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Cytotoxic and Antimicrobial Constituents of the Bark of *Diospyros maritima* Collected in Two Geographical Locations in Indonesia

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Bioactivity-directed fractionation of extracts of two *Diospyros maritima* bark samples from Indonesia, one collected at sea level in a beach forest in Java and the other collected at a slight elevation away from the sea shore on the island of Lombok, yielded a diverse set of secondary metabolites. The naphthoquinone plumbagin (**1**), although found in extracts of both specimens, constituted a much larger percentage of the former sample, which also yielded a series of plumbagin dimers, maritinone (**2**), chitranone (**3**), and zeylanone (**4**). The latter sample yielded a new naphthoquinone derivative, (4*S*)-shinanolone (**5**), and a new natural product coumarin, 7,8-dimethoxy-6-hydroxycoumarin (**6**), along with three other analogues of plumbagin, 2-methoxy-7-methyljuglone (**7**), 3-methoxy-7-methyljuglone (**8**), and 7-methyljuglone (**9**). The structures of compounds **5** and **6** were elaborated by physical, spectral, and chemical methods. All of the isolates were evaluated in both cytotoxicity and antimicrobial assays, and structure−activity relationships of these naphthoquinones are proposed. Plumbagin (**1**) and maritinone (**2**) were evaluated also for in vivo antitumor activity in the hollow fiber assay, but both were found to be inactive.

Diospyros maritima Blume (Ebenaceae) is a shrub indigenous to Southeast Asia.[1] The sap of this species has been used as a vesicant in Indonesia,[2] and the stems are used traditionally in Taiwan as a treatment for rheumatic diseases.[3] Crude extracts of *D. maritima* have displayed a range of biological effects, such as ichthyotoxicity[4] and antibacterial activity.[5,6] Previous phytochemical studies on *D. maritima* have resulted in the isolation of structurally diverse secondary metabolites, including alkanol ester,[7] aliphatic lactone,[8] benzenoid,[9] coumarin,[2,9,10] kaurane diterpene,[9] naphthoquinone,[2,5,8−16] phenylpropanoid,[17] sterol,[10,15] and triterpenoid types.[3,5,7,9,10,18−20] Recently, several purified naphthoquinones from this plant were found to exhibit activity against bacteria,[5] fungi,[9] guppy fish,[1,9] human tumor cells,[15] and seed germination of *Lactuca sativa* L. var. Great Lakes[1,9] and to effect platelet aggregation.[21]

As a part of our collaborative program to search for new anticancer compounds from plants,[22] two samples of the bark from *D. maritima* were collected from different geographical locations in Indonesia. One of these was gathered in July 1996 near sea level in a beach forest on the western side of Java (A3919), while the other was gathered in October 2001 at elevation, farther from the beach, on Lombok Island (A5248). Extracts of these geographically distinct samples displayed promising anticancer activity, and thus, each was investigated individually via bioactivity-directed fractionation, using KB cells (A3919) and Lu1 cells (A5248) to monitor the chromatographic purifications.

The cytotoxic compound plumbagin (**1**) along with the inactive compounds lupeol and scopoletin were found to be common to both accessions. Several active compounds that can be considered dimers of **1**, maritinone (**2**), chitranone (**3**), and zeylanone (**4**), were isolated from the A3919 sample. Two new compounds were isolated from the A5248 sample: a naphthoquinone derivative, (4*S*)-shinanolone (**5**), and a new natural coumarin, 7,8-dimethoxy-6-hydroxycoumarin (**6**); these two compounds were not active in either the anticancer or antimicrobial assays. Further, three known active naphthoquinones, all of which are analogues of **1**, but not dimers, 2-methoxy-7-methyljuglone (**7**), 3-methoxy-7-methyljuglone (**8**), and 7-methyljuglone (**9**), were isolated from A5248, along with the inactive compounds betulin and methyl α-orcinolcarboxylate. The identification of compounds **1**−**4**, the structure elucidation data of **5** and **6**, and the biological evaluation of all the isolates performed in anticancer and antimicrobial assays are described herein.

