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Isolation and structural elucidation of novel cholestane glycosides and spirostane saponins from *Polygonatum odoratum*

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ABSTRACT

Much attention has been paid to cholestane-type steroidal glycosides because of their importance from the perspectives of both chemical diversity and significant biological activities. A phytochemical investigation of the rhizomes of *Polygonatum odoratum* (Liliaceae) resulted in the isolation of three novel cholestane-type steroidal glycosides (**1**–**3**) with unique $\Delta^{14,16}$-unsaturated D-ring structures as well as two novel spirostane-type steroidal saponins (**4** and **5**) and three known steroidal glycosides (**6**–**8**). Their structures were determined by various spectroscopic methods and chemical reactions. Steroidal saponin **7** showed significant antifungal activity against *Candida albicans* JCM1542 (MIC 3.1 μg/mL) and *Aspergillus fumigatus* JCM1738 (MIC 6.3 μg/mL).

1. Introduction

Steroidal glycosides are an important class of natural products, including cardiac glycosides, steroidal saponins, and pregnane glycosides, that are isolated from plants and have been reported to show various biological activities. Recently, much attention has been paid to steroidal glycosides with aglycones that have cholestane skeletons, which are not included in the aforementioned types, because of their importance from the perspectives of both chemical diversity and significant biological activities. However, the number of known compounds of this type is still quite limited [1].

Polygonatum odoratum (Mill.) Druce is a perennial herbaceous plant in the Liliaceae family and is widespread in China and Eurasia. Its rhizomes have been used in traditional Chinese medicines for the treatment of diabetes, hypoimmunity, and rheumatic heart disease [2]. Previous phytochemical studies have reported that this natural medicine contains spirostane- and furostane-type steroidal saponins [3].

During our ongoing studies concerning the discovery of novel steroidal glycosides from traditional Chinese medicines [4], we performed a further phytochemical investigation of the rhizomes of *P. odoratum*. Herein, we report the isolation and structural determination of three novel cholestane glycosides (**1**–**3**), two novel spirostane saponins (**4** and **5**), and three known compounds, (25*S*)-(3β,14α)-dihydroxy-spirost-5-ene-3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galacopyranoside (**6**) [5], 3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galacopyranosyl-yamogenin (**7**) [6], and (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol-1-*O*-α-L-rhamnopyranosyl-16-*O*-β-D-glucopyranoside (**8**) [7] (Fig. 1).

2. Experimental

2.1. General

Melting points were determined in a SGW X-4 micro-melting point (INESA Instrument, Shanghai, China) apparatus and were uncorrected. The IR spectra were measured using a Nexus 670 FT-IR spectrometer (Thermo Nicolet, Madison, WI, USA) with the KBr disk method. The specific rotations were measured on a P-2200 polarimeter (JASCO, Tokyo, Japan) in a 0.5-dm cell. The ^{1}H and ^{13}C NMR spectra were measured on an ECP-500 spectrometer (JEOL, Tokyo, Japan) using TMS as the internal reference, and the chemical shifts are expressed in δ (ppm). The ESIMS was taken on a Trap VL mass analyzer (Agilent Technologies, Palo Alto, CA, USA), and the HRESIMS was taken on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Preparative HPLC was performed on an Agilent 1100 apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1314A

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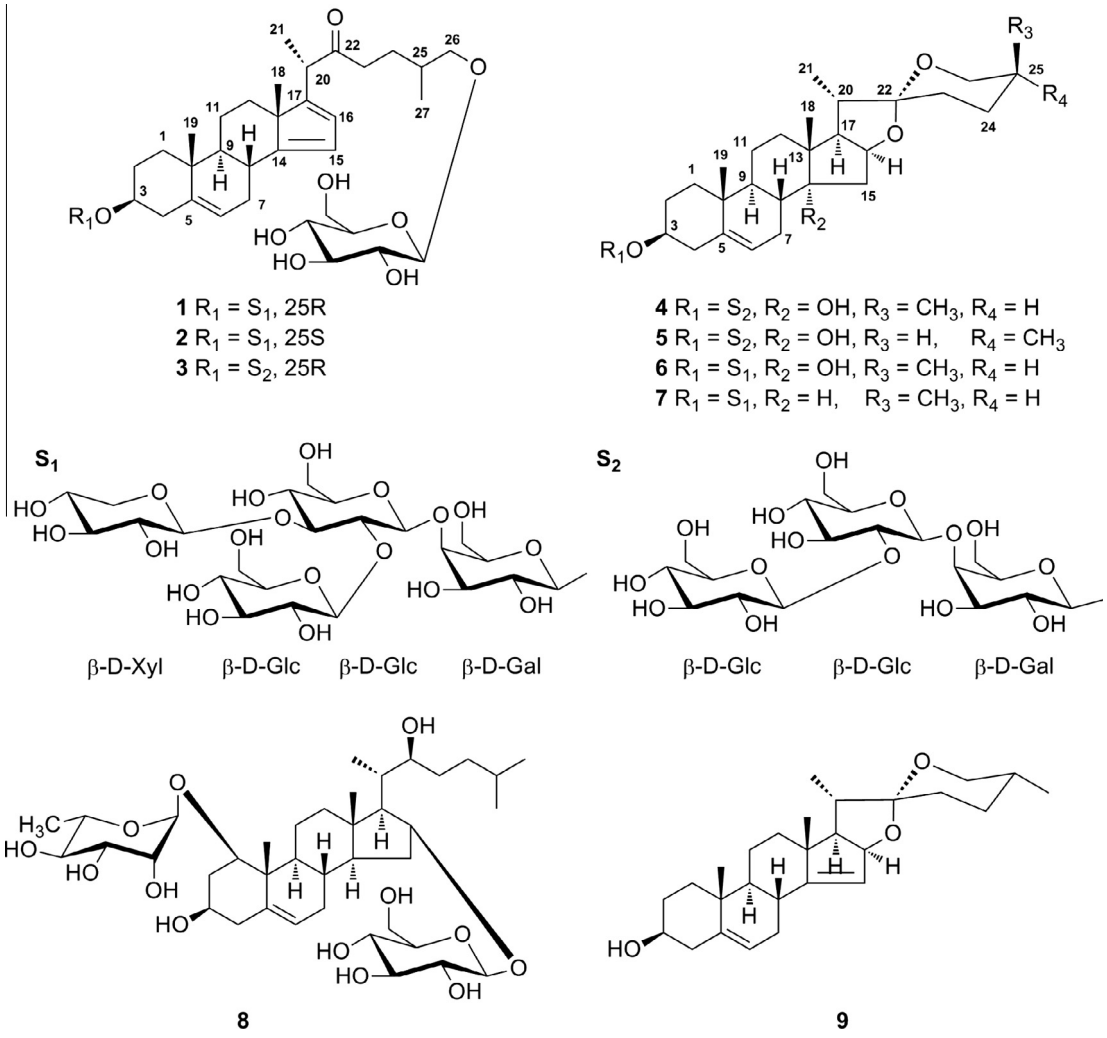

