Differentiation- and Apoptosis-Inducing Activities by Pentacyclic Triterpenes on a Mouse Melanoma Cell Line

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In a study to investigate the relationship between the chemical structure and the differentiation-inducing activity of pentacyclic triterpenes, several lupane, oleanane, and ursane triterpenes were prepared and their effects on B16 2F2 melanoma cell differentiation and growth were examined. Eleven lupane triterpenes used in this study acted on the melanoma cells as a melanogen, but no induction of melanogenesis of B16 2F2 cells by oleanane and ursane was detected. The differences at C-17 of the lupane series and acetylation of the OH group at C-3 did not markedly influence their activities. However, the ED_{50} value for up-regulation of melanin biosynthesis was markedly decreased by the oxidation of the OH group at C-3 of lupeol (**1**). Betulinic acid (**11**), its methyl ester (**12**), lup-28-al-20(29)-ene-3β-ol (**9**), and lup-28-al-20(29)-en-3-one (**10**) inhibited B16 2F2 cell proliferation by induction of apoptosis. These findings suggested that the carbonyl group at C-17 might be essential for the apoptotic effects of these compounds on B16 2F2 cells.

Lupane series triterpenes are widely distributed in Compositae plants, and their biological activities have attracted a great deal of attention. A number of studies on their antiinflammatory activities have been reported.[1,2] More recently, the anticarcinogenic activity of *Taraxacum* plants was investigated, and the triterpenes such as lupeol (**1**) were shown to exhibit inhibitory effects on the induction of Epstein−Barr virus early antigen by tumor promoters.[3] Furthermore, betulinic acid (**11**), a lupane triterpene, has been shown to possess anti-AIDS activity, and the C-3 hydroxy group and C-17 carboxylic group of **11** contributed to enhancement of this activity.[4] It was reported that **11** inhibited cell proliferation by induction of apoptosis in human melanoma and other cancer cell lines.[5,6] The carboxylic group at C-17 was essential for the apoptotic effects of both **11** and its analogue, ceanothic acid, on cancer cells.[7]

The mouse melanoma cell line B16 can be induced to differentiate into mature melanocyte-like cells by treatments with α-melanocyte-stimulating hormone (α-MSH) or signal transduction pathway inhibitors. In the differentiation of B16 cells, up-regulation of melanin biosynthesis was observed.[8−10] In our previous study, we isolated a B16-derived subclone with high differentiation capability (B16 2F2) and screened differentiation-inducing activities of naturally occurring substances in food materials on melanoma cells using this clone.[11] The alcoholic extracts of *Taraxacum* plants were found to markedly up-regulate melanogenesis of B16 2F2 cells, and we isolated lupeol as the active compound by bioassay-guided fractionation of the ethanol extract of Chinese dandelion root.[11] Here we evaluated the effects of 11 lupane, 5 oleanane, and 5 ursane triterpenes on B16 2F2 cell differentiation and growth.

Results and Discussion

Briefly, B16 2F2 cells were incubated with 1.0 or 10 μM **1** (lupeol) or **2** (lupenone) for 3 days. Following addition of 10 μM **1** or **2**, the melanin biosynthesis of B16 2F2 melanoma cells was up-regulated as compared with untreated controls (Figure 1). No up-regulation was observed with 1.0 μM **1**. However 1.0 μM **2** induced cell melanogenesis to the same extent as stimulation with 10 μM. These results indicated that the structural differences between these molecules at C-3 influenced their activities. To investigate the relationships between the structures of these triterpenes and their activities, B16 2F2 cells were treated with 11 lupane, 5 oleanane, and 5 ursane series triterpenes for 72 h, and the intracellular melanin content and the growth of B16 2F2 cells were measured. The results are summarized in Table 1. Eleven lupane series triterpenes used in this study induced the up-regulation of B16 2F2 cell melanin biosynthesis, an indicator of melanoma cell differentiation.[8] ED_{50} values of **1**, **3**, **4**, **8**, **9**, **11**, and **12** for the induction of melanogenesis ranged from 2.5 to 9.9 μM. These observations indicated that the differences at C-17 had little influence on differentiation-inducing activity. Acetylation at C-3 of **1** or **4** showed no effect on the activity. However, oxidation of the OH group at C-3 markedly influenced their ED_{50} values. From these results, we speculated that ketone formation at C-3 plays an important role in the induction of B16 2F2 cell melanin synthesis by lupane series triterpenes. On the other hand, the differentiation of B16 2F2 cells was not induced by other pentacyclic triterpenes such as oleanane (**14**−**18**) and ursane (**19**−**23**).

