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12-Keto-Porrigenin and the Unique 2,3-Seco-Porrigenin, New Antiproliferative Sapogenins from *Allium porrum*

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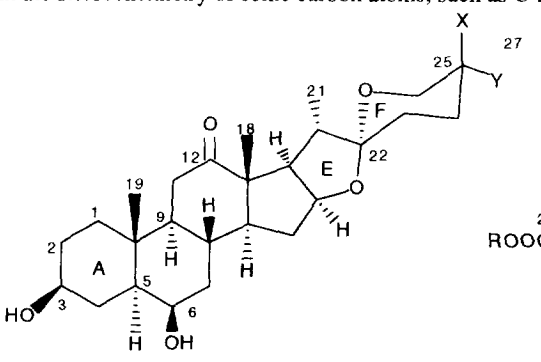
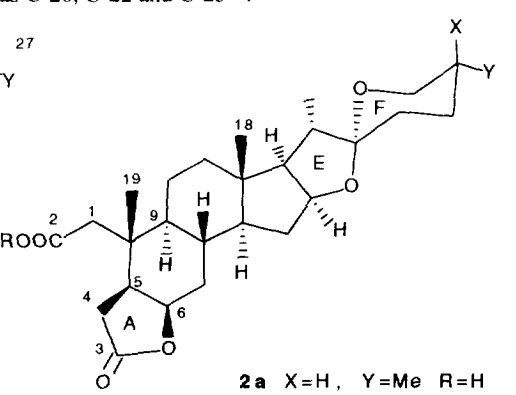
Abstract: Two new sapogenins, 12-keto-porrigenin (**1a**) and 2,3-seco-porrigenin (**2a**), have been isolated from the organic extract of *Allium porrum* and identified as (25R)-5α-spirostan-3β, 6β-diol-12-one (**1a**) and (25R)-5α-2,3-secospirostan-2,3-dioic acid-6β–hydroxy-3,6-γ-lactone (**2a**), the latter based on a unique 2,3-seco-spirostane skeleton. Small amounts of the 25S epimers, **1b** and **2b**, have been also found in the extract. Chemical structures were elucidated by a detailed spectroscopic analysis. In addition, **1a** and **2a** exhibited antiproliferative activity on four tumor cell lines *in vitro*.

The steroidal saponins are divided into two main groups, spirostanol glycosides and furostanol glycosides. The first (and the largest) group, named spirostanol saponins, which comprises aglycones of the spyrostan type with the sugar moiety generally linked at position 3, has been extensively investigated on account of their interesting bioactivities, as well as of their economic importance as starting material in the industrial synthesis of steroid hormones. A number of different spirostanol aglycones have been so far isolated from seeds, bulbs and roots of plants; they mainly differ in the oxidation pattern of the polycyclic skeleton and in the stereochemistry of some carbon atoms, such as C-20, C-22 and C-25[1,2].



1a X=H, Y=Me
1b X=Me, Y=H



2a X=H, Y=Me R=H
2b X=Me, Y=H R=H
3a X=H, Y=Me R=Me

As a part of our ongoing search for novel bioactive substances from *Allium* species[3,4] we have now examined *Allium porrum*, leek. The investigation resulted in the isolation of two novel spirostanol sapogenins, 12-keto-porrigenin (**1a**) and 2,3-seco-porrigenin (**2a**). The latter compound appears to be particularly interesting because it contains a seco structure, which, to the best of our knowledge, is an unprecedented feature among the sterols having a spirostane skeleton. Minor amounts of the 25S epimers of **1a** and **2a** (**1b** and **2b**, respectively) have been also found in the extract. This paper deals with isolation, structure elucidation and biological activity of the isolated compounds.

Samples of *Allium porrum* were collected and extracted with *n*-hexane followed by $CHCl_3$, $CHCl_3$/MeOH (9:1) and MeOH. Repeated column chromatographies of the $CHCl_3$/MeOH (9:1) extract yielded pure sapogenins.

