Remangilones A−C, New Cytotoxic Triterpenes from *Physena madagascariensis*

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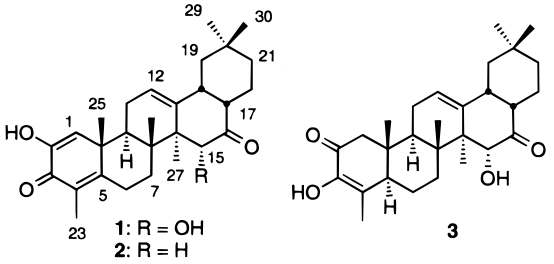
Three new 24,28-dinorolean-3-one derivatives, the remangilones A−C (**1**−**3**), were isolated from the dried leaves of *Physena madagascariensis* using a human mammary carcinoma cell line to guide the isolation. The structures of **1**−**3** were deduced primarily from NMR studies. Compounds **1** and **3**, remangilones A and C, respectively, were found to be cytotoxic against two human breast cancer cell lines and induced apoptosis at concentrations of 2.3 μM.

Physena madagascariensis Noronha ex Thouars (*Capparaceae*), locally known as "Remangily", is a tree endemic to Madagascar. The bark of the plant is rubbed on clothing as an effective repellent of terrestrial leeches, which are abundant in the region of the island where the tree is also found.[1] The petroleum ether-soluble portion of the crude methanolic extract of the dried leaves of *P. madagascariensis* was found to be cytotoxic against two breast cancer cell lines, MDA-MB-435 and MDA-MB-231, inducing programmed cell death in cell culture. Using activity against the MDA-MB-435 cell line to guide the isolation, three new dinoroleanane derivatives were obtained following repeated chromatography. No prior reports of natural products have appeared in the literature on this species, a member of a family with only two members, both endemic to Madagascar.[2] The structure determination and biological activity of these three triterpenes, which we have called the remangilones A−C (**1**−**3**), is described herein.



Results and Discussion

White crystalline remangilone A (**1**) showed 28 carbons in its ^{13}C NMR spectrum, analyzing for six methyl, six methylene, six methine, and 10 quaternary carbons in the DEPT spectrum. The ^{1}H NMR spectrum showed the presence of two exchangeable (D_2O) resonances (δ 6.39, s, OH-2, and 3.66, d, J = 3.7 Hz, OH-15) thereby indicating the presence of 38 protons in **1**. The high resolution mass spectrum (EI, 70 eV) displayed a molecular ion at *m*/*z* 438.2792, requiring a molecular formula of $C_{28}H_{38}O_4$ (calcd for $C_{28}H_{38}O_4$, *m*/*z* 438.2770). Key functionality readily discernible in the preliminary spectra included two carbonyl groups, one assignable to a nonconjugated ketone (IR 1710 cm^{-1}; ^{13}C NMR δ 213.8, C-16) while the second had spectral features reminiscent of a quinoidal carbonyl (IR 1626 cm^{-1}; ^{13}C NMR δ 181.4, C-3), though the colorless nature of **1** and the UV spectrum [λ_{max} 212 nm (ϵ 6600), 260 (11 100), 300 (sh)] eliminated the possibility of a quinone. Also present were the two aforementioned hydroxyl groups with distinct peaks in the IR spectrum (3414 and 3402 cm^{-1}) and three double bonds: ^{13}C NMR δ 124.3 (C-1) and 124.8 (C-12), both methines, and δ 125.8 (C-4), 141.9 (C-13), 144.5 (C-2), and 166.8 (C-5), quaternary carbons. That the three double bonds did not constitute an aromatic system was evident from the vinyl proton chemical shifts: ^{1}H NMR δ 6.26 (s, H-1, correlating with the carbon resonance at 124.3 in the HMQC spectrum) and δ 5.60 (d, J = 4.9, 3.1 Hz, H-12, correlating with the carbon at 124.8). The lack of coupling between the vinyl protons indicated that two of the double bonds were trisubstituted while the third was fully substituted.

