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New Cyclic Peptides from the Seeds of *Annona squamosa* L. and Their Anti-inflammatory Activities

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Two new cyclic peptides, cyclosquamosin H (**1**) and I (**2**), together with six known cyclic peptides, squamin A (**3**), squamin B (**4**), cyclosquamosin A (**6**), cyclosquamosin D (**7**), cyclosquamosin E (**8**), and cherimolacyclopeptide B (**9**), were isolated from the seeds of *Annona squamosa*. All structures were confirmed by 2D nuclear magnetic resonance, chemical evidence, and electrospray ionization−mass spectromotry/mass spectrometry (ESI-MS/MS). Compound **9** was isolated from this plant for the first time. In the anti-inflammatory assay, compound **7** showed an inhibitory effect on the production of pro-inflammatory cytokines within lipopolysaccharide and Pam3Cys-stimulated J774A.1 macrophages.

KEYWORDS: Annona squamosa; cyclic peptides; cyclosquamosin H; cyclosquamosin I; Anti-inflammatory activity; TNFα; IL-6

INTRODUCTION

Annona squamosa, sugar apple or sweetsop, is native to the tropic America. The Spaniards probably carried seeds from the New World to the Philippines, and the Portuguese are assumed to have introduced it to southern India before 1590 (*1*). Now it is cultivated in the tropic areas worldwide. Netherlanders introduced *A. squamosa* to Taiwan in the 17 century. First, it was planted around south of Taiwan. Because of small fruits and low yields, the cultivation of *A. squamosa* was not popular. In 1980, *A. squamosa* began to be cultivated in Taitung, a beautiful southeastern county of Formosa, which has suitable weather and soil for this plant. Until 2000, 81.8% of the cultivation area (over 4500 ha) of *A. squamosa* of Taiwan was grown in Taitung County (*2*). Now it is one of the most popular fruits in Taiwan. The fruits can be collected twice (July−September and December−February) per year.

In addition to edible fruits, traditionally, each part of *A. squamosa* could be used as folk medicines. The crushed leaves are sniffed to overcome hysteria and fainting spells, they are also applied on ulcers and wounds, and a leaf decoction is taken in cases of dysentery. The seeds are grounded and macerated in water as an insecticide. In India, the seeds are used to eliminate head lice. The decoction of seeds is also used as an enema for children with dyspepsia. The crushed ripe fruits, mixed with salt, are applied on tumors. The bark and root are both highly astringent. The bark decoction is given as a tonic and to halt diarrhea. The root, because of its strong purgative action, is administered as a drastic treatment for dysentery and other ailments (*3*). Phytochemical studies on *A. squamosa* were begun in 1924 (*4*). Up to now, more than 100 compounds have been yielded (*5*). Most of these compounds can be classified into four major groups: alkaloids, annonaceous acetogenins, cyclic peptides, and *ent*-kaurane diterpenoids. Some of them showed interesting pharmacological activities (*5, 6*). Since the first cyclic peptide, annosquamosin A, was isolated from this plant, ten cyclic peptides have been reported from *A. squamosa* (*7–9*). Among them, cyclosquamosin B showed a vasorelaxant effect on rat aorta (*10*). In the present study, eight cyclic peptides, including two new ones, cyclosquamosin H (**1**) and I (**2**), and six known ones, squamin A (**3**), squamin B (**4**), cyclosquamosin A (**6**), cyclosquamosin D (**7**), cyclosquamosin E (**8**), and cherimolacyclopeptide B (**9**), were isolated from the seeds (see **Scheme1**). Compound **9**, previously reported from the seeds of *Annona cherimola* (*11*), was obtained from this plant for the first time. All structures were confirmed by spectroscopic data interpretation and chemical evidence.

Inflammation is an early host immune reaction mediated via immune cells and their cytokines. The gram-negative bacterial cell wall, in particular lipopolysaccharide (LPS), can stimulate monocyte and macrophage immune cells to release inflammatory cytokines. TNFα is considered a key mediator of innate immunity because it activates neutrophils, promotes adhesion molecule expression on vascular endothelial cells, and enhances the secretion and synthesis of other pro-inflammatory cytokines

