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Metal-Ion-Mediated Tuning of Duplex DNA Binding by Bis(2-(2-pyridyl)-1*H*-benzimidazole)

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Abstract: Studies of double-stranded-DNA binding have been performed with three isomeric bis(2-(*n*-pyridyl)-1*H*-benzimidazole)s ($n=2$, 3, 4). Like the well-known Hoechst 33258, which is a bisbenzimidazole compound, these three isomers bind to the minor groove of duplex DNA. DNA binding by the three isomers was investigated in the presence of the divalent metal ions Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. Ligand–DNA interactions were probed with fluorescence and circular dichroism spectroscopy. These studies revealed that the binding of the 2-pyridyl derivative to DNA is dramatically reduced in the presence of Co^{2+}, Ni^{2+}, and Cu^{2+} ions and is abolished completely at a ligand/metal-cation ratio of 1:1. Control experiments done with the isomeric 3- and 4-pyridyl derivatives showed that their binding to DNA is unaffected by the aforementioned transition-metal ions. The ability of 2-(2-pyridyl)benzimidazole to chelate metal ions and the conformational changes of the ligand associated with ion chelation probably led to such unusual binding results for the *ortho* isomer. The addition of ethylenediaminetetraacetic acid (EDTA) reversed the effects completely.

Keywords: benzimidazoles · chelates · divalent metal ions · DNA · fluorescence

Introduction

The benzimidazole moiety is structurally related to purine bases and is found in several biologically relevant natural compounds such as vitamin B_{12}. Benzimidazole derivatives manifest a variety of pharmacological effects, which include antitumoral,[1,2] antiparasitic,[1] and antihelminthic[3] activity. Additionally, this unit is the key building block for a variety of derivatives that show high-affinity sequence-selective binding to double-stranded (ds) DNA.[4,5]

Hoechst 33258, a bisbenzimidazole derivative that binds to the adenine/thymine (AT)-rich minor grooves of ds B-DNA,[4,5] has emerged as a model for DNA-targeted drug design. This molecule has the essential attributes of a typical DNA groove binder, namely, an overall crescent shape to match the helical pitch of the minor groove and suitable hydrogen-bond donors provided by the benzimidazole protons.[6] These DNA-binding benzimidazole derivatives have the ability to modulate gene expression by inhibiting the action of many regulatory proteins, including helicase,[7] transcription factors,[8] and topoisomerases,[9] often by binding to their target sites on DNA. Hence, these molecules have been the focus of extensive research in the arena of medicinal chemistry.

Several analogues of Hoechst 33258 have been synthesized over the years to enhance its functional properties.[10,11] Neidle and co-workers reported the first examples of symmetric head-to-head bisbenzimidazole derivatives.[12,13] Based on the same scaffold, we have designed a series of symmetrical bis(2-(*n*-pyridyl)-1*H*-benzimidazoles) ($n=2$, 3, 4), abbreviated as 2-, 3-, and 4-pyrben, respectively. These derivatives were shown to bind in the minor groove of ds DNA. However, the binding of 2-pyrben to duplex DNA is weaker than that of its isomers. The theoretical basis for this differential DNA interaction has been discussed in an earlier report.[14]

Herein we report a unique observation for 2-pyrben. The otherwise weak ds DNA binding shown by this compound could be completely "switched off" in the presence of metal cations, and this effect was totally reversed by the addition of ethylenediaminetetraacetic acid (EDTA). Similar experiments done with the isomeric 3- and 4-pyridyl derivatives

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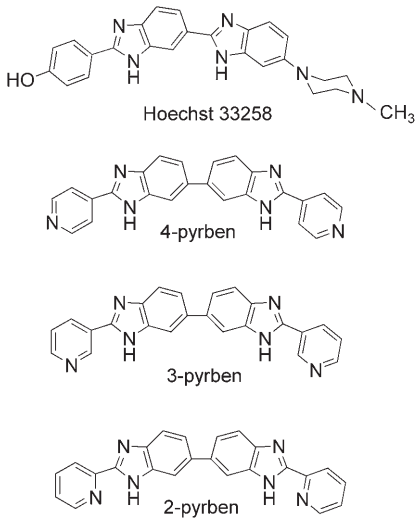
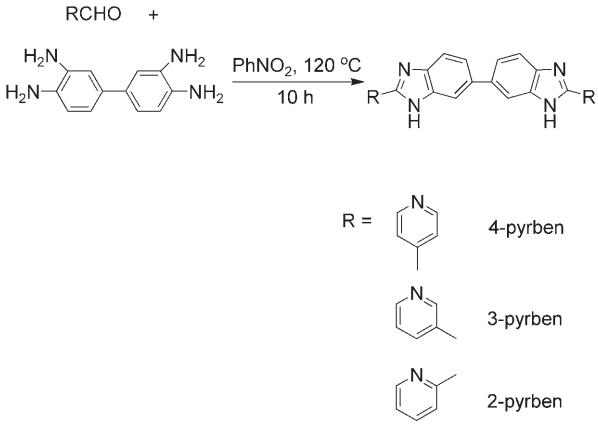
showed that their binding to duplex DNA was unaffected by the aforementioned metal ions.

2-Pyrben is a dimer in which two 2-(2-pyridyl)benzimidazole moieties are connected by a phenyl–phenyl bond. 2-(2-Pyridyl)benzimidazole itself is a well-known bidentate metal chelator, able to chelate a variety of metal ions through its two N centers, one on the pyridine ring and the other on the benzimidazole ring.[15–17] Metal complexes of benzimidazole derivatives have triggered interest because of their widespread biological applications. Transition-metal complexes of 2-substituted benzimidazole ligands have been found to act as antitumoral[18] and antiviral[19] agents. They have also been studied in relation to their interaction with nucleic acids.[20,21] It is known that metal ions beyond a threshold concentration often trigger structural transitions in the secondary structure of DNA.[22]

In the present study, ds DNA binding by the three isomers was probed in the presence of divalent metal cations (Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}). Our investigations reveal for the first time the unique metal-ion-mediated tunability of DNA binding exhibited by one of the isomers studied.



