JOURNAL OF NATURAL PRODUCTS

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Cyclic Heptapeptides, Cordyheptapeptides C–E, from the Marine-Derived Fungus *Acremonium persicinum* SCSIO 115 and Their Cytotoxic Activities

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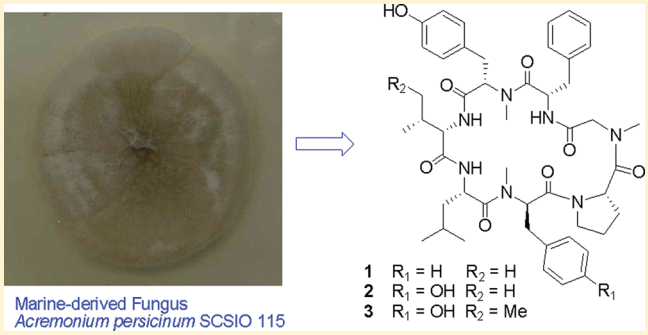
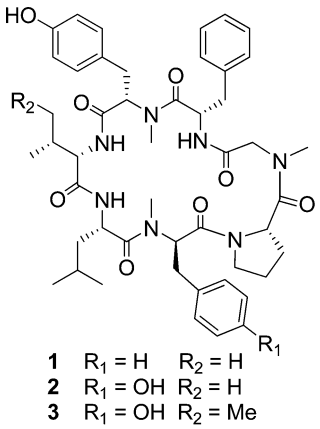
Supporting Information

ABSTRACT: Three new cycloheptapeptides, cordyheptapeptides C–E (**1**–**3**), were isolated from the fermentation extract of the marine-derived fungus *Acremonium persicinum* SCSIO 115. Their planar structures were elucidated on the basis of extensive MS, as well as 1D and 2D (COSY, HMQC, and HMBC) NMR spectroscopic data analyses. The absolute configurations of the amino acid residues were determined by single-crystal X-ray diffraction, Marfey's method, and chiral-phase HPLC analysis. Compounds **1** and **3** displayed cytotoxicity against SF-268, MCF-7, and NCI-H460 tumor cell lines with IC_{50} values ranging from 2.5 to 12.1 μM.



Marine fungi have attracted increasing attention from those in search of new pharmaceutically useful natural compounds in the past decade. So far, more than one thousand structurally unique and biologically active compounds have been isolated from marine-derived fungi.[1] Accordingly, marine fungi have been considered as an emerging resource for drug discovery.[2] For instance, plinabulin (NPI-2358), a synthetic analogue of halimide deriving from the marine fungus *Aspergillus* sp. CNC-139, is now in phase II clinical trial for the treatment of cancer.[3] In our efforts to identify novel structures and bioactive metabolites from South China Sea-derived fungi, we screened the fermentation extracts of 180 fungi for cytotoxic activity and have reported that the fungus *Xylaria* sp. SCSIO 156 produces new cytotoxic cytochalasins.[4] Another fermentation extract of strain SCSIO 115, identified as *Acremonium persicinum*, showed strong lethality against brine shrimp (*Artemia salina*) and cytotoxicity toward a panel of tumor cell lines. Chemical investigation of an *A. persicinum* SCSIO 115 extract has led to the identification of three new cycloheptapeptides, designated cordyheptapeptides C–E (**1**–**3**). Here we report the fermentation, isolation, structure elucidation, and cytotoxic activities of these compounds against three cancer cell lines, SF-268, MCF-7, and NCI-H460.

The organic extract of the fungal culture was subjected to column chromatography on silica gel, reversed-phase silica gel (ODS), and Sephadex LH-20 guided by brine shrimp lethality assays[4] to yield compounds **1**–**3**. Compound **1** was isolated as colorless crystals. Its molecular formula, $C_{48}H_{63}N_7O_8$, was established by HRESIMS, indicating 21 degrees of unsaturation. The ^{1}H and ^{13}C NMR spectra of **1** exhibited characteristic



signals for a heptapeptide. Seven carbonyl resonances at δ_C 174.1, 172.3, 170.9, 170.5, 170.4, 168.4, and 168.2 together with the seven α-amino acid carbon resonances between δ_C 69.2 and 47.6 in the ^{13}C NMR spectrum indicated the presence of seven amino acid residues. The ^{1}H NMR spectrum of **1** displayed seven methyl signals in the upfield region, three of which were assigned as *N*-Me groups at δ_H 3.03, 2.61, and 2.91. Comprehensive analysis of the 1D (^{1}H and ^{13}C, DEPT) and 2D (COSY, HMQC, and HMBC) NMR spectroscopic data revealed that **1** was a heptapeptide containing *N*-MeTyr, Phe, *N*-MeGly, Pro, *N*-MePhe, Leu, and Val residues (Table 1). The

