Bioactive Friedolanostanes and 11(10→8)-Abeolanostanes from the Bark of *Garcinia speciosa*

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A new friedolanostane, **7**, and three triterpenes, **8**, **9a**, and **10**, possessing the new 11(10→8)-abeolanostane carbon skeleton were isolated from the bark of *Garcinia speciosa*. Structures were elucidated by spectroscopic and spectrometric studies and the structure of **8** by X-ray crystallographic analysis, thus forcing structure revision of a triterpene from the same source previously assumed to be a friedolanostane. These and several friedo- and lanostanes earlier isolated from the same source were evaluated for cytotoxicity against three human cell lines. Most were moderately active, with three friedolanostanes effective in inducing apoptosis in the MCF-7 cell line.

In an earlier article[1] we described the isolation and structure determination of friedolanostanes **1**, **2a**, **3**, and **4** and five lanostanes, **5a**−**d** and **6**, from the bark of *Garcinia speciosa* Wall., a tree indigenous to Thailand and Myanmar. Interpretation of the ^{1}H and ^{13}C NMR spectra of the substance assigned friedolanostane formula **3** presented certain difficulties that were not resolved satisfactorily because the substance could not be obtained in crystalline form. Further examination of some of the subfractions remaining from the earlier work has now led to isolation of another new friedolanostane, **7**, and of three compounds containing a new rearranged triterpene carbon skeleton, the 11(10→8)-abeolanostanes **8**, **9a**, and **10**, structure **8** being established by X-ray crystallography. These results permitted reformulation of the compound earlier assigned formula **3** as the 11(10→8)-abeolanostane **11**. Triterpenes **1**, **2a**,**b**, **4**, **5b**−**d**, **8**, **9a**, and **11** were evaluated for their ability to inhibit the in vitro growth of three human tumor cell lines. All except **5b** were moderately active, with **1**, **2b**, and **4** more effective than the others in inducing apoptosis in the MCF-7 cell line.

Results and Discussion

We begin with a discussion of the structure of the new friedolanostane **7**. Chemical shifts and coupling constants within the side chain attached to C-17 were essentially identical with those in friedolanostanes of the same type reported earlier,[1,2] while the carbon chemical shifts of the α,β-unsaturated cyclopentenone portion corresponded to those in **4**[1] with H-7β at the same unusually low field (δ 4.00) for the reasons cited earlier but with C-9 now a singlet and further downfield at δ 74.60 due to the attachment of a new hydroxyl group. The presence of a carbonyl instead of a hydroxyl group at C-3 was also obvious from the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2). All assignments in this and later instances are based on extensive HSQC, COSY, NOESY, and HMBC experiments.

In the case of **8** evidence for the attachment of a 2*E*,4*Z*-2,6-dimethylhexadienoic acid side chain to C-17 of a triterpene skeleton and for the presence of a β-oriented hydroxyl group on C-3 (δ 2.89 dd, J = 10.5, 6 Hz) was also clear, but the problem of elucidating the nature of rings B, C, and D, which obviously contained a cyclohexanone carbonyl and an epoxide linking one secondary and one tertiary carbon, was similar to that encountered previously in the case of the presumed **3**.[1] In this instance, however, the substance was crystalline, and X-ray analysis (Figure 1) showed that its structure was 14β,15β-epoxy-3β-hydroxy-9-oxo-11[10→8]-abeolanostan-22-*cis*,24-*trans*-dien-28-oic acid (**8**), i.e., that of an 11(10→8)-abeolanostane possessing a modified triterpene skeleton, which has not been encountered previously. A plausible route to the formation of **8** from an 8,14-lanostadiene precursor is illustrated in Scheme 1. A factor that had complicated elucidation of the structure of **8** and that of its congeners by the usual NMR techniques is the absence of coupling between H-15 and its neighbors H-16α and H-16β, a phenomenon not readily deduced by inspection of Figure 1. The correspondence between the relevant carbon and proton shifts of **8** and those of the compound previously attributed formula **3** then required that the latter be reformulated as **11**. For comparison the ^{13}C NMR spectrum of **11** is included in Table 2.

