Antiproliferative Triterpenes from *Melaleuca ericifolia*

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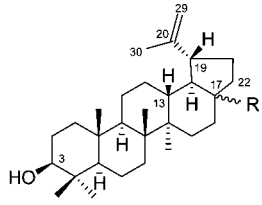
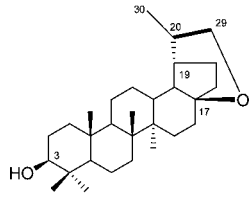
Three new 28-norlupane triterpenes, 28-norlup-20(29)-en-3β-hydroxy-17β-hydroperoxide (**1**), 28-norlup-20(29)-en-3β-hydroxy-17α-hydroperoxide (**2**), and 20*S*-17β,29-epoxy-28-norlupan-3β-ol (**3**), were isolated from the leaves of *Melaleuca ericifolia* along with eight known pentacyclic triterpenes. The structures of the new compounds were elucidated by spectroscopic methods including 1D and 2D NMR spectroscopy and mass spectrometry. The isolated triterpenes were evaluated for antiproliferative activity against the malignant +SA mammary epithelial cell line.

Research interest in the genus *Melaleuca* (tea tree species) has been focused on the antimicrobial activity of its essential oil.[1−3] Nevertheless, diverse biological activities have been attributed also to the nonvolatile constituents of members of this genus. These include antiviral activity against herpes simplex virus type 1[1] and antioxidant,[2] antihistaminic,[3] vasorelaxant,[4] and antiproliferative activities.[5] Phytochemical work of the nonvolatile secondary metabolites of the genus *Melaleuca* has revealed flavonoids,[3,6,7] triterpenoids,[8−11] hydrolyzable tannins, and other polyphenols,[2,5,11] as well as miscellaneous glycosides including monoterpene glucosides.[4]

The essential oil of *M. ericifolia* Sm. (Myrtaceae) has been reported to exhibit antibacterial, antifungal, and antiviral activities.[12,13] The phenolic constituents of the leaves of this species have been investigated most thoroughly.[14] The present study reports three new 28-norlupane derivatives, 28-norlup-20(29)-en-3β-hydroxy-17β-hydroperoxide (**1**), 28-norlup-20(29)-en-3β-hydroxy-17α-hydroperoxide (**2**), and 20*S*-17β,29-epoxy-28-norlupan-3β-ol (**3**), from the leaves of *M. ericifolia*, as well as eight known pentacyclic triterpenes, 28-norlup-20(29)-en-3β,17β-diol (**4**),[9] betulinic acid, betulinaldehyde, betulin,[15] platanic acid,[16] ursolaldehyde,[17] ursolic acid,[15] and 2α,23-dihydroxyursolic acid (asiatic acid).[18]





3

	R
1	β-OOH
2	α-OOH
4	β-OH

The HRESIMS data of **1** were consistent with a molecular formula of $C_{29}H_{48}O_3$ and six degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **1** (Tables 1 and 2) suggested its close similarity to the known 28-norlup-20(29)-en-3β,17β-diol (**4**), with the replacement of the C-17β hydroxy substituent with a hydroperoxy group.[9] The ^{13}C NMR spectrum of **1** showed a quaternary carbon signal at δ_C 91.6, assigned to C-17. This was based on HMBC data (Figure 1) that showed ^3J- and ^2J-HMBC correlations between both the H-21b and H-22b protons (δ_H 2.16 and 2.12, respectively) and C-17. The relative configuration determination of **1** was aided by interpretation of the NOESY data (Figure 2). The ^{1}H and ^{13}C NMR data (Tables 1 and 2) and HREIMS of **2** suggested a structure similar to **1** except for the configuration at C-17. The quaternary hydroperoxide-bearing C-17 (δ_C 93.4) was assigned in a similar way to **1**. The HMBC spectrum of **2** aided the unambiguous assignment of the methine carbon C-13 (δ_C 44.2), which showed a ^3J-HMBC correlation with the methyl singlet H$_3$-27 (δ_H 0.94).

