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Spirocyclic Lignans from *Guaiacum* (Zygophyllaceae) Induce Apoptosis in Human Breast Cancer Cell Lines

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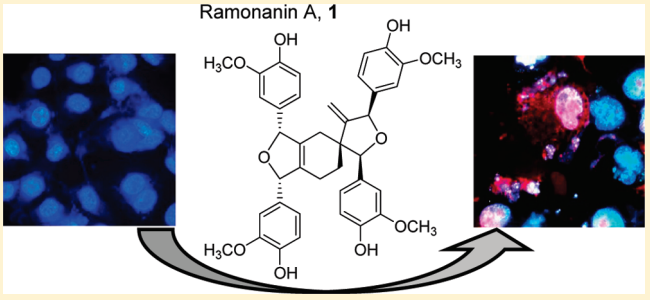
Supporting Information

ABSTRACT: We investigated the composition of extracts derived from *Guaiacum* spp. (Zygophyllaceae), a group of neotropical tree species with varied uses in Central and South American traditional medicine. Activity-guided fractionation of *Guaiacum* heartwood extracts led to the identification of four new spirocyclic lignans, named ramonanins A−D (**1**−**4**). The ramonanins exhibit cytotoxic activity against human breast cancer cell lines with an IC_{50} value of 18 μM and induce cell death via apoptotic mechanisms. The ramonanins are derived from four units of coniferyl alcohol and feature an unusual spirocyclic ring system.



Several plant-derived cancer drugs were discovered by examining the use of plants in traditional medicine,[1,2] for example, etoposide and teniposide, which are derived from the lignan podophyllotoxin, isolated from *Podophyllum* spp. (Berberidaceae). *Podophyllum* was used by several American and Asian cultures for the treatment of skin cancers and warts.[3,4] Here we report results from our chemical analysis of two neotropical tree species of the genus *Guaiacum*, which have a long history of use by indigenous societies of Central and South America to treat inflammatory diseases and cancer.[5,6] In addition, *Guaiacum* extracts and "gum" have been employed extensively as antioxidant food additives.[7] Correspondingly, the chemistry of *Guaiacum* species has been subjected to numerous studies.[8,9]

We tested methanol and chloroform extracts from *G. officinale* and *G. sanctum* heartwood for cytotoxicity in a series of human cancer cell lines including lung, colon, cervical, and breast cancer cell lines. Alamar blue assays indicated that *G. sanctum* chloroform extracts affected cell viability of breast cell cancer lines at concentrations as low as 16 μg/mL, whereas lung, colon, and cervical cell lines were affected only at much higher concentrations of 125−250 μg/mL. Methanol extracts were generally less active. Cytotoxicity of the *G. sanctum* chloroform extracts was further characterized using sulforhodamine B (SRB) assays, which indicated strong antiproliferative activity with an IC_{50} of 20 μg/mL for human breast cancer cell line MB-MDA 231. Analysis of MB-MDA 231 cells treated with *G. sanctum* extract at 20 μg/mL by fluorescence microscopy after staining with Hoechst 33342 and propidium iodide revealed 70% apoptotic cell death after 12 h of exposure.

Chromatographic fractionation of *G. officinale* and *G. sanctum* heartwood extracts produced several active subfractions, suggesting that not a single compound, but several different compounds were responsible for the observed activity. *G. officinale* and *G. sanctum* heartwood extracts showed very similar HPLC-MS chromatograms and activity profiles. As *G. officinale* wood was much more readily available, *G. officinale* extracts were used for further study. The most active *G. officinale* fractions were selected for additional fractionation via HPLC, which led to isolation of two active compounds, named ramonanin A and ramonanin B. Ramonanins exhibit modest cytotoxic activity against human breast cancer cell line MD-MBA 231 with an IC_{50} value of 18 μM. Because crude *Guaiacum* extracts appeared to induce apoptosis, we examined MD-MBA 231 cells treated with the more abundant ramonanin A for morphological features consistent with apoptotic cell death.[10,11] Ramonanin A-treated MD-MBA 231 cells showed an increase in segregated and condensed chromatin, condensation of the cytoplasm and nucleus, and loss of membrane symmetry, characteristic features of apoptotic cell death,[12,13] which appeared in a time- and dose-dependent manner. Control cells retained an intact plasma membrane with normal nuclear morphology (Figure S1).

