

Bioactive Compounds from the Aerial Parts of *Brachystemma calycinum* and Structural Revision of an Octacyclopeptide

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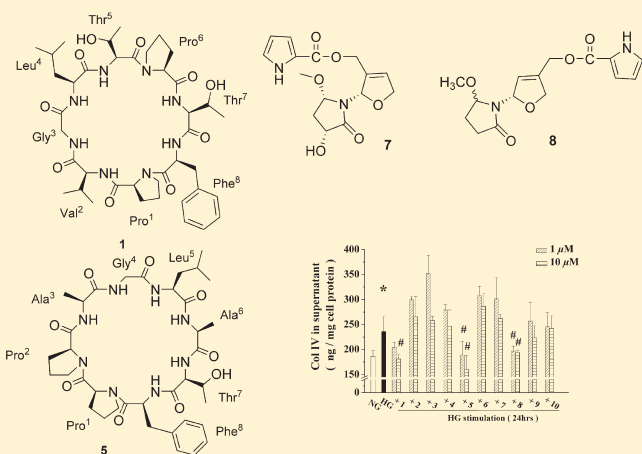
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S Supporting Information

ABSTRACT: Four new cyclic peptides, brachystemins F–I (1–4), and 11 known compounds were isolated from the aerial parts of *Brachystemma calycinum*. The absolute configurations of compounds 1–4 were assigned using Marfey's method. The structure of compound 5 was revised from *cyclo*-(Pro¹–Phe²–Leu³–Ala⁴–Thr⁵–Pro⁶–Ala⁷–Gly⁸) to *cyclo*-(Pro¹–Pro²–Ala³–Gly⁴–Leu⁵–Ala⁶–Thr⁷–Phe⁸) with QTOF/MS and X-ray diffraction analysis. The N-containing compounds were assessed for their inhibitory effects on the secretion of monocyte chemokine ligand 2 (CCL-2), interleukin 6 (IL-6), and collagen IV against high-glucose-stimulated mesangial cells. Compound 5 was evaluated for its effects on collagen I, reactive oxygen species (ROS), superoxide anion (O₂^{•-}) production, and cell viability in mesangial cells, and on nitric oxide (NO) production in macrophage cells.



Diabetic nephropathy (DN) has become a public health concern in recent years because it can develop into renal failure, with a high risk of morbidity and mortality. Diabetes mellitus is a primary driver of DN. With changes in lifestyle and dietary patterns, it is estimated that 7% of the United States population suffers from diabetes, and the incidence of diabetes mellitus continues to grow worldwide, which means that the number of patients with DN will increase in the coming years.¹ Although some preventive measures, such as glycemic and blood pressure control, are currently used to reduce the kidney damage attributable to kidney disease, the cause of DN is still not known, which makes the treatment of DN challenging. Recent experimental and clinical studies demonstrated that inflammation and oxidative stress are involved in the development and progression of DN.^{1–5} This pathogenic perspective will open new therapeutic approaches to DN, targeting inflammatory processes and oxidative stress.

Brachystemma calycinum D. Don (Caryophyllaceae) is the single species of the genus *Brachystemma*. It is a Chinese folk medicine used to treat rheumatoid arthritis, limb numbness, impotence, and gonorrhea.⁶ Previous studies showed that it contains alkaloids and cyclic peptides.^{7–9} An extract of the whole plant was recently found to be able to reduce disease symptoms

and the development of cartilagenous lesions in experimental dog osteoarthritis by inhibiting protease-activated receptor 2.¹⁰ Inflammatory and immune processes are implicated in rheumatism, and it may be possible to isolate anti-inflammatory or immunoregulatory compounds from *B. calycinum* that may be useful in the treatment of DN. Immunosuppressive alkaloids were characterized from the roots of this plant.⁹ As a continuation of our search for bioactive agents that will benefit patients with DN,^{11,12} 17 compounds were isolated from the aerial parts of this plant. The structure of a previously reported cyclic peptide was revised. The selected compounds were evaluated for their inhibitory effects on CCL-2, IL-6, and collagen IV secretion. In addition, the most active compound, 5, was further tested for the effects on collagen I, ROS, O₂^{•-} inhibition, and cell viability in mesangial cells and on NO production inhibition in macrophage cells.

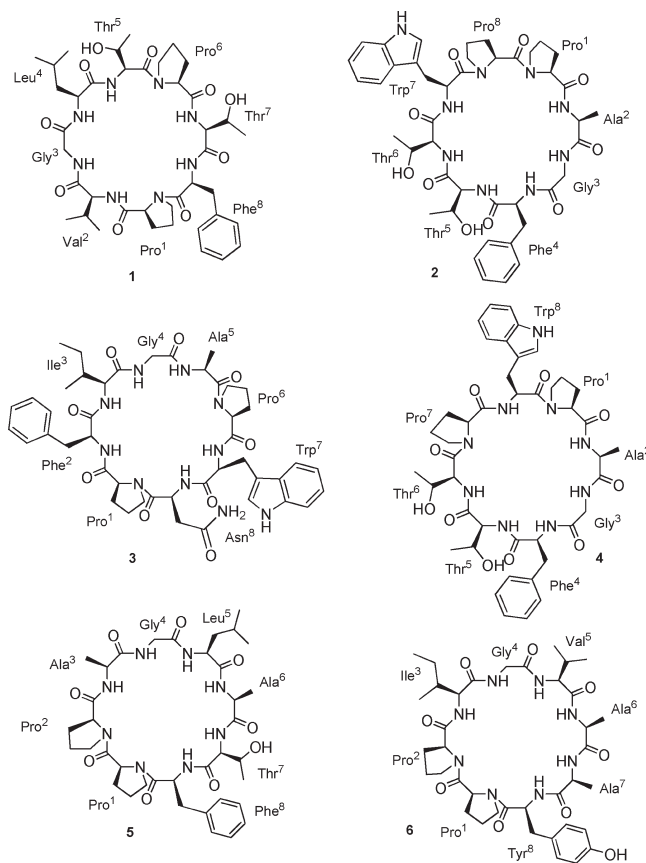
RESULTS AND DISCUSSION

Compound 1 was isolated as a white powder. Its molecular formula was determined to be C₄₀H₆₀N₈O₁₀ by the HRESIMS at

