Noducyclamides A1-A4 and B1-B2 from the cyanobacterium *Nodularia* sp. NIES-3585

Jakia Jerin Mehjabin,[b,▽] Chin-Soon Phan,[b,#,▽] Tatsufumi Okino[a,b,*]

[a]Graduate School of Environmental Science and [b]Faculty of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan. [#]Current affiliation at Latvian Institute of Organic Synthesis, Aizkraukles street 21, LV-1006 Riga, Latvia.

*Phone: +81 11 706 4519; Email: okino@ees.hokudai.ac.jp; Fax: +81 11 706 4867

ABSTRACT

A chemical investigation of the hydrophilic fraction of the cultured *Nodularia* sp. (NIES-3585) afforded six new cyclic lipopeptides, noducyclamides A1-A4 (**1**-**4**) containing 10 amino acid residues and dodecapeptides noducyclamides B1-B2 (**5**-**6**). The planar structures of these lipopeptides were elucidated based on the combination of HRMS and 1D and 2D NMR spectroscopic data analyses. These peptides are structurally analogous to laxaphycins and contain the non-proteinogenic amino acids 3-hydroxyvaline, 3-hydroxyleucine, and β-amino decanoic acid residue. The absolute configurations of noducyclamides (**1**-**6**) were determined by acid hydrolysis, followed by advanced Marfey's analysis. Noducyclamide B1 (**5**) showed cytotoxic activities against MCF7 breast cancer cell lines with an IC_{50} value of 3.0 μg/mL (2.2 μM).

Natural products from cyanobacteria frequently show interesting biological activities such as antimicrobial, biosurfactant, cytotoxic, and enzyme inhibitory activities.[1-5] Cyanobacteria are known to produce diverse cyclic lipopeptides that often contain β-amino fatty acids and form macrolactone or macrolactam rings.[6] These cyclic lipopeptides can be categorized into different structural classes,[7] among them the cyclic peptides containing 8-12 amino acids with long saturated β-amino fatty acids are limited to the hormothamnins,[8,9] hassallidins,[10,11] puwainaphycins/minutissamides,[12,13] and laxaphycins.[14] Both puwainaphycins and laxaphycins share structural similarities. The main difference is the number of residues in the cyclic peptide, where puwainaphycins have 10 residues and laxaphycins have 11 residues (type-A) and 12 residues (type-B), as shown in Figure 1. The puwainaphycin biosynthetic origin involves a hybrid PKS/NRPS enzyme with a 56 kb gene cluster,[15] while laxaphycin is encoded in a single 96 kb gene cluster with a shared PKS branching to two distinct NRPS pathways.[16] Our recent search for cyanobacterial compounds has led to the isolation of six new cyclic lipopeptides, noducyclamides (**1-6**) from the cyanobacterium *Nodularia* sp. NIES-3585 (Figure 1). This study reports the isolation, structure elucidations, and cytotoxic properties of noducyclamides A1-A4 (**1-4**) and noducyclamides B1-B2 (**5, 6**).

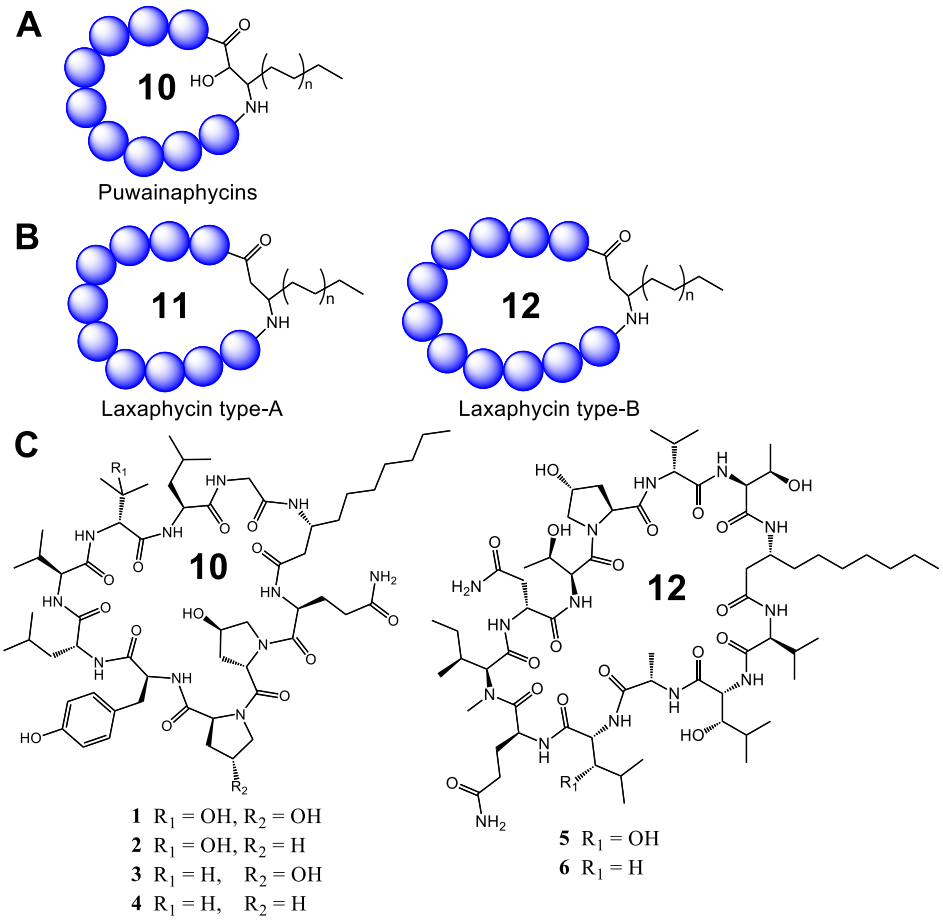

Figure 1. (A, B) The structures of puwainaphycins and laxaphycins. (C) The structures of noducyclamides A1-A4 (**1**-**4**) and B1, B2 (**5, 6**).

