A New Sterol From Sporoderm-Broken
Ganoderma sinense Spores and Its Anticancer Activity

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Abstract
A new sterol, ganoderaside E (1), and 4 known sterols, (22E,24R)-3β,5x-dihydroxyergosta-7,22-diene-6-one (2), (22E,24R)-3β,5x,9α-trihydroxyergosta-7,22-diene-6-one (3), (22E,24R)-ergosta-7,9(11),22-triene-3β,5x,6β-triol (4), and (22E,24R)-ergosta-7,22-diene-3β,5x,6α-triol (5), were isolated for the first time from the sporoderm-broken spores of Ganoderma sinense Zhao, Xu et Zhang. Their structures were determined by spectroscopic techniques such as nuclear magnetic resonance spectroscopy and mass spectrometry. Furthermore, all the compounds were evaluated for their in vitro cytotoxicity and migration inhibition on human non-small-lung cancer A549 cells. Compound 1 exhibited cytotoxicity with a half-maximal inhibitory concentration value of 21.12 ± 1.46 µM. Compound 5 exhibited the strongest and most significant antimetastatic activity at concentrations of 100 and 200 µM.

Keywords
Ganoderma sinense, spores, steroids, cytotoxic activity, migration inhibition

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The members of the genus Ganoderma (lingzhi) have been widely used as a crude drug and health food in East Asia for thousands of years. The fruiting bodies of Ganoderma sinense Zhao, Xu et Zhang and Ganoderma lucidum (Leyss. exFr.) Karst, 2 of the most well-known species, have been certified as authoritative medical materials in the Chinese Pharmacopoeia 2015, owing to their medicinal values such as anticancer activity1-3 and immunomodulating activity.4,5

In light of the development of spores collection method and sporoderm-breaking technology, spores can be collected at a larger scale, and a greater number of bioactive substances can be extracted.6 As a result, more and more chemical and pharmacological research are being carried out on G. lucidum spores.7-9 However, there are only a few reports on G. sinense. A study showed that the extract obtained from G. sinense spores by supercritical CO2 fluid extraction had tumoricidal activity, thus imparting antitumor effect. The extract could also stimulate the activity of human monocytes.10 However, there are no studies yet on the chemical structures and bioactivities of the components of the extract from G. sinense spores. Therefore, in this study, we explored the extract from the G. sinense spores in terms of the above 2 factors, namely, structure and biological activity, and this led to the identification of a new highly oxygenated sterol (22E,24R)-9x,15α-dihydroxyergosta-4,6,8(14),22-tetraen-3-one (1) (Figure 1), together with 4 known sterols, (22E,24R)-3β,5x-dihydroxyergosta-7,22-diene-6-one (2), (22E,24R)-3β,5x,9α-trihydroxyergosta-7,22-diene-6-one (3), (22E,24R)-ergosta-7,9(11),22-triene-3β,5x,6β-triol (4), and (22E,24R)-ergosta-7,22-diene-3β,5x,6α-triol (5) (Figure 1). Herein, we reported the isolation, structural elucidation, and tumoricidal activities of these compounds.

Compound 1 (Figure 1) was isolated as a colorless oil. The compound had an absorption maximum at 335 nm, and it fluoresced upon irradiation with UV light of 365 nm, indicating the presence of a highly conjugated system. The molecular formula of 1 was determined to be C35H56O3 by high-resolution electrospray ionization mass spectrometry (HRESIMS; m/z 525.3964 [M + H]+, calculated for 525.3966) (supplemental figure S1) and 425.3046 [M + H]+, calculated for 425.3056) (supplemental figure S1 and supplemental table S1). The 1H nuclear magnetic resonance (NMR) spectrum indicated 6 methyl protons at δH 1.17 (s), 1.11 (s), 0.96 (s), 0.89 (d, J = 6.8 Hz), and 0.87 (d, J = 6.8 Hz). The 1H and 13C nuclear magnetic resonance (NMRS) spectra indicated 6 methyl protons at δH 1.17 (s), 1.11 (d, J = 6.8 Hz), 0.96 (s), 0.89 (d, J = 6.8 Hz), and 0.87 (d, J = 6.8 Hz). The 1H and 13C, and distortionless enhancement by polarization transfer-135 NMR spectra indicated 28 carbons, confirming the presence of 6 methyl groups.

