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

Pei-Wen HSIEH,[a] Fang-Rong CHANG,[a] Ching-Chung WU,[a] Chien-Ming LI,[b] Kuen-Yuh WU,[b] Su-Li CHEN,[a] Hsin-Fu YEN,[c] and Yang-Chang WU*,[a]

[a] *Graduate Institute of Natural Products, Kaohsiung Medical University; Kaohsiung 807, Taiwan:* [b] *Divisions of Environmental Health and Occupational Medicine, National Health Research Institute; Kaohsiung 807, Taiwan: and* [c] *National Museum of Natural Science; Taichung, 404, Taiwan.* Received October 23, 2004; accepted January 5, 2005

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(Gly[1]-Phe[2]-Tyr[3]-Pro[4]-Phe[5]-) 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. *longicalysinus* 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. *longicalysinus*. The isolation and structural elucidation of the new compound are reported herein.

The methanolic extracts of *D. superbus* var. *longicalysinus* were partitioned between *n*-hexane and 80% aqueous MeOH. The latter extract was further partitioned between H_2O 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, dianosdie 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.

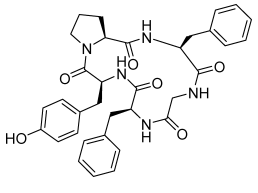

Fig. 1. The Structure of **1**

Absorptions at 3407, 1685, and 1518 cm^{-1} in the IR were characteristic of amide, carbonyl, and aromatic functions, respectviely. 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-MS^3 data demonstrated that the amino acid residues are Gly, Pro, Try, and Phe×2. The sequence of the amino acid residues was deduced from ESI-MS^3 analysis (Fig. 2). As shown, the collisional induced decomposition (CID) experiment on the $[M+H]^+$ ion of **1** gave preferential ring opening at the Phe[2]-Tyr[3] 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 Tyr[3]-Pro[4]-Phe[5]-Gly[1]-, and was followed by the subsequent losses of Gly[1] and Phe[2]. 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 Phe[2]-Gly[1]-Phe[5]-Pro[4]-Tyr[3]. Thus the structure of **1** was established as cyclo(Gly[1]-Phe[2]-Tyr[3]-Pro[4]-Phe[5]). The difference of ^{13}C-NMR chemical shifts of Pro[4] ($\Delta\delta_{C\beta\text{-}C\gamma}$=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 IC_{50} value 13.52 μg/ml.

* To whom correspondence should be addressed. e-mail: yachwu@kmu.edu.tw

Table 1. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR Data of **1** in C_5D_5N

		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)	43.6 (t)	3.62
		3.60 (dd, 16.0, 8.8)		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)	40.0 (t)	3.85
		3.85 (m)		3.62
	Ar	7.00—7.41 (m)	140.2 (s)	
			130.2 (d)	
			128.9 (d)	
			126.8 (d)	
Tyr[3]	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)	
		7.56 (d, 8.4)	130.7 (d)	7.34
		7.34 (d, 8.4)	116.4 (d)	7.56
			157.9 (s)	
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)	
		1.65 (m)		
	δ	3.80 (m)	48.8 (t)	
		3.42 (m)		
Phe[5]	C=O		171.2 (s)	
	NH	8.53 (br s)		
	α	5.05 (m)	57.0 (d)	10.90, 3.26, 3.20
	β	3.20 (m)	36.7 (t)	5.05, 3.26
		3.26 (m)		5.05, 3.20
	Ar	7.00—7.41 (m)	138.5 (s)	
			129.8 (d)	
			128.8 (d)	
			126.6 (d)	

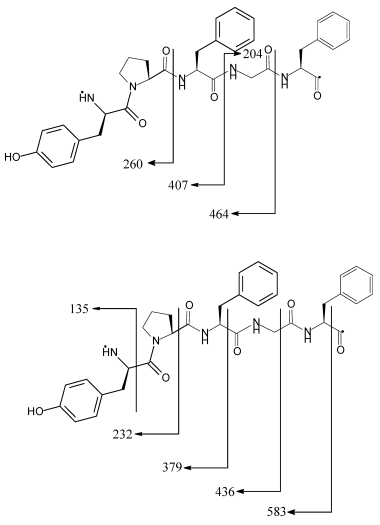

Fig. 2. Longicalycinin A (**1**) ESI-MS^3 Fragments Analysis

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 C_5D_5N as solvents) spectra were obtained on a Varian Unity Plus 400 FT-NMR. ESI-MS^n was recorded on an API 3000^{TM} (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 μm (250×4.6 mm i.d.) and preparative ODS 5 μm (250×21.2 mm i.d.) columns were employed in a HPLC system.

Plant Material *D. superbus* var. *longicalysinus* 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. *longicalysinus* 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/H_2O 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 H_2O, 90% MeCN/H_2O, 70% MeCN/H_2O, 50% MeCN/H_2O, and 100% MeCN) and afford five fractions. The subfraction B-2 was purified by preparative reverse-phase HPLC (MeCN/H_2O=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/H_2O=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/H_2O=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.[5]

Longicalycinin A (**1**): Pale-yellow powder; $[\alpha]_D^{25}$ −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 $C_{34}H_{37}N_5O_6$: 612.2744); ^{1}H-NMR (400 MHz, C_5D_5N) and ^{13}C-NMR (100 MHz, C_5D_5N), 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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