

Tandem mass spectrometry of kahalalides: Identification of two new cyclic depsipeptides, kahalalide P and Q from *Elysia grandifolia*.

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ABSTRACT

Spectra obtained using electrospray ionization mass spectrometry (ESI-MS) of mollusk *Elysia grandifolia* showed a cluster of molecular ion peaks centered at a molecular mass of 1478 Da (kahalalide F, an anticancer agent). Two new molecules, kahalalide P (m/z 1464) and Q (m/z 1492) were characterized using tandem mass spectrometry. The mass differences of 14 Da suggest that they are homologous molecules. In addition, previously identified kahalalide D and kahalalide G were also reported. While, ESI-MS of its algal diet *Bryopsis plumosa* showed presence of only kahalalide F. The amino acid sequences of kahalalide P and Q were proposed using collision induced dissociation (CID) experiments of singly and doubly charged molecular ions and by comparison with the amino acid sequence of kahalalide F. The pathway is presented for the loss of amino acid residues in kahalalide F. It is observed that there is sequential loss of amino acids in the linear peptide chain but in the cyclic part, the ring opens at the amide bond rather than at the lactone linkage and the loss of amino acid residues is not sequential. The CID experiment of the alkali metal cationized molecular ions shows that the sodium & potassium ions coordinate to the amide nitrogen/oxygen in the linear peptide chain of the molecule and not lactone oxygen of the lactone. In the case of kahalalide D, CID of the protonated peptide opens the depsipeptide ring to form a linear peptide with acylium ion and fragment ions signals indicate losses of amino acids in sequential order. In this study, tandem mass spectrometry provides detailed information required to fully characterize the new peptides.

KEY WORDS: *Bryopsis plumosa*, Collision induced dissociation, Cyclic depsipeptide, *Elysia grandifolia*, ESI-MS/MS, Kahalalide and Tandem mass spectrometry.

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INTRODUCTION

Among the sacoglossans, *Elysia rufescens* and more recently *Elysia ornata* are known to accumulate toxic cyclic depsipeptides from their algal diet *Bryopsis*. These compounds, called kahalalides, show very promising biological activity, including antiviral, antimalarial and, primarily antitumor properties.¹ Eight cyclic depsipeptides, kahalalide A-F,²⁻⁷ K,⁸ O⁹, and three linear peptides, kahalalide G,³ H and J¹⁰, ranging from a C₃₁ tripeptide to a C₇₅ tridecapeptide have been isolated from this mollusk. Among these, kahalalide A exhibited antituberculosis activity¹¹ and was antimalarial against *Plasmodium falciparum*. Kahalalide E was selective in action against *Herpes simplex II virus* (HSV II).³ Kahalalide F (KF) showed anticancer activity *in vitro* and *in vivo* against breast cancer, colon cancer, non-small cell lung cancer (NSCLC), ovarian cancer and in particular against androgen-independent prostate cancer.¹² New formulation of kahalalide F for the early clinical studies is under development.¹³ It is also active against some pathogenic microorganisms that cause the opportunistic infection of HIV/AIDS. Its mode of action has been studied¹⁴ and it is currently in phase II clinical trials in Europe.¹⁵⁻¹⁷

Multiple promising activities of kahalalides have attracted attention of researchers and the search for new representatives from this genus is in progress. Although the cyclic depsipeptides from genus *Elysia* are diverse they possess similar physiochemical properties, making their separation and analysis difficult. Therefore, a need was felt to develop a reliable analytical technique for rapid detection of peptides from genus *Elysia*. Because of the sensitivity and selectivity, tandem mass spectrometry was found to be the most suitable method for characterization of trace amount of peptides, present in the biological system, using peptide sequencing.

In recent years, tandem mass spectrometry (MS/MS) has become a powerful technique for sequencing peptides and small proteins. Tandem mass spectrometry of alkali metal cationized peptides has been extensively studied by Grese,^{18,19} Teesch,^{20,21} Mallis,²² Russell,²³ Tang²⁴ and Lin.²⁵ Russell²³ suggested that the sodium ion interacted with the amino terminus of the amide nitrogen in a small tyrosine containing peptide. Grese^{18,19} and Tang²⁴ proposed that metal cation binding occurs at the carbonyl oxygen of the C-terminus and the adjoining amino acid residue; upon collisional activation, rearrangement of the alkali metal cationized peptide and subsequent expulsion of the C-terminal residue ensues. Ngoka^{26,27} also presented a strategy for the determination of amino acid sequences of cyclic depsipeptide antibiotics by

a highly specific sodium ion interaction with the backbone lactone opening the depsipeptide ring to form a linear acylium ion or isomeric equivalent and fragmentation is charge-driven deleting sequentially amino acid residues from the C-terminus of the acylium ion. According to Lin,²⁵ the sodium ion, which is bound to the carbonyl oxygen of the lactam bridge, acts as a fixed charge site and directs a charge-remote, sequence-specific fragmentation of the ring-opened peptide. Govaerts²⁸ studied fragmentation of complex linear-ring peptides by applying multistage tandem MS. The study showed that only the linear side chain could be sequenced stepwise while the cyclic ring could not be sequenced stepwise. Similar studies were applied for investigating impurities in gramicidin, polymyxin B, polymyxin E and bacotracin using hyphenated technique i.e liquid chromatography coupled with ion trap mass spectrometry.²⁹ Das and co-workers also proposed that protonation of lactone oxygen opens the ring to form a linear peptide with a C-terminal carboxylic acid that fragmented to furnish the amino acid sequence.³⁰

Several other studies have examined the dissociation pattern of metal-cationized cyclic peptides. Williams³¹ used MSⁿ experiments involving low energy collisionally activated dissociation (CAD) in a quadrupole ion trap to characterize the fragmentation of alkali, alkaline earth and transition metal complexes of five cyclic peptides (cyclosporin A, valinomycin, enniantin A, iturin A and surfactin). It showed that the diagnostic utility of the fragmentation information of the metal complexes is metal specific. Of all the metal ions tested, those which provide the most useful complementary structural information to that of the protonated peptides are Na⁺ and Pb²⁺ ions for the depsipeptides, and Ni²⁺, Sr²⁺, Ag⁺ and Li⁺ for the other cyclic peptides. A detailed description of the fragmentation behaviour of beauverolides using post source decay (PSD), MSⁿ in the quadrupole ion trap and in-source CID in APCI on a sector instrument has been reported. Contrary to laborious PSD and tricky on-line LC/MS experiments, the in-source CID in APCI represents a powerful and robust tool for providing unambiguous sequence results.³² The peptide amino acid sequence for aspergillicins A-E was unambiguously assigned by MSⁿ ion trap ESI mass spectrometry.³³ Jegorov³⁴ identified natural roseotoxins A-C and of minor destruxins A-B using an ion trap ESI mass spectrometry. He proposed the dissociation pathway which involved opening of the cyclopeptide ring via formation of oxazolone derivatives and fragmentation of the resulting linear species have a free N-terminus and an oxazolone ring at the C-terminus.

