Eucosterol Oligoglycosides Isolated from *Scilla scilloides* and Their Anti-tumor Activity

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Two new eucosterol oligoglycosides, 15-deoxo-30-hydroxyeucosterol 3-*O*-α-L-rhamnopyranosyl-(1→2)-[(β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (scillanoside L-1, 1) and 3β,31-dihydroxy-17α,23-epoxy-5α-lanost-8-en-23,26-olactone 3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (scillanoside L-2, 2), were isolated from the bulbs of *Scilla scilloides*, together with four that were known (3—6), have been isolated from the bulbs of *Scilla scilloides*. The structures of the new compounds were determined on the basis of spectroscopic and chromatographic methods, and some chemical transformations were discussed. Amongst the isolated compounds, 3 showed the most significant cytotoxicity against tumor cells tested several types with ED_{50} value of 1.53—3.06 nM. *In vivo* experiments, 3 apparently increased the life span of mice bearing Sarcoma 180 tumor cell with T/C value of 239% at dose of 3 mg/kg.

Key words *Scilla scilloides*; spirocyclic nortriterpenoide; anti-tumor activity

Scilla scilloides (LIND.) DRUCE (Liliaceae) is a traditional Chinese medicine used for the treatment of antidote, blood circulatory activator, and abscess reducer.[1] Eucosterol oligoglycosides[2] isolated from *S. scilloides* have been proved to have inhibitory effect on 12-*O*-tetradecanoylphobol-13-acetate (TPA) stimulated ^{32}P incorporation into phospholipids of HeLa cells.[3,4] On the course of our research for anti-tumor activity from natural sources, we studied the constituents of *S. scilloides*, and isolated two new eucosterol oligoglycosides (**1**—**2**) and four known compounds (**3**—**6**). Herein we present the structural elucidation of new compounds, as well as anti-tumor activities of six eucosterol oligoglycosides.

Results and Discussion

Repeat column chromatography of the butanol-soluble fraction of the MeOH extract of *S. scilloides* on Sephadex LH 20 and reversed-phase silica gel columns let to the isolation of six compounds (**1**—**6**). The known compounds were identified as scillascillosides E-1 (**3**), E-2 (**4**), E-3 (**5**) and G-1 (**6**), which had been isolated from this plant, on the basis of their spectral and physical data in comparison with those reported in literature (Chart 1).[2]

Compound **1** (scillanoside L-1) was isolated as a white amorphous powder, $[\alpha]_D$ −57.1°. A molecular formula of $C_{58}H_{94}O_{28}$ was established by high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS), which gave a mass of *m*/*z* 1239.6012 (Calcd for $C_{58}H_{95}O_{28}$, 1239.6010). The IR spectrum was showed with the presence of hydroxyl group (3350 cm^{-1}) and a carbonyl group (1725 cm^{-1}). The aglycon of **1** was shown to be a eucosterol derivative from ^1H-NMR signals for a methylene at δ_H 2.51 (q, J=7.3 Hz), three tertiary methyls (δ_H 0.90, 1.04, and 1.46), a primary methyl at δ_H 1.00 (t, J=7.3 Hz), a secondary methyl at δ_H 1.01 (d, J=6.6 Hz), and two hydroxymethyls at δ_H 4.34 and 4.61 (each 2H, s). The ^{13}C-NMR spectrum, in combination with distortion less enhancement by polarization transfer (DEPT) and ^1H-detected multiple quantum coherence (HMQC) experiments, showed signals for a carbonyl carbon at δ_C 212.6, two sp^2 carbons at δ_C 135.4 and 136.0, a methylene at δ_C 32.4, a primary methyl carbon at δ_C 7.7, and three oxygen-bearing carbons at δ_C 81.6, 82.1 and 97.1, including two hydroxymethyl carbons at δ_C 61.1 and 62.8. These data indicated spirocyclic nortriterpene close to the structure of scillascilloside E-1 (**3**) except for the presence a hydroxy methyl at C-30 (δ_H 4.34, δ_C 61.1) in **1**. The connectivity of the hydroxy methyl group was confirmed by heteronuclear multiple-bond correlation (HMBC) observed between δ_H 4.34 (H-30) and δ_C 48.3 (C-4).

