

# Spiralosides A–C, Three New C<sub>27</sub>-Steroidal Glycoalkaloids from the Fruits of *Solanum spirale*

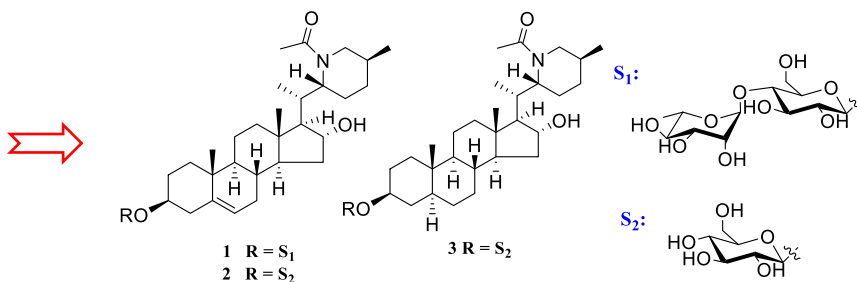


Dan Li · Yun-Li Zhao · Xu-Jie Qin ·  
Lu Liu · Xing-Wei Yang · Ying-Ying Chen ·  
Bei Wang · Xin Wei · Ya-Ping Liu ·  
Xiao-Dong Luo

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**Abstract** Three new C<sub>27</sub>-steroidal glycoalkaloids, spiralosides A–C (1–3), were obtained from the total alkaloids of *Solanum spirale* by chromatographic methods. On the basis of spectroscopic evidence, spiralosides A–C were elucidated as (22*R*,25*S*)-22,26-epiminocholest-5-ene-3 $\beta$ ,16 $\alpha$ -diol-*N*-acetyl-3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranosyl (1), (22*R*,25*S*)-22,26-epiminocholest-5-ene-3 $\beta$ ,16 $\alpha$ -diol-*N*-acetyl-3-*O*- $\beta$ -D-glucopyranosyl (2), (22*R*,25*S*)-22,26-epiminocholest-3 $\beta$ ,16 $\alpha$ -diol-*N*-acetyl-3-*O*- $\beta$ -D-glucopyranosyl (3), respectively. The total alkaloids of *S. spirale* have been screened for their antitussive and expectorant effects in intact animal model.

*Graphical Abstract*



**Keywords** Solanaceae · *Solanum spirale* · Spiralosides A–C

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D. Li · Y.-L. Zhao · X.-J. Qin · L. Liu · X.-W. Yang ·  
Y.-Y. Chen · B. Wang · X. Wei · Y.-P. Liu (✉) · X.-D. Luo (✉)  
State Key Laboratory of Phytochemistry and Plant Resources in  
West China, Kunming Institute of Botany, Chinese Academy of  
Sciences, Kunming 650201, China  
e-mail: liuyaping@mail.kib.ac.cn

X.-D. Luo  
e-mail: xdlo@mail.kib.ac.cn

D. Li · L. Liu · Y.-Y. Chen · B. Wang · X. Wei  
University of Chinese Academy of Sciences, Beijing 100039,  
China

## 1 Introduction

*Solanum spirale* (Solanaceae), an erect shrub, is widely distributed in Yunnan, Guangxi and Hunan provinces of China. It usually grows forest-shrub edge and wasteland at the altitude between 500 and 1900 m [1]. *S. spirale*, popularly named “Li Fei San”, has been used in “Yi” ethnopharmacy as anti-tussive and anti-inflammatory agent historically. Besides, its tender leaves and fruits can be edible as a wild vegetable by “Dai” ethnopharmacy [2]. Previously, the reported phytochemical studies of *S. spirale* led to the isolation of a series of components, such as oil

components, steroidal saponins, steroidal alkaloids and steroidal glycoalkaloids with antioxidant activity, and significant antibacterial activity and cytotoxicity [3–8]. The steroidal alkaloid glycosides from *Solanum* genus possess extensive activities including anticancer [9, 10], anti-acetylcholinesterase [11, 12], anticholesterol [13, 14], and antifungal [15] properties. In the course of searching for bioactive steroidal alkaloid glycosides from the fruits of *S. spirale*, three new steroidal glycoalkaloids named spiralosides A–C (**1–3**) were isolated. The total alkaloids were evaluated anti-tussive activity against ammonia liquor induced damage and the expectorant effects used by phenol red secretion test in mice. Reported herein are the isolation, structural elucidation, and the anti-tussive activities of the total alkaloids.

