Synthesis and photophysical evaluation of a pyridinium 4-amino-1,8-naphthalimide derivative that upon intercalation displays preference for AT-rich double-stranded DNA†

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The synthesis, characterisation and solid state crystal structure of a cationic 4-amino-1,8-naphthalimide derivative (1) are described. The photophysical properties of I are shown to vary with the solvent polarity and H-bonding ability. The fluorescence of I is enhanced and blue-shifted in its 1 : 1 complex with 5′-adenosine-monophosphate while it is partially quenched and red-shifted in its complex with 5′-guanosine-monophosphate. Linear and circular dichroism measurements show that I binds to double-stranded DNA by intercalation. Comparative UV-visible and fluorescence studies with double stranded synthetic polynucleotides poly(dA–dT)₂ and poly(dG–dC)₂ show that I binds much more strongly to the AT polymer; I also has a strong preference for A–T rich sequences in natural DNA. Thermal denaturation measurements also reveal a much greater stabilisation of the double-stranded poly (dA–dT)₂ than of natural DNA.

Introduction

The development of small molecules capable of binding to DNA and exhibiting anticancer activities has received much attention in recent times. 1–3 1,8-Naphthalimide derivatives represent an important family of DNA binders that have been shown to exhibit anti-tumour activities in vitro and in vivo. 4,5 Moreover, the naphthalimides have rich photophysical properties; which are dependent on the nature, as well as the location, of any aryl substituents. Photo-excitation of various naphthalimide derivatives has been also shown to induce sequence selective DNA strand cleavage. 6–8 For example, Saito et al. showed that unsubstituted as well as 4-nitro substituted L-lysine derived 1,8-naphthalimides caused sequence selective cleavage at the 5′-guanine (G) residue of 5′-GG sequence upon UV-irradiation and treatment with hot piperidine. The cleavage ability of these naphthalimide systems has been explained in terms of photoinduced electron transfer (PET), occurring from the most oxidizable DNA base G, to the triplet excited state of naphthalimide. 6 In a related study, Kelly and co-workers have reported the interaction of cationic unsubstituted naphthalimides functionalised with N-ethyl pyridinium group, with DNA and mononucleotides 8 and showed that laser flash photolysis could be employed to observe the formation of naphthalimide radical anion (NF⁻) in the presence of DNA.

While these unsubstituted naphthalimides absorb only at shorter wavelength, the 3- and 4-amino-1,8-naphthalimide derivatives by contrast absorb within the visible region (λmax ~ 450 nm) and fluoresce at long (λmax ~ 550 nm) wavelengths, with reasonably high quantum yield of emission in various organic and aqueous solvents. 9 Because of this, they have been extensively explored for the development of sensors for biologically relevant ions and molecules, etc. 9–15 Their excited states are characterized by a “push–pull” internal charge transfer (ICT) character, arising from the presence of the electron-withdrawing imide and the electron donating amino moiety; this being strongest where the amino group is in the 4-position of the ring. 9,15–19 Because of this they have also been employed as potential cellular imaging and antitumor agents. 20 In this context, we have also reported development of DNA photocleavage agents based on 4-amino-1,8-naphthalimide conjugated with ruthenium(ii) complex, 19 and we have prepared Tröger’s base derivatives of the 4-amino-1,8-naphthalimide structure and shown these to be efficient DNA binders, and cytotoxic agents. 20 In this article we report the synthesis of 1 (a novel cationic pyridinium based 4-amino-1,8-naphthalimide derivative), and examine its interaction with natural and synthetic double-stranded DNAs using UV-visible absorption, fluorescence, linear and circular dichroism spectroscopic methods. These demonstrate that unlike its unsubstituted analogue 2, the 4-amino-substituted compound 1 intercalates into double-stranded DNA and shows an apparent preference for AT sites.

†Electronic supplementary information (ESI) available: Fig. S1–S3. CCDC 843421. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2ob06898b

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**Results and discussion**

**Design, synthesis and solid state characterisation of 1**

In designing the molecule we foresaw that in 1 the pyridinium moiety would impart water solubility as well as favouring, electrostatic interaction with the negative phosphate backbone of the DNA. The substitution should also prevent protonation in the pH range used for studies with the biomolecule, simplifying the spectroscopic studies.

Compound 1 was synthesised in three steps as shown in Scheme 1. The first step involved formation of 3, which was achieved through a condensation reaction between the 4-nitro-1,8-naphthalic anhydride and ethanolamine in dry ethanol under an inert atmosphere and reflux. Product 3 was isolated as a brown solid in 75% yield and fully characterised (see ESI Fig. S2†). Compound 4 was then formed, as an orange solid in 90% yield by catalytic hydrogenation of 3 in DMF at 3 atm H₂. Refluxing 4 in the presence of p-toluene sulfonyl chloride, gave the desired product 1 as a tosylate salt. This was put into water, washed with CH₂Cl₂ and purified using silica flash column chromatography (88 : 11 : 1 CH₃CN : H₂O : NaCl). The product was then precipitated as its PF₆⁻ salt from acetone.21 Product 3 was isolated as a brown solid in 75% yield and fully characterised (see ESI, Fig. S2†). Compound 4 was then formed, as an orange solid in 90% yield by catalytic hydrogenation of 3 using 10% Pd/C in DMF at 3 atm H₂. Refluxing 4 in the presence of p-toluene sulfonyl chloride in pyridine, gave the desired product 1 as a tosylate salt. This was put into water, washed with CH₂Cl₂ and purified using silica flash column chromatography (88 : 11 : 1 CH₃CN : H₂O : NaCl). The product was then precipitated as its PF₆⁻ salt using NH₄PF₆ and converted to the chloride salt of 1 using 10% Pd/C in DMF at 3 atm H₂.

