Synthesis, photophysical and cytotoxicity evaluations of DNA targeting agents based on 3-amino-1,8-naphthalimide derived Tröger’s bases†

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The synthesis and characterisation of five bis-1,8-naphthalimide containing Tröger’s bases 1–5 formed from their corresponding 3-amino-1,8-naphthalimide precursors 6–10 is described. The photophysical investigations of 1–5 and 6–10 were carried out in several organic solvents as well as in water and as a function of pH using UV-Vis absorption and fluorescence spectroscopies. The DNA binding affinities of 1–5 in aqueous solution at pH 7.4 were also investigated using several UV-Vis absorption and fluorescence experiments by using calf thymus DNA (ct-DNA). These molecules exhibited significant DNA binding affinities; where large binding values (Kb) in the range of 10^6 M^-1 were determined, even in competitive media (50 mM and 160 mM NaCl at pH 7.4). Thermal denaturation measurements also showed that 1–5 significantly stabilised the DNA helix. Using linear and circular dichroism we further demonstrated that the DNA binding interaction occurs both by intercalation and by groove binding. The Tröger’s bases were further shown to be rapidly taken up into cells using confocal fluorescence spectroscopy; and cytotoxic studies in HeLa and MCF-7 cells showed that most of the Tröger’s bases were effective cytotoxic agents with EC50 values of between 1.1–12 μM and that all the active compounds induced programmed cell death by apoptosis, where up to 70% cellular death was observed after 24 h of incubation for 4.

Introduction

The design and development of small molecules which exert their biological activity by interacting with DNA and which exhibit photophysical properties that are sensitive to the binding event continue to be the focus of a significant amount of research within the field of medicinal chemistry.1–6 The 1,8-naphthalimide chromophore has been shown to bind to DNA and exhibit anti-cancer properties;1–6 but both the 4-amino and the 3-amino-1,8-naphthalimides possess spectral properties that are sensitive to their environment due to the “push–pull” internal charge transfer (ICT) character (due to the electron withdrawing imide and the electron donating amino group).6,7 Consequently, they have been employed as potential cellular imaging and anti-tumour agents.1,4,5,8–10 The 1,8-naphthalimide chromophore has been extensively used in our laboratory, both as cellular and DNA targeting probes,1–3 as well as supramolecular fluorescent and colorimetric probes and sensors for ions and molecules.6–11 The Tröger’s base unit (1,5-methanodiazocine), due to its C2 axis of symmetry, chiral nature and its rigid V-shape, has also been used in various such supramolecular structural designs.12,13 Recently, Deprez et al.,14 have developed Tröger’s bases using naphthalimide precursors. Similarly, we have shown, using 4-amino-1,8-naphthalimide Tröger’s bases derivatives, that such structures can be employed in the formation of bio-probes; the function of which is dictated by the incorporation of a functional moiety, such as polypyridyl units,16 at the imide site. In order to gain further insight into the use of the 1,8-naphthalimide moiety in the design of novel targeting biological Tröger’s based probes and therapeutics, we present herein the results of the synthesis and the photophysical characterisation of five new DNA targeting Tröger’s bases 1–5 (Scheme 1) derived from 3-amino-1,8-naphthalimide based precursors.
We demonstrate that these structures, using various tertiary alkyl amines, encode both different structural as well as photo-physical properties to those derived from the 4-azanaphthalimide; due to both different electronic as well as structural features of the 3-amino derivatives. We show that while these structures have high affinity for double stranded DNA, their binding occurs by a dual binding event involving both intercalation and groove binding, and that these structures are rapidly taken up into HeLa cells, localising within the cytosol, as demonstrated using confocal fluorescence microscopy, and that they give rise to apoptosis within a few hours of incubation.

Results and discussion

Synthesis and characterisation of the 3-amino-1,8-naphthalimides 6–10 and the Tröger’s bases 1–5

The bis-1,8-naphthalimide containing Tröger’s base derivatives 1–5 were prepared as (R,R; S,S) racemates in three steps from the commercially available 3-nitro-1,8-naphthalic anhydride as shown in Scheme 1. Reduction by catalytic hydrogenation using 10% Pd/C at 3 atm of H₂ in DMF gave the corresponding 3-amino-1,8-naphthalimide anhydride 11 in 92% yield. Treating 11 with the relevant alkyl amine and triethylamine resulted in the formation of the desired 3-amino-1,8-naphthalimide precursors 6–10 (see Experimental). All of the precursors were characterised using conventional techniques; but these precursors had previously been synthesised by Braña and co-workers as anticancer agents and were studied in HeLa cancer cell lines for their anticancer ability.17

Single crystals suitable for X-ray diffraction analysis were obtained for 6–9 by slow evaporation from CH₂Cl₂, while in the case of 10, crystals were obtained after a recrystallization from CH₃OH (see ESI†). To the best of our knowledge, the solid-state structures of these have not been reported before. Of these structures 6, 8 and 9 were of high enough quality for publication, while the crystal structure analysis proved the connectivity for 7 and 10. As can be seen in Fig. 1, molecule 9 crystallised in the monoclinic space group P2₁/c and contained one molecule in the asymmetric unit. The piperidine group adopts a chair like conformation while the dialkyl linker adopts a gauche conformation [N(1)–C(19)–C(18)–N(3) torsional angle = 61.9°] resulting in the piperidine group being oriented almost perpendicular to the plane of the naphthalimide. The orientation of the piperidine group resulted in the formation of a complementary hydrogen bonded dimer between the amino protons and the nitrogen atom of a piperidine on a neighbouring symmetry generated molecule. The dimer is then linked into a more complex H-bonding array through interactions between the amino group and the carbonyl oxygen atoms on a neighbouring molecule. Similar torsional angles and H-bonding patterns were also observed for 6–8 and 10 (see X-ray details in ESI†). The extensive π⋯π stacking interactions, shown in Fig. 1, result in the head to tail arrangement of molecules. The packing interactions are primarily dominated, as said above, by extensive hydrogen-bonding networks between the 3-amino group and the carbonyl oxygen atoms of the imide functionality and between the distal tertiary amino groups of the side arms of neighbouring naphthalimide units. Each of the 3-amino groups is also trans to its next neighbour, causing each of the naphthalimides to be slightly offset in the packing diagram.

Formation of the Tröger’s bases 1–5 as racemic mixtures from 6–10 was achieved by treating these precursors with 1.1 equivalents of paraformaldehyde and TFA. After stirring the resulting mixtures at room temperature for 3 hours, the desired products were extracted into CH₂Cl₂ and washed with alkaline solution, followed by further purification by column chromatography on flash silica or by using trituration or recrystallisation giving the desired compounds in moderate to high yields; 1 (54%), 2 (79%), 3 (54%), 4 (36%) and 5 (61%). All the compounds were characterised using conventional techniques (see Experimental). As an example, the ¹H NMR spectrum (see ESI† for 1–5) of 2 when recoded in CDCl₃ (600 MHz) confirmed its identity by the presence of a well separated doublet of doublets between 4.59 and 5.21 ppm as well
as a singlet at 4.60 ppm pertaining to the methylene protons of the diazocine ring while also clearly reflecting the $C_2$ plane of symmetry of the molecule (same was observed for $1$, $3$–$5$).

