# Two New Steroidal Derivatives from the Fruit Body of *Chlorophyllum molybdites*

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**Two new steroid derivatives, (22*E*,24*R*)-3$\alpha$-ureido-ergosta-4,6,8(14),22-tetraene (1) and (22*E*,24*R*)-5$\alpha$,8$\alpha$-epidioxyergosta-6,9,22-triene-3$\beta$-ol 3-*O*-$\beta$-D-glucopyranoside (2) were isolated from the fruit bodies of *Chlorophyllum molybdites* (Agaricaceae). The structures were established by spectroscopic and chemical methods. These compounds exhibited cytotoxicity against Kato III cells.**

**Key words** *Chlorophyllum molybdites*; Agaricaceae; uerido ergostene; Kato III cell

In the course of our research program aimed at the discovery of biologically active compounds from fungi, we have reported previously the isolation and structure elucidation of lanostane triterpenoids from the fruit bodies of *Laetiporus versisporus* (LLOYD) IMAZ. (Polyporaceae),[1,2] and benzofuran glycosides and a $C_{10}$ acetylenic acid from *L. sulphureus* (FR.) MURR. var. *miniatus* (JUNGH.) IMAZ.,[3] and a eudesmane sesquiterpene from *Lactarius laeticolorus* (IMAI) IMAZ. (Russulaceae).[4] We have initiated the chemical study for *Chlorophyllum molybdites* (MEYER : FR.) Massee belonging to the family Agaricaceae. It is a poisonous mushroom growing on the lawn and grass ground and is distributed in tropical and subtropical regions and a restricted area in Japan.[5,6] Chemical study on this fungus had led to the isolation of the pyrrolidine derivative alkaloids lepiotins A and B by Ohta *et al*.[7] The 70% EtOH extract of the fresh fruit bodies of the fungi was separated by successive chromatographic steps on a silica gel column followed by reversed-phase HPLC with 70—87% MeOH to afford two new steroidal compounds, **1** and **2**, together with six known ones. The known compounds, (22*E*,24*R*)-5$\alpha$,8$\alpha$-epidioxyergosta-6,9,22-triene-3$\beta$-ol (3), (22*E*,24*R*)-ergosta-8(14),22-diene-3$\beta$,7$\alpha$-diol (**4**), (22*E*,24*R*)-5$\alpha$,6$\alpha$-epoxyergosta-8(14),22-diene-3$\beta$,7$\alpha$-diol (**5**), (22*E*,24*R*)-ergosta-7,22-diene-3$\beta$,5$\alpha$,6$\beta$-triol (**6**),[8] (22*E*,24*R*)-5$\alpha$,8$\alpha$-epidioxyergosta-6,22-diene-3$\beta$-ol 3-*O*-$\beta$-D-glucopyranoside (**7**), (22*E*,24*R*)-ergosta-5,7,22-triene-3$\beta$-ol 3-*O*-$\beta$-D-glucopyranoside (**8**),[9] have previously been isolated from fungi and marine sponges.

Compound **1** was obtained as an amorphous powder, which showed an $[M+H]^+$ ion at *m/z* 437.3532 in the high-resolution (HR)-FAB-MS. This corresponds to a molecular formula $C_{29}H_{44}N_2O$, requiring nine unsaturation equivalents. The IR spectrum of **1** showed absorptions at 3450, 1655, and 1545 $cm^{-1}$. The UV spectrum of **1** exhibited absorption maximum at 285.5 ($\log \varepsilon$ 4.21) nm, implying the presence of a heteroannular triene system in the compound.[10] The $^1$H-NMR spectrum contained four doublets for secondary methyls at $\delta$ 1.06 (3H, d, $J$=6.6 Hz), 0.97 (3H, d, $J$=6.9 Hz), 0.88 (3H, d, $J$=6.9 Hz), and 0.87 (3H, d, $J$=6.9 Hz), and two singlets for tertiary methyls at $\delta$ 0.92 (3H, s) and 0.86 (3H, s), one methine proton at $\delta$ 4.87 (1H, m), in the low field, five ofefinic protons at $\delta$ 6.24 (1H, d, $J$=9.6 Hz), 5.97 (1H, d, $J$=9.6 Hz), 5.69 (1H, d, $J$=4.9 Hz), 5.29 (1H, dd, $J$=15.4, 7.4 Hz), and 5.24 (1H, d, $J$=15.4, 8.1 Hz), the latter two of which were assigned as H-23 and H-22 of a typical ergostene skeleton, and a broad $D_2O$ exchangeable proton at $\delta$ 6.24 (2H, m), and a doublet at $\delta$ 6.90 (1H, d, $J$=8.2 Hz). The last one was not correlated with a signal in the $^1$H-detected multiple quantum coherrence spectrum (HMQC). The 29 carbon signals were observed in the $^{13}$C-NMR spectrum and distortionless enhancement by polarization transfer (DEPT) experiment revealed the presence of six methyl, six methylene, six methine, two quaternary, and nine $sp^2$ carbons, five of which were protonated [$\delta$ 135.8 (d), 132.3 (d), 126.7 (d), 126.1 (d), 123.8 (d)] (Table 1). A combination of $^1$H–$^1$H correlation spectroscopy (COSY), $^1$H-detected heteronuclear multiple bond correlation spectrum (HMBC), and rotating frame nuclear Overhauser and exchange spectroscopy (ROESY) experiments enabled us to identify the (22*E*)-ergosta-4,6,8(14),22-tetraene nucleus.[11] The stereochemistry at the C-24 position for **1** was determined as *R* by comparison with the chemical shift values of **3**—**8**.[8,9,11] The remaining unassigned carbon at $\delta$ 159.2 (s) was given the long-range correlation from the doublet proton at $\delta$ 6.90, which was showing geminal coupling with H-3 at $\delta$ 4.87 (m). These observations recognized the presence of the urea junction at C-3 position, in addition to taking its molecular formula into consideration. Next, the nuclear Overhauser effect (NOE) interactions were observed between H-3/H-2$\alpha$, H-3/H-2$\beta$, H-3/NH, and H-3/H-4, in turn, NH/H-1$\alpha$, suggesting $\alpha$-orientation of the ureido group at C-3. Accordingly, these studies led to structure **1** as (22*E*,24*R*)-3$\alpha$-ureido-ergosta-4,6,8(14),22-tetraene.

