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Synthesis and characterization of novel tris-intercalators having potentially two different DNA binding modes

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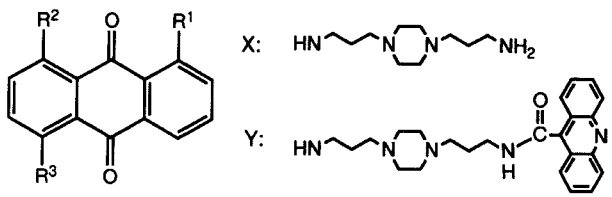
Novel DNA binding ligands (3 and its stereoisomer 4) which contain three potentially intercalating units in a linear molecular skeleton were prepared. The melting point of calf thymus DNA (T_m) was determined in the presence of 3 or 4 to show that these compounds possess a high binding affinity for DNA and that 3 stabilizes the double helix more than does isomeric 4. Upon mixing with DNA, circular dichroism spectra were induced for both the acridine and the anthraquinone chromophores of 3 and 4. A DNase I footprinting study indicated that these compounds intercalate in AT-rich DNA sequences. Viscometric analysis using sonicated calf thymus DNA and closed, circular, supercoiled plasmid DNA given an unwinding angle of 49° and 47° for 3 and 4, respectively. These results indicate that both compounds serve as trifunctional intercalators.



	R^1	R^2	R^3
1	X	H	X
2	X	X	H
3	Y	H	Y
4	Y	Y	H

INTRODUCTION

Intercalators are a class of polyaromatic compounds that insert between two adjacent base pairs of double helical DNA. They exhibit a variety of biological effects, and are pharmacologically important as antiparasitic and cytostatic agents.[1] Polyintercalating agents carrying several intercalating parts within the molecule are known to show both high affinity and high selectivity in DNA binding and have been studied in the search for new anti-tumour drugs.[2] However, polyintercalators so far described carry the same intercalating aromatic units within the molecule[3–6] and do not allow a detailed characterization of the topological mode of interaction between the poly-intercalators and DNA.

The present study concerns a novel DNA-binding ligand **3**, which contains an anthraquinone moiety bearing two acridine units at its 1 and 5 positions. In addition, its 1,8-isomer **4** was prepared for comparison. Both of these compounds carry three potentially intercalating units, but they differ in geometry; **3** is basically linear, while **4** is folded. Previous papers suggested that the two substituents of 1,5-disubstituted anthraquinone are positioned separately in the major and minor grooves, whereas the same substituents of the 1,8-isomer occupy the major groove only.[7,8] In principle, tris-intercalators can bind DNA in three distinctly different modes (Fig 1), which include (A) bis-intercalation in which the two terminal acridine units intercalate into the adjacent base pairs while the central anthraquinone unit lies outside the DNA strands, (B) tris-intercalation in which the terminal acridines insert into DNA, whereas the anthraquinone intercalates in the same groove, and (C) tris-intercalation in which all the three aromatic units of the ligand take part in intercalation with DNA. (A) and (B) are viewed as having only a single groove involved, whereas (C) is viewed as having both grooves involved. Compound **4** and all known tris-intercalators are supposed to intercalate through mechanism B, but this mode of intercalation is impossible with **3** because

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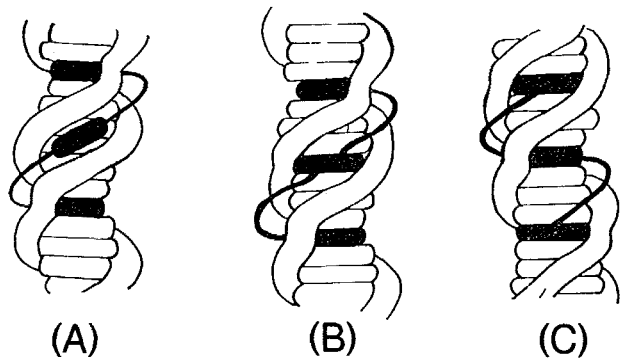


Figure 1 Schematic representation of (A) a covering mode for bis-intercalation, (B) a covering mode for tris-intercalation, and (C) a penetrating mode for tris-intercalation, of **3** and **4**.

of structural constraints. A more plausible intercalation mode for **3** is mechanism C, in which the molecular chain penetrates the DNA strands at the central anthraquinone moiety. This paper describes how compound **3** binds DNA tri-functionally, possibly through this unprecedented mechanism.

