Cytotoxic Dihydroagarofuranoid Sesquiterpenes from the Stem of *Microtropis fokienensis*

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Four dihydroagarofuran sesquiterpene polyesters (**1**−**4**) have been isolated from the stem of *Microtropis fokienensis*. The structures of these new compounds were determined through spectroscopic analyses. Compound **1**, **2**, **3**, and **4** exhibited cytotoxicities (IC_{50} values < 0.1 μg/mL) against P-388 and HT-29 cell lines in vitro.

Microtropis fokienensis Dunn (Celastraceae) is a small shrub that grows in high altitude forests throughout southern China and Taiwan.[1] Sesquiterpene alkaloids,[2] dihydroagarofuranoid sesquiterpenes,[3,4] triterpenes,[5,6] and their derivatives are widely distributed in plants of the family Celastraceae. Many of these compounds exhibit immunosuppressive,[2] insecticidal,[3] antiinflammatory,[4] antitumor,[5,6] and anti-AIDS[5] activities. However, the chemical constituents and biological activities of *M. fokienensis* have not been studied. Approximately 1000 species of Formosan plants have been screened for cytotoxicity, and *M. fokienensis* was shown to be one of the active species. Investigation of an EtOAc-soluble fraction of the stem of *M. fokienensis* has led to the isolation of four new dihydro-β-agarofuran sesquiterpene polyesters (**1**−**4**). The structures of these new compounds were determined through spectroscopic analyses including extensive 2D-NMR data. This paper describes the structural elucidation of these new compounds and the cytotoxic activities.

Repeated column chromatography of the ethyl acetate-soluble fraction from a methanol extract of the stem of *M. fokienensis* yielded four new sesquiterpene esters (**1**−**4**). Compound **1** was isolated as amorphous powder, $[\alpha]_D^{25}$ +32.4. The FABMS of **1** afforded the ion $[M + H]^+$ at m/z = 699, implying a molecular formula of $C_{40}H_{43}O_{11}$, which was confirmed by HRFABMS. UV absorptions at 231, 274, and 281 nm were similar to those of mutangin[7] and suggested the presence of aromatic rings. Ester carbonyl groups in the molecule were indicated by the bands at 1746 and 1716 cm^{-1} in the IR spectrum of **1** and were confirmed by signals at δ 165.2, 166.1, 166.8, 169.4, and 169.9 in the ^{13}C NMR spectrum. The ^1H NMR spectrum of **1** was also similar to that of mutangin[7] except that benzoyloxy-2 [δ 8.15 (2H, d, J = 7.5 Hz, H-2‴ and H-6‴), 7.52 (2H, t, J = 7.5 Hz, H-3‴ and H-5‴), and 7.60 (1H, t, J = 7.5 Hz, H-4‴)] of **1** replaced OAc-2 (δ 2.32) of mutangin.[7] This was supported by the HMBC correlations observed between H-2 (δ 5.92) and Ph*C*OO-2 (δ 166.1). From the $^1H-^1H$ COSY and NOESY spectra of **1**, the signals at δ 5.91 (1H, d, J = 4.0 Hz), 5.92 (1H, dd, J = 6.5, 4.0 Hz), 6.13 (1H, s), and 5.56 (1H, d, J = 7.2 Hz) were assigned as H_{ax}-1, H_{eq}-2, H_{ax}-6, and H_{eq}-9, respectively. The axial assignment of the C-9 benzoate was supported by NOESY experiments, which showed the interactions between H-2″,6″ (δ 8.07) of the C-9 benzoate and the C-12 methyl (δ 1.51) and H-1 (δ 5.91). NOESY correlations observed between the C-14 methyl and the OAc-2, H-6, and H-15 groups confirmed their axial assignments. The stereochemical assignments, which were also based on the splitting patterns and coupling constants of H-1 [δ 5.91 (1H, d, J = 4.0 Hz)], H-2 [δ 5.92 (1H, dd, J = 6.5, 4.0 Hz)], H-6 [δ 6.13 (1H, s)], and H-9 [δ 5.56 (1H, d, J = 7.2 Hz)], are in agreement with the stereochemistry observed at these positions in this class of natural products.[8,9] Thus, the structure of **1** was elucidated as 1α,6β-diacetoxy-2α,9β,15-tribenzoyloxy-β-dihydroagarofuran. The location of the ester groups was confirmed by the $^1H-^{13}C$ long-range correlation (HMBC) experiment, which exhibited cross-peaks between H-1 (δ 5.91), OAc-1 (δ 1.55) and the carbonyl of one acetate ester (δ 169.4); H-2 (δ 5.91), H-2′/6′ (δ 8.15) and the carbonyl of one benzoate ester (δ 166.1); H-6 (δ 6.13), OAc-6 (δ 2.10) and the carbonyl of the second acetate ester (δ 169.9); H-9 (δ 5.56), H-2″/6″ (δ 8.07) and the carbonyl of the second benzoate ester (δ 165.2); and H-15 (δ 4.95, 5.42), H-2‴/6‴ (δ 8.22) and the carbonyl of the tertiary benzoate ester (δ 166.8). Assignments of carbon signals are shown in Table 2 based on HSQC and HMBC techniques.

