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A Bisdesmosidic Cholestane Glycoside from the Rhizomes of *Polygonatum sibiricum*†

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Abstract – A bisdesmosidic cholestane glycoside was isolated from the rhizomes of *Polygonatum sibiricum* and the structure was elucidated by spectroscopic methods and acid hydrolysis as (22*S*)-cholest-5-ene-1β,3β,16β,22-tetrol 1-*O*-α-L-rhamnopyranosyl 16-*O*-β-D-glucopyranoside. This compound exhibited weak cytotoxic activity with the IC_{50} value, 63.6 μM in human MCF-7 breast cancer line, whereas it failed to show agonistic activity at 100 μM in TGR5 assay with Chinese hamster ovary (CHO) cells. This is the first report of a bisdesmosidic cholestane glycoside from *Polygonatum* species and the full assignments of ^{1}H, ^{13}C NMR by HMBC, TOCSY and NOESY experiments were provided.
Keywords – cholestane glycoside, *Polygonatum sibiricum*, spectroscopic methods, cytotoxic activity

Introduction

Polygonatum sibiricum (Liliaceae) is a perennial plant, which is native to eastern Asia and widely cultivated as a tonic or beverage with the roasted rhizomes. The dried rhizome is called Huang Jing or Siberian Solomon's Seal (Anonymous, 1978). It has been reported that the methanol extracts of these rhizomes showed hypoglycemic and cardiotonic activities (Kato and Miura, 1994; Miura *et al*., 1995; Hirai *et al*., 1997). A steroidal saponin has been reported to be a responsible compound for this hypoglycemic activity (Kato and Miura, 1993). In addition, this crude drug has been used as a folk medicine in Asia to treat cough, dizziness and pulmonary disease. Scientific research has revealed that it strengthen immune system and prevent our body from hyperlipidemia or aging (Liu *et al*., 1998; Zheng *et al*, 1998).

In our preliminary study, we have reported the structural assignment of four novel steroidal saponins, neosibiricosides A-D isolated from *P. sibiricum* (Ahn *et al*., 2006). Further analysis of the *n*-BuOH fraction of the MeOH extract resulted in the isolation of a bisdesmodic cholestane glycoside with a rhamnose and a glucose moiety. In this paper, we report the structural determination of this compound on the basis of spectroscopic analysis and the result of acid hydrolysis. Cytotoxic activity on with human MCF-7 breast cancer line exhibited by this compound is also described with the result at another biological assay, TGR5 assay.

Experimental

General Procedure – Optical rotations were obtained on a JASCO DIP-1000 digital polarimeter. Uncorrected melting point apparatus (Fischer Scientific, USA) was used to acquire the melting point. FT-IR spectra were recorded with a JASCO FT/IR-300 spectrophotometer. ^{1}H and ^{13}C NMR spectra were taken on a Bruker Avance-600 at 600 and 150 MHz, respectively. HRFAB/MS and ESI/MS were obtained on a JEOL JMS-AX505505WA and a Finnigan MAT LCQ ion-trap mass spectrometer, respectively. The experimental conditions for ESI/MS were as follows: sheath gas flow rate 80 au, auxiliary gas flow 20 au, source voltage 4.5 kV, capillary voltage 36.5 V, inter-octapole lens voltage 10 V, and capillary temperature 300 °C. The sodium cationized molecular ions

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were isolated with an isolation width of 2 *m/z* units and fragmented using a collision energy of 55% for MS^2 experiments. GC was conducted on a GC353B-FSL gas chromatograph (GL Sciences) with a flame ionization detector (FID). TLC was carried out on silica gel precoated plates (Art. No. 5715, Merck). Silica gel 60 (40 - 63 μm, Art. 9385, Merck) and ODS-A YMC Gel (12 nm - 150 μm, AA12SA5, Kyoto, Japan) were used for column chromatography. All chemicals used in bioassay were of biochemical reagent grade.

Plant Material – The rhizomes of *Polygonatum sibiricum* Redouté (Liliaceae) were collected in Kyeongki province of Korea in April, 2005. This plant was identified by Prof. Jong Hee Park of the College of Pharmacy, Pusan National University, Korea. A voucher specimen (SNUPH-0328) is deposited in the Herbarium of the College of Pharmacy, Seoul National University.

