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Identification and Characterization of a Library of Microheterogeneous Cyclohexadepsipeptides from the Fungus *Isaria*

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Ten new cyclic hexadepsipeptides, six isariins and four isaridins, from the fungus *Isaria* have been identified and characterized by high-performance liquid chromatography, coupled to tandem electrospray ionization mass spectrometry (LC-ESIMS/MS). The isariins possess a β-hydroxy acid residue and five α-amino acids, while isaridins contain a β-amino acid, an α-hydroxy acid, and four α-amino acids. One- and two-dimensional NMR spectroscopy confirmed the chemical identity of some of the isariin fractions. Mass spectral fragmentation patterns of $[M + H]^+$ ions reveal clear diagnostic fragment ions for the isariins and isaridins. Previously described cyclic depsipeptides, isarfelins from *Isaria felina* (Guo, Y. X.; Liu, Q. H.; Ng, T. B.; Wang H. X. *Peptides* **2005**, 26, 2384), are now reassigned as members of the isaridin family. Examination of isaridin sequences revealed significant similarities with cyclic hexadepsipeptides such as destruxins and roseotoxins. The structure of an isariin (isariin A) investigated by NMR spectroscopy indicated the presence of a hybrid $\alpha\beta$ C_{11} turn, formed by the β-hydroxy acid and glycine residues and a ^{D}Leu-^{L}Ala type II′ β-turn. Additionally, the inhibitory effect of isariins and an isaridin on the intra-erythrocytic growth of *Plasmodium falciparum* is presented.

Renewed recent interest in the structural characterization of naturally occurring cyclic peptides and depsipeptides stems from the recognition that this class of natural products possesses diverse biological activities that may be useful in the development of new therapeutics.[1] Nonribosomal synthesis provides a mechanism for enhancing the diversity of such natural peptides, thereby permitting organisms to produce molecular libraries that may afford distinctive ecological or developmental advantages.[2] Fungi are an extremely important source of biologically active peptides and other metabolites.[3]

We have earlier described two new cyclic depsipeptides from the fungus *Isaria*, the isaridins A and B, which possess several modified amino acids.[4] *Isaria*, isolated from soil, were described as early as 1959 by Taber and Vining.[5] A preliminary report of a new depsipeptide called isariin appeared in 1962, which established the sequence of isariin (isariin A).[6] Subsequently, three more cyclodepsipeptides, isariins B, C, and D from *Isaria felina*, were described by Baute and co-workers.[7] Metabolites derived from *Isaria* cultures are of particular interest following reports of potent immunosuppressant activity of nonpeptidic compounds derived from culture filtrates of *Isaria sinclairii*.[8]

The development of liquid chromatographic procedures coupled to mass spectrometry (LC-MS) permits relatively rapid characterization of complex natural product mixtures.[4,9] We have revisited the metabolites produced by an *Isaria* strain grown on a solid medium, which yields aerial hyphae that provide a complex peptide mixture as a powdery solid. We describe the characterization of several new cyclohexadepsipeptides and classify the peptide metabolites into two major groups, the isariins and isaridins. The isariins possess a β-hydroxy fatty acid and five α-amino acid residues, whereas the isaridins have an α-hydroxy acid and a

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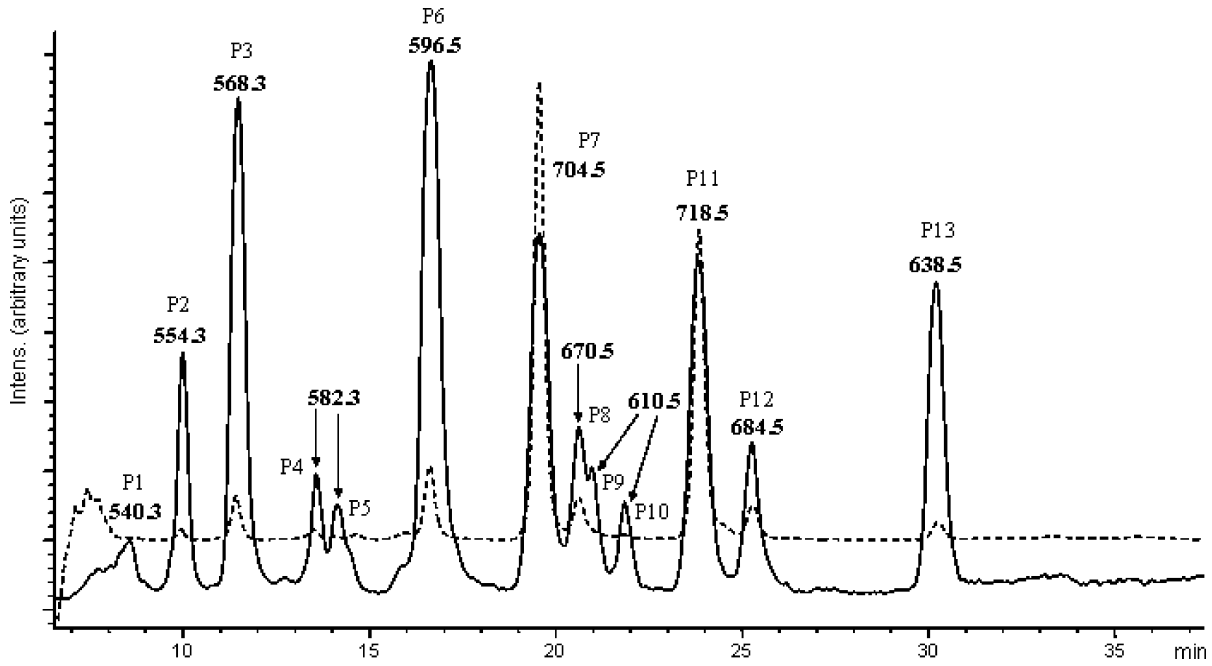


Figure 1. Overlay of total ion LC-ESIMS (black bold line) and LC-UV chromatograms (blue dotted line) of a MeOH/CH_3CN solution of crude hyphal extract.

β-amino acid, with a preponderance of N-alkylated residues. The conformational analysis of one member of the isariin class using two-dimensional NMR spectroscopy establishes an all-*trans* peptide backbone with a hybrid $\alpha\beta$ C_{11} reverse turn.[10] The peptides recently described from *I. felina* (isarfelins) are shown to belong to the isaridin family and not the isariin group, as reported in the literature.[11]

Results

Figure 1 shows the overlay of the UV-detected HPLC profile ($\lambda = 226$ nm) on the total ion chromatogram (mass spectrometric detection) obtained by passing a methanol/acetonitrile crude hyphal extract over a reversed-phase C_{18} column (see Experimental Section). The molecular ion mass ($[M + H]^+$) corresponding to each peak is indicated. The peaks P7 and P11 corresponding to *m*/*z* 704.5 and 718.5, respectively, are due to "isaridins", a class of cyclodepsipeptides, characterized earlier from our laboratory.[4] Isaridins are related to destruxins and roseotoxins, which possess unusual amino acids such as *N*-methylvaline (V), *N*-methylphenylalanine (F), hydroxyleucine (HyLeu), β-glycine (βG), and β-methylproline (β-MePro). The sequences of the two isaridins are almost identical, except that Pro in isaridin A (*m*/*z* 704.5) is replaced by β-MePro in isaridin B (*m*/*z* 718.5).

Isariins. In Figure 1, the peaks P1, P2, P3, P4, P5, P6, P9, P10, and P13, characterized by *m*/*z* 540.3, 554.3, 568.3, 582.3, 582.3, 596.5, 610.5, 610.5, and 638.5, respectively, indicate that all of these are very closely related molecules with the molecular mass ($[M + H]^+$) of each differing by an integral number of 14 Da. In order to confirm the cyclic depsipeptide nature of the isariins, the fraction P13 (*m*/*z* 638.5) was subjected to saponification in 2 N NaOH/CH_3OH. The resultant product yielded *m*/*z* 656.6 confirming the addition of water following the ring-opening. Figure 2 shows ESIMS/MS spectra of P1, P6, and P13, corresponding to precursor ions *m*/*z* 540.3, 596.5, and 638.5, respectively. The ESIMS/MS spectrum of P3, precursor ion *m*/*z* 568.3, is shown in Figure 4a. The peaks at *m*/*z* 522.2, 578.3, 620.4 (Figure 2) and 550.3 (Figure 4a) are due to the loss of an H_2O molecule from their respective precursor ions ($[M + H - H_2O]^+$).

Further, the peaks at *m*/*z* 512.2, 568.3, 610.3 (Figure 2) and 540.3 (Figure 4a), which are due to CO loss from their respective precursor ions, $[M + H - CO]^+$, confirm the cyclic nature of the peptide.[12] The observation of CO loss concomitant with the loss of H_2O from the protonated precursor ion confirms that P1, P3, P6, and P13 are indeed cyclodepsipeptides, more specifically, isariins. Cyclodepsipeptides, isolated from *Isaria*, characterized by the presence of a D-β-hydroxy acid ($^D\beta$-HA), ^{D}Leu, and absence of aromatic amino acid residues have been named as isariins.[6,7] Further analysis of the MS/MS spectra suggests that the peaks P1, P3, P6, and P13 are isariin D (*m*/*z* 540.3), isariin C (*m*/*z* 568.3), isariin B (*m*/*z* 596.5), and isariin A (*m*/*z* 638.5), respectively.[6,7b]

Scheme 1 illustrates two possible modes of ring-opening upon protonation of the cyclic structure **1**, giving rise to acyclic structures **2** and **3**. The observation of loss of H_2O suggests that the ester oxygen atom is the preferred site of protonation. The ester oxygen atom has been assigned as the preferred site of protonation, prior to opening of the cyclic structure, in earlier mass spectrometric studies of cyclic depsipeptides.[13] Examination of other product ions in Figure 2 (see also Figure 4a) suggests that subsequent to ring-opening, in the acyclic forms **3** and **4**, sequential loss of residues occurs until residue 4 (^{D}Leu) from the C-terminus end (as indicated in Scheme 1), while there is no evidence of fragmentation occurring from the $^D\beta$-HA residue (residue 1) end. In other words, a- and b-type fragment ions are observed from the structures **3** and **4**. Further, the peaks at *m*/*z* 373.1 (Figure 2a) and *m*/*z* 401.2 (Figure 2b, c) are assigned to internal fragment ions $[V^DLAA\text{-}OH + H]^+$ and $[V^DLAV\text{-}OH + H]^+$, respectively, arising from the acyclic form **2** (indicated in Scheme 1). A notable feature of the isariins is that their sequences are almost identical except for variations at residue 6, which is linked to the hydroxyl oxygen of the β-hydroxy acid residue 1 (Scheme 1).[7b] The variation at residue 6 is limited to Ala and Val, accompanied by the variability in the number of methylene units $[-(CH_2)_n]$ in the side chain of residue 1 (Scheme 1).[7b]

Having confirmed the presence of the four previously reported isariins A−D in the hyphal extract, we now turn to newly detected components. Analysis of the fragmentation patterns observed in the ESIMS/MS spectra of depsipeptides of known sequence readily permitted mass spectrometric identification of additional components.