Compound **2** was isolated as an amorphous powder. The ESIMS of **2** afforded the ion $[M + Na]^+$ at m/z = 601, implying a molecular formula of $C_{33}H_{38}O_9$, which was confirmed by the HRESIMS. The UV absorptions of **2** at 231, 274, and 281 nm were similar to those of **1**. The presence of ester carbonyl groups in the molecule was revealed by the band at 1725 cm^{-1} in the IR spectrum. The ^1H and ^{13}C NMR (Tables 1 and 2) spectra of **2** were similar to those of **1** except that H-2 of **2** replaced the 2-benzoyloxy group of **1**. The stereochemical assignments, which were based on the splitting patterns and coupling constants of H-1 [δ 5.64 (dd, J = 12.5, 4.5 Hz)], H-6 [δ 6.13 (s)], and H-9 [δ 5.51 (d, J = 7.0 Hz)], are in agreement with the stereochemistry observed at these positions in other members of this class of natural products.[8,10] On the basis of the above data, the structure of **2** was elucidated as 1α,6β-diacetoxy-9β,15-dibenzoyloxy-β-dihydroagarofuran.

Compound **3** was also isolated as an amorphous powder. The molecular formula of **3** was established as $C_{38}H_{46}O_{11}$ by HR-FABMS. The UV absorptions of **3** at 231, 274, and 281 nm were similar to those of **1**. The presence of ester carbonyl groups in the molecule of **3** was revealed by the band at 1720 cm^{-1} in the IR spectrum. The ^1H NMR spectrum of **3** was similar to those of fokienagarofuran A (**1**) except that the 15-isobutanoyloxy group [δ 1.04 (3H, d, J = 6.8 Hz), 1.05 (3H, d, J = 6.4 Hz), 2.24 (1H m), 2.41 (1H, d, J = 5.2 Hz), 2.43 (1H, d, J = 6.0 Hz)] of **3** replaced 15-benzoyloxy group of **1**. The stereochemical assignments, which were based on coupling constants of H-1 [δ 5.84 (d, J = 4.0 Hz)], H-2 [δ 5.87 (dd, J = 4.0, 3.4 Hz)], H-6 [δ 6.13 (s)], and H-9 [δ 5.51 (d, J = 7.4 Hz)], are in agreement with the stereochemistry observed at these positions in this class of natural products.[8,9] Thus, **3** was elucidated as 1α,6β-diacetoxy-2α,9β-dibenzoyloxy-15-isobutanoyloxy-β-dihydroagarofuran.

Compound **4** was isolated as an amorphous powder. The ESIMS of **4** afforded the positive ion $[M + Na]^+$ at m/z = 783, implying a molecular formula of $C_{45}H_{44}O_{11}$, which was confirmed by the HRESIMS. The UV absorptions of **4** were similar to those of **1**.

