Cytotoxic Activity of Ergosta-4,6,8(14),22-tetraen-3-one from the Sclerotia of *Polyporus umbellatus*

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Polyporus umbellatus (also called *Grifola umbellata*) is a mushroom causing a white rot in hardwoods. The sclerotia of *P. umbellata* are bumpy and rugged in shape and dark brown to black in color. They are used as a diuretic in Chinese medicine.[1] It was reported that the water extracts of the sclerotia of *P. umbellata* have diuretic effects and its methanol extracts have cytotoxic effects against human gastric cancer cell. However, the active components were unknown.[2] Erogostrol, erogosterol peroxide, glucan, and ergsta-7,22-dien-3-ol have been identified from sclerotia of *P. umbellatus*.[3-4] However there is no report on the cytotoxic activity of the compounds isolated from the sclerotia of *P. umbellatus*.

In this study, we report the isolation and identification of cytotoxic compound from the sclerotia of *P. umbellatus*. Its structure was elucidated by FT-IR, 1-D and 2-D NMR spectroscopy and we finally demonstrated the cytotoxic activities of the compound.

Experimental Section

Materials. The sclerotia of *P. umbellatus* were collected from the market at Seoul and were identified by Dr. K. H. Ka (Korea Forest Research Institute, Korea). A voucher specimen was deposited at the Korea Forest Research Institute, Seoul, Korea.

Instrumentals. MPs: uncorr. IR: KBr disc. ^{1}H and ^{13}C NMR in $CDCl_3$ with TMS as internal standard. NMR spectra were obtained using a Varian UI 500 spectrometer at the operating frequency of 500 MHz (^{1}H) and 125 MHz (^{13}C) at Korea Basic Science Institute in Seoul. EI-MS: JEOL JMS-600W, direct inlet at 70 eV.

Extraction and purification. Air-dried and powdered sclerotia of *P. umbellatus* (6 kg) were extracted with EtOH at room temperature for 72 h. The EtOH extract was partitioned between water and *n*-hexane. The hexane soluble fraction was subjected to column chromatography on silica gel column (62.5 × 3.2 cm) eluted with benzene-EtOAc (70 : 1, v/v) to yield 3 sets of fraction (G1-G3). Fraction G2 was rechromatographed on Sephadex LH-20 column with MeOH-H_2O (1 : 1, v/v) to give 3 subfractions (G2-1-G2-3). Compound 1 was obtained from fraction G2-2 (43 mg) as pale yellow amorphous powder, EI-MS *m/z*: 392 (M+), 375, 349, 293, 267 (base ion), 253, 214, 173, 69. IR (KBr) ν_{max} 2055, 2850, 1665, 1585, 1460, 970 cm^{-1}, ^{1}H-NMR (500 MHz, $CDCl_3$): δ0.80 (3H, d, J = 6.7 Hz, H_3-26), 0.82 (3H, d, J = 6.7 Hz, H_3-27), 0.90 (3H, d, J = 6.7 Hz, H_3-28), 0.93 (3H, s, H_3-18), 0.97 (3H, s, H_3-19), 1.03 (3H, d, J = 6.7 Hz, H_3-21), 1.23 (1H, m, H-17), 1.26 (1H, m, H-12), 1.45 (1H, m, H-24), 1.46 (1H, m, H-16), 1.59 (1H, m, H-11), 1.67 (1H, m, H-11), 1.77 (1H, m, H-16), 1.78 (1H, m, H-2), 1.83 (1H, m, H-25), 1.98 (1H, m, H-1), 2.08 (2H, m, H-9, 12), 2.12 (1H, m, H-20), 2.35 (2H, m, H-2, 15), 2.45 (1H, m, H-15), 2.51 (1H, m, H-1), 5.18 (1H, dd, J = 7.6, 15.2 Hz, H-22), 5.23 (1H, dd, J= 7.6, 15.2 Hz, H-23), 5.71 (1H, s, H-4), 6.01 (1H, d, J = 9.4 Hz, H-6), 6.58 (1H, d, J = 9.4 Hz, H-7). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 16.64 (q, C-19), 17.62 (q, C-28), 18.94 (q, C-18), 18.96 (t, C-11), 19.65 (q, C-26), 19.97 (q, C-27), 21.21 (q, C-21), 25.36 (t, C-15), 27.70 (t, C-16), 33.07 (d, C-24), 34.10 (t, C-1), 34.12 (t, C-2), 35.57 (t, C-12), 36.75 (s, C-10), 39.27 (d, C-20), 42.86 (d, C-25), 43.98 (s, C-13), 44.31 (d, C-9), 55.68 (d, C-17), 122.96 (d, C-4), 124.40 (d, C-6), 124.44 (s, C-8), 132.52 (d, C-23), 134.03 (d, C-7), 134.99 (d, C-22), 156.11 (s, C-14), 164.44 (s, C-5), 199.57 (s, C-3).