On acid hydrolysis,[5] **1** afforded the monosaccharide units which were identified by co-TLC with authentic samples such as glucose, arabinose, and rhamnose; their absolute configurations were determined as D-glucose , L-rhamnose, and L-arabinose by liquid chromatography method developed by Oshima *et al*.[6] In the acid hydrolysate of **1**, D-glucose, L-rhamnose, and L-arabinose were confirmed by comparison of the retention times of their 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate (AMBA) derivatives with those of authentic monosaccharides prepared in similar way, which showed retention times of 33.4, 20.0, and 26.3 min in the HPLC, respectively. In the correlation spectroscopy (COSY), HMQC, and total correlation spectroscopy (TOCSY) spectra of **1**, correlations were observed between the anomeric signals at δ_H 5.84 and signals δ_H 4.61, 4.54, 4.25, 4.92, and 1.78. The series of signals were considered to be due to the protons of a rhamnose, because correlations were found between the methyl signal at δ 18.8 and two of the proton signals at δ 4.25 (rha″″-H-4) and 4.92 (rha″″-H-5) in the HMBC spectrum. Similarly, the spin system for the glucoses and arabinose were assigned, since the anomeric proton signals at δ 5.02, 5.11, 5.14, and 5.28 were correlated with the signals as Table 2. Furthermore, the connectivity of the sugars were determined on the presence of correlations between a proton signal at δ_H 5.02 (glc-H-1′) and a carbon signal at δ_C 82.1 (C-3), δ_H 5.28 (ara-H-1″) and δ_C 68.6 (glc-C-6′), δ_H 5.14 (glc-H-1‴) and δ_C 77.7 (ara-C-2″), δ_H 5.84 (rha-H-1″″) and δ_C 77.9 (glc-C-2‴), δ_H 5.11 (glc-H-1⁗) and δ_C

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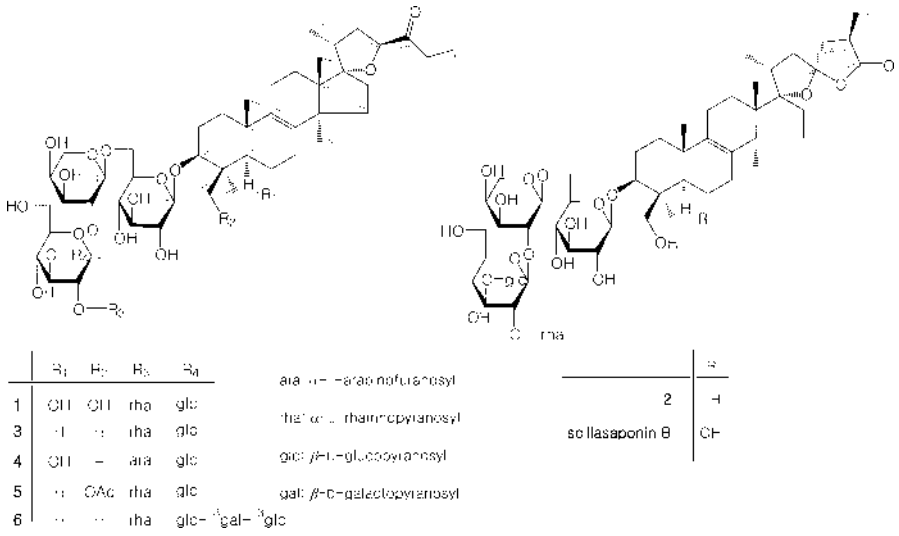