In the present communication, the use of the ESI-MS/MS technique to characterize two new cyclic depsipeptides, kahalalide P (**4**) and Q (**5**) along with the known kahalalides D (**1**), F (**2**), and G (**3**) present in *Elysia grandifolia*, has been discussed. Its algal diet, *Bryopsis plumosa*, showed only the presence of kahalalide F (**2**). Structures for all the kahalalides are represented in Fig 1. The complete fragmentation of all the cyclic depsipeptides is reported here for the first time. Kahalalides P (**4**) and Q (**5**) contain the same cyclic core structure as that of kahalalide F (**2**) and differ only in the side chains attached to the backbone through the amino group of threonine. The strategy is presented for ring opening of the cyclic part of the molecule. The fragmentation pattern of the alkali cationized molecular ion of kahalalide F and P is also discussed. The tandem mass spectrometry technique used in this study is much faster as compared to NMR, highly sensitive and does not require a highly purified material and thus, detection of minor peptides was possible.

EXPERIMENTAL

Biological material: The mollusk *Elysia grandifolia* and green seaweed *Bryopsis plumosa* were collected from Malvan (16° 50'N, 17° 35'E), west coast of India.

Extraction and fractionation of mollusk *Elysia grandifolia*:

The mollusk *Elysia grandifolia* was finely ground and extracted with MeOH (1L). The extract was decanted off and concentrated under vacuum to obtain crude extract, which was partitioned with ethyl acetate. The ethyl acetate fraction was subjected to gel chromatography (Sephadex LH-20) using methanol as mobile phase. The fractions obtained were monitored by thin layer chromatography (TLC) on alumina backed sheets (Si gel 60 F254, 0.25mm thick) using butanol:acetic acid:water (5:1:4) mobile phase and visualization with ninhydrin reagent. The fraction rich in peptides was used for the MS analysis.

Extraction and fractionation of green algae *Bryopsis plumosa*:

Immediately after collection, seaweed was freed from extraneous matter and extracted with methanol. The fractionation and purification procedure was carried out in the same manner as given for *Elysia*.

ESI-QTOF MS/MS spectrometry:

The mass spectrometer used was a QTOF-XL MS/MS Applied Biosystem instrument equipped with MDS Sciex Analyst software (Concord, Ontario, Canada). The sample dissolved in 1:1 MeOH: 0.1% TFA and was directly infused at a constant flow rate of

10 μ L/min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode with the following settings: Nebulizer gas (N₂) 28 (arbitrary units); curtain gas (N₂) 18 (arbitrary units); Ion spray voltage-5700 V; Declustering potential (DP)-120V; Focusing potential (FP)-365V; Declustering potential (DP2)-14V and Collision gas (CAD) 3 (arbitrary units). Full-scan data acquisition was performed, scanning from m/z 100 to m/z 2000 in profile mode and using a cycle time of 1s. The collision energy (CE) 25 V was selected in case of kahalalide D to obtain the most intense precursors and 40 V for kahalalides F, G, P and Q and mass analyzed using the TOF analyzer of the instrument.

RESULTS AND DISCUSSION

Parent ion scan (ESI-MS) for the detection of peptides:

The ESI-MS of the peptide rich fraction of the *E. grandifolia* showed clusters of molecular ion peaks (Fig 2). Closer examination of the spectra revealed the presence of both singly (+1) and doubly (+2) charged molecular ion peaks in the mass range of 1400-1550 amu (Fig 2a) and 700-800 amu (Fig 2b), respectively. The ethyl acetate fraction of *B. plumosa* was also subjected to ESI-MS, which showed a peak for only one peptide, kahalalide F (m/z 1477.9350).

The theoretical monoisotopic m/z values of the peptides in various forms of +1 and +2 charges at m/z 596.3554, 1463.9706, 1477.9356, 1491.9870 and 748.0010 were calculated. Based on the theoretical calculations singly and doubly charged ion peaks including sodium and potassium adduct for five peptides could be detected in the full scan spectra, which are shown in bold in Table 1. The literature reveals that [M+H]⁺ at m/z 596.3554, 1477.9346 and doubly charged ion at m/z 748.0010 correspond to earlier reported cyclic depsipeptides, kahalalide D (**1**), F (**2**) and linear peptide kahalalide G (**3**), respectively from *E. rufescens* and its algal diet *Bryopsis sp.*³ The compounds with [M+H]⁺ at m/z 1463.9706 and 1491.9870 are new to the literature. The relative abundance reveals kahalalide D as the major (100%) cyclic depsipeptide followed by kahalalide F (**2**) with 40.33%. The other two new peptides, designated as kahalalide P (**4**) (13.56%) and Q (**5**) (0.76%), are present as minor constituents.

The fragment ion peaks are labeled according to the nomenclature proposed by Roepstorff and Fohlman,³⁵ subsequently modified by Johnson *et al.*³⁶ To retrieve a sequence from the mass spectrometric data of the new peptides, we applied the criteria that the mass difference between two adjacent peaks should precisely fit the mass of an amino acid residue.

Tandem mass spectrometry (MS/MS) analysis:

1) MS/MS at m/z 596.3722 of kahalalide D (1):

Fig 3 represents the MS/MS spectrum of a protonated peptide at m/z 596.3722. It is evident from the spectrum that protonated lactone oxygen opens the ring to form the linear acylium ion and the amino acid residues are sequentially deleted. The side fatty chain with the loss of water gives a peak at m/z 140.0807 designated as b_1^0 . The corresponding y_3 fragment ion is observed at m/z 454.2920. The fragment ions b_2 and y_2 were significantly observed at m/z 325.1985 and 272.1771 for the cleavage between tryptophan and N-terminal of the proline. The fragment ions b_3 and y_1 were observed at m/z 422.2538 and 175.1205, respectively. The peak at m/z 579.3461 was due to loss of ammonia while m/z 568.3784 was due to loss of CO. The combined loss of CO and NH_3 gives a peak at m/z 551.3499. Peaks at m/z 187.0908 and 159.0918 were observed for the tryptophan and its immonium ion, respectively. It is observed that in case of kahalalide D (1) the protonation of lactone oxygen opens the ring to form a linear peptide and the amino acids are deleted sequentially.

