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A Cytotoxic Secocycloartenoid from *Abies koreana*

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Two triterpenoids, 24-methylene-3,4-seco-cycloart-4(28)-en-3-oic acid (**1**) and 3-oxo-9β-lanosta-7,22*Z*,24-trien-26,23-olide (**6**) were isolated from *Abies koreana*, together with β-sitosterol (**2**), maltol (**3**), β-sitosterol-*O*-β-D-glucoside (**4**), and hexacosylferulate (**5**). The structures of the compounds were established based on the spectroscopic data. The cytotoxic activities of triterpenoids have been evaluated using the sulforhodamine B (SRB) method. Compound **1** showed moderate cytotoxicities against human lung carcinoma (A549), ovarian carcinoma (SK-OV-3), malignant melanoma (SK-MEL-2), and colon carcinoma (HCT-15) cell lines.

Key words: *Abies koreana*, Pinaceae, 24-Methylene-3,4-seco-cycloart-4(28)-en-3-oic acid, 3-Oxo-9β-lanosta-7,22*Z*,24-trien-26,23-olide, Cytotoxicity

INTRODUCTION

Abies koreana Wilson (Korean name: Kusangnamu) (Pinaceae) is a tall evergreen tree grown indigenously at 500-1000 meters above sea level on the high mountains including Mt. Chiri and Mt. Halla in southern Korea (Kim, 1996). Faurie and his colleagues were the first to collect this tree on Mt. Halla in 1909, and thereafter it has been named *Abies koreana* by Wilson from the results of a plant survey around Mt. Chiri and Cheju-do (Kim *et al.*, 1994a). Several species of the plant genus *Abies* have been used as folk medicine against cold, stomachache, indigestion, vascular, pulmonary, and venereal diseases (Yesilada *et al.*, 1995; Fujita *et al.*, 1995), and have shown the antibacterial (Janssen *et al.*, 1986; Richardson *et al.*, 1992; Bagci *et al.*, 1996), antifungal (Maruzzella *et al.*, 1959), antiinflammatory, and antiulcer activities (Singh *et al.*, 1998). Although several monoterpenes and lignans have been isolated from this plant (Kim *et al.*, 1994a; Kim *et al.*, 1994b), *A. koreana* has not been investigated in detail on phytochemical analysis or biological evaluation. The components of the wood as well as leaves, bark, seeds and resin have been studied in other species of *Abies* grown in Korea (Li *et al.*, 1982; Kaneko *et al.*, 1985; Hasegawa *et al.*, 1987a; Hasegawa *et al.*, 1987b; Tanaka *et al.*, 1990; Tanaka *et al.*, 1991).

As a part of our research program on the chemical composition and biological evaluation of this plant, we have isolated six components including 24-methylene-3,4-seco-cycloart-4(28)-en-3-oic acid and 3-oxo-9β-lanosta-7,22*Z*,24-trien-26,23-olide from its leaves and branches. In the present investigation we report the isolation and identification of two triterpenenoids of *A. koreana* and the cytotoxicity of these compounds against a panel of human tumor cell lines.

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MATERIALS AND METHODS

General experimental procedures

^{1}H (500 MHz), ^{13}C (125 MHz) and 2D NMR spectra were obtained on Varian Unity INOVA 500 spectrometer. Chemical shifts were expressed in parts per million (ppm) relative to TMS as the internal standard, and coupling constants (*J*) were given in Hertz. MS were determined on a Micromass Platform II GC/LC mass spectrometer. IR spectra were obtained on a Jasco FT/IR-300E spectrometer and UV spectra were recorded on a Jasco V-530 UV/Vis spectrophotometer. TLC was carried out on Merck Silica gel F_{254}-precoated glass plates and RP-18 F_{254S} plates. MPLC was carried out with Silica gel 60 (230-

400 mesh) and Lobar® Größe A (240-10) Lichroprep® RP-18 (40-63 μm) (Merck).