Results and Discussion

The naphthoquinone plumbagin (**1**) was the most abundent compound isolated from the *D. maritima* bark sample A3919, accounting for more than 0.25% of the weight of the dried plant material, and this confirms earlier reports of its high concentration in this taxon.[9] Even though the ^{13}C NMR data exactly matched the reported literature values,[9] complete ^{1}H and ^{13}C NMR data are shown in Supplemental Table 1 (see Supporting Information) for two

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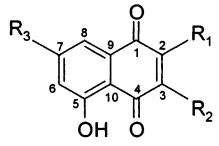
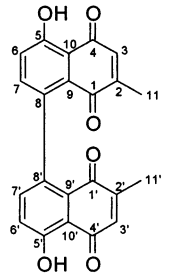
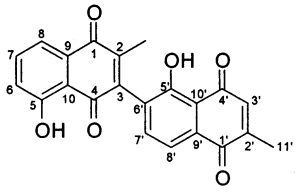


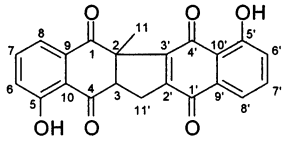
plumbagin (**1**) R_1=CH_3, R_2=R_3=H
2-methoxy-7-methyljuglone (**7**) R_1=OCH_3, R_2=H, R_3=CH_3
3-methoxy-7-methyljuglone (**8**) R_1=H, R_2=OCH_3, R_3=CH_3
7-methyljuglone (**9**) R_1=R_2=H, R_3=CH_3



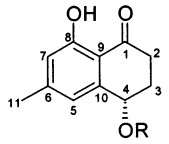
maritinone (**2**)



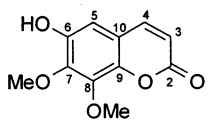
chitranone (**3**)



zeylanone (**4**)



(4*S*)-shinanolone (**5**) R=H
(**5s**) R=(S)-MTPA
(**5r**) R=(R)-MTPA



6-hydroxy-7,8-dimethoxycoumarin (**6**)

reasons. These ^{1}H NMR data are superior to the earlier literature,[10] largely due to advances in instrumentation in the past 30 years. Moreover, the ^{13}C NMR data for the structurally related plumbagin dimers, maritinone (**2**) and zeylanone (**4**), have not been published, and their identification was facilitated greatly by comparison with **1**.

A comparison of the NMR and mass spectral data between compounds **1** and **2** suggested that **2** was a dimer of **1** with C_2 symmetry (Supplemental Table 1).[10] The splitting pattern for H-6 and H-7 was simplified due to the absence of H-8, which established the dimer linkage as C-8 to C-8′.

Another plumbagin dimer, chitranone (**3**), was isolated, and its ^{13}C NMR data were reported recently.[1] However, the assignments for positions C-7 and C-7′ could be distinguished by using a combination of the HSQC data, the observed HMBC correlations from H-7′ to C-3, and the *ortho*-coupled, doublet splitting patterns of 7.5 Hz in the ^{1}H NMR data for both H-7′ and H-8′, as opposed to the more complex splitting for H-6, H-7, and H-8. Moreover, a combination of the HSQC data and the allylic splitting pattern observed for H-11′ in the ^{1}H NMR data led to the reassignment of positions C-11 and C-11′.[1] As with **1**, the ^{1}H NMR data for **3** are included to update the literature,[23] especially for positions H-6, H-7, and H-8 versus H-7′ and H-8′ (Supplemental Table 2, see Supporting Information).

Zeylanone (**4**) is a dimer of **1** where the C-11′ methyl group of one unit has been condensed at the C-3 position of a second unit. Together with a bond between positions C-2 and C-3′, this results in the formation of a five-membered ring.[24] Sankaram et al.[24] presented a structure elucidation of **4** using ^{1}H NMR data and chemical shift reagents, but, to the best of our knowledge, the ^{13}C NMR data for **4** have not been reported previously. Thus, a combination of the HSQC, HMBC, and ^{1}H NMR data was used to assign the ^{13}C NMR data (Supplemental Table 2, see Supporting Information). Each half of the molecule could be distinguished by a series of key HMBC correlations (Supplemental Figure 1, see Supporting Information). For instance, correlations from the H-11 methyl protons to the C-1 quinone, along with correlations from H-8 to C-1, the C-5-hydroxyl proton to C-6 and C-10, and cross correlations between positions H-6 and H-8 were used to assign one-half of the molecule, while similar HMBC correlations from H-8′ to the C-1′ quinone, from the C-5′-hydroxyl proton to C-6′, and cross correlations between H-6′ and H-8′ led to the assignments for the other half of the molecule. Other key observations are the HMBC correlations from both H-11′ and H-3 to C-4, whereas there were no HMBC correlations to C-4′, as would be predicted due to being located four bonds from the nearest proton. The stereochemistry at positions C-2 and C-3 was not assigned due to the paucity of compound isolated.