Received: March 2, 2012
Published: May 29, 2012

dx.doi.org/10.1021/np300152d | *J. Nat. Prod.* 2012, 75, 1215–1219

Table 1. Summary of ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectroscopic Data for Compounds 1−3

	1[a]		2[b]		3[a]	
position	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)	δ_C	δ_H, mult. (J in Hz)
*N*MeTyr			*N*MeTyr[1]		*N*MeTyr[1]	
CO	170.4, C[d]		170.7, C		170.4, C	
α	69.2, CH	3.40, dd (11.5, 3.5)	69.0, CH	3.40, m	69.3, CH	3.39, m
β	32.4, CH_2	3.16, m	32.4, CH_2	3.13, m	32.1, CH_2	3.16, d (11.5)
		2.72, m		2.72, m		2.70, m
1	127.0, C		126.7, C[e]		127.0, C	
2,6	129.8, CH	6.22, d (8.1) 155.3, C	129.7, CH	6.21, d (8.4)	130.0, CH	6.21, d (6.5)
3,5	115.7, CH	6.53, d (8.1)	115.6, CH	6.47, d (8.4)	115.8, CH	6.53, d (6.5)
4			155.3, C		155.7, C	
*N*Me	40.4, CH_3	2.61, s	40.3, CH_3	2.59, br s	40.5, CH_3	2.56, s
Phe			Phe		Phe	
CO	170.5, C[d]		170.2, C		170.3, C	
α	50.1, CH	5.36, m	49.8, CH	5.26, m	50.2, CH	5.29, m
β	38.1, CH_2	3.05, dd (12.3, 11.7)	38.0, CH_2	3.04, dd (12.5, 11.5)	38.1, CH_2	3.06, d (12.5)
		2.82, dd (12.3, 3.0)		2.74, dd (12.5, 3.0)		2.71, d (12.5)
1	137.3, C		137.3, C		137.4, C	
2, 6	128.6, CH	7.31, br d (7.3)	130.1, CH	7.29, m	130.1, CH	7.25, t (6.5)
3, 5	130.1, CH	7.40, m	128.5, CH	7.36, m	128.6, CH	7.33, t (6.5)
4	126.8, CH	7.36, m	126.6, CH[e]	7.30, m	126.7, CH	7.31, m
NH		8.61, d (9.5)		8.55, d (8.0)		8.59, d (10.0)
*N*MeGly			*N*MeGly		*N*MeGly	
CO	168.2, C		168.1, C		168.1, C	
α	50.8, CH_2	5.40, d (18.0)	50.8, CH_2	5.29, d (17.5)	50.8, CH_2	3.30, d (17.5)
		3.33, d (18.0)		3.28, d (17.5)		5.34, d (17.0)
*N*Me	35.5, CH_3	2.91, s	35.5, CH	2.85, s	35.8, CH_3	2.87, s
Pro			Pro		Pro	
CO	172.3, C		172.5, C		172.6, C	
α	57.8, CH	4.39, m	58.1, CH	4.38, dd (9.2, 3.2)	57.7, CH	4.37, dd (9.0, 2.5)
β	31.4, CH_2	2.42, m; 2.04, m	31.4, CH_2	2.40, m; 1.98, m	31.5, CH_2	2.42, m; 2.02, m
γ	22.0, CH_2	1.78, m; 1.85, m	22.0, CH_2	1.84, m; 1.77, m	22.2, CH_2	1.87, m; 1.77, m
δ	48.4, CH_2	3.60, m; 3.77, m	48.4, CH_2	3.70, m; 3.55, m	48.6, CH_2	3.75, m; 3.58, m
*N*MePhe			*N*MeTyr[2]		*N*MeTyr[2]	
CO	168.4, C		168.7, C		168.6, C	
α	54.5, CH	5.56, dd (12.0, 4.5)	54.6, CH	5.40, dd (11.5, 5.0)	54.9, CH	5.46, dd (11.5, 5.0)
β	35.2, CH_2	3.28, dd (14.7, 12.0)	34.1, CH_2	3.11, m	34.3, CH	3.18, dd (15.0, 5.0)
		3.02[c]		2.90, dd (15.0, 5.0)		2.97, dd (15.0, 5.0)
1	136.8, C		127.3, C		127.2, C	
2, 6	129.7, CH	7.15, t (7.0)	130.6, CH	6.93, d (8.3)	130.7, CH	6.98, d (8.0)
3, 5	128.6, CH	7.12, t (7.0)	115.3, CH	6.54, d (8.3)	115.7, CH	6.59, d (8.0)
4	126.8, CH	7.04, t (7.0)	155.3, C		155.3, C	
*N*Me	30.1, CH_3	3.03, s	29.9, CH_3	2.97, s	29.8, CH_3	3.02, s
Leu			Leu		Leu	
CO	174.1, C		174.1, C		174.3, C	
α	47.6, CH	4.92, t (9.5)	47.6, CH	4.87, m	47.8, CH	4.97, dd (12.5, 9.0)
β	39.9, CH_2	1.34, t (12.0)	39.9, CH_2	1.39, t (11.5)	40.0, CH_2	1.44, t (12.5)
		0.12, t (12.0)		0.21, t (11.5)		0.23, t (12.5)
γ	24.8, CH	1.55, m	24.8, CH	1.51, m	24.9, CH	1.56, m
δ_A-Me	23.7, CH_3	0.91, d (7.0)	23.5, CH_3	0.91, d (7.0)	23.8, CH_3	0.95, d (6.5)
δ_B-Me	20.8, CH_3	0.84, d (7.0)	19.5, CH_3	0.86, d (7.0)	21.0, CH_3	0.85, d (6.5)
*N*H		8.20, d (9.5)		8.16, d (8.0)		8.20, d (9.0)
Val			Val		L-*allo*-IIe	
CO	170.9, C		171.1, C		171.4, C	
α	58.1, CH	4.40, dd (9.5, 2.5)	58.2, CH	4.33, dd (9.5, 3.2)	56.0, CH	4.57, dd (9.5, 3.0)
β	28.5, CH	2.63, m	28.4, CH	2.60, m	35.2, CH	2.39, m
γ	16.3, CH_3	0.80, d (7.0)	20.7, CH_3	0.81, d (7.0)	26.6, CH_2	1.22, m
δ-Me	19.6, CH_3	0.89, d (7.0)	16.2, CH_3	0.78, d (7.0)	12.0, CH_3	0.89, t (7.4)
β-Me					14.4, CH_3	0.80, d (7.0)
NH		5.88, d (9.5)		5.94, d (9.5)		5.87, d (9.5)