Two further 11(10→8)-abeolanostanes, **9a** and **10**, which differed only in orientation of the C-3 hydroxyl group, were also isolated from other previously unstudied fractions of the extract. Their ^{1}H and ^{13}C NMR spectra are listed in Tables 1 and 2. The side chains attached to C-17 of **9a** and **10** are identical with the side chain of a lanostane (**6**) that

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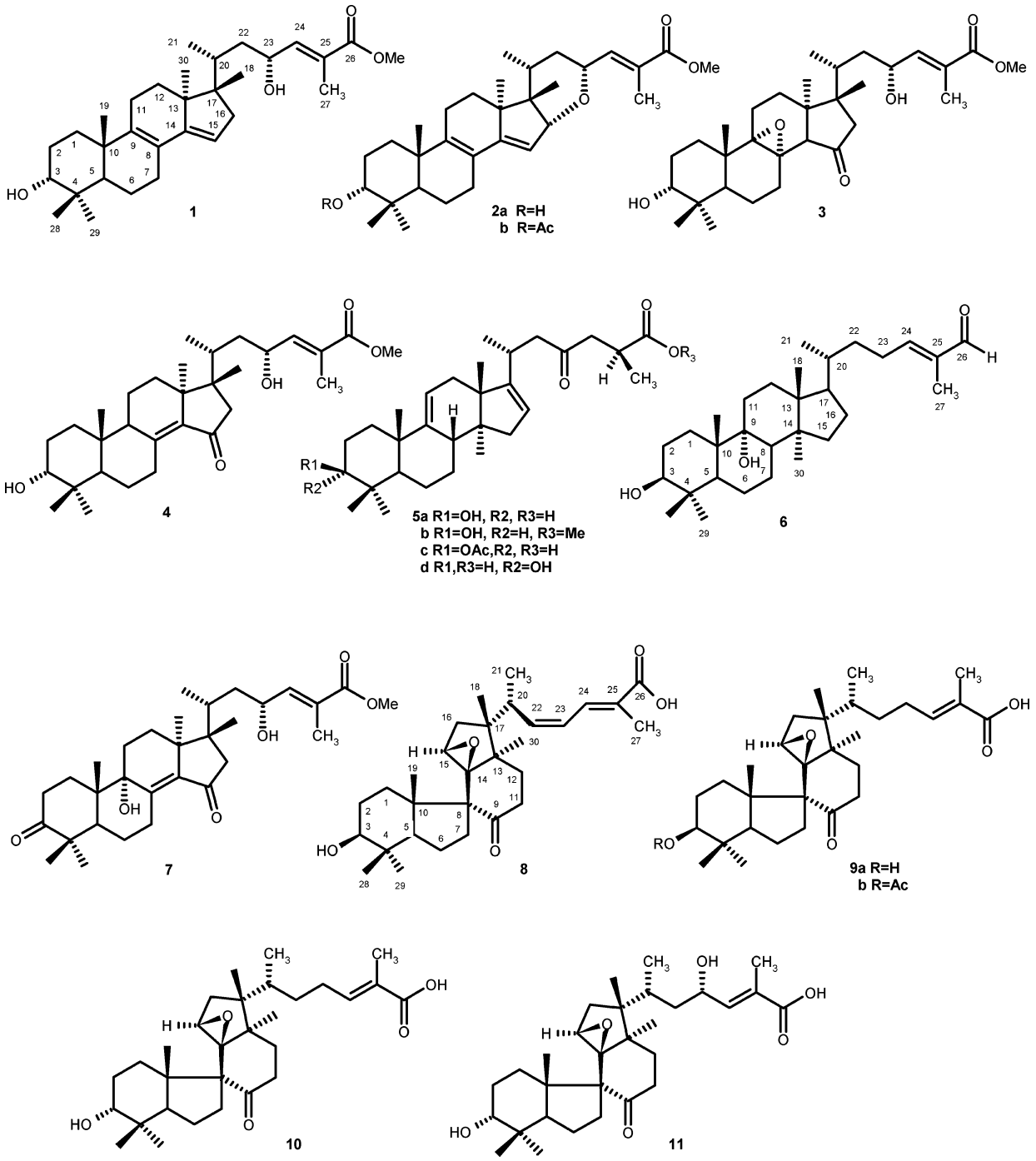
Chart 1



some of us have previously reported from *Garcinia speciosa*;[1] in other respects the ^{1}H and ^{13}C NMR spectra of **9a** and **10** duplicate those of **8** and require no further comment.

