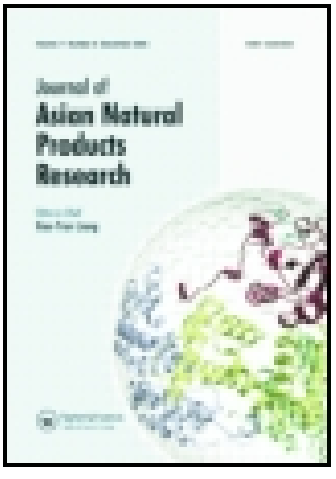

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A new cyclolignan glycoside from the tubers of *Pinellia ternata*

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A new 2,7′-type cyclolignan glycoside, cyclolignanyingoside A (**1**), together with six known compounds (**2**–**7**) were isolated from the tubers of *Pinellia ternata* (Thunb.) Breit. The structure of **1** was elucidated on the basis of chemical and spectral analysis, including 1D, 2D NMR analyses, HR-ESI-MS, and CD spectrometry. The cytotoxic, antioxidant and tyrosinase-inhibiting activities of all the isolates were determined. However, all the isolates exhibited no activity on the selected cell lines (Hep-3B, Bcap-37, and MCF-7). In addition, compounds **1**–**3** and **7** exhibited strong 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity, and compounds **2** and **4** showed a moderate mushroom tyrsinase inhibitory activity.

Keywords: Araceae; *Pinellia ternata*; cyclolignan glycoside; cytotoxic activity; antioxidant activity; tyrosinase-inhibiting activity

1. Introduction

Pinellia ternata (Thunb.) Breit, a member of the family Araceae, is widely distributed in China including Hubei, Anhui, and Sichuan Provinces. Its tubers are one of the most important original plants in Traditional Chinese Medicine which have been used for antitussive, antiemetic, anti-inflammatory, analgesic, antiulcer, and anticancer effects in oriental medicine [1–3]. Previous phytochemical studies on *P. ternata* showed that many kinds of compounds have been isolated from this plant, such as alkaloids, lectins, fatty acids, cerebrosides, volatile oils, flavonoids, sterols, and phenylpropanoids [4–8]. *P. ternata* is also widely used in many traditional medicine preparations, such as Xiao Chai Hu Decoction and Xiao Qing Long Decoction [9,10]. Our research is a part of the chemical constituents basic study for Xiao Chai Hu Decoction (the National Natural Sciences Foundation of China). In addition, there have been a lot of counterfeit drugs used instead of *P. ternata*. The chemical constituents study can provide a basis for the quality control of *P. ternata*. In this paper, we describe the isolation and structural elucidation of a new cyclolignan glycoside, along with six known compounds obtained from the 75% EtOH extract of *P. ternata*. Their structures (Figure 1) were established by extensive spectroscopic data analysis and comparison with literature values. Furthermore, the cytotoxic, antioxidant and tyrosinase-inhibiting activities of all the isolated compounds were evaluated.

2. Results and discussion

Compound **1** was obtained as a yellowish gum, and the HR-ESI-MS exhibited a molecular ion peak at *m/z* 677.2414 $[M + Na]^+$, corresponding to the molecular

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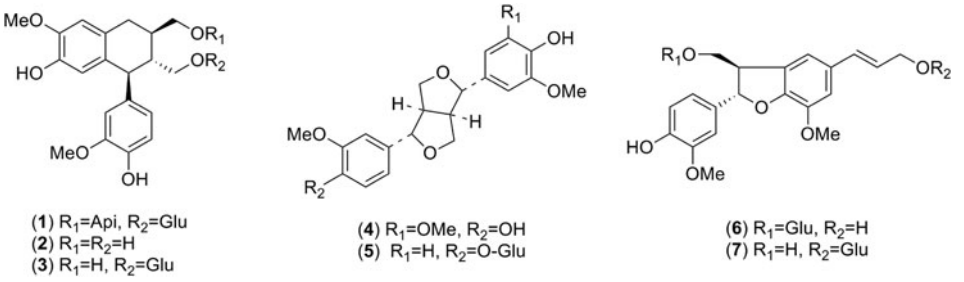

