Department of Natural Products Chemistry[1], Shenyang Pharmaceutical University Shenyang , China, Marine Drug and Food Institute[2], Qingdao Ocean University, Qingdao , China, and Institute of Molecular & Cellular Biosciences[3], The University of Tokyo, Tokyo, Japan

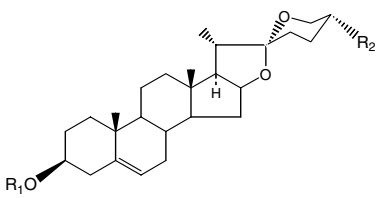
Bioactive saponins from *Dioscorea futschauensis*

H. W. Liu[1], K. Hu[1], Q. C. Zhao[1], C. B. Cui[2], H. Kobayashi[3] and X. S. Yao[1]

A new anti-neoplastic spirostanol saponin, (25*S*)−spirost-5-en-3β, 27-diol-3*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→3)]-β-D-glucopyranoside and three known compounds viz. prosapogenin A of dioscin, dioscin and gracilin were isolated from *Dioscorea futschauensis* by bioactivity-guided fractionation. Their structures were elucidated mainly by means of spectroscopic analysis. Their bioactivity against *Pyricularia oryzae* and cytotoxic activity on ts-FT210 cell line was evaluated.

1. Introduction

A screening bioassay detecting deformation of mycelia germinated from conidia of *Pyricularia oryzae* P-2b was first developed for quantitative application to screen antifungal and antineoplastic agents by. Kobayashi et al. [1]. Hu et al. first introduced and applied this bioassay in the search of bioactive agents from Traditional Chinese Medicine [2–4]. The rhizomes of *Dioscorea futschauensis* R. Kunth (Dioscoreaceae) are used as a traditional Chinese medicine "Mian Bi Xie" for the treatment of rheumatism and urinary tract disease. It is widely distributed in Southeast China and included in the Pharmacopoeia of People's Republic of China (2000). In our on-going program of screening the bioactive agents from Traditional Chinese Medicine, the ethanol extract of the plant showed strong activity against the growth of *Pyricularia oryzae* P-2b. This paper reports the isolation and structure elucidation of a new spirostanol glycoside from the rhizomes of this plant.



4 R_1= β–D-Glc(2→α–L-Rha, 3→β–D-Glc) $R_2 = CH_2OH$

2. Investigations, results and discussion

The n-butanol soluble fraction of the ethanol extract of *D. futschauensis* was subjected to repeated silica gel column chromatography and reversed phase HPLC to afford saponin **1**–**4**. Saponins **1**–**3** were identified as prosapogenin A of dioscin, dioscin and gracilin, based on their spectral data and comparison with literature data [5].

Saponin **4** was obtained as white amorphous powder, positive to the Libermann-Burchard reaction and Molish test, but it did not respond to Ehrlich reagent. In its IR spectrum, the broad absorption bands at 3420 and 1046 cm^{-1} indicated a glycosidic structure. The positive FAB-MS gave the quasimolecular ion peak $[M + H]^+$ at m/z 901 and three fragments M + H-Glc$^+$ at m/z 739, M + H-Rha-Glc$^+$ at m/z 593, M + H-Glc × 2-Rha$^+$ at m/z 431. The molecular formula was established as $C_{45}H_{72}O_{18}$ on the basis of its NMR data and FAB-MS. The ^{1}H NMR spectrum of **4** confirmed the presence of four methyl groups at δ 0.82 (s, Me-18), 1.05 (s, Me-19), 1.75 (d, J = 6.0 Hz, Rha Me-6″) and 1.15 (s, Me-21), and revealed additional three anomeric protons at δ 6.40 (br.s, Rha-1″), 4.93 (d, J = 6.8 Hz, Glc-1′) and 5.10 (d, J = 8.0 Hz, Glc-1‴), together with one olefinic proton at δ 5.30 (br. d, H-6). In addition to the carbon signals from sugar moieties, 27 carbon signals consisting three methyls, eight methines, eleven methylenes, three quaternary carbons and a pair of olefinic carbon were observed in its ^{13}C NMR spectrum. Comparing the NMR data of **4** with that of **3**, a great similarity was observed among A-E ring. However, the methyl carbon signal at δ 17.3 in **3** was absent in **4,** and replaced by a oxygenated methylene signal at δ 64.4 that was correlated with two-proton signals at δ 3.65 and 3.72 in HMQC of **4.** In the meantime, the chemical shift change at C-25 (+8.6), C-24 (−5.1) and C-26 (−2.9) in the ^{13}C NMR spectrum of **4** was observed as well. Therefore, it is suggested that a CH_2OH group is connected at C-25 in saponin **4**. The 25*S*-configuration (equatorial orientation of the CH_2OH group) of **4** was indicated by the ^{1}H NMR parameters of the C-26 proton (δ 3.86, 1 H, t, $J_{26ax,\ 26eq} = 11.2$ Hz, $J_{26ax,\ 25ax} = 11.2$ Hz, H-26α and δ 4.13, 1 H, dd, $J_{26eq,\ 26ax} = 11.2$ Hz, $J_{26eq,\ 25ax} = 4.0$ Hz, H 26-β). All the evidence tends to determine the structure of aglycone as (25*S*)-spirost-5-en-3β, 27-diol.

