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A New Furostanol Saponin from *Asparagus cochinchinensis*

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A new furostanol saponin, (25S)-26-O-β-D-glucopyranosyl-5β-furost-20(22)-en-3β, 15β,26-triol-3-O-[α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyranoside, namely, aspacochioside D (**1**) were isolated from *Asparagus cochinchinensis* (Lour.) Merr, along with three known saponins, aspacochioside C (**2**), (25S)-5β-spirostan-3β-yl-O-[O-α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyranoside (**3**), and pseudoprotoneodioscin (**4**). The structure of **1** was elucidated on the basis of chemical reactions and spectral analysis (IR, GC, ESI-MS, ^{1}H-NMR, ^{13}C-NMR, DEPT, HMBC, HMQC and NOESY). The antiproliferative effects of **1**-**4** were evaluated in a cytotoxicity assay against the human tumor cell line, A549. Compound **2** (Aspacochioside C) exhibited moderate cytotoxicity against A-549, with an IC_{50} value of 3.87 μg/mL.

Key words: *Asparagus cochinchinensis*, Furostanol glycosides, Cytotoxicity

INTRODUCTION

Asparagus cochinchinensis (Chinese name: Tiandong), a Traditional Chinese Medicine, is the tuberous root of *A. cochinchinensis* (Lour.) Merr (Liliaceae) which is distributed in many provinces of China. *A. cochinchinensis* was often used for treatments of fever, cough, hemoptysis, diabetes, constipation, swelling, throat pain and lung cancer (Jiangsu Medical College, 1985). A few substances such as β-sitosterol, some amino acids, some monosaccharides, oligosaccharides, polysaccharides and several furostanol saponins have been isolated from the plant (Aquino et al., 1986; Ju et al., 1994; Meng et al., 1998a; Shi et al., 2004). In recent years, some steroidal saponins which possess cytotoxic activities in a human lung carcinoma (A549) tumor cell line have been isolated from other *Asparagus* species, such as *A. filicinus* (Cong et al., 2000). In search for the bioactive constituents from Chinese herbs, compounds from *A. cochinchinensis* were isolated in the present study from the extract of the tuberous roots. Extraction, separation, structural identification and cytotoxicity of a new furostanol glycoside are discussed in this paper.

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MATERIALS AND METHODS

General procedures

Melting points were determined on an RY-2 melting point apparatus and were uncorrected. IR spectra were recorded on a Bruker Vector-22 spectrometer with KBr pellets. 1D and 2D NMR spectra were recorded on a Bruker DRX500 NMR spectrometer in pyridine-d5. GC was performed with a HP-5892 II gas chromatograph. An Agilent mass spectrometer (for ESI) and Q-Tof micro mass spectrometer (for HR-ESI) were used (in *m/z*). Column chromatography (CC) was performed with silica gel (200-300 mesh) and Sephadex LH-20 (40-70 μm, GE Healthcare Biosciences AB). Macroporous resin was from Zhen Tiancheng Technology Co. Ltd. TLC and preparative TLC silica gel plates were from Huiyou Silical Gel Development Co. Ltd. and visualized by spraying with 5% H_2SO_4 in ethanol followed by heating.

Plant material

Roots of *A. cochinchinensis* were collected in Huangshi city (Hubei province) in June 2006 and authenticated by Prof. Han-Chen Zheng, Department of Pharmacognosy, Second Military Medical University. A voucher

specimen (No. 20060764) was kept in the Herbarium of Second Military Medical University.