Figure 1. The structures of compounds **1**–**7**.

formula $C_{31}H_{42}O_{15}$. Compound **1** afforded isolariciresinol, glucose, and apiose by an acid hydrolysis experiment [11,12] that was identified by comparison of its NMR spectral data with those in the literature [13]. In the ^{1}H NMR spectrum, one 1,3,4-trisubstituted aromatic ring [δ_H 6.79 (1H, d, $J = 1.9$ Hz), 6.48 (1H, dd, $J = 8.0$, 1.9 Hz) and 6.68 (1H, d, $J = 8.0$ Hz)], a tetrasubstituted aromatic ring [δ_H 6.59 (1H, s) and δ_H 6.07 (1H, s)], two anomeric protons [δ_H 4.85 (1H, d, $J = 2.9$ Hz) and 3.94 (1H, d, $J = 7.7$ Hz)], and two methoxyl groups [δ_H 3.70 (3H, s) and δ_H 3.72 (3H, s)] were observed (Table 1). The ^{13}C NMR spectrum exhibited one methylene (δ_C 32.5), two oxygenated methylenes (δ_C 67.4 and 67.6), three methines (δ_C 37.4, 44.1 and 45.5), two anomeric carbons (δ_C 109.3 and 103.9), twelve aromatic carbons (δ_C 111.9, 113.9, 115.5, 116.3, 121.1, 127.7, 132.7, 136.9, 144.0, 144.5, 145.5, and 147.2), two methoxy groups (δ_C 55.5 and 55.6), and one glucopyranoyl unit (δ_C 103.9, 73.5, 76.6, 70.2, 75.8, and 62.7). The ^{1}H and ^{13}C NMR spectra of **1** were in part very similar to those of isolariciresinol 9-*O*-β-D-glucopyranoside [7] except for the presence of an apiofuranosyl unit (δ_C 109.3, 75.5, 78.8, 73.8, 63.2) [13]. Comparison of the ^{13}C

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

No.	δH	δC	No.	δH	δC
1		136.9	7′	2.72 (2H, overlapped)	32.5
2	6.79 (1H, d, $J = 1.9$ Hz)	113.9	8′	1.89 (1H, overlapped)	37.4
3		147.2	9′a	3.38–3.40 (1H, m)	67.6
			9′b	3.80 (1H, overlapped)	
4		144.5	Glu-1″	3.94 (1H, d, $J = 7.7$ Hz)	103.9
5	6.68 (1H, d, $J = 8.0$ Hz)	115.5	2″	2.99 (1H, overlapped)	73.5
6	6.48 (1H, dd, $J = 8.0$, 1.9 Hz)	121.1	3″	3.12–3.14 (1H, m)	76.6
7	4.03 (1H, d, $J = 10.6$ Hz)	45.5	4″	3.17–3.19 (1H, m)	70.2
8	1.69 (1H, overlapped)	44.1	5″	3.68 (1H, overlapped)	75.8
9a	2.99 (1H, overlapped)	67.4	6″	3.59 (1H, overlapped)	62.7
				3.47–3.49 (1H, m)	
9b	3.88–3.90 (1H, m)		Api-1‴	4.85 (1H, d, $J = 2.9$ Hz)	109.3
1′		127.7	2‴	3.17 (1H, overlapped)	75.5
2′	6.59 (1H, s)	111.9	3‴		78.8
3′		145.5	4‴	3.81 (1H, overlapped)	73.8
				3.67 (1H, overlapped)	
4′		144.0	5‴	3.28–3.30 (2H, m)	63.2
5′	6.07 (1H, s)	116.3	3-OCH_3	3.72 (3H, s)	55.5
6′		132.7	3′-OCH_3	3.70 (3H, s)	55.6

Note: Coupling constants (*J*) in Hz are given in parentheses; Chemical shift values are expressed in ppm.

