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A NOVEL TETRAIRIDOID GLUCOSIDE FROM *PTEROCEPHALUS HOOKERI*

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Abstract – Pterocephanoside (**1**), a novel iridoid glucoside derivative with three glucosides and an aromatic ring, were isolated from *Pterocephalus hookeri*, along with eight known compounds (**2**–**9**). The structure of the new compound was elucidated on the basis of extensive spectroscopic analysis, including 1D and 2D NMR experiments. Compounds **1**, **2**, **5** and **6** were evaluated for their cytotoxicity.

Pterocephalus hookeri (C. B. Clarke) Höeck belonging to the Dipsacaceae family, one of the most popular Tibetan herbs, has been widely applied in Tibetan medicine prescriptions. *P. hookeri* named "Bang-zi-du-wu" in Tibetan language, has multiple traditional uses in the treatment of illnesses such as cold, flu, rheumatoid arthritis, and enteritis in China.[1] Previous chemical investigations of this plant led to isolation of several triterpenoids,[2] iridoids,[3] pyridine alkaloids[4] and tetrahydrofuranoid lignans.[4] Iridoids can be found in a number of medicinal plants, usually as glycosides, and may be responsible for some of the pharmaceutical activities of these species.[3] In our study, a novel iridoid glucoside oligomer named pterocephanoside (**1**), were isolated from the 95% ethanol extract of aerial parts of *P. hookeri*, along with eight known compounds (**2**–**9**). The structure of the new compound was elucidated on the basis of extensive spectroscopic analysis, including 1D and 2D NMR experiments. To the best of our knowledge, pterocephanoside (**1**) is the first-reported iridoid derivative with three glucosides and an aromatic ring. The structures of known compounds, dipsanosides A (**2**),[5] dipsanosides B (**3**),[5] triplostoside A (**4**),[6]

cantleyoside (**5**),[7] loganin (**6**),[6] sweroside (**7**),[6] loganol (**8**)[8] and hookeroside D (**9**)[2] were identified by comparison of their spectral data with those reported in the literature. Herein, we report the isolation and structural elucidation of the new compound and investigated part of the isolated compounds for their cytotoxicity in vitro against H460, MCF-7 and Hep G2 human cancer cell lines.

The EtOH extract of *P. hookeri* was treated in the usual procedures to give the petroleum ether extract, the EtOAc extract, and the *n*-butanol extract, respectively. The *n*-butanol extract fraction was sequentially subjected to column chromatography over D101 macroporous resin and silica gel to give the new compound.

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{57}H_{74}O_{30}$ by HR-ESI-MS at *m/z* 1261.4169 $[M + Na]^+$ (calcd. for $C_{57}H_{74}O_{30}Na$, 1261.4163). The IR spectrum showed absorption bands for hydroxyl groups (3411 cm^{-1}) and the α,β-unsaturated ester carbonyl groups (1701, 1634 cm^{-1}). The ^{13}C NMR spectrum (Table 1) show three glucopyranoside units (δ_C 100.8, 100.3, 100.2, 74.7, 74.7, 74.7, 78.5, 78.4, 78.4, 78.0, 78.0, 78.0, 71.6, 71.6, 71.6, 62.9, 62.8 and 62.8).[4] Acid hydrolysis of **1** also gave D-glucose, which was identified by GC analysis. In the NMR spectra (Table 1), three singlets at δ_H 7.52 (1H, s), 7.50 (1H, s) and 7.36 (1H, s) together with δ_C 154.7, 152.7, 152.4, 113.2, 113.1 and 110.7, three carbonyl signals at δ_C 167.8, 169.2 and 169.3, which were indicators of the presence of three olefinic protons of the enols.[3] Also, signals at δ_H 5.22 (1H, d, J = 4.0 Hz), 5.33 (1H, d, J = 4.0 Hz) and 5.81 (1H, d, J = 8.4 Hz), together with δ_C 97.8, 97.6 and 97.3, established the existence of three protons on glycosylated carbons.[5] The 1D and 2D NMR spectroscopic data for compound **1** showed four distinct parts, indicated as units A, B, C and D. For units B and D were easily assigned to two loganin-like segments, due to two methyl groups in ^1H-NMR spectrum at δ_H 0.88 (3H, d, J = 5.4 Hz, H-10b) and δ_H 1.13 (3H, d, J = 6.6 Hz, H-10d), two olefinic signals at δ_H 7.36 (s, H-3b) and 7.50 (s, H-3d). The ^{13}C NMR and DEPT spectra, which were complete assined by 2D NMR experiments, showed two trisubstituted double bonds signals at δ_H 152.4, 152.7, 113.1, 113.2 and carbonyl signals at δ_C 169.2 and 169.3, which can be ascribed to C-3b, C-3d, C-4b, C-4d, C-11b, and C-11d, respectively.[5] Two sets of carbon signals in the ^{13}C NMR spectrum at δ_C 32.3, 40.0, 78.3, 40.7 and 47.0, and at δ_C 32.9, 40.5, 79.8, 41.2 and 47.1, along with the corresponding protons correlated to each other in the ^1H-^1H COSY spectrum, future supported that the existence of two loganin-like segments esterified at C-7.