Scheme 1. Structure of Hoechst 33258 and bis(2-(*n*-pyridyl)-1*H*-benzimidazole) ($n=2, 3, 4$).



Scheme 2. Synthesis of bis(2-(*n*-pyridyl)benzimidazole) ($n=2, 3, 4$).

Results

The similarity of these molecules to the parent Hoechst 33258 molecule lies in the nature of the bisbenzimidazole core unit. On the other hand, the features that distinguish them from Hoechst are many. Unlike Hoechst, they are symmetrical, with the benzimidazole units linked by the phenyl–phenyl bond. Also, instead of a piperazine unit (an sp^3-hybridized secondary amine), which gives a net positive charge at physiological pH (≈ 7),[4,5] these molecules bear aromatic pyridine moieties (sp^2-hybridized basic N) at the two termini and, hence, are partially protonated at pH 7. The molecules are isomeric, differing only in the position of the nitrogen atoms on the terminal pyridine rings (Scheme 1).

A common synthetic path was followed for the synthesis of the three isomers. The requisite 2-, 3-, or 4-pyridinecarboxaldehyde was dissolved in dry nitrobenzene, and half an equivalent of 3,3′-diaminobenzidine was added. The solution was heated at 120 °C for 8–10 h. Precipitation with hexane followed by column chromatography afforded the final compounds in 65–70 % yield (Scheme 2).

The inherent emission behavior of the molecules were examined in a range of solvents that included polar solvents such as water (at pH 7), methanol, and *n*-propanol, as well as aprotic solvents such as dioxane and nonpolar toluene. Like Hoechst 33258, all three isomers showed minimal fluorescence in neutral aqueous medium.[23] The emission intensity increased with decreasing solvent polarity accompanied by a blue shift in the emission maxima indicative of an increasingly hydrophobic environment.[24] This became apparent when the emission characteristics of the compounds in buffer or other protic solvents such as methanol were compared with that in aprotic solvents such as dioxane or toluene (Figure 1). It was also observed that the emission intensity of free 2-pyrben was higher than its isomers in all solvents.

Next, we wanted to investigate the effects of metal cations on the emission spectra of the free ligand. The solutions of the salts, $MgSO_4{\cdot}7H_2O$, $NiSO_4{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, and $ZnCl_2.2H_2O$ were added separately to solutions of the individual ligands in water/methanol (3:1). All the compounds were fluorescent in methanol/water in the absence of metal ions. However, Ni^{2+} and Cu^{2+} very efficiently quenched the fluorescence of 2-pyrben. Zn^{2+} was less effective, whereas Mg^{2+} had no significant effect on the emission of the free ligand (Figure 2). None of the above metal ions, however, significantly affected the fluorescence characteristics of 4- or 3-pyrben (see Supporting Information, Figure S1). Hence, the transition-metal ions Ni^{2+} and Cu^{2+} quenched the fluorescence of 2-pyrben, presumably by complexation with the ligand.

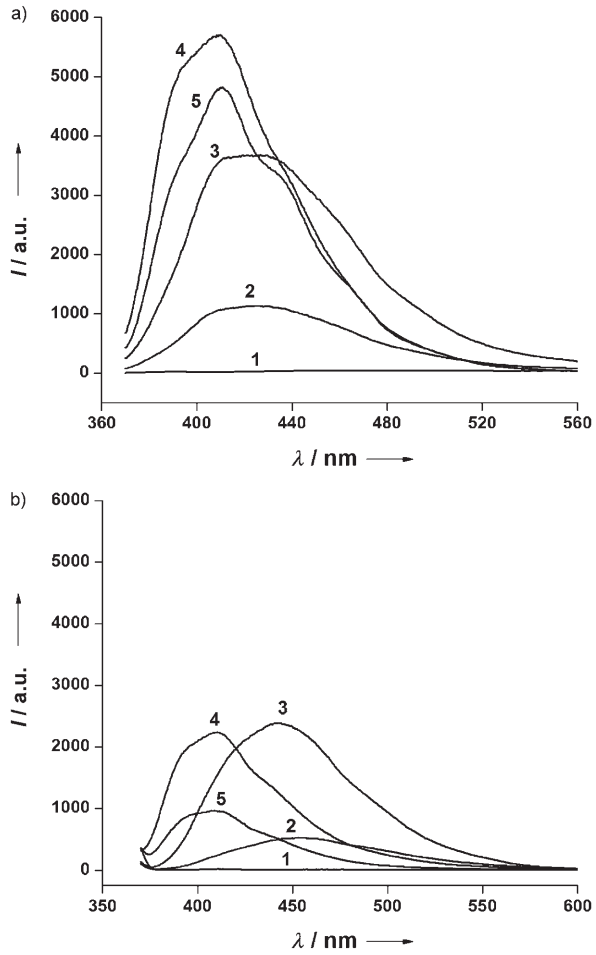


Figure 1. Emission behavior of a) 2-pyrben and b) 4-pyrben in solvents of different polarities. 1 = Tris/HCl buffer (pH 7.4), 2 = methanol, 3 = *n*-propanol, 4 = dioxane, 5 = toluene. Concentrations of compounds were 0.6 μM in solutions of dioxane and toluene and 3.0 μM in solutions of methanol, *n*-propanol, and tris/HCl buffer.

