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Pentacyclic Triterpenoids and Their Cytotoxicity from the Stem Bark of *Styrax japonica* S. et Z.

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The methylene chloride soluble fraction of MeOH extract from the stem bark of *Styrax japonica* S. et Z. (Styracaceae) showed significant cytotoxicity by SRB method against five human tumor cell lines. Four known pentacyclic triterpenoids, oleanolic aldehyde acetate (**1**), erythrodiol-3-acetate (**2**), euphorginol (**3**), and anhydrosophoradiol-3-acetate (**4**) were isolated by activity-guided fractionation. Their structures were determined by chemical and spectral analysis. Compounds **1-4** were isolated from *S. japonica* for the first time.

Key words: *Styrax japonica*, Styracaceae, Stem bark, Pentacyclic triterpenoids, Cytotoxicity

INTRODUCTION

Styrax japonica S. et Z. is a member of the Styracaceae family, a shrub found in Central America, Mexico, and the Mediterranean region including West and South Anatolia (Davis, 1972). The resin from the species has been used in traditional medicine to treat inflammatory diseases (Costa, 1968) and also used by Romans, Egyptians, Phoenicians and Ionians as incense and in therapeutics (Vardar and Oflas, 1973).

Chemical studies on seeds and leaves of several *Styrax* species have revealed them to be a rich source of benzofurans, benzofuran esters, benzofuran glycosides (Akgul and Anil, 2003; Anil, 1980; Pauletti *et al.*, 2000; Takanashi and Takizawa, 1988), and several sapogenins (Anil, 1979; Kitagawa *et al.*, 1975; Kitagawa *et al.*, 1983). But, phytochemical and biological studies of the stem bark of *Styrax* species have not been reported.

In an ongoing investigation into biologically active compounds from natural products, a methylene chloride soluble fraction of *S. japonica* showed significant cell cytotoxicity *in vitro*. By means of an activity-guided column chromatographies, oleanolic aldehyde acetate (**1**), erythrodiol-3-acetate (**2**), euphorginol (**3**), and anhydrosophoradiol-3-acetate (**4**) were isolated. Four pentacyclic triterpenoids have been isolated from this plant for the first time. This paper reports herein the isolation, characterization, and cytotoxicity of four isolates against A549, SK-OV-3, SK-MEL-2, MES-SA, and HCT-15 human tumor cell lines for the first time.

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MATERIALS AND METHODS

General procedure

The melting point was obtained with a Fisher Scientific melting point apparatus and uncorrected. The EI-MS (70 eV) spectra were determined using a JEOL JMS-AX 505H. NMR spectra were recorded on a Varian Unity Inova 500, and JEOL JNM-LA 300 spectrometer. Chemical shifts were expressed in parts per million (ppm) relative to TMS as an internal standard, and coupling constants (*J*) were given in hertz. TLC was carried out on precoated Silica gel 60 F_{254} (Merck, art. 5715) and RP-18 F_{254}s (Merck, art. 15389) plates. Column chromatography was carried out Silica gel 60 (Merck, 40-63 and 63-200 μm) and Sephadex LH-20 (Sigma, 25-100 μm).

Plant material

The stem bark of *S. japonica* was collected from Jogyesan, Suncheon, Chonnam, Korea, in September 2002. Voucher specimens were deposited in the Herbarium of College of Pharmacy, Chosun University, Korea (CSU-964-17).

Extraction and isolation

The air-dried stem bark of *S. japonica* (654 g) was cut and extracted with methanol for 4 h (×3) at 70°C. The MeOH extract (120.3 g) was suspended in water and then partitioned by methylene chloride, ethyl acetate, and *n*-butanol in turn. The CH_2Cl_2 fraction (2 g) was subjected to column chromatography over a silica gel (210 g, 3.8×68 cm) eluting with *n*-hexane-acetone (100:1→2.5:1, v/v) and CH_2Cl_2-MeOH (50:1→1:1, v/v) gradient system. Fractions were combined based on their TLC pattern to yield subfraction designated as C1-C7. Subfraction C1 (350 mg), C2 (180 mg), and C3 (230 mg) were recrystallized from *n*-hexane to afford compound **1** (150 mg), **2** (80 mg), and **3** (100 mg), respectively. Subfraction C4 (190 mg) was further purified by column chromatography over a silica gel (135 g, 2.3 × 55 cm) eluting with *n*-hexane-acetone (10:1, v/v) isocratic system to afford seven subfraction (C4.1-C4.7). Among these subfractions, C4.1 (71.7 mg) and C4.2 (52.7 mg) were combined and repeated column chromatography on Sephadex LH-20 (50 g, 1.8 × 45 cm; MeOH-H_2O=83:17, v/v) and silica gel (100 g, 1.8 × 45 cm; *n*-hexane-acetone=25:1, v/v) to give compound **4** (55 mg).

