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Isolation and Structural Elucidation of Proline-Containing Cyclopentapeptides from an Endolichenic *Xylaria* sp.

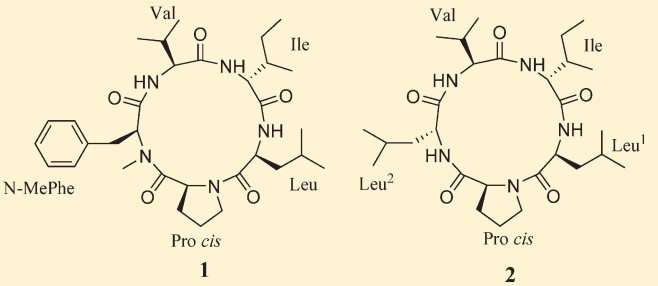
Wen Wu,[†,‡] Huanqin Dai,[†] Li Bao,[†] Biao Ren,[†] Jingcai Lu,[*,‡] Yuanming Luo,[†] Liangdong Guo,[†] Lixin Zhang,[†] and Hongwei Liu[*,†]

[†]Key Laboratory of Systematic Mycology & Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, People's Republic of China

[‡]College of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, No. 103 Wenhua-Road, Shenyang 110016, People's Republic of China

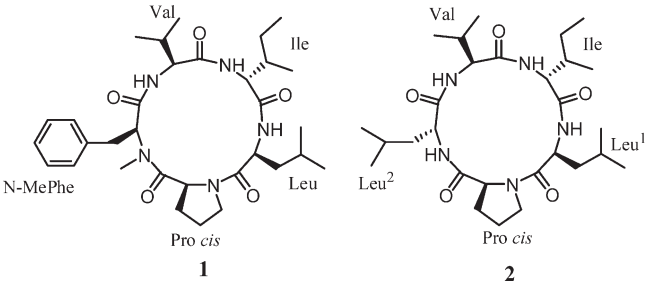
Supporting Information

ABSTRACT: Two new cyclic pentapeptides (**1** and **2**) and the known blazein (**3**), ganodesterone (**4**), ergosterin (**5**), cerevisterol (**6**), 24-methylcholesta-4,6,8(14),22-tetraen-3-one (**7**), 5,8-epidioxyergosta-6,22-dien-3-ol (**8**), 16-α-D-mannopyranosyloxyisopimar-7-en-19-oic acid (**9**), and 16-hydroxy isopimar-7-en-19-oic acid (**10**) have been isolated from the crude extract of an endolichenic *Xylaria* sp. The structures of **1** and **2** were elucidated primarily by NMR and MS methods. The absolute configurations of **1** and **2** were assigned using Marfey's method on their acid hydrolysate. Compounds **1**−**10** were evaluated for activity against fungi and for synergistic antifungal activity. Compound **1** showed synergistic antifungal activity against *Candida albicans* SC5314 with 0.004 μg/mL ketoconazole.



Xylaria is a genus of ascomycetes commonly found as wood-decaying and endophytic fungi. The genus *Xylaria* has been proven to be a valuable source of bioactive agents. Diverse secondary metabolites have been isolated, such as the chemokine receptor (CCR5) antagonist 19,20-epoxycytochalasin Q,[1] the antifungal metabolites multiplolides A and B,[2] the NPY Y5 receptor antagonists xylarenals A and B,[3] and the cytotoxic eremophilanolides.[4] We have recently embarked on a research program looking for new chemistry and bioactive metabolites from the genus *Xylaria* collected in China.

Endolichenic fungi living in the thalli of lichens are analogous to the plant endophytes inhabiting the intercellular spaces of the hosts. Limited chemical investigations have been conducted on metabolites of endolichenic fungi, but they have demonstrated great potential as a new source of novel bioactive natural products.[5−8] Examination of the lichen *Leptogium saturninum* (Dicks.) Nyl. (Collemataceae) afforded several endolichenic fungi, one of which was identified as *Xylaria* sp. (75-1-3-1). Thin-layer chromatography and HPLC analysis of a solid-substrate fermentation indicated the fungus produced many secondary metabolites. An organic solvent extract of its solid-substrate fermentation culture showed synergistic antifungal activity against *Candida albicans* (SC5314) by a high-throughput synergistic screening assay.[9] Two new cyclicpeptides (**1** and **2**) along with eight known metabolites were isolated from the extract by a combination of column chromatography over silica gel, LH-20, and ODS, followed by semipreparative HPLC. Details of the isolation, structure elucidation, and biological activities of these compounds are reported herein.