Chart 1. Structures of Compounds Isolated from *S. scilloides*

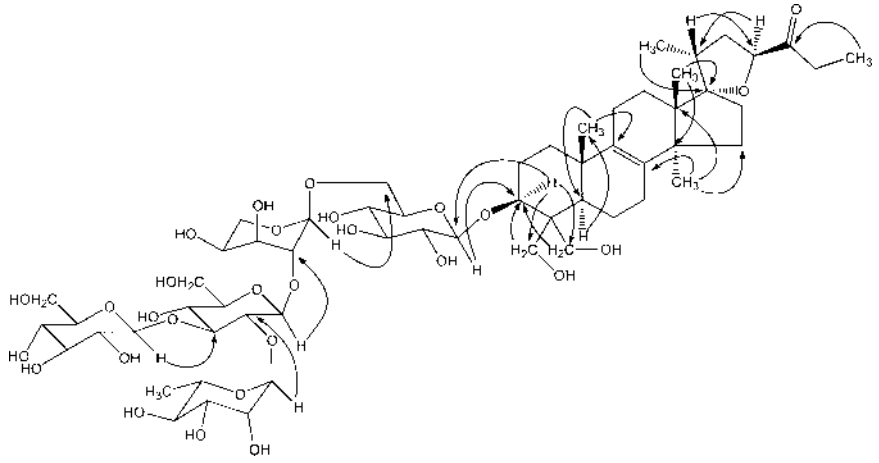

Fig. 1. HMBC-Correlations of Compound **1**

89.1 (glc-C-3‴) in HMBC spectrum.

An equatorial hydroxyl group at C-3 (β-orientation) was deduced from the multiplicity of H-3 (δ 4.49, dd, J=11.7, 4.5 Hz). The configuration of the glycosidic linkage of the three glucopyranoses in **1** were determined to be β on the basis of the $J_{1,2}$ values 7.8 Hz (at δ 5.02, 5.14) and 7.7 Hz (δ 5.11) of the anomeric protons, while that of the arabinofuranose was α form from the chemical shifts of C-1″, C-2″, C-3″ (δ 101.0, 77.7, 71.5)[2] and rhamnopyranose was α form from the chemical shift of C-5 (δ 69.8),[5] respectively. Finally, **1** was determined as 15-deoxo-30-hydroxyeucosterol 3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside.

Compound **2** (scillanoside L-2) was isolated as a white amorphous powder. **2** has a molecular formula of $C_{59}H_{94}O_{28}$ from the HR-FAB-MS ($[M+H]^+$ peak at *m/z* 1251.6003, Calcd for $C_{59}H_{95}O_{28}$ 1251.6010). Its IR spectrum revealed the presence of hydroxyl (3350 cm^{-1}) and carbonyl in lactone (1770 cm^{-1}). The latter band was further confirmed by a carbon signal at δ_C 179.3 in the ^{13}C-NMR spectrum. ^{1}H- and ^{13}C-NMR (Tables 1, 2) spectra of **2** were similar to those of **1**. Careful examination of the spectral data, however, revealed several significant differences. The most noticeable changes were δ_C 113.8 (C-23), 45.3 (C-24), 36.2 (C-25), and 179.3 (C-26) by comparison with the corresponding signals of **1** in the ^{13}C-NMR data. The signal indicated the presence of a spirocyclic-γ-lactone group in the side chain of nortriterpene, when compared with that of the ^{13}C-NMR spectra of scillasaponin B from *S. peruviana*.[7,8] This was further supported by HMBC experiment, which showed HMBC correlations observed between δ_H 1.22 (H-27) and δ_C 45.3 (C-24)/179.3 (C-26), and δ_H 2.62 (H-24) and δ_C 179.3 (C-26).[9] The absolute configurations at C-23 and C-25 were confirmed by ^{13}C-NMR chemical shift of δ_C 99.0 (C-17), 44.4 (C-20), 45.2 (C-22), 113.8 (C-23), 45.3 (C-24), and 36.2 (C-25) compared with scillasaponin B.[7,8] Furthermore, compound **2** showed a hydroxy methyl signal δ_H 4.66 (H-31) and δ_C 63.5 (C-31). The connectivity of the hydroxy methyl group was confirmed by HMBC correlation observed between δ_H 1.53 (H-30) and δ_C 89.2 (C-3)/52.1 (C-5). Therefore, the aglycon part of **2** was 3β,31-dihydroxy-17α,23-epoxy-5α-lanost-8-en-23,26-olactone. The monosaccharides of **2** were determined as D-glucose, L-rhamnose, and L-arabi-

