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Hymenamide F, New Cyclic Heptapeptide from Marine Sponge *Hymeniacidon* sp.

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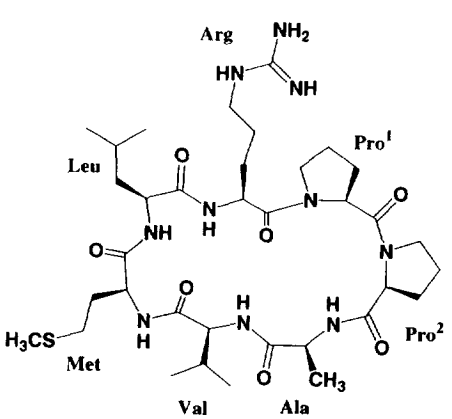
Abstract: A new cyclic heptapeptide, hymenamide F (**1**), was isolated from an Okinawan marine sponge *Hymeniacidon* sp. and the structure was elucidated on the basis of spectroscopic data of [Mso]-hymenamide F (**2**). The absolute configuration of each amino acid residue was determined by Marfey's method. The conformation in the solution of **2** is discussed on the basis of NMR data as well as molecular calculation.

Marine sponges belonging to the genus *Hymeniacidon* have been shown to be a rich source of bromopyrrole alkaloids[1] and cyclic peptides.[2] In our search for bioactive substances from marine organisms,[3] we previously isolatied tetrahydropyrimidine alkaloids manzacidins A ~ C[1], proline-rich cyclic heptapeptides hymenamides A ~ E,[4,5] and cyclic octapeptides hymenamides G, H, J, and K[6] from an Okinawan marine sponge *Hymeniacidon* sp. Further investigation on constituents of this sponge resulted in isolation of a new cyclic heptapeptide, hymenamide F (**1**), with a prolylproline and an arginine residues. Here we describe the isolation and structure elucidation of **1**.

The sponge *Hymeniacidon* sp. collected off Manza, Okinawan Island, was extracted with MeOH, and the MeOH extract was partitioned between EtOAc and H_2O. The *n*-BuOH soluble material of the aqueous layer was subjected to a silica gel column eluted with $CHCl_3$/*n*-BuOH/AcOH/H_2O (1.5:6:1:1) and then *n*-BuOH/AcOH/H_2O (2:1:1). The residue eluted with *n*-BuOH/AcOH/H_2O was



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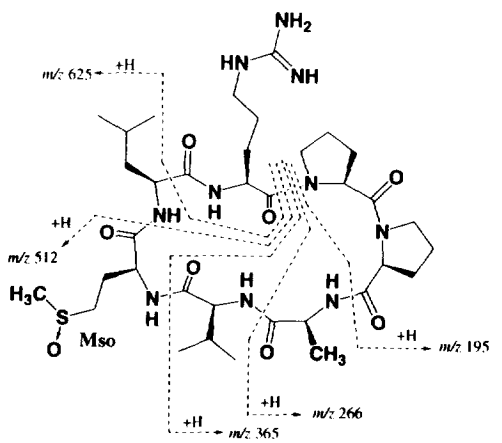

Fig. 1. FABMS/MS Fragmentations (Parent Ion: *m/z* 781) of [Mso]-Hymenamide F (**2**)

purified on a Sephadex LH-20 column (MeOH) followed by C_{18} HPLC ($CH_3CN/H_2O/CF_3CO_2H$, 20:80:0.1) to yield the *S*-oxide form (Mso) of hymenamide F (**2**, 0.003%, wet weight), in which methionine (Met) residue was oxidized, together with a trace[7] of hymenamide F (**1**). Although compound **2** ([Mso]-hymenamide F) seemed to have been generated from hymenamide F (**1**) through autooxidation of the Met residue during purification, the structure elucidation of **1** was carried out mainly using **2**.