RESULTS AND DISCUSSION

1,5-Disubstituted- and 1,8-disubstituted-9,10-anthraquinone, **1** and **2**, were prepared by the reaction of 1,4-bis(aminopropyl)piperazine with 1,5-dichloro- and 1,8-dichloro-9,10-anthraquinone, respectively. Tris-intercalators, **3** and **4**, were synthesized by the reaction of **1** or **2** with acridine-9-carbonyl chloride and were purified by silica gel chromatography (methanol) followed by crystallization. They showed a single peak on HPLC and gave a correct elemental analysis.

Spectroscopic properties of compounds **1**–**4** were studied in 0.3 mM sodium acetate buffer (pH 4.5). The molar extinction coefficient for **3** is smaller than that for **1** in the anthraquinone chromophore (Table 1). The same is true for **2** and **4**, suggesting strongly that intramolecular stacking of the anthraquinone and acridine rings is taking place in **3** and **4**. The magnitude of this hypochromicity is greater in **3** than in **4**. This implies that the stacking interaction is more extensive in **3** than in **4**, presumably because the spatial disposition of the aromatic rings is more favourable for stacking in the former compound.

The acridine and/or anthraquinone chromophores in **1**–**4** bound to sonicated calf thymus DNA exhibit hypochromic and bathochromic shifts (Table 1). The hypochromicity seen in the anthraquinone chromophores of **3** and **4** is less than that seen in **1** and **2**, respectively. These phenomena may be explained as follows. As described above, the aromatic rings of **3** and **4** stack intramolecularly. Hence, in order for these compounds to intercalate into DNA, this stacked conformation needs to be dissolved. This interpretation may be substantiated by the fact that **4**, whose intramolecular stacking is weaker than that of **3**, shows greater hypochromicity.

Circular dichroism (CD) spectra of sonicated calf thymus DNA were determined in the absence and presence of **3** or **4**. The CD spectrum of DNA alone is composed of positive and negative Cotton effects of similar intensity and is characteristic of B form DNA (Fig 2A, spectrum *a*). The CD spectra of its complexes