2) MS/MS at m/z 1477.9669 $[M+H]^+$ and 739.4820 $[M+2H]^{+2}$ of kahalalide F (2):

The MS/MS spectrum of kahalalide F (2) at m/z 1477.9666 (Fig 4a) showed intense series of 'b' and 'y' type fragment ions in the linear region. The spectrum shows the fragment at m/z 212.1449 which is consistent with D-val-(5-methylhexanoic acid), designated as b_1 ion, and results from the cleavage of the amide bond between the first valine and threonine residue in the linear region of the molecule. The corresponding y_{12} ion was observed at m/z 1266.8085. Similarly, the fragments at m/z 313.2144, 412.2842, 511.3546, 609.4066, 723.4777 and 835.5762 were assigned as b_2 , b_3 , b_4 , b_5 , b_6 and b_7 , respectively. The corresponding y ions were observed at m/z 1165.7494 (y_{11}), 1066.6857 (y_{10}), 967.6124 (y_9), 868.5424 (y_8), 756.4777 (y_7), and 643.3934 (y_6). The y_9 ion due to the cleavage of the amide between the valine at 4th position and proline was most prominent. This is because proline residue contains a tertiary nitrogen atom and is thus more basic than other residues, and hence more likely to be protonated, thereby leading to enhanced peptide-bond cleavage.³⁷ The above fragment ion observed confirms the peptide sequence 5-Me-Hex-V-T-V-V-P-Orn-I-, containing seven amino acids with short fatty chain. Earlier studies by E. Stokis *et al.*³⁸ report the quantitative analysis of KF in human plasma done during a Phase I clinical trial, in patients with androgen refractory prostate cancer using high performance liquid chromatography (HPLC) coupled to API 2000 triple-quadrupole mass spectrometer equipped

with a electrospray ionization source (ESI-MS/MS). The study reported the fragmentation leading to the formation of only b_1 to b_4 and y_9 (969) and y_{10} (1068) fragment ions. This reveals that the sensitivity is much better on QTOF instruments than on triple-quadrupole mass spectrometers. The recently introduced Q_2 -pulsing function enhances the transmission of fragment ions of a selected m/z window from the collision cell into the TOF part which improves the sensitivity of precursor ion scans on a quadrupole TOF instrument.³⁹⁻⁴⁰

In the present study, it is noted that there is sequential loss of amino acid residues up to isoleucine (b_7 & y_6) in the linear region while fragment ions y_1 to y_5 and its corresponding b_8 to b_{12} were not detected, which suggests that there is no further sequential loss of amino acids in the cyclic region in KF (Fig 4a).

In addition to 'b' and 'y' fragment ions 'a' and 'c' type of fragment ions were also observed. Fragment ions at m/z 184.1705, 693.4750 and 850.5354 were assigned to a_1 , a_6 , c_5 and c_7 , respectively. Peaks at m/z 1469.9568 and 1460.9655 were observed due to the loss of H_2O and NH_3 . The fragmentation pathway for the loss of amino acid residues in the cyclic region of KF is discussed below.

Fragmentation pathway in the cyclic region of kahalalide F (2):

For any cyclic depsipeptides the first dissociation step involves the opening of the cyclic ring by cleavage of either the lactone group or one of the amide bonds. In most cases the most basic site gets protonated first resulting in the weakening of the C-N or C-O bond.

In the case of KF, two pathways for the ring opening are possible as depicted in Scheme 1, designated as 'I' and 'II'. Pathway 'I' is for the ring opening at the lactone oxygen with the first cleavage of L-Val, which would result in the formation of fragment ion at m/z 541, which is not observed in the spectra. Similarly the sequential loss of other amino acid residues Dhb, F, V, T would result in the fragment ions at m/z 558, 311, 212 and 199, respectively which are also not observed in the spectra. This indicates that ring opening at the lactone oxygen i.e. pathway 'I' is ruled out. Pathway 'II' is favored with the initial loss of phenylalanine residue leading to the formation of the peak at m/z 494.3450 [y_6-F]⁺ designated as 'A'. This loss can be attributed to N-C ring opening. The second loss of D-Val gave a peak at m/z 394.2727 [$A-V$]⁺ designated as 'B'. Species 'B' undergoes internal cyclization and the loss L-Val which gives a very intense peak at m/z 295.2055 [$B-V$]⁺ designated as 'C'. The pathway proposed for the loss of the amino acids in the macrolidic region is outlined in Scheme 1. The electron donating aromatic ring makes the phenylalanine group a more basic

residue, and hence it gets protonated in preference to lactone oxygen. Thus, the ring opens at the amide bond of the phenylalanine group. It is also evident from Scheme 1 that loss of amino acid residues from the cyclic region is not sequential.

The tandem mass spectra of doubly charged ion at m/z 739.4803 are remarkably similar to Fig 4a differing only in the additional peaks being at m/z 1331.8930 and 1378.7940, which are accounted for by elimination of F and V residues from the parent ion. Fragment ion peaks b_5 , b_7 , y_7 and y_8 could not be detected or rather they may be less intense.

MS/MS of $[M+Na]^+$ at m/z 1499.9488, $[M+H+Na]^{+2}$ at m/z 750.4661 and $[M+H+K]^{+2}$ at m/z 758.4938 of kahalalide F (2):

In addition to the protonated molecular ion $[M+H]^+$, CID was also obtained for the sodium and potassium adducts of KF. Kahalalide F (2) displayed sodium adducts $[M+Na]^+$ at m/z 1499.9488 (Fig 4b) and $[M+H+Na]^{+2}$ at m/z 750.4661. The potassium adduct $[M+H+K]^{+2}$ was observed at m/z 758.4938 (Fig 4c). The MS fragmentation pattern for the alkali metal (Na/K) catalyzed molecular ion peaks differ significantly from the protonated molecular ion. The sodium or potassium ion affects the fragmentation pattern depending upon the location of the metal ion in the molecule.

