Cyclopeptides with Anti-inflammatory Activity from Seeds of *Annona montana*

Pei-Hsuan Chuang,[†] Pei-Wen Hsieh,[‡] Yu-Liang Yang,[§] Kuo-Feng Hua,[§] Fang-Rong Chang,[†] Jentaie Shiea,[⊥] Shih-Hsiung Wu,[§] and Yang-Chang Wu*[,†,⊥,∥]

Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China, Graduate Institute of Natural Products, Chang Gung University, Tao-Yuan 333, Taiwan, Republic of China, Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan, Republic of China, National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center, Kaohsiung 804, Taiwan, Republic of China, and Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China

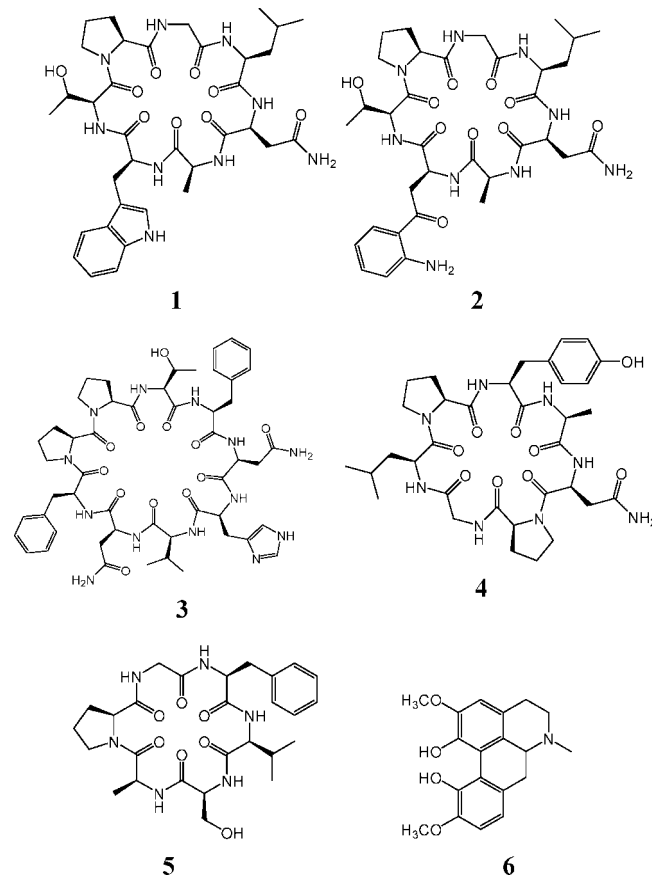
Received February 26, 2008

Four new cyclopeptides, cyclomontanins A−D (**1**−**4**), annomuricatin C (**5**), and (+)-corytuberine were isolated from a methanol extract of *Annona montana* seeds. Their structures were elucidated by 2D NMR analysis, ESIMS/MS fragment evidence, and chemical means. The structure of **1** was confirmed by synthesis. Compounds **1**, **3**, and **4** exhibited anti-inflammatory activity *in vitro* using the J774.1 macrophage model.

Annonaceae is a large family of tropical and subtropical trees and shrubs, comprising 130 genera and about 2300 species.[1,2] Many natural products, including alkaloids,[3–6] *ent*-kauranes,[7,8] flavonoids,[9,10] annonaceous acetogenins,[2] and cyclopeptides,[11] have been isolated from annonaceous plants. These products show biological effects such as cytotoxicity,[2,12] antimalarial,[2,3] pesticidal,[2,13] antiplatelet,[9] anti-inflammatory,[7,8] and vasorelaxant activities.[14] Recently, we reported two new cyclopeptides, cyclosquamosins H and I, from the seeds of *Annona squamosa* and found that cyclosquamosin D showed anti-inflammatory activity using the *in vitro* J774.1 macrophages model.[15]

Annona montana Macf. (Annonaceae), also called mountain soursop, is distributed mainly in tropical America, the West Indies, and southern Taiwan.[1] No cyclopeptides have previously been reported from this species. In our continuing research on annonaceous cyclopeptides, four new cyclopeptides, **1**−**4**, one known cyclopeptide, annomuricatin C (**5**), and one known alkaloid, (+)-corytuberine (**6**), were obtained from a methanol extract of *A. montana* seeds. Compound **5** was isolated from this plant for the first time.[16] All structures were confirmed by spectroscopic and chemical evidence. The structure of **1** was also established by total synthesis using a solid-phase synthetic method.

Lipopolysaccharide (LPS) is an outer membrane component of Gram negative bacteria that binds to toll-like receptor 4 (TLR4). LPS stimulation effects severe biological responses within host cells, including fever, procoagulant activity, septic shock, and death.[17–19] However, the stimulation of LPS involved in nitrogen intermediates, prostaglandins, and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, both *in vivo* and *in vitro*, is mediated through activation of the host immune and inflammatory cells. TNF-α and IL-6 are mediators in the inflammatory response involved in host defense.[17,20–23] In this study we investigated the anti-inflammatory activity of cyclopeptides using LPS- and Pam3Cys-stimulated macrophages, an *in vitro* model.



Results and Discussion

Cyclomontanin A (**1**) was obtained as a white powder. The molecular formula was determined to be $C_{35}H_{49}N_9O_9$ by HRESIMS at *m/z* 762.3555 $[M + Na]^+$ (calcd 762.3551). The IR absorptions at 3315, 1659, and 1522 cm^{-1} were attributed to amino, amide carbonyl, and aromatic groups, respectively. The ^1H spectrum had eight amide NH signals and an indole NH at δ 8.37 (d), 8.56 (s), 8.73 (d), 8.76 (d), 9.16 (d), 9.28 (d), 9.42 (s), 10.34 (dd), and 11.75 (d), respectively, and the ^{13}C NMR spectrum (pyridine-d_5) showed eight amide carbonyls at δ 169.6, 170.0, 172.0, 172.4, 172.6, 172.8, 172.9, and 174.8, indicating that **1** was a peptide. Marfey's method indicated that the amino acids all had L-configuration.[7,8] Complete assignments for ^1H and ^{13}C NMR signals (Table 1) were assigned using a combination of 2D NMR experiments (COSY, TOCSY, and HMQC spectra).

