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The Cytotoxic Properties of Natural Coumarins Isolated from Roots of *Ferulago campestris* (Apiaceae) and of Synthetic Ester Derivatives of Aegelinol

Sergio Rosselli[a], Antonella Maria Maggio[a], Nicoletta Faraone[a], Vivienne Spadaro[b], Susan L. Morris-Natschke[c], Kenneth F. Bastow[d], Kuo-Hsiung Lee[c] and Maurizio Bruno[a,*]

[a] *Dipartimento Chimica Organica "E. Paternò"*, *Università di Palermo*, *Viale delle Scienze*, *Parco d'Orleasn II*, *90128 Palermo*, *Italy*

[b]*Dipartimento di Scienze Botaniche*, *Università degli Studi di Palermo*, *Via Archirafi 38*, *90123 Palermo*, *Italy*

[c]*Natural Products Research Laboratories*, [d]*Division of Medicinal Chemistry*, *Eshelman School of Pharmacy*, *University of North Carolina at Chapel Hill*, *Chapel Hill, North Carolina 27599*, *USA*

bruno@dicpm.unipa.it

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Grandivittin (**1**), agasyllin (**2**), aegelinol benzoate (**3**) and felamidin (**20**), four natural coumarins isolated from *Ferulago campestris*, and several synthetic ester derivatives of aegelinol (**4**) were tested against four tumor cell lines. Some of them were shown to be marginally cytotoxic against the A549 lung cancer cell line.

Keywords: Apiaceae, *Ferulago campestris*, coumarins, aegelinol derivatives, cytotoxicity.

Since ancient times, *Ferulago* species have been well known as folk medicines due to their several biological properties, as sedatives, tonics, digestive remedies and aphrodisiacs, and also in the treatment of intestinal worms and hemorrhoids [1]. Furthermore, they are also useful against ulcers and snake bites, as well as for headaches and diseases of the spleen [2]. The gum (*galbanum*) obtained by incision of the roots of several species [3] is additionally used as a spice and fragrance in perfumes. *F. campestris* (Besser) Grec., (*F. galbanifera* (Mill) Kock. = *Ferula ferulago* L.), commonly known as *finocchiazzo*, is an annual or perennial herb with small flowers, widespread in the Mediterranean area. Our phytochemical investigation of the roots of this species, collected in Sicily, resulted in the isolation of several pyranocoumarins and one furanocoumarin [4]. Their stereochemical assessment was reported, as well as the antibacterial and antioxidant activities of the three most abundant constituents [grandivittin (**1**), agasyllin (**2**) and aegelinol benzoate (**3**)] and of the hydrolysis product [aegelinol, (**4**)] [4].

Decursinol (**5**) and decursin (**6**) are the enantiomers of aegelinol (**4**) and grandivittin (**1**), respectively, and were isolated from species of the genus *Angelica* [5,6]. They possess significant cytotoxic activity [7,8], and furthermore, decursinol (**5**), when administered orally, shows an antinociceptive effect in a dose-dependent manner [9] and high inhibitory activity toward AChE *in vitro* [10]. Decursin (**6**) and decursinol angelate (**7**), the enantiomer of agasyllin (**2**), showed *in vitro* cytotoxic and protein kinase C activating activities [11], as well as platelet anti-aggregatory effects [12]. Moreover, they possess anti-oxidant and hepatoprotective properties in rats [13], as well as antitumor [14,15] and antibacterial [16] activities. Furthermore, all three pyranocoumarins (**5**-**7**) exhibited significant neuroprotective properties [17]. In contrast, apart from the recently reported antibacterial and antioxidant activities [4], to the best of our knowledge, no other biological properties have been published for compounds **1**-**4**. Consequently, as part of our ongoing research on compounds with cytotoxic activity [18-21], we decided to prepare