[a]Recorded in $CDCl_3$. [b]Recorded in $CDCl_3$−CD_3OD. [c]Overlapped with 3.03. [d,e]The assignments bearing the same superscript can be interchanged.

dx.doi.org/10.1021/np300152d | *J. Nat. Prod.* 2012, 75, 1215−1219

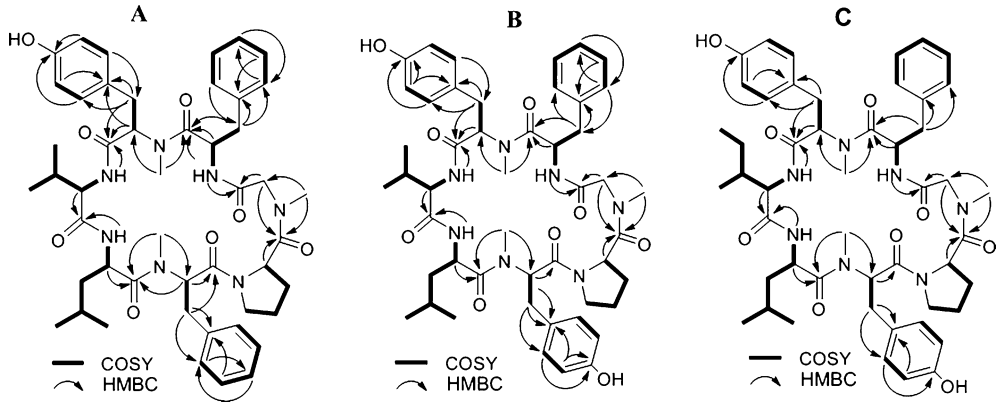


Figure 1. COSY and HMBC correlations of compounds **1**–**3**.