**Spectroscopic investigation of $1$–$5$ (and $6$–$10$) in various solvents**

As discussed above, the amino-$1,8$-naphthalimides are known to possess ICT based excited states. However, in the case of the $3$-amino derivatives, the ICT is significantly less pronounced than normally seen for the $4$-amino systems; an effect that we have recently investigated in details for the development of thiourea based anion sensors.$^7$ To investigate the effect of solvent polarity on $1$–$5$, the absorption and fluorescence emission spectra were recorded in both protic and aprotic solvents of varying polarity$^{10}$ and compared to that of the precursors $6$–$10$.

The absorption spectra of $1$–$5$ when recorded in $\text{CH}_3\text{OH}$ exhibited a high-energy transition at $238$ nm, a lower energy transition at $347$ nm with a shoulder with a $\lambda_{\text{max}} = 390$ nm. In general the absorption spectra were not significantly affected by the solvent medium as can be seen in Fig. 2a, for compound $1$ (see ESI† for $2$–$5$); particularly when compared to that observed for $6$, which showed a strong correlation between $\lambda_{\text{max}}$ and solvent polarity. This clearly demonstrated that the diazocine ring has a significant effect on the $3$-amino moiety to delocalise its electron pair into the naphthalimide ring. However, when recorded in water, the shape of the shoulder at $390$ nm was significantly altered for $1$–$5$; being blue shifted relative to the other solvents investigated. A similar effect has also been reported by Brown et al.$^{18}$ for $4$-amino-$1,8$-naphthalimide based systems; this being explained as being due to the strong hydrogen bonding with water, leading to the formation of hydrogen bonded solvated clusters that absorb at a lower wavelength (compared to other solvents investigated).

As shown in Fig. 2b, the absorption spectrum of $6$ was recorded in the same solvents for comparison purposes. Here the absorption spectrum of $6$ in $\text{CH}_3\text{OH}$ was characterised by a high energy structural band at $245$ nm, whereas, the region between $300$ and $500$ nm consists of a less intense band at ca. $349$ nm and a broad band centred at $426$ nm. Upon changing the solvent from $\text{CH}_3\text{OH}$ to $\text{CH}_2\text{Cl}_2$ a hypsochromic shift of $17$ nm was observed which demonstrates that $6$ possess an ICT excited state. However, when the solvent was changed from $\text{CH}_3\text{OH}$ to water a hypsochromic shift of $18$ nm was observed similar to that seen for $1$–$5$. These results seem to indicate that the ICT nature of $6$ is to a greater extent lost in the formation of the Tröger’s bases $1$–$5$; a phenomenon not seen for the Tröger’s bases formed from the use of $4$-amino-$1,8$-naphthalimide derivatives.$^{15,16}$ Consequently, the influences of solvent polarity on the photophysical behaviour of $1$–$5$ was further evaluated using fluorescence emission spectroscopy.

When excited in $\text{CH}_2\text{Cl}_2$ $1$–$5$ exhibited broad unsymmetrical bands at ca. $480$ nm that tailed off to longer wavelengths which supports the characterisation of such bands as having contributions from ICT transitions. As can be seen in Fig. 3, an increase in solvent polarity resulted in bathochromic and hypochromic shifts. For example, in the case of $1$, the largest bathochromic shift, of $67$ nm, was observed when changing the medium from $\text{CH}_2\text{Cl}_2$ to water. Such a shift was not observed in the absorption spectra indicating that the solvent stabilisation is more pronounced in the excited states compared to the ground states with similar behaviour also being observed for $6$ (see ESI†). In addition to these changes, the decrease in the emission intensity of $1$–$5$ as a function of solvent polarity was also accompanied by a reduction in their

![Fig. 2](image-url)  
Fig. 2 (a; top) Solvent effects on the absorption properties of $1$ in $\text{CH}_2\text{Cl}_2$ (●), $\text{CH}_3\text{OH}$ (▲), $\text{H}_2\text{O}$ (▲), $\text{CH}_2\text{CN}$ (▲), Acetone (▲) and DMF (▲) where $\lambda_{\text{max}} = 390$ nm. (b; bottom) Solvent effects on the absorption properties of $6$ in $\text{CH}_2\text{Cl}_2$ (●), $\text{CH}_3\text{OH}$ (▲), $\text{H}_2\text{O}$ (▲), $\text{CH}_2\text{CN}$ (▲), Acetone (▲) and DMF (▲) where $\lambda_{\text{max}} = 409, 426, 408, 416, 422$ and $434$ nm, respectively.

![Fig. 3](image-url)  
Fig. 3 Solvent effects on the fluorescence emission properties of $1$ in $\text{CH}_2\text{Cl}_2$ (●), $\text{CH}_3\text{OH}$ (▲), $\text{H}_2\text{O}$ (▲), $\text{CH}_2\text{CN}$ (▲), Acetone (▲) and DMF (▲) with an excitation at $390$ nm.
fluorescence quantum efficiencies (being 1% in CH₂Cl₂, while being 0.1% in water). This effect was first reported by Deprez et al.¹⁴ and is also in agreement with the low fluorescent quantum efficiencies obtained for the 4-amino-naphthalimide Tröger’s bases synthesised by Veale et al.¹⁵ and being reported as being due to the presence of an ICT excited state in those examples.

**Spectroscopic investigation of 1–5 (and 6–10) as function of pH**

Having established that the fluorescence emission properties of 1–5 were highly solvent dependent, it was anticipated that upon binding of these molecules to DNA, significant changes would be expected to be observed in their emission spectra as a function of increasing DNA concentration. To achieve this, compounds 1–5 all possess dialkyl tertiary amino moieties at the N-imide sites. In accordance to our previous work, this should facilitate the binding of these structures to DNA; the idea being that these amines should be protonated at physiological pH, making 1–5 both highly soluble in aqueous buffered solution, as well as allowing for electrostatic binding between 1–5 and the negatively charged phosphate backbone of DNA to take place. Furthermore, due to the almost orthogonal nature of the two naphthalimides within the Tröger’s base structures, it was further anticipated that these compounds could have the potential to bind to DNA in a bi-modal fashion where one of the naphthalimide units would be able to intercalate within the double stranded DNA structure while the second unit would give rise to groove binding.

As the aforementioned electrostatic binding would strengthen the binding process, the pKₐ values of the tertiary amines of 1–10 were first determined by recording their absorption and emission spectra as a function of pH in water using 100 mM NaCl to maintain constant ionic strength.

