Acid-base properties of functionalised tripodal polyamines and their interaction with nucleotides and nucleic acids

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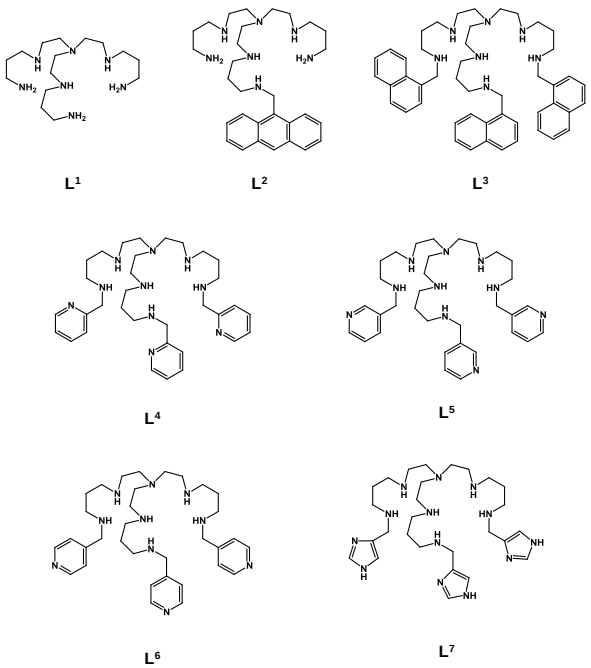
Novel, highly positively charged tripodal polyamines with appended heterocyclic moieties revealed an intriguing panel of protonation species within the biologically relevant range. Studied compounds bind nucleotide monophosphates by mostly electrostatic interactions but only the imidazole analogue showed selectivity toward UMP in respect to other nucleotides. Strong binding of all the studied compounds to both ds-DNA and ds-RNA is to some extent selective toward the latter, showing rather rare RNA over DNA preference.

Introduction

Current challenges in diagnostics and emerging therapies for treating genetic diseases call for novel, improved technologies for *in vitro* and *in vivo* targeting of nucleic acids. The rational design of new molecules able to interact selectively with nucleic acids has an immense practical application in several fields ranging from construction of nanomaterials to drug design and delivery.[1] Over the past few decades, small molecules that bind to DNA have shown significant promise as diagnostic probes, reactive agents and therapeutics. Much attention has focused on the design of organic DNA-binding agents as well as on the improvement of DNA detection methods in real time with high sensitivity.[2,3] Despite the large number of cellular roles that RNA plays in biological processes, this macromolecule has been considered only recently an attractive target for therapeutic intervention.[4] RNA is essential for replication,[5] transcription[6] and regulation processes,[7] protein function[8] and catalysis.[9] The development of molecules that bind specifically to RNA opens exciting new ways in therapeutic strategies.[4,10]

It is well-known that the natural polyamines spermidine and spermine and their diamine precursor putrescine are ubiquitous small basic molecules found in all eukaryotic cells which are implicated in many aspects of cellular physiology.[11] Polyamines are essential for mammalian cell growth and development but their specific functions at the molecular level are still far from clear. Interactions of polyamines with nucleic acids have been studied since the early 1960s[12] when it was found that they were bound to various cellular anions including DNA, RNA, proteins, and phospholipids.[11,13] Some of us had previously reported on different studies dealing with the affinities of some tripodal polyamines (**L**^{1}-**L**^{3} in Scheme 1) towards RNA and DNA models.[14] The high positive charge density coupled with high ligand flexibility allowed particularly deep and undistorted groove binding. Tripodal polyamines **L**^{1}-**L**^{3} showed RNA groove preference. Also, the unfolding effects of Cu^{2+} in those ligands held promise for the potential use of such complexes for RNA cleavage. In order to obtain tripodal ligands in which the functionalities at the terminal positions of the three arms could participate in the coordination of metal ions, we have prepared new receptors by attaching pyridine and imidazole units to the primary nitrogens of the enlarged tripodal polyamine **L**^{1}. Here we report on the interaction with nucleotide monophosphates and nucleic acids of the tripodal polyamines **L**^{4}-**L**^{7}.



Scheme 1 Structures of previously studied compounds (**L**^{1}-**L**^{3})[14] and of derivatives (**L**^{4}-**L**^{7}) here analysed

Results and discussion

Acid-base behaviour

Table 1 collects the stepwise basicity constants for the tripodal ligands **L**^{4}-**L**^{7} determined in NaCl or $NaClO_4$ 0.15 mol·dm^{-3} at 298.0 ± 0.1 K as well as those for **L**^{1}-**L**^{3} previously reported and determined at 298.1 K using 0.15 mol·dm^{-3} NaCl as ionic strength.[15] Figure 1 gives an example

Table 1 Logarithms of the protonation constants of tripodal ligands **L**[4]-**L**[7] determined in NaCl and **L**[5]-**L**[6] determined in $NaClO_4$ 0.15 mol·dm^{-3} at 298.0 ± 0.1 K. For comparison, this table includes logarithms of the protonation constants of tripodal ligands **L**[1]-**L**[3] determined in NaCl 0.15 mol·dm^{-3} at 298.0 ± 0.1 K.

