Identification of α- and β-Hydroxy Acid Containing Cyclodepsipeptides in Natural Peptide Mixtures Using Negative Ion Mass Spectrometry

Suman S. Thakur, Rappal S. Ranganayaki, Kallol Gupta, and Padmanabhan Balaram

Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India

Natural peptide libraries often contain cyclodepsipeptides containing α- or β-hydroxy residues. Extracts of fungal hyphae of *Isaria* yield a microheterogenous cyclodepsipeptide mixture in which two classes of molecules can be identified by mass spectral fragmentation of negative ions. In the case of isaridins, which contain an α-hydroxy residue and a β-amino acid residue, a characteristic product ion corresponding to a neutral loss of 72 Da is obtained. In addition, neutral loss of water followed by a 72 Da loss is also observed. Two distinct modes of fragmentation rationalize the observed product ion distribution. The neutral loss of 72 Da has also been obtained for a roseotoxin component, which is also an α-hydroxy residue containing cyclodepsipeptide. In the case of isariins, which contain a β-hydroxy acid residue, ring opening and subsequent loss of the terminal residue as an unsaturated ketene fragment, rationalizes the observed product ion formation. Fragmentation of negative ions provide characteristic neutral losses, which are diagnostic of the presence of α-hydroxy or β-hydroxy residues. (J Am Soc Mass Spectrom 2009, 20, 2221–2228) © 2009 American Society for Mass Spectrometry

Natural product libraries provide an excellent starting point for the discovery of molecules which target specific biological receptors. Secondary metabolites produced by plants and microorganisms provide a rich source of leads in pharmaceutical research. The greater success rate in identifying compounds with interesting biological properties from natural product libraries, as compared to molecular libraries prepared by combinatorial synthesis, has been attributed to the fact that natural molecular skeletons may be considered to be "privileged" [1–3]. The characterization of natural product libraries, the structure determination and separation of individual components and the identification of potential molecular receptors can be a formidable task. Natural products may be classified into several structure classes with peptides forming a major group.

Cyclic peptides are abundantly produced by microorganisms and plants, predominantly by nonribosomal peptide synthesis [4, 5] although gene encoded synthesis followed by post-translational modification has also been established [2]. Among the cyclic peptides, cyclodepsipeptides which contain ester linkages in addition to amide bonds, are widely distributed in nature [6, 7]. Both α- and β-hydroxy acids have been found in cyclodepsipeptides. For example, the well characterized heterodetic peptides, enniatins, obtained from *Fusarium*, contain an α-hydroxy acid residue, while surfactins from *Bacillus subtilis* and enterobacterin from *E. coli* contain β-hydroxy acid residues [4, 5]. Fungi are potentially valuable sources of depsipeptide libraries [8]. The soil fungus *Isaria* has been shown to produce diverse metabolites [9–12].

During the course of investigations on isarial peptides, produced by the fungus *Isaria*, we obtained two groups of cyclohexadepsipeptides [13, 14]. The isariins contain a single β-hydroxy residue, while isaridins contain an α-hydroxy residue and a single β-amino acid residue. The peptides from *Isaria* occur as microheterogenous mixtures in fungal hyphae. In an earlier study, we have used LC-ESI-MS procedures to characterize as many as 14 components in a complex peptide mixture [14]. The isariins and isaridins are undoubtedly produced by distinct biosynthetic pathways. The presence of peptides with both α-hydroxy and β-hydroxy residues in the natural peptide library prompted us to examine methods for rapidly classifying the components of cyclodepsipeptide mixtures. We describe in this report characteristic fragmentation patterns for the two classes of peptides obtained under conditions of negative ion mass spectrometry. The fragmentation of positively charged peptide ions occur along the peptide backbone by well established pathways. As a consequence, positive ion mass spectrometry has been widely

Address reprint requests to Professor P. Balaram, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India. E-mail: pb@mbu.iisc.ernet.in

