Steroids 155 (2020) 108557

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Cytotoxic steroidal saponins from the rhizome of *Anemarrhena asphodeloides*

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ARTICLE INFO

Keywords:
Anemarrhena asphodeloides
Steroidal saponin
Timosaponin V
Timosaponin W
Cytotoxic activity

ABSTRACT

Two novel steroidal saponins, timosaponin V and W (**1** and **2**), together with seven known steroidal saponins (**3**–**9**), were isolated from the rhizomes of *Anemarrhena asphodeloides* Bunge. Their structures were elucidated by extensive 1D NMR and 2D NMR (HSQC, HMBC, ^{1}H–^{1}H COSY, and NOESY), and MS analyses. The cytotoxic activities of the isolates were evaluated. Compound **1** showed a significant cytotoxic activity against MCF-7 and HepG2 cell lines with IC_{50} values of 2.16 ± 0.19 μM and 2.01 ± 0.19 μM, respectively.

1. Introduction

The rhizome of *Anemarrhena asphodeloides* Bunge, a traditional Chinese medicine called "Zhi-Mu", is widely cultivated in China such as Anhui and Hebei province. *A. asphodeloides* has the effects of clearing away heat, purging fire, nourishing yin and moistening dryness [1]. Previous phytochemical investigation showed that it contains a series of compounds including steroidal saponins [2–6], polysaccharides [7], flavonoids [8], lignans [9], and xanthones [10]. Pharmacological studies have found that steroidal saponins from this plant have a variety of biological activities, such as anti-tumor [11,12], anti-inflammatory [13] and anti-fungal [14] activities.

In our previously study, we have reported the isolation and structural elucidation of several new bioactive steroids from the *A. asphodeloides* which collected in Anhui province. In this paper, in order to find novel biologically active steroidal saponins in *A. asphodeloides* from different habitats, we carried out detailed phytochemical investigation on the *A. asphodeloides* which collected in Hebei province. Herein, we reported the isolation and structure elucidation of two novel steroidal saponins: timosaponin V (**1**), timosaponin W (**2**) and seven known steroidal saponins: timosaponin BIII (**3**) [15], timosaponin AII (**4**) [16], timosaponin P (**5**) [17], timosaponin AIII (**6**) [18], anemarrhenasaponin I (**7**) [19], anemarrhenasaponin Ia (**8**) [19], and timopregnane A (**9**) [18] (Fig. 1). In addition, the cytotoxic activities of the isolates were evaluated, and compounds **1** and **6** showed significant cytotoxic activities.

2. Experimental

2.1. General experimental procedures

Optical rotations were determined with a JASCO P-1020 digital polarimeter (Tokyo, Japan). NMR spectra were performed on a Bruker AV-300 instrument (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR, Zurich, Switzerland) in pyridine-d_5 with TMS as the internal standard. ESI-MS spectra were recorded on an Agilent 1100 Series LC/MSD Trap SL mass spectrometer (Santa Clara, USA). HR-ESI-MS spectra were recorded on an Agilent 6520B Q-TOF spectrometer (Santa Clara, USA). Column chromatography (CC) was performed on macroporous resin D101 (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), silica gel (200-300 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), ODS-AQG12S50 (YMC, Tokyo, Japan) and HW-40 (Tosoh, Tokyo, Japan). TLC was carried out on silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) and silica gel 60 RP-18 F_{254}S plates (Merck, Darmstadt, Germany). TLCs spots were visualized by heating after spraying with 10% H_2SO_4-EtOH aqueous. Sugar analysis was performed on an Agilent 1200 liquid chromatography (Santa Clara, USA) with a UV detector and a YMC-Pack ODS-A column (250 × 4.6 mml, S-5 μm, 12 nm, YMC, Tokyo, Japan).

