Fitoterapia 102 (2015) 102–108

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Three spirostanol saponins and a flavane-*O*-glucoside from the fresh rhizomes of *Tupistra chinensis*

Yan-Hua Xiao [a,b], Hai-Long Yin [c], Li Chen [a], Yin Tian [a], Shi-Jun Liu [a], Guang-Jie Zhang [a], Heng-Wen Chen [d], Hong Jin [e], Bin Li [a,*], Jun-Xing Dong [a,*]

[a] *Beijing Institute of Radiation Medicine, Beijing 100850, China*
[b] *Institute of Chemical Defense, Beijing 102205, China*
[c] *Humanwell Healthcare Group Co., Ltd, Wuhan 430075, China*
[d] *Guanganmen Hospital of China Academy of Chinese Medical Sciences, Beijing 100053, China*
[e] *Beijing Institute of Disease Prevention and Control, 100071, China*

ARTICLE INFO

Article history:
Received 24 December 2014
Accepted in revised form 12 February 2015
Accepted 13 February 2015
Available online 21 February 2015

Keywords:
Tupistra chinensis
Spirostanol saponins
Flavane-*O*-glucoside
Tupistroside
Tupichiside
Cytotoxicity

ABSTRACT

Four new compounds, including three new spirostanol saponins [tupistroside G-I (**1**–**3**)] and a new flavane-*O*-glucoside [tupichiside A (**4**)], together with ten known compounds, were isolated from the fresh rhizomes of *Tupistra chinensis*. The structures of the new compounds were elucidated by spectroscopic analysis and chemical evidence. All compounds were tested in vitro for their cytotoxic activities against the Human LoVo and BGC-823 cell lines, and six of them were found to possess potent cytotoxicity. Compounds **2**, **8** and **9** showed significant cytotoxicity against the tested tumor cell lines with IC_{50} values ranging from 0.2 to 0.9 μM.

1. Introduction

Tupistra chinensis (Liliaceae), mainly distributed in southwestern China, is a rich source of steroidal sapogenins, saponins and flavans [1–6]. These components exhibit diverse biological activities such as anti-inflammatory, ion channel-blocking, immune-stimulating, anti-fungal [7,8] and anti-tumor properties [9]. As a folk medicine, this species has usually been used to reduce carbuncles and ameliorate pharyngitis [10]. In our screening for cytotoxic agents from Chinese medicinal plants, the ethanol extract from the fresh rhizomes of *T. chinensis* showed inhibitory effect towards several human cancer cell lines.

As a part of a continuing study for the discovery of novel cytotoxic agents, four new compounds, including three new spirostanol saponins [tupistroside G-I (**1**–**3**)] and a new flavane-*O*-glucoside [tupichiside A (**4**)], together with ten known compounds identified as isorhodeasapigenin 3-*O*-β-D-glucopyranoside (**5**), rhodeasapogenin 3-*O*-β-D-glucopyranoside (**6**) [11], (25R)-spirostane-1β,3β,5β-triol-3-*O*-β-D-glucopyranoside (**7**) [12], 3-*O*-β-D-glucopyranosyl-3-epiruscogenin (**8**), 3-*O*-β-D-glucopyranoside-3-ruscogenin (**9**) [13], (2R,3R)-3,4′-dihydroxy-7-methoxyflavane (**10**) [14], tupichinol A (**11**), tupichinol B (**12**), tupichinol C (**13**) [3], and (2R)-7,4′-dihydroxy-8-methylflavane (**14**) [15], were isolated from the fresh rhizomes of *T. chinensis* (Fig. 1). All compounds were tested for cytotoxicity against Human LoVo and BGC-823 cell lines in vitro using the MTT assay method. This paper deals with the isolation, structure elucidation and cytotoxic activities of these compounds.

* Corresponding authors. Tel.: +86 10 66931314; fax: +86 10 68164257.
E-mail address: djx931314@163.com (J.-X. Dong).

http://dx.doi.org/10.1016/j.fitote.2015.02.008
0367-326X/

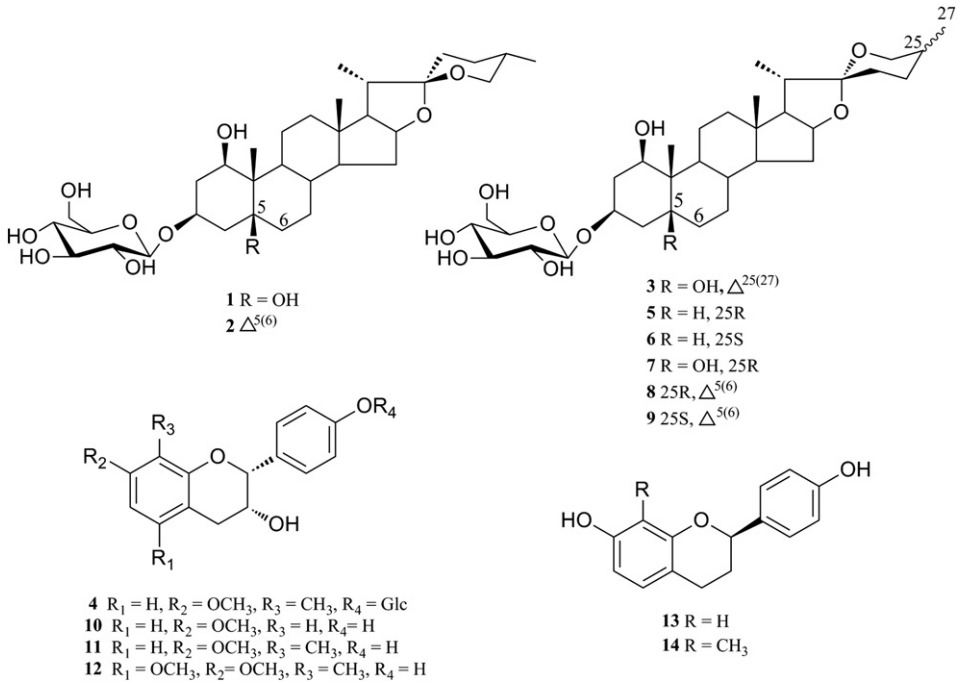

Fig. 1. Structures of compounds **1**–**14**.

