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TETRAHEDRON

Cytotoxic Constituents of the Roots of *Exostema acuminatum*

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Abstract—Bioassay-guided phytochemical investigation of the roots of *Exostema acuminatum* (Rubiaceae) using human oral epidermoid carcinoma (KB) cells as a monitor, led to the isolation of two norditerpenoids, namely, (16*R*)-*ent*-16,17-dihydroxy-19-nor-kaur-4-en-3-one (**1**) and (16*S*)-*ent*-16,17-dihydroxy-19-nor-kaur-4-en-3-one (**2**), and six previously known 4-phenylcoumarins (**3**–**8**). The structure and relative stereochemistry of the novel compound **1** were determined by X-ray crystallography. All isolates were tested against a panel of human tumor cell lines, and the 4-phenylcoumarins showed significant cytotoxicity, with 3′-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (**5**) and 8-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (**6**) exhibiting the most potent activity. Two of the 4-phenylcoumarins, 5,7,4′-trimethoxy-4-phenylcoumarin (**3**) and **6**, were evaluated in an in vivo P388 murine leukemia model.

Introduction

Exostema acuminatum Urb. (Rubiaceae), is a tree indigenous to Central America and northern South America, for which there is no information in the literature on its biological activity or constituents. The stem bark of some species of this genus, such as *Exostema caribaeum* and *Exostema mexicanum*, are used in traditional folk medicine as antimalarial agents.[1] The crude extract of *E. caribaeum* has been reported to exhibit antimalarial activity,[2] a blood glucose-lowering effect in diabetic mice,[3] and chloroplast DNA restriction site mutations.[4] The bark of *E. caribaeum* has afforded several phenylcoumarins and phenylcoumarin glycosides.[1,5,6] In addition, triterpenoids have been isolated from *E. mexicanum*[7] and *E. coulteri*.[8] Among the constituents of plants in this genus, 5-*O*-β-D-glucopyranosyl-3′,4′-dihydroxy-7-methoxy-4-phenylcoumarin and 7-methoxy-5,3′,4′-trihydroxy-4-phenylcoumarin, isolated from *E. caribaeum*, have been shown to inhibit ATP synthesis and proton uptake in spinach chloroplasts.[9] 5-*O*-β-D-Glucopyranosyl-3′,4′-dihydroxy-7-methoxy-4-phenylcoumarin, also isolated from *E. caribaeum*, has been found to act as an energy-transfer inhibitor in spinach chloroplasts.[10] Two other isolates from *E. caribaeum*, 8-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (exostemin I) and 5,7,8,4′-tetramethoxy-4-phenylcoumarin (exostemin III) were determined as inhibitors of adenosine 3′,5′-cyclic monophosphate (cAMP) phosphodiesterase.[11]

As a part of our ongoing program for the discovery of the anticancer agents from plants, a chloroform-soluble extract of the roots of *Exostema acuminatum* was found to exhibit significant cytotoxic activity against a panel of human cancer cell lines. Bioassay-guided phytochemical investigation of this extract, using human oral epidermoid carcinoma (KB) cells, led to the isolation of six cytotoxic 4-phenylcoumarins (**3**–**8**) together with two novel norditerpenoids, **1** and **2**, from an active extract. Compounds **3**–**8** were identified on the basis of physical and spectral data comparison with literature values.[1,4–6,9,10] The structure and relative stereochemistry of **1** were determined by X-ray crystallography. The structure of **2** was determined as a stereoisomer of **1** based on spectroscopic data, including a NOE difference experiment. These eight compounds, along with **9**, the methyl derivative of **5**, were evaluated for their activity against human cancer cells in culture. Based on their cytotoxic potency and availability, two of the phenylcoumarins, 5,7,4′-trimethoxy-4-phenylcoumarin (**3**) and 8-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (**6**), were evaluated in the in vivo P388 murine leukemia model. This paper deals

Keywords: *Exostema acuminatum*; Rubiaceae; norditerpenoids; 4-phenylcoumarins; X-ray crystallography; cytotoxic activity; in vivo evaluation.
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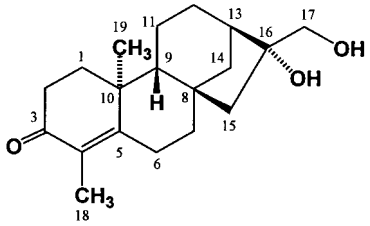
Table 1. ^{1}H and ^{13}C NMR data of compounds **1** and **2** (in $CDCl_3$; TMS as internal standard; spectra taken at 125 and 500 MHz for carbon and proton, respectively. Coupling values given in Hz; chemical shift values given in ppm)

