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RESEARCH ARTICLE

157 nm Photodissociation of Dipeptide Ions Containing N-Terminal Arginine

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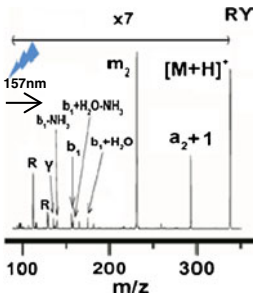

Abstract. Twenty singly-charged dipeptide ions with N-terminal arginine were photodissociated using 157 nm light in both a linear ion-trap mass spectrometer and a MALDI-TOF-TOF mass spectrometer. Analogous to previous work on dipeptides containing C-terminal arginine, this set of samples enabled insights into the photofragmentation propensities associated with individual residues. In addition to familiar products such as a-, d-, and immonium ions, m_2 and m_2+13 ions were also observed. Certain side chains tended to cleave between their β and γ carbons without necessarily forming d- or w-type ions, and a few other ions were produced by the high-energy fragmentation of multiple bonds.

Key words: Photodissociation, Peptide fragmentation

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Introduction

A variety of peptide activation methods exist, including collision-induced dissociation (CID) [1–4], electron capture/transfer dissociation (ECD/ETD) [5, 6], surface-induced dissociation [7, 8], and photodissociation [9]. Different photodissociation wavelengths of light have been applied ranging from infrared [10–12] to ultraviolet [13–19]. Ultraviolet light excites electronic transitions and provides a well-defined energy that is sufficient to induce bond cleavage [20].

Light with a wavelength below 200 nm is strongly absorbed by backbone amide groups [21]. The absorption spectrum of polyalanine film displays two main bands around 190 and 165 nm [22]. Similar VUV action spectra of protein polyanions have recently been recorded [23]. The VUV two absorption bands can be excited by the ArF excimer laser at 193 nm (6.4 eV) and the F_2 laser at 157 nm (7.9 eV). Although the former wavelength is more convenient to use, in our experience, 157 nm light tends to generate a wider distribution of fragment ions [14, 24, 25]. Trypsin produces peptides with C-terminal arginine or lysine, and their vacuum ultraviolet photodissociation leads to high-energy x-, v-, and w-type ions. Peptides containing N-terminal arginine or guanidinated lysine yield a plethora of a- and d-type ions [14, 26]. In addition to even-electron a-type ions, odd-electron a + 1 radical ions are also produced. Observation of these radical ions suggests that 157 nm photodissociation involves a homolytic bond cleavage [26]. The appearance of d-type ions depends upon the amino acids present in the peptide sequence. A library of peptides can facilitate data analysis and the recognition of fragmentation propensity trends [28]. However, even for peptides of modest length, libraries are typically (and necessarily) incomplete because of the large number of combinations of sequences that they contain. A library of dipeptides has a very manageable number of sequences and is relatively easy to analyze, yet it should display many of the important residue-dependent fragmentation features. We have previously investigated the photodissociation of all 20 naturally occurring dipeptides that contain C-terminal arginine [29]. For completeness, N-terminal arginine-containing dipeptides are now analyzed. Their spectra display a number of common features along with fragment ions whose appearance and mass depend on the C-terminal residue. Althogh most of the observed product ions are familiar peptide photofragments, some unusual cleavages involving the C-terminal residue also occur. Deriving assignments and structures for the latter is facilitated by accurate mass measurements and correlating the observed ions with the C-terminal residue of the precursor. Observations about dipeptide photodissociation

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spectra are summarized and structures of some unusual fragments are proposed.

Experimental

Materials

Acetonitrile and *N,N*-dimethylformamide (DMF) were supplied by EMD Chemicals (Gibbstown, NJ, USA). Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Piperidine, *N,N*-diisopropylethylamine and α-cyano-4-hydroxycinnamic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Amino acids, Wang resin, and DEPBT were purchased from Midwest Biotech (Erie, IL, USA). The peptide Arg-Pro was purchased from Bachem Americas, Inc. (Torrance, CA, USA).

