Recognition of Nucleotides and Polynucleotides by Phenanthridinium and Diazapyrenium Derivatives in Aqueous Media

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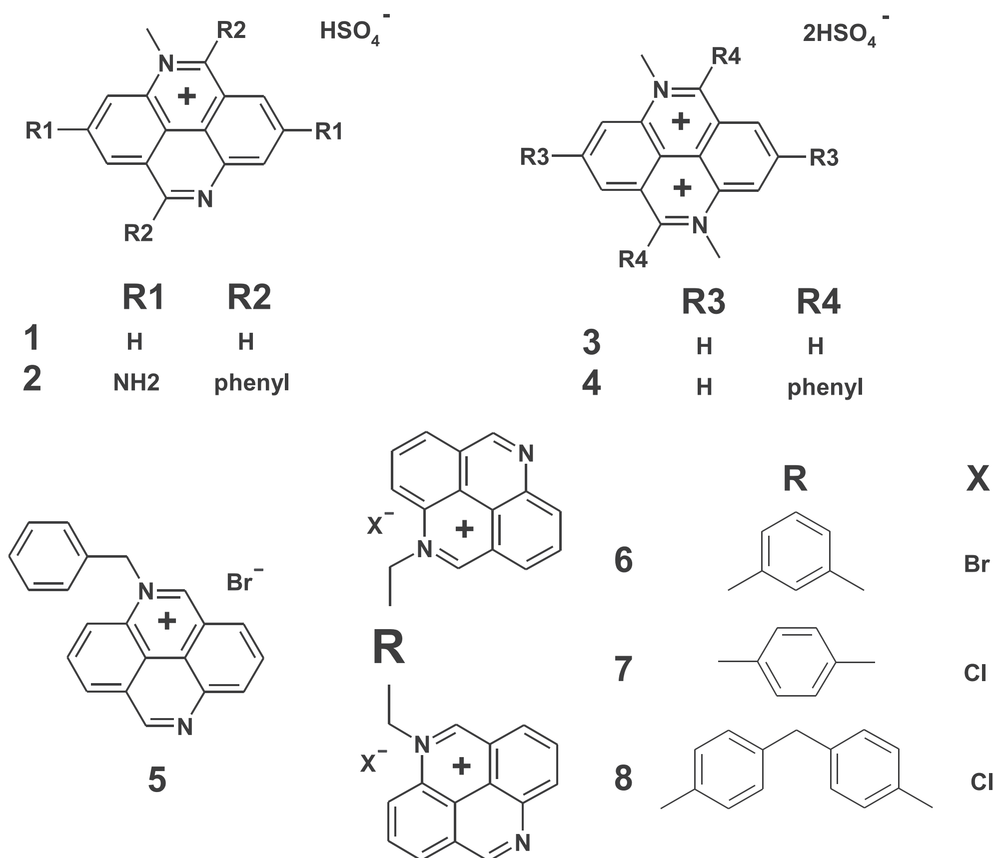
Interactions of 4,9-diazapyrenium cations with nucleotides and polynucleotides were studied in buffered aqueous solution (pH 5 or 7) by UV-Vis, fluorescence, NMR and viscometry. The formation of stacked nucleotide / 4,9-diazapyrenium and nucleotide-phenanthridinium complexes of 1 : 1 stoichiometry with log K_s 1.6 – 2.8 and log K_s < 1 to 2.3, respectively, suggests that larger surface of the former cationic heteroaromatics does not considerably enhance nucleotide binding. The stability constants were found independent on nucleotide charge suggesting the dominance of aromatic stacking over coulombic interactions in such complexes. Monomeric 4,9-diazapyrenium cations bind to *ss*- and *ds*-polynucleotides by intercalation. Affinities were found to be essentially independent on the number of permanent charges present in the aromatic system but strongly dependent on a type of substituents attached. 2,7-Diamino substituted 4,9-diazapyrenium derivative has shown the highest affinity toward polynucleotides, the highest double helix stabilisation and highly specific fluorescence response on binding to double-stranded G – C (quenching) and A – U(T) (emission increase) polymers. Interactions of 2,7-diamino-4,9-diazapyrenium derivative with *ss*-polynucleotides, in contrast to other derivatives, depend strikingly on the nucleobase composition and in some cases on pH. The bis(4,9-diazapyrenium) dicationic derivatives, differing in rigidity and length of the linkers connecting diazapyrenium units, were found to bind *ds*-polynucleotides by monointercalation; some of them exhibited strong additional interactions of the non-intercalated diazapyrenium unit with the outer surface of a polynucleotide.

The novel phenanthridinium – nucleobase conjugates were prepared and studied by spectroscopic methods. An analysis of ^{1}H NMR, UV-Vis and fluorescence spectra in aqueous media revealed intramolecular aromatic stacking interaction between phenanthridinium unit and nucleobase, resulting with folded conformation of molecules. Fluorimetric titrations showed that phenanthridinium-nucleobase conjugates **9, 10, 15** and **16** and reference phenanthridinium compounds **9** and **14** form 1 : 1 non-covalent complexes with nucleotides in water with binding constants ranging from 10^1 to 10^2 mol^{-1} dm^3, while compounds **11** and **13** form intercalative type of 1 : 1 complexes with nucleotide aromatic unit inserted between phenanthridinium and covalently attached nucleobase, yielding binding constants of 10^3 –10^4 mol^{-1} dm^3. Conjugates are binding either complementary or non-complementary nucleotides, showing a lack of any base recognition. Aromatic $\pi \ldots \pi$ stacking interactions are found to be dominant in complexes of all studied compounds with nucleotides. Adenine containing *N*-protonated conjugates **10** and **11** exhibit specific spectroscopic changes and two orders of magnitude higher affinity toward poly-U than uracil conjugates **12** and **13** and the reference compound **9** due to the existence of specific interactions between adenine and uracil, possibly Watson-Crick hydrogen bonding between the bases stacked on the phenanthridinium moiety. Contrary to **10** and **11,** the *N*-methylphenanthridinium--adenine conjugate **16** exhibited at pH 5 preferred binding to double stranded (*ds*-) poly-AH$^+$. The binding studies of *N*-methylated compounds **14-16** with *ss*-polynucleotides showed no preference of conjugates to polynucleotides containing complementary nucleobases. The results reveal that the presence of protonated or permanently charged intercalator units in the conjugates dramatically changes their binding preferences for polynucleotides.

Keywords: *Phenanthridinium, diazapyrenium, DNA, RNA, intercalation*

Introduction

The search for new nucleic acid intercalators[1] is stimulated by a variety of reasons. Exploiting the relatively high association constants of intercalators one can couple such compounds e.g. to selected oligonucleotides useful for preparation of stable triple helices[2] or to catalytic units for production of synthetic nucleases.[3] With new intercalators, capable of binding mono- or oligonucleotides, one can also hope to reach deeper understanding of intercalation mechanisms.[4] Recently discovered selective interaction of ethidium bromide (**EB**) with certain HIV-related sequences of RNA[5] stirs



F i g . 1 – *4,9-Diazapyrenium and bis(4,9-diazapyrenium) derivatives*

additional interest for development of new aromatic systems with even higher selectivity. Besides ethidium bromide, related 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.

Although for the first time prepared almost 50 years ago,[8] physico-chemical properties and noncovalent interactions of 4,9-diazapyrenium derivatives in any biologically relevant system were never studied. The 4,9-diazapyrenium derivatives (Fig. 1) possess a large aromatic surface, comparable to pyrene. At variance to the latter, these compounds have two nitrogens within the aromatic system, which upon methylation can provide positively mono- or doubly-charged derivatives. If compared to phenanthridine, the dizapyrene possesses larger aromatic surface and two nitrogens, which upon protonation or alkylation can provide monopositively charged like phenathridinium or doubly-charged derivatives. On the other hand, different positions of positive charges on the isomeric 2,7- and 4,9-diazpyrenium systems, and hence directions of the permanent dipoles could result in different binding and/or recognition of nucleotide bases by these systems.

Most of the intercalators known to date exhibited rather strong binding to DNA, however, with poor or even absent any base composition selectivity.[1] In the last decade several groups reported on the preparation of small synthetic molecules constructed of an intercalator unit and a linker bearing covalently attached nucleobase. Such intercalator-nucleobase conjugates were predicted to act as the multifunctional receptors, providing a complementary nucleobase recognition site through base-to-base hydrogen-bonding and strong stabilisation by aromatic stacking between the intercalator unit and the formed base pair. Until now, such conjugates were constructed from acridine,[9] phenanthridine,[10] naphthalene diimide[11] or porphyrine[12], and in most cases from adenine or thymine. With such systems only binding to complementary polynucleotides was studied. Although significant specific interactions were reported in some cases, the base recognition through hydrogen bonding was difficult to assess only on the basis of enhanced binding to the complementary polynucleotide, since the latter may be also the consequence of different types of weak binding interactions such as the electrostatic interactions between positively charged linkers and polynucleotide phosphates, and also by the hydrophobic interactions which could considerably contribute to the overall binding energy.[1] To the best of our knowledge only one publication describes the study of the simple system in aqueous media comprising the acridine – nucleobase conjugate and various nucleobase derivatives.[9c] According to the binding constants determined, the 3-fold preference for the complementary nucleobase derivative was reported but the hydrogen bonding between the complementary bases has not been explicitly proven. To shed more light on the possibility of nucleobase recognition by the intercalator-nucleobase conjugates we have prepared a series of phenanthridinium – adenine and -uracil conjugates possessing uncharged aliphatic linkers of different lengths and the *N*-5 protonated or methylated intercalator unit (Fig. 2).