The sequence and connectivity of the amino acid residues were deduced by ESIMS/MS analysis (Figure 1) and HMBC experiment

* To whom correspondence should be addressed. Tel: (+886)-(0)7-312-1101, ext. 2197. Fax: (+886)-(0)7-311-4773. E-mail: yachwu@kmu.edu.tw.
† Graduate Institute of Natural Products, Kaohsiung Medical University.
‡ Chang Gung University.
§ Academia Sinica.
⊥ National SunYat-Sen University−Kaohsiung Medical University Joint Research Center.
∥ Center of Excellence for Environmental Medicine, Kaohsiung Medical University.

10.1021/np8001282 CCC: $40.75 © 2008 American Chemical Society and American Society of Pharmacognosy
Published on Web 08/08/2008

Table 1. ^{1}H and ^{13}C NMR Data of **1** and **2** (pyridine-d_5)

		1				**2**	
		δ_H (mult., *J* in Hz)	δ_C (mult.)			δ_H (mult., *J* in Hz)	δ_C (mult.)
Gly[1]	C=O		169.7 (s)	Gly[1]	C=O		169.8 (s)[a]
	NH	10.34 (dd,8.0,4.4)			NH	10.35 (br s)	
		3.59−3.99 (m)	43.8 (t)			3.87−3.97 (m)	43.8 (t)
		4.78 (dd,16.8,8.4)				4.77 (dd,16.8,8.4)	
Pro[2]	C=O		172.6 (s)	Pro[2]	C=O		172.6 (s)[a]
	α	4.41 (t,8.4)	61.8 (d)		α	4.46 (t,8.4)	61.8 (d)
	β	2.03 (m)	29.5 (t)		*β*	1.96 (m)	29.6 (t)
	γ	1.30 (m)	24.8 (t)		*γ*	1.30 (m)	24.8 (t)
		1.71 (m)				1.73−1.87 (m)	
	δ	3.59−3.99 (*m*)	48.7 (t)		*δ*	3.61−3.82 (m)	48.6 (t)
Thr[3]	C=O		170.0 (s)	Thr[3]	C=O		170.0 (s)[a]
	NH	8.37 (d,9.6)			NH	8.28 (d,9.6)	
	α	5.41 (t,8.8)	57.8 (d)		α	5.41 (t,8.8)	58.0 (d)
	β	4.54 (m)	69.0 (d)		*β*	4.57 (m)	69.4 (d)
	γ	1.63 (d,6.4)	20.2 (q)		*γ*	1.64 (d,6.0)	20.1 (q)
Trp[4]	C=O		172.9 (s)	Kyn[4]	C=O		172.9 (s)[a]
	NH	8.76 (d,7.2)			NH	8.91 (d,9.6)	
	α	5.73 (td,10.4,4.4)	55.6 (d)		α	6.01 (td,9.6,3.2)	51.6 (d)
	β	3.59−3.99 (m)	29.4 (t)		*β*	3.59−3.99 (m)	42.4 (t)
	Ar		109.9 (s)		*γ*C=O		199.5 (s)
		7.52 (d,2.0)	123.5 (d)		Ar		117.8 (s)
		7.55 (d,7.6)	111.9 (d)			7.86 (d,8.0)	132.0 (d)
		7.18 (t, 7.6)	119.0 (d)			6.50 (dd,8.0)	115.1 (d)
		7.25 (t,7.6)	121.5 (d)			7.23−7.28 (m)	134.6 (d)
		7.83 (d,7.6)	119.1 (d)			6.92 (t,8.0)	117.5 (d)
			137.3 (s)				152.4 (s)
			128.9 (s)		NH_2	7.80 (br s)	
		11.75 (d,1.6)					
Ala[5]	C=O		172.8 (s)	Ala[5]	C=O		172.8 (s)[a]
	NH	9.28 (d,3.2)			NH	9.48 (br s)	
	α	4.47 (m)	52.9 (d)		α	4.57 (m)	53.1 (d)
	β	1.13 (d,7.2)	16.6 (q)		*β*	1.35 (d,7.2)	17.0 (q)
Asn[6]	C=O		172.0 (s)	Asn[6]	C=O		172.0 (s)[a]
	NH	9.16 (d,5.2)			NH	9.13 (d,4.8)	
	α	5.05 (d,2.8)	51.1 (d)		α	5.05 (m)	51.3 (d)
	β	3.59−3.99 (m)	36.0 (t)		*β*	3.61−3.97 (m)	36.3 (t)
	*γ*C=O		174.8 (s)		*γ*C=O		174.9 (s)
	NH_2	8.56 (s)			NH_2	8.75 (m)	
		9.42 (s)				9.41 (s)	
Leu[7]	C=O		172.4 (s)	Leu[7]	C=O		172.4 (s)[a]
	NH	8.73 (d,7.2)			NH	8.75 (m)	
	α	5.41 (t,8.8)	54.3 (d)		α	5.4 (t,8.8)	54.4 (d)
	β	1.71 (m)	44.4 (t)		*β*	1.73−2.10 (m)	44.2 (t)
		1.91 (m)			*γ*	1.96 (m)	25.0 (d)
	γ	1.91 (m)	24.9 (d)		*δ*	0.89 (d,6.0)	22.4 (s)
	δ	0.89 (d,6.0)	22.3 (s)			0.90 (d,6.0)	22.4 (s)
		0.91 (d,6.0)	22.5 (s)				

[a] Interchangeable.

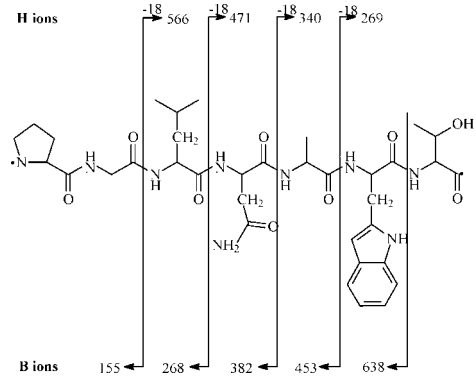


Figure 1. ESIMS/MS fragments of **1**.