Peptide Synthesis

Twenty dipeptides RX (where X represents each one of the 20 amino acids except proline) were synthesized in-house via solid phase reactions [30, 31]. Eleven mg of Wang resin with preloaded Fmoc amino acids were weighed out and placed in a cell for synthesis. One mL of DMF was added and the resin swelled for 5 min. After draining DMF, 250 µL of 20 % piperidine in DMF (vol/vol) was used to cleave the Fmoc group. The piperidine was drained after 10 min and the resin was washed five times using DMF. Nine mg DEPBT and Fmoc arginine equivalent to a four times molar excess relative to the resin were dissolved in 250 µL of 4.2 %*N,N*-diisopropylethylamine in DMF (vol/vol). This solution was immediately added to the Wang resin to attach the N terminal amino acid. The mixture was stirred several times during the 45-min reaction period. The resin was then dried and washed five times using DMF; 250 µL of 20 % piperidine in DMF were added to cleave the Fmoc group. After the resin was washed using DMF, 1 mL of 95 % TFA and 5 % methanol were transferred to the cell to cleave the peptide from the resin. After 2 h, the cleaved peptide was drained into a glass tube. The peptides were collected and stored in a freezer for future analysis.

Mass Spectrometry

Solutions to electrospray were diluted to 0.1 mg/mL of peptide in 50 % water/50 % acetonitrile with 2 % acetic acid. ESI mass spectra were recorded on a LTQ Velos Instrument (Thermo

