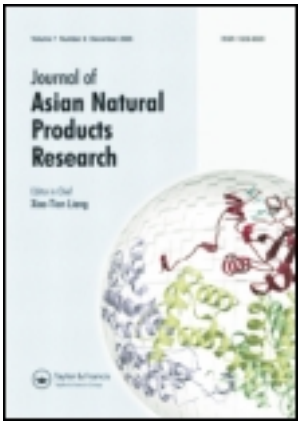

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A new pregnane glycoside and oligosaccharide from *Parabarium huaitingii*

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Two new compounds, along with two known compounds, were isolated from the barks of *Parabarium huaitingii*, and their structures were determined as 5α-pregn-6-ene-3β,17α,20(*S*)-triol-20-*O*-β-D-digitoxopyranoside (**1**), cymaropyranurolactone 4-*O*-β-D-digitalopyranosyl-(1 → 4)-*O*-β-D-cymaropyranosyl-(1 → 4)-*O*-β-D-oleandropyranosyl-(1 → 4)-*O*-β-D-cymaropyranoside (**2**), 3β,17α,20(*S*)-trihydroxy-5α-pregn-6-ene (**3**), and 5α-pregn-6-ene-3β,17α,20(*S*)-triol-3-*O*-β-D-digitalopyranoside (**4**) by spectroscopic methods.

Keywords: Apocynaceae; *Parabarium huaitingii*; pregnane glycoside; oligosaccharide

1. Introduction

Parabarium huaitingii (Apocynaceae) has been used by folk to treat rheumatoid arthritis and bruises [1]. It is mainly distributed in southern and southwestern areas of China. In previous investigations of the plant, three phenylpropanoid-substituted epicatechin glycosides, along with three phenolic acids, have been separated from *P. huaitingii*, which showed good antioxidative activity [2]. In this paper we describe the isolation and structural elucidation of a new pregnane glycoside, 5a-pregn-6-ene-3β,17α,20(*S*)-triol-20-*O*-β-D-digitoxopyranoside (**1**), and of a new oligosaccharide, cymaropyranurolactone 4-*O*-β-D-digitalopyranosyl-(1 → 4)-*O*-β-D-cymaropyranosyl-(1 → 4)-*O*-β-D-oleandropyranosyl-(1 → 4)-*O*-β-D-cymaropyranoside (**2**). Their structures were elucidated on the basis of spectroscopic methods, especially 2D NMR techniques, including $^{1}H-^{1}H$ COSY, NOESY, HMQC, and HMBC experiments. In addition, two known pregnane derivatives were also isolated from this plant and identified by comparing their physical and spectroscopic data with those reported in the literature.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder, and its molecular formula was established as $C_{27}H_{44}O_6$ by HR-TOF-MS at *m/z* 487.2598 $[M + Na]^+$ and ^{13}C NMR spectrum. Its IR spectrum featured a strong absorption at 3448 cm^{-1} due to hydroxyl groups. The ^{1}H NMR spectrum showed two angular methyl groups at δ 0.67 and 0.71 (each, s), and two secondary methyl proton signals at δ 1.13 (d, $J = 6.4$ Hz) and 1.16 (d, $J = 6.4$ Hz), and one C=C double bond protons at δ 5.26 (d, $J = 10.0$ Hz) and 5.43

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(d, $J = 10.0$ Hz). The ^{13}C NMR spectrum further revealed 27 carbon resonances that were classified by DEPT experiment into 4 methyl, 8 methylene, 12 methine, and 3 quaternary carbons. It showed three signals at lower field than 100 ppm. The signals at δ 131.5 and 129.1 were due to olefinic carbons, and the signal at δ 101.8 was assignable to anomeric carbon C-1′. There are seven carbons connected with oxygen in the structure (δ 101.8, 84.3, 81.8, 77.4, 71.9, 70.9, and 70.0).

Its ^{13}C NMR and NOESY spectroscopic data established that the aglycone of compound **1** was identical to that of the known natural product 3β,17α,20(*S*)-trihydroxy-5α-pregn-6-ene [4] (Table 1). The coupling constants of anomeric proton were 1.6 and 9.6 Hz, indicating that the sugar unit was β-linkage. It was identified as β-D-digitoxopyranose by NMR data and comparison with authentic sample. The sugar connected to the C-20 of the aglycone was deduced from the correlation of anomeric H-1′ (δ 4.54) with the carbon signal at δ 81.8 (C-20) in the HMBC spectrum. In conclusion, the structure of compound **1** was elucidated as 5α-pregn-6-ene-3β,17α,20(*S*)-triol-20-*O*-β-D-digitoxopyranoside (Figure 1).

