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PAPER

Effect of ester chemical structure and peptide bond conformation in fragmentation pathways of differently metal cationized cyclodepsipeptides†

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Fragmentation behavior of two classes of cyclodepsipeptides, isariins and isaridins, obtained from the fungus *Isaria*, was investigated in the presence of different metal ions using multistage tandem mass spectrometry (MS^n) with collision induced dissociation (CID) and validated by NMR spectroscopy. During MS^n process, both protonated and metal-cationized isariins generated product ions belonging to the identical 'b-ion' series, exhibiting initial backbone cleavage explicitly at the β-ester bond. Fragmentation behavior for the protonated and metal-cationized acyclic methyl ester derivative of isariins was very similar. On the contrary, isaridins during fragmentation produced ions belonging to the 'b' or/and the 'y' ion series depending on the nature of interacting metal ions, due to initial backbone cleavages at the α-ester linkage or/and at a specific amide linkage. Interestingly, independent of the nature of the interacting metal ions, the product ions formed from the acyclic methyl ester derivative of isaridins belonged only to the 'y-type'. Complementary NMR data showed that, while all metal ions were located around the β-ester group of isariins, the metal ion interacting sites varied across the backbone for isaridins. Combined MS and NMR data suggest that the different behavior in sequence specific charge-driven fragmentation of isariins and isaridins is predetermined because of the constituent β-hydroxy acid residue in isariins and the *cis* peptide bond in isaridins.

Introduction

Naturally occurring cyclic peptides exhibit a diverse spectrum of biological activities and are useful in the development of new therapeutics.[1,2] Pharmacologically potent and toxicologically safe cyclodepsipeptides, members of the cyclic peptide family, are usually obtained from marine sponges, bacteria or fungi. Members of the cyclodepsipeptide family often exhibit antiplasmodial, antiviral, insecticidal, cytotoxic and antiproliferative properties in addition to their use as potent lead compounds for cancer therapy.[3–7] In cyclodepsipeptides, one or more amino acid(s) are replaced by hydroxy acid(s) resulting in the appearance of at least one ester bond in the core ring structure. Diversity in composition (in terms of amino acids and hydroxyacids) and conformation (*cis-trans* geometry along the peptide bond) of these molecules are to be properly characterized, especially in the context of their structures.

High-performance liquid chromatographic techniques, coupled with electrospray ionization mass spectrometry (LC-ESI MS), have been successfully used for rapid identification and characterization of micro-heterogeneous cyclodepsipeptides for screening of new metabolites.[6,8–11] Advantage of mass spectrometry (MS) over other competing biochemical and spectroscopic techniques is in the substantial reduction in detection and analysis time. MS also requires small quantity of sample for detailed analysis.[6,11–16] Moreover, the ability of mass spectrometers to handle mixture of samples by selective operation is an added advantage. Although tandem mass spectrometric technique (MS/MS) is a powerful tool for structural identification of linear peptides and proteins,[17,18] characterization of cyclic peptides possess some difficulties due to lack of any termini. Thus sequencing of cyclic peptides by one step MS/MS is not straight forward, rather it is complicated due to multiple and indiscriminate ring opening pathways along the peptide backbone leading to many acylium/oxazolone ions with identical m/z.[19,20] An approach to overcome this limitation is to increase the dimensionality of the mass spectral data *via* multi-stage fragmentation (MS^n) experiments until the identity of the cyclic-peptide is satisfactorily determined. Studies that focus on the fragmentation of cyclic and cyclodepsipeptides using dissociation techniques, such as collision induced dissociation (CID) (fragmentation occurs due to cleavage at backbone CO–NH bond generating 'b/y-ions'[21]) and electron capture dissociation (ECD) (fragmentation occurs due to cleavage at backbone N–C^{α} bond generating mainly 'c/z˙-fragments', arising from the

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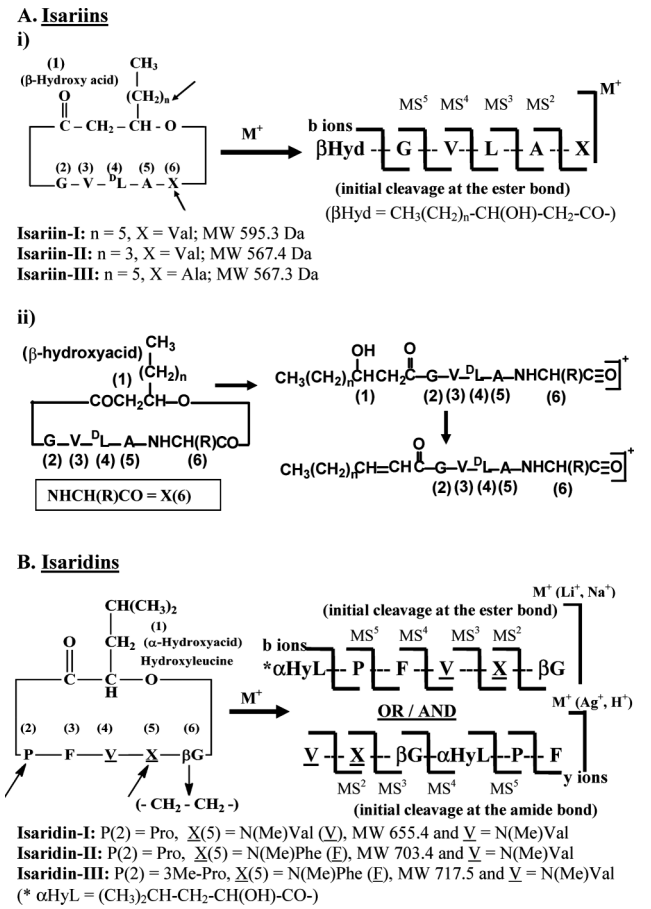
generation of carbon free radical species propagating along the backbone) for generating sequence information are available in literature.[19,22–25]

To improve the quality of mass spectra of cyclic and cyclodepsipeptides, a variety of metal ions such as Li^+, Na^+, K^+, Ag^+, Cs^+, Ca^{2+}, Ni^{2+} *etc.* are complexed with the peptides to achieve improved sequence information.[20,26–29] These studies further suggest that metal-cationized cyclic-peptides undergo a "charge-remote fragmentation" on CID where the location of the metal ion remains unchanged at all stages of the MS^n process,[20,26,28] thereby providing a gas-phase analogy of solution phase peptide sequencing.[26]

In this context, multi-stage tandem mass spectrometry experiments can offer new opportunities for studying the fragmentation pattern of cyclic-peptides with constrained conformation (*viz. cis*/*trans* peptide bond) on the basis of its sequence/structural context. However, the role of the peptide geometry (*cis-trans* conformation) in influencing fragmentation pathways, either as protonated or metal-cationized cyclic peptides, is not reported. The present study focuses on the role of composition and peptide bond conformation of cyclodepsipeptides in modulating fragmentation pathways as observed through tandem mass spectrometry using CID. The role of metal ions and its interacting sites on cyclodepsipeptides was investigated by NMR spectroscopy to validate the "sequence specific charge-driven fragmentation" analogy. To this end, we chose several compounds from two classes of cyclodepsipeptides: isariins and isaridins,[6,11,30] obtained from the fungus *Isaria.* Isariins and isaridins differ not only in sequence-composition but also in their backbone geometries.[6] While isarins possess one β-hydroxyacid residue (β-ester) and five α-amino acids (Gly, Val, ^{D}Leu, Ala, Ala/Val) with uniform '*trans*'-peptide geometry, isaridins contain one α-hydroxyacid residue (α-ester), one β-amino acid (βGly: NH_2–CH_2–CH_2–COOH) and four α-amino acid residues (Pro/β-(Me)Pro, Phe, N–(Me)Val, N–(Me)Val/N–(Me)Phe) with two backbone '*cis*' peptide bonds.[6,11] In this work fragmentation pathways for protonated and metal ions added isariins and isaridins were probed by Electro Spray Ionization Mass Spectrometry (ESI-MS) using CID and the location of Li^+ and Ag^+ in the metal cationized peptides was examined by 2D-NMR spectroscopy.



