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Insecticidal Cyclodepsipeptides from *Beauveria felina*

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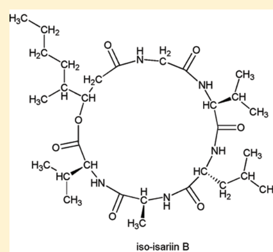
Supporting Information

ABSTRACT: A novel cyclodepsipeptide, iso-isariin B (**1**), and the known isaridin E (**2**) were isolated from the entomopathogenic fungus *Beauveria felina.* Their structures were elucidated using MS/MS fragmentation and extensive 2D-heteronuclear NMR. The X-ray structure of isaridin E was obtained, showing two potent intramolecular H bonds and a type-VI turn with the HyLeu[1]-Pro[2] amide bond in a *cis* conformation. Iso-isariin B (**1**) was active against the pest-insect *Sitophilus* spp. with an LD_{50} value of 10 μg/mL. This observation also gives some clues for ecological interpretation of cyclodepsipeptide production by *B. felina*.



The chemical ecology of fungi implies the study of their interactions with congeners and also with potential hosts. In such studies, it is important to identify classes of metabolites that could be mediators in these interactions. We have investigated the metabolite production and associated insecticidal activities of *Beauveria felina* (DC.) J. W. Carmich. isolated from the soil of an Algerian cavern but also known as entomopathogenic. In culture, *B. felina* produces luxurious morphological structures named synnemata (a group of hyphae-bearing conidia) described by Taber and Vining.[1] The ecological significance of these large structures, produced by diverse fungi including *Beauveria*, *Aspergillus*, and *Penicillium* for example, from humid habitats in tropical rainforest to drier environments in agricultural soils and grains has been discussed by Samson and Seifert.[2] *B. felina* was previously named *Isaria felina* (DC.) Fr.[3] The first depsipeptide isolated from this fungus was reported in 1962 and named isariin A in reference to this former nomenclature, and subsequently, three additional cyclodepsipeptides, isariins B, C, and D, were described by Baute and co-workers.[4,5] More recently, Balaram and co-workers reported the X-ray crystal structures of isaridins A and B, two new hexadepsipeptides featuring several modified amino acid residues.[6] The same group reported the identification and characterization of six isariins and four isaridins.[7]

We wish to report herein the isolation and sequence characterization of iso-isariin B (**1**), based on tandem mass spectroscopy and extensive 2D-heteronuclear NMR. This is a new cyclic hexadepsipeptide characterized by the presence of a 3-hydroxy-4-methyloctanoic acid moiety (HMA), so far unprecedented in the isariin series. We also report for the first time the X-ray structure of isaridin E (**2**), a cyclohexadepsipeptide previously isolated by Balaram et al.[7] Insecticidal activity of the compounds was demonstrated using the pest insects *Sitophilus* spp. and *Callosobruchus maculatus*, giving clues for ecological interpretation of cyclodepsipeptide production by *B. felina*.

RESULTS AND DISCUSSION

Sequence Determination by Mass Spectrometry. The molecular weight of iso-isariin B (**1**) was obtained from a positive ESI-qTOF mass spectrum, showing the protonated molecular $[M + H]^+$ ion at m/z 596.4034 and the $[M + Na]^+$ adduct ions at m/z 618.3851 corresponding to the molecular formula $C_{30}H_{53}N_5O_7$ (calcd for $C_{30}H_{54}N_5O_7$: 596.4023). Similarly, the molecular formula $C_{35}H_{53}N_5O_7$ was assigned to compound **2**, taking into account a protonated molecular $[M + H]^+$ ion observed at m/z 656.4044 and a $[M + Na]^+$ adduct ion at m/z 678.3859 as well as the $[M + K]^+$ adduct ion at m/z 694.3640 (calcd for $C_{35}H_{54}N_5O_7$: 656.4023).