The known metabolites isolated from the crude extract were identified as blazein (**3**), ganodesterone (**4**), ergosterin (**5**), cerevisterol (**6**), 24-methylcholesta-4,6,8(14),22-tetraen-3-one (**7**), 5,8-epidioxyergosta-6,22-dien-3-ol (**8**), 16-α-D-mannopyranosyloxyisopimar-7-en-19-oic acid (**9**), and 16-hydroxyisopimar-7-en-19-oic acid (**10**) by comparison of their NMR and MS data with those reported.[10−15]

Compound **1** was obtained as a colorless powder. It was assigned a molecular formula of $C_{32}H_{49}N_5O_5$ on the basis of its HR-TOF-ESIMS. Analysis of the ^1H, ^{13}C, and HMQC NMR spectroscopic data of **1** (Table 1) revealed the presence of three

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Table 1. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), COSY, HMBC, and NOESY Correlations for 1

amino acid	position	δ_C (multi)	δ_H, mult (J in Hz)	^{1}H−^{1}H COSY	HMBC	NOESY
L-*N*-methyl phenylalanine	CO	170.9			α, NHVal	
	α	56.5	5.07 (m)	*β*, N-CH_3	N-CH_3	*β*, N-CH_3, NHVal
	β	34.4	2.89 (dd, 14.2, 5.7)	α	α, ortho	α, ortho
			3.02 (m)			
	γ	137.8			*meta*	
	ortho	129.0	7.20 (m)	*meta, para*	*meta, para*	α, *β*
	meta	128.6	7.24 (m)	*ortho, para*	*para*	
	para	126.8	7.17 (m)	*ortho, meta*	*ortho*	
	N-CH_3	30.6	3.05 (s)	α	α, *β*	α, *β*Pro
L-valine	CO	171.5			α, NHIle	
	α	58.1	3.95 (t, 9.8)	*β*, NH	*γ*, NH	*β*, NH, NHIle, αN-MePhe
	β	26.9	1.98 (m)	α, *γ*	α, *γ*	α, *γ*
	γ	18.9	0.71 (d, 6.8)	*β*	α, *γ*	*β*
		20.2	0.81 (d, 4.6)			
	NH		8.12 (d, 9.5)	α	α, CON-MePhe	α, *β*, αN-MePhe, NHIle
D-isoleucine	CO	170.8			α, NHLeu	
	α	55.7	4.23 (m)	*β*, NH	*β*-CH_3	*β*, *γ*, *δ*, NH, NHLeu
	β	38.1	1.53 (m)	α, *γ*, *β*-CH_3	α, *δ*, *β*-CH_3	α, *δ*, *β*-CH_3
	γ	26.4	1.00 (m)	α, *β*, *γ*, *β*-CH_3	*β*, *δ*, *β*-CH_3	
			1.26 (m)			
	δ	11.9	0.83 (d, 7.5)	*γ*	*γ*, *β*-CH_3	
	β-CH_3	14.7	0.69 (d, 6.8)	α, *β*, *γ*	α, *γ*	
	NH		6.92 (d, 8.9)	α	COVal	α, αVal
L-leucine	CO	169.4			α	
	α	47.1	4.70 (m)	*β*, NH	NH	*β*, *δ*, αPro, NH
	β	41.7	1.40 (m)	α, *γ*	α, *δ*	α, *δ*
	γ	24.6	1.40 (m)	*β*, *δ*	*β*, *δ*	
	δ	22.7	0.78 (d, 5.4)	*γ*		α, *β*
		23.3	0.81 (d, 4.6)			
	NH		8.43 (d, 9.4)	α	α, COIle	α, *β*, αIle
L-proline	CO	172.5			α, *β*, αN-MePhe	
	α	59.2	5.07 (m)	*β*	*β*	*β*, αLeu
	β	30.6	0.98 (m), 1.84 (m)	α	α	α
	γ	20.9	1.30 (m), 1.63 (m)	*β*	α, *β*	
	δ	46.2	3.28 (m)	*γ*	α	

amide *N*-H protons (δ_H 6.92, 8.12, and 8.43, respectively), seven methyl groups (including one *N*-methyl), six methylenes, eight methines (five of which are heteroatom-bonded), a monosubstituted benzene, and five carboxylic carbons.