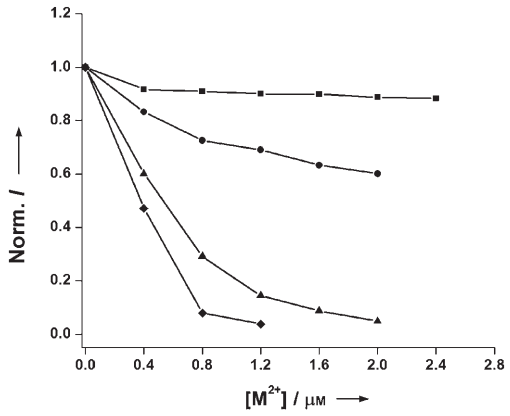


Figure 2. Emission intensity of 2-pyrben (2 μM) in H_2O/MeOH (3:1) with increasing metal-ion (M^{2+}) concentration. ■ = Mg^{2+}, ● = Zn^{2+}, ▲ = Ni^{2+}, ◇ = Cu^{2+}.

The bisbenzimidazole class of dyes such as Hoechst 33258 are nonfluorescent in aqueous solution at neutral pH, but on binding to ds DNA show a remarkable enhancement of fluorescence intensity, with quantum yield increasing by about 20–30-fold. This fluorescence increase upon binding to ds DNA may be attributed to exclusion of solvent water molecules or restricted internal mobility of the fluorophore upon binding to the minor groove of double-helical DNA.[23] All the ligands were minimally fluorescent in buffer at pH 7 but showed a remarkable emission enhancement on binding to ds DNA, thus implying a hydrophobic environment in the DNA minor groove (Figure 3).[23,24] However, the enhancements in emission intensity on DNA addition were different

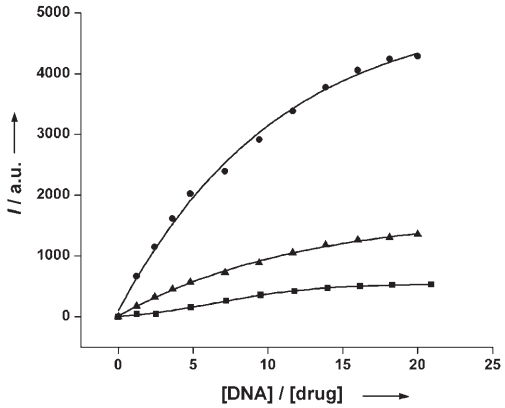


Figure 3. Fluorescence intensity of 3-pyrben (●), 4-pyrben (▲), and 2-pyrben (■) plotted with respect to increasing [CT DNA]/[drug] ratio.

for the three isomers. Whereas 3-pyrben showed maximum increase in fluorescence intensity, 2-pyrben showed the least. 4-Pyrben, on the other hand, exhibited an intermediate increase. The intrinsically similar emission behavior of the compounds (in solvents of different polarities) indicates that the low fluorescence enhancement of the *ortho* isomer on DNA addition (with respect to its *meta* and *para* analogues) arose mainly due to weak DNA-binding interactions and not its inherently low emission. Calculation of binding constants showed that the binding of 2-pyrben to DNA was indeed the weakest (Table 1).

Table 1. Association constants (K) for drug–DNA binding.[a]

Compound	K [10^7 M^{-1}]
4-pyrben	10.1 ± 0.8
3-pyrben	9.1 ± 0.4
2-pyrben	2.2 ± 0.6

[a] Association constants calculated for binding to CT DNA. Results are the average of two experiments.

Next, we examined DNA binding in the presence of divalent metal cations. We added incrementally calf thymus (CT) DNA to a 1 μM solution of ligand in buffer containing different concentrations (0.05, 0.1, 0.5, or 1 μM) of Cu^{2+}, Zn^{2+}, or Mg^{2+} cations. With increasing metal-ion concentration (both Cu^{2+} and Mg^{2+}), the magnitude of fluorescence enhancement on DNA addition decreased for 2-pyrben,

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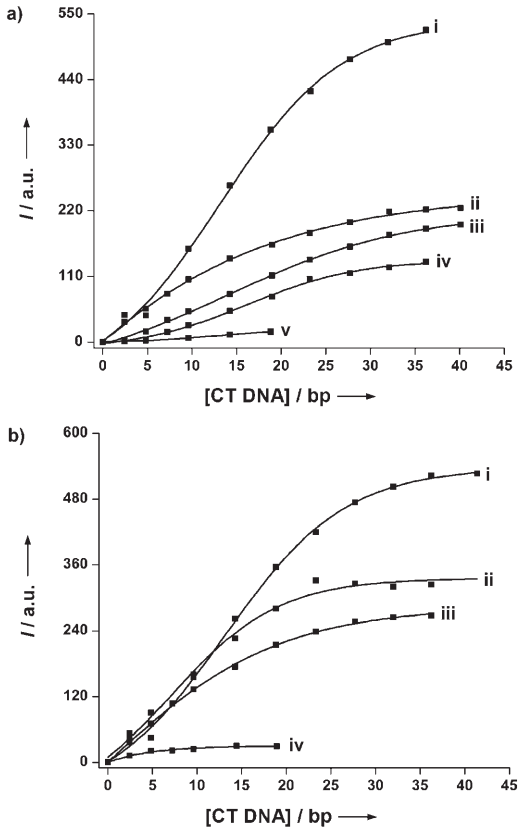

Figure 4. Fluorescence titration of 2-pyrben (1 μM) with CT DNA in the presence of a) Cu^{2+} at concentrations of 0.0 (i), 0.05 (ii), 0.10 (iii), 0.50 (iv), and 1.0 μM (v), and b) Mg^{2+} at concentrations of 0.0 (i), 0.1 (ii), 0.50 (iii), and 1.0 μM (iv). Emission intensity at 440 nm plotted versus DNA concentration.