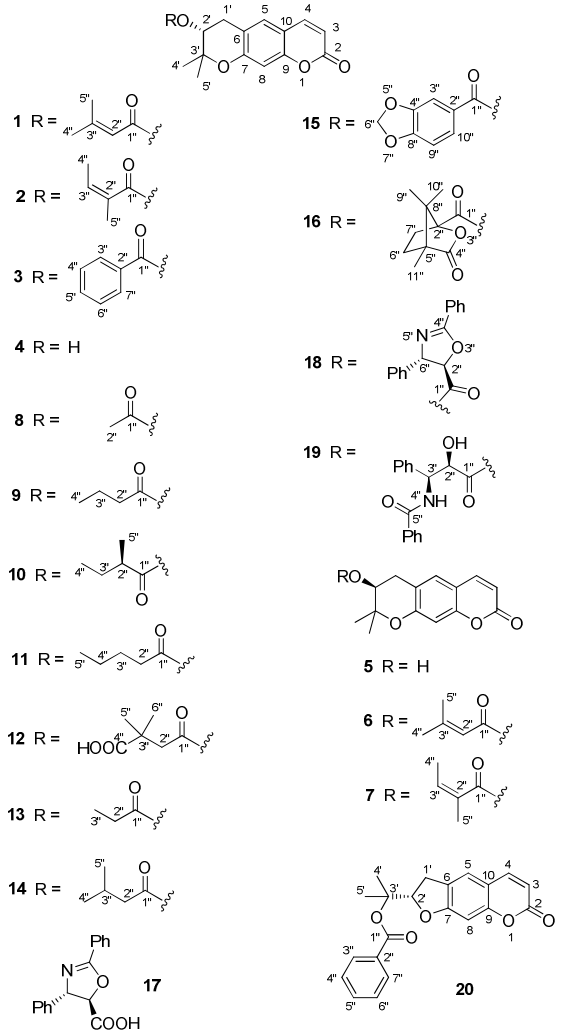

Figure 1: Structures of compounds **1-20**.

some C-2' ester derivatives of aegelinol (**4**) and to test the natural and synthetic compounds against various tumor cell lines.

The ester chains were chosen on the basis of our previous observations [19,20] that certain acyloxy groups were able to enhance biological properties. The three natural esters (**1**-**3**) were hydrolyzed under basic conditions to give aegelinol (**4**) [21]. Treatment of aegelinol (**4**) with triethylamine (TEA) or 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 and either various anhydrides (acetic, butyric, (2*R*)-2-methylbutyric, valeric, 2,2-dimethylsuccinic) or acyl chlorides (propionyl, isovaleryl, piperonyl, (1*S*)-camphanyl) gave the esters **8**-**16**.

According to a previously reported procedure [22], the commercially available (2*R*,3*S*)-3-phenylisoserine hydrochloride was converted to compound **17**, which was then esterified with aegelinol (**4**). Acidic hydrolysis of the resulting ester (**18**) gave compound **19**, with the same side chain as paclitaxel.

Compounds **1**-**4**, **8**-**16**, **18**, **19** and felamidin (**20**), a natural coumarin co-occurring in the same plant [4], were screened against a panel of human tumor cell lines including A549 (lung), PC-3 (prostate), KB (nasopharyngeal), and KB-VIN (multidrug-resistant KB subline) in order to explore their anticancer properties. The results against the A549 tumor cell line are shown in Table 5. Except for felamidin (**20**), which was marginally cytotoxic against KB-VIN [EC_{50} = 14.9 μM; doxorubicin: EC_{50} = 1.7 μM], none of the compounds were active against the other three tumor cell lines. The identity of the ester side chain was important to the cytotoxic activity, as only benzoyl (**3**), piperonyl (**15**), isovaleryl (**14**), and 3,3-dimethylacrylyl (**1**) led to slightly active ester derivatives of aegelinol (**4**), the inactive parent coumarin. However, the coumarin backbone was also important as felamidin (**20**), with a benzoate ester, but different coumarin skeleton, was active. Our compound (grandivittin, **1**) seems to have a worse cytotoxic activity towards PC-3 cell line than its enantiomer (decursin, **6**), showing a moderate activity (EC_{50} = 25.0 μM, 96h) [8]. On the other hand, compound **1** has a better response against the A549 tumor cell line (EC_{50} = 15.2 μM, 72h).

Table 1: ^{1}H NMR spectroscopic data of compounds **8**-**13** in $CDCl_3$, δ_H *J*(Hz).