Reaction	L^4	L^5	L^6	$L^{7\,c}$	$L^{1\,d}$	$L^{2\,d}$	$L^{3\,d}$
$L + H \leftrightarrows HL$[a]	10.41(2)[b]	10.02 (9)	9.79 (3)	9.78(2)	10.34 (7)	10.41 (3)	9.08 (6)
$HL + H \leftrightarrows H_2L$	9.46(1)	9.19 (6)	9.43 (3)	9.53(1)	10.26 (2)	9.87 (2)	8.70 (5)
$H_2L + H \leftrightarrows H_3L$	8.69(1)	8.44 (6)	8.43 (5)	8.64(1)	9.52 (4)	9.17 (3)	8.48 (5)
$H_3L + H \leftrightarrows H_4L$	7.61(1)	7.48 (6)	7.65 (6)	7.83(1)	8.68 (4)	8.02 (3)	7.76 (4)
$H_4L + H \leftrightarrows H_5L$	7.09(1)	6.89 (6)	6.81 (7)	7.35(1)	7.91 (5)	7.20 (3)	7.09 (5)
$H_5L + H \leftrightarrows H_6L$	6.35(1)	6.41 (6)	6.76 (7)	6.72(1)	7.37 (4)	5.78 (8)	6.80 (4)
$H_6L + H \leftrightarrows H_7L$	-	4.02 (9)	5.24 (1)	4.36(1)	2.21 (1)	< 2.00	2.25 (9)
$H_7L + H \leftrightarrows H_8L$	-	3.11 (9)	4.08 (1)	4.17(1)	-	-	-
$H_8L + H \leftrightarrows H_9L$	-	3.00 (2)	3.48 (1)	2.78(6)	-	-	-
log β[e]	49.63	58.60	63.90	60.72	56.28	52.45	50.16

[a] Charges omitted. [b] Numbers in parentheses are standard deviations in the last significant figure. [c] Taken from reference 16. [d] Taken from reference 15. [e] $\log \beta = \Sigma \log K_{HjL}$.

of distribution diagram for the species existing in equilibrium for the protonation of receptor **L**[5]. Figure S1 (ESI†) includes the distribution diagram for the species existing in equilibrium for all receptors **L**[4]-**L**[7]. The trend of the protonation constants can be largely interpreted in terms of minimization of coulombic repulsion between same sign charges.[17] All ligands **L**[4]-**L**[7] present six relatively high basicity constants in agreement with the protonation of the secondary amine nitrogen atoms. (Table 1 and Figure S1, ESI†).

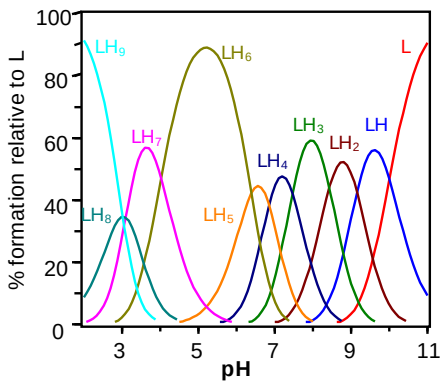


Figure 1 Distribution diagram for the species existing in equilibrium for the protonation of receptor **L**[5]

It is well established that electrostatic repulsion between positive charges separated by propylenic chains is considerably lower than when the separation is by ethylenic chains.[18] This is the reason for the relatively small decrease in basicity observed in every one of the six first protonations of all three ligands. These stepwise protonation constants are in all cases lower than those reported for precursor **L**[1], which can be attributed to the electron withdrawing character of the pyridine and imidazole rings [17,18,19] The next three basicity constants of **L**[5]-**L**[7] can be ascribed to the protonation steps of the pyridine and imidazole rings attached to the arms. Acid-base behavior of ligand **L**[4] has been previously reported.[20] The most important difference between them resides on the higher basicity of the pyridine nitrogens of **L**[5]-**L**[6]. The nitrogens of the imidazole moieties in **L**[7] have a basicity between the 3- and 4-substituted pyridines in **L**[5] and **L**[6]. For **L**[4] the values determined spectroscopically for the last three protonation steps are below 2 logarithmic units. In all ligands the apical nitrogen atom would not bear any neat protonation.

Interaction with Nucleotides

Detection of nucleosides and nucleotides in aqueous medium is of paramount importance as they form the fundamental units of all the life forms. However, differentiation among naturally occurring nucleobases based on different hydrogen bonding patterns within the artificial receptor is strongly limited due to competitive hydrogen bonding of water.[21] Therefore, although many artificial receptors have been reported, most of them lack of base selectivity. As a matter of fact, until now there are only a few receptors able to selectively bind specific nucleobases in water. Lhomme *et al.* showed the capacity of aryl-nucleobase conjugates to recognize certain nucleobases in water,[22] while Kimura *et al.* demonstrated that zinc(II) complexes of the macrocyclic tetraamine 1,4,7,10-tetraazacyclododecane (cyclen) have a unique propensity to bind with deprotonated imides like thymine and, uracil, by forming non-covalent stable complexes in biologically relevant conditions.[23] Moreover, cyclen units appended with aromatic rings such as acridine and ditopic receptors yielded binding constants for TMP and UMP up to $K = 10^7\ M^{-1}$.[24] In order to explore the possibility to use metal complexes for simultaneous detection of nucleotides and to better understand how the interaction with the nucleic acids occurs, an analysis of the interaction of the receptors **L**[4] and **L**[7] with nucleotide monophosphates (AMP, CMP, GMP, TMP and UMP) was carried out. The electrostatic interactions between the positively charged receptors and the negatively charged mononucleotides are expected to lead to the formation of complexes.

