

Antiproliferative Constituents of Geopropolis from the Bee *Melipona scutellaris*

Authors

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Key words

- Clusiaceae
- geopropolis
- *Melipona scutellaris*
- cytotoxicity
- coumarins

Abstract

Fractionation of geopropolis from *Melipona scutellaris*, guided by antiproliferative activity against two colon cancer cell lines (COLO205 and KM12), led to the isolation of two new cinnamic acid esters, mammea-type coumarins 5,7-dihydroxy-6-(3-methyl-2-butenyl)-8-(4-cinnamoyl-3-methyl-1-oxobutyl)-4-propyl-coumarin (**1**) and 5,7-dihydroxy-6-(4-cinnamoyl-3-methyl-1-oxobutyl)-4-phenylcoumarin (**2**), along with five known coumarins, mammeigin (**3**), hydroxymammeigin (**4**), mammeisin (**5**), cinnamoyloxy-mammeisin (**6**),

and mammein (**7**), and the prenylated benzophenone *ent*-nemorosone (**8**). Among the isolated compounds, **5** and **7** showed the highest cell growth inhibition against COLO205 (GI₅₀ 9.7 and 10.7 μM, respectively) and KM12 (GI₅₀ 12.0 and 10.9 μM, respectively). The presence of these compounds suggests that plants of Clusiaceae family, especially the genera *Kielmeyera* and *Clusia*, are likely to be major sources of geopropolis produced by *M. scutellaris*.

Supporting information available online at <http://www.thieme-connect.de/products>

Introduction

Propolis, a resin collected by bees from several plants, has been reported to possess a great variety of biological activities. The wide range of activities is a consequence of its complex chemical composition, which can vary according to plant source, season, and bee species [1,2]. Most of the studies available in the international literature concern propolis collected by *Apis mellifera*, whereas other types of propolis collected by different species of bees have been sparsely studied. *Melipona scutellaris* Latreille 1811, a native Brazilian stingless bee, is an important pollinator and recently has been considered threatened. This primitive bee produces a different type of propolis made of plant resins, wax, and soil, called geopropolis [3]. Our group has previously demonstrated a promising range of biological activities including anti-inflammatory [4], antimicrobial as well as antiproliferative [5]. Our previous studies have suggested the presence of cinnamic acid derivatives as well as prenylated compounds in this geopropolis [5], although no study has yet described the chemical composition of geopropolis from *M. scutellaris*. Therefore, a bioassay-guided fractionation and isolation based on the antiproliferative activity against colon cancer cell lines was undertaken, which yielded two new compounds and six known compounds. The compounds were tested in the NCI 60-cell screen in order to assess their cytotoxic profile.

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Results and Discussion

In order to isolate and identify the compounds present in geopropolis, we carried out a fractionation of the ethanolic extract of geopropolis (EEGP) from *M. scutellaris* guided by growth inhibitory activity against the colon cancer cell lines COLO205 and KM12. Bioguided fractionation of the EEGP using diol, Sephadex LH-20, and normal-phase HPLC separation led to the isolation of one new 4-propyl coumarin (**1**), one new 4-phenyl coumarin (**2**), five known coumarins (**3–7**), and one known benzophenone (**8**) (● Fig. 1). The structures were determined by spectroscopic analysis, including 1D and 2D NMR (COSY, HSQC and HMBC) and HRESIMS experiments. The structures of the known compounds were determined by comparing their spectroscopic data with the literature.

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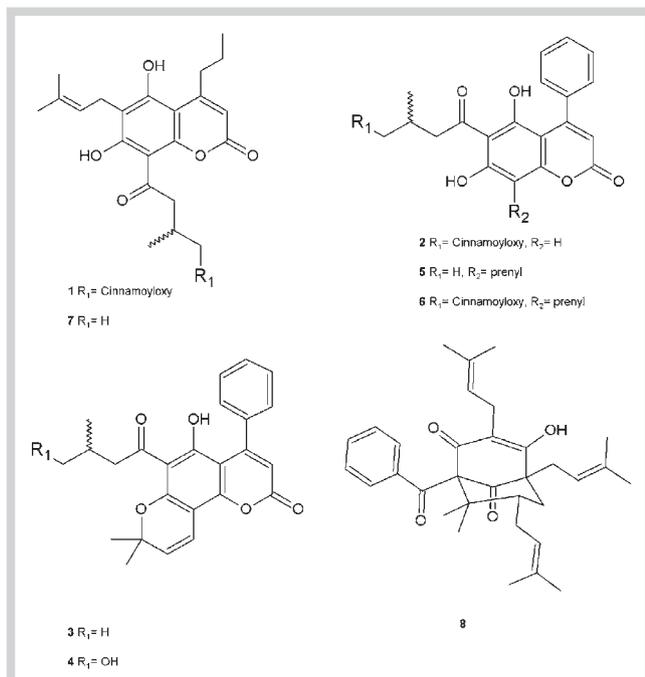


