Six New Cyclic Peptides from the Roots of *Gypsophila oldhamiana*

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Six new cyclic peptides, gypsophin A–F (1–6), were isolated from *Gypsophila oldhamiana*. Their structures were elucidated by extensive NMR and chemical degradation. Compound 3 exhibited moderate activity of antiplatelet aggregation *in vitro*.

Key words *Gypsophila oldhamiana*; Caryophyllaceae; cyclic peptide; antiplatelet aggregation activity

Plants of the Caryophyllaceae family are a rich source of cyclopeptide alkaloids. These Caryophyllaceae-type cyclopeptides showed interesting biological properties, including cytotoxic,^{1–3} antimalarial,^{4,5} antiplatelet,⁶ immunosuppressive,⁷ immunomodulating,⁸ Ca²⁺ antagonistic,⁹ inhibiting cyclooxygenase^{10,11} and tyrosinase, enhancing rotamase,^{10,11} and estrogen-like activity.^{12,13}

Gypsophila oldhamiana (Miq.) (Caryophyllaceae) is widely distributed in the north of China, and its roots have been used as a traditional folk medicine Shan-Yin-Chai-Hu for the treatment of fever, consumptive disease and infantile malnutrition syndrome.¹⁴ Previous phytochemical studies on the roots of this plant have afforded triterpenoid saponins, sterols, and glycosides.^{15–17} We also have reported gypsophin,¹⁸ an unusual new cyclic peptide with a pyrrolidine-2,5-dione unit isolated from this plant. Further investigations on this plant led to the isolation of six new cyclic peptides, gypsophin A–F (1–6) (Fig. 1). Herein we describe the isolation and structure elucidation of 1–6. In addition, the isolates were tested for antiplatelet aggregation activity and cytotoxicity in Bel7402, SMMC-7721, HCT 116 and H460 cancer cell lines.

Results and Discussion

Gypsophin A (1), obtained as a white powder, has a molecule formula of C₅₁H₆₇N₉O₁₁ (23 unsaturations) based on its positive high resolution-electrospray ionization (HR-ESI)-MS, showing a [M+H]⁺ ion peak at *m/z* 982.5034 (Calcd for 982.5033). The IR absorptions at 3408, 1651, and 1516 cm^{−1} were ascribable to amino, amide carbonyl, and aromatic groups, respectively. The ¹H- and ¹³C-NMR data of 1 (Table 1) displayed signals of nine amide carbonyls (δ_C 174.7, 171.3, 171.2, 171.1, 171.0, 170.9, 170.6, 170.0, 169.7), eight normal α -amino acid carbon resonances (δ_C 59.2, 58.3, 56.8, 56.4, 56.0, 56.0, 53.7, 50.2), and nine amide protons (δ_H 8.51, 8.46, 8.05, 8.04, 8.01, 7.85, 7.12, 7.07, 6.89), indicating that compound 1 is a peptide. HPLC analysis of the acid hydrolysates and the ¹H-NMR data showed that 1 consisted proline (Pro), tyrosine (Tyr), serine (Ser), phenylalanine (Phe), valine (Val), isoleucine (Ile) and glutamine (Gln). Eight amino acid residues of Pro¹, Tyr², Ser³, Phe⁴, Phe⁵, Val⁶, Ile⁷ and Gln⁸ were assigned by analysis of the total correlation spectroscopy (TOCSY), ¹H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments (Table 1). The HMBC correlations between the carbonyls and the α - or β -protons of the same

amino acid residue, the carbonyl resonances of the amino acid residues were determined as δ_C 171.3 (Pro¹), 171.0 (Tyr²), 170.0 (Ser³), 169.7 (Phe⁴), 171.1 (Phe⁵), 170.6 (Val⁶), 170.9 (Ile⁷) and 171.2 (Gln⁸), respectively. In addition, another carbonyl signal at δ_C 174.7 was assigned as δ -carbon of glutamine residue (Gln), which was illuminated by HMBC cross-peaks for this carbon with H γ (2.12) of glutamine residue (Fig. 2). All the amino acid residues accounted for 22 degrees of unsaturation, indicating that 1 is a cyclic octapeptide. The sequence of the amino acid residues was deduced with HMBC and rotating frame nuclear Overhauser effect correlation spectroscopy (ROESY). The HMBC spectrum showed correlations of Tyr²-NH (δ_H 8.05)/Pro¹-CO (δ_C 171.3), Phe⁵-NH (δ_H 8.51)/Phe⁴-CO (δ_C 169.7), Ile⁷-NH (δ_H 7.12)/Val⁶-CO (δ_C 170.6) and Gln⁸-NH (δ_H 8.46)/Ile⁷-CO (δ_C 170.9) (Fig. 2), corresponding to the peptide fragments Pro¹-Tyr² and Val⁶-Ile⁷-Gln⁸. The observed ROESY correlations of Ser³-H β (δ 3.98) and the Phe⁴ *o*-methylidyne resonance at δ_H 7.28,¹⁹ Tyr²-Ha/Ser³-Ha, Phe⁴-Ha/Phe⁵-Ha and Val⁶-NH/Phe⁵-NH, suggested the fragment Ser³-Phe⁴, Tyr²-Ser³, Phe⁴-Phe⁵-Val⁶, respectively (Fig. 2). Thus, 1 was determined as cyclo-(Pro¹-Tyr²-Ser³-Phe⁴-Phe⁵-Val⁶-Ile⁷-Gln⁸) and named gypsophin A. The geometry of the amide bond of Pro¹ was *cis*, on the basis of the difference in the ¹³C-NMR chemical shifts of Pro¹ ($\Delta\delta_{C\beta-C\gamma}$ =9.0 ppm).^{20,21}