Table 2 Logarithms of the stability constants for the interaction of monophosphate nucleotides ($MP^{2-} \equiv A$) with tripodal polyamine $\mathbf{L}^4$ determined at 298.0 ±0.1 K in 0.15 mol•dm^{-3} NaCl.

Reaction	AMP	CMP	Reaction	GMP	TMP	UMP
$A + HL \leftrightarrows HAL$	3.56 (3)	2.96 (1)	$H_{-1}A + HL \leftrightarrows AL$	-	3.88 (1)	3.59 (1)
$A + H_2L \leftrightarrows H_2AL$	3.86 (3)	3.06 (1)	$H_{-1}A + H_2L \leftrightarrows HAL$	4.66 (2)	4.11 (1)	3.77 (1)
$A + H_3L \leftrightarrows H_3AL$	4.10 (4)	3.13 (1)	$H_{-1}A + H_3L \leftrightarrows H_2AL$	-	-	-
$A + H_4L \leftrightarrows H_4AL$	4.35 (3)	3.25 (1)	$H_{-1}A + H_4L \leftrightarrows H_3AL$	-	-	-
$A + H_5L \leftrightarrows H_5AL$	4.33 (4)	3.14 (1)	$H_{-1}A + H_5L \leftrightarrows H_4AL$	-	-	-
$A + H_6L \leftrightarrows H_6AL$	4.71 (3)	3.47 (1)	$H_{-1}A + H_6L \leftrightarrows H_5AL$	-	-	-
$HA + HL \leftrightarrows H_2AL$	-		$A + HL \leftrightarrows HAL$	4.47 (2)	3.78 (1)	3.35 (1)
$HA + H_2L \leftrightarrows H_3AL$	-	-	$A + H_2L \leftrightarrows H_2AL$	4.08 (3)	3.45 (1)	3.12 (2)
$HA + H_3L \leftrightarrows H_4AL$	-	-	$A + H_3L \leftrightarrows H_3AL$	4.50 (2)	3.36 (1)	3.01 (2)
$HA + H_4L \leftrightarrows H_5AL$	-	-	$A + H_4L \leftrightarrows H_4AL$	4.68 (2)	3.51 (1)	3.30 (2)
$HA + H_5L \leftrightarrows H_6AL$	4.99 (3)	3.83 (1)	$A + H_5L \leftrightarrows H_5AL$	4.84 (2)	3.40 (1)	3.20 (1)
$HA + H_6L \leftrightarrows H_7AL$	3.97 (4)	-	$A + H_6L \leftrightarrows H_6AL$	5.22 (2)	-	3.60 (1)
	-	-	$A + H_7L \leftrightarrows H_7AL$	-	-	-
	-	-	$HA + H_5L \leftrightarrows H_6AL$	-	3.77 (1)	3.80 (1)
	-	-	$HA + H_6L \leftrightarrows H_7AL$	4.89 (2)	-	-

[a] Charges omitted.[b] Numbers in parentheses are standard deviations in the last significant figure.

Table 3 Logarithms of the stability constants for the interaction of monophosphate nucleotides ($MP^{2-} \equiv A$) with tripodal polyamine $\mathbf{L}^7$ determined at 298.0 ±0.1 K in 0.15 mol·dm^{-3} NaCl.

Reaction	AMP	CMP	Reaction	GMP	TMP	UMP
$A + HL \leftrightarrows HAL$	3.22 (1)	2.44 (1)	$H_{-1}A + HL \leftrightarrows AL$	3.66 (3)	3.30 (1)	4.40 (1)
$A + H_2L \leftrightarrows H_2AL$	3.23 (1)	2.21 (1)	$H_{-1}A + H_2L \leftrightarrows HAL$	3.44 (4)	3.72 (1)	5.03 (1)
$A + H_3L \leftrightarrows H_3AL$	3.57 (1)	2.41 (2)	$H_{-1}A + H_3L \leftrightarrows H_2AL$	-	-	-
$A + H_4L \leftrightarrows H_4AL$	3.80 (1)	2.80 (1)	$H_{-1}A + H_4L \leftrightarrows H_3AL$	-	-	-
$A + H_5L \leftrightarrows H_5AL$	3.90 (1)	2.86 (1)	$H_{-1}A + H_5L \leftrightarrows H_4AL$	-	-	-
$A + H_6L \leftrightarrows H_6AL$	4.40 (1)	3.52 (1)	$H_{-1}A + H_6L \leftrightarrows H_5AL$	-	-	-
$HA + L \leftrightarrows HAL$	-	-	$A + L \leftrightarrows AL$	3.80 (3)	2.98 (1)	4.81 (1)
$HA + HL \leftrightarrows H_2AL$	-	-	$A + HL \leftrightarrows HAL$	3.31 (4)	3.14 (1)	4.67 (1)
$HA + H_2L \leftrightarrows H_3AL$	-	-	$A + H_2L \leftrightarrows H_2AL$	3.27 (4)	2.92 (1)	4.32 (1)
$HA + H_3L \leftrightarrows H_4AL$	-	-	$A + H_3L \leftrightarrows H_3AL$	3.16 (3)	3.25 (1)	4.34 (1)
$HA + H_4L \leftrightarrows H_5AL$	-	-	$A + H_4L \leftrightarrows H_4AL$	3.40 (3)	3.43 (1)	4.41 (1)
$HA + H_5L \leftrightarrows H_6AL$	4.61 (1)	-	$A + H_5L \leftrightarrows H_5AL$	3.46 (2)	3.56 (1)	4.40 (1)
$HA + H_6L \leftrightarrows H_7AL$	3.72 (1)	1.87 (3)	$A + H_6L \leftrightarrows H_6AL$	3.90 (2)	3.93 (1)	4.67 (1)
$HA + H_7L \leftrightarrows H_8AL$	3.55 (1)	-	$A + H_7L \leftrightarrows H_7AL$	-	-	
$H_2A + H_5L \leftrightarrows H_7AL$	3.55 (1)	-	$HA + H_5L \leftrightarrows H_6AL$	-	3.93 (3)	4.81 (1)
			$HA + H_6L \leftrightarrows H_7AL$	2.94 (3)	2.89 (2)	3.69 (1)
			$HA + H_7L \leftrightarrows H_8AL$			3.41 (1)