Extraction and Isolation – The fresh rhizomes (30 kg) of *P. sibiricum* were extracted twice with 100% MeOH (30 L) and evaporated *in vacuo*. The MeOH extract (3.8 kg) was dissolved in water and partitioned with *n*-butanol. The *n*-butanol layer (75 g) was concentrated *in vacuo* and divided into five fractions (F.1 - F.5) on silica gel column chromatography (Merck, 230 - 400 mesh, 1 kg) using $CHCl_3$-MeOH-H_2O mixtures of increasing polarity (30 : 5 : 1 (F.1), 15 : 5 : 1 (F.2), 10 : 5 : 1 (F.3, F.4), 6 : 5 : 1 (F.5), 3 L of each). The F.4 fraction (12 g) was then chromatographed on ODS silica gel to give five subfractions. Compound **1** (11 mg) was purified from subfraction 2 by Sep-Pack cartilage (Waters C_{18}, USA) and crystallization in aqueous MeOH solution.

Analysis of Sugar Components of Compound 1 – The acidic hydrolysis of compound **1** (1 mg) and the trimethylsilyl L-cysteine derivatization of the monosaccharide were carried out according to the previous published paper (Ahn *et al*., 2006). Under these conditions, standard sugars gave peaks at t_R (min); 11.38 and 12.23 for D- and L-glucose, and 8.42 for L-rhamnose, respectively.

Compound 1 – amorphous powder; $[\alpha]^{20}_D$ –45.8° (*c* 0.13, MeOH); IR (KBr) ν_{max} 3430 (OH), 2926 (CH), 1453, 1371, 1265, 1067, 824 cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS (positive mode) *m/z* 765 $[M + Na]^+$; HRFABMS *m/z* 765.4421 (calcd for $C_{39}H_{66}O_{13}Na$, 765.4401); mp: 177 - 179 °C

Cytotoxicity assay – Cytotoxic activity was evaluated with MCF-7 cells according to the previous published paper (Ahn *et al*., 2006).

TGR5 assay – Chinese hamster ovary (CHO) cells stably transfected with CREB-luc were obtained from Panomics, and were maintained in F12K supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and hygromycin (100 μg/ml). For the TGR5 assay, a stable cell line was obtained by transfection with TGR5 expression plasmid (pCMV6/

Table 1. ^1H and ^{13}C NMR Spectral Data for Compound **1** (in C_5D_5N)[a]

position	δ_H	δ_C	HMBC
1	3.72 dd (11.5, 3.6)	81.1	H-1'
2	2.78 br d, 2.47	35.7	
3	3.81 m	67.8	H-4
4	2.64, 2.52	43.4	H-6
5		138.8	
6	5.46 m	124.9	
7	1.73, 1.35	31.2	H-6
8	1.25	33.2	H-6, H-15
9	1.24	50.5	H-1, H-19
10		42.6	H-1, H-6, H-9, H-11, H-19
11	2.58, 1.55	24.6	
12	2.05, 1.45	40.3	H-17, H-18
13		42.0	
14	0.84	55.0	
15	2.28, 1.74	36.9	
16	4.50	82.3	H-1"
17	1.96 dd (10.8, 7.6)	57.8	H-14, H-15, H-18, H-21
18	0.97 s	13.6	H-12, H-17
19	1.14 s	14.3	H-1
20	1.85	35.6	H-17, H-21
21	1.12 d (7.2)	12.3	
22	4.29	72.8	
23	1.83, 1.77	33.7	
24	1.89, 1.59	36.4	H-25, H-26, H-27
25	1.58	28.7	H-24, H-26, H-27
26	0.85	22.8	H-25
27	0.86	22.9	H-25
1'	5.60 (br s)	97.5	H-1
2'	4.49 dd (br d)	72.7	H-1'
3'	4.44 dd (7.6)	72.6	H-1'
4'	4.25 dd	73.3	
5'	4.22 m	70.4	H-1', H-4', H-6'
6'	1.60 d (6.1)	18.5	H-5'
1"	4.71 d (7.2)	106.7	
2"	3.97 dd (7.7, 7.2)	75.3	H-1"
3"	4.13 dd (7.7, 7.5)	78.5	H-1"
4"	4.23 dd (7.5, 7.3)	71.3	
5"	3.80 m	77.9	H-1"
6"a	4.47 br d	62.5	H-4", H-5"
6"b	4.37 dd (11.8, 4.5)		

[a]Signals for positions 26 and 27 are exchangeable each other.

