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Diabetic nephropathy-related active cyclic peptides from the roots of *Brachystemma calycinum*

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ABSTRACT

Three new cyclic peptides, namely duanbanhuains A–C (**1**–**3**), were isolated from the roots of *Brachystemma calycinum* which is a traditional medicine used to treat rheumatic diseases. Their structures were identified by means of a suite of MS and NMR experiments. These compounds were purposely evaluated for their inhibitory effects on the release of MCP-1, IL-6, collagen IV and reactive oxygen species (ROS) against high-glucose-stimulated mesangial cells. The results showed that compounds **1** and **2** exhibited potent inhibition on the production of IL-6, collagen IV and ROS at the concentration of 10 μM.
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Cyclic peptides (CPs) are a fascinating family of compounds. Many antibiotics, antimycotics, toxins, ion-transport regulators, protein binding inhibitors, enzyme inhibitors, and immunosuppressants in nature are present in CPs.[1–5] Due to the restricted conformation and the lack of flexibility, CPs are also useful in studying interaction between small molecules and receptors. However, so far there have not been more CPs examples for clinic application apart from cyclosporin A, a well-known metabolite produced by *Tolypocladium inflatum*. In the last decades, more CPs have been found in plants, fungi, and marine organisms. Their potential for drug development remained to be further explored.

Increasing evidence showed that inflammation and immune response has been implicated in the pathogenic processes of rheumatic diseases such as osteoarthritis and rheumatoid arthritis.[6] Thus, herbs with therapeutic effects on rheumatoid arthritis are rationally hypothesized to possess possible anti-inflammatory or immunosuppressive substances. *Brachystemma calycinum* D. Don (Caryophyllaceae) is a Chinese folk medicine for the treatment of rheumatoid arthritis, limb numbness, impotence, and gonorrhea.[7] The extract of the whole plant was recently found to reduce disease symptoms and the development of cartilage lesions in experimental dog osteoarthritis via inhibition of protease-activated receptor 2.[8] Our previous study revealed a novel immunosuppressive alkaloid from the roots of this plant. Cyclic peptides have also been characterized from the title plant, however, their biological functions remained unknown. As our continuous study for diabetic nephropathy, a disease related with inflammation,[9] we re-investigated the plant roots and isolated three new CPs (Fig. 1). Their biological effects on inhibition of IL-6, MCP-1, Collagen IV, and ROS were evaluated.

Compound **1**[10] was isolated[11] as a white powder. Its molecular formula was assigned as $C_{43}H_{58}N_8O_{11}$ by the positive ion HR-ESI-MS peak at *m*/*z* 885.4042 ($[M+Na]^+$, calcd for 885.4123). The IR absorptions at 3432, 1727, 1701, 1638 cm^{-1} were ascribable to amino, amide carbonyl and aromatic groups. The ^1H NMR spectrum showed six amide NH signals, and the ^{13}C NMR exhibit eight amide carbonyls, eight α-amino acid carbons (Table 1), indicative of **1** being a peptide. A negative response to ninhydrin reagent but positive after hydrolyzed by 6 N aq HCl implied that **1** is a cyclic peptide. Detailed interpretation of 2D NMR data including COSY, HSQC, and HMBC spectra assigned the ^1H and ^{13}C NMR resonances of **1**. The individual amino acid residue was elucidated as Pro (2×), Ser (2×), Ile, Ala, Tyr, and Phe. The sequence of amino acid residues of **1** was deduced mainly by MS analysis because of the lack of HMBC correlations between NH^i and $C{=}O^{i+1}$ when measured in pyridine-d_5 or DMSO-d_6 (at 8 or 4 Hz). The protonated molecular ion $[M+H]^+$ of **1** (*m*/*z* 863) was subjected to MALDI/CID analysis, and gave a series of adjacent $b_n(+1)$ ions at *m*/*z* 776, 704, 558, 470, 358, and 261, corresponding to the successive loss of Ser, Ala, Phe, Ser, Ile, Pro, and the terminal dipeptide ion Pro-Tyr. In addition, a series of $a_n(+1)$ ions at *m*/*z* 136, 233, 330, 443, 529, 677, 748, 835 was also observed, in accordance with the interpretation result from

