Lupane-Type Triterpenoids from *Microtropis fokienensis* and *Perrottetia arisanensis* and the Apoptotic Effect of 28-Hydroxy-3-oxo-lup-20(29)-en-30-al

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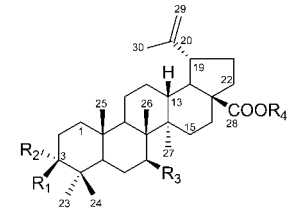
Seven new lupane triterpenoids were isolated from bioactive methanol extracts of *Microtropis fokienensis* (**1**−**4**) and *Perrottetia arisanensis* (**4**−**7**), along with 18 known compounds. The structures of the new compounds were elucidated on the basis of spectroscopic data analysis. All triterpenoids were evaluated for their in vitro cytotoxicity toward seven human cancer cell lines. Compound **8** (28-hydroxy-3-oxo-lup-20(29)-en-30-al) was among the most cytotoxic substances obtained and was found to induce apoptosis of human leukemia HL60 cells and mediate cleavage of PARP and up-regulation of Bax proteins.

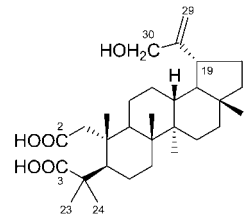
The family Celastraceae has almost 100 genera and about 1300 species[1] that are widespread in the tropical and subtropical regions of North Africa, South America, and East Asia.[2] Previous studies have revealed several bioactive secondary metabolites from this family including sesquiterpenes, diterpenes, and triterpenes, which have reported analgesic,[3,4] antiasthmatic,[5,6] anti-HIV,[7] anti-inflammatory,[8] antimicrobial,[5,6] antiseptic,[5,6] antispasmodic,[9] antispermatogenetic,[10] antiulcer,[3,4] cytotoxic,[11] and insecticidal[12,13] activities. On screening of bioactive Celastraceous species, *Microtropis fokienensis* Dunn. and *Perrottetia arisanensis* Hayata (epidemic to Taiwan),[14] preliminary data showed the crude extracts to have growth inhibitory effects toward HepG2, Hep3B, and MCF7 cells at a concentration of 20 μg/mL. Previously we have reported the cytotoxicity of ursane and oleanane triterpenoids from the leaves of *M. fokienensis*.[15]

In an ongoing search for bioactive triterpenoids from Celastraceous plants, the MeOH extracts of the stems of both *M. fokienensis* and *P. arisanensis* were selected for further investigation. Two new C-7-substituted lupane-type triterpenes, 7β-hydroxymethyl betulinate (**1**) and 7β-senecioyl-3-*epi*-betulinic acid (**2**), a new 2,3-*seco*-lupane derivative, 30-hydroxy-2,3-*seco*-lup-20(29)-ene-2,3-dioic acid (**3**), and a new lupane lactone, 3-*epi*-thurberogenin (**4**), were isolated from stems of *M. fokienensis*. In turn, 3-*epi*-thurberogenin (**4**) as well as the additional new lupane triterpenes, 3-*epi*-thurberogenin-22β-dodecanoate (**5**), 3-*epi*-thurberogenin-22β-tetradecanoate (**6**), and 3-*epi*-thurberogenin-22β-hexadecanoate (**7**), were isolated from the stems of *P. arisanensis*.

Programmed cell death, or apoptosis, is involved in a wide range of biological and pathological processes such as homeostasis, immune responses, and the progression of tumor cells.[16–19] It is characterized by striking morphological changes, such as membrane blebbing, collection of chromatin, and nuclear breakdown, and terminates with cell and internucleosomal DNA fragmentation as well as cleavage of poly(ADP-ribose) polymerase (PARP).[20] In this study, the cytotoxicity of all isolates, except for the mixture of β-sitosterol and β-stigmasterol, was assessed against seven human



	R_1	R_2	R_3	R_4
1	OH	H	OH	CH_3
2	H	OH		H
9	OH	H	H	H
10	H	OH	H	H



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cancer cell lines, namely, HepG2 and Hep3B (hepatoma), MCF7 and MDA-MB-231 (breast), A549 (lung), Ca9-22 (gingival), and HL60 (leukemia). Referencing to NCI criteria, IC_{50} values of less than 4.0 μg/mL were considered to represent activity in the cytotoxicity assay. The isolates **8**−**11** showed cytotoxicity for one or more cell lines. The active compound 28-hydroxy-3-oxo-lup-20(29)-en-30-al (**8**) was selected for further mechanic investigation of survival suppression and proliferation of HL60 cells.

