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Tuning urea–phenanthridinium conjugates for DNA/RNA and base pair recognition

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ABSTRACT

A series of novel urea–phenanthridine conjugates was prepared. The variation of linker length connecting two urea–phenanthridinium conjugates regulated their binding mode toward double stranded polynucleotides, consequently controlling selectivity of compounds toward ds-RNA over ds-DNA stabilization as well as selective fluorescence response toward addition of G–C base pair and A–U(T) base pair containing polynucleotides.

1. Introduction

Numerous drugs base their biological activity on interaction of low weight organic molecules with DNA and/or RNA. Small molecules of that kind are of special interest because they can more easily cross biological membranes than large molecules, and can even be delivered to cells that are strongly resistant to exogenous matter.[1] For example, brain cells resist the entry of molecules with MW larger than approximately 600, thus hampering any disease treatment.[1] Therefore design, synthesis, and biological evaluation of novel compounds that target DNA/RNA are of high interest. In general, there are three main modes of non-covalent binding of small molecules to DNA/RNA: (i) minor groove binding, (ii) intercalation, and (iii) electrostatic interaction of highly positively charged molecules with nucleotide phosphate backbone.[2] Many authors combined more modes of interaction in the same molecule targeting very specific goals. Very recently, even thoroughly studied molecules as classical DNA/RNA intercalator ethidium bromide had to be re-evaluated,[3–5] since it became obvious that mechanisms of non-covalent interactions between small molecule and DNA/RNA are not completely understood. In addition, it was shown that the chemical modulation of the ethidium exocyclic amines is a profitable option to tune the nucleic acid recognition properties of phenanthridinium dyes.[3,6] Very recent reports about numerous applications point toward versatility of the phenanthridinium core,[7] including even intriguing biological activity.[8] A huge number of bis-phenanthridinium derivatives were prepared with the aim of not only enhanced affinity due to the bis-intercalation into DNA/RNA but also with the idea of introducing selectivity.[9] Our recent results have pointed out that selectivity of bis-phenanthridinium derivatives toward various DNA/RNA sequences could be controlled by the steric effects[10,11] or by electrostatic (pH controlled) interactions with DNA/RNA backbone and/or nucleobases.[12] As a continuation of this research we have looked for linkers between two phenanthridines, which could be able to control interactions with DNA/RNA by combination of steric effects and specific interactions with nucleobases and/or phosphate backbone.

Urea presents an electron-rich aromatic scaffold ideal for multiple hydrogen bond formation. Large condensed aromatic moieties with variously positioned urea substituents have shown a number of intriguing properties. Anthracene derivatives bearing two urea groups on the 1,8 and 9,10-positions were found to be efficient anion fluorescent chemosensors,[13] xanthene tetraureas by intercalating into DNA efficiently inhibited binding of two transcription factors to DNA,[14] and ethidium bromide—urea conjugates proved to be strong DNA binders.[15] Some urea-containing molecules have also shown intriguing anti-HIV activity.[16] To take advantage of the ability of urea to form multiple hydrogen bonds for nucleobase recognition, many urea-substituted compounds were prepared and studied in organic solvents as model systems.[17] For example, urea-substituted compounds have recently shown strong recognition of the C–G base pairs in organic solvent by formation of three

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hydrogen bonds on the major groove side of the base pair.[18] Unfortunately, the same authors have reported that upon incorporation of the efficient model system into the oligonucleotide, strong hydrogen bonding in organic solvent was not translated into effective binding within a DNA triple helix in aqueous solution.[19] On the other hand, bis intercalators bridged by urea-containing linkers could be considered as analogues (regarding the potential to form hydrogen bonds with nucleobases) of the polyamide bridged bis intercalators selectively interacting with DNA/RNA.[20] Therefore, urea-containing aromatics could offer recognition of various DNA/RNA sequences by hydrogen bonding, either when co-planar to the G–C base pair[17,18] or interacting with more consequent base pairs from one of the DNA/RNA grooves like polyamides.[20] All aforementioned research urged us to the conclusion that bis-urea linker connecting two phenanthridines would be an ideal choice for our ongoing research. Therefore, here we present synthesis of bis-phenanthridine derivatives bridged by bis-urea linkers of variable length, and study of their interactions with DNA and RNA.

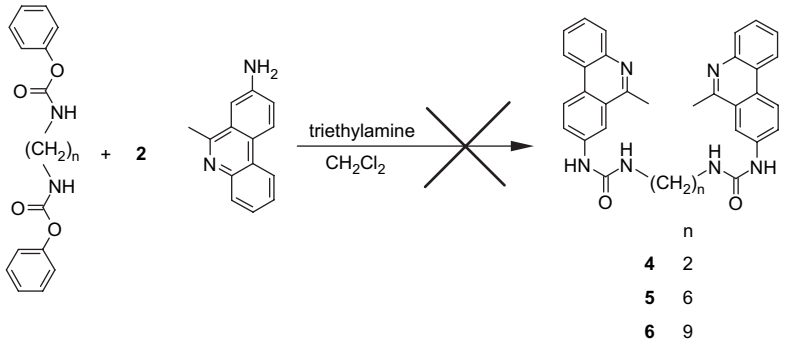
2. Results and discussion

2.1. Synthesis

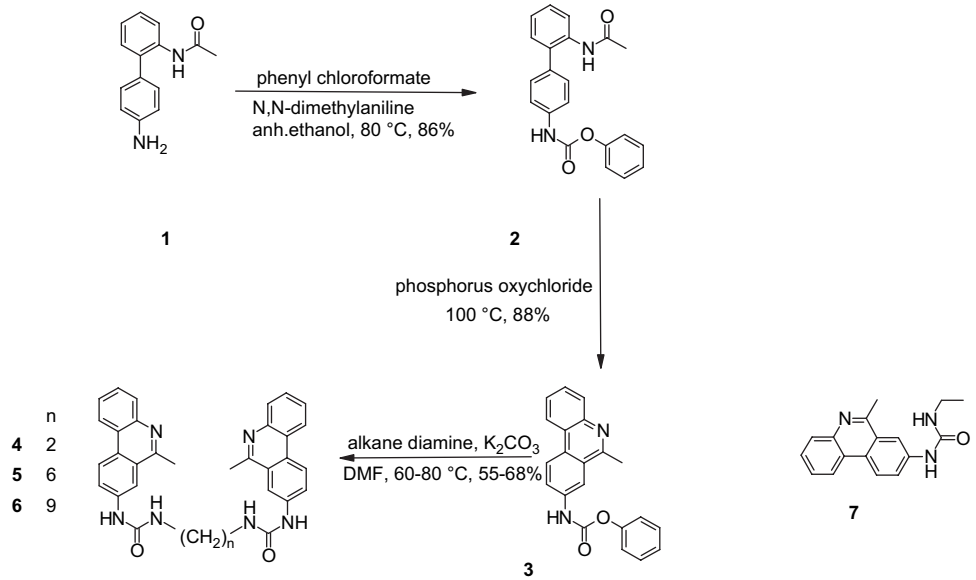
Structures composed of two urea groups connected by a spacer group are attractive building blocks, since they are readily obtained from simple starting materials. Our first attempt to prepare desired compounds **4**, **5**, and **6** according to the previously published procedure[21] (Scheme 1) was unsuccessful. Instead of targeted compounds we obtained partially decomposed starting material and the remainder was a complex mixture of unidentified reaction products. This initial synthesis attempt starting from diaminoalkanes protected with phenyl or 4-nitrophenyl carbamate group and 8-amino-6-methylphenanthridine failed, probably because the exocyclic amines of phenanthridine are poor nucleophiles and only weakly basic.[3] Also, poor solubility of some reactants in used solvents might have hampered the synthesis of desired products.