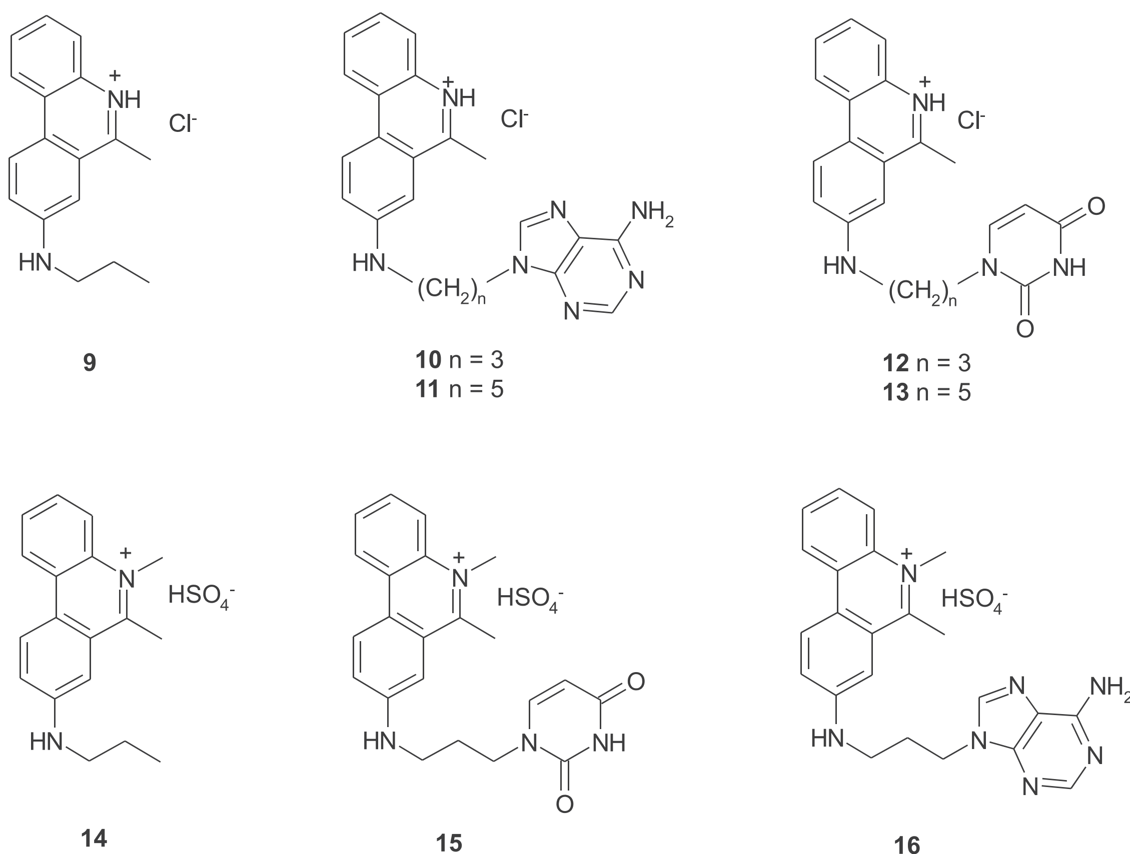


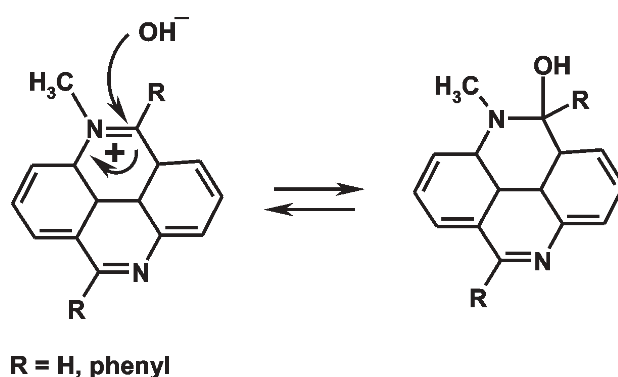
Fig. 2 – *N-5-protonated or methylated phenantridinium-nucleobase conjugates with varying lengths of the linkers*

In this account we present the studies of the physico-chemical properties of new 4,9-diazapyrenium systems and the phenanthridinium-nucleobase conjugates, their binding properties toward the complementary and non-complementary nucleotides and, *ds*- and *ss*-polynucleotides of DNA and RNA types. The results shed more light on the relative importance of hydrogen bonding, aromatic $\pi \ldots \pi$ stacking and the intercalator size and charge on the recognition of nucleotides and polynucleotides, and provide new guidance for construction of the nucleobase selective ligands.

Physico-chemical properties of 4,9-diazapyrenium cations (DAPs)

The self association constants determined for monocationic 4,9-diazapyrenium derivatives **1** and **2**[13,14] are close to that of ethidium bromide[15] (K_{ass} = 350–400 mol^{-1}dm^{3}). Dicationic bis(4,9-diazapyrenium) derivatives **6–8** showed no evidence of self-association in the concentration dependent NMR experiments nor in the UV spectra taken in the concentration ranges of 10^{-2} – 10^{-3} and 10^{-4} – 10^{-6} mol dm^{-3}, respectively. The absence of shielding effects in the NMR spectra and any appreciable hypochromicity in UV leads to the conclusion that the diazapyrenium units of **6–8** adopt an *anti*-orientation.

The electronic absorption, ^{1}H NMR and fluorescence spectra of most of the mono- and di-cationic 4,9-diazapyrenium salts taken in water showed strong pH dependence. These observations were explained by the reversible formation of the corresponding diazapyrenenium mono-pseudobases DMOH (Scheme 1).[13]



Scheme 1 – *Mechanism of reversible formation of the diazapyrenenium mono-pseudobase DMOH*

The equilibrium constants (expressed as pK_{DMOH}) were calculated from the pH dependent changes in the electronic absorption spectra (Table 1). The pK_{DMOH} values are strongly dependent on the type and position of the substituents on the diazapyrenium ring.

Some dicationic DAP derivatives (**3**) have shown to be chemicaly unstable in aqueous solutions of physiologically relevant pH (pH 5-8), probably due to the low p*K* values and high reactivity of corresponding diazapyrenenium pseudo-

bases. Their analogue (**4**) due to the influence of phenyl substituents at 5- and 10- position of DAP system exhibited higher p*K* value and much better chemical stability of both opened and closed form. An interesting feature of **2** is the absence of a pH dependent formation of 5-hydroxy-4,9-diazapyrene pseudobase in the pH range 3–10 (Scheme 1). Apparently, the presence of electron donating 5,10-diphenyl and 2,7-diamino-substituents of **2** strongly increase its stability against pseudobase formation in neutral and weakly basic conditions.

Table 1 – pK_{DMOH} *values for mono-pseudobase (DMOH) formation from 4,9-diazapyrenium compounds* **1, 3, 4, 5, 6–8**[a]

	λ_{max}/nm	pK_{DMOH}
1	236	9.6
3	239	5.4
4	440	6.5
5	235	8.3
6	393	7.6
7	393	7.3
8	392	7.4

[a] Concentration of each 4,9-diazapyrenium salt was 2×10^{-5} mol dm^{-3}. Changes of absorbance at specified λ_{max} occurring upon titration with 0.1 mol dm^{-3} HCl or NaOH (pH range 2 to 10) were used for calculations.