The C-17 epimeric compounds **1** and **2** are excellent examples to show the significance of the γ-substituent effect on ^{13}C NMR chemical shifts based on relative configuration. The chemical shifts of the C-13 and C-19 carbons in **1** and **2** were compared with those reported compounds based on the 17β-lupeol and 17-*epi*-lupeol triterpene skeletons.[9,19] The high observed $\Delta\delta$ values for C-13 and C-19 in **1** and **2** supported the fact that these compounds are C-17 epimers (Table 2). The β-configuration of C-17 in **1** was suggested on the basis of matching the chemical shift value of its C-13 (δ_C 36.8) and C-19 (δ_C 48.0) resonances with those of 3-*O*-acetyl-17β-lupeol (δ_C 38.0 and 48.0, respectively) and 28-norlup-20(29)-en-3β,17β-diol (δ_C 37.7 and 48.1, respectively).[9,19] The α-configuration of the C-17 hydroperoxide group in **2** was supported by the similarity of the chemical shift values of its C-13 (δ_C 44.2) and C-19 (δ_C 53.4) methine carbons to those of 17-*epi*-lupenyl acetate [C-13 (δ_C 43.4) and C-19 (δ_C 54.2)].[19] A literature survey revealed that the ^{1}H and ^{13}C NMR data of **2** differed from those of 19α-lupeol (H-18α, H$_3$-28β, and H-19α),[20] nepehinol (H-18β, H$_3$-28β, and H-19α),[21] and other lupane epimers.[22] Therefore, the structure of **1** was determined as 28-norlup-20(29)-en-3β-hydroxy-17β-hydroperoxide, and its C-17-epimer analogue, **2**, must thus be 28-norlup-20(29)-en-3β-hydroxy-17α-hydroperoxide.

The HREIMS of **3** suggested the molecular formula $C_{29}H_{48}O_2$, with six degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **3** (Tables 1 and 2) were consistent with a saturated nortriterpene skeleton. The oxygenated methine carbon C-3 (δ_C 79.0) was assigned on the basis of its ^3J-HMBC correlation with the methyl singlets H$_3$-23 and H$_3$-24 (Table 1). In the COSY spectrum, the methyl doublet at δ_H 0.71 (J = 6.4 Hz) correlated with the proton signal at δ 1.83 (H-20) and was assigned for H$_3$-30. The HMBC data of **3** (Figure 3) showed a ^2J-HMBC correlation of H$_3$-30 with the methine C-20 (δ_C 36.7) signal, in addition to ^3J-HMBC correlations with both the C-19 methine (δ_C 42.6) and the methylene C-29 (δ_C 67.0) resonances, and indicated an isopropyl rather than an isopropenyl side chain. In turn, the HMQC spectrum correlated the methylene C-29 (δ_C 67.0) with the oxygenated protons H-29a (δ_H 3.18) and H-29b (δ_H 3.61). The presence of a ^3J-HMBC

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Table 1. ^{1}H NMR Data (δ_H) of Compounds **1**−**3**[a]

position	1	2	3
1	0.94, m 1.71, m	0.94, m 1.73, m	0.95, m 1.76, m
2	1.59, m 1.67, m	1.52, m 1.56, m	1.56, m 1.61, m
3	3.16, dd (11.4, 5.1)	3.17, dd (11.4, 4.4)	3.17, dd (11.4, 5.1)
4			
5	0.66, m	0.68, m	0.68, m
6	1.34, m 1.60, m	1.33, m 1.40, m	1.38, m 1.52, m
7	1.38, m	1.28, m	1.24, m 1.45, m
8			
9	1.23, m	1.34, m	1.34, m
10			
11	1.16, m 1.46, m	1.24, m 1.39, m	1.25, m
12	1.62, m	0.84, m 1.72, m	1.53, m 1.60, m
13	1.82, m	1.97, ddd (13.2, 12.8, 5.1)	1.38, m
14			
15	1.35, m 1.99, m	0.97, m 1.67, m	1.23, m 1.64, m
16	1.05, m 1.83, m	1.51, m 1.82, m	1.44, m 1.88, m
17			
18	1.65, m	1.68, m	1.55, m
19	2.57, ddd (10.6, 10.4, 5.9)	2.34, ddd (8.1, 7.7, 3.7)	1.86, m
20			1.83, m
21	1.37, m 2.16, m	1.58, m 1.61, m	1.32, m 1.58, m
22	1.29, m 2.12, m	1.24, m 1.75, m	1.45, m 1.95, m
23	0.95, 3H, s	0.94, 3H, s	0.96, 3H, s
24	0.75,3H, s	0.75, 3H, s	0.75, 3H, s
25	0.81, 3H, s	0.83, 3H, s	0.83, 3H, s
26	1.01, 3H, s	0.96, 3H, s	0.92, 3H, s
27	0.92, 3H, s	0.94, 3H, s	0.96, 3H, s
28			
29	4.58, dd (2.2, 1.4) 4.69, d (2.2)	4.67, dd (1.8, 1.4) 4.78, d (1.8)	3.18, dd (11.4, 11.4) 3.24, dd (11.3, 5.8)
30	1.65, 3H, s	1.73, 3H, s	0.71, 3H, d (6.4)

