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NOVEL CYTOTOXIC PRINCIPLES OF FORMOSAN *GANODERMA LUCIDUM*

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ABSTRACT.—Two new steryl esters, ergosta-7,22-dien-3β-yl linoleate [**1**] and 5α,8α-epidioxyergosta-6,22-dien-3β-yl linoleate [**3**], and a novel steroid, ergosta-7,22-diene-2β,3α,9α-triol [**5**], have been isolated from the fruiting bodies of Formosan *Ganoderma lucidum* and characterized. A new lanostanoid, 3β-hydroxy-26-oxo-5α-lanosta-8,24-dien-11-one, and the new steroid exhibited potent inhibition of KB cells and human PLC/PRF/5 cells in vitro.

In the course of a search for the bioactive principles of Formosan *Ganoderma lucidum* (Fr.) Karst (Polyporaceae), we reported the isolation of a new steryl ester, ergosta-7,22-dien-3β-yl palmitate; a new lanostanoid, ganoderic aldehyde A (3β-hydroxy-26-oxo-5α-lanosta-8,24-dien-11-one); and several known steroids (1). During continued work on the $CHCl_3$ extract of this same fungus, two new steryl esters, ergosta-7,22-dien-3β-yl linoleate [**1**], 5α,8α-epidioxyergosta-6,22-dien-3β-yl linoleate [**3**], a novel steroid, ergosta-7,22-diene-2β,3α,9α-triol [**5**], and a known steroid, 5α,8α-epidioxyergosta-6,9(11),22-trien-3β-ol [**7**], were obtained and are reported in this paper. Since *G. lucidum* has been used as a Chinese drug to treat hepatopathy (2), the isolates and derivatives from this fungus, grown on *Acacia confusa* Merr. (Leguminosae), were screened for cytotoxic effects against human hepatoma PLC/PRF/5 and KB cells in vitro.

RESULTS AND DISCUSSION

The ester **1** had an ir spectrum which indicated the presence of an ester (1720 cm^{-1}) and cis double bonds (680 cm^{-1}). An ei mass spectrum showed characteristic fragmentations for a Δ^7-monoene steroid at *m/z* 255, 229, and 213 (3) and a peak at *m/z* 280 corresponding to a $C_{18}H_{32}O_2$ moiety. In the nmr spectra, in addition to steroid signals similar to those of ergosta-7,22-dien-3β-yl palmitate (1), proton signals at δ 5.34 (4H, m), 2.76 (2H, t), 2.25 (2H, t), 2.03 (4H, m), and 0.89 (3H, t) were attributed to the olefinic protons of double bonds, methylene protons between double bonds, methylene protons of C-2′, methylene protons of C-8′, C-14′, and terminal (C-18′) methyl protons, respectively (4), and the carbon signals at δ 128.3, 128.4, 130.3, and 130.4 were attributed to the four olefinic carbons of the linoleoyl moiety. On alkaline hydrolysis, **1** yielded linoleic acid and ergosta-7,22-dien-3β-ol [**2**], thus establishing **1** as ergosta-7,22-dien-3β-yl linoleate [**1**]. Ir, ms, and nmr spectra of the reaction product of linoleoyl chloride with ergosta-7,22-dien-3β-ol were identical to those of the natural product **1**.

The ester, **3**, a colorless oil, had ir signals for an ester (1720 cm^{-1}), peroxide (860 cm^{-1}), and cis double bonds (680 cm^{-1}). The ei mass spectrum had peaks for an epidioxy steroid at *m/z* 378 $[M-C_{18}H_{32}O_2-32]^+$, 253, and 251 (5) and a peak at *m/z* 280 corresponding to a $C_{18}H_{32}O_2$ moiety. The nmr spectrum of **3** was similar to that of 5α,8α-epidioxyergosta-6,22-dien-3β-ol [**4**] acetate (5–8) with the proton sig-