Fig. 1. The structures of steroidal glycosides isolated from *P. odoratum*.

Variable Wavelength Detector and a YMC-Pack RP-C_{18} column (150 × 20 mm, i.d., YMC, Kyoto, Japan). Diaion HP-20 (20–60 mesh, Mitsubishi Chemical, Tokyo, Japan), silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and ODS (50 μm, YMC, Kyoto, Japan) were used for column chromatography.

2.2. Plant material

The rhizomes of *P. odoratum* used in this study were collected from Hunan province, PR China in Aug. 2008 and identified by Dr. Wei Yingqin (Shandong Polytechnic University). A plant specimen (PO200808) is deposited in the herbarium of Institute of Materia Medica, Shandong Academy of Medical Sciences.

2.3. Extraction and isolation

The air-dried rhizomes of *P. odoratum* (10 kg) were extracted with 70% aqueous EtOH at the boiling point for 2 h for three times. The extract (780 g) was concentrated under reduced pressure and then partitioned between *n*-BuOH and H_2O. The *n*-BuOH extract (688 g) was subjected to Diaion HP-20 column chromatography and eluted with gradients of H_2O and 20%, 70% and 95% EtOH, successively. The 70% EtOH eluate was concentrated to yield a fraction (57 g) rich in steroidal glycosides. This fraction was chromatographed on a silica-gel column with a gradient of $CHCl_3$–MeOH–H_2O to yield ten subfractions (A1–A10). Three subfractions, A6 (2.6 g), A7 (13.2 g) and A8 (5.8 g), were further separated by repeated silica gel, Sephadex LH-20 and ODS column chromatography before being finally purified by preparative HPLC to afford compound **8** (12 mg) from subfraction A6, compounds **4** (26 mg), **5** (3 mg), **6** (6 mg) and **7** (45 mg) from subfraction A7, and compounds **1** (23 mg), **2** (23 mg) and **3** (15 mg) from subfraction A8.

2.3.1. Polygonatumoside A (**1**)

Amorphous powder, mp 226–227 °C; $[\alpha]_D^{20}$ +46.0 (*c* 1.26, MeOH); IR (KBr) ν_{max}: 3386, 2932, 1701, 1632, 1384, 1160, 1074, 1036, 895 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz), see Tables 1 and 2; positive-ion ESIMS *m*/*z* 1215 $[M+Na]^+$; positive-ion HRESIMS *m*/*z* 1215.5436 (calcd for $C_{56}H_{88}O_{27}Na$, 1215.5405).

2.3.2. Polygonatumoside B (**2**)

Amorphous powder, mp 219–220 °C; $[\alpha]_D^{20}$ −28.6 (*c* 1.33, MeOH); IR (KBr) ν_{max}: 3417, 2933, 1701, 1635, 1364, 1159, 1074, 1038, 894 cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz), see Tables 1 and 2; positive-ion ESIMS *m*/*z* 1215 $[M+Na]^+$; positive-ion HRESIMS *m*/*z* 1215.5418 (calcd for $C_{56}H_{88}O_{27}Na$, 1215.5405).

Table 1
^{13}C NMR data of **1–5** (125 MHz in pyridine-d_5).

Aglycone	1	2	3	4	5
1	37.8	37.8	37.8	37.7	37.7
2	30.1	30.1	30.2	30.3	30.3
3	78.3	78.3	78.2	78.2	78.1
4	39.2	39.2	39.3	39.3	39.3
5	140.5	140.6	140.5	140.5	140.6
6	121.5	121.4	121.5	122.3	123.1
7	29.4	29.4	29.4	26.7	26.8
8	32.1	32.1	32.1	35.6	35.5
9	53.7	53.6	53.8	43.6	43.6
10	37.6	37.6	37.6	37.4	37.4
11	21.2	21.3	21.3	20.4	20.4
12	35.4	35.7	35.4	32.0	32.1
13	54.1	54.2	54.1	45.1	45.6
14	159.8	159.9	159.8	86.4	85.8
15	119.4	119.4	119.4	40.0	40.8
16	125.3	125.7	125.4	82.0	81.3
17	156.5	156.3	156.6	59.8	59.5
18	18.2	18.0	18.3	20.1	20.4
19	19.4	19.4	19.4	19.3	19.3
20	45.1	45.7	45.2	42.6	42.2
21	18.4	18.3	18.5	15.2	16.8
22	210.4	210.5	210.4	110.1	110.9
23	38.1	38.3	38.1	26.6	28.0
24	28.4	28.5	28.4	26.3	28.0
25	33.4	33.6	33.5	27.6	30.8
26	75.1	75.0	75.1	65.1	69.9
27	17.0	17.3	17.1	16.3	17.3
Sugar	β-D-gal	β-D-gal	β-D-gal	β-D-gal	β-D-gal
1	102.8	102.8	102.8	102.7	102.6
2	73.1	73.2	73.3	73.3	73.3
3	75.5	75.6	75.6	75.6	75.6
4	79.8	79.8	81.0	81.0	81.0
5	75.1	75.1	75.1	75.1	75.1
6	60.5	60.6	60.5	60.5	60.5
	β-D-glc′	β-D-glc′	β-D-glc′	β-D-glc′	β-D-glc′
1	105.1	105.1	105.2	105.2	105.2
2	81.3	81.3	86.2	86.1	86.1
3	86.8	86.8	78.5	78.5	78.5
4	70.5	70.5	71.9	71.9	71.9
5	77.6	77.6	78.2	78.2	78.2
6	63.0	63.0	63.2	63.2	63.2
	β-D-glc″	β-D-glc″	β-D-glc″	β-D-glc″	β-D-glc″
1	104.8	104.8	107.0	107.0	106.9
2	76.2	76.2	76.7	76.8	76.8
3	77.7	77.7	77.7	77.7	77.7
4	71.0	71.1	70.3	70.4	70.4
5	78.7	78.7	79.0	79.0	79.0
6	62.5	62.5	61.6	61.6	61.6
	β-D-xyl	β-D-xyl			
1	104.9	104.9			
2	75.3	75.3			
3	78.6	78.6			
4	70.7	70.7			
5	67.3	67.3			
	β-D-glc‴	β-D-glc‴	β-D-glc‴		
1	105.0	105.0	105.0		
2	75.2	75.2	75.2		
3	78.6	78.6	78.6		
4	71.7	71.7	71.8		
5	78.5	78.5	78.5		
6	62.9	62.9	62.9		