All compounds were characterized by conventional 1H, 13C NMR (see ESI Fig. S2†). Compound 4 was then formed, as an orange solid in 90% yield by catalytic hydrogenation of 3 using 10% Pd/C in DMF at 3 atm H₂. Refluxing 4 in the presence of p-toluene sulfonyl chloride in pyridine, gave the desired product 1 as a tosylate salt. This was put into water, washed with CH₂Cl₂ and purified using silica flash column chromatography (88 : 11 : 1 CH₃CN : H₂O : NaCl). The product was then precipitated as its PF₆⁻ salt using NH₄PF₆ and converted to the chloride salt of 1 using 10% Pd/C in DMF at 3 atm H₂.

**UV-visible, fluorescence and NMR studies of 1**

The absorption and emission spectra of 1 (8.3 μM) in 10 mM phosphate buffer solution (pH 7.0) are shown in Fig. 2a. 1 exhibits a broad absorption band 435 nm (ε = 13 200 M⁻¹ cm⁻¹), which is, as discussed above, due to an ICT transition.9,23,24 Higher energy π–π transitions were also observed at wavelength ca. 250 nm (ε = 21 800 M⁻¹ cm⁻¹) in the same solvent (pH 7.0). 1 exhibits a broad ICT fluorescence emission band centred at 552 nm, when excited at 435 nm. As anticipated the photo-physical properties of 1 were found to be pH independent in the range pH 3–11 (see ESI, Fig. S3†), which is useful for its application as a DNA targeting molecule.

![Scheme 1](image)

**Scheme 1** Synthesis of 1.

**Fig. 1** (A) The X-ray crystal structure of 1 with thermal ellipsoids shown at 50% probability. (B) The packing of 1 when viewed down the crystallographic a-axis, showing the π–π interactions between the stacked naphthalimide rings which adopt a ‘head-to-tail’ type orientation.
Compound 1 shows good water solubility, and obeys the Beer–Lambert law in aqueous solution at concentrations up to 100 μM as expected if the molecule exists in its monomeric form under these conditions. However, as we wish to study the compound in higher concentrations in further studies (e.g. cellular studies), it was important to determine whether the compound might aggregate under such conditions. To investigate this we carried out 1H NMR studies (600 MHz, D2O) within the concentration range of 1 μM → 10 mM. These showed (see ESI†) that the naphthalimide ring protons were shifted up-field upon increasing concentration above 100 μM (even though no new band was observed in the UV/vis absorption spectrum). The NMR results are consistent with the formation of small aggregates at these higher concentrations. This is not too unexpected given the ability of 1 to undergo π–π stacking as was observed in the solid state structure in Fig. 1. Importantly, these results also show that the compound is monomeric at the concentrations of 1 employed in the photophysical studies herein.

To investigate the effect of solvent polarity, the absorption and fluorescence emission spectra of 1 were recorded in alcohols of varying polarity and in water. Representative spectra are shown in Fig. 2, and the results are summarised in Table 1. In all the solvents, 1 exhibited a broad absorption band, which showed a slight blue shift (ca. 8–9 nm) on moving from the less polar butan-2-ol to highly polar water. The emission properties were found to be much more strongly dependent on the polarity of the medium, showing ca. 22 nm red shift on moving from butan-2-ol to water and a significant decrease in the quantum yield of emission and fluorescence lifetime. The radiative (k_r) and non radiative (k_nr) decay constants were estimated from the quantum yield and lifetime values, respectively. This showed that for the alcohols k_r is not significantly altered with increase in solvent polarity, while k_nr increased substantially between butan-2-ol and ethanol. By contrast in water, k_r was found to be significantly lower compared to the alcohols.

Similar behaviour has previously been observed for related 4-amino-1,8-naphthalimides, which suggests that the excited states of these molecules have ICT character.23 The decrease of quantum yield of emission and lifetime in more polar and H-bonding solvents can be explained in terms of specific solvent–solute interactions resulting in the increase in non radiative decay rates. Since 1 exhibits significant solvatochromism and as its emission quantum yield is strongly dependent on solvent polarity we may expect to observe substantial changes in the photophysical properties of 1 upon binding to nucleic acids.26

### Interaction of 1 with mononucleotides

**Ground state interaction of 1 with mononucleotides.** We first investigated its ability to bind mononucleotides. As can be seen from Fig. 3a and 3b the UV-vis spectrum of 1 is significantly altered in the presence of purine mononucleotides adenosine 5′-monophosphate (AMP) (0 → 120 mM) and guanosine 5′-monophosphate (GMP) (0 → 60 mM). Addition of both AMP and GMP caused a decrease in the absorbance of 1, with a concomitant red shift of the λ_max. However, the extent of hypochromicity was different with each mononucleotide (Table 2).

