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HYMENAMIDES C ~ E, NEW CYCLIC HEPTAPEPTIDES WITH TWO PROLINE RESIDUES FROM THE OKINAWAN MARINE SPONGE *HYMENIACIDON* SP.

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Abstract: Three new cyclic heptapeptides containing two proline residues, hymenamides C ~ E (**1** ~ **3**), have been isolated from the Okinawan marine sponge *Hymeniacidon* sp. and the structures elucidated on the basis of two dimensional NMR data.

Recently, many peptides isolated from various marine sources have been reported such as hymenistatin 1[1a], motuporin[1b], discokinolide[1c], and polydiscamide[1d] from marine sponges, and diazonamides[2a] and cycloxazoline[2b] from tunicates. We have also isolated some unique peptides with calmodulin antagonistic activity[3a], inhibitory activity of superoxide generation[3b], stimulating activity of nerve growth factor synthesis[3c,d], and cytotoxicity[3e] from marine sponges or a fish-possessing fungus. In our continuing search for bioactive substances from marine organisms[4], we encounterd extracts of the Okinawan marine sponge *Hymeniacidon* sp. and obtained two new proline-rich cyclic heptapeptides, hymenamides A and B[5]. Further investigation on constituents of the same sponge resulted in isolation of three new cyclic heptapeptides with two proline residues, named hymenamides C ~ E (**1** ~ **3**). Here we describe the isolation and structure elucidation of **1** ~ **3**.

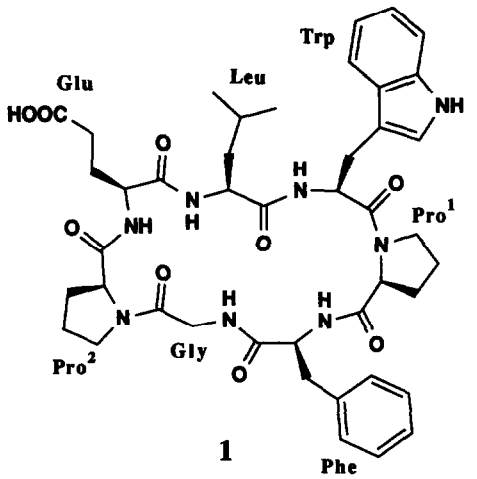
The sponge of the genus *Hymeniacidon* was collected off Manza, Okinawa Island and kept frozen until used. The methanolic extract of this sponge was partitioned with ethyl acetate and water. The ethyl acetate soluble material was subjected to a silica gel column ($CHCl_3$/*n*-BuOH/AcOH/H_2O) to give crude peptide fractions, which were separated repeatedly by Sephadex LH-20 column chromatographies (MeOH and $CHCl_3$/MeOH). Further purification using a Sep-Pak C_{18} cartridge ($CH_3CN/H_2O/CF_3CO_2H$) and/or HPLC on ODS ($CH_3CN/H_2O/CF_3CO_2H$) afforded hymenamides C (**1**, 0.003 %, wet weight), D (**2**, 0.0001 %), and E (**3**, 0.0006 %) as colorless amorphous solids together with known cyclic peptides, axinastatin 1[6] and phakellistatin 1[7].

Table 1. ^{1}H and ^{13}C NMR Data of Hymenamide C (**1**) in DMSO-d_6.

position	^{1}H[a]		J(Hz)	^{13}C[a]		position	^{1}H[a]		J(Hz)	^{13}C[a]	
Trp						Gly					
α	4.45	m		53.45	d	α	4.10	dd	3.9, 17.6	42.61	t
β	3.12	m		26.53	t		3.96	dd	3.9, 17 6		
	3.08	m				CO				167.99	s
1	10.77	d	2.0			NH	7.56	t	3.9		
2	7.31	d	2.0	123.94	d						
3				108.65	s	Pro[2]					
4				136.01	s	α	4.23	dd	5.9, 8.3	61.39	d
5	7.34	d	8.0	117.96	d	β	2.26	m		29.52	t
6	6.91	t	8.0	118.25	d		1.81	ddd	2.7, 5.9, 18.6		
7	7.04	t	8.0	121.08	d	γ	1.93[b]	qin.	6.1	24.27	t
8	7.34	d	8.0	111.23	d	δ	3.62	dt	6.4, 9.8	45.88	
9				126.88	s		3.53	dt	6.4, 9.8		
CO				170.37	s	CO				171.45	s
NH	9.34	d	4.4								
						Glu					
Pro[1]						α	4.26	m		52.62	d
α	3.45	d	6.8	59.75	d	β	2.12	m		26.35	t
β	1.53	dd	5.4, 11.7	28.69	t		1 75	m			
	0.28	m				γ	2.25[b]	m		30.43	t
γ	1.04	m		20.44	t	γCO				173.57	s
	0.34	m				CO				170.94	s
δ	2.93	b.dd	5.9, 13.2	45.30	t	NH	7.90	d	8.8		
	2.41	b.t	10.3								
CO				169.58	s	Leu					
						α	4 44	m		51.33	d
Phe						β	1.70	m		40.16[d]	t
α	4.17	ddd	3.9, 7.9, 8.8	56.31	d		1.47	m			
β	3.20	dd	3.9, 13.2	36.98	t	γ	1.63	m		24.27	d
	2.96	dd	8.8, 13.2			CH_3	1.01	d	8.0	21.78	q
1				137.51	s	CH_3	0.91	d	8.0	23.44	q
2, 6	7.11[b]	d	6.8	128.40[c]	d	CO				171.36	s
3, 5	7.24[b]	dd	6.8, 7.3	128.20[c]	d	NH	7.10	d	5.9		
4	7.18	t	7.3	126.42	d						
CO				170.79	s						
NH	8.27	d	7.9								