RESULTS AND DISCUSSION

Nodularia sp. (NIES-3585) was grown in BG-11 medium in 10 L bottles. The freeze-dried cells (12 g) from the 5-6 weeks culture were extracted with MeOH, and the MeOH extract was partitioned between H_2O and EtOAc. The hydrophilic fraction was further separated by octadecylsilyl (ODS) column chromatography, and the fractions were monitored by liquid chromatography-mass spectrometry (LC-MS) analysis. The major compounds noducyclamides

A1 (**1**), A2 (**2**), and B1 (**5**) and minor compounds noducyclamides A3 (**3**), A4 (**4**), and B2 (**6**) eluted in the 80% MeOH fraction. Noducyclamide A1 (**1**) was acquired as a white amorphous powder, and the structure was determined by NMR, MS, and Marfey's methodology (Table 1 and Figures S1-S8). The HRESIMS analysis showed a protonated molecule $[M + H]^+$ at *m/z* 1184.6926, indicative of the molecular formula $C_{58}H_{93}N_{11}O_{15}$. The ^1H and ^{13}C NMR spectra in DMSO-d_6 (Table 1) showed eight signals for the Cα methines of α-amino acids, one Cα methylene, Cα and Cβ positions of a β-amino acid (δ_C 44.1, 39.3; δ_H 4.29, 1.81, 1.72) and 10 signals for NH protons suggested a cyclic decapeptide. One *p*-substituted benzene was determined from 1D NMR carbon signals at δ_C 129.8 and 115.1; hydrogen peaks at δ_H 7.05 and 6.66, and COSY and HMBC correlations that connected these signals (Figure 2). The presence of two Leu units was detected from four doublet methyl signals at δ_H 0.93, 0.87, 0.70, and 0.67, COSY correlations of δ_H 0.93/0.87 to Cγ methine (δ_H 1.63) and δ_H 0.70/0.67 to Cγ methine (δ_H 1.30), and HMBC peaks of δ_H 0.93/0.87 to Cβ methylene (δ_C 39.5; δ_H 1.52, 1.32) and δ_H 0.70/0.67 to Cβ methylene (δ_C 38.4; δ_H 1.15). Besides Leu1 and Leu2, the other isopropyl moieties from Val and OHVal were detected in a similar manner, where methyl signals of Val at δ_H 0.82 and 0.68 have HMBC peaks to Cβ and Cα (δ_C 32.8, δ_H 2.17; δ_C 55.2, δ_H 4.79) methines and those of OHVal at δ_H 1.14 have HMBC peaks to the Cβ quaternary carbon (δ_C 71.4) and Cα methine (δ_C 59.3; δ_H 4.53). The presence of two OHPro was deduced from COSY/TOCSY correlations from Cα methines at δ_H 4.34 to Cβ (δ_H 2.00, 1.38), Cγ (δ_H 3.76) and Cδ units (δ_H 3.48, 3.18) and 4.35 to Cβ (δ_H 2.08, 1.88), Cγ (δ_H 4.39) and Cδ units (δ_H 3.91, 3.48). The Gly was observed at Cα methylene (δ_C 42.4; δ_H 3.80, 3.28). The Gln was determined from HMBC correlations of Cα methine at δ_H 4.54 to Cβ and Cγ methylenes (δ_C 26.4, δ_H 2.06, 1.67; δ_C 30.3,

δ_H 2.19) and ROESY correlations of NH_2 (δ_H 7.46, 6.81) to Cγ methylene (δ_H 2.19). The last residue, 3-aminodecanoic acid (Ada), was deduced from COSY/total correlation spectroscopy (TOCSY) correlations of δ_H 1.81/1.72, 4.29, 1.32, 1.37, 1.24, 1.23, 1.27, and 0.86 (Figure 2).

The HMBC spectrum (Figure 2) allowed the connection of the Leu2-Gly-Ada-Gln and OHPro2-Tyr-Leu1-Val-OHVal fragments. Subsequently, OHPro1 was connected to the C-terminal of Gln to establish the Leu2-Gly-Ada-Gln-OHPro1 fragment by ROESY correlations (Figure 2) of Gln Cα methine (δ_H 4.54) to OHPro1 Cδ methylene (δ_H 3.91, 3.48), then this fragment was extended to Leu2-Gly-Ada-Gln-OHPro1-OHPro2-Tyr-Leu1-Val-OHVal based on ROESY correlations of the OHPro2 Cα methine (δ_H 4.34) to OHPro1 Cβ methylene (δ_H 1.88); finally, ROESY correlations of the OHVal Cα methine (δ_H 4.53) to the Leu2 NH proton (δ_H 8.15) allowed this linear fragment to be closed at the peptide bond between Leu2-OHVal. The conformations between Gln-OHPro1 and OHPro1-OHPro2 were assigned to be *trans* and *cis*, respectively, based on three ROESY correlations (Figure 2) of OHPro1 Cδ (δ_H 3.91, 3.48) to Gln Cα (δ_H 4.54), OHPro2 Cα (δ_H 4.34) to OHPro1 Cβ (δ_H 1.88) and OHPro1 Cα (δ_H 4.35) to Tyr NH (δ_H 7.84). The Cα methines in OHPro1 (δ_H 4.35) and OHPro2 (δ_H 4.34) in **1** have poor resolution, but these methines have a better resolution (Figure S16) in **2** with OHPro (δ_H 4.35), and Pro (δ_H 4.38) and these three ROESY correlations are consistent in **1**-**4**. In addition, a large shift difference between Cβ and Cγ ($\Delta\delta_{C\text{-}3}$ - $\delta_{C\text{-}4}$ = 9.5 ppm) determined a *cis*-OHPro-Pro in **2** and **4**; this also supported a *cis*-conformation in **1** and **3**.[1,17]

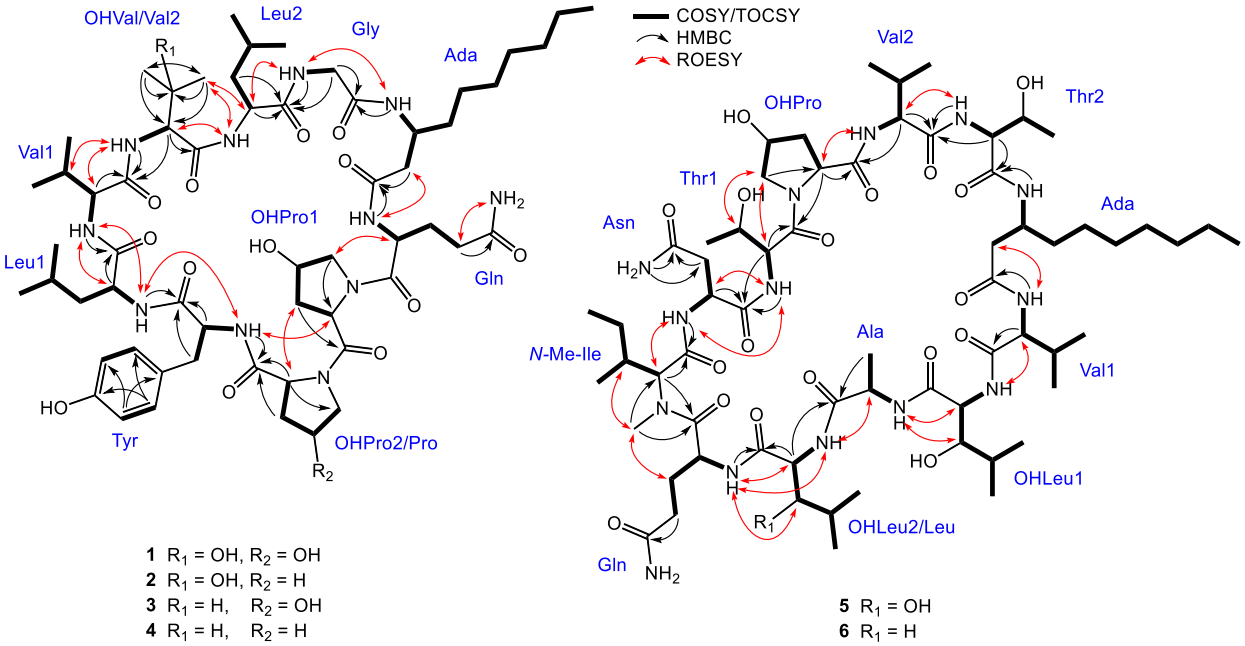

Figure 2. 2D NMR analyses of **1**-**6**.

