Cyclic Peptides from an Endophytic Fungus Obtained from a Mangrove Leaf (*Kandelia candel*)

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Two new cyclic depsipeptides, 1962A (**1**) and 1962B (**2**), along with the three known cyclodipeptides cyclo-(Leu-Tyr) (**3**), cyclo-(Phe-Gly) (**4**), and cyclo-(Leu-Leu) (**5**) were isolated from the fermentation broth of the mangrove endophytic fungus (No. 1962) isolated from an old leaf of *Kandelia candel* collected in Hong Kong. Through spectroscopic experimentation, X-ray crystallographic analysis, and acid hydrolysis followed by chiral HPLC analysis, their structures were established to be 1962A, cyclo-(D-Leu-Gly-L-Tyr-L-Val-Gly-S-O-Leu) (**1**), and 1962B, cyclo-(D-Leu-Gly-L-Phe-L-Val-Gly-S-O-Leu) (**2**), respectively. Both of these new cyclo-depsipeptides were found to contain one D-amino acid. In the MTT bioassay, 1962A (**1**) showed weak activity against human breast cancer MCF-7 cells.

Peptides have played a significant role in pharmaceutical research as biomedically useful agents or as lead compounds for drug development.[1,2] Many natural cyclopeptides have novel structures and exhibit significant bioactivity.[3–7] On the other hand, a growing number of biologically active metabolites from marine microorganisms, including terpenoids, cyclopeptides, and lactones, have been isolated during the last two decades.[8–12] As a part of our ongoing search for novel and potent antitumor natural products from marine mangrove fungi,[13–17] we carried out chemical investigations on the crude extract of a mangrove endophytic fungus, No. 1962. This paper reports two new cyclohexpeptides, 1962A (**1**) and 1962B (**2**), together with three known compounds, cyclo-(Leu-Tyr) (**3**),[18] cyclo-(Phe-Gly) (**4**),[19] and cyclo-(Leu-Leu) (**5**),[20] from the fermentation broth of this fungus.

Results and Discussion

A 100 L fermentation broth was concentrated and extracted with ethyl acetate. The extract was repeatedly chromatographed over silica gel. Compound **1** was obtained as colorless block crystals, mp 180–182 °C, $[\alpha]^{20}_D = -63.4$ (*c* 0.005, MeOH), and determined to have the molecular formula $C_{30}H_{45}N_5O_8$ by HRESIMS data (*m/z* $[M + 1]^+$ 604.3317, calcd 604.3346; $[M + Na]^+$ 626.3110, calcd 626.3150). The number of hydrogen and carbon atoms observed in the ^{1}H and ^{13}CNMR spectra were in agreement with this molecular formula (see Table 1).

The IR spectrum of **1** showed the presence of ester and amide carbonyl groups with bands at 1758 and 1661 cm^{-1} and typical benzene C=C absorption bands at 1555, 1457, and 1377 cm^{-1}. The ^{1}H and ^{13}C NMR spectra of **1** showed typical signals for a cyclopeptide. In the ^{13}C NMR spectrum there was one ester carbonyl carbon (δ_C172.3), five amide carbonyl carbons (δ_C 170.9, 170.7, 170.2, 169.8, and 169.6), one oxygen-bearing methine carbon (δ_C 72.0), two nitrogen-bearing methylene carbons (δ_C 40.6 and 41.3), and six methyl groups (δ_C 22.9, 22.3, 21.7, 20.9, 18.6, and 18.4). The ^{1}H NMR spectrum of 1962A (**1**) in DMSO-d_6 revealed five NH proton signals (δ_H 8.95, 8.02, 7.87, 7.73, and 7.06) and the proton signals for a *p*-disubstituted benzene (δ_H 7.00, 6.60, 4H). Combining the above information with HMQC and ^{1}H–H COSY data, we could easily deduce that **1** was a cyclic hexapeptide composed of Tyr, Leu, O-Leu, 2 Gly, and Val residues.

The sequence of the amino acid residues in **1** was established by the analysis of HMBC data (see Table 1), which showed correlations from the α-methine and α-methylene protons of each amino acid residue to the carbonyl carbon of the neighboring residues (Figure 1).

