Steroidal Oligoglycosides from the Seeds of *Allium tuberosum*

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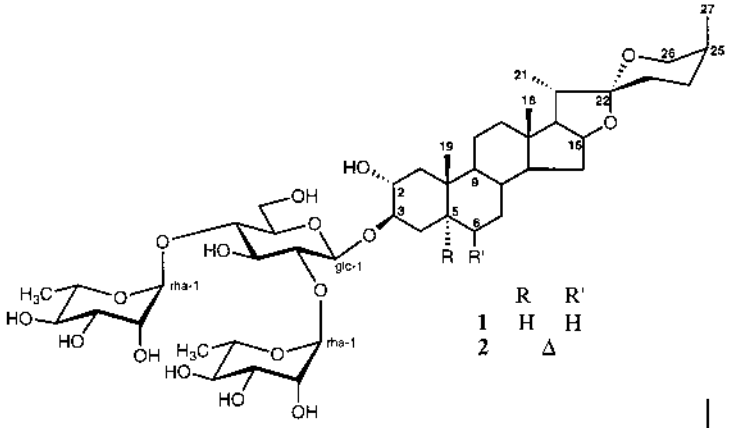
Three new spirostanol steroidal oligoglycosides, together with a known oligoglycoside, were obtained from the seeds of *Allium tuberosum* after enzymatic hydrolysis of furostanol saponin fraction by β-glucosidase. On the basis of spectroscopic analysis, the structure of new spirostanol oligoglycosides were elucidated as (25*S*)-spirost-5-ene-2α,3β-diol 3-*O*-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside, (25*S*)-spirostane-3β,5β,6α-triol 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside, and (25*S*)-5β-spirostane-3β,6α-diol 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside.

Key words *Allium tuberosum*; steroidal oligoglycoside; spirostanol; furostanol; Liliaceae; β-chacotriose

Our survey of the anti-herpes[1] and cytotoxic[2] active compounds among solanaceous plants has produced an interim result that the spirostanol glycoside is more effective than the coexisting furostanol, salasodane, or nuatigenin glycosides. Therefore, in order to obtain more detailed information about the relationship between structures and bioactivities, we studied the constituents of the seeds of *Allium tuberosum* (Liliaceae), which is a plant material known to include two spirostanol glycosides.[3] *A. tuberosum* is widely distributed in Asia and is cultivated as a foodstuff. The seeds of *A. tuberosum* are used as a tonic in China.[4] This paper deals with the isolation and structural elucidation of three new spirostanol steroidal saponins, together with a known saponin, on the basis of spectroscopic analysis.

The methanolic extract of the seeds (285.8 g) of *A. tuberosum* was partitioned between hexane and 80% MeOH. The 80% MeOH fraction was subjected to Diaion HP-20 and octadecylsilanized (ODS) silica gel column chromatographies to provide a furostanol saponin fraction. The furostanol saponin fraction was too complicated to separate, and the furostanol type steroidal saponins tended to be less active than the corresponding spirostanol type saponins for cytotoxicity.[2] Thus, we converted the furostanol saponin fraction to the corresponding spirostanol saponin by enzymatic hydrolysis with β-glucosidase. The obtained spirostanol saponin fraction was further purified on Chromatorex ODS and silica gel column chromatographies to give compounds **1** (31.2 mg), **2** (16.6 mg), **3** (5.0 mg), and **4** (2.7 mg).

Compound **1** was isolated as an amorphous powder, $[\alpha]_D$ −78.4° (MeOH). It showed a quasi-molecular ion peak of $[M-H]^-$ at *m/z* 885, and fragment ion peaks due to $[M-H-\text{deoxy-pentose}]^-$ at *m/z* 739 and $[M-H-2\times\text{deoxy-pentose}]^-$ at *m/z* 593 in the negative FAB-MS. The high-resolution (HR) FAB-MS gave a $[M+Na]^+$ ion at *m/z* 909.4836, which corresponds to the composition $C_{45}H_{74}O_{17}Na$. The ^{1}H-NMR spectrum in pyridine-d_5 displayed signals due to four steroidal methyl groups at δ 0.80, 0.89 (each 3H, s), 1.08 (3H, d, *J*=6.7 Hz), 1.14 (3H, d, *J*=6.7 Hz), two secondary