amino acid sequence of **1** was determined on the basis of HMBC experiments (Figure 1A). Paramount HMBC correlations of *N*-Me (*N*-MePhe)/C═O (Leu), NH (Leu)/C═O (Val), NH (Val)/C═O (*N*-MeTyr), *N*-Me (*N*-MeTyr)/C═O (Phe), NH (Phe)/C═O (*N*-MeGly), and *N*-Me (*N*-MeGly)/C═O (Pro) established that the heptapeptide contained the sequence −*N*-MePhe-Leu-Val-*N*-MeTyr-Phe-*N*-MeGly-Pro−, accounting for 20 out of the 21 calculated degrees of unsaturation. The cyclic nature of **1** accounted for the remaining degree of unsaturation not satisfied by an acyclic peptide. The amino acid sequence of **1** was also confirmed by analyses of the ESIMS/MS data of the quasi-molecular ion (Supporting Information, Figure S1c).[5] Consequently, the planar structure of **1** was elucidated as cyclo-(-*N*-MeTyr-Phe-*N*-MeGly-Pro-*N*-MePhe-Leu-Val).

The absolute configuration of **1** was determined by single-crystal X-ray crystallography using Cu Kα radiation (Figure 2).

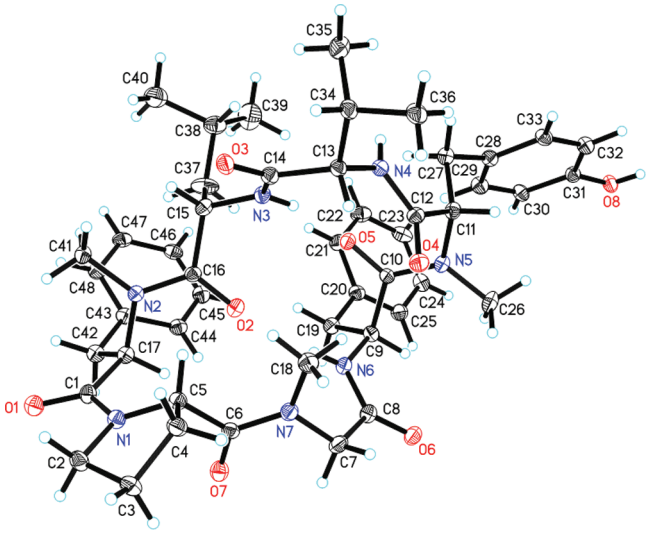


Figure 2. X-ray crystal structure of cordyheptapeptide C (**1**).

The X-ray diffraction pattern not only confirmed the amino acid sequence but also allowed assignment of the six chiral amino acids as *N*-Me-D-Phe, L-Leu, L-Val, *N*-Me-L-Tyr, L-Phe, and L-Pro through refinement of Flack's parameter [x = 0.05(15)].[6] These results were in agreement with HPLC analyses of the acid hydrolysates of **1** generated using Marfey's method and chiral-phase HPLC analyses.[7,8] A literature search disclosed that the structure of **1** is very similar to those of the reported cordyheptapeptides A and B, which were isolated from the insect pathogenic fungi *Cordyceps* sp. BCC 1788 and 16176.[9,10] The L-Ile residue in cordyheptapeptide A was replaced by an L-Val in **1**. Hence, compound **1** was accordingly named cordyheptapeptide C.

Compound **2** was obtained as white needles. The molecular formula of **2** was determined by HRESIMS as $C_{48}H_{63}N_7O_9$, suggesting that **2** had one more oxygen atom than **1**. The ^{1}H NMR data of **2** resembled those of **1**, except that two pairs of *ortho*-coupled aromatic proton signals at δ_H 6.93 and 6.54 (d, J = 8.3 Hz) were observed instead of five aromatic proton signals between δ_H 7.04 and 7.15. The ^{13}C NMR spectrum of **2** was characterized by two oxygen-bearing quaternary carbon signals at δ_C 155.3. These data suggested the presence of two Tyr residues in **2**. Detailed interpretation of the COSY, HMQC, and HMBC spectra revealed that the *N*-MePhe in **1** was replaced by a *N*-MeTyr in **2** (Figure 1B). The amino acid sequence of **2** was also supported by analyses of the ESIMS/MS data of the quasi-molecular ion (Supporting Information, Figure S2c). The absolute configurations of the amino acid residues composing cyclopeptide **2** were determined by Marfey's method and found to be identical to those identified in **1**. Therefore, the structure of **2** was established as cyclo-(-*N*-Me-L-Tyr^1-L-Phe-*N*-MeGly-L-Pro-*N*-Me-D-Tyr^2-L-Leu-L-Val-) and named cordyheptapeptide D.

