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Rapid cyclopeptide analysis by microwave enhanced Akabori reaction

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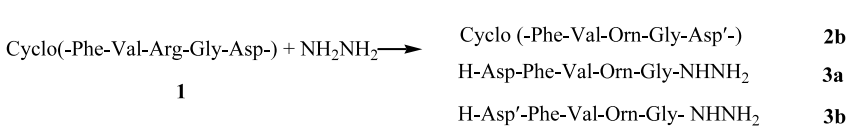
Abstract—The application of microwave assisted modification of the Akabori hydrazinolysis reaction has been found to cleave cyclic oligopeptides in a selective fashion to produce mainly the hydrazide of a specific linear peptide. This ring cleavage requires a few minutes of reaction in a domestic microwave oven. The linear peptide hydrazide can be analyzed by ESI-MS/MS, FAB-MS, and FAB-MS/MS methods for the determination of the amino acid sequence.

In recent years, cyclic peptides, which are widely distributed in nature, have been drawing increasing attention of medicinal chemists and chemical biologists for their potential as lead compounds to drug targets.[1,2] Although linear peptides can readily be sequenced by using either classical Edman degradation[3] or mass spectrometry methods,[4,5] these approaches have not been easy for analyzing cyclic peptides.[5] Because of the lack of free N-terminus, selective hydrolysis of peptide bonds by classical method is difficult to achieve. Characterization of cyclic peptides by tandem mass spectrometry (MS/MS) is also difficult, because the indiscriminate ring-opening pathways often give a set of acylium ions of the same mass to charge ratio.[5d,e] During the last two decades, however, there have been many reports in the literature where tandem mass spectrometry (MS^n) have been applied for the sequence determination of cyclic peptides.[5] Cyclic peptides have also been characterized by the use of metal ion,[5f] as well as chemical ring opening,[5g] followed by the MS analysis of the resulting linear peptides. Other methods used are extensive 2D NMR studies[5e] or X-ray crystallography of a suitable derivative.[5h]

The Akabori reaction,[6] devised over 50 years ago for the identification of the C-terminus of peptides, depends on the cleavage of amide (peptide) bonds in peptides by hydrazine when heated in a sealed tube at 125°C for several hours. The carboxy (C)-termius is liberated as a free amino acid and thus easily identified. In the course of our studies on microwave assisted chemical and biochemical reactions,[6–11] we became interested in the Akabori reaction. We found that irradiation in a domestic microwave oven of a mixture of a linear peptide and hydrazine, for 5–10 min (20–60 min with hydrazine and dimethyl-sulfoxide), led to the rapid identification of C-terminus amino acids including the sequence information of the polypeptide.[10] We have also demonstrated that certain amino acids and some carboxy functions are very rapidly modified by hydra-



Scheme 1.

Keywords: cyclopeptides; amino acid sequence; microwave enhanced reaction; hydrazinolysis; Akabori reaction.
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zine under microwave irradiation. For example, arginine is converted to orinthine and the β-carboxy group of aspartic acid and glutamic acid is transformed into a $-CONHNH_2$ group.[10]

We wish to report here the extension of microwave enhanced Akabori reaction techniques to cyclic oligopeptides for the rapid corroboration (or determination) of the sequence of amino acids present. We have observed that in the case of several glycine containing cyclic oligopeptides, microwave assisted hydrazinolysis led in a few minutes to selective ring-opening at glycine residue to produce the corresponding open chain hydrazide(s). This open chain hydrazide is analyzed by mass spectrometry for sequence determination.

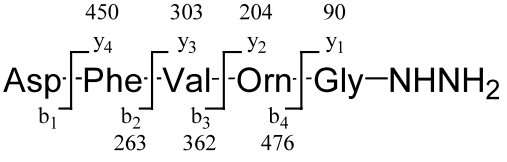
The powerful MS/MS approach was used in case of the cyclic pentapeptide **1**. This peptide which is cyclo(-Phe-Val-Arg-Gly-Asp-) showed the $[M+H]^+$ ion m/z 575. On hydrazinolysis **1** (Scheme 1) was changed in a few minutes to **2a** m/z 533. The loss of 42 Da indicated that the arginine residue had been converted to the ornithine residue in **2a** (and **2b**).[10] The molecular weight of **2b** was 14 Da higher than that of **2a**. This increase is characteristic of the conversion of the carboxy group of aspartic acid to a $CONHNH_2$ group to form a new amino acid residue Asp′. After continuation of microwave irradiation, for 8–30 min, the Akabori reaction mixture containing **1** was subjected to HPLC ESI-MS identification. Several fractions were displayed in the total ion chromatogram (TIC) that were analyzed by MS/MS methods. The LC fraction eluted at 10 min (approximately 33% of the major peak) with the $[M+H]^+$ ion m/z 533 corresponded to **2a**. One of the minor LC fractions ($R_t = 11$ min) that showed the $[M+H]^+$ ion m/z 575 corresponded to the starting cyclic peptide while a second minor LC fraction ($R_t = 8$ min) that displayed the $[M+H]^+$ ion m/z 547 was compatible with structure **2b**. The MS/MS analysis of **2a** and **2b** (Scheme 1), however, provided inadequate information for sequencing.