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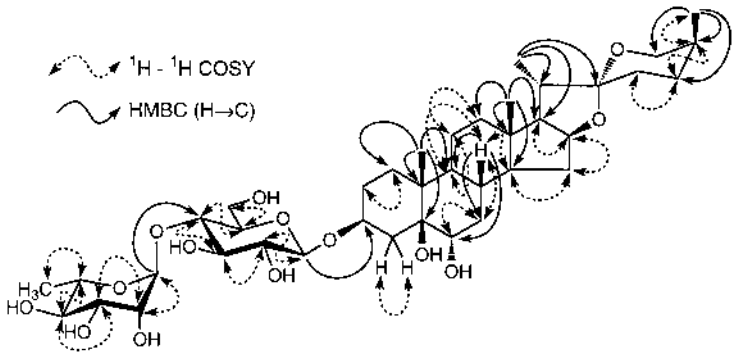

Fig. 1. ^{1}H–^{1}H COSY and HMBC Observed for **3**

methyl groups of 6-deoxypentose at δ 1.62 (3H, d, J=6.0 Hz) and 1.68 (3H, d, J=6.1 Hz), and three anomeric protons at δ 5.00 (1H, d, J=7.0 Hz), 5.81 (1H, br s) and 6.34 (1H, br s). In the ^{13}C-NMR spectrum, signals due to an aglycone part of **1** were superimposed on those of neogitogenin,[5] and signals due to the sugar moiety was assigned as α-L-rhamnopyranosy-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranose (β-chacotriose)[6] (Table 1). Thus, the structure of **1** was determined to be β-chacotriosyl neogitogenin. This compound was identified to be tuberoside D, which was recently obtained from the title plant as a genuine saponin.[3]

Compound **2** was isolated as an amorphous powder, $[\alpha]_D$ −94.2° (MeOH). It showed a quasi-molecular ion peak of $[M-H]^-$ at *m/z* 883 and fragment ion peaks due to [M−H−deoxy-pentose]$^-$ at *m/z* 737 and [M−H−2×deoxy-pentose]$^-$ at *m/z* 591 in the negative FAB-MS. The HR-FAB-MS gave a $[M+Na]^+$ ion at *m/z* 907.4679, which corresponds to the composition $C_{45}H_{72}O_{17}Na$. It suggested that the molecular ion of **2** was smaller by 2 mass units than that of **1**. When the ^{1}H- and ^{13}C-NMR spectra of **2** were compared with those of **1**, signals ascribable to the sugar moiety and the C, D, E, and F rings of the aglycone were identical. On the other hand, an olefinic proton signal appeared at δ 5.34 (1H, br s) and the signal of H_3-19 was shifted by +0.19 ppm. In the ^{13}C-NMR spectrum of **2**, trisubstituted double bond carbon signals appeared at δ 122.0 (d) and 140.0 (s) (Table 1). When the ^{13}C-NMR data of the aglycone part of **2** was compared with that of yuccagenin [(25*R*)-spirost-5-ene-2α,3β-diol],[7] it was identical except for the signals due to the F ring. Thus, the aglycone part of **2** was identified as (25*S*)-spirost-5-ene-2α,3β-diol, lilagenin.[8] The structure of **2** was therefore determined to be β-chacotriosyl lilagenin. This is the first time the ^{1}H- and ^{13}C-NMR data of lilagenin has been presented.

