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Inhibition of Tumor Cells Interacting with Stromal Cells by Xanthones Isolated from a Costa Rican *Penicillium* sp.

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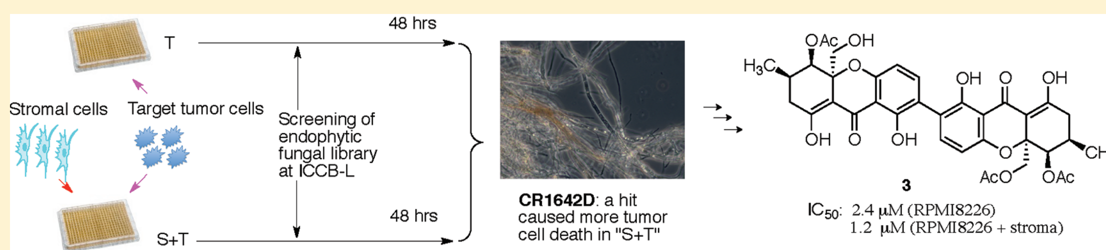
Supporting Information



ABSTRACT: CR1642D, an endophytic isolate of *Penicillium* sp. collected from a Costa Rican rainforest, was identified through a high-throughput approach to identify natural products with enhanced antitumor activity in the context of tumor−stromal interactions. Bioassay-guided separation led to the identification of five xanthones (**1**−**5**) from CR1642D. The structures of the xanthone dimer penexanthone A (**1**) and monomer penexanthone B (**2**) were elucidated on the basis of spectroscopic analyses, including 2D NMR experiments. All of the compounds were tested against a panel of tumor cell lines in the presence and absence of bone marrow stromal cells. Compound **3** was the most active, with IC_{50} values of 1−17 μM, and its activity was enhanced 2-fold against tumor cell line RPMI8226 in the presence of stromal cells (IC_{50} 1.2 μM, but 2.4 μM without stromal cells).

Identification of agents for the treatment of cancer typically involves target-based screens or phenotypic screens against isolated cancer cell lines. However, tumor cells in patients interact with diverse types of nonmalignant cells, which can stimulate tumor cell survival, proliferation, and resistance to diverse classes of established anticancer drugs and investigational agents. Recently, we have also shown that the presence of nonmalignant accessory cells, such as bone marrow stromal cells (BMSCs), can sensitize tumor cells to other novel agents.[1] Therefore, in vitro screening for novel anticancer agents has to be performed in cocultures of tumor cells with nonmalignant accessory cells, in order to detect whether an agent is subject to microenvironment-dependent drug resistance or sensitization. In our continuing search for biologically active natural products from Costa Rican tropical rainforests as part of an International Cooperative Biodiversity Group (ICBG) program,[2] we screened endophytic fungal extracts from Costa Rica at Harvard Medical School's high-throughput screening facility (ICCB-L) against a panel of cancer cell lines with and without BMSCs (Scheme 1). One extract, designated as CR1642D and identified as being from *Penicillium* sp., had enhanced antitumor activity against MM.1S myeloma cells with 40.2% viability in the presence of BMSCs, but 66.8% without BMSCs at 1 μg/mL. Bioassay-guided fractionation using C_{18} open column and phenyl-hexyl HPLC yielded five xanthones (**1**−**5**). Herein we report the structure elucidation of compounds **1** and **2** and the antitumor properties of compounds **1**−**5**.