2.2. Plant material

The rhizomes of *Anemarrhena asphodeloides* Bunge were collected in August 2016 from Chengde of Hebei province. The plant material was identified by Dr. Minjian Qin, Department of Resources Science of Traditional Chinese Medicines, China Pharmaceutical University, China. A voucher specimen (No.2016080001) was deposited at the

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https://doi.org/10.1016/j.steroids.2019.108557
Received 18 September 2019; Received in revised form 4 December 2019; Accepted 12 December 2019
Available online 20 December 2019
0039-128X/

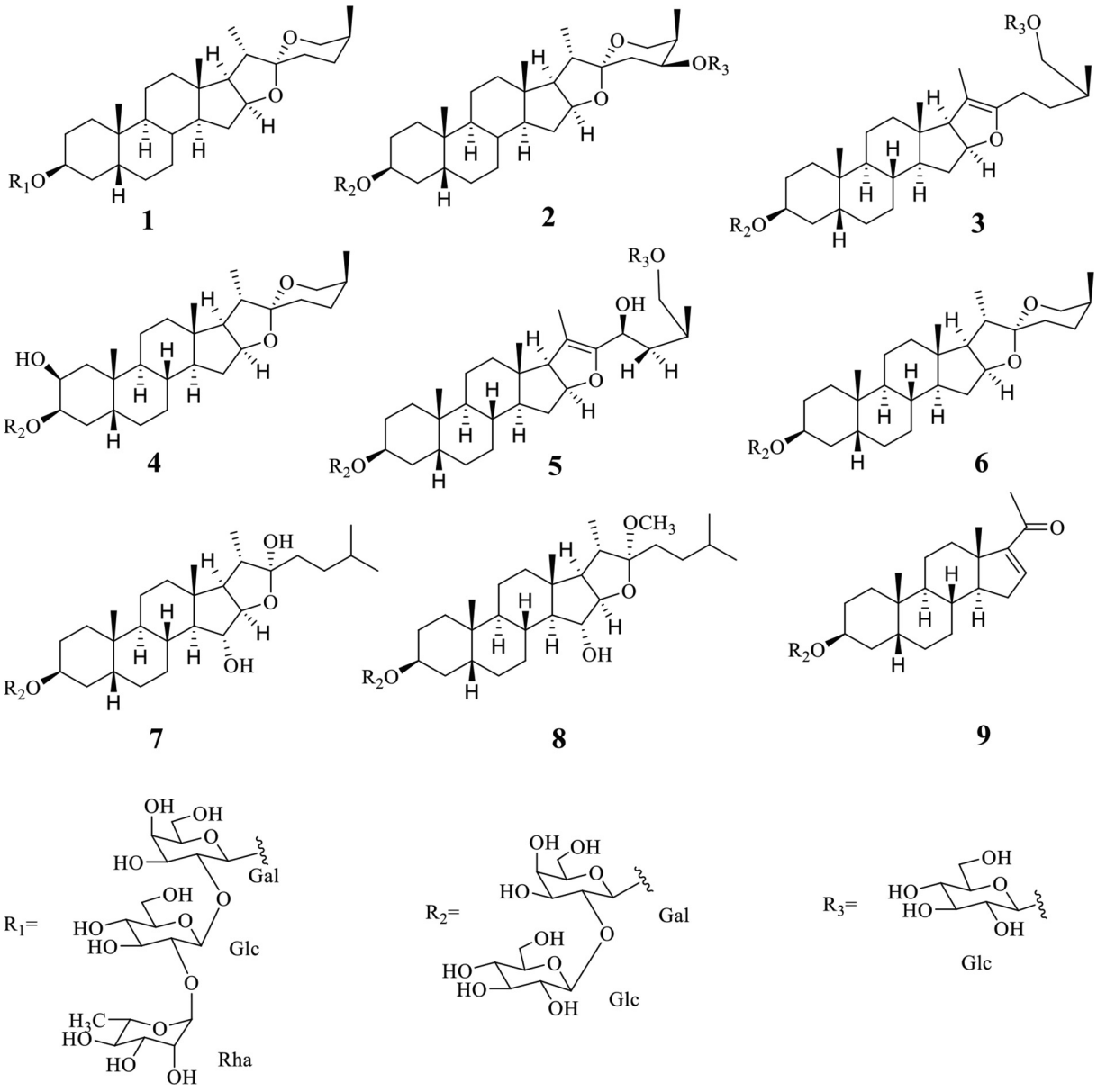

Fig. 1. Structures of compounds **1**–**9**.

