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Hx, a Novel Fluorescent, Minor Groove and Sequence Specific Recognition Element: Design, Synthesis, and DNA Binding Properties of *p*-Anisylbenzimidazole-imidazole/pyrrole-Containing Polyamides

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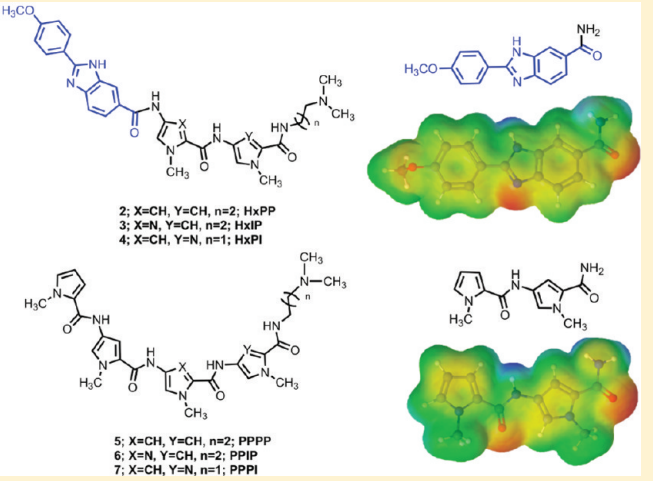
Supporting Information

ABSTRACT: With the aim of incorporating a recognition element that acts as a fluorescent probe upon binding to DNA, three novel pyrrole (P) and imidazole (I)-containing polyamides were synthesized. The compounds contain a *p*-anisylbenzimidazolecarboxamido (Hx) moiety attached to a PP, IP, or PI unit, giving compounds HxPP (**2**), HxIP (**3**), and HxPI (**4**), respectively. These fluorescent hybrids were tested against their complementary nonfluorescent, non-formamido tetraamide counterparts, namely, PPPP (**5**), PPIP (**6**), and PPPI (**7**) (cognate sequences 5′-AAATTT-3′, 5′-ATCGAT-3′, and 5′-ACATGT-3′, respectively). The binding affinities for both series of polyamides for their cognate and noncognate sequences were ascertained by surface plasmon resonance (SPR) studies, which revealed that the Hx-containing polyamides gave binding constants in the 10^6 M^{-1} range while little binding was observed for the noncognates. The binding data were further compared to the corresponding and previously reported formamido-triamides f-PPP (**8**), f-PIP (**9**), and f-PPI (**10**). DNase I footprinting studies provided additional evidence that the Hx moiety behaved similarly to two consecutive pyrroles (PP found in **5**−**7**), which also behaved like a formamido-pyrrole (f-P) unit found in distamycin and many formamido-triamides, including **8**−**10**. The biophysical characterization of polyamides **2**−**7** on their binding to the abovementioned DNA sequences was determined using thermal melts (ΔT_M), circular dichroism (CD), and isothermal titration calorimetry (ITC) studies. Density functional calculations (B3LYP) provided a theoretical framework that explains the similarity between PP and Hx on the basis of molecular electrostatic surfaces and dipole moments. Furthermore, emission studies on polyamides **2** and **3** showed that upon excitation at 322 nm binding to their respective cognate sequences resulted in an increase in fluorescence at 370 nm. These low molecular weight polyamides show promise for use as probes for monitoring DNA recognition processes in cells.



Pyrrole (P) and imidazole (I)-containing polyamide analogues of the naturally occurring distamycin A (**1**) bind in the minor groove of DNA in a stacked, antiparallel 2:1 (ligand/DNA) motif.[1−3] Early studies have led to the establishment of a set of pairing rules for DNA base pair recognition; a P/P stacking recognizes an A/T or T/A base pair, an I/P stacking binds to a G/C base pair, while a P/I stacking recognizes a C/G base pair. In addition, a stacked I/I pairing targets a T/G mismatch.[4−6] As a result of the high degree of sequence specificity that these molecules exhibit, their potential use as regulators of gene expression has been widely explored.[7] However, attempts to inhibit the transcription of genes that code for a disease in cell lines have been met with moderate success which presumably points toward poor cellular uptake of some of these polyamides and their limited ability to achieve nuclear localization.[8,9] Some success in this area has been achieved through the attachment of