Compound **2** was obtained as a white amorphous powder, and its molecular formula was established as $C_{35}H_{60}O_{17}$ by the data of HR-TOF-MS at *m*/*z* 775.3103 $[M + Na]^+$ and the ^{13}C NMR spectrum. The ^{1}H NMR spectrum showed five methyl

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data for compound **1** in DMSO-d_6.

No.	^{1}H NMR (J in Hz)	^{13}C NMR (DEPT\HMQC)
1	1.49 (1H, m), 0.97 (1H, m)	34.7 (CH_2)
2	1.69 (1H, m), 1.36 (1H, m)	31.7 (CH_2)
3	3.41 (1H, m)	70.0 (CH)
4	1.57 (1H, m), 1.19 (1H, m)	36.4 (CH_2)
5	1.82 (1H, m)	45.1 (CH)
6	5.43 (1H, d, $J = 10.0$)	129.1 (CH)
7	5.26 (1H, d, $J = 10.0$)	131.5 (CH)
8	1.86 (1H, m)	37.9 (CH)
9	0.91 (1H, m)	52.5 (CH)
10		34.2
11	1.45 (1H, m), 1.16 (1H, m)	20.7 (CH_2)
12	1.61 (1H, m), 1.36 (1H, m)	31.5 (CH_2)
13		46.7
14	1.86 (1H, m)	48.6 (CH)
15	1.61 (1H, m), 1.16 (1H, m)	23.1 (CH_2)
16	1.89 (1H, m), 1.57 (1H, m)	36.5 (CH_2)
17		84.3
18	0.67 (3H, s)	14.9 (CH_3)
19	0.71 (3H, s)	11.7 (CH_3)
20	3.59 (1H, q, $J = 6.4$)	81.8 (CH)
21	1.13 (3H, d, $J = 6.4$)	18.0 (CH_3)
1′	4.54 (1H, dd, $J = 9.6, 1.6$)	101.8 (CH)
2′	1.95 (1H, m), 1.30 (1H, m)	40.5 (CH_2)
3′	3.31 (1H, m)	70.9 (CH)
4′	2.70 (1H, dd, $J = 8.8, 3.2$)	77.4 (CH)
5′	3.12 (1H, dq, $J = 8.8, 6.4$)	71.9 (CH)
6′	1.16 (3H, d, $J = 6.4$)	18.7 (CH_3)
4′-OH	4.85 (1H, d, $J = 4.4$)	
3′-OH	4.77 (1H, br s)	
3-OH	4.51 (1H, br s)	
17-OH	3.64 (1H, s)	

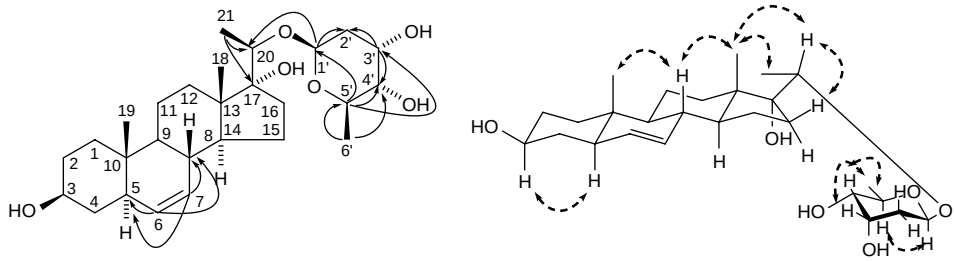

Figure 1. Key HMBC and NOESY correlations of compound **1**.

groups (δ 1.30, 1.20, 1.18, 1.15, and 1.14), four anomeric protons (δ 4.85, 4.79, 4.55, and 4.16), and five methoxyl groups (δ 3.35, 3.34, 3.32, 3.32, and 3.30). The ^{13}C NMR spectrum further revealed 35 resonances that were classified by DEPT experiment into 10 CH_3, 4 CH_2, and 20 CH. The signal at δ 170.8 was a carbonyl carbon, and the signals at δ 105.8, 101.2, 100.1, and 97.8 were anomeric carbons. The ^{13}C NMR spectrum showed five methyl carbon signals at δ 18.7, 18.6, 18.5, 18.3, and 17.2, and five methoxyl carbon signals at δ 58.4, 58.0, 57.0, 56.8, and 56.6. There were 16 carbons connected with oxygen in the structure except the anomeric carbons (δ 83.7, 82.7, 82.3, 82.3, 81.3, 78.8, 78.2, 77.3, 76.9, 75.0, 70.9, 70.2, 69.6, 69.0, 68.7, and 67.2). The ^{1}H and ^{13}C NMR spectra of compound **2** revealed the existence of four sugar residues in its structure.

