New Lanostanoids of *Ganoderma tsugae*

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Three new compounds, (24*R*,*S*)-3α-acetoxy-24-hydroxy-5α-lanosta-8,25-dien-21-oic acid, named tsugaric acid C (**1**); 3α-acetoxy-5α-lanosta-8,24-diene-21-*O*-*β*-D-xyloside, named tsugarioside B (**2**); and 3α-acetoxy-(*Z*)-24-methyl-5α-lanosta-8,23,25-trien-21-oic acid ester *β*-D-xyloside, named tsugarioside C (**3**), and a mixture of two known steroids were isolated from the fruit bodies of *Ganoderma tsugae*. The structures of **1**−**3** were determined by spectral and chemical methods. The cytotoxic activity of the lanostanoid constituents of this fungus was evaluated against several different cancer cell lines.

Previously, we isolated and characterized three new lanostanoids, tsugaric acid A (3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid) (**5**), tsugaric acid B (3α-acetoxy-16α-hydroxy-24ξ-methyl-5α-lanosta-8,25-dien-21-oic acid) (**6**), and tsugarioside A, 3α-acetoxy-5α-lanosta-8,24-dien-21-oic acid ester *β*-D-glucoside (**7**), along with four known compounds, 3*β*-hydroxy-5α-lanosta-8,24-dien-21-oic acid (**8**), 3-oxo-5α-lanosta-8,24-dien-21-oic acid (**10**), ergosta-7,22-dien-3*β*-ol, and 2*β*,3α,9α-trihydroxy-5α-ergosta-7,22-diene from the fruit bodies of *Ganoderma tsugae* Murr. (Polyporaceae).[1,2] In continued studies on the consitutents of this fungus, three new lanostanoids were obtained from a MeOH extract of the fruit body: tsugaric acid C (**1**), tsugariosides B (**2**) and C (**3**), and a mixture of the already known compounds 5α,8α-epidioxyergosta-6,22-dien-3*β*-ol and 5α,8α-epidioxyergosta-6,9(11),22-trien-3*β*-ol. Recently, we reported that **7** and 2*β*,3α,9α-trihydroxy-5α-ergosta-7,22-diene mediate their cytotoxicity through apoptosis and cell-cycle inhibition,[2] respectively. In the present paper, the structural characterization of **1**−**3** and the cytotoxic effects against a small cancer-line panel of several of the *G. tsugae* constituents and their derivatives are reported.

The HREIMS of **1** indicated a molecular ion peak at *m*/*z* 514.3662, which corresponded to a molecular formula $C_{32}H_{50}O_5$. IR absorptions were indicative of a hydroxyl group (3465 cm^{-1}), an ester (1730 cm^{-1}), and a C=C double bond (1656 cm^{-1}). The EIMS of **1** showed significant peaks at *m*/*z* 496 $[M - H_2O]^+$, 481 $[496 - Me]^+$, 421 $[481 - CH_3COOH]^+$, and 281 $[421 - (\text{side chain}) - H_2O)]^+$. The ^1H NMR spectrum of **1** showed signals for six tertiary methyl groups, an acetyl proton signal, two oxygen-bearing methine proton signals at *δ* 4.01 (m) and 4.61 (1H, br s, H-3*β*), and two olefinic proton signals a *δ* 4.75, 4.89 (1H, br s, H-27 of 24*R*,24*S*) and 4.75, 4.92 (1H, br s, H-27 of 24*R*,24*S*).[1−3] In addition to the above evidence, the presence of correlations between the proton signal at *δ* 4.01 and C-24 and between H-27 and C-27 (Table 1) in the HMQC spectrum, and the interactions between the proton signal at *δ* 4.01 and H-27 and between Me-26 and H-27 in the NOESY spectrum suggested that **1** is a 24-epimeric pair of 3α-acetoxy-24-hydroxy-lanostanoids with a Δ^{25} double bond. The proton signal at *δ* 4.01 was assigned to H-24.[3] The ratio of these epimers was assumed to be 1:1 based on the signal intensity of H-27. The information obtained from the 2D NMR and comparison of ^{13}C NMR data with those of lanostanoids allowed the assignment of the ^{13}C NMR signals of **1** as shown in Table 1. The signals of C-23 to C-27 appeared as a doublet (Table 1), supporting the fact that **1** is a mixture of C-24 epimers.[3,4] Therefore, **1** was