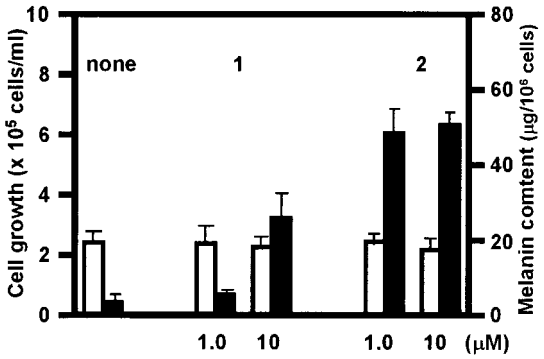

Figure 1. Effects of **1** and **2** on B16 2F2 cell growth and melanogenesis following treatment with test compounds at 1.0 or 10 μM for 72 h. The cell growth (□) and melanin contents (■) were measured.

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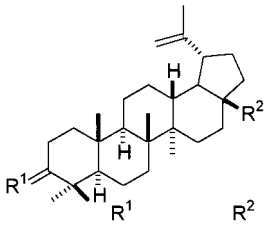
Table 1. Effects of Some Triterpenes on B16 2F2 Cell Differentiation and Growth

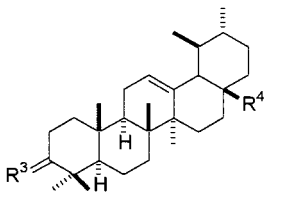
compound	skeleton	ED_{50} (μM)[a]	IC_{50} (μM)[b]
1	lupane	9.9	38.0
2		0.35	25.4
3		7.5	22.7
4		4.8	27.4
7		0.19	29.3
8		3.4	36.8
9		3.3	6.4
10		0.35	4.1
11		4.1	7.9
12		2.5	4.9
13		0.13	5.6
14	oleanane	>50	48.8
15		>50	38.7
16		>50	33.4
17		-[c]	6.4
18		-	4.8
19	ursane	>50	50.0
20		>50	40.3
21		>50	46.8
22		-	5.5
23		-	3.1

[a] ED_{50} values were determined from the results of dose-response experiments in which the up-regulation of melanogenesis by the compound was monitored. [b] IC_{50} value represents the concentration that inhibited cell growth by 50%. [c] The value for the compound could not be determined because of its cytotoxicity at 10 μM, and the up-regulation of melanogenesis was not induced by concentrations under 10 μM.

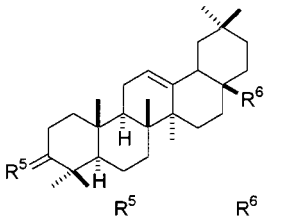


	R^1	R^2
1	α-H, β-OH	CH_3
2	=O	CH_3
3	α-H, β-OAc	CH_3
4	α-H, β-OH	CH_2OH
5	α-H, β-OH	CH_2OTr
6	=O	CH_2OTr
7	=O	CH_2OH
8	α-H, β-OAc	CH_2OCOCH_3
9	α-H, β-OH	CHO
10	=O	CHO
11	α-H, β-OH	COOH
12	α-H, β-OH	$COOCH_3$
13	α-H, β-OAc	$COOCH_3$

Previously, **11** was reported to show selective anticancer effects against human melanoma cells.[5] We examined the cytotoxic effects of these triterpenes on B16 2F2 cells by comparing their IC_{50} values for B16 2F2 cell growth. Compounds **9**, **10**, **11**, **12**, **13**, **17**, **18**, **22**, and **23**, all of which have a carbonyl group at C-17, inhibited B16 2F2 cell proliferation at lower concentrations than other compounds. Following incubation of B16 2F2 cells with 10 μM **9** or **17** for 48 h, the cells were stained with Hoechst 33258 dye. The condensation and fragmentation of nuclei were observed in B16 2F2 cells treated with **9** (Figure 2B) or **17** (Figure 2C). These compounds showed apoptotic effects on B16 2F2 cells, and 70.4% and 62.5% of the cells underwent apoptosis by 10 μM **9** and **17** for 48 h, respectively. The carboxyl group at the C-17 position of **11** and ceanothic acid was reported to be essential for the apoptotic activities against some cancer cell lines.[7] In this study, **11**, **12**, and **13**, all of which possess a carboxyl group at C-17, showed



