Two New Pregnane Glycosides from *Dioscorea futschauensis* R. KUNTH

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Two new pregnane glycosides (1, 2) together with two known saponins were isolated from the rhizomes of *Dioscorea futschauensis* R. KUNTH. The structures of 1 and 2 were established as 16α-methoxyl-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl)oxy]pregn-5-en-20-one and 21-methoxyl-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl)oxy]pregn-5,16-en-20-one, respectively, on the basis of two-dimension NMR (2D NMR) and other spectral analysis. Their *in vitro* bioactivity against plant pathogenic fungus *Pyricularia oryzae* and osteoblastic proliferation stimulatory activity in the UMR106 cell line were evaluated.

Key words pregnane glycoside; *Dioscorea futschauensis*; anti-fungal activity; osteoblastic proliferation stimulatory activity; UMR106 cell line

In our ongoing study on the biologically active steroidal saponins from *Dioscorea* genus, a series of studies on the isolation, structural elucidation, and biological activity of saponins from *Dioscorea* species has been conducted by our group.[1—10] In the study of saponins of *Dioscorea futschauensis* R. KUNTH, we reported the isolation and structure elucidation of three new steroidal saponins together with 14 known saponins.[8—10] Continuing the preceding studies, two new pregnane glycosides (**1**, **2**) together with two known saponins (**3**, **4**) were obtained from the rhizomes of *D. futschauensis*. Herein, we would like to describe the isolation and characterization of these saponins, as well as their anti-fungal activity against *Pyricularia oryzae* and osteoblastic proliferation stimulatory activity on the UMR106 cell line.

The *l*-butanol soluble fraction of the ethanol extract of the plant was subjected to column chromatography over silica gel and the combined saponin containing fractions were then subjected to prep.HPLC. Compounds **1**—**4** were isolated from saponin-containing subfraction 25. Compounds **3** and **4** were identified as the known pregnane glycosides, 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl)oxy]pregn-5,16-en-20-one (**3**) and 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-glucopyranosyl-(1→3)]-β-D-glucopyranosyl)oxy]pregn-5,16-en-20-one (**4**), by detailed analysis of spectroscopic data and comparison with the early reports.[5,11]

On acid hydrolysis, GC-MS analysis of the pertrimethylsilylated sugars in the hydrolysate of **1** and **2** showed L-rhamnose and D-glucose to be present in a ratio of 2 : 1 in comparison with the authentic sugar. β-Configuration of glucose was inferred from the values of the coupling constants (**1**, J=7.5 Hz; **2**, J=7.2 Hz). The α-configuration of the rhamnose was assured by comparison of the chemical shift values of carbons Rha-3 and 5 with those of the corresponding carbons of methyl α- and β-rhamnopyronoside.[12]

Compound **1** was isolated as white amorphous powder and responded positively to the Libermann–Burchard reaction and Molish reagents. The molecular formula, $C_{40}H_{64}O_{16}$, was deduced from the high resolution (HR)-FAB-MS spectrum. The IR spectrum showed absorption bands at 3400 (OH), 2950 (CH), 1710 (C=O), 1040 (C–O) cm^{-1}. Its ^{1}H-NMR spectrum showed signals for five methyl groups at δ 0.60 (3H, s), 1.01 (3H, s), 1.62 (3H, d, J=6.0 Hz), 1.75 (3H, d, J=6.0 Hz) and 2.15 (3H, s), one methoxyl group at δ 3.21 (3H, s), one olefinic proton at δ 5.30 (1H, br s), and three anomeric protons at δ 6.40 (1H, br s), 5.85 (1H, br s), 4.95 (d, J=7.5 Hz) that suggested the presence of two rhamnoses and one glucose unit. The signals at δ 1.62 and 1.75 were due to the methyl groups of the two rhamnoses, respectively. The signals at δ 4.55 (1H, m) in the ^{1}H-NMR spectrum and the corresponding carbon signal at δ 81.8 indicated the presence of an oxygenated group in the aglycon. In the ^{13}C-NMR spectrum, 40 carbon signals were observed, 22 assigned to the aglycone moiety and 18 from one glucose and two rhamnoses. The signals for the aglycone moiety of **1** were in good agreement with those of 3β-hydroxy-16α-methoxyl-pregn-5-en-20-one.[13] The α-configuration of 16-OCH$_3$ was confirmed by the nuclear Overhauser effect spectroscopy (NOESY) correlation between 16-H and 18-CH$_3$.

