A Pair of Diastereoisomeric Steroidal Saponins from Cytotoxic Extracts of *Tupistra chinensis* Rhizomes

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A pair of diastereoisomeric steroidal saponins were obtained from the saponin fraction (SF) of methanol extracts from *Tupistra chinensis* rhizomes, collected in Shennongjia Forest District, China. Based on the chemical and spectroscopic evidences, their structures were determined as shown in Fig. 1. The sample SF displayed marked inhibitory action *in vitro* towards HeLa and HL-60 cancer cell lines at 10 μg/ml by MTT method.

Key words *Tupistra chinesis*; steroidal saponin; cytotoxicity; folk medicine

More than 1800 species of medicinal plants grow in Shennongjia Forest District, a National Natural Protection Region of China. Most of them were recorded to be used as anti-inflammatory agents in the folk.[1)] Kaikoujian, the Chinese name of rhizomes of *Tupistra chinensis* Bak., is a reputed folk medicine in that district because of its potent to markedly reduce carbuncles and to ameliorate pharyngitis. In the course of our studies on the native folk medicines of Shennongjia Forest District,[2—4)] a pair of diastereoisomeric steroidal saponins were isolated and their structures were elucidated by means of chemical and spectroscopic methods. Although studies on the lipophilic constituents, including the sapogenins of this plant, were reported in several papers,[5—7)] the steroidal saponins, especially those showing anti-tumor activities, were hardly elucidated yet.[8)] This paper reported the isolation and structural elucidation of two steroidal saponins from cytotoxic extracts of this plant.

Compounds **1** and **2**, two distinctly separate peaks appearing on the analytical HPLC, were separated by means of preparative HPLC and purified by repeated semi-preparative HPLC. **1** was obtained as a colorless powder. Its HR-FAB-MS showed a quasi-molecular ion peak at *m/z* 1103.5252 $[M+Na]^+$, which indicated a formula $C_{51}H_{84}O_{24}Na$ (Calcd: 1103.5250). Positive coloration reactions were observed when **1** was subjected to Ehrlich, Molish and Liebermann–Buchard tests, which suggested that **1** have a steroidal saponin skeleton. ^{1}H-NMR data of **1** contained an olefinic proton at δ 5.28 (br s), a methenyl proton at δ 4.98 (m), two doublet methyl groups at δ 1.01(d, J=7.0 Hz) and 1.31(d, J=7.0 Hz), two singlet methyl groups at δ 0.88 and 0.86 (each s), attributable to a steroidal aglycone moiety. Furthermore, the furostanol glycosidic nature of **1** was suggested by the strong absorption bands at 3432 and 1050 cm^{-1} in the IR spectrum, and a semiketal carbon signal at δ 110.65 in the ^{13}C-NMR spectrum.[9)]

The above ^{1}H-NMR spectral data and a comparison of the ^{13}C-NMR signals of the aglycone moiety of **1** with those described in the literature[10)] showed the structure of the aglycone to be (3β,22α,25*S*)-furost-5-en-3,22,26-triol. The α-configuration of C-22 hydroxyl group of the aglycone moiety was deduced from the semiketal carbon signal at δ 110.65, instead of δ 115.5 for β-configuration, and it was further confirmed by the ROESY correlation between H-20 (2.21, dq, J=6.5, 7.5 Hz) and H-23 (1.95, m).[11)] The 25*S* stereochemistry was inferred by the resonance of H-27 methyl protons at δ 1.01, a few bigger than 1.00[12)]; and also by the resonance difference between equatorial proton signal (3.46, dd, J=6.5, 9.5 Hz) and axial proton signal (4.06, m) of H-26: 0.60 ppm, a few bigger than 0.57 ppm.[13)] Yamogenin,[14)] an isomer of diosgenin, was obtained from the hydrolysate of compound **1**, which further confirmed a 25*S* configuration for compound **1**.