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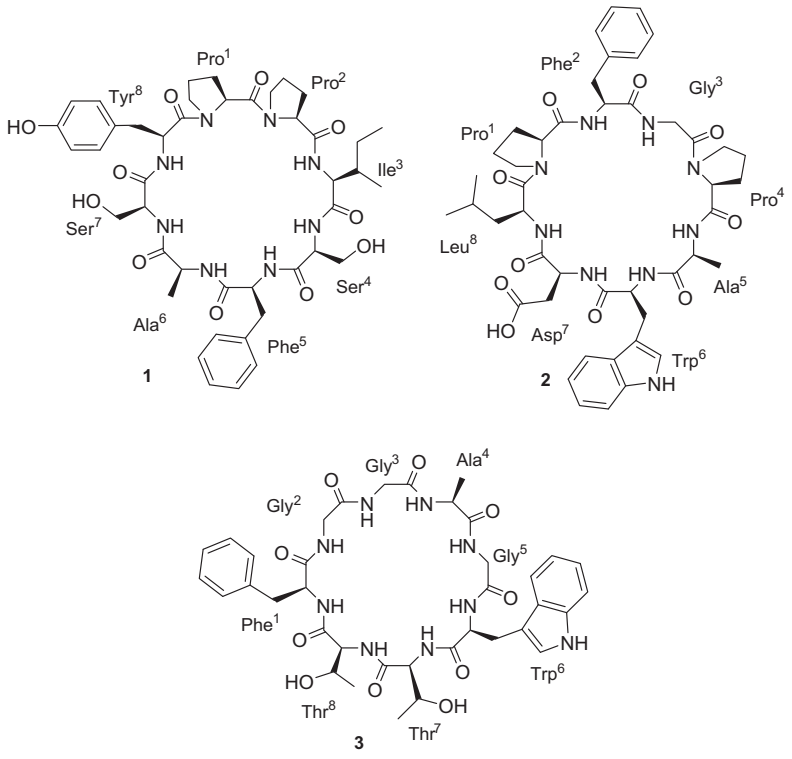

Figure 1. The structures of duanbanhuains A–C.

Table 1
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **1** in DMSO-d_6

		1					
		δ_H	δ_C			δ_H	δ_C
Pro^1	α	4.14, overlap	58.8		NH	8.48, overlap	
	β	2.16, m	27.9		C=O		170.6
		1.68, m		Phe^5	α	3.96, overlap	57.7
	γ	1.90, overlap	24.3		β	3.10, overlap	35.4
		1.83, overlap			1		137.8
	δ	3.42, overlap	46.6		2,6	7.20, overlap	129.1
					3,5	7.28, t, 7.4	128.2
	C=O		170.1		4	7.20, overlap	126.4
Pro^2	α	4.31, overlap	60.0		NH	8.48, overlap	
	β	1.83, overlap	30.8		C=O		171.2
				Ala^6	α	4.14, overlap	50.1
	γ	1.54, m	20.9		β	1.21, d, 7.2	17.4
		0.77, t, 7.0			NH	8.00, d, 5.9	
	δ	3.24, m	46.2		C=O		171.8
		3.10, overlap		Ser^7	α	4.51, dd, 7.6, 12.8	52.5
	C=O		170.8		β	3.71, dd, 7.1, 16.7	60.2
Ile^3	α	4.14, overlap	57.8			3.42, overlap	
	β	1.83, overlap	36.2		NH	7.47, d, 6.7	
	γ	1.50, m	24.5		C=O		168.2
		1.05, m		Tyr^8	α	4.31, overlap	56.5
		0.87, d, 6.7	15.1		β	2.98, m	35.4
	δ	0.84, t, 7.4	10.6				
	NH	7.44, d, 7.9			1		127.7
	C=O		172.6		2,6	7.02, d, 8.4	129.7
Ser^4	α	3.95, br s	56.9		3,5	6.66, d, 8.4	114.9
	β	3.61, overlap	60.6		4		155.9
		3.43, overlap					
					NH	8.18, br s	
	OH	5.20, br s			C=O		171.5

