

## Cytotoxic Ergosterol Derivatives from the Mushroom *Naematoloma fasciculare*<sup>†</sup>

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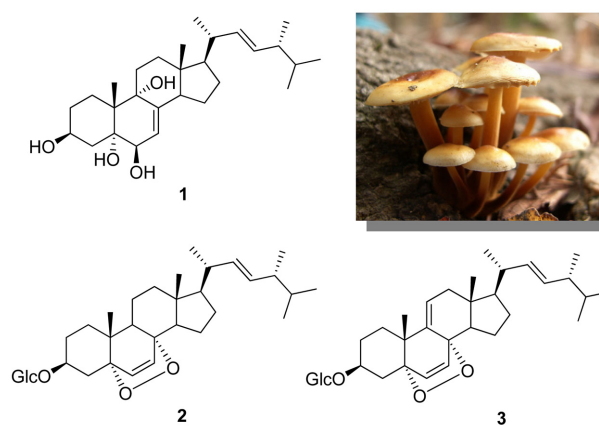
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**Abstract** – In our ongoing search for structurally interesting and biologically active metabolites from Korean wild mushrooms, bioassay-guided fractionation and a chemical investigation of the MeOH extracts of the fruiting bodies of the mushroom *Naematoloma fasciculare* resulted in the isolation of three ergosterol derivatives, (22*E*,24*R*)-ergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ ,9 $\alpha$ -tetrol (**1**), (22*E*,24*R*)-5 $\alpha$ ,8 $\alpha$ -epidioxyergosta-6,22-diene-3 $\beta$ -ol 3-*O*- $\beta$ -D-glucopyranoside (**2**), and (22*E*,24*R*)-5 $\alpha$ ,8 $\alpha$ -epidioxyergosta-6,9,22-triene-3 $\beta$ -ol 3-*O*- $\beta$ -D-glucopyranoside (**3**). The structures of **1** - **3** were determined by comparison of their spectroscopic and physical data with reported values. The isolated steroid derivatives **1** and **3** were reported for the first time from this mushroom. Compounds **1** - **3** were tested for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15).

**Keywords** – *Naematoloma fasciculare*, Strophariaceae, ergosterol derivatives, cytotoxicity

### Introduction

In our ongoing search for structurally interesting and biologically active metabolites from Korean wild mushrooms,<sup>1-6</sup> we have collected scores of endemic Korean mushroom species in the mountainous areas during the hot humid summer and prepared MeOH extracts of the mushrooms for anti-tumoral screening tests. Among the collected wild mushrooms, we found that the extract of the mushroom *Naematoloma fasciculare* (Strophariaceae) displayed a significant cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines in our screening test. The bitter poisonous mushroom *N. fasciculare* (Japanese name: Nigakuritake), which is widely distributed worldwide, grows on the stumps of old trees in tufts.<sup>7</sup> Previous phytochemical studies on this mushroom resulted in the isolation of diverse metabolites (ergosterols, triterpenoids, and sesquiterpenoids),<sup>7-14</sup> particularly, the isolation of lanostane triterpenoids as the toxic components of this mushroom.<sup>9-13</sup> In addition, pharmacological studies with lanostane triterpenoids



**Fig. 1.** The chemical structures of **1** - **3** and the mushroom *N. fasciculare*.

reported to have antimicrobial and calmodulin inhibitory activities.<sup>9-13</sup> Recently, we reported the isolation of four new lanostane triterpenoids, together with 11 known compounds, and their antiproliferative and anti-inflammatory activities.<sup>14</sup> Our ongoing search for active constituents from this mushroom *N. fasciculare* led to the isolation of three ergosterol derivatives (**1** - **3**) (Fig. 1). Their structures were determined by comparison of their spectroscopic and physical data with reported values. Compounds **1** - **3** were tested for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3,

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SK-MEL-2, and HCT-15). We describe here the isolation, structures, and cytotoxic activities of these steroid derivatives.

