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Pyrroloacridine Alkaloids from *Plakortis quasiamphiaster*: Structures and Bioactivity

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Abstract

A re-collection of *Plakortis quasiamphiaster* from Vanuatu in 2003 resulted in the isolation of three known compounds, plakinidine A (**1**) and amphiasterins B1 (**6**) and B2 (**7**). Also isolated was a new bis-oxygenated pyrroloacridine alkaloid, plakinidine E (**8**), with a unique *O*-substitution versus *N*-substitution at position C-12 in **1**. The biological evaluation of the active compounds in two assays provided complementary data. Plakinidine A (**1**) exhibited cytotoxicity against human colon H-116 cells with an IC_{50} of 0.23 μg/mL, but there were no effects against the yeast *Saccharomyces cerevisiae* diploid homozygous deletion strain of topoisomerase I (*top1*Δ). By contrast, **8** was inactive against H-116 cells but was potent in the yeast halo screen.

Marine invertebrate-derived polycyclic heteroaromatic alkaloids with 16–18 π-electron containing cores are relevant for therapeutic development, as they interfere with many biological disease targets.[1] At the forefront of much continuing research are these marine-derived aromatic classes shown below as general structures, **I** for pyridoacridines and **II** for pyrroloacridines. Both structural types are derived from sponges and tunicates, and there are 10 times as many examples known from the former versus the latter.[2] The molecular structures of these tetra- and pentacyclic alkaloids appear to incorporate tryptophan and dopamine building blocks and derive further diversity via functionalization by OH and NH ring substituents.[3] We have engaged in prior discussion about how the pyridoacridines with these substituents can exist in tautomeric forms and recently used the results of *ab initio* calculations to comment on the possibility of their further biosynthetic transformations, especially by the action of methyltransferases.[4] Of even greater significance are the impressive biological effects of the pyridoacridines especially regarding DNA intercalation[5] and topoisomerase I and II inhibition.[6] Such properties have heightened interest to develop the therapeutic potential of this class. Thus neoamphimidine, a selective topoisomerase II inhibitor, can be considered as a current lead compound, as it is under preclinical investigation.[7]

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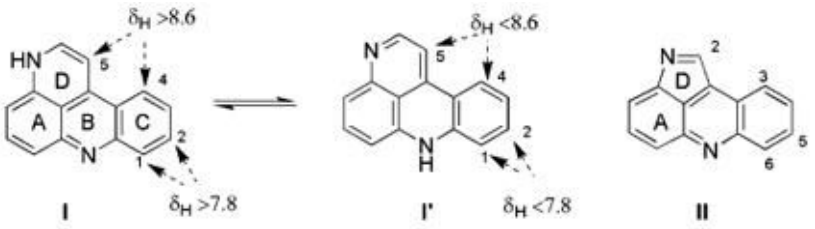
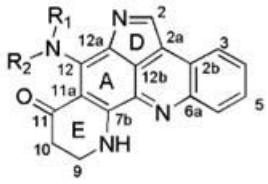
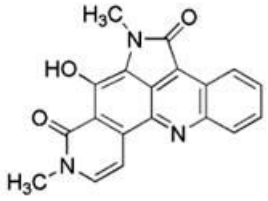
Supporting Information Available: General experimental procedures, underwater and above water photographs, isolation scheme, ^{1}H, ^{13}C NMR, gHMQC, and gHMBC data for **8**, and gCOSY for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.



Perhaps surprising at this point in time is that there are only five known pyrroloacridines. This implies that the organism sources of this class seem to be uncommon. Our investigation of a *Plakortis* species collected during a 1987 expedition to Vanuatu resulted in the first report of pyrroloacridines that we called plakinidines A (**1**) and B (**2**).[8] A new and known analogues were quickly added to the record by the report of plakinidine C (**3**) obtained from a Fijian *Plakortis*.[9] Seven years later this list was further expanded to include plakinidine D (**4**) isolated from two ascidians of the genus *Didemnum*.[10] Discovery of the latter compound came in 2002 from our work on an Indo-Pacific *Xestospongia* cf. *carbonaria*, which resulted in the isolation of alpkinidine (**5**).[11]



plakinidine A, R_1 = H, R_2 = CH_3 (**1**)
B, R_1 = CH_3, R_2 = CH_3 (**2**)
C, R_1 = H, R_2 = CH_3 (9, 10-didehydro) (**3**)
D, R_1 = R_2 = H (**4**)