b series of ions. The sequence obtained from MS was confirmed by the HMBC correlation of Ser^4-αH/Ile^3-CO and the following ROESY correlations: Pro^1-βH/Pro^2-αH, Pro^1-αH/Tyr^8-H-2 (weak), Tyr^8-H-3/Ser^7-αH, Phe^5-αH/Ala^6-βH, Ala^6-βH/Ser^7-NH (weak). Taken together, the sequence of **1** was elucidated as cyclo(Pro^1-Pro^2-Ile^3-Ser^4-Phe^5-Ala^6-Ser^7-Tyr^8). Furthermore, the ^{13}C NMR chemical shift

difference of Pro[1] ($\Delta\delta_{C\beta\text{-}C\gamma}$ = 3.6 ppm) and Pro[2] ($\Delta\delta_{C\beta\text{-}C\gamma}$ = 9.9 ppm) indicated the amide bond in the Pro[1] residue was *trans*, and that in the Pro[2] was *cis*.[12,13]

Compound **2**[14]was obtained as an amorphous solid. It had molecular formula $C_{45}H_{57}N_{10}O_9$ deduced from its HR-ESI-MS spectrum, requiring 22 degrees of unsaturation. HPLC analysis indicated that compound **2** was isolated as a pure form. However, poor resolved ^{1}H and ^{13}C NMR spectra recorded either in pyridine-d_5 or DMSO-d_6 made it difficult to assign its NMR signals. Whereas, the resolution of NMR spectra were much improved when recorded in methanol-d_4 at the cost of the disappearance of NH signals. The NMR signal assignments of **2** were thus performed according to the spectra measured in methanol-d_4. Due to the scarce of useful HMBC and ROESY correlations, the structure of **2** was mainly identified by MS experiments. The sequence of **2** was determined by MALDI/CID sequence analysis, which gave a b_n(+1) series of ions at *m*/*z* 769, 582, 512, 415, 358, and 210, corresponding to the successive loss of Asp, Trp, Ala, Pro, Gly, Phe, and the terminal dipeptide ion Pro-Leu. In addition, two series of y_n(+1) ions at *m*/*z* 301, 372, 469, 526, 673, and 770, and *m*/*z* 169, 226, 470, 583, and 698 confirmed the peptide sequence. This conclusion was also in accordance with the observed HMBC correlations of Gly[3]-αH/Phe[2]-CO and Trp[6]-αH/Ala[5]-CO. Unfortunately, HMBC correlation of OH/Asp-βC or ROESY correlation of Asp[7]-αH/Leu[8]-NH were not observed in the present experiments, which made it difficult to differentiate which carboxylic acid of Asp being linked with Leu-NH. However, the most possible linkage between Asp and Leu should be as indicated from the biogenetic pathway. Indeed, the chemical shift of γC of Asp[7] is also in consistence with literature data.[15,16] Thus the structure of **2** was identified as cyclo (Pro[1]-Phe[2]-Gly[3]-Pro[4]-Ala[5]-Trp[6]-Asp[7]-Leu[8]).

The molecular formula of compound **3**[17] was established as $C_{37}H_{47}N_9O_{10}$ by HR-ESI-MS, which showed a pseudomolecular ion peak at *m*/*z* 778.3575 ([M+H]$^+$, calcd for 778.3524). The peptide nature of **3** was suggested from the observation of eight amide protons and eight amide carbonyl signals in the ^{1}H and ^{13}C NMR spectra. It was noted that the NMR spectra of **3** were much better resolved in DMSO-d_6 than in pyridine-d_5, Thus the NMR signal assignments were carried out by analysis of COSY, HSQC and HMBC spectra in DMSO-d_6, which revealed the amino acid residues as

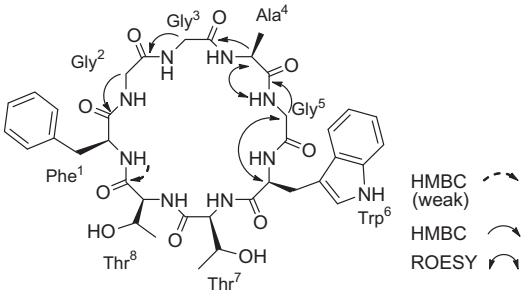

Figure 2. Key HMBC and ROESY correlations of duanbanhuain C.