Binding of nucleotides

Interactions of 4,9-diazapyrenium derivatives **1 – 8** (Fig. 1) with nucleotides in buffered aqueous solution (pH 5 and for **2** at pH 7 also) at constant ionic strength (NaCl or Na_2SO_4, 0.01 mol dm^{-3}) have been studied by fluorescence.[13,18] Titration of DAPs with nucleotides induced total quenching of their fluorescence emission. The only exception is 2,7--diamino-substituted analogue **2**. Addition of AMP, ATP and CMP in excess does not quench fluorescence emission down to zero as in the case of analogues **1** and **4**[13], obviously due to influence of amino substituents. Only addition of GMP yielded total quenching of **2** emission. Similar differences in fluorimetric response were reported for proflavine and explained by more pronounced electron donating properties of guanine compared to other nucleic bases.[16]

The stability constants (Table 2) were calculated from titration data by the non-linear least squares fitting using the SPECFIT[17] program. In the case of monomeric derivatives **1, 2, 4, 5** the best fit was obtained for 1 : 1 stoichiometry of the complexes, while for dimeric analogues **6–8** the best fit was obtained for the formation of two complexes; the one of 1 : 1 **DAPdimer** / nucleotide stoichiometry exhibiting fluorescence emission, and the other of 1 : 2 stoichiometry being non-fluorescent.[13,14,18] Calculated $K_{s_{11}}$ values for **DAPdimer** / nucleotide complexes (Table 2) are of the same order of magnitude as those determined for monomer DAPs (**1, 2, 4, 5**) being in agreement with the independent binding of one nucleotide by each of the diazapyrenium units. In most cases values of $K_{s_{12}}$ were not possible to calculate accurately, due to the low percentage to complex formed.

Table 2 – *Binding constants log* K_{11} *(log* K_{12}*)*[a] *of* ***DAP's*** *with different nucleotides*

Compd.	AMP[b]	ATP[b]	GMP[b]	CMP[b]
1	2.21	2.24	2.11	1.37
2	2.16	2.39	2.30	1.61
4	1.67	1.78	1.66	< 1[d]
5	2.40	2.37	2.11	1.33
6[c]	2.25(d)	2.21(d)	2.10(d)	1.42(d)
7[c]	2.60(d)	2.85(d)	2.23(d)	1.63(d)
8[c]	2.56(2.18)	2.92(2.08)	2.68(2.27)	(d)

[a] Calculated by processing titration data with SPECFIT

[b] AMP^{2-} = adenosine monophosphate; ATP^{4-} = adenosine triphosphate; GMP^{2-} = guanosine monophosphate; CMP^{2-} = cytidine monophosphate

[c] for dimeric DAPs best fit was found for the equilibria $L + N \rightleftharpoons LN$ and $LN + N \rightleftharpoons LN_2$ (L = ligand, N = nucleotide), K_{11} and K_{12} respectively;

[d] Not determined due to low percentage to complex formed

Comparing **DAP** analogues with **EB** it appears that neither the presence of two positive charges (**4**) nor the larger aromatic surface (**2**) leads to considerable increase of binding of nucleotides. Similar binding affinity of **DAP's** 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 stacking interactions between **DAP** and nucleobases as dominant interaction stabilizing the complexes.

Interaction of 4,9-diazapyrenium cations with DNA and RNA

To elucidate interactions of simple single charged 4,9-diazapyrenium system with DNA we used **1**. Since its double charged analogue **3** has shown to be chemicaly unstable in aqueous solutions, for study of impact of double charge in DAP system on interactions with DNA, we have used compound **4**. To study influence of different substituents attached to DAP system we have used **2**, **4** and **5**. Bis-diazapyrenium derivatives **6**, **7**, and **8** were designed to assess their bis-intercalation potential by variation of spacer length and rigidity.[19]

Thermal melting experiments, UV-Vis and fluorescence titrations, viscometry and NMR experiments were used to characterize complexes of studied DAPs with *ds*-polynucleotides.[20] Stability constants calculated by means of Scatchard equation[20, 21] of most monomeric DAP (**1, 4, 5**)[19] complexes with *ds*-polynucleotides were found to be of the same order of magnitude as those reported for a number of other classical intercalators (**EB**, acridine dyes) (Table 3).

Spectroscopic properties of afore mentioned complexes (hypochromic and bathochromic effect in UV-Vis spectra, fluorescence quenching) and thermal stabilisations of *ds*-polynucleotide helices (ΔT_m), induced by their binding also agree with intercalative binding mode. Final proofs for intercalation of **1, 4** and **5** into *ds*-polynucleotides are characteristic changes in the NMR spectra[19] such as the line width increases of up to 70 Hz, and viscometry results showing a rather uniform length increase of the calf thymus

DNA double helix with slopes similar to those found for known mono-intercalator ethidium bromide (α = 1.0).[5,19] Comparing the values of binding constants (log K_s) and ratios $n_{ligand\ /\ DNA\ phosphate}$ (Table 3) it can be concluded that mono (**1, 2** and **5**) and double charged (**4**) DAPs do not interact with DNA similarly but strongly depend on a type of substituents attached. It seems that there are affinities essentially independent of the number of charges present in the DAP system, in line with negligible electrostatic binding contributions and with the corresponding affinities towards nucleotides.

T a b l e 3 – *Binding constants (log K_s) and bound ratio n (ligand to DNA phosphate) for diazapyrenium compounds, ethidium bromide (EB) and calf thymus DNA obtained from Scatchard binding isotherms*

	EB	1	2[c]	4	5	6	7	8
n	0.17	0.12	0.34[d]	0.16	0.11	0.14	0.07	0.3
log K_s ($dm^3\ mol^{-1}$)[a]	6.0	5.0	6.4[d]	4.6	5.3	5.8	6.9	6.0

[a] Accuracy: $n \pm$ 10–20 %, K_s in the order of magnitude

[b] pH 4.5, citric acid / Na_2HPO_4 buffer, I = 0.01 mol dm^{-3}, data processed by means of Scatchar equation

[c] pH 5.0 (buffer citric acid, I = 0.025 mol dm^{-3})

[d] Cumulative value due to unknown contribution of non-intercalative mode of binding at ratios *n* ([**6**] / [polynucleotide]) > 0.2.

Ligands containing two diazapyrenium rings bridged either by a 2,2′-(methylenedibenzyl) (**8**) or by para-xylylene (**7**) spacer showed, both, a significantly higher affinities for *ds*-polynucleotides and stronger thermal stabilization than the monomeric **5** suggesting bis-intercalation. In contrast, the dimeric **6** exhibited the affinity characteristic for mono-intercalation. Spectroscopic effects of all bis-DAP derivatives (**6, 7, 8**) observed upon their binding to *ds*-polynucleotides (hypochromic and bathochromic effect in UV-Vis spectra, fluorescence quenching) agree with intercalative binding mode. Viscometry, however, for **6, 7** and **8** showed rather uniform length increase of the calf thymus DNA double helix with slopes of α = 1.1, similar to those found for the common mono-intercalators.[19,10] Therefore, the results of binding and thermal denaturation studies with **7** and **8** suggest their mono-intercalation with additional, possibly electrostatic interaction of the non-intercalated DAP unit with negatively charged phosphates of polynucleotide. In contrast, for **6** having short and rigid 1,3-xylylene linker the results are typical for the mono-intercalative binding.

The binding constants determined for **2** and **EB** and double stranded DNA and RNA polymers are mostly of the same order of magnitude, although, somewhat higher than those found for other DAP analogues; the constants do not depend significantly on pH, base composition or structural differences between homo- and alternating polymers (Table 4).

The fluorimetric properties of **2**/nucleotide complexes were found to be essentially different from those of other DAPs (paragraph 3). Following this tendency, **2** has also shown differences upon binding to polynucleotides.[14] Namely, the striking difference in fluorescence response is observed for **2 –** poly GC and **2 –** poly AU complexes. Upon addition of poly GC, the emission intensity of **2** decreased by 80 % while poly AU yielded the increase by 100 % (Fig. 3). Similar effects are observed for DNA homopolymer poly dAdT, the alternating polymers poly $(dAdT)_2$ – poly $(dAdT)_2$ and the poly $(dGdC)_2$ – poly $(dGdC)_2$. Considering different fluorescence properties of **2-**AMP complex (fluorescent) and **2-**/GMP complex (non-fluorescent), it is likely that **2** gives different fluorescence response depending on the presence of A or G in DNA and RNA polymers. Some acridine derivatives also exhibited similar nucleic base selective spectroscopic response; the effect was correlated with electron donating properties of guanine.[16, 22] The observed specific fluorescence response of **2** 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.