Results and Discussion

The molecular formula of compound **1** was assigned as $C_{31}H_{50}O_4$ (*m/z* 509.3607 $[M + Na]^+$, calcd 509.3604, DBU 7) by HRESIMS and from its NMR spectroscopic data. The IR spectrum indicated the occurrence of hydroxyl (3398 cm^{-1}) and ester (1691 cm^{-1}) moieties. ^{13}C NMR and DEPT experiments exhibited 31 carbons including seven singlet methyl groups (six tertiary methyl groups and one methoxyl group), 10 methylenes, seven methines, and seven quaternary carbons, which revealed the basic skeleton of a

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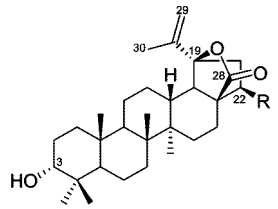
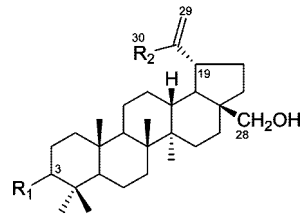


	R
4	H
5	$OCO(CH_2)_{10}CH_3$
6	$OCO(CH_2)_{12}CH_3$
7	$OCO(CH_2)_{14}CH_3$



	R_1	R_2
8	=O	CHO
11	α-OH	CH_3

triterpenoid. From the NMR spectra, an isopropenyl group [δ_H 1.78 (3H, s), 4.77 (1H, brs), and 4.93 (1H, brs); δ_C 19.5, 110.0, and 151.1] and one carbonyl group [δ_C 176.6] were observed. The remaining five degrees of unsaturation suggested **1** to be a pentacyclic triterpene of the lupane family.[16,21,22] The proton signal at δ_H 3.38 (1H, td, J = 11.2, 4.8 Hz) further revealed a typical H-19$_\beta$ lupane structure.[22] Moreover, the results of the ^{1}H, ^{13}C, and HMBC spectra for **1** were similar to those for methyl betulinate, except for the presence of one more oxymethine group.[22,23] On comparison of the ^{13}C NMR shifts of **1** with those of methyl betulinate, downfield shifts of signals for C-6 (δ_C 18.4 → 30.3), C-7 (δ_C 34.6 → 74.4), and C-8 (δ_C 41.0 → 47.2) were found. The hydroxyl group was then assigned at C-7 from the signals of δ_H 1.32 (H-26), 1.82 (H-6), and 2.00 (H-6) and the HMBC correlation of H-26 and H-6/C-7. The NOESY spectrum showed a NOE effect between H-3 and H-7 with H-5 and H-9, respectively (Figure 1), and indicated the relative configuration of the two hydroxyl groups at C-3 and C-7 as β. Therefore, the structure of **1** was determined as 7β-hydroxymethyl betulinate.

Compound **2** was obtained as a white, amorphous solid. The molecular formula was established as $C_{35}H_{54}O_5$ by HRESIMS (*m/z* 577.3869 [M + Na]$^+$, calcd 577.3866). Analysis of the IR spectrum of **2** suggested a hydroxyl group (3423 cm^{-1}) and an ester group (1698 cm^{-1}) in the structure. The NMR signals resembled those of **1**, except for the presence of a senecioyl ester group (δ_C 117.6 and 156.4)[24] and the absence of a methoxyl group. Assignments of the ^{1}H and ^{13}C signals performed by extended 2D NMR methods indicated **2** is an *epi*-betulinic acid derivative bearing a senecioyl moiety.[22,25] By comparing the ^{13}C NMR signals with those of 3-*epi*-betulinic acid, downfield shifts of C-7 (δ_C 76.7, $\Delta\delta$ +41.9), C-6 (δ_C 26.1, $\Delta\delta$ +7.5), and C-8 (δ_C 46.3, $\Delta\delta$ +5.1) and an upfield shift of C-26 (δ_C 12.1, $\Delta\delta$ −4.2) were evident.[25] The location of the senecioyl moiety was assigned at C-7 and was confirmed from the HMBC spectrum. In the NOESY spectrum of **2** (Figure S3, Supporting Information), a correlation of H-5 and H-7 was found. The relative configuration of the senecioyl moiety was ascertained as being in the 7β form. To the best of our knowledge, this is the first instance of a naturally occurring lupane-type triterpene ester substituted at C-7 isolated from a species in the family Celastraceae. On the basis of all of the evidence described above, the structure 7β-senecioyl-3-*epi*-betulinic acid was assigned for **2**.

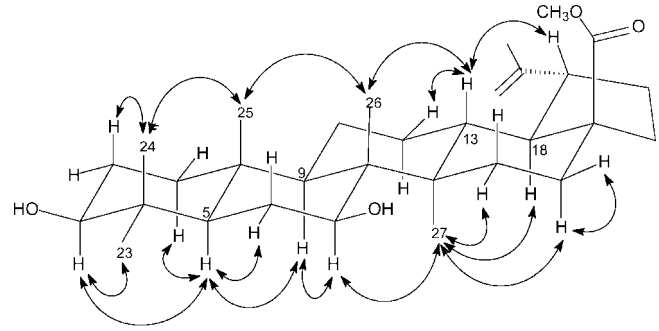


Figure 1. Key NOESY correlations of **1**.