Compound **1a** shows in the HREIMS spectrum a molecular ion peak at m/z 446.3018 in accordance with the empirical formula $C_{27}H_{42}O_5$, also deduced on the basis of the ^{13}C NMR data. The spirostane structure of **1a** was suggested from its ^1H NMR spectrum, which showed methyl signals at δ 1.09 (3H, s), 1.13 (3H, s), 0.79 (3H, d, J=6.4 Hz), and 1.06 (3H, d, J=7.1 Hz), assigned to methyls 19, 18, 27, and 21 of a spirostane skeleton, respectively. Analysis of NMR data indicated the presence of three oxymethines (^1H: δ 3.66, 3.87 and 4.34; ^{13}C: δ 71.3, 71.3 and 79.1), an oxymethylene (^1H: δ 3.35 and 3.48; ^{13}C: δ 66.9), a ketal (^{13}C: δ 109.3), and a ketone group (^{13}C: δ 213.4). Although the high field region in the ^1H NMR spectrum in $CDCl_3$ contained several overlapping signals, the ^1H-^1H COSY experiment provided useful informations enough to assign all the proton resonances, delineating two spin systems. The first one was a large system comprising the protons of rings A-E [C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8 (C-9, C-11), C-14, C-15, C-16, C-17, C-20, C-21]. The second one was the segment including the protons of ring F [C-23, C-24, C-25, (C-26), C-27].

Using as anchorage site of the first segment the signal of H_3-21 (δ 1.06) comparative analysis of ^1H-^1H COSY, HOHAHA and HMQC spectra allowed the identification, in sequence, of three methine (H-20, δ 1.76; H-17, δ 2.54; and H-16, δ 4.34) one methylene (H_2-15, δ 2.12 and 1.56), and two methine groups (H-14, δ 1.47; and H-8, δ 2.32). The last proton signal (H-8) was in turn coupled with one methine (H-9, δ 1.16) and one methylene protons (H_2-7, δ 1.19 and 1.92), thus indicating a branching at C-8. Vicinal couplings between H-9 and H_2-11 signals (δ 2.48 and 2.23) permitted us to further extend the branching to methylene 11. The chemical shifts in the ^1H and ^{13}C NMR spectra of the H_2-11 (δ 2.48 and 2.23), C-11 (δ 37.6), and C-13 (δ 55.2) signals are characteristic of a 12-oxo-spirostane[1,5]. Correlation peaks observed in the HMBC experiment between C-12 and H_2-11 confirmed this structural feature.

Going back to the branching at C-8, we extended the spin system from H_2-7 to the remaining protons of rings A-B. The chemical shift values observed for H-3 (δ 3.66), and H-6 (δ 3.87) signals indicated the presence of two hydroxyl groups at these positions, respectively. The coupling constants of H-3 signal (tt, J=12.0, 12.0, 5.0, 5.0) indicated its axial orientation, while, the small couplings observed for H-6 signal defined its equatorial nature. 1,3-Diaxial interactions of H-5 with H-3 and H-9, deduced by the ROESY spectrum, determined the α-orientation for H-5 and consequently a *trans* junction of rings A and B.

Through analysis of 2D homo- and etero-nuclear NMR experiments the second spin system was easily attributed to the proton signals of the ring F, a part structure of general occurrence in spirostanol sapogenins.

According to the above data the structure of **1a** was established as reported in formula.

The molecular formula of **2a** was determined by HREIMS providing a molecular ion at m/z 460.2810 appropriate for the molecular formula $C_{27}H_{40}O_6$. ^{13}C NMR spectrum confirmed the presence of 27 carbon

atoms, including two carbonyls (s, δ 175.5 and s, δ 177.7), one ketal (s, δ 109.4), one oxymethylene (t, δ 66.9), and two oxymethine (d, δ 79.7 and d, 80.6) functions.

The presence of four methyl signals easily identifiable both by ^{13}C and ^{1}H NMR spectra together with the presence of the ketal function suggested a spirostane-like structure.