[a] In $CDCl_3$, at 400 MHz. Coupling constants (J) are in Hz.

Table 2. ^{13}C NMR Data (δ_C) of Compounds **1**−**3**[a]

position	1	2	3
1	38.8, CH_2	38.9, CH_2	38.9, CH_2
2	27.5, CH_2	27.5, CH_2	27.4, CH_2
3	79.1, CH	79.0, CH	79.0, CH
4	38.9, qC	38.9, qC	38.9, qC
5	55.4, CH	55.6, CH	55.5, CH
6	18.4, CH_2	18.3, CH_2	18.3, CH_2
7	34.5, CH_2	34.0, CH_2	34.0, CH_2
8	40.9, qC	40.6, qC	40.7, qC
9	50.7, CH	51.2, CH	50.9, CH
10	37.3, qC	37.3, qC	37.3, qC
11	21.0, CH_2	21.4, CH_2	21.2, CH_2
12	25.3, CH_2	26.9, CH_2	25.7, CH_2
13	36.8, CH	44.2, CH	35.8, CH
14	42.1, qC	40.7, qC	38.9, qC
15	29.6, CH_2	28.8, CH_2	30.7, CH_2
16	27.0, CH_2	28.2, CH_2	28.5, CH_2
17	91.6, qC	93.4, qC	82.9, qC
18	49.2, CH	46.8, CH	54.3, CH
19	48.0, CH	53.4, CH	42.6, CH
20	150.0, qC	150.5, qC	36.7, CH
21	27.4, CH_2	27.4, CH_2	21.3, CH_2
22	32.3, CH_2	33.4, CH_2	28.2, CH_2
23	28.1, CH_3	28.0, CH_3	28.1, CH_3
24	15.4, CH_3	15.4, CH_3	15.5, CH_3
25	16.3, CH_3	16.5, CH_3	16.5, CH_3
26	16.2, CH_3	15.6, CH_3	15.8, CH_3
27	14.1, CH_3	14.7, CH_3	13.7, CH_3
28			
29	109.8, CH_2	108.9, CH_2	67.0, CH_2
30	19.4, CH_3	21.1, CH_3	15.3, CH_3

[a] In $CDCl_3$, at 100 MHz. Carbon multiplicities were determined by APT experiment. qC = quaternary, CH = methine, CH_2 = methylene, CH_3 = methyl carbons.

correlation between the protons H_2-29 and the quaternary oxygenated C-17 (δ_C 82.9) suggested a cyclic structure through an ether bridge between C-29 and the C-17β hydroxy group of the 28-norlupane nucleus. This was confirmed by a TOCSY experiment, in which the segments [(H_2-29)-(H-20)-(H-19)-(H_3-30)-(H-18)] were

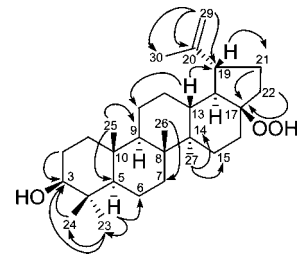

Figure 1. Selected HMBC correlations of compound **1**.

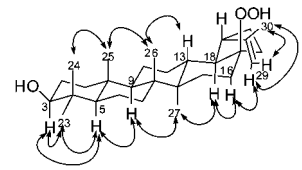

Figure 2. Important NOESY correlations of compound **1**.