## 2 Result and Discussion

Compound **1** was obtained as a white, amorphous powder;  $[\alpha]_D^{23} -46.6$  ( $c$  0.03, MeOH). It displayed a positive reaction to Dragendorff's reagent and gave the molecular formula of  $C_{41}H_{67}NO_{12}$  by HRESIMS at  $m/z$  788.4553  $[M+Na]^+$  (calcd for 788.4561), corresponding to nine degrees of unsaturation. The  $^1H$ -,  $^{13}C$ -NMR and DEPT spectra displayed two sugar units on basis of three proton signals at  $\delta_H$  4.36 (1H, d,  $J = 7.8$  Hz), 4.81 (1H, br s), and 1.23 (3H, d,  $J = 6.2$  Hz), two anomeric carbons [ $\delta_C$  102.3 (d), and 102.9 (d)], a methyl group ( $\delta_C$  17.8), a methylene carbon ( $\delta_C$  62.1), and other 8 methines signals between  $\delta_C$  70.6 and  $\delta_C$  79.9. The coupling constant ( $J = 7.8$  Hz) of the anomeric proton at  $\delta_H$  4.36 (1H, d, H-1) indicated the  $\beta$ -configuration of the glucosyl residues [16]. Likewise the other anomeric configuration of the rhamnopyranosyl was confirmed as  $\alpha$ -orientated on the basis of the chemical shift values of C-3'' ( $\delta_C$  72.2), C-5'' ( $\delta_C$  70.6) with those of the corresponding carbons of methyl  $\alpha$ - and  $\beta$ -rhamnopyranoside [17]. The identification of the sugar residues were continued by hydrolysis with 10 % HCl to afford D-glucose and L-rhamnose, which were confirmed by GC chromatographic analysis of their L-cysteine methyl ester-TMS derivatives. Besides of two sugar units,  $^{13}C$ -NMR and DEPT spectra also showed 29 carbons, five methyl groups, ten methylenes, ten methines, and four quaternary carbons (Table 1). Comparison of above data with those of capsimine [18] and baikeine [19], pingbeine [20] showed that the aglycone of **1** was similar to capsimine with exception of an additional acetyl group at  $\delta_C$  22.2 (q) and 172.4 (s) in **1** (Fig. 1). This acetyl group ( $\delta_C$  22.2, q and 172.4, s) was located at N in last ring by correlations of  $\delta_H$  2.08 (3H, s, H-29) with  $\delta_C$  172.4 (s, C-28) and 50.7 (t, C-26), of  $\delta_H$  3.60 (1H, overlap, H-26a), 2.91 (1H, t,

$J = 12.4$  Hz, H-26b), and 4.46 (1H, dt,  $J = 6.1, 6.0$  Hz, H-22) with  $\delta_C$  172.4 (s, C-28). Signal at  $\delta_H$  4.22 (1H, t,  $J = 7.4$  Hz) corresponding to  $\delta_C$  76.3 (t) in its HSQC spectrum showed cross peaks with  $\delta_C$  61.9 (d, C-17), 55.2 (d, C-14), and 37.4 (t, C-15), which suggested that the hydroxyl substitute at C-16. The other oxygenic proton signal at  $\delta_H$  3.53 (1H, m) placed at C-3, which was confirmed by HMBC correlations of  $\delta_H$  1.84 (1H, m, H-1a), 1.03 (1H, d,  $J = 4.3$  Hz, H-1b), 2.38 (1H, dd,  $J = 2.1, 13.1$  Hz, H-4a), and 2.22 (1H, t,  $J = 12.4$  Hz, H-4b) with  $\delta_C$  79.9 (d, C-3) (Fig. 2). The glycositatic position was unambiguously ascribed to be at C-3 from the HMBC correlation of  $\delta_H$  4.36 (1H, d,  $J = 7.8$  Hz, H-1') with C-3 ( $\delta_C$  79.9). The  $^1H$ - $^1H$  COSY spectrum of **1** displayed five partial fragments a–e (Fig. 2). The signals of fragments a and b were from  $\delta_H$  4.36 (1H, d,  $J = 7.8$  Hz, H-1') to  $\delta_H$  4.76 and 3.62 (1H each, H-6'), from  $\delta_H$  4.81 (1H, br s, H-1'') to  $\delta_H$  1.23 (3H, q,  $J = 7.8$  Hz, H-6''), respectively, were further suggested a rhamnose and a glucose appeared. On the basis of  $\delta_H$  4.81 (1H, br s, H-1'') correlating with C-4' ( $\delta_C$  79.6), the presence of a rhamnopyranosyl (1 $\rightarrow$ 4)-glucopyranosyl moiety was deduced (Fig. 2).