Table 1. ^{13}C- (75 MHz) and ^{1}H-NMR (600 MHz) Spectral Data for Aglycon Parts of **1** and **2** in Pyridine-d_5

C No	**1**		**2**	
	δ_C	δ_H, (J in Hz)	δ_C	δ_H (J in Hz)
1	35.8	2.37, m	36.1	2.26, m
2	27.3	1.72, m	27.8	1.16, m
3	82.1	4.49, dd (11.7, 4.5)	89.2	4.52, dd (11.6, 4.6)
4	48.3		44.8	
5	43.8	1.98, m	52.1	1.30, m
6	18.8	1.00, m	19.1	—
7	26.6	1.98, m	27.2	1.28, m
8	135.4		135.1	
9	136.0		134.8	
10	36.7		37.2	
11	21.2	2.17, m	21.3	1.94, m
12	25.4	2.41, m	25.3	—
13	49.0		49.0	
14	50.9		51.0	
15	32.2	1.33, m	32.2	—
16	39.8	2.11, m	37.8	—
17	97.1		99.0	
18	19.4	0.90, s	19.0	0.85, s
19	19.7	1.04, s	19.9	0.91, s
20	43.5	—	44.4	2.09, m
21	17.3	1.01, d (6.6)	19.1	1.00, d (6.8)
22	36.9	1.73, m	45.2	—
23	81.6	—	113.8	
24	212.6		45.3	2.62, m
25	32.4	2.51, q (7.3)	36.2	—
26	7.7	1.00, t (7.3)	179.3	
27			15.5	1.22, s
30	61.1	4.34, s	23.5	1.53, s
31	62.8	4.61, s	63.5	4.66, s
32	26.4	1.46, s	26.3	1.23, s

Table 2. ^{13}C- (75 MHz) and ^{1}H-NMR (600 MHz) Spectral Data for Oligosaccharides Parts of **1** and **2** in Pyridine-d_5

C No	**1**		**2**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
Glc′ 1	106.9	5.02, d (7.8)	106.4	4.94, d (7.8)
2	75.4	3.77	75.3	3.96
3	78.2	4.15	78.8	4.15
4	72.5	4.18	72.6	4.18
5	76.9	3.60	78.9	3.97
6	68.6	4.16	68.9	4.24
		4.42		4.52
Ara″ 1	101.0	5.28, d (2.7)	101.1	3.31, br s
2	77.7	4.64	77.9	4.66
3	71.5	4.27	71.9	4.67
4	66.7	4.53	66.6	4.61
5	62.8	4.35	62.7	4.37, dd (11.1, 8.1)
		3.90, dd (11.2, 3.6)		3.89, dd (11.1, 4.0)
Glc‴ 1	102.6	5.14, d (7.8)	102.9	5.17, d (7.7)
2	77.9	4.20	77.3	4.20
3	89.1	4.08	89.4	4.05
4	69.1	4.85	70.1	4.84
5	77.9	3.53	78.2	3.58
6	62.3	4.26	63.0	4.35, dd (11.1, 8.0)
		3.98		3.84, dd (11.1, 4.0)
Rha⁗ 1	102.1	5.84, br s	102.4	5.85, br s
2	72.1	4.61	72.6	4.64
3	72.5	4.54	72.9	4.66
4	74.9	4.25	74.5	4.27
5	69.8	4.92	70.1	4.87
6	18.8	1.78, d (5.7)	18.9	1.79, d (6.0)
Glc⁗ 1	104.3	5.11, d (7.7)	104.6	5.02, d (8.1)
2	74.1	4.58	75.8	4.17
3	82.1	4.25	79.0	4.01
4	69.1	4.11	69.4	4.10
5	77.9	3.88	78.6	3.98
6	61.1	4.13	62.1	4.15
		4.22		4.23

The assignment of the signals are based on HMQC, HMBC, and TOCSY spectral data.

