Some novel fluorescent azo compounds as intercalators for calf thymus DNA

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Two new fluorescent azo compounds, viz. 4-(diazenyl-N-phenyl-carbazolyl) phenol and 4,6-di(diazenyl-N-phenyl-carbazolyl) resorcinol have been synthesized, characterized and their noncovalent interaction studied with calf thymus DNA both in non-ionic and ionic media. Their mode of binding is mainly intercalative.

DNA-ligand interaction has been characterized by intercalation, groove-binding or electrostatic interaction. Ability of some sequences to recognize DNA double helices by chemical modification of DNA or inhibition of DNA binding protein or by triple helix formation via Hoogsteen base pairing has been utilized in cleavage. However, several aspects including DNA sequence and structural specificity, chemical structure and biological activity relationships and the nature of site exclusion still remain largely unclear. The changes induced in the electronic absorption spectra and fluorescence characteristics of ligands upon intercalation have been efficiently used to determine the kinetics of ligand-DNA system and also evidenced by earlier reports from our laboratory on intercalation of substituted naphthalimide with CT-DNA, supercoiled pBR 322 plasmid and synthetic polynucleotides.

We have now chosen substituted carbazole unit, as another promising fluorochrome system. In this paper the synthesis and characterization of two new carbazolyl azo compounds 4-(diazenyl-N-phenyl-carbazolyl) phenol 1 and 4,6-di(diazenyl-N-phenyl-carbazolyl) resorcinol 2 has been reported and their intercalation studies have been carried out with calf thymus DNA both in the absence and in the presence of different strengths of NaCl.

Results and Discussion

The azo compounds 1 and 2 synthesized as outlined in Scheme 1 are planar molecules containing nucleophilic -N=N- and -OH having available electrons for linking. These are highly fluorescent molecules and under appropriate conditions these two ligands could be successfully employed for study of specificity in their intercalative mode of binding to DNA exploiting the enhancement or quenching of fluorescence as a measure of the intensity of binding. Fluorescence intensity of compounds 1 and 2 keeping their concentration constant at 0.05 OD was recorded in different solvents viz. dioxane, aq. amm. acetate buffer, DMF, aq. sodium carbonate, methanol and water whereas for ligand 2 water is the best solvent showing maximum fluorescence followed by aq. sod. carbonate (Table I) for ligand 1 the best solvent is DMF. Both the compounds showed least fluorescence in dioxane which is found to be least useful solvent (Figures 1 & 2). The results along with wavelengths for excitation and emission are given in Table I.

Interaction of the compounds 1 and 2 with CT-DNA was carried out in the absence and in the presence of different strengths of NaCl (0.1 M, 0.2 M and 0.5 M). Since fluorescence was used as a parameter for assessing the extent of binding, the fluorescence quenching or enhancement was taken to be proportional to the extent of binding.

In the absence of NaCl, a sharp quenching of fluorescence was observed at all the DNA/ligand ratios of 0.5:1, 1:1, 1.5:1 and 2:1 respectively for both the compounds (Figures 3-6, Table II).

This significant quenching may be attributed to the slipping in of the ligand between the base pairs thus causing restricted mobility of the ligand. In both the cases there is initial quenching when ligand is mixed with CT-DNA and there is gradual decrease in the
extent of quenching with increasing concentration of the later. This can be explained by the fact that the increased concentration of CT-DNA inhibits access of ligand to slip into the spaces between bases which are concentrated on the surface. The conformational alterations of DNA may also contribute to this phenomenon. However, the intensity of quenching is relatively very high in case of 2 as compared to 1 which obviously indicates higher binding in case of 2 because in this compound there is more surface for intercalation and more sites for binding. It may intercalate between two different base pairs instead of one (as in case of compound 1) with proper spacing and folding of molecules. The addition of NaCl in both the cases results in enhancement of fluorescence which gradually increases with increasing concentration of NaCl. However, the relative enhancement in case of 2 is more significant than 1. Addition of salt is obviously having a denaturing effect and opening of the helical structure exposes more ligand resulting in fluorescence enhancement. However, this enhanced fluorescence intensity of the DNA-ligand conjugate in the presence of NaCl still remains less than that of the free intercalator except in the case where the ratio of DNA is twice than that of the ligand. In this case the fluorescence intensity of the conjugate is much greater than even that of the free ligand. This again points out to a different mode of binding of the ligand to DNA at this concentration.

