Hantupeptin A, a Cytotoxic Cyclic Depsipeptide from a Singapore Collection of *Lyngbya majuscula*

Ashootosh Tripathi,[†] Jonathan Puddick,[‡] Michele R. Prinsep,[‡] Peter Peng Foo Lee,[†] and Lik Tong Tan*[,†]

Natural Sciences and Science Education, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore, and Department of Chemistry, University of Waikato, Private Bag 3105, Hamilton 3205, New Zealand

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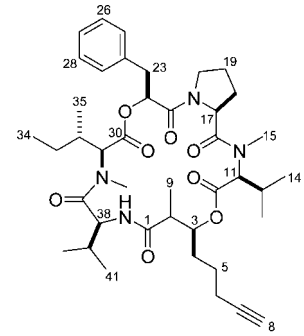
Chemical investigation of the marine cyanobacterium *Lyngbya majuscula* from Pulau Hantu Besar, Singapore, has led to the isolation of a cyclodepsipeptide, hantupeptin A (**1**). The planar structure of **1** was assigned on the basis of extensive 1D and 2D NMR spectroscopic experiments. The absolute configuration of the amino and hydroxyl acid residues in the molecule was determined by application of the advanced Marfey method, chiral HPLC analysis, and Mosher's method. Hantupeptin A showed cytotoxicity to MOLT-4 leukemia cells and MCF-7 breast cancer cells with IC_{50} values of 32 and 4.0 μM, respectively.

Filamentous marine cyanobacteria are an established source for a prodigious array of unique bioactive secondary metabolites.[1,2] These metabolites exhibit a variety of biological activities including immunosuppressant, antiproliferative, and antimicrobial activities.[4,5] Interestingly, approximately 40% of the cyanobacterial compounds possess anticancer/antitumor activity, making them invaluable as potential therapeutic leads.[6] Recent examples of potent marine cyanobacterial metabolites include coibamide A,[7a] largazole,[7b] and somocystinamide A.[7c] The benthic filamentous cyanobacterium *Lyngbya majuscula*, in particular, has proven to be an exceptional source of novel potential pharmaceuticals.[2] Among the diverse classes of compounds being discovered from this species, a substantial amount belongs to either the polypeptide or hybrid polyketide−polypeptide structural classes.[1−3] In spite of the chemical richness of these marine microorganisms, no studies have been conducted on the natural products chemistry of marine cyanobacteria from Singapore.

In our ongoing efforts toward finding novel and pharmacologically active marine cyanobacterial metabolites, a detailed biological investigation of *L. majuscula* sampled from intertidal regions at different islands off the southern coast of Singapore was undertaken. Preliminary data from the brine shrimp toxicity and MTT cytotoxicity (based on the MOLT-4 cell line) assays revealed a high incidence of biological activities in these cyanobacterial organic extracts (unpublished data). Furthermore, *L. majuscula* procured from the western lagoon of Pulau Hantu Besar, Singapore, displayed the most significant activities. Chemical investigation of this strain resulted in the isolation of two new fatty acid amides, besarhanamides A and B, recently reported in the literature.[8] Further examination of other active fractions has now led to the isolation of a new cyclic depsipeptide. Herein, we wish to report a bioassay-guided isolation and structure elucidation of hantupeptin A (**1**), which was also shown to possess high levels of cytotoxicity toward cancer cells.

Results and Discussion

Samples of the marine cyanobacterium *Lyngbya majuscula* were collected by hand during low tides from the western lagoon of Pulau Hantu Besar, Singapore, and stored in 70% ethanol. The organic extract was prepared using $CHCl_3$/MeOH (1:1) and subsequently fractionated by vacuum flash chromatography (VFC) based on a combination of hexanes, EtOAc, and MeOH with increasing polarity. The fraction eluted with 80% EtOAc/20% hexane was subjected to SEP PAK C_{18} solid-phase fractionation followed by reversed-phase HPLC to yield hantupeptin A (**1**).



