



Cite this: *RSC Adv.*, 2018, 8, 2498

Received 12th November 2017
 Accepted 21st December 2017

DOI: 10.1039/c7ra12363a

rsc.li/rsc-advances

Novel steroidal saponins with cytotoxic activities from the roots of *Ophiopogon japonicus* (L. f.) Ker-Gawl†

Yan Wu,† Su-Xia Bi,† Zhen Huang, Jin Qi* and Bo-Yang Yu *

Six new steroidal saponins (1–6) and one known steroidal saponin (7) were obtained from the roots of *Ophiopogon japonicus* (L. f.) Ker-Gawl. Their structures were determined by the detailed analysis of extensive nuclear magnetic resonance and mass spectroscopic data. The *in vitro* cytotoxic activities of these compounds against MDA-MB-435, HepG2 and A549 cell lines were also investigated.

Introduction

Ophiopogon japonicus (L.f.) Ker-Gawl is an evergreen perennial in the family Liliaceae and mainly distributed in the Southern of China, Japan, Vietnam and India. *O. japonicus* is widely artificially cultivated in Zhejiang and Sichuan Provinces of China.¹ The tuber of *O. japonicus*, commonly known as Maidong, is a famous herb used to treat coughs, sore throats, constipation and insomnia for thousands of years in Traditional Chinese Medicine.²

Pharmacological studies indicated that the tubers of *O. japonicus* exhibit various biological activities such as immunomodulation, anti-diabetes, cardiovascular protection, anti-oxidation and anti-cancer.^{3–7} In recent years, the significant pharmacological effects of the tubers of *O. japonicus* on cardiovascular disease and cancer have drawn much attention from medicinal researchers. Previous phytochemical investigations on the tubers of *O. japonicus* resulted in the isolation of homoisoflavonoids,^{8–13} organic acids,^{14–16} saccharides^{17,18} and steroidal saponins.^{19–23} However, the bioactive ingredients of this herb are still not fully elucidated. As part of our ongoing progress to search for bioactive constituents from traditional Chinese medicine, a 75% EtOH extract of the roots of *O. japonicus* was investigated which led to the isolation of six new steroidal saponins (1–6) and one known steroidal saponin (7) (Fig. 1). The *in vitro* cytotoxic activities of these compounds against MDA-MB-435, HepG2 and A549 cell lines were also investigated. In this paper, we describe the isolation, structural elucidation and cytotoxic activities evaluation of these steroidal saponins.

Results and discussion

The phytochemical investigation of a 75% EtOH extract of the roots of *Ophiopogon japonicus* (L. f.) Ker-Gawl resulted in the isolation of seven steroidal saponins (1–7), including six new steroidal saponins (1–6) and one known steroidal saponin (7). The known compound (7) was identified as (25*R*)-ruscogenin-1-*O*- α -*L*-rhamnopyranosyl-(1 \rightarrow 2)-[β -*D*-xylopyranosyl-(1 \rightarrow 3)]- α -*L*-arabinopyranoside by comparison of its MS, ¹H NMR and ¹³C NMR data with published data in the literature.²⁴

Compound 1 was isolated as an amorphous solid and gave a positive Liebermann–Burchard reaction. Its molecular formula was assigned to be C₄₄H₇₀O₁₇ by the HRESI-QTOF-MS ion peak at *m/z* 893.4447 [M + Na]⁺ (calcd for C₄₄H₇₀O₁₇Na, 893.4505). The ¹H and ¹³C spectra of 1 (Tables 1–4) displayed characteristic signals for four steroidal methyl groups [δ _H 0.68 (3H, d, *J* = 4.5 Hz), 0.96 (3H, s), 1.09 (3H, s) and 1.24 (3H, d, *J* = 7.0 Hz)], an olefinic group [δ _C 141.2, 122.2; δ _H 5.31 (1H, d, *J* = 5.0 Hz)], three anomeric protons [δ _H 4.92 (1H, d, *J* = 7.5 Hz), 5.90 (1H, d, *J* = 3.0 Hz) and 6.24 (1H, br s)] and three anomeric carbon signals at δ _C 100.5, 111.6, 102.4. These spectra data together with the observation that three anomeric protons signals at δ _H 4.92, 5.90 and 6.24 giving correlations with three anomeric carbon signals at δ _C 100.5, 111.6, 102.4 in the HSQC spectrum, implied that 1 was a steroidal derivative containing three sugar units.

The ¹³C-NMR chemical shifts at δ _C 32.8 (C-23), 29.2 (C-24), 32.9 (C-25) and 67.1 (C-26) as well as the characteristic absorptions of a 25(*R*) spiroketal unit at 981, 918, 892, 836 (intensity 918 < 892 cm⁻¹) in the IR spectrum indicated that the C-25 configuration of the aglycone was *R*. The aglycone moiety of 1 was further deduced to be pennogenin by comparison its ¹H and ¹³C NMR data to those reported in the literature.^{25–27} Complete assignments of each sugar unit were achieved by analyses of their chemical shifts and coupling constants obtained from extensive 1D and 2D NMR experiments, allowing the identification one β -glucopyranosyl (Glc), one

Jiangsu Key Laboratory of TCM Evaluation and Translational Research, China Pharmaceutical University, Nanjing 211198, People's Republic of China. E-mail: boyangyu59@163.com; yaoyuelingxing@163.com; Fax: +86-25-86185158; Tel: +86-25-86185157

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ra12363a

‡ Co-first author.