Interpretation of the ^{1}H, ^{13}C, and 2D NMR data of **1** revealed its peptidic nature. The independent spin system of the type X-CH-CH_2-CH_2-CH_2-X′ was defined using COSY and HSQC (Table 1), indicating the presence of one proline unit. The spin systems X-CH-CH$(CH_3)_2$, X-CH-CH(CH_3)-CH_2-CH_3, and X-CH-CH_2-CH$(CH_3)_2$ were identified, suggesting the existence of valine, isoleucine, and leucine residues. The remaining independent spin system of the type X-CH-CH_2-X′, together with the HMBC correlations from βH_2(N-MePhe) to the *ortho* carbons of the benzene, from αH(N-MePhe) to C-NMe (δ_c 30.6) and CO(N-MePhe), and from H_3-NMe (δ_H 3.05) to αC(N-MePhe), established the *N*-methylphenylalanine (*N*-MePhe) unit. The amino acid composition was further confirmed by HPLC analysis of the acid hydrolysate of **1** after derivatization with the Marfey reagent (FDAA), allowing the absolute configurations at the α-carbons to be assigned as L for valine, leucine, *N*-methylphenylalanine, and proline residues and D for the isoleucine residue.[16] Because only 10 of the calculated 11 degrees of unsaturation could be accounted for, it became clear that **1** was a cyclic peptide. Upon extensive analysis of these data, **1** was assigned as a cyclic pentapeptide containing one equivalent each of valine, leucine, isoleucine, *N*-methylphenylalanine, and proline.

The carbonyl carbons within each residue were assigned from HMBC correlations between the C═O and their respective α-protons (Table 1), whereas each peptide-bond amide proton was identified from ^{1}H−^{1}H COSY correlation to its adjacent α-proton and by reference to known values for these residues. The two bond ^{1}H−^{13}C correlations of NH(Leu)/CO (Ile), NH(Ile)/CO (Val), NH (Val)/CO (N-MePhe), and H_3-NMe-(N-MePhe)/CO (Pro), along with the NOE correlations of αH(Pro)/αH(Leu), NH(Leu)/αH(Ile), NH(Ile)/αH(Val), αH(Val)/αH(N-MePhe), NH(Val)/αH(N-MePhe), and H_3-NMe(N-MePhe)/*β*H(Pro) (Figure 1), established the complete

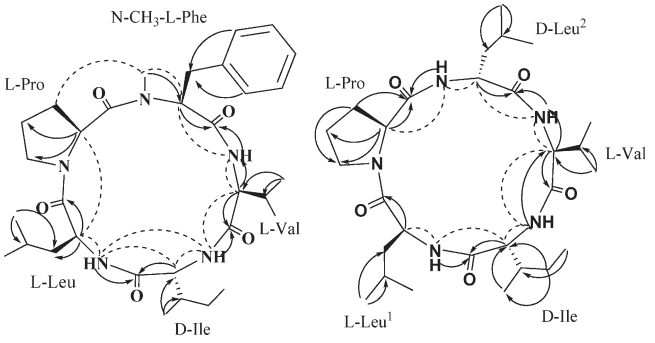

Figure 1. HMBC (H→C) and NOE correlations (dashed arrows) for **1** and **2**.

