Antifungal Cyclic Peptides from *Psammosilene tunicoides*

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Received May 30, 2010

Three new cyclic peptides, tunicyclins B−D, and a known cyclic peptide, psammosilenin B, were isolated from the root of *Psammosilene tunicoides*. The structures of new cyclic peptides were elucidated by extensive NMR and MS analysis. Tunicyclin B contains an unusual α,β-dehydrotryptophan (Δ^Z-Trp) residue, previously reported from marine sponges and bacteria. Tunicyclin D showed a broad spectrum of antifungal activity against *Candida albicans* (SC5314), *Candida albicans* (Y0109), *Candida tropicalis*, *Candida parapsilosis*, and *Cryptococcus neoformans* (BLS108) with MIC_{80} values of 4.0, 16.0, 0.25, 1.0, and 1.0 μg mL^{-1}, respectively.

Psammosilene tunicoides W. C. Wu *et*. C. Y. Wu, a monotype genus plant of the Caryophyllaceace family, is a well-known medicinal herb used as an anodyne and hemastatic agent in southwest China.[1,2] Previous phytochemical studies on this plant have afforded triterpenoid saponins and cyclopeptides.[3,4] We have previously reported tunicyclin A,[5] a unique cycloheptapeptide isolated from the same plant. Further investigations on this plant led to the isolation of three structurally novel and potent antifungal cyclic peptides, tunicyclins B−D (**1**−**3**), together with a known cyclic peptide, psammosilenin B (**4**) (Figure 1). Among these cyclopeptides, tunicyclin B contains an unusual α,β-dehydrotryptophan (Δ^Z-Trp) residue, previously reported only from marine sponge and bacteria,[6,7] while tunicyclin D exhibited potent antifungal activity against *Candida tropicalis* with an MIC_{80} value of 0.25 μg/mL. Herein we describe the isolation and structure elucidation of **1**−**3** as well as the antifungal effects of these cyclopeptides.

Results and Discussion

An 80% alcohol extract of the roots of *P. tunicoides* was partitioned between H_2O and $CHCl_3$. The $CHCl_3$- and H_2O-soluble fractions were repeatedly subjected to column chromatography on silica gel, macroporous adsorbent resin (Diaion HP-20), reversed-phase silica gel (ODS), and Sephedex LH-20 to yield three new cyclic pepetides (**1**−**3**) and a known cyclic peptide, psammosilenin B (**4**) (Figure 1).

Compound **1** was isolated as a colorless solid ($[\alpha]_D^{20}$ −107, *c* 0.065, MeOH). The molecular formula was established as $C_{35}H_{48}N_8O_9$ by negative HR-ESIMS (*m/z* $[M - H]^-$ 723.3469; calcd 723.3466). The ^1H and ^{13}C NMR spectra exhibited the typical features of cycloheptapeptides, i.e., seven amide carbonyl resonances (δ_C 176.0, 172.5, 172.5, 171.9, 171.1, 170.1, and 167.1), together with six normal α-amino acid carbon resonances (δ_C 62.1, 62.0, 58.2, 53.2, 52.9, and 44.0) in the ^{13}C NMR spectrum and five amide proton signals (δ_H 10.15, 10.11, 9.10, 8.68, and 8.04) in the ^1H NMR spectrum (Table 1). In addition, the 1D NMR spectra of **1** indicated the presence of four methyls due to two isopropyl groups, three methylene groups, two methine groups, one CH_2N group, two CH_2OH groups, one 3-substituted indolyl, and one trisubstituted double bond (δ_C 122.7, the α-, and 127.7, the β-carbon). From $^1H-^1H$ COSY and TOCSY experiments, six amino acid spin systems of Pro, Ser, Leu, Val, Gly, and Ser were determined (Figure 2).[8,9] The assignments of the protonated carbons were obtained from the HMQC spectrum, in combination with inspection of the HMBC spectrum (Table 1). By comparison of the UV spectrum (λ_{max} 338 nm, in MeOH) of **1** with that of keramamide F[10] (λ_{max} 339 nm, in MeOH), it was suggested that **1** contained an α,β-dehydrotryptophan (Δ-Trp) residue. The result was further confirmed by the HMBC correlations (Figure 2) between the β-H (δ_H 8.82) of the trisubstituted double bond and the carbon resonances at δ_C 130.6 (CH-2′ of indolyl group) and 167.1 (carbonyl group of Δ-Trp residue). The carbonyl carbons of Pro, Ser^a, Δ-Trp, Val, Gly, and Ser^b were clearly assigned to δ_C 172.5, 171.1, 167.1, 172.5, 170.1, and 171.9 on the basis of the observed correlations between carbonyl groups and α- or β-protons of the same amino acid residue in the HMBC spectrum of **1**, respectively. The carbonyl carbon of Leu was thus assigned to δ_C 176.0 despite the lack of HMBC correlation between the carbonyl group and the α- or β-protons of the same residue.