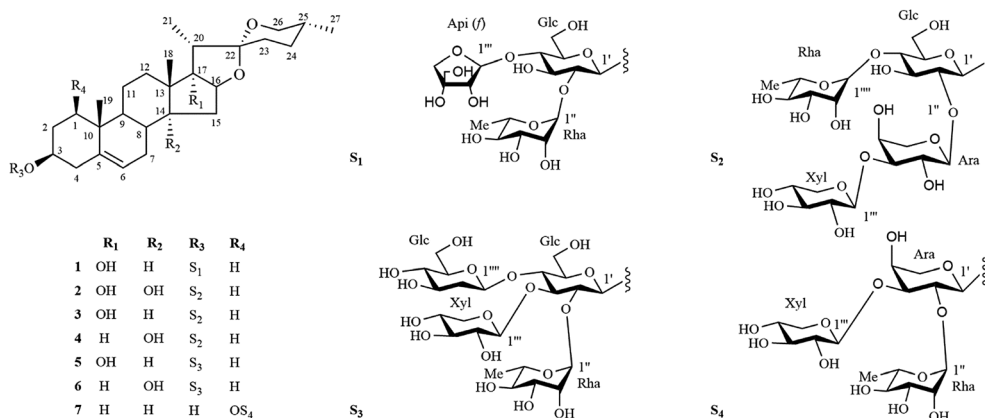


Fig. 1 Chemical structures of compounds 1–7.

Table 1 ¹³C NMR data for aglycone moieties of compounds 1–6^a (δ in ppm, pyridine-d₅)

Position	1	2	3	4	5	6
1	37.6	38.1	38.0	37.9	37.5	38.1
2	30.8	30.4	31.0	30.8	30.8	30.4
3	78.5	78.2	78.3	78.3	78.3	78.2
4	39.4	39.0	39.1	40.3	39.7	39.1
5	141.2	140.6	141.2	140.7	141.2	140.7
6	122.2	122.7	122.8	122.3	122.2	122.8
7	32.2	26.5	32.8	27.1	32.2	27.1
8	30.6	36.6	30.8	36.0	30.4	36.0
9	50.7	43.9	50.7	44.0	50.6	44.0
10	38.0	37.8	37.6	37.6	37.9	37.9
11	21.4	20.5	21.4	20.8	21.3	20.8
12	32.2	26.9	32.8	32.2	32.4	32.4
13	45.2	48.7	45.5	45.5	45.2	45.4
14	53.5	88.1	53.5	86.8	53.4	86.8
15	32.5	40.7	32.9	39.1	32.7	40.3
16	90.5	90.9	90.5	82.3	90.4	82.2
17	90.6	91.5	90.6	60.3	90.5	60.3
18	17.5	21.0	17.5	20.4	17.5	20.4
19	19.8	19.8	19.8	19.7	19.8	19.7
20	45.5	45.5	45.2	42.5	45.5	42.4
21	10.1	10.1	10.1	15.7	10.0	15.7
22	110.2	109.9	110.2	110.0	110.1	109.9
23	32.8	32.5	32.4	31.0	32.8	31.0
24	29.2	29.6	29.2	29.8	29.2	29.7
25	32.9	30.8	30.4	30.4	32.8	30.3
26	67.1	67.2	67.1	67.2	66.2	67.2
27	17.7	17.6	17.7	17.5	17.6	17.7

^a NMR data were measured at 500 MHz for ¹H and at 125 MHz for ¹³C in pyridine-d₅. Assignments are based on HSQC and HMBC experiments.

α-rhamnopyranosyl (Rha) and one β-apiofuranosyl (Api) units in 1. Their absolute configurations were further determined to be D for Glc/Api and L for Rha by GC comparison of their corresponding trimethylsilylthiazolidine derivatives to the authentic samples prepared in the same procedure. In the HMBC spectrum (Fig. 2), the correlations between δ_H 6.24 (br s, Rha-1'') and δ_C 78.0 (Glc-2'), between δ_H 5.90 (d, J = 3.0 Hz, Api-1''') and δ_C 79.8 (Glc-4'), between δ_H 4.92 (d, J = 7.5 Hz, Glc-1') and δ_C 78.5 (Agly C-3), characterized that the sequence of the sugar chain

Table 2 ¹³C NMR data for sugar moieties of compounds 1–6^a (δ in ppm, pyridine-d₅)

Position	1	2	3	4	5	6
1'-Glc-1'	100.5	100.2	100.3	100.3	100.3	100.3
2'	78.0	79.3	79.3	79.3	76.3	76.3
3'	77.8	74.2	74.3	74.3	80.7	80.7
4'	79.8	79.4	79.4	79.4	80.7	80.7
5'	77.0	77.6	77.7	77.7	78.3	78.5
6'	61.7	61.4	61.4	61.4	62.4	62.4
2''-Ara-1''		103.6	103.9	103.9		
2''		73.5	73.6	73.6		
3''		75.8	75.9	75.9		
4''		71.2	71.3	71.3		
5''		66.4	66.5	66.5		
2''-Rha-1''	102.4				102.5	102.5
2''	72.9				72.8	72.8
3''	73.2				72.8	72.8
4''	74.6				73.8	73.9
5''	69.9				70.3	70.3
6''	19.0				19.0	19.0
3'''-Api (f)-1'''	111.6					
2'''	78.1					
3'''	80.5					
4'''	75.4					
5'''	65.2					
3'''-Xyl-1'''		103.8	103.7	103.7	102.9	102.9
2'''		74.2	74.3	74.3	73.8	73.1
3'''		77.6	77.0	77.0	78.3	78.9
4'''		71.1	71.1	71.1	70.9	70.9
5'''		65.8	65.9	65.9	64.8	66.2
4'''-Rha-1'''	102.9	102.9	102.9			
2'''	73.1	72.8	72.8			
3'''	73.1	73.2	73.2			
4'''	74.3	74.4	74.4			
5'''	70.3	70.3	70.3			
6'''	19.0	19.1	19.1			
4''-Glc-1''''					102.9	102.9
2''''					74.3	74.3
3''''					75.6	75.6
4''''					71.1	71.1
5''''					77.2	77.2
6''''					61.8	62.4

^a NMR data were measured at 500 MHz for ¹H and at 125 MHz for ¹³C in pyridine-d₅. Assignments are based on HSQC and HMBC experiments.