Extraction and isolation

Air-dried and ground roots of *A. cochinchinensis* (9.5 kg) were extracted twice with 75% ethanol for 3 days each time at room temperature. The solvent was removed under reduced pressure to yield a dark residue (1516 g). The residue was suspended in water and then partitioned with $CHCl_3$. The aqueous layer was subjected to column chromatography over macroporous resin, eluting first with H_2O then successively with 30%, 50%, 80%, and 90% EtOH. Combining 50% partition with 80% partition, the new partition was evaporated under reduced pressure to yield a residue (612 g). The residue (600 g) was subjected to silica gel column chromatography eluting with a $CHCl_3$-MeOH-H_2O gradient (40:10:1-65:35:10) to afford 5 fractions (Fr.1-Fr.5). Fraction 3 (18 g) was rechromatographed on a silica gel column with $CHCl_3$-MeOH-H_2O (40:10:1 -20:10:1) as the eluent to give the known compounds **2** (24 mg) and **3** (17 mg). Fraction 4 (14) was purified by column chromatography over silica gel using a $CHCl_3$-MeOH-H_2O gradient (90:35:6-65:35:10) and further purified with a C-18 silica gel column by using MeOH-H_2O (65:35) as the eluent to give compound **1** (53 mg). Compound **4** (24 mg) was obtained from Fraction 5 (21 g) by using a C-18 silica gel column with acetonitrile-H_2O (28:72) as the eluent.

(25S)-26-*O*-β-D-glucopyranosyl-5β-furost-20(22)-en-3β, 15β, 26-triol-3-*O*-[α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyranoside (1)

White amorphous powder, m.p. 210-212°C; ^{1}H-NMR (600 MHz, in C_5D_5N): δ 4.94 (1H, H-16), 4.27 (1H, H-3), 4.12 (1H, *dd*, *J* = 10.0 Hz, 7.3, H-26), 4.05 (1H, *m*, H-15), 3.48 (1H, *dd*, *J* = 10.0 Hz, 7.3, H-26), 2.50 (1H, *d*, *J* = 10.1 Hz, H-17), 1.72 (3H, *s*, H-21), 1.18 (3H, d, *J* = 6.0 Hz, H-27), 1.16 (1H, *m*, H-14), 0.85 (3H, *s*, H-19), 0.70 (3H, *s*, H-18); sugar portions: 5.90 (1H, *brs*, H-1"), 4.82 (1H, *d*, *J* = 7.0 Hz, H-1'), 4.81 (1H, *d*, *J* = 7.0 Hz, H-1'"), 1.70 (3H, *d*, *J* = 6.2 Hz, H-6"); ^{13}C-NMR (150 MHz, in C_5D_5N) see Table I; ESI-MS *m/z* 925 [M+Na]$^+$, HR-ESI-MS *m/z* 925.9913 [M+Na]$^+$ (Calcd. for $C_{45}H_{74}O_{17}Na$, 926.0615).

Aspacochioside C (2)

White amorphous powder. $C_{45}H_{75}O_{17}$. ESI-MS *m/z* 909 [M+Na]$^+$ (Shi and Bhutani, 2004).

(25S)-5β-spirostan-3β-yl-O-[O-α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyranoside

White amorphous powder. $C_{39}H_{64}O_{12}$. ESI-MS *m/z* 747[M+Na]$^+$ (Jadhav and Bhutani, 2006).

Table I. ^{13}C-NMR data of compound **1** in C_5D_5N (150 M)

NO	δ (C)	NO	δ (C)
1	30.6	3-Glu	
2	26.6	1'	102.7
3	74.9	2'	75.2
4	29.9	3'	78.2
5	36.6	4'	78.1
6	25.5	5'	76.8
7	26.6	6'	61.3
8	34.8		
9	39.8	4'-Rha	
10	34.9	1"	102.4
11	21.0	2"	72.3
12	39.7	3"	72.5
13	43.6	4"	73.7
14	54.4	5"	70.0
15	84.4	6"	18.2
16	90.1		
17	64.6	26-Glu	
18	14.2	1'"	104.9
19	23.5	2'"	74.9
20	105.0	3'"	78.1
21	11.4	4'"	71.4
22	153.8	5'"	76.5
23	34.1	6'"	62.5
24	39.4		
25	30.7		
26	75.4		
27	17.7		

Pseudoprotoneodioscin (4)

White amorphous powder. $C_{51}H_{82}O_{21}$. ESI-MS *m/z* 1053 [M+Na]$^+$ (Yoshikawa et al., 2007).