From sample A5248, a new compound (**5**), $[\alpha]^{25}_D$ +14° (*c* 0.1, MeOH), was obtained as a pale yellowish amorphous powder, and the molecular formula was deduced as $C_{11}H_{12}O_3$ by HREIMS (obsd *m*/*z* 192.0778 for $[M]^+$). Compound **5** exhibited a yellowish fluorescence on silica gel TLC plates under UV light at 365 nm, and it showed UV absorption maxima at 217, 267, and 329 nm, suggesting the presence of an acetophenone chromophore.[25] In the IR spectrum, absorption bands at 3526−3109 cm^{-1} (hydrogen-bonded OH) and 1641 cm^{-1} (carbonyl) were apparent.[25] The ^{1}H NMR and COSY spectra showed three isolated spin systems, inclusive of a hydrogen-bonded hydroxyl at δ_H 12.44 (1H, s, OH-8), a methyl group at δ_H 2.38 (3H, br s, CH_3-6) coupled with two aromatic protons at δ_H 6.76 (1H, br s, H-7) and 6.87 (1H, br s, H-5), and a methine at δ_H 4.89 (1H, dd, J = 7.4 and 3.8 Hz, H-4) coupled with a methylene at δ_H 2.20 and 2.36 (each 1H, m, H_2-3), which were further coupled with another methylene unit at δ_H 2.67 and 2.97 (each 1H, m, H_2-2). We were not able to assign the respective α and β orientations of the methylene signals H_2-2 and H_2-3; neither of the J values at H-4 were large enough to indicate a β relationship to either of the H_2-3 protons, the NOE difference spectrum of H-4 showed correlations to both H-3a and H-3b, and the results of chemical shift difference experiments in $CDCl_3$ and C_5D_5N were inconclusive. One hydroxyl group was placed at C-4

Table 1. ^{1}H, ^{13}C, DEPT-135, and HMBC NMR Data for Compounds **5**, **5s**, and **5r**

	(4*S*)-shinanolone (**5**)					**5s**	**5r**	
position	δ_C ($CDCl_3$)	DEPT	δ_H ($CDCl_3$)[a]	HMBC (H→C)	δ_H (C_5D_5N)[a]	δ_H (C_5D_5N)[a]	δ_H (C_5D_5N)[a]	$\Delta\delta_{H((S-R)}$
1	203.9	C						
2a	34.8	CH_2	2.97, ddd (18, 8.1, 4.8)	68.1, 203.9	2.98, ddd (18, 6.6, 4.6)	2.59, m	2.69, m	−0.10
2b			2.67, ddd (18, 8.3, 4.8)	68.1, 203.9	2.63, ddd (18, 9.7, 4.9)	2.71, m	2.84, m	−0.13
3a	31.7	CH_2	2.36, m		2.30, m	2.28, m	2.35, m	−0.07
3b			2.20, m	68.1, 145.9, 203.9	2.22, m	2.28, m	2.28, m	0
4	68.1	CH	4.89, dd (7.4, 3.8)		5.02, dd (8.4, 3.8)	6.41, dd (4.3, 4.0)	6.42, dd (6.7, 3.8)	*S*[b]
5	118.9	CH	6.87, s	22.6, 68.1, 113.8, 118.1	7.17, s	6.86, s	6.57, s	+0.29
6	149.1	C						
7	118.1	CH	6.76, s	22.6, 113.8, 118.9	6.81, s	6.86, s	6.79, s	+0.07
8	163.2	C						
9	113.8	C						
10	145.9	C						
CH_3-6	22.6	CH_3	2.38, s	118.1, 118.9, 149.1	2.18, s	2.15, s	2.09, s	+0.06
OH-8			12.44, s	113.8, 118.1, 163.2	12.98, s			

[a] δ_H, multiplicity (*J* in Hz). [b] Absolute configuration.

as a result of correlations between H-5 (δ_H 6.87) and C-4 (δ_C 68.1) in the HMBC spectrum (Table 1) and the spatial correlation between H-4 and H-5 observed in the NOE difference spectra. The absolute configuration was determined by a convenient Mosher ester procedure carried out in NMR tubes,[26,27] in which **5** was treated with (*R*)-(−)- and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride to obtain the (*S*)- (**5s**) and (*R*)-MTPA (**5r**) esters, respectively. Analysis of the $\Delta\delta_{H(S-R)}$ data between these compounds (Table 1) showed a positive difference in chemical shift for the protons at C-5 and CH_3-6 and a negative effect for the protons at C-2 and C-3, and this indicated that the absolute configuration at C-4 was *S*. Accordingly, the structure of **5** was assigned as 4*S*,8-dihydroxy-6-methyl-1-tetralone, to which the trivial name (4*S*)-shinanolone has been accorded. The structure of shinanolone, with $[\alpha]^{20}_D$ −22.8°, was reported by Kuroyanagi et al. in 1971,[25] and its absolute configuration at C-4 was proposed as *R* through its dibenzoate by utilizing an aromatic chirality method.