A single isosbestic point was observed with both AMP and GMP, suggesting the formation of 1:1 supramolecular ground state complex between 1 and either of the purine mononucleotides. Similar behaviour has been previously observed for unsubstituted naphthalimides and has been attributed to naphthalimide–nucleotide stacking interactions.25 In contrast, the pyrimidine mononucleotides, cytidine 5′-monophosphate (CMP) and thymidine 5′-monophosphate (TMP) did not cause any significant change in the UV-vis spectrum of 1.

### Table 1 UV-vis absorption and emission data for 1 in a range of solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>λ_max (Abs) (nm)</th>
<th>λ_max (Flu) (nm)</th>
<th>φ_F</th>
<th>τ (ns)</th>
<th>k_r×10^7 (s⁻¹)</th>
<th>k_nr×10^8 (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>436</td>
<td>552</td>
<td>0.08</td>
<td>2.25</td>
<td>3.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>440</td>
<td>536</td>
<td>0.16</td>
<td>2.60</td>
<td>6.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Propan-1-ol</td>
<td>444</td>
<td>534</td>
<td>0.23</td>
<td>3.21</td>
<td>7.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>444</td>
<td>532</td>
<td>0.29</td>
<td>3.90</td>
<td>7.43</td>
<td>1.8</td>
</tr>
<tr>
<td>Butan-2-ol</td>
<td>445</td>
<td>530</td>
<td>0.40</td>
<td>5.10</td>
<td>7.8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*a All spectra were recorded at optical density (OD) of ~0.1 upon excitation at λ_max for each of these solvents.
Assuming a 1:1 association between 1 and the nucleotide monophosphates (XMP), the association constants for their formation were first determined using linear Scatchard analysis (see ESI† for details).27 Good linearity was observed for the association of 1 with GMP. However, for the interaction of 1 with AMP, deviation from linearity was observed at higher nucleotide concentration, which possibly indicates that higher order supra-molecular complexes also exist under these conditions. The binding data were therefore also analysed using the nonlinear expression developed by Deranleau (see eqn (3), ESI†) considering the formation of 1:1 and 1:2 complexes between 1 and XMP as shown in eqn (1).28 The derived binding constants (Table 2) indicates that 1 complexes more strongly to AMP than to GMP. Furthermore, these are almost twice as high as that previously observed for the unsubstituted naphthalimide derivative 2,8b clearly demonstrating the importance of the ICT character, which makes 1 highly polar and as such more capable of π–π stacking with these mononucleotides.

**Singlet excited state interactions with mononucleotides.** Having established a strong association of 1 with AMP and GMP in the ground state, we next evaluated changes in the excited state of 1. A significant blue shift (7 nm) in the emission maximum is observed for 1 in the presence of AMP (120 mM) (Fig. 3c) and concurrently the quantum yield of emission of 1 ($\Phi_{\text{free}} = 0.08$) is approximately doubled ($\Phi_{\text{bound}} = 0.14$) when excited at the isosbestic point ($\lambda_{\text{ex}} = 463$ nm). This behaviour was further supported by time resolved fluorescence measurements, as the fluorescence lifetime increased from 2.2 ns for free 1 to 4.8 ns in the presence of AMP (70 mM), Table 3. The increase in relative quantum yield of emission and singlet state lifetime of 1 in its AMP complex can be attributed primarily to a decrease of the

![Image](https://i.imgur.com/3.png)

**Table 2** Summary of binding parameters obtained from absorption spectra of 1 in presence of AMP and GMP

<table>
<thead>
<tr>
<th>XMP</th>
<th>% Hypo</th>
<th>$K_1$ (M$^{-1}$)</th>
<th>$K_2$ (M$^{-1}$)</th>
<th>$K_1$ (M$^{-1}$)</th>
<th>$K_2$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>19</td>
<td>138 ± 10</td>
<td>37 ± 5</td>
<td>155 ± 7</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>GMP</td>
<td>12</td>
<td>117 ± 11</td>
<td>—</td>
<td>135 ± 5</td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 3 Changes in the absorption spectrum of 1 (7.2 μM) in the presence of increasing concentration of (a) AMP (0–120 mM) in 10 mM phosphate buffer (pH 7.0), (b) GMP (0–60 mM). Inset: plot of Δ$\lambda$/[NI] vs. concentration of AMP/GMP and fit (—) according to eqn (3) ($\Delta\lambda = \lambda_{\text{max}}$ of free 1). Changes in the steady state emission spectra of 1 (7.2 μM) in the presence of increasing concentration of (c) AMP (0–120 mM) ($\lambda_{\text{ex}} = 463$ nm) and (d) GMP (0–60 mM) ($\lambda_{\text{ex}} = 470$ nm) in 10 mM phosphate buffer (pH 7.0). Inset: plot of $I/I_0$ vs. concentration of XMP.
rate of non radiative decay processes as it is less accessible to solvent molecules and is in a relatively more rigid orientation. Similar changes in non radiative decay processes have been reported for the interaction of various porphyrin and ethidium bromide derivatives with nucleobases and DNA.

