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Cytotoxic Constituents of *Amanita subjunquillea*

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As part of our systematic study of Korean toxic mushrooms, we have investigated the constituents of *Amanita subjunquillea*. The column chromatographic separation of the MeOH extract of *A. subjunquillea* led to the isolation of four ergosterols, two cerebrosides and four cyclopeptides. Their structures were determined by spectroscopic methods to be (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9,22-triene-3β-ol (**1**), (22*E*,24*R*)-5α,8α-epidioxyergosta-6,22-dien-3β-ol (**2**), (22*E*,24*R*)-5α,6α-epoxyergosta-8,22-diene-3β,7β-diol (**3**), (24*S*)-ergost-7-en-3β-ol (**4**), 8,9-dihydrosoyacerebroside I (**5**), soyacerebroside I (**6**), β-amanitin (**7**), phalloin (**8**), α-amanitin (**9**), and phalloidin (**10**). The compounds **1**-**6** and **8** were isolated for the first time from this mushroom. The isolated compounds were evaluated for the cytotoxicity against A549, SK-OV-3, SK-MEL-2 and HCT15 cells. Compound **9** exhibited significant cytotoxic activity against A549, SK-OV-3, SK-MEL-2 and HCT15 with ED_{50} values of 1.47, 0.26, 1.57 and 1.32 μM, respectively.

Key words: *Amanita subjunquillea*, Ergosterol, Cyclopeptide, Cytotoxicity

INTRODUCTION

Amanita subjunquillea, known as 'the East Asian death cap', is a poisonous mushroom of the genus *Amanita*, which is widely distributed throughout Korea and other East Asian countries. *A. subjunquillea* has been reported to have similar toxic effects as *A. phalloides*, such as delayed gastrointestinal symptoms, hepatotoxicity and mortality (Rho *et al*., 2000). A phytochemical study of *A. subjunquillea* described the isolation of α-amanitin, β-amanitin and phalloidin (Bao *et al*., 2005) and demonstrated that *A. subjunquillea* inhibited angiotensin I converting enzyme (Tsuda *et al*., 2000).

In this study, we report the isolation of four ergosterols, two cerebrosides and four cyclopeptides from the MeOH extract of *A. subjunquillea*. Their structures were determined by spectroscopic methods. The compounds **1**-**6** and **8** were isolated for the first time from this mushroom. The isolated compounds were tested for cytotoxicity against four human tumor cells *in vitro* by SRB assay.

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MATERIALS AND METHODS

General

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 Polarimeter. NMR spectrums were recorded on a Varian UNITY INOVA 500 NMR spectrometer. FAB-MS data were obtained on a JEOL JMS700 mass spectrometer. Preparative HPLC used a Gilson 306 pump with a Shodex refractive index detector and an Apollo Silica 5 m column (250×22 mm) or Econosil® RP-18 10 m column (250×22 mm). Silica gel 60 (Merck, 70-230 mesh and 230-400 mesh) was used for column chromatography. Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates were used for TLC. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). Low pressure liquid chromatography was carried out over either Merck LiChroprep Lobar®-A Si 60 (240×10 mm) or LiChroprep Lobar®-A RP-18 (240×10 mm) column with a FMI QSY-0 pump (ISCO).

Plant materials

Amanita subjunquillea was collected at Mt. Gwanggyo, Suwon, Korea in September, 2005. A voucher specimen

(SKKU-2005-9) of the mushroom was deposited at the College of Pharmacy at Sungkyunkwan University, Korea.

Extraction and isolation

Half dried *A. subjunquillea* (100 g) were extracted with 80% MeOH at room temperature and evaporated under reduced pressure to give a residue (200 g), which was dissolved in water (800 mL) and solvent partitioned to give *n*-hexane (2.5 g), $CHCl_3$ (170 mg), and *n*-BuOH fractions (1.6 g).

The *n*-hexane fraction (2.5 g) was chromatographed over a silica gel column with *n*-hexane:EtOAc = 1:1 as the eluent to give three fractions (H1-H3). Fraction H2 (90 mg) was subjected to silica Lobar A®-column (*n*-hexane:EtOAc = 2:1) and purified with silica gel preparative HPLC (Apollo Silica 5 μ column, 250×22 mm; *n*-hexane:EtOAc = 2:1, retention time : 15 and 17 minute, respectively) to yield compounds **1** (3 mg) and **2** (5 mg). Fraction H3 (300 mg) was also subjected to a RP-C_{18} silica gel column with 95% MeOH as the eluent and purified with silica gel preparative HPLC (Apollo Silica 5 μ column, 250×22 mm; *n*-hexane:EtOAc = 3:1, retention time : 16 and 18 minute, respectively) to yield compounds **3** (4 mg) and **4** (8 mg). Fraction H1 (1.0 g) was subjected to a silica gel column with *n*-hexane:EtOAc = 10:1 as the eluent and purified with silica gel preparative HPLC (Apollo Silica 5 μ column, 250×22 mm; *n*-hexane:EtOAc = 3:1, retention time : 18 and 21 minute, respectively) to yield compounds **5** (5 mg) and **6** (6 mg).