After acid hydrolysis, the sugar moieties were determined as glucose, rhamnose by TLC comparison with the authentic samples. The ^{13}C NMR spectral data for three sugars in **4** were also in good agreement with that of **3**. By HMBC analysis, the anomeric proton signals at δ 6.39 (H-1″ of the terminal rhamnose attached to C-2′ of the inner glucose), 5.11 (H-1‴ of the terminal glucose attached to C-3′ of the inner glucose), and 4.94 (H-1′ of the 2′, 3′-substituted inner glucose attached to C-3 of the aglycone) showed cross-peaks with the carbon signals at δ 77.0 (C-2′ of the 2′, 3′-substituted inner glucose), 89.6 (C-3′ of the 2′, 3′-substituted inner glucose), 77.9 (C-3 of the aglycone), respectively. β-Configuration at the anomeric position may be inferred from the values of the coupling constants for both glucopyranosyl units (6.8, 8.0 Hz). The α-configuration of the anomeric carbon of the rhamnose was assured by comparison of the chemical shift values of carbons 3″and 5″with those of the corresponding carbons of methyl α- and β-rhamnopyronoside [6]. These signals were sufficient to determine the linkages by which the sugars were connected. Based on the data mentioned above, **4** was determined to be (25*S*)-spirost-5-en-3β, 27-diol-3-*O*-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→3)]-β-D-glucopyranoside.

Table 1: ^{1}H NMR data for saponin 3 and 4 in pyridine-d_5 (δ-values)

	3 δ_H	**4** δ_H		**3** δ_H	**4** δ_H
1	0.96; 1.75 (o)[a]	0.94; 1.74 (o)	3-O-Glc		
2	1.87; 2.10 (o)	1.86; 2.07 (o)	1′	4.94 (d, 7.5)	4.93 (d, 6.8)
3	3.95 (m)	3.95 (m)	2′	4.20 (o)	4.20 (o)
4	2.74; 2.80 (m)	2.72; 2.78 (o)	3′	4.18 (o)	4.18 (o)
5			4′	4.06 (o)	4.06 (o)
6	5.32 (br.s)	5.30 (br.s)	5′	3.82 (o)	3.82 (o)
7	1.50;1.87 (o)	1.48; 1.88 (o)	6′	4.24; 4.43 (o)	4.24; 4.43 (o)
8	1.54 (o)	1.56 (o)	Rha(1→2)		
9	0.90 (o)	0.88 (o)	1″	6.39 (d,1.0)	6.40 (br.s)
10			2″	4.89 (o)	4.90 (o)
11	1.42 (o)	1.45 (o)	3″	4.57 (o)	4.58 (o)
12	1.08; 1.66 (o)	1.08; 1.70 (o)	4″	4.32 (o)	4.32 (o)
13			5″	4.93 (o)	4.93 (o)
14	1.06 (o)	1.07 (o)	6″	1.75 (d, 6.5)	1.75 (d, 6.0)
15	1.43; 2.03 (o)	1.43; 2.02 (o)	Glc(1→3)		
16	4.53 (m)	4.53 (m)	1‴	5.11 (d, 7.5)	5.10 (d, 8.0)
17	1.79 (o)	1.78 (o)	2‴	4.01 (o)	4.02 (o)
18	0.82 (s)	0.82 (s)	3‴	4.16 (o)	4.16 (o)
19	1.05 (s)	1.05 (s)	4‴	4.12 (o)	4.12 (o)
20	1.94 (o)	1.94 (o)	5‴	4.02 (o)	4.02 (o)
21	1.13 (d, 7.0)[b]	1.15 (o)	6‴	4.28; 4.55 (o)	4.28; 4.55 (o)
22					
23	1.65 (o)	1.68 (o)			
24	1.56 (o)	1.57 (o)			
25	1.57 (o)	2.04 (o)			
26	3.49; 3.57 (m)	3.86 (dd, 11.2, 11.2) 4.13 (dd, 11.2, 4.0)			
27	0.69 (d, 6.5)	3.65; 3.72 (o)			

