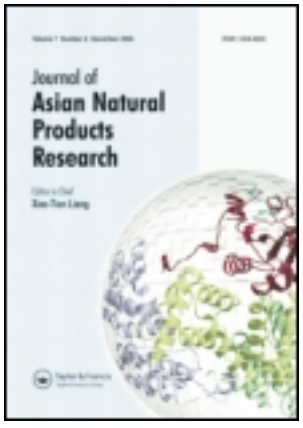

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Steroidal saponins from *Anemarrhena asphodeloides*

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Two new steroidal saponins, named anemarnoside A (**1**) and anemarnoside B (**2**), along with three known compounds, timosaponin J (**3**), timosaponin B II (**4**), and timosaponin B (**5**), have been isolated from *Anemarrhena asphodeloides*. Their structures were established by spectroscopic techniques (IR, MS, 1D NMR, and 2D NMR) and by comparison with published data.

Keywords: *Anemarrhena asphodeloides*; steroidal saponins; NMR

1. Introduction

Anemarrhena asphodeloides Bunge belongs to the family Liliaceae widely distributed in China. It has been reported that steroidal saponins were the main components of *Rhizoma anemarrhenae* [1]. Studies showed that they had a broad range of pharmacological effects such as improving senile dementia [2,3], preventing cerebral ischemia [4], anti-coagulated blood [5], anti-oxidant [6,7], anti-tumor [8,9], anti-osteoporosis [10], anti-inflammation [11], lowering blood pressure [12], and lowering blood sugar [13]. Therefore, a phytochemical investigation on the saponin constituents of *A. asphodeloides* was carried out. Two new steroidal saponins, anemarnoside A (**1**) and anemarnoside B (**2**), along with three known compounds, timosaponin J (**3**), timosaponin B II (**4**), and timosaponin B (**5**) (Figure 1), were obtained. In this paper, we report the isolation and structural elucidation of these compounds.

2. Results and discussion

Compound **1** was isolated as a white amorphous solid and showed a positive reaction in Lieberman–Burchard and Ehrlich reagent tests. The IR spectrum showed absorption bands for hydroxyl (3386 cm^{-1}), carbonyl of ester (1727 cm^{-1}), and a glycosidic linkage (1000–1100 cm^{-1}). Its spectral features and physicochemical properties suggested **1** to be a furostanol saponin. Its molecular formula $C_{45}H_{76}O_{19}$ was deduced from the HR-ESI-TOF-MS at *m/z* 943.4873 $[M + Na]^+$. The ^1H NMR spectrum of **1** showed two angular methyl signals at δ 0.98 and 0.74 (each 3H, s); one secondary methyl signal at δ 0.97 (3H, d, $J = 6.6$ Hz); one terminal methyl group of an alkane at δ 0.90 (3H, t, $J = 7.2$ Hz); three anomeric signals appearing at δ 5.28 (1H, d, $J = 7.2$ Hz), 4.91 (1H, d, $J = 7.2$ Hz), and 4.81 (1H, d, $J = 7.8$ Hz). The ^{13}C NMR spectrum of **1** revealed characteristic signals for four methyl groups at δ 24.1, 17.1, 13.7, and 13.4; one ester carbonyl at δ 173.4; three anomeric carbons of sugars at δ 106.2,