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Table 1. ^{1}H NMR Data of Compounds **1**−**4**

H	**1**[a]	**2**[b]	**3**[b]	**4**[b]
1	5.91 d (4.0)	5.64 dd (12.5, 4.5)	5.84 d (4.0)	5.95 d (4.0)
2	5.92 dd (6.5, 4.0)	1.64 m	5.87 dd (6.5, 4.0)	5.95 m
		1.99 dd (14.0, 4.0)		
3 (ax)	2.03 br d (16.0)	1.52 m	1.97 br d (16.0)	2.07 dd (15.2, 2.4)
3 (eq)	2.62 ddd (16.0, 6.5, 3.3)	2.31 m	2.57 ddd (16.0, 6.5, 3.4)	2.70 m
4	2.51 m	2.40 m	2.45 m	2.69 m
6	6.13 s	6.01 s	5.95 s	6.36 s
7	2.41 dd (3.8, 2.8)	2.35 dd (3.0, 3.0)	2.30 dd (3.6, 2.8)	2.57 dd (3.6, 2.8)
8 (ax)	2.29 dd (16.3, 2.8)	2.25 dd (16.0, 3.0)	2.24 (15.6, 2.8)	2.34 dd (16.2, 2.8)
8 (eq)	2.70 ddd (16.3, 7.2, 3.8)	2.59 ddd (16.0, 7.0, 3.0)	2.57 ddd (15.6, 7.4, 3.6)	2.79 ddd (16.2, 7.2, 3.6)
9	5.56 d (7.2)	5.51 d (7.0)	5.47 d (7.4)	5.60 d (7.2)
12	1.51 s	1.47 s	1.48 s	1.53 s
13	1.48 s	1.44 s	1.44 s	1.52 s
14	1.33 d (7.6)	1.07 d (8.0)	1.24 d (8.0)	1.34 (7.6)
15	4.95 d (12.5)	4.72 d (12.3)	4.48 d (12.4)	5.00 d (12.8)
	5.42 d (12.5)	4.99 d (12.3)	5.29 d (12.4)	5.42 d (12.8)
OAc-1	1.55 s	1.53 s	1.52 s	1.56 s
OAc-6	2.10 s	2.10 s	2.10	
2′/6′	8.15 d (7.5)	8.06 d (7.5)	8.12 d (7.5)	8.14 d (7.6)
3′/5′	7.52 t (7.5)	7.45 t (7.5)	7.50 t (7.5)	7.50 t (7.6)
4′	7.60 t (7.5)	7.58 t (7.5)	7.58 t (7.5)	7.59 t (7.6)
2″/6″	8.07 d (7.5)	8.22 d (7.5)	8.06 d (7.5)	8.03 d (7.6)
3″/5″	7.46 t (7.5)	7.53 t (7.5)	7.45 t (7.5)	7.46 t (7.6)
4″	7.58 t (7.5)	7.63 t (7.5)	7.58 t (7.5)	7.58 t (7.6)
2‴/6‴	8.22 d (7.5)			8.08 d (7.6)
3‴/5‴	7.54 t (7.5)			7.46 t (7.6)
4‴	7.63 t (7.5)			7.58 t (7.6)
2⁗/6⁗				8.27 d (7.6)
3⁗/5⁗				7.58 t (7.6)
4⁗				7.65 t (7.6)
Me$_2$CHCH$_2$CO			1.04 d (6.8)	
			1.05 d (6.4)	
Me$_2$C*H*CH$_2$CO			2.24 m	
Me$_2$CHC*H*$_2$CO			2.41 d (5.2)	
			2.43 d (6.0)	

[a] Recorded in $CDCl_3$ at 500 MHz. [b] Recorded in $CDCl_3$ at 400 MHz. Values in ppm (δ). J (in Hz) in parentheses.

The presence of ester carbonyl groups in the molecule of **4** was revealed by the band at 1749, 1721 cm^{-1} in the IR spectrum. The ^{1}H NMR and ^{13}C NMR spectra of **4** were similar to those of fokienagarofuran A (**1**), except that in **4**, at C-6, a benzoyloxy group [δ = 8.03 (2H, d, J = 7.6, H-2″ and 6″), 7.46 (2H, t, J = 7.6 Hz, H-3″ and 5″), 7.58 (1H, t, J = 7.6 Hz, H-4″)] replaced the acetoxy group [δ = 6.13 (3H, s)] in **1**. In the ^{1}H NMR spectrum of **4**, signals at δ 5.95 (d, J = 4.0 Hz), 5.95 (m), 6.36 (s), and 5.56 (d, J = 7.2 Hz) due to the methine bearing secondary ester groups were assigned as H_{ax}-1, H_{eq}-2, H_{ax}-6, and H_{eq}-9, respectively, from the ^{1}H−^{1}H COSY and NOESY spectra. The axial assignment of the C-9 benzoate was supported by NOESY experiments, which showed the interactions between H-2‴,6‴ (δ 8.08) of the C-9 benzoate and the C-12 methyl (δ 1.53) and H-1 (δ 5.95). NOESY correlations observed between the C-14 methyl and H-6, H-15 confirmed the axial assignments of the C-14 methyl, H-6, and 15-benzoyloxy groups. Thus, **4** was elucidated to be 1α-acetoxy-2α,6β,9β,15-tetrabenzoyloxy-β-dihydroagarofuran.