The ROESY correlations of Me-19/H-4b/H-2b/H-11b/H-8, H-3/H-2a, and H-11a/H-9/H-14 indicated the  $\alpha$ -orientation of H-3, H-9 and H-14,  $\beta$ -orientation of H-8 (Fig. 3). ROESY correlations of Me-18 with H-20, of H-20 with H-16 positioned  $\alpha$ -orientation for both Me-21 and OH-16,  $\beta$ -orientation for H-20. ROESY correlations of Me-18 with H-20 also indicated a 17 $\beta$ -side chain, as well as on the biogenetical derivation of C-27 steroidal alkaloids [21]. And a missing effect between H-17 and H-20 displayed, at least approximately, antiperiplanar positions of H-17 and H-20. The coupling constant  $^3J_{17,20} = 12.4$  Hz was in agree with this assumption [7]. Furthermore, the coupling constant  $^3J_{20,22} = 6.0$  Hz suggested that they should be on the same side and the C-22 of configuration is *R* [22]. Besides, ROESY correlations of H-22 with H-23b, of Me-27 with H-23a indicated H-22 and H-25 both are axial bonds and the C-25 of configuration is *S*. So compound **1** was determined as (2*2R*,2*5S*)-22,26-epiminocholest-5-ene-3 $\beta$ ,16 $\alpha$ -diol-*N*-acetyl-3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranosyl, named spiraloside A. All of the signals of  $^1H$ - and  $^{13}C$ -NMR were assigned by HSQC, HMBC,  $^1H$ - $^1H$  COSY and ROESY spectra (Table 1).

Compound **2** was isolated as a white, amorphous powder;  $[\alpha]_D^{23} -43.9$  ( $c$  0.03, MeOH). The molecular formula  $C_{35}H_{57}NO_8$  was established by the HRESIMS quasi-molecular ion at  $m/z$  642.3975  $[M+Na]^+$  (calcd for  $m/z$  642.3976), indicating eight degree of unsaturation. Its IR spectrum revealed the presence of hydroxyls (3440  $cm^{-1}$ ) and an acylamino group (1630  $cm^{-1}$ ). The 1D-NMR data (Table 1) were similar to those of **1** except the absence of

**Table 1** <sup>1</sup>H and <sup>13</sup>C NMR Spectroscopic Data of Compounds **1–3** (methanol-*d*<sub>4</sub> δ in ppm)

