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Cytotoxic steroids from the mushroom *Ganoderma australe* collected in Laos

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Abstract

Five steroids named stigmasterol (**1**), ergosterol peroxide (**2**), ganodertriol M (**3**), lucidumol B (**4**) and kansenone (**5**) were purified from the ethyl acetate extract of fruit bodies of the mushroom *Ganoderma australe* collected in Savannakhet province, Laos. Their structures were characterized by the combination of HR-MS, one and two dimensional NMR spectroscopic analyses. In addition, ergosterol peroxide and kansenone showed good cytotoxicity against four cancer cell lines, KB (human epidermal carcinoma), MCF7 (human breast carcinoma), SK-LU-1 (human lung carcinoma) and Hep-G2 (hepatocellular carcinoma). This is the first report on the chemical constituents and cytotoxic steroids from *Ganoderma* sp. in Laos.

Keywords. *Ganoderma australe*, steroid, cytotoxic.

1. INTRODUCTION

Ganoderma australe (Ganodermataceae family) is a common perennial bracket fungus that causes white heart rot in trees of the genera *Tilia* (limes), *Quercus* (oaks), *Fagus* (beech, birch etc), *Platanus* (Sycamore etc)... It distributes most common in central and northern Europe. It also can be found in Nghean, Thuathienhue provinces, Vietnam and Dongphouvieng National forest, Sanvannaket province, Laos [1]. Phytochemical investigation on the fruit bodies of this mushroom revealed lanostane triterpenoids with antimicrobial activity [2-5]. In addition, *Ganoderma australe* collected in Nghean province, Vietnam was reported to have ergosta-4,22-diene-3-one and ergosterol peroxide [6]. Recently, the crude ethyl acetate extract of Laos *Ganoderma australe* showed good cytotoxicity against KB cells (IC_{50} value of 7.27 μg/ml) that encourages us to investigate its chemical constituent. This paper describes the isolation, structural elucidation, and cytotoxicity of five steroids from *Ganoderma australe* collected in Savannakhet province, Laos.

2. EXPERIMENTAL

2.1. General experimental procedures

TLC was carried out on precoated Si gel GF_{254} (Merck Co., Germany) and TLC spots were viewed at 254, 302 and 366 nm and visualized by spraying with vanilline-10 % H_2SO_4 solution followed by heating. Silica gel 0.04-0.20 mm (Merck Co., Germany) was used for column chromatography. Preparative HPLC was performed on a Jasco PU-2087 instrument with a UV-2070 and RI-2031 detectors using a Waters 5 SL-II column (10.0 x 250 mm), flow rate of 1.0 ml/min. ESI-MS spectra were recorded on an Agilent 1200 LC mass spectrometer. NMR (^1H, ^{13}C NMR, DEPT, HSQC and HMBC) spectra were recorded on a Bruker Avance 500MHz. The chemical shift (δ) values are given in ppm with TMS as internal standard, coupling constant J is expressed in Hz.

2.2. Fungal material

The fruit bodies of *Ganoderma australe* were collected in Savannakhet province, Laos in July 2015 by V. A. Khamko. The fungal material was identified by Prof. Dr. Trinh Tam Kiet, Center of Biotechnology, VNU. A voucher specimen (ONK1501) has been deposited at the Faculty of Chemistry, Hanoi National University of Education.

2.3. Extraction and isolation

The dried fruit bodies of *Ganoderma australe* (945 g) were extracted with EtOAc (3Lx3) at room temperature in a ultrasonic bath to give a crude extract (13.3 g), which was subjected to silica gel column, using *n*-hexane/EtOAc from 15/1 (v/v) to 100% EtOAc to afford 6 fractions. Compound **1** (615.8 mg) was precipitated from Fr. 2 (1.27 g) in methanol. Compound **2** (48.7 mg) was obtained from fraction 3 (2.1 g) by silica gel column chromatography, using *n*-hexane/acetone (7/1, v/v). Compound **5** (6.9 mg) was also isolated from Fr. 3. by prep. HPLC, *n*-hexane/EtOAc (3/1, v/v). Fr. 4 (2.5 g) was purified by silica gel column chromatography, using *n*-hexane/EtOAc (2/1, v/v) to afford compounds **3** (50.4 mg) and **4** (64.9 mg).