Table 3 ^1H NMR data for aglycone moieties of compounds 1–6^a (δ in ppm, pyridine-*d*₅, *J* in Hz)

Position	1	2	3	4	5	6
1	0.97 1.78	1.06 1.78	0.98 1.76	1.01 1.81	0.96 1.79	0.96 1.78
2	1.87 m 2.10	2.03 1.83	1.85 2.00	1.84 2.01	1.83 2.03	1.83 2.02
3	3.96 m	3.84	3.82	3.82	3.85	3.80
4	2.77 m	2.76	2.72	2.73	2.71	2.75
6	5.31 d (5.0 Hz)	5.40 br.s	5.34 d (4.5 Hz)	5.42 br.s	5.35 d (6.0 Hz)	5.42 d (5.0 Hz)
7	1.54 2.26 m	1.87 2.58	1.56 2.29	1.86 2.53	2.23 1.56	2.46 1.86
8	1.61 m	2.06	1.65	2.01	1.62	2.05
9	0.98 m	1.79	0.97	1.82	0.98	1.78
11	1.60	1.12	1.60	1.57	1.62	1.57
12	1.54 1.74	1.35	1.53 1.73	1.46 1.74	1.49 1.72	1.73 1.45
14	2.01 m	—	2.01	—	2.06	—
15	1.64 m	1.87 2.57	1.65	1.90 2.35	1.68	1.88 2.33
16	4.46	4.79	4.44	5.01	4.42	5.06
17	—	—	—	2.78	—	2.75
18	0.96 s	1.11	0.97 s	1.07 s	0.96 s	1.08
19	1.09 s	1.12	1.09 s	1.13 s	1.09 s	1.06
20	2.29 d (7.0 Hz)	2.40 d (7.0 Hz)	2.23 d (7.5 Hz)	2.10 m	2.27 d (7.5 Hz)	2.06 m
21	1.24 d (7.0 Hz)	1.27 d (7.0 Hz)	1.23 d (7.5 Hz)	1.27 d (7.0 Hz)	1.23 d (7.5 Hz)	1.19 d (7.0 Hz)
23	1.95	1.70	1.95	1.28	1.98	1.27
24	1.61	1.59	1.59	1.32	1.60	1.32
25	1.95 2.16	1.85 2.05	1.83 2.01	2.03	1.98 2.16	2.02
26	3.54 m	3.51	3.55	3.52	3.55	3.47
27	0.68 d (4.5 Hz)	0.69 d (4.0 Hz)	0.70 d (4.5 Hz)	0.68 d (5.0 Hz)	0.68 d (6.5 Hz)	0.68 d (6.0 Hz)

^a NMR data were measured at 500 MHz for ^1H and at 125 MHz for ^{13}C in pyridine-*d*₅. Assignments are based on HSQC and HMBC experiments.

was α -L-Rha-(1 \rightarrow 2)-[β -D-Api-(1 \rightarrow 4)]- β -D-Glc, and that the glycosidic site was at C-3. Therefore, the structure of **1** was established as pennogenin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 2)-[β -D-apiofuranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside.

Compound **2** gave a sodium adduct ion at m/z 1041.4809 [$M + \text{Na}$]⁺ (calcd for C₄₉H₇₈O₂₂Na, 1041.4877) in the HRESI-QTOF-MS, corresponding to the molecular formula C₄₉H₇₈O₂₂. Comparison of the NMR data of **2** obtained from 1D and 2D NMR spectra (Tables 1–4) to those of lirigramoside A²⁸ indicated that their chemical structures were closely similar. The major difference was that the chemical shifts at δ_{C} 32.8 (C-23), 29.2 (C-24), 32.9 (C-25) and 67.1 (C-26) in the ^{13}C -NMR spectrum had lower field resonances than those of lirigramoside A, as well as an additional hydroxyl group in the aglycone moiety of **2**. These observations suggested a (25*R*)-spirostanol aglycone moiety containing three hydroxyl groups for **2**. The aglycone moiety of **2** was further identified as ophiogenin by comparison its spectroscopic data to those reported in the literature.²⁹ The HMBC correlations (Fig. 2) between δ_{H} 6.05 (brs, Rha-1''') and δ_{C} 79.4 (Glc-4'), between δ_{H} 5.48 (d, $J = 5.5$ Hz, Xyl-1''') and δ_{C} 75.8 (Ara-3''), between δ_{H} 5.46 (d, $J = 5.5$ Hz, Ara-1'') and δ_{C} 79.3 (Glc-2'), between δ_{H} 4.93 (d, $J = 7.5$ Hz, Glc-1') and δ_{C} 78.2 (Agly C-3) indicated **2** contained the same sugar moiety of α -L-Rha-(1 \rightarrow 4)-[β -D-Xyl-(1 \rightarrow 3)- α -L-Ara(1 \rightarrow 2)]- β -D-Glc at C-3 as lirigramoside A. Compound **2** was therefore assigned as ophiopogenin-3-

O- α -L-rhamnopyranosyl-(1 \rightarrow 4)-[β -D-xylopyranosyl-(1 \rightarrow 3)- α -L-arabinopyranosyl-(1 \rightarrow 2)]- β -D-glucopyranoside.