The FABMS and ESIMS of **1** supported this analysis.The mass spectrometry data showed the loss of Tyr (163), Val (99), Gly (57), O-Leu (114), Leu (113), and then Gly (57) fragments. Finally, the structure of **1** was also confirmed by X-ray crystallographic analysis (Figure 2) and showed that the relative configurations of the Tyr, Val, and O-Leu residues were *S** and that the Leu residue was *R**.

To confirm the stereochemistry of the amino acid residues, 2 mg of 1962A (**1**) was dissolved in 6 N HCl (2 mL) and heated at 110°C for 24 h in a sealed glass tube. After cooling to room temperature, the solution was dried under N_2 for 24 h, dissolved in $HClO_4$ (pH 2.0), and then analyzed by chiral HPLC column along with the appropriate standards of L-Tyr, L-Val, L-Leu, DL-Tyr, DL-Val, and DL-Leu (Table 3). On the basis of this analysis the peptide **1** was elucidated as cyclo-(D-Leu-Gly-L-Tyr-L-Val-Gly-S-O-Leu).

The cyclodepsipeptide 1962B (**2**) was obtained as colorless block crystals, mp 165–167 °C. The ^{1}H and ^{13}C NMR were similar to that of 1962A (**1**) and showed signals typical of cyclopeptides. The ^{1}H NMR spectrum of 1962B (**2**) in DMSO-d_6 revealed five NH proton signals (δ_H 8.96, 8.04, 7.82, 7.73, and 7.04) and five protons of a monosubstituted phenyl ring (δ_H 7.16–7.22, 5H). The FABMS showed that the molecular weight of 1962B (**2**) was 16 amu less than that of 1962A (**1**). Thus, it became apparent that the Tyr residue in **1** was substituted with a Phe residue in **2**. Therefore, the structure of 1962B (**2**) was elucidated as a cyclic hexpeptide composed of Phe, Val, 2 Gly, Leu, and O-Leu residues.

The FABMS fragmentation pattern for **2** showed sequential loss of Phe (147), Val (99), Gly (57), O-Leu (114), Leu (113), and then Gly (57). The sequence of amino acid residues in **2** was also established by X-ray crystallographic analysis (Figure 3), which showed that the relative configurations of the Phe, Val, and O-Leu residues were *S**, and the Leu residue was *R**. Similar to 1962A (**1**), 1962B (**2**) was elucidated as cyclo-(D-Leu-Gly-L-Phe-L-Val-Gly-S-O-Leu).

Cyclo-(Leu-Tyr) (**3**) was obtained as colorless needle crystals, mp 242–243 °C. FABMS of **3** showed a molecular ion peak at $[M + H]^+$ 277. The ^{1}H NMR spectrum showed proton signals for a *p*-disubstituted benzene at δ_H 7.00 (2H, d, 8.5) and 6.70 (2H, d, 8.5) and two methyls at δ_H 0.74 (6H, d, 6.5), signals typical of cyclodipeptides. The ^{13}C NMR spectrum showed two amide carbonyl carbons (δ_C 167.3 and 166.2) and two nitrogen-bearing

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Table 1. NMR Data (500 MHz, DMSO-d_6) of **1** (δ in ppm, J in Hz)