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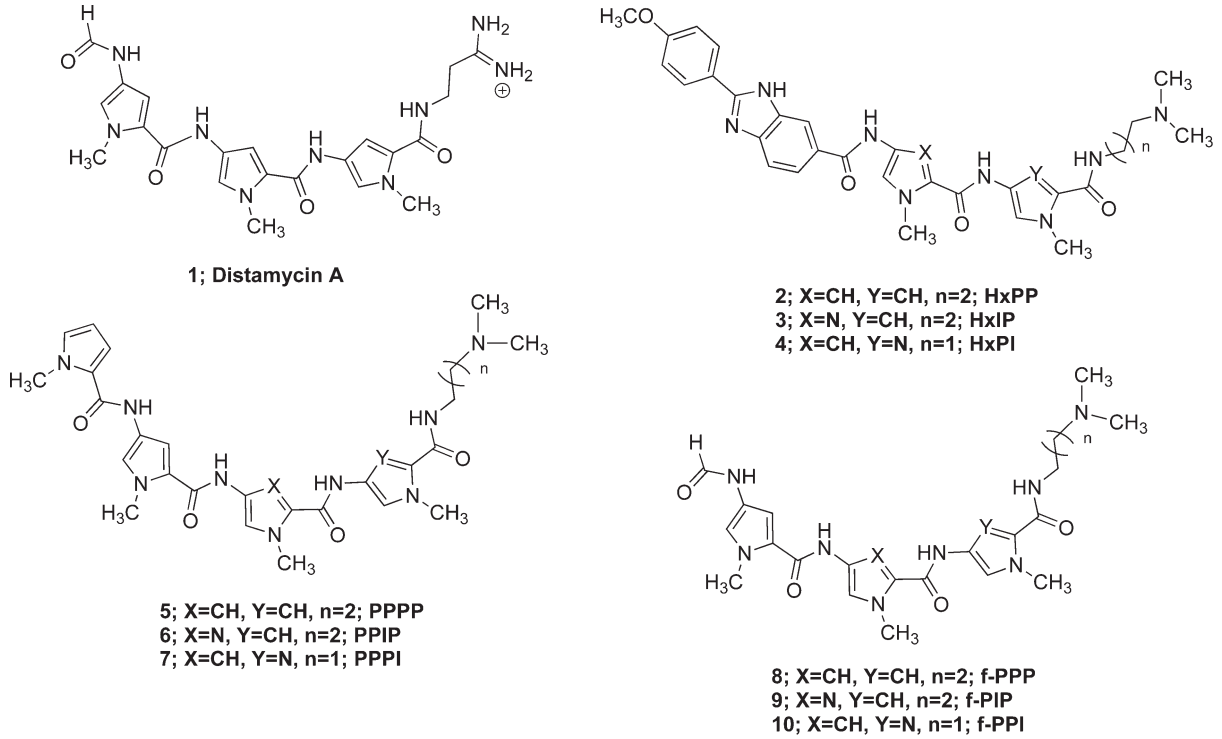


Figure 1. Structures of distamycin A (**1**); target fluorescent Hx polyamides: HxPP (**2**), HxIP (**3**), HxPI (**4**); their nonfluorescent nonformamido tetraamide counterparts PPPP (**5**), PPIP (**6**), PPPI (**7**); and the formamido-triamides f-PPP (**8**), f-PIP (**9**), and f-PPI (**10**).

specific chemical groups that facilitate cell uptake, and these pendant groups include fluorescein (FITC)[9a−c] and isophthalic acid.[9d,e] Over the past decade, the need for the development of DNA recognition elements that exhibit fluorescence, in addition to high sequence specificity and binding affinity, upon binding to their target DNA sequences has become evident. Such compounds would provide an intrinsic probe for monitoring the cellular and nuclear uptake of the molecule, movement through the cell, and ultimately binding to nuclear DNA.

A large number of molecules are known to display an increase in fluorescence upon binding to DNA with a prime example being the dye 4′,6-diamidino-2-phenylindole (DAPI).[10] However, the usefulness of DAPI as a tracking element is hampered due to the multiple binding modes it possesses in the minor groove,[11,12] major groove,[13] and as an intercalator.[14] The incorporation of fluorescent dyes such as thiazole orange,[15] fluorescein,[16] and bodipy[17] into hairpin polyamides is well documented. The results from the DNA binding studies of such fluorophore-polyamide hybrids have demonstrated that such compounds can induce fluorescence while maintaining a high degree of specificity and affinity upon binding to their cognate DNA sequences. However, nuclear localization of such molecules has been subject to a number of restrictions, which include DNA sequence, position of imidazole residues, dye choice, position of attachment, and hairpin linker composition.[8,9a−9c] In addition, the behavior of the nonfluorescing hairpins did not always mimic the behavior of its fluorescing counterpart thus limiting the usefulness of this approach. Thus, there is clearly a need for the development of polyamide fluorophores that have increased cell and nuclear penetration abilities. To address this issue, a number of hairpin polyamides have been reported wherein the fluorescing component is incorporated as a heterocyclic recognition element.[18]