The $CHCl_3$ fraction (170 mg) was chromatographed over a Sephadex LH-20 column with solvent system of CH_2Cl_2:MeOH (1:1) as the eluent to give four fractions (C1-C4). Fraction C2 (50 mg) was purified by RP-C_{18} preparative HPLC (Econosil® RP-18 10 μ column, 250×22 mm; 40% MeCN, retention time : 17 and 25 minute, respectively) to afford compounds **7** (7 mg) and **8** (5 mg).

The *n*-BuOH fraction (1.6 g) was chromatographed over a RP-C_{18} silica gel column with solvent system of MeOH: Water (2:8 - 1:1) as the eluent to give eight fractions (B1-B8). Fraction B5 (100 mg) was purified by RP-C_{18} preparative HPLC (Econosil® RP-18 10 μ column, 250×22 mm; 20% MeCN, retention time : 20 minute) to yield compound **9** (60 mg). Fraction B7 (70 mg) was also purified by RP-C_{18} preparative HPLC (Econosil® RP-18 10 μ column, 250×22 mm; 20% MeCN, retention time : 19 minute) to give compound **10** (50 mg).

(22*E*,24*R*)-5α,8α-Epidioxyergosta-6,9,22-triene-3β-ol (1)
Amorphous powder, mp. 164-166; $[\alpha]_D^{20}$: +8.0° (c 0.01, $CHCl_3$); FAB-MS m/z: 427 $[M+H]^+$; ^{1}H-NMR (C_5D_5N, 500 MHz): δ 6.67 (1H, d, *J* = 8.5 Hz, H-7), 6.31 (1H, d, *J* = 8.5 Hz, H-6), 5.44 (1H, dd, *J* = 6.0, 1.9 Hz, H-11), 5.26 (1H, dd, *J* = 15.4, 7.7 Hz, H-23), 5.17 (1H, dd, *J* = 15.4 , 8.5 Hz, H-22), 4.40 (1H, m, H-3), 1.05 (3H, s, H-19), 0.99 (3H, d, *J* = 6.6 Hz, H-21), 0.95 (3H, d, *J* = 6.9 Hz, H-28), 0.86 (6H, d, *J* = 6.8 Hz, H-26, 27), 0.74 (3H, s, H-18); ^{13}C-NMR (C_5D_5N, 125 MHz): δ 143.7 (C-9), 136.1 (C-23), 135.2 (C-6), 132.4 (C-22), 131.0 (C-7), 119.3 (C-11), 82.7 (C-5), 78.4 (C-8), 66.3 (C-3), 56.8 (C-17), 48.6 (C-14), 43.8 (C-13), 43.0 (C-24), 41.3 (C-12), 40.4 (C-20), 38.7 (C-10), 35.9 (C-1), 33.8 (C-4), 33.3 (C-25), 30.7 (C-2), 29.0 (C-16), 25.4 (C-19), 21.3 (C-15), 20.3 (C-21), 20.1 (C-27), 19.8 (C-26), 17.8 (C-28), 13.4 (C-18).

(22*E*,24*R*)-5α,8α-Epidioxyergosta-6,22-dien-3β-ol (2)
White powder, mp. 176-178; $[\alpha]_D^{20}$: -29.1° (c 0.29, $CHCl_3$); FAB-MS m/z: 429 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 6.45 (1H, d, *J* = 8.5 Hz, H-7), 6.20 (1H, d, *J* = 8.5 Hz, H-6), 5.15 (1H, dd, *J* = 15.4, 7.7 Hz, H-23), 5.12 (1H, dd, *J* = 15.4, 8.5 Hz, H-22), 3.91 (1H, m, H-3), 1.19 (3H, d, *J* = 6.6 Hz, H-21), 0.95 (3H, d, *J* = 6.9 Hz, H-28), 0.86 (3H, s, H-19), 0.83 (6H, d, *J* = 6.8 Hz, H-26, 27), 0.81 (3H, s, H-18); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 135.4 (C-6), 135.1 (C-22), 132.2 (C-23), 130.6 (C-7), 82.1 (C-5), 79.3 (C-8), 66.2 (C-3), 56.1 (C-17), 51.5 (C-14), 50.9 (C-4), 44.5 (C-13), 42.7 (C-24), 39.7 (C-20), 39.2 (C-12), 36.8 (C-1, C-10), 34.6 (C-9), 33.0 (C-25), 29.9 (C-2), 28.6 (C-15), 23.3 (C-16), 20.8 (C-11), 20.5 (C-27), 19.9 (C-26), 19.6 (C-21), 18.1 (C-19), 17.5 (C-28), 12.8 (C-18).