Compounds **3** and **4** possessed the same molecular formula C₄₉H₇₈O₂₁ as deduced from their HRESI-QTOF-MS data (m/z 1025.4871 [$M + \text{Na}$]⁺, calcd for C₄₉H₇₈O₂₁Na, 1025.4928). Detailed analysis of their ^1H and ^{13}C NMR data (Tables 1–4) suggested that they possessed the same sugar chain at C-3 as **2**, but differed slightly in the aglycone moiety. The only difference was the absent of a hydroxyl group in the aglycone moiety of **3** and **4**, respectively. The differences in chemical shifts of C-7 ($\Delta\delta + 6.3$), C-8 ($\Delta\delta - 5.8$), C-9 ($\Delta\delta + 6.8$), C-12 ($\Delta\delta + 5.9$), C-13 ($\Delta\delta - 3.2$), C-14 ($\Delta\delta - 34.6$) and C-15 ($\Delta\delta - 7.8$) observed from comparative analysis of the ^{13}C NMR data of **2** and **3**, proved the absent hydroxyl group was attached at C-14 in **2**, indicating a pennogenin^{25–27,30} aglycone moiety for **3**. Similarly, the differences in chemical shifts of C-12 ($\Delta\delta + 5.3$), C-13 ($\Delta\delta - 3.2$), C-14 ($\Delta\delta - 1.3$), C-16 ($\Delta\delta - 7.4$), C-17 ($\Delta\delta - 31.2$) and C-21 ($\Delta\delta + 5.6$) in the ^{13}C NMR spectra of **4**, compared with those for **2** implied the absent hydroxyl group was at C-17 in **2**, the aglycone moiety of **4** was therefore identified to be prazerigenin A.³¹ The structures of the aglycone moiety and the sequence of the sugar chains of **3** and **4** were further confirmed by the correlations in their HMBC spectrum (Fig. 2). Thus, the structures of **3** and **4** were determined to be pennogenin-3-*O*- α -L-rhamnopyranosyl-(1 \rightarrow 4)-[β -D-xylopyranosyl-(1 \rightarrow 3)- α -L-arabinopyranosyl-(1 \rightarrow 2)]- β -D-glucopyranoside and praze rigenin A-3-*O*- α -L-



Table 4 ^1H NMR data for sugar moieties of compounds 1–6^a (δ in ppm, pyridine-*d*₅, *J* in Hz)

Position	1	2	3	4	5	6
1'-Glc-1'	4.92 d (7.5 Hz)	4.93 d (7.5 Hz)	4.91 d (7.5 Hz)	4.91 d (7.0 Hz)	4.88 d (7.5 Hz)	4.89 d (8.0 Hz)
2'	4.16	4.15	4.17	4.16	4.14	4.14
3'	4.74	4.30	4.30	4.30	4.68	4.64
4'	3.85	4.51	4.52	4.52	4.68	4.64
5'	3.74	3.73	3.73	3.73	3.73	3.72
6'	4.22	4.31	4.31	4.31	4.30	4.29
	4.29	4.47	4.48	4.48	4.38	4.39
2''-Ara-1''		5.46 d (5.5 Hz)	5.46 d (5.5 Hz)	5.46 d (6.0 Hz)		
2''		4.20	4.20	4.20		
3''		4.13	4.13	4.13		
4''		4.12	4.13	4.13		
5''		3.67	3.67	3.67		
		4.52	4.52	4.52		
2''-Rha-1''	6.24 br.s				6.23 br.s	6.21 br.s
2''	4.77				4.83	4.83
3''	4.60				4.54	4.52
4''	4.31				4.31	4.19
5''	4.91				4.81	4.81
6''	1.75 d (6.5 Hz)				1.73 d (6.5 Hz)	1.74 d (7.0 Hz)
3'''-Api (f)-1'''	5.90 d (3.0 Hz)					
2'''	4.74					
3'''	4.18					
4'''	4.30					
5'''	4.13					
3'''-Xyl-1'''		5.48 d (5.5 Hz)	5.49 d (5.5 Hz)	5.48 d (6.0 Hz)	5.51 d (6.0 Hz)	5.50 d (6.0 Hz)
2'''		4.12	4.13	4.13	4.12	4.10
3'''		4.13	4.15	4.15	4.28	4.27
4'''		4.13	4.13	4.13	4.16	4.12
5'''		3.67	3.67	3.67	3.59	3.65
		4.65	4.68 d (8.0 Hz)	4.68 d (8.5 Hz)	4.67	4.57
4''''-Rha-1''''		6.05 br.s	6.07 br.s	6.07 br.s		
2''''		4.80	4.83	4.83		
3''''		4.52	4.56	4.56		
4''''		4.31	4.32	4.32		
5''''		4.86	4.83	4.83		
6''''		1.74 d (6.5 Hz)	1.74 d (6.5 Hz)	1.74 d (6.0 Hz)		
4''''-Glc-1''''					5.42 d (8.0 Hz)	5.42 d (8.0 Hz)
2''''					4.29	4.32
					4.11	4.02
3''''					4.05	4.14
4''''					4.20	4.19
					4.13	4.09
5''''					3.83	3.80
6''''					4.37	4.80

^a NMR data were measured at 500 MHz for ^1H and at 125 MHz for ^{13}C in pyridine-*d*₅. Assignments are based on HSQC and HMBC experiments.

rhamnopyranosyl-(1 → 4)-[β -D-xylopyranosyl-(1 → 3)- α -L-arabinopyranosyl-(1 → 2)]- β -D-glucoopyranoside, respectively.

The molecular formulas of compounds 5 and 6 were established as C₅₀H₈₀O₂₂ from their HRESI-QTOF-MS ion peak at *m/z* 1055.4968 (calcd for C₅₀H₈₀O₂₂Na, 1055.5033). A comprehensive analysis of the ^1H and ^{13}C NMR spectra (Tables 1–4) of 5 disclosed that these data were in good agreement with those of Cixi-ophiopogon B.²⁷ The only difference was the absent of a hydroxyl group in the aglycone moiety of 5. The aglycone moiety of 5 was further assigned as pennogenin by comparison its ^1H and ^{13}C NMR data to those of 3. In the HMBC spectrum (Fig. 2), the correlations between δ_{H} 6.23 (brs, Rha-1'') and δ_{C} 76.3 (Glc-2'), between δ_{H} 5.51 (d, *J* = 6.0 Hz, Xyl-1''') and δ_{C} 80.7