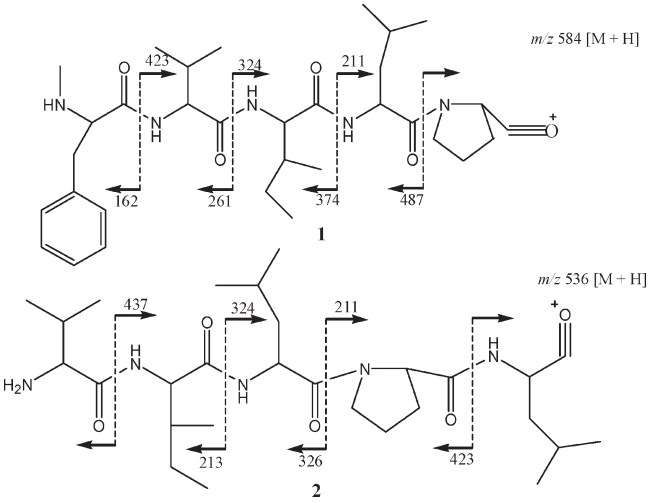

Figure 2. MALDI-TOF/TOF sequence ions (m/z) for protonated molecular $[M + H]^+$ ions of **1** and **2**.

amino acid sequence of **1** as *cyclo*-(-NMePhe-Pro-Leu-Ile-Val-). The complete amino acid sequence of **1** was also confirmed on the basis of the results of MALDI-TOF/TOF experiments. Although there was more than one possible ring-opening position for the peptide, the preferred ring-opening of **1** occurred at the N-MePhe-Pro amide bond. One ion series started with loss of 97 amu due to Pro, leaving m/z 487 (-Leu-Ile-Val-NMePhe plus H), which then lost 113 amu (Leu), affording m/z 374 (-Ile-Val-NMePhe plus H). The latter fragment lost 113 amu (Ile), yielding m/z 261 (-Val-NMePhe plus H), then lost 99 amu (Val) to give m/z 162 (-NMePhe plus H) (Figure 2). Another pathway left the major fragment m/z 423 $[M + H - N\text{-MePhe}]^+$ after loss of 161 amu (N-MePhe) from m/z 584 $[M + H]^+$, which then lost sequentially 99 amu (Val) and 113 amu (Ile), leaving m/z 324 $[M + H - N\text{-MePhe-Val}]^+$ and 211 $[M + H - N\text{-MePhe-Val-Ile}]^+$, respectively.

The geometry of the peptidic linkages was assigned on the basis of the differences in ^{13}C chemical shifts of the Cβ and Cγ of the proline residues.[17,18] The ^{13}C NMR data of **1** indicated that the proline peptide bonds were *cis*, as shown by the large chemical shift difference of Pro $\Delta\delta$ Cβ−Cγ = 9.7 (Table 1).

The molecular formula of cycloaspeptide G (**2**) was determined to be $C_{28}H_{49}N_5O_5Na$ on the basis of its HR-TOF-ESIMS. Analysis of the ^1H and ^{13}C NMR spectroscopic data of **2** (Table 2) revealed structural features similar as those found in **1**, except that the signals for the N-MePhe moiety were replaced by those from Leu. These observations were confirmed by relevant $^1H-^1H$ COSY, HMBC, and NOESY correlations and MALDI-TOF/TOF sequence analysis (Figures 1, 2). The HPLC analyses of the acid hydrolysate of **2** after derivatization with FDAA revealed that L-valine, L-leucine, D-leucine, D-isoleucine, and L-proline were present in **2**. The NMR data of the leucine residue between proline and isoleucine residues were quite similar to those of **1**, indicating the L configuration for Leu.[1] The configuration for another leucine residue was thus determined to be D. Compounds **1** and **2** are classified as proline-containing cyclopentapeptides. The proline-containing cyclopeptides are usually found from the invertebrate animals and microorganisms typically associated with marine habitats. Examples of this kind of bioactive natural product are the axinellins,[19] axinastatins,[20] phakellistatins,[21] and others.[22] The role of proline in these molecules has been associated with control of conformation of the molecule in solution because of the restricted dihedral angle of proline.[23]

Compounds **1**−**10** were tested for antifungal activity against *C. albicans* (SC5314). None of the compounds showed antifungal activity at the concentraton of 100 μg/mL. However, compound **1** showed strong synergistic antifungal activity against *C. albicans* at 6.25 μg/mL with 0.004 μg/mL ketoconazole (FICI < 0.3125). Compounds **2**−**10** did not show synergistic antifungal activity at 100 μg/mL with 0.004 μg/mL ketoconazole. Our previous study has demonstrated that the depsipeptide beauvericin is a potent synergistic antifungal agent at 1 μg/mL with 0.004 μg/mL ketoconazole (FICI < 0.375).[9] The synergistic antifungal activity of **1** is relatively weaker than that of beauvericin.

