New dicyclopeptides from *Dianthus chinensis*

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Abstract: One new dicyclopeptide cyclo-(*L*-*N*-methyl Glu-*L*-*N*-methyl Glu) (**1**), together with one new natural dicyclopeptide cyclo-(*L*-methyl Glu ester-*L*-methyl Glu ester) (**2**), and two known dicyclopeptides cyclo-(*L*-methyl Glu ester-*L*-Glu) (**3**), and cyclo-(*L*-Glu-*L*-Glu) (**4**), were isolated from the aerial parts of *Dianthus chinensis* L. Their structures were determined by spectroscopic analyses and chemical methods.

Key words: *Dianthus chinensis*; Caryophyllaceae; dicyclopeptides

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石竹中一个新的环二肽

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摘要: 对石竹 (*Dianthus chinensis*) 地上部分进行了化学成分研究, 经过一系列正相和反相色谱柱和高效液相色谱等现代分离技术, 分离得到 4 个环二肽 (**1**～**4**), 并通过 MS、NMR、IR 和 LC-MS 等波谱学方法对这些化合物进行结构鉴定, 其中 cyclo-(*L*-*N*-methyl Glu-*L*-*N*-methyl Glu) (**1**) 为一个新的环二肽。

关键词: 石竹; 石竹科; 环二肽

The genus *Dianthus* plants (Caryophyllaceae) are widely distributed as garden plants in Europe and Asia[1]. Cyclopeptides (CPs) are one of the markers of secondary metabolites for the family[2]. Caryophyllaceae-type CPs are homomono-cyclopeptides with mainly five to twelve *α*-amino acid residues, in which some CPs exhibited activities as cytotoxic, antiplatelet and inhibiting tyrosinase and melanogenesis[3].

The aerial parts of *Dianthus chinensis* L. have been used as a traditional Chinese medicine (TCM) Dianthi Herba (Chinese name 'Qu-Mai') in Chinese Pharmacopoeia (2010 Version), and are widely used for the treatment of diuresis and strangury[4]. Only eight saponins[5−7] and one monosaccharide[8] have been reported from this species. Over 20 dicyclopeptides were isolated from the Caryophyllaceae plants[3], in which some exhibited antitumor, antibacterial, and antifungal activities[9]. Herein we report one new dicyclopeptide cyclo-(*L*-*N*-methyl Glu-*L*-*N*-methyl Glu) (**1**), together with one new natural dicyclopeptide cyclo-(*L*-methyl Glu ester-*L*-methyl Glu ester) (**2**)[10], and two known dicyclopeptides cyclo-(*L*-methyl Glu ester-*L*-Glu) (**3**), and cyclo-(*L*-Glu-*L*-Glu) (**4**)[11] isolated from the aerial parts of *D. chinensis* (Figure 1). All compounds were tested for cytotoxicity and antibacterial activities.

Results and discussion

Compound **1**, was positive to the ninhydrin reaction

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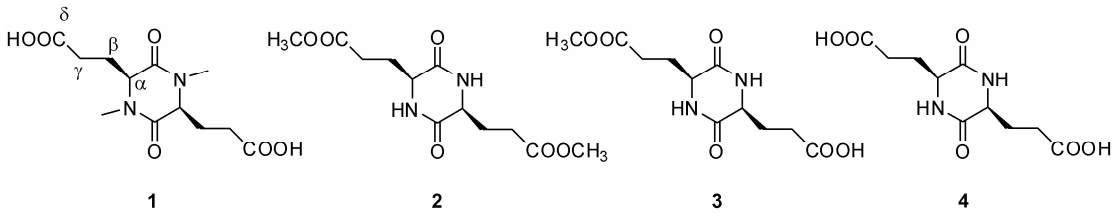

1 **2** **3** **4**

Figure 1 Structures of compounds **1**−**4**

Table 1 ^{1}H and ^{13}C NMR data for compounds **1**−**4**. † Recorded at 500 MHz for δ_H and 125 MHz for δ_C in pyridine-d_5, ‡ Recorded at 600 MHz for δ_H and 150 MHz for δ_C in pyridine-d_5 (J in Hz)

