A New Sterol From the Polypore Fungus
Ganoderma luteomarginatum and
Its Cytotoxic Activities

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Abstract
A new sterol, ganolutol A (1), together with 4 known compounds 2 to 5, were isolated from the polypore fungus Ganoderma luteomarginatum collected in Myanmar. The chemical structures of the isolated compounds were determined based on extensive spectroscopic analyses in conjunction with comparisons with published data. Compounds 1 to 4 exhibited moderate cytotoxic activities against A549 (lung), MCF-7 (breast), and HeLa (cervical) human cancer cell lines, with IC₅₀ values ranging from 10.1 to 86.6 μM.

Keywords
Ganoderma luteomarginatum, Ganodermataceae, polypore fungus, ergostane, cytotoxicity

Introduction
Ganoderma luteomarginatum is a rare polypore fungus belonging to the family Ganodermataceae. It is mainly distributed throughout tropical and subtropical areas including China, Laos, Myanmar, Thailand, and Vietnam.¹,² The G luteomarginatum fruiting bodies have been utilized in traditional medicine to treat and prevent various diseases such as hypertension, diabetes, hepatitis, and cancers in China, Japan, and Korea.³,⁴ The fruiting bodies are known as “Lin zhi” in Myanmar and their extracts have long been used locally for the purposes of liver protection, blood purification, and detoxification, as well as tumor treatment.⁵ A few previous phytochemical investigations of ethanol extracts of G luteomarginatum fruiting bodies collected in China led to the isolation of 12 lanostane-type triterpene acids, a pair of alkaloid enantiomers, and 9 lanostane triterpenoids.¹,⁶,⁷ Among them, lanostane triterpenoids such as (17Z)-3β,7β,15β-trihydroxy-11,23-dioxolanost-8,17(20)-dien-26-oate, (20E)-15β-hydroxy-3,7,11,23-tetraoxolanost-20(22)-en-26-oate, and (5α,24E)-3β-acetoxy-26-hydroxylanosta-8,24-dien-7-one exhibited strong cytotoxicities against gastric HGC-27, cervical HeLa, and lung A549 human cancer cell lines.¹,⁶ In the course of our search for cytotoxic compounds from traditional folk medicines in Myanmar, phytochemical investigations of the ethyl acetate-soluble fraction of a 70% ethanolic extract of the Myanmar G luteomarginatum fruiting bodies revealed the presence of one new ergostane-type steroid and four known compounds. Herein, we report the isolation and structure elucidation of the compounds and their cytotoxicities.

Results and Discussion
Various chromatographic procedures on the ethyl acetate-soluble fraction of the 70% ethanolic extract of G luteomarginatum led to the isolation of 5 compounds, including 1 new ergostane-type steroid, named ganolutol A (1) and 4 known compounds (Figure 1). The known compounds were identified as ergosterol (2),⁸ ergosterol peroxide (3),⁹ (3β,5α,6β,22E)-6-methoxyergost-7,22-diene-3,5-diol (4),¹⁰ and 4-hydroxy-2′,4′-dimethoxydihydrochalone (5),¹¹ by comparison of their 1D NMR spectroscopic data with those in the literature.

Compound 1 was obtained as a white amorphous powder. Its molecular formula was determined as C₂₈H₄₄O₅ from HR-ESI-MS data (m/z 459.3108, [M-H]⁻, calcd. for C₂₈H₄₄O₅ 459.3104). In the course of our search for cytotoxic compounds from traditional folk medicines in Myanmar, phytochemical investigations of the ethyl acetate-soluble fraction of a 70% ethanolic extract of the Myanmar G luteomarginatum fruiting bodies revealed the presence of one new ergostane-type steroid and four known compounds. Herein, we report the isolation and structure elucidation of the compounds and their cytotoxicities.