Compound **1** had the molecular formula $C_{36}H_{36}O_{15}$ by HRESIMS. The UV spectrum of **1** was very similar to that of phomoxanthone B, a xanthone dimer.[3] The IR spectrum of **1** supported the presence of carbonyl groups (1744 and 1605 cm^{-1}). The proton NMR spectrum of **1** showed two aromatic AB spin systems, six protons bound to oxygenated carbons, two methines, two methylenes, three acetyl methyl groups, and two chelated phenolic hydroxy groups. In ring A of monomer I, H-4 exhibited ^3J HMBC correlations to C-2 and C-9a. Peri to a carbonyl group, the C-1 hydroxyl group correlated to C-1, C-2, and C-9a. H-3 was ortho coupled with H-4 and showed ^3J HMBC correlations to C-1, C-4a, and C-4′. Therefore ring A was determined as a 2,3,6-trisubstituted phenol (2,4a,9a-trisubstituted phenol). H-2′ and H-3′ showed ^3J HMBC correlations to C-4′ and C-9a′, and C-1′ and C-4a′, respectively; hence ring A′ in monomer II was determined as a 2,3,4-trisubstituted phenol (4′,4a′,9a′-trisubstituted phenol), and rings A and A′ were connected through the C-2−C-4′ bond. Since 1-

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Scheme 1. High-Throughput Screening to Identify Compounds Active against Tumor Cells Interacting *in Vitro* with Bone Marrow Stromal Cells

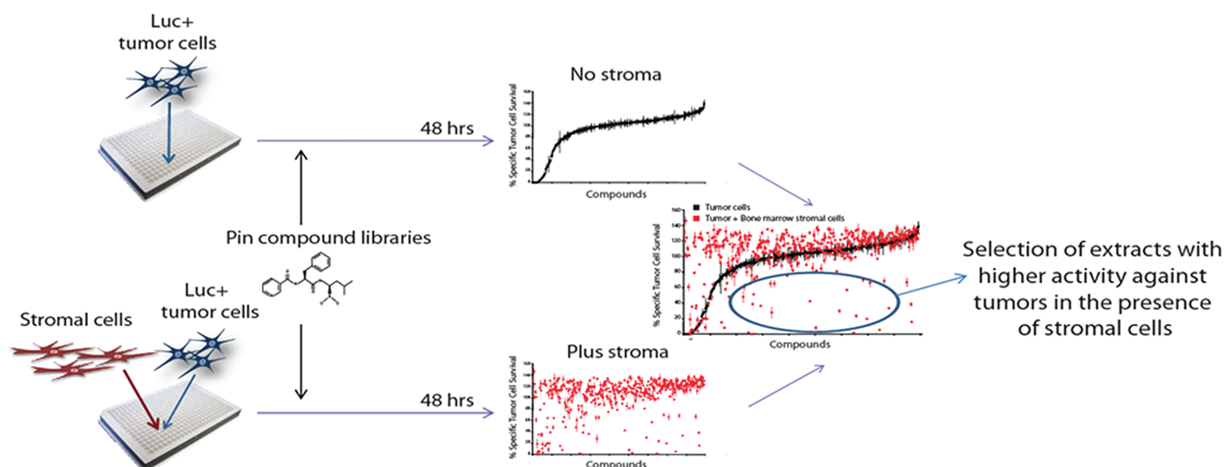
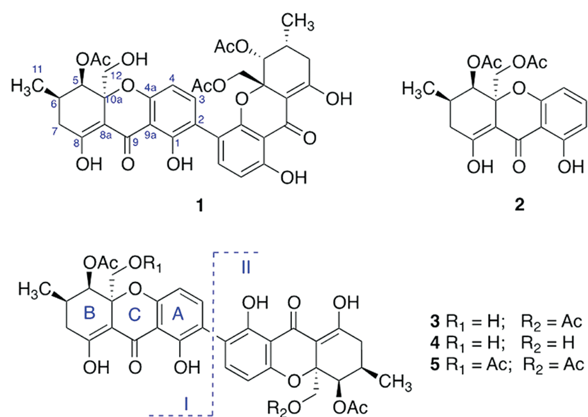


Chart 1. Structures of Compounds 1−5



OH was chelated and C-4a was oxygenated, it could be deduced that A/A′ joined C/C′ to form a 4-chromanone.