Gypsophin B (2) was isolated as an amorphous solid. It showed a quasimolecular ion at *m/z* 1029.5385 [M+Na]⁺ in HR-ESI-MS spectrum, in conjunction with the NMR experiments, suggesting the molecular formula C₅₀H₇₄N₁₀O₁₂Na (Calcd for 1029.5380) with 19 degrees of unsaturation. The IR spectrum showed absorption bands of amine (3419 cm^{−1}), amide carbonyl (1644 cm^{−1}) and aromatic (1546 cm^{−1}) groups. The ¹H-NMR spectrum showed six amide NH resonances, and the ¹³C-NMR spectrum displayed ten amide carbonyl carbons. These data were indicative of a peptide. Using ROESY, TOCSY, HMQC, and HMBC spectra, ten amino acid residues were identified as Phe, Ser, Leu (leucine), Thr (threonine), Gly (glycine), Ile and Pro (×4) (Fig. 1, Table 1), which was further confirmed by HPLC analysis of the acid hydrolysates. These residues accounted for 18 degrees of unsaturation, suggesting that 2 is also a cyclic decapeptide. The sequence of 2 was assembled with HMBC and ROESY experiments. The HMBC spectrum showed correlations of Phe²-NH (δ_H 8.73)/Pro¹-CO (δ_C 171.7), Ser⁵-NH (δ_H 8.29)/Pro⁴-CO (δ_C 174.4), Thr⁶-NH (δ_H 7.78)/Ser⁵-CO (δ_C 170.6), Gly⁷-NH (δ_H 7.85)/Thr⁶-CO (δ_C 171.1), Leu⁸-NH (δ_H 6.92)/Gly⁷-CO (δ_C 167.5) and Ile¹⁰-NH (δ_H 8.10)/Pro⁹-CO (δ_C 172.3) (Fig. 2), corresponding to the peptide fragments Pro¹-Phe², Pro⁴-Ser⁵-Thr⁶-Gly⁷-Leu⁸ and Pro⁹-Ile¹⁰.

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Fig. 1. Structures of Compounds 1–6

The observed ROESY correlations of Phe²-Ha/Pro³-Ha, Pro³-Ha/Pro⁴-H γ , Leu⁸-Ha/Pro⁹-H γ , Ile¹⁰-Ha/Pro¹-Ha and Pro⁴-Ha/Ser⁵-NH indicated the three peptide units Phe²-Pro³-Pro⁴, Leu⁸-Pro⁹ and Ile¹⁰-Pro¹ (Fig. 2). Therefore, the structure of **2** was elucidated as cyclo-(Pro¹-Phe²-Pro³-Pro⁴-Ser⁵-Thr⁶-Gly⁷-Leu⁸-Pro⁹-Ile¹⁰) and named gypsophin B. The differences in the ¹³C-NMR chemical shifts of Pro¹ ($\Delta\delta_{C\beta-C\gamma}$ =9.6 ppm), Pro³ ($\Delta\delta_{C\beta-C\gamma}$ =3.0 ppm), Pro⁴ ($\Delta\delta_{C\beta-C\gamma}$ =4.2 ppm) and Pro⁹ ($\Delta\delta_{C\beta-C\gamma}$ =4.7 ppm) suggested that the amide bonds in the Pro³, Pro⁴ and Pro⁹ residues were *trans*, and that in the Pro¹ was *cis*.^{20,21)}

Gypsophin C (**3**), a colorless solid, had a molecular formula C₄₂H₆₆N₈O₁₀, established by positive HR-ESI-MS (m/z 865.4814 [M+Na]⁺; Calcd 865.4818). It was identified as a peptide on the basis of the presence of seven amide protons (δ_H 7.18–8.70), eight carbonyls (δ_C 173.7–168.3) and eight α -amino acid carbon resonances (δ_C 61.4–42.9) in the ¹H- and ¹³C-NMR spectrum. A negative response to the ninhydrin reagent but a positive response after its hydrolysis with 6N aqueous HCl implied that **3** is a cyclic peptide. Eight amino acid

residues of **3** were determined as Pro¹, Gly², Ile³, Phe⁴, Thr⁵, Ile⁶, Ile⁷ and Thr⁸ on the basis of detailed analysis of one-dimensional (1D)- and two-dimensional (2D)-NMR, and their proton and carbon resonances were completely assigned (Fig. 1, Table 1). The amino acid sequence of **3** was established by HMBC cross-peaks: Gly²-NH (δ_H 8.70)/Pro¹-CO (δ_C 173.7), Ile³-NH (δ_H 7.69)/Gly²-CO (δ_C 168.3), Phe⁴-NH (δ_H 7.92)/Ile³-CO (δ_C 171.4), Thr⁵-NH (δ_H 7.25)/Phe⁴-CO (δ_C 170.3), Ile⁶-NH (δ_H 7.98)/Thr⁵-CO (δ_C 170.5), Ile⁷-NH (δ_H 7.47)/Ile⁶-CO (δ_C 171.1) and Thr⁸-NH (δ_H 7.18)/Ile⁷-CO (δ_C 171.7). The gross structure was further supported by ROESY data, as indicated in Fig. 2. Thus, **3** was determined as cyclo-(Pro¹-Gly²-Ile³-Phe⁴-Thr⁵-Ile⁶-Ile⁷-Thr⁸) and named gypsophin C. Furthermore, the ¹³C-NMR chemical shifts difference of Pro¹ ($\Delta\delta_{C\beta-C\gamma}$ =4.3 ppm) indicated that the amide bond in the Pro¹ residue was *trans*.^{20,21)}