2.3.3. Polygonatumoside C (**3**)

Amorphous powder, mp 222–223 °C; $[\alpha]_D^{20}$ +32.2 (*c* 0.47, MeOH); IR (KBr) ν_{max}: 3405, 2933, 1702, 1639, 1384, 1161, 1073, 896 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz), see Tables 1 and 2; positive-ion ESIMS *m*/*z* 1083 [M+Na]$^+$; positive-ion HRESIMS *m*/*z* 1083.5003 (calcd for $C_{51}H_{80}O_{23}Na$, 1083.4983).

2.3.4. Polygonatumoside D (**4**)

Amorphous powder, mp 241–242 °C; $[\alpha]_D^{20}$ −57.8 (*c* 1.61, MeOH); IR (KBr) ν_{max}: 3386, 2934, 1631, 1371, 1157, 1070, 921, 894 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz), see Tables 1 and 2; positive-ion ESIMS *m*/*z* 939 [M+Na]$^+$; positive-ion HRESIMS *m*/*z* 939.4575 (calcd for $C_{45}H_{72}O_{19}Na$, 939.4560).

2.3.5. Polygonatumoside E (**5**)

Amorphous powder, mp 224–225 °C; $[\alpha]_D^{20}$ −38.1 (*c* 0.29, MeOH); IR (KBr) ν_{max}: 3417, 2933, 1631, 1384, 1158, 1071, 920, 894 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz), see Tables 1 and 2; positive-ion ESIMS *m*/*z* 939 [M+Na]$^+$; positive-ion HRESIMS *m*/*z* 939.4578 (calcd for $C_{45}H_{72}O_{19}Na$, 939.4560).

2.3.6. Compound **6**

Amorphous powder, mp 258–259 °C (lit. [5]: mp 258–260 °C); $[\alpha]_D^{24}$ −45.7 (*c* 0.50, MeOH) [lit. [5]: $[\alpha]_D^{20}$ −63.0 (*c* 0.15, MeOH)]. ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data agree well with the values reported for (25*S*)-(3β,14α)-dihydroxy-spirost-5-ene-3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galacopyranoside.

2.3.7. Compound **7**

Amorphous powder, mp 277–278 °C (lit. [5]: mp 278–279 °C; lit [6]: mp 280–283 °C); $[\alpha]_D^{29}$ −74.6 (*c* 0.50, C_5H_5N) (lit. [6]: $[\alpha]_D^{20}$ −63.0 (*c* 1, C_5H_5N)). ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data agree well with the values reported for 3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galacopyranosyl-yamogenin.

2.3.8. Compound **8**

Amorphous powder, mp 245–246 °C; $[\alpha]_D^{27}$ −24.1 (*c* 0.50, MeOH) (lit. [7]: $[\alpha]_D^{27}$ −36.0 (*c* 0.29, MeOH)). ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data agree well with the values reported for (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol-1-*O*-α-L-rhamnopyranosyl-16-*O*-β-D-glucopyranoside.

2.4. Acid hydrolysis of compounds **1–5**

Compounds **1** (8 mg), **2** (8 mg), **3** (1 mg), **4** (1 mg) and **5** (1 mg) were separately dissolved in 1 M HCl (dioxane–H_2O, 1:1, 25 mL) and then heated at 100 °C for 2 h. After the dioxane was removed, the solutions were extracted with EtOAc (10 mL × 3).

The aqueous layers were neutralized with Ag_2CO_3 and filtered, and the filtrates were concentrated under reduced pressure to yield the sugar fractions. The sugar fractions were dissolved in H_2O (4 mL), and *S*-(−)-α-methylbenzylamine (14 μL) and $NaBH_3CN$ (12 mg) in EtOH (2 mL) was added. The solution was stirred at 40 °C for 4 h, after which glacial acetic acid (0.4 mL) was added before evaporating to dryness. The resulting solids were acetylated with acetic anhydride (0.6 mL) in pyridine (0.6 mL) for 24 h at room temperature. After evaporation, H_2O (2 mL) was added to the residue, and the solutions were filtered through a Cleanert C_{18}-SPE cartridge (Agela, Tianjin, China) and washed with H_2O and 20% and 50% CH_3CN (each 15 mL), successively. The 50% CH_3CN eluate was analyzed, and the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxy-alditol acetate derivatives of the monosaccharides were identified by HPLC analysis with the derivatives of standard sugars prepared under the same conditions. The HPLC conditions were as follows: column, Agilent SB-C_{18} (4.6 × 250 mm, Agilent Technologies, Palo Alto, CA, USA); solvent, 40% CH_3CN; flow rate, 0.8 mL/min; and detection, DAD 230 nm. The identification of the monosaccharides present in the sugar fraction was carried out by a comparison of the retention times t_R (min) of their derivatives with those of authentic samples: 21.80 (derivative of D-glucose),

Table 2
^{1}H NMR data of **1**–**5** (500 MHz in pyridine-d_5).

