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Evidence for cross-linking DNA by bis-intercalators with rigid and extended linkers is provided by knotting and catenation

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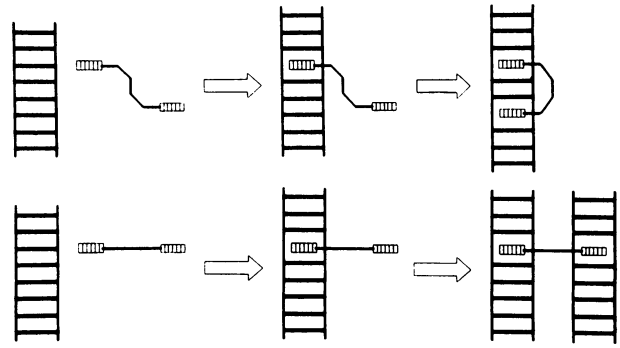
ABSTRACT

A new series of DNA bis-intercalators is reported in which acridine moieties are connected by rigid and extended pyridine-based linkers of varied length. Cross-linking of DNA by bis-intercalation is inferred from the unwinding and folding of linear DNA induced by the compounds; after ligation and removal of the bis-intercalator, superhelical circles, catenanes and knots that bear a residual imprint of the bis-intercalator are observed. These novel bis-intercalators are of interest because they can be used to probe the spatial organization of DNA, especially near sites of replication, recombination or topoisomerase action where two duplexes must be in close proximity. Preliminary results on the effects of the various compounds on the cloning efficiency of bacteria and replication by permeabilized human cells are also presented.

INTRODUCTION

Although a large number of DNA bis-intercalators are known,[1,2,3,4] the two intercalating moieties usually bind to the same DNA duplex because they can rotate freely about the connecting linker. Binding of one intercalating moiety inevitably leaves the other in close proximity to other binding sites in the same duplex, leading to intramolecular cross-linking (Scheme 1, upper). However, if the linker is rigid with an extended configuration, binding of both intercalators into the same duplex will be impossible, unless the duplex is long enough to fold back on itself. Therefore, bis-intercalators with rigid and extended linkers should cross-link DNA duplexes, forming intermolecular links (Scheme 1, lower). Previously, we made two series of such bis-intercalators with rigid, extended and cleavable linkers connecting the two intercalating groups, based on the phenanthridinium and acridinium ions respectively.[5] Although these compounds were all intercalating agents, they were only weak cross-linking agents: they were also not very water soluble. We now report a new series of compounds based on acridine as the intercalator which have improved cross-linking power and solubility. The rigid linkers terminate in pyridine at each end and are cleavable. Such molecules are of interest because they can be used to probe the spatial organization of DNA, especially near sites of replication, recombination or topoisomerase action where two duplexes must be in close proximity. We also present preliminary results on the effects of these compounds on the cloning efficiency of bacteria and on replication by permeabilized human cells.

The detection of intermolecular bis-intercalation poses a special problem. Hydrodynamic methods (including electrophoretic methods) generally provide strong circumstantial evidence for intercalation,[6,7],8 before formal proof is obtained using X-ray crystallography or n.m.r. spectroscopy.[9,10,11] However, bis-intercalators that cross-link different DNA molecules affect the hydrodynamic properties of DNA in ways that complicate analysis. After mono-intercalation, the second intercalating group protrudes from DNA so that mobility depends on the number



Scheme 1. Both intercalating moieties of bis-intercalators with flexible linkers generally bind to the same DNA molecule, because binding of one leaves the other close to other binding sites in the same duplex (upper). But if the linker connecting the two moieties is rigid and points the intercalating groups in opposite directions, binding to different duplexes is favoured (lower); binding to the same duplex is impossible, unless the duplex is long enough to fold back on itself.

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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 bis-intercalators is difficult.[5,12,13]

We have developed an indirect, but decisive, assay for unwinding (and so intercalation) and knotting (and so cross-linking).[5] 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 Cozzarelli have reviewed the structure of knots and catenanes.[14]] 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, but we deliberately use a low DNA concentration to minimize their formation. Cross-linking distant parts of the same molecule stimulates intertwining of the two parts so that subsequent circularisation produces a knot. The various different structures (i.e. supercoils, catenanes, knots, etc.) are resolved by gel electrophoresis,[14,15] **after removal** of the test compound.

This assay has an important advantage. Intermolecular bis-intercalators can be expected to be only weak cross-linkers as the entropic factor involved in bringing two DNA molecules together, or the ends of a linear duplex, is large. The bis-intercalators we have reported are indeed relatively weak cross-linking agents and not very soluble in aqueous buffers, which precluded testing them at high concentrations.[5] 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 DNA ligase.[16] Using this assay we have demonstrated that a new series of bis-intercalators have improved cross-linking ability.

MATERIALS AND METHODS

NMR spectra were recorded on a Varian Gemini 200 MHz 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.

9-Acridine-pyridinium chloride, *AP* (2)

9-Chloroacridine (2.0g, 9.36mmol) was dissolved in dry pyridine (20ml) and the solution heated, with stirring, at 100°C for 0.5h. The solution was allowed to cool to about 80°C, benzene (20ml) was added to the solution and the product crystallised out on cooling to room temperature. The product was collected by filtration and washed with benzene and finally diethyl ether to remove traces of any starting materials. No further purification was necessary (2.76g, 99%), m.p. 152−153°C (Lit.[17] 152−154°C) d_H (200 MHz, d_6-DMSO) 7.5 (d J 9Hz, 2H, 4- and 5-H), 7.75 (m, 2H, 3- and 6-H), 8.05 (m, 2H, 2- and 7-H), 8.4 (d J 9 Hz, 2H, 1- and 8-H), 8.6 (m, 2H, 3'- and 5'-H), 9.1 (m, lH, 4'H) and 9.55 (d J 5.5 Hz, 2H, 2'- and 6'-H); m/z (+ve FAB) 257 (positive ion, M+).

