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Secondary Metabolites of *Volvariella bombycina* and Their Inhibitory Effects on Melanogenesis

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Four compounds were isolated from the culture broth of *Volvariella bombycina* and they were identified as ergosta-4,6,8(14),22-tetraene-3-one (1), ergosterol peroxide (2), indole-3-carboxaldehyde (3), and indazole (4) by interpretation of spectroscopic data. Among them, compound 2 exhibited melanogenesis inhibitory effect in cultured B16 mouse melanoma cells.

Keywords: Melanogenesis, structure elucidation, *Volvariella bombycina*

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial and nontoxic medicines. They have been used in folk medicine throughout the world since ancient times. Many pharmaceutical secondary metabolites have been isolated from medicinal mushrooms and distributed worldwide [8]. *Volvariella bombycina* (Schaeff.: Fr.), a commonly available edible mushroom with a dry yellowish to white fibrillose cap, belongs to the well-known Pluteaceae family and grows abundantly in South East Asia. Biological activities such as antioxidant and antitumor activities of *V. bombycina* have been reported [1, 11]. However, there are limited phytochemical studies on *V. bombycina* so far, except that polysaccharide components have been isolated from it [3]. Previously, bioassay-guided fractionation on anti-elastase activity led to the isolation of a novel cuparene-type sesquiterpenoid, isodeoxyhelicobasidin, from this fungus [19]. In the present paper, we describe the isolation, structure elucidation, and melanogenesis inhibitory activities of two steroids and two alkaloids from the culture broth of *V. bombycina*.

The strain of *V. bombycina* (MKACC 53745) was provided by the Korea Agricultural Culture Collection of the National Institute of Agricultural Biotechnology, Suwon, Republic of Korea. The producing strain of *V. bombycina* pregrown on a PDA slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of YPS medium consisting of 2% glucose, 0.5% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 6.6), and cultured on a rotary shaker (153 rpm) for 7 days at 27°C. For fermentation, the seed culture was aseptically transferred into a 5-l jar fermenter containing 3.5 l of the above medium, and cultivation was carried out at 28°C for 7 days with aeration of 2 l/min and agitation of 250 rpm [15]. The collected mycelial cake from the whole fermented broth (10 l) was extracted with acetone and the extract was concentrated *in vacuo* to an aqueous solution, which was then extracted with an equal volume of EtOAc three times. The EtOAc layer (5.2 g) was loaded on a silica gel column and eluted with CH_2Cl_2/MeOH in a gradient mode (20:1→1:1) to give 12 fractions (VB1–12). The fraction VB4 (700 mg) was rechromatographed over a silica gel column and eluted with *n*-hexane/EtOAc (9:1→1:1) to give 3 fractions (VB41–43), and then the subfraction VB42 (100 mg) was further purified by preparative HPLC (YMC-Pack ODS column, 20×250 mm; MeOH:H_2O=9:1; flow rate, 4.0 ml/min) to afford compound **1** (R_t, 21 min, 5.5 mg). The fraction VB8 (350 mg) was subjected to Sephadex LH-20 column chromatography and eluted with CH_2Cl_2/MeOH (1:1) to give 3 fractions (VB81–83), and the fraction VB81 was recrystallized from MeOH to yield compound **2** (10 mg). The fraction VB11 (320 mg) was applied to Sephadex LH-20 column chromatography and eluted with MeOH to give 4 fractions (VB111–114), and then the subfractions VB112 and 113 were purified by preparative HPLC to afford compounds **3** (R_t, 15 min, 2.5 mg) and **4** (R_t, 24 min, 1.6 mg), respectively.

Ergosta-4,6,8(14),22-tetraen-3-one (1): Yellowish powder; UV ($CHCl_3$) λ_{max}: 239, 282, and 352 nm; ESI–MS *m/z* 415 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz,) δ 6.61 (1H, d,

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J=9.2 Hz, H-7), 6.04 (1H, d, *J*=9.2 Hz, H-6), 5.74 (1H, s, H-4), 5.27 (1H, dd, *J*=15.4, 7.2 Hz, H-23), 5.21 (1H, dd, *J*=15.4, 7.2 Hz, H-22), 1.07 (3H, d, *J*=6.8 Hz, H-21), 1.01 (3H, s, H-19), 0.97 (3H, s, H-18), 0.94 (3H, d, *J*=6.8 Hz, H-28), 0.86 (3H, d, *J*=6.4 Hz, H-27), 0.84 (3H, d, *J*=7.2 Hz, H-26); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ 199.7 (C-3), 164.6 (C-5), 156.3 (C-14), 135.2 (C-22), 134.2 (C-7), 132.8 (C-23), 124.7 (C-8), 124.6 (C-6), 123.2 (C-4), 55.9 (C-17), 44.6 (C-9), 44.2 (C-13), 43.1 (C-25), 39.5 (C-20), 36.9 (C-10), 35.8 (C-12), 34.3 (C-1,2), 33.3 (C-24), 27.9 (C-16), 25.5 (C-15), 21.4 (C-21), 20.2 (C-26), 19.9 (C-27), 19.2 (C-18), 19.2 (C-11), 17.9 (C-28), 16.9 (C-19).