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m/z 835.4314 $[M + Na]^+$ (calcd 835.4330) and ^{13}C NMR and DEPT spectra, indicating 15 degrees of unsaturation. The IR absorptions at 3419, 1657, and 1526 cm^{-1} were ascribable to amino, amide carbonyl, and aromatic groups, respectively. The 1H NMR spectrum showed six amide NH resonances, and the ^{13}C NMR spectrum indicated eight amide carbonyls and eight α -amino acid carbons (Table 1), suggesting that **1** is a peptide. A negative response to the ninhydrin reagent but a positive response after its hydrolysis with 6 N aqueous HCl implied that **1** is a cyclic peptide. Analysis of 2D NMR data (COSY, HMQC, HMBC, and NOESY-ROESY spectra) permitted the assignment of the 1H and ^{13}C NMR resonances and also allowed the diagnosis of the eight amino acid residues as Pro ($\times 2$), Thr ($\times 2$), Leu, Gly, Val, and Phe.



The sequence of the amino acid residues was deduced with HMBC, ROESY, and QTOF/MS experiments. A peptide fragment, Gly–Leu–Thr, was evident from the HMBC correlations of Thr–NH/Leu–CO and Leu–NH/Gly–CO (Supporting Information, S29). The ROESY spectrum demonstrated the correlation Phe–NH/Pro– δ H (Supporting Information, S36). The protonated molecular ion $[M + H]^+$ of **1** (m/z 813) was subjected to QTOF/MS analysis. The preferred ring-opening occurred at Phe–Pro and gave a main series of adjacent $b_n(+1)$ ions at m/z 666, 565, 468, 367, 254, and 197 (Figure 1), corresponding to the successive loss of Phe, Thr, Pro, Thr, Leu, Gly, and the terminal dipeptide ion Pro–Val. Taking these data together, the sequence of **1** was determined to be *cyclo*-(Pro¹–Val²–Gly³–Leu⁴–Thr⁵–Pro⁶–Thr⁷–Phe⁸). Furthermore, the ^{13}C NMR chemical shift differences of Pro¹ ($\Delta\delta_{C\beta-C\gamma} = 3.2$ ppm) and Pro⁶ ($\Delta\delta_{C\beta-C\gamma} = 3.5$ ppm) indicated that the amide bonds in the two Pro residues were *trans*.^{13,14}

Compound **2** was isolated as an amorphous solid. It showed a quasimolecular ion at m/z 880.3976 $[M + Na]^+$ in the HRESIMS spectrum, in conjunction with the ^{13}C NMR and DEPT spectra, suggesting the molecular formula $C_{43}H_{55}N_9O_{10}Na$ (calcd 880.3969) with 21 degrees of unsaturation. The IR spectrum showed absorption bands of amine (3417 cm^{-1}), amide carbonyl (1659 and 1628 cm^{-1}), and aromatic (1513 cm^{-1}) groups. The 1H NMR spectrum showed seven amide NH resonances, and the ^{13}C NMR spectrum displayed eight amide carbonyl carbons. These data were indicative of a cyclic peptide. Using COSY, HMQC, and HMBC spectra, the eight amino acid residues were identified as Phe, Trp, Gly, Ala, Thr ($\times 2$), and Pro ($\times 2$). These residues accounted for 20 degrees of unsaturation, suggesting that **2** is a cyclic octapeptide. The sequence of **2** was assembled with HMBC and ROESY experiments. The HMBC spectrum showed correlations of Ala²–NH/Pro¹–CO, Gly³–NH/Ala²–CO, Phe⁴–NH/Gly³–CO, Thr⁵–NH/Phe⁴–CO, Thr⁶–NH/Thr⁵–CO, and Trp⁷–NH/Thr⁶–CO (Figure 2), corresponding to the peptide fragment Pro¹–Ala²–Gly³–Phe⁴–Thr⁵–Thr⁶–Trp⁷. The observed ROESY correlations of Trp⁷–H α /Pro⁸–H δ , Pro⁸–H α /Pro¹–H δ , and Pro¹–H α /Ala²–NH suggested the fragment Trp⁷–Pro⁸–Pro¹–Ala² (Figure 2). These data implied that the sequence of **2** is *cyclo*-(Pro¹–Pro²–Ala³–Gly⁴–Phe⁵–Thr⁶–Thr⁷–Trp⁸). This conclusion was confirmed with QTOF/MS data, which demonstrated a series of adjacent $b_n(+1)$ ions at m/z 672, 571, 470, 323, and 195, corresponding to the successive loss of Trp, Thr, Thr, Phe, Ala–Gly, and the terminal dipeptide ion Pro–Pro. In addition, $b_n(+1)$ ions at m/z 266 and 195 were observed (Figure 1), in accordance with the peptide sequence Pro–Pro–Ala (Figure 2). The geometry of the amide bonds of both Pro¹ and Pro⁸ was *trans*, on the basis of the difference in the ^{13}C NMR chemical shifts of Pro¹ ($\Delta\delta_{C\beta-C\gamma} = 2.9$ ppm) and Pro⁸ ($\Delta\delta_{C\beta-C\gamma} = 3.0$ ppm).^{13,14}