The modified (reversed) procedure of Luedtke et al.[21] (Scheme 2) has given targeted **4**, **5**, and **6** in acceptable yields. Protection of aminobiphenyl-2-yl-acetamide with phenyloxycarbonyl group and cyclization via Morgan–Walls reaction[22] gave carbamate **3**, electrophilic enough to effectively react with chosen alkane diamines. Thus, the procedure turned out to be successful when the reaction of primary aliphatic amines as good nucleophiles with electrophilic phenanthridine carbamate was employed. Accordingly to the above elaborated synthetic procedure (Scheme 2) monomer **7** was prepared for comparison reasons.

All compounds presented here have satisfactory elemental analyses or mass spectra and their structures were verified by detailed 1D and 2D NMR analysis.



Scheme 1. Unsuccessful attempt of the synthesis of bis-urea bridged bis-phenanthridines **4**–**6**.



Scheme 2. Efficient synthetic route to bis-urea bridged bis-phenanthridines **4**–**6**, and the structure of corresponding monomer **7**.

2.2. Spectroscopy

Linear dependence of both UV/vis and fluorescence spectra on the concentration of all studied compounds covering the range c(**4**–**7**)=1×10^{-6}–1×10^{-5} mol dm^{-3} does not support intermolecular interactions or aggregation of any of the studied compounds, supported by negligible temperature dependent changes of UV/vis spectra (25–50 °C, c(**4**–**7**)=1×10^{-5} mol dm^{-3}, $\Delta Abs_{264\ nm}$<2%) and excellent reproducibility upon cooling to 25 °C. Low solubility of studied compounds in aqueous media hampered spectroscopic experiments at higher concentration. The electronic absorption spectra of **4**–**7** in the buffered aqueous solutions and corresponding molar extinction coefficients (ε) were found to be quite similar (Table 1). The fluorescence excitation spectra of **4**–**7** are in good accordance with the corresponding UV/vis spectra in the region where emission and excitation spectra do not overlap.

Table 1
Electronic absorbtion maxima and molar extinction coefficients of **1**–**4** (c=2×10^{-5} mol dm^{-3}); relative fluorescence emission intensities (c(**2**–**4**)=1.4×10^{-6} mol dm^{-3}) in citric acid buffer, I=0.03 M, pH=5

	$\lambda_{max}/\varepsilon$ ×10^3 (mmol^{-1} cm^2)	λ_{em} (nm)/Rel fluo int.	Int (**4**)/Int(**X**)
4	264/33.40	470/41	1
5	265/37.94	480/55	1.33
6	262/36.02	468/364	8.86
7	268/33.08	470/443[a]	110

[a] Ten times lower concentration.

Intriguingly, the fluorescence emission of monomer **7** was 1–2 orders of magnitude stronger than emission of bis-phenanthridinium analogues **4**–**6** at the same experimental conditions (Table 1), possibly due to some kind of intramolecular interactions between two phenanthridinium subunits of bis-compounds **4**–**6**. This is in line with observation that fluorescence intensity of **4**–**6** is proportional to the length of the aliphatic linker connecting two urea–phenanthridinium systems (Table 1). However, these intramolecular interactions are not obvious from the UV/vis spectra since corresponding ε values of all compounds are almost identical (Table 1).

It is well known that heterocyclic nitrogen of phenanthridine is protonated under acidic conditions. Therefore, by measuring the fluorescence emission intensity of studied compounds as a function of pH we have determined pK values characteristic for the presented phenanthridine–phenanthridinium system (pK(**4**)=4.8, pK(**5**)=4.7, pK(**6**)=5.2). Comparison of the obtained pK values with those previously determined for other phenanthridine analogues (p$K\approx$6),[23] pointed to considerable impact of urea substituent on the protonation affinity of the phenanthridine heteroaromatic nitrogen. That observation is in accord with the electron withdrawing properties of the urea substituent.

2.3. Interactions with double stranded (ds-) DNA and RNA

Due to the low solubility of phenanthridines at pH=7 and also for easier comparison with results of previous research,[11,12] further experiments in aqueous media were done at pH=5, all compounds being about 50% in a protonated (phenanthridinium) form. However, even at pH 5 poor solubility of **4**–**7** at c>5×10^{-5} mol dm^{-3} hampered NMR and viscometry experiments with DNA and RNA, as well as the UV/vis titrations at λ>300 nm due to the low ε values of **4**–**7**. Nevertheless, strong fluorescence emission of the **4**–**7** aqueous solutions allowed more detailed studies of interactions with DNA and RNA at conditions of an excess of polynucleotide over **4**–**7**.

2.3.1. Fluorimetric titrations

Fluorimetric titrations of studied compounds with ds-DNA and RNA revealed intriguing differences between **4**, **5** and **6**, **7** in changes of fluorescence spectra.

Addition of any polynucleotide resulted exclusively in strong emission increase of **4** and **5** (Fig. 1, Table 2). However, the fluorimetric maxima of **4**, **5** were significantly red shifted upon binding to poly G–poly C, while addition of other studied ds-DNA and ds-RNA caused almost no shift of emission maxima (Fig. 1).

