Cyclization of Peptide b_9 Ions

Alex G. Harrison

Department of Chemistry, University of Toronto, Toronto, Canada

The product ion mass spectra obtained by CID of the b_9 ions derived by loss of neutral alanine from the MH^+ ion of the peptides Tyr(Ala)$_9$, (Ala)$_4$Tyr(Ala)$_5$, and (Ala)$_8$TyrAla are essentially identical, indicative of full cyclization reaction to a common intermediate before fragmentation. This leads to abundant nondirect sequence ions in the product ion mass spectra of the b_9 ions. The product ion mass spectra of the b_8 ions from the first two peptides also are essentially identical. The fragmentation of the MH^+ ions also leads to low intensity nondirect sequence ions in the product ion mass spectra. N-terminal acetylation blocks the cyclization and eliminates nondirect sequence fragment ions in the product ion mass spectra. (J Am Soc Mass Spectrom 2009, 20, 2248–2253) © 2009 American Society for Mass Spectrometry

Tandem mass spectrometry plays an important role in the sequencing of peptides [1–3]. The most common approach has involved collision-induced dissociation (CID) of the protonated (or multiply-protonated) gas-phase peptide ions produced by soft ionization techniques. As a result of many studies, the fragmentation of protonated peptides is known, at least in a phenomenologic sense, as illustrated in Scheme **1** [4, 5]. Under the frequently-used low-energy collision conditions, protonated peptides most often fragment at amide bonds. In the ideal case this leads to a series of b and/or y ions, which contain, respectively, the N-terminus and C-terminus residues. It is these series of b and y ions, which provide the sequence information, and any nondirect sequence ions [6] may lead to uncertainty in interpretation of the observed spectra.

It has been clearly established [7, 8] that the y ions are protonated amino acids (y_1) or protonated truncated peptides (y_n). Although it was originally proposed [4, 5] that b ions had an acylium ion structure, extensive studies [9–14], mainly of b_2 and b_3 ions, have presented strong evidence that, in many cases, cyclization has occurred at the C-terminus to form a protonated oxazolone. Recently, infrared multiple photon dissociation (IRMPD) experiments [15, 16] have provided definitive evidence for a protonated oxazolone structure for the b_4 ion derived from Leu-enkephalin. More recently, several IRMPD studies [17–19] have shown that relatively simple b_2 ions also have a protonated oxazolone structure. On the other hand, when there is a strong nucleophile in the side chain, alternative cyclization reactions involving this nucleophile may occur [20–24].

Early studies [25, 26] reported the observation of nondirect sequence ions in the fragmentation of doubly-protonated b ions containing lysyl or ornithyl residues, which was interpreted in terms of cyclization/reopening reactions before fragmentation. More recently, several groups [6, 27–31] have presented evidence that b_5 ions form fully cyclic structures; ab initio calculations [6, 30] indicate that the pathway to these cyclic structures involves attack of the N-terminal amine function on the C-terminal oxazolone formed in the initial fragmentation process as shown in Scheme **2** for a b_5 ion. Acetylation at the N-terminus appears to eliminate this cyclization process [31]. Ion mobility experiments [32–35] also have presented evidence for more than one structure for b_5 and larger b ions. The fully cyclic structure may reopen at a variety of positions to form a variety of oxazolones, thus achieving scrambling of the initial amino acid sequence [6, 27–31]. Since b ions with protonated oxazolone structures fragment, in part, to form lower mass b ions [36], further fragmentation of these rearranged oxazolones will lead to nondirect sequence fragment ions [6] that would not be expected on the basis of the original sequence of the peptide and, thus, have the potential to lead to confusion when sequencing an unknown peptide.

The majority of work on full cyclization of b ions have involved studies of b_5 ions. (Stable b_4 ions appear to undergo only minor cyclization [15, 16], although CID studies [37] suggest a much greater extent of cyclization for activated ions before fragmentation.) Although there is some evidence for at least partial cyclization of larger b ions [27, 28, 34], no systematic study has been carried out to probe the extent of full cyclization of larger b ions. In the present work, we have examined the fragmentation of b_9 and b_8 fragment ions derived from the decapeptides Tyr(Ala)$_9$, (Ala)$_4$Tyr(Ala)$_5$, and (Ala)$_8$TyrAla. Essentially identical product ion mass spectra are observed for the b_9 ions derived from these peptides as well as for the b_8 ions derived from the first two peptides. The results indicate that full cyclization has occurred for these larger b ions before fragmentation. The occurrence of nondirect sequence fragment ions in the product ion mass spectra of the MH^+ ions also is examined.