Gypsophin D (**4**), a colorless solid, had a molecular formula of C₃₆H₆₂N₈O₁₁, established by HR-ESI-MS (m/z 805.4423 [M+Na]⁺; Calcd 805.4430). The ¹H- and ¹³C-NMR data were similar to those of compound **3**. The octapeptide

Fig. 2. The Key Selective HMBC and ROESY Correlations of Compounds **1**, **2**, **3** and **6**

skeleton was suggested by the presence of seven amide protons (δ_{H} 7.25–8.80) and eight amide carbonyl resonances (δ_{C} 173.5–168.5) in the ^1H - and ^{13}C -NMR spectra. Eight amino acid residues were determined as Pro¹, Gly², Leu³, Ser⁴, Thr⁵, Ile⁶, Leu⁷ and Thr⁸ on the basis of detailed analysis of 1D- and 2D-NMR. The amino acid sequence of **4** was established by HMBC and ROESY data. HMBC correlations were observed between Leu³-NH (δ_{H} 7.73)/Gly²-CO (δ_{C} 168.5), Thr⁵-NH (δ_{H} 7.34)/Ser⁴-CO (δ_{C} 170.0), Thr⁸-NH (δ_{H} 7.25)/Leu⁷-CO (δ_{C} 171.0), indicating the peptide fragments Gly²-Leu³, Ser⁴-Thr⁵ and Leu⁷-Thr⁸. Correlations in the ROESY spectra of Thr⁸-Ha/Pro¹-H γ , Pro¹-Ha/Gly²-NH, Leu³-NH/Ser⁴-NH and Thr⁵-Ha/Ile⁶-NH indicated the three peptide units Thr⁸-Pro¹-Gly², Leu³-Ser⁴ and Thr⁵-Ile⁶. Thus, **4** was determined as cyclo-(Pro¹-Gly²-Leu³-Ser⁴-Thr⁵-Ile⁶-Leu⁷-Thr⁸) and named gypsophin D. The *trans* configuration of the amide bond in the Pro¹ residue was deduced from chemical shift differences in $\Delta\delta_{\text{C}\beta-\text{C}\gamma}$ value (4.2 ppm).^{20,21)}

Gypsophin E (**5**) was isolated as amorphous powder with a molecular formula of $\text{C}_{31}\text{H}_{51}\text{N}_7\text{O}_7$ based on the pseudomolecular ion peak at m/z 656.3745 $[\text{M}+\text{Na}]^+$ (Calcd 656.3742) in HR-ESI-MS spectrum. The peptidic nature was suggested by the presence of five amide protons and seven amide carbonyl signals in the ^1H - and ^{13}C -NMR spectra. Analysis of 2D-NMR data (TOCSY, HMQC, HMBC and ROESY spectra) permitted the assignment of the ^1H - and ^{13}C -NMR resonances and also allowed the diagnosis of the seven amino acid residues as Pro ($\times 2$), Gly ($\times 2$), Leu, Ile and Val. The HMBC spectrum showed correlations of Gly²-NH/Pro¹-CO, Leu³-NH/Gly²-CO, and Ile⁶-NH/Pro⁵-CO, corresponding to the peptide fragment Pro¹-Gly²-Leu³ and Pro⁵-Ile⁶, respectively. The observed ROESY correlations of Pro¹-Ha (δ_{H} 4.08) and the Gly⁷ α -methylene resonance at δ 3.98,²²⁾ Val⁴-Ha/Pro⁵-Ha, Ile⁶-NH/Gly⁷-NH suggested the fragment Pro¹-Gly⁷-Ile⁶, Val⁴-Pro⁵,

respectively. These data implied that the sequence of **5** is cyclo-(Pro¹-Gly²-Leu³-Val⁴-Pro⁵-Ile⁶-Gly⁷), named gypsophin E. The *trans* configuration of the amide bonds in the two Pro residues was deduced from their chemical shift differences in $\Delta\delta_{\text{C}\beta-\text{C}\gamma}$ values (4.2 ppm for Pro¹ and 5.2 ppm for Pro⁵).^{20,21)}

Gypsophin F (**6**) was isolated as amorphous powder. Its molecular formula was determined to be $\text{C}_{41}\text{H}_{55}\text{N}_7\text{O}_9$ by the HR-ESI-MS at m/z 788.3999 $[\text{M}-\text{H}]^-$ (Calcd 788.3988), indicating 18 degrees of unsaturation. The ^1H - and ^{13}C -NMR were similar to those of gypsophin.¹⁸⁾ However, the HR-ESI-MS showed that the molecular weight of compound **6** was 18 amu more than that of gypsophin. The C-H long range correlation of Ha of Phe⁵ to the γ -carbonyl carbon of Asp⁴ (aspartic acid) observed in HMBC spectrum of gypsophin was not detected in compound **6**. Alternatively, the ^1H -NMR spectrum of compound **6** in DMSO- d_6 revealed an additional NH proton, which was attributed to Asp residue by HMBC experiment. These data indicated that the dehydrated Asp unit in gypsophin was liberated in compound **6**. Therefore, the structure compound **6** was elucidated as cyclo-(Pro¹-Gly²-Phe³-Asp⁴-Phe⁵-Ile⁶-Leu⁷) and named gypsophin F.