1,4-bis(4-vinylpyridyl)benzene (PEBEP) (3)

Benzoyl chloride (6g, 43mmol) was added slowly to a solution of 4-methylpyridine (4g, 0.043mol) in dimethylformamide (50ml) at room temperature. After stirring for 0.5h at room temperature the solution was heated at 50°C for 0.5h, a solution of terephthaldicarboxaldehyde (2.88g, 21.5mmol) in DMF (20ml) was then added and the reaction mixture heated under reflux for 12h. The resulting solution was cooled to room temperature and poured into water (300ml) which was rendered basic by addition of 0.88 ammonia. The crude product was collected by filtration and dissolved in hydrochloric acid (2M, 200ml). Impurities were removed by filtration and the acidic solution neutralised by addition of 0.88 ammonia. The resulting precipitate was collected and recrystallised from toluene to give 1,4-bis(4-vinylpyridyl) benzene (3.96g, 65%) m.p.272−273°C; (Found: C,83.6; H, 5.7; N, 9.4. $C_{20}H_{16}N_2$ requires C, 84.2; H, 5.6; N, 9.8%); δ_H (d_6 DMSO, 200MHz) 7.27 (lH, J 16.5Hz, vinylic-H), 7.52 (lH, J 16.5Hz, vinylic-H), 7.41 and 8.56 (8H, A_2B_2 J 20.1 Hz, pyridinyl-H), 7.7 (4H, s, benzenoid-H); m/z (EI) 284 (M^+).

N,N'-Bis(9-acridine)-4,4'-(vinyl-p-phenylene-vinyl)dipyridinium dichloride, *APEBEPA* (4)

9-Chloroacridine (0.230g, 1.1 mmol) was dissolved in nitrobenzene (5ml) and the solution was heated to 130°C. A solution of 1,4-bis(4-vinylpyridyl)benzene (0.153g, 0.53mmol) was added and the mixture heated at 140°C for 3h. After cooling to room temperature the nitrobenzene solution was added dropwise to benzene (100ml) and the solid precipitate collected by filtration. The crude material was purified by recrystallisation from ethanol to give the desired product as its tetrahydrate (0.23g, 55%), m.p. >250°C; (Found: C,70.8; H,4.7; N,7.3. $C_{46}H_{32}N_4Cl_2.4H_2O$ requires C, 70.8, H, 5.1; N, 7.2%); δ_H(200 MHz d_6 DMSO) 7.63 (d J 8.5 Hz, 4H, 4-,5-,4'-,5'-H) 7.81 (m, 4H, 3-,6-,3'-,6'-H), 7.69 and 8.16 (AB J 16Hz,4H,vinylic-H), 8.05 (m, 8H, 2-,7-,2'-,7'-and phenylene-H), 8.44 (d J 9.2Hz, 4H, 1-,8-,1'-,8'-H), 8.70 and 9.39 (A_2B_2, J 14.0Hz, 4H pyridinyl H).

N,N'-Bis(9-acridine)-4,4'-dipyridinium dichloride, *APPA* (5)

9-Chloroacridine (0.214 g, 1.0 mmole) and 4,4'-dipyridyl (0.078 g, 0.50 mmole) were intimately mixed before heating in an oil bath at 120°C until the mixture melted and then solidified. The temperature of the oil bath was then raised to 150°C for 20 min. After the reaction mixture had cooled it was dissolved in hot ethanol and the solution kept at 0°C. The crystals were filtered off and recrystallised from ethanol to give an orange powder (0.22g, 68%), (Found: C, 66.9; H, 4.7; N, 8.5. $C_{36}H_{24}Cl_2N_4.3.5H_2O$ requires C, 66.9; H, 4.7; N, 8.8%), δ_H (200MHz, d_6-DMSO) 10.00 (d, 4H), 9.49 (d,4H), 8.50 (d, 4H), 8.13 (t, 4H), 7.90 (t,4H), 7.70 (d, 4H).

N,N'-Bis(9-acridine)-4,4'-trans-vinylidene-dipyridinium dichloride, *APEPA* (6)

9-Chloroacridine (0.214 g, 1.0 mmole) and 4,4'-*trans*-vinylidene-dipyridine (0.091 g, 0.50 mmole) were intimately mixed before heating in an oil bath at 120°C until the mixture melted and then solidified. The temperature of the oil bath was then raised to 150°C for 30 min. After the reaction mixture had cooled it was dissolved in hot ethanol and the solution kept at 0°C. The crystals were filtered off and recrystallised from ethanol to give a red-brown powder (0.15g, 46%), (Found: C, 70.4; H, 4.9; N, 8.9. $C_{38}H_{26}Cl_2N_4.2H_2O$ requires C, 70.7; H, 4.7; N, 8.7%), δ_H (200MHz, d_6-DMSO) 9.64 (d, 4H), 8.95 (d, 4H), 8.80 (s, 2H), 8.42 (d, 4H), 8.07 (t, 4H), 7.81 (t, 4H), 7.69 (d, 4H).