(Figure 2). The protonated molecular ion $[M + H]^+$ of **1** (*m*/*z* 740) was subjected to ESIMS/MS. The ring opening began at the Thr-Pro amide bond and gave a series of adjacent *B* ions at *m*/*z* 638, 453, 382, 268, and 155 corresponding to the successive loss of Thr, Trp, Ala, Asn, and Leu from C-terminal to N-terminal and yielded the fragment of N-terminal dipeptide [Gly-Pro]. Another series of adjacent *H* ions (i.e., *Y* ion-H_2O) at *m*/*z* 566, 471, 340, and 269 were obtained from N-terminal to C-terminal of the breaking Thr-Pro amide bond, corresponding to the successive loss of [Gly-Pro], Leu, Asn, and Ala, yielding the fragment of C-terminal

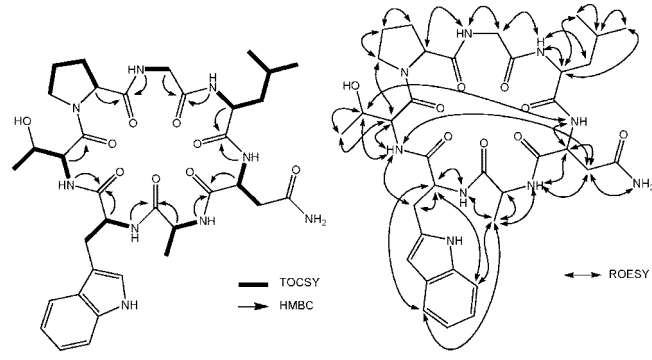
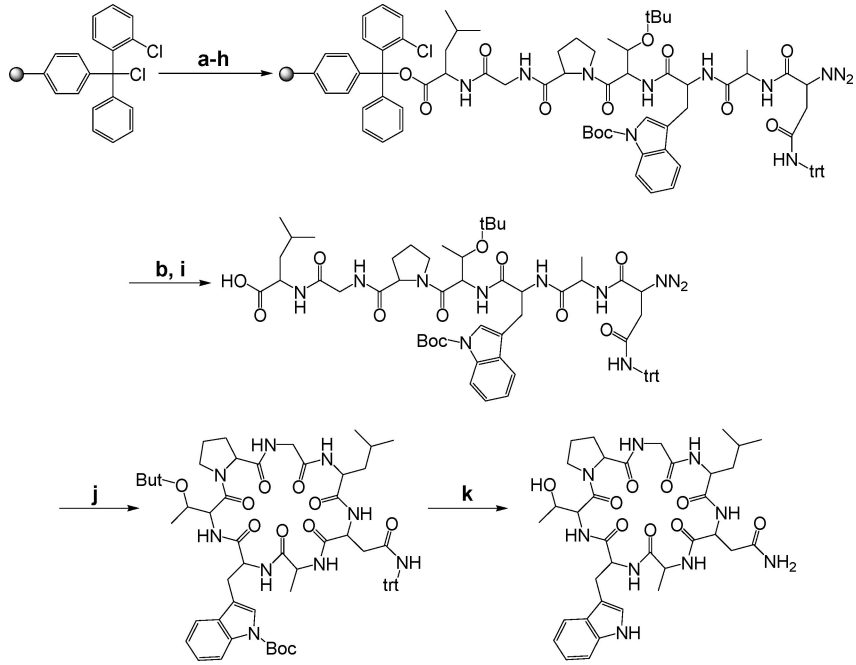


Figure 2. Significant TOCSY, HMBC, and ROESY correlations of **1**.

Scheme 1. Synthetic Route for **1**[a]



[a] Reagents and conditions: (a) Fmoc-Leu-OH, DIEA, DCM, room temperature, 2 h; (b) 20% piperidine in DMF; (c) Fmoc-Gly-OH, HBTU, HOBt, DMF, room temperature, 3 h; (d) Fmoc-Pro-OH, HBTU, HOBt, DMF, room temperature, 3 h; (e) Fmoc-Thr(tBu)-OH, HBTU, HOBt, DMF, room temperature, 3 h; (f) Fmoc-Trp(Boc)-OH, HBTU, HOBt, DMF, room temperature, 3 h; (g) Fmoc-Ala-OH, HBTU, HOBt, DMF, room temperature, 3 h; (h) Fmoc-Asn(Trt)-OH, HBTU, HOBt, DMF, room temperature, 3 h; (i) AcOH/TFE/DCM, room temperature, 2 h; (j) HATU, DIEA, DCM, room temperature, 24 h; (k) TFA/TIS/H_2O, room temperature, 1 h.

dipeptide [Trp-Thr]-H_2O. These results suggested that the partial amino acid sequence of **1** was Thr-Trp-Ala-Asn-Leu. The sequence of **1** was determined by HMBC and ROESY correlations (Figure 2), which elucidated the amino acid sequence of **1** as Gly^1-Pro^2-Thr^3-Trp^4-Ala^5-Asn^6-Leu^7. In addition, the difference of ^{13}C NMR chemical shifts of Pro^2 ($\Delta_{C\beta\text{-}C\gamma} = 7.6$ ppm) suggested that the amide bond in the Pro^2 residue was *trans*.[24] Thus, the structure of **1** was assigned as *cyclo*(-Gly^1-*trans*Pro^2-Thr^3-Trp^4-Ala^5-Asn^6-Leu^7-).

In accordance with the same amino acid sequence as mentioned above, cyclomontanin A (**1**) was synthesized using Fmoc/t-Bu chemistry and a 2-chlorotrityl chloride resin as solid support (Scheme 1).[25,26] The knit sequence was coupled with hydroxybenzotriazole/*O*-(benzotriazol-1-yl)-*N,N,N′,N′* -tetramethyluronium hexafluorophosphate (HOBt/HBTU) in *N,N*-dimethylformamide (DMF), and the cyclization was allowed to proceed in solution using *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIEA) in dichloromethane (DCM). After the side-chain protecting groups were removed with 95% aqueous trifluoroacetic acid (TFA), the crude cyclopeptide was purified by preparative RP-HPLC, which yielded **1**, and was identified on the basis of ESIMS/MS and ^1H NMR data.