The synergistic antifungal activity is an interesting new bioactivity for this class of compounds. In this work, the discovery of new cyclopeptides from endolichenic fungus *Xylaria* strain further expanded structural diversity of the secondary metabolites produced by endolichenic fungi.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ^1H and ^{13}C NMR data were acquired with Bruker Avance-500 and -600 spectrometers using solvent signals (DMSO-d_6; δ_H 2.49/δC39.7) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. MALDI-TOF/TOF spectra were recorded on a 4700 Proteomics analyzer (Applied Biosystems, USA), and HR-TOF-ESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

Fungal Material. The culture of *Xylaria* sp. (Xylariaceae) was isolated by one of the authors (L.G.) from the lichen *Leptogium saturninum* (Dicks.) Nyl. collected from Zixi Mountain, Yunnan, People's Republic of China, in November 2006. The fungus was identified by L.G. and assigned the accession no. 75-1-3-1 in L.G.'s culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The fungal strain was cultured on slants of potato dextrose agar at 25 °C for 10 days. Agar plugs were inoculated in 500 mL Erlenmeyer flasks containing 120 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract; the final pH of the media was adjusted to 6.5 before sterilization) and incubated at 25 °C on a rotary shaker at 170 rpm for one week. Large-scale fermentation was carried out in thirty 500 mL Fernbach flasks each containing 80 g of rice and 120 mL of

Table 2. ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), COSY, HMBC, and NOESY Correlations for 2

amino acid	position	δ_C (multi)	δ_H, mult (J in Hz)	$^1H-^1H$ COSY	HMBC	NOESY
L-valine	CO	170.5			α, NHIle	
	α	58.5	3.85, dd (10.8, 9.0)	β	β, γ	β, γ, NH, NHIle
	β	26.0	2.02, m	α, γ	γ	α, γ, NH
	γ	19.7	0.82	β, γ	α, β, γ	α, β, NH
		18.6	0.80	γ	α, β, γ	
	NH		8.56, d (8.9)	α	α, COLeu[2]	α, β, γ, αLeu[2]
D-isoleucine	CO	170.2			α, NHLeu[1]	
	α	55.7	4.20, dd (8.4, 7.2)	β	γ, β-CH_3	β, β-CH_3, NH, NHLeu[1]
	β	37.6	1.54, m	α, β-CH_3	α, γ, δ, β-CH_3	α, NH
	γ	25.6	1.00, m	β, γ, δ	α, β-CH_3	
			1.26, m	β, γ, δ		
	δ	11.5	0.81	γ	γ	
	β-CH_3	14.6	0.72, d (6.8)	β	α, γ	α, NH
	NH		7.17, d (8.7)	α	COVal	α, αVal
L-leucine[1]	CO	168.9			α, β	
	α	47.8	4.58	β	β	β, NH
	β	41.4	1.42, m	α, β, γ	α, γ, δ	
			1.50, m	α, β, γ		
	γ	24.4	1.36, m	β, δ	β, δ	
	δ	22.5	0.83	γ	β, δ	
		22.7	0.81	γ	β, δ	
	NH		8.32, d (8.3)	α	COIle	α, αIle
L-proline*cis*	CO	170.5			α, β, NHLeu[2]	
	α	60.0	4.68, d (7.8)	β	β	β, NHLeu[2]
	β	32.5	1.89, m	α, β, γ	α	α, γ
			2.05, m	α, β, γ		
	γ	21.2	1.64, m;	β, γ, δ	α, δ	β
			1.77, m	β, γ, δ		
	δ	45.8	3.34, m	γ	α, β	
D-leucine[2]	CO	172.6			α, NHVal	
	α	51.4	4.11	β	β	β, NH
	β	39.6	1.39, m	α	α, γ, δ	
			1.43, m	α		α
	γ	24.4	1.47, m	δ	β, δ	
	δ	22.3	0.89, d (6.4)	γ	β, δ	
		22.3	0.79	γ	β, δ	
	NH		8.24, d (7.5)		COPro	α, αPro

distilled H_2O. Each flask was inoculated with 5.0 mL of the culture medium and incubated at 25 °C for 40 days.