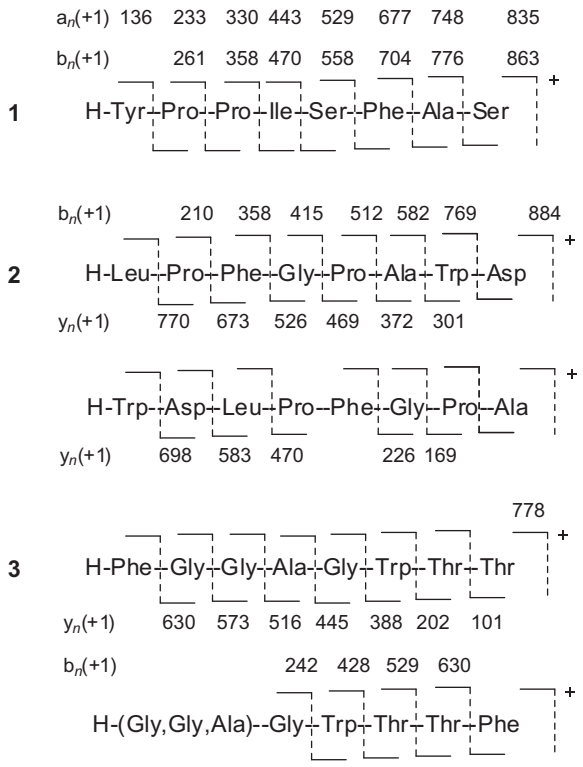

Figure 3. Peptide sequence ions (*m*/*z*) of protonated molecular ions of compounds **1**–**3**.