HMBC correlations: H-1 → C-2/C-3/C-5/C-10, H-2 → C-1/C-3/C-10, H-4 → C-2/C-6/C-10, H-6 → C-3/C-4/C-8/C-10, H-7 → C-5/C-6/C-8/C-9, H-9 → C-11/C-14, H-11 → C-8/C-9/C-12/C-13, H-12 → C-9/C-11/C-13/C-14/C-17, H-15 → C-8/C-14/C-16, H-16 → C-13/C-14, H-17 → C-12/C-13/C-16/C-18/C-20/C-21/C-22, H-20 → C-17/C-21/C-26/C-28, H-25 → C-23/C-24/C-26/C-27/C-28, H_3-18 → C-12/C-13/C-14/C-17, H_3-19 → C-1/C-5/C-9/C-10, H_3-21 → C-17/ C-20/C-22, H_3-26 → C-24/C-25/C-27, H_3-27 → C-24/C-25/C-26, H_3-28 → C-23/C-24/C-25.

Antitumor assay. HT-29, HeLa 229, Hep3B, and AGS are human colon cancer cell line, human cervix cancer cell line, human liver cancer cell line, and human stomach cancer cell line, respectively and were obtained from Korean Cell Line Bank of Seoul National University. HT-29 and AGS were maintained in RPMI 1640 medium and HeLa 229 and Hep3B were maintained in DMEM medium, respectively, supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin). Cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air. Purified compound was dissolved in EtOH and diluted to 5, 10, 15, 20, 25, 30 and 35 μg/mL. For the growth inhibition, tumor cells (4 × 10^4 cells/well for HT-

29, 1×10^4 cells/well for Hep3B, and 5×10^3 cells/well for HeLa 229 and AGS) were cultured in 96-well culture plate in the presence or absence of the compound. After one day culture, cell growth inhibition was measured by XTT assay kit (Roche, Germany). Briefly, XTT mixing solution (XTT labeling reagent : electron coupling reagent, 5 : 1) was added to each well. After 6 hr reaction, optical density was measured by microplate reader (BIO-RAD) at 450 nm. Cytotoxic activity was measured by the following formula : Inhibition ration (%) = 100 × (OD_{450} of control − OD_{450} of test)/OD_{450} of control.

Results and Discussion

The structure of compound purified from the sclerotia of *P. umbellatus*. The structure was elucidated by spectral data such as IR, MS, ^{1}H- and ^{13}C-NMR spectra. Compound obtained from the hexane soluble part of the ethanol extract of sclerotia of *P. umbellatus* was isolated as yellow amorphous powder and showed molecular ion peak at *m/z* 392 $[M]^+$ and 267 $[M\text{-}C_9H_{17}]^+$ in the EI-MS spectrum. The ^{1}H-NMR spectrum showed the presence of two tertiary methyl groups at δ 0.93 (3H, s, H_3-18) and 0.97 (3H, s, H_3-19) and four secondary methyl groups at δ 0.80 (3H, d, J = 6.7 Hz, H_3-26), 0.82 (3H, d, J = 6.7 Hz, H_3-27), 0.90 (3H, d, J = 6.7 Hz, H_3-28), and 1.30 (3H, d, J = 6.7 Hz, H_3-21). A total of 28 carbons appeared in the ^{13}C-NMR spectrum, which induced six methyl, six methylene, ten methine and five tertiary carbons. The ^{13}C-NMR spectrum showed carbon signal of C=O at δ 199.57 (C-3) and four C=C at δ 122.96 (d, C-4), 164.44 (s, C-5), 124.40 (d, C-6), 134.03 (d, C-7), 124.44 (s, C-8), 156.11 (s, C-14), 134.99 (d, C-22), and 132.52 (d, C-23). Carbons with protons and their protons were precisely matched by the HMQC experiment. In the HMBC spectrum (Figure 1), major correlations were observed between H_3-19 and C-1, C-5, C-9, and C-10, between H-7 and C-5, C-6, C-8, and C-9, and between H_3-18 and C-12, C-13, C-14, and C-17. From the above evidence, the structure of compound was concluded to be ergosta-4,6,8(14),22-tetraen-3-one (ergone) (Figure 2). All the spectral data of this compound were in good agreement with those of Tanaka *et al*.[5]

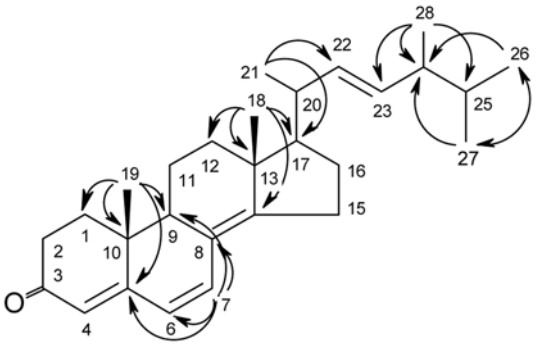

Figure 1. HMBC correlations for the compound isolated from the sclerotia of *P. umbellatus*.