Cyclomontanin B (**2**) was obtained as a pale yellow powder and showed a pseudomolecular ion peak at *m/z* 766.3495 $[M + Na]^+$ (calcd 766.3500) on HR-ESIMS, corresponding to the molecular formula $C_{35}H_{49}N_9O_9$. IR absorptions indicated the presence of amine (3316 cm^{-1}), amide carbonyl (1662 cm^{-1}), and aromatic groups (1521 cm^{-1}). Ten amide (NH) and eight amide carbonyl signals were observed in its ^1H and ^{13}C NMR spectra (Table 1), indicating a peptide. Analysis of COSY, TOCSY, and HMQC spectra demonstrated that the amino acid residues were Gly, Pro, Thr, Ala, Asn, Leu, and Kyn (kynurenine; a tryptophan metabolite[27]). Using Marfey's method, the amino acid residues were established to have L-configurations.[7,8] The chemical shift difference between C_β and C_γ of Pro^2 was 4.8 ppm; thus, the Pro^2 residue in **2** had *trans* geometry.[24]

The ESIMS/MS data of **2** at *m/z* 744 $[M + H]^+$ indicated preferential opening at the Thr-Pro amide bond level to obtain one series of adjacent *B* ions (at *m/z* 643, 453, 382, 268, and 155) and one series of adjacent *H* ions (*Y* ion-H_2O at *m/z* 571, 458, and 291). The amino acid residues were lost in the order Thr, Kyn, Ala, Asn, Leu, and the terminal dipeptide [Gly-Pro] sequentially from the C-terminal to N-terminal. Furthermore, the ions at *m/z* 545, 356, and 284 were attributed to the fragments [Kyn-Ala-Asn-Leu-Gly], [Ala-Asn-Leu-Gly], and [Asn-Leu-Gly], respectively. The ion at *m/z* 425 indicated the fragment [Pro-dehydroThr-Kyn-Ala]-NH. Correlation of HMBC and ROESY spectra proved that the sequence of **2** was *cyclo*(-Gly^1-*trans*Pro^2-Thr^3-Kyn^4-Ala^5-Asn^6-Leu^7-). According to the negative CD bands at 222.3 and 198.9 nm, compound **2** was proposed to have an α-helix conformation.[28] The amino acid sequence of **2** was similar to that of **1**, except for Kyn and Trp.

The molecular formula of **3** was established as $C_{51}H_{67}N_{13}O_{12}$ by HR-ESIMS, which showed a pseudomolecular ion peak at *m/z* 1054.5106 $[M + H]^+$ (calcd 1054.5110). IR absorptions at 3291, 1670, and 1524 cm^{-1} indicated the presence of amino, amide carbonyl, and aromatic groups, respectively.

Since the ^1H NMR spectrum of **3** showed better resolution at 320 K than at room temperature, the following NMR experiments were carried out under this condition. However, correlations of 2D NMR spectra were difficult to interpret, especially HMBC and ROESY; thus the sequence of **3** was elucidated by ESIMS/MS data. ESIMS/MS on the $[M + H]^+$ ion of **3** yielded preferential ring opening at the Phe-Asn amide bond and gave relative *B* ions of peptide fragments at *m/z* 1055, 939, 841, 704, 509, 443, and 342; one series of adjacent *H* ions at *m/z* 921, 823, 687, 572, and 425; and one series of adjacent *A* ions at *m/z* 910, 813, 562, 413, and 314. On the basis of the fragment data, amino acid residues were lost sequentially from the C-terminal to N-terminal, corresponding to the successive loss of Asn, Val, His, Asn, Phe, Thr, and the terminal tripeptide [Pro-Pro-Phe]. Opening at the same amide bond level, a series of *Y* ions at *m/z* 713, 612, and 465 agreed with successive loss of [Phe-Pro-Pro], Thr, and Phe from N-terminal to

Table 2. ^{1}H and ^{13}C NMR Data of **3** (320 K; pyridine-d_5)

		δ_H (mult., *J* in Hz)	δ_C (mult.)			δ_H (mult., *J* in Hz)	δ_C (mult.)
Phe[1]	C=O		173.9 (s)	Phe[5]	C=O		171.8 (s)[a]
	NH	8.77 (m)			NH	9.57 (br s)	
	α	5.05 (m)	56.6 (d)		α	5.28 (m)	57.2 (d)
	β	3.19 (m)	38.0 (t)		*β*	3.73 (m)	35.1 (t)
		3.48 (m)			Ar		139.2 (s)
	Ar		138.4 (s)			7.51 (d, 7.5)	130.5 (d)
		7.34 (d, 7.5)	129.6 (d)			7.29 (t, 7.5)	128.5 (d)
		7.23 (t, 7.5)	128.8 (d)			7.16 (m)	126.5 (d)
		7.16 (m)	126.9 (d)	Asn[6]	C=O		173.6 (s)
Pro[2]	C=O		170.9 (s)		NH	8.52 (m)	
	α	3.63 (m)	58.9 (d)		α	5.28 (m)	51.9 (d)
	β	1.67 (m)	28.6 (t)		*β*	3.19 (m)	38.3 (t)
		1.89 (m)				3.30 (m)	
	γ	1.75 (m)	25.2 (t)		*γ*C=O		173.2 (s)[a]
		1.89 (m)			NH_2		
	δ	3.63 (m)	48.0 (t)	His[7]	C=O		171.8 (s)[a]
		3.68 (m)			NH	9.49 (m)	
Pro[3]	C=O		171.3 (s)[a]		α	5.23 (m)	55.7 (d)
	NH				*β*	3.63 (m)	27.6 (t)
	α	4.35 (m)	61.4 (d)			3.68 (m)	
					Ar		136.0 (s)
	β	1.89 (m)	30.8 (t)			7.41 (*br s*)	118.3 (d)
						8.58 (br s)	134.4(d)
		2.58 (m)			NH	7.70 (br s)	
	γ	1.53 (m)	22.3 (t)	Val[8]	C=O		170.5 (s)
		1.75 (m)			NH	7.95 (d, 8.5)	
	δ	3.30 (m)	47.1 (t)		α	4.95 (t, 8.0)	56.2 (d)
		3.48 (m)			*β*	2.28 (m)	32.7 (d)
Thr[4]	C=O		172.2 (s)		*γ*	1.11 (d, 6.5)	18.4 (q)
	NH	8.27(br s)				1.21 (d, 6.5)	19.6 (q)
	α	4.47 (t, 5.0)	62.5 (d)	Asn[9]	C=O		172.6 (s)
	β	4.36 (m)	66.9 (d)		NH	9.26 (d, 6.5)	
	γ	1.32 (d, 6.5)	21.2 (q)		α	5.68 (m)	52.7 (d)
					β	3.33 (m)	39.2 (t)
					*γ*C=O		173.9 (s)[a]
					NH_2		

[a] Interchangeable.

