# A new 4,9-diazapyrenium intercalator for single- and double-stranded nucleic acids: distinct differences from related diazapyrenium compounds and ethidium bromide †

**Ivo Piantanida,[a] Barbara Susanne Palm,[b] Mladen Žinić[a] and Hans-Jörg Schneider *[b]**

[a] *Laboratory of Supramolecular and Nucleoside Chemistry, Department of Chemistry and Biochemistry, Rudjer Boskovic Institute, HR 10000, Zagreb, POB 1016, Croatia. E-mail: zinic@rudjer.irb.hr*
[b] *FR Organische Chemie der Universität des Saarlandes, D 66041 Saarbrücken, Germany. E-mail: ch12hs@rz.uni-sb.de*

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The new ligand **6** is structurally related to ethidium bromide (**EB**) and other diazapyrenium compounds (**1**, **2**) studied earlier, but exhibits significant differences. In contrast to ligands such as **1** and **2** the new **6** is stable over a large pH range. At concentrations > $10^{-4}$ mol $dm^{-3}$ **6** forms a dimer, which is characterised by NMR analysis. The association constants of **6** are larger with purine than with pyrimidine nucleotides and are the same for AMP, ADP or ATP, with an absence of any charge effects. Surprisingly, under conditions close to saturation of intercalation binding sites association of **6** with single-stranded poly U and poly A is 100 to 1000 times more efficient than that of **EB** or analogue **2**. Also the affinity of **6** toward poly U is significantly higher than toward poly C. CD spectra indicate that stacking of **6** with poly U induces a strong helicity in the usually disordered structure of this single strand. The binding of **6** to poly C is pH dependent, as a consequence of the known formation of a poly $CH^+$–poly $CH^+$ double strand at pH < 5. With double-stranded polynucleotides the binding affinity of **6** and **EB** is similar for RNA homopolymers. Striking fluorescence differences, however, are observed with complexes of **6**–poly GC (*de*crease of emission intensity of **6** by 80%), and **6**–poly AU (*in*crease by 100%). Similar effects are also observed for the DNA polynucleotides. At higher ligand to phosphate ratios ($r$ > 0.2) **6** shows with double strands electrostatic binding in addition to intercalation, with ensuing opposite effects in fluorescence emission. The biphasic melting profiles of poly dA–poly dT with **6** differ sharply from those observed with **1** and **EB** and are in line with the dependence of binding modes obvious from the fluorescence studies. Melting analyses show with calf thymus DNA and with poly dA–poly dT an increase ($\Delta\Delta T$) in melting temperature with **6** about twice as high as that with **1** or **EB**. With the RNA model poly A–poly U the $\Delta T_m$ values of **6** at low ionic strength are about seven times higher than those observed with **2** or with **EB**. In contrast to other 4,9-diazapyrenium compounds which show with poly A–poly U a destabilising and a stabilising step, **6** gives only positive $\Delta T$ values at both pH = 5.0 and pH = 7.0. Extensive supporting data for this paper are available as supplementary material † in electronic form, allowing re-evaluation of experimental results with suitable programs for curve fitting.

## Introduction

The search for new nucleic acid intercalators[1] is stimulated by a variety of reasons. One can couple such intercalators, *e.g.* to oligonucleotides, or to catalytic units for cleaving nucleic acids, for the development of stable triple helices[2] or of synthetic nucleases,[3] exploiting the relatively high association constants of intercalators. With new systems, capable of binding mono- or oligonucleotides, one can also hope to reach a deeper understanding of intercalation mechanisms.[4] The recently discovered relatively selective interaction of ethidium bromide (**EB**) with particular RNA, HIV–related sequences[5] stirs new interest in the development of new aromatic systems with possibly higher selectivity. Besides ethidium bromide, related compounds with extended aromatic systems, such as 2,7-diazapyrenium and 2,7-diazaperopyrenium[6] cations, as well as acridinium cations,[7] have been studied in detail. For systems which exhibit photo-induced cleavage of double-stranded polynucleotides,[8] it was found that small structural variations strongly influence the binding affinity to polynucleotides.[6,9]

Recently we have reported on the interaction of a series of new 4,9-diazapyrenium derivatives (**1**, **2**, Chart 1) with nucleotides and with double-stranded (ds) nucleic acids.[10,11] Some of these 4,9-diazapyrenium derivatives exhibit *in vitro* antitumor activity.[12] However, a more detailed examination was hampered by their instability at neutral pH.[11] In the present work, we describe the synthesis *via* intermediates **3** to **5** of a novel 4-methyl-2,7-diamino-5,10-diphenyl-4,9-diazapyrenium analogue **6** (Scheme 1) which is stable over a wide pH range (pH = 3–10), including physiological pH conditions. Although **6** is structurally very similar to ethidium bromide **EB** it possesses several features distinct from classical intercalators. The larger aromatic surface could enhance the intercalation strength, the 5,10-diphenyl substituents are expected to be oriented orthogonal to the diazapyrene plane, which should sterically restrict orientation possibilities upon intercalation. We report on the surprising spectroscopic properties of the new intercalation complexes of **6**, the binding to nucleotides and on affinities toward single- and double-stranded DNA/RNA polynucleotides, based on UV/Vis, fluorescence and CD measurements as well as on melting experiments and viscometry. The data are compared to the ones obtained with **EB** and the previously reported ones of two closely related 4,9-diazapyrenium derivatives **1** and **2**[11] (Chart 1).

† Electronic supplementary information (ESI) available: NMR data, fluorimetric titration data, thermal melting curves. See http://www.rsc.org/suppdata/p2/b1/b103214n/

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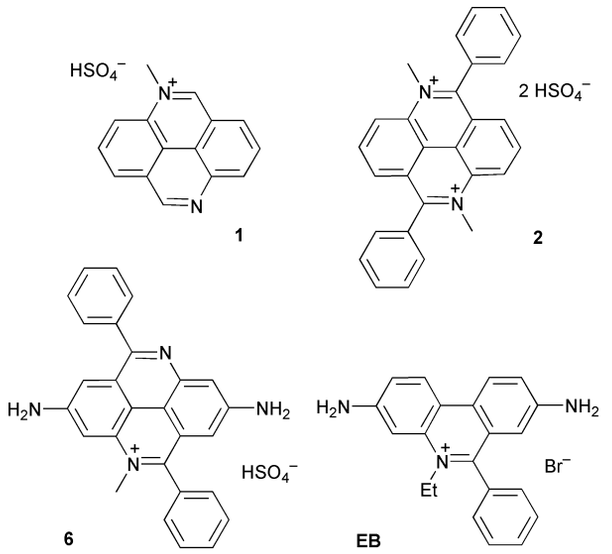
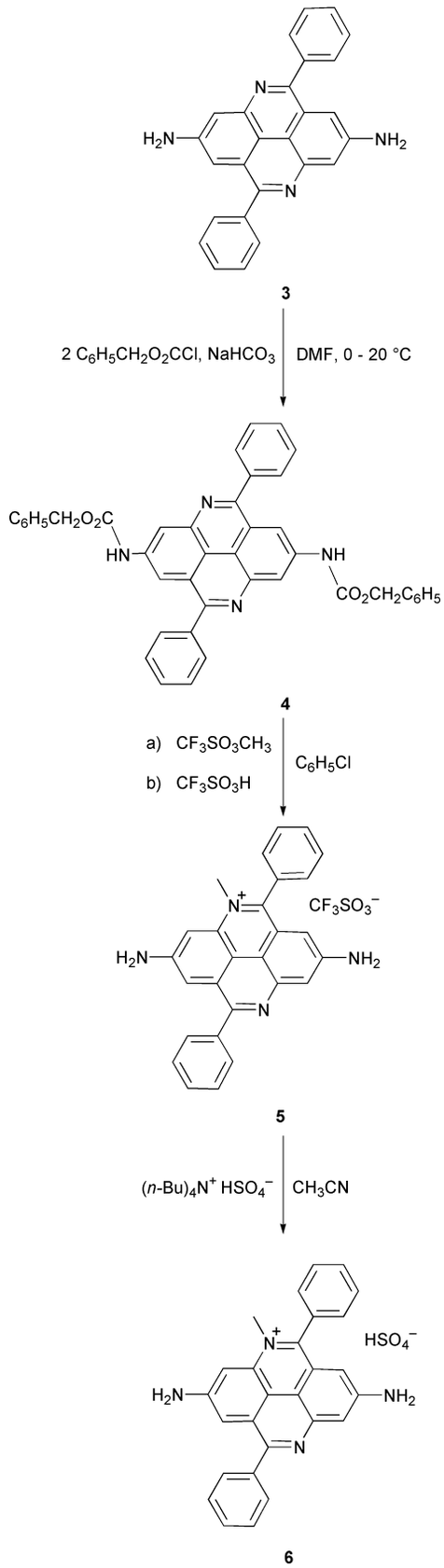