	position	δ_C	δ_H	^{1}H–H COSY	HMBC
Tyr	1	170.7 (C=O)			7.06 (NH), H (2), H (3)
	2	53.8 (CH)	4.50 (ddd, 3.5, 9.0, 2.0)	7.73, H (3)	H (3)
	3	34.9 (CH_2)	3.20 overlap 2.72 (dd, 12.0, 14.0)	H (2)	H (2), H (5), H (9)
	4	128.2 (C)			H (6), H (8)
	5, 9	129.5 (CH)×2	7.00 (d, 8.5)	H (6), H (8)	7-OH, H (3)
	6, 8	115.0 (CH)×2	6.60 (d, 8.5)	H (5), H (9)	7-OH
	7	155.6 (C)			H (5), H (6), H (8), H (9), 7-OH
	7-OH		9.05 (s)		
	NH		7.73 (d, 9.0)	H (2)	
Val	10	170.9 (C=O)			7.73 (NH), H (11)
	11	61.5 (CH)	3.48 (dd, 5.0, 10.5)	8.02, H (12)	H (13), H (14), H (12)
	12	28.7 (CH)	1.72 (overlap)	H (11), H (13), H (14)	H (11), H (13), H (14)
	13	18.6 (CH_3)	0.55 (d, 7.0)	H (12), H (14)	H (12), H (14)
	14	18.4 (CH_3)	0.70 (d, 7.0)	H (12), H (13)	H (12), H (13)
	NH		8.02 (d, 5.0)	H (11)	
Gly-1	15	170.2 (C=O)			8.02 (NH), H (16)
	16	40.6 (CH_2)	4.40 (dd, 9.0, 17.0) 3.76 (dd, 2.5, 17.0)	7.87	
	NH		7.87 (d, 2.5, 9.0)	H (16)	
O-Leu	17	169.6 (C=O)			7.87 (NH), H (18)
	18	72.0 (CH)	5.10 (dd, 7.0, 13.5)	H (19)	H (19)
	19	39.9 (CH_2)	1.73 (overlap) 1.74 (overlap)	H (18), H (20)	H (18), H (20), H (21), H (22),
	20	24.0 (CH)	1.69 (overlap)	H (19), H (21), H (22)	H (19), H (21), H (22)
	21	22.9 (CH_3)	0.92 (d, 6.5)	H (20), H (22)	H (20), H (22)
	22	20.9 (CH_3)	0.87 (d, 6.5)	H (20), H (21)	H (20), H (21)
Leu	23	172.3 (C=O)			H (18), H (25)
	24	51.8 (CH)	4.20 (ddd, 5.0, 7.0, 12.5)	8.96, H (25)	H (25), H (26)
	25	38.5 (CH_2)	1.60 (overlap) 1.58 (overlap)	H (24), H (26)	H (27), H (28)
	26	24.3 (CH)	1.74 (overlap)	H (25), H (27), H (28)	H (25), H (27), H (28)
	27	22.3 (CH_3)	0.89 (d, 6.5)	H (26), H (28)	H (25), H (26), H (28)
	28	21.7 (CH_3)	0.94 (d, 6.5)	H (26), H (27)	H (25), H (26), H (27)
	NH		8.96 (d, 5.0)	H (24)	
Gly-2	29	169.8 (C=O)			8.96 (NH), H (30)
	30	41.3 (CH_2)	4.10 (dd, 5.0, 15.0) 3.60 (d, 15.0)	7.06	
	NH		7.06 (d, 5.0)	H (30)	

methylene carbons (δ_C 55.6 and 52.2). Thus, the structure of **3** was identical with that reported in the literature.[18]

The structure of cyclo-(Phe-Gly) (**4**) was determined by FABMS, ^{1}H NMR, and ^{13}C NMR data. The FABMS data showed a molecular peak at $[M + H]^+$ 205. The ^{1}H NMR spectrum showed five protons of a monosubstituted phenyl ring (δ_H 7.17–7.27, 5H) and other signals typical of cyclodipeptides. The ^{13}C NMR spectrum showed two amide carbonyl carbons (δ_C 166.9 and 165.4), only one nitrogen-bearing methylene carbon (δ_C 55.5), and two higher field methylenes (δ_C 43.5 and 39.1). These data were identical to those in the literature.[19]

The structure of cyclo-(Leu-Leu) (**5**) was also determined by FABMS, ^{1}H NMR, and ^{13}C NMR data. The ^{13}C NMR spectrum showed one amide carbonyl carbon (δ_C 167.3), one nitrogen-bearing methylene carbon (δ_C 53.2), one higher field methylene (δ_C 43.5), and two methyls (δ_C 23.3 and 21.0). The ^{1}H NMR spectrum showed seven signals typical of cyclodipeptides. The FABMS data showed a molecular peak at $[M + H]^+$ 227. Thus, the structure of **5** was identical with that reported in the literature.[20]