Compound 1: Amorphous solid; ESI-MS (m/z): 413.1 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H (ppm): 5.57 (1H, m, H-6), 5.38 (1H, m, H-22), 5.19 (m, H-23), 3.57 (1H, m, H-3), 1.04 (3H, d, *J* = 6.5 Hz, H-21), 0.94 (3H, s, H-19), 0.91 (3H, d, *J* = 6.8 Hz, H-26), 0.84 (3H, d, *J* = 6.8 Hz, H-27), 0.81 (3H, t, *J* = 7.0 Hz, H-29), 0.63 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) (δ_C ppm): 141.33 (C-5), 139.55 (C-22), 131.88 (C-23), 119.59 (C-6), 71.05 (C-3), 55.79 (C-14, C-17), 49.46 (C-8, C-24), 43.30 (C-13), 42.82 (C-4), 40.79 (C-20), 40.27 (C-12), 37.15 (C-1), 37.03 (C-10), 31.98 (C-8), 31.46 (C-2, C-7, C-25), 29.65 (C-16), 23.00 (C-28), 22.93 (C-15), 21.55 (C-26), 21.11 (C-11, C-21), 19.95 (C-19), 19.65 (C-27), 12.09 (C-29), 12.05 (C-28).

Compound 2: White crystals; ESI-MS (m/z): 429.2 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H (ppm): 6.50 (1H, d, *J* = 8.5 Hz, H-7), 6.24 (1H, d, *J* = 8.5 Hz, H-6), 5.22 (1H, dd, *J* = 7.5, 7.5 Hz, H-23), 5.14 (1H, dd, *J* = 8.5, 8.5 Hz, H-22), 3.97 (1H, m, H-3), 0.99 (3H, d, *J* = 6.5 Hz, H-21), 0.91 (3H, d, *J* = 7.0 Hz, H-28), 0.88 (3H, s, H-19), 0.84 (3H, d, *J* = 7.0 Hz, H-26), 0.82 (3H, d, *J* = 7.0 Hz, H-27), 0.81 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) (δ_C ppm): 135.42 (C-6), 135.20 (C-22), 132.32 (C-23), 130.75 (C-7), 82.16 (C-5), 79.43 (C-8), 66.47 (C-3), 56.21 (C-17), 51.69 (C-14), 51.10 (C-9), 44.56 (C-13), 42.78 (C-24), 39.71 (C-20), 39.35 (C-12), 36.97 (C-4), 36.92 (C-10), 34.69 (C-1), 33.06 (C-25), 30.10 (C-2), 28.63 (C-16), 23.40 (C-11), 20.87 (C-21), 20.63 (C-15), 19.94 (C-26), 19.64 (C-27), 18.17 (C-19), 17.56 (C-28), 12.87 (C-18).

Compound 3: Amorphous solid; ESI-MS (m/z): 475.2 $[M+H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$) δ_H (ppm): 3.28 (2H, m, H-3, H-24), 2.42 (2H, m, H-6), 1.22 (3H, s, H-27), 1.17 (3H, s, H-26), 1.16 (3H, s, H-19), 0.99 (3H, s, H-29), 0.93 (3H, d, *J* = 6.0 Hz, H-21), 0.92 (3H, s, H-30), 0.88 (3H, s, H-28), 0.66 (3H, s, H-18); ^{13}C-NMR ($CDCl_3$, 125 MHz) (δ_C ppm): 199.09 (C-7), 164.82 (C-9), 138.99 (C-8), 79.59 (C-24), 77.97 (C-3), 73.22 (C-25), 49.87 (C-5), 49.02 (C-17), 47.78 (C-14), 44.96 (C-13), 39.80 (C-10), 38.94 (C-4), 36.65 (C-20), 36.60 (C-6), 34.84 (C-1), 33.53 (C-22), 32.01 (C-15), 30.18 (C-12), 28.73 (C-16), 28.61 (C-23), 27.45 (C-29, C-2), 26.56 (C-27), 25.02 (C-30), 23.68 (C-11), 23.27 (C-26), 18.95 (C-21), 18.36 (C-19), 15.84 (C-18), 15.29 (C-28).