Hantupeptin A (**1**)

Hantupeptin A (**1**) was isolated as an amorphous solid, and HRESIMS of the molecule provided a molecular formula of $C_{41}H_{60}N_4O_8$ (14 degrees of unsaturation) based on the $[M + H]^+$ and pseudomolecular ion $[M + Na]^+$ peaks at *m*/*z* 737.4484 and 759.4310, respectively. The molecule seems to adopt at least two conformers as observed in the 1D NMR data when measured in $CDCl_3$. Chemical signals due to the minor conformer were diminished when ^{1}H NMR data were acquired at higher temperature (at 50 °C) during the variable-temperature experiments (see below). The presence of a peptidic molecule was evident from the ^{1}H NMR spectrum, which showed two tertiary amide *N*-Me 3H singlets at δ 3.07 and 2.60 as well as a characteristic secondary amide *N*H resonance occurring as a doublet at δ 6.31. Furthermore, the ^{13}C NMR spectrum of **1** exhibited the presence of six carbonyl carbons attributable to ester/amide functionalities and a monosubstituted phenyl ring (δ 136.6, 129.9, 128.8, and 127.1) system (Table 1). These functionalities accounted for 10 of the 14 degrees of unsaturation.

Extensive NMR analyses based on a combination of 1D and 2D NMR data of **1** allowed construction of six partial structures. Four standard amino acid residues were deduced as *N*-methylisoleucine (*N*-Me-Ile), *N*-methylvaline (*N*-Me-Val), valine (Val), and proline (Pro) (Figure 1). A fifth residue exhibited ^{1}H NMR resonances at δ 7.21−7.53 (H-25 to H-29), 2.94 (H-23a), 3.18 (H-23b), and 5.51 (H-22), which are characteristic of a phenylalanine residue. However, the HSQC spectrum showed that H-22 was attached to an oxymethine carbon (δ 73.2) and thus was more consistent with a 3-phenyllactic acid (Pla) moiety (Figure 1). The sixth and final residue required a composition of $C_9H_{12}O_2$ to complete the molecular formula of **1**. From TOCSY, HMBC, and HSQC data,

* Corresponding author. Tel: +65 6790 3820. Fax: +65 6896 9432. E-mail: liktong.tan@nie.edu.sg.
[†] Nanyang Technological University.
[‡] University of Waikato.

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Table 1. 1D NMR Data for the Major Conformer of Hantupeptin A (**1**) in $CDCl_3$