The absolute configurations of the amino acid residues of **1** were determined by acid hydrolysis, followed by advanced Marfey's analysis (Figures S42-S49). Comparing LC-MS retention times for the DLA derivatives of authentic standards and the hydrolysate of **1**, the absolute configurations were assigned as L for the Gln and Tyr residues. Racemization was noticed for the Val unit during Marfey's analysis for **1**. The possibility of this racemization was explored by performing acid hydrolysis using deuterium chloride (DCl) in deuterium oxide (D_2O) solution. Similar racemization was observed in polytheonamide B for Asn residues and solved by the hydrolysis using DCl in D_2O, and the hypothesis behind this analysis was to detect the racemization of α-amino acids that might be produced by the exchange of α-hydrogens to the hydrogen derived from solvent.[18] Marfey's analysis in deuterated solution for **1** showed a higher ion peak by 1 Da for D-Val than L-Val in LC-MS analysis, indicating D-Val was the product of inversion under acidic conditions (Figure S45). A. Broberg *et al*. reported that

racemization occurred in the Phe residue of the cyclic lipodepsipeptide isopedopeptin B containing a OHVal adjacent to a Phe unit, and this racemization might be the result of forming an oxazoline intermediate.[19] Oxazoline ring was formed between the OHVal and the N-terminal neighboring amino acid (Val1 in this study), by loss of the Val1-Hα and subsequent ring formation from the Val1 carbonyl oxygen to the tertiary carbocation. When the reaction was reversed, oxazoline ring opening can led to either L- or D-Val1. Since the Leu2 was located at C-terminal of OHVal, racemization was not observed in D_2O/DCl analysis (Figure S45), indicated the presence of D-Leu is not from racemization. Hence, we concluded that **1** contains both L- and D-Leu. However, one thing found in common for all existing laxaphycin type-A peptides including **1**-**4** is a partial structure of Leu^7-X^8-Y^9-Leu^{10}-Gly^{11} for positions 7-11, whereas positions 7 and 10 were D-Leu and L-Leu, respectively as observed in trichormamides A, and D,[20,21] laxaphycins A, A2 and E,[22,23] acyclolaxaphycin A,[24] hormothamnin A,[9] and scytocyclamides A and A2 (Table S1).[16] Based on this consistency of reported laxaphycin type-A analogues, we proposed that noducyclamide biosynthetic gene cluster should synthesize D-Leu1 and L-Leu2. The absolute configuration of OHVal was determined by comparing the LC-MS retention times of L- and D-DLA derivatives of L-OHVal and hydrolysates of **1** that exhibited D-configuration for OHVal (Figure S47). Based on the LC-MS retention time of L-DLA derivatives of four OHPro isomers, (2*S*,4*S*)/(2*R*,4*S*)/(2*S*,4*R*)/(2*R*,4*R*)-4-hydroxy-Pro, **1** contained (2*S*,4*R*)-4-hydroxy-Pro (Figure S48). By comparing the elution order (L→D) of L- and D-DLA derivatives of the acid hydrolysate of **1** with those reported in the literature,[20] the configuration of β-amino decanoic acid (Ada) was assigned as 3*R* (Figure S49). Therefore, **1** contained L-Tyr, L-Gln, L-Val, D-OHVal, D-Leu1, L-Leu2, (2*S*,4*R*)-OHPro and (3*R*)-Ada.

Table 1 NMR Spectroscopic Data (DMSO-d_6) of Noducyclamides A1-A4 (**1**-**4**)

	Position	Noducyclamide A1 (**1**)		Noducyclamide A2 (**2**)		Noducyclamide A3 (**3**)		Noducyclamide A4 (**4**)	
		δ_C[a], type	δ_H (J in Hz)[b]	δ_C[a], type	δ_H (J in Hz)[b]	δ_C[a,c], type	δ_H (J in Hz)[b]	δ_C[a,c], type	δ_H (J in Hz)[b]
Ada	1	168.7, C		168.8, C		168.6, C		n.d.[d]	
	2	39.3, CH_2	1.81 m	39.4, CH_2	1.79 m	39.5, CH_2	1.77 m	n.d.[d]	
			1.72 m		1.71 m		1.60 m		
	3	44.1, CH	4.29 m	44.1, CH	4.29 m	44.0, CH	4.28 m	44.0, CH	4.28 m
	4	35.2, CH_2	1.32 m	35.2, CH_2	1.32	35.1, CH_2	1.31 m	35.1, CH_2	1.38 m
			1.37 m		1.35 m		1.37 m		
	5	28.8, CH_2	1.24 m	28.8, CH_2	1.24 m	28.9, CH_2	1.23 m	28.5, CH_2	1.23 m
	6	28.5, CH_2	1.24 m	28.5, CH_2	1.23 m	28.5, CH_2	1.23 m	28.5, CH_2	1.23 m
	7	25.5, CH_2	1.23 m	25.5, CH_2	1.22 m	25.6, CH_2	1.22 m	25.6, CH_2	1.22 m
	8	31.2, CH_2	1.24 m	31.3, CH_2	1.24 m	31.3, CH_2	1.23 m	31.3, CH_2	1.23 m
	9	22.1, CH_2	1.27 m	22.1, CH_2	1.26 m	22.1, CH_2	1.26 m	22.1, CH_2	1.26 m
	10	13.9, CH_3	0.86 t (6.9)	14.0, CH_3	0.85 t (6.9)	13.9, CH_3	0.85 t (6.9)	13.9, CH_3	0.85 m
	NH		6.93 d (10.0)		6.94 d (8.4)		6.80 d (9.5)		6.80 m
Gln	1	171.8, C		172.2, C				n.d.[d]	
	2	48.9, CH	4.54 m	49.1, CH	4.50 m	48.9, CH	4.54 m	49.1, CH	4.47 m
	3	26.4, CH_2	2.06 m	26.5, CH_2	2.06 m	26.4, CH_2	2.08 m	26.4, CH_2	2.08 m
			1.67 m		1.74 m		1.60 m		1.70 m
	4	30.3, CH_2	2.19 m	30.3, CH_2	2.22 m	30.3, CH_2	2.25 m	30.3, CH_2	2.28 m
							2.19 m		2.22 m
	5	173.8, C		173.8, C		173.8, C		n.d.[d]	
	NH		6.91 dd (8.6, 2.1)		6.92 dd (8.9, 2.1)		6.95 d (8.6)		6.95 m
	NH_2		7.46 s		7.48 s		7.48 s		n.d.[d]
			6.81 s		6.84 s		6.77 s		
OHPro1	1	170.5, C		169.8, C		170.5, C		n.d.[d]	
	2	56.8, CH	4.35 m	57.4, CH	4.35 m	56.8, CH	4.33 m	57.4, CH	4.33 m
	3	36.3, CH_2	2.08 m	36.3, CH_2	2.06 m	36.3, CH_2	2.06 m	36.3, CH_2	2.06 m
			1.88 dt (12.9, 5.2)		1.94 m		1.87 m		1.93 m
	4	68.2, CH	4.39 m	68.2, CH	4.40 m	68.2, CH	4.38 m	68.2, CH	4.38 m
	5	54.5, CH_2	3.91 dd (10.5, 5.7)	54.7, CH_2	3.93 dd (10.3, 5.7)	54.5, CH_2	3.92 m	54.5, CH_2	3.92 m
			3.48 m		3.58 dd (10.3, 4.6)		3.46 m		3.56 m
Pro/	1	171.9, C		171.0, C		172.0, C		n.d.[d]	
OHPro2	2	58.9, CH	4.34 m	60.0, CH	4.38 m	58.9, CH	4.32 m	60.0, CH	4.35 m
	3	40.0, CH_2	2.00 ddd (12.9, 8.6, 4.3)	31.3, CH_2	1.96 m	40.0, CH_2	1.98 m	36.0, CH_2	2.89 m
			1.38 m		1.74 m		1.36 m		2.86 m
	4	66.8, CH	3.76 m	20.8, CH_2	1.51 m	66.8, CH	3.76 m	20.8, CH_2	1.50 m
					0.79 m				
	5	54.4, CH_2	3.48 m	46.4, CH_2	3.20 m	54.4, CH_2	3.49 m	48.6, CH_2	3.15 m
			3.18 dd (11.9, 4.6)		3.13 m		3.17 m		
Tyr	1	171.7, C		171.8, C		171.6, C		n.d.[d]	
	2	56.9, CH	4.21 ddd (11.5, 7.4, 3.0)	56.8, CH	4.22 ddd (11.0, 7.1, 3.6)	56.9, CH	4.20 ddd (11.5, 7.1, 3.0)	56.7, CH	4.20 m
	3	35.6, CH_2	3.06 dd (11.5, 3.0)	36.1, CH_2	3.10 m	35.6, CH_2	3.04 dd (12.7, 3.3)	36.0, CH_2	3.08 m