The carbonyl and olefinic groups account for five of the ten units of unsaturation required by the molecular formula, and thus, **1** was determined to be pentacyclic, suggesting a dinortriterpene. From the ^{1}H NMR spectrum it was clear that all six methyl groups were bonded to quaternary carbons, and one of these was a vinyl methyl group (δ 0.82, 0.84, 0.86, 1.30, 1.36, and 1.98, all singlets). From the HMBC spectrum, which showed heteronuclear long-range coupling between two methyls (H-29/C-29, δ 0.82/33.1, H-30/C-30 δ 0.86/23.1) as well as between both methyl protons with the relatively high-field quaternary carbon at δ 30.8 (C-20), it could be deduced that these formed a *gem*-dimethyl pair. Also apparent in the HMBC spectrum was coupling from both of these methyl singlets to two methylene carbons (δ 46.4, C-19, 34.2, C-21), indicating that the *gem*-dimethyl pair was flanked by two methylene groups. This observation eliminated the possibility of the *gem*-dimethyl pair at the classic C-4 position, leading to two suggestions ultimately comfirmed by more detailed NMR studies. First, one of the "nor" positions was most likely C-24, and second, the triterpene was suggested to be a member of the triterpenoid class with a *gem*-dimethyl group at C-20 of the E-ring, as found in the oleananes.[3]

The ^{1}H,^{1}H-COSY and TOCSY spectra delineated the proton scalar coupled spin networks. Thus, beginning with the methylene protons at C-19 and C-21 flanking the *gem*-dimethyl group (located from the HMQC spectra by their correlation with the methylene carbons that showed coupling with the *gem*-dimethyl protons in the HMBC spectrum), the isolated E-ring proton spin system was identified

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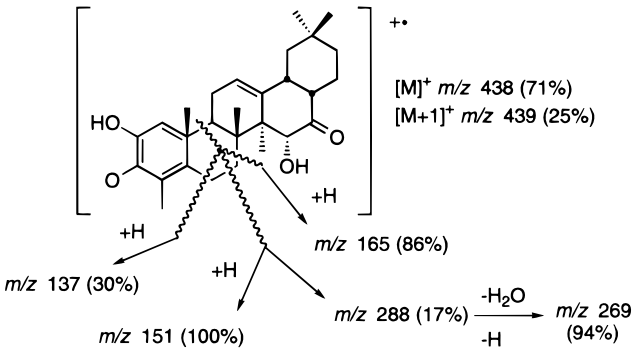
(H-19/19′ to H-18 to H-17 to H-22/22′ to H-21/21′). The presence of a methine proton at C-17 indicated that the second nor-position was located there; thus, **1** was a 24,-28-dinoroleanane derivative.

The α-hydroxyketone, most readily identified by the heteronuclear long-range coupling (HMBC) between the nonconjugated carbonyl carbon (δ 213.8) and the exchangeable hydroxyl proton at δ 3.66, was located at C-15(OH)/C-16 by further heteronuclear couplings between the C-16 carbonyl and H-17, H-18, and both C-22 protons. Since H-15 (δ 4.32, dd, J = 3.7, 1.2 Hz, C-15: δ 75.4 from the HMQC) coupled only with the hydroxyl proton, coupling which disappeared upon D_2O exchange, and only very weakly with H-17, C-14 was therefore suggested to be a quaternary carbon. Since all four sp^3 hybridized carbons must bear methyl groups to account for the five nonolefinic methyl singlets (including the *gem*-dimethyl group), a methyl group must be a C-14, in support of the oleanane structure.[4] This conclusion was confirmed by long-range heteronuclear couplings observed (HMBC) between the quaternary carbon at δ 54.9 (C-14) with H-15, OH-15, and H-18, and couplings between H-15 and the methyl carbon at δ 19.2 (C-27). Couplings between C-14 and the methyl singlet at δ 0.84 (H-27), which in turn also coupled with C-15, were also observed in accord with this structure.

A second proton spin system revealed by the DQ-COSY and TOCSY spectra was a simple $CHCH_2CH$ (H-9, H-11, H-12) sequence terminating with the vinyl proton at δ 5.60. Long-range heteronuclear couplings (HMBC) between H-18 and two olefinic carbons, the methine carbon at δ 124.9 lying at one end of this proton spin system and the quaternary carbon at δ 141.8 (which also showed coupling with both allylic methylene protons of this spin system) completed the carbon connectivity of the D-ring with the C-13 nonprotonated sp^2-hybridized carbon. The chemical shift (which ruled out an allylic methine) and simple multiplicity of the sp^3 methine at the other terminus (H-9) of this proton spin system (δ 1.69, dd, J = 11.6, 5.5 Hz, correlating with the methine carbon δ 44.5 in the HMQC spectrum) suggested that this position (C-9) bears an axial proton and is flanked by two methyl-bearing quaternary carbons. This was confirmed by long-range heteronuclear couplings (HMBC) between H-9 and two methyl carbons (δ 17.7 and 21.8, correlating with methyl singlets at δ 1.36 and 1.30, respectively, HMQC). Equally important was the coupling between H-9 and the C-14 quaternary carbon, thereby completing the C-ring and establishing methyl groups at C-8, C-10, and C-14 of the triterpene skeleton.[5]