The molecular formula of compound **3** was established to be $C_{45}H_{58}N_{10}O_9$ by HRESIMS, ^{13}C NMR, and DEPT spectra. The IR absorptions at 3415, 3342, 1663, and 1524 cm^{-1} indicated the presence of amino, hydroxy, amide carbonyl, and aromatic groups, respectively. The peptidic nature was suggested by the presence of eight amide protons and nine amide carbonyl signals in the 1H and ^{13}C NMR spectra. The amino acid residues were identified as Phe, Ile, Gly, Ala, Trp, Asn, and Pro ($\times 2$) by analysis of the COSY, HMQC, and HMBC spectra. HMBC correlations were observed between Phe²–NH/Pro¹–CO, Ile³–NH/Phe²–CO, Gly⁴–NH/Ile³–CO, Ala⁵–NH/Gly⁴–CO, and Gly⁴–H α /Ile³–CO, indicating the peptide fragment Pro¹–Phe²–Ile³–Gly⁴–Ala⁵. Correlations in the ROESY spectra of Asn⁸–H α /Pro¹–H δ , Pro⁶–H δ /Ala⁵–H α , and Pro⁶–H α /Trp⁷–NH indicated the two peptide units Ala⁵–Pro⁶–Trp⁷ and Asn⁸–Pro¹. The QTOF/MS data demonstrated a series of adjacent $b_n(+1)$ ions at m/z 769, 583, 486, 415, 358, and 195, corresponding to the successive loss of Asn, Trp, Pro, Ala, Gly, Ile, and the terminal dipeptide ion Pro–Phe (Figure 1). Thus, the structure **3** was determined to be *cyclo*-(Pro¹–Phe²–Ile³–Gly⁴–Ala⁵–Pro⁶–Trp⁷–Asn⁸). The *trans* configuration of the amide bonds in the two Pro residues was deduced from their chemical shift differences in $\Delta\delta_{C\beta-C\gamma}$ values (4.4 ppm for Pro¹ and 4.2 ppm for Pro⁶).^{13,14}

Compound **4** had the molecular formula $C_{43}H_{56}N_9O_{10}$, deduced from the ion at m/z 858.4132 $[M + H]^+$ in its HRESIMS spectrum, ^{13}C NMR, and DEPT spectra. The 1H and ^{13}C NMR data were similar to those of compound **2**. The octapeptide skeleton was suggested by the presence of seven

Table 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data for 1 and 2 in Pyridine- d_5

		1				2	
		δ_{H} (J in Hz)	δ_{C}			δ_{H} (J in Hz)	δ_{C}
Pro ¹	α	5.03, overlap	64.3, CH ^a	Pro ¹	α	4.57, t (8.4)	63.9, CH
	β	2.30, m	28.3, CH ₂		β	2.26, m	29.3, CH ₂
		1.80, m				1.80, m	
	γ	2.27, m	25.1, CH ₂		γ	1.35, m	26.4, CH ₂
	δ	4.09, m	47.4, CH ₂ ^b		δ	2.88, t (4.8)	47.0, CH ₂
2.27, overlap			1.92, m				
Val ²	C=O		172.8, qC ^c	Ala ²	CO		172.0, qC
	α	5.35, m	57.2, CH		α	5.18, m	48.8, CH
	β	2.97, m	29.9, CH		β	1.88, d (7.6)	19.2, CH ₃
		1.16, d (6.5)	19.2, CH ₃			NH	8.20, d (9.6)
	Me γ	1.42, overlap	17.7, CH ₃		C=O		173.2, qC
Gly ³	NH	7.85, d (10.4)		Gly ³	α	4.52, d (7.2)	43.5, CH ₂
	C=O		172.2, qC ^c		α	3.76, dd (7.2, 6.4)	
	α	4.64, overlap	43.9, CH ₂ ^d			NH	8.88, t (6.4)
		3.79, dd (17.2, 6.0)			C=O		
	NH	8.88, t (6.0)			Phe ⁴	α	5.89, m
C=O		169.8, qC	β	3.69, d (13.6)		41.4, CH ₂	
Leu ⁴	α	5.65, t (10.0)		50.1, CH		1	3.31, t (13.6)
	β	3.44, m	43.8, CH ₂ ^d	2,6			7.17, d (7.2)
		2.28, overlap				3,5	7.23, t (7.2)
	γ	1.92, m	24.8, CH	4	7.12, d (7.2)	128.9, CH	
	Me δ	0.89, d (5.6)	23.7, CH ₃	NH	7.52, d (10.4)	127.0, CH	
0.56, d (5.6)		20.6, CH ₃	C=O			176.65, qC	
Thr ⁵	NH	7.18, d (8.8)		Thr ⁵	α	4.46, m	64.6, CH
	C=O		177.8, qC		β	4.72, m	65.9, CH
	α	4.42, d (8.0)	64.5, CH ^a			γ	1.39, d (6.4)
		4.73, m	65.9, CH		NH		10.8, d (2.8)
	β	1.39, d (6.4)	21.9, CH ₃		C=O		172.5, qC
Pro ⁶	NH	10.8, d (2.8)		Thr ⁶	α	4.91, d (6.8)	60.5, CH
	C=O		172.7, qC ^c		β	5.15, overlap	66.6, CH
	α	4.64, dd (9.5, 5.5)	64.4, CH ^a			γ	1.50, d (6.4)
		2.38, m	29.8, CH ₂		NH		7.64, d (7.6)
	1.64, m		C=O				172.8, qC
Thr ⁷	γ	1.56, m	26.3, CH ₂	Trp ⁷	α	5.39, m	53.9, CH
	δ	1.44, m	47.3, CH ₂ ^b		β	3.68, d (13.6)	27.2, CH ₂
		3.38, overlap				NH	3.52, t-like (11.2)
	3.18 d, (8.0)		2		11.99, s		
	C=O		172.5, qC ^c		3	7.40, s	124.3, CH
Phe ⁸	α	4.83, dd (7.2, 0.8)	60.2, CH	Pro ⁸	α	4.99, m	64.5, CH
	β	5.03, overlap	66.5, CH		β	2.24, m	28.0, CH ₂
		1.44, overlap	21.7, CH ₃			1.67, m	
	γ	1.44, overlap	21.7, CH ₃		3a		128.2, qC
	NH	7.48, d (7.2)			4	7.89, d (7.6)	120.3, CH
Phe ⁸	C=O		172.2, qC ^c	5	7.39, t (5.0)	119.8, CH	
	α	5.03, overlap	55.9, CH	6	7.36, t (5.0)	122.4, CH	
		3.44, overlap	36.1, CH ₂	7	7.64, d (7.6)	112.1, CH	
	1	139.3, qC	7a		137.7, qC		
	2,6	7.62, d (7.2)	129.8, CH	NH	7.89, d (5.2)		
	3,5	7.32, t (7.2)	128.8, CH	C=O		170.4, qC	
	4	7.27, d (7.2)	127.2, CH	Pro ⁸	α	4.99, m	64.5, CH
	NH	7.81, d (5.6)			β	2.24, m	28.0, CH ₂
	C=O		169.5, qC	1.67, m			

Table 1. Continued

	1		2	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
γ			2.21, m 1.85, m	25.0, CH ₂
δ			4.06, m 3.17, dd (6.4, 1.6)	47.5, CH ₂
CO				172.2, qC

a,b,c,d Resonances with the same superscripts are interchangeable.