Compound **3** was determined by HRESIMS to have the molecular formula $C_{49}H_{66}N_7O_9$, possessing one CH_2 beyond that of **2**. A comparison of the ^{1}H and ^{13}C NMR spectroscopic data for **3** with those of **2** revealed close structural similarity with the exception of one additional methylene (δ_H 1.22 and δ_C 26.6) in **3**. Furthermore, the ^{13}C NMR signal of a Val methine in **2** was shifted downfield from δ_C 28.4 to δ_C 35.2 in **3**, and two Val methyl signals at δ_C 20.7 and 16.2 in **2** were shifted to δ_C 12.0 and 14.4 in **3**, respectively. These data suggested that the Val residue in **2** was substituted by an Ile in **3**. Detailed analyses of the COSY, HMQC, and HMBC spectroscopic data of **3** confirmed this hypothesis (Figure 1C). Evidence in support of the amino acid sequence of **3** was also obtained from the ESIMS/MS data of the quasi-molecular ion (Supporting Information, Figure S3c). It was noticed that the ^{13}C NMR spectroscopic data of the Ile residue in **3** were significantly different from those of the L-Ile in cordyheptapeptides A and B,[9,10] but very similar to those reported for L-*allo*-Ile unit,[11] thereby suggesting the presence of an L-*allo*-Ile residue in **3**. Through Marfey's analysis, the L-Ile and L-*allo*-Ile residues

dx.doi.org/10.1021/np300152d | *J. Nat. Prod.* 2012, 75, 1215–1219

could not be differentiated because the retention times of their FDAA derivatives were identical. Subsequently, chiral-phase HPLC analysis of the acid hydrolysates of **3** was carried out, which successfully clarified the configuration of the Ile residue in **3** as L-*allo*-Ile. The absolute configurations of the other amino acid residues were determined to be the same as those in **2** using Marfey's method. Thus, compound **3** was identified as cyclo-(-*N*-Me-L-Tyr[1]-L-Phe-*N*-MeGly-L-Pro-*N*-Me-D-Tyr[2]-L-Leu-L-*allo*-Ile-), possessing an L-*allo*-Ile residue instead of the L-Ile residue as well as an *N*-Me-D-Tyr residue instead of the *N*-Me-D-Phe residue in cordyheptapeptide A. Compound **3** was named cordyheptapeptide E.

The cytotoxicities of cordyheptapeptides C–E (**1**–**3**) were evaluated using human glioblastoma (SF-268), human breast cancer (MCF-7), and human lung cancer (NCI-H460) cell lines and the sulforhodamine B (SRB) method (Table 2).

Table 2. In Vitro Cytotoxicities (IC_{50}, μM) of Compounds 1–3 ($x \pm s$, $n = 3$)

	SF-268	MCF-7	NCI-H460
1	3.7 ± 0.3	3.0 ± 0.2	11.6 ± 0.5
2	45.6 ± 5.4	82.7 ± 1.8	>100 μM
3	3.2 ± 0.2	2.7 ± 0.2	4.5 ± 0.3
cisplatin[a]	4.6 ± 0.1	10.2 ± 0.4	1.6 ± 0.1

[a]Positive control.