The molecular formula of compound **3** was deduced to be $C_{30}H_{48}O_5$ on the basis of HRESIMS (*m/z* 511.3399 [M + Na]$^+$, calcd 511.3396). Signals for two carbonyls (δ_C 174.4 and 182.4) and one allylic hydroxymethylene group (δ_H 4.42/δ_C 64.3) were observed from the ^{1}H and ^{13}C NMR spectra. The seven degrees of unsaturation implied by the molecular formula, together with the presence of two carbonyls and one terminal olefin, suggested that **3** is based on a tetracyclic system. The NMR data (Tables 1 and 2) indicated that **3** belongs to the lupane family of triterpenoids.[16,21,22] In the HMBC spectrum of **3**, correlations were observed from the proton signals at δ_H 5.05 and 5.42 (H-29) to the carbon signals at δ_C 43.8 (C-19), 156.5 (C-20), and 105.9 (C-30), between the proton signal at δ_H 3.09 (H-1a) and the carbon signal at δ_C 174.4 (C-2), and between the proton signals at δ_H 1.54 (H-23)/1.59 (H-24) and the carbon signal at δ_C 182.4 (C-3). From the HMQC and HMBC spectra, compound **3** was observed to be a 30-hydroxylupeol-type triterpenoid with cleavage in ring A.[15] Prominent differences in the ^{1}H and ^{13}C NMR data between **3** and known 30-hydroxylupeols were observed only in those spectroscopic data pertaining to ring A. Two carboxyl groups and a geminal dimethyl group at C-4 indicated a *seco*-lupane structure having a cleavage between C-2 and C-3 rather than C-3 and C-4.[26,27] In general, ring-A opened triterpenoids have a cleavage between C-3 and C-4, while 2,3-*seco*-triterpenoids, such as compound **3**, are rare. In the past, no clear assignments of ^{13}C and 2D NMR data were given for these compounds.[28] The mass spectrum of **3** showed two diagnostic fragmentations[26] at *m/z* 207 [M − $C_{16}H_{25}O_4$]$^+$ and 341 [M − $C_6H_{11}O_4$]$^+$, corresponding to a typical lupane-type skeleton cleavage (Figure S5, Supporting Information). The relative configuration of **3** was assigned by the NOESY correlations H-1α/H-5; H-5/H-7α, H-9α; H-7α/H-27; H-9α/H-27; H-13/H-26; H-18/H-27; and H-19/H-28. From the HMBC spectrum, the structure of compound **3** was concluded to be a 2,3-*seco*-lupane derivative and was assigned as 30-hydroxy-2,3-*seco*-lup-20(29)-ene-2,3-dioic acid. This is the first time a 2,3-*seco*-lupane derivative has been isolated from a Celastraceous plant.

Compound **4** was obtained as a white, amorphous solid and gave the molecular formula $C_{30}H_{46}O_3$ (*m/z* 477.3345 [M + Na]$^+$, calcd 477.3344, DBU 8) by HRESIMS. The IR spectrum of **4** exhibited hydroxyl (3600 cm^{-1}) and γ-lactone (1775 cm^{-1}) absorptions. An oxygenated carbon [δ_C 92.2 (s)], a terminal olefinic group (δ_C 141.4 (s)/112.2 (t)), and a carbonyl group (δ_C 178.6 (s)) were evident in the ^{13}C NMR spectrum. These data indicated that **4** is based on a hexacyclic system of the lup-20(29)-ene or hop-22(29)-ene type.[29–31] Furthermore, in comparison with the ^{1}H NMR data for 3-*epi*-betulinic acid, the presence of two downfield-shifted olefinic proton signals (δ_H 5.01 and 5.50) and the lack of a lupenol H-19$_\beta$ proton signal were apparent.[25] The carbon signals for C-16, -20, and -22 were observed shifted upfield (δ_C 32.9 → 22.9, δ_C 151.4 → 141.4, and δ_C 37.5 → 29.3), and the carbons at C-18, -19, and -29 were shifted downfield from δ_C 47.8 → 55.5, δ_C 49.3 → 92.2, and δ_C 109.9 → 112.2, respectively. Except for the carbonyl and alkene groups, the six degrees of unsaturation remaining revealed a hexacyclic structure for compound **4**. The sixth ring was assigned

Table 1. ^{1}H NMR Spectroscopic Data for Compounds **1**−**4** and **6** (in C_5D_5N, 400 MHz)

