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Sequencing peptides by electrospray ion-trap mass spectrometry: A useful tool in synthesis of Axinastatin 3

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Abstract: Axinastatin 3 as a potential anticancer agent was synthesized by chemical methods. In an electrospray ion-trap mass spectrometer, using one stage of tandem mass spectrometry (MS/MS), the linear peptide intermediate was sequenced via the complementarities of y and b ions. Then, using multistep MS/MS (to MS^6), the cyclic peptide was sequenced through sequentially removing one amino acid residue in each stage of MS/MS. The difference of the fragmentation mechanisms and the sequencing approaches between them is discussed.

Keywords: Tandem mass spectrometry, cyclic peptides, fragmentation pathways, collisionally activated decomposition

1 Introduction

Cyclic peptides are very common among natural products and exhibit a wide range of biological activities [1]. In 1994, Pettit *et al.* [2] reported a cyclic peptide Axinastatin 3, cyclo(Leu-Pro-Val-Asn-Pro-Phe-Ile), isolated from the marine sponge *Axillna sp.*, which showed significant activity as a cell growth suppressant therefore, showed promise as a potential anticancer drug. In order to further study its bioactivity, we synthesized this cyclopeptide by chemical methods. The determination of the peptide sequence was essential to ensure the correctness of the synthesis protocol.

Mass spectrometry is a highly effective tool for identification of peptide sequences.

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In comparison with Edman's degradation or NMR, sequencing peptides by tandem mass spectrometry (MS/MS) has some distinct advantages [3, 4] *viz* (a) a peptide mixture can be sequenced; (b) the necessary sample amount is less than 1 pmol; (c) peptides without free N-termini can be analyzed; and (d) the experimental operation is noticeably simple. As a result, MS/MS is very suitable for analyzing synthetic crude peptides.

The fragmentation mechanisms for protonated linear and cyclic peptides are dissimilar under low-energy collision activated decomposition (CAD) [5] hence, the strategies for sequencing them are different. In general, one stage of MS/MS is sufficient to sequence linear peptides, whereas multiple stages of MS/MS are required for sequencing cyclic peptides. This unique feature of MS^n ion-trap mass spectrometry is quite relevant for cyclic peptide sequencings, because a further CAD can, in principle, be performed as long as the parent ion signal is abundant [6].

In 1999, Ngoka and Gross [7], using multistep MS/MS first sequenced some protonated cyclic peptides in an ion-trap mass spectrometer and proposed a nomenclature system [8] for labeling the fragment ions of protonated cyclic peptides, which indeed represents the 'state of the art'. Subsequently, a series of [9–12] reports focused on characterization of protonated cyclic peptides and metal complexes using multistep MS/MS. The challenge we faced in our studies to sequence the cyclic heptapeptide Axinastatin 3 was that a large number of fragment ions with the same or similar m/z value are present and at least MS^6 experiments would be needed to decipher the sequencing. However, it would be very instructive to systematically discuss the difference between sequencing linear and cyclic peptides.

We report here, our results of sequencing the linear precursor of the cyclic peptide i.e the linear peptide Leu-Pro-Val-Asn-Pro-Phe-Ile and its corresponding cyclic peptide using electrospray-ionization (ESI) ion-trap mass spectrometry and enlighten its usefulness as a tool in the synthesis of Axinastatin 3. In the process of synthesis of cyclic peptides, a practical method was developed for sequencing peptides.

2 Experimental

2.1 Materials and reagents

Wang-resin, Fmoc-amino acids and coupling reagents for peptide synthesis were purchased from GL Biochem (Shanghai, China). All solvents for peptide synthesis were commercial analytical grade and were redistilled before use. HPLC-grade formic acid and acetonitrile were supplied by Merck (Germany), and the HPLC-grade water was redistilled and subjected to ion-exchange process in a Milli-Q unit (Millipore) for use in mass spectrometry analysis.

2.2 Synthesis of Axinastatin 3

The linear peptides were prepared manually using Wang resin as solid support following the Fmoc methodology as we reported previously[13]. The cyclizations of linear peptides were carried out in solution using the following procedure. The linear peptides (0.10 mmol) were dissolved in DMF (7.8×10^{-4} M), and the pH was adjusted to 8-9 by addition of DIEA. PyBOP (0.20 mmol, 2 equiv.) was added, and the solution was stirred at room temperature for 2 h. Then the solvent was removed under reduced pressure and ether was added to precipitate the peptide. After triturating and washing 3 times with ether, the cyclic peptides were obtained as white powders. They were purified by semipreparative reversed-phase HPLC.