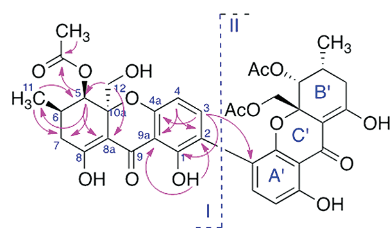


Figure 1. Key HMBC correlations in monomer I of **1**.

The remaining parts of the molecule to be determined are rings B and B′. The methyl group at δ_H 1.07 (H_3-11) showed HMBC correlations to C-5 (δ_C 72.1), C-6 (28.0), and C-7 (33.7). The oxygenated methylene at δ_H 3.94 and 3.75 (H_2-12) correlated to C-10a (δ_C 80.7), C-5, and C-8a (101.5), while the oxygenated methine at δ_H 5.55 (H-5) exhibited correlations to C-10a, C-8a, C-6, C-7, C-12 (δ_C 65.6), C-11 (17.9), and 5-OCOCH$_3$ (170.9). Therefore, this ring was determined to be 4-acetoxyl-3-hydroxymethyl-5-methyl cyclohexenol, with the other two substituents at the 2,3-positions of this fragment, which could be ring B or ring B′. Similarly, the last ring was determined to be 4-acetoxyl-3-acetoxymethyl-5-methyl cyclohexenol, with the other two substituents at the 2,3-positions of this fragment, which could be ring B′ or B. The only difference between ring B and ring B′ is the substituent at the oxygenated methylene: one is a hydroxy while the other is an acetoxy. A ROESY experiment showed that the oxygenated methylene (12-position) was on one face of the hemichair ring, while both the methyl (11-position) and the acetoxyl group (5-OAc) were on the other face (Figure 2). Molecular modeling with

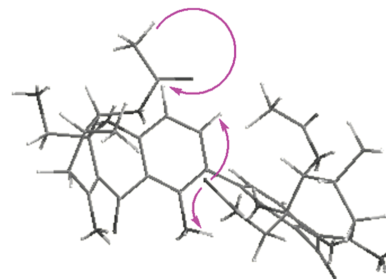


Figure 2. Key NOESY correlations of **1**.

Chem3D Ultra (9.0) suggested that the substituent at the 10a-position was far away (>5 Å) from the aromatic protons of ring A. In the NOESY spectrum of compound **1**, both 1-OH (δ_H 11.62) and H-3 (δ_H 7.16) correlated with the signal at δ_H 1.83 (12′-OCOCH$_3$), and H-4 (δ_H 6.43) showed a correlation with the signal at δ_H 2.11 (5-OCOCH$_3$). Since the signals at δ_H 1.83 (12′-OCOCH$_3$) and 2.05 (5′-OCOCH$_3$) were in the same ring system, the signal at δ_H 2.11 must be the 5-acetoxyl methyl. Hence, the structure of compound **1** including its relative stereochemistry was determined as shown. Both monomers were assigned the relative stereochemistry (R^*,R^*,R^*) based on the assumption that they share the same biosynthetic pathway. All the ^{13}C NMR chemical shifts except C-9 and C-9′ were obtained from the HSQC and HMBC spectra due to insufficient sample for a good ^{13}C NMR spectrum. The planar structure of **1** was reported in a Korean patent, but with an unclean ^{1}H NMR spectrum, which most likely resulted from an impure sample.[5]

The molecular weight of compound **2** was 376.1158, as suggested by HRESIMS, which was 1 unit more than that of monomer II of compound **1**. The proton NMR data of compound **2** were similar to those of monomer II of compound **1**, but with an extra aromatic proton signal. The planar