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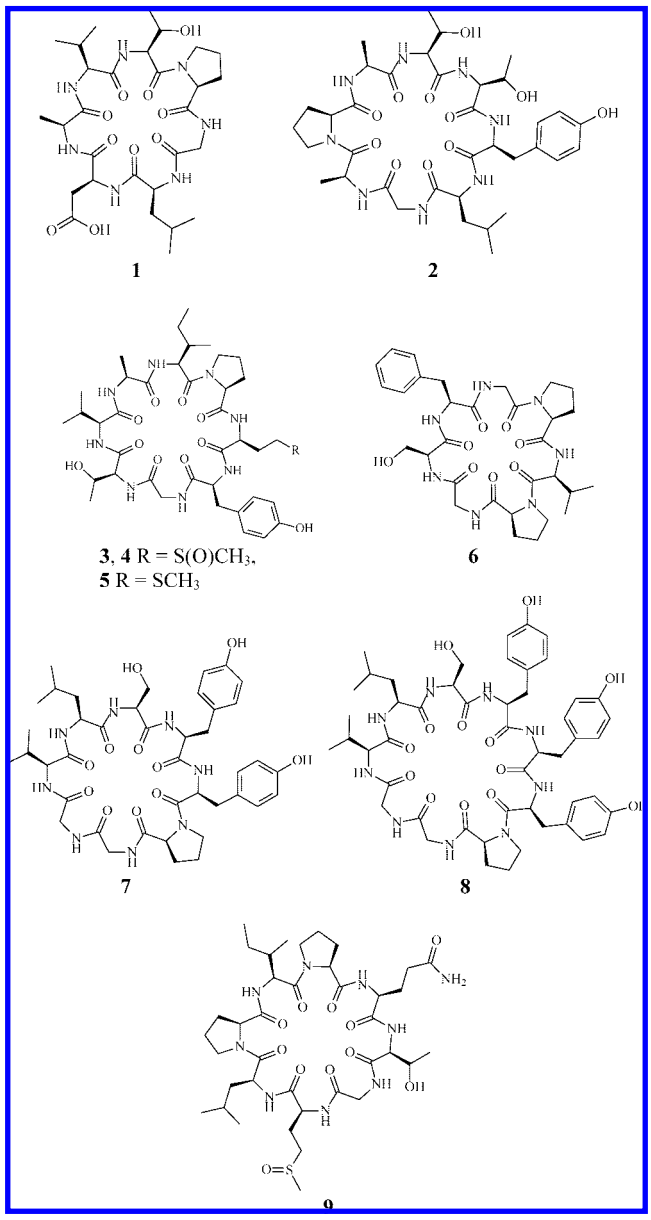
Scheme 1



and acute-phase proteins (*12*). IL-6 is mainly involved in macrophage and osteoclast differentiation, T- and B-cell proliferation, synthesis of acute-phase proteins, suppression of albumin production, and enhancement of tumor cell growth (*13*). The release of these inflammatory cytokines is essential for host survival from infection and also is required for repair of tissue injury. Various in vivo and in vitro experimental models have been established to assess the inhibitory effects of various natural products on the synthesis and releasing of inflammatory cytokines.

In this study we investigated the effects of cyclic peptides on anti-inflammatory activity using in vitro model LPS- and Pam3Cys-stimulated macrophages and monitoring of the production of TNFα and IL-6.

MATERIALS AND METHODS

General Experimental Procedures. Melting points were determined using a Yanagimoto micromelting point apparatus and were uncorrected. Optical rotations were measured with a JASCO P-1020 digital polarimeter. The ultraviolet (UV) spectra were obtained on a JASCO V-530 spectrophotometer. Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer. ^{1}H Nuclear magnetic resonance (^{1}H NMR, 400 and 500 MHz, using pyridine- d_5 and CD_3CN as solvent for measurement), ^{13}C NMR, DEPT, ^{1}H−^{1}H COSY, TOCSY (mixing time = 120 ms), HMBC ($^2J_{1H-13C}$ or $^3J_{1H-13C}$ = 5 and 8 Hz), HSQC ($^1J_{1H-13C}$ = 145 Hz), and ROESY (mixing time = 400 ms) spectra were obtained on Varian (Unity Plus 400 and Unity INOVA-500) and Bruker (AVANCE II 500) NMR spectrometers. HRMS-ESI spectra were measured on a Micromass Q-Tof micro mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography. A preparative column (Develosil C30-UG-5, 250 × 20 mm i.d.) was used for a preparative HPLC JASCO PU-1580 with an UV-1575 detector. The TLC spots were detected by spraying with 50% H_2SO_4 and then heated on a hot plate.

