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A cytotoxic cardenolide and a saponin from the rhizomes of *Tupistra chinensis*

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ABSTRACT

A new cardenolide tupichinolide (**1**) and a new steroidal saponin tupichinin A (**2**), together with seven known compounds, were isolated from the rhizomes of *Tupistra chinensis*. Their structures were established using spectroscopic analysis and chemical methods. Compound **1** was the first cardenolide isolated from *Tupistra chinensis* and exhibited potent cytotoxicity against five human cancer cell lines: HL-60, SMMC-7721, A-549, MCF-7 and SW480.

1. Introduction

Tupistra chinensis (Liliaceae), widely distributed in southwestern China, is commonly used in Chinese traditional medicine to treat throat irritation, rheumatic diseases and snake-bite [1,2]. According to the results of modern pharmacological experiments, the extracts of this species possessed significant antitumor activity [3,4]. In our screening for cytotoxic agents from Chinese medicinal plants, the ethanol extract from the rhizomes of *Tupistra chinensis* showed inhibitory effect towards several human cancer cell lines. Previous phytochemical investigations showed *Tupistra chinensis* is a rich source of steroidal sapogenins and saponins [5–14]. As a part of our search for new biologically active metabolites from traditional Chinese medicines, a new cardenolide tupichinolide (**1**) and a new steroidal saponin tupichinin A (**2**), together with seven known steroids, were isolated from the rhizomes of *Tupistra chinensis*. All compounds were also tested for cytotoxicity against HL-60, SMMC-7721, A-549, MCF-7 and SW480 human cancer cell lines. This paper deals with the isolation, structure elucidation, and cytotoxic activity of these compounds (Fig. 1).

2. Experimental procedure

2.1. General

Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained on a Shimadzu UV-2401A spectrophotometer. IR spectroscopy was measured in a Bio-Rad FTS-135 spectrometer with KBr pellets. ESI-MS and HRESI-MS were recorded on an API QSTAR Pulsar 1 spectrometer. The NMR spectra were recorded on Bruker DRX-500 spectrometers with TMS as internal standard, and chemical shifts (δ) were expressed in ppm with reference to the solvent signals. Silica gel (200−300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), RP-18 gel (40−63 μm; Merck, Darmstadt, Germany), and Sephadex LH-20 (Amersham Pharmacia biotech, Sweden) were used for column chromatography. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. Solvents were distilled before use.

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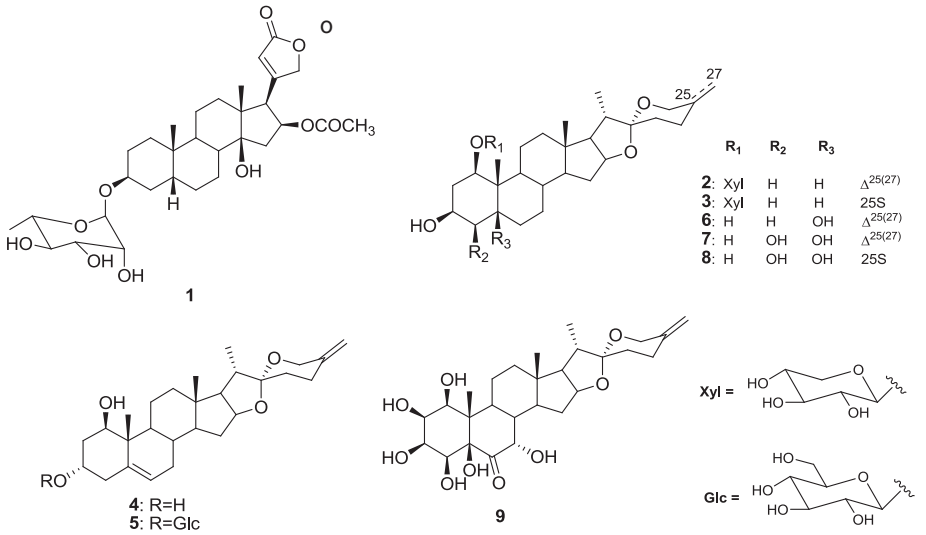

Fig. 1. Structures of compounds **1**–**9**.

2.2. Plant material

The rhizomes of *Tupistra chinensis* were purchased in Zhejiang province, China, in October 2010 and identified by one of the authors (Professor Yong-Xin Wen). A voucher specimen (CTM201001) was deposited at the Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization, Guangxi Institute of Botany, Chinese Academy of Sciences, China.