H	8	9	10	11	12	13
3	6.23 (1H) d (9.6)	6.22 (1H) d (9.3)	6.22 (1H) d (9.3)	6.23 (1H) d (9.3)	6.22 (1H) d (9.6)	6.22 (1H) d (9.3)
4	7.59 (1H) d (9.6)	7.58 (1H) d (9.3)	7.58 (1H) d (9.3)	7.59 (1H) d (9.3)	7.82 (1H) d (9.6)	7.58 (1H) d (9.3)
5	7.16 (1H) s	7.15 (1H) s	7.15 (1H) s	7.15 (1H) s	7.35 (1H) s	7.15 (1H) s
8	6.79 (1H) s	6.78 (1H) s	6.79 (1H) s	6.79 (1H) s	6.74 (1H) s	6.77 (1H) s
1′a	3.19 (1H) dd (17.4, 4.8)	3.18 (1H) dd (17.4, 4.8)	3.18 (1H) dd (17.1, 5.1)	3.18 (1H) dd (17.4, 4.8)	3.19 (1H) dd (17.1, 5.1)	3.18 (1H) dd (17.1, 5.1)
1′b	2.85 (1H) dd (17.4, 4.8)	2.83 (1H) dd (17.4, 4.8)	2.82 (1H) dd (17.1, 5.1)	2.84 (1H) dd (17.4, 4.8)	2.89 (1H) dd (17.1, 5.1)	2.83 (1H) dd (17.1, 5.1)
2′	5.05 (1H) t (4.8)	5.05 (1H) t (4.8)	5.03 (1H) t (5.1)	5.05 (1H) t (4.8)	5.09 (1H) t (5.1)	5.05 (1H) t (5.1)
4′	1.38 (3H) s	1.37 (3H) s	1.37 (3H) s	1.37 (3H) s	1.37 (3H) s	1.36 (3H) s
5′	1.35 (3H) s	1.35 (3H) s	1.36 (3H) s	1.36 (3H) s	1.36 (3H) s	1.34 (3H) s
2"	2.07 (3H) s	2.30 (2H) t (7.5)	2.37 (1H) m	2.29 (2H) t (7.2)	2.59 (2H) s	2.33 (2H) q (7.6)
3"a		1.61 (2H) m	1.61 (1H) m	1.59 (2H) m		1.11 (3H) t (7.6)
3"b			1.44 (1H)m			
4"		0.91 (3H) t (7.4)	0.84 (3H) t (7.4)	1.60 (2H) m		
5"			1.11 (3H) d (7.0)	0.88 (3H) t (7.2)	1.28 (3H) s	
6"					1.28 (3H) s	

Table 2: ^{1}H NMR spectroscopic data of compounds **14-16**, **18**, **19** in $CDCl_3$, δ_H *J* (Hz).

H	14	15	16	18	19
3	6.22 (1H) d (9.3)	6.25 (1H) d (9.6)	6.24 (1H) d (9.6)	6.23 (1H) d (9.6)	6.23 (1H) d (9.6)
4	7.58 (1H) d (9.3)	7.59 (1H) d (9.6)	7.59 (1H) d (9.6)	7.54 (1H) d (9.6)	7.59 (1H) d (9.6)
5	7.15 (1H) s	7.17 (1H) s	7.17 (1H) s	7.11 (1H) s	7.52 (1H) s
8	6.78 (1H) s	6.85 (1H) s	6.79 (1H) s	6.76 (1H) s	6.80 (1H) s
1′a	3.18 (1H) dd (17.1, 4.8)	3.29 (1H) dd (17.4, 4.8)	3.28 (1H) dd (17.4, 4.8)	3.24 (1H) dd (17.4, 4.8)	3.14 (1H) dd (16.5, 5.1)
1′b	2.83 (1H) dd (17.1, 4.8)	2.99 (1H) dd (17.4, 4.8)	2.96 (1H) dd (17.4, 4.8)	2.89 (1H) dd (17.4, 4.8)	2.92 (1H) dd (16.5, 5.1)
2′	5.04 (1H) t (4.8)	5.26 (1H) t (4.8)	5.15 (1H) t (4.8)	5.24 (1H) t (4.8)	5.10 (1H) t (5.1)
4′	1.37 (3H) s	1.46 (3H) s	1.42 (3H) s	1.45 (3H) s	1.43 (3H) s
5′	1.35 (3H) s	1.42 (3H) s	1.39 (3H) s	1.42 (3H) s	1.34 (3H) s
2"	2.19 (2H) d (6.9)			4.90 (1H) d (5.7)	4.63 (1H) d (1.8)
3"	2.08 (1H) m	7.39 (1H) s			5.70 (1H) dd (9.0, 1.8)
4"	0.91 (3H) d (6.6)				
5"	0.91 (3H) d (6.6)				
6"a		6.04 (2H) s	2.37 (1H) ddd (13.3, 10.5, 4.2)	5.44 (1H) d (5.7)	
6"b			2.04 (1H) ddd (13.3, 10.8, 4.5)		
7"a			1.87 (1H) ddd (13.3, 10.8, 4.2)		
7"b			1.67 (1H) ddd (13.3, 10.5, 4.5)		
9"		6.82 (1H) d (8.1)	0.83 (3H) s		
10"		7.60 (1H) d (8.1)	0.78 (3H) s		
11"			1.06 (3H) s		
Ar				8.10-8.04 (2H)	7.77-7.73 (2H)
Ar				7.60-7.20 (8H)	7.48-7.28 (8H)
NH					7.00 (1H) d (9.0)