2. Experimental part

2.1. General experimental procedures

UV spectra were recorded using the UV-2500PC spectrometer (Shimadzu, Japan). IR spectra were recorded on the Bio-Rad FTS-65A spectrometer (Bio-Rad, Richmond, VA, USA). Optical rotations were measured with a Perkin-Elmer 343 polarimeter (PerkinElmer, Waltham, MA, USA). ^{1}H and ^{13}C NMR spectra were obtained on a Bruker ECA-400 MHz and Varian ^{UNITY}*INOVA* 600 (Varian, Palo Alto, CA, USA), and the chemical shifts were given on δ (ppm) scale with TMS as an internal standard. The HR-ESI-MS were measured on a 9.4 T Q-FT-MS Apex Qe (Bruker Co. Billerica, MA, US). Macroporous resin AB-8 (Nan Kai College Chemical Inc., Tianjin, China), silica gel (60–120 mesh, 200–300 mesh, Qingdao Marine Chemical Group Co. China), Rp-C18 silica gel (100–200 mesh, YMC, Japan) and Sephadex LH-20 (Pharmacia, Sweden) were employed for column chromatography. TLC was carried out using silica gel 60 (>230 mesh, Qingdao Marine Chemical Group Co.) and GF254 plates precoated with silica gel 60. Spots on TLC were visually observed under UV light and/or by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

2.2. Plant material

The fresh rhizomes of *T. chinensis* were collected from Shennongjia Forest District, Hubei province of China in May 2013 and identified by Dr. Bin Li (Beijing Institute of Radiation Medicine). A voucher specimen (No. 2013-0501) was deposited in the Herbarium of Beijing Institute of Radiation Medicine.

2.3. Extraction and isolation

The fresh rhizomes of *T. chinensis* (80 kg) were cut into pieces and extracted with 60% EtOH for three times under reflux. After removal of the solvent in a vacuum, the concentrated residue was partitioned with petroleum ether, $CHCl_3$ and *n*-BuOH successively. The *n*-BuOH phase (400 g out of 1350 g) was dissolved in water (1.0 L), and then was subjected to macroporous resin column chromatography, eluting with a gradient of EtOH in water (100% water to 95% EtOH). The 75% EtOH eluate (70 g out of 135 g) was subjected to silica gel column chromatography (CC) eluted with $CHCl_3$–MeOH gradient (100% $CHCl_3$ to 100% MeOH) to yield 110 fractions. Fractions 9–10 (31 mg) and 41–43 (85 mg) were chromatographed by Sephadex LH-20 (with $CHCl_3$-MeOH, 1:1) to obtain compound **13** (25 mg) and compound **11** (65 mg) respectively. Fraction 21–35 (6.2 g) was subjected to silica gel CC eluted with $CHCl_3$–MeOH gradient (from 50:1 to 10:1) to yield twenty fractions, A1–A20. Fraction A7–A9 (87 mg) was chromatographed by Sephadex LH-20 (with $CHCl_3$–MeOH, 1:1) to obtain compound **10** (21 mg) and compound **14** (17 mg). Fraction A16 (31 mg) was filtered and chromatographed by Sephadex LH-20 (with MeOH) to obtain compound **12** (11 mg). Fraction 56–64 (4.8 g) was purified by CC ($CHCl_3$–MeOH, 35:1 to 5:1) to give seventeen fractions, B1–B17. Fraction B6–B10 (245 mg) was further separated by repeated preparative HPLC eluted with 65% methanol, giving compound **5** (13 mg) and compound **6** (11 mg). Fraction B13 (36 mg) was purified by Rp-C18 silica gel (55% methanol) to obtain compound **7** (19 mg). Fraction 71–80 (7.3 g) was applied to silica gel CC eluted with $CHCl_3$–MeOH (from 25:1 to 3:1) to give fifteen fractions, C1–C15. Fraction C11–C13 (285 mg) was further separated by repeated preparative

HPLC eluted with 65% methanol to afford compound **3** (15 mg), compound **8** (10 mg) and compound **9** (11 mg). Fraction 86–87 (185 mg) was purified by Sephadex LH-20 (with MeOH) and preparative HPLC eluted with 60% methanol to obtain compound **4** (13 mg). Fraction 91–94 (2.1 g) was subjected to silica gel CC eluted with $CHCl_3$–MeOH (from 15:1 to 1:1) to give nine fractions, D1–D9. Fraction D4–D5 was repeatedly chromatographed by Sephadex LH-20 (with MeOH) and preparative HPLC eluted with 60% methanol to afford compound **1** (15 mg) and compound **2** (17 mg).