**Chart 1**

## Results and discussion

### Synthesis

2,7-Diamino-5,10-diphenyl-4,9-diazapyrene **3** was prepared according to the previously published procedure.[13] The synthesis of **6** from **3** is outlined in Scheme 1. The amino groups of **3** were protected using benzyloxycarbonyl chloride in DMF–$NaHCO_3$ suspension at room temperature giving **4**. Heating of **4** with an equimolar amount of methyl trifluoromethanesulfonate (methyl triflate) in chlorobenzene, followed by subsequent *in situ* amino deprotection by trifluoromethanesulfonic acid at room temperature gave **5** as the triflate salt. The attempted preparation of the 4,9-dimethyl derivative of **5** failed despite the use of 2–5 molar excess of methyl triflate and prolonged heating. Under the same reaction conditions dimethylated analogue **2** was prepared in high yield,[10] which points to a pronounced deactivation effect of the 2,7-benzyloxycarbonylamino substituents in **4**. To improve the water solubility of **5**, the triflate anion was exchanged with hydrogensulfate giving, after recrystallization from DMSO–dichloromethane, a dark blue precipitate of **6**. The hygroscopic nature of the precipitates hampered elementary analysis; however, the structures of **5** and **6** were confirmed by (1D and 2D) NMR and ES-MS data.




**Scheme 1** Synthesis of 2,7-diamino-5,10-diphenyl-4-methyl-4,9-diazapyrenium hydrogensulfate (**6**).

### Spectroscopic properties of 6

**$^1$H-NMR spectra.** Changing the concentration of **6** from $1 \times 10^{-4}$ to $5 \times 10^{-3}$ mol dm$^{-3}$ all signals exhibited upfield shifts by up to 0.7 ppm (supplementary data),† indicating self-stacking. Assuming predominant formation of a dimer, the stability constant $K_{s,dim.}$ of ≈400 ± 100 dm$^3$ mol$^{-1}$ is calculated[14] (Fig. 1). The association constant is similar to those calculated for phenanthridinium and acridinium dimers (**EB**, proflavine $K_{s,dim.}$ ≈ 180 and ≈ 350 dm$^3$ mol$^{-1}$, respectively).[15] The signals of **6** protons at $5 \times 10^{-3}$ mol dm$^{-3}$ concentration were assigned on the basis of NOESY and ROESY (suppl ementary data)† cross peaks. At this concentration **6** is considerably self-stacked and intermolecular ROESY interactions were observed between the $H_6$ proton of the diazapyrenium ring and the *o*-protons of the 10-phenyl substituent, and also between $H_1$ of the diazapyrenium ring and the *o*-protons of the 5-phenyl substituent. The observed intermolecular ROESY cross peaks suggest the formation of a stacked dimer with offset ADAP molecules and the second molecule rotated by 180° around the longer axes (Fig. 2).

**Electronic absorption and fluorescence spectra.** In comparison with the previously prepared 4,9-diazapyrenium derivatives[10] **1** [$\lambda_{max}$/nm, ($\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$): 236, (55800); 351 (9000); 389 (10000)] and **2** [$\lambda_{max}$/nm, ($\varepsilon$): 243 (45400); 335 (12800); 400 (24000)] the amino substituents of **6** induce pronounced red shifts of absorption maxima in UV/Vis spectra of the 4,9-diazapyrenium ring [$\lambda_{max}$/nm ($\varepsilon$): 266 (37700); 368 (5050); 517 (5750)]. Absorption of **6** in buffered solution (pH = 5 and 7;

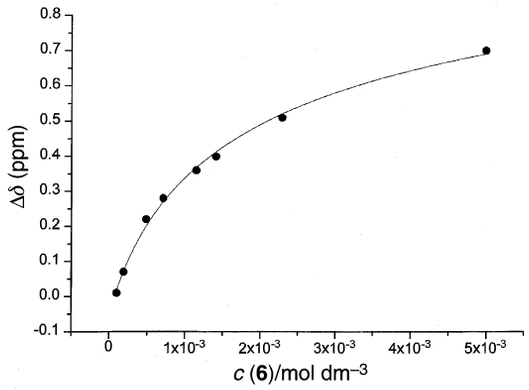



**Fig. 1** Experimental (●) and calculated (——) data of $\Delta\delta$ (ppm) for proton $H_6$ according to dimerisation equation.

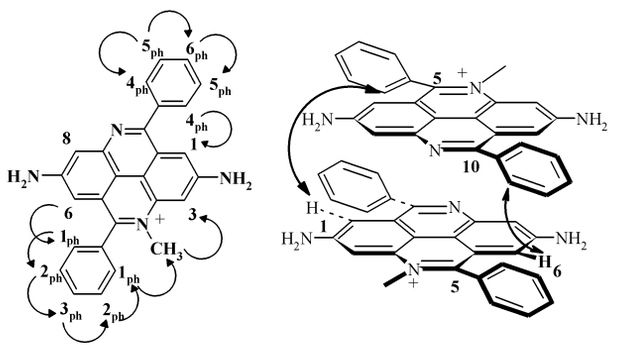


**Fig. 2** Observed intra- (left) and inter-molecular (right) ROESY interactions in possible **ADAP** dimer with indicated intermolecular $H_6$–$H_{ortho}$ (C10-phenyl) and $H_1$–$H_{ortho}$ (C5-phenyl) interactions.

0.01 mol dm$^{-3}$) is found to be linearly concentration dependent up to $8 \times 10^{-5}$ mol dm$^{-3}$. In the concentration range $8 \times 10^{-5}$–$2 \times 10^{-4}$ mol dm$^{-3}$ a small hypochromicity was observed, indicating self-stacking of **6**, in accord with the self-association constant $K_{s,dim.} \approx 400 \pm 100$ dm$^3$ mol$^{-1}$ determined by $^1$H-NMR.

The fluorescence emission intensity of **6** is significantly lower than those of analogues[10] **1** and **2**, with the emission maxima strongly red-shifted by almost 200 nm ($\lambda_{max}$ = 613 nm). Excitation spectra of **6** agree with the absorption spectra. Fluorescence intensity is found to be linearly concentration dependent up to $5 \times 10^{-6}$ mol dm$^{-3}$.