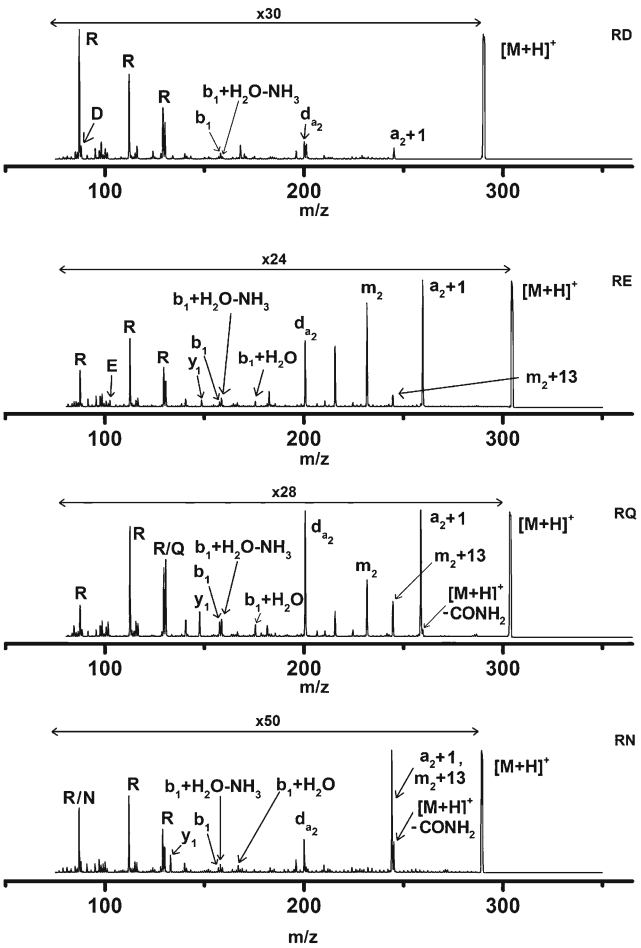


Figure 1. Ion-trap photodissociation spectra of dipeptides RD, RE, RQ, and RN

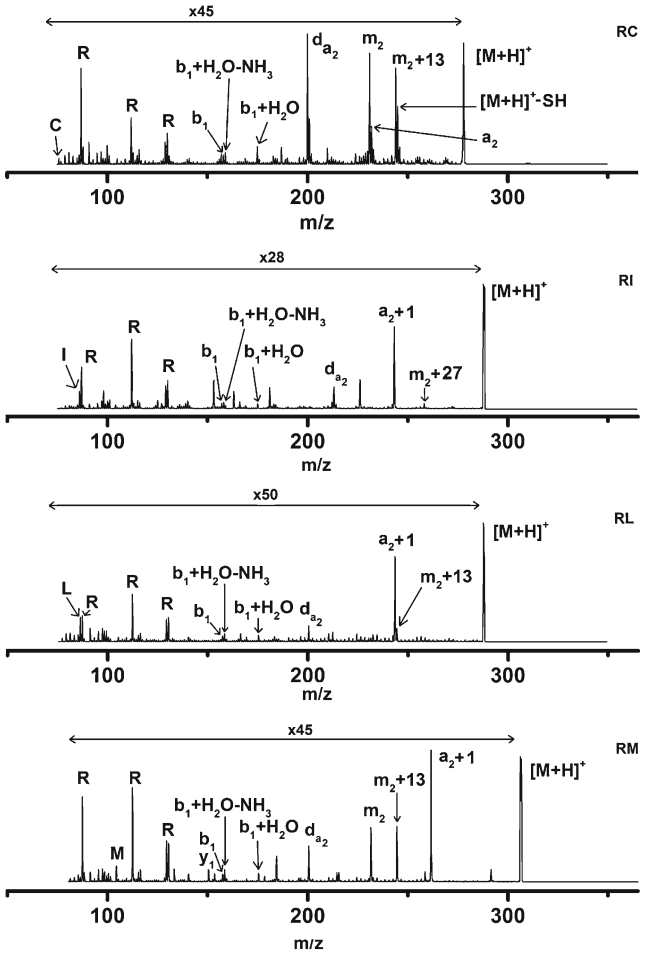


Figure 2. Ion-trap photodissociation spectra of dipeptides RC, RI, RL, and RM

Electron, San Jose, CA, USA) with a custom source and a F_2 laser (EX100HF-60; GAM Laser, Orlando, FL, USA) connected to the back end of the trap [26, 32]. The laser's pulse length was 18 ns and the fluence was 2.6 mJ/cm^2. All ESI samples were infused at a rate of 300 nL/min from a needle biased at ~2 kV. Ions were photodissociated about once every second at the beginning of the normal activation stage. Photodissociation mass spectra were averaged for 3 min.

For MALDI experiments, each dipeptide was diluted to 2 pmol/μL with H_2O, and 0.5 μL of this solution was spotted onto a stainless steel target plate. After drying, 0.5 μL of matrix solution (10 mg/mL CHCA in 50 % ACN/50 % H_2O/0.1 % trifluoroacetic acid) was added on top of each spot. MALDI mass spectra were recorded with an ABI 4700 TOF-TOF mass spectrometer (Foster City, CA, USA) in positive reflectron mode. Another F_2 laser (Coherent Lambda Physik, Gottingen, Germany) was connected to the TOF-TOF mass spectrometer as previously described [33]. The laser's pulse length was 10 ns with a fluence of 6 mJ/cm^2. Its timing is automatically controlled by a programmable delay generator. It is important to note that in the TOF-TOF apparatus, fragment ions are analyzed on a microsecond time scale, while they are scanned out of the trap over tens of milliseconds.

Results and Discussion

All 157 nm ion trap photodissociation spectra of dipeptide ions are displayed in Figures 1, 2, 3, and 4, and in Supplementary Figure 1. In general, peaks that appeared in these ion trap mass spectra were also observed in TOF-TOF data. Most of the major differences could be attributed to neutral losses, proton transfers, or double bond formations that were observed on the ion trap, but not the TOF-TOF. This can be rationalized by the difference in time scales of the two experiments: milliseconds on the ion trap, and microseconds with the TOF [34]. The TOF-TOF signal was dominated by immonium ions and convoluted with post source decay (PSD) and matrix cluster peaks, making direct analysis somewhat more difficult. Likewise, precursor ion isolation was better with the ion trap. Therefore, ion trap data were primarily relied upon, unless exact masses were required to determine an ion's identity. Table 1 summarizes