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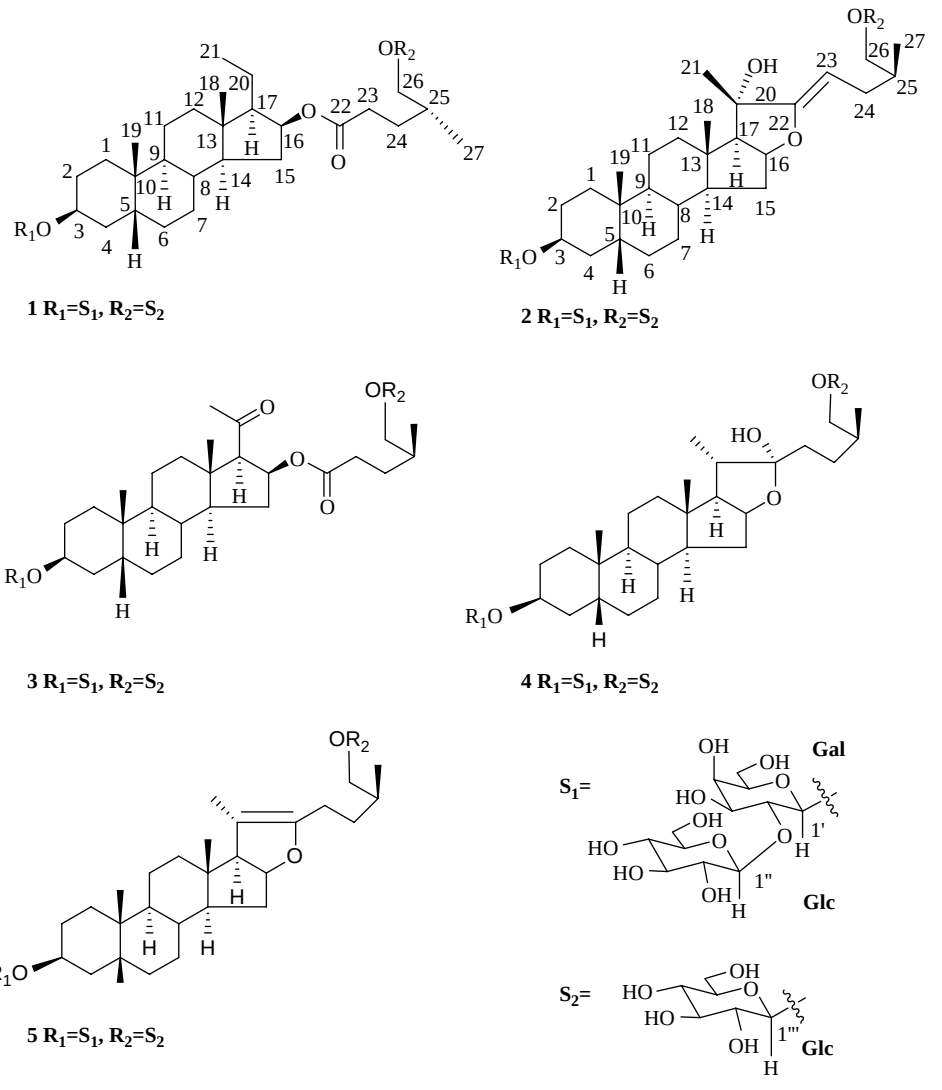

Figure 1. The structures of compounds **1**–**5**.

105.2, and 102.6. The above evidences indicated that **1** was a furostanol saponin with three sugar units. The NMR data of **1** (Table 1) were very similar to those of timosaponin J [14], indicating the same partial structure with rings A, B, C, and D. A feature differing from that of timosaponin J was the substitution of the carbonyl group C-20 in timosaponin J by a new methylene carbon signal C-20 (δ 17.3) in **1**. That was confirmed by HMBC spectrum which showed the correlations between the methyl signal H-21 (δ 0.90) and the carbon signals C-20 (δ 17.3), C-17 (δ 57.5). The 25*R* configuration of **1** was deduced on the basis of differences in chemical shifts for the geminal protons of H_2-26 appearing at δ 3.97 (H_a-26) and 3.49 (H_b-26) ($\triangle ab = 0.48$), since the difference is usually ≥ 0.57 for 25*S* compounds and ≤ 0.48 for 25*R* compounds [15,16]. The nuclear overhauser effect spectroscopy (NOESY) spectrum of **1** showed nuclear overhauser effect (NOE) correlations

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (125 MHz) spectral data of the aglycone moieties of compounds **1** and **2** in pyridine-d_5.

