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Triple recognition of B-DNA

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ABSTRACT

A novel conjugate of Hoechst 33258, pyrene and neomycin was synthesized and examined for its binding and stabilization of A-T rich DNA duplexes using spectroscopic and viscometric techniques. The conjugate, containing three well known ligands that bind nucleic acids albeit in different binding modes, was found to significantly stabilize DNA over parent conjugates containing only one or both of the other recognition elements. The study represents the first example of DNA molecular recognition capable of minor/major groove recognition in conjunction with intercalation.

Molecular recognition of nucleic acids is an important endeavor for our understanding of biological processes. A better understanding of nucleic acid recognition may ultimately provide us with the ability to regulate cellular events. For example, a better understanding of DNA shape recognition (A, B or Z-forms) is crucial for defining the principles for development of gene-regulatory drugs.

B-Form DNA recognition is traditionally accomplished through the minor groove, major groove or stacking between the base pairs (intercalation). A number of natural products have been shown to possess structures characteristic of both groove-binding and intercalation.[1,2] Selected examples include rebeccamycin (indolocarbazole family), nogalamycin (anthracycline family), altromycin (pluramycin family), and mithramycin (aureolic acid family; see Supplementary data for chemical structures). Furthermore, quite a few natural products have been shown to display multiple DNA binding modes. For example, nogalamycin has been[3] reported to thread DNA, exhibiting intercalation by the central chromophore, with saccharide regions extending from both ends to bind both major and minor grooves.[3]

We have reported on the design of molecules that recognize DNA using simultaneous recognition of the minor and the major groove.[4–8] Previous attempts have been made to design molecules that recognize DNA using intercalation and minor groove binding simultaneously.[1,9,10] No successful attempts have been made to combine the three recognition motifs, to the best of our knowledge. This report describes one such endeavor in the preparation and DNA-binding analysis of a novel neomycin–Hoechst 33258–pyrene conjugate, termed 'NHP'. The goal of this work was to establish a general multi-recognition scaffold utilizing three well-established DNA binders. Once a 'proof of concept' approach is deemed feasible, as shown in this manuscript, ligand based triple-recognition of biologically-relevant DNA sequences could be addressed.

Recently, neomycin, an aminoglycoside antibiotic, has been shown to be a versatile scaffold in probing nucleic acid recognition.[7,11] From its well known ability to bind RNA and recent reports of DNA triplex and nucleic acid hybrid stabilization,[12] neomycin has since been shown to stabilize both A-form (or A-like)[5] and B-form[4,11] nucleic acid structures via conjugation with such ligands as intercalators (pyrene,[13] BQQ,[14] ethidium[15,16]) or groove binders (Hoechst 33258,[4,11] nucleic acids.[6,17,18]) Herein, we expand the recognition of DNA using these multi recognition motifs. A neomycin–Hoechst 33258–pyrene conjugate, 'NHP', was synthesized. Conjugates of neomycin and pyrene, Hoechst 33258 and pyrene, and neomycin and Hoechst 33258 ('NP', 'HPA', and 'NH', respectively), were also synthesized and utilized as controls in these experiments. All structures of the conjugates used in the study are shown in Figure 1.

UV melting experiments exhibit the increased stabilization of DNA by NHP. T_m enhancements by individual recognition elements were resolved using control conjugates containing only one or both of the other recognition elements. Fluorescence techniques indicate enhanced binding over parent conjugates, with a binding site size larger than that of Hoechst 33258. Induced Circular Dichroism studies were used to identify the groove binding of Hoechst 33258 and intercalation of pyrene into DNA. Classical viscometric techniques also detail the binding mode of the pyrene moiety, supporting the intercalative nature of pyrene in NHP.