T a b l e 4 – *Stability constants (log K_s) and ratios (n)[a] ([bound **2**] / [polynucleotide]) calculated from fluorimetric titrations of **2** with ds polynucleotides at pH 5.0 (buffer citric acid, (0.01 mol dm^{-3}), I = 0.025 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
[c]poly dAdT – poly dAdT	0.09	6.1	0.14	5.9
[c]poly dGdC – poly dGdC	0.21	6.1[b]	0.47	6.9[b]
[c]calf thymus DNA	0.34	6.4[b]	–	–

[a] Accuracy of $n \pm$ 10 – 30 %, consequently log K_s values vary in the same order of magnitude

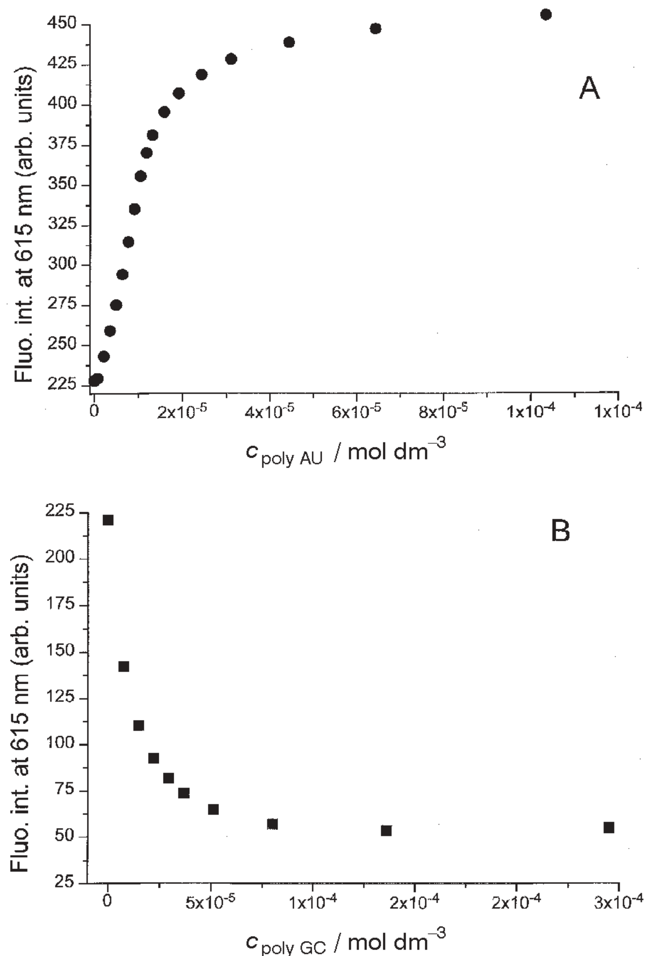
[b] Cumulative value due to unknown contribution of non-intercalative mode of binding at ratios *n* ([**2**] / [polynucleotide]) > 0.2

[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}.

As found for **EB**[23], **2** also exhibited additional, non-intercalative binding at ratio $n_{[\mathbf{2}\ /\ polynucleotide]}$ > 0.2. The non-intercalative binding contribution is more pronounced for **2** / DNA complexes (especially for the **2-**polydAdT) than for the RNA complexes. Viscometric titrations of **2** with ct-DNA gave a slope of α = 1.1 close to that of **EB** (α = 1.0)[19,5] and consistent with monointercalation at ligand to phosphate ratios $n_{[\mathbf{2}\ /\ polynucleotide]}$ < 0.12. Due to the presence of two orthogonally oriented phenyl substituents in **2** it seems plausible to propose its threading intercalation found for other intercalators with bulky substitutents.[1]

Interactions of **2** with *ss*-polynucleotides depend strikingly on the nucleobase composition and in some cases on pH (Table 5).

The variation of ratio *n* ($c_{\mathbf{2}}$ / $c_{poly\ U}$) from 0 to 0.8 induces large increase of CD intensity of poly U spectra (Fig. 4A). 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. The observed CD effects strongly suggest the increase



F i g . 3 – *Fluorimetric titration of* **2**, *c* = 2 × 10^{-6} *mol dm*$^{-3}$ *with poly AU (•) and poly GC (■), pH 7 (buffer Na cacodilate, c = 0.02 mol dm*$^{-3}$*)*

T a b l e 5 – Stability constants (K_s) *and ratios (n)*[a] *([bound compound] / [polynucleotide]) calculated for UV/Vis and fluorimetric titrations of* **2** *or* **EB** *with ss-polynucleotides at pH 5.0 (buffer citric acid, (0.01 mol dm*$^{-3}$*), I = 0.025) and pH 7.0 (buffer Na cacodylate, I = 0.02 mol dm*$^{-3}$*)*

		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
2	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	$> 5^d$
2	5	b	b	0.6	4.7	1^c	2.9^c	1	3.9b
(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	$< 3^c$

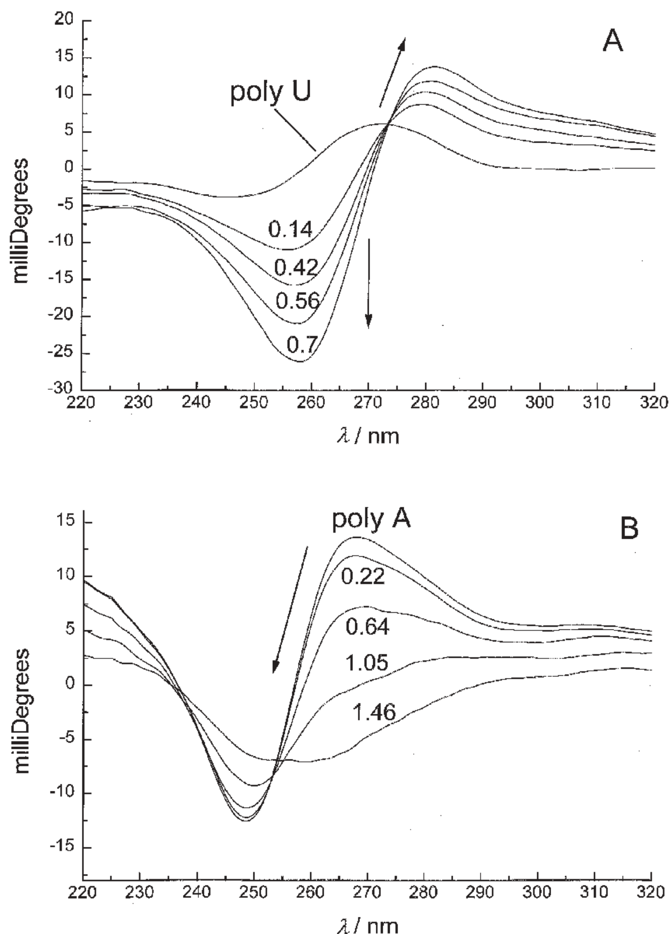
[a] Accuracy of *n* ± 10–30 %, consequently log K_s values vary within the same order of magnitude

[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

of chirality in **2** – poly U complex compared to free polymer and can be explanied by induction of polyU helicity by intercalation of **2**. In contrast to poly U, addition of **2** as well as **EB** to solutions of poly A (Fig. 4B), poly G and poly C produced decrease of CD intensities in accord with partial unwinding of the helical polynucleotides. However, the CD effects in the spectra of poly A produced by **2** are more pronounced that those produced by **EB** being in accord with somewhat stronger binding of **2**.[14]



F i g . 4 – *CD titration of poly U (**A**, c = 4.5 × 10*$^{-5}$ *mol dm*$^{-3}$*) and poly A (**B**, c = 3 × 10*$^{-5}$ *mol dm*$^{-3}$*) with* **2**; *ratio n([**2**] / [poly X]) shown for each spectrum; pH 7, buffer PIPES, 0.01 mol dm*$^{-3}$

In the range of concentrations used in CD and UV/Vis titrations, the **2**-poly U complex is formed close to the saturation conditions ($n_{[2]\ /\ polynucleotide]} \approx 1$); the complex is much more stable than the one formed at large excess of polyU as in the experimental conditions used in the fluorimetric titrations. In the latter concentration range, **2** binds on the "isolated" binding sites of polyU with an affinity similar to **EB**.

To the best of our knowledge such unique binding as found for **2** and polyU which results by increased helicity of the complex compared to free *ss*-polynucleotide was not observed earlier. Also, the intercalators which as **2** are capable to differentiate the *ds*-polynucleotides giving the specific fluorimetric response depending on the basepair composition are also quite rare. Such molecules provide the potential for development of fluorescence sensors.

Physico-chemical properties of phenantridinium-nucleobase conjugates

Results of ^{1}H NMR, UV-Vis and fluorescence studies show that, both, *N*-protonated **10–13**[24] and *N*-methylated conjugates **15** and **16**[25] form folded conformations in aqueous solution with intramolecular base-on-phenathridinium stacking. The magnitudes of the shielding effects in the ^{1}H NMR spectra are higher for the adenine **10**, **11** and **16** than for the uracil conjugates **12**, **13** and **15** in accord with stronger intercalator-purine than the intercalator-pyrimidine stacking intercations.[13,26] Intramolecular base-on-phenathridinium stacking is also supported by the molecular modelling executed for **10** (Fig. 5).