It is a common observation that the intercalative binding to DNA is diminished by raising the ionic strength of the medium. The effect of ions is probably attributable to several factors related to influence of electrostatic interaction between...
Table I—Relative fluorescence emission of compounds (1) and (2) in different solvents.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Dioxane</th>
<th>Amm. acetate buffer</th>
<th>DMF</th>
<th>aq. Sod. carbonate</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\lambda_{em}$</td>
<td>368</td>
<td>374</td>
<td>370</td>
<td>340</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>$\lambda_{ex}$</td>
<td>373</td>
<td>378</td>
<td>405</td>
<td>345</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>$\gamma_r$</td>
<td>38</td>
<td>111</td>
<td>185</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>$\lambda_{em}$</td>
<td>438</td>
<td>445</td>
<td>430</td>
<td>410</td>
<td>432</td>
</tr>
<tr>
<td></td>
<td>$\lambda_{ex}$</td>
<td>446</td>
<td>450</td>
<td>435</td>
<td>415</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>$\gamma_r$</td>
<td>21</td>
<td>20</td>
<td>48</td>
<td>53</td>
<td>24</td>
</tr>
</tbody>
</table>

Table II—Relative fluorescence of CT-DNA-ligand conjugates at different molar ratios {for ligand 1 $\gamma_r$ 150 and for ligand 2 $\gamma_r$ 95 at 0.06 OD}.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Molar ratios</th>
<th>0.5:1</th>
<th>1:1</th>
<th>1.5:1</th>
<th>2:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>CT-DNA-ligand conjugate</td>
<td>100</td>
<td>99</td>
<td>69</td>
<td>81</td>
</tr>
<tr>
<td>(ii)</td>
<td>in 0.1M NaCl</td>
<td>110</td>
<td>113</td>
<td>116</td>
<td>175</td>
</tr>
<tr>
<td>(iii)</td>
<td>in 0.2M NaCl</td>
<td>125</td>
<td>129</td>
<td>136</td>
<td>182</td>
</tr>
<tr>
<td>(iv)</td>
<td>in 0.5M NaCl</td>
<td>135</td>
<td>135</td>
<td>140</td>
<td>194</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i)</td>
<td>CT-DNA-ligand conjugate</td>
<td>17</td>
<td>21</td>
<td>27</td>
<td>52</td>
</tr>
<tr>
<td>(ii)</td>
<td>in 0.1M NaCl</td>
<td>38</td>
<td>48</td>
<td>55</td>
<td>109</td>
</tr>
<tr>
<td>(iii)</td>
<td>in 0.2M NaCl</td>
<td>45</td>
<td>60</td>
<td>64</td>
<td>164</td>
</tr>
<tr>
<td>(iv)</td>
<td>in 0.5M NaCl</td>
<td>47</td>
<td>66</td>
<td>74</td>
<td>174</td>
</tr>
</tbody>
</table>

Figure 1 — Comparative fluorescence spectra of 4-(diazenyl-N-phenyl-carbazolyl) phenol in (i) DMF ($\lambda_{ex}$ 370, $\lambda_{em}$ 405), (ii) aq. sodium carbonate ($\lambda_{ex}$ 340, $\lambda_{em}$ 345), (iii) methanol ($\lambda_{ex}$ 370, $\lambda_{em}$ 375), (iv) aq. amm. acetate buffer ($\lambda_{ex}$ 374, $\lambda_{em}$ 378), (v) dioxane ($\lambda_{ex}$ 368, $\lambda_{em}$ 373), (vi) water ($\lambda_{ex}$ 340, $\lambda_{em}$ 346).
Figure 2 — Comparative fluorescence spectra of 4,6-di(diazenyl-N-phenyl-carbazolyl) resorcinol in (i) dioxane ($\lambda_{ex}$ 438, $\lambda_{em}$ 446), (ii) aq. amm. acetate buffer ($\lambda_{ex}$ 445, $\lambda_{em}$ 450), (iii) DMF ($\lambda_{ex}$ 430, $\lambda_{em}$ 435), (iv) aq. sod. carbonate ($\lambda_{ex}$ 410, $\lambda_{em}$ 415), (v) methanol ($\lambda_{ex}$ 432, $\lambda_{em}$ 440), (vi) water ($\lambda_{ex}$ 400, $\lambda_{em}$ 415).

Negatively charged phosphates of DNA backbone. The interactions are influenced by conformational alterations induced in the helix by binding process and would be sensitive to qualitative changes in the character of the interaction at variable ionic strengths. In addition, the increase in ionic strength can also effect ligand interaction by decreasing water activity around the double helix which is the major factor stabilizing double helical conformation of DNA.