The absolute configurations of all the amino acids in **1–6** were determined to be L-configuration by slightly modified Marfey's method.²³⁾ The hydrolysates were treated with Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA), and the resulting derivatives were analyzed by reversed-phase HPLC. The peaks on the chromatogram were identified by comparing the retention times with those of the FDAA derivatives of the authentic amino acids.

Since some cyclopeptides exhibited antiplatelet aggregation activity, the isolates were evaluated for their effects against ADP-induced platelet aggregation. As shown in Table 3, compound **3**, gypsophin C, showed moderate inhibition with 30.80% inhibitory rate at the concentration of $100\mu\text{g mL}^{-1}$.

Table 1. ¹H- and ¹³C-NMR Signal Assignments of Compounds **1–3** in DMSO-*d*₆ at 298 K

1			2			3		
Residue	δ_{H} [int., mult., <i>J</i> (Hz)]	δ_{C}	Residue	δ_{H} [int., mult., <i>J</i> (Hz)]	δ_{C}	Residue	δ_{H} [int., mult., <i>J</i> (Hz)]	δ_{C}
Pro ¹			Pro ¹			Pro ¹		
α -CH	4.68 (1H, d, 6.5)	58.3	α -CH	4.03 (1H, d, 8.0)	59.8	α -CH	4.42 (1H, m)	61.4
β -CH ₂	1.99 (1H, m)	30.7	β -CH ₂	1.98 (1H, m)	31.0	β -CH ₂	1.71 (1H, m)	28.9
	2.03 (1H, m)			2.03 (1H, m)			2.16 (1H, m)	
γ -CH ₂	1.80 (1H, m)	21.7	γ -CH ₂	1.01 (1H, m)	21.4	γ -CH ₂	1.82 (1H, m)	24.6
	1.95 (1H, m)			1.61 (1H, m)			1.92 (1H, m)	
δ -CH ₂	3.41 (1H, m)	46.2	δ -CH ₂	3.15 (1H, m)	46.4	δ -CH ₂	3.48 (1H, m)	47.2
	3.41 (1H, m)			3.30 (1H, m)			3.56 (1H, m)	
CO		171.3	CO		171.7	CO		173.7
Tyr ²			Phe ²			Gly ²		
α -CH	4.03 (1H, m)	56.4	α -CH	4.28 (1H, m)	55.1	α -CH ₂	3.59 (1H, m)	42.9
β -CH ₂	2.96 (1H, m)	34.5	β -CH ₂	2.63 (1H, d, 12.0)	34.2		3.62 (1H, m)	
	3.05 (1H, m)			2.86 (1H, t, 13.5)		NH	8.70 (1H, s)	
<i>i</i> -C		127.9	<i>i</i> -C		138.8	CO		168.3
<i>o</i> -CH	6.99 (2H, d, 9.0)	129.3	<i>o</i> -CH	7.35 (2H, d, 8)	129.1	Ile ³		
<i>m</i> -CH	6.66 (2H, d, 9.0)	115.0	<i>m</i> -CH	7.24 (2H, t, 8)	127.7	α -CH	4.32 (1H, m)	50.2
<i>p</i> -CH		155.8	<i>p</i> -CH	7.16 (1H, t, 8)	126.1	β -CH	1.48 (1H, m)	39.2
NH	8.05 (1H, m)		NH	8.73 (1H, d, 8.0)		γ -CH ₂	1.44 (1H, m)	24.1
CO		171.0	CO		168.7		1.65 (1H, m)	
Ser ³			Pro ³			γ -CH ₃	0.85 ^{a)}	20.9
α -CH	4.20 (1H, m)	56.0	α -CH	4.63 (1H, t, 7.0)	57.4	δ -CH ₃	0.79 (3H, d, 6.5)	20.9
β -CH ₂	3.83 (1H, d, 7.5)	61.7	β -CH ₂	2.02 (1H, m)	28.5	NH	7.69 (1H, d, 9.5)	
	3.98 (1H, d, 7.5)			2.32 (1H, m)		CO		171.4
NH	8.04 (1H, m)		γ -CH ₂	1.97 (1H, m)	25.5	Phe ⁴		
CO		170.0		2.14 (1H, m)		α -CH	3.96 (1H, m)	55.8
Phe ⁴			δ -CH ₂	3.91 (1H, m)	46.8	β -CH ₂	3.18 (1H, m)	33.9
α -CH	4.49 (1H, s)	53.7		3.62 (1H, m)			3.30 (1H, m)	
β -CH ₂	2.83 (1H, t, 12.5)	36.8	CO		173.7	<i>i</i> -C		139.0
	3.25 (1H, t, 12.5)		Pro ⁴			α -CH	7.15 (2H, d, 8.0)	128.7
<i>i</i> -C		138.0	α -CH	5.02 (1H, t, 9.0)	61.4	<i>m</i> -CH	7.25 (2H, t, 8.0)	128.1
<i>o</i> -CH	7.28 (2H, m)	128.1	β -CH ₂	1.82 (1H, m)	29.1	<i>p</i> -CH	7.17 (1H, t, 8.0)	125.9
<i>m</i> -CH	7.28 (2H, m)	128.0		2.22 (1H, m)		NH	7.92 (1H, s)	
<i>p</i> -CH	7.18 (1H, m)	126.3	γ -CH ₂	1.92 (1H, m)	24.9	CO		170.3
NH	8.01 (1H, m)			1.97 (1H, m)		Thr ⁵		
CO		169.7	δ -CH ₂	4.28 (1H, m)	48.1	α -CH	4.70 (1H, d, 7.5)	56.4
Phe ⁵				3.82 (1H, m)		β -CH	4.40 (1H, m)	67.7
α -CH	4.40 (1H, s)	56.0	CO		174.4	γ -CH ₃	1.05 (3H, d, 6.0)	19.3
β -CH ₂	2.93 (1H, m)	36.4	Ser ⁵			NH	7.25 (1H, s)	
	3.20 (1H, m)		α -CH	3.94 (1H, m)	58.1	CO		170.5
<i>i</i> -C		138.2	β -CH ₂	3.74 (2H, brs)	39.7	Ile ⁶		
<i>o</i> -CH	7.23 (2H, m)	128.9	NH	8.29 (1H, s)		α -CH	3.83 (1H, m)	59.5
<i>m</i> -CH	7.23 (2H, m)	128.8	CO		170.6	β -CH	1.79 (1H, m)	35.3
<i>p</i> -CH	7.18 (1H, m)	126.1	Thr ⁶			γ -CH ₂	1.18 (1H, m)	24.8
NH	8.51 (1H, d, 7)		α -CH	4.02 (1H, m)	59.9		1.48 (1H, m)	
CO		171.1	β -CH	4.15 (1H, m)	67.9	γ -CH ₃	0.85 ^{a)}	11.1
Val ⁶			γ -CH ₃	1.25 (3H, d, 6.5)	19.5	δ -CH ₃	0.85 ^{a)}	15.3
α -CH	3.95 (1H, m)	59.2	NH	7.78 (1H, d, 7.5)		NH	7.98 (1H, s)	
β -CH	1.67 (1H, m)	30.3	CO		171.1	CO		171.1
γ -CH ₃	0.45 (3H, d, 6.5)	19.3	Gly ⁷			Ile ⁷		
γ -CH ₃	0.56 (3H, d, 6.5)	18.0	α -CH ₂	3.25 (1H, m)	42.1	α -CH	4.05 (1H, m)	52.7
NH	7.85 (1H, d, 6)			4.03 (1H, m)		β -CH	1.62 (1H, m)	39.3
CO		170.6	NH	7.85 (1H, d, 7.5)		γ -CH ₂	1.47 (1H, m)	24.3
Ile ⁷			CO		167.5		1.49 (1H, m)	
α -CH	4.12 (1H, t, 7.5)	56.8	Leu ⁸			γ -CH ₃	0.83 ^{a)}	22.9
β -CH	1.62 (1H, m)	36.4	α -CH	4.45 (1H, t, 10.5)	48.0	δ -CH ₃	0.90 (3H, d, 6.0)	22.7
γ -CH ₂	1.07 (1H, m)	24.5	β -CH ₂	1.02 (1H, m)	39.7	NH	7.47 (1H, s)	
	1.50 (1H, m)			1.25 (1H, m)		CO		171.7
γ -CH ₃	0.83 (3H, t, 7)	10.9	γ -CH	1.25 (1H, m)	23.7	Thr ⁸		
δ -CH ₃	0.80 (3H, d, 7.5)	16.0	δ -CH ₃	0.18 (3H, d, 6.5)	22.1	α -CH	4.44 (1H, m)	54.8
NH	7.12 (1H, d, 6)		δ -CH ₃	0.55 (3H, d, 6.5)	20.5	β -CH	3.89 (1H, q, 5.5)	66.3
CO		170.9	NH	6.92 (1H, d, 9.0)		γ -CH ₃	0.98 (3H, d, 6.5)	20.0