C-terminal and yielded the fragment of C-terminal tetrapeptide [Asn-His-Val-Asn]. Furthermore, another series of adjacent *Y* ions at *m*/*z* 955, 858, 758, 612, and 496 were obtained from N-terminal to C-terminal of the breaking Pro-Phe amide bond, corresponding to the successive loss of Pro, Pro, Thr, Phe, and Asn and yielding the C-terminal tertapeptide [His-Val-Asn-Phe]. Thus, the structure of **3** was established as *cyclo*(-Phe[1]-Pro[2]-Pro[3]-Thr[4]-Phe[5]-Asn[6]-His[7]-Val[8]-Asn[9]-). The differences in the ^{13}C NMR chemical shifts of Pro[2] ($\Delta_{C\beta\text{-}C\gamma}$ = 3.4 ppm) and Pro[3] ($\Delta_{C\beta\text{-}C\gamma}$ = 8.5 ppm) suggested that the amide bonds in the Pro[2] and Pro[3] residues in **3** were *trans* and *cis*, respectively.[24] Finally, based on Marfey's analysis, each amino acid residue configuration in **3** was L.[7,8]

IR absorptions at 3321 and 1676 cm^{-1} indicated amide bonds in the structure of **4**. The molecular formula was determined to be $C_{34}H_{48}N_8O_9$ by HR-ESIMS. Analysis of 2D NMR spectra, COSY, TOCSY, and HMQC demonstrated that the amino acid residues of **4** were Pro × 2, Ala, Tyr, Leu, Gly, and Asn, and all were L configuration based on Marfey's analysis.[7,8]

The ESIMS/MS data on the $[M + H]^+$ of **4** showed preferential ring opening at the Pro-Asn amide bond and gave relative *Y* ions of peptide fragments at *m*/*z* 614, 557, 446, 348, 186, and 115, together with a series of adjacent *B* ions at *m*/*z* 598, 268, and 155, corresponding to the successive loss of Pro, Gly, Leu, Pro, Tyr, and Ala from N-terminal to C-terminal and yielded a fragment of Asn. Furthermore, one series of adjacent *A* ions at *m*/*z* 569, 500, 335, 240, and 127 and adjacent *X* ions series at *m*/*z* 585, 472, and 142 were obtained from C-terminal to N-terminal of the breaking of the same amide bond, corresponding to the successive loss of Asn, Ala, Tyr, Pro, Leu, Gly, and yielded Pro. Therefore, the structure of **4** was determined to be *cyclo*-(Pro[1]-Gly[2]-Leu[3]-Pro[4]-Tyr[5]-Ala[6]-Asn[7]). The *trans* amide bond in the Pro[2] residue was indicated by the ^{13}C NMR chemical shift of Pro[2] ($\Delta\delta_{C\beta\text{-}C\gamma}$ = 8.6 ppm).[24]

Annomuricatin C (**5**) was initially isolated from *A. muricata* seeds in 2004.[16] According to this report, the major conformation (>85%, 318 K, in DMSO-d_6) of **5** was determined and was found to be cytotoxic against KB cells with an IC_{50} of 1.0 μM. However, our compound **5** showed a 2:1 ratio between major/minor conformations (318 K, in DMSO-d_6) and showed no cytotoxicity against Hep G2, Hep 3B, MDA-MB-231, MCF-7, A549, and Ca9-22 cell lines.

In order to investigate whether cyclopeptides **1**, **3**, **4**, and **5** exhibited immunomodulation activity in cultured murine macrophage J774A.1 cells, the cells were pretreated with various cyclopeptides (30 μg/mL) for 30 min at 37 °C, followed by LPS challenge for 6 h. DMSO-treated J774A.1 cells were used as controls. LPS was noted to stimulate significant levels of TNF-α and IL-6 secretions; in cyclopeptide **1**, **3**, **4**, and **5** pretreated cells, LPS-induced secretion of TNF-α and IL-6 was decreased by about 50−80% (Figure 3).

To further investigate the dose-dependent activity of cyclopeptides **1**, **3**, **4**, and **5**, J774A.1 cells were pretreated with various concentrations of cyclopeptide **1**, **3**, **4**, or **5** for 30 min, followed by stimulation with LPS or Pam3Cys, a synthetic component binding to TLR2, for an additional 6 h. Cyclomontanin A (**1**) showed significant inhibition of TNF-α and IL-6 production at 1 and 3 μg/mL, respectively, even within LPS- or Pam3Cys-stimulated J774A.1 cells. Specifically, cyclomontanin C (**3**) showed a marked inhibition of TNF-α and IL-6 production in LPS- or Pam3Cys-stimulated J774A.1 cells at 3−50 μg/mL. Cyclomontanin D (**4**) and annomuricatin C (**5**) decreased the secretion of TNF-α and IL-6 in LPS-stimulated cells at 30 μg/mL. On stimulation with

Table 3. ^{1}H and ^{13}C NMR Data of **4** (pyridine-d_5)

		δ_H (mult., J in Hz)	δ_C (mult.)
Pro[1]	C=O		172.4 (s)[a]
	α	4.49 (m)	64.7 (d)
	β	2.26 (m), 2.05 (m)	30.2 (t)
	γ	1.92 (m), 1.69 (m)	26.4 (t)
	δ	3.80 (m), 3.63 (m)	48.3 (t)
Gly[2]	C=O		170.7 (s)[a]
	NH	10.04 (d, 6.0)/9.40 (br s)	
	α	4.93 (m)/4.49 (m)	43.7 (t)/44.2 (t)
		3.75 (m)/4.20 (m)	
Leu[3]	C=O		172.5 (s)[a]
	NH	8.01 (m)	
	α	4.93 (m)	54.8 (d)
	β	3.07 (m)/2.91 (m)	32.5 (t)/30.9 (t)
	γ	2.51 (m)	32.9 (d)/33.3 (d)
	δ	1.19 (d, 6.0)	19.2 (q)/19.0 (q)
		1.18 (d, 6.0)	20.5 (q)/21.8 (q)
Pro[4]	C=O		172.1 (s)[a]
	α	4.49 (m)	63.5 (d)/62.9 (d)
	β	2.05 (m), 1.92 (m)	30.1 (t)
	γ	1.69 (m), 1.56 (m)	25.6 (t)
	δ	3.68 (m), 3.59 (m)	48.6 (t)
Tyr[5]	C=O		173.6 (s)[a]
	NH	9.21 (br s)	
	α	5.38 (m)	58.9 (d)
	β	3.39 (dd, 13.0, 4.0), 3.27 (m)	40.2 (t)
	Ar		130.9/130.1 (s)
		7.98 (m)/7.98 (m)	131.0/131.3 (d)
		7.08 (d, 7.5)/7.19 (m)	116.2/116.3 (d)
			157.3/157.9 (s)
Ala[6]	C=O		172.2 (s)[a]
	NH	8.91 (br s)	
	α	4.84 (m)	51.4 (d)/53.2 (d)
	β	1.82 (d, 8.0)	18.3 (q)/18.2 (q)
Asn[7]	C=O		173.9 (s)[a]
	NH	8.68 (br s)	
	α	5.28 (m)	56.2 (d)
	β	3.65 (m)/3.12 (*dd*, 13.0, 4.0)	36.8 (t)
	γC=O		176.8 (s)[a]
	NH_2		