(Glc-3'), between δ_{H} 5.42 (d, *J* = 8.0 Hz, Glc-1''') and δ_{C} 80.7 (Glc-4'), between δ_{H} 4.88 (d, *J* = 7.5 Hz, Glc-1') and δ_{C} 78.3 (Agly C-3) suggested a α -L-Rha-(1 → 2)-[β -D-Xyl-(1 → 3)]-[β -D-Glc-(1 → 4)]- β -D-Glc sugar moiety at C-3 for 5, which was identical to that of Cixi-ophiopogon B. The structure of 5 was therefore established to be pennogenin-3-*O*- α -L-rhamnopyranosyl-(1 → 2)-[β -D-xylopyranosyl-(1 → 3)]-[β -D-glucoopyranosyl-(1 → 4)]- β -D-glucoopyranoside. The aglycone moiety of compound 6 was deduced to be prazerigenin A by detailed comparison its NMR data (Tables 1–4) to those of 4. Moreover, 6 was established to own the same sugar moiety at C-3 as 5 by the correlations between δ_{H} 6.21 (brs, Rha-1'') and δ_{C} 76.3 (Glc-2'), between δ_{H} 5.50 (d, *J* = 6.0 Hz, Xyl-1''') and δ_{C} 80.7 (Glc-3'), between δ_{H} 5.42 (d, *J* = 8.0 Hz, Glc-1''')



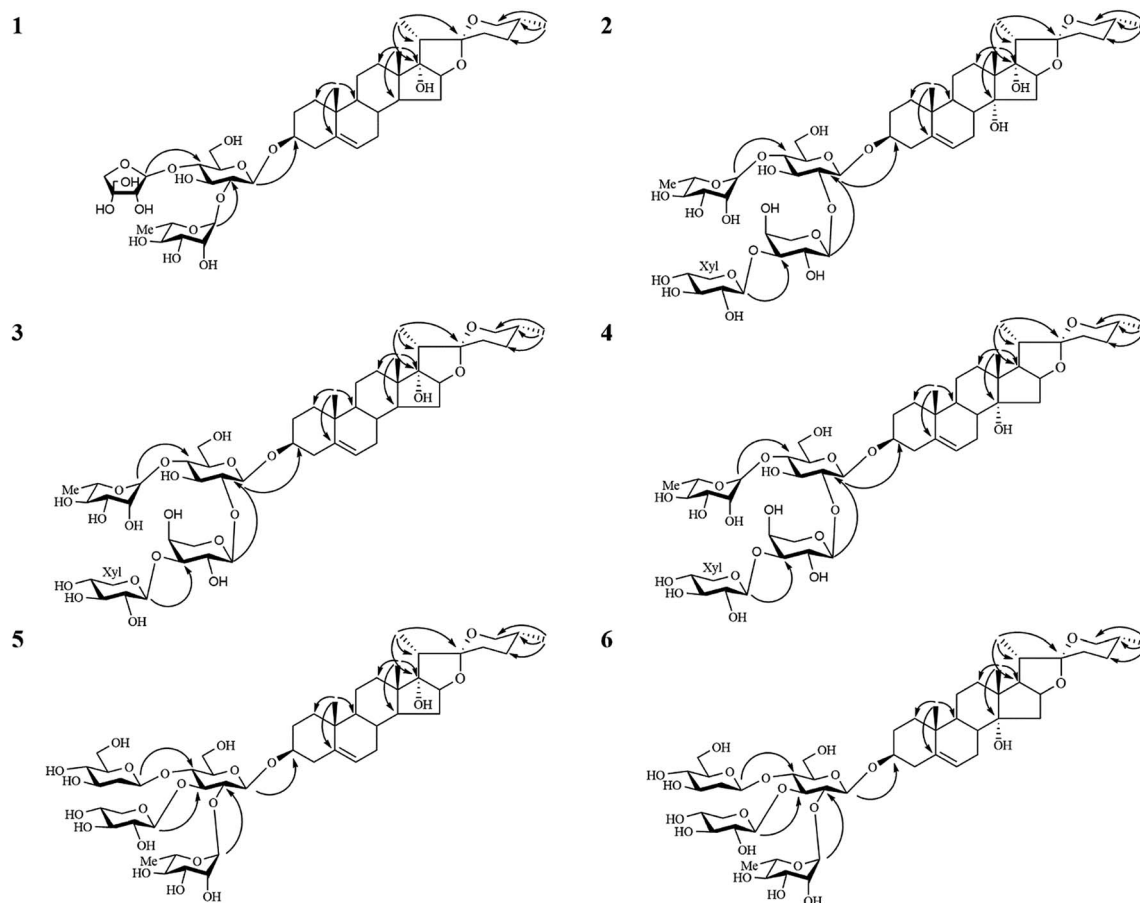


Fig. 2 Key HMBC correlations of compounds 1–6.

Table 5 Cytotoxic effects of compounds 1–7 on human cancer cells (mean \pm SD, $n = 6$)

Compounds	IC ₅₀ (μ M)		
	HepG2	MDA-MB-435	A549
1	1.69 \pm 0.18	1.90 \pm 0.17	4.39 \pm 0.37
2	NA ^a	NA ^a	NA ^a
3	— ^b	— ^b	— ^b
4	— ^b	— ^b	— ^b
5	21.18 \pm 1.87	9.13 \pm 1.43	21.27 \pm 2.53
6	NA ^a	NA ^a	NA ^a
7	NA ^a	10.32 \pm 2.37	29.12 \pm 4.66
5-Fluorouracil	87.3 \pm 12.10	120.5 \pm 15.53	256.8 \pm 19.03

^a No activity (IC₅₀ > 50 μ M). ^b Not measured due to insufficient amount of compounds.

and δ_C 80.7 (Glc-4'), between δ_H 4.89 (d, $J = 8.0$ Hz, Glc-1') and δ_C 78.2 (Agly C-3) in the HMBC spectrum (Fig. 2). The structure of **6** was therefore established to be prazigerenin A-3-O- α -L-rhamnopyranosyl-(1 \rightarrow 2)-[β -D-xylopyranosyl-(1 \rightarrow 3)]-[β -D-glucopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside.

All the isolates were biologically evaluated for their *in vitro* cytotoxic activities against MDA-MB-435, HepG2 and A549 cell lines except for compounds **3** and **4** due to insufficient amount

of compounds. According to the cytotoxicity data summarized in Table 5, compound **1** exhibited the best cytotoxicity against three tested cell lines with IC₅₀ values ranging from 1.69 to 4.39 μ M, compounds **5** and **7** showed moderate cytotoxicity with IC₅₀ values ranging from 9.13 to 29.12 μ M, whereas compounds **2** and **6** were nearly inactive (IC₅₀ > 50 μ M).

