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ASPERELINES G AND H, TWO NEW PEPTAIBOLS FROM THE MARINE-DERIVED FUNGUS *TRICHODERMA ASPERELLUM*

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Abstract – Two new peptaibols, asperelines G (**1**) and H (**2**), together with five known compounds, asperelines A (**3**), C (**4**), D (**5**), E (**6**), and F (**7**) were isolated from the marine-derived fungus *Trichoderma asperellum*. The structures of these compounds were determined through spectroscopic methods, X-ray diffraction analysis, and chemical derivatization. This study is the first report on asperelines with an acetylated *C*-terminus and a crystal structure.

Marine environment is an excellent source of novel compounds with higher pharmaceutical applications.[1] To search for new anticancer compounds, more than 300 microbial strains isolated from sediment samples collected from the Min River estuary in China were screened for cytotoxicity against HL-60 cells. Among these strains, a fungal strain identified as *Trichoderma asperellum* showed significant cytotoxic activity. The active constituents of this strain were investigated through a bioassay-guided isolation procedure. The investigation resulted in the isolation of two new peptaibols (**1** and **2**), together with five known peptaibols (**3**–**7**).

Peptaibols are fungal metabolites that have been mainly isolated from *Trichoderma*.[2] They have attracted increasing attention for their unusual structural features, such as the presence of α-aminoisobutyric acid residues (Aib) at a high ratio, an *N*-acyl terminus and a *C*-terminus with an amino alcohol residue, and a wide range of biological activities including antibacterial,[3] antifungal,[4] antiviral,[5] antiparasitic,[6] anticancer,[7] and neuroleptic effects.[8] Aspereline is a subgroup of peptaibols with nine amino acid residues.

To date, only six asperelines have been reported, which have a structurally unique prolinol residue at the *C*-terminus and an acetylated *N*-terminus.[9] Our study is the first report on asperelines with an acetylated *C*-terminus and a crystal structure.

