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Cyclosquamosins A - G, Cyclic Peptides from the Seeds of *Annona squamosa*

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Abstract: Seven new cyclic peptides, cyclosquamosins A - G (**1** - **7**), have been isolated from the seeds of *Annona squamosa*. Their structures and conformation of cyclosquamosin A in solution were elucidated by extensive 2D NMR methods and chemical degradation.

Recently, higher plants have been demonstrated to be a rich source of cyclic peptides as well as microorganisms and marine organisms.[1, 2] In view of our extensive results to date with cyclic peptides from higher plants, it is tempting to consider that the distribution of cyclic peptides is unbalanced and different by species and site.[2] From Annonaceae plants of *A. muricata* [3] and *A. glabra* ,[4] which may be regarded as one of the rich sources of cyclic peptides in higher plants, cyclic peptides have been reported. As part of our continuing investigation, we have focused our attention to the isolation of new cyclic peptides from the Annonaceae plants. *Annona squamosa*, the custard apple, belonging to this plant family, is a fruit tree, and the seeds are famous to contain many acetogenins, waxy substances consisting of long chain fatty acids. They showed antimalarial, immunosuppressive, antifeedant, and particularly remarkable cytotoxic activity.[5] Chromatographic purification of the *n*-BuOH soluble fraction resulted in the isolation of seven new cyclic peptides, cyclosquamosins A (**1**), B (**2**), C (**3**), D (**4**), E (**5**), F (**6**), and G (**7**). Here, we report the structure elucidation of **1** - **7** by extensive 2D NMR methods and chemical degradation.

The methanolic extract of the seeds of *Annona squamosa* was partitioned between hexane, EtOAc, *n*-BuOH, and H_2O. The *n*-BuOH soluble material was subjected to a Diaion HP-20 column (H_2O - MeOH), and 80% MeOH eluted fraction was chromatographed on a silica gel column. The final purification by HPLC on ODS afforded seven cyclic peptides, cyclosquamosins A (**1**: 0.003 %), B (**2**: 0.002 %), C (**3**: 0.02 %), D (**4**: 0.08 %), E (**5**: 0.002 %), F (**6**: 0.001 %), and G (**7**: 0.002 %) together with a known cyclic peptide, annosquamosin A (**8**, 0.005%).[6]

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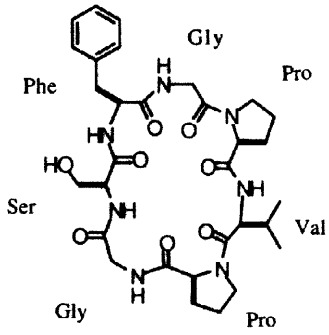

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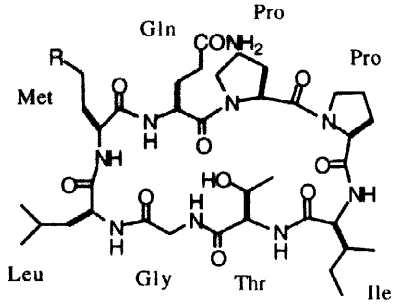

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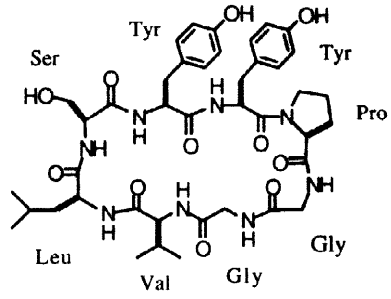

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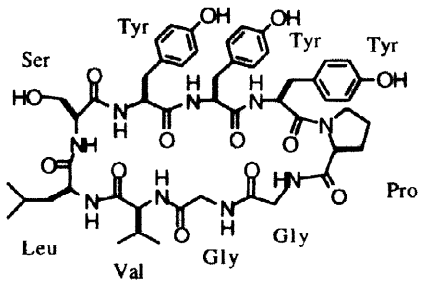

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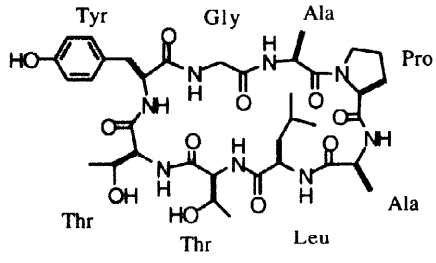

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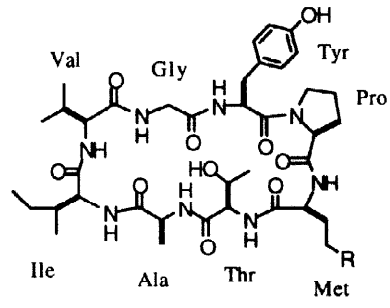

7: R= SMe
8: R= S(O)Me

Cyclosquamosin A (**1**) was isolated as colorless powder, $[\alpha]_D^{20}$ -74.7° (*c* 0.83, MeOH), showed a quasimolecular ion peak at *m/z* 642.3271 [$(M+H)^+$, Δ+2.0 mmu] in HRFABMS, corresponding to the molecular formula, $C_{31}H_{43}N_7O_8$. IR absorptions at 3421 and 1676 cm^{-1}, ascribable to amino and amide carbonyl groups, respectively, led to hydrolyze with 6N HCl at 110 °C for 24 h in a sealed tube. HPLC analysis of the hydrolysate showed that **1** consisted of two Gly, two Pro, one Ser, one Val, and one Phe per molecule, indicating a heptapeptide. The molecular formula of **1** corresponds well to the above amino acid composition, if **1** is a cyclic peptide. Detailed analysis of the COSY and HOHAHA spectra recorded in pyridine-*d*5 revealed the individual spin system of the above amino acids (Table 1). The gross structure including the sequence was elucidated on the basis of connectivity observed in the NOESY and HMBC experimental results (Fig. 1). Thus the structure of **1** was identified as *cyclo* (-Gly-Ser-Phe-Gly-Pro-Val-Pro-). All of amino acids in **1** were proved to be L-amino acids by chiral HPLC analysis after acid hydrolysis.