In the MS/MS at m/z 1499.9488 of KF (2), an intense series of fragment ions with Na^+ attached to the y and b ions was observed. The fragment ion peak with metal ion is denoted by an asterisk (*) (Fig 4b). The fragment ion peaks from b_1 to b_5 were exactly matching with CID of the protonated molecular ion. Its corresponding y ions, y_{12}^* (1288.8334), y_{11}^* (1187.7263), y_{10}^* (1088.6648), y_9^* (989.5904) and y_8^* (890.5554) were ionized by sodium ion attachment. It also shows peak at 1271.8 for $[y_{12}^*-NH_3]^+$. Peaks at m/z 505.3588, 647.4149, 700.4432, 744.4685 and 829.5602 were attributed to the sodiated fragment ions a_4^* , c_5^* , $[a_6^*-NH_3]^+$, $[b_6^*-NH_3]^+$ and a_7^* , respectively. No change in the fragmentation of the cyclic region was observed. It was identical to the fragmentation observed in MS/MS of $[M+H]^+$ ion. This indicated that Sodium is attached either to amide nitrogen or amide carbonyl oxygen of the linear chain V-P-Orn-I. The Na^+ does not bind to the lactone oxygen of the cyclic ring, which opens the ring to give a linear peptide as described by Ngoka.²⁶ This suggests that the lactone linkage is stronger than any other amide bond in the linear as well as cyclic part of the molecule and hence the new fragmentation pattern was observed. Fig 4b also summarizes the fragmentation pattern for the sodium adduct. Combined loss of CO and NH_3 from sodiated molecular ion gives a peak at m/z 1455.9123 $[M^*-(CO+NH_3)]^+$. Peaks

were also observed at m/z 1382.8642 and 1356.8642 for $[M^*-(H_2O+V)]^+$ and $[M^*-(CO+NH_3+V)]^+$, respectively. Doubly charged ion $[M+H+Na]^{+2}$ at m/z 750.4661 also displayed similar peaks.

A similar fragmentation pattern was observed during CID of the potassium adduct $[M+H+K]^{+2}$ at m/z 758.4938 (Fig 4c). Fragment ion peaks from b_1 to b_4 were identical to that of protonated molecular ion but the y fragment ions were ionized by potassium. The y^* fragment ion peaks were observed at m/z 1304.8310 (y_{12}^*), 1202.7209 (y_{11}^*), 1104.6974 (y_{10}^*), 1005.6188 (y_9^*), 906.5515 (y_8^*) and 794.4641 (y_7^*) but the y_6 fragment is observed at m/z 643.3944 without addition of potassium ion. No change was observed in the fragmentation pattern of the cyclic ring. This indicated that potassium ion is not attached to the lactone oxygen of the cyclic region but may be attached to the amide nitrogen or carbonyl oxygen in the linear chain of the molecule.

3) Mass fragmentation of kahalalide G (3):

The doubly charged molecular ion at m/z 748.0010 for $[M+2H]^{+2}$ corresponds to the molecular mass 1496, which is 18 Da higher than kahalalide F. This suggest it is a ring opened form i.e kahalalide G (3).¹² It could also due to the formation of water adduct of kahalalide F. But based on the literature we presumed it to be kahalalide G, which is reported from the same source. The MS/MS experiment for the doubly charged ion m/z 748.0010 was carried out at 25V, which showed identical b and y fragment ions as that of kahalalide F (2). This suggests that KF is derived from kahalalide G (3) with the initial loss of water resulting in the cyclisation via a lactone bond between the threonine (sixth residue from the C-terminal) and carboxylic group. Thus, the fragmentation pattern is identical to that of (2).

4) Elucidation of the peptide sequence of new cyclic depsipeptides, kahalalide P (4) and Q (5):

The CID spectra of $[M+H]^+$ at m/z 1463.9541 (Fig 4a), $[M+2H]^{+2}$ at m/z 732.4854 and sodium adduct $[M+H+Na]^{+2}$ at m/z 743.4582 (Fig 4b) of kahalalide P (4) were studied. Molecular ion $[M+H]^+$ showed peaks at m/z 212.1447 (b_1) 313.2171 (b_2), 412.2843 (b_3), 511.3578 (b_4), 609.3909 (b_5) and 723.4675 (b_6) identical to those observed in the MS/MS spectrum of KF. The corresponding y ions were observed at 1252.7944 (y_{12}), 1151.7452 (y_{11}), 1052.6714 (y_{10}), 953.6054 (y_9), 839.5439 (y_8-NH_3) and 742.4637 (y_7). The fragment ion y_6 at m/z 643.3909 (y_6) reveals the presence of a cyclic part of the structure identical to that observed in KF. Further it is also evident that the sequence of amino acids (5-MeHex-V-

T-V-V-P-Orn-) in the linear portion from b_1 to b_6 is identical to that of KF. The difference was observed in the amino acid linking the cyclic moiety. The mass difference between the 821.5634 (b_6) and 723.4675 (b_7) is 99, which could be accounted for the amino acid valine instead of isoleucine in KF. Also, the difference between y_7 and y_6 was exactly 99 amu confirming the presence of valine residue (Fig 5a).

Thus the CID mass spectrum reveals a valine substitution for isoleucine at number 7. The one line representation of the proposed structure of kahalalide P is given as Cyclo-8,13-(5MeHex-V-T-V-V-P-Orn-V-T-I-V-F-Dhb-V) [Dhb-dehydroaminobutyric acid). In analogy with the reported KF, similar stereochemistry may be assigned to this molecule. The CID of doubly charged ion at m/z 732.4723 also showed similar peaks that were less intense. The peak at 1316.8573 was assigned to the loss of phenylalanine from the parent ion.

In the CID of doubly charged sodiated molecular ion $[M+H+Na]^{+2}$ at m/z 743.4682, (Fig 5b), sodium ion was found attached to y fragment ions from y_{12}^* to y_7^* in the linear peptide chain. The y_6 fragment ion is devoid of sodium ion. This confirms that sodium ion is bound either to nitrogen or oxygen of the valine residue attached to the cyclic ring. The immonium ion of phenylalanine is observed at m/z 120.0816. The cyclic ring shows fragment ions identical to those found in $[M+H]^+$.

In a similar fashion the MS/MS of the $[M+H]^+$ at m/z 1491.9876 (Fig 6) showed identical fragment ions b_1 to b_5 , indicating linear peptide chain 5-MeHex-V-T-V-V-P- and the y_6 peak at m/z 643.3909 showed there is no change in the cyclic ring. This indicated that 14 units extra should be present in the linear region of the molecule. The corresponding b fragment ion for y_6 (m/z 643.3909) was observed at m/z 849.5965 (b_7). The mass difference between 643.3909 (y_6) and newly observed peak at m/z 756.4822 was 113, which can be accounted by the presence of isoleucine. Thus, the peak at m/z 756.4822 was assigned as the y_7 fragment ion. The corresponding b_6 fragment ion could not be detected in the spectrum. The corresponding y_8 was observed at m/z 882.5586 for 609.3977 (b_5). The peak m/z 864.5491 was attributed for loss of ammonia $[y_8-NH_3]^+$. The mass difference between y_8 (882.5586) and y_7 (756.4822) gives 128, which can be fitted by placing amino acid lysine at position six. The peak at m/z 226.1566 was observed for $[P+K]^+$ which was not observed in peptides previously discussed.