Figure 3 shows the ESIMS/MS spectra of P2, P5, and P10 corresponding to precursor ions *m*/*z* 554.3, 582.3, and 610.5, respectively. Observation of several common features between the fragmentation spectra in Figure 3 and in Figure 2 indicates that

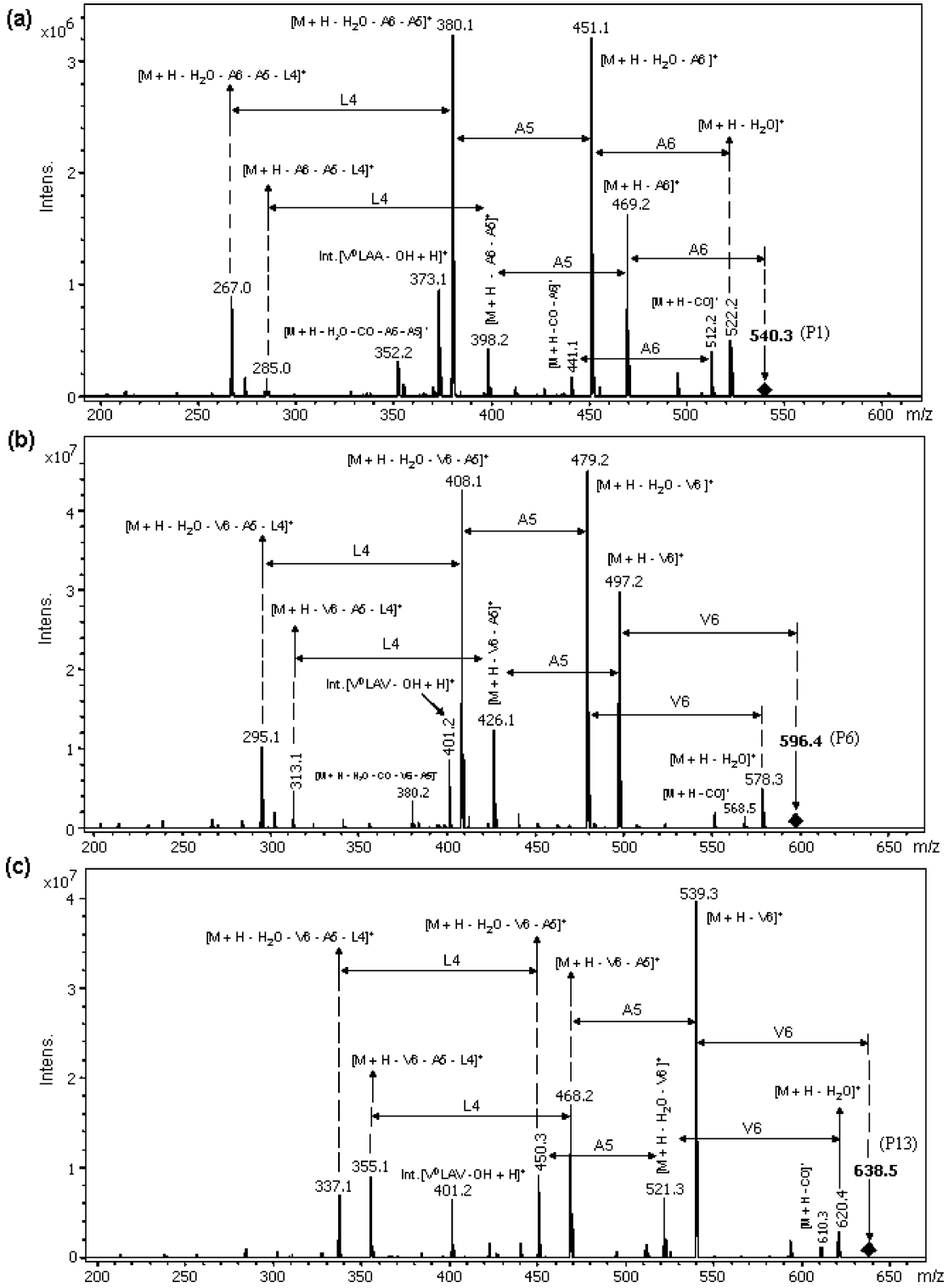


Figure 2. ESIMS/MS spectra of previously reported isariins:[6,7b] (a) isariin D (P1: precursor ion *m*/*z* 540.3), (b) isariin B (P6: precursor ion *m*/*z* 596.4), (c) isariin A (P13: precursor ion *m*/*z* 638.5).

these components also belong to the isariin class of cyclodepsipeptides. From examination of MS/MS spectra of $[M + H]^+$ adducts shown in Figure 3 and following the fragmentation mechanism as described in Scheme 1, the sequences of the molecular ions, *m*/*z* 554.3, 582.4, and 610.4 are derived. The sequences of isariins identified thus far from the fungus *Isaria* are listed in Table 1. The fractions P2, P5, and P10 are hereafter designated as isariins E, F2, and G2, respectively. Subtle compositional variations corresponding to changes at residue 6, accompanied by a change in the number of methylene units (*n*) of the side chain of residue 1, can be observed.

Figure 4a shows the ESIMS/MS of P3 with a precursor ion at *m*/*z* 568.3. The peak P3 corresponds to isariin C, whose sequence was reported to be cyclo-($^p\beta$-HA (n = 5) G V ^{p}L A A).[7b] Comparison of the ESIMS/MS spectrum of P3 with the spectra of other isariins strongly suggests that this fraction contains two components having an identical mass. This is readily deduced from the appearance of two peaks at *m*/*z* 497.3 and 469.2 in Figure 4a, suggesting the two components differ only at residue 6 (Ala/Val). The mass difference arising from the replacement of Ala of isariin C by Val is compensated for by the change in *n* of residue 1; n = 5 in isariin C, while n = 3 in the newly identified peptide, isariin

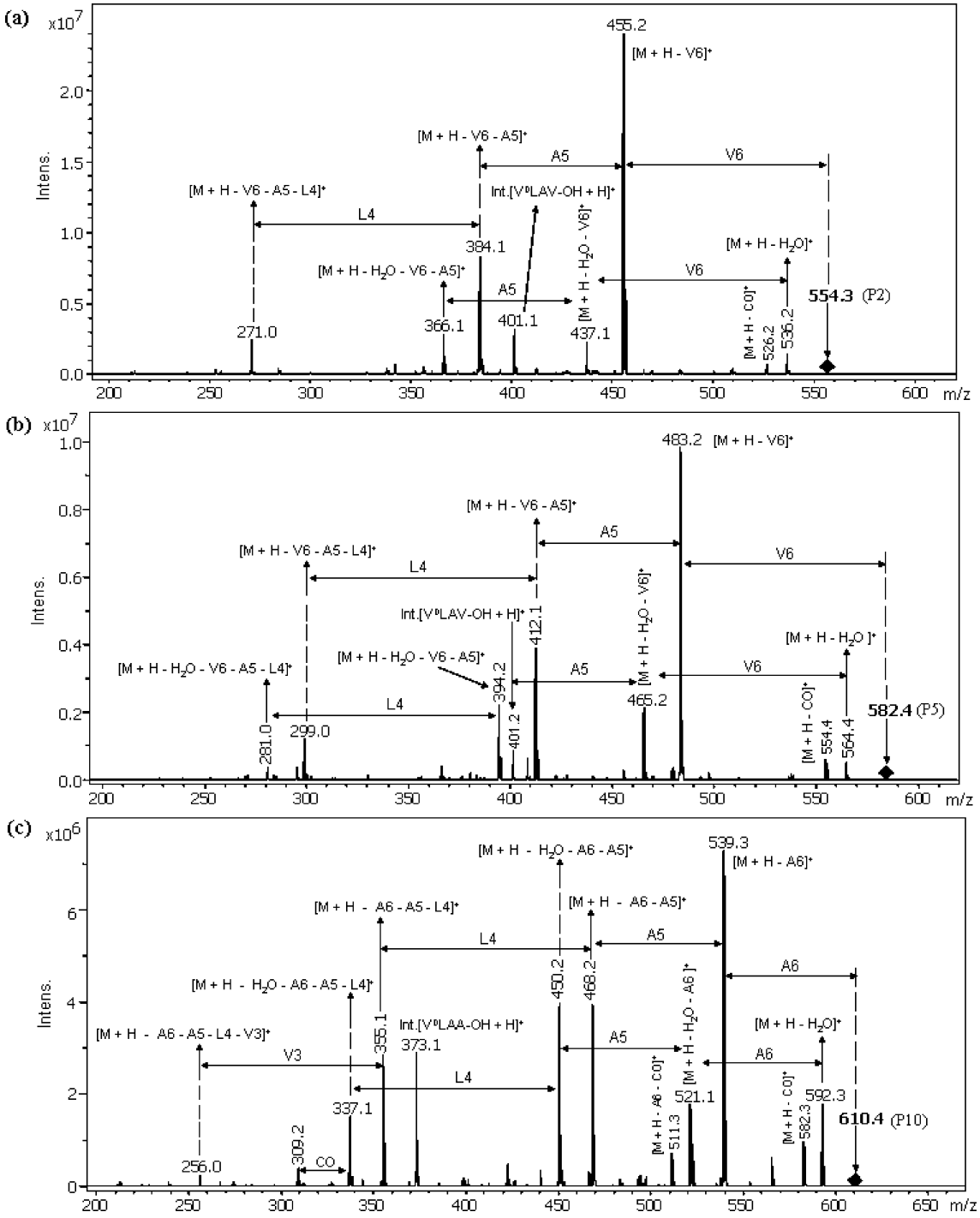


Figure 3. ESIMS/MS spectra of isariins identified in this study: (a) isariin E (P2: precursor ion *m/z* 554.3), (b) isariin F2 (P5: precursor ion *m/z* 582.4), (c) isariin G2 (P10: precursor ion *m/z* 610.4).

C2. Specifically, the product ion peak at *m/z* 401.2, which is assigned to an internal fragment ion, $[V^{D}LAV\text{-}OH + H]^+$, arising from the structure **2** (indicated in Scheme 1), supports the presence of Val as residue 6 in isariin C2. In addition, the peak at *m/z* 373.1, which is assigned to an internal fragment ion, $[V^{D}LAA\text{-}OH + H]^+$ from the structure **2** (Scheme 1), indicates the presence of isariin C. The product ions arising from the fragmentation of isariin C are marked M_1, while the fragment ions obtained from the new peptide, isariin C2, are marked as M_2 (Figure 4a). The presence of two components is further confirmed by NMR spectroscopy. Figure 4b shows the expanded region of the 500 MHz ^{1}H NMR spectrum of the fraction P3 recorded in $CDCl_3$ containing 4% (v/v) DMSO-d_6. The partial TOCSY spectrum is also shown (left panel of Figure 4b). It is clearly seen that the number of NH resonances exceeds the number anticipated for a single cyclohexadepsipeptide. Assignment of resonances using sequential scalar connectivities, determined using TOCSY, confirms the presence of two coeluting peptides. The partial TOCSY spectrum illustrates the presence of two distinct components, one of which contains only one Ala residue instead of the two that are anticipated in isariin C. Further analysis of TOCSY connectivities in the second component revealed the presence of two Val residues. Thus, sequence analysis of these coeluting peptides, which are very closely related, is facilitated by use of MS/MS and NMR methods.