In contrast to what is found for AMP, addition of GMP (up to 60 mM) caused a red shift of 4 nm in $\lambda_{\text{max}}$ and about 60% quenching of the emission intensity of I when excited at 470 nm (Fig. 3). This may be attributed to PET from guanine to the photoexcited naphthalimide moiety in the preformed complex, as has been for other naphthalimides. Quenching of the emission intensity of I at lower concentration of GMP occurs in a 1 : 1 complex. The residual fluorescence at very high concentration of GMP, where I is completely bound can be explained by considering fluorescence emission from the NI : GMP complexes. At very high concentration of GMP, formation of higher order complexes cannot be ruled out.

The nature of the interaction of I with GMP was also supported by time correlated single photon counting measurements. In aerated 10 mM phosphate buffer the fluorescence of I decays by single exponential kinetics with a lifetime of 2.2 ± 0.2 ns, while in the presence of GMP, two components are observed. The first one has a lifetime of ca. 2 ns and the second one has a lifetime of 0.7 ± 0.1 ns. The relative contribution of this short-lived component increased with increasing concentration of GMP up to 66 mM. This short-lived component is presumed to arise from a 1 : 1 excited state complex between I and GMP. However, it is intriguing to note that at the highest concentration used, the long-lived component (1.9 ns) still represents 33% of the emitting species, even though calculations from the UV-vis absorption titrations suggest that the concentration of free I should be much lower. A possible explanation is that this emitting species is I : (GMP)$_2$. A longer lifetime for the I : (GMP)$_2$ would be similar to the behaviour recently reported for Pt(t)(meso-tetrakis(4-N-methyl-pyridyl))porphyrin, where the Por(GMP)$_2$ has a lifetime approximately five times longer than that for the 1 : 1 Por(GMP) excited state.

The fluorescence enhancement of I in the presence of AMP is in contrast to the behaviour of the corresponding unsubstituted naphthalimide 2, where the steady state emission of the naphthalimide derivative was quenched in the presence of both AMP and GMP. This was suggested to be due to PET from the nucleotide to the photoexcited naphthalimide chromophore. The failure to observe fluorescence quenching of I with AMP is presumably a consequence of the lower reduction potential of the excited state of the 4-amino derivative compared to the unsubstituted analogue, 2.

### Table 3. Fluorescence lifetime data as a function of mononucleotide concentration in 10 mM phosphate buffer (pH 7)

<table>
<thead>
<tr>
<th>[XMP] (mM)</th>
<th>$\phi_F$</th>
<th>$\lambda_{\text{max}}$ (Flu) (nm)</th>
<th>$\tau_1$ (ns)</th>
<th>$\tau_2$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>0.08</td>
<td>552</td>
<td>2.2 (100%)</td>
</tr>
<tr>
<td>I + GMP</td>
<td>66</td>
<td>0.02</td>
<td>556</td>
<td>1.9 (33%) 0.7 (67%)</td>
</tr>
<tr>
<td>I + AMP</td>
<td>71</td>
<td>0.14</td>
<td>545</td>
<td>2.6 (29%) 4.8 (71%)</td>
</tr>
<tr>
<td>AMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 4** The LD spectrum of free st-DNA (400 μM) and st-DNA in presence of I at varying P/D in 10 mM phosphate buffer (pH 7.0).

**Interaction of I with st-DNA**

Having explored the ability of I to interact with mononucleotides, we next explored the binding of I to double stranded salmon testes DNA (st-DNA) using various spectroscopic techniques, including LD, CD, UV-vis absorption and fluorescence spectroscopy, as well as thermal denaturation.

**Linear dichroism measurements.** LD spectroscopy, which is increasingly being found to be a powerful method for probing the binding of small molecules to nucleic acids, was used to investigate the mode of binding of I to st-DNA. The DNA was flow-oriented in a Couette cell at 2000 rpm. The spectrum of free st-DNA (400 μM) exhibits a negative LD signal around 260 nm characteristic of B-DNA, which arises from the orientation of transition dipole of the base pairs approximately perpendicular to the helical axis of DNA. The reduced linear dichroism (LD = LD/(isotropic absorbance)) was calculated to be $-0.027 \pm 0.003$ (a value similar to that obtained for B-DNA samples by other researchers). The LD was also measured for st-DNA in the presence of I at nucleotide/drug ratio P/D = 2.5, 5 and 10, Fig. 4. Most significantly, a new negative band appears at 440 nm corresponding to the ICT absorption band of I. The reduced LD for this feature for the P/D = 5 sample is 0.033 ± 0.003, which is similar to that of the DNA sample, indicating that the naphthalimide is at approximately the same angle to the direction of flow as the base-pairs. This is as would be expected for an intercalative mode of binding by the naphthalimide (This calculation assumes that the orientation factor S is the same for both DNA and the naphthalimide–DNA sample. It is possible that the interaction of I slightly stiffens the biomolecule and hence improves the orientation of the DNA molecules along the direction of flow, which would lead to a slightly higher LD$^t$). At P/D = 2.5 unbound ligand makes a significant contribution to the overall absorbance value, thereby making LD$^t$ for the 440 nm band quite low compared to the nucleobase.