The amino acid sequence of **1** was mainly established by the following ROESY cross-peaks: Ser^a-NH/αH-Pro, Δ-Trp-2′H/αH-Ser^a, Val-NH/αH-Leu, Gly-NH/αH-Val, Ser^b-NH/NH-Gly, and Pro-δ_a,δ_bH/αH-Ser^b (Figure 3). In conjunction with the HMBC correlation of Leu-NH with CO-Δ-Trp, the planar structure of **1** was determined as cyclo-(Pro-Ser^a-Δ-Trp-Leu-Val-Gly-Ser^b).

The geometrical configuration of the Δ-Trp residue was assigned as *Z* on the basis of the chemical shift of the β-proton (δ_H 8.82).[7] This result was supported by the ROESY correlation between Δ-Trp-2′CH (δ_H 9.15) and Ser^a-αH (δ_H 5.09). The strong NOE correlation between Ser^b-αH and both δ_a and δ_b H-atoms of Pro suggested the amide bond of Ser^b-Pro being *trans*. This stereoconfiguration was further confirmed by the ^{13}C chemical shifts of β- and γ-carbons of Pro at δ_C 29.4 and 25.3 ppm, respectively, in agreement with those of *trans*-Pro.[11]

The absolute configurations of Pro, Ser^a, Leu, Val, and Ser^b were identified as L on the basis of HPLC-ESIMS analysis of the retention times and *m/z* values of the chiral derivatives of the amino acid residues in the acid hydrolysate of **1** (see Supporting Information).[12] Thus, **1** was determined as cyclo-(L-Pro-L-Ser^a-Δ^Z-Trp-L-Leu-L-Val-Gly-L-Ser^b) and named tunicyclin B. As far as we are aware, tunicyclin B is the first plant cyclic peptide reported to contain the

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10.1021/np100363a
Published on Web 11/11/2010

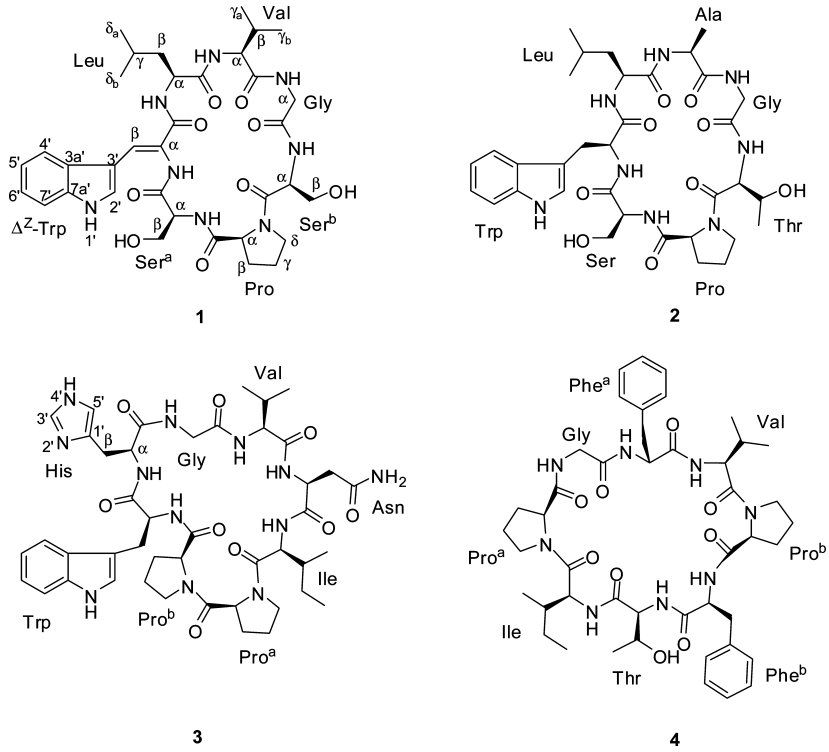

Figure 1. Chemical structures of **1**, **2**, **3**, and **4**.

Table 1. NMR Spectroscopic Data (500 MHz, C_5D_5N) for Tunicyclin B (**1**)[a]