Electrospray Ionization Tandem Mass Spectrometry. A triple-quadruple mass spectrometer, API 3000 (Applied Biosystems), operating with a TurboIonSpray source (PE-SCIEX, Concord, Ortario, Canada) was used. Each cyclic peptide was directly infused into the mass spectrometer (flow-rate = 10 μL/min) to acquire the full-scan and product ion mass spectra. A Q1 full scan spectrum of each analyte was first conducted to obtain their corresponding protonated molecular ions. Their product ion scan spectrum was further acquired by transmitting the protonated molecular ion via Q1 and scanning for products resulting from fragmentation in the collision cell. The electrospray voltage at the spraying needle was optimized at 4500 V. The TurboIonSpray source was operated with nitrogen as the nebulizing (set to 10), curtain (set to 10). Low-energy collision-activated dissociation (CAD) experiments were performed using nitrogen (CAD gas valve set to 4) as collision gas, and a collision energy of 40 eV was used.

Plant Materials. Air-dried seeds of *A. squamosa* were collected from Luye, Taitung County, Taiwan, in September 2000. The material was collected and identified by Dr. Ching-Shan Yang (Taitung District Agricultural Research and Extension Station, Council of Agriculture, Taitung, Taiwan). The voucher specimens (Annona 6a) were deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. The air-dried seeds of *A. squamosa* (5 kg) were grounded and then extracted with CH_3OH to yield 1000 g of extract. The extract was partitioned with 50% aq-CH_3OH and $CHCl_3$ (1:1) to give three parts: $CHCl_3$ layer (720 g), aqueous layer (200 g), and insoluble part (30 g). The $CHCl_3$ layer was separated into 38 fractions by Si gel chromatography (*n*-Hexane to CH_3OH). Fraction 34 was further purified by RP-HPLC (C-30, CH_3CN-0.01% aq-TFA = 20:80) to give compounds **1**–**4** and **6**–**9** (8, 25, 52, 56, 45, 105, 10, and 33 mg, respectively).

Acid Hydrolysis of Cyclic peptides. Cyclic peptides were hydrolyzed by heating 1 mg of the respective peptide in 2 mL of 6 N HCl at 110 °C for 16 h. After cooling, the solution was evaporated to dryness and redissolved in 250 μL of H_2O.

Preparation and Analysis of Marfey Derivatives. To 50 μL of a 50 mM solution of the respective amino acid (or to 50 μL of the acid hydrolysate solution) was added 100 μL of a 1% (w/v) solution of FDAA (Marfey's reagent, N^{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) in acetone. After addition of a 1 M $NaHCO_3$ solution (20 μL), the mixture was incubated for 1 h at 50 °C. The reaction was stopped by the addition of 10 μL of 2 N HCl. The solvents were evaporated to dryness, and the residue was redissolved in 1 mL of CH_3OH-H_2O (1:1). An aliquot of this solution (5 μL) was analyzed by HPLC.

Cell Culture. Murine macrophage J774A.1 cells (1×10^6/mL) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Co., Logan, UT) and 2 mM L-glutamine (Life Technologies, Inc., MD) in a 37 °C, 5% CO_2 incubator (the cultured medium volume was 2 mL), unless otherwise indicated. J774A.1 cells were pretreated with the cyclic peptides (dissolved in dimethyl sulfoxide, DMSO) for 30 min, followed by LPS (0.1 μg/mL) or Pam3Cys (1 μg/mL) treatment for an additional 6 h. The sample was dissolved in DMSO, and the final DMSO concentration was 0.1% in all cultures containing this agent.

Enzyme-Linked Immunosorbent Assay (ELISA). To investigate the inhibitory effect of the cyclic peptides on TNFα and IL-6 secretion from LPS- or Pam3Cys-stimulated J774A.1 cells, cells were pretreated with cyclic peptides for 30 min at 37 °C, followed by LPS (0.1 μg/

Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Data of **1** in Pyridine-d_5

position		δ_H (J in Hz)	δ_C	position		δ_H (J in Hz)	δ_C
Gly	α	3.83, dd (17.2, 4.4)	43.8	**Ala**	α	4.65, qd (7.4, 3.6)	53.0
		4.81, dd (17.2, 8.2)			β	1.53, d (7.4)	17.6
	NH	10.42, dd (8.2, 4.4)			NH	9.26, d (3.6)	
	C=O		169.6		C=O		173.3
Pro	α	4.44, m	61.8	**Asp**	α	5.00, ddd (5.2, 4.8, 4.4)	51.3
	β	1.20, m	29.6		β	3.87, dd (16.8, 4.8)	35.9
		2.03, m				3.96, dd (16.8, 4.4)	
	γ	1.26, m	24.8		γ	9.51, s	174.9
		2.07, m			NH	9.19, d (5.2)	
	δ	3.65, dt (7.2, 9.7)	48.7		C=O		172.0
		4.06, m		**Leu**	α	5.46, m	54.3
	C=O		172.7		β	1.80, m	44.5
Thr	α	5.36, t (9.4)	57.7			2.05, m	
	β	4.48, dq (9.4, 6.4)	68.3		γ	1.90, m	24.9
	γ	1.61, d (6.4)	20.3		δ	0.90, d (6.4)	22.3
	NH	8.29, d (9.4)				0.92, d (6.0)	22.5
	C=O		170.2		NH	8.73, d (14.0)	
Val	α	5.23, dd (10.4, 6.4)	59.6		C=O		172.6
	β	2.81, sextet (6.4)	31.1				
	γ	1.09, d (6.4)	18.6				
		1.10, d (6.4)	19.7				
	NH	8.53, d (10.4)					
	C=O		171.9				