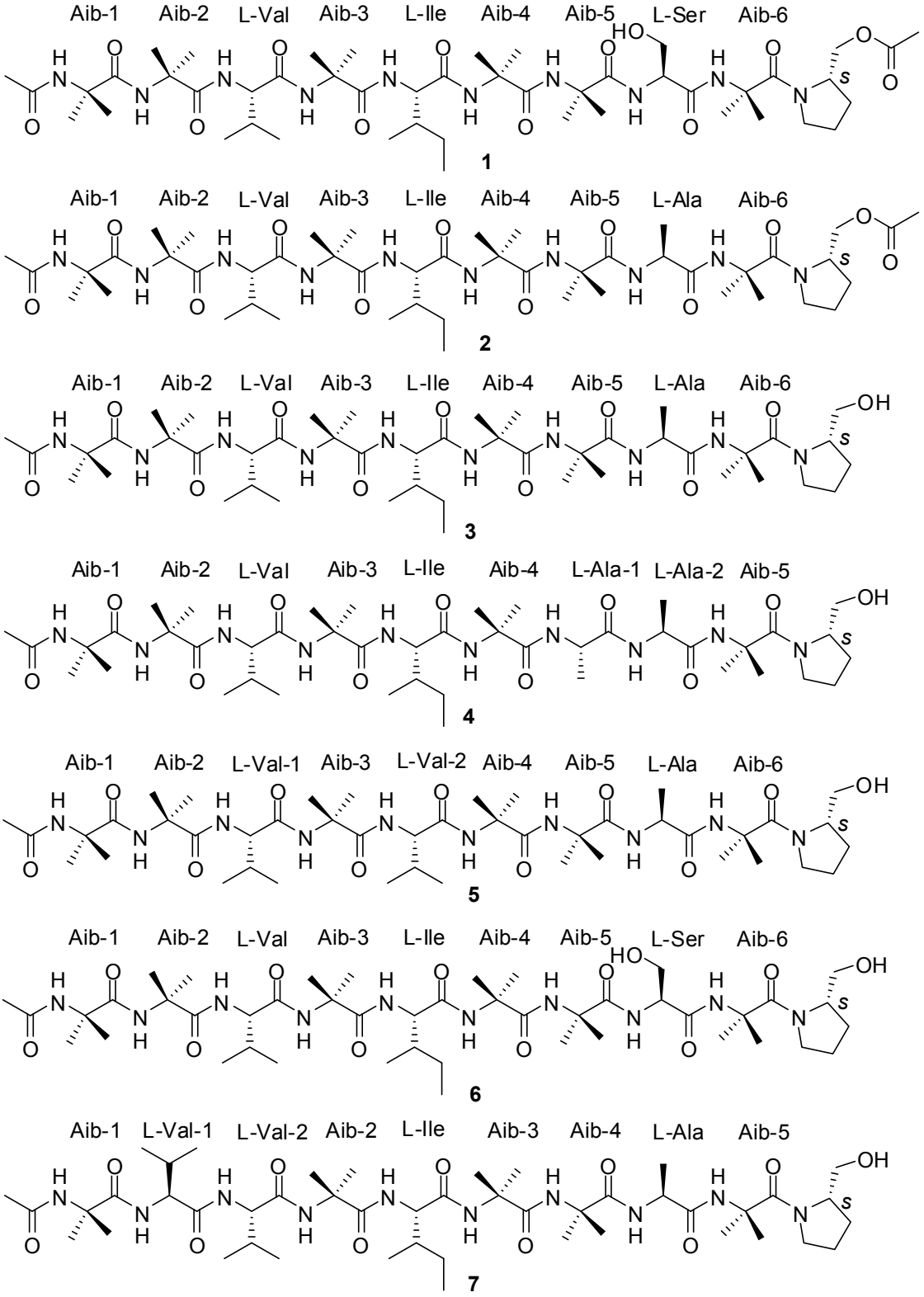

Figure 1. Structures of Compounds **1**–**7**

Aspereline G (**1**) was isolated as a colorless crystal with a molecular formula of $C_{47}H_{82}N_{10}O_{13}$ by using HRESIMS (*m/z* 995.6172 $[M + H]^+$) and NMR data. The results indicated that **1** had 12 degrees of unsaturation. The ^1H NMR spectrum in DMSO-d_6 displayed nine resonances in the downfield range between δ_H 7.20 and 8.58, indicating the probable presence of NH protons of amides. The ^{13}C NMR spectrum included 11 carbonyl signals between δ_C 176.8 and 166.8, confirming **1** to be a peptide. Chemical shift assignments for each amino acid residue were based on the interpretation of ^1H, ^{13}C NMR, DEPT, and 2D NMR data, which revealed the presence of nine amino acid residues, including an isoleucine (Ile), a valine (Val), a serine (Ser), and six 2-aminoisobutyric acids (Aib-1 to Aib-6), as well as a prolinol residue and two acetyls. For instance, the spin systems of the α-H and NH resonances of Val (δ_H 3.48/δ_H 7.71), Ile (δ_H 3.61/δ_H 7.20), and Ser (δ_H 4.06/δ_H 7.41) and the correlations from the α-methine resonances to the side chains of the individual amino acid residues were clearly observed in the COSY spectrum. Six NH singlets were attributed to the Aib residues, which were proven by the HMBC correlation between NH resonances and the quaternary carbons resonating at around δ_C 55.5, which was characteristic of the α-C of Aib residues. Two acetyl groups were represented by two methyl singlets (δ_H 1.98, 1.92) and their HMBC correlations to carbonyl carbons (δ_C 170.0, 170.6). In addition, a prolinol residue was recognized from the COSY couplings of H-1 (δ_H 4.15)/H_2-2 (δ_H 1.81, 1.62), H_2-2/H_2-3 (δ_H 1.79, 1.62), H_2-3/H_2-4 (δ_H 3.61, 3.40), and H_2-5 (δ_H 4.01, 3.92)/H-1. The sequence of amino acid residues and two acetyl groups was established throughand the HMBC correlations (Figure 2) and ESIMS/MS experiments (Figure 3). The positive and negative ESIMS/MS spectra of **1** exhibited the successive fragment ions that were generated by X-type fragmentation from the *C*-terminus and B-type fragmentation from the *N*-terminus, respectively.[9] These findings provided important evidence for assigning the sequence of **1** as Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ser-Aib-prolinol-Ac. The L-configuration of amino acid residues and the *S*-configuration of prolinol were determined by acid hydrolysis and derivatization of **1** using Marfey's reagent, followed by HPLC analysis.[10]

The molecular formula of aspereline H (**2**) was determined as $C_{47}H_{82}N_{10}O_{12}$, based on the HRESIMS (*m/z* 979.6203 $[M + H]^+$) and NMR data. The molecular weight of **2** was thus 16 amu lower than that of **1**. Analysis of 1D and 2D NMR spectroscopic data revealed that the structure of **2** was similar to **1**, except for the absence of a Ser residue and the presence of an additional alanine (Ala) residue, which was consistent with the HRESIMS result. The positive and negative ESIMS/MS fragments verified the sequence of **2** to be Ac-Aib-Aib-Val-Aib-Ile-Aib-Aib-Ala-Aib-prolinol-Ac. The configurations of the amino acid residues and the prolinol unit of **2** were determined to be the same with **1**, by the same method.