Cyclic peptides are molecules which exhibit a wide range of biological activity.[1] Conformational determination of such cyclic peptides is an important step, because their biological activities are known to be closely related to their conformational states. Recently, we have reported the conformations of a series of cyclic heptapeptides, such as hymenamide,[7] pseudostellarin D,[8] and yunnanin A,[9] and segetalins D and E.[10] To examine whether conformational characteristics observed in these cyclic heptapeptides are favorable and common features for cyclic heptapeptides consisting of all L-amino acids, conformational state in solution of **1** was elucidated.

The NOE relationships measured by its phase sensitive NOESY spectrum are shown in Fig. 1. The NOE correlations between Val^6-Hα and Pro^7-Hδ, and between Gly^4-Hα and Pro^5-Hδ indicated a *trans* geometry for the two proline amide bonds. The chemical shifts of Cβ and Cγ of Pro^5 and Pro^7 (Pro^5: Cβ δ29.2, Cγ δ24.5; Pro^7: Cβ δ29.4, Cγ δ25.6) also supported the *trans* geometry of the both Pro amide bonds.[11] The presence

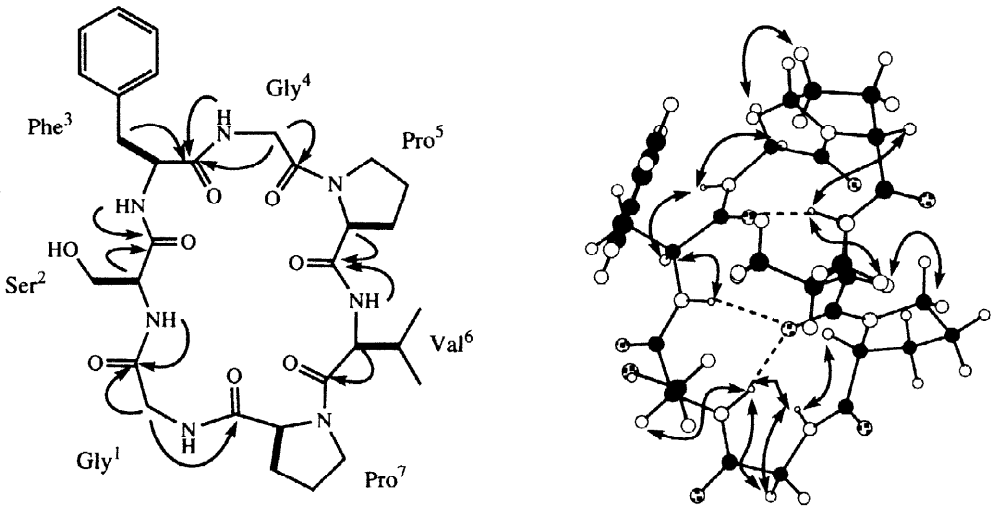

Fig. 1 Structure and proposed conformation of cyclosquamosin A (**1**) in solution. Arrows in the left structure show selected HMBC correlations. The right-hand conformation of **1** is deduced by the NOE correlations (arrows) and three intramolecular hydrogen bonds (broken lines).

Table 1. ^{1}H and ^{13}C NMR Data of Cyclosquamosin A (**1**) in Pyridine-d_5.

assignment		δ_H (int. mult, J(Hz))	δ_C		δ_H	δ_C
Gly[1]				Pro[5]		
	α	4.61 (1H, dd, 8.3, 17.1)	43.9	α	4.55 (1H, dd, 2.5, 8.3)	61.7
		3.86 (1H, dd, 4.4, 17.1)		β	1.90 (2H, m)	29.2
	NH	9.97 (1H, dd, 4.4, 8.3)				
	C=O		169.4	γ	1.78 (2H, m)	24.5
Ser[2]				δ	3.38 (1H, m)	47.5
	α	5.00 (1H, ddd, 4.4, 8.0, 9.0)	53.6		3.82 (1H, m)	
	β	4.23 (1H, dd, 4.4, 9.0)	62.6	C=O		171.5
		3.92 (1H, t, 9.0)				
	NH	8.08 (1H, d, 8.0)		Val[6]		
	C=O		173.0	α	4.50 (1H, t, 8.0)	58.0
Phe[3]				β	2.37 (1H, m)	30.9
	α	4.86 (1H, m)	56.6	γ	1.11 (3H, d, 6.8)	19.1
	β	3.33 (1H, dd, 5.9, 13.7)	38.1		1.05 (3H, d, 6.3)	19.6
		3.13 (1H, dd, 8.8, 13.7)		NH	7.51 (1H, d, 8.0)	
	γ		137.4	C=O		173.4
	δ	7.22 - 7.33 (5H, m)	129.0	Pro[7]		
	ε		129.5	α	4.78 (1H, t, 7.0)	61.3
	ζ		127.3	β	2.00 (2H, m)	29.4
	NH	9.19 (1H, d, 5.0)		γ	1.68 (1H, m)	25.6
	C=O		173.7		1.70 (1H, m)	
Gly[4]				δ	3.65 (1H, m)	48.6
	α	3.76 (1H, dd, 5.9, 16.1)			4.18 (1H, m)	
		4.42 (1H, dd, 5.0, 16.1)	42.5	C=O		173.3
	NH	10.00 (1H, dd, 5.0, 5.9)				
	C=O		170.9			