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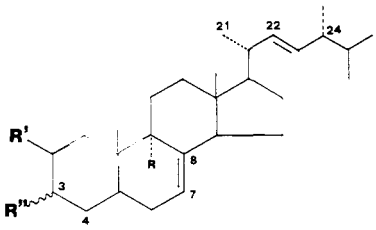
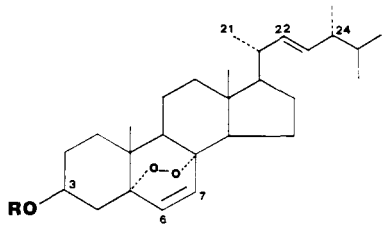


1 R=R′=H, R″=β-O-linoleoyl
2 R=R′=H, R″=β-OH
5 R=R′=OH, R″=α-OH
6 R=Oh, R′=OAc, R″=α-OAc



3 R=linoleoyl
4 R=H

nals at δ 5.31 (4H, m), 2.73 (2H, t), and 0.84 (3H, t) attributed to the olefinic protons of double bonds, methylene protons between double bonds, and terminal protons, respectively (4). ^{13}C-nmr signals at δ 127.9, 128.0, 130.0, and 130.2 were assigned to the four olefinic carbons of the linoleoyl moiety. Alkaline hydrolysis yielded linoleic acid and 5α,8α-epidioxyergosta-6,22-dien-3β-ol [**4**]. Ir, ms, and nmr spectra of the reaction product of linoleoyl chloride with **4** were identical to those of the natural product **3**.

Compound **5** gave a positive Liebermann-Burchard reaction and hydroxyl (3400 cm^{-1}) absorption in its ir spectrum. The ei mass spectrum showed a molecular ion peak at *m/z* 430 and significant peaks at *m/z* 412 $[M-H_2O]^+$, 287 $(M-SC-H_2O]^+$, 269 $[M-SC-2H_2O]^+$, and 251 $[M-SC-3H_2O]^+$, indicating **5** was a monoene steroid that lost the side chain and three hydroxyl groups located in rings A, B, C, and D (3). The ^{1}H nmr ($CDCl_3$ + CD_3OD) indicated signals for 2H-C-O- (δ 3.57 and 3.98), and -CH=CH- and >C=CH- (δ 5.25, 3H, m) as well as for the five methyl groups typical of a sterol [Me-18 (s, δ 0.62), Me-19 (s, δ 1.06), Me-21 (d, δ 1.03, J = 6.6 Hz), Me-26 (d, δ 0.84, J = 6.6 Hz), and Me-27 (d, δ 0.83, J = 6.6 Hz)]. Appearance of the C-18 methyl signal in the rather high-field position (δ 0.62) indicated **5** was a Δ^7 sterol (9,10). In the ^{13}C-nmr spectrum (Table 1), the chemical shifts of C-4 to C-7, C-12 to C-18, and C-20 to C-28 were almost superimposable with those for the ester of ergosta-7,22-dien-3β-ol [**2**] (1,11); thus the three hydroxyl groups of **5** were located at rings A and B. Since the signals ascribable to C-8, C-10, and C-11 shifted to low field, the quarternary carbon signal at δ 76.3 (Table 1) could be assigned to C-9, with an α-hydroxy group, by comparison with the chemical shifts of the ester of **2** (1,11). The C-19 signal was located downfield (5.5 ppm) compared with the one in the ester of **2** (1,11) because of 1,3-diaxial interaction (12) with a β-axial hydroxyl group at C-2, C-4, C-6, or C-11. Based on the above results, the downfield shift of C-1, and the carbinol protons shown to couple by the COSY spectrum of the diacetate **6**, the remaining two tertiary carbon signals at δ 73.4 and 67.5 were assigned to C-2 (β-axial OH) and C-3 (α-OH), respectively, by comparison with the chemical shifts of the ester of **2** (1,11) and reported data in the literature (13). The ^{13}C-nmr spectrum of **6** (Table 1) was assigned by ^{1}H-decoupling spectra, DEPT pulse sequence, and comparison with the chemical shifts of the esters of **2**, **5**, (25*R*)-5α-spirosta-2β,3α-diol, and 5α-cholesta-2β,3α-diol 3α-acetate (1,11,14). The ^{1}H-^{13}C shift correlation spectra of **6** further supported the above characterization of **5** and indicated the proton signals of **5** at δ 3.98 (1H, W 1/2 = 18 Hz) and 3.57 (1H, W½ = 7 Hz) (shifted on acetylation to δ 5.13 and 4.83, respectively), could be assigned to the H-3β and H-2α, respectively. Since polyoxygenated derivatives of ergosterol showed cytotoxic effect against hepatoma cells (HCT) in vitro (15), the inhibitory activity of the isolates and isolated derivatives