	Compound **1**			Compound **2**	
Position	δ_C	δ_H	HMBC[a]	δ_C	δ_H
1	36.6 t	2.07 d (12.2) 1.76–1.50 (overlapped)	C-2, C-3 C-3, C-5, C-10	36.7 t	2.14 (overlapped) 1.67–1.47 (overlapped)
2	36.4 t	2.07 d (12.2) 1.80 dd (12.2, 4.0)	C-1, C-3 C-1, C-3, C-4, C-10	36.3 t	2.14 (overlapped) 1.86 m
3	199.1 s			199.1 s	
4	128.4 s			128.3 s	
5	163.9 s			164.0 s	
6	26.6 t	2.71 ddd (11.2, 3.1, 3.1) 2.24 brddd (11.2, 11.2, 2.8)	C-4, C-7, C-8, C-10 C-5, C-7	26.5 t	2.67 ddd (14.2, 3.3, 3.3) 2.17 m
7	39.1 t	1.76–1.50 (overlapped)	C-5, C-9	39.0 t	1.67–1.47 (overlapped)
8	40.6 s			40.6 s	
9	54.6 d	1.26 m	C-8, C-11, C-19	54.8 d	1.22 m
10	44.3 s			43.2 s	
11	18.8 t	1.76–1.50 (overlapped)	C-9, C-12	19.2 t	1.67–1.47 (overlapped)
12	25.9 t	1.76–1.50 (overlapped)	C-9, C-11, C-13	26.0 t	1.67–1.47 (overlapped)
13	44.9 d	2.15 m	C-11, C-16	40.7 d	2.20 m
14	33.2 t	2.48 m 2.35 m	 C-8, C-12	33.3 t	2.49 m 2.37 m
15	51.9 t	1.76–1.50 (overlapped)	C-8, C-14, C-16, C-17	51.4 t	1.67–1.47 (overlapped)
16	81.8 s			79.7 s	
17	66.1 t	3.81 d (11.0) 3.69 d (11.0)	C-16	69.8 t	3.52 d (10.7) 3.44 d (10.7)
18	11.1 q	1.78 s	C-3, C-4, C-5	11.1 q	1.73 s
19	19.9 q	1.29 s	C-1, C-2, C-5, C-10	20.0 q	1.31 s

[a] ^{13}C NMR signal correlation with ^{1}H resonance.

with the isolation, structural identification, and biological evaluation of these compounds.



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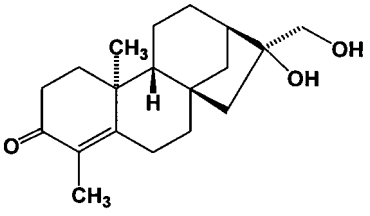

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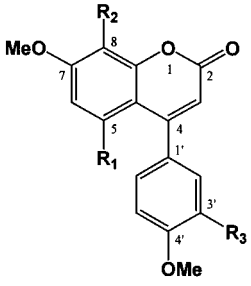

	R_1	R_2	R_3
3	OMe	H	H
4	OH	H	H
5	OMe	H	OH
6	OMe	OH	H
7	OMe	OH	OH
8	OH	H	OH
9	OMe	H	OMe

Results and Discussion

The HREIMS of compound **1** showed a molecular ion peak at *m/z* 304.2045, indicating an elemental formula of $C_{19}H_{28}O_3$. The EIMS showed a molecular ion peak at *m/z* 304 along with major fragment ions at *m/z* 286, 273, 197, 176, 150, 138, and 121. The presence of a cyclic α,β-unsaturated ketone moiety was suggested from the UV λ_{max} appearing at 209 nm. In the ^{13}C NMR spectrum of **1** (Table 1), 19 signals were observed, including a carbonyl at δ_C 199.1, two olefinic carbons at δ_C 163.9 and δ_C 128.4, two methyl carbons at δ_C 19.9 and δ_C 11.1, eight methylene

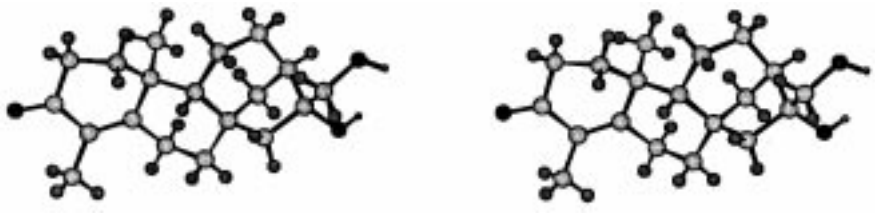

Figure 1. Stereochemical views of the structure and configuration of compound **1** as determined by X-ray diffraction.