5α,6α-Epoxyergosta-8,22-diene-3β,7β-diol (3)
White powder, mp. 174-175; $[\alpha]_D^{20}$: -52.0° (c 0.01, $CHCl_3$); FAB-MS m/z: 429 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 5.23 (1H, dd, *J* = 15.1, 7.7 Hz, H-23), 5.16 (1H, dd, *J* = 15.4, 7.8 Hz, H-22), 4.39 (1H, br s, H-7), 3.93 (1H, m, H-3), 3.14 (1H, d, *J* = 2.9 Hz, H-6), 2.20 (1H, dd, *J* = 12.9, 11.5 Hz, H-4a), 1.28 (3H, s, H-19), 1.02 (3H, d, *J* = 6.6 Hz, H-21), 0.91 (3H, d, *J* = 6.8 Hz, H-28), 0.83 (6H, d, *J* = 6.3 Hz, H-26, 27), 0.63 (3H, s, H-18); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 137.0 (C-9), 135.5 (C-22), 132.2 (C-23), 126.5 (C-8), 68.6 (C-3), 67.0 (C-7), 63.2 (C-5), 60.1 (C-6), 54.3 (C-17), 51.1 (C-14), 42.8 (C-24), 41.9 (C-13), 40.5 (C-20), 39.0 (C-4), 37.9 (C-10), 36.2 (C-12), 33.1 (C-25), 30.8 (C-1), 29.7 (C-2), 29.2 (C-16), 23.6 (C-15), 23.0 (C-11), 22.9 (C-19), 21.0 (C-21), 19.9 (C-27), 19.6 (C-26), 17.6 (C-28), 11.5 (C-18).

(24*S*)-Ergost-7-en-3β-ol (4)
Amorphous powder, mp. 168-169; $[\alpha]_D^{20}$: +12.5° (c 0.02, $CHCl_3$); FAB-MS m/z: 401 $[M+H]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 5.16 (1H, m, H-7), 3.61 (1H, m, H-3), 0.92 (3H, d, *J* = 6.3 Hz, H-21), 0.85 (3H, d, *J* = 6.8 Hz, H-27), 0.79 (3H, s, H-19), 0.78 (3H, d, *J* = 6.9 Hz, H-26), 0.77 (3H, d, *J* = 6.8 Hz, H-28), 0.53 (3H, s, H-18); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 139.6 (C-8), 117.4 (C-7), 71.08 (C-3), 56.0 (C-17), 55.05 (C-14), 49.4 (C-4), 43.3 (C-13), 40.2 (C-5),

39.5 (C-12), 39.1 (C-24), 38.3 (C-4), 37.1 (C-1), 36.6 (C-20), 34.2 (C-10), 33.6 (C-22), 31.5 (C-2), 31.4 (C-25), 30.7 (C-23), 29.6 (C-6), 27.9 (C-16), 22.9 (C-15), 21.5 (C-11), 20.5 (C-26), 19.0 (C-21), 17.6 (C-27), 15.4 (C-28), 13.0 (C-19), 11.8 (C-18).

8,9-Dihydrosoyacerebroside I (5)

Colorless gum, $[\alpha]_D^{20}$: -12.3° (c 0.01, MeOH); FAB-MS m/z: 738 $[M+Na]^+$; ^{1}H-NMR (CD_3OD, 500 MHz): δ 5.73 (1H, br dt, *J* = 15.5, 6.0 Hz, H-5), 5.47 (1H, dd, *J* = 15.5, 7.0 Hz, H-4), 4.28 (1H, d, *J* = 7.7 Hz, glc-1"), 4.12 (1H, t, *J* = 7.5 Hz, H-3), 4.10 (1H, dd, *J* = 10.3, 5.5 Hz, H-1b), 4.00 (1H, dd, *J* = 8.3, 3.5 Hz, H-2'), 3.98 (1H, ddd, *J* = 7.5, 5.0, 3.5 Hz, H-2), 3.81 (1H, dd, *J* = 12.0, 1.4 Hz, glc-6"b), 3.71 (1H, dd, *J* = 10.1, 3.5 Hz, H-1a), 3.67 (1H, dd, *J* = 12.0, 5.1 Hz, glc-6"a), 3.36 (1H, t, *J* = 9.0 Hz, gla-3"), 3.31-3.28 (2H, m, glc-4", glc-5"), 3.19 (1H, m, glc-2"), 2.06 (1H, m, H-6), 1.70 (1H, m, H-3'b), 1.58 (1H, m, H-3'a), 1.42 (2H, m, H-4'), 1.31-1.28 (44H, m, H-7-17, H-5'-15'), 0.90 (6H, t, *J* = 6.7 Hz, H-16', H-18); ^{13}C-NMR (CD_3OD, 125 MHz): δ 177.3 (C-1'), 136.2 (C-5), 129.5 (C-4), 104.8 (C-glc-1"), 78.1 (glc-3", glc-5"), 75.2 (glc-2"), 73.2 (C-2'), 72.9 (C-3), 71.7 (glc-4"), 69.8 (C-1), 62.8 (glc-6"), 54.7 (C-2), 35.9 (C-3'), 33.2 (C-5), 32.5 (C-16, C-14'), 29.8-30.3 (C-7-15, C-5'-13'), 23.8 (C-17, C-4', C-15'), 14.3 (C-18, C-16').