The remaining methylenes (shown to be adjacent in the DQ-COSY spectrum), two olefins, and carbonyl group could only be reasonably assembled into a 2-hydroxy-$\Delta^{1,4}$-dien-3-one subunit in the A-ring to account for the chromophore observed in the UV spectrum.[6] This subunit was also apparent from the mass spectral fragmentation (EIMS, 70 eV) producing a base peak of m/z 151 (Scheme 1).[7] Other fragments of m/z 165 (86%) and 137 (30%) are also in accord with this subunit, while the major fragment ion at m/z 269 (94%), which presumably forms from the fragment ion at m/z 288 (17%), supported the C-15/C-16 α-hydroxyketone. These mass spectral interpretations were supported by HRMS analysis (see the Experimental Section). The B-ring is then completed with a C-6/C-7 pair of methylene carbons. Long-range heteronuclear coupling (HMBC) between the remaining vinyl proton (δ 6.26, s, correlating with the sp^2-hybridized methine δ 124.3, HMQC) with C-9 and the methyl carbon at δ 21.8 (C-25), the oxygenated olefinic carbon at δ 144.5 (C-2), the conjugated carbonyl δ 181.4, (C-3), and the low-field olefinic carbon at δ 166.8 (C-5), typical for the α-carbon of an α,β-unsaturated ketone, were also observed. These couplings linked the A-ring chromophore with the C-ring, distinguished the ^1H and ^{13}C NMR resonances for the methyl groups on C-8 and C-10 (both showed long-range heteronuclear couplings with H-9), and supported the $\Delta^{1,4}$-dien-3-one system. Coupling between the low-field hydroxyl proton (δ 6.39) and the carbonyl carbon at C-3 as well as with C-1 and C-2 confirmed the α-hydroxy-α,β-unsaturated ketone. Long-range couplings from the vinyl methyl protons to C-3, C-4, and C-5 confirmed the location of this group at C-4. Finally, confirmation of the C-6/C-7 methylene groups was also apparent from long-range heteronuclear couplings (Table 1).

Scheme 1. Mass Spectral Fragmentations of 1 (EIMS).



With the carbon connectivity completed, stereochemical definitions at C-15, C-17, and C-18 were resolved from coupling constants and NOE's (Table 1). It should be noted that at this point, while the oleanane skeleton was considered correct, the relatively rare germanicane framework, which differs from the oleananes only in the stereochemistry at C-18, could not be ruled out on the basis of carbon connectivity alone.[8,9] In DNOE spectra, both H-15 and H-18 showed strong NOE's with H-17, indicating that these three protons lie on the same face of the molecule. In addition, H-18 also showed an NOE with CH_3-30 (distinguished from CH_3-29 on the basis of their ^{13}C shifts),[10] while H-15 showed a strong NOE with CH_3-26. Thus, these protons lie on the β-face of **1**, and the oleanane structure was confirmed. Other NOE's (Table 1), such as that between H-9 and CH_3-27, were in accord with the assigned structure.

With the detailed structure analysis of **1** completed, structure assignments for **2** and **3** were relatively simple. Remangilone B (**2**) (HREIMS m/z 422.2843, calcd for $C_{28}H_{38}O_3$, 422.2821) showed only a single, exchangeable (enolic) proton (δ 6.38) with the secondary OH proton and the accompanying carbinol proton and carbon resonances found in **1** were no longer present in the ^1H and ^{13}C NMR spectra, respectively, thereby accounting for the loss of an oxygen upon comparison of the molecular formulas of **1** and **2**. An isolated AB system in the ^1H NMR spectrum (δ 1.93 and 2.56, J_{AB} = 14.3 Hz), with chemical shifts typical for protons adjacent to a carbonyl group, and the correlating (HMQC) methylene carbon (δ 46.8) in the ^{13}C NMR spectrum indicated that the C-15 hydroxyl group of **1** was not present in **2** but was replaced by a methylene group. The ^{13}C NMR chemical shifts near this substitution site reflected this difference: the C-27 methyl (δ 26.1), C-17 methine (δ 47.1), and C-13 olefinic (δ 142.8) carbons were shifted downfield in **2** relative to **1** (Δ_{27} = 6.9 ppm, Δ_{17} = 2.9 ppm, Δ_{13} = 0.9 ppm) due to the γ-effect (in **1**), while C-14 in **2** (δ 48.3) was upfield from the corresponding

