Bisintercalators of DNA with a Rigid Linker in an Extended Configuration†

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ABSTRACT: A new class of DNA bisintercalators is reported in which phenanthridinium or acridinium rings are connected by rigid and extended linkers of varied length. Cross-linking of DNA by bisintercalation is inferred from the unwinding and folding of linear DNA induced by the compound; after ligation and removal of the bisintercalator, superhelical circles, catenanes, and knots that bear an imprint of the bisintercalator are observed. These novel bisintercalators are of interest because they can be used to probe the organization of DNA in three-dimensional space, especially near sites of replication, recombination, or topoisomerase action, where two duplexes must be in close proximity.

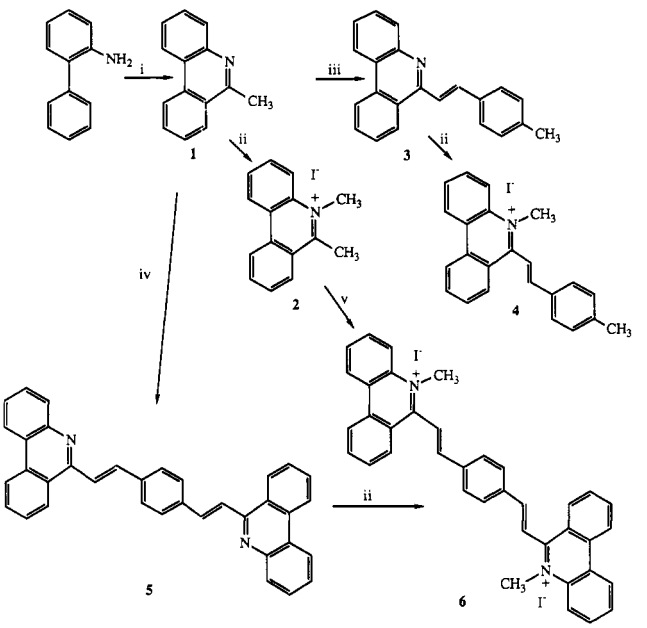
A large number of DNA bisintercalators are known [see, for example, LePecq et al. (1975), Cannellakis et al. (1976), Gaugain et al. (1978), and Welsh and Cantor (1987)], but the two intercalating moieties of such compounds usually bind to the same DNA duplex because they can rotate freely about the connecting linker. Binding of one moiety inevitably leaves the other in close proximity to other binding sites in the same duplex, leading to intramolecular cross-linking. However, if the linker is rigid with an extended configuration (i.e., points the two intercalating groups in opposite directions), binding of both intercalators into the same duplex will be impossible unless the duplex is long enough to fold back on itself. Therefore, we might expect bisintercalators with rigid linkers to cross-link DNA duplexes more frequently, i.e., form intermolecular links. We now report the synthesis of a new class of bisintercalators with rigid and cleavable linkers connecting the two intercalating groups; one group is based on the phenanthridinium ion, i.e., **6** (synthesized as outlined in Scheme I) and **9** (synthesized as outlined in Scheme II), the other on the acridinium ion, i.e., **15** (synthesized as outlined in Scheme III). Such molecules are of interest because they can be used to probe the organization of DNA in three-dimensional space, especially near sites of replication, recombination, or topoisomerase action where two duplexes must be in close proximity. Analogous compounds lacking the second intercalating group, e.g., **2** and **4** (Scheme I) or **11** and **13** (Scheme III), provide appropriate monointercalators for control experiments. The olefinic bonds in the bisintercalators are potential sites of cleavage when DNA cross-linking has been achieved.

The antitumor antibiotic luzopeptin A (BBM-928A) provides the only example for which intermolecular bisintercalation has been demonstrated. However, the linker connecting the two intercalating groups is flexible so that intermolecular cross-links gradually revert in solution to intramolecular links (Huang et al., 1983). Moreover, in the crystalline state luzopeptin was found by X-ray analysis (Arnold & Clardy, 1981) to have a bent conformation, and in the complex formed with a self-complementary oligonucleotide it was shown by NMR spectroscopy (Searle et al., 1989) to bisintercalate intramolecularly.

The development of a facile assay to detect intermolecular bisintercalation by compounds with rigid linkers posed a special problem. Hydrodynamic methods (including electrophoretic methods) generally provide strong circumstantial evidence for intercalation [reviewed by Waring (1981), Waring & Fox (1983), and Saenger (1984)] before formal proof is obtained using X-ray crystallography or NMR spectroscopy (Ughetto et al., 1985; Gao & Patel, 1988; Searle et al., 1989). However, bisintercalators that cross-link *different* DNA molecules affect the hydrodynamic properties of DNA in ways that complicate analysis. After monointercalation, the second intercalating group protrudes from DNA so that mobility depends on the number of bound intercalators. In addition, intermolecular cross-links yield aggregates with different sizes and shapes and, hence, mobilities. Consequently, analysis of mobility changes induced by luzopeptin or the bisintercalators reported here proved difficult (Huang et al., 1983; Fox et al., 1988; P. R. Cook, unpublished work).

We have developed an indirect, but decisive, assay for unwinding (and so intercalation) and knotting (and so cross-linking). The test compound is allowed to bind to linear DNA molecules and alter their shape. Then, the linear molecules are treated with DNA ligase; some are ligated end-to-end, others into circles, catenanes, or knots. [Wasserman and

Scheme I[a]



[a] Reagents: (i) (a) AcCl or (b) $POCl_3$; (ii) CH_3I; (iii) PhCOCl, *p*-$CH_3C_6H_4CHO$; (iv) PhCOCl, DMF, $C_6H_4(CHO)_2$; (v) $C_6H_4(CHO)_2$, piperidine.

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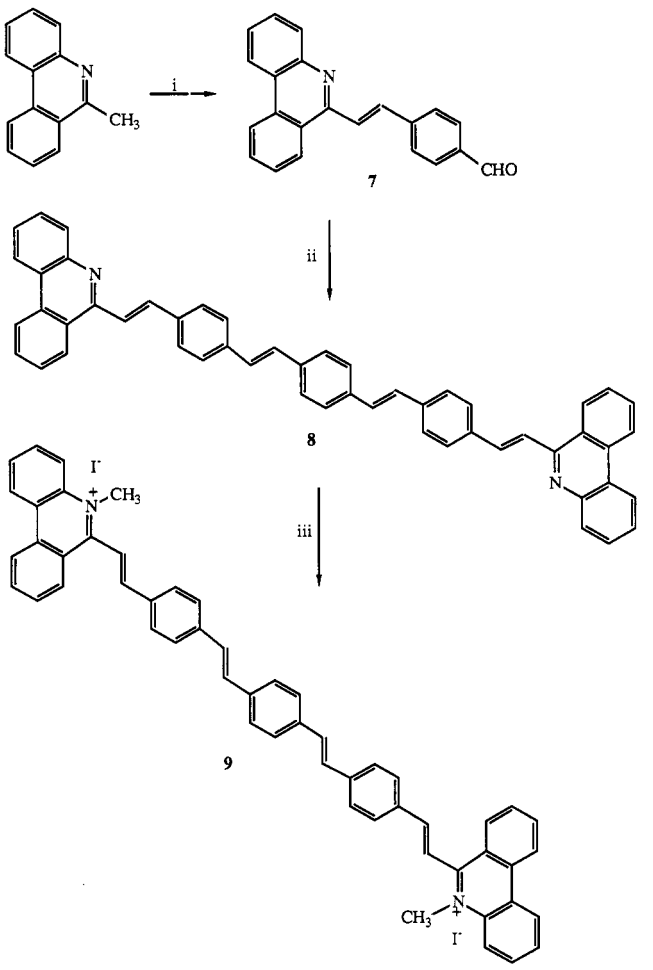
Scheme II[a]