2.3 Mass spectrometry

Experiments were performed using a Finnigan LCQ ion-trap mass spectrometer (San Jose, CA, USA) equipped with an ESI source operated in positive mode. The spray needle was set at a potential of 4.3 kV, and a nitrogen sheath gas flow of 30 (arbitrary units) was used to stabilize the spray. The capillary temperature was maintained at 200 °C. The tube-lens offset was 20 V and the electron multiplier voltage was -800 V. Helium gas was introduced into the ion-trap at a pressure of 1mTorr to improve the trapping efficiency of the analyte ions introduced into the ion trap. The background helium gas also served as the collision gas during the CAD event. A typical experimental protocol consisted of infusing a 50 pmol/μl solution of peptides in 50:50 (v/v) acetonitrile/H_2O containing 1% formic acid into the mass spectrometer at a flow-rate of 2 μl/minby syringe pump.

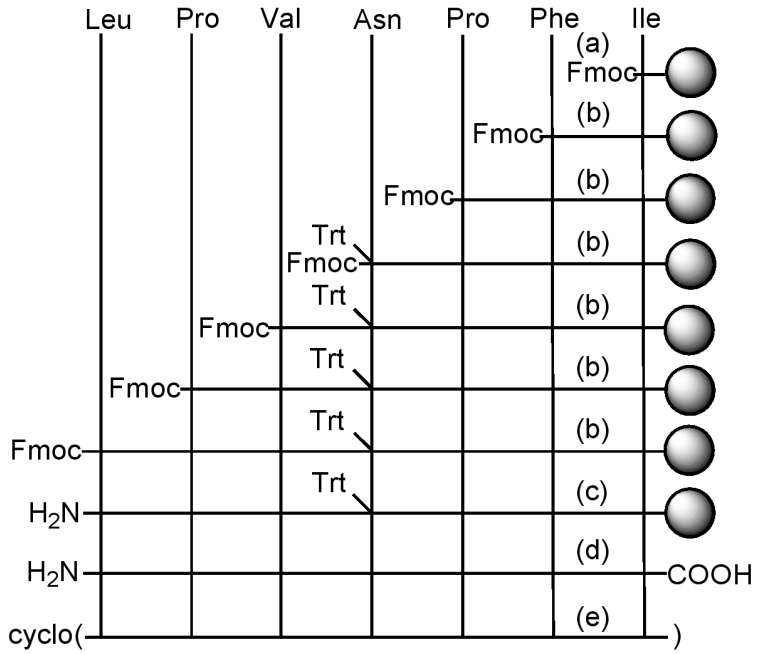
2.4 Nomenclatures

The widely accepted protocol of nomenclature as described by Roepstotff and Fohlman was used for labeling the fragment ions of linear peptides [14] and the nomenclature proposed by Ngoka and Gross [8] was used for designating the fragment ions of cyclic peptides. The nomenclature involves tagging the ions with a four-part descriptor with the general formula x_{nJZ}, where 'x' is the designation for the ion and n is the number of amino acid residues in the ion. J and Z are the one-letter codes for the two amino acid residues connecting the backbone amide bond, J-Z, that is broken to form the linear ion. J is the N-terminal amino acid residue and Z is the C-terminal amino acid residue.

3 Results and discussion

The linear peptide was prepared using solid phase synthesis, and then cyclized in solution to yield the cyclic peptide Axinastatin 3 (Scheme 1). In the synthesis process, the amino acid sequences of the linear and cyclic peptide both need be identified by mass

spectrometry so as to ensure the exact structure of the synthetic products.