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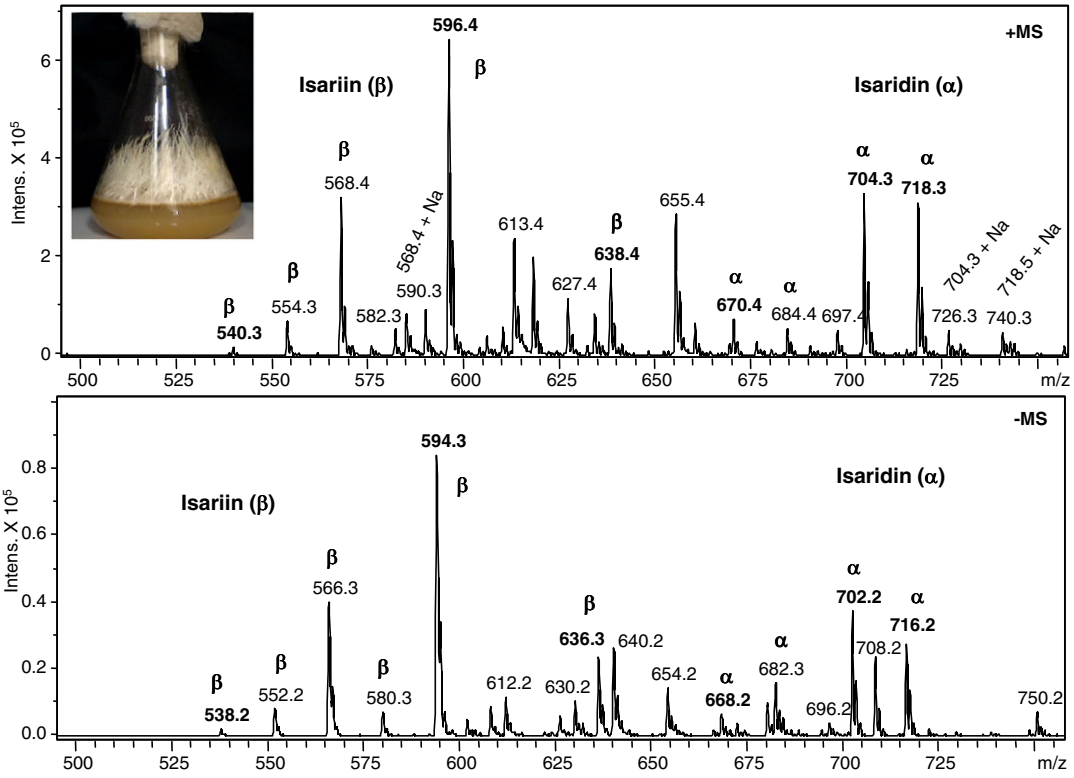


Figure 1. ESI-MS spectra (direct injection) of a methanol extract of fungal hyphae of *Isaria* grown on solid medium, (top) positive ion MS, (bottom) negative ion MS. Peaks corresponding to the isariins, which contains β-hydroxy acids and isaridins, which contain α-hydroxy acids, are marked. Inset: Fungal hyphae.

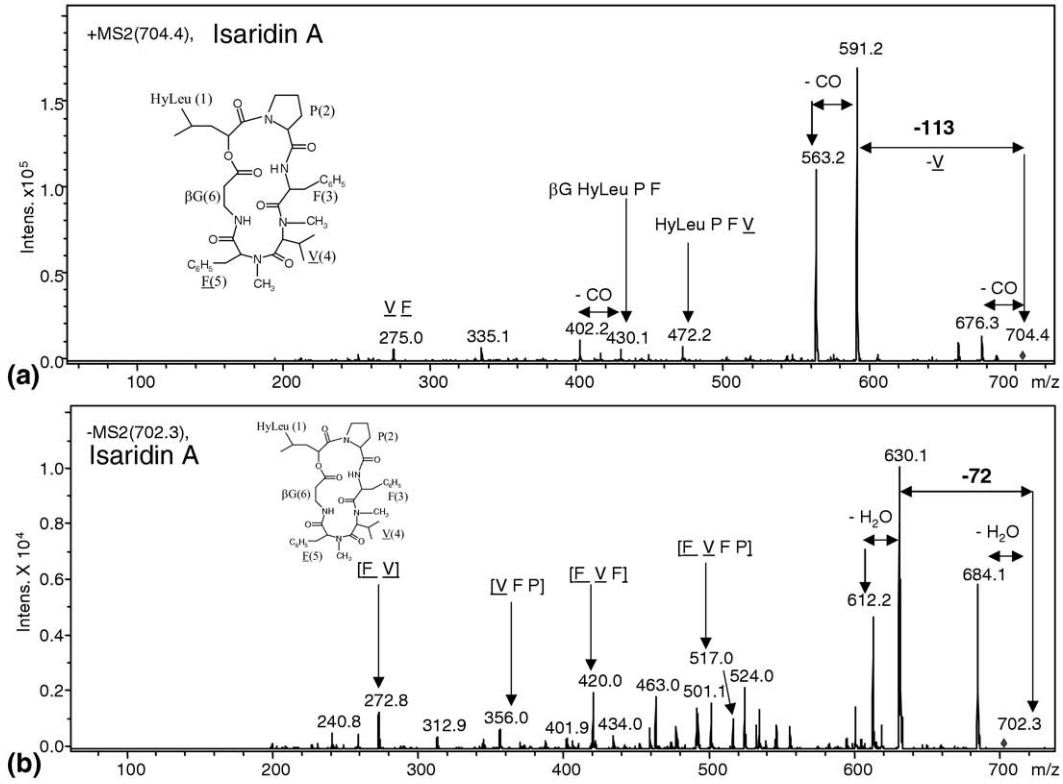


Figure 2. (**a**) Positive ion ESI-MS/MS of isaridin A ($M + H^+$, *m/z* 704.4). (**b**) Negative ion ESI-MS/MS of isaridin A ($M - H^-$, *m/z* 702.3). *V*: N-methylvaline, *F*: N-methylphenylalanine.