Table 1. $^1H^a$ and $^{13}C^b$ NMR Data of Compounds 1 and 2

	1				2	
#	δ_C, type (I)	δ_C, type (II)	δ_H (J in Hz) (I)	δ_H (J in Hz) (II)	δ_C, type	δ_H (J in Hz)
1, 1′	160.2, C	161.9, C			162.0, C	
2, 2′	118.6, C	110.3, CH		6.58, d (8.4)	108.1, CH	6.35, d (8.4)
3, 3′	139.4, CH	139.4, CH	7.16, d (8.4)	7.32, d (8.4)	139.4, CH	7.39, t (8.4)
4, 4′	107.6, CH	117.4, C	6.43, d (8.4)		109.7, CH	6.47, d (8.4)
4a, 4a′	157.6, C	155.0, C			158.2, C	
5, 5′	72.1, CH	69.8, CH	5.55, br s	5.30, br s	70.6, CH	5.90, br s
6, 6′	28.0, CH	27.9, CH	2.47, m	2.28, m	27.5, CH	2.57, m
7, 7′	33.7, CH_2	33.3, CH_2	2.30−2.50, m	2.30−2.50, m	33.4, CH_2	2.57, m; 2.37, m
8, 8′	177.9, C	177.9, C			179.5, C	
8a, 8a′	101.5, C	100.8, C			100.3, C	
9, 9′	–, C	–, C			–, C	
9a, 9a′	106.8, C	106.7, C			106.5, C	
10a, 10a′	80.7, C	80.3, C			80.8, C	
5,5′-O<u>CO</u>CH$_3$	170.9, C	170.8, C			169.8, C	
5,5′-OCO<u>CH$_3$</u>	21.3, CH_3	21.3, CH_3	2.11, s	2.05, s	20.3, CH_3	2.03, s
11, 11′	17.9, CH_3	17.6, CH_3	1.07, d (6.0)	0.97, d (6.0)	17.0, CH_3	1.03, d (6.0)
12, 12′	65.6, CH_2	64.2, CH_2	3.94, m	4.52, d (12.0)	65.0, CH_2	4.51, d (12.0)
			3.75, m	3.86, d (12.0)		4.27, d (12.0)
12,12′- O<u>CO</u>CH$_3$		170.1, C			169.5, C	
12,12′- OCO<u>CH$_3$</u>		21.1, CH_3		1.83, s	20.1, CH_3	1.99, s
1,1′-OH			11.62, s	11.20, s		

$^a\delta$ (ppm) 600 MHz; multiplicities; J values (Hz) in parentheses. $^b\delta$ (ppm) 150 MHz; chemical shifts from gHSQC and gHMBC.

structure and relative stereochemistry of compound **2** were determined by 2D NMR experiments as shown. A structure identical to compound **2** was used for CD calculation, but never isolated.[6]

Compounds **3**−**5** were identified as dicerandrols B, A, and C, respectively, by comparison of physical and spectroscopic data (UV, IR, ^{1}H NMR, $[\alpha]_D$, and MS) with literature values.[4i] These were previously purified from the endophytic fungus *Phomopsis longicolla* isolated from an endangered mint from Florida.[4i] Compounds **4** and **5** are homodimers with a 2−2′ connectivity. The heterodimer **3** has the same two monomers as **1**, but they are dimerized through a 2−2′ linkage, while the two different monomeric units of **1** are coupled through the 2−4′ positions. Many such xanthone dimers with a methyl at the 6-position and an oxygenated methylene at the 10a-position have been reported, but most of them are symmetrical homodimers as in **4** and **5**.[4] Usually, the unsymmetrical xanthone heterodimers are different only at either ring A/A′ as in phomoxanthone B[3] or ring C/C′ as in **3**. Curiously the monomeric units in these xanthone dimers (**1** and **3**−**5**) had not been previously isolated from natural sources.