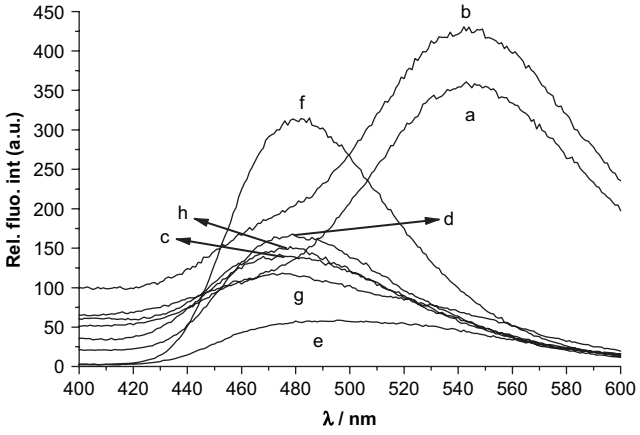


Figure 1. Fluorescence spectra of complexes at pH=5: (a) **4**+poly G–poly C, $r_{[4]/[pG-pC]}$=0.26; (b) **5**+poly G–poly C, $r_{[5]/[pG-pC]}$=0.36; (c) **4**+ct-DNA, $r_{[4]/[ct-DNA]}$=0.01; (d) **5**+ct-DNA, $r_{[5]/[ct-DNA]}$=0.008; (e) **4**+poly A–poly U, $r_{[4]/[pA-pU]}$=0.005; (f) **5**+poly A–poly U, $r_{[5]/[poly\ A-poly\ U]}$=0.004; (g) **4**+poly dA–poly dT, $r_{[4]/[pA-pT]}$=0.005; (h) **5**+poly dA–poly dT, $r_{[5]/[pA-pT]}$=0.005.

Table 2
Binding constants[a] (log K_s) calculated from the fluorescence titrations of **4**–**7** with ds-polynucleotides at pH=5.0 (citric acid buffer, I=0.03 mol dm^{-3})

	4		**5**		**6**		**7**	
	I/I_0[b]	log K_s	I/I_0[b]	log K_s	I/I_0[b]	log K_s	I/I_0[b]	log K_s
ct-DNA	8	5.5	5	6.3	0.2	6.0	0	5.6
poly dA–poly dT	1.7	5.8	2.6	5.7	1.7	6.1	1.2	5.1
poly A–poly U	7	5.2	15	5.2	2.1	6.0	2.0	5.2
poly G–poly C	3	6.6[c]	2	6.8[c]	0	5.0	0	4.9

[a] Processing of titration data by means of Scatchard equation[24] gave values of ratio $n_{[bound\ 4-7]/[polynucleotide]}$=0.1–0.05, for easier comparison all log K_s values were recalculated for fixed n=0.1.

[b] I_0—starting fluorescence intensity of **4**–**7**; I—fluorescence intensity of **4**–**7**/polynucleotide complex calculated by Scatchard equation.

[c] Cumulative biding constants for mixed binding mode, calculated ratios n=0.9(**4**) and n=0.8(**5**).

On the other hand, while additions of poly A–poly U and poly dA–poly dT yielded strong fluorescence increase of **6** and **7**, most intriguingly, titrations with ct-DNA and poly G–poly C strongly quenched **6** and **7** fluorescence (Fig. 2, Table 1). Furthermore, opposite to **4** and **5**, in all titrations no significant shift of emission maxima of **6** and **7** was observed.

Processing of the titration data by means of Scatchard equation[24] gave the log K_s values (Table 2), which pointed to the comparable binding affinity of **4**–**7** toward most of the studied ds-polynucleotides. The only exception was titrations of **4** and **5** with poly G–poly C (Table 2), in which the sigmoidal shape of titration curves close to equimolar **4**,**5**/polynucleotide ratios and values $n\gg$0.2 suggest coexistence of more binding modes.

2.3.2. Thermal denaturation experiments

In thermal denaturation experiments at conditions close to the equimolar **4**–**7**/polynucleotide ratio, stabilization of ds-polynucleotides is outstandingly different (Table 3).

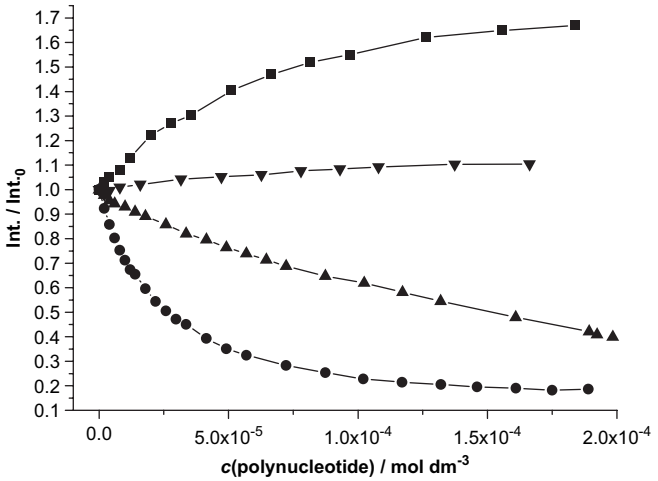


Figure 2. Fluorescence changes of **7** ($c=1\times10^{-6}$ mol dm^{-3}; $\lambda_{ex}=320$ nm) upon titration with ct-DNA (●), poly dA–poly dT(▼), poly A–poly U (■), poly G–poly C(▲).

Table 3
ΔT_m-Values[a] (°C) of different ds-polynucleotides with **4**–**7** at pH=5, (citric acid buffer, I=0.03 mol dm^{-3})

	r>[b]	4	5	6	7
ct-DNA	0.1	1.9/12.5[d]	1.79	5.16	—
	0.2	2.0/22.6[d]	2.88	7.68	—
	0.3	3.4/25.7[d]	3.85	9.61	2.4
poly A–poly U[c]	0.1	0/0.4[c]	0.5/0.6[c]	7.5/−2.3[c]	—
	0.2	0/1.1[c]	0/0.9[c]	10.6/−2.0[c]	—
	0.3	ND[e]	0/1.7[c]	12.3/−1.4[c]	3.4/0[c]
Poly dA–poly dT	0.1	3.2/22.1[d]	3.4	2.4	—
	0.2	3.3/27.7[d]	3.4	3.8	—
	0.3	3.3/33.3[d]	3.4	4.0	0.7

[a] Error in ΔT_m: ±0.5 °C.
[b] r=[**4**–**7**]/[polynucleotide].
[c] Biphasic transitions: the first transition at T_m=28.5 °C is attributed to denaturation of poly A–poly U and the second transition at T_m=80.1 °C is attributed to denaturation of poly AH$^+$–poly AH$^+$ since poly A at pH=5 is mostly protonated and forms ds-polynucleotide.[32]
[d] Biphasic thermal denaturation transitions.
[e] Not possible to determine.