Aglycone	**1**	**2**	**3**	**4**	**5**
1a	1.71 (m)	1.70[a]	1.71 (m)	1.77[a]	1.74 (m)
1b	0.95[a]	0.96[a]	0.97 (m)	1.03 (m)	1.01 (m)
2a	2.10 (m)	2.10[a]	2.10 (m)	2.11 (m)	2.10 (m)
2b	1.75 (m)	1.70[a]	1.75 (m)	1.74[a]	1.72[a]
3	3.90[a]	3.86[a]	3.90[a]	3.88 (m)	3.87 (m)
4a	2.68 (m)	2.67[a]	2.68 (m)	2.70 (dd, 13.7,4.8)	2.69 (m)
4b	2.44 (t, 13.0)	2.44 (t, 11.2)	2.47 (t,11.4)	2.46[a]	2.46[a]
6	5.40 (br s)	5.39 (br s)	5.41 (br s)	5.38 (br d, 4.8)	5.38 (d, 4.8)
7a	2.17 (m)	2.15 (m)	2.17 (m)	2.50[a]	2.46[a]
7b				1.86[a]	1.83[a]
8	2.32 (m)	2.31 (m)	2.32 (m)	2.04[a]	2.00 (td, 11.2, 5.3)
9	0.86 (m)	0.88 (m)	0.86 (m)	1.80 (td, 11.3, 3.8)	1.81 (m)
11	1.49 (m)	1.52 (m)	1.49 (m)	1.54[a]	1.53[a]
12a	1.92 (m)	2.07[a]	1.92 (m)	2.25 (m)	2.26 (m)
12b	0.77 (m)	0.91 (m)	0.77 (m)	1.45 (m)	1.46 (m)
15a	5.89 (br s)	5.91 (d, 1.8)	5.89 (br s)	2.31 (dd, 12.7, 7.8)	2.34 (dd, 12.5, 7.2)
15b				1.88 (dd, 12.7, 6.0)	1.94 (dd, 12.5, 6.2)
16	6.17 (d,1.8)	6.23 (d, 1.8)	6.17 (d, 1.6)	5.07 (ddd, 7.8, 6.8, 6.0)	4.88[a]
17				2.77 (dd, 7.8, 6.8)	2.54 (dd, 8.0, 6.4)
18	1.10 (s)	1.03 (s)	1.10 (s)	1.07 (s)	1.21 (s)
19	0.96 (s)	0.96 (s)	0.96 (s)	0.98 (s)	0.96 (s)
20	3.52 (q, 6.9)	3.52 (q, 6.9)	3.52 (q, 6.9)	2.05[a]	2.46[a]
21	1.34 (d, 6.9)	1.36 (d, 6.9)	1.34 (d, 6.9)	1.20 (d, 7.1)	1.07 (d, 7.1)
23a	2.62 (m)	2.67[a]	2.62 (m)	1.95 (td, 13.5, 4.8)	1.70 (m)
23b	2.56 (m)	2.54 (m)	2.56 (m)	1.50 (m)	
24a	1.96 (m)	1.97 (m)	1.96 (m)	2.17 (tt, 13.5, 4.8)	1.53[a]
24b	1.56 (m)	1.55 (m)	1.56 (m)	1.38 (m)	
25	1.90 (m)	1.88 (m)	1.90 (m)	1.55[a]	1.63 (m)
26a	3.95[a]	3.97[a]	3.95[a]	4.05 (dd, 10.7, 1.8)	3.75 (t, 10.7)
26b	3.47 (dd, 9.4, 6.1)	3.48 (dd, 9.4, 6.2)	3.47 (dd, 9.4, 6.0)	3.44 (d, 10.7)	3.74 (d, 10.7)
27	0.92 (d, 6.8)	0.94 (d, 6.9)	0.92 (d, 6.6)	1.08 (d, 5.9)	0.69 (d, 6.4)
Sugar	β-D-gal	β-D-gal	β-D-gal	β-D-gal	β-D-gal
1	4.89 (d, 7.6)	4.87 (d, 7.5)	4.91 (d, 7.8)	4.88 (d, 7.8)	4.88 (d, 7.6)
2	4.39[a]	4.39[a]	4.50 (dd, 9.4, 7.8)	4.49 (dd, 8.6, 7.8)	4.49 (dd, 9.5, 7.6)
3	4.11[a]	4.11[a]	4.10[a]	4.10[a]	4.10[a]
4	4.60 (br d, 3.0)	4.59 (br d, 3.0)	4.57[a]	4.57 (br d, 2.3)	4.57 (br d, 2.7)
5	3.95[a]	3.95[a]	3.97[a]	3.97[a]	3.97[a]
6a	4.67 (m)	4.65 (m)	4.76 (m)	4.74 (m)	4.74 (m)
6b	4.17[a]	4.17[a]	4.18[a]	4.18 (m)	4.18 (m)
	β-D-glc′	β-D-glc′	β-D-glc′	β-D-glc′	β-D-glc′
1	5.19 (d, 7.8)	5.18 (d, 8.1)	5.14 (d, 7.8)	5.14 (d,7.8)	5.14 (d,7.8)
2	4.39[a]	4.39[a]	4.15 (dd, 8.7, 7.8)	4.15 (dd, 8.4, 7.8)	4.15 (dd, 8.7, 7.8)
3	4.12[a]	4.12[a]	4.28 (dd, 8.7, 8.4)	4.27 (dd, 8.4, 8.2)	4.27 (dd, 8.7, 8.2)
4	3.82 (t, 9.1)	3.81 (t, 9.0)	3.97[a]	3.97[a]	3.97[a]
5	3.85[a]	3.85[a]	3.97[a]	3.97[a]	3.97[a]
6a	4.50[a]	4.50[a]	4.63 (br d, 10.8)	4.62 (br d, 10.3)	4.62 (br d, 10.5)
6b	4.02[a]	4.02[a]	4.09[a]	4.09[a]	4.09[a]
	β-D-glc″	β-D-glc″	β-D-glc″	β-D-glc″	β-D-glc″
1	5.57 (d, 7.5)	5.57 (d, 7.6)	5.23 (d, 7.8)	5.23 (d, 7.5)	5.23 (d,7.6)
2	4.06[a]	4.06[a]	4.06[a]	4.06 (dd, 8.5, 7.5)	4.06 (dd, 9.4, 7.6)
3	4.08[a]	4.08[a]	4.12 (t, 8.7)	4.12 (dd, 8.9, 8.5)	4.12 (dd, 9.4, 9.1)
4	4.20[a]	4.20[a]	4.22[a]	4.22 (dd, 8.9, 8.1)	4.22 (dd, 9.1, 8.7)
5	3.87[a]	3.87[a]	3.80 (m)	3.80 (m)	3.80 (m)
6a	4.38[a]	4.38[a]	4.37[a]	4.58 (br d, 11.4)	4.58 (br d, 11.7)
6b	4.55[a]	4.55[a]	4.58[a]	4.37 (br d, 11.4)	4.37 (br d, 11.7)
	β-D-xyl	β-D-xyl			
1	5.24 (d, 7.8)	5.23 (d, 7.8)			
2	3.95[a]	3.95[a]			
3	4.06[a]	4.06[a]			
4	4.09[a]	4.09[a]			
5a	4.22[a]	4.22[a]			
5b	3.67 (t, 10.5)	3.67 (t, 10.3)			
	β-D-glc‴	β-D-glc‴	β-D-glc‴		
1	4.81 (d, 7.6)	4.80 (d, 7.8)	4.81 (d, 7.8)		
2	3.98[a]	3.98[a]	3.98[a]		
3	4.23[a]	4.23[a]	4.23[a]		
4	4.21[a]	4.21[a]	4.21[a]		
5	3.93[a]	3.93[a]	3.93[a]		
6a	4.55[a]	4.55[a]	4.55[a]		
6b	4.38[a]	4.38[a]	4.38[a]		