NMR spectral data (DMSO-d_6) of **1** with those of isolariciresinol 9-*O*-β-D-glucopyranoside [7] revealed a downfield shift of C-9′ (4.9 ppm), indicating that an additional sugar was located at C-9′. The β-anomeric configuration for the apiofuranosyl group was determined from its C-1‴ data (109.3) [14]. The configuration of the anomeric proton of glucose was proposed as β on the basis of its coupling constants ($J = 7.7$ Hz). The absolute configurations of the monosaccharide units mentioned above were successively determined as D and D, with the help of GC analysis of their respective derivatives [12]. The arrangements of the hydroxyl, methoxyl, apiose, and glucose moieties were determined by analysis of the proton coupling patterns, HMQC, and HMBC data (Figure 2). In the HMBC spectrum, the correlation of the anomeric proton at δ 4.85 (1H, d, $J = 2.9$ Hz, apiofuranosyl H-1‴) with C-9′ at δ 67.6 revealed that the apiose was linked to C-9′; the correlation of the anomeric proton at δ 3.94 (1H, d, $J = 7.7$ Hz, glucopyranosyl H-1″) with C-9 at δ 67.4 revealed that the glucose was linked to C-9 (Figure 2).

The configurations at C-7 and C-8 were confirmed by the large coupling constants of H-7 ($J = 10.6$ Hz) [15,16], and there was no NOESY correlation between H-7 and H-8, reflecting the *trans* configuration at C-7 and C-8. Furthermore, there was a correlation between H-7 and H-8′ from the NOESY spectrum. Thus, compound 1 had a *trans*–*trans* configuration at C-7, C-8, C-8′. The (7*S*, 8*R*, 8′*R*)-stereochemistry was elucidated by comparison of its circular dichroism (CD) and optical rotation with the reported compound urinatetralin [16] (see Experimental). Based on the above evidence, the structure of **1** was unambiguously established as shown in Figure 2 and it was given the trivial name cyclolignanyingoside A.

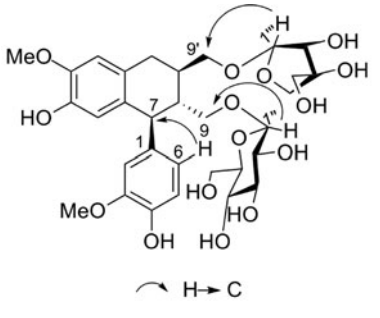

Figure 2. Key HMBC correlations of compound **1**.

The known compounds were readily identified as isolariciresinol (**2**) [7], isolariciresinol-9-*O*-β-D-glucopyranoside (**3**) [7], medioresinol (**4**) [17], pinoresinol-4′-*O*-β-D-glucopyranoside (**5**) [18], dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**6**) [19] and dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**7**) [19] by comparing NMR spectral data with those reported in the literature.

All the isolated compounds were evaluated for their cytotoxic activities against breast cancer (Bcap-37), hepatoma (Hep-3B) and breast cancer (MCF-7) cell lines by the MTT Method [20], using 5-fluorouracil as a positive control. However, none of the isolated compounds exhibited any cytotoxicity. It has been reported that alkaloids and lectins may be responsible for the anti-tumor activity of *P. ternata* [8]. Thus, lignanoids may contribute to other effects of *P. ternata*.

Lignanoids have been claimed to possess antioxidant activity and could serve as lead compounds for the development of antioxidant agents [21]. The antioxidant effects of the isolates were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays. It has been reported that the DPPH radical scavenging assay is usually used to evaluate the ability of new compounds or extracts to capture free radicals, whereas the ABTS cation is another synthetic radical which is more versatile than DPPH, and the ABTS cation model can be used to assess the scavenging

Table 2. Antioxidant activities of compounds **1**–**7**.

