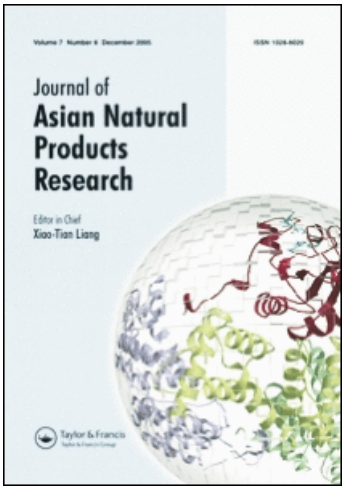

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ORIGINAL ARTICLE

Steroidal glycosides with anti-tumor activity from the roots of *Cynanchum wallichii* Wight

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Two new steroidal glycosides characterized with 2,6-dideoxypyranoses as component sugars have been isolated from roots of *Cynanchum wallichii* Wight. Their structure elucidation and cytotoxic activities against HL-60 and PC-3 cells are reported.

Keywords: *Cynanchum wallichii* Wight; C21 steroids; deoxypyranoses; cytotoxicity

1. Introduction

Cynanchum wallichii Wight, also named Duanjieshen, is a traditional Chinese medicine distributed extensively over southwest China. It is used as the primary drug in the famous Chinese prescription 'hulisan', which is used to treat arthrophlogosis and injury from fall or fracture. The main components of *C. wallichii* Wight had been reported as C21 steroidal glycosides [1]. In order to find out more about C21 steroids with bioactivity, we carried out the research on the roots of *C. wallichii* Wight and two new steroidal glycosides were found. In this paper, we report the cytostatic activity against HL-60 and PC-3 cells as well as the isolation and structural elucidation of two new glycosides (Figure 1).

2. Results and discussion

Compound **1** was obtained as a white amorphous powder with $[\alpha]_D^{20} + 2.6$ ($c = 0.59$, MeOH). The molecular formula was determined to be $C_{42}H_{60}O_{14}$ by HR-ESI-MS at *m/z* 811.3874 $[M + Na]^+$. Liebermann–Burchard reaction and Keller–Kiliani reaction proved to be positive, which suggested the presence of steroidal glycoside with 2-deoxysugars. The IR spectrum showed the absorption bands for hydroxyl (3443 cm^{-1}), carbonyl (1715 cm^{-1}), and benzyl (1610 and 1591 cm^{-1}) groups. The ^{13}C NMR spectrum of **1** showed two anomeric carbon signals at δ 96.4 and 102.2. The carbon signals assignable to the aglycone moiety were similar to those of qinyangshengenin [1], with glycosylation shifts at C-3 (+5.2), C-2 (−2.1), C-4 (−4.0). Hence, compound **1** was considered to be qinyangshengenin-3-*O*-diglycoside. Proton signals of the sugar moiety were also assigned to two secondary methyl groups at δ_H 1.45 (d, $J = 6.0$ Hz) and 1.55 (d, $J = 6.0$ Hz), two methoxyl groups at δ_H 3.46 (s) and 3.57 (s), and two anomeric protons at δ_H 4.76 (dd, $J = 9.5$, 1.3 Hz) and 5.27 (dd, $J = 9.5$, 1.3 Hz), whose multiplicities showed the presence of two 2,6-dideoxy-sugar units and β-configuration

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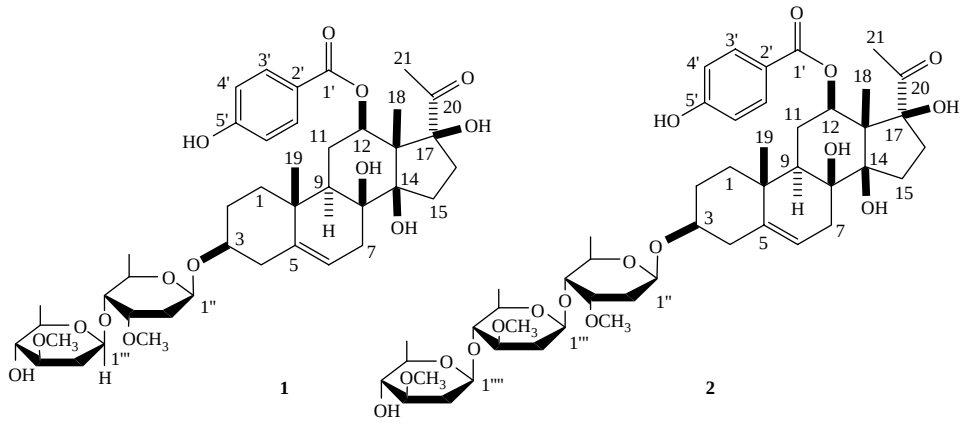