TALBE 1. ^{13}C-nmr Spectra Data of Compounds **5** and **6**.[a]

Carbon	**5**[b]	**6**[c]
C-1	39.3	35.7
C-2	73.4	73.8
C-3	67.5	71.0
C-4	33.0	32.2
C-5	42.1	43.0
C-6	30.7	29.8
C-7	117.8	114.3
C-8	143.5	146.1
C-9	76.3	75.0
C-10	37.3	37.3
C-11	23.2	27.0
C-12	39.6	39.3
C-13	43.4	43.8
C-14	55.0	55.0
C-15	22.2	22.9
C-16	28.3	28.0
C-17	56.3	56.0
C-18	12.4	12.4
C-19	18.4	18.1
C-20	40.7	40.6
C-21	21.3	21.2
C-22	135.8	135.8
C-23	132.3	132.5
C-24	43.1	43.2
C-25	33.4	33.2
C-26	20.1	20.0
C-27	19.6	19.7
C-28	17.6	17.7
$COCH_3$		21.5, 21.9
*C*OMe		171.0, 171.2

[a]The number of directly attached protons to each carbon was vearified with the DEPT pulse sequence.

[b]Measured in $CDCl_3 + CD_3OD$.

[c]Measured in $CDCl_3$.

against human hepatoma PLC/PRF/5 and KB cells in vitro were studied (16,17). The results are listed in Table 2. Both 3β-hydroxy-26-oxo-5α-lanosta-8,24-dien-11-one and **5** showed potent inhibitory activity against human hepatoma PLC/PRF/5 and KB cells in vitro, supporting the cytotoxic effects of polyoxygenated derivatives of ergosterol.

TABLE 2. Cytotoxicity[a] of Compounds Against Various Tumor Cells.

Compound	ED_{50} (μg/ml)	
	PLC/PRF/5	KB
5α,8α-Epidioxyergosta-6,22-dien-3β-ol [**4**]	10.99	9.79
Ganoderic aldehyde A (3β-hydroxy-26-oxo-5α-lanosta-8,24-dien-11-one)	2.54	1.25
2H-Ganoderic aldehyde A (3β,26-dihydroxy-5α-lanosta-8,24-dien-11-one)	10.74	8.27
Ergosta-7,22-diene-2β,3α,9α-triol [**5**]	1.17	0.89

[a]For significant activity of the pure compound, an ED_{50} <4.0 μg/ml is required (19); $n = 8$.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All mp's are uncorrected. Ft-nmr spectra were performed on a Varian VXR-300/51 Superconducting High Resolution FT NMR System; ir spectra on a Hitachi model 260-30; ms on a JMS-HX 110 Mass Spectrometer; and optical rotations on a Jasco model dip-181 digital polarimeter.

EXTRACTION AND SEPARATION.—*G. lucidum*, grown on the stem of *A. confusa*, was collected at Liu-Kuei Shian, Kaohsiung Hsien, Taiwan, Republic of China, during June 1987. A voucher specimen is deposited in our laboratory, Air-dried fruiting bodies were extracted and chromatographed as before (1). Elution of $CHCl_3$ extract with cyclohexane-C_6H_6 (4:1) yielded **1**, with C_6H_6 and C_6H_6-EtOAc (4:1), **3** and **7** (5–8, 18), respectively, and with $CHCl_3$-MeOH (9:1), **7**.