a All of the signals were assigned by ^{1}H-^{1}H COSY, HMQC and HMBC spectra. Overlapped signals are indicated by "(o)".
b *J* values (in parentheses) are reported in Hz

Complete assignment of saponin 4 was achieved with the aid of the ^{1}H-^{1}H COSY and HMQC and HMBC spectra (Tables 1 and 2).

Table 2: ^{13}C NMR data for saponin 3 and 4 in pyridine-d_5 (δ-values)

	3 δ_C	**4** δ_C		**3** δ_C	**4** δ_C
1	37.5	37.5	3-O-Glc		
2	30.1	30.1	1′	100.0	100.0
3	77.9	77.9	2′	77.0	77.0
4	38.7	38.7	3′	89.6	89.6
5	140.8	140.8	4′	69.6	69.6
6	121.9	121.9	5′	77.7	77.9
7	32.2	32.3	6′	62.4	62.4
8	31.7	31.7	Rha(1→2)	102.2	102.2
9	50.3	50.3	1″	72.5	72.5
10	37.1	37.1	2″	72.8	72.8
11	21.1	21.1	3″	74.1	74.1
12	39.9	39.9	4″	69.6	69.6
13	40.5	40.5	5″	18.7	18.7
14	56.6	56.6	6″		
15	32.2	32.3	Glc (1→3)		
16	81.1	81.1	1‴	104.6	104.6
17	62.9	62.9	2‴	75.0	75.0
18	16.3	16.3	3‴	78.5	78.5
19	19.4	19.4	4‴	71.5	71.5
20	42.0	42.0	5‴	78.7	78.6
21	15.0	15.0	6‴	62.4	62.4
22	109.7	109.7			
23	31.7	31.7			
24	29.1	24.0			
25	30.6	39.2			
26	66.9	64.0			
27	17.3	64.4			

Saponins **1**–**4** induced morphological deformation *P. oryzae* mycelia with MMDC (minimum morphologic deformation concentration) values of 6.0, 2.0, 10.0, 12.0 μM, respectively. A mouse ts-FT210 cell line, which is a temperature-sensitive p34^{cdc2} mutant isolated from the mouse mammary carcinoma cell line FM3A, was used for antineoplastic test. In our study, Saponins **1**–**4** showed cytotoxic activity on ts-FT210 cell line with the MIC values at 4.32, 3.59, 3.53, 3.46 μM by morphological observation with the aid of flow cytometry.

3. Experimental

3.1. Instruments

Melting points: Yanaco MP-S3 micro-melting point apparatus, uncorr. Optical rotations: Perkin-Elmer 241 polarimeter at 13°. IR: Jasco A-102 (KBr). ^{1}H and ^{13}C NMR: JNM Alpha-500 (^{1}H 500 MHz, ^{13}C 125 MHz) spectrometer with TMS as internal standard). Positive-ion FAB-MS: JEOL JMS-DX302. Prep. HPLC: Liquid Chromatograph LC-10 (Japan Analytical Industry Co., Ltd.) using an ODS column (Waters, 20 × 250 mm, 5 μm) with flow rate of mobile phase 4.0 ml · min^{-1}. C. C. silica gel H (10–40 μm, Qingdao Haiyang Chemical Factory). TLC: silica gel G (10–40 μm, Qingdao Haiyang Chemical Factory). Spots were visualized by spraying with 10% H_2SO_4 followed by heating.