Plant materials

The leaves and stems of *Abies koreana* were collected in November, 1999, in Mt. Chiri, Korea. The plant material was identified by Prof. KiHwan Bae of the College of Pharmacy, Chungnam National University. Voucher specimens have been deposited in the herbarium of the College of Pharmacy, Chonnam National University (CNUNPDDL-99-001).

Extraction and isolation

The dried leaves of *A. koreana* (2.7 kg) were extracted at room temperature with MeOH to give a MeOH extract (813 g). This was diluted with H_2O, and extracted with hexane, EtOAc, and BuOH, successively. The hexane extract (28 g) was subjected to silica gel column chromatography (CC) with a hexane-EtOAc gradient system to provide 11 fractions. Fraction 2 was purified to afford compound **1** (52 mg), and fraction 5 (2.4 g) was further subjected to MPLC with a hexane-Et_2O gradient system (10:1 →5:1) to give compound **2** (203 mg). Fraction 8 (2.0 g) was subjected to silica gel column chromatography with $CHCl_3$-acetone mixtures (15:1 →1:1) to give 9 fractions. Subfractions 2, 3 and 4 were combined (340 mg) and separated by MPLC with a hexane-Et_2O-$CHCl_3$ gradient system (50:3:2 →4:3:2), providing compound **3** (140 mg). The EtOAc extract (288 g) was chromatographed on silica gel with hexane-EtOAc (20:1 →EtOAc 100%) and EtOAc-MeOH mixtures (1:5) to provide 7 fractions. Fraction 5 (51 g) was subjected to silica gel CC eluted with $CHCl_3$-MeOH (20:1 →1:5, MeOH 100%) gave 9 fractions. Subfraction 4 (1.6 g) was further chromatographed on silica gel eluting with CH_2Cl_2:EtOH (20:1 →1:1) and recrystallized to afford compound **4** (66 mg).

Dried stems of *A. koreana* (2.8 kg) were extracted repeatedly with MeOH. The concentrated MeOH extract (294 g) was dissolved in H_2O, and partitioned with hexane and EtOAc, successively. The EtOAc extract of stems (150 g) was submitted to silica gel column chromatography, eluting with hexane-EtOAc and EtOAc-MeOH mixtures provide to 7 fractions. Fraction 2 (30 g) was subjected to MPLC eluting with a hexane-EtOAc gradient system (20:1 →1:5) to give 14 subfractions. The purification of subfraction 2 afforded compound **5** (7.4 mg). Subfraction 4 (320 mg) was chromatographed with Lobar® Größe A (240-10) Lichroprep RP-18 (40-63 m) (Merck) using EtOH 45% solution to give compound **6** (18 mg).

