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Synthesis and Cytotoxic Activity of *N*,*N*-bis-{3-[*N*-(4-Chlorobenzo[*g*]-phthalazin-1-yl)]aminopropyl}-*N*-methylamine: A New Potential DNA Bisintercalator

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Abstract—The synthesis of a new series of mono- and dinuclear 1-alkylamino-4-chlorobenzo[*g*]phthalazine derivatives **7**–**10** containing flexible polyaminic chains is reported. It has been achieved by the reaction of 1,4-dichlorobenzo[*g*]phthalazine with the corresponding polyamines. In vitro antitumoral activity against HT-29 human colon carcinoma cells was evaluated and showed best results for compound **10**, in which two heteroaromatic units are linked by a *N*-methylsubstituted polyaminic chain. Molecular modelling of the complexes of **9** and **10** with DNA strongly suggests the possibility of bisintercalation, and also that the *N*-methyl group of **10** plays an important role in the formation of a specially stable DNA complex.

Introduction

The intercalation of planar aromatic molecules with the DNA double helix is considered to be important in the medicinal action of antineoplastic drugs.[1,2] Intercalators contain a planar chromophore with two to four fused aromatic rings, with an optimum of three, but they also ought to posses groups available for hydrogen bonding,[3] and flexible cationic side chains are usually needed for activity, as happens with the polyaminic moieties of the classical mitoxantrone derivatives.[4]

Furthermore, a rational approach in the search of antitumor agents is based on the design of bisintercalating compounds, which present higher DNA binding affinities and slower dissociation rates than the corresponding monomers.[5,6] On the other hand, increasing the global size occupied by the ligand could afford greater opportunities for sequence selectivity.

The dimerization of well-known monointercalators through polyaminic linking chains has been shown to be a successful strategy for the preparation of antineoplastic compounds. That is the case of the ditercalinium analogue **1**, (Fig. 1) which exhibits pharmacological activity in eukaryotic cells and behaves as a true bisintercalator.[7] Further studies established that the role of symmetry in the design of bisintercalators was important,[8] because drastic reductions in antitumor potency were observed when asymmetrical parameters were introduced.

Studies performed in the field of bisacridines have shown that the chromophore shape and size are determinant of bifunctional binding, and that the capacity of forming complexes depends sensitively on the linker chain conformation.[9–11] Functionalization in the chromophore is also a critical factor: the introduction of chlorine atoms in the heteroaromatic moieties of **2** significantly improves activity against Lewis lung carcinoma cell lines.[12] Spacing between the electronegative sites in the flexible chain should also be taken into account, and the indenoquinoline dimers **3** and **4**

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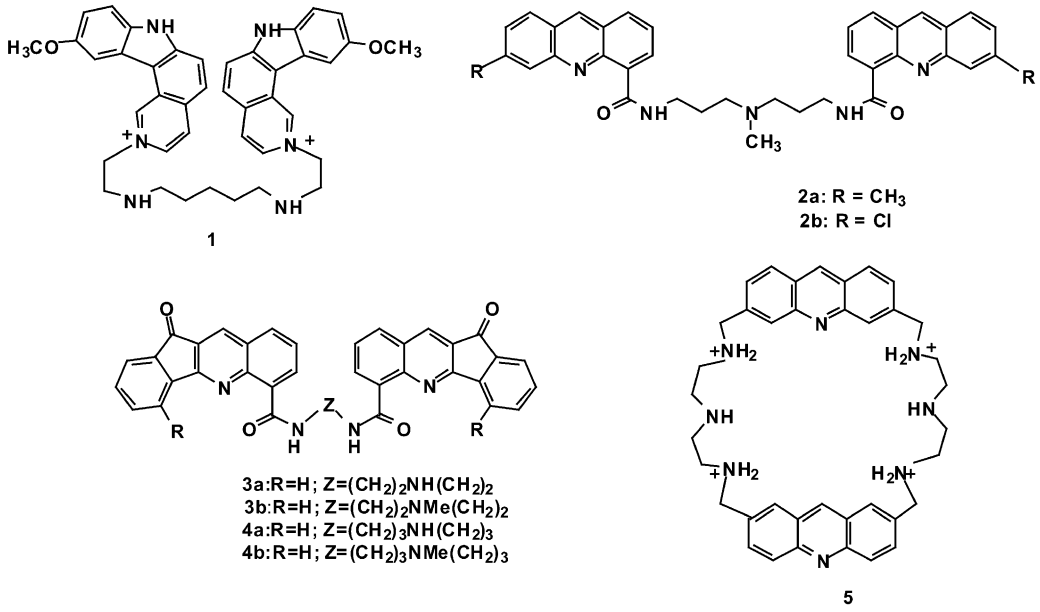


