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THREE NEW DITERPENE ALKALOIDS FROM *SPIRAEA JAPONICA*

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Abstract - Chemical investigation of the basic fraction from the roots of *Spiraea japonica* var. *acuta* has resulted in the isolation of three new diterpene alkaloids, spiramines X (**1**), Y (**2**) and Z (**3**), along with five known compounds, spiradine F (**4**), and spiramines A, B, P (**5**), and U (**6**). The structures of **1-3** were determined by spectral and chemical methods. Antiplatelet aggregation activities of compounds (**1- 6**) were tested.

INTRODUCTION

Spiraea japonica L. (Rosaceae) and its varieties are widespread in Yunnan Province, People's Republic of China. The young leaves, fruits, and roots of some of these plants have been used as diuretic, detoxicant, and analgesic agents and for the treatment of inflammation, cough, headache, and toothache in traditional Chinese medicine.[1, 2] Previous investigations on the roots of *S. japonica* var. *acuta* have led to the report of two new diterpene alkaloids, spiramines U and T, and other known constituents.[3] During the course of the continued studies on biologically active and/or structurally novel compounds from *S. japonica* and its varieties,[3-11] we collected a large amount of the roots of *S. japonica* var. *acuta* from Li Jiang of Yunnan Province. Chemical investigation on the root material led to the isolation of three new alkaloids of the spiramine series, spiramines X (**1**), Y (**2**), and Z (**3**), together with the known compounds, spiradine F (**4**), and spiramines A, B, P (**5**), and U (**6**) (Figure 1). This paper describes the isolation, structural elucidation, and antiplatelet aggregation activity of these isolated alkaloids.

RESULTS AND DISCUSSION

The 95% EtOH extract of the roots of *S. japonica* var. *acuta* was separated into an alkaloid and a non-alkaloid fractions. The alkaloid fraction was chromatographically separated over silica gel to afford eight diterpenoid alkaloids. The structures of the known compounds, spiradine F (**4**) [12] and spiramines A,[4, 13] B,[4, 13] P (**5**) [9] and U (**6**) [3] were established by comparison with published spectral data. Here it deserve to mention that the hydroxyl group in spiramine P and acetoxyl group in spiramine U have been reassigned to attach at C-6 position as shown in Figure 1, respectively.[14]

Spiramine X (**1**) was determined to have the molecular formula $C_{26}H_{35}NO_6$ by HRMS and showed the presence of carbonyl group (1743 cm^{-1}) and carbon-carbon double bond (1658 cm^{-1}) in its IR spectrum. The ^1H NMR spectrum of **1** showed signals for a tertiary methyl group (δ 1.13) and two acetate methyls (δ 2.02 and 2.04). The ^{13}C NMR and DEPT spectra of **1** showed twenty-six carbon signals, including three methyls, ten methylenes, six methines, and seven quaternary carbon atoms. Comparison of the ^1H and ^{13}C NMR spectral data of **1** with those of spiramine R (**7**) [9] (Figure 1) showed that the two compounds possess similar carbon skeleton with a different pattern of substituents.