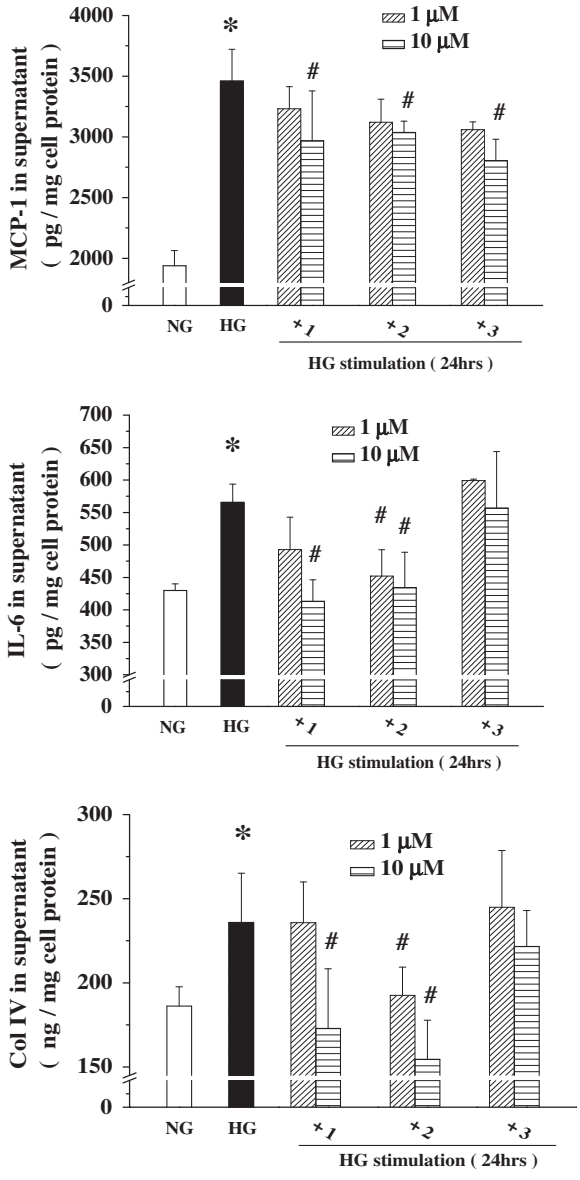

Figure 4. Inhibitory effects of the compounds **1**–**3** on MCP-1, IL-6 and collagen IV secretion (*P <0.05 vs NG, #P <0.05 vs HG).

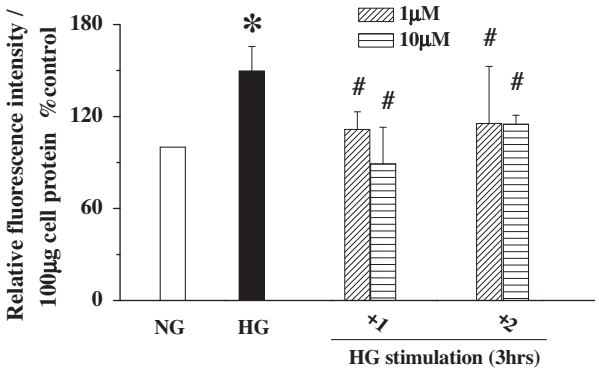

Figure 5. Inhibition of ROS production in mesangial cells by duanbanhuains A and B ($^{*}P$ <0.05 vs NG, $^{\#}P$ <0.05 vs HG).

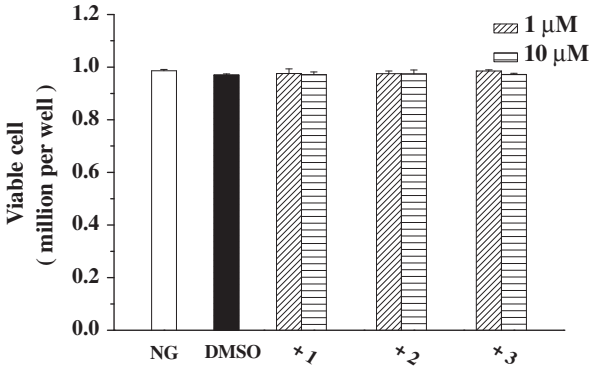

Figure 6. Cytotoxicity of duanbanhuains A–C against mesangial cells.

Phe, Gly (3×), Ala, Trp, Thr (2×). HMBC correlations between Gly^2-αH/Phe^1-CO, Gly^3-αH/Gly^2-CO, Gly^5-αH/Ala^4-CO, Phe^1-NH/Thr^8-CO (weak), and ROESY correlations between Gly^5-αH/Trp^6-αH, Ala^4-αH/Gly^5-NH (Fig. 2) were observed, which suggested the peptide sequences of Thr^8-Phe^1-Gly^2-Gly^3 and Ala^4-Gly^5-Trp^6. Further, HMBC correlations between Gly^3-NH/Gly^2-CO and Thr^8-NH/Thr^7-CO were detected when recorded in pyridine-d_5, showing the peptide fragments of Gly^2-Gly^3 and Thr^7-Thr^8. Taken the above data together, the peptide sequence could be determined as Phe^1-Gly^2-Gly^3-Ala^4-Gly^5-Trp^6-Thr^7-Thr^8. MALDI/CID sequence analysis gave y_n(+1) ions at m/z 101, 202, 388, 445, 516, 573, and 630, and b_n(+1) ions at m/z 630, 529, 428, and 242 (Fig. 3), indicative of a peptide sequence of Phe-Gly-Gly-Ala-Gly-Trp-Thr-Thr, agreed well with the conclusion from NMR spectra. Thus, the structure of **3** was elucidated as cyclo(Phe^1-Gly^2-Gly^3-Ala^4-Gly^5-Trp^6-Thr^7-Thr^8).