Table 1. ^{1}H and ^{13}C NMR Data for **1** Recorded in $CDCl_3$

position	^{13}C (m)	^{1}H (m, *J*)	HMBC correlations	NOE
1	124.3 (d)	6.26 (s)	C-2, C-3, C-5, C-9, C-10, C-25	H-9, H-11α, H-25
2	144.5 (s)			
3	181.4 (s)			
4	125.8 (s)			
5	166.8 (s)			
6α	25.5 (t)	2.75 (ddd, *J* = 13.6, 3.7, 3.7 Hz)	C-4, C-5, C-7, C-8, C-10	H-6*β*, H-7*β*, H-23
6*β*		2.34 (ddd, *J* = 13.6, 14.0, 3.7 Hz)	C-4, C-5, C-7	H-6α, H-7*β*, H-25, H-26
7α	37.0 (t)	1.60 (ddd, *J* = 14.0, 13.8, 3.7 Hz)	C-5, C-6, C-8, C-14, C-26	H-7*β*, H-27
7*β*		2.07 (ddd, *J* = 13.8, 3.7, 3.7 Hz)	C-5, C-6, C-8, C-9, C-26	H-6α, H-6*β*, H-7α, H-26
8	40.8 (s)			
9	44.5 (d)	1.69 (dd, *J* = 11.6, 5.5 Hz)	C-1, C-8, C-10, C-11, C-14, C-25, C-26	H-11α, H-27
10	43.3 (s)			
11α	25.3 (t)	2.22 (ddd, *J* = 17.7, 5.5, 4.9 Hz)	C-8, C-9, C-12, C-13	H-1, H-9, H-12
11*β*		2.31 (ddd, *J* = 17.7, 11.6, 3.1 Hz)	C-9, C-12, C-13	H-12, H-25, H-26
12	124.8 (d)	5.60 (dd, *J* = 4.9, 3.1 Hz)	C-9, C-11, C-14, C-18	H-9, H-11α, H-11*β*, H-18
13	141.9 (s)			
14	54.9 (s)			
15	75.4 (d)	4.32 (dd, *J* = 3.7, 1.2 Hz)	C-8, C-14, C-16, C-27	H-7*β*, H-17, H-26
16	213.8 (s)			
17	44.2 (d)	2.76 (br)	C-16, C-19, C-21, C-22	H-15, H-18, H-22α,*β*
18	45.8 (d)	3.00 (ddd, *J* = 14.2, 7.3, 4.2 Hz)	C-12, C-13, C-14, C-16, C-17, C-19	H-12, H-17, H-30
19α	46.4	1.26 (dd, *J* = 14.2, 14.2 Hz)[a]		
19*β*		1.12 (m)		
20	30.8 (s)			
21α[b]	34.2 (t)	1.46 (m)		
21*β*[b]		1.17 (m)		
22[c]	20.1 (t)	1.46 (m)	C-16	
22[c]		1.98 (m)	C-16	
23	10.6 (q)	1.98 (s)	C-3, C-4, C-5	H-6α
25	21.8 (q)	1.30 (s)	C-1, C-5, C-9, C-10	H-6*β*, H-11*β*, H-26
26	17.7 (q)	1.36 (s)	C-7, C-8, C-9, C-14	H-6*β*, H-7*β*, H-11*β*, H-15, H-25
27	19.2 (q)	0.84 (s)	C-7, C-13, C-15, C-14	H-9
29	33.1 (q)	0.82 (s)	C-19, C-20, C-21, C-30	
30	23.1 (q)	0.86 (s)	C-19, C-20, C-21, C-29	H-18
2-OH		6.39 (s)	C-1, C-2, C-3	
15-OH		3.66 (d, *J* = 3.7 Hz)	C-14, C-15, C-16	

[a] Coupling constants obtained from a homo 2D-J experiment. [b] Protons 21α and 21*β* were tentatively assigned by the observed of ^4J-coupling (*W*-coupling) in an LRCOSY experiment (Δ = 300 ms). [c] Due to overlap, the 22α and 22*β* protons could not be unambiguously assigned in $CDCl_3$.

resonance in **1** ($\Delta_{14} = -6.5$ ppm). Virtually no change was observed in the shift of the C-16 carbonyl resonance (*δ* 213.5, $\Delta_{16} = -0.3$ ppm). All other proton and carbon resonances, UV and IR absorptions, and A-ring fragmentations in the MS matched very closely (almost identical) with those recorded for **1**. Of note was the appearance of the fragment ion *m*/*z* 272.2142 (7%, calcd for $C_{19}H_{28}O$, 272.2141) in the HREIMS, which corresponds to the fragment ion of *m*/*z* of 288 observed in the EIMS of **1** minus the oxygen atom at C-15. Detailed analysis of the NMR spectroscopic data (Table 2) beginning with the *gem*-dimethyl group as described above for **1** confirmed the structure of **2**. Long-range heteronuclear couplings from the H-15 methylene pair to C-27, C-16, and C-17 highlighted these studies, with an NOE of H-15α upon irradiation of the C-27 methyl singlet in a DNOE experiment distinguishing the two C-15 methylene resonances.