Tupistroside G (**1**): white amorphous powder; $[\alpha]_D^{20}$ −11.0 (*c* 0.10, Pyridine); IR (KBr) ν_{max} 3396, 2925, 2852, 1647, 1468, 1376, 1087, 1046, 625 cm^{-1}; HR-ESI-MS *m/z* 611.3789 $[M + H]^+$ (calcd for $C_{33}H_{55}O_{10}$, 611.3790); ^{1}H- and ^{13}C-NMR (Pyridine-d_5) spectroscopic data, see Tables 1 and 2, respectively.

Tupistroside H (**2**): white amorphous powder; $[\alpha]_D^{20}$ 0.0 (*c* 0.10, Pyridine); IR (KBr) ν_{max} 3428, 2953, 1637, 1457, 1362, 1272, 1164, 1073, 992, 919 cm^{-1}; HR-ESI-MS *m/z* 593.3690 $[M + H]^+$ (calcd for $C_{33}H_{53}O_9$, 593.3684); ^{1}H- and ^{13}C-NMR (Pyridine-d_5) spectroscopic data, see Tables 1 and 2, respectively.

Tupistroside I (**3**): white needles; $[\alpha]_D^{20}$ −65.0 (*c* 0.10, Pyridine); IR (KBr) ν_{max} 3396, 2923, 2851, 1647, 1468, 1084, 1043, 924, 894, 876 cm^{-1}; HR-ESI-MS *m/z* 609.3615 $[M + H]^+$ (calcd for $C_{33}H_{53}O_{10}$, 609.3633); ^{1}H- and ^{13}C-NMR (Pyridine-d_5) spectroscopic data, see Tables 1 and 2, respectively.

Tupichiside A (**4**): yellow amorphous powder; $[\alpha]_D^{20}$ −52.5 (*c* 0.04, MeOH); UV [MeOH] λ_{max} (log ε) 276 (3.66), 245 (4.16) nm, 204(4.69); IR (KBr) ν_{max} 3396, 2921, 2849, 1646, 1513, 1494, 1468, 1419, 1218, 1165, 1129, 1076, 1046, 788 cm^{-1}; HR-ESI-MS *m/z* 471.1629 $[M + Na]^+$ (calcd for $C_{23}H_{28}O_9$, 471.1626); ^{1}H- and ^{13}C-NMR (CD_3OD) spectroscopic data, see Table 3.

2.4. Cell cultures

Human LoVo cell line from American Type Culture Collection (ATCC, Rockville, MD) and BGC-823 cell line from Cancer Institute and Hospital of Chinese Academy of Medical Sciences were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% (*v/v*) fetal calf serum (Gibco, USA), penicillin G (Macgene, China) 100 units mL^{-1} and streptomycin (Macgene, China) 100 μg mL^{-1}, at 37 °C under 5% CO_2.

2.5. Cell viability assay

Cells were plated at a density of 2×10^4 cells mL^{-1} on 96-well plates and treated with different concentrations of the

Table 1
^{1}H (600 MHz) NMR data for compounds **1**, **2**, **3** and **7** (pyridine-d_5).