[Mso]-Hymenamide F {**2**, $[\alpha]_D^{20}$ -25° (*c* 0.22, MeOH)} showed the pseudomolecular ion peak at *m/z* 781 $(M+H)^+$ in the FABMS spectrum. The molecular formula, $C_{35}H_{60}N_{10}O_8S$, of **2** was established by HRFABMS [*m/z* 781.4417 $(M+H)^+$, $C_{35}H_{61}N_{10}O_8S$, Δ -2.2 mmu]. IR absorptions (3300 and 1660 cm^{-1}) in addition to ^1H and ^{13}C NMR (Table 1) spectra of **2** were suggestive of a peptide. Amino acid analysis of the acid hydrolysate of **2** revealed the presence of 2 mol of proline (Pro) and 1 mol each of arginine (Arg), valine (Val), leucine (Leu), alanine (Ala), and methionine sulfoxide (Mso) residues. The presence of the sulfoxide group was also supported from IR absorption at 1030 cm^{-1}. The ^1H and ^{13}C chemical shifts of each amino acid residue of **2** (Table 1) were assigned from detailed analyses of the 2D NMR data (^1H-^1H COSY, HSQC, and HMBC spectra in CD_3OH). The chemical shift of γC-Pro^2 (δ_C 22.9) was located at higher field than that of γC-Pro^1 (δ_C 25.8), indicating that the Pro^1–Pro^2 amide bond had *cis*-geometry.[6] Proton signals due to the γ-methylene and the *S*-methyl groups in Mso residue were observed as a pair of signals {γ-H_2: δ 2.88 (1H, m), and 2.97 (0.5H, m) and 2.74 (0.5H, m), and *S*-CH_3: δ 2.64 (1.5H, s) and 2.62 (1.5H, 3)} in a ratio of 1:1, implying that the sulfoxide group in the Mso residue was racemic.[8]

The amino acid sequence of [Mso]-hymenamide F (**2**) was elucidated by analyses of the NOESY and HMBC data in CD_3OH as well as the FAB MS/MS spectrum. The two segments of Pro^2-Ala-Val and Mso-Leu-Arg were implied by two-bond ^1H-^{13}C correlations of NH(Ala)/CO(Pro^2), NH(Val)/CO(Ala), NH(Leu)/CO(Mso) and NH(Arg)/CO(Leu) in the HMBC spectrum. The NOESY spectrum indicated the presence of a segment of Pro^2-Ala-Val-Mso-Leu-Arg-Pro^1 by the following NOE's: βH(Pro)/NH(Ala), NH(Ala)/NH(Val), βH(Val)/αH(Mso), αH(Mso)/NH(Leu), NH(Leu)/NH(Arg), αH(Arg)/δH_2(Pro^1), and γH_2(Arg)/δH_2(Pro^1). The connection between Pro^1 and Pro^2 to form a cyclic peptide was deduced from the presence of daughter ions at *m/z* 195 (Pro–Pro)$^+$ and 167 (Pro–Pro–CO)$^+$ in FAB MS/MS spectrum of the pseudomolecular ion (*m/z* 781) of **2**. The FAB MS/MS spectrum showed other fragment ions supporting the proposed structure as follows: *m/z* 625 (Pro–Pro–Ala–Val–Mso–Leu)$^+$, 512 (Pro–Pro–Ala–Val–Mso)$^+$, 365 (Pro–Pro–Ala–Val)$^+$, and 266 (Pro–Pro–Ala)$^+$. Thus the structure of [Mso]-hymenamide F (**2**) was concluded to be cyclo-(Pro^1–Pro^2–Ala–Val–Mso–Leu–Arg).

The absolute stereochemistry of each amino acid residue in **2** was determined to be all L-configuration by gradient C_{18} HPLC analyses (Inatosil ODS-2 column; CH_3CN/50 mM triethylamine phosphate buffer, 10:90 to 40:60) of 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) derivative of the acid hydrolysate.[8]

The solution conformation of [Mso]-hymenamide F (**2**) in MeOH was elucidated from NOE's (Table 1) and temperature coefficients (Δδ/ΔT values: Ala, -2.3; Val, +1.1; Mso, -4.5; Leu, -3.9; Arg, +1.1) of chemical shifts of the α-amide protons to have two β-turns {type I at Mso–Leu and type VI(a) at Pro^1–Pro^2} and three transannular hydrogen bonds at NH(Ala)/CO(Arg), NH(Val)/CO(Arg), and NH(Arg)/CO(Val) incorporating a classical β-bulge motif.[9] The solution conformation corresponded well to the most stable conformation of **2** (Chart I) evaluated by combination of systematic