This completes the peptide sequence of kahalalide Q (**5**) and the one line representation is given as Cyclo-8,13-(5MeHex-V-T-V-V-P-K-I-T-I-V-F-Dhb-V) with the cyclic residue. Peak at m/z 1374.9 was assigned to combined loss of water and valine $[M-(H_2O+V)]^+$.

CONCLUSION

The unreported peptides, kahalalide P (**4**) & Q (**5**) are close analogues of kahalalide F. In kahalalide P, number 7th amino acid isoleucine is replaced by valine while in kahalalide Q, number 6th amino acid ornithine is replaced by lysine. The presence of lysine was previously reported in kahalalide J.¹⁰ ESI-MS/MS experiments provided data that was difficult to obtain by the NMR technique, because of their low concentration. This technique requires about ten thousand times less raw material than that of the NMR based procedure and all experiments, including extraction, take only a few days. On the other hand, the basic limitation of the mass spectrometric procedure is to determine the configuration of amino acid residues. The present study also indicates that the loss of amino acid residues from depsipeptide is not always sequential.

KF (**2**) exhibits significant bioactivity and is currently in Phase II clinical trials in Europe. Hence it elicits maximum interest chemically. The two new cyclic depsipeptides, kahalalide P and Q that were identified as being chemically similar to kahalalide F, may exhibit similar bioactivity. Since the synthetic methods are well known for KF, the new cyclic depsipeptides can be prepared to check for biological activity. As these compounds are typically administered at very low doses, considerable demands are placed upon the sensitivity of analytical methods to characterize their pharmacokinetic behaviour in humans. Thus, ESI-MS/MS is the detection method of choice for the analysis of cyclic depsipeptides.

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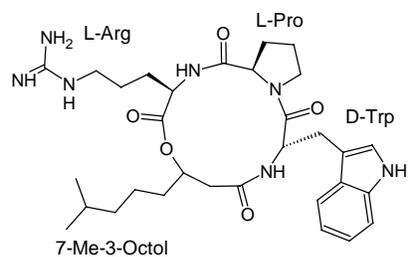
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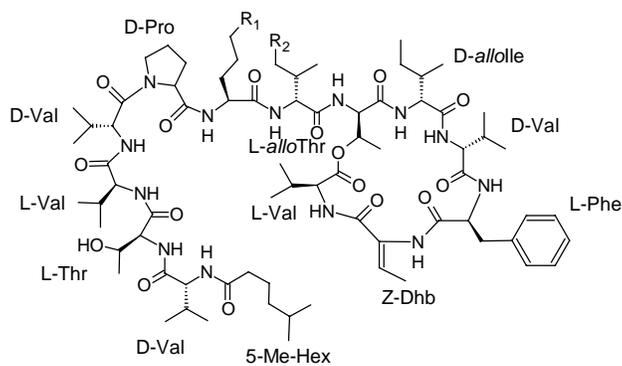
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Table 1: Theoretical monoisotopic m/z values of peptides in various forms of +1 and +2 charges. Molecular ion peaks of the peptides detected in ESI-MS experiment of *Elysia grandifolia* are shown in bold.

Peptides	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[M+Na+K] ⁺	[M+2H] ⁺²	[M+H+Na] ⁺²	[M+H+K] ⁺²	[M+2Na] ⁺²
Kahalalide D	596.45	618.3	634.3	657.3	298.22	309.22	317.72	320.72
Kahalalide P	1463.97	1485.9	1501.9	1524.9	732.45	743.45	751.45	754.45
Kahalalide F	1477.93	1499.9	1515.9	1538.9	739.46	750.46	758.46	761.46
Kahalalide Q	1492.00	1515.0	1530.0	1553.0	746.00	757.50	765.50	768.50
Kahalalide G	1497.00	1519.0	1558.0	1536.0	748.00	760.00	768.00	771.00



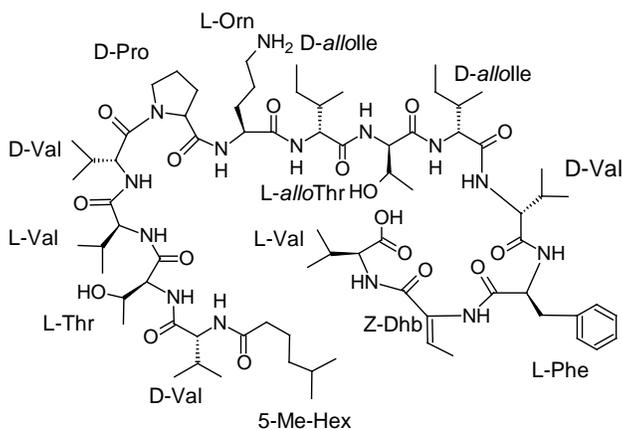
Kahalalide D (1)



Kahalalide F (2): R₁= NH₂; R₂=Me

Kahalaide P (4): R₁= NH₂; R₂=H

Kahalaide Q (5): R₁= -CH₂NH₂; R₂=Me



Kahalalide G (3)

Fig 1: Structures of kahalalides.