Address reprint requests to Dr. A. G. Harrison, Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON M5S 3H6, Canada. E-mail: aharriso@chem.utoronto.ca

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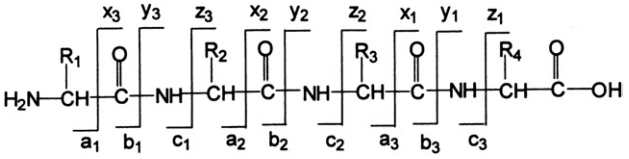


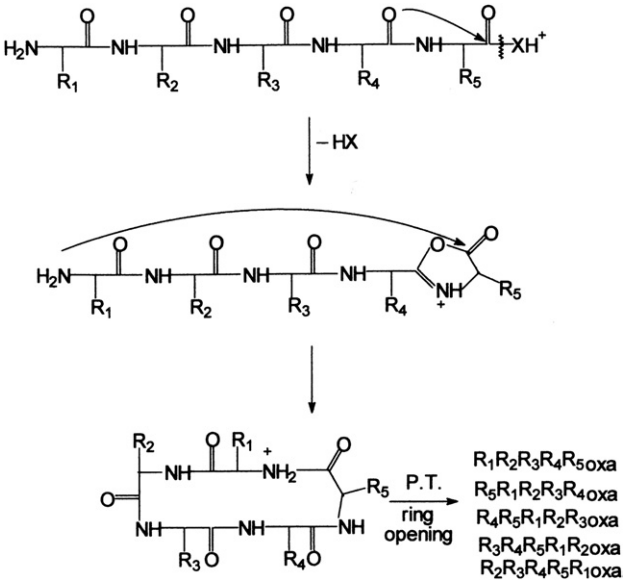
Scheme 1

Experimental

All experimental work was carried out using an electrospray/quadrupole/time-of-flight (QqTOF) mass spectrometer (QStarXL; SCIEX, Concord, Canada). MS^2 experiments were carried out in the usual fashion for MH^+ ions by mass-selecting the ions of interest with the mass analyzer Q with CID in the quadrupole collision cell q and mass analysis of the ionic products with the time-of-flight analyzer. In the study of the b fragment ions, CID in the interface region produced fragment ions with those of interest being selected by the quadrupole mass analyzer for fragmentation and analysis in the usual manner. The cone voltage in the interface region was varied to achieve the best yield of the fragment ions of interest; typically a cone voltage of 50–60 V was found to be optimum. The product ion mass spectra were independent of the cone voltage employed.

Ionization was by electrospray with the sample at micromolar levels dissolved in 1:1 CH_3OH:1% aqueous formic acid and introduced into the ion source at a flow rate of 10 μL min^{-1}. Nitrogen was used as nebulizing and drying gas and as collision gas in the quadrupole collision cell.

The peptides were obtained from Celtek Peptides (Nashville, TN, USA); they showed no impurities in their mass spectra and were used as received. N-acetylation was achieved by reaction of the peptide with a 1:1 mixture of acetic anhydride and methanol [38].