Table I. ^{1}H NMR assignments ($CDCl_3$) for compounds **1a-2a**

	1a		**2a**	
Pos.	δ_C (mult.)	δ_H (mult., *J* [Hz])	δ_C (mult.)	δ_H (mult., *J* [Hz])
1 a	38.0 (CH_2)	0.98 [a] (dt, 3.5, 13.0, 13.0)	42.4 (CH_2)	2.22 (d, 14.5)
b		1.52 [a]		2.30 (d, 14.5)
2 β	31.2 (CH_2)	1.44 [b]	175.5 (C)	
α		1.83 (bd, 12.4)		
3	71.3 (CH)	3.66 (tt, 12.0, 12.0, 5.0, 5.0)	177.7 (C)	
4α	35.1 (CH_2)	1.69 [b]	34.7 (CH_2)	2.61 (dd, 7.4, 17.3)
β		1.74 [b]		2.49 (d, 17.3)
5	47.3 (CH)	1.18 [b]	41.7 (CH)	2.80 (dd, 4.8, 7.4)
6	71.3 (CH)	3.87 (bs)	79.7 (CH)	4.57 (bt, 4.7)
7 α	39.1 (CH_2)	1.19 [b]	32.3 (CH_2)	1.21 [b]
β		1.92 (td, 3.0, 3.0, 13.4)		2.29 [b]
8	29.4 (CH)	2.32 (dq, 3.0, 11.0,11.0, 11.0)	29.1 (CH)	1.79 [b]
9	55.6 (CH)	1.16 [b]	45.8 (CH)	1.22 [b]
10	36.0 (C)		38.0 (C)	
11β	37.6 (CH_2)	2.48 (t, 13.5)	21.0 (CH_2)	1.48 [b]
α		2.23 (dd, 5.0, 13.5)		1.62 [b]
12α	213.4 (C)		39.4 (CH_2)	1.19 [b]
β				1.74 [b]
13	55.2 (C)		40.2 (C)	
14	55.6 (CH)	1.47 [b]	56.2 (CH)	1.08 [b]
15α	31.4 (CH_2)	2.12 (m)	31.4 (CH_2)	2.02 m
β		1.56 [b]		1.32 [b]
16	79.1 (CH)	4.34 (bq, 7.5)	80.6 (CH)	4.41 (bq, 7.5)
17	53.4 (CH)	2.54 (dd, 7.1, 8.5)	62.0 (CH)	1.77 [b]
18	15.2 (CH_3)	1.13 (s)	16.2 (CH_3)	0.76 (s)
19	16.1 (CH_3)	1.09 (s)	17.0 (CH_3)	1.00 (s)
20	42.2 (CH)	1.76 [b]	41.6 (CH)	1.86 (q, 7.0)
21	13.2 (CH_3)	1.06 (d. 7.1)	14.4 (CH_3)	0.96 (d, 7.0)
22	109.3 (C)		109.4 (C)	
23β	31.2 (CH_2)	1.60 [b]	31.3 (CH_2)	1.60 [b]
α		1.68 [b]		1.68 [b]
24α	28.7 (CH_2)	1.43 [b]	28.7 (CH_2)	1.43 [b]
β		1.62 [b]		1.62 [b]
25	30.2 (CH)	1.63 [b]	30.2 (CH)	1.63 [b]
26α	66.9 (CH_2)	3.35 (t, 11.0)	66.9 (CH_2)	3.37 t, 11.0)
β		3.48 (ddd, 1.5, 4.0, 11.0)		3.47 (ddd, 1.5, 4.0, 11.0)
27	17.1 (CH_3)	0.79 (d, 6.4)	17.1 (CH_3)	0.79 (d, 6.6)

[a] 1a = α-oriented; 1b = β-oriented; [b] submerged by other signal

Analysis of 2D COSY, HOHAHA, HMBC and HMQC experiments delineated three spin systems. The first one was based on the large segment C-4, C-5, C-6, C-7, C-8, (C-9, C-11, C-12), C-14, C-15, C-16, C-17, C-20, C-21. The second one was an isolated methylene group (C-1) and the third segment included the ring F protons : C-23, C-24, C-25, (C-26), C-27.