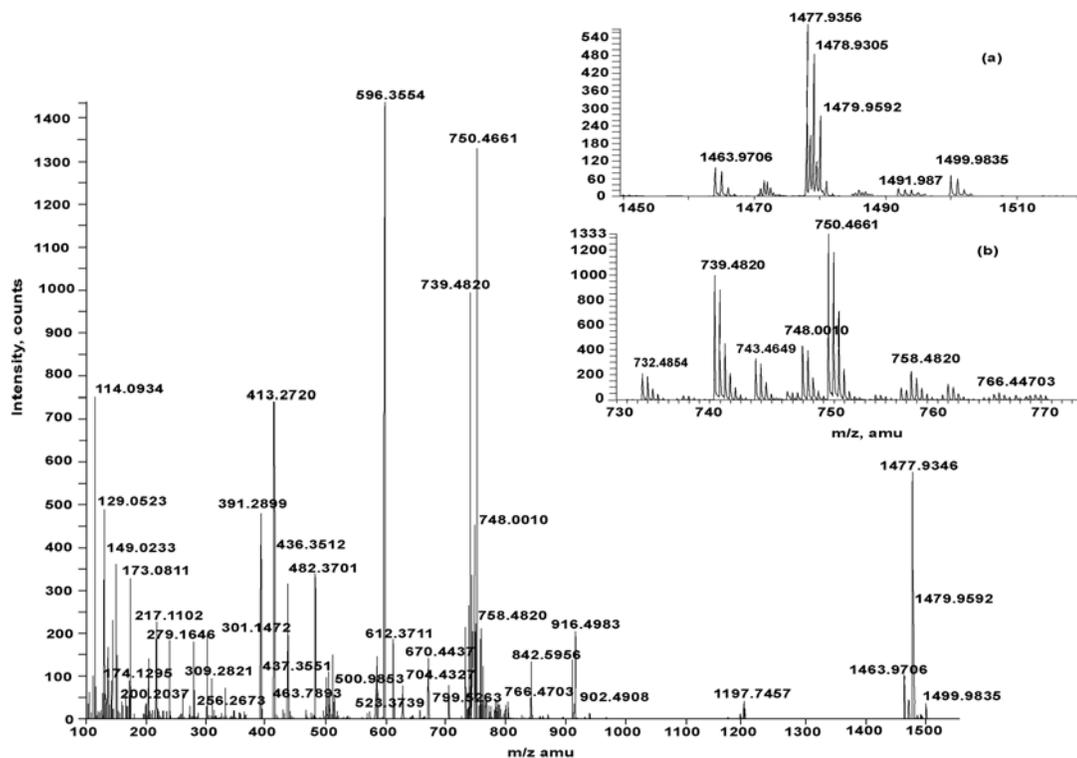


Fig. 2: Full scan ESI-MS spectra in positive ion mode of peptide rich fraction of *Elysia grandifolia*; (a) Singly charged molecular ion peaks (b) Doubly charged molecular ion peaks

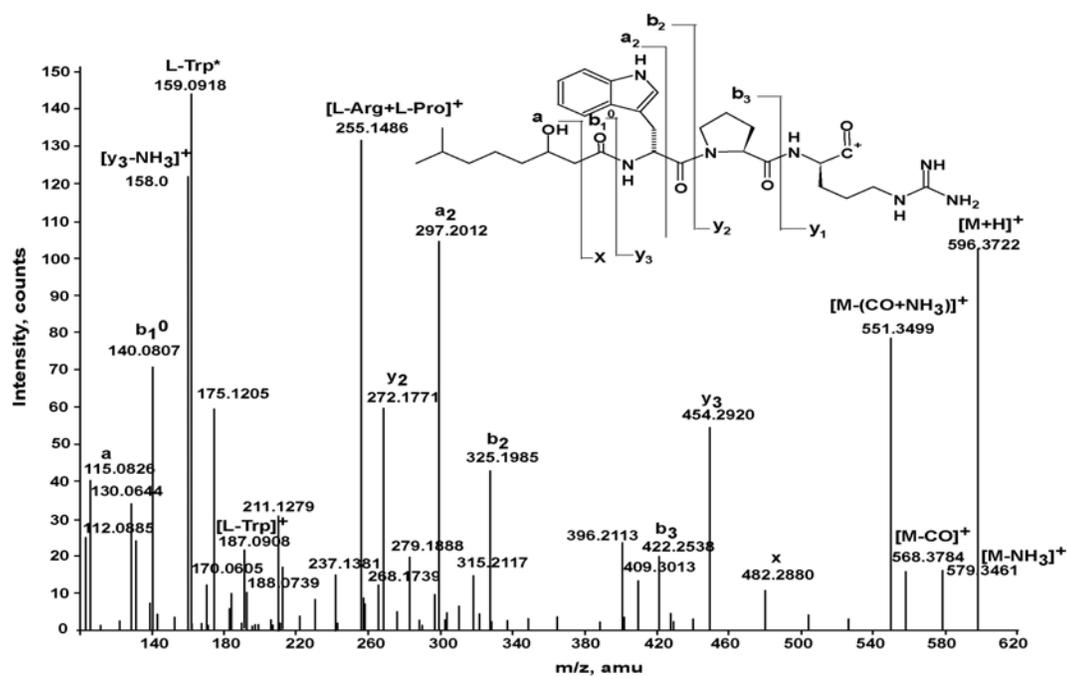


Fig. 3: MS/MS spectra of kahalalide D (2) at m/z 596.3722 along with the proposed fragmentation.