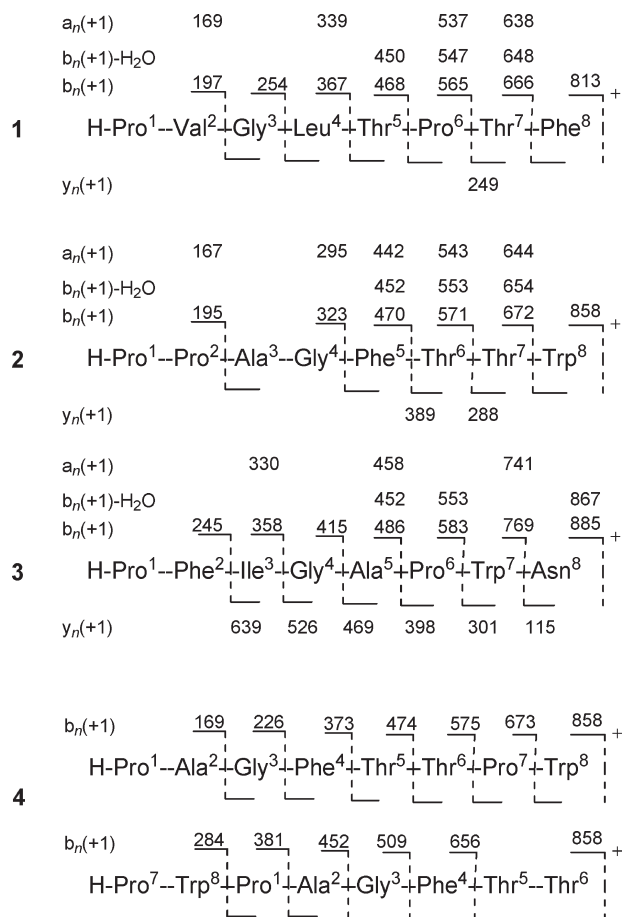


Figure 1. QTOF/MS sequence ions (m/z) of the protonated molecular ions of compounds 1–4.

amide protons (δ 7.49–10.52) and eight amide carbonyl resonances in the ^1H and ^{13}C NMR spectra. Analysis of the COSY, HMQC, and HMBC spectra demonstrated that the amino acid residues were Phe, Trp, Gly, Ala, Thr ($\times 2$), and Pro ($\times 2$), which are the same individual amino acids as those of 2. HMBC correlations were observed between Ala²-NH/Pro¹-CO, Gly³-NH/Ala²-CO, Phe⁴-NH/Gly³-CO, Thr⁵-NH/Phe⁴-CO, Thr⁶-NH/Thr⁵-CO, and Trp⁸-NH/Pro⁷-CO, indicative of the partial peptide sequences Pro¹-Ala²-Gly³-Phe⁴-Thr⁵-Thr⁶ and Trp⁷-Pro⁸. The ROESY correlations of Ala²-NH/Gly³-NH/Phe⁴-NH, Phe⁴-H $_{\alpha\beta}$ /Thr⁵-NH/Thr⁶-NH, and Trp⁸-H $_{\alpha}$ /Pro¹-H $_{\delta}$ supported the interpretation of the HMBC spectrum (Figure 2). Finally, two main series of $b_n(+1)$ ions were observed: one at m/z 673, 575, 474, 373, 226, and 169 (Figure 1),

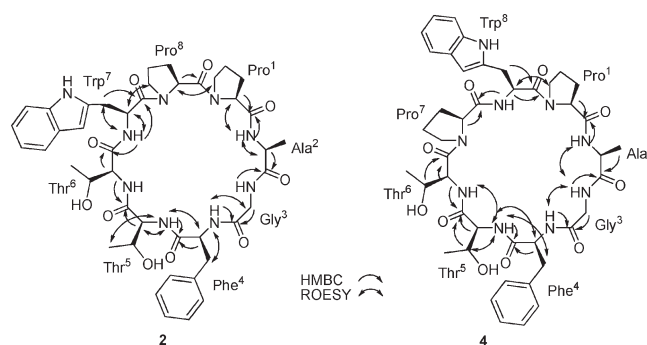


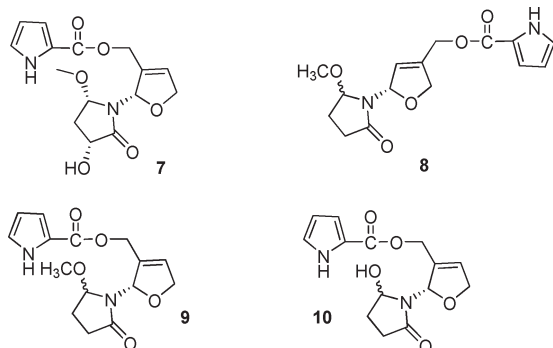
Figure 2. HMBC and ROESY correlations in compounds 2 and 4.

corresponding to the successive loss of Trp, Pro, Thr, Thr, Phe, Gly, and the terminal dipeptide ion Pro–Ala, and the other at m/z 656, 509, 452, 381, and 284, corresponding to the successive loss of Thr–Thr, Phe, Gly, Ala, Pro, and the terminal dipeptide ion Pro–Trp (Figure 1). These data confirmed the structure of peptide 4 as *cyclo*(Pro¹-Ala²-Gly³-Phe⁴-Thr⁵-Thr⁶-Pro⁷-Trp⁸). The differences in the ^{13}C NMR chemical shifts of Pro¹ ($\Delta\delta_{\text{C}\beta-\text{C}\gamma} = 2.8$ ppm) and Pro⁷ ($\Delta\delta_{\text{C}\beta-\text{C}\gamma} = 2.9$ ppm) suggested that the amide bonds in the Pro¹ and Pro⁷ residues are *trans*.^{13,14}

The absolute configuration of the amino acid residues of compounds 1–4 were determined by acid hydrolysis and analysis of the hydrolysates with Marfey's method.¹⁵ The Marfey's derivatives of authentic amino acids were prepared as standards and compared with those of the hydrolysates by their co-injection onto an HPLC apparatus. The results showed that all the amino acids had L-configurations, except Gly. Because the Trp residues in the structures of 2–4 were destroyed during hydrolysis, it was difficult to clarify the configuration of Trp residues in these structures. However, considering that naturally occurring amino acids from higher plants are all L configured, the Trp residues in the compounds 2–4 were tentatively proposed to be L.