[a] Overlapped.

20.31 (derivative of L-glucose), 18.48 (derivative of D-galactose), 19.78 (derivative of L-galactose), 16.28 (derivative of D-xylose) and 15.69 (derivative of L-xylose). The sugars in compounds **1**–**5** were determined to be D-glucose, D-galactose and D-xylose.

The EtOAc extracts obtained from compounds **1** and **2** were purified by semi-preparative HPLC with gradient elution of 80–100% MeOH, respectively, and afforded the same compound **9** (0.7 mg from **1** and 0.6 mg from **2**).

*(25R)-3ß-Hydroxy-spirost-5,14-diene (**9**):* Positive-ion ESIMS *m/z* 412.9 $[M+H]^+$. ^{1}H NMR (500 MHz, pyridine-d_5): 1.90 (1H, m, H-1a), 1.14 (1H, overlapped, H-1b), 2.10 (1H, m, H-2a), 1.79 (1H, overlapped, H-2b), 3.84 (1H, m, H-3), 2.61 (2H, m, H-4), 5.40 (1H, br d, J = 5.0 Hz, H-6), 2.18 (1H, m, H-7a), 2.00 (1H, m, H-7b), 2.20 (1H, m, H-8), 1.05 (1H, overlapped, H-9), 1.53 (2H, m, H-11), 1.65 (1H, overlapped, H-12a), 1.05 (1H, overlapped, H-12b), 5.44 (1H, br s, H-15), 5.21 (1H, br d, J = 7.5 Hz, H-16), 2.52 (1H, dd, J = 10.0, 7.5 Hz, H-17), 1.13 (3H, s, H-18), 1.05 (3H, s, H-19), 1.90 (1H, overlapped, H-20), 1.15 (3H, d, J = 7.0 Hz, H-21), 1.65 (2H, overlapped, H-23), 1.79 (1H, overlapped, H-24a), 1.65 (1H, overlapped, H-24b), 1.65 (1H, overlapped, H-25), 3.64 (2H, overlapped, H-26), 0.73 (3H, d, J = 6.5 Hz, H-27). ^{13}C NMR (125 MHz, pyridine-d_5): 37.6 (C-1), 32.6 (C-2), 71.3 (C-3), 43.4 (C-4), 141.3 (C-5), 120.8 (C-6), 31.9 (C-7), 31.3 (C-8), 50.5 (C-9), 37.4 (C-10), 21.3 (C-11), 33.6 (C-12), 46.9 (C-13), 159.8 (C-14), 121.8 (C-15), 85.4 (C-16), 60.4 (C-17), 28.4 (C-18), 19.3 (C-19), 44.4 (C-20), 13.8 (C-21), 106.4 (C-22), 30.2 (C-23), 29.3 (C-24), 30.9 (C-25), 67.3 (C-26), 17.4 (C-27).

2.5. Antimicrobial activity

Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* ATCC 9341), Gram-negative bacteria (*Salmonella enterica* serovar Typhimurium ATCC 14028 and *Escherichia coli* ATCC 25922), yeast (*Candida albicans* JCM 1542) and fungus (*Aspergillus fumigatus* JCM 1738) were used as indicator microbes for antimicrobial evaluation. The antimicrobial activity of isolated glycosides was assayed by the micro-broth dilution method using Mueller–Hinton broth (Becton, Dickinson and Company, USA) for bacteria and potato dextrose broth (Becton, Dickinson and Company, USA) for yeast and fungus according to previous reports [8,9]. The lowest antibiotic concentration that prevented the growth of a given test organism was determined as the minimal inhibitory concentration (MIC).

3. Results and discussion

A 70% EtOH extract from the rhizomes of *P. odoratum* was partitioned between *n*-BuOH and H_2O. The *n*-BuOH fraction was separated by Diaion HP-20, silica gel, ODS, and Sephadex LH-20 column chromatography, as well as preparative HPLC, to afford four cholestane-type steroidal glycosides (**1**–**3**, **8**) and four spirostane-type steroidal saponins (**4**–**7**). The structures of known compounds (**6**–**8**) were identified by detailed NMR analysis and comparison with literature data. The structures of the new compounds were determined by various spectroscopic analyses and chemical reactions.