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The collision-induced dissociation (CID) spectrum of the $[M + H]^+$ ion of **1** at *m*/*z* 596 showed a main series of adjacent b_n ions at *m*/*z* 497, 426, 313, 214, and 157 corresponding to the successive loss of Val, Ala, Leu/Ile, Ala, Gly, and a $C_9H_{16}O_2$ fragment, thus suggesting the sequence $C_9H_{16}O_2$-Gly^2-Val^3-Leu/Ile^4-Ala^5-Val^6 for the linear peptide ion derived from **1**.

A second series of ions was observed at 568, 469, 398, 285, and 186 and was assigned to the related adjacent a_n ion. A third series of ions was observed at *m*/*z* 578, 479, 408, 295, 196, and 139, having 18 mass units less than the preceding b_n ion and corresponding to the loss of a water molecule. The mass spectra results are consistent with an isariin and suggested a provisional structure similar to the described isariin B:[7] *cyclo*(βHA^1-Gly^2-Val^3-Leu/Ile^4-Ala^5-Val^6). However, the nature of the $C_9H_{16}O_2$ fragment could not be elucidated by this method, and an NMR study was then undertaken as described below.

The CID spectrum of the $[M + H]^+$ ion of compound **2** at *m*/*z* 656 showed a main series of adjacent b_n peaks at 412, 227, and 114 corresponding to the successive loss of (Phe-Pro), (HyLeu-β-Ala), *N*Me-Val, and *N*Me-Val.

A second linear peptide was formed from the $[M + H]^+$ ion at *m*/*z* 656 due to the cleavage at the *N*Me-Val^5-βAla^6 amide bond level, and the resulting b_n ions were detected at *m*/*z* 543, 430, and 283, corresponding to the successive loss of *N*Me-Val^5, *N*Me-Val^4, and Phe^3. A third significant series of ions was observed at 628, 515, 402, and 255, which were assigned to an adjacent a_n ion of the above b_n.

A fourth series of peaks, due to the ease of cleavage of the *N*Me-Val-*N*Me-Val amide bond, was observed at *m*/*z* 543, 396, 299, 185, and 114. These ions correspond to the successive loss of *N*-Me-Val, Phe, Pro, HyLeu, β-Ala, and *N*-Me-Val and suggested the sequence *cyclo*(HyLeu^1-Pro^2-Phe^3-*N*-Me-Val^4-*N*-Me-Val^5-βala^6) of isaridin E, which was further established by the 2D NMR studies.

NMR Studies. The ^{1}H and ^{13}C spectrum of iso-isariin B (**1**) in DMSO-d_6 solution (Table 1) displayed a single stable conformational state (>95%). In agreement with a hexapeptide structure, five amide protons were seen in the ^{1}H NMR spectrum, as well as six carbonyl groups in the range δ_C 169.0−171.8 in the ^{13}C NMR spectrum. Additional ^{1}H NMR and ^{13}C NMR signals suggesting the presence of a linear alkyl chain were also recorded. The assignment of the proton and carbon signals to the amino acid residues was achieved using COSY, TOCSY, HSQC, and HMBC experiments. Thus, the five amide NH's that resonate at δ_H 7.39 (d, *J* = 7.7 Hz), 7.87 (dd, *J* = 3.8; 6.7 Hz), 7.99 (d, *J* = 7.9 Hz), 8.06 (d, *J* = 7.6 Hz), and 8.62 ppm (d, *J* = 6.5 Hz) correlated to their corresponding CHα: δ_C 57.6 (δ_H 4.07, m), δ_C 41.9 (δ_H 4.10, 3.45, m), δ_C 47.9 (δ_H 4.17, m), δ_C 58.7 ppm (δ_H 4.03, m), and δ_C 51.8 ppm (δ_H 4.02, m), respectively. From the CHα signals, further COSY and HMBC correlations led to the identification of the amino acid side chains. The following five standard amino acid residues were thus identified: glycine (Gly), valine (Val), leucine (Leu), alanine (Ala), and valine (Val).