The bis-benzimidazole fluorophores, such as bisbenzimide or Hoechst 33258, are known to selectively recognize A/T-rich sequences in the DNA minor groove and are capable of crossing certain cellular and nuclear membranes.[19] To this end, Bruice and co-workers have developed hairpin tripyrrole/imidazole-Hoechst conjugates and have shown their capability of passing through NIH 3T3 cell membranes and inhibiting DNA transcription factor (TF) binding.[19] Although a number of predetermined DNA sequences can be targeted through the design of hairpin polyamides through obeying the established pairing rules, their poor cellular uptake still remains a drawback. On the basis of the sequence specificity and high affinity of formamido-containing polyamides for the minor groove as ascertained from our previous studies,[20] we envisioned the incorporation of a *p*-anisylbenzimidazolecarboxamido (Hx) moiety of Hoechst 33258 attached to imidazole−pyrrole, pyrrole−pyrrole, or pyrrole−imidazole heterocyclic units (**2**, **3**, and **4** respectively; Figure 1). Because of the aforementioned specificity and affinity of Hoechst 33258 for A/T-rich sequences, we anticipated that the Hx component of the hybrid polyamide should behave similarly to a non-formamido, terminal diheterocyclic unit (PP), thus, binding over two A/T base pairs. In effect, the Hx component also behaves similarly to a formamido-pyrrole (f-P) unit found in formamido-containing polyamides since a stacked f-P unit also recognizes two A/T base pairs similar to a PP unit but with enhanced binding affinity.[20b,f]

Because of the absence of an *N*-terminus formamido group as found in distamycin (**1**), and with reference to the aforementioned pairing rules, polyamides **2**, **3**, and **4** (Figure 1) are anticipated to form an overlapped stacked dimer and bind in a 2:1 fashion to their cognate sequences 5′-AAATTT-3′, 5′-ATCGAT-3′, 5′-ACATGA-3′ respectively.[20] Three control nonfluorescing polyamides PPPP (**5**),[21,22] PPIP (**6**), and PPPI (**7**)

were also synthesized which should target the same sequences. The binding affinities, minor groove and sequence specificity and fluorescence properties of **2**−**7** were studied against their cognate sequences and a negative control sequence 5′-ACGCGT-3′ using thermal melts (ΔT_M), circular dichroism (CD), surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), DNase I footprinting, and fluorescence emission studies. In addition, the binding properties of the Hx compounds were also compared to their formamido−triamide counterparts, f-PPP **8**,[20f] f-PIP **9**,[20c,f] f-PPI **10**.[20f]

EXPERIMENTAL SECTION

Thermal Denaturation (ΔT_M) Studies. DNA oligomers were purchased from Operon with the following sequences: **A_3T_3_10**; 5′-CGAAATTTCCCTCTGGAAATTTCG-3′; **TCGA_10**, 5′-5′-GAATCGATTGCTCTCAATCGATTC-3′; **ACGCGT**; 5′-GAACGCGTCGCTCTCGACGCGT-TC-3′; **CATG_10**; 5′-GAACATGTTGCTCTCAACATGTTC-3′ and **CTAG_10**; 5′-GAACT-AGTTGCTCTCAACTAGTTC-3′. Thermal denaturation studies were performed by following an earlier published procedure.[23] Experiments for polyamides **2**−**7** were performed at a concentration of 3 μM ligand and 1 μM DNA. All experiments were run in $PO_4$0 buffer (10 mM sodium phosphate, 1 mM EDTA, pH 6.2). For the salt-effect experiments, NaCl was added accordingly to obtain Na^+ concentrations of 25, 200, and 400 mM. Oligonucleotide samples were reannealed prior to denaturation studies by heating at 70 °C for 1 min then allowing them to cool to rt. Experiments were typically performed between 25 and 95 °C, with a heating rate of 0.5 °C min^{-1} while continuously monitoring the absorbance at 260 nm (digitally sampled at 200 ms intervals). All melts were performed in 10-mm path length quartz cells. T_M values were determined as the maximum of the first derivative.

Circular Dichroism (CD) Studies. CD studies were performed using previously reported procedures[20b,24] and were conducted at ambient temperature in a 1-mm path length quartz cell using $PO_4$0 buffer. Buffer and stock DNA were added to the cuvette to give a final DNA concentration of 9 μM. Each polyamide (in 500 μL in double distilled H_2O) was titrated in 1 molar equivalents into the relevant DNA (160 μL of 9 μM DNA) until saturation was achieved. Each run was performed over 400−220 nm. The CD response at the λ_{max} of the induced peak was plotted against the mole ratio of ligand/DNA.