Scheme 1 Sequence and molecular masses of different *isariins* (A) and *isaridins* (B) [Sabareesh, V. *et al. J. Nat. Prod.* 2007] showing possible fragmentation pathways for generating product ions from Isariins (A. i)) and Isaridins (B). Formation of conjugated enone form from β-hydroxy acid group in isariins is shown in A.ii.

Results and discussion

Mass spectrometry study of isariins and isaridins

In order to probe the role of composition and peptide bond conformation of cyclodepsipeptides, along the fragmentation pathway, protonated and several metal cationized isariins and isaridins were subjected to collision induced dissociation. Based on literature survey,[20,26–29] metal ions (Li^+, Na^+, K^+ and Ag^+) having potential for generating better sequence information were chosen for formation of metal ion complexes of isariins and isaridins in MS^n studies. The peptides and the metal ions used for complex formation in this study are listed in Scheme 1, Table 1 and Table 2.

MS^n analysis of *Isariins* (β-hydroxy acid residue containing peptides)

Protonated species of isariin-I [cyclo(β-hydroxyacid-Gly-Val-^{D}Leu-Ala-Val)][6] (*m*/*z* 596.4), on CID fragmentation generated product ions at *m*/*z* 578.3 (due to loss of H_2O) and 568.3 (due to loss of CO) thereby confirming the cyclic nature of the peptide.[6,31] However, appearance of the product ion at *m*/*z* 497.2 due to concomitant loss of 99 Da from the precursor ion readily confirmed the presence of a valine residue, V, in the sequence. Subsequent isolation and fragmentation (MS^3) of the product ion at *m*/*z* 497.2 generated a peak at *m*/*z* 426.1 due to loss of 71 Da and confirmed the presence of alanine, A. This product ion (*m*/*z* 426.1) on next stage of fragmentation (MS^4) yielded a product ion at *m*/*z* 313.0, due to loss of 113 Da, leucine, L. In MS^5, a peak at *m*/*z* 214.1 was generated from *m*/*z* 313.0, due to loss of 99 Da, valine, V (Table 1A, Fig. 1A). On a closer look at the fragmentation pattern and the product ions, it could be concluded that during MS/MS process, a linear acylium ion or its equivalent ion was generated due to initial ring opening occurred specifically at the cyclic β-ester (lactone) bond between Val(6) and β-hydroxyacid (β-Hyd)(1) residues (Scheme 1A). Amino acid residues were then sequentially deleted from the C-terminus of the linear acylium ion or its equivalent (oxazolonium ion) form of the immediate precursor ions in this multistage fragmentation (MS^n) process. The MS^n process thus would furnish a partial information of amino acid sequence as V(3)–L(4)–A(5)–V(6) (Scheme 1A). This observation was at par with the MS/MS data (Fig. 1A)

Table 1 (A) Formation of product ions (m/z) from protonated and several metal ions added isariin-I (MW 595.3 Da) during MS^n experiments along with the partially assigned sequence information obtained from fragmentation study (B) Formation of product ions (m/z) from protonated and several metal ions added isaridin-II (MW 703.4 Da) during MS^n experiments along with the partially assigned sequence information from fragmentation study

A)

Metal ions	MS (m/z)	MS^2 (m/z)	MS^3 (m/z)	MS^4 (m/z)	MS^5 (m/z)	MS^6 (m/z)	Partial sequence
H^+	596.4	497.2	426.1	313.0	214.1		V-L-A-V (b-ions)
Na^+	618.2	519.2	448.2	335.1	236.1		V-L-A-V (b-ions)
K^+	634.4	535.2	464.1	351.2			L-A-V (b-ions)
Li^+	602.2	503.2	432.3	319.1	220.2		V-L-A-V (b-ions)
Ag^+	704.4 (702.4)	605.1	534.0	420.9	303.8 (322.1- H_2O)[a]	246.9 (265.1- H_2O)[a]	G-V-L-A-V (b-ions)

[a]Obtained from precursor ion 403.0 which was (420.9-H_2O) and 303.8 (322.1- H_2O).

B)

Metal ions	MS (m/z)	MS^2 (m/z)	MS^3 (m/z)	MS^4 (m/z)	MS^5 (m/z)	MS^6 (m/z)	Partial sequence
H^+	704.4	591.1	430.2	359.1	245.2		V-F-βG-αHyL(y-ions)[b]
	704.4	633.2	472.2	359.2	212.1		F-V-F-βG(b-ions)
Na^+	726.4	585.2 (613.2-H_2O)	424.2	353.1	239.1		V-F-βG-αHyL(y-ions)
	726.4	654.3	494.2	381.1	233.9		F-V-F-βG(b-ions)[b]
K^+	742.4	629.3	468.1	397.0			V-F-βG(y-ions)
	742.4	510.1	397.0				V-F-βG(b-ions)
Li^+	710.4	638.2	478.2	365.2	218.1		F-V-F-βG(b-ions)
Ag^+	810.0 (812.0)	697.0	536.1	447.1 (465.1- H_2O)			V-F-βG-αHyL(y-ions)

[b] Indicated higher intensity for product ions peak in case of mixed b- and y-ions).

Table 2 (A) Formation of product ions (m/z) during MS^n process from protonated and several metal ions added acyclic methyl ester derivative of isariin-I (MW 627.3) along with the partially assigned sequence information obtained from fragmentation study (B) Formation of product ions (m/z) during MS^n process from protonated and several metal ions added acyclic methyl ester derivative of isaridin-II (MW 735.4 Da) along with the partially assigned sequence information obtained from fragmentation study

A)

Metal ions	MS (m/z)	MS^2 (m/z)	MS^3 (m/z)	MS^4 (m/z)	MS^5 (m/z)	MS^6 (m/z)	Partial sequence
H^+	628.2	497.3	426.1	313.1			L-A-V (b-ions)
Na^+	650.4	519.2	448.1	335.1	236.0		V-L-A-V (b-ions)
Li^+	634.4	503.2	432.3	319.2	220.1		V-L-A-V (b-ions)
Ag^+	736.4 (734.4)	605.0	534.0	421.0	321.9		V-L-A-V (b-ions)

B)

Metal ions	MS (m/z)	MS^2 (m/z)	MS^3 (m/z)	MS^4 (m/z)	MS^5 (m/z)	MS^6 (m/z)	Partial sequence
H^+	736.4	622.3	524.9				αHyL-P (y-ions)
Na^+	758.4	644.2	547.1	400.0	286.9		αHyL-P-F-V (y-ions)
Li^+	742.2	628.1	531.1	384.0	271.2		αHyL-P-F-V (y-ions)
Ag^+	842.2 (844.4)	728.0	631.1	484.0	371.0	210.1	αHyL-P-F-V-F (y-ions)

where the product ions could be designated as 'b-ions'.[20] The assignment of 'b-ions' was further supported by the presence of 'a-ions'.

Similar results were obtained during the MS^n process of the other two isarins: isariin-II and isariin-III. Appearance of product ions at m/z 550.2 (due to the loss of H_2O) and 540.2 (due to the loss of CO) from the protonated species of both the isariins (m/z 568.5 and 568.5) confirmed their cyclic nature.[6,31] Through multi-stage fragmentation process, partial sequences from the protonated species of isariin-II and isariin-III were derived respectively as L(4)–A(5)–V(6) and V(3)–L(4)–A(5)–A(6) (Supplementary Material Table-S1a, Figure S1†). In both the cases, similar to isariin-I, the generated product ions belonged to only 'b-ion' series where initial ring opening occurred specifically at the β-ester bond. Assignment of 'b-ions' were further strengthened by the presence of 'a-ions'.