The peptide sequence determination is based on the data from the HMBC experiments. This heteronuclear method was preferred to the $d_{NN}(i, i+1)$ and $d_{\alpha N}(i+1)$ connectivity obtained with the ROESY/NOESY experiments because of the small size of this cyclic peptide. Indeed for such small size cyclopeptides, conformational NOE effects have been reported to interfere with sequential correlations.[8] The sequence of compound **1** was determined by analysis of HMBC correlations between the carbonyl of the residue *i* and the amide and/or the α-protons of the residue *i*+1. Thus amide protons of Gly^2, Val^3, Leu^4, Ala^5, and Val^6 were correlated respectively to the carbonyls C-1, Gly^2, Val^3, Leu^4, and Ala^5, leading to the following peptidic sequence: Gly^2-Val^3-Leu^4-Ala^5-Val^6. The HMBC spectrum shows a correlation between the additionnal diastereotopic methylene (H_2-2) at δ_H 2.51, 2.22 and the carbonyl C-1 (δ_C 169.8). Moreover this diasteroisotopic methylene displays COSY and HMBC correlations

Table 1. NMR Spectroscopic (DMSO-d_6, 298 K, ^{1}H 400.13 MHz; ^{13}C 75.04 MHz) Data for Iso-isariin B (1)

position	δ_C, mult.	δ_H, mult, *J* (Hz)
HMA^1		
1	169.8, qC	
2	37.5, CH_2	2.51, dd (14.7; 9.3)
		2.22, dd (14.7; 2.1)
3	75.5, CH	4.90, ddd (9.3; 3.9; 2.1)
4	36.2, CH	1.66, m
5	31.2, CH_2	1.35, m
		1.01, m
6	28.7, CH_2	1.25, m
		1.16, m
7	22.3, CH_2	1.23, m
8	13.8, CH_3	0.85, m
9	14.8, CH_3	0.82, m
Gly^2		
CO	169.0, qC	
α	41.9, CH_2	4.10, m
		3.45 m
NH		7.87, dd (3.8; 6.7)
Val^3		
CO	171.6, qC	
α	58.7, CH	4.03, m
β	29.5, CH	1.85, m
γ	22.9, CH_3	0.88, m
γ′	18.7, CH_3	0.82, m
NH		8.06, d (7.6)
Leu^4		
CO	171.2, qC	
α	51.8, CH	4.02, m
β	38.6, CH_2	1.49, m
γ	24.1, CH	1.62, m
δ	20.9, CH_3	0.78, m
δ′	19.03, CH_3	0.88, m
NH		8.62, d (6.5)
Ala^5		
CO	171.8, qC	
α	47.9, CH	4.17, m
β	17.30, CH_3	1.20, m
NH		7.99, d (7.9)
Val^6		
CO	170.6, qC	
α	57.6, CH	4.07, m
β	29.5, CH	2.07, m
γ	17.5, CH_3	0.85, m
γ′	18.9, CH_3	0.86, m
NH		7.39, d (7.7)

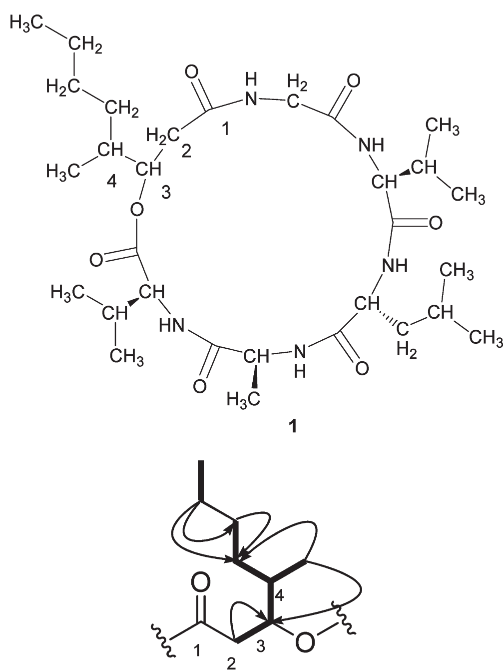

Figure 1. HMA moiety of **1** with $^{1}H-^{1}H$ COSY and HMBC correlations (H→C).