[a] Charges omitted. [b] Numbers in parentheses are standard deviations in the last significant figure.

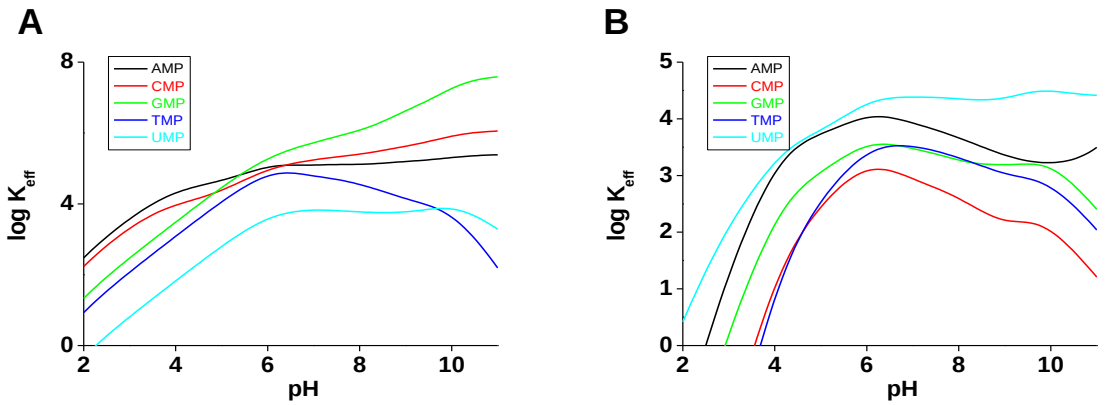


Figure 2 Plot of the effective conditional constants *vs.* pH for the interaction of tripodal polyamines A) $\mathbf{L^4}$ and B) $\mathbf{L^7}$ with monophosphate nucleotides AMP, CMP, GMP, TMP and UMP.

Tables 2 and 3 collect the corresponding data for the interaction of monophosphate nucleotides with the tripodal receptors $\mathbf{L^4}$ and $\mathbf{L^7}$ respectively. Previously it was necessary to determine the protonation constants of the different nucleotides under the experimental conditions used in this work. The results are collected in Table S1, (ESI†). GMT, TMP and UMP show a deprotonation process of the imide nitrogen in the heterocyclic base.[23] AMP and CMP bear a protonation of the nitrogen N1 in the aromatic ring.

By examining the different values of binding constants, it is interesting to notice that all tripodal receptors are able to form mononuclear complexes of significant stability with the studied nucleotides. Figure S2 (ESI†) includes distribution diagrams for the studied systems and shows that the adduct species clearly predominate in a wide pH range. Ligand $\mathbf{L^4}$ forms species with stoichiometries H_xLA where x varies from 1 to 7 and receptor $\mathbf{L^7}$ gives species with a higher protonation degree where x varies from 0 to 8. Formation of these protonated species can be explained by means of the basicity of the ligands. The ligand with imidazole groups is more basic, so the protonation of the aromatic nitrogen is produced at a higher pH. Ligand $\mathbf{L^4}$ has their nitrogen atoms in pyridine moieties protonated at more acidic pH. To analyze the A:L adduct-formation constants for the different systems shown in Table 2, care must be exerted in comparing the right equilibria and values of stability constants. Since both the substrate and the receptors participate in overlapping proton-transfer processes, translating the cumulative stability constants into representative stepwise constants is not always straightforward. To do so, one has to consider the basicities of the nucleotides and of the different ligands and assume that the interaction will not affect much the pH range of existence of the protonated species of nucleotides and L. If this is taken into account, stepwise constants can be deduced. However, the most unambiguous way to compare the relative stabilities of the different systems and to establish selectivity ratios is to use effective constants. The effective constants K_{eff} are calculated at each pH value as the quotient between the overall amount of complexed species and the overall amounts of free receptor and substrate independently of their protonation degree.

$$K_{eff} = \frac{\sum[H_{i+j}AL]}{\sum[H_iA]\sum[H_jL]}$$

Figure 2 represents the plot of the logarithms of the effective conditional constant *vs.* pH for the interaction of tripodal polyamine $\mathbf{L^4}$ and $\mathbf{L^7}$ with nucleotide monophosphates AMP, CMP, GMP, TMP and UMP.

The present results demonstrate the ability of these tripodal polyamine receptors to strongly bind nucleotides, giving a variety of complex species. ^{1}H-NMR and ^{31}P-NMR experiments were done in order to confirm the existence of the complexes. All spectra show only small variations of the signals (Figures S3 and S4, ESI†).