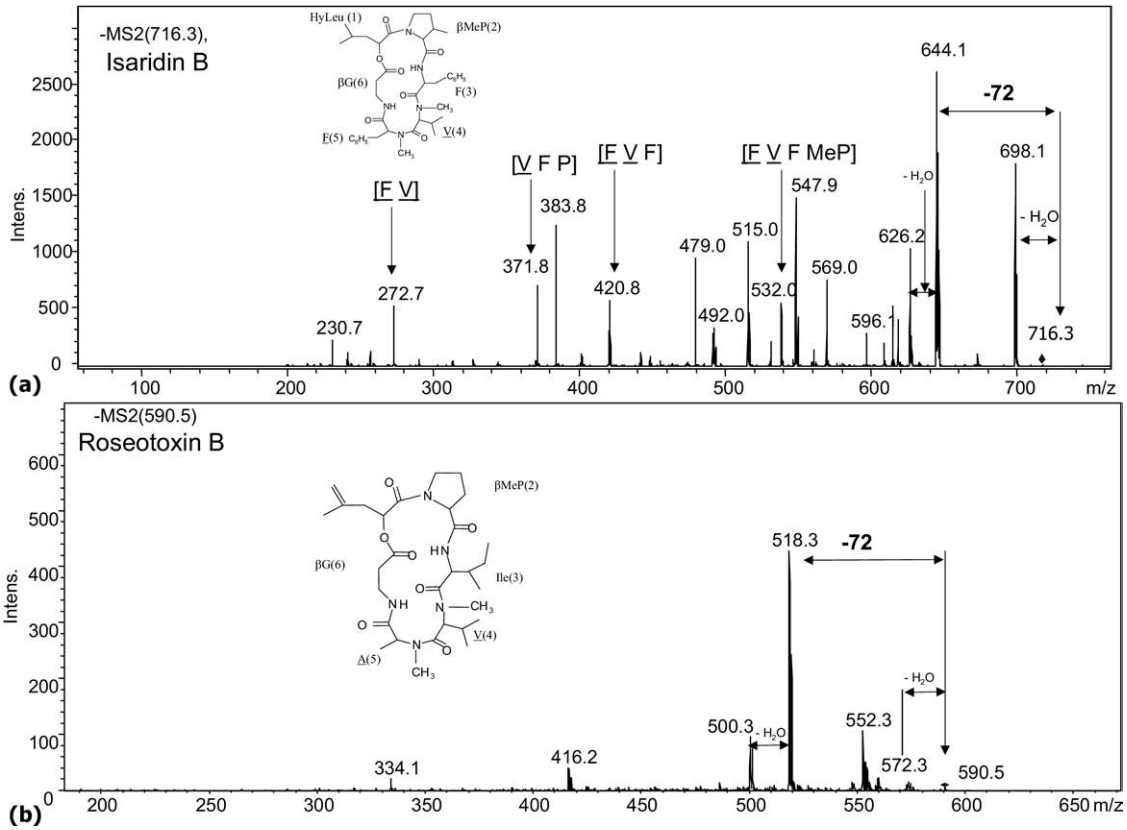


Figure 3. (**a**) Negative ion ESI-MS/MS of isaridin B ($M - H^-$, *m/z* 716.3). (**b**) Negative ion ESI-MS/MS of roseotoxin B ($M - H^-$, *m/z* 590.5). *V*: N-methylvaline, *F*: N-methylphenylalanine, *β*MeP: beta methylproline, *A*: N-methylalanine, *β*G: betaglycine.