Oleanolic aldehyde acetate (1)

White crystal from *n*-hexane; m.p. 216-218°C; EI-MS *m/z* (rel.int.%): 452 (9), 422 (10), 407 (14), 393 (10), 392 (8), 253 (10), 239 (80), 211 (100), 210 (90), 195 (99), 181 (55), 109 (60); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ : 9.40 (1H, s, C<u>H</u>O), 5.34 (1H, t, *J*=3.5 Hz, H-12), 4.49 (1H, dd, *J*=9.6, 5.0 Hz, H-3), 2.63 (1H, br dd, *J*=13.5, 6.7 Hz, H-18), 2.04 (3H, s, OCOC<u>H</u>$_3$), 1.98 (1H, dt, *J*=14.0 Hz, H-11), 1.88 (2H, m, H-2, 22), 1.45 (1H, dd, *J*=13.5, 6.5 Hz, H-9), 1.14 (3H, s, H-27), 0.93, 0.92 and 0.91 (each 3H, s, H-25, 29, 30), 0.86 (3H, s, H-24), 0.85 (3H, s, H-23), 0.74 (3H, s, H-26); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ : 207.48 (<u>C</u>HO, C-28), 170.98 (O<u>C</u>OCH$_3$), 142.98 (C-13), 123.15 (C-12), 80.87 (C-3), 55.27 (C-17), 49.07 (C-5), 47.47 (C-9), 45.54 (C-14), 41.68 (C-8), 39.56 (C-19), 38.15 (C-21), 33.14 (C-4), 33.04 (C-15), 32.67 (C-18), 30.62 (C-7, 10), 28.03 (C-1), 27.72 (C-29, 30), 26.70 (C-16), 25.48 (C-11), 23.50 (C-20), 23.40 (C-2), 22.08 (C-25), 21.28 (C-22), 18.17 (C-26), 17.00 and 16.67 (C-6, 27), 15.38 (OCO<u>C</u>H$_3$).

Erythrodiol-3-acetate (2)

White crystal from *n*-hexane; m.p. 243-244°C; EI-MS *m/z* (rel.int.%): 484 (5), 466 (5), 234 (23), 203 (100); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ : 5.19 (1H, t, *J*=3.5 Hz, H-12), 4.51 (1H, dd, *J*=9.0, 8.0 Hz, H-3), 3.55 and 3.22 (each 1H, d, *J*=11.0 Hz, H-28), 2.05 (3H, s, OCOC<u>H</u>$_3$), 1.16 (3H, s, H-27), 0.96 and 0.94 (each 3H, s, H-29, 30), 0.89, 0.88 and 0.86 (12H, s, H-23, 24, 25, 26); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ : 171.01 (O<u>C</u>OCH$_3$), 144.21 (C-13), 122.27 (C-12), 80.90 (C-3), 69.70 (C-28), 55.24 (C-5), 47.49 (C-9), 46.41 (C-19), 42.33 (C-18), 41.71 (C-14), 38.26 (C-17), 37.70 (C-1), 36.83 and 36.81 (C-8, 10), 34.08 (C-21), 33.17 (C-29), 32.50 (C-7), 31.02 (C-20), 30.94 (C-22), 28.01 (C-23), 25.88 (C-27), 25.52 (C-15), 23.57, 23.54 and 23.52 (C-2, 11, 30), 21.99 (C-16), 21.29 (C-25), 18.23 (C-6), 16.72 (C-24), 16.67 (C-26), 15.56 (OCO<u>C</u>H$_3$).