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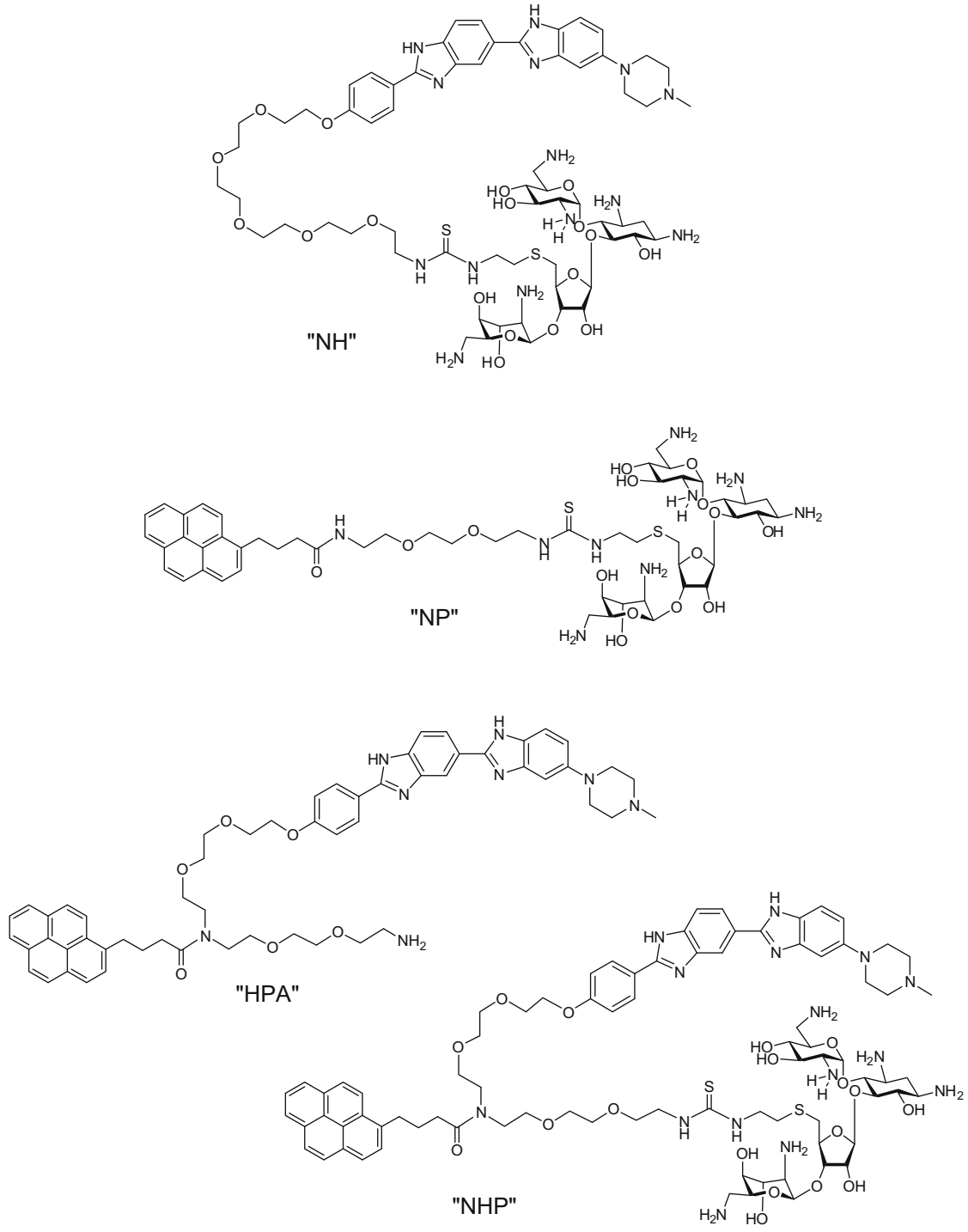
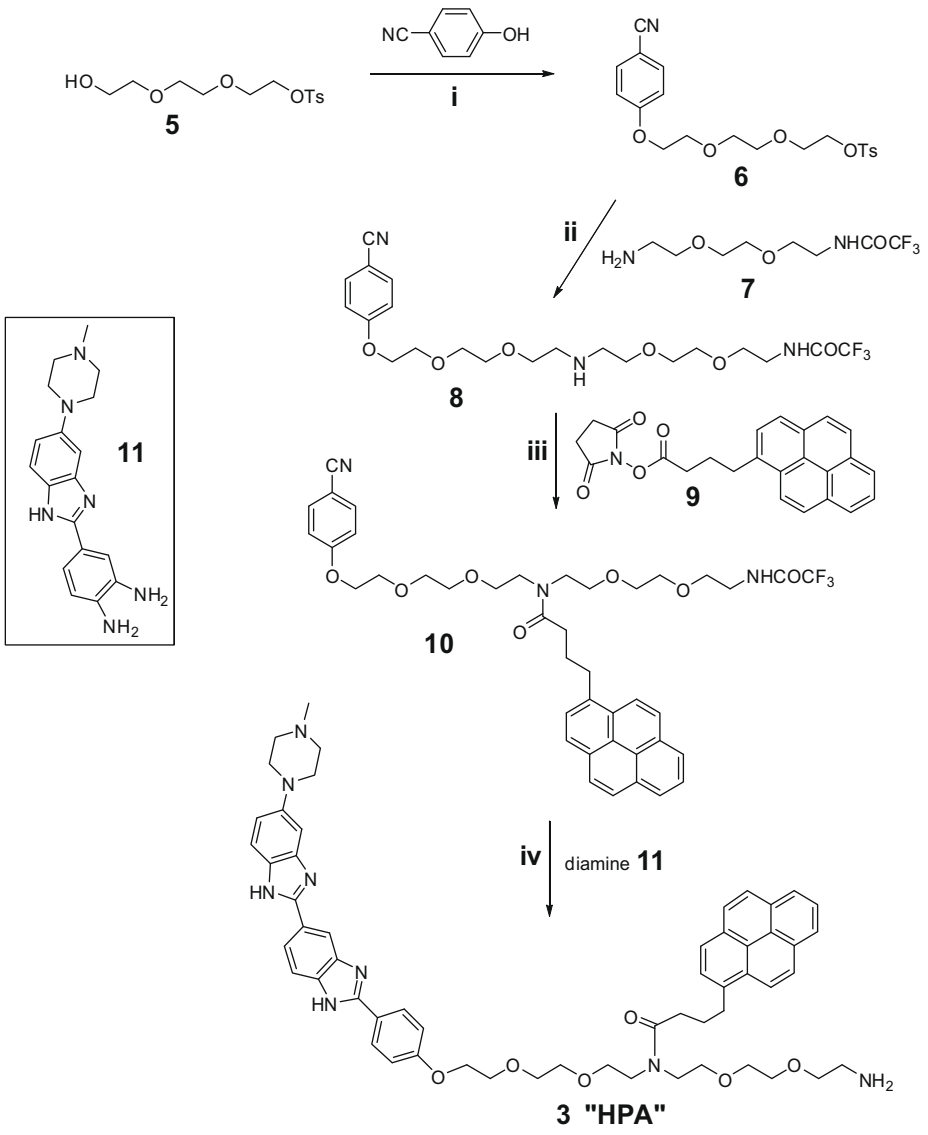