Compounds **6** and **7** more efficiently stabilized ds-RNA than ds-DNA helices, which resembled the ds-RNA selectivity of ethidium bromide.[25] Weaker stabilization effect of **6** and **7** on the poly dA–poly dT than ct-DNA is most likely a result of the peculiar twisted structure of the former polynucleotide, which has to unwind significantly to allow intercalation.[26] It is noteworthy that dimmer **6** stabilized all studied polynucleotides 4 times more strongly than monomer **7**. Opposite to **6** and **7**, compounds **4** and **5** stabilized only ct-DNA and poly dA–poly dT but didn't show any impact on the thermal denaturation of poly A–poly U. Such a behavior is characteristic for small molecules that bind into the minor groove of ds-polynucleotides,[25] since the broad, shallow minor groove of ds-RNA does not support small molecule binding as well as the deep, narrow minor groove of the ds-DNA.

It should be stressed that stabilization of ds-DNA's by **4** is an order of magnitude stronger than the stabilization effect of monomer **7**, and several times stronger than stabilization effects of **5** and **6**.

2.3.3. *Circular dichroism experiments*

So far, non-covalent interactions at 25 °C were studied by monitoring the spectroscopic properties of studied compound upon addition of the polynucleotides. In order to get insight into the changes of polynucleotide properties induced by small molecule binding, we have chosen CD spectroscopy as a highly sensitive method toward conformational changes in the secondary structure of polynucleotides.[27] In addition, achiral small molecules like **4**–**7** can eventually acquire an induced CD spectrum (ICD) upon binding to polynucleotides, from which, mutual orientation of small molecule and polynucleotide chiral axis could be derived, consequently giving useful information about modes of interaction.[28]

Close to the equimolar **4**–**7**/polynucleotide ratios, addition of **4**–**7** resulted in the significant decrease of CD spectra of all studied polynucleotides (Figs. 3 and 4, λ<300 nm). Again, **6** and **7** induced similar changes in the CD spectra of both, ct-DNA and poly A–poly U. A clear isoelliptic point at λ=256 nm (ct-DNA) and λ=248 nm (poly A–poly U) pointed toward formation of one dominant type of **6**/polynucleotide complex.[28] Additionally, weak positive induced CD (ICD) band in the region between λ=300 and 350 nm was observed for both, ct-DNA and poly A–poly U. Since **6** and **7** do not have any intrinsic CD spectrum, but phenanthridinium has UV/vis spectrum in corresponding region, the observed ICD band strongly suggested uniform orientation of phenanthridinium(s) of **6** and **7** in respect to the chiral axis of polynucleotide duplex.[28] The positive sign of ICD spectrum at λ>300 nm observed for **6**, **7** suggests that the longer axis of phenanthridinium moiety is approximately perpendicular to the long axis of the base-pair pocket but still in plane with the adjacent base pairs.[27] That would agree well with the bulkiness of the urea substitutent attached to the 3-position of phenanthridinium moiety, thus hampering the positioning of phenanthridinium longer axis parallel to the long axis of the base pair pocket.

The results of the CD experiments obtained for **4** (Fig. 3A) were substantially different from those observed for other studied compounds. The most remarkable feature observed among all studied compounds exclusively for **4** was inversion of the strong

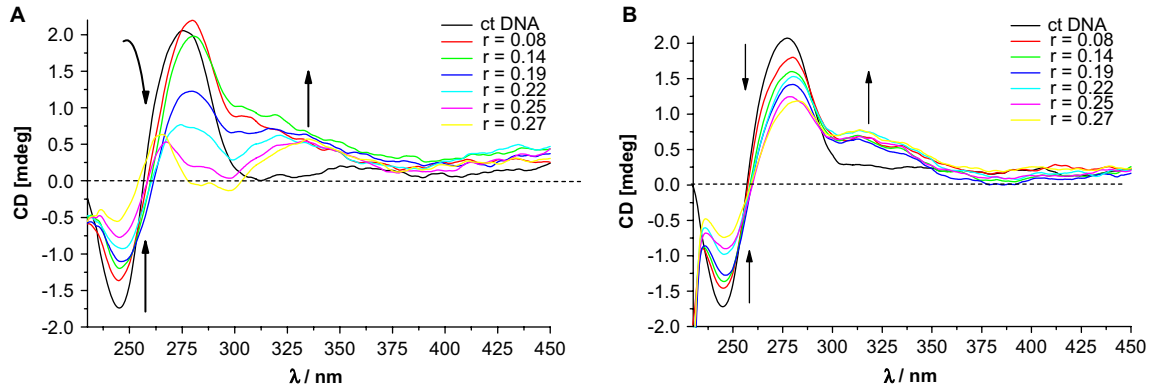


Figure 3. CD titrations of ct-DNA ($c=2.0\times10^{-5}$ mol dm^{-3}) with **4** (A, r[**4**]/[DNA]), **6** (B, r[**6**]/[DNA]), at pH=5, (citric acid buffer, I=0.03 mol dm^{-3}).

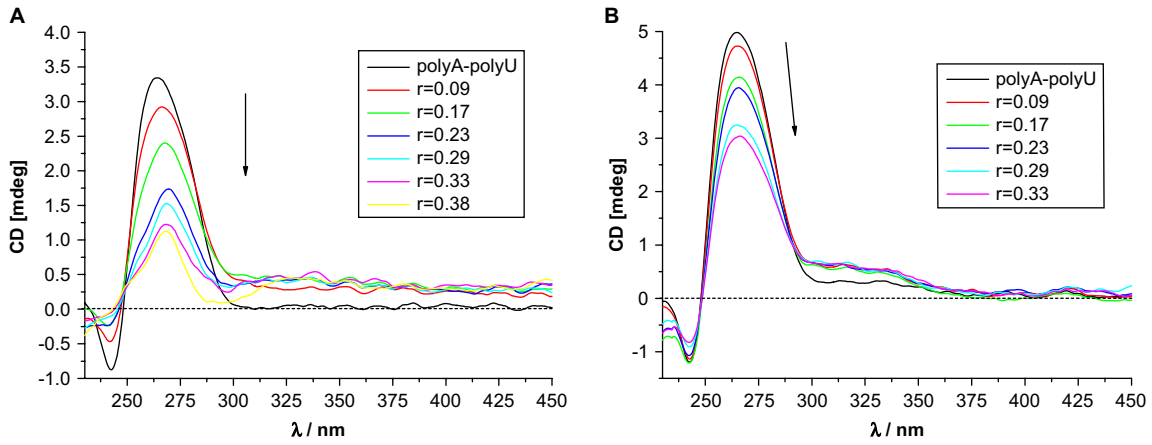


Figure 4. CD titrations of poly A–poly U (c=1.0×10^{-5} mol dm^{-3}) with **4** (A, r[4]/[pA–pU]), **6** (B, r[**6**]/[pA–pU]) at pH=5 (citric acid buffer, I=0.03 mol dm^{-3}).

