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Aplicyanins A–F, new cytotoxic bromoindole derivatives from the marine tunicate *Aplidium cyaneum*

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ABSTRACT

Six new bromoindole derivatives, aplicyanins A–F (**1**–**6**), have been isolated from the CH_2Cl_2/MeOH extract of the tunicate *Aplidium cyaneum* collected in Antarctica. Their structures were determined by extensive analysis of their spectroscopic features, particularly 1D and 2D NMR spectra, and comparison with related compounds. Cytotoxic and antimitotic activities were found for compounds **2** and **4**–**6**.

1. Introduction

Marine tunicates of the genus *Aplidium* have been the source of numerous nitrogen-containing metabolites belonging to unprecedented structural families of natural products.[1] Some of these compounds possess interesting biological properties, with the cyclodepsipeptide aplidin isolated from *Aplidium albicans* being perhaps the most renowned due to its interesting antitumor activity.[2] Other cytotoxic nitrogenous metabolites isolated from ascidians of the genus *Aplidium* include the lobatamides, macrocycles with an uncommon methylated oxime moiety isolated from *Aplidium lobatum*,[3] a group of iodotyrosine-derived compounds obtained from an unidentified species of an Australian tunicate of this genus,[4] a 1,2,3-trithiane derivative isolated from a New Zealand species of *Aplidium*,[5] pantherinine, a pyridoacridine from *Aplidium pantherinum*,[6] the haouamines from *Aplidium haouarianum*,[7] and the conicaquinones[8] and thiaplidiaquinones[9] obtained from *Aplidium conicum*. The presence in this genus of indole alkaloids is restricted to the isolation of the meridianins, a family of compounds with a brominated and/or hydroxylated indole nucleus and a 2-aminopyrimidine substituent at C-3 from *Aplidium meridianum*,[10,11] and the histamine antagonist conicamycin from the Mediterranean *A. conicum*.[12] Moderate cytotoxicity toward murine mamarian adenocarcinoma cells as well as protein kinase inhibitory properties have been described for the meridianins, making these compounds promising scaffolds for the development of new protein kinase inhibitors.[10,13]

In the course of our ongoing program for the search of new antitumor agents from marine organisms, the CH_2Cl_2/MeOH extracts of the hitherto uninvestigated ascidian *Aplidium cyaneum* were found to display cytotoxicity against the human tumor cell lines A-549, HT-29, and MDA-MB-231 as well as antimitotic properties. Bioassay-guided fractionation of these extracts led to the isolation of aplicyanins A–F (**1**–**6**), a group of cytotoxic and antimitotic alkaloids containing a bromoindole nucleus and a 6-tetrahydropyrimidine substituent at C-3. Herein, we report the isolation, structural characterization, and cytotoxic and antimitotic properties of this new family of marine metabolites.

2. Results and discussion

Samples of *A. cyaneum* were collected by bottom trawling at the Weddell sea (Antarctica) and kept frozen until used. The crude CH_2Cl_2/MeOH extract of the frozen tunicate was subjected to reversed phase C_{18} chromatography. Semipreparative reversed phase HPLC of selected active fractions from this chromatography led to the isolation of compounds **1**–**6** as their TFA salts.

The most polar compound of the extract, aplicyanin A (**1**), was isolated as an optically active pale yellow oil. A pseudomolecular

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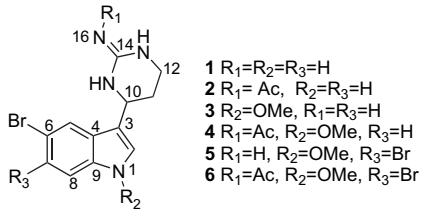

1 $R_1=R_2=R_3=H$
2 R_1= Ac, $R_2=R_3=H$
3 R_2=OMe, $R_1=R_3=H$
4 R_1=Ac, R_2=OMe, $R_3=H$
5 $R_1=H$, R_2=OMe, R_3=Br
6 R_1=Ac, R_2=OMe, R_3=Br