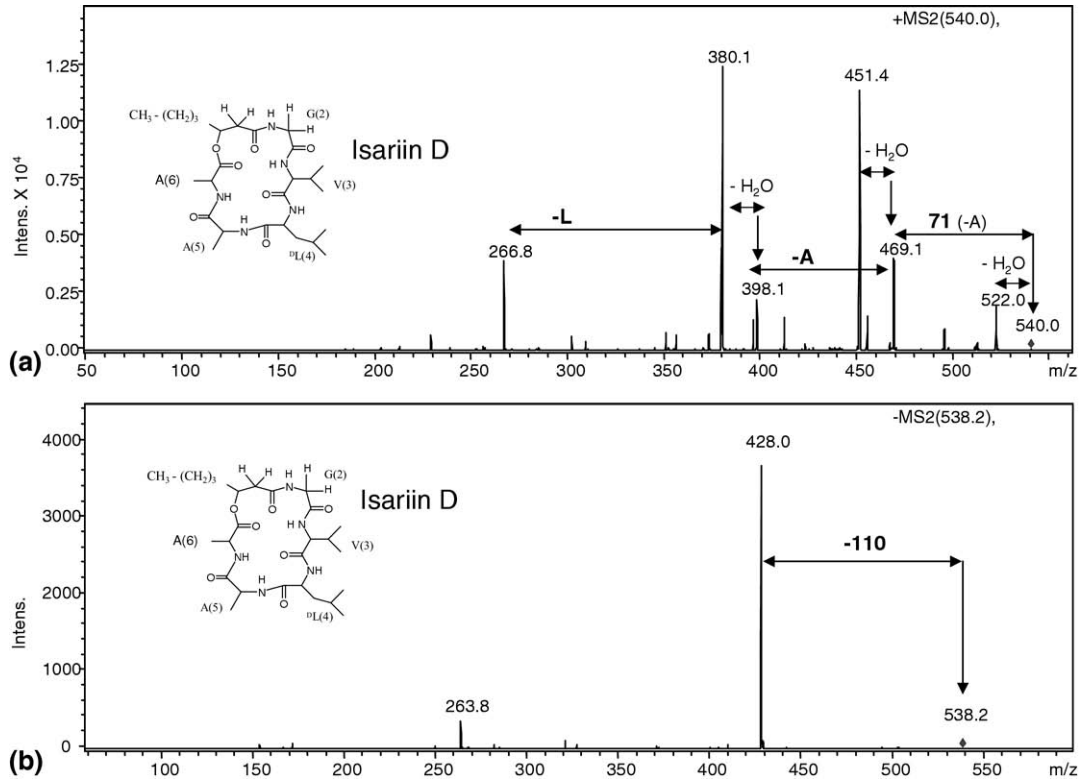


Figure 4. (**a**) Positive ion ESI-MS/MS of isariin D ($M - H^-$, *m/z* 540.0). (**b**) Negative ion ESI-MS/MS of isariin D ($M - H^-$, *m/z* 538.2).

used for peptide sequencing [15]. In contrast, negative ions, derived from peptides, fragment poorly along backbone bonds. The fragmentation of negative peptide ions in the gas-phase has been effectively used to study peptide disulfides and post translationally modified sequences [16]. In these cases, the abstraction of an acidic proton can lead to novel cleavages which provide valuable structural insights [17]. In the case of cyclic depsipeptides protonation of the ester bond has been shown to result in linearization, permitting mass spectrometric sequencing [14]. Under the conditions of negative ion mass spectrometry we anticipated that cleavage patterns arising from fragmentation at the hydroxyacid residue may yield insights into the chemical nature of the substituent.

Materials and Methods

Extraction and Purification of Depsipeptides

Conditions for growth of fungal cultures and extracts of hyphae have been described earlier [13, 14]. The crude hyphal extract was dissolved in MeOH, centrifuged to obtain a clear solution, and subsequently used for mass spectrometry and also for reverse-phase (RP) chromatographic purification. Semipreparative fractionation was achieved using a RP column (Zorbax C_{18}, 9.4 –250 mm, 5–10 um particle size) coupled to an LKB HPLC system. A MeOH/H_2O solvent system was used following a linear gradient of 60%–90% MeOH in 60 min while the flow rate was maintained at 1.5 mL/min.

Mass Spectrometry

Electrospray ionization (ESI) mass spectra were recorded in positive/negative ion mode using an Esquire 3000-plus mass spectrometer (Bruker Daltonics, Bremen, Germany) consisting of two octapoles followed by an ion trap. The direct injection of crude and purified samples was achieved using a syringe pump (Cole-Parmer, Vernon Hills, IL, USA) operated at a flow rate of 200 to 240 μL h^{-1}. The extract was dissolved in methanol using methanol/water (MiliQ, Millipore, France)/0.1% formic acid (CH_3OH/H_2O/0.1% HCOOH) in the positive ion mode. In the positive ion mode, the conditions are: capillary voltage 3 kV, nebulizing gas pressure 10 psi, drying gas flow rate 4 L/min, drying gas temperature 300°C, target mass *m/z* 500. Negative ion mode spectra were recorded using methanol/water/ammonium acetate (10 mM) as the solvent. In the negative ion mode, the conditions are: capillary voltage 4.2 kV, nebulizing gas pressure 10 psi, drying gas flow rate 4 L/min,