	1		**2**	
No.	δ_C	δ_H	δ_C	δ_H
1	31.0	1.85 O, 1.48 m	31.0	1.85 O, 1.46 m
2	27.1	1.86 m, 1.23 m	27.1	1.84 m, 1.20 m
3	75.4	4.28 m	75.4	4.32 br s
4	31.0	1.85 O	31.1	1.85 O
5	37.2	2.16 m	37.1	2.15 m
6	26.9	1.98 m, 1.49 m	26.9	1.97 m, 1.48 m
7	26.7	1.23 m, 0.97 O	26.7	1.19 m, 0.91 m
8	35.5	1.36 m	35.0	1.43 m
9	40.8	1.29 m	40.2	1.21 m
10	35.4		35.3	
11	21.0	1.29 m, 1.18 m	20.8	1.23 m, 1.20 m
12	38.6	1.66 m, 0.93 m	39.9	1.89 m, 1.18 m
13	42.6		40.9	
14	54.1	0.78 m	57.0	0.92 m
15	35.5	2.31 m, 1.35 m	35.0	2.10 m, 1.43 m
16	74.7	5.42 m	84.5	5.27 m
17	57.5	1.26 m	68.2	2.26 d (5.4)
18	13.7	0.74 s	13.9	0.87 s
19	24.1	0.98 s	24.2	0.97 s
20	17.3	1.50 m, 1.33 m	76.9	
21	13.4	0.90 t (7.2)	22.0	1.72 s
22	173.4		163.9	
23	32.6	2.48 m, 2.41 m	91.4	4.55 O
24	29.4	2.01 m, 1.64 m	29.8	2.51 m, 2.15 m
25	33.8	1.92 m	35.3	1.89 O
26	74.9	3.97 O, 3.49 t (7.8)	75.7	4.13 O, 3.53 dd (9.6, 7.2)
27	17.1	0.97 d (6.6)	17.6	1.07 d (6.0)

Note: Overlapped signals are indicated by 'O.'

(Figure 3) between α-oriented proton H-14 (δ 0.78), H-16 (δ 5.42), and H-17 (δ 1.26), which indicated the α-configurations of H-16 and H-17. Thus, the aglycone of **1** was identified as (25*R*)-20,22-seco-5β-furost-22-one-3β,26-diol. Acid hydrolysis of **1** afforded D-glucose and D-galactose in a ratio of 2:1 by thin layer chromatography (TLC) and gas chromatography (GC) analyses. The β-anomeric configurations for the two glucoses were determined from their large $^{3}J_{1,2}$ coupling constants (J = 7.8, 7.2 Hz), meanwhile the β-anomeric configuration for the galactose was determined from their large $^{3}J_{1,2}$ coupling constant (J = 7.2 Hz) [17]. The sugar sequences and its linkage to C-3 and C-26 of the aglycone were ascertained by HMBC correlations (Figure 2) between the anomeric proton signal H-1′ (δ 4.91) and the carbon signal C-3 (δ 75.4), between H-1″ (δ 5.28) and C-2′ (δ 81.9), and between H-1‴ (δ 4.81) and C-26 (δ 74.9). On the basis of the foregoing evidence, the structure of **1** was determined as (25*R*)-26-*O*-β-D-glucopyranosyl-20,22-seco-5β-furost-22-one-3β,26-diol-3-*O*-β-D-galactopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside, named anemarnoside A.

Compound **2** was isolated as a white amorphous solid and showed a positive reaction in Lieberman–Burchard and Ehrlich reagent tests. The IR spectrum showed absorption bands for hydroxyl (3392 cm^{-1}) and a glycosidic linkage

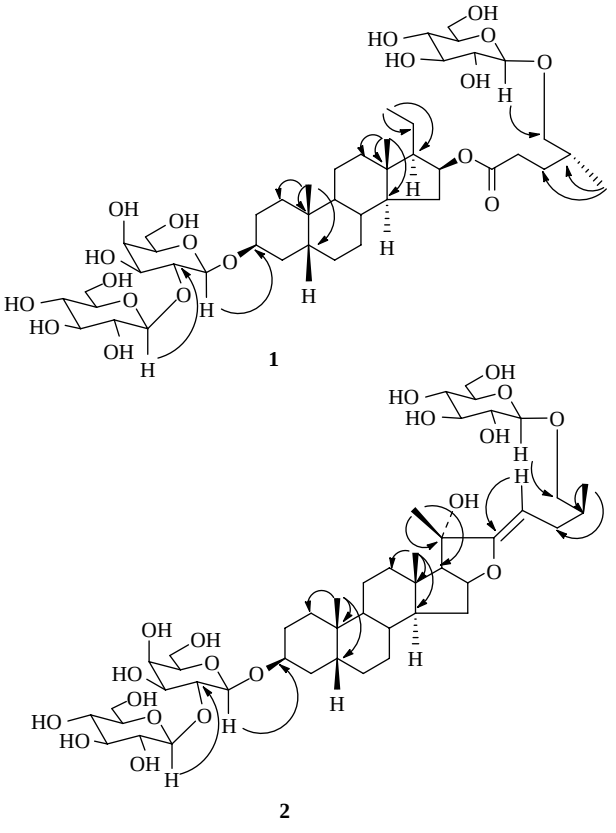

Figure 2. Key HMBC correlations of compounds **1** and **2**.