Interaction with Nucleic Acids

Physico- chemical properties of aqueous solutions

In order to decide which was the most appropiate pH to carry out the experiments we took into account the previously discussed acid-base properties of the compounds. It was obvious that within the biologically relevant pH range (pH = 5-8), only at pH = 5 most of the studied compounds are present in one dominant protonation form, except $\mathbf{L^6}$, see Figures 1 and S1 (ESI†) for a plot of the distribution diagrams. The number of positive charges that each one of the ligands bears at the pH of study is as follows: $\mathbf{L^4}$ (6+), $\mathbf{L^5}$ (6+), $\mathbf{L^6}$ (6-7+) and $\mathbf{L^7}$ (6-7+).

Therefore, all further experiments were done at pH = 5.0, in citrate buffer, I = 0.05 mol dm^{-3}. All the stock solutions of the compounds were prepared in re-distilled water and kept in dark and cold place (+8 °C). While in in these conditions the solutions were stable for about 2-3 weeks (checked by UV/vis spectroscopy), at room temperature they were stable only for several days. Changes of the UV/Vis spectra of compounds upon the temperature increase up to 98 °C were negligible and reproducibility of UV/Vis spectra upon cooling back to 25 °C was excellent.

Study of the interactions of L^4-L^7 *with ds-DNA and ds-RNA in aqueous media*

The UV/vis titration experiments were hampered by instant precipitation upon addition of the ct-DNA to solutions of any of the studied compounds ($c \approx 10^{-5}$ mol dm^{-3}). As an alternative method for estimation of affinity, at least as a comparison of ability of studied molecules to compete for binding with classical intercalators already bound to ds-polynucleotides,[25] we have performed ethidium bromide (**EB**) displacement assays (Figure S5, ESI†).

The obtained IC_{50} = 1.2 - 0.15 suggest that affinities of $\mathbf{L^4}$-$\mathbf{L^7}$ toward ct-DNA and poly A-poly U are comparable to the affinity of **EB**. Since the structures of $\mathbf{L^4}$-$\mathbf{L^7}$ do not support intercalation into ds-DNA/RNA as a binding mode but more likely an electrostatic interactions, the obtained IC_{50} values cannot be used for accurate calculation of binding constants but only as a measure of high affinity (log*K*s > 5).

Table 4 The [a]ΔT_m values (ºC) of ct-DNA upon addition of different ratios [b]***r*** of $\mathbf{L^4}$-$\mathbf{L^7}$ at pH = 5.0 (citrate buffer I = 0.05 mol·dm^{-3})

	ct-DNA			
[b]***r*** =	0.1	0.2	0.3	0.5
$\mathbf{L^4}$	5.95	7.55	10.15	10.7
$\mathbf{L^5}$	5.30	10.35	12.30	11.85
$\mathbf{L^6}$	4.55	6.10	12.30	12.65
$\mathbf{L^7}$	3.0	7.4	11.0	20.0

[a] Error in ΔT_m: ± 0.5°C; [b] ***r*** = [compound]/[ct-DNA].

It is well known that upon heating, ds-helices of polynucleotides at well-defined temperature (T_m value) dissociate into two single stranded polynucleotides. Non-covalent binding of small molecules to ds-polynucleotides usually has certain effect on the thermal stability of helices thus giving different T_m values. Difference between T_m value of free polynucleotide and complex with a small molecule (ΔT_m value) is an important factor in the characterisation of small molecule/ds-polynucleotide interactions Addition of any of the studied compounds strongly stabilised the double helix of ct-DNA (Table 4). The pronounced nonlinear dependence of ΔT_m values on the ratio $\boldsymbol{r}_{[\text{compound}]/[\text{ct-DNA}]}$ obtained for $\mathbf{L^4}$-$\mathbf{L^6}$ suggested saturation of binding sites at about ***r*** = 0.3. Intriguingly, no saturation of binding sites was observed for $\mathbf{L^7}$ even up to $\boldsymbol{r}_{[\text{compound}]/[\text{ct-DNA}]}$ =0.5.

Impact of the ionic strength of aqueous solution on the binding of small molecules to DNA/RNA depends heavily on a type of non-covalent interactions. Namely, under experimental conditions similar to those applied in this work, increase of ionic strength for one order of magnitude resulted in diminished (but still measurable) stabilization effect of classical intercalator ethidium bromide on ct-DNA.[26] At variance to that, comparable increase of ionic strength (addition of 0.1 mol·dm^{-3} NaCl to conditions presented in Table 1) completely abolished the stabilisation effect of $\mathbf{L^4}$ on ct-DNA, pointing toward dominant role of electrostatic interactions in binding of $\mathbf{L^4}$-$\mathbf{L^7}$ to polynucleotides.

Thermal denaturation of poly A-poly U at pH = 5.0 yielded biphasic transition. The first transition at about T_m = 30 ± 1 ºC is attributed to denaturation of poly A-poly U and the second transition at about T_m = 79 ± 1 ºC is attributed to denaturation of poly AH$^+$- poly AH$^+$, since poly A at pH = 5.0 is mostly protonated and forms ds-polynucleotide (poly AH$^+$- poly AH$^+$).[27,28] For comparison, thermal denaturation of only poly AH$^+$- poly AH$^+$ as well as of the DNA analogue (poly dA – poly dT) were performed.