Identical trends of formation of 'b-ions' were also observed when several metal cationized isariin-I were subjected to multistage fragmentation using CID (Table 1A, Fig. 1B and C). However, the extent of sequence coverage varied depending upon the nature of metal ions participating in the complex formation. For example, lithium (Table 1A, Fig. 1B) and sodium cationized (Table 1A) isariin-I generated a stretch of amino acid sequence V(3)–L(4)–A(5)–V(6). Conversely, potassium cationized isariin-I yielded a limited sequence information: L(4)–A(5)–V(6) (Table 1A). Product ions formed from the silver cationized isariin-I readily yielded a sequence information of G(2)–V(3)–L(4)–A(5)–V(6) (Fig. 1C, Table 1A). All these data unambiguously

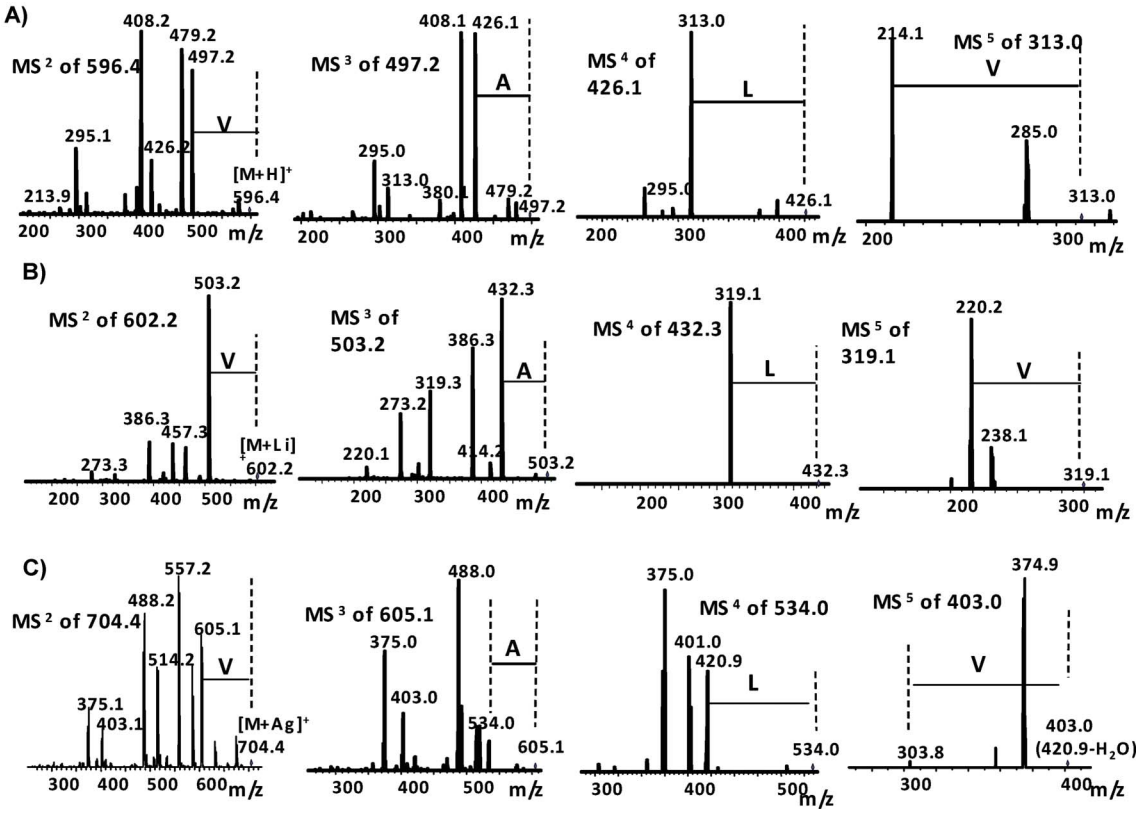


Fig. 1 Formation of exclusive 'b'-type product ions in MS^n experiments (ESI-MS using CID) of β-hydroxyacid residue containing peptide Isariin-I (MW 595.3 Da) as protonated and several metal ions adduct: A) MH^+ (*m/z* 596.4), B) MLi^+ (*m/z* 602.2) and C) MAg^+ (*m/z* 704.4).

pointed that, like protonated species during MS/MS fragmentation of the metal ion added cyclodepsipeptide isariin-I, initial backbone cleavage occurred specifically at the cyclic β-ester (lactone) bond between Val(6) and β-hydroxyacid (β-Hyd)(1) residues, yielding an acylium ion or its equivalent (oxazolonium ion), as shown in Scheme 1A. Fragmentation occurred in a sequence specific charge-driven manner and the amino acid residues were sequentially deleted from the C-terminus of the linear acylium ion or its equivalent form. The sequence specific product ions thus formed, belonged to 'b-ion' (and 'a') series as the fragmentation ensued from carbonyl to amine direction. Identical results were obtained from metal ion complexes of both isariin-II and isariin-III (Supplementary Material Table S1a, Figure S1†).

It was argued and established in many previous reports[19,20,23,26] that due to lack of well defined termini, cyclic peptides undergo nonspecific fragmentation (initial cleavage of the ring system) during the process of collision induced dissociation. However, our study on isariins using CID technique indicated a clear and specific trend that during MS/MS fragmentation, the initial ring opening took place explicitly at the cyclic β-ester bond among all other theoretically possible fragmentation pathways producing only 'b-ions' in a sequence specific charge-driven manner (Scheme 1A). This specific fragmentation pattern could be attributed to the generation of conjugated enone system at the β-hydroxy acid group of isariins (Scheme 1A) which acted as the driving force for determining the charge-remote fragmentation pathway in metal cationized peptides.

MS^n analysis of *Isaridins* (α-hydroxy acid residue containing peptides)

Similar to isariins, multistage CID-fragmentation of protonated species of isaridin-II [cyclo(α-hydroxyLeu-Pro-Phe-N–(Me)Val–N–(Me)Phe–βGly)][6,11] (*m/z* 704.4), generated product ions at *m/z* 686.1 (due to loss of H_2O) and 676.1 (due to loss of CO), confirming the cyclic nature of this peptide.[6,23,31] Moreover, appearance of the product ion at *m/z* 660.2 due to loss of 44 Da (one CO_2 molecule) indicated the presence of α-hydroxy acid residue.[6] Appearance of product ion at *m/z* 591.1, due to subsequent loss of 113 Da from the precursor ion (*m/z* 704.4) confirmed the presence of N-methyl valine (V) residue. Subsequent stages of fragmentation produced product ions at *m/z* 430.2 (MS^3), 359.1 (MS^4) and 245.2 (MS^5) and were respectively due to successive losses of constituting residues: N-methyl phenylalanine (F, residue mass 161Da), β-glycine (βG: -NH–CH_2–CH_2–CO-, residue mass 71Da) and α-hydroxyleucine (αHyL, residue mass 114 Da) from the immediate precursor ion (Table 1B, Fig. 2A). Considering the amino acid sequence of isaridin-II, it could be concluded that during fragmentation, initial ring opening took place at the amide bond between residues, phenylalanine [F(3)] and N-methyl valine [V(4)] (Scheme 1B) and fragmentation followed from amine to carbonyl direction as observed in MS/MS data (Fig. 2A). According to Williams and Brodbelt,[20] the product ions thus obtained could be designated as 'y-ions' and would suggest the partial sequence information as V(4)–F(5)–βG(6)–αHyL(1). However, it would be mentioned here that N-methyl