Table 2. Cytotoxic activity of compounds **1**–**9** (results are expressed as ED_{50} values ($\mu g\ mL^{-1}$). Key to cell lines used: BC1=human breast cancer; Lu1=human lung cancer; Col2=human colon cancer; KB=human oral epidermoid carcinoma; KB-V^+=multidrug-resistant KB assessed in the presence of vinblastine (1 $\mu g\ mL^{-1}$); KB-V^-=multidrug-resistant KB assessed in the absence of vinblastine; LNCaP=hormone-dependent human prostate cancer; SW626=human ovarian cancer; SKNSH=human neuroblastoma cancer, M109=mouse lung cancer)

Compound	BC1	Lu1	Col2	KB	KB-V^+	KB-V^-	LNCaP	SW626	SKNSH	M109
1	>20	>20	13.7	>20	>20	>20	>20	>20	>20	>20
2	14.8	>20	>20	7.7	11.9	14.5	4.8	10.8	6.1	7.4
3	10.1	>20	5.5	2.5	6.1	7.0	>20	>20	4.7	>20
4	6.1	6.3	4.8	3.3	4.1	3.9	>20	>20	4.1	6.6
5	5.0	0.6	0.8	NT[a]	0.1	0.09	NT[a]	0.63	0.03	0.05
6	0.5	1.4	0.1	0.02	0.1	0.1	>20	>20	0.2	0.1
7	1.6	4.1	1.8	0.2	1.8	1.8	0.3	0.4	0.7	2.1
8	4.3	7.9	4.8	0.3	1.8	2.2	0.3	0.5	1.8	1.4
9	4.0	0.6	2.9	0.1	0.1	0.2	>20	0.06	0.6	1.0

[a] NT: not tested.

carbons (δ_C 51.9, 39.1, 36.6, 36.4, 33.2, 26.6, 25.9, and 18.8), a hydroxymethyl carbon at δ_C 66.1, and two methine carbons at δ_C 54.6 and δ_C 44.9. The index of hydrogen deficiency was determined as six, suggesting the presence of four rings in the structure of **1** accompanied by a double bond and a ketone. The HMBC spectrum showed cross-peaks between the H-18 methyl protons signal and the C-3 carbonyl carbon and the C-4 and C-5 olefinic carbons, as well as cross-peaks between the H-19 methyl proton signal and C-1, C-2, C-5, and C-10. These interactions gave evidence for the relative positions of the methyl groups as well as the functionality of ring A, which included an α,β-unsaturated ketone moiety. Further correlations in the HMBC spectrum between H-15 and C-8, C-14, C-16, and C-17, and between H-13 and C-16, supported the presence in the molecule of **1** of a five-membered ring moiety with both a hydroxyl group and a hydroxymethyl group affixed to C-16. Further HMBC correlations between H_2-6 and C-4, C-5, C-7, C-8, and C-10, between H-7 and C-5 and C-9, between H-9 and C-8, C-11, and C-19 and between H-12 and C-9, C-11 and C-13 were consistent with rings B and C both being six-membered rings. Since data obtained from the COSY NMR spectrum were not additionally informative, the unambiguous structure and relative stereochemistry of the novel isolate **1** were determined finally by single-crystal X-ray crystallography (Fig. 1) as (16*R**)-16,17-dihydroxy-19-nor-kaur-4-en-3-one.

Compound **2** showed a molecular ion peak at *m/z* 304.2035, indicating the same elemental formula as **1** ($C_{19}H_{28}O_3$). The EIMS of **2** exhibited an identical spectrum to **1**. The ^{13}C NMR spectrum of **2** (Table 1) was closely comparable to that of **1**, but showed upfield shifts for C-13, C-15, and C-16 (4.2, 0.5, and 2.1 ppm, respectively), accompanied by downfield shifts for C-17 (3.7 ppm). In addition, in an NOE difference experiment on **2**, no NOE was observed between the H-17 methylene proton and H-13, although a positive NOE was observed between these protons in the case of **1**. Accordingly, it was inferred that the only difference between **2** and **1** was the opposite stereochemistry at C-16. Thus, the structure of compound **2** was determined as (16*S**)-16,17-dihydroxy-19-nor-kaur-4-en-3-one.