Preliminary experiments with poly A - poly U revealed much stronger stabilisation effects caused by addition of all studied compounds than observed in ct-DNA experiments. In addition, at ratio $\boldsymbol{r}_{[\text{compound}]/[\text{polynucleotide}]} > 0.2$ for most of compounds precipitation was observed, thus hampering the measurements. Therefore, more detailed experiments with poly A – poly U, poly dA – poly dT and poly AH$^+$- poly AH$^+$ were done at ratios ***r*** < 0.1 (Table 5).

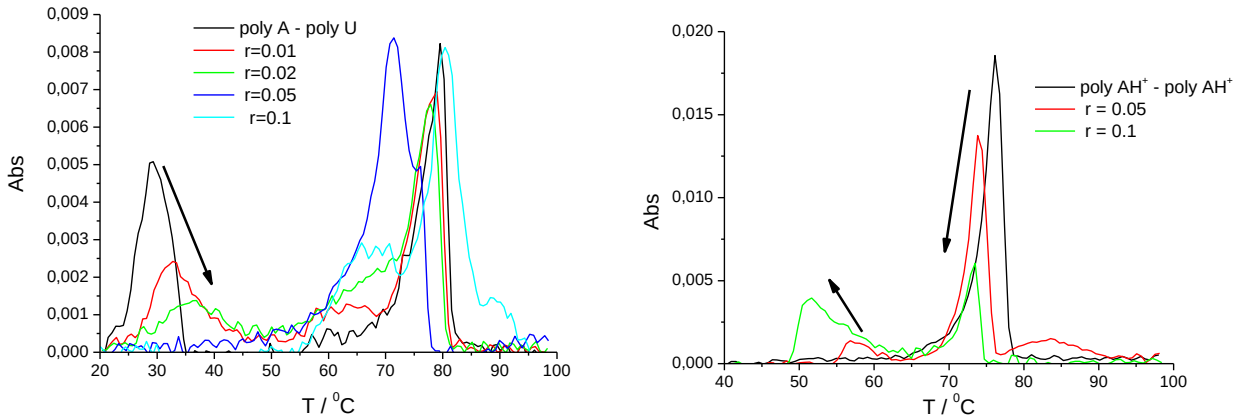
Even at ratio $r_{[\text{compound}]/[\text{polynucleotide}]}$ =0.01, addition of all studied compounds caused measurable stabilisation of poly A – poly U by ΔT_m values roughly comparable to those obtained for ds-DNA's at 10 times higher ratios (Tables 4 and 5).

Further increases of the $\mathbf{L^4}$-$\mathbf{L^7}$ concentration ($r_{[\text{compound}]/[\text{polynucleotide}]}$ = 0.05-0.1) stabilised even more poly A-poly U, shifting the melting transitions in the range between 70-90 ºC. Consequently the denaturation curve of poly A-poly U overlapped with the thermal transition of poly AH$^+$-poly AH$^+$.[27,28] Comparison of thermal denaturation curves for the same ratio ***r*** obtained for poly A-poly U and AH$^+$-poly AH$^+$ (Table 5, Figures 4 and 5), respectively, allowed in the most cases for an accurate assignation of thermal transitions to corresponding polynucleotides.

Table 5 The [a]ΔT_m values (°C) of poly A – poly U, poly AH^+-poly AH^+ and poly dA-poly dT upon addition of different ratios [b]***r*** of $\mathbf{L^4}$-$\mathbf{L^7}$ at pH = 5.0 (citrate buffer I = 0.05 mol·dm^{-3}).

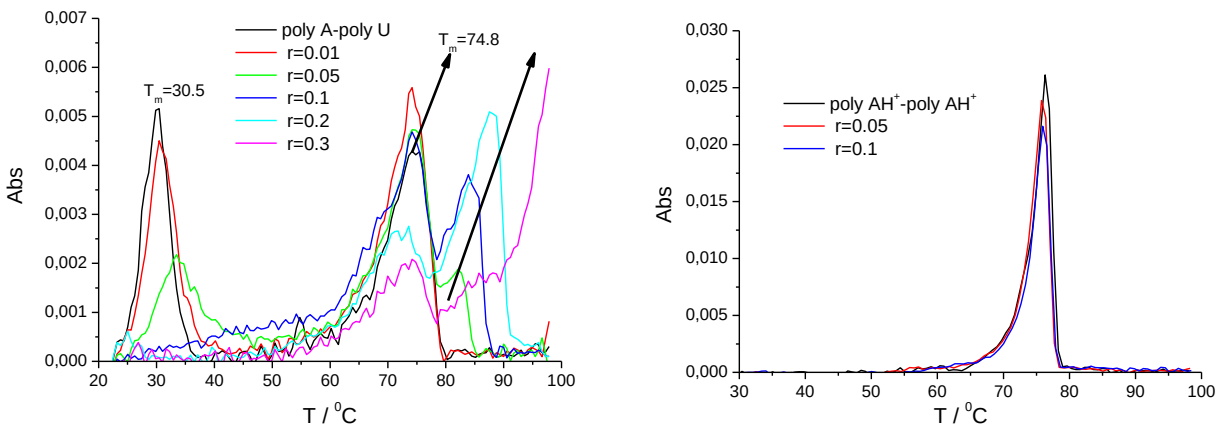
	[b]**r** =	$\mathbf{L^4}$	$\mathbf{L^5}$	$\mathbf{L^6}$	$\mathbf{L^7}$
poly A-polyU	0.01	[c] +2.9 / -0.5	[c] +3.4 / -0.6	[c] +2.2 / -1.0	[c] +0.5 / -0.6
	0.05	[c] +42.1 / -4.2	[c] +46.5 / 0	[c] +51. 4 / -2.8	[c] +3.5 and +51.6 / 0
	0.1	+51.1 / -11.4	[c] +46.1/ 0	d	[c] +53.4 / 0
	0.2	d	d	d	[c] +57.0 / 0
poly AH^+-poly AH^+	0.05	-2.4 / -18.8	-1.0	-1.1	0
	0.1	-2.7 / -24.5	-2.7 / -21.5	-1.8 / -22.1	0
poly dA-poly dT	0.1	+2.2 / +26.0	-	-	+9.1 / +26.5