Table 1. ^{13}C NMR Data for **1**−**4**[a]

carbon	**1** [$(CD_3)_2CO$][b]	**2** ($CDCl_3$)[b]	**3** ($CDCl_3$)	**4** ($CDCl_3$)[b]
1	30.5	30.2	30.4	30.1
2	24.6	23.3	23.3	25.7
3	78.8	78.1	78.1	76.0
4	38.1	36.7	36.7	36.9
5	46.9	45.3	45.3	44.3
6	19.4	18.0	18.0	18.1
7	27.3	26.0	25.9	28.0
8	135.6	134.0	133.7	134.0
9	136.3	134.6	134.7	135.0
10	38.4	36.9	36.9	37.5
11	22.2	21.0	20.8	21.0
12	30.8	30.8	30.8	30.7
13	45.8	44.3	44.3	44.2
14	51.0	49.9	49.5	50.8
15	28.3	27.5	27.0	28.0
16	28.7	29.7	28.9	29.8
17	48.6	40.6	47.0	42.9
18	17.0	16.1	16.3	16.0
19	20.0	19.0	19.0	19.0
20	49.0	44.9	47.8	49.9
21	178.1	70.2	175.2	62.6
22	34.3	30.7	32.9	30.5
23	32.3 (24*R*) 31.8 (24*S*)	24.7	123.5	24.3
24	76.5 (24*R*) 75.6 (24*S*)	124.8	132.3	124.0
24[1]			25.7	
25	150.0 (24*R*) 149.8 (24*S*)	131.4	155.4	132.0
26	110.8 (24*R*) 111.5 (24*S*)	17.7	106.7	17.8
27	18.7 (24*R*) 18.1 (24*S*)	25.7	17.7	26.0
28	28.7	27.6	27.6	27.7
29	22.9	21.8	21.8	22.2
30	25.3	24.4	24.4	25.0
1′		102.7	94.5	
2′		71.9	72.3	
3′		73.7	75.9	
4′		69.7	69.5	
5′		63.7	65.8	
$COCH_3$	171.3	170.9	170.9	
$COCH_3$	21.8	21.4	21.4	

[a] The number of protons directly attached to each carbon was verified by DEPT experiments. [b] Signals obtained by $^1H-^1H$ COSY, HMBC, NOESY, or ROESY techniques.

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Table 2. Cytotoxicity of Lanostanoids Isolated from *G. tsugae* (ED_{50} values in μG/mL)

compound	cell line[a]					
	PLC/PRF/5	T-24	212	HT-3	SiHa	CaSKi
3	6.5	8.6	NS[b]	7.2	9.5	*c*
5	6.8	3.1	*c*	*c*	*c*	NS
6	NS	NS	10.3	*c*	*c*	NS
7	NS	1.73	*c*	6.8	8.4	*c*
8	*c*	4.4	*c*	3.5	5.5	6.2
9	9.2	5.8	NS	*c*	*c*	3.9
10	12.1	8.2	*c*	6.9	5.1	7.2
11	*c*	NS	*c*	*c*	NS	NS
cisplatin	5.3	*c*	1.3	*c*	*c*	*c*
actinomycin D	1.4×10^{-3}	1.5×10^{-3}	*c*	5.6×10^{-4}	8.1×10^{-4}	1.9×10^{-3}

[a] For significant activity of the pure compounds, an ED_{50}<4.0 μg/mL is required. [b] NS, no significant activity of the pure compounds. [c] Not determined.

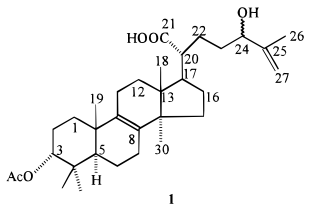
characterized as a 1:1 mixture of the C-24 epimers of 3α-acetoxy-24-hydroxy-5α-lanosta-8,25-dien-21-oic acid (**1**).