residue	δ_H	δ_C	residue	δ_H	δ_C
Pro			7a′ C		137.2
CO		172.5	Leu		
α	4.82 (dd, 8.0, 6.0)	62.0	CO		176.0
β_a	2.22 (m)	29.4	NH	8.04 (d, 9.0)	
β_b	2.16 (m)		α	5.34 (m)	52.9
γ_a	1.83 (m)	25.3	β_a	1.98 (m)	41.5
γ_b	1.63 (m)		β_b	1.88 (m)	
δ_a	3.95 (m)	48.5	γ	2.02 (m)	25.0
δ_b	3.62 (m)		δ_a	0.83 (3H, d, 6.0)	23.2
Ser[a]			δ_b	0.69 (3H, d, 6.0)	21.5
CO		171.1	Val		
NH	8.68 (s)		CO		172.5
α	5.09 (s)	58.2	NH	10.15 (s)	
β_a	4.70 (d, 10.0)	63.0	α	4.20 (s)	62.1
β_b	4.30 (d, 10.0)		β	2.60 (m)	29.8
Δ^Z-Trp			γ_a	1.27 (3H, d, 7.0)	20.1
CO		167.1	γ_b	1.18 (3H, d, 7.0)	19.9
NH			Gly		
α		122.7	CO		170.1
β	8.82 (s)	127.7	NH	10.11 (s)	
1′ NH	13.25 (s)		α_a	4.89 (dd, 17.0, 7.0)	44.0
2′ CH	9.15 (s)	130.6	α_b	3.95 (dd, 17.0, 7.0)	
3′ C		110.4	Ser[b]		
3a′ C		129.2	CO		171.9
4′ CH	7.91 (d, 8.0)	118.6	NH	9.10 (s)	
5′ CH	7.30 (t, 8.0)	120.8	α	5.66 (d, 6.0)	53.2
6′ CH	7.36 (t, 8.0)	122.6	β_a	4.56 (dd, 11.0, 6.0)	64.0
7′ CH	7.67 (d, 8.0)	112.5	β_b	4.49 (dd, 11.0, 6.0)	

[a] All proton signals integrate to 1H, unless otherwise indicated.

unusual α,β-dehydrotryptophan (Δ^Z-Trp) residue, which was previously reported only from marine sponge and bacteria.[6,7] The Trp dehydrogenation of tunicyclin B might be catalyzed by L-tryptophan 2′,3′-oxidase (LTO) previously found in *Chromobacterium violaceum*.[13]

Compound **2**, a colorless solid ($[\alpha]_D^{20}$ −41, *c* 0.060, H_2O), had a molecular formula a $C_{34}H_{48}N_8O_9$, established by negative HR-ESIMS (*m*/*z* $[M - H]^-$ 711.3461; calcd 711.3466). It was also identified as a cycloheptapeptide on the basis of the presence of seven carbonyls (δ_C 175.2, 173.9, 172.7, 171.7, 171.5, 170.4, and 169.6) and seven α-amino acid carbon resonances (δ_C 60.3, 57.0, 56.7, 55.0, 51.9, 51.3, and 43.5) in the ^{13}C NMR spectrum. Seven amino acid residues of **2** were determined as Pro, Ser, Trp, Leu, Ala, Gly, and Thr on the basis of detailed analysis of 1D and 2D NMR, and their proton and carbon resonances were completely assigned (Figure 2, Table 2). The amino acid sequence of **2** was established by HMBC cross-peaks: Ser-NH/CO-Pro, Trp-NH/CO-Ser, Leu-NH/CO-Trp, Ala-NH/CO-Leu, Gly-NH/CO-Ala, and Thr-NH/CO-Gly. The gross structure was further supported by NOESY data, as indicated in Figure 3. The

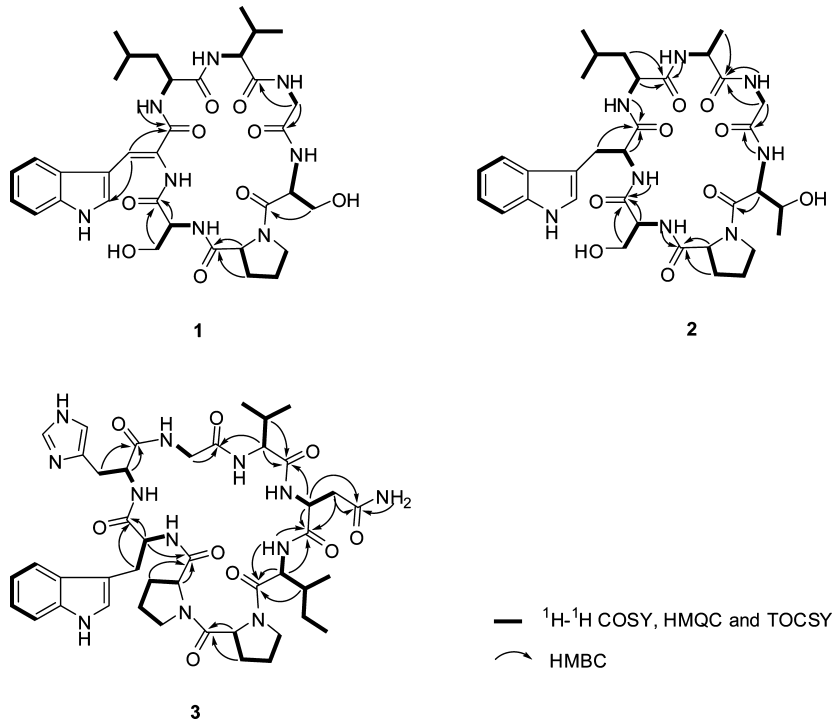

Figure 2. Selected 2D NMR correlations for **1**, **2**, and **3**.