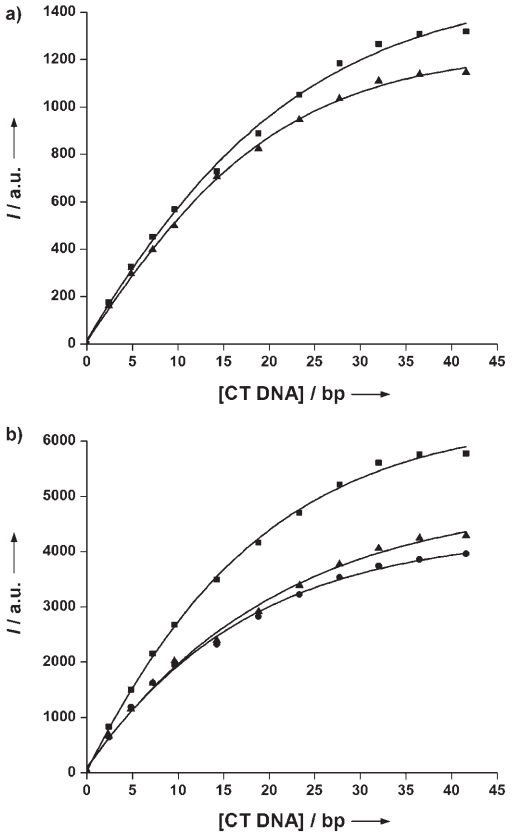

Figure 5. Fluorescence titration of 1 μM of a) 4-pyrben (λ_{em} = 444 nm) and b) 3-pyrben (λ_{em} = 420 nm) with CT DNA in the absence of divalent cation (■) and in the presence of 1 μM Cu^{2+} (▲) and 1 μM Mg^{2+} (●).

until finally, at 1 μM of M^{2+}, there was no fluorescence enhancement on DNA addition (Figure 4).

Fluorescence quenching by divalent copper has been already shown, but what was surprising was the quenching in the presence of Mg^{2+}. This observation suggested that the decreased fluorescence intensity of 2-pyrben on DNA addition, in the presence of metal ions, was not merely an outcome of quenching effects. It probably also signifies reduced DNA binding for 2-pyrben on metal coordination. However, when the DNA–ligand–M^{2+} solution was incubated for 24 h, nearly complete recovery of fluorescence intensity was observed in the presence of Mg^{2+}. This suggests that Mg^{2+} coordination by the ligand was not too efficient and preferential binding to DNA took place on incubation with the latter for 24 h. Such metal-ion-induced effects on DNA binding were not observed for 3- and 4-pyrben (Figure 5 a and b). In the presence of 1 μM metal ion (Cu^{2+} or Mg^{2+}), no fluorescence enhancement was observed for 2-pyrben on DNA addition; however, for 4- or 3-pyrben, even at that metal-ion concentration, there was only a slight lowering of emission intensity. This indicates little or no effect of metal ion on DNA binding of the *para* and *meta* pyridine derivatives.

Next, the reverse titration was done for 2-pyrben, in which the ligand in buffer was first saturated with DNA, and then the metal salts were added allowing a 10-min equilibration. Neither Cu^{2+} nor Mg^{2+} caused any attenuation of emission intensity of the ligand already complexed to DNA (not shown).

As the metal cations, especially Cu^{2+}, Ni^{2+}, and Co^{2+}, also had a pronounced effect on the emission intensity of the free 2-pyrben, it was difficult to investigate accurately by fluorescence spectroscopy the effect of these metal ions on ligand–DNA binding interactions. For a better understanding, we studied the phenomena by circular dichroism spectroscopy.

CD spectroscopy provides a powerful tool for the detection and characterization of the complexes of DNA duplexes with ligands.[25] Neither the duplex DNA nor the ligand alone exhibited CD signals at the wavelengths of absorption of the ligands. However, the intrinsically achiral molecules acquired an induced CD (ICD) on complexing with the chiral polynucleotide.[26] To investigate ligand–DNA interaction by CD spectroscopy, the polynucleotide $[poly(dA\text{-}dT)]_2$ was incubated with the ligands for 24 h prior to recording. The positive Cotton effect observed at about 350 nm indicates strong ligand–DNA interactions (see Supporting Information, Figure S2).

Next, a 1:1 solution of ligand/M^{2+} (3 μM each) was incubated with $[poly\ (dA\text{-}dT)]_2$ (12 μM) for 24 h, in buffer with-

out EDTA, at room temperature before CD spectra were recorded. Whereas 4- and 3-pyrben showed virtually unchanged ICD signals at 350 nm in the presence and absence of divalent metal salts (see Supporting Information, Figure S3), 2-pyrben exhibited dramatically different behavior (Figure 6a). In the presence of Mg^{2+}, drug–DNA incubation