Table 2. ESI-MS/MS Data for **1** and **2**

1				**2**			
$[M + Na]^+$ m/z 675		$[M + Na - CO]^+$ m/z 647		$[M + H]^+$ m/z 775		$[M + Na - CO]^+$ m/z 769	
mass m/z	residue	mass m/z	residue	mass m/z	residue	mass m/z	residue
604	Ala	546	Thr	674	Thr	668	Thr
505	Val	447	Val	573	Thr	567	Thr
404	Thr	376	Ala	410	Tyr	404	Tyr
307	Pro	261	Asp	297	Leu	291	Leu
250	Gly			240	Gly	234	Gly
				168	Ala		

mL) or Pam3Cys (1 μg/mL) treatment for the indicated times, and the cell culture medium was collected. For assaying purposes, 50 μL of biotinylated antibodies reagent was added to antimouse TNFα and IL-6 precoated stripwell plates, respectively, with 50 μL of supernatant concentrate from tested samples for various times and incubated at room temperature for 3–6 h. After the plate was washed three times with washing buffer provided in kit components, 100 μL of diluted streptavidin-HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated, and 100 μL of premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL of provided stop solution to each well to stop the reaction, the absorbance of the plate was measured by a MRX microplate reader (Dynex Tech. Inc., VA) at 450–550 nm wavelengths.

Statistical Analysis. All values are given as means ± SD. Data analysis involved one-way ANOVA with subsequent Scheffe's test.

Cyclosquamosin H (1). White powder; mp 275–276 °C; $[\alpha]_D^{23}$ −40.0° (*c* 0.04, CH_3CN); IR (Neat) V_{max} 3310, 1655 cm^{-1}; UV λ_{max}^{MeCN} 201 nm; ^{1}H and ^{13}C NMR data, see **Table 1**; ESI-MS/MS data, see **Table 2**; HRESIMS *m/z* 653.3461 $[M]^+$ (calcd. for $C_{29}H_{47}O_{10}N_7$, 653.3384).

Cyclosquamosin I (2). Syrup; $[\alpha]_D^{23}$ + 52.3° (*c* 0.04, CH_3CN); IR (Neat) V_{max} 3315, 1651 cm^{-1}; UV λ_{max}^{MeCN} 202, 221 (sh), 277 nm; ^{1}H and ^{13}C NMR data, see **Table 3**; ESI-MS/MS data, see **Table 2**; HRESIMS *m/z* 775.3956 $[M + H]^+$ (calcd. for $C_{36}H_{55}O_{11}N_8$, 775.3990).

RESULTS AND DISCUSSION

Cyclosquamosin H (**1**), obtained as white powder, had the molecular formula $C_{29}H_{47}O_{10}N_7$ from the analysis of HRMS-ESI. The IR absorptions at 3310 and 1655 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. In the ^{13}C NMR spectrum, eight carbonyl carbons, including amide and acid carbonyl carbons, were revealed. However, six NH and one COOH signals were observed in the ^{1}H NMR spectrum. To elucidate the amino acid composition, **1** was subjected to complete acidic hydrolysis by 6N HCl at 110 °C for 16 h in a sealed tube. The hydrolysate was then analyzed by HPLC (Marfey's method) (*14*), and the amino acid composition was showed as one Gly, one Pro, one Thr, one Val, one Ala, one Asp, and one Leu per molecule. The ^{1}H−^{1}H COSY and TOCSY spectra allowed the coupling sequence of each amino acid resonance, and the corresponding carbon resonances were elucidated by HMQC and are shown in **Table 1**. The above results and a negative Ninhydrin reaction indicated compound **1** to be a cyclic heptapeptide.

