

## Two New Cholestanol Glycosides from the Roots and Rhizomes of *Smilacina henryi*

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**Abstract:** Two new cholestanol glycosides (**1** and **2**) were obtained from the roots and rhizomes of *Smilacina henryi*. Their structures were determined as 5 $\alpha$ -cholest-9(11)-ene-3 $\beta$ , 26-dihydroxy-16, 22-dione 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-galactopyranoside (**1**), 5 $\alpha$ -cholest-9(11)-ene-3 $\beta$ , 26-dihydroxy-16, 22-dione 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-[ $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-galactopyranoside (**2**), by physicochemical properties and spectroscopic methods. In addition, the isolated glycosides were tested for their cytotoxic activity against human HepG2 tumor cells and compound **2** showed moderate activity with IC<sub>50</sub> value of 59.32  $\mu$ M.

**Keywords:** *Smilacina henryi*; cholestanol glycosides; structure determination; cytotoxicity. © 2019 ACG Publications. All rights reserved.

### 1. Introduction

*Smilacina henryi* (bekev) Wang et Tang, mainly distributed in Asia, Europe and north America, is a perennial herb in the genus *Smilacina* (Liliaceae) [1, 2]. As one of about 16 species grown in China, *S. henryi* is widely distributed in Shaanxi, Hebei, and Yunnan province. Its roots, named Pian Tou Qi in the region of Qinba Mountains of Shaanxi province, are usually used as traditional Chinese medicines (TCMs) for the treatment of rheumatism, traumatic injury and impotence [3-7]. Some chemical constituents have been isolated from *S. henryi* such as steroidal saponins and flavonoids [3, 8]. In our research project of searching for the bioactive constituents from TCMs [9, 10], an investigation

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of secondary metabolites of *S. henryi* was carried out and two new cholestanol glycosides were obtained. Additionally, their cytotoxic activities against human HepG2 tumor cells were also studied. The isolation, structural elucidation and cytotoxic evaluation for the glycosides were reported in this paper.

## 2. Materials and Methods

### 2.1. General Experimental Procedures

Optical rotation was measured using a Rudolph Autopol VI polarimeter (Rudolph, USA); IR spectra were recorded on a Nicolet iS10 instrument (Thermo Fisher Scientific, USA); 1D and 2D NMR spectra were recorded on a Bruker-Avance 400 instrument (Bruker Corp. Karlsruhe, Germany); Semipreparative HPLC was performed on Agilent infinity II system equipped with a UV detector and a YMC-Pack-ODS-A (10 mm × 250 mm, 5 μm particles) column. The HR-ESI-MS spectra were taken on an Agilent Technologies 6650 Q-TOF (Agilent Technologies). Sephadex LH-20 gel and ODS C<sub>18</sub> (5 μm) silica gel was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China).

### 2.2. Plant Material

The roots and rhizomes of *Smilacina henryi* (bekev) Wang et Tang were collected on August in 2017 from Qinba Mountains in Shaanxi Province of China, and were authenticated by one of our co-authors Prof. Jing Sun (Shaanxi University of Chinese Medicine). A voucher specimen (herbarium No. SH-201708) is deposited in School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China.

### 2.3 Extraction and Isolation

The air-dried roots and rhizomes of *S. henryi* (6.6 kg) were extracted with 80% EtOH under reflux for three times (2h, 2h, 1h, successively). The concentrated residue was partitioned with petroleum ether (PE) and *n*-BuOH successively. The *n*-BuOH extract (130.2 g) was subjected to column chromatography (CC) on silica gel (1 kg), eluting with gradient solvent system (CH<sub>2</sub>Cl<sub>2</sub>-MeOH-H<sub>2</sub>O, 100:0:0 – 60:40:10) to give six fractions (Fr.1 – Fr.6). Fr.4 (19.1 g) was subjected to CC on silica gel (200 g), eluting with (CH<sub>2</sub>Cl<sub>2</sub>-MeOH-H<sub>2</sub>O, 100:10:0 – 80:20:5) to give six subfractions (Fr.4-1 – Fr.4-6). Fr.4-3 (3.1 g) subjected to CC on Sephadex LH-20 gel (100 g) eluting with (CH<sub>2</sub>Cl<sub>2</sub>-MeOH 100:100) to give ten subfractions (Fr.4-3-1 – Fr.4-3-10). Fr.4-3-2 (55.7 mg) was purified by HPLC (YMC-Pack-ODS-A, 10 mm × 250 mm, 5 μm particles, flow rate: 1.5 mL/min) with MeCN-H<sub>2</sub>O (70:30) as mobile phase to afford compound **2** (16.9 mg; *t<sub>R</sub>* = 19.7 min), Fr.4-3-4 (31.1 mg) was purified by HPLC (YMC-Pack-ODS-A, 10 mm × 250 mm, 5 μm particles, flow rate: 1.5 mL/min) with MeCN-H<sub>2</sub>O (72:28) as mobile phase to afford compound **1** (10.4 mg; *t<sub>R</sub>* = 28.4 min).