[a] Reagents: (i) PhCOCl, DMF, p-$C_6H_4(CHO)_2$; (ii) p-$C_6H_4(CH_2PMe_3^+)_2Br^-_2$, NaOEt, DMF; (iii) CH_3I, nitrobenzene.

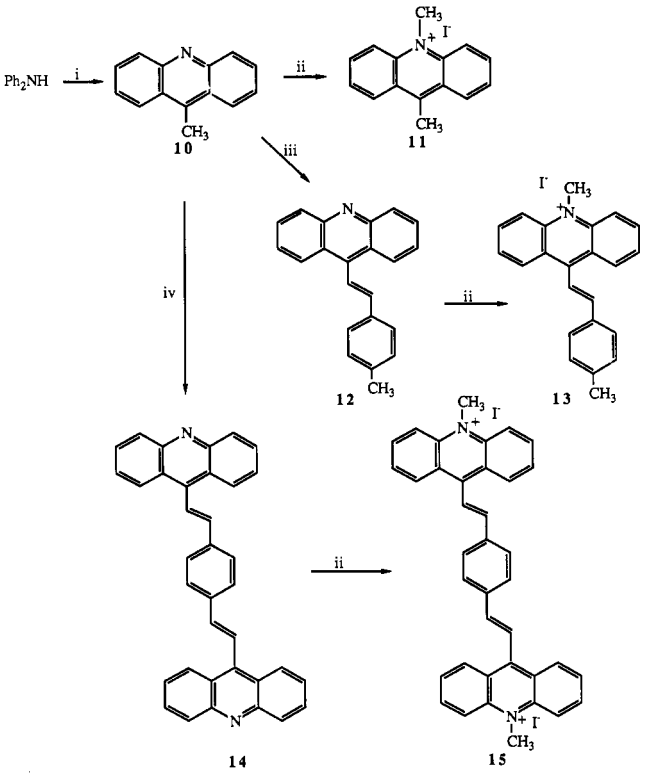
Cozzarelli (1986) have reviewed the structure of knots and catenanes.] If the test compound is an intercalator (either mono- or bis-), its removal has little effect on the overall shape of the linear forms but it compacts the circular forms by inducing compensatory supercoiling in them. If the test compound also cross-links, ligation of entwined linear molecules yields catenanes. Cross-linking distant parts of the same molecule inevitably leads to knotting; as binding sites are helically arranged, cross-linking tends to intertwine the two parts and subsequent circularization then produces a knot. The various different structures (i.e., supercoils, catenanes, knots, etc.) are resolved by gel electrophoresis after *removal* of the test compound (Keller, 1975; Wasserman & Cozzarelli, 1986).

This assay has an important advantage over others. Intermolecular bisintercalators can be expected to be only weak cross-linkers as the entropic factor involved in bringing two DNA molecules together is large. Our bisintercalators **6**, **9**, and **15** were indeed relatively weak cross-linking agents and were only soluble in aqueous buffers at concentrations up to about 10 μg/mL. Fortunately, we could demonstrate intercalation and cross-linking in the accessible concentration range because a minor fraction of a supercoiled or knotted form can be detected in the presence of an excess of other forms. However, the assay is limited if the agents inhibit the DNA ligase (Montecucco et al., 1990).

Materials and Methods

The Ligation Assay. pSVtkneo, a 5.3-kbp plasmid (Townsend et al., 1984), was linearized with *Hin*dIII (2

Scheme III[a]



[a] Reagents: (i) CH_3COOH, $ZnCl_2$; (ii) CH_3I; (iii) PhCOCl, DMF, p-$CH_3C_6H_4CHO$; (iv) PhCOCl, DMF, p-$C_6H_4(CHO)_2$.

units/μg of DNA; 1 h; 37 °C), ethanol precipitated, and redissolved. Ligations generally contained 250–750 ng of linear DNA in a solution containing 1 mM ATP, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 unit of T4 DNA ligase (Boehringer), and various concentrations of test compound in a final volume of 250 μL. Test compounds were dissolved in dimethyl sulfoxide, and control experiments showed that this solvent had no effect on the mobility of the ligated products at the concentrations used. After incubation on ice overnight, the ligated DNA was ethanol precipitated to remove the test compound and then subjected to electrophoresis in 0.8% agarose gels containing 40 mM Tris, 2 mM EDTA, and 20 mM sodium acetate (pH 8.3), and the gel was stained with ethidium and photographed (Keller, 1975; Maniatis et al., 1982). This assay requires that all test compound is removed, but putting limits on how much might remain during electrophoresis is technically difficult, given the low concentrations used. All the compounds are soluble in ethanol, so precipitating and dissolving the compounds in sample buffer necessarily dilutes them >50-fold and then electrophoresis dilutes them further. Moreover, electrophoretic profiles of samples prepared by redissolving precipitated samples in 50 μL of 10 mM Tris (pH 8.0)/1 mM EDTA/1% SDS followed by reprecipitation and electrophoresis are identical, so any residual compound does not influence electrophoretic mobility. In some cases samples were γ-irradiated (1180 J/kg; Cook et al., 1976) in the sample buffer used for electrophoresis; control experiments showed that this dose nicked >99% supercoiled molecules.