In the HMQC spectrum, the protons at δ_H 2.89/2.51, 3.85, 3.58, 4.20, 3.32, and 1.30 showed correlations with the carbons at δ_C 33.8, 78.8, 81.3, 75.0, 57.0, and 18.7, respectively. The long-range correlations of H-2 (δ 2.89) with C-3 (δ 78.8) and C-1 (δ 170.8), H-3 (δ 3.85) with C-6 (δ 57.0) and C-5 (δ 75.0), H-4 (δ 3.58) with C-7 (δ 18.7), C-5 (δ 75.0), and H-5 (δ 4.20) with C-7 (δ 18.7) and C-4 (δ 81.3) were found in the HMBC spectrum. In the ^{1}H NMR spectrum, the peak of H-3 (δ 3.85) was broad single and the coupling constant of H-4 (δ 3.58) was 8.4 Hz, indicating H-3 should be at *e* bond and H-4, H-5 at *a* bond. The above data suggested the presence of structural fragment as shown in Figure 2. This is a new structure, named cymaropyranurolactone.

The coupling constants of anomeric protons indicated that four sugar units were β-linkage. They were identified as two β-D-cymaropyranosyl, one β-D-oleandropyranosyl, and one β-D-digitalopyranosyl by NMR spectral data (Table 2) and comparison with authentic samples. The long-range correlations of H-4 (δ 3.58) with C-1′ (δ 100.0), H-4′ (δ 3.22) with C-1″ (δ 101.2), H-4″ (δ 3.07) with C-1‴ (δ 97.5), and H-4‴ (δ 3.14) with C-1⁗ (δ 105.8) in the HMBC spectrum revealed a linear linkage of the sugar chain. Therefore, the structure of compound **2** was unequivocally assigned as cymaropyranurolactone 4-*O*-β-D-digitalopyranosyl-(1 → 4)-*O*-β-D-cymaropyranosyl-(1 → 4)-*O*-β-D-oleandropyranosyl-(1 → 4)-*O*-β-D-cymaropyranoside (Figure 3).

The structures of compounds **3** and **4** were elucidated as 3β,17α,20(*S*)-tri-

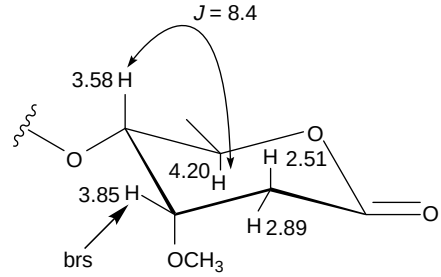

Figure 2. The structure of cymaropyranurolactone.

Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data for compound **2** in DMSO-d_6.

No.	^{1}H NMR (J in Hz)	^{13}C NMR (DEPT\HMQC)
1		170.83
2	2.89 (1H, dd, $J = 16.0, 4.0$), 2.51 (1H, dd, $J = 15.6, 2.0$)	33.8 (CH_2)
3	3.85 (1H, br s like)	78.8 (CH)
4	3.58 (1H, d, $J = 8.4$)	81.3 (CH)
5	4.20 (1H, m)	75.0 (CH)
6	3.30 (3H, s)	56.6 ($-OCH_3$)
7	1.30 (3H, d, $J = 4.0$)	18.6 (CH_3)
	β-D-Cym	
1′	4.85 (1H, dd, $J = 9.6, 1.6$)	100.1 (CH)
2′	2.04 (1H, m), 1.50 (1H, m)	35.9 (CH_2)
3′	3.72 (1H, m)	76.9 (CH)
4′	3.22 (1H, dd, $J = 9.6, 2.8$)	82.3 (CH)
5′	3.75 (1H, m)	68.7 (CH)
6′	3.35 (3H, s)	58.4 ($-OCH_3$)
7′	1.15 (3H, d, $J = 6.4$)	18.3 (CH_3)
	β-D-Ole	
1″	4.55 (1H, dd, $J = 9.6, 1.2$)	101.2 (CH)
2″	1.26 (1H, m), 2.21 (1H, m)	37.0 (CH_2)
3″	3.30 (1H, m)	78.2 (CH)
4″	3.07 (1H, t, $J = 8.8$)	82.3 (CH)
5″	3.27 (1H, m)	70.9 (CH)
6″	3.32 (3H, s)	57.0 ($-OCH_3$)
7″	1.18 (3H, d, $J = 6.4$)	18.7 (CH_3)
	β-D-Cym	
1‴	4.79 (1H, dd, $J = 9.6, 1.2$)	97.8 (CH)
2‴	1.44 (1H, m), 2.04 (1H, m)	35.9 (CH_2)
3‴	3.69 (1H, m)	77.3 (CH)
4‴	3.14 (1H, dd, $J = 9.6, 2.8$)	82.7 (CH)
5‴	3.74 (1H, m)	69.0 (CH)
6‴	3.34 (3H, s)	58.0 ($-OCH_3$)
7‴	1.20 (3H, d, $J = 6.4$)	18.5 (CH_3)
	β-D-Digta	
1⁗	4.16 (1H, d, $J = 7.6$)	105.8 (CH)
2⁗	3.34 (1H, m)	69.6 (CH)
3⁗	2.97 (1H, dd, $J = 9.6, 3.2$)	83.7 (CH)
4⁗	3.63 (1H, like-br s)	67.2 (CH)
5⁗	3.48 (1H, q, $J = 6.4$)	70.2 (CH)
6⁗	3.32 (3H, s)	56.8 ($-OCH_3$)
7⁗	1.14 (3H, d, $J = 6.4$)	17.2 (CH_3)
2⁗-OH	4.93 (1H, d, $J = 4.8$)	
4⁗-OH	4.38 (1H, d, $J = 4.4$)	