The proton and carbon signals of sugar moiety of **1** were quite consistent with those of **3**, revealing the same sugar chain connected at C-3. The locations of the glycosidic linkage were also confirmed by ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectrum analysis. Complete assignments of the ^{1}H- and ^{13}C-NMR signals of **1** were made with the aid of ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) and ^{1}H-detected heteronuclear multiple-quantum coherence (HMQC) and HMBC spectra analysis. Consequently, the structure of compound **1** is proposed as 16α-methoxyl-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl)oxy]pregn-5-en-20-one.

Compound **2** was isolated as amorphous powder, positive to Libermann–Burchard reaction and Molish reagents. The molecular formula, $C_{40}H_{62}O_{16}$, was determined on the basis of the HR-FAB-MS spectrum. The ^{1}H-NMR spectrum of **2** showed signals for four methyl groups at δ 0.95 (3H, s), 1.04 (3H, s), 1.62 (3H, d, J=6.0 Hz), 1.75 (3H, d, J=6.0 Hz), one methoxy group at δ 3.41 (3H, s), a primary alcohol group at δ 4.37 (1H, d, J=18.0 Hz), 4.45 (1H, d, J=18.0 Hz), two olefinic protons at δ 5.30 (1H, br s) and 6.74 (1H, m), and three anomeric protons at δ 6.39 (1H, br s), 5.84 (1H, br s), 4.92 (d, J=7.2 Hz). Comparing the ^{13}C-NMR data of **2** and **3**,

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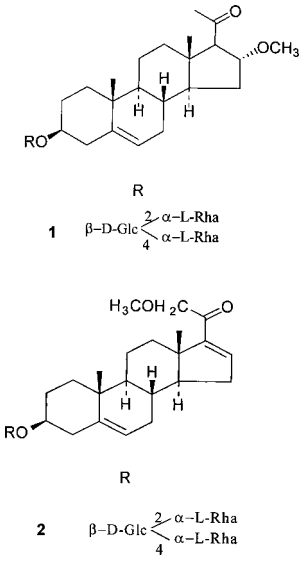

Fig. 1. Structures of Compounds **1** and **2**

Table 1. Stimulative Effect of the Isolated Compounds on UMR106 Cell Proliferation

Compound	Increase in cell proliferation (%)[a]		
	1 μM	10 μM	100 μM
Prosapogenin A of dioscin	7.1	—[b]	—
Prosapogenin B of dioscin	11.6	8.3	—
Dioscin	5.3	17.0	—
Gracillin	12.2	9.9	—
Pseudoprotodioscin	16.4	19.7	19.7
Pseudoprotogracillin	11.5	11.5	—
Protodioscin	17.5	14.9	3.6
Methyl protodioscin	46.4*	46.8*	50.6*
Protogracillin	48.9*	39.2*	25.7*
Methyl protogracillin	—	—	—
Compound **1**	16.7	4.4	—
Compound **2**	9.9	34.4*	17.6
Compound **3**	15.6	28.2*	30.9*
Compound **4**	37.1*	59.5*	50.6*
NaF		36.6*	

a) The increase in cell proliferation was determined with reference to a blank experiment. *b*) Inactive; $*p<0.05$.

a great similarity was observed except for H/C-21 and the methoxyl group. The 21-CH_3 of **3** disappeared and was replaced by a primary alcohol in **2**, which was confirmed by the HMBC correlations between the primary alcohol protons and C-20 and C-17. The methoxyl group of **2** was attached to C-21 due to its HMBC correlation with the carbon of the primary alcohol group. Unambiguous assignments of proton and carbon signals for **2** were achieved by ^{1}H–^{1}H COSY, HMQC and HMBC spectra analysis and comparison with those of known compound **3**. Based on the above analysis, compound **2** was identified as 21-methoxyl-3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl)oxy]pregn-5,16-en-20-one.

Compounds **1**—**4** exhibited weak activity against the plant pathogenic fungus *Pyricularia oryzae* with the minimal morphological deformation concentration (MMDC) values of 250, 265, 245, and 260 μM, respectively. Previously isolated spirostanol glycosides (prosapogenin A of dioscin, prosapogenin B of dioscin, dioscin, gracillin), furostanol glycoside (pseudoprotodioscin, pseudoprotogracillin, protodioscin, methyl protodioscin, protogracillin, methyl protogracillin), and pregnane glycosides **1**, **2**, **3** and **4**, were screened by an osteroblastic proliferation assay in the UMR106 cell line. The results suggested that these saponins might stimulate bone formation or have potential activity against osteoporosis. Initially structure–activity relationship analysis suggested that the aglycon part of saponins influences the activity more greatly than the sugar moiety. Pregnane glycosides showed the strongest stimulative effect on UMR106 cell proliferation, followed by furostanl glycosides. The spirostanol glycosides presented the weakest activity, possibly due to their strong cytotoxicity.