Fig. 1 Chemical structures of compounds **1–8** isolated from *M. scutellaris* geopropolis.

Compound **1** was isolated as a yellowish powder with $[\alpha]_D^{27} - 0.8$ (c, 0.1, MeOH). The HREIMS of **1** showed a molecular peak ion at m/z 517.2269 $[M - H]^-$ supporting a molecular composition of $C_{31}H_{34}O_7$, with 15 degrees of unsaturation. On the basis of the 1H and ^{13}C NMR spectra and HMBC correlations of **1** (Table 1, Fig. 2), it was possible to observe a characteristic singlet at δ_H 5.99 and δ_C 108.4 (C-3), which correlated to a carbonyl at C-2 (δ_C 160.8) and to an aromatic carbon at C-4a (δ_C 103.7), along with two hydroxyl groups at the carbons C-5 and C-7 at δ_C 161.6 and δ_C 165.6, respectively, suggesting a 5,7-dihydroxy coumarin skeleton [6, 7]. In addition, the NMR data also showed the presence of an acyl side chain characterized by the presence of 1H - 1H COSY and HMBC correlations between the methylene group at H-2' (dd, δ_H 3.20, $J = 15.5, 16.0$ Hz and δ_H 3.38, $J = 15.5, 7.4$ Hz, respectively), the methine at H-3' (m, δ_H 2.66), the methyl group at H-5' (d, δ_H 1.13, $J = 6.8$ Hz), and the methylene at H-4' (t, δ_H 4.20, $J = 6.1$ Hz). The cinnamoyl moiety linked to C-4' (δ_C 68.9) was observed by the presence of the protons H-7' (d, δ_H 6.37, $J = 16.0$ Hz) and H-8' (d, δ_H 7.55, $J = 16.0$ Hz) showing long-range correlations to the aromatic ring signals at C-9' (δ_C 134.5) and C-10' (δ_C 127.9). The attachment of this acyl side chain at C-8 was proposed due to the small bathochromic shift in the UV spectra after alkali addition, as described for other acylcoumarins [7].

The presence of a prenyl group at position 6 was supported by the characteristic doublets at δ_H 3.29 ($J = 7.2$ Hz, H-1'') correlating to the hydroxyl carbons C-5 (δ_C 161.6) and C-7 (165.6) (Table 1). The signal assigned to H-2'' (m, δ_H 5.02), which exhibits long-range correlations to the vinylic carbon at δ_C 131.8 (C-3'') and the methyl groups at δ_C 24.7 (C-4'') and δ_C 16.7 (C-5''), confirmed the prenyl moiety. Besides that, compound **1** showed a propyl group at position 4 of the 5,7-dihydroxy coumarin ring, suggested by the HMBC correlations between the methylene protons at δ_H 3.01 (t, $J = 7.2$ Hz, H-1''') and the carbons C-3 (δ_C 108.4) and C-4a (δ_C 103.7), along with the long-range correlation between the

proton at H-3 (s, δ_H 5.99) to the methylene carbon at C-1''' (δ_C 38.9). The additional signals at δ_H 1.65 (m, H-2''') and δ_H 1.62 (t, $J = 7.3$ Hz, H-3''') confirmed the structure of the propyl group placed at position 4. All of these assignments led to the structure of **1** as 5,7-dihydroxy-6-(3-methyl-2-butenyl)-8-(4-cinnamoyl-3-methyl-1-oxobutyl)-4-propyl-coumarin, a cinnamoyl ester of **7** (mammein).