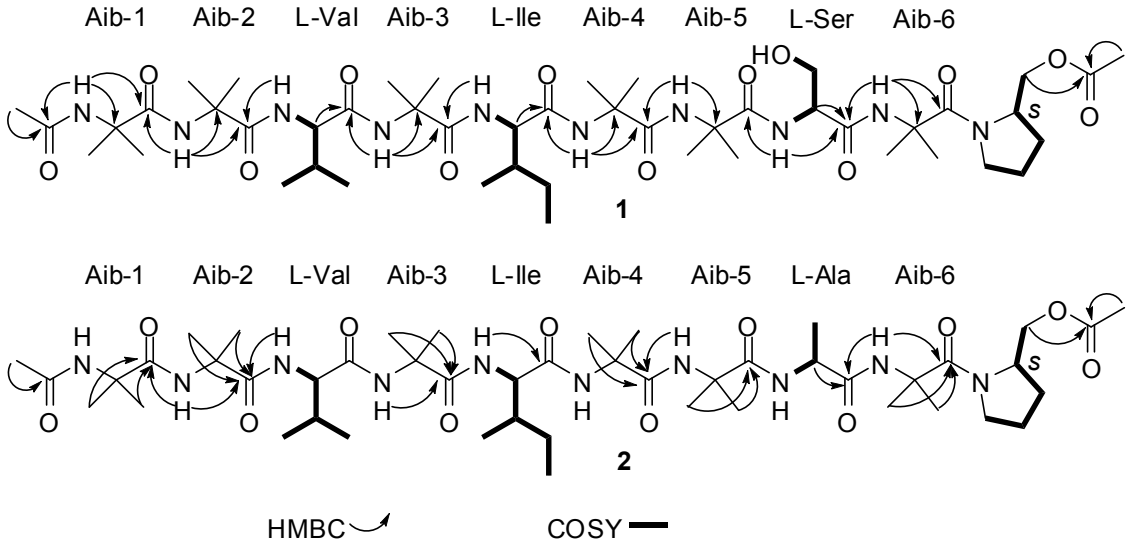

Figure 2. Key ^{1}H–^{1}H COSY and HMBC Correlations of Compounds **1** and **2**