			2.91 t (11.5)		2.87 t (13.1)		2.89 t (12.7)		2.86 m
	4	128.0, C		127.3, C		128.0, C		127.3, C	
	5/9	129.8, CH	7.05 d (8.3)	129.6, CH	7.01 d (8.3)	129.8, CH	7.04 d (8.3)	129.8, CH	7.00 m
	6/8	115.1, CH	6.66 d (8.3)	115.3, CH	6.68 d (8.3)	115.3, CH	6.65 d (8.3)	115.3, CH	6.67 m
	7	156.0, C		156.2, C		156.0, C		156.3, C	
	NH		7.84 d (7.4)		7.55 d (7.1)		7.84 d (7.1)		7.53 m
Leu1	1	171.8, C		171.5, C		171.8, C		n.d.[d]	
	2	52.6, CH	4.09 ddd (11.9, 7.9, 4.5)	52.2, CH	4.07 ddd (11.5, 7.7, 4.3)	52.6, CH	4.08 ddd (12.0, 7.6, 4.5)	52.3, CH	4.06 m
	3	38.4, CH_2	1.15 m	38.7, CH_2	1.18 m 1.11 m	38.4, CH_2	1.12 m	38.4, CH_2	1.16 m
	4	24.4, CH	1.30 m	24.6, CH	1.33 m	24.4, CH	1.28 m	24.6, CH	1.32 m
	5	20.5, CH_3	0.67 d (6.5)	20.6, CH_3	0.70 d (6.5)	20.5, CH_3	0.66 d (6.5)	20.6, CH_3	0.69 m
	6	23.1, CH_3	0.70 d (6.5)	23.0, CH_3	0.73 d (6.5)	23.1, CH_3	0.69 d (6.5)	23.1, CH_3	0.72 m
	NH		7.91 d (7.7)		7.85 d (7.7)		7.90 d (7.6)		7.85 m
Val1	1	172.4, C		172.4, C		172.6, C		n.d.[d]	
	2	55.2, CH	4.79 dd (9.5, 2.9)	55.2, CH	4.76 dd (9.5, 2.8)	55.2, CH	4.82 dd (9.5, 2.9)	55.2, CH	4.78 m
	3	32.8, CH	2.17 m	32.8, CH	2.17 m	32.3, CH	2.15 m	33.3, CH	2.15 m
	4	15.5, CH_3	0.68 d (6.9)	15.5, CH_3	0.66 d (6.9)	15.9, CH_3	0.68 d (6.5)	15.9, CH_3	0.68 m
	5	19.4, CH_3	0.82 d (6.9)	19.5, CH_3	0.82 d (6.9)	19.5, CH_3	0.82 d (6.5)	19.5, CH_3	0.82 m
	NH		6.86 d (9.8)		6.83 d (9.5)		6.84 d (9.5)		6.80 m
OHVal/ Val2	1	170.6, C		170.6, C				n.d.[d]	
	2	59.3, CH	4.53 m	59.4, CH	4.53 m	56.4, CH	4.52 m	56.4, CH	4.52 m
	3	71.4, C		71.4, C		31.0, CH	2.26 m	31.0, CH	2.26 m
	4	24.1, CH_3	1.14 s	24.2, CH_3	1.13 s	16.2, CH_3	0.82 d (6.5)	16.2, CH_3	0.82 m
	5	28.4, CH_3	1.14 s	28.3, CH_3	1.13 s	19.5, CH_3	0.82 d (6.5)	19.5, CH_3	0.82 m
	NH		8.69 d (9.6)		8.70 d (9.6)		8.54 d (9.28)		8.54 m
Leu2	1	172.7, C		172.7, C		171.9, C		n.d.[d]	
	2	52.9, CH	4.03 m	53.0, CH	4.02 dd (8.4, 6.5)	53.1, CH	3.98 dd (10.6, 5.8)	53.1, CH	3.98 m
	3	39.5, CH_2	1.52 m	39.5, CH_2	1.52 m	39.0, CH_2	1.53 m	39.0, CH_2	1.56 m
			1.32 m		1.40 m		1.34 m		1.36 m
	4	24.1, CH	1.63 m	24.1, CH	1.62 m	24.2, CH	1.60 m	24.2, CH	1.60 m
	5	21.4, CH_3	0.87 d (6.5)	21.4, CH_3	0.87 d (6.5)	21.5, CH_3	0.83 d (6.5)	21.5, CH_3	0.83 m
	6	22.8, CH_3	0.93 d (6.5)	22.8, CH_3	0.93 d (6.5)	22.6, CH_3	0.90 d (6.5)	22.6, CH_3	0.90 m
	NH		8.15 br s		8.17 br s		8.61 br s		n.d.[d]
Gly	1	166.6, C		166.7, C		166.4, C		n.d.[d]	
	2	42.4, CH_2	3.80 dd (16.8, 7.2)	42.4, CH_2	3.81 dd (17.0, 7.0)	42.4, CH_2	3.79 m	42.6, CH_2	3.80 m
			3.28 dd (16.8, 5.0)		3.27 m		3.22 m		3.22 m
	NH		8.92 dd (7.2, 5.0)		8.91 dd (7.0, 5.0)		8.99 br s		8.99 m