Materials and Methods

All melting points reported are uncorrected, UV absorption and emission spectra were recorded on Hitachi 220 S Spectrophotometer and emission spectra on Kontron SFM 25 spectrofluorometer; $^1$H NMR spectra (chemical shifts in $\delta$, ppm) on Brooker AMX 500 and IR spectra on Perkin-Elmer spectrophotometer. The silica gel column was used for column chromatography. HPLC was done on LKB-DBF Pharmacia using RPC C18 column. Methanol, nitrobenzene, hexane, phenol and resorcinol were purchased from E. Merck India Ltd., Bombay. CT-DNA was obtained from Astra Research Centre, Bangalore. Water used was triple distilled and autoclaved. All the glasswares were also autoclaved prior to their use. The stock solutions of the CT-DNA and the compounds were prepared in Tris-EDTA buffer (pH 8.0). p-Iodonitrobenzene was prepared by established procedure.

$N$-(4-Nitrophenyl) carbazole. Carbazole (1g; 0.005 mole), p-iodo-nitrobenzene (1.8g; 0.0075 mole), nitrobenzene (60 mL), anhyd. potassium carbonate (1.03g; 0.0075 mole) and copper powder (0.030g) were vigorously refluxed for 12 hr. After cooling, the reaction mixture was steam distilled to remove nitrobenzene and then filtered. The residue obtained was column chromatographed (eluant, hexane). Yield 61%, m p 185°C. Rf 0.5 (Hex:Bz:6:4 v/v); UV(MeOH): $\lambda_{max}$ 280, 380 and $\lambda_{max}$ 300 nm; $^1$H NMR (CDCl3): 8.5 (d, 4H, Ar-H), 8.15 (d, 4H, Ar-H), 7.8 (d, 2H, Ar-H), 7.3-7.6 (m, 2H, Ar-
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Figure 3 — Relative fluorescence emission values of 4-(diazenyl-N-phenyl-carbazolyl) phenol (I) (λ<sub>ex</sub> 374, λ<sub>em</sub> 378) at CT-DNA ligand ratio 0.5:1 (i) free ligand (ii) DNA-ligand conjugate (iii) DNA-ligand conjugate with 0.1 M NaCl (iv) DNA-ligand conjugate with 0.2 M NaCl (v) DNA-ligand conjugate with 0.5 M NaCl.

\[
\begin{align*}
H), \ 5.3-5.8 (m, Ar-NH_2). \text{ Anal. Caled: C, 71.42; N, 11.11; H, 4.76. Found: C, 70.2; N, 10.85; H, 4.54%.}
\end{align*}
\]

\[N-(4-\text{aminophenyl}) \text{ carbazole.} \]

\[N-(4-\text{nitrophenyl}) \text{ carbazole (0.500 g; 0.001 mole) was dissolved in hot methanol (2 mL). To this solution tin pellets were added and concentrated HCl (3 mL) was added gradually with stirring. The solution was gently refluxed for 2hr. The mixture was filtered and evaporated to dryness under vacuum and crude solid was dissolved in methanol. The solution was made alkaline and then filtered. Brown product was purified by column chromatography (eluant, DCM-methanol). Yield 48%, R<sub>f</sub> 0.5 (Bz:Hex::8:2 v/v); UV(MeOH): λ<sub>max</sub> 290 and λ<sub>min</sub> 305; IR (KBr): 3500 (N-H; str.), 1600 cm<sup>-1</sup> (C-N; str.), <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.2-7.6 (m, 12H, Ar-H), 5.3-5.8 (m, Ar-NH<sub>2</sub>). Anal. Caled: C, 81.08; N, 12.61; H, 6.31. Found: C, 80.2; N, 11.52; H, 6.1%.]