Table 1. ^1H - and ^{13}C -NMR Signal Assignments of Compounds **1–3** in $\text{DMSO}-d_6$ at 298 K

1			2			3		
Residue	δ_{H} [int., mult., J (Hz)]	δ_{C}	Residue	δ_{H} [int., mult., J (Hz)]	δ_{C}	Residue	δ_{H} [int., mult., J (Hz)]	δ_{C}
Gln ⁸			CO		168.7	NH	7.18 (1H, s)	
α -CH	4.22 (1H, m)	50.2	Pro ⁹			CO		170.1
β -CH ₂	1.69 (1H, m)	27.3	α -CH	4.40 (1H, dd, 4.0, 9.0)	57.9			
	1.82 (1H, m)		β -CH ₂	2.03 (1H, m)	29.1			
γ -CH ₂	2.12 (1H, m)	31.1		2.14 (1H, m)				
	2.27 (1H, m)		γ -CH ₂	1.82 (1H, m)	24.4			
δ -CO		174.7		1.92 (1H, m)				
NH	8.46 (1H, s)		δ -CH ₂	3.35 (1H, m)	46.3			
NH ₂	6.89 (1H, s)			3.52 (1H, m)				
	7.07 (1H, s)		CO		172.3			
CO		171.2	Ile ¹⁰					
			α -CH	4.60 (1H, d, 11.0)	51.2			
			β -CH	1.49 (1H, t, 11.5)	38.4			
			γ -CH ₂	1.81 (1H, m)	24.4			
				1.91 (1H, m)				
			γ -CH ₃	0.92 (3H, t, 5.0)	23.5			
			δ -CH ₃	1.25 (3H, d, 6.5)	20.4			
			NH	8.10 (1H, d, 3)				
			CO		171.0			

a) Signal pattern unclear due to overlapped.