Polygonatumoside A (**1**) was isolated as an amorphous powder. Its molecular formula was determined to be $C_{56}H_{88}O_{27}$ by positive-ion HRESIMS analysis. The ^{13}C NMR spectroscopic data revealed 56 carbon resonances, of which 29 were assigned to the sugar portions and the remaining 27 were assigned to the C_{27}-steroidal aglycone. Upon acid hydrolysis, the component sugar composition was determined to consist of D-glucose, D-xylose and D-galactose in a ratio of 3:1:1 by HPLC analysis after conversion of monosaccharides to their corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxy-alditol acetate derivatives [10]. The ^{1}H and ^{13}C NMR spectroscopic data also revealed the presence of five sugar units, whose β-anomeric configurations were determined by the large coupling constant values of their corresponding anomeric protons (7.5–7.8 Hz). Further detailed analyses of the DQFCOSY, TOCSY, HMQC, HMBC and NOESY spectroscopic data established the presence of a β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranosyl moiety and a β-D-glucopyranosyl moiety. The C_{27}-steroidal aglycone structure was inferred from the presence of a cholestane skeleton, as shown by two typical methyl singlets at δ 1.10 (s, H_3-18) and 0.96 (s, H_3-19) and two methyl doublets at δ 1.34 (d, J = 6.9 Hz, H_3-21) and 0.92 (d, J = 6.8 Hz, H_3-27) in the ^{1}H NMR spectrum. In addition, three trisubstituted C=C bonds were found, and their positions were determined to be C-5(6), C-14(15) and C-16(17) by the HMBC correlations from δ_H 1.71 (H-1) and 0.96 (H_3-19) to δ_C 140.5 (C-5), δ_H 2.32 (H-8) to δ_C 121.5 (C-6), δ_H 1.10 (H_3-18) to δ_C 159.8 (C-14) and 156.5 (C-17), δ_H 1.34 (H_3-21) to δ_C 156.5 (C-17), and δ_H 5.89 (H-15) to δ_C 32.1 (C-8) (Fig. 2). In addition, the carbonyl carbon at δ 210.4 was determined to be C-22 by the HMBC correlations from δ_H 1.34 (H_3-21) to 210.4 (C-22) and δ_H 1.96, 1.56 (H_2-24) to δ_C 210.4 (C-22). The relative configurations in the cholestane A–D ring system were typically confirmed by the 1,3-diaxial NOE correlations of H-1α/H-3, H-4β/H_3-19, H-8/H_3-19 and H-8/H_3-18. Furthermore, the HMBC correlations of Gal-H-1/C-3 and Glc‴-H-1/C-26 established the bisdesmoside structure. However, the absolute configuration of C-25 remained unsolved.

Polygonatumoside B (**2**) was isolated in the same fraction as compound **1** and their retention times for preparative HPLC were very similar. They have the same molecular formula, $C_{56}H_{88}O_{27}$, which was determined by positive-ion HRESIMS. These two compounds showed a high similarity in their ^{1}H and ^{13}C NMR spectroscopic data, although they exhibited some slight differences (Tables 1 and 2). However, their optical rotation values {$[\alpha]_D^{20}$ +46.0 for **1** and −28.6 for **2**} differed greatly. All of these data suggested that compound **2** is a C-25 diastereomer of compound **1**.

It is always difficult to assign the C-25 absolute configuration in cholestane-type compounds. In this study, several approaches were utilized to solve this problem. An empirical rule by calculating the chemical shift difference between H_2-26 (δ_{ab} ⩾0.57 ppm for 25*S*, and δ_{ab} ⩽0.48 ppm for 25*R*, when in pyridine-d_5) [11] was applied to determine the C-25 absolute configuration of cholestane glycosides [12,13], however, compounds **1** and **2** did not follow this rule. Furthermore, on acid hydrolysis of compounds **1** and **2**, only the same compound (25*R*)-3β-hydroxy-spirost-5,14-diene (**9**) could have been isolated as the major product, possibly due to the equilibrium of C-25 epimerization [14] (Fig. 3). Thus, comparison of the chemical shifts of H_3-27 between **1** and **2** was utilized to assign the 25*R*/25*S*-configuration because for most furostane saponins, the resonance from H_3-27 in the 25*R* configuration appears at a higher field than that in the 25*S* configuration [15]. Using this empirical observation, compound **1** was determined to possess the 25*R* configuration, and compound **2** possesses the 25*S* configuration. Thus, polygonatumoside A (**1**) was determined to be (25*R*)-26-*O*-β-D-glucopyranosyl-3β-hydroxy-cholest-5,14,16-trien-22-one-3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside, and the structure of polygonatumoside B (**2**) is (25*S*)-26-*O*-β-D-glucopyranosyl-3β-hydroxy-cholest-5,14,16-trien-22-one-3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl (1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Polygonatumoside C (**3**) was obtained as an amorphous powder. HRESIMS analysis (m/z 1083.5003 $[M+Na]^+$) indicated a molecular formula of $C_{51}H_{80}O_{23}$. Careful comparison of the NMR data of compound **3** with those of **1** suggested that **3** had the same aglycone as that of **1** but different sugar moieties. The presence of three β-D-glucopyranosyl moieties and one β-D-galactopyranosyl in **3** was