Emission Studies. The experiments were performed using a SPEX Fluoro-Log 2 spectrofluorometer to measure the emission spectrum on the binding of Hx-polyamides **2** and **3** to their respective cognate sequences. A 20 μM solution of DNA in $PO_4$0 buffer was introduced to a 1 mm path length cuvette. The polyamides were subsequently added to the DNA solutions in 1 mol equivalent aliquots past the point of saturation. The emission spectrum was recorded upon excitation of the Hx moiety in the complex at 322 nm; the λ_{max} of emission spectrum was measured at 370 nm. KaleidaGraph was then used to analyze and plot the data.

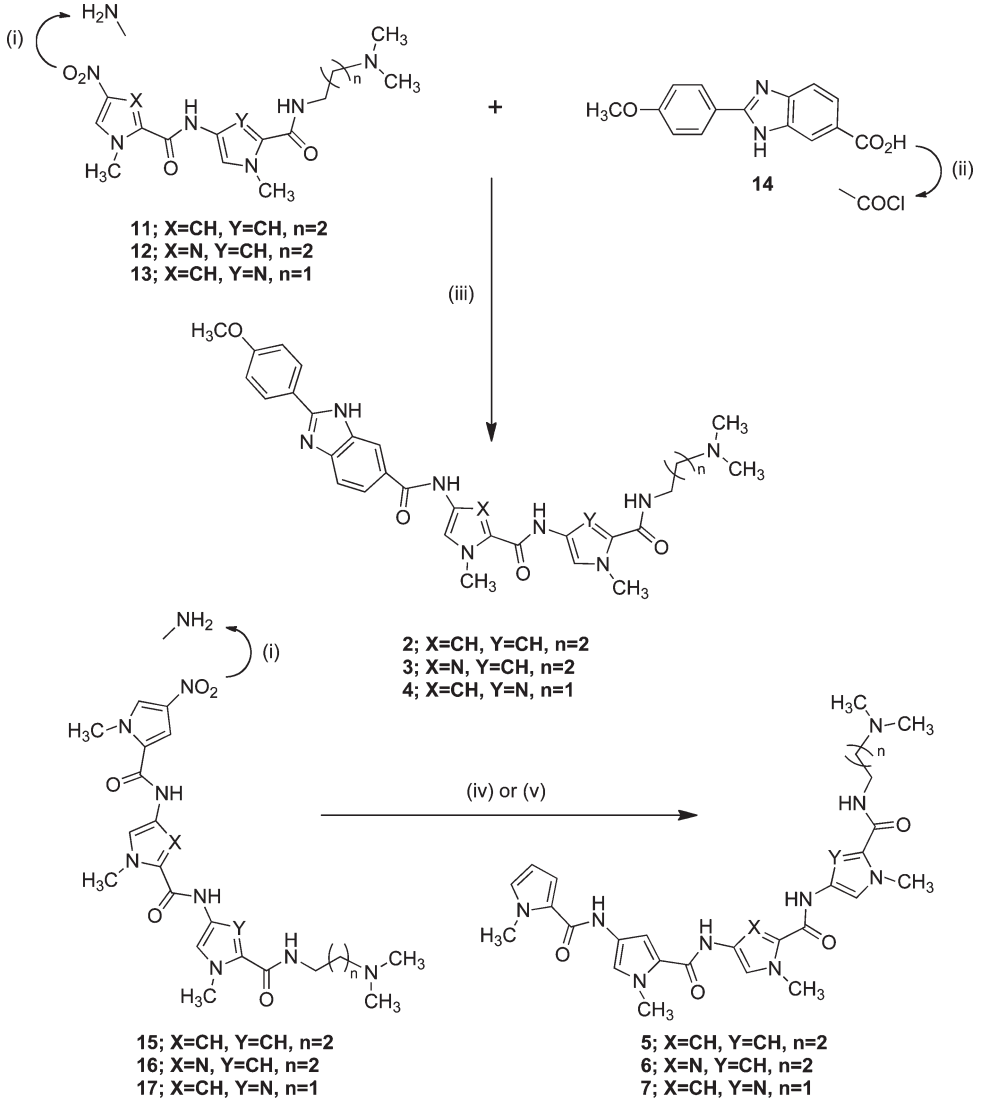
Biosensor-Surface Plasmon Resonance (SPR). SPR measurements were performed with a four-channel BIAcore T100 optical biosensor system (Biacore, GE Healthcare Inc.). 5′-Biotin-labeled DNA hairpin duplex samples were immobilized onto streptavidin−coated sensor chips (BIAcore SA) as previously described.[20] Three flow cells were used to immobilize the DNA oligomer samples, while a fourth cell was left blank as a control. The SPR binding experiments were performed in 0.01 M cacodylic acid (CCA) solution at pH 6.25 containing 0.001 M EDTA (disodium ethylenediamine tetraacetate), 0.1 M NaCl, and 0.005% v/v surfactant P20 (Biacore AB) was used to reduce the nonspecific binding of polyamides to the fluidics and sensor chip surface. A 0.01 M *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) solution at pH 7.4 containing 0.15 M NaCl, 0.003 M EDTA, and 0.005% v/v surfactant P20 was used during the DNA immobilization process. All buffers were degassed and filtered prior to experiments. DNA sequences were obtained from Integrated DNA Technologies (San Diego, CA) with HPLC purification and were used without further purification. The lyophilized 5′ biotin-labeled DNA hairpin constructs were dissolved in the appropriate amount of DI H_2O to create 1.0 mM DNA stock solutions. Further dilutions were made using 0.01 M HEPES buffer during the DNA immobilization step. DNA concentrations were determined spectrophotometrically using molar absorptivity coefficients calculated for each individual DNA sequence by using the nearest neighboring method for the singled-stranded DNA method. Stock solutions of **4** and **5** were prepared by dissolving the solid compound in the necessary amount of distilled H_2O to create a stock solution with a concentration of approximately 1.0×10^{-3} M. Stock solutions were kept frozen at 4 °C until experimental use to minimize any degradation of the compound. Samples for SPR experiments were prepared by a series of dilutions from the stock solution using a 0.01 M cacodylic acid buffer solution. The SPR experiments were conducted using a four-flow cell BIAcore 2000 biosensor instrument (GE Life Sciences). DNA hairpin constructs labeled with biotin at the 5′ end were immobilized onto a streptavidin-coated sensor chip (BIAsensorSA) as previously reported.[20a,b,24] The 5′-biotin-labeled DNA hairpins were immobilized on three of the four flow cells. The fourth was left blank and used as a control. All SPR experiments were performed at 25 °C and used 0.01 M CCA as the running buffer. The amount of DNA immobilized was approximately 400 response units (RU). This was achieved by continuously injecting ∼20 μL of an approximately 50 nM DNA solution at a rate of 2 μL/min onto the sensor chip surface until a relative response of 400 units was reached. Binding data were obtained by injecting known concentrations and were analyzed with one or two site binding models as previously described.[20a,b,24]