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2.3. Extraction and isolation

The dried rhizomes of *A. asphodeloides* (10 kg) were extracted three times with 75% EtOH at reflux (50 L × 3 h). The combined extract was filtered and concentrated under vacuum to give a residue (3.09 kg, not thoroughly dry). Then, the crude extract was suspended in H_2O and extracted with petroleum ether, EtOAc and 1-butanol, respectively. The 1-butanol extract (600 g) was subjected to a D101 macroporous resin column and eluted with EtOH-H_2O (1:9, 3:7, 1:1, 7:3, 19:1, v/v) to obtain five fractions (ZA-1∼ZA-5). ZA-3 was subjected to an ODS column using MeOH-H_2O (30:70–100:0, v/v) as eluent to afford six fractions (ZA-3-1∼ZA-3-6). ZA-3–5 (MeOH-H_2O, 7:3, v/v) precipitated a large amount of white powder, then compound **3** (1.0 g) was obtained by filtrating and washing with MeOH. ZA-3-2 was purified on an ODS CC using MeOH-H_2O (30:70–100:0, v/v) as eluent to give compound **5** (8.4 mg). ZA-3-3 was separated by repeated ODS CC with MeOH-H_2O (30:70–100:0, v/v) and silica gel CC with CH_2Cl_2-MeOH-H_2O (100:20:2–100:50:8, v/v/v) to yield compound **2** (4.0 mg) and compound **9** (9.0 mg). ZA-4 was subjected to a silica gel CC using CH_2Cl_2-MeOH (20:1–1:0, v/v) as eluent to afford five fractions (ZA-4-1∼ZA-4–5). ZA-4–3 (CH_2Cl_2-MeOH, 5:1, v/v) precipitated a large amount of white powder, then compound **6** (5.0 g) was purified by filtrating and washing with MeOH. ZA-4–2 was fractionated by an ODS CC and eluted with MeOH-H_2O (30:70–100:0, v/v) to give compounds **7** (78.7 mg) and **8** (19.1 mg). ZA-4–4 was separated by an ODS CC with MeOH-H_2O (30:70–100:0, v/v) to afford compound **1** (8.0 mg), then the residue was purified on a HW-40 column with CH_2Cl_2-MeOH (1:1, v/v) to yield compound **4** (26.5 mg).

2.3.1. Timosaponin V (1)

White powder; $[\alpha]_D^{25}$ −3.39 (*c* = 0.10, pyridine); ^{1}H NMR (pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz) spectra data see Table 1; HR-ESI-MS *m/z* 885.4873 $[M-H]^-$ (calcd for $C_{45}H_{73}O_{17}$, 885.4853).

2.3.2. Timosaponin W (2)

White powder; $[\alpha]_D^{25}$ −4.67 (*c* = 0.10, pyridine); ^{1}H NMR

Table 1
NMR data of compounds **1** and **2** in pyridine-d_5 (δ in ppm, J in Hz).