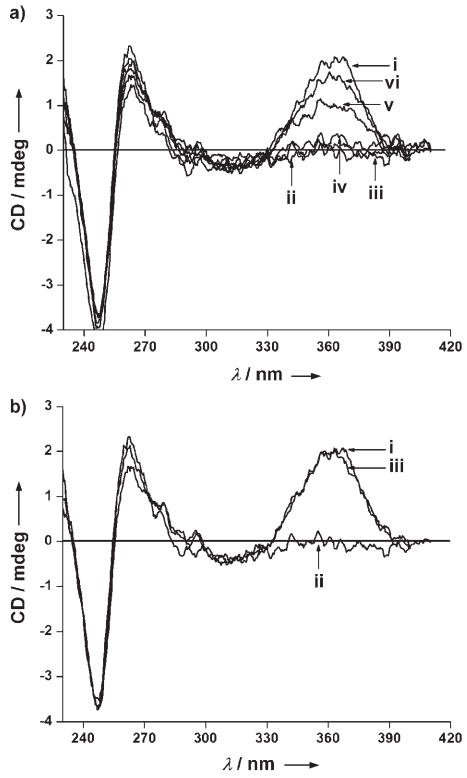

Figure 6. a) CD spectra of $[poly(dA\text{-}dT)]_2$+2-pyrben (i), $[poly(dA\text{-}dT)]_2$+2-pyrben+Co^{2+} (ii), $[poly(dA\text{-}dT)]_2$+2-pyrben+Ni^{2+} (iii), $[poly(dA\text{-}dT)]_2$+2-pyrben+Cu^{2+} (iv), $[poly(dA\text{-}dT)]_2$+2-pyrben+Zn^{2+} (v), and $[poly(dA\text{-}dT)]_2$+2-pyrben+Mg^{2+} (vi) incubated for 24 h prior to measurement. b) CD spectra of $[poly(dA\text{-}dT)]_2$+2-pyrben (i), $[poly(dA\text{-}dT)]_2$+2-pyrben+Cu^{2+} (ii), and $[poly(dA\text{-}dT)]_2$+2-pyrben+Cu^{2+}+EDTA (10 μM) (iii) incubated for 24 h prior to measurement. Trace iii shows the recovery of the 2-pyrben ICD signal upon incubation with EDTA.

for 24 h produced a nearly unchanged ICD signature for the ligand; with Zn^{2+} there was a minor reduction of the same, but in the presence of Co^{2+}, Cu^{2+}, and Ni^{2+}, the ligand ICD at 350 nm was totally abolished (Figure 6a). We then incubated the DNA–drug–M^{2+} ($M^{2+}=Co^{2+}$, Cu^{2+}, Ni^{2+}) solution along with EDTA (10 μM), again for 24 h, and observed complete recovery of the ICD signature of the ligand (Figure 6b, trace iii). The unchanged ICD signals for 4- and 3-pyrben proves that the divalent cations used in the study had no effect on their binding interactions with the minor groove of ds DNA. These data concurred with the fluorescence titration results. However, for 2-pyrben, the cations Co^{2+}, Ni^{2+}, and Cu^{2+} were found to inhibit its DNA binding drastically. The reduced DNA binding presumably arose from the ability of the ligand to chelate a wide array of transition-metal ions. Incubation with EDTA completely reversed the effects, and full recovery of the ICD signal was obtained.

We carried out thermal denaturation of duplex DNA by following the 260-nm DNA-absorption band with respect to temperature.[27] 2-Pyrben caused very weak stabilization of duplex DNA even in the absence of metal ions ($\Delta T_m \approx 0.5$–$0.7\,^{\circ}C$). The presence of metal ions did not significantly alter the ability of the ligand to stabilize the DNA double helix (see Supporting Information, Figure S4). The thermal denaturation of ds DNA was also carried out in the presence of divalent metal salts in the absence of the ligands (see Supporting Information, Figure S5). The helix-to-coil transition temperature was not affected significantly. This proves that the concentration of divalent metal ions used in the studies did not cause any significant helix stabilization or destabilization.

Discussion

Fluorescence titration indicated that all three molecules interact with ds DNA. However, the binding of 2-pyrben to the minor groove of DNA was comparatively weak. Computational studies reported elsewhere show that in the minimum-energy conformation of 2-pyrben, the pyridine nitrogen atom and the benzimidazole proton are involved in intramolecular hydrogen bonding. This makes the benzimidazole protons in 2-pyrben less available for hydrogen bonding with the AT bases in the DNA minor groove.[14] Nevertheless, the molecule showed some interaction with ds DNA.

Our present studies revealed that the DNA binding of 2-pyrben could be completely "switched off" in the presence of transition-metal cations. We attempted to justify the totally abolished minor-groove binding of 2-pyrben in the presence of metal cations, as indicated by fluorescence and CD spectroscopic studies. Before investigating the DNA binding of the molecules in the presence of metal ions, we studied the effect of divalent cations (Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) on the emission behavior of the individual molecules. Cu^{II} is a known fluorescence quencher, being a paramagnetic cation with an incomplete d shell (d^9). When a quenching metal ion binds tightly to a ligand, intracomplex quenching takes place.[28,29] Indeed, earlier studies reported in the literature testify to the fact that chelating agents of the 2-picolylamidine type, such as 2-(2′-pyridyl)imidazole and 2-(2′-pyridyl)benzimidazole, complex efficiently with many transition-metal ions.[30]

Complexation between 2-pyrben and the cation is probably the reason why the fluorescence of the free ligand in solution was so effectively quenched by Cu^{2+}. Quenching was also observed with Ni^{2+} and to a lesser extent with Zn^{2+} (closed d^{10} shell). Quite expectedly, divalent Mg^{2+} did not cause any quenching of fluorescence.[29] The fluorescence behavior of 4- and 3-pyrben were not significantly affected by transition-metal ions, presumably because of their inability

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to form such complexes (see Supporting Information, Figure S1).