Very recently, after our work had been completed, Makino et al.[3] reported from a *Juliania* species among various tirucallanes a modified tirucallane. The presence of a 9-oxo function in this molecule suggests that it is formed by a route similar to that shown in Scheme 1.

Friedolanostanes **1**, **2a**,**b**, **4**, lanostanes **5b**−**d**, and 11-(10→8)-abeolanostanes **8**, **9a**, and **11** were screened for in vitro cytotoxicity against three human tumor cell lines: MCF-7, NCI-H460, and SF-268. The results, given in concentrations causing 50% cell growth inhibition (GI_{50}), are summarized in Table 3. All compounds except **5b** exhibited moderate growth inhibitory effects after a continuous exposure of 48 h. Compounds **1**, **2a**,**b**, **4**, **5c**,**d**, and **11** were more active than **8** and **9a** and also more effective against the MCF-7 cell line. To determine whether this effect was due to apoptosis, MCF-7 cells were exposed for 48 h to GI_{50} concentrations of **1**, **2a**,**b**, **4**, **5c**,**d**, and **11** followed by submission to the in situ terminal deoxynucleotidyl transferase (TUNEL) assay to assess DNA fragmentation at a single cell level. Examination by fluorescence microscopy of treated cells after DAPI staining revealed, when compared with untreated control cells, a great number of cells with nuclear condensation, which stained intensively green after the TUNEL assay, indicating DNA fragmentation, a characteristic of apoptotic cells. Quantification (Table 4) showed that **1**, **2b**, and **4** were more effective in inducing apoptosis of MCF-7 cells than **2a**, **5c**,**d**, and **11**.

Table 1. ^{1}H NMR Data for Compounds **7**, **8**, **9a**,**b**, and **10** ($CDCl_3$, 500 MHz)

position	7	8	9a	9b	10
1α	1.76m	1.01td(12.8,4)	1.57−1.63m	1.59−1.69m	1.46td(12.6,3.2)
1*β*	2.35td (15, 5.6)	1.77dt(12.8,3)	2.2−2.3m	2.18−2.35m	1.77dt(12.5,3.2)
2α			1.54−1.65m		1.85m
	2.52m	1.36−1.50m		2.06m	
2*β*			1.54−1.65		1.60m
3		2.87dd(10.5,6)	3.11dd(11,5)	4.37dd(10.5,6)	3.36brs
5	2.41dd(12,3)	1.54dd(12.7,7)	1.63 dd(12,7)	1.76dd(12.5,7)	1.98dd(12.7,7.3)
6α	1.61dq(13.5,3)				1.61−1.66m
		1.40−1.46m	1.54−1.65m	1.59−1.69m	
6*β*	1.37qd(14,4)				1.39−1.49m
7α	2.23td(14,5.5)	1.68ddd(13.5,10,3)	1.06−1.15m	1.08−1.15m	1.56td(13.5,2)
7*β*	4.09dt(14,3)	2.09ddd(13.5,10,6.5)	1.91m	2.09m	2.24−2.28m
11α	1.70td(15,5)	2.14td(11,7)	2.23td(11,7)	1.98m	2.29td(11.5,7)
11*β*	2.29dt(15,4)	2.84q(11)	2.95q(11)	2.99q(11)	3.05q(11)
12α	1.76m	1.11m	1.37ddd(12,11.5,7)	1.49td(12,6)	1.42ddd(12,11.5,7)
12*β*	1.95td(14,3)	2.32m	2.41m	2.46m	2.46m
15		3.53s	3.43s	3.43s	3.47s
16α	2.40d(18.5)	1.84d(14)	1.56d(14)	1.60d(14)	1.61d(14.5)
16*β*	2.12d(18.5)	1.90d(14)	1.85d(14)	1.90d(14)	1.90d(14.5)
18[a]	0.87s	1.15s	1.00s	1.05s	1.06s
19[a]	0.99s	0.94s	0.94s	0.93s	0.96s
20	2.2−2.3m	2.72dq(10,7)	1.29−1.37m	1.29−1.37m	1.40m
21[a]	0.95d(7)	0.85d(7)	0.79d(6.6)	0.84d(6.6)	0.84d(6.6)
22α	1.76m	5.86t(11)	1.03−1.21m	1.06−1.15m	1.10m
22*β*	1.09td(12.5,2)		1.03−1.21m	1.06−1.15m	1.18−1.26m
23	4.57td(9,2)	6.25t(12)	2.0m,2.15m	2.0m,2.18−2.35m	2.05m,2.21m
24	6.70dq(9,1.2)	7.31d(12)	6.73t(7.3)	6.84t(7.2)	6.85t(7.2)
27[a]	1.87d(1.2)	1.85brs	1.76brs	1.81brs	1.81brs
28[a]	1.02s	0.77s	0.82s	0.86s	0.89s
29[a]	1.13s	0.84s	0.91s	1.01s	0.93s
30[a]	1.22s	0.69s	0.75s	0.80s	0.81s
OMe[a]	3.76s				
3-OH		4.35d(5)			
26-OH		12.37br			
Ac[a]				2.02s	