Experimental

Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20°. IR spectra (KBr) were taken on a JASCO A-102 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL JNM-GX400 (^{1}H 300 MHz, ^{13}C 75 MHz) spectrometer. FAB-MS and HR-MS were obtained using a JEOL JMS-DX302 spectrometer. GC was done on a HP-5890 SERIES II spectrometer, with a SE30 capillary column (12 m, 0.22 mm i.d.), hydrogen flame ionization detector (FID, 270°), the column temperature was 170—250 °C with the rate of 5 °C/min, and the carrier gas was N_2 (30 ml/min). Preparative HPLC was carried out on a liquid chromatograph LC-10 (Japan Analytical Industry Co., Ltd.) with RI detection, using an ODS column (Waters, 20×250 mm, 5 μm) with the flow rate of mobile phase 4.0 ml min^{-1}. Silica gel H (10—40 μm, Qingdao Haiyang Chemical Factory) was used for column chromatography. Analytical TLC and preparative TLC were performed on Merck precoated silica gel 60 F_{254}S plates and RP-18 F_{254}S plates, respectively, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. Minimum essential medium (MEM) was obtained from Gibco and fetal calf serum (FCS) from Tianjin Chuanyie Bio-engineering Co. (Tianjin, China).

Extraction and Isolation Air-dried rhizomes (3000 g) of *D. futschauensis* were refluxed with 75% EtOH. The EtOH was evaporated and the residue was partitioned between H_2O and *l*-BuOH to afford *l*-BuOH soluble fraction DB (100 g). DB (50 g) was subjected to column chromatography on silica gel H (500 g) and eluted stepwisely with $CHCl_3$/MeOH (100 : 1, 100 : 2, 97 : 3, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 70 : 30 and 60 : 40 v/v, each 5000 ml) to give 27 corresponding fractions. Saponin-containing fractions DB-15—DB-25 were subjected to prep.HPLC. Compounds **1** (8 mg), **2** (7 mg), **3** (200 mg), and **4** (300 mg) were isolated from fr. DB-25 (2.0 g) by prep. HPLC (column, ODS, 5 μM, 20×250 mm; solvent, 65% MeOH–H_2O; flow rate, 4.0 ml/min).

Compound **1**: Amorphous powder, $[\alpha]_D^{24}$ −47.8° (*c*=0.2, pyridine). HR-FAB-MS *m/z* [M+Na]$^+$ 823.5186 (Calcd for $C_{40}H_{64}O_{16}Na$: 823.5165). IR (KBr) cm^{-1}: 3420 (OH), 2950 (CH), 1710 (C=O), 1380, 1040 (glycosyl C–O). ^{1}H-NMR (pyridine-d_5): 3.86 (1H, m, H-3), 2.75 (2H, m, H-4), 5.30 (1H, br s, H-6), 4.51 (1H, m, H-16), 2.65 (1H, d, *J*=6.6 Hz, H-17), 0.60 (3H, s, Me-18), 1.01 (3H, s, Me-19), 2.15 (3H, s, Me-21), 3.21 (3H, s, MeO-16), 4.95 (1H, d, *J*=7.5 Hz, Glc-1), 6.40 (1H, br s, Rha(1→2)-1′), 1.75 (3H, d, *J*=6.0 Hz, Rha-6′), 5.85 (1H, br s, Rha(1→4)-1″), 1.62 (d, *J*=6.0 Hz, Rha-6″). ^{13}C-NMR (pyridine-d_5): 37.4 (C-1), 30.2 (C-2), 78.0 (C-3), 38.7 (C-4), 140.8 (C-5), 121.7 (C-6), 32.2 (C-7), 31.6 (C-8), 50.1 (C-9), 37.1 (C-10), 20.9 (C-11), 38.9 (C-12), 44.4 (C-13), 54.6 (C-14), 38.9 (C-15), 81.8 (C-16), 71.6 (C-17), 14.5 (C-18), 19.4 (C-19), 207.5 (C-20), 31.5 (C-21), 56.9 (21-OMe), 100.3 (Glc-1), 77.8 (Glc-2), 78.0 (Glc-3), 78.6 (Glc-4), 77.0 (Glc-5), 61.3 (Glc-6), 102.0 (Rha-1′), 72.6 (Rha-2′), 72.9 (Rha-3′), 74.1 (Rha-4′), 69.5 (Rha-5′), 18.7 (Rha-6′), 102.9 (Rha-1″), 72.6 (Rha-2″), 72.8 (Rha-3″), 73.9 (Rha-4″), 70.4 (Rha-5″), 18.5 (Rha-6″).