[a] Error in ΔT_m: ± 0.5 ºC; [b] ***r*** = [compound]/[polynucleotide]; [c] Biphasic transitions: the first transition at Tm = 30 ºC is attributed to denaturation of poly A-poly U and the second transition at Tm = 79 ºC is attributed to denaturation of poly AH^+-poly AH^+ since poly A at pH = 5 is mostly protonated and forms ds-polynucleotide; [d] precipitation.



A **B**

Figure 4 Comparison of thermal denaturation experiments (1st derivatives of denaturation curves, maxima presenting T_m values) of $\mathbf{L^4}$ with poly A-poly U (A) and poly AH^+-poly AH^+ (B) at various ratios r = [$\mathbf{L^4}$]/[polynucleotide].



A **B**

Figure 5 Comparison of thermal denaturation experiments (1st derivatives of denaturation curves, maxima presenting T_m values) of $\mathbf{L^7}$ with poly A-poly U (A) and poly AH^+-poly AH^+ (B) at various ratios r = [$\mathbf{L^7}$]/[polynucleotide]..

For example, all compounds either destabilised or had no effect on poly AH^+-poly AH^+ denaturation, thus transitions higher than T_m > 80 ºC could not be attributed to that polynucleotide but are assigned to denaturation of the compound/poly A-poly U complex. However, ΔT_m values > 30 °C are not common for poly A-poly U, thus a possible formation of very stable triple helical polynucleotide (like the ones observed for DNA analogues)[29] cannot be neglected.

In order to get insight into the changes of polynucleotide secondary structure induced by small molecule binding, we have chosen Circular Dichroism (CD) spectroscopy.[30] In addition, achiral small molecules can eventually acquire induced CD spectrum (ICD) upon binding to polynucleotides, which could give useful information about modes of interaction.[30] It should be noted that the studied compounds are achiral and therefore do not possess intrinsic CD spectrum. Addition of any of the studied compounds didn't induce any significant change in the CD spectra of DNA and RNA (Figures S6 and S7 ESI†). Since previous experiments (thermal denaturation, **EB** displacement) revealed significant affinity of the studied compounds toward DNA/RNA, the only explanation of such minor CD effects could be that the structural flexibility of the studied compounds allows their easy adjustment to the secondary structure of the polynucleotide, thus not disturbing significantly the helicity of DNA/RNA. In addition, for $\mathbf{L^4}$-$\mathbf{L^7}$/ DNA complexes no ICD signal between 220-280 nm was observed, thus excluding formation of only one dominant binding orientation with respect to the DNA or RNA chiral axis.[30] Small changes of the poly A-poly U CD spectrum upon binding of studied compounds excluded formation of any triple helical structure (mentioned in thermal denaturation experiments)[29], at least at room temperature.

Conclusions

We have described the protonation and nucleotide coordination properties of new tripodal receptors containing pyridine and imidazole units. The studied compounds bind nucleotide monophosphates in aqueous medium with high affinity, most likely due to the strong electrostatic interactions between positively charged amines and negatively charged phosphates. It is also interesting to point out the formation of stable mononuclear complexes with high stability constant values. Exceptionally strong thermal denaturation effects and efficient displacement of ethidium bromide from DNA/RNA point toward strong interactions of $\mathbf{L^4}$-$\mathbf{L^7}$ with double stranded DNA/RNA. In all experiments $\mathbf{L^4}$-$\mathbf{L^6}$ yielded comparable results, while $\mathbf{L^7}$ presents somewhat higher ΔT_m values, most likely due to the higher protonation state that $\mathbf{L^7}$ exhibits at pH 5. Because of the flexible structure, the compounds efficiently adjusted to the polynucleotides (weak CD effects) and absence of any ICD signal suggested that there is no specific binding site within polynucleotide structure.[30] The aforementioned results suggest that compounds "wrap" around the polynucleotides, forming strong interactions with negatively charged DNA/RNA backbone. However, evidently stronger stabilisation of ds-RNA in comparison with analogue DNA-polynucleotide points toward some type of interaction selective toward RNA. Since ds-DNA and ds-RNA significantly differ in the secondary structure (β-helix of DNA vs α-helix of RNA),[28] one could speculate that the negatively charged backbone of RNA-double helix gives a better structural match with the positive charges of the compounds than the DNA-double helix. Consequently, the studied compounds exhibit rather rare but therefore even more intriguing ds-RNA over ds-DNA selectivity, which makes interesting further studies in respect to RNA targeting small molecules.[31] Moreover, due to their high affinity toward DNA and the multiple positive charges, the studied compounds could be considered as analogues of spermidine and similar polyamines with significantly increased DNA polyanion neutralisation and therefore could offer a promising potential to act as artificial histone modulators.