Ergosterol peroxide (2): White powder; UV ($CHCl_3$) λ_{max}: 240 nm; ESI-MS *m/z* 451 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$, 400 MHz,) δ 6.51 (1H, d, *J*=8.4 Hz, H-7), 6.25 (1H, d, *J*=8.4 Hz, H-6), 5.23 (1H, dd, *J*=15.6, 7.2 Hz, H-23), 5.14 (1H, dd, *J*=15.6, 7.2 Hz, H-22), 3.97 (1H, m, H-3), 1.01 (3H, d, *J*=6.8 Hz, H-19), 0.91 (3H, d, *J*=6.8 Hz, H-21), 0.89 (3H, s, H-18), 0.85 (3H, d, *J*=6.4 Hz, H-28), 0.83 (3H, d, *J*=6.7 Hz, H-26) 0.81 (3H, d, *J*=7.0 Hz, H-27); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ 135.6 (C-6), 135.4 (C-22), 132.5 (C-23), 130.9 (C-7), 82.4 (C-5), 79.6 (C-8), 66.7 (C-3), 56.4 (C-17), 51.9 (C-14), 51.3 (C-9), 44.8 (C-13), 42.9 (C-24), 39.9 (C-20), 39.6 (C-12), 37.2 (C-10), 37.1 (C-4), 34.9 (C-1), 33.3 (C-25), 30.3 (C-2), 28.9 (C-16), 23.6 (C-11), 21.1 (C-21), 20.8 (C-15), 20.2 (C-26), 18.8 (C-27), 18.4 (C-19), 17.8 (C-28), 13.1 (C-18).

Indole-3-carboxaldehyde (3): Pale yellowish powder; UV (MeOH) λ_{max}: 244, 260, and 299 nm; ESI-MS *m/z* 168 $[M+Na]^+$; ^{1}H-NMR (methanol-d_4, 400 MHz,) δ 9.90 (1H, s, CHO), 8.17 (1H, dd, *J*=6.8, 1.6 Hz, H-4), 8.11 (1H, s, H-2), 7.49 (1H, dd, *J*=7.2, 1.6 Hz, H-7), 7.29 (1H, td, *J*=6.8, 1.6 Hz, H-5), 7.25 (1H, td, *J*=7.2, 1.6 Hz, H-6); ^{13}C-NMR (methanol-d_4, 125 MHz) δ 187.6 (C-8), 139.8 (C-2), 139.1 (C-7a), 125.9 (C-3a), 125.2 (C-6), 123.8 (C-5), 122.5 (C-4), 120.3 (C-3), 113.3 (C-7).

Indazole (4): Pale brown powder; UV (MeOH) λ_{max}: 244, 260, and 298 nm; ESI-MS *m/z* 141 $[M+Na]^+$; ^{1}H-NMR (methanol-d_4, 400 MHz,) δ 8.08 (1H, dd, *J*=6.8, 1.6 Hz, H-4), 7.96 (1H, s, H-3), 7.45 (1H, dd, *J*=7.2, 1.2 Hz, H-7), 7.21 (1H, td, *J*=6.8, 1.6 Hz, H-5), 7.18 (1H, td, *J*=7.2, 1.6 Hz, H-6); ^{13}C-NMR (methanol-d_4, 125 MHz) δ 138.4 (C-7a), 133.6 (C-3), 127.8 (C-3a), 123.7 (C-6), 122.5 (C-4), 122.2 (C-5), 113.0 (C-7).

Melanogenesis inhibitory activity was evaluated according to the method previously reported [17] with minor modifications. The B16F10 murine melanoma cell line, strain KCLB 80008, was purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The cells were cultured in 6-well plates at a density of 1×10^5 cells/ml and were maintained in 2 ml of DMEM containing 10% (v/v) FBS for 24 h, and then the cells were washed twice with 2 ml of phosphate-buffered saline (PBS), fed 2 ml of fresh medium (phenol red free DMEM), distributed as 2-μl samples to the dishes in a dilution series, and treated with α-MSH (Sigma, St. Louis, MO, U.S.A.) at 100 nM. After incubation for 48 h, the supernatant was added to a 96-well plate, and the amounts of melanin in the medium were measured at 405 nm with the ELISA microplate reader.