All five pure compounds were tested against a panel of tumor cell lines (from myeloma, lymphoma, leukemia, as well as breast and prostate cancer) in the presence and absence of BMSCs. In many cases, the activity was either decreased or enhanced in the context of tumor−stromal interactions. As can be seen in Table 2, the cytotoxic activity of these compounds increases upon dimerization and acetylation of 12′-OH (**4**) to 12′-OAc (**3**). The C-12 and C-12′ diacetate (**5**) exhibits less activity than **3**, and the 2−4′ linked monoacetate (**1**) is much less active than the 2−2′ linked monoacetate (**3**). The most active compound, **3**, exhibits moderate activity against Dox40, Farage, H929, HT, OPM2, and RPMI8226 in the presence of stromal cells with IC_{50} values of 2.3, 1.3, 3.4, 1.3, 1.5, and 1.2 μM, respectively. The activity of **3** against cancer cell lines RPMI8226 and H929 is doubled or tripled in the presence of stromal cells, results that would not have been identified through a traditional cell-based screen. On the other hand, compound **3** is much less toxic against human immortalized nonmalignant cells, such as HS-5 bone marrow stromal cells, HOBIT osteoblast-like cells, THLE-3 hepatocytes, and SVGp12 astrocytes, with IC_{50} values of 13.0, 9.2, 10.0, and 13.7 μM, respectively (Figure 2S). The relative selectivity of these compounds, especially compound **3**, warrants further investigation.

■ EXPERIMENTAL SECTION

General Experimental Procedures. All NMR experiments were carried out on a Varian INOVA 600 MHz spectrometer. IR spectra were measured on a Bruker Alpha-P spectrometer, and UV spectra on an Amersham Biosciences Ultrospec 5300 Pro spectrophotometer. All the compounds (**1**−**5**) were purified from CR1642D on an Agilent 1100 series HPLC (Agilent Technologies) using a semipreparative Phenomenex Luna Phenyl-hexyl column (Luna, 25 cm × 10 mm, 5 μm particle size) and a Phenomenex Luna C_{18} HPLC column (250 × 10 mm, 5 μm particle size). Optical rotations were obtained using a Jasco polarimeter.

Culturing. The isolated strain CR1642D is deposited at INBio, Costa Rica. Agar plugs of CR1642D were initially grown at 25 °C on yeast malt agar plates supplemented with 30 μg/mL streptomycin and 12 μg/mL chlortetracycline. After one week, three macerated agar plugs from these plates were placed in 75 mL of rich seed medium [tryptone peptone (5 g/L), dextrose (10 g/L), yeast extract (3 g/L), and malt extract (10 g/L)] in a 125 mL Erlenmeyer flask (×2) with a pH value of 6.2. The culture was grown at 25 °C and 150 rpm for 6 days. Then 150 mL of 0.66% (w/v) malt extract and 5 g of HP-20 resin were added to each Erlenmeyer (250 mL each, × 8), which was inoculated with 15 mL of the rich seed media, and the fungi were cultured under the same conditions for 16 days. The fungal cultures were then held at 25 °C without shaking for 5 days.

Sequencing and Species Identification. For identification by internal transcribed spacer sequencing, CR1642D was cultured in the above-mentioned rich seed medium (see the "Culturing" section) for 6 days. Mycelia were then retrieved by filtration and ground to a fine powder in liquid N_2. Genomic DNA was extracted using the SurePrep

Table 2. Assay Results of Compounds 1−5[a]