Results

The Phenanthridinium Series: Intercalation. Figure 1 illustrates a typical ligation assay. Linear DNA runs as a single band (form III, lane 2), and most is ligated in the absence of any test compounds into a complicated set of

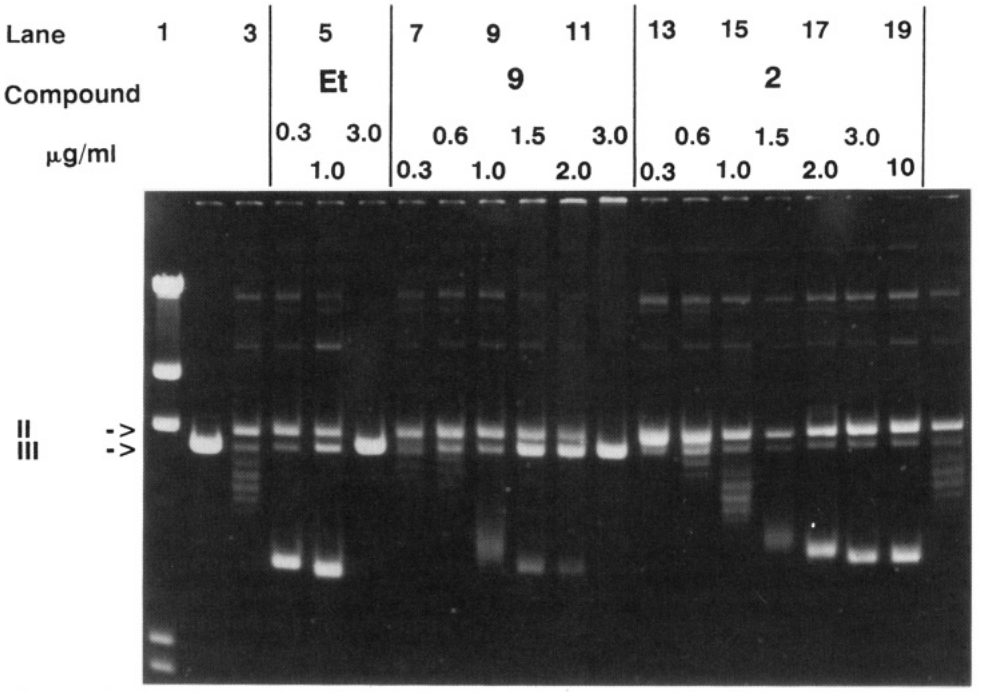


FIGURE 1: Gel showing a typical ligation assay where ethidium (Et), **9**, and **2** are intercalating agents. Linear DNA (500 ng) was ligated in the presence of different concentrations of the compounds, the compounds were removed, and the ligation products were resolved electrophoretically before the resulting gel was stained and photographed. Lane 1: λ/*Hin*dIII markers. Lane 2: unligated DNA. Lane 3: linear DNA ligated in the absence of any test compound. Lane 20: ligation in 2% dimethyl sulfoxide, the maximum concentration of solvent present during ligation. The positions of forms II and III are indicated.

products (lane 3). At the DNA concentration used, the majority of these are circles. As some linear molecules originally contained nicks, some circles are nicked and run just behind the linear molecules. Such relaxed circles (i.e., form II molecules) constitute a background present whenever the ligase is active. However, about half of the circles contain no nicks and run as a number of bands slightly ahead of the nicked circles. These are topoisomers containing positive supercoils generated after ligation by the duplex unwinding that occurs on transfer of DNA from the ligation buffer (containing a high Mg^{2+} concentration) into the electrophoresis buffer (Depew & Wang, 1975). There are additional, but minor, bands at the top of the gel formed by end-to-end ligation which give linear molecules of two or more unit lengths together with their circular and catenated counterparts (both supercoiled and relaxed). (As these forms are difficult to identify, DNA is ligated at a low concentration to minimize their formation.)

If the known intercalating agent, ethidium, is present during ligation, a different pattern of ligation products is obtained. At lower concentrations of ethidium than those shown (i.e., 0.1 μg/mL), there is little intercalation and the double helix is only slightly unwound. When ethidium is removed after ligation and the DNA is transferred into electrophoresis buffer, intact circles, already slightly unwound, need to unwind less. As a result, the topoisomers are less positively supercoiled than those in lane 3 and run more slowly. As the concentration of ethidium is increased, its unwinding eventually balances that due to transfer between buffers and the topoisomers are centered around the mobility of the relaxed circle. At higher ethidium concentrations, intercalative unwinding becomes larger than the slight effects due to transfer between buffers. Then, DNA unwound by intercalation *rewinds* on transfer to electrophoresis buffer, inducing *negative* supercoils. These run rapidly as an unresolved group of topoisomers (lane 4). After ligation in 1 μg/mL ethidium, this group of topoisomers is even more negatively supercoiled (lane 5). At a high concentration of ethidium, 3 μg/mL, ligase is inhibited (lane 6; Montecucco et al., 1990). Intercalating agents are known to have these characteristic effects on DNA supercoiling (Wang et al., 1983).

This shift from positively supercoiled topoisomers through a relaxed group to negatively supercoiled forms is shown clearly by the monointercalator, **2**, as its concentration is increased. In lane 13, most topoisomers are fully relaxed and as the concentration is increased, they become more negatively supercoiled, migrating faster (lanes 14–19). Unlike ethidium, ligase is not inhibited at 10 μg/mL (lane 19) but is inhibited by higher concentrations (results not shown). The bifunctional compound, **9**, shows a similar pattern but begins to inhibit at 1.5 μg/mL (lane 10), with almost complete inhibition occurring at 3 μg/mL (lane 12). Between 1.5 and 3 μg/mL, some DNA has aggregated (presumably as catenanes) and cannot migrate far into the gel (lanes 10–12), providing circumstantial evidence for cross-linking. These results, and others for the remaining members of this series (Figure 3), show that all unwind like ethidium. Unfortunately, inhibition of the ligase precludes testing at higher concentrations.

The Phenanthrinium Series: Evidence for Knotting. A cross-linking agent would be expected to promote catenation and knotting by DNA ligase (Figure 2A). It is clear from Figure 1 that the pattern produced by the monointercalator, **2**, is broadly similar to that produced by the possible bisintercalator, **9**; therefore, the latter cannot be a powerful bisintercalator. However, close inspection of Figure 1, lane 11, shows there to be an extra faint band just below the linear molecule (i.e., form III), in the position characteristic of a relaxed knot with three nodes, the trefoil (Dean et al., 1985; Wasserman & Cozzarelli, 1986). This suggests that **9** might be acting as a weak cross-linking agent, even at these low concentrations.