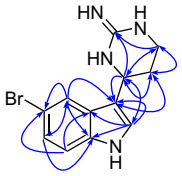

Figure 1. HMBC (H–C) correlations for aplicyanin A (**1**).

ion in the (+)-HRESIMS at *m*/*z* 293.0399, with an isotopic cluster for one bromine atom, and the presence of 12 signals in the ^{13}C NMR spectrum (Table 1) were consistent with a molecular formula of $C_{12}H_{13}BrN_4$. The ^{13}C NMR spectrum contained nine low field signals, eight of them attributable to olefinic carbons. The remaining low field signal was assigned to the presence of a guanidine group in the molecule on the basis of its chemical shift (δ_C 155.7 ppm). Signals in the UV, ^{13}C NMR, and the low field region of the ^1H NMR spectra (Table 1) accounted for the presence of a 3-substituted indole in the molecule. In addition, the proton coupling pattern [7.75 (d, 2.0), 7.26 (dd, 9.0, 2.0), and 7.34 (d, 9.0)] indicated a substitution either at C-6 or C-7. A bromine atom was placed at C-6 based on the correlations observed in the HMBC spectrum (Fig. 1), the downfield shift of proton H-5, which appeared as a *m*-coupled doublet, and comparison with reference data for similar compounds.[11] The nature of the substituent at C-3 was deduced from the analysis of the high field region of the ^1H NMR spectrum and correlations observed in the HSQC and HMBC spectra. Signals for a nitrogenated methine and two methylenes, one of them also nitrogenated, were observed in the ^1H NMR spectrum. Correlations observed in the COSY spectrum established the sequence from C-10 to C-12, and HMBC correlations from H-10 and both H-12 protons to the guanidine carbon C-13 confirmed the presence of a 6-substituted tetrahydropyrimidin-2(1*H*)-imine in the molecule. Finally, HMBC cross-peaks between H-10 and C-2 and C-3, between both H-11 protons and C-3, and between H-2 and C-10 confirmed the attachment of C-3 to C-10 establishing the structure of the compound as depicted in **1**.

Compound **2** had a molecular formula of $C_{14}H_{15}BrN_4O$, according to its (+)-HRESIMS (*m*/*z* $[M+H]^+$ 335.0508, calcd for $C_{14}H_{16}{}^{79}BrN_4O$, 335.0501) and ^{13}C NMR spectra (Table 1). The two additional carbon atoms with respect to **1** were assigned to an acetyl group based on the observation of signals for a carbonyl at δ_C 173.9 ppm and a methyl group at δ_H/δ_C 2.21/24.1 ppm. ^{15}N HMBC studies carried out on aplicyanin F (see below) located this acetyl group at N-16. To the best of our knowledge, this is the first report of the presence of *N*-acetylguanidines in marine natural products.

The third compound in the series, aplicyanin C (**3**), was isolated as an optically active yellowish oil of molecular formula $C_{13}H_{15}BrN_4O$, according to the (+)-HRESIMS and ^{13}C NMR spectra. A direct comparison of the ^1H and ^{13}C NMR spectra (Table 1) of this compound with those of aplicyanin A revealed the presence of a methoxyl group in **3** as the only noticeable difference between both compounds. That function was placed at the indole nitrogen based on correlations observed in the ROESY spectrum between the methoxyl singlet at δ_H 4.10 ppm and protons H-2 and H-8. *N*-Methoxyindoles are fairly uncommon in marine natural products. Only two compounds containing that functionality have been previously reported: pibocin B, an ergoline alkaloid isolated from an *Eudistoma* species ascidian,[14] and the nematocide convolutindole A, obtained from dichloromethane extracts of the bryozoan *Amathia convoluta*.[15] The ^1H and ^{13}C NMR chemical shifts reported for the *N*-methoxy functionality of these compounds are well in agreement with those observed for aplicyanin C.