Addition of 20% of $D_2O$ to an aqueous solution of **6** induced a 30% increase in fluorescence intensity. A similar effect was previously found for **EB** and explained by an amino group NH–ND exchange which decreases the quenching by amino proton transfer to water molecules in the excited state.[16]

An interesting feature of **6** is the absence of pH dependent formation of 5-hydroxy-4,9-diazapyrene pseudobase which one finds with all previously prepared derivatives including **1** and **2** (Fig. 3).[10] Monitoring the changes in UV spectra with variation of pH (pH range 3–10) only a slight variation of $\varepsilon$ can be observed (less than 5%) between pH 4.5 and 7. Amino groups are protonated at pH < 4 as is apparent from pH dependent absorbance changes. The similar chemical shift pattern of diazapyrenium protons in $^1$H NMR spectra at pD 2.4 and 6.2 also exclude, formation of pseudobase. Apparently, the presence of the electron donating 5,10-diphenyl and 2,7-diamino substituents of **6** strongly increases its stability to pseudobase formation in neutral and weakly basic conditions.

### Interactions of 6 with nucleotides in aqueous media

Fluorimetric titration of **6** with nucleotides exhibited interesting nucleic base specific spectroscopic changes. Addition of AMP, ATP and CMP in excess does not quench fluorescence emission down to zero as in the case of analogues **1** and **2**,[10]

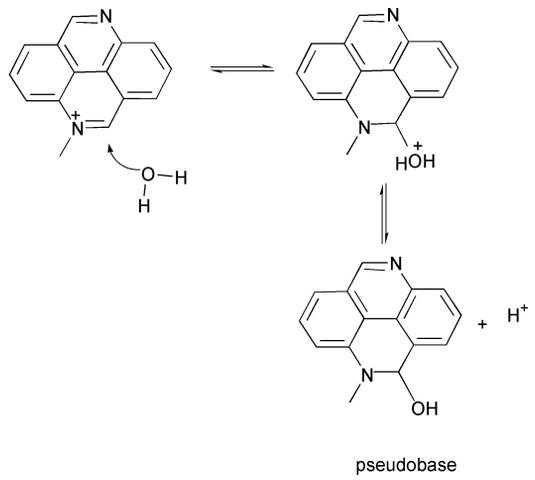



**Fig. 3** Formation of 4,9-diazapyrene pseudobase.

which is obviously due to the influence of the amino substituents. Only addition of GMP yielded total quenching of **6** emission. Similar differences in fluorimetric response were reported for proflavine and explained by the more pronounced electron donating properties of guanine compared to other nucleic bases.[17]

Stability constants ($K_s$) were calculated by processing fluorimetric titration data with the program SPECFIT;[18] the best fit for a 1 : 1 stoichiometry of complexes was obtained in all cases. Titration of **6** with UMP gave too small changes of emission intensity for accurate calculation of the binding constant. However, a constant similar to that of CMP can be estimated. Compared to previously reported binding constants for **2** (nucleotide, log $K_s$: AMP, 1.67; ADP, 1.74; ATP, 1.78; GMP, 1.66; CMP, <1)[10] and **EB** (GMP, 2.01; AMP, 1.92),[19] binding with **6** is somewhat stronger (CMP, 1.61; GMP, 2.30; AMP, 2.16; ATP, 2.39) but similar to **1**[10] (CMP, 1.37; GMP, 2.11; AMP, 2.21; ATP, 2.24).

Comparing **2** and **6** with **EB** it appears that neither the presence of two positive charges (**2**) nor the larger aromatic surface (**6**) leads to a considerable increase of binding strength. Similar binding affinity of **6** for AMP, ADP and ATP with 2, 3 and 4 negative charges respectively, and larger $K_s$ values for purinic than for pyrimidinic nucleotides point to the aromatic π–π stacking interactions between **6** and nucleobases as the dominant interaction in the complexes. As in related cases[20] there is a striking absence of nucleotide charge effects.

### Interactions of 6 with single-stranded RNA polynucleotides

Single-stranded RNA polymers of homogenous structure (poly A, G, C, U) are good models for testing the nucleic base dependent affinity of selected molecules and also the spectroscopic response of the studied compound induced by binding to polymers. Most single-stranded (ss) RNA polynucleotides (except poly U) form rather well organised helical structures in water at ionic strength $I$ = 0.01–0.1 mol dm$^{-3}$, due to stacking of nucleobases.[21]

**UV/Vis titrations.** Additions of ss polymers (poly A, G, C, U) at pH 5 and 7 induce bathochromic shifts in the spectra of **6** for 20–40 nm and hypochromic effects up to 50%. Similar changes observed earlier for most of the classical intercalators were explained by their intercalative binding mode.[22] In most titration experiments isosbestic points are observed, suggesting that only two spectroscopically active species are present at equilibrium. Only in the titration with poly A at pH = 5, is a clear deviation from the isosbestic point observed, due to protonation of poly A. Under such conditions a double helix of poly AH$^+$–poly AH$^+$ is partially formed,[21] resulting in two different complexes with **6**.

**Table 1** Stability constants ($K_s$) and ratios ($n$)[a] ([bound compound] : [polynucleotide]) calculated for UV/Vis and fluorimetric titrations of **6** or **EB** with ss polynucleotides at pH = 5.0 (buffer citric acid (0.01 M), $I$ = 0.025 M) and pH = 7.0 (buffer Na cacodylate, $I$ = 0.02 M)

| | | poly A | | poly G | | poly C | | poly U | |
|---|---|---|---|---|---|---|---|---|---|
| | pH | $n$ | log $K_s$ | $n$ | log $K_s$ | $n$ | log $K_s$ | $n$ | log $K_s$ |
| **6** | 5 | [b] | [b] | 0.6 | 4.5 | 0.5 | 4.5 | 1 | >5[d] |
| UV | 7 | 1 | >5[d] | 0.8 | 4.8 | 1[c] | 3[c] | 1 | >5[d] |
| **6** | 5 | [b] | [b] | 0.6 | 4.7 | 1[c] | 2.9[c] | 1 | 3.9[b] |
| Fluo | 7 | 0.5 | 4.1 | 1 | 4.5 | 1[c] | 2.6[c] | 1 | 4.4[b] |
| **EB** | 5 | 0.5 | 3.3[b] | 0.3 | 3.8 | 1[c] | 3.3[c] | 1[c] | 3[c] |
| UV | 7 | 0.5 | 3.9 | 0.5 | 3.1 | 1[c] | <3[c] | 1[c] | <3[c] |

[a] Accuracy of $n$ ± 10–30%, consequently log $K_s$ values vary within the same order of magnitude. [b] Due to formation of different types of complexes only estimation of cumulative log $K_s$ was possible. [c] Small spectroscopic changes allowed only estimation of $n$ and log $K_s$. [d] Linear change of absorbance with [polynucleotide] allowed only estimation of log $K_s$.

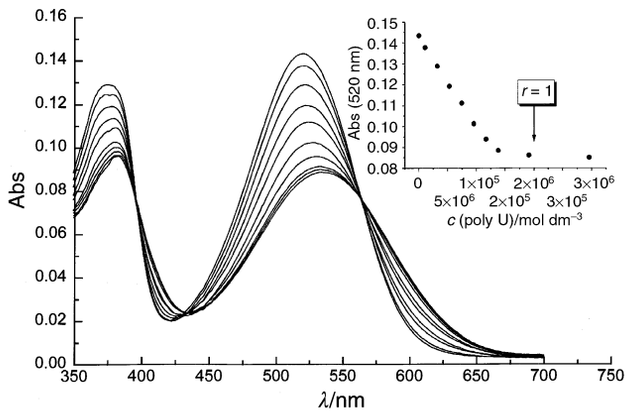



**Fig. 4** UV/Vis titration of **6**, $c = 2 \times 10^{-5}$ mol dm$^{-3}$ with poly U; pH = 7 (PIPES buffer, $c$ = 0.01 mol dm$^{-3}$).