DNA binding was investigated both by fluorescence and CD spectroscopic techniques. Fluorescence titration revealed comparatively weaker DNA binding for 2-pyrben than its isomers, whereas the CD spectra showed nearly similar ICD for all three ligands at saturation. Notably, for the fluorescence studies, DNA was titrated into the ligand solution with an interval of 5–7 min between each addition to allow equilibration. On the other hand, CD spectra were recorded after the ligands were incubated with DNA (at saturated [ligand]/[DNA] ratio) for 24 h. The short time interval between each addition during fluorescence titration was probably not enough for the binding interaction of 2-pyrben with DNA to reach equilibrium.

Next, we investigated the effect of metal ions on DNA binding by fluorescence methods. In the presence of Cu^{2+}, the fluorescence enhancement of 2-pyrben on DNA addition was significantly lower than that in the absence of the metal ion. At a concentration of the metal salt of 1 μM, the fluorescence was completely quenched. The results were expected as Cu^{II} has already been established as an efficient fluorescence quencher of 2-pyrben. But surprisingly similar results were also observed in the presence of Mg^{2+}. Now it had already been proved that Mg^{2+} did not affect the emission behavior of the free ligand. This led us to think that the metal cations not only quenched the fluorescence intensity, but also reduced the DNA-binding affinity of the ligand. The DNA binding of 4- and 3-pyrben, on the other hand, were not significantly affected by either Mg^{2+} or Cu^{2+}. Hence, the ability of 2-pyrben to chelate metal ions is clearly responsible for its total lack of DNA-binding affinity in the presence of metal cations.

When DNA was titrated into a solution of 2-pyrben–M^{2+}, both the metal ion and DNA competed for the ligand. As the molecule has an inherently weak affinity towards DNA, it preferentially bound to the metal cation. Next, the DNA–2-pyrben–M^{2+} solution was incubated for 24 h. With Mg^{2+}, complete recovery of emission intensity of 2-pyrben was observed. This suggests that Mg^{2+} coordination by the ligand was not too efficient and preferential binding to DNA took place on incubation with the latter for 24 h.

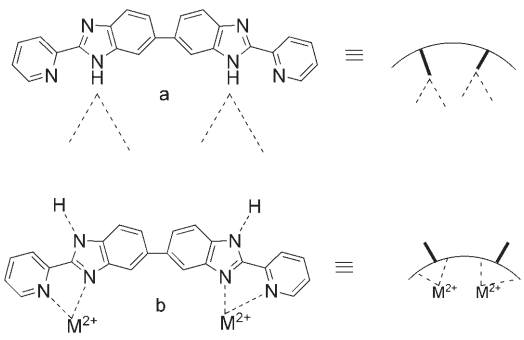
For a better insight into the matter, we analyzed the metal-ion-dependent DNA-binding behavior by CD spectroscopy. Importantly, the CD profile of DNA did not change significantly in the presence of the metal cations at the concentrations at which the studies were performed (see Supporting Information, Figures S2 and S3). All the compounds showed ICD at around 350 nm when incubated with ds DNA for 24 h, thus indicating significant interaction.

However, incubation of the DNA–2-pyrben–M^{2+} solution (at 2-pyrben/M^{2+} = 1:1) showed complete disappearance of the ICD band of the ligand at 350 nm (when $M^{2+} = Co^{2+}$, Ni^{2+}, Cu^{2+}). This indicated completely abolished DNA-minor-groove binding of 2-pyrben in the presence of the transition-metal cations indicated above. Incubation with EDTA (of the DNA–2-pyrben–M^{2+} solution) completely reversed the effects, and full recovery of the ICD signal was obtained. EDTA being an efficient metal chelator sequestered the metal cations, thereby releasing the ligand and making it free for DNA binding. In the presence of Zn^{2+}, there was some attenuation of the ICD intensity that indicates reduced binding, whereas with Mg^{2+}, DNA binding was not significantly affected as evident from the unchanged ICD signature. The absence of significant thermal stabilization of duplex DNA in presence of 2-pyrben/M^{2+} (1:1) proved that no other binding modes (electrostatic, intercalative) are operative.

The DNA binding of 4- and 3-pyrben, on the other hand, were not affected by any of the divalent metal ions mentioned above. Hence, it was established beyond doubt that the affinity of 2-pyrben for DNA-minor-groove binding was significantly reduced in the presence of the transition-metal ions Co^{2+}, Ni^{2+}, and Cu^{2+}. The differential binding behavior of the ligand clearly arises from its ability to form complexes with the aforementioned metal ions. We endeavored to justify the finding.

As reported in the literature, an essential factor governing minor-groove binding is hydrogen-bond formation between the DNA bases and benzimidazole protons on the inner concave surface of the crescent-shaped molecules, which face the wall of the groove. These protons are involved in forming bifurcated hydrogen bonds with N3 of adenine and O2 of thymine.[6,31,32] However, metal-ion coordination requires the pyridine and benzimidazole nitrogen atoms of 2-pyrben to be spatially close (conformation b in Scheme 3); hence, in the metal-coordinated form, the hydrogen atoms attached to the benzimidazole N atom are probably on the outer convex edge facing away from the groove wall. Not only that, metal binding lowers the pK_a of the said protons, which become acidic;[15,30] hence, at physiological pH, the molecule is expected to be partially deprotonated and thus devoid of the crucial protons essential for H-bonding to the DNA bases.