Remangilone C (**3**) was readily identified as a dihydro derivative of **1** from the HREIMS (*m*/*z* 440.2930, calcd for $C_{28}H_{40}O_4$, 440.2926). The ^{1}H NMR spectrum showed the appearance of a new methylene proton pair as an isolated AB system (*δ* 2.07 and 2.65, both doublets, $J_{AB} = 16.5$ Hz) and a new methine (*δ* 2.36, bd, *J* = 12.8 Hz) with the loss of the H-1 vinyl proton in comparison to the spectrum of **1**, and the ^{13}C NMR spectrum also showed new methylene and methine carbons (*δ* 51.6, C-1, and *δ* 48.5, C-5), with the loss of a methine and a nonprotonated olefinic carbon in comparison to **1** (Table 2). The downfield shift of the conjugated carbonyl carbon resonance to *δ* 193.6 in **3** (compared to 181.4 in **1**), the UV spectrum in comparison to **1** [λ_{max} 213 nm (*ϵ* 3400), 280 (9600)], and the appearance of α-hydroxy-α,*β*-unsaturated carbonyl stretching frequencies at 1662 and 1636 cm^{-1} in the IR spectrum all indicated the loss of one of the two double bonds from the chromophore of **1**.[11] Also of note was the appearance of two, distinct OH stretching bands (3480, 3434 cm^{-1}) in the IR spectrum as observed with **1**; in contrast, only a single band was observed for **2**.

The ^{1}H and ^{13}C NMR spectra of **3** were nearly identical to those of **1** with the exception of A-ring resonances and those in the immediate vicinity. Long-range heteronuclear couplings (HMBC) between the vinyl methyl protons (*δ* 1.85 correlating with the methyl carbon resonance at *δ* 13.4 in the HMQC spectrum) and the remaining A-ring olefinic carbons (*δ* 143.5, C-3, and *δ* 130.9, C-4) and with C-5 suggested the 3-hydroxy-3,4-en-2-one enolic system, supported by both the ^{1}H (*δ* 5.96, OH-3, exchangeable) and ^{13}C (*δ* 130.9, C-4, and 143.5, C-3) NMR chemical shifts. Other long-range heteronuclear couplings (of note, coupling between the 3-OH proton and C-2, C-3, and C-4, between both H-1 protons and C-2, C-5, C-10, and C-25, and H-1 with C-3, between H-5 and C-3, C-4, and C-25, and between H-25 and C-1, C-5, C-9, and C-10), and detailed analysis of the NMR spectroscopic data beginning with the *gem*-dimethyl group, as described above for **1**, confirmed the structure of **3**.

The effects of **1** and **3**, the only two natural products purified in sufficient quantities for the studies, on three human mammary epithelial cell lines (two cancer lines and one normal) were investigated using an SRB assay to monitor cell viability[13] and fluorescence microscopy to reveal apoptosis.[14] Treatment with both compounds re-