(1000–1100 cm^{-1}). Its spectral features and physicochemical properties suggested **2** to be a furostanol saponin. Its molecular formula $C_{45}H_{74}O_{19}$ was deduced from the HR-ESI-TOF-MS at *m/z* 941.4717 $[M + Na]^+$. The ^1H NMR spectrum of **2** showed three angular methyl signals at δ 1.72, 0.97, and 0.87 (each 3H, s); one secondary methyl signal at δ 1.07 (3H, d, $J = 6.0$ Hz); one olefinic proton at δ 4.55 (1H, O); three anomeric signals appearing at δ 5.27 (1H, d, $J = 7.2$ Hz), 4.90 (1H, d, $J = 7.2$ Hz), and 4.82 (1H, d, $J = 7.2$ Hz). The ^{13}C NMR spectrum of **2** revealed the characteristic signals for four methyl groups at δ 24.2, 22.0, 17.6, and 13.9; one pair of olefinic carbons at δ 163.9 and 91.4; three anomeric carbons of sugars at δ 106.3, 105.3, and 102.7. The above evidences indicated that **2** was a furostanol saponin with three sugar units. Comparison of the NMR spectral data of **2** (Table 1) with that of timosaponin B II [18] revealed that they have the same partial structure with rings A, B, C, and D. However, significant differences were recognized in the signals from the ring E part and the side chain moiety, where a tertiary oxygenated carbon (δ 76.9) and an oxygen-bearing trisubstituted olefinic groups (δ 163.9 and 91.4) were supposed to be located. These were confirmed by the HMBC spectrum which showed the correlations between the methyl proton H-21 (δ 1.72) and the carbon signal C-20 (δ 76.9), and between the olefinic proton H-23 (δ 4.55) and the carbon

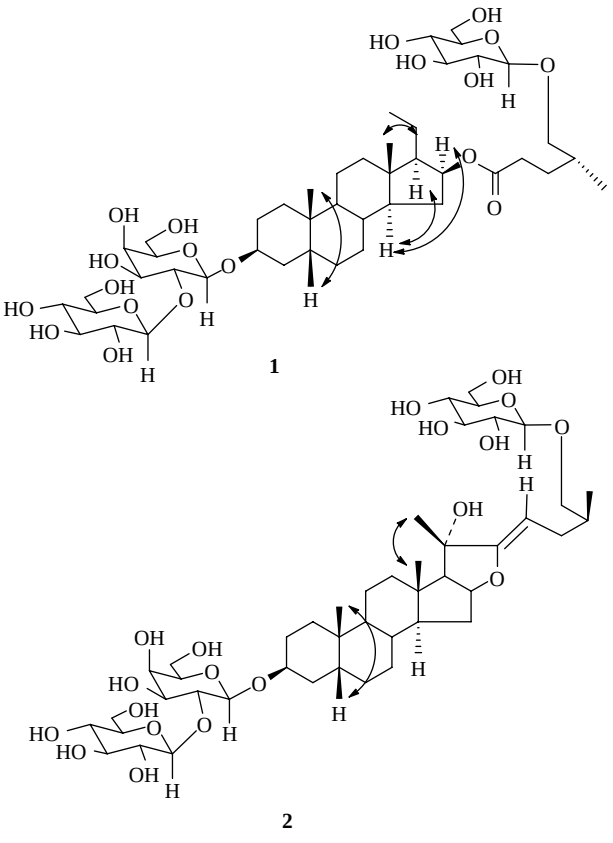

Figure 3. Key NOE correlations of compounds **1** and **2**.