24-Methylene-3,4-seco-cycloart-4(28)-en-3-oic acid (1): White amorphous powder, MS *m/z*: 455 $[M+1]^+$. IR ν_{max} cm^{-1}: 2919 (CH), 1705 (C=O), 1641 (C=C). ^1H NMR ($CDCl_3$): δ 2.06 (1H, *m*, H-1), 1.38 (1H, *m*, H-1), 2.54 (1H, *m*, H-2), 2.30 (1H, *m*, H-2), 2.43 (1H, *dd*, *J*=12, 5 Hz, H-5), 1.51 (1H, *m*, H-6), 1.08 (1H, *m*, H-6), 1.31 (1H, *m*, H-7), 1.10 (1H, *m*, H-7), 1.56 (1H, *m*, H-8), 2.10 (1H, *m*, H-11), 1.26 (1H, *m*, H-11), 1.65 (2H, *m*, H-12), 1.30 (2H, *m*, H-15), 1.93 (1H, *m*, H-16), 1.29 (1H, *m*, H-16), 1.60 (1H, *m*, H-17), 0.96 (3H, *s*, H-18), 0.41 (1H, *d*, *J*=4 Hz, H-19), 0.74 (1H, *d*, *J*=4 Hz, H-19), 1.41 (1H, *m*, H-20), 0.90 (3H, *d*, *J*=6.5 Hz, H-21), 1.58 (1H, *m*, H-22), 1.14 (1H, *m*, H-22), 2.12 (1H, *m*, H-23), 1.89 (1H, *m*, H-23), 2.24 (1H, *septet*, *J*=6.8 Hz, H-25), 1.03 (3H, *d*, *J*=6.8 Hz, H-26), 1.03 (3H, *d*, *J*=6.8 Hz, H-27), 4.74 (1H, *brs*, H-28), 4.82 (1H, *brs*, H-28), 1.68 (3H, *s*, H-29), 0.94 (3H, *s*, H-30), 4.67 (1H, *brs*, H-31), 4.72 (1H, *brs*, H-31). ^{13}C NMR: δ 29.0 (C-1), 31.7 (C-2), 180.5 (C-3), 149.7 (C-4), 46.1 (C-5), 28.0 (C-6), 25.2 (C-7), 47.9 (C-8), 21.6 (C-9), 27.2 (C-10), 27.2 (C-11), 33.3 (C-12), 45.4 (C-13), 49.2 (C-14), 35.9 (C-15), 28.3 (C-16), 52.5 (C-17), 18.3 (C-18), 30.2 (C-19), 36.4 (C-20), 18.5 (C-21), 35.2 (C-22), 31.6 (C-23), 157.1 (C-24), 34.0 (C-25), 22.2 (C-26), 21.9 (C-27), 111.8 (C-28), 20.2 (C-29), 19.6 (C-30), 106.19 (C-31).

β-Sitosterol (2): White needle, MS *m/z*: 414 $[M]^+$, IR ν_{max} cm^{-1}: 3432 (OH), 1464, 1381, 1063.

Maltol (3-Hydroxy-2-methyl-4*H*-pyran-4-one) (3): Pale red needle. MS *m/z*: 126 $[M]^+$. IR ν_{max} cm^{-1}: 3260 (OH), 1650, 1615, 1620 and 1560. ^1H NMR ($CDCl_3$): δ 7.24 (OH, *brs*, H-3), 6.43 (1H, *d*, *J*=5.5 Hz, H-5), 7.72 (1H, *d*, *J*=5.5 Hz, H-6) and 2.37 (3H, *s*, O-CH_3). ^{13}C NMR: δ 149.3 (C-2), 143.2 (C-3), 173.0 (C-4), 113.1 (C-5), 154.1 (C-6), 14.2 (O-CH_3).

β-Sitosterol-3-*O*-β-D-glucoside (4): IR ν_{max} cm^{-1}: 3435 (OH), 1654. ^1H NMR (pyridine-d_5): δ 5.36 (1H, *m*, H-6), 5.02 (1H, *d*, *J*=7.5 Hz, H-1'). ^{13}C NMR: δ 37.5 (C-1), 30.3 (C-2), 78.6 (C-3), 40.0 (C-4), 141.0 (C-5), 121.9 (C-6), 32.2 (C-7), 31.2 (C-8), 50.4 (C-9), 37.0 (C-10), 21.3 (C-11), 39.4 (C-12), 42.5 (C-13), 56.9 (C-14), 24.5 (C-15), 28,6 (C-16), 56.3 (C-17), 12.0 (C-18), 19.3 (C-19), 36.4 (C-20), 19.1 (C-21), 34.2 (C-22), 26,4 (C-23), 46.1 (C-24), 29.5 (C-25), 20.2 (C-26), 19.5 (C-27), 23.4 (C-28), 12.2 (C-29), 102.6 (C-1') 75.4 (C-2'), 78.1 (C-3'), 71.7 (C-4'), 78.5 (C-5'), 62.9 (C-6').