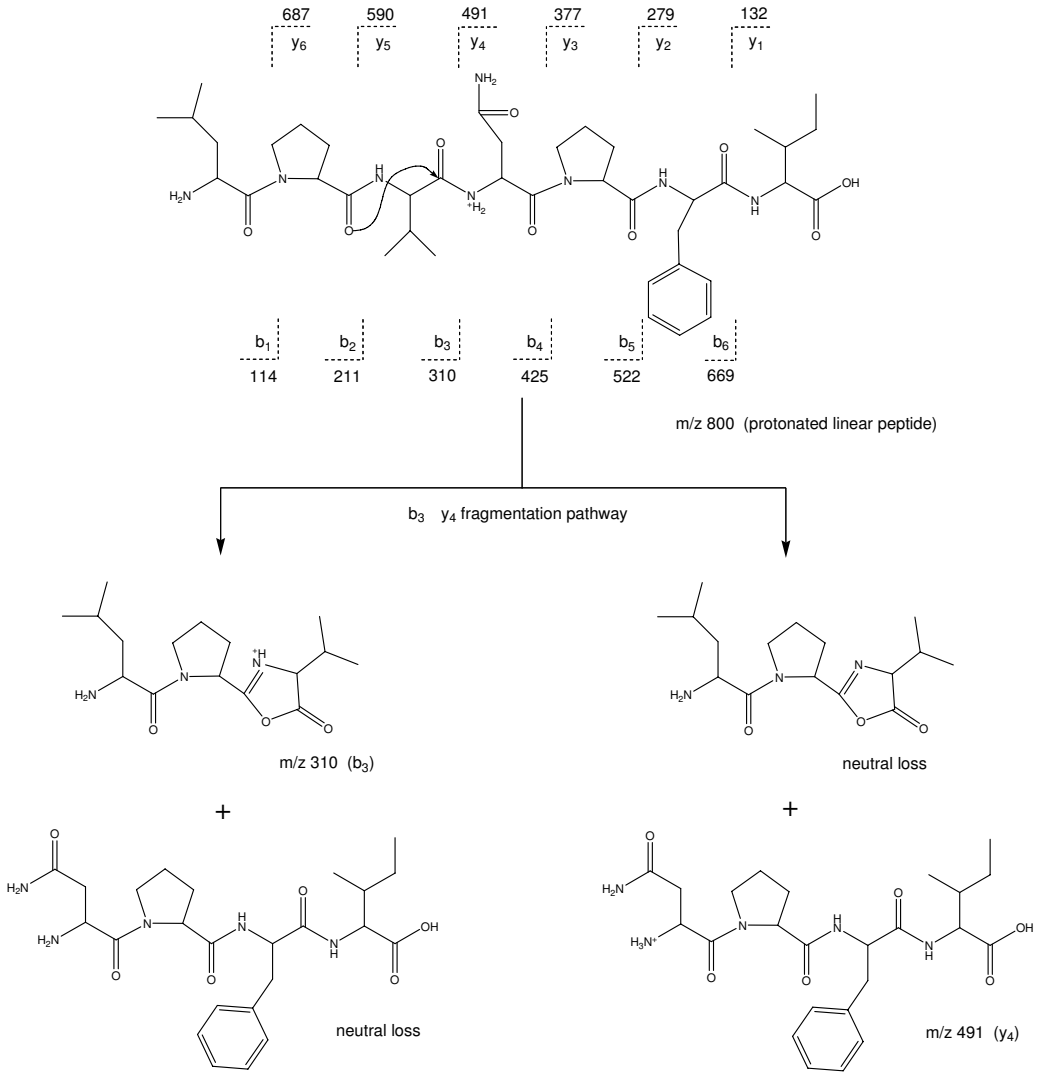


Scheme 1 Synthesis of Axinastatin 3. (a)DIC coupling; (b) 20% piperidine deprotection; HBTU coupling; (c) 20% piperidine deprotection; (d) trifluoroacetic acid/H_2O/p-cresol cleavage from resin; (e) PyBOP cyclization.

3.1 Sequencing the linear peptide

Compared with cyclic peptides, sequencing linear peptides is simpler, which can be accomplished by a single stage of MS/MS. Based on the mobile proton model [15], the added proton may move along the peptide chain and locate on a protonation site. If the proton locates itself on the nitrogen of the Val-Asn amide bond as shown in Scheme 2, the charge is fixed on the nitrogen, which causes the oxygen of the neighbouring Pro residue to attack the carbonyl carbon of the Val-Asn amide bond. The Val-Asn amide bond is then broken to generate the b_3 ion and a loss of neutral species or the y_4 ion and a loss of neutral species. This is the b_3-y_4 fragmentation pathway [5, 16]. If the proton perches on the nitrogen of all the amide bonds, all the amide bonds will cleave to form whole sets of b and y ions as labeled around the protonated linear peptide in Scheme 2. We can detect the two sets of ions in single stage of MS/MS, which yields complementary sequence information.

As shown in the Fig. 1, two sets of fragment ions, y_n and b_n ions (n=2-6), arising from CAD of an ESI-produced $[M+H]^+$ ion at m/z 800 were clearly detected. The m/z values of the b and y ions were compared to their theoretical values in the Scheme 2. Nevertheless, in ion-trap mass spectrometry, the so-called low mass cut-off feature limited the detection of y_1 (m/z 132) and b_1(m/z 114) ions. Even if the $b_1$and y_1 ions in low mass range were observed by means of lowering the Activation Q value to 0.16, it was still difficult to



Scheme 2 Proposed fragmentation pathways of the protonated linear peptdie.