Compound **2** was isolated as a yellowish powder with $[\alpha]_D^{27} - 5$ (c, 0.1, MeOH). The HREIMS of **2** showed a molecular ion at m/z 485.1602 $[M + H]^+$ supporting a molecular composition of $C_{29}H_{24}O_7$, with 18 degrees of unsaturation. The 1H and ^{13}C NMR spectra (Table 1, Fig. 2) of **2** showed characteristic signals of the 5,7-dihydroxy coumarin skeleton, as described for compound **1**. An acyl side chain was characterized by the COSY 1H - 1H correlations between the proton H-3' (m, δ_H 2.68) to the methylene groups H-4' (qd, δ_H 4.21, $J = 10.8, 6.2$ Hz) and H-2' (dd, δ_H 3.23, $J = 15.8, 7.3$ Hz and δ_H 3.42, $J = 15.8, 6.1$ Hz, respectively), and an isolated methyl resonating at δ_H 1.03 (d, $J = 6.8$ Hz, H-5'). The HMBC correlations of the protons H-3' and H-2' to C-1' (δ_C 204.1) supported the position of the carbonyl group linked to C-6 (δ_C 103.6) on the dihydroxy 4-phenyl coumarin skeleton. The O-cinnamoyl group was characterized by the trans-vinyl protons at δ_H 6.43 (d, $J = 16.0$ Hz, H-7') and δ_H 7.60 (d, $J = 16.0$ Hz, H-8'), which correlate to the aromatic carbon at δ_C 134.4 (C-9') and the ester carbon at δ_C 167.0 (C-6'). In contrast to compound **1**, which showed a prenyl group at position 8, compound **2** exhibited no substituent at this position, which was confirmed by the presence of a singlet at δ_H 6.06 (H-8) correlated to the aromatic carbon C-4a (δ_C 102.3). The large bathochromic shift after alkali addition confirmed the position of the side chain at C-6 [7]. On the basis of these assignments, the structure of **2** was established as 5,7-dihydroxy-6-(4-cinnamoyl-3-methyl-1-oxobutyl)-4-phenyl-coumarin.

The other known compounds were isolated and their structures were determined by comparing spectroscopic data with literature values. They were identified as mammeigin (**3**) [8,9], hydroxymammeigin (**4**) [9], mammeisin (**5**) [6], cinnamoyloxymammeisin (**6**) [7], mammein (**7**) [10], and the benzophenone ent-nemorosone (**8**) [11]. All compounds were tested in the NCI 60-cell panel at an initial concentration of 10^{-5} M. As shown in Table 2, at this concentration, compounds **5** and **7** showed a higher mean percent of inhibition, with 56 and 83% growth inhibition, respectively, and were submitted to the full five-dose screen; however, their cell line selectivity was modest (see Supporting Information). Nonetheless, a COMPARE [12,13] study demonstrated a substantial correlation between the cell growth inhibition pattern of the crude geopropolis extract and that of compounds **5** and **7** (Table 3). Some studies have demonstrated that the antiproliferative activity of synthetic coumarins might be attributed to the presence of the hydroxyl group at C-7 [14]. However, there is no report about the influence of phenyl or propyl at C-4 and how those groups would change the activity of these compounds. In the same way, the presence of the cinnamoyl moiety seems to reduce the antiproliferative activity of those coumarins.

Both coumarins and benzophenones have been reported to have antiproliferative activity [15–17]. However, the cinnamic acid esters of coumarins have no reported biological activity.

The elucidation of the compounds present in geopropolis hints at the possible botanical origin of the geopropolis. The known coumarins reported herein were previously isolated from plants of the genus *Mammea* (Clusiaceae) [6] and recently reported as ma-