positive CD bands in the range λ=280–300 nm (ct-DNA and poly A–poly U) into almost negative bands. However, from obtained results it is not clear whether that inversion is the consequence of the severe changes of polynucleotide helices or the strongly negative ICD spectrum of **4** in the range λ=250–300 nm. Upon mixing **4** with ct-DNA clear deviation of the isoelliptic point revealed coexistence of more binding modes of **4** to ct-DNA close to conditions of polynucleotide saturation with **4**, and agreeing nicely with biphasic transitions in the thermal denaturation experiments (Table 3). Opposite to **4**, mixing of **5** with ct-DNA yielded a clear isoelliptic point, as well as a positive ICD spectrum at λ>300 nm. However, for **5**/poly A–poly U mixing, clear deviation of the isoelliptic point may indicate different binding modes.

2.3.4. *Discussion of results*

The results obtained for **6** and **7** (strong affinity, thermal stabilization of both ds-DNA and ds-RNA, weak ICD effects) suggested intercalation as a dominant binding mode to polynucleotides.[29] Opposite changes in fluorescence of **6** and **7** seem to be related to the different electronic properties of A–U(T) and G–C base pairs, respectively. Among only few compounds known to exhibit opposite changes of fluorescence upon addition of A–U(T) and G–C base pairs, for acridine derivatives this effect was correlated with the property of guanine being the most electron-donating of all four nucleobases.[30] It should be stressed that efficient fluorescence quenching of electron-accepting fluorophore can be achieved either, by direct aromatic stacking interactions with guanine or by remote G sites over the electron-transfer mechanism through the π-stacked DNA helix when the fluorophore is efficiently stacked within the DNA double helix.[31] Both modes of quenching presume strong aromatic stacking interactions of fluorophore with polynucleotide and therefore additionally support intercalation of monomer **7** and dimer **6** into ds-DNA and ds-RNA.

Unlike **6** and **7**, compounds **4** and **5** do not stabilize ds-RNA at all, thus excluding an intercalative binding mode.[29] However, the high affinity of **4** and **5** toward ds-RNA observed in fluorimetric titration experiments (Table 2), as well as pronounced CD effects, suggest agglomeration of **4**, **5** along ds-RNA polynucleotides, most likely inside the hydrophobic major groove.[32] Such agglomerates could be stabilized by aromatic stacking interactions between phenanthridinium moieties of two different molecules of **4** (or **5**) similar as found for some acridinium dyes.[33] Since **4** and **5** do not intercalate into poly G–poly C, there is no efficient fluorescence quenching by guanine, which usually happens over the electron-transfer mechanism through the π-stacked DNA helix when the fluorophore is efficiently stacked within the DNA double helix. Consequently, the fluorescence of **4** and **5** was increased due to the agglomeration. In addition, the strong bathochromic shift of emission maxima of **4** and **5** observed exclusively upon addition of poly G–poly C (Fig. 1) could be the result of the recognition of G–C base pairs by bis-urea linkers of **4** and **5**, as shown before for some analogous compounds.[17]

Compound **4** binds to ds-DNA's by mixed binding modes. The thermal stabilization effect of **4** on the ds-DNA is several times stronger than effect of dimeric analogue **6**, as well as monomer **7** (Table 3) and therefore cannot be attributed to intercalation. In addition, increase of the fluorescence of **4** upon addition of ct-DNA excluded aromatic stacking interactions with dG–dC base pairs, otherwise fluorescence quenching should occur as observed for **6**, **7**. These results suggest that dominant interactions of **4** are formed within ds-DNA minor groove, most likely as a result of hydrogen bonding between urea groups of **4** and polynucleotide backbone and/or base pairs (as found for polyamides[34] and bis-guanidinium derivative of ethidium[6]).

Although most of the results of interactions with ds-DNA obtained for **5** and for **6**, **7** are quite comparable, increase in fluorescence **5** upon addition of ct-DNA excluded efficient aromatic stacking interactions with dG–dC base pairs. Since **5** does not intercalate into ds-RNA, it seems more likely that it binds within ds-DNA minor groove similarly as proposed for **4**.

Obviously, the reason for the switch of DNA/RNA binding mode from DNA minor groove binding/RNA groove agglomeration (**4**, **5**) to intercalation (**6**) is the length of the aliphatic linker connecting two urea–phenanthrininium subunits. The intrinsic fluorescence emission intensities, Int($\mathbf{7}_{\text{monomer}}$)≫Int(**6**)>Int(**5**)>Int(**4**), are obviously reversely proportional to the length of the aliphatic linker connecting two urea–phenanthridinium subunits, and therefore most likely related to the efficiency of intramolecular interactions between two phenanthridinium subunits **4**–**6**. Accordingly, the shortest linker (**4**) would allow highly efficient intramolecular interaction between phenanthridinium units. Most likely, such a small folded molecule fits tightly into ds-DNA minor groove, yielding the strongest ds-DNA stabilization (Table 3). Although the longer linker (**5**) weakens intramolecular interactions between phenanthridinium units, it seems that **5** is still mostly present in the folded form, not supporting intercalation into ds-polynucleotides, and thus giving no thermal stabilization of ds-RNA. Since the folded form of **5** is larger than **4**, it does not fit so tightly into ds-DNA minor groove, and therefore yielded an order of magnitude weaker ds-DNA stabilization than **4**. Finally, the longest linker (**6**) does not support intramolecular stacking of urea–phenanthridine subunits and consequently allowed independent binding of urea–phenanthridinium subunits to DNA/RNA by intercalation.

3. Conclusions

In conclusion, it is important to stress that none of the previously known bis-phenanthridinium analogues showed such linker-length dependent switch of the DNA/RNA binding mode. Obviously a fine interplay between intramolecular aromatic stacking and DNA/RNA intercalation combined with the potential of hydrogen bonding interactions (urea) could have a dramatic impact on the interaction of small molecule with DNA/RNA. Together with previously shown recognition of polynucleotides by modified phenanthridines[3,10,12,23] and related compounds, the results presented here add significantly to the information pool available for the design of DNA/RNA selective small molecules. In addition, **6** and **7** are, to the best of our knowledge, the first phenanthridine-based intercalators able to differentiate between A–U(T) and G–C base pairs by sign of fluorimetric response.