	Compound **1**	Compound **2**	Compound **3**	Compound **7**
	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1	4.14 (br s)	4.36 (br s)	4.15 (br s)	4.15 (br s)
2	2.54 (br d, 13.2)	1.88 (br d, 12.6)	2.56 (br d, 13.8)	2.55 (br d, 14.4)
	2.04 (br d, 13.2)	1.43 (br d, 12.6)	2.05 (br d, 13.8)	2.01 (br d, 14.4)
3	4.57 (br s)	4.49 (br s)	4.59 (d, 2.4)	4.56 (br s)
4	2.35 (d, 15.6)	2.63 (d, 14.4)	2.34 (dd, 13.8, 2.4)	2.33 (br d, 15.6)
	2.21 (d, 15.6)	2.52 (d, 14.4)	2.19 (d, 13.8)	2.18 (br d, 15.6)
6	1.96 (m), 1.48 (m)	5.63 (d, 6.0)	1.97 (m), 1.48 (m)	1.92 (m), 1.42 (m)
7	1.46 (m), 0.97 (m)	2.54 (m), 1.97 (m)	2.03 (m), 1.44 (m)	1.44 (m), 1.03 (m)
8	1.64 (m)	1.60 (m)	1.65 (m)	1.64 (m)
9	1.14 (m)	1.33 (m)	1.15 (m)	1.11 (m)
11	2.02 (dd, 9.6, 4.8)	2.85 (d, 11.4)	2.01 (dd, 9.6, 4.8)	1.99 (dd, 9.6, 4.2)
	1.37 (m)	1.71 (m)	1.37 (m)	1.35 (m)
12	1.64 (m), 1.01 (m)	1.72 (m), 1.12 (m)	1.65 (m), 1.02 (m)	1.66 (m), 1.01 (m)
14	0.93 (m)	0.93 (m)	1.01 (m)	0.98 (m)
15	2.04 (m), 1.49 (m)	1.98 (m), 1.50 (m)	2.03 (m), 1.78 (m)	2.05 (m), 1.42 (m)
16	4.38 (d, 9.0)	4.22 (d, 8.4)	4.6 (d, 7.8)	4.55 (d, 9.0)
17	1.52 (m)	1.52 (d, 7.2)	1.82 (d, 9.0)	1.79 (d, 7.8)
18	0.98 (s)	1.02 (s)	0.85 (s)	0.84 (s)
19	1.53 (s)	1.27 (s)	1.53 (s)	1.52 (s)
20	2.31 (m)	2.30 (d, 6.9)	1.97 (m)	1.93 (m)
21	1.07 (d, 6.6)	0.94 (d, 7.2)	1.09 (d, 7.2)	1.14 (d, 7.2)
23	1.76 (m), 1.54 (m)	1.61 (m), 1.53 (m)	2.23 (m), 1.46 (m)	1.84 (m), 1.46 (m)
24	1.56 (m), 1.47 (m)	1.54 (m), 1.42 (m)	2.71 (m), 0.98 (m)	2.13 (m), 1.32 (m)
25	1.63 (m)	1.62 (m)		1.57 (m)
26	3.69 (d, 10.8)	3.68 (d, 9.9)	4.47 (d, 12.0)	4.06 (d, 10.8)
	3.64 (d, 10.8)	3.63 (m, 9.9)	4.03 (d, 12.0)	3.36 (d, 10.8)
27	0.68 (d, 6.6)	0.68 (d, 6.6)	4.81 (s), 4.78 (s)	1.07 (d, 7.2)
1′	5.00 (d, 7.8)	4.94 (d, 8.4)	5.00 (d, 7.8)	4.99 (d, 7.8)
2′	3.96 (dd, 9.0, 6.6)	3.94 (dd, 9.0, 7.2)	3.96 (dd, 10.2, 7.8)	3.95 (dd, 8.4, 6.6)
3′	3.97 (dd, 9.0, 6.6)	3.91 (dd, 9.0, 7.2)	3.98 (dd, 10.2, 7.8)	3.98 (dd, 8.4, 6.6)
4′	4.23 (dd, 9.6, 7.2)	4.19 (dd, 9.0, 7.2)	4.21 (dd, 9.0, 7.8)	4.22 (dd, 9.0, 8.4)
5′	4.25 (dd, 9.6, 7.2)	4.20 (dd, 9.0, 7.2)	4.26 (dd, 9.0, 7.8)	4.26 (dd, 9.0, 8.4)
6′	4.55 (dd, 11.4, 5.4)	4.55 (dd, 11.4, 4.8)	4.55(dd, 13.8, 6.6)	4.56 (d, 9.0)
	4.41 (dd, 11.4, 5.4)	4.41 (dd, 11.4, 4.8)	4.40 (dd, 13.8, 6.6)	4.40 (d, 9.0)

Table 2
^{13}C (150 MHz) NMR data for compounds **1**, **2**, **3** and **7** (pyridine-d_5).

	Compound **1**	Compound **2**	Compound **3**	Compound **7**
	δ_C	δ_C	δ_C	δ_C
1	73.0	74.0	73.0	73.1
2	33.1	32.5	33.2	33.1
3	75.4	74.8	75.4	75.4
4	35.4	38.9	35.4	35.4
5	75.2	139.8	75.2	75.2
6	36.7	124.3	36.7	36.7
7	29.0	36.8	32.2	28.9
8	34.7	30.7	34.9	34.9
9	45.7	50.7	45.6	45.6
10	44.0	44.3	44.0	44.0
11	21.6	24.3	21.5	21.5
12	40.5	40.7	40.7	40.7
13	41.2	41.0	40.0	40.0
14	55.4	56.2	56.2	56.2
15	33.3	33.4	33.0	32.2
16	80.9	80.7	81.7	81.2
17	62.7	62.7	63.0	62.8
18	17.0	17.0	16.6	16.6
19	13.5	13.3	13.6	13.6
20	42.2	42.1	41.9	42.5
21	16.8	16.6	15.0	14.9
22	110.6	110.6	109.5	109.7
23	28.3	28.1	29.0	26.4
24	28.2	28.0	28.9	26.2
25	30.7	32.3	144.4	27.6
26	69.6	69.6	65.0	65.1
27	17.3	17.3	108.7	16.3
1′	102.9	103.0	102.9	102.9
2′	75.4	75.0	75.5	75.5
3′	78.7	78.5	78.9	78.9
4′	71.4	71.7	71.4	71.4
5′	78.5	78.7	78.4	78.4
6′	62.7	62.9	62.7	62.7

test compounds for 4 days. Cytotoxicity was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [16]. After addition of 10 μL MTT solution (5 mg/mL), cells were incubated at 37 °C for 4 h. After adding 150 μL DMSO, cells were shaken to mix thoroughly. The absorbance of each well was measured at 540 nm in a Multiscan photometer. The IC_{50} values were calculated by Origin software and listed in Table 4.

Table 3
^1H (600 MHz) and ^{13}C (150 MHz) for compounds **4** (CD_3OD).

	Compound 4					
	δ_H (*J* in Hz)	δ_C			δ_H (*J* in Hz)	δ_C
2	5.02 (s)	79.8		4′		158.5
3	4.22 (br s)	67.5		5′	7.11 (d, 8.7)	117.4
4	3.20 (dd, 16.2, 4.2)	34.4		6′	7.45 (d, 8.7)	128.8
	2.77 (dd, 16.2, 4.2)			1″	4.91 (d, 7.8)	102.5
5	6.87 (d, 8.4)	128.2		2″	3.45 (dd, 9.0, 6.0)	75.0
6	6.52 (d, 8.4)	104.7	3″		3.43 (dd, 9.0, 6.0)	78.2
7		158.3	4″		3.38 (dd, 10.8, 7.2)	71.5
8		114.3	5″		3.44 (dd, 10.8, 7.2)	78.0
9		154.1	6″		3.88 (dd, 12.0, 3.6)	62.6
10		113.0			3.70 (dd, 12.0, 5.4)	
1′		134.9	7-OMe		3.78 (s)	56.2
2′	7.45 (d, 8.7)	128.8	8-Me		2.10 (s)	8.4
3′	7.11 (d, 8.7)	117.4				

Table 4
Cytotoxic activity of compounds against LoVo and BGC-823 cell lines in vitro.