We further investigated whether the ramonanins affect cell cycle progression by using fractional DNA content analysis. MD-MBA-231 cells were treated with ramonanin A or camptothecin, and cell cycle distribution was monitored via flow cytometry using fluorescence-activated cell sorting. We found that ramonanin A caused concentration-dependent decreases in S-phase events from 13% for untreated control cells to less than 4% for cells treated with 30 μg/mL ramonanin A or B (Figure 1).

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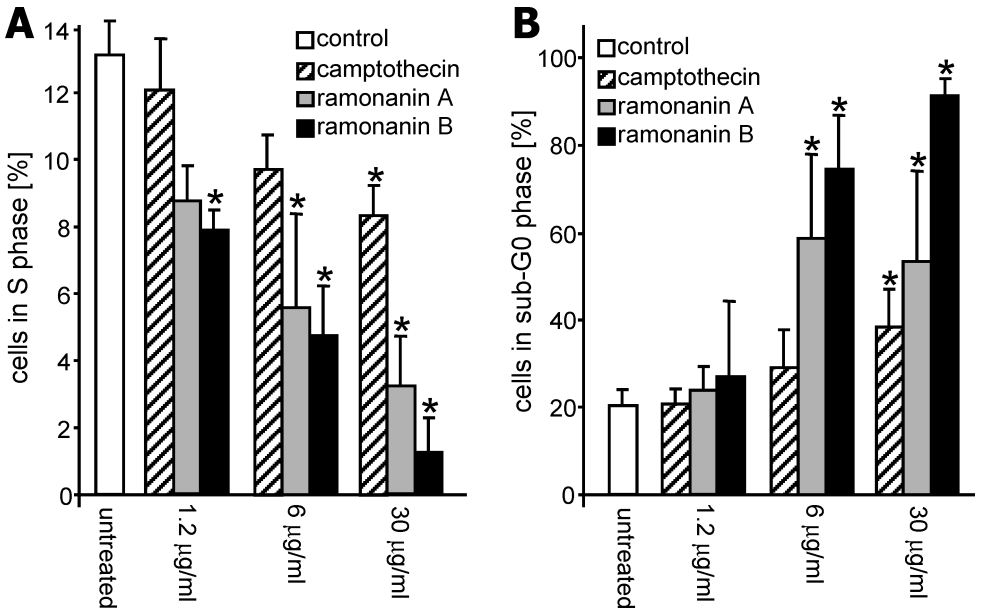

Figure 1. Effect of ramonanins A (**1**) and B (**2**) on cell cycle distribution compared to camptothecin. Human breast cancer cell line MD-MBA 231 was treated at the indicated concentrations for 24 h and analyzed via fluorescence-activated cell sorting (FACS). (A) Percentage of cells in S phase. (B) Percentage of cells in sub-G0 phase.

Furthermore, the decrease of S-phase populations was accompanied by an increase in the percentage of cells in sub-G0 phase. Therefore, it appears that the ramonanins strongly disrupt cell cycle progression at the G1/S phase transition. Furthermore, the results indicate that the ramonanins activate apoptotic cell death at a rate similar to that induced by camptothecin.

For structure elucidation, samples of ramonanins A (**1**) and B (**2**) were analyzed using NMR spectroscopy and mass spectrometry. Analysis of dqfCOSY, HSQC, and HMBC spectra revealed the presence of four 4-hydroxy-3-methoxyphenyl moieties in both ramonanins A and B, which along with a molecular formula determined as $C_{40}H_{40}O_{10}$ suggested tetrameric structures derived from four phenylpropanoid units (Table 1 and Table S1). Analysis of the HMBC spectra confirmed this hypothesis and revealed a furano-2-oxaspiro[4,5]decane core bearing the 4-hydroxy-3-methoxyphenyl substituents in positions 1 and 3 of the spirocycle and 1′ and 3′ of the annelated furan. Important HMBC correlations are shown in Figure 2A. Placement of the four methines in positions 1, 3, 8, and 10 follows from HMBC cross-peaks of the methine protons with carbons of the four aromatic rings. HMBC correlations of the methylene protons and of H-8 and H-10 determined placement of the two double-bond carbons forming the 7−11 double bond. Lastly, HMBC correlations of the methine protons H-1 and H-3 in conjunction with HMBC signals of the 4-CH_2, C-6, and C-13 methylenes enabled assembly of the spirocyclic portion of the molecule.