The other compounds exhibited weak inhibitory rate between 8% and 15%. Aspirin was used as a positive control with 38.78% inhibitory rate at the same concentration. In addition, the cytotoxicity of compounds **1–6** against four human cancer cell lines Bel7402 (human liver cancer), SMMC-7721 (human liver cancer), HCT 116 (human colon cancer) and H460 (human lung cancer) was tested using the methyl thiazol tetrazolium (MTT) method.²⁴⁾ All of the compounds showed no cytotoxic (IC_{50} values $>100\mu\text{M}$) against the tested tumor cells.

Experimental

General Experimental Procedures IR (KBr-disc) spectra were recorded by Bruker Tensor 27 spectrometer. NMR spectra were recorded on Bruker Avance-III NMR instrument (^1H : 500MHz, ^{13}C : 125MHz) with TMS as internal standard. Chemical shifts were given in values of ppm and coupling constants in Hertz. HR-ESI-MS were obtained on an Agilent 6520B Q-TOF spectrometer (Agilent, CA, U.S.A.). Semi-preparative HPLC was carried out on a Shimadzu LC-8A instrument (flow rate set at 10mL/min) with a SPD-20A detector using a Shim-pack PRC-ODS column (5 μm , 250 $\times$ 20mm). Silica gel (100–200 mesh and 200–300 mesh, Qingdao Haiyang Chemical Co., Ltd., China), Sephadex LH-20 (Pharmacia, U.S.A.), and RP-C₁₈ (40–63 μm , YMC, Japan) were used for column chromatography. All solvents used were of analytical grade (Jiangsu Hanbon Science and Technology Co., Ltd., China).

Plant Material The roots of *G. oldhamiana* were collected in Lianyungang City, Jiangsu Province, China, and identified by Prof. Minjian Qin, the Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing, China. A voucher specimen (No. 20110616) was deposited in the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

Extraction and Isolation The air-dried roots (4.5kg) were refluxed with 80% EtOH. The residue obtained by

concentrating alcohol was partitioned between H₂O and EtOAc, and then the EtOAc-soluble portion (20g) was subjected to a silica gel column (CHCl_3 -MeOH, 25:1–1:1), in which a fraction eluted with CHCl_3 -MeOH (10:1) was further separated by RP-C₁₈ column (MeOH-H₂O, 6:4–8:2). The fraction (MeOH-H₂O, 6:4) was further separated by HPLC (MeOH-H₂O, 62:38, UV detection at 210nm), to yield pure **1** (10mg, t_{R} =20min), and **2** (12mg, t_{R} =26.2min), respectively. The fraction (MeOH-H₂O, 8:2) was subjected to a silica gel column (200–300 mesh), which was eluted with CHCl_3 -MeOH (10:1, 5:1, 1:1) to give three fractions (fractions 1–3). The fraction 1 was further separated by HPLC (MeOH-H₂O, 70:30, UV detection at 210nm), and yielded pure **3** (10mg, t_{R} =15min), **4** (17mg, t_{R} =21min), and **5** (12mg, t_{R} =28.5min), respectively. Fraction 2 was subjected to HPLC (MeOH-H₂O, 80:20) to afford **6** (10mg, t_{R} =25min).

Gypsophin A (1): White powder; $[\alpha]_{\text{D}}^{23}$ -77.2 (c =0.192, MeOH); IR (KBr) ν_{max} 3408, 2965, 1651, 1516, 1452, 1244, 701 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 1; ESI-MS (positive) m/z 982 $[\text{M}+\text{H}]^+$; HR-ESI-MS (positive) m/z 982.5034 $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{51}\text{H}_{68}\text{N}_9\text{O}_{11}$, 983.5033).

Gypsophin B (2): Amorphous solid; $[\alpha]_{\text{D}}^{23}$ -30.1 (c =0.300, MeOH); IR (KBr) ν_{max} 3419, 2954, 1644, 1546, 1450, 1204, 1027 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 1; ESI-MS (positive) m/z 1029 $[\text{M}+\text{Na}]^+$; HR-ESI-MS (positive) m/z 1029.5385 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{50}\text{H}_{74}\text{N}_{10}\text{O}_{12}\text{Na}$, 1029.5380).

Gypsophin D (3): Colorless solid; $[\alpha]_{\text{D}}^{23}$ -30.1 (c =0.158, MeOH); IR (KBr) ν_{max} 3410, 2962, 1645, 1530, 1455, 1224 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 1; ESI-MS (positive) m/z 843 $[\text{M}+\text{H}]^+$; HR-ESI-MS (positive) m/z 865.4814 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{42}\text{H}_{66}\text{N}_8\text{O}_{10}\text{Na}$, 865.4818).

Gypsophin E (4): Colorless solid; $[\alpha]_{\text{D}}^{23}$ -31.3 (c =0.142, MeOH); IR (KBr) ν_{max} 3414, 2963, 1640, 1530, 1400, 1246 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 2; ESI-MS (positive) m/z 783 $[\text{M}+\text{H}]^+$; HR-ESI-MS (positive) m/z 805.4423 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{36}\text{H}_{62}\text{N}_8\text{O}_{11}\text{Na}$, 805.4430).