Compound	IC_{50} (μM)	
	LoVo	BGC-823
2	0.267 ± 0.012**	0.327 ± 0.146**
4	NE	4.707 ± 0.127
8	0.616 ± 0.279**	0.797 ± 0.074**
9	0.731 ± 0.157**	0.677 ± 0.083**
10	2.637 ± 0.098	5.347 ± 0.310
11	2.577 ± 0.132	2.747 ± 0.127
Cisplatin	5.920 ± 0.363	4.593 ± 0.845

NE—no effect.
Other inactive compounds were not listed in the table.
Values were mean ± SD.
Cisplatin, positive control.
Cell lines: LoVo colon cancer; BGC-823 stomach cancer.
** $p < 0.01$ as compared with the respective positive control value.

2.6. Statistical analysis

Values were expressed as mean ± SD. Statistical analyses were performed using the Student's *t*-test. Differences were considered significant when associated with a probability of 5% or less ($p \leq 0.05$).

3. Results and discussion

Compound **1** was obtained as white amorphous powder. Its molecular formula was assigned as $C_{33}H_{55}O_{10}$ by HR-ESI-MS *m/z* 611.3789 $[M + H]^+$ (calcd for $C_{33}H_{55}O_{10}$, 611.3790), corresponding to the same molecular formula ($C_{33}H_{55}O_{10}$) as that of (25R)-spirostane-1β,3β,5β-triol-3-*O*-β-D-glucopyranoside (compound **7**) [12]. Comparable ^{13}C and ^1H chemical shifts for the A–D rings were observed for **1** and **7**, but with a noticeable difference for the carbons of rings E and F, suggesting that the aglycone of **1** might be different in stereostructure from those of **7** with respect to the E- and F- parts (Tables 1 and 2). The proton signal for H-27 moved upfield from δ 1.07 to δ 0.68, and the carbon signal for C-27 downfield from δ 16.3 to δ 17.3, suggestive of a 25S configuration in **1** [17], instead of 25R configuration as in **7**. The carbon signal for C-22 moved downfield from δ 109.7 to δ 110.6, and the other ^{13}C chemical shifts for the F ring downfield from δ 26.4 (C-23), δ 26.2 (C-24), δ 27.6 (C-25), δ 65.1 (C-26) to δ 28.3 (C-23), δ 28.2 (C-24), δ 30.7 (C-25), and δ 69.6 (C-26), respectively. The ^{13}C shifts reported for the E/F rings are in excellent agreement with those reported 22-S configuration which has been observed before in a saponin isolated from the tubers of *Dichelostemma multiflorum* [18] and *Asparagus racemosus* [19]. Additionally, the chemical shifts and coupling constants of H-26a (δ 3.64, d, J = 10.8 Hz) and H-26b (δ 3.69, d, J = 10.8 Hz) on the F ring of **1** were different from those (δ 3.36, d, J = 10.8 Hz for H-26a; δ 4.06, d, J = 10.8 Hz for H-26b) on the F ring of **7**. The signals of both protons at the 26 position of **1** closely appeared at δ 3.64–3.69, different from those of **7**, which indicates that compound **1** has a different F-ring structure [20]. The phase-sensitive NOESY spectrum provided certain information for the stereostructure assignments. All ^1H NMR signals were assigned by the ^1H–^1H COSY spectrum before inspection of the NOESY spectrum (Table 1). The clear NOE correlations, H-14/H-17 and H-16,

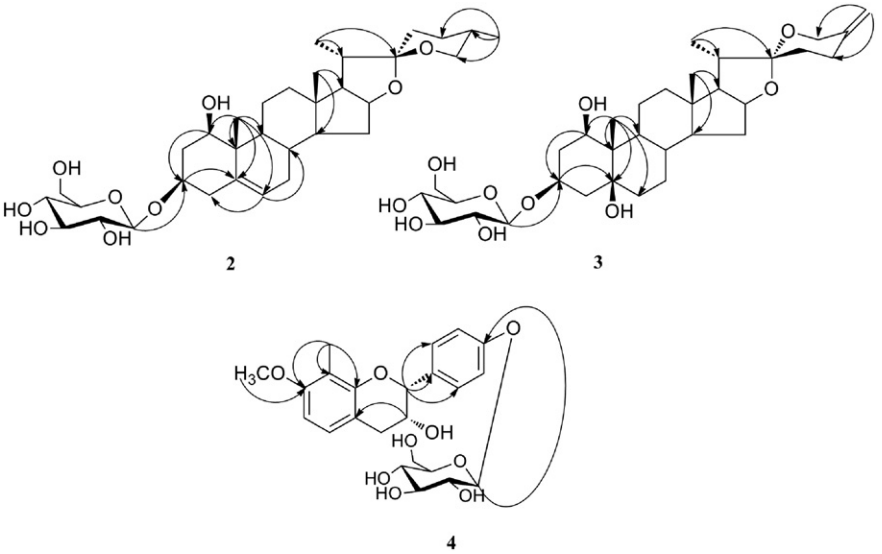

Fig. 2. Key HMBC correlations of compounds **2**, **3** and **4**.