Two major LC fractions displayed the $[M+H]^+$ ions at m/z 565 and 579 in the TIC. MS/MS analysis of m/z 565 and 579 with computer assisted sequence determination[12] established these fractions to be the open chain pentapeptide hydrazides (H-Asp-Phe-Val-Orn-Gly-$NHNH_2$ or **3a**) and (H-Asp′-Phe-Val-Orn-Gly-$NHNH_2$ or **3b**), respectively (Fig. 1). Thus, conclusive evidence was obtained for the correct sequence of amino acids in a cyclic peptide by a combination of the microwave enhanced Akabori reaction and MS/MS analysis.

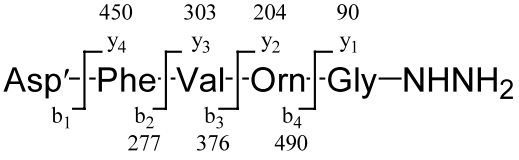
The FAB-MS method was also applied to the analysis of the reaction sample at 15 min irradiation, which showed abundant protonated molecules at m/z 579 and 565, corresponding to **3b** and **3a**, respectively (Scheme 1). The FAB spectra contained several pairs of abundant ions (m/z: 490, 476; 375, 361; 277, 263, etc.) that differ by 14 Da, possibly representing fragments generated from the protonated molecules. These ions provided evidence for amino acids sequence in **3b** and **3a**. Moreover, the FAB product ion spectra of the hydrazides (**3b** and **3a**) were dominated by uninterrupted series of b- and y-ions, thus the sequence of the peptides can be easily determined (spectra not shown). These data are consistent with those of the ESI-MS/MS results. When the reaction sample was irradiated for a longer period of time (>30 min), a series of Akabori cleavage products were detected in the FAB mass spectra.

During further studies it was observed that much more intense spectra were obtained if lithium bromide was added to the mixture of hydrazine and the cyclic peptide before microwave irradiation. In some cases, the prominent ions in the spectra were almost all lithiated ions corresponding to the various hydrazides formed during the cleavage of peptides. Even more important than this simplification of the spectra was the finding that the hydrazinolysis was complete in 10 min or less (compared to about 30 min by the earlier method). This acceleration of the Akabori cleavage reaction can be rationalized by noting that lithium bromide being a salt was more effective in absorbing microwave energy than the dipoles of various peptide fragments.[8b]

We record here the data obtained by applying these different methods of microwave assisted hydrazinolysis to a nonapeptide **4**, cyclo(-D-Phe-His-Trp-Ala-Val-Gly-His-Leu-Leu-). In the first experiment, the cyclic peptide **4** was treated with 98% hydrazine. At the initial point, FAB-MS showed the expected $[M+H]^+$ m/z 1061. This peptide did not contain arginine, aspargine, cysteine and other amino acids that are rapidly modified by hydrazine under microwave irradiation. Therefore no satellite peaks (such as, M+14, or M−42) were expected or found. An additional ion appeared at m/z 1093 after the first few minutes of microwave irradiation indicating the formation of a linear oligopeptide hydrazide by opening the nine-membered ring (spectrum not shown). Preliminary work with some other cyclopeptides, including the cyclopentapeptide dis-



3a



3b

Figure 1.

cussed above, had shown that usually the amide bond of glycine is the cyclic peptide bond to undergo cleavage (Scheme 2). Therefore, we assigned tentatively the open chain structure **5** to the peptide hydrazide (Scheme 2). A scrutiny of the FAB-MS showed ions representing both the C-terminus and the N-terminus cleavages for compound **5**. These data revealed the sequence of all nine amino acid residues correctly.

The ESI-MS/MS of the cyclic peptide **4** produced ions due to random cleavage that were not useful for sequence determination. The ESI-MS/MS spectrum of the protonated linear nonapeptide hydrazide ion at *m/z* 1093, however, shows most of the 'y' and 'b' ions (Fig. 2). These 'y' and 'b' ions reveal the sequence of amino acids in the cyclononapeptide **4**.

Cyclo(-Phe-His-Trp-Ala-Val-Gly-His-Leu-Leu-) + NH_2NH_2 ⟶

4

H-His-Leu-Leu-Phe-His-Trp-Ala-Val-Gly-$NHNH_2$

5 (major product)

Scheme 2.

In a separate Akabori style experiment the cyclopeptide **4**, hydrazine and lithium bromide were mixed together and exposed to microwave irradiation for only 10 min. The FAB-MS spectrum of the sample at this time-point shows that almost all the ions are lithiated ions that correspond to the multiple Akabori cleavages starting at the C-terminus. It is to be noted that the other set of ions starting at the N-terminus were mostly too weak to be seen in this spectrum.