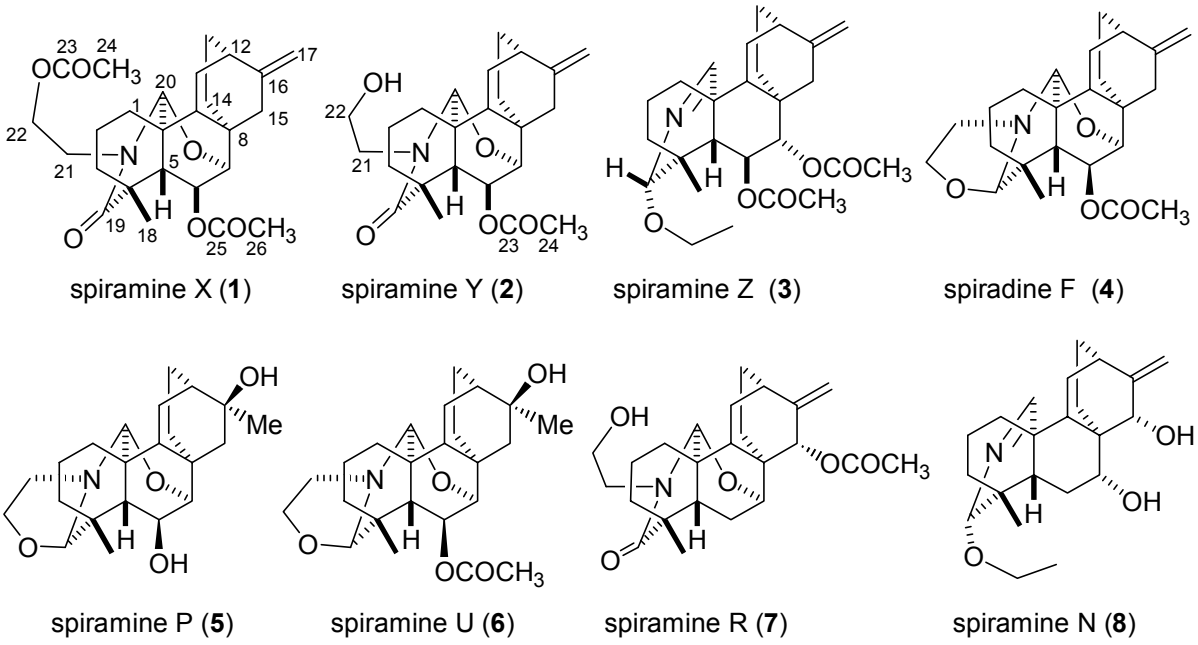


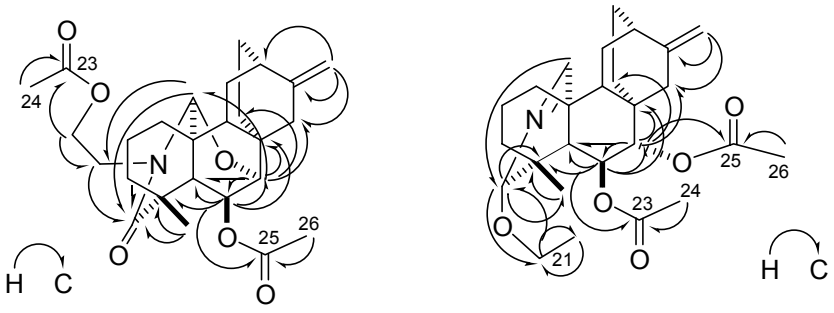
Figure 1. Structures of **1-8**

The ^1H NMR and associated ^{13}C NMR resonances of **1** were assigned by a HMQC experiment (see Table 1). The locations of the acetoxyl groups were determined by NMR comparison of **1** to **7**, the resonance assignments of the HMQC experiment, and a HMBC experiment. The ^{13}C NMR of **1** displayed four resonances (δ 62.4, 70.3, 71.8 and 85.7) for carbon linked to ether/ester oxygens. Two of these resonances (δ 70.3 and 71.8) correlated with methine protons in the ^1H NMR spectrum at δ 3.65 and 5.12

in HMQC experiment. These two methine protons exhibited cross peaks (J=3.8 Hz) in the ^{1}H-^{1}H COSY spectrum, confirming the protons on vicinal dioxy substituted carbons. A HMBC experiment (Figure 2) established the δ 3.65 resonance to be H-7 through long-range correlations between the proton and C-5, C-6, C-8, C-9, C-14, C-15, and C-20 (δ 85.7). The HMBC experiment further correlates the δ 5.12 proton to C-4, C-5, C-7, C-8 and C-25, confirming its vicinal relationship to the δ 3.65 proton and placing an acetoxyl group at C-6 (Figure 2).