Soyacerebroside I (6)

Colorless gum, $[\alpha]_D^{20}$: +15.3° (c 0.04, MeOH); FAB-MS m/z: 736 $[M+Na]^+$; ^{1}H-NMR (CD_3OD, 500 MHz): δ 5.73 (1H, br dt, *J* = 15.5, 6.0 Hz, H-5), 5.47 (1H, dd, *J* = 15.5, 7.0

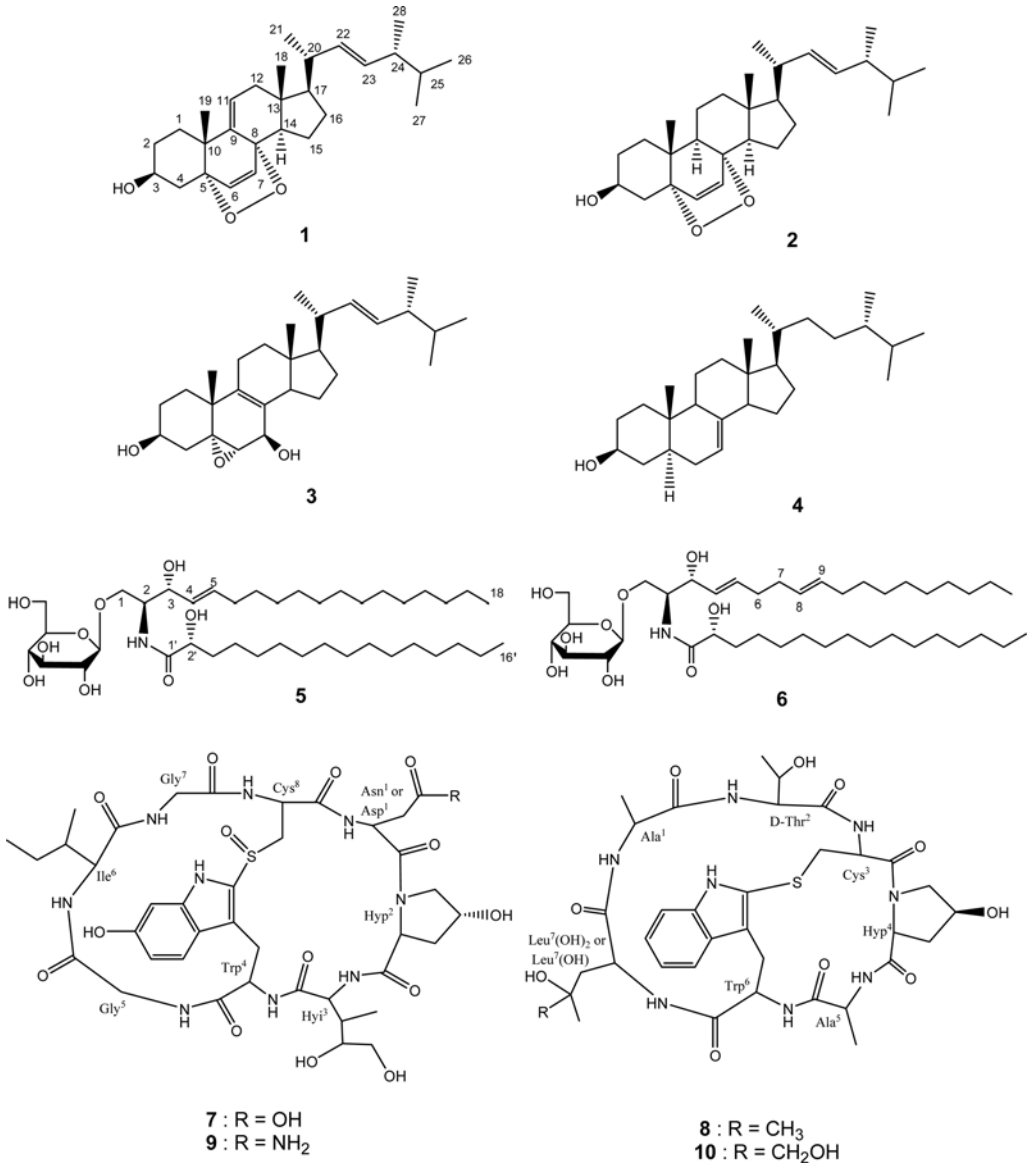

Fig. 1. The Structures of **1**-**10** from of *A. subjunquillea*

Hz, H-4), 5.42 (2H, t like, *J* = 4.5 Hz, H-8, H-9), 4.27 (1H, d, *J* = 7.7 Hz, glc-1"), 4.12 (1H, t, *J* = 7.5 Hz, H-3), 4.10 (1H, dd, *J* = 10.3, 5.5 Hz, H-1b), 4.00 (1H, dd, *J* = 8.3, 3.5 Hz, H-2'), 3.98 (1H, ddd, *J* = 7.5, 5.0, 3.5 Hz, H-2), 3.81 (1H, dd, *J* = 12.0, 1.4 Hz, glc-6"b), 3.71 (1H, dd, *J* = 10.1, 3.5 Hz, H-1a), 3.67 (1H, dd, *J* = 12.0, 5.1 Hz, glc-6"a), 3.36 (1H, t, *J* = 9.0 Hz, gla-3"), 3.31-3.28 (2H, m, glc-4", glc-5"), 3.19 (1H, m, glc-2"), 2.07 (1H, m, H-7), 2.06 (1H, m, H-6), 1.97 (1H, m, H-10), 1.70 (1H, m, H-3'b), 1.58 (1H, m, H-3'a), 1.42 (2H, m, H-4'), 1.31-1.28 (36H, m, H-11-17, H-5'-15'), 0.90 (6H, t, *J* = 6.7 Hz, H-16', H-18); ^{13}C-NMR (CD_3OD, 125 MHz): δ 177.2 (C-1'), 134.2 (C-5), 131.6 (C-8), 130.0 (C-9), 129.8 (C-4), 103.8 (C-glc-1"), 77.1 (glc-3", glc-5"), 74.2 (glc-2"), 72.6 (C-2'), 72.4 (C-3), 70.8 (glc-4"), 69.0 (C-1), 62.1 (glc-6"), 53.9 (C-2), 35.2 (C-3'), 33.1 (C-7), 32.8 (C-10), 32.5 (C-6, C-16, C-14'), 29.8-30.3 (C-11-15, C-5'-13'), 23.2 (C-17, C-4', C-15'), 14.3 (C-18, C-16').