Position	**1**†		**2**†		**3**‡		**4**‡	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Amino acid 1								
α	4.51 (m)	56.8, CH	4.38 (dd, 7.5, 4.3)	56.1, CH	4.53 (dd, 12.0, 4.8)	56.9, CH	4.53 (dd, 10.4, 3.2)	57.0, CH
β	2.28 (m)	25.9, CH_2	2.08 (m)	25.4, CH_2	2.35 (m)	26.3, CH_2	2.34 (m)	26.3, CH_2
γ	2.39 (m)	30.3, CH_2	2.33 (m)	30.0, CH_2	2.53 (m)	30.7, CH_2	2.44 (m)	30.8, CH_2
δ		178.7, qC		178.3, qC		178.5, qC		178.7, qC
COOH	9.25 (br s)							
NH			9.20 (br s)		9.36 (br s)		10.70 (br s)	
C=O		176.2, qC		173.9, qC		174.2, qC		176.5, qC
NCH_3	2.03 (s)	30.7, CH_3						
OCH_3			3.62 (s)	52.3, CH_3	3.62 (s)	52.6, CH_3		
Amino acid 2								
α	4.51 (m)	56.8, CH	4.38 (dd, 7.5, 4.3)	56.1, CH	4.53 (dd, 12.0, 4.8)	56.9, CH	4.53 (dd, 10.4, 3.2)	57.0, CH
β	2.28 (m)	25.9, CH_2	2.08 (m)	25.4, CH_2	2.35 (m)	26.3, CH_2	2.34 (m)	26.3, CH_2
γ	2.39 (m)	30.3, CH_2	2.33 (m)	30.0, CH_2	2.53 (m)	30.7, CH_2	2.44 (m)	30.8, CH_2
δ		178.7, qC		178.3, qC		178.5, qC		178.7, qC
COOH	9.25 (br s)							
NH			9.20 (br s)		9.36 (br s)		10.70 (br s)	
C=O		176.2, qC		173.9, qC		176.5, qC		176.5, qC
NCH_3	2.03 (s)	30.7, CH_3						
OCH_3			3.62 (s)	52.3, CH_3				

of CPs[12]. The molecular formula was determined as $C_{12}H_{18}N_2O_6$ by HR-EI-MS at *m/z* 286.116 6 $[M]^+$, indicating 5 degrees of unsaturation. The IR spectrum showed NH and C=O absorptions at 3 419 cm^{-1} and 1 679 cm^{-1}. The ^{13}C NMR spectrum exhibited six signals corresponding to 12 carbons (Table 1), *i.e.* four carbonyls (δ_C 178.7×2, 176.2×2), two *α*-methines (δ_C 56.8×2), four methenyls (δ_C 30.3×2, 25.9×2), and two methyls (δ_C 30.7×2), indicating **1** is a cyclic dipeptide. Further, the ^{1}H NMR spectrum showed the presences of *α*-H (δ_H 4.51, m), N-CH_3 (δ_H 2.03, s), CH_2 (δ_H 2.39, m; 2.28, m), and COOH (δ_H 9.25, br s) signals, suggested **1** contained two *N*-methyl glutamic acid residues.

The absolute configurations of **1** were identified as *S* and *S* by the amino acid analysis and LC-MS according to the Marfey's method[13] (Table 2). The retention time of FDLA derivative of acid hydrolysate

Table 2 Analysis of FDLA derivatives of acid hydrolysates of **1**−**4**. [a]Standard of amino acids

No.	*L*-FDLA derivative	*D*, *L*-FDLA derivative		Absolute configuration
	t_R	t_{R1}	t_{R2}	
1	31.90	25.22	31.90	*L*
2	31.53	25.05	31.52	*L*
3	32.73	25.53	32.72	*L*
4	31.95	25.25	31.90	*L*
Glu[a]	31.75	25.16	31.71	*L*
N-CH_3 Glu[a]	32.65	24.36	32.67	*L*

of **1** indicated that *N*-methyl Glu in **1** is *L*-form. Therefore, compound **1** is determined as cyclo-(*L*-*N*-methyl Glu-*L*-*N*-methyl Glu).

Compounds **1**−**2** were natural products confirmed by detecting the Me_2CO-H_2O crude extract by the LC/MS. All compounds were tested for cytotoxicity and antibacterial activities, but showed no activity.

Experimental section

General experimental procedures Optical rotations were measured with a Horiba SEPA-300 polarimeter. IR spectra were obtained on a Tenor 27 spectrophotometer with KBr pellets. 1D and 2D NMR spectra were run on a Bruker AV-600 or Bruker DRX-500 spectrometer with TMS as internal standard. Mass spectra were recorded on a VG Autospec-3000 spectrometer or an API QSTAR Pulsar TOF spectrometer. LC/MS spectra were run on a Waters XEVO TQ-S MS spectrometer. Analytical or semi-preparative HPLC was performed on an Agilent 1100 with the Zorbax Eclipse-C_{18} (4.6 mm×150 mm; 9.4 mm×250 mm; 5 μm). Column chromatographies were performed on silica-gel (200−300 mesh, Qingdao Yu-Ming-Yuan Chemical Co. Ltd., Qingdao, China), Sephadex LH-20 (Pharmacia Fine Chemical Co., Uppsala, Sweden), or Lichroprep RP-18 (40−63 μm, Merck, Darmstadt, Germany). Fractions were monitored by TLC (GF254, Qingdao Yu-Ming-Yuan Chemical Co. Ltd., Qingdao, China), and spots were detected by spraying with ninhydrin reagent for cyclopeptides[12].