**Circular dichroism measurements.** CD spectroscopy was also used to investigate the mode of interaction of I with st-DNA (150 μM) (Fig. 5) by monitoring the changes in the CD spectrum upon addition of increasing amounts of I (P/D = 0 →...
The ellipticity of the positive band at 275 nm band doubled, while that of the negative band at 240 nm decreased slightly. These changes are consistent with the alteration of the helical structure of B-DNA in the presence of I. Significantly, no induced CD signal was observed for the bound naphthalimide. An induced signal would be expected if it were groove-bound to the DNA as has previously been reported for its unsubstituted analogue 2. While these CD results do not explicitly demonstrate that I binds to DNA via intercalation, they clearly show that the interaction (particularly in light of the results obtained in the LD-measurements) alters the secondary structure of DNA significantly. These results might also support our objective that the pyridinium moiety is also essential to achieving high binding affinity for DNA via electrostatic interactions.

**Thermal denaturation studies.** The influence of I on the thermal stability of st-DNA was next investigated by monitoring the temperature induced change in absorbance at 260 nm arising from the conversion of double stranded DNA to the single stranded DNA.

![Fig. 5](image1)

**Fig. 5** The CD spectra of st-DNA (150 μM) in 10 mM phosphate buffer in presence of I at varying P/D ratios: 0 (—), 2.5 (——), 10 (—), 20 (——).

In the presence of I (P/D = 10), stabilisation of st-DNA was indicated by a 5° increase of the DNA melting temperature of st-DNA in 10 mM buffer solution (Fig. 6). It is perhaps significant that the extent of stabilisation is smaller than the effect observed for well-established intercalators such as ethidium bromide. However such moderate stabilization has been observed for other mono-naphthalimide based DNA intercalators previously and perhaps suggests a weaker intercalation interaction of I.

The thermal denaturation profile of the synthetic polynucleotide poly(dA–dT)2 recorded in the presence of I shows that I stabilises poly(dA–dT)2 (ΔT_m = 12 °C) to a much greater extent than it does st-DNA, which contains 58% AT basepairs. This is indicative of a higher affinity of I towards AT rich sequences. (Unfortunately due to the high melting temperature of poly(dG–dC)2 (~90 °C) in 10 mM phosphate buffer, it was not possible to study the thermal melting of this polynucleotide in the presence of I.)

**UV-visible measurements.** The binding of I with st-DNA was investigated from the changes in absorption of I in 10 mM phosphate buffer solution at pH 7.0 (Fig. 7a). In the presence of st-DNA, a large hypochromicity and ca. 10 nm red shift is observed in the ICT absorption band, accompanied by a single isosbestic point at ca. 480 nm for all DNA/I (P/D) concentrations suggesting the presence of only two spectroscopically distinct species.

The absorbance data were fitted to the non-cooperative binding model of McGhee–von Hippel to determine the intrinsic binding constant (K) and the binding site size (n) (inset Fig. 7a)

![Fig. 6](image2)

**Fig. 6** Melting profile of (a) st-DNA and (b) poly(dA–dT)2 in absence of I (■) and in presence of I (▲) (P/D = 10), (▲) (P/D = 5) in 10 mM phosphate buffer (pH 7.0).

![Fig. 7](image3)

**Fig. 7** (a) UV-vis spectra of I (7.2 μM) in the presence of increasing concentration of st-DNA (0–780 μM) in 10 mM phosphate buffer (pH 7.0); Inset: plot of r/C vs. r (■) and the best fit of the data (—) using the McGhee–von Hippel model. (b) Steady state emission of I (7.2 μM) in presence of increasing concentration of st-DNA (0–780 μM) (λ_ex = 480 nm) in 10 mM phosphate buffer (pH 7.0); Inset: plot of I/I_0 vs. DNA nucleotide phosphate/ligand (P/D).
Table 4  Summary of binding parameters obtained from the absorption spectra of 1 in presence of st-DNA in 10 mM phosphate buffer solution containing 0, 50, 100 mM NaCl

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>% Hypo</th>
<th>( \Delta \lambda_{\text{max}} ) (nm)</th>
<th>( K \times 10^5 \text{ M}^{-1} )</th>
<th>( n ) (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44</td>
<td>12</td>
<td>1.85 ± 0.05</td>
<td>2.65 ± 0.22</td>
</tr>
<tr>
<td>50</td>
<td>41</td>
<td>12</td>
<td>0.64 ± 0.08</td>
<td>3.83 ± 0.32</td>
</tr>
<tr>
<td>100</td>
<td>36</td>
<td>12</td>
<td>0.26 ± 0.01</td>
<td>4.49 ± 0.12</td>
</tr>
</tbody>
</table>

and Table 4).35 In the 10 mM phosphate buffer solution the intrinsic binding constant for the association of 1 with st-DNA was found to be ca. 1.85 \times 10^5 \text{ M}^{-1}, which is comparable to the affinity of phenothiazine dyes for DNA.36 Notably this is an order of magnitude higher than that seen for the unsubstituted analogue 2, demonstrating once more the importance of the 4-amino moiety and ICT character of 1.8 Similar DNA binding affinity has recently been reported by Qian and co-workers for thio-heterocyclic rings fused naphthalimides.5

To investigate the role of electrostatic interaction in the binding process, the binding of 1 with st-DNA was investigated in the presence of increasing NaCl concentration. An overall decrease in the magnitude of binding constant was observed with increasing ionic strength of the buffer, indicating significant role in the overall binding affinity of 1 for DNA.