ERGOSTA-7,22-DIEN-3β-YL LINOLEATE [**3**].—Colorless oil: positive to Liebermann-Burchard reaction; ir ν max ($CHCl_3$) cm^{-1} 1720 (CO), 680; ^1H nmr ($CDCl_3$) δ 0.53 (3H, s, H_3-18), 0.81 (3H, s, H_3-19), 0.83 (3H, d, $J = 6.6$ Hz, H_3-27), 0.84 (3H, d, $J = 6.6$ Hz, H_3-26), 0.92 (3H, d, $J = 6.6$ Hz, H_3-28), 1.03 (3H, d, $J = 6.6$ Hz, H_3-21), 4.69 (1H, br s, W½ = 20 Hz, H-3), 5.18 (3H, m, olefinic protons); eims (12 eV) *m/z* (rel. int.) $[M+1]^+$ 661 (70), $[M-C_9H_{17}-2H]^+$ 533 (20), $[M-C_{18}H_{32}O_2+H]^+$ 381 (100), $[C_{18}H_{32}O_2]^+$ 280 (26), $[M-C_{18}H_{32}O_2-C_9H_{17}]^+$ 255 (68), $[M-C_{18}H_{32}O_2-C_{11}H_{19}]^+$ 229 (17), $[381-C_{12}H_{21}-3H]^+$ 213 (13). *Anal.* calcd for $C_{46}H_{76}O_2$: 660.5841; found (ms) 660.5827.

HYDROLYSIS OF **1**.—Compound **1** (50 mg) was refluxed with 5% alcoholic KOH (20 ml) for 4 h. The solvent was reduced to half its volume and then diluted with H_2O (10 ml), extracted with Et_2O, washed with H_2O, and dried (Na_2SO_4). Removal of solvent furnished alcohol **2** (15 mg) identical to authentic **2** by comparison of mp, ir, ms, and nmr. The mother liquor from the above extraction was acidified with dilute HCl and extracted with Et_2O, washed with H_2O, and dried (Na_2SO_4). Removal of solvent furnished linoleic acid, $C_{18}H_{32}O_2$, $[M]^+$ at 280, 5 mg, colorless oil (ms, nmr).

5α,8α-EPIDIOXYERGOSTA-6,22-DIEN-3β-YL LINOLEATE [**3**].—Colorless oil: positive to Liebermann-Burchard reaction; ir ν max ($CHCl_3$) cm^{-1} 1720 (CO), 860, 680; ^1H nmr ($CDCl_3$) δ 0.78 (3H, d, $J = 6.6$ Hz, H_3-26), 0.78 (3H, s, H_3-18), 0.80 (3H, d, $J = 6.6$ Hz, H_3-27), 0.85 (3H, s, H_3-19), 0.88 (3H, d, $J = 6.6$ Hz, H_3-28), 3.95 (1H, m, H-3), 5.15 (2H, m, olefinic protons), 6.19 (1H, d, $J = 8.5$ Hz, H-6), 6.47 (1H, d, $J = 8.5$ Hz, H-7); eims (12 eV) *m/z* (rel. int.) $[M]^+$ 690 (5), $[M-C_{18}H_{32}O_2]^+$ 410 (49), $[M-C_{18}H_{32}O_2-32]^+$ 378 (31), $[M-C_{18}H_{32}O_2-32-H]^+$ 377 (100), $[377-C_9H_{17}+H]^+$ 253 (6), $[377-C_9H_{17}-H]^+$ 251 (60). *Anal.* calcd for $C_{46}H_{74}O_4$: 690.5583; found (ms) 690.5534.

HYDROLYSIS OF **3**.—Compound **3** (50 mg) was dissolved in a 0.1 M MeOH solution (5 ml) of K_2CO_3 and heated at 80° for 1 h. The mixture was partitioned between Et_2O (10 ml) and H_2O (20 ml). The Et_2O layer was dried with Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on Si gel, and the C_6H_6 eluates yielded **4** (15 mg), mp 154°, colorless needles (Me_2CO), identified as 5α,8α-epidioxyergosta-6,22-dien-3β-ol **4** by comparison of mp, ir, ms, and nmr with those of the authentic sample. The mother liquor from the above extraction was treated and identified as in the hydrolysis of **1**.

