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Steroidal Alkaloids from the Marine Sponge *Corticium niger* That Inhibit Growth of Human Colon Carcinoma Cells

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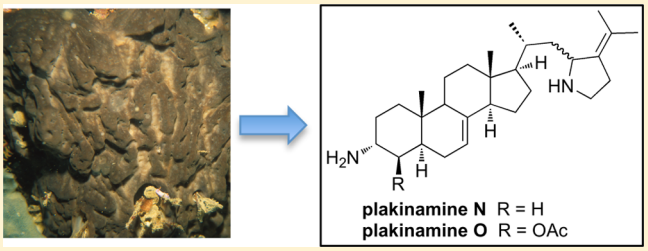
Supporting Information

ABSTRACT: Bioinformatic analysis of data from the NCI-60 cell cytotoxicity screen revealed a subset of extracts that showed selective cytotoxic activity toward human colon carcinoma cell lines. Bioassay-guided fractionation of a colon cancer selective extract from a Philippines collection of the marine sponge *Corticium niger* provided two new steroidal alkaloids, plakinamines N (**1**) and O (**2**), along with two known compounds of the plakinamine class (**3**, **4**). The structures of these compounds were elucidated by interpretation of combined MS and NMR spectroscopic data. Plakinamines N (**1**), O (**2**), and J (**4**) were tested for antiproliferative activity in the NCI-60 screen, and they showed enhanced inhibitory effects against all of the colon cell lines with mean GI_{50} values of 11.5, 2.4, and 1.4 μM, respectively.



Colorectal cancer is the third leading cause of cancer-related deaths in both men and women in the United States.[1] While surgical resection of early stage colorectal carcinoma can provide substantial benefit, adjuvent chemotherapy is required for more advanced disease or when tumors are inoperable due to their size or dissemination. The antimetabolite 5-fluorouracil is a prominent conventional treatment for colon cancer, but combination chemotherapy regimes that also include leucovorin, oxaliplatin, or irinotecan are now standard treatments.[2−4] New biologic agents have recently been introduced such as the targeted monoclonal antibodies cetuximab and panitumumab, which target the epidermal growth factor receptor, and bevacizumab, which inhibits vascular endothelial growth factor A.[5] In spite of some improvements in clinical outcomes, about 50% of diagnosed patients are estimated to ultimately die from colon cancer,[6] thus highlighting the need for improved chemotherapeutic agents and treatment options.

As part of an ongoing effort to discover natural products with potential anticancer activity, bioinformatic analysis of data from the National Cancer Institute (NCI) 60 human tumor cell line anticancer drug screen[7] was employed to identify extracts from the extensive NCI natural products repository with selective growth inhibitory activity against a panel of colon cancer cell lines. Using Student's *t* test and a Kruskal−Wallis test, candidate extracts were identified that achieved statistical significance in both tests ($p \leq 0.05$) for growth inhibition within the NCI-60 colon tumor panel, when compared to the remaining noncolon tumor cell lines. Selected extracts that had not been examined previously were considered for this study. Our laboratory has successfully utilized this selective cytotoxicity screening and analysis method to identify new antitumor agents from a variety of natural sources.[8−10] Of particular note was the discovery of the guaiane sesquiterpene englerin A as a specific inhibitor of renal cancer growth,[11,12] which has recently been shown to act on protein kinase C-*theta* (PKC-θ) to inhibit insulin signaling while promoting glucose dependence in cancer cells.[13]

The organic solvent extract of a Philippines collection of the marine sponge *Corticium niger* showed significant differential growth inhibition toward the colon cell line panel and was therefore selected for more detailed chemical study. While nonsteroidal metabolites such as halogenated cyclic peptides[14] and the heterocyclic alkaloid meridine[15] have been reported from various *Corticium* collections, these sponges are best known for producing the plakinamine and cortistatin families of steroidal alkaloids. Plakinamines have modified ergostane-type steroidal cores with nitrogen substitution in the A ring and linear or cyclized nitrogenous side chains.[16−26] Cortistatins, on the other hand, have rearranged steroidal skeletons with A-ring