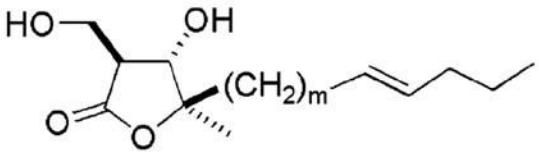
alpkinidine (**5**)

The project described herein was begun with the goal of reisolating plakinidine A (**1**) accompanied by the search for additional congeners. The goal was to probe ring system **II** for its potential to exhibit selective cytotoxicity and to gain some insights about its possible action on topoisomerase targets. For years this proved to be problematic, as it appeared that the only sponge source of the plakinidines was an organism we eventually identified as *Plakortis quasiamphiaster* (order Homosclerophorida, family Plakinidae),[12] but this species did not appear to be cosmopolitan in the Indo-Pacific. Moreover, a survey of the literature showed that *P. quasiamphiaster* was the least studied of the seven different *Plakortis* genera (family Plakinidae) and its 14 species.[12] Our efforts to re-collect this elusive sponge succeeded during a 2003 expedition to Vanuatu. Reported below are the reisolation of plakinidine A (**1**), the characterization of a new plakinidine analogue, plakinidine E (**8**), the reisolation of amphiasterins B1 (**6**) and B2 (**7**), and the results of a broad-based bioactivity assessment of these compounds.

Results and Discussion

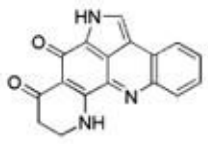
The sponge *Plakortis quasiamphiaster* (coll. no. 03404) was processed by accelerated solvent extraction (ASE), which afforded three fractions. The CH_2Cl_2-soluble fraction coded as XFD (3.8 g) was chosen for further purification due to selective cytotoxicity against human colon

H-116 cells. This extract was subjected to silica gel column chromatography to yield 24 fractions, labeled as F1–F24 (Figure S2, Supporting Information). Fraction F8 was interesting by MS analysis, and a subsequent HPLC run yielded 12 new fractions (labeled H1–H12). Fractions H5 and H7 from this HPLC purification were pure and contained, by NMR analysis, the known compounds amphiasterins B1 (**6**) and B2 (**7**),[13] respectively. In addition, fractions F17 and F19 were also targeted for further purification because diagnostic resonances of framework **II** could be observed by ^{1}H NMR. Fraction F17 was potent and exhibited selective cytotoxicity against H-116 cells, and it was shown by LC-MS to contain impure plakinidine A (**1**), *m*/*z* 303.2 $[M + H]^+$. This fraction was further purified by preparative HPLC (Figure S2, Supporting Information) and yielded **1** (29 mg), which was subjected, as described below, to further biological testing in the disk diffusion assay.[14] Next, LC-MS showed that F19 contained a mixture of compound **1** and an unknown metabolite having *m*/z 290.1 $[M + H]^+$ and 312.1 $[M + Na]^+$. Further purification by reversed-phase Biotage gave 13 fractions (labeled B1–B13). By ^{1}H NMR it could be seen that fractions B5 and B8 were pure and contained the new compound plakinidine E (**8**) and more of **1**, respectively. Unfortunately, compound **8** completely degraded after standing for a few days at room temperature, but this happened after acquisition of 1D and 2D NMR data.