	R^3	R^4
14	α-H, β-OH	CH_3
15	=O	CH_3
16	α-H, β-OH	CH_2OH
17	α-H, β-OH	CHO
18	α-H, β-OH	COOH



	R^5	R^6
19	α-H, β-OH	CH_3
20	=O	CH_3
21	α-H, β-OH	CH_2OH
22	α-H, β-OH	CHO
23	α-H, β-OH	COOH

strong cytotoxic effects against B16 2F2 cells, but **9** and **10** inhibited B16 2F2 cell growth to the same extent as **11**, **12**, and **13**. These results suggested that the carbonyl group and not the carboxyl group at C-17 was essential for their apoptotic effects, and similar effects were observed following treatment with other pentacyclic triterpenes with a carbonyl group at C-17. In addition, the ketone function at C-3 of lupane series triterpenes enhanced their B16 2F2 cell differentiation-inducing activities. We concluded that the different moieties of the lupane skeleton separately regulated the induction of differentiation and apoptosis of B16 2F2 melanoma cells.

Experimental Section

Materials. Lupeol (**1**), lupenone (**2**), lupenyl acetate (**3**), betulin (**4**), betulin diacetate (**8**), betulinic acid methylester (**12**), β-amyrin (**14**), erythrodiol (**16**), oleanolic acid (**18**), α-amyrin (**19**), uvaol (**21**), and ursolic acid (**23**) were purchased from Extrasynthese S.A. Hoechst 33258 and betulinic acid (**11**) were obtained from Sigma Co. Ltd., (dimethylamino)pyridine and triphenylmethyl chloride (trytyl chloride) were from Nakalai Tesque Co. Ltd., and pyridium chlorochromate (PCC) was from Tokyo Kasei Kogyo Co., Ltd.

General Experimental Procedures. Melting points were determined on a Yanagimoto micro-melting point apparatus and recorded as observed. Optical rotations were measured in a 0.5 cm length cell tube with a JASCO DIP-1000 polarimater. All the mass spectra (EIMS, negative-FABMS, HRE-IMS, and negative-HRFABMS) were obtained on a JEOL JMS BU-20 gas chromatograph mass spectrometer. Infrared (IR) spectra were obtained on a JASCO FT-IR 300 spectrometer. Ultraviolet (UV) spectra were obtained on a JASCO 270-30 UV−vis spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity plus 400 spectrometer (^1H, 400 MHz; ^{13}C, 100 MHz), with tetramethylsilane (TMS) as an internal standard. Chemical shifts are given on a δ scale (ppm). The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Coupling constants (J value) are given in hertz (Hz).

Tritylation of Betulin (4). A solution of betulin (**4**, 100 mg) in dry dichloromethane (3 mL) was treated with trityl chloride (200 mg) and *N*,*N*-(dimethylamino)pyridine (50 mg),