Experimental

General

IR data (KBr disks, in cm^{-1}) were recorded on a Bruker Tensor 27 spectrometer (Bruker Corporation, Faellanden, Switzerland). Optical rotations were acquired using a JASCO P-1020 digital polarimeter (JASCO Corporation, Easton, MD, USA). NMR spectra were obtained on a Bruker Avance 500 NMR spectrometer (Bruker Corporation, Faellanden, Switzerland) using pyridine-*d*₅ and tetramethylsilane as solvent and internal standard, respectively. Melting points were measured on a Buchi melting point B-545 apparatus (Buchi Instrument, Switzerland) without correction. ESI-MS and HRESI-Q-TOF-MS spectroscopic data were acquired on an Agilent 1100 Series MSD Trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) and Agilent 6520 ESI-Q-TOF spectrometer (Agilent Technologies, Santa Clara, CA, USA), respectively. D101 macroporous resin (Shaanxi Lanshen Special Resin Co., Xian, China), silica gel



(100–200 mesh and 200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), and C18 reversed-phase silica gel (50 mesh, YMC, Tokyo, Japan) were used for column chromatography (CC). Preparative high-performance liquid chromatography (PHPLC) experiments were performed on an Agilent 1100 Series HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with an evaporative light scattering detector and a diode array detector. Fractions obtained from CC were analyzed by TLC using silica gel GF₂₅₄ (Qingdao Marine Chemical Factory, Qingdao, China) plates. GC analysis was measured with an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). Standard L-rhamnose, D-xylose, L-arabinose, D-glucose, D-apiofuranose, hexamethyldisilazane, L-cysteine methyl ester hydrochloride, and trimethylchlorosilane were purchased from Sigma-Aldrich Trading Co. Ltd. (Shanghai, China).

Plant material

Dried tubers of *O. japonicas* were collected from Cixi city, Zhejiang Province, People's Republic of China, in May 2013 and authenticated by one of the authors (B. Y. Y.). A voucher specimen (no. 20130510) was deposited at the herbarium of Jiangsu Key Laboratory of TCM Evaluation and Translational Research, China Pharmaceutical University, Nanjing, People's Republic of China.

Extraction and isolation

Dried roots of *O. japonicas* (15.5 kg) were powdered and extracted with 75% EtOH (1.5 h, 3 × 60 L), and then the solvent was removed by evaporation to afford a crude residue (825 g). The crude residue was suspended in H₂O and then subjected to D101 macroporous resin CC, eluted successively with EtOH–H₂O (0 : 100, 30 : 70, 70 : 30, 95 : 5, v/v) to afford four sub-fractions (Fr. A–D). After concentration under vacuum, Fr. B and Fr. C were suspended in H₂O and extracted successively with ethyl acetate (10 L × 3) and *n*-butyl alcohol (10 L × 3). The *n*-butyl alcohol extract of Fr. B was subjected to silica gel CC eluted with a gradient of CHCl₃–MeOH–H₂O (90 : 10 : 1 to 10 : 90 : 5, v/v) to afford five sub-fractions (Fr. Ba–e). Fr. Ba was further purified using a Waters Xbridge OBD Prep C-18 column, eluted with CH₃CN–H₂O (38 : 62, v/v) to yield compounds 2 (23 mg), 5 (9 mg), 6 (11 mg) and 7 (35 mg). The *n*-butyl alcohol part of Fr. C was subjected to silica gel CC eluted with a gradient of CHCl₃–MeOH–H₂O (90 : 10 : 1 to 30 : 70 : 5, v/v) to afford Fr. Ca–i. Fr. Ca was chromatographed on an ODS column with a gradient of acetone–H₂O (20 : 80 to 100 : 0, v/v) as the mobile phase to afford four sub-fractions (Fr. Ca1–4). Fr. Ca1 was further purified using a Waters Xbridge OBD Prep C-18 column, eluted with CH₃CN–H₂O (47 : 53, v/v) to yield compounds 3 (12 mg) and 4 (17 mg). Compound 1 (25 mg) was obtained from Fr. Ca3 using a Waters Xbridge OBD Prep C-18 column, eluted with CH₃CN–H₂O (50 : 50, v/v).

Characterization of new compounds

Compound 1. White amorphous powder; Mp 308–309 °C; [α]_D²³ –85.71 (c 0.10, MeOH); IR (KBr) ν_{\max} : 3423, 2932, 1057, 981,

918, 892, 836 (intensity 918 < 892, (25*R*)-spiroketal) cm⁻¹; ¹H-NMR (pyridine-*d*₅, 500 MHz) data and ¹³C-NMR (pyridine-*d*₅, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 893 [M + Na]⁺; HRESI-QTOF-MS *m/z* 893.4447 [M + Na]⁺ (calcd for C₄₄H₇₀O₁₇Na, 893.4505).

Compound 2. White amorphous powder; Mp 276–277 °C; [α]_D²³ –64.51 (c 0.10, MeOH); IR (KBr) ν_{\max} : 3441, 2932, 1048, 922, 897, 837 (intensity 922 < 897, (25*R*)-spiroketal) cm⁻¹; ¹H-NMR (pyridine-*d*₅, 500 MHz) data and ¹³C-NMR (pyridine-*d*₅, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 1041 [M + Na]⁺; HRESI-QTOF-MS *m/z* 1041.4809 [M + Na]⁺ (calcd for C₄₉H₇₈O₂₂Na, 1041.4877).

Compound 3. White amorphous powder; Mp 279–280 °C; [α]_D²³ –200.00 (c 0.10, MeOH); IR (KBr) ν_{\max} : 3427, 2930, 1045, 978, 918, 892, 837 (intensity 918 < 892, (25*R*)-spiroketal) cm⁻¹; ¹H-NMR (pyridine-*d*₅, 500 MHz) data and ¹³C-NMR (pyridine-*d*₅, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 1025 [M + Na]⁺; HRESI-QTOF-MS *m/z* 1025.4871 [M + Na]⁺ (calcd for C₄₉H₇₈O₂₁Na, 1025.4928).