Furthermore, due to the capability of urea substituents to form transition metal complexes,[35] which are in this work combined with phenanthridine fluorophores, compounds **4**–**7** could be of great interest for researchers in the fields of inorganic chemistry and biochemistry. Moreover, preliminary results of our current studies point toward promising antiproliferative activity of urea–phenanthridinium compounds toward human tumor cell lines.

4. Experimental

4.1. Synthesis

*4.1.1. 4′-(Phenyloxycarbonyl)aminobiphenyl-2-yl-acetamide (**2**)*

N,*N*-Dimethylaniline (1.15 ml, 1.1 g, 9.07 mmol) was added to a stirred solution of 4′-aminobiphenyl-2-yl-acetamide (1.14 g, 5.04 mmol) in anhydrous ethanol (25 ml). To the resulting mixture a solution of phenyl chloroformate (0.925 ml, 0.947 g, 6.05 mmol) in ethanol (2 ml) was added dropwise and the reaction was carried out under reflux for 2 h. Subsequently, the solvent was evaporated under reduced pressure, the remaining solid was dissolved in ethyl acetate (20 ml), resulting solution washed with water (2×20 ml) and crude product was obtained by evaporation of solvent under reduced pressure. The product was purified by recrystallization from dichloromethane–petroleum ether to give 1.51 g of brown-gray crystals (86%).

Mp=183 °C; ^{1}H NMR (DMSO-d_6) δ/ppm: 1.83 (s, CH_3, 3H), 7.16–7.28 (m, 5H, biphenyl, 3H, PhOCO), 7.36–7.40 (m, 1H, biphenyl, 2H, PhOCO), 7.50–7.51 (s, 2H, biphenyl), 9.15 (s, 1H, $NHCOCH_3$), 10.27 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ/ppm: 23.02 (CH_3), 118.40, 121.85, 125.39, 125.77, 127.03, 127.34, 129.20, 129.40, 130.05, 133.59, 134.89, 136.07, 137.73, 150.53, 151.70, 168.57; IR (KBr) ν (cm^{-1}): 3454.26, 3394.47, 3238.24, 3172.67, 3093.59, 3041.52, 2937.37, 2856.37, 2790.79, 2362.63, 2335.62, 2219.90, 1951.82, 1930.60, 1733.88, 1666.37, 1608.51, 1589.23, 1483.15, 1446.51, 1407.93, 1321.14, 1298.00, 1228.57, 1157.20, 1068.49, 1022.20, 1010.63, 916.20, 835.12, 811.97, 796.54, 756.04, 719.40, 663.46, 561.24, 507.24, 466.74, 379.95, 329.80, 287.37. For X-ray structure see Ref. 36.

*4.1.2. Phenyl-6-methylphenanthridin-8-yl carbamate hydrochloride hydrate (**3**)*

4′-(Phenyloxycarbonyl)aminobiphenyl-2-yl-acetamide (**2**) (0.97 g, 2.80 mmol) was dissolved in phosphorus oxychloride (4.1 ml) and heated at 100 °C for 1 h. The reaction mixture was poured into ice water to give a brownish oil, which was upon stirring converted into yellow precipitate. The resulting mixture was slowly neutralized by additions of 5 M NaOH, the solid was filtered off, washed with water and dried. After recrystallization from methanol, the pure product (**3**) was obtained as a yellow powder or crystals (0.81 g, 88%). Mp 228–229 °C; ^{1}H NMR (DMSO-d_6) δ/ppm: 3.11 (s, 3H, CH_3), 7.21–7.24 (m, 3H, PhOCO), 7.38–7.41 (m, 2H, PhOCO), 7.78–7.83 (m, 2H, phen-H2, phen-H3), 8.23 (dd, 1H, phen-H9, J_{7-9}=2.0 Hz, J_{9-10}=9.0 Hz), 8.41 (d, 1H, phen-H4, J_{3-4}=7.5 Hz), 8.64 (d, 1H, phen-H7, J_{7-9}=1.6 Hz), 8.74 (d, 1H, phen-H1, J_{1-2}=7.5 Hz), 8.86 (d, 1H, phen-H10, J_{9-10}=9.0 Hz), 10.82 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ/ppm: 18.83(CH_3), 115.44 (phen-H7), 121.68 (phen-H4); 122.22 (Ph); 123.02 (phen-H1); 123.82; 124.42 (phen-H10), 124.92, 125.60 (Ph), 127.19 (phen-H9), 128.83, 129.03 and 129.41 (phen-H2 and phen-H3), 129.86 (Ph), 133.81, 139.81, 150.49, 151.91, 160.28. IR (KBr) ν (cm^{-1}): 3425.33, 3240.17, 3109.02, 3066.59, 3041.52, 2921.94, 2854.44, 2671.21, 2370.34, 2345.27, 2023.18, 1944.10, 1735.81, 1625.94, 1566.08, 1531.37, 1488.94, 1448.43, 1371.29, 1321.14, 1292.21, 1234.35, 1193.85, 1176.49, 1070.41, 1026.05, 844.76, 784.97, 754.11, 717.47, 682.75, 557.39, 414.67, 316.30. Anal. Calcd for $C_{21}H_{19}N_2O_3Cl$ ($C_{21}H_{16}N_2O_2\cdot H_2O\cdot$ HCl): C, 65.88; H, 5.00; N, 7.32. Found: C, 65.95; H, 4.92; N, 7.37.

*4.1.3. 1-(6-Methylphenanthridin-8-yl)-3-[2-(3-(6-methylphenanthridin-8-yl)ureido)ethyl] urea trihydrate (**4**)*