In a HMQC experiment, C-H direct correlations were observed between the proton signal at δ 4.18 and the oxygenated methylene carbon at δ 62.4 (C-22). The proton signal at δ 4.18 (2H, m, H-22) in the ^{1}H NMR was correlated with signals of a methylene at δ 3.28 (1H, m, H-21a) and 4.01 (1H, m, H-21b) in the ^{1}H-^{1}H COSY spectrum. These methylene protons showed no other ^{1}H-^{1}H correlations thereby defining an isolated substituted ethyl group in **1**. Long-range correlations between the acetoxyl methyl protons at δ 2.02 (H-24) and the quaternary carbon at δ 170.6 (C-23), between the methylene protons at δ 4.18 and C-23 and C-21, and between methylene protons at δ 3.28, 4.01 (H-21) and C-19 (δ 174.6), C-20 (δ 85.7), and C-22 (δ 62.4) in the HMBC spectrum established the C-22 substitution of the second acetoxyl group in **1**.

The existence of a C-19 carbonyl group was confirmed by long-range correlations between the proton signals of H-3, H-5, H-18, H-20, H-21 and the carbonyl carbon signal at δ 174.6 (C-19) in the HMBC spectrum (Figure 2).



spiramine X (1) **spiramine Z** (3)

Figure 2. Selected HMBC correlations of spiramine X (**1**) and Z (**3**)

The stereochemistry of **1** was determined from the NOESY spectrum, in which the H-5 signal showed cross peaks with H-18 and H-7, indicating that H-5, H-7, and H-18 should be in *syn*- and β-positions. The H-5 and H-7 proton signals also had cross peaks with the acetyl methyl group at δ 2.04 (H-26), suggesting that the acetyl group at C-6 was at the β-position and H-6 at the α-position.

These data designated the structure of spiramine X (**1**) as depicted.

Table 1. ^{1}H, ^{13}C NMR, and DEPT Spectral Data for Compounds (**1-3**) (δ in ppm, $CDCl_3$)

No	**1** [a]		**2** [b]		**3** [a]	
	H	**C**	**H**	**C**	**H**	**C**
1	1.38, 1.68, each 1H, m	28.9 t	1.37, 1.70, each 1H, m	28.8 t	1.09, 1.66, each 1H, m	34.2 t
2	1.46, 1.71, each 1H, m	25.4 t	1.48, 1.75, each 1H, m	25.3 t	0.98, 1.94, each 1H, m	36.1 t
3	1.52, 1.58, each 1H, m	19.9 t	1.55, 1.61, each 1H, m	19.8 t	1.34, 2 H, m	19.4 t
4		43.7 s		43.8 s		36.7 s
5	1.49, 1H, br s	55.1 d	1.50, 1H, br s	55.1 d	1.52, 1H, d, *J* = 10.2 Hz	51.9 d
6	5.12, 1H, t, *J* = 3.8 Hz	71.8 d	5.13, 1H, t, *J* = 4.6 Hz	71.6 d	5.08, 1H, t, *J* = 10.2 Hz	69.2 d
7	3.65, 1H, d, *J* = 3.8 Hz	70.3 d	3.73, 1H, d, *J* = 4.8 Hz	70.7 d	4.65, 1H, d, *J* = 10.2 Hz	79.4 d
8		36.1 s		36.0 s		37.7 s
9	1.40, 1H, m	46.3 d	1.44, 1H, m	46.2 d	1.50, 1H, m	45.5 d
10		34.3 s		34.3 s		43.6 s
11	2.07, 2.36, each 1H, m	39.3 t	2.08, 2.38, each 1H, m	39.2 t	1.78, 2H, m	27.8 t
12	2.33, 1H, m	36.5 d	2.35, 1H, m	36.4 d	2.27, 1H, m	35.6 d
13	1.37, 1.87, each 1H, m	26.7 t	1.36, 1.90, each 1H, m	26.6 t	1.52, 1.58, each 1H, m	25.7 t
14	1.40, 1.90, each 1H, m	26.6 t	1.35, 1.85, each 1H, m	26.7 t	1.48, 1.56, each 1H, m	21.4 t
15	1.29, 1.79, each 1H, m	39.4 t	1.33, 1.77, each 1H, m	39.6 t	1.92, 2.19, each 1H, d, *J* = 17.2 Hz	41.3 t
16		150.1 s		149.8 s		149.3 s
17	4.66, 4.82, each 1H, br s	108.4 t	4.66, 4.82, each 1H, br s	108.4 t	4.54, 4.71, each 1H, s	105.9 t
18	1.13, 3 H, s	20.7 q	1.13, 3 H, s	20.6 q	0.84, 3 H, s	26.4 q
19		174.6 s		175.3 s	4.56, 1 H, s	94.1 d
20	4.78, 1H, d, *J* = 1.8 Hz	85.7 d	4.78, 1H, d, *J* = 1.6 Hz	86.1 d	7.74, 1H, s	163.2 d
21	3.28, 4.01, each 1H, m	45.4 t	3.20, 3.63, each 1H, m	51.7 t	3.59, 4.03, each 1H, m	64.2 t
22	4.18, 2H, m	62.4 t	3.79, 2H, m	61.9 t	1.13, 3H, t, *J* = 7.0 Hz	15.1 q
23		170.6 s		169.4 s		170.4 s
24	2.02, 3H, s	20.8 q	2.04, 3H, s	21.1 q	1.95, 3H, s	20.6 q
25		169.3 s				170.4 s
26	2.04, 3H, s	21.1 q			1.89, 3H, s	21.4 q