Cordyheptapeptide E (**3**) demonstrated cytotoxicity against all three cell lines, with IC_{50} values of 3.2, 2.7, and 4.5 μM, respectively. Cordyheptapeptide C (**1**) also was found to possess cytotoxicity against SF-268 and MCF-7 cells with IC_{50} values of 3.7 and 3.0 μM, respectively, and weaker cytotoxicity against the NCI-H460 cell line. The most polar compound, cordyheptapeptide D (**2**), displayed no activity against all three cell lines. The results of the biological assays are shown in Table 2. In the literature, cordyheptapeptides A and B were reported to possess cytotoxicities against KB (oral human epidermoid carcinoma), BC (human breast cancer), NCI-H187 (human small cell lung cancer), and Vero (African green monkey kidney fibroblasts) cell lines with IC_{50} values of 0.78, 0.20, 0.18, 14 μM and 2.0, 0.66, 3.1, 1.6 μM, respectively.[10] These results provide a vivid demonstration of how subtle differences in structure among cordyheptapeptides A–E profoundly impact their biological activities.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on an SFW-X-4 apparatus and are uncorrected. Optical rotations were obtained with an Anton Paar MCP 300 polarimeter. UV spectra were recorded on a U-2910 spectrometer (Hitachi). IR spectra were measured on an IRAffinity-1 Fourier transform infrared spectrophotometer (Shimadzu). 1D and 2D NMR spectra were recorded with a Bruker Avance-500 spectrometer using TMS as an internal standard. ESIMS spectra were measured using a Bruker Esquire 3000[plus] spectrometer. HR-ESIMS spectra were measured with a Waters Q-TOF-micromass spectrometer. ESIMS[2] spectra were measured using a Bruker Maxis spectrometer. TLC was performed on precoated plates with silica gel GF_{254} (10–40 μm). Column chromatography (CC) was performed using silica gel (100–200 mesh, Qingdao Marine Chemical Chemicals) and Sephadex LH-20 (Amersham Pharmacia Biotech). MPLC was performed using a CHEETAH MP 100 (Bonna-Agela Technologies) equipped with an ODS column (20 × 90 mm, 40–63 μm, YMC). Semipreparative HPLC was performed using a Hitachi L-2000 with an ODS column (250 × 10 mm, 5 μm, YMC-Pack ODS-A). Single-crystal data were collected with an Oxford Xcalibur Onyx Nova diffractometer using Cu Kα radiation. The common amino acids were purchased from Sangon Biotech Co., Ltd (Shanghai). *N*-Me-D-Phe and *N*-Me-D-Tyr were synthesized by Binhai Hanhong Biochemical Co., Ltd (Shanghai), and *N*-Me-L-Phe and *N*-Me-L-Tyr were purchased from Sigma-Aldrich Co.

Fungal Material. Fungus SCSIO 115 was isolated from a marine sediment sample collected in the South China Sea at E 1114°47.802′, N 18°47.922′ and at a depth of 205 m. This fungus was characterized as *Acremonium persicinum* SCSIO 115 on the basis of morphology[12] and analysis of the ITS region[13] sequence with Genbank accession number JQ599382. This fungus was deposited in RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences (Guangzhou, China).

Fermentation and Isolation. *A. persicinum* SCSIO 115 was maintained on potato dextrose agar at 25 °C. Agar plugs were inoculated into a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose broth supplemented with 3% sea salt. Flask cultures were incubated at 28 °C on a rotary shaker at 200 rpm for two days as seed culture. Thirty 1 L Erlenmeyer flasks, each containing 250 mL of liquid medium (20% peeled potatoes, 2% glucose, 3% sea salt, and 0.5% bacterial peptone), were individually inoculated with 25 mL of seed culture and then incubated at 28 °C on a rotary shaker at 200 rpm for seven days. The fermented broth was filtered through cheesecloth to separate the supernatant and mycelia. The supernatant was processed with XAD-16 resin three times, and the XAD-16 resin was eluted with EtOH to afford the broth extract. The mycelia were extracted with acetone (3 × 1 L) to afford the mycelial extract. These two extracts were combined (affording 8.28 g) and applied to Si CC; gradient elution with $CHCl_3$–MeOH afforded six fractions (Fr.1–Fr.6). Fr.2 (1.60 g), eluted with $CHCl_3$–MeOH (95:5), was further refined by Sephadex LH-20 CC eluted with $CHCl_3$–MeOH (1:1) to give four fractions (Fr.2-1–Fr.2-4). Fr.2-1 was purified by Si CC, eluted with gradient ratios of $CHCl_3$–MeOH (100:0, 99:1, 98:2, 97:3, 96:4, and 95:5), to give seven fractions (Fr.2-1-1–Fr.2-1-7). Fr.2-1-6, eluted by $CHCl_3$–MeOH (96:4), was further purified by MPLC with an ODS column eluted with MeOH–H_2O (30:70 to 80:20 over 50 min, 15 mL/min) to yield **1** (15.6 mg). Fr.2-1-7, eluted with $CHCl_3$–MeOH (95:5), was subjected to Si CC eluted with EtOAc–MeOH to give four fractions (Fr.2-1-7-1–Fr.2-1-7-4). Fr.2-1-7-2, eluted with EtOAc–MeOH (97:3), was isolated by semipreparative HPLC with an ODS column eluted with MeCN–H_2O (50:50 to 100:0 over 30 min, 2.5 mL/min) to yield compound **3** (6.7 mg, t_R 14.9 min). Fr.2-1-7-3, eluted with EtOAc–MeOH (96:4), was further purified by MPLC with an ODS column eluted with MeOH–H_2O (40:60 to 100:0 over 60 min, 15 mL/min) to yield compound **2** (86.2 mg).