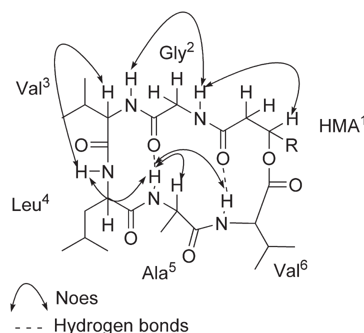

Figure 2. Proposed solution structure of iso-isariin B (**1**) in DMSO-d_6 solution.

with the oxymethine (δ_H 4.90; δ_C 75.5, C-3), suggesting the attachment of this oxymethine C-3 to C-2. The absence of correlation between the CO (Val^{6}) and an amide proton associated with the shift of the oxymethine suggests that the ester linkage is at this position.

The rest of the ^{1}H and ^{13}C signals were assigned to a 3-hydroxy-4-methyloctanoic acid moiety. Indeed, the ^{13}C spectrum clearly shows the presence of two additional methyls at δ_C 13.8 and 14.8 (apart from the four standard amino acid residues Ala, Val, Val, Leu). A COSY correlation between the methyl at δ_H 0.82 and the methine at δ_H 1.66 (H-4) demonstrates the existence of this methyl on C-4. This was confirmed by the HMBC correlation between the carbon at δ_C 75.5 (C-3) and the proton of the methyl at δ_H 0.82. Similarly, an HMBC correlation is observed between a methylene at δ_C 31.2 (C-5) and the methyl at δ_H 0.82, thus fully establishing the methyl position on C-4. As depicted in Figure 1, the rest of this alkyl chain is easily characterized with the COSY and HMBC correlations. All the data are thus consistent with the structure *cyclo*[3-hydroxy-4-methyloctanoyl-Gly^{2}-Val^{3}-Leu^{4}-Ala^{5}-Val^{6}] for compound **1**.

The absolute configurations of the amino acid residues of compound **1** were also determined using Marfey's method.[9] The hydrolysate of **1** was derivatized with 1-fluoro-2,4-dinitro-5-L-alanine amide (FDAA) and analyzed by HPLC (at 340 nm). This showed the presence of L-Gly, L-Ala, L-Val, and D-Leu. Interestingly, the composition and sequence of compound **1** is in agreement with the general structures of isariin cyclodepsipeptides described for *Isaria*, more precisely with isariin of the B type.[7]

Nevertheless, isariins are probably synthesized by the nonribosomal peptide biosynthesis pathway, and the existence of two kinds of isomers could be explained by a lack of selectivity of one of the enzymes involved in the process for the substrate. The biosynthesis of cyclooligomer depsipeptide bassianolide and beauvericin is by the programmed iterative use of modules for the assembly of dipeptidol monomers from amino acid and hydroxycarboxylic acid precursors, and the formation of cyclic ester peptide occurs by unusual recursive condensation of this monomer.[10,11] The usual isomer isariin B was not found during this study, and the occurrence of iso-isariin B in *B. felina* is related to the existence of a polyketide route leading to the HMA residue. The HMA moiety is unusual in this kind of isariin peptide but has been found in beauveriolide cyclopeptides isolated from *Beauveria* sp.[12,13]

Attempts to isolate the corresponding 3-hydroxy-4-methyloctanoic acid by acidic hydrolysis of **1** were unsuccessful. Therefore the absolute configurations of the oxymethine (C-3) and of the C-4 remain undefined. It should be mentioned that Tomada et al. have demonstrated that the absolute stereochemistry of the HMA residue in natural beauveriolide is (3*S*,4*R*).[14]