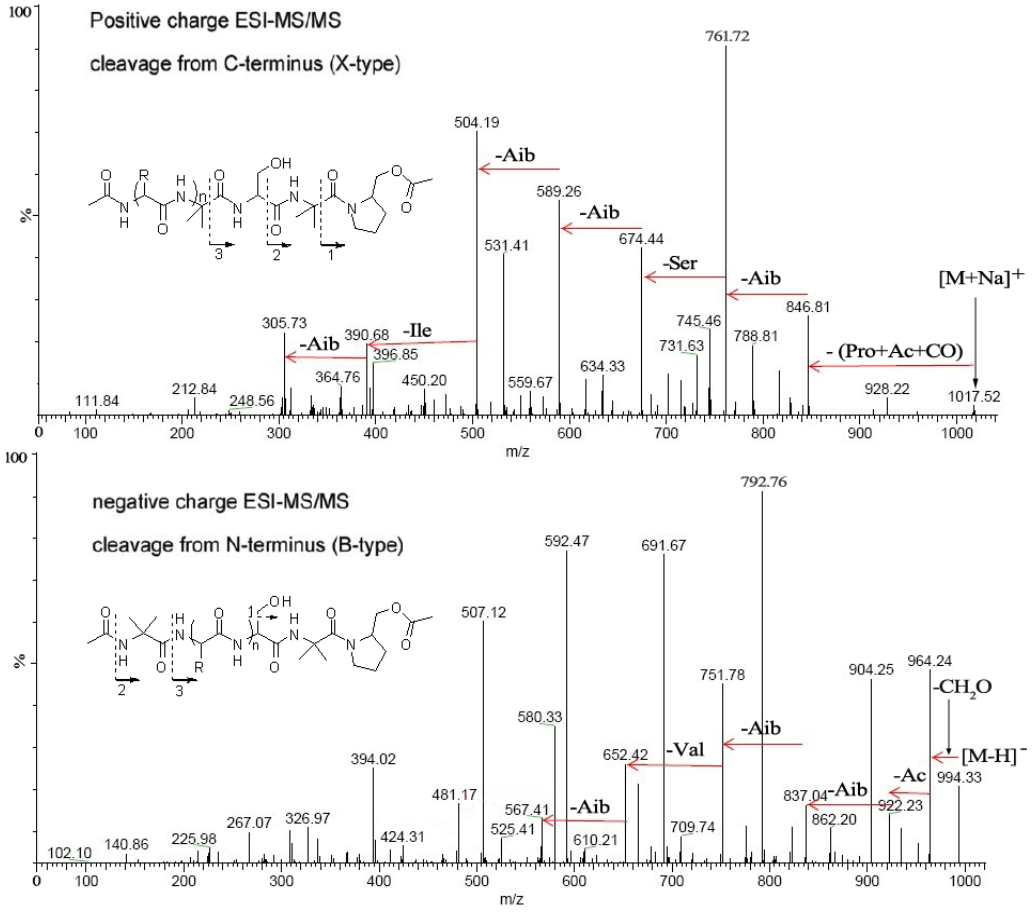

Figure 3. Positive and Negative ESIMS/MS Fragmentation of Compound **1**

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** and **2**[a]

residue	position	**1**		**2**	
		δ_C	δ_H	δ_C	δ_H
Ac-1	C=O	170.6 s		170.9 s	
	CH_3	22.8 q	1.92 (3H, s)	23.0 q	1.93 (3H, s)
Aib-1	C=O	174.6 s		175.0 s	
	1	55.5 s		[b]55.9 s	
	2	25.6 q	1.36 (3H, s)	26.6 q	1.36 (3H, s)
	3	22.6 q	1.33 (3H, s)	25.9 q	1.34 (3H, s)
	NH		8.58 (H, s)		8.58 (H, s)
Aib-2	C=O	176.8 s		177.0 s	
	1	55.5 s		[b]55.7 s	
	2	27.1 q	1.33 (3H, s)	27.4 q	1.34 (3H, s)
	3	23.7 q	1.35 (3H, s)	22.5 q	1.36 (3H, s)
	NH		8.39 (H, s)		8.39 (H, s)
Val	C=O	172.2 s		172.3 s	
	1	62.2 d	3.48 (H, dd, 8.2, 6.4)	62.4 d	3.51 (H, dd, 8.0, 6.2)
	2	28.5 d	2.13 (H, m)	28.7 d	2.14 (H, m)
	3	19.3 q	0.96 (3H, d, 6.5)	19.6 q	0.98 (3H, d, 6.6)
	4	18.4 q	0.90 (3H, d, 6.4)	18.7 q	0.91 (3H, d, 6.8)
	NH		7.71 (H, d, 6.4)		7.76 (H, d, 6.2)
Aib-3	C=O	175.3 s		175.0 s	
	1	55.5 s		[b]55.7 s	
	2	26.4 q	1.34 (3H, s)	25.2 q	1.41 (3H, s)
	3	22.4 q	1.41 (3H, s)	22.5 q	1.42 (3H, s)
	NH		7.52 (H, s)		7.79 (H, s)
Ile	C=O	173.0 s		173.0 s	
	1	60.5 d	3.61 (H, dd, 9.5, 7.0)	60.6 d	3.62 (H, dd, 9.6, 7.1)
	2	34.2 d	1.86 (H, m)	34.6 d	1.82 (H, m)
	3	25.2 t	1.52 (H, m)	25.6 t	1.55 (H, m)
			1.13 (H, m)		1.14 (H, m)
	4	9.7 q	0.76 (3H, t, 7.2)	10.0 q	0.77 (3H, t, 7.3)
	5	14.4 q	0.81 (3H, d, 6.7)	14.8 q	0.83 (3H, d, 6.7)
	NH		7.20 (H, d, 7.0)		7.09 (H, d, 7.1)
Aib-4	C=O	174.7 s		173.7 s	
	1	55.6 s		[b]56.0 s	
	2	25.6 q	1.33 (3H, s)	27.0 q	1.34 (3H, s)
	3	24.5 q	1.29 (3H, s)	22.7 q	1.38 (3H, s)
	NH		7.94 (H, s)		7.52 (H, s)
Aib-5	C=O	173.9 s		175.0 s	
	1	55.8 s		[b]55.9 s	
	2	26.9 q	1.42 (3H, s)	25.8 q	1.33 (3H, s)
	3	22.1 q	1.39 (3H, s)	23.1 q	1.31 (3H, s)