The ligation assay

In our earlier study, the relatively insoluble compounds were dissolved in dimethylsulphoxide as stock concentrates.[5] However, we found that dissolution of **APEBEPA** (4), **APPA** (5) and **APEPA** (6) in dimethylsulphoxide cleaves the linker, generating the acridone. The rate of cleavage is, however, markedly different. **APPA** (5) is cleaved to the mono-intercalator **APP** (7) and acridone in only 3 hours at ambient temperature, whereas **APEBEPA** (4) only decomposes to the extent of about 5% after 3 days; (6) has intermediate stability. There was no effect on the mono-acridine derivative **AP** (2). Therefore we routinely dissolved (5) and (6) in water and (4) in ethanol immediately before use. When **APEBEPA** (4) was dissolved in dimethylsulphoxide or ethanol, control experiments showed that the solvent had no effect on the mobility of the ligated products at the concentrations used. The highest concentration of ethanol used during ligation (2%) was much lower than the minimum concentration known to induce effects on supercoiling.[18]

pSVtkneo, a 5.3kbp plasmid,[19] was linearized with *Hind*III (2 units/μg DNA; lh; 37°C) ethanol precipitated and redissolved. Ligation solutions generally contained 250−750ng linear DNA in lmM ATP, 10mM $MgCl_2$, 50mM Tris-HCl (pH7.5), 1 mM dithiothreitol, 1 unit T4 DNA ligase (Boehringer) and various concentrations of test compound in a final volume of 250 μl. After incubation on ice overnight, the ligated DNA was ethanol precipitated to remove the test compound, redissolved in a sample buffer containing 1% sodium dodecyl sulphate and then subjected to electrophoresis in a 0.8% agarose gel containing 40mM Tris, 2mM EDTA and 20mM sodium acetate (pH 8.3) at 4°C, and the gel stained with ethidium and photographed.[15,20] In some cases samples were gamma-irradiated (1180J/kg)[21] in the sample buffer used for electrophoresis; control experiments showed that this dose nicked >99% of the supercoiled molecules.

Electrophoretic mobilities

Lambda/*Hind*III fragments or supercoiled Bluescript plasmid DNA (pBS) were run (lh; 6v/cm) in a 0.8% agarose 'mini-gel' in TEB buffer;[20] in some cases both gel and electrophoresis buffer were supplemented with the test compound. Prior to staining with ethidium and photography as above, gels were soaked in distilled water for two days to remove any added compound that quenched fluorescence.

Effects on cloning efficiency of bacteria

Bacterial toxicity was tested by growing *Echerichia coli* strain DH5 to an optical density at 600nm of 0.5−0.6 in L broth. Aliquots (0.5ml) were incubated (30 or 60 min; 37°C) in the presence or absence of various compounds, diluted and plated on L plates and grown overnight at 33°C.[20] The number of colonies surviving treatment (averages of at least 2 experiments) were expressed as a fraction relative to controls treated with an equivalent concentration of solvent.

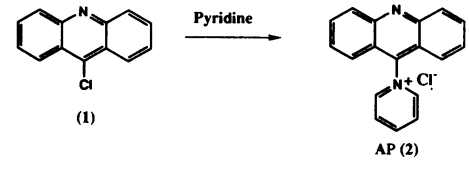
Effects on replication in human cells

Initial rates of replication by permeabilized HeLa cells in the presence of various compounds were measured using a slight modification of published procedures.[22,23] Unsynchronized cells were prelabelled by growing them overnight in [^{3}H]thymidine; this uniformly labels the DNA and allows corrections to be made for slight variations in recoveries. The cells were then encapsulated ($5-10\times10^7$/ml) in agarose microbeads, lysed using Triton in a 'physiological' buffer (pH 7.4) and washed. Rates of incorporation of [α-^{32}P]TTP into acid-insoluble material were then measured over a 15min. period; 500ml samples of microbeads (in an equal volume of the 'physiological' buffer) were pre-incubated (5min; 0°C) with each of the test compounds (10 μg/ml) and then for 5min. at 37°C, before the reaction was started by the addition of a 10×concentrated solution of 'triphosphates'. Final concentrations of constituents in the reaction mixture were (in addition to those in the buffer): 100 μM CTP, GTP, UTP; 250 μM dATP, dCTP, dGTP; 15−20 μCi/ml [α-^{32}P]TTP (3000Ci/mmol); 5mM potassium phosphate (pH 7.4); 5mM $MgCl_2$. The low TTP concentration (i.e. nM) used to conserve label leads to inefficient DNA synthesis, which is ~1/1000 that found at the optimum. Initial rates of replication were measured between 15s. and 5min. and expressed as a ratio relative to the rate in the absence of the test compound. The averages of at least two experiments with each compound are shown. Controls showed that solvents (dimethylsulphoxide or ethanol) had no effect on the replication rate at the concentrations used.

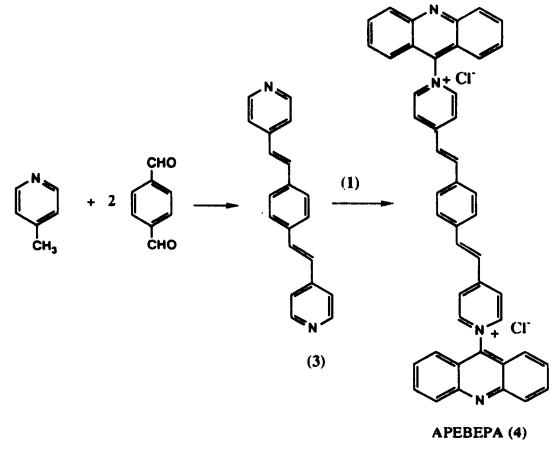
RESULTS

Synthesis and stability of the mono- and bis-intercalators

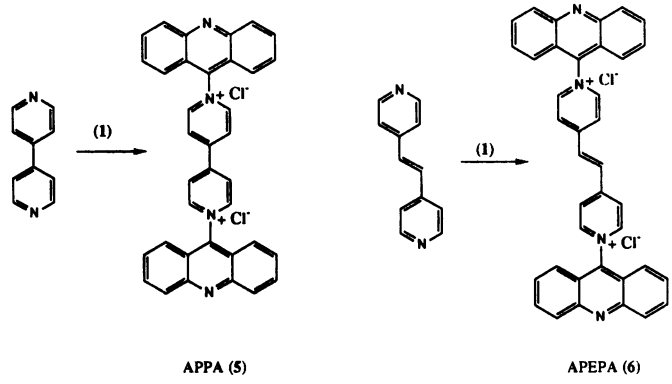
The mono-intercalator, **AP** (2) was readily prepared from 9-chloro-acridine by reaction with pyridine as outlined in Scheme 2. 1,4-Bis(4-vinylpyridyl)-benzene (3) was prepared from 4-picoline and two equivalents of terephthaldicarboxaldehyde in the presence of benzoyl chloride. On reaction with 9-chloro-acridine, the bis-intercalator **APEBEPA** (4) was obtained (Scheme 3). **APPA** (5) and **APEPA** (6) were prepared similarly by reacting 9-chloro-acridine with 4,4′-dipyridyl and 4,4′-*trans*-vinylidene-