The pattern of hydrogen bonding as shown in Figure 2 is deduced from the thermal coefficient values of the amide proton measured in DMSO-d_6 (Supporting Information, Table S1). The low value of the thermal coefficient of amide protons of Ala^{5} and Val^{6} ($\Delta\delta/\Delta T$ respectively -3.4 and -0.8 ppb·K^{-1}) suggests that they are involved in intramolecular hydrogens bonds, whereas the NH's of Leu^{4} and Val^{3} are exposed to the solvent ($\Delta\delta/\Delta T$ respectively -6.6 and -5.6 ppb·K^{-1}). An ambiguity persists in the case of the amide proton of Gly^{2}, as its thermal coefficient is intermediate. However, the NOE correlation seen between the NH (Gly^{2}) and the NH (Val^{3}) suggests that the NH of Gly^{2} is exposed to the solvent. The strong d_{NN} NOE between NH (Ala^{5}) and NH (Val^{6}) and the NOE between NH (Gly^{2}) and NH(Val^{3}) provide the location of the turn segment in the cyclic peptide. In the case of compound **1**, the Val^{3}-^{D}Leu^{4}-Ala^{5} segment is likely to form a β-turn with an intramolecular hydrogen bond between CO (Gly^{2}) and NH (Ala^{5}). A second intramolecular hydrogen bond between CO (HMA^{1}) and NH (Val^{6}) would stabilize this structure.

The ^{1}H and ^{13}C spectra of compound **2** in DMSO-d_6 (Table 2) solution also displayed a single stable conformational state (>95%). The two amide protons were identified in the ^{1}H NMR spectrum, as well as the presence of six carbonyl groups in the range δ_C 168.6−173.9 in the ^{13}C NMR spectrum, in agreement with a hexapeptide structure. The two intense singlets at δ_H 2.97 and 3.12 are indicative of two *N*-methylamino acids.

Assignment of protons and carbons to amino acid residues was achieved, with the help of COSY, TOCSY, HSQC, and HMBC experiments, leading to the identification of the six amino acid residues as leucine (Leu), proline (Pro), phenylalanine (Phe), valine (Val), valine (Val), and β-alanine (βAla). The peptide sequence determination was based on the HMBC experiment,

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Table 2. NMR Spectroscopic Data ($CDCl_3$-*d6*, 298 K, ^1H 400.13 MHz; ^{13}C 75.04 MHz) for Isaridin E (**2**)

position	δ_C, mult.	δ_H, mult, *J* (Hz)
HyLeu^1		
CO	169.8, qC	
α	73.4, CH	5.32, dd (11.3; 1.8)
β	38.8, CH_2	1.95, m
		1.23, m
γ	24.7, CH	1.95, m
δ	23.3, CH_3	0.98, d (3.3)
δ′	20.5, CH_3	0.98, d (3.3)
Pro^2		
CO	172.0, qC	
α	60.9, CH	4.08, dd (8.6; 2.0)
β	32.2, CH_2	2.20, m
		2.11, m
γ	21.9, CH_2	1.75, m
		1.32, m
δ	47.1, CH_2	3.48, m
Phe^3		
CO	173.7, qC	
α	53.8, CH	4.64, ddd (7.5; 5.1; 2.6)
β	35.0, CH_2	2.97, m
Φ1′	136.4, CH	
Φ3′, Φ5′	128.7, CH	7.25, m
Φ2′, Φ5′, Φ4′	127.2, CH	7.20, m
NH		8.12, d (7.5)
N-Me-Val^4		
CO	169.7, qC	
N-CH_3	29.6, CH_3	3.12, s
α	57.6, CH	5.11, d (10.7)
β	27.6, CH	2.35, m
γ	18.8, CH_3	0.85, d (6.8)
γ′	20.2, CH_3	0.86, d (6.8)
N-Me-Val^5		
CO	168.6, qC	
N-CH_3	28.9, CH_3	2.97, s
α	66.5, CH	4.27, d (10.7)
β	27.6, CH	2.40, m
γ	19.4, CH_3	0.91, d (6.4)
γ′	19.7, CH_3	0.85, d (6.6)
β-Ala^6		
CO	173.9, qC	
α	35.5, CH_2	4.14, m
		3.12, m
β	35.3, CH_2	2.60, dt (14.5; 3.1)
		2.47, m
NH		7.39, d (10.1)

which provided the following peptide sequence: Leu^1-Pro^2-Phe^3-*N*-Me-Val^4-*N*-Me-Val^5-*β*-Ala^6. All the data are thus consistent with a *cyclo*[HyLeu^1-Pro^2-Phe^3-*N*-Me-Val^4-*N*-Me-Val^5-*β*-Ala^6] structure for compound **2**, which has been previously reported as isaridin E.[7] However, to go beyond the described primary structure, we achieved a conformational analysis of isaridin E (**2**) using X-ray diffraction studies (Figure 3). X-ray quality crystals of