and H-20/Me-18, and the downfield shift for H-20, from δ 1.93 to δ 2.31, indicated the usual D/E cis-ring junction and C-20s configuration. The H-16 proton showed an intense NOE with 23 eq-H, giving evidence for an equatorial position for the F-ring and the (S) configuration of C-22 (Fig. 3). In fact, the aglycone of the saponin from *A. racemosus* differs from **1** only in the configuration of C-1 and C-5, which results in significantly different chemical shifts for the carbons of the A/B rings in these two compounds. Both **1** and **7** could presumably form from a common intermediate which undergoes cyclisation to give an F ring with differing stereochemistry at C-22. Maintenance of the anomeric effect then results in the differing disposition of the C-27 methyl group. In addition, the presence of a β-D-glucopyranosyl moiety in **1** was readily recognized by the appearance of an anomeric proton signal at 5.00 (d, $J = 7.8$ Hz), in the ^{1}H NMR spectrum and also by the characteristic six signals at δ 102.9 (CH), 75.4 (CH), 78.7 (CH), 71.4 (CH), 78.5 (CH), and 62.7 (CH_2) in the ^{13}C NMR spectrum (Table 1). This conclusion is in agreement with the sugar TCL analysis after acid hydrolysis of **1** with 2 N HCl. In the HMBC spectrum, a cross peak between the ^{1}H NMR anomeric proton signal at δ 5.00 and the carbon signal at δ 75.4 (C-3, aglycone) indicated glycosylation of the aglycone at C-3. Compound **1** was therefore established as (22S,25S)-1β,3β,5β-triol-spirost-3-*O*-β-D-glucopyranoside (tupistroside G).

Compound **2** was a white amorphous powder. The molecular formula was deduced as $C_{33}H_{53}O_9$ from the $[M + H]^+$ peak at *m/z* 593.3690 (calcd for $C_{33}H_{53}O_9$, 593.3684) in the HR-ESI-MS spectrum. All spectral properties of compound **2** were closely similar to those of **1**. The major differences were observed for their B-ring. Signals due to C-5 and C-6 in the ^{13}C NMR spectrum of **1**, which were observed at δ 75.2 and 36.7 respectively, were replaced by the signals assignable to the olefinic carbons at δ 139.8 and 124.3 in **2**. This was confirmed by the long range correlations of δ 5.63 (H-6) with δ 32.5 (C-2), 38.9 (C-4) and 44.3 (C-10), δ 2.52 (H-4) and 1.97 (H-7) with δ 124.3 (C-6), δ 2.52 (H-4), 1.97 (H-7) and 1.27 (H-19) with δ 139.8 (C-5) in the HMBC spectrum (Fig. 2). Acid hydrolysis of **2** with 2 N HCl gave D-glucose which was confirmed on TCL. Thus, compound **2** was deduced to be (22S,25S)-1β,3β,5β-triol-spirost-5-ene-3-*O*-β-D-glucopyranoside (tupistroside H).

Compound **3** was obtained as white needles. The HR-ESI-MS showed a quasi-molecular ion peak at *m/z* 609.3615 $[M + H]^+$ (calcd for $C_{33}H_{53}O_{10}$, 609.3633). Its ^{1}H NMR spectrum showed three methyl signals at δ 0.85 (3H, s), 1.09 (3H, $J =$ 7.2 Hz) and 1.53 (3H, s), and instead of a doublet methyl proton signal, the presence of the olefinic carbon signals at δ 144.4 and 108.7 [21] and a quaternary carbon signal at δ 109.5 in the ^{13}C NMR spectrum [22] indicated that **3** was a $\triangle^{25}$-spirostanol derivative, which was confirmed by combination of ^{1}H–^{1}H COSY, HSQC and HMBC spectrum. And the geminal protons at

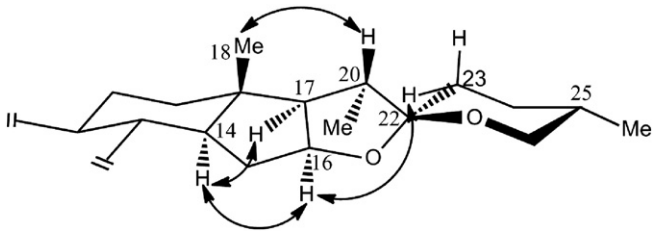

Fig. 3. NOE correlation of **1** in pyridine-d_5.