Compound **3** was isolated as an amorphous powder, $[\alpha]_D$ −61.1° (MeOH). It showed a quasi-molecular ion peak due to $[M-H]^-$ at *m/z* 755 and fragment ion peaks due to [M−H−deoxy-pentose]$^-$ at *m/z* 609 and [M−H−deoxy-pentose-hexose]$^-$ at *m/z* 447 in the negative FAB-MS. The HR-FAB-MS gave a $[M+Na]^+$ ion at *m/z* 779.4178, which corresponds to the composition $C_{39}H_{64}O_{14}Na$. These data suggest that **3** is a steroidal glycoside with a terminal methylpentosyl moiety and an inner hexosyl moiety. The ^{1}H-NMR spectrum displayed signals due to four steroidal methyl groups at δ 0.83, 1.15 (each 3H, s), 1.08 (3H, d, J=7.3 Hz), 1.16 (3H, d, J=6.7 Hz), one secondary methyl group of 6-deoxypentose at δ 1.71 (3H, d, J=6.1 Hz), and two anomeric protons at δ 5.00 (1H, d, J=7.9 Hz) and 5.88 (1H, br s). When the ^{13}C-NMR spectrum of **3** was compared with that of **1**, the carbon signals appearing from the D, E, F rings on the aglycone were identical (Table 1). This suggested that **3** is a 25*S*-spirostane skeleton. On the other hand, two hydroxyl methine carbon signals appeared at δ 66.1 and 79.2, and one hydroxy-bearing quaternary carbon signal was observed at δ 73.0. When the ^{13}C-NMR data of the sugar part of **3** was compared with that of **1**, the presence of a de-rhamnosyl chacotriosyl moiety was deduced for the sugar moiety of **3**. To determine the location of the hydroxyl group on the aglycone part and the glycosidic linkage, the ^{1}H–^{1}H shift correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments were made in order to assign the respective proton and carbon signals, as shown in Fig. 1. In the sugar moiety, the rhamnosyl H-1 at δ 5.88 correlated with the glucosyl C-4 at δ 78.5 in the HMBC, so the sugar moiety of **3** was disclosed to be an α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside. Furthermore, the glucosyl H-1 at δ 5.00 correlated with the aglycone carbon signal at δ 79.2, in which the carbon was correlated to the proton signal at δ 4.63 (1H, br s, W_H=7.6 Hz) in the HMQC. Moreover, the H_3-19 (δ 1.15) and carbon signals [δ 73.0 (s), δ 42.5 (d), δ 35.9 (t)] were correlated in the HMBC, so that the hydroxy bearing quaternary carbon (δ 73.0) was deduced to be attached at C-5 or C-9. On the other hand, the hydroxyl methine carbon signal appeared at δ 66.1 corresponded to the proton signal at δ 4.04 (1H, br d, J=12.2 Hz) in the HMQC. Starting from the proton signal at δ 4.04, the ^{1}H–^{1}H COSY and HMQC measurements revealed a sequence of connectivities through respective signals at δ 4.04 [δ 66.1 (d)]→δ 2.25, 1.82 [δ 35.6 (t)]→δ 2.12 [δ 34.7 (d)]→δ 1.90 [δ 42.5 (d)]. Thus, the location of the hydroxyl groups were assigned to C-5 (δ 73.0) and C-6 (δ 66.1). A nuclear Overhauser exchange spectroscopy (NOESY) between H-7β (δ 2.25) and both H-6 (δ 4.04) and H-8 (δ 2.12) indicated a 6α hydroxy group (Fig. 2). And the correlation between H-9 (δ 1.90) and H-2α (δ 1.45), and 4α (δ 1.58) suggested an A/B *cis* ring configuration. When the ^{1}H-NMR spectrum of **3** measured in pyridine-d_5 was compared with that of methanol-d_4, the H_3-19 signal was shifted by +0.19 ppm owing to the anisotropic effect of pyridine. This observation also suggested a 5β hydroxyl group. A small W_H value of the H-3 proton at δ 4.63 (1H, br s, W_H=7.6 Hz) suggested a 3β axial hydroxyl group.