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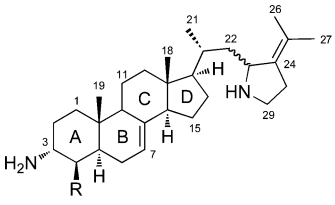
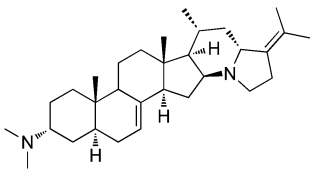
nitrogen substitution, an expanded B ring that incorporates an oxabicyclo functionality, and a side chain comprising various nitrogen heterocyclic functionalities.[27−29] The cortistatins have demonstrated antiangiogenic properties,[30] while the plakinamines have been reported to exhibit diverse biological effects including anti-HIV,[19] antimicrobial,[20] and cytotoxic activities.[17,19,21−23,26] The *C. niger* extract identified in the current study was subjected to a bioassay-guided fractionation procedure that monitored cytotoxic activity against COLO205 and KM12 colon cancer cell lines. Sequential chromatographic separations employing diol SPE, Sephadex LH-20, and C_{18} HPLC led to the isolation of two new metabolites, plakinamine N (**1**) and plakinamine O (**2**), along with two previously described plakinamines, **3** and **4**.[22]



1 R = H
2 R = OAc



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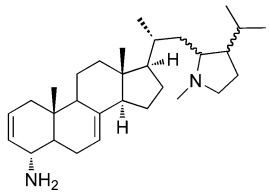

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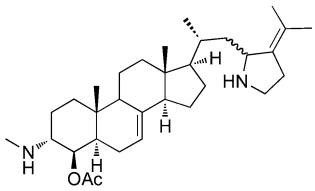

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RESULTS AND DISCUSSION

Corticium niger specimens were collected near Luzon, on the west side of the Calumpan Peninsula of the Philippines in July 1994, and extracted using the standard NCI protocol for marine invertebrate animals.[31] A portion (224 mg) of the sponge organic solvent extract was initially fractionated by step gradient elution through an SPE cartridge containing diol solid-phase media, which concentrated the cytotoxic activity into fractions eluted with EtOAc−MeOH (5:1) and 100% MeOH. These materials were combined and subjected to size-exclusion chromatography on LH-20 (MeOH−H_2O, 9:1) and C_{18} HPLC to yield two new plakinamines, N (**1**) and O (**2**), along with the known plakinamines, I (**3**) and J (**4**). The structures of the new compounds were unambiguously assigned by comprehensive analysis of their 1D and 2D NMR data, in conjunction with accurate mass measurements (HRESIMS), and by comparison of their spectroscopic data to appropriate literature values.[16−25]

Plakinamine N (**1**) was obtained as a pale yellow oil, and a preliminary examination of the ^1H and ^{13}C NMR spectra revealed the steroidal nature of the compound, as they were very similar to those reported for plakinamine K (**5**).[22] The HRESIMS spectrum established a molecular formula of $C_{29}H_{48}N_2$, requiring seven degrees of unsaturation. Two of these were attributed to olefinic groups assigned to $\Delta^{7,8}$ (δ_C 118.5, 140.4) and $\Delta^{24,25}$ (δ_C 128.4, 130.6) on the basis of their HMBC correlations (Table 1). Analysis of COSY and HSQC data allowed assignment of the C-1 to C-7, C-9 to C-12, and C-14 to C-17 spin systems, which were characteristic of a tetracyclic steroidal backbone with a C-3 amine substituent. The chemical shift of C-3 (δ_C 48.7), together with COSY correlations from H-3 (δ_H 3.53) to methylene resonances assigned to H_2-2 (δ_H 1.71, 1.91) and H_2-4 (δ_H 1.75), confirmed placement of the amine at this position. HMBC correlations from the methyl singlet at δ_H 0.85 (Me-19) to C-1, C-5, C-9, and C-10 helped define the composition and junction of rings A and B. The fused nature of rings B, C, and D was established by HMBC correlations from the olefinic methine resonance (H-7, δ_H 5.22) to the ring junction methine carbons C-5, C-9, and C-14 and from the methyl singlet (Me-18, δ_H 0.63) to C-12, C-13, C-14, and C-17. HMBC correlations from the methyl doublet (Me-21, δ_H 1.12) to C-17, C-20, and C-22 helped elaborate the steroidal side chain. Deshielded carbon and proton resonances at C-23 (δ_C 59.2, δ_H 4.40) and C-29 (δ_C 45.1, δ_H 3.43 and 3.32), which were indicative of nitrogen substitution, and characteristic $^1H-^1H$ vicinal coupling between H_2-28 and H_2-29 were consistent with the presence of a pyrrolidine ring system. An exocyclic tetrasubstituted olefinic group was assigned to $\Delta^{24,25}$ based on HMBC correlations from the coincident vinyl methyl protons of Me-26 and Me-27 (δ_H 1.71, 6H) to C-23, C-24, and C-28. Homoallylic coupling observed from Me-26/27 to the H_2-28 methylene resonances at δ_H 2.61 and 2.66 supported this assignment. Characteristic HMBC correlations from H-29 to C-23, C-24, and C-28 helped confirm that compound **1** has the same substituted heterocyclic ring system that is found in plakinamine K (**5**).[22]