Hexacosylferulate (5): White amorphous powder. MS *m/z:* 558 $[M]^+$, 544, 530, 516, 502, 194, 177, 150 and 137. ^1H NMR ($CDCl_3$): δ 7.04 (1H, d, *J*=1.5 Hz, H-2), 5.85 (OH, *brs*), 6.92 (1H, *d*, *J*=8 Hz, H-5), 7.07 (1H, *dd*, *J*=8, 1.5 Hz, H-6), 3.93 (3H, *s*, H-7), 7.61 (1H, *d*, *J*=16 Hz, H-8), 6.29 (1H, *d*, *J*=16 Hz, H-9), 4.19 (2H, *t*, *J*=7 Hz, H-11), 1.70 (2H, *quint*, *J*=7 Hz, H-12), 1.39 (2H, *m*, H-13), 1.25-1.26, 0.88 (3H, *t*, *J*=7 Hz, H-36). ^{13}C NMR : δ 127.1 (C-1), 109.2 (C-2), 146.7 (C-3), 147.9 (C-4), 114.7 (C-5), 123.0 (C-6), 55.9 (C-7), 144.6 (C-8), 115.7

(C-9), 167.4 (C-10), 64.6 (C-11), 28.8 (C-12), 26.0 (C-13), 29.3-29.7, 31.9 (C-34), 22.7 (C-35), 14.1 (C-36)

3-Oxo-9-lanosta-7,22Z,24-trien-26,23-olide (6): White amorphous powder. MS *m/z*: $[M]^+$ 450. IR ν_{max} cm^{-1}: 2948, 1775, 1754, 1459, 1102, 1059. UV λ_{max} (nm): 279. ^1H NMR ($CDCl_3$): δ 1.62 (1H, *m*, H-1), 1.71 (1H, *m*, H-1), 2.50 (2H, *dd*, *J*=9.3, 6.3 Hz, H-2), 1.41 (1H, *dd*, *J*=12, 3 Hz, H-5), 1.82 (1H, *ddd*, *J*=12, 3, 2.5 Hz, H-6), 1.91 (1H, *m*, H-6), 5.64 (1H, *dt*, *J*=7.5, 3 Hz, H-7), 2.23 (1H, *m*, H-9), 1.65 (1H, *m*, H-11), 1.64 (1H, *m*, H-12), 1.85 (1H, *m*, H-12), 1.37 (1H, *m*, H-15), 1.58 (1H, *m*, H-15), 1.29 (2H, *m*, H-16), 1.68 (1H, *m*, H-17), 0.85 (3H, *s*, H-18), 1.00 (1H, *s*, H-19), 2.89 (1H, *ddq*, *J*=10.5, 6.5, 6.5 Hz, H-20), 1.05 (3H, *d*, *J*=6.5 Hz, H-21), 4.98 (1H, *d*, *J*=10.5 Hz, H-22), 6.97 (1H, *d*, *J*=1.0 Hz, H-24), 1.99 (3H, *d*, *J*=1.0 Hz, H-27), 1.10 (3H, *s*, H-28), 1.09 (3H, *s*, H-29), 1.02 (3H, *s*, H-30). ^{13}C NMR : δ 34.1 (C-1), 34.3 (C-2), 218.9 (C-3), 47.0 (C-4), 52.4 (C-5), 23.0 (C-6), 121.7 (C-7), 148.4 (C-8), 45.3 (C-9), 35.8 (C-10), 20.7 (C-11), 34.0 (C-12), 44.3 (C-13), 51.9 (C-14), 33.1 (C-15), 28.0 (C-16), 53.0 (C-17), 22.6 (C-18), 23.1 (C-19), 34.5 (C-20), 19.8 (C-21), 120.8 (C-22), 146.6 (C-23), 138.1 (C-24), 128.8 (C-25), 171.3 (C-26), 10.5 (C-27), 21.2 (C-28), 27.9 (C-29), 27.3 (C-30).