Fluorescence studies. In parallel with the absorption spectra, the steady state emission spectra of 1 were measured upon increasing concentration of st-DNA (Fig. 7b). It was found that the fluorescence emission of 1 was enhanced in the presence of st-DNA by a factor of two with a concomitant blue shift in \( \lambda_{\text{max}} \) of ca. 5 nm. This enhancement in fluorescence of 1 probably results from a decreased rate of non-radiative decay of the excited state due to increased rigidity and the less polar nature of the binding site.

Time resolved emission measurements were also undertaken and these studies showed that in the presence of st-DNA, the emission decay of 1 is consistent with a bi-exponential process with a short component of \( \tau_1 = 3.73 \text{ ns (30\%)} \) and a longer lived component of \( \tau_2 = 9.23 \text{ ns (70\%)} \) at a P/D of 100, Table 5, which presumably represents two different localization of 1 within the DNA helix.

Table 5  Summary of binding parameters obtained from the absorption and fluorescence spectra of 1 in the presence of st-DNA, poly(dA–dT)2 and poly(dG–dC)2 in 10 mM phosphate buffer solution (pH 7)

<table>
<thead>
<tr>
<th></th>
<th>st-DNA</th>
<th>Poly(dA–dT)2</th>
<th>Poly(dG–dC)2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} ) (Abs) (nm)</td>
<td>447</td>
<td>447</td>
<td>445</td>
</tr>
<tr>
<td>( \Delta \lambda_{\text{max}} ) (nm)</td>
<td>+12</td>
<td>+12</td>
<td>+10</td>
</tr>
<tr>
<td>% Hypo</td>
<td>44</td>
<td>49</td>
<td>52</td>
</tr>
<tr>
<td>( K \times 10^5 \text{ M}^{-1} )</td>
<td>1.85 ± 0.05</td>
<td>5.15 ± 0.07</td>
<td>0.82 ± 0.02</td>
</tr>
<tr>
<td>( n ) (bp)</td>
<td>2.65 ± 0.22</td>
<td>2.54 ± 0.12</td>
<td>3.62 ± 0.09</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} ) (Flu) (nm)</td>
<td>547</td>
<td>547</td>
<td>544</td>
</tr>
<tr>
<td>( \Delta \lambda_{\text{max}} ) (nm)</td>
<td>−5</td>
<td>−5</td>
<td>−8</td>
</tr>
<tr>
<td>( \tau_1 ) (ns)</td>
<td>3.7 (30%)</td>
<td>3.1 (6%)</td>
<td>1.5 (51%)</td>
</tr>
<tr>
<td>( \tau_2 ) (ns)</td>
<td>9.2 (70%)</td>
<td>9.9 (94%)</td>
<td>3.7 (49%)</td>
</tr>
</tbody>
</table>

\*Fluorescence intensity after correcting for the absorbance decrease at 435 nm.

Interaction of 1 with synthetic polynucleotides

UV-visible absorption studies. It has been demonstrated above that 1 has a high affinity for DNA and that binding causes significant changes in its photophysical properties. As we showed earlier that complexing of 1 to AMP leads to increased excited state lifetime while GMP causes a partial quenching, it was of interest to study the behaviour of 1 in the presence of the synthetic polynucleotides poly(dA–dT)2 and poly(dG–dC)2.

As before the changes in the UV-vis absorption spectra of 1 with increasing concentration of poly(dA–dT)2 and poly(dG–dC)2 were monitored (Fig. 8a and 8b, respectively). Addition of either polynucleotide results in large hypochromicity and a significant red shift in the ICT absorption band of 1. In the case of poly(dA–dT)2, a single isosbestic point was observed at 480 nm for the entire polynucleotide concentration range (Fig. 8a). By contrast for poly(dG–dC)2 (Fig. 8b) an isosbestic point was observed (at 480 nm) only at low concentration of the polynucleotide (\([\text{poly(dG–dC)}_2]/[\text{ligand}] \approx 3\) indicating the possible existence of more than one bound form of ligand at higher concentration. The equilibrium binding constants values for the association of 1 with polynucleotides were determined as described above, from the fit of the absorbance data to non-cooperative model of McGhee and von Hippel and are summarized in Table 5. This shows that 1 exhibits a very much higher affinity for poly(dA–dT)2. It may be noted that this behaviour contrasts with the lack of sequence preference for the unsubstituted compound 2.8c

Fluorescence studies. The variation in the steady state emission intensity of 1 in the presence of increasing concentration of poly(dA–dT)2 is shown in Fig. 8c. At high concentration of poly(dA–dT)2, the emission intensity of 1 was approximately doubled, with a 5 nm blue shift similar to that observed for the titration of 1 with st-DNA. Time resolved emission measurements revealed that in the presence of poly(dA–dT)2 at a P/D = 30 a long lived component with \( \tau = 9.9 \text{ ns (94\%)} \) is observed corresponding to the bound form of 1 (Table 5).