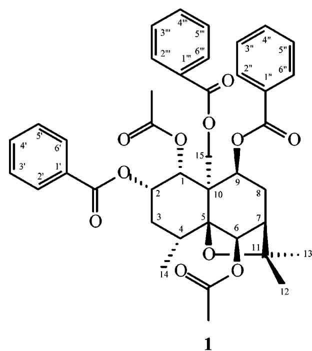
The isolates **1**−**4** from the stem of *M. fokienensis* were tested in vitro against P-388 and HT-29 cell lines. The cytotoxicity data are shown in Table 3. The anticancer agent mithramycin was used as a positive control. Compounds **1**, **2**, **3**, and **4** exhibited significant cytotoxicities (IC_{50} values < 0.1 μg/mL, respectively) against the P-388 and HT-29 cell lines. Compounds **1**, **2**, and **4** (all with a 15-benzoyloxy group) exhibited greater cytotoxicities than **3** (with the 15-isobutanoyloxy group) against the P-388 and HT-29 cell lines. Compounds **1** and **4** were the most cytotoxic, with IC_{50} values = 0.032 ± 0.004 and 0.036 ± 0.005 μg/mL, respectively, against the P-388 cell line, and with IC_{50} values = 0.054 ± 0.006 and 0.058 ± 0.007 μg/mL, respectively, against the HT-29 cell line.

Experimental Section

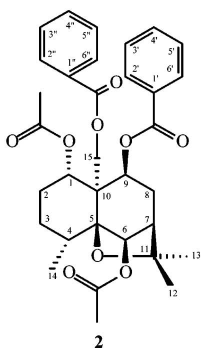
General Experimental Procedures. All melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. IR spectra (KBr or neat) were taken on a Perkin-Elmer system 2000 FT-IR spectrometer. UV spectra were obtained on a Jasco UV-240 spectrophotometer. EI, ESI, and HRESI mass spectra were recorded on a Bruker APEX II mass spectrometer. HREI, FAB, and HRFAB mass spectra were recorded on a JEOL JMX-HX 110 mass spectrometer. NMR spectra, including COSY, NOESY, HMBC, and HSQC experiments, were recorded on a Varian Unity 400 or a Varian Inova 500 spectrometer operating at 400 and 500 MHz (^{1}H) and 100 and 125 MHz (^{13}C), respectively, with chemical shifts given in ppm (δ) using TMS as an internal standard. Silica gel (70−230, 230−400 mesh) (Merck) was used for CC. Silica gel 60 F-254 (Merck) was used for TLC and preparative TLC.

Plant Material. The stem of *M. fokienensis* was collected from Chunrih, Pingtung County, Taiwan, in November 2004 and identified by one of the authors (I.S.C.). A voucher specimen (Sheng-Zehn Yang 023531) was deposited in the herbarium of the Department of Forest Resource, Management and Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan.

Extraction and Separation. The dried stem (3.2 kg) was extracted with cold MeOH, and the extract concentrated under reduced pressure. The MeOH extract (315 g), when partitioned between H_2O−EtOAc (1:1), afforded a EtOAc-soluble fraction (fraction A, 38 g). The H_2O-soluble fraction was further extracted with *n*-BuOH to afford an *n*-BuOH fraction (fraction B, 85 g) and a H_2O fraction (fraction C, 168 g). Fraction A (38 g) was chromatographed on silica gel (70−230 mesh, 1.52 kg), eluting with CH_2Cl_2, gradually increasing the polarity with MeOH to give 13 fractions: A1−A3 (each 6 L, CH_2Cl_2), A4−A5 (each 10 L, CH_2Cl_2−MeOH, 20:1), A6−A7 (each 3 L, CH_2Cl_2−MeOH, 10:1), A8−A9 (each 2 L, CH_2Cl_2−MeOH, 5:1), A10−11 (each 2 L, CH_2Cl_2−MeOH, 2:1), A12 (each 2 L, CH_2Cl_2−MeOH, 1:1), A13