#### 2.4. Spectroscopic Data

*5 $\alpha$ -cholest-9(11)-ene-3 $\beta$ , 26-dihydroxy-16, 22-dione 3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-galactopyranoside (1)*: A white amorphous powder;  $[\alpha]_D^{20} -49.4^\circ$  (*c* 0.18, MeOH); IR (KBr)  $\nu_{\max}$ : 3419, 2926, 1736, 1614, 1455, 1067, 890, 770  $\text{cm}^{-1}$ ;  $m/z$  777.4025  $[\text{M} + \text{Na}]^+$  (calcd for 777.4037  $\text{C}_{39}\text{H}_{62}\text{O}_{14}\text{Na}^+$ ).  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  data (400 MHz and 100 MHz in pyridine-*d*<sub>5</sub>) see Table 1.

*5 $\alpha$ -cholest-9(11)-ene-3 $\beta$ , 26-dihydroxy-16, 22-dione 3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-[ $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-galactopyranoside (2)*: A white amorphous powder;  $[\alpha]_D^{20} -20.0^\circ$  (*c* 0.1, MeOH); IR (KBr)  $\nu_{\max}$ : 3417, 2926, 1736, 1704, 1603, 1456, 1068, 688  $\text{cm}^{-1}$ ;  $m/z$  939.4532  $[\text{M} + \text{Na}]^+$  (calcd for 939.4565  $\text{C}_{45}\text{H}_{72}\text{O}_{19}\text{Na}^+$ ).  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  data (400 MHz and 100 MHz in pyridine-*d*<sub>5</sub>) see Table 1.

#### 2.5. Acid Hydrolysis

Solutions of **1** and **2** (5 mg each) were hydrolyzed in 2 M hydrochloric acid (5 mL) at 80 °C for 2 h. After cooling, each solution was concentrated under vacuum, dissolved with water, and extracted twice with dichloromethane ( $\text{CH}_2\text{Cl}_2$ ). The aqueous parts were subjected to CC on ODS  $\text{C}_{18}$  silica gel (10 g), eluting with (MeCN- $\text{H}_2\text{O}$ , 5:95) to give two products. The D configurations of the galactose and the glucose moieties in **1** and **2** were confirmed through their optical rotation data (Gal:  $[\alpha]_D^{20} +29.0^\circ$ , MeOH and Glc:  $[\alpha]_D^{20} +48.8^\circ$ , MeOH) and  $R_f$  values (BuOH-AcOH- $\text{H}_2\text{O}$ , 4:1:5 upper layer, Gal: 0.39 and Glc: 0.36) with the authentic sugar samples [11].