Firstly, the pH dependence of the 3-amino-1,8-naphthalimides 6–10 was investigated where minor changes were observed in the absorption spectra. For example, the absorption spectrum of 7, consisting of a main ICT band centred at 409 nm and a plot of the absorbance versus pH (inset) exhibited only minor changes and can be considered to be independent of pH (see ESI†). Larger changes were, however, observed in the emission spectra at basic pH where a fluorescence enhancement as a function of increasing DNA concentration. To achieve this, would be expected to be observed in their emission spectra as a function of increasing DNA concentration. To achieve this, 409 nm and a plot of the absorbance by 0.1% in water). This effect was first reported by Deprez et al.¹⁴ and is also in agreement with the low fluorescent quantum efficiencies obtained for the 4-amino-naphthalimide Tröger’s bases synthesised by Veale et al.¹⁵ and being reported as being due to the presence of an ICT excited state in those examples.

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\[ pK_a(S_i) = pH - \log(I_{AHi} - I_{Ai})/(I_{Ai} - I_{AHi}) \]

where \( I_{AHi} \) and \( I_{Ai} \) are the emission intensities in acidic and basic solution, respectively, and \( I_{Ai} \) is the emission intensity at each pH value.²¹

Having established the pH behaviour of the precursors 6–10, the pH dependence of Tröger’s bases 1–5 was analysed using the same conditions and techniques. In contrast to the pH independence observed in the absorption spectra of precursors 6–10, the Tröger’s bases 1–5 exhibited pH dependence as can be seen in the case of 2 in Fig. 4, where upon the addition of base, the ICT band centred at 347 nm decreased in absorbance by ca. 60%. Such behaviour may possibly be attributed to the ability of the tertiary amine of the N-imide side chain to donate a proton into the ring and may further arise due to the 3-amino moiety becoming twisted as a consequence of the V-shaped structure of the methano-1,5-diazocine ring, as is evident from the inset in Fig. 4, when recorded at 347 nm for 2.¹⁵

The fluorescence emission behaviour of 1–5 was also analysed as a function of pH; in general, fluorescence enhancement was observed upon the addition of base except in the case of 3 where the emission was quenched. Such behaviour may arise because of the ability of the terminal amine to function as an electron donor as upon deprotonation, emission quenching occurs due to photoinduced electron transfer (PET), which upon protonation is removed due to increased oxidation potential for the tertiary amine (i.e. the terminal amine donates an electron to the Tröger’s base excited state). Due to the reduced ICT character in these Tröger’s base structures, compared to that of the 1,8-naphthalimide precursors, the PET comes active, despite its directional electron transfer nature.¹⁵ The normalised changes observed in both the ground and excited states were analysed and assigned to the protonations of their respective terminal amines, cf. inset in Fig. 4. The pKₐ value estimated for 3 was ca. 6.2 and will not be fully protonated at physiological pH and thus is not expected to give rise to electrostatic interactions in the presence of DNA to the same extend as the other derivatives (and, hence, this should be reflected in their binding affinity, see later). Conversely, the pKₐ values determined for the terminal amines of 1 and 2, 4 and 5 ranges from 7.5–8.9 and are expected to be substantially protonated at physiological pH enabling electrostatic interactions to occur between these molecules and the negatively charged phosphate backbone of DNA.

![Fig. 4](image-url) The overall changes in the absorption spectra of 2 as a function of pH. The decreasing changes seen in the ICT band as a function of pH were also mirrored at higher energy. *Inset:* The changes at 347 nm vs. pH.
Spectroscopic DNA titrations of 1–10

Having established that 1, 2, 4 and 5 would possess pH dependent properties that favour DNA binding, the DNA binding affinities of 1–5 were investigated at pH 7.4 (10 mM phosphate buffer) by observing the changes in their absorption and emission spectra in the presence of 0 mM, 50 mM and 160 mM NaCl. As anticipated, then with the exception of compound 3, the absorption spectra of these compounds were significantly affected upon the addition of calf thymus (ct) DNA. The changes observed for 2 in 10 mM phosphate buffer at pH 7.4, are shown in Fig. 5, whereby the addition of ct-DNA (0 to 12.4 µM) resulted in a significant hypochromism of ca. 34% in the ICT band centred at 347 nm. This was further accompanied by a bathochromic shift of 2 nm; occurring at a phosphate to dye (P/D) ratio of greater than 3. However, upon further addition of ct-DNA (14.3 µM to 56.1 µM) the absorption of 2 was enhanced by ca. 14%, with no further changes being seen at higher concentrations.

The results obtained for all these compounds are summarised in Table 1. The behaviour observed here is perhaps indicative of a second mode of binding. Upon binding to ct-DNA at medium ionic strength, 50 mM NaCl, the changes observed in the absorption spectrum of 2 were similar to those observed in Fig. 5, in the absence of added salt. However, at 160 mM NaCl ionic strength, only a hypochromic effect of ca. 28% was observed between a P/D of 0 → 17. Further additions of ct-DNA resulted in a negligible effect. The above results show that at high ionic strength, a single binding interaction occurred, implying that the electrostatic interactions do no longer play as strong a role in the DNA binding event as observed at lower ionic strength. The spectral changes observed for the other compounds were similar (see ESI†).

Analysis of the changes observed in the absorption spectra using the Bard binding model resulted in large binding constants, \( K_b \), of ∼10⁶ M⁻¹ being determined for 1, 2, 4 and 5 as summarised in Table 2.¹⁵,²² The fitting observed for the changes in 10 mM phosphate buffer at pH 7.4, is shown in ESI†. Furthermore, in more competitive 160 mM NaCl salt media, these binding constants still remained in the range of 10⁵ M⁻¹, clearly demonstrating that these molecules possess strong affinities for DNA.

The DNA binding of the 3-amino-1,8-naphthalimide precursors 6–10 was also investigated in an analogous manner to that described above in 10 mM phosphate buffer at pH 7.4. In the case of 7 (the precursor to 2) a 47% hypochromism at the \( \lambda_{\text{max}} \) at 408 nm was observed, which was accompanied with a 22 nm bathochromic shift. In addition, during the course of the titration an isosbestic point at 443 nm was observed indicating the presence of two spectroscopically distinct free and ct-DNA bound chromophores (see ESI†). The significant decrease and red shift in the absorbance indicates that naphthalimide 7 has a strong binding affinity for ct-DNA. However, the bi-phasic DNA binding exhibited by 2 was not observed for 7 suggesting that the presence of the Tröger’s base moiety is influencing the manner in which the naphthalimide chromophore interacts with DNA. On all occasions the binding site size (n) was close to unity, being in the order of \( n = 1–2.4 \) (see ESI†).