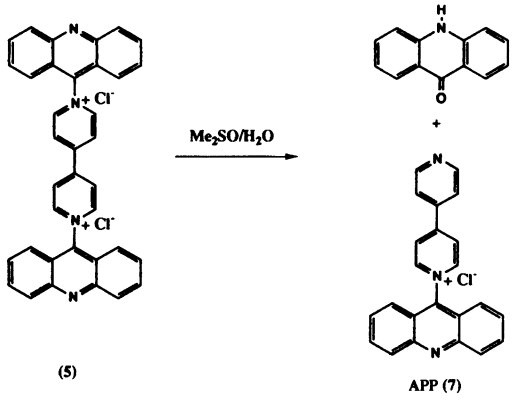
Scheme 2. Synthesis of AP (2).



Scheme 3. Synthesis of APEBEPA (4)



Scheme 4. Synthesis of APPA (5) and APEPA (6).



Scheme 5. The rapid break down of APPA (5) in dimethyl sulphoxide solution.

dipyridine respectively (Scheme 4). It is possible to obtain the corresponding mono-intercalators by using less forcing conditions. For example, APP (7) can be prepared in excellent yield by reacting 9-chloro-acridine with 4,4'-dipyridyl at a lower temperature. Clearly introducing the second intercalating group is more difficult than the first, especially in the case of APPA (5) where the electrostatic repulsion must be considerable. In accord with this, APPA (5) when dissolved in dimethylsulphoxide (which presumably contained traces of water) decomposed to APP (7) and acridone completely in about 3 hours at ambient temperature. APEPA (6) decomposed more slowly under these conditions taking several days to reach completion, whereas APEBEPA (4) decomposed only about 5% in 3 days under the same conditions. AP (2) was perfectly stable in dimethylsulphoxide solution. All the compounds had sufficient solubility in water, except APEBEPA (4) which was stable enough in aqueous dimethylsulphoxide or aqueous ethanol for the biochemical experiments. APPA (5) was considerably more stable in aqueous solution than in dimethylsulphoxide.

Intercalation

Fig. 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 compound into a complicated set of products (lane 3). At the low DNA concentration used, the majority of these are circles. As some linear molecules originally contained nicks, some of the resulting circles are nicked and run just behind the linear molecules. Such relaxed form II circles constitute a

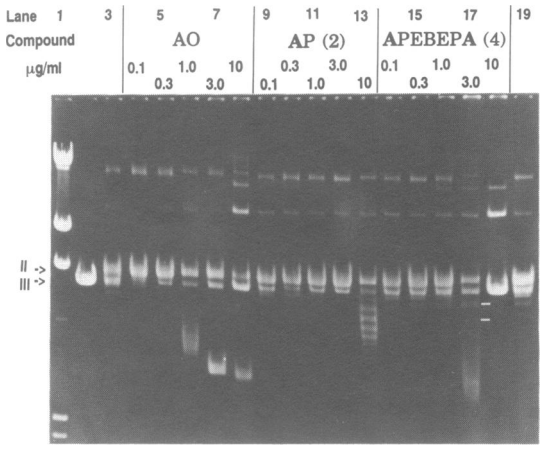


Fig. 1. Acridine orange (AO), AP (2) and APEBEPA (4) are intercalating agents. Linear DNA (500ng) was ligated in the presence of different concentrations of the compounds, the compounds removed and the ligation products resolved electrophoretically before staining and photographing the resulting gel. Lane 1: lambda/*Hind*III markers. Lane 2: unligated DNA. Lane 3: linear DNA ligated in the absence of any test compound. Lane 19: ligation in 0.1% dimethylsulphoxide, the maximum concentration of organic solvent present during ligation. The positions of forms II and III are indicated. White lines to the right of lane 17 indicate the positions of simple knots lacking supercoils.

background present whenever the ligase is active. However, some of the circles contain no nicks and run as a number of faint bands slightly ahead of the nicked circles. These are topoisomers containing increasing numbers of positive supercoils generated after ligation by the duplex unwinding on transfer of DNA from the ligation buffer (containing a high Mg^{2+} concentration) into the electrophoresis buffer.[24] 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 usually ligated at a low concentration to minimize their formation.]

If the intercalating agent, acridine orange (AO), is present during ligation, a different pattern of ligation products is obtained. At 0.1 μg/ml (lane 4), there is little intercalation and the double helix is only slightly unwound. When the dye is removed after ligation and DNA transferred into electrophoresis buffer, the positive supercoiling due to the buffer change is offset by negative supercoiling induced by removing the intercalator. As a result, the topoisomers are less positively supercoiled than those in lane 3 and run slightly more slowly. As the concentration of the acridine orange is increased, the negative supercoiling induced by removal of the intercalator eventually balances the positive supercoiling due to transfer between buffers and the topoisomers are centred around the mobility of the relaxed circle (lane 5). At higher concentrations, the effect of the intercalator 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 a group of topoisomers (lane 6). After ligation in 3 μg/ml acridine orange, this group of topoisomers is even more negatively supercoiled and are not resolved under these conditions (lane 7). At 10 μg/ml, ligase is partly inhibited, resulting in a stronger band due to form III molecules (lane 8). Intercalating agents are known to have these characteristic effects on DNA supercoiling.[5,25]