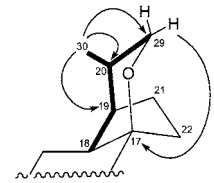

Figure 3. Selected TOCSY (bold bonds) and HMBC (arrows) correlations of compound **3**.

connected (Figure 3). The C-17β configuration was suggested on the basis of the chemical shift value of C-13 (δ_C 35.8), in a similar fashion to **1**.[9,19] The C-20*S* configuration was established from the positive sign of the optical rotation for **3** (+18.1) compared to 3β,28-acetoxy-29-lupanal epimers (20*R*, $[\alpha]_D = -103$; 20*S*, $[\alpha]_D = +70$).[23] This was consistent with the splitting pattern and J values of the proton H-20. Both the H_2-29 protons were geminally coupled

Table 3. Antiproliferative Activity of Isolated Triterpenes against the Malignant +SA Mouse Mammary Epithelial Cells

compound	IC_{50} (μM)
1	15.5
2	>20.0
3	18.1
4	18.7
asiatic acid	>20.0
betulin	>20.0
betulinaldehyde	19.4
betulinic acid	>20.0
platanic acid	>20.0
ursolaldehyde	19.9
ursolic acid	>20.0

with $J = 11.4$ Hz, and therefore the other couplings of H-29a and H-29b were with H-20 (Table 1). Proton H-20 showed one large and one small coupling value with H-29a ($J = 11.4$) and H-29b ($J = 5.8$), respectively, indicating its pseudoaxial orientation. A modeling study suggested that the pseudoaxially oriented substituent should be in the α-orientation. Therefore, H_3-30 was presumed to be β- oriented. Thus, the structure of **3** was determined to be 20*S*-17β,29-epoxy-28-norlupan-3β-ol.

The effect of various concentrations of triterpenes of *M. ericifolia* on the proliferation of malignant mouse +SA mammary epithelial cells is shown in Table 3.[24] Simvastatin was used as a positive control with known antiproliferative activity (IC_{50} value 2.0 μM).[25] Pravastatin was used as a negative control because it showed no antiproliferative activity at the tested doses.[25] 28-Norlupanes **1**−**4** showed IC_{50} values of 15.5, 20.6, 18.1, and 18.7 μM, respectively (Table 3). All other tested compounds showed IC_{50} values > 20.0 μM.

Experimental Section

General Experimental Procedures. Measurements of optical rotation were carried out on a Rudolph Research Analytical Autopol III polarimeter. IR spectra were recorded on a Varian 800 FT-IR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, using TMS as an internal standard, on a JEOL Eclipse NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The HREIMS and HRESIMS experiments were conducted at the University of Michigan on a Micromass LCT spectrometer. TLC analysis was carried out on precoated silica gel 60 F_{254} 500 μm TLC plates, using the developing systems *n*-hexane−EtOAc (9:1), *n*-hexane−EtOAc (8:2), or $CHCl_3$−MeOH (9:1).

Plant Material. Branches of *M. ericifolia* grown in Mansoura, Egypt, were collected in January 2006 and shade-dried, and then the leaves were separated from the stems. The plant was identified by Professor S. F. Khalifa, Faculty of Sciences, Ain Shams University, Cairo, Egypt. A voucher specimen was deposited at the Pharmacognosy Department, Mansoura University, Egypt (06-Mansoura-1).

Extraction and Isolation. The powdered leaves (2.5 kg) were extracted with MeOH (7 × 2 L) and MeOH−H_2O (7.5:2.5, 2 × 2 L). The combined MeOH extract was then concentrated under vacuum. The MeOH extract (915 g) was partitioned successively using *n*-hexane and $CHCl_3$. The *n*-hexane fraction (138 g) was chromatographed over silica gel and eluted with *n*-hexane−EtOAc mixtures of increasing polarity to give 14 fractions. Fraction 5 (5 g), eluted with *n*-hexane−EtOAc (9.5:0.5), was purified further by repeated column chromatography and medium-pressure liquid chromatography (MPLC) using C_{18} silica gel with H_2O−MeOH gradient elution, to give **1** (24 mg), **2** (8 mg), **3** (8 mg), 28-norlup-20(29)ene-3β,17β-diol (**4**, 20 mg), betulinaldehyde (18 mg), and ursolaldehyde (10 mg). Fraction 6 (11 g), eluted with *n*-hexane−EtOAc (9:1), was further purified using silica gel and *n*-hexane−EtOAc gradient elution to give betulin (40 mg). The $CHCl_3$ extract (156 g) was fractionated using a flash silica gel column with *n*-hexane, *n*-hexane−$CHCl_3$, and $CHCl_3$−MeOH mixtures to give 12 fractions. Fraction 8, eluted with *n*-hexane−$CHCl_3$ (5:5), was further purified on silica gel 60 using toluene−EtOAc (8:2) to afford betulinic acid (500 mg) and ursolic acid (20 mg). Fraction 9, eluted with *n*-hexane−$CHCl_3$ (25:75), was purified on C_{18} silica gel using H_2O−MeOH (2:8) to give platanic acid (7 mg). Fraction 12, eluted with $CHCl_3$, was chromatographed over silica gel 60 and eluted with $CHCl_3$−MeOH mixtures of increasing polarity to give eight fractions. Fraction 3, eluted with $CHCl_3$−MeOH (9.5: 0.5), was purified further on Sephadex LH20 using $CHCl_3$−MeOH (9.5: 0.5) to give asiatic acid (50 mg).