Acidic hydrolysis of compound 1 and preparation of the aglycon

A solution of compound **1** (5 mg) in 2 M HCl methanol solution (3 mL) was heated for 4 h at 70°C. After the reaction was completed and checked by HPTLC, 1 M NaOH was added dropwise until neutral pH (7) was achieved. Removal of methanol was done under reduced pressure, the water solution was extracted with $CHCl_3$ (2 mL × 3), the organic layers were combined and solvent was removed under reduced pressure to give the aglycon (Meng et al., 1998b).

Hydrolysis of compound 1, preparation of nitrile acetate derivatives and GC analysis

A solution of each compound (5 mg) in 2 M HCl methanol solution (3 mL) was heated for 4 h at 70°C.

After the reaction was completed checked by HPTLC, methanol was removed under reduced pressure. Pyridine (0.5 mL) and hydroxylamine hydrochloride (5 mg) was added, the mixture was shaken, for 0.5 h at 90°C and then cooled to RT. Acetic anhydride (0.5 mL) was added in, heated for 0.5 h at 90°C and the mixture was then analyzed by GC (Zhang, 1999). Glucose, xylose, galactose and rhamnose standards were prepared by the same method. GC condition: HP-5892 II gas chromatograph, FID, HP-20M (Carbowx 20 M), 25 m × 0.32 mm × 0.3 μm, sample temperature: 220°C, detector temperature: 280°C, column temperature: 210°C (constant temperature), column pressure: 60 psi.

Bioassay for cytotoxic activity

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide] colorimetric method to determine the cytotoxic activity against human cell cultures (A-549 cells) *in vitro* was performed; doxorubicin hydrochloride (DOX) was used as a positive control. The samples (purity > 95%) were individually dissolved in DMSO and diluted with Phosphate Buffered Saline (PBS) to a final concentration 1000 μg/mL. The solutions were serially diluted with PBS to obtain the lower concentrations from 100-1.5625 μg/mL. The cells were incubated in 96-well microtitre plates (Nunc and Tarson) at a density of 4×10^4 - 6×10^4 cell/mL. Samples of different concentrations (10 μL/well) were added 24 h after cell seeding. The microtitre plates were incubated for 72 h in a humidified atmosphere with 5% CO_2 at 37°C. The cellular viability was determined using the MTT assay (Li et al., 2002). The optical densities (OD) were read on an ELISA plate reader (Denley MK-2) at a wavelength of 570 nm.

RESULTS AND DISCUSSION

Chemical structure elucidation of compound 1

Compound **1**, white amorphous powder, m.p. 210-212°C. Liebermann-Burchard reaction was positive. Ehrlich reagent reaction showing a red color indicated that the compound was a furostanol saponin. ESI-MS