As in the case of 1–5, the binding constants for these precursors were determined according to the Bard model and were found to be in the range of 10⁴ to 10⁶ M⁻¹; but all had lower affinity for DNA than their corresponding Tröger’s base systems.¹⁸

The fluorescence emission spectra of 1–10 were also monitored upon binding of these systems to DNA. In the cases of 1–5, significant changes were observed in the emission (Fig. 5) spectra upon the addition of ct-DNA. As an example the fluorescence emission spectra of 2 and 5 in 10 mM phosphate buffer (pH 7.4) are shown in Fig. 6A and B. Upon excitation at 347 nm and the successive addition of ct-DNA, the emission intensity at 543 nm decreased by ca. 70%. The substantial fluorescence quenching observed may be attributed to an efficient

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**Table 1** Various photophysical properties of 1–5

<table>
<thead>
<tr>
<th>Property</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( e_\lambda ) (M⁻¹ dm³ cm⁻¹)</td>
<td>14 825</td>
<td>19 332</td>
<td>14 934</td>
<td>13 412</td>
<td>16 762</td>
</tr>
<tr>
<td>( \lambda_{\text{Abs,max}} ) (nm)</td>
<td>347</td>
<td>347</td>
<td>347</td>
<td>347</td>
<td>347</td>
</tr>
<tr>
<td>( \lambda_{\text{Emission}} ) (nm)</td>
<td>350</td>
<td>349</td>
<td>347</td>
<td>350</td>
<td>349</td>
</tr>
<tr>
<td>Bathochromic shift (nm)</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Hyperchromism (%)</td>
<td>39</td>
<td>34</td>
<td>7</td>
<td>57</td>
<td>35</td>
</tr>
<tr>
<td>Hyperchromism (%)</td>
<td>16</td>
<td>14</td>
<td>0</td>
<td>23</td>
<td>12</td>
</tr>
</tbody>
</table>

\( ^a \) \( e_\lambda \) is the extinction coefficient of 1–5. \( ^b \) \( \lambda_{\text{Abs,max}} \) is the wavelength of maximum absorbance of 1–5. \( ^c \) \( \lambda_{\text{Emission}} \) is the wavelength of maximum absorbance of DNA bound 1–5.

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**Table 2** Binding constants \( K_b \) and binding site sizes \( n \) determined for 1, 2, 4 and 5 bound to ct-DNA using the Bard binding model²²

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_b ) (×10⁶ M⁻¹)</th>
<th>( n ) (bp)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.68 ±(4.8)</td>
<td>0.23 ±(0.10)</td>
<td>0.99</td>
</tr>
<tr>
<td>2</td>
<td>8.51 ±(5.34)</td>
<td>0.22 ±(0.01)</td>
<td>0.99</td>
</tr>
<tr>
<td>4</td>
<td>9.69 ±(3.96)</td>
<td>0.17 ±(0.1)</td>
<td>0.99</td>
</tr>
<tr>
<td>5</td>
<td>6.59 ±(2.33)</td>
<td>0.27 ±(0.01)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

\( ^a \) Changes were too small to determine \( K_b \) for 3. \( ^b \) The binding site size is lower than would be expected and could be a reflection of the complex changes seen in the absorption spectra.
photoinduced electron transfer (PET) quenching from the DNA bases to the excited state of the naphthalimide chromophore.\textsuperscript{10,19,20} For comparison purposes, the interactions of the precursors 6–10 with ct-DNA were also investigated in 10 mM phosphate buffer (pH 7.4) using fluorescence emission spectroscopy. Upon excitation at 408 nm, the emission spectrum of 6 exhibited a broad band centred at 582 nm, which upon addition of ct-DNA decreased in intensity by up to ca. 56% with a concomitant blue shift of 24 nm. Similar changes were also observed for derivatives 7–10 showing that the excited states of the precursors were affected differently upon binding to DNA (see ESI for changes in the emission spectra for compounds 6–10). The significant changes highlight once again the strong affinities for DNA possessed by 6–10.

The binding of 1–5 to ct-DNA was further analysed at pH 7.4 in the presence of both 50 mM and 160 mM NaCl using fluorescence emission spectroscopy. In the case of 2, in the presence of 50 mM NaCl an excitation at 347 nm resulted in the appearance of an ‘unsymmetrical band’ with a \( \lambda_{\text{max}} \) at 543 nm. Upon the successive addition of ct-DNA, the intensity of this band decreased by ca. 45% and was accompanied by a bathochromic shift of 7 nm. However, in the presence of 160 mM NaCl, the intensity of this band decreased by 35%; but was still red shifted by 7 nm. The overall changes in the ICT band as a function of DNA (as P/D ratio) in these three ionic strength environments is shown in Fig. 7.

The changes observed in the emission spectra in the absence and presence of salt were further used to derive Scatchard plots which were analysed according to the model of McGhee and von Hippel\textsuperscript{23} to determine the \( K_b \). In the absence of any NaCl salt, the \( K_b \) values determined for 1–5 were in the range of \( 10^6 \text{ M}^{-1} \) (see ESI\textsuperscript{†}) complementing the results from the absorption titrations above, and were found to decrease only slightly in competitive media. Here, the binding site size was determined to be between \( n = 1.3–2.5 \) depending on the NaCl salt concentration. The binding constants \( K_b \) were also determined for 6–10 using this model and were also found to be in all cases lower than that see for 1–5, being in the range of \( 10^5–10^6 \text{ M}^{-1} \); clearly demonstrating the advantages of the formation of the Tröger’s base systems over that of the naphthalimide precursors themselves.

**Ethidium bromide displacement assays and DNA thermal denaturation studies**

In order to further quantify the binding affinity of the Tröger’s bases 1–5 in comparison to their respective precursors 6–10, ethidium bromide (EtBr) displacement assays were also carried out in 10 mM phosphate buffer (pH 7.4) according to the procedure developed by Boger et al.,\textsuperscript{24} where the changes in the emission spectra of EtBr bound to ct-DNA were monitored upon the successive addition of the relevant Tröger’s base naphthalimide or the 4-aminonaphthalimide precursor.

As can be seen in Fig. 8, the emission intensity of EtBr bound to ct-DNA at ca. 602 nm significantly decreased upon addition of ct-DNA (0.44 µM: 0.88 µM) in 10 mM phosphate buffer + 160 mM NaCl (red) versus P/D at 543 nm.

**Fig. 6** The fluorescence emission changes in 2 (A) and 5 (B), respectively, upon titrating with ct-DNA (0–47.9 µM) in 10 mM phosphate buffer (pH 7.4); being quenched in both cases upon binding to DNA.

**Fig. 7** Plot of changes in the emission intensity of 2 in 10 mM phosphate (blue), 10 mM phosphate + 50 mM NaCl (green) and in 10 mM phosphate buffer + 160 mM NaCl (red) versus P/D at 543 nm.

**Fig. 8** The overall quenching in the emission spectra of ethidium bromide, free and bound to ct-DNA (0.44 µM: 0.88 µM) in 10 mM phosphate buffer (pH 7.4) upon the addition of 1 with an excitation at 545 nm. Top curve is the fully bound E\textsubscript{B}r (prior to addition of 1) while the lowest one is free E\textsubscript{B}r in solution (after addition of 1).
the addition of 1, indicating that 1 is highly capable of substituting EtBr for DNA. Similar results were seen for 2, 4 and 5, while compounds 3 and 6–10 only showed limited ability to displace EtBr; once again demonstrating the advantages of the Tröger’s bases binding motive over the 3-amino-1,8-naphthalimide precursors and the effect the pK_a of the terminal amines has on the binding to DNA for these systems.