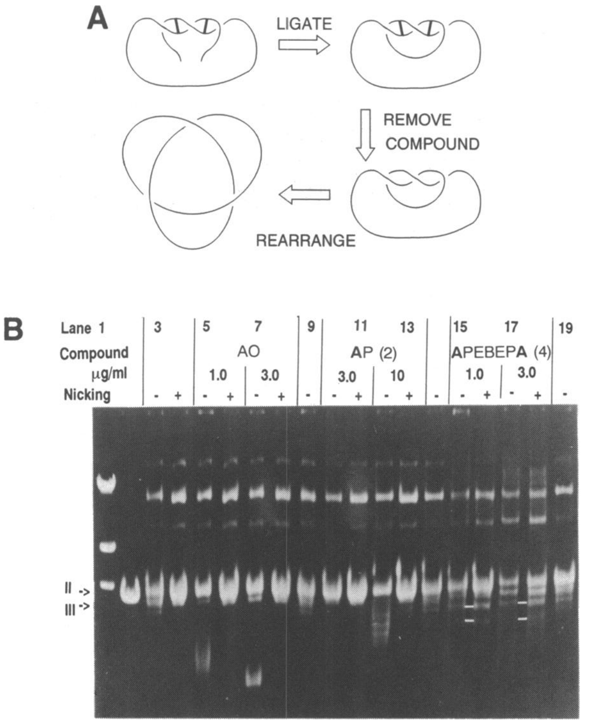


Fig. 2. **APEBEPA** (4), but not acridine orange (AO) or **AP** (2), knots DNA. A. Cross-linking followed by ligation induces knotting. Two bis-intercalating molecules (short thick lines) cross-link distant parts of one linear molecule (thin line). Cross-linking promotes entwining of the two ends. After ligation and removal of the bis-intercalators, 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; the more nodes, the more compact the molecule and the faster it migrates during electrophoresis. Increasing the flexibility of DNA (e.g. by reducing its persistence length) might also stimulate intertwining of the ends of the same molecule (and so knotting) but would not usually stimulate entwining of different molecules (and so catenation). Both mono- and bis-intercalation would be expected to have similar intercalation-induced effects on flexibilty (and so knotting). B. Linear DNA was ligated (750ng in 250 µl) in the presence of different concentrations of the compounds, the compounds removed, some samples nicked and the ligation products resolved electrophoretically before staining and photography. Lane 1: lambda/*Hind*III markers. Lane 2: linear DNA. Lanes 3,4: linear DNA ligated in the absence of any test compound. + and − indicate whether samples are nicked or not. The positions of forms II and III are indicated on the left. White lines to the right of lanes 15 and 17 indicate the position of simple relaxed knots with increasing numbers of nodes. Samples in lanes 3, 9, 14 and 19 are identical and serve as markers.

This shift from positively supercoiled topoisomers, through a relaxed group to negatively supercoiled forms is shown clearly by the mono-functional compound, **AP** (2), as its concentration is increased (lanes 9−13). However, as it is not as powerful an unwinding agent as acridine orange at the concentrations used, fewer superhelical turns are induced by 10 µg/ml (lane 13) and 30 µg/ml are needed to give the most rapidly-migrating forms (Fig. 3, lane 8). **AP** (2) inhibits the ligase less than acridine orange (compare Fig. 1, lane 8 with 13; see also Fig. 3, lane 8). The bifunctional compound, **APEBEPA** (4), gives a different pattern. After ligation in 3 µg/ml (lane 17), a significant fraction of the DNA runs faster than the most rapidly-migrating forms given by acridine orange. These must be very compact and are probably superhelical forms condensed even further by knotting. At 10 µg/ml, ligase is almost completely inhibited (lane 18), precluding testing at higher concentrations. In lanes 17 and 18, some DNA remains at the top of the gel, providing circumstantial evidence for cross-linking into large catenanes. These results, show that **AP** (2) unwinds DNA like acridine orange and that **APEBEPA** (4) additionally gives more complicated forms, probably knots.

Similar results are presented in Fig. 3 for the bifunctional compounds **APPA** (5) and **APEPA** (6); both behave like **APEBEPA** (4), but act more powerfully (see below).

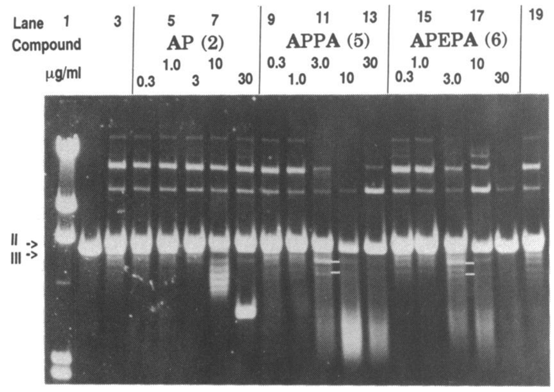


Fig. 3. **AP** (2), **APPA** (5) and **APEPA** (6) are all intercalating agents. Linear DNA (500ng in 250 µl) was ligated in the presence of different concentrations of the compounds, the compounds removed, and the ligation products resolved electrophoretically before staining and photographing the resulting gel. Lane 1: lambda/*Hind*III markers. Lane 2: unligated DNA. Lanes 3 and 19: linear DNA ligated in the absence of any test compound. The positions of forms II and III are indicated. White lines to the right of lanes 11 and 16 indicate the position of simple relaxed knots with increasing numbers of nodes.