Trefoils—as well as more complicated knots—are formed as the result of any ligation, but their concentration is usually too low to detect. Even if their concentration is artificially increased, their presence in gels is often obscured by the

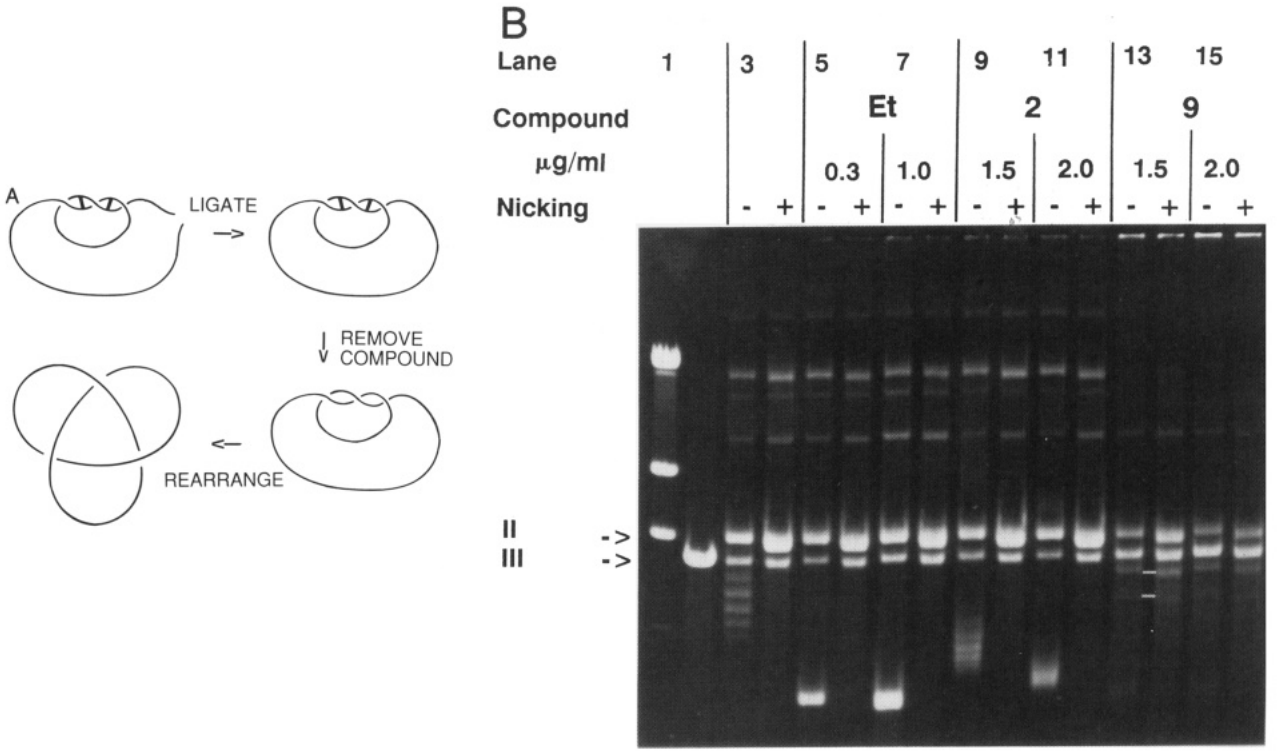


FIGURE 2: Diagram and gel showing that **9** knots DNA. (A) Cross-linking followed by ligation induces knotting. Two bisintercalating molecules (short thick lines) cross-link distant parts of one linear molecule (thin line). As binding sites are helically arranged, cross-linking will entwine the two duplexes so that they will wind around each other helically. After ligation and removal of the bisintercalators, the resulting structure can be rearranged to reveal a knot (trefoil) with three nodes. Increasing the number of windings induced by the cross-linking agent increases the number of nodes in the resulting knots. (B) Linear DNA was ligated (750 ng in 250 μL) in the presence of different concentrations of the compounds, the compounds were removed, some samples were nicked, and the ligation products were resolved electrophoretically before staining and photography. Lane 1: λ/*Hin*dIII markers. Lane 2: linear DNA. Lanes 3 and 4: linear DNA ligated in the absence of any test compound. Plus and minus indicate whether the samples are nicked or not. The positions of forms II and III are indicated on the left. White lines between lanes 13 and 14 indicate the position of knots.

presence of supercoiled topoisomers that have similar mobilities. Therefore, they are usually detected after the supercoiled forms are removed by nicking (Wasserman & Cozzarelli, 1986). Figure 2B illustrates such an experiment where the products formed by ligation have been nicked by γ-irradiation.

Linear DNA was ligated in the presence of ethidium, **2**, or **9**, and the products were analyzed as before. (Note that in this experiment the DNA concentration was increased 3-fold to allow visualization of minor species, and this inevitably leads to more intermolecular ligation.) Half of each sample was then irradiated with a dose of γ-rays sufficient to nick >99% of the circles. Ligation of linear DNA (lane 2) in the absence of any compound produced a set of positively supercoiled topoisomers (lane 3) which were relaxed by irradiation (lane 4). (The faint smear extending below the unit-length DNA is due to a continuous range of smaller linear fragments produced when two nicks occur opposite each other.) The presence of ethidium or **2** (odd-numbered lanes 5–11) during ligation led to rapidly migrating negatively supercoiled topoisomers; these, too, were relaxed by irradiation (even-numbered lanes 6–12). The pattern of the products generated by ligation in the presence of **9** (lanes 13 and 15) included bands (marked by white lines) below that of linear DNA that were out of register with the topoisomers in lane 3; this is characteristic of knots lacking supercoils and which have an increasing number of nodes (Wasserman & Cozzarelli, 1986). Ligation of nicked linear molecules produces such relaxed knots; ligation of intact linear molecules forms supercoiled trefoils and other knots with more nodes which migrate faster. Irradiation destroyed most rapidly migrating material but left these out-of-register bands, again characteristic of relaxed knots containing different numbers of nodes. As these out-of-register bands are undetectable in irradiated samples ligated in the presence of ethidium or **2**, we conclude that **9** is able to cross-link DNA to a small extent at the concentrations used here. Note also that some DNA has been ligated into a form unable to migrate far into the gel (lanes 13–16), again providing circumstantial evidence for cross-linking. Unfortunately, higher concentrations of **9** inhibit the ligase and so cannot be tested using this assay. The concentration of **9** generating knots depends on the DNA concentration (results not shown); this is to be expected as its molar concentration roughly equals the concentration of base pairs in the assay.

Results for the remaining members of the group are presented in Figure 3. Ligating the linear DNA in the presence of the phenanthridinium salt **4** or the bisphenanthridinium salt **6** yields rapidly migrating supercoiled circles (lanes 2 and 5). Note that the supercoiled forms generated with **6** (lane 5) migrate slightly faster than the corresponding forms generated with **4**, as expected for highly supercoiled knots. However, only the supercoiled forms produced by **6** when nicked give out-of-register bands (lane 6, white lines). These results show that all members of this group (i.e., **2**, **4**, **6**, and **9**) unwind, and so intercalate, but that only the bisphenanthridinium salts (i.e., **6** and **9**) knot, and so cross-link.