C-27 were observed at δ 4.78 and 4.81 as two singlets. Acid hydrolysis of **3** with 2 N HCl afforded D-glucose, which was recognized by the appearance of an anomeric proton signal at δ 5.00 (1H, d, J = 7.8 Hz) in the ^{1}H NMR spectrum and the characteristic six carbon signals at δ 102.9 (CH), 75.5 (CH), 78.9 (CH), 71.4 (CH), 78.4 (CH), and 62.7 (CH_2) in the ^{13}C NMR spectrum (Table 1). After having excluded signals due to the glucose residue, the remaining carbon and proton signals implied a spirostanetriol moiety due to the presence of two oxymethine [δ_H 4.15 (br s), δ_C 73.0; δ_H 4.59 (br d, J = 2.4 Hz), δ_C 75.4] and an oxygenated quaternary carbon (δ 75.2) resonances. In the ^{1}H–^{1}H COSY spectrum, the methylene protons at δ 2.56 (1H, br d, J = 13.8 Hz) and 2.05 (1H, br d, J = 13.8 Hz) were shown to be coupled to both of the two oxymethine protons at δ 4.15 and δ 4.59. The oxymethine proton at δ 4.59 was further coupled to the methylene protons at δ 2.34 (1H, dd, J = 13.8, 2.4 Hz) and 2.19 (1H, br d, J = 13.8 Hz). In the HMBC spectrum, the long-range correlations from the oxymethine proton at δ 4.15 to the carbons at δ 75.4 (CH), 75.2 (C), 44.0 (C), 33.2 (CH_2), and 13.6 (CH_3), from the oxymethine proton at δ 4.59 to the carbons at δ 73.0 (CH) and 75.2 (C), and from the exchangeable proton at δ 6.56 (br s) to the carbons at δ 75.2 (C), 44.0 (C), and 35.4 (CH_2) were observed. These findings allowed the placement of these three hydroxyl groups at C-1, C-3 and C-5 positions. HMBC correlation of the anomeric proton signal at δ 5.00 with δ 75.4 (C-3) proved the location of the glucopyranosyl moiety at C-3 of aglycone (Fig. 2). The β-axial orientations of all these hydroxyl groups were indicated by the NOESY spectrum in which the NOE correlations were observed between H-1/H-19, H-1/H_2-2, H-3/H_2-2, and H-3/H_2-4, and between the proton of the hydroxyl group at C-5 and H-4 eq and H-19. On the basis of the above evidence, compound **3** was determined to be 1β,3β,5β-triol-spirost-25(27)-ene-3-*O*-β-D-glucopyranoside (tupistroside I).

Compound **4** was obtained as yellow amorphous powder. The HR-ESI-MS showed a $[M + Na]^+$ ion at m/z 471.1629 (calcd 471.1626), consistent with the molecular formula $C_{23}H_{28}O_9$. In the ^{1}H NMR spectrum of **4**, signals that are characteristic of the 8-methylflavan-3-ol aglycone skeleton were observed [23]. Two signals at δ 2.10 (3H, s) and 3.78 (3H, s) were assigned to the methyl group on C-8 and the methoxyl group attached to C-7 in ring A, respectively. The oxymethine protons at δ 5.02 (1H, s) and 4.22 (1H, br s) were assigned to H-2 and H-3, respectively. The protons at δ 6.87 (1H, d, J = 8.4 Hz) and 6.52 (1H, d, J = 8.4 Hz) were assigned to H-5 and H-6, respectively. Furthermore, the aromatic protons at δ 7.45 (2H, d, J = 8.7 Hz) and 7.11 (2H, d, J = 8.7 Hz) were assigned to H-2′/6′ and H-3′/5′, respectively [23]. The ^{13}C NMR spectrum showed the characteristic flavan-3-ol aglycone signals at δ 79.8, 67.5, and 34.4, corresponding to C-2 (OCH), C-3 (OCH), and C-4 (CH_2), respectively [24,25]. Moreover, this spectrum also indicated the required 12 aromatic carbons (δ 104.7-158.5), one methoxyl carbon at δ 56.6, and one methyl carbon at δ 9.2. In addition, the presence of a β-D-glucopyranosyl moiety in **4** was readily recognized by the appearance of an anomeric proton signal at δ 4.91 (1H, d, J = 7.8 Hz) in the ^{1}H NMR spectrum and also by the characteristic six signals at δ 102.5 (CH), 75.0 (CH), 78.2 (CH), 71.5 (CH), 78.0 (CH) and 62.6 (CH_2) in the ^{13}C NMR spectrum (Table 2). Acid hydrolysis of **4** with 2 N HCl gave D-glucose which was confirmed on TCL and tupichinol A (compound **11**) which was confirmed by direct comparison of the ^{1}H and ^{13}C NMR chemical shifts with literature data [3]. The HMBC correlation of the anomeric proton signal at δ 4.91 with δ 158.5 (C-4′) proved the location of the glucopyranosyl moiety at C-4′ of aglycone (Fig. 2). Therefore, by combination of ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY spectra, the structure of compound **4** was determined to be (+)-(2R,3R)-3,4′-dihydroxy-7-methoxy-8-methylflavan-4′-*O*-β-D-glucopyranoside (tupichiside A).

All compounds (**1**–**14**) obtained were evaluated for their in vitro growth inhibitory effects against Human LoVo and BGC-823 cell lines using the MTT method as reported previously, and six of them were found to possess potent cytotoxicity. The cytotoxicity of the compounds against the LoVo and BGC-823 cell lines was compared with the respective positive control group Cisplatin, and finally, the result was summarized in Table 3. The new compound **2** exhibited potent toxicity effects against LoVo and BGC-823 cell lines, with IC_{50} values of 0.267 and 0.327 μM, respectively. Meanwhile, compounds **8**–**9** also showed significantly cytotoxicity against the selected cells, and the IC_{50} values were much lower than that of Cisplatin on LoVo and BGC-823 cell lines, respectively. Comparison of the cytotoxic activities and structures of compounds **1**–**9** suggested that the ethylenic linkage at C-5 position increased their cytotoxic activities. In addition, comparing compound **4** with **11**, the flavane aglycone tupichinol A (compound **11**) possesses more cytotoxic activity than its glucoside.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

We are grateful to Mrs. Yan Xue, Mrs. Mei-feng Xu and Mrs. Yu-mei Zhao of the National Center of Biomedical Analysis for the measurements of the MS and NMR spectra.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.02.008.