of type II β-turn was implied by the NOE correlations between Pro[7]-Hα and Gly[1]-NH. The NOE correlations between Gly[4]-NH and Gly[4]-Hα, and between Gly[4]-Hα and Pro[5]-Hδ were indicative of the presence of II' β-turn structure from Phe[3] to Val[6]. The other NOEs indicated by the arrows in Fig. 1 were also noted in the the conformation like yuunanin A, which takes type II β-turn between Pro and Gly, and type II' β-turn between Gly and Pro.[9] The presence of two β-turns, one β-bulge,[12] and three intramolecular hydrogen bonds characteristic of cyclic heptapeptides was also implied by temperature dependent effects on NH protons.[13] The

Ser[2], Phe[3], and Val[6] NH protons exhibited low temperature dependence (Gly[1]-NH 5.43×10^{-3}, Ser[2]-NH 2.29×10^{-3}, Phe[3]-NH 3.43×10^{-3}, Gly[4]-NH 4.00×10^{-3}, and Val[6]-NH 3.14×10^{-3} ppm/K), suggesting that they were involved in the formation of intramolecular hydrogen bonding.

Table 2. ^{1}H and ^{13}C NMR Data of Cyclosquamosin B (**2**) in Pyridine-d_5.

assignment	δ_H (int. mult, J(Hz))	δ_C		δ_H	δ_C
Gly[1]			Pro[5]		
α	4.81 (1H, dd, 7.8, 17.1)	43.5	α	4.39 (1H, m)	64.0
	3.86 (1H, dd, 4.9, 17.1)		β	2.15 (1H, m)	29.8
NH	8.60 (1H, dd, 4.9, 7.8)			2.01 (1H, m)	
C=O		169.2	γ	1.96 (2H, m)	25.3
Leu[2]			δ	4.23 (2H, m)	48.7
α	5.30 (1H, m)	51.9	C=O		172.3
β	2.52 (2H, m)	38.9	Pro[6]		
γ	1.85 (1H, m)	22.5	α	4.74 (1H, d, 7.3)	61.4
δ	0.78 (3H, d, 6.3)	21.4	β	2.10 (1H, m)	31.6
	0.71 (3H, d, 6.3)	23.1		2.47 (1H, m)	
NH	8.25 (1H, d, 8.8)		γ	1.77 (2H, m)	24.9
C=O		173.2	δ	3.80 (1H, m)	47.3
Met[3]				3.65 (1H, m)	
α	4.41 (1H, m)	52.2	C=O		171.1
β	2.03 (1H, m)	30.0	Ile[7]		
	2.18 (1H, m)		α	5.23 (1H, m)	56.1
γ	2.72 (2H, m)	30.1	β	2.51 (1H, m)	34.9
ε	1.98 (3H, s)	14.9	γ	1.65 (2H, m)	24.7
NH	9.85 (1H, d, 5.4)			1.25 (3H, d, 6.3)	16.9
C=O		174.6	δ	0.83 (3H, t, 7.3)	10.3
Gln[4]			NH	8.95 (1H, brs)	
α	4.54 (1H, m)	57.8	C=O		172.1
β	2.39 (2H, m)	26.1	Thr[8]		
γ	2.70 (1H, m)	32.7	α	5.01 (1H, dd, 2.9, 9.3)	60.3
	2.81 (1H, m)		β	4.96 (1H, m)	69.0
δ		177.6	γ	1.40 (3H, d, 7.8)	19.9
NH_2	8.86 and 8.41 (each 1H, s)		NH	8.58 (1H, d, 9.3)	
NH	10.50 (1H, d, 3.4)		C=O		172.9
C=O		172.0			

Cyclosquamosin B (**2**), colorless powder, $[\alpha]_D^{20}$ -53.8° (c 0.58, MeOH), showed a quasimolecular ion peak at *m/z* 838.4491 [$(M+H)^+$, Δ-0.7 mmu] in HRFABMS, corresponding to the molecular formula, $C_{38}H_{63}N_9O_{10}S$. The IR absorptions at 3424 and 1676 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The octapeptide nature was evident from its ^{13}C NMR spectrum, showing nine amide carbonyl groups as shown in Table 2. In ^{1}H NMR spectrum, however, the signals due to six amide protons were only observed. To elucidate the amino acid composition, **2** was subjected to complete hydrolysis with 6N HCl at 110 °C for 24 h in a sealed tube. The hydrolysate was then analyzed by HPLC and the amino acid composition was shown to be one Thr, one Gln, one Gly, one Met, one Ile, one Leu, two Pro per molecule. These eight amino acid units accounted for the above observed amide protons and amide carbonyl groups. Further, the lack of terminal amino group protons in the ^{1}H NMR and the observed mass molecular weight suggested that **2** might be a cyclic octapeptide. The ^{1}H-^{1}H COSY spectrum allowed the coupling sequence of each amino acid resonance, and the corresponding carbon resonances were elucidated on the basis of the HMQC spectrum as shown in Table 2. The gross structure including the sequence of the amino acids was assembled by connecting the individual amino acids on the basis of connectivities observed in a phase sensitive