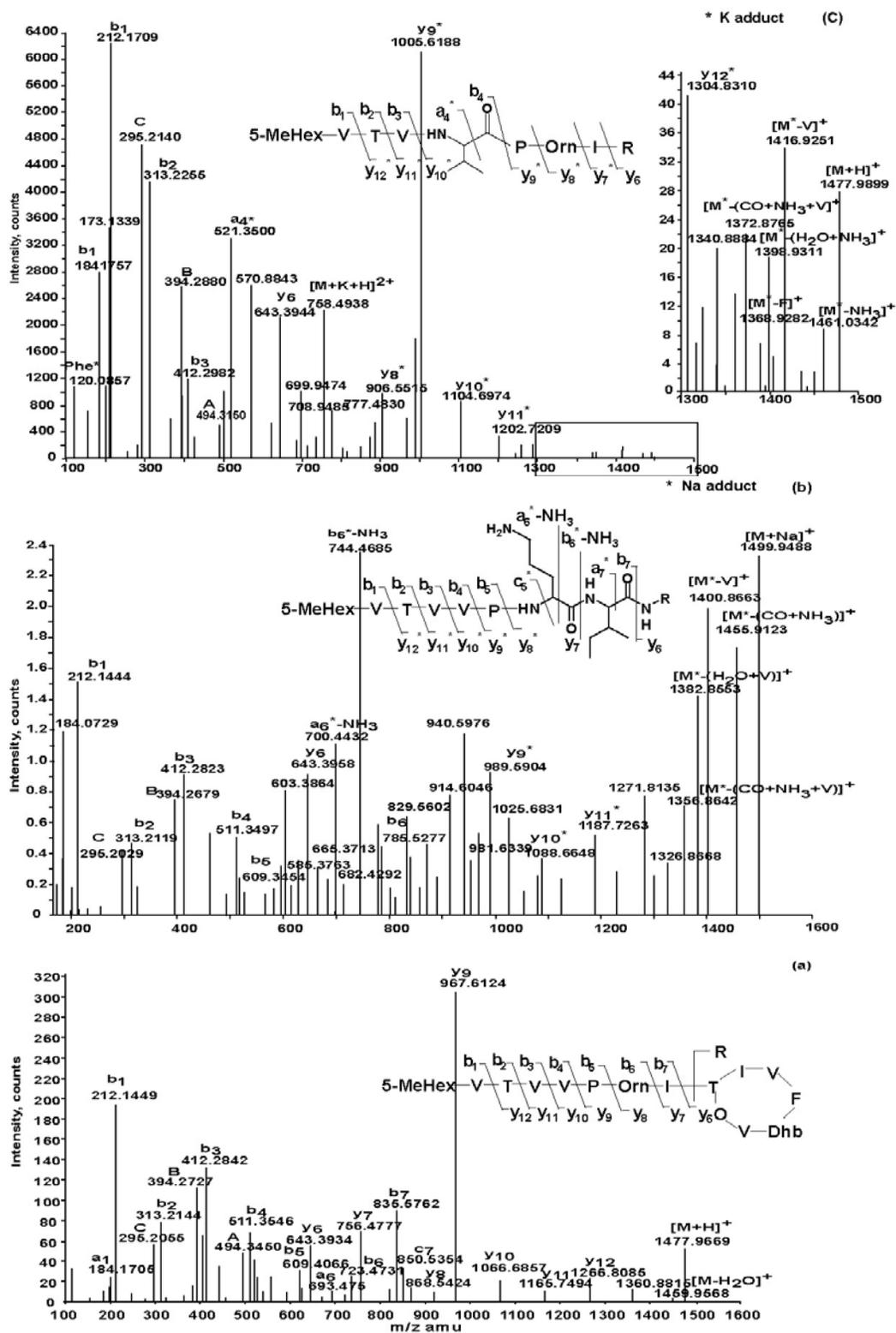


Fig. 4: MS/MS spectra of kahalalide F (2) at (a) m/z 1477.9669 $[M+H]^+$, (b) 1499.9488 $[M+Na]^+$ and (c) 758.4938 $[M+H+K]^+2$ along with the proposed fragmentation pattern.

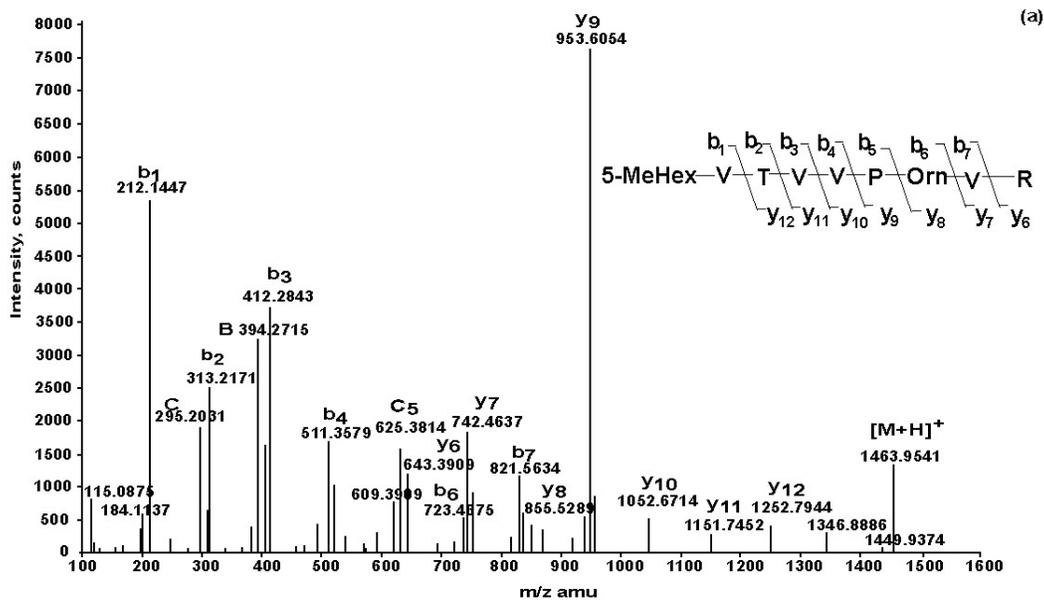
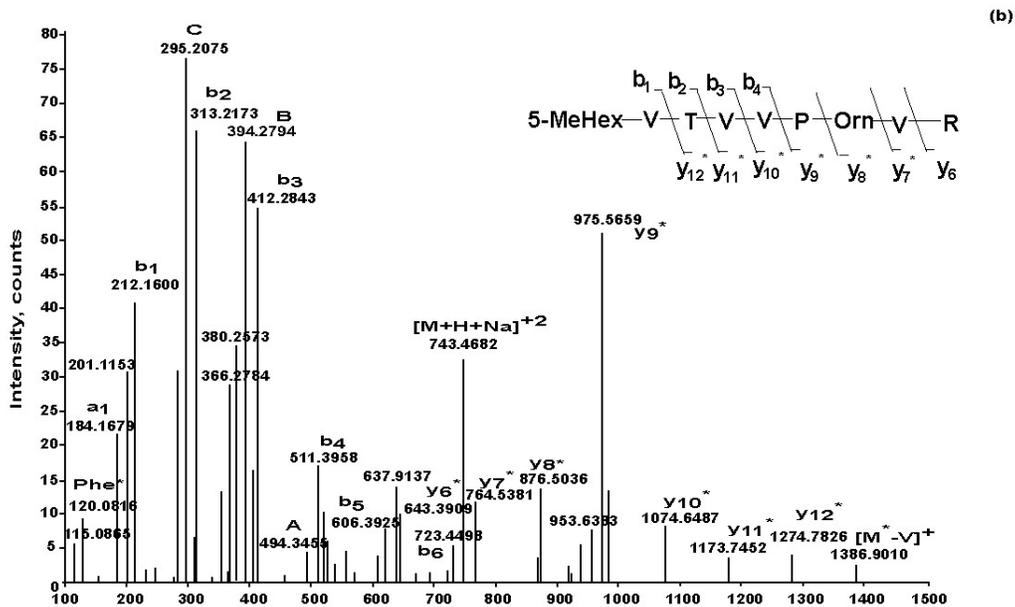


Fig. 5: MS/MS spectra of kahalalide P (4) at (a) m/z 1463.541 $[M+H]^+$ and (b) m/z 743.4682 $[M+H+Na]^{+2}$ along with the proposed fragmentation pattern.