observe integral sets of b_n and y_n in the same spectrum due to arbitrary loss of ions in high mass range. However, this drawback did not affect the correct sequence assignment. The b_n ions (n=2-6) indicated the connectivity of the amino acid residues as Leu-Pro-Val-Asn-Pro-Phe-Ile or Pro-Leu-Val-Asn-Pro-Phe-Ile, because the first two amino acids (whether Leu-Pro or Pro-leu) could not be ascertained. Furthermore, the y_n ions (n=2-6) revealed the connectivity of the amino acid residues as Leu-Pro-Val-Asn-Pro-Phe-Ile or Leu-Pro-Val-Asn-Pro-Ile-Phe, allowing the order of the first two amino acids to be set as Leu-Pro. To sum up, the original sequence of the linear peptide is identified as Leu-Pro-Val-Asn-Pro-Phe-Ile. It is worth noting that the distinction between Ile and Leu relies on observations of w or d ions formed via amino acid side-chain cleavages [3, 17, 18]. Unfortunately, the side-chain cleavages usually do not occur under the low-energy CAD in an ion trap [19]. So it is difficult to distinguish Ile and Leu. Therefore, the Ile and Leu were labeled for clarity.

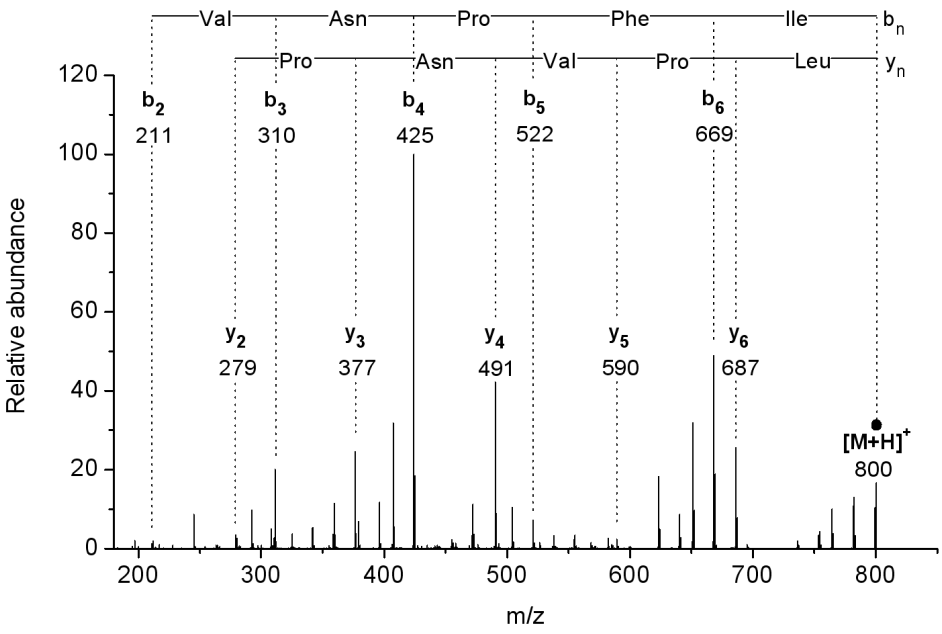
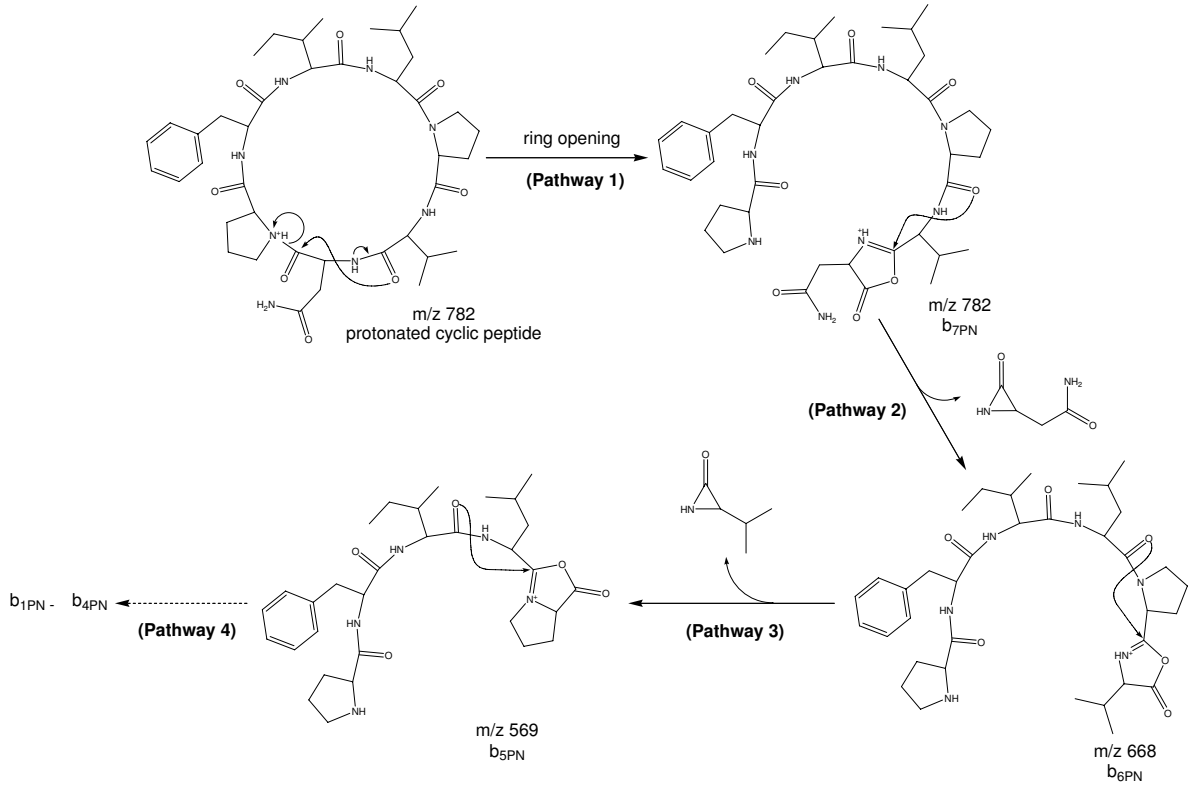