Table 3: ^{13}C NMR spectroscopic data of compounds **8-13** in $CDCl_3$.

C	8	9	10	11	12	13
2	161.2 C	161.3 C	161.3 C	161.3 C	161.2 C	161.3 C
3	113.3 CH	113.4 CH	113.4 CH	113.4 CH	113.3 CH	113.3 CH
4	143.1 CH	143.2 CH	143.2 CH	143.2 CH	143.1 CH	143.2 CH
5	128.6 CH	128.6 CH	128.6 CH	128.6 CH	128.6 CH	128.7 CH
6	115.6 C	115.8 C	115.8 C	115.8 C	115.6 C	115.8 C
7	156.3 C	156.4 C	156.4 C	156.4 C	156.3 C	156.4 C
8	104.7 CH	104.8 CH	104.7 CH	104.8 CH	104.7 CH	104.7 CH
9	154.2 C	154.2 C	154.2 C	154.2 C	154.2 C	154.2 C
10	112.9 C	112.9 C	112.9 C	113.0 C	112.9 C	112.9 C
1′	27.7 CH_2	27.8 CH_2	27.9 CH_2	27.8 CH_2	27.7 CH_2	27.7 CH_2
2′	70.2 CH	70.0 CH	69.9 CH	70.0 CH	70.2 CH	70.1 CH
3′	76.4 C	76.6 C	76.6 C	76.6 C	76.4 C	76.6 C
4′	24.9 CH_3	25.1 CH_3	25.1 CH_3	26.8 CH_3	24.9 CH_3	26.7 CH_3
5′	23.1 CH_3	23.1 CH_3	22.9 CH_3	23.1 CH_3	23.1 CH_3	25.0 CH_3
1"	170.4 C	173.1 C	176.1 C	173.3 C	172.2 C	173.9 C
2"	21.0 CH_3	36.3 CH_2	41.0 CH	34.1 CH_2	44.7 CH_2	27.8 CH_2
3"		18.5 CH_2	26.6 CH_2	25.0 CH_2	41.5 C	23.0 CH_3
4"		13.6 CH_3	11.5 CH_3	22.2 CH_2	180.4 C	
5"			16.6 CH_3	13.7 CH_3	25.3 CH_3	
6"					25.3 CH_3	

Table 4: ^{13}C NMR spectroscopic data of compounds **14-16**, **18**, **19** in $CDCl_3$.

C	14	15	16	18	19
2	161.3 C	161.2 C	161.1 C	161.2 C	161.1 C
3	113.4 CH	113.0 CH	113.7 CH	113.5 CH	113.4 CH
4	143.2 CH	143.1 CH	143.0 CH	143.1 CH	143.0 CH
5	128.6 CH	128.7 CH	128.5 CH	128.6 CH	128.6 CH
6	115.8 C	115.7 C	115.0 C	114.9 C	114.9 C
7	156.4 C	156.4 C	156.2 C	156.8 C	156.8 C
8	104.7 CH	104.8 CH	104.8 CH	104.7 CH	104.6 CH
9	154.2 C	154.0 C	154.3 C	154.2 C	154.3 C
10	112.9 C	113.4 C	113.0 C	112.9 C	112.9 C
1′	27.8 CH_2	27.9 CH_2	27.8 CH_2	27.9 CH_2	27.9 CH_2
2′	70.0 CH	70.8 CH	71.9 CH	72.0 CH	72.0 CH
3′	76.5 C	76.7 C	76.6 C	76.6 C	76.5 C
4′	25.7 CH_3	25.0 CH_3	25.0 CH_3	25.1 CH_3	25.1 CH_3
5′	23.1 CH_3	23.4 CH_3	23.4 CH_3	23.5 CH_3	23.5 CH_3
1"	172.5 C	165.1 C	166.8 C	169.7 C	172.4 C
2"	43.4 CH_2	123.5 C	90.8 C	82.9 CH	73.4 CH
3"	25.1 CH	108.1 CH			54.6 CH
4"	22.4 CH_3	147.8 C	177.7 C	164.2 C	
5"	22.3 CH_3		54.7 C		166.3 C
6"		101.9 CH_2	30.7 CH_2	74.7 CH	
7"			28.9 CH_2		
8"		152.0 C	54.1 C		
9"		109.5 CH	16.6 CH_3		
10"		125.7 CH	16.5 CH_3		
11"			9.6 CH_3		
Ar				142.2-126.4	138.6-126.9