SYNTHESIS OF **1** AND **3**.—Compound **2** (50 mg) or **4** (50 mg), pyridine (5 ml), and linoleoyl chloride (200 mg) were reacted at room temperature for 24 h. Reaction mixtures were decanted into H_2O to yield a precipitate which was chromatographed on Si gel. The C_6H_6 eluates yielded **1** (25 mg) and **3** (10 mg), respectively. Synthetic **1** and **3** were identical to the natural products, by comparison of mp, ir, ms, and nmr.

ERGOSTA-7,22-DIENE-2β,3α,9α-TRIOL [**5**].—Colorless needles (MeOH): mp 253°; $[\alpha]^{25}D$ −7.2° (MeOH, $C = 0.125$); ir ν max (KBr) cm^{-1} 3400, 1460, 1390, 1375; ^1H nmr ($CDCl_3 + CD_3OD$) see text; ^{13}C nmr ($CDCl_3 + CD_3OD$) see Table 1; eims (12 eV) *m/z* (rel. int.) $[M]^+$ 430 (2), $[M-H_2O]^+$ 412 (100), 394 (54), 383 (43), 379 (41), $[M-\text{side chain}-H_2O]^+$ 287 (11), $[M-\text{side chain}-2H_2O]^+$ 269 (29), $[M-\text{side chain}-3H_2O]^+$ 251 (25), 233 (9), 227 (7), 215 (11), 125 (15), 69 (15), 57 (7), 18 (9). *Anal.* calcd for $C_{28}H_{46}O_3$: 430.3445; found (ms) 430.3447.

ACETYLATION OF **5**.—Compound **5** was acetylated by the usual method. The acetylated product was purified by cc (Si gel). Elution with C_6H_6-EtOAc (2:1) gave ergosta-7,22-diene-2β,3α,9α-triol 2β,3α-diacetate [**6**]: colorless needles (MeOH); mp 175°; ir ν max (KBr) cm^{-1} 3450 (OH), 1740 (CO-O), 1720 (CO-O), 1300 (C-O), 1280 (C-O), 1250 (C-O); ^1H nmr ($CDCl_3$) δ 0.58 (3H, s, H_3-18), 0.81 (3H, d, $J = 6.6$ Hz, H_3-27), 0.83 (3H, d, $J = 6.6$ Hz, H_3-26), 0.91 (3H, d, $J = 6.6$ Hz, H_3-28), 1.02 (3H, d, $J = 6.6$ Hz, H_3-21), 1.05 (3H, s, H_3-19), 2.03 (3H, s, OAc), 2.06 (3H, s, OAc), 4.83 (1H, br s, W 1/2 = 7 Hz, H-2), 5.13 (1H, m, W½ = 18 Hz, H-3); ^{13}C nmr ($CDCl_3$) see Table 1; eims (12 eV) (rel. int.) $[M-H_2O]^+$ 496 (2), $[M-H_2O-42]^+$ 454 (12), $[454-H_2O]^+$ 436 (59), $[454-H_2O-42]^+$

394 (56), [394 − H_2O]$^+$ 376 (100), [394 − side chain]$^+$ 269 (7), [376 − side chain]$^+$ 251 (28), 125 (10), 69 (14).

BIOLOGICAL ASSAY.—PLC/PRF/5 cells were established from human hepatoma and are known to produce HBs Ag continuously in culture fluids (16). The cells were grown as continuous cultures in a growth medium consisting of Dulbecco's modified Eagle medium (DMEM, GIBCO, Grand Island, NY), 10% fetal bovine serum (FBS, GIBCO), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The KB cells were maintained on DMEM containing with 10% FBS, L-glutamine, and antibiotics. For microassay, the growth medium was supplemented further with 10 mM Hepes buffer, pH 7.3.

The microassay for anticellular effect was performed as previously (17,20). The ED_{50} values were calculated from a semilog plot of the drug concentration vs. the percentage of viable cells on day 4.

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