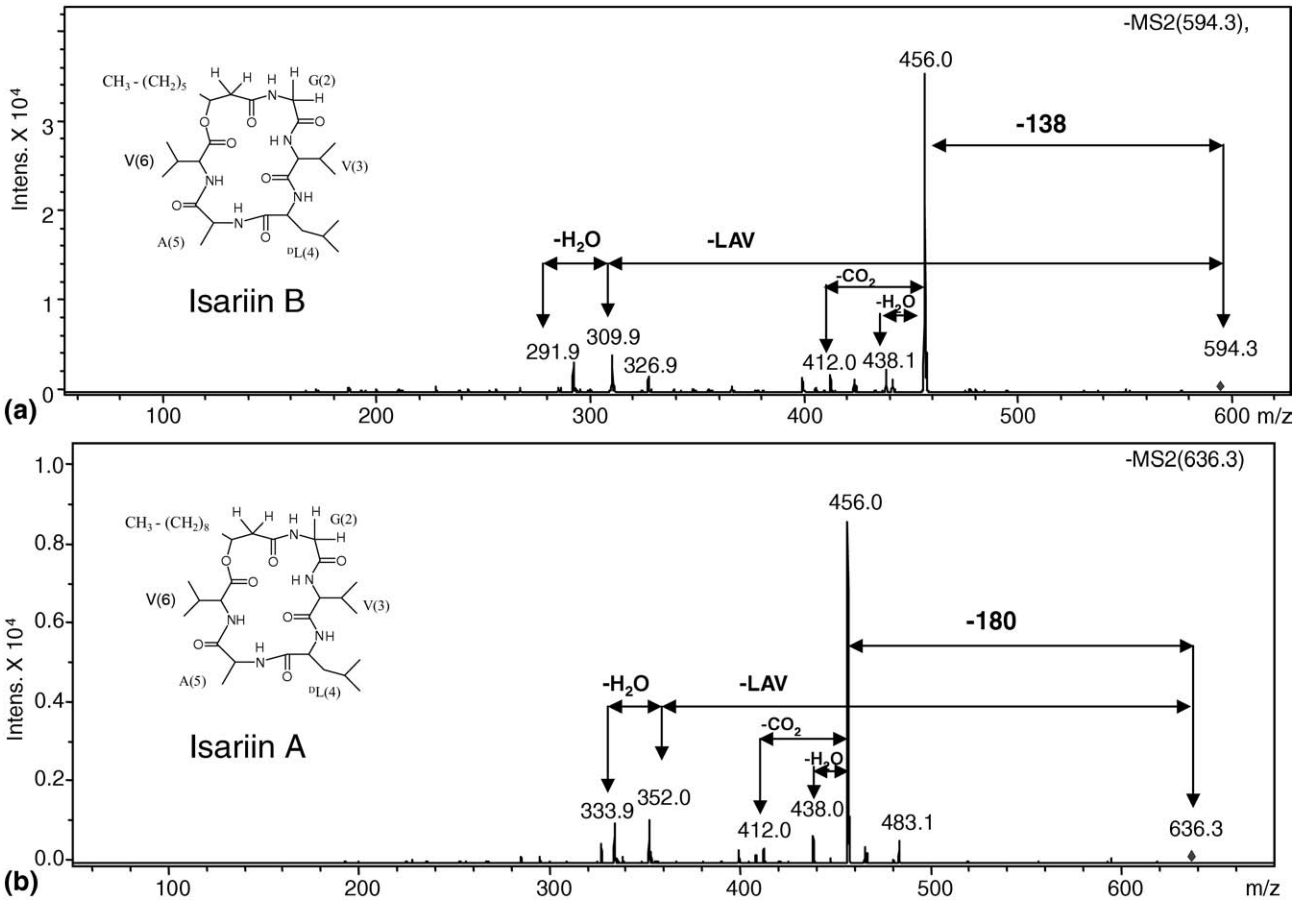


Figure 5. (**a**) Negative ion ESI-MS/MS of isariin B ($M - H^-$, *m/z* 594.3). (**b**) Negative ion ESI- MS/MS of isariin A ($M - H^-$, *m/z* 636.3).

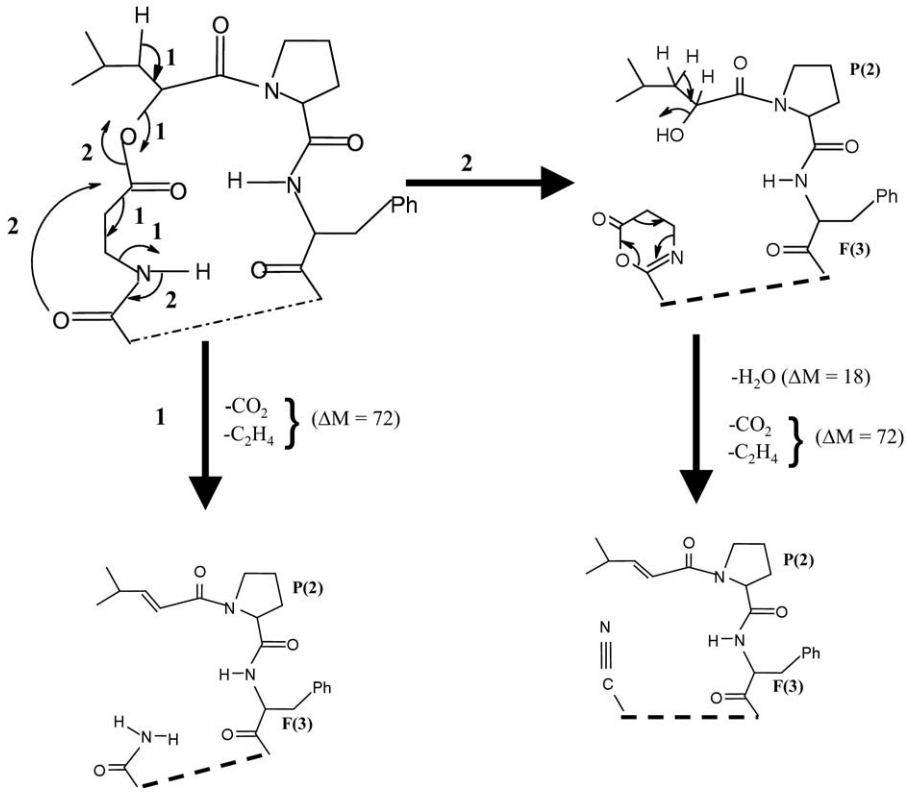
drying gas temperature 300°C, target mass *m/z* 500. Helium was used as the collision gas for collision induced dissociation (CID) experiments. The data were processed using Esquire data analysis software, version 3.1, Bruker Daltonics, Bremen, Germany. All mass spectral data shown are obtained from peptide mixtures. Identical spectra were obtained with individual components after preparative purification.