Owing to the greatly predominant occurrence of this enantiomeric form in the plant kingdom, compounds **1** and **2** have been assigned to the *ent*-kaurene series, as (16*R*)-*ent*-16,17-dihydroxy-19-nor-kaur-4-en-3-one and (16*S*)-*ent*-16,17-dihydroxy-19-nor-kaur-4-en-3-one, respectively. 19-Nor-kaurene derivatives appear to be extremely rare[12–14] and compound **1** is the first structure of this type to have been supported by X-ray crystallography.

Compounds **3**–**8** were identified on the basis of physical and spectral data comparison with literature values as 5,7,4′-trimethoxy-4-phenylcoumarin,[15] 7,4′-dimethoxy-5-hydroxy-4-phenylcoumarin,[16] 3′-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin,[17] 8-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (exostemin I),[5] 8,3′-dihydroxy-5,7,4′-trimethoxy-4-phenylcoumarin,[18] and 5,3′-dihydroxy-7,4′-dimethoxy-4′-phenylcoumarin,[6] respectively. Also, the methyl derivative of **5**, the permethylated 5,7,3′,4′-tetramethoxy-4-phenylcoumarin (**9**), was prepared for biological testing.

As summarized in Table 2, compounds **1**–**9** were evaluated against a panel of human tumor cell lines. The 4-phenylcoumarins **3**–**9** showed good activity against most cell lines, with 3′-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (**5**) and 8-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (**6**) showing the most potent activity. On the basis of their availability and cytotoxic potency, two of the phenylcoumarins, 5,7,4′-trimethoxy-4-phenylcoumarin (**3**) and 8-hydroxy-5,7,4′-trimethoxy-4-phenylcoumarin (**6**) were evaluated in the in vivo P388 murine leukemia model (injected ip once daily for five days). However, neither compound was active (T/C ≥125%) at the doses tested.

Experimental

General procedures

Melting points were determined using a Fisher-Johns melting point apparatus, and are uncorrected. Optical rotations were obtained on a Perkin-Elmer model 241 polarimeter. UV spectra were measured on a Beckman DU-7 spectrometer. IR spectra were taken on a Nicolet MX-1 FT-IR spectrophotometer. ^1H NMR, ^{13}C NMR, and ^1H-^1H COSY (including DEPT) spectra were measured on a Bruker DRX-500 instrument operating at 500.1 and 125.7 MHz, respectively. Compounds were analyzed in $CDCl_3$, with tetramethylsilane (TMS) as internal standard. ^{13}C NMR multiplicity was determined using DEPT experiments.

EIMS and HREIMS were recorded on a Finnigan MAT-90 instrument.

Plant material

The roots of *Exostema acuminatum* were collected at Llanura Oriental, in the Dominican Republic, in January 1996. A voucher specimen (A3303) has been deposited at the Field Museum of Natural History, Chicago, IL.

Extraction and isolation

The dried roots of *E. acuminatum* (450 g) were extracted three times with MeOH at room temperature, and the solution was evaporated in vacuo, with the dried MeOH extract resuspended in 10% MeOH in H_2O and partitioned with hexane (3×300 mL). On drying, the aqueous MeOH layer was redissolved in H_2O, and partitioned with 20% MeOH/$CHCl_3$ (3×300 mL). The 20% MeOH/$CHCl_3$ partition was combined and washed with 1% NaCl solution twice. The cytotoxic 20% MeOH/$CHCl_3$-soluble extract (12.5 g; ED_{50} 1.7 μg mL^{-1} against the KB cell line) was subjected to Si gel column chromatography and eluted using mixtures of $CHCl_3$/MeOH (50:1–4:1) to give eight fractions. Fractions 2–5 were active in the KB cytotoxicity assay (ED_{50} 1.6, <0.16, 0.57, and <0.16 μg mL^{-1}, respectively). Additional chromatographic separation of the active fraction 2 over Si gel with 5% MeOH in $CHCl_3$, yielded compounds **3** (300 mg) and **4** (17 mg). Further chromatography of fraction 3 over Si gel with 5% MeOH in $CHCl_3$ afforded compound **5** (24 mg). Compound **6** (350 mg) was obtained from fraction 4 by Si gel column chromatography using 10% MeOH in $CHCl_3$ for elution. After further chromatographic purification procedure on fraction 5 over Si gel, using mixtures of $CHCl_3$–MeOH (20:1–4:1) for elution, compounds **7** (8 mg), **8** (6 mg), and a mixture of **1** and **2** were obtained. Compounds **1** (12 mg; *Rt* 33 min) and **2** (8 mg; *Rt* 39 min) were finally purified by HPLC with an ODS-AQ column (250×20 mm; YMC, Inc., Wilmington, NC) using MeOH/H_2O (65:35) as eluant at a flow rate of 6 mL min^{-1}.