Figure 1. Structures of novel conjugates used in this study.

To synthesize the target conjugate (NHP), the Hoechst–pyrene–amine portion was first prepared (Scheme 1). Monotosylate **5** was coupled with *p*-cyanophenol using Mitsunobu conditions to afford **6** in good yields. Tosylate substitution with mono-protected diamine **7** under basic conditions provided intermediate **8**. Reaction of the secondary amine of **8** with pyrene succinimide ester **9**, followed by standard coupling with **11** in an ethanol/acetic acid mixture provided the desired Hoechst–pyrene–amine (HPA, **3**) after deprotection (K_2CO_3, MeOH/H_2O) of the terminal amine. Reaction of **3** with neomycin isothiocyanate **12**, followed by Boc deprotection of coupled product **13** gives the desired triple recognition agent **4** (Scheme 2) as a trifluoroacetate salt.

To test the effect of the conjugates on oligomeric DNA duplex stability, UV melting profiles of $dA_{22}{\cdot}dT_{22}$ in the absence and presence of various ligands were gathered. The resulting T_m associated with each DNA complex is listed in Figure 2 (right). A much more significant and noticeable shift in the T_m for

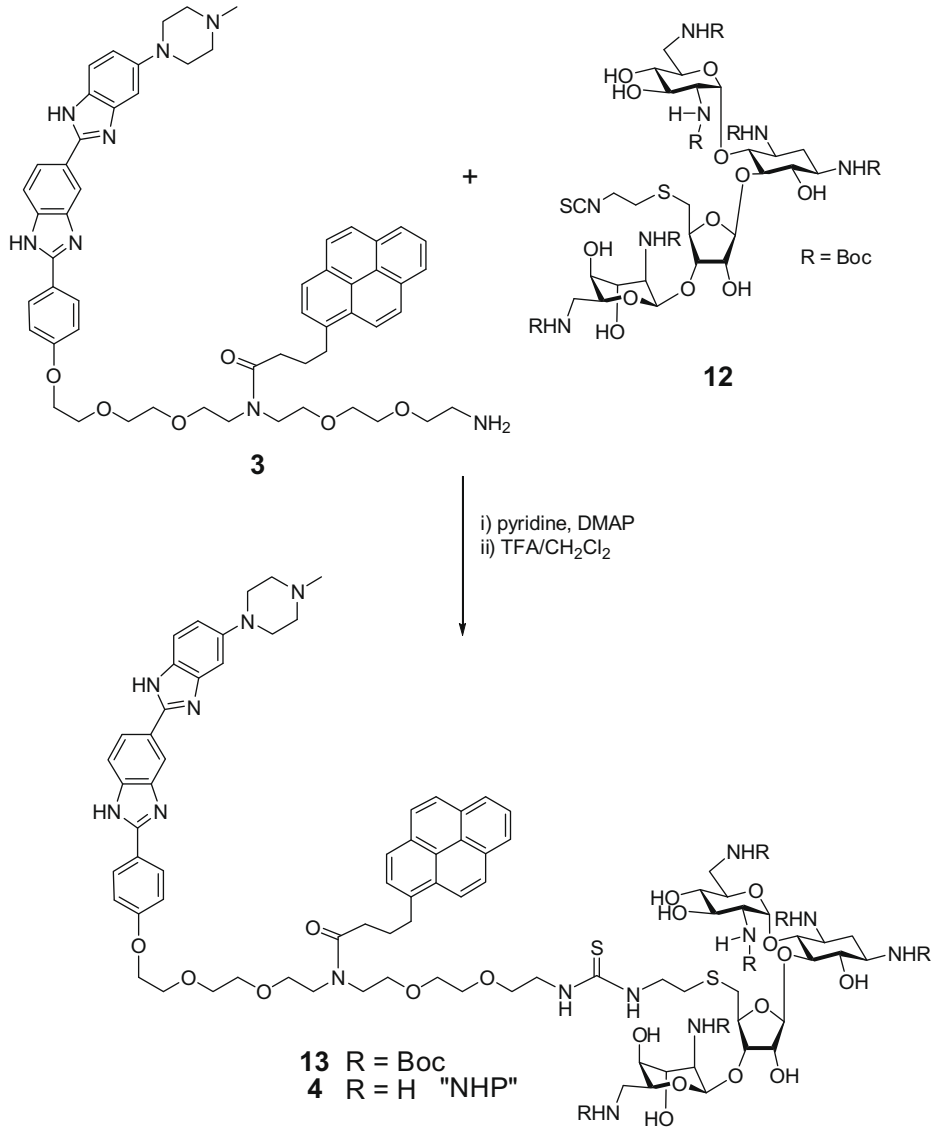


Scheme 1. Preparation of Hoechst 33258–pyrene–amine. Reagents and conditions: (i) *p*-cyanophenol, PPh_3, DIAD, dioxane, 78%; (ii) **7**, DMF, NaI, 61%; (iii) **9**, DMAP, DMF, 65%; (iv) (a) **11**, HOAc, EtOH, 41%; (b) K_2CO_3, MeOH, H_2O, 90%.

$dA_{22}{\cdot}dT_{22}$ with NHP was apparent. When compared with other ligands, such as Hoechst 33258, NH, NP, and HPA, the triple-binding agent NHP was clearly the strongest stabilizer of this DNA oligomer, with a $\Delta T_m = 35\ ^\circ C$. A concentration–dependent melting study (Fig. 2) illustrates the gradual shift in T_m values as ligand concentrations are increased. These results indicate a clear effect of each recognition element of NHP on DNA stability, as solutions containing a combination of individual ligands (NH + aminoypyrene; neomycin + HPA; NP + Hoechst 33258) exhibited significantly lower T_m values.