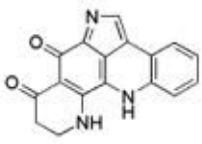


amphiasterin B1(**6**) m = 10
B2 (**7**) m = 12

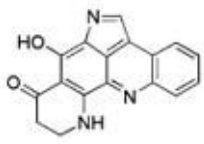
The structural elucidation of plakinidine E (**8**) began with the formula established by HRMS of $C_{17}H_{11}N_3O_2$ (Δ 0.9 mmu vs calcd) requiring 14 degrees of unsaturation. Comparing this formula to that of plakinidine D (**4**, $C_{17}H_{12}N_4O$) showed that the overall unsaturation numbers were the same but that there was a difference of +O and −HN. In addition, as shown in Table 1, the ^{13}C and ^{1}H NMR data of **8** were similar to those of **4**, exhibiting signals characteristic of the C and E rings of plakinidine D (**4**). We immediately envisioned structure **8c** as the simplest way to explain these differences on the basis of the following logic. First, the gradient HMBC data were consistent with substructures **A** and **B** shown in Figure 1. Second, a side-by-side comparison of the ^{13}C NMR data of **4** and **8** in Figure 2 further highlighted the minimal differences for carbons of the C and E rings between these two compounds. Third, the differences evident in Figure 2 were observed in three regions (corresponding to sites C-2–2b, C-7a–7b, and C-11–12a) belonging to the A, B, and D ring chromophore, with the largest change being at C-12 (Δδ 13.9). Finally, on the basis of our previous experience with the tautomeric forms of pyridoacridines (**I** vs **I′**), the consideration of two additional isomeric possibilities, **8a** and **8b**, was required.