	1			2			3			4			5		
	IC_{50} (μM)[b]	IC_{50} (μM)[c]	diff[d]	IC_{50} (μM)[b]	IC_{50} (μM)[c]	diff[d]	IC_{50} (μM)[b]	IC_{50} (μM)[c]	diff[d]	IC_{50} (μM)[b]	IC_{50} (μM)[c]	diff[d]	IC_{50} (μM)[b]	IC_{50} (μM)[c]	diff[d]
Dox40[e]	15	21.6	6.7	11.6	24.2	12.6	2.2	2.3	0.1	22.8	36.9	14.1	10	13.9	3.9
Farage[f]	12.4	10.5	−1.9	199.8	38.7	161.1	1.6	1.3	−0.3	15.6	14.8	−0.8	3.5	3.3	−0.3
H929[e]	54.5	35.2	−19.3	187.5	94.8	−92.7	10.2	3.4	−6.8	42.5	22.9	−19.6	10.8	5.4	−5.4
HT[f]	10.8	9.9	−0.9	100.0	34.9	−65.2	1.2	1.3	0.1	ND	ND		2.1	4.0	1.9
KMS34[e]	22.6	55.6	33	152.9	199.1	46.1	5.5	9.3	3.8	ND	ND		ND	ND	
KU812F[g]	14.6	24.2	9.5	44.9	95.9	51.0	2.7	3.6	0.9	24.6	46.6	22	7.1	14.3	7.2
L363[e]	66.0	79.4	13.4	464.2	198.6	265.6	16.7	94	−7.3	ND	ND		ND	ND	
MDA-MB-231[h]	43.4	34.2	−9.3	163.9	118.5	−45.4	8.5	5.5	−2.9	43.3	38.4	−4.9	18.1	13.3	−4.8
MM1S[e]	37.1	47.1	10.0	168.5	185.6	17.1	7.3	8.0	0.7	28.9	33.3	4.4	14.7	20.8	6.1
OCILY17R[f]	31.1	47.4	16.3	1616	293.4	−1323	4.6	5.2	0.6	ND	ND		ND	ND	
OCIMY5[e]	18.8	47.7	28.8	158.0	383.9	225.8	2.8	5.7	2.9	ND	ND		ND	ND	
OPM2[e]	12.7	10.5	−2.2	42.8	44.8	1.9	1.9	1.5	−0.3	ND	ND		4.8	7.5	2.6
PC3[i]	122.3	66.3	−56.0	1382.	216.0	1166.	34	14.1	−19.9	100.	71.7	−28.4	121	45.2	−75.4
RPMI8226[e]	12.9	8.9	−4	71.6	34	−37.6	2.4	1.2	−1.2	ND	ND		4.8	2.8	−2.0

[a]Activity of five compounds from the CR1642D extract across various tumor types in the presence and absence of bone marrow stromal cells. Tumor cells of various types were cultured in the presence and absence of HS-5 stromal cells treated with increasing concentrations of compounds. IC_{50} was calculated in both the presence and absence of stroma. [b]No stroma. [c]Plus stroma. [d]Difference in IC_{50} values. [e]Myeloma. [f]Lymphoma. [g]Leukemia. [h]Breast. [i]Prostate.

RNA/DNA/protein purification kit (Fisher Bioreagents), and large subunit rDNA was amplified by PCR using primers LR5 (5′-TCCTGAGGGAAACTTCG-3′) and LROR (5′-ACCCGCTGAACT-TAAGC-3′). PCR products were sequenced at Genewiz (http://www.genewiz.com/). The DNA sequence data obtained from the fungal strain CR1642D have been deposited at GenBank with accession number JQ778844.

Extraction and Separation. The cultures were filtered, the mycelial mat and HP-20 were extracted with 90% EtOH three times, and the extract was concentrated under vacuum. The crude extract (1.2 g) of CR1642D in 90% H_2O−MeOH was passed through a C_{18} SPE and then washed with MeOH. The MeOH wash was evaporated to dryness on a rotary evaporator, redissolved, and fractionated using a phenyl-hexyl column (2 mL/min; 70% CH_3CN for 20 min then to 100% CH_3CN in 10 min). Fractions 2, 4, 5, and 7 yielded compounds **4** (SC2-30-2, t_R 13.5 min, 1.0 mg/L), **3** (SC2-30-4, t_R 19 min, 19 mg/L), **1** (SC2-30-5, t_R 23.5 min, 1.3 mg/L), and **5** (SC2-30-7, t_R 27 min, 0.8 mg/L), respectively. Further purification of the most polar fraction, fraction 1, using a Phenomenex Luna C_{18} HPLC column (2 mL/min; 60% CH_3CN for 30 min then to 100% CH_3CN in 10 min) yielded compound **2** (SC2-32-3, t_R 22.5 min, 0.4 mg/L).