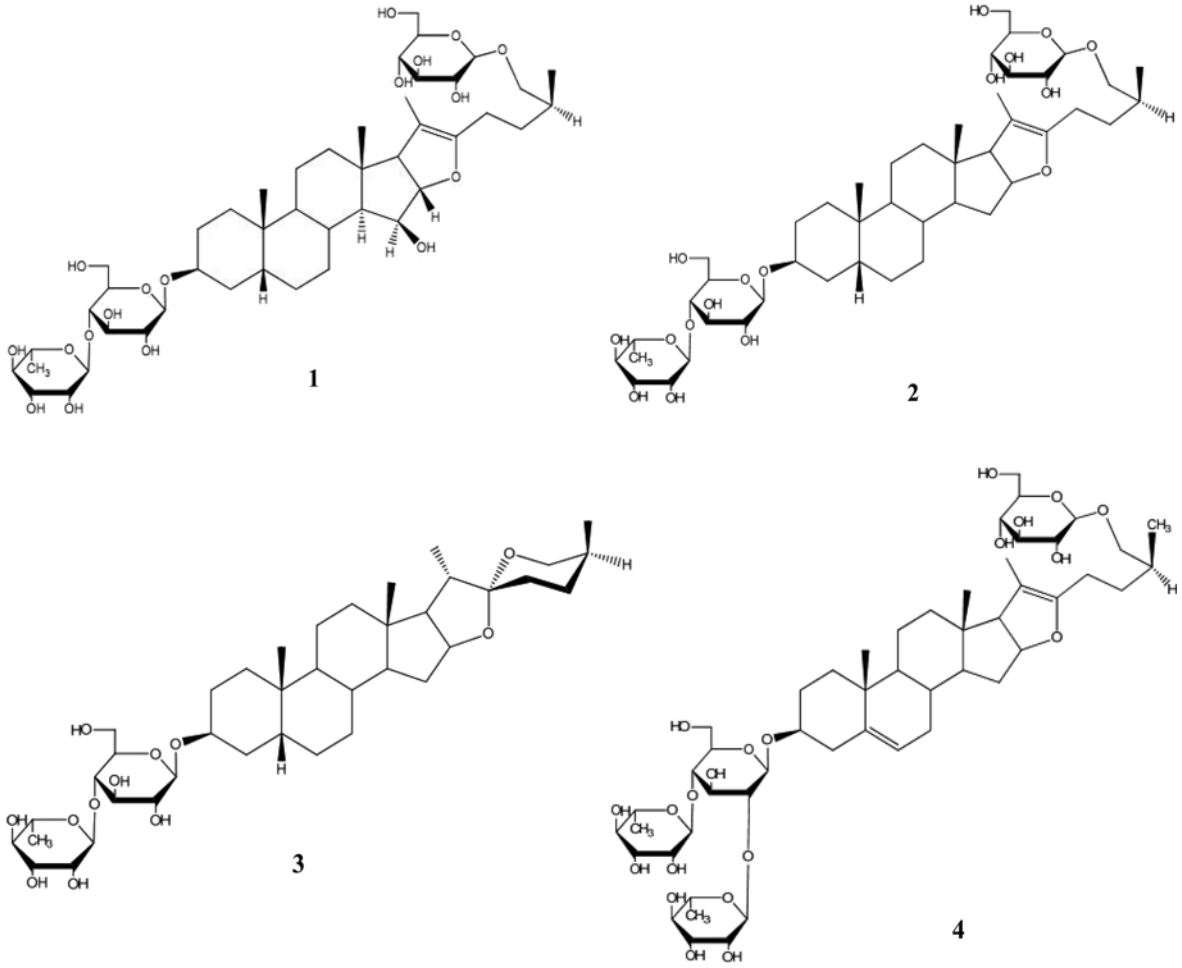

Fig. 1. Chemical structures of compounds **1**-**4**

Table II. GC results of hydrolyzed products of compound **1**

Comp.\t_R Sugar Standard	t_R1 (min) 4.398 (Rha)	t_R2 (min) 6.489 (Xyl)	t_R3 (min) 12.983 (Glu)	t_R4 (min) 14.840 (Gal)
I	4.438	–	12.473	–

t_R: relative retention time

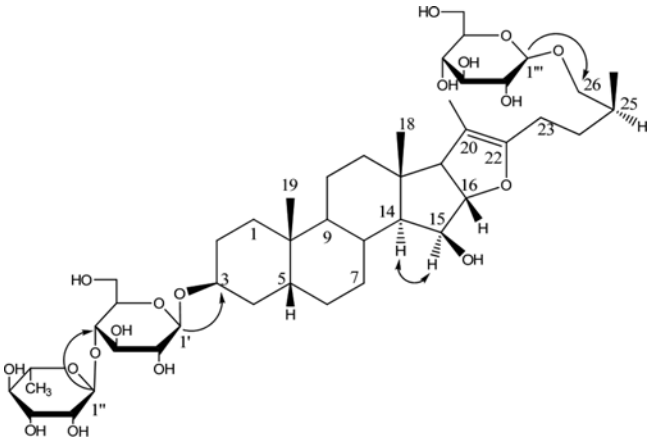

Fig. 2. Chemical structure and Key HMBC correlations (H → C) and NOESY correlations (H-H) of compound **1**