NOESY and HMBC experiments. The HMBC correlations between each amide carbonyl carbon and neighboring amide NH and Hα protons indicated the partial sequence of -Pro-Ile-Thr-Gly-Leu-Met-Gln-. In addition, a strong NOE correlation between α proton of each Pro residue suggesting a *cis* amide bond indicated the structure of **2** to be *cyclo* (-Gly-Leu-Met-Gln-Pro-Pro-Ile-Thr-). The absolute stereochemistry of the component amino acids was determined to be L-configuration by chiral HPLC analysis after acid hydrolysis of **2**.

Cyclosquamosin C (**3**) showed a quasimolecular ion peak at *m/z* 854.4436 [$(M+H)^+$, Δ-1.0 mmu] in HRFABMS, corresponding to the molecular formula, $C_{38}H_{63}N_9O_{11}S$, larger than **2** by 16 mass unit. The same amino acid composition as **2** was indicated by acid hydrolysis, followed by amino acid analysis. In the NMR spectra, the methyl signal [^1H: δ 2.47; ^{13}C: δ 36.6] assigned to methionine sulfoxide (Mso) was observed. Judging from the ^1H and ^{13}C NMR signal pattern, as like those of **8**,[6] **3** was considered to contain the Mso residue instead of the Met one in **2**. Reductive transformation of **3** by thioglycolic acid into **2** provided an evidence for the absolute structure of **3**: *cyclo* (-Gly-Leu-Mso-Gln-Pro-Pro-Ile-Thr-).[14]

Cyclosquamosins D (**4**), E (**5**), and F (**6**) were obtained as colorless powder, and each molecular formula was shown to be $C_{41}H_{56}N_8O_{11}$, $C_{50}H_{65}N_9O_{13}$, and $C_{36}H_{54}N_8O_{11}$, by each quasimolecular ion peak at *m/z* 837.4161 [$(M+H)^+$, Δ+1.4 mmu], 1000.4800 [$(M+H)^+$, Δ+2.0 mmu], and 775.3975 [$(M+H)^+$, Δ-1.5 mmu], respectively, in HRFABMS. The ^1H NMR spectra of **4**, **5**, and **6** in conventional NMR solvents such as pyridine-d_5 or DMSO-d_6 gave broad signals resulting from the slow rate of interconversion. In addition, the ^1H NMR spectra of **4** and **5** showed two and three sets of signals, respectively, arising from a major conformer, in slow equilibrium with minor one. Therefore, the identification of the individual amino acids and sequence analysis were deduced from the Edman sequencing method. The acyclic peptide fragment [*m/z* 855 $(M+H)^+$], generated from **4** by digestion with α-chymotrypsin, was analyzed by peptide sequencer. The sequence of this acyclic peptide was determined to be Ser-Tyr-Tyr-Pro-Gly-Gly-Val-Leu. Therefore, the structure of **4** was unequivocally established to be *cyclo* (-Ser-Tyr-Tyr-Pro-Gly-Gly-Val-Leu-). As for amino acid composition , the molecular weight of **5** was larger than that of **4** by a Tyr residue. Two acyclic peptides [*m/z* 768 $(M+H)^+$ and 432 $(M+H)^+$, respectively] were obtained by α-chymotrypsin digestion and Edman sequencing of each acyclic peptide indicated the sequence to be Tyr-Tyr-Pro-Gly-Gly-Val-Leu as the same in **4** and Ser-Tyr-Tyr, respectively. Therefore, the structure of **5** was elucidated to be *cyclo* (-Gly-Gly-Val-Leu-Ser-Tyr-Tyr-Tyr-Pro-). Furthermore, the structure of **6** was also elucidated by the same method, enzymatic digestion and Edman sequencing, to be *cyclo* (-Gly-Ala-Pro-Ala-Leu-Thr-Thr-Tyr-). Absolute configurations of amino acids in **4** - **6** were verified to be L by chiral HPLC analysis.

Cyclosquamosin G (**7**) gave a quasi-molecular ion at *m/z* 833.4258 [$(M+H)^+$, Δ+2.7 mmu] in HRFABMS, appropriate for a molecular formula of $C_{39}H_{60}N_8O_{10}S$, less molecular weight than that of **8** by 16 mass unit. Amino acid analysis of **7** showed the presence of one of each Thr, Pro, Gly, Ala, Val, Tyr, Met, and Ile, which were all L-configuration by chiral HPLC analysis. The ^1H and ^{13}C NMR spectral pattern of **7** resembled those of **8**.[6] In the ^1H NMR spectra, a singlet methyl signal (δ 1.87) in **7**, in place of the methyl signal (δ 2.56) assigned to Mso in **8**, was observed. In addition, the ^{13}C signals ascribable to γ and methyl carbons of Met in **7** were resonated at higher field [δ 29.9 (γ), 14.8 (Me)] than those of **8** [δ 49.4 (γ), 37.7 (Me)]. Therefore, **7** was concluded to contain Met instead of Mso in **8**. Reductive transformation of **8** by thioglycolic acid into **7** provided additional support for the structure of **7**: *cyclo* (-Gly-Tyr-Pro-Met-Thr-Ala-Ile-Val-).[14]