8a

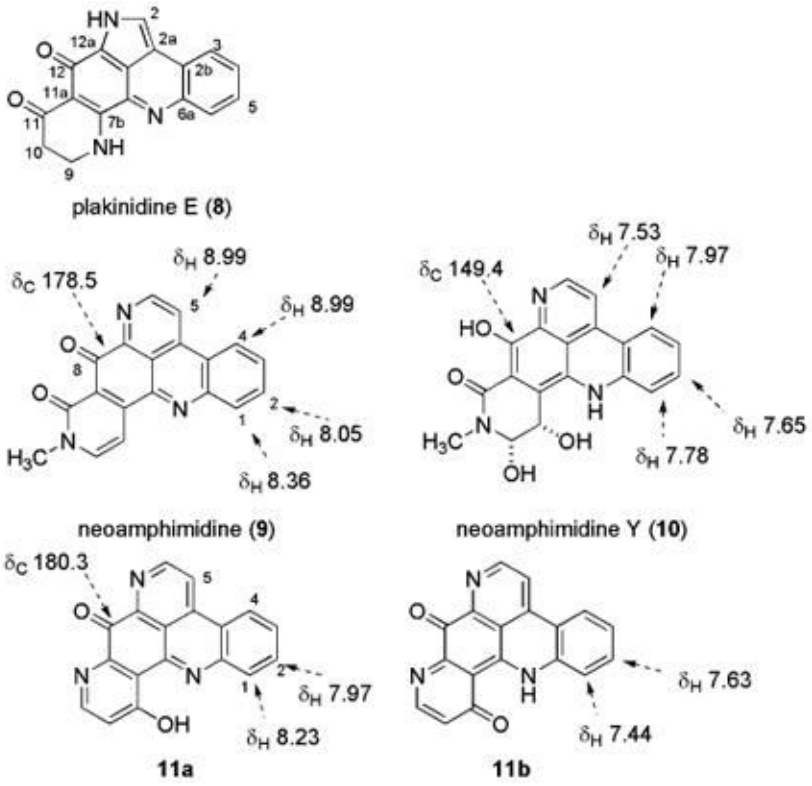


8b



8c

The choice in favor of tautomer **8a** over **8b** and **8c** for plakinidine E began by making NMR comparisons to model systems, then progressed to employing subtle yet diagnostic ^{1}H NMR shift trends we previously published,[11] and concluded with an analysis of molecular mechanics calculation predictions. The NMR data indicated that only one tautomer was present for plakinidine E (**8**), and this was also the case for plakinidine A (**1**). The HMBC correlations shown in Figure 1 justified the assignments of the eight quaternary carbons of the A/B/D rings of **8**, making it possible to unambiguously designate C-12 at δ 171.5. The C-8 shift of δ 149.4 of neoamphimidine Y (**10**)[11] provided the chemical shift expected for this site in tautomer **8c**, which allowed for its elimination. By contrast, the δ_C 178.5 for C-8 of neoamphimidine (**9**)[11] provided a good match to the value expected at C-12 for both **8a** and **8b**. Eventually, we recognized that it might be possible to distinguish between these two tautomers by using ^{1}H NMR shift trends characteristic of **I** (shifts > δ 7.8 for H1/H2 and > δ 8.6 for H4/H2) versus **I** ′ (shifts < δ 7.8 for H1/H2 and < δ 8.6 for H4/H2) shown next to their structures.[11] These data trends also tracked the structural changes in neoamphimidine (**9**), neoamphimidine Y (**10**), meridine (**11a**), and meridin-12(13H)-one (**11b**), as reflected in ^{1}H NMR shifts accompanying each structure. Accordingly, the D ring proton shifts of **8** of δ 8.19 (H-6) and δ 7.72 (H-5) were in agreement with the B/C ring constitution of **8a** and not **8b**, concluding the final structure of plakinidine E as shown in **8**.



An attempt to use gas-phase theoretical energy estimates to assess the most stable of these tautomers was also considered. The results of semiempirical molecular orbital calculations carried out employing the AM-1 force field are shown in Figure 3. The goal of the first entry was to compare these data to those we had previously obtained from the semiempirical approach for pyridoacridine structures **I** and **I′**. The second entry shows that the data for meridines **11a** and **11b** match the trends obtained from higher-level calculations we published (but not shown here) in a different study of oxygenated pyridoacridines.[11] Both calculations predict that the lowest energy tautomer has the B ring *N*H as observed by NMR for

neoamphidine Y (**10**). The tautomers of **11** have been isolated, and in agreement with the experimental observation, **11b** is calculated to be the more stable species.[11] The consistency in such data for the pyrroloacridines is less straightforward. In the case of alpkinidine, form **5a**, predicted to be favored energetically, was observed by X-ray crystallography. Unfortunately, the ^{1}H NMR data of **5** cannot be used to distinguish between the two possible tautomers (**8a** and **8b**), and the ^{13}C NMR data essential for verifying this point could not be obtained.

Further, the experimental data, X-ray data,[9] and ^{1}H-^{1}H COSY correlation between the geminal *N*H/CH_3 show that plakinidine A (**1**) exists as the energetically less favored **1c** (Figure 3). To date, we have been unable to find conditions to transform **1c** into the other tautomers. In addition, the dimethylated isomer, plakinidine B (**2**), exists as tautomer **2c** (not shown), and on the basis of NMR data comparisons to **1** and **2**, plakinidines C (**3**) and D (**4**) also exist as the calculated higher energy tautomers **3c** (not shown) and **4c**, respectively. Finally, form **8a** observed in solution for plakini-dine E is calculated to be energetically favored.

A multifaceted approach was used to further explore the biological properties of plakinidines A (**1**) and E (**8**) and amphiasterins B1 (**6**) and B2 (**7**). The first undertaking involved using a disk diffusion and a growth inhibition (clonogenic) assay to explore the cytotoxicity of these compounds against human and murine cancer cell lines.[14] In the second approach, the compounds were profiled against the *Saccharomyces cere*V*isiae* diploid homozygous deletion strain of topoisomerase I (*top1*Δ) in an agar halo assay to identify novel DNA damaging agents and potential inhibitors of DNA damage checkpoint processes and DNA repair enzymes.[15] The cytotoxicity screen showed that **1** was potent and selective against H-116 cells, while no activity was observed for **6**, **7**, or **8**. In contrast, the yeast halo assay revealed that **8** was active against the *top1*Δ strain, while **1**, **6**, and **7** were inactive.

The disk diffusion assay showed that **1** was potent and selective against human colon H-116 cells. Consequently, the growth inhibition assay in liquid culture gave an IC_{50} of 0.23 μg/mL.[14] In addition, a concentration-clonogenic cell survival experiment was carried out for exposure of **1** at durations of 2, 24, and 168 h (continuous over the 7-day incubation). The surviving fraction at 10% (S_{10}) is used as a parameter of cytotoxicity. For the 2, 24, and 168 h exposures, these values were 20 μg/mL, 1.5 μg/mL, and 150 ng/mL, respectively as shown in Figure 4. The therapeutic implication of these results is that either a single bolus or a daily in vivo dosing schedule for **1** could produce an effective therapeutic outcome in the tumor-bearing animal if plasma levels were at or above these respective S_{10} levels for the specified time periods.