Analysis of ROESY spectra established the relative configuration of ramonanins A (**1**) and B (**2**), which differ with regard to the relative configurations at C-1 and C-3 (Figure 2B). ROESY cross-peaks between 4-CH_2 and the H-8 and H-10 methines established the configuration at the spirocyclic center relative to that at C-8 and C-10 and *cis*-substitution of the dihydrofuran ring for both ramonanins A and B. Further, *cis*-substitution of the tetrahydrofuran ring follows from ROESY cross-peaks between H-1 and H-3 observed for both compounds. The two compounds are distinguished in that ramonanin A shows ROESY cross-peaks between H-1 and the C-6 protons, whereas ramonanin B shows cross-peaks between H-1 and the C-13 protons, establishing the relative configurations as shown (Figure 2B).

Subsequent analysis of *G. officinale* HPLC fractions adjacent to those containing ramonanins A and B revealed smaller amounts of two additional ramonanin stereoisomers, ramonanins C (**3**) and D (**4**). Analysis of the ROESY spectra of ramonanins C and D indicated that they feature the same relative configuration as ramonanin A at C-1, C-3, and C-5, but differ with regard to the orientation of the substituents at C-8 and C-10. As opposed to ramonanin A, ramonanin C does not show ROESY cross-peaks between 4-CH_2 and H-8 or H-10 and instead shows cross-peaks between 4-CH_2 and protons of the two phenyl substituents at C-8 and C-10, indicating that 4-CH_2 and these two aromatic substituents are oriented *cis* with respect to the furanobenzene bicycle (see Tables S2 and S3). Similarly, the presence of ROESY correlations between H-8 and 4-CH_2 as well as between the aromatic substituent at C-10 and 4-CH_2 established the relative configuration of ramonanin D (Scheme 1).

The furano-2-oxaspiro[4,5]decane core of the ramonanins is unprecedented among known natural products; nonetheless, these compounds appear to be derived from fairly straightforward oligomerization of four units of coniferyl alcohol-like precursors. Intriguingly, the ramonanins could be considered as dimers of two identical 1,3-di-(4-hydroxy-3-methoxyphenyl)-substituted furanes such as **7** (Scheme 1). Dimerization of **7**, perhaps via a Diels−Alder-like mechanism, would directly lead to ramonanins A−D. Although compound **7** was not found in the current study and has not been identified previously from any other source, partially reduced derivatives such as nectandrin B (**6**) have been identified from many plant species,[8] including *Guaiacum* spp,[9] and were also found to be present in our *G. officinale* heartwood extracts. In conclusion, we have identified a new group of lignans that induce cell death via apoptotic mechanisms. The established use of *Guaiacum* extracts in

Table 1. NMR Spectroscopic Data (600 MHz, $CDCl_3$) for Ramonanin A (1)