proton	1	2	3	4	6
1	0.94 (m, H-1β)	1.43 (m)	3.09 (d, 18.0, H-1a)	1.44 (m)	1.44 (m)
	1.67 (dt, 12.8, 3.2, H-1α)	1.70 (m)	2.73 (d, 18.0, H-1b)	1.70 (m)	1.75 (m)
2	1.88 (2H, m)	1.14 (m)		2.00 (m, H-2β)	1.79 (m)
		1.80 (m)		1.78 (m, H-2α)	1.99 (m)
3	3.49 (brt, 6.0, H-3α)	3.60 (brs, H-3β)		3.61 (m, H-3β)	3.61 (m, H-3β)
5	1.00 (m)	1.94 (d, 12.8)	3.13 (m)	1.63 (m)	1.65 (m)
6	1.82 (m, H-6β)	1.80 (m)	1.72 (m)	1.32 (m, H-6β)	1.44 (2H, m)
	2.00 (m, H-6α)	1.99 (dd, 12.8, 10.8)	1.74 (m)	1.45 (m, H-6α)	
7	4.12 (m)	5.48 (dd, 10.8, 4.8)	1.42 (m, H-7β)	1.33 (m)	1.30 (m)
			1.73 (m, H-7α)	2.13 (m)	2.34 (m)
9	1.35 (m)	1.60 (d, 3.0)	3.00 (d, 10.4)	1.49 (m)	1.50 (m)
11	1.45 (2H, m)	1.33 (dd, 12.9, 4.3)	1.14 (m)	1.16	1.16 (m)
		1.54 (m)	1.56 (m)	(dd, 12.4, 4.0, H-11β)	1.46 (m)
				1.43 (m, H-11α)	
12	1.95 (m, H-12β)	1.62 (2H, m)	1.34 (m)	1.82 (m, H-12β)	1.04 (m)
	1.16 (m, H-12α)		1.63 (m)	1.03	1.80 (m)
				(td, 12.4, 3.6, H-12α)	
13	2.60 (td, 11.2, 3.2)	2.77 (td, 12.1, 3.5)	1.68 (dd, 13.2, 3.2)	1.66 (m)	1.80 (m)
15	2.00 (m, H-15β)	1.45 (m)	1.01 (brd, 13.6)	1.15 (m, H-15β)	1.21 (m)
	2.41 (m, H-15α)	2.27 (dd, 13.5, 3.6)	1.63 (m)	1.69 (m, H-15α)	1.72 (m)
16	2.44 (m, H-16β)	2.60 (dt, 13.5, 4.3)	1.36 (m)	1.44 (m, H-16β)	1.70 (m)
	1.57 (m, H-16α)	1.53 (m)	1.44 (m)	2.11 (m, H-16α)	2.36 (m)
18	1.76 (t, 11.2)	1.68 (m)	1.65 (d, 10.4)	1.81 (d, 10.8)	2.13 (d, 11.2)
19	3.38 (td, 11.2, 4.8)	3.51 (td, 10.9, 4.6)	2.43 (td, 10.8, 4.8)		
21	1.47 (m)	1.46 (m)	1.48 (dd, 13.2, 4.4)	1.61 (m)	1.72
	2.00 (m)	2.19 (m)	2.12	2.13 (m)	(dd, 14.2, 3.2, H-21β)
			(dddd, 13.2, 11.6, 9.6, 4.4)		2.89
					(dd, 14.2, 9.0, H-21α)
22	1.49 (m)	1.52 (m)	1.29 (t, 10.8)	1.60 (m)	5.50
	1.98 (m)	2.18 (m)	1.37 (brt, 3.6)	1.64 (m)	(dd, 9.0, 3.2, H-22α)
23	1.23 (s)	1.20 (s)	1.54 (s)	1.21 (m)	1.22 (s)
24	1.03 (s)	0.85 (s)	1.59 (s)	0.88 (s)	0.89 (s)
25	0.90 (s)	0.89 (s)	1.10 (s)	0.82 (s)	0.83 (s)
26	1.32 (s)	1.40 (s)	1.10 (s)	0.87 (s)	0.90 (s)
27	1.23 (s)	1.12 (s)	1.13 (s)	0.75 (s)	0.77 (s)
28			0.81 (s)		
29	4.77 (brs)	4.75 (brs)	5.05 (brs)	5.01 (brs)	5.06 (brs)
	4.93 (brs)	4.91 (brs)	5.42 (brs)	5.50 (brs)	5.55 (brs)
30	1.78 (s)	1.74 (s)	4.42 (2H, brs)	1.74 (s)	1.76 (s)
2′		5.77 (s)			
4′		1.62 (s)			
5′		2.18 (s)			
2″					2.34 (2H, m)
3″					1.65 (2H, m)
13″					1.24 (2H, brs)
14″					0.86 (brt, 6.8)

as a lactone between the C-19 oxygen and the C-28 carbonyl group,[30,31] which was supported by the key HMBC correlations: H-13/C-18, C-19; H-18/C-17, C-28; H-30/C-19, C-20, C-29 (Figure S6, Supporting Information). On the basis of NOESY correlations (Figure S6, Supporting Information), the relative configuration of **4** was assigned. The correlation between H-3 and H-24 confirmed the β-orientation of H-3. Therefore, the structure of **4** was assigned as 3-*epi*-thurberogenin.