In the yeast halo assay, compounds **1**, **6**, and **7** had no effect on the growth of the *top1*Δ yeast, nor the wild-type yeast. However, **8** was toxic against the *top1*Δ yeast, resulting in a halo 7 mm in diameter, yet not toxic to wild-type yeast. In liquid culture, plakinidine E (**8**) exhibited an LC_{50} of 33 μM. Topoisomerase I (Top1) is a type IB topoisomerase in yeast, which forms a transient DNA-enzyme complex that relaxes both positively and negatively supercoiled DNA by breaking and rejoining single strands of DNA. Top1 exists in a synthetic lethal relationship with a number of DNA damage checkpoint and DNA repair genes, including Mre11, Rad50, and Rad52, in addition to the other yeast topoisomerases, Top2 and Top3. Compounds that exhibit selective toxicity toward the *top1*Δ deletion strain are thus expected to target one of these gene products or induce DNA damage by a mechanism that depends on the activity of Top1 for its repair. The selective toxicity of plakinidine E (**8**) toward *top1*Δ yeast is consistent with the literature[5,6] since compounds similar to the plakinidines have been shown to cause DNA damage or target topoisomerase II-mediated DNA activity. In particular, ascididemin causes thiol-dependent reductive DNA cleavage and deoxyamphimidine damages DNA

through generation of reactive oxygen species.[16,17] Neoamphimedine (**9**), in contrast, has been shown to induce topoisomerase II-dependent DNA catenation.[18]

The discovery of **8** from *P. quasiamphiaster* presents a new member to the plakinidine family with its uniquely positioned oxygen functionality on C-12. The iminoquinone moiety of **8** is distantly related to the B ring of ascididemin[19] and the C rings of neoamphimidine (**9**) and 5-methoxyneoamphimidine.[8] These marine acridine alkaloids are currently in advanced preclinical evaluations as topoisomerase II inhibitors, and further work has led to semisynthetic derivatives that exhibit submicromolar activities against human cancer cell lines.[2,4] In addition, their potent cytotoxicities are the result of their unique pharmacophoric domains, which are as follows: (1) planar chromophores that have been implicated in DNA intercalation and topoisomerase II inhibition; (2) phenanthroline bay nitrogens that are important for metal complexation; and (3) iminoquinone moieties that produce reactive oxygen species that may interfere with certain metabolic pathways.[4] Plakinidine E (**8**) possesses each of these pharmacophores in that it has a planar structure, bay nitrogens, and an iminoquinone chromophore.

It was also interesting to note that **1** has activity opposite that of **8**; it does not demonstrate *top1*Δ yeast toxicity but is toxic against H-116 cells. This result may indicate that plakinidine A (**1**) targets a unique pathway in H-116 cells and does not target one of the Top 1 synthetic lethal gene products or induce DNA damage in a manner dependent upon Top 1 activity. This selectivity could be ascribed to their structural differences. Both **1** and **8** have similar polycyclic, aromatic scaffolds, but plakinidine A (**1**) has a C-12 nitrogen, while plakinidine E (**8**) has a C-12 oxygen. This difference does not affect the overall planarity of the polycyclic scaffold; however, it does change the A, B, D ring chromophore, which could affect its ability to bind to a molecular target. Currently, we are comparing the activity of a variety of pyrroloacridines and pyridoacridines against both yeast deletion strains and H-116 cells with the goal of determining the specific requirements for this difference in activity.