[a] Interchangeable.

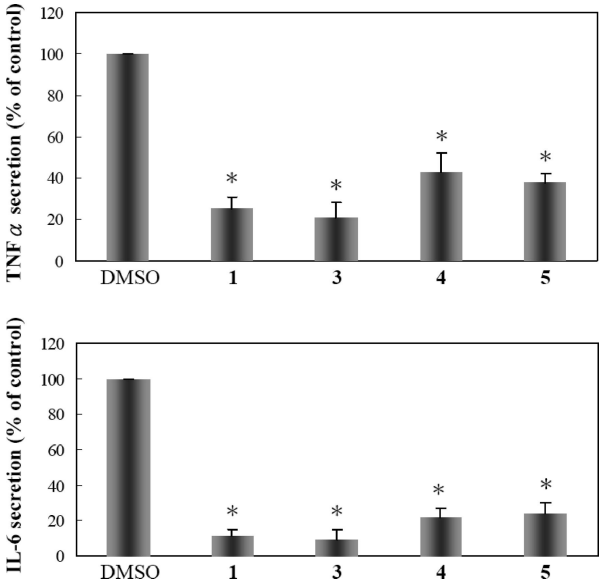


Figure 3. Anti-inflammatory effects of cyclopeptides **1**, **3**, **4**, and **5** at 30 μg/mL toward LPS-stimulated J774A.1 cells. *$p < 0.05$ versus DMSO.

Pam3Cys, cyclomontanin D (**4**) showed approximately 20%, 50%, 60%, 85%, and 90% inhibition of TNF-α production at 3, 5, 10, 30, and 50 μg/mL, respectively. Additionally, cyclomontanin D (**4**) and annomuricatin C (**5**) exhibited a dose−response relationship with IL-6 secretion in Pam3Cys-stimulated cells.

In conclusion, we isolated and elucidated four novel cyclopeptides (**1**−**4**) and demonstrated that cyclomontanin A (**1**), cyclomontanin C (**3**), cyclomontanin D (**4**), and annomuricatin C (**5**) exhibited anti-inflammatory effects within the LPS- or Pam3Cys-stimulated J774A.1 cells. These finding are worthy of further research to determine the mechanisms of these anti-inflammatory effects and to suggest additional synthetic analogues of cyclopeptides **1**, **3**, **4**, and **5** for further drug development investigation.

Experimental Section

General Experimental Procedures. Optical rotations were measured with Jasco DIP 370 and P-1020 digital polarimeters. IR and UV spectra were obtained on Mattson Genesis II infrared and Jasco UV-530 ultraviolet spectrophotometers, respectively. The CD spectra were measured on a Jasco J-720 spectropolarimeter. NMR (400 and 500 MHz, using pyridine-d_5 as solvent) spectra were collected on Varian Unity 400 MHz NMR, Varian Unity INOVA-500 NMR, and Bruker AVANCE II 500 NMR spectrometers. Low-resolution and high-resolution ESIMS were recorded on a Bruker APEX II spectrometer (FT-ICR/MS, FTMS). ESIMS^n were collected on a MicroMass Q-TOF ultima global mass spectrometer or a Bruker Esquire 3000 ion trap mass spectrometer. Shimadzu LC-10AT pumps, Shimadzu SPD-10A UV−vis detector, Ascentis C18 (250 × 4.6 mm i.d.; 5 μm)/preparative Ascentis C18 (250 × 21.2 mm i.d.; 5 μm) columns, and Develosil C30-UG-5 (250 × 4.6 mm i.d.; 5 μm)/preparative Develosil C30-UG-5 (250 × 20 mm i.d.; 5 μm) columns were employed for HPLC.

Plant Material. Seeds of *A. montana* were offered from the Taitung District Agricultural Research and Extention Station (TDARES), Taitung, Taiwan in September, 2006. The plant material was identified by Dr. Ching-Shan Yang (TDARES). A voucher specimen is deposited at the Graduate Institute of Natural Products (Annona-05), Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. The air-dried seeds (ca. 1.4 kg) of *A. montana* were crumbled, then extracted with MeOH at room temperature. The MeOH extract (122.5 g) was partitioned between *n*-hexane and 20% aqueous MeOH to yield *n*-hexane and 20% MeOH(aq) layers. The MeOH(aq) layer (105.8 g) was further partitioned between *n*-butanol and H_2O to give *n*-butanol, insoluble, and water layers. The *n*-butanol layer (60.8 g) was separated on Diaion HP-20 with a gradient of 10% to 100% MeOH and acetone to yield five fractions (A−E). Fraction C (2.7 g) was separated using a flash column with 85% MeCN(aq) to afford three subfrations (C-1−3). Subfraction C-1 was further separated on a flash column eluting with 85% MeCN(aq) to afford five subfrations (C-1-1−5). Subfractions C-1-2 and C-1-4 were purified by an open column and HPLC (Develosil C30-UG-5, 70% MeCN(aq) with 0.5% TFA) to yield **6** (18.2 mg) and **3** (3.1 mg), respectively. Subfraction C-2 was purified by preparative reversed-phase HPLC (Ascentis C18 column, 75% MeCN(aq)) to obtain **1** (44.3 mg) and **2** (4.3 mg). Subfraction C-3 was separated to four fractions (C-3-1−4) by preparative RP-HPLC (Ascentis C18 column, 65% MeCN(aq)) and than separated by HPLC (Develosil C30-UG-5 column, 75% MeCN(aq) with 0.5% TFA) to afford **3** (126.9 mg), **4** (15.6 mg), and **5** (14.4 mg).