Compound	Antioxidant activities	
	DPPH	ABTS
1	$>100^{b}$	7.4 ± 0.7^{b}
2	75.6 ± 3.7^{b}	2.9 ± 0.4^{b}
3	$>100^{b}$	7.4 ± 0.8^{b}
4	$>100^{b}$	22.7 ± 1.4^{b}
5	$>100^{b}$	9.9 ± 1.1^{b}
6	78.9 ± 3.8^{b}	9.1 ± 1.3^{b}
7	88.7 ± 3.5^{b}	4.5 ± 0.6^{b}
Trolox[a]	9.5 ± 0.8^{b}	5.3 ± 0.7^{b}

Note: The IC_{50} greater than 100 μg/ml was considered to indicate no antioxidant activities.
[a] Trolox was used as positive control in test of antioxidant activities.
[b] IC_{50} (μg/ml) represents means ± SD of three independent replicates.

activity of both polar and nonpolar samples [21]. The results of the DPPH and ABTS radical scavenging activities of all the isolates are summarized in Table 2. All isolates ($IC_{50} > 75.6$ μg/ml) were found to be less active than the positive control trolox ($IC_{50} = 9.5$ μg/ml) in the DPPH assay. However, in the ABTS assay, most of the isolated lignanoids showed significant activity with $IC_{50} < 9.9$ μg/ml, in comparison with the positive control trolox ($IC_{50} = 5.3$ μg/ml).

It has been reported that antioxidants can prevent or delay pigmentation by several different mechanisms, such as scavenging ROS, reducing *o*-quinones, and other intermediates in melanin biosynthesis [21]. Thus, there is a link between the antioxidant-scavenging of ROS and tyrsinase inhibitory effects [21]. Tyrosinase inhibition by the isolates (**1**–**7**) was examined using mushroom tyrosinase as described previously with minor modifications, and arbutin was used as a positive control [22]. The inhibition data of mushroom tyrosinase are summarized in Figure 3. Coumpounds **1**, **3**, and **5**–**7** exhibited weak inhibitory activity against mushroom tyrosinase with 31.5%, 30.6%, 36.5%, 33.2%, and 32.6% inhibition at 500 μg/ml, respectively. However, compounds **2** and **4** exhibited moderate inhibitory activity against mushroom tyrosinase with 45.3% and 53.3% inhibition, respectively, at the same concentration.