The MS and NMR data of compound 5 were in accordance with those of brachystemin A, an octacyclopeptide previously isolated from the roots of the same plant. The structure of brachystemin A was erroneously identified as *cyclo*(Pro¹-Phe²-Leu³-Ala⁴-Thr⁵-Pro⁶-Ala⁷-Gly⁸).⁸ The structure of 5 should be revised to *cyclo*(Pro¹-Pro²-Ala³-Gly⁴-Leu⁵-Ala⁶-Thr⁷-Phe⁸) according to QTOF/MS analysis and X-ray diffraction data. QTOF/MS data showed a series of $b_n(+1)$ ions at m/z 608, 507, 436, 323, 266, and 195, indicating that the preferred ring-opening occurred at the Phe–Pro amide bond, and corresponded to the successive loss of Phe, Thr, Ala, Leu, Gly, Ala, and the terminal dipeptide Pro–Pro. X-ray data further confirmed the structure and assigned the relative configuration of

5 (Figure 3). The difference between the revised and previously reported structure is that the sequence of Pro–Phe and Phe–Leu should be revised to Thr–Phe and Gly–Leu. Such confusion arises because of the fact that the carbonyl resonances of Thr (δ 172.2), Pro (δ 172.4), Phe (δ 169.8), and Gly (δ 169.6) are often wrongly assigned.⁸



The known compounds were identified as brachystemin C (6),¹⁶ brachystemidines C (7),⁷ A (8),⁷ B (9),⁷ and D (10),⁷

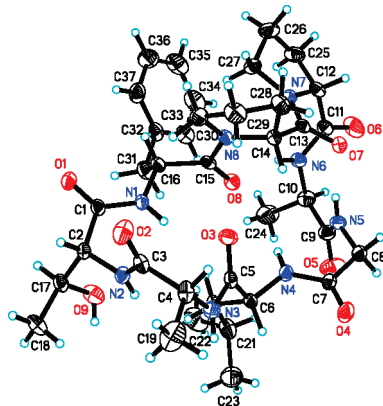


Figure 3. ORTEP drawing of 5.

loliolide (11),¹⁷ annuonone D (12),¹⁸ 9-*O*-*D*-glucopyranoside (13),¹⁹ 7 α -hydroxylambertianic acid (14),²⁰ 2-minaline (15),²¹ and 6,9-dihydroxy-4,7-megastigmadien-3-one (16)²² by comparison with literature data. Compounds 12–17 were isolated from this plant for the first time.

On the basis of the traditional medicinal knowledge of this herb, the cyclic peptides and alkaloids were evaluated *in vitro* for their inhibitory effects on the secretion of CCL-2, IL-6, and collagen IV by high-glucose-stimulated mesangial cells. The results showed that compounds 1, 5, and 8 significantly inhibited the secretion of IL-6, CCL-2, and collagen IV at concentrations of 10 μ M, compound 5 being the most active (Figure 4). There is evidence showing that cytokines and ROS are interconnected,²³ and high-glucose-induced ROS overproduction has been directly implicated in the pathogenic progress of DN. Thus, the antioxidant effect of 5 was further investigated. The results showed that high-glucose-induced ROS and $O_2^{\bullet-}$ generation could be significantly attenuated by 5 at 1 or 10 μ M (Supporting Information, S31, S32). Transforming growth factor- β (TGF- β), one of the key mediators of extracellular matrix genes in mesangial cells, has been implicated in the development of DN. Elevated TGF- β levels have been shown to be related to the accumulation of collagen I.^{24,25} Thus, the inhibitory effect of 5 on collagen I secretion in mesangial cells was tested and showed that 5 significantly decreased collagen I levels at 1 or 10 μ M (Supporting Information: S33). To exclude the possibility that the effects of 5 were related to its cell toxicity, the trypan blue exclusion test was carried out. It showed that 5 exhibited no toxic effect in mesangial cells at 10 μ M (Supporting Information, S34).

The role of NO in the DN is complicated. There are studies showing that the increased NO release contributes to the hyperfiltration and microalbuminuria in the early stages of DN. Several pathogenic factors of DN including hyperglycemia, oxidative stress, and activation of protein kinase C may decrease NO production in advanced nephropathy.²⁶ In this sense, compound 5 was further investigated for its effect on NO production inhibition using LPS-stimulated RAW 264.7 macrophage cells. The results showed that compound 5 is inactive