The Acridinium Series: Unwinding and Knotting. A similar series of experiments showed that the monofunctional members of the group based on acridine unwind DNA, but only the bisacridinium salt, **15**, knots. Thus, acridine orange and the monoacridinium salts **11** and **13** produced rapidly migrating supercoils (Figure 4), while the bisacridinium salt **15** produced a few even more rapidly migrating forms (Figure 4, lanes 15 and 16; Figure 3, lane 12), again characteristic of supercoiled

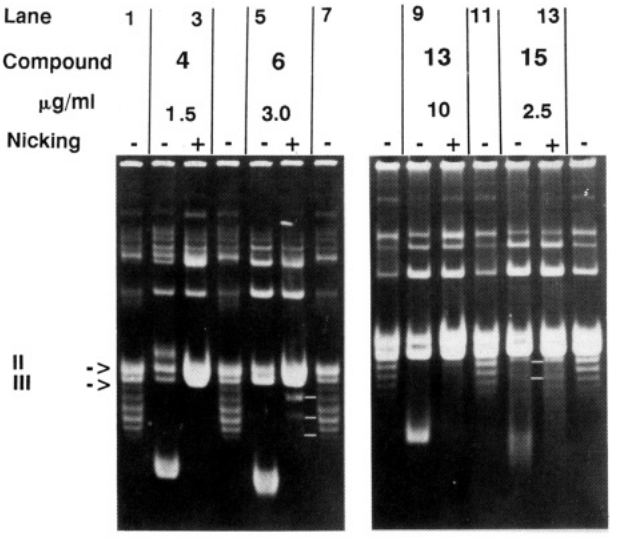


FIGURE 3: Gel showing that **6** and **15** knot DNA. Linear DNA was ligated (750 ng for lanes 1–7 and 1000 ng for lanes 8–14) in the presence of different concentrations of test compounds, the compounds were removed, some samples were nicked, and the ligation products were resolved electrophoretically before staining and photography. Lanes 1, 4, 7, 8, 11, and 14: markers provided by ligating linear DNA in the absence of any test compound. The positions of forms II and III are indicated on the left. The white lines between lanes 6 and 7 and between 12 and 13 indicate the position of knots.

knots. It also produced out-of-register bands (Figure 4, lane 15, white lines). The concentration range over which **15** yielded even these few supercoiled knots was narrow; ligase was inhibited at higher concentrations (Figure 4, lane 17), and as with other weak intercalators, the precise range critically depended on the DNA concentration (compare Figure 4, lane 19, with Figure 3, lane 12). This made the demonstration of intercalation and knotting difficult. Therefore, higher concentrations of DNA were used to confirm the presence of knots after nicking (Figure 3, lanes 8–14). This meant that trefoils obtained with the monoacridinium salt **13**, which were too low in concentration to be detected in Figure 4, lane 13, became visible after nicking (Figure 3, lane 10). However, no knots with more nodes could be seen. In contrast, bisacridinium salt **15** yielded a higher concentration of trefoils and knots with more nodes (i.e., the out-of-register bands in Figure 3, lanes 12 and 13, white lines). This confirms that all members of this series (i.e., **11**, **13**, and **15**) unwind, and so intercalate, but only the bisacridinium salt **15** increases the percentage of knots, and so cross-links.

Discussion

Assay for Intermolecular Bisintercalation. In order to screen compounds for intermolecular bisintercalating activity, we needed to develop a facile assay. Monointercalation is easily demonstrated using hydrodynamic assays, but bisintercalators form aggregates that complicate the analysis in the presence of the compound (Huang et al., 1983; Fox et al., 1988). Therefore, we allowed the test compound to interact with linear DNA and distort its structure, locked the structure by ligation into circles, and then removed the test compound; the resulting structures, which now carry a residual imprint of the test compound, are resolved by gel electrophoresis. As the electrophoretic behavior of unit-length circles and knots is known, ligation is conducted at a low DNA concentration which favors their formation. Higher concentrations yield complex catenanes which are difficult to analyze.

Cross-Linking by Bisintercalation. Our results show that all the phenanthridinium salts (i.e., **2**, **4**, **6**, and **9**) and acridinium salts (i.e., **11**, **13**, and **15**) unwind DNA (Figures 1–4). Ligating linear molecules in the presence of the bifunctional agents (i.e., **6**, **9**, and **15**)—but not the analogous monofunctional compounds—also increases the proportion of complex structures (perhaps catenanes) that do not migrate far into the gel. More significantly, only these molecules also increase the proportion of knots in the ligation mixture (Figures 2–4).

Evidence of unwinding, though circumstantial, is generally accepted as proof of intercalation (Waring, 1981; Waring & Fox, 1983). Therefore, our results provide strong evidence that

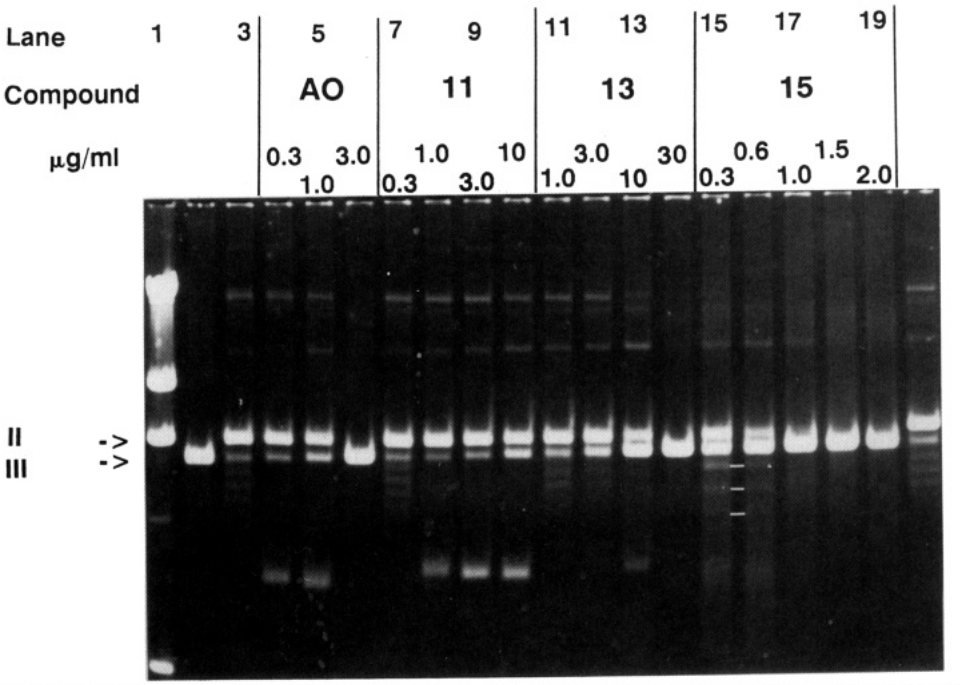


FIGURE 4: Gel showing a ligation assay where acridine orange (AO), **11**, **13**, and **15** are intercalating agents. Linear DNA (250 ng) was ligated in the presence of various concentrations of test compounds, the compounds were removed, and the ligation products were resolved electrophoretically before staining and photography. Lane 1: λ/*Hin*dIII markers. Lane 2: linear DNA. Lane 3: linear DNA ligated in the absence of any test compound. Lane 20: ligation in 2% dimethyl sulfoxide. Plus and minus indicate whether the samples are nicked or not. The positions of forms II and III are indicated. White lines between lanes 15 and 16 indicate the position of knots.

all these compounds are, at least, monointercalators. However, proof of bisintercalation requires the demonstration that both groups intercalate *simultaneously*. Such evidence is much more difficult to obtain. Formation of catenanes and knots by the bifunctional agents provides evidence only for cross-linking of some sort. It remains possible that they might do this nonintercalatively, for example, in the same way that a spermine molecule might link two duplexes. However, in view of our demonstration that the phenanthridinium and acridinium groups intercalate, it seems probable that the bifunctional molecules (i.e., **6**, **9**, and **15**) do bisintercalate simultaneously and so cross-link. Although knotting provides circumstantial evidence for cross-linking, formal proof of bisintercalation must await evidence from X-ray crystallography or NMR spectroscopy.