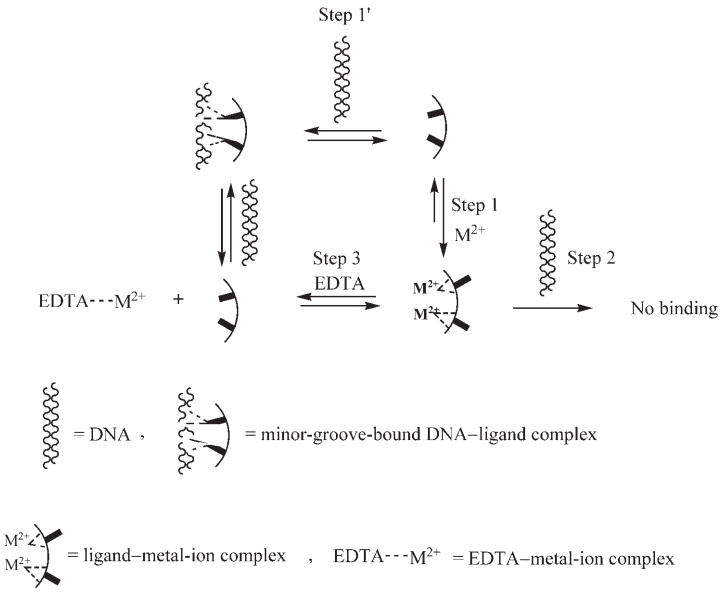
However, when 2-pyrben and DNA were allowed to equilibrate first and then metal ions (Cu^{2+} or Mg^{2+}) were added



Scheme 3. Conformations of 2-pyrben. Conformation a is favored for DNA binding, as the benzimidazole protons are able to participate in hydrogen-bonding interactions (shown as dotted lines) with the DNA bases. Conformation b predominates in metal-ion complexation. The acidity of the benzimidazole protons increases as they are on the convex edge of the molecule away from the floor of the minor groove.

to a saturated solution of the same, there was no effect on the fluorescence intensity of the ligand. This suggests that once the ligands are bound to the DNA minor groove, the metal ions probably cannot bring about the aforementioned changes in the ligand characteristics.

The above discussions are summarized in Scheme 4. As the binding of 2-pyrben to DNA is weak, Step 1′ is slow.



Scheme 4. Schematic representation of the equilibrium steps involved in the complexation of 2-pyrben with DNA and divalent metal ion M^{2+}.

This has been substantiated by the fluorescence experiment. On the other hand, the molecule is an extremely efficient chelator of transition-metal ions such as Co^{2+}, Ni^{2+}, and Cu^{2+}, hence when the ligand, metal ion, and DNA are simultaneously incubated, the ligand chelates the cations preferentially. Metal chelation causes the aforementioned tautomeric change in 2-pyrben, thus making the hydrogen-bond donors unavailable to the DNA bases in the minor groove. Therefore, once the molecule is complexed with the metal ion, DNA-groove binding cannot occur, and this is reflected in the completely abolished ICD signal for the ligand. When the DNA–ligand–M^{2+} solution is incubated with EDTA, metal chelation by EDTA occurs, and the ligand is released once again for DNA binding.

Conclusions

The results of the present study show the unusual DNA-binding behavior of bis(2-(2-pyridyl)-1*H*-benzimidazole). In the presence of divalent metal ions, the DNA binding of this molecule can be completely "turned off". This was established by fluorescence and circular dichroism studies. This observation is of interest because metal-chelating compounds often bind to DNA better when they are complexed with metals rather than as a free ligand because of the favorable electrostatics that arise from metal ligation. Retention of DNA binding for the *meta* and *para* isomers, in the presence of metal ions, indicate that whatever unusual behavior was observed for 2-pyrben was due to its ability to chelate metal ions and the associated changes in ligand characteristics that arise from it. Importantly, the effect of metal ions can be completely reversed by the addition of EDTA. Indeed, with such tunable molecular-recognition characteristics, bis(2-(2-pyridyl)-1*H*-benzimidazole) has immense potential for gene regulation, and the concept may be developed for controlled modulation of gene expression.

Experimental Section

Materials

3,3′-Diaminobenzidine and *o*, *m*, and *p*-pyridinecarboxaldehyde were purchased from Fluka and were used without further purification. Nitrobenzene used for the coupling reactions was stirred over anhydrous P_2O_5 prior to vacuum distillation. Thin-layer chromatography (TLC) was carried out on silica gel GF254 precoated glass slides with detection under UV light or I_2 vapor. Column chromatography was performed with silica gel mesh 60–120. ^{1}H NMR spectra were recorded on a Jeol JNM λ-300 (300 MHz for ^{1}H) spectrometer, and chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). Mass spectra were recorded on a Micromass Q-TOF Water spectrometer.

Syntheses

A common synthetic pathway was followed for all three compounds. The respective pyridinecarboxaldehyde (2.5 equiv) was dissolved in nitrobenzene (5 mL). 3,3′-Diaminobenzidine (1 equiv) was then added. The solution was heated at 120 °C for 8–10 h. Once all the 3,3′-diaminobenzidine had reacted, the reaction mixture was cooled to room temperature. Petroleum ether was added to precipitate the crude solid. The solvent was decanted off. This process was repeated several times until almost all the nitrobenzene was removed. The compounds were purified by column chromatography over silica gel (mesh 60–120). The pure product was obtained as a yellow powdery solid (R_f = 0.4 in 6% MeOH in $CHCl_3$ on TLC plate coated with silica GF254). The compound was further purified by dissolving it in $CHCl_3$/MeOH mixture and precipitating it with petroleum ether. The pure compounds were obtained in 65–70% yield.

4-Pyrben: ^{1}H NMR (300 MHz, $CDCl_3$/[D_6]DMSO, 25 °C, TMS): δ = 8.65 (d, J = 4.8 Hz, 4H), 8.04 (d, J = 4.8 Hz, 4H), 7.84 (s, 2H), 7.6–7.5 ppm (m, 4H); HR-MS: *m/z* calcd: 389.1514 [M+H]$^+$; found: 389.1520.