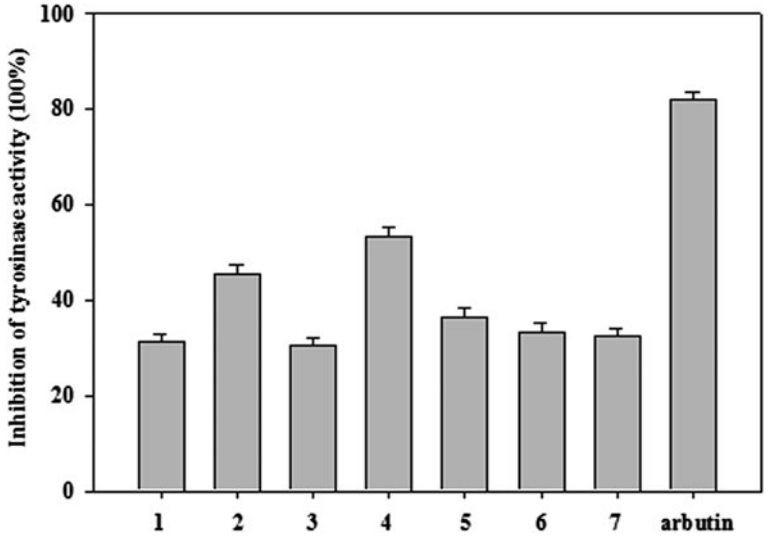

Figure 3. Tyrosinase inhibition of compounds **1**–**7** (at 500 μg/ml). Arbutin was used as positive control; each column represents the mean ± SD of triplicate determinations.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a JASCOP-1020 Polarimeter (Jasco Co., Tokyo, Japan). UV spectra were carried out on a Shimadzu UV-1700 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). IR spectra were obtained on a Shimadzu ftir-8400s spectrophotometer (Shimadzu Corporation). NMR spectra were recorded on Bruker ARX-400 and ARX-600 instruments (Bruker Co., Billerica, MA, USA). HR-ESI-TOF-MS experiments were performed on a Micro TOF spectrometer (Bruker Co., Karlsruhe, Germany). CD spectra were measured by MOS 450 detector (Bio-Logic Co., Claix, France). High-performance liquid chromatography (HPLC) preparation was carried out on an Agilent 1100 instrument (Agilent, Santa Clara, CA, USA) equipped with prep-ODS (10 mm × 250 mm). GC was done on an Agilent 7890A Gas Chromatograph (Agilent) equipped with HP-5 capillary column (30 m × 320 mm × 0.25 μm). Silica gel (200–300 mesh, Qingdao Marine Chemistry Ltd, Qingdao, China), macroporous resin (D101, Cangzhou Bon Adsorber Technology Co., Ltd, Cangzhou, China), and Cosmosil octadecyl silane (ODS; 40–80 μm, Nacalai Tosoh, Inc., Uetikon, Switzerland) were used for column chromatography (CC). TLC was conducted on silica gel GF254 (Qingdao Marine Chemistry Ltd).

3.2 Plant material

The air-dried tubers (15 kg) of *P. ternata* were collected in November 2013 from The China National GAP Base of the Chinese Materia for *P. ternata* in Guizhou, China. The species was identified by Professor Jin-Cai Lu, Department of Pharmacognosy, Shenyang Pharmaceutical University. A voucher specimen (No. 20131116) has been deposited in the Herbarium of Shenyang Pharmaceutical University, Liaoning, China.

3.3 Extraction and isolation

The air-dried and powdered tubers of *P. ternata* (15 kg) were extracted with 75% EtOH for three times. The solvent was evaporated under vacuum. The extract (400 g) was suspended in H_2O (5 L). Then, the extract (400 g) was chromatographed over D101 macroporous resin column using H_2O–EtOH as eluent. The H_2O–EtOH (v/v 30:70) fraction (100 g) was subjected to ODS CC and eluted with H_2O–CH_3OH (from v/v 90:10 to 0:100) to give four fractions (Fr. A–D). Fraction B (4 g) was further separated over silica gel CC by CH_2Cl_2–CH_3OH (v/v 25:1–3:1) to yield six subfractions (B_1–B_6). Subfraction B_3 (300 mg) was subjected to semipreparative HPLC [Agilent 1100 instrument; YMC 5 μm C18 column (250 mm × 10 mm)], eluted with CH_3CN–H_2O (v/v 18:82, 3.5 ml/min) to yield compound **2** (41 mg, $t_R = 52$ min). Subfraction B_5 (202 mg) was further purified by semipreparative HPLC (CH_3CN–H_2O, v/v 20:80, 3.5 ml/min) to produce compound **5** (4.7 mg, $t_R = 43$ min). Similarly, subfraction B_6 (670 mg) was subjected to semipreparative HPLC, eluted with CH_3CN–H_2O (v/v 22:78, 3.5 ml/min) to afford compounds **1** (13.6 mg, $t_R = 30$ min) and **3** (27 mg, $t_R = 44$ min). Fr. C (6 g) was fractionated by silica gel CC eluted with a step-wise gradient of CH_2Cl_2–CH_3OH (v/v 30:1–6:1) to afford five subfractions (C_1–C_5). Subfraction C_1 (780 mg) was subjected to preparative HPLC [Agilent 1100 instrument; Acchrom-XAqua 5 μm C18 column (250 mm × 20 mm)], eluted with CH_3OH–H_2O (v/v 15:85, 7 ml/min), to produce four subfractions (C_{1-1}–C_{1-4}). Subfraction C_{1-3} (139 mg) was further purified by semipreparative HPLC (CH_3CN–H_2O, v/v 25:75, 3.5 ml/min) to produce compound **4** (8 mg, $t_R = 50$ min). Subfraction C4 (223 mg) was purified in the same way using CH_3CN–H_2O (v/v 25:75, 3.5 ml/min) as the eluent to afford compounds **6** (8 mg, $t_R = 45$ min) and **7** (5 mg, $t_R = 47.1$ min).