\[4-(\text{Diazenyl-N-carbazolyl}) \text{ phenol 1.} \]

\[N-(4-\text{Amino-phenyl}) \text{ carbazole (0.100 g; 0.00015 mole) was taken in conec. HCl (1.5 mL) and water (1 mL) and the solution was stirred in a bath of crushed ice. The temp. was maintained at 5°C. Cold aq. solution of NaN<sub>3</sub> (0.002 g; 0.0003 mole) was added to this solution. To this cold diazonium salt solution an ice cold solution of phenol (0.015 g; 0.00015 mole) in 10% NaOH (1 mL) was added very slowly. The mixture was allowed to stand for about 1 hr and then filtered. The crude product was purified by column chromatography (eluant, DCM). Yield 55%, m.p 295°C, R<sub>f</sub> 0.3 (DCM:Hex::5:5 v/v); UV(MeOH): λ<sub>max</sub> 260, 380 and λ<sub>min</sub> 320; <sup>1</sup>H NMR (CDCl<sub>3</sub>): 7.8-8.0 (d, 2H, Ar-H), 7.75 (s, 1H, Ar-H), 7.2-7.5 (m, 8H, Ar-H). HPLC retention time 4.2 min (C<sub>18</sub> RP C, MeOH). Its fluorescence was recorded in dioxane, amm. acetate buffer, DMF, aq. sodium carbonate, methanol and water at 0.05 OD concentration (see Table I and Figure 1). Anal. Caled: C, 76.19; N, 13.35; H, 5.39. Found: C, 75.2; N, 13.2; H, 4.8%.]

\[4,6-\text{Di-(diazenyl-N-phenyl-carbazolyl) resorcinol (2).} \]

Compound 2 was prepared similarly as compound 1 above by adding cold solution of
resorcinol (0.020 g; 0.0002 mole) to the diazonium salt solution. Crude product was purified by column chromatography (eluants, DCM). Yield 57%, m.p. 155°C, Rf 0.5 (DCM:Hex:6:4 v/v) HPLC retention time 4.0 min (C18 RPC, MeOH); UV(MeOH): \( \lambda_{\text{max}} \) 285, 500 and \( \lambda_{\text{em}} \) 420; \( ^1 \text{H NMR (CDCl}_3 \): 8.1-8.5 (d, 1H, Ar-H), 7.85-7.95 (d, 1H, Ar-H), 7.6-7.75 (d, 8H, Ar-H), 7.2-7.5 (m, 16H, Ar-H). Its comparative fluorescence was recorded in different solvents i.e. dioxane, amm. acetate buffer, DMF, aq. sodium carbonate, methanol and water at 0.05 OD concentration (see Table I and Figure 2). Anal. Calcd: C, 74.46; N, 14.89; H, 4.96. Found: C, 74.00; N, 14.2; H, 4.54%.

The stock solutions of the CT-DNA and the azo compounds 1 and 2 were prepared in Tris-EDTA buffer (pH 8.0). The buffer containing 10 mm Tris and EDTA was prepared by dissolving 0.24 g Tris in 200 mL water containing 400 μl (0.5 M) EDTA solution (pH was adjusted to 8.0). The desired concentrations were obtained by further diluting the stock solutions of DNA and the azo compounds. The concentration of both the ligands were kept constant at 0.05 OD in all the experimental sets. Observations were made by varying the concentration of DNA 0.025, 0.05, 0.075 and 0.01 OD with the DNA/ligand ratio of 0.5:1, 1:1, 1.5:1 and 2:1 respectively. Excitation wavelength for ligand 1 was 374 nm while for ligand 2 it was 425 nm and emission wavelengths were found to be 378 and 432 nm respectively.

To each of these sets of 0.1, 0.2 and 0.5 M solutions of NaCl were added one at a time and subsequently fluorescence measurements were carried out. The results are reported in Table II.

From all these studies, a quantitative relation can be worked out between intercalative binding and fluorescence intensity changes.

**Acknowledgement**

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Figure 5 — Relative fluorescence emission values of 4,6-di(diazenyl-N-phenyl-carbazolyl) resorcinol (II) ($\lambda_{ex}$ 445, $\lambda_{em}$ 450) at CT-DNA/ligand ratio 0.5:1 (i) free ligand (ii) DNA-ligand conjugate (iii) DNA-ligand conjugate with 0.1 M NaCl (iv) DNA-ligand conjugate with 0.2 M NaCl (v) DNA-ligand conjugate with 0.5 M NaCl.

Figure 6 — Relative fluorescence emission values of 4,6-di(diazenyl-N-phenyl-carbazolyl) resorcinol (II) ($\lambda_{ex}$ 445, $\lambda_{em}$ 450) at CT-DNA/ligand ratio 2:1 (i) free ligand (ii) DNA-ligand conjugate (iii) DNA-ligand conjugate with 0.1 M NaCl (iv) DNA-ligand conjugate with 0.2 M NaCl (v) DNA-ligand conjugate with 0.5 M NaCl.
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