Sequence homology of cyclic peptides in seeds of Annonaceae and the species of the other family such as Caryophyllaceae and Compositae[2] seems especially disparate. But such inclination containing Pro residues richly in higher plants is found. In the seeds of *A. squamosa*, one or two Pro residues are contained in cyclosquamosins A - G (**1** - **7**). In general, cyclic peptides are constrained as they contain turns in the backbones, which have been implicated in the bioactivity of naturally occurring peptides, and these turns are often stabilized by intramolecular hydrogen bonds. The presence of Pro residues without amide protons involved in intramolecular hydrogen bonds in the primary sequence could lead to a number of possible stable conformations due to *cis-trans* isomerization of a Pro amide bond. On the other hand, prolines would reduce the conformational space available to the peptides. In this way, conformations of cyclic peptides containing Pro residues are very complicated, and are dependent on the environment greatly. Actually, the ^{1}H NMR spectra of cyclosquamosins A (**1**), B (**2**), and C (**3**) with two Pro residues gave clear sharp signals, while cyclosquamosins D (**4**), E (**5**), and F (**6**) containing one Pro residue showed broad signals.

Attempts to obtain a single crystal for each cyclosquamosin as well as details from NMR studies and to examine various biological activities are currently being done.

Experimental Section

General Methods. Optical rotations were measured on a Jasco DIP-370 polarimeter. The IR and UV spectra were taken on JASCO FT/IR-5300 and JASCO Ubest-35 spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX-500 and JEOL EX-400 spectrometers, with chemical shifts (δ) reported in ppm. The spectra were recorded at 300°K. FABMS was measured on a JEOL HX-110 spectrometer by using glycerol matrix. Amino acid analysis was carried out using JLC-500/V(JEOL) amino acid analyzer.

Material. The seeds of *Annona squamosa* were collected from Penang, Malaysia, in 1997. The botanical identification was made by Prof. Kit-Lam Chan, School of Pharmaceutical Sciences, Universiti Sains Malaysia. A voucher specimen has been deposited in the herbarium of Hokkaido University.

Extraction and Isolation. The seeds of *Annona squamosa* (1.36 kg) were crashed and extracted with MeOH (3 L) three times. The MeOH extract (225 g) was treated with Hexane, EtOAc, *n*-BuOH, and H_2O, successively. The *n*-BuOH soluble fraction (18.8 g) was subjected to Diaion HP-20 column chromatography using a H_2O-MeOH gradient system (6:4-0:1). The fraction eluted with 80% MeOH was further subjected to Si gel column chromatography using a $CHCl_3$-MeOH gradient system (1:0-1:1). The fraction eluted with 10 ~ 30% MeOH was subjected to ODS HPLC with CH_3CN : H_2O (3:7) containing 0.05% TFA solvent system to give cyclosquamosins A (**1**, 0.003%), B (**2**, 0.002%), C (**3**, 0.02%), D (**4**: 0.08 %), E (**5**: 0.002 %), F (**6**: 0.001 %), and G (**7**: 0.002 %) with annosquamosin A (**8**, 0.005%).

Cyclosquamosin A (1). Colorless powder; $[\alpha]_D^{20}$ -74.7° (*c* 0.83, MeOH); ^{1}H-NMR and ^{13}C-NMR data, see Table 1; FABMS *m/z* 642 $(M+H)^+$; HRFABMS m/z 642.3271 $(M+H)^+$, calcd for $C_{31}H_{44}N_7O_8$, 642.3251; IR (KBr) ν_{max} 3421 and 1676 cm^{-1}.

Cyclosquamosin B (2). Colorless powder; $[\alpha]_D^{20}$ -53.8° (*c* 0.58, MeOH); ^{1}H-NMR and ^{13}C-NMR data, see Table 2; FABMS *m/z* 838 $(M+H)^+$; HRFABMS *m/z* 838.4491 $(M+H)^+$, calcd for $C_{38}H_{64}N_9O_{10}S$, 838.4498; IR (KBr) ν_{max} 3424 and 1676 cm^{-1}.