	NH		7.86 (H, s)		7.83 (H, s)
Ser/Ala	C=O	166.8 s		171.0 s	
	1	56.6 d	4.06 (H, m)	49.1 d	4.00 (H, dq, 8.1, 6.5)
	2	61.4 t	3.82 (H, m)	17.1 q	1.32 (3H, d, 6.5)
			3.68 (H, m)		
	OH		4.53 (H, t, 6.7)		
	NH		7.41 (H, d, 6.7)		7.36 (H, d, 8.1)
Aib-6	C=O	168.3 s		171.3 s	
	1	55.7 s		[b]55.8 s	
	2	25.0 q	1.33 (3H, s)	24.7 q	1.34 (3H, s)
	3	22.2 q	1.32 (3H, s)	23.9 q	1.33 (3H, s)
	NH		7.40 (H, s)		7.46 (H, s)
prolinol	1	56.0 d	4.15 (H, m)	56.2 d	4.15 (H, m)
	2	25.8 t	1.81 (H, m)	26.1 t	1.80 (H, m)
			1.62 (H, m)		1.66 (H, m)
	3	24.4 t	1.79 (H, m)	25.6 t	1.79 (H, m)
			1.62 (H, m)		1.66 (H, m)
	4	46.9 t	3.61 (H, m)	47.2 t	3.57 (H, m)
			3.40 (H, m)		3.44 (H, m)
	5	63.1 t	4.01 (H, dd, 10.3, 3.2)	63.4 t	4.03 (H, dd, 10.4, 3.1)
			3.92 (H, dd, 10.3, 7.4)		3.91 (H, dd, 10.4, 7.4)
Ac-2	C=O	170.0 s		170.3 s	
	CH_3	20.4 q	1.98 (3H, s)	20.7 q	1.99 (3H, s)

[a] Spectra were recorded in DMSO-d_6 at 500 and 125 MHz for ^{1}H and ^{13}C, respectively.
[b] Carbon chemical shifts may be interchanged.

Aspereline D (**5**) had the molecular formula $C_{44}H_{78}N_{10}O_{11}$, as determined by the HRESIMS and NMR data, and its structure was elucidated via 1D NMR, 2D NMR spectra and ESIMS/MS. Although this structure had been reported,[9] we successfully obtained the crystal from the solvent of MeOH/H_2O, and the crystal structure was determined by X-ray diffraction method. Figure 4 showed the perspective view of the molecule with the atomic numbering scheme. The results indicated that the molecule had a helix-like structure after calculating of the torsion angles (Table 2). Since peptidbols related to asperelines expressed various biological activities, the 3-dimensional structure obtained from the crystallography will be useful to understand the asperelines' conformation-activity relationships.

The cytotoxic effects of compounds **1**–**7** were evaluated using the MTT method on HL-60, A-375, and P388 cancer cell lines. Unfortunately, the results showed that all of their IC_{50} values were larger than 50 μM.

Table 2. Selected Torsion Angles(°)