[a]Measured at 150 MHz. [b]Measured at 600 MHz. [c]^{13}C signals obtained from HMBC. [d]Not detected

Noducyclamides A2 (**2**) and A3 (**3**) have the same molecular formulas of $C_{58}H_{93}N_{11}O_{14}$. The ^{1}H and ^{13}C NMR spectra indicated that **2** and **3** share structural similarity to **1** (Table 1, Figures S9-S22). Detailed analysis of the 2D NMR spectra (Figure 2) revealed that **2** and **3** possess the same macrocyclic scaffold as **1**, except that **2** has one Pro and one OHPro instead of two OHPro as in **1**, and **3** has two OHPro, but lacked OHVal. Based on the retention times of the DLA derivatives of authentic standards of amino acids and hydrolysates of **2**, the configurations were assigned as L-Gln, L-Pro, and L-Tyr (Figures S50-S52). The absolute configuration of Val was determined as L similar to **1** using DCl in D_2O for hydrolysis (Figures S45, S53). The presence of both L and D isomers for Leu was detected in **2** (Figure S54). Based on the known analogues, the mixture L/D-Leu can be assigned as D-Leu1 and L-Leu2. The configuration of OHVal was determined as D by comparing the retention times of L- and D-DLA derivatives of the hydrolysate and the standards (Figure S47). The absolute configurations of OHPro and Ada were determined as (2*S*,4*R*)-4-hydroxy-Pro and 3*R*-Ada (Figures S48 and S49). Based on the retention times of the DLA derivatives of standards and the hydrolysate of **3**, the absolute configurations for **3** were determined as L-Gln and L-Tyr (Figures S55 and S56). Mixtures of L/D-Leu and L/D-Val were detected from the hydrolysate of **3** (Figures S57 and S58). Based on the structures of **1** and **2**, we can assume that configurations of **3** are L-Val1, D-Val2, D-Leu1, and L-Leu2. The same absolute configurations of (2*S*,4*R*)-OHPro and 3*R*-Ada were determined for **3** (Figures S48 and S49). Therefore, **2** contained L-Tyr, L-Gln, L-Val, D-OHVal, D-Leu1, L-Leu2, L-Pro, (2*S*,4*R*)-OHPro and 3*R*-Ada, while **3** contained L-Tyr, L-Gln, L-Val1, D-Val2, D-Leu1, L-Leu2, (2*S*,4*R*)-OHPro and 3*R*-Ada.

Noducycalmide A4 (**4**) exhibited a protonated molecule $[M + H]^+$ at *m/z* 1152.7004 with the molecular formula, $C_{58}H_{93}N_{11}O_{13}$, and detailed analysis of 1D and 2D NMR data revealed the

similarities of this minor analogue with **2** except having Val instead of OHVal (Table 1, Figures S23-28). Based on the advanced Marfey's method of the acid hydrolysate of **4** and the standards, the absolute configuration of **4** was determined as L-Tyr, L-Gln, L-Val1, D-Val2, D-Leu1, L-Leu2, L-Pro, (2*S*,4*R*)-OHPro and 3*R*-Ada (Figures S66-S71).

Noducycalmide B1 (**5**) has a protonated molecule $[M + H]^+$ at *m/z* 1381.8309 for the molecular formula of $C_{64}H_{112}N_{14}O_{19}$, and this molecular weight is larger than **1**-**4** (10-residue peptides). Upon analyses of ^{1}H and ^{13}C NMR spectra in DMSO-d_6 (Table 2, Figures S29-35), 11 signals for the Cα methines of α-amino acids, Cα-β positions of β-amino fatty acid (δ_C 45.8, 40.0; δ_H 4.11, 2.43, 2.36), five oxymethines of α-amino acids, 10 signals for amide NH protons and one *N*-Me signal at δ_C 30.2; δ_H 3.05 suggested a 12-residue cyclic peptide. A detailed analysis of the 2D NMR data (Figure 2) established the structure composed of Ada, Val1, OHLeu1, Ala, OHLeu2, Gln, *N*-MeIle, Asn, Thr1, OHPro, Val2, and Thr2. The HMBC, COSY, and TOCSY spectra allowed the connection of the four fragments *N*-MeIle-Asn-Thr1-OHPro-Val2-Thr2-Ada, Ala-OHLeu2-Gln, Val1, and OHLeu1. These fragments were connected into a head-to-tail 12-residue cyclic peptide by ROESY correlations. The *trans* conformation of the OHPro amide bond was assigned based on ROESY correlations.

The absolute configurations of **5** were determined as L for the Gln and Ala residues (Figures S59 and S60). The configuration of Asn was confirmed as D, and the presence of both L- and D-Val in the hydrolysate confirmed that the two Val residues present in **5** had opposite configurations (Figures S61 and S62). Similar to laxaphycin type A, the existing laxaphycin type-B analogues also have high conservation of the D-amino acids at positions 8 and 11, as observed in

trichormamides B and C,[20,21] laxaphycins B, B2, B3, B5 and B6,[4,25] acyclolaxaphycins B and B3,[26,27] lyngbyacyclamides A and B,[28] and scytocyclamides B, B2, B3, and C (Table S2).[16] Based on the conserved D-amino acids at positions 8 and 11 in reported laxaphycin type-B analogues, we concluded that the noducyclamide biosynthetic gene cluster should synthesize L-Val1 and D-Val2. Based on the retention times of L- and D-DLA derivatives of hydrolysates of **5** and authentic standards of 3-OHLeu such as (2*S*,3*S*)-3-OHLeu-L-DLA, (2*S*,3S)-3-OHLeu-D-DLA, (2*S*,3*R*)-3-OHLeu-L-DLA and (2*S*,3*R*)-3-OHLeu-D-DLA, the configuration was assigned as (2*R*,3*S*)-3-OHLeu (Figure S63). The absolute configurations of *N*-MeIle and Thr have been confirmed as L-*N*-MeIle and L-Thr by comparing the LC-MS retention times of L- and D-DLA derivatives of L-*N*-MeIle, L-*allo*-*N*-MeIle, L-Thr and L-*allo*-Thr, and hydrolysates of **5** (Figures S64 and S65). The configurations of OHPro and Ada were determined as (2*S*,4*R*)-4-hydroxy-Pro and 3*R*-Ada, similar to noducyclamides A1-A3 (**1**-**3**) (Figures S48 and S49). Therefore, **5** contained L-Ala, L-Gln, L-*N*-MeIle, D-Asn, L-Thr, L-Val1, D-Val2, (2*S*,4*R*)-OHPro, (2*R*,3*S*)-OHLeu, and 3*R*-Ada.