NO.	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
1	31.21	1.49 m 1.91 m	31.34	1.47 m 1.80 m
2	27.59	1.59 m 1.83 m	27.20	1.09 m 2.00 m
3	75.25	4.32 m	75.89	4.34 m
4	30.88	1.83 m 1.96 m	31.34	1.80 m 1.82 m
5	36.44	2.45 d (12.5)	37.34	2.16 m
6	27.41	1.79 m 1.85 m	27.39	1.23 m 1.84 m
7	26.86	1.93 m 2.17 m	27.15	0.95 m 1.86 m
8	36.05	1.47 m	35.90	1.49 m
9	40.72	1.33 m	40.61	1.28 m
10	35.71	–	35.64	–
11	21.60	1.18 m 1.33 m	21.53	0.92 m 1.20 m
12	40.72	1.10 m 1.71 m	40.66	1.06 d (6.2) 1.66 d (12.3)
13	41.32	–	41.20	–
14	56.93	1.10	56.83	1.04 m
15	32.62	1.45 m 2.00 m	32.39	1.33 m 1.97 m
16	81.80	4.63 dd (15.0, 7.3)	82.08	4.55 m
17	63.44	1.86 m	62.84	1.80 m
18	16.98	0.84 s	16.95	0.77 s
19	24.38	1.01 s	24.40	0.98 s
20	42.91	1.93 m	42.86	1.96 m
21	15.30	1.17 d (6.8)	15.13	1.12 d (6.8)
22	110.10	–	111.60	–
23	27.30	1.17 m 1.24 m	34.52	1.95 m 2.12 m
24	26.64	1.36 m 1.41 m	73.24	4.84 dt (10.4, 5.0)
25	27.97	1.59 m	32.14	2.28 m
26	65.53	3.38 d (10.8) 4.08 m	64.44	3.53 brd (10.6) 3.95 brd (9.8)
27	16.70	1.09 d (6.2)	10.32	1.34 d (7.1)
Gal-1′	102.00	4.95 d (7.1)	102.99	4.93 d (7.6)
Gal-2′	77.02	4.81 m	82.32	4.69 m
Gal-3′	76.80	4.50 m	75.63	4.29 m
Gal-4′	71.09	4.40 m	70.25	4.59 m
Gal-5′	77.45	4.01 t (5.8)	77.02	4.05 t (6.0)
Gal-6′	62.95	4.37 m 4.40 m	62.60	4.43 m 4.46 m
Glc-1″	102.40	5.77 d (7.0)	106.59	5.31 d (7.7)
Glc-2″	79.79	4.20 m	77.38	4.11 m
Glc-3″	79.71	4.21 m	78.44	4.22 t (9.0)
Glc-4″	73.61	4.08 m	71.92	4.33 m
Glc-5″	77.67	3.68 m	78.83	3.87 m
Glc-6″	63.92	4.27 m 4.40 m	62.90	4.49 m 4.52 m
Glc-1‴			101.58	5.06 d (7.7)
Glc-2‴			75.77	4.08 m
Glc-3‴			79.10	4.28 m
Glc-4‴			72.12	4.33 m
Glc-5‴			78.87	3.95 m
Glc-6‴			63.19	4.43 m 4.49 m
Rha-1‴	102.79	6.35 s		
Rha-2‴	72.91	4.83 m		
Rha-3‴	73.19	4.78 m		
Rha-4‴	74.85	4.40 m		
Rha-5‴	69.98	5.06 dd (9.4, 6.2)		
Rha-6‴	19.51	1.86 d (6.2)		

(pyridine-d_5, 300 MHz) and ^{13}C NMR (pyridine-d_5, 75 MHz) spectra data see Table 1; HR-ESI-MS m/z 917.4757 $[M-H]^-$ (calcd for $C_{45}H_{73}O_{19}$, 917.4752).

2.4. Acid hydrolysis of compounds 1–2 and sugar analysis

Compounds **1** and **2** (each 2.0 mg) were individually dissolved in 2 M HCl-1, 4-dioxane (1:1, 1.0 mL) and held at 80 °C for 6 h. The liberated aglycone was extracted with $CHCl_3$ (0.5 mL × 3). The aqueous residue was evaporated to dryness under nitrogen protection to yield the monosaccharides. The residue was dissolved in 1, 4-dioxane (80 μL), then 0.5 M PMP-CH_3OH (80 μL) and 0.3 M NaOH (80 μL) were added to the mixture. After reacting at 70 °C for 0.5 h, 0.3 M HCl was added to neutralize and then the mixture was extracted with $CHCl_3$ (3 × 0.5 mL). The aqueous residue was dried and then dissolved with MeOH-H_2O for analysis of the sugar moieties by HPLC. The following was the analysis condition: mobile phase A: acetonitrile-20 mM ammonium acetate solution (15:85, v/v), mobile phase B: acetonitrile-20 mM ammonium acetate solution (40:60, v/v), gradient elution: 0% B to 60% B for 30 min with the rate of 1 mL/min, injection volume: 10 μL, detection wavelength: 254 nm. As a result, sugars obtained from compounds were confirmed to be D-galactose, D-glucose, L-rhamnose by comparison of the retention times of standard monosaccharides: D-galactose (t_R = 24.375 min), D-glucose (t_R = 23.380 min), L-rhamnose (t_R = 19.653 min).