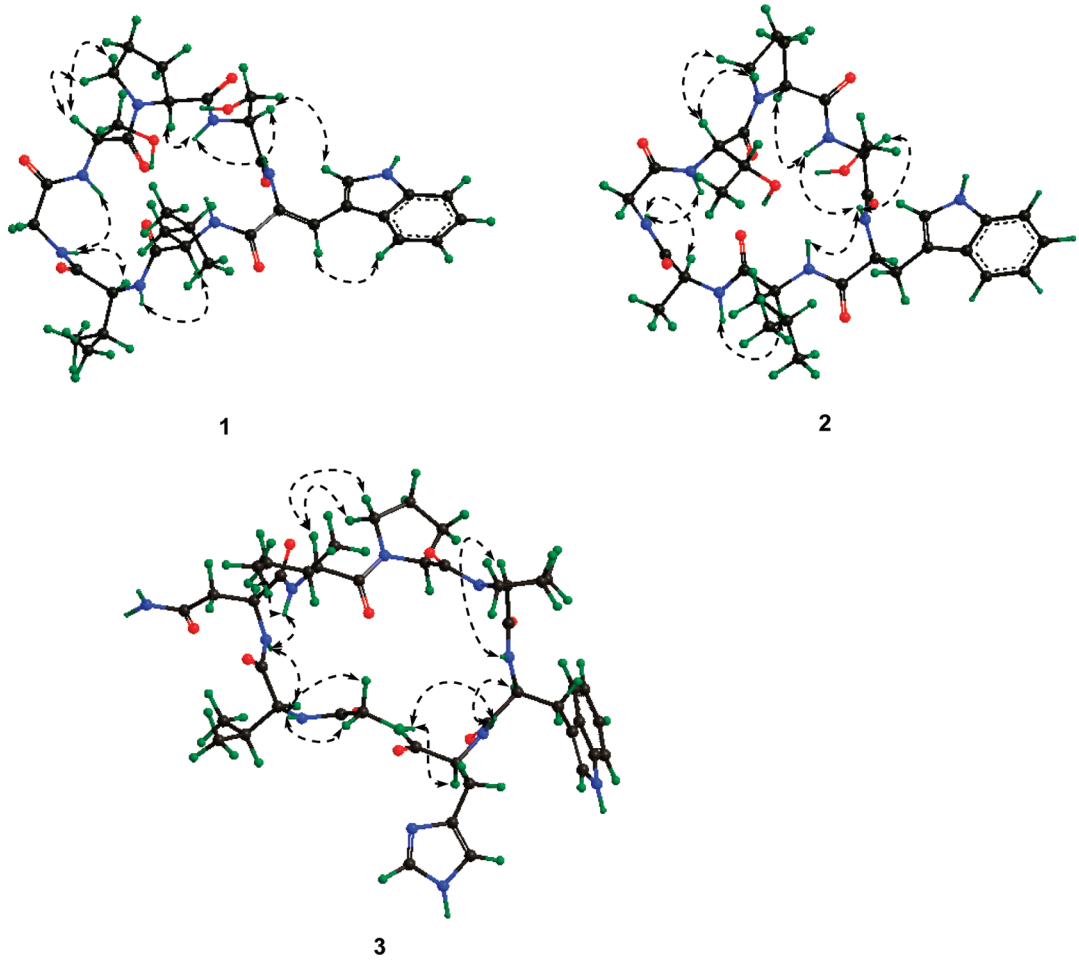

Figure 3. Key NOESY correlations of **1**, **2**, and **3**.

strong NOE correlations between Thr-αH and both δ_a and δ_b protons of Pro suggested the presence of a *trans* amide bond of Thr-Pro. The stereoconfiguration was further confirmed by the ^{13}C chemical shifts of β- and γ-carbons of Pro at 27.6 and 25.0 ppm, respectively, in agreement with the presence of *trans*-Pro.[11]