When **1** was hydrolyzed with 2.0 M HCl, only glucose was detected in the hydrolysate on TLC and PC. Among the 51 carbon signals in the ^{13}C-NMR spectrum, 27 signals were assignable to the aglycone, the remaining 24 signals were indicative of the presence of four glucose moieties, in good agreement with the four anomeric proton signals appearing at δ 5.21 (d, J=7.5 Hz), 5.12 (d, J=8.0 Hz), 4.89 (d, J=7.5 Hz), and 4.78 (d, J=7.5 Hz), and the corresponding anomeric carbon signals at δ 106.92, 105.20, 102.68 and 105.13, respectively.

The absence of any glycosidation shift for two β-D-glucopyranosyl moieties which contained anomeric protons at δ 4.78 and 5.21 as obtained from HMQC and TOCSY data, suggested that there be at most two sugar chains in compound **1**. A sugar chain was determined to be attached to C-26 by an observation of glycosidation shift of C-26 signal from δ 66.00 to 75.37, and this linkage was further indicated by the cross peak between the carbon-13 signal at δ 75.37 and the anomeric proton signal at δ 4.78 of β-D-glucopyranosyl moiety in the HMBC spectrum of **1**.

Similarly, a cross peak between the C-3 signal of aglycone at δ 78.13 and the anomeric proton signal at δ 4.89 in the HMBC spectrum of **1**, together with a correlation between H-3 proton at δ 3.98 and the anomeric proton at δ 4.89 in the NOESY spectrum of **1**, indicated that another sugar chain was located at the C-3 position of aglycone. A 1$\rightarrow$4 linkage of the middle sugar moiety to the inner one was revealed by a cross peak between the C-4 signal of the inner glucose at δ 81.11 and the anomeric proton signal of the middle glucose at δ 5.12 in the HMBC spectrum of **1**, and further determined by the correlation between the H-4 proton signal of the inner moiety at δ 4.57 and the anomeric proton signal at δ 5.12 in the NOESY spectrum of **1**. Finally, the terminal sugar residue was assigned to be located at the C-2 position of the middle moiety, which was determined by a correlation between the C-2 signal of the middle moiety at δ 86.12 and

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Table 1. ^{13}C- and ^{1}H-NMR Data for Compounds **1** and **2** (125, 500 MHz in Pyridine-d_5)

Position	1		2		Position	1		2	
	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)		δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)
1	37.47	1.69, m	37.48	1.69, m	Glc′				
		0.96, m		0.95, m	1	102.68	4.89	102.68	4.89
2	30.25	2.06, m	30.24	2.06, m			d (7.5)		d (7.5)
		1.69, m		1.70, m	2	75.57	3.98, m	75.57	3.98, m
3	78.13	3.98, m	78.12	3.98, m	3	76.75	4.05, m	76.75	4.05, m
4	39.27	2.65, d (13.0)	39.27	2.65, d (13.0)	4	81.11	4.57, m	81.09	4.58, m
		2.43, t (13.0)		2.43, t (13.0)	5	73.27	4.48, m	73.27	4.47, m
5	140.97		140.97		6	60.42	4.73, m	60.42	4.73, m
6	121.67	5.28, br s	121.67	5.28, br s			4.18, m		4.18, m
7	31.63	1.51, m	31.62	1.51, m	Glc″				
8	32.28	1.82, m	32.28	1.82, m	1	105.20	5.12	105.21	5.12
9	50.31	0.86, m	50.31	0.86, m			d (8.0)		d (8.0)
10	37.14		37.14		2	86.12	4.14, m	86.12	4.14, m
11	21.09	1.41, m	21.09	1.41, m	3	77.63	4.09, m	77.63	4.08, m
12	39.92	1.09, m	39.92	1.09, m	4	70.31	4.20, m	70.31	4.20, m
		1.73, m		1.73, m	5	78.61	4.23, m	78.60	4.23, m
13	40.76		40.76		6	61.59	4.58, m	61.59	4.57, m
14	56.57	1.06, m	56.57	1.06, m			4.34, m		4.34, m
15	32.43	1.44, m	32.43	1.44, m	Glc‴				
16	81.09	4.98, m	81.08		1	106.92	5.21	106.94	5.21
17	63.78	1.94, m	63.78	1.94, m			d (7.5)		d (7.5)
18	16.46	0.86, s	16.46	0.86, s	2	75.11	4.00, m	75.11	4.00, m
19	19.37	0.88, s	19.37	0.88, s	3	78.46	4.24, m	78.47	4.24, m
20	40.58	2.21	40.68	2.21	4	71.71	4.21, m	71.70	4.21, m
		dq (6.5, 7.5)		dq (6.5, 7.6)	5	78.94	3.78, m	78.94	3.77, m
21	16.46	1.31, d (7.0)	16.46	1.31, d (7.0)	6	62.81	4.56, m	62.81	4.56, m
22	110.65		110.65				4.34, m		4.35, m
23	37.03	2.04, m	37.03	2.02, m	26-Glc				
		1.95, m		1.95, m	1	105.13	4.78	105.14	4.78
24	28.31	1.74, m	28.31	1.74, m			d (7.5)		d (7.5)
		2.00, m		1.99, m	2	75.21	4.01, m	75.21	4.00, m
25	34.42	1.91, m	34.27	1.91, m	3	78.19	4.26, m	78.19	4.26, m
26	75.37	3.46, dd	75.37	3.61, dd	4	71.83	4.23, m	71.83	4.23, m
		(6.5, 9.5)		(5.5, 9.5)	5	78.47	3.96, m	78.47	3.96, m
		4.06, m		4.06, m	6	62.81	4.55, m	62.81	4.56, m
27	17.43	1.01, d (7.0)	17.44	0.97, d (7.0)			4.35, m		4.35, m