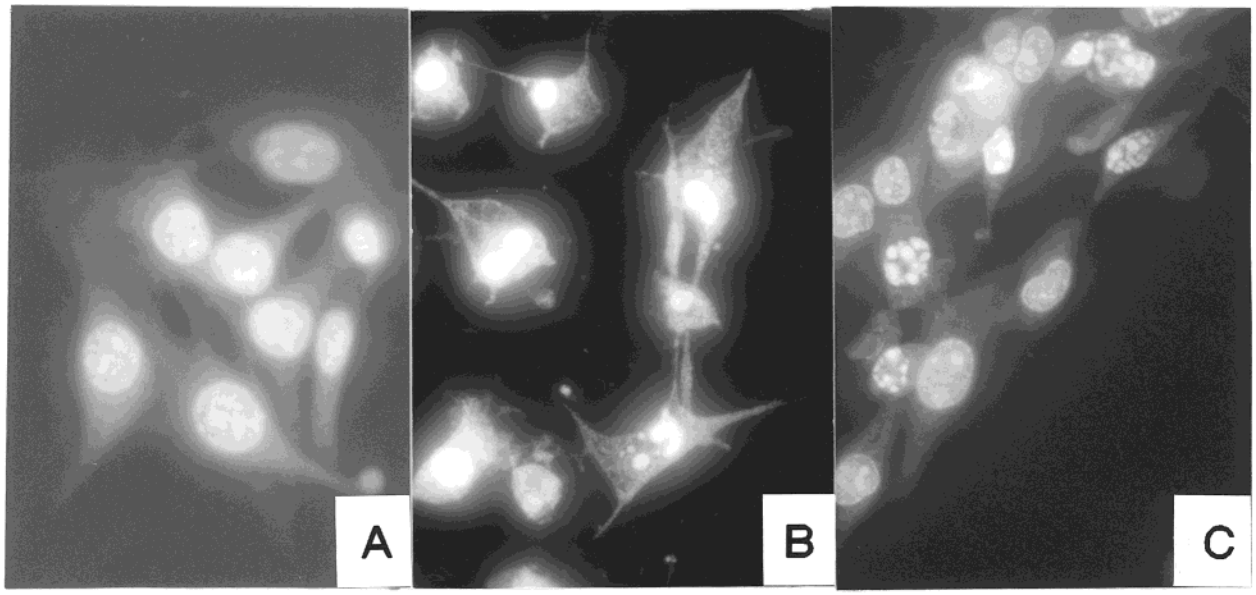


Figure 2. Induction of apoptosis of B16 2F2 cells by **9** and **17** B16 2F2 cells stained with Hoechst 33258 dye after incubation without (A) or with 10 μM **9** (B) or **17** (C) for 48 h.

and the mixture was stirred at 60 °C for 2 h. The reaction mixture was poured into ice−water and extracted with EtOAc. The EtOAc extract was washed with saturated saline, then dried over $MgSO_4$ and filtered. Evaporation of the solvent from the filtrate under reduced pressure afforded the product, which was purified by silica gel column chromatography [SiO_2 50 g, *n*-hexane−EtOAc (10:1)] to furnish **5** [28-*O*-trityl-lup-20(29)-en-3β,28-diol (28-*O*-trityl betulin), 263 mg].

5: colorless needles (MeOH); mp 155−156 °C; $[\alpha]^{25}_D$ −5.4° (*c* 0.3,$CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 208 (sh) (4.50) nm; IR (KBr) ν_{max} 3500−3200, 2942, 1640, 1597, 1490, 1449, 1388, 1374, 1216, 1064, 983, 884, 760, 706, 632 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.51 (6H, d, J = 7.6 Hz, trityl 2′,6′-protons), 7.36 (6H, dd, J = 7.6, 7.6 Hz, trityl 3′,5′-protons), 7.24 (3H, dd, J = 7.6, 7.6 Hz, trityl 4′-protons), 4.61, 4.56 (each 1H, br s, H_2-29), 3.20 (1H, dd, J = 10.0, 4.2 Hz, 3α-H), 3.17, 2.96 (each 1H, d, J = 8.6 Hz, H_2-28), 2.30−2.17 (3H, m, H_2-2 and H-19), 1.66 (3H, s, H_3-30), 1.00 (3H, s), 0.95 (3H, s), 0.79 (3H, s), 0.78 (3H, s), 0.57 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 150.8 (s, C-20), 149.5 (s, trityl C-1′), 128.7 (s, trityl C-3′,5′), 127.7 (s, trityl C-2′,6′), 126.8 (s, trityl C-4′), 109.3 (t, C-29), 85.8 (s, C-17), 78.9 (d, C-3), 59.4 (t, C-28), 55.2 (d, C-5), 50.2 (d, C-9), 48.8 (d, C-19), 47.7 (d, C-18), 47.5 (s, trityl C-α), 42.4 (s, C-14), 40.5 (s, C-8), 38.8 (s, C-4), 38.6 (t, C-1), 37.2 (d, C-13), 37.1 (s, C-10), 35.2 (t, C-22), 34.1 (t, C-7), 30.1 (t, C-16), 29.9 (t, C-15), 28.0 (q, C-23), 27.3 (t, C-2), 26.4 (t, C-21), 25.1 (t, C-12), 20.7 (t, C-11), 19.1 (q, C-30), 18.3 (t, C-6), 16.0 (q, C-25), 15.8 (q, C-26), 15.3 (q, C-24), 14.7 (q, C-27); negative-FABMS (diethanolamine as a matrix) *m*/*z* 683 (1.5, $[M - H]^-$), 502 (7), 259 (100); negative-HRFABMS (diethanolamine as a matrix) *m*/*z* 683.4842 (calcd for $C_{49}H_{63}O_2$, 683.4828).