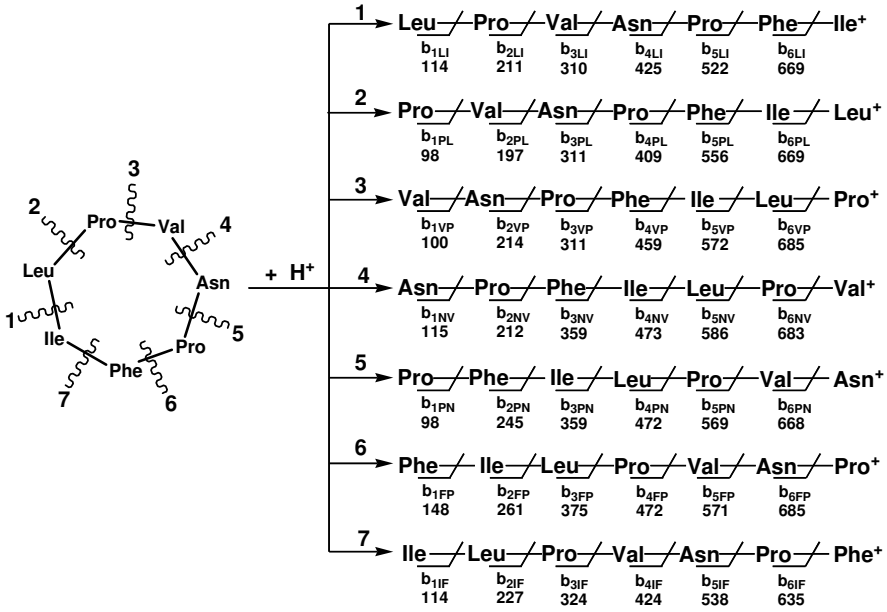
Fig. 1 Product-ion spectrum of the protonated linear peptide generated by one stage of MS/MS. For clarity, just the b and y ions are labeled. The parent ion is marked with "•".

3.2 Sequencing the cyclic peptide

In comparison with linear peptides, sequencing cyclic peptides is more complex and should be completed in multiple stages of MS/MS. As shown in Scheme 3, the added proton moves around the peptide ring. If the proton locates on the nitrogen of the Pro-Asn amide bond, the charge is fixed on the nitrogen, which causes the oxygen of the neighboring Val residue to attack the carbonyl carbon of the Pro-Asn amide bond. Then the Pro-Asn amide bond cleaves, resulting in ring opening to give the b_{7PN} ion as an oxazolone ring (pathway 1) [5, 20]. Subsequently, the oxygen of the neighboring Pro residue attacks the -C=N$^+$- carbon center of the oxazolone ring, which cause a loss of neutral Leu residue to generate the b_{6PN} ion as an aziridinone ring (pathway 2). This is the $b_{7PN} \rightarrow b_{6PN}$ pathway [5, 21]. In a similar fragmentation pattern following Pathway 2, the b_{6PN} ion keep on dissociating to produce the remaining b_{nPN} (n=1-5) ions through the $b_{nPN} \rightarrow b_{(n-1)PN}$ (n=2-6) pathways (pathway 3 and 4). This is just one set of b ions, arising from one ring opening pathway. If ring opening pathways occur at each amide bond of the peptide ring, all the b ions of the cyclic peptide obtained from all ring opening pathways (Scheme 4) will probably present in one MS^2 spectrum. So it is difficult to avoid assignment mistakes in a single stage of MS/MS, and multistage MS/MS is needed for sequencing cyclic peptides. In principle, the approach allows one $b_n \rightarrow b_{(n-1)}$ fragmentation pathway to take place at each stage of MS/MS; in other words, one amino acid residue is sequentially eliminated from C-terminus at each stage of MS/MS.