Table 2. ¹H- and ¹³C-NMR Signal Assignments of Compounds 4–6 in DMSO-*d*₆ at 298 K

4			5			6		
Residue	δ_{H} [int., mult., <i>J</i> (Hz)]	δ_{C}	Residue	δ_{H} [int., mult., <i>J</i> (Hz)]	δ_{C}	Residue	δ_{H} [int., mult., <i>J</i> (Hz)]	δ_{C}
Pro ¹			Pro ¹			Pro ¹		
α -CH	4.40 (1H, t, 9.0)	61.4	α -CH	4.08 (1H, m)	60.9	α -CH	4.18 (1H, m)	60.8
β -CH ₂	1.75 (1H, m)	28.9	β -CH ₂	1.75 (1H, m)	28.7	β -CH ₂	1.79 (1H, m)	28.8
	2.16 (1H, m)			2.12 (1H, m)			2.13 (1H, m)	
γ -CH ₂	1.85 (1H, m)	24.7	γ -CH ₂	1.86 (1H, m)	24.5	γ -CH ₂	1.88 (1H, m)	24.7
	1.95 (1H, m)			1.93 (1H, m)			2.04 (1H, m)	
δ -CH ₂	3.55 (1H, m)	47.3	δ -CH ₂	3.50 (1H, m)	45.7	δ -CH ₂	3.57 (1H, m)	47.6
	3.59 (1H, m)			3.30 (1H, m)			3.88 (1H, m)	
CO		173.5	CO		171.6	CO		171.4
Gly ²			Gly ²			Gly ²		
α -CH ₂	3.64 (1H, m)	43.0	α -CH ₂	3.55 (1H, m)	42.3	α -CH ₂	3.25 (1H, m)	42.3
	3.75 (1H, m)						3.97 (1H, m)	
NH	8.80 (1H, s)		NH	8.49 (1H, m)		NH	8.76 (1H, s)	
CO		168.5	CO		168.3	CO		169.2
Leu ³			Leu ³			Phe ³		
α -CH	4.49 (1H, m)	50.3	α -CH	4.42 (1H, m)	51.0	α -CH	4.53 (1H, m)	55.3
β -CH ₂	1.49 (1H, m)	39.1	β -CH ₂	1.28 (1H, m)	40.0	β -CH ₂	2.78 (1H, m)	39.1
	1.69 (1H, m)			1.53 (1H, m)			2.97 (1H, m)	
γ -CH	1.48 (1H, m)	24.3	γ -CH	1.42 (1H, m)	24.5	<i>i</i> -C		137.2
δ -CH ₃	0.81 (3H, d, 8.5)	21.1	δ -CH ₃	0.79 (3H, d, 7.5)	21.8	<i>o</i> -CH	7.20–7.26 (1H, m)	128.1
δ -CH ₃	0.83 (3H, d, 8.5)	23.0	δ -CH ₃	0.85 (3H, d, 7.5)	23.2	<i>m</i> -CH	7.20–7.26 (1H, m)	128.0
NH	7.73 (1H, d, 9.5)		NH	7.43 (1H, d, 7)		<i>p</i> -CH	7.17–7.25 (1H, m)	126.3
CO		170.5	CO		171.6	NH	8.08 (1H, d, 10.0)	
Ser ⁴			Val ⁴			CO		169.9
α -CH	3.83 (1H, m)	56.8	α -CH	4.05 (1H, m)	58.0	Asp ⁴		
β -CH ₂	3.84 (1H, m)	59.5	β -CH	1.98 (1H, m)	29.4	α -CH	4.16 (1H, m)	50.3
	3.87 (1H, m)		γ -CH ₃	0.88 (3H, d, 6.5)	18.3	β -CH ₂	2.72 (1H, m)	35.1
NH	7.91 (1H, s)		γ -CH ₃	0.99 (3H, d, 6.5)	19.0		2.98 (1H, m)	
CO		170.0	NH	8.90 (1H, d, 3.5)		NH	8.47 (1H, d, 5.0)	
Thr ⁵			CO		170.4	γ -CO		172.8
α -CH	4.45 (1H, m)	56.8	Pro ⁵			CO		170.0
β -CH	4.33 (1H, m)	67.3	α -CH	4.74 (1H, d, 7.5)	60.6	Phe ⁵		
γ -CH ₃	1.03 (3H, d, 6.5)	19.5	β -CH ₂	1.74 (1H, m)	29.6	α -CH	4.22 (1H, m)	56.6
NH	7.34 (1H, d, 8.5)			2.42 (1H, dd, 6.5, 12)		β -CH ₂	2.98 (1H, m)	36.1
CO		171.0	γ -CH ₂	1.50 (1H, m)	24.4		3.05 (1H, m)	
Ile ⁶				1.87 (1H, m)		<i>i</i> -C		137.7
α -CH	3.88 (1H, m)	59.1	δ -CH ₂	3.18 (1H, m)	45.3	<i>o</i> -CH	7.15–7.25 (1H, m)	128.8
β -CH	1.79 (1H, m)	35.7		3.56 (1H, m)		<i>m</i> -CH	7.15–7.25 (1H, m)	129.0
γ -CH ₂	1.12 (1H, m)	24.6	CO		170.1	<i>p</i> -CH	7.17–7.25 (1H, m)	126.3
	1.47 (1H, m)		Ile ⁶			NH	7.93 (1H, s)	
γ -CH ₃	0.85 (3H, t, 6.5)	11.4	α -CH	3.97 (1H, m)	59.6	CO		170.8
δ -CH ₃	0.82 (3H, d, 8)	15.4	β -CH	2.02 (1H, m)	35.5	Ile ⁶		
NH	7.78 (1H, s)		γ -CH ₂	1.12 (1H, m)	24.9	α -CH	3.85 (1H, m)	59.6
CO		170.0		1.42 (1H, m)		β -CH	1.90 (1H, m)	35.4
Leu ⁷			γ -CH ₃	0.77 (3H, d, 7.0)	10.5	γ -CH ₂	1.01 (1H, m)	24.7
α -CH	4.03 (1H, m)	52.7	δ -CH ₃	0.87 (3H, d, 6.5)	15.7		1.34 (1H, m)	
β -CH ₂	1.46 (1H, m)	39.0	NH	8.47 (1H, m)		γ -CH ₃	0.79 (3H, t, 6)	10.6
	1.60 (1H, m)		CO		170.9	δ -CH ₃	0.80 (3H, 5.5)	15.3
γ -CH	1.59 (1H, m)	24.1	Gly ⁷			NH	7.70 (1H, m)	
δ -CH ₃	0.80 (3H, d, 6.5)	21.1	α -CH ₂	3.93 (1H, m)	42.5	CO		170.1
δ -CH ₃	0.90 (3H, d, 6.5)	22.7		3.98 (1H, m)		Leu ⁷		
NH	7.43 (d, 6.5)		NH	7.60 (1H, s)		α -CH	4.79 (1H, m)	47.8
CO		171.0	CO		167.5	β -CH ₂	1.42 (1H, m)	40.8
Thr ⁸							1.58 (1H, m)	
α -CH	4.45 (1H, m)	55.1				γ -CH	1.57 (1H, m)	23.9
β -CH	3.87 (1H, m)	66.5				δ -CH ₃	0.78 (3H, d, 6)	22.0
γ -CH ₃	1.00 (3H, d, 6.5)	19.9				δ -CH ₃	0.82 (3H, d, 6)	22.6
NH	7.25 (1H, d, 8.5)					NH	7.65 (1H, m)	
CO		171.0				CO		170.0