Scheme 2

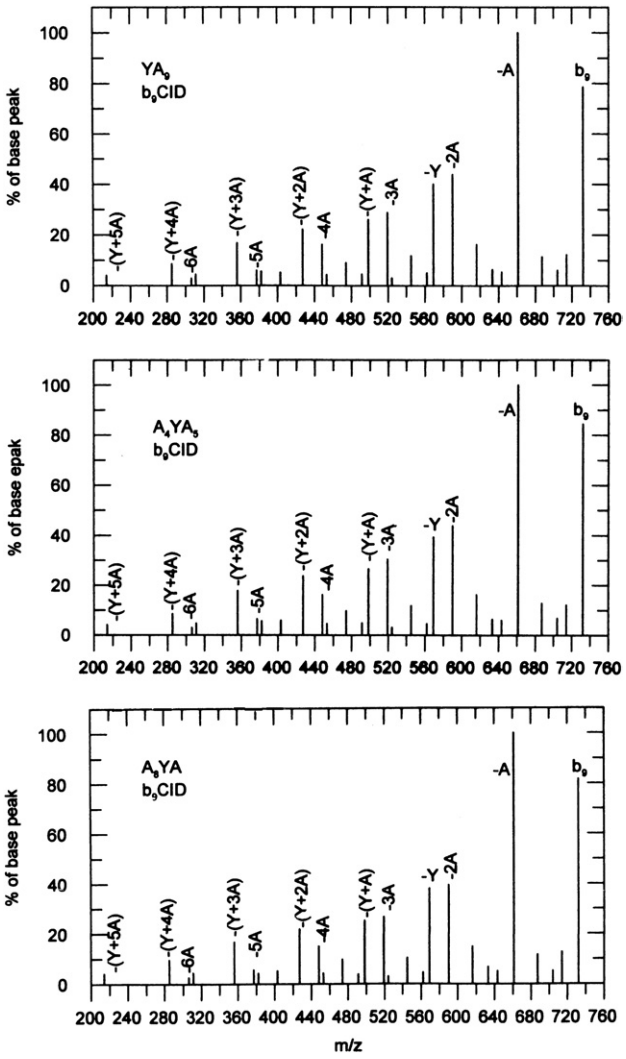


Figure 1. Product ion mass spectra of the b_9 ions derived from YA_9, A_4YA_5, and A_8YA. 30 eV collision energy.

Results and Discussion

Figure 1 shows the product ion mass spectra obtained by CID at 30 eV collision energy of the b_9 ions derived by loss of neutral alanine from the MH^+ ions of YA_9, A_4YA_5, and A_8YA. Only the b ion series are identified; the lower intensity signals correspond to the respective a and a* (a-NH_3) ions plus, in a few cases, signals corresponding to loss of H_2O from the b ions. An extensive series of b ions is observed originating by direct and sequential fragmentation of the b_9 ions by loss of amino acid residues. In the absence of full cyclization and reopening at various sites one would not expect to observe loss of the tyrosine residue (Y) from the b_9 ions of YA_9 and A_4YA_5 nor dominant loss of the alanine residue (A) from the b_9 ion of A_8YA. Thus, it is evident that full cyclization and reopening has occurred; the fact that the three product ion mass spectra are essentially identical implies that, in each case, the entire assembly of b ions has undergone

cyclization and subsequent "scrambling" of the original sequence before fragmentation.

In a protonated cyclic A_8Y ion there are nine amide bonds, which can cleave to form nine different protonated oxazolones as per Scheme **2**; these oxazolones will differ in the position of the tyrosine residue. Only one of these will place the tyrosine residue at the C-terminus and, consequently, in the position to be eliminated. If the probability of cleavage of all amide bonds was the same as well as the probability of fragmentation of the resulting oxazolones, one would expect a loss of A:loss of Y = 8:1. Experimentally, the ratio is found to be ~2.5:1 over a wide range of collision energies for all three b_9 ions. Clearly, there is a distinct preference for ring opening and subsequent fragmentation, which results in loss of the tyrosine residue Y. Ab initio calculations [30] relevant to the fragmentation of protonated cyclo-(YAGFL) showed that loss of the tyrosine residue (Y) was slightly favored energetically over the loss of other residues. It should be noted that the labeling of the fragment b ions in Figure 1 does not indicate the pathway forming the ions. Thus, the ion labeled −(Y + A) probably originates in part by loss of A from b_9 − Y and, in part, by loss of Y from b_9 − A. The breakdown graphs for the b_9 ions derived from the three peptides were essentially identical. A typical breakdown graph, showing only the b ions, is presented in Figure 2. The sequential nature of the fragmentation leading to the low mass ions is clearly evident.