Phenyl-6-methylphenanthridin-8-yl carbamate (**3**) (0.206 g, 0.627 mmol) was dissolved in dimethyl formamide (3 ml). To this, K_2CO_3 (0.087 g, 0.627 mmol) and 1,2-diaminoethane (0.020 ml, 0.018 g, 0.298 mmol) were added. The reaction was stirred at 60–80 °C for 3–4 h and then at room temperature overnight. Upon addition of water into the reaction mixture, a white precipitate was formed and filtered off. The crude product was recrystallized from methanol to give 0.092 g of a pale yellow solid (58%). Mp 297 °C; ^{1}H NMR (DMSO-d_6) δ/ppm: 2.88 (s, 6H, CH_3), 3.34–3.35 (m, 4H, CH_2), 6.40–6.42 (m, 2H, NH), 7.56–7.66 (m, 4H, phen-H2, phen-H3), 7.90–7.95 (m, 4H, phen-H4, phen-H9), 8.42 (d, 2H, phen-H7, J_{7-9}=1.3 Hz), 8.58 (d, 2H, phen-H1, J_{1-2}=7.4 Hz), 8.65 (d, 2H, phen-H10, J_{9-10}=8.9 Hz), 9.06 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) δ/ppm: 24.25 (CH_3), 41. 06 (–(CH_2)$_2$–), 113.86 (phen-H7), 123.12 (phen-H1), 123.76 (phen-H9), 124.58 (phen-H10), 124.76, 127.46, 127.58 and 128.73 (phen-H2, phen-H3), 130.15 (phen-H4), 141.43, 143.65, 156.79, 159.16; IR (KBr) ν (cm^{-1}): 3315.39, 3064.66, 2939.30, 2362.63, 2345.27, 1635.52, 1591.16, 1562.23, 1529.44, 1483.15, 1463.86, 1379.00, 1325.00, 1299.93, 1263.28, 1228.57, 1149.49, 1116.70, 1008.70, 993.27, 950.84, 865.97, 837.04, 761.83, 723.25, 642.25, 622.96, 540.03, 518.81, 464.81, 405.02, 364.52, 329.80, 295.09. Anal. Calcd for $C_{32}H_{34}N_6O_5$ ($C_{32}H_{28}N_6O_2\cdot 3H_2O$): C, 65.96; H, 5.88; N, 14.43. Found: C, 66.15; H, 5.77; N, 14.59.

*4.1.4. 1-(6-Methylphenanthridin-8-yl)-3-[6-(3-(6-methylphenanthridin-8-yl)ureido)hexyl] urea trihydrate (**5**)*

The product (**5**) was synthesized as described for **4**, starting from phenyl-6-methylphenanthridin-8-yl carbamate (**3**) (0.273 g, 0.832 mmol), 1,6-diaminohexane (0.046 g, 0.396 mmol), K_2CO_3 (0.115 g) in dimethyl formamide (7 ml). The product was then recrystallized from methanol to yield 0.157 g of a pale yellow solid (68%). Mp 287–288 °C; ^{1}H NMR (DMSO-d_6) δ/ppm: 1.30–1.44 (m, 8H, CH_2), 2.81 (s, 6H, CH_3), 3.08–3.11 (m, 4H, N–CH_2), 6.27 (t, 2H, NH, J_{NH-CH2}=5.5 Hz), 7.51–7.58 (m, 4H, phen-H2, phen-H3), 7.81 (dd, 2H, phen-H9, J_{7-9}=2.0 Hz, J_{9-10}=8.9 Hz), 7.87 (d, 2H, phen-H4, J_{3-4}=7.98 Hz), 8.34 (d, 2H, phen-H7), 8.53 (d, 2H, phen-H1, J_{1-2}=8.2 Hz), 8.60 (d, 2H, phen-H10, J_{9-10}=9.0 Hz), 8.88 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) δ/ppm: 23.01 (CH_3), 26.14 and 29.73 (–(CH_2)$_4$–), 39.09 (N(–CH_2–)$_2$), 112.26 (phen-H7), 121.84 (phen-H1), 122.32 (phen-H9), 123.32 (phen-H10), 123.46, 125.96, 126.16, 126.30, and 127.42 (phen-H2, phen-H3), 128.83 (phen-H4), 140.24, 142.28, 155.19, 157.89; IR (KBr) ν (cm^{-1}): 3325.03, 3105.17, 3056.95, 2925.80, 2864.08, 2364.55, 2339.48, 1942.18, 1888.17, 1643.23, 1560.30, 1475.44, 1438.79, 1380.93, 1359.72, 1296.07, 1245.92, 1203.49, 1114.77, 1010.63, 946.98, 875.62, 862.12, 823.54, 757.97, 700.11, 653.82, 621.03, 540.03, 520.74, 466.74, 430.09, 393.45, 358.73, 335.59, 316.30, 273.87. Anal. Calcd for $C_{36}H_{42}N_6O_5$ ($C_{36}H_{36}N_6O_2\cdot 3H_2O$): C, 67.69; H, 6.63; N, 13.16. Found: C, 67.41; H, 6.63; N, 13.13.

4.1.5. 1-(6-Methylphenanthridin-8-yl)-3-[9-(3-(6-methylphenanthridin-8-yl)ureido)nonyl] urea (**6**)

The product (**6**) was synthesized as described for **4**, starting from phenyl-6-methylphenanthridin-8-yl carbamate (**3**) (0.174 g, 0.531 mmol), 1,9-diaminononane (0.04 g, 0.253 mmol), and K_2CO_3 (0.073 g, 0.531 mmol) in dimethyl formamide (4 ml). The product was then recrystallized from methanol to give 0.087 g of a pale yellow solid (55%). Mp 230 °C; ^{1}H NMR (DMSO-d_6) δ/ppm: 1.23–1.40 (m, 14H, CH_2), 2.81 (s, 6H, CH_3), 3.05–3.09 (m, 4H, N–CH_2), 6.24 (t, 2H, NH, J_{NH-CH2}=5.3 Hz), 7.51–7.57 (m, 4H, phen-H2, phen-H3), 7.81 (d, 2H, phen-H9, J_{9-10}=8.9 Hz), 7.87 (d, 2H, phen-H4, J_{3-4}=8.0 Hz), 8.33 (s, 2H, phen-H7), 8.52 (d, 2H, phen-H1, J_{1-2}=7.9 Hz), 8.59 (d, 2H, phen-H10, J_{9-10}=8.9 Hz), 8.87 (s, 2H, NH); ^{13}C NMR (DMSO-d_6) δ/ppm: 23.00 (CH_3), 26.39, 28.75, 29.04 and 29.73 (CH_2, 14H), 39.13 (4H, N–CH_2), 112.24 (phen-H7), 121.83 (phen-H1), 122.31 (phen-H9), 123.31 (phen-H10), 123.46, 125.95, 126.15, 126.29 and 127.41 (phen-H2, phen-H3), 128.82 (phen-H4),140.23, 142.27, 155.19, 157.89; IR (KBr) ν (cm^{-1}): 3315.39, 3294.18, 3058.88, 2927.73, 2848.65, 2364.55, 2335.62, 1631.66, 1591.16, 1560.30, 1533.30, 1481.22, 1463.86, 1438.79, 1377.07, 1323.07, 1298.00, 1226.64, 1205.42, 1114.77, 1037.63, 993.27, 946.98, 931.55, 865.97, 833.19, 757.97, 723.25, 653.82, 540.03, 489.88, 464.81, 420.45, 378.02, 289.30; ES-MS *m*/*z* for $C_{39}H_{42}N_6O_2$ (M_r=626.81 g mol^{-1}): 627.1 $[M+H]^+$, 314.3 $[M+2H]^{2+}$. Anal. Calcd for $C_{39}H_{44}N_6O_3$ ($C_{39}H_{42}N_6O_2\cdot CH_3OH$): C, 72.92; H,7.04; N, 12.76. Found: C, 72.72; H, 6.91; N, 12.42.