Compounds **5**−**7** gave identical ^{1}H and ^{13}C NMR spectra. They were separated using a reversed-phase HPLC system (250 × 4 mm, Hypersil, MeOH−H_2O (90:10); t_R **5**, 12 min; **6**, 16 min; and **7**, 23 min). However, only compound **6** was obtained in a sufficient amount for full NMR interpretation. In addition, compounds **5** and **7** displayed almost identical NMR signals to those of **6**. The molecular formulas of **5** (*m*/*z* 675.4960, calcd 675.4964; $C_{42}H_{68}O_5Na$), **6** (*m*/*z* 703.5273, calcd 703.5277; $C_{44}H_{72}O_5Na$), and **7** (*m*/*z* 731.5587, calcd 731.5590; $C_{46}H_{76}O_5Na$) were established by HRESIMS data. The ^{1}H and ^{13}C NMR, COSY, HMQC, and HMBC spectra of **6** were similar to **4** and assigned to a hexacyclic system of a 19,28-lactone lupane-type triterpenoid.[29−31] However, the absorption of carboxylic ester groups at 1735 cm^{-1} appeared in the IR spectra of **5**−**7**, indicating an ester group in each case. In compound **6**, the characteristic signals of long-chain fatty acids were found in the ^{1}H and ^{13}C NMR spectra and confirmed by the HMBC cross-peaks between the carbonyl carbon (δ_C 173.5) and the protons of $^{2''}CH_2{}^{3''}CH_2$ [δ_H 2.34 (2H, m, H-2″) and 1.65 (2H, m, H-3″)]. In the HMBC spectrum (Figure S7, Supporting Information), the signal at δ_H 5.50 (H-22) showed correlations with carbon signals at δ_C 174.8 (C-28) and 173.5, suggesting that a long-chain ester in the structure of **6** is located at C-22. Furthermore, the configuration of the oxymethine proton at the C-22 position was assigned as α on the basis of the NOESY spectrum (Figure S7, Supporting Information). On the basis of all spectroscopic data and the different molecular weights of **5**−**7**, various chain lengths (C_{12}, C_{14}, and C_{16}) of long-chain fatty acids were evident on C-22. Therefore, the structures of **5**−**7** were assigned as 3-*epi*-thurberogenin-22β-dodecanoate, 3-*epi*-thurberogenin-22β-tetradecanoate, and 3-*epi*-thurberogenin-22β-hexadecanoate, respectively.

Besides compounds **1**−**7**, 18 known compounds were also isolated from *M. fokienensis* and *P. arisanensis*, namely, 28-hydroxy-3-oxo-lup-20(29)-en-30-al (**8**),[32] betulinic acid (**9**),[25] 3-*epi*-betulinic acid (**10**),[25] 3-*epi*-betulin (**11**),[16] 30-hydroxylupeol,[33] 30-hydroxylup-20(29)-en-3-one,[33] 30-hydroxybetulin,[33] 28,30-dihydroxylup-20(29)-en-3-one,[33] 13β,28-epoxy-3β-hydroxyolean-11-ene,[15] 13β,28-epoxy-3β-hydroxyurs-11-ene,[15] 3β-methoxybetulinic acid,[34] lupeol,[35] 3-*epi*-betulinaldehyde,[36] thurberogenin,[30,31] thurberogenone,[30,31] β-amyrin,[37] and a mixture of β-sitosterol and β-stigmasterol.[28] The structures of the known compounds were determined by a combination of spectroscopic analysis and comparison with previously reported data.

Table 2. ^{13}C NMR Spectroscopic Data for Compounds **1**−**4** and **6** (in C_5D_5N, 100 MHz)

carbon	**1**	**2**	**3**	**4**	**6**
1	39.3	34.0	42.6	34.1	34.1
2	28.3	26.2	174.4	26.6	26.5
3	78.0	75.0	182.4	75.1	75.1
4	39.3	38.1	46.9	38.1	38.1
5	53.2	46.4	48.4	49.2	49.2
6	30.3	26.1	21.6	18.5	18.5
7	74.4	76.7	33.8	34.5	34.5
8	47.2	46.3	41.1	41.1	41.0
9	51.1	51.2	42.0	50.8	50.7
10	37.6	37.8	42.3	37.7	37.7
11	21.3	20.8	22.2	20.8	20.7
12	26.3	26.5	27.3	26.1	26.0
13	39.3	39.1	38.6	35.2	35.1
14	44.0	44.4	43.5	41.4	41.2
15	34.0	33.0	27.9	28.2	27.7
16	32.8	33.4	35.6	22.9	21.3
17	56.7	56.3	43.2	54.0	58.2
18	49.7	49.6	48.9	55.5	54.2
19	47.7	48.0	43.8	92.2	90.9
20	151.1	151.4	156.5	141.4	140.3
21	30.9	31.2	32.2	34.4	42.3
22	37.2	37.7	40.0	29.3	73.9
23	28.5	29.0	27.8	29.2	29.3
24	16.4	22.4	24.8	22.8	22.5
25	16.3	16.1	20.4	16.5	16.5
26	10.9	12.1	16.3	15.7	15.7
27	15.1	15.1	15.0	13.6	13.5
28	176.6	179.0	17.9	178.6	174.8
29	110.0	109.9	105.9	112.2	112.8
30	19.5	19.5	64.3	19.3	19.2
1′		166.3			
2′		117.6			
3′		156.4			
4′		27.0			
5′		20.2			
1″					173.5
2″					34.2
3″					25.2
4″−12″					29.2−30.0
13″					22.9
14″					14.3

Table 3. Cytotoxic Activity of Compounds **8**−**11** for Various Human Cancer Cell Lines

	IC_{50} (μg/mL)						
compound	HepG2	Hep3B	MDA-MB-231	MCF7	A549	Ca9-22	HL60
8	12.6	4.7	7.9	2.9	16.4	1.4	1.6
9	3.1	1.7	3.5	4.0	2.8	>20	2.0
10	9.3	8.1	9.2	9.7	9.7	NT[a]	2.3
11	6.7	9.3	>20	16.0	15.7	>20	1.7
doxorubicin[b]	0.17	0.27	0.15	0.32	0.52	0.09	0.003

[a] Not tested. [b] Positive control.