Cytotoxicity was determined using MTT assays. Briefly, after treatment of the cells with or without chemicals for 24 h, 5 mg/ml MTT in PBS was added to each well. Cells were incubated at 37°C for 3 h and DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with a microplate reader.

Compound **1** was obtained as a yellow amorphous powder and showed a molecular ion peak at *m/z* 415 $[M+Na]^+$ in the ESI-MS spectrum. The ^{1}H-NMR spectrum of **1** showed the presence of two tertiary methyl groups at δ 0.97 (3H, s, H-18) and 1.01 (3H, s, H-19), and four secondary methyl groups at δ 1.07 (3H, d, *J*=6.8 Hz, H-21), 0.94 (3H, d, *J*=6.8 Hz, H-28), 0.86 (3H, d, *J*=6.4 Hz, H-27), and 0.84 (3H, d, *J*=7.2 Hz, H-26) as required by the ergostane-type steroid [20]. It also displayed signals for five olefinic protons at δ 6.61 (1H, d, *J*=9.2 Hz, H-7), 6.04 (1H, d, *J*=9.2 Hz, H-6), 5.74 (1H, s, H-4), 5.27 (1H, dd, *J*=15.4, 7.2 Hz, H-23), and 5.21 (1H, dd, *J*=15.4, 7.2 Hz, H-22). The ^{13}C-NMR spectrum of **1** exhibited 28 resonances for six methyl groups, six methylenes, ten methines, five quaternary carbons, and one carbonyl carbon. On the basis of the above evidences, the structure of **1** was determined as ergosta-4,6,8(14),22-tetraene-3-one by comparison of its spectral data with those reported in the literature [6].

Compound **2** was obtained as a white powder and showed a molecular ion peak at *m/z* 451 $[M+Na]^+$ in the ESI-MS spectrum. The ^{1}H-NMR spectrum of **2** showed proton signals for two tertiary methyl groups at δ 0.89 (3H, s, H-18) and 1.01 (3H, d, *J*=6.8 Hz, H-19), and four secondary

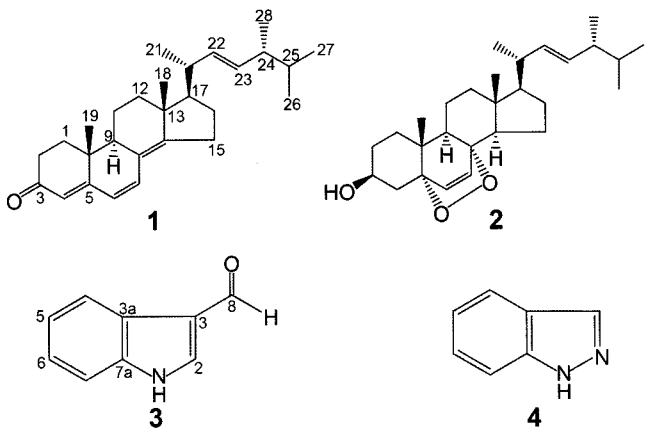

Fig. 1. Chemical structures of isolated compounds **1**–**4**.

methyl groups at δ 1.07 (3H, d, *J*=6.8 Hz, H-21), 0.94 (3H, d, *J*=6.8 Hz, H-28), 0.86 (3H, d, *J*=6.4 Hz, H-27), and 0.84 (3H, d, *J*=7.2 Hz, H-26). In the aromatic region, two olefinic methine (δ 6.51 and 6.25) signals exhibited vicinal coupling with a coupling constant of 8.4 Hz, and two other olefinic methine (δ 5.23 and 5.14) signals exhibited *trans* vicinal coupling with a coupling constant of 15.6 Hz. In addition, an oxygenated methine signal was observed at δ 3.97 (1H, m, H-3). The ^{13}C-NMR spectrum of **2** displayed 28 carbon signals including four olefinic methines, four quaternary carbons, seven methines, seven methylenes, and six methyl groups. Furthermore, two oxygen-bearing quaternary carbons at δ 79.6 (C-8) and 82.4 (C-5) suggested the presence of a 5α,8α-peroxide bond [2]. Thus, compound **2** was identified as 5α,8α-epidioxy-22*E*,24*R*-ergosta-6,22-dien-3β-ol (ergosterol peroxide) by direct comparison of its spectral data with those reported in the literature [14].