position	δ_C	proton	δ_H	J_{HH}, Hz[a]	ROESY[b,c]	HMBC[c,d]
1	87.8	1-H	4.58		3-H, 6-H_a, 6-H_b, 2‴-H, 6‴-H	4, 5, 6, 13, 1‴, 6‴, 2‴
3	82.7	3-H	5.38	$J_{3\text{-}H,4\text{-}CH_aH_b}$ = $J_{3\text{-}H,4\text{-}CH_aH_b}$ = 2.3	1-H, 2⁗-H, 6⁗-H, 4-CH_aH_b	4, 4-CH_2, 5 (wk), 1⁗, 2⁗, 6⁗
4	157.6					
4-CH_2	106.9	4-CH_aH_b	5.13		6-H_b, 8-H, 10-H, 12-H_a, 4-CH_aH_b	3, 4, 5, 13 (wk), 1⁗ (wk)
		4-CH_aH_b	4.92		3-H, 2⁗-H, 6⁗-H, 4-CH_aH_b	3, 4, 5, 13 (wk), 1⁗ (wk)
5	47.6					
6	30.1	6-H_a	2.12	$J_{6\text{-}Ha,6\text{-}Hb}$ = 16.8, $J_{6\text{-}Ha,12\text{-}Ha} \approx 3$, $J_{6\text{-}Ha,12\text{-}Hb} \approx 1.5$	1-H, 13-H_b, 2‴-H, 6‴-H	1, 4, 5, 7, 11
		6-H_b	2.00	$J_{6\text{-}Hb,12\text{-}Ha} \approx 2.5$, $J_{6\text{-}Hb,12\text{-}Hb} < 1$ $J_{6\text{-}Hb,13\text{-}Ha} \approx 1$	1-H, 8-H, 12-H_b, 4-CH_aH_b	4, 5, 7, 8, 11, 13
7	135.9					
8	88.9	8-H	5.58	$J_{8\text{-}H,10\text{-}H}$ = 3, $J_{8\text{-}H,6\text{-}Ha} \approx 1.5$, $J_{8\text{-}H,6\text{-}Hb} \approx 1$	6-H_b, 4-CH_aH_b, 2″-H, 6″-H	7, 11, 1″, 2″, 6″
10	88.5	10-H	5.53	$J_{10\text{-}H,6\text{-}Ha} \approx 2$, $J_{10\text{-}H,6\text{-}Hb} \approx 3$	12-H_a, 12-H_b, 4-CH_aH_b, 2′-H, 6′-H_b	7, 11, 1′, 2′, 6′
11	132.8					
12	18.5	12-H_a	1.96	$J_{12\text{-}Ha,12\text{-}Hb}$ = 17.3, $J_{12\text{-}Ha,13\text{-}Ha}$ = 5.6, $J_{12\text{-}Ha,13\text{-}Hb}$ = 10.9	10-H, 4-CH_aH_b, 12-H_b, 13-H_a, 2⁗-H, 6⁗-H	
		12-H_b	1.78	$J_{12\text{-}Hb,13\text{-}Ha} \approx 2.5$, $J_{12\text{-}Hb,13\text{-}Hb}$ = 5.5	10-H (wk), 12-H_a, 13-H_a (wk), 13-H_b, 2′-H, 6′-H	5, 7, 10 (wk), 11, 13
13	26.1	13-H_a	1.62	$J_{13\text{-}Ha,13\text{-}Hb}$ = 13.7	4-CH_aH_b, 12-H_a, 12-H_b, 13-H_b, 2‴-H, 6‴-H, 2⁗-H, 6⁗-H	1, 4, 5, 6, 11, 12
		13-H_b	0.91		1-H (wk), 6-H_a, 12-H_b, 13-H_a, 2′-H, 6′-H, 2‴-H, 6‴-H	1, 4, 5, 6, 11, 12
1′	132.07					
2′	109.92	2′-H	6.73	$J_{2'\text{-}H,6'\text{-}H}$ = 2.0	10-H, OCH_3′, 12-H_b, 13-H_b	1′, 3′, 4′, 6′, 10
3′	146.49					
4′	145.42					
5′	113.91	5′-H	6.81	$J_{5'\text{-}H,6'\text{-}H}$ = 8.0		2′, 3′, 4′, 6′
6′	119.77	6′-H	6.67		10-H, 12-H_b, 13-H_b	2′, 3′(wk), 4′, 5′, 10
1″	132.07					
2″	110.28	2″-H	6.79	$J_{2''\text{-}H,6''\text{-}H}$ = 2.0	6-H_a, 6-H_b, 8-H, 13-H_b (wk), OCH_3″	1″, 3″, 4″, 6″, 8
3″	146.49					
4″	145.43					
5″	114.43	5″-H	6.88	$J_{5''\text{-}H,6''\text{-}H}$ = 8.0		1″, 3″, 6″(wk), 8 (wk)
6″	120.35	6″-H	6.81		6-H_a, 6-H_b, 8-H	1″, 3″, 4″, 6″, 8
1‴	128.32					
2‴	109.69	2‴-H	6.77	$J_{2'''\text{-}H,6'''\text{-}H}$ = 2.0	1-H, 6-H_a, 13-H_a, 13-H_b, OCH_3‴	1‴, 3‴, 4‴, 6‴, 1
3‴	146.00					
4‴	145.12					
5‴	113.59	5‴-H	6.82	$J_{5'''\text{-}H,6'''\text{-}H}$ = 8.0		1‴, 2‴, 3‴, 1 (wk)
6‴	120.12	6‴-H	6.75		1-H, 6-H_a, 13-H_a, 13-H_b	2‴, 4‴, 5‴(wk), 1
1⁗	133.00					
2⁗	110.04	2⁗-H	6.964	$J_{2''''\text{-}H,6''''\text{-}H}$ = 2.0	3-H, 4-CH_aH_b, OCH_3⁗, 12-H_a, 13-H_a	1⁗, 3⁗, 4⁗, 6⁗, 5⁗(wk), 3
3⁗	146.29					
4⁗	145.20					
5⁗	114.14	5⁗-H	6.90	$J_{5''''\text{-}H,6''''\text{-}H}$ = 8.0		1⁗, 2⁗, 3⁗, 4⁗, 3 (wk)
6⁗	120.12	6⁗-H	6.94		3-H, 4-CH_aH_b, 12-H_a, 13-H_a	2⁗, 4⁗, 3⁗(wk), 3
OCH_3′	55.66		3.73		2′	3′
OCH_3″	55.69		3.75		2″	3″
OCH_3‴	55.66		3.76		2‴	3‴
OCH_3⁗	55.72		3.88		2⁗	3⁗