Oxidation of 5 with Pyridinium Chlorochromate (PCC). PCC (50 mg) was added to a solution of **5** (100 mg) in dry dichloromethane (2 mL), and the mixture was stirred vigorously at room temperature for 2 h. The reaction mixture was dissolved with diethyl ether (5 mL) and suspended in 10 volumes of diethyl ether, then passed through a short Florisil (Nakalai Tesque, 60−100 mesh) column. The solvent was removed from the eluate to furnish **6** [28-*O*-trityl lup-20(29)-en-28-ol-3-one, 78.4 mg].

6: colorless needles (MeOH); mp 142−143 °C; $[\alpha]^{25}_D$ 16.2° (*c* 0.3,$CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 210 (sh) (4.32) nm; IR (KBr) ν_{max} 2944, 2868, 1705, 1640, 1597, 1490, 1449, 1384, 1375, 1216, 1065, 1031, 986, 897, 883, 759, 707, 632 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.49 (6H, d, J = 7.5 Hz, trityl 2′,6′-protons), 7.35 (6H, dd, J = 7.5, 7.5 Hz, trityl 3′,5′-protons), 7.22 (3H, dd, J = 7.5, 7.5 Hz, trityl 4′-protons), 4.60, 4.57 (each 1H, br s, H_2-29), 3.17, 2.94 (each 1H, d, J = 8.2 Hz, H_2-28), 2.50−2.20 (3H, m, H_2-2 and H-19), 1.62 (3H, s, H_3-30), 1.05 (3H, s), 1.02 (3H, s), 0.91 (3H, s), 0.89 (3H, s), 0.58 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.2 (s, C-3), 150.7 (s, C-20), 144.4 (s, trityl C-1′), 128.7 (s, trityl C-3′,5′), 127.7 (s, trityl C-2′,6′), 126.8 (s, trityl C-4′), 109.4 (t, C-29), 85.8 (s, C-17), 59.4 (t, C-28), 54.8 (d, C-5), 49.6 (d, C-9), 48.8 (d, C-19), 47.7 (s, trityl C-α), 47.5 (d, C-18), 47.3 (s, C-4), 42.5 (s, C-14), 40.5 (s, C-8), 39.5 (t, C-1), 37.3 (d, C-13), 36.8 (s, C-10), 35.2 (t, C-22), 34.1 (t, C-2), 33.4 (t, C-7), 30.0 (t, C-16), 29.9 (t, C-15), 26.8 (t, C-21), 26.7 (q, C-23), 25.1 (t, C-12), 21.2 (t, C-11), 21.0 (q, C-24), 19.6 (t, C-6), 19.1 (q, C-30), 15.9 (q, C-25), 15.6 (q, C-26), 14.6 (q, C-27); negative-FABMS (diethanolamine as a matrix) *m*/*z* 682 (0.6, $[M(^{13}C \times 1) - H]^-$), 681 (2.2, $[M - H]^-$), 502 (5.5), 259 (100); negative-HRFABMS (diethanolamine as a matrix) *m*/*z* 681.4675 (calcd for $C_{49}H_{61}O_2$, 681.4671).

Acidic Treatment of 6. A solution of **6** (35 mg) in tetrahydrofuran (1 mL) was treated with *p*-toluenesulfonic acid (100 mg), and the mixture was stirred at 60 °C for 5 h. The reaction mixture was poured into ice−water and extracted with EtOAc. The EtOAc extract, obtained after workup as described above, was purified by column chromatography [SiO_2 20 g, *n*-hexane−AcOEt (5:1)] to give compound **7** [lup-20(29)-en-28-ol-3-one (betulone), 13.2 mg].[12]