Position	<b>1</b>		<b>2</b>		<b>3</b>	
	δ <sub>H</sub>	δ <sub>C</sub>	δ <sub>H</sub>	δ <sub>C</sub>	δ <sub>H</sub>	δ <sub>C</sub>
1a	1.84 (1H, m)	38.5 (t)	1.83 (1H, m)	38.5 (t)	1.59 (1H) <sup>o1</sup>	37.4 (t)
1b	1.03 (1H, d, 4.3)		1.04 (1H, br s)		1.47(1H) <sup>o2</sup>	
2a	1.89 (1H) <sup>o1</sup>	30.7 (t)	1.89 (1H) <sup>o1</sup>	30.7 (t)	1.83 (1H) <sup>o3</sup>	30.5 (t)
2b	1.57 (1H) <sup>o2</sup>		1.57 (1H) <sup>o2</sup>		1.73 (1H) <sup>o4</sup>	
3	3.53 (1H, m)	79.9 (d)	3.54 (1H, m)		3.66 (1H, m)	79.3 (d)
4a	2.38 (1H, dd, 2.1, 13.1)	39.7 (t)	2.38 (1H, dd, 2.4, 13.2)		1.66 (1H) <sup>o5</sup>	38.2 (t)
4b	2.22 (1H, t, 12.4)		2.21 (1H, t, 12.6)		0.95 (1H, m)	
5		142.0 (s)		142.0 (s)	1.05 (1H, m)	46.0 (d)
6a	5.34 (1H, br s)	122.6 (d)	5.34 (1H, br s)	122.7 (d)	1.49 (1H) <sup>o2</sup>	30.2 (t)
6b					1.27 (1H) <sup>o6</sup>	
7a	1.92 (1H) <sup>o1</sup>	33.0 (t)	1.93 (1H) <sup>o1</sup>	33.0 (t)	1.62 (1H) <sup>o5</sup>	33.3 (t)
7b	1.53 (1H) <sup>o2</sup>		1.53 (1H) <sup>o2</sup>		0.91 (1H,m)	
8	1.44 (1H) <sup>o3</sup>	32.5 (d)	1.45 (1H) <sup>o3</sup>	32.5 (d)	1.33 (1H, m)	36.0 (d)
9	0.93 (1H, d, 4.5)	51.7 (d)	0.93 (1H, d, 5.4)	51.6 (d)	0.65 (1H, m)	55.8 (d)
10		37.9 (s)		37.9 (s)		36.7 (s)
11a	1.48 (1H) <sup>o3</sup>	21.7 (t)	1.48 (1H) <sup>o3</sup>	21.7 (t)	1.58 (1H) <sup>o1</sup>	21.9 (t)
11b	1.30 (1H) <sup>o4</sup>		1.23(1H) <sup>o4</sup>		1.46 (1H) <sup>o2</sup>	
12a	1.92 (1H) <sup>o1</sup>	41.2 (t)	1.91 (1H) <sup>o1</sup>	41.1 (t)	1.87 (1H) <sup>o3</sup>	41.5 (t)
12b	1.23 (1H) <sup>o4</sup>		1.23 (1H) <sup>o4</sup>		1.18 (1H, m)	
13		44.9 (s)		44.8 (s)		45.2 (s)
14	1.31 (1H) <sup>o4</sup>	55.3 (d)	1.31 (1H) <sup>o4</sup>	55.3 (d)	1.30 (1H, m)	55.0 (d)
15a	1.61 (1H) <sup>o5</sup>	37.5 (t)	1.61 (1H) <sup>o5</sup>	37.5 (t)	1.64 (1H) <sup>o5</sup>	35.3 (t)
15b	1.49 (1H) <sup>o3</sup>		1.47 (1H) <sup>o3</sup>		1.55 (1H) <sup>o3</sup>	
16	4.22 (1H, dt, 7.0, 7.4)	76.3 (d)	4.22 (1H, t, 7.4)	76.3 (d)	4.22 (1H, t, 7.4)	76.3 (d)
17	1.18 (1H, dd, 7.0, 12.4)	61.9 (d)	1.18 (1H, t, 7.8)	62.2 (d)	1.16 (1H, t, 7.2)	62.2 (d)
18	0.72 (3H, s)	14.7 (q)	0.72 (3H, s)	14.7 (q)	0.69 (3H, s)	14.8 (q)
19	0.98 (3H, s)	19.8 (q)	0.98 (3H, s)	19.8 (q)	0.79 (3H, s)	13.1 (q)
20	2.27 (1H, ddq, 6.0, 7.1, 12.4)	35.7 (d)	2.27 (1H, ddq, 6.0, 7.1, 14.2)	35.8 (d)	2.27 (1H, ddq, 6.1, 7.2, 14.4)	35.8 (d)
21	0.96 (3H, d, 7.1)	15.1 (q)	0.97 (3H, d, 7.1)	15.1 (q)	0.94 (3H, d, 6.6)	15.1 (q)
22	4.46 (3H, ddd, 6.0, 6.0, 12.0)	55.4 (d)	4.46 (3H, ddd, 6.0, 6.0, 12.1)	55.4 (d)	4.44 (3H, ddd, 6.1, 6.1, 12.6)	55.5 (d)
23a	1.72 (1H) <sup>o6</sup>	22.2 (t)	1.72 (1H) <sup>o6</sup>	22.3 (t)	1.72 (1H) <sup>o4</sup>	22.1 (t)
23b	1.62 (1H) <sup>o5</sup>		1.61 (1H) <sup>o5</sup>		1.61 (1H) <sup>o5</sup>	
24a	1.53 (1H) <sup>o2</sup>	28.6 (t)	1.52 (1H) <sup>o2</sup>	28.6 (t)	1.50 (1H) <sup>o2</sup>	28.6 (t)
24b	1.29 (1H) <sup>o4</sup>		1.29 (1H) <sup>o4</sup>		1.28 (1H) <sup>o6</sup>	
25	1.75 (1H) <sup>o6</sup>	31.3 (d)	1.75 (1H) <sup>o6</sup>	31.3 (d)	1.73(1H) <sup>o4</sup>	31.2 (d)
26a	3.60 (1H) <sup>o7</sup>	50.7 (t)	3.59 (1H) <sup>o7</sup>	50.7 (t)	3.58 (1H) <sup>o7</sup>	50.8 (t)
26b	2.91 (1H, t, 12.4)		2.91 (1H, t, 13.8)		2.90 (1H, t, 13.8)	
27	0.89 (3H, d, 6.6)	19.4 (q)	0.89 (3H, d, 6.6)	19.5 (q)	0.88 (3H, d, 6.6)	19.5 (q)
28		172.4 (s)		172.5 (s)		172.4 (s)
29	2.08 (3H, s)	22.2 (q)	2.07 (3H, s)	22.3 (q)		
1'	4.36 (1H, d, 7.8)	102.3 (d)	4.34 (1H, d, 7.8)	102.4 (d)	4.34 (1H, d, 7.8)	102.2 (d)
2'	3.15 (1H, t, 8.5)	75.2 (d)	3.10 (1H, t, 8.4)	75.1 (d)	3.09 (1H, t, 7.8)	75.1 (d)
3'	3.42 (1H, t, 8.9)	76.7 (d)	3.31 (1H, t, 8.4)	78.0 (d)	3.30 (1H, t, 8.4)	78.1 (d)
4'	3.49 (1H, t, 9.1)	79.6 (d)	3.23 (1H) <sup>o8</sup>	77.8 (d)	3.21 (1H) <sup>o8</sup>	77.8 (d)
5'	3.29 (1H) <sup>o8</sup>	76.8 (d)	3.22 (1H) <sup>o8</sup>	71.5 (d)	3.22 (1H) <sup>o8</sup>	71.6 (d)
6'a	3.76 (1H, d, 12.0)	62.1 (t)	3.81 (1H, d, 12.0)	62.7 (t)	3.81 (1H, d, 12.0)	62.8 (t)
6'b	3.62 (1H) <sup>o7</sup>		3.61 (1H) <sup>o7</sup>		3.60 (1H) <sup>o7</sup>	