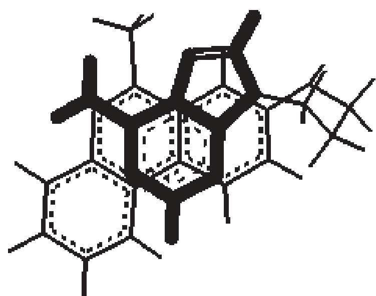


Fig. 5 – *Intramoleculary stacked conformation of the conjugate **10** obtained by molecular modelling using SYBYL of TRIPOS INC. molecular modelling software*

^{1}H NMR chemical shifts (δ, ppm) of aromatic protons of the *N*-protonated compounds **9** – **13** and *N*-methylated compounds **14** – **16** were found to be concentration dependent due to intermolecular stacking. The significant changes of the phenantridinium chemical shifts of **9** – **13** allowed estimation of the self-association constants of $K_{sa} < 10^3$ dm^3 mol^{-1}, being close to the previously reported data for ethidium bromide (K_{sa} = 180 dm^3 mol^{-1}).[27]

UV-Vis spectra of the *N*-protonated compounds **9–13** are strongly pH dependent, exhibiting a one step change with pK ≈ 6, attributed to protonation of phenanthridine. Due to the low solubility of phenanthridines **9–13** at pH 7, the experiments were done at pH 5, where the compounds are in a fully protonated form. With the *N*-methylated conjugates **14–16** no changes in their UV spectra were observed in the pH range of 3–8.

No self-association of compounds **9–16** were found in the concentration range of 1×10^{-6} to 4×10^{-5} mol dm^{-3}.[24, 25] It was observed that the electronic absorption and the fluorescence emission spectra of former compounds **9–16** obey the Lambert-Beer law in this concentration range.

Interaction of phenantridinium conjugates with nucleotides

The addition of nucleotides to aqueous solutions of the conjugates **9–13** as well as *N*-methylated conjugates **14–16** induced significant changes of their fluorescence emission (Fig. 6), allowing determination of the binding constants (K_s) and stoichiometries of the conjugate-nucleotide complexes (Table 6, Table 7).[24, 25] Processing the fluorescence titration data of the *N*-protonated compounds **9**, **10** and **12** and the *N*-methylated conjugates **14–16** with various nucleotides gives the best fit for the 1 : 1 stoichiometry of the complexes and binding constants (K_s) of the same order of magnitude as those found for ethidium bromide.[13,26] No significant differences in affinity were observed for complexes of **10** and **12,** and complexes of **15** and **16** with the complementary nucleotides. Also no charge dependence was observed in binding of AMP^{2-} – ADP^{3-} – ATP^{4-} series, indicating a dominant role of stacking interactions between the phenanthridinium unit of the conjugate and the base of the nucleotide in the complex, and only a minor contribution of the electrostatic binding.

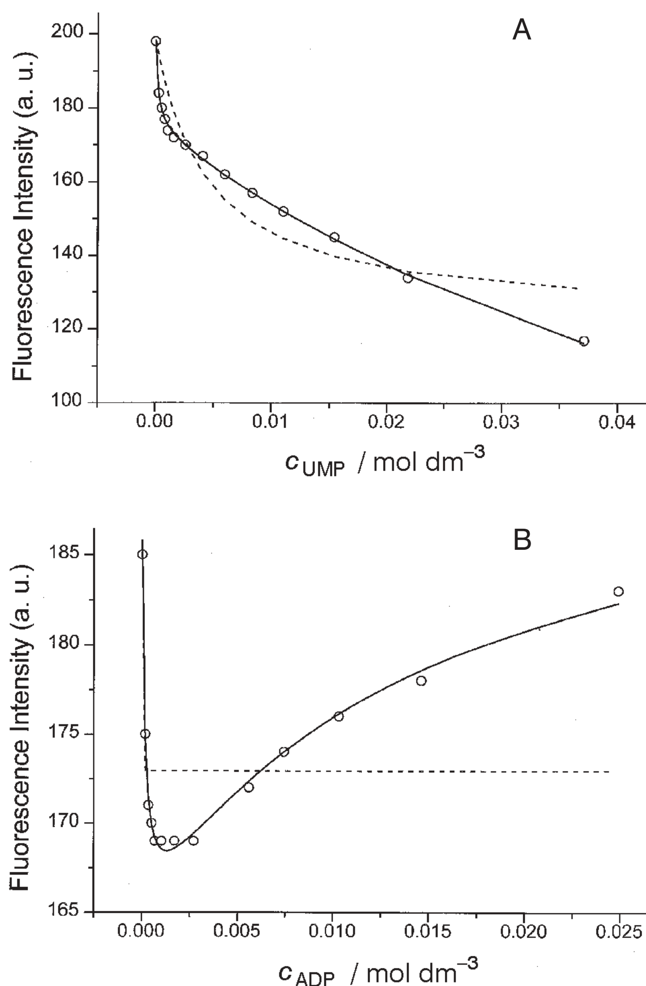


Fig. 6a – *Experimental (○) and calculated data (λ_{exc} = 460 nm, λ_{ems} = 553 nm) of **11** obtained for a model of 1 : 1 (dashed line) and model of 1 : 1 + 1 : 2 (solid line) stoichiometry as a function of UMP^{2-} concentration*

6b – *Experimental (○) and calculated fluorescence intensities (λ_{exc} = 460 nm, λ_{ems} = 548 nm) of **11** obtained for a model of 1 : 1 (dashed line) and model of 1 : 1 + 1 : 2 (solid line) stoichiometry as a function of ADP^{3-} concentration*

The analysis of the titration data for **11** and **13** and nucleotides (Fig. 7) gives the best fit for formation of 1 : 1 and 1 : 2 stoichiometry complexes (L : N; L = ligand, N = nucleotide). The binding constants of **11** – and **13** – nucleotide 1:1 complexes (Table 6; K_1) are more than one order of magnitude higher than those of the ligands **9**, **10** and **12**, as well as those measured for ethidium bromide.[16] The enhanced

Table 6 – *Stability constants (log K_i) for various ligand-substrate complexes*[a,b]

	log K_1, log K_2[c]				
L/N	**9**	**10**	**11**	**12**	**13**
AMP^{2-}	1.73, -[d]	1.66, -[d]	3.78, 2.02	1.84, -[d]	4.18, 1.99
ADP^{3-}	1.78, -[d]	2.58, -[d]	3.79, 1.84	1.87, -[d]	[d]
ATP^{4-}	2.29, -[d]	2.79, -[d]	2.29, -[d]	[d]	[d]
GMP^{2-}	1.72, -[d]	1.72, -[d]	3.91, 1.03	2.05, -[d]	3.82, 1.66
GMP^{2-}	1.93, -[d]	1.58, -[d]	4.25, 1.06	1.45, -[d]	3.63, 1.36
UMP^{2-}	1.59, -[d]	1.47, -[d]	3.7, 1.1	1.50, -[d]	3.84, 1.32
TMP^{2-}	1.34, -[d]	1.54, -[d]	4.01, < 1	1.29, -[d]	3.91, 1.3

[a] Fluorimetric titrations were performed at pH 5 (I = 0.1 mol dm^{-3}, sodium chloride, Na cacodylate buffer, 0.05 mol dm^{-3}

[b] AMP^{2-} = adenosine monophosphate; ADP^{3-} = adenosine diphosphate; ATP^{4-} = adenosine triphosphate; GMP^{2-} = guanosine monophosphate; CMP^{2-} = cytidine monophosphate; UMP^{2-} = uridine monophosphate; TMP^{2-} = thymidine monophosphate

[c] K_1 and K_2 refer to the equilibria L + N $\rightleftharpoons$ LN and LN + N $\rightleftharpoons$ LN_2 (L = ligand. N = nucleotide), respectively

[d] Small spectroscopic changes hampered determination of K_s

Table 7 – *Stability constants (log K_s) for **14–16** toward nucleotides*[a,b]

	AMP	ATP	GMP	UMP
14	1.6 ± 0.04	2.0 ± 0.1	2.00 ± 0.13	1.6[c]
15	1.7 ± 0.07	1.9 ± 0.04	2.1± 0.04	1.5[c]
16	2.1 ± 0.05	–	1.8 ± 0.06	1.7 ± 0.1
EB	1.6	–	1.6	1.2

[a] Fluorimetric titrations were performed at pH 6 (I = 0.1 mol dm^{-3}, sodium sulphate, Na cacodylate buffer, 0.05 mol dm^{-3})

[b] AMP^{2-} = adenosine monophosphate; ATP^{4-} = adenosine triphosphate; GMP^{2-} = guanosine monophosphate; UMP^{2-} = uridine monophosphate

[c] Only 50 % of complexation was reached allowing only estimation of binding constant

binding of nucleotides by **11** and **13** having longer pentamethylene linkers and the fact that the complementary and the non-complementary nucleotides were bound by similar strengths[28] suggest formation of structurally distinct complexes. The molecular modelling on **11** and uridine shows that the penamethylene spacer is of a sufficient length to allow formation of the intercalative complex with the nucleotide base intercalated between the intercalator and the base of the conjugate (Fig. 7a and c). The 1 : 2 conjugate-to-nucleotide complex is formed at higher excess of a nucleotide. Binding constant K_2 (equilibrium 1 : 1 complex of **11** and **13** + nucleotide $\rightleftharpoons$ 1 : 2 complex) is of the same order of a magnitude as found for binding constants K_1 for **9** : nucleotide complexes of 1 : 1 stoichiometry. So, it can be concluded that in such complex the base of the second nucleotide may stack on the remaining free surface of the intercalator unit of the 1 : 1 complex, in the same way as in **9** : nucleotide complexes (Fig. 7b).