Although the novel compounds reported here were moderately effective intercalators when compared with ethidium (Figure 1) or acridine orange (Figure 4), they cross-link only weakly at the concentrations tested. Unfortunately, high concentrations of the compounds inhibited the ligase, preventing the formation of large catenanes. However, only weak cross-linking between two different DNA molecules is to be expected since the entropic factor involved in bringing them together must be considerable. This factor should be reduced if two parts of the same molecule are brought together, and this is probably the reason why knots in unit-length molecules are relatively abundant. These molecules should be even more effective cross-linkers where two duplexes are forced to lie together, for example, at replication forks, sites of topoisomerase action, or in sperm heads.

Appendix

NMR spectra were recorded on either a Varian Gemini 200 MHz, a Bruker WM300 MHz, or AM 500 MHz FT spectrometer all with internal reference. Mass spectral data were obtained in either EI or positive FAB mode on a VG Micromass 30FD or 16F spectrometer. All solvents were dried by distillation from suitable drying agents prior to use. Melting points are uncorrected.

5,6-Dimethylphenanthridinium Iodide (**2**). 6-Methylphenanthridine (**1**) (0.89 g, 4.6 mmol, white needles, mp 84 °C, prepared in 70% yield) (Morgan & Walls, 1931) was dissolved in nitrobenzene (1 mL); methyl iodide (1 mL) was added and the mixture was heated at 140 °C (oil bath temperature) for 5 h. After being cooled to room temperature the resulting crystalline product was collected and washed with copious amounts of benzene followed by diethyl ether to give yellow needles of 5,6-dimethylphenanthridinium iodide [1.3 g, 84%; mp 260 °C (decomp)]. Anal. Found (calcd for $C_{15}H_{14}NI$): C, 53.5 (53.7); H, 4.1 (4.2); N, 4.1 (4.2). ν_{max} (KBr disc): 3080 (aromatic C–H stretch), 3040 (aromatic C–H stretch), 1605 (C=C stretch), 1570 (aromatic C=C stretch). NMR [300 MHz, $(CD_3)_2SO$]: δ_H 3.44 (3 H, s, Me), 4.57 (3 H, s, Me-N^+), 8.02–8.11 (3 H, m, H-2, H-3, and H-8), 8.34 (1 H, m, H-9), 8.61 (1 H, d, J = 8 Hz, H-7), 8.90 (1 H, d, J = 8 Hz, H-4), 9.13 (2 H, d, J = 8 Hz, H-1 and H-10). m/z (+ve FAB): 208 (M^+ cation).

6-((p-Methylbenzylidene)methyl)phenanthridine (**3**). Benzoyl chloride (3.1 mL, 3.75 g, 0.026 mol) was added to a solution of 6-methylphenanthridine (5.0 g, 0.026 mol) in dry DMF (60 mL), and the mixture was stirred at ambient temperature for 0.5 h. *p*-Tolualdehyde (3.0 g, 0.026 mol) was added, and the mixture was refluxed for 4 h. After being cooled to room temperature, the DMF was removed under reduced pressure and the resulting residue was dissolved in ethyl acetate and put in the refrigerator to crystallize. The yellow crystals were recrystallized in ethyl acetate to give bright yellow crystals [2.34 g, 31%; mp 149–151 °C (decomp)]. Anal. Found (calcd for $C_{22}H_{17}N$): C, 89.6 (89.5); H, 5.8 (5.8); N, 4.7 (4.7). NMR (300 MHz, $CDCl_3$): δ_H 8.73 (1 H, d, H-1), 8.69 (1 H, d, H-10), 8.51 (1 H, d, H-4), 8.25 (1 H, d, H-7), 8.03 (1 H, d, J = 15 Hz, olefinic-H), 8.15 (1 H, d, J = 15 Hz, olefinic-H), 7.87–7.73 (4 H, m, H-2, H-3, H-8, and H-9), 7.64 (2 H, d, benzenoid-H), 7.25 (2 H, d, benzenoid-H), 2.37 (3 H, s, Me).

5-Methyl-6-((p-methylbenzylidene)methyl)phenanthridinium Iodide (**4**). Methyl iodide was added to a solution of 6-((*p*-methylbenzylidene)methyl)phenanthridine (0.2 g, 0.678 mmol) in nitrobenzene (30 mL), and the mixture was refluxed for 2 h. The reaction mixture was cooled to ambient temperature and poured into diethyl ether and cooled in a dry ice/acetone bath. The bright yellow solid was filtered and washed with copious amounts of ether and recrystallized from ethanol [0.25 g, 85%; mp 243–244 °C (decomp)]. Anal. Found (calcd for $C_{23}H_{20}IN$): C, 63.4 (63.2); H, 4.5 (4.6); N, 3.0 (3.2). NMR [300 MHz, $(CD_3)_2SO$]: δ_H 9.24 (1 H, d, H-1), 9.16 (1 H, d, H-10), 8.65 (1 H, m, H-4), 7.82–8.43 (6 H, m, H-7, H-8, H-9, H-2, H-3, and benzenoid-H), 7.64 (1 H, d, benzenoid-H), 7.38 (1 H, t, benzenoid-H), 7.19 (1 H, d, benzenoid-H), 6.96–6.89 (2 H, q, olefinic-H), 4.62 (3 H, s, Me-N^+), 2.14 (3 H, s, Me).

1,4-Bis(phenanthridin-6-ylvinyl)benzene (**5**). 6-Methylphenanthridine (5 g, 0.026 mol) was dissolved in dry DMF (60 mL). Benzoyl chloride (3.1 mL, 3.75 g, 0.026 mol) was added, and the mixture was stirred at room temperature for 0.5 h. Terephthaldicarboxaldehyde (1.74 g, 0.013 mol) was then added, and the mixture was heated under reflux for 5 h. After being cooled to room temperature, the DMF solution was poured into excess hydrochloric acid and steam distilled until 1200 mL of distillate was collected. The acidic residue was made alkaline by the addition of 0.88 g/mL ammonia, and the yellow precipitate was collected and recrystallized from chlorobenzene (200 mL) to give **5** as a mat of fine bright yellow, feathery needles [4.73 g, 75%; mp >300 °C]. Anal. Found (calcd for $C_{35}H_{24}N_2$): C, 89.3 (89.2); H, 5.0 (5.0); N, 5.8 (5.8). m/z (EI): 484 (M^+, 100%). Due to the lack of solubility of the compound, it proved impossible to obtain an NMR spectrum. It was N-methylated without further purification.