Cyclosquamosin C (3). Colorless powder; $[\alpha]_D^{20}$ -94.0° (*c* 0.10, MeOH); FABMS *m/z* 854 $(M+H)^+$; HRFABMS *m/z* 854.4436 $(M+H)^+$, calcd for $C_{38}H_{64}N_9O_{11}S$, 854.4446; ^{1}H-NMR (pyridine-*d*5): 4.81(1H, q, 4.7, 10.1, Gly-Hα), 3.88 (1H, dd, 5.0, 10.1, Gly-Hα), 8.60 (1H, dd, 4.7, 5.0, Gly-NH), 5.49 (1H, t, 8.0, Leu-Hα), 2.70 (2H, m, Leu-Hβ), 1.90 (1H, m, Leu-Hγ), 0.75 (3H, d, 5.0, Leu-Hδ), 0.85 (3H, d, 7.4, Leu-Hδ), 8.17 (1H, t, 9.6, Leu-NH), 4.25 (1H, m, Mso-Hα), 2.10 (2H, m, Mso-Hβ), 2.82 (2H, m, Mso-Hγ), 2.47 (3H, s, Mso-Hε), 9.84 (1H, d, 5.1, Mso-NH), 4.55 (1H, t, 3.4, Gln-Hα), 2.39 (2H, m, Gln-Hβ), 2.69 (2H, m, Gln-Hγ), 8.47 and 8.72 (each 1H, s, Gln-NH_2), 10.57 (1H, d, 3.4, Gln-NH), 4.24 (1H, m, Pro1-Hα), 2.14 (2H, m, Pro1-Hβ), 1.94 (2H, m, Pro1-Hγ), 4.21 (2H, m, Pro1-Hδ), 4.74 (1H, d, 5.8, Pro2-Hα), 2.10 (2H, m, Pro2-Hβ), 1.72 (2H, m, Pro2-Hγ), 3.63 (1H, t, 10.0, Pro2-Hδ), 3.84 (1H, t, 5.2, Pro2-Hd), 5.23 (1H, brs, Ile-Hα), 2.35 (1H, m, Ile-Hβ), 1.80 (2H, m, Ile-Hγ), 1.26 (3H, d, 3.3, Ile-Hγ), 0.86 (3H, t, 7.3, Ile-Hδ), 8.94 (1H, brs, Ile-NH), 5.02 (1H, m, Thr-Hα), 4.96 (1H, m, Thr-Hβ), 1.41 (3H, d, 6.2, Thr-Hγ), 8.55 (1H, d, 9.4, Thr-NH). ^{13}C-NMR data (pyridine-*d*5): 43.4 (Gly-Cα), 169.4 (Gly-CO); 51.1 (Leu-Cα), 38.5 (Leu-Cβ), 22.4 (Leu-Cγ), 20.7 (Leu-Cδ), 23.1 (Leu-Cδ), 172.9 (Leu-CO); 52.3 (Mso-Cα), 28.1 (Mso-Cβ), 29.9 (Mso-Cγ), 36.6 (Mso-Cε), 174.7 (Mso-CO); 57.8 (Gln-Cα), 26.8 (Gln-Cβ), 32.6 (Gln-Cγ), 177.6 (Gln-Cδ), 171.6 (Gln-CO); 63.9 (Pro1-Cα), 31.5 (Pro1-Cβ), 26.1 (Pro1-Cγ), 49.5 (Pro1-Cδ), 172.5 (Pro1-CO); 61.5 (Pro2-Cα), 31.6 (Pro2-Cβ), 25.2 (Pro2-Cγ), 48.6 (Pro2-Cδ), 170.9 (Pro2-CO); 55.9 (Ile-Cα), 35.0 (Ile-Cβ), 24.9 (Ile-Cγ), 16.8 (Ile-Cγ), 10.3 (Ile-Cδ), 171.9 (Ile-CO); 60.2 (Thr-Cα), 68.6 (Thr-Cβ), 20.0 (Thr-Cγ), 172.4 (Thr-CO); IR (KBr) ν_{max} 3380 and 1654 cm^{-1}.

Cyclosquamosin D (4). Colorless powder; $[\alpha]_D^{20}$ -36.4° (*c* 0.11, MeOH); FABMS *m/z* 837 $(M+H)^+$; HRFABMS *m/z* 837.4161 $(M+H)^+$, calcd for $C_{41}H_{57}N_8O_{11}$, 837.4147; ^{1}H-NMR (pyridine-*d*5): 0.87 (6H, brs, Leu-Me×2), 1.08 (6H, d, 6.6, Val-Me×2), 1.64 (m), 1.95 (m), 2.14 (m), 2.37 (m), 2.52 (m), 3.42 (m), 3.83 (m), 4.24 (m), 4.43 (m), 4.68 (m), 4.77 (m), 5.06 (m), 5.22 (m), 5.29 (m), 7.03 (2H, brd, 5.2), 7.22 (2H, brd), 7.34 (1H, d, 8.1), 7.39 (1H, d, 8.1), 7.51 (br s); IR (KBr) ν_{max} 3300 and 1668 cm^{-1}; UV (MeOH) λmax 278nm (ε 2560).

Cyclosquamosin E (5). Colorless powder; $[\alpha]_D^{20}$ -10.9° (*c* 1.38, MeOH); FABMS *m/z* 1000 $(M+H)^+$; HRFABMS *m/z* 1000.4800 $(M+H)^+$, calcd for $C_{50}H_{66}N_9O_{13}$, 1000.4780; ^{1}H-NMR (pyridine-*d*5): 0.83 (brs), 1.05 (d, 6.7), 1.11 (d, 6.8), 1.55 (m), 1.83 (m), 2.15 (m), 2.55(m), 3.05-3.68 (m), 4.08 (m), 4.35 (m), 4.45 (m), 4.60 (m), 4.73 (m), 4.98 (m), 5.21 (m), 5.33 (m), 5.53 (m), 6.97 (d, 8.1), 7.03 (d, 8.0), 7.12 (d, 7.7), 7.23 (d, 9.4), 7.33 (d, 7.8), 7.65 (d, 7.9), 8.16 (m), 8.41 (m), 8.65 (m), 8.93-9.52 (m), 10.4 (m); IR (KBr) ν_{max} 3301 and 1668 cm^{-1}; UV (MeOH) λmax 278nm (ε 7440).