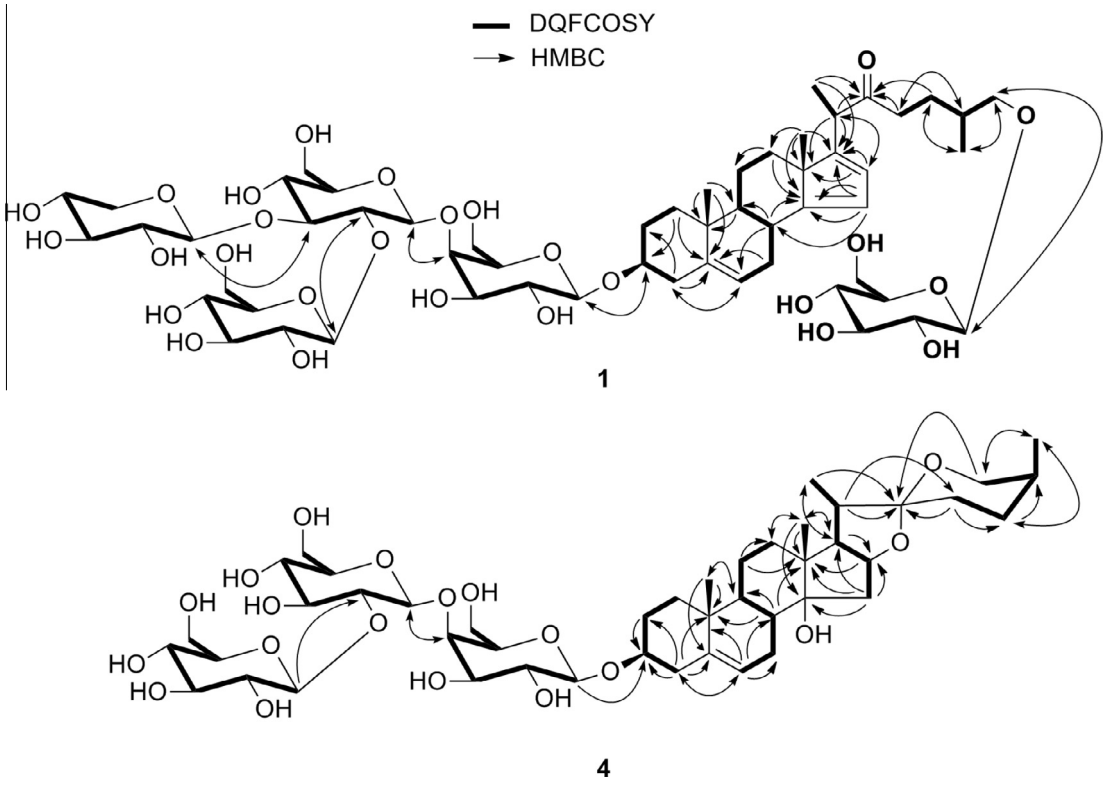

Fig. 2. Key DQFCOSY and HMBC correlations of compounds **1** and **4**.

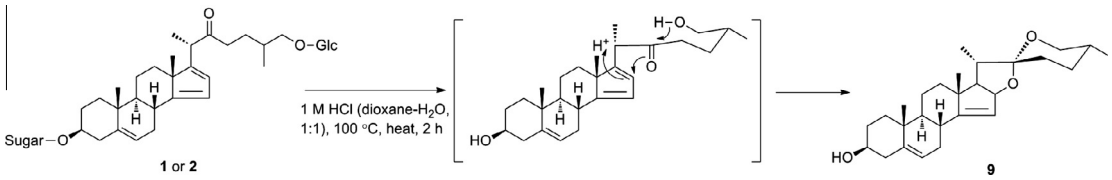

Fig. 3. Acid hydrolysis of compounds **1** and **2**.

suggested by the observation of four anomeric proton doublets at δ 4.91, 5.14, 5.23 and 4.81 in the ^{1}H NMR spectrum and by the acid hydrolysis results. The structures of the sugar moieties and the location of the glycosidic bonds were determined by further detailed analyses of 2D NMR spectroscopic data, in particular, the HMBC correlations of Glc″-H-1/Glc′-C-2, Glc′-H-1/Gal-C-4, Gal-H-1/C-3 and Glc‴-H-1/C-26 indicated the bisdesmoside structure of a β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranosyl moiety connected at C-3 and a β-D-glucopyranosyl moiety connected at C-26. Thus, the structure of polygonatumoside C (**3**) was determined to be (25*R*)-26-*O*-β-D-glucopyranosyl-3β-hydroxy-cholest-5,14,16-trien-22-one-3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Polygonatumoside D (**4**) and polygonatumoside E (**5**) were isolated as amorphous powders with the same molecular formula, $C_{45}H_{72}O_{19}$. They are spirostane-type steroidal saponins; this conclusion was suggested by the characteristic proton resonances for two tertiary angular methyl and two secondary methyl groups in the ^{1}H NMR spectra and by the quaternary carbon at ca. δ 110 in the ^{13}C NMR spectra (Tables 1 and 2). Compounds **4** and **5** are a pair of C-25 diastereomers, as shown by their superimposable NMR spectra, but differences were observed in the E and F ring resonances. In the aglycone, the presence of a Δ^5-unsaturated A/B ring fusion was indicated by the HMBC correlations of H_3-19/C-5, H-6/C-4, H-6/C-8 and H-6/C-10. The position of the 14α-hydroxy moiety was determined by comparing the downfield-shifted resonances of H-9, H-11 and H-17 with those of the 14α-H compound [16], as well as the HMBC correlations from H_3-18/C-14 and H_2-12/C-14. The 25*S* configuration in **4** and 25*R* configuration in **5** were inferred from the differences in the chemical shifts of the H_2-23, H_2-24 and H_2-26 geminal protons, which were >0.35 ppm in compound **4** and <0.20 ppm in compound **5**, as well as the fact that the H_3-27 resonance appears 0.39 ppm upfield in compound **5** compared to that of **4** [17]. The component sugars in compounds **4** and **5** were determined to be a β-D-galactopyranosyl unit and two β-D-glucopyranosyl units through detailed NMR spectroscopic analyses and acid hydrolysis. The connection and the sequence of the sugar chain at C-3 were determined through the same HMBC correlations as in **3** (Fig. 2). Thus, the structure of polygonatumoside D (**4**) is (25*S*)-(3β,14α)-dihydroxy-spirost-5-en-3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside, and the structure of polygonatumoside E (**5**) was determined to be (25*R*)-(3β,14)-dihydroxy-spirost-5-en-3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Although our knowledge of cholestane-type steroidal glycosides is still quite limited, this class of compounds has been recognized