7: colorless needles (*n*-hexane); mp 94−96 °C; $[\alpha]^{25}_D$ 52.7° (*c* 0.3,$CHCl_3$); IR (KBr) ν_{max} 3600−3250, 2944, 2868, 1704, 1641, 1457, 1375, 1242, 1134, 1111, 1026, 881, 755 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.78, 4.62 (each 1H, br s, H_2-29), 3.17, 2.94 (each 1H, d, J = 8.2 Hz, H_2-28), 2.50−2.20 (3H, m, H_2-2 and H-19), 1.62 (3H, s, H_3-30), 1.05 (3H, s), 1.02 (3H, s), 0.91 (3H, s), 0.89 (3H, s), 0.58 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.2 (s, C-3), 150.4 (s, C-20), 109.7 (t, C-29), 60.5 (t, C-28), 54.9 (d, C-5), 49.7 (d, C-9), 48.7 (d, C-19), 47.7 (s and d, C-17 and C-18), 47.3 (s, C-4), 42.7 (s, C-14), 40.8 (s, C-8), 39.6 (t, C-1), 37.4 (d, C-13), 36.8 (s, C-10), 34.1 (t, C-2), 33.9 (t, C-22), 33.5 (t, C-7), 29.7 (t, C-19), 29.0 (t x 2, C-16 and C-21), 27.0 (t, C-15), 26.6 (q, C-23), 25.2 (t, C-12), 21.3 (t, C-11), 21.0 (q, C-24), 19.6 (t, C-6), 19.1 (q, C-30), 15.9 (q, C-25), 15.8 (q, C-26), 14.6 (q, C-27); EIMS *m*/*z* 440 (19.2 $[M^+]$), 422 (15), 409 (92), 245 (40), 203 (92), 189 (90), 121 (100), 107 (97), 95 (93); HREIMS *m*/*z* 440.3659 (calcd for $C_{30}H_{48}O_2$, 440.3654).

Oxidation of 4 with PCC. PCC (100 mg) was added to a solution of **4** (100 mg) in dry dichloromethane (2 mL), and the mixture was stirred vigorously at room temperature for 2 h. The residue obtained after workup as described above was purified by column chromatography [SiO_2 20 g, *n*-hexane−EtOAc (5:1)] to provide compound **9** [lup-28-al-20(29)-en-3β-ol, 30 mg] and compound **10** [lup-28-al-20(29)-en-3-one, 20.8 mg].

9: colorless needles (MeOH); mp 167−169 °C; $[\alpha]^{25}_{D}$ 15.9° (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 3650−3200, 3402, 2942, 1726, 1642, 1452, 1376, 1215, 1106, 1043, 984, 886,757, 668 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.68 (1H, s, −C*H*O), 4.70, 4.57 (each 1H, br s, H_2-29), 3.20 (1H, dd, J = 10.4, 4.0 Hz, 3α-H), 2.82 (1H, dt, J = 6.0, 11.2 Hz, H-19), 1.68 (3H, s, H_3-30), 0.98 (3H, s), 0.97 (3H, s), 0.91 (3H, s), 0.82 (3H, s), 0.77 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 206.7 (s, C-28), 149.7 (s, C-20), 110.2 (t, C-29), 79.0 (d, C-3), 59.3 (s, C-17), 55.3 (d, C-5), 50.4 (d, C-9), 48.0 (d, C-19), 47.5 (d, C-18), 42.5 (s, C-14), 40.8 (s, C-8), 38.8 (s, C-4), 38.70 (t, C-1), 38.67 (d, C-13), 37.1 (s, C-10), 34.3 (t, C-7), 33.2 (t, C-22), 29.8 (t, C-21), 29.2 (t, C-16), 28.8 (t, C-15), 27.9 (q, C-23), 27.4 (t, C-2), 25.5 (t, C-12), 20.7 (t, C-11), 19.0 (q, C-30), 18.2 (t, C-6), 16.1 (q, C-25), 15.8 (q, C-26), 15.3 (q, C-24), 14.2 (q, C-27); EIMS *m*/*z* 440 (31 [M^+]), 422 (12), 412 (28), 220 (25), 207 (68), 189 (100), 175 (41), 135 (49); HREIMS *m*/*z* 440.3647 (calcd for $C_{30}H_{48}O_2$, 440.3654).