[a] ^{1}H,^{1}H-Coupling constants were estimated from the 1D ^{1}H NMR or the dqfCOSY spectrum. [b] ROESY correlations were observed using a mixing time of 300 ms. [c] Abbreviation: wk = weak, but clearly discernible HMBC or ROESY correlation. [d] HMBC correlations (optimized for 6 Hz) are from the proton(s) stated to the indicated carbon.

traditional medicine and as a food additive may encourage more detailed study of these compounds' mode of action.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded on a Varian INOVA 500 MHz (500 MHz for ^{1}H and 126 MHz for ^{13}C) and a Varian INOVA 600 NMR (600 MHz for ^{1}H, 151 MHz for ^{13}C) using DBG (^{13}C-direct detection) and HCN indirect detection probes. Nongradient phase-cycled dqfCOSY spectra were acquired using the following parameters: 0.6 s acquisition time, 500−600 complex increments, 8−32 scans per increment. Gradient and nongradient HSQC, HMQC, and HMBC spectra were acquired with these parameters: 0.25 s acquisition time, 300−600 increments, 4−32 scans per increment. HMBC spectra were optimized for $J_{H,C}$ = 6 Hz. Susceptibility-matched NMR tubes (Shigemi) were used for sample amounts smaller than 2 mg. NMR spectra were processed using Varian VNMR and MestreLabs MestReC and MNOVA software packages. HRMS was performed on a LTQ Orbitrap Velos (Thermo Scientific). HPLC-MS was performed using an Agilent 1100 Series HPLC system equipped with a diode array detector and connected to a Quattro II spectrometer (Micromass/Waters) operated in positive-ion

