

Longicalycinin A, a New Cytotoxic Cyclic Peptide from *Dianthus superbus* var. *longicalycinus* (MAXIM.) WILL.

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A new cyclic peptide, longicalycinin A (1), and six known compounds, vaccaroside A, dianoside A, dianoside G, 3-(4-hydroxy-3-methoxy-phenyl)propionic acid methyl ester, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde were isolated from the MeOH extract of *Dianthus superbus* var. *longicalycinus*. The amino acid sequences of 1 was elucidated as cyclo(Gly¹-Phe²-Tyr³-Pro⁴-Phe⁵-) on the basis of ESI tandem mass fragmentation analysis, chemical evidence, and extensive 2D NMR methods. Furthermore, compound 1 showed cytotoxicity to Hep G2 cancer cell line.

Key words *Dianthus superbus* var. *longicalycinus*; cyclic peptide; cytotoxicity

The species *Dianthus superbus* var. *longicalycinus* is an Oriental drug for treating diuretic, carcinoma, and inflammatory.¹⁾ In previous studies, nine new triterpene saponins were isolated, and showed anti-hepatotoxic and anti-inflammatory activities.^{1–4)} As part of our research for bioactive constituents of *Dianthus* sp., we previously investigated the extract of *Dianthus superbus*, a Chinese medicine, and isolated four new cyclic peptides dianthins C–F, along with a new dianthramide.⁵⁾ Among them, a cyclic peptide, dianthin E, and dianthramide, 4-methoxydianthramide B, showed selectively cytotoxicity to Hep G2 cancer cell line. In a continuing research of this genus plants, a new cyclic peptide, longicalycinin A (1), together with six known compounds, vaccaroside A,⁶⁾ dianoside A,¹⁾ dianoside G,³⁾ 3-(4-hydroxy-3-methoxy-phenyl)propionic acid methyl ester,⁷⁾ *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde, were isolated from *D. superbus* var. *longicalycinus*. The isolation and structural elucidation of the new compound are reported herein.

The methanolic extracts of *D. superbus* var. *longicalycinus* were partitioned between *n*-hexane and 80% aqueous MeOH. The latter extract was further partitioned between H₂O and *n*-BuOH. Among them, the *n*-BuOH layer showed the peptide signals in its NMR spectrum. Therefore, this layer was further separated, and gave a new compound (Fig. 1), longicalycinin A (1), together with six known compounds, vaccaroside A, dianoside A, dianoside G, 3-(4-hydroxy-3-methoxy-phenyl)propionic acid methyl ester, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde. The known compounds were identified by comparisons of spectral data with those reported.^{1,3,6,7)}

Longicalycinin A (1) was obtained as pale yellow powder.

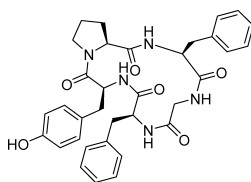


Fig. 1. The Structure of 1

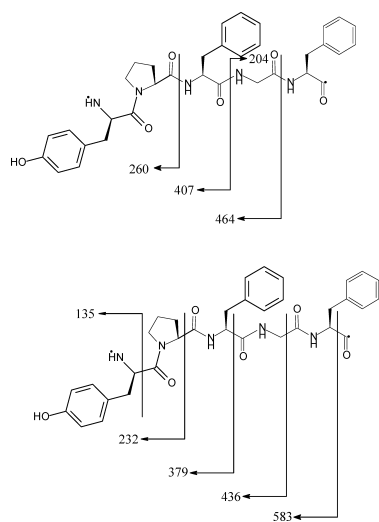
Absorptions at 3407, 1685, and 1518 cm⁻¹ in the IR were characteristic of amide, carbonyl, and aromatic functions, respectively. The NMR spectra of 1 (Table 1) showed four amide N–H signals and five carbonyls, which indicated that 1 might belong to a peptide class of compound. A negative ninhydrin test indicated its cyclic nature. The molecular weight 611 Da was obtained from ESI mass spectrum, which showed the pseudomolecular ion [M+H]⁺ at *m/z* 612, and the sodium adduct ion at *m/z* 634, respectively. Analysis of 2D NMR data (TOCSY and ROESY) and ESI-MS³ data demonstrated that the amino acid residues are Gly, Pro, Tyr, and Phe×2. The sequence of the amino acid residues was deduced from ESI-MS³ analysis (Fig. 2). As shown, the collisional induced decomposition (CID) experiment on the [M+H]⁺ ion of 1 gave preferential ring opening at the Phe²-Tyr³ amide bond, and gave relative B ions (a peptide fragmented at a single peptide bond retaining the positive charge at the N-terminus) of peptide fragments. The fragment ion at *m/z* 465 could be attributed to Tyr³-Pro⁴-Phe⁵-Gly¹-, and was followed by the subsequent losses of Gly¹ and Phe². Furthermore, a series of A ion (a peptide fragmented at a C–C=O bond retaining the positive charge at the N-terminus) fragments also obtained at *m/z* 584, 437, 380, 233, and 136, which were assigned to Phe²-Gly¹-Phe⁵-Pro⁴-Tyr³. Thus the structure of 1 was established as cyclo(Gly¹-Phe²-Tyr³-Pro⁴-Phe⁵). The difference of ¹³C-NMR chemical shifts of Pro⁴ ($\Delta\delta_{\beta-\gamma} = 7.6$ ppm) provided evidence that the amide bond in the Pro residue is *cis*.⁸⁾ The configuration of each amino acid residue was assigned as L, which was deduced by acid hydrolysis and Marfey's analysis of the individual amino acids.^{9,10)} The secondary structure of 1 was not included helix, turn, and β -sheet, and was confirmed by CD spectrum which showed three negative Cotton effect at 217, 212, and 196 nm.^{11–13)} However, it still needs more evidence to establish the conformation of 1.