For Tröger’s bases 1–5 the displacement capability was found to be in the order of 2 > 1 > 5 > 4 with apparent binding constants K_{app} of 15.7, 11.5 10.4 and 7.6 × 10^6 being determined for 2, 1, 5 and 4, respectively; once again demonstrating high affinity of these compounds for ct-DNA. Unfortunately, in the case of 3, the changes were too small for a K_{app} to be determined suggesting that electrostatic interactions may play a role in the displacement of EtBr from DNA.

With the view of further evaluating the DNA binding affinity of 1 and 2, 4 and 5 (see ESI†) thermal denaturation (T_m) experiments were conducted. For such measurements the T_m of ct-DNA (150 µM) alone and in the presence of the relevant Tröger’s base naphthalimide at a P/D of 10 were carried out in 10 mM phosphate buffer (pH 7.4), where 1, 2, 4 and 5 were shown to stabilise the ct-DNA structure significantly with changes in the T_m of >15 °C (see ESI†). In general, such large changes were expected as the naphthalimide structure has been well documented as interacting strongly with DNA.15

Circular and linear dichroism (CD) measurements

Circular dichroism (CD) measurements were conducted to evaluate the potential of the Tröger’s bases to effect conformational changes in the double helical structure of ct-DNA, Fig. 9. The CD measurements were carried out using 1, 2, 4 and 5 in which the concentration of ct-DNA (150 µM) was kept constant and that of the Tröger’s base were varied giving rise to a range of P/D ratios (see ESI†). Upon the addition of the relevant Tröger’s base, positive and negative induced signals appeared outside the region of absorption of the ct-DNA (i.e. for the ICT based naphthalimide based transitions) confirming the strong nature of the interactions of such molecules with ct-DNA, as shown for 1, Fig. 9. Moreover, these two transitions corresponded well with the absorption spectra of the Tröger’s bases ICT band upon binding to DNA (cf. Fig. 5).

The nature of the interaction of this series of Tröger’s bases with ct-DNA was further analysed by linear dichroism (LD). In the absence of any Tröger’s bases, a negative LD signal was observed for the DNA, characteristic of B-DNA, due to the nearly perpendicular orientation of the transition moments of DNA bases relative to the DNA helical axis, Fig. 10 (as P/D = 0). However, upon the addition of each of the Tröger’s bases to DNA, both positive and negative signals were observed in the LD spectra within the Tröger’s base absorptions (i.e. for the ICT absorption band between 300–450 nm), while the LD signal for the 260 nm DNA bands was still negative. This is demonstrated in Fig. 10 for 4 at different P/D values, where a positive band is seen at ca. 350 nm and a negative band centred at ca. 400 nm, again, comparing well with that seen in the absorption spectra in Fig. 5, upon binding to DNA.

Analysis of the CD and LD data might suggest that the Tröger’s bases bind to ct-DNA in a bimodal fashion; whereby, one naphthalimide unit acts as a groove binder while the second unit intercalates between the base pairs of DNA. This would support our hypothesis outlined in our discussion above on the changes in the absorption and the emission spectra, respectively. This hypothesis also stems from the fact that in the case of the 3-amino-1,8-naphthalimides precursors 6–10 no induced CD signal was observed for the ICT based naphthalimide transitions, however, a negative LD signal was seen in the ICT based naphthalimide transitions (see ESI†), which would support an intercalative mode of binding for these 3-amino precursors. This we have also seen in our previous work where we have used 4-amino-1,8-naphthalimide analogues as DNA binders, i.e. only a single negative LD band was observed for the ICT transition, no induced CD was observed. Moreover, when using 4-amino-1,8-naphthalimide based Tröger’s bases, only a positive changes have been recorded within for the ICT LD absorption, upon binding to DNA; an indication of groove binding interaction of these

![Fig. 9](image1.png) CD spectra of ct-DNA (150 µM) in 10 mM phosphate buffer in the absence and presence of 1 at P/D ratios of 2.5 (-), 5 (-), 10 (-) and 20 (-).

![Fig. 10](image2.png) The LD spectra of ct-DNA (400 µM) in 10 mM phosphate buffer in the absence and presence of 4 at different P/D ratios. Insert: The reduced LD spectrum at P/D = 10 where 4 is fully bound to DNA.
chiral molecules. Hence, it is clear that the 3-amino-1,8-naphthalimide based Tröger’s bases 1–5 interact differently upon binding to DNA than that seen for either the 3-, or the 4-amino-1,8-naphthalimides and that seen for 4-amino-1,8-naphthalimide Tröger’s bases, which all give support to intercalating binding interactions with DNA. We believe that this can only be due to the different shape of the Tröger’s bases, forced by the substitution pattern on the naphthalimide structures (i.e. 3- vs. 4-amino). To probe this difference further, we obtained the reduced LD spectrum (LD^r) of the 4, shown as an inset in Fig. 10, when recoded at P/D = 10. Here, the LD^r was calculated as −0.093 in the absence of 4 at 260 nm, for the band corresponding to the transitions of DNA bases. However, in the presence of 4, the LD^r values for 340 and 392 nm bands were found to be 0.037 and −0.045, respectively, while the 260 nm band was still negative. It is important to point out that the two naphthalimide moieties of 4 have overlapping absorptions (due to the C_2 symmetry), and hence, a quantitative analysis (such as the determining of the angle α values for both naphthalimide moieties within DNA) is difficult to obtain accurately. While these results don’t exclusively proof our hypothesis of a dual intercalating/groove binding interactions they strongly indicate that 1–5 all bind to DNA in a bi-modal manner.

Molecular modelling

With the aim of investigating the potential relationship between the shape of the Tröger’s base and its DNA binding mode further, the conformations of compound 2 and the corresponding 4-amino analogue were analysed using MM2 molecular mechanics studies in gas-phase. The two minimised energy structures are shown in Fig. 11. It is clear from these two structures (when viewed sideways) that the substitution (3-amino vs. 4-amino) has a major effect on the dihedral angle between the naphthalimide fluorophores. From these studies, dihedral angles of 96° and 72° were determined for the 4-amino-1,8-naphthalimide Tröger’s base analogue and 2, respectively. We have recently managed to obtain a X-ray crystal structure of a 4-amino-1,8-naphthalimide Tröger’s base, which showed a highly ordered packing giving rise to the formation of self-assembly structures that form helical channels. This packing arrangement we believe gives rise to a rather tighter dihedral angle of 82° between the two naphthalimide units; which is somewhat narrower than seen here, but still significantly larger than seen for 2. In fact, viewing the two structures in Fig. 11 face on (e.g. viewing from the plane of the aromatic rings) demonstrates that the 3-amino analogue has a helical twist, which might explain its ability to participate in the dual mode of binding to DNA as demonstrated in the LD experiments. Hence, the substitution pattern (3- vs. 4-amino) has a pivotal role in determining the DNA affinity and DNA binding mode of these molecules; but this new family of compounds showed lesser changes in the absorption and the emission spectra for the ICT transition, than their corresponding 4-amino-1,8-naphthalimide derivatives, which might reflect the smaller dihedral angle. This we have also recently seen in the use of Ru(n) polypyridyl complexes based on the 4-amino-1,8-naphthalimide Tröger’s base structure, where indeed no significant changes were seen in the MLCT emission of these complexes upon binding to DNA, but the structures were shown to greatly stabilise (using DNA denaturation studies) double stranded DNA.