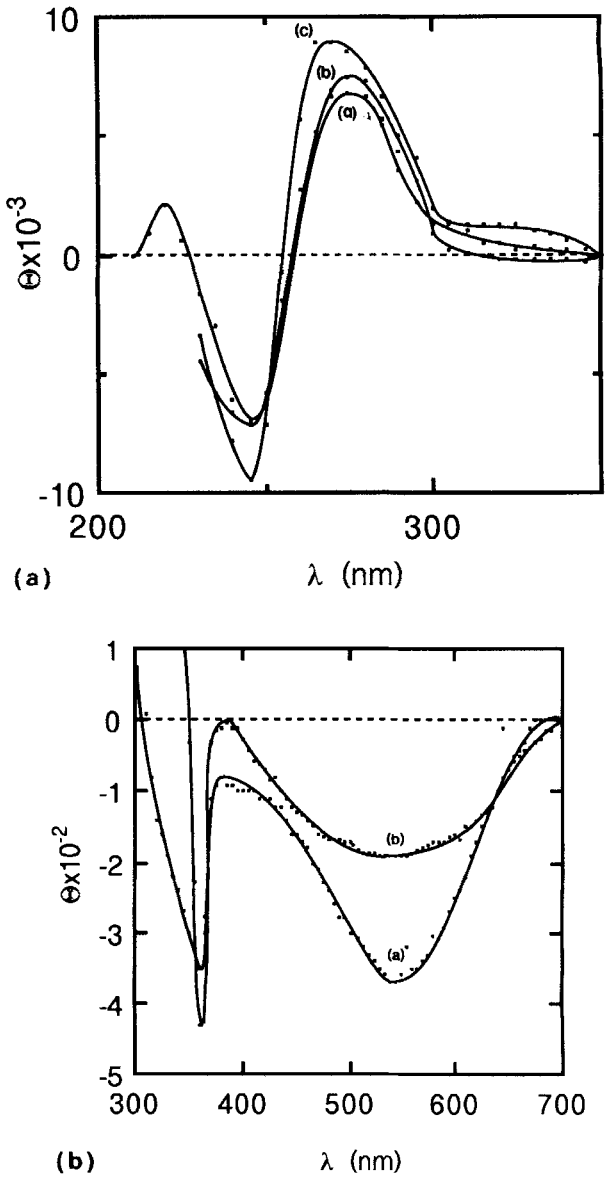


Figure 2 (A) CD spectra of 1 mM sonicated calf thymus DNA in the absence (a) and presence of **3** (b) and **4** (c). (B) DNA-induced CD spectra of the complexes with **3** (a) and **4** (b) with DNA in the visible region. The concentrations of **3** and **4** were 50 and 35 μM, respectively. All experiments were performed in 0.3 mM sodium acetate buffer (pH 4.5).

Table 1 Spectroscopic properties of compounds **1–4** in free and DNA-bound form

	UV-visible absorption[a]				
	Free ligand		*Bound ligand*		
Compound	λ_f (nm)	ε_f ($M^{-1}cm^{-1}$)	λ_b (nm)	ε_b ($M^{-1}cm^{-1}$)	H[b]
1	536	11,450	541	7,850	31
2	554	9,550	567	5,950	38
3	360	8,690	362	8,200	6
	539	4,650	540	3,890	16
4	360	17,450	363	14,950	14
	555	7,800	562	6,250	20

[a] Experiments were conducted at pH 4.5 in 0.3 mM sodium acetate buffer. Spectra were measured for 20 μM compound in the presence of 400 μM calf thymus DNA (phosphate unit). [b] H stands for percentage of hypochromicity [$\%H = (1 - \varepsilon_b/\varepsilon_f) \times 100$].

with **3** or **4** are also nearly superimposable (Fig 2A, spectra *b* and *c*), suggesting that the DNA structure remains intact upon binding of **3** and **4**. In addition to this CD band of DNA, new bands were induced at around 360 and 540 nm, which are ascribable, respectively, to the acridine and anthraquinone chromophores of **3** and **4**. This observation reinforces the view that the central anthraquinone moiety of **3** and **4** does participate in intercalation into DNA. The shape and sign of the CD bands are similar for the two complexes, suggesting that their intercalating behaviour is basically the same, regardless of the detailed mechanism of interaction with DNA.

Thermal denaturation profiles for solutions of sonicated calf thymus DNA were determined at several ratios of compound (dye)/DNA phosphate (D/P) in 0.5 mM Tris/HCl containing 50 μM EDTA (pH 7.4), 1% DMSO, and 4% methanol. Figure 3 shows the effects of binding of **3** and **4** on the ΔT_m of DNA.* The ΔT_m values for **3** kept increasing over the D/P range studied (0–0.4). The ΔT_m values for **4** were increased up to D/P = 0.1 and then leveled off. The maximum ΔT_m values were 30°C for **3** and 25°C for **4**. These results indicate that both compounds stabilize the double helix of DNA through intercalation. Although the binding affinity of **3** for DNA is lower than that of **4**, the former compound stabilizes the double helix more than does isomeric **4**. The lower affinity of **3** might be derived from the stronger intramolecular stacking of the chromophores in **3** which impares intercalation into DNA.

Illustrative results of viscometric titrations of sonicated calf thymus DNA with quinacrine, **3** and **4** are shown in Figure 4. All compounds increased the relative specific viscosity (η) of DNA, as the molar