Euphorginol (3)

White crystal from *n*-hexane; m.p. 168-170°C; EI-MS *m/z* (rel.int.%): 426 (12), 411 (20), 408 (23), 393 (10), 302 (30), 287 (26), 269 (12), 204 (100), 189 (42); ^{1}H-NMR (pyridine-d_5, 500 MHz) δ : 5.65 (1H, dd, *J*=8.0, 4.0 Hz, H-15), 3.47 (1H, q, *J*=4.0 Hz, H-6β), 1.15, 1.10 and 1.03 (each 3H, s, H-25, 26, 29), 1.02 and 1.01 (each 3H, s, H-27, 30), 0.98 and 0.93 (9H, s, H-23, 24, 28); ^{13}C-NMR (pyridine-d_5, 125 MHz) δ : 158.91 (C-14), 117.57 (C-15), 78.64 (C-6), 56.45 (C-5), 50.01 (C-18), 49.59 (C-9), 42.14 (C-19), 39.87 (C-17), 38.75 (C-8), 38.40 (C-4), 38.26 (C-1), 37.36 (C-10, 13), 36.50 (C-16), 35.80 (C-7), 34.47 (C-21), 33.89 (C-29), 33.88 (C-12), 30.52 (C-26), 29.44 (C-28), 29.12 (C-20), 28.53 (C-23), 26.63 (C-2, 22, 27), 21.99 (C-3), 19.67 (C-30), 18.29 (C-24, 25), 16.89 (C-11).

Anhydrosophoradiol-3-acetate (4)

White crystal; m.p. 233-234°C; EI-MS *m/z* (rel.int.%): 466 (1), 216 (100); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ : 5.27 and 5.24 (3H, H-12, 21, 22), 4.49 (1H, t, *J*=10.2 Hz, H-3), 2.05 (3H, s, OCOC<u>H</u>$_3$), 1.25, 1.12, 1.07, 0.95, 0.94, 0.92, 0.87 and 0.85 (each 3H, s, H-23, 24, 25, 26, 27, 28, 29, 30); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ : 170.91 (O<u>C</u>OCH$_3$), 143.75 (C-13), 138.01 (C-21), 125.60 (C-22), 122.47 (C-12), 80.90 (C-3), 55.32 (C-5), 47.56 (C-9), 47.45 (C-18), 45.89 (C-14), 41.88 (C-19), 39.49 (C-8), 36.98 (C-16), 36.91 (C-17), 32.54 (C-15), 30.66 (C-4, 10), 29.71 (C-7), 28.07 (C-1), 28.04 (C-29, 30), 27.67 (C-20), 25.89 (C-11), 23.61 (C-28), 23.55 (C-2), 21.32 (C-23, 24), 21.20 (C-25), 18.18 (C-26), 17.12 (C-6), 17.03 (C-27), 16.70 (OCO<u>C</u>H$_3$).

Cytotoxicity assay

The *in vitro* cytotoxicity of compounds **1-4** was evaluated according to the standard procedures (Skehan *et al.*, 1990) of the NCI on cell line panel consisting of 5 lines, A549 (non small cell lungcarcinoma), SK-OV-3 (adenocarcinoma, ovary malignant ascites), SK-MEL-2 (malignant melanoma, metastasis to skin of thigh), MES-SA (uterine sarcoma), and HCT-15 (colon adenocarcinoma) by sulforhodamin B (SRB) assay (Monks *et al.*, 1991).

RESULTS AND DISCUSSION

Activity-guided column chromatographies of a CH_2Cl_2

soluble fraction from the stem bark of *S. japonica* led to the isolation of compounds **1-4** (Fig. 1).

Compound **1** was obtained as white crystal from *n*-hexane. The EI-MS spectrum showed an [M$^+$] ion at *m/z* 482. The ^{1}H-NMR spectrum exhibited an aldehyde proton at δ 9.40, an olefinic proton at δ 5.34 (1H, t, *J*=3.5 Hz, H-12), and an acetyl methyl proton at d 2.04. The ^{13}C-NMR spectrum showed an aldehyde carbon at δ 207.48 (C-28), an acetyl carbonyl carbon at δ 170.98, two olefinic carbons at δ 142.98 (C-13), and δ 123.15 (C-12), and an acetyl methyl carbon at δ 15.38. Based on the foregoing observations and a comparison of the data with the literature (Kircher, 1980; Nomura *et al.*, 1981; Samaraweera *et al.*, 1983), compound **1** was determined to be oleanolic aldehyde acetate (3β-acetoxyolean-12-en-28-al).

Compound **2** was obtained as white crystal from *n*-hexane. The EI-MS of **2** showed an [M$^+$] ion at *m/z* 484. The ^{1}H-NMR and ^{13}C-NMR spectra were very similar to those of **1** except for the presence of three signals (δ_H 3.55 and 3.22, each 1H, d, *J*=11.0 Hz, H-28; δ_C 69.70, C-28). Based on the NMR spectral evidences, and a comparison of the data with the literature (Nomura *et al.*, 1981), compound **2** was determined to be erythrodiol-3-acetate (3β-acetoxy-28-hydroxyolean-12-ene).