Scheme 3 Proposed fragmentation pathways of the protonated cyclic peptdie. (Pathway **1**) ring opening pathway; (Pathway **2-4**) $b_{nPN} \rightarrow b_{(n-1)PN}$ (n=3-7) pathways.



Scheme 4 All the b ions of the protonated cyclic peptide obtaining from all the ring opening pathways. The symbol and theoretical m/z value is labeled under each b ion.

The theoretical values of the b ions from the cyclic peptide are shown in Scheme 4, which are used in analyzing the b ions in the mass spectra obtained in the MS/MS experiments. In the first stage (MS^2), an ESI-produced protonated cyclic peptide at m/z 782 was subjected to CAD to produce the first-generation, product-ion mass spectrum (Fig. 2a). Fortunately, the superpositions of spectra from fragmentation of the various ring-opened forms are not obvious. Just one set of b_{nPN} (n=2-6) dominate the spectrum, originating from the acylium ion Pro-Phe-Ile-Leu-Pro-Val-Asn$^+$. The zoom scan mode was utilized to determine the m/z values of fragment ions in the range of m/z 665.5-671.5. In the high resolution spectrum acquired (Fig. 2b), the b_{6PN} (calculated 667.9, found 667.8) and the b_{6PL} or b_{6LI} (calculated 668.8, found 668.7) ions were detected. Subsequently, in the second stage (MS^3), with an isolated width of 0.5 Da, the b_{6PN} ion was chosen from the first-generation spectrum and then subjected to CAD to generate the second-generation product-ion mass spectrum (Fig. 3a). The b_{5PN} ion was seen at m/z 569. Next, in the third stage (MS^4), the b_{5PN} ion was selected from the second-generation spectrum and then subjected to CAD to furnish the third-generation, product-ion mass spectrum (Fig. 3b). The b_{4PN} ion was obtained at m/z 569. This procedure was repeated until the b_{2PN} ion (m/z 245) was observed (Fig. 3c and 3d) and further CAD was exhausted. Nevertheless, according to the spectra obtained, the first two amino acids, whether Pro-Phe or Phe-Pro, can not be ordered owing to the incapability to detect b_{1PN}.