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5 methylene groups, 5 methine groups (1 hydroxylated secondary carbon at δ C 69.8 and 5 sp² methines at δ C 136.4, 134.0, 133.5, 126.3, and 126.1), 6 quaternary carbons (1 hydroxylated tertiary carbon at δ C 73.6, and 3 sp² carbons at δ C 165.8, 159.6, and 132.8), and 1 carbonyl carbon at δ C 202.2 (supplemental figures S2–S4). Heteronuclear multiple bond correlation (HMBC) from H-17, H-21 to C-22 (δ C 136.4), and from H-25, H-28 to C-23 (δ C 134.0) suggested the presence of a C₂₂-C₂₃ double bond. The coupling constant between H-22 (dd, \( J = 15.2, 7.8 \) Hz) and H-23 (dd, \( J = 15.2, 7.2 \) Hz) indicated an E-configuration of the double bond at C-22. Generally, natural sterols isolated from fungi possess the 24β configuration, indicating a phylogenetic significance.¹⁵ Thus, compound 1 was presumed to possess the 24R configuration because of the presence of a \( \Delta^{22} \) unsaturated side chain. The 24R configuration was also confirmed from the corresponding \( ^{1} \)H and \( ^{13} \)C chemical shifts of the side chain of 9α,11α-dihydroxyergosta-4,6,8(14),22-tetraen-3-one,¹⁶ whose structure was determined by X-ray crystallography. The analysis of the NMR data and a comparison with the compounds reported previously revealed that 1 contained the (22E,24R)-ergosta-4,6,8(14),22-tetraen-3-one skeleton.¹⁶⁻¹⁸ The structure was further supported by 2-dimensional NMR experiments (correlation spectroscopy [COSY], heteronuclear single quantum coherence, and HMBC) (supplemental figures S5-S8). The HMBC signals arose from the correlations of methyl protons at δ H 0.96 (H-18) to C-12, C-13, C-14, and C-17; methyl protons at δ H 1.17 (H-19) to C-1, C-5 (δ C 165.8), C-9 (δ C 73.6), and C-10; H-1, H-2 to C-3 (δ C 202.2); H-4 to C-10; H-6 to C-5 and C-10; H-7 to C-8, C-9, and C-14; and hydroxylated methine at δ H 4.82 (H-15) to C-13 and C-14. Thus, the hydroxylated secondary and quaternary carbon atoms were assigned to C-15 and C-9, respectively. In addition, the COSY spectrum revealed that the partial structure was C-1 to C-2, C-6 to C-7, C-11 to C-12, and C-15 to C-28. The key correlations, as obtained from the HMBC and COSY spectra, are indicated by the arrows and bold bonds, respectively, in Figure 2.

The relative configuration of compound 1 was assigned based on the results of nuclear Overhauser effect spectroscopy (NOESY) (Figure 2). A NOESY correlation between H₃-18 and H-20 revealed that they were on the same side of compound 1. The chemical shifts of H-20 (δ H 2.17) and H₃-21 (δ H 1.11) also supported the R-configuration at C-20.¹⁶ The 17R configuration was determined from the NOESY correlations of H-17/H-21. The cross-peaks of H₃-18/H-15,
H₃-18/H-11β (δ₁₉ 2.10), and H-11β/H₃-19 in the NOESY spectrum indicated that they were on the same side. Thus, the relative configuration of 15-OH was α-oriented. The stereo-chemical orientation of 9-OH was presumed to be α, because the B and C rings of all natural steroids are trans-fused. Furthermore, the 9α-OH configuration was confirmed by the positive Cotton effect at about 361 and 250 nm and negative Cotton effect at about 314 nm (Figure 3). This conclusion was arrived at by comparing the calculated electronic circular dichroism (ECD) spectrum with the experimental spectrum. The calculated molecular orbitals (MOs) of (9α)-1 contributing to the rotatory strengths, indicated the crucial role of the stereochemical orientation of 9-OH in generating the ECD spectrum (supplemental figure S9). The experimentally observed Cotton effect around 361 nm primarily arose owing to the positive rotatory strengths at 353 and 343 nm, resulting from the

Figure 2. Structure of compound 1 with key HMBC, ¹H-¹H COSY, and NOESY correlations. COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; NOESY, nuclear Overhauser effect spectroscopy.

Figure 3. Comparison of the calculated electronic circular dichroism spectra with the experimental spectrum of compound 1 in MeOH.
Table 1. Effect of the Test Compounds on the Migration of A549 Cells Induced by Serum Starvation.

| Group        | Concentration (µM) | Number of migrated cells |
|--------------|--------------------|--------------------------|
| Blank control| -                  | 947 ± 43                 |
| Model        | -                  | 1247 ± 44\(^a\)          |
| Positive control\(^b\) | 10   | 135 ± 18\(^c\)          |
|              | 5                  | 1185 ± 56                |
|              | 10                 | 1170 ± 22                |
|              | 20                 | 1164 ± 33                |
| 2            | 50                 | 1154 ± 47                |
|              | 100                | 1130 ± 85                |
|              | 200                | 1009 ± 47\(^d\)         |
| 3            | 50                 | 1250 ± 49                |
|              | 100                | 1400 ± 52\(^d\)         |
|              | 200                | 1271 ± 80                |
| 4            | 50                 | 1220 ± 57                |
|              | 100                | 1160 ± 37                |
|              | 200                | 706 ± 43\(^e\)          |
| 5            | 50                 | 1168 ± 75                |
|              | 100                | 216 ± 29\(^e\)          |
|              | 200                | 3 ± 2\(^e\)             |

Data are reported as the mean ± standard error of the mean of at least 3 independent experiments.