Table 2. ^{1}H and ^{13}C Data for **2** and **3** Recorded in $CDCl_3$

	2		3	
position	^{13}C (m)	^{1}H (m, *J*)	^{13}C (m)	^{1}H (m, *J*)
1α	124.4 (d)	6.26 (s)	51.6 (t)	2.07 (d, *J* = 16.5 Hz)
1β				2.65 (d, *J* = 16.5 Hz)
2	144.4 (s)		193.6 (s)	
3	181.4 (s)		143.5 (s)	
4	126.2 (s)		130.9 (s)	
5	166.2 (s)		48.5 (d)	2.36 (bd, *J* = 12.8 Hz)
6α	24.9 (t)	2.76 (ddd, *J* = 13.4, 3.7, 3.7 Hz)	20.7 (t)	1.86 (m)
6β		2.34 (ddd, *J* = 13.6, 13.4, 3.7 Hz)		1.43 (m)
7α	34.29[a]	1.46 (m)	34.6 (t)	1.72 (m)
7β		1.54 (m)		1.82 (m)
8	39.6 (s)		40.5 (s)	
9	44.0 (d)	1.79 (dd, *J* = 11.3, 6.4 Hz)	43.4 (d)	1.73 (m)
10	43.4 (s)		41.3 (s)	
11α	25.4 (t)	2.26 (m, 2H)	23.7 (t)	1.99 (m, 2H)
11β				
12	122.3 (d)	5.52 (dd, *J* = 3.7, 3.7 Hz)	124.7 (d)	5.56 (dd, *J* = 3.1, 3.1 Hz)
13	142.8 (s)		141.9 (s)	
14	48.3 (s)		54.5 (s)	
15α	46.8 (t)	1.93 (d, *J* = 14.3 Hz)	75.5 (d)	
15β		2.56 (d, *J* = 14.3 Hz)		4.33 (d, *J* = 3.7 Hz)
16	213.5 (s)		214.3 (s)	
17	47.1 (d)	2.61 (br)	44.1 (d)	2.76 (br)
18	44.9 (d)	2.93 (ddd, *J* = 14.0, 6.7, 3.7 Hz)	45.9 (d)	2.99 (ddd, *J* = 13.8, 7.3, 3.4 Hz)
19α	46.4 (t)	1.33 (m)	46.7 (t)	1.28 (dd, *J* = 13.8, 13.8 Hz)
19β		1.16 (m)		1.16 (m)
20	30.9 (s)		30.8 (s)	
21α	34.22[a] (t)	1.38 (m)[b]	34.2 (t)	1.46 (m)[b]
21β		1.16 (m)[b]		1.18 (m)[b]
22α	20.6 (t)	1.38 (m)[c]	20.2 (m)	1.43 (m)[c]
22β		2.02 (m)[c]		1.96 (m)[c]
23	10.7 (q)	1.99 (s)	13.4 (q)	1.85 (d, *J* = 1.8 Hz)
25	21.4 (q)	1.29 (s)	14.4 (q)	0.97 (s)
26	17.4 (q)	1.22 (s)	18.0 (q)	1.14 (s)
27	26.1 (q)	1.02 (s)	19.8 (q)	1.04 (s)
29	33.2 (q)	0.83 (s)	33.1 (q)	0.84 (s)
30	23.3 (q)	0.87 (s)	23.2 (q)	0.87 (s)
OH-2/3		6.38 (s)		5.96 (s)
OH-15				3.67 (d, *J* = 3.7 Hz)

[a] Assignments of C-7 and C-21 in **2** may be reversed. [b] Due to overlap, the 21α and 21β protons of **2** and **3** could not be unambiguously assigned in $CDCl_3$. [c] Due to overlap, the 22α and 22β protons of **2** and **3** could not be unambiguously assigned in $CDCl_3$.

Table 3. Cytotoxicity of Remangilones A (**1**) and C (**3**) Against Human Mammary Epithelial Cells

	IC_{50} (μM)	
cell line	**1**	**3**
MDA-MB-435[a]	8.5	2.0
MDA-MB-231[a]	6.6	1.6
70N[b]	40.5	51.6

[a] Breast carcinoma cell lines. [b] Normal mammary epithelial cell line.

sulted in a significant decrease in the total cell number of two established breast carcinoma cell lines MDA-MB-231 and MDA-MB-435 with IC_{50} values in the μM range (Table 3). In addition, dramatic changes from polarized to spherical cellular morphology was associated with the treatment, indicative of induced cell death. In parallel experiments, MDA-MB-435 cells were treated with **1** and **3** at a concentration of 2.3 μM for 24 h (37 °C) and subsequently cultured in "drug-free" DFCI-1 medium for 96 h. Significant cell death was still detected by the SRB assay (data not shown). Interestingly, both **1** and **3** were significantly less toxic toward normal mammary epithelial cells 70N in the same assay.

Consistent with the prolonged and irreversible cytotoxicity, **1** and **3** were shown to induce apoptosis (programmed cell death) at concentrations of 2.3 μM. Cells undergoing apoptosis typically exhibit distinct nuclear morphological changes: the nuclei shrink, and the chromatin becomes condensed or fragmented.[15] For comparison in the same assay, paclitaxel induced apoptosis[16] at a concentration of 50 nM.[17]

In conclusion, bioassay-guided isolation of the anticancer principles of the methanolic extract of the leaves of *P. madagascariensis*, a tree endemic to Madagascar, has led to the isolation and structural identification of three new dinortriterpenes, remangilones A−C (**1**−**3**). The cyctotoxicity paralleled that reported for other triterpene-derived quinone methides.[18] While the molecular mechanism for the cytotoxicity of **1** and **3** remains the subject of further investigation, it can be noted that the normal mammary epithelial cells 70N express wild-type p53, whereas the mammary carcinoma cells MDA-MB-435 and MDA-MB-231 produce mutant p53.[19] Since p53 plays an important role in the apoptotic pathways induced by various pathogenic stimuli,[20] it is possible that the different p53 status among the three cell lines tested contributes to the observed differential cytotoxicity of **1** and **3**.