Table 1. ^{1}H and ^{13}C NMR Data of [Mso]-Hymenamide F (**2**) in CD_3OH.

positn.		δ_C	δ_H [J (Hz)]		HMBC (H)	NOESY (H)
Pro^1	α	61.0 (d)	4.46 (m)			NH–Val
	β	29.5 (t)	2.32 (m)	1.86 (m)		γH–Pro^1
	γ	25.8 (t)	2.03 (m)[b]			δH–Pro^1
	δ	49.8 (t)[c]	3.59 (m)	3.56 (m)		αH–Arg, βH–Arg
	CO	172.5 (s)			βH–Pro^1	
Pro^2	α	62.2 (d)	4.43 (m)			
	β	32.8 (t)	2.26 (m)	1.80 (m)	αH–Pro^2	NH–Ala, γH–Pro^2
	γ	22.9 (t)	1.98 (m)	1.70 (m)	αH–Pro^2	NH–Ala
	δ	49.6 (t)[c]	3.64 (m)	3.54 (m)		NH–Ala
	CO	173.4 (s)				αH–Pro^2, βH$_2$–Pro^2, NH–Ala
Ala	α	54.0 (d)	4.21 (m)		CH_3–Ala	NH–Ala, CH_3–Ala, NH–Val
	CH_3	18.4 (q)	1.55 (d, 7.3)		NH–Ala	NH–Ala, NH–Val
	CO	173.4 (s)			CH_3–Ala, NH–Val	
	NH		7.99 (d, 6.4)			NH–Val
Val	α	58.1 (d)	4.52 (d, 3.4, 5.9)		CH_3–Val	
	β	34.4 (d)	2.33 (m)		CH_3–Val	NH-Mso
	CH_3	20.9 (q)	1.00 (d, 6.8)		CH_3–Val	NH–Val
	CH_3	18.2 (q)	0.89 (d, 6.8)		CH_3–Val	NH–Val
	CO	175.2 (s)			αH–Val	
	NH		7.30 (d, 9.0)			
Mso	α	57.8 (d)	3.99 (br.t, 6.8)		βH$_2$–Mso	βH$_2$–Mso, γH–Mso, NH–Leu
	β	25.1 (t)	2.19 (m)[b]		αH–Mso	γH$_2$–Mso, NH-Leu
	γ[d]	50.8 (t)	2.88 (m)[b]		βH$_2$–Mso, CH_3–Mso	
		51.2 (t)	2.95 (m)	2.74 (m)		
	CH_3[d]	38.1 (q)	2.64 (s)			
		38.1 (q)	2.62 (s)			
	CO	174.4 (s)				αH–Mso, βH–Mso, NH–Leu
	NH		8.53 (3.5)			
Leu	α	54.9 (d)	4.19 (m)			βH$_2$–Leu, γH–Leu, NH–Leu
	β	40.6 (t)	1.76 (m)	1.70 (m)	CH_3–Leu	NH–Leu
	γ	26.1 (d)	1.72 (m)		CH_3–Leu	NH–Leu
	CH_3	23.3 (q)	0.95 (d, 5.4)		CH_3–Leu	
	CH_3	21.3 (q)	0.93 (d, 5.4)		CH_3–Leu	
	CO	174.4 (s)			NH–Arg	
	NH		7.79 (d, 10.6)			NH–Arg
Arg	α	52.3 (d)	4.57 (dt, 5.4, 7.3)			
	β	29.4 (t)	1.87 (m)	1.68 (m)		NH–Arg
	γ	26.1 (t)	1.66 (m)[b]			
	δ	42.2 (t)	3.18 (m)[b]		γH$_2$–Arg	
	δNH		6.88 (br.s)			
	guanidine	158.8 (s)	6.90 (br.)			
	CO	173.8 (s)				
	NH		7.48 (d, 7.3)			

a) δ in ppm. b) 2H. c) The carbon signals were overlapped with CD_3OH signal and assigned by HSQC data.
d) These protons were observed as a pair of signals in the ^{1}H NMR spectrum.