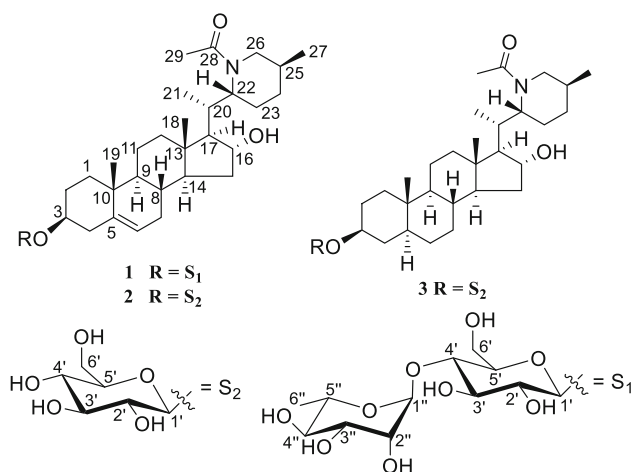
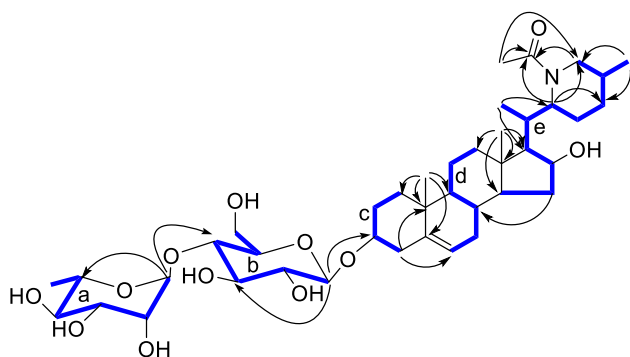
**Table 1** continued

Position	<b>1</b>		<b>2</b>		<b>3</b>	
	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1''	4.81 (1H, br s)	102.9 (d)				
2''	3.80 (1H, br s)	72.4 (d)				
3''	3.59 (1H) <sup>a7</sup>	72.2 (d)				
4''	3.36 (1H, t, 9.5)	73.7 (d)				
5''	3.93 (1H, dq, 6.2, 12.4)	70.6 (d)				
6''	1.23 (3H, d, 6.2)	17.8 (q)				

<sup>1</sup> H-NMR Recorded at 600 MHz, <sup>13</sup>C-NMR Recorded at 150 MHz

<sup>a1-a7</sup> Overlapped signals

<sup>a8</sup> Overlapped CD<sub>3</sub>OD

**Fig. 1** The chemical structures of spiralosides A–C (**1–3**)**Fig. 2** Key <sup>1</sup>H–<sup>1</sup>H COSY (—) and HMBC (—) correlations of **1**

the rhamnopyranosyl moiety, as supported by the HMBC correlations of  $\delta_{\text{H}}$  4.34 (1H, d,  $J = 7.8$  Hz, H-1') with  $\delta_{\text{C}}$  79.8 (d, C-3). Other parts of the structure were identical to those of **1** by detailed analysis of 2D-NMR and acid hydrolysis of **2**. Consequently, spiraloside B (**2**) was determined as (22*R*,25*S*)-22,26-epiminocholest-5-ene-3 $\beta$ ,16 $\alpha$ -diol-*N*-acetyl-3-*O*- $\beta$ -D-glucopyranosyl (Fig. 1).