In all cases, the nature of the D-β-hydroxy fatty acid ($^{D}\beta$-HA) was inferred from the mass of the residue. Further confirmation of the $^{D}\beta$-HA structure is obtained from ^{1}H−^{13}C HSQC spectra, which

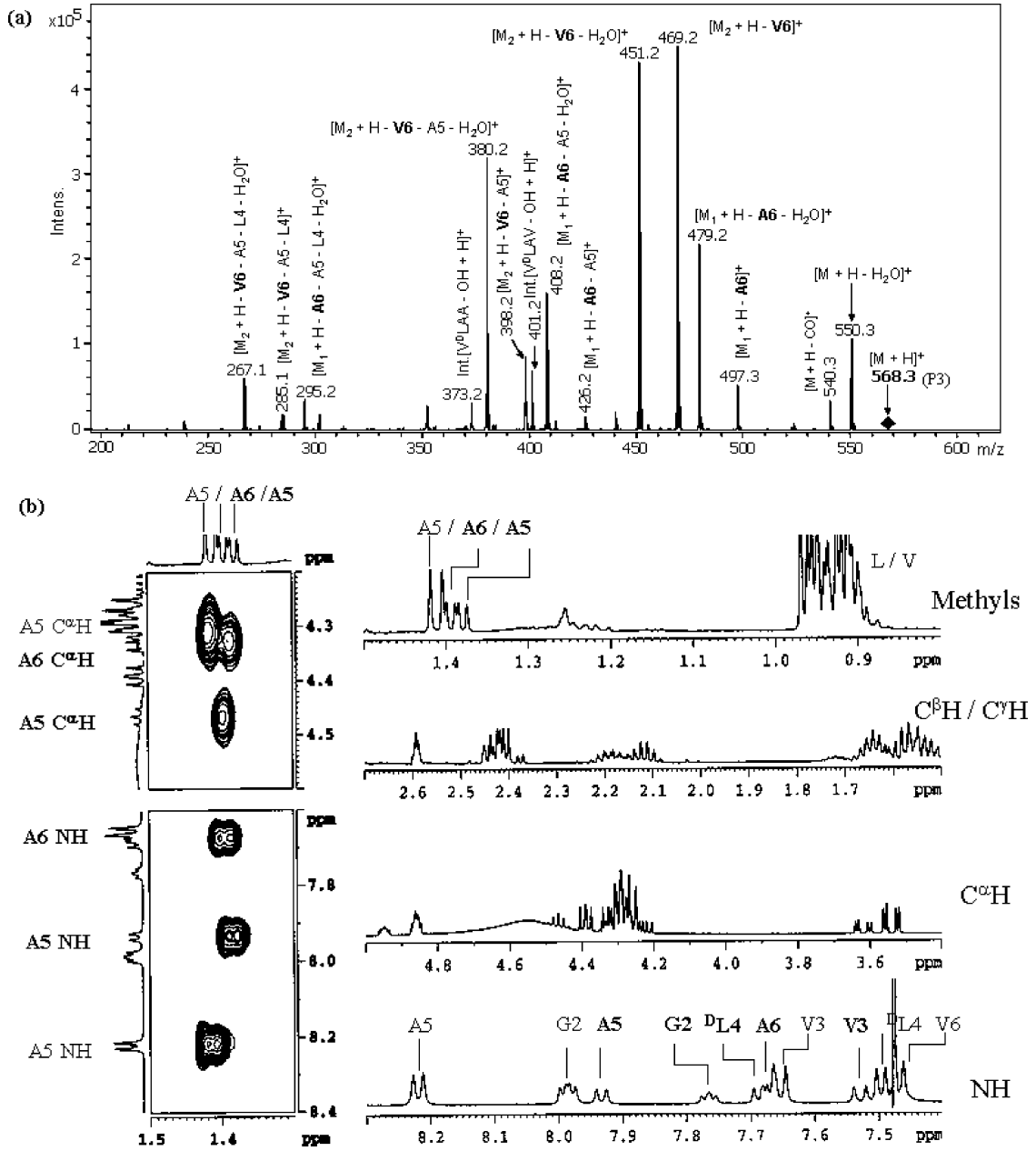


Figure 4. (a) ESIMS/MS spectrum of $[M + H]^+$ of isariin fraction P3 (isariin C/C2, precursor ion *m*/*z* 568.3). The product ion peaks from isariins C and C2 are assigned as M_1 and M_2, respectively. (b) Expanded region of the 500 MHz ^{1}H NMR spectrum of isariin fraction P3 recorded in $CDCl_3$ containing 4% (v/v) DMSO-d_6. Left panel: Partial 500 MHz TOCSY spectrum, showing correlations between side chain protons and C$^\alpha$H (top); correlations between the side chain protons and NH (bottom).

permit identification of the number of methylene units (Figures 5 and 6). Figure 5 shows partial 700 MHz ^{1}H−^{13}C HSQC spectra of isariin A.

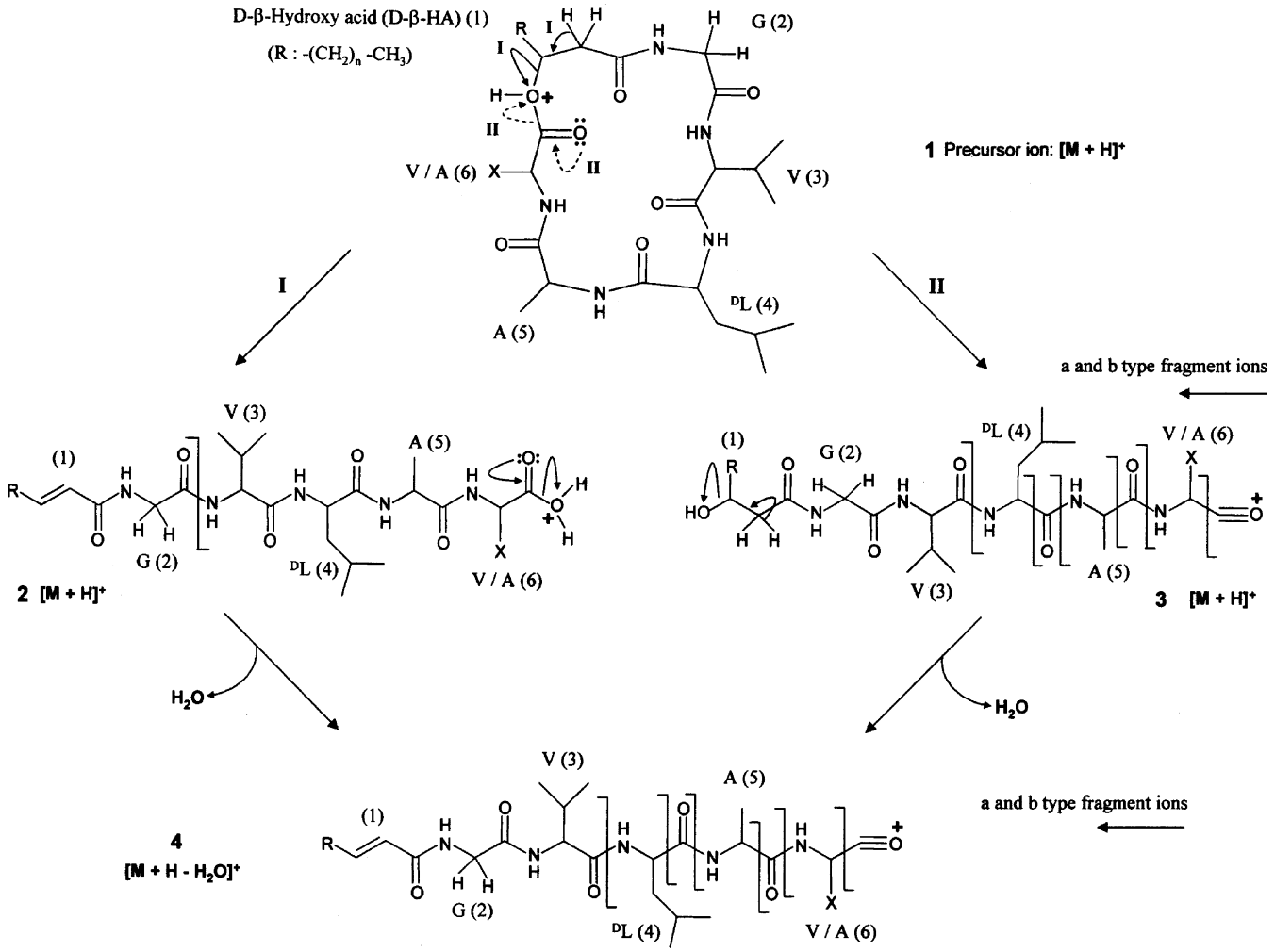
The correlations corresponding to the methylene units of $^D\beta$-HA are enclosed in a triangle (right panel of Figure 5), which proves useful in distinguishing different isariins on the basis of the number of methylene units (*n*) of $^D\beta$-HA.

Figure 6 shows the expansion of the region enclosed in a triangle in Figure 5 (700 MHz ^{1}H−^{13}C HSQC spectra) for isariins A, B, and C/C2. The number of horizontal lines marked in each frame of Figure 6 is indicative of the number of methylene units (*n*) of $^D\beta$-HA.

P4 (*m*/*z* 582.3) and P9 (*m*/*z* 610.5) present an interesting situation, where their masses are identical to that observed for components with different retention times, isariins F2 (P5, *m*/*z* 582.3) and G2 (P10, *m*/*z* 610.5).

The MS/MS spectrum of P4 (isariin F1) revealed an initial loss of 85 Da, resulting in a fragment ion at *m*/*z* 497.2 (Supporting Information, Figure S1-a). This may be contrasted with the case of the isobaric isariin F2 (P5, *m*/*z* 582.3), in which the initial loss corresponds to 99 Da, readily assignable to a Val residue (Figure 3b). Three possibilities may be considered for a residue mass of 85 Da. These are aminobutyricacid (Abu), α-aminoisobutyricacid (Aib), and *N*-methylalanine, all of which have been observed in microbial peptides. There is as yet no evidence for metabolites containing Abu or Aib in *Isaria*. However, N-alkylated residues, such as *N*-methylleucine, *N*-methylvaline, and *N*-methylphenylalanine have been observed in the isaridins (*vide infra*).[4] Distinction between these possibilities could not be achieved due to the limited availability of this fraction, which precluded NMR analysis. Assuming the presence of Abu or Aib or *N*-methylalanine, the peaks corresponding to other fragment ions are readily assigned, thereby yielding $n = 5$ for the side chain of $^D\beta$-HA (Table 1). The MS/MS spectrum of isariin G2 (P10) reveals an initial loss of an Ala residue (position 6) to yield the fragment ion at *m*/*z* 539.3 (Figure 3c).

Scheme 1. Plausible Ring-Opening Mechanism of $[M + H]^+$ Adduct of Isariin and Subsequent Generation of Product Ions from the Linear Structures



However, fraction P9 (isariin G1), which has an identical mass, reveals an initial loss of 99 Da, corresponding to the fragment ion at *m*/*z* 511.3, which thus may be assigned to the loss of a Val residue (position 6) (Supporting Information, Figure S1-b). The identity of the remaining amino acid residues is readily established, suggesting that P9 must contain a β-hydroxy acid residue with $n = 6$ (Table 1).