the anomeric proton signal of the terminal sugar at δ 5.21 in the HMBC spectrum of **1**, as well as by a cross peak due to a correlation between the H-2 proton signal of the middle sugar residue at δ 4.14 and the anomeric signal of the terminal residue at δ 5.21 in the NOESY spectrum of **1**.

On the basis of all the evidences, compound **1** was identified as (25*S*)-26-*O*-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside.

Compound **2** was obtained from a peak closely behind that of compound **1** on the HPLC. The coloration tests, IR, 1D and 2D NMR spectral data of **2** were almost superimposable on those of compound **1**, with an exception that the proton signals of **2** due to H-26 [δ 3.61 (dd, J=5.5, 9.5 Hz, H-equatorial); 4.06 (m, H-axial)] and H-27 [δ 0.97 (d, J=7.0 Hz)] of the aglycone were slightly but distinctly different from those of **1**. The chemical shift difference (0.45 ppm) between the equatorial proton and the axial one was smaller than 0.48 ppm, indicating a 25*R* configuration of **2**,[13] which was consistent with the fact that the chemical shift of H-27 in compound **2** was less than 1.00 ppm.[12] Furthermore, diosgenin was obtained from the acid hydrolysate of **2**, which confirmed the 25*R* configuration of **2**. Accordingly, compound **2** was identified as (25*R*)-26-*O*-(β-D-glucopyranosyl)-furost-5-en-3β,22α,26-triol 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside.

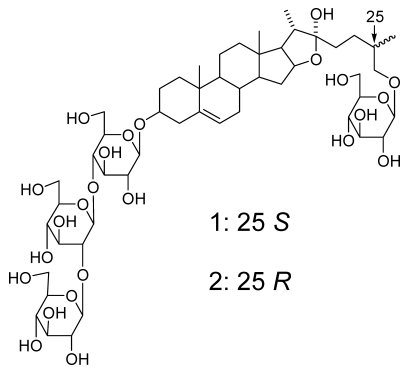

Fig. 1. The Structures of Compounds **1** and **2**

The separation of a steroidal mixture of 25*R* and 25*S* epimers is very difficult,[15] however, compounds **1** and **2** were obtained with a satisfactory purity in this experiment, *ca.* 99.9% for compound **1** and *ca.* 98% for compound **2**.

Compounds **1** and **2** were isolated from SF (a sample, see Extraction and isolation), which showed marked inhibitory action towards HeLa (inhibition 81%) and HL-60 (inhibition

92%) cancer cell lines in MTT method at 10 μg/ml.

