Mediation of the Cytotoxicity of Lanostanoids and Steroids of *Ganoderma tsugae* through Apoptosis and Cell Cycle

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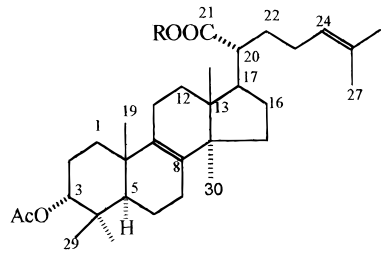
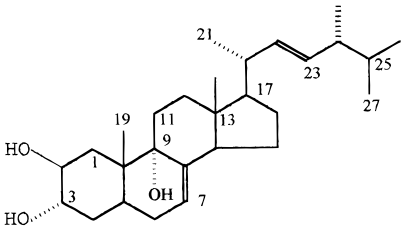
A new lanostanoid ester glucoside, 3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid ester β-D-glucoside (**1**), and a known steroid, 2β,3α,9α-trihydroxy-5α-ergosta-7,22-diene (**2**), were isolated from the fruit bodies of *Ganoderma tsugae* and their structures determined by spectroscopic methods. To study the cytotoxicity of **1** and **2**, the changes of DNA content in human hepatocytes (Hep 3B) were studied. A sub-G_1 cell stage was drastically increased after 24-h incubation with **1** (24 μg/mL). Compound **2** (100 μg/mL) inhibited the cell cycle progression of Hep 3B cells at the G2/M phase with an IC_{50} value of about 87.1 μg/mL. These results indicate that **1** causes cell death by apoptosis and **2** may possess the activity of cell cycle inhibition.

Crude extracts of *Ganoderma tsugae*, a traditional Chinese medicine, have been demonstrated to enchance splenic natural killer cell activity and serum interferon production in mice.[1] Recently, we have isolated and characterized two new lanostanoids, 3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid (**3**) and 3α-acetoxy-16α-hydroxy-24ξ-methyl-5α-lanosta-8,25-dien-21-oic acid, two known lanostanoids, and a known steroid from the fruit bodies of *G. tsugae*.[2] Continuing our studies on Formosan *Ganoderma* fungus, we further investigated the $CHCl_3$ extracts of its fruit bodies. The $CHCl_3$ extract was chromatographed over silica gel. Elution with $CHCl_3$−MeOH (9:1) yielded 3α-acetoxy-5α-lanosta-8,-24-dien-21-oic acid ester β-D-glucoside (**1**) and a known cytotoxic steroid, 2β,3α,9α-trihydroxy-5α-ergosta-7,22-diene.[3]



1: R=β-D-glucosyl

3: R=H



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Results and Discussion

Compound **1**, colorless needles, showed $[\alpha]^{25}_D$ +21° (*c* = 0.1, MeOH). It gave a positive Liebermann−Burchard reaction and possesses the molecular formular $C_{38}H_{60}O_9$ as determined from EI mass spectra ($[M]^+$ at *m/z* 660) and from ^1H and ^{13}C counting in NMR spectra. IR absorptions were indicative of a hydroxyl group (3404 cm^{-1}), an ester (1749 and 1712 cm^{-1}), and a C=C double bond (1641 cm^{-1}). The ^1H NMR spectrum of **1** showed signals for seven tertiary methyl groups at δ 0.80 (Me-18), 0.88 (Me-28), 0.94 (Me-29), 0.94 (Me-30), 1.03 (Me-19), 1.63 (Me-26), and 1.67 (Me-27), an acetyl proton signal at δ 2.06, an oxygen-bearing methine proton signal at δ 4.64 (1H, bs), an olefinic proton signal at δ 5.09 (t, *J* = 7.6 Hz, H-24), and three aliphatic proton signals at δ 1.52 (2H, m, H-22) and 2.38 (1H, m, H-20).[2] The signal at δ 4.64 that was shifted to δ 3.43 after alkaline hydrolysis, as observed in related lanostanoid-type compounds, was assigned to the methine group bearing the α-acetoxy group at C-3.[4] In addition to the above evidence, the absence of two secondary methyl signals and the presence of an anomeric proton signal at 5.47 (d, *J* = 8.0 Hz) in the ^1H NMR spectrum of **1**[5] and a tertiary carbon signal at δ 125.0, four-quarternary carbon signals at δ 133.0, 135.2, 136.2, and 177.4, and six glucosyl carbon signals at δ 95.8, 78.7, 78.4, 73.9, 71.3, and 62.2 in the ^{13}C NMR spectrum of **1** clearly indicated that **1** was a 3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid ester β-D-glucoside (**1**).[6] On alkaline hydrolysis, it gave glucose, detected by TLC, and 3α-hydroxy-5α-lanosta-8,24-dien-21-oic acid, identified by comparison with an authentic sample.[2] The ^{13}C NMR spectrum of **1** (Table 1) was assigned by comparison with those of the corresponding data for **3** and methyl β-D-glucopyranoside.[2] The ^{13}C NMR and mass spectrum (Experimental Section) also supported the characterization of **1**.