1				2		
Pos.	δ_C , type	δ_H , m (J in Hz)	HMBC	δ_C , type	δ_H , m (J in Hz)	HMBC
2	160.8, C			160.0, C		
3	108.4, CH	5.99, s	2, 4a, 1'''	110.4, CH	5.91, s	2, 4a, 1''
4	142.6, C			157.9, C		
4a	103.8, C			102.3, C		
5	161.6, C			163.7, C		
6	93.0, C			103.6, C		
7	165.5, C			167.2, C		
8	112.3, C			99.3, CH	6.06, s	4a
8a	156.7, C			158.2, C		
1'	204.1, C			204.0, C		
2'	48.4, CH ₂	3.20, dd (15.5, 6.0) 3.38, dd (15.5, 7.4)	1', 3', 4', 5'	48.1, CH ₂	3.23, dd (15.8, 7.3) 3.42, dd (15.8, 6.1)	1', 3', 5', 4'
3'	30.6, CH	2.66, m	2', 4', 5'	30.4, CH	2.68, m	1', 2', 5', 4'
4'	69.0, CH ₂	4.20, t (6.1)	2', 3', 4', 6'	68.9, CH ₂	4.21, t (6.1)	2', 3', 5', 6'
5'	16.3, CH ₃	1.13, d (6.8)	2', 3', 5'	16.3, CH ₃	1.03, d (6.8)	2', 3', 4'
6'	167.2, C			167.2, C		
7'	117.4, CH	6.37, d (16.0)	6', 8', 9'	117.4, CH	6.43, d (16.0)	6', 8', 9'
8'	144.8, CH	7.55, d (16.0)	6', 7', 9', 10', 14'	144.9, CH	7.60, d (16.0)	5', 7', 9', 10', 14'
9'	134.4, C			134.5, C		
10'/14'	128.0, CH	7.53	8', 12'	128.0, CH	7.57	
11'/13'	128.7, CH	7.39*	9'	128.7, CH	7.39*	
12'	130.1, CH	7.38*		130.2, CH	7.35*	
1''	21.0, CH ₂	3.29, d (7.2)	5, 7, 8, 2'', 12	139.9, C		
2''	121.7, CH	5.02, m	1'', 5'', 3'', 4''	127.0, CH	7.27*	
3''	131.8, C			127.2, CH	7.34*	
4''	24.6, CH ₃	1.65, s	2'', 3'', 5''	127.8, CH	7.34*	
5''	16.8, CH ₃	1.75, s	2'', 3'', 4''	127.2, CH	7.34*	
6''				127.0, CH	7.27*	
1'''	38.9, CH ₂	3.01 t (7.2)	3, 4a, 5, 2''', 3'''			
2'''	23.2, CH ₂	1.65, m	1''', 3'''			
3'''	13.1, CH ₃	1.02, t (7.3)	1''', 2'''			

* Overlapped signals

major components of *Kielmeyera* (Clusiaceae) [7–9]. Ent-nemorosone (**8**) was recently synthesized; its enantiomer nemorosone is well known from the Clusiaceae [11]. The genus *Kielmeyera* is native to the state of Bahia, which is the place of collection of our geopropolis samples, which leads us to propose that the bees preferentially visit Clusiaceae plant species in this area to collect the resin with which they make geopropolis. The NCI 60 data also supports this preference, as a COMPARE study using the geopropolis extract data as a seed returned a predominance (10/14) of Clusiaceae extracts out of all plant extracts tested, with Pearson coefficients of > 0.60 (Table 1 S, Supporting Information). Interestingly, the presence of prenylated benzophenones from Clusiaceae was previously reported in *A. mellifera* propolis type 6 [18], indicating that bees from different species may utilize the same plant sources for collecting propolis. Also, the type of coumarins we found have been reported as insecticidal compounds, indicating that the bees may use coumarin-containing resins to protect the hive from intruding insects [19]. Further, this is the first report describing coumarins as major components of any kind of Brazilian propolis. Last, most of the compounds described here have no previous report of biological activity, so this is the first report on their pharmacological properties.

Materials and Methods

General procedures

Optical rotations ($[\alpha]_D$) were measured on a Perkin-Elmer 241 polarimeter in a 100 × 2 mm cell (units 10⁻¹ deg cm² g⁻¹). LCMS data were obtained using a Hewlett-Packard Series 1100 MSD, whereas HREIMS 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. The NMR experiments were performed on a Bruker 600 MHz NMR spectrometer. ¹H and ¹³C spectra were referenced to deuterated solvent peaks. The 60 × 2.5 cm i. d. Sephadex LH-20 columns attached to a model UA-6 UV detector and Foxy 200 fraction collector (Teledyne Isco) were used for fractionation of the extract, whereas purification of the compounds was performed using a Varian ProStar 210/215 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 were of analytical grade.