The major differences found in the ^1H and ^{13}C NMR spectra of aplicyanin D (**4**) with respect to **3** were the presence of signals attributable to the presence of an acetyl group in the molecule. It was placed at N-16 based on ^{15}N HMBC studies carried out on the structurally related aplicyanin F (see below). A parent ion at *m*/*z* 365.0611 ($[M+H]^+$) in the (+)-HRESIMS with an isotopic cluster for one bromine, accounting for a molecular formula of $C_{15}H_{17}BrN_4O_2$, corroborated the structural proposal.

Compound **5** had a molecular formula of $C_{13}H_{14}Br_2N_4O$ according to the (+)-HRESIMS (*m*/*z* $[M+H]^+$ 400.9609 (calcd for $C_{13}H_{15}{}^{79}Br_2N_4O$, 400.9607)) and ^{13}C NMR spectra (Table 2). The major differences in the NMR spectra of this compound compared to **3** were found in the aromatic region of the proton spectrum. Three singlet signals accounted for the presence of a 3,6,7-trisubstituted indole in the molecule. Substituents at C-6 and C-7

Table 1
^1H and ^{13}C NMR data (CD_3OD, 500/125 MHz) for aplicyanins A–C (**1**–**3**)[a]

No	**1**		**2**		**3**	
	^{13}C	^1H (multiplicity, *J*)	^{13}C	^1H (multiplicity, *J*)	^{13}C	^1H (multiplicity, *J*)
2	125.3 d	7.31 (s)	125.6 d	7.37 (s)	123.8 d	7.56 (s)
3	113.6 s		113.8 s		112.0 s	
4	124.1 s		127.8 s		124.3 s	
5	121.9 d	7.75 (d, 2.0)	121.8 d	7.80 (d, 2.0)	122.5 d	7.80 (d, 2.0)
6	115.2 s		114.3 s		114.4 s	
7	125.9 d	7.26 (dd, 9.0, 2.0)	126.1 d	7.29 (dd, 8.5, 2.0)	126.9 d	7.37 (dd, 8.5, 2.0)
8	114.5 d	7.34 (d, 9.0)	114.6 d	7.36 (d, 8.5)	111.4 d	7.42 (d, 8.5)
9	137.2 s		137.2 s		132.7 s	
10	48.1 d	4.95 (dd, 6.5, 6.0)	48.2 d	5.14 (dd, 6.5, 6.0)	47.6 d	4.94 (dd, 8.0, 4.5)
11	28.3 t	2.25 (m) 2H	26.9 t	2.34 (m), 2H	28.3 t	2.26 (dddd, 13.5, 8.5, 8.0, 5.5); 2.18 (dddd, 13.5, 5.5, 4.5, 4.5)
12	38.6 t	3.46 (ddd, 12.5, 6.5, 6.5); 3.41 (ddd, 12.5, 5.0, 5.0)	38.6 t	3.60 (ddd, 13.0, 7.5, 6.5); 3.53 (ddd, 13.0, 5.5, 5.0)	38.4 t	3.45 (ddd, 12.5, 8.5, 4.5); 3.40 (ddd, 12.5, 5.5, 5.0)
14	155.7 s		152.3 s		155.7 s	
O*Me*					66.8 q	4.10 (s)
CH_3CO			173.9 s			
CH_3CO			24.1 q	2.21 (s)		

[a] Assignments were made with the help of edited HSQC and HMBC experiments.

Table 2
^{1}H and ^{13}C NMR data (CD_3OD, 500/125 MHz) for aplicyanins D–F (**4**–**6**)[a]