Thurberogenin, with a 19,28-lactone lupane-type skeleton, was first isolated by Djerassi et al. from *Lemaireocereus thurberi* (Cactaceae),[30,31] but the structure elucidation was based mainly on data obtained from chemical transformations, and only incomplete NMR data were reported. In addition, the trivial name of thurberogenin was ambiguously presented in previous literature.[38,39] This is the first report to include full ^1H and ^{13}C NMR elucidation on a 19,28-lactone lupane-type triterpenoid.

According to the literature, lupane-derived triterpenoids have been investigated recently for their various biological properties.[40–43] In the present study, all isolates were subjected to cytotoxic assay against HepG2, Hep3B, MDA-MB-231, MCF7, A549, Ca9-22, and HL60 cancer cell lines (Table 3); only data with IC_{50} less than 4 μg/mL were listed. Compounds **9**−**11** are well-known in structure, and their activities have been studied.[40,44,45] In this study, compounds **9** and **10** were active against six cancer cell lines, with the 3-*epi* hydroxy function of **10** resulting in decreased activity when compared to **9**. Compound **11**, with a 28-hydroxymethylene was only active against the HL60 cell line. The results also showed that 28-hydroxy-3-oxo-lup-20(29)-en-30-al (**8**) had specific cytotoxicity against Ca9-22 and HL60 with IC_{50} values of 1.4 and 1.6 μg/mL, respectively, and showed generally better activity than the 30-hydroxy compound **11**. Although previous studies reported that **8** showed cytotoxic effects,[17] its selectivity and mechanism of action toward specific cancer cells has not yet been demonstrated. Therefore, it was decided to determine its growth inhibition and apoptotic induction by flow cytometry and Western blotting analysis.

To investigate the potential effects of **8** on growth inhibition of HL60 cells, the cells were treated at a concentration range of 0.625−5 μg/mL. The antiproliferative effect of **8** on apoptosis of HL60 cells is shown in Table S1 (Supporting Information), and this compound induced apoptosis in a dose-dependent manner. Compound **8** produced cleavage of PARP and upregulated Bax, but it did not effect Bcl-2 (Figure 2).

In addition to various medicinal properties, including anti-HIV,[46] anti-inflammatory,[47] and antimalarial[48] activities, betulinic acid (**9**)

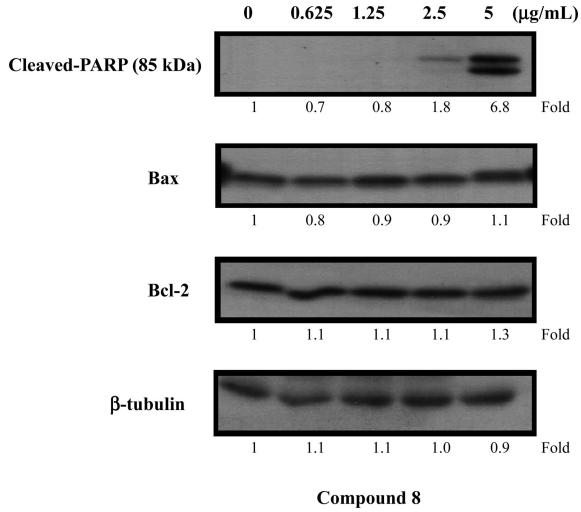


Figure 2. Western blot analysis of PARP, Bax, Bcl-2, and protein levels after exposure to **8** in HL60 cells. Cells were incubated in the indicated concentrations of compound **8** for 24 h. After treatment, the cytosolic fractions were resolved by SDS-PAGE, transferred onto cellulose membranes, and then probed with specific antibodies. The amount of β-tubulin was measured as an internal control.

possesses in vitro cytotoxicity against several human cancers such as malignant brain,[49] neuroectodermal,[50] and several melanoma-derived cell lines,[51] as well as in vivo activity in human melanoma-bearing mice.[51] Therefore, we further investigated the cytotoxicity of three selective compounds, 30-hydroxylup-20(29)-en-3-one, 28,30-dihydroxylup-20(29)-en-3-one, and betulinic acid (**9**), toward mouse melanoma (B16). However, betulinic acid (**9**) and 30-hydroxylup-20(29)-en-3-one showed mild cytotoxicity, with IC_{50} values 5.0 and 9.2 μg/mL, respectively.[52]