C5-C3-C6-O2	-124.6(2)	C27-C25-C28-N7	172.43(16)	C11-C15-N4-C16	3.4(3)
C4-C3-C6-O2	-2.4(3)	N7-C29-C32-O8	-66.2(2)	C18-C16-N4-C15	-174.97(17)
N1-C3-C6-N2	117.3(2)	C31-C29-C32-O8	-144.78(19)	C17-C16-N4-C15	-64.9(2)
C5-C3-C6-N2	57.5(2)	C30-C29-C32-O8	-21.4(3)	C19-C16-N4-C15	173.78(17)
C4-C3-C6-N2	179.81(18)	N7-C29-C32-N8	98.9(2)	O5-C19-N5-C20	57.5(2)
N2-C7-C10-O3	-60.5(2)	C31-C29-C32-N8	39.7(2)	C16-C19-N5-C20	2.0(3)
C8-C7-C10-O3	-140.01(18)	C30-C29-C32-N8	163.05(18)	C24-C20-N5-C19	179.32(16)
C9-C7-C10-O3	-16.9(3)	N8-C33-C35-O9	-76.6(2)	C21-C20-N5-C19	60.8(2)
N2-C7-C10-N3	103.0(2)	C34-C33-C35-O9	-149.58(18)	O6-C24-N6-C25	-175.39(17)
C8-C7-C10-N3	44.0(2)	N8-C33-C35-N9	88.7(2)	C20-C24-N6-C25	-1.1(3)
C9-C7-C10-N3	167.08(17)	C34-C33-C35-N9	33.7(2)	C26-C25-N6-C24	177.34(16)
N3-C11-C12-C13	-73.0(2)	N9-C36-C39-O10	-88.1(2)	C27-C25-N6-C24	-69.5(2)
C15-C11-C12-C13	-58.6(2)	C38-C36-C39-O10	-139.33(18)	C28-C25-N6-C24	169.55(18)
N3-C11-C12-C14	70.2(2)	C37-C36-C39-O10	-19.4(2)	O7-C28-N7-C29	52.0(2)
C15-C11-C12-C14	67.6(2)	N9-C36-C39-N10	100.3(2)	C25-C28-N7-C29	-4.5(3)
N3-C11-C15-O4	-163.53(17)	C38-C36-C39-N10	43.0(2)	C31-C29-N7-C28	172.05(16)
C12-C11-C15-O4	-143.95(18)	C37-C36-C39-N10	162.88(17)	C30-C29-N7-C28	-59.7(2)
N3-C11-C15-N4	87.0(2)	N10-C40-C41-O11	-77.3(2)	C32-C29-N7-C28	178.90(17)
C12-C11-C15-N4	34.5(2)	C42-C40-C41-O11	47.7(2)	O8-C32-N8-C33	63.0(2)
N4-C16-C19-O5	-94.5(2)	N10-C40-C42-C43	-68.5(2)	C29-C32-N8-C33	-2.4(3)
C18-C16-C19-O5	-134.81(19)	C41-C40-C42-C43	21.1(2)	C35-C33-N8-C32	173.06(17)
C17-C16-C19-O5	-12.8(3)	C40-C42-C43-C44	141.60(19)	C34-C33-N8-C32	80.6(2)
N4-C16-C19-N5	108.7(2)	C42-C43-C44-N10	-38.1(2)	O9-C35-N9-C36	-157.48(19)
C18-C16-C19-N5	47.9(2)	O1-C2-N1-C3	39.8(2)	C33-C35-N9-C36	-11.4(3)
C17-C16-C19-N5	169.82(17)	C1-C2-N1-C3	-5.3(3)	C38-C36-N9-C35	165.28(17)
N5-C20-C21-C23	-68.6(2)	C5-C3-N1-C2	173.28(19)	C37-C36-N9-C35	-65.3(2)
C24-C20-C21-C23	-171.80(17)	C4-C3-N1-C2	-67.6(2)	C39-C36-N9-C35	175.42(18)
N5-C20-C21-C22	-48.4(2)	C6-C3-N1-C2	172.12(19)	O10-C39-N10-C44	54.9(2)
C24-C20-C21-C22	65.5(2)	O2-C6-N2-C7	53.9(2)	C36-C39-N10-C44	-172.36(19)
N5-C20-C24-O6	-171.08(16)	C3-C6-N2-C7	-8.4(3)	O10-C39-N10-C40	5.3(3)
C21-C20-C24-O6	-133.59(18)	C8-C7-N2-C6	169.48(17)	C36-C39-N10-C40	7.2(3)
N5-C20-C24-N6	102.9(2)	C10-C7-N2-C6	-61.8(2)	C43-C44-N10-C39	-175.17(17)
C21-C20-C24-N6	48.0(2)	C9-C7-N2-C6	61.6(2)	C43-C44-N10-C40	151.7(2)
N6-C25-C28-O7	-75.6(2)	O3-C10-N3-C11	178.24(18)	C41-C40-N10-C39	-27.9(2)
C26-C25-C28-O7	-132.02(19)	C7-C10-N3-C11	-2.5(3)	C42-C40-N10-C39	62.6(2)
C27-C25-C28-O7	-11.0(3)	C15-C11-N3-C10	173.59(16)	C41-C40-N10-C44	-175.31(17)
N6-C25-C28-N7	110.4(2)	C12-C11-N3-C10	73.7(2)	C42-C40-N10-C44	-117.78(18)
C26-C25-C28-N7	51.4(2)	O4-C15-N4-C16	-157.34(18)	C5-C3-C6-O2	4.3(2)