The cytotoxicities of isolates were evaluated against the cell lines of human hepatocellular carcinoma Hep G2 and Hep 3B, human breast carcinoma MCF-7 and MDA-MB-231, and human lung carcinoma A-549, respectively. However, only compound 1 showed activity against Hep G2 cancer cell line with IC₅₀ value 13.52 μ g/ml.

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Table 1. ^1H - (400 MHz) and ^{13}C - (100 MHz) NMR Data of **1** in $\text{C}_5\text{D}_5\text{N}$

		δ_{H} , mult. (J in Hz)	δ_{C} , mult.	ROESY (δ_{H})	
Gly ¹	C=O		170.7 (s)		
	NH	8.29 (br d, 8.8)			
	α	4.84 (dd, 16.0, 8.8) 3.60 (dd, 16.0, 8.8)	43.6 (t)	3.62 4.84	
Phe ²	C=O		171.2 (s)		
	NH	10.90 (d, 7.2)		5.05	
	α	4.37 (m)	57.7 (d)		
	β	3.62 (m) 3.85 (m)	40.0 (t)	3.85 3.62	
	Ar		7.00—7.41 (m)	140.2 (s) 130.2 (d) 128.9 (d) 126.8 (d)	
		C=O		171.3 (s)	
NH		8.43 (br d, 9.6)			
α		5.34 (ddd, 14.4, 9.6, 9.6)	54.3 (d)	7.56, 7.34, 4.37, 3.95, 3.43	
β		3.43 (m)	35.5 (t)	7.56, 7.34, 5.34, 3.95	
		3.95 (m)		7.56, 7.34, 3.43	
	Ar		127.5 (s) 130.7 (d) 116.4 (d) 157.9 (s)	7.34 7.56	
Pro ⁴	C=O		170.7 (s)		
	α	4.40 (t, 8.0)	60.3 (d)		
	β	1.81 (m)	30.5 (t)		
	γ	1.90 (m)	22.9 (t)		
	δ		1.65 (m) 3.80 (m) 3.42 (m)	48.8 (t)	
		C=O		171.2 (s)	
Phe ⁵	NH	8.53 (br s)			
	α	5.05 (m)	57.0 (d)	10.90, 3.26, 3.20	
	β		3.20 (m) 3.26 (m)	36.7 (t)	5.05, 3.26 5.05, 3.20
		Ar	7.00—7.41 (m)	138.5 (s) 129.8 (d) 128.8 (d) 126.6 (d)	

Fig. 2. Longicalycinin A (**1**) ESI-MS³ Fragments Analysis

Experimental

Optical rotations were measured with a JASCO P-1020 digital polarimeter. The UV spectra were obtained on a Hitachi 200-20 spectrophotometer, and IR spectra were measured on a Hitachi 260-30 spectrophotometer. CD spectra were measured on a Jasco J-810 circular dichroism spectrometer (using 0.5 cm length cell). NMR (using $\text{C}_5\text{D}_5\text{N}$ as solvents) spectra were obtained on a Varian Unity Plus 400 FT-NMR. ESI-MSⁿ was recorded on an API 3000TM (Applied Biosystems). Low-resolution EI-MS were collected on a Quattro GC/MS spectrometer having a direct inlet system. High-resolution FAB-MS were collected on a Finnigan/Thermo Quest MAT 95XL spectrometer. High-resolution EI-MS were collected on a JEOL JMS SX/SX 102A spectrometer. Shimadzu LC-10AT pumps, SPD-10A UV-VIS detector, and Hypersil ODS $5\ \mu\text{m}$ ($250 \times 4.6\ \text{mm}$ i.d.) and preparative ODS $5\ \mu\text{m}$ ($250 \times 21.2\ \text{mm}$ i.d.) columns were employed in a HPLC system.