unit	C/H no.	δ_H (*J* in Hz)	δ_C	COSY	HMBC
Hmoya	1		171.1, qC		
	2	3.31, m (6.5, 2.8)	42.6, CH	H-3, H-9	1, 3, 9
	3	4.91, dt (10.6, 2.8)	77.5, CH	H-4a, H-4b, H-2	
	4a	1.67, m	25.6, CH_2	H-3, H-4b, H-5	
	4b	2.13, m		H-4a	3
	5	1.68, m	25.4, CH_2	H-4b, H-6a, H-6b	
	6a	2.24, m	18.4, CH_2	H-5, H-6b	5, 7, 8
	6b	1.50, m			
	7		83.9, qC		
	8	1.98, t	69.1, CH		6, 7
	9	1.31, d	20.1, CH_3	H-2	1, 2, 3
N-Me-Val	10		165.8, qC		
	11	4.07, d (10.2)	66.2, CH	H-12	10
	12	2.16, m	27.8, CH	H-13, H-14	
	13	0.92	14.5, CH_3	H-12	12
	14	0.92	11.5, CH_3	H-12	12
	15	2.60, s	29.2, CH_3		11, 16
Pro	16		173.8, qC		
	17	4.98, dd (8.4, 4.7)	57.9, CH	H-18a, H-18b	
	18a	2.29, m	29.5, CH_2	H-19b, H-18b	19
	18b	1.85, m		H-18a	
	19a	2.00, m	27.9, CH_2	H-20, H-19b	
	19b	2.09, m		H-19a, H-18b	
	20	3.60, m	47.4, CH_2	H-19a, H-19b	18, 19
Pla	21		171.4, qC		
	22	5.51, dd (9.8, 4.1)	73.2, CH	H-23a, H-23b	23
	23a	2.94, dd (14.8, 9.8)	37.4, CH_2	H-22, H-23b	22, 24, 25/29
	23b	3.18, dd (14.8, 4.1)		H-22, H-23a	22, 25/29, 21
	24		136.6, qC		
	25/29	7.53, m	129.9, CH	H-26, H-28	24, 26, 28
	26/28	7.28, m	128.8, CH	H-25, H-29, H-27	25, 27, 29
	27	7.21, m	127.1, CH	H-26, H-28	26, 27, 28
N-Me-Ile	30		169.3, qC		
	31	4.05, d (11.0)	64.5, CH	H-32	30, 32, 35
	32	2.13, m	35.0, CH	H-31, H-35	35
	33a	2.23, m	26.1, CH_2	H-33b, H-34	
	33b	1.51, m		H-32, H-33a, H-34	32, 34
	34	0.90	16.6, CH_3	H-33a, H-33b	32, 33
	35	0.92	18.0, CH_3	H-32	32
	36	3.07, s	29.5, CH_3		31, 37
Val	37		172.8, qC		
	38	4.78, dd (8.8, 6.4)	53.2, CH	H-39, NH	37, 39
	39	2.02, m	32.7, CH	H-38, H-40, H-41	37, 38, 40, 41
	40	0.97	20.5, CH_3	H-39	38, 39
	41	0.97	20.1, CH_3	H-39	38, 39
	*N*H	6.31, d (8.8)		H-38	1

the remaining spin system was shown to possess a methine group at δ 3.31 (H-2)/42.6 (C-2) connected to a methyl group at δ 1.31 (H-9)/20.1 (C-9) as well as to an oxygen-bearing methine at δ 4.91 (H-3)/77.5 (C-3), which was bonded to a methylene group at C-4. From COSY and TOCSY data, C-4 was shown to be further extended by two methylene carbons at C-5 and C-6. In addition, HMBC experiments confirmed the presence of a terminal acetylenic group with correlations from δ 2.24 (H-6) to carbons at δ 69.1 (C-8) and 83.9 (C-7). These data led to the identification of this spin system as a 3-hydroxy-2-methyloctynoic acid (Hmoya) residue (Figure 1). Since Hmoya and Pro accounted for three of four remaining degrees of unsaturation and with only a single degree of unsaturation remaining, compound **1** must be a cyclic depsipeptide. In addition, HMBC correlations were used to unambiguously connect these residues, completing its planar structure as shown in **1**. Furthermore, the MS/MS spectrum of the molecule provided *m*/*z* peaks that corresponded with fragments obtained from **1**, such as *m*/*z* 228 (Val/*N*-Me-Ile), 246 (Pla/Pro), and 611 (Pla/Pro/*N*-Me-Val/Hmoya/Val).

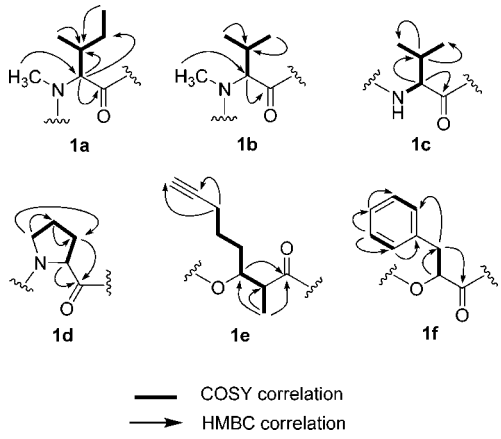

Figure 1. Subunits of hantupeptin A (**1**) with COSY (bold bonds) and selected HMBC correlations (arrow).