## Experimental

**General experimental procedures** – Optical rotations were measured on a JASCO P-1020 polarimeter. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz ( $^1\text{H}$ ) and 125 MHz ( $^{13}\text{C}$ ), respectively. ESIMS spectra were recorded on a Micromass QTOF2-MS. Semi-preparative HPLC was performed using a Gilson 306 pump with a Shodex refractive index detector. Chromatographic separation was performed on Econosil RP-18 10  $\mu$  column (250  $\times$  10 mm i.d.). Silica gel 60 (Merck, 230 - 400 mesh) and RP-C<sub>18</sub> silica gel (Merck, 230 - 400 mesh) were used for column chromatography. Silica Waters Sep-Pak Vac 6 cc and C<sub>18</sub> Waters Sep-Pak Vac 6 cc cartridges were also used for column chromatography. TLC was performed using Merck pre-coated Silica gel F<sub>254</sub> plates and RP-18 F<sub>254s</sub> plates. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

**Mushroom materials** – Fresh fruiting bodies of *N. fasciculare* were collected at Donggureung, Guri of GyeongGi-do, Korea, in August, 2009. A voucher specimen (SKKU-2009-08) of the mushroom was authenticated by one of the authors (K.H.K.) and was deposited at the herbarium of the School of Pharmacy, Sungkyunkwan University, Korea.

**Extraction and isolation** – Air-dried and powdered *N. fasciculare* fruiting bodies (88 g) were extracted twice with 80% aqueous MeOH (each 2.0 L  $\times$  3 days) at room temperature and filtered. The filtrate was evaporated under vacuum to obtain a crude MeOH extract (9.4 g), which was suspended in distilled water and then successively partitioned with *n*-hexane, CHCl<sub>3</sub>, and *n*-BuOH, yielding 900 mg, 1.8 g, and 2.8 g of residues, respectively. Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using the SRB bioassay. We selected the CHCl<sub>3</sub>-soluble fraction for the current phytochemical study, since the CHCl<sub>3</sub>-soluble fraction was the most cytotoxic against the tested tumor cell lines. The CHCl<sub>3</sub>-soluble fraction (1.8 g) was separated by a silica gel (230 - 400 mesh) column chromatography [65 g, 1.5  $\times$  32 cm, eluted with CHCl<sub>3</sub>-MeOH, 20:1 (1.8 L) and 4:1 (1.0 L)] to yield 10 fractions (Fr. A, 20:1, 0.3 L; Fr. B, 20:1, 0.3 L; Fr. C,

20:1, 0.3 L; Fr. D, 20:1, 0.3 L; Fr. E, 20:1, 0.3 L; Fr. F, 20:1, 0.3 L; Fr. G, 4:1, 0.2 L; Fr. H, 4:1, 0.2 L; Fr. I, 4:1, 0.2 L; and Fr. J, 4:1, 0.4 L). Fraction F (115 mg) was subjected to fractionation with passage over a C<sub>18</sub> Waters Sep-Pak Vac 6 cc (80% aq. MeOH) to give 13 subfractions (F1 – F13).

Subfraction F11 (10 mg) was purified by semi-preparative HPLC (95% aq. MeOH) with a Shodex refractive index detector, using an Econosil RP-18 10  $\mu$  column (250  $\times$  10 mm i.d.), to yield compound **1** (6 mg). Subfraction F12 (7 mg) was purified by separation with semi-preparative HPLC (95% aq. MeOH) to give compound **3** (4 mg). Compound **2** (5 mg) was obtained from subfraction F13 (12 mg) by separation with semi-preparative HPLC (98% aq. MeOH).

**(22E,24R)-Ergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ ,9 $\alpha$ -tetrol (1)** – A white powder, ESI-MS *m/z*: 447.4 [M + H]<sup>+</sup>;  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 500 MHz):  $\delta$  0.69 (3H, s, H-18), 0.84 (3H, d, *J* = 6.8 Hz, H-26), 0.85 (3H, d, *J* = 6.8 Hz, H-27), 0.94 (3H, d, *J* = 6.8 Hz, H-28), 1.06 (3H, d, *J* = 6.8 Hz, H-21), 1.60 (3H, s, H-19), 4.43 (1H, d, *J* = 4.0 Hz, H-6), 4.81 (1H, m, H-3), 5.21 (2H, m, H-22, H-23), 5.82 (1H, d, *J* = 4.0 Hz, H-7).  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 125 MHz):  $\delta$  143.04 (C-8), 136.2 (C-22), 132.2 (C-23), 121.3 (C-7), 78.7 (C-9), 75.0 (C-5), 73.8 (C-6), 67.3 (C-3), 56.3 (C-17), 51.3 (C-14), 44.2 (C-13), 43.1 (C-24), 42.0 (C-4), 41.3 (C-10), 40.7 (C-20), 36.0 (C-12), 33.3 (C-25), 32.4 (C-2), 29.1 (C-1), 28.5 (C-11), 28.2 (C-16), 23.5 (C-15), 22.4 (C-19), 21.4 (C-21), 20.1 (C-27), 19.8 (C-26), 17.6 (C-28), 12.1 (C-18).