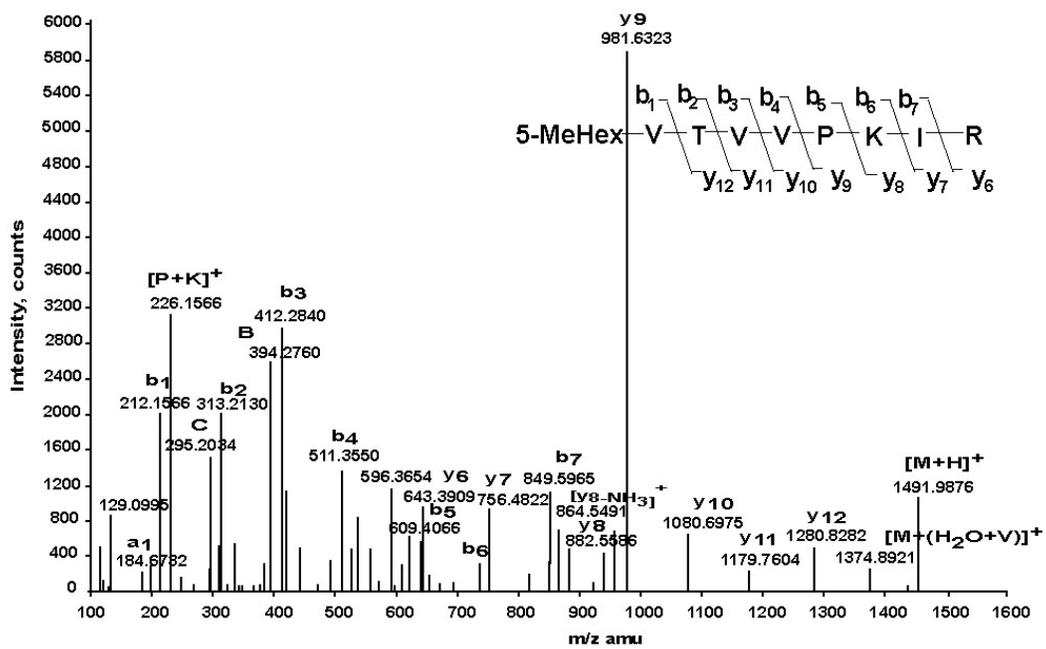
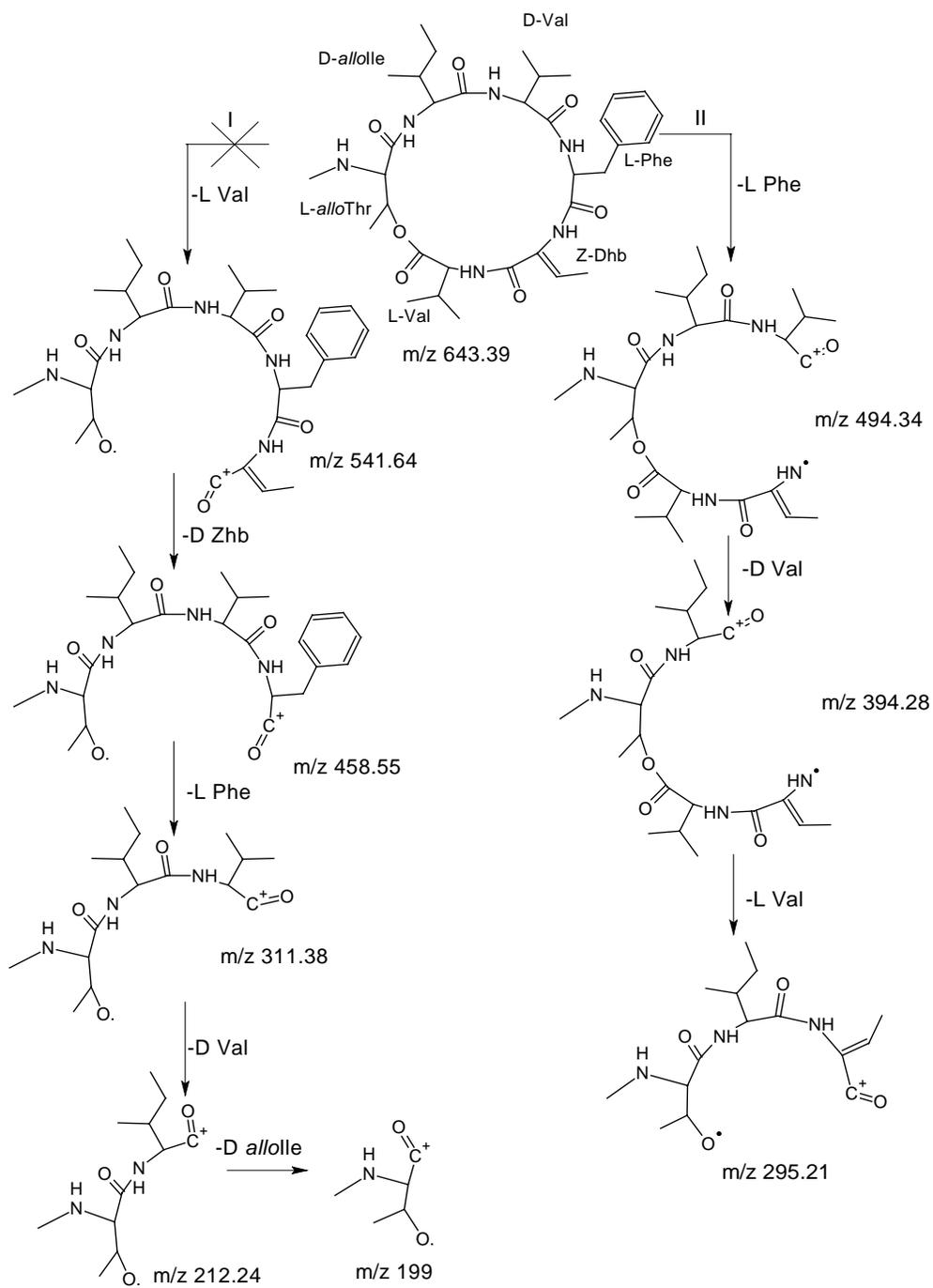


Fig. 6: MS/MS spectra of kahalalide Q (5) at m/z 1491.9876 [M+H]⁺ along with the fragmentation pattern.



Scheme 1: The proposed fragmentation pathway for loss of amino acid residues from the cyclic region of kahalalide F (2).

LIST OF FIGURE LEGENDS

Fig. 1: Structures of kahalalide D, F, G, P & Q.

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