signal C-22 (δ 163.9). The 25*S* configuration of **2** was deduced on the basis of differences in chemical shifts for the geminal protons of H_2-26 appearing at δ 4.13 (H_a-26) and 3.53 (H_b-26) ($\triangle ab = 0.600 > 0.57$ for 25*S* compound) [14,19]. The orientation of the C-20 hydroxyl group was determined to be α-configuration by the observed NOE correlation (Figure 3) between β-oriented methyl proton H-18 (δ 0.87) and methyl proton H-21 (δ 1.72). Hence, the aglycone of **2** was identified as (25*S*)-5β-furost-22(23)-en-3β,20α, 26-triol. Acid hydrolysis of **2** afforded D-glucose and D-galactose in a ratio of 2:1 by TLC and GC analysis. The β-anomeric configurations for the two glucoses were determined from their large $^3J_{1,2}$ coupling constants ($J = 7.2$, 7.2 Hz), meanwhile the β-anomeric configuration for the galactose was determined from their large $^3J_{1,2}$ coupling constant ($J = 7.2$ Hz) [17]. The sugar sequences and its linkage to C-3 and C-26 of the aglycone were ascertained by HMBC correlations (Figure 2) between the anomeric proton signal H-1′ (δ 4.90) and the carbon signal C-3 (δ 75.4), between H-1″ (δ 5.27) and C-2′(δ 82.0), and between H-1‴ (δ 4.82) and C-26 (δ 75.7). On the basis of the foregoing evidence, the structure of **2** was determined as (25*S*)-26-*O*-β-D-glucopyranosyl-5β-furost-22(23)-en-3β,20α,26-triol-3-*O*-β-D-galactopyranosyl-(1 → 2)-*O*-β-D-glucopyranoside, named anemarnoside B.

The known compounds were readily identified as timosaponin J (**3**) [14], timosaponin B II (**4**) [19], and timosaponin B (**5**) [19] by comparing NMR spectral data with those reported in the literature.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter (Jasco Co., Tokyo, Japan). IR spectra were obtained on a Shimadzu ftir-8400s spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR spectra were recorded on Bruker ARX-300 and ARX-600 instruments (Bruker Co., Billerica, MA, USA). HR-ESI-TOF-MS experiments were performed on a *MicroTOF* spectrometer (Bruker Co., Karlsruhe, Germany). High performance liquid chromatography (HPLC) preparation was performed on a Hitachi preparative HPLC system (Hitachi Ltd, Tokyo, Japan) equipped with Refractive Index Detector (L-2490) and prep-ODS (5 μm × 10 mm × 250 mm). GC was done on an Agilent 7890A Gas Chromatograph (Agilent technologies, Inc., Santa Clara, CA, USA) equipped with HP-5 capillary column (30 m × 320 mm × 0.25 μm). Sephadex LH-20 (20–100 μm, Pharmacia Fine Chemical Co. Ltd, NJ, USA), silica gel (200–300 mesh, Qingdao Marine Chemistry Ltd, Qingdao, China), and Cosmosil octadecyl silane (ODS) (40–80 μm, Nacalai Tosoh, Inc., Uetikon, Switzerland) were used for column chromatography (CC). TLC was conducted on silica gel GF254 (Qingdao Marine Chemistry Ltd, Qingdao, China).

3.2 Plant material

The rhizome of *A. asphodeloides* was collected from Liaoning Province of China in September 2010. The plant material was identified by Prof. Qishi Sun (Department of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). A voucher specimen (No. 20100907) has been deposited in the Herbarium of Shenyang Pharmaceutical University, Liaoning, P.R. China.

3.3 Extraction and isolation

The fresh rhizome of *A. asphodeloides* (6 kg) was extracted three times (3 × 2 h) with 70% EtOH at 80°C. The EtOH extract was evaporated under reduced pressure to give a residue (387.0 g), which was suspended in water and then extracted with petroleum ether, ethyl acetate, and *n*-butanol successively. The *n*-butanolic fraction (92.8 g) was subjected to macroporous adsorption resin D101 CC, and eluted with gradient system EtOH–H_2O (30:100 to 90:100) to afford four fractions (Frs 1–4). Fr. 3 (15.4 g) was subjected to CC on Sephadex LH-20 eluting with 70% MeOH, and five fractions (Frs 3-1–5) were obtained. Fr. 3-2 (1.75 g) was subjected to CC on reversed-phase ODS, using MeOH–H_2O (20:100 to 80:100) as the eluent to afford four fractions (Frs 3-2-1–4). Fr. 3-2-3 (75 mg) was purified by HPLC (column: 10 mm × 250 mm; RP-18, 5 μm; flow rate: 2.0 ml/min) with MeOH–H_2O (80:20) as mobile phase to afford compound **1** (20 mg; $t_R = 23.7$ min). Fr. 3-2-2 (109 mg) was further purified by HPLC (column: 10 mm × 250 mm; RP-18, 5 μm; flow rate: 2.0 ml/min) with MeOH–H_2O (70:30) as mobile phase to afford compound **2** (20 mg; $t_R = 33.4$ min) and compound **3** (18 mg; $t_R = 36.7$ min). Fr. 3-2-4 (213 mg) was further purified using silica gel CC eluting with a solvent system of CH_2Cl_2–MeOH–H_2O (8:2:0.25) to yield compound **4** (40 mg) and compound **5** (37 mg).