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. The IR spectra were measured on a Mattson Genesis II spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA 500, Varian Unity Plus 400, or Varian Gemini 200 NMR spectrometers. Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in hertz. LREIMS, LRESIMS, and LRFABMS were measured on a VG Biotech Quattro 5022 mass spectrometer. HRESIMS were measured on a Bruker Daltonics APEX II mass spectrometer. Silica gel 60 (Merck, 230−400 mesh) and Sephadex LH-20 were used for column chromatography, while TLC analysis was carried out on silica gel GF_{254} precoated plates with detection using 50% H_2SO_4 followed by heating on a hot plate. HPLC was performed with a Hitachi L-7100 pump and D-7000 interface equipped with a Bischoff RI detector using ODS (Hypersil, 250 × 4 mm; Hypersil, 250 × 10 mm) columns.

Plant Material. The dried stems of *Microtropis fokienensis* and *Perrottetia arisanensis* were collected from Taichung County, Taiwan, in June 2004, and identified by a botanist, Dr. Hsin-Fu Yen. Two voucher specimens (Microtropis-01 and Perrottetia-01) were deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. The dried stems (2.85 kg) of *M. fokienensis* were extracted four times with MeOH overnight at room temperature, followed by removal of the solvent under reduced pressure, to yield a dried MeOH extract (130 g). The MeOH extract was dissolved in H_2O and extracted with EtOAc. Using *n*-hexane and 80% MeOH, the EtOAc-soluble fraction was divided into *n*-hexane and aqueous MeOH layers. The aqueous MeOH layer (38.0 g) was chromatographed on silica gel using mixtures of *n*-hexane−EtOAc of increasing polarity as eluants to afford 18 fractions. Fraction 7 (289.13 mg) was purified on silica gel, eluting with mixtures of $CHCl_3$−MeOH, to give six subfractions. Fraction 7-2 (20.90 mg) was subjected to passage over an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 88:12) to give **4** (0.70 mg, t_R 22 min, flow rate 2 mL/min). Fraction 8 (676.90 mg) was chromatographed on Sephadex LH-20 with $CHCl_3$−MeOH (1:1) to give seven subfractions. Fraction 8-4 (326.40 mg) was purified using an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 87:13) to give **2** (3.18 mg, t_R 22 min, flow rate 2 mL/min). Fraction 10 (2.5 g) was chromatographed on Sephadex LH-20 with $CHCl_3$−MeOH (1:1) to give six subfractions. Fraction 10-3 (132.88 mg) was further separated using an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 78:22) to afford **1** (4.33 mg, t_R 57 min, flow rate 2 mL/min). Fraction 12 (5.5 g) was chromatographed on Sephadex LH-20 eluting with $CHCl_3$−MeOH (1:1) to give six subfractions. Fraction 12-3 (4.2 g) was subjected to column chromatography over silica gel ($CHCl_3$−MeOH of increasing polarity) to give 12 subfractions. Fraction 12-3-11 (560.64 mg) was then purified by separation over an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 95:5) to give **3** (45.86 mg, t_R 12 min, flow rate 2 mL/min).

The dried stems (1.58 kg) of *P. arisanensis* were extracted five times with MeOH overnight at room temperature to give an extract (51.0 g). The residue was suspended in H_2O and partitioned sequentially with *n*-hexane, CH_2Cl_2, MeOH, and *n*-BuOH. The MeOH layer (5.8 g) was divided into 14 fractions using silica gel column chromatography, with gradient mixtures of *n*-hexane, EtOAc, and MeOH of increasing polarity as solvents. Fraction 4 (305.50 mg) was chromatographed on Sephadex LH-20 with $CHCl_3$−MeOH (1:1) to give 10 subfractions. Compound **4** (6.51 mg) was obtained from Fraction 4-5 (110.70 mg) with silica gel column chromatography using mixtures of $CHCl_3$−EtOAc. Fraction 5 (68.94 mg) was chromatographed by Sephadex LH-20 with $CHCl_3$−MeOH (1:1) and further purified using an ODS HPLC column (250 × 4 mm, Hypersil, MeOH−H_2O, 90:10) to give **5** (1.20 mg, t_R 12 min, flow rate 1 mL/min), **6** (1.30 mg, t_R 16 min, flow rate 1 mL/min), and **7** (0.80 mg, t_R 23 min, flow rate 1 mL/min).

7β-Hydroxymethyl betulinate (1): white, amorphous solid; mp 93−94 °C; $[\alpha]^{23}_D$ −7.7 (*c* 0.43, MeOH); IR (neat) ν_{max} 3398 (OH), 2926, 2858, 1691, 1453, 1376 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Tables 1 and 2; EIMS *m/z* 486 $[M]^+$ (7), 341 (20), 267 (27); HRESIMS *m/z* 509.3607 $[M + Na]^+$ (calcd for $C_{31}H_{50}O_4Na$, 509.3604).