Figure 1.

containing ethylenic and propylenic units linked to the central NH and NMe have been reported as a new class of topoisomerase inhibitors.[13] Molecular modeling techniques have shown to be very useful in studying the selectivity of binding, as demonstrated for the macrocyclic bisacridine **5**[14] or aminoacridine carboxamide dimers related to **2**.[15]

We have described in previous work[16] the synthesis of the 1,4-bis(alkylamino)benzo[*g*]phthalazines **6a–c** (Fig. 2) in order to investigate their DNA intercalating properties. These compounds contain a tricyclic heteroaromatic system with two of the four conjugated nitrogen atoms located in the aromatic moiety and the other two in an exocyclic disposition. It was shown that these compounds behave as proton-ionizable chromophores[17] that bind to DNA by intercalation, causing a positive superhelicoidal twist in closed, circular DNA, with unwinding angles comparable to that one originated by the intercalation of ethidium bromide.[18] On the other hand, the 1,4-bis(butylamino) substituted compound **6c** was able to inhibit protein biosynthesis,[19] and showed in vivo and in vitro antitumor activity on the growth and differentiation of U-937 human promocytic leukemia cells. In fact, when compared with the well-known DNA intercalator amsacrine,[20] **6c** showed a more prominent inhibition of RNA and protein synthesis and a similar grade of activity against cell proliferation.

With these results in mind, we have planned the synthesis of the mono and dinuclear alkylamino derivatives **7–10**, in order to evaluate their respective abilities as DNA mono- and bisintercalators. When designing the flexible aliphatic linker, a polyaminic chain with propylenic sequences bound to a basic nitrogen was selected, in order to afford a nine-atoms linker because it corresponds to an interchromophore distance between 9 and 10 Å, which has been reported as adequate for