[a] Intensity three protons.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at ambient temperature on a Bruker DRX instrument operating at 500 and 125 MHz, respectively. EI mass spectra were measured on a Hitachi Perkin-Elmer RMV-6 M instrument. HRMS spectra were acquired using + FAB ionization with Xe gas at GKV on a KRATOS Concept III 2 sector mass spectrometer. The accelerating voltage was 8 kV. Rotations were determined using a Polarotronic Universal Schmidt and Haensch polarimeter. Si gel for chromatography was Si gel 60 (0.2−0.5 mm) Merck, and for analytical and preparative TLC, Si gel G 60 GF 254 Merck.

Plant Material. Source of the collection and its authentication have been described earlier.[1]

Isolation of the New Constituents. Extraction and fractionation of the bark extract of *Garcinia speciosa* Wall. from Narathiwat Province, Southern Thailand, were described earlier.[1] Further elution of fractions 259−414 (21 g) with petroleum ether−$CHCl_3$ (1:4) (170 subfractions of 500 mL each) and purification of subfractions 54−58 (512 mg) by preparative TLC (Si gel, toluene−EtOAc−$CHCl_3$−HCO_2H, 70:20:10:1) gave **8** (17 mg). Purification of subfractions 68−81-(734 mg) by preparative TLC (Si gel, toluene−EtOAc−$CHCl_3$−HCO_2H, 60:30:10:1) gave **5a** (56 mg), whose properties were described earlier,[1] and **9a** (148 mg). Subfractions 89−126 were combined (1.9 g) and purified by preparative TLC (Si gel, $CHCl_3$−EtOAc−$(CH_3)_2O$−HCO_2H, 85:10:5:1) to give 160 mg of a mixture of **7** and **10**, which was further purified by TLC (Si gel, toluene−EtOAc−$CHCl_3$−HCO_2H, 50:35:15:1) to give 16 mg of **7** and 43 mg of **10**.

Methyl (24*E*)-9α,23α-dihydroxy-3,15-dioxo-17,15-friedolanostan-8(14),24-dien-26-oate (7): yellowish gum; $[\alpha]_D^{24}$ −20.9° ($CHCl_3$, *c* 0.24 g/100 mL); HRFABMS *m/z* 515.33729 [M + H$^+$] (calcd for $C_{30}H_{47}O_5$ 515.33726); ^{1}H NMR in Table 1, ^{13}C NMR in Table 2.