Results and Discussion

Figure 1 shown as ESI-MS spectrum of a methanol extract of hyphal powder. The inset to Figure 1 illustrates fungal hyphae obtained over a solid medium, which can be directly extracted with organic solvents to yield predominantly cyclodepsipeptide libraries. The assignments of the isaridins (α hydroxy) and isariins (β hydroxy) are based on previous mass spectrometric, NMR and X-ray crystallographic investigations [13, 14]. While there is a distinct difference in the molecular weight ranges of isariins and isaridins, it is evident that the higher mass components of the former and lower mass components of the latter may overlap.

Figure 2 compares the ESI-MS/MS spectra of isaridin A (M = 703) obtained by fragmentation of the positive ion M + H^+ = 704.4 Da and negative ion M − H^- = 702.4 Da. In the positive ion mode, protonation has been postulated to occur preferentially at the N-methyl residue with subsequent ring opening, resulting in loss of N-methylvaline (*V*) (ΔM = 113) [14. In contrast, fragmentation of the negative ion M − H^- (*m/z* 702.3) results in three prominent product ions at *m/z* 684.1 (−18 Da, H_2O), *m/z* 630.1 (−72 Da), and *m/z* 612.2 (−18 Da, −72 Da).

Figure 3a shows the product ions obtained upon fragmentation of the negative ions of isaridin B, (M − H^-, *m/z* 716.3). The results for isaridin C (M − H^-, *m/z* 668.3), isaridin D (M − H^-, *m/z* 682. 3, and isaridin E (M − H^-, *m/z* 654.3) are shown as supplementary data (see Supplementary Figure S1 and Table S1, which can be found in the electronic version of this article). Interestingly, in all the cases the neutral loss of H_2O (−18 Da) and 72 Da are observed. Apparently the intense product ion arising from neutral loss of 72 Da is common to all the isaridins (Table S1). To establish that these fragment ions are diagnostic of α-hydroxyacid containing cyclodepsipeptides we examined a mixture of roseotoxins [18], produced by *Trichothecium roseum*. Fragmentation of roseotoxin B (M − H^-: 590.5) in negative ion mode, shown in Figure 3b, results in an intense fragment ion at *m/z* 518.3, confirming that this feature can indeed serve as



Scheme 1. Fragmentation modes to rationalize neutral loss during mass spectral fragmentation of the negative ions of isaridins. Pathway 1 depicts direct loss of 72 Da (CO_2 and C_2H_4) by abstraction of the C$^\alpha$H proton of the α-hydroxy acid. Pathway 2 rationalizes the observed neutral loss of H_2O, in addition to CO_2 and C_2H_4. Abstraction of the amide proton of the β-Gly 6 residue with results in formation of a 1,3-oxazine and cleavage of the ester bond, followed by subsequent neutral losses.

a robust diagnostic for the identification of α-hydroxyacid containing cyclodepsipeptides.

Figure 4 compares fragmentation pattern obtained from the positive and negative ions of isariin D (M = 539), illustrating dramatic differences in the two cases. For positive ions, protonation at the ester linkage results in ring opening and linearization, with subsequent fragmentation [14]. In the case of negative ions, an intense product ion is observed at *m/z* 428 corresponding to a neutral loss of 110 Da.