The absolute configuration of all regular and *N*-Me-Val units in **1** was established as L on the basis of the advanced Marfey method.[9−11] An L-configuration was determined for the Pla moiety using chiral HPLC analysis. Although, Marfey's analysis clearly suggested the L-configuration of the *N*-Me-Ile moiety, we were unable to distinguish *N*-Me-L-*allo*-Ile and *N*-Me-L-Ile by this method due to the lack of standard *N*-Me-L-*allo*-Ile. In order to confirm the L-configuration of the *N*-Me-Ile in **1**, racemization of the *N*-Me-L-Ile standard was carried out, and the reaction mixture, containing both *N*-Me-L-Ile and *N*-Me-D-*allo*-Ile, was subjected to the advanced

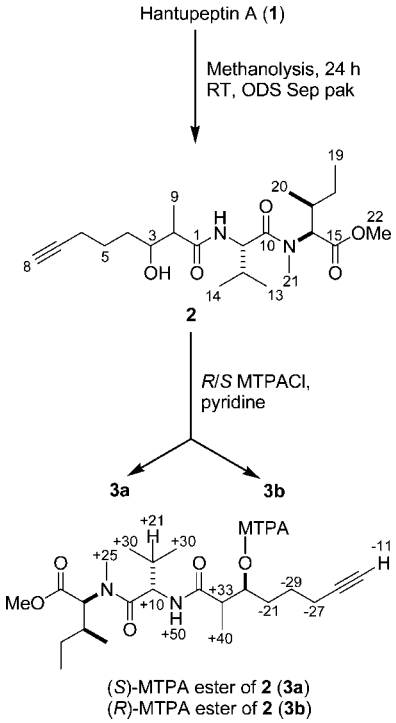

Figure 2. Preparation of **3a** and **3b** and $\Delta\delta$ values ($\times 10^{-3}$ ppm).

Marfey analysis using LC-MS. Both the retention times of the L-Marfey-derivatized *N*-Me-L-Ile standard and the *N*-Me-Ile unit in the hydrolyzate of **1** were similar, and this was confirmed by coelution.

The absolute stereochemistry at C-3 was determined using Mosher's analysis[12] following methanolysis of **1** and isolation of fragment **2** (Figure 2), the structure of which was confirmed by ^{1}H NMR data. Subsequently, treatment of **2** with Mosher's reagents showed unambiguously that C-3 had the *S*-configuration (for $\Delta\delta$ values, see Figure 2). The Hmoya unit has also been reported in a number of marine-derived compounds such as onchidin B, kulomo'opunalide 1, kulomo'opunalide 2, and trungapeptin A, having ^{1}H and ^{13}C NMR signals well within the comparable limits of **1**.[13−15]

Variable-temperature NMR experiments were performed on hantupeptin A (**1**) in order to probe the degree and nature of the amide proton (Val) participating in intramolecular hydrogen bonding within the molecule.[16] Proton NMR spectra of **1** were acquired over a range of temperatures in $CDCl_3$, and the respective $\Delta\delta/\Delta T$ (in ppb/°C) value of the amide proton was calculated as −0.1. This value is small enough to suggest that the *N*H of Val is involved in an intramolecular H-bond.

The solution confirmation of hantupeptin A (**1**) was predicted in $CDCl_3$ on the basis of several 2D ROESY experiments using different mixing times. The observed ROE correlations for **1** included Val-N^H (*i* position) to Hmoya-H$^{\beta 1}$ (*i*+1), Val-N^H (*i* position) to Hmoya-H$^\alpha$ (*i*+2), Val-N^H (*i* position) to *N*-Me-Val-H$^\alpha$ (*i*+3), and Val-H$^\alpha$ (*i*−1) to *N*-Me-Val-H$^{N\text{-}Me}$ (*i*+4), suggesting a single turn within the backbone due to the intramolecular H-bonding between the amide proton of Val and the carbonyl oxygen atom of *N*-Me-Val.