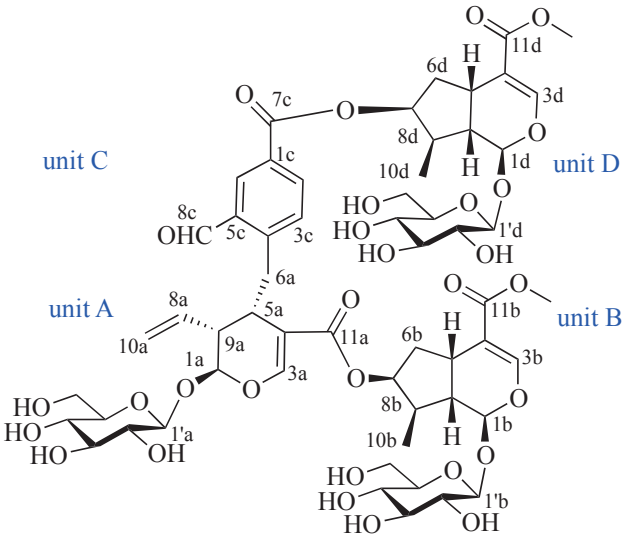

Figure 1. Structure of compound **1**

Furthermore, the ^{13}C NMR spectrum of **1** showed signals due to one α,β-unsaturated ester carbonyl group at δ_C 167.8, and one double bond at δ_C 154.7 and 110.7. In the ^{13}C NMR spectrum, one anomeric carbon signal at δ_C 97.8 was assigned readily. One set of terminal carbon-carbon double bond signals at δ_C 135.8 and 119.7 were characteristic for one secoiridoid glucoside unit. These data indicated clearly that unit A of **1** is a secoiridoid glucoside moiety.[5] In addition, the ^{13}C NMR and 2D NMR experiments of **1** showed signals due to one α,β-unsaturated ester carbonyl group at δ_C 167.8, and one double bond at δ_C 154.7 and 110.7, and one anomeric carbon signal at δ_C 97.8 was assigned readily. These data indicated clearly that unit A of **1** is a secoiridoid glucoside moiety.[5] Complete structure assignments were obtained from exhaustive analysis of the HMQC, HMBC and COSY data.

After the removal of the signal of units A, B and D, three singlets of aromatic protons at δ_H 8.09 (1H, dd, J = 7.8, 1.8 Hz), 7.42 d (1H, d, J = 7.8 Hz), 8.44 d (1H, d, J = 1.8 Hz), along with six carbon signals at δ_C 130.5 s, 134.8 d, 133.9 d, 136.1 s, 148.9 s and 133.3 d, supporting the presence of a 1,3,4-tirsubstituted aromatic ring. Further more, the NMR spectra exhibited signal of an aldehyde (δ_H 10.3, 1H, s; δ_C 193.5) and a carbonyl group (δ_C 166.5). The locations of the aldehyde group at C-5c and a carbonyl group at C-1c were determined by the correlations in the HMBC experiment. HMBC also supported unit A and unit C as shown in Figure 1 by showing long-range correlations from H-5a to C-4c, H-6a to C-3c, C-4a and C-5c.

Further information was obtained from 2D NMR experiments. The linkage was also ascertained by long-range correlations between H-7b at δ_H 4.77 and C-11a at δ_C 167.8, H-7d at δ_H 5.40 and C-7c at δ_C 166.5 in the HMBC spectrum (for other important HMBC correlations, see Figure 2).