The amino acid sequence of compound **1** was revealed by the source CID ESI-MS/MS and 2D NMR. In the source CID ESI-MS/MS, the sodium adduct ion at *m/z* 675 $[M + Na]^+$ was selected as a precursor ion, and it showed two set of amino acid sequences, Ala-Val-Thr-Pro-Gly and Thr-Val-Ala-Asp (**Table 2**). The HMBC correlations between each amide carbonyl carbon and neighboring amide NH and Hα protons indicated two sets of partial sequences: Pro-Gly-Leu-Asp and Ala-Val (**Figure 1**). These data indicated the sequence of **1** as cyclic-Gly-Pro-Thr-Val-Ala-Asp-Leu.

The ROE correlations of compounds were obtained by the ROESY spectrum (**Figure 2**). The strong ROE correlations between amide NH and neighboring H_α protons could confirm the sequence. The ROE correlations between Thr-H_β/H_γ and

Table 3. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data of **2** in Pyridine-d_5

position		δ_H (J in Hz)	δ_C	position		δ_H (Jin Hz)	δ_C
Thr	α	5.02, dd (7.0, 4.0)	59.2	**Gly**	α	3.90, dd (16.8, 4.8)	43.7
	β	4.70, qd (6.5, 4.0)	68.0			4.73, dd (16.8, 6.8)	
	γ	1.47, d (6.5)	19.6		NH[a]	9.16, br d	
	NH	8.04, d (7.0)			C=O[b]		172.0
	C=O[b]		169.6	**Ala**	α	5.20, dq (8.0, 6.5)	47.3
Thr	α	4.51, m	61.9		β	1.60, d (6.5)	17.4
	β	4.94, m	66.3		NH	8.30, d (8.0)	
	γ	1.56, d (6.5)	21.5		C=O[b]		173.3
	NH	8.57, d (6.5)		**Pro**	α	4.63, m	63.0
	C=O[b]		171.6		β	2.01, m	29.4
Tyr	α	4.79, dd (13.0, 7.5)		57.5		2.18, m	
	β	3.16, dd (13.8, 7.5)	36.6		γ	1.67, m	25.2
		3.46, dd (13.8, 7.5)				1.86, m	
	γ		127.7		δ	3.74, m	47.5
	δ	7.25, d (8.5)	130.6			3.84, m	
	ϵ	7.06, d (8.5)	116.1		C=O		174.3
	ζ		157.5	**Ala**	α	4.67, m	51.4
	NH	9.16, br d (13.0)			β	1.66, d (7.0)	17.0
	C=O		173.6		NH[a]	9.66, s	
Leu	α	4.97, m	52.3		C=O		174.1
	β	2.18, m	39.3				
	γ	1.86, m	25.0				
	δ	0.81, d (7.0)	21.6				
		0.86, d (7.0)	23.0				
	NH[a]	8.82, br d					
	C=O[b]		172.0				

[a] Interchangeable. [b] Interchangeable.

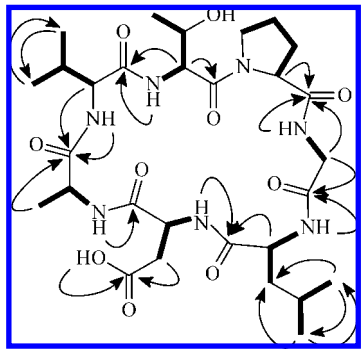


Figure 1. $^{1}H-^{1}H$ COSY (—) and HMBC (→) correlations of **1**.

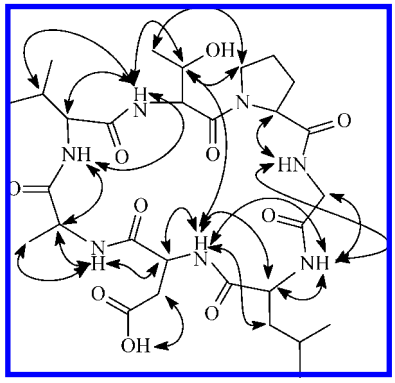


Figure 2. ROESY Correlations of **1**.

Pro-H_δ together with the ^{13}C NMR chemical shifts of Pro-C_β(δ 29.6) and Pro-C_γ(δ 24.8) revealed a trans geometry for the proline amide bone (*15*). The presence of type II β-turn (Thr-Pro-Gly-Leu) was implied by a strong ROE cross peak between Pro-H_α and Gly-NH and by an ROE correlation between Gly-NH and Leu-NH (*8*). The absolute stereochemistry of each amino acid was determined as the L-configuration by HPLC analysis after acid hydrolysis of **1**.

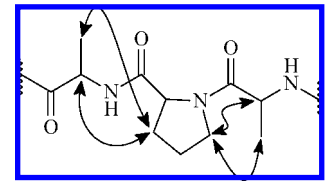


Figure 3. Key ROESY Correlations of the -Ala-Pro-Ala- Residues in **2**.