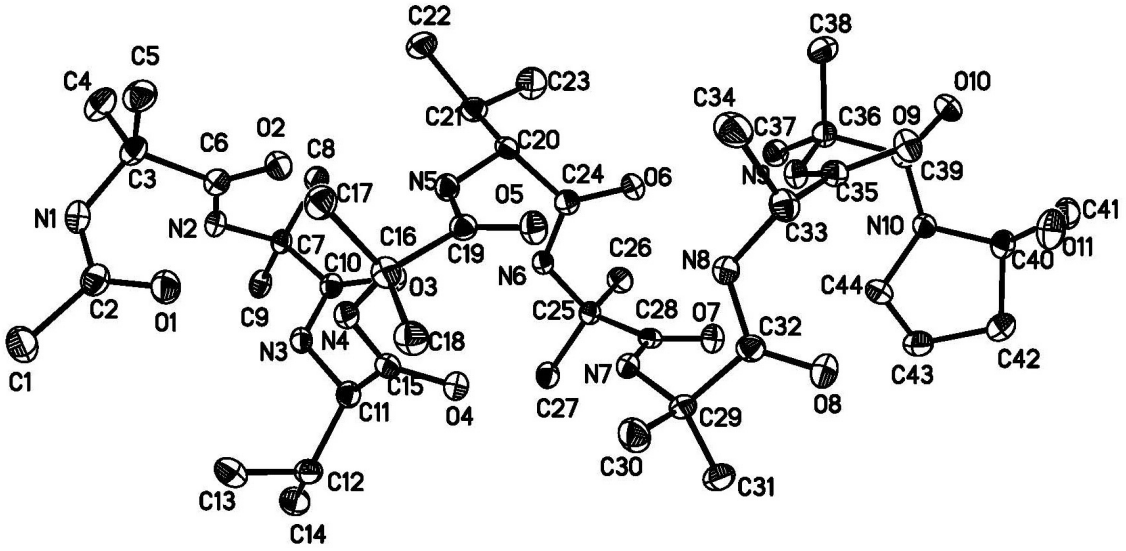

Figure 4. X-Ray Structure of Compound **5**

EXPERIMENTAL

General Experimental Procedures. Optical rotations were obtained from a Shenguang SGW-1 digital polarimeter. ^{1}H NMR, ^{13}C NMR, DEPT spectra and 2D NMR were recorded on a BRUKER BIOSPIN AVANCE III spectrometer using TMS as the internal standard. HRESIMS were obtained by an Agilent Q-TOF 6520 LC mass spectrometer. ESIMS/MS were obtained by a Thermo Scientific TSQ Quantum Access Max mass spectrometer. Semipreparative HPLC was performed using an ODS column (ODS-A, 10×250 mm, 5 μm) at 5 mL/min.

Fungal Material. The fungus *T. asperellum* was isolated from marine sediments collected from Langqi Island, Fujian, China. It was identified according to its morphological characteristics and ITS by Beijing Sunbiotech Co. Ltd, and preserved in our laboratory at −80 ˚C. The producing strain was prepared on Martin medium and stored at 4 ˚C.

Fermentation and Extraction. The fungus was cultured under static conditions at 28 ˚C for 30 days in 1000 mL conical flasks containing the liquid medium (400 mL/flask), composed of glucose (10 g/L), maltose (20 g/L), mannitol (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.3 g/L), yeast extract (3 g/L), and seawater. The fermented whole broth (90 L) was filtered through cheese cloth to separate supernatant from mycelia. The former was extracted two times with EtOAc to yield an EtOAc solution that was concentrated under reduced pressure to give a broth extract (33.2 g).

Purification. The broth extract (33.2 g) was separated into five fractions on a Si gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Fraction A (2.7 g) eluted with CH_2Cl_2/MeOH (100:1) was further purified on a Si gel column and a Sephadex LH-20 column to obtain four subfractions (A1 to A4). Subfraction A2 (890 mg) was purified by a reversed-phase column (MeOH:H_2O, 3:2) and semipreparative HPLC (45% MeCN), yielding compounds **4** (8.3 mg) and **5** (22 mg). Fraction B (5.6 g) eluted with CH_2Cl_2/MeOH (20:1) was separated into four subfractions (B1 to B4) on a Si gel column using a step gradient elution of petroleum ether, CH_2Cl_2, and MeOH. Subfraction B2 (785 mg) was purified by a reversed-phase column (MeOH:H_2O, 3:2) and semipreparative HPLC (55% MeCN), yielding compound **6** (28.5 mg). Subfraction B3 (403 mg) was subsequently purified by a Sephadex LH-20 column, a reversed-phase column (MeOH:H_2O, 3:2) and semipreparative HPLC (50% MeCN) to give compounds **1** (4.3 mg) and **2** (4.8 mg). Subfraction B4 (1.4 g) was purified by a reversed-phase column (MeOH:H_2O, 3:2) and semipreparative HPLC (55% MeCN) to give compounds **3** (261.3 mg) and **7** (12.0 mg).

Aspereline G (**1**): colorless needles (MeOH); $[\alpha]^{25}_D$ –22° (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data (see Table 1); (–)ESIMS/MS *m/z*: 994, 964, 922, 837, 752, 652, 567; (+)ESIMS/MS *m/z*: 1018 $[M + Na]^+$, 847, 762, 674, 589, 504, 391, 306; HRESIMS *m/z*: 995.6172 $[M + H]^+$ (calcd for $C_{47}H_{83}N_{10}O_{13}$: 995.6141).