References

[1] Pan WB, Chang FR, Wu YC. Spirostanol sapogenins from the underground parts of *Tupistra chinensis*. Chem Pharm Bull 2000;48:1350–3.
[2] Pan WB, Chang FR, Wu YC. Tupichigenin A, a new steroidal sapogenin from *Tupistra chinensis*. J Nat Prod 2000;63:861–3.
[3] Pan WB, Chang FR, Wei LM, Wu YC. New flavans, spirostanol sapogenins, and a pregnane genin from *Tupistra chinensis* and their cytotoxicity. J Nat Prod 2003;66:161–8.
[4] Zou K, Wu J, Du M, Liu C, Tu GZ, Wang JZ. Diastereoisomeric saponins from the rhizomes of *Tupistra chinensis*. Chin Chem Lett 2007;18:65–8.
[5] Zou K, Wang JZ, Wu J, Zhou Y, Liu C, Dan FJ, et al. Furostanol saponins with inhibitory action against COX-2 production from *Tupistra chinensis* rhizomes. Chin Chem Lett 2007;18:1239–42.
[6] Liu CX, Guo ZY, Xue YH, Cheng J, Huang NY, Zou K, et al. Five new furostanol saponins from the rhizomes of *Tupistra chinensis*. Fitoterapia 2012;83:323–8.
[7] Wang XK. Natural medicine chemistry. Beijing: People's medical publishing house; 1988.
[8] Lanzotti V. Bioactive saponins from *Alliumand Asterplants*. Phytochem Rev 2005;4:95–110.
[9] Sparg SG, Light ME, Staden J. Biological activities and distribution of plant saponins. J Ethnopharmacol 2004;94:219–43.

[10] Zhan YH. China Shenongjia resources of medicinal plants. Wuhan: Hubei Scientific and Technological Press; 1994.
[11] Miyahara K, Kudo K, Kawasaki T. Co-occurrence and high-performance liquid chromatographic separation of the glycosides of rhodeasapogenin and its analogs which differ in the F-ring structure. Chem Pharm Bull 1983;31:348–51.
[12] Wu GX, Wei XY, Chen WX. Spirostane steroidal saponins from the underground parts of *Tupistra chinensis*. Chin Chem Lett 2005;16(7): 911–4.
[13] Takahira M, Kondo Y, Kusano G, Nozoe S. Studies on the constituents of "*Senshokushichikon*". II. Isolation and structural elucidation of spirostane derivatives. Yakugaku Zasshi 1979;99(5):528–32.
[14] Maki J, Akihito Y, Hiroshi S, Yoshihiro M. Chemical constituents of the bulbs of *Habranthus brachyandrus* and their cytotoxic activities. Chem Pharm Bull 2009;57(10):1153–7.
[15] Luo Y, Dai HF, Wang H, Mei WL. Chemical constituents from dragon's blood of *Dracaena cambodiana*. Chin J Nat Med 2011;9(2):0112–4.
[16] Wang BJ, Won SJ, Yu ZR, Su CL. Free radical scavenging and apoptotic effects of *Cordyceps sinensisfractionated* by supercritical carbon dioxide. Food Chem Toxicol 2005;43:543–52.
[17] Agrawal PK. 25R/25S stereochemistry of spirostane-type steroidal sapogenins and steroidal saponins via chemical shift of germinal protons of ring-F. Magn Reson Chem 2003;41:965–8.
[18] Inoue T, Mimaki Y, Sashida Y, Nikaido T, Ohmoto T. Steroidal saponins from *Dichelostemma multiflorumtubers* and their inhibitory activity on cAMP phosphodiesterase. Phytochemistry 1995;39:1103–10.
[19] Patricia YH, Aisyah HJ, Reg L, Kerry P, William K, James JD. Steroidal saponins from the roots of *Asparagus racemosus*. Phytochemistry 2008;69: 796–804.
[20] Tobari A, Teshima M, Koyanagi J, Kawase M. Spirostanols obtained by cyclization of pseudosaponin derivatives and comparison of antiplatelet agglutination activities of spirostanol glycosides. Eur J Med Chem 2000; 35:511–27.
[21] Yokosuka A, Mimaki Y, Sashida Y. Steroidal saponins from *Dracaena surculosa*. J Nat Prod 2000;63:1239–43.
[22] Agrawal PK, Jain DC, Gupta RK, Thakur RS. Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. Phytochemistry 1985;24: 2479–96.
[23] Coxon DT, O'Neill TM, Mansfield JW, Porter AEA. Identification of three hydroxyflavan phytoalexins from *daffodil bulbs*. Phytochemistry 1980;19: 889–91.
[24] Takasugi M, Niino N, Nagao S, Anetai M, Masamune T, Shirata A, et al. Studies on the phytoalexins of the Moraceae. 13. Eight minor phytoalexins from *diseased paper mulberry*. Chem Lett 1984;5:689–92.
[25] Morimoto S, Nonaka GI, Nishioka I, Ezaki N, Takizawa N. Tannins and related compounds. XXIX. Seven new methyl derivatives of flavan-3-ols and a 1,3-diarylpropan-2-ol from *Cinnamomum cassia*, *Cobtusifolium* and *Lindera umbellata* var. membranacea. Chem Pharm Bull 1985;33:2281–6.