2.3. Extraction and isolation

The air-dried and powdered sample (4.8 kg) was extracted with 95% EtOH (3×8 L) at room temperature. The EtOH extract was evaporated in a vacuum to yield a residue, which was partitioned between H_2O (2 L) and EtOAc (3×1 L). The EtOAc extract (300 g) was subjected to column chromatography over silica gel, eluting with a gradient of acetone in petroleum ether, to yield six fractions (Fr. 1—6). Fr. 3 (32 g) was further purified by CC (silica gel, petroleum ether:EtOAc, 9:1 to 0:1) and Sephadex LH-20 column (1 g; $CHCl_3$:MeOH, 1:1) to yield **4** (120 mg) and **6** (82 g). Fr. 4 (36 g) was repeatedly subjected to CC (silica gel, petroleum ether:acetone, 8:1 to 0:1) and Sephadex LH-20 column (1 g; MeOH:H_2O, 50:50 to 100:0) to afford **7** (60 mg), **8** (3 mg) and **9** (115 mg); Fr. 5 (26 g) was further purified by CC (silica gel, CCl_3:MeOH, 20:1 to 0:1) and RP-18 column (MeOH:H_2O, 0:100 to 100:0) to yield **1** (10 mg), **2** (30 mg), **3** (8 mg) and **5** (50 mg).

Tupichinolide (**1**): white needles; mp 242—244°C; $[\alpha]_D^{18.3}$ −59.58 (*c* 0.16, MeOH); IR (KBr) ν_{max}: 3431, 2932, 1738, 1736, 1632, 1048 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 601 $[M+Na]^+$; negative HRESIMS *m/z* 613.2779 $[M+Cl]^-$ (calcd for $C_{31}H_{46}O_{10}Cl$, 613.2779).

Tupichinin A (**2**): white amorphous powder; $[\alpha]_D^{19.3}$ −122.34 (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3440, 2927, 1630, 1045 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; negative ESIMS *m/z* 561 $[M-H]^-$; negative HRESIMS *m/z* 561.3440 $[M-H]^-$ (calcd for $C_{32}H_{49}O_8$, 561.3427).

2.4. Acid hydrolysis of compounds **1** and **2**

Each compound (2 mg) was hydrolyzed with 1 M HCl–dioxane (1:1, 1 mL) at 80 °C for 4 h. The reaction mixture was partitioned between EtOAc and H_2O three times. The aqueous layer was neutralized with 2 M $NaHCO_3$ and then dried to in vacuo. The residue was dissolved in pyridine (0.5 mL), to which L-cysteine methyl ester hydrochloride in pyridine (0.1 M, 0.5 mL) was added. After reacting at 60 °C for 1 h, trimethylsilylimidazole (0.5 mL) was added to the reaction mixture and kept at 60 °C for another 30 min. The mixture was partitioned between *n*-hexane and H_2O, and the *n*-hexane extract was analyzed on Shimadzu GC-14C gas chromatograph equipped with a HP-5MS 30 m×0.32 mm column with the following conditions: column temperature, 210 °C; injector and detector temperature, 270 °C. By comparison of the retention times of the corresponding derivatives with that of standard sugar, the monosaccharides of compounds **1** and **2** were determined to be L-rhamnose, D-xylose, of which the retention time was 10.25, and 8.36 min, respectively.

2.5. Cytotoxicity assay

The cytotoxicity of the compounds against HL-60, SMMC-7721, A-549, MCF-7 and SW480 cell lines was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method [15], with cisplatin as positive control. Cell was maintained in RPMI 1640 medium and seeded in 96-well plates. After 12 h incubation at 37 °C, the test compounds were added and the plate was further incubated. After 48 h, 20 μL of MTT solution was added to each well, which was incubated for a further 4 h. Then 20% SDS (100 μL) was added to each well. After 12 h at room temperature, the OD value of each well was recorded at 595 nm. The IC_{50} value was calculated by the Reed and Muench method [16].

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compounds **1**, **2** and **5**.