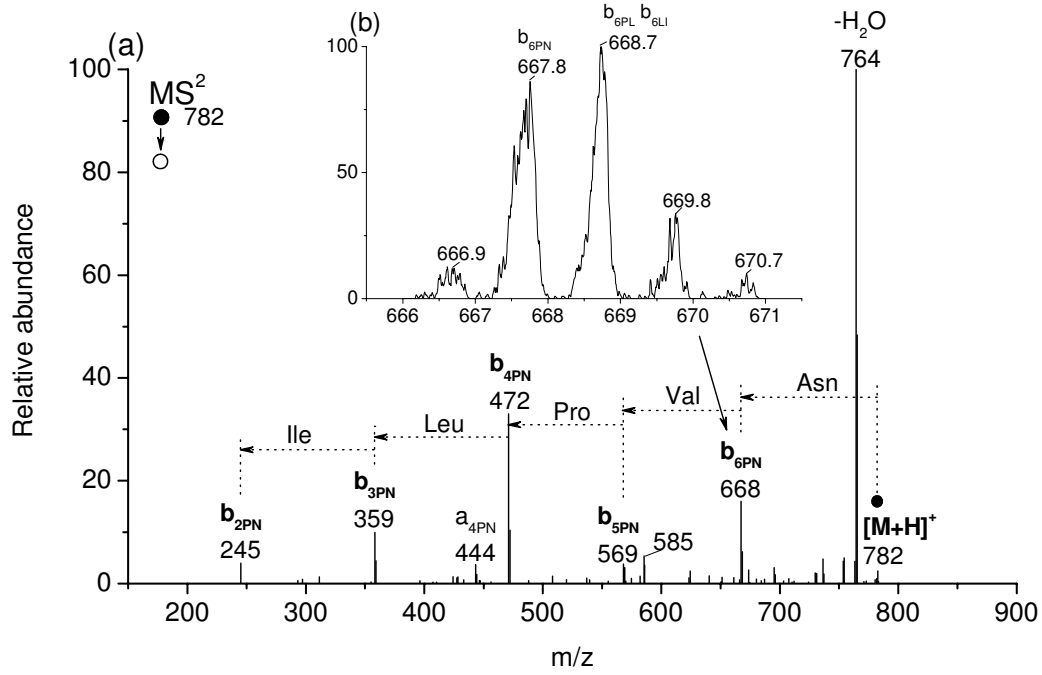


Fig. 2 (a) Product-ion spectra of the protonated cyclic peptide produced by one stage of MS/MS; (b) zoom scan spectrum in the range of m/z 665.5-671.5. The parent ion is marked with "•".