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C$_{28}$H$_{41}$(O$_3$, 459.3116) and interpretation of the $^{13}$C NMR data, which indicated 7 degrees of unsaturation (Supplementary Figures S7). The IR spectrum showed absorption bands corresponding to hydroxy (3418 cm$^{-1}$) and olefinic (1633 cm$^{-1}$) functionalities. The $^1$H NMR data (Table 1 and Supplementary Figure S1) showed characteristic signals corresponding to 2 tertiary methyl protons [$\delta$H 0.63 (s, H$_3$-18), 1.37 (s, H$_3$-19)], 4 secondary methyl protons [$\delta$H 1.03 (d, $J$ = 6.4 Hz, H$_3$-21), 0.87 (d, $J$ = 6.8 Hz, H$_3$-26), 0.88 (d, $J$ = 6.8 Hz, H$_3$-27), 0.96 (d, $J$ = 6.8 Hz, H$_3$-28)], 3 olefinic protons [$\delta$H 5.95 (t, $J$ = 3.6 Hz, H-7), 5.23 (dd, $J$ = 15.4, 7.2 Hz, H-22), 5.22 (dd, $J$ = 15.4, 8.0 Hz, H-23)], and 2 oxygenated methine protons [$\delta$H 4.47 (m, H-3), 4.64 (t, $J$ = 3.6 Hz, H-6)]. The $^{13}$C NMR data (Table 1 and Supplementary Figures S2 and S4) revealed 28 carbon signals, consisting of 2 oxygenated methine carbons [$\delta$C 66.6 (C-3), 86.4 (C-6)], 2 oxygenated carbons [$\delta$C 87.7 (C-5), 85.4 (C-9)], 2 quaternary carbons [52.2 (C-10), 42.6 (C-13)], 4 olefinic carbons [$\delta$C 121.3 (C-7), 143.0 (C-8), 136.3 (C-22), 132.8 (C-23)], 5 methine carbons [$\delta$C 53.1 (C-14), 56.0 (C-17), 41.1 (C-20), 43.5 (C-24), 33.8 (C-25)], 7 methylene carbons [$\delta$C 29.6 (C-1), 33.2 (C-2), 37.3 (C-4), 24.0 (C-11), 37.4 (C-12), 23.7 (C-15), 28.9 (C-16)], and 6 methyl carbons [$\delta$C 12.1 (C-18), 17.6 (C-19), 21.7 (C-21), 20.3 (C-26), 20.6 (C-27), 18.3 (C-28)].

The above NMR data closely resembled those of the ergostane-type steroid isolated from Panellus serotinus, 5α,9α-epidioxy-(22E)-ergosta-7,22-diene-3β,6β-diol (1′), with a lower molecular weight than that of 1 by 16-mass units.$^{12}$ The main difference between 1 and 1′ was the oxygenated methine resonance at C-6, where the oxygenated methine proton and carbon signals were shifted upfield from $\delta$H$_1$ 4.26 (m) and $\delta$C 72.2 in 1′ to $\delta$H$_1$ 4.64 (t, $J$ = 3.6 Hz) and $\delta$C 86.4 in 1, respectively. Considerations of the differences between the NMR data of 1 and 1′ and the 16-mass unit higher molecular weight of 1 than 1′ suggested that compound 1 is a hydroperoxy analog of 1′ at C-6. The $^1$H-$^1$H COSY correlations of H-14 ($\delta$H 2.29)/H-15β ($\delta$H 1.36)/H-16β ($\delta$H 1.93)/H-17 ($\delta$H 1.19)/H-20 ($\delta$H 2.00)/H$_2$-21 ($\delta$H 1.03)/H-22 ($\delta$H 5.23) and the HMBC correlations from H-11β ($\delta$H 1.84) to C-8 ($\delta$C 143.0) and C-13 ($\delta$C 42.6), from H-12β ($\delta$H 1.89) to C-9 ($\delta$C 143.0) and C-14 ($\delta$C 53.1), from H$_3$-28 to C-23, C-24, and C-25, and from H$_3$-26 and H$_3$-27 to C-24 and C-25 (Figure 2 and Supplementary Figures S3 and S5) indicated that 1 possessed the same planar structures as those of 1′ at the C and D rings and the side-chain unit at C-17. The $^1$H-$^1$H COSY cross-peaks of H$_2$-1 ($\delta$H 1.43, 2.21)/H$_2$-2 ($\delta$H 2.22, 1.77)/H-3 ($\delta$H 4.47)/H$_2$-4 ($\delta$H 2.86, 2.76) and the HMBC correlations from H-4α ($\delta$H 2.86) to C-5 ($\delta$C 87.7) and C-10 ($\delta$C 52.2) and from H$_3$-19 to C-1 ($\delta$C 29.6), C-5 ($\delta$C 87.7), and C-9 ($\delta$C 85.4) supported the presence of the hydroxy and methyl groups at C-3 and C-19, respectively, as well as the peroxide bridge between C-5 and C-9, as in the case of 1′ (Figure 2). The HMBC correlations from H$_3$-18 ($\delta$H 0.63) to C-12 ($\delta$C 37.4), C-14 ($\delta$C 53.1), and C-17 ($\delta$C 56.0) confirmed the presence of the methyl group at the same C-18 position as that of 1′. Furthermore, the $^1$H-$^1$H COSY cross-peak of H-6 ($\delta$H 4.64)/H-7 ($\delta$H 5.95) and the HMBC correlations from H-7 ($\delta$H 5.95) to C-9 ($\delta$C 85.4) and C-14 ($\delta$C 53.1) indicated the existence of the oxygenated group at C-6, suggesting that a hydroperoxy group was attached to C-6.