10: colorless oil; $[\alpha]^{25}_{D}$ 35.2° (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 2943, 2867, 1705, 1642, 1453, 1376, 1248, 1139, 1043, 883, 755 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 9.68 (1H, s, −C*H*O), 4.77, 4.64 (each 1H, br s, H_2-29), 2.87 (1H, dt, J = 6.2, 11.0 Hz, H-19), 2.38−2.58 (3H, m, H_2-2 and H-16eq), 1.70 (3H, s, H_3-30), 1.07 (3H, s), 1.03 (3H, s), 0.99 (3H, s), 0.95 (3H, s), 0.93 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.1 (s, C-3), 206.5 (s, C-28), 149.6 (s, C-20), 110.2 (t, C-29), 59.3 (s, C-17), 54.9 (d, C-5), 49.8 (d, C-9), 48.0 (d, C-19), 47.5 (d, C-18), 47.3 (s, C-4), 42.6 (s, C-14), 40.8 (s, C-8), 39.6 (t, C-1), 38.8 (d, C-13), 36.9 (s, C-10), 34.1 (t, C-2), 33.6 (t, C-7), 33.2 (t, C-22), 29.8 (t, C-21), 29.1 (t, C-16), 28.8 (t, C-15), 26.6 (q, C-23), 25.4 (t, C-12), 21.3 (t, C-11), 21.0 (q, C-26), 19.6 (t, C-6), 19.0 (q, C-30), 16.0 (q, C-25), 15.7 (q, C-24), 14.2 (q, C-27); EIMS *m*/*z* 438 (31 [M^+]), 410 (73), 232 (32), 219 (47), 205 (97), 189 (100), 175 (70); HREIMS *m*/*z* 438.3507 (calcd for $C_{30}H_{46}O_2$, 438.3498).

Oxidation of 12 with PCC. PCC (50 mg) was added to a solution of **12** (100 mg) in dry dichloromethane (3 mL), and the mixture was stirred vigorously at room temperature for 2 h. The residue obtained after workup as described above was purified by column chromatography [SiO_2 20 g, solvent system; *n*-hexane−$CHCl_3$ (1:3)] to furnish compound **13** [methyl lup-20(29)-en-3-one-28-oic acid, 88 mg].

13: colorless needles (MeOH); mp 158−159 °C; $[\alpha]^{25}_{D}$ 39.7° (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 2947, 2868, 1726, 1705, 1642, 1458, 1376, 1319, 1187, 1156, 984, 883, 755 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.75, 4.60 (each 1H, br s, H_2-29), 3.67 (3H, s, COOC*H*$_3$), 3.05 (1H, dt, J = 5.6, 11.0 Hz, H-19), 2.20−2.55 (3H, m, H_2-2 and H-16eq), 1.70 (3H, s, H_3-30), 1.08 (3H, s), 1.04 (3H, s), 0.97 (3H, s), 0.96 (3H, s), 0.91 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.2 (S, C-3), 176.6 (s, C-28), 150.5 (s, C-20), 109.6 (t, C-29), 56.5 (s, C-17), 55.0 (d, C-5), 51.3 (q, COO*C*H$_3$), 49.8 (d, C-9), 49.4 (d, C-19), 47.3 (d, C-18), 46.9 (s, C-4), 42.4 (s, C-14), 40.6 (s, C-8), 39.0 (t, C-1), 38.3 (d, C-13), 36.92 (t, C-22), 36.88 (s, C-10), 34.1 (t, C-2), 33.6 (t, C-7), 32.1 (t, C-21), 30.5 (t, C-16), 29.6 (t, C-15), 26.6 (q, C-23), 25.5 (t, C-12), 21.3 (t, C-11), 21.0 (q, C-24), 19.6 (t, C-6), 19.3 (q, C-30), 15.9 (q, C-25), 15.7 (q, C-26), 14.6 (q, C-27); EIMS *m*/*z* 468 (61, [M^+]), 453 (10), 409 (44), 262 (59), 249 (45), 205 (46), 203 (47), 189 (100), 175 (35); HREIMS *m*/*z* 468.3632 (calcd for $C_{31}H_{48}O_3$, 468.3603).

Oxidation of β-Amyrin (14), Erythrodiol (16), α-Amyrin (19), and Uvaol (21) with PCC. PCC was added to the solutions of **14**, **16**, **19**, and **21** in dry dichloromethane, and mixtures were stirred vigorously at room temperature. Residues obtained after workup in the same manner as described above were purified by SiO_2 column chromatography to give **15** [olean-12-en-3-one (β-amyrenone)],[13] **17** [olean-28-al-12-en-3β-ol (oleanoaldehyde)],[14] **20** [ursan-12-en-3-one (α-amyrenone)],[13] and **22** [ursan-28-al-12-en-3β-ol (ursolaldehyde)],[15] respectively. These structures were identified by IR, ^1H NMR, and ^{13}C NMR spectrometry.