hydroxy-5α-pregn-6-ene [3] and 5α-pregn-6-ene-3β,17α,20(*S*)-triol-3-*O*-β-D-digitalopyranoside [4], respectively, by spectral analysis.

The antitumor activity of compounds **1**, **3**, and **4** was studied, but neither of them showed cytotoxicites against Hela, HepG2, DU145, and MCF-7 cell lines.

3. Experimental

3.1 General experimental procedures

Melting points were determined on an X-6 micro melting point apparatus and are uncorrected. FT-IR spectra were obtained on a Nicolet 6700 FT-IR spectrometer. NMR spectra were recorded on VARIAN INOVA-500 with TMS as an internal

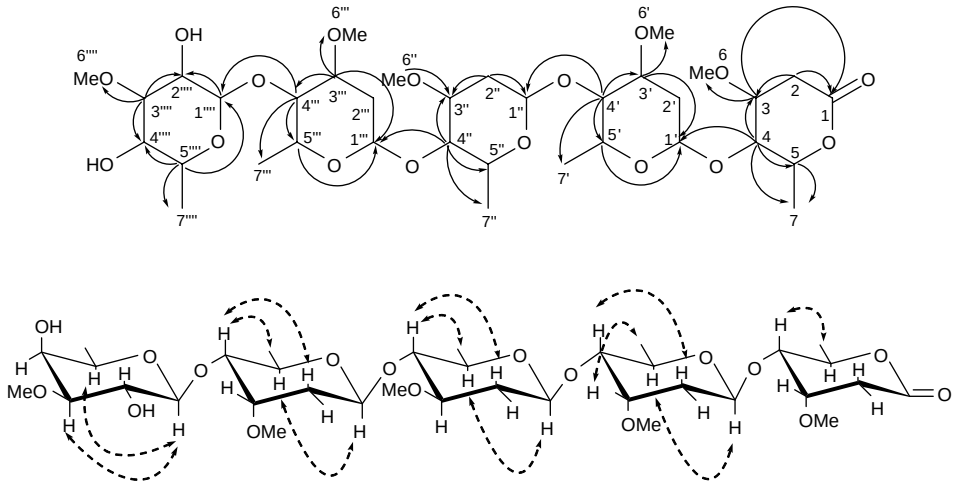

Figure 3. Key HMBC and NOESY correlations of compound **2**.

reference. The ESI-MS were measured on ABI4000 Q TRAP. HR-TOF-MS were obtained on Acquity UPLC-Q-TOF Micro MS. Optical rotations were measured on a JASCO P-1020 polarimeter. Toyopearl HW-40C was provided by Tosoh Corporation (Tokyo, Japan). Silica gel used for column chromatography (CC) was supplied by Qingdao Ocean Chemical Factory (Qingdao, China).

3.2 Plant material

The barks of *P. huaitingii* were purchased in Nanning City, Guangxi Province, China, and identified by Yue-Wen Cai, Guangdong Food and Drug Vocational College. A voucher specimen (PH081114) is deposited in the Natural Medicines Research, Department of Chemistry, Jinan University, China.