Table 2. Backbone Dihedral Angles (ϕ) of [Mso]-Hymenamide F (**2**) Calculated from NH-CαH Coupling Constants (Hz) and the Most Stable Conformation of **2** Obtained by MD Calculation.

residues	$^3J_{NH\text{-}C\alpha H}$ (Hz)	dihedral angles (ϕ)[a]				dihedral angles ϕ[b]
Ala	6.4	-78	29	**-162**[c]	91	-161
Val	9.2	**-97**[c]	-144			-95
Mso	3.5	-61	9	**-179**[c]	111	-172
Leu	10.6	**-110**[c]	-129			-118
Arg	7.3	**-83**[c]	36	-156	83	-71

[a] Calculated by using Karplus-Bystrov equation: $^3J_{NH\text{-}C\alpha H} = 9.4\cos^2|60\text{-}\phi| - 1.1\cos|60\text{-}\phi| + 0.4$
[b] Dihedral angles of the the most stable conformation obtained by MD calculation
[c] Dihedral angles closest to ϕ[b]

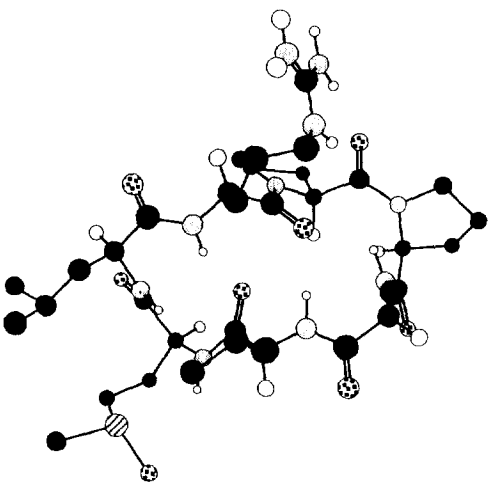

Chart I. Three-dimensional Structure of the Lowest Energy Conformer of [Mso]-Hymenamide F (**2**) Evaluated by the MD Calculations

conformational searching and molecular dynamics (MD) calculation using Macromodel Program (Ver. 4.5).[10] MD calculation was performed for 18 conformers generated by the Montecarlo method, and all sampling conformers (3600 conformers) were subsequently minimized. The global minimum structure (total energy, -160.8 kcal/mol) was shown in Chart I. This conformation satisfied proton-proton distances deduced from the NOESY data of **2**. The dihedral angles (ϕ) of NH-CαH in the lowest energy conformation were very close to the torsion angles calculated by using Karplus-Brystov equation[11] from the NH-CαH coupling constants of **2** (Table 2). In this most stable conformation three intramolecular hydrogen bonds were found for NH(Ala)/CO(Arg), NH(Val)/CO(Arg), and NH(Arg)/CO(Val), corresponding well to those indicated by temperature coefficients of chemical shifts of the α-amide protons.

Hymenamide F (**1**) is a new cyclic heptapeptide containing an arginine and a prolylproline residues. Conformation in the solution of the backbone of [Mso]-hymenamide F (**2**) containing two β-turns, one of which consists of *cis* amide bond, and three intramolecular hydrogen bonds, was closely

similar to crystal structure of a known cyclic heptapeptide with one Pro residue, evolidine[12] [cyclo-(Pro–Val–Asn–Leu–Ser–Phe–Leu)] possessing two β-turns (types I and VI). On the other hand, the types of β-turns of **2** differed from those of other cyclic heptapeptides such as hymenamides B ~ E (types II and VI) and pseudostellarin D [cyclo-(Gly–Tyr–Gly–Pro–Leu–Ile–Leu)] (types I and II).[13]