When these studies were carried out in the presence of a DNA (dT) third strand, no triplex formation was observed with NH or NHP, due to neomycin binding in the major groove, consistent with previous reports.[4,7,11] Of importance also is the fact that while aminopyrene, NP, or HPA show no contribution of pyrene to an increase in duplex T_m, its conjugation to neomycin and Hoechst 33258 in NHP clearly leads to a increase of about 11 °C in T_m over NH. It is well known that pyrene favors the alternating purine–pyrimidine sequence over the non-alternating purine–pyrimidine sequence polymers.[19] DNA intercalation involves both unstacking of base pairs to accommodate the intercalants and unwinding of the double helix. The energy requirement for creating the intercalation sites is lower for alternating pyrimidine (3′–5′)-purine than for a nonalternating homocopolymer used in this study. This energetic cost for unwinding and unstacking is likely paid by the concurrent binding of neomycin and Hoechst 33258 in the two grooves.

Circular dichroism (CD) was also utilized to probe the changes in ligand and DNA conformation attributable to complexation. Several CD studies have indicated the tight and rigid conformational preference of Hoechst 33258 when bound to A/T rich sequences, typically characterized by an increase in CD signal in the region of Hoechst 33258 λ_{max}. The current study supports that observed previously by us and others[4,11]: a pronounced induced (+) CD band around 360 nm as ligand concentrations increased, consistent with previous CD reports of minor groove binding of Hoechst 33258 and as also observed previously with Hoechst 33258-neomycin conjugates[4,11] (Fig. 3). A noticeable overlap with

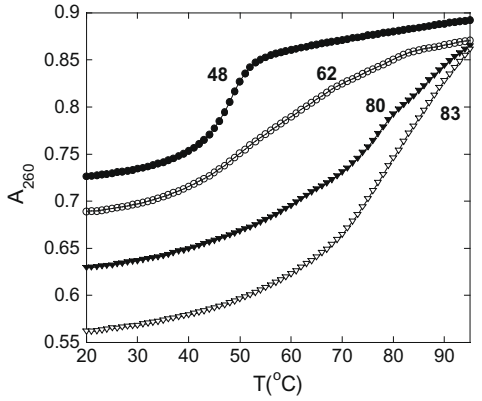


Scheme 2. Preparation of triple recognition agent NHP. Reagents and conditions: (i) pyridine, DMAP, 52%; (ii) 1:1 TFA/CH_2Cl_2, quantitative.

a new induced CD signal is apparent between 270 and 400 nm, indicative of an induced CD band likely due to a π to π^* absorption of the pyrene moeity.[9] Since asymmetry is induced to both chromophores of NHP upon binding to DNA, these data support the hypothesis that both the Hoechst 33258 and pyrene moieties are interacting with DNA.