Isaridins. The two major peptide components of hyphal powder from *Isaria* correspond to the HPLC fractions P7 and P11 (Figure 1), both of which show intense UV absorption. These two metabolites, isaridins A and B, have been earlier characterized by NMR and X-ray diffraction (see Table 2).[4]

In our initial investigation of *Isaria* metabolites, the identification of the isaridins was rapidly achieved due to the serendipitous formation of diffraction quality crystals from HPLC fractions. Systematic characterization of related peptides from complex mixtures is more tractable using mass spectral methods. The isaridins possess an α-hydroxy acid, a β-amino acid residue, and several N-methylated residues. To examine whether the hyphal peptide mixture contained additional isaridins, we undertook a mass spectral investigation of isaridins A and B in order to establish their characteristic fragmentation patterns.

Figure 7 shows ESIMS/MS spectra of isaridins A (precursor ion *m*/*z* 704.4) and B (precursor ion *m*/*z* 718.5). The intense fragment ions observed at *m*/*z* 591.3 and *m*/*z* 605.2 correspond to the loss of the *N*-methylvaline residue from the respective linear precursor ions. This suggests that the cyclic hexadepsipeptide fragments selectively at the peptide bond between *N*-methylvaline and *N*-methylphenylalanine. Notably, this peptide unit adopts a *cis* conformation in both crystals and solution.[4] Peptide geometry may thus influence the site of protonation. In contrast to the isariins, the peak corresponding to loss of H_2O from the precursor ions of isaridins A and B is of very low intensity. The peaks at *m*/*z* 676.4, 660.4 (Figure 7a) and at *m*/*z* 690.4, 674.4 (Figure 7b) correspond to neutral losses of CO and CO_2, respectively, from their respective precursor ions.

The cyclodepsipeptide ring can open in multiple ways yielding different kinds of linear precursor ions (see Scheme 2). In the case of isaridins, the preferential cleavage occurs at the site between two *N*-methyl residues with an additional fragmentation pathway involving cleavage at the depsipeptide linkage, resulting in generation of a C-terminal carboxylic acid, which further undergoes a neutral loss of CO_2. Scheme 2 shows that the product ions containing Pro can be readily distinguished from the β-MePro fragment ions, using the *m*/*z* values of the characteristic fragment ions. The observed fragmentation pattern of isaridins of known sequence permits exploration of the hyphal mixture for variant sequences.

Comparison of the ESIMS/MS spectra of the precursor ion *m*/*z* 670.5 (P8) (Figure 8a) with that of isaridins A and B (Figure 7) reveals several common features, suggesting that P8 may be designated as isaridin C1. Examination of fragment ions (at *m*/*z* 591.3, 563.4, 275.1) from isaridins A and P8 (at *m*/*z* 557.3, 529.4, 241.1) and following the fragmentation mechanism depicted in Scheme 2 clearly suggests variation of only one residue between

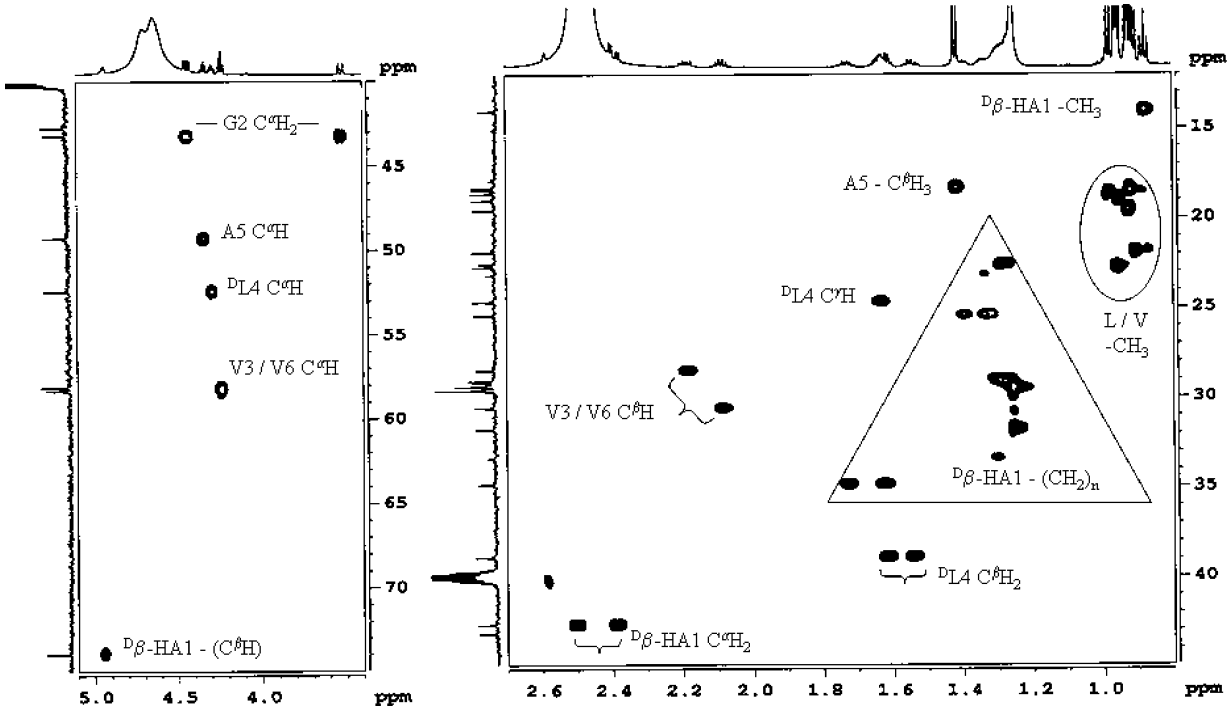
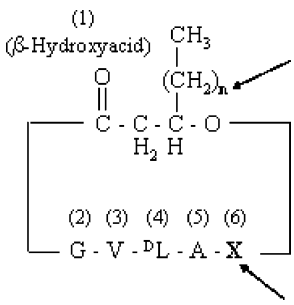


Figure 5. Partial 700 MHz $^1H-^{13}C$ HSQC spectrum of isariin A in $CDCl_3$ containing 4% (v/v) DMSO-d_6. Left panel shows $C^{\alpha}-H$ correlations. The correlations between the remaining carbons and protons are shown in the right panel. The correlations corresponding to the methylene $-(CH_2)_n-$ units of D-β-hydroxy acid ($^D\beta$-HA) are enclosed in the triangle. Although the dispersion of ^1H chemical shifts is very limited, the better dispersion of ^{13}C chemical shifts can be noted in this region. The expansion of this region is shown in Figure 6, which aids in distinguishing between different isariins, on the basis of the number of methylene units $-(CH_2)_n-$ of the $^D\beta$-HA.

Table 1. Isariin Sequences[a]



X (6)	*n* (1)	$[M + H]^+$ (*m/z*) (peak no.)	reference
A	3	540.3 (P1) (isariin D)	7b, this work
V	2	554.3 (P2) (isariin E)	this work
A	5	568.3 (P3) (isariin C)	7b, this work
V	3	568.3 (P3) (isariin C2)	this work
Abu/Aib	5	582.3 (P4) (isariin F1)	this work[b]
V	4	582.3 (P5) (isariin F2)	this work
V	5	596.5 (P6) (isariin B)	7b, this work
V	6	610.5 (P9) (isariin G1)	this work
A	8	610.5 (P10) (isariin G2)	this work
V	8	638.5 (P13) (isariin/isariin A)	6, 7b, this work

[a] Abu: aminobutyric acid; Aib: α-aminoisobutyric acid. [b] Not certain.

these two sequences. Replacement of F in isaridin A by Lxx results in a 34 Da shift (Lxx: *N*-methylleucine/*N*-methylisoleucine/*N*-methylalloisoleucine), thereby yielding the sequence of isaridin C1 as cyclo-(HyLeu-P-F-V-Lxx-βG) (Table 2). The product ions from the precursor ion *m/z* 684.5 (P12) revealed an isaridin signature, permitting designation as isaridin D (Figure 8b). Comparison of the MS/MS spectrum of P12 (*m/z* 684.5) with that of isaridin B (P11, *m/z* 718.5) clearly indicated variation of only one residue: replacement of F in isaridin B with Lxx resulting in a 34 Da shift, as evident from the peaks at *m/z* 275.1, 577.4, 605.4 (Figure 7b) and *m/z* 241.1, 543.4, 571.3 (Figure 8b). The observation of fragment ions at *m/z* 486.3, 444.3, 416.3 all indicate the presence of β-MePro in isaridin D, consistent with the sequence cyclo-(HyLeu-β-MePro-F-V-Lxx-βG) (Table 2).

In addition, a minor component of identical mass to that of isaridin C1 (P8, *m/z* 670.5) was detected, which eluted closely with P8 (not indicated in Figure 1). This was obtained upon reinjection of an HPLC purified fraction of the hyphal extract. Although it had the same mass as that of isaridin C1, the sequence of this minor component differed slightly. This distinction was realized from the fragment ions at *m/z* 486.3, 444.2, 416.2, obtained from the fragmentation of the minor component (precursor ion *m/z* 670.5, Supporting Information, Figure S2-a), while the fragmentation of P8 resulted in product ions at *m/z* 472.3, 430.2, 402.2, 241.0 (precursor ion *m/z* 670.5, Figure 8a). The product ion peaks at *m/z* 486.1, 444.1, 416.2 were also noted in the fragmentation spectrum of isaridin B, and all of these ions indicated the presence of β-MePro (Figure 7b). Taken together, these observations confirmed the minor component to be isaridin C2, possessing β-MePro (cyclo-(HyLeu-β-MePro-F-V-V-βG)), whose molecular mass is identical to isaridin C1. The 14 Da difference arising due to the replacement of Pro of isaridin C1 with β-MePro in isaridin C2 is compensated by the replacement of Lxx in isaridin C1 by V in isaridin C2.

Furthermore, another minor peptidic component was found to coelute with P7 whose $[M + H]^+$ was at 656 Da (not indicated in Figure 1). Examination of the product ions obtained from the precursor ion at *m/z* 656.5 and from the knowledge of the fragmentation mechanism as shown in Scheme 2 revealed that this component was also an isaridin, designated as isaridin E (Supporting Information, Figure S2-b). Notably, isaridin E (*m/z* 656.5) differed from C1 (*m/z* 670.5) by the replacement of Lxx with V, accounting for the 14 Da difference. Table 2 lists the sequences of isaridins identified thus far from the fungus *Isaria*. While βG, F, V, and HyLeu are the conserved amino acid residues in all of these isaridins, the microheterogeneity arises due to P ↔ β-MePro, Lxx ↔ V, and F ↔ Lxx replacements, rationalizing the 14 and 34 Da differences between their molecular masses.