UV/Vis titration data of **6** with poly U (Fig. 4) point to surprisingly high complex stability. The linear dependence of absorbance changes *vs.* concentration of poly U observed did not allow calculation of $K_s$ and $n$ by the Scatchard equation. The end value at the **6** : poly U ratio of 1 suggests a value of $n \approx 1$ and $K_s > 10^5$ dm$^3$ mol$^{-1}$. Similar results are obtained for titration of **6** with poly A at pH = 7. Some 100–1000 times higher concentrations of poly A and poly U, respectively, need to be added to the solution of **EB** or **2** in order to induce spectroscopic changes comparable to those of **6** under the same experimental conditions. This clearly shows much weaker binding of **EB** and **2** compared to **6** (Table 1). $K_s$ values for **6** and **EB** for the complexes with poly G and poly C are comparable except for the $K_s$ for **6** and poly C at pH = 5 which is close to two orders of magnitude higher than that at pH = 7 (Table 1). The latter can be explained by protonation of poly C at pH = 5 and formation of a double helix[21] which increases the electrostatic interactions of **6** and polyphosphates (see the following paragraph on non-intercalative interactions with double-stranded polynucleotides).

**Fluorescence titrations.** Addition of almost all polynucleotides quenched fluorescence emission of **6** (with poly A by 30%, and with poly G by 70%) with a similar trend to that observed in titrations with nucleotides. Results obtained from titration of **6** with poly C appeared to be strongly pH dependent, which is not the case for **EB** (Table 1). Binding of **6** with poly C at pH = 5 results in a unique fluorescence increase (35%) while at pH = 7 fluorescence decreases. However, the fluorescence gives one order of magnitude lower $K_s$ for **6** and poly C at pH = 5 than that determined by UV/Vis measurements. This can be explained by a much lower electrostatic contribution (between **6** and the phosphates of poly CH$^+$–poly CH$^+$ formed at pH = 5[21]) to overall binding (intercalative and electrostatic) in the fluorescence titration due to a lower concentration range ($c_6 = 2 \times 10^{-6}$ and $2 \times 10^{-5}$ mol dm$^{-3}$ for fluorescence and UV/Vis, respectively). Consequently, in fluorescence titration first spectroscopic changes are observed at ratio $r$ ($c_6/c_{\text{polynucl.}}$) << 1 where the intercalative mode of binding prevails. In line with that, similar stability constants are obtained by UV/Vis and fluorescence measurements at pH = 7 where protonation of poly C is negligible.

Stability constants $K_s$ calculated from titration data of **6** with poly A and poly G at large excess of either polynucleotide ($r$ << 1) were of the same order of magnitude (Table 1). Different behaviour was observed, however, when **6** was in excess ($r$ > 1); addition of poly A produced small but significant quenching while addition of poly G had no effect. Taking into account the UV/Vis titration experiments where changes of absorbance were quite different for poly A and poly G one can assume formation of two different complexes with poly A and only one with poly G.

Addition of poly U (pH 5 and 7) induced rather small but significant fluorescence quenching (7–9%) of **6** with excess of the compound (ratio $r$ > 1). With excess of poly U (10–100 times) dominant fluorescence quenching (15%) of **6** was observed. It was not possible to clearly differentiate the concentration range where only one binding mode prevails. Therefore, the cumulative stability constant was calculated, being significantly larger than for poly C (pH = 7).

**CD spectra.** It is known that intercalation of some flat aromatic molecules into single-stranded polynucleotides induces large chirality changes and consequently significant effects in their CD and LD spectra.[7,23] The CD and LD changes are useful for determination of mobility and orientation of intercalated dye in double helical polynucleotides.[24] Most of the ss polynucleotides (poly A, G, C) form helical structures in water due to stacking interactions between adjacent bases and it is possible to monitor their conformational changes using CD spectroscopy.[23]

The variation of ratio $r$ ($c_6/c_{\text{poly U}}$) from 0 to 0.8 induces a large increase of CD intensity of poly U spectra (Fig. 5). The observation of the isosbestic point at 272 nm suggests formation of only one complex at equilibrium with free polymer. Under the same conditions addition of **EB** had no effect. Poly U and poly T under these experimental conditions, in contrast to other single-stranded polynucleotides, do not form organised helical structures in water due to lack of stacking interactions between bases.[21] Therefore, the observed CD effects strongly suggest an increase of chirality in the **6**–poly U complex compared to free polymer. The rational explanation can be the induction of helicity by intercalation of **6**. Structural features of **6** and uracils accompanied by strong stacking interactions can provide the driving force for helical organisation of the complex. In contrast to poly U, addition of **6** as well as **EB** to solutions of poly A, poly G and poly C produced a decrease of CD intensities in accord with a decrease of helicity upon intercalation. The effects of **6** in CD spectra of poly A are more pronounced than those of **EB**; this observation is in accord

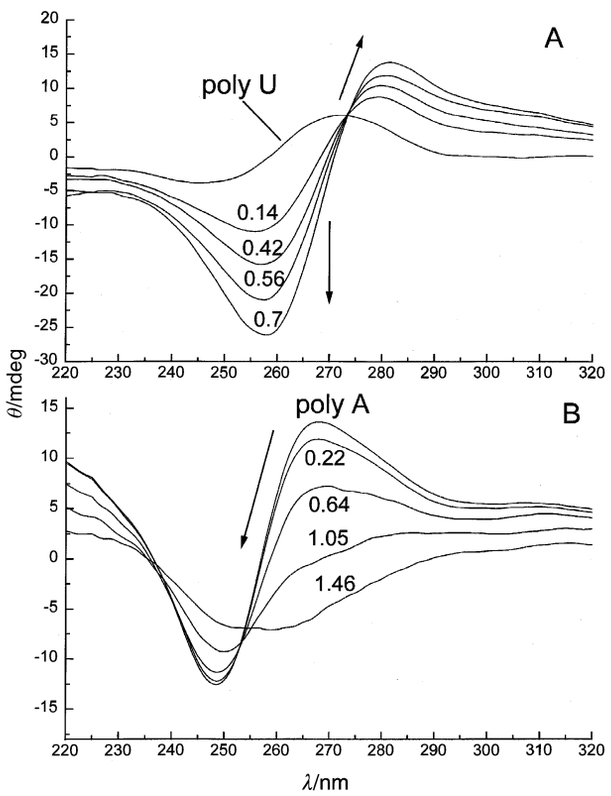



**Fig. 5** CD titration of poly U (**A**, $c = 4.5 \times 10^{-5}$ mol dm$^{-3}$) and poly A (**B**, $c = 3 \times 10^{-5}$ mol dm$^{-3}$) with **6**; ratio $r$ ([**6**] : [poly X]) shown for each spectrum; pH = 7, buffer PIPES, 0.01 M.

**Table 2** Stability constants ($K_s$) and ratios ($n$)[a] ([bound **6**] : [polynucleotide]) calculated from fluorimetric titrations of **6** with ds polynucleotides at pH = 5.0 (buffer citric acid (0.01 M), $I = 0.025$ M) and pH = 7.0 (buffer Na cacodylate (0.02 mol dm$^{-3}$), $I = 0.02$ mol dm$^{-3}$)

| | pH = 5 | | pH = 7 | |
|---|---|---|---|---|
| | $n$ | log $K_s$ | $n$ | log $K_s$ |
| poly A–poly U | 0.1 | 6.5 | 0.16 | 6.5 |
| poly G–poly C | 0.16 | 6.8 | 0.16 | 6.1 |
| poly dA–poly dT | 0.16 | 5.4 | 0.14 | 5.6 |
| poly dAdT–poly dAdT[c] | 0.09 | 6.1 | 0.14 | 5.9 |
| poly dGdC–poly dGdC[c] | 0.21 | 6.1[b] | 0.47 | 6.9[b] |
| Calf thymus DNA[c] | 0.34 | 6.4[b] | — | — |

[a] Accuracy of $n \pm 10$–30%, consequently log $K_s$ values vary in the same order of magnitude. [b] Cumulative value due to unknown contribution of non-intercalative mode of binding at ratios $r$ ([**6**] : [polynucleotide]) > 0.2. [c] High ionic strength buffers used: pH = 5, citric acid buffer, $I = 0.13$ mol dm$^{-3}$; pH = 7, buffer Na cacodylate, $I = 0.12$ mol dm$^{-3}$.

with the larger aromatic surface and somewhat stronger binding of **6** compared to **EB**.