Figure 5 shows the fragmentation of the negative ions obtained from isariin B ($M - H^-$, *m/z* 594.3) and isariin A, ($M - H^-$, *m/z* 636.3). In both cases, an intense fragment ion is observed at *m/z* 456 that corresponds to neutral loss of 138 Da in the case of isariin B and 180 Da in the case of isariin A. The observation of neutral loss of 110, 138, and 180 Da suggests elimination of a modified fragment derived from the β-hydroxy acid residue (Table S1). In isariins, microheterogeneity occurs because of variable lengths of the alkyl chain of the β-hydroxy acid residue. Negative ion mass spectrometric fragmentation was carried out for all the isariins present in a crude hyphal extracts and similar results were obtained (see Supplementary Figures S3 and S4). The magnitude of the neutral losses 110, 138, and 180 Da is consistent with the interpretation that the moiety that is lost contains the alkyl chain, with the differences of 28 and 70 Da arguing for the presence of two additional CH_2 groups and five additional CH_2 groups, respectively. The above results suggest that the fragmentation of the negative ions of isaridin (α-hydroxy) and isariin (β-hydroxy) result in unique, abundant product ions. While in α-hydroxy acid containing peptides, all components of the mixture yield a neutral loss of 72 Da, in the case of β-hydroxy acid peptides, the neutral loss may be rationalized by elimination of a fragment containing the alkyl chain of variable length.

Scheme **1** presents a mechanism that rationalizes the observed neutral loss in the case of the α-hydroxy acid containing peptides. The observed product ions arise by two distinct fragmentation pathways. In pathway 1, abstraction of the C^{β} proton of the α-hydroxyleucine residues, with subsequent elimination of CO_2 and ethylene (C_2H_4), results in a linear fragment which undergoes further backbone cleavages. Interestingly, the observed loss of water (−18 Da) from the parent negative ion suggests that a second pathway for the fragmentation must be operative. Pathway 2, rationalizes the loss of water. Abstraction of the amide NH proton of the β-Gly residue, with subsequent formation of a six membered azlactone, 5,6 dihydro-4H-1,3-oxazin–6-one [[19] and cyclodepsipeptide ring opening yields a terminal hydroxyleucine residue. Neutral losses of water (ΔM = 18) and CO_2 and C_2H_4 (ΔM = 72) are readily explained as shown in Scheme **1**. The formation of six oxazinones requires nucleophilic attack of the carbonyl oxygen atom of residue (5) on the carbonyl carbon of β-Gly 6. The molecular conformation of isaridin has been determined in crystals for two distinct members of this class of cyclodepsipeptides [13]. Significant differences have been observed in the conformation at the β-Gly residues in the case of isaridin A and isaridin B, as illustrated in Figure 6. In isaridin B, the distance between the two reacting atoms is ~4 Å. Significant flexibility is present in the macrocyclic ring to permit the formation of 6-oxazinones, with subsequent cleavage of the macrocycle.

Scheme **2** illustrates the mechanistic pathway that rationalizes the neutral loss of 110 Da from isariin D ([$M - H^-$], *m/z* 538.2). Abstraction of the $C^{\alpha}H$ proton from the β-hydroxy acid and ring opening results in linear precursor ions, which undergo subsequent cleavages as shown, resulting in the neutral loss of an unsaturated ketene fragment. A heterolytic version of a McLafferty type rearrangement, which involves a cyclic six-membered transition-state and proton abstraction from the α-methylene group of the β-hydroxy acid by the carbonyl group of residue 6, may also be invoked, as suggested by the referee, to obtain a neutral loss of 110 Da. The direct ring opening, shown in Scheme **2**, may be more readily permitted by the conformation of the peptide shown in Figure 6, which involves the forma-