Spiraloside C (**3**), obtained as an amorphous powder, had the molecular formula of C<sub>35</sub>H<sub>59</sub>NO<sub>8</sub> as revealed by HRESIMS (calcd for C<sub>35</sub>H<sub>59</sub>NO<sub>8</sub> at  $m/z$  621.4241), corresponding to seven degrees of unsaturation. The IR data showed the presence of hydroxys (3440 cm<sup>-1</sup>) and an acylamino group (1619 cm<sup>-1</sup>). Comparison of 1D-NMR data of **3** with those of **2** (Table 1), showed that the two compounds were similar with exception of the absence of a double bond at C-5/6 in **3**, which was suggested by the HMBC correlations of  $\delta_{\text{H}}$  0.79 (3H, s, H-19) with  $\delta_{\text{C}}$  46.0 (d, C-5), and 1.27 (1H, m, H-6b) with  $\delta_{\text{C}}$  46.0 (d, C-5), as well as one less degree of unsaturation. The ROESY correlations of Me-19/H-2b, H-3/H-2a/H-5 indicated the  $\alpha$ -orientation of H-3 and H-5. Other parts of the structure were identical to those of **2**, by detailed analyses of 2D-NMR and acid hydrolysis of **3**. Thus, spiraloside C (**3**) was determined as (22*R*,25*S*)-22,26-epiminocholest-3 $\beta$ ,16 $\alpha$ -diol-*N*-acetyl-3-*O*- $\beta$ -D-glucopyranosyl (Fig. 1).

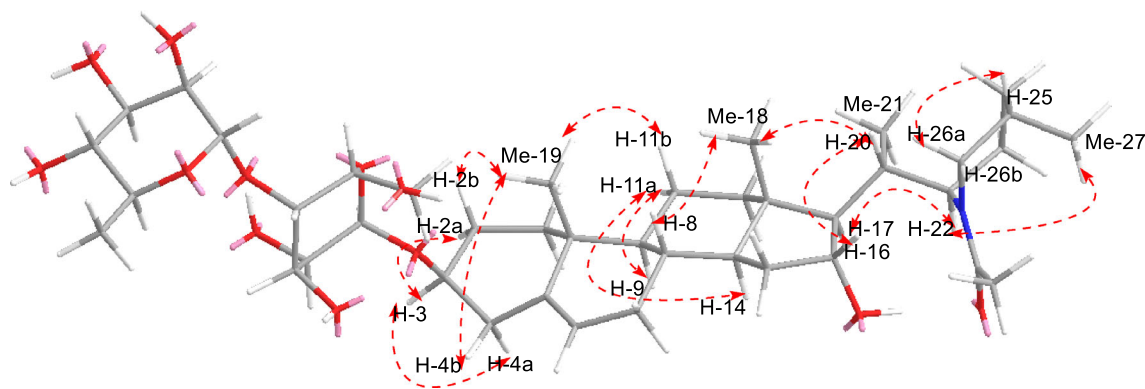
The total alkaloids of *S. spirale* have been screened for the protective anti-tussive effect against ammonia liquor induced cough and the protective expectorant activity used by phenol red secretion test in mice. The results showed that the total alkaloids exhibited an inhibited tendency on antitussive in mice (Tables 2, 3).

Compounds **1–3** were evaluated for their cytotoxicity against five human cancer cell lines using the MTT method as reported previously [23]. Cisplatin (sigma, USA) was used as the positive control. Unfortunately, the results showed that all compounds were inactive (IC<sub>50</sub> values >40  $\mu$ M).

## 3 Experimental Section

### 3.1 Plant Material

Air-dried fruits of *S. spirale* were collected in December 2014 from Shuangbai county, Yunnan province, P.R china,



**Fig. 3** Key ROESY (→) correlations of **1**

**Table 2** Effect of the total alkaloids on the ammonia liquor induced cough in mice

Groups	Dose (mg/kg)	Treatment	Frequency of cough	Inhibition (%)
Control	–	ig	23.5 ± 10.3	–
Codeine phosphate	30	ig	9.2 ± 4.1**	60.9
The total alkaloids	10	ig	16.4 ± 4.8	30.2

Values expressed as mean ± SEM (n = 10)