1,4-Bis((N-methylphenanthridinium-6-yl)vinyl)benzene Diiodide (**6**). (a) 1,4-Bis(phenanthridin-6-ylvinyl)benzene (148 mg, 3.1 mmol) was dissolved in hot nitrobenzene (30 mL). Methyl iodide (2 mL) was cautiously added, and the mixture was heated at 140 °C (oil bath temperature) for 4 h. After being cooled to room temperature, the resulting crystals were collected and washed with diethyl ether. Proton NMR of this crystalline solid shows it to be the desired product; however, it contains one molecule of nitrobenzene as solvent of crystallization per molecule of **6**. Treating the solid with boiling ethanol removes the nitrobenzene to give a pure sample [185 mg, 79%; mp >300 °C]. Anal. Found (calcd for $C_{38}H_{30}N_2I_2$): C, 59.5 (59.4); H, 3.9 (3.9); N, 3.5 (3.6); NMR [300 MHz, $(CD_3)_2SO$]: δ_H 4.67 (6 H, s, Me-N^+), 7.5 (2 H, d, J = 17 Hz, vinylic-H), 8.07–8.21 (12 H, m, H-2, H-2′, H-3, H-3′, H-8, H-8′, benzene-H, and vinylic-H), 8.41 (2 H, m, H-9 and H-9′), 8.68 (4 H, m, H-4, H-4′, H-7, and H-7′), 9.22 (4 H, d, J = 8 Hz, H-1, H-1′, H-10, and H-10′). m/z (+ve FAB): 514 (M^+ reduced cation).

(b) 5,6-Dimethylphenanthridinium iodide (0.113 g, 0.337 mmol) was dissolved in hot ethanol (30 mL), and terephthaldicarboxaldehyde (22 mg, 0.168 mmol) was added

followed by piperidine (0.5 mL). The mixture was heated under reflux for 2 h, and then half the solvent was removed by distillation. On cooling to room temperature, the desired product **6** precipitated as a dark red powder which was collected and dried (50 mg, 39%). Analysis showed this material to be identical to that synthesized in (a).

4-(Phenanthridin-6-ylvinyl)-1-benzenecarboxaldehyde (**7**). 6-Methylphenanthridine (1.17 g, 6 mmol) was dissolved in dry DMF (20 mL). Benzoyl chloride (1 mL) was added, and the solution was stirred at room temperature for 30 min during which time a precipitate formed. Terephthaldicarboxaldehyde (0.811 g, 6 mmol) was then added, and the mixture was heated under reflux for 5 h. After being cooled to room temperature, the DMF solution was added dropwise to water (400 mL) and the resulting yellow precipitate was collected by filtration and recrystallized from ethanol to give the aldehyde **7** [1.2 g, 65%; mp >250 °C]. NMR (300 MHz, $CDCl_3$): δ_H 7.68 (1 H, m, H-8), 7.74 (1 H, m, H-3), 7.76 (1 H, m, H-2), 7.87 (1 H, m, H-9), 7.87 (4 H, AA'BB', benzene ring-H), 8.09 (1 H, d, J = 16 Hz, vinyl-H), 8.17 (1 H, d, J = 16 Hz, vinyl-H), 8.22 (1 H, d, J = 8 Hz, H-7), 8.44 (1 H, d, J = 8 Hz, H-4), 8.65 (1 H, d, J = 8 Hz, H-10), 8.67 (1 H, d, J = 8 Hz, H-1), 10.04 (1 H, s, aldehyde-H). m/z (EI): 309 (M^+ 100%).

1,4-Bis((p-((N-methylphenanthridinium-6-yl)vinyl)-phenyl)vinyl)benzene (**9**). Sodium ethoxide (0.374 g, 5.5 mmol, as a 21% w/v solution in ethanol) was added slowly to a solution of 4-(phenanthridin-6-ylvinyl)-1-benzenecarboxaldehyde (**7**) (1.7 g, 5.5 mmol) and *p*-xylylenebis(triphenylphosphonium bromide) (2.17 g, 2.75 mmol; Aldrich Chemical Co., Ltd.) in dry DMF (50 mL) at room temperature. The red coloration generated on addition of sodium ethoxide solution was allowed to dissipate before the addition of a further drop of the ethoxide solution. After addition was complete, the mixture was stirred at room temperature for 3 h and poured into water (500 mL) and the resulting solid was collected and washed with copious amounts of hot methanol. The resulting solid was recrystallized from chlorobenzene to give **8** [0.62 g, 33%; mp >250 °C]. m/z (EI): 688 (M^+).

Methyl iodide (3 mL) was added cautiously to a solution of **8** (0.3 g, 0.44 mmol) in nitrobenzene (10 mL) at 140 °C. The mixture was heated with stirring at 140 °C (oil bath temperature) for 5 h. After being cooled to room temperature, the mixture was added dropwise to diethyl ether (500 mL), and the red precipitate was collected by filtration. The product was recrystallized from methanol (100 mL) to yield the bisphenanthridinium salt **9** [0.3 g, 69%; mp >250 °C]. NMR [300 MHz, $(CD_3)_2SO$]: δ_H 4.65 (6 H, s, Me-N^+), 7.42 (2 H, d, J = 14 Hz, vinyl-H), 7.47 (4 H, s, central benzene ring), 7.72 (4 H, m, vinyl-H), 7.85 (8 H, AA'BB', benzene ring-H), 8.03–8.25 (8 H, m, H-2, H-2', H-3, H-3', H-8, H-8', and vinyl-H), 8.38 (2 H, m, H-9, H-9'), 8.64 (2 H, d, J = 8 Hz, H-7 and H-7'), 8.70 (2 H, d, J = 8 Hz, H-4 and H-4'), 9.19 (4 H, d, J = 8 Hz, H-1, H-1', H-10, and H-10'). m/z (EI): 359 (M^{2+}).

9,10-Dimethylacridinium Iodide (**11**). 9-Methylacridine (**10**) (3.7 g, 19 mmol), a yellow crystalline solid (mp 117–118 °C) prepared in 66% yield (Tsugo et al., 1963), and methyl iodide (3.7 mL) were heated together under reflux for 48 h. After being cooled to room temperature, the mixture was added dropwise to diethyl ether (200 mL), and the resulting precipitate was collected by filtration and washed with boiling diethyl ether (2 × 400 mL). The solid was recrystallized from water to give the desired product [4.6 g, 72%; mp 245 °C (decomp)]. Anal. Found (calcd for $C_{15}H_{14}NI$): C, 53.4 (53.7); H, 4.2 (4.2); N, 4.0 (4.2). NMR [200 MHz, $(CD_3)_2SO$]: δ_H 3.5 (3 H, s, Me), 4.8 (3 H, s, Me-N^+), 8.05 (2 H, m, H-2 and H-7), 8.4 (2 H, m, H-3 and H-6), 8.75 (2 H, d, J = 8.7 Hz, H-4 and H-5), 8.95 (2 H, d, J = 8.7 Hz, H-1 and H-8). m/z (+ve FAB): 208 (M^+).