#### 2.6. Cytotoxic Activity Assay

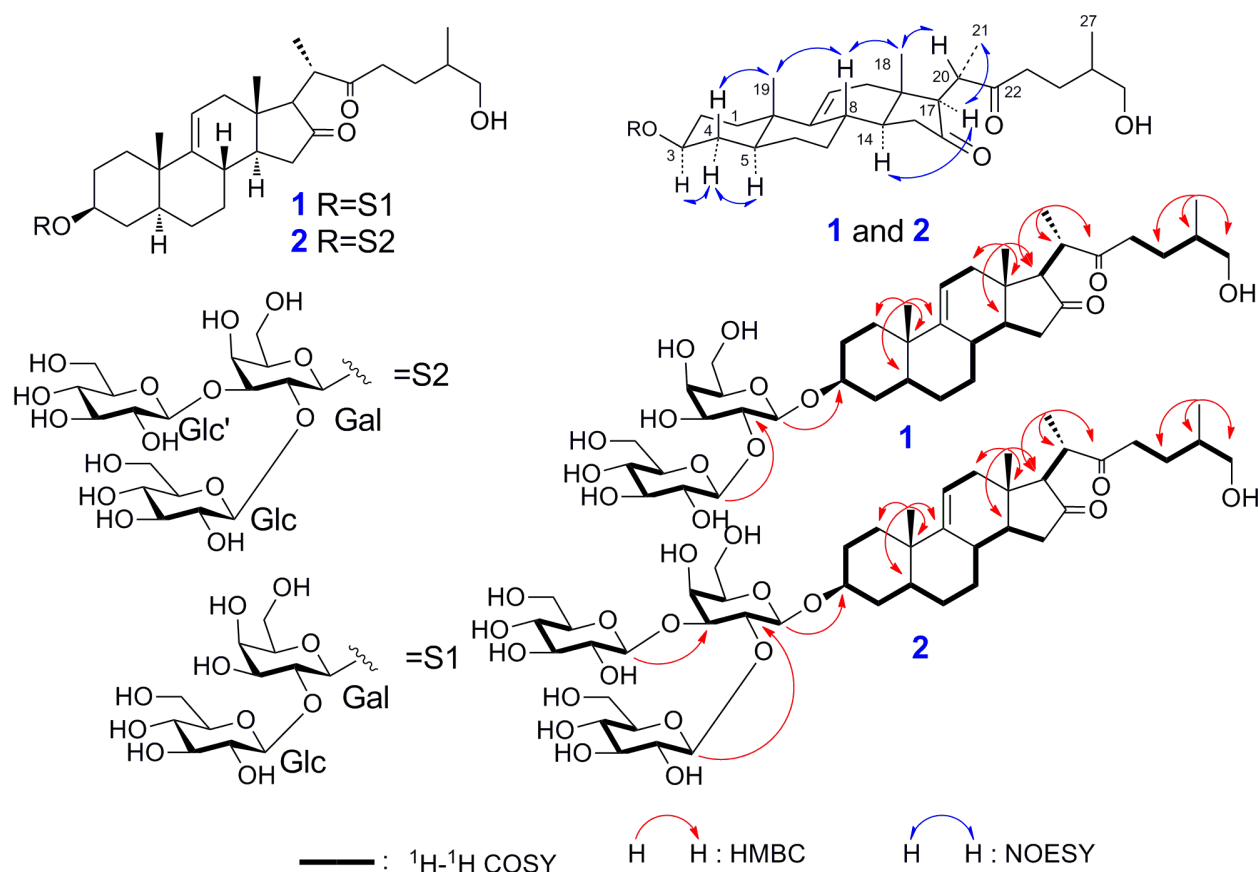
The cytotoxic activity assay toward the human HepG2 cell lines was measured by the MTT method (positive control: 5-fluorouracil,  $\text{IC}_{50}$  12.07  $\mu\text{M}$ ). Briefly,  $1 \times 10^4/\text{mL}$  cells were seeded into 96-well plates and allowed to adhere for 24 h. Compounds **1** and **2** were dissolved in DMSO and diluted with complete medium to 6 degrees of concentration for inhibition rate determination. After incubation at 37.8 °C for 4 h, the supernatant was removed before adding DMSO (100  $\mu\text{L}$ ) to each well [8].

### 3. Results and Discussion

Compound **1**, white amorphous powder, with  $[\alpha]_D^{20} -49.4^\circ$  (*c* 0.18, MeOH), has a molecular formula of  $\text{C}_{39}\text{H}_{62}\text{O}_{14}$  which was deduced from the HR-ESI-MS positive molecular ion peak at  $m/z$  777.4025  $[\text{M} + \text{Na}]^+$  (calcd for 777.4037  $[\text{M} + \text{Na}]^+$ ). Four steroid methyl groups at  $\delta_{\text{H}}$  0.66 (s, H-18), 0.85 (s, H-19), 1.14 (d,  $J = 6.7$  Hz, H-21) and 1.07 (d,  $J = 6.8$  Hz, H-27) along with an olefinic proton 5.29 (d,  $J = 5.3$  Hz, H-11) and two anomeric protons at  $\delta_{\text{H}}$  4.88 (d,  $J = 7.8$  Hz, Gal-H1) and 5.33 (d,  $J = 7.8$  Hz, Glc-H1) were observed in the  $^1\text{H-NMR}$  spectrum of **1**. The  $^{13}\text{C-NMR}$  spectrum displayed 39 carbon signals, in which a double bond carbons at  $\delta_{\text{C}}$  147.3 (C-9), 115.3 (C-11), and two carbonyl carbon at  $\delta_{\text{C}}$  217.1 (C-16) and 213.1 (C-22) and two oxygen-carbon signals at  $\delta_{\text{C}}$  76.7 (C-3) and 67.3 (C-26) were observed. These data compared with the literature [8] supported the