Compound **2**, colorless needles, showed $[\alpha]^{27}_D$ +7.6°(*c* 0.1, $CHCl_3$). It also gave a positive Liebermann−Burchard reaction and possesses the molecular formula $C_{37}H_{60}O_7$ as determined from positive FABMS ($[M + 1]^+$ at *m/z* 617) and ^1H and ^{13}C NMR data. It failed to show a molecular ion peak under high-resolution conditions. IR absorptions were indicative of a hydroxyl group (3425 cm^{-1}), an ester (1745 cm^{-1}), and a C═C double bond (1645 cm^{-1}).The ^1H NMR spectrum of **2** showed signals for seven tertiary methyl groups, an acetyl proton signal, an oxygen-bearing methine proton signal at δ 4.66 (1H, br s, H-3β), and an olefinic proton signal at δ 5.09 (1H, t, J = 6.8 Hz, H-24).[2,5] In addition to the above evidence, the presence of an anomeric proton signal at δ 4.39 (1H, d, J = 7.2 Hz) and signals corresponding to a secondary carbon (δ 70.2), a tertiary carbon (δ 124.8), three quaternary carbons (δ 131.4, 134.0, and 134.6), and five xylosyl carbons (δ 102.7, 73.7, 71.9, 69.7, and 63.7) in the ^1H and ^{13}C NMR spectra of **2**, as well as significant peaks at *m/z* 391 [M − CH_3COOH − CH_3 − xylose]$^+$ and 149 [xylose − H]$^+$ in the FABMS of **2**, clearly indicated that **2** is 3α-acetoxy-5α-lanosta-8,24-diene-21-*O*-β-D-xyloside (**2**).[2,6,7]

Acidic methanolysis of **2** with HCl/MeOH gave the 3α-*O*-acetate of **4** and methyl α- and β-D-xylopyranoside. On alkaline hydrolysis, the 3α-*O*-acetate of **4** gave **4**, which was further identified by comparison with the product obtained from the reduction of **7** with $LiAlH_4$. The information obtained from 2D NMR and ^{13}C NMR data further supported the characterization of **2** and allowed the assignment of ^{13}C NMR signals of **2** as shown in Table 1.[1,2,6,7]

The HRFABMS of **3** indicated a molecular ion peak at *m/z* 643.3791 ([M + 1], +42 mmu error), which corresponded to a molecular formula of $C_{38}H_{58}O_8$. IR absorptions were indicative of a hydroxyl group (3445 cm^{-1}), an ester (1745 and 1720 cm^{-1}), and a C═C double bond (1645 cm^{-1}). The EIMS of **3** showed significant peaks at *m/z* 512 [M − (xylose-H_2O) − 2H]$^+$, 483 [512 − CO − H]$^+$, and 423 [483 − CH_3COOH]$^+$. The ^1H NMR spectrum of **3** showed signals for seven tertiary methyl groups, an acetyl proton signal, an oxygen-bearing methine proton signal at δ 4.66 (1H, t, J = 5.6 Hz, H-3β), and three olefinic proton signals at δ 4.65 (1H, s, H-26), 4.75 (1H, s, H-26), and 5.08 (1H, t, J = 6.8 Hz).[2,5] In addition to the above evidence, the presence of an anomeric signal at δ 5.56 (1H, d, J = 7.2 Hz) in the ^1H NMR spectrum of **3**, signals corresponding to a tertiary carbon (δ 125.3), five quaternary carbons (δ 132.3, 133.7, 134.7, 155.4, and 175.2), and five xylosyl carbons (δ 94.5, 75.9, 72.3, 69.5, and 65.8) in the ^{13}C NMR spectrum of **3**, as well as correlations between Me-24[1] and C-23 and C-24, and between H-27 and C-24 in the HMBC spectrum, and an interaction between Me-24[1] and H-23 in the ROESY spectrum, clearly indicated that **3** is a 3α-acetoxy-(*Z*)-24-methyl-5α-lanosta-8,23,25-trien-21-oic acid ester β-D-xyloside (**3**).[2,6−8] Therefore, the tertiary methyl and olefinic proton signals at δ 1.67 and 5.08 were assigned to Me-24[1] and H-23, respectively. The information obtained from 2D NMR and ^{13}C NMR data further supported the characterization of **3** and allowed the assignment of ^{13}C NMR signals of **3** as shown in Table 1.[1,2,6−8]

The cytotoxic activity of the constituents isolated from this fungus against PLC/PRF/5, T-24, 212, HT-3, SiHa, and CaSKi cells was studied in vitro. The results are listed in Table 2. Compound **7** showed significant activity against T-24 cells, while **5**, **8**, and **9** showed significant activity against T-24, HT-3, and CaSKi cells, respectively. Cisplatin and actinomycin D were used as positive controls.