9-((p-Methylbenzylidene)methyl)acridine (**12**). Benzoyl chloride (1.5 g, 1.2 mL, 10.5 mmol) was added to 9-methylacridine (5.2 g, 10.5 mmol) dissolved in dry DMF (25 mL). The mixture was stirred at ambient temperature for 20 min. *p*-Tolualdehyde (1.26 g, 10.5 mmol) was added, and the reaction mixture was refluxed for 5 h. After being cooled to room temperature, the solvent was removed under vacuum and dilute ammonia solution was added to neutrality. The organic material was extracted with ethyl acetate, and the extract was washed with brine and sodium bicarbonate solution and dried ($MgSO_4$). The ethyl acetate was removed to give a brown oil which was column chromatographed (silica, 3:1 hexane/ethyl acetate). The second fraction was collected and crystallized from hexane/ethyl acetate to give yellow crystals of **12** [1.89 g, 25%; mp 167–169 °C (decomp)]. Anal. Found (calcd for $C_{22}H_{17}N$): C, 89.4 (89.5); H, 5.7 (5.7); N, 5.0 (4.8). NMR (300 MHz, $CDCl_3$): δ_H 8.35 (2 H, d, H-1 and H-8), 8.27 (2 H, d, H-4 and H-5), 7.81 (2 H, m, H-3 and H-6), 7.61 (2 H, d, benzenoid-H), 7.55 (2 H, m, H-2 and H-7), 7.29 (2 H, d, benzenoid-H), 7.05 (1 H, d, olefinic-H), 6.99 (1 H, d, olefinic-H), 2.45 (3 H, s, Me).

10-Methyl-9-((p-methylbenzylidene)methyl)acridinium Iodide (**13**). 9-((*p*-Methylbenzylidene)methyl)acridine (**12**) (0.2 g) was dissolved in excess nitrobenzene (20 mL), methyl iodide (10 mL) was added, and the mixture was refluxed for 2 h. The reaction mixture was cooled and poured into ether cooled in a dry ice/acetone bath, and the deep maroon solid was filtered and washed with copious amounts of ether. The solid was recrystallized from ethanol [0.24 g, 82%; mp 260–263 °C]. Anal. Found (calcd for $C_{23}H_{20}IN$): C, 67.7 (67.7); H, 4.7 (4.6); N, 2.9 (3.2). NMR [300 MHz, $(CD_3)_2SO$]: δ_H 8.80 (2 H, d, H-1 and H-8), 8.55 (2 H, d, H-4 and H-5), 8.43 (2 H, t, H-2 and H-7), 7.89–7.94 (2 H, t, H-3 and H6), 7.43–7.55 (2 H, q, olefinic-H), 6.71–6.84 (4 H, m, benzenoid-H), 4.88 (3 H, s, Me-N^+), 2.15 (3 H, s, Me).

1,4-Bis(acridin-9-ylvinyl)benzene (**14**). 9-Methylacridine (**10**) (5.2 g, 10.5 mmol) was dissolved in dry DMF (25 mL), benzoyl chloride (1.5 g, 1.2 mL, 10.5 mmol) was then added, and the mixture was stirred at room temperature for 20 min. Terephthaldicarboxaldehyde (0.7 g, 5.25 mmol) was added, and the reaction mixture was heated under reflux for 5 h. After being cooled to room temperature, the solvent was removed by evaporation under reduced pressure, hydrochloric acid was added to the residue, and the solvent was evaporated to dryness. The gum was triturated with dilute ammonia, and the resulting solid was collected and washed with more dilute ammonia and then water. The crude product was recrystallized from chlorobenzene to give **14** as an orange solid [0.7 g, 28%; mp >300 °C]. Anal. Found (calcd for $C_{36}H_{24}N_2$): C, 89.2 (89.2); H, 5.2 (5.0); N, 5.3 (5.7). NMR (300 MHz, $CDCl_3$) δ_H 7.12 (2 H, d, J = 17 Hz, vinylic-H), 7.57 (4 H, m, H-2, H-2', H-7, and H-7'), 7.8 (4 H, d, J = 9 Hz, H-3, H-3', H-6, and H-6'), 8.0 (2 H, d, J = 17 Hz, vinylic-H), 8.28 (4 H, d, J = 9 Hz, H-4, H-4', H-5, and H-5'). m/z (DCI NH_3): 485 (M^+ + 1).

1,4-Bis((N-methylacridinium-9-yl)vinyl)benzene Diiodide (**15**). Methyl iodide (2 mL) was added cautiously to a hot solution of **14** (18 mg) in nitrobenzene (1.5 mL). The solution was heated at 140 °C (oil bath temperature) for 6 h. After being cooled to room temperature, the mixture was added dropwise to dry benzene and the resulting precipitate was

collected, washed with benzene and diethyl ether, and dried ($MgSO_4$). The bisacridinium salt **15** was obtained as a dark red powder [21.2 mg, 74%; mp >250 °C]. Anal. Found (calcd for $C_{38}H_{30}N_2I_2$): C, 59.2 (59.4); H, 3.9 (3.9); N, 3.8 (3.6). NMR [300 MHz, $(CD_3)_2SO$]: δ_H 4.84 (6 H, s, Me-N^+), 7.35 (2 H, d, J = 17 Hz, vinylic-H), 8.03 (4 H, m, H-2, H-2′, H-7, and H-7′), 8.18 (4 H, s, benzene-H), 8.46 (4 H, m, H-3, H-3′, H-6, and H-6′), 8.75 (2 H, d, J = 17 Hz, vinylic-H), 8.79 (4 H, d, J = 8.8 Hz, H-4, H-4′, H-5, and H-5′), 8.89 (4 H, d, J = 8.8 Hz, H-1, H-1′, H-8, and H-8′). m/z (+ve FAB): 514 (M^+ reduced cation), 499 (M^+-Me).

Registry No. **1**, 3955-65-5; **2**, 16511-48-1; **3**, 137868-53-2; **4**, 137868-54-3; **5**, 137868-55-4; **6**, 137868-56-5; **7**, 137868-57-6; **8**, 137868-58-7; **9**, 137868-63-4; **10**, 611-64-3; **11**, 951-00-8; **12**, 137868-59-8; **13**, 137868-60-1; **14**, 137868-61-2; **15**, 137868-62-3; terephthaldicarboxaldehyde, 623-27-8.

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