Compound **3** was obtained as a yellowish powder and showed a molecular ion peak at *m/z* 168 $[M+Na]^+$ in the ESI-MS spectrum. The UV absorption maxima were at 244, 260, and 299 nm, suggesting the presence of a 3-substituted-indole chromophore [12]. The ^{1}H-NMR spectrum of **3** displayed four proton signals at δ 8.17 (1H, dd, *J*=6.8, 1.6 Hz, H-4), 7.49 (1H, dd, *J*=7.2, 1.6 Hz, H-7), 7.29 (1H, td, *J*=6.8, 1.6 Hz, H-5), and 7.25 (1H, td, *J*=7.2, 1.6 Hz, H-6), indicating a 1,2-disubstituted benzene ring. The ^{13}C-NMR spectrum exhibited nine resonances for one carbonyl group and eight olefinic carbons. The described data led to the identification of **3** as an indole alkaloid [16]. In addition, an olefinic methine signal at δ 8.11 (1H, s, H-2) was observed, and its downfield shift of 1 ppm suggested a 3-substituted indole moiety [21]. Furthermore, the singlet signal at δ 9.90 (1H, s, CHO) was attributed to a formyl group. Thus, compound **3** was elucidated as indole-3-carboxaldehyde and confirmed by comparison of the spectroscopic data with those of previously reported [7].

Compound **4** was obtained as a pale white powder and showed a molecular ion peak at *m/z* 141 $[M+Na]^+$ in the ESI-MS spectrum. The UV spectrum of **4** showed absorption maxima at 244, 260, and 299 nm, indicating a 3-substituted-indole chromophore as in **3**. The ^{1}H-NMR spectrum of **4** displayed proton signals for the aromatic ABCD system at δ 8.08 (1H, dd, *J*=6.8, 1.6 Hz, H-4), 7.45 (1H, dd, *J*=7.2, 1.2 Hz, H-7), 7.21 (1H, td, *J*=6.8, 1.6 Hz, H-5), and 7.18 (1H, td, *J*=7.2, 1.6 Hz, H-6), and an olefinic methine signal at δ 7.96 (1H, s, H-3). The ^{13}C-NMR spectrum exhibited seven resonances for five methine and two quaternary carbons. Thus, compound **4** was identified as indazole and its spectral data were in good agreement with reported literature [4].

Table 1. Melanogenesis inhibitory activities and cytotoxicities of compounds **1**–**4** in B16 mouse melanoma cells.

Compound	Melanogenesis	Cytotoxicity
	IC_{50} (μM)	LD_{50} (μM)
1	80.9	50.6
2	21.6	>100
3	NA[b]	>100
4	NA	>100
Arbutin[a]	300	>100

[a]Positive control.
[b]NA represents no inhibitory effect at a final concentration.

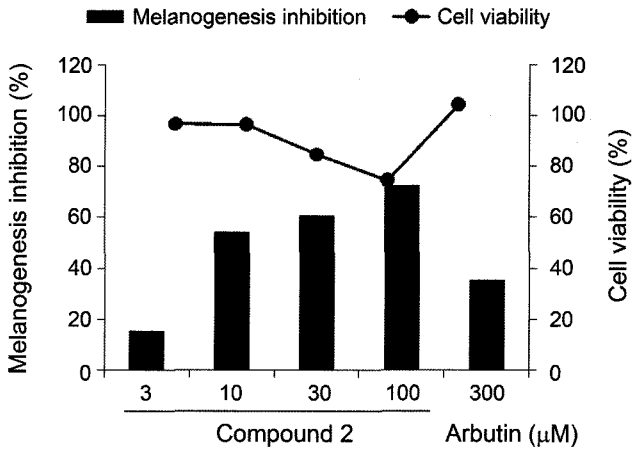

Fig. 2. Dose-dependent melanogenesis inhibitory effect and cytotoxicity of compound **2**.
Cells were cultured for 24 h in medium and treated with 3–100 μM of sample and 100 nM of α-MSH for 24 h. After incubation for 48 h, the OD of the supernatants was measured at 405 nm using an ELISA reader.

The isolated compounds were evaluated for inhibitory effects on melanogenesis and cytotoxicities in B16 melanoma cells, and the results are summarized in Table 1. As a result, the inhibitory activity of compound **1** on melanogenesis was thought to be due to its cytotoxic action, since it reduced significantly cell viability with an LD_{50} value of 50.6 μM. Compound **2** dose-dependently inhibited melanin synthesis with an IC_{50} value of 21.6 μM and showed weak cytotoxicity in cultured B16 mouse melanoma cells (Fig. 2), whereas the positive control, arbutin, exhibited 35% inhibitory effect at the concentration of 300 μM. The other two compounds (**3** and **4**) showed no inhibitory activities on melanogenesis. Previously, it has been reported that compound **2** possessed a variety of biological properties such as immunosuppressive, anti-atherosclerosis, anti-sulfatase, DNA topoisomerase I inhibitory, and antitumor activities [5, 9, 10, 13, 18]. To the best of our knowledge, all of the isolated compounds were identified for the first time from *V. bombycina*.

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