It has been recognized that apoptotic cells have reduced DNA stainability with a variety of fluorochromes.[7,8] The appearance of cells with low DNA stainability forms a "sub-G_1 peak", which has been

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Table 1. ^{13}C NMR Chemical Shift Assignment of **1**[a]

carbon	**1** (CD_3OD)	**3**[b] ($CDCl_3$)	carbon	**1**	**3**[b]
1	31.5	30.4	20	48.2	47.2
2	24.2	23.4	21	177.4	182
3	79.6	78.0	22	33.9	32.5
4	37.8	36.8	23	26.8	25.9
5	46.7	45.3	24	125.0	123.6
6	19.1	18.0	25	133.0	132.2
7	27.1	26.0	26	17.9	17.6
8	135.2	133.8	27	25.9	25.6
9	136.3	134.6	28	28.1	27.6
10	38.1	36.9	29	22.3	21.8
11	21.9	20.8	30	24.7	24.3
12	32.0	30.8	1′	95.8	
13	45.6	44.3	2′	73.9	
14	50.7	49.6	3′	78.4	
15	28.0	27.0	4′	71.3	
16	29.9	29.0	5′	78.7	
17	46.7	47.2	6′	62.6	
18	16.7	16.0	$COCH_3$	21.1	21.3
19	19.4	18.9	$COCH_3$	172.6	170.8

[a] The number of protons directly attached to each carbon was verified by DEPT experiment. [b] Data in these columns are from ref 2.

Table 2. Cell Populations of Cell Cycle of Hep 3B Cells[a]

dose (μg/mL)	G_0/G_1 (%)	S (%)	G_2/M (%)
control	56.1	31.1	12.8
20	50.6	36.8	12.6
35	55.3	35.0	9.8
50	55.3	35.5	9.2
100	57.8	37.0	5.2

[a] Flow cytometric analysis of the DNA histograms of propidium (PI)-stained Hep 3B cells. The cells were incubated with various concentrations of **2** for 24 h. After incubation, the cells were fixed and incubated with PI and RNase before reading red fluorescence excited by blue light. Control, untreated cells. The cell populations of cell cycle were analyzed by LYSIS II software.

considered to be the marker of cell death by apoptosis.[9] In the study, **1** (24 μg/mL) was incubated with Hep 3B cells for different intervals. A sub-G_1 peak was observed at 24 h in the DNA histograms of **1**-treated Hep 3B cells by flow cytometry. Various concentrations (10, 20, and 30 μg/mL) of **1** were also incubated with Hep 3B cells for 24 h, respectively. A sub-G_1 peak was detected in the DNA histograms of **1** (20 or 30 μg/mL)-treated Hep 3B cells by flow cytometry, respectivly. The shift of G_0/G_1 and G_2/M cell cycles to the sub-G_1 phase is increased dose-dependently in the Hep 3B cells treated by **1**. Thus, these results show that **1** mediates its cytotoxicity through apoptosis. The cell populations of the cell cycle for **2**, obtained from the flow cytometric histograms, were given in Table 2. Compound **2** slightly inhibited the cell cycle progression of Hep 3B cells in the G2/M phase at concentrations of 35 and 50 μg/mL and strongly inhibited the cell cycle progression of Hep 3B cells in the G2/M phase at the concentration of 100 μg/mL. Compound **2** inhibited dose-dependently the cell cycle progression of Hep 3B cells in the G2/M phase with an IC_{50} value of about 87.1 μg/mL. These results show that **2** may exert its cytotoxicity through the inhibition of cell cycle progression of Hep 3B cells. The present study is the first report of a lanostanoid ester glucoside (**1**) and a steroid (**2**), isolated from *G. tsugae*, mediating their cytotoxicity through apoptosis and possibly cell cycle inhibition, respectively. Further experiments are needed to evaluate their cytotoxic effect and elucidate their exact mechanism of action.