Proton coupling, ROESY correlations, and ^{13}C comparisons were used to define the relative configuration of **1**. The small vicinal couplings observed for H-3 (δ_H 3.53, br s), together with its ROESY correlations (Figure 1) to H-2β (δ_H 1.91) and H-4β (δ_H 1.75), were consistent with a β-(equatorial)-orientation of this proton. The chair conformation and *trans*-ring-fusion of the A and B rings were established from 1,3-diaxial ROESY correlations between Me-19β (δ_H 0.85), H-2β (δ_H 1.91), H-4β (δ_H 1.75), and H-11β (δ_H 1.52) and a correlation between H-5α (δ_H 1.62) and H-9α (δ_H 1.84). A ROESY cross-peak between Me-18 (δ_H 0.63) and one of the H-11 protons (δ_H 1.52) revealed these two substituents had a 1,3-diaxial relationship, or β-orientation. A ROESY correlation between H-14 and H-7 supported an α-orientation for H-14 and, thus, *trans*-fusion of the C and D rings. All of the previously reported plakinamine steroidal alkaloids possess a *trans*-fused C/D ring junction, a β-oriented side chain at C-17, and an α-methyl group at C-20. Thus, a comparison of the ^{13}C NMR data recorded for **1** with the data reported for other structurally related plakinamines allowed assignment of the relative configuration at these centers. There are nine compounds in the plakinamine class with a pyrrolidine or dehydropyrrolidine ring in the side chain, and the ^{13}C chemical shifts for C-13, C-14, C-17, and C-20 in these compounds all fall within very characteristic ranges (C-13, δ_C 44.3 ± 0.7; C-14, δ_C 56.8 ± 1.7; C-17, δ_C 58.0 ± 0.9; C-20, δ_C 35.2 ± 0.7).[16,17,19−23] The ^{13}C NMR data for **1** were in close correspondence with these values (C-13, δ_C 44.7; C-14, δ_C 56.2; C-17, δ_C 57.8; C-20, δ_C 34.7), which indicated it also had a β side chain and an α methyl group at C-20. A ROESY correlation between Me-18β and Me-21 (δ_H 1.12) and additional correlations between H-23 (δ_H 4.40), H-20β (δ_H 1.56), Me-21 (δ_H 1.12), and Me-26 were all

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Table 1. ^{1}H (600 MHz), ^{13}C (150 MHz), and HMBC NMR Data for Compounds 1 and 2 in CD_3OD