### Interactions of 6 with double-stranded RNA and DNA polynucleotides

**Fluorescence titrations.** Binding constants for **6** and **EB** with double-stranded DNA and RNA polymers (Table 2) are mostly of the same order of magnitude and do not depend significantly on pH, base composition or structural differences between homo- and alternating polymers. A striking difference in fluorescence response is observed for **6**–poly GC and **6**–poly AU complexes. Upon addition of poly GC the emission intensity of **6** decreased by 80% while poly AU yielded an increase of 100% (Fig. 6). Similar effects are observed for DNA homopolymer poly dAdT and alternating polymers poly $(dAdT)_2$–poly $(dAdT)_2$ and poly $(dGdC)_2$–poly $(dGdC)_2$. Considering the different fluorescence properties of **6**–AMP complex (fluorescent)

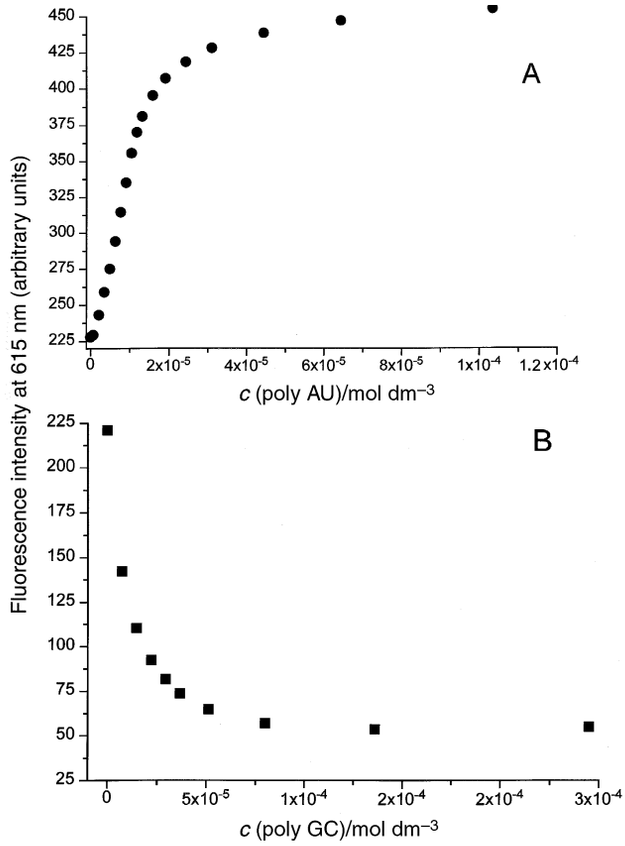



**Fig. 6** Fluorimetric titration of **6**, $c = 2 \times 10^{-6}$ mol dm$^{-3}$ with poly AU (●) and poly GC (■), pH = 7 (buffer Na cacodylate, $c = 0.02$ mol dm$^{-3}$).

and **6**–GMP complex (non-fluorescent) it is likely that **6** gives different fluorescence responses depending on the presence of A or G in DNA/RNA polymers. Some acridine derivatives also exhibited a similar nucleic base selective spectroscopic response; the effect was correlated with the electron donating properties of guanine.[17,25]

For both **6** and **EB**[26] additional, non-intercalative binding is present at $r > 0.2$. The non-intercalative binding contribution is more pronounced for **6**–DNA complexes (especially for the **6**–poly dAdT complex) than for the RNA complexes. It is interesting to note that addition of poly dAdT at low ionic strength and ratio $r > 0.2$ quenches the fluorescence of **6** (Fig. 7, **A**) while the emission decrease is not present if the titration is performed at high ionic strength ($I = 0.1$ mol dm$^{-3}$) (Fig. 7, **B**). The latter suggests that the observed emission decrease is the consequence of electrostatic interactions between **6** and polymer backbone phosphates. At higher $r$ ratios ($r < 0.05$) the intercalation of **6** in poly dAdT prevails, causing an increase in its fluorescence emission. Binding constants $K_s$ and $n$ values ($r < 0.1$) determined at different ionic strengths ($I = 0.01$ and 0.1 mol dm$^{-3}$) were practically the same (log $K_s/n$: 5.6/0.14 and 5.7/0.1, respectively) in accord with low sensitivity of intercalative binding to ionic strength.

Somewhat larger binding constants were obtained from titrations of **6** with poly GC, poly dGdC and calf thymus (CT)-DNA (Table 2). With these polymers both non-intercalative (electrostatic) and intercalative binding modes seem to cause quenching of **6**. At $r > 0.2$ already *ca.* 70% of emission intensity is quenched, preventing accumulation of sufficient data points for accurate calculations at $r < 0.1$ where the intercalation binding mode is prevalent. Therefore the calculated values of $K_s$ and $n$ are cumulative, comprising both binding modes; the latter can also explain the values of $n$ being larger than those theoretically possible for intercalation.

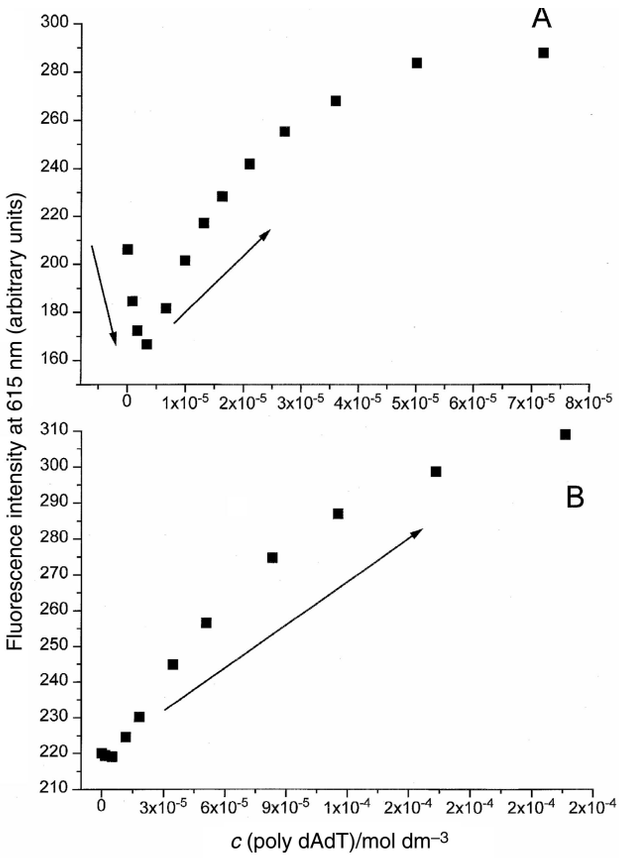



**Fig. 7** Fluorimetric titration of **6**, $c = 2 \times 10^{-6}$ mol dm$^{-3}$ with poly dAdT, pH = 7, at $I = 0.01$ M (**A**) and $I = 0.1$ M (**B**).