In the MTT bioassay, **1** showed activity against human breast cancer MCF-7 cells with an IC_{50} value of 100 μg/mL.[21]

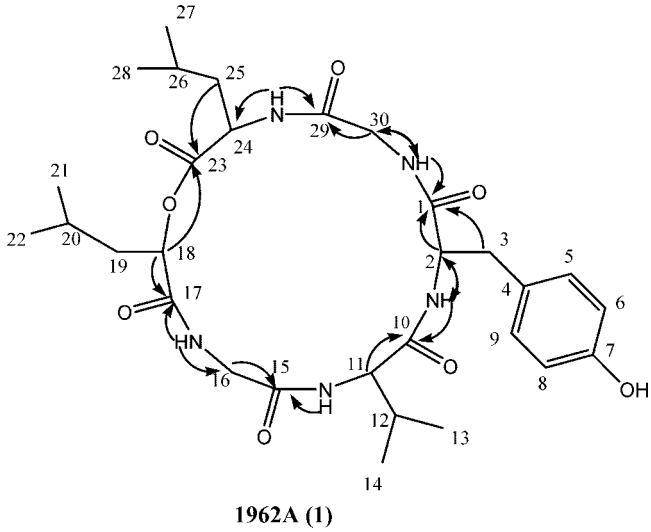


Figure 1. Selected HMBC and ^{1}H–H COSY for 1962A (**1**) in DMSO-d_6.

Experimental Section

General Experimental Procedures. Melting points were determined on an X-4 micromelting point apparatus (uncorrected), Bei Jing Tech Instrument Co, Ltd. Optical rotations were measured on a Polartronic HHW5 digital polarimeter. IR spectra were recorded on a Bruker VECTOR22 FT-IR spectrometer using KBr pellets. NMR spectra were taken on a Varian Inova-500 and a Varian Inova-300 spectrometer, using DMSO-d_6 as solvent and TMS as internal standard. FABMS was measured on a VG-ZAB-HS mass spectrometer. HRES-

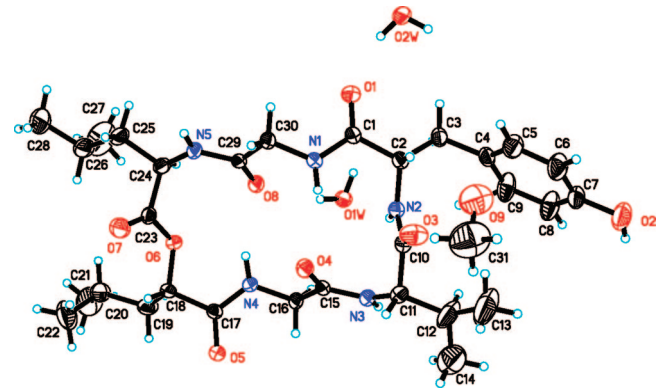


Figure 2. X-ray structure of 1962A (**1**) parent molecule with two water molecules and one solvate methanol drawn by ORTEP.

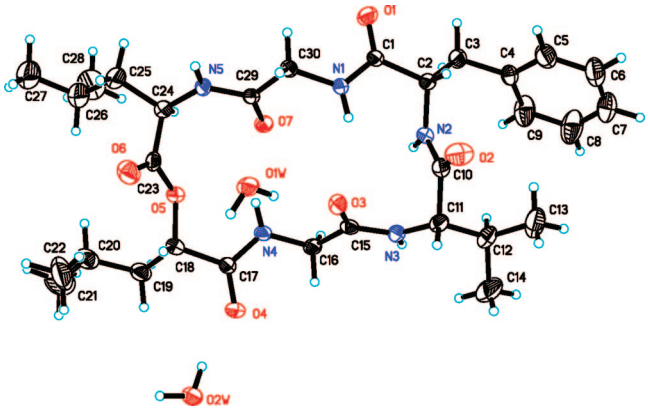


Figure 3. X-ray structure of 1962B (**2**) parent molecule with two water molecules drawn by ORTEP.