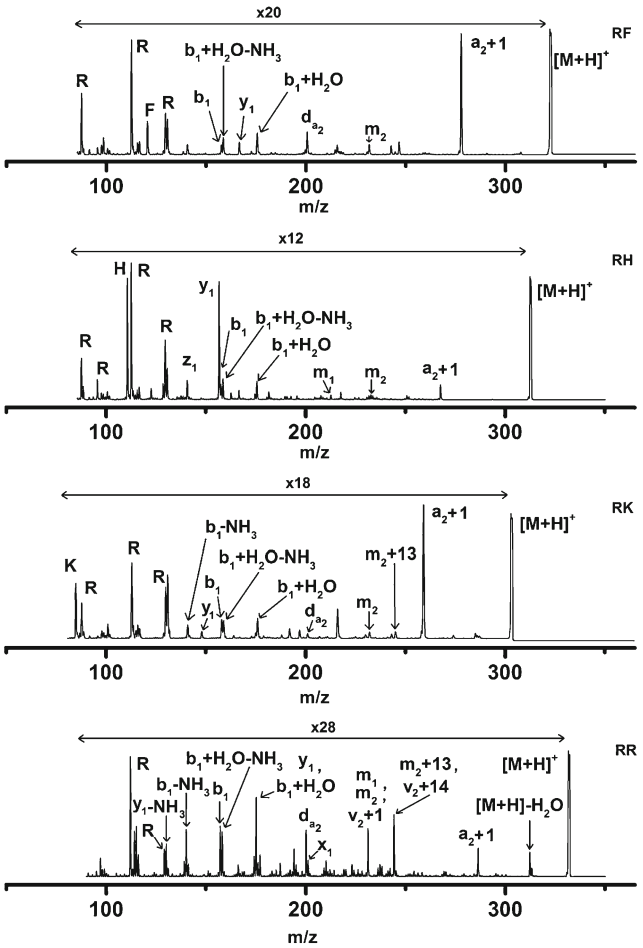


Figure 3. Ion-trap photodissociation spectra of dipeptides RF, RH, RK, and RR

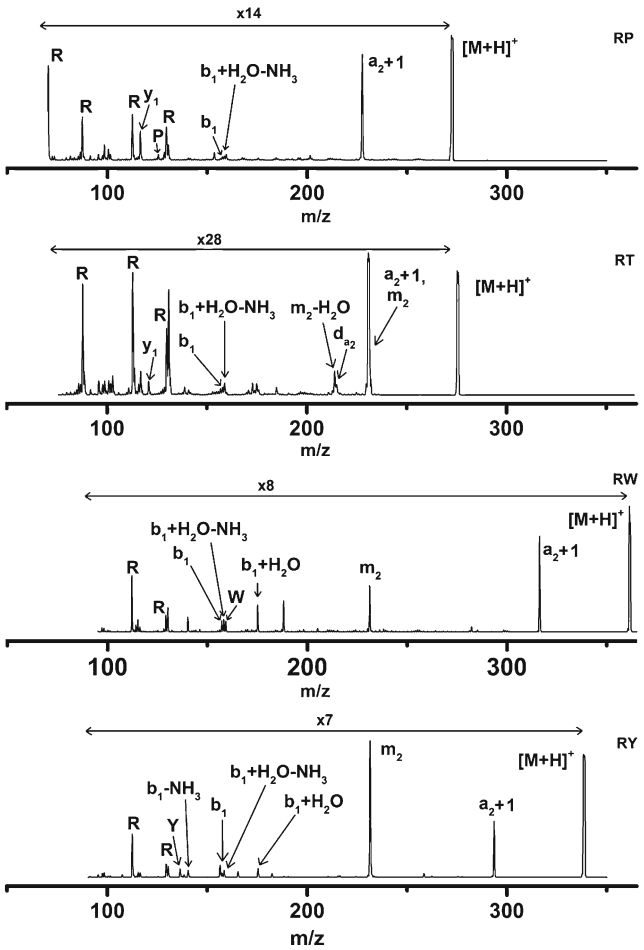


Figure 4. Ion-trap photodissociation spectra of dipeptides RP, RT, RW, and RY

Table 1. Summary of 157 nm Photodissociation Fragments Observed from Dipeptide Ions with N-Terminal Arginine