$$r = (K_1{*}C_{free} + 2{*}K_1{*}K_2{*}C_{free}{}^2)/(1 + K_1{*}C_{free} + K_1{*}K_2{*}C_{free}{}^2)$$

where r represents the moles of bound compound per mole of DNA hairpin duplex, K_1, K_2 are macroscopic binding constants, and C_{free} is the free compound concentration in equilibrium with the complex.

Isothermal Titration Calorimetry (ITC). ITC analysis was performed at 25 °C using a VP-ITC microcalorimeter (MicroCal, Northampton, MA) as previously reported.[25] After the system was equilibrated to 25 °C, and after an initial delay of 300 s, the polyamide (50 μM) was titrated 50 times (3 μL for 7.2 s, repeated every 240 s) into 2 μM DNA. The solution was constantly mixed at 300 rpm. The reference cell contained $PO_4$0 or $PO_4$20 as appropriate for the experiment. Origin 7.0 was used to analyze the data, where the integration of each titration was plotted against ligand concentration. A linear fit was then employed, and this was subtracted from the reaction integrations to normalize for nonspecific heat components. A two-site, nonsequential model was then fitted to the curve and used to determine the binding constant, K_{eq}.[25]

Scheme 1. Synthesis of Polyamides 2−7[a]



[a] Conditions and reagents (i) **15**, **16**, or **17**; 5% Pd/C, H_2, MeOH, rt 12 h; (ii) **14** in $SOCl_2$/THF (1:1), 90°C, 1.5 h; (iii) add Hx acid chloride to amine, Et_3N, DCM, 0 °C then stir 12 h rt; (iv) *N*-methylpyrrole-2-carboxylic acid, EDCI, DMAP, DCM, stir rt 3 days; (v) add 1-methylpyrrole-2-carbonyl chloride to amine, Et_3N, DCM, 0 °C to rt, 12 h.

DNase1 Footprinting Studies with DNase I and Micrococcal Nuclease. Radiolabeled DNA fragments of 131 base pairs each, designed to contain the cognate sites of the Hx compounds and their nonfluorescent tertaamide counterparts were generated by polymerase chain reaction, as described previously.[26] The amplified fragments were purified on a Bio-Gel *P6* column followed by agarose gel electrophoresis and isolated using the Mermaid Kit (MP biomedicals) according to the manufacturer's instructions.

