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C_{21} steroidal glycosides and oligosaccharides from the root bark of *Periploca sepium*

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ABSTRACT

Four new C_{21} steroidal glycosides (**1**–**4**), named perisepiumosides F—I (**1**–**4**) together with six known steroidal glycosides (**5**–**10**) and four oligosaccharides (**11**–**14**), were isolated from the root bark of *Periploca sepium*. Their structures were characterized on the basis of 1D and 2D–NMR spectroscopic data as well as HR-ESI-MS analysis. The evaluation of inhibition activity against human A-549 and HepG2 cell lines indicated that compounds **2**, **8**, **10** and **13** showed different levels of cytotoxic activities with IC_{50} values ranging from 0.61 to 7.86 μM.

1. Introduction

Periploca sepium Bge., a perennial herbaceous plant belonging to the family of Asclepiadaceae, is widely distributed from the Northeast to the Southwest in China. The root of *P. sepium*, a traditional Chinese herb medicine called 'xiangjiapi', has been used for many years [1]. Pharmacological studies on this plant have demonstrated its anti-inflammatory and immunosuppressive effects [2]. More recently, the root bark of *P. sepium* has been widely used for relieving rheumatic conditions and slaking dropsy, for strengthening the bone and the musculature, and to suppress tumor and treat chronic congestive heart failure [3–4]. In addition, the crude extracts of root bark of this plant had insecticidal activity against *Pieris rapae* and *Plutella xylostella* [5]. Previous phytochemical studies of the plant were mainly carried out by some Japanese research groups, which have led to the isolation of C_{21} steroidal glycosides, cardenolides, oligosaccharides, coumarins, flavonoids, and triterpenoids [6–7]. The C_{21} steroidal glycosides were determined as effective ingredients of this plant, and some interesting biological activities have been reported. Periplocoside A has the therapeutic potential for treatment of human autoimmune-related hepatitis [8]. Recently, periplocoside E was reported as an immunosuppressant which could directly suppress T-cell activation *in vitro* and *in vivo* [9]. As a part of a program to search for novel bioactive steroidal glycosides from traditional Chinese medicines, a methanol extract of the root bark of *P. sepium* was investigated. This procedure led to the isolation of four new steroidal glycosides, named perisepiumosides F—I (**1**–**4**), together with six known steroidal glycosides (**5**–**10**) and four oligosaccharides (**11**–**14**). In this paper, we describe the isolation and structural elucidation of the new steroidal glycosides, along with the evaluation of the cytotoxic activities of the 14 compounds against two human cancer cell lines, A-549 and HepG2.

2. Experimental

2.1. General

Optical rotations were determined by using a Perkin-Elmer 341 polarimeter at room temperature. IR spectra were measured on a Perkin-Elmer 1725X-FT spectrometer with KBr disks. UV spectra were run on a Shimadzu UV-2550 spectrophotometer. NMR spectra were recorded on a Bruker Avance-400 spectrometer. The chemical shift (δ) values are given in ppm with TMS as internal standard, and coupling constants (J) are in Hz. High resolution electrospray ionization mass spectrometry (HR-ESI-MS) were performed on a MicrOTOF QII mass spectrometer. Analytical HPLC was carried out on LabAlliance Series III with a model 201 (SSI) detector and Ultimate C18 column (250 mm × 4.60 mm, 5 μm). Preparative HPLC was carried out on P3000 with a UV3000 detector (Beijing ChuangXinTongHeng Science and Technology Co., Ltd) and Ultimate C18 column (250 mm × 30 mm, 10 μm). Silica gel (100–200 mesh and 200–300 mesh, Qingdao Marine Chemical Factory) and RP-

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C18 (150–200 mesh, Merck, Germany) were used for column chromatography (CC). TLC was carried out on precoated silica gel GF254 plates and spots were visualized under UV light and by spraying with 5% H_2SO_4 in ethanol followed by heating.

2.2. Plant material

The root bark of *P. sepium* were purchased from Sichuan Neautus Traditional Chinese Medicine Co., Ltd., People's Republic of China in October 2014. The plant identification was verified by Prof. W.K. Bao of Chengdu Institute of Biology, Chinese Academy of Sciences. A voucher specimen (CIB-A-403) has been deposited at the Laboratory of Phytochemistry, Chengdu Institute of Biology, Chinese Academy of Sciences. The purity (>96%) of compounds **1**–**14** used for biological assay was determined by HPLC.