The absolute configurations of the amino acid residues of compounds **1**–**3** were determine by acid hydrolysis followed by Marfey's method.[18,19] The Marfey's derivatives of authentic amino acids (L- and DL-configurations) were prepared as standards and compared with those of the hydrolysates by their injection onto an HPLC apparatus. The results showed that all the amino acids had L-configurations, except Gly, in consistent with those of naturally occurring amino acids from higher plants.

Considering the traditional uses of *B. calycinum* for the treatment of rheumatic diseases, which are considered to be closely related with immune and inflammation response, the isolated compounds were purposely screened for their anti-inflammatory effects by observing their inhibition on the secretion of IL-6, MCP-1, and collagen IV in high-glucose-stimulated mesangial cells.[20–23] The results showed that compounds **1** and **2** could significantly inhibit the secretion of IL-6 and MCP-1 at 10 μM. Especially, compound **1** is most active on collagen IV inhibition at 1 μM (Fig. 4). Oxidative stress are implicated in the progression of diabetic nephropathy, further investigation on ROS inhibitory effects

Table 2
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of **2** in methanol-d_4

		2					
		δ_H	δ_C			δ_H	δ_C
Pro^1	α	4.27, dd, 8.7, 5.6	63.9		C=O		175.7
	β	2.24, m	31.0	Ala^5	α	3.93, q, 7.2	53.1
		1.88, m			β	1.41, d, 7.3	16.9
	γ	2.04, m	25.8		C=O		175.3[a]
		1.96, m		Trp^6	α	4.62, overlap	55.5
	δ	3.80, m	48.7		β	3.58, dd, 14.7, 3.8	26.3
		3.69, m				3.26, dd, 14.7, 5.3	
					2	7.13, s	125.3
	C=O		176.1		3		109.0
Phe^2	α	4.62, overlap	56.3		3a		129.0
	β	3.33, overlap	39.3		4	7.61, d, 7.6	118.7
		2.75, d, 12.1			5	7.18, overlap	120.8
	1		139.0		6	7.18, overlap	123.0
	2,6	7.34, d, 7.4	130.6		7	7.43, d, 7.8	112.9
	3,5	7.29, t, 7.6	129.4		7a		138.1
	4	7.18, overlap	127.5		C=O		173.4
	C=O		172.9	Asp^7	α	4.96, overlap	50.9
Gly^3	α	4.62, overlap	45.0		β	2.69, dd, 11.9, 3.7	40.0
		3.47, d, 15.3				2.52, br d, 14.1	
					γ		177.5
	C=O		169.7		C=O		172.4
Pro^4	α	4.05, overlap	61.3	Leu^8	α	4.05, overlap	55.4
	β	1.18, overlap	29.8		β	1.53, m	40.7
		0.29, br s				1.18, overlap	
	γ	1.58, m	26.5		γ	1.47, overlap	25.9
		1.47, m			δ	0.89, d, 6.5	23.0
	δ	3.35, overlap	48.7			0.79, d, 6.5	21.8
					C=O		175.2[a]

[a] Signals with the same superscripts are interchangeable.

Table 3
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data of **3** in DMSO-d_6