Micrococcal nuclease digestions were conducted in a total volume of 8 μL. The labeled DNA fragment (2 μL, 200 counts s^{-1}) was incubated for 30 min at room temperature in 4 μL TN binding buffer (10 mM Tris Base, 10 mM NaCl, pH 7) containing the required drug concentration. Cleavage by MNase (NEB) was initiated by addition of 2 μL of MNase (5U) diluted in 1× micrococcal nuclease reaction buffer (50 mM Tirs-HCl, 5 mM CaCl, pH 7.9) followed by incubation at 37 °C and stopped after 5 min by snap freezing the samples on dry ice.

DNase I digestions were conducted in a total volume of 8 μL. In each case, the labeled DNA fragment (2 μL, 200 counts s^{-1}) was incubated for 30 min at room temperature in 4 μL of TN binding buffer (10 mM Tris Base, 10 mM NaCl, pH 7) containing the required drug concentration. Cleavage by DNase I was initiated by addition of 2 μL of DNase I solution (20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, DNase I 0.02U, pH 8.0) and stopped after 3 min by snap freezing the samples on dry ice.

The nuclease-digested samples were subsequently lyophilized to dryness and resuspened in 5 μL of formamide loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Following heat denaturation for 5 min at 90 °C, the samples were loaded on a denaturing polyacrylamide (10%) gel (Sequagel, National Diagnostics, UK) containing urea (7.5 mM). Electrophoresis was carried out for 2 h at 1650 V (~70 W, 50 °C) in 1× TBE buffer. The gel was then transferred onto Whatman 3MM and dried under a vacuum at 80 °C for 2 h.

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The gel was exposed overnight to Fuji medical X-Ray film and developed on a Konica Medical Film Processor SRX-101A.

Molecular Modeling Studies. The Gaussian 09 software package was used to perform geometry optimizations, generate electrostatic potential surfaces, and calculate dipole moments of Hx and PP.[27a] Specifically, the calculations were conducted on the carboxamide derivative of Hx-acid **14** (Hx-carboxamide), and 4-(1-methylpyrrole-2-carboxamido)-1-methylpyrrole-2-carboxamide (PP-carboxamide). All calculations were performed at the B3LYP level with the 6-31G(d) basis set. WebMO was used as the interface to prepare all calculations, which were conducted on the MU3C cluster computer at Hope College.[27b]

RESULTS AND DISCUSSION

Chemistry. The three Hx-substituted polyamides **2**, **3**, and **4** were synthesized as described in Scheme 1. Reduction of the nitro group of the diheterocyclic intermediates O_2N-PP-*N,N*-dimethyl-3-aminopropylamine **11**, O_2N-IP-*N,N*-dimethyl-3-aminopropylamine **12**, and O_2N-PI-*N,N*-dimethyl-2-aminoethylamine **13** over 5% Pd/C in methanol furnished the amine intermediates, which were immediately coupled to *p*-anisylbenzimidazole-2-carbonyl chloride which, in turn, was synthesized by refluxing the corresponding carboxylic acid **14**[28] in a 1:1 mixture of thionyl chloride and dry THF. The desired polyamides **2**−**4** were isolated in ∼20% yield. The low yields can be attributed to the instability of the amine intermediates.[29] For the synthesis of **5** (PPPP), an EDCI-mediated coupling of *N*-methylpyrrole-2-carboxylic acid to the reduced form of the nitro triamide **15** was performed. The other two nonfluorescent control non-formamido tetraamides **6** and **7** were synthesized utilizing Schotten-Baumann conditions from the reduction of their respective nitro-triamides **16** and **17** followed by coupling to *N*-methylpyrrole-2-carbonyl chloride (which in turn was synthesized from *N*-methylpyrrole-2-carboxylic acid and $SOCl_2$).