Table 5: Growth-inhibitory activity of **1-4**, **8-16**, **18-20** against A549 tumor cell line replication.

Compound	EC_{50} (μM)	Compound	EC_{50} (μM)
1	15.2	**13**	NA
2	NA	**14**	18.5
3	13.6	**15**	19.2
4	NA	**16**	NA
8	NA	**18**	NA
9	NA	**19**	NA
10	NA	**20**	11.6
11	NA	doxorubicin	0.9
12	NA		

Experimental

General experimental procedures: Optical rotations were determined on a JASCO P-1010 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance series 300 MHz spectrometer, using the residual solvent signal (δ = 7.27 in ^{1}H and δ = 77.00 in ^{13}C for $CDCl_3$) as reference. ^{13}C NMR signal multiplicities were determined by DEPT spectra. ESI-MS were obtained with an Applied Biosystem API-2000 mass spectrometer. Merck silica gel (70-230 mesh), deactivated with 15% H_2O, was used for column chromatography.

Plant material: The roots of *Ferulago campestris* (Besser.) Grec. (700 g) were collected at Alimena, Palermo province, Italy in July 2007 and identified by Professor F. M. Raimondo, Department of Botanic Sciences, University of Palermo (Italy). Voucher specimens were deposited at the Herbarium of the Botanical Gardens of Palermo (Italy) under the number PAL 07-621 (Raimondo, Schimmenti & Scafidi).

Extraction and isolation: The extraction of the roots and the isolation of phytochemicals were performed as previously reported [4].

Synthesis of aegelinol (4): The esters **1-3** (500 mg, about 1.5 mmol) were added to a solution of KOH in dioxane (75 mL, 16.8 g, 0.3 mol, 4 M). The reaction mixture was refluxed for 0.5 h, and was monitored by TLC (2:3 EtOAc-light petroleum). After cooling, the reaction mixture was quenched and portion-wise acidified with 10% H_2SO_4 solution. The solution was extracted with dichloromethane, dried over Na_2SO_4 and evaporated *in vacuo*. Compound **4** was purified by crystallization (EtOAc/*n*-hexane) to obtain white crystals (340 mg, 93 %) [23].

Esterification-General procedure: Aegelinol (**4**, 20 mg) was dissolved in 10 mL of dry CH_2Cl_2 and added to 1 equiv of DMAP (12 mg), 25 equiv of TEA (0.3 mL), and the appropriate acyl chloride/anhydride (4 equiv) at room temperature under an argon atmosphere. After stirring overnight, the reaction was subjected to the usual workup by adding H_2O and extracting with EtOAc. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. Generally, the residue was purified by column chromatography (Si gel, 4:1 light petroleum-EtOAc as eluent). This procedure gave the following ester derivatives.

Compound 8

White solid (90% yield).
$[\alpha]_D^{25}$: –69.8 (*c* 1.09 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{16}H_{16}O_5$.
ESI MS (positive mode) *m/z* (%): 327 $[M+K]^+$ (24), 311 $[M+Na]^+$.

Compound 9

White solid (82% yield).
$[\alpha]_D$: –62.9 (*c* 3.65 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{18}H_{20}O_5$.
ESI MS (positive mode) *m/z* (%): 355 $[M+K]^+$ (10), 339 $[M+Na]^+$ (100), 317 $[M+H]^+$ (5).

Compound 10

White solid (85% yield).
$[\alpha]_D$: –61.7 (*c* 1.29 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{19}H_{22}O_5$.
ESI MS (positive mode) *m/z* (%): 369 $[M+K]^+$ (24), 353 $[M+Na]^+$ (100).

Compound 11

White solid (95% yield).
$[\alpha]_D$: –59.1 (*c* 3.21 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{19}H_{22}O_5$.
ESI MS (positive mode) *m/z* (%): 369 $[M+K]^+$ (17), 353 $[M+Na]^+$ (100).