	3						
		δ_H	δ_C			δ_H	δ_C
Phe[1]	α	4.51, overlap	54.5		C=O		168.9
	β	3.13, dd, 13.7, 3.9	36.6	Trp[6]	α	4.51, overlap	53.8
		2.95, dd, 13.7, 10.8			β	3.21, dd, 14.8, 5.6	26.9
	1		137.3			3.06, dd, 14.6, 8.6	
	2,6	7.25, overlap	129.1		2	7.12, d, 1.9	123.2
	3,5	7.25, overlap	128.1		3		109.9
	4	7.19, m	126.3		3a		127.1
	NH	8.06, overlap			4	7.54, d, 7.9	118.2
	C=O		171.9[a]		5	6.97, t, 7.3	118.2
Gly[2]	α	3.82, dd, 16.3, 6.2	42.8		6	7.06, t, 7.5	120.9
		3.63, overlap			7	7.31, d, 8.1	111.3
	NH	8.18, overlap			7a		136.0
	C=O		169.2		NH	7.70, overlap	
					Ar-NH	10.83, d, 1.9	
Gly[3]	α	3.73, overlap	42.2		C=O		171.8[a]
		3.46, overlap		Thr[7]	α	4.00, overlap	59.5
	NH	8.06, overlap			β	4.00, overlap	65.7
	C=O		170.1		γ	0.96, d, 6.3	20.1
Ala[4]	α	4.17, m	48.9		OH	5.07, d, 5.4	
	β	1.22, d, 7.1	17.2		NH	7.70, overlap	
					CO		169.5
	NH	8.18, overlap		Thr[8]	α	4.22, br s	58.8
	C=O		172.9		β	4.10, m	66.1
Gly[5]	α	3.73, overlap	42.4		γ	1.05, d, 6.3	19.5
		3.63, overlap			OH	5.22, br s	
					NH	7.99, br s	
	NH	8.24, br s			CO		170.5

[a] Signals with the same superscripts are interchangeable.

of compounds **1** and **2** in mesangial cells indicated that they could significantly decrease ROS production (Fig. 5).[24–27] The significant inhibition of IL-6, MCP-1, collagen IV, and ROS in high-glucose-stimulated mesangial cells and without cytotoxicity (Fig. 6)[28,29] at 10 μM of peptide **1** may suggest its potential for the development of diabetic nephropathy therapeutic drugs, which may arise further interest of medicinal chemists.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.004.

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10. Duanbanhuain A (**1**): white powder; $[\alpha]_D^{23}$ −99.5 (*c* 0.70, MeOH); UV λ_{max} (MeOH) nm (log ε): 255 (4.32); IR (KBr) ν_{max} cm^{-1}: 3432, 2967, 2931, 1727, 1701, 1638, 1377, 1231, 1039; ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS (positive) *m/z*: 863 [M+H]$^+$; HR-ESI-MS (positive) *m/z*: 885.4042 [M+Na]$^+$ ($C_{43}H_{58}N_8O_{11}$Na, calcd for 885.4123).
11. *Isolation*: The air-dried roots (13 kg) were ground into powders, and extracted with 95% EtOH (50 L × 3) under reflux. The combined extracts were evaporated in vacuo followed by successive partition with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc extracts (50 g) were divided into five fractions by silica gel column chromatography eluted with increasing MeOH in $CHCl_3$. Fraction 4 (2.5 g) was subjected to C_{18} silica gel column with gradient aqueous MeOH to yield subfractions 4.1–4.3. Fraction 4.2 (600 mg) was passed through Sephadex LH-20 column (MeOH) to give compounds **1** (12 mg) and **2** (14 mg). Fraction 4.3 (500 mg) was separated on silica gel column with $CHCl_3$–MeOH (9:1) as solvents to afforded subfractions 4.3.1 and 4.3.2. Fraction 4.3.1 (35 mg) was purified by Sephadex LH-20 column to yield **3** (8 mg).
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14. Duanbanhuain B (**2**): white powder; $[\alpha]_D^{25}$ −30.1 (*c* 0.15, pyridine); UV λ_{max} (MeOH) nm (log ε): 283 (3.55), 201 (3.31); IR (KBr) ν_{max} cm^{-1}: 3402, 3363, 2930, 1671, 1645, 1523; ^{1}H and ^{13}C NMR data, see Table 2; FAB-MS (positive) *m/z*: 884 [M+H]$^+$; HR-ESI-MS (positive) *m/z*: 906.4239 [M+Na]$^+$ ($C_{45}H_{57}N_9O_{10}$Na, calcd for 906.4252).
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17. Duanbanhuain C (**3**): white powder; $[\alpha]_D^{24}$ −21.5 (*c* 0.28, MeOH); UV λ_{max} (MeOH) nm (log ε): 261 (3.76); IR (KBr) ν_{max} cm^{-1}: 3396, 2977, 2930, 1656, 1527, 1456, 1380 1339, 1246, 1103; ^{1}H and ^{13}C NMR data, see Table 3; ESI-MS (positive) *m/z*: 800 [M+Na]$^+$; HR-ESI-MS (positive) *m/z*: 778.3575 [M+H]$^+$ ($C_{37}H_{48}O_9O_{10}$, calcd for 778.3524).
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19. *Marfey's derivatization of compounds **1**–**3** analyzed by HPLC*: Each compound (0.5 mg) was dissolved in 6 N HCl (1 mL) and heated at 110 °C for 18 h. The reaction mixture was dried and then was added to 20 μL of 1 M $NaHCO_3$ solution and 100 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA) in acetone. The solution was reacted at 40 °C for 1 h. The Marfey's derivatives were analyzed by HPLC (ODS, 5 μm, 250 × 9.4 mm i.d.; MeCN/H_2O (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.