**UV/Vis titrations.** Bathochromic shifts of $\lambda_{max}$ at 520 nm of 25–35 nm and hypochromic effect of up to 50–60% observed in titrations of **6** with all examined DNA and RNA double-stranded polymers strongly suggest intercalation of **6** as the dominant binding mode.[22]

### Melting temperature UV studies with 6

For CT-DNA and poly dA–poly dT, the melting point increases ($\Delta T_m$) caused by **6** (in low ionic strength buffers $I \approx 0.02$ M) do not show significant pH dependence between pH 5 and 7 within the error limit (±0.5 °C). This is consistent with the almost identical melting point increases of poly dA–poly dT with **EB** at pH = 5 ($r = 0.3$: 14.0 °C) and pH = 7 ($r = 0.3$: 13.5 °C) (at low ionic strength) and the stability constants of **6** with poly dA–poly dT in both buffers (log $K_s = 5.4$, pH = 5; 5.6, pH = 7) and also poly $(dAdT)_2$ (log $K_s = 6.1$, pH = 5; 5.9, pH = 7), here at high ionic strength ($I = 0.125$ M).

A comparison of the melting point increases of CT-DNA by **6** and by its structurally closest analogue **2**[11] at pH = 5.0 in low ionic strength buffer shows at all ratios $r$ about 5–7 °C higher values of **6** with only one charge but two amino groups (*e.g.* $r = 0.3$: 23.5 °C for **6** and 16.1 °C for **2**). This is in line with the binding constants of both ligands to CT-DNA (log $K_s = 6.4$ for **6** and 4.7 for **2**). As it is known for **EB**,[27] the amino groups can give additional interactions with *e.g.* the 5′-oxygen atoms of the phosphate backbone which is probably the reason for the increased stabilisation. The diazapyrenium system of **6** with an additional phenyl ring also causes a difference of 6 °C compared to the phenanthridinium moiety of **EB** ($r = 0.3$: 17.3 °C; at low ionic strength). The same feature of a better binding of **6** compared to **1** and **2** and to **EB** is also seen at high ionic strength (pH = 5); **6** shows the $\Delta T_m$-values for CT-DNA are more than twice as large as those for **1**[11] or those for **EB** (*e.g.* $r = 0.3$: **6**, 6.5 °C; **1**, 3.0 °C; and **EB**, 3.2 °C); the log $K_s$ values of the three compounds and CT-DNA are 6.4 (**6**), 5.04 (**1**) and 5.8 (**EB**).

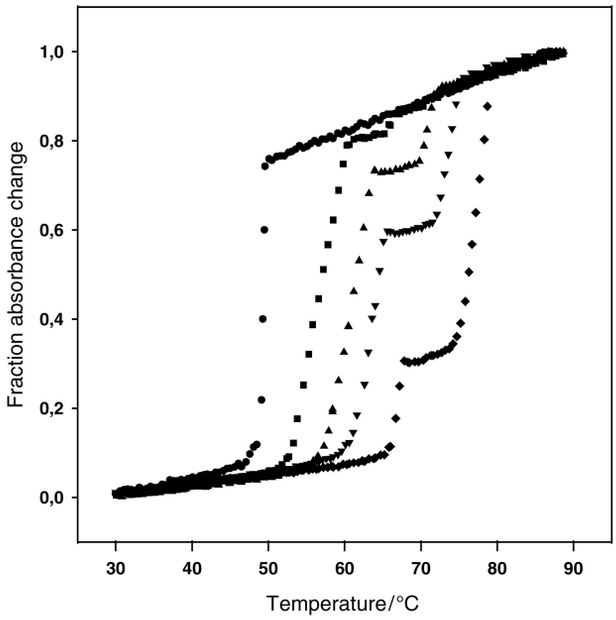



**Fig. 8** Melting curves of poly dA–poly dT and **6** at low ionic strength $I$ and pH = 7.0. For measuring conditions see footnotes to Tables 3 and 4 and the Experimental section; the ligand : nucleic acid ratios $r$ are: 0.0; 0.1; 0.2; 0.3; 0.5 (from left to right).

The melting profiles of poly dA–poly dT with **6** (supplementary material,† Fig. 8) in the buffers of low ionic strength ($I \approx 0.02$ M) show at both pH = 5 and pH = 7 an unusual behaviour absent with other diazapyrenium monointercalators like **2**[11] and also **EB**. The curves are clearly separated into two transitions with both midpoints lying above the melting temperature of the homopolymer-duplex alone; going from lower to higher ligand : phosphate ratios $r$, the absorbance of the second step becomes more and more pronounced until at $r \approx 0.7$ (pH = 5) or $r \approx 0.8$ (pH = 7) one sees more or less only one transition. An increase of both denaturation temperatures is seen with increasing ratio $r$ in a constant difference between each other of ≈ 10 °C. This is in contrast to the biphasic melting with some other ligands, due to insufficient saturation below $r = 0.16$, as was described earlier[11] for poly dA–poly dT (pH = 5). There the first midpoint increases faster than the second, merging finally in one single curve. This distinct biphasic behaviour of **6** with poly dA–poly dT below high ratios $r$ (in low ionic strength buffer) is in line with the fluorescence titrations with the same polymer (Fig. 7A,B). The first transitions in the melting profiles can be assigned to the intercalative complex; the latter is supported by the comparison of the $\Delta T_m$ values at *e.g.* $r = 0.3$ for **6** (I transition 12.5/II transition 22.8 °C) and for **EB** (14.0 °C). Therefore, the second transition should correspond to the non-intercalative complex. However, at high ionic strength both **6** and **EB** give monophasic melting profiles with similar stabilisation ($\Delta T_m$ values at *e.g.* $r = 0.3$: **6** 4.0 °C and **EB** 3.0 °C, Tables 4 and 5).

As with CT-DNA, **6** also shows higher melting temperature increases with poly dA–poly dT in low ionic strength buffer compared to the other diazapyrenium monointercalators.[11] Even at the lowest $r$ the $\Delta T_m$ is 5–8 times (6–10 °C) higher than the $\Delta T_m$'s of **2** at the highest ratios (*e.g.* $r = 0.3$: **6**, 12.5/22.8 °C corresponding to **2**, 2.7 °C); the second transition appears with a difference of 15–20 °C. The observed large stabilisation by **6** can be attributed to the additional binding interactions involving the amino substituents being absent in **2**. The same holds for the interactions with the RNA model poly A–poly U at all ratios (Table 3). The $\Delta T_m$ values of **6** are about seven times higher than the stabilising transitions in the profiles of the

**Table 3** $\Delta T_m$ values (°C) of **6** in *low* ionic strength buffers pH = 5 and pH = 7[a]

| pH | *r*= | 0.1 | 0.2 | 0.3 | 0.5 |
|---|---|---|---|---|---|
| 5 | CT-DNA | 14.5 | 19.6 | 23.5 | 28.8 |
| | poly dA–poly dT | 6.8/16.3 | 10.3/20.3 | 12.5/22.8 | 15.7/26.7 |
| | poly A–poly U | 28.2 | 35.6 | 39.0 | 43.6 |
| 7 | CT-DNA | 13.9 | 19.4 | 23.2 | >26.5 |
| | poly dA–poly dT | 7.1/16.7 | 11.3/21.6 | 13.8/24.3 | 17.5/27.9 |
| | poly A–poly U | 10.9/23.3 | 25.5 | 29.0 | 32.9 |

[a] *r*: molar ratio of ligand : nucleic acid phosphates; CT-DNA: calf thymus DNA; ionic strength $I = 0.025$ M (pH = 5.0) and $I = 0.016$ M (pH = 7); error in $\Delta T_m$: ±0.5 °C.

**Table 4** $\Delta T_m$ values (°C) of **6** in *high* ionic strength buffer pH = 5[a]

| *r*= | 0.1 | 0.2 | 0.3 |
|---|---|---|---|
| CT-DNA | 2.7 | 5.6 | 6.5 |
| poly dA–poly dT | 1.9 | 3.0 | 4.0 |
| poly A–poly U | 4.2 | 8.4 | 10.6 |

[a] See footnotes to Table 3, except: $I = 0.125$ M.

