Steroidal Saponins from Rhizomes of *Tupistra wattii* Hook. f.

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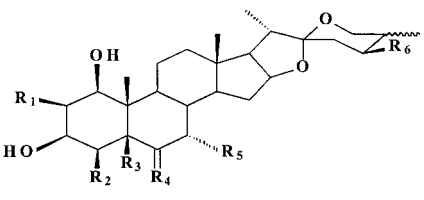
Chemical examination of the fresh rhizomes of *Tupistra wattii* Hook. f. led to the isolation of three new steroidal saponins, wattoside G (1), H (2), and I (3), together with one known steroidal saponin, (25*S*)-1β,3β,4β-trihydroxyspirotan-5β-yl-*O*-β-D-glucopyranoside (4). The structures of 1—3 were established to be (25*R*)-1β,2β,3β,5β-tetrahydroxyspirostan-4β-yl-*O*-β-D-xylopyranoside (1), (24*S*,25*S*)-24-[(β-D-glucopyranosyl)oxy]-1β,2β,3β,4β,5β,7β-hexahydroxyspirostan-6-one (2), and (24*S*,25*S*)-1β,3β-dihydroxy-5β-spirostan-24-yl-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (3) on the basis of detailed analyses of physical, chemical, and spectral data. The isolated compounds were evaluated for cytotoxic activity against the cancer cell line K562 *in vitro*.

Key words *Tupistra wattii*; steroidal saponin; wattoside G; wattoside H; wattoside I; cancer cell line K562

There are several species of the *Tupistra* genus (Liliaceae) in southwestern China, possessing similar morphologic characteristics, and some can be substituted for each other as a folk medicine.[1] However, according to reports on the chemical structure of the *Tupistra* genus,[2—8] these species have different chemical components showing various bioactivities. Therefore further investigation of the chemical constituents of *Tupistra* appears worthwhile. *Tupistra wattii* Hook. f. is widely distributed in Yunnan Province, China, and the fresh rhizomes are used as a folk herbal medicine for the treatment of several inflammatory diseases such as pharyngitis, tonsillitis, bronchitis, and cystitis.[9] The isolation of a new steroid sapogenin and several new steroidal glycosides has been reported from this plant.[5,8] In this paper, we continue to report the isolation and structure elucidation of three new spirostanol saponins, (25*R*)-1β,2β,3β,5β-tetrahydroxyspirostan-4β-yl-*O*-β-D-xylopyranoside (**1**), (24*S*,25*S*)-24-[(β-D-glucopyranosyl)oxy]-1β,2β,3β,4β,5β,7β-hexahydroxyspirostan-6-one (**2**), and (24*S*,25*S*)-1β,3β-dihydroxy-5β-spirostan-24-yl-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**3**), as well as one known steroidal saponin, (25*S*)-1β,3β,4β-trihydroxyspirotan-5β-yl-*O*-β-D-glucopyranoside (**4**)[10] based on detailed physical, chemical, and spectral analyses. The activity of the isolated compounds against the cancer line K562 was also investigated.