The relative configuration of 1 was assigned from the NOESY spectrum analyses and the coupling constant value (Figure 3 and Supplementary Figure S6). The NOESY correlations starting from H$_3$-18 ($\delta$H 0.63) to H-1β ($\delta$H 2.21) via H-11β ($\delta$H 1.84)/H$_3$-19 ($\delta$H 1.37) and from H$_3$-18 ($\delta$H 0.63) to H-4β ($\delta$H 2.76) via H-11β/H$_3$-19/H-2β ($\delta$H 1.77) indicated that
the methyl groups at C-10 and C-13, H-1β, and H-4β were β-oriented. The NOESY correlations from H-1α (δH 1.43) to H-3 (δH 4.47) and from H-4α (δH 2.86) to H-6 (δH 4.64) and the lack of the NOESY correlation between H-6 and H3-19 confirm the α-orientations of H-3 and H-6 as well as β-orientations of the hydroxyl group at C-3 and the hydroperoxy group at C-6. Furthermore, the NOESY correlations from H-12β (δH 1.89) to H-14 (δH 2.29) and H-14 to H-17 (δH 1.19) indicated the α-orientations of H-14 and H-17. The Δ22-double bond was elucidated to have the E configuration, based on the 1H-1H coupling constant (J = 15.4 Hz) between H-22 and H-23.

Primary CD calculation analyses of the (3S,5R,6R,9R,10R,13R,14R,17R)-1 model with each set of 20S and 24S, 20S and 24R, 20R and 24S, and 20R and 24R indicated that the side-chain did not affect the CD spectra, suggesting that the absolute configurations at C-20 and C-24 could not be determined by CD analysis (Supplementary Figure S8). Thus, the absolute configuration of 1 was elucidated by comparisons of its CD spectrum with the calculated CD spectra of (3S,5R,6R,9R,10R,13R,14R,17R,20R,24R)-1 and its enantiomer (Figure 4) and the chemical shift values between 1 and ergosterol (2), as well as from a biogenetic viewpoint. Compound 1 showed positive and negative Cotton effect values at 265 and 218 nm, respectively, in the

Table 1. 1H (400 MHz) and 13C (100 MHz) NMR Data of 1 in pyridine-d5.