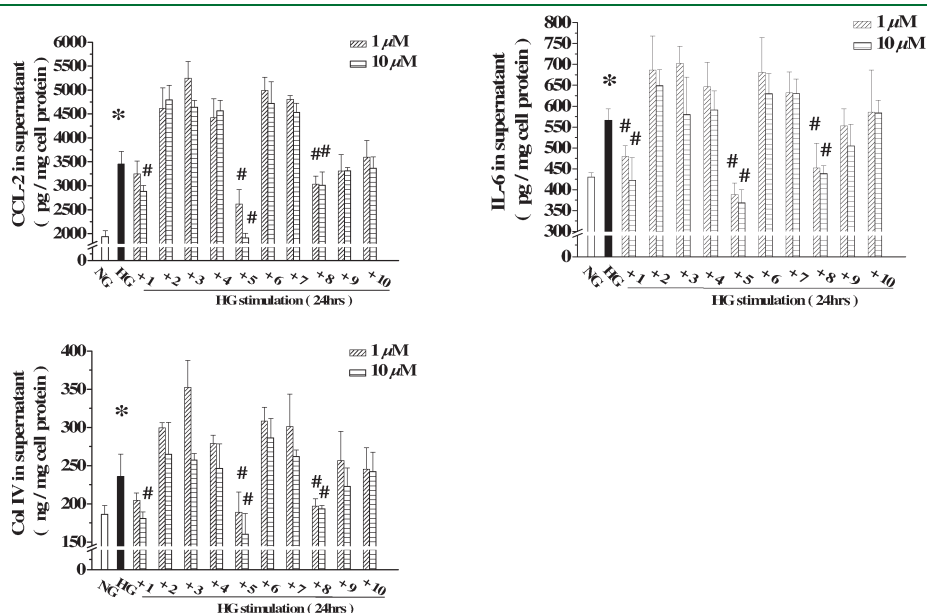


Figure 4. Inhibitory effects of compounds 1–11 on CCL-2, collagen IV, and IL-6 secretion. * P < 0.05 vs normal glucose; # P < 0.05 vs high glucose.

Table 2. ¹H (500 MHz) and ¹³C (100 MHz for 3 and 125 MHz for 4) NMR Data for 3^a and 4^b

		3				4		
		δ_H (J in Hz)	δ_C			δ_H (J in Hz)	δ_C	
Pro ¹	α	4.14, t (9.5)	61.7, CH	Pro ¹	α	4.92, overlap	65.3, CH	
	β	2.02, m	29.3, CH ₂		β	2.41, m	28.9, CH ₂	
		1.63, m				2.35, m		
	γ	1.94, m	24.9, CH ₂		γ	2.35, m	26.1, CH ₂	
		1.85, m			δ	2.18, m		
	δ	3.72, m	47.2, CH ₂		4.05, t (9.0)	48.6, CH ₂		
		3.37, m			3.22, m			
Phe ²	C=O		174.0, qC	Ala ²	C=O		174.2, qC	
	α	3.93, m	55.5, CH		α	5.12, m	49.9, CH	
	β	2.24, m	23.4, CH ₂		β	1.93, d (7.0)	19.9, CH ₃	
		2.11, m		NH	8.20, d (9.5)			
	1		136.2, qC	C=O		174.8, qC		
	2, 6	7.17, d (7.2)	123.3, CH	Gly ³	α	4.40, dd (16.5, 6.5)	44.2, CH ₂	
	3, 5	7.23, t (7.2)	123.3, CH			3.79, dd (16.5, 5.5)		
	4	7.12, d (7.2)	127.0, CH	NH	8.80, t (6.5)			
	NH	7.94, t (7.5)		C=O		171.2, qC		
	C=O		171.7, qC	Phe ⁴	α	5.86, overlap	54.5, CH	
α	5.41, d (12.5)	56.4, CH	β		4.03, overlap	41.7, CH ₂		
β	2.06, m	35.5, CH			3.29, dd (13.5, 11.0)			
γ	1.23, m	23.6, CH ₂	1			137.3, qC		
	1.07, m		2,6		7.59, d (7.5)	130.9, CH		
Ile ³	Me γ	0.71, d (6.5)	15.3, CH ₃	3,5	7.42, m	130.0, CH		
	Me δ	0.76, t (7.5)	12.1, CH ₃	4	7.27, d (7.5)	128.0, CH		
	NH	7.09, m		NH	7.49, d (10.0)			
	C=O		171.1, qC	C=O		176.9, qC		
	Gly ⁴	α	3.82, dd (20.5, 7.5)	42.9, CH ₂	Thr ⁵	α	4.47, dd (7.5, 2.5)	65.7, CH
			3.28, overlap			β	4.83, m	67.3, CH
		NH	7.46, t (9.0)			γ	1.69, d (6.0)	22.0, CH ₃
		C=O		167.7, qC	NH	10.52, d (2.5)		
Ala ⁵	α	4.67, m	44.9, CH	Thr ⁶	α	4.90, overlap	61.6, CH	
	β	0.72, d (7.6)	17.2, CH ₃		β	5.16, m	67.7, CH	
	NH	7.01, m			γ	1.62, d (6.0)	22.4, CH ₃	
	C=O		172.6, qC	NH	8.05, d (7.0)			
Pro ⁶	α	3.92, m	62.3, CH	Pro ⁷	α	4.64, t (9.5)	173.7, qC	
	β	2.05, m	28.5, CH ₂		β	2.66, m	64.8, CH	
		1.62, m				1.81, m	30.2, CH ₂	
	γ	1.79, m	24.3, CH ₂	γ	2.01, m	27.3, CH ₂		
	δ	3.51, m	46.5, CH ₂		1.64, m			
Trp ⁷	C=O		171.8, qC	Trp ⁸	δ	2.93, m	48.2, CH ₂	
	α	4.33, m	53.8, CH		C=O		174.0, qC	
	β	3.24, d (11.0)	25.5, CH ₂		α	5.43, m	54.7, CH	
		3.17, d (11.0)		β	3.68, m	28.3, CH ₂		
	NH	10.87, s			3.59, t (13.0)			
	2	7.02, s	123.3, CH	NH	11.96, s			
	3		111.6, qC	2	7.87, s	113.4, CH		
	3a		127.2, qC	3		112.3, qC		
	4	7.04, m	121.3, CH	3a		128.8, qC		
	5	7.45, d (9.5)	118.0, CH	4	7.89, d (7.6)	120.6, CH		
	6	6.98, m	118.6, CH	5	8.61, d (7.5)	120.9, CH		
	7	7.28, d (9.0)	111.6, CH	6	7.40, m	123.3, CH		
	7a		137.7, qC					
NH	7.18, d (5.2)							

Table 2. Continued

	3		4	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
	C=O	171.4, qC	7	125.0, CH
Asn ^s	α	3.93, t (9.5)	7a	138.6, qC
	β	2.61, m	NH	7.89, d (5.5)
		2.48, m	C=O	171.0, qC
	γ			
	NH ₂	172.6, qC		
	NH	7.08, m		
		7.03, m		
	C=O			
		170.2, qC		

^a In DMSO-*d*₆. ^b In Pyridine-*d*₅.

toward these cells. As mentioned above, the level of NO decreased in advanced nephropathy, whereas the proper level of NO is important for maintaining normal physical function. Therefore, the negative effect of **5** on NO production may be instead good for the later stages of DN patients.