3-Pyrben: ^{1}H NMR (300 MHz, $CDCl_3$/[D_6]DMSO, 25 °C, TMS): δ = 9.3 (s, 2H), 8.67 (d, J = 5.1 Hz, 2H), 8.55 (d, J = 8.3 Hz, 2H), 7.83 (s, 2H), 7.67–7.53 ppm (m, 6H); HRMS: *m/z* calcd: 389.1514 [M+H]$^+$; found:389.1523.

2-Pyrben: ^{1}H NMR (300 MHz, $CDCl_3$/[D_6]DMSO, 25 °C, TMS): δ = 8.66 (d, J = 4.2 Hz, 2H), 8.45 (d, J = 7.5 Hz, 2H), 7.89–7.78 (m, 2H), 7.70–7.60 (m, 6H), 7.41–7.30 ppm (m, 2H); HRMS: *m/z* calcd: 389.1514 [M+H]$^+$; found: 389.1520.

DNA Binding

DNA-binding experiments were performed with CT DNA and [poly(dA-dT)]$_2$. These were obtained from Sigma Chem. Co. (St. Louis, MO) and used as received. The concentrations of the nucleic acids were determined spectrophotometrically and expressed as base pairs with ε values ($M^{-1}cm^{-1}$) as indicated: CT DNA, ε_{258} = 13600; poly[d(A-T)]$_2$, ε_{262} = 13200. Buffers used for all the studies were Tris/HCl (50 mM) and NaCl (100 mM, pH 7.4) with or without EDTA (0.1 mM). MilliQ water was used to prepare the buffers. The divalent metal salts used for the spectroscopic studies, $MgSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 7H_2O$, $NiSO_4 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, and $ZnCl_2 \cdot 2H_2O$, were obtained from Ranbaxy Chemicals.

Fluorescence

Fluorescence spectra were recorded on a Hitachi F4500 spectrofluorimeter, with excitation and emission slit widths both fixed at 10 nm. The molecules were excited at a wavelength of about 350 nm, and emission was centered at about 450 nm. All measurements were done at 25 °C.

To investigate the effect of divalent cations on the emission characteristics of the free ligands, a solution of the ligand (2 μM) in H_2O/MeOH (3:1 *v*/*v*) was titrated with incremental concentrations of the salts $MgSO_4{\cdot}7H_2O$, $NiSO_4{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, and $ZnCl_2{\cdot}2H_2O$.

DNA binding by the three isomers were then investigated by titrating a constant concentration of the ligand (2 μM) with increasing concentrations of CT DNA. Binding constants were determined by using the Scatchard equation. The nonlinear Scatchard plot obtained in the case of CT DNA was interpreted by considering the special case of two independent binding sites. The larger of the two association constants so obtained was attributed to specific binding of the drug molecule in the AT-rich stretches of the DNA minor groove.[27]

Next, to study the effect of metal cations on DNA binding, CT DNA was titrated into a solution of the ligand of constant concentration in the presence of increasing concentrations of divalent metal ion M^{2+} (M^{2+} = Cu^{2+}, Zn^{2+}, or Mg^{2+}) in Tris/HCl (50 mM)/NaCl (100 mM) buffer without EDTA. In separate sets of experiments, ligand solutions (1 μM), each containing 0.05, 0.1, 0.5, or 1 μM of M^{2+}, were titrated with DNA. Between each DNA addition, an interval of 5 min was given before the emission spectra were recorded.

Circular Dichroism

CD spectra were recorded on a JASCO J-810 spectropolarimeter. The wavelength range between 220–420 nm was scanned at a rate of 50 nm min^{-1}. All spectra presented herein were averaged over two acquisitions and were recorded in optical cells of path length 1 cm. The CD values are expressed in millidegrees.

The polynucleotide $[poly(dA\text{-}dT)]_2$ (12 μM) was incubated for 24 h in the presence of saturating concentrations of ligand (3 μM) in Tris/HCl (50 mM) containing NaCl (100 mM) and EDTA (0.1 mM). For experiments done in the presence of divalent cations, the metal salts used were $MgSO_4{\cdot}7H_2O$, $CoSO_4{\cdot}7H_2O$, $NiSO_4{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, and $ZnCl_2{\cdot}2H_2O$. A 1:1 solution of ligand/salt (3 μM each) was incubated with $[poly(dA\text{-}dT)]_2$ (12 μM) for 24 h in buffer without EDTA at room temperature before CD spectra were recorded.

Thermal Denaturation

DNA melting-point (T_m) measurements were performed by following the changes in UV/Vis absorbance at 260 nm as a function of temperature. Experiments were carried out in quartz cuvettes stoppered with teflon caps in a Beckman 640 spectrophotometer attached to a programmable temperature controller. Samples were heated at the rate of 0.5 °C min^{-1}, and the absorbance was recorded with every 0.5 °C rise in temperature. The concentration of DNA used was 12 μM (base-pair molarity). The concentration of 2-pyrben used was 3 μM. All the experiments were carried out in Tris/HCl buffer (50 mM, pH 7.4) and at an NaCl concentration of 100 mM, in the presence of 3 μM of metal salt ($MgSO_4{\cdot}7H_2O$, $CoSO_4{\cdot}7H_2O$, $NiSO_4{\cdot}6H_2O$, $CuCl_2{\cdot}2H_2O$, or $ZnCl_2{\cdot}2H_2O$). Melting points were determined by first-derivative analysis of the heating scans. Under similar conditions, DNA melting was also carried out in the presence of the metal salts but in the absence of the ligand.

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