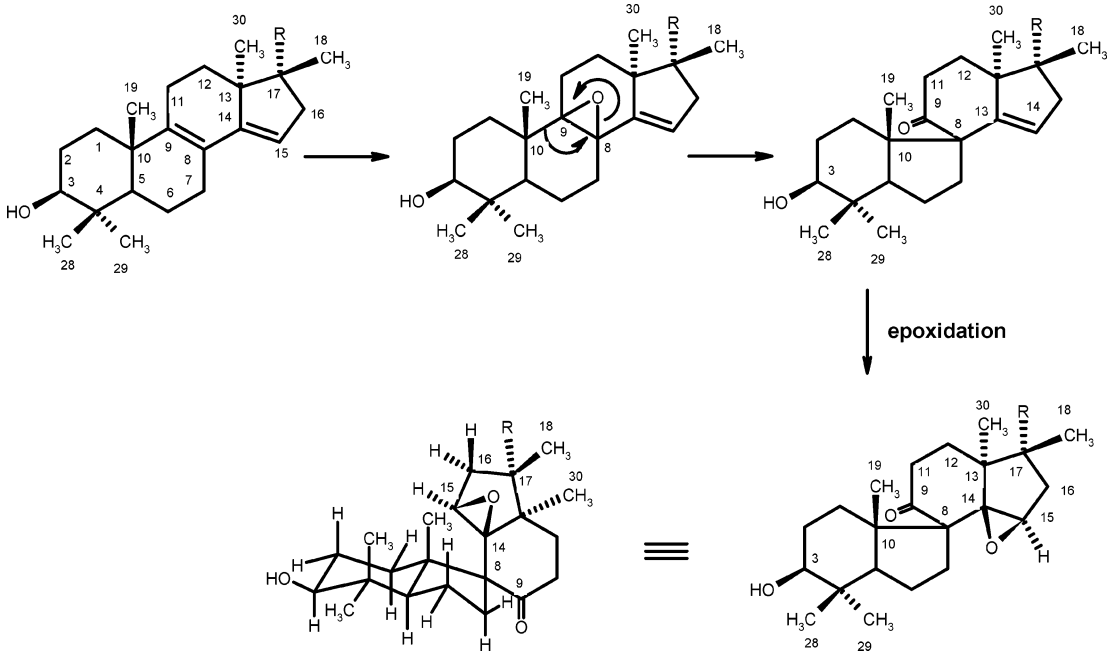
14*β*,15*β*-Epoxy-3*β*-hydroxy-9-oxo-11(10→8)-abeolanosta-22-*cis*,24-*trans*-dien-26-oic acid (8): white needles from MeOH; mp 210 °C (with decomposition); $[\alpha]_D$ 169.0° (MeOH),

Table 2. ^{13}C NMR Data of Compounds **7**, **8**, **9a**, **9b**, **10**, and **11** ($CDCl_3$, 125 MHz)

	7	8[f]	9a	9b	10	11[a]
1	31.13t	34.04t	34.60t	34.52t	29.88t	29.98t
2	34.46t	28.16t	28.06t	24.63t	26.25t	26.23t
3	217.40s	77.61d	79.20d	80.86d	74.74d	74.74d
4	47.09s	37.68s	37.95s	37.18s	37.41s	37.43s
5	45.87d	49.02d	49.21d	49.29d	42.99d	43.02d
6	23.33t	21.45t	21.62t	21.59t	21.99t	21.95t
7	23.33t	34.22t	34.49t	33.96t	33.81t	33.90t
8	150.30s	63.59s	64.14s	64.15s	64.67s	64.76s[b]
9	74.60d	218.30s	219.52s	219.00s	219.32s	220.00s[c]
10	43.97s	50.26s	50.76s	50.54s	50.78s	50.88s
11	30.89t	39.46t	40.14t	40.18t	40.15t	40.27t
12	32.55t	29.29s	30.92t	31.03t	31.00t	30.88t
13	46.02s	46.38s	46.60s	46.73s	46.71s	46.71s
14	140.64s	79.18s	79.85s	79.95s	80.07s	80.23s[d]
15	207.81s	57.72d	58.21d	58.35d	58.30d	58.25d[e]
16	52.23t	41.21t	41.90t	42.03t	42.02t	42.07t
17	44.58s	43.03s	44.42s	44.56s	44.50s	43.91s
18	16.86q	21.27q	20.66q	20.79q	20.75q	20.77q
19	18.05q	14.53q	14.82q	15.00q	14.48q	14.48q
20	33.33d	39.14d	39.49d	39.62d	39.58d	34.94d
21	15.39q	17.55q	14.77q	14.89q	14.84q	14.78q
22	39.15t	141.32d	31.19t	31.28t	31.24t	39.19t
23	66.48d	122.52d	26.95t	27.02t	27.02t	66.65d
24	144.05d	131.34d	144.14d	144.50d	144.33d	144.37d
25	127.36s	128.35s	127.14s	127.11s	127.14s	126.91s
26	168.36s	169.21s	172.65s	172.69s	172.84s	168.49s
27	12.77q	12.39q	11.87q	11.97q	11.91q	12.65q
28	21.58q	16.82q	16.40q	17.48q	22.35q	22.36q
29	27.17q	29.98q	29.52q	29.60q	28.49q	28.45q
30	21.06q	21.80q	22.83q	22.93q	22.91q	22.36q
-OMe	52.06q					51.99q
Ac				171.04q		
				21.22q		

[a] Compound assigned formula **3** of ref 1. [b] Formerly assigned to C-9 of old structure. [c] Formerly assigned to C-15 of old structure. [d] Formerly assigned to C-8 of old structure. [e] Formerly assigned to C-14 of old structure [f] In DMSO.