Table 2. NMR Data (300 MHz, DMSO-d_6) of **2** (δ in ppm, J in Hz)

	position	δ_C	δ_H
Phe	1	170.4 (C═O)	
	2	54.3 (CH)	4.56 (m)
	3	34.5 (CH_2)	3.28 overlap 2.84 (d, 12.9)
	4	127.8 (C)	
	5, 9	128.6 (CH)×2	7.16–7.22 (5H)
	6, 8	129.4 (CH)×2	
	7	137.6 (CH)	
	NH		7.73 (s)
Val	10	170.6 (C═O)	
	11	60.7 (CH)	3.44 (d, 9.6)
	12	29.2 (CH)	1.78 (overlap)
	13	19.4 (CH_3)	0.50 (d, 6.6)
	14	19.2 (CH_3)	0.63 (d, 6.6)
	NH		8.04 (s)
Gly-1	15	170.1 (C═O)	
	16	410 (CH_2)	4.38 (dd, 8.4, 17.4) 3.72 (d, 17.4)
	NH		7.82 (s)
O-Leu	17	169.5 (C═O)	
	18	70.6 (CH)	5.06 (d, 10.8)
	19	39.1 (CH_2)	1.73 (overlap) 1.74 (overlap)
	20	24.0 (CH)	1.71 (overlap)
	21	23.2 (CH_3)	0.92 (d, 6.6)
	22	21.0 (CH_3)	0.88 (d, 5.7)
Leu	23	171.5 (C═O)	
	24	51.4 (CH)	4.20 (m)
	25	38.1 (CH_2)	1.60 (overlap) 1.58 (overlap)
	26	24.8 (CH)	1.70 (overlap)
	27	22.7 (CH_3)	0.90 (d, 6.5)
	28	21.7 (CH_3)	0.94 (d, 6.5)
	NH		8.96 (d, 5.0)
Gly-2	29	169.7 (C═O)	
	30	42.3 (CH_2)	4.10 (m) 3.60 (m)
	NH		7.04

IMS was measured on a VG Autospec-500 mass spectrometer. X-ray diffraction was measured on a Bruker Smart 1000 CCD. CD spectra were recorded on a JASCO J-810 spectrometer, using MeOH as solvent. Silica GF254 for TLC and silica gel (200–300 mesh) for CC were produced by Qingdao Marine Chemical Company, Qingdao, China. Solvents and chemicals were of analytical grade and purchased from Guangzhou Chemical Company, Guangdong, China.

Fungal Strain. Strain No. 1962, a mangrove endophytic fungus, was isolated from an old leaf of *Kandelia candel* from an estuarine mangrove collected in Hong Kong, and a specimen was deposited in the Department of Applied Chemistry, Zhongshan University, Guangzhou, China.

Culture Conditions. Starter cultures were maintained on cornmeal seawater agar. Plugs of agar supporting mycelial growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of GYT (glucose 10 g/L, peptone 2 g/L, yeast extract 1 g/L, NaCl 2 g/L, pH 7.0). The flask was incubated at 28 °C on a rotary shaker for 5–7 days, and the mycelium was aseptically transferred to a 500 mL Erlenmeyer flask containing culture liquid (300 mL). The flask was then incubated at 28 °C for 25 days.

Extraction and Separation of Metabolites. The cultures (100 L) were filtered through cheesecloth. The filtrate was concentrated to 5 L below 50 °C and extracted three times with EtOAc. The organic solvent was evaporated under reduced pressure to give an extract. The combined organic extracts were chromatographed repeatedly on silica gel using gradient elution from ether to EtOAc, to obtain **1** (20 mg), **2** (4 mg), cyclo-(Leu-Tyr) **3** (20 mg), cyclo-(Phe-Gly) **4** (20 mg), and cyclo-(Leu-Leu) **5** (30 mg). 1962A (**1**) and 1962B (**2**) were then recrystallized from MeOH by slow evaporation, and colorless plate crystals were formed and were stable at room temperature.