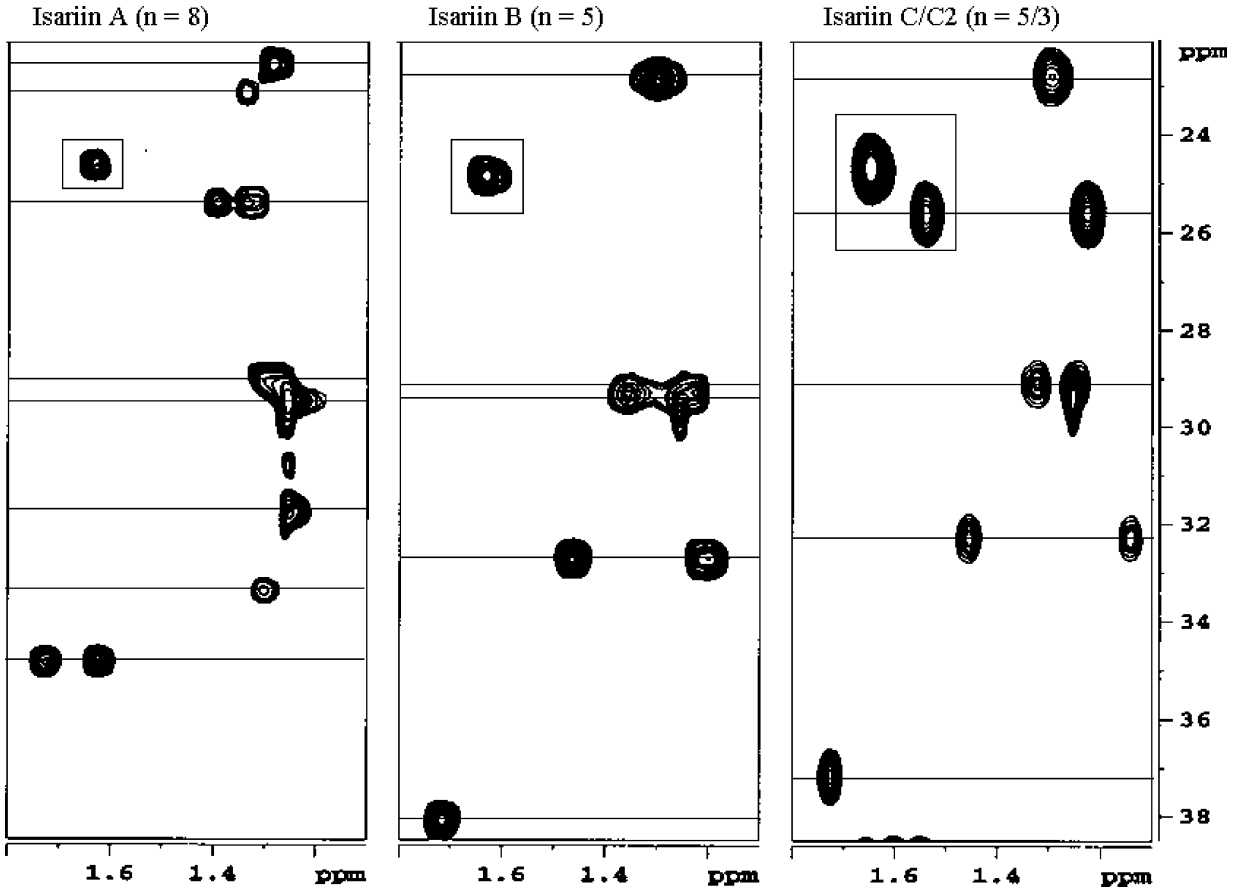
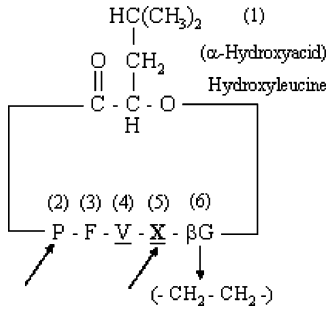


Figure 6. Partial 700 MHz $^1H-^{13}C$ HSQC spectra (expansion of the triangle box indicated in Figure 5) of isariins A, B, C/C2 showing the correlations corresponding to methylene units of D-β-hydroxy acid ($^D\beta$-HA) in $CDCl_3$ containing 4% (v/v) DMSO-d_6. The number of horizontal lines in each frame corresponds to the number of methylene units '*n*' in isariins A, B, and C/C2, which is 8, 5, and 5/3, respectively. (Boxed cross-peaks are from C$^\gamma$H of leucine.)

Table 2. Isaridin Sequences[a]



P (2)	X (5)	$[M + H]^+$ (peak no.) (*m/z*)	reference
P	F	704.5 (P7) (isaridin A)	4
P′	F	718.5 (P11) (isaridin B)	4
P	Lxx	670.5 (P8) (isaridin C1)	this work
P′	V	670.5 (isaridin *C*2)	this work
P′	Lxx	684.5 (P12) (isaridin D)	this work
P	V	656.5 (isaridin E)	this work

[a] P′: 3-methylproline (β-methylproline); X: N-methylated residue; F: N-MePhe; V: N-MeVal; Lxx: N-Me Leu/Ile/Allo-Ile; βG: beta-glycine (also referred as beta-alanine (βA)).

Isarfelins and Isaridins: The Cases of Isaridins C1/C2 and E. Recently two cyclohexadepsipeptides were reported from *I. felina*, isarfelins A and B, of molecular masses $[M + H]^+ = 670$ and 656 Da, respectively, and were shown to possess antifungal and insecticidal properties.[11]

The sequencing of the isarfelins was facilitated by the determination of the amino acid composition by acid hydrolysis studies and tandem mass spectral analysis of the sodium ion adducts. Guo et al. assumed isarfelins to possess a β-hydroxy acid residue on the basis of the reported sequences of isariins isolated from *I. felina* by Baute et al.[7b] Interestingly, the isaridins C1/C2 ($[M + H]^+ = 670$ Da) and E ($[M + H]^+ = 656$ Da) characterized in this study also have the same mass as that of isarfelins A and B, respectively. It was striking to find that the ESIMS/MS spectra of sodium adducts of isaridins C1/C2 and E (data not shown) were very similar to the ESIMS/MS spectra of sodium adducts of isarfelins A and B as presented in the report of Guo et al.[11] This suggested that the isaridins C1/C2 and E might be identical to isarfelins A and B. The sequence determination of isaridins C1, C2, and E was in fact facilitated by the interpretation of the mass spectrometric fragmentation behavior of isaridins A and B (Figure 7 and Scheme 2), which were independently characterized earlier in our laboratory.[4] Inspection of the isarfelin sequences proposed by Guo et al. revealed significant differences from those of isariins. The presence of a β-hydroxy acid with a $-(CH_2)_7-CH_3$ chain has been inferred on the basis of an unlikely side chain fragmentation resulting in a loss of 113 Da. Therefore, we believe that isarfelins A and B isolated from *I. felina* by Guo et al. are indeed isaridins C1/C2 and E, respectively. Our reassignment of the isarfelins as isaridins is consistent with all available mass spectrometric data. Thus, all the cyclodepsipeptides characterized thus far from *Isaria* species fall into two distinct classes, the isariins and the isaridins.

Conformation of the Isariins. Considerable structural evidence is available on the conformation of the isaridins and the related peptides, roseotoxins and destruxins.[4,14] In these cases, the 19-atom heterocycle has been characterized by X-ray diffraction and NMR spectroscopy. In contrast, no three-dimensional structural information is available for the isariins. The key difference between the isaridins and the isariins is the former possess a backbone

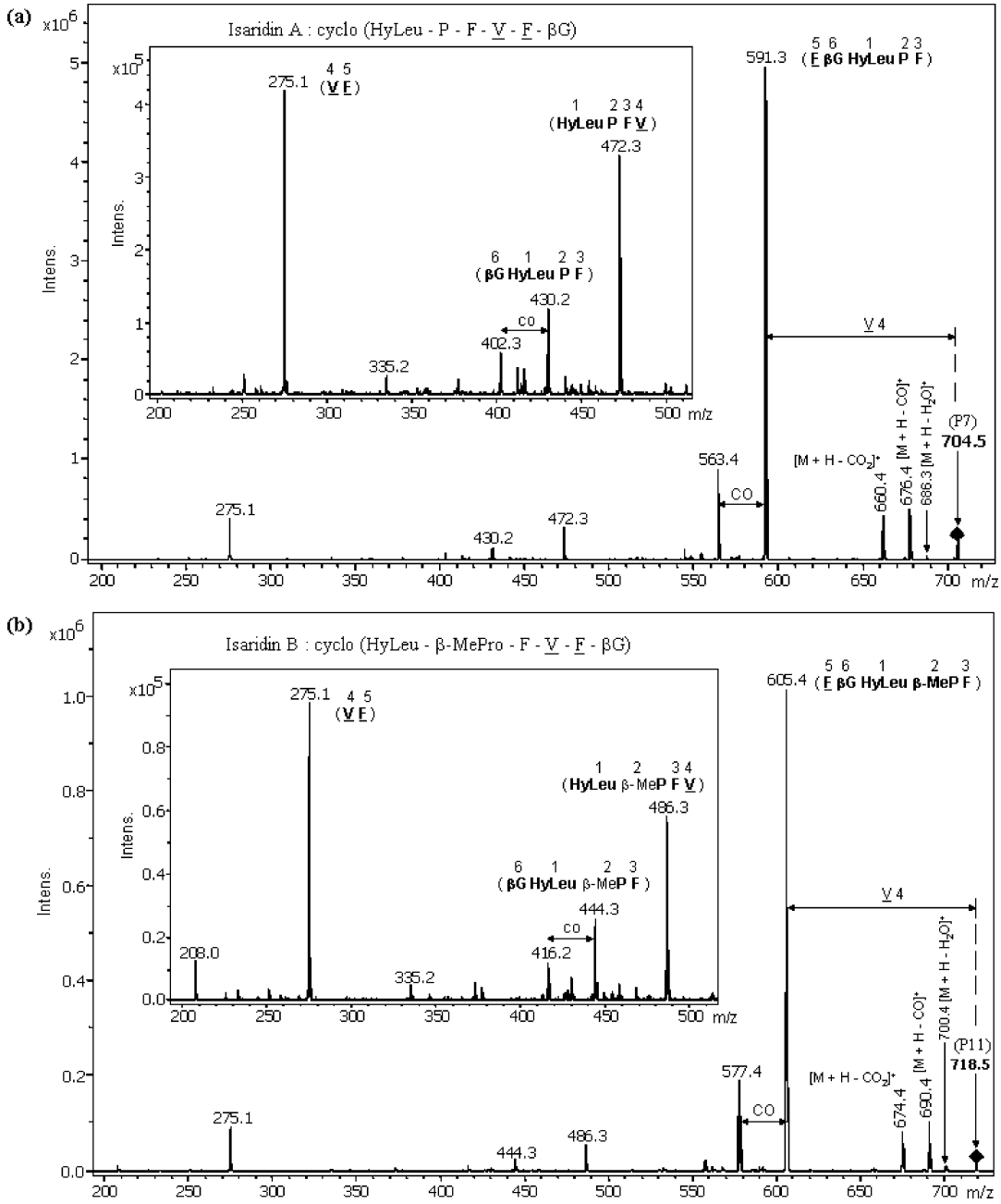
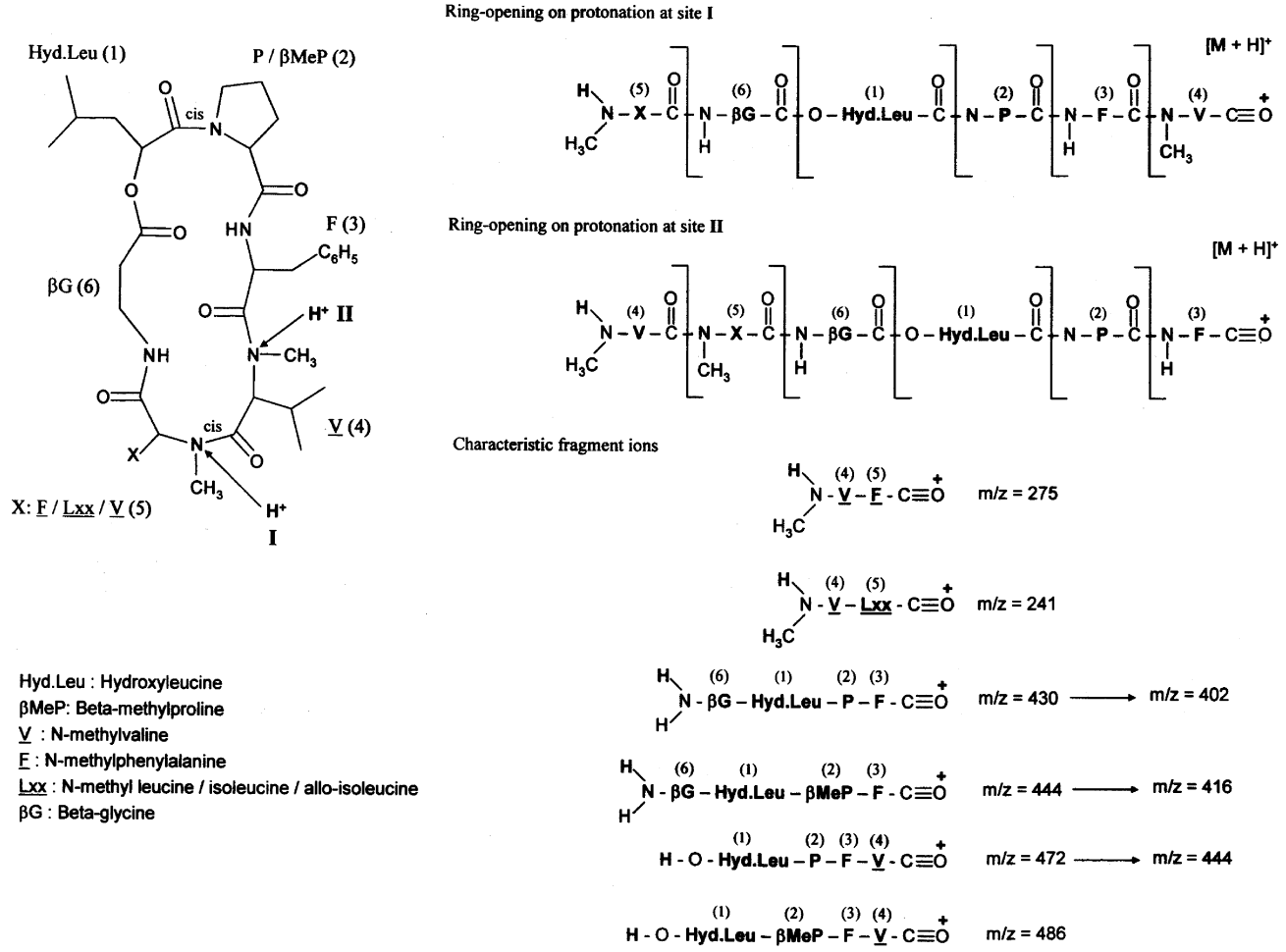