7β-Senecioyl-3-*epi*-betulinic acid (2): white, amorphous solid; mp 110−111 °C; $[\alpha]^{26}_D$ −16.0 (*c* 0.32, MeOH); IR (neat) ν_{max} 3423 (OH), 2934, 2867, 1698, 1643, 1452, 1373 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Tables 1 and 2; ESIMS *m/z* 577 $[M + Na]^+$; HRESIMS *m/z* 577.3869 $[M + Na]^+$ (calcd for $C_{35}H_{54}O_5Na$, 577.3866).

30-Hydroxy-2,3-*seco*-lup-20(29)-ene-2,3-dioic acid (3): white, amorphous solid; mp 107−109 °C; $[\alpha]^{23}_D$ −1.3 (*c* 5.4, MeOH); IR (neat) ν_{max} 3417 (OH), 2940, 2872, 1702, 1651, 1542, 1454, 1367 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Tables 1 and 2; ESIMS *m/z* 511 $[M + Na]^+$; HRESIMS *m/z* 511.3399 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3396).

3-*epi*-Thurberogenin (4): white, amorphous solid; mp 264−265 °C; $[\alpha]^{24}_D$ +34.1 (*c* 0.62, MeOH); IR (neat) ν_{max} 3600 (OH), 2943, 2870, 1775, 1713, 1447, 1382, 1357, 1170 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Tables 1 and 2; FABMS *m/z* 477 $[M + Na]^+$; HRESIMS *m/z* 477.3345 $[M + Na]^+$ (calcd for $C_{30}H_{46}O_3Na$, 477.3344).

3-*epi*-Thurberogenin-22β-dodecanoate (5): white, amorphous solid; mp 52−54 °C; $[\alpha]^{24}_D$ −26.2 (*c* 0.11, MeOH); IR (neat) ν_{max} 3700 (OH), 2927, 2855, 1785, 1736, 1449, 1165 cm^{-1}; ESIMS *m/z* 675 $[M + Na]^+$; HRESIMS *m/z* 675.4960 $[M + Na]^+$ (calcd for $C_{42}H_{68}O_5Na$, 675.4964).

3-*epi*-Thurberogenin-22β-tetradecanoate (6): white, amorphous solid; mp 42−44 °C; $[\alpha]^{24}_D$ −17.7 (*c* 0.12, MeOH); IR (neat) ν_{max} 3700 (OH), 2922, 2856, 1781, 1735, 1451 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Tables 1 and 2; ESIMS *m/z* 703 $[M + Na]^+$; HRESIMS *m/z* 703.5273 $[M + Na]^+$ (calcd for $C_{44}H_{72}O_5Na$, 703.5277).

3-*epi*-Thurberogenin-22β-hexadecanoate (7): white, amorphous solid; mp 37−38 °C; $[\alpha]^{24}_D$ −50.4 (*c* 0.05, MeOH); IR (neat) ν_{max} 3700 (OH), 2924, 2849, 1787, 1738, 1455 cm^{-1}; ESIMS *m/z* 731 $[M + Na]^+$; HRESIMS *m/z* 731.5587 $[M + Na]^+$ (calcd for $C_{46}H_{76}O_5Na$, 731.5590).

Cytotoxicity Assays. Test compounds were assayed for cytotoxicity against the human hepatoma (HepG2 and Hep3B), breast cancer

(MCF-7 and MDA-MB-231), lung cancer (A549), gingival cancer (Ca9-22), human leukemia cancer (HL60), and mouse melanoma (B16) cell lines, using the MTT method performed according to a previously published protocol.[53] In brief, freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000−10 000 cells per well, and the test compounds were added from DMSO stock solutions. After 3 days in culture, the attached cells were incubated with MTT (0.5 μg/mL, 1 h) and subsequently solubilized in DMSO. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions. The results represent the mean of two to three separate experiments, each performed in triplicate.

Flow Cytometric Analysis. Controlled and treated cells were harvested, washed in cold PBS, fixed in 70% ethanol, and stored at 4 °C. DNA was treated with RNase-A solution (500 unit/mL) at 37 °C for 15 min and stained by propidium iodide (50 μg/mL) in 1.12% sodium citrate at room temperature before analysis. Flow cytometric determination of DNA content was analyzed using a Coulter Epics XL flow cytometer (Coulter Corp., Miami, FL). The fractions of the cells in Sub G1, G0/G1, S, and G2/M phase were analyzed using Multicycle cell-cycle analysis software (Phoenix Flow System, San Diego, CA).

Western Blot Analysis. Cells were washed in PBS, suspended in lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and placed on ice for 30 min. After centrifugation at 20000*g* for 30 min at 4 °C, the supernatant was collected. The protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce, Rockford, IL). Whole lysate (50 μg) was resolved by 12% SDS-PAGE, transferred onto PVDF membranes (Roche) by electroblotting, and probed with anti-PARP, -Bax, -Bcl-2, and -β-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The blot was developed by enhanced chemiluminescence.[54]

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Supporting Information Available: Spectroscopic data and additional information of known compounds as well as the biological information are available free of charge via the Internet at http://pubs.acs.org.

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