*Cordyheptapeptide C (**1**):* colorless crystals ($CHCl_3$–MeOH, 3:1); mp 256–257 °C; $[\alpha]^{25}_D$ −80 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 228 (4.10), 278 (3.24) nm; IR (KBr) ν_{max} 3277, 2910, 1630, 1541, 1510 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 1; HR-ESIMS *m/z* 864.4648 $[M - H]^-$ (calcd for $C_{48}H_{63}N_7O_8$, 864.4665).

*Cordyheptapeptide D (**2**):* white needles ($CHCl_3$–MeOH, 1:1); mp 207–208 °C; $[\alpha]^{25}_D$ −85 (*c* 0.2, $CHCl_3$–MeOH, 1:1); UV (MeOH) λ_{max} (log ε) 228 (4.18), 278 (3.42) nm; IR (KBr) ν_{max} 3291, 2960, 1628, 1539, 1510 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 1; HR-ESIMS *m/z* 882.4777 $[M + H]^+$ (calcd for $C_{48}H_{63}N_7O_9$, 882.4760).

*Cordyheptapeptide E (**3**):* white needles ($CHCl_3$–MeOH, 1:1); mp 189–190 °C; $[\alpha]^{25}_D$ −60 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 225 (4.11), 281 (3.39); IR (KBr) ν_{max} 3292, 2957, 1628, 1539, 1505 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 1; HR-ESIMS *m/z* 896.4925 $[M + H]^+$ (calcd for $C_{49}H_{65}N_7O_9$, 896.4917).

X-ray Structure Determination of 1. A colorless crystal of **1** was obtained in $CHCl_3$–MeOH (3:1). The crystal data of **1** were recorded on an Oxford Xcalibur Onyx Nova single-crystal diffractometer with Cu Kα radiation (λ = 1.5418 Å). The structure was solved by direct methods (SHELXS-97) and refined using full-matrix least-squares difference Fourier techniques.[14] Crystallographic data for **1** have been deposited in the Cambridge Crystallographic Data Center with the

deposition number CCDC 853793. A copy of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44(0)-1233-336033 or e-mail: deposit@ccdc.cam.ac.uk].

Crystal data of **1**: monoclinic, $C_{49}H_{69}N_7O_{10}$, space group $P2_1$, a = 12.4995(14) Å, b = 13.3874(14) Å, c = 14.8616(15) Å, α = 90°, β = 99.9697(11)°, γ = 90°, V = 2449.31(5) Å^3, Z = 2, D_{calcd} = 1.242 g/cm^3, μ = 0.711 mm^{-1}, and $F(000)$ = 984. Crystal size: 0.34 × 0.23 × 0.18 mm^3. Independent reflections: 8779 [R_{int} = 0.0881]. The final indices were R_1 = 0.0426, wR_2 = 0.1061 [$I > 2\sigma(I)$].

HPLC Analysis of the Acid Hydrolysates of 1−3 Using Marfey's Method. Compounds **1** (0.88 mg), **2** (0.88 mg), and **3** (0.35 mg) were each dissolved in 6 N HCl (1 mL) and heated at 110 °C for 18 h. After cooling to room temperature (rt), the solvent was removed under reduced pressure. The remaining hydrolysate was resuspended in 50 μL of H_2O and treated with 100 μL of 1% (w/v) 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in acetone and 25 μL of 1 M $NaHCO_3$. The mixture was heated at 40 °C for 1.5 h. After cooling to rt, the contents were neutralized with 25 μL of 1 M HCl, and the resulting mixture was added to 300 μL of MeOH to afford a final hydrolysate volume of 500 μL. From this sample was then withdrawn a 50 μL aliquot, and its solvent removed followed by redissolution in 50 μL of MeOH. Ten microliters of this sample was then analyzed by HPLC (Alltima C_{18} column; 4.6 × 250 mm, 5 μm) using a solvent gradient from 10% to 70% solvent B (solvent A: 15:85 MeCN−H_2O w/0.1% TFA; solvent B: 90:10 MeCN−H_2O w/0.1% TFA) over the course of 40 min at 1 mL/min with detection at 340 and 210 nm. Amino acid standards (4 μM) were prepared by dissolving amino acids in 10 μL of H_2O followed by addition of 20 μL of FDAA and 5 μL of 1 M $NaHCO_3$, reaction at 40 °C for 1.5 h, and neutralization with 5 μL of 1 M HCl. Mixtures were then processed for HPLC in a fashion similar to that used for cyclopeptide analyses; the retention times for FDAA derivatives of L-Pro, D-Pro, L-Val, D-Val, L-Ile, L-*allo*-Ile, D-Ile, D-*allo*-Ile, L-Leu, D-Leu, L-Phe, D-Phe, *N*-Me-L-Phe, *N*-Me-D-Phe, *N*-Me-L-Tyr, and *N*-Me-D-Tyr were 17.8, 18.7, 22.5, 26.7, 25.7, 25.7, 29.0, 29.0, 26.4, 29.5, 26.3, 28.6, 26.1, 26.1, 15.5, and 15.1 min, respectively. Accordingly, the amino acids were assigned in **1** as *N*-Me-L-Tyr (15.5 min), L-Leu (26.4 min), L-Val (22.5 min), L-Phe (26.3 min), L-Pro (17.8 min), *N*-Me-D-Phe or *N*-Me-L-Phe (26.1 min), in **2** as *N*-Me-L-Tyr^1 (15.5 min), *N*-Me-D-Tyr^2 (15.1 min), L-Leu (26.4 min), L-Val (22.5 min), L-Phe (26.3 min), L-Pro (17.8 min), and in **3** as *N*-Me-L-Tyr^1 (15.5 min) and *N*-Me-D-Tyr^2 (15.1 min), L-Leu (26.4 min), L-Phe (26.3 min), L-Pro (17.8 min), L-Ile or L-*allo*-Ile (25.7 min), respectively.