Biological evaluations of 1–5 in HeLa cells

Having investigated the various photophysical properties of 1–5 and their ability to bind to DNA in competitive media, their biological properties were next investigated. It was found that compounds 1–5 (10 µM concentration) were rapidly taken up into HeLa cells as demonstrated in Fig. 12, using confocal fluorescence imaging where the emission from the compounds themselves was monitored. The compounds were shown to localise within the cytosol of HeLa cells (also shown in Fig. 12 are the same cells co-stained with the far-red emitting nuclear stain DRAQ5, and the overlay of both) but after 2 hours of incubation, significant apoptosis was observed, which prevented further analysis. This indicates, significant cytotoxicity for these compounds except compound 3.

The cytotoxicity was confirmed by evaluating their cytotoxic potential after 24 h incubation, in HeLa cells using Alamar blue viability assays; from which EC_{50} values were calculated. Here, 1–2 and 4–5 induced sharp decreases in cell viability with EC_{50} values of 1.14, 2.98, 1.42 and 3.98 µM, respectively, as summarised in Fig. 12. However, in the case of 3, a much higher EC_{50} value of >25 µM was determined. As 3 does not possess a positively charged side chain at pH 7.4 and thus is not expected to interact as strongly with DNA as 1–2 and 4–5 as previously discussed, this may be a potential reason for its reduced cytotoxicity when compared to the other compounds. However further investigations would be required to confirm direct DNA targeting within cells for compounds 1–2 and 4–5.

Similar potent cytotoxicity results were also demonstrated in a second cancer cell line, MCF-7, the results are shown in table in Fig. 12. In addition, the ability of 1–5 to induce apoptosis in HeLa cells was investigated using flow cytometry as demonstrated in Fig. 13a. Such studies were carried out at a range of concentrations and have shown that 1–2 and 4–5 potently induce apoptosis in HeLa cells in the order of 4 > 2 > 1 > 5. As expected, 3 induced minimal apoptosis and proved to
be the least cytotoxic compound in this study, showing that the design of these molecules was well founded.

The most potent compound (as determined by flow cytometry), 4, was also subjected to a time-course analysis at 10 µM as shown in Fig. 13b and demonstrated apoptosis to be rapidly induced after 2 h of treatment, correlating with the confocal microscopy results. These results clearly show the therapeutic possibility of these naphthalimide based Tröger’s base structures.

**Conclusion**

In this article we have presented our results from the synthesis of new 3-amino-1,8-naphthalimide based Tröger’s base structures, 1–5, formed from the corresponding 3-amino-1,8-naphthalimides 6–10. We have demonstrated that these structures show substantially different spectroscopic behaviour to that of the corresponding 4-amino analogues; being both blue shifted in their absorption and the emission spectra. We further demonstrate that their luminescence is highly pH dependent, and that they possess ICT excited state character proven by carrying out solvent dependent luminescent studies. Importantly, we show (by carrying out spectroscopic titrations, observing the changes in both the ICT centred UV-Vis absorption and fluorescence emission spectra) that these structures, bind to ct-DNA with high binding affinity; matching that seen for many transition metal ion complexes, even in the presence of highly competitive ionic background. In comparison to the 4-amino analogues the changes in these spectra were in general overall less dramatic. However, their binding affinities are comparable; which was also demonstrated by using EtBr displacement assays. To share some light on the DNA binding mode of these systems, we also used both CD and LD spectroscopy; the latter indicated that the binding was most

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**Fig. 12** Confocal microscopy and cellular viability EC₅₀ values. The green naphthalimide emission of 1–5 was observed (left hand panels). The nuclear stain DRAQ5 (1,5-bis([2-(di-methylamino) ethyl]amino)-4, 8-dihydroxyanthracene-9,10-dione) was also used (middle panels) as a co-stain (right hand panels).

**Table 1**

<table>
<thead>
<tr>
<th>Compound #</th>
<th>EC₅₀ Value (µM): HeLa</th>
<th>EC₅₀ Value (µM): MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.14</td>
<td>4.36</td>
</tr>
<tr>
<td>2</td>
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<td>6.97</td>
</tr>
<tr>
<td>3</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<td>7.15</td>
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<tr>
<td>5</td>
<td>3.98</td>
<td>12</td>
</tr>
</tbody>
</table>
likely through both groove and intercalation mode. This is indeed different to that seen for the 4-amino analogues where the binding was dominantly shown to occur by intercalation mode.

The ability of structures 1-5 to function as cytotoxic agents was also evaluated in both HeLa and MCF-7 cell lines. The analysis demonstrated that, with the exception of compound 3, these cells rapidly took up all the compounds, and that these were luminescent, as shown using confocal scanning fluorescence microscopy, within these cells. Importantly, these studies also showed that out of these compounds, 1, 2, 4 and 5 were active cytotoxic agents with EC$_{50}$ values ranging between 1.14-12 μM; and that this was highly dependent on the nature of the substituents at the imide side, as compound 3, has throughout these investigations been shown to be a relatively poor DNA binder. Hence, the cationic nature (and hence their ability to be protonated at physiological pH) of these complexes is also important, not only for their ability to bind to DNA, but also in their ability to be taken up by cells. This was further, reflected in the fact that all the active compounds were shown to induce apoptosis; where up to 70% cellular death was observed after 24 h incubation, while 3 was unable to do so.

In summary, we have extended the possibility of using the Tröger’s base unit as a novel and highly efficient binding motive for DNA and we have shown that the incorporation of this moiety into a 3-amino-1,8-naphthalimide chromophore gives rise to fluorescent molecules, that possess excited state properties that are dominantly ICT based, solvent dependent, are taken rapidly up into cancer cells and give rise to programmed cell death within 24 hours. We are currently evaluating their binding modes with DNA and the biological properties of these and related Tröger’s base structures in greater detail.

Experimental details

All reagents and solvents were purchased commercially from Sigma-Aldrich, Fluka, TCI and Apollo Ltd. and unless specified, were used without the need for further purification. Anhydrous solvents were prepared using standard procedures with distillation under argon prior to each use.

All NMR spectra were recorded using a Bruker DPX-400 Avance spectrometer, operating at 400.1 MHz for $^1$H NMR and 100.6 MHz for $^{13}$C NMR, or a Bruker AV-600 spectrometer operating at 600.1 MHz for $^1$H NMR and 150.2 MHz for $^{13}$C NMR. Shifts are referenced relative to internal solvent signals. Electrospray mass spectra were recorded on a Micromass LCT spectrometer, running Mass Lynx NT V 3.4 on a Waters 600 controller connected to a 996 photodiode array detector with HPLC grade methanol or chloroform. High resolution mass spectra were determined by a peak matching method, using leucine Enkephalin, (Tyr-Gly-Gly-Phe-Leu) as the standard reference (m/z = 556.2771). Melting points were determined using an IA9000 digital melting point apparatus. Infrared spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer fitted with a Universal ATR Sampling Accessory. Elemental analysis was conducted at the Microanalytical Laboratory, School of Chemistry and Chemical Biology, University College Dublin. X-Ray diffraction studies were carried out using a Bruker SMART APEX single crystal CD diffractometer.