Compound **1** was obtained as an amorphous powder, which was positive in the Leibermann–Burchard reaction and Molish test. The molecular formula of $C_{32}H_{52}O_{11}$ was determined by the pseudo-molecular peak at *m/z* 611.3466 $[M-H]^-$ (Calcd for 611.3431) in the negative high resolution (HR)-FAB-MS and distortionless enhancement by polarization transfer (DEPT) spectra. The IR spectrum of **1** showed a strong absorption band of hydroxyl groups at 3412 cm^{-1} and characteristic bands due to the F-ring of a spirostanol derivative at 982, 920<899 and 851 cm^{-1} (25*R*). In the FAB-MS (negative), in addition to the anion peak at *m/z* 611 $[M-H]^-$, a strong peak at *m/z* 479 $[M-H-132]^-$ indicated the existence of one pentose unit in the molecule. The ^{1}H-NMR spectrum showed signals for two angular methyl groups at δ 1.58 (3H, s) and 0.85 (3H, s), two secondary methyl groups at δ 1.18 (3H, d, J=6.5 Hz) and 0.73 (3H, d, J=6.8 Hz), and one anomeric proton signal at δ 5.18 (1H, d, J=7.3 Hz), along with IR absorptions, suggesting **1** to be a spirostanol glycoside. Complete acid hydrolysis of **1** gave an aglycon (**1a**), which was identified as pentologenin [(25*R*)-spirostane-1β,2β,3β,4β,5β-pentol][11] by analysis of its IR, FAB-MS, and NMR data. The hydrolysate was trimethylsilated with trimethyl-chlorsilan, and the GC-retention time (506 s) of the derived sugar was compared with those of authentic samples prepared in the same manner, indicating that the sugar was a xylose derivative (506 s). The ^{13}C-NMR spectrum exhibited a total of 27 carbon signals arising from the aglycone moiety, and according to the literature[12] the other five signals were assigned to the pentopyranosyl moiety [δ 103.2 (C-1), 75.4 (C-2), 78.3 (C-3), 71.4 (C-4), 67.5 (C-5)] corresponding to a β-D-xylopyranosyl group. In the ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectrum of **1**, the anomeric proton signal (δ 5.18) correlated with the carbon signal at δ 74.1, which could be assigned to C-4 by combined analysis of the HMBC, ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC), and ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H correlation spectroscopy (COSY)) spectra. Accordingly, the structure of **1** was determined to be (25*R*)-1β,2β,3β,5β-tetrahydroxyspirostan-4β-yl-*O*-β-D-xylopyranoside and called wattoside G.

Compound **2** was obtained as colorless needles (AcOEt/acetone/H_2O). The IR spectrum showed a band at 1706 cm^{-1}, indicating a carbonyl group in the molecule. The HR-FAB-MS (negative) gave a pseudo-molecular ion peak at *m/z* 687.3278 $[M-H]^-$ (Calcd for 687.3228), corresponding to the molecular formula $C_{33}H_{52}O_{15}$. The fragment at *m/z* 525 $[M-H-162]^-$ indicated the existence of one hexose unit in



1	R_1=OH	R_2=O-Xyl	R_3=OH	R_4=H_2	R_5=H	R_6=H	25R
1a	R_1=OH	R_2=OH	R_3=OH	R_4=H_2	R_5=H	R_6=H	25R
2	R_1=OH	R_2=OH	R_3=OH	R_4=O	R_5=OH	R_6=O-Glc	25S
3	R_1=H	R_2=H	R_3=H	R_4=H_2	R_5=H	R_6=O-Glc-$^{\beta}$-Glc	25S
4	R_1=H	R_2=OH	R_3=O-Glc	R_4=H_2	R_5=H	R_6=H	25S

Fig. 1. Structures of **1**, **1a**, **2**, **3**, and **4**

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the molecule.

Unambiguous assignments for the ^{1}H- and ^{13}C-NMR signals in **2** were made by a combination of the DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and nuclear Overhauser effect spectroscopy (NOESY) spectra. The ^{1}H-NMR spectrum of **2** showed four methyl signals at δ 0.80 (3H, s, Me-18), 1.44 (3H, s, Me-19), 1.04 (3H, d, J=6.8 Hz, Me-21), and 1.12 (3H, d, J=6.4 Hz, Me-27) and one anomeric proton signal at δ 4.92 (d, J=7.7 Hz). Acid hydrolysis and GC analysis of **2** indicated that the hexose was D-glucose. The J value of the anomeric proton signal suggested that the glucose was β-oriented. Comparing the NMR data of **2** with that of a known compound (20*S*,22*R*)-spirost-25(27)-ene-1β,2β,3β,4β,5β,7β-hexahydroxyl-6-one,[6] the chemical shifts of the A, B, C, D, and E rings were found to be identical to each other, except those belonging to F-ring. In the HMBC spectrum of **2**, carbon signals resonating at δ 38.3, 65.2, and 81.5 were correlated with Me-27 (δ 1.12). From the ^{1}H–^{1}H COSY correlations, the above three signals were assignable to C-25, C-26, and C-24, respectively. In the HMBC spectrum, the correlation between the anomeric proton of glucose and the carbon signal at δ 81.5 indicated that the sugar chain was linked to the C-24 hydroxyl group. In the NOESY spectrum of **2**, the proton signal at δ 4.02 (1H, m, H-24) correlated with three proton signals at δ 1.12 (Me-27), 3.52 (1H, H-26$_{ax}$), and 2.67 (1H, H-23$_{eq}$), indicating that H-24 was *cis* to them. Comparing the NMR data of the F-ring with those of a known compound, (24*S*,25*S*)-5α-spirostane-2α,3β,5α,6β,24-pentaol-2,24-di-*O*-β-D-glucopyranoside,[13] they showed identical NMR data, indicating they had the same F-ring configuration. The glycosylation shifts of the α carbon (+10.9) and the anomeric carbon (+9.8) also confirmed the above conclusion. On the basis of the above analysis, **2** was finally assigned to be (24*S*,25*S*)-24-[(β-D-glucopyranosyl)oxy]-1β,2β,3β,4β,5β,7β-hexahydroxyspirostan-6-one (**2**), and called wattoside H.