Noducyclamide B2 (**6**) appeared as a minor analogue and showed a protonated molecule $[M + H]^+$ at *m/z* 1365.8330 having a molecular formula $C_{64}H_{112}N_{14}O_{18}$. The planar structure of **6** was determined based on the detailed analyses of 2D NMR data and showed a similar structure with noducyclamide B1 (**5**), except for one Leu and one OHLeu instead of two OHLeu (Table 2, Figures S36-41). The absolute configurations of the amino acid residues were determined as L-Ala, L-Gln, L-*N*-MeIle, D-Asn, L-Thr, L-Val1, D-Val2, (2*S*,4*R*)-OHPro, D-Leu, (2*R*,3*S*)-OHLeu, and 3*R*-Ada (Figures S71-81).

Table 2. NMR Spectroscopic Data (DMSO-d_6) of Noducyclamides B1 (**5**) and B2 (**6**)

	Position	Noducyclamide B1 (**5**)		Noducyclamide B2 (**6**)	
		δ_C[a], type	δ_H (J in Hz)[b]	δ_C[a], type	δ_H (J in Hz)[b]
Ada	1	171.6, C			n.d.[c]
	2	40.0, CH_2	2.43 m	n.d.[c]	n.d.[c]
			2.36 m		
	3	45.8, CH	4.11 m	46.0, CH	4.06 m
	4	33.6, CH_2	1.35 m	n.d.[c]	n.d.[c]
			1.26 m		
	5	28.7, CH_2	1.18 m	28.6, CH_2	1.18 m
	6	28.5, CH_2	1.19 m	28.6, CH_2	1.18 m
	7	25.3, CH_2	1.24 m	n.d.[c]	n.d.[c]
			1.19 m		
	8	31.2, CH_2	1.19 m	31.2, CH_2	1.18 m
	9	22.0, CH_2	1.23 m	22.0, CH_2	1.23 m
	10	13.9, CH_3	0.84 t (6.5)	13.9, CH_3	0.83 m
	NH		7.69 d (9.8)		7.66 m
Val1	1	171.5, C			n.d.[c]
	2	58.7, CH	4.17 m	59.3, CH	4.03 m
	3	29.5, CH	1.99 m	29.5, CH	1.99 m
	4	18.3, CH_3	0.89 d (6.5)	18.5, CH_3	0.89 m
	5	19.0, CH_3	0.85 d (6.5)	19.0, CH_3	0.85 m
	NH		8.30 d (7.2)		8.33 m
OHLeu1	1	171.6, C			n.d.[c]
	2	55.8, CH	4.42 m	55.6, CH	4.33 m
	3	76.9, CH	3.49 m	76.9, CH	3.46 m
	4	30.8, CH	1.58 m	30.8, CH	1.58 m
	5	18.6, CH_3	0.78 d (6.5)	18.6, CH_3	0.75 m
	6	19.4, CH_3	0.92 d (6.5)	19.4, CH_3	0.92 m
	NH		8.07 d (8.6)		8.30 m
Ala	1	172.6, C			n.d.[c]
	2	49.5, CH	4.20 m	49.0, CH	4.16 m
	3	17.7, CH_3	1.34 d (6.5)	17.7, CH_3	1.24 m
	NH		8.04 d (4.3)		7.96 m
OHLeu2/	1	170.8, C			n.d.[c]
Leu	2	55.5, CH	4.30 m	51.5, CH	4.20 m
	3	75.9, CH	3.45 m	40.6, CH_2	1.46 m
					1.34 m
	4	29.8, CH	1.56 m	24.9, CH	1.53 m
	5	18.8, CH_3	0.89 d (6.5)	21.4, CH_3	0.78 m
	6	18.9, CH_3	0.76 d (6.5)	22.8, CH_3	0.83 m
	NH		7.71 d (9.8)		7.81 m
Gln	1	172.5, C			n.d.[c]
	2	48.9, CH	4.56 m	48.2, CH	4.57 m
	3	26.2, CH_2	1.98 m	26.4, CH_2	1.89 m
			1.56 m		1.66 m
	4	30.4, CH_2	2.30 m	30.6, CH_2	2.12 m
			2.15 m		2.10 m
	5	174.6, C			n.d.[c]
	NH		7.60 d (6.7)		7.91 m
	NH_2		7.51 br s		n.d.[c]
			7.06 br s		
N-Me Ile	1	169.7, C			n.d.[c]
	2	59.6, CH	4.67 d (10.8)	59.7, CH	4.67 m
	3	32.2, CH	1.90 m	32.1, CH	1.88 m
	4	23.8, CH_2	1.26 m	23.8, CH_2	1.25 m
			0.86 m		0.84 m
	5	10.4, CH_3	0.76 d (6.5)	10.4, CH_3	0.76 m
	6	15.1, CH_3	0.76 d (6.5)	15.3, CH_3	0.77 m
	N-Me	30.2, CH_3	3.05 s	30.3, CH_3	2.98 m
Asn	1	170.5, C			n.d.[c]
	2	49.8, CH	4.64 m	50.0, CH	4.59 m
	3	35.9, CH_2	2.51 m	36.1, CH_2	2.50 m
			2.41 m		2.43 m
	4	171.4, C			n.d.[c]

	NH		8.46 d (7.4)		8.30 m
	NH_2		7.24 s		n.d.[c]
			6.85 s		
Thr1	1	168.6, C			n.d.[c]
	2	55.0, CH	4.43 m	55.8, CH	4.42 m
	3	66.9, CH	3.83 m	66.8, CH	3.85 m
	4	19.6, CH_3	1.04 d (6.5)	19.0, CH_3	1.03 m
	NH		7.37 d (7.9)		7.37 m
OHPro	1	171.7, C			n.d.[c]
	2	58.5, CH	4.54 t (7.9)	58.6, CH	4.52 m
	3	38.1, CH_2	2.02 m	37.8, CH_2	2.03 m
			1.83 m		1.82 m
	4	68.6, CH	4.30 m	68.6, CH	4.28 m
	5	55.6, CH_2	3.69 dd (10.5, 3.3)	55.4, CH_2	3.69 m
			3.57 m		3.56 m
Val2	1	170.8, C			n.d.[c]
	2	57.9, CH	4.26 t (8.0)	57.8, CH	4.25 m
	3	30.9, CH	1.99 m	30.6, CH	1.97 m
	4	18.2, CH_3	0.82 d (6.5)	18.1, CH_3	0.81 m
	5	19.2, CH_3	0.81 d (6.5)	19.2, CH_3	0.81 m
	NH		7.97 d (8.1)		7.97 m
Thr2	1	168.6, C			n.d.[c]
	2	58.3, CH	4.13 m	58.2, CH	4.14 m
	3	66.4, CH	3.95 m	66.5, CH	3.95 m
	4	19.0, CH_3	1.05 d (6.5)	19.5, CH_3	1.01 m
	NH		7.86 (7.6)		7.58 m

[a]Measured at 150 MHz. [b]Measured at 600 MHz. [c]Not detected.