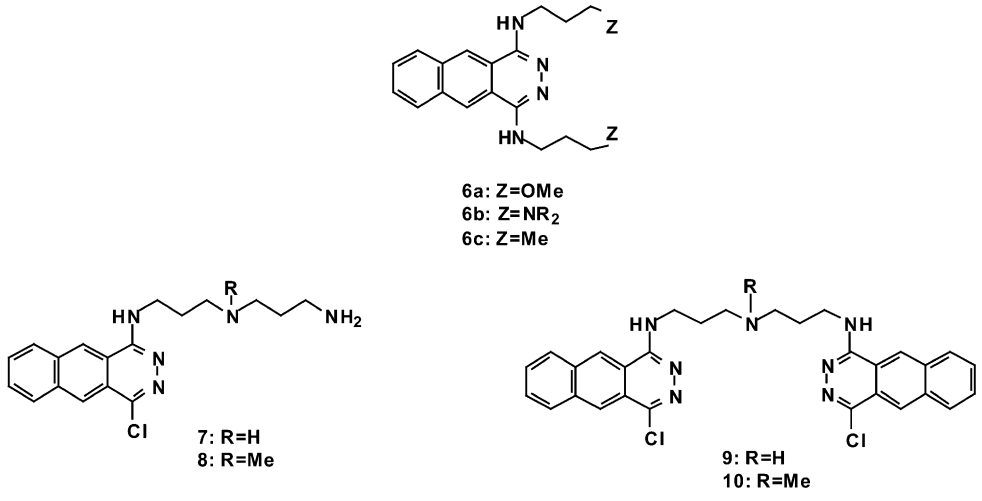


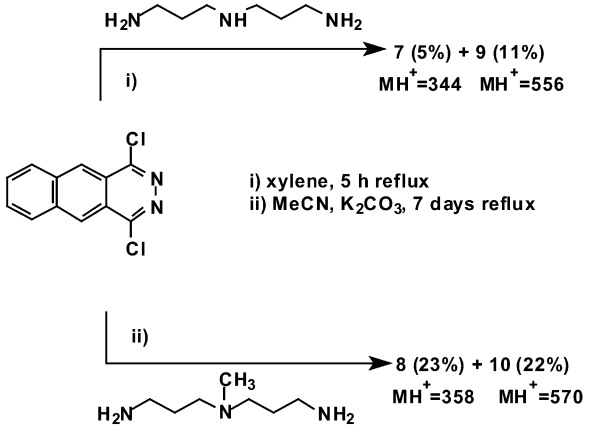
Figure 2.

allowing bifunctional intercalation according to an one base-pair sandwich model.[21] Furthermore, related linking chains were used in bisacridine derivatives like **2**, that exhibit a DNA bisintercalating behavior.[22] We chose to prepare both the N–H (**7**, **9**) and N–Me (**8**, **10**) compounds because that kind of variation in the substitution at the central electronegative chain site could modify the conformational features and consequently the DNA complexation ability. The in vitro activity of these compounds against HT-29 human colon carcinoma cells was evaluated, and results obtained were interpreted with the help of molecular modelling of the DNA complexes performed by using the AMBER method.[23]

Results and Discussion

The synthesis of compounds **7**–**10** was performed by the nucleophilic substitution of 1,4-dichlorobenzo[*g*]-phthalazine with bis(3-amino-propyl)amine or 3,3′-diamino-*N*-methyldipropyl-amine according to the reaction conditions shown in Scheme 1. Ligands **8** and **10** were prepared by following the classical procedure previously described by Körmendy[24] under relatively mild conditions. However, the synthesis of **7** and **9** required stronger conditions, and lower yields (5 and 11% as opposed to 23 and 22%) were consequently obtained due to the increase of polymerization products.

Molecular ions obtained from mass spectra agree with the proposed structures and are displayed in Scheme 1. ^{1}H and ^{13}C NMR data are also shown in the Experimental. Asymmetry in the substitution at the ring A of the heteroaromatic moiety can be easily deduced from the different chemical shifts obtained for both the H_5/H_{10} and the C_1/C_4 signals in, respectively, the ^{1}H and ^{13}C spectra. It is also shown that the alkylamino chains substantially unshields the H_5 and C_1 signals with respect to those neighboring to the chlorine atoms. On the other hand, dinuclear compounds **9** and **10** exhibit only three different methylenes, every signal integrating for four protons. Methylene groups nearest to the pyridazine rings are more deshielded in the ^{1}H spectra than those nearest to the NH and NMe central groups, whereas the opposite occurs in the ^{13}C spectra, being the $C_{1'}$ carbons the most deshielded.



Scheme 1.

The effect of compounds **7**–**10** on cell proliferation was determined with HT-29 Human Colon Carcinoma Cell Lines by using a standard MTT-based colorimetric assay, according to a method previously described.[25] Results are summarized in Table 1. The four polyaminic systems were active in the described assays, although activity was in every case less than that found for doxorubicine. The dinuclear NH substituted compound **9** exhibits a similar activity to that found for its mononuclear analogue **7**. However, between the N–Me substituted compounds, dinuclear **10** is one order of magnitude more active than mononuclear **8**. It looks surprising that the simple substitution of a hydrogen atom by a methyl group in the middle of the aliphatic chain linking the two heteroaromatic units increase so significatively the cytotoxic activity. On the basis of previous work concerning the activity of DNA intercalating compounds,[21,22] these differences agree with the hypothesis that dimerization of potential monointercalants is an useful strategy in the design of antineoplastic drugs, and also that the conformational features of the polyaminic chains can have a noteworthy influence on activity patterns. According to that, the *N*-methyl group of **10** could be efficiently favouring the bisintercalation geometry.