2.5. Cytotoxic activity

MCF-7 (human breast cancer cells) and HepG2 (human hepatocellular carcinoma cells) were obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Royal, Lanzhou, China), 100U/ml benzyl penicillin, and 100U/ml streptomycin in a humidified environment with 5% CO_2 at 37 °C.

Cell toxicity was measured by a colorimetric assay using MTT as described previously [20]. Each experiment was carried out in triplicate. Control group was cultured with culture media containing 0.1% dimethylsulfoxide. Absorbance (A) was measured at 570 nm. Cell viability (I) was calculated according to the following equation:

$$\text{Cell viability}(I) = (1-A_{\text{experimental group}}/A_{\text{control group}}) \times 100\%$$

Paclitaxel was used as a positive control.

3. Results and discussion

Compound **1** was obtained as a white powder. Its HR-ESI–MS showed quasi-molecular ion peak at m/z 885.4873 ($[M-H]^-$), (calcd for $C_{45}H_{73}O_{17}$ is 885.4853, −2.26 ppm) indicating nine degrees of unsaturation. The ^{1}H NMR spectrum of compound **1** (Table 1) displayed three anomeric proton signals at δ_H 4.95 (1H, d, J = 7.1 Hz, H-1′), 5.77 (1H, d, J = 7.0 Hz, H-1″) and 6.35 (1H, br s, H-1‴), two angular methyl signals at δ_H 0.84 (3H, s, H-18) and 1.01 (3H, s, H-19), and three doublet signals at δ_H 1.17 (3H, d, J = 6.8 Hz, H-21), 1.09 (3H, d, J = 7.1 Hz, H-27) and 1.86 (3H, d, J = 6.2 Hz, Rha-H-6‴). The ^{13}C NMR spectrum of compound **1** (Table 1) showed three anomeric carbon signals at δ_C 102.00 (C-1′), 102.40 (C-1″) and 102.79 (C-1‴), and five methyl carbon signals at δ_C 16.98 (C-18), 24.38 (C-19), 15.30 (C-21), 16.70 (C-27) and 19.51 (Rha-C-6‴). The observations above suggested that compound **1** was a steroidal glycoside with three sugar moieties. Acid hydrolysis and HPLC analysis of compound **1** afforded D-galactose, D-glucose and L-rhamnose and the relative ratio was 1:1:1. The β-configuration of the galactose and glucose were determined according to the coupling constants ($^3J_{1,2}$ > 7 Hz) of the anomeric protons. The α-configuration of the rhamnose was inferred by δ_C 73.19 (C-3‴) and 69.98 (C-5‴) [21]. After acid hydrolysis of **1**, the NMR and MS spectrum of the aglycone were similar to sarsasapogen [22]. The configuration of H-5 was confirmed as β based on the NOESY correlations of H-19 (δ_H 1.01) with H-5 (δ_H 2.45) (Fig. 2). In the HSQC spectrum,