Cyclosquamosin I (**2**), obtained as syrup, showed a pseudomolecular ion peak at *m/z* 775.3956 in HRMS-ESI, corresponding to the molecular formula $C_{36}H_{54}O_{11}N_8$. The IR absorptions at 3315 and 1651 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. In NMR spectra, eight amide carbonyl carbons and seven amino protons were observed (**Table 3**). In the analysis of the $^{1}H-^{1}H$ COSY and TOCSY spectra, two Thr, two Ala, one Pro, one Gly, one Leu, and one Tyr were determined. The HPLC analysis (Marfey's method) (*14*) on the hydrolysate of **2** confirmed the amino acid composition and indicated absolute stereochemistry of all amino acids as the L-configuration. The HRMS-ESI, together with negative Ninhydrin reaction, indicated **2** to be a cyclic octapeptide.

In the analysis of the source CID ESI-MS/MS, two ion peaks, *m/z* 775 $[M + H]^+$ and *m/z* 798 $[M + Na]^+$, were selected as precursor ions. Both spectra showed the partial sequence to be Thr-Thr-Tyr-Leu-Gly-Ala (**Table 2**). In the fragmentation analysis of *m/z* 775 $[M + H]^+$, an X ion peak, *m/z* 704 $[M - Ala]^+$, was also observed. The aforementioned results together with the key ROESY correlations around two Alanines and one Proline (**Figure 3**) revealed compound **2** as cyclic-Thr-Thr-Tyr-Leu-Gly-Ala-Pro-Ala.

In 1999 and 2000, squamin A and B, a pair of conformational isomers, were isolated from *A. squamosa* by research in China (*9*). The authors identified the structures of squamin A and B by 2D NMR and FABMS, and the molecular structures were also revealed by X-ray. In 2003, squamtin A (the same structure as squamin A mentioned by the authors) was found to be crystallized in two pseudopolymorphisms (*16*). The authors investigated the molecular structure of squamtin A by X-ray

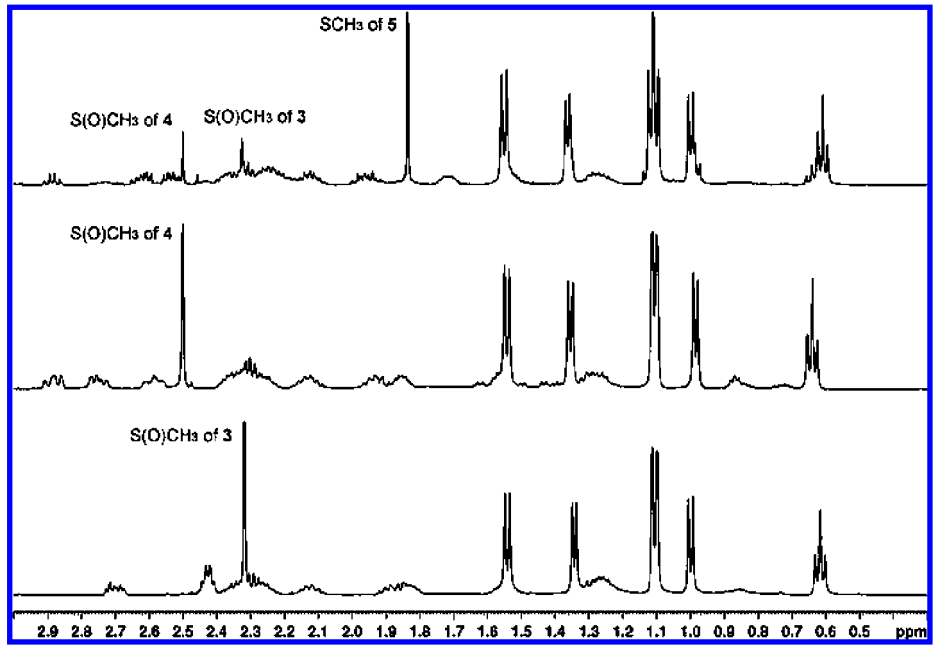


Figure 4. ^{1}H NMR spectra of **3**, **4**, and the mixtures from reductive transformation of **3** and **4** (from buttom to top). The signals of methyl groups of methionine sulfoxide in **3** and **4** and methyl group of methionine in **5** are marked.