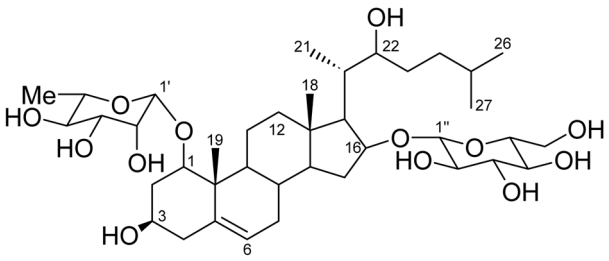

Fig. 1. Chemical structure of compound **1**.

TGR5, Origene) using Lipofectamine 2000 reagent (Invitrogen). The transfected cells were selected with 600 μg/mL G418 sulfate, and single clones were grown in a 96-well plate, independently. Samples were tested by luminescence on the selected CHO/CREB-luc/TGR5 cell line. The biological effects of samples on TGR5 were compared to those of lithocholic acid (Sigma, USA) used as an internal control in the luciferase assay. TGR5-expressing CHO cells were treated for 5 h with 10 μM lithocholic acid or 100 μM sample, followed by a luciferase assay. Luminescence was determined with Fusion alpha (PerkinElmer).

Results and Discussion

The concentrated MeOH extract of the rhizomes of *P. sibiricum* was partitioned between *n*-BuOH and water. The *n*-BuOH-soluble fraction was chromatographed on Si gel, octadecylsilanized (ODS) Si gel to give compound **1** (Fig. 1).

Compound **1**, obtained as an amorphous powder, showed a positive dark-green color by Lieberman-Burchard reagent on TLC plate. In the positive-ion ESIMS of **1**, a quasimolecular ion peak at *m/z* 765 $[M+Na]^+$ was observed, and HRFABMS analysis revealed the molecular formula to be $C_{39}H_{66}O_{13}$.

The ^{1}H-NMR spectrum of **1** showed signals for two tertiary methyl groups at δ 0.97 and 1.14 (each 3H, s), three secondary methyl groups at δ 1.12 (d, J = 7.2 Hz), δ 0.85 and 0.86, and an olefinic proton at δ 5.46 (m). The signal at δ 1.60 (d, J = 6.1 Hz) was due to the methyl group of 6-deoxyhexopyranose. Two anomeric proton signals were also found at δ 5.60 (br s) and 4.71 (d, J = 7.2 Hz). From the ^{13}C NMR spectrum showing 39 resonance lines, 27 of them were attributed to aglycone part and 12 to two monosaccharides. These NMR data supported the fact that **1** has a steroidal skeleton and two saccharide moieties. All the carbon signals of its aglycone were consistent with those of previously reported (22*S*)-cholest-5-ene-1*β*,3*β*,16*β*,22-tetrol moiety (Mimaki *et al.*, 1999).

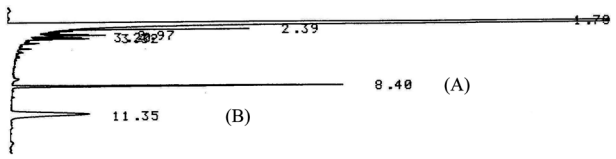

Fig. 2. GC chromatogram of acid hydrolysate (trimethylsilyl L-cysteine derivatives) of compound **1**. Two major peaks indicate L-rhamnose (A) and D-glucose (B), respectively.