Cell lines

The human tumor cell line panel consisted of non small cell lung carcinoma (A549), ovarian adenocarcinoma (SK-OV-3), malignant melanoma (SK-MEL-2), and colon adenocarcinoma (HCT-15). All stock cultures were grown in T-75 flasks containing 20 mL of RPMI-1640 medium supplemented with 0.2% (w/v) sodium bicarbonate, 1% penicillin-streptomycin, and 10% (v/v) fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity test *in vitro*

Cellular viability was determined using the SRB method, currently adopted in the NCI(USA)'s *in vitro* anti-cancer drug screening (Skehan *et al.*, 1990). Freshly trypsinized tumor cell suspensions were seeded in 96-well microtiter plates and preincubated at 37°C for 24 h. Tumor cells were further incubated for 48 h after treatment with the test materials. At the termination of incubation, attached cells were fixed with cold 10% trichloroacetic acid (TCA) and then stained with 0.4% (w/v) sulforhodamine B (SRB). The absorbance at 520 nm was measured using a microculture plate reader after solubilizing the bound dye with 10 mM Trizma base. All samples were performed in triplicate, and the ED_{50} values (μg/mL), the concentration that caused 50% inhibition of cell proliferation, were interpolated from dose-response data.

RESULTS AND DISCUSSION

Fractionation and separation of the extract of *A. koreana* led to the isolation of six major compounds.

Compounds **2** ($C_{29}H_{50}O$), **3** ($C_6H_6O_3$), **4** ($C_{35}H_{60}O_6$) and **5** ($C_{36}H_{62}O_4$) were identified as β-sitosterol, maltol, β-sitosterol-3-*O*-β-D-glucoside and hexacosylferulate, respectively, by direct comparison with reported spectroscopic data (Goad *et al.*, 1997; Aoyagi *et al.*, 1974; Chang *et al.*, 1981; Chatterjee *et al.*, 1977; Tanaka *et al.*, 1990).

Compound **1** was isolated from hexane extract of leaves with column chromatography. The number of carbons and carbon multiplicities were determined by HSQC, and the signals in the ^{13}C NMR (δ 180.5) and IR spectra (ν_{max} 1705 cm^{-1}) showed the presence of a carboxylic acid. Also, **1** showed in its EI mass spectrum a $[M+1]^+$ at *m/z* 455 ($C_{31}H_{50}O_2$) and the seven degrees of unsaturation. The ^1H NMR spectrum displayed signals for a cyclopropane methylene at δ 0.41 and 0.74 (*d*, *J*=4 Hz, H-19), two tertiary methyls at δ 0.94 (H-30) and 0.96 (H-18), three secondary methyls at δ 0.90 (*d*, *J*=6.5 Hz, H-21) and two methyls at δ 1.03 (*d*, *J*=6.8 Hz, H-26 and H-27) and one vinyl methyl at δ 1.68 (H-29). Four broad singlets at δ 4.74 (1H, *brs*, H-28), 4.82 (1H, *brs*, H-28), 4.67 (1H, *brs*, H-31) and 4.72 (1H, *brs*, H-31) were ascribed to two sp^2 exomethylene groups. In order to obtain more information regarding the skeleton of **1**, HMBC experiments were performed. In this experiment, the protons of the C-18 methyl group showed correlation with C-12 and C-13, while the C-30 methyl protons exhibited long range coupling with C-8, C-14 and C-15. Correlation peaks were observed between the C-31 exomethylene protons and carbon peaks C-23 through C-27. The other important correlations were also shown between the vinyl methyl at δ 1.68 (H-29) and the carbons C-4 and C-28. On the basis of the spectroscopic evidences, the structure of **1** ($C_{31}H_{50}O_2$) was assigned as 24-methylene-3,4-seco-cycloart-4(28)-en-3-oic acid.

Chromatography of the stem EtOAc extract from *A. koreana* led to the isolation of compound **6**. It was tested positive with Liebermann-Burchard reagent, and the presence of a conjugated -lactone carbonyl function (ν_{max} 1754 cm^{-1}) was indicated in the IR spectrum. The ^1H and ^{13}C NMR of **6** suggested the presence of a vinyl methyl at δ 1.99 (H-27) and 10.5 (C-27), a secondary methyl at δ 1.05 (*d*, *J*=6.5 Hz, H-21), and five tertiary methyl groups at δ 0.85 (H-18), 1.00 (H-19), 1.02 (H-30), 1.09 (H-29), and 1.10 (H-28). Also, the ^{13}C NMR spectrum exhibited the signals for a saturated six-membered ring ketone at δ 218.9 and three trisubstituted double bonds at δ 121.7 (=CH-), 148.3 (=C<), 120.8 (=CH-), 146.6 (=C<), 138.1 (=CH-) and 128.8 (=C<), in addition to the lactone carbonyl (δ 171.3). Based on the HSQC,