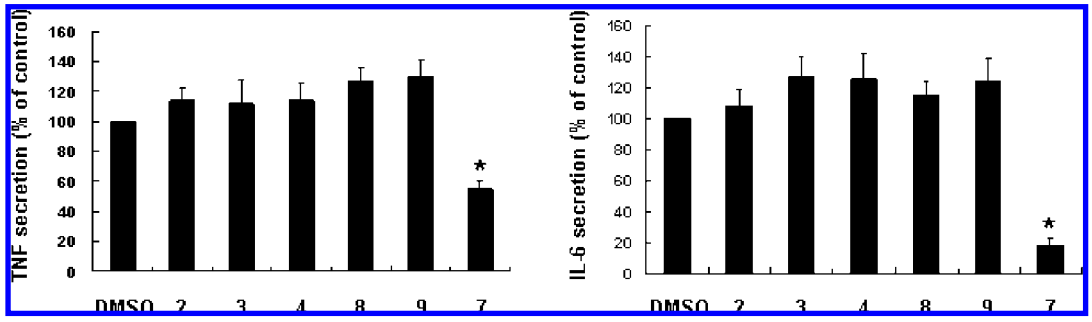


Figure 5. Inhibitory effect of cyclic peptides on the production of TNFα and IL-6 within LPS-stimulated J774A.1 Cells. *$p < 0.05$ vs DMSO.

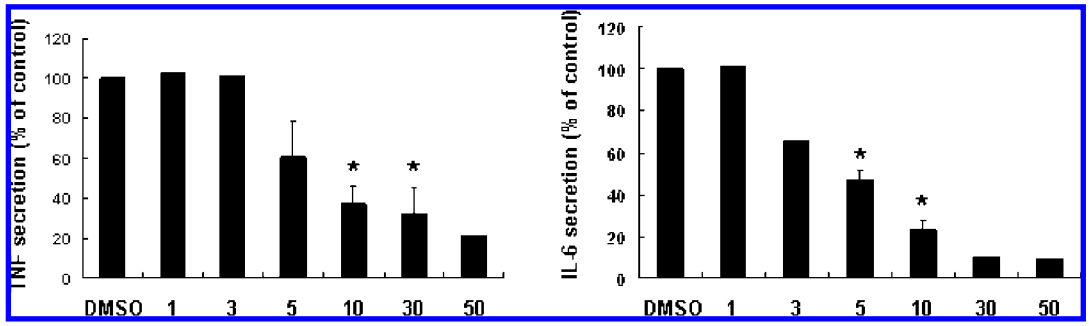


Figure 6. Inhibitory effect of **7** (μg/mL) on the production of TNFα and IL-6 within LPS-stimulated J774A.1 Cells. *$p < 0.05$ vs DMSO.

and indicated that it can form two kinds of hydrates, $C_{39}H_{60}O_{11}N_8S\cdot(H_2O)_{3.5}$ and $C_{39}H_{60}O_{11}N_8S\cdot(H_2O)_{3.9}$. Interestingly, the X-ray data of the pseudopolymorphisms of squamtin A, squamin A [$C_{39}H_{60}O_{11}N_8S\cdot(H_2O)_{3.15}$], and squamin B [$C_{39}H_{60}O_{11}N_8S\cdot(H_2O)_{3.4}$], showed that they have the same molecular structure except for the positions and occupancies of the water molecules (*9, 16*). Generally, the conformers of cyclic peptides can give various sets of signals or broad signals resulting from the slow rate of interconversion in NMR spectra. Whatever the pseudopolymorphisms and conformers of cyclic peptides, they can not be separated as pure compounds in liquid state. In our study, **3** and **4** were isolated by HPLC and identified as squamin A and B by spectroscopic methods, respectively (Supporting Information). In the HPLC and NMR analysis of **3** and **4**, we did not find the interconversion phenomena in various liquid states (CH_3OH, CH_3CN, and pyridine), which suggests that **3** and **4** should be configurational isomers. The absolute stereochemistry of the amino acids in **3** and **4** was determined by Marfey's method (*14*) to be L, except for the configuration of sulfoxide. Reductive transformation of **3** and **4** by thioglycolic acid gave compound **5** (*17*), containing the methionine in the place of methionine sulfoxide in **3** and **4**, providing evidence that they are configurational isomers (**Figure 4**).

Compounds **6–9** were reported as known compounds. The structures were determined by comparing spectroscopic data with those in the literatures (*8, 9, 11*).