No	**4**		**5**		**6**	
	^{13}C	^{1}H (multiplicity, *J*)	^{13}C	^{1}H (multiplicity, *J*)	^{13}C	^{1}H (multiplicity, *J*)
2	124.1 d	7.61 (s)	124.7 d	7.60 (s)	125.0 d	7.67 (s)
3	111.2 s		112.3 s		111.5 s	
4	124.1 s		123.5 s		123.2 s	
5	122.4 d	7.83 (d, 1.5)	124.6 d	7.99 (s)	124.5 d	8.05 (s)
6	114.6 s		119.3 s		119.5 s	
7	127.1 d	7.38 (dd, 8.5, 1.5)	116.4 s		116.6 s	
8	111.5 d	7.42 (d, 8.5)	114.6 d	7.85 (s)	114.6 d	7.88 (s)
9	132.7 s		133.6 s		133.6 s	
10	47.7 d	5.11 (dd, 7.5, 5.0)	47.4 d	4.94 (dd, 8.0, 5.0)	47.6 d	5.13 (dd, 7.0, 5.0)
11	26.6 t	2.32 (dddd, 13.5, 8.0, 7.5, 6.5); 2.18 (dddd, 13.0, 5.0, 5.0, 4.5)	28.3 t	2.26 (dddd, 14.0, 8.0, 8.0, 5.5); 2.18 (dddd, 14.0, 5.0, 5.0, 4.5)	26.8 t	2.26 (dddd, 13.5, 8.0, 7.0, 6.5); 2.18 (dddd, 13.5, 5.0, 4.5, 4.5)
12	38.2 t	3.57 (ddd, 13.5, 8.0, 5.0); 3.48 (ddd, 13.5, 6.5, 4.5)	38.3 t	3.45 (ddd, 13.0, 8.0, 4.5); 3.39 (ddd, 13.0, 5.5, 5.0)	38.1 t	3.59 (ddd, 13.5, 8.0, 4.5); 3.49 (ddd, 13.5, 6.5, 4.5)
14	152.4 s		155.7 s		152.3 s	
O*Me*	66.7 q	4.11 (s)	67.0 q	4.11 (s)	67.1 q	4.13 (s)
CH_3CO	174.0 s				174.0 s	
CH_3CO	24.1 q	2.21 (s)			24.1 q	2.22 (s)

[a] Assignments were made with the help of edited HSQC and HMBC experiments.

were identified as bromine atoms based on their ^{13}C chemical shifts, assigned through correlations observed in the HMBC spectrum with protons H-8 and H-5, respectively. An isotopic cluster for the presence of two bromine atoms in the mass spectrum of the molecule corroborated this proposal. The rest of the signals in the NMR spectra of aplicyanin E accounted for the presence in the molecule of an *N*-methoxy group located at the nitrogen indole and a 6-substituted tetrahydropyrimidin-2(1*H*)-imine that was placed at the C-3 position of the indole on the basis of HMBC correlations and by analogy with the rest of the compounds of the family.

The last compound of the series, aplicyanin F (**6**), had a molecular formula of $C_{15}H_{16}Br_2N_4O_2$, based on a pseudomolecular ion at *m*/*z* 442.9734 in its (+)-HRESI mass spectrum (calcd for $C_{15}H_{17}{}^{79}Br_2N_4O_2$, 442.9712). The major differences between the NMR spectra of **6** and those of aplicyanin E were attributed to the presence of an acetyl group, placed at N-16 on the basis of ^{15}N HMBC correlations (Fig. 2). Signals for all four of the nitrogen atoms present in the molecule were indirectly detected through 2 and 3-bond distance correlations. A signal at δ_N 195.7 ppm (liquid ammonia reference) was assigned to the indole nitrogen N-1 on the basis of its chemical shift and HMBC correlations observed with H-2 and the *N*-methoxy group. Both H-11 protons displayed 3-bond correlations with two signals at δ_N 90.7 and 98.0 ppm, attributable to N-13 and N-15. Finally an additional 3-bond correlation observed between N-16 (δ_N 130.3 ppm) and the methyl of the acetyl group at δ_H 2.22 ppm confirmed the location of the latter group at the N-16 nitrogen atom. To corroborate the structural relationship between aplicyanins E and F, **5** was readily converted to **6** in 49% yield by treatment with Ac_2O/Pyr at room temperature.