Compound **6**, mp 145−146 °C, was obtained as pale yellowish needles, and the molecular formula was determined as $C_{11}H_{10}O_5$ by HREIMS (obsd *m*/*z* 222.0535). This compound exhibited UV absorption maxima at 209, 330, and 383 nm, suggesting the presence of considerable conjugation in the molecule. In the IR spectrum, absorption bands at 1690 (α,*β*-unsaturated ester), 1641 and 1546 cm^{-1} (aromatic ring) were apparent. The ^{1}H NMR spectrum showed four isolated spin systems, inclusive of two methoxy groups at δ_H 3.94 and 4.00 (each 3H, s), one pair of coupled vinylic protons at δ_H 6.24 (1H, d, J = 9.6 Hz, H-3) and 7.93 (1H, dd, J = 9.6 and 0.4 Hz, H-4), and one aromatic proton at δ_H 6.71 (1H, d, J = 0.4 Hz, H-5). A coumarin skeleton was determined for **6** on the basis of the characteristic AB spin system for H-3 and H-4 in the ^{1}H NMR spectrum and seven degrees of unsaturation. The two methoxy groups in **6** were placed at C-7 and C-8 due to their appearance at ca. δ_C 61, typical for methoxy moieties neighboring two *O*-substituted groups in an aromatic ring, instead of at ca. δ_C 56, as found for an isolated methoxy or a methoxy group neighboring only one *O*-substituted functionality.[28] Furthermore, the above assignments were supported by the spatial correlations between H-4 and H-3 and H-5, along with OCH_3-7 and OCH_3-8 observed in NMR NOE difference experiments, and 6,7,8-trimethoxycoumarin was generated after compound **6** was treated directly with diazomethane in diethyl ether solution.[28] Accordingly, compound **6**, a new natural product, was assigned as 7,8-dimethoxy-6-hydroxycoumarin. This compound was reported previously to be a synthetic substance, but the structure was elucidated only by ^{1}H NMR data,[29] which are somewhat inconsistent with the values reported herein.

Table 2. Cytotoxic Activity of Constituents from the Bark of *D. maritima*[a−d]

compound	KB	Lu1	LNCaP	HUVEC
1	0.3	0.3	0.4	0.4
2	0.3	1.4	2.2	2.2
3	0.3	1.1	2.2	2.1
4	2.1	14.5	3.3	3.3
7	1.9	0.2	3.4	2.0
8	2.4	1.8	4.1	5.5
9	4.1	13.2	3.7	5.7

[a] Results are expressed as EC_{50} values (μg/mL). [b] Key to cell lines used: KB = human oral epidermoid carcinoma; Lu1 = human lung cancer; LNCaP = hormone-dependent human prostate cancer; HUVEC = human umbilical vein endothelial cells. [c] Compounds **5** and **6**, betulin, lupeol, methyl α-orcinolcarboxylate, and scopoletin were regarded as inactive (ED_{50} values >5 μg/mL in all cell lines). [d] Typical average EC_{50} value for camptothecin against KB cells is 0.01 μg/mL.

Eight other compounds of known structure were isolated from *D. maritima* sample A5248, as described in the Experimental Section. A much smaller quantity of **1** was obtained relative to sample A3919. Also, whereas analogues of **1** that represented dimers of the parent compound were found in the A3919 specimen, A5248 yielded monomeric analogues that were methylated in an alternate manner, such as 2-methoxy-7-methyljuglone (**7**),[30] 3-methoxy-7-methyljuglone (**8**),[30] and 7-methyljuglone (**9**).[9] Lupeol and scopoletin were common to both samples, and betulin and methyl α-orcinolcarboxylate[31] were identified only in A5248 by comparison of their physical and spectral data with reported values. Among these isolates, **7**, **8**, and methyl α-orcinolcarboxylate were obtained from this species for the first time.