a) δ in ppm. b) 2H. c) 2C. d) This carbon signal was overlapped in DMSO-d_6 signal and assigned by HSQC data.

Hymenamide C (**1**) showed the pseudomolecular ion at *m/z* 827 in the FABMS spectrum, and the molecular formula, $C_{43}H_{54}N_8O_9$, of **1** was established by the HRFABMS (*m/z* 827.4109, M^++H, Δ +1.7 mmu). The IR absorptions at 3400 and 1670 cm^{-1} suggested the presence of NH and amide carbonyl groups, respectively. The ^{13}C NMR (Table 1) spectrum of **1** contained 41 resonances including two sets of symmetrical phenyl carbons at δ 128.40 and 128.20. The ^{1}H NMR (Table 1) spectrum of **1** was indicative of peptide. Standard amino acid analysis of the acid hydrolysate of **1** revealed the presence of 1 mol each of glutamic acid (Glu), glycine (Gly), leucine (Leu), phenylalanine (Phe), and tryptophan (Trp), and two mol of proline (Pro) residues. Hymenamide C (**1**) was negative to ninhydrin test, indicating a cyclic peptide or an *N*-terminus-modified peptide. The absolute stereochemistry of each amino acid in **1** was



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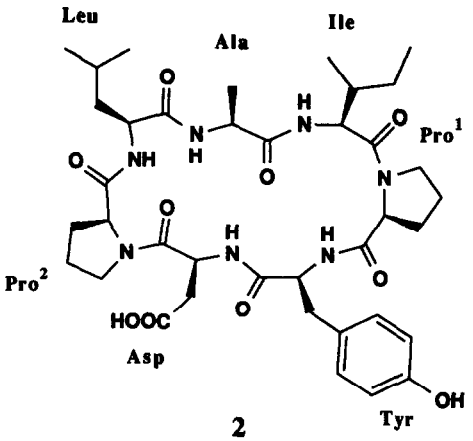

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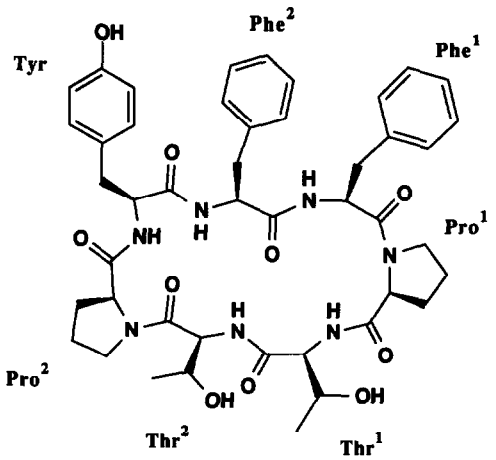

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determined to be L-configuration by C_{18} HPLC analyses of the acid hydrolysate using Marfey's procedure[8]. Detailed analyses of the ^1H-^1H COSY[9] and HOHAHA[10] spectra in DMSO-d_6 allowed assignments of the proton signals due to each amino acid residue, while HSQC[11] experiment enabled assignments of the protonated carbon resonances (Table 1). Eight carbonyl carbons of **1** were assigned on the basis of correlations observed in the HMBC[12] spectrum. The HMBC correlations of NH(Phe)/CO(Pro^1), NH(Gly)/CO(Phe), NH(Glu)/CO(Pro^2), NH(Leu)/CO(Glu), and NH(Trp)/CO(Leu) revealed the presence of two segments of Pro^1–Phe–Gly and Pro^2–Glu–Leu–Trp. The

ROESY[13] correlations observed for αH-Trp/αH-Pro^1 and αH$_2$-Gly/δH$_2$-Pro^2 suggested the connectivities between Trp and Pro^1 and between Gly and Pro^2, respectively. Thus the structure of **1** was concluded to be cyclo-(Trp–Pro^1–Phe–Gly–Pro^2–Glu–Leu).