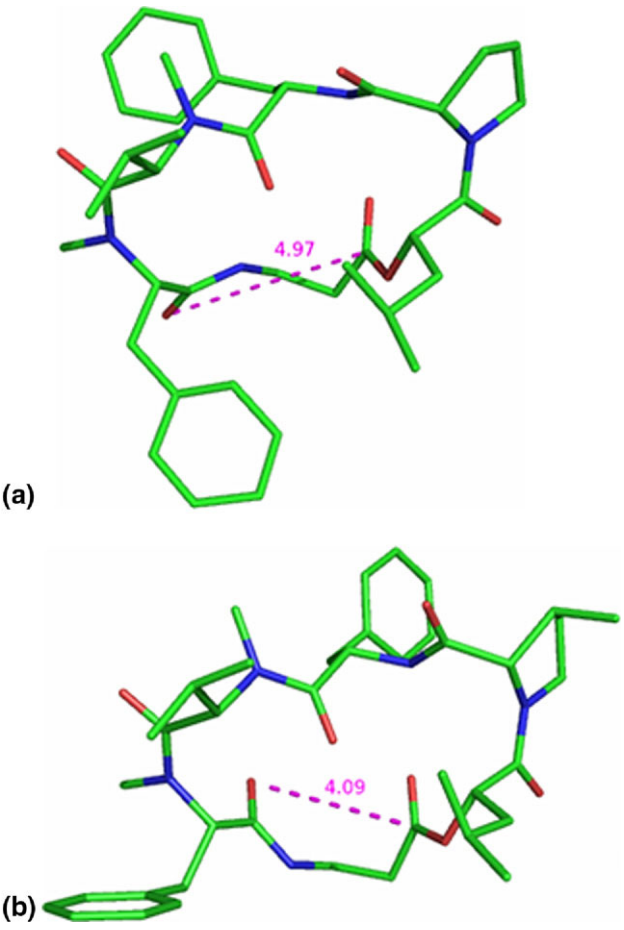
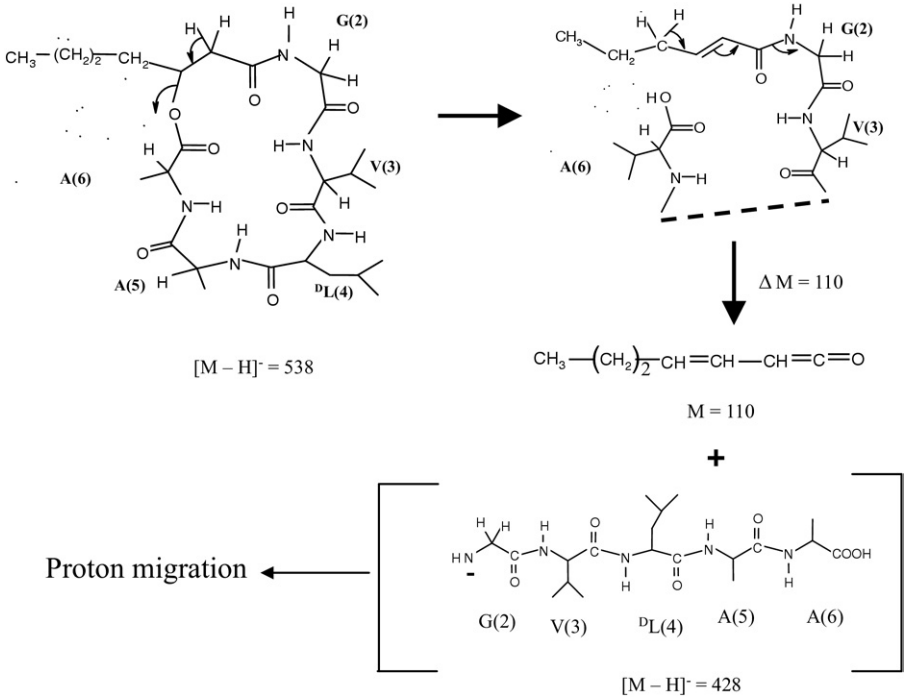


Figure 6. Crystal structure of (**a**) isaridin A, and (**b**) isaridin B. The distance (A°) between the carbonyl carbon atom of β-Gly 6 and the carbonyl oxygen atom of the N-methylphenylalanine (residue 5) is marked.



Scheme 2. Rationalization of the neutral loss of 110 Da in isariin D ($M - H^-$, *m/z* 540) containing an *n*-propyl chain in the β-hydroxy fatty acid. Neutral loss of 138 Da (R = $(CH_2)_5$ CH_3) and 180 Da (R = $(CH_2)_8$ CH_3) are observed in isariin B ($M - H^-$, *m/z* 596) and isariin A ($M - H^-$, *m/z* 638), respectively.

tion of a backbone expanded β turn mimicking structural feature involving the β-hydroxy acid residue and the Gly (2) residue at the (i + 1) and (i + 2) position [14]. This conformation places the α-methylene group of the β-hydroxy acid residue and carbonyl group of the ester bond in an orientation which appears unfavorable for the McLafferty type mechanism. However, the possibility of significant conformational changes in the gas-phase cannot be ruled out. The linear peptide fragment negative ions undergo further proton migration, followed by backbone cleavages (see Supplementary Figure S2 for the MS^3 spectra). In the other members of the isariin class, the mass of this fragment depends on the number of methylene groups of the β-hydroxy residues.

Conclusions

The results presented above establish that the α-hydroxy and β-hydroxy acid containing cyclodepsipeptides produced in cultures of the fungus *Isaria* can be readily distinguished by inspection of the neutral losses obtained upon the fragmentation of negative ions under mass spectrometric conditions. These observations facilitate rapid classification of the two classes of heterodetic cyclic peptides in complex mixtures of natural origin. The studies also emphasize the utility of negative ion mass spectrometry in providing structural information that may be complementary to that obtained under conventional positive ion conditions. The potential of negative ion mass spectrometry in studies of acyclic peptides [20, 21] and disulfide bonded peptides [16, 22–25] has been previously demonstrated. In evaluating the utility of peptide negative ion fragmentation compared with conventional studies with positive ions, it is useful to note that in sequences containing unusual residues and novel post-translational modifications, proton abstraction from specific sites can promote cleavages resulting in identifiable fragments and neutral losses, which may facilitate structure determination. The full potential of these methods in characterizing diverse natural peptide metabolites merits further investigation.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2009.08.010.

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