All of the isolates were examined with a panel of cytotoxicity and antimicrobial assays (Tables 2 and 3). With respect to the former, **1** was potently cytotoxic against all four of the cancer cell lines tested, and a preliminary structure−activity relationship (SAR) may be inferred. On the basis of data obtained with KB cells, for example, compounds **1**−**3**, which all have an allylic methyl group at the 2 (and 2′) position(s), were more cytotoxic by approximately an order of magnitude than those naphthoquinones lacking such a moiety (**4** and **7**−**9**). Against the

Table 3. Antimicrobial Activity of Constituents from the Bark of *D. maritima*[a−c]

	antibacterial activity (μg/mL)								antifungal activity (μg/mL)							
	Gram-positive				mycobacteria				yeast						filamentous	
	M. luteus		*S. aureus*		*M. smegmatis*		*M. avium*		*C. albicans*		*C. neoformans*		*S. cerevisiae*		*A. niger*	
compd	C	I	C	I	C	I	C	I	C	I	C	I	C	I	C	I
1	600	80	600	100	>1000	300	>1000	600	>1000	9	40	20	40	10	400	20
2	>1000	200	300	9	80	20	>1000	600	40	40	200	20	80	9	>1000	40
3	>1000	10	>1000	90	20	6	90	5	>1000	10	10	3	10	3	>1000	6
4	>800	50	>800	6	400	50	>800	200	3	3	100	10	500	10	>800	30
7	800	50	400	200	800	50	400	50	800	100	200	50	200	50	>1000	100
8	>1000	700	>1000	>1000	>1000	>1000	>1000	700	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
9	1000	20	>1000	200	600	40	300	5	20	0.3	1	0.3	1	0.3	300	5
nitidine[b]	5000	200	5000	300	>5000	1000	>5000	3000	200	20	300	80	3000	300	>5000	3000

[a] C = cidal = 99.9% inhibition of growth; I = inhibitory = 50% inhibition of growth. [b] Used as a positive control against some yeast species. [c] Compounds **5** and **6**, lupeol, methyl α-orcinolcarboxylate, and scopoletin were regarded as inactive (values >1000 μg/mL against all organisms).

other three cell lines, **1** displayed consistently strong cytotoxicity, although the proposed SAR did not necessarily hold true. The new compound, **5**, a naphthoquinone analogue in which one of the quinone carbonyls was reduced to an alcohol, was completely inactive, suggesting that an intact quinone may be necessary for potency. Recent studies have reported the chemopreventive effects of **1**.[32] Thus, the compound was evaluated for chemotherapeutic efficacy via an in vivo antitumor hollow fiber assay.[33,34] No significant growth inhibition of tumor cells was observed at either the intraperitoneal or subcutaneous sites, with KB, LNCaP, or Lu1 cancer cell lines, when the mice were treated with doses of **1** that ranged from 1.25 to 10 mg/kg. Doses of 7.5 and 10 mg/kg were lethal to one and two out of six mice, respectively, and a dose of 5 mg/kg caused significant loss of body weight. In a similar fashion, **2** was tested in the hollow fiber assay and also found to be inactive. Interestingly, the mice could tolerate doses of **2** as high as 25 mg/kg, although death was observed after two injections when the concentration of **2** was increased to 50 mg/kg.

Compound **1** is well known to have strong antimicrobial activity.[9,35] Mahoney et al.[36] found that **1** inhibited the germination of spores of *Aspergillus flavus* more effectively than juglone, an analogous naphthoquinone that lacks an allylic methyl group at the 2 position. Against a related fungus, *A. niger*, a clear SAR was not observed among the series of structurally related naphthoquinones isolated in this study, although **1** was one of the most potent compounds tested against this fungus (Table 3). Against the three yeast species, **9** was the most potent, while in the antibacterial assays, **3** was the most potent. In general, the isolated compounds displayed broad antimicrobial activity. Of note was the strong antimycobacterial activity observed against both slowly growing (*Mycobacterium avium*) and rapidly growing (*M. smegmatis*) species. Further, strong antimicrobial activity was detected against pathogenic yeasts, *Candida albicans* and *Cryptococcus neoformans*, and against the filamentous fungus *A. niger*.

Experimental Section

General Experimental Procedures. For sample A3919 (see below), melting points were determined on a Bristoline hot-stage apparatus and are uncorrected. UV spectra were measured on a Varian ProStar 330 photodiode array detector. All NMR experiments were performed in $CDCl_3$ with TMS as an internal standard; gs-COSY, ROESY, gs-HSQC, gs-HMBC, and ^{1}H NMR spectra were run on a Bruker AMX-500 instrument using a Bruker 5 mm broad-band inverse probe with z-gradient or a Nalorac 3 mm microinverse broad-band probe with z-gradient, while a Bruker DPX-300 instrument was utilized for the DEPT-135 and ^{13}C NMR spectra using a Bruker 5 mm QNP probe. Low-resolution ESIMS were determined on a Finnigan LCQ instrument with an electrospray interface. Column chromatography was carried out on silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany), and fractions were monitored via TLC (silica gel 60 F254 plates, 0.25 mm thickness) visualized with vanillin−sulfuric acid in EtOH. A Varian ProStar HPLC system was utilized for preparative-scale HPLC.