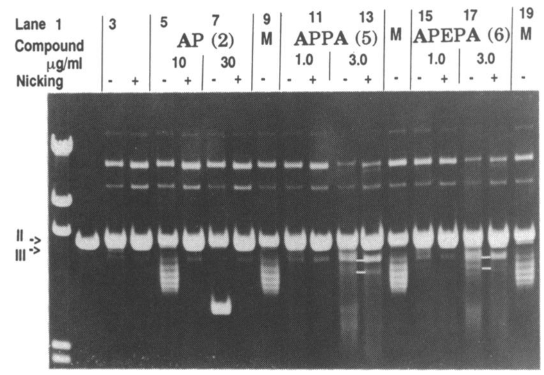


Fig. 4. **APPA** (5) and **APEPA** (6) knot DNA. Linear DNA was ligated (1 µg in 250 µl) in the presence of different concentrations of the compounds, the compounds removed, some samples nicked and the ligation products resolved electrophoretically before staining and photography. Lane 1: lambda/*Hind*III markers. Lane 2: linear DNA. Lanes 3,4: linear DNA ligated in the absence of any test compound. + and − indicate whether samples are nicked or not. The positions of forms II and III are indicated on the left. White lines to the right of lanes 12 and 17 indicate the position of simple relaxed knots with increasing numbers of nodes. Samples in lanes 9, 14 and 19 are identical to those in lane 5 and serve as markers (M).

Knotting

Trefoils, as well as more complicated knots and catenanes, are formed as the rèsult of any ligation, but their concentration is usually too low to be detected (but see Fig. 4, lanes 3 and 4 where a higher DNA concentration is used). A cross-linking agent would

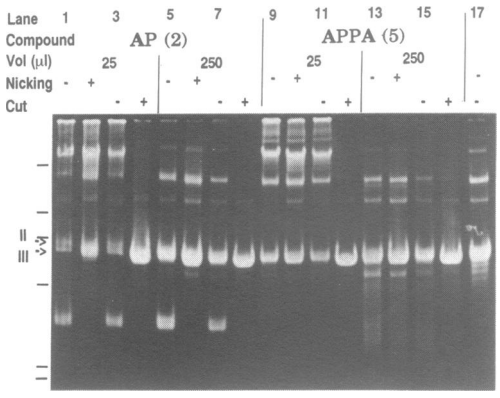


Fig. 5. Effects of ligation volume, nicking and cutting of knots and supercoils generated in the presence of AP (2) and APPA (5). Linear DNA was ligated (500ng in 25 or 250 μl) in the presence of AP (2) (30 μg/ml) or APPA (5) (3 μg/ml) and the compounds removed by ethanol precipitation. Some samples (lanes 1,2,5,6,9,10,13,14,17) were redissolved directly in sample buffer, others (3,4,7,8,11,12,15,16) were redissolved in 50 μl 50mM Tris-HCl (pH8.0), 50mM NaCl, 5mM $MgCl_2$ and incubated with or without 2.5 units *Hind*III for 1h at 37°C before ethanol precipitation and dissolving in sample buffer. Some samples were nicked and ligation products were resolved electrophoretically before staining and photography. Lane 17: linear DNA ligated in the absence of any test compound. + and − indicate whether samples are nicked or not, or cut with *Hind*III or not. The positions of lambda/*Hind*III markers and forms II and III are indicated on the left.

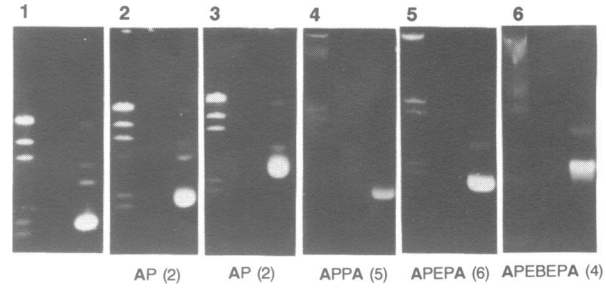


Fig. 6. The effects of AP (2), APEBEPA (4), APPA (5) and APEPA (6) on electrophoretic mobilities. Lambda/*Hind*III fragments (left-hand lanes) and supercoiled circles (pBS; right-hand lanes) were applied to wells at the top of 6 gels, each gel containing the compound indicated; after electrophoresis the gels were stained and photographed. The bands in the right-hand lanes (in order of decreasing mobility) are: supercoiled monomer, relaxed monomer, supercoiled dimer, relaxed dimer. 1: no compound. 2: 3 μg/ml AP (2). 3: 10μg/ml AP (2). 4: 3 μg/ml APPA (5). 5: 3 μg/ml APEPA (6). 6: 3 μg/ml APEBEPA (4).

be expected to promote such knotting (Fig. 2A). Indeed, a close inspection of Fig. 1, lane 17 (white markers) shows there to be extra faint bands below the linear form III, in the positions characteristic of simple relaxed knots with 3 or more nodes.[14,26] Note that these bands are not in register with those given by the topoisomers produced by mono-intercalating agents like acridine orange and AP (2). This suggests that APEBEPA (4) acts as a cross-linking agent.

Detecting such knots is difficult because their presence is often obscured by supercoiled topoisomers that have similar mobilities. Therefore they are usually detected after removing the supercoiled forms by nicking.[14] Fig. 2B illustrates such an experiment where the products formed by ligation have been treated with a dose of gamma rays sufficient to nick >99% of the circles. Here the DNA concentration has also been increased to allow visualization of minor species and this inevitably leads to more intermolecular ligation. It also slightly reduces the degree of knotting and supercoiling obtained at a given drug concentration, as the molar concentration of test compound roughly equals that of the base-pairs in the assay.

Ligation of linear DNA (Fig 2B; lane 2) in the absence of any compound produced a set of (slightly) positively-supercoiled topoisomers (lane 3) which were relaxed by irradiation to give form II (lane 4). Acridine orange at 1 or 3 μg/ml during ligation led to rapidly migrating negatively-supercoiled topoisomers (lanes 5,7); these, too, were relaxed by irradiation (lanes 6,8). Ligation in the presence of 3 μg/ml AP (2) produced relaxed circles (lane 10), which were, as expected, relatively unaffected by nicking (lane 11). As with the low concentration of acridine orange, 10 μg/ml AP (2) produced negatively supercoiled topoisomers (lane 12) which were relaxed by irradiation (lane 13). After nicking, essentially no forms are visible below linear form III; all these rapidly migrating forms were originally supercoiled, but not knotted, as nicking produced form II molecules.