Thus, the structure of **3** was determined to be a (25*S*)-

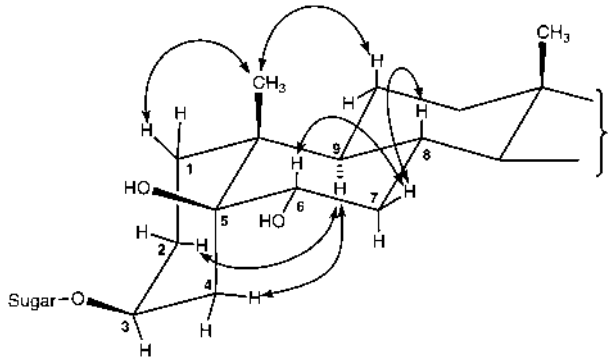

Fig. 2. NOEs Observed for **3** (NOESY)

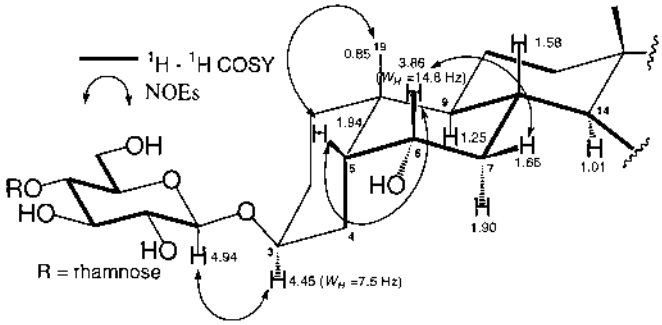

Fig. 3. ^{1}H–^{1}H COSY and NOEs Correlations Observed for **4**

Table 1. ^{13}C-NMR Data for **1**—**4** in Pyridine-d_5

	1	**2**	**3**	**4**		**1**	**2**	**3**	**4**
C- 1	45.8	45.9	35.9	30.0	glc-1	100.9	101.1	101.9	104.0
- 2	70.4	70.2	29.1	26.7	-2	78.8	78.6	74.9	75.0
- 3	85.0	85.2	79.2	80.1	-3	77.1	77.1	76.8	76.6
- 4	33.4	37.2	35.1	31.7	-4	78.0	77.9	78.5	78.3
- 5	44.6	140.0	73.0	36.4	-5	77.8	77.6	77.5	77.4
- 6	28.1	122.0	66.1	66.9	-6	61.2	61.7	61.2	61.4
- 7	32.1	32.2	35.6	40.5					
- 8	34.6	31.2	34.7	35.6	rha-1	102.9	101.9	102.8	103.0
- 9	54.4	50.2	42.5	41.4	-2	72.5	72.8	72.6	72.6
-10	36.9	37.9	43.1	36.9	-3	72.7	72.5	72.8	72.8
-11	21.4	21.2	21.8	21.4	-4	73.9	74.1	74.0	74.0
-12	40.1	39.8	40.1	40.2	-5	70.6	70.4	70.4	70.5
-13	40.8	40.4	40.6	40.8	-6	18.5	18.5	18.5	18.6
-14	56.4	56.5	56.4	56.4					
-15	32.3	32.3	32.2	32.1	rha′-1	102.1	102.9		
-16	81.2	81.2	81.2	81.3	-2	72.4	72.7		
-17	62.8	62.7	62.9	63.0	-3	72.7	72.4		
-18	16.6	20.4	16.5	16.6	-4	74.1	73.9		
-19	13.5	14.2	17.6	23.8	-5	69.5	69.5		
-20	42.5	42.4	44.7	42.5	-6	18.5	18.5		
-21	14.9	14.9	14.9	14.9					
-22	109.7	109.7	109.8	109.7					
-23	26.2	26.2	26.4	26.2					
-24	26.4	26.4	26.2	26.4					
-25	27.5	27.5	27.6	27.6					
-26	65.1	65.1	65.1	65.1					
-27	16.3	16.3	16.3	16.3					

spirostane-3β,5β,6α-triol 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside.