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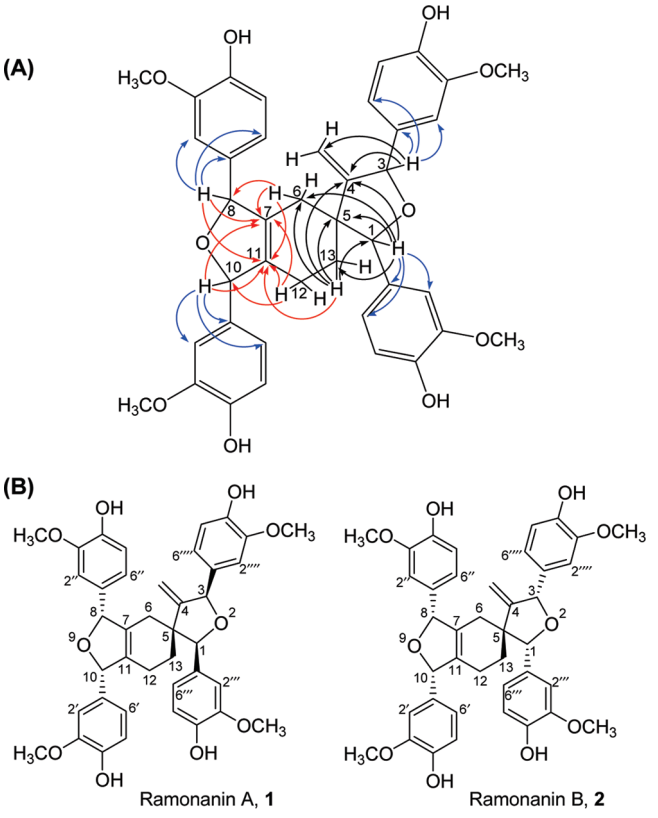
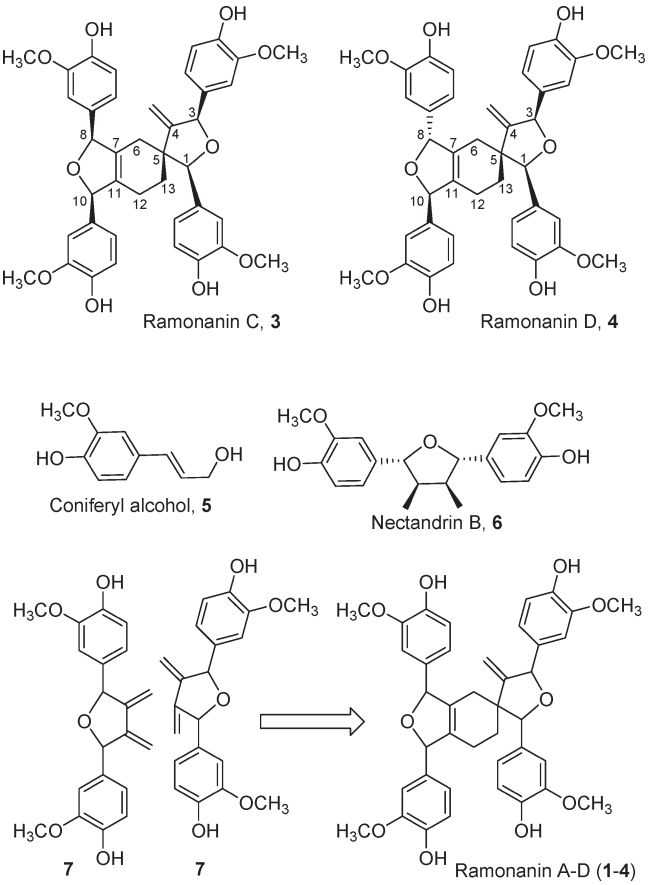


Figure 2. (A) Important HMBC correlations used for structural assignments. Blue arrows signify correlations used for placing the aromatic substituents, red arrows represent correlations determining placement of C-7 and C-11, and black arrows indicate correlations establishing connectivity around the C-5 spirocenter. (B) Relative configuration in ramonanins A (**1**) and B (**2**).

Scheme 1. Structures of Ramonanin C (3), Ramonanin D (4), Coniferyl Alcohol (5), and Nectandrin B (6) and Possible Biogenesis of the Ramonanins (1−4) from Putative Diene Precursor 7



electrospray ionization mode. Data acquisition and processing for HPLC-MS was controlled by Waters MassLynx software. Optical rotations were measured using a Perkin-Elmer 241 polarimeter and a 1 mL cuvette.

Solvents and Sephadex LH-20 were obtained from Sigma-Aldrich and Acros. Silica gel (KG60, 40−63 μm) and precoated TLC plates (silica gel KG60-F254) were obtained from Merck (Darmstadt, Germany) and Teledyne ISCO. For thin-layer chromatography, compounds were visualized using UV light and/or vanillin/H_2SO_4 reagent. Flash chromatography was performed using a Teledyne ISCO CombiFlash system. For semipreparative HPLC, a Dionex LC system with a P580 pump, ASI-100 autosampler, UVD 170U detector, and Gibson 206 fraction collector was equipped with a preparative reversed-phase Phenomenex Aqua 5 μ C18 125 (250 × 10.00 mm) column.