Table 3. Cytotoxic Activities of the Constituents of *S. scilloides* on Tumor Cell Growth

Tumor cells	ED_{50} (nM)[a]						
	1[b]	**2**	**3**	**4**	**5**	**6**	AD[c]
HT1080	2.62±1.79	2.34±0.11	1.66±0.04	2.30±0.04	1.69±1.65	2.28±1.29	0.09±0.03
B16(F-10)	2.38±2.10	5.27±2.71	2.66±0.21	7.06±4.07	3.82±1.79	4.91±2.80	0.06±0.10
3LL	4.07±1.05	3.53±3.53	2.59±0.49	3.83±3.98	2.61±0.26	2.94±2.52	0.09±0.03
MCF7	7.96±4.83	>10	3.06±2.35	4.80±1.72	4.34±2.46	4.14±0.78	0.38±0.34
PC-3	5.08±3.95	4.82±3.52	1.53±0.28	3.95±0.43	2.33±0.95	1.73±1.57	0.83±0.18
HT29	6.56±5.21	>10	3.00±2.76	3.23±3.09	5.96±2.73	3.22±2.00	1.07±0.12
LOX-IMVI	3.82±1.68	4.51±1.82	2.44±0.43	3.70±4.32	4.89±0.12	2.04±0.42	0.38±0.33
A549	4.51±3.23	5.60±2.99	1.98±1.80	3.54±0.74	3.09±1.98	3.20±0.29	0.67±0.21

a) ED_{50} is defined as the concentration which resulted in a 50% decrease in cell number. *b*) Results are means±S.D. of 3 to 5 independent replicates. *c*) Adriamycin as positive control.

nose by the liquid chromatography method developed by Oshima *et al*.[6] Detailed analysis of the ^{1}H-, ^{13}C-NMR, HMQC, and HMBC revealed that sugars of **2** were the same to those of **1**. Consequently, the structure of **2** was established as 3β,31-dihydroxy-17α,23-epoxy-5α-lanost-8-en-23,26-olactone 3-*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (scillanoside L-2).

Compounds **1**—**6** were evaluated for their cytotoxic activity against eight cancer lines. As summarized in the Table 3, **1**—**6** showed cytotoxic activity against all tested cancer cell lines, although **2** was inactive on MCF7 and HT29 cell lines. Of these, scillascilloside E-1 (**3**) showed most significant cytotoxic effect. In the cancer cell lines tested, HT1080 and PC-3 cell lines showed high sensitivity on all tested compounds. Furthermore, we examined the anti-tumor activity of **3**

Table 4. *In Vivo* Effect of Scillscilloside E-1 (**3**) and Cisplatin on B16 (F-10) Melanoma and Sarcoma 180 Cell-Bearing Mice

Tumor	Sample	Dose (mg/kg)	Survival days (mean±S.D.)	T/C (%)[a,b]
B16 (F-10)	Control[c]		16.8±2.3	100.0
	Cisplatin	3	25.2±3.4	150.0
	Scillascilloside E-1 (**3**)	1.5	13.2±2.1	78.6
		3	16.5±2.7*	98.2
Sarcoma 180	Control		19.5±2.6	100.0
	Cisplatin	3	30.1±4.3*	154.3
	Scillascilloside E-1 (**3**)	1.5	25.6±3.1*	131.2
		3	46.7±4.4	239.4

a) The efficacy of the tumor treatment was determined by the increase in the survival day of the treated mice (T) as compared to that of the control group (C) using the expression T/C (%). *b*) T/C (%); >125 for significant anti-tumor activity. *c*) Each group consists of 7 mice. *: $p<0.01$.

against B16 (F-10)- and Sarcoma 180-bearing mice. The results of the *in vivo* anti-tumor evaluation of **3**, when given intraperitoneally once daily for the 20 d at dosages of 1.5 and 3 mg/kg, and vehicle (0.9% saline), used as a negative control, were shown in Table 4. Compound **3** prolonged the life span of B16 (F-10)-bearing mice (T/C values, 82, 103%) and Sarcoma 180-bearing mice (T/C values, 131, 239%) at doses of 1.5 and 3 mg/kg/day, respectively. The anti-tumor effect of **3** at 3 mg/kg showed a significant anti-tumor activity against Sarcoma 180. From these results, scillascilloside E-1 (**3**) showed a significant anti-tumor activity *in vitro* and *in vivo* experiments.