UV-visible absorption spectra were recorded on a Varian Cary 50 spectrometer. Emission spectra were recorded on a Cary Eclipse Luminescence spectrometer. Linear and circular dichroism spectra were recorded at concentrations corresponding to optical densities of approximately 2.5 and 1.0, respectively, on a J-815 Circular Dichroism Spectropolarimeter equipped with a Linear Dichroism Accessory (LD) or a Jasco J-810-150S CD spectropolarimeter (CD). Thermal denaturation experiments were performed on a thermoelectrically coupled Perkin Elmer LAMBDA 25 UV/Vis Spectrophotometer. Fluorescence lifetime experiments were carried out on a Horiba Scientific Fluorolog – Modular Spectrofluorimeter equipped with Time Correlated Single Photon Counting (TCSPC) capability.

Cell culture

HeLa (cervical cancer) and MCF-7 (breast cancer) cells were grown in Dulbecco’s Modified Eagle Medium (Glutamax) supplemented with 10% fetal bovine serum and 50 μg ml$^{-1}$ penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO$_2$. 

![Graph](image-url)
Confocal microscopy

HeLa cells were seeded at a density of $1 \times 10^5$ cells per well in glass bottom wells and treated with the indicated compounds for up to 2 h. Cells were washed followed by the addition of fresh media and DRAQ5 (red nuclear stain), followed by viewing using an Olympus FV1000 confocal microscope with a 60× oil immersion lens. Image analysis was performed using Fluoview Version 7.1 Software. Compounds were excited by a 405 nm argon laser, emission 500–550 nm, DRAQ5 was excited by a 633 nm red helium–neon laser, emission >650 nm.

Alamar blue viability assay

HeLa and MCF-7 cells were seeded at a density of $5 \times 10^3$ cells per well and $1 \times 10^4$ cells per well respectively, in a 96-well plate and treated with the indicated compounds for 24 h. Alamar blue (20 µl) was then added to each well and incubated at 37 °C for 4 h. Plates were then read on a fluorescence plate reader (SpectraMax Gemini, Molecular Devices) with excitation and emission wavelengths of 544 nm and 590 nm respectively. Experiments were performed in triplicate on three independent days with activity expressed as percentage cell viability compared to vehicle treated controls.

Flow cytometry

HeLa cells were seeded at a density of $1 \times 10^5$ cells per well in a 6-well plate and treated with the indicated compounds for up to 24 h. Samples were then centrifuged at 650g for 5 min and resuspended in 100 µl ice-cold PBS. Ice-cold 70% (v/v) ethanol to 24 h. Samples were then centrifuged at 650g and the cell pellets were resuspended in 100 µl ice-cold PBS. Ice-cold 70% (v/v) ethanol to 24 h. Samples were then centrifuged at 650g, washed with 1 ml PBS and treated with the indicated compounds for up to 24 h. Samples were then centrifuged at 650g and the cell pellets were resuspended in 100 µl ice-cold PBS. Ice-cold 70% (v/v) ethanol to 24 h. Samples were then centrifuged at 650g.

General synthetic procedures

Procedure 1: formation of the bis-1,8-naphthalimide

Tröger’s base derivatives 1–5. A mixture of the relevant 3-amino-1,8-naphthalimide (1 eq.) and paraformaldehyde (1.1 eq.) in neat TFA was stirred at room temperature for 3 hours under an argon atmosphere. The reaction was initially neutralised and further basified using 6 M NaOH. The aqueous solution was extracted several times with CH2Cl2. The organic extracts were combined and the solvent was removed under reduced pressure. The crude product was purified and dried under vacuum.

Synthesis

N-[1-Pyrrolidino-ethyl]-3-amino-1,8-naphthalimide 6. Compound 6 was synthesised using 1-(2-aminoethyl)-pyrrolidine (153 mg, 0.17 mL, 1.3 mmol) according to Procedure 1 and was yielded as a yellow solid (198 mg, 67%) after trituration with CH3OH. m.p. 191–193 °C (ref. 25 195–197 °C); HRMS: 310.1557 ([M + H] +.C 16H18N3O2 requires 310.1556); δH (600 MHz, (CD3)2SO), 8.08 (1H, d, J = 7.1 Hz, Ar-H), 8.04 (1H, d, J = 8.3 Hz, Ar-H), 7.97 (1H, d, J = 2.3 Hz, Ar-H), 7.62 (1H, app, t, Ar-H), 7.29 (1H, d, J = 2.2 Hz, Ar-H), 5.98 (2H, br. s, NH2), 4.17 (2H, t, J = 7.1 Hz, NCH2), 2.67 (2H, t, J = 7.1 Hz, CH2N), 2.54 (4H, s, N(CH2)2), 1.66 (4H, s, (CH2)2O); δC (150 MHz, (CD3)2SO), 163.7, 163.5, 147.8, 133.5, 131.4, 126.9, 125.4, 122.5, 121.7, 120.5, 111.7, 53.7, 53.0, 38.5, 23.1; δN (40.8 MHz, (CH3)2SO), 65.6 (NH2); r<sub>max</sub> (neat sample)/cm<sup>-1</sup> 3406, 3314, 1693, 1653, 1451.

N-[1-Dimethylamino-ethyl]-3-amino-1,8-naphthalimide 7. Compound 7 was synthesised by reacting N,N-dimethylhexylamine (210 mg, 0.32 mL, 2.98 mmol), N1 (455 mg, 2.13 mmol) and triethylamine (431 mg, 0.60 mL, 4.26 mmol) in toluene (100 mL) according to procedure 1, and was yielded as yellow needles (448 mg, 74%) after trituration with CHCl3, m.p. 171–172 °C (ref. 25 169–171 °C); HRMS: 284.1400 ([M + H] +.C 16H18N3O2 requires 284.1399); δH (600 MHz, (CD3)2SO), 8.08 (1H, d, J = 7.2, Ar-H), 8.04 (1H, d, J = 7.9, Ar-H), 7.97 (1H, d, J = 2.3 Hz, Ar-H), 7.63 (1H, app, t, Ar-H), 7.29 (1H, d, J = 2.3, Ar-H), 5.98 (2H, br. s, NH2), 4.14 (2H, t, J = 6.8, NCH2), 2.49 (2H, t, J = 7.2, CH2N), 2.09 (6H, s, N(CH3)2); δC (150 MHz, (CD3)2SO), 163.6, 163.5, 147.8, 133.5, 131.4, 126.9, 125.4, 122.5, 121.7, 120.5, 111.7, 53.7, 53.0, 38.5, 23.1; δN (60.8 MHz, (CH3)2SO), 65.4 (NH2); r<sub>max</sub> (neat sample)/cm<sup>-1</sup> 3406, 3314, 1693, 1653, 1451.