	Immonium ion from C-terminal residue	a_2+1	m_2+13	m_2	d_{a2}	b_1+H_2O	b_1+H_2O-NH_3 and b_1	y_1	z_1	loss of $CONH_2$
RA		✔								
RG		✔								
RP	✓	✔					✓	✔		
RK	✔	✔	✓	✓	✓	✔	✓	✓		
RL	✓	✔	✓		✓	✓	✓			
RF	✔	✔		✓	✔	✔	✓	✓		
RI	✓	✔	✓		✔	✓	✓			
RT		✔		✓	✔		✓	✓		
RV		✔			✓					
RS		✔		✔	✓					
RC	✓		✔	✔	✔	✔	✓			
RE	✓	✔	✓	✔	✔	✓	✓	✓		
RQ	✓	✔	✔	✔	✔	✓	✓	✔		✓
RH	✔	✔		✓		✔	✓	✔	✔	
RM	✓	✔	✔	✔	✔	✓	✓	✓		
RW	✔	✔		✔		✔	✓			
RY	✔	✔		✔		✓	✓			
RD	✓	✓			✓		✓			
RN	✓	✔	✔		✔	✓	✓	✓		✔
RR	✔	✔	✔	✔	✔	✔	✓	✔		

Light check marks correspond to relatively weak peaks; darker checks indicate more intense peaks.

the commonly observed spectral features. Light check marks correspond to relatively weak peaks while darker checks indicate more intense peaks. It was noted that for these dipeptide ions that fragment yield varied with the C-terminal residue. Fragmentation efficiency, defined as the integrated fragment ion intensity divided by the integrated intensities of all ions including the precursor, is summarized by the bar graph in Figure 5. As one might predict, aromatic residues that include chromophores likely to absorb in the vacuum ultraviolet tend to produce the highest fragment ion yields.

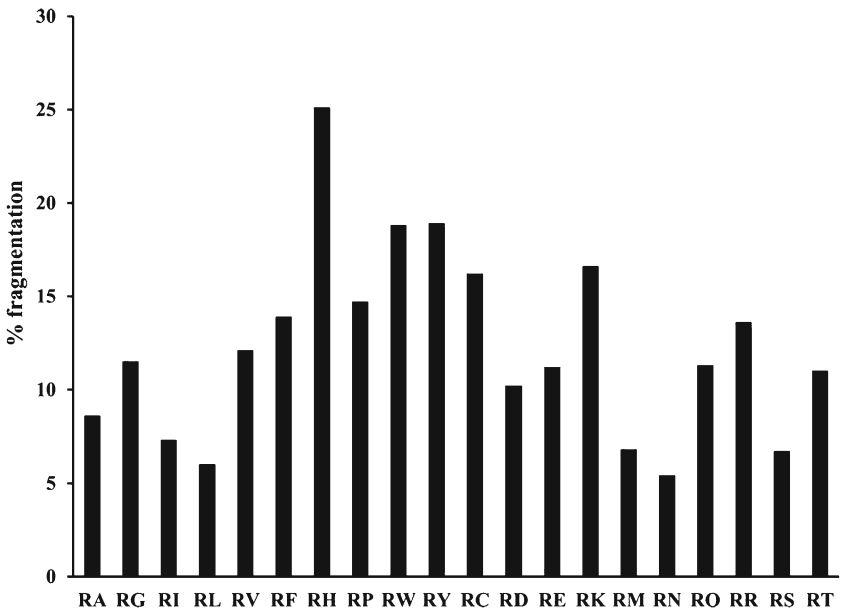


Figure 5. Relative photofragmentation efficiency for all of the RX dipeptides; % fragmentation is defined as (ion fragments)/(all ions), where all ions include the precursor

Immonium Ions

Photodissociation of dipeptide ions yielded a plethora of immonium ions, particularly those associated with the N-terminal arginine. The exceptions to this were several of the smaller residues (glycine, alanine, serine, etc.) whose immonium ions were not observed because of their small masses. Phenylalanine, histidine, tryptophan, and tyrosine residues produced particularly large immonium ion peaks, presumably because their aromatic groups absorb 157 nm vacuum ultraviolet light very strongly [37].