Cell Culture. The B16-derived subclone B16 2F2[11] was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/mL of streptomycin, and 100 U of penicillin.

Effect of Triterpenes on Melanogenesis and Cell Growth of B16 2F2Cells. Aliquots of 1 mL of B16 2F2 cells (1×10^5 cells) were incubated with various concentrations of triterpenes for 3 days, and the melanin contents and viable cell number were measured as described previously.[9] ED_{50} values for melanin biosynthesis were calculated from the results of dose−response studies for up-regulation of melanogenesis. IC_{50} values, representing the concentration that inhibited melanoma cell growth by 50%, were measured.

Detection of Cell Apoptosis. B16 2F2 cells were incubated with or without various triterpenes at 10 μM for 48 h. Following collection and washing with PBS, the cells were fixed in 1% glutaraldehyde and stained with 50 μM Hoechst 33258 dye for 30 min. The apoptotic appearance of B16 2F2 melanoma cells was confirmed by fluorescence microscopy, and percentages of apoptosis-induced cells were counted manually on a minimum of 200 cells.

References and Notes

(1) Hasmeda, M.; Okai, G. K.; Macrides, T.; Polya, G. M. *Planta Med*. **1995**, *65*, 14−18.
(2) Recio, C. M.; Giner, R. M.; Manez, S.; Rios, J. L. *Planta Med*. **1995**, *61*, 182−185.
(3) Takasaki, M.; Konoshima, T.; Tokuda, H.; Masuda, K.; Arai, Y.; Shiojima, K.; Ageta, H. *Biol. Pharm. Bull*. **1999**, *22*, 606−610.
(4) Fujioka, Y.; Kashiwada, Y. *J. Nat. Prod*. **1994**, *57*, 243−247.
(5) Pisha, E.; Chai, H.; Lee, I. S.; Chagwedera, T. E.; Fransworth, N.; Cordell, G. A., Beecher, C. W. W.; Fong, H. H. S.; Kinghorn, A. D., Brown, D. M.; Wani, M. C.; Wall, M. E.; Hieken, T. J.; Dupta, T. K. D.; Pezzuto, J. M. *Nature Med*. **1995**, *1*, 1046−1051.
(6) Fulda, S.; Scaffidi, C.; Susin, S. A.; Krammer, P. H.; Kroemer, G.; Peter, M. E.; Debatin, K. M. *J. Biol. Chem*. **1998**, *273*, 33942−33948.
(7) Lee, S. S.; Chen, W. C.; Huang, C. F.; Su, Y. *J. Nat. Prod*. **1998**, *61*, 1343−1347.
(8) Englaro, W.; Rezzonico, R.; Clement, M. D.; Lallemand, D.; Ortonne, J. P.; Ballotti, R. *J. Biol. Chem*. **1995**, *270*, 24315−24320.
(9) Busca, R.; Bertolotto, C.; Ortonne, J. P.; Bellotti, R. *J. Biol. Chem*. **1996**, *271*, 31824−31830.
(10) Engalo, W.; Bertolotto, C.; Busca, R.; Brunet, A.; Pages, G.; Ortonne, J. P.; Ballotti, R. *J. Biol. Chem*. **1998**, *273*, 9966−9970.
(11) Hata, K.; Ishikawa, K.; Hori, K.; Konishi, T. *Biol. Pharm. Bull*. **2000**, *23*, 962−967.
(12) Cole, B. J. W.; Bentley, M. D.; Hua, Y. *Holzforschung* **1991**, *45*, 265−268.
(13) Wahlberg, I.; Karlsson, K.; Enzell, C. R. *Acta Chem. Scand*. **1972**, *26*, 1383−1388.
(14) Kamiya, K.; Yoshikawa, K.; Sakai, Y.; Ikuta, A.; Satake, T. *Phytochemistry* **1997**, *44*, 141−144.
(15) Dagna, L.; Gasparini, G.; Ichardi, M. L.; Sesia, E. *Am. J. Enol. Vitic*. **1982**, *33*, 201−206.

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