Extraction and isolation

Crude samples of geopropolis from *M. scutellaris* (native stingless bee) were obtained from the coastal area of the city of Entre Rios (12°22'S and 37°54'W), state of Bahia, Northeast Brazil. Samples of *M. scutellaris* bee were deposited in the Paulo Nogueira Neto

Table 1 ¹H and ¹³C NMR data for compounds **1** and **2** (600 and 150 MHz, CD₃OD).

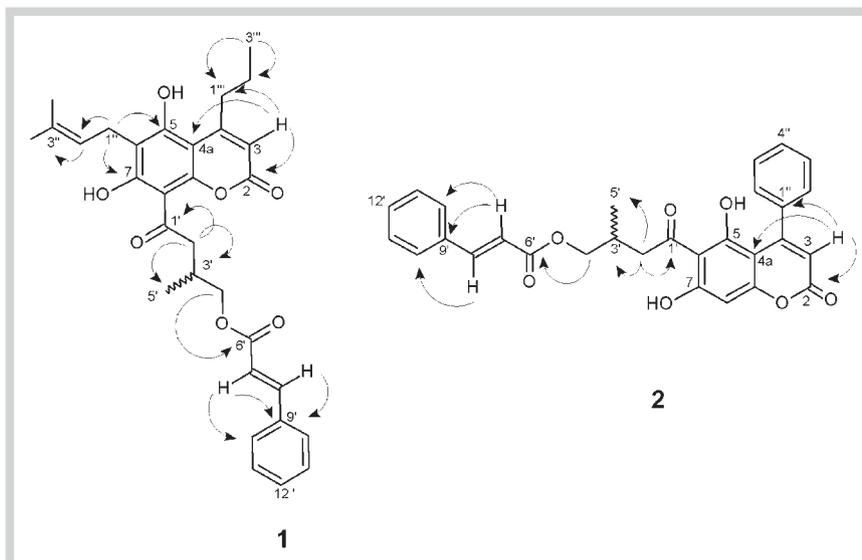


Fig. 2 Key HMBC correlations for compounds **1** and **2**.

Compound	Mean percent inhibition at 10^{-5} M	Percent range at 10^{-5} M	GI ₅₀ against COLO205 (μ M)	GI ₅₀ against KM12 (μ M)
1	0	42	NT ^a	NT
2	4	55	NT	NT
3	0	28	NT	NT
4	8	46	NT	NT
5	56	126	9.7	12.0
6	21	73	NT	NT
7	83	103	10.7	10.9
8	7	48	NT	NT
Adriamycin ^b	40.0 ± 1.3^b (n = 16)	105.5 ± 5.6^b	0.098 (n = 2)	0.162 (n = 2)

Table 2 Cytotoxicity of compounds in the NCI 60-cell screen.

^a NT: Not tested; ^b one dose test at 2.5×10^{-7} M

Table 3 Pearson correlation coefficients at the GI₅₀ level for geopropolis extract and compounds **5** and **7** in the NCI 60-cell screen.

	N192723 extract	5	7
N192723 extract	1	0.73	0.73
5	0.73	1	0.85
7	0.73	0.85	1

Entomological Collection of the Biosciences Institute at the State University of São Paulo (CEPANN – IBUSP, Brazil) and identified under the voucher number CEPANN 42.863. In addition, this research had authorization and remittance of genetic heritage components granted by the Brazilian National Council of Technological and Scientific Development – CNPq # 010666/2014–1. Geopropolis samples were extracted using ethanol 70% (1 : 7, w/v) and dried as described elsewhere [20]. Two grams of this ethanolic extract of geopropolis (EEGP, NSC# N192723) was coated on diol bonded phase media and eluted with a series of solvents of increasing polarity (hexane, dichloromethane, ethyl acetate, acetone, and methanol) yielding five fractions (A–E) of 40, 500, 170, 60, and 870 mg, respectively. Fraction B was the most active and was selected for further fractionation and isolation of compounds.