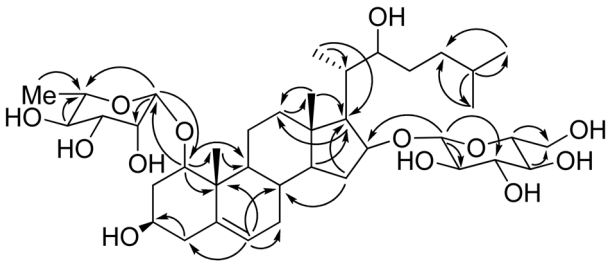

Fig. 3. HMBC correlations of compound **1**.

The monosaccharides obtained from the acidic hydrolysis of **1** were identified as D-glucose and L-rhamnose with the molar ratio of 1 : 1 by GC of their respective trimethylsilyl L-cysteine derivatives (Fig. 4) (Agrawal *et al.*, 1985; Hara *et al.*, 1987). The *β*-orientations of the glucose moiety was supported by the relatively large J values of the anomeric proton (J = 7.2 Hz for H-1") and the chemical shift (δ 106.7) of the anomeric carbon. The α-configuration of the L-rhamnose was confirmed by their ^{13}C shifts because remarkable differences in the ^{13}C shifts at C-3 and C-5 were recognized between α-, and *β*-L-rhamnopyranosides (Agrawal *et al.*, 1985; Agrawal *et al.*, 1992).

Each monosaccharide was considered to be directly attached to the aglycone and was not substituted because no glycosylation shifts could be observed among the assigned ^{13}C NMR shifts (Mimaki *et al.*, 2000). This fact was revealed by the following correlations in the HMBC spectrum (Fig. 3). The H-1 proton (δ 3.72) of the aglycone and the anomeric proton (δ 4.71) of glucose showed correlations with the anomeric carbon of rhamnose (δ 97.5) and the C-16 carbon (δ 82.3) of the aglycone, respectively. These correlations were also confirmed by NOESY spectrum and anomeric carbon signals (δ 97.5 and 106.7 for rhamnose and glucose, respectively) different from those (δ 104.9 and 101.3) of (22*S*)-cholest-5-ene-1*β*,3*β*,16*β*,22-tetrol 1-*O*-*β*-D-glucopyranosyl 16-*O*-*α*-L-rhamnopyranoside. 1D-selective TOCSY experiment allowed the sequential assignments of all resonances for each monosaccharide, starting from the anomeric protons (Fig. 4).