Hymenamide C (**1**) existed as almost a single conformation in DMSO-d_6. In the ROESY spectrum of **1** a cross-peak observed for αH-Trp/αH-Pro^1 suggested that the amide bond at CO(Trp)/N(Pro^1) was cis geometry {type VI(a) β-turn}[14], while cross-peaks observed for αH$_2$-Gly/δH-Pro^2 indicated that the amide bond at CO(Gly)/N(Pro^2) was trans geometry. The ROESY correlations of αH-Trp/NH-Phe and αH-Trp/NH-Gly suggested that both amide protons of Phe and Gly were oriented inside the peptide ring. The temperature coefficients of the chemical shifts of α-amide protons of Phe, Gly, and Leu (Δδ/ΔT: -0.5, -0.6, and -0.4, respectively) were smaller than that of Trp (Δδ/ΔT: -6.5) of **1**, implying that the three amide protons of Phe, Gly, and Leu participated in intramolecular hydrogen bonds[15], and that the amide proton of Trp was oriented outside the peptide ring. The lower-field chemical shift (δ 9.34) of the α-amide proton of Trp in DMSO-d_6 indicated that the amide proton of Trp was exposed to the solvent[16]. The

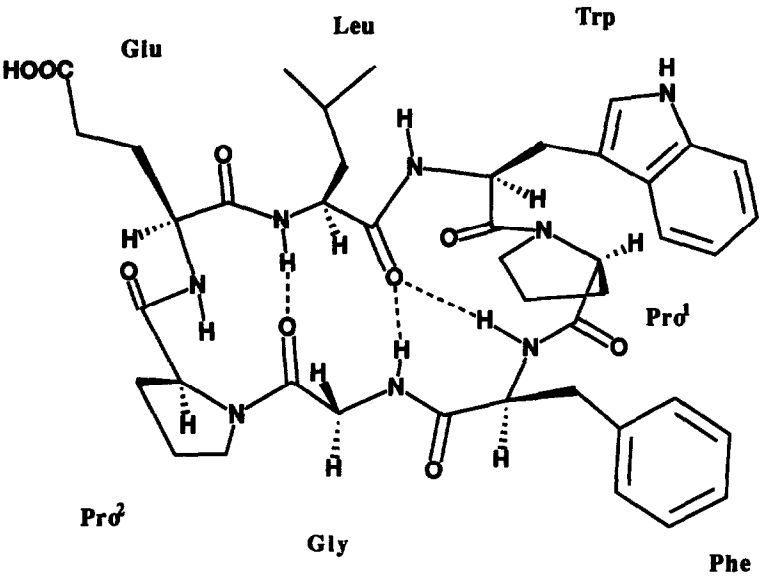

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Fig. 1 Plausible Conformation of Hymenamide C (1) Proposed on Basis of NMR Data.
Hash lines showed transannular hydrogen bonds.

presence of type II β-turn at CO(Pro^2)/NH(Glu)[17] was revealed by the ROESY cross-peaks for NH-Glu/αH-Pro^2 and NH-Glu/NH-Leu. Model consideration of **1** also supported the presence of three transannular hydrogen bonds at NH(Phe)/CO(Leu), NH(Gly)/CO(Leu), and NH(Leu)/CO(Gly), which composed β-bulge motif[18]. Thus the backbone structure of hymenamide C (**1**) was implied to have two types of β-turns, namely type II at Pro^2–Glu and type VI(a) at Trp–Pro^1, and three transannular hydrogen bonds at NH(Phe)/CO(Leu), NH(Gly)/CO(Leu), and NH(Leu)/CO(Gly), incorporating β-bulge motif (Fig.1).