[a] Assignments based on the analysis of ^{1}H-^{1}H COSY, HMQC, and HMBC spectra. [b] Assigned by comparison with those of **1**.

Spiramine Y (**2**) was assigned a molecular formula of $C_{24}H_{33}NO_5$ as determined by HRMS. Comparison of the ^{1}H, ^{13}C NMR data of **2** to those of **1** suggested that **2** was a diterpene alkaloid with the same carbon skeleton as **1** with only one acetyl substituent. The ^{1}H NMR displayed a single acetyl methyl resonance

(δ 2.04) and the ^{13}C NMR spectrum of **2** differed from **1** with changes in chemical shifts for C-22 and C-21, and the absence of a carbonyl and an acetyl methyl group.
The structural designation for **2** was further confirmed by treatment with pyridine/Ac_2O to give **1**, whose MS and co-TLC behavior were identical with the authentic sample, spiramine X.

The molecular formula of spiramine Z (**3**) was determined to be $C_{26}H_{37}NO_5$ by HRMS. The IR spectrum showed the presence of double bond (1650 cm^{-1}) and ester carbonyl (1746 cm^{-1}) groups in the molecule. A detailed inspection of the ^1H and ^{13}C NMR spectra of **3** with those reported for spiramine N (**8**), a diterpenoid alkaloid bearing an ethoxyl group at C-19 and an imine group at C-20, previously isolated from *Spiraea japonica* var. *acuminata* Franch, [8] showed that the structures of these two compounds are very similar. A difference of more 84 mass units in the MS spectra of the latter compound compared to that of **8** suggested a possible acetylation of the two hydroxyl groups in ^1H and ^{13}C differences **3** *vs* **8**. The locations of the two acetoxyl groups at C-6 and C-7 in **3** were confirmed by the COSY and HMBC experiments. In the COSY spectrum, a cross peak was observed between the low field acetoxyl methine protons at δ 5.08 (H-6) and 4.65 (H-7). The H-6 signal was further coupled to the doublet of H-5 at δ 1.52. In HMBC spectrum (Figure 2), both of the H-6 and C-6 acetoxyl methyl proton signals showed long-range cross peaks with the carbonyl carbon signal of the C-6 acetoxyl group at δ 170.4 (C-23), suggested that an acetoxyl group was attached at the C-6 position. Similarly, the long-range correlations between H-7 and C-7 acetoxyl methyl signals with the carbonyl carbon signal of the C-7 acetoxyl group at δ 170.4 (C-25) indicated that another acetoxyl group should be attached to C-7 position.
The relative stereochemistry of **3** was deduced using a combination of the ^1H-^1H coupling constants and NOE interactions from a NOESY experiment. The large couplings observed between H-5 and H-6 (J = 10.2 Hz), between H-6 and H-7 (J = 10.2 Hz) required that H-5, H-6, and H-7 were located in *trans*-axial positions (β, α, and β) and the corresponding acetoxyl groups at C-6 and C-7 should be in β and α configurations, respectively. The observed cross peak between H-5 and H-7 in NOESY spectrum was in agree with these designations. The assignment of S-configuration for the C-19 in **3** was based on the ^{13}C signal at δ 94.1 (C-19) according to a literature data (at δ 95 for C-19S and δ 91 for C-19R), [3] thereby defining the structure of spiramine Z as **3**. Here it should be noted that since ethanol has been used for extraction, maybe spiramine Z was an artifact formed during the extraction procedure.