Extraction and Isolation. The fermented rice substrate was extracted with EtOAc by exhaustive maceration (3 × 4 L), and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (5 g). The EtOAc extracts were subjected to silica gel column chromatography using a gradient of *n*-hexane−EtOAc (1:0, 100:1, 50:1, 30:1, 20:1, 10:1, 9:1, 5:1, 2:1, v/v) and CH_2Cl_2−MeOH gradient elution (50:1, 20:1, 10:1, 9:1, 4:1, 2:1, v/v). Fractions were analyzed by TLC and grouped into 27 fractions (E-1−E-27). Fraction E-6 was separated by ODS column chromatography using a gradient of MeOH in H_2O (60−100%) to give 12 subfrations (E-6-1−E-6-12). Compound **7** (2.0 mg, t_R 18.9 min) was obtained from subfraction E-6-2 by reversed-phase HPLC (100% MeOH). Fraction E-10 was separated by Sephadex LH-20 column chromatography (CC) using CH_2Cl_2−MeOH (1:1, v/v) to give 10 subfrations (E-10-1−E-10-10). Compounds **3** (2.5 mg, t_R 26.9 min), **4** (5.2 mg, t_R 24.3 min), and **8** (2.0 mg, t_R 28.4 min) were obtained from subfraction E-10-7 by reversed-phase HPLC (95% MeOH in water). E-10-8 was subjected to silica gel column chromatography using a gradient of *n*-hexane−EtOAc (20:1, 10:1, 9:1, 8:2, 7:3, 6:4, v/v); the subfraction eluted with *n*-hexane−EtOAc (10:1) afforded **5** (5.2 mg) by recrystalliztion. Fraction E-17 was first separated by a silica gel column using CH_2Cl_2−MeOH gradient elution (1:0, 100:1, 50:1, 20:1, 10:1, 9:1, 4:1, 2:1, v/v) to give 10 subfractions (E-17-1−E-17-10). Subfraction E-17-5 was further purified by reversed-phase HPLC (72% MeOH−H_2O) to yield **1** (22.8 mg, t_R 31.3 min) and **2** (2.5 mg, t_R 44.8 min). Fraction E-18 was purified by reversed-phase HPLC (95% MeOH−H_2O) to afford **6** (3.5 mg, t_R 18.2 min). Fraction E-20 was first isolated on an ODS column using MeOH−H_2O gradient elution (40−100%) to give 18 subfrations (E-20-1−E-20-18). Purification of the resulting subfractions with CH_2Cl_2−MeOH (1:1, v/v) by Sephadex LH-20 column chromatography (CC) gave **10** (10.2 mg) from subfraction E-20-3 and **9** (17.4 mg) from subfraction E-20-5, repectively.

Compound 1 (cyclo(*N*-methyl-L-Phe-L-Val-D-Ile-L-Leu-L-Pro)): white powder; $[\alpha]^{25}_D$ −46.8 (*c* 1.36, MeOH); IR (neat) ν_{max} 3260,

3064, 2961, 2875, 1636, 1533, 1450, 1408, 1384, 1346, 1305, 1204, 1174, 1133, 1083, 699 cm^{-1}; NMR data, see Table 1; MALDI-TOF/TOF data, see Figure 2; HR-TOF-ESIMS m/z 606.3627 $[M + Na]^+$ (calcd for $C_{32}H_{49}N_5NaO_5$, 606.3626).

Compound 2 (cyclo(L-Val-D-Ile-L-Leu-L-pro-D-Leu)): white powder; $[\alpha]^{25}_D$ −16.8 (*c* 0.2, MeOH); IR (neat) ν_{max} 3284, 2961, 2930, 1667, 1548, 1386, 1203, 1135, 1027, 959, 835, 800, 720 cm^{-1}; NMR data, see Table 2; MALDI-TOF/TOF data, see Figure 2; HR-TOF-ESIMS m/z 558.3641 $[M + Na]^+$ (calcd for $C_{28}H_{49}N_5NaO_5$, 558.3626).