In an effort to throw some light over this matter, we have performed the molecular modelling of the bisintercalation complexes of **9** and **10** with DNA.

Models were built by using the Hyperchem 5.0 (Hypercube Inc.) package capabilities. Monointercalation was studied on tetramer duplexes following the Newlin et al. methodology[26] and looking for specificity among the different base pair sequences. The treatment of bisintercalation was done in a similar way on duplexes of five base pairs, as the chain length of **9** and **10** prevents intercalation with two base pairs in between. We selected the AMBER method[23] modified by the inclusion of the appropriate parameters. When available, the parameters came from analogous parameters used in the literature.[27] All others were developed following Kölman[28] and Hopfinger[29] procedures. The equilibrium bond length and angle values came from experimental ones on reasonable reference compounds. Solvent effects were simulated by the use of a distance-dependent dielectric function of $\varepsilon = 4r$ in all calculations. In order to achieve electrical neutrality an appropriate number of Na^+ counterions were included, placed at 6 Å distance from each phosphate-oxygen bisector. The

Table 1. Cytostatic activity against HT-29 human colon carcinoma cell line (EC_{50}, molar)

Compd	Assay 1	Assay 2	Mean
7	1.30×10^{-5}	4.70×10^{-5}	3.00×10^{-5}
8	1.60×10^{-5}	1.80×10^{-5}	1.70×10^{-5}
9	1.30×10^{-5}	1.40×10^{-5}	1.35×10^{-5}
10	2.40×10^{-6}	2.60×10^{-6}	2.50×10^{-6}
Doxorubicine	5.20×10^{-7}	4.20×10^{-7}	4.70×10^{-7}

intercalation energies were calculated as the sum of the energy required for opening of the DNA sequence (ΔE_i) and the intermolecular interaction energy between the intercalator and DNA (ΔE_c).[30]

Figures 3 and 4 show the most stable geometries obtained for the bisintercalation complexes of, respectively, ligands **9** and **10**. In the NH dinuclear compound **9** one of the two planar chromophores is inserted in a 'head on' fashion with its long axis oriented nearly at right angle to the long axis of the adjacent base pairs, but the second one is laterally oriented and parallel to the long axis of the base pairs. The last one is an usually unfavored orientation in intercalator chromophores (i.e., anthracyclines).[31] The intercalation site sequence is the favoured d(GCGG). The chromophore is situated at an average distance of about 3.5 Å with respect to the base pairs, so that effective π–π 'face to face' stacking may occur. However, no effective hydrogen bonding was found between the ligand and DNA constituents. Intercalation energy was calculated as $\Delta H = -17.59$ kcal/mol.

The preferred geometry found for the **10**-DNA complex, for which a ΔH value of −63.01 kcal/mol was obtained, corresponds to an arrangement of the two heterocyclic units in a 'head on' orientation that is in contrast with that one found for **9**-DNA and could account for a greater stability. On the other hand, the intercalation site sequence obtained is now d(CGCGC), a preferential feature for cytostatic active principles.[32] Stabilizing hydrogen bondings between the exocyclic NH group close to the upper heterocyclic system and two oxygen atoms belonging to a sugar unit attached to the third base pair and to a neighboring phosphate (3.18 and 2.98 Å) were detected. Similar hydrogen bondings have been found in the intercalation complexes of active anthraquinonic anticancer drugs like mitoxantrone.[33] All the previous remarks seem to support the good values of intercalation energy and cytotoxic activity obtained for **10**. It looks surprising that substitution of the NH group by NMe may lead to remarkable differences both in the experimental biological activity and the theoretical intercalation geometry, but it is not unusual to find substantial modifications in the DNA bisintercalation geometries induced by small structural differences in the flexible chains of the intercalators.[2] Anyway, from available data compound **10** seems to possess DNA bisintercalating properties.