Figure 7. ESIMS/MS spectra of $[M + H]^+$ of isaridins that were identified earlier:[4] (a) isaridin A (P7: precursor ion *m*/*z* 704.5), (b) isaridin B (P11: precursor ion *m*/*z* 718.5).

consisting of an α-hydroxy acid, four α-amino acids, and one *β*-amino acid, while the latter contain one *β*-hydroxy acid and five α-amino acids. Both heterocycles have the same number of backbone atoms (19).

Isariin A, which was chosen as the representative member from this class, yields a well-resolved ^{1}H NMR spectrum in $CDCl_3$ containing 4% (v/v) DMSO-d_6 (Supporting Information, Figure S3 and Table S1). In pure $CDCl_3$ broad resonances were obtained indicative of peptide self-association. Sequence-specific assignments were readily achieved using a combination of TOCSY (Supporting Information, Figure S4) and ROESY spectra (Figure 9). The temperature dependence of the NH chemical shifts revealed high $\Delta\delta/\Delta T$ values for G(2) (9.1 ppb/K), A(5) (9.1 ppb/K), and ^{D}L(4) NH (7.2 ppb/K) resonances (Supporting Information, Figure S5). The NH groups of V(3) and V(6) showed much lower coefficients, 4.3 and 6.5 ppb/K, respectively, indicative of their possible involvement in transannular hydrogen bonds. Figure 9 shows the partial ROESY spectrum of isariin A, illustrating key inter-residue nuclear Overhauser effects (NOEs). The strong d_{NN} NOEs, G(2) NH ↔ V(3) NH and A(5) NH ↔ V(6) NH, confirm the location of the obligatory turn segments in the cyclic depsipeptide. The schematic view of the molecular conformation shown in Figure 9 is compatible with the NOE data and the observed solvent shielding of V(3) and V(6) NH. In cyclic hexapeptides composed of an all α-amino acid backbone, the favored conformation consists of two *β*-turns with two stabilizing intramolecular 4−1 hydrogen bonds.[15] In the case of isariin A, the ^{D}L(4)−A(5) segment is likely to form a type II′ *β*-turn, commonly observed in heterochiral DL sequences.[16] The strong NOE observed between ^{D}L(4) C$^\alpha$H and A(5) NH is supportive of $\psi \approx -120°$ for the residue ^{D}L(4), compatible with the i+1 position of type II′ *β*-turn.[17] The second reverse turn in the molecule is formed by the ^{D}*β*-HA(1) and G(2), with an intramolecular hydrogen bond between V(6) C═O and V(3) NH, resulting in a 11-atom (C_{11}) structure. Similar C_{11} hydrogen bonds

Scheme 2. Schematic Description of Plausible Ring-Opening Modes on Protonation (at Sites I and II) of Isaridin and Subsequent Generation of Product Ions from the Linear Structures[a]



[a] The characteristic fragment ions (and their *m/z* values) obtained from the cleavage of the linear structures are listed.

have been well characterized in hybrid sequences of α- and β-amino acids, in $\alpha\beta$ or $\beta\alpha$ segments.[10] The observed $\beta\alpha$ C_{11} turn in isariin A can be viewed as a hydroxy analogue of the previously characterized C_{11} turn in all amide backbones. The notable differences between the structures of isariin A and the isaridins are as follows: (i) in the isaridins two *cis* peptide bonds are formed and occupy the central position in the facing turn segments; in contrast, an all-*trans* peptide/ester backbone is observed for the isariins. (ii) In the isaridins, the β-amino acid residue occupies a nonturn (extended) position; in contrast, in isariin A the $^{D}\beta$-HA(1) occupies the $i+1$ position of the two-residue turn.

Antiplasmodial Activity. The isariins were originally shown to have insecticidal activity.[7a] No biological studies have been reported thus far on the isaridins. In the course of studies aimed at screening natural products for antiplasmodial activity, we investigated the effects of these two classes of cyclohexadepsipeptides isolated from *Isaria* for their effects on malarial parasites grown in culture. The effects of three HPLC fractions were tested on the intra-erythrocytic growth of *Plasmodium falciparum* (Supporting Information, Figure S6). It should be noted that one of the HPLC fractions contains two coeluting peptidic components, isariins C/C2 (P3 of Figure 1), as described earlier (Figure 4 and Table 1a). This fraction was used directly for the antiplasmodial activity studies, as continued purification of this fraction did not result in separation of isariin C from C2. Therefore, the activity exhibited by this HPLC fraction could be from both of the peptidic components. The IC_{50} values for the peptides were isariin C/C2, 87 μM; isariin B, 118 μM; and isaridin A, 230 μM. The results from microscopy examination (data not shown) agree well with that from radioactive label incorporation. At the IC_{50} concentration, the surviving parasites appeared pyknotic on microscopic examination. All three peptide fractions at the highest concentration (300 and 600 μM) tested did not lyse erythrocytes.

Discussion

High-performance liquid chromatography coupled to electrospray mass spectrometry (LC-ESIMS), especially tandem mass spectrometry (LC-ESIMS/MS), permitted the rapid identification of a library of microheterogeneous cyclic depsipeptide metabolites produced by the fungus *Isaria*. In fact, LC-ESIMS/MS methods have proved to be of great use in the screening of new metabolites of microbial origin.[9e−h] The cyclic hexadepsipeptides found from the fungus *Isaria* thus far fall mainly into two classes, isariin and isaridin. The former group of peptides is characterized by the presence of a β-hydroxy acid, while the latter class possesses an α-hydroxy acid and a β-amino acid.

Homonuclear and heteronuclear NMR experiments were useful in confirming the chemical identities of these cyclic hexadepsipeptides. In the case of P3, a combination of mass spectrometry and NMR methods aided in the detection of a new coeluting peptidic component, isariin C2, an isobar of isariin C (Table 1). In this investigation, 10 isariins have been identified, out of which six are new and four were reported earlier.[6,7b] Six isaridin molecules have been detected, and among them, four are new while two have been previously characterized from our laboratory.[4] The identification of the six new isariins was based on the presence of the four already reported sequences of isariins from hyphal extracts of the