Table 2. ^{1}H and ^{13}C NMR Data of Hymenamide D (**2**) in DMSO-d_6.

position	^{1}H[a]		J(Hz)	^{13}C[a]		position	^{1}H[a]		J(Hz)	^{13}C[a]	
Ile						Asp					
α	3.91	dt	4.4, 5.8	47.95[b]	d	α	4.79	m		50.58[b]	d
β	1.72	m		35.22	d	β	3.10	dd	3.9, 15.6	36.19	t
γ	1.53	m		24.45	t		2.79	m			
	1.28	m				βCO				172.62	s
βCH$_3$	0.80	d	6.8	14.65	q	NH	7.76	d	7.8		
γCH$_3$	0.81	t	6.8	10.77	q	CO				170.41[c]	s
NH	8.68	d	4.9								
CO				170.84[c]	s	Pro^2					
						α	4.04	dd	7.3, 9.8	62.98	d
Pro^1						β	2.28	m		30.28	t
α	4.34	d	7.8	60.35	d		1.60	m			
β	2.09	m		29.37	t	γ	1.96	m		25.05	t
	1.66	m					1.83	m			
γ	1.58	m		20.88	t	δ	3.79	m		47.38	d
	0.81	m					3.58	m			
δ	3.26	m		45.65	t	CO				172.11[c]	s
	2.85	m									
CO				170.79[c]	s	Leu					
						α	4.29	m		57.04	d
Tyr						β	1.68	m		39.5[f]	t
α	4.12	m		48.90[b]	d		1.56	m			
β	3.00	m		35.48	d	γ	1.55	m		24.56	d
	2.93	m				CH$_3$	0.88	d	6.4	24.45	q
1				127.50	s	CH$_3$	0.82	d	6.4	20.79	q
2, 6	6.95[d]	d	8.8	129.57[e]	d	NH	7.22	d	9.8		
3, 5	6.66[d]	d	8.8	115.07[e]	d	CO				170.31[c]	d
4				155.94	s						
OH	9.21	b.s				Ala					
NH	8.42	d	7.8			α	4.42	qin.	6.4	57.44[b]	d
CO				170.44[c]	d	CH$_3$	1.13	d	6.4	16.04	q
						NH	8.68	d	4.9		
						CO				169.88	s

a) δ in ppm. b) and c) These carbon signals were interchangeable. d) 2H. e) 2C.
f) This carbon signal was overlapped in DMSO-d_6.

Hymenamide D (**2**) showed the pseudomolecular ion at *m/z* 770 in the FABMS spectrum. The molecular formula, $C_{38}H_{55}N_7O_{10}$, of **2** was established by the HRFABMS (*m/z* 770.4047, M^++H, Δ -4.2 mmu). The ^1H NMR (Table 2) spectrum of **2** showed five amide NH and seven α-methine protons, while the ^{13}C NMR spectrum of **2** showed eight carbonyl carbons, seven of which were amide ones, and one of which was a carboxyl one, indicating that hymenamide D (**2**) was also a cyclic heptapeptide. Combination of standard amino acid analysis and HPLC analyses using Marfey's procedure of the acid hydrolysate of **2** revealed the presence of 1 mol each of L-asparatic acid (Asp), L-alanine (Ala), L-Leu, L-isoleucine (Ile), and L-tyrosine (Tyr), and two mol of L-Pro residues. The ^1H-^1H COSY spectrum of **2** disclosed all spin systems of each amino acid residue. The presence of a segment, Tyr–Asp–Pro^2–Leu–Ala–Ile–Pro^1, was deduced from the following ROESY correlations of **2** in DMSO-d_6; αH-Ile/αH-Pro^1, NH-Tyr/NH-Asp, αH-Asp/δH-Pro^2, αH-Pro^2/NH-Leu, αH-Leu/NH-Ala, NH-Leu/NH-Ala, and αH-Ala/NH-Ile. The cross-peaks observed for αH-Ile/NH-Tyr and αH-Ile/NH-Asp in the ROESY spectrum implied connectivity between Pro^1 and Tyr residues. Thus the structure of hymenamide D (**2**) was elucidated to be cyclo-(Ile–Pro^1–Tyr–Asp–Pro^2–Leu–Ala).

Hymenamide D (**2**) was considered to exist as almost a single conformer in DMSO-d_6. The cis amide bond at CO(Ile)/N(Pro^1) was indicated from the ROESY correlation of αH-Ile/αH-Pro^1. The temperature coefficients {Δδ/δT: -5.0 (Ile), +0.1 (Asp), -2.8 (Leu), and -1.0 (Ala)} of the α-amide proton chemical shifts of **2** suggested that the amide protons of Asp and Ala participated in intramolecular hydrogen bonds, and that the amide proton of Ile was oriented outside the peptide ring. ROESY correlations of αH-Pro^2/NH-Leu, αH-Leu/NH-Ala, and NH-Leu/NH-Ala were indicative of the presence of type II β-turn at CO(Pro^2)/NH(Leu). Although the temperature coefficient (Δδ/δT: -2.8) of the α-amide proton chemical shift of Tyr in **2** was slightly larger than those of Asp and Ala, ROESY correlations observed for αH-Ile/NH-Tyr, αH-Ile/NH-Asp, and NH-Tyr/NH-Asp suggested that orientations of NH-Tyr and NH-Asp in **2** were similar to those of NH-Phe and NH-Gly in **1**.