\*\*  $P < 0.01$  for comparison of treated groups with control

**Table 3** Effects of the total alkaloids on tracheal phenol red output in mice

Groups	Dose (mg/kg)	Treatment	Absorbance	Phenol red secretion (μg/mL)
Control	–	ig	0.140 ± 0.058	2.66 ± 1.64
Carbocisteine	10	ig	0.214 ± 0.063*	4.75 ± 1.77*
The total alkaloids	10	ig	0.175 ± 0.053	3.67 ± 1.49

Values expressed as mean ± SEM (n = 10)

\*  $P < 0.05$  for comparison of treated groups with control

and identified by one of the author Yun-Li Zhao, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 20141225) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

### 3.2 Extraction and Isolation

The air-dried fruits from *S. spirale* (5 kg) were crushed and extracted with 20 L 90 % MeOH for five times under reflux for a total 3 h, and then combined extract was concentrated under reduced pressure to afford an extract. The extract was partitioned between EtOAc and 0.5 % HCl solution, getting a nor-alkaloid fraction. The acidic water-soluble, adjusted pH to 9–10, was extracted with EtOAc to give total alkaloids and water-soluble fraction. The total alkaloid fraction (84 g) was subjected to silica gel column (200–300 mesh; gradient CHCl<sub>3</sub>–MeOH 20:1, 10:1, 8:1, 6:1, 4:1, 3:1, 1:1, 0:1 v/v) to afford fractions 1–6,

respectively. Fr.3 (5 g) was chromatographed over silica gel CC (200–300 mesh), eluted with repeated CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (10:1:0.1 to 0:1:1) to obtain three subfractions (Fr.3-1 to Fr.3-3). Fr.3-1 (66 mg) was then separated by repeated RP-18 CC with MeOH:H<sub>2</sub>O (5:5 to 10:0) and Sephdex LH-20 (MeOH) to give **3** (9 mg). Fr.4 (8.4 g) was submitted to RP-18 CC and elicited with MeOH:H<sub>2</sub>O (1:9 to 8:2) to afford five subfractions (Fr.4-1 to Fr.4-5). Fr.4-3 (900 mg) was chromatographed over silica gel CC (200–300 mesh), eluted with CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (8:2:0.2 to 0:1:1) to obtain three subfractions (Fr.4-3-1 to Fr.4-3-3). Fr.4-3-2 (35 mg) was further purified by Sephdex LH-20 (MeOH/H<sub>2</sub>O, 1:9) to obtain **2** (10 mg). Fr.6 (6 g) was separated by RP-18 CC and elicited with MeOH:H<sub>2</sub>O (1:9 to 10:0) to yield five subfractions (Fr.6-1 to Fr.6-5). Fr.6-3 (1.5 g) was further separated by repeated silica gel with CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O (7:3:0.3 to 0:1:1), then followed by Sephdex LH-20 with MeOH:H<sub>2</sub>O (1:9 to 0:1) to obtained three subfractions (Fr.6-3-1 to Fr.6-3-3), Fr.6-3-2 (25 mg) was further

purified by semi-preparative HPLC (28 % CH<sub>3</sub>CN) to yield **1** (13 mg).

Spiraloside A (**1**): White amorphous powder;  $[\alpha]_D^{23}$   $-46.6$  (*c* 0.03, MeOH); IR (KBr)  $\nu_{\max}$  3441, 2934, 2870, 1623, 1442, 1383, 1343, 1266, 1127, 1056, 1038, 809 cm<sup>-1</sup>; <sup>1</sup>H, <sup>13</sup>C-NMR spectroscopic data see Table 1; Positive ESIMS *m/z* 788 [M+Na]<sup>+</sup>; HREIMS *m/z* 788.4553 [M+Na]<sup>+</sup> (calcd for C<sub>41</sub>H<sub>67</sub>NO<sub>12</sub>, 788.4561).

Spiraloside B (**2**): white amorphous powder;  $[\alpha]_D^{23}$   $-43.9$  (*c* 0.03, MeOH); IR (KBr)  $\nu_{\max}$ : 3440, 2969, 2928, 1630, 1553, 1445, 1383, 1267, 1197, 1079, 1046 cm<sup>-1</sup>; <sup>1</sup>H, <sup>13</sup>C-NMR spectroscopic data see Table 1; Positive ESIMS *m/z* 642 [M+Na]<sup>+</sup>; HREIMS *m/z* 642.3975 [M+Na]<sup>+</sup> (calcd for C<sub>35</sub>H<sub>57</sub>NO<sub>8</sub>, 642.3976).