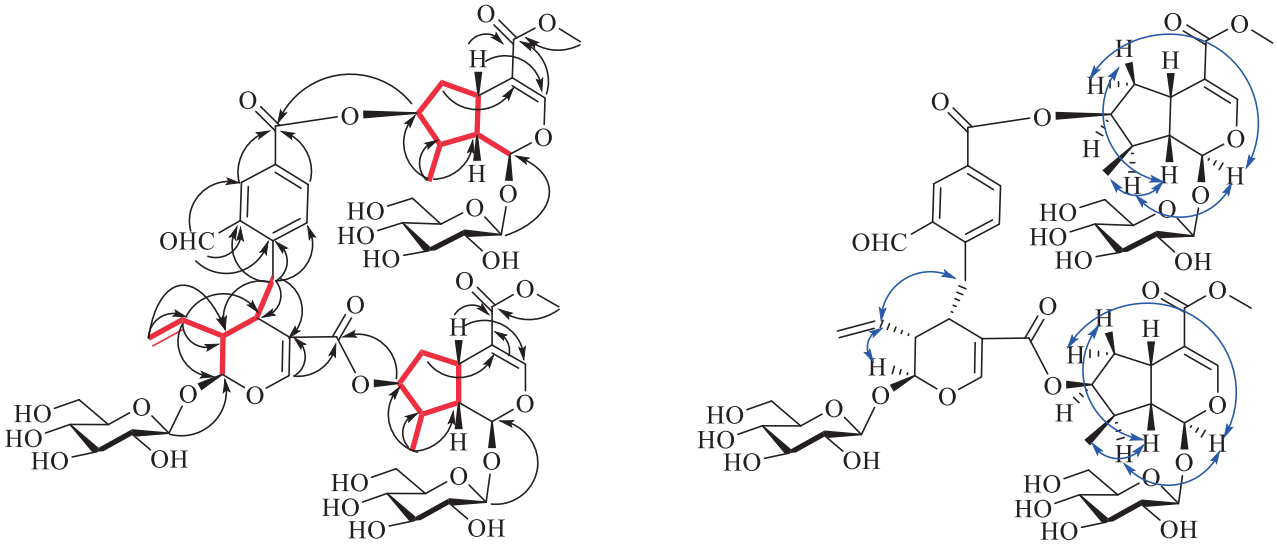

Figure 2. Key HMBC, ^{1}H-^{1}H COSY and NOESY correlations of compound **1**

Table 1. ^{1}H and ^{13}C NMR data of compound **1**[a,b,c]

	δ_H	δ_C		δ_H	δ_C
1a	5.81 d (8.4)	97.8	1c	—	130.5
3a	7.52 s	154.7	2c	8.09 dd (7.8, 1.8)	134.8
4a	—	110.7	3c	7.42 d (7.8)	133.9
5a	3.24 m	36.8	4c	—	136.1
6a	3.19 m; 3.54 m	33.8	5c	—	148.9
7a	—	—	6c	8.44 d (1.8)	133.3
8a	5.94 ddd (7.8, 8.4, 18.0)	135.8	7c	—	166.5
9a	2.61 m	45.3	8c	10.3 s	193.5
10a	5.33 ov	119.7	—		
11a		167.8			
1'a	4.77 d (7.8)	100.8	—		
2'a	3.20 m	74.7	—		
3'a	3.30 m	78.5	—		
4'a	3.27 m	71.6	—		
5'a	3.36 m	78.0	—		
6'a	3.89 ov; 3.66 ov	62.9	—		
1b	5.22 d (4.0)	97.3	1d	5.33 d (4.0)	97.6
3b	7.36 s	152.4	3d	7.50 s	152.7
4b	—	113.2	4d	—	113.1
5b	2.91 br q (7.2)	32.3	5d	3.19 ov	32.9
6b	1.93 ov	40.0	6d	2.42 br dd (7.8, 13.0)	40.5
	1.56 ddd (5.4, 8.4, 13.2)			1.86 ddd (4.0, 7.2, 13.8)	
7b	4.77 ov	78.3	7d	5.40 t (4.8)	79.8
8b	1.94 ov	40.7	8d	2.24 m	41.2
9b	1.95 ov	47.0	9d	2.19 ddd (4.8, 9.0, 8.4)	47.1
10b	0.88 d (5.4)	13.8	10d	1.13 d (6.6)	13.9
11b	—	169.2	11d	—	169.3
MeO	3.66 s	51.8	MeO	3.68 s	51.8
1'b	4.63 d (7.8)	100.3	1'd	4.68 d (7.8)	100.2
2'b	3.20 m	74.7	2'd	3.20 m	74.7
3'b	3.30 m	78.4	3'd	3.30 m	78.4
4'b	3.27 m	71.6	4'd	3.27 m	71.6
5'b	3.36 m	78.0	5'd	3.36 m	78.0
6'b	3.89 ov; 3.66 ov	62.8	6'd	3.89 ov; 3.66 ov	62.8