Experimental

Melting points were determined on electrothermal melting point apparatus 9100. Optical rotations were measured on DIP-370 digital polarimeter (JASCO). IR spectra were measured on IR Report-100 infrared spectrometer (JASCO). HR-FAB-MS spectra were measured on JMS 700 mass (JEOL) and ESI mass spectra were obtained on a VG Quattro 400 mass (FISONS). ^{1}H- and ^{13}C-NMR spectra were recorded on an AC 300 and DMS 600 (BRUKER) the chemical shifts being represented as part per million (ppm) referenced to pyridine signal as solvent (8.71 ppm at ^{1}H- and 150.3 ppm at ^{13}C-NMR spectrum). Column chromatography was carried out using Kieselgel 60, 400—230 mesh, (MERCK). TLC was performed on aluminium backed Kieselgel 60 F_{254} plates (MERCK) developed with BuOH/MeOH/H_2O (4 : 1 : 1) and spots were visualized under UV light and by 10% sulfuric acid (in H_2O) followed by heating.

Plant Material The bulbs of *S. scilloides* (Lind.) Druce were collected during August 2000 at Taejon, Korea and identified by Dr. S. M. Lee. Voucher specimen (KRIBB1700) is deposited at the Herbarium of Korea Research Institute of Bioscience and Biotechnology, Korea.

Isolation The fresh bulbs of *S. scilloides* (3 kg) were extracted with MeOH at room temperature (7 d×3) to give an extract (35 g). The MeOH extract was suspended in H_2O (1 l) and then shaken with EtOAc (1 l×2, each time), BuOH saturated with H_2O (1 l×3, each time), successively. The BuOH-soluble fraction (10 g) was divided into twenty fractions (Fr. 1—Fr. 20, each 100 ml) by gel filtration on Sephadex LH 20 (3.0×70 cm, 210 g) eluting with MeOH. The fractions 3—5 (6 g) were preparative-HPLC on YMC-Pak ODS-AQ column (300×10 mm I.D.) eluting with aq. MeOH (75%, 1.5 ml/min, det. at 210 nm) to give fifteen sub fractions (Fr. 1′—15′, each 50 ml). These sub fractions were further purified on preparative-HPLC eluting with 75% aq. MeOH obtained compounds **1**—**6**. Yields: **1** (98 mg, from Fr. 2′), **2** (110 mg, from Fr. 2′, 3′), **3** (130 mg, from Fr. 5′, 6′), **4** (25 mg, from Fr. 8′), **5** (17 mg, from Fr. 10′, 11′) and **6** (14 mg, from Fr. 11′).

Scillanoside L-1 (**1**): White amorphos powder. $[\alpha]_D^{25}$ −57.1° (c=0.08, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1725 (CO). HR-FAB-MS (*m*/*z*): 1239.6012 $(M+H)^+$ (Calcd for $C_{58}H_{95}O_{28}$ 1239.6010). Electrospray ionization (ESI)-MS (*m*/*z*): 1261.6 $(M+Na)^+$, 1237.6 $(M-H)^-$. ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Scillanoside L-2 (**2**): White amorphous powder. $[\alpha]_D^{25}$ −34.7° (c=0.08, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1770 (CO). HR-FAB-MS (*m*/*z*): 1251.6003 $(M+H)^+$ (Calcd for $C_{59}H_{95}O_{28}$ 1251.6010). ESI-MS (*m*/*z*): 1274.6 $(M+Na)^+$, 1250.7 $(M-H)^-$. ^{1}H- and ^{13}C-NMR: see Tables 1 and 2.

Sciscllascilloside E-1 (**3**): White amorphous powder (aq. MeOH). mp 221—223 °C, $[\alpha]_D^{25}$ −57.1° (c=0.08, MeOH). ESI-MS (*m*/*z*): 1245.6 $(M+Na)^+$, 1221.7 $(M-H)^-$.