| Position | δH (J in Hz) | δC | Type | Reference 1’ |
|----------|--------------|-----|------|--------------|
| 1α       | 1.43, m      | 29.6| CH2  | 28.7         |
| 1β       | 2.21, m      |     |      |              |
| 2α       | 2.22, m      | 33.2| CH2  | 31.7         |
| 2β       | 1.77, m      |     |      |              |
| 3        | 4.47, m      | 66.6| CH   | 66.8         |
| 4α       | 2.86, dd (14.8, 11.2) | 37.3| CH2  | 34.8         |
| 4β       | 2.76, dd (14.8, 3.6) |     |      |              |
| 5        | 87.7         |     | C    | 86.6         |
| 6        | 4.64, t (3.6) | 86.4| CH   | 72.2         |
| 7        | 5.95, t (3.6) | 121.3| CH  | 122.5        |
| 8        | 143.0        |     | C    | 141.8        |
| 9        | 85.4         |     | C    | 84.7         |
| 10       | 52.2         |     | C    | 51.0         |
| 11α      | 1.80, m      | 24.0| CH2  | 28.0         |
| 11β      | 1.84, m      |     |      |              |
| 12α      | 1.34, m      | 37.4| CH2  | 34.7         |
| 12β      | 1.89, m      |     |      |              |
| 13       | 42.6         |     | C    | 41.9         |
| 14       | 2.29, m      | 53.1| CH   | 52.1         |
| 15α      | 1.71, m      | 23.7| CH2  | 23.2         |
| 15β      | 1.36, m      |     |      |              |
| 16α      | 1.71, m      | 28.9| CH2  | 28.1         |
| 16β      | 1.93, m      |     |      |              |
| 17       | 1.19, m      | 56.0| CH   | 55.5         |
| 18       | 0.63, s      | 12.1| CH3  | 11.7         |
| 19       | 1.37, s      | 17.6| CH3  | 17.3         |
| 20       | 2.00, m      | 41.1| CH   | 39.5         |
| 21       | 1.03, d (6.4)| 21.7| CH3  | 21.1         |
| 22       | 5.23, dd (15.4, 7.2) | 136.3| CH  | 135.2        |
| 23       | 5.22, dd (15.4, 8.0) | 132.8| CH  | 132.2        |
| 24       | 1.88, m      | 43.5| CH   | 42.8         |
| 25       | 1.48, m      | 33.8| CH   | 33.1         |
| 26       | 0.87, d (6.8)| 20.3| CH3  | 19.7         |
| 27       | 0.88, d (6.8)| 20.6| CH3  | 20.0         |
| 28       | 0.96, d (6.8)| 18.3| CH3  | 17.6         |

Figure 2. Key HMBC (arrows) and 1H-1H COSY (bold lines) correlations of 1.

Figure 3. Key NOESY correlations (dashed arrows) of 1.

Figure 4. Calculated and experimental ECD spectra of 1.
Table 2. Cytotoxic Activities of Compounds 1 to 5.

| Sample  | IC_{50} (µg/mL, µM) |
|---------|---------------------|
| A549    | MCF-7               | HeLa    |
| EsOAc   | 47.6<sup>a</sup>    | 65.6<sup>a</sup> | 55.2<sup>a</sup> |
| 1       | 63.1<sup>b</sup>    | 51.6<sup>b</sup> | 86.6<sup>b</sup> |
| 2       | 19.1<sup>b</sup>    | 69.3<sup>b</sup> | 20.2<sup>b</sup> |
| 3       | 19.3<sup>b</sup>    | 18.1<sup>b</sup> | 10.1<sup>b</sup> |
| 4       | 18.7<sup>b</sup>    | 19.6<sup>b</sup> | 26.9<sup>b</sup> |
| 5       | 64.8<sup>b</sup>    | 124.3<sup>b</sup> | 95.3<sup>b</sup> |
| 5-fluorouracil<sup>d</sup> | 5.0<sup>b</sup> | 3.2<sup>b</sup> | 18.3<sup>b</sup> |

Note: <sup>a</sup>µg/mL. <sup>b</sup>µM. <sup>c</sup>Ethyl acetate-soluble fraction. <sup>d</sup>Positive control.

CD spectrum. The CD spectrum was closely similar to that of the (3,5R,6R,9R,10R,13R,14R,17R,20R,24R)-1 model, revealing that 1 possessed the same absolute configurations as those of 2 at C-3, C-9, C-10, C-13, C-14, and C-17. In light of the same absolute configurations at these positions between 1 and 2 and the quite similar carbon chemical shift values between these compounds, the remaining absolute configurations of C-20 and C-24 in 1 were biogenetically determined to be the same R and R as those in 2, respectively. Hence, compound 1 was assigned to be (3R,5R,6R,9R,10R,13R,14R,17R,20R,24R)-5,9-epidioxy-3-hydroxy-(22E)-ergosta-7,22-dien-6-hydroperoxide, and was named ganolutol A.