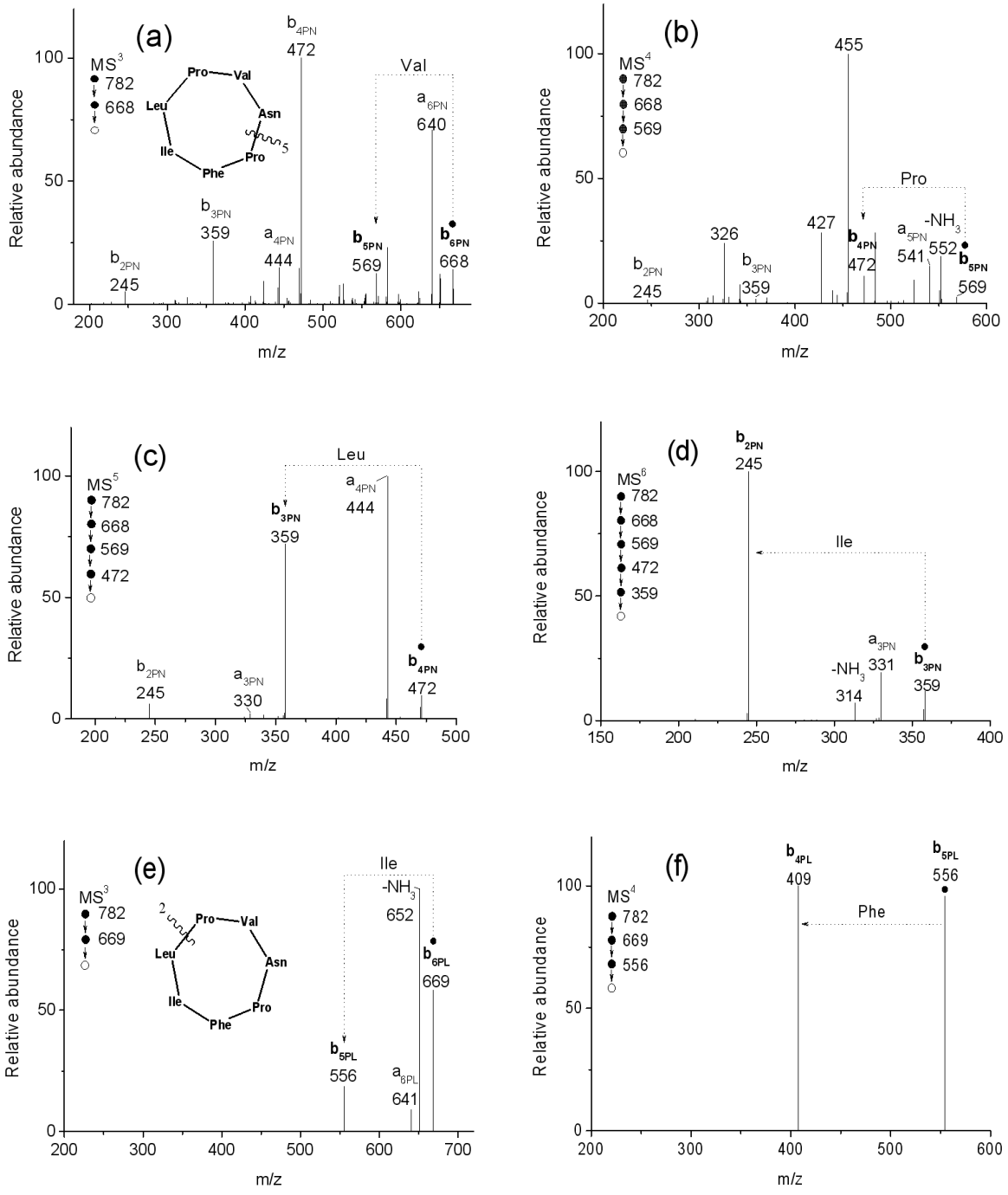


Fig. 3 Product-ion spectra generated by multiple stages of MS/MS. (a-d) MS^n spectra of the b_{6PN}, Pro-Phe-Ile-Leu-Pro-Val$^+$; (e-h) MS^n spectra of the b_{6PL}, Pro-Val-Asn-Pro-Phe-Ile$^+$. The simple schemes of the ring-opening pathways to form the b_{6PN} and b_{6PL} are shown in panel (a) and (e), respectively. In the two sets of mass spectra, the amino acid residues are sequentially eliminated from C-termini one at each stage of MS/MS. The parent ions are marked with "•".

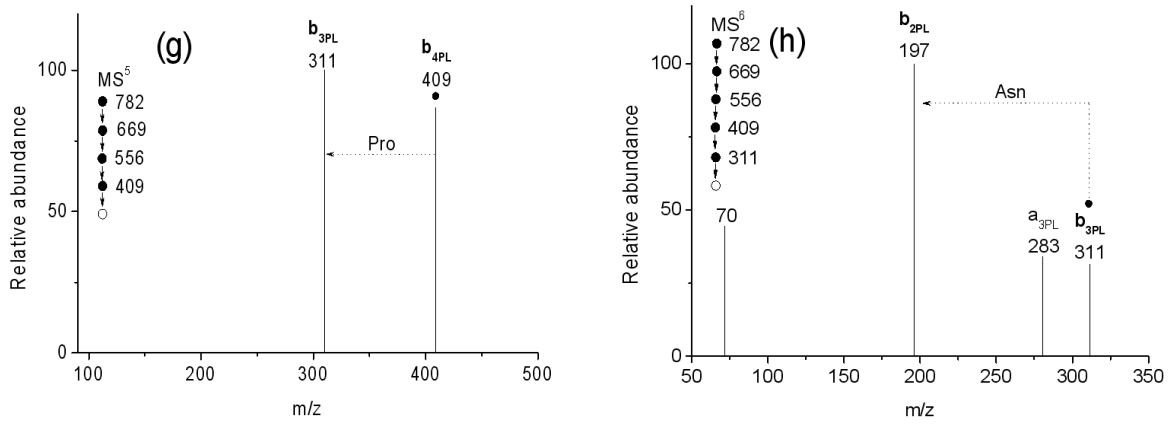


Fig. 3 (continued) Product-ion spectra generated by multiple stages of MS/MS. (a-d) MS^n spectra of the b_{6PN}, Pro-Phe-Ile-Leu-Pro-Val$^+$; (e-h) MS^n spectra of the b_{6PL}, Pro-Val-Asn-Pro-Phe-Ile$^+$. The simple schemes of the ring-opening pathways to form the b_{6PN} and b_{6PL} are shown in panel (a) and (e), respectively. In the two sets of mass spectra, the amino acid residues are sequentially eliminated from C-termini one at each stage of MS/MS. The parent ions are marked with "•".

In order to solve this problem, another set of b ions had to be determined in the same way. The MS^2 experiment was done with an isolation width of 0.5 Da. The b_{6PL} or b_{6LI} ion was chosen from the first-generation, product-ion mass spectrum and subjected to another stage of CAD. Further, a series of CAD experiments were performed to produce a desired set of b_{nLI} (n=2-5) shown in Figs. 3e-h, which enabled us to conclude the order of the first two amino acids as Pro-Phe and also suggest the existence of acylium ion Pro-Val-Asn-Pro-Phe-Ile-Leu$^+$. In summary, the original sequence of cyclic peptide is determined and as expected corresponded to Axinastatin 3 peptide sequence, cyclo(Leu-Pro-Val-Asn-Pro-Phe-Ile).

4 Conclusions

This investigation aims to solve the problem of sequencing peptides in the synthesis process of cyclic peptides. The differences of the fragmentation mechanisms and the sequencing protocols between sequencing linear and cyclic peptides were discussed. Initially, using one stage of MS/MS, the linear peptide was sequenced through the complementarities of y and b ions in the same MS^2 spectrum. Next, using multiple stages of MS/MS (to MS^6), the cyclic peptide was sequenced through sequentially cleaving one amino acid residue from the C-terminus to the N-terminus in each stage of MS/MS. Furthermore, this practical method can also be applicable to sequence peptides in the field of natural product isolation.

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