β-Amanitin (7)
Colorless gum, $[\alpha]_D^{20}$: -130.5° (c 0.02, MeOH); FAB-MS m/z: 942 $[M+Na]^+$; ^{1}H- and ^{13}C-NMR: see Table I.

Phalloin (8)
Colorless gum, $[\alpha]_D^{20}$: -56.3° (c 0.2, MeOH); FAB-MS m/z: 772 $[M]^+$; ^{1}H- and ^{13}C-NMR: see Table I.

α-Amanitin (9)
Colorless gum, $[\alpha]_D^{20}$: +79.3° (c 0.93, MeOH); FAB-MS m/z: 919 $[M+H]^+$; ^{1}H- and ^{13}C-NMR: see Table I.

Phalloidin (10)
Colorless gum, $[\alpha]_D^{20}$: +73.2° (c 0.32, MeOH); FAB-MS m/z: 788 $[M]^+$; ^{1}H- and ^{13}C-NMR: see Table I.

Test for cytotoxicity *in vitro*

Sulforhodamin B bioassay (SRB) was used as for cytotoxicity screening (Skehan *et al*., 1990). The *in vitro* cytotoxicity of each compound against four cultured human tumor cells was assessed at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells).

RESULTS AND DISCUSSION

The column chromatographic separation of the MeOH extract of *A. subjunquillea* led to the isolation of four ergosterols (**1**-**4**), two cerebrosides (**5**-**6**), and four cyclopeptides (**7**-**10**).

The ^{1}H- and ^{13}C-NMR spectra of compounds **1**-**4** showed spectral patterns typical of sterols. The ^{13}C-NMR spectra of **1** showed 28 signals including six peaks due to three double bonds [δ 143.7 (C-9), 136.1 (C-23), 135.2 (C-6), 132.4 (C-22), 131.0 (C-7), 119.3 (C-11)], two oxygenated quaternary carbons [δ 82.7 (C-5), 78.4 (C-8)] and one oxygenated methine peak [δ 66.3 (C-3)]. In the ^{1}H-NMR spectrum of **1**, two sets of an AB coupling system were shown at δ 6.31 (1H, d, *J* = 8.5 Hz, H-6), 6.67 (1H, d, *J* = 8.5 Hz, H-7), and at δ 5.17 (1H, dd, *J* = 15.4, 7.7 Hz, H-22), 5.26 (1H, dd, *J* = 15.4, 7.7 Hz, H-23). In addition, an olefinic proton signal was detected at δ 5.44 (1H, dd, *J* = 6.0, 1.9 Hz, H-11). Based on the above and the comparison of the ^{1}H-, ^{13}C-NMR and MS spectral data reported in a previous paper (Kazuko *et al*., 2001), the structure of **1** was identified as (22*E*,24*R*)-5α,8α-epidioxyergosta-6,9,22-triene-3β-ol. The ^{1}H- and ^{13}C-NMR spectral data of **2** were very similar to those of **1**, except for the absence of a double bond at C-9 in **2**. Based on the comparison of the ^{1}H-, ^{13}C-NMR and MS spectral data with those reported in a previous paper (Xu *et al*., 2007), the structure of **2** was determined to be (22*E*,24*R*)-5α,8α-epidioxyergosta-6,22-dien-3β-ol. The structures of the compounds **3** and **4** were identified as 5α,6α-epoxyergosta-8,22-diene-3β,7β-diol (Yasunori *et al*., 1999) and (24*S*)-ergost-7-en-3β-ol (Noboru *et al*., 2007) respectively by comparison of ^{1}H-, ^{13}C-NMR and MS spectral data with those previously reported in the literature. These ergosterols are commonly isolated from various natural sources, including mushroom and also known for exhibiting cytotoxicity (Kazuko *et al*., 2001; Kwon *et al*., 2002).