Experimental Section

General Experimental Procedures. ^{1}H NMR and ^{13}C NMR spectra were recorded at 93.94 kG (^{1}H 400 MHz), 70.5 kG (^{13}C 75 MHz), and 63.41 kG (^{13}C 67.5 MHz) in $CDCl_3$ at ambient temperature. Chemical shifts are expressed in ppm relative to internal reference: for ^{1}H NMR spectra, the residual $CHCl_3$ resonance at δ 7.24, for ^{13}C NMR, the center line of the solvent triplet at δ 77.0. The HMQC spectra were optimized for an average $^1J_{C,H}$ of 135 Hz; the HMBC spectra were

optimized for $J_{C,H}$ of 4.5 and 7 Hz. Difference NOE (DNOE) spectra were recorded with a minimum of 1024 transients each for the on-resonance (saturation) and off-resonance (negative) spectra acquired in repetitive blocks of 16 transients each. The irradiation interval was set to 8 s. LRMS and exact mass measurements were taken on a Finnigan MAT-90 spectrometer as indicated. IR spectra were recorded after depositing an $CDCl_3$ solution of sample on an NaCl plate and evaporating the solvent. Melting points were determined on a capillary melting point apparatus and are uncorrected. Optical rotations were measured on a Rudolph Research Autopol III polarimeter. All chromatography solvents were distilled before use.

Plant Material. The leaves of *P. madagascariensis* were collected in the Fort Dauphin area of Madagascar in November 1993. A voucher speciman is on deposit at ESSD, Antananarivo, Madagascar.

Extraction and Isolation. The air-dried leaves (7.5 kg) were extracted sequentially in batches with petroleum ether (bp 35−60 °C, 2 × 2 L/kg), MeOH (2 × 2 L/kg), and H_2O/MeOH (95/5, 2 × 2 L/kg) for a minimum of 48 h/extraction. After each extraction, the sample was filtered and the solvent removed from the filtrate *in vacuo* to yield the crude residues for bioassay. The active MeOH extract was then partitioned between equal portions of EtOAc and H_2O (1 L each), the layers were separated, the solvents were removed *in vacuo*, and the residues were assayed for activity. The active EtOAc-soluble fraction was further partitioned between equal portions (2 L) of petroleum ether (bp 35−60 °C) and aqueous MeOH (30% H_2O). The residue (19 g) from the active petroleum ether-soluble fraction was then subjected to flash chromatography on silica gel in 2 g batches using the bioassay with the MDA-MB-35 cell line to guide the isolation. Initial chromatography eluting with a step gradient of EtOAc in hexanes (5% EtOAc, 500 mL; 10% EtOAc, 1 L; 20% EtOAc, 1 L; 30% EtOAc, 500 mL; 50% EtOAc, 500 mL) produced five fractions, with fraction 2 (142 mg, eluting with 10% EtOAc) being active. Subsequent flash chromatography (Si gel) of this fraction eluting with another step gradient of EtOAc in hexanes (2% EtOAc, 400 mL; 5% EtOAc, 500 mL; 10% EtOAc, 500 mL; 20% EtOAc, 300 mL; 50% EtOAc, 200 mL) gave 5 fractions, with fractions 2 ("F2", 60 mg, eluting with 5% EtOAc, containing 3) and 3 ("F3", 35 mg, eluting with 10% EtOAc, containing **1** and **2**) being active. Final purification of **3** from F2 was accomplished by HPLC [Si gel (10 μm), 10 × 250 mm; flow rate 3 mL/min; UV detection 270 nm] eluting with hexanes−*n*-Bu_2O−Et_2O (95:5:5, retention time 60 min, 20 mg/2 g of petroleum ether-soluble residue). Final purification of **1** (15 mg/2 g of petroleum ether-soluble resiude) and **2** (4 mg/2 g petroleum ether-soluble residue) from F3 was accomplished by preparative TLC (Si gel) eluting with hexanes−Et_2O (4:1, R_f 0.23 and 0.18 for **1** and **2**, respectively).

Remangilone A (1): colorless needles; mp 186−187 °C; $[\alpha]^{24}_D$ +38.3° (*c* 0.6 g/100 mL, MeOH); CD (*c* 2.74 mM, MeOH) 218 nm ($\Delta\epsilon$ +1.67), 284 (−3.22); UV (MeOH) λ_{max} (ϵ) 212 (6600), 261 (11 100), 300 (sh) nm; IR (NaCl) 3414, 3402, 1710, 1626 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS (70 eV) *m/z* 439 ([M + 1]$^+$, 25), 438 (M$^+$, 71), 288 (17), 269 (93), 165 (86), 151 (100), 137 (30); HREIMS (70 eV) *m/z* 438.2792 (M$^+$, 11, calcd for $C_{28}H_{38}O_4$, 438.2770), 288.2092 (5, calcd for $C_{19}H_{28}O_2$, 288.2090), 269.1874 (8, calcd for $C_{19}H_{25}O$, 269.1906), 165.0909 (19, calcd for $C_{10}H_{13}O_2$, 165.0916), 151.0758 (100, calcd for $C_9H_{11}O_2$, 151.0760), 137.0596 (1.4, calcd for $C_8H_9O_2$, 137.0603).