By contrast, rapidly-migrating molecules produced by APEBEPA (4) (Fig. 2B; lanes 15 and 17) continue to migrate faster than form II and form III molecules even after nicking (lanes 16 and 18); they must be knots. Ligation of nicked linear molecules produces relaxed knots, faintly visible as the discrete bands in lanes 15 and 17 marked by the white lines. These are out of register with topoisomers in lane 12 and are characteristic of relaxed knots with increasing numbers of nodes.[14] Ligation of intact linear molecules forms supercoiled trefoils and other knots with more nodes that migrate faster as an unresolved smear. Irradiation nicks some of this rapidly-migrating material, increasing the concentration of the relaxed knots marked with the white lines in lanes 16 and 18. Nicking has little effect on the more complicated knots in the faint and unresolved smear (compare lanes 15 with 16, and 17 with 18); releasing their supercoils has little effect on compaction. We conclude that APEBEPA (4) acts as both an unwinding and weak cross-linking agent to generate these forms.

Results for the other bifunctional agents, APPA (5) and APEPA (6) are presented in Figs. 3 and 4; results for the mono-functional agent AP (2) are included for comparison. Both APPA (5) and APEPA (6) prove to be more powerful knotting agents than APEBEPA (4). For example, 10 μg/ml APPA (5) or APEPA (6) convert a significant fraction of DNA into unresolved forms that migrate even more rapidly than the highly supercoiled topoisomers generated by 30 μg/ml AP (2) (Fig. 3, compare lanes 12 and 17 with 8); knotting compacts them even further. This occurs even though the ligase is partially inhibited. Relaxed knots are clearly visible in lanes 11, 12, 16 and 17 (white markers). Nicking has relatively little effect on such rapidly migrating forms (Fig. 4, compare lane 12 with 13 and lane 17 with 18); it reduces only slightly the intensity of the smear with a corresponding increase in the intensity of the relaxed knots (white markers). These results show that all the bifunctional agents unwind (and so intercalate) and knot (and so cross-link). As the connecting linker becomes shorter, knots with a greater number of nodes are produced for a given reagent concentration.

Catenation

In the above experiments a low DNA concentration was used in order to maximize intramolecular ligation and minimize

Table I. The effects of the various compounds on the cloning efficiency of bacteria. Bacteria were exposed to 10μg/ml test compound for 30 or 60 min. and then plated. The number of colonies surviving treatment (averages of at least 2 experiments) were expressed as a fraction relative to controls treated with an equivalent concentration of solvent. *; compounds of the previous series.[5]

COMPOUND	FRACTION SURVIVING 30min EXPOSURE	FRACTION SURVIVING 60min EXPOSURE
Ethidium	1.00	0.62
Ph (2*)	1.01	0.83
PhEB (4*)	0.71	0.47
PhEBEPh (6*)	0.38	0.43
PhEBEBEBEPh (9*)	0.11	0.10
Acridine orange	0.96	1.04
A (11*)	1.62	1.29
AEB (13*)	1.84	1.52
AEBEA (15*)	1.96	2.36
AP (2)	0.88	0.91
APPA (5)	0.21	0.09
APEPA (6)	0.76	0.59
APEBEPA (4)	1.28	0.75

Table II. The effects of the various compounds on the initial rate of replication by permeabilized HeLa cells. Rates were measured in the presence of 10μg/ml test compound and expressed relative to the rate in the absence of the compound. *; compounds in the previous series.[5]

COMPOUND	RELATIVE REPLICATION RATE
Ethidium	0.04
Ph (2*)	0.56
PhEB (4*)	0.25
PhEBEPh (6*)	0.04
PhEBEBEBEPh (9*)	0.80
Acridine orange	0.07
A (11*)	0.42
AEB (13*)	1.03
AEBEA (15*)	1.01
AP (2)	0.80
APPA (5)	0.87
APEPA (6)	0.35
APEBEPA (4)	0.99

formation of dimeric and higher forms that are difficult to analyze. As expected, increasing the DNA concentration (by ligating in one-tenth the volume) increases the proportion of dimers and higher forms produced in the presence of both AP (2) and APPA (5) (Fig 5; compare lanes 1 with 5, and 9 with 13). Any supercoiled forms present can be nicked and relaxed by irradiation (lanes 2, 6, 10, 14) and all forms can be linearized by cutting with *Hind*III (lanes 4, 8, 12, 16).

Even at the high DNA concentration, a significant fraction of ligations in the presence of AP (2) remain intramolecular and yield rapidly-migrating supercoiled monomers (lane 1). However, ligation in the presence of APPA (5) yields few monomers running faster than form III molecules (Fig. 5, lane 9); instead most ligations are intermolecular and give catenanes that run slowly. Nicking these has little effect on the overall pattern, as these catenanes are not well-resolved (lanes 9 and 10). At such high DNA concentrations essentially similar results (not shown) were obtained with APEBEPA (4) and APEPA (6); they concatenate two molecules of DNA equally effectively. It is difficult to envisage how such catenation could be promoted by any process other than cross-linking.

Our assay requires that all test compound is removed after ligation; if not, residual bound molecules would alter mobilities, confounding interpretation. Establishing exactly how much might remain during electrophoresis is technically difficult, given the low concentrations used. However, samples are first ethanol precipitated (all compounds are soluble in ethanol) before redissolving them in sample buffer, which necessarily dilutes the sample >50 fold prior to loading on the gel. The sample buffer contains sodium dodecyl sulphate, which would be expected to enhance dissociation of any remaining test compound. The best evidence that essentially all test compound has been removed is provided by the similarity of the electrophoretic profiles of samples prepared routinely and after redissolving and reprecipitating them (Fig. 5; compare lanes 1 with 3, 5 with 7, 9 with 11 and 13 with 15).