Cyclosquamosin F (6). Colorless powder; $[\alpha]_D^{20}$ -38.2° (*c* 1.11, MeOH); FABMS *m/z* 775 $(M+H)^+$; HRFABMS *m/z* 775.3975 $(M+H)^+$, calcd for $C_{36}H_{55}N_8O_{11}$, 775.3990; ^{1}H-NMR (pyridine-*d*5): 0.89 and 0.90 (each 3H, d, Leu-Me), 1.46 (3H, d, 6.3, Ala-Hβ), 1.53 (3H, d, 6.2, Ala-Hβ), 1.60 (3H, d, 7.2, Thr-Hβ), 1.62 (3H, d, 7.2, Thr-Hβ), 3.19 (1H, dd, 8.2, 13.7, Tyr-Hβ), 3.49 (1H, m, Pro-Hδ), 3.52 (1H, m, Tyr-Hβ), 3.66 (1H, m, Pro-Hδ), 3.90 (1H, dd, 4.5, 16.8, Gly-Hα), 4.63-4.81 (3H, m, Gly, Thr, Ala-Hα), 4.94-5.04 (4H, m, Ala, Thr, Tyr, Leu-Hα), 7.06 (2H, d, 8.1, NH), 7.32 (2H, d, 8.1, NH), 8.07 (1H, brs, NH), 8.24 (1H, brs, NH), 8.81 (2H, brs, NH), 8.90 (1H, brs, NH), 9.29 (1H, brs, NH), 9.73 (1H, brs, NH); IR (KBr) ν_{max} 3344 and 1655 cm^{-1}; UV (MeOH) λmax 278nm (ε 1750).

Cyclosquamosin G (7). Colorless powder, $[\alpha]_D^{20}$ -37.1° (*c* 0.14, MeOH); FABMS *m/z* 833 $(M+H)^+$; HRFABMS *m/z* 833.4258 $(M+H)^+$, calcd for $C_{39}H_{61}N_8O_{10}S$, 833.4231; ^{1}H-NMR (pyridine-*d*5): 3.98 and 4.73 (each 1H, dd, 6.4, 17.0, Gly-Hα), 8.75 (1H, t, 6.4, Gly-NH), 5.65 (1H, m, Tyr-Hα), 3.29 (1H, dd, 12.2, 15.1, Tyr-Hβ), 4.23 (1H, brd, 8.5, Tyr-Hβ), 7.16 (2H, d, 8.6, Tyr-Hδ), 7.37 (2H, d, 8.6, Tyr-Hε), 8.63 (1H, d, 9.8, Tyr-NH), 5.25 (1H, dd, 5.3, 10.3, Pro-Hα), 1.98 and 2.35 (each 1H, m, Pro-Hβ), 2.14 and 1.75 (each 1H, m, Pro-Hγ), 3.45 and 3.99 (each 1H, m, Pro-Hδ), 4.79 (1H, m, Met-Hα), 2.26 (2H, m, Met-Hβ), 2.56 and 2.62 (each 1H, m, Met-Hγ), 1.87 (3H, s, Met-SMe), 8.80 (1H, d, 3.4, Met-NH), 5.57 (1H, dd, 2.9, 9.8, Thr-Hα), 4.94 (1H, m, Thr-Hβ), 1.39 (3H, d, 5.9, Thr-Hγ), 7.95 (1H, d, 9.8, Thr-NH), 4.77 (1H, m, Ala-Hα), 1.58 (3H, d, 7.3, Ala-Hβ), 7.70 (1H, d, 6.8, Ala-NH), 4.76 (1H, m, Ile-Hα), 2.34 (1H, m, Ile-Hβ), 1.30 and 1.56 (each 1H, m, Ile-Hγ), 1.03 (3H, d, 6.8, Ile-Hγ), 0.67 (3H, t, 7.3, Ile-Hδ), 7.63 (1H, d, 8.8, Ile-NH), 4.18 (1H, dd, 3.4, 6.4, Val-Hα), 2.34 (1H, m, Val-Hβ), 1.13 (3H, d, 6.8, Ile-Hγ), 1.11 (3H, d, 7.3, Ile-Hγ), 9.18 (1H, d, 3.4, Val-NH). ^{13}C-NMR data (pyridine-*d*5): 44.4 (Gly-Cα), 170.4 (Gly-CO); 56.7 (Tyr-Cα), 36.8 (Tyr-Cβ), 129.1 (Tyr-Cγ), 116.0 (Tyr-Cδ), 129.7 (Tyr-Cε), 157.4 (Tyr-Cζ), 173.0 (Tyr-CO); 64.1 (Pro-Cα), 30.3 (Pro-Cβ), 25.2 (Pro-Cγ), 47.8 (Pro-Cδ), 176.3 (Pro-CO); 56.0 (Met-Cα), 29.7 (Met-Cβ), 29.9 (Met-Cγ), 14.8 (Met-Cε), 172.2 (Met-CO); 53.3 (Thr-Cα), 70.5 (Thr-Cβ), 19.6 (Thr-Cγ), 172.5 (Thr-CO); 56.4 (Ala-Cα), 17.9 (Ala-Cβ), 173.8 (Ala-CO); 52.1 (Ile-Cα), 36.7 (Ile-Cβ), 24.3 (Ile-Cγ), 17.4 (Ile-Cγ), 11.1 (Ile-Cδ), 172.2 (Ile-CO); 62.9 (Val-Cα), 30.5 (Val-Cβ), 19.4 × 2 (Val-Cγ), 172.4 (Val-CO). IR (KBr) ν_{max} 3329 and 1665 cm^{-1}; UV (MeOH) λ_{max} 278 nm (ε 1840).