(16*R*)-*ent*-16,17-Dihydroxy-19-nor-kaur-4-en-3-one (1). Needles (MeOH); mp 122–123°C; $[\alpha]_D$=28.8° (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ϵ) 209 (4.42), 221 (4.40), 250 (4.41) nm; IR (NaCl) ν_{max} 3413, 2936, 2865, 1661, 1607 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1; EIMS *m/z* $[M]^+$ 304 (40), 286 (65), 273 (27), 197 (20), 176 (24), 150 (32), 138 (100), 121 (56); HREIMS *m/z* 304.2045 (calcd for $C_{19}H_{28}O_3$, 304.2038).

(16*S*)-*ent*-16,17-Dihydroxy-19-nor-kaur-4-en-3-one (2). Needles (MeOH); mp 158–159°C; $[\alpha]_D$=−21.9° (*c* 0.15, MeOH), UV (MeOH) λ_{max} (log ϵ) 209 (4.41), 221 (4.40), 249 (4.40) nm; IR (NaCl) ν_{max} 3413, 2936, 2865, 1661, 1607 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) see Table 1; ^{13}C NMR (500 MHz, $CDCl_3$) see Table 1; EIMS *m/z* $[M]^+$ 304 (33), 286 (53), 273 (18), 197 (14), 176 (17), 150 (22), 138 (100), 121 (44); HREIMS *m/z* 304.2035 (calcd for $C_{19}H_{28}O_3$, 304.2038).

5,7,4′-Trimethoxy-4-phenylcoumarin (3). Prisms (MeOH); mp 150–152°C (lit.[15] mp 151–152°C); UV, IR, ^1H NMR, and EIMS consistent with literature values.[15] ^{13}C NMR (500 MHz, $CDCl_3$) δ 163.6 (s, C-7), 161.3 (s, C-2), 160.0 (s, C-4′), 158.6 (s, C-5), 157.6 (s C-4), 158.6 (s, C-5), 132.4 (s, C-1′), 129.1 (d, C-2′,6′), 113.1 (d, C-3′,5′), 112.8 (d, C-3), 103.9 (s, C-4a), 96.0 (d, C-6), 94.0 (d, C-8), 56.1 (q, OMe-7), 55.8, 55.7 (q, OMe-5, 4′).

7,4′-Dimethoxy-5-hydroxy-4-phenylcoumarin (4). Prisms (MeOH); mp 210–212°C; (lit.[16] mp 212°C); UV, IR, ^1H NMR, and EIMS consistent with literature values.[16] ^{13}C NMR (500 MHz, $CDCl_3$) δ 163.3 (s, C-7), 161.1 (s, C-2), 158.3 (s, C-4′), 157.3 (s, C-4), 155.6 (s C-5), 155.5 (s, C-8a), 132.4 (s, C-1′), 128.9 (d, C-2′,6′), 114.3 (d, C-3′,5′), 112.6 (d, C-3), 103.7 (s, C-4a), 95.9 (d, C-6), 93.6 (d, C-8), 55.8, 55.4 (q, OMe-7, 4′).

5,7,4′-Trimethoxy-3′-hydroxy-4-phenylcoumarin (5). Prisms (MeOH); mp 153–155°C (lit.[15] mp 153–154°C); UV, IR, ^1H NMR, and EIMS consistent with literature values.[15] ^{13}C NMR (500 MHz, $CDCl_3$) δ 163.6 (s, C-7), 161.0 (s, C-2), 160.0 (s, C-5), 158.3 (s, C-4), 157.2 (s C-4), 155.4 (s, C-8a), 146.6 (s, C-4′), 144.6 (s, C-3′), 133.1 (s, C-1′), 119.1 (d, C-6′), 114.2 (d, C-2′), 112.6 (d, C-5'), 109.6 (d, C-3), 103.6 (s, C-4a), 95.9 (d, C-6), 93.6 (d, C-8), 56.0, 55.7, 55.6 (q, OMe-5,7,4′).