Table 2. NMR Spectroscopic Data (600 MHz, C_5D_5N) for Tunicyclin C (**2**)[a]

residue	δ_H	δ_C	residue	δ_H	δ_C
Pro			7′ CH	7.50 (d, 7.9)	111.9
CO		171.7	7a′ C		137.3
α	4.87 (dd, 8.4, 4.7)	60.3	Leu		
β_a	2.23 (m)	27.6	CO		175.2
β_b	1.89 (m)		NH	7.86 (d, 7.6)	
γ_a	1.73 (m)	25.0	α	4.90 (m)	51.9
γ_b	1.51 (m)		β_a	1.77 (m)	40.5
δ_a	3.50 (dt, 9.1, 6.9)	47.1	β_b	1.66 (m)	
δ_b	3.37 (dt, 9.1, 6.9)		γ	1.80 (m)	24.3
Ser			δ_a	0.78 (3H, d, 6.3)	22.6
CO		171.5	δ_b	0.68 (3H, d, 6.1)	21.9
NH	8.42 (d, 8.4)		Ala		
α	5.01 (m)	55.0	CO		173.9
β_a	4.24 (dd, 11.5, 4.3)	62.0	NH	10.22 (d, 3.7)	
β_b	4.19 (dd, 11.5, 5.9)		α	4.51 (dq, 3.7, 7.0)	51.3
Trp			β	1.47 (3H, d, 7.0)	16.5
CO		172.7	Gly		
NH	8.95 (d, 6.0)		CO		169.6
α	4.97 (m)	57.0	NH	9.89 (dd, 7.6, 4.7)	
β_a	3.79 (dd, 14.9, 8.3)	27.0	α_a	4.74 (dd, 17.1, 7.6)	43.5
β_b	3.67 (dd, 14.9, 4.4)		α_b	3.91 (dd, 17.1, 4.7)	
1′ NH	11.80 (d, 3.6)		Thr		
2′ CH	7.61 (d, 3.6)	124.5	CO		170.4
3′ C		110.4	NH	8.44 (d, 9.6)	
3a′ C		128.0	α	5.34 (dd, 9.6, 3.3)	56.7
4′ CH	7.81 (d, 7.9)	118.8	β	4.43 (m)	68.6
5′ CH	7.04 (t, 7.9)	119.1	γ	1.42 (3H, d, 5.9)	19.6
6′ CH	7.15 (t, 7.9)	121.7			

[a] All proton signals integrate to 1H, unless otherwise indicated.

Table 3. NMR Spectroscopic Data (400 MHz, C_5D_5N) for Tunicyclin D (**3**)[a]

residue	δ_H	δ_C	residue	δ_H	δ_C
Pro[a]			β_a	3.68 (m)	37.5
CO		171.0	β_b	3.49 (m)	
α	4.61 (d, 7.7)	59.9	1′ C		134.8[b]
β_a	2.18 (m)	29.0	2′ NH		
β_b	1.85 (m)		3′ CH	8.01 (s)	136.2
γ_a	2.02 (m)	25.4	4′ NH		
γ_b	1.85 (m)		5′ CH	7.46 (s)[b]	118.5[b]
δ_a	3.84 (m)	48.1	Gly		
δ_b	3.84 (m)		CO		170.6
Pro[b]			NH	9.55 (m)	
CO		172.3	α_a	4.47 (m)	44.4
α	4.61 (d, 7.7)	61.7	α_b	4.16 (m)	
β_a	2.33 (m)	31.5	Val		
β_b	1.85 (m)		CO		172.6
γ_a	1.35 (m)	22.0	NH	8.95 (m)	
γ_b	1.00 (m)		α	4.91 (m)	60.1
δ_a	3.43 (m)	47.0	β	2.65 (m)	30.5
δ_b	3.34 (m)		γ_a CH_3	1.22 (3H, d, 6.6)	18.7
Trp			γ_b CH_3	1.17 (3H, d, 6.7)	20.0
CO		173.8	Asn		
NH	9.25 (m)		CO		172.0
α	5.30 (m)	56.2	NH	9.27 (m)	
β_a	4.04 (m)	26.4	α	5.44 (m)	53.2
β_b	3.80 (m)		β_a	3.68 (m)	37.5
1′ NH	11.89 (s)		β_b	3.49 (m)	
2′ CH	7.53 (s)	124.9	γ CO		173.9
3′ C		111.7	δ_a NH	8.62 (s)	
3a′ C		128.9	δ_b NH	7.90 (s)	
4′ CH	8.02 (d, 7.5)	119.4	Ile		
5′ CH	7.26 (d, 7.5)	119.6	CO		170.4
6′ CH	7.34 (d, 7.5)	121.9	NH	8.12 (m)	
7′ CH	7.66 (d, 7.5)	112.0	α	5.05 (t, 8.3)	56.4
7a′ C		137.6	β_a	2.41 (m)	37.5
His			β_b CH_3	1.31 (3H, d, 6.5)	16.4
CO		174.1	γ_a	2.18 (m)	24.9
NH	9.05 (m)		γ_b	1.52 (m)	
α	5.22 (m)	56.2	δ CH_3	1.03 (3H, t, 7.2)	11.9

[a] All proton signals integrate to 1H, unless otherwise indicated. [b] NMR spectroscopic data (500 MHz) for tunicyclin D (**3**) were recorded in D_2O with several drops of C_5D_5N as internal standard.