1



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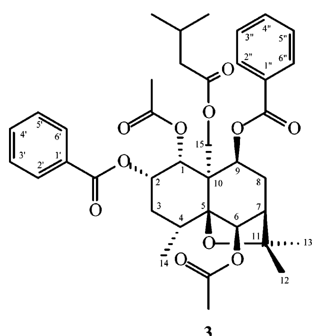

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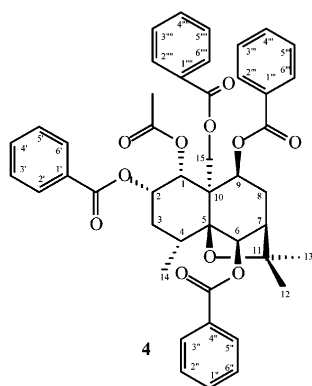

4

(4 L, MeOH). Fraction A2 (2.3 g) was chromatographed further on silica gel (230−400 mesh, 85 g) eluting with *n*-hexane−EtOAc (10:1) to give 14 fractions (each 800 mL, A2-1−A2-14). Fraction A2-5 (203 mg) was purified further by preparative TLC (*n*-hexane−acetone, 3:1) to obtain **4** (4.3 mg) (R_f = 0.55). Fraction A2-8 (213 mg) was purified further by preparative TLC (*n*-hexane−acetone, 3:1) to obtain **3** (4.2 mg) (R_f = 0.46). Fraction A2-9 (231 mg) was purified further by preparative TLC ($CHCl_3$−acetone, 50:1) to obtain **2** (3.5 mg) (R_f = 0.45). Fraction A2-10 (175 mg) was purified further by preparative TLC (*n*-hexane−acetone, 3:1) to obtain **1** (5.3 mg) (R_f = 0.36).

Compound 1: amorphous powder; $[\alpha]_D^{25}$ +32.4 (*c* 0.15, $CDCl_3$); UV (MeOH) λ_{max} (log ϵ) 231 (4.12), 274 (3.13), 281 (3.06) nm; IR (KBr) ν_{max} 1746 (C═O), 1716 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m/z* (rel int) 699 ([M + H]$^+$, 28); HRFABMS *m/z* 699.2802 (calcd for $C_{40}H_{43}O_{11}$, 699.2806).

Table 2. ^{13}C NMR Data of Compounds **1**−**4**

C	1[a]	2[b]	3[b]	4[b]
1	71.6	73.6	71.5	71.6
2	70.3	22.2	70.3	70.3
3	30.9	26.4	30.9	31.0
4	33.1	33.5	33.0	33.5
5	89.2	89.5	89.2	89.4
6	78.7	78.9	78.4	79.3
7	48.7	48.6	48.8	48.8
8	34.7	34.4	34.8	34.8
9	69.2	69.6	69.4	69.2
10	53.5	53.4	53.4	53.6
11	82.8	82.5	82.7	82.9
12	26.0	26.0	26.0	26.0
13	30.4	30.4	30.4	30.6
14	18.3	16.7	18.1	18.4
15	66.4	65.6	65.7	66.4
1′	130.0[c]	129.6[c]	129.7[c]	129.7[c]
2′/6′	129.8	130.0	129.8	129.8
3′/5′	128.7	128.3	128.7	128.7
4′	133.3	133.4	133.2	133.2
1″	129.8[c]	129.3[c]	129.3[c]	129.7[c]
2″/6″	130.1	129.8	130.1	129.5
3″/5″	128.3	128.6	128.3	128.7
4″	133.4	133.4	133.4	133.4
1‴	129.1[c]			129.9[c]
2‴/6‴	129.7			130.2
3‴/5‴	128.6			128.3
4‴	133.2			133.5
1⁗				129.1[c]
2⁗/6⁗				129.8
3⁗/5⁗				128.7
4⁗				133.3
CO_2-1	169.4	169.8	169.4	169.5
CO_2-2	166.1		166.1	166.1
CO_2-6	169.9	170.0	169.8	165.4
CO_2-9	165.2	165.3	165.3	165.3
CO_2-15	166.8	166.7	172.8	166.8
MeCO_2-1	20.4	20.8	20.4	20.5
MeCO_2-6	21.3	21.3	21.3	
Me$_2$CHCH$_2$CO			22.5	
			22.5	
Me$_2$*C*HCH$_2$CO			25.6	
Me$_2$CH*C*H$_2$CO			43.6	

[a] Recorded in $CDCl_3$ at 125 MHz. [b] Recorded in $CDCl_3$ at 100 MHz. Values in ppm (δ). [c] Values superscripted with *c* are interchangeable in every column.