Using as starting point of the first segment the doublet at δ 0.96 (H_3-21), we were able to identify, in sequence, three methine (H-20, δ 1.86; H-17, δ 1.77; and H-16, δ 4.41), a methylene (H_2-15, δ 2.02 and δ 1.32) and two methine groups (H-14, δ 1.08; and H-8, δ 1.79). The last proton signal resulted to be coupled with a methine a δ 1.22 (H-9) and a methylene proton signals at δ 1.21 and δ 2.29 (H_2-7). When considering H-9, analysis of the cross-peaks in the 2D experiments allowed us to extend the structure to H_2-11 (δ 1.48 and δ 1.62) and H_2-12 (δ 1.19 and δ 1.74). On the other hand, starting from the H_2-7 signals we were able to identify in sequence H-6 (δ 4.57), H-5 (δ 2.80) and H_2-4 (δ 2.61 and 2.49). The chemical shift value and splitting pattern of H_2-4 signals, together with their correlations in the 2D HMBC with the carbonyl signal at δ 177.7 in the ^{13}C NMR, located a CO-O function at position 3, thus accounting for two further oxygen atoms present in the molecular formula. The H-6 proton signal resonating at δ 4.57 appeared to be downfield shifted when compared to the corresponding one of **1a**. These data together with the cross peak observed in the HMBC experiment between C-3 and H-6 strongly suggested the presence of a γ-lactone moiety. The J values of H_2-4, H-5 and H-6 signals ($J_{4\alpha\text{-}5}$=7.4, $J_{4\beta\text{-}5}$=0 and $J_{5\text{-}6}$=4.8 Hz) were consistent with the hypothesized five membered lactone ring. Intense nOe effects in a ROESY experiment of H_3-19 with H_β-4, and H-9 with H-5 indicated the α-orientation of H-5. Furthermore nOe contact between H_α-4 and H-6 pointed to the α-orientation of H-6 as shown in formula.

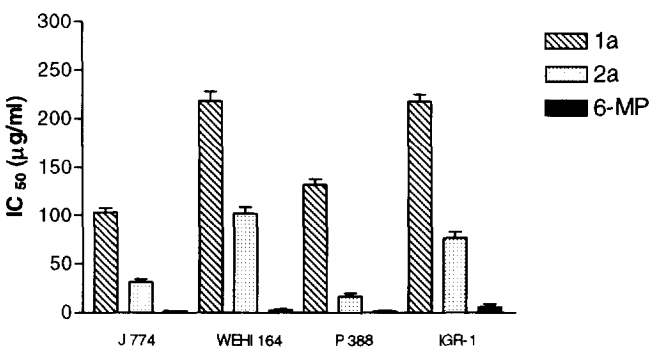


Figure 1. *In vitro* antiproliferative activity (IC_{50} µg/ml)of **1a**, **2a**, and 6-MP (6-mercaptopurine) on IGR-1 (human melanoma), J774 (murine monocyte/macrophage), WEHI 164 (murine fibrosarcoma), and P388 (murine leukemia) cell lines. Results are expressed as mean ± S.E.M. of three separate experiments in triplicate.

The AB system resonating at δ 2.22 and 2.30 in the ^1H NMR spectrum, constituting the second fragment, was attributed to the methylene at position 1 on the basis of the following consideration : i) intense dipolar interactions in a ROESY experiment between the AB system protons and H_3-19 signal, ii) correlation peaks in the HMBC experiment of H_2-1 both with C-10 (δ 38.0) and C-19 (δ 17.0). The chemical shift values of H_2-1 and C-1 in the ^1H and ^{13}C NMR spectra, respectively, together with the splitting pattern of H_2-1

suggested the presence of a carboxyl function (implied by the molecular formula) at C-2. This was confirmed by a cross peak in the HMBC spectrum between H_2-1 signals and the C-2 signal. The acidic nature of the C-2 functionality was proved by its reaction with diazometane to afford the corresponding methyl ester **3a** (see experimental).

Regarding the atoms constituting the third spin system of **2a**, comparative analysis of the ^1H and ^{13}C resonance with the corresponding ones in **1a**, permitted us to easily identify the ring F proton system in **2a** (assignments are reported in Table I).

Compounds **1a**, **2a** were tested for their antiproliferative activity on four tumor cell lines *in vitro*. Both compounds **1a** and **2a** inhibited the growth of all cell lines evaluated at 96 h. The effects of these compounds are reported in Figure I as IC_{50} (μg/ml). Results indicate that **2a** exhibits a higher activity compared to that of **1a** on all the cell lines and particularly on the murine leukemia (P388) cell line.