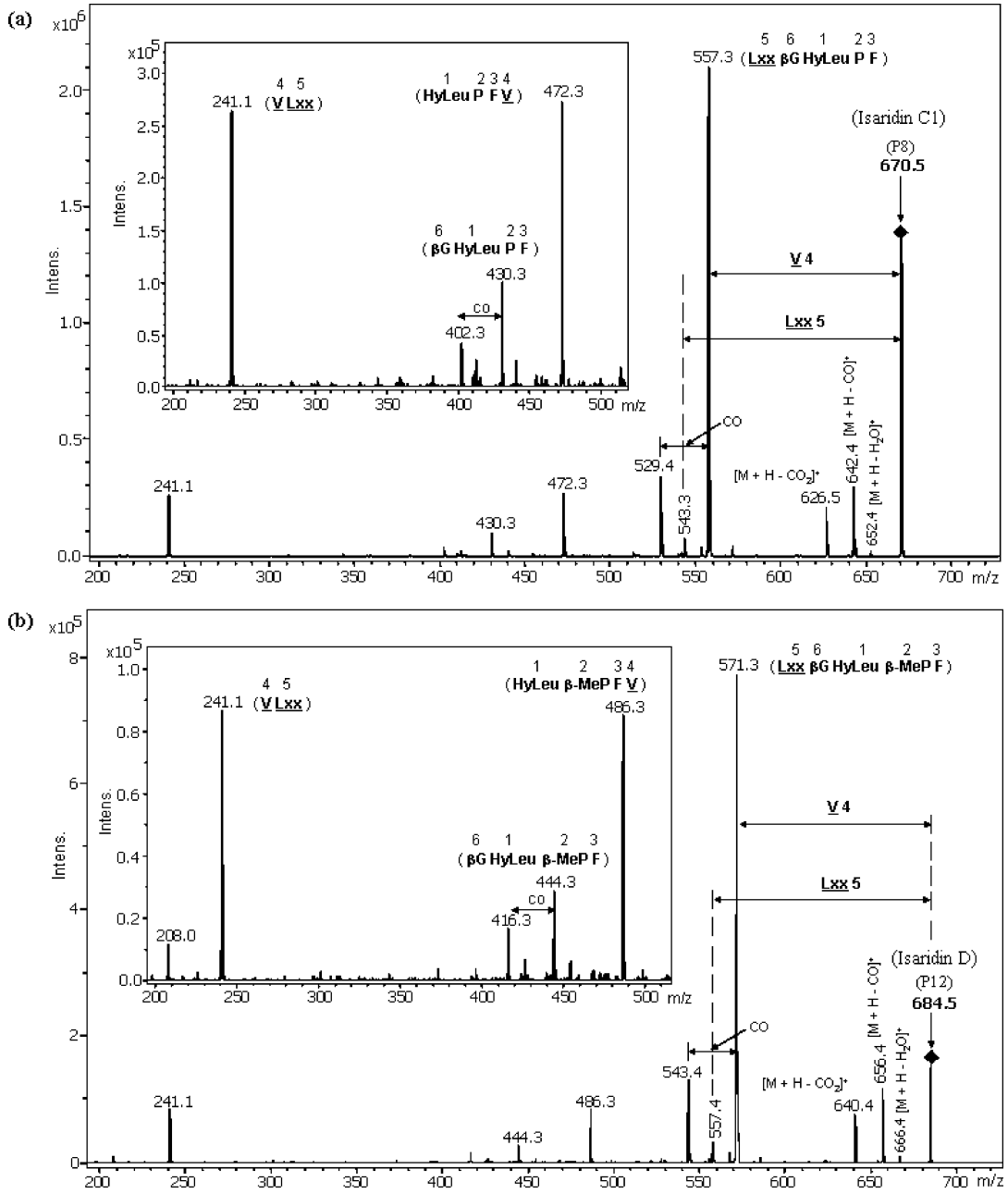


Figure 8. ESIMS/MS spectra of $[M + H]^+$ of isaridins identified in this study: (a) isaridin C1 (P8: precursor ion *m*/*z* 670.5), (b) isaridin D (P12: precursor ion *m*/*z* 684.5).

Isaria strain used in this study.[6,7b] Likewise, the sequences of the four new isaridins were facilitated having prior knowledge of the sequences of isaridins A and B, previously characterized in our laboratory. Hence, the configuration of the amino acids of the newly detected peptides is assumed to be the same as that of the already reported peptides. Nevertheless, determination of the configuration of the constituent amino acids in these new peptides is presently underway.

The structure of isariin A was investigated by temperature-dependent 1D ^{1}H NMR spectroscopy and ROESY and suggested the possibility of a transannular hydrogen bond, a hybrid $\alpha\beta$ C_{11} turn, formed by the $^D\beta$-HA(1) and G(2), and a plausible reverse-type II β turn consisting of ^{D}L(4) and A(5) in a 19-membered macrocyclic ring. Since the sequences of the isariins vary only at residues 1 and 6 (Table 1), it is likely that the other isariins also adopt a structure similar to that of isariin A, assuming that the size of the side chain of the $^D\beta$-HA(1) has little or no influence on the overall structure of the backbone.

Sequence Comparison between Isarial Metabolites and Related Cyclic hexadepsipeptides. The nonribosomal synthesis of microbial peptides requires a well-orchestrated pattern of expression of gene clusters that code for the modular multienzyme complexes involved in peptide and ester bond formation.[2] Comparison between the sequences of peptide secondary metabolites produced by diverse organisms can provide insights into taxonomical and evolutionary relationships.

A search of the literature of cyclohexadepsipeptides containing a β-hydroxy acid and five α-amino acids as in the isariins did not yield appropriate matches. However, a metabolite isolated from cultures of *Bacillus subtilis*, "surfactin", is a "cyclooctadepsipeptide" (lipopolypeptide) containing a β-hydroxy acid residue.[19] Surfactin possesses a large heterocyclic ring composed of 25 backbone atoms. The isariins thus appear to belong to a family of which the number of presently characterized members is extremely limited, derived only from the fungus *Isaria*.

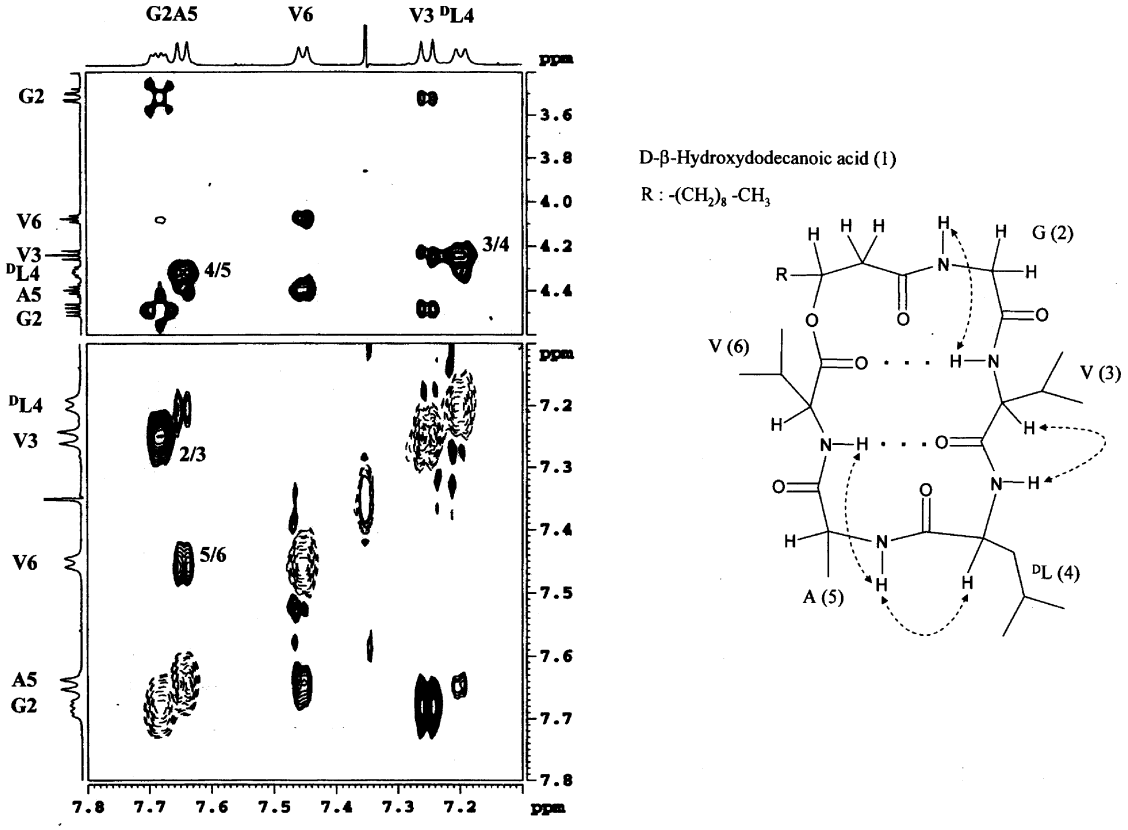


Figure 9. Partial expansion of the 500 ^{1}H ROESY spectrum of isariin A in $CDCl_3$ containing 4% (v/v) DMSO-d_6. The observed NOEs marked in the spectrum, $d_{\alpha N}$ (top) and d_{NN} (bottom), are indicated by double-headed arrows in the schematic shown on the right.

Interestingly, in the case of isaridins, four classes of related peptides have been isolated from different fungi species. The sequences of the destruxins, roseotoxins, bursaphelocides, and pseudodestruxins are listed in Table 3 along with the isaridin sequences identified in this study.[4,9a,e,14d,18] These peptides bear great similarity to the isaridins in the nature of the amino acids and differ substantially only in the side chains of the α-hydroxy acids (not indicated in Table 3). The sequence alignment of these peptides clearly shows the complete conservation of the *β*-amino acid residue, *β*G(6) (*β*G, *β*-glycine, also referred as *β*-alanine or 3-aminopropanoic acid). At position 2, proline is largely conserved, although the proline analogues, 3-methylproline, 4-methylproline, and pipecolic acid, are also present. Position 3 is occupied by a hydrophobic residue, Ile or Val, in the roseotoxins and destruxins, while the pseudodestruxins and the isaridins contain a phenylalanine at this position. Interestingly, the sequences of newly detected isaridins C1, C2, D, and E are similar to pseudodestruxins; in particular, the pseudodestruxin B and isaridin C1 are identical (assuming that isaridin C1 possesses *N*-methylleucine). The sequence of pseudodestruxin B was deduced using two-dimensional NMR spectroscopy and FABMS techniques,[14d] while the MS fragmentation data as described in an earlier section strongly support the proposed sequence for isaridin C1. Residues 4 and 5 are occupied exclusively by nonpolar amino acids I, V, L, and A. N-Methylation is almost invariably observed at these positions except in destruxin-protoB, where residue 5 is Ala. The isaridins A and B are the only cases in which two aromatic residues are present.

The presence of the largely conserved residues noted across all of these classes of peptides prompted us to look for relationships among the fungal species from which these peptides were isolated. The cyclohexadepsipeptides listed in Table 3 are produced by widely different species of fungi. For example, the fungi *Nigrosabulum globosum* and *Isaria* produce identical and very closely related peptides. *Nigrosabulum* forms sexually produced ascocarps bearing ascospores and belongs to the order Eurotiales under Ascomycetes. Its asexual spore form (conidia) is not prominent. In *Isaria*, the spore form is the asexual conidial stage, and its sexual stage is rarely known. The fungus *Isaria* is classified under the order Hypocreales in the Ascomycetes, a taxonomic group very distinct and unrelated to the Eurotiales. Further, another hyphomycetous genus, *Trichothecium*, is known to produce peptides such as destruxin, roseotoxin, and roseocardin.[9e,14c,18] It is noteworthy that the cyclodepsipeptides from these unrelated taxa are very similar. Thus, the ability to produce similar depsipeptides, and indeed secondary metabolites in general, transcends taxonomic barriers.