**(22E,24R)-5 $\alpha$ ,8 $\alpha$ -Epidioxyergosta-6,22-diene-3 $\beta$ -ol 3-O- $\beta$ -D-glucopyranoside (2)** – An amorphous powder, ESI-MS *m/z*: 613.2 [M + Na]<sup>+</sup>;  $^1\text{H}$  NMR (pyridine-*d*<sub>5</sub>, 500 MHz):  $\delta$  0.77 (3H, s, H-18), 0.79 (3H, s, H-19), 0.87 (3H, d, *J* = 6.8 Hz, H-26), 0.88 (3H, d, *J* = 6.8 Hz, H-27), 0.97 (3H, d, *J* = 6.8 Hz, H-28), 1.03 (3H, d, *J* = 6.8 Hz, H-21), 4.46 (1H, m, H-3), 5.19 (1H, dd, *J* = 15.4, 7.5 Hz, H-22), 5.27 (1H, dd, *J* = 15.4, 7.5 Hz, H-23), 6.23 (1H, d, *J* = 8.5 Hz, H-6), 6.51 (1H, d, *J* = 8.5 Hz, H-7), Glc; 4.94 (1H, d, *J* = 7.5 Hz, H-1'), 4.05 (1H, dd, *J* = 9.0, 7.7 Hz, H-2'), 4.25 (1H, dd, *J* = 9.0, 8.8 Hz, H-3'), 4.32 (1H, dd, *J* = 9.3, 8.8 Hz, H-4'), 3.86 (1H, ddd, *J* = 9.3, 4.9, 2.5 Hz, H-5'), 4.41 (1H, dd, *J* = 11.9, 4.9 Hz, H-6'a), 4.50 (1H, dd, *J* = 11.9, 2.5 Hz, H-6'b).  $^{13}\text{C}$  NMR (pyridine-*d*<sub>5</sub>, 125 MHz):  $\delta$  136.1 (C-6), 136.0 (C-22), 132.3 (C-23), 131.0 (C-7), 103.0 (C-1'), 82.0 (C-5), 79.3 (C-8), 78.6 (C-5'), 78.3 (C-3'), 75.3 (C-2'), 73.8 (C-3), 71.5 (C-4'), 62.7 (C-6'), 56.3 (C-17), 52.0 (C-14), 51.8 (C-9), 44.7 (C-13), 43.0 (C-24), 40.1 (C-20), 39.5 (C-12), 37.4 (C-10), 35.1 (C-1), 34.6 (C-4), 33.3 (C-25), 29.0 (C-2), 29.0 (C-16),

23.6 (C-15), 21.1 (C-11), 21.1 (C-21), 20.2 (C-26), 19.9 (C-27), 18.1 (C-19), 17.9 (C-28), 13.0 (C-18).