The ethyl acetate-soluble fraction and the isolated compounds 1 to 5 were assessed for their cytotoxicities against A549, MCF-7, and HeLa human cancer cell lines. Compounds 1 to 4 did not show any cytotoxicities against the tested cancer cell lines as shown in Table 2 and Supplementary Figure S9.

Conclusions

A new sterol, ganolutol A (1), and four known compounds 2 to 5 were isolated from the polypore fungus, *G luteomarginatum*. Compounds 1 to 4 did not show any cytotoxicity against the tested A549, MCF-7, and HeLa cancer cell lines.

Experimental Section

General Experimental Procedures

NMR spectra were recorded on a JEOL ECA400II spectrometer. Pyridine-d<sub>5</sub> and CDCl<sub>3</sub> were used to solve all isolated compounds. δ<sub>H</sub> 8.74 and δ<sub>C</sub> 150.35 for pyridine-d<sub>5</sub> and δ<sub>H</sub> 7.26 and δ<sub>C</sub> 77.0 for CDCl<sub>3</sub> of the residual solvent peaks were used to calibrate the signals. HR-ESI-MS data were obtained with a Shimadzu LCMS-IT-TOF spectrometer. Optical rotation and CD measurements were performed on a JASCO P2100 polarimeter and a JASCO J-805 spectropolarimeter, respectively. UV and IR spectra were measured on an Implen NP80 nanospectrometer and a JASCO FT/IR-460 Plus spectrometer (KBr pellets), respectively. Silica gel 60N (spherical, neutral, 40-50 µm, Kanto Chemical) and Cosmosil 75C18-OPN (Nacalai Tesque) were used to perform normal phase and reverse phase open column chromatography, respectively. Thin-layer chromatography (TLC) was performed on silica gel GF<sub>254</sub> pre-coated plates (Merck). The compounds were detected under a UV lamp (254 and 365 nm) and by spraying with a p-anisaldehyde stain solution followed by heating at 170 °C for 10 min in a drying cabinet.

Fungal Material

The fruiting bodies of *G luteomarginatum* were purchased from the local medicinal market in Aungban, Shan State of Myanmar and were identified by Dr Ni Lar Cho, a botanist at the Department of Botany, Meiktila University. A voucher specimen (31185) has been deposited in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

Extraction and Isolation

The fruiting bodies of *G luteomarginatum* (1.49 kg) were chopped and extracted with 70% ethanol under sonication (12 L, 3 h, ×5) at 30 °C after drying. The solvent was then evaporated, and the resultant 70% ethanolic extract (45.9 g) was suspended in water and partitioned with ethyl acetate to give an ethyl acetate-soluble fraction (31.5 g). The ethyl acetate-soluble fraction was chromatographed on a silica gel column eluted with n-hexane – ethyl acetate (90:10 to 0:100) and ethyl acetate – methanol (90:10 to 0:100) to give eleven fractions (F<sub>1</sub>–F<sub>11</sub>). Fraction F<sub>1</sub> (2.8 g) was rechromatographed on a silica gel column eluted with n-hexane – dichloromethane – ethyl acetate (40:40:20 to 50:50:50) to afford 4 subfractions, F<sub>1</sub>–1 (180 mg), F<sub>1</sub>–2 (166 mg), F<sub>1</sub>–3 (54 mg), and F<sub>1</sub>–4 (2.4 g). Subfraction F<sub>1</sub>–1 (180 mg) was subjected to a silica gel column eluted with n-hexane – ethyl acetate (80:20 to 0:100) to afford 2 (12.0 mg) and 3 (16.0 mg). Purification of subfraction F<sub>1</sub>–3 (54 mg) on a silica gel column eluted with n-hexane – ethyl acetate (60:40 to 50:50) gave 4 (11 mg). Subfraction F<sub>1</sub>–4 (2.4 g) was rechromatographed on a silica gel column eluted with n-hexane – ethyl acetate (60:40 to 50:50) to give 7 subfractions (F<sub>4</sub>–1 to F<sub>4</sub>–7). Subfraction F<sub>4</sub>–1 to F<sub>4</sub>–4 was subjected to chromatography on a silica gel column eluted with n-hexane – ethyl acetate (66:34 to 50:50) to afford 1 (10.5 mg). Fraction 5 (1.6 g) was subjected to chromatography on an ODS column, eluted with water – methanol (100:0 to 0:100) to give 7 subfractions (F<sub>5</sub>–1 to F<sub>5</sub>–7). Compound 5 (7.7 mg) was obtained from the subfraction F<sub>5</sub>–3 by chromatography on a silica gel column, eluted with n-hexane – ethyl acetate (66:34 to 50:50).