Compound 12

White solid (75% yield).
$[\alpha]_D$: –38.0 (*c* 0.59 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{20}H_{22}O_7$.
ESI MS (positive mode) *m/z* (%): 411 $[M+K]^+$ (13), 295 $[M+Na]^+$ (100).

Compound 13

White solid (81% yield).
$[\alpha]_D$: –80.8 (*c* 1.98 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{17}H_{18}O_5$.
ESI MS (positive mode) *m/z* (%): 341 $[M+K]^+$ (37), 325 $[M+Na]^+$ (100), 303 $[M+H]^+$ (8).

Compound 14

White solid (80% yield).
$[\alpha]_D$: –51.7 (*c* 3.38 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4
$C_{19}H_{22}O_5$.
ESI MS (positive mode) *m/z* (%): 369 $[M+K]^+$ (10), 353 $[M+Na]^+$ (100).

Compound 15

White solid (78% yield).
$[\alpha]_D$: –43.3 (*c* 0.46 $CHCl_3$).
^{1}H NMR ($CDCl_3$): see Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{22}H_{18}O_7$.
ESI MS (positive mode) *m/z* (%): 433 $[M+K]^+$ (52), 417 $[M+Na]^+$ (100).

Compound 16

White solid (70% yield).

$[\alpha]_D$: –86.5 (*c* 0.42 $CHCl_3$).
^1H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{24}H_{26}O_7$.
ESI MS (positive mode) *m/z* (%): 465 $[M+K]^+$ (9), 449 $[M+Na]^+$ (100).

Synthesis of compound 18: Aegelinol (**4**, 20 mg) was dissolved in 5 mL of dry CH_2Cl_2, and to this solution were added 1.2 equiv. of DCC and 2 equiv. of oxazoline **17**. After stirring overnight at room temperature, the reaction mixture was filtered and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography (Si gel, 7:3 light petroleum-EtOAc as eluent) to give compound **18** (95% yield).

Compound 18

White solid.
$[\alpha]_D$: –89.7 (*c* 0.35 $CHCl_3$).
^1H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{30}H_{25}O_6N$.
ESI MS (positive mode) *m/z* (%): 518 $[M+Na]^+$ (5), 496 $[M+H]^+$ (100).

Synthesis of compound 19: Compound **18** (20 mg), dissolved in CH_2Cl_2 (5 mL), was stirred at room temperature with 2 equiv. of *p*-toluene-sulfonic acid. After stirring overnight at room temperature, the reaction was neutralized with saturated aqueous $NaHCO_3$, diluted with water (10 mL), and extracted 3 times with $CHCl_3$ (15 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure, leaving a residue, which was purified by column chromatography (Si gel, 4:1 light petroleum-EtOAc as eluent) to give compound **19** (72% yield).

Compound 19

White solid.
$[\alpha]_D$: –46.5 (*c* 0.13 $CHCl_3$).
^1H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{30}H_{27}O_7N$.
ESI MS (positive mode) *m/z* (%): 552 $[M+K]^+$ (18), 536 $[M+Na]^+$ (100), 514 $[M+H]^+$ (20).

***In vitro* cytotoxicity assay:** All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates with compounds added from DMSO-diluted stock. The plates were incubated for an additional 72 h after attachment and drug addition, and the assay was terminated by 10% TCA. Then, 0.4% SRB dye in 1% HOAc was added to stain the cells for 10 min. Unbound dye was removed by repeated washing with 1% HOAc and the plates were air dried. Bound stain was subsequently solved with 10 mM trizma base, and the absorbance read at 515 nm. Growth inhibition of 50% (EC_{50}) was calculated as the drug concentration that caused a 50% reduction in the net protein increase in control cells during the drug incubation. The mean EC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations. Variation between replicates was no more than 5% of the mean. The following human tumor cell lines were used in the assay: A549 (non-small cell lung cancer), PC-3 (prostate cancer), KB (nasopharyngeal carcinoma), KB-VIN (vincristine-resistant KB subline). All cell lines were obtained from either the Lineberger Cancer Center (UNC-CH) or from ATCC (Rockville, MD). Cells propagated in RPMI-1640 supplemented with 10% FBS, penicillin-100 IU/mL, streptomycin-1μg/mL, and amphotericin B-0.25μg/mL, were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2.

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