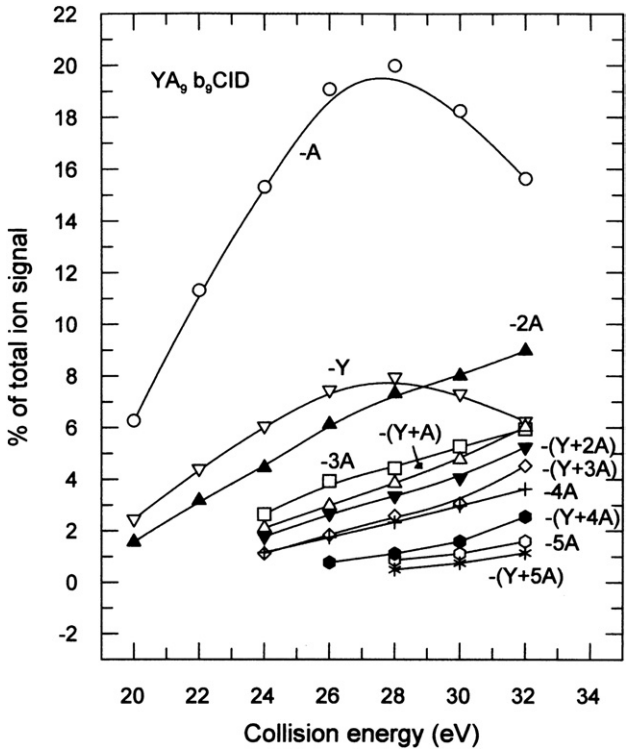


Figure 2. Breakdown graph for b_9 ion derived from YA_9. Only b ions shown.

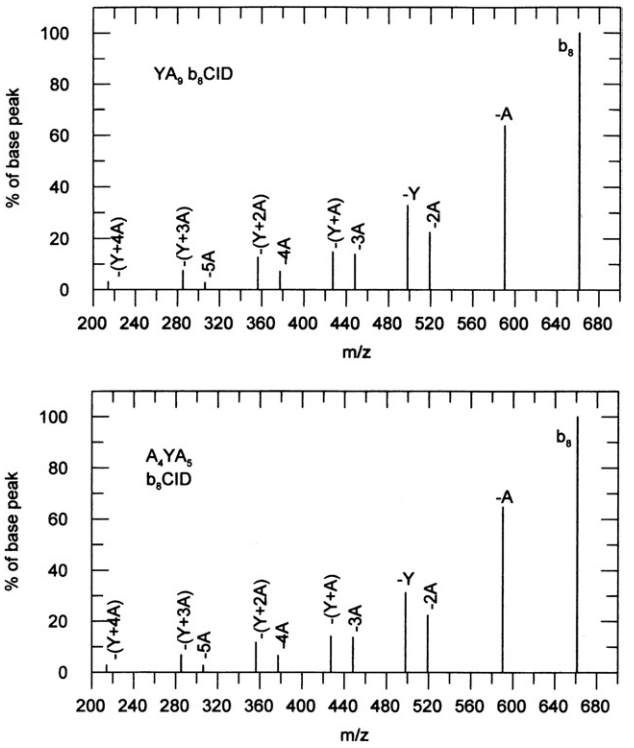


Figure 3. Product ion mass spectra of b_8 ions derived from YA_9, and A_4YA_5. Only b ions shown. 26 eV collision energy.

Figure 3 shows the product ion mass spectra (CID at 26 eV collision energy) obtained for the b_8 ions derived from YA_9 and A_4YA_5. Only the b ion series are shown. Again, one would not expect to observe direct loss of the tyrosine residue from these b_8 ions in the absence of full cyclization; the observation that the CID mass spectra are essentially identical indicates that all b_8 ions have undergone full cyclization and reopening before fragmentation under our experimental conditions. In a A_7Y protonated cyclic peptide there are seven amide bond cleavages, which put an alanine residue at the C-terminus compared with one that puts the tyrosine residue at the C-terminus. The observed loss of A:loss of Y of ~2:1 again indicates a substantial preference for cleavage and fragmentation which results in loss of the tyrosine residue. The b_7 ions from YA_9 and A_4YA_5 also showed essentially identical product ion mass spectra (not shown), but in these cases it is probable that considerable sequence scrambling has occurred in their formation by fragmentation of b_9 and b_8 ions (see Figures 1 and 3).