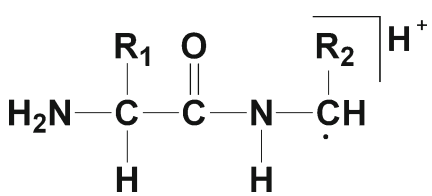
a-Type Ions

Loss of the C-terminal carboxylic acid group (45 Da) to produce a_2 + 1 ions was commonly observed (Scheme 1). These radical species have previously been observed in 157 nm photodissociation spectra of peptide ions that have highly basic N-terminal residues [26, 27]. RC is the only case for which an a_2 ion peak of significant intensity appeared; a_1 ions were observed ubiquitously, but these are just arginine immonium ions.

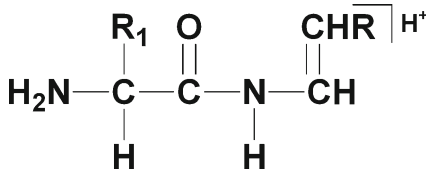
Although loss of the carboxylic acid group was dominant for all sequences, there were a few cases with a loss of 44 Da, which suggested a loss of carbon dioxide. However, this peak was only significant in the spectrum of RN and to a lesser extent RQ. In a previous 157 nm photodissociation study, loss of CO_2 was reported to be favored when a zwitterionic salt bridge was present in the gas phase structure [35].The salt bridge would ordinarily involve the positive N-terminus, the negative C-terminus, and a positive amino acid side chain. As recently demonstrated, internal calibration of TOF-TOF data can yield highly accurate mass spacings. Using this approach, we found that the 44 Da loss peak actually corresponded to a loss of 44.010 Da. This is more consistent with a loss of $CONH_2$ (44.014 Da) than CO_2 (43.990 Da), thereby explaining why this feature appears in the spectra of RN and RQ.

d-Type Ions

Side-chain fragmentation of the C-terminal residue producing d_{a2} ions was observed for several amino acids. This fragment usually appears at 200 Da. However, for RI and



Scheme 1. Structural formula of an a_2 + 1 ion. R_1 and R_2 are the side chains of arginine and the C-terminal residue, respectively



Scheme 2. Structural formula of a d_2 ion. R_1 is the arginine side chain

RT, its mass shifts to 214 Da because of the branched side chains. As depicted in Scheme 2, production of d_2 ions involves loss of the C-terminal carboxylic acid group (45 Da), cleavage between the β and γ carbons of the amino acid side chain, and formation of a double bond between the α and β carbon. These are not produced if the side-chain residue is too small (e.g., glycine or alanine) or if the side chain is proline. Usually, d-type ions are not observed terminating at an aromatic residue because of the strength of the bond between the β carbon and the ring. It was therefore somewhat surprising that photodissociation of the dipeptide RF ion yielded a d_{a2} ion. Nevertheless, VUV light provides substantial excitation energy and the aromatic chromophore absorbs very strongly in this wavelength region. It is interesting that analogous w_2 ions were only formed in the photodissociation of two XR dipeptide ions, NR and DR [28]. The w_2 ions form because of the side chain of NR and DR hydrogen bonding with the N-terminal amine, and it is possible that an analogous process occurs for the d_{a2} ions with the C-terminal carboxylic acid and a wider array of amino acid side chains.

b_1 + H_2O Ions

In peptides with C-terminal arginine, 175 Da corresponds to the commonly observed y_1 ion fragment so it was surprising

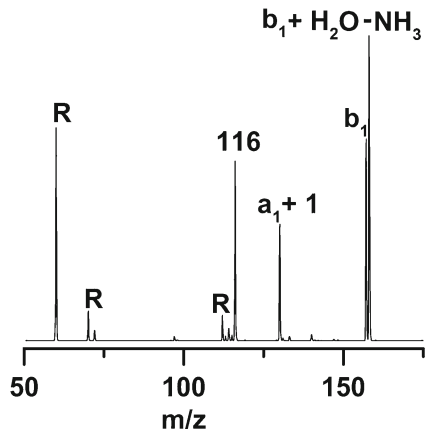
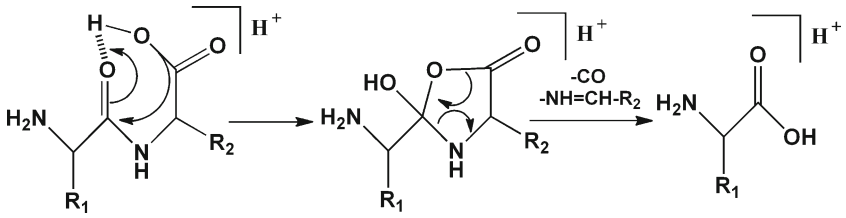