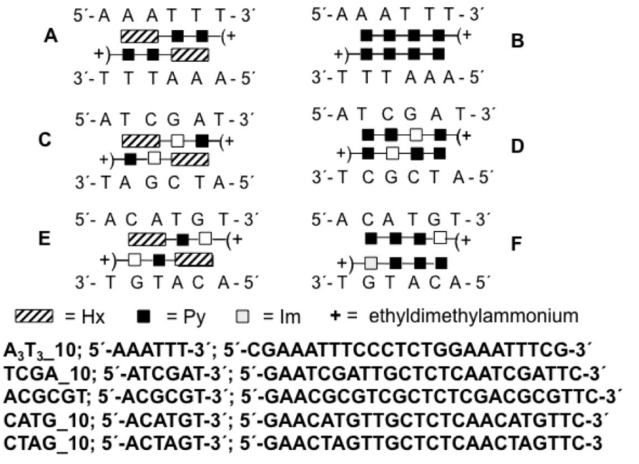

Figure 2. Proposed sequence specific recognition of polyamides **2**−**7** to their target (cognate) DNA sequences. Molecules are shown binding in an antiparallel, stacked motif. **A** = HxPP (**2**), **B** = PPPP (**5**), **C** = HxIP (**3**), **D** = PPIP (**6**), **E** = HxPI (**4**), and **F** = PPPI. A_3T_3_10 is also referred as 5′-AAATTT-3′; likewise TCGA_10 is 5′-ATCGAT-3′; ACGCGT is 5′-ACGCGT- 3′; CATG_10 is 5′-ACATGT- 3′; and CTAG_10 is 5′-ACTAGT-3′.

Thermal Denaturation Studies. The binding of the Hx-substituted polyamides **2**−**4** along with their nonfluorescing non-formamido tetraamides **5**−**7** was tested using the DNA sequences shown in Figure 2 in thermal denaturation experiments. An excess of polyamides (3 μM) to DNA (1 μM) was used in these studies, and in all cases the concentration of DNA used was identical. The DNA sequences examined contained the following sequences: 5′-AAATTT-3′ (A_3T_3_10), the cognate site for distamycin A and that anticipated for **2**, **5**, and **8**, 5′-ATCGAT-3′ (TCGA_10) (anticipated to be the preferred binding sequence for **3**, **6** and **9**) and 5′-ACATGT- 3′ (CATG_10) along with 5′-ACTAGT-3′ (CTAG_10) (anticipated to be the preferred binding sequences for **4**, **7**, and **10**). In addition, a GC-rich sequence 5′-ACGCGT- 3′ (ACGCGT) was used as a negative control. These thermal denaturation experiments screened the preferred binding sequences of the fluorescent hybrid polyamides **2**−**4** along with their nonfluorescing counterparts **5**−**7** by measuring their ability to stabilize each double-stranded DNA upon binding. ΔT_M values were determined from the differences in melting temperature of the DNA−polyamide complexes and duplex DNA alone and are shown in Table 1. For comparative purposes, the reported ΔT_M value for distamycin A against its cognate sequence A_3T_3_10 is 14 °C.[25]

The data presented in Table 1 indicate that the Hx-substituted polyamides **2**−**4** bind more strongly to their cognate sequences ($\Delta T_M \gg 10$ °C) relative to their nonfluorescent counterparts, non-formamido tetraamides **5**−**7**, and the formamido-triamides **8**−**10**[20c,f] (both $\Delta T_M \ll 10$ °C). For HxPP (**2**) and HxIP (**3**) (ΔT_M 19 and 15 °C respectively), this is a greater than 3-fold

Table 1. Thermal Denaturation Data and SPR Binding Constants for Polyamides 2−10

	A3T3_10 ΔT_M (°C)[a]; [K_{eq} M^{-1}][b]	TCGA_10 ΔT_M (°C); [K_{eq} M^{-1}]	ACGCGT ΔT_M (°C); [K_{eq} M^{-1}]	CATG_10 ΔT_M (°C); [K_{eq} M^{-1}]	CTAG_10 ΔT_M (°C); [K_{eq} M^{-1}]
HxPP (**2**)	**19; [1 × 10^6]**[c]	10; [<10^3]	0;	- [<1 × 10^3]	
HxIP (**3**)	5; [4 × 10^6][d]	**15; [3 × 10^6]**	0;	- [1 × 10^4]	
HxPI (**4**)	18; [5 × 10^6]	14; [2 × 10^6]	5;	**19; [7 × 10^6]**	**17;**
PPPP (**5**)	**5; [3 × 10^6]**	2;	0; [<1 × 10^5]		
PPIP (**6**)	0; [2 × 10^4]	**4; [4 × 10^4]**	0;		
PPPI (**7**)	4; [3 × 10^6]	0;	0; [<1 × 10^4]	**8; [3 × 10^6]**	**8;**
f-PPP (**8**)[20f]	**9; [3 × 10^6]**				
f-PIP (**9**)[20f]	3; [2 × 10^5]	**0; [2 × 10^5]**			
f-PPI (**10**)[20f]	6; [4 × 10^5]				**1; [1 × 10^5]**

[a] $\Delta T_M = T_M$ (bound DNA) − T_M (free DNA). [b] $K_{eq} = (K_1K_2)^{1/2}$, determined at 25 °C. [c] Cognate sequences are typed in bold face. [d] Denotes a 1:1 binding mode.