Compound 4. White amorphous powder; Mp 293–294 °C; [α]_D²³ –80.82 (c 0.10, MeOH); IR (KBr) ν_{\max} : 3429, 2925, 1046, 980, 920, 897, 839 (intensity 920 < 897, (25*R*)-spiroketal) cm⁻¹; ¹H-NMR (pyridine-*d*₅, 500 MHz) data and ¹³C-NMR (pyridine-*d*₅, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 1025 [M + Na]⁺; HRESI-QTOF-MS *m/z* 1025.4867 [M + Na]⁺ (calcd for C₄₉H₇₈O₂₁Na, 1025.4928).

Compound 5. White amorphous powder; Mp 269–270 °C; [α]_D²³ –85.71 (c 0.10, MeOH); IR (KBr) ν_{\max} : 3423, 2930, 1051, 978, 917, 892, 836 (intensity 917 < 892, (25*R*)-spiroketal) cm⁻¹; ¹H-NMR (pyridine-*d*₅, 500 MHz) data and ¹³C-NMR (pyridine-*d*₅, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 1055 [M + Na]⁺; HRESI-QTOF-MS *m/z* 1055.4968 [M + Na]⁺ (calcd for C₅₀H₈₀O₂₂Na, 1055.5033).

Compound 6. White amorphous powder; Mp 262–263 °C; [α]_D²³ –166.67 (c 0.10, MeOH); IR (KBr) ν_{\max} : 3427, 2928, 1050, 982, 922, 900, 841 (intensity 922 < 900, (25*R*)-spiroketal) cm⁻¹; ¹H-NMR (pyridine-*d*₅, 500 MHz) data and ¹³C-NMR (pyridine-*d*₅, 300 MHz) data: see Tables 1–4; ESI-MS *m/z* 1055 [M + Na]⁺; HRESI-QTOF-MS *m/z* 1055.4977 [M + H]⁺ (calcd for C₅₀H₈₀O₂₂Na, 1055.5033).

Acid hydrolysis

Compounds 1–6 (2 mg each) were individually refluxed with 2 mL of 2 M HCl (HCl–H₂O–dioxane, 1 : 2 : 3) at 95 °C for 4 h in a water bath and then dioxane was removed by evaporation. Each solution was then diluted with H₂O (2 mL) and further extracted with EtOAc (2 mL), with the aqueous layer repeatedly evaporated until a neutral residue was obtained (10–15 times). The residue was then analyzed by TLC over silica gel together with authentic sugar samples using EtOAc–pyridine–absolute EtOH–H₂O (8 : 1 : 1 : 2) and aniline–diphenylamine–phosphoric acid (5 : 5 : 1) as developing solution and detection solution, respectively. The remaining residue in pyridine (300 μ L) was mixed with L-cysteine methyl ester hydrochloride (4 mg) and then heated at 60 °C in an oil bath for 1.5 h. Then 300 μ L of hexamethyldisilazane–trimethylchlorosilane (2 : 1) was added



and the mixture was kept at 60 °C for a further 30 min. After centrifugation, the supernatant was analyzed by GC under the following conditions: capillary column, HP-5 (0.32 mm × 30 m × 0.5 μm); flame ionization detection; detector temperature, 280 °C; injection temperature, 250 °C; initial temperature, 150 °C and an initial time of 5 min, 1.1 °C min⁻¹ to 260 °C and then held for 10 min; carrier, N₂; split ratio, 1/25. The monosaccharides of compound **1** were identified as D-glucose, L-rhamnose and D-apiofuranose by comparing the retention times (*T_R*) of monosaccharide derivatives with the derivatives prepared in the same procedure from standard sugars. Similarly, D-glucose, D-xylose, L-rhamnose and L-arabinose were identified from compounds **2**, **3** and **4**, D-glucose, D-xylose and L-rhamnose were identified from compounds **5** and **6**.

Cytotoxicity assay

The *in vitro* cytotoxic activities of the isolates against MDA-MB-435, HepG2 and A549 cell lines were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in the literature³² and 5-fluorouracil was used as positive control. All cells lines were purchased from the Cell Bank of the Shanghai Institute of Chinese Academy of Sciences and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂, with high glucose DMEM medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Life Technologies Corporation, Carlsbad, CA, USA) used as culture medium. The cells in logarithmic phase were seeded in 96-well plates for 12 h, then the medium was replaced by fresh medium containing test compounds at various concentrations and treated for an additional 48 h. At the end of the treatment period, the medium was replaced by 100 μL of fresh medium containing 0.5 mg mL⁻¹ MTT and the cells were cultured for a further 3 h. The medium solution was then removed and 150 μL of DMSO was added to dissolve the MTT reduction product (formazan crystals). The optical density was measured by detection of the absorbance at 570 nm and reference wavelength of 650 nm on a microplate reader.

Conclusions

Steroidal constituents exhibit various biological activities due to diversity of their chemical structures. The clear anti-tumorigenic properties of steroidal constituents have attracted the attention of pharmaceutical researchers for the development of anticancer drugs. In this work, six new steroidal saponins (**1–6**) and one known steroidal saponin (**7**) were obtained and characterized from the root of *O. japonicas*. Cytotoxicity data of these compounds against MDA-MB-435, HepG2 and A549 cancer cell lines *in vitro* indicated that compound **1** exhibited significant cytotoxicity with IC₅₀ values ranging from 1.69 to 4.39 μM, compounds **5** and **7** showed moderate cytotoxicity with IC₅₀ values ranging from 9.13 to 29.12 μM. These compounds may have a possibility for use in anticancer drug development.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was funded by the National Natural Science Foundation of China (No. 81473317 and 81673555), the Major National Science and Technology Project of China for Significant New Drugs Development (No. 2012ZX09102201-015), Qing Lan Project, the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 2011' Program for Excellent Scientific and Technological Innovation Team of Jiangsu Higher Education, and the Major Project Program of State Key Laboratory of Natural Medicines, China Pharmaceutical University (No. SKLNMZZ201203).