2.3. Extraction and isolation

The air-dried root bark of *P. sepium* (40 kg) were extracted with MeOH (3 × 80 L, 1d, each) at room temperature. The solvent was evaporated, and the residue suspended in water and then successively extracted with EtOAc and n-BuOH, to yield EtOAc (1.5 kg) and n-BuOH extracts (820 g). The EtOAc extract (1.5 kg) was suspended in 95% MeOH and then was extracted with petroleum ether, yielding a MeOH extract. The MeOH extract (980 g) was subjected to silica gel column eluting with a petroleum ether/ EtOAc gradient system (50:1, 30:1, 10:1, 1:1, *v*/v) to give fractions 1–12. Fraction 2 (123 g) was chromatographed on a silica gel column eluted with CH_2Cl_2/MeOH (100:1, 70:1, 5:1, 1:1, *v*/v) to afford nine subfractions, 2a-2i. Compound **13** (124 mg) was isolated from subfractions 2a (1.7 g) by crystallization from acetone. Subfractions 2c (4.6 g) was further purified by reversed phase preparative HPLC (MeCN − H_2O, 60:40, *v*/v) to obtain compound **6** (37 mg, Rt = 11 min), compound **14** (20 mg, Rt = 13 min), compound **2** (22 mg, Rt = 20 min), and compound **3** (24 mg, Rt = 33 min). Subfractions 2e (1.3 g) was crystallized by acetone to give compound **8** (216 mg). Subfractions 2i (1.1 g) was also crystallized by MeOH and CH_2Cl_2 to give compound **9** (198 mg). Fraction 3 (29 g) was subjected to a silica gel column chromatography using gradient solvents of CH_2Cl_2/MeOH (100:1, 80:1, 2:1, 1:1, *v*/v) to get four subfractions, 3a-3d. Subfractions 3b (430 mg) was crystallized by acetone to give compound **11** (57 mg). Subfractions 3d (502 mg) was purified by reversed phase preparative HPLC and eluted with MeCN − H_2O (55:45, *v*/v) to give compound **1** (11 mg, Rt = 16 min) and compound **5** (9 mg, Rt = 24 min). Fraction 7 (20.4 g) was applied to a RP-C18 column, eluting with MeOH − H_2O (35:65, 50:50, 65:35, 80:20, 100:0, *v*/v) to afford subfractions, 7a-7e. Subfraction 7b (1.1 g) and 7c (1.3 g) were crystallized by acetone to give compound **12** (158 mg) and compound **4** (24 mg), respectively. Fraction 8 (12.5 g) was chromatographed on a silica gel column eluted with CH_2Cl_2/MeOH to afford compound **10** (37 mg) and compound **7** (43 mg).

2.3.1. Perisepiumoside F (**1**)

White, amorphous powder; $[\alpha]_D^{20}$ −20.5 (c 0.1 , MeOH); IR (KBr) ν_{max} 3445, 2933, 1623, 1410, and 1072 cm^{-1}; for ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 533.3101 $[M + Na]^+$ (calcd for $C_{28}H_{46}O_8Na$, 533.3085).

2.3.2. Perisepiumoside G (**2**)

White powder; $[\alpha]_D^{20}$ −32.5 (c 0.1, $CHCl_3$); IR (KBr) ν_{max} 3421, 2933, 1725, 1623, 1447, 1195, 1001, and 619 cm^{-1}; for ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 977.5445 $[M + Na]^+$ (calcd for $C_{50}H_{82}O_{17}Na$, 977.5444).

2.3.3. Perisepiumoside H (**3**)

White powder; $[\alpha]_D^{20}$ −9.0 (c 0.1, $CHCl_3$); IR (KBr) ν_{max} 3435, 2933, 1726, 1621, 1450, 1106, 1002, and 616 cm^{-1}; for ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 1121.6270 $[M + Na]^+$ (calcd for $C_{57}H_{94}O_{20}Na$, 1121.6231).

2.3.4. Perisepiumoside I (**4**)

White needles; $[\alpha]_D^{20}$ −30.5 (c 0.1, MeOH); IR (KBr) ν_{max} 3429, 2936, 1623, 1410, and 1072 cm^{-1}; for ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 647.3759 $[M + Na]^+$ (calcd for $C_{34}H_{56}O_{10}Na$, 647.3766).