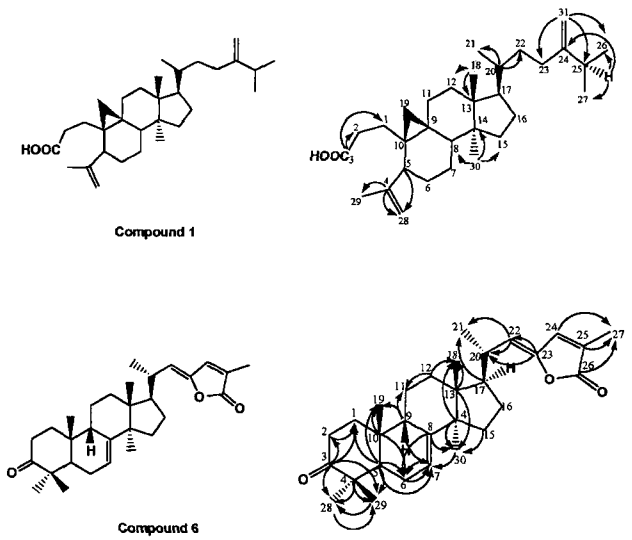

Fig. 1. Structures of compounds **1** and **6**, and their HMBC correlations

HMBC and NOESY spectral data, the exact structure of **6** was established. Thus, the vinyl methyl function (δ 1.99, 3H, *d*, *J*=1 Hz), next to the lactone carbonyl, showed allylic coupling with the olefinic hydrogen (δ 6.97, 1H, *d*, *J*=1 Hz) at C-24. In the HMBC spectrum, the carbon signals of C-8 and C-9 correlated with the proton signals of C-30 and C-19, respectively. Also, the carbon signals at C-3, C-13 and C-14 showed correlation with the proton signals of C-28 and C-29, C-18 and C-30, and C-18 and C-30, respectively. The stereochemistry at C-23 position has been shown to be in *Z* configuration. It was proved that the olefinic proton at C-22 showed correlation with C-24 proton in the NOESY experiment. Consequently, the structure of **6** ($C_{30}H_{42}O_3$) was confirmed to be 3-oxo-9β-lanosta-7,22Z,24-trien-26,23-olide.

These two triterpenoids, **1** and **6** have been first isolated from *Abies koreana*, although they had been reported earlier from *Abies sibirica* (Raldugin *et al.*, 1987; Raldugin *et al.*, 1989). Fig. 1 shows the structures of these compounds and some important correlations observed in long range optimized HMBC experiment.

The cytotoxicities of six compounds from *A. koreana* were evaluated *in vitro* against cultured human tumor cell lines, including A549, SK-OV-3, SK-MEL-2, and HCT-15. Among them, compounds **1** and **6** exhibited moderate cytotoxicity against four human cancer cell lines. The results are shown in Table I. In the test, **1** displayed general cytotoxic activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 tumor cell lines, with ED_{50} values of 2.93, 3.01, 3.18 and 2.96 g/mL, respectively, whereas compound **6** showed marginal activity.

Table I. Cytotoxicity (ED_{50}) of compounds **1** and **6** against human tumor cell lines[a]

compound	A549	SK-OV-3	SK-MEL-2	HCT-15
1	2.93	3.01	3.18	2.96
6	56.02	43.56	25.11	65.54
doxorubicin	0.09	0.05	0.07	0.06

[a]Cytotoxicity expressed as ED_{50} in μg/mL for each cell line is the concentration of a compound that causes a 50% reduction in absorbance at 520 nm relative to untreated cells using SRB assay.

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