Compound **2** was obtained as an amorphous powder, and the molecular formula was established as $C_{34}H_{52}O_8$ on the basis of a HR-FAB-MS [*m/z* 589.7876 $(M+H)^+$]. The $^1$H-

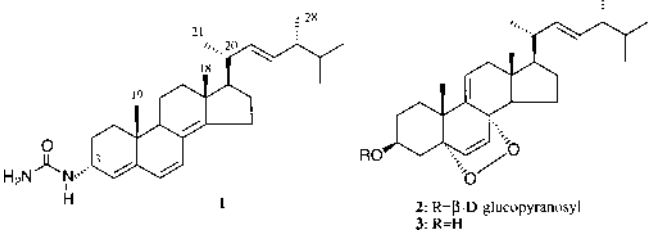


Chart 1

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Table 1. $^{1}$H- and $^{13}$C-NMR Data for Compounds **1** and **2** in Pyridine-$d_5$

| Position | **1** | | Position | **2** |
|---|---|---|---|---|
| | $^{13}$C | $^{1}$H, mult ($J$ in Hz) | | $^{13}$C |
| 1$\alpha$ | 30.3 t | 1.42 m | 1$\alpha$ | 33.0 t |
| 1$\beta$ | | 1.45 m | 1$\beta$ | |
| 2$\alpha$ | 26.7 t | 2.09 m | 2$\alpha$ | 30.0 t |
| 2$\beta$ | | 2.00 m | 2$\beta$ | |
| 3 | 44.2 d | 4.87 m | 3 | 73.7 d |
| 4 | 123.8 d | 5.69 (d, 4.9) | 4$\alpha$ | 33.8 t |
| 5 | 145.7 s | | 4$\beta$ | |
| 6 | 126.7 d | 6.24 (d, 9.6) | 5 | 82.7 s |
| 7 | 126.1 d | 5.97 (d, 9.6) | 6 | 135.2 d |
| 8 | 124.0 s | | 7 | 131.0 d |
| 9 | 45.5 d | | 8 | 78.4 s |
| 10 | 35.9 s | | 9 | 143.7 s |
| 11$\alpha$ | 19.7 t | 1.44 m | 10 | 38.5 s |
| 11$\beta$ | | 1.44 m | 11 | 119.3 d |
| 12$\alpha$ | 36.8 t | 1.13 m | 12$\alpha$ | 41.3 t |
| 12$\beta$ | | 1.94 m | 12$\beta$ | |
| 13 | 43.7 s | | 13 | 43.8 s |
| 14 | 149.2 s | | 14 | 48.6 s |
| 15$\alpha$ | 25.1 t | 2.36 m | 15$\alpha$ | 21.3 t |
| 15$\beta$ | | 2.27 m | 15$\beta$ | |
| 16$\alpha$ | 28.2 t | 1.72 m | 16$\alpha$ | 29.0 t |
| 16$\beta$ | | 1.40 m | 16$\beta$ | |
| 17 | 56.1 d | 1.08 m | 17 | 55.9 d |
| 18 | 19.3 q | 0.92 s | 18 | 13.1 q |
| 19 | 17.5 q | 0.86 s | 19 | 25.4 q |
| 20 | 39.7 d | 2.11 m | 20 | 40.2 d |
| 21 | 21.4 q | 1.06 (d, 6.6) | 21 | 20.9 q |
| 22 | 135.8 d | 5.24 (dd, 15.4, 8.1) | 22 | 132.4 d |
| 23 | 132.3 d | 5.29 (dd, 15.4, 7.4) | 23 | 136.1 d |
| 24 | 43.1 d | 1.89 m | 24 | 43.0 d |
| 25 | 33.3 d | 1.47 m | 25 | 33.3 d |
| 26 | 19.3 q | 0.87 (d, 6.9) | 26 | 19.8 q |
| 27 | 20.2 q | 0.88 (d, 6.9) | 27 | 20.1 q |
| 28 | 17.8 q | 0.97 (d, 6.9) | 28 | 17.8 q |
| C=O | 159.2 s | | Glc-1′ | 103.0 d |
| –NH | | 6.90 (d, 8.2) | 2′ | 75.3 d |
| –NH$_2$ | | 6.24 (2H, m) | 3′ | 78.6 d |
| | | | 4′ | 71.5 d |
| | | | 5′ | 78.3 d |
| | | | 6′ | 62.6 t |