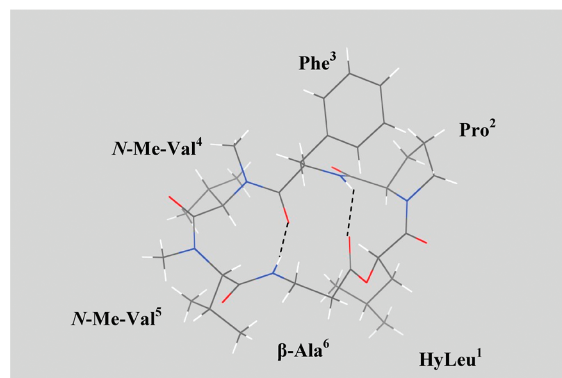

Figure 3. Crystal structure of isaridin E (**2**) (dotted lines indicate hydrogen bonds).

compound **2** were obtained from CH_3CN, 65% yield at 24 °C. The values of the backbone torsion angles and the H bonds are summarized in Tables S2 and S3. Notably, the HyLeu^1-Pro^2 and the *N*-Me-Val^3- *N*-Me-Val^4 amide bonds adopt a *cis* conformation ($\omega \pm 0$). Two potent intramolecular H bonds, NHPhe^3→CO*β*Ala^6 and NH*β*Ala^6→COPhe^3, are observed with a O−HN hydrogen bond distance of 2.19 and 2.08 Å, respectively. Thus, the X-ray structure of isaridin E is closely related to that described for isaridins A and B and also provides an example of type-VI turns, as defined for protein, in which the turning segments have the central peptide unit in a *cis* conformation.[16]

The crystal structure of isaridin E seems to be consistent with the solution structure in DMSO-d_6. Indeed, a strong NOE correlation between the Hα of the Pro^2 (δ_H 4.08) and the Hα of the HyLeu^1 (δ_H 5.32) indicates that the HyLeu^1-Pro^2 amide bonds are in a *cis* configuration. This stereochemistry is further confirmed by the *γ*-carbon ^{13}C chemical shifts of C_γ of Pro^2 at δ_C 21.9, in agreement with the presence of a *cis* proline.[17] Moreover, the relatively low thermal coefficient values of the amide proton of Phe^3 and *β*Ala^6 (respectively $-\Delta\delta/\Delta T$ = 0.06 and 1.90 ppb·K^{-1}) confirm the presence of two potent intramolecular H bonds, NHPhe^3→CO*β*Ala^6 and NH*β*Ala^6→COPhe^3.

Insecticidal Studies. *B. felina* is a facultative entomopathogen fungus, as is *B. bassiana*, which has been used as a biopesticide against a broad range of insects in agriculture. Since several cyclopeptides from *B. felina* featured interesting insecticidal activities,[4,5,17] our compounds **1** and **2** were evaluated for their insecticidal properties against *Sitophilus* spp. and *Callosobruchus maculatus*, two pest insects responsible for seed and crop infestations. After five days of surface contact with isaridine E (**2**), relatively weak lethal doses 50 (LD_{50}) of 300 and 220 μg/mL were found against these two species. However, a remarkable LD_{50} of 10 μg/mL was measured for compound **1** against *Sitophilus* spp. Thus, compound **1** can be considered, like bassianolide, beauvericin, and eniantins, which have been shown to act as virulence factors for *B. bassiana* in pathogenic context,[10] as an agent of competition, defending the niche of the producer fungi against competing parasites, thus providing a possible explanation for the ecological role of these metabolites.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer model 341 polarimeter, and the $[\alpha]_D$ values are given in deg cm^2 g^{-1}. Melting points were determined on a Büchi melting point B-545 apparatus. Mass spectra were recorded on an