For sample A5248 (see below), melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations, UV spectra, CD spectra, and IR spectra were measured with a Perkin-Elmer 241 polarimeter, a Beckman DU-7 spectrometer, a JASCO-710 spectropolarimeter, and an ATI Mattson FT-IR spectrometer, respectively. NMR spectra were recorded on a Bruker DPX-360 NMR spectrometer. HREIMS and LREIMS were recorded on a Finnigan MAT 95 (70 eV) and JEOL GC-mate II mass spectrometer. Compounds were visualized by dipping TLC plates into a phosphomolybdic acid reagent (Aldrich, Milwaukee, WI) followed by charring at 110 °C for 5−10 min.

Plant Material. Two collections of the bark of *D. maritima* were obtained in Indonesia, and voucher specimens have been deposited at the Field Museum of Natural History, Chicago, IL (accession no. A3919 and A5248). Sample A3919 was collected at 5−10 m above sea level in July 1996 at Sukabumi, West Java, while A5248 was obtained at ∼350 m above sea level in October 2001 at West Lombok County, Lombok Island.

Extraction and Isolation. For sample A3919, the dried bark (892 g) was percolated with hot MeOH overnight (2 × 3 L). This concentrated extract was partitioned between 10% (aqueous) MeOH and hexane, and then the aqueous MeOH fraction was partitioned further between $CHCl_3$−MeOH (4:1) and H_2O. The organic layer was washed with 1% saline to remove tannins.[37] This organic fraction (51.2 g) was separated further on a flash silica gel column using a gradient of 100% hexane to 100% $CHCl_3$ to 20% MeOH. The fractions were combined into 13 pools based on TLC properties and examined against the KB assay. Pools 2−4 were highly cytotoxic (0% survival tested at 2 μg/mL), and no further purification of pool 2 was required to isolate compound **1** (2.27 g, yield 0.25%, 95% pure by HPLC). A large percentage of pools 3 and 4 was determined to be **1** as well. A small quantity of **1** was found also in pools 5 and 6, contributing to their bioactivity, and the inactive compound lupeol (41.5 mg, yield 0.047%) was isolated from these pools using HPLC with an isocratic solvent system of 100% MeOH on an 8 μm Inertsil ODS-3 column (10 mm i.d. × 50 mm guard and 20 mm i.d. × 250 mm column; Metachem Technologies, Torrance, CA). Pool 7, having strong cytotoxicity (∼480 mg), was separated on a second flash silica gel column using a gradient of 100% hexane to 100% EtOAc to 20% MeOH. Fractions were combined into 12 pools (7-1 through 7-12) based on TLC properties and tested versus the KB assay. Pools 7-4 through 7-7 (∼150 mg) were combined and purified via RP-HPLC using a gradient of 80:20 MeOH−H_2O up to

100% MeOH over 50 min using the aforementioned Metachem column to isolate **3** (23.3 mg isolated here along with 23.5 mg isolated from pools 8-2-5 and 8-2-6, mentioned below, total yield 0.0052%). Another fraction from this HPLC experiment (~26 mg) was purified further using a step MeOH−$CHCl_3$ gradient that started at 0:100 up to 10:90 over 30 min, remained isocratic for 30 min, then increased to 20:80 over 10 min and remained isocratic thereafter, using a 5 μm YMC-Pack DIOL-120 NP column (20 mm i.d. × 250 mm column; YMC Inc., Milford, MA) to give compounds **4** (3.4 mg, yield 0.00038%) and **2** (16.4 mg isolated here along with 38.5 mg isolated from pools 8-2-5 and 8-2-6, mentioned below, total yield 0.0062%). Pool 8 from the initial column (~15 g) was separated on a second flash silica gel column using a gradient of 100% hexane to 100% EtOAc to 60% MeOH to give 10 pools (8-1 through 8-10). Scopoletin (1.2 g, yield 0.13%) was isolated by filtering a MeOH suspension of pool 8-7 (~3.7 g) through a fritted funnel. Bioactive pool 8-2 (~1.3 g) was separated on a flash diol column using a gradient of 100% hexane to 100% $CHCl_3$ to 100% MeOH to yield 13 pools (8-2-1 through 8-2-13). Pool 8-2-5 (~50 mg) was purified using a gradient of 70:30 MeOH−H_2O up to 100% MeOH over 90 min on a 5 μm YMC ODS-A column (20 mm i.d. × 50 mm guard and 20 mm i.d. × 250 mm column) to give compounds **2** and **3**; the same purification system was used on pool 8-2-6 (~50 mg) to give **2** and **3** also.