Notes and references

- 1 Editorial Committee of Flora of China, *Flora of China*, Science Press, Beijing, 2014.
- 2 National Pharmacopoeia Committee, *Pharmacopoeia of Peoples Republic of China 2015, Part 1*, China Medical Science Press, Beijing, 2015.
- 3 X. Lu, W. Tong, S. Wang, J. Li, J. Zheng and X. Fan, *J. Pharm. Biomed. Anal.*, 2016, **134**, 60–70.
- 4 Y. Wang, Y. Zhu, K. Ruan, H. Wei and Y. Feng, *Carbohydr. Polym.*, 2014, **114**, 183–189.
- 5 M. Zhao, W. F. Xu, H. Y. Shen, P. Q. Shen, J. Zhang and D. D. Wang, *J. Pharm. Biomed. Anal.*, 2017, **138**, 134.
- 6 Y. L. Zhang, M. Z. Xi, Y. B. Choi and B. H. Lee, *J. Med. Food*, 2017, **20**, 637–645.
- 7 J. Chen, J. Yuan, L. Zhou, M. Zhu, Z. Shi, J. Song, Q. Xu, G. Yin, Y. Lv, Y. Luo, X. Jia and L. Feng, *Biomed. Pharmacother.*, 2017, **87**, 118–126.
- 8 A. Tada, R. Kasai, T. Saitoh and J. Shoji, *Chem. Pharm. Bull.*, 1980, **28**, 2039–2044.
- 9 A. Tada, R. Kasai, T. Saitoh and J. Shoji, *Chem. Pharm. Bull.*, 1980, **28**, 1477–1484.
- 10 J. M. Chang, C. C. Shen, Y. L. Huang, M. Y. Chien, J. C. Ou, B. J. Shieh and C. C. Chen, *J. Nat. Prod.*, 2002, **65**, 1731–1733.
- 11 N. Li, J. Y. Zhang, K. W. Zeng, L. Zhang, Y. Y. Che and P. F. Tu, *Fitoterapia*, 2012, **83**, 1042–1045.
- 12 C. L. Duan, Z. Y. Kang, C. R. Lin, Y. Jiang, J. X. Liu and P. F. Tu, *J. Asian Nat. Prod. Res.*, 2009, **11**, 876–879.
- 13 C. Zhou, L. Zou, J. Mo, X. Wang, B. Yang and Q. He, *Helv. Chim. Acta*, 2013, **96**, 1397–1405.
- 14 Z. Iqbal, S. Isojima, A. Noda and Y. Fujii, *J. Weed Sci. Technol.*, 2003, **47**, 160–161.
- 15 C. Jiang, Z. H. Liu, L. Li, B. B. Lin, F. Yang and M. J. Qin, *J. Asian Nat. Prod. Res.*, 2012, **14**, 491–495.
- 16 C. L. Duan, Y. Jiang, Y. Jiang, C. R. Lin, J. X. Liu and P. F. Tu, *J. Chin. Pharm. Sci.*, 2009, **18**, 236–239.
- 17 M. Tomoda and S. Kato, *Jpn. J. Pharmacogn.*, 1966, **20**, 6–9.
- 18 M. Tomoda and S. Kato, *Chem. Pharm. Bull.*, 1968, **16**, 113–116.



- 19 N. Li, L. Zhang, K. W. Zeng, Y. Zhou, J. Y. Zhang, Y. Y. Che and P. F. Tu, *Steroids*, 2013, **78**, 1–7.
- 20 C. L. Duan, Y. J. Li, P. Li, Y. Jiang, J. X. Liu and P. F. Tu, *Helv. Chim. Acta*, 2010, **93**, 227–232.
- 21 J. Qi, Z. F. Hu, Y. F. Zhou, Y. J. Hu and B. Y. Yu, *Chem. Pharm. Bull.*, 2015, **63**, 187–194.
- 22 Z. H. Cheng, T. Wu and B. Y. Yu, *J. Asian Nat. Prod. Res.*, 2006, **8**, 555–559.
- 23 Y. F. Zhou, J. Qi, D. N. Zhu and B. Y. Yu, *Chin. Chem. Lett.*, 2008, **19**, 1086–1088.
- 24 M. Kuroda, Y. Mimaki, K. Ori, H. Sakagami and Y. Sashida, *J. Nat. Prod.*, 2004, **67**, 1690–1696.
- 25 L. Tang, Z. Wang, H. Wu, A. Yokosuka and Y. Mimaki, *Phytochemistry*, 2014, **107**, 102–110.
- 26 J. Z. Wang, L. M. Ye and X. B. Chen, *Chin. Chem. Lett.*, 2008, **19**, 82–84.
- 27 M. A. Fernandez-Herrera, M. G. Hernandez-Linares, G. Guerrero-Luna, S. Meza-Reyes, S. Montiel-Smith and J. Sandoval-Ramirez, *Lett. Org. Chem.*, 2011, **8**, 341–346.
- 28 K. W. Wang, H. Zhang, L. Q. Shen and W. Wang, *Carbohydr. Res.*, 2011, **346**, 253–258.
- 29 J. J. Chen, Z. L. Zhu and S. D. Luo, *Acta Bot. Yunnanica*, 2000, **22**, 97–102.
- 30 X. Zhou, X. He, G. Wang, H. Gao, G. Zhou, W. Ye and X. Yao, *J. Nat. Prod.*, 2006, **69**, 1158–1163.
- 31 M. Sugiyama, K. Nakano, T. Tomimatsu and T. Nohara, *Chem. Pharm. Bull.*, 2008, **32**, 1365–1372.
- 32 Y. Wu, X. M. Wang, S. X. Bi, W. Zhang, R. M. Li, R. J. Wang, B. Y. Yu and J. Qi, *RSC Adv.*, 2017, **7**, 13696–13706.