Experimental

General

The starting amines were purchased from Aldrich and used without further purification. 1,4-Dichlorobenzo[*g*]phtalazine was obtained from 2,3-naphthalenedicarboxylic acid following a method previously described.[16] All the reactions were monitored using layer chromatography (TLC) on precoated aluminium sheets of silica gel, and compounds were detected with UV light (245 nm). Flash column chromatography was performed in the indicated solvent and supported on silica gel (particle size 0.040–0.063 mesh). Melting points were determined in a Gallenkamp or Köfler apparatus and are uncorrected. The ^{1}H NMR spectra were recorded at 300 MHz and the ^{13}C spectra were recorded at 75 or 100 MHz at room temperature, employing $CDCl_3$ or CD_3OD as the solvent. Chemical

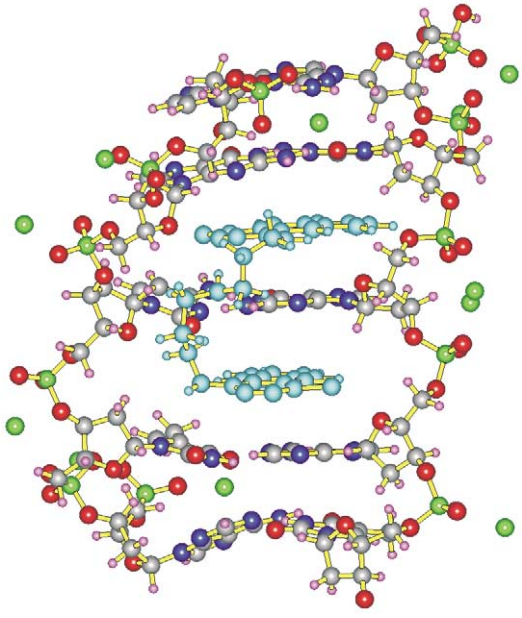


Figure 3. Image of the **9**-DNA complex obtained by molecular modeling. In the DNA oligomer carbon atoms are colored in grey, nitrogens in deep blue, oxygens in red, hydrogens in pink and phosphorus and counterions in green. All the atoms of the intercalating molecule are colored in light blue.

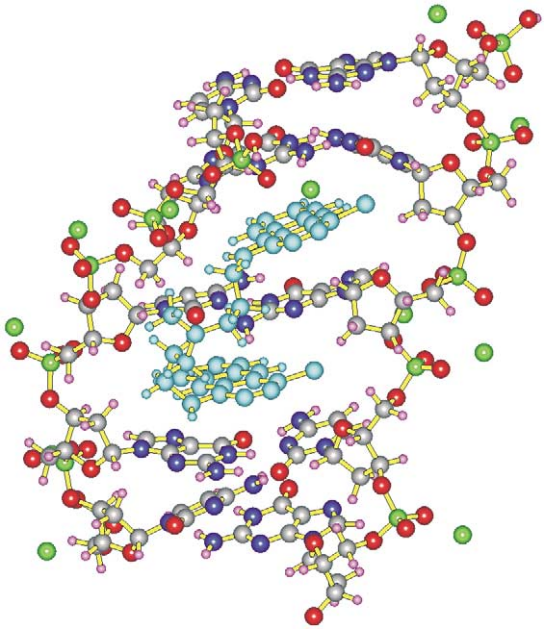


Figure 4. Image of the **10**-DNA complex obtained by molecular modeling. In the DNA oligomer carbon atoms are colored in grey, nitrogens in deep blue, oxygens in red, hydrogens in pink and phosphorus and counterions in green. All the atoms of the intercalating molecule are colored in light blue.

shifts are reported in ppm from TMS (δ scale). Mass spectra were recorded by electronic impact (EI) at 70 eV, or by the fast atomic bombardment (FAB) technique using a *m*-nitrobenzyl alcohol matrix.