**(22*E*,24*R*)-5 $\alpha$ ,8 $\alpha$ -Epidioxyergosta-6,9,22-triene-3 $\beta$ -ol 3-*O*- $\beta$ -D-glucopyranoside (**3**)** – An amorphous powder,  $[\alpha]_{25}^D$ : +5.6° (*c* 2.0, MeOH). IR  $\nu_{\max}$  cm<sup>-1</sup>: 3400, 1060, 1040. ESI-MS *m/z*: 589.7 [M + H]<sup>+</sup>; <sup>1</sup>H NMR (pyridine-*d*<sub>5</sub>, 500 MHz):  $\delta$  0.74 (3H, s, H-18), 0.86 (6H, d, *J* = 6.8 Hz, H-26, H-27), 0.95 (3H, d, *J* = 6.9 Hz, H-28), 0.99 (3H, d, *J* = 6.6 Hz, H-21), 1.05 (3H, s, H-19), 4.49 (1H, m, H-3), 5.17 (1H, dd, *J* = 15.4, 8.5 Hz, H-22), 5.26 (1H, dd, *J* = 15.4, 7.7 Hz, H-23), 5.44 (1H, dd, *J* = 6.0, 1.9 Hz, H-11), 6.31 (1H, d, *J* = 8.5 Hz, H-6), 6.67 (1H, d, *J* = 8.5 Hz, H-7), Glc; 4.93 (1H, d, *J* = 7.7 Hz, H-1'), 4.04 (1H, dd, *J* = 9.0, 7.7 Hz, H-2'), 4.24 (1H, dd, *J* = 9.0, 8.8 Hz, H-3'), 4.31 (1H, dd, *J* = 9.3, 8.8 Hz, H-4'), 3.84 (1H, ddd, *J* = 9.3, 4.7, 2.5 Hz, H-5'), 4.40 (1H, dd, *J* = 11.9, 4.7 Hz, H-6'a), 4.46 (1H, dd, *J* = 11.9, 2.5 Hz, H-6'b). <sup>13</sup>C NMR (pyridine-*d*<sub>5</sub>, 125 MHz):  $\delta$  143.7 (C-9), 136.1 (C-23), 135.2 (C-6), 132.4 (C-22), 131.0 (C-7), 119.3 (C-11), 103.0 (C-1'), 82.7 (C-5), 78.6 (C-3'), 78.4 (C-8), 78.3 (C-5'), 75.3 (C-2'), 73.7 (C-3), 71.5 (C-4'), 62.6 (C-6'), 55.9 (C-17), 48.6 (C-14), 43.8 (C-13), 43.0 (C-24), 41.3 (C-12), 40.2 (C-20), 38.5 (C-10), 33.8 (C-4), 33.3 (C-25), 33.0 (C-1), 30.0 (C-2), 29.0 (C-16), 25.4 (C-19), 21.3 (C-15), 20.9 (C-21), 20.1 (C-27), 19.8 (C-26), 17.8 (C-28), 13.1 (C-18).

**Cytotoxicity testing** – The cell lines used were A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma). These cancer cell lines such as A549, SK-OV-3, SK-MEL-2, and HCT-15 cells were provided by the National Cancer Institute (NCI). A SRB bioassay was used to determine the cytotoxicity of each compound against the cell lines mentioned above.<sup>15</sup> The assays were performed at the Korea Research Institute of Chemical Technology. Doxorubicin was used as a positive control. Doxorubicin cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines was IC<sub>50</sub> 0.02, 0.01, 0.01, and 0.13  $\mu$ M, respectively.

## Results and Discussion

Compound **1** was isolated as a white powder. The ESI-MS (*m/z* 447 [M + H]<sup>+</sup>) and <sup>13</sup>C NMR spectral data of **1** gave a molecular formula of C<sub>28</sub>H<sub>46</sub>O<sub>4</sub>. The <sup>13</sup>C NMR spectrum demonstrated 28 carbon signals comprising six methyls, eight methylenes, nine methines, and five quaternary carbons as indicated in DEPT experiments. The signals for six methyl groups at  $\delta$  0.69 (s), 0.84 (d, *J* = 6.8 Hz), 0.85 (d, *J* = 6.8 Hz), 0.94 (d, *J* = 6.8 Hz), 1.06

(d, *J* = 6.7 Hz), and 1.60 (s) in the <sup>1</sup>H NMR spectrum implied a sterol nucleus. Four of the carbon signals at  $\delta$  67.3, 73.8, 75.0, and 78.7 in the <sup>13</sup>C NMR spectrum were assigned to C-3, C-6, C-5, and C-9, respectively, bearing hydroxyl groups. One typical proton signal at  $\delta$  4.81 (m) for H-3 $\alpha$  indicated the presence of a 3 $\beta$ -hydroxyl group. The proton signal at  $\delta$  4.43 (d, *J* = 4.0 Hz) assigned to H-6 $\alpha$  inferred the presence of a 6 $\beta$ -hydroxyl group. One tertiary and one quaternary carbon signals at  $\delta$  121.3 and 143.0, respectively, were attributable to C-7 and C-8. The olefinic proton at  $\delta$  5.82 (d, *J* = 4.0 Hz) was assigned to H-7 based on chemical shift and coupling constant with H-6 $\alpha$ . The remaining tertiary carbon signals at  $\delta$  132.2 and 136.2 were characteristic of a double bond of C-22/C-23 in the ergosterols. On the basis of the further comparison with reported values,<sup>16</sup> the structure of **1** was determined to be (22*E*,24*R*)-ergosta-7,22-diene-3 $\beta$ ,5 $\alpha$ ,6 $\beta$ ,9 $\alpha$ -tetrol.