Compound **3** was obtained as an amorphous powder. The HR-FAB-MS of **3** showed an $[M-H]^-$ at m/z 771.4153 (Calcd for 771.4167), consistent with the molecular formula of $C_{39}H_{64}O_{15}$. The ^{1}H-NMR spectrum contained two anomeric proton signals at δ 5.02 (1H, d, J=7.6 Hz) and 4.86 (1H, d, J=7.7 Hz), as well as four steroidal methyl proton signals at δ 1.26 (3H, s), 1.16 (3H, d, J=6.8 Hz), 1.10 (3H, d, J=6.4 Hz), and 0.81 (3H, s). The ^{1}H- and ^{13}C-NMR assignments of **3**, derived from analyses of the ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY spectra, showed that **3** consisted of a C-27 steroidal aglycone and two glucose groups. The structure of the aglycone was very similar to that of rhodeasapogenin [(25*S*)-5β-spirostane-1β,3β-diol],[12] with the exception of the signals due to the F-ring (C-22 to C-27). Using the method applied in the case of **2**, C-24 (δ 81.6) was found to have a hydroxyl group with the sugar chain attached, and **2** and **3** were found to have the same configuration of the F-ring. In the HMBC spectrum of **3**, one anomeric proton at δ 4.86 correlated with C-24, and the other anomeric proton signal at δ 5.02 correlated with the methylene carbon signal at δ 70.0, which could be assigned to be C-6 of the inner glucose residue by analyzing the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra of **3**. Acid hydrolysis and GC analysis confirmed that the sugars were both glucose, with the same retention time (685 s) as the standard sugar. The structure of **3** was accordingly determined to be (24*S*,25*S*)-1β,3β-dihydroxy-5β-spirostan-24-yl-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**3**) and called wattoside I.

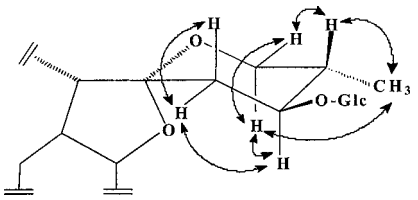

Fig. 2. ^{1}H–^{1}H COSY Correlations of the F-Ring of **2**

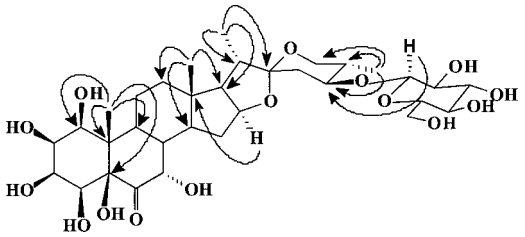

Fig. 3. Main HMBC Correlations of **2**

Table 1. ^{13}C-NMR Data of **1**, **1a**, **2**, **3**, and **4**[a]