Synthesis of the bis(3-aminopropyl)amine derivatives 7 and 9

A solution of 1,4-dichlorobenzo[*g*]phthalazine (224 mg, 0.89 mmol) and bis(3-aminopropyl)amine (1.75 g, 13.35 mol) in xylene (50 mL) was refluxed for 5 h. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The oily residue was purified by flash column chromatography ($CHCl_3$/MeOH/aq 25% NH_4OH in a 1/1/0.5 relation).

The appropriate fractions were monitored by TLC and combined to give two products of R_f 0.66 and 0.92.

1-{3-[3-(Aminopropyl)amino]propylamino}-4-chlorobenzo[*g*]phthalazine, 7. The most retained fraction afforded 15 mg (5% yield) of a yellow solid with mp 168–170 °C. Anal. calcd for $C_{18}H_{22}N_5Cl{\cdot}H_2O$: C, 59.74; H, 6.68; N, 19.35. Found: C, 59.60; H, 7.01; N, 19.22; IR (KBr, cm^{-1}), 3500–3300, 1570, 1518, 1428, 1372, 1300; ^1H NMR (CD_3OD, 300 MHz), δ 8.68 (s, 1H), 8.45 (s, 1H), 8.05 (m, 2H), 7.64 (m, 2H), 3.70 (t, 2H), 2.84 (m, 6H), 2.10 (m, 2H), 1.88 (m, 2H); ^{13}C NMR ($CDCl_3$, 300 MHz),δ 155.84, 146.14, 135.94, 135.71, 129.95, 129.79, 126.41, 123.53, 119.05, 47.80, 47.52, 40.14, 39.97, 29.80, 28.91; MS (FAB, *m*/*z*) 344 (MH^+, 71), 301 (90), 258 (5), 229 (38).

Bis-{3-[*N*-(4-chlorobenzo[*g*]phthalazin-1-yl)]-aminopropyl}amine, 9. The less retained fraction (R_f 0.92) gave 70 mg (11%) of a brown solid with mp 168–170 °C. Anal. calcd for: $C_{30}H_{27}N_7Cl_2{\cdot}2H_2O$: C, 60.81; H, 5.27; N, 16.55. Found: C, 60.73; H, 5.56; N, 16.75; IR ($CHCl_3$, cm^{-1}), 3600–3200, 3020; 1430, 1370, 1220, 760; ^1H NMR (CD_3OD, 300 MHz), δ 8.46 (s, 2H), 7.93 (s, 2H), 7.92 (m, 2H), 7.67 (m, 2H), 7.53 (m, 4H), 3.64 (m, 4H), 3.05 (t, 4H), 2.12 (m, 4H); ^{13}C NMR ($CDCl_3$, 300 MHz), δ, 154.76, 145.95, 135.22, 134.73, 129.68, 125.27, 124.01, 121.99, 117.87, 4691, 47.52, 39.25, 26.38. MS (FAB, *m*/*z*) 556 (MH^+, 86), 325 (15), 270 (100).

Synthesis of the 3,3′-diamine-*N*-methyldipropyl-amine derivatives 8 and 10

To a refluxed suspension of 1,4-dichlorobenzo-[*g*]phthalazine (250 mg, 1.0 mmol) and anhydrous potassium carbonate (1.39 g, 10.0 mmol) in anhydrous acetonitrile (130 mL), 3,3′-diamine-*N*-methyldipropyl-amine (146 mg, 1.0 mmol) in acetonitrile (70 mL) was slowly added for a period of 2 h. The reaction mixture was further refluxed and monitored by TLC plates until the reaction was completed (7 days). After cooling to room temperature, potassium carbonate was eliminated by filtration and the solution evaporated under reduced pressure. The oily brown residue was purified by flash column chromatography ($CHCl_3$/EtOH/aq 25% NH_4OH in a 3/1/0.1 relation).The appropriate fractions monitored by TLC were combined to give two products of R_f 0.12 and 0.72.