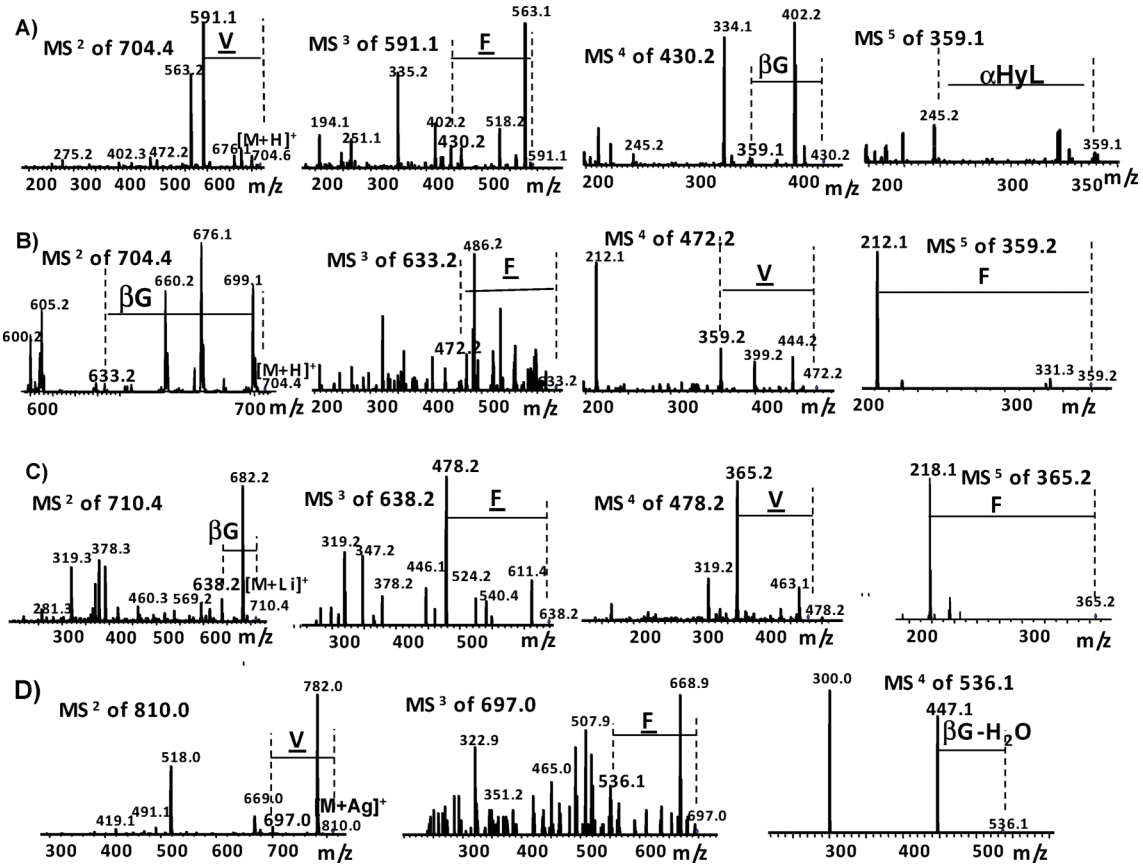


Fig. 2 Formation of product ions in MS^n experiments (ESI-MS using CID) of α-hydroxyacid and N-methylated residue containing peptide Isaridin-II (MW 703.4 Da) as protonated and several metal ions adduct: A) & B) MH^+ (*m/z* 704.4, showing 'y' and 'b'-type ions respectively), C) MLi^+ (*m/z* 710.4, showing exclusive 'b'-type ions) and D) MAg^+ (*m/z* 810.0, showing exclusive 'y'-type ions).

amide bond is tertiary type which is relatively more labile and basic in comparison to secondary amide bond. On the contrary, fragmentation of protonated isaridin-II simultaneously produced much less intense product ions at *m/z* 633.2, 472.2, 359.2 and 212.1; due to successive loss of βG, F, V and F respectively (Table 1B, Fig. 2B). This indicated that the initial ring opening occurred at the α-ester bond between residues, β-Glycine [βG(6)] and α-hydroxyleucine [αHyL(1)] generating a linear acylium ion or its equivalent (oxazolonium ion) (Scheme 1B). These ions could be designated as 'b-ions' as the fragmentation followed by sequential deletion of amino acid residues from C-terminus corroborating the sequence information as F(3)–V(4)–F(5)–βG(6), complementary to the earlier one.

Similar observations were obtained during the MS^n process of the other two protonated isaridins, isaridin-I (*m/z* 656.4) and isaridin-III (*m/z* 718.4). Majority of the intense product ions belonged to 'y-ions' (initial cleavage at the amide bond between phenylalanine [F(3)] and N-methyl valine [V(4)] residues, with subsequent losses of amino acid residues from N-terminus of the linear peptide ion; Scheme 1B) as observed for isaridin-II; while less intense 'b-ions' were also formed due to the fragmentation of the backbone at the ester bond (Scheme 1B, Supplementary Material Table S1b and Figure S2†).

An interesting trend was observed in multistage CID-fragmentation of metal cationized isaridins, where unlike isariins, fragmentation occurred in a metal ion dependent manner. In Li-cationized isaridin-II (*m/z* 710.4), the appearance of intense peaks at *m/z* 638.2, 478.2, 365.2, 218.1 were due to consequent loss of βG, F, V and F respectively (Table 1B, Fig. 2C), during subsequent stages of MS^n process. It clearly indicated that the initial ring opening took place specifically at the cyclic α-ester (lactone) bond (Scheme 1B), which happened due to specific interaction of Li-ion with the backbone cyclic α-ester (lactone) system.[27] This would facilitated the depsipeptide ring opening to form a linear acylium ion or its isomeric equivalent (Supplementary Material Scheme S1†) which on low energy collision had undergone fragmentation in a charge-driven sequence specific manner.[27,28] The subsequent product ions (Table 1B) could be designated as 'b-ions' as the amino acid residues were sequentially deleted from the C-terminus of the linear peptide ion during fragmentation. On the other hand, in the multistage fragmentation process Ag-cationized isaridin-II (*m/z*, 810.0) produced only 'y-ions' [*m/z*: 697.0, 536.1, 447.1 (465.1–H_2O) due to respective losses of V, F, βG from the N-terminus of the linear peptide ion] in a charge-directed manner[32] (Fig. 2D, Table 1B). This would corroborate that during MS^2 fragmentation process, initial cleavage in the cyclic system took place unequivocally at the amide bond between phenylalanine [F(3)] and N-methylvaline [V(4)] as shown in Scheme 1B and Supplementary Material Scheme S1. The sequence information derived from Li-cationized isaridin-II was βG(6)–F(5)–V(4)–F(3)

(from carbonyl to amino terminal) while that obtained from Ag-cationized isaridin-II was V(4)–F(5)–βG(6)–αHyL(1) (from amino to carbonyl terminal), rationalizing the cyclic sequence of isaridin (Scheme 1B, Table 1B).

Na^+ adducted isaridin-II (*m/z* 726.4), produced peaks of intense product ions at *m/z* 654.3 (from MS^2); 494.2 (from MS^3); 381.1(from MS^4) and 233.9 (from MS^5) (Table 1B), in a charge-driven manner like Li^+ with the initial cleavage mainly at the cyclic ester bond. This occurred due to specific interaction of Na^+ with cyclic α-ester (lactone) system as observed for other Na-cationized cyclodepsipeptides[28] with production of mostly 'b-ions'. Appearance of 'a-ions' strengthened the assignment. However, simultaneous appearance of peaks of product ions with much lower intensity at *m/z* 585.2 (613.2 – H_2O) (from MS^2); 424.2 (from MS^3); 353.1(from MS^4) and 239.1 (from MS^5) suggested that these product ions formed *via* fragmentation at amide bond between F(3) and V(4) as shown in Scheme 1B and belonged to 'y-ions' series (Table 1B). From K^+ added isaridin-II, both 'b-ions' and 'y-ions' were observed (Table 1B), but the extent of sequence information, which could be extracted, was less due to lower efficiency in MS/MS.

Similar trends in formation of 'b-ions' and/or 'y-ions', in a metal ion dependent manner, were also observed for the other two isaridin molecules, isaridin-I and isaridin-III (A detailed representation of the product ions is outlined in supplementary materials, Table S1b and Figure S2†). Based on these observations, a consensus could be generated that the fragmentation pattern of isaridins was metal ion dependent while that of the isariins was not. Such different behavior in fragmentation process in sequence specific manner along with discriminate ring opening of the metal ion complexes for isariins and isaridins might occur due to the difference in composition (ester chemical structure) and backbone geometry of these cyclodepsipeptides. The presence of '*cis* peptide bond' between N-methyl valine (4) and N-methylated amino acid residue [X(5)] in the cyclic structure of isaridins imposing constraints in the system played a crucial role in the ring cleavage at its immediate preceding peptide bond between phenylalanine(3) and N-methyl valine(4) residues. This idea could be supported by the work of Paizs and Suhai[33] which reports that in fragmentation process '*cis-trans*' sequential peptide bond arrangement at the N-terminus is kinetically favored over the '*trans-trans*' arrangement in the linear sequence. Moreover, peptide composition (β-hydroxy or α-hydroxy acid residue) of two classes of peptides along with the chemistry of the added metal ions might also act as determinant for such discrepancy in charge-driven and charge directed sequence specific fragmentation of the metal cationized cyclodepsipeptides.