Cyclomontanin A (1): white powder; $[\alpha]^{26}_D$ −37.7 (*c* 0.0017, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.50), 218 (4.50), 279 (3.66) nm; CD ($c = 1.7 \times 10^{-4}$ M, MeOH) λ_{max} (Δε) 252.2 (+1.67), 219.2 (−1.37), 197.5 (−7.72) nm; IR (neat) ν_{max} 3315, 1659, 1622, 1522, 1458 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS/MS *m*/*z* 638, 566, 471, 453, 382, 340, 269, 268, 155; HRESIMS *m*/*z* 762.3555 [M + Na]$^+$ (calcd for $C_{35}H_{49}N_9O_9Na$, 762.3551).

Cyclomontanin B (2): pale yellow powder; $[\alpha]^{26}_D$ −60.0 (*c* 0.006, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.54), 228 (4.33), 259 (3.71), 363 (3.49) nm; CD ($c = 1.5 \times 10^{-4}$ M, MeOH) λ_{max} (Δε) 275.6 (+1.24), 249.8 (+1.40), 222.3 (−4.80), 198.9 (−4.46) nm; IR (neat) ν_{max} 3316, 1662, 1521, 1450 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; ESIMS/MS *m*/*z* 643, 571, 545, 458, 453, 425, 382, 356, 291, 284, 268, 155; HRESIMS *m*/*z* 766.3495 [M + Na]$^+$ (calcd for $C_{34}H_{49}N_9O_{10}Na$, 766.3500).

Cyclomontanin C (3): pale yellow power; $[\alpha]^{27}_D$ −70.0 (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ε) 212 (3.91), 254 (3.07) nm; CD ($c = 1.4 \times 10^{-4}$ M, MeOH) λ_{max} (Δε) 255.1 (+1.34), 230.8 (−8.95), 222.8

(−9.96), 205.1 (−11.39) nm; IR (neat) ν_{max} 3291, 1670, 1524, 1439 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; ESIMS/MS *m/z* 955, 939, 921, 910, 858, 841, 823, 813, 758, 713, 704, 687, 612, 572, 562, 509, 496, 465, 443, 425, 413, 342, 314; HRESIMS *m/z* 1054.5106 $[M + H]^+$ (calcd. for $C_{51}H_{68}N_{13}O_{12}$, 1054.5110).

Cyclomontanin D (4): white power; $[\alpha]^{27}_D$ −41.2 (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ε) 216 (3.89), 255 (3.15), 278 (3.04) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 254.7 (+1.67), 221.2 (−10.23), 200.0 (+10.81) nm; IR (neat) ν_{max} 3321, 1676 cm^{-1}; ^1H and ^{13}C NMR data, see Table 3; ESIMS/MS *m/z* 614, 598, 585, 569, 557, 500, 472, 446, 348, 335, 268, 240, 186, 155, 142, 127, 115; HRESIMS *m/z* 735.3438 $[M + Na]^+$ (calcd. for $C_{34}H_{48}N_8O_9Na$, 735.3442).

Hydrolysis and Derivatization of 1−5 (Marfey's Method).[29,30] Compounds **1**−**5** (0.1 mg) were each dissolved in 6 N HCl (0.5 mL) in a sealed tube and heated at 130 °C for 12−16 h. After cooling and drying, the total hydrolysates were dissolved in 300 μL of 1 M $NaHCO_3$ solution and reacted with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA or Marfey reagent, 1% in acetone, 250 μL) at 50 °C for 1 h. Finally, the Marfey's derivatives were analyzed by HPLC (Ascentis C18, 5 μm, 250 × 4.6 mm i.d.; MeCN/H_2O (0.5% TFA) = 30/70; UV detection at 340 nm) and compared to Marfey's derivatives of amino acid standards.

Synthesis of 1 (Solid-Phase Synthesis Method).[25,26] 2-Chlorotrityl chloride resin was purchased from Aldrich. The Fmoc-L-amino acids with or without other protecting groups (such as Boc, t-Bu, and trityl groups) and the coupling reagents (HOBt, HBTU, HATU) were supplied by Novabiochem, Aldrich, or Fluka. The procedure is described below: (1) The resin (0.6 g), Fmoc-Leu-OH (219 mg), and DIEA (0.3 mL) in DCM (10 mL/g of resin) were reacted for 2 h, then capped with 20 mL of DCM, MeOH, and DIEA (17:2:1) and finally washed with 3 mL of DCM and 3 mL of DMF at least three times. (2) The Fmoc group of the mixture was deprotected by 20% piperidine in DMF (3 mL) for 1.5 min and 3 mL of 20% piperidine in DMF for 10 min and finally washed in 3 mL of DCM and 3 mL of DMF at least three times. (3) HOBt (85 mg, 4 equiv), HBTU (234 mg, 4 equiv), and Fmoc-AA-OH (4 equiv) were added to the mixture in DMF (3 mL) for 3 h, then washed with 3 mL of DCM and 3 mL of DMF at least three times and deprotected. The coupling and deprotecting steps were repeated, and the expected amino acid sequence was elongated. (4) The expected sequence was cleaved from the resin using AcOH/TFE(trifluoroethanol)/DCM (2:2:6) solution for 2 h. The cleavage mixture was filtered off and washed twice with the same solution. The filtrate was then dried and weighed and was found to be 89 mg. (5) The mixture was separated on Sephadex LH-20 and eluted with MeOH to yield the crude product (79 mg). (6) Cyclization was carried out using the crude product, HATU (53 mg), and DIEA (39 μL) was dissolved in DCM (1 L) under an ice bath for 1 h and then warmed to room temperature followed by continuous stirring for 24 h. (7) Solvent was evaporated to afford the product (160 mg). The product was treated with TFA/TIS(triisopropylsilane)/H_2O (95:2.5:2.5) for 1 h. (8) The final product was dried and purified by preparative RP-HPLC [solvent system 75% MeCN(aq); UV detection at 230 nm; Ascentis C18 (250 × 10.0 mm i.d.; 5 μm) column; flow rate 4.0 mL/min], which then gave **1** (t_R 35.6 min).