NMR spectrum showed the presence of the three secondary methyl signals [$\delta$ 0.99 (3H, d, $J$=6.6 Hz), 0.95 (3H, d, $J$=6.9 Hz), 0.86 (3H, d, $J$=6.8 Hz), 0.85 (3H, d, $J$=6.8 Hz)], and two singlet methyls [$\delta$ 1.05 (3H, s), 0.74 (3H, s)], two sets of olefinic protons [$\delta$ 6.67, 6.31 (each 1H, d, $J$=8.5 Hz), 5.26 (1H, dd, $J$=15.4, 7.7 Hz), 5.17 (1H, dd, $J$=15.4, 8.5 Hz)], and an isolated olefinic proton [$\delta$ 5.44 (1H, dd, $J$=6.0, 1.9 Hz)], and one methine proton [$\delta$ 4.49 (1H, m)], in addition, one anomeric proton [$\delta$ 4.93 (1H, d, $J$=7.7 Hz)]. Its $^{13}$C-NMR spectrum contained 34 signals, of which 28 were assigned to a steroid moiety and six signals due to a hexose unit (Table 1). Acid hydrolysis of **2** afforded D-glucose, which was confirmed by specific rotation using chiral detection by HPLC analysis.[1,3] The H-1, H-2 vicinal coupling constant 7.7 Hz for glucose indicated that this sugar occurred in **2** as the $\beta$-anomer. All of the above spectral data for **2** apparently indicated (22*E*,24*R*)-5$\alpha$,8$\alpha$-epidioxyergosta-6,22-diene-3$\beta$-ol 3-*O*-$\beta$-D-glucopyranoside and an additional trisubstituted double bond in the molecular.[8] The position of the third double bond was determined to be C-9 and C-11, by the long-range coupling from H$_3$-19 at $\delta$ 1.05 to the olefinic carbon at $\delta$ 143.7 (s), which was assigned to C-9. The *R* configuration at C-24 was determined by comparison of chemical shift values as in the case of **1**. Accordingly, the structure of **2** was formulated as (22*E*,24*R*)-5$\alpha$,8$\alpha$-epidioxyergosta-6,9,22-triene-3$\beta$-ol 3-*O*-$\beta$-D-glucopyranoside.

To the best of our knowledge, compound **1** is the first report of the isolation of a steroidal compound in which the urea was condensed from a natural source.[12]

Compounds **1**—**3** were tested for cytotoxic activities against Kato III cells.[13,14] Compounds **1**—**3** showed moderate cytotoxicities *in vitro* with IC$_{50}$ values of 7.60, 14.27, and 2.69 $\mu$g/ml, respectively (positive control, hinokitiol 0.6 $\mu$g/ml).

## Experimental

Optical rotations were taken on a JASCO DIP-1000 polarimeter. IR and UV spectra were recorded on JASCO FT-IR 5300 and Hitachi U-3000 spectrometers, respectively. NMR spectra were recorded on a Varian UNITY 600 spectrometer in $C_5D_5N$ solution. NMR experiments included $^{1}$H–$^{1}$H COSY, DEPT, HMQC, HMBC, and ROESY. Coupling constants ($J$ values) are given in Hertz (Hz). HR-FAB-MS was measured on a JEOL AX-500 mass spectrometer. Silica gel 60 (230—400 mesh, Merck) was used for column chromatography, and Silica gel 60F-254 (Merck) for TLC.