NMR analysis of isariins and isaridins

The MS^n data presented above showed different fragmentation patterns depending upon the nature of peptides and metal ions. Complementary spectroscopic techniques could further be employed to gather more information for similar consequences. Since metal ions added peptides on MS^n process undergo sequence specific fragmentation; NMR spectroscopy, able to detect changes at residue level, was employed to investigate the location of metal ions in the peptide-metal ion complexes in solution state. Different ^1H, ^{13}C, ^{15}N 2D-NMR experiments (TOCSY, ROESY, ^1H-^{13}C HSQC and ^1H-^{15}N HSQC) were employed with standard protocol[34] for identifying the individual chemical shift values of residues in cyclodepsipeptides in the absence as well as in presence of different metal ions. We chose one peptide from each group of isariin and isaridin family: isariin-I (molecular mass 595.3 Da) and isaridin-II (molecular mass 703.4 Da) respectively for detailed NMR studies. We also studied the properties of Ag^+ and Li^+ ion adducts of these peptides through NMR analysis for identifying the location of metal ion in the peptide-metal ion complexes. It may be noted that for isaridins these two metal ion adducts showed entirely opposite fragmentation behavior in MS^n experiments. The difference in chemical shift values for the residues of metal ion added isariin-I and isaridin-II in comparison to metal free peptide [$\delta_{M\text{-}pep} - \delta_{H\text{-}pep}$, where M-pep indicated as metal adducted and H-pep indicated metal free peptide] shown in Fig. 3, is a good measure for identifying the interacting site(s) of metal ion(s) in the peptide-metal ion complexes. From different 2D NMR spectra, it was observed that while in presence of both the metal ions (Li^+ and Ag^+), the residues of isariin-I exhibited similar changes in chemical shift pattern (for Li^+ the changes were greater) (Fig. 3a-d), a complete different pattern was obtained for isaridin-II (Fig. 3e-h). For isariin-I, in presence of metal ions maximum changes in the chemical shift values were observed for residues βHyd(1) [$^{13}C\alpha$, CαH]; G(2) and V(6) [^{15}N, NH of main chain amino group along with $^{13}C\alpha$, CαH]. When compared to metal ion free system except for CαH of G(2) and V(6), all the other nuclei showed an appreciable down field shift in both the metal ion adducts (Fig. 3a–d; Supplementary Material Figure S3, S4†). As for other residues the changes were nominal or negligible, this clearly corroborated that both the metal ions were located closely to the ester group of the cyclic moiety and as a consequence, identical fragmentation pattern was observed in MS^n experiments. But for isaridin-II, a peptide containing two '*cis*' peptide bonds,[6,11] it was found that the interacting site of peptide-metal ion differed depending upon the nature of the interacting metal ion. For Li^+ added isaridin-II, it was observed that αHyL(1), P(2) and βG(6) residues suffered maximum shift (at βG(6) the changes were maximum) in comparison to other residues; while for Ag^+ added isaridin-II, the changes were most prominent around F(3) residue (Fig. 3e–h; Supplementary Material Figure S3, S5†). Interestingly no change in chemical shift values for αHyL residue was observed at all in the Ag^+ added isaridin-II when compared to that of metal-free peptide. This would establish that Li^+ was closely associated with the α-ester group of isaridin, while Ag^+ ion was located near the F(3) residue. The observed downfield shift for NH of βG(6) residue in presence of Ag^+ (in comparison to free peptide like NH of F(3)), suggested that Ag^+ might reside in the cavity of the isaridin molecule and was connected through these amide-N in an array, which was not feasible for other metal ions due to their smaller size (NN distance between F(3) and βG(6) is 4.88 Å as observed in the crystal structure[11]).

Further support could be obtained from ^{13}C–CO spectra which showed that for Li^+ adduct of isaridin-II, three CO groups were shifted downfield with respect to free peptide while for Ag^+-adduct only one showed such downfield shift (Supplementary Material Figure S5†). Moreover, the NMR spectral patterns for Ag^+-adduct and protonated isaridin-II were quite similar but that of Li^+ was different (Supplementary Material Figure S3†). On the contrary, these changes were quite similar for both the metal-ion adducts of isariin-I. Thus the NMR data helped to conclude

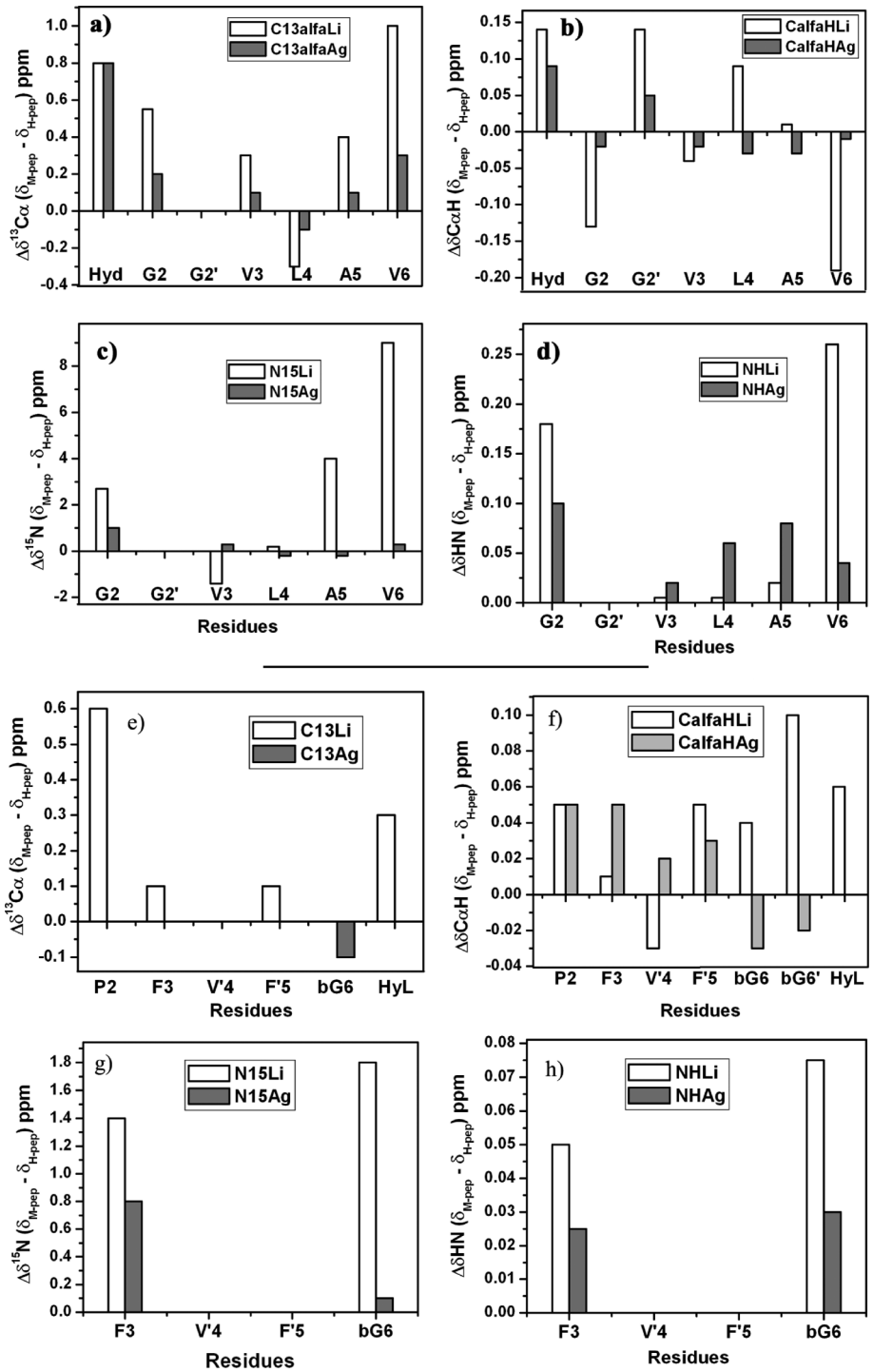


Fig. 3 Difference of NH, ^{15}N, $^{13}C\alpha$ and CαH chemical shift values [$\delta_{M\text{-}pep} - \delta_{H\text{-}pep}$: M-pep as metal ion added and H-pep as metal free peptide] upon addition of metal ions (Li^+ and Ag^+) to isariin-I (a-d) and isaridin-II (e-h) indicating the residue specific interaction of cyclodepsipeptides with metal ions.