For A5248, the plant material (500 g) was extracted by maceration with MeOH (1 L × 3). The resultant extract was diluted with H_2O to afford an aqueous MeOH solution (90%) and then partitioned with *n*-hexane (0.8 L × 3). The lower layer was concentrated and partitioned between 5% MeOH in H_2O (1 L) and $CHCl_3$ (1 L × 3). The $CHCl_3$-soluble extract (10 g, ED_{50} 4.4 μg/mL against the Lu1 cell line) was subjected to silica gel column chromatography by elution with increasing concentrations of MeOH in $CHCl_3$ to give six pooled fractions. Fractions 1, 2, and 3 were active when tested against the Lu1 cell line (ED_{50} 0.19, 3.2, and 5.9 μg/mL, respectively). 7-Methyljuglone (**9**, 6 mg) and **1** (4 mg) were purified from a portion (12 mg) of fraction 1 (400 mg, $CHCl_3$−MeOH, 50:1) using preparative TLC developed twice by *n*-hexane−EtOAc (20:1). Further purification of fraction 2 (1.5 g, $CHCl_3$−MeOH, 45:1) over silica gel by elution with gradient mixtures of *n*-hexane−EtOAc (stepwise, 40:1 to 8:1) gave three subfractions (i−iii). Methyl α-orcinolcarboxylate (5 mg) was obtained from subfraction i (27 mg) using preparative TLC developed twice by *n*-hexane−$CHCl_3$−MeOH (6:1:0.4). Similarly, 2-methoxy-7-methyljuglone (**7**, 10 mg), 3-methoxy-7-methyljuglone (**8**, 10 mg), and lupeol (25 mg) were purified from subfraction ii (65 mg) by preparative TLC developed twice by *n*-hexane−$CHCl_3$−EtOAc (3:1:0.4). In turn, fraction 3 (5.2 g, $CHCl_3$−MeOH, 40:1) was further fractionated (SiO_2, stepwise, *n*-hexane−acetone, 9:1 to 1:1) to afford betulin (2.1 g), scopoletin (1.5 g), and a subfraction iv (240 mg, *n*-hexane−acetone, 6:1), which was purified by preparative TLC developed twice by *n*-hexane−acetone (3:2) to afford compounds **5** (15 mg) and **6** (36 mg).

Plumbagin (1): orange needles; mp 75−76 °C (lit.[9] 75−76 °C); UV spectrum consistent with literature values.[38] ^{1}H, ^{13}C, and DEPT-135 NMR data are given in Supplemental Table 1 (see Supporting Information) and are consistent with literature values.[9,10]

Maritinone (2): orange-red amorphous powder; mp 197−198 °C (lit.[9] 199−200 °C); UV spectrum consistent with literature values.[10] ^{1}H, ^{13}C, and DEPT-135 NMR data are given in Supplemental Table 1 (see Supporting Information).

Chitranone (3): orange amorphous powder; mp 112−115 °C (lit.[39] 116−118 °C); UV spectrum consistent with literature values.[23] ^{1}H, ^{13}C, and DEPT-135 NMR data are given in Supplemental Table 2 (see Supporting Information).

Zeylanone (4): orange amorphous powder; mp 208 °C (lit.[40] 208 °C); UV spectrum consistent with literature values.[24] ^{1}H, ^{13}C, and DEPT-135 NMR data are given in Supplemental Table 2 (see Supporting Information).

4*S*,8-Dihydroxy-6-methyl-1-tetralone [(4*S*)-shinanolone] (5): pale yellowish powder; $[\alpha]^{25}_{589}$ +14°, $[\alpha]^{25}_{578}$ +17°, $[\alpha]^{25}_{546}$ +21°, $[\alpha]^{25}_{436}$ +34°, $[\alpha]^{25}_{365}$ +29° (*c* 0.1, MeOH); CD nm (MeOH) $\Delta\epsilon_{216}$ +7.87, $\Delta\epsilon_{234}$ −1.55, $\Delta\epsilon_{271}$ −3.03, $\Delta\epsilon_{325}$ +0.44; UV (MeOH) λ_{max} (log ϵ) 217 (4.19), 267 (3.95), 329 (3.51) nm; IR (dried film) ν_{max} 3526−3109, 1641, 1362, 1048 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 192 $[M]^+$ (71), 164 (20), 149 (14), 135 (100), 107 (19); HREIMS *m*/*z* 192.0778 $[M]^+$ (calcd for $C_{11}H_{12}O_3$, 192.0790).