The earlier biological studies of laxaphycins found that trichormamide B (laxaphycin type-B) showed cytotoxicity against MDA-MB-435 and HT-29 cancer cell lines with IC_{50} values at 0.8 and 1.5 µM, respectively.[20] Another report also found that the laxaphycin type-B peptide, trichormamide C exhibited more potent cytotoxicity than laxaphycin type-A peptides (trichormamides A and D) against MDA-MB-435 and HT-29 cancer cell lines with IC_{50} values at 1.0 and 1.7 µM, respectively.[20,21] In our study, a similar pattern of cytotoxicity was observed, where noducyclamide B1 (**5**) exhibited cytotoxicity against the MCF7 breast cancer cell line with an IC_{50} of 3.0 µg/mL (2.2 µM), which is more potent than laxaphycin type-A noducyclamides A1 (**1**) and A2 (**2**) with IC_{50} values of 18 µg/mL (15.2 µM) and 9.6 µg/mL (8.2 µM), respectively (Figure S82). This may be due to some converted residues such as OHLeu2, *N*-Me-Ile, and Thr2 in laxaphycin type-B peptides contributing to greater potency than laxaphycin type-A peptides as suggested in a previous report.[21] Cytotoxicity of B-type laxaphycins is mediated by an apoptotic process,[26] and it has been suggested that they are

involved in depolarizing the mitochondrial membrane and reducing ROS and ATP levels.[26] Synergistic effects were observed in antibacterial, antifungal, and cytotoxic activities for laxaphycins, lobocyclamides, heinamides, and scytocyclamides,[16,25,29-32]. Due to the limited amount of compounds, we were unable to determine statistically significant results for synergistic effects. We observed the concentrations of 10, 7.5, 5, 2.5, and 1µg/mL to see the effects of a combination of both type-A and type-B compounds (i.e., **1** and **5**; **2** and **5**), however, there's no combined effect for increasing the percentage of cytotoxicity against the MCF7 breast cancer cell line. Due to the insufficient amounts, compounds **3**, **4**, and **6** were not tested for their cytotoxic activities.

To date, puwainaphycins (10-residue) and laxaphycins (type-A: 11-residue and type-B: 12-residue) have never been isolated from the same cyanobacterium.[6] In this study, structures **1**-**4** (10-residue) are not categorized into the puwainaphycin class but are considered to be laxaphycin type-A due to amino acid sequence similarity. The structures of type-A laxaphycins consistently have segregation of hydrophobic and hydrophilic residues,[22] and this feature is matched to structure **3** with the segregation of hydrophobic residues (Leu-Val-Val-Leu-Gly-Ada) and hydrophilic residues (Gln-OHPro-OHPro-Tyr). This also applies to structures **1**, **2** and **4**. Laxaphycin type-B variants have alternating hydrophobic and hydrophilic residues,[20] as observed in structures **5** and **6**. In addition, many studies reported both type-A and type-B laxaphycins are produced in the same organism,[16,20-22,29,30] except one report that showed the cyanobacterium *Phormidium* sp. UIC 10484 produced only type-B.[4] Unique characteristics of the puwainaphycins are differences in the lengths and substitutions of β-amino fatty acids.[11,12,33-35] On the other hand, laxaphycins type-A/B shared the same length of β-amino fatty acid as observed in this study for structures **1**-**6**.[20-21,28,29] Taken together with the above discussion,

structures **1**-**4** belong to type-A laxaphycins with a rare feature of 10-residue instead of typical 11-residue. Two acyclic shorter versions of type-A laxaphycins with 10- and 9-residue were reported from the cyanobacterium *Anabaena torulosa*,[24] but the mechanism involved to generate acyclic laxaphycin in cyanobacteria was remained elusive. However, a closer example to such acyclic mechanism was D-peptidase from the sea hare *Stylocheilus striatus* that fed on the laxaphycin-producing cyanobacterium *Anabaena torulosa*, and biotransformed laxaphycin type-B peptides (cyclic, 12-residue) into linear or acyclic shorter peptides (acyclic, 10-residue).[26,36]

In summary, an LC-MS guided investigation of the cultured *Nodularia* sp. (NIES-3585) resulted in the isolation of six new cyclic lipopeptides, noducyclamides A1-A4 (**1**-**4**) and B1-B2 (**5, 6**). The cyclic lipopeptides **1**-**6** are analogues of the laxaphycins. This is the first example of type-A laxaphycins that contain only 10-residue, and the first occurrence of the non-proteinogenic amino acid OHVal in a peptide from the laxaphycin family. Noducyclamide B1 exhibited cytotoxicity against the MCF7 breast cancer cell line with an IC_{50} value of 3.0 µg/mL (2.2 µM), whereas noducyclamides A1 and A2 had higher IC_{50} values of 18 µg/mL (15.2 µM) and 9.6 µg/mL (8.2 µM), respectively.

EXPERIMENTAL SECTIONS

General Experimental Procedures

Optical rotations were measured on a JASCO P-1030 polarimeter. ^{1}H and ^{13}C NMR spectra were acquired on a JEOL ECA 600 NMR spectrometer**,** and chemical shifts were referenced to the DMSO-d_6 solvent peaks as internal standards (δ_H 2.50, δ_C 39.5). The mixing times of TOCSY

and ROESY are 90 ms and 250 ms, respectively. HPLC separations were conducted with a JASCO HPLC system equipped with a PU-980 pump and UV-970 UV/vis detector. The high-resolution ESI mass spectra were obtained on an Agilent 1100 Series HPLC system coupled with a micrOTOF-HS mass spectrometer (Bruker Daltonics). The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 μm, 25 °C, 0.2 mL/min) under the following conditions: 0 - 30 min, gradient elution of 20 - 80% MeCN with 0.1% (v/v) formic acid in Milli-Q H_2O; 30 - 45 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in Milli-Q H_2O.

Biological material

The *Nodularia* sp. NIES-3585 was obtained from the NIES collections (Microbial Culture Collection, the National Institute for Environmental Studies, Japan). The species was grown in a BG-11 medium with continuous aeration at 25 °C under 80 $\mu Em^{-2}s^{-1}$ illuminations on a 12L:12D cycle for 5-6 weeks. Cells were collected using continuous centrifugation at 10,000 rpm, and lyophilized cells were stored at -30 °C until extraction.