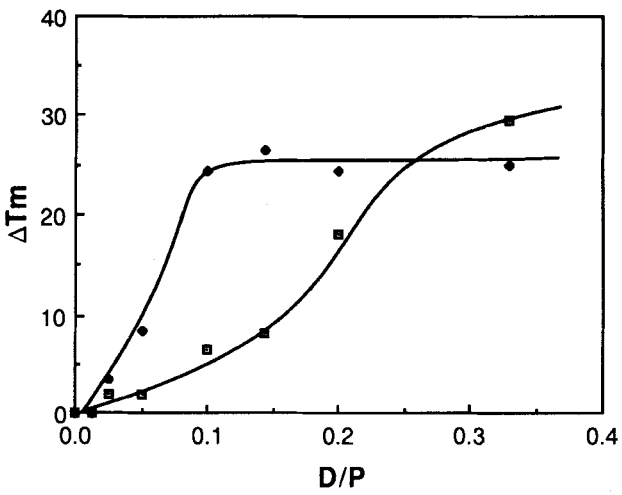


Figure 3 Changes in the denaturation temperature of the sonicated calf thymus DNA as a function of the concentration of compounds **3** (□) and **4** (◆). All experiments were performed in 0.5 mM Tris/HCl containing 50 μM EDTA (pH 7.4), 1% DMSO, and 4% methanol.

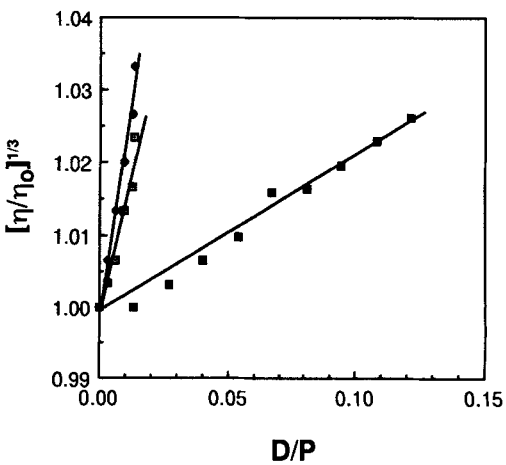
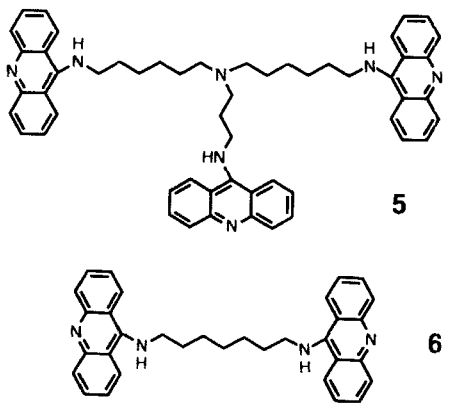


Figure 4 Representative experiment showing an increase in the viscosity of sonicated calf thymus DNA by intercalating compounds **3** (◆), **4** (□), and quinacrine (■). Experiments were conducted at 25 ± 0.05°C in 1 mM Tris/HCl, 0.1 mM EDTA (pH 7.4), and 1 mM NaCl.

* The T_m curves for these compounds are composed of two phases. The higher T_m was 82°C for **3** and 72°C for **4**. Wilson and co-workers[16] observed similar phenomena for bis-intercalators and explained that the intercalators bind to different regions of DNA.

ratio of the compound to DNA base pairs was increased. The slope obtained from a plot of $[\eta/\eta_0]^{1/3}$ vs. compound/DNA phosphate ratio for **3** and **4** was almost the same and was nearly 3-times that seen for quinacrine (mono-intercalator). As the helix-extension of DNA rods caused by intercalating ligands is directly related to the viscosity observed,[9] the results obtained indicate that all three intercalating units are bound simultaneously to DNA. The ability of **3** and **4** to unwind closed, circular, supercoiled DNA was also evaluated from viscometric measurements. Addition of increasing amounts of **3** or **4** to DNA caused relaxation and then reversal of the supercoils. A typical example is shown for **3** in Figure 5. These data were analysed by the method of Révet *et al.*[10] to give an unwinding angle of 49° for **3** and 47° for **4**. These values compare well with the unwinding angle of 44° for **5** (tris-intercalator)[11] and are roughly 3-times that for mono-intercalators: 17° for quinacrine[12] and 18° for 1,5- and 1,8-disubstituted anthraquinones.[7] (Bis-intercalator **6** exhibited an unwinding angle of 35°.[2]) This provides more evidence to support the notion that both **3** and **4** serve as tris-intercalators.