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of 5. Compound **5** (1.5 mg) and 4-(dimethylamino)-pyridine (0.2 mg) were transferred into a clean NMR tube, and this mixture was dried under vacuum. Deuterated pyridine (0.5 mL) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (6 μL) were added into the NMR tube immediately under a N_2 gas stream, then the NMR tube was sealed and shaken to mix the sample and MTPA chloride evenly. The reaction NMR tube was permitted to stand at room temperature and monitored every 20 min by ^{1}H NMR. (*R*)-MTPA monoester derivative **5r** was afforded completely after 3 h, and then the reaction was stopped by shaking the unsealed NMR tube under air. Treatment of **5** (1.5 mg) with (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, as described above, yielded the (*S*)-MTPA ester derivative **5s**. ^{1}H NMR data for **5s** and **5r** are presented in Table 1 (data were obtained from the reaction NMR tube directly and were assigned on the basis of the correlations of the ^{1}H−^{1}H COSY spectrum).[26]

7,8-Dimethoxy-6-hydroxycoumarin (6): pale yellowish needles; mp 145−146 °C; UV (MeOH) λ_{max} (log ϵ) 209 (4.50), 330 (4.04), 383 (3.45) nm; IR (dried film) ν_{max} 3348, 1690, 1641, 1546 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.94 (3H, s, OCH_3-7), 4.00 (3H, s, OCH_3-8), 6.24 (1H, d, J = 9.6 Hz, H-3), 6.71 (1H, d, J = 0.4 Hz, H-5), 7.93 (1H, dd, J = 0.4, 9.6 Hz, H-4); ^{13}C NMR ($CDCl_3$) δ 61.6 (q, OCH_3-7), 61.9 (q, OCH_3-8), 99.2 (d, C-5), 107.5 (s, C-10), 112.6 (d, C-3), 136.8 (s, C-7), 139.2 (d, C-4), 148.9 (s, C-8), 151.8 (s, C-9), 154.0 (s, C-6), 162.0 (s, C-2); EIMS *m*/*z* 222 $[M^+]$ (100), 207 (99), 179 (41), 151 (19), 136 (20), 69 (59); HREIMS *m*/*z* 222.0535 $[M]^+$ (calcd for $C_{11}H_{10}O_5$, 222.0514).

Preparation of 6,7,8-Trimethoxycoumarin. 6,7,8-Trimethoxycoumarin (5 mg) was generated in 100% yield by treating **6** directly for 10 min with diazomethane in diethyl ether solution, which was prepared using Diazald KIT diazomethane generator (Aldrich-Sigma). Pale yellow powder; mp 100−102 °C (lit.[28] 102−104 °C). The UV, ^{1}H NMR, ^{13}C NMR, and EIMS data are consistent with literature values.[28,41]

2-Methoxy-7-methyljuglone (7): brown amorphous powder; mp 171−173 °C (dec); UV, ^{1}H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[30]

3-Methoxy-7-methyljuglone (8): orange needles; mp 209−211 (lit.[42] 209−210 °C); UV, ^{1}H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[30]

7-Methyljuglone (9): brown amorphous powder; mp 114−116 °C (lit.[43,44] 116 °C); UV, ^{1}H NMR, ^{13}C NMR, and EIMS data, consistent with literature values.[9,44]

Cytotoxic Potential. All of the compounds were evaluated for cytotoxicity against the KB (human oral epidermoid carcinoma), Lu1 (human lung cancer), LNCaP (hormone-dependent human prostate cancer), and HUVEC (human umbilical vein endothelieal cells) cell lines using established procedures.[45,46]

In Vivo Hollow Fiber Assay. Compounds **1** and **2** were tested against this assay according to established protocols with some modification.[33,34] The compounds were regarded as inactive if <50% cell growth inhibition was observed when compared with PBS control.

Measurement of Antimicrobial Activities. Antimicrobial activity of pure compounds was measured by a zone-of-inhibition assay[47] against strains of *Micrococcus luteus*, *Staphylococcus aureus*, *Mycobacterium avium*, *M. smegmatis*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus niger*. The minimal inhibitory concentration (MIC) was defined as the lowest concentration resulting in inhibition of growth; the term "cidal" was used for 99.9% inhibition, which was evidenced by clearing, and the term "inhibitory" was used for 50% inhibition, which was evidenced by a hazy spot.

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Supporting Information Available: Tables of NMR data for compounds **1**−**4** and a figure that graphically represents the key HMBC correlations for compound **4** are available free of charge via the Internet at http://pubs.acs.org.

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