Figure 1. Structures of compounds **1** and **2**.

of the two units. The ^{13}C shifts of each sugar unit were assigned unambiguously by HMBC and HMQC analyses. The existence of one oleandropyranosyl unit and one cymaropyranosyl unit was confirmed by the comparison of their spectroscopic data with those in the literatures [2,3] and their absolute configuration was considered to be D-form by their hydrolysis experiment. The sequence of the two sugar units was demonstrated by the HMBC spectrum, in which distinct correlations from H-1″ (5.27) to C-3 (77.6) of the aglycone and from H-1‴ (4.76) to C-4″ (83.5) were observed. Thus, the structure of compound **1** was established as qinyangshengenin-3-*O*-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **2** was obtained as a white amorphous powder with $[\alpha]_D^{20} + 7.6$ ($c = 0.88$, MeOH). The molecular formula was determined to be $C_{49}H_{72}O_{17}$ by HR-ESI-MS at *m/z* 955.4660 $[M + Na]^+$. The ^{13}C NMR spectrum of **2** showed three anomeric carbon signals at δ 96.4, 100.2, and 101.9. The carbon signals assignable to the aglycone moiety were the same as those of compound **1**. Hence, compound **2** was considered to be qinyangshengenin-3-*O*-triglycoside. Proton signals of the sugar moiety were also assigned to three secondary methyl groups [1.45 (d, $J = 6.0$ Hz), 1.47 (d, $J = 6.0$ Hz), and 1.59 (d, $J = 6.0$ Hz)], three methoxyl groups [3.49 (s), 3.52 (s), and 3.59 (s)], and three anomeric protons [4.93 (br d, $J = 9.0$ Hz), 4.98 (br d, $J = 9.0$ Hz), and 5.28 (br d, $J = 9.0$ Hz)], whose multiplicities showed the presence of three 2,6-dideoxy-sugar units and β-configuration of the three units. The ^{13}C shifts of each sugar unit were assigned unambiguously by HMBC and HMQC analyses. The existence of two oleandropyranosyl and one cymaropyranosyl units was confirmed by the comparison of their spectroscopic data with those in the literatures [2,3] and their absolute configuration was considered to be D-form by the hydrolysis experiment. The sequence of these three sugar units was demonstrated by the HMBC spectrum, in which distinct correlations from H-1″ (5.28) to C-3 (77.6) of the aglycone, from H-1‴ (4.98) to C-4″ (83.4) and from H-1⁗ (4.93) to C-4‴ (82.6) were observed. Thus, the structure of compound **2** was established as qinyangshengenin-3-*O*-β-D-oleandropyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra

were taken on a Bruker IFS-55 infrared spectrophotometer. The NMR spectral data were recorded on Bruker AV-600 (600 MHz for ^{1}H and 150 MHz for ^{13}C) in C_5D_5N with TMS as an internal standard. The HR-FAB-MS data were obtained on the Micross Mass Autospec-UltimaE TOF mass spectrophotometer. Chromatography was performed on silica gel (200–300 mesh, Qingdao Haiyang Chemical Factory, Qingdao, China), Sephadex LH-20 (Pharmacia, Peapack, NJ, USA), and reversed-phase HPLC (Shimadzu LC-8A vp, Kyoto, Japan).