showed that the quasi-molecular ion peak was 925 $[M+Na]^+$ indicating that its moleculer weight was 902. HR-ESI-MS showed that quasi-molecular ion peak was 925.9913 $[M+Na]^+$ (cal. 926.0615) which indicated that the molecular formula was $C_{45}H_{74}O_{18}$. Aglycon was obtained after acid hydrolysis and its absorption band at 917 cm^{-1} was higher than 899 cm^{-1} in the IR spectrum indicating that C25 was in an S configuration. ^{1}H-NMR (C_5D_5N) of Compound **1** at high field revealed 4 methyl groups [δ_H 0.70 (3H, s, 18-CH_3), 0.85 (3H, s, 19-CH_3), 1.18 (3H, d, J = 6 Hz, 27-CH_3), 1.72 (3H, s, 21-CH_3)], 3 signals for sugar [δ_H 5.90 (1H, brs), 4.82 (1H, d, J = 7.0 Hz), 4.81 (1H, d, J = 7.0 Hz)]. Glucose and rhamnose were detected by GC analysis: a β-configuration of glucose and an α-configuration of rhamnose were confirmed due to the coupling constant of the end group proton [δ_H 4.83 (1H, d, J = 7.0 Hz), δ_H 5.90 (1H, brs), δ_H 4.81 (1H, d, J = 7.0 Hz)]. ^{13}C-NMR and DEPT spectrums displayed 3 carbon signals of the end group [δ_C 104.9, 102.7 and 102.4], 2 characteristic signals of a double bond (both were quaternary carbons) at δ_C 153.8 and 105.0. Further, the correlations of H-21 with C-22 (δ_C 153.8) and H-17 with C-20 (δ_C 105.0) indicated that the double bond was at C-20 (22). The β-configuration of H-5 was determined by the ^{13}C-NMR data for C-18 (δ_C 14.2) and C-19 (δ_C 23.5). The signals assigned to the aglycon moiety were in good agreement with that of (25S)-5β-furostane-20(22)-en-3β, 26-diol glycosylated at C-3 and C-26. Furthermore, these data indicated that compound **1** possessed a triglycosidic structure. In the HMBC spectrum (see Fig. 1), long range correlations from H-1' to C-3, H-1" to C-4' and H-1'" to C-26 unequivocally revealed a disaccharide [α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyra-nosyl moiety and the remaining β-D-glucopyranosyl unit was located at C-3 and C-26 of the aglycon. The NMR data of **1** were similar to the known compound aspacochioside C (**2**) which isolated from *A. cochinchinensis* by Shi et al. (2004) except that compound **1** had a hydroxy moiety. The OH group was positioned at C-15 (δ_C 84.4) due to the HMBC cross-peaks of H-15 (δ_H 4.05) with C-14 (δ_C 54.4) and C-16 (δ_C 90.1). The relative configuration δ of OH (C-15) was deduced by the NOESY correlations of H-15 (δ_H 4.05) and H-14 (δ_H 1.16). Accordingly, the structure of **1** was confirmed as (25S)-26-O-β-D-glucopyranosyl-5β-furost-20(22)-en-3β,15β,26-triol-3-O-[α-L-rhamnopyranosyl-(1-4)]-β-D-glucopyranoside. It is a novel compound and was named aspacochioside D.

Compounds **1-4** were tested for their cytotoxicities against a human lung adenocarcinoma cell line, A-549. Compounds **1-4** exhibited moderate cytotoxicities against A-549 cells with IC_{50} values of 21.3, 3.87, 17.4, and 25.8 μg/mL, respectively.

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