The antiplatelet aggregation activities of compounds (**1-6**) have been assayed [15] using ginkgolide B and acetylsalicylic acid (ASA) as positive controls. Compounds (**1-4**) and (**6**) showed inhibition against platelet activating factor (PAF)-induced platelet aggregation. Compounds (**1-4**) also exhibited modest

inhibition against adenosine diphosphate (ADP)-induced platelet aggregation.

Table 2. Percentage Inhibition of Compounds (**1**-**6**) (240 μM) Against the Aggregation of Rabbit Platelets Induced by Platelet-Activating Factor (PAF), Arachidonic Acid (AA), and Adenosine Diphosphate (ADP)[a]

Compounds	Percentage Inhibition (%)		
	PAF (4.5 nM)	AA (350 μM)	ADP (5 μM)
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	29.2 ± 7.7 [b]	13.2 ± 5.9	37.4 ± 7.7 [b]
2	46.0 ± 8.0 [b]	0.3 ± 0.7	31.1 ± 7.4 [b]
3	41.6 ± 8.3 [b]	2.1 ± 1.9	35.3 ± 10.8 [b]
4	58.5 ± 8.2 [b]	3.1 ± 3.0	34.5 ± 6.7 [b]
5	6.3 ± 2.0	6.8 ± 3.5	8.2 ± 3.2
6	49.3 ± 6.3 [b]	15.6 ± 3.8	4.9 ± 1.9
Ginkgolide B [c]	80.2 ± 4.4 [b]		
Acetylsalicylic acid [c]		86.0 ± 1.8 [b]	

[a] Platelets were preincubated with control (5 % polyethylene glycol) and each compound (240 μM) was tested at 37 °C for 5 min, and then the inducer was added. The data were expressed as means ± SD (n = 4). [b] $P < 0.01$ as compared with control (*t*-test). [c] Positive control used at a concentration of 120 μM.

EXPERIMENTAL

General Experimental Procedures. Melting points were determined using a Kofler micro-melting point apparatus and are uncorrected. Optical rotations were determined on a Horiba SEPA-300 polarimeter. IR spectra were obtained on KBr pellets using a Bio-Rad FTS-135 spectrophotometer. 1D and 2D NMR spectra were recorded on a Bruker AM-400 and Bruker DRX-500 spectrometers, respectively, using TMS as internal standard. EIMS and HRFABMS measurements were carried out on a VG Auto Spec-3000 spectrometer. TLC were performed on plates precoated with silica gel F_{254} (Qingdao Marine Chemical Ltd., People's Republic of China). Solvents were distilled prior to use.

Plant Material. The roots of *Spiraea japonica* var. *acuta* were collected in Li Jiang, Yunnan Province, in July, 1998, and the specimen was identified by Prof. Zheng-Wei Lu of Kunming Botanical Garden. A voucher specimen has been deposited in the Herbarium of Kunming Institute of Botany, Chinese

Academy of Sciences.