Absolute Configuration of Amino Acids (ref 16). Compound **1** (0.5 mg) or **2** (0.3 mg) was dissolved in 6 N HCl (1.0 mL) and heated at 110 °C for 17 h. Upon removal of excess HCl under vacuum, the hydrolysate was placed in a 4 mL reaction vial and treated with a 1 g/100 mL solution of FDAA (200 μL) in acetone, followed by 1.0 N $NaHCO_3$ (40 μL). The reaction mixtures were heated at 40 °C for 1.5 h, cooled to room temperature, and then acidified with 2.0 N HCl (20 μL). In a similar fashion, standard L-Ile, D-Ile, L-Leu, D-Leu, L-Val, D-Val, L-Pro, D-Pro, N-Me-L-Phe, and N-Me-D-Phe were derivatized separately. The derivatives of the acid hydrolysate and the standard amino acids were subjected to RP HPLC analysis (Kromasil C18 column; 5 μm, 4.6 × 250 mm; 1.0 mL/min; UV detection at 340 nm) with a linear gradient of (A) water (TFA, pH 3.0) and (B) CH_3CN from 35% B to 50% B over 40 min. The retention times for the FDAA derivatives of L-Ile, D-Ile, L-Leu, D-Leu, L-Val, D-Val, L-Pro, D-Pro, N-Me-L-Phe, and N-Me-D-Phe were 18.0, 25.8, 19.4, 26.8, 12.9, 18.4, 8.2, 9.0, 19.8, and 19.1 min, respectively, whereas those for the FDAA derivatives of Ile, Leu, Val, Pro, and N-CH_3-L-Phe in the hydrolysate of **1** were 25.5 (D-Ile), 19.3 (L-Leu), 12.9 (L-Val), 8.2 (L-Pro), and 19.9 (N-Me-L-Phe) min, and Ile, Leu, Val, and Pro in the hydrolysate of **2** were 25.6 (D-Ile), 26.8 (D-Leu), 19.4 (L-Leu), 12.9 (L-Val), and 8.3 (L-Pro) min, respectively.

Antifungal and Synergistic Antifungal Assay. *Candida albicans* SC5314 was used as a test strain for antifungal and synergistic antifungal bioassay.[9] All experiments were carried out in flat-bottom, 96-well microtiter plates (Greiner, Germany), using a broth microdilution protocol modified from the Clinical and Laboratory Standards Institute M-27A methods. Overnight cultures were picked to prepare the strain suspension with RPMI 1640 medium at the concentration of 1×10^4 cfu/mL. To the test wells in 96-well plates, 2 μL of the samples was added, followed by an additional 80 μL of the strain suspension. The test plates were incubated at 35 °C for 16 h. The antifungal positive control was ketoconazole, and antifungal MICs were determined by measuring and comparing the optical densities of the blank control and test wells. For the synergistic antifungal assay, a quarter of the normal antifungal MIC of ketoconazole (MIC = 0.016 μg/mL) was supplemented into the strain suspension, and the other procedures were the same as the antifungal assay. The synergistic antifungal MICs were determined, and FICIs (fractional inhibitory concentration indices) were calculated; the FICI represents the sum of the FICs of each drug tested, where the FIC is determined by dividing the MIC of each drug when used in combination by the MIC of each drug when used alone. FICI = [MIC(drug A in combination)/MIC(drug A alone)] + [MIC(drug B in combination)/MIC(drug B alone)].[24] In the case that drug A has no antifungal activity, the value of MIC(drug A in combination)/MIC(drug A alone) will be very low, and the FICI value just depends on the value of MIC(drug B in combination)/MIC(drug B alone). In our assay, **1** showed strong antifungal activity against *C. albicans* at 6.25 μg/mL with 0.004 μg/mL ketoconazole. The value of MIC(drug A in combination)/MIC(drug A alone) is lower than 0.0625 since the MIC value of **1** is higher than 100 μg/mL; the value of MIC(drug B in combination)/MIC(drug B alone) is 0.25. Thus, the FICI value is lower than 0.3125 (<0.0625 + 0.25). FICI < 0.5 means synergy, 0.5 < FICI < 4 means additive, and FICI > 4 means antagonism. All experiments were carried out in triplicate.

■ ASSOCIATED CONTENT

Supporting Information. NMR data for new compounds **1** and **2** are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel/Fax: +86 10 62566577. E-mail: (H.L.) Liuhw@im.ac.cn; (J.L) jincailu@yahoo.com.cn.

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