1-{3-[*N*-(3-Aminopropyl)-*N*-methylamino]-propylamino}-4-chlorobenzo[*g*]phthalazine, 8. The most retained fraction afforded 80 mg (23% yield) of a pure oil identified as **8.** Anal. calcd for $C_{19}H_{24}N_5Cl{\cdot}H_2O$: C, 60.71; H, 6.97; N, 18.63. Found: C, 61.05; H, 6.75; N, 18.40; IR (KBr, cm^{-1}), 3500–3000, 1627, 1560, 1508, 1431, 1323, 1222; ^1H NMR ($CDCl_3$ 300 MHz), δ 8.50 (s, 1H), 8.40 (s, 1H), 8.00 (m, 2H), 7.60 (m, 2H), 3.74 (t, 2H), 2.85 (t, 2H), 2.60 (t, 2H), 2.54 (t, 2H), 2.38 (s, 3H), 1.93 (q, 2H), 1.78 (q, 2H); ^{13}C NMR ($CDCl_3$, 300 MHz), δ, 154.00, 145.35, 134.41, 134.30, 128.71, 125.51, 122.95, 122.29, 118.55, 57.28, 56.74, 42.40, 42.37, 40.93, 29.76, 24.85; MS (FAB, *m*/*z*) 358 (MH^+, 50), 269 (13), 242 (15), 229 (6), 179 (29).

***N*,*N*-Bis-{3-[*N*-(4-chlorobenzo[*g*]phthalazin-1-yl)]aminopropyl}-*N*-methylamine 10.** The less retained fraction (R_f 0.72) gave 125 mg (22% yield) of a yellow solid with mp 178–180 °C. Exact mass calcd for $C_{31}H_{30}N_7Cl_2$ (MH^+): 570.193229. Found: 570.193975. IR (KBr, cm^{-1}), 3600–3000, 2940, 1660, 1560, 1520, 1420, 1380, 1290; ^1H NMR (CD_3OD, 300 MHz), δ 8.37 (s, 2H), 8.32 (s, 2H), 7.88 (m, 4H), 7.49 (m, 4H), 3.62 (t, 4H), 2.75 (t, 4H), 2.47 (s, 3H), 2.03 (q, 4H); ^{13}C NMR (CD_3OD, 300 MHz), δ 155.46, 146.22, 135.70, 135.51, 131.52, 129.65, 126.32, 123.80, 123.39, 119.01, 56.62, 42.24, 41.35, 26.53. MS (FAB, *m*/*z*) 570 (MH^+, 39), 569 (M^+, 33), 339 (11), 301 (12), 270 (100), 242 (10).

Cytotoxicity essays

The effect of compounds 7–10 on cell proliferation was determined using a standard MTT-based colorimetric assay, according to the method previously reported by De Arruda et al.[25] Diluted drug (50 μL, 10^{-4}–10^{-12} M, final concentrations) or medium alone was added to exponentially growing cells (3.5×10^3/well) in a 96-well plate. After incubation for 72 h at 37 °C, 5% CO_2, MTT (50 μL, 3 mg/mL, Sigma) was added to each well and incubated for additional 5 h at 37 °C. SDS (25%; 50 μL; pH 2) was then added to the cultures and incubated for 3–4 h at 37 °C to allow formazan solubilization The absorbance of each well at 550 nm was measured using a microplate reader (Molecular Devices) interfaced with a computer. Data were analyzed and the drug concentration necessary to cause 50% of cell death (ED_{50}) was determined by an in-house computer program. Two different essays were performed for each compound, and the medium value was selected and is shown in Table 1.

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