Experimental Section

General methods. HREIMS were obtained by electron impact at 55eV on a VG Prospec Fisons mass spectrometer. Optical rotations were determined on a Perkin Elmer 192 polarimeter. All NMR spectra were recorded on a Bruker AMX-500 spectrometer in $CDCl_3$ solution. ^1H and ^{13}C chemical shifts were referenced to the residual solvent signals. The multiplicities of ^{13}C resonances were determined by DEPT experiments. ^1H connectivities were determined by using COSY and HOHAHA[6] experiments; ^1H-^{13}C connectivities were determined with 2D HMQC experiments[7], interpulse delays were adjusted for an average $^1J_{CH}$ of 135 Hz. Two and three bond heteronuclear ^1H-^{13}C connectivities were determined with 2D HMBC experiments[8], optimized for $^{2-3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (nOe) measurements were performed by 2D ROESY experiments. Medium-pressure liquid chromatography (MPLC) was performed on a Buchi 861 apparatus using a SiO_2 (230-400 mesh) and RP-8 columns. High-performance liquid chromatography (HPLC) separations were performed on a Varian apparatus equipped with an RI-3 refractive index detector using Hibar LiChrospher SiO_2 columns.

*Compound **1b*** : Yield 0.8 mg; $[\alpha]^{25}{}_D$ = - 31° (c 0.003, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 3.66 (1H, m, H-3), 3.87 (1H, bs H-6), 4.34 (1H, bq, J=7.5 Hz, H-16), 1.13 (3H, s, H_3-18), 1.09 (3H, s,

Extraction and isolation. Specimens of *Allium porrum* were collected in May 1995 near Salerno (Campania, Italy); a reference specimen has been deposited at the Dipartimento di Chimica delle Sostanze Naturali, Universita' di Napoli, Italy. The plants were air dried immediately after collection (820g, dry weight) and extracted at room temp. with the following solvents : *n*-hexane, $CHCl_3$, $CHCl_3$/MeOH (9:1), MeOH. The $CHCl_3$/MeOH (9:1) extract (81g) was concentrated *in vacuo* to afford 8.54 g of a crude organic extract which was chromatographed by MPLC on a RP-8 column using a gradient solvent system from H_2O to MeOH. Fraction eluted with MeOH/H_2O 8:2 was rechromatographed by MPLC on a SiO_2 column using sequential mixtures of increasing polarity from $CHCl_3$ 100% to $CHCl_3$/MeOH 8:2. Fractions eluted with $CHCl_3$/MeOH 95:5, (65 mg), were purified by HPLC on a Hibar LiChrospher Si60 column with mobile phase $CHCl_3$/MeOH 9:1, to give a mixture of **1a** and **1b** and a mixture of **2a** and **2b**. Mixture of **1a,b** subjected to HPLC (eluent: *n*-hexane/EtOAc 1:9) afforded compounds **1a** and **1b**. Mixture of **2a,b** subjected to the same treatment gave compounds **2a** and **2b**.

*Compound **1a*** : Yield 4.5 mg; $[\alpha]^{25}{}_D$ = -13° (c 0.03, $CHCl_3$; ^1H and ^{13}C NMR spectra see Table I; HREIMS: obsd. m/z 446.3018, $C_{27}H_{42}O_5$, calcd m/z. 446.3021. H_3-19), 1.06 (3H, d, J=7.1 Hz, H_3-21), 3.35 (1H, t, J=11.0 Hz, Hα -26), 3.48 (1H, ddd, J= 1.5, 4.0, 11.0 Hz, Hβ -26), 0.79 (3H, d, J=6.4 Hz, H_3-27); HREIMS: obsd. m/z 446.3016, $C_{27}H_{42}O_5$, calcd m/z 446.3021.

*Compound **2a*** : Yield 4.2 mg; $[\alpha]^{25}{}_D$ = -16° (c 0.03, $CHCl_3$); ^1H and ^{13}C NMR spectra see Table I; HREIMS: obsd. m/z 460.2810, $C_{27}H_{40}O_6$calcd m/z 446.2814.