Compound **2**: Amorphous powder, $[\alpha]_D^{24}$ −40.5° (*c*=0.2, pyridine). HR-FAB-MS *m/z* [M+Na]$^+$ 821.4568 (Calcd for $C_{40}H_{62}O_{16}Na$, 821.4542). IR (KBr) cm^{-1}: 3385 (OH), 2930 (CH), 1700 (C=O), 1040 (C–O) cm^{-1}. ^{1}H-NMR (pyridine-d_5): 3.85 (1H, m, H-3), 5.32 (1H, d, *J*=5.0 Hz, H-6), 2.56 (2H, m, H-12), 6.74 (1H, m, H-16), 0.95 (3H, s, Me-18), 1.04 (3H, s, Me-19), 4.37 (1H, d, *J*=18.0 Hz, H-21), 4.45 (1H, d, *J*=18.0 Hz, H-21), (3H, s, Me-21), 3.41 (3H, s, MeO-21), 4.92 (1H, d, *J*=7.2 Hz, Glc-1), 6.39 (1H, br s, Rha(1→2)-1′), 1.75 (3H, d, *J*=6.0 Hz, Rha-6′), 5.84 (1H, br s, Rha(1→4)-1″), 1.62 (d, *J*=6.0 Hz, Rha-6″). ^{13}C-NMR (pyridine-d_5): 37.4 (C-1), 30.2 (C-2), 78.1 (C-3), 39.0 (C-4), 141.3 (C-5), 121.5 (C-6), 31.8 (C-7),

30.3 (C-8), 50.8 (C-9), 37.2 (C-10), 20.9 (C-11), 35.0 (C-12), 46.8 (C-13), 56.1 (C-14), 32.6 (C-15), 144.1 (C-16), 152.5 (C-17), 15.9 (C-18), 19.3 (C-19), 195.4 (C-20), 75.4 (C-21), 59.0 (21-OMe), 100.3 (Glc-1), 77.8 (Glc-2), 78.0 (Glc-3), 78.6 (Glc-4), 77.0 (Glc-5), 61.3 (Glc-6), 101.9 (Rha-1′), 72.6 (Rha-2′), 72.9 (Rha-3′), 74.1 (Rha-4′), 69.5 (Rha-5′), 18.6 (Rha-6′), 102.9 (Rha-1″), 72.6 (Rha-2″), 72.7 (Rha-3″), 73.9 (Rha-4″), 70.4 (Rha-5″), 18.5 (Rha-6″).

GC-MS Analysis Compounds **1** and **2** (about 2 mg each) were refluxed with 1 N H_2SO_4 in 50% Me_2CO for 5 h. The reaction mixtures were extracted with AcOEt, the hydrosylate was dried by blowing N_2 gas over it at room temperature. For GC analysis, the residue was trimethylsilyated with hexamethyldisilazane and trimethylchlorosilane in a 2 : 1 ratio at room temperature.[5] Two peaks corresponding to L-rhamnose (t_R: 3.78 min) and D-glucose (t_R: 7.15 min) were detected consistent with the authentic samples prepared in the same manner.

Anti-fungal Bioassay Anti-fungal assay against plant pathogenic fungus *P. oryzae* was carried out as previously reported.[14] The dioscin was used as a positive control with the MMDC value of 2.3 μM.

Osteoblastic Proliferation Stimulation Assay As described in the previous report,[15] UMR106 cells were plated in 96-well tissue culture plates and cultured for 24 h in minimum essential medium (MEM) supplemented with 7% fetal calf serum (FCS). The cells were then cultured with a serum-like medium containing compounds (dissolved in 1% dimethyl sulfoxide) at various concentrations for 36 h. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added and the cultures were incubated at 37 °C for another 4 h. Finally, the formed formazan crystals were dissolved in DMSO. The absorbance was recorded on a plate reader (Model 3550 enzyme immunoassay plate reader; BIO-RAD, U.S.A.) at two wavelengths of 595 nm (determination) and 655 nm (reference). The positive control, NaF, showed an increase of 36.6% in osteoblastic proliferation at the concentration of 10 μM.

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