The observed antiplasmodial activities of isariins and an isaridin requires comment. Diverse peptides from microorganisms, from insect venoms, and of the innate immunity system have been shown to possess antiplasmodial activity. Scorpine, a peptide from scorpion venom, was shown to inhibit development of *P. berghei* ookinete and gamete stages.[20] Hybrid peptides based on the defensin sequence were found to be lethal to early sporogonic stages of *P. berghei* and *P. yeolii*.[21] Psalmopeotoxins I and II present in the venom of the Trinidad chevron tarantula *Psalmopoeus cambridgei* kill intra-erythrocytic stages of *P. falciparum* in the low micromolar range while remaining nontoxic to mammalian cells.[22] A hybrid peptide of melittin and cecropin was found to be antiparasitic, with an IC_{50} in the micromolar range.[23] Dermaseptin S4 exhibited antiparasitic activity through its hemolytic action.[24] Antiamoebin and efrapeptin, acyclic peptides of fungal origin, have been shown to be potent inhibitors of the growth of intra-erythrocytic *P. falciparum*.[25] Micrococcin, a cyclic peptide from *Staphylococcus*, has been found to be a potent inhibitor of *P. falciparum* and as good as that of the classical antimalarial drugs pyrimethamine and mefloqine.[26]

A recent study establishes that malaria-causing mosquitoes are prone to fungal infection by *Beauveria bassiana* and *Metarhizium anisopliae*, thereby causing decreased malarial transmission by these

Table 3. Amino Acid Sequences of Cyclodepsipeptides Related to Isaridins

c(D-HA X X X X X)	Peptides*	Species*
1 2 3 4 5 6		
P I V A βG	Dxs[a] and Bursa A	*Metarhizium* anisopliae and other species[b]
P' I V A βG	Ros A, B, C, Roseocardin	*Trichothecium roseum*, *Trichothecium roseum* TT103
P'' I V A βG	Bursa B	Imperfect fungus strain D1084
P I V A βG	Dxs: A_1, B_1, D_1, E_1 and Ed_1	*Metarhizium anisopliae*
A I V A βG	Dx A_3	*Metarhizium anisopliae*
P I I A βG	Dxs[c]	Fungal culture OS-F68576, Aschersonia sp., *Alternaria brassicae*[d]
P' I I A βG	Dx A_5 ; [MeLxx[4]]Ros A, B	*Aschersonia* sp., *Trichothecium roseum*
P I V A βG	Dxs: DesmA, DesmB, DesmC	*Metarhizium anisopliae*, *Alternaria brassicae*[d]
P' I V A βG	[Val[4]]RosA	*Trichothecium roseum*
P I V A βG	Dx ProtoB	*Metarhizium anisopliae*
P V V A βG	Dxs A_2-E_2 and E_2 Chlorohydrin	*Metarhizium anisopliae*
P V V A βG	Dx $DesmB_2$	*Metarhizium anisopliae*
P F I L βG	Pseudo Dx A	*Nigrosabulum globosum*
P F V L βG	Pseudo Dx B	*Nigrosabulum globosum*
P F V F βG	Isaridin A	*Isaria*
P' F V F βG	Isaridin B	*Isaria*
P F V L βG[e]	Isaridin C1	*Isaria* (This study)
P' F V V βG	Isaridin C2	*Isaria* (This study)
P' F V L βG[e]	Isaridin D	*Isaria* (This study)
P F V V βG	Isaridin E	*Isaria* (This study)

D-HA: D-α-Hydroxy acid; P': 3-Methylproline; P'': 4-Methylproline; P: Pipecolic acid; V: N-Methylvaline;

I: N-Methylisoleucine; βG: Beta-glycine (also referred as Beta-alanine); F: N-Methylphenylalanine;

L: N-Methylleucine; MeLxx: L or I

Dx: Destruxin; Ros: Roseotoxin; Bursa: Bursaphelocide.

[a] Dxs: Destruxins: A-F, Ed, HyroxyB, E Chlorohydrin, DihydroA, β-D-Glucosyl HydroxyB

[b] *Trichothecium roseum* TT103, *Ophiosphaerella herpotricha* (Fr.) Walker, Fungal culture OS-F68576, Imperfect fungus D1084, *Sinapis alba*, *Brassica* sp.

[c] Dxs: Destruxins: A_4, A_4 Chlorohydrin, HomoB, HydroxyHomoB

[d] *Alternaria brassicae* (Berkeley) Saccardo

[e] L: L or I or N-Methylallo-isoleucine (see Table 2)

* See Refs. (4), (9e), (14d), (18)

mosquitoes.[27] Blanford et al. have demonstrated the effect of oil-based formulations of fungal entomopathogens, in particular, *Beauveria bassiana*, on the growth of adult *Anopheles stephensi* infected with a clone of the rodent malaria parasite *Plasmodium chabaudi*.[27a] From a field study in a rural village in Tanzania, Scholte and co-workers demonstrated the virulent effect of *Metarhizium anisopliae* on the adult *Anopheles gambiae*, an African malarial vector.[27b]

Interestingly, the genus *Isaria* is very closely related to *Beauveria*, and members of both genera have representatives that are pathogenic to insects and occur saprophytically in soil, dung, etc. The genus *Isaria* is characterized by conidiogenous cells without elongation and prominent synnemata, while elongated conidiogenous cells and absence of synnemata are the characteristics of *Beauveria*. Our fungal strain resembles *I. felina*, which possesses flask-shaped conidiogenous cells and often curved and broadly

ellipsoidal conidia. Further, the species *Isaria cretacea* is treated as a synonym of *I. felina*, and all of the dung-inhabiting strains have been referred to as *I. felina*/*I. cretacea* in the literature.[28]

It would be of special interest to establish the entomopathogenicity of the fungi *Isaria* toward the malarial mosquito vectors. Moreover, the *in vitro* intra-erythrocytic antiplasmodial activity exhibited by both classes of isarial peptides suggests that further studies of isarial metabolites may be of value. Additionally, LC-ESIMS methodologies could be not only of tremendous use in the rapid screening of fungal metabolites but also applicable for the identification of fungal infection markers from clinically significant fungi and novel metabolites from other microorganisms.[29]

Experimental Section

Extraction and Purification of Depsipeptides. The *Isaria* fungal strain was grown and the depsipeptides were extracted as described earlier.[4] The crude hyphal extract was dissolved in CH_3CN/MeOH, centrifuged to obtain a clear solution, and subsequently taken for reversed-phase (RP) chromatographic purification. Semipreparative fractionation was achieved using a RP column (Zorbax C_{18}, 9.4 mm × 250 mm, 5−10 μm 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. For the LC-MS experiments, an analytical RP column (Zorbax C_{18}, 4.6 mm × 150 mm) was utilized using a CH_3CN/H_2O solvent system at a flow rate of 0.2 mL/min following a linear gradient of 60−90% CH_3CN in 30 min (detection at $\lambda = 226$ nm).

Mass Spectrometry. The LC-ESIMS traces, UV ($\lambda = 226$ nm), and total ion chromatograms were acquired on a Hewlett-Packard 1100 MSD series mass spectrometer equipped with a single quadrupole. The data were recorded in the range *m*/*z* 200−3000. The data were processed using HP LC/MSD Chemstation software.

The LC-ESIMS/MS spectra were recorded on a Esquire 3000 plus (Bruker Daltonics) mass spectrometer housed with an ion trap using the Auto-MS(n) option. For certain HPLC-purified fractions, MS/MS spectra were acquired by direct injection mode, using a syringe pump (Cole-Parmer) operated at a flow rate of 200 μL/h. The data were acquired over the range *m*/*z* 50−3000 in positive ion mode. The data were processed with Esquire data analysis, version 3.1 software.

NMR Spectroscopy. NMR experiments were carried out on Bruker AV500/AV700 spectrometers. The $^1H-^{13}C$ HSQC spectra were acquired on the 700 MHz spectrometer, while the other spectra were acquired at 500 MHz. A peptide concentration of ~5 mM was used. Spectra were recorded in $CDCl_3$ containing 4% (v/v) DMSO-d_6, as the amide proton resonances showed considerable broadening in $CDCl_3$ solution due to aggregation. The probe temperature was maintained at 300 K for 1D and 2D data collection. Hydrogen-bonding information of amide protons was delineated by recording 1D spectra at five different temperatures between 283 and 323 K at 10 K intervals. All 2D experiments were recorded in phase-sensitive mode using the States-TPPI method. A data set of 1024 × 450 was used for acquiring the data. The same data set was zero filled to yield a data matrix of size 2048 × 1024 before Fourier transformation. A spectral width of 6000 and 9000 Hz was used in ^1H homonuclear experiments on 500 and 700 MHz spectrometers, respectively. Mixing times of 100 and 250 ms were used for TOCSY and ROESY, respectively. Shifted sine bell windows were used for data processing, and all data processing was done using Bruker XWINNMR software.

Biological Activity Studies. Test for *in Vitro* Antiplasmodial Activity. The 3D7 strain of *P. falciparum* was cultivated *in vitro* using the method described by Trager and Jensen.[30] Parasites were maintained in human O^+ cells, obtained from O^+ human blood collected from healthy volunteers, at 5% hematocrit in RPMI 1640 containing 10% human serum. Serum for culture medium was separated from clotted O^+ human blood. The antiplasmodial activity test was based on previously reported methods.[31] All peptides were dissolved in dimethyl sulfoxide (DMSO). Drugs were serially diluted 2-fold over the concentration range 300 to 2.34 μM for isariins B and C/C2, while isaridin A was diluted 2-fold from 600 to 1.17 μM. Each well contained 200 μL of cell suspension with 2% parasitemia and 2% hematocrit in culture medium. Parasitized and nonparasitized erythrocytes and solvent controls were incorporated in all the tests. The plates were incubated at 37 °C in a candle jar. After 15 h, each well was pulsed with 10 μL of culture medium containing 1.0 μCi of [8-^{3}H] hypoxanthine, and plates were incubated for a further 13 h. The contents of each well were then harvested onto glass fiber filters using a Skaktron automated cell harvester, washed extensively with distilled water, and dried. The incorporated radioactivity was measured as disintegrations per minute using a Wallac 1409 (Wallac Oy, Turku, Finland) liquid scintillation counter. All experiments were repeated at least three times. For test samples the percent incorporated with respect to the control was plotted against the logarithm of the drug concentration. The concentration causing 50% inhibition of radioisotope incorporation (IC_{50}) was determined by interpolation. A parallel experiment by microscopy, using Giemsa-stained smears, was also conducted. These wells containing parasites were incubated with the drug for a total period of 24 h. The data obtained from the parasite counts were subjected to the same analysis as that from the radioactive hypoxanthine incorporation. All data were analyzed using the software Prism (GraphPad Software Inc., San Diego, CA).

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Supporting Information Available: ESIMS/MS spectra of $[M + H]^+$ species of isariins F1 and G1; ESIMS/MS spectra of $[M + H]^+$ species of isaridins C2 and E; 500 MHz ^1H NMR spectra of isariin A in $CDCl_3$ and in $CDCl_3$ containing 4% (v/v) DMSO-d_6, at 300 K; 500 MHz TOCSY spectra of isariin A in $CDCl_3$ containing 4% (v/v) DMSO-d_6 at 300 K; temperature-dependent 500 MHz ^1H NMR spectra showing amide chemical shifts of isariin A in $CDCl_3$ containing 4% (v/v) DMSO-d_6; inhibitory effect of isariins and an isaridin on the intra-erythrocytic growth of *Plasmodium falciparum*; Table listing 500 MHz ^1H NMR chemical shifts and scalar coupling constants of isariin A in $CDCl_3$ containing 4% (v/v) DMSO-d_6. This material is available free of charge via the Internet at http://pubs.acs.org.

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