3.2 Plant material

The roots of *C. wallichii* Wight were obtained in October 2005 in Kunming, Yunnan Province, China. A voucher specimen (No. 6040) has been identified by Prof. Qishi Sun and has been deposited in the School of Traditional Chinese Materia Medica of Shenyang Pharmaceutical University.

3.3 Extraction and isolation

The dried roots (10 kg) of *C. wallichii* Wight were extracted with EtOH (95%, 20 liters) at 85°C three times, for 2 h each. The extract (1300 g) was successively partitioned with EtOAc, *n*-BuOH, and H_2O. The EtOAc-soluble fraction (150 g) was subjected to silica gel column and eluted with $CHCl_3$–CH_3OH (100:1 to 0:1), yielding 12 fractions. Fraction 5 (30 g) was purified by Sephadex LH-20 (CH_3OH) to yield two fractions. Four grams of the second fraction (15 g) were subjected to HPLC and eluted with CH_3OH (75%), which afforded compounds **1** (110 mg) and **2** (150 mg).

Table 1. The ^{1}H NMR spectral data for sugar moieties of compounds **1**and **2** (δ in ppm, C_5D_5N, 600 MHz).

No.	**1** (*J*, Hz)	**2** (*J*, Hz)
	β-D-Cym	β-D-Cym
1″	5.27 (dd, *J* = 9.5, 1.3)	5.28 (br d, *J* = 9.0)
2″	–[a]	–[a]
3″	4.08 (m)	4.07(m)
4″	3.54 (br d, *J* = 9.0)	3.54[b]
5″	4.24 (m)	4.23 (m)
6″	1.45 (d, *J* = 6.0)	1.45 (d, *J* = 6.0)
3″-OMe	3.57 (s)	3.52 (s)
	β-D-Ole	β-D-Ole
1‴	4.76 (dd, *J* = 9.5, 1.3)	4.98 (br d, *J* = 9.0)
2‴	–[a]	–[a]
3‴	3.47[b]	3.57[b]
4‴	3.48[b]	3.55[b]
5‴	3.58 (m)	3.50[b]
6‴	1.55 (d, *J* = 6.0)	1.47 (d, *J* = 6.0)
3‴-OMe	3.46 (s)	3.59(s)
		β-D-Ole
1⁗		4.93 (br d, *J* = 9.0)
2⁗		–[a]
3⁗		3.50[b]
4⁗		3.50[b]
5⁗		3.61 (m)
6⁗		1.59 (d, *J* = 6.0)
3⁗-OMe		3.49 (s)

Notes: [a] Overlapped.
[b] Overlapped sugar signals.

3.3.1 Compound **1**

A white amorphous powder, $[\alpha]_D^{20} + 2.6$ ($c = 0.59$, MeOH). IR (KBr) ν_{max} (cm^{-1}) 3443, 2933, 1715, 1610, 1591, 1504, 1276, 991, and 773. ^1H and ^{13}C NMR spectral data, see Tables 1–3. HR-ESI-MS *m/z*: 811.3874 $[M + Na]^+$ (calcd for $C_{42}H_{60}O_{14}Na$, 811.3875).

3.3.2 Compound **2**

A white amorphous powder, $[\alpha]_D^{20} + 7.6$ ($c = 0.88$, MeOH). IR (KBr) ν_{max} (cm^{-1}) 3443, 2933, 1715, 1611, 1590, 1503, 1275, 991, and 773. ^1H and ^{13}C NMR spectral data, see Tables 1–3. HR-ESI-MS *m/z*: 955.4660 $[M + Na]^+$ (calcd for $C_{49}H_{72}O_{17}Na$, 955.4662).

Table 2. The ^{13}C NMR spectral data for aglycone moieties of compounds **1** and **2** (δ in ppm, C_5D_5N, 150 MHz).