API Q-STAR PULSAR i (Applied Biosystems). For the CID spectra, the collision energy was 40 eV and the collision gas was nitrogen. The ^{13}C NMR spectra were recorded on a Bruker AC 300 spectrometer operating at 75.47 MHz (for ^{13}C). The ^{1}H and 2D NMR spectra were recorded at 298 K on a Bruker AVANCE 400 spectrometer operating at 400.13 MHz. ^{1}H and ^{13}C chemical shifts were reported with respect to the residual signal of dimethylsulfoxide, whose shifts relative to TMS were taken at 2.49 ppm and at 39.5 ppm, respectively. For the HMBC experiments the delay ($1/2J$) was 70 ms, and for the ROESY experiments the mixing time was 150 ms. TOCSY experiments were acquired with 40 and 80 ms spin-lock time using a MLEV-17 sequence. Temperature coefficients of amide protons were obtained in the range 298−318 K by the acquisition of five 1D ^{1}H spectra using 5 K temperature increments.

X-ray Crystal Structure Determination. A colorless crystal, $0.25 \times 0.11 \times 0.08$ mm^3 was glued to a glass fiber. Intensity data were collected at 200 K with a Bruker-Enraf Kappa-CCD diffractometer equipped with a CCD two-dimensional detector [λ Mo Kα = 0.71073 Å]. Data reduction was performed with EVALCCD software. Data were corrected for Lorentz and polarization effects, and a semiempirical absorption correction based on symmetry equivalent reflections was applied by using the SADABS program. Lattice parameters were obtained from least-squares analysis of 109 reflections. The structure was solved by direct method and refined by full matrix least-squares, based on F^2, using the Crystals software package. All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were located with geometrical restraints in the riding mode.

Crystal structure analysis: monoclinic, space group $P2_1$; dimensions a = 13.0206(5) Å, b = 9.8032(7) Å, c = 15.4303(15) Å, β = 94.550(6)°; V = 1963.4(2) Å^3; Z = 2; the data were collected in the $h\,k\,l$ range −18 to 17, −13 to 13, −21 to 20. Total reflections collected: 20 570; independent reflections: 6006 (4409 $I > 2\sigma(I)$); data was collected up to a $2\theta_{max}$ value of 60°. Number of variables: 452; $R[I > 2\sigma(I)]$ = 0.0519, wR_2(all) = 0.144, S = 0.953; highest residual electron density 0.47e Å^{-3}. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, on quoting the depository number CCDC-795741. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

Fungal Materiel. The fungus used in this study was isolated from the soil of an Algerian cavern in 2006 and is now maintained at the LCP culture collection (culture collection of the Muséum National d'Histoire Naturelle) under the number LCP 5299.

Fermentation and Isolation. The fungus *Beauveria felina* was maintained in potato dextrose agar at 25 °C. The agar was cut into small plugs and inoculated into 16 Erlenmeyer flasks (750 mL) containing medium defined by Riley[18] (30.0 g glucose (Sigma Aldrich), 5.0 g tryptone (Difco), 3.0 g yeast extract (Merck), 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, and 0.3 g $MgSO_4 \cdot 7H_2O$ for 1 L). After incubation at 25 °C for 10 days on a rotary shaker (200 rpm), the culture was centrifuged (7000 rpm, 20 min) to separate the mycelium and the filtrate. After lyophilization, the mycelium (66.4 g) was macerated in MeOH (4 × 800 mL, 2 days each) and filtered. The MeOH phase was concentrated under reduced pressure to leave a brown gum (9.40 g).

This crude extract was passed through a Sephadex LH-20 column with MeOH as eluant to obtain 12 fractions, F1−F12. Fraction F3 mainly contained iso-isariin B (**1**) (100 mg). Fraction F5 was subjected to column chromatography on silica gel (cyclohexane/EtOAc fom 50:50 to 100), and subfraction 7 contained isaridin E (**2**) (75 mg).