Scillascilloside E-2 (**4**): White amorphous powder (aq. MeOH). mp 210—216 °C. $[\alpha]_D^{25}$ −38.1° (c=0.08, MeOH), ESI-MS (*m*/*z*): 1247.7 $(M+Na)^+$, 1223.7 $(M-H)^-$.

Scillascilloside E-3 (**5**): White amorphous powder (aq. MeOH). mp 218—221 °C. $[\alpha]_D^{25}$ −51.9° (c=0.08, MeOH), ESI-MS (*m*/*z*): 1303.6 $(M+Na)^+$, 1219.6 $(M-H)^-$.

Scillascilloside G-1 (**6**): White amorphous powder (aq. MeOH). mp 240—245 °C. $[\alpha]_D^{25}$ −46.8° (c=0.08, MeOH), ESI-MS (*m*/*z*): 1570.2 $(M+Na)^+$, 1546.2 $(M-H)^-$.

Determination of Sugar in Compound 1, 2 Each sample (10 mg) was refluxed with 1 N HCl–dioxan (1 : 1, 5 ml) for 2 h. After cooling, the mixture was extracted with EtOAc (5 ml×3). The residual water layer was neutralized with Amberlite IRA-93ZU (OH^- form) (Organo Co. Ltd.) and dried to give a residue. The residue (2 mg) was treated with *S*-(−)-α-methylbenzylamine (10 mg) and Na[BH_3CN] (12 mg) at 40 °C for 4 h, followed by acetylation with Ac_2O in pyridine containing a catalytic amount of 4-(dimethylamino) pyridine (5 mg). The reaction solution was concentrated under reduced pressure, and applied to preparative TLC with hexane/Me_2CO (4 : 1) to yield the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate (AMBA) derivatives of the monosaccharides, which were analyzed by HPLC under the following conditions: solvent, hexane/EtOH (19 : 1) flow rate, 1.25 ml/min; detection, UV 230 nm, and YMC-Pack SIL (4.6 mm i.d.×150 mm, silica gel).

Cytotoxicity Assay Aliquots of 2×10^5 cells were seeded into each well of a 96-well flat microtiter plates in RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). The compounds dissolved in MeOH at various concentrations were added to culture and adjusted to a final concentration of 0.1% (v/v). Above cells were grown in a humidified atmosphere of 95% air and 5% CO_2, and the cells were seeded in plates after three passages. The cell viability was determined in the presence or absence of a compound or adriamycin, as a positive control, using the standard MTT assay. Briefly, 24 h after seeding, 100 μl new media or a test compound was added and the plate was incubated for 48 h. Cells were washed once before adding 50 μl FBS-free medium containing 5 mg/ml MTT. After incubation for 4 h at 37 °C, the medium was discarded and formazan blue formed in the cells was dissolved with 50 μl DMSO. Optical density was measured at 570 nm. The concentration required to reduce absorbance by 50% (ED_{50}) in comparison to control cells was determined.

Animals and Tumors Specific pathogen free male inbred ICR mice (18—20 g), purchased from DaiHan Bio Ringk (Korea). Groups of 10 mice were fed with a commercial pellet chow in a standard laboratory conditions (25±2 °C and water *ad. libitum*). Sarcoma 180 and B16 (F-10) cells were maintained by weekly subcutaneous passages in mice. For *in vivo* assays, each mouse was subcutaneous inoculated with 5×10^6 tumor cells, which were harvested from tumor-bearing mice 7 d after tumor inoculation.

Administration of Compounds and Anti-tumor Evaluation Each compound was administered intraperitoneally once a day for consecutive 20 d 24 h after tumor inoculation. The control animals were received vehicle (saline) alone by i.p. injection. The anti-tumor effect was determined by the increase in survival time of treated mice (T) as compared to that of the control group (C), and expressed as T/C as described earlier.[7]

Statistics The results were expressed as the mean±standard deviation. The significance of the difference between the control and the drug treated

groups was analyzed by Dunnett's *t*-test; $p<0.01$ was considered significant.

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