Hymenamide E (**3**) showed the pseudomolecular ion at *m/z* 854 in the FABMS spectrum. The molecular formula, $C_{45}H_{55}N_7O_{10}$, of **3** was determined by the HRFABMS (*m/z* 854.4092, M^++H, Δ +0.3 mmu). Standard amino acid analysis of the acid hydrolysate of **3** revealed two mol each of Pro, Phe, and threonine (Thr), and one mol of Tyr residues. Each amino acid residue was assigned as L-configuration by Marfey's procedure. The ^1H NMR spectrum of **3** showed five amide NH, seven α-methine, and two doublet methyl protons (Table 3). Extensive analysis of the NMR data of **3** including ^1H-^1H COSY and HSQC spectra allowed assignments of the proton chemical shifts of all amino acid residues. The connectivities of amino acid residues of **3** were deduced from the following ROESY correlations; αH-Phe^1/αH-Pro^1, αH-Phe^1/NH-Thr^1, NH-Thr^1/NH-Thr^2, αH-Thr^2/δH-Pro^2, αH-Pro^2/NH-Tyr, NH-Tyr/NH-

Phe^2, and αH-Phe^2/NH-Phe^1. Thus the structure of hymenamide E (**3**) was determined to be cyclo-(Phe^1–Pro^1–Thr^1–Thr^2–Pro^2–Tyr–Phe^2).

Table 3. ^{1}H and ^{13}C NMR Data of Hymenamide E (**3**) in DMSO-d_6.

position	^{1}H[a]		J(Hz)	^{13}C[a]		position	^{1}H[a]		J(Hz)	^{13}C[a]	
Phe^1						Pro^2					
α	4.48	m		53.83[f]	d	α	4.11	t	8.0	61.63	d
β	2.99	dd	6.3, 14.2	35.83	t	β	2.08	m		28.76	t
	2.88	dd	8.3, 14.2				1.16	m			
1				138.13[g]	s	γ	1.79[b]	m		25.02	t
2, 6	7.22[b]	d	6.8	128.81[c]	d	δ	3.82	m		47.48	t
3, 5	7.28[b,d]	t	6.8	128.54[c,h]	s		3.59	dd	7.3, 16.1		
4	7.22[e]	t	6.8	126.61[i]	s	CO				170.01[j]	s
NH	8.82	d	3.9								
CO				170.58[j]	s	Tyr					
						α	4.46	m		52.91	d
Pro^1						β	3.01	dd	7.8, 13.2	35.54	t
α	3.75	d	7.8	60.42	d		2.56	dd	4.9 13.2		
β	1.85	m		30.42	t	1				128.15	s
	1.05	m				2, 6	7.02[b]	d	8.3	130.17[c]	d
γ	1.64	m		21.07	t	3, 5	6.64[b]	d	8.3	114.82[c]	d
	1.46	m				4				155.48	s
δ	3.40	m		45.65	t	4OH	9.02	b.s			
	3.35	m				NH	7 34	d	7.8		
CO				170.58[j]	s	CO				169.70[j]	s
Thr^1						Phe^2					
α	3.89	dd	6.1, 7.8	62.34	d	α	4.33	ddd	3.4, 3.9, 12.2	53.65[f]	d
β	4.07	q	6.1	66.07	d	β	3.06	dd	3.4, 13.7	37.05	t
CH_3	1.13	d	6.1	21.07	q		2.70	dd	12.2, 13.7		
βOH	4.33	b.s				1				136.39[g]	s
NH	7.82	d	7.8			2, 6	7.14[b]	d	6.8	128.81[d]	d
CO				170.42[j]	s	3, 5	7.29[b,d]	t	6.8	128.05[d,h]	d
						4	7.20[e]	t	6.8	126.22[i]	t
Thr^2						NH	7.23	d	9 4		
α	4.91	dd	3.4, 7.8	55.41	d	CO				169.70[j]	s
β	4.54	m		66.89	d						
CH_3	1.21	d	6.4	19.70	q						
βOH	5.46	b.s									
NH	7 99	d	7.8								
CO				170 28[j]	s						

a) δ in ppm b) 2H. c) 2C. d) ~ j) These proton and carbon signals were interchangeable.