The ^{1}H- and ^{13}C-NMR spectra of compounds **5**-**6** showed spectral patterns typical of cerebrosides (Laurence *et al*., 1999; Hirotaka *et al*., 1993). The ^{13}C-NMR spectrum of **6** showed 40 signals including a ketone group [δ 177.2 (C-1')], two double bonds [δ 134.2 (C-5), 131.6 (C-8), 130.0 (C-9), 129.8 (C-4)], three oxygenated carbons [δ 72.6 (C-2'), 72.4 (C-3), 69.0 (C-1)], a methine attached to nitrogen [δ 53.9 (C-2)], three vicinal methylenes [δ 33.1 (C-7), 32.8 (C-10), 32.5 (C-6)], and a glucosyl moiety [δ 103.8 (C-1"), 77.1 (C-3", C-5"), 74.2 (C-2"), 70.8 (C-4"), 62.1 (C-6")]. In the ^{1}H-NMR spectrum of **6**, two double bonds at δ 5.73 (1H, br dt, *J* = 15.5, 6.0 Hz, H-5), 5.47 (1H, dd, *J* = 15.5, 7.0 Hz, H-4), 5.42 (2H, t like, *J* = 4.5 Hz, H-8, H-9) and two terminal methyl groups (H-16', H-18) at δ 0.90 (6H, t, *J* = 6.7 Hz) were observed. The coupling constant (*J* = 7.7 Hz) of the anomeric proton at δ 4.27 of D-glucose indicated it to be the β-form (Stephen *et al*., 1977). Thus, the structure of compound **6** was identified as soyacerebroside I based on the above and the comparison of the data with those in a previous paper (Laurence *et al*., 1999). The ^{1}H- and ^{13}C-NMR spectral data of **5** were very similar to those of **6**, except for the absence of a double bond group at C-8, C-9 in **5**. Based on the comparison of the ^{1}H-, ^{13}C-NMR and MS spectral data reported in a previous paper

Table I. ^{1}H- and ^{13}C-NMR data for **7** - **10** (δ in ppm, 500 MHz for ^{1}H and 125 MHz for ^{13}C in DMSO)

Amino acid residue		7		9		Amino acid residue		8		10	
		δ_H	δ_C	δ_H	δ_C			δ_H	δ_C	δ_H	δ_C
Asn[1] (Asp[1])	NH	8.39		8.41		Ala[1]	NH	7.30		7.31	
	α	4.56	51.4	4.63	50.8		α	4.45	49.6	4.48	49.6
	β	3.14*, 2.88	36.6	3.50*, 2.95	33.5		β	1.20	19.4	1.21	19.4
	γ		172.1		172.6		CO		174.5		174.8
	CO		171.7		170.7	D-Thr[2]	NH	8.56		8.57	
	NH_2			8.31, 7.51			α	3.99	59.6	3.96	59.8
Hyp[2]	α	4.30	61.5	4.27	61.9		β	4.22	64.5	4.26	64.4
	β	2.21, 1.80	37.8	2.18, 1.84	37.4		γ	1.05	20.2	1.06	20.8
	γ	4.35	68.5	4.37	68.7		CO		170.8		171.2
	δ	3.70*, 3.67*	55.9	3.95*, 3.80*	55.8	Cys[3]	NH	7.66		7.69	
	CO		171.7		170.2		α	4.72	50.6	4.73	50.5
Hyi[3]	NH	8.36		7.77			β	3.50*, 3.22*	38.5	3.52*, 3.22*	38.1
	α	4.34	55.8	4.42	55.2		CO		172.2		172.0
	β	2.32	37.8	2.11	38.0	Hyp[4]	α	4.14	60.8	4.14	61.8
	CH_3	0.81	14.6	0.86	13.4		β	2.28, 1.80	37.0	2.28, 1.80	37.5
	γ	3.36*	71.7	3.52*	72.2		γ	4.33	68.4	4.33	68.4
	δ	3.39*, 3.25*	63.9	3.40*, 3.31*	63.3		δ	3.75*	53.8	3.75*, 3.50*	53.9
	CO		171.0		170.7		CO		173.2		173.0
Trp[4]	NH	7.90		7.83		Ala[5]	NH	7.75		7.70	
	α	5.07	52.5	4.91	53.0		α	3.98	49.5	3.98	49.5
	β	3.03, 2.91	28.6	3.19*, 2.73	28.6		β	0.78	17.1	0.79	17.1
	C-2'		129.7		129.8		CO		172.5		172.5
	C-3'		111.9		111.6	Trp[6]	NH	7.25		7.25	
	C-3'a		120.7		120.7		α	4.81	52.5	4.81	52.7
	C-4'	7.40	122.0	7.42	122.2		β	3.35*, 3.11*	28.3	3.32*, 3.12*	28.6
	C-5'	6.59	110.5	6.59	110.7		C-2'		132.5		132.5
	C-6'		154.6		154.6		C-3'		111.1		111.3
	C-7'	6.74	96.6	6.74	96.7		C-3'a		128.5		128.0
	C-7'a		138.8		138.8		C-4'	7.71	120.5	7.71	120.6
	CO		170.0		170.1		C-5'	6.95	119.4	6.97	119.4
	NH (indole)	11.13		11.18			C-6'	7.11	123.2	7.10	123.3
Gly[5]	NH	7.97		7.94			C-7'	7.23	111.5	7.23	111.3
	α	4.23, 3.44*	41.3	4.30, 3.38*	41.2		C-7'a		137.8		137.4
	CO		170.2		170.3		CO		170.5		170.3
Ile[6]	NH	8.47		8.44			NH (indole)	11.10		11.20	
	α	3.68*	59.1	3.66*	59.2	Leu[7](OH)$_2$ (Leu[7](OH))	NH	8.40		8.41	
	β	1.57	34.5	1.56	34.6		α	4.28	52.0	4.08	52.5
	γ	1.53, 1.11	25.1	1.50, 1.11	25.2		β	2.05, 1.85	42.5	2.15, 2.02	39.5
	γ-CH_3	0.79	14.7	0.79	14.8		γ		67.2		72.0
	δ-CH_3	0.82	10.6	0.82	10.7		δ	0.95	31.4	4.28, 3.20*	69.8
	CO		171.6		171.6		γ-CH_3	0.96	31.6	1.00	25.8
Gly[7]	NH	8.71		8.69			CO		172.5		172.4
	α	3.90, 3.43*	42.3	3.90, 3.44*	42.4						
	CO		167.6		167.8						