The absolute configurations of Pro, Ser, Leu, Ala, and Thr were identified as L on the basis of the HPLC-ESI-MS analysis of the retention times and *m/z* values of the chiral derivatives of the amino acid residues in the acid hydrolysate of **2** (see Supporting Information).[12] Although there is no direct evidence, considering that all naturally occurring amino acids from higher plants have an L configuration, together with the weak ROESY correlations of Leu-γH with Trp-β_a,β_bH, the absolute configuration of Trp still could be assigned as L. Thus, **2** was determined as cyclo-(L-Pro-L-Ser-L-Trp-L-Leu-L-Ala-Gly-L-Thr) and named tunicyclin C.

Compound **3** was isolated as a colorless solid ($[\alpha]_D^{20}$ −115, *c* 0.070, H_2O) with a molecular formula of $C_{44}H_{60}N_{12}O_9$ based on the pseudomolecular ion peak at *m/z* 901.4616 $[M + H]^+$. Nine amide carbonyls (δ_C 174.1, 173.9, 173.8, 172.6, 172.3, 172.0, 171.0, 170.6, and 170.4) and eight α-amino acid carbon resonances (δ_C 61.7, 60.1, 59.9, 56.4, 56.2, 56.2, 53.2, and 44.4) suggested that **3** should be a cyclic octopeptide. By analysis of the $^1H-^1H$ COSY, TOCSY, HMQC, and HMBC spectra, seven amino acid residues of Pro, Pro, Trp, Gly, Val, Asn, and Ile were unambiguously assigned (Figure 2, Table 3). Considering the molecular formula of **3**, the unidentified amino acid residue could be deduced as His. Only part of the resonances of His, i.e., His-NH (δ_H 9.05), His-αCH (δ_H 5.22; δ_C 56.2), His-βCH_2 (δ_H 3.68, 3.49; δ_C 37.5), and His-3′CH (δ_H 8.01; δ_C 136.2), were observed in deuterated pyridine. The lack of other aromatic protons (His-2′NH, His-4′NH, and His-5′CH) and aromatic carbons (His-1′C and His-5′CH) in C_5D_5N was probably because of fast exchange between the two equivalent tautomeric forms of the imidazole group of the His residue. 1D NMR spectra of **3** were recorded again in D_2O with several drops of deuterated pyridine as internal standard. The resonances observed (δ_C 134.8, His-1′C; δ_H 8.09, δ_C 136.9, His-3′CH; and δ_H 7.46, δ_C 118.5, His-5′CH) strongly supported the presence of a His residue. On the basis of the HMBC correlations between the carbonyls and the α- or β-protons of the same amino acid residue, the carbonyl resonances of the amino acid residues were determined as 171.0 (Pro[a]), 172.3 (Pro[b]), 173.8 (Trp), 174.1 (His), 170.6 (Gly), 172.6 (Val), 172.0 (Asn), and 170.4 (Ile), respectively. The amino acid sequence of **3** was established by ROESY correlations between α-protons and neighboring residue NH protons: Trp-NH/αH-Pro[b], His-NH/αH-Trp, Gly-NH/αH-His, Val-NH/α_a,α_bH-Gly, Asn-NH/αH-Val, and Ile-NH/αH-Asn (Figure 3). The strong NOE correlation between Ile-αH and the both δ_a and δ_b protons of Pro[a] suggested the presence of a *trans* amide bond of Ile-Pro[a]. Although the ROESY correlation between the α-proton of Pro[a] and that of Pro[b] could not be observed because they heavily overlapped each other, the amide bond of Pro[a]-Pro[b] was still deduced as having a *cis* configuration on the basis of no strong NOE correlation between Pro[a]-αH and the both δ_a and δ_b protons of Pro[b], in combination with the ^{13}C chemical shifts of β- and γ-carbons of Pro[b] at 31.5 and 22.0 ppm, respectively.[11]

The absolute configurations of Pro[a], Pro[b], His, Val, Asn, and Ile were identified as L on the basis of the HPLC-ESIMS analysis of the retention times and *m/z* values of the chiral derivatives of the amino acid residues in the acid hydrolysate of **3** (see Supporting Information).[12] Although there is no direct evidence, the Trp residue was still assigned as having an L configuration on the basis of the weak NOE correlations of Pro[b]-β_bH/β_bH-Trp, Trp-β_aH/β_aH-His, and Trp-6′H/β_a,β_bH-His and the biosynthesis pathway. Thus, **3** was determined as cyclo-(L-Pro[a]-L-Pro[b]-L-Trp-L-His-Gly-L-Val-L-Asn-L-Ile) and named tunicyclin D.