Anti-inflammatory Activity Assays. 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). For anti-inflammatory activity assay, cells (1 × 10^6/mL) were preincubated with various concentrations of cyclopeptides **1**, **3**, **4**, and **5** at 37 °C for 30 min, followed by stimulating with LPS (0.1 μg/mL) for an additional 6 h. TNF-α and IL-6 concentration in culture media were assayed by enzyme-linked immunosorbent assay (ELISA). In addition, J774A.1 cells were challenged with Pam3Cys (1 μg/mL), and the effect of **1**, **3**, **4**, and **5** on cytokine production was examined as explained above. All values are given as means ± SD. Data analysis involved one-way ANOVA with subsequent Scheffe's test.

Acknowledgment. This work was supported by a grant from the National Science Council of the Republic of China. The authors would like to thank Dr. C.-S. Yang, Taitung District Agricultural Research and Extension Station, Council of Agriculture, Taiwan, for collection and identification of *A. montana*.

Supporting Information Available: ESIMS/MS fragments for **2**, **3**, and **4**. The dose−response results of cyclopeptides **1**, **3**, **4**, and **5** on TNF-α and IL-6 production within LPS- and Pam3Cys-stimulated J774A.1 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Chen, W. C. *J. Trop. Subtrop. Bot.* **1995**, *3*, 19–35.
(2) Bermejo, A.; Figadere, B.; Zafra-Polo, M. C.; Barrachina, I.; Estornell, E.; Cortes, D. *Nat. Prod. Rep.* **2005**, *22*, 269–303.
(3) Padma, P.; Khosa, R. L.; Sahai, M. *Indian J. Nat. Prod* **1995**, *11*, 3–15.
(4) Bracher, F. *PZ Wiss.* **1992**, *5*, 109–117.
(5) Cavé, A.; Leboeuf, M.; Waterman, P. G.; Pelletier, S. W., Eds. In *Alkaloids: Chemical and Biological Perspectives*; Wiley: New York, 1987; Vol. 5, pp 133−270.
(6) Da Rocha, A. I.; Reis Luz, A. I.; Rodrigues, W. A. *Acta Amazon* **1981**, *11*, 537–546.
(7) Yeh, S. H.; Chang, F. R.; Wu, Y. C.; Yang, Y. L.; Zhuo, S. K.; Hwang, T. L. *Planta Med.* **2005**, *71*, 904–909.
(8) Yang, Y. L.; Chang, F. R.; Hwang, T. L.; Chang, W. T.; Wu, Y. C. *Planta Med.* **2004**, *70*, 256–258.
(9) Chang, F. R.; Wei, J. L.; Teng, C. M.; Wu, Y. C. *J. Nat. Prod.* **1998**, *61*, 1457–1461.
(10) Seetharaman, T. R. *Fitoterapia* **1986**, *57*, 198–199.
(11) Morita, H.; Sato, Y.; Kobayashi, J. *Tetrahedron* **1999**, *55*, 7509–7518.
(12) Alali, F. Q.; Liu, X. X.; McLaughlin, J. L. *J. Nat. Prod.* **1999**, *62*, 504–540.
(13) Fang, X. P.; Rieser, M. J.; Gu, Z. M.; Zhao, G. X.; McLaughlin, J. L. *Phytochem. Anal.* **1993**, *4*, 27–48.
(14) Morita, H.; Iizuka, T.; Choo, C. Y.; Chan, K. L.; Takeya, K.; Kobayashi, J. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4609–4611.
(15) Yang, Y. L.; Hua, K. F.; Chuang, P. H.; Wu, S. H.; Wu, K. Y.; Chang, F. R.; Wu, Y. C. *J. Argi. Food Chem.* **2008**, *56*, 386–392.
(16) Wélé, A.; Zhang, Y.; Caux, C.; Brouard, J. P.; Pousset, J. L.; Bodo, B. *C. R. Chim.* **2004**, *7*, 981–988.
(17) Martinez, C.; Delgado, M.; Pozo, D.; Leceta, J.; Calvo, J. R.; Ganea, D.; Gomariz, R. P. *J. Leukocyte Biol.* **1998**, *63*, 591–601.
(18) Meng, F.; Lowell, C. A. *J. Exp. Med.* **1997**, *185*, 1661–1670.
(19) Raetz, C. R. H.; Whitfield, C. *Annu. Rev. Biochem.* **2002**, *71*, 625–700.
(20) Ma, X. *Microbes. Infect.* **2001**, *3*, 121–129.
(21) Yoon, H. J.; Moon, M. E.; Park, H. S.; Im, S. Y.; Kim, Y. H. *Biochem. Biophys. Res. Commun.* **2007**, *358*, 954–959.
(22) Taga, T.; Kishimoto, T. *Annu. Rev. Immunol.* **1997**, *15*, 797–819.
(23) Marino, M. W.; Dunn, A.; Grail, D.; Inglese, M.; Noguchi, Y.; Richards, E. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8093–8098.
(24) Dorman, D. E.; Bove, F. A. *J. Org. Chem.* **1973**, *38*, 2379–2383.
(25) Napolitano, A.; Rodriquez, M.; Bruno, I.; Marzocco, S.; Autore, G.; Riccio, R.; Gomez-Paloma, L. *Tetrahedron* **2003**, *59*, 10203–10211.
(26) Norgren, A. S.; Buttner, F.; Prabpai, S.; Kongsaeree, P.; Arvidsson, P. I. *J. Org. Chem.* **2006**, *71*, 6814–6821.
(27) Wang, J.; Simonavicius, N.; Wu, X.; Swaminath, G.; Reagan, J.; Tian, H.; Ling, L. *J. Biol. Chem.* **2006**, *281*, 22021–22028.
(28) Johnson, W. C. *Annu. Rev. Biophys. Biophys. Chem.* **1988**, *17*, 145–166.
(29) Bhushan, R.; Bruckner, H. *Amino Acids* **2004**, *27*, 231–247.
(30) B'Hymer, C.; Montes-Bayon, M.; Caruso, J. A. *J. Sep. Sci.* **2003**, *26*, 7–19.

NP8001282