Aspereline H (**2**): colorless needles (MeOH); $[\alpha]^{25}_D$ –45° (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data (see Table 1); (–)ESIMS/MS *m/z*: 978, 936, 851, 765, 666, 581, 468; (+)ESIMS/MS *m/z*: 1002 $[M + Na]^+$, 831, 745, 674, 589, 504, 391; HRESIMS *m/z*: 979.6203 $[M + H]^+$ (calcd for $C_{47}H_{83}N_{10}O_{12}$: 979.6192).

Hydrolysis and L-FDAA Derivatization of Asperelines G and H (1 and 2). Pure asperelines G and H (0.25 mg each) were hydrolyzed with 1 mL of 6 N HCl for 4 h in a sealed and thick-walled vial at 110 °C, respectively. The hydrolysates were evaporated under nitrogen and then derivatized by treatment first with 50 μL of 1 M $NaHCO_3$ and second with 50 μL of *N*-α-(2,4-dinitro-5-flourophenyl)-L-alaninamide (L-FDAA) solution (10 mg/mL in acetone). Each reaction mixture was heated to 80 °C for 30 min, cooled to room temperature, and finally quenched with 50 μL of 2 N HCl. MeCN (400 μL) was then added for subsequent HPLC analysis. Each standard amino acid (0.25 mg) was derivatized using the same procedure with L-FDAA. For each reaction product, a linear gradient of 5% to 100% MeCN (0.1% TFA) over 60 min was used to separate the derivatized products by HPLC (ODS column, 4.6 mm × 250 mm, UV 340 nm, flow rate 1 mL/min). The retention times of the derivatized hydrolysates were monitored by UV (340 nm) and compared with those of commercially available standards. The retention times of FDAA derivatives of the amino acid standards were identified at 19.55 (*S*-prolinol), 23.05 (L-Ser), 25.57 (L-Ala), 28.16 (L-Val), and 29.70 (L-Ile) min. The FDAA derivatives of hydrolysate from aspereline G showed peaks at 19.55, 23.05, 28.16, and 29.70 min, which corresponded to *S*-prolinol, L-Ser, L-Val, and L-Ile, respectively. For aspereline H, the peaks were at 19.55, 25.57, 28.16, and 29.70 min, indicating the

existence of *S*-prolinol, L-Ala, L-Val, and L-Ile. The co-injection experiment of hydrolyzate-FDAA derivatives and standard-FDAA derivatives further confirmed the results.

X-Ray Crystallography of 5. $C_{44}H_{78}N_{10}O_{11}\cdot 3H_2O$, MW = 977.21, space group $P2_12_12_1$, a = 10.297(2) Å, b = 16.785(3) Å, c = 30.395(6) Å, V = 5253.3(18) Å^3, Z = 4. The X-ray diffraction intensity data of **5** was collected on a Rigaku Saturn 724 CCD diffractometer with graphite-monochromater Mo Kα radiation (λ= 0.71073 Å) by the ω scan technique [$2\theta \leq 51°$]. A total of 11998 reflections were collected, of which 11631 with $F_0 \geq 4\sigma F_0$ were observed. The structure was solved by direct methods and refined by full-matrix least-squares procedure to R_1 = 0.0432 and wR_2 = 0.0989. H atoms bonded to C atoms were refined in idealized positions using the riding-model. All calculations were carried out on a personal computer using the SHELX-97 program system.

Supporting Information Available. The X-ray crystallographic data for the structure of **5** has been deposited at the Cambridge Crystallographic Data Centre (CCDC 907001). Copy of the data can be obtained free of charge by applying to CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (fax: +44 1223 762911; e-mail: deposit@ccdc.cam.ac.uk).

Biological Assay. Cytotoxic activity was evaluated by the MTT method using HL-60, A-375, and P388 cell lines. The cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37 ˚C. Those cell suspensions (200 μL) at a density of 5×10^4 cell mL^{-1} were plated in 96-well microtiter plates and incubated for 24 h at the above condition. The test compound solution (2 μL in DMSO) at different concentrations was added to each well and further incubated for 72 h in the same condition. Then 20 μL of the MTT solution (5 mg/mL in RPMI-1640 medium) was added to each well and incubated for 4 h. The old medium containing MTT (150 μL) was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Spectra Max Plus plate reader at 540 nm.

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