Given the low value of the optical rotation measured for the aplicyanins and in order to preclude the possibility that some racemization at C-10 might have occurred during the isolation process due to the presence of both an aromatic ring and a nitrogen atom vicinal groups, aplicyanin E was subjected to chiral HPLC analysis. The observation of a single peak is in favor of the isolation of the aplicyanins in their optically pure form.

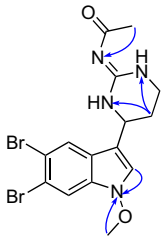

Figure 2. ^{15}N-HMBC (H–N) correlations observed for aplicyanin F (**6**).

Two biological activities were evaluated in parallel during the fractionation of the tunicate extracts: cytotoxicity, evaluated against a panel of three human tumor cell lines, including colon (HT-29), lung (A-549), and breast (MDA-MB-231),[16] and antimitotic activity, measured using a specific microplate immunoassay (ELISA). Both activities were evaluated for all six of the compounds isolated and the results are shown in Table 3. Cytotoxic activity in the submicromolar range as well as antimitotic properties were found for compounds **2**, **4**, and **6**, whereas compounds **1** and **3** proved to be inactive at the highest concentrations tested and compound **5** displayed only mild cytotoxic properties. These results clearly suggest a key role for the presence of the acetyl group at N-16 in the biological activity of this family of compounds.

Table 3
Cytotoxicity (GI_{50} values (μM)) and antimitotic activity (IC_{50}, μM) of compounds **2** and **4**–**6**

Compound	Cell lines			Antimitotic activity
	A-549	HT-29	MDA-MB-231	
2	0.66	0.39	0.42	1.19
4	0.63	0.33	0.41	1.09
5	8.70	7.96	7.96	—
6	1.31	0.47	0.81	0.18–0.036

3. Conclusions

In conclusion, six members of a new family of indole alkaloids, some of them with cytotoxic and antimitotic properties, have been isolated from extracts of the tunicate *A. cyaneum*. These compounds are reduced forms of the structurally related meridianins isolated from *A. meridianum* and can therefore be considered their biogenetic precursors. Three of the new compounds incorporate an acetyl group at one of the guanidine nitrogens, an element of structural novelty that confers these molecules improved cytotoxic and antimitotic properties with respect to the other members of the series. Our findings are also further proof that marine organisms can produce structurally novel chemical entities that may be lethal to cancer cells and are therefore potential new drugs for the treatment of this disease.

4. Experimental section

4.1. General experimental procedures

Optical rotations were determined in MeOH using a Jasco P-1020 Polarimeter. IR spectra were recorded on a Perkin Elmer 881 Infrared Spectrophotometer. NMR spectra were recorded in CD_3OD on a Varian 'Unity 500' spectrometer at 500/125 MHz ($^1H/^{13}C$). Chemical shifts were reported in parts per million using residual non-deuterated CD_3OD signals (δ 3.31 for ^1H and 49.0 for ^{13}C) as internal reference. ^{15}N-HMBC experiments were optimized for a $^{2,3}J_{NH}$ of 8 Hz. Accurate mass analyses were performed by (+)-HRESIMS on an Applied Biosystems API QStar pulsar i Spectrometer.

4.2. Sample collection and identification

Samples of *A. cyaneum* were collected in December 2003 by bottom trawling at the Weddell Sea (10° 31′ 59″ W, 71° 55′ 59″S) at depths ranging between 220 and 300 m, and kept frozen until used. The material was identified by Dr. Alfonso Ramos from the University of Alicante (Spain). Voucher specimens with numbers ASC.ANT.EQ433-1 and ASC.ANT.EQ1097-1 are deposited at the Department of Environmental Sciences (Marine Biology Unit) of the University of Alicante (Spain).

