI/PSD spectra of all seven derivatized peptides studied in this work we observe fragment ions at *m/z* 176 and 221. These fragment ions are assigned to the *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue. The *m/z* 221 fragment ion corresponds to the acylium ion [Scheme 2(A)], which was assigned as *b*₁ in the case of Pyo location at the *N*-terminus. When the Pyo residue is located at the *C*-terminus the *m/z* 221 fragment ion was assigned as *y*₁ - 18. The assignment for the *m/z* 221 fragment ion is (*b*₃*y*₃)₁ when the Pyo location is in the middle of the sequence Lys-Glu-Pyo-Val-Tyr. In the case of [Pyo 1, Pyo 9]-bradykinin, this ion can come from either the *N*-terminus or the *C*-terminus so it can be either *b*₁ or *y*₁ - 18. The most abundant signals at *m/z* 221 were detected in the spectra of [Pyo 1]-[1-5]-bradykinin and [Pyo 1]-splenopentin, which has the Pyo at the *N*-terminus of the molecule [see Figs 2(B) and 3(B)].

The *m/z* 176 fragment ion is probably formed by loss

of NH₃ and CO from the *m/z* 221 ion. The vinylic cation structure derived from generally accepted fragmentation mechanisms should be very unstable, yet this ion is abundant in the spectra and is the dominant ion in some cases. We suggest that this vinylic cation cyclizes to yield the biheterocyclic structure with the charge located on nitrogen [Scheme 2(B)]. Loss of NH₃ from the *m/z* 221 ion is supported by the corresponding ion (*b*₃*z*₃)₁ (204.00) which is observed in the MI spectrum of Lys-Glu-Pyo-Val-Tyr. The *m/z* 176 fragment ion is assigned as *a*₁ - 17 in the case when Pyo is at the *N*-terminus of the sequence. When Pyo is located at the *C*-terminus, the assignment for this ion is (*a*_{*n*}*z*₁) (*n* is the number of residues in the sequence). The assignment for the *m/z* 176 fragment ion is (*a*₃*z*₃)₁ in the case of Lys-Glu-Pyo-Val-Tyr. The ion at *m/z* 176 is especially abundant when the Pyo residue is at the *N*-terminus or in the middle of the sequence [see Figs 1(B), 2(B), 3(B) and 5(B)].

Another fragmentation process specific for the *N*⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine residue is observed in the PSD spectra of four out of the seven derivatized peptides. This process involves loss of 123 u, corresponding to loss of 2-amino-4,6-dimethylpyrimidine, as shown in Scheme 3. The [*M* + *H*]⁺ - 123 is abbreviated as - O in the spectra. This ion is detected in the



Scheme 3

spectra of [Pyo 1]-bradykinin [Fig. 1(B)], [Pyo 1, Pyo 9]-bradykinin [Fig. 1(D)], [Pyo 1]-[1-5]-bradykinin [Fig. 2(B)] and Lys-Glu-Pyo-Val-Tyr [Fig. 5(B)].

CONCLUSIONS

We have demonstrated an increase in the fragmentation efficiency (by a factor of 2.0–3.5) of arginine-containing peptides by derivatizing the guanidino groups with acetylacetone. The increase in the fragmentation efficiency is higher when the derivatized residue (Pyo) is at the *N*-terminus or on both ends of the sequence. The relative abundance of some fragment ions is higher than or the same as the abundance of the protonated parent ion in the derivatized peptides, whereas in the non-derivatized peptides the relative abundance of the protonated parent ion is higher than that of any fragment ion. For peptides that contain more than one arginine, derivatization of all arginine residues is needed to observe the maximum effect. The proposed explanation for this phenomenon is based on the assumption that backbone amide nitrogens are competing for the

ionizing proton with N(5) of N⁵-(4,6-dimethyl-2-pyrimidinyl)ornithine and this residue is significantly less basic than the guanidino group of arginine. Hence the charge-directed fragmentation mechanism is more probable for derivatized peptides as opposed to charge-remote fragmentation for non-derivatized peptides.

Metastable ion decomposition MALDI/PSD spectra of derivatized peptides exhibit fewer ions resulting from the loss of small neutral molecules, which do not provide sequence information. Those ions are of lower relative abundance in comparison with non-derivatized peptides as reflected in the decrease in the neutral loss factor by 3–5-fold. Fragment ions characteristic for the Pyo residue were identified at *m/z* 176 and 221, in addition to the ion $[M + H]^+ - 123$.

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