Solution conformation of hymenamide E (**3**) in DMSO-d_6 was provided by detailed analysis of the ROESY spectrum and temperature coefficients of the α-amide proton chemical shifts. The ROESY correlations observed for αH-Phe^1/αH-Pro^1, αH-Pro^2/NH-Tyr, and NH-Tyr/NH-Phe^2 verified the presence of two types of β-turns at CO(Phe^1)/N(Pro^1) and CO(Pro^2)/NH(Tyr), which attributed to type VI(a) and type II,

respectively. ROESY cross-peaks for αH-Phe[1]/NH-Thr[1], αH-Phe[1]/NH-Thr[2], and NH-Thr[1]/NH-Thr[2] suggested that the amide protons of Thr[1] and Thr[2] were oriented inside the peptide ring. The temperature coefficients {Δδ/δT: -5.3 (Phe[1]), -1.5 (Thr[1]), -0.5 (Thr[2]), -2.6 (Tyr), and -1.8 (Phe[2])} of the amide proton chemical shifts in **3** indicated that the amide protons of Thr[1], Thr[2], and Phe[2] participated in intramolecular hydrogen bonds, and that the amide proton of Phe[1] was oriented outside the peptide ring. Model consideration of **3** also supported to contain three intramolecular hydrogen bonds at CO(Phe[1])/NH(Thr[1]), CO(Phe[1])/NH(Thr[2]), and CO(Thr[2])/NH(Phe[2]). Thus the solution conformation of **3** was elucidated to consist of two types of β-turns, type VI(a) at Phe[1]–Pro[1] and type II at Pro[2]–Tyr, and three intramolecular hydrogen bonds at CO(Phe[1])/NH(Thr[1]), CO(Phe[1])/NH(Thr[2]), and CO(Thr[2])/NH(Phe[2]).

Hymenamides C ~ E (**1** ~ **3**) are new cyclic heptapeptides containing one or two hydrophilic amino acid and two Pro residues, one of which participates in a cis X-Pro amide bond. Solution conformations of **1** ~ **3** are characterized to have two β-turns and three intramolecular hydrogen bonds in each backbone, although differences in bulkiness of side-chains slightly affects on each backbone conformation. It is noted that solution conformations of **1** ~ **3** with a Pro–X–X–Pro segment are close to that of hymenamide B {cyclo-(Pro[1]–Pro[2]–Asn–Phe[1]–Val–Glu–Phe[2])}, a cyclic heptapeptide with a Pro–Pro segment[5]. Solid and solution conformations of evolidine {cyclo-(Leu[1]–Pro–Val–Asn–Leu[2]–Ser–Phe)}[19], a known cyclic heptapeptide with one Pro residue, are reported to contain three intramolecular hydrogen bonds and two β-turns {type I and VI(a)} in the backbone structure[20], while crystaline structure of phakellistatin 1 {cyclo-(Leu[1]–Pro[1]–Leu[2]–Phe–Pro[2]–Tyr–Pro[3])}[7], a cyclic heptapeptide containing three Pro residues, has two cis amide bonds and hymenamide A, {cyclo-(Pro[1]–Pro[2]–Val–Pro[3]–Phe–Trp–Arg), a cyclic heptapeptide containing three Pro residues possesses one cis amide bond at the prolylproline sequence[5]. These observations suggest that both position and number of Pro residue play important roles to generate solution conformations of such cyclic peptides.

Hymenamides C (**1**) and E (**3**) showed antifungal activity against *Cryptococcus neoformans* (MIC 133 μg/mL each), while axinastatin 1 {cyclo-(Val[1]–Pro[1]–Val[2]–Asn–Pro[2]–Phe–Val[3])}[6] exhibited antibacterial activity against *Sarcina lutea* (MIC 66 μg/mL). Hymenamides C ~ E (**1** ~ **3**), axinastatin 1, and phakellistatin 1 were not cytotoxic against both murine lymphoma L1210 cells and human epidermoid carcinoma KB cells in vitro (IC_{50} >10 μg/mL).

EXPERIMENTAL

General Methods. The optical rotation was observed using a JASCO DIP-370 polarimeter. UV and IR spectra were taken on a JASCO Ubest-35 and a JASCO IR Report-100 spectrometer, respectively. ^1H and ^{13}C NMR spectra were conducted with a JEOL EX-400 spectrometer in DMSO-d_6 at 24.5, 30.0, 40.0, 50.0, and 60.0 °C. The

resonaces of residual DMSO at δ_H 3.30 and δ_C 49.0 were using as internal references for ^1H and ^{13}C NMR spectra, respectively. Standard amino acid analysis was performed with a Hitachi amino acid autoanalyzer (Model 835) using a column (4.0 x 250 mm, #2617) at a flow rate of 0.275 mL/min with 0.2N Na buffer and detected at 570 and 440 nm for Pro. FAB mass spectra were recorded employing a JEOL HX-110 spectrometer.