	1			2		
position	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC
1a	32.4, CH_2	1.72, m	3, 5, 10	31.9, CH_2	1.77, m	2, 5, 9, 10, 19
1b		1.34, m	2, 9, 10		1.39, m	2, 9, 10, 19
2a	25.2, CH_2	1.91, m	1	21.5, CH_2	2.12, m	1, 9, 10
2b		1.71, m	10		1.75, m	
3	48.7, CH	3.53, br s		50.3, CH	3.45, br s	1, 4, 5
4a	31.6, CH_2	1.75, m	5	71.9, CH	5.00, br s	3, 5, 10, *C*-OAc, *Me*-OAc
4b		1.61, m	5			
5	35.7, CH	1.62, m		39.8, CH	1.80, m	6, 9, 10, 19
6a	30.2, CH_2	1.77, m	5	25.9, CH_2	2.06, m	
6b		1.76, m			1.74, m	10
7	118.5, CH	5.22, br d (2.6)	5, 6, 9, 14	118.5, CH	5.25, br d (2.6)	6, 5, 9, 14
8	140.4, C			140.1, C		
9	50.2, CH	1.84, m	11	51.0, CH	1.87, m	5, 7, 8, 13
10	35.7, C			35.5, C		
11a	22.3, CH_2	1.64, m		22.0, CH	1.60, m	8, 13
11b		1.52, m	9, 10, 12		1.53, m	9, 10, 12
12a	40.8, CH_2	2.12, m	9, 13, 14	40.6, CH_2	2.12, m	13, 14, 17, 18
12b		1.32, m	11, 13, 14, 17, 18		1.30, m	11, 13, 14, 17, 20, 18
13	44.7, C			44.7, C		
14	56.2, CH	1.89, m	13	56.1, CH	1.88, m	8, 13
15a	24.0, CH_2	1.57, m		24.0, CH_2	1.58, m	8, 13, 14
15b		1.47, m	8, 14, 16		1.46, m	8, 14, 16
16a	28.8, CH_2	1.93, m	13, 15, 17	28.7, CH_2	1.92, m	15, 17
16b		1.39, m			1.38, m	15, 17
17	57.8, CH	1.26, m	12, 13, 16, 20, 22, 18	57.7, CH	1.26, d (9.6)	12, 13, 20, 22, 18
18	12.4, CH_3	0.63, s	12, 13, 14, 17	12.4, CH_3	0.63, s	12, 13, 14, 17
19	12.5, CH_3	0.85, s	1, 5, 9, 10	15.0, CH_3	1.07, s	1, 5, 9, 10
20	34.7, CH	1.56, m		34.7, CH	1.56, m	13, 17, 18
21	18.3, CH_3	1.12, d (6.4)	17, 20, 22	18.3, CH_3	1.12, d (6.3)	17, 20, 22
22a	39.1, CH_2	1.77, m	23, 21	39.1, CH_2	1.78, m	23, 21
22b		1.34, m	17		1.34, m	17, 20, 21
23	59.2, CH	4.40, br (11.7)	24	59.1, CH	4.40, d (11.8)	20, 22, 24, 25, 28, 29
24	130.6, C			130.6, C		
25	128.4, C			128.4, C		
26	21.1, CH_3	1.71, br s	23, 24, 25, 28	21.1, CH_3	1.71, s	23, 24, 25, 28, 27
27	21.7, CH_3	1.71, br s	23, 24, 25, 28	21.7, CH_3	1.71, s	23, 24, 25, 28, 26
28a	28.0, CH_2	2.66, m	24, 25, 29	28.0, CH_2	2.67, m	23, 24, 25, 29
28b		2.61, m	24, 29		2.61, m	24, 25, 29
29a	45.1, CH_2	3.43, m	24, 28	45.1, CH_2	3.41, m	23, 24, 28
29b		3.32, obs	23, 24, 28		3.3, obs m	23, 24, 28
Ac				171.5, C		
				20.8, CH_3	2.10, s	*C*-OAc, 4

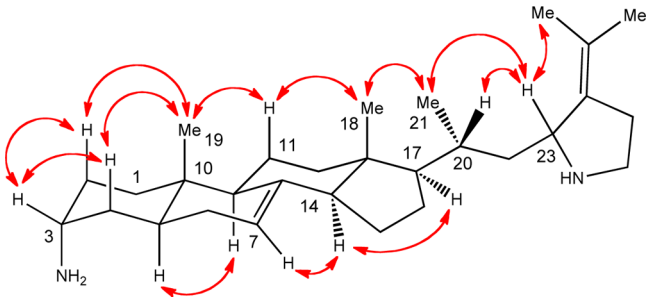

Figure 1. Key ROESY correlations for 1.

consistent with these assignments, but they were not sufficient to define the relative configuration at C-23.