Fraction B (89.6 mg) was chromatographed on a 60×2.5 cm i.d. Sephadex LH-20 column and eluted with $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1 : 1, v/v), with 300 drop fractions collected in each tube. On the basis

of TLC and UV traces, they were combined into three fractions (B1, B2, and B3). Fractions B2 and B3 showed activity and were further purified by HPLC. The compounds were isolated using a semipreparative (10×250 mm, $5 \mu\text{m}$) cyano column with a hexane/isopropanol gradient (0–3 min: 95% hexane; 3–24 min: 95–80% hexane, 24–26 min: 80% hexane, 26–29 min: 80–95% hexane, 29–31 min: 95% hexane, flow rate 4 mL/min) as the solvent and the UV detector at $\lambda = 230$ nm. One unknown 4-propyl-coumarin, **1** (0.4 mg), one unknown 4-phenyl-coumarin, **2** (1.1 mg), five known coumarins, **3** (1.5 mg), **4** (2.2 mg), **5** (1.5 mg), **6** (11.4 mg), and **7** (0.5 mg), and the benzophenone **8** (0.6 mg) were obtained.

Isolates

5,7-dihydroxy-6-(3-methyl-2-butenyl)-8-(4-cinnamoyl-3-methyl-1-oxobutyl)-4-propylcoumarin (**1**; NSC# 781 047): yellow solid; $[\alpha]_D^{27} - 0.8$ (c, 0.1, MeOH); ^1H and ^{13}C (600 MHz, CD_3OD) NMR data, see **Table 1**; HREIMS $[\text{M} - \text{H}]^-$ at m/z 517.2269 (calcd. for $\text{C}_{31}\text{H}_{33}\text{O}_7$, 517.2232).

5,7-dihydroxy-6-(4-cinnamoyl-3-methyl-1-oxobutyl)-4-phenylcoumarin (**2**; NSC# 781 048): yellow solid; $[\alpha]_D^{27} + 5$ (c, 0.1, MeOH); ^1H and ^{13}C (600 MHz, CD_3OD) NMR data, see **Table 1**; HREIMS $[\text{M} + \text{H}]^+$ at m/z 485.1602 (calcd. for $\text{C}_{29}\text{H}_{25}\text{O}_7$, 485.1595). *Mammeigin* (**3**; NSC# 781 045): a yellow solid; $[\alpha]_D^{27} - 0.8$ (c, 0.12, MeOH); the ^1H and ^{13}C NMR data were identical with those reported elsewhere [8,9].

Hydroxymammeigin (4; NSC# 781 050): a yellow solid; $[\alpha]_D^{27} + 6.1$ (c, 0.23, MeOH); the ^1H and ^{13}C NMR data were identical with those reported elsewhere [9].

Mammeisin (5; NSC# 781 046): a yellow solid; $[\alpha]_D^{27} - 0.8$ (c, 0.63, MeOH); the ^1H and ^{13}C NMR data were identical with those reported elsewhere [6].

Cinnamoyloxy-mammeisin (6; NSC#781051): a yellow solid; the ^1H and ^{13}C NMR data were identical with those reported elsewhere [7].

Mammein (7; NSC# 781 049): a yellow solid; the ^1H and ^{13}C NMR data were identical with those reported elsewhere [10].

ent-Nemorosone (8; NSC# 781 044): a white solid; $[\alpha]_D^{27} - 45$ (c, 0.05, CHCl_3); the ^1H and ^{13}C NMR data were identical with those reported recently [11].

Cytotoxicity assay on colon cancer cells

The isolation of the compounds was bioguided by the activity against colon cancer cell lines COLO205 and KM12 in a two-day drug exposure with a formazan (XTT) endpoint, developed by the MTL Assay Development and Screening Section. Cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum, and held at 37 °C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air. Cells used in the assay were harvested with RPMI-1640 medium, without phenol red, and supplemented with 2 mM L-glutamine and 10% fetal bovine serum without antibiotics. After harvest, cells were counted using a Cellometer Auto T4 cell counter, plated in 384-well flat-bottom polystyrene microtiter plates at a density of 5000 cells/well and then were incubated in a 5% CO_2 , 95% air, and 37 °C incubator for 24 h. After incubation, 2-fold serial dilutions of the samples were added to plates using a Biomek FX robotic liquid handling workstation. After a 48-h incubation period, cell viability was accessed with a formazan (XTT reagent) endpoint [13].

NCI 60 data was generated as previously reported [12]. The positive control standard was adriamycin (NSC#123 127). The historic mean GI_{50} value for the control was 93.5 nM (n = 1816).

Supporting information

NMR spectra of compounds **1** and **2** and NCI 60 data for all compounds are available as Supporting Information.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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