In addition, positive charge of here studied aliphatic amines can be tuned (reversibly) by simple external stimuli like e.g. pH, thus in future studies this can be related to the property of tumor cells in solid tumors, which consistently have lower extracellular pH levels than normal tissues because of the inefficient clearance of metabolic acids from chronically hypoxic cells.[32] Tumors of the bladder, kidney and gastrointestinal system in particular are exposed to extremes of pH. However this difference (0.6–0.8 pH unit) is small in chemical terms and has proved difficult to exploit. Nevertheless, uptake of weakly ionizing drugs by tumours is greatly influenced by the interstitial and intracellular pH, as well as the ionization properties of the drug. Therefore strategies for enhancing and exploiting pH gradients to drive the uptake of weak acid drugs into tumors are under investigation.[33]

Experimental

Materials and methods

All chemicals and solvents were obtained from commercial sources and used without further purification. Nucleotide monophosphates were purchased as follows: Adenosine 5′-monophosphate disodium salt ≥ 99% (AMP) from Fluka, Cytidine 5′-monophosphate disodium salt ≥ 99% (CMP) from Sigma, Guanosine 5′-monophosphate disodium salt hydrate ≥ 99% (GMP) from Sigma, Thymidine 5′-monophosphate disodium salt hydrate ≥ 99% (TMP) from Sigma and Uridine 5′-monophosphate disodium salt ≥ 98% (UMP) from Sigma.

Tripodal ligands $\mathbf{L^5}$ - $\mathbf{L^7}$ have been prepared following the general synthetic strategy previously described.[15] Amine $\mathbf{L^1}$ reacted with the corresponding pyridine or imidazole

carbaldehydes to give the corresponding pyridine or imidazole functionalized tripodal polyamines. In all cases, a molar ratio carbaldehyde:$\mathbf{L^1}$ 3:1, was used. The overall yield is large enough to obtain all compounds in a gram scale. Elemental microanalysis gave satisfactory values for all ligands. Synthesis of $\mathbf{L^5}$ - $\mathbf{L^7}$ will be reported elsewhere.[16]

Electromotive Force Measurements. Potentiometric Measurements.

The potentiometric titrations were carried out in water at 298.1±0.1 K using NaCl (for the ligands $\mathbf{L^4}$ and $\mathbf{L^7}$) or $NaClO_4$ for the ligands $\mathbf{L^5}$ and $\mathbf{L^6}$) 0.15 mol·dm^{-3} as supporting electrolyte. The experimental procedure (burette, potentiometer, cell, stirrer, microcomputer, etc.) has been fully described elsewhere.[34] The acquisition of the emf data was performed with the computer program PASAT.[35] The reference electrode was an Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as an hydrogen-ion concentration probe by titration of previously standardized amounts of HCl with CO_2-free NaOH solutions and determining the equivalent point by the Gran´s method,[36] which gives the standard potential, E$^{o\prime}$, and the ionic product obtained were 13.73(1) in pure water.[37] Concentration of the ligand solutions were about 1×10^{-3} mol·dm^{-3}.

The computer program HYPERQUAD was used to calculate the protonation and stability constants.[38] The pH range investigated (pH = -log[H$^+$]) was 2.0-11.0. The different titration curves for each ligand were treated as separated curves without significant variations in the values of the stability constants. Finally, the sets of data were merged together and treated simultaneously to give the final stability constants.

NMR measurements.

The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance DPX 300 MHz spectrometer operating at 299.95 MHz for ^{1}H and at 75.43 for ^{13}C. For the ^{13}C NMR spectra, dioxane was used as a reference standard (δ = 67.4 ppm) and for the ^{1}H spectra, the solvent signal. The ^{31}P NMR spectra were recorded on a Bruker Avance DPX 300 MHz operating at 121.495 MHz. Chemical shifts are relative to an external reference of 85% H_3PO_4. Adjustments to the desired pH were made using drops of DCl or NaOD solutions. The pD was calculated from the measured pH values using the correlation, pH = pD - 0.4.[39]

Spectroscopic measurements

The electronic absorption spectra were obtained on Varian Cary 100 Bio spectrometer, CD spectra on JASCO J815 spectrophotometer and fluorescence spectra on the Varian Eclipse fluorimeter, all in quartz cuvettes (1 cm). Spectroscopic studies were performed in aqueous buffer solution (pH = 5, citrate buffer, I = 0.05 mol·dm^{-3}). Under the experimental conditions absorbance of $\mathbf{L^4}$, $\mathbf{L^5}$, $\mathbf{L^6}$ and $\mathbf{L^7}$ was proportional to their concentrations. Polynucleotides were purchased as noted: poly A-poly U, poly dA-poly dT, (Sigma) and calf thymus (*ct*)-DNA (Aldrich). Polynucleotides were dissolved in sodium cacodylate buffer, I = 0.05 mol·dm^{-3}, pH = 7. Calf thymus (*ct*)-DNA was additionally sonicated and filtered through a 0.45 μm filter.[40,41] Polynucleotide concentration was determined spectroscopically[41] as the concentration of phosphates.

Thermal melting curves for DNA, RNA and their complexes with studied compounds were determined as previously described[41] 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. The *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.[41] Δ*T*m values were calculated subtracting *Tm* of the free nucleic acid from *Tm* of the complex. Every Δ*Tm* value here reported was the average of at least two measurements, the error in Δ*Tm* is ± 0.5 °C.

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Notes and references

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