A cursory examination of the ^{1}H and ^{13}C NMR spectroscopic data (Table 1) of plakinamine O (**2**) showed that the chemical shifts from C-1 to C-19 were virtually identical to those recorded for compound **1**, thus establishing the presence of a closely related steroidal alkaloid. The HRESIMS spectrum of **2** defined a molecular formula of $C_{31}H_{50}N_2O_2$, which differed from **1** by the substitution of an OAc group. The presence of an acetate functionality was confirmed by an HSQC correlation from a methyl proton singlet at δ_H 2.10 to a carbon resonance at δ_C 20.8 and an HMBC correlation from this methyl signal to an ester carbonyl carbon at δ_C 171.5. COSY correlations from both H-3 (δ_H 3.45, br s) and H-5 (δ_H 1.80) to an oxymethine group (δ_H 5.00, br s, δ_C 71.9) positioned the acetate moiety at C-4. This was confirmed by an HMBC correlation from the acetate methyl protons to C-4. The remainder of the tetracyclic backbone was defined using COSY spin systems from C-1 to C-

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7, C-9, to C-12 and from C-14 to C-17 and a comprehensive set of HMBC correlations (Table 1). Both the carbon and proton NMR chemical shifts for C-20 to C-29 in compound **2** were virtually identical to those of **1**, which indicated they shared the same side chain and substituted pyrrolidine moiety. HMBC correlations from H-23 (δ_H 4.40) to C-20, C-22, C-24, C-25, C-28, and C-29 supported this assignment.

The relative configuration of **2** was assigned primarily from ROESY cross-peaks and ^{13}C NMR data that were similar to those observed in **1**. ROESY correlations between H-4 and H-5 and those between Me-Ac and Me-19 (δ_H 1.07) were used to unequivocally assign a β-orientation for the C-4 acetate group (Figure 2). The corresponding β-orientation for H-3 (δ_H 3.45, br s) was confirmed by the very small magnitude of its vicinal couplings and ROESY correlations with Me-Ac (δ_H 2.10) and H-2β (δ_H 2.12). All of the other ROESY cross-peaks observed in **2** were analogous to those seen in **1**, and the ^{13}C data were also very similar, which indicated that they shared the same relative configuration throughout the tetracyclic steroidal core and the pyrrolidine-containing side chain.

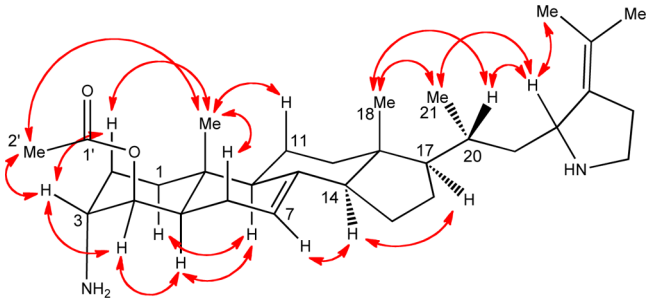

Figure 2. Some key ROESY correlations for **2**.