Position	**1**[a]		**2**[b]		**5**[b]	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	31.5	1.52 (m)	79.4	4.06 (br s)	76.2	3.76 (dd, 12.0, 4.2)
2	27.4	1.62 (m)	29.3	2.31 (m), 1.64 (m)	36.6	1.98 (m), 1.69 (m)
3	73.5	3.95 (br s)	66.6	4.34 (br s)	75.4	4.04 (br s)
4	30.8	1.84 (m), 1.46 (m)	34.5	2.08 (m), 1.64 (m)	41.3	1.70 (m), 1.21 (m)
5	38.1	1.66 (m)	31.5	2.40 (br d, 12.5)	139.8	
6	27.7	1.90 (m), 1.26, (m)	26.4	1.76 (m), 1.13 (m)	125.5	5.47 (d, 5.4)
7	22.2	1.80 (m), 1.19 (m)	26.2	1.31 (m), 1.05 (m)	33.0	1.97(m), 1.29 (m)
8	42.7	1.61 (m)	35.6	1.62 (m)	33.7	1.60 (m)
9	36.6	1.71 (m)	41.7	1.26 (m)	51.6	1.29 (m)
10	36.2		39.4		44.9	
11	22.0	1.44 (m), 1.24 (m)	21.3	1.25 (m)	24.9	2.28 (m), 1.55 (m)
12	39.9	1.54 (m), 1.42 (m)	40.2	1.71 (m), 1.11 (m)	39.1	2.49 (d, 15.0)
						2.24 (m)
13	51.4		40.8		41.1	
14	84.6		56.3	1.12 (m)	58.0	1.17 (overlap)
15	41.3	2.78 (m), 1.79 (m)	32.1	2.04 (m), 1.43 (m)	33.0	1.97 (m), 1.30 (m)
16	75.9	5.46 (m)	81.5	4.59 (m)	82.5	4.45 (m)
17	57.4	3.27 (d, 8.5)	63.1	1.86 (m)	64.1	1.74 (t, 7.2)
18	16.4	0.92 (s)	16.7	0.84 (s)	17.0	0.84 (s)
19	24.3	0.94 (s)	19.7	1.33 (s)	13.3	1.03 (s)
20	171.6		41.8	1.96 (m)	42.8	1.93 (m)
21	77.5	5.02 (dd, 13.5, 1.5)	15.0	1.13 (d, 7.0)	14.9	0.95 (d, 7.2)
		4.96 (dd, 13.5, 1.5)				
22	121.7	5.98 (s)	109.4		110.7	
23	176.7		33.2	1.78 (m)	34.0	1.71 (m)
24			29.0	2.72 (m), 2.24 (m)	29.5	2.51 (m), 2.25 (m)
25			144.4		145.2	
26			65.0	4.04 (m)	65.8	4.26 (d, 12.0), 3.82 (d, 12.0)
27			108.8	4.79 (d)	108.9	4.77 (br s), 4.74 (br s)
Ac	172.1, 20.9	1.93 (s)				
1′	99.8	4.77 (d, 1.0)	102.3	4.85 (d, 7.5)	102.9	4.32 (d, 7.8)
2′	72.5	3.68 (m)	75.2	4.01 (m)	74.9	3.16 (t, 7.8)
3′	72.9	3.75 (br s)	78.9	4.19 (m)	78.2	3.34 (m)
4′	74.0	3.36 (t, 11.5, 9.5)	71.2	4.24 (m)	71.6	3.27 (m)
5′	70.0	3.65 (m)	67.6	4.38 (dd, 11.0, 5.0), 3.73 (t, 10.5)	77.9	3.25 (m)
6′	17.9	1.23 (t, 6.5)			62.8	3.85 (dd, 12.0, 2.4), 3.65 (dd, 12.0, 5.4)

[a] CD_3OD was used for NMR solvent.
[b] C_5D_5N was used for NMR solvent.

3. Results and discussion

Compound (**1**), obtained as white needles, exhibited the molecular formula $C_{31}H_{46}O_{10}$ with nine degrees of unsaturation as deduced from negative HRESIMS *m/z* 613.2779 $[M+Cl]^-$ (calcd for $C_{31}H_{46}O_{10}Cl$, 613.2779). The IR spectrum of **1** showed the presence of hydroxyl (3431 cm^{-1}) and α, β-unsaturated γ-lactone (1738 and 1632 cm^{-1}) groups. Acid hydrolysis liberated L-rhamnose, which was determined by GC analysis.

In the ^{1}H NMR spectrum, the characteristic signals for cardenolide were observed: two angular methyls at δ_H 0.92 and 0.94 (3H each, both s, H-18, 19), an oxygenated methylene group at δ_H 4.96 and 5.04 (1H each, both dd, 13.5, 1.5 Hz, H-21) and an olefinic proton at δ_H 5.98 (1H, s, H-22). The aglycone of **1** was identified as oleandrigenin by comparison of the detailed analysis of NMR data with literature value [17]. The NMR data of **1** were almost identical to those of (5α)-sarmentogenin 3-(α-L-rhamnopyranoside) [18], except for the chemical shift of C-5. The upfield shift of C-5 (δ_C 38.1) implied that H-5 was β-orientation, which was consistent with the previous literature [19]. The sugar unit was linked to C-3 confirmed by the HMBC correlation from δ_H 4.77 (H-1′) and δ_C 73.5 (C-3) (Fig. 2) and has an α-anomeric orientation deduced from the small coupling constant (J = 1.0 Hz) of the anomeric proton H-1′. Thus, compound **1** was elucidated unambiguously as oleandrigenin 3-(α-L-rhamnopyranoside), which was named as tupichinolide.