Compound **3** was obtained as white crystal. The EI-MS of **3** showed an [M$^+$] ion at *m/z* 426. The ^{1}H-NMR spectrum of **3** exhibited eight methyl groups at δ 1.15, 1.10, 1.03, 1.02, 1.01, 0.98, and 0.93 (6H). One double of doublets at δ 5.65 (1H, dd, *J*=8.0, 4.0 Hz, H-15) were assigned to be olefinic proton while another one proton quintet at δ 3.47 (1H, q, *J*=4.0 Hz, H-6β) could be attributed to a carbinylic proton. The ^{13}C-NMR spectrum showed thirty carbon signals. Eight methyl carbons appeared at δ 33.89, 30.52, 29.44, 28.53, 26.63, 19.67, and 18.29 (C-29, 26, 28, 23, 27, 30, 24 and 25, respectively). Two olefinic carbons appeared at δ 158.91 (C-14), and δ 117.57 (C-15), and one oxygenated carbon at δ 78.64, respectively. Based on the NMR spectral evidences, and a comparison of the data with the literature (Rasool *et al.*, 1989), compound **3** was determined to be euphorginol (6α-hydroxytaraxer-14-ene).

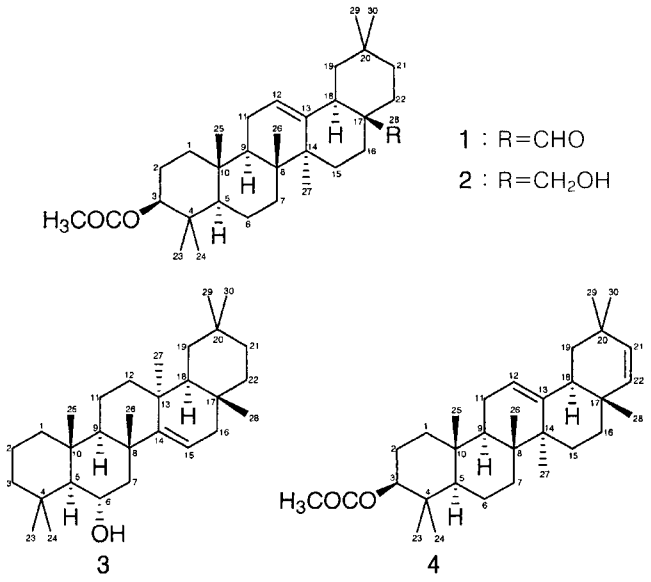

Fig. 1. *Chemical structures of compounds* **1-4** *from S. japonica.*

Table I. Cytotoxicity of compounds **1-4** from *S. japonica.*

Compounds	IC_{50} (μg/mL)*				
	A549	SK-OV-3	SK-MEL-2	MES-SA	HCT-15
1	5.16	9.86	9.78	5.07	6.86
2	32.19	51.95	49.53	42.44	31.68
3	>100	>100	>100	>100	>100
4	3.42	7.81	7.65	7.37	4.07
Doxorubicin**	0.18	0.27	0.21	0.09	0.69

*IC_{50} value of compound against each cancer cell line, which was defined as a concentration (μg/mL) that caused 50% inhibition of cell growth *in vitro*
**Used as a positive control

Compound **4** was obtained as white crystal. The EI-MS of **4** showed an [M$^+$] ion at *m/z* 466. The ^{1}H-NMR and ^{13}C-NMR spectra of **4** were very similar to those of **2**. The main differences were the presence of δ 138.01 (C-21), and 125.60 (C-22), instead of δ 34.08, and 30.94 of **2** in ^{13}C-NMR spectrum, which can be assigned to be olefinic carbons. Based on the analysis of the ^{1}H-NMR and ^{13}C-NMR, and a comparison of the data with the literature (Kitagawa *et al.*, 1983), compound **4** was determined to be anhydrosophoradiol-3-acetate (3β-acetoxyolean-12, 21-diene). Compounds **1-4** have been isolated from this plant for the first time.

The *in vitro* cytotoxicity of isolates against cultured five human cancer cell lines, A549, SK-OV-3, SK-MEL-2, MES-SA, and HCT-15 was evaluated by sulforhodamine B (SRB) assay. The results were summarized in Table I. Among them, compounds **1** and **4** exhibited potent cytotoxicity against five tumor cell lines (IC_{50} : 5.07~9.86, and 3.42~7.81 mg/mL, respectively), and compound **2** exhibited marginal activity (IC_{50} : 31.68~51.95 μg/mL).

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