Hantupeptin A (**1**) showed 100% brine shrimp toxicity at 100 and 10 ppm, which was significantly higher than the activity reported for its close analogue trungapeptin A, which exhibited mild brine shrimp toxicity.[14] Furthermore, *in vitro* cytotoxicity testing of **1** against the leukemia cell line MOLT-4 exhibited an IC_{50} value of 32 nM. Compound **1** also displayed significant cytotoxicity in the breast cancer cell line MCF-7, with an IC_{50} value of 4.0 μM. In comparison, a derivative of hantupeptin A, trungapeptin A, was reported to be inactive when tested at 10 μg/mL against KB or LoVo cells.[15]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Bellingham Stanley ADP 440 polarimeter. UV and IR spectra were measured on a Varian Cary 50 UV visible spectrophotometer and a PerkinElmer spectrum 100 FT-IR spectrophotometer, respectively. Proton, ^{13}C, and 2D NMR spectra were recorded in $CDCl_3$ on a 400 MHz Bruker NMR spectrometer using the residual solvent signal (δ_H at 7.26 and δ_C at 77.0) as internal standards. Both HRESIMS and LRMS/MS data were obtained using a Microtof series 89 MALDI TOF mass spectrometer equipped with an ESI multimode ion source detector. HPLC isolation of hantupeptin A (**1**) was conducted on a Shimadzu LC-8A preparative LC and Shimadzu SPD-M10A VP diode array detector, while an Agilent 1100 series coupled with an Agilent LC/MSD trap XCT mass spectrometer equipped with an ESI interface system was used for the detection of the Marfey-derivatized L/D-valine, proline, *N*-methylvaline moiety from hantupeptin A. Chiral analysis was carried out using a Waters 2790 Alliance HPLC (Milford, MA) equipped with a photodiode array detector. Cell viability in 96-well plates was measured using a Bio Rad Benchmark plus microplate reader.

Biological Material. About 1.0 L of the filamentous benthic marine cyanobacterium *Lyngbya majuscula* was collected by hand from the western lagoon of Pulau Hantu Besar (1°13′31.44″ N, 103°44′55.89″ XXXX) during low tides on June 29, 2005, and stored in 70% aqueous EtOH at −20 °C before extraction. A voucher specimen of this microalga is maintained at the National Institute of Education, Singapore, under the code TLT/PHB/002.

Isolation and Purification. Extraction of *Lyngbya majuscula* was carried out repeatedly using $CHCl_3$/MeOH (1:1) to produce about 1.0 g of crude organic extract. The extract was then subjected to vacuum flash chromatography (VFC) on normal-phase Si using a combination of hexanes, EtOAc, and MeOH of increasing polarity. Nine fractions were obtained, and solvents were removed *in vacuo* using a rotary evaporator before storage in 4 dram vials in $CHCl_3$. All fractions were assayed at 100 and 10 ppm in the brine shrimp (*Artemia salina*) toxicity assay.

Fraction 7 (100% toxicity at 10 ppm in the brine shrimp toxicity assay) obtained from VFC of the organic extract of *L. majuscula* was subjected to further fractionation on a SEP-PAK RP-18 cartridge using a combination of MeOH and H_2O into four subfractions. The brine shrimp active subfraction eluted with 10% H_2O in MeOH and was subjected to purification by preparative HPLC [Phenomenex Sphereclone 5 μm ODS, 250 × 10.00 mm, 8:2 MeOH/H_2O in 50 min at 4.0 mL/min, detected at 215 nm) to elute hantupeptin A (**1**, 56.2 mg, t_R = 15.4 min), yielding approximately 0.5% of crude extract.

Hantupeptin A (1): white, amorphous powder; $[\alpha]^{25}_D$ −41.5 (*c* 1.0, MeOH); UV (MeOH) λ_{max} 204 nm (log ε 4.4); IR (neat) 3584, 2963, 1736, 1649, 1531, 1452, 1405, 1242, 1184, 1123 cm^{-1}; ^{1}H NMR (400.13 MHz, $CDCl_3$) and ^{13}C NMR (100.62 MHz, $CDCl_3$) data, see Table 1; HRESIMS *m*/*z* $[M + H]^+$ 737.4484 (calcd for $C_{41}H_{61}N_4O_8$, 737.4489), $[M + Na]^+$ 759.4310 (calcd for $C_{41}H_{60}N_4O_8Na$, 759.4313).