5

6

Figure 6 shows the DNase I footprinting patterns observed for a 5′-end ^{32}P-labelled 156-base pair DNA fragment in the presence of **3** or **4** at several concentrations. Compound **3** maskes selectively about ten base pair stretches of DNA that contain AT-rich sequences, and compound **4** also protected the same region as that masked by **3**, with the latter having a slightly higher affinity for DNA. This result suggests that these compounds shown an AT base pair preference in their interactions with DNA. A Corey–Pauling–Koltun model reveals that **3** and **4**, when bound properly, can mask a ten base pair stretch of DNA, in good agreement with experiment.

In conclusion, all the data presented above are consistent with the view that novel intercalators **3** and **4** do serve as tris-intercalators. We propose that, because of its structural constraints, **3** interacts with DNA in the penetration mode (C), in which the intercalator creeps into the DNA double strands, though unambiguous proof for this mechanism remains to be obtained. Zimmermann and co-workers[5,6] previously reported a catenated complex of a macrocyclic bis-intercalator with DNA. This structure is made possible only when the intercalator penetrates into the DNA double strands.

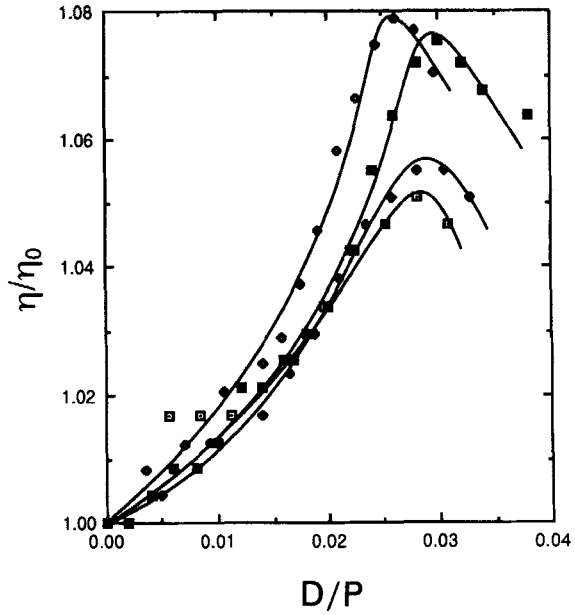


Figure 5 Representative experiment showing unwinding of closed, circular, superhelical plasmid DNA by compound **3**. The concentrations of **3** were 88, 105, 123, and 141 μM (top to bottom curves). Experimental conditions were the same as those in Figure 4. Data were analysed by the method given in reference 10.

EXPERIMENTAL SECTION

Materials

Calf thymus DNA was obtained from Sigma Chemical Co. and, following sonication, was used in the thermal denaturation and linear viscosity experiments.[13] The closed, circular, supercoiled plasmid DNA of 8800 base pairs was made of vector pUC9 and a portion of *Escherichia coli* DNA replication termination sequence (unpublished). This plasmid was propagated in *E. coli* strain JM105 and isolated by a standard method.[14] A 156 base pair fragment of yeast ribosomal RNA gene was used for the footprinting studies. Both of the DNA samples were provided by Prof. T. Horiuchi, National Institute for Basic Biology, Japan. The DNA concentration was determined using $\varepsilon = 6600\ M^{-1}\ cm^{-1}$, at 260 nm. Stock solutions of compounds **1**–**4** were made with 25% DMSO in methanol and stored at −20°C.