Effects on electrophoretic mobility

The effects of the various compounds on the mobility of linear and supercoiled DNA are illustrated in Fig 6. Unlike the experiments described above, samples are run in the presence of the test compounds. Increasing the concentration of AP (2) during electrophoresis progressively reduces the mobility of all lambda/*Hind*III fragments (Fig. 6, gels 1–3, left-hand lanes); this is to be expected of an agent that slightly increases the length of linear molecules on intercalation. AP (2), like acridine orange (not shown) also reduces the mobility of supercoiled circles by removing some supercoils (gels 2 and 3, right-hand lane). The bifunctional agents APEBEPA (4), APPA (5) and APEPA (6) have relatively little effect on the smaller supercoiled forms (gels 4–6, right-hand lanes) or on the smaller lambda/*Hind*III fragments, but they do dramatically reduce the mobility of the largest fragment, which barely enters the gels (gels 4–6, left-hand lane). [The topmost lambda/*Hind*III band (gel 1, left-hand side) contains, in fact, two unresolved fragments; the largest (minor) one is formed by annealing of the cohesive ends of lambda DNA.]

These effects are most simply interpreted if cross-linking is a minor event under these conditions. If it were a major occurrence the mobility of the circles would be reduced more than that of the linear molecules since after mono-intercalation into a circle, the other intercalating moiety will lie relatively close to more binding sites on the other side, favouring cross-linking and compaction–and so increasing mobility. But this does not occur, all fragments migrate more slowly, with the mobility of the longest linear fragment being reduced the most. This can be explained if the number and length of the groups that protrude from the side of the linear fragments largely determines their mobility. Mono-intercalation into the long fragment would leave a sufficient number of protruding groups to reduce mobility and the longer the protrusion, the greater the effect (Fig. 6). Streaking would result from molecules with different numbers of protruding groups. By contrast, the other fragments and the circles are too small to bind sufficient compound to have any marked effect.

Biological effects

We have begun to characterize the biological effects of both the present and previous series of bifunctional agents.[5] Bacteria were exposed to 10 μg/ml of the compounds for one or two generations and then plated; only 1,4-bis[N-methylphenanthridinium-6-(1,4-vinylphenylenevinyl)phenylene diiodide (compound 9 of the previous series,[5]) and APPA (5) had any significant effect on the cloning efficiency (Table I). We also tested for inhibitory effects on the initial rate of replication by permeabilized human cells (Table II). None of the current series proved as inhibitory as 1,4-(n-methylphenanthridinium-6-vinyl) phenylene diiodide (compound 6 of the previous series,[5]), or the ethidium and acridine orange used as controls.

DISCUSSION

Intermolecular bis-intercalation

The results show that all the compounds of this new series AP (2), APEBEPA (4), APPA (5) and APEPA (6) unwind DNA (Figs. 1, 3). Ligating linear DNA molecules in the presence of the bifunctional agents APEBEPA (4), APPA (5) and APEPA (6), but not the analogous mono-functional compound AP (2), also increases the proportion of knots in the ligation mixture (Figs. 2, 4). Evidence of unwinding, though circumstantial, is generally accepted as proof of intercalation.[6,7] Therefore our results provide strong evidence that all these compounds are, at least, mono-intercalators. However, proof of bis-intercalation requires the demonstration that both groups intercalate **simultaneously.** Such evidence is more difficult to obtain. Formation of catenanes and knots by the bifunctional agents provides circumstantial evidence only for cross-linking of some sort. It remains possible that they might do this non-intercalatively, for example, in the same way that a spermine molecule might link two duplexes. However, because they also intercalate, it seems probable that the bifunctional molecules do bis-intercalate simultaneously and so cross-link. Although knotting and catenation provide circumstantial evidence for cross-linking, formal proof of bis-intercalation must await evidence from X-ray crystallography or n.m.r. spectroscopy.

The compounds reported here were more effective knotting-agents than those analyzed previously.[5] Perhaps surprisingly, the shorter the linker connecting the two intercalating groups, the more effective knotting-agents they become. Under the appropriate conditions in the ligation assay, APPA (5) can effectively cross-link essentially all DNA intermolecularly (Fig. 5). This suggests that the agents might be relatively powerful cross-linking agents. However, electrophoresis in the presence of the agents suggests that very few molecules are cross-linked together (Fig. 6). These apparently conflicting results can be reconciled as follows. Linear DNA molecules are predominately mono-intercalated by the bifunctional molecules but in the small population of molecules which are transiently cross-linked by bis-intercalation their ends are often drawn closer together and consequently ligate more rapidly (Fig.2A). The residual pool of predominately mono-intercalated molecules re-equilibrate, replenishing the small population of bis-intercalated molecules which again are more rapidly ligated and withdrawn from the pool. This continual selective removal of the bis-intercalated molecules which are replenished from the much greater population of mono-intercalated molecules, leads to a significant fraction of the whole population becoming knotted. Indeed the success of this novel assay in detecting cross-linking appears to depend, at least in part, on this amplification mechanism, since electrophoresis of linear DNA in the presence of the bifunctional agents provides little evidence for cross-linking. Even relaxed circles where opposite sides lie in relatively close proximity are not significantly cross-linked at equilibrium suggesting that the agents are only weak cross-linkers. This, however, is to be expected as the entropic factor involved in bringing the two ends of linear DNA molecules together, or opposite sides of a circular DNA molecule together, is large. They would be expected, however, to be much more effective cross-linking agents where two duplexes lie in close proximity, for example at sites of replication, recombination or topoisomerase action. Therefore we hope to use these reagents to probe the DNA structure at the active site of these enzymes.

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