that upon complex formation with isariins, both Li^+ and Ag^+ ions were located near the peptide ester group and induced identical fragmentation pattern in MS^n process, generating only 'b-ions' in a charge-driven manner (Scheme 1A). But for isaridins, Ag^+ located near the F(3) residue, helped in ring cleavage at the F(3)–V(4) peptide bond during MS/MS process with exclusive production of 'y-ions' upon fragmentation in a charge-directed manner, while Li^+, attached to the ester group produced exclusively 'b-ions' in charge remote fragmentation with an initial cleavage only at the cyclic ester bond (Scheme 1B).

Moreover, in ^7Li NMR ($LiClO_4$ in CD_3CN using LiCl in H_2O as external reference) change in chemical shift of the ^7Li resonance was observed upon addition of isaridin-II to free $LiClO_4$ in CD_3CN. Upon successive addition of peptide the continuous down field shift of ^7Li from –0.79 ppm to –0.54 ppm (the metal ion became deshielded) (Supplementary Material Figure S6†)

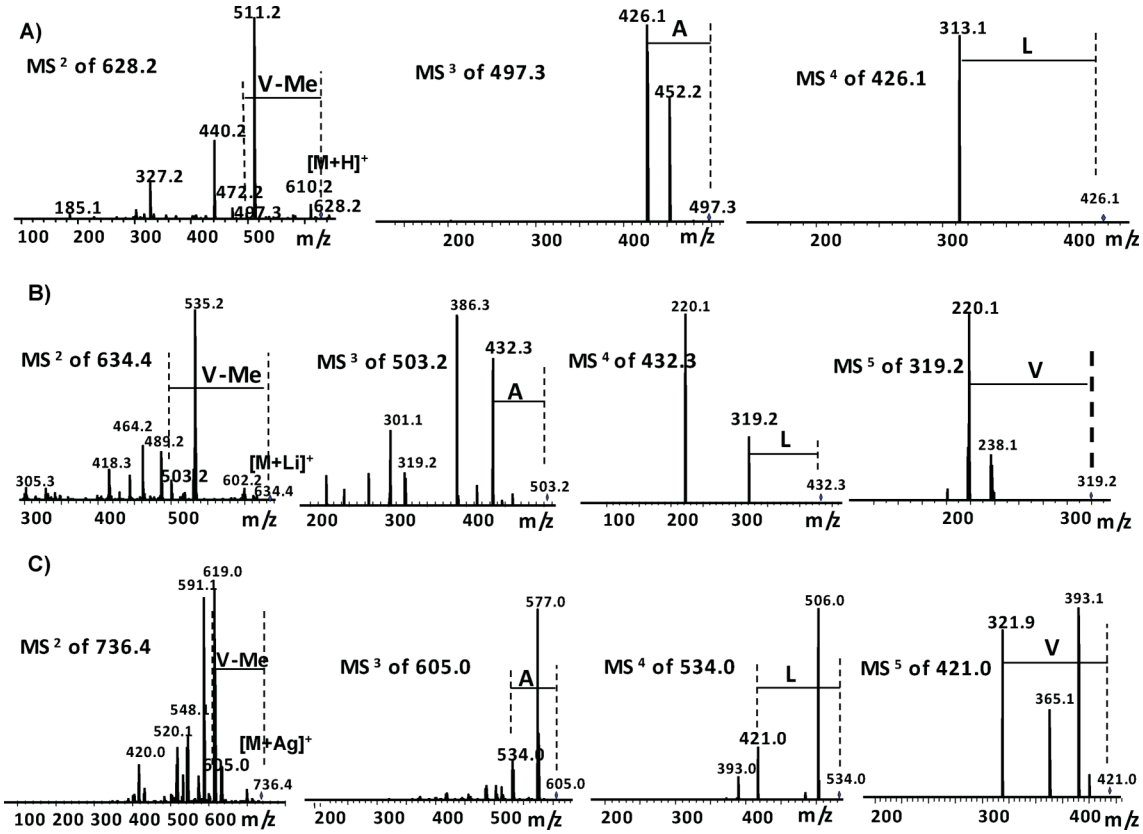


Fig. 4 Formation of exclusive 'b'-type product ions in MS^n experiments (ESI-MS using CID) of acyclic methyl ester derivatives of β-hydroxyacid residue containing peptide Isariin-I (MW 595.3 Da) as protonated and several metal ions adduct: A) MH^+ (*m/z* 628.2), B)MLi^+ (*m/z* 634.4) and C) MAg^+ (*m/z* 736.4).

strongly indicated that Li^+ had interacted with the less negatively charged/electronically dense cyclic peptide moiety through electrostatic interaction replacing ClO_4^- ion. Similar down field shift of ^7Li resonance was also observed upon addition of isariin-I to free $LiClO_4$, where chemical shift of ^7Li changed from –0.79 ppm to –0.4 ppm.

MS^n analysis of protonated and metal cationized acyclic methyl ester derivative of isariins and isaridins

For linear peptide it is found that a *cis-trans* sequential arrangement of peptide bond at the N-terminus play a specific role in fragmentation process in terms of energetics and site for protonation.[33] As isaridins contain combination of *cis* & *trans* peptide bond while isariin has uniform *trans* peptide geometry, to justify the role of peptide geometry (*cis*/*trans*), if any, in the fragmentation process of cyclodepsipeptides during MS^n experiments using CID technique, pure and lyophilized compound of isariin-I (molecular mass 595.3 Da) and isaridin-II (molecular mass 703.4 Da) were separately treated with absolute anhydrous methanol at 55 °C and monitored as a function of time (3 h, 4 h, 6 h, 12 h and 14 h). The aim was to open the depsipeptide ring through methanolysis for generating a linear peptide with C-terminal methyl ester without disturbing the peptide (amide) bonds (as reported for a few cyclodepsipeptides[35]) and study the fragmentation pathways of the respective protonated and metal ion added acyclic derivatives through tandem mass spectrometry as described for isariins and isaridins. The addition of 32 Da on precursor mass confirmed the formation of the acyclic methyl ester derivative of the treated compounds. Although the expected product was formed in all time course reactions, 12 and 14 h incubation time reaction produced the maximum yield.

The cationized acyclic methyl ester derivative of isariin-I demonstrated completely identical fragmentation pattern during multi stage fragmentation (Fig. 4, Table 2A), generating exclusively 'b-ions' in a metal ion independent manner as shown by its cyclic analogue. However, the acyclic methyl ester derivative of isaridin-II showed quite distinct and interesting result in MS^n process (Fig. 5, Table 2B). Contrary to different fragmentation pattern of isaridin-II in a metal ion dependent manner (generating either 'b' or/and 'y ions'), product ions, produced due to fragmentation of its acyclic methyl ester derivative (both protonated and metal ions added), belonged to exclusively 'y-ion' series with consequent loss of αHyL(1), Pro(2), Phe(3), N(Me)–Val(4) and N(Me)–Phe(5) respectively from the N-terminus end of the linear peptide ion (Table 2B).

Similar results were also obtained for protonated and metal ion added acyclic methyl ester derivatives obtained from isariin-II and isaridin-III.