Collection, Extraction, and Separation. The sponge *Hymeniacidon* sp. was collected off Manza, Okinawa Island and kept frozen until used. The sponge (1.5 kg, wet weight) was extracted with methanol (3 L x 2). Evaporation of the extract afforded a residue (69.5 g), which was dissolved in a mixture of ethyl acetate (1 L) and water (1 L). The aqueous layer was extracted with ethyl acetate (900 mL x 3). The ethyl acetate soluble fraction (6.20 g) was subjected to a silica gel column (Wako gel C-300, Wako Pure Chemical, 4.8 x 45 cm) with chloroform/*n*-butanol/acetic acid/water (1.5:6:1:1) to give two fractions containing peptides. The fraction (1860 mg) eluting with 440 ~ 800 mL was chromatographed on a Sephadex LH-20 column (Pharmacia Fine Chemical, 2 x 100 cm) with MeOH and then $CHCl_3$/MeOH (1:1) followed by a silica gel column (Wako gel C-300, 0.5 x 13 cm) with $CHCl_3$/MeOH (90:10) to a crude peptide fraction (80 mg). The crude fraction was passed through a Sep-Pak C_{18} cartridge (Waters, CH_3CN/H_2O/TFA, 40:60:0.1) and then C_{18} HPLC (Asahipak ODP-50, Asahi Chemical Industry, 10 x 250 mm; eluent, CH_3CN/H_2O/TFA, 45:55:0.1; flow rate, 2.0 mL/min; UV detection at 254 nm) to afford hymenamides C (**1**, 47 mg, 0.003 % wet weight, t_R 12.5 min) and D (**2**, 1.7 mg, 0.0001 %, t_R 7.8 min) as colorless amorphous solids. The fraction (710 mg) eluting with 380 ~ 420 mL in the first silica gel column was chromatographed on a silica gel column (Wako gel C-300, 1 x 40 cm) with $CHCl_3$/MeOH (85:15) followed by a Sephadex LH-20 column (2 x 100 cm) with $CHCl_3$/MeOH, (1:1). Further purification on a Sep-Pak C_{18} cartridge (CH_3CN/H_2O/TFA, 55:45:0.1) followed by C_{18} HPLC (Asahipak ODP-50, 10 x 250 mm; eluent, CH_3CN/H_2O/TFA, 40:60:0.1; flow rate, 2.0 mL/min; UV detection at 254 nm) afforded hymenamide E (**3**, 9.2 mg, 0.0006 %, t_R 10.8 min) as a colorless amorphous solid.

Hymenamide C (1). A colorless amorphous solid; $[\alpha]_D^{17}$ -138° (*c* 0.41, MeOH); IR (KBr) ν_{max} 3400, 2950, 1630, 1520, 1440, and 1010 cm^{-1}; UV (MeOH) λ_{max} 291 (ε 2700), 282 (3200) and 218 (sh.) nm; ^1H and ^{13}C NMR (see Table 1); FABMS (diethanolamine matrix) *m/z* 827 $(M+H)^+$, 849 $(M+Na)^+$, and 932 $(M+DEA+H)^+$; FABMS {dithiothreitol/dithioerythritol (3:1) matrix} *m/z* 827 $(M+H)^+$, 849 $(M+Na)^+$, and 865 $(M+K)^+$; HRFABMS (diethanolamine matrix) *m/z* 827.4109 [$(M+H)^+$, calcd for $C_{43}H_{55}N_8O_9$, 827.4092].

Hymenamide D (2). A colorless amorphous solid; $[\alpha]_D^{17}$ -70.8° (*c* 0.15, MeOH); IR (KBr) ν_{max} 3400, 2950, 1680, 1630, 1510, 1440, 1200, 1180, 1130, and 1010 cm^{-1}; UV (MeOH) λ_{max} 279 (ε 1400) and 224 (sh.) nm; ^1H and ^{13}C NMR (see Table 2); FABMS (diethanolamine matrix) *m/z* 770 $(M+H)^+$, 792 $(M+Na)^+$, and 875

$(M+DEA+H)^+$; HRFABMS (diethanolamine matrix) *m/z* 770.4047 [$(M+H)^+$, calcd for $C_{38}H_{56}N_7O_{10}$, 770.4089].