Annosquamosin A (8). Colorless powder; $[\alpha]_D^{20}$ -76.0° (*c* 0.10, MeOH); FABMS *m/z* 849 $(M+H)^+$; HRFABMS *m/z* 849.4186 $(M+H)^+$, calcd for $C_{39}H_{61}N_8O_{11}S$, 849.4181; IR (KBr) ν_{max} 3313 and 1663 cm^{-1}; UV (MeOH) λ_{max} 279 nm (ε 1000).

Amino Acid Analysis of 1 - 8. Solutions of **1** - **8** (each containing 1 mg of peptide) in 6N HCl were heated at 110°C for 24h in a sealed tube. After cooling, each solution was concentrated to dryness. The hydrolysates were dissolved in 0.02N HCl and were submitted amino acid analyzer.

Absolute Configuration of Amino Acids. Each solution of **1** - **7** (0.5 mg) in 6 N HCl (1 mL) was heated at 110° for 24 h. The solution was concentrated to dryness. The residue was dissolved in H_2O (100 μL) and chiral HPLC analyses were carried out using a SUMICHIRAL OA-5000 column (Sumitomo Chemical Industry; 150 mm; 25°C, detection at 254 nm). Retention times (min) of authentic amino acids were as follows: L-Ala (2.4), D-Ala (3.5), L-Ser (2.5), D-Ser (2.8), L-Val (9.3), D-Val (16.2), L-Pro (7.1), D-Pro (14.8), L-

Gln (2.8), D-Gln (5.6), L-Met (24.8), and D-Met (34.2) (eluent: H_2O containing 1.0 mM $CuSO_4$, flow rate 1.0 mL/min); L-Tyr (7.4), D-Tyr (10.8), L-Ile (7.0), D-Ile (11.2), L-Leu (7.5), D-Leu (9.4), L-Phe (19.3), and D-Phe (27.3) [eluent: MeOH/H_2O (15:85) containing 2.0 mM $CuSO_4$, flow rate 1.0 mL/min]; L-Thr (4.9) and D-Thr (5.6) [eluent: H_2O containing 0.5 mM $Cu(OAc)_2$, flow rate 1.0 mL/min]. Retention times of the hydrolysates of **1** - **7** were as follows: **1**, L-Ser (2.4), L-Pro (7.0), L-Val (9.7), and L-Phe (19.3); **2**, L-Gln (3.1), L-Pro (7.6), L-Met (25.3), L-Ile (6.8), L-Leu (7.8), and L-Thr (4.1); **3**, L-Gln (3.2), L-Pro (7.5), L-Met (25.2), L-Ile (6.8), L-Leu (7.8), and L-Thr (4.1); **4**, L-Ser (2.6), L-Pro (7.6), L-Val (10.2), L-Tyr (7.6), and L-Leu (8.0); **5**, L-Ser (2.5), L-Pro (7.5), L-Val (10.2), L-Tyr (7.6), and L-Leu (7.6); **6**, L-Tyr (7.6), and L-Leu (8.0), L-Ala (2.8), L-Pro (7.5), and L-Thr (4.0); **7**, L-Ala (2.5), L-Pro (7.6), L-Val (10.4), L-Met (25.9), L-Ile (6.9), L-Tyr (7.5), and L-Thr (4.3).

Reduction of 8 and 3 with Thioglycolic Acid. A solution of **8** (4.8 mg) in 40% thioglycolic acid (0.5 ml) was heated at 50°C for 24h. The reaction mixture was subjected to HP-20 column to give the reduction product (3.1 mg), whose spectroscopic data such as ^1H and ^{13}C-NMR data and optical rotation were completely identical with those of cyclosquamosin G (**7**). Similarly, reduction product (4.0 mg) of **3** obtained by the same manner was identical with those of cyclosquamosin B (**2**).

Enzymatic Hydrolysis of 4 - 6. α-Chymotrypsin (1.0 mg dissolved in 100 μL of 0.001% HCl, Merck) was added to NH_4HCO_3 solution (1%, 0.9 mL) of **4** - **6** (1.0 mg) and the digestion was performed at 37 °C with the pH maintained at 8.0 by addition of 0.1N HCl. After 11 h (**4**), 26 hr (**5**), and 46 hr (**6**), the reaction was stopped by adjusting the solution to pH 2.2 with 1N HCl. Each digestion mixture was lyophilized to dryness and subjected to HPLC (Develosil ODS-HG-5 column, 10 mm i.d. × 250 mm, Nomura Chemical, eluted with 20% CH_3CN/0.05%TFA for **4** and **6**, 22% CH_3CN/0.05%TFA for **5**, flow rate 2 mL/min) to give acyclic peptide as amorphous powder, respectively: ESI MS *m/z* 855 $(M+H)^+$ for **4** (0.1 mg, R_t 44.1 min), ESI MS *m/z* 768 $(M+H)^+$ (0.1 mg, R_t 19.6 min) and 432 (0.1 mg, Rt 8.0 min) for **5**, and ESI MS *m/z* 793 $(M+H)^+$ for **6** (0.1 mg, R_t 8.4 min).

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