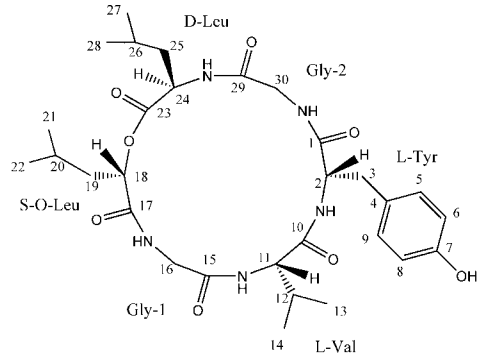
Compound 1962A (1): colorless block crystals; mp 180–182 °C; $[\alpha]^{20}_D$ −63.4 (*c* 0.005, MeOH); IR (KBr) 3347, 3069, 2929, 1758, 1661, 1555, 1457, 1376, 1264, 1171, 1060, 810, 611 cm^{-1}; CD (MeOH, *c* 0.00435) $\Delta\varepsilon$ (λ nm) 209 (−2.68), 229 (−1.09), 243 (+0.07); ^1H and ^{13}C NMR, see Table 1; FABMS *m/z* 604 $[M + H]^+$, 441 $[M + H - Tyr]^+$, 341 $[M + H - Tyr - Val]^+$, 284 $[M + H - Tyr - Val - Gly]^+$, 170 $[M + H - Tyr - Val - O\text{-}Leu]^+$, 57 $[Gly]^+$; HRESIMS *m/z* $[M + H]^+$ 604.3317, calcd 604.3346; $[M + Na]^+$, 626.3110, calcd 626.3150.

Compound 1962B (2): colorless block crystals; mp 165–167 °C; ^1H and ^{13}C NMR, see Table 2; FABMS *m/z* 588 $[M + H]^+$, 441 $[M + H - Phe]^+$, 341 $[M + H - Phe - Val]^+$, 284 $[M + H - Phe - Val - Gly]^+$, 170 $[M + H - Phe - Val - O\text{-}Leu]^+$, 57 $[Gly]^+$.

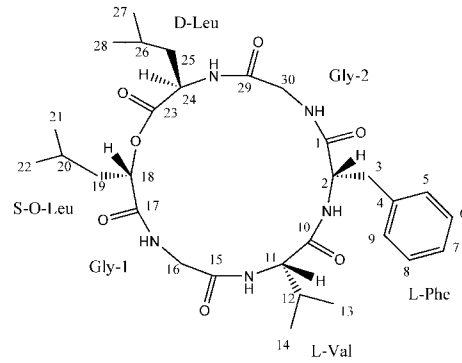
Cyclo-(Leu-Tyr) (3): colorless needle crystals; mp 242–243 °C; IR (KBr) ν 3441, 3214, 2984, 1670, 1540, 1515, 1468, 1380, 1105, 850 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ_H 0.14 (ddd, 13.5, 9, 4.5), 0.74 (d, 6.5), 0.89 (ddd, 14, 9.5, 4.5), 1.43 (m), 2.82 (dd, 4.5, 14), 3.20 (dd, 3.5, 14), 3.63 (dd, 3.5, 10), 4.21 (m), 6.70 (d, 8.5), 7.00 (d, 8.5); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 167.3 (C), 166.2 (C), 156.3 (C), 131.1 (CH), 125.8 (C), 114.8 (CH), 55.6 (CH), 52.2 (CH), 43.6 (CH_2), 37.5 (CH_2), 22.9 (CH), 22.7 (CH_3), 1.3 (CH_3).

Cyclo-(Phe-Gly) (4): colorless needle crystals; ^1H NMR (DMSO-d_6, 500 MHz) δ_H 8.07 (1H, brs), 7.80 (1H, brs), 7.17–7.27 (5H, m), 4.05 (1H, m), 3.361H, dd, 17.5, 2.5, 3.17 (1H, dd, 13.0, 5.5), 3.03 (1H, dd, 13.0, 5.0), 2.80 (1H, d, 17.5); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C 166.9 (C), 165.4 (C), 135.0 (C), 129.5 (C), 127.7 (C), 126.3 (C), 55.5 (CH), 43.5 (CH_2), 39.1 (CH_2); FABMS *m/z* 205 $[M + H]^+$.