3β-Hydroxy-28-norlup-20(29)-en-17β-hydroperoxide (1): colorless, amorphous solid, $[\alpha]_D^{25}$ +24.5 (*c* 0.40, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3535, 2945, 2866, 1455, 1376, 1139, 1103, 983, 891 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; HRESIMS *m/z* 467.3478 $[M + Na]^+$ (calcd for $C_{29}H_{48}O_3Na$, 467.3501).

3β-Hydroxy-28-norlup-20(29)-en-17α-hydroperoxide (2): colorless, amorphous solid, $[\alpha]_D^{25}$ +14.1 (*c* 0.12, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3396, 2928, 2855, 1457, 1377, 1262, 1100, 1009, 886, 864, 841 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2; HREIMS *m/z* 444.3590 $[M]^+$ (calcd for $C_{29}H_{48}O_3$, 444.3603).

20S-17β,29-Epoxy-28-norlupan-3β-ol (3): colorless, amorphous solid, $[\alpha]_D^{25}$ +18.1 (*c* 0.16, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3603, 3535, 2931, 2864, 1716, 1456, 1377, 1143, 1089, 1008, 986, 915 cm^{-1}; ^{1}H and ^{13}C NMR see Tables 1 and 2; HREIMS *m/z* 428.3654 $[M+]^+$ (calcd for $C_{29}H_{48}O_2$, 428.3654).

Cell Antiproliferation Assay. The effects of triterpenes of *M. ericifolia* on malignant +SA mouse mammary epithelial cell proliferation were determined in 24-well culture plates (6 wells/group) by a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay, as described previously.[24] Cells were maintained on serum-free media containing 10 ng/mL EGF and 10 μg/mL insulin as mitogens. Cells were plated at a density of 5×10^4 cells/well (6 wells/group) in 24-well culture plates and fed media containing various concentrations (0.01−1000 μM) of each compound. After a 4-day culture period, the viable +SA cell number was determined by MTT colorimetric assay.[24] On the day of assay, treatment medium was removed and cells were incubated at 37 °C for 4 h with fresh control medium containing 0.83 mg/mL MTT. Afterward, the medium was removed and MTT crystals were dissolved in 1 mL of 2-propanol. The optical density of each sample was determined at 570 nm on a microplate reader (SpectraCount, Packard BioScience Company, Meriden, CT) against a blank prepared from cell-free cultures. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometer, at the start of each experiment.[24]

In order to dissolve the highly lipophilic triterpenes in aqueous culture media, these compounds were conjugated to bovine serum albumin (BSA) as previously described.[24] Briefly, an appropriate amount of each compound was placed in a 1.5 mL screw top glass vial and dissolved in 100 μL of 100% ethanol. Once dissolved, this ethanol/triterpene solution was added to a small volume of sterile 10% BSA in water and incubated overnight at 37 °C. This solution was then used to prepare various concentrations (0−1000 μM) of triterpene-supplemented treatment media such that all control and treatment media had a final concentration of 5 mg/mL BSA. Ethanol was added to all treatment media such that the final ethanol concentration was the same in all treatment groups within a given experiment and was always less than 0.1%.

Statistical Analysis. Differences among the various treatment groups were determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test. Differences were considered to be statistically significant at a value of $P < 0.05$, as compared to vehicle-treated controls.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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