Experimental Section

General Experimental Procedures

NMR spectra were collected using a Varian Inova NMR spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Multiplicities of ^{13}C NMR peaks were determined using DEPT and gHMQC data. Low- and high-resolution mass spectrometry was performed on a benchtop Mariner electrospray ionization time-of-flight instrument (ESTOF). Preparative HPLC was carried out using a single-wavelength (γ = 254 nm) UV detector and evaporative light scattering detector (ELSD) in series with a Waters reversed-phase 5 μm particle column. Computer modeling and semiempirical calculations were done using PC Spartan Pro1.0 with AM1 force field.

Animal Material

Sponge specimens (UCSC sample code 03404) were collected in waters near Hideaway Island in the Mele Bay off the coast of Efate in Vanuatu at depths of 30–60 ft. The specimens were found hanging under rocks, caves, or coral. The sponge was identified by Dr. R. W. M. van Soest as *Plakortis quasiamphiaster*, and a voucher sample has been deposited at the Zoological Museum of Amsterdam (ZMAPOR 17710). Voucher specimens and photographs are available from the Crews Laboratory.

Extraction and Isolation

The sponge *P. quasiamphiaster* (0.85 kg wet weight) was extracted using an accelerated solvent extraction (ASE) process to afford three fractions. The CH_2Cl_2-soluble fraction was subjected to passage over a silica gel column using a step gradient of 10% EtOAc/hexanes to 100%

EtOAc and finally to 100% MeOH to afford 24 fractions, coded F1–F24 (Figure S2, Supporting Information). Fraction F8 (66.7 mg) was further purified by HPLC (10%–100% CH_3-CN/H_2O) to afford 12 fractions, labeled H1–H12. Fractions H5 and H7 contained the known compounds amphiasterins B1 (**6**, 2.7 mg) and B2 (**7**, 2.7 mg). Fraction F17 was further purified by HPLC (10%–100% CH_3CN/H_2O) to afford plakinidine A (**1**). Fraction F19 was subjected to a reversed-phase biotage column with a stepwise gradient solvent system of 30%–60% MeOH/H_2O. This afforded 13 fractions, of which fractions B5 and B8 were pure and contained the new plakinidine E (**8**, 5.7 mg) and more of **1** (44 mg), respectively.

Disk Diffusion and Clonogenic Assay

This assay was carried out as previously described.[14]

Growth Inhibition Measurement from Yeast Halo Assay

A solution of 2× YPD-H (YPD medium buffered with HEPES) was prepared by dissolving 10 g of yeast extract, 20 g of peptone, and 20 g of dextrose in 500 mL of 25 mM HEPES at pH 7.5. The medium was autoclaved, the pH was readjusted to pH 7 with NaOH, and the medium was filter-sterilized. A solution of 2× agar was prepared by autoclaving 1.5 g of granulated agar (BP1423-500) in 100 mL of H_2O. YPD-H-agar was prepared by mixing 10 mL of 2× YPD-H at 50–55 °C with 10 mL of 2× agar at 50–55 °C. The warm medium was inoculated with 400 μL of an overnight yeast culture (diploid strain BY4743, *top1*Δ, and wild type), poured into an OmniTray, and cooled for 30 min.

Compound DMSO stocks plated in 384-well polypropylene trays were transferred into the solidified agar using notched pins that deliver 200 nL (±8%) each with a pin-tool robot (VP903B, V&P Scientific). Absorbance readings were taken of the agar plates using a plate reader at 544 nm. Growth of the *top1*Δ versus the wild-type yeast was compared to identify potent agents.

Growth Inhibition Measurement in Liquid Culture

Yeast (diploid strain BY4743, *top1*Δ, and wild type) was incubated with six 2-fold dilutions of **8** and DMSO controls in 100 μL cultures in 96-well plates. Absorbance readings at 544 nm were collected every 30 min using a plate reader. Yeast doubling times at each compound concentration were calculated and compared to the doubling time with DMSO as a control. The LC_{50} was defined as the concentration in which the doubling time of the DMSO control divided by the doubling time with compound was equal to 0.5.

Plakinidine E (8)

5.7 mg; dark red-purple solid; ^1H and ^{13}C NMR data, see Table 1 and Figures S2 and S3 (Supporting Information); HRMS *m*/*z* 290.0933 (calcd for $C_{17}H_{12}N_3O_2$ $[M + H]^+$ 290.0924).