N-[1-Morpholino-ethyl]-3-amino-1,8-naphthalimide 8. Compound 8 was synthesised by reacting 4-(2-aminoethyl)pyrrolidine (119 mg, 0.12 mL, 0.20 mmol) with 11 (140 mg, 0.65 mmol) and triethylamine (132 mg, 0.18 mL, 1.30 mmol) in toluene (100 mL) according to procedure 1, and was yielded as yellow solid (143 mg, 67%) after triturating with CH3OH. m.p. 230–231 °C (ref. 25 228 °C); HRMS: 326.1520 ([M + H] +.C 18H20N3O2 requires 326.1505); δH (600 MHz, (CD3)2SO), 8.08 (1H, d, J = 7.14 Hz, Ar-H), 8.04 (1H, d, J = 7.9 Hz, Ar-H), 7.97 (1H, d, J = 2.3 Hz, Ar-H), 7.62 (1H, app, t, Ar-H), 7.29 (1H, d, J = 2.3 Hz, Ar-H), 5.98 (2H, br. s, NH2), 4.17 (2H, t, J = 7.1, NCH2), 3.54 (4H, s, N(CH2)2), 2.56 (2H, s, CH2N), 2.47 (4H, s, (CH2)2O); δC (150 MHz, (CD3)2SO), 163.7, 163.5, 147.8, 133.5, 131.4, 126.9, 125.4, 122.5, 121.7, 120.5, 111.7, 53.7, 53.0, 38.5, 23.1; δN (60.8 MHz, (CH3)2SO), 65.4 (NH2); r<sub>max</sub> (neat sample)/cm<sup>-1</sup> 3415, 3322, 1692, 1656, 1445.
126.9, 125.4, 122.5, 121.7, 121.7, 120.5, 111.7, 66.2, 59.36; H, 4.84; N, 11.28; HRMS: 687.2931 ([M + H]+).

**C35H34N6O4·1.5CH2Cl2:** C, 60.05; H, 5.11; N, 11.20. Found C, 60.05; H, 5.01; N, 11.28; HRMS: 687.2931 ([M + H]+).

**C39H38N6O6·0.75CH2Cl2:** C, 63.62; H, 5.77; N, 14.73; HRMS: 713.3568 ([M + H]+·C41H43N6O4 requires 713.3564).

**[N-{1-pyrrolidino-ethyl}]-9,18-methano-1,8-naphthalimido-19,23-bis-{1,5-diazocine] 5.** Compound 5 was synthesised according to procedure 2 by stirring 9 (670 mg, 2.07 mmol) with paraformaldehyde (68 mg, 2.28 mmol) in trifluoroacetic acid (4 mL), and yielded as a yellow solid (254 mg, 36%) after purification by trituration with ethyl acetate. m.p. 234–235 °C; HRMS: 683.3332 ([M + H]+·C41H43N6O4 requires 683.3346); δH (600 MHz, CDCl3), 8.45 (2H, d, J = 8.1 Hz, Ar-H), 8.43 (2H, s, Ar-H), 8.03 (2H, d, J = 9.3 Hz, Ar-H), 7.73 (2H, app, t, Ar-H), 5.18 (2H, d, J = 17.3 Hz, diazocine-H), 4.94 (2H, d, J = 17.3 Hz, diazocine-H), 4.58 (2H, s, diazocine-H), 4.31 (4H, m, CD2N); δC (150 MHz, CDCl3), 163.0, 162.2, 161.7, 160.6, 55.1, 54.7, 52.7, 45.7, 37.0; δN (60.8 MHz, (CD3)2SO), 65.4 (NH2); νmax (neat sample)/cm⁻¹ 2955, 2850, 1698, 1655, 1339.

**[N-{1-pyrrolidino-ethyl}]-9,18-methano-1,8-naphthalimido-19,23-bis-{1,5-diazocine] 4.** Compound 4 was synthesised according to procedure 2 by reacting 9 (670 mg, 2.07 mmol) with paraformaldehyde (68 mg, 2.28 mmol) in trifluoroacetic acid (4 mL), and yielded as a yellow solid (254 mg, 36%) after purification by trituration with ethyl acetate. m.p. 234–235 °C; HRMS: 683.3332 ([M + H]+·C41H43N6O4 requires 683.3346); δH (600 MHz, CDCl3), 8.45 (2H, d, J = 8.1 Hz, Ar-H), 8.43 (2H, s, Ar-H), 8.03 (2H, d, J = 9.3 Hz, Ar-H), 7.73 (2H, app, t, Ar-H), 5.18 (2H, d, J = 17.3 Hz, diazocine-H), 4.94 (2H, d, J = 17.3 Hz, diazocine-H), 4.58 (2H, s, diazocine-H), 4.31 (4H, m, CD2N); δC (150 MHz, CDCl3), 163.0, 162.2, 161.7, 160.6, 55.1, 54.7, 52.7, 45.7, 37.0; δN (60.8 MHz, (CD3)2SO), 65.4 (NH2); νmax (neat sample)/cm⁻¹ 2955, 2850, 1698, 1655, 1339.

**Bis-[N-{1-pyrrolidino-ethyl}]-9,18-methano-1,8-naphthalimido-19,23-bis-{1,5-diazocine] 5.** Compound 5 was synthesised according to procedure 2 by stirring 10 (1.32 mg, 3.90 mM) and paraformaldehyde (152 mg 5.07 mM) in trifluoroacetic acid (4 mL) and yielded as an orange solid (852 mg, 61%) after dissolving in CH2Cl2 (2 mL) and precipitating from diethyl ether (50 mL). m.p. 191–192 °C; Calculated for C41H42N6O4·0.8CH2Cl2: C, 64.30; H, 5.89; N, 14.35; Found C, 63.95; H, 5.77; N, 14.73; HRMS: 713.3568 ([M + H]+).

**C41H42N6O4 requires 713.3564; δH (600 MHz, CDCl3), 8.44...
(2H, d, J = 7.3 Hz, Ar-H), 8.42 (2H, s, Ar-H), 8.02 (2H, d, J = 8.5 Hz, Ar-H), 7.73 (2H, app. t, Ar-H), 5.18 (2H, d, J = 17.4 Hz, diazocine-H), 4.93 (2H, d, J = 17.4 Hz, diazocine-H), 4.57 (2H, s, diazocine-H), 4.29 (4H, m, N(CH$_2$)$_2$, CH$_2$N), 2.40 (8H, br. s, (CH$_2$)$_2$N), 2.24 (6H, s, NCH$_3$); $\delta$C (150 MHz, CDCl$_3$), 163.9, 163.7, 146.4, 130.1, 129.7, 129.5, 128.7, 127.7, 127.3, 125.6, 123.1, 122.7, 66.5, 56.1, 55.5, 55.1, 53.2, 45.9, 37.5; $\nu_{\text{max}}$ (neat sample)/cm$^{-1}$ 2935, 2794, 1699, 1598, 1336.

Acknowledgements

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Notes and references