2.4. Cytotoxicity assay

The cytotoxicity of all isolated compounds against human A-549 and HepG2 cell lines were evaluated by means of the MTT assay [10]. Cells were incubated at 37 °C for 24 h in a humidified incubator with 5% CO_2 and 95% air. It was performed in a 96-well format, and each tumor cell line was exposed to each test compound at various concentrations for 48 h, with cisplatin used as positive control. At the end of the treatment period, 20 μL (5 mg/mL) of the MTT was added to each well and the plates were incubated for 4 h at 37 °C. The medium was removed and MTT reduction product (formazan crystals) was dissolved in dimethylsulfoxide (DMSO) (150 μL for each well). The optical density was measured at 490 nm. Cell viability (%) was measured and cell growth curve was plotted. IC_{50} values were calculated by the Reed and Muench method [11].

3. Results and discussion

A MeOH extract from the root bark of *P. sepium* Bge. was extracted with EtOAc and n-BuOH, successively. The EtOAc portion was further resolved using silica gel, ODS, and preparative HPLC to yield four new steroidal glycosides (**1**–**4**) and six previously identified steroidal glycosides, four known oligosaccharides, as shown in Fig. 1.

Compound **1** was obtained as a white amorphous powder with a molecular formula of $C_{28}H_{46}O_8$, which was determined by HRESIMS at *m/z* 533.3101 ($[M + Na]^+$, calcd for $C_{28}H_{46}O_8Na$, 533.3085). The IR spectrum showed the absorption bands for hydroxy (3445 cm^{-1}) and olefinic (1623 cm^{-1}) groups. In the ^1H NMR spectrum of **1**, two singlet methyl signals at δ_H 1.20 (3H, s) and δ_H 1.00 (3H, s), one doublet methyl signal at δ_H 1.29 (3H, d, J = 6.24 Hz), one methoxyl signal at δ_H 3.39 (3H, s), and one olefinic proton signal at δ_H 5.41 (1H, br. s), as well as one anomeric proton signal at δ_H 4.95 (dd, J = 9.56, 1.72 Hz) were observed. The ^{13}C NMR, DEPT and HSQC spectra exhibited 28 carbon signals, comprising three methyls, a methoxyl, ten methylenes, ten methines and four quaternary carbons (Tables 1 and 2). The above information coupled with literature references [12], indicated **1** was characteristic for a C-21 steroidal glycoside. Comparing the 1D NMR data of **1** to those of the known compound perisepiumoside B (**5**) [13], it was suggested that they possessed the same aglycone, identified as (3*β*,14*β*,17*β*,20*S*)-3,14,17,20-tetrahydroxy-21-methyoxypregn-5-ene [14]. The difference was the sugar chain located at C-3, which consisted of only one sugar residue instead of tree sugar units in perisepiumoside B. The assignment of all the carbon and proton signals of the aglycone and the sugar moiety was determined from HSQC and HMBC experiments, and the sugar unit was identified as a *β*-digitoxopyranose unit on the basis of comparison of their ^1H and ^{13}C NMR data with those in the literature [15]. The *β*-configuration of the anomeric proton of sugar moiety was determined by the large coupling constant (J = 9.56 Hz) [16]. The *β*-digitoxopyranose unit was located at C-3 on the basis of the carbon signal at C-3 shifted downfield due to the glycosidation shifts, and the ^1H—^{13}C long-range correlation signal between the anomeric proton of digitoxose at δ_H 4.95 (dd, J = 9.56, 1.72 Hz) and C-3 at δ_C 77.4 (Fig. 2). In addition, in the HMBC experiment, the long-range correlations were observed from δ_H 2.36 (H-4) to δ_C 37.1 (C-10); from δ_H 5.41 (H-6) to δ_C 37.1 (C-10); from δ_H 1.65 (H−12) to δ_C 46.1 (C-9); from δ_H 1.81 (H-15) to δ_C 50.8 (C-13); from δ_H 1.00 (H-18) to δ_C 139.5 (C-5), δ_C 46.1 (C-9) and δ_C 37.1 (C-10); from δ_H 1.20 (H-19) to δ_C 32.3 (C-12), δ_C 50.8 (C-13) and δ_C 88.1 (C-