**Table 5** $\Delta T_m$ values (°C) of **EB** in *low* and *high* ionic strength buffers pH = 5 and pH = 7.0[a]

| pH | *r* = 0.3 | $I = 0.025$ M | $I = 0.125$ M |
|---|---|---|---|
| 5 | CT-DNA | 17.3 | 3.2 |
| | poly dA–poly dT | 14.0 | 3.0 |
| | poly A–poly U | 36.1 | 10.7 |
| 7 | CT-DNA | — | — |
| | poly dA–poly dT | 13.5 | — |
| | poly A–poly U | 29.1 | — |

[a] See footnotes to Tables 3 and 4.

related diazapyrenium derivative **2** (pH = 5, *e.g.* *r* = 0.3: **2**, −4.0/+5.9 °C and **6**, 39.0 °C). Ethidium bromide with two amino substituents shows stabilisation similar to that of **6** in both low and high ionic strength buffers (*r* = 0.3: **EB**, 36.1 °C compared to **6**, 39.0 °C at $I = 0.025$ M and at $I = 0.125$ M, **EB**, 10.7 °C and **6**, 10.6 °C; Tables 3–5).

In contrast to the behaviour of all other examined 4,9-diazapyrenium compounds towards poly A–poly U with one destabilising and one stabilising step,[11] **6** gives only positive values at both pH = 5 and pH = 7 ($I \approx 0.02$ M). The poly A–poly U denaturation curves with **6** (data not shown) exhibit two phases only at low ratios ($r < 0.1$) which is due rather to insufficient saturation than to two distinct binding modes (as discussed above in the case of poly dA–poly dT). In contrast to poly dA–poly dT, in the fluorescence titrations with poly A–poly U a decrease of emission due to the non-intercalative binding could not be observed. The observed non-intercalative binding of **6** to poly dA–poly dT could be the consequence of its more narrow and deeper minor groove compared to that of poly A–poly U.[28]

In contrast to CT-DNA and to poly dA–poly dT, the $\Delta T_m$ values for **6** and poly A–poly U are about 5–11 °C higher at pH = 5 than at pH = 7 (*e.g.* *r* = 0.3: 39.0 °C at pH = 5 and 29.0 °C at pH = 7, Table 3), although the stability constants are comparable (log $K_s = 6.5$ at both pH, Table 2). It is known that single-stranded poly A at acidic pH[21,29] forms stable poly $AH^+$–poly $AH^+$ duplexes. During thermal denaturation of poly A–poly U at pH 5, the poly A released is expected to form a duplex to which the ligand can intercalate. The $\Delta T_m$ then reflects a mixed binding to poly A–poly U and poly $AH^+$–poly $AH^+$. The pH dependence of the poly A–poly U melting temperatures is also observed with **EB** (*r* = 0.3 : 36.1 °C at pH = 5 compared to 29.1 °C at pH = 7, $I \approx 0.02$ M, Table 5).

In order to evaluate RNA/DNA selectivity for **6**, the $\Delta T_m$ poly A–poly U : $\Delta T_m$ poly dA–poly dT ratio was calculated using the data for the first transition in the melting curves of poly dA–poly dT attributed to the intercalation mode. At all *r* ratios, the selectivity factor was ≈ 3 at pH = 5 and ≈ 2 at pH = 7. Such a preference of **6** for the poly A–poly U polymer is comparable to that of **EB** (2.4)[5] and to the other 4,9-diazapyrenium compounds[11](1.8–2.7).

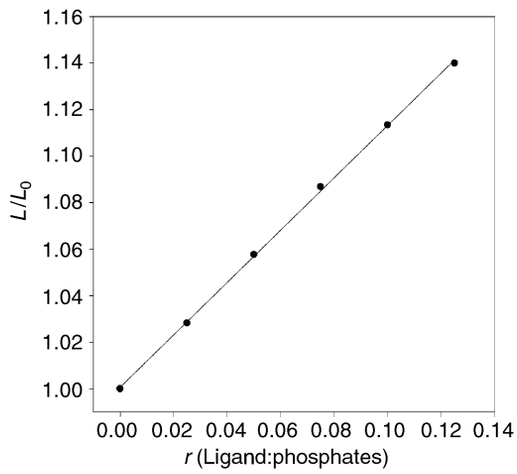



**Fig. 9** Helix length extension ($L/L_0$) *vs.* ligand : DNA phosphate ratio *r*; plot for **6** at pH = 5.0 (low ionic strength).

### Viscometry

Viscometric titrations of **6** with CT-DNA in low salt citric acid buffer gave a slope of $\alpha = 1.1$ (Fig. 9); this is close to that of **EB** ($\alpha = 1.0$)[5,11] and consistent with monointercalation at ligand to phosphate ratios $r < 0.12$.

## Conclusions

The results demonstrate that relatively small structural variations in intercalating ligands can lead to dramatic differences in their interactions with nucleic acids. This study also shows how different methods such as UV/Vis, fluorescence and CD measurements and melting analyses lead, when applicable, to a consistent picture of binding differences.

Although the binding affinity of **6** toward nucleotides is similar to that of **EB** and the previously studied 4,9-diazapyrenium analogue **1**, a smaller affinity of **2** toward nucleotides is probably due to the larger hydration shell of this doubly charged ligand.

The observed specific fluorescence response of **6** on binding to double-stranded G–C (quenching) and A–U(T) (emission increase) polymers could be of interest for development of sensors for such polynucleotides.

In view of the bulky phenyl substituents placed at both ends of the short 4,9-diazapyrenium axes of **6** it seems reasonable to propose threading intercalation into double-stranded polynucleotides as a main binding mode, with the intercalator and adjacent base pairs' long axes being parallel. Stability constants of **6**–ds polynucleotide complexes are similar to those of **EB**, somewhat larger than found for analogue **1**, and almost two

orders of magnitude larger than those for the analogue **2**. Such a difference in affinity along with the significantly larger stabilisation of the double strand by addition of **6** than found for analogues **1** and **2** points to additive interactions of amino substituents of **6** and **EB** with polynucleotides. Additional non-intercalative (electrostatic) interactions of **6** with ds polymers under conditions close to saturation of polymer ($r > 0.2$) are more pronounced for DNA than for RNA derivatives.

Interactions of **6** with ss polynucleotides depend strikingly on the nucleobase composition and in some cases on pH. The complex of **6** with poly U formed close to saturation of poly U ($r \approx 1$) has a well organised, possibly helical structure (CD evidence); it is much more stable than the other complex dominating at large excess of poly U, where **6** binds on "isolated" binding sites with an affinity similar to that of **EB**. Until now none of the intercalators had exhibited such different modes of binding exclusively on one ss polymer combined with a specific change in chiral properties of the polynucleotide. Fluorimetric properties of **6**–poly C are found to be strongly pH dependent, pointing to the sensitivity of the **6** chromophore system to electron donor–acceptor surrounding conditions.

## Experimental

$^1$H-NMR spectra were recorded on Varian-Gemini 300 MHz and Bruker 500 MHz Avance DRX instruments with tetramethylsilane as internal standard for organic solvents and the acetone signal as external, or acetate buffer as internal, standard for aqueous media. Chemical shifts ($\delta$) are expressed in ppm, with numbering of protons according to Fig. 2. Signal multiplicities are denoted by s (singlet), d (doublet), t (triplet), pt (pseudotriplet—formed from overlapping double doublet), q (quartet) and m (multiplet). Electronic absorption spectra were obtained on a Varian Cary 1 spectrometer using quartz cuvettes (1 cm). Fluorescence spectra were recorded on a Perkin-Elmer LS 50 fluorimeter. CD spectra were recorded on JASCO J-715 spectropolarimeter in quartz cuvettes (1 cm), total absorbance at the end of titration being $Abs_{max} = 1.5$. Electron spray mass spectra (ES-MS) were obtained using a Varian MAT 711 spectrometer. For chromatographic purification of the prepared compounds silica gel $HF_{254}$ (Merck) for preparative thin layer chromatography was used.