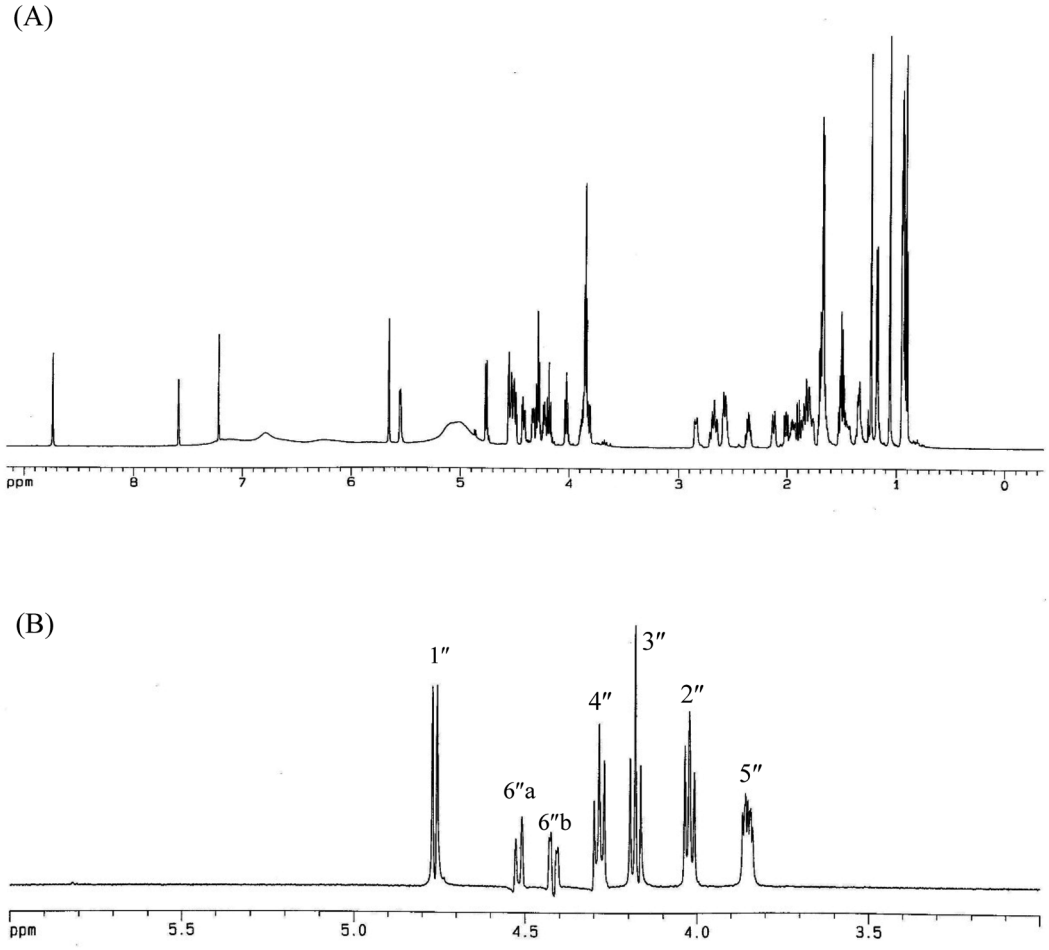

Fig. 4. ^{1}H-NMR spectrum (A) and δ 4.75 selective TOCSY spectrum (B) of compound **1** (600 MHz, in C_5D_5N).

Meanwhile, compound **1** displayed a quasimolecular ion peak at *m/z* 765 $[M + Na]^+$ and *m/z* 781 $[M + K]^+$. The MS/MS spectrum of the $[M + Na]^+$ ion showed the peaks at *m/z* 747 $[M + Na - H_2O]^+$, 601 $[M + Na - Rhm - H_2O]^+$ and 585 $[M + Na - Glc - H_2O]^+$ resulting from loss of a hydroxy group (18 Da) in aglycone moiety, a rhamnose unit (146 Da) and a glucose unit (162 Da), respectively. In addition, it exhibited the peaks at *m/z* 403 $[M + Na - Glc - Rhm - 3H_2O]^+$ due to the successive loss of two saccharide moieties and three hydroxy groups. The peaks at *m/z* 695 $[M + Na - C_5H_{10}]^+$ resulted from the cleavage of side chain of the aglycone (Fig. 5).

On the basis of the obtained data, the structure of compound **1** was assigned as (22*S*)-cholest-5-ene-1*β*,3*β*,16*β*,22-tetrol 1-*O*-*α*-L-rhamnopyranosyl 16-*O*-*β*-D-glucopyranoside (Mimaki *et al*., 1999). This compound belongs to rare cholestane bisdesmosides possessing disaccharide residues linked to a polyhydroxycholesterol aglycone. Bisdesmosidic cholestane glycosides have been isolated from several Liliaceae plants. Since compound **1** was firstly isolated from a plant source, *Allium jesdianum*, it has been reported to be isolated from tubers or bulbs or seed of Liliaceae plants (Dai *et al*., 2000; Fattorusso *et al*., 2000; Sang *et al*., 2000; Higano *et al*., 2007). Partial assignment or overlapped signals in CD_3OD has been previously reported (Fattorusso *et al*., 2000). However, this is the first report of a bisdesmodic cholestane glycoside from the genus *Polygonatum*. In this study, the full assignment of ^{1}H-NMR spectrum by HSQC, TOCSY and NOESY experiments was also provided.