\(^a\)P < 0.01, compared to the blank control group.

\(^b\)Cisplatin was used as a positive control.

\(^c\)P < 0.01, compared to the model group.

\(^d\)P < 0.05, compared to the model group.

\(^e\)P < 0.01, compared to the blank control group.

electronic transition from MO114 and HOMO (MO116) to LUMO (MO117), respectively. The diagnostic Cotton effect around 314 and 250 nm mainly arose owing to the negative rotatory strength at 314 and 250 nm mainly arose owing to the negative rotatory strength at 314 nm (MO114 to MO117) and positive rotatory strength at 250 nm (MO115 to MO117). Thus, compound 1 was confirmed to be (22E,24R)-9α,15α-dihydroxyergosta-4,6,8(14),22-tetraen-3-one and was named ganoderaside E.

By comparing the NMR spectra and electrospray ionization-mass spectra of the compounds reported previously,\(^{11,14}\) the other 4 compounds were confirmed to be (22E,24R)-3β,5α-dihydroxyergosta-7,22-dien-6-one (2),\(^{11}\) (22E,24R)-3β,5α,9α-trihydroxyergosta-7,22-diene-6-one (3),\(^{12}\) (22E,24R)-ergosta-7,9(11),22-triene-3β,5α,6β-triol (4),\(^{13}\) and (22E,24R)-ergosta-7,22-diene-3β,5α,6α-triol (5).\(^{14}\)

Compound 1 exhibited cytotoxic activity against human non-small-cell lung cancer A549 cells with a half-maximal inhibitory concentration (IC\(_{50}\)) value of 21.12 ± 1.46 µM. Doxorubicin, which was used as a positive control, exhibited cytotoxic activity with an IC\(_{50}\) value of 0.68 ± 0.02 µM. Compounds 2, 3, 4, and 5 were considered inactive as the IC\(_{50}\) values were greater than 100 µM (supplemental table S2).

Besides uncontrolled multiplication, metastasis of the cancer cells also plays an important role in tumor development. In view of the in vivo nutritional deficiency of the cancer cells, herein, an in vitro tumor metastasis model was established by serum starvation stimulation. Table 1 shows that 2, 4, and 5 significantly suppressed the migration of A549 cells induced by serum starvation at a concentration of 200 µM. Among these, only 5 could notably suppress the migration at a concentration of 100 µM. Considering that 1 exhibited significant cytotoxic activity (90.33% ± 4.26%) at a concentration of 30 µM, the concentrations of 1 for the transwell assay were adjusted to 5, 10, and 20 µM. However, 1 showed no significant inhibition for the migration of A549 cells at the 3 concentrations.

Compounds 1 to 5 were isolated for the first time, to the best of our knowledge, from G. sinense. The pharmacological assays showed that compounds 1 and 5 could notably inhibit the proliferation and metastasis, respectively, of A549, suggesting their potential as antineoplastic agents. In vivo studies and further investigations on the specific molecular mechanisms will be carried out to confirm the antiproliferative and antimetastatic effects of the isolated compounds on A549 cells.

**Experimental Section**

**General Procedures**

NMR spectra were acquired on a Bruker AVANCE III HD 600 MHz spectrometer. HRESIMS data were recorded on an LTQ Orbitrap Elite spectrometer. Optical rotations were measured with an MCP 500 Modular Circular Polarimeter. The high-pressure liquid chromatography (HPLC) system consisted of a binary HPLC pump (Waters 1525) and a photodiode array detector (W2996). Medium-pressure liquid chromatography was performed on a Buchi Sepacore Chromatography set comprising a pump controller (C-605), control unit (C-620), UV detector (C-635), and fraction collector (C-660). Silica gel (200-300 mesh, Qingdao Marine Chemical Factory), ODS-A (50 µm, YMC), and Sephadex LH-20 (25-100 µm, Pharmacia) were used for column chromatography.