Table I. Continued

Amino acid residue		7		9		Amino acid residue	8		10	
		δ_H	δ_C	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
Cys^8	NH	8.21		8.25						
	α	4.92	49.9	4.94	50.1					
	β	3.06, 2.97	58.7	3.08, 2.95	58.9					
	CO		166.7		167.1					

*Overlapped signals.

Asn: asparagine; Asp: aspartic acid; Hyp: hydroxyproline; Hyi: hydroxyisovaleric aicd; Trp: tryptophan; Gly: glycine; Ile: isoleucine; Cys: cysteine; Ala: alanine; D-Thr: D-threonine; $Leu(OH)_2$: 4,5-dihydroxyleucine; Leu(OH): 4-hydroxyleucine

(Hirotaka *et al*., 1993), the structure of **5** was determined to be 8,9-dihydrosoyacerebroside I. Several ceramides have been isolated from mushrooms (Yaoita *et al*., 2002) and have shown anti-ulcerogenic (Okuyama *et al*., 1983) and enzyme inhibitory effect (Kong *et al*., 2001).

The ^{1}H-NMR and ^{13}C-NMR spectra of compounds **7**-**10** showed spectral patterns typical of cyclopeptides (Pehk *et al*., 1989; Anderson *et al*., 2005; Kobayashi *et al*., 1995). The amatoxins (**7** and **9**) are bicyclic octapeptides with a bridge of 6"-hydroxytryptathionine-(*R*)-sulfoxide. The phallotoxins (**8** and **10**), are bicyclic heptapeptides with a thioether bridge. The positive ion FAB-MS spectrum of **7** showed an $[M + Na]^+$ peak at m/z 942. The ^{13}C-NMR spectrum of **7** showed 39 signals, including nine ketone groups [δ 172.1, 171.7 (Asn^1), 171.7 (Hyp^2), 171.6 (Ile^6), 171.0 (Hyi^3), 170.2 (Gly^5), 170.0 (Trp^4), 167.6 (Gly^7), 166.7 (Cys^8)], an indole skeleton of the tryptophane moiety [δ 154.6 (C-6"), 138.8 (C-7"a), 129.7 (C-2"), 122.0 (C-4"), 120.7 (C-3"a), 111.9 (C-3"), 110.5 (C-5"), 96.6 (C-7")], three oxygenated carbons [δ 71.7 (Hyi^3, γ), 68.5 (Hyp^2, γ), 63.9 (Hyi^3, δ)], eight methines between the nitrogen and ketone groups [δ 61.5 (Hyp^2), 59.1 (Ile^6), 55.8 (Hyi^3), 52.5 (Trp^4), 51.4 (Asn^1), 49.9 (Cys^8), 42.3 (Gly^7), 41.3 (Gly^5)], and three terminal methyl groups [δ 14.7 (Ile^6), 14.6 (Hyi^3), 10.6 (Ile^6)]. The ^{1}H-NMR spectrum of **7** had eight NH peaks at δ 11.13 (Trp^4, indole), 8.71 (Gly^7), 8.47 (Ile^6), 8.39 (Asn^1), 8.36 (Hyi^3), 8.21 (Cys^8), 7.97 (Gly^5) and 7.90 (Trp^4), three double bond protons at δ 7.40 (1H, d, *J* = 8.5 Hz, H-4"), 6.74 (1H, d, *J* = 1.5 Hz, H-7") and 6.59 (1H, dd, *J* = 8.5, 1.5 Hz, H-5") and three terminal methyl groups at δ 0.82 (3H, d, *J* = 7.5 Hz, Ile^6-δ-CH_3), 0.81 (3H, d, *J* = 7.5 Hz, Hyi^3-γ-CH_3), and 0.79 (3H, m, Ile^6-γ-CH_3). The connectivity of eight amino acids of **7** was confirmed by analysis of 2D NMR experiments (^{1}H-^{1}H COSY, HMQC, HMBC and ROESY). Thus, the structure of **7** was identified as β-amanitin based on the above and by comparison of spectral data with those reported in a previous paper (Pehk *et al*., 1989). The positive ion FAB-MS spectrum of **8** showed an $[M]^+$ peak at m/z 772. The ^{13}C-NMR spectrum of **8** showed 35 signals, including seven ketone groups [δ 174.5 (Ala^1), 173.2 (Hyp^4), 172.5 (Ala^5), 172.5 (Leu^7 (OH)), 172.2 (Cys^3), 170.8 (D-Thr^2), 170.5 (Trp^6)], an indole skeleton of the tryptophane moiety [δ 137.8 (C-7"a), 132.5 (C-2"), 128.5 (C-3"a), 123.2 (C-6"), 120.5 (C-4"), 119.4 (C-5"), 111.5 (C-7"), 111.1 (C-3")], three oxygenated carbons [δ 68.4 (Hyp^4, γ), 67.2 (Leu^7 (OH), γ), 64.5 (D-Thr^2, β)], seven methines between the nitrogen and ketone groups [δ 60.8 (Hyp^4), 59.6 (D-Thr^2), 52.5 (Trp^6), 52.0 (Leu^7 (OH)), 50.6 (Cys^3), 49.6 (Ala^1), 49.5 (Ala^5)], and five terminal methyl groups [δ 31.6, 31.4 (Leu^7(OH)), 20.2 (D-Thr^2), 19.4 (Ala^1), 17.1 (Ala^5)]. The ^{1}H-NMR spectrum of **8** had seven NH peaks at δ 11.10 (Trp^6, indole), 8.56 (D-Thr^2), 8.40 (Leu^7 (OH)), 7.75 (Ala^5), 7.66 (Cys^3), 7.30 (Ala^1), 7.25 (Trp^6), four double bond protons at δ 7.71 (1H, d, *J* = 8.0 Hz, H-4"), 7.23 (1H, d, *J* = 8.0 Hz, H-7"), 7.11 (1H, t like, *J* = 8.0 Hz, H-6"), and 6.95 (1H, t like, *J* = 8.0 Hz, H-5") and five terminal methyl groups at δ 1.20 (3H, d, *J* = 7.0 Hz, Ala^1-β-CH_3), 1.05 (3H, d, *J* = 7.0 Hz, D-Thr^2-γ-CH_3), 0.96 (3H, s, Leu^7 (OH)-γ-CH_3), 0.95 (3H, s, Leu^7 (OH)-δ-CH_3), and 0.78 (3H, d, *J* = 7.0 Hz Ala^5-β-CH_3). The connectivity of seven amino acids of **8** was determined by analysis of 2D NMR experiments (^{1}H-^{1}H COSY, HMQC, HMBC and ROESY). Thus, the structure of **8** was identified as phalloin based on the above and by comparison of spectral data with those reported in previous studies (Anderson *et al*., 2005; Kobayashi *et al*., 1995). The ^{1}H- and ^{13}C-NMR spectral data of **9** were very similar to those of **7,** except that the OH group at the aspartic acid moiety in **7** was replaced with an amine group at the asparagine moiety in **9**. Based on 2D NMR experiments (^{1}H-^{1}H COSY, HMQC, HMBC, and ROESY) and the comparison of the ^{1}H-, ^{13}C-NMR, and MS spectral data reported in a previous paper (Pehk *et al*., 1989), the structure of **9** was determined to be α-amanitin. The ^{1}H- and ^{13}C-NMR spectral data of **10** were very similar to those of **8**, except for replacement of the CH_3 group at the 4-hydroxyleucine moiety in **8** with an CH_2OH group at the 4,5-dihydroxyleucine moiety in **10**. Based on 2D NMR experiments (^{1}H-^{1}H COSY, HMQC, HMBC, and ROESY) and the comparison of the ^{1}H-, ^{13}C-NMR, and MS spectral data reported in