Compound **2** was isolated as a white amorphous powder with the molecular formula $C_{32}H_{50}O_8$, based on the negative HRESIMS 561.3440 $[M-H]^-$ (calcd for $C_{32}H_{49}O_8$, 561.3427). The IR spectrum showed absorptions for hydroxyl (3440 cm^{-1}), and double bond (1630 cm^{-1}). The five characteristic ^{13}C NMR signals (δ_C 102.3, 75.2, 78.9, 71.2, 67.6) further confirmed the presence of a xylose moiety and its β-anomeric orientation was determined by the large coupling constant (J = 7.5 Hz) of the anomeric proton in the ^{1}H NMR spectrum. The ^{13}C NMR (Table 1) and HSQC spectra revealed that the remaining signals of **2** contain three methyls, eleven methylenes including an olefinic one (δ_C 108.8) and an oxygenated one (δ_C 65.0), nine methines including three oxygenated ones (δ_C 66.6, 79.4, 81.5), and four quaternary carbons including an olefinic one (δ_C 144.4) and an oxygenated one (δ_C 109.4). The above data were almost identical with rhodeasapogenin 1-(β-D-xylopyranoside) (**3**) [20], except for the presence of an exocyclic double bond [δ_C 144.4 (s), 108.8 (t)] between C-25 and C-27 in **2**. The HMBC spectrum of **2** also confirmed that the xylose unit was

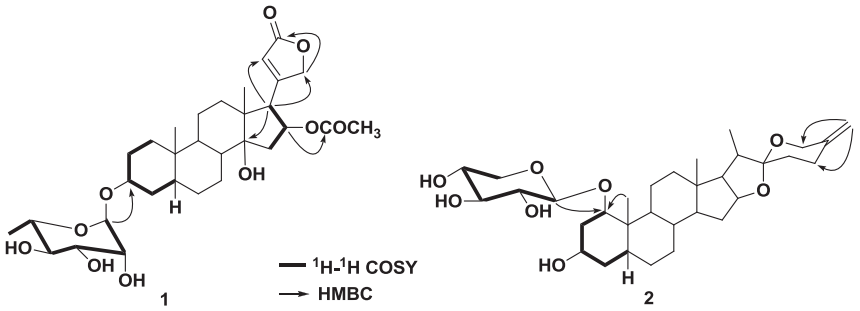

Fig. 2. Key selective $^{1}H-^{1}H$ COSY and HMBC correlations of compounds **1** and **2**.

attached at C-1 by the observed cross-peak between the anomeric H-1′ and C-1 (Fig. 2). From the above evidence, compound **2** was determined to be spirosta-25(27)-en-1β,3β-diol 1-(β-D-xylopyranoside), and named as tupichinin A.

Because there were no detailed ^{1}H and ^{13}C NMR spectral data of 3-epi-neoruscogenin 3-β-D-glucopyranoside (**5**) in the literature [21], we report the complete 1D NMR spectral assignments for this compound (Table 1). Other known compounds were identified as rhodeasapogenin 1-(β-D-xylopyranoside) (**3**) [20], 3-epi-neoruscogenin (**4**) [14], tupichigenin E (**6**) [14], ranmogenin A (**7**) [12], convallagenin B (**8**) [22], (20S,22R)-spirost-25(27)-ene-1β,2β,3β,4β,5β,7α-hexaol-6-one (**9**) [13], respectively, by comparison of the spectroscopic data with those reported in the literature.

Some cardenolides [23,24] and steroidal saponins [25,26] have been reported to show cytotoxic activity against cultured tumor cells. Considering this point, all compounds obtained were evaluated for their in vitro growth inhibitory effects against five human cancer cell lines (HL-60, SMMC-7721, A-549, MCF-7, and SW480) using the MTT method as reported previously, with cisplatin as the positive control. As seen from Table 2, the new compound **1** exhibited potent toxicity effects against HL-60, SMMC-7721, A-549, MCF-7, and SW480 cell lines, with IC_{50} values of 0.06, 0.06, 0.06, 0.06 and 0.12 μM, respectively. Meanwhile, two steroidal saponins **2** and **5** also showed moderate cytotoxicity against most of the selected cells (see Table 2), while the other compounds were inactive in these bioassays ($IC_{50}>40$ μM), which indicated that the steroidal saponins possess more cytotoxic activity than their sapogenins and the sugar moiety plays a key role in the activity. The results suggested that cardenolide and steroidal saponins were the antineoplastic constituents of this plant.

Table 2
Cytotoxicity of compounds **1**, **2** and **5** against cancer cell lines with IC_{50} (μM).[a]

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	0.06	0.06	0.06	0.06	0.12
2	18.58	>40	19.99	11.01	10.78
5	10.02	12.76	11.40	5.02	28.26
Cisplatin[b]	2.03	13.54	12.56	18.65	19.70

[a] Cell lines: HL-60 acute leukemia; SMMC-7721 liver cancer; A-549 lung cancer; MCF-7 breast cancer; SW480 colon cancer.
[b] Positive control.

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