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5 $\alpha$ -cholest-9(11)-ene-3 $\beta$ , 26-dihydroxy-16, 22-dione aglycone of **1**. This inference was deduced from 2D-NMR data analysis including HSQC, HMBC, NEOSY and  $^1\text{H}$ - $^1\text{H}$  COSY experiments (Figure 1). Detailed considering the  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra showed a galactose moiety with anomeric proton at  $\delta_{\text{H}}$  4.88 and carbon signals (102.4, 79.9, 75.3, 73.2, 75.7, 60.8) and a glucose with anomeric proton at  $\delta_{\text{H}}$  5.33 and carbon signals (106.9, 75.0, 78.5, 72.1, 78.3, 62.9) in **1**, respectively [3]. The sugar sequence and its linkage site to the aglycone moiety were determined from the HMBC spectra. HMBC correlations observed between Gal-H1 ( $\delta_{\text{H}}$  4.88) and C-3 ( $\delta_{\text{C}}$  76.7), and between Glc-H1 ( $\delta_{\text{H}}$  5.33) and Gal-C2 ( $\delta_{\text{C}}$  79.9), which disclosed that the D-galactose unit was linked at C-3 of aglycone, the D-glucose unit was linked at C-2 of the D-galactose. Acid hydrolysis of **1** resulted in the products of D-galactose and D-glucose which were confirmed by their optical rotation data (Gal:  $[\alpha]_{\text{D}}^{20} +29.0^\circ$ , MeOH and Glc:  $[\alpha]_{\text{D}}^{20} +48.8^\circ$ , MeOH) and  $R_f$  values (BuOH-AcOH-H<sub>2</sub>O, 4:1:5 upper layer, Gal: 0.39 and Glc: 0.36) with the authentic sugar samples [9]. Coupling constants of the anomeric proton signals (7.8 Hz each) suggested the  $\beta$ -configurations for the D-galactose and D-glucose, respectively [12-13]. Then the structure of **1** was deduced as 5 $\alpha$ -cholest-9(11)-ene-3 $\beta$ , 26-dihydroxy-16, 22-dione 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-galactopyranoside (**1**, Figure 1).