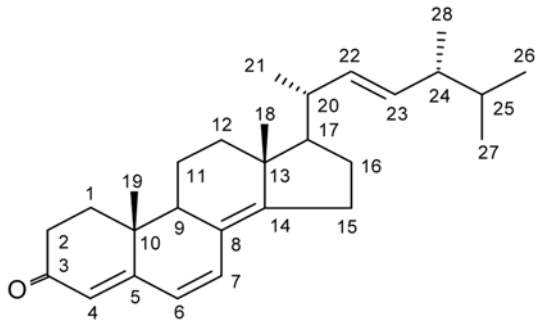

Figure 2. Chemical structure of compound isolated from the sclerotia of *P. umbellatus*.

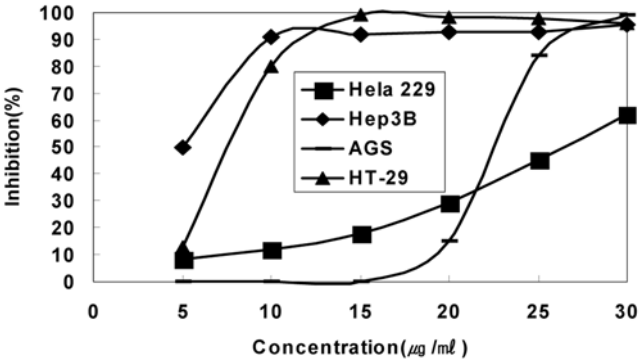

Figure 3. Growth inhibition of ergone on HT-29, HeLa 229, Hep3B and AGS.

Cytotoxic activities of ergone. Cytotoxic activities of the isolated compound against human cancer cell lines, HT-29 (colon cancer), HeLa 229 (cervix cancer), Hep3B (liver cancer), and AGS (stomach cancer), were compared one another. As shown in Figure 3, ergone inhibited all the cell lines as dose increased. In case of Hep3B and HT-29 cell line, maximal cytotoxic activities of ergone were achieved at the concentration of 10 and 15 μg/mL, respectively. However, the cytotoxic activities of ergone against Hela 229 and AGS were much weaker than those against Hep3B and HT-29 cell line.

Values of 50% inhibitory concentrations (IC_{50}) of ergone against Hep3B, HT-29, HeLa 229, and AGS were 5, 7.2, 26.3, and 22 μg/mL, respectively (Table 1). From the results above, cytotoxic activities of ergone against Hep3B and HT-29 were stronger than those against AGS and HeLa 229.

Table 1. IC_{50} values of ergone isolated from the sclerotia of *P. umbellatus* against various tumor cell lines

Cell line	Tumor type	IC_{50} (μg/mL)[a]
Hep3B	Hepatocellular carcinoma	5.0 ± 0.41 (12.7 uM)[b]
HT-29	Colorectal carcinoma	7.2 ± 0.17 (18.4 uM)
Hela229	Cerix carcinoma	26.3 ± 0.93 (67 uM)
AGS	Gastric carcinoma	22 ± 0.16 (56.1 uM)

[a]IC_{50} value was defined as a concentration (μg/mL) that caused 50% inhibition of cell proliferation in vitro and measured by XTT assay after one day culture. [b]Data are mean ± SD of four replications.

Ergone is a fungal metabolite derived from ergosterol. It has been previously isolated and identified from a sponge, *Dysidea herbacea*.[6] According to Yuan *et al.*,[7] ergone may be used as a marker component for the chemical standardization of Polyporus sclerotium. It was also reported that ergone has an anti-aldosteronic diuretic effect.[1]

Ergosterol peroxide isolated from *Paecilomyces tenuipes* and *Armillariella mellea* was active against various tumor cell lines and showed potent inhibition on lipid peroxidation.[8,9] Compared the structures of ergone and ergosterol peroxide, they are similar each other except the presence of epidioxy group on C_5 and C_8 and the absence of two double bonds on C_4, C_5 and C_8, C_{14} in ergosterol peroxide. Although the various biological activities such as antiviral, anti-inflammatory, and antitumor activities[10] of ergosterol peroxide have been investigated, such activities of ergone have not been reported. Cytotoxic activities of ergone against various tumor cell lines were evaluated in this study for the first time. Since only a few compounds, cordycepin, ergosterol peroxide, and acetoxyscirpenediol which have cytotoxic activities, were isolated from mushrooms, it is necessary to investigate and isolate cytotoxic compounds from different kinds of mushrooms.

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