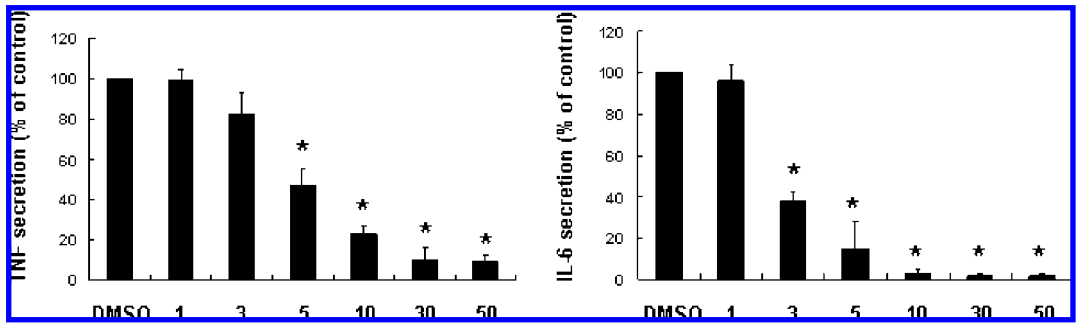


Figure 7. Inhibitory effect of **7** (μg/mL) on the production of TNFα and IL-6 within Pam3Cys-stimulated J774A.1 Cells. *$p < 0.05$ vs DMSO.

Certain pathogen infections can cause significant mortality for infected hosts by the overexpression of pro-inflammatory cytokines, including TNFα and IL-6. To investigate whether the cyclic peptides isolated from *A. squamosa* exhibit certain immunomodulation activity within cultured murine macrophage J774A.1 cells, in particular, the cells were pretreated with various types of cyclic peptides (30 μg/mL) for a period of 30 min at 37 °C, followed by challenge with LPS, a gram-negative bacteria cell wall component that binds to toll-like receptor 4 (TLR4), for 6 h. For vesicle (DMSO) treated control J774A.1 cells, LPS was noted to stimulate significant TNFα secretion from J774A.1 cells within compound **7** pretreated cells, whereas LPS-induced TNFα secretion by J774A.1 cells to culture supernatants was concomitantly clearly reduced (**Figure 5**). However, compounds **2**–**4**, **8**, and **9** were not able to reduce LPS-induced TNFα secretion by J774A.1 cells (**Figure 5**). Further, to investigate the effect of these cyclic peptides on IL-6 secretion from LPS-stimulated J774A.1 cells, IL-6 secretion was monitored within J774A.1 cells stimulated by LPS in the presence or absence of cyclic peptides for 6 h. Similar to the results of TNFα secretion, only compound **7** significantly reduced IL-6 secretion from LPS-stimulated J774A.1 cells (**Figure 5**).

Following this, we tested the dose–response of compound **7** on reducing TNFα and IL-6 secretion from LPS-stimulated J774A.1 cells. We found that LPS-induced TNFα concentration in supernatant dropped to levels of about 60, 40, 30, and 20% by **7** pretreatment at a levels of 5, 10, 30, and 50 μg/mL, respectively, as compared to vesicle pretreatment (**Figure 6**). In addition, upon treatment with **7** (5, 10, 30, and 50 μg/mL), the IL-6 production by J774A.1 cells was inhibited in a dosage-dependent fashion (50, 30, 10, and 10%, respectively, as compared to vesicle pretreated cells; **Figure 6**).

We investigated whether the inhibitory effect of compound **7** is specific for LPS-induced responses. Studies have revealed that TLRs are the key molecules for recognizing pathogen-associated molecular patterns in order to elicit inflammatory responses. Therefore, J774A.1 cells were stimulated with Pam3Cys, a synthetic component that binds to TLR2, and the effect of compound **7** on cytokines production was examined. As shown in **Figure 7**, compound **7** inhibited TNFα and IL-6 secretion from Pam3Cys-stimulated J774A.1 cells in a dosage-dependent fashion.

Although several cyclic peptides have been isolated from Annonaceous plants, the biological function studies of these peptides are still rare. In this study, we elucidated two new cyclic peptides (**1** and **2**) and the configuration isomers, squamin A (**3**) and B (**4**), by spectroscopic interpretation. The previous studies told us the major active components in the seeds of *A. squamosa* are annonaceous acetogenins, which showed strong antitumor activities (*5*). The anti-inflammatory effect of **7,** the major cyclic peptide in the seeds of *A. squamosa*, was revealed. The results deduce that **7** can inhibit TNFα and IL-6 production by J774A.1 cells that were stimulated with LPS and Pam3Cys, and the action target is suggested to be the TLR2/4 receptors. This hint could inspire us to synthesize the analogues of compound **7** for further pharmaceutical and medicinal chemical investigations.

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Supporting Information Available: ^{1}H NMR and HSQC spectra of **1** and **2**; ^{1}H and ^{13}C NMR data of **3**-**5**; ESI-MS/MS data, ^{1}H-^{1}H COSY, and HMBC correlations of **3** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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