20. *Inhibition of IL-6, MCP-1, and collagen IV secretion*: Rat mesangial cells (RMC; ATCC no. CRL-2573) were grown in DMEM (Invitrogen, Carlsbad, CA) containing 5.6 mM D-glucose (Sigma Chemical Co., St. Louis, MO), pH 7.4, supplemented with 20% FCS (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM HEPES. After confluence reached 80%, mesangial cells were growth-arrested in 0.5% FCS for 24 h. Exposure of mesangial cells to media containing high concentration of glucose induced overproduction of MCP-1, IL-6 and Collagen IV as described in the previous reports.[19,20] To determine whether the compounds could inhibit MCP-1, IL-6 and Collagen IV overproduction triggered by high glucose, mesangial cells were pre-treated respectively with 1 or 10 μM compounds for 1 h, and then stimulated with high concentration of glucose for 24 h. The level of supernatant MCP-1, IL-6, and Collagen IV were measured using a solid phase quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit for MCP-1 (BD Biosciences, San Diego, CA; sensitivity 10 pg/mL). The concentration in culture supernatant was normalized to the total amount of cell protein, analyzed by the BCA method (Pierce, Rockford, IL, USA).[21] A similar protocol was used for rat IL-6 (R & D Systems, Abingdon, UK; sensitivity 0.25 ng/mL), and rat Collagen IV (R & D Systems; sensitivity 0.13 ng/mL).
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24. *Analysis of intracellular ROS production*: Intracellular ROS production was measured as previous methods.[23,24] The intracellular formation of ROS was detected by loading cultured cells with the fluoroprobe carboxymethyl-H2-dichlorofluorescein diacetate (CM-H2-DCF-DA, Sigma Chemical Co., St. Louis, MO), a nonpolar compound that is converted into a nonfluorescent polar derivative 2′,7′-dichlorofluorescin (DCFH) by cellular esterase after incorporation into cells. DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of a proper oxidant.[26] Briefly, mesangial cells were incubated for 30 min at 37 °C with 1 μM CM-H2-DCF-DA in normal or high glucose. Fluorescence intensity was determined immediately by a flow cytometer (excitation $\lambda = 488$ nm, emission $\lambda = 515$ nm, BD FACSCalibur system, Franklin Lakes, NJ). The average fluorescence intensity was normalized by total cell protein contents for each experimental group of cells as described previously.[25]
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28. *Trypan blue exclusion test for cell viability*: Rat mesangial cells were plated as a density of 5×10^5 per well, then stimulated by 0.1% DMSO or compounds **1**, **2**, and **3** for 24 h at indicated dose. Cells from the late experimental time points were trypsinized from the cell culture plates ($1 \times$ trypsin/EDTA, Life Technologies, Inc., Eggenstein, Germany), and diluted in fresh DMEM. One part of the mesangial cell suspension was mixed with one part of 0.4% trypan blue (Life Technologies) and incubated for 3 min at room temperature. Subsequently, unstained (viable) and stained (nonviable) cells were counted by using a Neubauer chamber, as described previously.[27]
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