Experimental

Optical rotations were recorded with a Perkin-Elmer 241 spectropolarimeter. IR spectra were measured on a Nicolet FT360 instrument as samples in pressed KBr disks. 1D and 2D NMR spectra were recorded using Bruker AM 500 and instruments with Me_4Si as the intestinal standard. FAB and ESI mass spectra were obtained using a VG AUTO Spec-300 mass spectrometer and Finnigan-MAT LCQ DECA XP plus mass spectrometer, respectively. HPLC was performed using a Varian ProStar 1510 system for analytical (YMC-Pack ODS-AQ column: 5 μm, 60 Å, 250×4.6 mm i.d.), preparative (YMC-Pack ODS-AQ column: 5 μm, 120 Å, 250×20 mm i.d.) and semi-preparative (μBondpak C_{18} column: 6 μm, 60 Å, 7.8×300 mm i.d.) HPLC. Macroporous resins (AB-8, Nankai), Silica gel (10—40 μm, 200—300 mesh, Qingdao), Sephadex LH-20 (OUYA, Pharmacia), RP C_{18} Silica Gel (100—200 mesh, YMC) were used as packing materials for column chromatography.

Plant Material The rhizomes of *T. chinensis* were collected near Muyu, a town of Shennongjia Forest District of China in August 2004 and identified by Professor Chen Faju. A voucher specimen (Herbarium No.: 2002ZW07408) has been deposited in the Herbarium of Department of Medicinal Plants, College of Chemistry and Life Science, China Three Gorges University, Yichang.

Extraction and Isolation Air-dried powdered rhizomes (7.85 kg) was extracted with methanol under reflux. After the removal of solvent *in vacuo* and freeze-drying, the methanol extract (3659 g) was obtained. The extract was suspended in water (3.6 l), and then extracted with $CHCl_3$, EtOAc and *n*-BuOH, respectively. The *n*-BuOH-soluble extract (300 g out of 1383 g) was dissolved in water (2.0 l), and then was subject to macroporous resin column chromatography with gradient elution (100% water→100% methanol). The 30% methanol eluate (60 g, SF) was dissolved in methanol (100 ml), and then poured drop wise into acetone (1.5 l). The resulting precipitates (58 g) were separated by repeated Rp-C_{18} silica gel column chromatography in elution with gradient solvent system (100% water→60% acetonitrile) to give rise to 83 fractions. Fractions 70—72 (1.8 g) was further separated by repeated preparative HPLC eluted with gradient solvent (15%→30% acetonitrile within 60 min, 6.0 ml/min, 203 nm detection), and then repeated semi preparative HPLC eluted with gradient solvent (20%→25% acetonitrile within 30 min, 1.5 ml/min, 203 nm detection), giving rise to compounds **1** (76 mg) and **2** (28 mg).

Bioassay The cytotoxic activity of the sample SF (see Extraction and isolation) was evaluated according to the same protocol as the literature.[16)]

Acid Hydrolysis Compounds **1** (18 mg) and **2** (11 mg) were refluxed with 2.0 M HCl, respectively. The hydrolysate was neutralized with $NaHCO_3$, and then extracted with chloroform. The chloroform-solvable part was repeatedly purified by using preparative TLC, giving rise to yamogenin (3.5 mg) and diosgenin (2.1 mg), respectively.

Yamogenin: White needles, mp 180—182 °C, $[\alpha]_D^{20}$ −127° (c=0.015, chloroform). EI-MS *m/z*: 414 $[M]^+$. IR (KBr) ν_{max} cm^{-1}: 3400, 2923, 1640, 1450, 1033, 968, 911, 850. ^{1}H-NMR (500 MHz, $CDCl_3$) was in good agreement with those reported.[17)]

Diosgenin: White needles, mp 204—207 °C, $[\alpha]_D^{20}$ −126° (c=0.010, chloroform). EI-MS *m/z*: 414 $[M]^+$. IR (KBr) ν_{max} cm^{-1}: 3401, 2924, 1638, 1448, 1032, 960, 915, 856. ^{1}H-NMR (500 MHz, $CDCl_3$) was in good agreement with those reported.[18)]