Compounds **1**, **2**, and **3** were extensively tested for bioactivities including cytotoxicity, anti-inflammatory, and antifungal effects. None of them displayed cytotoxicity or anti-inflammatory effects (data not shown). However, compound **3** exhibited potent antifungal effects against *Candida albicans* (SC5314), *Candida albicans* (Y0109), *Candida tropicalis*, *Candida parapsilosis*, and *Crypto-*

coccus neoformans (BLS108) with MIC_{80} values of 4.0, 16.0, 0.25, 1.0, and 1.0 μg mL^{-1}, respectively.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. UV spectra were taken on a Varian CARY 300 Bio spectrometer (**1**) and a UV-2550 UV−visible spectrophotometer (Shimadzu, Japan) (**2** and **3**), respectively. IR spectra were recorded on a Bruker Vector 22 spectrometer with KBr pellets. ^1H and ^{13}C NMR spectra of **1** and **2** were recorded on a Bruker Avance 500 NMR spectrometer in C_5D_5N and Bruker Avance 600 NMR spectrometer in C_5D_5N, respectively, with chemical shifts (δ) reported in ppm. ^1H and ^{13}C NMR spectra of **3** were recorded on a Bruker DPX 400 spectrometer in C_5D_5N and a Bruker DRX 500 spectrometer in D_2O with several drops of C_5D_5N as internal standard. ESIMS were measured on a LC/MSD Trap XCT (Agilent, USA). HR-ESIMS were measured on a Q-TOF micro mass spectrometer (Waters USA) (**1** and **2**) and an Accurate-Mass-Q-TOF LC/MS 6520 (Agilent, USA) (**3**), respectively.

Plant Material. The roots of *Psammosilene tunicoides* (40 kg) were collected in Lijiang, Yunnan Province, China, in 2006. The botanical identification was made by Prof. Lishan Xie, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (herbarium no. 2006071015) is deposited in the School of Pharmacy, Second Military Medical University, China.

Extraction and Isolation. The air-dried powdered material was refluxed with 80% alcohol. The residue obtained by concentrating alcohol was partitioned between H_2O and $CHCl_3$, and then the $CHCl_3$-soluble extract (285 g) was chromatographed by a silica gel (100−200 mesh) column eluted successively with petroleum ether−EtOAc (1%, 5%, 10%, 20%, 30%, and 40%), then eluted with 10% CH_3OH−$CHCl_3$ to yield nine fractions (F1 to F9). The H_2O-soluble extract was chromatographed by a macroporous resin (Diaion HP-20) column eluted successively with H_2O, 70% alcohol, and acetone to yield two fractions (acetone fraction and 70% alcohol fraction, 7.5 kg). The 70% alcohol fraction (1 kg) was subjected to column chromatography on reversed-phase silica gel (ODS) eluted successively with gradient EtOH−H_2O to afford six fractions (S1 to S6). Fraction S2 was further separated into three fractions (S2-1, S2-2, and S2-3) by column chromatography on reversed-phase silica gel (ODS). Fraction S2-2 was subject to column chromatography over silica gel with gradient $CHCl_3$−CH_3OH to afford 14 fractions (S2-2-1 to S2-2-14). Fraction S2-2-2 was further purified by repeated reversed-phase silica gel (ODS) and Sephadex (LH-20) columns to afford compounds **1** (21 mg) and **2** (52 mg). Fraction S2-2-11 was further purified by repeated reversed-phase silica gel (ODS) and Sephedex (LH-20) columns to afford compound **3** (68 mg). The acetone fraction was combined with the F9 fraction to afford fraction M. Fraction M was chromatographed by a MCI gel column eluting successively with H_2O, 70% CH_3OH, and CH_3OH to yield two fractions (M-1 and M-2). Fraction M-1 was subjected to column chromatography over silica gel eluting with gradient CH_3OH−$CHCl_3$ (5%, 10%, 15%, 20%, and 30%) to give four subfractions (M-1-1 to M-1-4). Fraction M-1-2 was further purified by repeated reversed-phase silica gel (ODS) and Sephedex (LH-20) column chromatography to afford compound **4** (25 mg).

Tunicyclin B (1): colorless solid; $[\alpha]_D^{20}$ −107 (*c* 0.065, MeOH); UV (MeOH) λ_{max} 225, 338 nm; IR (KBr) ν_{max} 3307, 3061, 2959, 2926, 2873, 2854, 1653, 1575, 1559, 1541, 1437, 1368, 1338, 1269, 1234, 1063, 924, 746, 650 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; ESIMS (negative) *m/z* 723 $[M - H]^-$, 759 $[M + Cl]^-$; HR-ESIMS (negative) $[M - H]^-$ *m/z* 723.3469 (calcd for $C_{35}H_{47}N_8O_9$, 723.3466).

Tunicyclin C (2): colorless solid; $[\alpha]_D^{20}$ −41 (*c* 0.060, MeOH); UV (MeOH) λ_{max} 218, 290 nm; IR (KBr) ν_{max} 3294, 3061, 2957, 2873, 1645, 1539, 1438, 1235, 1063, 747, 705 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 2; ESIMS (negative) *m/z* 711 $[M - H]^-$, 747 $[M + Cl]^-$; HR-ESIMS (negative) $[M - H]^-$ *m/z* 711.3461 (calcd for $C_{34}H_{47}N_8O_9$, 711.3466).