Spiraloside C (**3**): white amorphous powder;  $[\alpha]_D^{24}$   $-30.1$  (*c* 0.09, MeOH); IR (KBr)  $\nu_{\max}$ : 3427, 2928, 2858, 1727, 1619, 1444, 1382, 1075, 1046 cm<sup>-1</sup>; <sup>1</sup>H, <sup>13</sup>C-NMR spectroscopic data see Table 1; Positive ESIMS *m/z* 644 [M+Na]<sup>+</sup>; HREIMS *m/z* 621.4231 [M+H]<sup>+</sup> (calcd for C<sub>35</sub>H<sub>59</sub>NO<sub>8</sub>, 621.4241).

### 3.3 Acid Hydrolysis of compounds **1–3** and GC Analysis

Compounds **1–3** (each 3 mg) were refluxed with 2 M HCl (1,4 dioxane/H<sub>2</sub>O 1:1, 2 mL) on water bath for 2 h. After cooling, the reaction mixture was neutralized with 1 M NaOH. The reaction mixture was extracted with CHCl<sub>3</sub> (3 × 5 mL). The aqueous layer was evaporated to dryness. The dried residue was dissolved in 1 mL anhydrous pyridine and treated with L-cysteine methyl ester hydrochloride (1.5 mg) stirred at 60 °C for 1 h. Trimethylsilylimidazole (1.0 mL) was added to the reaction mixtures, and they were kept at 60 °C for 30 min. The supernatants (4 μL) were analyzed by GC, respectively, under the following conditions: H<sub>2</sub> flame ionization detector. Column: 30QC2/AC-5 quartz capillary column (30 m × 0.32 mm). Column temperature: 180–280 °C with the rate of 3 °C/min, and the carrier gas was N<sub>2</sub> (1 mL/min) injector temperature: 250 °C; and split ratio: 1/50. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-glucose and L-rhamnose after treatment with trimethylchlorosilane (TMCS) in pyridine. The absolute configurations of the compounds **1–3** were determined by comparison of the retention times of the corresponding derivatives with those of standard D-glucose and L-rhamnose giving a single peak at 19.01 and 15.43 min, respectively.

### 3.4 Animals

ICR mice of either sex (20–22 g) were purchased from Kunming Medical College (License number SYXK2014-

0004). All animals were housed at room temperature (20–25 °C) and constant humidity (40–70 %) under a 12 h light–dark cycle in SPF grade laboratory. The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

### 3.5 Anti-tussive Activity Assay

ICR mice of either sex weighing 21–24 g were divided randomly, 10 mice per group. The negative control of animals was treated with distilled water orally, and the positive control was treated with codeine phosphate, the remaining groups treated were with test samples respectively. Anti-tussive activity was investigated on a classical mouse cough model induced by ammonia liquor [24]. Briefly, each mouse was placed in a 300 mL special glass chamber and exposed to 40 μL 25 % NH<sub>4</sub>OH. The cough frequency produced during 2 min exposure period was counted. In the second assay for alkaloids, cough frequency and latent period of cough were recorded.

### 3.6 Expectorant Effect Assessment

The procedures were performed as described previously [25]. Male and female mice were randomly allotted and treated with a single dose 30 min before intraperitoneal injection of phenol red solution (5 % in saline solution, w/v, 0.1 mL/10 g body weight). Mice were sacrificed by cervical dislocation 30 min after application of phenol red. After dissected free from adjacent organs, the trachea was removed from the thyroid cartilage to the main stem bronchi and put into 2 mL normal saline immediately. After ultrasonic for 5 min, 0.1 mL of 1 M NaOH solution was added to the saline and optical density of the mixture were measured at 546 nm using enzyme standard instrument.

### 3.7 Cytotoxicity Assay

Five human cancer cell lines, lung cancer A-549, human myeloid leukemia HL-60, hepatocellular carcinoma SMMC-7721, breast cancer MCF-7, and colon cancer SW480 cells, were used in the cytotoxic assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10 % fetal bovine serum (Hyclone, USA) in 5 % CO<sub>2</sub> at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method in 96-well microplates [26]. Briefly, 100 μL of adherent cells was seeded to each well of a 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while

suspended cells were seeded just before drug addition with an initial density of  $1 \times 10^5$  cells/mL in 100  $\mu$ L of medium. Each tumor cell line was exposed to the test compound dissolved in DMSO at concentrations of 0.064, 0.32, 1.6, 8, and 40  $\mu$ M in triplicates for 48 h, with cisplatin (Sigma, USA) as a positive control. After compound treatment, cell viability was detected, and the cell growth curve was graphed. The IC<sub>50</sub> value was calculated by Reed and Muench's method [27].

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#### Compliance with Ethical Standards

**Conflict of Interest** The authors declare no conflict of interest.

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