Plant material The aerial parts of *D. chinensis* were collected from Kunming, Yunnan Province, PRC, in May, 2009. The material was identified by Prof. Zhe-kun Zhou at Kunming Institute of Botany. The voucher specimen (No. 4146) has been deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation The air-dried and powdered aerial parts (75.0 kg) were extracted with 95% MeOH (4×100 L) for four times. After removal of the solvent under reduced pressure, the MeOH extract (21 kg) was suspended in H_2O and partitioned successively with EtOAc and *n*-BuOH, giving an EtOAc-soluble portion (6.4 kg) and a *n*-BuOH-soluble portion (8 kg). The EtOAc fraction (6.4 kg) was subjected to silica gel column chromatography eluting with $CHCl_3$−MeOH (1∶0, 95∶5, 9∶1, 8∶2, 0∶1) to afford a CP-containing fraction (1.2 kg, $CHCl_3$−MeOH, 95∶5, 9∶1, 8∶2) and a CP-non-containing fraction (2.5 kg, $CHCl_3$−MeOH, 1∶0, 0∶1). The CP-containing fraction was loaded onto a silica gel column and eluted with gradient $CHCl_3$−$CH_3COOC_2H_5$ (30∶1−1∶1). Seven fractions (I−VII) were obtained, and fractions Ⅲ and VI contained CPs. Fraction Ⅲ (100 g) was subjected to silica gel column ($CHCl_3$−Me_2CO 10∶1, 5∶1), sub-fraction 3 was subjected to Sephadex LH-20 ($CHCl_3$−MeOH, 1∶1 and 2∶1), then Lichroprep RP-18 (MeOH−H_2O, 70∶30−100∶0), and further purified by HPLC (Zorbax Eclipse-C_{18}, 5 μm, 9.4 mm× 250 mm, 1.0 mL·min^{-1}, UV detection at 215, 230, 254 and 280 nm) eluting with 30%−36% CH_3CN which contained 0.04% TFA to get **1** (21.1 mg) and **2** (12.4 mg). Fraction VI (24.0 g) was subjected to CC on silica gel ($CHCl_3$−Me_2CO, 3∶1, 5∶1), Sephadex LH-20 (MeOH), and Lichroprep RP-18 (MeOH−H_2O, 50∶50−70∶30), and further purified by HPLC (Zorbax Eclipse-C_{18}, 5 μm, 9.4 mm × 250 mm, 1.0 mL·min^{-1}, UV detection at 215, 230, 254 and 280 nm) eluting with 30% CH_3CN which contained 0.04% TFA to provide **3** (11.2 mg) and **4** (17.6 mg).

Cyclo-(*L*-*N*-methyl Glu-*L*-*N*-methyl Glu) (1) White powder; $[\alpha]_D^{15}$ −13.9 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3 419, 2 986, 2 904, 1 679, 1 647, 1 607, 1 420, 1 205, 1 139 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) spectroscopic data (Table 1); HR-EI-MS *m/z* 286.116 6 $[M]^+$ (calcd for $C_{12}H_{18}N_2O_6$, 286.116 5).

Cyclo-(*L*-methyl Glu ester-*L*-methyl Glu ester) (2) Yellow oil; $[\alpha]_D^{16}$ +1.0 (*c* 0.50, MeOH); IR (KBr) ν_{max} 3 258, 2 959, 2 607, 2 531, 1 711, 1 463, 1 422, 1 384, 1 203 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz) spectroscopic data (Table 1); positive ESI-MS *m/z* 287 $[M+H]^+$ (80); HR-EI-MS *m/z* 286.112 5 $[M]^+$ (calcd for $C_{12}H_{18}N_2O_6$, 286.116 5).

Cyclo-(*L*-methyl Glu ester-*L*-Glu) (3) White powder; $[\alpha]_D^{16}$ −7.2 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3 418, 2 924, 2 854, 1 681, 1 645, 1 453, 1 420, 1 234 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) and ^{13}C NMR (C_5D_5N, 150 MHz) spectroscopic data (Table 1); positive ESI-MS *m/z* 272 $[M+H]^+$ (5).

Cyclo-(*L*-Glu-*L*-Glu) (4) White powder; $[\alpha]_D^{15}$ +31.9 (*c* 0.10, MeOH); IR (KBr) ν_{max} 3 402, 3 000, 2 888, 1 719, 1 648, 1 421, 1 331, 1 232 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) and ^{13}C NMR (C_5D_5N, 150 MHz) spectroscopic data (Table 1); EI-MS *m/z* 281 $[M+Na]^+$ (10); positive HR-ESI-MS *m/z*: 281.074 3 $[M+Na]^+$ (calcd for $C_{10}H_{14}N_2O_6$, 281.074 9).