5,7,4′-Trimethoxy-8-hydroxy-4-phenylcoumarin (Exostemin I, 6). Yellow prisms (MeOH); mp 158–160°C (lit.[5] mp 157–160°C); UV, IR, ^1H NMR, ^{13}C NMR, and EIMS consistent with literature values.[5] ^{13}C NMR (500 MHz, $CDCl_3$) δ 160.4 (s, C-4′), 160.1 (s, C-1), 156.2 (s, C-4), 151.3 (s, C-7), 150.3 (s, C-5), 143.4 (s C-8a), 132.2 (s, C-8), 129.2 (d, C-2′,6′), 127.9 (s, C-1′), 113.2 (d, C-3′,5′), 113.0 (d, C-3), 93.6 (d, C-6), 56.9, 56.7, 55.7 (q, OMe-5,7,4′).

5,7,4′-Trimethoxy-8,3′-dihydroxy-4′-phenylcoumarin (7). Prisms (MeOH); mp 190–192°C (lit.[17] mp 191–192°C); UV, IR, ^1H NMR, ^{13}C NMR, and EIMS consistent with literature values.[17]

7,4′-Dimethoxy-5,3′-dihydroxy-4′-phenylcoumarin (8). Prisms (MeOH); mp 225–226°C (lit.[6] mp 225–226°C); UV, IR, ^1H NMR, and EIMS consistent with literature values.[6] ^{13}C NMR (500 MHz, $CDCl_3$) δ 163.0 (s, C-2), 159.0 (s, C-4), 158.8 (s, C-5), 152.9 (s, C-7), 152.3 (s C-4′), 145.1 (s, C-8a), 132.3 (s, C-1′), 130.0 (d, C-6′), 129.4 (s, C-8), 115.3 (d, C-2′, 5′), 112.5 (s, C-4a), 104.8 (d, C-3), 94.9 (d, C-6), 56.9, 56.7 (q, OMe-5,7).

Preparation of methyl ether derivative of 5

Compound **5** was dissolved in MeOH; to the solution was added an excess of CH_2N_2 in Et_2O, yielding 5,7,3′,4′-tetramethoxy-4′-phenylcoumarin (**9**) on removal of the solvents. Prisms (MeOH); mp 167–170°C (lit.[15] mp 169–170°C); ^1H NMR data consistent with literature values.[15]

X-Ray crystal structure analysis of compound 1

Crystal data: $C_{18}H_{29}O_3 \cdot H_2O$, monoclinic, space group $P2_1$,

a=10.5620(8), b=7.3845(4), c=11.7915(6) Å, β=106.904(5)°, V=879.94(9) Å^3, Z=2, D_x=1.308 g cm^{-3}, CuKα radiation, absorption coefficient μ=0.578 mm^{-1}. A colorless thin plate crystal of dimensions 0.05×0.12×0.70 mm^3 was used for X-ray measurements at 295 K on an Enraf–Nonius CAD4 diffractometer with a graphite monochromator. The total number of independent reflections measured was 1958, of which 1724 were considered to be observed (I 3α). The absorption correction was 0.91–0.99 ($T_{min}-T_{max}$). The structure was solved by direct methods and refined by full-matrix least-squares.[18] Final agreement factors were $R(F)$=0.044; $wR(F)$=0.055, where w=1/[σ^2(F)+0.04F^2], S=1.755.

Bioassay evaluation

Compounds **1**–**9** were screened for cytotoxicity against a panel of human cancer cell lines, according to an established protocol.[19] ED_{50} values of >5 μg mL^{-1} are regarded as inactive. Comparative cytotoxicity data in an analogous tumor cell panel for ellipticine, podophyllotoxin, taxol (palitaxel), vinblastine and other standard antitumor agents have been published.[20]

In vivo testing

The samples were injected ip (once a day for five days) into mice bearing ip-implanted P388 leukemia. An active result represents an increase in lifespan of test mice over control mice×100%, where T/C 125% is the threshold for activity. Compounds **3** and **6** showed 100% T/C at 24 and 135 mg kg^{-1} per injection, respectively.

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