For **11** and **13**, as well as for **10**, **12, 15** and **16** no preferential binding of the complementary nucleotides was observed. These results are at variance to the previously reported recognition of propyladenine by proflavine–tymine conjugate in aqueous media.[9c] Apparently, the recognition of nucleotides by hydrogen bonding of complementary bases on the surface of the intercalator unit is not possible due to strongly competitive hydrogen bonding with water molecules. However, such a conjugates may exhibit base pairing in the more lipophilic microenvironment existing within the complementary sequences of the single stranded regions of DNA and RNA.[29]

Interaction of phenantridinium conjugates with DNA and RNA

Interaction of **9–16** with *ct*-DNA as the representative of doublestranded polynucleotides at pH 5 were studied by fluorimetric titrations. The addition of *ct* DNA increased the emission of the referent compound **9** 2.8 times and those of the *N*-protonated derivatives **10–13** 1.1 to 1.4 times, while the fluorescence of **EB**[14] was increased 20 times. Titrations with *ct*-DNA resulted by a 3.4 times increased emission of the reference compound **14** and only by 1.7 and 1.6 times increase of the *N*-methylated conjugates **15** and **16**, respectively. The binding constants for, both, the *N*-protonated and the *N*-methylated derivatives **9–16** (log K_s = 5.2 – 6 dm^3 mol^{-1}), as well as the [bound ligand]/[polynucleotide phosphate] ratios (n = 0.05 – 0.2) calculated from the titration data according to the Scatchard equation[21] are comparable to those determined for **EB** (log K_s = 6.1 dm^3 mol^{-1}, n = 0.2) within the error of the method.[30] These results suggest that the conjugates bind to *ct*-DNA by intercalation with similar affinity as **EB** and that the presence of a spacer and a tethered nucleobase does not significantly alter the

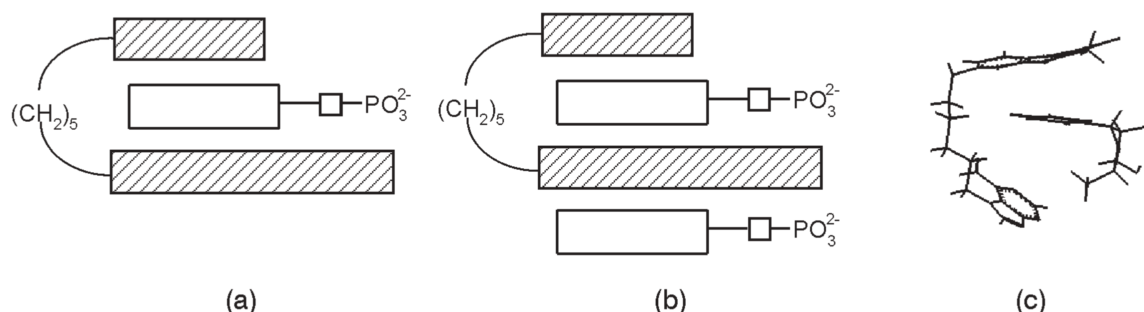


Fig. 7 – *Shematic representation of (a) the conjugate-nucleotide complex of 1 : 1 stoichiometry (b) of the complex of 1 : 2 stoichiometry and (c) the side view of the minimized structure of 1 : 1 **11** – uridine complex generated by molecular modelling*

intercalation ability of the phenanthridinium unit. Only slightly lower n values determined for **10–13** and **16** relative to references **9** and **14,** and to **EB** indicate a somewhat less dense intercalation than predicted by the "neighbour exclusion" principle,[31] very likely due to a steric hindrance imposed by the linker and the adenine.

Table 8 – *Spectroscopic properties of* ***9–13*** *complexes with singlestranded polynucleotides*

	Poly A		Poly U	
	UV-Vis $\Delta\lambda_{max}/\Delta A^b$	Fluorescence ΔI^b	UV-Vis $\Delta\lambda_{max}/\Delta A^b$	Fluorescence ΔI^b
9	30 / –20 %	+ 330 %	c	c
10	10/<–5 %	+ 110 %	10/–15 %	–10 %
11	10/<–15 %	+ 120 %	10/–15%	–11 %
12	25/–15 %	+ 260 %	c	–2 %
13	20/–20 %	+ 288 %	c	c

a Spectroscopic titrations were performed at pH 5 (I = 0.1 mol dm^{-3}, Na cacodyate buffer), λ_{max}(Abs) = 440 nm

b Calculated as $\Delta A(\Delta I) = (A_0(I_0) - A(I) / A_0(I_0)) \times 100$

c Spectroscopic changes close to the error of the instrument

Table 9 – *Binding affinities (log K_s) and ratios n ($c_{bound\ 9–13}$ / $c_{phosphate}$)*[a] *for* ***9–13*** *toward singlestranded polynucleotides*[b]

	Poly A		Poly U	
	n	log K_s	n	log K_s
9	0.1 ± 0.07	5.1 ± 0.4	c	< 3[c]
10	0.1 ± 0.03	5.3 ± 0.4	0.1 ± 0.03	4.5 ± 0.4
11	0.8 ± 0.11	5.2 ± 0.1	0.1 ± 0.03	5.5 ± 0.4
12	0.1 ± 0.07	5.4 ± 0.2	c	< 3[c]
13	0.1 ± 0.05	5.3 ± 0.4	c	< 3[c]

a The correlation coefficients > 0.999 correspond to given ranges of n and log K_s calculated according to Scatchard equation[12,13]

b Fluorimetric titrations were performed at pH 5 (I = 0.1 mol dm^{-3}, Na cacodyate buffer)

c Estimated value due to less than 20 % of complex formed

In contrast to the titrations with *ct*-DNA, the effects in UV spectra induced by additions of ss-polynucleotides were strongly dependent on both, a type of the nucleobase present in **10–13** and a type of the polynucleotide added (Table 8). The bathochromic shifts and the hypochromicity effects induced by poly A are stronger for **9**, **12** and **13** than for **10** and **11** and can be explained as in the case of *ct* DNA by stronger intramolecular base stacking in the latter conjugates. In contrast, poly U induced significantly stronger effects in the UV spectra of **10** and **11** bearing the complementary adenine than in those of the reference **9** and the non-complementary **12** and **13**. This difference points to important additional interactions between the uracils of poly U and the adenines of **10** and **11**. The fluorimetric titrations of **9-13** with poly U reveal significantly higher affinity of the adenine conjugates **10** and **11** than those of the reference **9**, the non-complementary uracil conjugates **12** and **13** and **EB** (log K_s < 3)[14] (Table 9). Quenching of phenanthridinium emission by poly U is much stronger for **10** and **11** than for **9**, **12** and **13** (Table 8) pointing to the stronger stacking interactions in the former conjugate – poly U complexes. Since in the intramolecularly stacked conformations of **10** and **11** the adenines cover only a part of the phenanthridinium surface, the insertion of the uncovered part between the uracils of poly U can be expected (Fig. 8).

It has been shown that **11** could form the intercalative type of complex with nucleotides (Fig. 7a).[24] Based on these results the bis-intercalative binding of **11** to poly U (Fig. 8) cannot be excluded especially due to its somewhat higher affinity compared to **10**. However, for **10** the monointercalative binding mode with subsequent A–U pairing (Fig. 8, right) remains as the most possible explanation.

The addition of poly A resulted in significant bathochromic and hypochromic effects in the UV-Vis spectra of **9–13** (Table 8), that increase in order **10, 11**< **12, 13** < **9**, depending on the type of covalently attached nucleobase. In the fluorimetric titrations, the emission increase follows the same tendency (Table 8). The observed UV-Vis and fluorescence effects point toward intercalation of phenanthridinium unit into poly A as the dominant binding mode for all of the conjugates; for **12** and **13** bearing uracil no increased affinity toward poly A could be observed.