Table 3. Cytotoxic Effects of Compounds Isolated from the Stem of *Microtropis fokienensis* against P-388 and HT-29 Cell Lines

compound	IC$_{50}$ [μg mL^{-1}]	
	P-388	HT-29
fokienagarofuran A (**1**)	0.032 ± 0.004	0.054 ± 0.006
fokienagarofuran B (**2**)	0.043 ± 0.006	0.071 ± 0.008
fokienagarofuran C (**3**)	0.051 ± 0.008	0.082 ± 0.009
fokienagarofuran D (**4**)	0.036 ± 0.005	0.058 ± 0.007
mithramycin[a]	0.06 ± 0.01	0.08 ± 0.01

[a] Mithramycin was used as a positive control.

Compound 2: amorphous powder; $[\alpha]_D^{25}$ +46.8 (*c* 0.12, $CDCl_3$); UV (MeOH) λ_{max} (log ϵ) 231 (4.20), 274 (3.14), 281 (3.07) nm; IR (KBr) ν_{max} 1725 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; ESIMS *m/z* (rel int) 601 ([M + Na]$^+$, 100); HRESIMS *m/z* 601.2417 (calcd for $C_{33}H_{38}O_9Na$, 601.2413).

Compound 3: amorphous powder; $[\alpha]_D^{25}$ +34.5 (*c* 0.18, $CDCl_3$); UV (MeOH) λ_{max} (log ϵ) 231 (4.25), 274 (3.15), 281 (3.07) nm; IR (KBr) ν_{max} 1720 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; FABMS *m/z* (rel int) 679 ([M + H]$^+$, 100); HRFABMS *m/z* 679.3122 (calcd for $C_{38}H_{47}O_{11}$, 679.3119).

Compound 4: amorphous powder; $[\alpha]_D^{25}$ +35.4 (*c* 0.13, $CDCl_3$); UV (MeOH) λ_{max} (log ϵ) 231 (4.11), 273 (3.12), 280 (3.05) nm; IR (KBr) ν_{max} 1749 (C═O), 1721 (C═O) cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; ESIMS *m/z* (rel int) 783 ([M + Na]$^+$, 100); HRESIMS *m/z* 783.2784 (calcd for $C_{45}H_{44}O_{11}Na$, 783.2781).

Cytotoxicity Assay. P-388 (mouse lymphocytic leukemia) cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; HT-29 (human colon carcinoma) was purchased from the American Type Culture Collection (ATCC). P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (FCS, GIBCO). HT-29 cells were maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium (GIBCO) containing 10% heat-inactivated FCS. All cell lines were maintained in an incubator at 37 °C in humidified air containing 5% CO_2.

The cytotoxic activities of compounds against P-388 and HT-29 were assayed by a modification of the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[11] For P-388 cells, 200 μL cultures were established at 1500 cells/well in 96-well tissue culture plates (Falcon). Compounds (dissolved in 0.5% DMSO) were dispensed to establish cultures at eight concentrations in triplicate. After 3 days of incubation, P-388 cells were enumerated with MTT (Sigma).

To measure the cytotoxic activities of purified compounds against HT-29 cells, each cell line was initiated at 1000 cells/well in 96-well microtiter plates. Eight concentrations (triplicate) of test compounds (dissolved in 0.5% DMSO) encompassing a 128-fold range were added to each cell line. HT-29 cells were enumerated using MTT after exposure to test compounds for 6 days. Fifty microliters of 1 mg/mL MTT was added to each well, and plates were incubated at 37 °C for a further 5 h. Formazan crystals were redissolved in DMSO (Merck) for 10 min with shaking, and the plate was read immediately on a microtiter plate reader (Dynatech) at a wavelength of 540 nm. The IC_{50} was defined as the concentration of the test compound resulting in a 50% reduction of absorbance compared to untreated cells in the MTT assay. The assays were repeated three times. The anticancer agent mithramycin and 0.5% DMSO were used as the positive control and solvent control, respectively.

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