3.3.1 Cyclolignanyingoside A (*1*)

Yellow oil, $[\alpha]_D^{25}$ − 43.1 (*c* 0.1, MeOH). IR (KBr) ν_{max} (cm^{-1}): 3376, 1645, 1573, 1514, 1449, and 1417; UV (MeOH) λ_{max} ($\Delta\varepsilon$): 210 (0.945) and 283 (0.166) nm. ^1H and ^{13}C NMR spectral data (DMSO-d_6), see Table 1; HR-ESI-MS: *m/z* 677.2414 $[M + Na]^+$ (calcd for $C_{31}H_{42}O_{15}Na$, 677.2416); CD (MeOH) $\Delta\varepsilon_{313.0nm}$ 0, $\Delta\varepsilon_{293.1nm}$ − 21.982, $\Delta\varepsilon_{280.1nm}$ + 8.543, $\Delta\varepsilon_{270.7nm}$ + 9.576, $\Delta\varepsilon_{254.2nm}$ 0, $\Delta\varepsilon_{248.1nm}$ + 12.461, $\Delta\varepsilon_{228.4nm}$ − 4.134, $\Delta\varepsilon_{213.3nm}$ + 16.139, $\Delta\varepsilon_{204.7nm}$ − 5.832.

3.4 Acid hydrolysis of compound 1

Each compound (3.0 mg) was hydrolyzed with 2 M HCl (5.0 ml), heated for 4 h at 95°C and extracted with $CHCl_3$ (3 × 5.0 ml). Then the aqueous layer was concentrated *in vacuo* to appropriate volume, and the solution was examined by TLC (EtOAc–BuOH–H_2O–HOAc, 4:4:1:1) and comparison with the authentic sample, and thus glucose and apiose were detected. Each remaining aqueous layer was concentrated to dryness to give a residue, which was dissolved in pyridine (1.0 ml), and then L-cysteine methyl ester hydrochloride (2.0 mg) was added to the solution. The mixture was heated at 60°C for 2 h, and 0.5 ml *N*-(trimethylsily)imidazole was added, followed by heating at 60°C for 2 h. The reaction product was subjected to GC analysis on Agilent 7890 A (HP-5, 30 m × 320 mm, 0.25 μm) with flame ionization detector. Column temperature was set at 120–280°C with the rate of 8°C/min, and the carrier gas was N_2 (1.4 ml/min), injection temperature 250°C; injection volume 1 μl. The absolute configurations of the monosaccharides were confirmed to be D-apiose and D-glucose by comparison of the retention times of its Me_3Si ethers with those of standard samples [t_R (D-apiose) 12.80 min, t_R (D-glucose) 25.87 min].

3.5. Antioxidant assays

In this assay, trolox (purity > 98%; Sigma, Los Angeles, CA, USA) was used as a positive control. A 0.1-mM solution of DPPH radical in ethanol was prepared, and 100 μl of this solution was mixed with 100 μl of sample solution. The mixture was incubated for 30 min in a darkroom at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 517 nm. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol, to an absorbance of 0.7 ± 0.02 at 734 nm. An ethanolic solution (50 μl) of the samples at various concentrations was mixed with 150 μl diluted $ABTS^{\cdot+}$ solution. After reaction at room temperature for 20 min, the absorbance was measured at 734 nm using a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA).

The capability to scavenge the DPPH/$ABTS^{\cdot+}$ was calculated using the formula given below: DPPH/$ABTS^{\cdot+}$ scavenging activity (%) = $[1-(S-S_B)/(C-C_B)] \times 100$, where S, S_B, C, and C_B are the absorbencies of the sample, the blank sample, the control, and the blank control, respectively [23].

3.6. Inhibitory activity to tyrosinase

This assay was performed according to the procedure of Dai et al. [22] with slight modifications, using L-tyrosine as a substrate. Arbutin (purity > 98%; Sigma) was used as a positive control.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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