Importantly, it is known that pyrene binding to homocopolymers such as poly(dA)·poly(dT) also occurs via an 'external', non-intercalative binding mode that is characterized by little change in T_m, viscosity, or circular dichroism in pyrene's absorption region.[21,19] Our observations with NHP in the presence of poly(dA)·poly(dT) suggest an intercalation mode similar to that observed with intercalation events in poly(dA-dT)$_2$.[9,21] Therefore, it is likely that a combination of conformational restrictions in NHP and the resulting unwinding of the duplex by concerted groove binding of neomycin and Hoechst 33258 moieties designate pyrene to an intercalative mode of binding. Almost no change is seen in the CD spectrum between 200 and 260 nm, suggesting that the DNA conformation is relatively unperturbed upon ligand binding.

Viscometry has long been utilized for investigating ligand–substrate binding modes, particularly to confirm intercalation events. Viscometric titrations of neomycin, Hoechst 33258, NH, and NHP with poly(dA)·poly(dT) were thus carried out (Fig. 4). In all cases except Hoechst 33258, the DNA solution viscosity *decreased* when ligand was added. The most marked decrease was with NH, whereas NHP was clearly higher. Similar to that observed before[11,20] the decrease in viscosity can be attributed to groove binding, which, in contrast to intercalation, can shorten the helix by compaction. This compacting of DNA is clearly a result of neomycin interaction. Difference plots of NH–DNA binding with NHP–DNA binding in the viscosity experiments were generated to study the effect of the *pyrene* moiety in NHP. By overlaying the subtracted data (NHP minus NH, Fig. 5), a clear resemblance to theoretical intercalation is observed for the pyrene moiety in NHP. The slightly lower slope can be attributed to intercalation occurring less periodically within the DNA lattice due to the large binding site size (one pyrene per every nine base pairs) of NHP.[11] Furthermore, the mere observation of viscosity *decrease*, as seen in previous accounts,[11] supports neomycin bind-



Ligand	$T_{m2\rightarrow1}$	$\Delta T_{m2\rightarrow1}$
None	48	0
Neomycin	48	0
Hoechst 33258	70	22
aminopyrene	50	2
NP	53	5
NH	72*	24*
HPA	64*	16*
NHP	83	35
NH + aminopyrene	73	25
HPA + neomycin	61*	13*
NP + Hoechst 33258	69	21

Figure 2. UV melting profiles of $dA_{22}{\cdot}dT_{22}$ with NHP. Samples of DNA (1 μM in duplex) were mixed with ligand (0–3 μM from left to right) in buffer (10 mM sodium cacodylate, 0.5 mM EDTA, 150 mM KCl, pH 6.8) before heating at 95 °C for 5 min and slow annealing to 20 °C before UV analysis at 260 nm from 20 to 95 °C at heating rate of 0.2°/min. T_m reported is the midpoint temperature of $dA_{22}{\cdot}dT_{22}$ denaturation (right) T_m data for $dA_{22}{\cdot}dT_{22}$ melting in the presence of indicated ligands (3 μM). *For compounds NH and HPA, biphasic transitions were observed (at unbound T_m of 48 °C and indicated T_m). See Supplementary data for solution conditions and UV melting profiles.