[a] Data were obtained in CD_3OD. [b] Ov means overlapped. [c] ^{1}H NMR (600 MHz, δ, J in Hz in parentheses), ^{13}C NMR (150 MHz, δ).

In the literature, all of the reported compounds have the β-configuration at C-1 of the loganin and secologanin moieties.[4] Units B and D were estimated as loganin moieties by comparison of the NMR spectroscopic data with the literature.[4] This being confirmed further by NOE experiments and coupling constants. The configuration of H-1b and H-1d were determined as α-orientation as well based on the vicinal coupling constants of H-1b (δ_H 5.22, 1H, d, J = 4.0 Hz) and H-1d (δ_H 5.33, 1H, d, J = 4.0 Hz) in the ^{1}H-NMR spectrum.[5] The NOE cross-peaks between H-1b / H-6bα, H-1b / H-8b, H-6bβ / H-9b, and H-10b / H-9b, suggested that H-5b, H-9b and the methyl group at C-8b in the β-orientation, and H-7b and H-8b in the α-orientation, which was in good agreement with that of loganin. The same relative

configuration was also determined for unit D. For unit A, the cross-peak between H-1a / H-8a and H-8a / H-6a, indicating that H-5a and H-7a in the β-orientation. Thus, the structure of **1** was determined as shown in Figure **1**, named pterocephanoside.

Compounds **1**, **2**, **5** and **6** were evaluated for their cytotoxicity against MCF-7, Hep G2 and H460 human cancer cell lines, but none of them showed any significant activity (IC_{50} > 50 μM, n = 3).

EXPERIMENTAL

General experimental procedure

Optical rotations were measured on a Perkin-Elmer 341 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker AV 500 or 600 NMR spectrometers with TMS as an internal standard. IR spectra were obtained with a Thermo Fisher Nicolet 6700 spectrometer, KBr pellets in cm^{-1}. HR-ESI-MS were measured using a Q-TOF micro mass spectrometer (Waters, USA). Silica gel (Qingdao Haiyang Chemical Co., Ltd., China, 200-300 mesh), RP-18 silica gel (Merck, 40-60 μm), and D-101 macroporous resin (Rohm & Haas) were used for column chromatography (CC). Semi-preparative HPLC was carried out on a Waters SymmetryPrepTMC-18 column (7 μm, ∅ 19.0 × 300 mm) with a Waters 600 controller and Waters 2487 detector. GC analyses were performed using a Hewlett Packard GC6890 instrument on a L-CP-Chirasil-Val column (0.32 mm × 25 m). TLC plates were precoated with silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd., China) and visualized under a UV lamp at 254 nm or by spraying 5% vanillin-H_2SO_4 (w/v) or by iodine.

Plant Material

P. hookeri were collected in Lhasa, Tibet, China, in July 2010. The plant was identified by Prof. Yi Zhang in College of Ethnic Medicine, Chengdu University of Traditional Chinese Medicine, China. A voucher specimen (No. Z3615201003) was deposited in the School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China.

Extraction and isolation

The shade dried *P. hookeri* (5.0 kg), powered and extracted with 95% EtOH (20 L) at room temperature for three times (3 × 3 days). After removal of the solvent by evaporation, the ethanol extract (560 g) was recovered. The extract was then suspended in 50 ^oC water (1 L), extracted with petroleum ether (1 L × 3), EtOAc (1 L × 3) and *n*-butanol (1 L × 3) to obtain the petroleum ether extract (120 g), the EtOAc extract (100 g), and the *n*-butanol extract (150 g), respectively.