Scheme 1



1962A (1)



1962B (2)

Cyclo-(Leu-Leu) (5): colorless powder; ^{1}H NMR (DMSO-d_6, 500 MHz) δ_H 6.14 (2H, brs), 3.96 (2H, dd, 9.5, 4.5), 1.97 (2H, m), 1.8 (2H, m), 1.59 (2H, m), 1.00 (6H, d, 6.5), 0.97 (6H, d, 6.5); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C 167.3 (C), 53.2 (CH), 43.7 (CH_2), 24.3 (CH), 23.3 (CH_3), 21.0 (CH_3); FABMS *m/z* 227 $[M + H]^+$.

Stereochemistry of 1962A (1). The configurations of the amino acid were determined by analysis of the acid hydrolysate from compound **1**. Two milligrams of 1962A (**1**) was dissolved in 6 N HCl (2 mL) and heated at 110 °C for 24 h in a sealed glass tube. After cooling to room temperature, the solution was dried under N_2 for 24 h, redissolved in $HClO_4$ (pH 2.0), and then analyzed by chiral HPLC (Crownpak CR (+), Daicel Chemical Industries, Ltd) with an aqueous $HClO_4$ pH 2.0 gradient (0.4 mL/min, UV detection at 200 nm) on a 515 Waters HPLC, along with appropriate standards of DL-amino acids purchased from Fluka Chemika and L-amino acids purchased from Fluka BioChemika. The results are shown in the Supporting Information, and the absolute configurations of Tyr and Val were all L, the Leu was D, and the O-Leu was *S*.

X-ray Crystallographic Data of 1962A (1) Dihydrate Methanol Solvate. A plate-shaped crystal was obtained via slow evaporation of a methanol solution of the compound. Crystal data: $C_{30}H_{45}N_5O_8 \cdot 2H_2O \cdot CH_3OH$, space group monoclinic, $P2_1$; unit cell dimensions a = 7.156(2) Å, b = 30.155(9) Å, c = 8.678(3) Å, β = 103.888(5)°, V = 1818.0(10) Å^3, Z = 2, D_{calcd} = 1.227 mg/m^3, μ = 0.093 mm^{-1}, $F(000)$ = 724. All single-crystal data were collected using the hemisphere technique on a Bruker AXS SMART 1000 CCD diffractometer with graphite-monochromated Mo Kα radiation λ = 0.71073 Å at 293(2) K. The structures were solved by direct methods using SHELXTL V6.14 (Bruker AXS Inc., Madison, WI)[22] and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the Siemens Area Detector Absorption Program (SADABS).[23] The final value of R was 0.0566, wR_2 = 0.1572 [$I > 2\sigma(I)$].

X-ray Crystallographic Data of 1962B (2) Dehydrate. A plate-shaped crystal was obtained via slow evaporation of a methanol solution of the compound. Crystal data: $C_{30}H_{45}N_5O_7 \cdot 2H_2O$, space group monoclinic, $P2_1$; unit cell dimensions a = 7.089(3) Å, b = 28.710(14) Å, c = 8.770(4) Å, β = 103.606(7)°, V = 1734.9(14) Å^3, Z = 2, D_{calcd} = 1.194 mg/m^3, μ = 0.088 mm^{-1}, $F(000)$ = 672. All single-crystal data were collected using the hemisphere technique on a Bruker AXS SMART 1000 CCD system diffractometer with graphite-monochromated Mo Kα radiation λ = 0.71073 Å at 293(2) K. The structures were solved by direct methods using SHELXTL V6.14 (Bruker AXS Inc., Madison, WI)[22] and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the Siemens Area Detector Absorption Program (SADABS).[23] The final value of R was 0.0702, wR_2 = 0.1643 [$I > 2\sigma(I)$].

Crystallographic data for the structures 1962A (**1**) and 1962B (**2**) reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (deposition numbers 259380–259381).

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Supporting Information Available: X-ray crystallographic data of 1962A (**1**) and 1962B (**2**) and table of HPLC results for amino acid configurations are available free of charge via the Internet at http://pubs.acs.org.

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