**Figure 1.** Structures and key  $^1\text{H}$ - $^1\text{H}$  COSY, HMBC and NOESY correlations of **1** and **2**

**Table 1.** <sup>1</sup>H and <sup>13</sup>C-NMR data<sup>a</sup> ( $\delta$  in ppm, *J* in Hz) of **1** and **2**

NO	<b>1</b>		<b>2</b>		NO	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$		$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1	1.31 m	35.4	1.31 m	35.4	Gal-1	4.88, d (7.8)	102.4	4.91, d (7.8)	102.3
	1.63 m		1.62 m		2	4.73 m	79.9	4.58 ov	80.8
2	1.73 m	29.8	1.72 m	29.8	3	4.11ov	75.3	4.13ov	85.9
	2.19 m		2.17 m		4	4.42, d (9.2)	73.2	4.50, d (9.2)	73.1
3	3.93 m	76.7	4.03 m	76.5	5	4.28 ov	75.7	3.97 ov	77.4
4	1.77 m	38.0	1.74 m	38.0	6	4.31 ov	60.8	4.29 ov	60.3
	2.22 m		2.23 m			4.78 m	4.79 m		
5	1.08 ov	42.8	1.06 ov	42.8	Glc-1	5.33, d (7.8)	106.9	5.16 ov	105.0
6	1.21 m	28.3	1.22 m	28.3	2	4.09 ov	75.0	4.08 ov	75.4
	1.25 m		1.24 m		3	4.25 ov	78.5	4.26 ov	78.7
7	0.97 m	33.0	0.96 m	33.0	4	4.07 ov	72.1	4.24 ov	71.6
	1.65 m		1.64 m		5	4.05 ov	78.3	4.07 ov	76.5
8	1.98 m	34.7	1.99 m	34.7	6	4.22 ov	62.9	4.38, d (10.8)	61.3
9	—	147.3	—	147.3		4.64, d (10.8)		4.59 ov	
10	—	38.0	—	38.0	Glc-1'			5.23, d (7.8)	106.7
11	5.29, d (5.3)	115.3	5.28, d (5.3)	115.2	2			4.09 ov	75.0
12	1.63 ov	35.3	1.63 ov	35.3	3			3.96 ov	78.2
	1.88 ov		1.88 ov		4			4.23 ov	70.1
13	—	39.6	—	39.6	5			4.12 ov	78.0
14	1.61 ov	47.8	1.61 ov	47.8	6			4.11 ov	63.0
15	2.91, dd (11.2, 8.9)	40.3	2.92, dd (11.2, 8.9)	40.3				4.65 ov	
	2.98, dd (11.2, 8.9)		2.96, dd (11.2, 8.9)						
16	—	217.1	—	217.2					
17	2.83, d (10.9)	65.8	2.83, d (10.9)	65.8					
18	0.66 s	12.3	0.66 s	12.4					
19	0.85 s	17.8	0.84 s	17.8					
20	2.78, dd (10.9, 6.7)	43.3	2.78, dd (10.9, 6.7)	43.3					
21	1.14, d (6.7)	15.3	1.14, d (6.7)	15.3					
22	—	213.1	—	213.1					
23	2.11 m	40.1	2.10 m	40.1					
	2.14 m		2.14 m						
24	1.77 m	27.5	1.77 m	27.5					
	2.22 m		2.22 m						
25	1.81 m	35.9	1.81 m	35.9					
26	3.74, d (10.5)	67.3	3.74, d (10.5)	67.3					
	3.81, dd (10.5, 6.8)		3.82, dd (10.5, 6.8)						
27	1.07, d (6.8)	17.1	1.08, d (6.8)	17.1					

<sup>a</sup> <sup>1</sup>H-NMR and <sup>13</sup>C-NMR were measured at 400 MHz and 100 MHz in pyridine-*d*<sub>5</sub>, and the assignments were based on the HSQC, HMBC, NOESY and <sup>1</sup>H-<sup>1</sup>H COSY experiments; ov: overlap signals.

Compound **2**, was obtained as white amorphous powder, with  $[\alpha]_D^{20} -20.0^\circ$  (*c* 0.10, MeOH), which has the molecular formula of  $C_{45}H_{72}O_{19}$  supporting by the HR-ESI-MS positive molecular ion peak at  $m/z$  939.4532  $[M + Na]^+$  (calcd for 939.4565  $[M + Na]^+$ ). When comparing the NMR data of **2** and **1**, they showed almost the similar NMR spectroscopic features of the aglycone. Therefore, the aglycone of **2** was deduced as  $5\alpha$ -cholest-9(11)-ene- $3\beta$ , 26-dihydroxy-16, 22-dione [8] which was further supported by 2D-NMR data analysis (Figure 1). The difference between **2** and **1** was the sugar moiety in **1** (3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-galactopyranoside) which was replaced by 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $[\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-galactopyranoside in **2** [3, 4]. This inference was determined from the HMBC correlations from 4.91 (Gal-H1) to 76.5 (C-3), from 5.16 (Glc-H1) to 80.8 (Gal-C2) and from 5.23 (Glc'-H1) to 85.9 (Gal-H3). The  $\beta$ -configurations for the D-galactose and D-glucose in **2** were identified as the same method as **1**. Compound **2** was thus deduced as  $5\alpha$ -cholest-9(11)-ene- $3\beta$ , 26-dihydroxy-16, 22-dione 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $[\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)]- $\beta$ -D-galactopyranoside (**2**, Figure 1).

Steroidal glycosides, possessing various cytotoxic activities, have been isolated from the genus *Smilacina* [2, 4-8]. In this paper, the cytotoxic activity against human HepG2 tumor cells of compounds **1** and **2** were evaluated by the MTT method. The results showed that only compound **2** exhibited moderate effect with  $IC_{50}$  value of 59.32  $\mu$ M.

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## Supporting Information

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