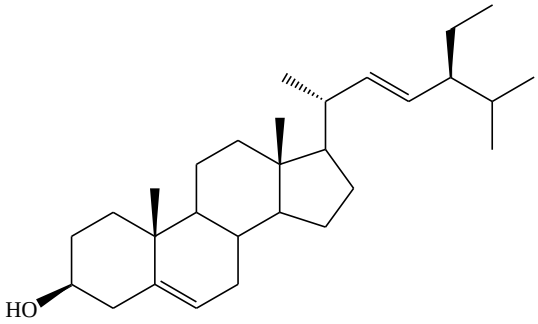
Compound 4: Amorphous solid; ESI-MS (m/z): 459.2 $[M+H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 5.48 (1H, bd, *J* = 6 Hz, H-7), 5.31 (1H, bd, *J* = 5 Hz, H-11), 3.29 (1H, d, *J* = 10 Hz, H-24), 3.25 (1H, dd, *J* = 4.5, 4 Hz, H-3), 1.22 (3H, s, H-27), 1.17 (3H, s, H-26), 1.00 (3H, s, H-28), 0.998 (3H, s, H-19), 0.90 (1H, d, *J* = 6.5 Hz, H-21), 0.88 (6H, s, H-29, H-30), 0.57 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) (δ_C ppm): 145.95 (C-9), 142.65 (C-8), 120.28 (C-7), 116.25 (C-11), 79.63 (C-24), 78,98 (C-3), 73.24 (C-25), 50.99 (C-17), 50.32 (C-14), 49.14 (C-5), 43.78 (C-13), 38.72 (C-4), 37.85 (C-12), 37.38 (C-10), 36.55 (C-20), 35.73 (C-1), 33.51 (C-22), 28.73 (C-23), 28.15 (C-28), 27.89 (C-2), 27.82 (C-16), 26.56 (C-27), 25.59 (C-30), 23.23 (C-26), 23.02 (C-6), 21.51 (C-15), 18.64 (C-21), 15.80 (C-29), 15.69 (C-18).

Compound 5: Amorphous solid; ESI-MS (m/z): 441.3 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H (ppm): 5.09 (1H, bt, *J* = 7 Hz, H-24), 3.28 (1H, dd, *J* = 4.5, 4.5 Hz, H-3), 2.41 (2H, m, H-6), 1.68 (3H, s, H-26), 1.60 (3H, s, H-27), 1.16 (3H, s, H-19), 0.99 (3H, s, H-28), 0.92 (3H, s, H-30), 0.92 (3H, d, *J* = 7.5 Hz, H-21), 0.88 (3H, s, H-29), 0.65 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) (δ_C ppm): 199.08 (C-7), 164.81 (C-9), 139.02 (C-8), 130.98 (C-25), 125.13 (C-24), 77.97 (C-3), 49.85 (C-5), 49.01 (C-17), 47.78 (C-14), 44.94 (C-13), 39.78 (C-10), 38.93 (C-4), 36.64 (C-6), 36.30 (C-20), 36.15 (C-22), 34.83 (C-1), 32.02 (C-15), 30.15 (C-12), 28.75 (C-16), 28.74 (C-2), 27.44 (C-28), 25.70 (C-26), 24.99 (C-23), 24.89 (C-30), 23.69 (C-11), 18.72 (C-21), 18.35 (C-19), 17.63 (C-27), 15.79 (C-18), 15.28 (C-30).

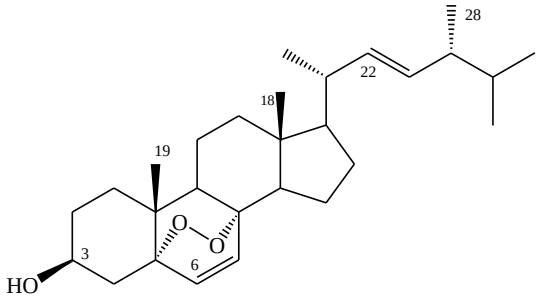
3. RESULTS AND DISCUSSION

Compound **1** was obtained as amorphous solid. Its ^{1}H NMR spectrum shows the presence of three olefinic protons at δ_H = 5.57 (1H, m, H-6), 5.38 (1H, m, H-22), 5.19 (m, H-23), together with one carbinol proton (3.57 ppm) and other methyl signals typically for sterol structures [2-4]. The ^{13}C NMR spectrum of compound **1** has 28 carbon signasl, including four olefinic carbons at 139.75, 135.67, 131.88 and 119.59 ppm. Its spectral data are identical to those of stigmasta-5,22-diene-3β-ol (stigmasterol) [7],

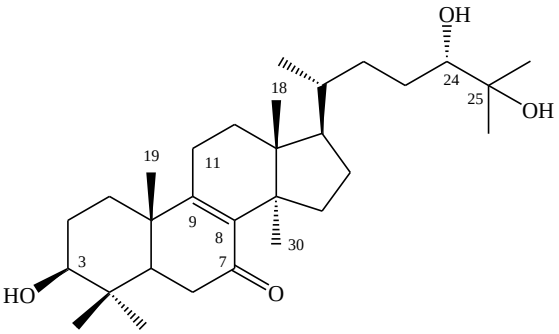
therefore, compound **1** is characterized as stigmasterol. This compound is available in many plants and mushrooms with a inhibition activity of skin carcinoma [8].