Figure 6. Spectrum produced by collision-induced dissociation of the 175 m/z photofragment ion derived from dipeptide RY

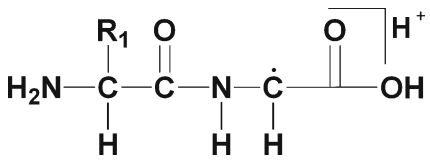


Scheme 3. Rearrangement of the C-terminus to form a $b_1 + H_2O$ ion. R_1 is the arginine side chain

to see this photofragment in almost every one of these RX spectra. The MS^3 capability of the ion trap was used to establish the identity of this peak. One hundred seventy-five Da photofragment ions from several dipeptides were isolated and subjected to CID. The resulting mass spectra were all similar and a typical one, obtained with dipeptide RY, appears in Figure 6. The key fragments are the arginine immonium ions and peaks at 157 and 158 Da. The appearance of the former suggests that the arginine is completely intact. $b_{N-1} + H_2O$ fragment ions (where N is the number of residues in the peptide) are commonly observed from larger peptide precursors when the loss of the C-terminal amino acid occurs with oxygen migration [36, 38, 39]. The analogous formation of $b_1 + H_2O$ ions from our dipeptides is depicted in Scheme 3. It is apparent from this picture that a $b_1 + H_2O$ ion originating from a RX dipeptide is identical to the y_1 ion from a XR dipeptide, and they are both simply protonated arginine. Harrison and coworkers have demonstrated that collisionally activated protonated arginine produces 157 and 158 Da fragments through the loss of H_2O and NH_3 [40]. The peak at 116 Da corresponds to the loss of guanidine (CN_3H_5) from the 175 Da ion. These observations are all consistent with the 175 Da feature being a $b_1 + H_2O$ ion.

m-Type Ions

Complete homolytic cleavage of the C-terminal amino acid side chain from the peptide backbone occurred frequently and produced 231 Da m_2 ions, whose structure is depicted in Scheme 4 (where R_1 represents the arginine side chain). This cleavage occurred in a variety of cases, including RC, RE, RF, RH, RK, RM, RQ, RS, RT, RW, and RY, and a few generalizations can be made. All of the aromatic amino acids were cleaved from the backbone following 157 nm photoexcitation. However, the aliphatic residues



Scheme 4. Structural formula of a 231 Da m_2 ion. R_1 is the arginine side chain

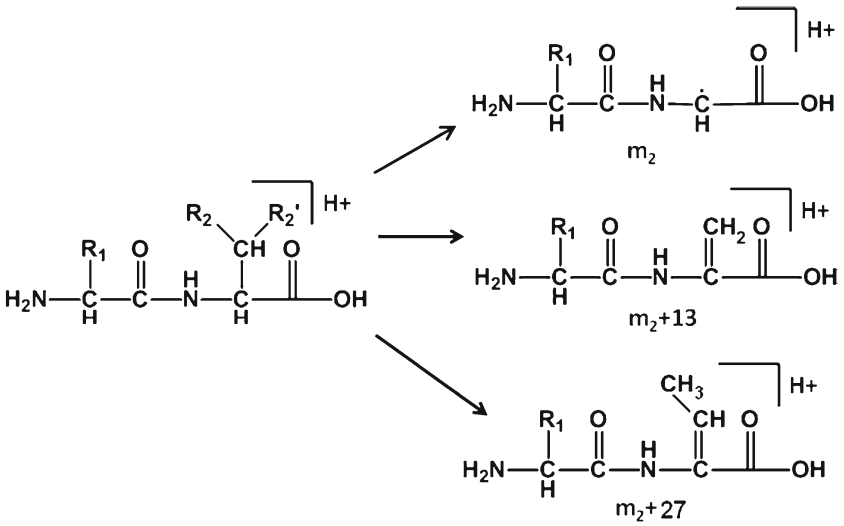
leucine, isoleucine, and valine did not form m_2 ions. Proline also did not undergo this cleavage, presumably because it is attached to the peptide backbone in two places. Interestingly, aspartic acid and asparagine did not undergo this cleavage, whereas glutamic acid and glutamine did. The extra carbon in the side chain of glutamic acid and glutamine allows for the formation of a conjugated leaving group, stabilizing its structure, but this is not possible for aspartic acid and asparagine. These m-type fragmentation propensities involving residues L, I, V, P, D, N, E, and Q are identical to those previously reported for XR peptides (and labeled in that work as v + 1 ions) [28].