4.1.6. 1-Ethyl-3-(6-methylphenanthridin-8-yl)urea (**7**)

The product (**7**) was synthesized as described for **4**, starting from phenyl-6-methylphenanthridin-8-yl carbamate (**3**) (0.0556 g, 0.1695 mmol), aminoethane hydrochloride (0.025 g, 0.307 mmol), and K_2CO_3 (0.0424 g, 0.307 mmol) in dimethyl formamide (2 ml). The product was then recrystallized from methanol to give 0.019 g of a white solid (40%). Mp 215–216 °C; ^{1}H NMR (DMSO-d_6) δ/ppm: 1.018 (pst, 3H, CH_3), 2.81 (s, 3H, phen-CH_3), 3.07–3.12 (m, 2H, N–CH_2), 6.22 (t, 2H, NH, J_{NH-CH2}=5.5 Hz), 7.51–7.58 (m, 2H, phen-H2, phen-H3), 7.81 (d, 1H, phen-H9, J_{9-10}=8.9 Hz), 7.87 (d, 1H, phen-H4, J_{3-4}=8.0 Hz), 8.33 (d, 1H, phen-H7, J_{7-9}=2.1 Hz), 8.53 (d, 1H, phen-H1, J_{1-2}=8.1 Hz), 8.60 (d, 1H, phen-H10, J_{9-10}=8.9 Hz), 8.89 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ/ppm: 15.41 (CH_3), 22.96 (phen-CH_3), 34.02 (2H, N–CH_2), 112.29 (phen-H7), 121.86 (phen-H1), 122.40 (phen-H9), 123.32 (phen-H10), 123.45, 125.97, 126.12, 126.34 and 127.46 (phen-H2, phen-H3), 128.73 (phen-H4),140.24, 142.15, 155.11, 157.93; IR (KBr) ν (cm^{-1}): 3458.11, 2929.66, 2364.55, 1666.37, 1593.09, 1550.65, 1521.72, 1508.22, 1481.22, 1463.86, 1380.93, 1326.93, 1307.64, 1249.78, 1076.20, 881.40, 835.12, 761.83, 723.25, 688.54, 655.75, 551.60, 464.81; ES-MS *m*/*z* for $C_{17}H_{17}N_3O$ (M_r=279.34 g mol^{-1}) 280.2 $[M+H]^+$; HRMS (FAB), for $C_{17}H_{18}N_3O$ $[M+H]^+$ M_r calcd=280.1444; M_r found=280.1446.

4.2. Materials and methods

Materials were obtained from commercial suppliers and were used without purification unless noted otherwise. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker AV300 or Brucker AV600 (at 300 and 600 MHz) at 25 °C. Chemical shifts (δ) were given in parts per million (ppm) relative to tetramethylsilane as an internal standard and coupling constants (*J*) in hertz. The splitting patterns in the ^{1}H NMR spectra are denoted as follows: s (singlet), d (doublet), t (triplet), pst (pseudotriplet), m (multiplet). Melting points were determined on a Kofler melting points apparatus and are uncorrected. Infrared spectra were recorded on a BOMEM MB 102 spectrophotometer and spectral bands are expressed in 'wave numbers' with the unit cm^{-1}. Elemental analyses were carried out with a Perkin Elmer HCNS/O analyzer Series II 2400. Mass spectra were obtained using Waters Micromass ZQ spectrometer. The pH measurement was carried out using Mettler TOLEDO MP220 pHmeter calibrated with commercially available buffered aqueous solutions of pH standards 4.00 and 7.00.

The UV/vis spectra were recorded on a Varian Cary 100 Bio spectrophotometer, CD spectra on JASCO J815 spectrophotometer and fluorescence spectra on a Varian Cary Eclipse spectrophotometer at 25 °C using appropriate 1 cm path quartz cuvettes. For study of interactions with DNA and RNA, aqueous solutions of compounds buffered to pH=5 with citric acid buffer, I=0.03 mol dm^{-3} were used. Buffered aqueous solutions of **4**–**7** were stable for more days on the room temperature and after a week they tend to precipitate. Under the experimental conditions UV/vis, CD and fluorescence spectra of **4**–**7** were proportional to their concentrations up to c(**4**–**7**)=5×10^{-5} mol dm^{-3}.

Materials. Polynucleotides were purchased as noted: poly A–poly U, poly G–poly C, poly dA–poly dT (Sigma), *calf thymus* (*ct*)-DNA (Aldrich). Polynucleotides were dissolved in Na-cacodylate buffer, I=0.05 mol dm^{-3}, pH=7. The calf thymus (*ct*)-DNA was additionally sonicated and filtered through a 0.45 μm filter.[37] Polynucleotide concentration was determined spectroscopically[38] as the concentration of phosphates.

Spectrophotometric titrations were performed at pH=5 (I=0.03 mol dm^{-3}, sodium citrate/HCl buffer) by adding portions of polynucleotide solution into the solution of the studied compound for UV/vis and fluorimetric experiments and for CD experiments were done by adding portions of compound stock solution into the solution of polynucleotide. In fluorimetric experiments excitation wavelength of λ_{exc}>300 nm was used to avoid the inner filter effect caused due to increasing absorbance of the polynucleotide. Emission was collected in the range λ_{em}=400–600 nm. Processing titration data by means of Scatchard equation[24] gave values of ratio n=0.1±0.05. For easier comparison all K_s values were re-calculated for fixed n=0.1. Values for K_s given in Table 2 all have satisfactory correlation coefficients (>0.99). Thermal melting curves for DNA, RNA and their complexes with studied compounds were determined as previously described[38] by following the absorption change at 260 nm as a function of temperature. Absorbance of the ligands was subtracted from every curve and the absorbance scale was normalized. T_m values are the midpoints of the transition curves determined from the maximum of the first derivative and checked graphically by the tangent method.[38] The ΔT_m values were calculated subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value here reported was the average of at least two measurements. The error in ΔT_m is ±0.5 °C.

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