Compounds **1**–**4** were evaluated for cytotoxicity in the NCI-60 anticancer screen, which includes a panel of seven human colon carcinoma cell lines (COLO 205, HCT-15, SW-620, HCC-2998, HT29, HCT-116, KM12).[7] Plakinamines N (**1**), O (**2**), and J (**4**) showed sufficient growth-inhibitory activity when tested at a single concentration to warrant full dose–response testing against all 60 cell lines. Plakinamine I (**3**) had only modest activity and was not subjected to further testing. While compounds **1**, **2**, and **4** contain a substituted pyrrolidine ring in the steroidal side chain, the pyrrolidine nitrogen in compound **3** forms an additional fused piperidine ring system, which may affect the cytotoxic activity of these alkaloids. Results from the complete 60-cell study revealed that plakinamines O (**2**) and J (**4**) were the most potent, with mean GI_{50} (50% growth inhibition) values of 2.4 and 1.4 μM, respectively, while plakinamine N (**1**) was less potent, with a mean GI_{50} of 11.5 μM. All three of these plakinamines showed an enhanced antiproliferative effect against the entire panel of colon cell lines at the mean GI_{50}, mean TGI (total growth inhibition), and mean LC_{50} (50% cell death) levels (Supporting Information, S15–S17). While their potencies were only low micromolar, the observation of greater colon cancer sensitivity at all three of the standard indices of inhibition was striking, and it provided strong evidence that these plakinamines are indeed colon cell line selective. While the differential cytotoxic activity of the plakinamines was real and highly reproducible, the degree of colon selectivity was relatively modest, as the total variation about the mean for all three indices was approximately $10^{0.8}$ to $10^{-0.8}$. In our experience, it is extremely rare to find a class of compounds that is more active against all cell lines in a particular tumor panel, at all three indices of inhibition. These findings indicate that the plakinamine steroidal alkaloid skeleton provides a structural scaffold that could potentially be modified to generate analogues with greater potency and enhanced colon-selective antiproliferative properties.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations ($[\alpha]_D$) were measured on a PerkinElmer 241 polarimeter in a 100 × 2 mm cell (units 10^{-1} deg cm^2 g^{-1}). UV absorption spectra were obtained using a Varian Cary 50 Bio UV–visible spectrophotometer. NMR spectra were acquired on a Bruker Avance III spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C and equipped with a 3 mm cryogenically cooled probe. ^{1}H and ^{13}C spectra were referenced to the residual deuterated solvent peaks. LCMS data were obtained using a Hewlett-Packard Series 1100 MSD, and HRMS data were acquired on an Agilent 6520 Accurate Mass Q-TOF instrument with internal reference masses calibrated at 121.050 87 and 922.009 79, both within 5 ppm. Diol SPE fractionation of the extract was performed on DIO Spe-ed SPE cartridges, and subsequent fractions were separated on a Sephadex LH-20 column attached to a model UA-6 UV detector and Foxy 200 fraction collector (Teledyne Isco). Final purifications were performed using a Varian ProStar 218 solvent delivery module HPLC equipped with a Varian ProStar 325 UV–vis detector, operating under Star 6.41 chromatography workstation software. All solvents and chemicals used were of analytical grade.

Sponge Material. The sponge *Corticium niger* Pulitzer-Finali 1996 was collected using scuba at a depth of −15 to −20 m at Luzon (west side of Calumpan Peninsula), Philippines, in 1994, and it was immediately frozen and stored at −20 °C until extraction. Taxonomic identification of the sponge was provided by Michele Kelly (National Institute of Water and Atmospheric Research, New Zealand), and a voucher specimen is maintained at the Smithsonian Institution, Washington, D.C. (collection no. 0CDN2615).

Extraction and Isolation. The frozen sponge was ground and processed using the standard NCI method for marine samples.[31] The sponge material (203 g) was first extracted with 100% H_2O to give an aqueous extract (39.9 g) and then sequentially extracted with CH_2Cl_2–MeOH (1:1) followed by 100% MeOH. Removal of the combined organic solvents under reduced pressure provided 6.1 g of organic extract (NSC no. C013619). A portion of the organic extract (224 mg) was utilized for the colon cancer cell based bioassay-guided isolation of the cytotoxic compounds. The extract was loaded onto two separate diol DIO Spe-ed SPE cartridges (112 mg each), and each cartridge was sequentially eluted with 6.0 mL of hexane–CH_2Cl_2 (9:1), CH_2Cl_2–EtOAc (20:1), 100% EtOAc, EtOAc–MeOH (5:1), and 100% MeOH to give five fractions (A–E), which weighed 42.5, 34.5, 2.0, 13.5, and 98.5 mg, respectively. The colon cancer cytotoxicity of fractions D and E was more potent than the other fractions, so they were combined (92.0 mg total) and then chromatographed on a 90 × 2.0 cm i.d. column of Sephadex LH-20 eluted with MeOH–H_2O (9:1), with 275 drop fractions retained in each collection tube. On the basis of their UV absorption at 220 nm, seven fractions (A–G) were collected by combining tubes 12–17, 18–20, 21/22, 23/24, 25/26, 27/28, and 29–45, respectively. Among these, fractions D, E, and F exhibited colon cancer cytotoxicity.