Investigation of the role of composition and peptide bond conformation of isariins and isaridins on fragmentation pathway

Cyclic peptides during tandem mass spectrometry fragmentation either through CID or ECD generate complex spectra as the

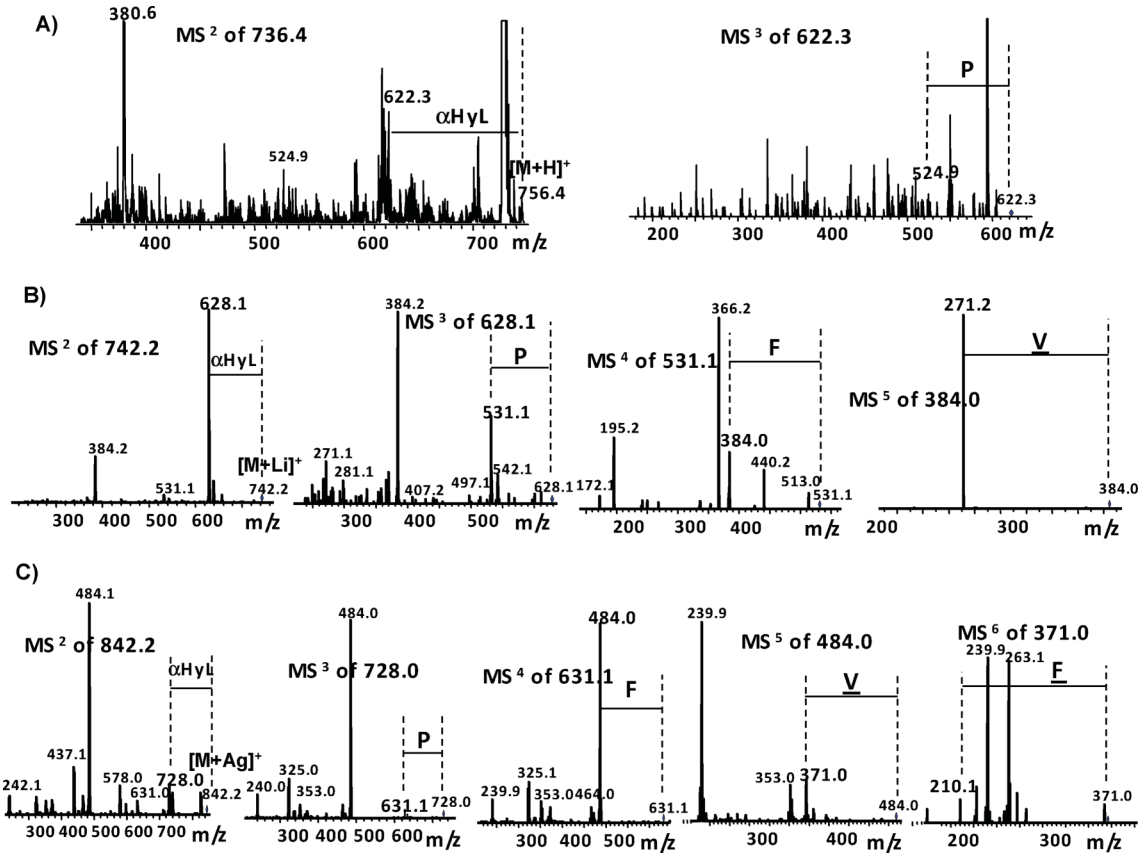


Fig. 5 Formation of exclusive 'y'-type product ions in MS^n experiments (ESI-MS using CID) of acyclic methyl ester derivative of α-hydroxyacid and N-methylated residue containing peptide Isaridin-II (MW 703.4 Da) as protonated and several metal ions adduct: A) MH^+ (*m/z* 736.4), B) MLi^+ (*m/z* 742.2) and C) MAg^+ (*m/z* 842.2).

initial ring openings are statistically distributed around the peptide.[19,20,23–25] Fragmentation of protonated or metal cationized cyclic peptides (cyclodepsipeptides) in low energy collision is initiated by mobilization of added proton or metal ion to reach the reactive conformation for dissociation. Protonation or metal ion adduct can be occurred either on oxygen of C–O of cyclic ester (lactone)[6] (subsequent opening of the cyclic ester bond to generate linear peptide) or at any amide bond (subsequent cleavage of the amide bond in b_x–y_z/a_1–y_x pathway).[33] In both the cases subsequent formation of the linear peptide ion affords an acylium ion or contains oxazolone ring at the C-terminus. This on subsequent stages of fragmentation can produce either b or y ions.[20,28,32,33] Although in cyclic peptides the most stable protonation site on amide bond is the amide oxygen, however, on mobilization of added proton, the amide nitrogen may become the protonated species. This is because protonation on amide nitrogen leads to considerable weakening of the amide bond facilitating ring opening and subsequent deletion of amino acid residues.[36]

Analysis of the mass spectral fragmentation pattern obtained through collision induced dissociation (CID) unequivocally demonstrated that isariins, whether protonated or metal-cationized, generate product ions belonging to the identical 'b-ion' series and exhibiting initial backbone cleavage explicitly at the cyclic ester bond. Similar results in fragmentation behavior were observed for its acyclic C-terminal methyl ester derivatives. Isaridins, on the other hand, illustrated a completely different fragmentation profile in a metal-ion dependent manner. Both 'b' and 'y-ions' were produced, which occurred due to initial backbone cleavages either at the cyclic ester linkage or at a specific amide linkage or at both the positions. Structurally, isariins and isaridins differ in composition (constituent residues) as well as in backbone conformation.[6,11] Isariins contain a β-hydroxy acid residue that can generate conjugated enone system, while isaridins contain an α-hydroxy acid residue and two N-methylated α-amino acids in the sequence. Moreover, all the peptide bonds in isariins are in the *'trans'* conformation, whereas isaridins contain two *'cis'* peptide bonds present between αHyL(1)–P(2) and V(4)–X(5) residues (where X is either F or V). All of the metal ions, including H^+ were observed to be attached at the conjugated enone system formed by the β-hydroxy acid group in isariins (in cyclic as well as in acyclic methyl ester derivative) (Scheme A (ii)) either at the carbonyl oxygen (for H^+, Li^+, Na^+) or through the overall conjugated enone moiety (donation of π-HOMO from conjugated system to metal ion followed by π-withdrawal from the filled d-orbital of metal to the vacant π*–LUMO of the conjugated system; for Ag^+)[37,38] rendering sequence specific charge remote fragmentation to produce identical 'b-ions'.

Conversely, in the case of isaridins, during fragmentation a clear dependence of metal ion on the nature of added cation was observed. On fragmentation, Ag^+ added isaridins produced only 'y-ions', whereas Li^+ added isaridins produced only 'b-ions'. On the other hand, H^+, Na^+ and K^+ added to isaridins

produced mixtures of 'b' and 'y-ions' on fragmentation (Table 1B, Supplementary Material Table S1b†). These results may be rationalized by postulating different binding/interacting sites on the peptide for different metal ions which is determined by the energetics of the system as well as nature of the participating metal ion.