Compound ***2b*** : Yield 0.7 mg; $[\alpha]^{25}_{D}$ = - 28° (c 0.003, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 2.22 (1H, d, J=14.5 Hz, Ha-1), 2.30 (1H, d, J=14.5 Hz, Hb-1), 2.61 (dd, J=7.4, 17.3 Hz, Hα-4), 2.49, d, J=17.3 Hz, Hβ-4), 4.57 (1H, bt, J=4.7 Hz, H-6), 4.41 (1H, bq, J=7.5 Hz, H-16), 0.86 (3H, s, H_3-18), 1.24 (3H, s, H_3-19), 1.02 (3H, d, J=7.0 Hz, H_3-21), 3.30 (1H, dd, J=3.0, 11.0 Hz, Hα-26), 3.47 (1H, dd, J= 3.0, 11.0 Hz, Hβ-26), 1.12 (3H, d, J=7.0 Hz, H_3-27); HREIMS: obsd. m/z 460.2807, $C_{27}H_{40}O_6$, calcd m/z 460.2814.

Methylation of ***2a*** : **2a** (1 mg) was treated with CH_2N_2 in diethyl ether and kept overnight at room temp. The reaction mixture, dried under vacuum, afforded **3a** (0.8 mg), analyzed by EIMS and ^1H NMR.

Compound ***3a*** : Yield 0.8 mg; $[\alpha]^{25}_{D}$ = - 32° (c 0.003, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 2.21 (1H, d, J=14.5 Hz, Ha-1), 2.28 (1H, d, J=14.5 Hz, Hb-1), 2.60 (dd, J=7.4, 17.3 Hz, Hα-4), 2.44, d, J=17.3 Hz, Hβ-4), 4.57 (1H, bt, J=4.7 Hz, H-6), 4.41 (1H, bq, J=7.5 Hz, H-16), 0.86 (3H, s, H_3-18), 0.98 (3H, s, H_3-19), 0.96 (3H, d, J=7.0 Hz, H_3-21), 3.30 (1H, dd, J=3.0, 11.0 Hz, Hα-26), 3.47 (1H, dd, J= 3.0, 11.0 Hz, Hβ-26), 1.12 (3H, d, J=7.0 Hz, H_3-27), 3.64 (3H, s, H_3-MeO); HREIMS: obsd. m/z 474.2962, $C_{28}H_{42}O_6$, calcd m/z 474.2970.

Cells. WEHI 164 cells (murine fibrosarcoma cell line) were maintained in adhesion on Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml). J774 cells (murine monocyte/macrophage cell line) were grown in suspension culture, in Techne stirrer bottles, spun at 25 rpm and incubated at 37°C in DMEM medium supplemented with 10% FBS, 25 mM Hepes, glutamine (2mM), penicillin (100U/ml) and streptomycin (100 µg/m). IGR-1 cells (human melanoma cell line) were grown in adhesion on Petri dishes with Minimum Essential Medium Eagle (MEM) supplemented with 10% FBS, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml). P388 cells (murine leukemia cell line) was grown in adhesion on Petri dishes with L-15 (Leibovitz) medium supplemented with 10% FBS, 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml).

Proliferation and cytotoxicity assay. WEHI 164, J774, IGR-1, P 388 ($1x10^4$ cells) were plated on 96-well microliter plates and allowed to adhere at 37 °C in 5% CO_2/95% air for 2 h. Thereafter the medium was replaced with 50 µl of fresh medium and 75 µl aliquot of 1.2 v/v serial dilution of each test compound **1a**, **2a** was added and then the cells incubated for 96 h. In some experiments 6-mercaptopurine (6-MP) was added. The cells viability was assessed through an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] conversion assay[9]. Briefly, 25 µl of MTT (5 mg/ml) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 100 µl of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5[10]. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with compounds **1a**, **2a**, and 6-MP was calculated as: % dead cells= 100 - (OD treated/OD control) x 100. Figure I shows the results expressed as IC_{50} (concentration that inhibited cell growth by 50%).

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