The recognition of poly U by **10** and **11** with attached complementary adenines and lack of any recognition of poly A by **12** and **13** with appended uracils point to the complexity

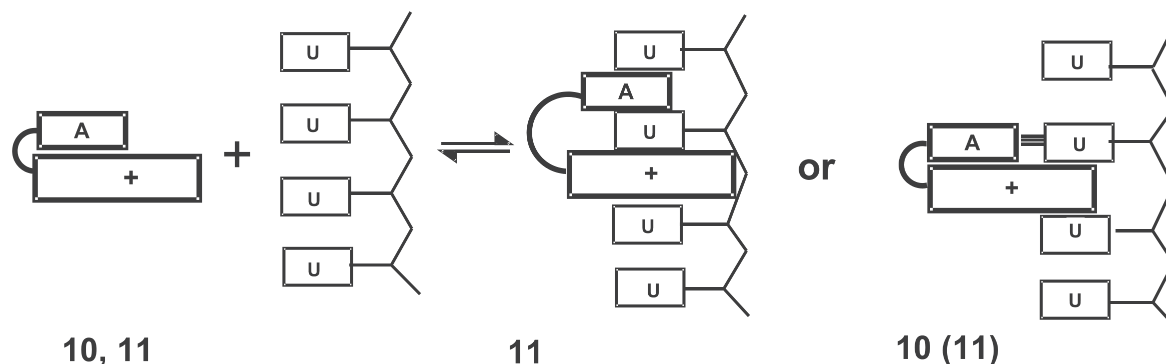


Fig. 8 – *Schematic presentation of possible bis-intercalative (middle) or mono-intercalative with A–U pairing (right) binding modes for* ***10*** *or* ***11*** *and poly U*

and sensitivity of these systems on the structural properties of both, the conjugates and the polynucleotides. Phenantridinium-adenine conjugates **10** and **11** with intramolecularly strongly stacked adenines are capable to intercalate into poly U only by a remaining free part of the phenanthridinium surface. The additional stabilisation of the complex can be provided by stacking interactions and the A–U hydrogen bonding supported by easy conformational adaptation of the random and flexible poly U. The obtained results clearly demonstrate that the conjugates **10** and **11** exhibit two types of binding, depending on the structural properties of the polynucleotides: *i)* the mono-intercalation for well organised helical *ds*- or *ss*-polynucleotides and *ii)* the intercalation supported by additional A–U interactions in the case of unorganised and flexible poly U.

UV-Vis and fluorimetric titrations of *N*-methylated compounds **14–16**, with single stranded (ss-) polynucleotides were performed at physiological conditions (pH 7) and also at pH 5 for comparison with protonated analogues **9–13**.[29] In all titrations, the addition of *ss*-polynucleotide to the solutions of **14–16** induced a hypochromic effect in UV-Vis spectra and an increase of fluorescence (Table 10).[25] In general, addition of poly A resulted in more pronounced spectroscopic changes than the addition of poly U. This observation can be explained by a larger aromatic surface and hence a stronger stacking between the phenanthridinium unit and purine nucleobases, but it also shows that the stacking interactions are dominant binding forces that stabilizes such complexes.

Table 10 – *Spectroscopic properties of* ***14–16*** *complexes with ss- polynucleotides*[a]

	pH	Poly A UV-Vis	Poly A Fluorescence ΔI^b	Poly U UV-Vis	Poly U Fluorescence; ΔI^b
		ΔA^b	$\Delta\lambda_{exc}$ = 325_{nm} / 440_{nm}	ΔA^b	$\Delta\lambda_{exc}$ = 325_{nm} / 440_{nm}
14		–35 %	+ 525 % / +170 %	– 8 %	c
15	**5**	–20 %	+ 310 % / +150 %	–15 %	c
16		–10 %	+ 260 % / +120 %	–17 %	c
14		–14 %	+ 200 % / + 60 %	–12 %	c
15	**7**	–20 %	+ 110 % / + 40 %	–14 %	c
16		–10 %	+ 90 % / + 38 %	– 3 %	c

[a] Spectroscopic titrations were performed at pH 5 and 7 (I = 0.1 mol dm^{-3}, Na cacodylate buffer), λ_{max}(**14, 15**) = 440 nm and λ_{max}(**16**)= 445 nm

[b] Calculated as $\Delta A(\Delta I) = \{[A_0(I_0) - A(I)] / A_0(I_0)\} \times 100$, where $A(I)$ is calculated value at 100 % of complex formed

[c] Due to the linear dependence of spectroscopic changes on the concentration of polynucleotide it was not possible to calculate $A(I)$ value at 100 % of complex formed

[d] Different excitation wavelengths (325 nm – abs. shoulder; 440 nm abs. maxima) are used for the comparison of the observed emission changes.

No significantly increased affinity of conjugates **15** and **16** toward complementary *ss*-polynucleotide relative to the non-complementary one could be observed (Table 11). The binding constants for **14–16** – poly U complexes can only be estimated (log K_s < 3, Table 11) due to the solubility problems at c(poly U) > 0.01 mol dm^{-3}. The affinities of the reference **14** and the uracil conjugate **15** toward poly A are the same at pH 5 and 7. In contrast, the affinity of adenine conjugate **16** toward *ds*-poly AH^+, that is formed at pH 5 from the protonated *ss*-poly AH^+[32], is an order of magnitude higher than that at pH 7 (Table 11). In the *ds*-helix, the base-pairs provide a larger surface for stacking with phenanthridinium than single bases in ss-poly A existing at pH 7. The K_s of **16** is also significantly higher than those of **15** and the reference **14**. Interestingly, the affinity order reverses at pH 7. These results strongly suggest that some additional specific interactions stabilize the complex of **16** and *ds*-poly AH^+. To prove this additional stabilization, the thermal denaturation experiments with **14–16** and poly A at pH 5 were performed.

Table 11 – *Binding constants (log* K_s*) and ratios n (*$c_{bound\ 14-16}$ / $c_{phosphate}$*)*[a] *for* ***14–16*** *toward singlestranded polynucleotides*[b]

	pH	Poly A n	Poly A log K_s	Poly U n	Poly U log K_s
14		0.1 ± 0.05	4.3 ± 0.1	c	< 3[c]
15	5	0.1 ± 0.05	4.8 ± 0.2	c	< 3[c]
16		0.1 ± 0.05	5.3 ± 0.2	c	< 3[c]
14		0.1 ± 0.05	4.8 ± 0.2	c	< 3[c]
15	7	0.1 ± 0.05	4.7 ± 0.2	c	< 3[c]
16		0.1 ± 0.05	4.4 ± 0.3	c	< 3[c]

[a] The correlation coefficients > 0.999 correspond to given ranges of n and log K_s

[b] Fluorimetric titrations were performed at pH 5 and 7 (I = 0.05 mol dm^{-3}, Na cacodylate buffer)

[c] Estimated value due to less than 20 % of complex formed, λ_{exc} = 320 nm and λ_{exc} = 440 nm used for titration

Addition of adenine conjugate **16** resulted in strong stabilization of *ds*-poly AH^+ (ΔTm / $n_{16/poly\ AH^+}$: 2.3/0.2; 4.6/0.3; 17.0/0.5), while addition of the reference compound **14** and the uracil conjugate **15** had no effect on the melting transition. Thus, the results of thermal denaturation experiments are fully in accord with those of fluorimetric titrations showing that **16** recognizes *ds*-poly AH^+ by the additional specific interaction (Table 11).[25]

The structure of the intercalative **16**-*ds*-poly AH^+ tetramer complex generated by molecular modelling is presented in Fig. 9. The model of the complex shows that besides the intermolecular stacking an additional interaction is possible through the hydrogen bonding between adenine *N*-1 hydrogen of *ds*-poly AH^+ and the adenine *N*-1 of **16**. This additional interaction could account for a stronger binding of **16** to poly AH^+.

Different type of binding interactions of *N*-protonated compounds **9-13** compared to *N*-methylated compounds **14–16** toward *ss*-polynucleotides, clearly demonstrate the importance of small variations of structure and charge in the conjugates for their binding properties. The presented results provide new experimental facts relating to the structu-

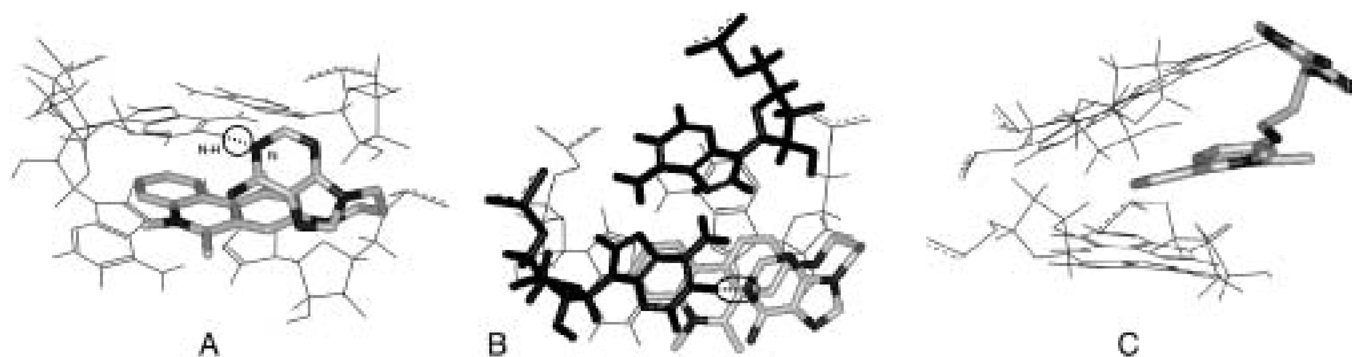


Fig. 9 – *Optimized structure of the intercalative **16**-ds-poly AH+ tetramer complex (only the middle two pairs of tetramers are shown; hydrogen atoms of **16** omitted for clarity) generated by molecular modelling. The structure reveals possible hidrogen bonding between adjacent adenine +N1–H and N1 of **16** as indicated in (A) and (B) (distance 2.65 Å). In (C) the side view of the complex showing intercalation of the phenantridinium unit of **16** is seen*

re and charge of nucleic acid binders of intercalator-nucleobase conjugate type that can be of high relevance for design of small molecules capable of recognizing specific *ss*-regions of DNA and RNA.