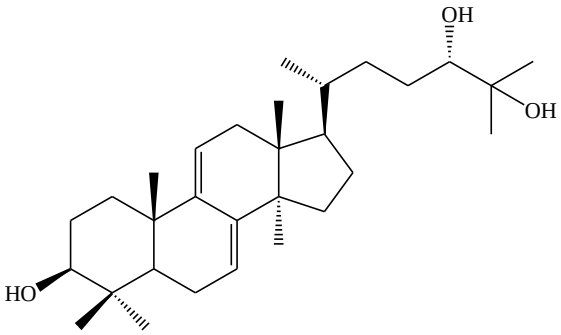
1 (Stigmasterol)



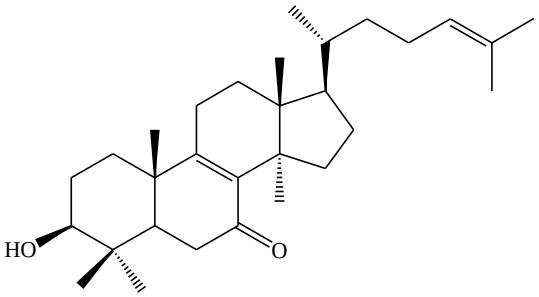
2 (Ergosterol peroxide)



3 (Ganodertriol M)



4 (Lucidumol-B)



5 (Kansenone)

Figure 1: Structures of compounds **1-5**

Compound **2** was isolated as white crystals. Analysis of its ^{1}H NMR spectrum revealed that it has two olefinic protons at 6.50 and 6.24 ppm with a coupling constant of 8.5 Hz, indicating that they are *cis*-configuration. The ^{13}C NMR spectrum of **2** displayed the presence of 28 carbon atoms, including carbons of two double bonds, three oxygen-bearing carbons (82.15, 79.43, and 66.47 ppm). From these spectral data, it is concluded that compound **2** is ergosterol peroxide [9].

The ^{1}H NMR spectrum of compound **3** has two carbinol protons at δ_H = 3.28 (m, 2H, H-3 and H-24) together with eight methyls δ_H = 0.66 (s, 3H, H-18), 0.88 (s, 3H, H-28), 0.91 (s, 3H, H-30), 0.92 (d, 3H, *J* = 6Hz, H-21), 0.99 (s, 3H, H-29), 1.16 (s, 3H, H-19), 1.21 (s, 3H, H-27) and 1.16 (s, 3H, H-26). Its ^{13}C NMR spectrum shows 30 carbon signals. Then, **3** was suggested as lanostane triterpenoid [2-5] by analysis of its 2D NMR (HSQC and HMBC spectra). A conjugated ketone (199.09 ppm) has HMBC correlation with H-6 indicating that it is located at C-7. An olefinic carbon at 164.82 (C-9) has long-range correlation with H-19, while the other olefinic carbon (138.99 ppm) couples to H-30 suggesting that the double bond is at C-8 and C-9. In addition, H-26 and H-27 coupled to C-24 (79.59 ppm) and C-25 (73.22 ppm) locating two hydroxyl groups at C-24 and C-25. From the above

discussion, compound **3** was found to be (24*S*)-lanosta-7-oxo-8-ene-3β,24,25-triol or ganodertriol M [10].

Compound **4** has similar NMR spectral data with those of compound **3**, except for the presesnce of two olefinic protons at 5.31 (bd, 1H, *J* = 5.0 Hz) and 5.47 (bd, 1H, *J* = 6.0 Hz) and the disappearance of the ketone group. Interpretation of its 2D NMR spectra concluded that two olefinic protons are located at C-7 and C-11. Thus, **4** is characterized as (24*S*)-lanosta-7,9-diene-3β,24,25-triol (lucidumol-B) [11]. Final steroid, compound **5** was also obtained as amorphous solid with very similar NMR spectral data with those of compound **3**, except for the disappearance of two hydroxyl group at C-24 and C-25 instead of a double bond at $C_{24\text{-}25}$. The location of this double bond is confirmed by HMBC correlations between i) H-26, H-27 and C-24, C-25; ii) H-24 and C-26, C-27. Consequently, compound **5** is lanosta-7-oxo-8,24-dien-3β-ol (Kansenone) [12].