No.	**1**	**2**
1	39.2	39.2
2	29.9	29.9
3	77.6	77.6
4	39.1	39.1
5	139.4	139.4
6	119.1	119.1
7	34.8	34.8
8	74.3	74.3
9	44.5	44.5
10	37.4	37.4
11	25.2	25.2
12	73.4	73.4
13	58.4	58.4
14	89.6	89.6
15	33.9	33.9
16	33.2	33.2
17	92.5	92.5
18	10.9	10.9
19	18.2	18.2
20	209.8	209.8
21	27.8	27.8
1′	165.4	165.4
2′	122.0	122.0
3′	132.4	132.4
4′	116.2	116.2
5′	163.6	163.6
6′	116.2	116.2
7′	132.4	132.4

3.4 Cell culture assay

HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The leukemia cells were washed and resuspended in the above medium to 5×10^4 cells/ml, and 2 ml of this cell suspension was placed in each well of a 24-well flat-bottomed plate. The cells were incubated in 5% CO_2/air for 24 h at 37°C. After incubation, 10 μl of EtOH solution containing the sample was added to give the final concentration of 20–80 μM, and 10 μl of EtOH was added into control wells. The cells were further incubated for 72 h in the presence of each agent, and then cell growth was evaluated using the cell counting method. Counting the number of cells of the control group and the administered group was performed by hemocytometer. The cell growth

Table 3. The ^{13}C NMR spectral data for sugar moieties of compounds **1** and **2** (δ in ppm, C_5D_5N, 150 MHz).

No.	**1**	**2**
	β-D-Cym	β-D-Cym
1″	96.4	96.4
2″	37.2	37.3
3″	77.9	78.0
4″	83.5	83.4
5″	69.0	68.9
6″	18.7	18.5
3″-OMe	58.8	57.3
	β-D-Ole	β-D-Ole
1‴	102.2	101.9
2‴	37.2	37.6
3‴	81.4	79.0
4‴	76.2	82.6
5‴	72.9	71.7
6‴	18.7	18.7
3‴-OMe	57.1	58.9
		β-D-Ole
1⁗		100.2
2⁗		37.0
3⁗		81.6
4⁗		76.3
5⁗		73.0
6⁗		18.1
3⁗-OMe		57.0

inhibition rate: 1 − (administered group cell number/control group cell number). Each assay was done in triplicate, and inhibition was expressed as an IC_{50} value, the concentration of inhibition of cell growth was 50%. The IC_{50} value for compounds **1** and **2** was 9.5 ± 1.1 and 9.5 ± 0.7 μg/ml, respectively. And the number was 35.5 ± 5.7 and 15.3 ± 3.7 μg/ml respectively, for the PC3 cells.

3.5 Acid hydrolysis of compounds 1 and 2

A solution of **1** and **2** (each 50 mg) in MeOH (50 ml) was treated separately with 0.1 N H_2SO_4 (50 ml) at 50°C for 15 min. After adding H_2O (50 ml), the mixture was evaporated to 50 ml under reduced pressure to remove MeOH and then it was kept at 60°C for another 30 min. The hydrolyzed mixture was neutralized to pH 7 with $Ba(OH)_2$ and condensed to dryness under reduced pressure. The monosaccharides were isolated by means of preparative TLC eluted by $CHCl_3$–MeOH–H_2O (20:3:1). The monosaccharides were determined to be cymarose and oleandrose by their ESI-MS and NMR spectral data. Their ESI-MS data were both 185.1, and thus in order to distinguish them, the NMR spectral data of one sample were obtained, which led to the identification of the sample as cymarose, and the other as oleandrose. Both of their absolute configurations were considered to be D-form by their optical values: cymarose $[\alpha]_D^{25}$ + 49.7 ($c = 0.04$, H_2O) and oleandrose $[\alpha]_D^{25}$ − 10.2 ($c = 0.03$, H_2O).

Cymarose: $[\alpha]_D^{25}$ + 49.7 ($c = 0.04$, H_2O), ^{13}C NMR (C_5D_5N, 75 MHz) for D-cymarose ($\alpha - : \beta - = 5:4$): δ 92.6, 89.2 (C-1), 81.8, 79.2 (C-3), 74.0, 70.9 (C-4), 68.4, 67.7 (C-5), 57.9, 56.5 (—OCH_3), 41.2, 37.2 (C-2), 20.2, 19.3 (C-6).

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