Position	**1**	**1a**	**2**	**3**	**4**
1	77.9	78.0	76.5	73.4	73.5
2	68.6	67.5	67.9	33.0	33.9
3	72.7	75.7	75.6	68.2	71.9
4	74.1	68.3	71.2	34.4	68.1
5	78.5	78.3	86.3	31.3	87.9
6	30.5	30.5	211.2	26.7	24.8
7	28.7	28.5	75.3	26.6	28.6
8	35.2	35.0	40.9	35.7	34.9
9	45.8	45.4	37.9	42.2	46.7
10	45.6	45.1	50.2	40.4	47.3
11	21.9	21.7	21.9	21.1	21.6
12	40.2	40.1	39.4	40.7	40.1
13	41.0	40.7	40.7	40.4	40.6
14	56.3	56.2	49.3	56.5	56.1
15	32.5	32.2	31.4	32.1	32.2
16	81.6	81.2	81.5	81.6	81.3
17	63.2	63.1	62.4	62.2	63.0
18	16.9	16.6	16.4	16.8	16.6
19	13.9	13.8	13.1	19.4	13.8
20	42.4	42.5	42.4	42.2	42.6
21	15.4	14.9	14.9	14.9	14.9
22	109.9	109.8	111.8	111.8	109.8
23	32.1	32.0	40.9	40.7	26.3
24	29.6	29.4	81.5	81.6	26.5
25	30.9	30.7	38.3	38.2	27.6
26	67.3	67.2	65.2	65.1	65.2
27	17.7	17.5	13.6	13.6	16.4
1′	03.2		106.5	106.1	97.5
2′	75.4		75.7	75.1	75.9
3′	78.3		78.2	78.5	78.7
4′	71.4		71.9	71.3	71.9
5′	67.5		78.7	76.7	78.8
6′			62.9	70.0	62.8
1″				105.6	
2″				75.5	
3″				78.4	
4″				71.6	
5″				78.5	
6″				62.8	

a) Spectra were measured in pyridine-d_5.

Compound **4** was identified to be (25*S*)-1β,3β,4β-trihydroxyspirotan-5β-yl-β-D-glucopyranoside (convallagenin B 5-*O*-β-D-glucopyranoside)[10] on the basis of mp, IR, MS, and NMR data.

Compounds **1**—**4** are all polyhydroxylated steroidal saponins, with 5β-H or OH as the common structural feature. Because of the small amounts of compounds **2** and **3**, the aglycones of the two saponins were not obtained by means of acid hydrolysis.

The cytotoxic activity of the isolated compounds against the cancer cell line K562 was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. All four saponins exhibited cytotoxicity, with IC_{50} values of 35.67, 76.16, 76.96, and 44.52 μmol/l, respectively.

Experimental

Melting points were determined on a Yanaco MP-S_3 micromelting point apparatus and were uncorrected. Optical rotations were measured on a HORIBA SEPA-300 polarimeter. IR spectra were recorded on Bio-Red FTS-135 spectrophotometer. NMR spectra were obtained on a Bruker DRX-500 (500 MHz for ^{1}H-NMR, 125 MHz for ^{13}C-NMR) spectrometer, using tetramethylsilane (TMS) as an internal standard. FAB-MS and electron impact (EI)-MS were recorded on a VG Autospec-3000 mass spectrometer. Column chromatography was carried out on Diaion HP-20 (Mitsubishi-Kasei, Japan), silica gel H (200—300 mesh, Qingdao Haiyang Chemical Factory), Rp-18 silica gel (Merck). Precoated Kieselgel 60 F_{254} silica gel plates (0.2 mm, Merck) and silica gel H (Qingdao Haiyang Chemical Factory) were used for analytical TLC. Spots were detected by spraying with 10% H_2SO_4/EtOH solution followed by heating. Middle pressure liquid chromatography (MPLC) was performed using a Buchi 681 MPLC system with a Buchi UV/Vis Filter-photometer detector. GC-MS analysis was performed on a GC-MS MD 800 chromatograph, with an HP AC-5 capillary column (0.25 m×30 m), FID (230 °C) detector, the column temperature was 180—240 °C (rate of increase 5 °C/min), and the carrier gas was He (30 ml/min). The materials for cell culture and MTT assay were: 96-well flat-bottomed plates (Iwaki Glass, Japan), microplate reader (Inter Med Immuno-Mini JN-2300, Japan), RPMI 1640 medium (GIBCO BRL, U.S.A), and MTT (Sigma, U.S.A.).