An important question is how much do these scrambling reactions of b ions affect the interpretation of the product ion mass spectra of MH^+ ions. Figure 4 displays the product ion mass spectra for the three MH^+ ions of the present study. Only the b ion series are identified. No significant y ions were observed and the low intensity ion signals correspond to various a and a* ions. In each case, a substantial series of b ions indicative of the peptide sequence is observed and the non-

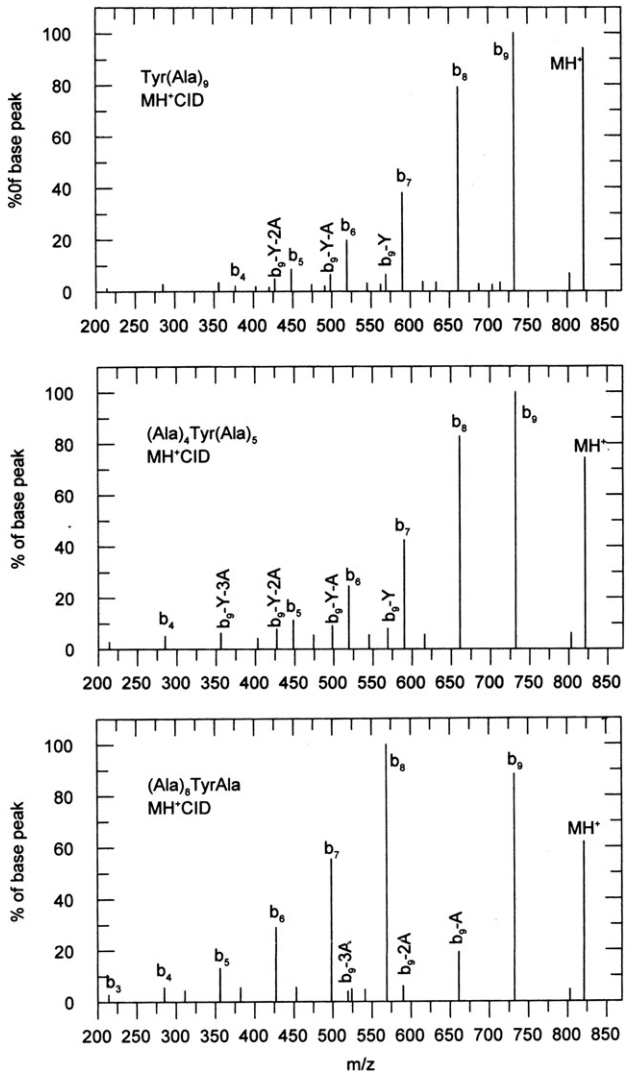


Figure 4. Product ion mass spectra of MH^+ ions of YA_9, A_4YA_5, and A_8YA. 30 eV collision energy.

direct sequence b ions are of relatively minor importance. The latter consist of $b_9 - Y$, $b_9 - Y - A$, and $b_9 - Y - 2A$ for the MH^+ ions of YA_9 and A_4YA_5, and $b_9 - A$, $b_9 - 2A$, and $b_9 - 3A$ for the MH^+ ion of A_8YA. In the latter case, the rather prominent $b_9 - A$ ion signal could be a source of confusion if the peptide was indeed an unknown. Figure 5 shows the breakdown graph for the MH^+ ion of A_8YA. As would be expected, the nondirect sequence b ions are observed primarily at higher collision energies, where sufficient internal energy is carried over in the first generation b ions to result in further fragmentation. Of course, the situation will be quite different if one carries out multi-stage collisional experiments, which are possible in ion trapping instruments. Olsen and Mann [39] have noted this problem when using multi-stage collisional activation experiments involving further fragmentation of primary b ions.

In our earlier study [31] of the fragmentation of b_5 ions, it was observed that acetylation of the N-terminus amine group stopped the cyclization reaction of Scheme 2 and, thus, the concomitant sequence scrambling. Figure 6 shows the product ion mass spectra of the b_9 ion and MH^+ ion of acetyl-A_8YA. Note the complete absence of the $b_9 - A$ and $b_9 - 2A$ ions which are major products observed (Figure 1) in the CID mass spectrum of the b_9 ion of the nonacetylated peptide. No nondirect sequence b ions are observed in the CID mass spectra of the b_9 and MH^+ ions, in agreement with the earlier observation [31]. Again in agreement with the earlier study, the acetylated peptide shows essentially no a or a* ions in fragmentation of the b_9 or MH^+ ions. A number of recent studies [16, 30, 33, 37, 40, 41] have established that a ions have more than one structure including a cyclic structure. Formation of such a cyclic structure requires a free N-terminal amino group with the result that such cyclization is not possible for the acetylated peptide. Such a cyclization apparently plays a major role in the formation of stable a ions. Formation of a* ions by loss of NH_3 from a ions also requires a free N-terminal amino group [37, 40, 41] and is blocked by the N-terminal acetylation.