Hymenamide E (3). A colorless amorphous solid; $[\alpha]_D^{20}$ -108° (*c* 0.94, MeOH); IR (KBr) ν_{max} 3400, 2950, 1680, 1630, 1510, 1440, 1200, 1180, 1130, and 1010 cm^{-1}; UV (MeOH) λ_{max} 279 (ε 1700) and 226 (sh.) nm; ^1H and ^{13}C NMR (see Table 3); FABMS (glycerol matrix) *m/z* 854 (M^++H) and 876 (M^++Na); HRFABMS (glycerol matrix) *m/z* 854.4092 [(M^++H), calcd for $C_{45}H_{56}N_7O_{10}$, 954.4089].

Amino Acid Analysis of Acid Hydrolysate of 1. Hymenamide C (**1**, 0.1 mg) in 4N methanesulfonic acid (100 μL) was hydrolyzed in a sealed tube at 115 °C for 24 h, and the solution was prepared for pH 2 with 1N NaOH aq. and subjected to automatic amino acid analyzer. Two mol of Pro and 1mol each of Glu, Gly, Leu, Phe, and Trp were found in the hydrolysate of **1**.

Amino Acid Analyses of Acid Hydrolysates of 2 and 3. Hymenamide D or E (**2** or **3**, each 0.1 mg) was dissolved in 6N HCl (100 μL) and heated in a sealed tube at 110 °C for 24 h. Each acidic solution was prepared for pH 2 with 1N NaOH aq. and subjected to automatic amino acid analyzer. Two mol of Pro and 1 mol each of Asp, Ala, Ile, Leu, and Tyr were found for the hydrolysate of **2**. Two mol each of Thr, Phe, and Pro and 1 mol of Tyr were detected for the hydrolysate of **3**.

1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) Derivatization of Hydroysates of 1 ~ 3. Hymenamide C (**1**, 100 μg) was treated with 4N methanesulfonic acid (100 μL) at 115 °C for 24 h, and the solution was subjected on Amberlite CG-120/Celite (1:9) with 1N HCl and then NH_3 aq. The fraction eluting with NH_3 aq. was evaporated under reduced pressure. The excess HCl was removed by N_2 gas, and an aqueous solution (20 μL) of the hydrolysate was reacted with 1% FDAA/acetone (5 μL) and 1 M $NaHCO_3$ (10 μL) at 40 °C for 1 h. After cooling to room temperature, the reaction mixture was neutralized with 2 M HCl (5 μL). The solvent was evaporated, and the residue was dissolved in DMSO (50 μL). Hymenamide D or E (**2** or **3**, each 100 μg) was treated with 6N HCl (100 μL) at 110 °C for 24 h. The excess HCl was removed by N_2 gas, and an aqueous solution (20 μL) of the hydrolysate was applied to the reaction with FDAA as described above.

C_{18} HPLC Analyses of the FDAA Derivatives of Amino Acids. The FDAA derivatives of standard amino acids were prepared by the same procedure described above. The FDAA derivatives of hydrolysates of **1** ~ **3** and standard amino acids were subjected to C_{18} HPLC analyses using an Inatosil ODS-2 column (GL Sciences Inc., 5μm, 4.6 x 150 mm) and the following gradient program; solvent A, acetonitrile; solvent B, 50 mM triethylamine phosphate buffer (pH 3.0); time (min)/A(%)/B(%), 0/10/90, 60/60/40; flow rate, 1 mL/min; detection at 340 nm; column temperature, 40 °C. The retention times (min) of FDAA derivatives of authentic L and D amino acids were as follows; L-Thr (15.2), D-Thr (20.4), L-Asp (15.4), D-Asp (17.8), Gly (18.3), L-Glu (19.8), D-Glu (21.6), L-Ala (19.1), D-Ala (23.6), L-Pro (20.9), D-Pro

(23.7), L-Tyr (21.5 and 39.0), D-Tyr (25.8 and 44.1), L-Trp (29.4), D-Trp (33.0), L-Ile (29.8), D-Ile (35.7), L-Phe (30.2), D-Phe (35.2), L-Leu (31.3), and D-Leu (37.1). The retention times (min) of FDAA derivatives of the hydrolysates of **1** ~ **3** were as follows; Gly (18.3), L-Glu (19.8), L-Pro (20.9), L-Trp (29.4), L-Phe (30.2), and L-Leu (31.3) in **1**; L-Asp (15.4), L-Ala (19.1), L-Pro (20.9), L-Tyr (21.5 and 39.0), L-Ile (29.8), and L-Leu (31.3) in **2**; L-Thr (15.2), L-Pro (20.9), L-Tyr (21.5 and 39.0), and L-Phe (30.2) in **3**.

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