Plant Material The fresh rhizomes of *T. wattii.* Hook.f. were collected in Wenshan, Yunnan, China, and identified by Professor Heng Li. A voucher specimen (No. 0303393) is deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation The fresh rhizomes (6.5 kg) were extracted with hot EtOH (10 l×3). The EtOH extract was concentrated under reduced pressure to give 853 g of residue, which was suspended in water to afford water-soluble part (637.3 g) and water-insoluble part (207.5 g). The former was further fractionated on Diaion HP-20 (water, 70% EtOH) to give the total saponins (114.3 g), which were continuously separated on a silica gel column and eluted with $CHCl_3$–MeOH mixtures of increasing polarity to yield six fractions. Fraction I (5.01 g) was further purified by MPLC on Rp-18 silica gel eluted with MeOH–H_2O (3 : 7, 3.5 : 6.5, 4 : 6, 4.5 : 5.5, 5 : 5), followed by silica gel with AcOEt–acetone–H_2O (8 : 2 : 0.5) to yield compound **2** (8 mg). The water-insoluble part was extracted with *n*-BuOH, and the BuOH extract (175.3 g) was fractionated on silica gel with $CHCl_3$–MeOH (100 : 1, 50 : 1, 25 : 1, 15 : 1, 10 : 1, 5 : 1, 2 : 1, 1 : 1) to give eight fractions. Fraction VII (3.6 g) was subjected to MPLC silica gel column using AcOEt–acetone–H_2O (10 : 1 : 0.5) as the eluting system to afford compound **4** (25 mg). Fractions VIII (3.76 g) and IX (1.04 g) were separately chromatographed with MPLC on silica gel eluted with AcOEt–acetone–H_2O (8 : 3 : 0.5, 6 : 2 : 1.5) to yield compounds **1** (15 mg) and **3** (10 mg), respectively.

Wattoside H (**1**): Amorphous powder (MeOH) (mp 214—216 °C), $[\alpha]_D^{20}$ −65.5° (c=0.03, MeOH). HR-FAB-MS (negative mode) *m/z*: 611.3466 $[M-H]^-$ (Calcd for $C_{32}H_{52}O_{11}$: 611.3431), FAB-MS (negative mode) *m/z*: 611 $[M-H]^-$, 479 $[M-H-132]^-$. IR ν_{max} (KBr) cm^{-1}: 3412 (OH), 2942, 1452, 1378, 1240, 1157, 1045, 982, 920, 899, 851. ^{1}H-NMR (pyridine-d_5) δ: 5.18 (1H, d, J=7.30 Hz, H-1′), 1.58 (3H, s, Me-19), 1.18 (3H, d, J=6.54 Hz, Me-21), 0.85 (3H, s, Me-18), 0.73 (3H, d, J=6.83 Hz, Me-27). ^{13}C-NMR (pyridine-d_5): see Table 1.

Acid Hydrolysis and GC Analysis of 1 The solution of **1** (10 mg) was treated with 1 M HCl in dioxane–H_2O (1 : 1, 5 ml) at 100 °C for 2 h. Then the reaction mixture was extracted with AcOEt, the AcOEt part was purified by MPLC on silica gel with AcOEt–acetone–H_2O (100 : 1 : 0.5) to afford **1a** [pentologenin] (5 mg) The hydrolysate of **1** was trimethylsilated with trimethyl-chlorsilan, and the GC retention time (506 s) of the derived sugar was compared with those of the authentic samples prepared by the same manner, indicating that the sugar was xylose (506 s).

(25*R*)-Spirostane-1β,2β,3β,4β,5β-pentol (Pentologenin) (**1a**): Colorless needles (AcOEt/acetone), mp *ca.* 300 °C, FAB-MS (negative) *m/z*: 479 $[M-H]^-$. EI-MS *m/z*: 480 $[M]^+$, 444, 412, 389, 372, 317, 258, 139. IR ν_{max} (KBr) cm^{-1}: 3437 (OH), 2944 (CH), 1449, 1380, 1274, 1200, 1177, 1028, 982, 956, 918<895, 855. ^{13}C-NMR (pyridine-d_5): see Table 1.