Cyclopeptides are an important group of compounds and have been isolated from plant, marine, and microbial sources.²⁷ Although the biological effects of some cyclopeptides were reported previously, few examples (such as cyclosporine A) have been developed for clinical applications. The place of cyclopeptides in drug discovery requires further exploration. The present study provides new perspectives on the biological significance of cyclopeptides.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were determined on a Shimadzu double-beam 210A spectrometer. IR spectra were measured on a Bio-Rad FTS-135 infrared spectrophotometer with KBr disks. 1D and 2D NMR spectra were obtained on a Bruker AM-400 or a DRX-500 spectrometer, respectively, with TMS as the internal standard. MS data were obtained on a VG Auto Spec-3000 mass spectrometer. QTOF/MS was performed on an Agilent 1290 Infinity-QTOF 6520 LC/MS system. Semipreparative HPLC was carried out on an Agilent 1200 series pump equipped with a diode-array detector and a Zorbax SB-C₁₈ column (5.0 μm , ϕ 9.4 \times 250 mm). Silica gel (80–100 and 300–400 mesh; Qingdao Marine Chemical Inc., People's Republic of China), C₁₈ silica gel (40–60 μm ; Daiso Co., Japan), and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography (CC). The plates were dipped into 5% H₂SO₄ (EtOH) and heated to visualize the TLC spots.

Plant Material. The aerial parts of *B. calycinum* were collected in Xishuangbanna, in Yunnan Province of People's Republic of China, at the end of March 2008, and identified by Prof. H. Peng at the Kunming Institute of Botany. A voucher specimen (CHYX0572) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, People's Republic of China.

Extraction and Isolation. The sun-dried aerial parts of *B. calycinum* (12 kg) were ground to a powder and extracted with 95% aqueous EtOH (3 \times 20 L, each 7 d) at room temperature. The crude extract was suspended in H₂O and then partitioned successively with EtOAc and *n*-BuOH. The EtOAc portion (42 g) was subjected to CC over silica gel (200–300 mesh) using increasing amounts (2%) of MeOH in CHCl₃ and finally MeOH as the eluent to produce fractions A–G. Fraction B (2.7 g) was subjected to reversed-phase CC on C₁₈

silica gel, eluted with an increasing gradient of aqueous MeOH (10–95%), to produce five fractions (B₁–B₅). Fraction B₃ (600 mg) was passed through a Sephadex LH-20 column (CHCl₃–MeOH, 6:4) and then a silica gel column (petroleum ether–*i*-PrOH, 15:1 and 10:1) to yield compounds **11** (11.5 mg) and **12** (10 mg). Fraction E (3.3 g) was subjected to reversed-phase CC over C₁₈ silica gel (MeOH–H₂O, 5:95–95:5) to produce two fractions, E₁ and E₂. Fractions E₁ (300 mg) and E₂ (180 mg) were purified by semipreparative HPLC (MeCN–H₂O, 65:35) to give (in this order) **10** (3.4 mg) from E₁ and **8** (21 mg) and **9** (8 mg) from E₂. Fraction F (6.0 g) was separated on reversed-phase CC over C₁₈ silica gel, eluted with aqueous MeOH (5–80%), to yield fractions F₁ and F₂. Fraction F₁ (1.3 g) was separated on a Sephadex LH-20 column (MeOH) to produce **13** (62 mg), and fraction F₂ (720 mg) was purified on a silica gel column (CHCl₃–MeOH, 10:1 and 5:1) to yield **7** (2.5 mg). Fraction G (8.5 g) was divided into six fractions (G₁–G₆) by reversed-phase CC over C₁₈ silica gel with a gradient of aqueous MeOH (5–75%). Fraction G₂ (400 mg) was separated on Sephadex LH-20 (MeOH), followed by vacuum liquid chromatography (VLC) on silica gel (CHCl₃–Me₂CO, 10:1), to produce **1** (64 mg) and **2** (5.5 mg). Fraction G₃ (4.0 g) was purified by VLC on silica gel (CHCl₃–*i*-PrOH, 7:1) to yield **5** (2.1 g). Compounds **3** (34 mg), **4** (39 mg), and **6** (30 mg) were purified from fraction G₄ (350 mg) by a combination of VLC on silica gel (CHCl₃–Me₂CO, 5:1) and Sephadex LH-20 (MeOH). Fraction G₆ (350 mg) was separated into subfractions G_{6A} and G_{6B} by silica gel CC (CHCl₃–MeOH, 5:1), and G_{6B} (110 mg) was passed through a Sephadex LH-20 (MeOH) column to yield **14** (6 mg), **15** (11 mg), and **16** (30 mg).

Brachystemin F (1): white powder; $[\alpha]_{\text{D}}^{25} -69$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 250 (3.32), 203 (4.22) nm; IR (KBr) ν_{max} 3419, 2964, 2932, 2875, 1657, 1628, 1526, 1511, 1444 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; FABMS (positive) *m/z* 813 [M + H]⁺; HRESIMS (positive) *m/z* 835.4314 [M + Na]⁺ (calcd for C₄₀H₆₀N₈O₁₀Na, 835.4330).

Brachystemin G (2): white powder; $[\alpha]_{\text{D}}^{25} -34$ (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 281 (3.61), 219 (4.36), 203 (4.43) nm; IR (KBr) ν_{max} 3417, 2933, 1659, 1628, 1512, 1449, 1304 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; FABMS (positive) *m/z* 858 [M + H]⁺; HRESIMS (positive) *m/z* 880.3976 [M + Na]⁺ (calcd for C₄₃H₅₅N₉O₁₀Na, 880.3969).