Scheme 1. Biogenesis of Compound **8**



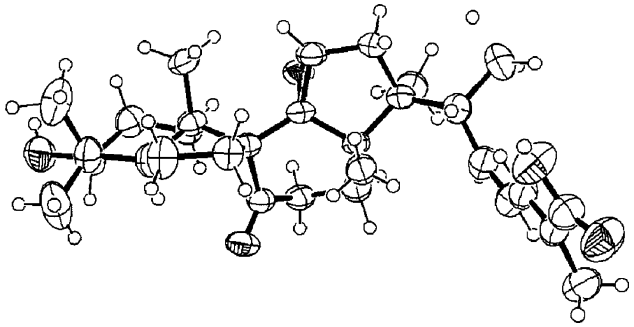


Figure 1. ORTEP view of compound **8**.

Table 3. Effect (GI_{50} in μM) of Triterpenes on Growth of Human Tumor Cell Lines[a]

compound	MCF-7	NCI-H460	SF-268
1	22.3 ± 1.6	37.9 ± 3.8	38.6 ± 1.8
2a	19.2 ± 0.5	30.4 ± 1.4	29.7 ± 3.0
2b	15.3 ± 0.4	34.4 ±1.9	28.6 ± 1.1
4	26.0 ± 2.0	42.7 ± 4.1	44.7 ± 5.2
5b	>100	>100	>100
5c	15.5 ± 0.3	22.1 ± 0.7	30.6 ± 1.7
5d	19.4 ± 1.1	36.9 ± 1.4	34.8 ± 3.9
8	69.6 ± 2.8	70.3 ± 4.1	95.7 ± 7.6
9a	63.8 ± 8.3	68.6 ± 10.7	86.4 ± 11.5
11	18.7 ± 0.5	27.8 ± 1.7	28.4 ± 1.7
doxorubicin	0.043 ± 0.008	0.094 ± 0.008	0.093 ± 0.007

[a] Results are means ± SEM of 3 independent experiments performed in duplicate.

c 0.12 g/100 mL; HRFABMS *m/z* 485.32671 [M + H$^+$] (calcd for $C_{30}H_{45}O_5$ 485.32670); ^{1}H NMR in Table 1, ^{13}C NMR in Table 2.

X-ray Crystal Structure of 8. Suitable crystals were obtained by slow evaporation of a solution in methanol and were triclinic, space group *P*1, cell volume $V = 1390.3(6)$ Å^3, cell dimensions $a = 17.712(5)$ Å, $b = 16.932(4)$ Å, $c = 7.3868(17)$ Å, $\alpha = 102.52(3)°$, $\beta = 78.09(3)°$, and $\gamma = 139.89(2)°$ (uncertainties in parentheses). There were two compound molecules per unit cell, calculated density 1.179 g/cm^3. X-ray analysis revealed that one H_2O molecule per cell was retained in the crystal structure, which displayed a network of hydrogen bonds involving H_2O and the molecules of **8**. Diffraction data were collected at 293 K with a Stoe IPDS plate equipped with Mo Kα radiation ($\lambda = 0.71073$ Å). A total of 10 981 reflections were measured, of which 9081 were independent and 6107 were observed ($I > 2\sigma(I)$). The structure was solved by direct methods using SHELXL-97[4] with atomic positions and displacement parameters refined with SHELXL-97.[5] Non-hydrogen atoms were refined anisotropically; the refinement converged to R(all data) = 8.51% and wR^2 (all data) = 15.52%. Full details of the data collection and refinement and tables of final atomic coordinates, anisotropic thermal parameters for all non-hydrogen atoms, hydrogen atomic coordinates, bond lengths and angles, and torsion angles have been deposited with the Cambridge Crystallographic Data Centre, CCDC n*o*. XXXXX.