As can be seen in Fig. 8d, the excited state behaviour of 1 with poly(dG–dC)2 is strikingly different from that seen for poly(dA–dT)2 or st-DNA, as the fluorescence is partially quenched rather than enhanced. Thus in the presence of poly(dG–dC)2, the emission intensity of 1 is reduced by ca. 30\% after correcting for the absorbance decrease at 435 nm. The fluorescence intensity plot (inset on Fig. 8d) again reveals the marked difference in the binding affinities for the two synthetic polynucleotides (note the different P/D scales in Fig. 8c and 8d). Time resolved measurements in the presence of excess poly(dG–dC)2 (at P/D = 105) showed that the fluorescence of 1 decays by a bi-exponential process with \( \tau_1 = 1.5 \text{ ns (51\%)} \) and \( \tau_2 = 3.8 \text{ ns (49\%)} \), so that the observed average singlet state lifetime of 1 bound to poly(dG–dC)2 remained practically unchanged from the free ligand. This behaviour may be contrasted with what was found for 5′-GMP.
(Table 3) where no longer-lived emission was observed and with that of poly(dA–dT)2, where a much longer-lived component was detected. It is probable that two conflicting trends are operative for the singlet excited state of 1 bound to poly(dG–dC)2. Firstly it is expected that there should be an increase in lifetime due to location within the non-aqueous and more rigid environment of the polynucleotide. However this will be balanced by a reduction in the excited state lifetime because of photo-induced electron transfer (PET) from guanine to the excited naphthalimide, as found with 5′-GMP.

Conclusions

This study shows that the cationic 4-amino-1,8-naphthalimide derivative 1, which possesses a pyridinium moiety, binds strongly to DNA and synthetic polynucleotides. Linear dichroism and the other spectroscopic studies are consistent with the compound binding by intercalating between the base-pairs of polynucleotide. It is also likely that the pyridinium moiety binds electrostatically within the groove and possibly with the phosphate group of the DNA. The strong interaction between the naphthalimide ring and the purine bases is further demonstrated by the complexation with the mononucleotides 5′-AMP and 5′-GMP.

1 shows a strong preference to bind to A–T regions of DNA, as is demonstrated by the six times larger binding constant determined for poly(dA–dT)2 than for poly(dG–dC)2. The similar emission enhancement in both double-stranded ss-DNA and in poly(dA–dT)2 strongly suggests that in the natural DNA the intercalator also seeks out AT sequences. It is also notable that thermal denaturation experiments show that binding of 1 to poly (dA–dT)2 causes a significantly higher stabilisation of the double-stranded form than is found for natural DNA, indicative of a thermodynamically stronger interaction with such alternating AT sequences. This could indicate that compounds such as 1 might therefore have a medicinal role for targeting AT rich DNA, as is for example found in the genome of the malaria parasite P. falciparum.37

It is interesting to note that 1 behaves quite differently in many respects to the analogous unsubstituted compound 4, which McMasters and Kelly have shown locates in the minor groove of double stranded DNA with the binding predominantly...
were carried out in quartz cuvettes (10 mm × 10 mm). 10 mM phosphate buffer of pH 7.0 was made up by mixing portions of 1 M stock solutions of Na2HPO4 and NaH2PO4. All solutions were prepared fresh prior to measurement. The association constants, K for nucleotides was determined from the slope of Scatchard plot (see ESI† for details).

For the thermal denaturation studies, semi-micro UV-vis cuvettes were used (path length of 1 cm and window width of 4 mm, from Starna). The temperature-based measurements were obtained using a Cary temperature controller in conjunction with the Varian Cary 300 UV-vis spectrometer. The temperature was ramped from 30–90 °C at a rate of 0.5 °C min⁻¹. All solutions were thoroughly degassed before the experiment.

**Fluorescence measurements.** Steady-state fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorimeter. Fluorescence titrations, were carried out using optically dilute solutions (absorbance < 0.1) following the same procedure as described for UV-vis titrations using slit widths 5 nm for both excitation and emissions. Fluorescence quantum yields were measured using dilute solutions (absorbance < 0.05) against fluorescein in 0.1 N NaOH solution (Φ = 0.9). Fluoresent lifetimes were measured and analysed using Fluorolog 3 time correlated single photon counting apparatus with a Nanolode (λex = 458 nm) used as the excitation source. Emission decays were recorded at 550 nm using slit width of 2 nm. All the decay traces were corrected for instrument response using a dilute solution of colloidal silica. In the absence of any mononucleotides, DNA or polynucleotides, decay kinetic of 1 was analysed by monoeponential model. In the presence of mononucleotides or polynucleotides, decay traces were best described by biexponential functions. Goodness of the fit was determined from the χ² value (1.0–1.2) and the average residual distributions.

**Linear dichroism measurements.** Flow oriented linear dichroism studies of were performed on a Jasco J-815 CD spectrometer fitted with a Diotica Scientific Ltd Linear Dichroism 2 apparatus. For an oriented sample, linear dichroism is defined as the differential absorption of light polarised parallel (Apar) and perpendicular (Aper) to a reference axis (eqn (2))

\[
\text{LD} = A_{\text{par}} - A_{\text{per}}
\]

Long flexible polymers such as peptides or DNA are usually oriented by the technique of flow orientation. In this technique, the molecules are oriented by shear force generated by a flow gradient in the solution flowing between the narrow gap between a spinning cell and a stationary cylindrical rod.