Extraction and Isolation. The air-dried roots of *Spiraea japonica* var. *acuta* (200 Kg) were extracted twice with 95% ethanol (250 L at 80 ^{o}C for 4 h each time) in a semi-plant scale equipment, Kunming Institute of Botany. The extracts were condensed *in vacuo* to afford a crude mixture (12 kg) which was dissolved in 3% HCl (50 L) solution and filtered. The acidic solution was basified with 5% NaOH aqueous to pH 11 and then extracted with $CHCl_3$. Evaporation of the $CHCl_3$ solution gave 1.2 kg of a crude alkaloid fraction which was all subjected to column chromatography on silica gel. Elution was carried out with mixtures of solvents of increasing polarity starting with petroleum ether-acetone-diethylamine (from 40:10:1 to 20:10:1). The fractions (60g) eluted with petroleum ether-acetone-diethylamine (40:10:1) were combined and concentrated, and then further purified by repeated flash column chromatography on silica gel using petroleum ether-ethyl acetate-methanol (32:4:1) to afford the major components spiradine F (**4**) (20 g), and spiramines A (3.5 g), B (3.0 g), and the minor constituent (**3**) (50 mg). The fraction (8g) eluted with petroleum ether-acetone-diethylamine (30:10:1) was also purified by flash column chromatography on silica gel using petroleum ether-diethyl ether (1:2) as solvent to give 60 mg of **1** and 40 mg of **2**. Spiramines P (**5**) (300 mg) and U (**6**) (1.0 g) were obtained from the fractions (12g) eluted with petroleum-acetone-diethylamine (20:10:1).

Bioassay. The assay were carried out by Born's method. 15 Results are given in Table 2.

***Spriramine X* (1)** $C_{26}H_{35}NO_6$: Colorless needles (acetone); mp 125-127 ^{o}C; $[\alpha]_D^{25}$ -139.7 ^{o} (*c* 0.23, $CHCl_3$); IR (KBr) ν_{max}: 2927, 1743, 1658, 1469, 1371, 1235, 1213, 1051, 1034, 1012 cm^{-1}; ^{1}H- and ^{13}C NMR (see Table 1); EIMS 70 eV *m/z* (rel. int.): 457 $[M]^+$ (15), 397 $[M\text{-}HOAc]^+$ (100), 384 (18), 255 (15), 243 (36); HRFABMS (positive) *m/z* 458.2548 $[M+1]^+$ (calcd for $C_{26}H_{36}NO_6$, 458.2543).

***Spiramine Y* (2)** $C_{24}H_{33}NO_5$: Colorless needles (acetone); mp 144-145 ^{o}C; $[\alpha]_D^{27}$ -152 ^{o} (*c* 0.75, $CHCl_3$); IR (KBr) ν_{max}: 3443, 2930, 2874, 2853, 1742, 1646, 1470, 1378, 1318, 1236, 1215, 1051, 1035, 1013 cm^{-1}; ^{1}H- and ^{13}C NMR (see Table 1); EIMS 70 eV *m/z* (rel. int.): 415 $[M]^+$ (39), 397 $[M\text{-}H_2O]^+$ (16), 384 $[M\text{-}CH_2OH]^+$ (52), 372 $[M\text{-}Ac]^+$ (100), 356 $[M\text{-}OAc]^+$ (23), 355 $[M\text{-}HOAc]^+$ (15), 312 (21), 243 (41); HRFABMS (positive) *m/z* 416.2415 $[M+1]^+$ (calcd for $C_{24}H_{34}NO_5$, 416.2437).

***Acetylation of* 2**: Compound (**2**) (10 mg) was acetylated using 2 mL of Ac_2O in 2 mL of pyridine and left at rt overnight. The acetylated product was purified by preparative TLC (petroleum ether-diethyl ether, 1:2, developed two times, R_f = 0.42) to give a monoacetate whose mp, EIMS, and co-TLC were identical with those of **1**.

***Spiramine Z* (3)** $C_{26}H_{37}NO_5$: Colorless waxy solid. $[\alpha]_D^{27}$ = + 81.7 ^{o} (*c* 0.35, $CHCl_3$); IR (KBr) υ_{max}: 2934, 2873, 1746, 1650, 1461, 1372, 1247, 1232, 1110, 1032 cm^{-1}; ^{1}H- and ^{13}C NMR (see Table 1); E IMS 70

eV *m/z* (rel. int.): 443 $[M]^+$ (100), 384 $[M\text{-}OAc]^+$ (92), 383 $[M\text{-}HOAc]^+$ (63), 372 (95), 354 (22), 324 (51), 312 (33), 294 (20), 267 (15); HRFABMS (positive) *m/z* 444.2781 $[M+1]^+$ (calcd for$C_{26}H_{38}NO_5$, 444.2750)

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