4.3. Extraction and isolation

The frozen organism (437 g) was triturated and exhaustively extracted with water (1 L+2×300 mL) and a mixture of MeOH/CH_2Cl_2 (1:1, 3×500 mL) at room temperature. The organic extract was evaporated under reduced pressure to yield a crude of 939.7 mg. This material was chromatographed (VLC) on Lichroprep RP-18 with a stepped gradient from H_2O to MeOH and subsequently MeOH/CH_2Cl_2 (1:1) and CH_2Cl_2. The fraction eluted with H_2O/MeOH 1:1 (51.3 mg) was subjected to semipreparative reversed phase HPLC (SymmetryPrep C18, 7.8×150 mm, gradient H_2O+0.1% TFA/CH_3CN+0.1% TFA, from 10 to 60% CH_3CN+0.1% TFA in 20 min, flow 2.3 mL/min, UV detection at 254 nm) to yield compounds **1** (1.6 mg), **2** (5.1 mg), **3** (5.3 mg), **4** (14.7 mg), **5** (10.5 mg), and **6** (11.4 mg). Semipreparative HPLC in the same conditions of the fraction eluted with H_2O/MeOH 1:3 (57.0 mg) yielded additional amounts of **1** (0.9 mg), **3** (4.1 mg), **5** (31.1 mg), and **6** (1.4 mg).

4.3.1. Aplicyanin A (**1**)

Pale yellow oil. $[\alpha]_D^{25}$ −0.8 (*c* 0.1, $CHCl_3$); IR (NaCl) ν_{max} 3369, 2922, 1668, 1627, 1459, 1198, 1134 cm^{-1}; (+)-HRESIMS *m/z* 293.0399 $[M+H]^+$ (calcd for $C_{12}H_{14}{}^{79}BrN_4$, 293.0396); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 1.

4.3.2. Aplicyanin B (**2**)

Pale yellow oil. $[\alpha]_D^{25}$ +8.7 (*c* 0.1, $CHCl_3$); IR (NaCl) ν_{max} 3430, 1661, 1436, 1257, 1200, 1138 cm^{-1}; (+)-HRESIMS *m/z* 335.0508 $[M+H]^+$ (calcd for $C_{14}H_{16}{}^{79}BrN_4O$, 335.0501); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 1.

4.3.3. Aplicyanin C (**3**)

Pale yellow oil. $[\alpha]_D^{25}$ +3.1 (*c* 0.1, $CHCl_3$); IR (NaCl) ν_{max} 3373, 1668, 1627, 1438, 1201, 1137 cm^{-1}; (+)-HRESIMS *m/z* 323.0516 $[M+H]^+$ (calcd for $C_{13}H_{16}{}^{79}BrN_4O$, 323.0501); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 1.

4.3.4. Aplicyanin D (**4**)

Pale yellow oil. $[\alpha]_D^{25}$ +9.5 (*c* 0.1, $CHCl_3$); IR (NaCl) ν_{max} 3433, 1670, 1451, 1259, 1200, 1134 cm^{-1}; (+)-HRESIMS *m/z* 365.0611 $[M+H]^+$ (calcd for $C_{15}H_{18}{}^{79}BrN_4O_2$, 365.0607); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 2.

4.3.5. Aplicyanin E (**5**)

Pale yellow oil. $[\alpha]_D^{25}$ +0.5 (*c* 0.1, $CHCl_3$); IR (NaCl) ν_{max} 3411, 1672, 1439, 1201, 1135 cm^{-1}; (+)-HRESIMS *m/z* 400.9609 $[M+H]^+$ (calcd for $C_{13}H_{15}{}^{79}Br_2N_4O_2$, 400.9607); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 2.

4.3.6. Aplicyanin F (**6**)

Pale yellow oil. $[\alpha]_D^{25}$ +1.9 (*c* 0.1, $CHCl_3$); IR (NaCl) ν_{max} 3440, 1680, 1440, 1260, 1201, 1135 cm^{-1}; (+)-HRESIMS *m/z* 442.9734 $[M+H]^+$ (calcd for $C_{15}H_{17}{}^{79}Br_2N_4O_2$, 442.9712); ^1H (500 MHz) and ^{13}C NMR (125 MHz) see Table 2.