Plant Materials. *G. sanctum* and *G. officinale* L. wood samples were collected in Punta Cana, Dominican Republic. Collected branches approximately 10 to 20 mm in diameter were stripped of their bark and stored. The wood was cut into small pieces, air-dried, and ground. Voucher specimens were deposited at the L. H. Bailey Hortorium Herbarium (BH) at Cornell University, Ithaca, NY, herbarium@cornell.edu (accession number BH000035546, authentication by Francisco Guanchez, 2000).

Extraction and Isolation. Dried and powdered *G. officinale* heartwood (500 g, origin Dominican Republic) was extracted exhaustively with $CHCl_3$ for three days in a Soxhlet apparatus followed by extraction with MeOH. The $CHCl_3$extract yielded 28.6 g of a yellow oil, which was fractionated further over Sephadex LH-20, using a mixture of CH_2Cl_2 and acetone (85:15 v/v) as eluent. Nineteen fractions were collected and tested for cytotoxicity and induction of apoptosis (FACS, see below). Activity (Alamar blue assay) was found to be highest in fraction F13, showing 75% apoptosis. Fraction F13 pooled with fraction F14 yielded 0.476 g of material, which was fractionated again over Sephadex LH-20, now using 100% acetone. Most active fractions (Alamar blue assay) were combined and purified further via semipreparative HPLC, yielding 0.27 g of a yellow oil (HPLC conditions: isocratic 70% MeOH in H_2O, run time of 35 min). Ramonanins A and B eluted at 22.5 and 23.6 min, respectively, and were obtained in amounts of 6.3 and 2.3 mg. Ramonanins C and D eluted at 24.2 and 24.8 min, respectively, and were obtained in amounts of 1.5 and 0.8 mg. Additional samples of ramonanins A, C, and D were obtained from processing of additional *G. officinale* wood samples. HPLC-MS analyses of $CHCl_3$ wood extracts indicated the presence of ramonanins A and C in all *G. sanctum* and *G. officinale* wood samples analyzed. Because of their lower abundance, ramonanins B and D could not always be detected in unfractionated wood extracts.

*Ramonanin A (**1**):* colorless wax; $[\alpha]_D$ −2.2 (*c* 0.6 MeOH, 22 °C); UV (MeOH) λ_{max} (log ε) 230 (4.1), 279 nm (3.6); ^{1}H NMR, ^{13}C NMR, ROESY, and HMBC data, see Table 1; HR-ESI$^+$MS obsd *m/z* 703.2533 $[M + Na]^+$, calcd for $C_{40}H_{40}NaO_{10}$, 703.2519.

*Ramonanin B (**2**):* colorless oil; $[\alpha]_D$ −4.0 (*c* 0.2, MeOH, 22 °C); UV (MeOH) λ_{max} (log ε) 230 (4.1), 279 nm (3.6); ^{1}H NMR, ^{13}C NMR, ROESY, and HMBC data, see Supporting Table 1; HR-ESI$^+$MS obsd *m/z* 703.2541 $[M + Na]^+$, calcd for $C_{40}H_{40}NaO_{10}$, 703.2519.

*Ramonanin C (**3**):* colorless oil; $[\alpha]_D$ −5.2 (*c* 0.2, MeOH, 22 °C); UV (MeOH) λ_{max} (log ε) 230 (4.1), 279 nm (3.6); ^{1}H NMR, ^{13}C NMR, ROESY, and HMBC data, see Supporting Table 2; HR-ESI$^+$MS obsd *m/z* 703.2544 $[M + Na]^+$, calcd for $C_{40}H_{40}NaO_{10}$, 703.2519.

*Ramonanin D (**4**):* colorless wax; $[\alpha]_D$ −5.1 (*c* 0.3, MeOH, 22 °C); UV (MeOH) λ_{max} (log ε) 230 (4.1), 279 nm (3.6); ^{1}H NMR, ^{13}C NMR, ROESY, and HMBC data, see Supporting Table 3; HR-ESI$^+$MS obsd *m/z* 703.2529 $[M + Na]^+$, calcd for $C_{40}H_{40}NaO_{10}$, 703.2519.