Table II. Cytotoxic activities of compounds (**1**-**10**) isolated from *A. subjunquillea*

Compound	ED_{50} (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	12.27	10.86	11.84	9.61
2	10.20	6.71	9.70	11.43
3	>30.0	29.55	>30.0	15.18
4	16.44	15.73	15.92	11.86
5	>30.0	>30.0	>30.0	>30.0
6	>30.0	24.17	>30.0	28.61
7	>30.0	22.76	>30.0	>30.0
8	>30.0	22.81	>30.0	>30.0
9	1.47	0.26	1.57	1.32
10	>30.0	>30.0	>30.0	>30.0
Doxorubicin	0.16	0.38	0.04	0.82

* ED_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

previous papers (Anderson *et al*., 2005; Kobayashi *et al*., 1995), the structure of **10** was determined to be phalloidin. The isolated compounds **1**-**6** and **8** were isolated from this mushroom for the first time.

α-Amanitin (**9**) and phalloidin (**10**) were previously reported to be responsible for a variety of poisoning and toxicity (Kaneko *et al*., 2001; Floersheim, 1975; Faulstich *et al*., 1985). The major poisoning by this mushroom manifests as delayed gastrointestinal symptoms, hepatotoxicity, and 12.5% mortality (Rho *et al*., 2000). We assumed that isolated four cyclicpeptides are responsible for the poisoning symptom of this source.

The isolated compounds were tested *in vitro* for cytotoxicity against four human tumor cells using the SRB assay. Compound **9** exhibited significant cytotoxic activity against A549, SK-OV-3, SK-MEL-2, and HCT15 (ED_{50}: 1.47, 0.26, 1.57, and 1.32 μM, respectively) as shown in Table II. Recently, several cytotoxic cyclic peptides were isolated from natural sources (Seo *et al*., 2007; Isaka *et al*., 2007; Weber *et al*., 2006). In addition, the compounds **1**, **2** and **4** exhibited moderate cytotoxicity against the four human tumor cell lines.

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