**Plant Material** The fruit bodies of *Chlorophyllum molybdites* were collected in Tokushima, Japan, in Autumn 1999. A voucher specimen (TB3018) is deposited in the Herbarium of Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan.

**Extraction and Isolation** The fresh fruit bodies (3.35 kg) of *C. molybdites* were extracted with 70% EtOH at room temperature for 6 weeks. The ethanolic extract was partitioned between EtOAc and H$_2$O. The EtOAc soluble portion (13.53 g) was repeatedly subjected to silica gel column chromatography with $CH_2Cl_2$–MeOH–H$_2$O (50 : 1 : 0—25 : 4 : 0.1) to afford six fractions (frs. 1—6). Fraction 2 (0.5 g) was purified by preparative HPLC (ODS, 70—80% $CH_3OH$) to afford **3** (12.4 mg). Fractions 3 (0.48 g) and 4 (0.44 g) were purified by preparative HPLC (octadecyl silica (ODS), 85—87% $CH_3OH$) to afford **4** (5.7 mg) from fr. 3, and **1** (3.2 mg), and **5** (2.9 mg) from fr. 4. Fraction 5 (4.0 g) was further subjected to silica gel column chromatography with $CH_2Cl_2$–MeOH–H$_2$O (25 : 8 : 0.1) to give four fractions (frs. 5-1—4). Fractions. 5-2 (0.2 g) and 5-3 (0.21 g) were purified by preparative HPLC (ODS, 85% $CH_3OH$) to afford **2** (7 mg) and **6** (5.5 mg) from fr. 5-2, and **7** (18 mg) and **8** (1.5 mg) from fr. 5-3, respectively.

Compound **1**: An amorphous powder, $[\alpha]_D^{25}$ +364.5° ($c$=0.3, MeOH). UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 202 (3.38), 274 (sh.), 285.5 (4.21), 297 (sh.) nm. FT-IR (film) cm$^{-1}$: 3450, 2995, 1655, 1545, 1460. HR-FAB-MS $m/z$: 437.3532 (Calcd for $C_{29}H_{44}N_2O$+H: 437.3532). $^{1}$H- and $^{13}$C-NMR ($C_5D_5N$) see Table 1.

Compound **2**: An amorphous powder, $[\alpha]_D^{25}$ +5.6° ($c$=2.0, MeOH). FT-IR (film) cm$^{-1}$: 3400, 1060, 1040. HR-FAB-MS $m/z$: 589.7876 (Calcd for $C_{34}H_{52}O_8$+H, 589.7879). $^{1}$H-NMR ($C_5D_5N$) $\delta$: 0.74 (3H, s, H$_3$-18), 0.86 (6H, d, $J$=6.8 Hz, H$_3$-26, -27), 0.95 (3H, d, $J$=6.9 Hz, H$_3$-28), 0.99 (3H, d, $J$=6.6 Hz, H$_3$-21), 1.05 (3H, s, H$_3$-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$_2$-6′), 4.46 (1H, dd, $J$=11.9, 2.5 Hz, H$_2$-6′). $^{13}$C-NMR ($C_5D_5N$) see Table 1.

**Acid Hydrolysis of 2** A solution of **2** (2 mg) in 5% $H_2SO_4$–dioxane (1 : 1) was heated at 100 °C for 2 h. The reaction mixture was diluted with H$_2$O, and then neutralized with Amberlite IRA-35 and evaporated *in vacuo* to dryness. The identification and the D or L configuration of the sugar was determined by using refraction index (RI) detection (Waters 410) and chiral detection (Shodex OR-1) by HPLC [Shodex RSpak NH2P-50 4E column, $CH_3CN$–H$_2$O–$H_3PO_4$ (95 : 5 : 1), 1 ml/min, 47 °C] by comparison with an authentic sugar (10 mmol of D-Glc). The sugar portion from **2** gave the peak of D-(+)-Glc at 20.7 min.

**Medium** Complete RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) containing 100 $\mu$g/ml streptomycin and 0.0035 $\mu$l/ml 2-mercaptoethanol and Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Japan) containing 50 $\mu$g/ml kanamycin sulfate and 4 mM L-glutamin were used throughout the study.

**Cells** Human stomach cancer cells Kato-III were maintained in the DMEM and RPMI-1640 (1 : 1) supplemented 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, U.S.A.). Cells were cultured in the medium at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air chamber throughout the study.

**Cytotoxic Assay** Kato III cells in the exponential growth phase were plated in 96-well flat-bottom microplates at a density of $3\times10^3$ cells per 100 $\mu$l in each well and grown for 24 h in the medium. After removal of medium, 100 $\mu$l of fresh medium with various concentration of test compounds was added. After 72 h culturing, cell growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Test compounds 1—3 dissolved in dimethyl sulfoxide (DMSO) were used.

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