Brachystemin H (3): white powder; $[\alpha]_{\text{D}}^{25} -42$ (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 290 (3.20), 280 (3.26), 203 (4.04) nm; IR (KBr) ν_{max} 3342, 2931, 1663, 1630, 1524, 1456, 1026 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; FABMS (positive) *m/z* 883 [M + H]⁺; HRESIMS (positive) *m/z* 905.4386 [M + Na]⁺ (calcd for C₄₅H₅₈N₁₀O₉Na, 905.4385).

Brachystemin I (4): white powder; $[\alpha]_{\text{D}}^{25} -13$ (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ϵ) 281 (3.02), 219 (3.83), 203 (3.92) nm. IR (KBr) ν_{max} 3431, 2923, 1656, 1639, 1629, 1512, 1451 cm⁻¹; ¹H and

^{13}C NMR data, see Table 2; FABMS (positive) m/z 858 $[\text{M} + \text{H}]^+$; HRESIMS (positive) m/z 858.4132 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{43}\text{H}_{55}\text{-N}_9\text{O}_{10}\text{Na}$, 858.4150).

Crystallographic data for compound 5: $\text{C}_{37}\text{H}_{54}\text{N}_8\text{O}_9$, $M_r = 754$, orthorhombic, space group $P2_12_12_1$, $a = 10.2490(8)$ Å, $b = 16.5450(12)$ Å, $c = 25.6543(19)$ Å; $V = 4350.2(6)$ Å³, $Z = 4$, $D_{\text{calcd}} = 1.263$ g cm⁻³, crystal size $0.403 \times 0.369 \times 0.195$ nm³, $F(000) = 1776$. The final R_1 value is 0.0542 ($wR_2 = 0.0627$) for 8074 reflections [$I > 2\sigma(I)$].

The crystallographic data for compound 5 have been deposited with the Cambridge Crystallographic Data Centre (deposit number CCDC 807929). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Marfey's Derivatization and HPLC Analysis of 1–4. Each compound (0.5 mg) was dissolved in 6 N HCl (1 mL) in a sealed container and heated at 110 °C for 18 h. After cooling, the reaction mixture was concentrated in vacuo to dryness. The hydrolysate was added to 20 µL of 1 M NaHCO₃ solution and 100 µL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide in acetone. The solution was reacted at 40 °C for 1 h. The Marfey's derivatives were analyzed by co-injection into an HPLC apparatus (ODS, 5 µm, 250 × 9.4 mm i.d.; MeCN–H₂O (0.05% TFA) = 10–60%; flow rate 1.5 mL/min; UV detection at 340 nm) and compared with the Marfey's derivatives of authentic amino acids.

Inhibition of IL-6, CCL-2, Collagen I, and Collagen IV Secretion. Rat mesangial cells (American Type Culture Collection no. CRL-2573) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 5.6 mM D-glucose (pH 7.4; Sigma Chemical Co., St Louis, MO), supplemented with 20% fetal calf serum (FCS; Invitrogen), 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES. After the mesangial cells reached 80% confluence, their growth was arrested in 0.5% FCS for 24 h. Exposure of the mesangial cells to medium containing high-concentration glucose induced the overproduction of CCL-2, IL-6, collagen I, and collagen IV, as described in the previous reports.^{28,29} To determine whether the selected compounds inhibited the CCL-2, IL-6, and collagen overproduction triggered by high glucose, the mesangial cells were pretreated with 1 or 10 µM of each compound for 1 h and then stimulated with high-concentration glucose for 24 h. The levels of supernatant CCL-2, IL-6, and collagen were measured with a solid-phase quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit for CCL-2 (BD Biosciences, San Diego, CA), specific for rat CCL-2 and sensitive to 10 pg/mL. The concentration in the culture supernatant was normalized to the total amount of cell protein, quantified with the BCA method (Pierce, Rockford, IL).³⁰ Similar protocols were used for rat IL-6 (R&D Systems, Abingdon, UK; sensitivity 0.25 ng/mL), rat collagen IV (R&D Systems; sensitivity 0.13 ng/mL), and rat collagen I (EIAab; sensitivity 0.39 ng/mL).

Analysis of Intracellular ROS Production. Cell culture of rat mesangial cells in this assay was carried out as above-described. To examine the antioxidant effect of compound 5, mesangial cells were exposed to either 5.6 mM (normal glucose, NG) or 25 mM (high glucose, HG) D-glucose for up to 24 h with or without 5 preincubated at 37 °C for 1 h, respectively at 1 and 10 µM. Intracellular ROS production was measured by reported methods.^{31,32} For details see the Supporting Information (S35).

NADPH-Dependent Superoxide Anion Production. NADPH-dependent superoxide anion production by homogenates from cultured mesangial cells was assessed by lucigenin-enhanced chemiluminescence using previous methods.^{33,34} The homogenates (100 µg/well) were added into a 96-well microplate. Immediately before recording, dark-adapted lucigenin (5 µM) with or without NADPH (100 µM) was added to the homogenates. The chemiluminescence value was recorded every minute for 30 min (VICTOR V Wallac 1420, PerkinElmer, Turku,

Finland). The readings in each of the last 5 min were averaged and expressed as counts per second. The data of each group were normalized to the NG group and expressed as percent of control.

Inhibition of NO Production in LPS-Stimulated RAW 264.7 Macrophage Cells. The inhibition of compound 5 on NO production in LPS-stimulated RAW 264.7 macrophage cells was carried out as was previously reported.³⁵ For details see the Supporting Information (S36).

Trypan Blue Exclusion Test for Cell Viability. For details see the Supporting Information (S37).

■ ASSOCIATED CONTENT

S Supporting Information. HMBC and ROESY correlations for compounds 1 and 3. Retention times for amino acids and X-ray crystallographic data in CIF format for compound 5. 1D and 2D NMR spectra and MS spectra for the new compounds. Figures of analysis of intracellular ROS production, NADPH-dependent superoxide anion production, and collagen I secretion in mesangial cells. Cytotoxicity test in mesangial cells. Procedure for determining ROS production and NO production. These materials are available free of charge via the Internet at <http://pubs.acs.org>.

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