Although compound **1** was reported not to have shown cytotoxic activity against HL-60 cells, this compound exhibited weak cytotoxic activity with the IC_{50} value, 63.6 μM in human MCF-7 breast cancer line (Mimaki *et al*., 1999). Meanwhile, TGR5 is an emerging bile acid G-protein-coupled receptor target for the potential treatment of metabolic disorders or inflammation (Atul and Pranab, 2009; Thijs *et al*., 2011). For example, TGR5 activation

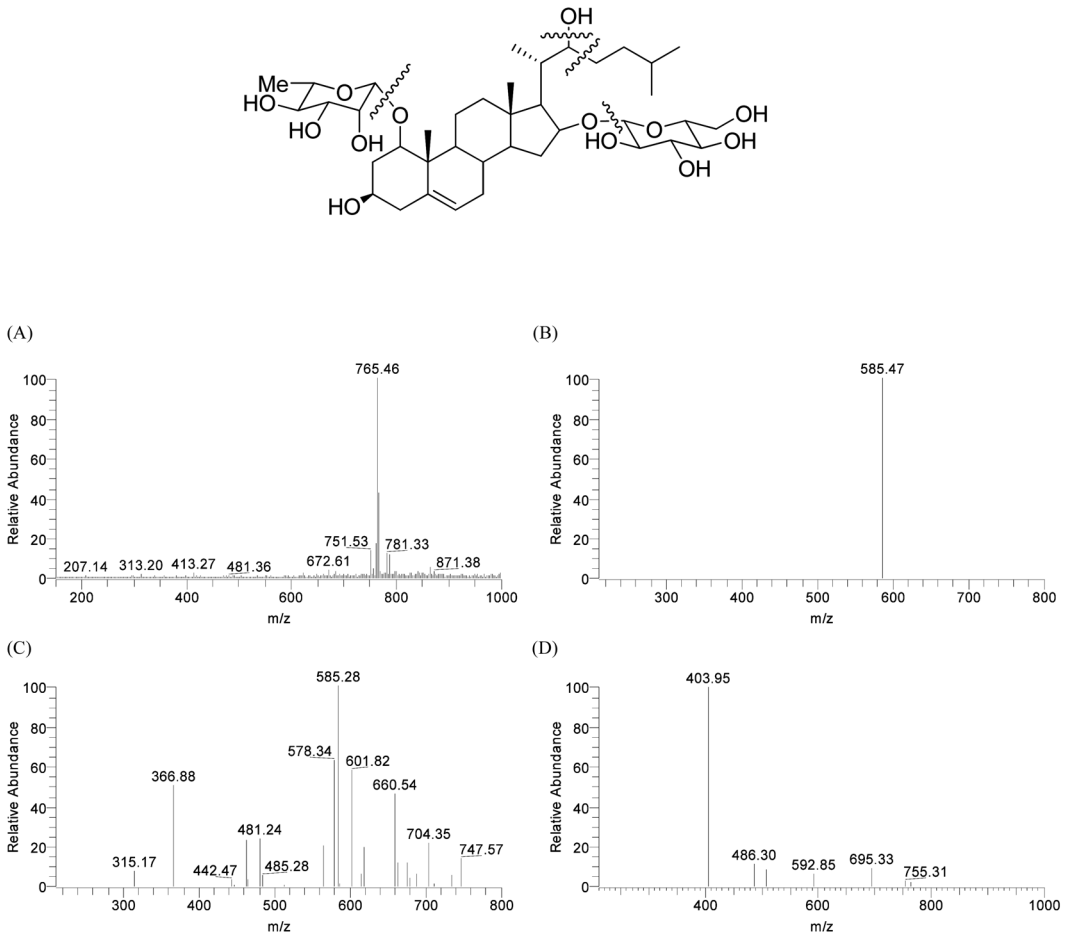

Fig. 5. ESI MS spectrum of the sodium cationized compound **1** (A) and MS/MS spectrum of the molecular ion at *m/z* 765 (B-D).

induces a significant reduction of the body weight of mice fed a high fat diet (Watanabe, 2006). Since compound **1** has the same cholestane structure with bile acids, ligands for this receptor, TGR5 assay was accomplished with this isolate using Chinese hamster ovary (CHO) cells and a bile acid, lithocholic acid as a positive control. However, this compound failed to show agonistic activity on TGR5 receptor. It was suggested that it could have resulted from lack of negatively charged functional groups or presence of a double bond to increase molecular rigidity or bulky saccharide moieties of this bisdesmosidic cholestane glycoside (Atul and Pranab, 2009).

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