Ganolutol A (1): white amorphous powder; [α]<sub>D</sub><sup>25</sup> = +31.5 (c 0.1, MeOH); UV (MeOH) λ<sub>max</sub> (log ε) 209 (3.62), 218 (3.64) nm; CD (MeOH) λ<sub>max</sub> (Δε) 218 (~6.30), 265 (+1.49) nm; IR (KBr) ν<sub>max</sub> 3418, 1720, 1633, 1457, 1388, 1103, 840 cm<sup>−1</sup>.
1H and 13C NMR data, see Table 1; negative HR-ESI-MS m/z [M-H]− 459.3108 (calcd. for C28H43O5, 459.3116).

ECD Calculations
The conformational search was conducted as previously described with some modifications.13 The Avogadro 1.2 program was used to obtain the MMFF94 molecular force field. The optimization of all possible conformers at the B3LYP level of theory was performed by using the 6-31G(d) basis set, and the minimum energy of the structure was ensured by calculating their optimized geometries. The geometries used for the ECD calculations were obtained from Gaussian 16 at the B3LYP/6-31G(d) level. GaussSum using a half bandwidth of 0.2 eV was used to generate the ECD curves. All spectra of the lowest energy conformations were averages.

Cell Culture and Cytotoxicity Assay
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay14 was used to evaluate the cytotoxicities of the isolated compounds in cell lines. The human cancer cell lines (A549, MCF-7, and HeLa) were cultured in α-Minimum essential medium with phenol red and L-glutamine (α-MEM, Wako).15-20 A 1% antibiotic antifungal solution (Sigma) and 10% fetal bovine serum (FBS, Sigma) were supplemented to all media. For the MCF-7 cells, 1% 1 mM sodium pyruvate (Gibco) and 1% 0.1 mM nonessential amino acids (NEAA, Gibco) were supplemented in the growth medium. Briefly, each cancer cell line was seeded at 2×10^3 cells per well in 96-well plates and incubated for 24 h in the respective medium at 37 °C, under a 5% CO2 and 95% air atmosphere. The cells were then washed with phosphate-buffered saline (PBS), and the tested samples were added to each well at 6 concentrations (6.25, 12.5, 25.0, 50.0, 100, and 200 µM). After a 72 h incubation, the cells were washed with PBS, and to each well, 100 µL of medium containing 10 µL of the MTT solution (5 mg/mL) was added and incubated for 3 h. Subsequently, each well was monitored to calculate cell viability at 570 nm, using the following equation (each set of cell viability at each concentration was the mean value of the data from 3 wells):

(%)Cell viability = 100 × \[
\frac{[\text{Abs}_{\text{test sample}} - \text{Abs}_{\text{blank}}]}{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}]}
\]

GraphPad Prism 8.0 software was used to evaluate the IC50 value of the cytotoxicity on a sigmoid dose–response model. 5-Fluorouracil (Wako) was used as a positive control.

Declaration of Conflicting Interests
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Ethical Approval
Not applicable, because this article does not contain any studies with human or animal subjects.

Informed Consent
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Trial Registration
Not applicable, because this article does not contain any clinical trials.

Supplemental Material
Supplemental material for this article is available online.
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