Compound **4** was isolated as an amorphous powder, $[\alpha]_D$ −60.3° (MeOH). It showed a quasi-molecular ion peak due to $[M-H]^-$ at *m/z* 739 and fragment ion peaks due to $[M-H-$deoxy-pentose$]^-$ at *m/z* 593 and $[M-H-$deoxy-pentose-hexose$]^-$ at *m/z* 431 in the negative FAB-MS. The HR-FAB-MS gave a $[M+Na]^+$ ion at *m/z* 763.4249, which corresponds to the composition $C_{39}H_{64}O_{13}Na$. When the ^{1}H- and ^{13}C-NMR spectra of **4** were compared with that of **3**, signals due to the sugar moiety and signals due to the D, E and F rings on the aglycone were identical. On the other hand, signals due to two hydroxyl methine protons were observed at δ 3.86 and 4.45, and the H_3-19 proton signal was shifted −0.30 ppm in **4**. In the ^{13}C-NMR spectrum of the aglycone moiety, three oxygen-bearing methine carbon signals appeared at δ 66.9, 80.1 and 81.3, the latter two of which were assigned as C-3 and C-16, respectively, by comparing their chemical shifts with those of **3** (Table 1). The ^{1}H–^{1}H COSY and HMQC data (Fig. 3) disclosed a sequence of connectivities through the respective signals at δ 1.25 [δ 41.4 (d)]→δ 1.58 [δ 35.6 (d)]→δ 1.66, 1.90 [δ 40.5 (t)]→δ 3.86 [δ 66.9 (d)]→δ 1.94 [δ 36.4 (d)]→δ 1.85, 1.96 [δ 31.7 (t)]. Thus, the location of the oxygen-bearing methine carbon at δ 66.9 was assigned as C-6. The NOESY between H-5 (δ 1.94) and H_3-19 (δ 0.85) indicated the presence of a 5β hydrogen, per the A/B *cis* ring configuration (Fig. 3). The correlation between H-6 (δ 3.86) and H-5, 7β (δ 1.66) indicated the presence of a 6α hydroxy group. A large W_H value (14.8 Hz) of the H-6 proton also suggested a 6α equatorial hydroxyl group. A small W_H value of the H-3 proton at δ 4.45 (1H, br s, W_H=7.5 Hz) suggested a 3β axial hydroxyl group. Furthermore, the W_H values of H-3 and H-6 were identical to (25*R*)-5β-spirostane-3β,6α-diol, ruizgenin.[7] Thus, the structure of **4** was determined to be a (25*S*)-5β-spirostane-3β,6α-diol (25*epi*-ruizgenin) 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside.

Although compounds **1**, **2**, **3**, and **4** consisted as furostanol glycosides in the authentic sample, compounds **2**, **3**, and **4** were regarded as new spirostanol oligoglycosides. Compounds **1** and **2** were chacotriosyl neogitogenin and lilagenin, respectively. In our research of cytotoxic steroidal oligoglycosides, the chacotriosyl spirostanol oligoglycosides were effective.[2,9] It seems worth subjecting **1** and **2** to tests for cytotoxicity against human cancer cell lines. Compounds **3** and **4** had an A/B *cis* ring configuration. There were fewer examples of this than the A/B *trans* ring configuration. The obtained oligoglycosides have a variety of A/B ring functions. Thus, these compounds (**1**—**4**) are useful for studying the structure–bioactivity relationship of A/B ring functions.

Work is in progress to use these oligoglycosides to study cytotoxicity against human cancer cell lines and anti-herpes simplex virus type 1 (HSV-1) activity.