3.3 Extraction and isolation

The barks of *P. huaitingii* (20 kg) were extracted three times with 80% ethanol at room temperature. After evaporation of ethanol *in vacuum*, the residue was suspended in water and then extracted successively with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$ extract (90 g) was subjected to CC over silica gel (200–300 mesh) and eluted with petroleum ether–acetone (0–100%) to get fractions 1–10. From fraction 7 (4 g), compound **1** (20 mg) was obtained by repeated silica gel CC with petroleum ether–EtOAc (7:3, 6:4, 3:7). Fraction 8 (8 g) was eluted with $CHCl_3$–MeOH (98:2) to get fractions A and B, and compound **2** (10 mg) was obtained by HW-40C with MeOH from fraction B. The EtOAc extract (300 g) was chromatographed on a silica gel (200–300 mesh) column, which was successively eluted with $CHCl_3$–MeOH (0–100%). According to the different TLC profiles, 10 crude fractions (1–10) were collected. Fraction 5 (10 g) was further purified by silica gel CC with $CHCl_3$–MeOH (100:5, 9:1) to yield compound **3** (6 mg). From fraction 4 (15 g), compound **4** (17 mg) was obtained by repeated silica gel CC with $CHCl_3$–MeOH (98:2).

*3.3.1 Compound **1***

This compound was obtained as a white amorphous powder; mp 238–240°C; $[\alpha]_D^{26}$ −117.8 ($c = 1.25$, CH_3OH); IR (KBr) ν_{max} 3448 (OH), 2928 and 2863 (CH), 1141 (C—O), 1067 (C—O), 951 (C═C—H), 863 (C═C—H) cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HR-TOF-

MS: m/z 487.2598 $[M + Na]^+$ (calcd for $C_{27}H_{44}O_6Na$, 487.3036).

3.3.2 Compound 2

This compound was obtained as a white amorphous powder; mp 185–187°C; $[\alpha]_D^{26}$ 30.2 ($c = 1.00$, CH_3OH); IR (KBr) ν_{max} 3415(OH), 2916 and 2845 (CH), 1705 (C═O), 1394 (CH_3), 1161 (C—O), 1075 (C—O) cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 2; HR-TOF-MS: m/z 775.3103 $[M + Na]^+$ (calcd for $C_{35}H_{60}O_{17}Na$, 775.3728).

3.4 Acid hydrolysis of compounds 1, 2, and 4

A solution of **1**, **2**, and **4** (each 5 mg) in 3 ml of 50% dioxane and 3 ml of 0.05 M H_2SO_4 was heated at 60°C for 2 h. After dioxane was removed *in vacuum*, the solution was extracted with $CHCl_3$. The H_2O layer of each compound was neutralized with salt aqueous $Ba(OH)_2$ solution and the precipitation was filtered off [5]. The filtrate was concentrated and analyzed by TLC with three solvent systems: solvent A, $CHCl_3$–CH_3OH (9:1); solvent B, CH_2Cl_2–C_2H_5OH (9:1); and solvent C, petroleum ether–acetone (3:2). The R_f values of authentic samples D-cymarose, D-oleandrose, D-digitoxose, and D-digitalose were in the order of 0.51, 0.45, 0.27, and 0.20 with solvent A; 0.58, 0.49, 0.32, and 0.23 with solvent B; and 0.41, 0.36, 0.28, and 0.18 with solvent C, respectively. D-Digitoxose was detected from compound **1**, D-cymarose, D-oleandrose, and D-digitalose from compound **2**, D-digitalose from compound **4**.

3.5 Antitumor activity

The inhibition effects of compounds **1**, **3**, and **4** on the Hela, HepG2, DU145, and MCF-7 cells were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]. The tumor cells were incubated on a 96-well cultivation plate at a concentration of 1×10^5 cells/ml. Each well was inoculated with 100 μl Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum solution containing the cells and 100 μl samples (at concentrations of 81, 27, 9, 0.03, 0.01, 0.003 μmol/ml, respectively) under an atmosphere of 5% CO_2 at 37°C for 48 h. The tumor cells were continuously inoculated for another 4 h after 10 μl MTT (5 mg/ml) had been added. The supernatant was removed by centrifugation, and then 200 μl of DMSO was added to terminate the reaction. MTT colorimetric method was used to observe the effect of growth inhibition of tumor cell. The sample groups were compared with control groups in the absence of the tested samples. All results were expressed as the inhibition ratio (A) of tumor cell proliferation as $A = (1 - N_t/N_c) \times 100\%$, where N_c and N_t represent the average number of viable tumor cells of the control group and test group, respectively.

Acknowledgements

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