Penexanthone A (1): yellow powder; $[\alpha]^{23}_D$ −36 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.23), 242 (sh), 337 (4.30) nm; IR (film) ν_{max} 3419, 2966, 2934, 1744, 1605, 1562, 1435, 1410, 1373, 1324, 1229, 1048 cm^{-1}; ^1H NMR (600 MHz, CD_3CN) δ 7.30 (1H, d, J = 7.8 Hz, H-3′), 7.18 (1H, d, J = 8.4 Hz), H-3, 6.50 (1H, d, J = 7.8 Hz, H-2′), 6.40 (1H, d, J = 8.4 Hz, H-4), 5.65 (1H, s, H-5), 5.35 (1H, s, H-5′), 4.53 (1H, d, J = 13.2 Hz, H-12α′), 3.99 (1H, d, J = 12.6 Hz, H-12α), 3.90 (1H, d, J = 12.6 Hz, H-12β′), 3.60 (1H, d, J = 13.2 Hz, H-12β), 2.30−2.50 (6H, m, H-6, H-6′, H_2-7, H_2-7′), 2.06 (3H, s, 5-$OCOCH_3$), 2.03 (3H, s, 5′-$OCOCH_3$), 1.76 (3H, s, 12′-$OCOCH_3$), 1.03 (3H, d, J = 6.6 Hz, CH_3-11), 0.95 (3H, d, J = 6.6 Hz, CH_3-11′); ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) see Table 1; HRESIMS m/z 709.2126 ($[M + H]^+$ calcd for $C_{36}H_{37}O_{15}$, 709.2132).

Penexanthone B (2): yellow powder; $[\alpha]^{23}_D$ +2.5 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.89), 222 (sh), 267 (sh), 276 (3.28), 332 (3.90) nm; IR (film) ν_{max} 3420, 1741, 1602, 1441, 1230, 1044 cm^{-1}; ^1H NMR (600 MHz, acetone-d_6) and ^{13}C NMR (150 MHz, acetone-d_6) see Table 1; HRESIMS m/z 377.1243 ($[M + H]^+$ calcd for $C_{19}H_{21}O_8$, 377.1236).

Biological Assay. As previously described,[1] stromal cells were plated and allowed to seed overnight for nonadherent tumor experiments. The following day, tumor cells were overlaid and treated with natural product extracts. Following 48 h of incubation, luciferin substrate was added, cultures were incubated for 30 min, and the bioluminescence signal was read on an Envision or Luminoskan luminometer. For adherent tumors, stromal cells were counted and plated with tumor cells, both allowed to seed overnight and treated with extracts the following day. Cultures were again incubated for 48 h in the presence of extracts, and the bioluminescence signal was read on a luminometer following addition of luciferin substrate. Conditions were normalized to each respective nontreated control, and each condition was performed in quadruplicate. High-throughput screening of natural product extract libraries was performed by screening MM.1S multiple myeloma tumor cells in the presence and absence of HS-5 bone marrow stromal cells. Natural product hits were identified as those extracts that resulted in enhanced antitumor killing in the presence of BMSCs compared to their absence, with minimal cytotoxic activity against the HS-5 BMSCs alone. Tumor cells were cocultured with stromal cells for 48 h unless otherwise stated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra of penexanthone A (**1**) and penexanthone B (**2**). These materials are available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

D.W.M. is a Founder and equity holder in Axios Biosciences. C.S.M. has received in the past Consultant honoraria from Millennium Pharmaceuticals, Novartis Pharmaceuticals, Bristol-Myers Squibb, Merck & Co., Kosan Pharmaceuticals, Pharmion and Celgene, licensing royalties from PharmaMar, and research funding from Amgen Pharmaceuticals, AVEO Pharma, EMD

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