Plant Material *D. superbus* var. *longicalycinus* was collected from Nan-Tao (Lu-Guo) and identified by Dr. Hsin-Fu Yen (National Museum of Natural Science, Taichung, Taiwan). The samples were authenticated and deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan (KMU-DS-002).

Extraction and Isolation The air-dried plant (180 g) of *D. superbus* var. *longicalycinus* was extracted with MeOH at room temperature. The methanol extract (16 g) was partitioned between *n*-hexane–80% MeOH/ H_2O to yield *n*-hexane and MeOH extracts. The MeOH extract (10 g) was further partitioned between H_2O and *n*-BuOH to yield H_2O and *n*-BuOH extracts. The *n*-BuOH extract (2 g) was separated on Sephadex LH-20 with 80% MeOH/ H_2O to give six fractions (A–F). Fraction C (300 mg) was further

purified by HPLC (MeCN/H₂O 30:70, flow rate: 3.6 ml/min, detection at 220 nm) to give **1** (2.4 mg). Fraction B (320 mg) was further separated using an RP-18 (LiChroprep, 40—63 mm, Merck) flash column (eluting with H₂O, 90% MeCN/H₂O, 70% MeCN/H₂O, 50% MeCN/H₂O, and 100% MeCN) and afford five fractions. The subfraction B-2 was purified by preparative reverse-phase HPLC (MeCN/H₂O=25:75, flow rate: 3.6 ml/min, detection at 225 nm) to obtain vaccaroside A (8.3 mg), dianoside A (4.8 mg), and dianoside G (3.2 mg). The subfraction B-3 was purified by preparative reverse-phase HPLC (MeCN/H₂O=30:70, flow rate: 3.6 ml/min, detection at 225 nm) to give dianoside A (4.8 mg). The fraction D (125 mg) was purified by preparative HPLC (MeCN/H₂O=36:64, flow rate: 3.5 ml/min, detection at 254 nm) to yield 3-(4-hydroxy-3-methoxy-phenyl)propionic acid methyl ester (2.3 mg). The fraction E (55 mg) was purified by silica gel column to yield *p*-hydroxybenzoic acid (4.2 mg), and *p*-hydroxybenzaldehyde (3.3 mg).

Hydrolysis and Derivatization of **1**^{11,12)} Compound **1** (0.1 mg) was hydrolysis following methods as described previously.^{5,10)}

Electrospray Ionization Tandem Mass Spectrometry This experimental was processed using methods as described previously.^{5,10)}

Cytotoxicity Assays This assay was performed using methods as described previously.³⁾

Longicalycinin A (**1**): Pale-yellow powder; [α]_D²⁵ -12° (*c*=0.01, MeCN); UV (MeCN) λ_{\max} (log ϵ) 202 (4.01), 230 (3.75), 264 (sh, 3.52) nm; CD (*c*=1.6×10⁻⁴ M, MeOH) λ_{\max} (mdeg) 217 (sh, -3.45), 212 (-3.80), 204 (-2.39), 196 (-4.72) nm; IR (KBr) ν_{\max} 3407, 2925, 2857, 1685, 1604, 1518, 1449, 1280, 1090, 1017, 847, 772, 752 cm⁻¹; HR-FAB-MS *m/z* 612.2750 ([M+H]⁺, Calcd for C₃₄H₃₇N₅O₆: 612.2744); ¹H-NMR (400 MHz, C₅D₅N) and ¹³C-NMR (100 MHz, C₅D₅N), see Table 1; ESI-MS (Full scan)

m/z 634 (100, [M+Na]⁺), 612 (25, [M+H]⁺); ESI-MS/MS *m/z* 612 (100, [M+H]⁺), 584 (33), 465 (20), 437 (9), 408 (12), 380 (9), 302 (15), 278 (14), 261 (48), 233 (62), 204 (6), 172 (6), 136 (6), 120 (12), 69 (5).

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