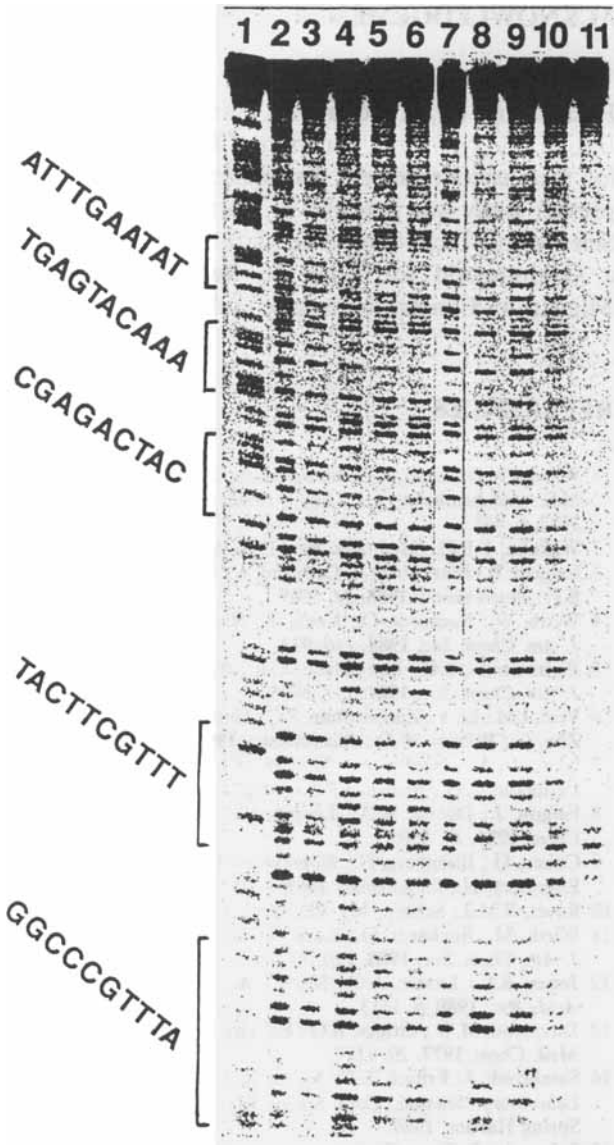


Figure 6 DNase I footprinting on a 156-base pair DNA fragment. Lanes 2 and 7 (control): DNase I digest in the absence of ligands. Lanes 3–6: DNase I digests in the presence of increasing concentrations (left to right) of **3** in 0.08, 0.16, 0.32, and 0.65 ligand/base ratio. Lanes 8–11: DNase I digests in the presence of increasing concentrations (left to right) of **4** in 0.08, 0.16, 0.32, and 0.65 ligand/base ratio. The concentration of DNA was 4×10^{-11} M (base). The square brackets indicate the areas of protection afforded to DNA by **3** from digestion by DNase I. Maxam–Gilbert sequencing reactions (A–G) are shown in lane 1.

Apparatus

Electronic absorption spectra were determined on a Hitachi U-3210 UV-visible spectrophotometer equipped with a temperature controller Hitachi SPR-10. In thermal denaturation experiments a heating rate was set at 0.33°C/min. The ΔT_m is defined as the difference in T_m of DNA in the presence and absence of ligand. Circular dichroism spectra were recorded at 230–700 nm on a Jasco-500 spectropolarimeter at 25°C. Quartz cells of 1 or 10 mm thickness were used for 230–400 and 300–700 nm regions, respectively. The viscometric titrations were carried out by using the method of Cohen and Eisenberg.[9] The unwinding angle was determined for each compound by using the technique described by Révent *et al.*[10] Footprinting analysis was performed as described in a previous paper.[15] HPLC was run with a dual plunger pump Hitachi L-6200 on an Inertsil ODS column (4.6 mm i.d. × 15 cm, GL Sciences Inc.). The eluent, consisting of 0.1% trifluoroacetic acid (TFA) (A) and 0.1% TFA in 70% acetonitrile (B), was run in a linear gradient of 3–97% B in 40 min at a flow rate of 1.0 ml/min.

1,5-Bis[[4-(3-aminopropyl)piperazinyl]propylamino]-9,10-anthraquinone (1)

1,5-Dichloro-9,10-anthraquinone (2.5 g, 10 mmol) was dissolved in 1,4-bis(aminopropyl)piperazineamine (20 ml) and the solution heated below 150°C for 4 h. The solution was allowed to cool and poured into ether (1 l). The precipitate formed was taken up in chloroform (300 ml) and washed with 3 M sodium hydroxide (300 ml). The organic layer was evaporated to dryness under reduced pressure and the residue was dissolved in a small amount of methanol and poured into ether (1 l). The solid obtained was dried under reduced pressure to yield 1.6 g (26%) of **1** as a crimson solid: m.p. > 300°C; TLC $R_f = 0.1$, 10% diethylamine/methanol; IR (KBr) 1620, 1600 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.75 (m, 8H), 1.95 (m, 4H), 2.3–3.80 (m, 20H), 2.94 (m, 8H), 3.40 (m, 4H), 7.10 (m, 2H), 7.50 (m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 26.6, 30.6, 40.9, 41.0, 53.2, 55.9, 56.5, 112.9, 114.6, 116.3, 135.0, 136.2, 151.3, 185.2. Anal. calcd. for $C_{34}H_{52}N_8O_2 \cdot H_2O$: C, 55.59; H, 8.68; N, 18.01. Found: C, 55.52; H, 8.67; N, 18.53.