Fraction D (30.4 mg) was further purified by HPLC onto a 250 × 10 mm Phenomenex Luna 5 μ C_{18} (2) 100 Å column. The detector wavelength was set to 220 nm, and the solvent flow rate was 2.5 mL/min. Elution began with 50% MeOH–H_2O (0.1% TFA), running isocratic for 10 min and then followed by a linear gradient of 50% to 80% MeOH–H_2O (0.1% TFA) over 20 min to yield compounds **3** (5.1 mg; t_R = 17.3 min) and **4** (3.6 mg; t_R = 25.6 min).

Fraction E (31.4 mg) was purified using the same C_{18} HPLC column and almost identical conditions to fraction D, except that the starting elution composition was 55% MeOH–H_2O (0.1% TFA). Of the five fractions (1–5) that were collected, only fractions 2 (8.5 mg; t_R = 23.3 min) and 4 (8.5 mg; t_R = 25.6 min) were found to be active

in the colon cancer cell bioassay. Fraction 4 was found to be solely composed of compound **4**, while fraction 2 appeared to be a mixture of two closely related compounds. It was then subjected to further HPLC purification on the same C_{18} column using an isocratic elution with 55% MeOH–H_2O (0.1% TFA) to yield the two new compounds **1** (2.3 mg; t_R = 23.3 min) and **2** (4.2 mg; t_R = 25.1 min).

Plakinamine N (1), NSC #775605: pale yellow oil; $[\alpha]_D^{25}$ +8.8 (*c* 0.17, MeOH); IR (NaCl) 3450, 2956, 2833, 2150, 1651, 1630, 1455, 1017 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data, see Table 1; HRESIMS found *m*/*z* 425.3903 $[M + H]^+$ (calcd for $C_{29}H_{49}N_2$, 425.3890).

Plakinamine O (2), NSC #775606: pale yellow oil; $[\alpha]_D^{25}$ +17.5 (*c* 0.40, MeOH); IR (NaCl) 3440, 2951, 2085, 1681, 1670, 1651, 1450, 1408, 1205, 1143, 1018 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data, see Table 1; HRESIMS found *m*/*z* 483.3952 $[M + H]^+$ (calcd for $C_{31}H_{51}N_2O_2$, 483.3945).

Antiproliferative Bioassay. Chromatography fractions were made up in DMSO and assayed in an *in vitro* cytotoxicity assay employing the COLO205 and KM12 colon cancer cell lines to direct purification. General experimental details of the 2-day antiproliferative assay for testing in a 96-well format have been published previously.[32] In the current study, the assay was modified for use in a 384-well plate. In brief, cells were harvested and plated (45 μL) at a seeding density of 3.0×10^5 cells per well for the COLO205 cell line and 2.5×10^5 cells per well for the KM12 cell line into a 384-well "assay plate" and then incubated for 24 h. DMSO solutions of the test materials (8 μL) were diluted 1:25 with medium (192 μL) and then subjected to five 2:1 serial dilutions (100 μL each) on a 96-well plate. Duplicate 40 μL aliquots of each sample concentration were then transferred to a 384-well "dilution plate", which could accommodate the duplicate samples from two 96-well plates. A 5 μL portion of each solution on the dilution plate was transferred to the cell cultures on the 384-well assay plate to give a final volume of 50 μL and a DMSO concentration of 0.4%. Cells were incubated for 48 h at 37 °C in the presence of the test samples and then treated with the tetrazolium salt XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide). Viable cells reduced the XTT to a colored formazan product, and after an additional 4 h incubation period the amount of formazan produced was quantified by absorption at 450 nm, using a 650 nm reference. Pure compounds were tested in the assay described above and in the NCI 60 cell assay.[7]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H NMR, ^{13}C NMR, COSY, HSQC, HMBC, and ROESY spectra, NCI 60 cell line assay results, together with HRESIMS data, for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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