Table 1: Cytotoxic activity of compounds **2-5**

Samples/ cells	IC_{50} (μg/mL)			
	KB	Hep-G2	Lu-1	MCF-7
2	23.5	26.5	62.6	76.2
3	>128	>128	>128	>128
4	>128	>128	>128	>128
5	19.5	23.7	69.3	72.0

Since the crude extract of *G. australe* showed significantly inhibition of cancer cells (KB), four compounds (**2-5**) were tested their cytotoxicity toward four cancer cell lines: KB (human epidermal carcinoma), MCF7 (human breast carcinoma), LU-1 (human lung carcinoma) and Hep-G2 (hepatocellular carcinoma). The result is shown in table 1. Accordingly, compounds **2** and **5** have moderate and non-selective cytotoxicity against all four cancer cells.

4. CONCLUSION

Stigmasterol (**1**), ergosterol peroxide (**2**), ganodertriol M (**3**), lucidumol-B (**4**) and kansenone (**5**) were purified and structurally characterized from the mushroom *Ganoderma australe* collected in Laos. Two of them (**2**, **5**) have good inhibition activity on four cancer cell lines. This report on the chemical constituents and their cytotoxic activity of Laos *Ganoderma australe*, suggests the possible application of this mushroom for pharmaceutical purposes.

REFERENCES

1. Tham L. X. *Ganodermataceae family*, Science-Technology Publisher, Hanoi (2005).
2. C. J. Amolak. *The isolation of lanosta-7, 9(11),24-trien-3β,21-diol from the fungus G. australe*. Phytochemistry, **23**, 686-687 (1984).
3. A. L. Gerber, A. Jr. Smania, F. Delle Monache, N. Jr. Biachi, E. F. A. Smania. *Triterpenes and sterols from Ganoderma australe (Fr.) Pat. (Aphyllophoromycetdae)*. Int. J. Med. Mushr., **2**, 303-311 (2000).
4. F. Leon, M. Valenica, A. Rivera, I. Nieto, J. Quintana, F. Estevez, J. Bermejo. *Novel cytostatic lanostanoid triterpenes from Ganoderma australe.* Helv. Chim. Acta, **86**, 3088-3095 (2003).
5. E. F. A. Smania, F. D. Monache, R. A. Yunes, R. Paulert, A. S. Junior. *Antimicrobial activity of methyl australate from Ganoderma australe*. Brazilian Journal of Pharmacognosy, **17**, 14-16 (2007).
6. H. T. H. Yen. *Studies on the chemical constituents of Ganoderma australe (Fr.) Pat. in Nghe An*, Master thesis, Vinh University, 2012.
7. M. Khatun, M. Billah, M. D. A. Quader. *Sterols and sterol glugoside from Phyllanthus species*, Dhaka Univ. J. Sci., **60**, 5-10 (2012).
8. H. Ali, S. Dixit, D. Ali, S. M. Alqahtani, S. Alkahtani, S. Alarifi. *Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma*, Drug Design, Development and Therapy, **9**, 2793-2800 (2015).
9. M.A. Wei-Guang, L. X. Cong, W. D. Zu, Y. C. Ren. *Ergosterol peroxides from Cryptoporus volvatus*. Acta Botanica Yunnanica, **16**, 196-200 (1994).
10. C. Man, Z. Mi, S. Shi, X. Bing, Z. H. Qing. *A new triterpene from the fruiting bodies of Ganoderma lucidum*. Acta Pharmaceutica Sinica, **44**, 768-770 (2009).
11. M. Isaka, P. Chinthanom, S. Kongthong, K. Srichomthong, R. Choeklin R. *Lanostane triterpenes from cultures of the basidiomycete Ganoderma orbiforme BCC 22324*, Phytochemistry, **87**, 133-139 (2013).
12. L. Zhang, L. Gao, Z. Li, X. Yan, Y. Yang, Y. Tang, Y. Cao, A. Ding. *Bio-Guided Isolation of the Cytotoxic Terpenoids from the Roots of Euphorbia kansui against Human Normal Cell Lines L-O2 and GES-1*. Int. J. Mol. Sci., **13**, 11247-11259 (2012).

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