Formation of 'b-ions' from Li^+, Na^+ or K^+ (alkali metals ions) complexes of isaridins can be explained considering their harder acidic oxyphilic nature and their preference to interact with the harder oxygen atom[27,28] of the α-ester group, which can not form conjugated enone like β-ester group present in isariins. Such interaction with the ester oxygen (C–O) generates a linear acylium ion or its equivalent which induces fragmentation in a charge-driven, sequence specific manner [Supplementary Material Scheme S1]. However, Ag^+, being soft in nature, prefers the amide group (amide nitrogen) as reported by several others.[39,40] The interaction between amide (CONH) bond and Ag^+ can be explained by the donation of filled π-HOMO of the CONH group to Ag^+, followed by π-withdrawal from the filled d-orbital of Ag^+ to the vacant π*-LUMO of the peptide (CONH) bond.[37,38] This interaction either an "a_1-y_x" pathway or an "aziridinone" pathway produces 'y-ions'[32,33] where the '*cis*-peptide' bond may regulate the ring opening process at a specific position. Ring opening occurred exclusively at the backbone CONH group between F(3) and V(4) (Scheme 1B) with the subsequent formation of 'y-ions'. This fragmentation may be due to the presence of an immediate succeeding '*cis*' peptide bond between V(4) and X(5) residues, imposing constraints to the peptide system. This observation may be in accordance to what is observed by Paizs and Suhai[33] that, in the fragmentation process of model linear peptides involving the formation of 'y-ions' a *cis-trans* sequential peptide bond arrangement at the N-terminus involving protonation at the nitrogen of the N-terminal amide is a favorable situation over the *trans-trans* arrangement. A similar observation for protonated isaridins can be explained on the basis of a higher 'proton affinity' of nitrogen in comparison to oxygen, where the tertiary amide group of either V(4) or X(5) offers the most suitable site for protonation. These amide groups are more labile than the CONH (secondary amides, peptide bond) group due to their higher relative basicity.[31,41] However, selective cleavage at the CONH group between F(3) and V(4) in MS/MS (Scheme 1B) (protonation at V(4) residue) may be preferred due to the presence of the immediate succeeding '*cis*' CONH bond between V(4) and X(5) which induces the subsequent formation of 'y-ions' as observed in case of Ag^+ adducts. The appearance of less intense 'y-ions' from the Na^+ and K^+-adducts may be due to their comparatively softer nature relative to Li^+ ($Li^+ > Na^+ > K^+$).[42]

Effect of composition and conformation of peptide bonds on the fragmentation pathway of isariins and isaridins can be further validated by the observation of the product ions obtained from the protonated and metal ion complexes of their acyclic C-terminal methyl ester derivatives. It was reported by several research groups that on fragmentation, protonated as well as metal cationized (Li^+, Na^+ and K^+) linear peptides with free N- and C-termini produced 'b' type ions, while such linear peptides with protected N- and C-termini or with an esterified/amidated C-terminus produced 'b-ions' as protonated species and 'y-ions' as metal ion complexes.[43–45] Contrary to these reports, our studies showed that both protonated and metal ions added acyclic methyl ester derivative of isariins (both N- and C- termini protected), produced only 'b-ions' (as observed in the cyclic system), while the acyclic methyl ester derivative of isaridins, either protonated or metal ions added, produced only 'y-ions'. This distinct phenomena observed in sequence specific, charge-driven fragmentation of isariin (only b-ions) using CID in ESI-MS^n can be attributed by considering the ability of the β-ester group of the isariin derivatives to interact with protons and metal ions through the formation of a conjugated enone system. However, for the acyclic derivative of isaridins the exclusive formation of y-ions, irrespective of the interacting metal ions, can be accredited considering the presence of a '*cis*' peptide bond between the αHyL(1) and P/P′(2) residues at the N-terminus as observed for the modeled G-G-G (*cis-trans*) peptide by Paizs and Suhai.[33] Moreover, it is reported in literature that in linear peptides, N-methylation induces the formation of b-ions from the C-terminal neighbor amide bond.[46] On the contrary, the acyclic methyl ester derivative of isaridins containing N-methylated residues produces only y-ions. Such an interesting contradiction can only be explained by the presence of a *cis*-peptide bond at the N-terminus.

Conclusion

The fragmentation behavior of protonated and metal ions added species of two classes of cyclodepsipeptides, isariins and isaridins have been investigated. It has been found that the composition (ester chemical structure) and peptide bond conformation (*cis*/*trans*) can modulate the fragmentation pattern in tandem mass spectrometry using CID. Isariins contain a β-hydroxy acid residue along with five α-amino acids as found in protein, while isaridins contain an α-hydroxy acid residue, a β-amino acid and N-methyl α-amino acid residues with two '*cis*' peptide bond. In MS^n analysis the presence of β-hydroxy acid residue in isariins leads to follow the identical fragmentation pathway in its cyclic as well as its acyclic methyl ester derivatives in metal ion independent manner. In contrast, for isaridins fragmentation occurs in a cation dependent manner and the presence of '*cis*' peptide bonds play a crucial role in the sequence-specific fragmentation. Moreover, the nature of metal ions (hard/soft) plays a decisive role in determining the metal-binding site on the peptide. This comparison of fragmentation patterns of metal ion adducts of two classes of cyclodepsipeptides helps in understanding the influence of sequence/structural context in fragmentation pathways and can provide valuable insights into peptide composition and peptide bond geometry in cyclic sequences.

Experimental

a) Isolation of different *isariins* and *isaridins*

Three isariins: Isariin-I (595.3 Da), Isariin-II (567.4 Da), Isariin-III (567.3 Da) and three isaridins: Isaridin-I (655.4 Da), Isaridin-II (703.4 Da), Isaridin-III (717.5 Da) were selected for this study. Sequence information of these molecules are shown in Scheme 1. Isolation of these peptides was described earlier.[11] Briefly, the fungus *Isaria* was grown in Potato-Carrot-Agar from which the fractions containing peptides were extracted using 100% chloroform. The crude extract after drying was re-dissolved

in acetonitrile and was centrifuged to obtain a light yellowish supernatant which was then injected into a semi-preparative C_{18} reversed-phase (RP) column [Merck LichroCART–Lichrospher 100] fitted to an LKB HPLC system. Separation was achieved using a linear gradient for 60 min at a flow rate of 1.5 ml min^{-1} using water and acetonitrile as mobile phase, where acetonitrile concentration varied from 60-90%.

b) Mass spectrometry

Mass spectra were recorded either on an Esquire 3000 plus or an HCT Ultra (Bruker Daltonics) ion trap ESI-mass spectrometer using collision induced dissociation (CID). All the spectral data were collected by direct infusion, using a syringe pump (Cole-Parmer, Vernon Hills, IL, USA) operated at a flow rate of 240 µl/h. Data were acquired in the positive ion mode over the range of *m/z* 50–1500, and were processed with *Esquire data analysis* software, version 3.1. Solution containing ~1–2 µM of individual peptide prepared in 1 : 1 H_2O/CH_3CN containing 0.1% formic acid was used for the studies of protonated species. The same concentration of peptide with ~10 fold molar excess of salt concentration was used to generate metal adducts. To generate Li^+-adducts of the peptides, either $LiClO_4$ or $LiNO_3$ was used, while Ag^+-adducts of the peptides were generated using $AgNO_3$ or $AgClO_4$ salts. KCl was used for the production of K^+-adduct of the peptides while Na^+-adduct were obtained from the protonated sample itself, during MS process.

c) NMR Spectroscopy

1D and 2D NMR spectra of ^1H, ^{13}C and ^{15}N were recorded on a Bruker AV 700 system attached with a cryo probe. Peptides, both in the free form and also in their metal complexed forms, were used at concentrations ~2 mM (for ^1H) and ~10 mM (for ^{13}C and ^{15}N) in CD_3CN at 300 K. Metal ions were added ~4 fold excess in comparison to free peptide. Li^+-complex of the peptides were generated using $LiClO_4$ while Ag^+-complex of the peptides were produced using $AgClO_4$ in CD_3CN. Occurrence of inter-molecular association was ruled out from the observed concentration independence of 1D ^1H NMR spectra (1 mM – 10 mM). Residue specific assignments were obtained from TOCSY experiments, while sequence specific assignments were done using ROESY experiments.[34] All 2D experiments were recorded in phase sensitive mode using STATES-TPPIalgorithm. The size of the acquired data set was 1024 × 450, which was zero filled before Fourier transformation to yield a data matrix of size 2048 × 1024. For ^1H, spectral width of 8700 Hz was used in both dimensions at 700 MHz. Mixing times of 100 and 200 ms were used for TOCSY and ROESY experiments respectively. Shifted square sine bell windows were used during processing. Spectra were referenced with TMS signal resonating at 0 ppm. Hetero nuclear correlation spectra of ^1H-^{13}C and ^1H-^{15}N (natural abundance), were recorded again on 700 MHz (cryo probe) spectrometer using standard HSQC, ECHO-ANTIECHO pulse sequence from Bruker.[7]Li spectra were recorded on AV400 MHz spectrometer with the help of Broad Band Inverse (BBI) probe using LiCl in H_2O as external reference. All data were processed off line using Bruker Topspin software and analyzed by using the program Sparky 3.114.[47]

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