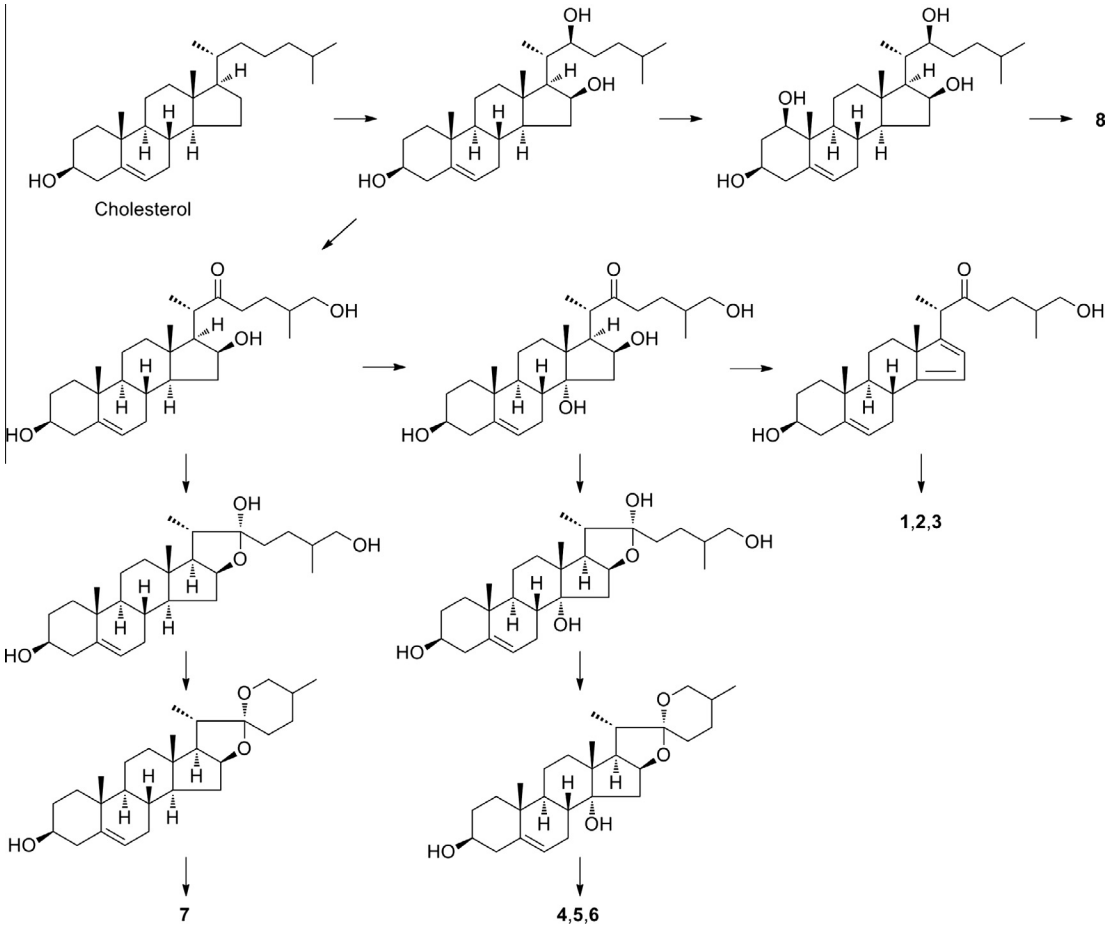

Fig. 4. Possible biosynthetic pathway for compounds **1**–**8**.

as the third class of C_{27}-steroidal glycosides [1]. The biosynthesis of cholestane-type glycosides has been proposed to involve independent pathways from furostane- and spirostane-type steroidal saponins, but in this study, the cholestane glycosides (**1**–**3**) and spirostane saponins (**4**–**7**) are proposed to share a common biosynthetic precursor, 16,26-dihydroxy-22-keto-cholesterol (Fig. 4). The high sugar-sequence similarity between the cholestane glycosides (**1**–**3**) and spirostane saponins (**4**–**7**) also indicated their close biosynthetic relationship, compared to another cholestane-type glycoside (**8**). The occurrence of different biosynthetic reactions at the 16-hydroxy group appears to be an important diverging point for their biosynthetic pathways. This conclusion is also supported by other phytochemical investigations that have used similar metabolic profiling [12,18].

All isolated compounds were evaluated for their antimicrobial activity against *S. aureus* ATCC 25923, *M. luteus* ATCC 9341, *S. enterica* serovar Typhimurium ATCC 14028, *E. coli* ATCC 25922, *C. albicans* JCM 1542 and *A. fumigatus* JCM 1738. Compound **7** showed significant antifungal activity against *C. albicans* JCM 1542 (MIC 3.1 μg/mL) and *A. fumigatus* JCM 1738 (MIC 6.3 μg/mL). However, the other seven compounds had very low activity against these yeast and fungus strains (MICs $\geqslant$100 μg/mL). Moreover, all isolated glycosides showed no activity against the bacterial strains using in this antimicrobial test at concentration of 100 μg/mL. Comparison of the structures of **7** and **6**, suggested that 14-hydroxylation greatly decreased the activity. In comparison of **7** with several structurally related antifungal spirostane saponins (MICs $\leqslant$10 μg/mL) [19–21], it suggested that the C-5(6) double bond, the C-25 absolute configuration and the sugar structures did not significantly influence the activity.

4. Conclusions

The phytochemical investigation of *P. odoratum* resulted in the isolation of four cholestane-type steroidal glycosides (**1**–**3**, **8**) and four spirostane-type steroidal saponins (**4**–**7**). Polygonatumosides A–C (**1**–**3**) have an unique $\Delta^{14,16}$-unsaturated D-ring structure, which extends the biodiversity of plant steroidal glycosides. From the biosynthetic perspective, the cholestane-type steroidal glycosides (**1**–**3**) are steroidal saponin-like glycosides, which is evident from their close biosynthetic relationship with and high sugar-sequence similarity to the steroidal saponins obtained in this study. However, further investigation is required to identify the biosynthetic enzymes and intermediates involved in C_{27}-steroidal glycoside biosynthesis. Furthermore, spirostane-type steroidal saponin **7** showed significant antifungal activity against *C. albicans* JCM 1542 and *A. fumigatus* JCM 1738.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.11.013.

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