Quantitative information about the orientation of a chromophore with respect to the reference axis can be obtained from reduced LD (LD') as described in eqn (3) below,

\[
\text{LD}' = \frac{\text{LD}}{A} = \frac{A_{\text{par}} - A_{\text{per}}}{A} = \frac{3}{2} S (3 \cos^2 \alpha - 1)
\]

where, A is the absorbance of the sample under isotropic condition (i.e., no orientation), S refers to the orientation factor (S = 1 for perfectly oriented sample, S = 0 for random orientation) and α is the angle between the transition dipole of the chromophore and the reference axis. The magnitude of S provides

**Experimental details**

All the solvents used for photophysical studies were spectroscopic grade. Freshly distilled acetonitrile was used for quantum yield measurement. Deuterated solvents for NMR were purified grade. Freshly distilled acetonitrile was used for quantum yield measurement. Deuterated solvents for NMR were purified grade. Freshly distilled acetonitrile was used for quantum yield measurement. Deuterated solvents for NMR were purified grade. Freshly distilled acetonitrile was used for quantum yield measurement. Deuterated solvents for NMR were purified grade. Freshly distilled acetonitrile was used for quantum yield measurement. Deuterated solvents for NMR were purified grade. Freshly distilled acetonitrile was used for quantum yield measurement.
information about structural changes in the macromolecule such as lengthening, stiffening, bending etc.

Circular dichroism measurements. CD spectra were recorded on a Jasco J810 spectropolarimeter. CD titrations were carried out by adding aliquots of solution of 1 to solution of st-DNA (150 μM) in 10 mM phosphate buffer at varying P/D.

Synthesis of N-(2-hydroxy ethyl)-4-nitro-1,8-naphthalimide (3)\textsuperscript{21}

4-Nitro-1,8-naphthalic anhydride (2.034 g, 8.365 mmol) was suspended in dry ethanol and ethanol amine (0.5 ml) was added dropwise. The mixture was refluxed for 24 h under an argon atmosphere and cooled to room temperature. The desired product was separated by filtration, washed with cold ethanol and obtained as a dark brown solid (1.8 g, 75%). m.p. 157–160 °C (ref. 21). HRMS: 304.2083 ([M + Na]+). C\textsubscript{14}H\textsubscript{10}N\textsubscript{2}O\textsubscript{5} requires 304.0976; δ\textsubscript{H} (600 MHz, d\textsubscript{6}-DMSO) 9.13 (2H, d, J = 6.0 Hz, H17), 8.73 (1H, d, J = 8.2 Hz, Ar–H5), 8.59 (1H t, J = 7.6 Hz, H17), 8.30 (1H, d, J = 7.3 Hz, Ar–H7), 8.07 (3H, m, H16, Ar–H2), 7.63 (1H, t, J = 8.0, Ar–H6), 7.59 (2H, s, NH2), 6.83 (1H, d, J = 8.6 Hz, Ar–H3), 4.94 (2H, t, J = 4.9 Hz, CH2), 4.57 (2H, t, J = 4.5 Hz, CH2); δ\textsubscript{C} (100 MHz, d\textsubscript{6}-DMSO) 164.4 (C–O), 163.7 (C), 133.8 (CH), 132.3 (CH), 131.7 (CH), 130.8 (C), 128.2 (CH), 124.9 (C), 124.8 (C), 123.6 (CH), 44.37 (CH2). ν\textsubscript{max}(neat sample)/cm\textsuperscript{-1}: 3375.42 (w, N–H stretch), 2920.58 (w, C–H asym. stretch), 2872.49 (w, C–H sym. stretch). 1679.93 (m, C–O stretch).

Synthesis of N-(2-hydroxy ethyl)-4-amino-1,8-naphthalimide (4)

Compound 4 was synthesised by catalytic hydrogenation of 3 (1.00 g, 3.494 mmol) in DMF using a Parr hydrogen shaker apparatus at 3 atm pressure in the presence of 10% Pd/C catalyst until the hydrogen consumption ceased. The reaction mixture was filtered through celite and washed with DMF. The filtrate and washings were concentrated under reduced pressure and the product was precipitated as an orange solid from ice (0.812 g, 60%). m.p. degraded at 201 °C (ref. 70). HRMS: 279.0746 ([M + Na]+). C\textsubscript{14}H\textsubscript{12}N\textsubscript{2}O\textsubscript{3} requires 279.0746. δ\textsubscript{H} (400 MHz, d\textsubscript{6}-DMSO) 9.13 (2H, d, J = 6.0 Hz, H17), 8.73 (1H, d, J = 8.2 Hz, Ar–H5), 8.59 (1H t, J = 7.6 Hz, H17), 8.30 (1H, d, J = 7.3 Hz, Ar–H7), 8.04 (3H, m, H16, Ar–H2), 7.62 (1H, t, J = 8.0, Ar–H6), 7.59 (2H, s, NH2), 6.83 (1H, d, J = 8.6 Hz, Ar–H3), 4.94 (2H, t, J = 4.9 Hz, CH2), 4.57 (2H, t, J = 4.5 Hz, CH2); δ\textsubscript{C} (150 MHz, d\textsubscript{6}-DMSO) 164.4 (C–O), 163.9 (C), 133.7 (CH), 132.3 (CH), 131.7 (CH), 130.8 (C), 128.2 (CH), 124.9 (C), 124.8 (C), 123.6 (CH), 44.37 (CH2). ν\textsubscript{max}(neat sample)/cm\textsuperscript{-1}: 3375.42 (w, N–H stretch), 2920.58 (w, C–H asym. stretch), 2872.49 (w, C–H sym. stretch). 1679.93 (m, C–O stretch).

References