Plakinidine A (1)

73.7 mg; dark red-purple solid; ^1H and ^{13}C NMR data as previously reported.[8,9]

Amphiasterin B1 (6)

2.7 mg; whitish powder; ^1H and ^{13}C NMR data as previously reported.[13]

Amphiasterin B2 (7)

2.7 mg; whitish powder; ^1H and ^{13}C NMR data as previously reported.[13]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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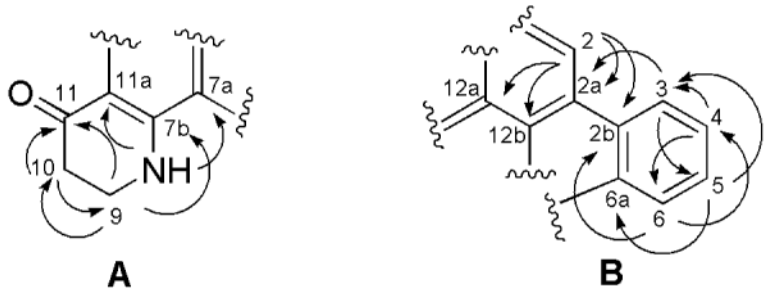

Figure 1.
gHMBC correlations for plakinidine E (**8**) substructures.

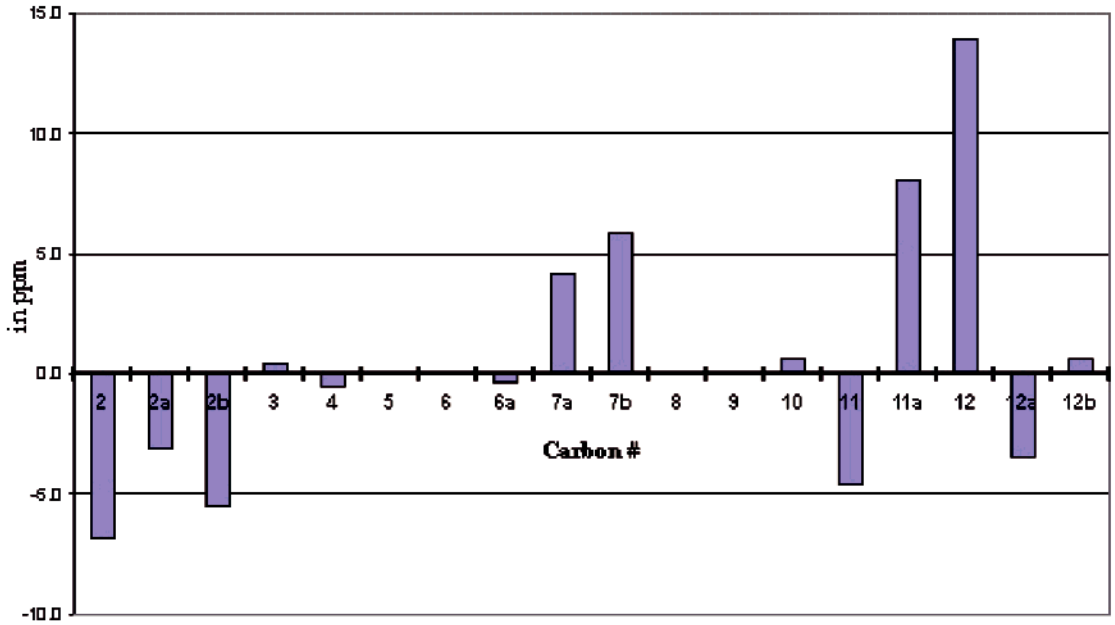

Figure 2.
^{13}C NMR chemical shift differences of plakinidine E (**8**) vs plakinidine D (**4**).[10]