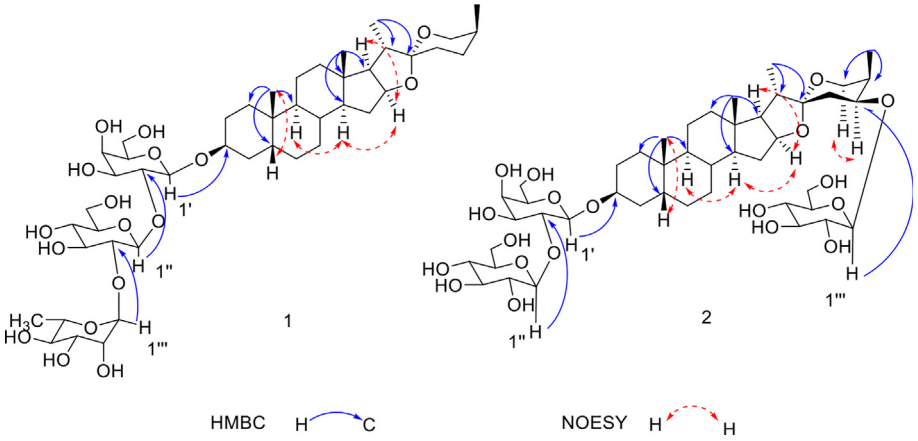

Fig. 2. Key HMBC and NOESY correlations of compounds **1** and **2**.

according to the two proton signals of δ_H 4.08 (m, 1H) and 3.38 (d, 1H, J = 10.8 Hz) of C-26, the 25*S* configuration was deduced [23]. In addition, the 25*S* configuration could also be confirmed by δ_C 16.70 (C-27) [24]. Therefore, the aglycone of compound **1** was determined as (25*S*)-5*β*-spirostane-3*β*-ol.

The HMBC correlations H-1′ (δ_H 4.95)/C-3 (δ_C 75.25); H-1″ (δ_H 5.77)/C-2′(δ_C 77.02) and H-1‴ (δ_H 6.35)/C-2″ (δ_C 79.79) confirmed the sequence of the sugar chain at C-3. So, the attachment sequence of the sugar chain at C-3 was elucidated to be *α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-galactopyranoside. Thus, the structure of compound **1** was determined as (25*S*)-5*β*-spirostane-3*β*-ol-3-*O*- *α*-L-rhamnopyranosyl-(1 → 2)-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-galactopyranoside, named tiomosaponin V.

Compound **2** was obtained as a white powder. Its HR-ESI–MS showed a quasi-molecular ion peak at *m/z* 917.4757 $[M-H]^-$, (calcd for $C_{45}H_{73}O_{19}$ is 917.4752, −0.54 ppm) indicating nine degrees of unsaturation. The ^{1}H NMR spectrum of compound **2** (Table 1) displayed three anomeric proton signals at δ_H 4.93 (1H, d, J = 7.6 Hz, H-1′), 5.06 (1H, d, J = 7.7 Hz, H-1‴) and 5.31 (1H, d, J = 7.7 Hz, H-1″); two angular methyl signals at δ_H 0.77 (3H, s, H-18) and 0.98 (3H, s, H-19); two doublet signals at δ_H 1.12 (3H, d, J = 6.8 Hz, H-21) and 1.34 (3H, d, J = 7.1 Hz, H-27). The ^{13}C NMR spectrum of compound **2** (Table 1) showed three anomeric carbon signals at δ_C 101.58 (C-1‴), 102.99 (C-1′) and 106.59 (C-1″); four methyl carbon signals at δ_C 16.95 (C-18), 24.40 (C-19), 15.13 (C-21) and 10.32 (C-27). The observations above suggested that compound **2** was a steroidal glycoside with three sugar moieties. Acid hydrolysis and HPLC analysis of compound **2** gave D-glucose and D-galactose and the relative ratio was 2:1. The *β*-configuration of the galactose and glucose were determined according to the coupling constants ($^3J_{1,2}$ > 7 Hz) of the anomeric protons. Comparing the NMR data of compound **2** with timosaponin A III [18], it displayed similarity except for variation at the ring F. These indicated that the hydroxyl group attached to C-24. In addition, the conclusion was further confirmed by the HMBC correlations from H-26a (δ_H 3.53) to C-24 (δ_C 73.24) and H-27 (δ_H 1.34) to C-24 (δ_C 73.24). The NOESY correlations of H-24 (δ_H 4.84)/H-26b (δ_H 3.95) suggested that H-24 was *α*-oriented (Fig. 2). Therefore, the hydroxyl group at C-24 was *β*-oriented, and the stereochemistry of C-24 was determined as *S*. Moreover, the data of ring F was consistent with literature [25], which can be proved that absolute configuration of C-25 is *R*-configuration. Therefore, the aglycone of compound **2** was elucidated as (24*S*, 25*R*)-5*β*-spirostane-3*β*, 24*β*-diol.