Configuration of 1−4 (Marfey's Method[13]) Compounds **1**−**4** (1 mg each) were separately dissolved in 6 mol·L^{-1} HCl (1 mL) in a sealed glass tube and incubated at 115 ℃ for 24 h. The hydrolyzate was divided into two portions (0.5 mg each), then dried. Each hydrolyzate was dissolved in 100 μL of acetone, 1 mol·L^{-1} $NaHCO_3$ (20 μL), and 1% 1-fluoro-2, 4-dinitrophenyl-5-*L*-leucinamide (-*L*- or -*D*-FDLA, Marfey's

reagent, Sigma Aldrich, 100 μL) were added. The mixture was incubated at 45 ℃ for 2 h. The reaction was quenched by adding 2 mol·L^{-1} HCl (10 μL) after being cooled, and the dried mixture was dissolved in 50% aqueous CH_3CN (600 μL). 10 μL of each solution of FDLA derivatives was analyzed by LC/MS.

The analysis of the *L*- and *D*, *L*-FDLA (mixture of *D*- and *L*-FDLA) derivatives was performed using an Agilent Eclipse XDB-C_{18} column (4.6 mm×150 mm, 5 μm) maintained at 40 ℃. Acetonitrile-0.1% HCOOH−H_2O was used as the mobile phase under a linear gradient elution mode (acetonitrile, 10%−70%, 50 min) at a flow rate of 1 mL·min^{-1}. A Waters Xevo TQ-S mass spectrometer was used for detection in ESI (negative) mode. The capillary voltage was kept at 2.5 kV and the ion source at 350 ℃. Nitrogen gas was used as a sheath gas at 400 L·h^{-1}. A mass range of *m/z* 100−1 000 was scanned in 0.2 s.

Detection of 1−2 The aerial parts of *D. chinensis* (100 g) were extracted three times with Me_2CO−H_2O (5∶1, 1.2 L), then subjected to Sephadex LH-20 (Me_2CO−H_2O, 10∶1). The fractions positive to the ninhydrin reaction and compounds **1−2** were analyzed by HPLC and LC/MS.

The analysis was performed using a Zorax Eclipse XDB-C_{18} column (4.6 mm×150 mm, 5 μm) maintained at 40 ℃. Acetonitrile-1‰ HCOOH/H_2O was used as the mobile phase under a linear gradient elution mode (acetonitrile, 0−10%, 45 min) at a flow rate of 1 mL·min^{-1}. A Bruker mass spectrometer was used for detection in ESI (positive) mode. The capillary voltage was kept at −4.0 kV and the ion source at 330 ℃. Nitrogen gas was used as a sheath gas at 10 L·min^{-1}. A mass range of *m/z* 100 − 1 000 was scanned in 0.2 s.

Cytotoxic assay The cytotoxicity of compounds **1−4** against A549, HeLa and BGC-823 cancer cell lines were evaluated by the SRB assay with taxol as a positive control[14]. The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum at 37 ℃ and 5% CO_2 for 48 h. Aliquots of 90 μL were seeded in 96-well flat-bottomed microtiter plates for 24 h and treated with compounds **1−4** at the maximum concentration of 20 μg·mL^{-1}. After incubation for another 48 h, cells were fixed with 25 μL of ice-cold 50% trichloroacetic acid and incubated at 4 ℃ for 1 h. After washing, air-drying, and staining for 15 min with 100 μL 0.4% SRB in 1% glacial acetic acid, excessive dye was removed by washing with 1% glacial acetic acid. SRB was resuspended in 100 μL 10 mmol·L^{-1} Tris buffer, and the absorbance was measured at 560 nm with a Plate Reader (Molecular Devices, SPECTRA MAX 340). The percentage inhibition of cell growth below 50% is regarded as inactive.

Antimicrobial assay Compounds **1−4** were tested for their antimicrobial activity against *Candida albicans* and *Staphylococcus aureus in vitro* using a turbidimetric method[14]. Miconazole nitrate was used as a positive control. *C. albicans* and *S. aureus* were inoculated in potato dextrose broth (formulated identically to potato dextrose agar (PDA), omitting the agar, prepared by ourselves) and Mueller Hinton Broth (Oxiod, CM0405, Hampshire, England) to McFarland 0.5 and diluted with the medium to 1×10^6 CFU·mL^{-1}. Aliquots of 90 μL were filled in 96-well U-bottomed microplates, and then treated compounds **1−4** with the maximum concentration of 20 μg·mL^{-1}. After cultured at 37 ℃ for 24 h, the absorbance was measured at 620 nm with the microplate reader mentioned above. The percentage inhibition of cell growth below 50% is regarded as inactive.

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