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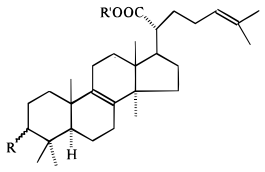
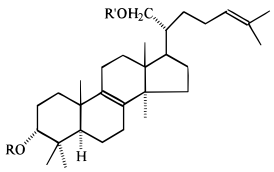
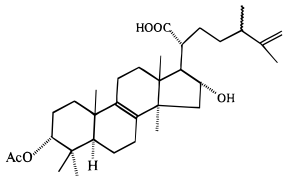


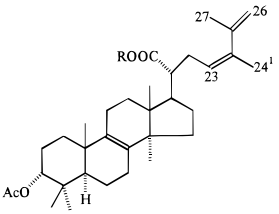
5 R= α−OAc, R'= H
7 R= α−OAc, R'= β−D-glucosyl
8 R= β−OH, R'= H
9 R= β−OAc, R'= H
10 R= O, R'= H
11 R= α-OH, R'= H



2 R= Ac, R'= β-D-xylosyl,
4 R=R'=H



6



3 R= β-D-xylosyl

Experimental Section

General Experimental Procedures. Melting points are reported uncorrected. The optical rotations were obtained on a JASCO model DIP-370 digital polarimeter. IR spectra were recorded on a Hitachi model 260-30 spectrophotometer. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a

Varian Unity-400 spectrometer, and MS were obtained on a JMS−HX 100 mass spectrometer.

Fungal Material. *G. tsugae* was collected at Liu-Kuei Shian, Kaohsiung Hsien, Taiwan, Republic of China, during July 1995. A voucher specimen (9501) is deposited in the laboratory of Medicinal Chemistry. It was identified by Dr. Ming-Hong Yen, School of Pharmacy, Kaohsiung Medical University. Compounds **5**−**8** and **10** were isolated and identified as reported previously.[1] Compound **8** was acetylated using Ac_2O in pyridine, and **7** was hydrolyzed in methanolic 5% KOH to give **9** and **11**, respectively.

Extraction and Isolation. Air-dried fruit bodies (10 kg) were extracted with $CHCl_3$ and MeOH, respectively. The MeOH extract was chromatographed on a Si gel column, and elution with cyclohexane−CH_2Cl_2 (1:4) yielded a mixture of 5α,8α-epidioxyergosta-6,22-dien-3β-ol and 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol (25 mg, 0.0005%). Elution with CH_2Cl_2−EtOAc (3:2) afforded **1** (20 mg, 0.0004%), **2** (10 mg, 0.0002%), and **4** (15 mg, 0.0003%). The known compounds were identified by spectroscopic methods and comparison with reported data.[9,10]

Tsugaric acid C (**1**): colorless needles (MeOH); mp 213−215 °C; IR (KBr) ν_{max} 3465, 1730, 1656 cm^{-1}; ^1H NMR [$(CD_3)_2$-CO, 400 MHz] δ 0.80 (3H, s, Me-18), 0.86 (3H, s, Me-28), 0.91 (3H, s, Me-29), 0.94 (3H, s, Me-30), 1.03 (3H, s, Me-19), 1.67 (3H, s, Me-27), 2.00 (3H, s, OAc), 2.26 (1H, m, H-20), 3.75 (1H, br s, OH-24), 4.01 (1H, m, H-24), 4.61 (1H, br s, H-3), 4.75, 4.89 (1H, br s, H-27 of 24*R*,24*S*), 4.75, 4.92 (1H, br s, H-27 of 24*R*,24*S*); ^{13}C NMR [$(CD_3)_2CO$, 100 MHz], see Table 1; EIMS *m/z* 514 $[M]^+$ (8), 496 (3), 481 (9), 421 (54), 281 (17), 187 (39), 81 (66), 69 (58), 43 (100); HREIMS *m/z* 514.3662 (calcd for $C_{32}H_{50}O_5$, 514.3658).

Tsugarioside B (**2**): colorless needles ($CHCl_3$); mp 135−137 °C; $[\alpha]^{27}_D$ +7.6° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3425, 1745, 1645 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.71 (3H, s, Me-18), 0.86 (3H, s, Me-28), 0.91 (3H, s, Me-29), 0.92 (3H, s, Me-30), 0.99 (3H, s, Me-19), 1.60 (3H, s, Me-26), 1.67 (3H, s, Me-27), 2.07 (3H, s, OAc), 2.33 (1H, m, H-20), 4.39 (1H, d, J = 7.2 Hz, H-1′), 4.66 (1H, br s, H-3β), 5.09 (1H, t, J = 6.8 Hz, H-24); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; FABMS (positive) *m/z* 617 $[M + 1]^+$ (0.2), 413 (3), 391 (12), 154 (39), 149 (100).