3.3.1 Anemarnoside A (*1*)

A white amorphous solid, positive Liebermann–Burchard test; $[\alpha]_D^{25}$ −46.5 ($c = 0.054$, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3386, 2928, 1727, 1635, 1447,

Table 2. ^{1}H NMR (300 MHz) and ^{13}C NMR (125 MHz) spectral data of the sugar moieties of compounds **1** and **2** in pyridine-d_5.

	1		**2**	
No.	δ_C	δ_H	δ_C	δ_H
(3-*O*)-β-D-Gal				
1′	102.6	4.91 d (7.2)	102.7	4.90 d (7.2)
2′	81.9	4.67 dd (9.0, 7.2)	82.0	4.65 dd (9.0, 7.2)
3′	75.3	4.29 brt (9.0)	75.3	4.26 brt (9.0)
4′	70.0	4.57 O	70.0	4.56 O
5′	76.7	4.03 m	76.8	4.02 O
6′	62.3	4.44 O, 4.42 O	62.3	4.42 O, 4.45 O
(Gal^2)-β-D-Glc				
1″	106.2	5.28 d (7.2)	106.3	5.27 d (7.2)
2″	77.0	4.09 brt (7.2)	77.0	4.08 brt (7.2)
3″	78.1	4.19 O	78.2	4.21 O
4″	71.8	4.30 O	71.8	4.25 O
5″	78.6	3.96 m	78.6	3.83 m
6″	62.9	4.56 d (11.4), 4.50 d (11.4)	62.9	4.54 m, 4.50 m
(26-*O*)-β-D-Glc				
1‴	105.2	4.81 d (7.8)	105.3	4.82 d (7.2)
2‴	75.3	4.02 O	75.5	4.02 O
3‴	78.7	4.20 O	78.8	4.28 brt (9.0)
4‴	71.8	4.22 O	71.9	4.32 O
5‴	78.5	3.86 m	78.5	3.86 m
6‴	63.0	4.43 m, 4.38 O	63.1	4.50 m, 4.45 m

Note: Overlapped signals are indicated by 'O'.

1381, 1167, 1074, 1045, and 636; ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2; HR-ESI-TOF-MS: *m/z* 943.4873 $[M + Na]^+$ (calcd for $C_{45}H_{76}O_{19}Na$, 943.4879).

3.3.2 *Anemarnoside B (2)*

A white amorphous solid, positive Liebermann–Burchard test; $[\alpha]_D^{25}$ −75.4 ($c = 0.052$, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3393, 2928, 1649, 1380, 1167, 1015, 894, and 636; ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2; HR-ESI-TOF-MS: *m/z* 941.4717 $[M + Na]^+$ (calcd for $C_{45}H_{74}O_{19}Na$, 941.4722).

3.4 *Acid hydrolysis of compounds 1 and 2*

Each compound (3.0 mg) was hydrolyzed with 2M HCl (5.0 ml), heated for 4 h at 95°C and extracted with $CHCl_3$ (3 × 5.0 ml). Then the aqueous layer was concentrated *in vacuo* to appropriate volume, and the solution was examined by TLC (EtOAc–BuOH–H_2O–HOAc, 4:4:1:1) and compared with the authentic samples, galactose and glucose were detected. Each remaining aqueous layer was concentrated to dryness to give a residue, which was dissolved in pyridine (1.0 ml), and then L-cysteine methyl ester hydrochloride (2.0 mg) was added to the solution. The mixture was heated at 60°C for 2 h, and 0.5 ml TMSI (*N*-tremethylsilyimidazole) was added, followed by heating at 60°C for 2 h. The reaction product was subjected to GC analysis on Agilent 7890A (HP-5, 30 m × 320 mm, 0.25 μm) with flame ionization detector (FID) detection. Column temperature: 120–280°C with the rate of 8°C/min, and the carrier gas was N_2 (1.4 ml/min), injection temperature: 250°C; injection volume: 1 μl. The absolute configur-

ations of the monosaccharides were confirmed to be D-galactose and D-glucose by comparison of the retention times of its Me_3Si ethers with those of standard samples [t_R (D-glucose) = 19.843 min, t_R (D-galactose) = 20.165 min)].

Acknowledgments

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