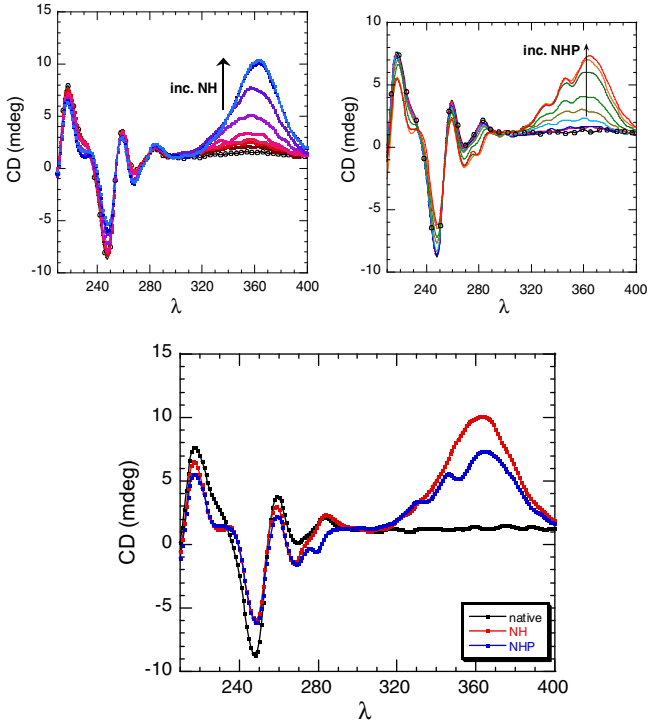


Figure 3. Circular dichroism of poly(dA)·poly(dT) titrated with NH and NHP (top) and an overlay of NH and NHP scans (below). A solution of DNA (30 μM) was titrated with small aliquots of concentrated ligand (200 μM) before equilibration and scanning from 450–210 nm. Peaks around 360 nm correspond to ligand–DNA complexation. Solutions were in 10 mM sodium cacodylate, 0.5 mM EDTA, 150 mM KCl, pH 7.2; T = ambient.

ing. These data, from a combination of UV, flourescence, CD and viscosity measurements, give strong candidacy for a DNA triple-recognition agent in NHP.[11] These studies should open the door for development of novel DNA binding agents with specificities and affinities far surpassing the current arsenal of DNA binding drugs.

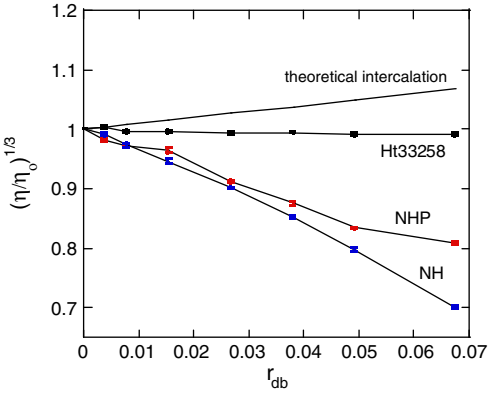


Figure 4. Viscometric analysis of poly(dA)·poly(dT) with various ligands: DNA solutions (100 μM) were titrated with respective drug (200 μM) and corresponding flow times were recorded in triplicate with deviation less than 0.1 s. Error bars are indicated for each titration point. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 150 mM KCl, 1 mM $MgCl_2$, pH 7.2; T = 27 ± 0.05 °C.

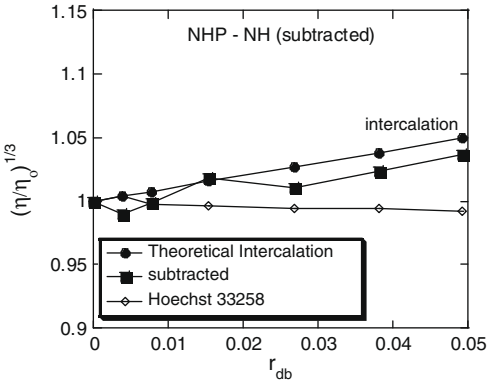


Figure 5. Subtracted viscosity data demonstrate pyrene effect on solution viscosity: DNA solutions (100 μM) were titrated with respective drug (200 μM) and corresponding flow times were recorded in triplicate with deviation less than 0.1 s. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 150 mM KCl, 1 mM $MgCl_2$, pH 7.2; T = 27 ± 0.05 °C.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.079.

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