Experimental Section

General Experimental Procedures. The melting point is reported uncorrected. Optical rotation was obtained on a JASCO model DIP-370 digital polarimeter, the IR spectrum was recorded on a Hitachi model 260-30 spectrophotometer, ^1H and ^{13}C NMR (400 MHz) spectra were recorded on a Varian Unity-400 spectrometer, and the MS was obtained on a JMS-HX 100 mass spectrometer.

Plant Material. *G. tsugae* was collected at Liu-Kuei Shian, Kaohsiung Hsien, Taiwan, R.O.C., during July 1995. A voucher specimen (9501) is deposited in our laboratory. It was identified by Dr. Ming-Hong Yen, School of Pharmacy, Kaohsiung Medical College.

Extraction and Isolation. Air-dried fruit bodies (10 kg) were extracted with $CHCl_3$. The $CHCl_3$ extract (0.26 kg) was chromatographed over silica gel. Elution with $CHCl_3$−MeOH (9:1) yielded **1** (0.12 g) and 2β,3α,9α-trihydroxy-5α-ergosta-7,22-diene (**2**) (0.045 g). Compound **2** was characterized by spectral methods and by comparison of its physical and spectral data with those of an authentic sample.[3]

3α-Acetoxy-5α-lanosta-8,24-dien-21-oic acid ester β-D-glucoside (1): colorless needles (50 mg); mp 188−190 °C; $[\alpha]^{27}_D$ +21° (MeOH; c 0.1); IR ν_{max} (KBr) cm^{-1} 3404, 1749, 1712, 1641; ^1H NMR (400 MHz, CD_3OD) see text; ^{13}C NMR (400 MHz, CD_3OD) see Table 1; EIMS (rel int) *m/z* 660 $[M]^+$ (11), 541 (28), 498 [M − glucose − $H_2O]^+$ (23), 483 [M − Me]$^+$ (16), 437 [483 − HCOOH]$^+$ (8), 423 [483 − $CH_3COOH]^+$ (58), 281 [423 − side chain]$^+$ (12), 43 (100).

Cell Lines and Cell Culture. Hepatocellular carcinoma (Hep 3B) cells were kindly provided by Dr. L. C. Chiang of the Department of Microbiology, Kaohsiung Medical College, Taiwan. The cells were cultured at 37 °C in a humidified atmosphere of carbon dioxide−air (5:95). The culture medium consisted of Dulbecco modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hazelton Product, Denver, PA), 100 μg/mL of streptomycin, and 100 unit/mL of penicillin. The cells (1×10^4 cells/mL) were each seeded in 1-mL wells of 24-well multidishes with DMEM−10% fetal bovine serum for at least 24 h prior to use.

Flow Cytometry. Compounds **1** (10, 20, 24, or 30 μg/mL) and **2** (20, 35, 50, or 100 μg/mL) were added to cells (1×10^7 cell/mL), respectively. At various time intervals, the reactions were terminated by washing with PBS. The cells were fixed with 4% paraformaldehyde/PBS[10] (pH 7.4) at room temperature for 30 min. After centrifugation at 1000 rpm for 10 min, the cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate at 4 °C for 2 min. Propidium iodide in PBS (10 μg/mL) was added to stain the cells at 37 °C for 30 min. The intensity of red fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A minimum of 5000 cell counts were collected for the analysis by LYSIS II software.[11]

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(10) PBS is a phosphate-buffered saline solution.
(11) LYSIS II (version 1.0) is a registered trademark of Becton Dickinson Inc., San Jose, CA.

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