Reduction of 7 with $LiAlH_4$. Compound **7** (0.06 mmol) was dissolved in anhydrous THF (5 mL). $LiAlH_4$ (0.6 mmol) was added to this solution, and the mixture was refluxed for 24 h. The excess $LiAlH_4$ was decomposed with wet diethyl ether, and the mixture was extracted with diethyl ether. The diethyl ether layer was washed with H_2O, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed on a Si gel column, and by elution with $CHCl_3$, afforded **4** (0.02 mmol) as colorless needles ($CHCl_3$): mp 71−72 °C; $[\alpha]^{27}_D$ −12.7° (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 3965 (OH), 1656 (C=C); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.71 (3H, s, Me-18), 0.87 (3H, s, Me-28), 0.89 (3H, s, Me-29), 0.90 (3H, s, Me-30), 0.98 (3H, s, Me-19), 1.61 (3H, s, Me-26), 1.68 (3H, s, Me-27), 3.43 (1H, br s, H-3), 3.71 (2H, m, H-21), 5.11 (1H, m, H-24); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS *m/z* 442 $[M]^+$ (3), 427 $[M - CH_3]^+$ (4), 409 $[427 - H_2O]^+$ (7), 281 [*m/z* 409 − side chain − H]$^+$ (4), 255 (5), 187 (13), 109 (96), 69 (100); HREIMS *m/z* 442.3789 (calcd for $C_{30}H_{50}O_2$, 442.3811).

Tsugarioside C (**3**): colorless needles ($CHCl_3$); mp 181−183 °C; $[\alpha]^{27}_D$ +10° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3445, 1745, 1720, 1656 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.74 (3H, s, Me-18), 0.86 (3H, s, Me-28), 0.91 (3H, s, Me-29), 0.93 (3H, s, Me-30), 0.97 (3H, s, Me-19), 1.57 (3H, s, Me-27), 1.67 (3H, s, Me-24[1]), 2.06 (3H, s, OAc), 4.65 (1H, s, H-26), 4.66, (1H, t, J = 5.6 Hz, H-3β), 4.75 (1H, s, H-26), 5.08 (1H, t, J = 6.8 Hz, H-23), 5.56 (1H, d, J = 7.2 Hz, H-1′); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; FABMS (positive) *m/z* 665 $[M + Na]^+$ (14), 551 (12), 543 (10), 311 (9), 237 (16), 193 (11), 108 (100); HRFABMS (positive) *m/z* 643.3791 $[M + 1]^+$ (calcd for $C_{38}H_{58}O_8$, 643.4210).

Tumor Cell Growth Inhibition Assays. A microassay for cytotoxicity was performed using a MTT (3-[4,5-dimethylthiazo-2-yl]-5-[3-carboxymethoxy-methoxyphenyl]- 2[4-sulfophenyl]-2*H*-tetrazolium bromide) assay.[11,12] Briefly, 1−3 × 10^3 cells/100 μL were seeded in 96-well microplates (Nunck, Roskilde, Denmark) and preincubated for 6 h to allow cell attachment. This medium was then aspirated, and 100 μL of fresh medium containing various concentrations of test drug were added to the cultures. The cells were incubated with each drug for 6 days. Cell survival was evaluated by adding 10 μL of tetrazolium salt solution (1 mg MTT/mL in PBS). After 4 h incubation at 37 °C, 100 μL DMSO were added to dissolve the precipitate of reduced MTT. Microplates were then shaken for 15 min, and the absorbance was determined at 550 nm with a multiwell scanning spectrophotometer (Dynex MR 5000, Chantilly, VA).

PLC/PRF/5 cells were established from a human hepatoma and known to produce HBs Ag continuously in culture fluids.[13] Human hepatoma PLC/PRF/5, T24 cells, human cervical carcinoma, HT-3, SiHa, and CaSki cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY)[11,12] containing 10% fetal bovine serum (FBS; Gibco BRL), 2 mM L-glutamine, 100 u/mL penicillin, and 100 μg/mL streptomycin. The 212 cells (an inducible Ha-*ras* oncogene transformed NIH/3T3 cell line) were maintained in minimum essential alpha medium (MEM; Gibco BRL), containing 10% calf serum (Gibco BRL), and antibiotics.[14] For the microassay, the growth medium was supplemented with 10 mM HEPES buffer pH 7.3 and incubated at 37 °C in a CO_2 incubator.

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