Table 4. Quantification of Apoptosis by Compounds in MCF-7 Cell Line by TUNEL Assay[a]

compound	apoptotic cells (%)
1	17.36
2a	2.97
2b	23.54
4	26.36
5c	5.33
5d	2.34
11	2.70
doxorubicin	26.02
untreated control cells	1.23

[a] Cells were treated with GI_{50} concentration of compound for 48 h. Cells exposed to 40 nM doxorubicin for a similar period were used as positive control.

14*β*,15*β*-Epoxy-3*β*-hydroxy-9-oxo-11(10→8)-abeolanosta-24-*trans*-en-26-oic acid (9a): white needles from MeOH; mp 205−207 °C; $[\alpha]_D^{27}$ −22.3° ($CHCl_3$), *c* 2.24 g/100 mL; HRFABMS *m/z* 487.34226 [M + H$^+$] (calcd for $C_{30}H_{47}O_5$ 487.34235); ^{1}H NMR in Table 1; ^{13}C NMR in Table 2. Acetylation in the usual manner (Ac_2O−pyridine) furnished acetate **9b** as a gum whose ^{1}H and ^{13}C NMR spectra are listed in Tables 1 and 2.

14*β*,15*β*-Epoxy-3α-hydroxy-9-oxo-11(10→8)-abeolanost-24-*trans*-en-26-oic acid (10): yellowish gum; $[\alpha]_D^{27}$ −53° ($CHCl_3$, *c* 0.66 g/100 mL); HRFABMS *m/z* 487.34231 [M + H$^+$] (calcd for $C_{30}H_{47}O_5$ 487.34235); ^{1}H NMR in Table 1; ^{13}C NMR in Table 2.

Tumor Cell Growth Assay. Stock solutions in DMSO (Sigma Chemical Co.) of compounds were prepared at 400 times the desired final maximum test concentration and stored at −20 °C. The frozen samples were diluted with cell culture medium immediately prior to the assay. Final concentrations

of DMSO did not interfere with tumor cell growth. The effect of the triterpenes on the growth of MCF-7, NCI-H-460, and SF-268 was evaluated by the procedure used by the National Cancer Institute, in the in vitro anticancer drug discovery screen, which used the protein-binding dye sulforhodamine B (Sigma Chemical Co.) to assess cell growth inhibition.[6] Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium (Gibco BRL) supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL), 2 mM glutamine (Sigma Chemical Co.), and 50 mg/mL of gentamicin (Sigma Chemical Co) at 37 °C in a humidified atmosphere containing 5% CO_2. The optimal plating density of each cell line ensuring exponential growth throughout the period of the experiment was identical with that published originally, 1.5×10^5 cells/mL to MCF-7 and SF-268, 7.5×10^4 cells/mL to NCI-H-460. Cells in 96-well plates were allowed to attach overnight and then exposed for 48 h to five concentrations of compounds beginning with a maximum concentration of 100 μM. After this incubation period the adherent cells were fixed in situ, washed, and dried with SRB. The bound stain was solubilized. A dose−response curve was generated for each compound tested and for each cell line, and GI_{50} was calculated as described.[7] Doxorubin used as a positive control was tested in the same manner.

Apoptosis Assay. Fragmentation of the genomic DNA was evaluated using the in situ cell death detection kit (TUNEL) and fluorescein (Boehringer, Mannheim, Germany). MCF-7 cells were plated at a density of 1.5×10^5 cells/mL into 12-well plates, allowed to grow for 24 h at 37°, and then continuously exposed for 48 h to the GI_{50} concentration of **1a**, **2a**, **2b**, **4**, **5c**, **5d**, and **11**. Afterward adherent and nonadherent cells were collected by trypsinization and centrifuged onto glass slides at 500 rpm (Cytospin 2, Shandon) for 5 min at room temperature, fixed with 4% paraformaldehyde for 1 h at room temperature, and prepared according to the protocol recommended by the supplier. Slides were mounted in Vectashield medium for fluorescence with DAPI (Vector Laboratories, UK) and observed under a fluorescence microscope (Nikon Eclipse E400). Apoptotic cells were quantified by counting a minimum of 400 cells from at least five different random areas of the slide. Cells exposed to 40 nM of doxorubin for a 48 h period were used as positive control.

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