**Fungal Materials**

The spores of G. sinense were collected on August 2018 from the Ganoderma base in an alpine forest of Fujian Province, south-eastern China. This region was developed by the Food Engineering Research Center of the State Ministry of Education, Sun Yat-Sen University. The spores were stored in a dry storage within a narrow temperature range between 5 and 10°C. They were identified by Dr Wenhua He, Institute of Microbiology, Chinese Academy of Sciences. A voucher specimen (HMAS 77207M) has been deposited in the same institution.

**Extraction and Isolation**

The spores of G. sinense (13.2 kg) were subjected to sporoderm-breaking treatment and then extracted by supercritical CO\(_2\) fluid extraction to give a crude extract, as described previously.\(^5\)
The crude extract (4.5 kg) was further subjected to silica gel column chromatography and eluted with petroleum ether, CH₂Cl₂, and CH₂Cl₂/MeOH (4:1) to afford 2 fractions (ZI and ZII). Fraction ZII (25.3 g) was suspended in petroleum ether (500 mL) and partitioned successively with 30% MeOH and 95% MeOH (500 mL × 3). After in vacuo evaporation of 95% MeOH, the residue (11.7 g) was subjected to a silica gel column and eluted with CH₂Cl₂/MeOH (1:1) to yield compound 1 (15 mg).

F1b was chromatographed on a silica gel column and eluted with petroleum ether/EtOAc gradient (from 1:1 to 2:3) to afford compound 2 (92 mg). F2 (2.1 g) was subjected to a reverse-phased silica gel column and eluted with MeCN/H₂O gradient (from 2:3 to 1:4). This was further subjected to silica gel column and eluted with petroleum ether/EtOAc gradient (from 1:1 to 2:3) to afford compound 3 (70 mg). F3 (1.2 g) was subjected to reverse-phased silica gel column and eluted with MeCN/H₂O gradient (from 2:3 to 9:1). This was further subjected to silica gel column and eluted with petroleum ether/EtOH gradient (from 19:1 to 93:7) to obtain compound 4 (151 mg). F5 (997 mg) was repeatedly subjected to silica gel column and eluted with petroleum ether/EtOH gradient (from 24:1 to 23:2). This was followed by recrystallization with CH₂Cl₂/MeOH (1:1) to yield compound 5 (224 mg).

### Cytotoxicity Assay

Compounds 1 to 5 were dissolved in dimethyl sulfoxide and stored at 4°C. Human non-small-cell lung cancer A549 cells were purchased from Shanghai Cell Bank in China and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Australia) with 10% fetal bovine serum (FBS, Gibco, Australia). The A549 cells were grown in 96-well microplates at 37°C for 24 hours in a humidified cell culture incubator with 5% CO₂; the cell density was 5 × 10⁴ cells/well. Thereafter, the A549 cells were treated with different concentrations (200, 100, 50, 25, 12.5, and 6.25 µM) of the isolated compounds. Doxorubicin (Sigma) was used as a positive control. After 24 hours, the cytotoxicity was tested using the Cell Counting Kit-8 (CCK-8, Beyotime, China). The absorbance was measured at 450 nm after 4 hours of incubation, and the IC₅₀ values were calculated using GraphPad Prism 5.

### Transwell Assay

The transwell assay was performed in a 24-well Boyden chamber with an 8 mm pore size polycarbonate membrane (Corning, NY, USA). The A549 cells were treated with compounds 1 to 5 at 3 concentrations in DMEM with 10% FBS for 24 hours and then cultured without serum for another 24 hours. Thereafter, the cells (5 × 10⁴) were harvested, resuspended in 100 µL DMEM without serum, and injected to the upper chamber. Meanwhile, the lower chamber was infused with 500 µL DMEM and 10% FBS to encourage migration. After 48 hours, the cells that migrated to the surface of the lower chamber were fixed with MeOH, stained with crystal violet, and counted under an optical microscope with 200× magnification. Throughout the transwell assay, the blank control group was the cells cultured with serum, while the model group was the cells treated only with serum starvation. Cisplatin was used as a positive control. The positive control group was the cells cultured with cisplatin and serum starvation. The differences were found to be significant for P < 0.05, 0.01.
**Characterization of Ganoderaside E**

Colorless oil; [α]_D^{25} +204.9 (c = 0.30, MeOH); UV (MeOH) λ_max (log ε): 333 (4.35) nm; 1H NMR and 13C NMR (CD_3OD): Table 2. HRESIMS m/z 425.3046 [M + H]^+ (calculated for C_{28}H_{41}O_3 [M + H]^+, 425.3056); CD (MeOH): δε (nm): +41.56 (361), –25.32 (314), +23.84 (250) (c 1.77 × 10^{-3} M).

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**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Supplemental Material**

Supplemental material for this article is available online.

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