Tunicyclin D (3): colorless solid; $[\alpha]_D^{20}$ −115 (*c* 0.070, MeOH); UV (MeOH) λ_{max} 211, 217, 282, 289 nm; IR (KBr) ν_{max} 3367, 2965, 2877, 1653, 1522, 1437, 1384, 746 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 3; ESIMS (positive) *m/z* 901 $[M + H]^+$, 923 $[M + Na]^+$; HR-ESIMS (positive) $[M + H]^+$ *m/z* 901.4616 (calcd for $C_{44}H_{61}N_{12}O_9$, 901.4679).

Absolute Configuration of Amino Acid Residues of 1, 2, and 3. To 50 μL of a 50 mM aqueous solution of the L-configurations of Ala, Asp, His, Ile, Leu, Pro, Ser, Thr, Trp, and Val were added 20 μL of 1 M sodium bicarbonate and then 100 μL of 25 mM L-FDLA (TCI, Japan) in acetone, respectively. The solutions were incubated at 37 °C for 60 min. Reactions were quenched by addition of 20 μL of 1 N HCl, respectively. Samples were diluted with 810 μL of acetonitrile, and 400 μL of these solutions was analyzed by HPLC-ESIMS, respectively.

A 100 μL amount of 1 mg/mL tunicyclins B−D (**1**−**3**) was hydrolyzed at 100 °C for 24 h by adding 200 μL of 10 N HCl, respectively. These solutions were evaporated to dryness. Then, the residues were dissolved in 50 μL of water, respectively. To these amino acid solutions were added 20 μL of 1 M sodium bicarbonate and 50 μL of 25 mM L-FDLA in acetone, respectively. The solutions were incubated at 37 °C for 60 min. Reactions were quenched by addition of 20 μL of 1 N HCl, respectively. Samples were diluted with 810 μL of acetonitrile, and 400 μL of this solution was analyzed by HPLC-ESIMS, respectively.

HPLC was performed on an Agilent 1100 system. Separations were carried out on a TSKgel ODS-100 V column (150 × 4.6 mm i.d., 3 μm, TOSOH) maintained at 40 °C. Acetonitrile−0.01 M trifluoroacetic acid (TFA) was used as mobile phase under a linear gradient elution mode (acetonitrile, 20−100%, 80 min). The flow rate was 1 mL/min with detection at 340 nm by photodiode array detection and ESIMS. The mass spectrometer used was a LC/MSD Trap XCT mass spectrometer (Agilent, USA). The auxiliary and sheath gas nitrogen pressure were set at 10 unit and 35 psi, respectively, and the capillary was heated to 350 °C. A mass range of *m/z* 100−1000 was covered with a scan time of 200 μs, and data were collected in negative ion mode.

The absolute configurations of Pro, Ser[a], Leu, Val, and Ser[b] residues of **1**, Pro, Ser, Leu, Ala, and Thr residues of **2**, and Pro[a], Pro[b], His, Val, and Ile residues of **3** were all identified as having L configurations by comparison of the retention times and *m/z* values of the chiral derivatives of the amino acid residues in the acid hydrolysate of **1**−**3** with the chiral derivatives of the corresponding standard L-configured amino acids, respectively (see detailed information in the Supporting Information).

Antifungal Assay. The ATCC standard fungal strains *Candida albicans* (SC5314) and *Cryptococcus neoformans* (BLS108) and clinical fungal strains *Candida parapsilosis*, *Candida tropicalis*, *Trichophyton rubrum*, *Candida kefyr*, *Candida albicans* (Y0109), and *Aspergillus fumigatus* were used. Fungal strains were stored at −4 °C and grown at 35 °C on SDA with 1 mL of YEPD. The suspension was diluted with RPMI 1640 to 1×10^3 to 5×10^3 units/mL, The cyclic peptides were dissolved in DMSO (6.4 g/L) and 2-fold diluted in the broth (64, 16, 4, 1, 0.25, 0.0625, 0.0156, 0.0039, 0.00097, and 0.00024 mg/L). Incubation was at 35 °C (24 h, 72 h, and one week), and the MIC was determined as the lowest concentration inhibiting fungal growth.

Acknowledgment. The work was supported by the NCET Foundation, program NSFC(30725045), the Special Program for New Drug Innovation of the Ministry of Science and Technology, China (2009ZX09311-001, 2008ZX09308-005), and Shanghai Leading Academic Discipline Project (B906), and in part by the Scientific Foundation of Shanghai China (09DZ1975700, 09DZ1971500).

Supporting Information Available: Additional information on determination of the absolute configuration of amino acid residues and copies of spectra are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP100363A