Advanced Marfey's Analysis of Amino Acids. Hydrolysis of hantupeptin A (**1**, 1.0 mg) was achieved in 1.0 mL of 6 N HCl placed in a sealed reaction vial at 110 °C for 18 h. Trace HCl was removed under an N_2 stream, and the resulting hydrolysate redissolved in 0.6 mL of H_2O. The aqueous hydrolysate was divided into two equal portions. To one portion were added a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA, Marfey's reagent, 100 μL) in acetone and 1 M $NaHCO_3$ (25 μL), and the mixture was heated at 40 °C for 45 min. To the second portion was added a racemic mixture of a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA, 50 μL) in acetone, a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-D-alaninamide (D-FDAA, 50 μL), and 1 M $NaHCO_3$ (25 μL), and the mixture was heated at 40 °C for 45 min. Both reaction mixtures were cooled to RT and quenched by addition of 2 N HCl (25 μL), dried, and dissolved in MeCN (500 μL). The aliquots were subjected to reversed-phase LCMS (Agilent 1100 series) according to the advanced Marfey method[10,11] [column: Phenomenex, Luna, 150 × 2.0 mm, 5 μm, 100 Å; mobile phase, MeCN in 0.1% (v/v) aqueous HCOOH; flow rate, 0.20 mL/min] using a linear gradient (10−50% MeCN over 60 min). An Agilent 1100 series MSD spectrometer was

used for detection in API-ES (positive mode). The retention times and ESIMS product ions (t_R in min, m/z $[M + H]^+$) of the L-FDAA monoderivatized amino acids in the hydrolysate of the first portion were observed to be Pro (34.9, 366.8), Val (43.0, 368.8), and *N*-Me-Val (48.4, 382.9), while the reaction with racemic DL-FDAA in the second portion gave rise to two peaks for each corresponding amino acid moiety. The retention times and ESIMS product ions (t_{R1}/t_{R2}, min, m/z $[M + H]^+$) were observed to be Pro (34.9/37.2, 366.8), Val (43.0/49.9, 368.8), and *N*-Me-Val (48.4/52.0, 382.8). Peaks eluted with longer t_R could be attributed to the D-FDAA derivative of the amino acids. Consequently, the absolute configuration of the moieties in the hydrolysate of **1** was confirmed as L-Pro, L-Val, and *N*-Me-L-Val. The advanced Marfey method indicated an *N*-Me-L-Ile unit in compound **1**. However, this was not conclusive since the *N*-Me-L-*allo*-Ile standard was not used. The *N*-Me-L-Ile unit in **1** was subsequently confirmed on the basis of extensive chiral HPLC analyses as well as chemical manipulation using the advanced Marfey method (see below).

Methanolysis of Hantupeptin A (1). A solution of hantupeptin A (**1**, 7.5 mg) in 5% methanolic KOH (0.75 mL) was stirred for 24 h at RT. The reaction mixture was diluted with ether (15 mL), and the organic layer was washed with brine, dried over Na_2SO_4, and subsequently dried under N_2. Purification of **2** (1.5 mg) was accomplished by C_{18} SEP PAK [MeOH/H_2O (1:1), MeOH/H_2O (3:2), MeOH/H_2O (7:3)].

α-Methoxy-α-trifluoromethyl-α-phenylacetic Acid (MTPA) Esters of 2. Compound **2** obtained from the methanolysis of **1** (see preceding section) was divided into two equal portions (0.75 mg each), and to each sample was added 0.75 mL of pyridine. To one portion was added 6.0 mg of *R*-MTPACl and to the other 6.0 mg of *S*-MTPACl, the reaction was carried out for 10 h at RT, and the solvent was evaporated under N_2. The corresponding esters, **3a** and **3b**, were subjected to NMR analysis.