Experimental

Optical rotations were measured on a JASCO DIP-360 automatic digital polarimeter. The NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a JEOL α-500 spectrometer and chemical shifts were given on a δ (ppm) scale with tetramethylsilane as the internal standard. Standard pulse sequences were employed for the distortionless enhancement by polarization transfer (DEPT), HMQC, and HMBC experiments. NOESY spectra were measured with mixing times of 600 ms. The FAB-MS were measured with a JEOL DX-300 and/or SX102A spectrometer. The HR-FAB-MS were measured with a JEOL DX-303 HF spectrometer and taken in a glycerol, triethylene glycol and *m*-nitrobenzyl alcohol matrix. GLC was performed on a HP5890A gas chromatograph with a flame ionization detector. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck). Column chromatography was carried out on Kieselgel 60 (70—230 mesh, 230—400

mesh), Diaion HP-20 (Mitsubishi Chemical, Ind.), Sephadex LH-20 (Pharmacia), and Chromatorex ODS-DU 3050MT (Fuji Silysia). β-Glucosidase from almonds was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Extraction and Isolation The seeds (285.8 g) of *Allium tuberosium* were ground and extracted with MeOH twice under reflux. The combined extract was concentrated (19.4 g) and partitioned between hexane and 80% MeOH. The 80% MeOH fraction (11.4 g) was subjected to Diaion HP-20 column chromatography using $H_2O \rightarrow$MeOH to give fractions 1 to 5. Fraction 4 (80% MeOH eluate; 656 mg) was further separated by Chromatorex ODS (50% MeOH→MeOH) to provide Ehrlich reagent (*p*-dimethylaminobenzaldehyde and hydrochloric acid)[10] positive furostanol saponin fraction (356 mg). To a solution of a part of the furostanol saponin fraction (210 mg) in acetate buffer (100 mM, pH 5.0, 4.5 ml) was added β-glucosidase (92 mg, 100 units), and the mixture was incubated at 37 °C for 18 h. The reaction mixture was heated at 80 °C for 1 min, and the precipitate was filtrated off and washed with MeOH. The filtrate and washing was combined and chromatographed on Diaion HP-20, then successively eluted with H_2O and MeOH to give a spirostanol saponin fraction (146 mg). The spirostanol saponin fraction was further purified on Chromatorex ODS (70% MeOH→MeOH) and silica gel column chromatography ($CHCl_3$: MeOH : H_2O = 8 : 2 : 0.2→7 : 3 : 0.5) to give **1** (31.2 mg), **2** (16.6 mg), **3** (5.0 mg) and **4** (2.7 mg), respectively.

Compound **1**: An amorphous powder, $[\alpha]_D^{29}$ −78.4° (c=0.26, MeOH). Negative FAB-MS (*m/z*) 885 $[M-H]^-$, 739 [M−H−deoxy-pentose]$^-$, 593 [M−H−2×deoxy-pentose]$^-$. HR-FAB-MS (*m/z*) 909.4836 $[M+Na]^+$ (Calcd for $C_{45}H_{74}O_{17}Na$; 909.4824). ^{1}H-NMR (pyridine-d_5) δ: 0.80 (3H, s, H_3-18), 0.89 (3H, s, H_3-19), 1.08 (3H, d, J=6.7 Hz, H_3-21), 1.14 (3H, d, J=6.7 Hz, H_3-27), 1.62 (3H, d, J=6.0 Hz, rha-H_3-6), 1.68 (3H, d, J=6.1 Hz, rha-H_3-6), 5.00 (1H, d, J=7.0 Hz, glc-H-1), 5.81 (1H, br s, rha-H-1), 6.34 (1H, br s, rha-H-1). ^{13}C-NMR (pyridine-d_5) δ: Table 1.

Analysis of Sugars Respective compounds **1**—**4** (1 mg) were hydrolyzed with 2 mol/l HCl in H_2O for 4 h at 80 °C. The reaction mixture was neutralized with 2 mol/l NaOH in H_2O and extracted with $CHCl_3$. The aqueous layer was concentrated to dryness *in vacuo* to give a residue which was dissolved in dry pyridine, to which was added L-cysteine methyl ester hydrochloride.[11] The reaction mixture was heated for 2 h at 60 °C and concentrated to dryness by blowing N_2 gas. To the residue was added trimethylsilylimidazole, followed by heating for 1 h at 60 °C. The residue was extracted with hexane and H_2O, and the organic layer was analyzed by GLC; column: OV-17 (0.32 mm×30 m), detector: FID, column temperature: 230 °C, detector temperature: 270 °C, injector temperature: 270 °C, carrier gas: He (2.6 kg/cm^2). Two peaks were observed at t_R (min): 11′78″ (D-Glc) and 8′12″ (L-Rha). The standard monosaccharides were subjected to the same reaction and GLC analysis under the same condition.