In addition to the m_2 ions that were observed at 231 Da, a peak at 244 Da was observed in the photodissociation mass spectrum of many of the peptides. In our previous study, a side-chain cleavage was observed where the β carbon was retained, producing v_2 + 14 ions [28]. In the present case, retention of the β carbon and formation of a double bond to the α carbon yield the observed m + 13 fragment ion (Scheme 5). (Note that this is like a d_2 ion but with retention of the C-terminal carboxylic acid).

To verify that the peaks at 244 Da were m_2+13 ions, an internal calibration was applied to each TOF-TOF photodissociation spectrum using ABI software Data Explorer 4.6. A similar procedure was previously performed to assign an unexpected dipeptide fragment ion [29]. A reference file was created using the exact masses of unambiguously assigned photofragmentation peaks. The internal calibration peaks were the arginine immonium ions, the d_2 ion, the m_2 ion, and the a_2 ion. The d_2, m_2, and a_2 ions did not appear in all of the mass spectra, but at least two of them were present in each spectrum. No intensity weighting factor

Table 2. Calibrated Accurate Masses for 244 and 258 Da and Their Matching Formulas

Nominal mass (Da)	Accurate measured mass (Da)	Possible formulas/ masses (Da)
244 RC, RE, RK, RL, RM, RN, RQ, RR	244.141	$C_9H_{18}N_5O_3$, 244.141
258 RI	258.154	$C_{10}H_{20}N_5O_3$, 258.157



Scheme 5. Proposed structures for the m_2 and related ions

was used as this would have amplified the contributions of the immonium ion peaks. The $b_1 + H_2O$ peak was used as an "unknown" peak to assess the accuracy of the calibration. The calibrated mass of the $b_1 + H_2O$ peak was 175.112 Da, which is within 0.007 Da of its correct mass. Applying the same approach to the 244 Da peak (and to a very small 258 Da peak that appears in the spectrum of RI) yielded results shown in Table 2. Data Explorer's elemental composition calculator was used to search for formulas whose masses were within 0.01 Da of the calibrated masses. The maximum number of atoms for each element was restricted based on the elemental composition of the dipeptides. The calibrated masses that were obtained (244.141 and 258.154 Da) were within 0.003 Da of the theoretical masses expected for $m_2 + 13$ and $m_2 + 27$ ions. Their structures appear in Scheme 2.

Conclusions

Numerous fragments were observed in the 157 nm photodissociation of dipeptides having N-terminal arginine, including structures formed from more than one bond cleavage. In addition to a-type ions, side-chain fragmentation occurs readily, producing m- and d-type ions. For sequencing of larger peptides, observing both of these ion types would be useful. A rearrangement of the peptide C-terminus to produce $b_1 + H_2O$ ions was also observed. This was confirmed through CID MS^3 experiments using the ion trap. This rearrangement would likely be observed in peptides produced by Lys-N that have N-terminal lysine [26].

The MALDI-TOF-TOF mass spectrometer and ESI ion trap mass spectrometers served complementary roles. The ion trap data made it easy to identify photofragments; in the TOF-TOF data, PSD and matrix peaks significantly hindered the analysis of photodissociation spectra. However, the overall photofragment signal was much higher with the TOF-TOF instrument, and this was useful for verifying the integrity of the ion trap data. Additionally, the TOF-TOF's mass accuracy is quite good when the data are internally calibrated, and this facilitates the confirmation of fragment ion assignments.

Acknowledgments

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