Remangilone B (2): colorless needles; $[\alpha]^{24}_D$ +60.0° (*c* 0.4 g/100 mL, MeOH); CD (*c* 2.37 mM, MeOH) 216 nm ($\Delta\epsilon$ +2.38), 249 (−1.52), 288 (−1.21); UV (MeOH) λ_{max} (ϵ) 214 (3100), 259 (3400) nm; IR (NaCl) 3400, 1710, 1624 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; EIMS (70 eV) *m/z* 422 (M$^+$, 15), 151 (100); HREIMS (70 eV) *m/z* 422.2843 (M$^+$, 9, calcd for $C_{28}H_{40}O_4$, 422.2821), 272.2142 (7, calcd for $C_{19}H_{28}O$, 272.2141), 151.0758 (100, calcd for $C_9H_{11}O_2$, 151.0759).

Remangilone C (3): colorless needles; mp 162−163 °C; $[\alpha]^{24}_D$ +75.0° (0.6 g/100 mL, MeOH); CD (MeOH) 223 nm ($\Delta\epsilon$ −0.34), 278 (+0.602), 315 (−0.65); UV (*c* 2.27 mM, MeOH) λ_{max} (ϵ) 213 (3400), 280 (9600); IR (NaCl) 3480, 3430, 1709, 1662, 1636 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; EIMS (70 eV) *m/z* 440 (M$^+$, 100); HREIMS (70 eV) *m/z* 440.2930 (M$^+$, 54, calcd for $C_{28}H_{40}O_4$, 440.2926).

Bioassays. Cell Culture. Human mammary carcinoma cells MDA-MB-435 and MDA-MB-231 (American Type Culture Collection, Rockville, MD) were maintained in α-medium supplemented with 5% fetal calf serum. Normal human mammary epithelial cells 70N[21] were maintained in DFCI-1 medium.[22] Cells were incubated at 37 °C with 6.5% CO_2 in a humidified chamber. Exponentially growing cells were harvested by trypsin/EDTA and used for the drug treatment studies. Cells were washed with PBS, counted using a Coulter counter (Coulter Electronic, Hialeah, FL), and resuspended in DFCI-1 medium, which supports the growth of both normal mammary epithelial and mammary carcinoma cells at a similar rate.[22] For the SRB procedure, cells were seeded in triplicate at 4000/0.2 mL/well in a 96-well microtiter plate. For microscopy assay of apoptosis, cells were plated in DFCI-1 medium at (3−5) × 10^4 cells/35-mm dish.

Drug Treatment. Stock solutions of remangilones A (**1**) and C (**3**) were prepared in DMSO at 1.0 mg/mL. Aliquots of the stock solution were added to the culture medium so that the final concentration of DMSO was less than 0.2%. Cells were allowed to attach to the culture plate for 24 h at 37 °C in a humidified chamber with 6.5% CO_2. For the SRB assay, cells were subsequently incubated with 0.2 mL of DCFI-1 medium containing 0.1, 0.2, 2.0, 5.0, 10.0, 20.0, and 40.0 μM **1** or **3**. The same volume of DMSO was added to the corresponding controls. The cells were incubated for another 48 h at 37 °C in the humidified chamber with 6.5% CO_2 and then screened by either the SRB assay or fluorescent microscopy. In the microscopy assay, MDA-MB-435 cells treated with 50 nM paclitaxel (Sigma) were used as a positive control for drug-induced apoptosis.

Measurement of Drug Effect by SRB Assay and Microscopy. The cells were washed twice with PBS and then stained with SRB by an established method (SRB stains for cellular proteins).[13] The SRB bound to the cells was redissolved in 10 mM Tris·HCl (0.1 mL/well) and was measured by the absorbance at 550 nm using a Benchmark microplate reader (BioRad, Hercules, CA). The relative cell number based on the absorbance of SRB was plotted against the drug concentration and the IC_{50} determined as the concentration required for 50% reduction of viable cells. The washing procedure and mechanical agitation during the SRB assay does not lead to significant removal of viable, yet less adherent cells (e.g., cells in the M-phase of the cell-cycle) that can recover from the drug treatment.[23] The apoptotic effect of **1** and **3** on attached cells was also examined by the fluorescent microscopic assay as described.[14]

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Supporting Information Available: Structurally definitive NMR spectra for **1**−**3** and table of HMBC correlations and NOE's for **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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