### Solutions

Low ionic strength citric acid buffer contained 0.01 mol dm$^{-3}$ citric acid, adjusted to pH = 5 ($I = 0.025$ mol dm$^{-3}$); low ionic strength PIPES buffer contained 0.01 mol dm$^{-3}$ PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)), adjusted to pH = 7 ($I = 0.016$ mol dm$^{-3}$). High ionic strength citric acid buffer contained 0.01 mol dm$^{-3}$ citric acid and 0.1 mol dm$^{-3}$ NaCl, adjusted to pH = 5 ($I = 0.125$ mol dm$^{-3}$). For NMR experiments acetate buffer ($CD_3COOD$–NaOD; 0.05 M; pD = 5.4) was used.

Polynucleotides were purchased as noted: poly A, poly C, poly U, poly dA–poly dT, poly $(dAdT)_2$–poly $(dAdT)_2$ and poly $(dGdC)_2$–poly $(dGdC)_2$ (Pharmacia); poly G, poly G–poly C, poly A–poly U (Sigma), calf thymus (CT)-DNA (Aldrich). Polynucleotides were dissolved in the respective buffer and their concentration determined spectroscopically[11] as the concentration of phosphates.

### Methods

All titration data were corrected for dilution, significant ones are available as supplementary material. Stability constants ($K_s$) of **6**–nucleotide complexes were calculated by processing fluorimetric titration data with the program SPECFIT.[18]

Determination of self-stacking by $^1$H-NMR experiments was done by changing the $c$(**6**) in acetate buffer. 2D NMR experiments were done in $D_2O$ with acetone as external standard.

UV/Vis and fluorescence titrations were used to determine the binding affinity of **6** and **EB** as reference intercalator toward polynucleotides. In fluorimetric titrations excitation wavelength of 385 nm was used and changes of emission were monitored at 615 nm. Where possible, using the titration data stability constants ($K_s$) and [bound intercalator] : [polynucleotide phosphate] ratios ($n$) were calculated according to the Scatchard equation[30] by non-linear least-squares fitting methods.[11] Values for $K_s$ and $n$ given in Tables 1 and 2 are results of calculations of absolute minima and all had satisfying correlation coefficients (> 0.999).

Thermal melting curves for DNA, RNA and their complexes were determined as previously described[11] by following the absorption change at 260 nm as a function of temperature. The absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalised. $T_m$ values are the midpoints of the transition curves, determined from the maximum of the first derivative or graphically by a tangent method.[31] $\Delta T_m$ values were calculated by subtracting $T_m$ of the free nucleic acid from $T_m$ of the complex. Every $\Delta T_m$ value reported here was the average of at least two measurements; the error in $\Delta T_m$ is ± 0.5 °C.

Viscometric titrations were conducted in an Ubbelohde micro-viscometer (Schott) as previously described.[11] The concentration of CT-DNA in low salt citric acid buffer was $5 \times 10^{-4}$ mol dm$^{-3}$ in phosphates, the ligand to DNA phosphate ratio $r$ less than 0.15. The viscosity index $a$ was obtained from the flow times at varying $r$ according to eqn. (1),[32] where $t_0$, $t_{DNA}$

$$L/L_0 = [(t_r - t_0)/(t_{DNA} - t_0)]^{1/3} = 1 + a^*r \qquad (1)$$

and $t_r$ denote the flow times of buffer, free DNA and DNA complex at ratio $r$, respectively:

### Synthesis

The starting compound 2,7-diamino-5,10-diphenyl-4,9-diazapyrene **3** was prepared by a published procedure.[13] The Hygroscopic nature of the precipitates hampered elementary analysis. However, compounds **5** and **6** were prepared according to a previously well studied procedure,[10] structures being confirmed by (1D and 2D) NMR and ES-MS data.

**2,7-Bis(benzyloxycarbonylamino)-5,10-diphenyl-4,9-diazapyrene 4.** Starting compound **3** (52 mg, 0.135 mmol) and $NaHCO_3$ (36 mg, 0.4 mmol) were suspended in dry DMF (2 ml). To a cooled suspension (0–5 °C) a 50% solution of benzyloxycarbonyl chloride was added (0.14 ml, 4 mmol) while stirring. After 2 hours of stirring at room temperature 5 ml of dry diethyl ether were added, and the precipitate was collected and washed with water. Recrystallization from hot methanol gave **4** (50–61% yield, mp. 280–282 °C). $^1$H NMR ($\delta_H$/ppm, DMSO-$d_6$): 5.23 (s, 4H, 2 × $OCH_2$), 7.36–7.49 (m, $10H_{benzyl}$), 7.67–7.69 and 7.92–7.94 (2m, $10H_{phenyl}$), 8.74 and 8.75 (2s, 4H, C1-H, C3-H, C6-H, C8-H), 10.55 (s, 2H, 2 × NH).

**2,7-Diamino-5,10-diphenyl-4-methyl-4,9-diazapyrenium triflate 5.** A solution in chlorobenzene (6 ml) of **4** (100 mg, 0.15 mmol) and methyl triflate (1.6 ml, 0.15 mmol) was heated under reflux for 2 hours. After cooling to room temperature triflic acid (0.05 ml, 0.5 mmol) was added to the dark red suspension and upon treatment of the oily residue in an ultrasound bath, the suspension was stirred overnight. Chlorobenzene was decanted and to the oily residue 5 ml of dry diethyl ether were added.The blue precipitate was collected, washed with dry ether and purified by preparative thin layer chromatography on silica gel using 10% methanol in dichloromethane for elution. Evaporation of solvent and recrystallization from acetonitrile gave **5** (40–50% yield).$^1$H NMR($\delta_H$/ppm, $CD_3CN$): 4.3 (s, 3H, $CH_3$), 5.16 (s, 2H, C7-$NH_2$), 5.52 (s, 2H, C2-$NH_2$),

7.18 (d, $J_{67}$ = 1.9 Hz, 1H, C6-H), 7.72–7.75 and 7.87–7.94 (2m, $10H_{phenyl}$), 8.0 and 8.15 (2s, 3H, C1-H, C3-H, C8-H); ES-MS (*m*/*z*) 401.1 ($M^+$), $CF_3SO_3^-$ 148.7 ($M^-$).

**2,7-Diamino-5,10-diphenyl-4-methyl-4,9-diazapyrenium hydrogensulfate 6.** To a solution of triflate **5** (16 mg, 0.03 mmol) in acetonitrile (1 ml), an acetonitrile solution of tetrabutylammonium hydrogensulfate (175 mg, 5 mmol) was added. After standing for 30 min at room temperature, the precipitate formed is collected and washed with 3 × 0.5 ml of dry dichloromethane followed by recrystallization from DMSO–dichloromethane giving a dark blue precipitate of **6** (76% yield).$^1$H NMR($\delta_H$/ppm, $D_2O$, $c$(**6**) = $5 \times 10^{-3}$ mol dm$^{-3}$): 3.89 (s, 3H, $CH_3$), 6.60 (s, 1H, C6-H), 6.85 (d, 2H, $H4_{phenyl}$), 6.87 (s, 1H, C8-H), 6.96 (d, 2H, $H1_{phenyl}$), 7.13 (s, 1H, C1-H), 7.38 (pt, 2H, $H5_{phenyl}$), 7.49 (s, 1H, C3-H), 7.54 (pt, 2H, $H6_{phenyl}$), 7.61 (pt, 2H, $H2_{phenyl}$), 7.78 (pt, 2H, $H3_{phenyl}$); ES-MS (*m*/*z*) 401.1 ($M^+$), $HSO_4^-$ 96.8 ($M^-$).

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