The *n*-butanol extract (130 g) was subjected to a column chromatography (∅ 8.0 × 50 cm) using D101 resin and eluted with H_2O, 25%, 65% and 95% EtOH sequentially, yielding four fractions (I, II, III, and IV).

Fraction II (9.0 g) was separated by RP-18 silica gel column CC (∅ 3.5 × 40 cm) using MeOH-H_2O in a gradient manner (1:60-1:0) to afford fractions (F_{II-1}-F_{II-4}) based on TLC analysis. F_{II-2} (600 mg) was subjected to CC (∅ 2.0 × 35 cm) on silica gel eluted with $CHCl_3$:MeOH (18:1) to yield compounds **7** (42 mg) and **9** (12 mg). F_{II-3} (1.6 g) was subjected to CC (∅ 3.5 × 40 cm) on silica gel eluted with $CHCl_3$:MeOH (25:1) to yield six fractions (F_{II-3-1}-F_{II-3-6}). F_{II-3-2} was subjected to Sephadex LH-20 column chromatography (∅ 1.5 × 60 cm) eluted with MeOH to yield compound **8** (13 mg). F_{II-3-3} was purified using Sephadex LH-20 column chromatography (∅ 1.5 × 60 cm) eluted with MeOH and then preparative HPLC, eluting with MeOH:H_2O (20:80) with a flow rate of 8 mL/min to afford compound **6** (9 mg).

Fraction III (6.0 g) was subjected to a silica gel column chromatography (∅ 5.0 × 50 cm) eluted with $CHCl_3$:EtOAc:MeOH (10:10:1) to yield six fractions (F_{III-1}-F_{III-6}). F_{III-3} (350 mg) was subjected to RP-18 silica gel CC (∅ 2.0 × 45 cm), and eluted with MeOH:H_2O (22:78) to yield compounds **4** (17 mg) and **5** (20 mg).

Fraction IV (23.0 g) was subjected to RP-18 silica gel CC (∅ 7.0 × 60 cm) eluted with MeOH:H_2O (0:1-1:0) to yield five fractions (F_{1-1}-F_{1-5}). F_{1-3} (4.0 g) was purified using Sephadex LH-20 column chromatography (∅ 1.5 × 60 cm, MeOH) and then with preparative HPLC, eluting with MeOH:H_2O (30:70) to afford compounds **1** (13 mg) and **3** (12 mg). F_{1-5} (1.7 g) subjected to was subjected to chromatographed on a silica gel column (∅ 3.5 × 45 cm) eluted with EtOAc:MeOH:H_2O (20:1:1) to yield compound **2** (16 mg).

Pterocephanoside (1)

White amorphous solid; $[\alpha]_D^{20}$ -110.2 (*c* 0.28, MeOH); IR (KBr) ν_{max} 3411, 2928, 1701, 1634, 1439, 1384, 1294, 1191, 1176, 1155, 1074, 953, 893, 766 cm^{-1}; ^1H and ^{13}C NMR data see **Table 1**. HR-ESI-MS at *m/z* 1261.4169 $[M + Na]^+$ (calcd. for $C_{57}H_{74}O_{30}Na$, 1261.4163).

Cell culture and cytotoxicity assay

The *in-vitro* growth inhibitory activities of compounds **1**, **2**, **5** and **6** were assayed by the MTT method.[9] H460 (human lung adenocarcinoma), MCF-7 (human breast carcinoma) and Hep G2 (hepatocellular carcinoma) cell lines were obtained from ATCC. The cells were cultured in a 96-well plate 24h before treatment and then exposed to different concentrations of compounds or control for 72 h. Three replicates were prepared for each treatment. DMSO (0.1% *v/v*) was used as negative control.

Acid Hydrolysis

Compound **1** (2 mg) was heated in 4 mL of 10% HCl / 1,4-dioxane (1:1) at 80 °C for 4 h. After cooling, the reaction mixture was neutralized with 1 M NaOH in MeOH and evaporated to dryness. H_2O (5 mL) was added and the solution was extracted with EtOAc (5 mL × 3), then the water layer was evaporated, the residue was silylated with HMDS:TMCS:pyridine (3:1:9). The thiazolidine derivatives were analyzed

by GC for sugar identification. D-glucose was confirmed by comparison with the retention time of the authentic standards at 13.04 min.[9]

ACKNOWLEDGEMENTS

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