Compound **1**: Amorphous white powder, $[\alpha]_D^{20}$ −43.6° (c=0.38, 30% acetonitrile). HR-FAB-MS (positive ion mode) *m/z*: 1103.5252 $[M+Na]^+$ (Calcd for $C_{51}H_{84}O_{24}Na$: 1103.5250). ESI-MS (positive ion mode) *m/z*: 1119 $[M+K]^+$, 1103 $[M+Na]^+$, (negative ion mode) *m/z*: 1079 $[M-H]^-$, 917 $[M-glc-H]^-$. IR (KBr) ν_{max} cm^{-1}: 3432, 2923, 1645, 1050 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) see Table 1.

Compound **2**: Amorphous white powder, $[\alpha]_D^{20}$ −28.7° (c=0.23, 30% acetonitrile). HR-FAB-MS (positive ion mode) *m/z*: 1103.5258 $[M+Na]^+$ (Calcd for $C_{51}H_{84}O_{24}Na$: 1103.5250). ESI-MS (positive ion mode) *m/z*: 1119 $[M+K]^+$, 1103 $[M+Na]^+$, (negative ion mode) *m/z*: 1079 $[M-H]^-$, 917 $[M-glc-H]^-$. IR (KBr) ν_{max} cm^{-1}: 3431, 2922, 1643, 1050 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) and ^{13}C-NMR (125 MHz, pyridine-d_5) see Table 1.

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References

1) Zhan Y. H. (ed.), "Resources of Medicinal Plants in Shennongjia of China," Hubei Scientific and Technologic Press, Wuhan, 1974, pp. 16—29.
2) Zou K., Liu Z. X., Zhu S., Cai S. Q., Komatsu K., *Acta Pharm. Sin.*, **39**, 385—388 (2004).
3) Huang L., Liao Q. B., Zou K., Ruan H., Nie D. D., Hu C. L., Hu Y. L., *J. China Three Gorges Univ. (Nat. Sci. Ed.)*, **25**, 562—564 (2003).
4) Yang C. Y., Yang X. H., Liu Y., Yue C. Y., Zou K., *J. Chin. Folk Ethnic Med.*, **73**, 103—106, 124 (2005).
5) Pan W. B., Chang F. R., Wu Y. C., *J. Nat. Prod.*, **63**, 861—863 (2000).
6) Pan W. B., Chang F. R., Wu Y. C., *Chem. Pharm. Bull.*, **48**, 1350—1353 (2000).
7) Pan W. B., Chang F. R., Wei L. M., Wu Y. C., *J. Nat. Prod.*, **66**, 161—168 (2003).
8) Wu G. X., Wei Y. X., Chen W. X., *Chin. Chem. Lett.*, **16**, 911—914 (2005).
9) Braca A., Prieto J. M., Tommasi N. D., Tome F., Morelli I., *Phytochemistry*, **65**, 2921—2928 (2004).
10) Pereira B. D. S., Bernardo R. R., Parente J. P., *Phytochemistry*, **51**, 931—935 (1999).
11) Fattorusso E., Iorizzi M., Lanzotti V., Targlialatelascafati O., *J. Agric. Food Chem.*, **50**, 5686—5690 (2002).
12) Murakami T., Kishi A., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **48**, 994—997 (2000).
13) Agrawal P., *Magn. Reson. Chem.*, **42**, 990—993 (2004).
14) Marker R. E., Wagner R. B., Ulshafer P. R., *J. Am. Chem. Soc.*, **65**, 1199—1209 (1941).
15) Zheng Q. A., Zhang Y. J., Li H. Z., Yang C. R., *Steroids*, **69**, 111—119 (2004).
16) Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. T., Bokesch H., Kenney S., Boyd M. R., *J. Natl. Cancer Inst.*, **82**, 1107—1109 (1990).
17) Saxena V. K., Shalem A., *J. Chem. Sci.*, **116**, 79—82 (2004).
18) Yahara S., Murakami N., Yamasaki M., Hamada T., Kinjo J., Nohara T., *Phytochemistry*, **24**, 2748—2750 (1985).