Table 3. Antiplatelet Aggregation Activity of Compounds 1–6 ($\bar{x} \pm s$, $n=3$)

Compounds	Dosage ($\mu\text{g mL}^{-1}$)	Inhibition ratio (%)
1	100	8.07 ± 0.87
2	100	14.10 ± 0.85
3	100	30.80 ± 0.47
4	100	12.91 ± 1.25
5	100	12.17 ± 0.88
6	100	15.32 ± 0.83
Aspirin (positive control)	100	38.78 ± 0.45

Gypsophin E (5): Amorphous powder; $[\alpha]_{\text{D}}^{23} -63.3$ ($c=0.140$, MeOH); IR (KBr) ν_{max} 3406, 2962, 1652 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 2; ESI-MS (positive) m/z 634 $[\text{M}+\text{H}]^+$; HR-ESI-MS (positive) m/z 656.3745 $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{51}\text{H}_{66}\text{N}_8\text{O}_{12}\text{Na}$, 656.3742).

Gypsophin F (6): Amorphous powder; $[\alpha]_{\text{D}}^{23} -31.5$ ($c=0.164$, MeOH); IR (KBr) ν_{max} 3442, 2962, 1626, 1527, 1455, 1384 cm^{-1} ; ^1H - and ^{13}C -NMR see Table 2; ESI-MS (positive) m/z 790 $[\text{M}+\text{H}]^+$; HR-ESI-MS (negative) m/z 788.3999 $[\text{M}-\text{H}]^-$ (Calcd for $\text{C}_{41}\text{H}_{54}\text{N}_7\text{O}_9$, 788.3988).

Marfey's Derivatization and HPLC Analysis of 1–6

Each compound (0.5 mg) was dissolved in 6 N HCl (1 mL) in a sealed container and heated at 110°C for 24 h. After cooling, the reaction mixture was concentrated *in vacuo* to dryness. The hydrolysate was added to 20 μL of 1 M NaHCO_3 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 (4.6 $\times$ 250 mm C_{18} column (5 μm); MeCN– H_2O (0.05% TFA)=32–45%; flow rate 1.0 mL/min; UV detection at 340 nm) and compared with the Marfey's derivatives of authentic amino acids.²³ The results showed that all the amino acids had L-configurations, except Gly. The standards derivatives gave the following t_{R} values (min): 5.67 (L-Glu-L-FDAA), 5.85 (L-Ser-L-FDAA), 6.40 (L-Asp-L-FDAA), 6.49 (L-Thr-L-FDAA), 8.17 (Gly-L-FDAA), 10.29 (L-Pro-L-FDAA), 12.29 (L-Tyr-L-FDAA), 16.29 (L-Val-L-FDAA), 20.40 (L-Ile-L-FDAA), 21.32 (L-Phe-L-FDAA), 21.70 (L-Leu-L-FDAA).

Platelet Aggregation in Rabbit Platelet-Rich Plasma (PRP) Rabbit common carotid artery was cut off to take a sample of blood, which was mixed with an anticoagulant (3.8% sodium citrate) in the proportion of 9 to 1, then centrifuged at 1000 rpm for 10 min at room temperature to give PRP. Centrifugation of the remaining blood at 3000 rpm for 10 min yielded platelet-poor plasma (PPP). Platelet count was adjusted to 3×10^5 with PPP. The samples were added to the PRP (0.200 mL), then the mixture was incubated at 37°C with stirring for 1 min before the addition of ADP (10 μM) as inducer of platelet aggregation. Aggregation was measured by a turbidimetric method. Changes in light transmission caused by platelet clotting were measured using an aggregometer. Platelet aggregation was expressed as the percentage change with the difference of light transmittance between PRP and PPP as 100%. The same experiment was done for the positive

control drug Aspirin and the blank solvent ethanol. Each analyte was tested three times, and an average value was applied. Antiplatelet activity was expressed as the percent inhibition of the control value.²⁵

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