Chiral-Phase HPLC Analysis of the Acid Hydrolysates of 1 and 3. To determine the absolute configurations of the *N*-MePhe in **1** and the Ile in **3**, chiral-phase HPLC analyses of the acid hydrolysates were conducted. Compounds **1** (0.30 mg) and **3** (0.30 mg) were hydrolyzed as mentioned above. The dried hydrolysate was dissolved in 100 μL of 2 mM $CuSO_4$−H_2O solution. Ten microliters of this sample were then analyzed by HPLC with a chiral column (MCIGEL CRS10W, 4.6 × 50 mm, Mitsubishi Chemical Corporation) using 2 mM $CuSO_4$−H_2O solution as the mobile phase at a flow rate of 0.5 mL/min with UV detection at 254 nm. *N*-Me-L-Phe and *N*-Me-D-Phe, L-Ile, and L-*allo*-Ile were detected as references. The retention times of the *N*-Me-D-Phe, *N*-Me-L-Phe, L-*allo*-Ile, and L-Ile were 25.2, 26.3, 11.6, and 14.3 min, respectively. Hence, the *N*-MePhe residue in **1** was assigned as *N*-Me-D-Phe (25.1 min), and the Ile residue in **3** was assigned as L-*allo*-Ile (11.4 min) (Supporting Information, Figures S19a and S19b).

Cytotoxic Activity Assays. Compounds **1**−**3** were evaluated for their cytotoxic activities against SF-268, MCF-7, and NCI-H460 cell lines with the SRB method.[15] Cells (180 μL) with a density of 3 × 10^4 cells/mL of media were seeded onto 96-well plates and incubated for 24 h at 37 °C, 5% CO_2. To plate wells were then added 20 μL of various concentrations of compounds, and plates were further incubated for 72 h. After incubation, cell monolayers were fixed with 50% (wt/v) trichloroacetic acid (50 μL) and stained for 30 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. Unbound dye was removed by washing repeatedly with 1% acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution (200 μL) for OD determination at 570 nm using a microplate reader. Cisplatin was used as a positive control, possessing potent cytotoxic activity. All data were obtained in triplicate and are presented as means ± SD. IC_{50} values were calculated with the SigmaPlot 10.0 software using a nonlinear curve-fitting method.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

1D and 2D NMR, HRESIMS, and ESIMS^2 spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the analytical facility center of South China Sea Institute of Oceanology for recording NMR and MS data. This work is supported, in part, by grants from the Knowledge Innovation Programs of the Chinese Academy of Sciences (KZCX2-YW-JC202 and KZCX2-EW-G-12), Science and Technology Planning Project of Guangdong Province (2010B030600010), National High Technology Research Program of China (2012AA092104), National Basic Research Program of China (2010CB833805), and Scientific Research Foundation for the Returned Overseas Chinese Scholars of the State Education Ministry. J.J. is a scholar of the "100 Talents Project" of Chinese Academy of Sciences (08SL111001).

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