Conclusions

The formation of stacked nucleotide/4,9-diazapyrenium complexes of 1 : 1 stoichiometry with log K_s = 1.6 – 2.8 (Table 1) suggest that larger surface compared to phenanthridinium analogues (ethidium bromide) does not considerably enhance nucleotide binding. The stability constants were found independent on nucleotide charge but dependent on the aromatic surface of nucleotide, suggesting the dominance of aromatic stacking over coulombic interactions in such complexes. Monomeric 4,9-diazapyrenium cations bind to *ss*- and *ds*-polynucleotides by intercalation. Affinities were found to be essentially independent on the number of permanent charges present in the aromatic system but strongly dependent on a type of substituents attached. 2,7-Diamino substituted 4,9-diazapyrenium derivative (**2**) has shown the highest affinity toward polynucleotides, the highest double helix stabilisation, and highly specific fluorescence response on binding to double-stranded G – C (quenching) and A – U(T) (emission increase) polymers. Interactions of 2,7-diamino-4,9-diazapyrenium derivative (**2**) with *ss*-polynucleotides, in contrast to other derivatives, depend strikingly on the nucleobase composition and in some cases on pH. The bis(4,9-diazapyrenium) dicationic derivatives **6–8**, differing in rigidity and length of the linkers connecting diazapyrenium units, were found to bind to *ds*-polynucleotides by monointercalation. Some of them exhibited strong additional interactions of the non-intercalated diazapyrenium unit with the outer surface of a polynucleotide.

Spectroscopic studies of aqueous solutions of phenantridinium compounds **9–16** revealed different intra- and intermolecular interactions. According to ^{1}H NMR and UV-Vis experiments, all compounds form intermolecular dimeric associates with binding constants similar to those reported for other phenanthridinium derivatives. Folded conformations with intramolecularly base-on-phenanthridinium stacking, that was more pronounced for the adenine conjugates, were found for both *N*-protonated and *N*-methylated conjugates.

Phenantridinium compounds **9-16** bind efficiently nucleotides in aqueous media.[24,25] In contrast with reported proflavine-nucleobase conjugates, it haven't been observed any significant preference of conjugates **10–13** and **45–16** toward complementary nucleotides, probably due to competition of hydrogen bonding of water molecules. The increased affinity of **11** and **13** toward nucleotides compared to **9–10**, **12** and **14–16** is due to the different linker length, the former conjugates with pentamethylene linker forming more stable intercalative type of complexes than the latter.

Spectrophotometric titrations of **9–16** with *ss*- and *ds*-polynucleotides indicate intercalation as the dominant binding interaction of the phenanthridinium unit.[24,25] At pH 5, the *N*-methylated adenine conjugate (**16**) / poly AH+ complex is significantly more stable than the corresponding complexes of uracil conjugate **15** and the reference **14** lacking the nucleobase, what can be explained by the additional interactions of the adenine of **16** with *ds*-polynucleotide. Adenine containing *N*-protonated conjugates **10** and **11** exhibit specific spectroscopic changes and two orders of magnitude higher affinity toward poly U, than other phenantridinium compounds due to the existence of specific interactions between adenine and uracil, possibly Watson-Crick hydrogen bonding between the bases stacked on the phenanthridinium moiety. Different preferences of *N*-methylated adenine conjugate **16** and its *N*-protonated analogue **10** could be a consequence of a different density and/or distribution of the positive charge on the phenanthridinium units of the protonated and methylated conjugates.

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SAŽETAK

Prepoznavanje nukleotida i polinukleotida pomoću derivata fenantridinija i diazapirenija u vodenom mediju

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Ispitane su interakcije 4,9-diazapirenijevih kationa s nukleotidima i polinukleotidima u puferiranim vodenim otopinama (pH 5 ili 7) uz uporabu UV-Vis spektrofotometrije, fluorescencije, NMR-spektroskopije i viskozimetrije. Pri tome nastaju kompleksi nukleotid/4,9-diazapirenij i nukleotid/fenantridinij u stehiometrijskom omjeru 1 : 1 s konstantama stabilnosti log K_s 1,6–2,8 i log K_s do 2,3, redom, što ukazuje da veća površina kationskog heteroaromata ne povećava vezanje nukleotida. Pokazalo se da konstante stabilnosti ne ovise o naboju nukleotida, što ukazuje na to da su u takvim kompleksima aromatske interakcije slaganja dominantne u odnosu na Coulombove interakcije. Monomerni 4,9-diazapirenijevi kationi interkaliraju u *ss*- i *ds*-polinukleotide. Afiniteti vezanja potpuno su neovisni o broju permanentnih naboja prisutnih u aromatskom sustavu, ali znatno ovise o tipu vezanog supstituenta. Diamino supstituirani derivati 4,9-diazapirenija pokazali su najveći afinitet prema polinukleotidima, najveću stabilizaciju dvostruke uzvojnice te visoko specifični fluorescencijski odgovor prilikom vezanja na dvolančane G – C (gašenje) i A – U(T) (porast emisije) polimere. Interakcije 2,7-diamino-4,9-diazapirenijevih derivata sa *ss*-polinukleotidima, za razliku od ostalih derivata, neuobičajeno ovise o vrsti polinukleotida te u nekim slučajevima o pH. Bis(4,9-diazapirenijevi) dikationski derivati, koji se međusobno razlikuju u krutosti i duljini premosnice koja povezuje diazapirenske jedinice, vežu se na *ds*-polinukleotide monointerkaliranjem, pri čemu neki od njih pokazuju snažne dodatne interakcije diazapirenijeve jedinice koja nije interkalirala s vanjskom površinom polinukleotida.

Pripravljeni su novi konjugati fenantridinija i nukleo-baza te su ispitani pomoću spektroskopskih metoda. Analiza ^{1}H NMR, UV-Vis i fluorescencijskih spektara u vodenim otopinama pokazala je da postoje unutarmolekulske interakcije između fenantridinijeve jedinice i nukleo-baze te da se molekule nalaze u slijepljenoj konformaciji. Fluorimetrijske titracije pokazale su da konjugati fenantridinija i nukleo-baza **9, 10, 15** i **16** te referentni spojevi **9** i **14** stvaraju komplekse s nukleotidima u vodi stehiometrije 1 : 1, s konstantama stabilnosti u rasponu 10^1 do 10^2 mol^{-1} dm^3, dok spojevi **11** i **13** formiraju interkalativni tip kompleksa, pri čemu je aromatska jedinica nukleotida stavljena između fenantridinija i kovalentno vezane nukleo-baze, te su konstante stabilnosti takvih kompleksa 10^3–10^4 mol^{-1} dm^3. Konjugati vežu komplementarne kao i nekomplementarne nukleotide, bez prepoznavanja komplementarne nukleo-baze. U svim kompleksima s nukleotidima pokazalo se da su dominantne aromatske $\pi\ldots\pi$ interakcije slaganja. *N*-protonirani konjugati fenatridinija i adenina **10** i **11** pokazali su specifične spektroskopske promjene i afinitet prema poli-U koji je dva reda veličine veći nego kod uracilnih konjugata **12, 13** i **15** te referentnih spojeva **9** i **14,** zbog specifičnih interakcija, vjerojatno Watson-Crickovih veza, između adenina i uracila. Za razliku konjugata **10** i **11**, *N*-metilirani konjugat fenantridinija i adenina **16** pokazao je pri pH 5 pojačani afinitet za dvolančani (*ds*-)poly-AH$^+$. *N*-metilirani spojevi **14**–**16** nisu prilikom vezanja ss-polinukleotida pokazali pojačan afinitet za vezanje komplementarnog polinukleotida. Ovi rezultati pokazuju da protoniranje odnosno permanentni naboj na interkalatoru značajno utječu na vezanje spojeva na polinukleotide.

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