Experimental

Isolation. 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). The methanolic extract (69.5 g) was patitioned between water and ethyl acetate (900 mL x 3), and the aqueous layer was subsequently extracted with *n*-butanol (900 mL x 3). The *n*-butanol soluble material (15.1 g) was subjected to a silica gel column with $CHCl_3$/*n*-BuOH/AcOH/H_2O (1.5:6:1:1) and then *n*-BuOH/AcOH/H_2O (2:1:1). The residue eluted with *n*-BuOH/AcOH/H_2O was purified by a Sephadex LH-20 column with MeOH followed by C_{18} HPLC [YMC-Pack AM-323, YMC, 10 x 250 mm; eluent, $CH_3CN/H_2O/CF_3CO_2H$ (20:80:0.1); UV detection at 220 nm] to yield [Mso]-hymenamide F (**2**, 5.0 mg, 0.0003% wet weight, t_R 9.6 min) together with hymenamide F (**1**, 0.2 mg, 0.000012 %, t_R 13.2 min).

[Mso]-Hymenamide F (2). A colorless amorphous solid; $[\alpha]_D^{20}$ -25° (*c* 0.22, MeOH); IR (KBr) ν_{max} 3430, 2920, 2840, 1660, 1525, 1450, 1380, 1200, 1135, and 1020 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); FABMS (Pos. glycerol matrix) *m/z* 803 $(M+Na)^+$, 781 $(M+H)^+$, and 765 $(M-O)^+$; HRFABMS *m/z* 781.4417 $(M+H)^+$, calcd for $C_{35}H_{61}N_{10}O_8S$, 781.4439.

Amino Acid Analysis of Hydrolysate of 2. Compound **2** (0.1 mg) was dissolved in 6N HCl (100 μL) and heated at 110 °C for 24 h. Standard amino acid analysis was performed with Hitachi amino acid autoanalyzer (Model 835) by using a column (4.0 x 250 mm, #2617) at flow rate of 0.275 mL/min with 0.2N Na buffer and detected at 570 and 440 nm for Pro. Two mol of Pro and 1 mol each of Ala, Val, Leu, Arg, and Mso were found in the hydrolysate.

1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) Derivatization and Absolute Stereochemistry. Compound **2** (50 μg) was treated with 6N HCl 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 reacted with 1% FDAA/acetone (3 μL) and 0.1 M $NaHCO_3$ (7 μL) at 40 °C for 1 h. After cooling to room temperature, the reaction mixture was neutralized with 0.2 M HCl (3.2 μL). The standard amino acids were derivatized by the same procedure as described above. The derivatized hydrolysate and standard amino acids were subjected to C18 HPLC analyses [Inatosil ODS-2 column, GL Sciences Inc., 5μm, 4.6 x 150 mm, CH_3CN/50 mM triethylamine phosphate buffer (pH 3.01), 10:90 to 40:60; flow rate, 1 mL/min; detection at 340 nm; column temperature, 35 °C]. The retention time (min) of each derivatized authentic L and D amino acid was appeared as follows; L-Arg (18.6), D-Arg (19.9), L-Mso (22.2 and 22.6), D-Mso (23.0 and 23.9), L-Ala (27.6), L-Pro (29.7), D-Pro (32.7), D-Ala (33.0), L-Val (37.4), L-Leu (44.5), D-Val (45.0), and D-Leu (51.9). The retention times (min) of derivatized amino acids in the hydrolysate of **2** were found to be L-Arg (18.6), L-Mso (22.2 and 22.6), L-Ala (27.6), L-Val (37.4), and L-Leu (44.5).

MD Calculation of [Mso]-Hymenamide F (2). Computer modeling was carried out with Macromodel program (version 4.5) using a Silicon Graphics Personal IRIS workstation (Indigo^{2TM}). 10000 randamly-generating structures were evaluated for **2** using Montecarlo method, yielding 245 unique conformers in the 0–3 kcal/mol energy region. 18 low-energy conformers remained by rejection of the rotamers around the bonds to the isopropyl group in the Leu residue, the guanidine group in the Arg residue, and the *S*-methyl group in the Mso residue. MD calculation was performed by using AMBER force field in Macromodel / Bachmin (Ver. 4.5) under the following conditions: solvent, water; intial temperature, 300 K; time step, 1.0 fs; duration for equilibrium, 100 ps. Each 200 sampling conformers generated by MD calculation were all minimized by the molecular mechanics calculation of AMBER force field to afford the lowest energy conformation (total energy, -180.8 kcal/mol).

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