3a: ^{1}H NMR ($CDCl_3$) δ 3.135 (H-2), 4.910 (H-3), 1.652 (H_1-4), 1.653 (H-5), 1.511 (H_2-6), 1.942 (H-8), 1.260 (H-9), 6.800 (NH), 4.754 (H-11), 2.061 (H-12), 0.980 (H-13), 0.950 (H-14), 2.965 (s, *N*-Me).

3b: ^{1}H NMR ($CDCl_3$) δ 3.102 (H-2), 4.922 (H-3), 1.673 (H_1-4), 1.682 (H-5), 1.538 (H_2-6), 1.953 (H-8), 1.220 (H-9), 6.750 (NH), 4.744 (H-11), 2.040 (H-12), 0.95 (H-13), 0.920 (H-14), 2.940 (s, *N*-Me).

Racemization of the *N*-Methyl-L-isoleucine Standard. Approximately 0.4 mg of the *N*-Me-L-Ile standard was dissolved in H_2O (100 μL), followed by the addition of triethylamine (40 μL) and acetic anhydride (40 μL). The mixture was heated at 60 °C for 1 h and evaporated to dryness in a stream of N_2. The residue was subsequently dissolved in 6 N HCl (200 μL), heated at 110 °C for 12 h, and then evaporated to dryness in a stream of N_2.

Advanced Marfey's Analysis of the Racemized *N*-Methyl-L-isoleucine Standard. After racemization of the *N*-Me-L-Ile standard [containing both *N*-Me-L-Ile and *N*-Me-D-*allo*-Ile], the advanced Marfey method (using both L-Marfey's and D-Marfey's reagents) was used to determine the retention times of the four stereoisomers of *N*-Me-L-Ile using LCMS [column, Phenomenex, Luna, 150 × 2.0 mm, 5 μ, 100 Å; mobile phase, MeCN in 0.1% (v/v) aqueous HCOOH; flow rate, 0.20 mL/min using a linear gradient (10−50% MeCN over 70 min)]. The retention times and ESIMS product ions (t_R in min) of the L/D-FDAA-derivatized amino acids were as follows: *N*-Me-L-Ile (56.1), *N*-Me-D-*allo*-Ile (60.3), *N*-Me-D-Ile (=D-FDAA-derivatized *N*-Me-L-Ile, 59.9), and *N*-Me-L-*allo*-Ile (=D-FDAA-derivatized *N*-Me-D-*allo*-Ile, 56.6). The t_R of the *N*-Me-Ile unit in the hydrolysate of **1** was similar to the L-FDAA-derivatized *N*-Me-L-Ile standard at 56.1 min, and this was confirmed with coelution of both molecules.

Absolute Configuration of the 3-Phenyllactic Acid Unit in 1. Hantupeptin A (**1**, 1.0 mg) was hydrolyzed in 6 N HCl at 110 °C for 12 h. The hydrolysate was concentrated to dryness and analyzed by chiral HPLC [column, Chiralpak MA(+) (0.46 × 5 cm), Daicel Chemical Industries Ltd., solvent, 2 mM $CuSO_4$/MeCN (85:15); flow rate, 0.7 mL/min; detection at 218 nm]. Phenyllactic acid eluted at t_R = 19.5 min, corresponding to the retention time of an authentic sample of L-3-phenyllactic acid and therefore indicating an *S*-configuration [t_R of D-3-phenyllactic acid = 14.7 min].

Cell Viability Assays. Cells were plated in 96-well plates (MOLT-4, 40 000 cells; MCF-7, 40 000 cells) and were treated 24 h later with various concentrations of hantupeptin A (**1**) and solvent control (10% DMSO). After 24 h of incubation, MTT was added to all wells followed by lysis buffer. Plates were incubated for another 24 h, and cell viability was measured by observing absorbance at 570 nm on a plate reader.

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Supporting Information Available: 1D and 2D NMR data and MS fragmentation pattern of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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