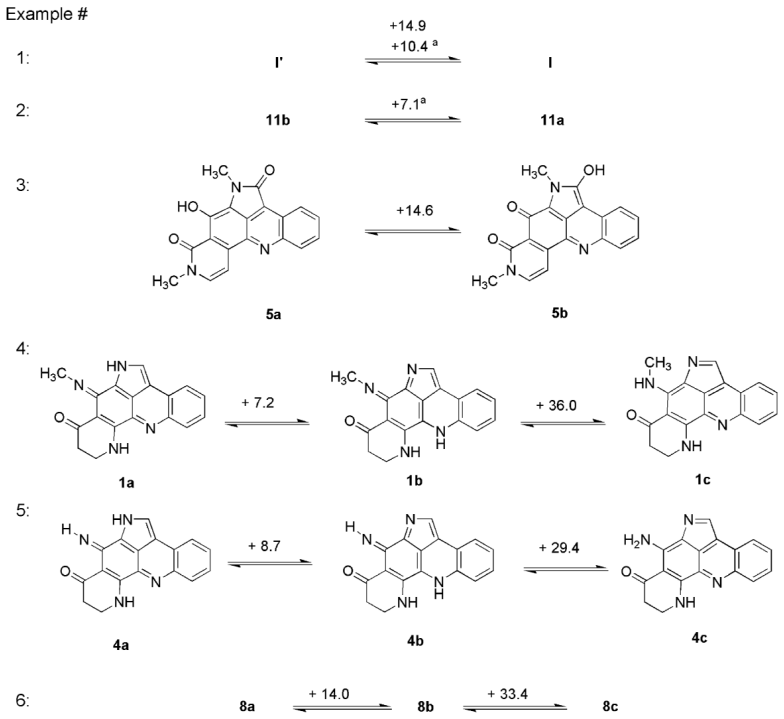

Figure 3.
Semiempirical AM-1 molecular orbital calculated tautomer equilibrium values (Δ*H*) for annulated acridines. [a]Calculations from ref [11].

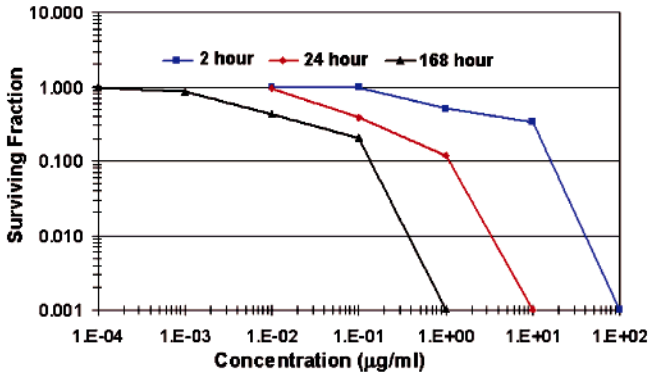

Figure 4.
Concentration–response curve for **1** against H-116 cells exposed for 2 h (blue), 24 h (red), and 168 h (black) in vitro.

Table 1

NMR Data of Plakinidines E (**8**)[a] and D (**4**)[b] in DMSO-d_6

	8				**4**
carbon	**δ_C**	**C mult.**	**δ_H mult. *J* (Hz), int.**	**gHMBC**	**δ_C**
2	124.2	C	8.17s, 1H	2a, 2b, 3, 12b	131.0
2a	118.6[c]	C			121.7
2b	119.3[c]	C			124.8
3	124.4	CH	8.36dd, 2.0, 8.0, 1H	2a, 5, 6a	124.0
4	128.8	CH	7.68dt, 2.0, 8.0, 1H	3, 6	129.3
5	127.1	CH	7.72dt, 1.5, 8.0, 1H	3, 6a	127.0
6	130.9	CH	8.19dd, 1.5, 8.0, 1H	2b, 4, 12b	130.8
6a	144.2	C			144.3
7a	143.9	C			140.0
7b	157.9	C			152.0
8			9.29s	7a, 11a	
9	40.1	CH_2	3.73t, 7.0, 2H	7b, 11, 10	40.0
10	36.5	CH_2	2.48t, 7.0, 2H	9, 11	35.9
11	189.2	C			193.8
11a	107.7	C			99.6
12	171.5	C			157.6
12a	121.6	C			125.1
12b	117.7	C			117.1

[a] Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C).

[b] Ref 10.

[c] Interchangeable.