Conclusions

The present results show that, at least for these relatively simple cases, b_9 and b_8 ions undergo cyclization and subsequent sequence scrambling before fragmentation. Consequently, MS^3 experiments involving fragmentation of the b ions formed by fragmentation of

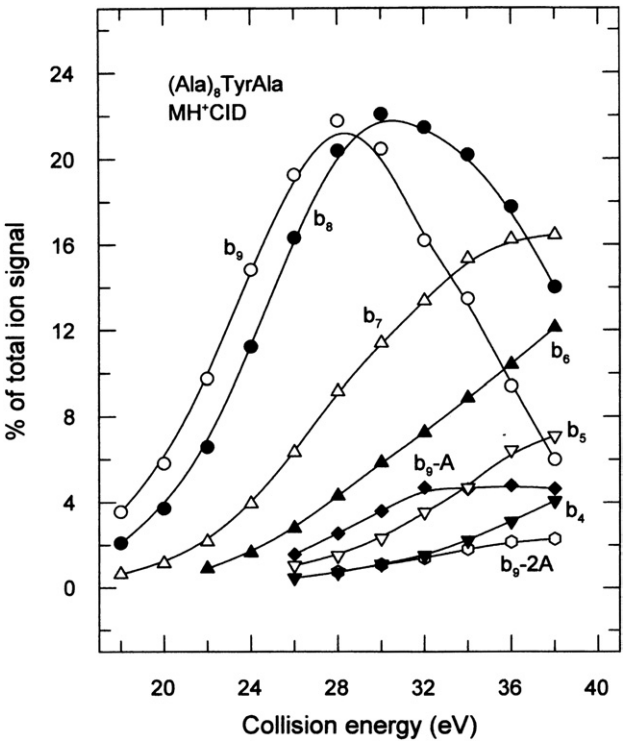


Figure 5. Breakdown graph for MH^+ ion of A_8YA. Only b ions shown.

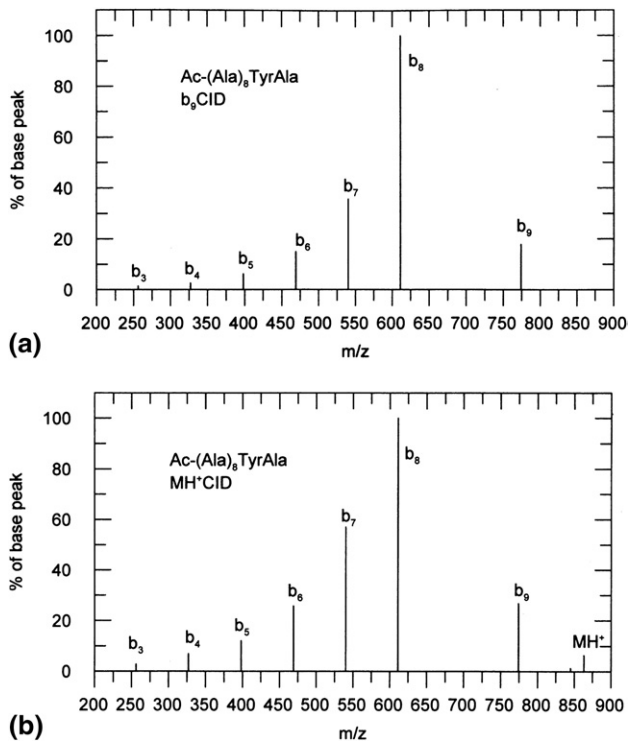


Figure 6. Product ion mass spectra of (**a**) b_9 and (**b**) MH^+ ions derived from Ac-A_8YA. Collision energy 26 eV for b_9 ions and 30 eV for MH^+ ions.

MH^+ ions will not lead to reliable sequence information. On the other hand, N-terminal acetylation eliminates the cyclization reaction, and MS^3 experiments on N-acetylated b ions are likely to be useful in sequence determination.

While the results to date suggest that cyclization and subsequent sequence scrambling is a common phenomenon for b_5 and larger b ions, it should be noted that b ions with strongly basic or acidic residues have not been studied in detail. Thus, the generality of cyclization is not yet fully established. This aspect is under study.

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