1,8-Bis[[4-(3-aminopropyl)piperazinyl]propylamino]-9,10-anthraquinone (2)

This was prepared from 1,8-dichloroanthraquinone in a manner identical to that for **1**. This crude material was further purified by HPLC. Yield 0.6 g (10%) as a violet solid: m.p. > 300°C; TLC $R_f = 0.1$, 10% diethylamine/methanol; IR (KBr) 1620, 1600 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD) δ 1.96 (m, 4H), 2.14 (m, 4H), 2.83 (m, 4H), 3.08 (m, 16H), 3.28 (m, 12H), 3.45 (m, 4H), 7.16 (m, 2H), 7.48 (m, 4H); ^{13}C-NMR (100 MHz, CD_3OD) δ 24.5, 25.2, 34.0, 40.8, 51.0, 52.4, 55.3, 55.9, 115.7, 116.2, 119.1, 135.6, 135.7, 152.2, 190.0. Anal. calcd. for $C_{34}H_{52}N_8O_2 \cdot 7CF_3COOH$: C, 42.86; H, 4.54; N, 8.70. Found: C, 42.31; H, 4.69; N, 8.63.

1,5-Bis[[4-[3-(9-acridinecarboxamido)propyl]-piperazinyl]propylamino]-9,10-anthraquinone (3)

A solution of acridine-9-carboxylic acid (1.2 g, 4.8 mmol) and thionyl chloride (50 ml, 690 mmol) was refluxed for 3 h. The excess thionyl chloride was distilled off and the last traces of it were removed by co-distillation with toluene. The resulting acid chloride, **1** (1 g, 2.1 mmol), and triethylamine (0.38 ml, 4.8 mmol) were dissolved in dichloromethane (100 ml) and refluxed for 3 h. After evaporation, the solid residue was purified by silica gel (Merck 60) chromatography (methanol). This solid showed a single peak on HPLC with a retention time of 18.7 min. Yield 0.8 g (40%) as a crimson solid: m.p. > 300°C; TLC $R_f = 0.1$, methanol; IR (KBr) 1640, 1620, 1600 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.75–1.85 (m, 8H), 2.34–2.45 (m, 24H), 3.37 (m, 4H), 3.53 (m, 4H), 7.18 (m, 2H), 7.43 (m, 2H), 7.60 (m, 2H), 7.67 (m, 2H), 7.88 (m, 4H), 8.00 (m, 4H), 8.20 (m, 4H), 9.05 (m, 2H), 9.70 (m, 2H). Anal. calcd. for $C_{62}H_{66}N_{10}O_4 \cdot 1/2H_2O$: C, 72.93; H, 6.54; N, 13.73. Found: C, 72.65; H, 6.53; N, 13.78.

1,8-Bis[[4-[3-(9-acridinecarboxamido)propyl]-piperazinyl]propylamino]-9,10-anthraquinone (4)

This was prepared from **2** in a manner identical to that for **3**. This solid showed a single peak on HPLC with a retention time of 20.5 min. Yield 0.7 g (9%) as a crimson solid: m.p. > 300°C; TLC $R_f = 0.2$, methanol; IR (KBr) 1670, 1600 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 1.25–1.48 (m, 12H), 1.80–2.00 (m, 9H), 2.25–2.65 (m, 8H), 3.20–3.50 (m, 8H), 3.70 (m, 4H), 4.20 (m, 4H), 7.18 (m, 2H), 7.46 (m, 2H), 7.50 (m, 2H), 7.64 (m, 4H), 7.70 (m, 2H), 7.86 (m, 4H), 8.04 (m, 4H), 8.18 (m, 4H). Anal. calcd. for $C_{62}H_{66}N_{10}O_4$: C, 73.35; H, 6.55; N, 13.80. Found: C, 73.21; H, 6.47; N, 13.44.

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