Extraction and Isolation

Freeze-dried cells (12 g from 120 L of culture in 5-6 weeks) were homogenized and extracted with MeOH for three times. The concentrated MeOH extract was partitioned between H_2O and ethyl acetate (EtOAc). The EtOAc fraction was further partitioned between hexane and 90% MeOH. The combined fraction of 90% MeOH and H_2O was subjected to ODS (YMC-Gel, 150 μm) column chromatography with a gradient of MeOH in H_2O to yield five fractions (20%, 40%, 60%, 80%, and 100% MeOH). The fractions eluted with 80% and 100% MeOH (200 mg) were applied to HPLC (Wakosil-II 5C18 AR, 20 x 250 mm, UV detection 215 nm, flow rate 4.0 mL/min, linear gradient condition 20 to 80% MeCN in H_2O) to yield compounds **1**-**6**. The

fraction containing compounds **1** and **5** was further purified by HPLC (Wakosil-II 5C18 AR, 20 x 250 mm, UV detection 215 nm, flow rate 4.0 mL/min, isocratic, 47% MeCN with 0.1% TFA) to yield **5** (19 mg). The resulting sub-fraction was further purified by HPLC with Cosmosil Cholester (10 x 250 mm, UV detection 215 nm, flow rate 2.0 mL/min) and isocratic conditions, (40% MeCN with 0.1% TFA) to yield **1** (4 mg). Compounds **2-4**, and **6** were purified using C18 column (Cosmosil 5C_{18}-MS-II, 10 x 250 mm, UV detection 215 nm, flow rate 3.0 mL/min, linear gradient condition 30 to 80% MeCN in H_2O) to yield **2** (10 mg), **3** (2 mg) **4** (1.2 mg) and **6** (1 mg).

*Noducyclamide A1 (**1**):* White amorphous powder; $[\alpha]^{20}_D$ -0.63 (*c* 0.11, MeOH); NMR (DMSO-d_6, 600 MHz), Table 1; HRESIMS *m/z* 1184.6926 $[M + H]^+$ (calcd for $C_{58}H_{94}N_{11}O_{15}$, 1184.6931).

*Noducyclamide A2 (**2**):* White amorphous powder; $[\alpha]^{20}_D$ -2.67 (*c* 0.11, MeOH); NMR (DMSO-d_6, 600 MHz), Table 1; HRESIMS *m/z* 1168.7000 $[M + H]^+$ (calcd for $C_{58}H_{94}N_{11}O_{14}$, 1168.6982).

*Noducyclamide A3 (**3**)*: White amorphous powder; NMR (DMSO-d_6, 600 MHz), Table 1; HRESIMS *m/z* 1168.6952 $[M + H]^+$ (calcd. for $C_{58}H_{94}N_{11}O_{14}$, 1168.6982).

*Noducyclamide A4 (**4**)*: White amorphous powder; NMR (DMSO-d_6, 600 MHz), Table 1; HRESIMS *m/z* 1152.7004 $[M + H]^+$ (calcd. for $C_{58}H_{94}N_{11}O_{13}$, 1152.7032).

*Noducyclamide B1 (**5**)*: White amorphous powder; $[\alpha]^{19}_D$ -28.4 (*c* 0.1, MeOH); NMR (DMSO-d_6, 600 MHz), Table 2; HRESIMS *m/z* 1381.8309 $[M + H]^+$ (calcd. For $C_{64}H_{113}N_{14}O_{19}$, 1381.8306).

*Noducyclamide B1 (**6**)*: White amorphous powder; NMR (DMSO-d_6, 600 MHz), Table 2; HRESIMS *m/z* 1365.8330 $[M + H]^+$ (calcd. for $C_{64}H_{113}N_{14}O_{18}$,1365.8357).

Acid Hydrolysis and Advanced Marfey's Analysis

Hydrolysis was carried out for compound **1-6** (0.2 mg) by adding 200 µL of 6 M HCl and heating in sealed vacuum hydrolysis tubes at 110 °C for 16 h. Hydrolysates were cooled to room temperature (rt), dried *in vacuo,* and resuspended in H_2O. The aqueous solutions of standards and hydrolysates were derivatized with FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) followed by the addition of 1 M $NaHCO_3$ and heating at 40 °C for 1 h.[37] The reaction mixture was cooled to rt, neutralized with 2 M HCl, and resuspended in MeCN for LC-MS analysis. The LC-MS analysis was performed using a Zorbax SB-C3 column (2.1 × 150 mm) with a linear gradient of 25% to 65% aqueous MeCN containing 0.1% formic acid for 45 min. For the hydrolysis in D_2O/DCl, compound, **1** and **2** (0.2 mg) was hydrolysed in 200 µL of DCl solution (35 wt. % in D_2O) for 18 h at 110 °C. The absolute configurations of Gln and Asn were determined based on the LC-MS retention times of DLA derivatives of glutamic acid (Glu) and aspartic acid (Asp).

Cytotoxicity Assay

A standard MTT assay was performed to check the cytotoxicity against the human MCF7 breast cancer cell line.[38] Cancer cells were cultured in RPMI-1640 (Wako) with L glutamine supplemented by 10% (v/v) fetal bovine serum (FBS, BioWest). Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. The cellular viability was evaluated by reducing the 3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole soluble in water, to purple formazan crystals that are insoluble in water. The reduction of MTT to formazan is directly proportional to mitochondrial activity and cell viability. Cells were seeded in 96-well culture plates (10,000 cells per well) in 90 µL of RPMI medium supplemented by 10% fetal bovine serum (FBS) for 24 h before treatment at 37 °C with 5% CO_2. Cisplatin was used as a positive control in this study with an IC_{50} value of 9.5 µM against the MCF7 cell line. 10 µL samples were added to the well and incubated for 72 h. After incubation, the medium (100

μL) was carefully aspirated from the well, and 100 μL of MTT solution (0.5 mg/mL) was added. After 3 h incubation, the MTT solution was removed, and 100 μL of DMSO was added to the well. The residual formazan was dissolved in DMSO, and the absorbance was measured at 570 nm in a microplate reader (Multiskan). The plate was shaken for 10 sec prior to the absorbance measurement. The solution would give a purple color for the existence of live cells. The percent of cytotoxic activity was determined by following the formula reported in the Joray et al., 2015.[39] IC_{50} values were calculated using 'R' software (version: R 4.2.2) with "drc" package.

ASSOCIATED CONTENT

Supporting Information

The ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, COSY, ROESY spectra of the compounds **1-6**, LC-MS chromatogram of the compounds **1-6.** Marfey's derivatives of the hydrolysates of **1**-**6**. The NMR data for 1-6 have been deposited in the Natural Products Magnetic Resonance Database (www.np-mrd.org) and can be found at NP0332608 (**1**), NP0332609 (**2**), NP0332610 (**3**), NP0332611 (**4**), NP0332612 (**5**), and NP0332613 (**6**).

AUTHOR INFORMATION

Author Contributions

$^{\nabla}$J.J.M. and C.-S.P. contributed equally

Notes

The authors declare no competing financial interest

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