Compound **2** was obtained as an amorphous powder, and the molecular formula was established as C<sub>34</sub>H<sub>54</sub>O<sub>8</sub> on the basis of ESI-MS (*m/z* 613.2 [M + Na]<sup>+</sup>). The <sup>1</sup>H NMR spectrum showed the presence of the four secondary methyl signals at  $\delta$  0.87 (d, *J* = 6.8 Hz), 0.88 (d, *J* = 6.8 Hz), 0.97 (d, *J* = 6.8 Hz), 1.03 (d, *J* = 6.8 Hz), two singlet methyls at  $\delta$  0.77 (s), 0.79 (s), two sets of olefinic protons at  $\delta$  5.19 (dd, *J* = 15.1, 7.5 Hz), 5.27 (dd, *J* = 15.1, 7.3 Hz), and 6.23 (d, *J* = 8.5 Hz), 6.51 (d, *J* = 8.5 Hz), and one anomeric proton at  $\delta$  4.94 (d, *J* = 7.3 Hz). Its <sup>13</sup>C NMR spectrum contained 34 signals, of which 28 were assigned to a steroid moiety and six signals due to a hexose unit. The sugar group was determined to be glucose by the comparison of chemical shifts values with reported values.<sup>17</sup> The vicinal coupling constant (7.3 Hz) between H-1' and H-2' for glucose indicated that this sugar occurred in **2** as the  $\beta$ -anomer. The *R* configuration at C-24 was determined by comparison of chemical shift values as in the case of **1**, and the stereochemistry of C-5/8 peroxide was established by comparison of <sup>13</sup>C NMR data with literature data.<sup>17</sup> Accordingly, the structure of **2** was elucidated as (22*E*,24*R*)-5 $\alpha$ ,8 $\alpha$ -epidioxyergosta-6,22-diene-3 $\beta$ -ol 3-*O*- $\beta$ -D-glucopyranoside.<sup>17,18</sup>

Compound **3** was isolated as an amorphous powder, and the molecular ion at *m/z* 589.7 [M + H]<sup>+</sup> in its ESI-MS and 34 carbon signals in its <sup>13</sup>C NMR spectrum suggested a molecular formula of C<sub>34</sub>H<sub>52</sub>O<sub>8</sub>. Analysis of the <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra revealed that compound **3** also possesses a 3 $\beta$ -hydroxyl group, glucose with the anomeric center of  $\beta$ -configuration, 5 $\alpha$ ,8 $\alpha$ -peroxide ring, and 22*E*,24*R*-methyl-ergostane-type side chain.<sup>18</sup> Comparison of the spectral features of **3** with those of **2** suggested the close relationship of two structures. The only difference

**Table 1.** Cytotoxicity of compounds **1** - **3** using the SRB bioassay

Compound	IC <sub>50</sub> (μM) <sup>a</sup>			
	A549	SK-OV-3	SK-MEL-2	HCT-15
<b>1</b>	10.83	12.16	10.39	13.20
<b>2</b>	14.21	15.11	9.01	17.49
<b>3</b>	15.42	18.26	12.96	19.32
Doxorubicin <sup>b</sup>	0.02	0.01	0.01	0.13

<sup>a</sup> IC<sub>50</sub> value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

<sup>b</sup> Doxorubicin as a positive control.

was the assignment of additional trisubstituted double bond (143.7 (C-9) and 119.3 (C-11)) to C-9/C-11 in **3**. The stereochemistry of C-5/8 peroxide was determined to be same as compound **2** by comparison of NMR data with those of **2**. Compound **3** was, therefore, identified as (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9,22-triene-3β-ol 3-*O*-β-D-glucopyranoside by the further comparison with published data.<sup>19</sup> To the best of our knowledge, the isolated steroid derivatives **1** and **3** were reported for the first time from this mushroom.

Compounds **1** - **3** were evaluated for their cytotoxic activities against the A549, SK-OV-3, SK-MEL-2, and HCT-15 human cancer cell lines using the SRB bioassay.<sup>15</sup> All isolated ergosterol derivatives showed moderate cytotoxic activity against the four human cancer cell lines (Table 1).

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