Hydrolysis of Amino Acid Residue. The iso-isariin B (**1**) sample (3.0 mg) was dissolved in 6 N HCl (1 mL) and heated at 110 °C for 24 h. The solvent was removed under reduced pressure, and the resulting material was subjected to further derivatization. The hydrolysate mixture (3.5 mg) or the amino acid standards (0.5 mg) were dissolved in 0.1 mL of water and treated with 0.2 mL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in acetone and 0.04 mL of 1.0 M sodium bicarbonate. The vials were heated at 50 °C for 90 min, and the contents after cooling at room temperature were neutralized with 1 N HCl. After degassing, an aliquot of the FDAA derivative was diluted in MeOH and purified by chromatography using a RP C-18 column (250 × 4.6 mm) and a linear gradient of acetonitrile and water containing 0.05% trifluoroacetic acid from 20:80 to 50:50 in 20 min and then isocratic. The flow rate was 1 mL/min, and the absorbance detection was at 340 nm. The chromatogram was compared with those of amino acid standards treated in the same conditions.

Amino acids in iso-isariin B (**1**): Gly (8.7 min), L-Ala (9.8 min), L-Val (13.0 min), D-Leu (18.7). t_R of standards: Gly (8.6 min), L-Ala (9.8 min), L-Val (13.1 min), D-Val (15.6 min), L-Leu (16.2 min), D-Leu (18.6).

Insecticidal Activity. The insecticidal effect of substances was determined in Petri dishes of diameter 90 mm. Products diluted in acetone were tested at five concentrations (100, 200, 400, 800, and 1000 mg/mL). A 50 μL aliquot of each solution was distributed in a dish before allowing the solvent to evaporate for 3 min. A batch of 20 insects between 24 and 48 h old from the mass rearing was introduced into each box and then left at room temperature. Adult mortality was monitored at 1 h after treatment and then every 12 h for 120 h (five days). Each treatment was repeated three times; control experiments with insects treated only with the solvent dilution and with untreated insects were also performed.

*Iso-isariin B (**1**):* $C_{30}H_{53}N_5O_7$; white needles (CH_3CN/H_2O, 80:20); mp 265−270 °C; $[\alpha]^{20}_{D}$ −10 (c 0.1, MeOH); ^{1}H and ^{13}C data, see Table 1; ESI-qTOF MS/MS on $[M + H]^+$ (ce 40 eV) m/z (%) 596 (15), 578 (4), 568 (10), 551 (7), 523 (2), 507 (1), 497 (43), 483 (8), 479 (25), 469 (3), 465 (1), 455 (6), 438 (3), 426 (65), 412 (4), 408 (100), 401 (13), 398 (9), 384 (20), 366 (4), 356 (5), 338 (4), 327 (5), 313 (92), 309 (5), 302 (20), 295 (99), 285 (13), 284 (56), 277 (1), 273 (7), 270 (11), 267 (83), 257 (15), 239 (22), 231 (8), 228 (3), 225 (10), 217 (8), 214 (7), 213 (96), 211 (11), 203 (11), 196 (12), 189 (84), 186 (6), 185 (99), 183 (2), 168 (11), 157 (11), 143 (46), 139 (1), 118 (7), 86 (58), 72 (85).

*Isaridin E (**2**):* $C_{35}H_{53}N_5O_7$; colorless crytal (CH_3CN/H_2O, 80:20); mp 140−145 °C; $[\alpha]^{20}_{D}$ −166 (c 0.1, MeOH); ^{1}H and ^{13}C see Table 2; ESI-qTOF MS/MS on $[M + H]^+$ (ce 40 eV) m/z (%). 656 (28), 628 (1), 612 (34), 543 (59), 515 (42), 499 (1), 472 (4), 430 (8), 412 (1), 402 (18), 396 (2), 368 (13), 331 (2), 299 (9), 287 (2), 283 (5), 255 (9), 227(4), 212 (9), 203 (6), 194 (100), 185 (17), 169 (1), 142 (6), 114 (2), 86 (13), 69 (1).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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