Compound **2**: An amorphous powder, $[\alpha]_D^{29}$ −94.2° (c=0.12, MeOH). Negative FAB-MS (*m/z*) 883 $[M-H]^-$, 737 [M−H−deoxy-pentose]$^-$, 591 [M−H−2×deoxy-pentose]$^-$. HR-FAB-MS (*m/z*) 907.4679 $[M+Na]^+$ (Calcd for $C_{45}H_{72}O_{17}Na$; 907.4667). ^{1}H-NMR (pyridine-d_5) δ: 0.81 (3H, s, H_3-18), 1.08 (3H, s, H_3-19), 1.08 (3H, d, J=6.7 Hz, H_3-21), 1.14 (3H, d, J=6.7 Hz, H_3-27), 1.63 (3H, d, J=6.1 Hz, rha-H_3-6), 1.69 (3H, d, J=6.1 Hz, rha-H_3-6), 4.98 (1H, d, J=7.0 Hz, glc-H-1), 5.34 (1H, br s, H-6), 5.82 (1H, s, rha-H-1), 6.39 (1H, s, rha-H-1). ^{13}C-NMR (pyridine-d_5) δ: Table 1.

Compound **3**: An amorphous powder, $[\alpha]_D^{29}$ −61.1° (c=0.14, MeOH). Negative FAB-MS (*m/z*) 755 $[M-H]^-$, 609 [M−H−deoxy-pentose]$^-$, 447 [M−H−deoxy-pentose-hexose]$^-$. HR-FAB-MS (*m/z*) 779.4178 $[M+Na]^+$ (Calcd for $C_{39}H_{64}O_{14}Na$; 779.4194). ^{1}H-NMR (pyridine-d_5) δ: 0.83 (3H, s, H_3-18), 1.08 (3H, d, J=7.3 Hz, H_3-27), 1.15 (3H, s, H_3-19), 1.16 (3H, d, J=6.7 Hz, H_3-21), 1.71 (3H, d, J=6.1 Hz, rha-H_3-6), 1.90 (1H, m, H-9), 3.38 (1H, br d, J=11.0 Hz, H-26), 4.04 (1H, br d, J=12.2 Hz, H-6), 4.07 (1H, m, H-26), 4.63 (1H, br s, W_H=7.6 Hz, H-3), 5.00 (1H, d, J=8.0 Hz, glc-H-1), 5.88 (1H, br s, rha-H-1). ^{13}C-NMR (pyridine-d_5) δ: Table 1.

Compound **4**: An amorphous powder, $[\alpha]_D^{30}$ −60.3° (c=0.27, MeOH). Negative FAB-MS (*m/z*) 739 $[M-H]^-$, 593 [M−H−deoxy-pentose]$^-$, 431 [M−H−deoxy-pentose-hexose]$^-$. HR-FAB-MS (*m/z*) 763.4249 $[M+Na]^+$ (Calcd for $C_{39}H_{64}O_{13}Na$; 763.4244). ^{1}H-NMR (pyridine-d_5) δ: 0.81 (3H, s, H_3-18), 0.85 (3H, s, H_3-19), 1.08 (3H, d, J=6.7 Hz, H_3-27), 1.15 (3H, d, J=7.3 Hz, H_3-21), 1.73 (3H, d, J=6.7 Hz, rha-H_3-6), 1.94 (1H, br d, J=6.7 Hz, H-5), 3.86 (1H, m, W_H=14.8 Hz, H-6), 4.45 (1H, m, W_H=7.5 Hz, H-3), 4.94 (1H, d, J=7.3 Hz, glc-H-1), 5.89 (1H, br s, rha-H-1). ^{13}C-NMR (pyridine-d_5) δ: Table 1.

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