Cell Line Culture Conditions and Reagents. The following human cancer cell lines were obtained from ATCC (Manassas, VA): CaLu-6 (lung), HCT-116 and HT-29 (colon), HeLa (cervical), SKBR-3 (breast), MD-MBA-468 (breast), and MD-MBA 231 (breast). Cells were maintained in RPMI 1640 medium (PAA Laboratories GmbH, Teddington Middlesex, UK) supplemented with 10% heat-inactivated tetracycline free fetal calf serum (PAA Laboratories) and 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY). Cells were propagated and cultured at 37 °C in a 100% relative humidity atmosphere consisting of 5% CO_2 and 95% air. Cells were trypsinized with 0.05% Trypsin in aqueous 0.2% EDTA (w/v) solution.

Cytotoxicity Assays. For Alamar blue cell viability assays, cells were seeded onto a standard 96-well microtiter plate at a density of 2 × 10^4 cells/well in 100 μL of tissue culture medium. After 24 h at densities of 5000 cells per well, cells were treated with *Guaiacum* extracts and fractions. All samples were added as solutions in DMSO (10 μg extract/1 μL DMSO). DMSO (0.1%) was used a negative control, and camptothecin (CPT) and 5-fluorouracil (5-FU) were used as positive controls. Following a 48 h incubation period, cells were evaluated immediately by visualizing color densities and spectrophotometrically at 540 nm.

Sulforhodamine (SRB) Blue Assay (ref 14). MB-MDA 231 cells were seeded as described above. After a 24 h incubation period, *Guaiacum* extracts and fractions were added as solutions in DMSO to achieve final concentrations of 200, 20, 2, and 0.2 μg extract/1 mL of media. Cultures were treated with trichloracetic acid (10%), washed with cold tap water, stained with SRB (100 μL, 0.4% (w/v) in 1% HOAc), and incubated at room temperature for 10 min. Unbound dye was removed by five washes with 1% HOAc. After drying, dye-stained protein was extracted with 10 mM TRIS base. Absorbance of stained protein was measured using an automated 96-well microtiter ELISA plate reader (SAFIRE) at 540 nm.

Apoptosis and Cell Cycle Analysis. The morphology of cell cultures was analyzed for apoptosis using a double-fluorescence staining technique with a Hoechst 33342/propidium iodide (H/PI) assay.[15] MD-MBA 231 was seeded at low density (1.0 × 10 4 cells/mL) in six-well plates. After 24 h growth, camptothecin (CPT, as solution in DMSO), the ramonanins (as solution in DMSO), or DMSO (as control) was added. Samples were collected after 6, 12, and 24 h, at which time both the supernatant and adhered cells were stained in 20 μg/mL propidium iodide (emitting red fluorescence) and 100 μg/mL Hoechst 33342 (emitting blue fluorescence) for 15 min at 37 °C in the dark. Double fluorescence was detected with a Zeiss fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) using an epifluorescence system with a long pass filter cube A.

For detecting apoptosis, cells were seeded in 24-well microtiter plates at a density of 1.5 × 10^4 cells per well in 3 mL of tissue culture medium. After 24 h, cells were treated with *Guaiacum* fractions, isolated ramonanins, or camptothecin. Test compounds were dissolved in DMSO (10−100 μg sample/1 mL DMSO). Following a second 24 h incubation period, cell cycle distribution was analyzed using a fluorescence-activated cell sorter (FACS Caliber Becton Dickinson, San Jose, CA), and double staining with Annexin V/propidium iodide.[10] For DNA content analysis, 24 h after exposure to ramonanin A, 1.5 mL of cells was resuspended. Cells were centrifuged at 1000 *g* at room temperature, washed in 0.5 mL of hypotonic staining solution, and analyzed by flow cytometry on the same day. Resulting DNA distributions were analyzed for the fraction of cells in apoptosis in G0/G1 and S phases of the cell cycle. Singlet events were gated, and 10 000−20 000 events were acquired within the gated region. All experiments were repeated at least three times, each in duplicate.

■ ASSOCIATED CONTENT

Supporting Information. NMR spectroscopic and mass spectrometric data for ramonanins A−D. This material is available free of charge via the Internet at http://pubs.acs.org.

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