Wattoside I (**2**): Colorless needles (AcOEt/acetone/H_2O), mp 200—203 °C, $[\alpha]_D^{20}$ −78.0° (c=0.014, MeOH). HR-FAB-MS (negative mode) *m/z*: 687.3278 $[M-H]^-$ (Calcd for $C_{33}H_{52}O_{15}$: 687.3228), FAB-MS (negative mode) *m/z*: 687 $[M-H]^-$, 525 $[M-H-162]^-$. IR ν_{max} (KBr) cm^{-1}: 3423 (OH), 2932, 1706 (C=O), 1634, 1455, 1373, 1113, 1052, 897. ^{1}H-NMR (pyridine-d_5) δ: 4.92 (1H, d, J=7.7 Hz, H-1′), 1.44 (3H, s, Me-19), 1.12 (3H, d, J=6.4 Hz, Me-27), 1.04 (3H, d, J=6.8 Hz, Me-21), 0.80 (3H, s, Me-18), 4.52 (1H, br s, H-7), 2.16 (1H, m, H-8), 2.44 (1H, br s, H-9), 1.95 (1H, dd, J=13.1, 9.6 Hz, H-23$_{ax}$), 2.67 (1H, dd, J=13.1, 4.4 Hz, H-23$_{eq}$), 4.02 (1H, m, H-24), 1.88 (1H, m, H-25), 3.52 (1H, br t, J=12.0 Hz, H-26$_{ax}$), 3.62 (1H, dd, J=12.0, 3.7 Hz, H-26$_{eq}$). ^{13}C-NMR (pyridine-d_5): see Table 1.

Wattoside J (**3**): Amorphous powder (MeOH) (mp 205—207 °C), $[\alpha]_D^{20}$ −76.2° (c=0.027, MeOH). HR-FAB-MS (negative mode) *m/z*: 771.4153 $[M-H]^-$ (Calcd for $C_{39}H_{64}O_{15}$: 771.4167). IR ν_{max} (KBr) cm^{-1}: 3396 (OH), 2929, 1594, 1381, 1165, 1055, 897. ^{1}H-NMR (pyridine-d_5) δ: 4.86 (1H, d, J=7.7 Hz, H-1′), 5.02 (1H, d, J=7.6 Hz, H-1″), 1.26 (3H, s, Me-19), 1.16 (3H, d, J=6.8 Hz, Me-27), 1.10 (3H, d, J=6.4 Hz, Me-21), 0.81 (3H, s, Me-18), 2.00 (1H, dd, J=13.0, 9.8 Hz, H-23$_{ax}$), 2.73 (1H, dd, J=13.0, 4.4 Hz, H-23$_{eq}$), 4.05 (1H, m, H-24), 1.89 (1H, m, H-25), 3.57 (1H, br t, J=11.7 Hz, H-26$_{ax}$), 3.65 (1H, dd, J=11.7, 3.5 Hz, H-26$_{eq}$). ^{13}C-NMR (pyridine-d_5) data: see Table 1.

Acid Hydrolysis and GC Analysis of 2 and 3 The solutions of **2** (2.0 mg) and **3** (2.0 mg) in 1 M HCl (dioxane–H_2O, 1 : 1, 2 ml) were heated at 100 °C for 2 h under an Ar atmosphere, respectively. After cooling, each hydrolysate was trimethylsilated and the GC retention time (685 s) of the derived sugar was compared with that of the authentic sample prepared in the same manner, indicating that the sugar was glucose.

Cytotoxicity Assay Cancer cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum. The cultures were incubated at 37 °C in a 5% CO_2 humidified incubator and subcultured every 2 d to maintain them in a state of logarithmic growth. Then the cells were seeded into 96-well flat-bottomed plates (2×10^4 cells/well). Compounds were dissolved in MeOH and added to the 96-well flat-bottomed plates 24 h after seeding. The cells were incubated for 2 d in the presence of sample. For the evaluation of *in vitro* cytotoxicity, the MTT assay was used. The anticancer drug *cis*-dichlorodiamine platinum (*cis*-DDP) was used as a positive control, with an IC_{50} value of 69.33 μmol/l.

Acknowledgments This work was supported by the State Key Basic Research and Development Plan of China (G1998051113), and State Key Laboratory of Phytochemistry & Western Plant Resources (PCWPR). We are grateful to the faculty of the instrument group of the Kunming Institute of Botany, Chinese Academy of Sciences, for the measurement of all spectra, and to Professor Heng Li for her identification of the plant material.

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