4.4. Conversion of 5 to 6

To a solution of **5** (40 mg, 0.1 mmol) in pyridine (5 mL), Ac_2O (1.5 mL) was added and the resulting mixture was stirred at room temperature for 15 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL) and saturated aqueous solution of $NaHCO_3$ (10 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (2×20 mL) and the combined organic extracts were washed with 5% HCl (2×20 mL), dried over Na_2SO_4, filtered, and concentrated to yield a crude of 60 mg. Semipreparative HPLC (same conditions as for the natural product isolation) of this crude yielded **6** (21.5 mg, 0.049 mmol, 49%).

4.5. Chiral HPLC analysis of compound 5

Aplicyanin E (**5**) was subjected to chiral HPLC analysis (Chiralpak AD-RH column, 4.6×150 mm, gradient 10 mM $AcONH_4$ pH= 3/EtOH, from 30 to 70% EtOH in 15 min, flow 0.8 mL/min, UV detection at 234 nm). A single peak with a retention time of 7.0 min was observed under these conditions.

4.6. Cytotoxicity assay

A-549 (ATCC CCL-185), lung carcinoma; HT-29 (ATCC HTB-38), colorectal carcinoma; and MDA-MB 231 (ATCC HTB-26), breast adenocarcinoma cell lines were obtained from ATCC. Cells are maintained in RPMI 1640 10% FBS, supplemented with 0.1 g/L penicillin and 0.1 g/L streptomycin sulfate and then incubated at 37 °C, 5% CO_2 and 98% humidity. For the experiments, cells were harvested from subconfluent cultures using trypsin and resuspended in fresh medium before plating. Cells are seeded in 96 well microtiter plates, at 5×10^3 cells per well in aliquots of 195 μL medium, and they are allowed to attach to the plate surface by growing in drug free medium for 18 h. Afterward, samples are added in aliquots of 5 μL ranging from 10 to 10^{-8} μg/mL, dissolved in DMSO/EtOH/PBS (0.5:0.5:99). After 48 h exposure, the antitumor effect is measured by the SRB methodology:[16] cells are fixed by adding 50 μL of cold 50% (w/v) trichloroacetic acid (TCA) and incubated for 60 min at 4 °C. Plates are washed with deionized water and dried. SRB solution (100 μL, 0.4% w/v in 1% acetic acid) is added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB is removed by washing with 1% acetic acid. Plates are air dried and bound stain is solubilized with Tris buffer. Optical densities are read on an automated spectrophotometric plate reader at a single wavelength of 490 nm.

4.7. Antimitotic assay protocol

The mitotic ratio of cell culture was determined using a specific microplate immunoassay (ELISA). HeLa cells (h-cervix carcinoma, ATCC# CCL-2) were incubated in the presence or absence of the

indicated compounds in 96 well microtiter plates. After 18 h, cells were washed with PBS and lysed on ice in 75 mL of freshly prepared lysis buffer (1 mM EGTA (pH 7.5), 0.5 mM PMSF, and 1 mM $NaVO_3$) for 30 min. An aliquot of the cell extract (60 μL) was transferred to a high-binding surface ELISA plate and dried in a speed-vac for 2 h at room temperature. Plates were then blocked in 100 μL PBS-1% BSA for 30 min at 30 °C and sequentially incubated with anti-MPM2 primary mouse monoclonal antibody (Upstate Biotechnology, cat # 05-368) for 18 h at 4 °C and appropriate peroxidase-conjugated secondary antibody for 1 h at 30 °C. After intensive washing in 0.02% Tween-20, peroxidase reaction was performed using 30 μL of TMB (3,3′,5,5′-tetramethyl-benzidine) for 30 min at 30 °C. Reaction was stopped by adding 30 μL of a 4% H_2SO_4 solution. Assay was quantified by measuring the O.D. at 450 nm in a microplate spectrophotometer. Results were expressed as compound concentration that produces 50% of the control (taxol) mitotic ratio.

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