3.2. Plant material, extraction and isolation

Rhizomes of *Dioscorea futschauensis* R. Kunth (Dioscoreaceae) were collected in 1999 from Fujian Province (China), and identified by Prof. Qishi Sun (Division of Pharmacognosy, Shenyang Pharmaceutical University). A voucher specimen is deposited at the herbarium of Shenyang Pharmaceutical University, Liaoning Province.

Air-dried powdered rhizomes (3000 g) of *D. futschauensis* were refluxed with 75% EtOH . The combined EtOH soln. were concd. *in vacuo* to give 330 g extract. A suspension of the resulting extract in H_2O was partitioned with n-BuOH to afford n-butanol soluble fraction DB (100 g) residues. Fr. DB (50 g) was subjected to CC on silica gel H (500 g) and eluted stepwisely by $CHCl_3$–MeOH (100 : 1, 100 : 2, 97 : 3, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 70 : 30 and 60 : 40, each 5000 ml) to give 27 corresponding frs. Fr.

DB-15-DB-25 were active against the growth of *Pyricularia oryzae*. DB-15 (100 mg, 90 : 10), DB-19 (900 mg, 80 : 15), DB-20 (400 mg, 80 : 20) were recrystallized to give known the saponins **1** (20 mg), **2** (600 mg) and **3** (100 mg), respectively. Fr. DB-21 (100 mg) was further separated by prep. HPLC (column, ODS, 5 μm , 20 × 250 mm; solvent, 70% aq. MeOH; flow rate, 4.0 ml · min^{-1}) to give saponin 4 (5 mg).

Saponin 4: Amorphous powder, m.p. 284–285°C (dec.); $[\alpha]_D^{24}$ −80.3° (pyridine; *c* 0.01). IR ν_{max} cm^{-1}: 3420 (OH), 2940, 1639, 1454, 1381, 1046 (glycosylic C−O), 912. Anal. calc. for $C_{45}H_{72}O_{18}$. 4 H_2O; C, 55.56; H, 8.23%; found C, 55.54; H, 8.28%. FAB-MS (positive) m/z: 901 $[M + H]^+$, 739 $[M + H\text{-Glc}]^+$, 593 $[M + H\text{-Rha-Glc}]^+$, 431 $[M + H\text{-Glc} \times 2\text{-Rha}]^+$. ^{1}H NMR: 0.82 (3 H, s, CH_3-18), 1.05 (3 H, s, CH_3-19), 1.15 (3 H, d, J = 6.5 Hz, CH_3-21), 1.75 (3 H,d, J = 6.0 Hz, H-Rha 6″), 4.93 (1 H, d, J = 6.8 Hz, H-Glc1′), 5.10 (1 H, d, J = 8.0 Hz, H-Glc1‴), 5.30 (1 H, br s, H-6), 6.40 (1H, br s, H-Rha1″). ^{13}C NMR: Table **2**.

3.5. Acid hydrolysis

Saponin **4** (2 mg) was heated with 2N HCl (2 ml) in a sealed tube at 100 °C for 4 h. The aglycone was extracted with ethyl acetate, the aqueous layer was concentrated to dryness. The sugar moieties were identified by co-TLC with an authentic sample using solvent system EtOAc/CH_3OH/H_2O/CH_3COOH (65 : 15 : 15 : 25). The plates were sprayed with naphtoresorcinol phosphoric reagent by heated at 100 °C.

3.6. Bioassay

Bioassay against *Pyricularia oryzae* was done as previously reported [7]. The bioassay of cytotoxic activities on a mouse ts-FT210 cell line was carried out on flow cytometry as described by Cui et al. [8].

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Dr. Xinsheng Yao
Department of Natural Products Chemistry
Shenyang Pharmaceutical University
Shenyang 110015
China
yaoxinsheng@hotmail.com