The above method has been successfully applied for several other glycine-containing cyclopeptides that includes cyclo(-Ala-Gly-Ala-Gly-), cyclo(-Gln-Trp-Phe-Gly-Leu-Met-) and cyclo(-Gly-Arg-Gly-Arp-Ser-Pro-Ala-). We have also analyzed a limited number of cyclic peptides that does not contain a glycine residue. Preliminary results indicate that this method is applicable to non-glycine containing cyclic peptides. These findings will be reported in the future.

In summary, our preliminary data show that practical and rapid methods have been devised for the sequence determination of cyclic peptides. The best approach appears to be the selective opening in a few minutes of a cyclic oligopeptide by microwave enhanced Akabori reaction followed by computer assisted MS/MS analysis

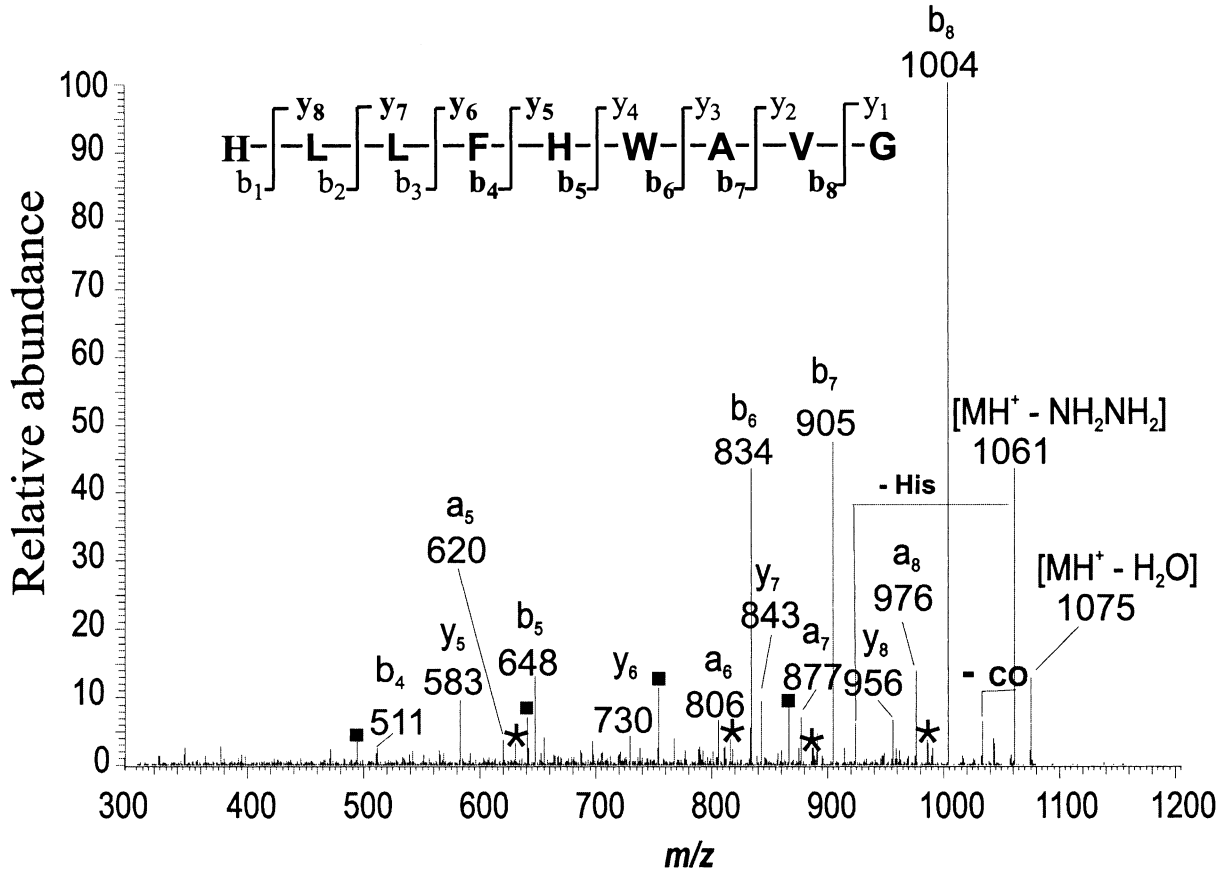


Figure 2. Product-ion spectrum of the linear nonapeptide **5** (*HLLFHWAVG-NHNH*$_2$). The peaks marked with a '*' represent 'b_n–H_2O' ions: (b_8–H_2O (*m/z* 986); b_7–H_2O (*m/z* 887); b_6–H_2O (*m/z* 816); b_5–H_2O (*m/z* 630), whereas, the peaks marked with a '■' represent the internal fragment ions (b_8–His (*m/z* 867); b_8–His–Leu (*m/z* 754); b_8–His–2Leu (*m/z* 641); b_8–His–2Leu–Phe (*m/z* 494)).

of the major linear oligopeptide hydrazide so obtained. More extended study of the FAB-MS of samples generated by aqueous hydrazine and by hydrazine and lithium bromide combination can provide corroboration of the structure of the cyclic peptide.

Acknowledgements

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