The connectivity of the sugar chain was deduced based on the HMBC correlations. Firstly, the HMBC cross-peaks between H-1‴ (δ_H 5.06) and C-24 (δ_C 73.24) indicated a glucosyl residue attached to C-24. Moreover, the linkages of the sugar units at C-3 were ascertained from the HMBC correlations H-1′ (δ_H 4.93)/C-3 (δ_C 75.89) and H-1″ (δ_H 5.31)/C-2′ (δ_C 82.32). Thus, the structure of compound **2** was determined as 24-*O*-*β*-D-glucopyranosyl-(24*S*,25*R*)-3*β*, 24*β*-dihydroxy-5*β*-spirostane-3-*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-galactopyranoside, named tiomosaponin W.

It has been reported that the sugars linked to the steroidal saponins in *A. asphodeloides* are mainly glucose, galactose, mannose, and xylose. In this study, the compound **1** was isolated for the first time from *A. asphodeloides*. Moreover, the particulary glycosylation at C-24 on the ring F observed for compound **2** is noteworthy.

The compounds (**1–9**) were evaluated for in vitro cytotoxic activities against MCF-7 and HepG2 cell lines (Table2), and paclitaxel was used as positive control. The results showed that compounds **1** and **6** exhibited significant cytotoxic activities against MCF-7 and HepG2 cell lines, with IC_{50} values ranging from 2.01 ± 0.19 μM to 2.22 ± 0.25 μM. Compound **4** showed a good cytotoxic activity against HepG2 cells with an IC_{50} values of 13.98 ± 0.43 μM. Compounds **3**, **5**, **7**, **8**, and **9** showed moderate cytotoxic activities against HepG2 cells with IC_{50} values below 65 μM. Compound **2** was inactive toward the two cell lines with IC_{50} > 100 μM. Comparing the cytotoxicity of compounds **1**, **2**, and **4**, it indicated that the aglycon structure, the type of monosaccharide and the position of sugar moiety have an effect on the activity. Compounds **3**, **5**, **7**, and **8** showed lower cytotoxicities than compounds **1**, **4**, and **6,** which suggested that the cytotoxicity of spirostanol saponins is stronger than that of saponins without F-ring.

4. Conclusion

In conclusion, two novel steroidal saponins **1** and **2** together with seven known steroidal saponins were isolated from the rhizomes of

Table 2
The cytotoxic activities of compounds **1–9**.[a] (IC_{50}/μM.)

Compounds	Cell lines	
	MCF-7	HepG2
1	2.16 ± 0.19	2.01 ± 0.19
2	> 100	> 100
3	> 100	61.91 ± 2.60
4	> 100	13.98 ± 0.43
5	> 100	56.28 ± 3.91
6	2.05 ± 0.12	2.22 ± 0.25
7	> 100	35.75 ± 3.98
8	> 100	42.22 ± 3.42
9	> 100	45.71 ± 4.22
Paclitaxel[b] (nM)	6.92 ± 0.82	4.71 ± 0.56

[a] IC_{50} valve based on triplicate five points, presented as the mean ± S.D.
[b] Positive control.

Anemarrhena asphodeloides Bunge. The cytotoxic activities of the isolates were evaluated. Compounds **1** and **6** exhibited significant cytotoxic activities against MCF-7 and HepG2 cell lines. The aglycon structure and the variations of the sugar moiety of furostanol saponins could affect their cytotoxicity. Compounds **1** and **6** have similar structures and pharmacological activities. In-depth studies and comparisons of their structure-activity relationships have a good effect on the antitumor mechanism of steroidal saponins.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (grant number 81573560). We are grateful to the members of the analytical group of China Pharmaceutical University for the spectral data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2019.108557.

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