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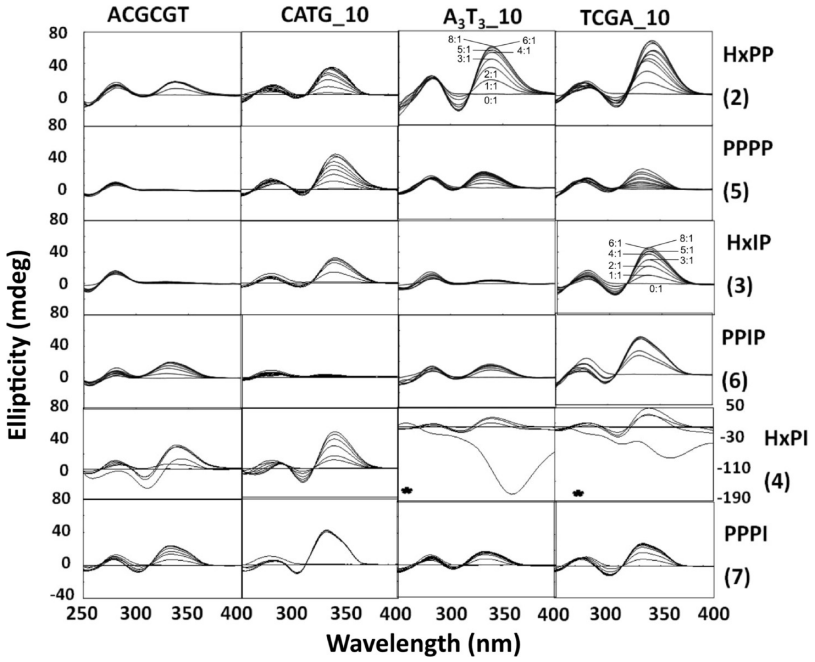

Figure 3. Circular dichroism results for polyamides **2**−**7** with cognate and noncognates DNA sequences. In each experiment, 9 μM DNA in $PO_4$0 was used, and the polyamides were titrated at ratios of 1, 2, 3, 4, 5, 6, and 8 mol equiv of polyamides relative to DNA. A ratio of 0:1 indicates DNA alone. Note that the CD (mdeg) scale on the right-hand-side of the figure is applicable to the spectra marked with an asterisk.

increase in melting temperature relative to that given by PPPP (**5**) and PPIP (**6**) with A_3T_3_10 (ΔT_M 5 °C) and TCGA_10 (ΔT_M 4 °C), respectively. The ΔT_M values for f-PPP (**8**) and f-PIP (**9**) were reported at 9 and 0 °C, respectively.[20c,f] These results indicate that the Hx group contributes favorable stabilizing properties when it is bound to its cognate DNA sequence. In addition, no ΔT_M was observed for the GC-rich sequence ACGCGT, which contains a flanking G/C base pair toward the 5′ and 3′ termini of the oligonucleotide. This suggests that the Hx group, when paired with a pyrrole unit, prefers A/T base pairs over G/C base pairs.

HxPI (**4**) gave a greater than 2-fold increase in melting temperature (ΔT_M > 10 °C) over 7 upon binding to the degenerate cognate sites 5′-ACATGT-3′ and 5′-ACTAGT-3′. For 5′-ACTAGT-3′, the ΔT_M for f-PPI (**10**) was reported at 1 °C.[20f] As f-PPI (**10**), HxPI (**4**) was found to bind to the noncognate 5′-AAATTT-3′ site (ΔT_M 18 °C at A_3T_3_10 versus 19 and 17 °C at 5′-ACATGT-3′ and 5′-ACTAGT-3′, respectively) indicating tolerance of AT/TA over CG/GC base pairs. In addition HxPI (**4**) gave a ΔT_M value of 14 °C upon binding to the noncognate alternative GC/CG containing 5′-ATCGAT-3′ site while in comparison, it gave a ΔT_M value of 5 °C for binding to 5′-ACGCGT-3′. This suggests that the benzimidazole (of Hx)/P pairing has preference for A/T base pairs at the 5′ and 3′-flanks of the hexamer sequence. The poor binding of **4** to ACGCGT provides additional support for the binding preference. Overall, the T_M data for polyamides **2**−**4** suggest that the *p*-anisylbenzimidazolecarboxamido (Hx) moiety binds to two AT or TA base pairs with a higher affinity than that of a PP heterocyclic unit and an f-P unit. Although it should be stated that the shift in T_M upon binding is a complicated function of the enthalpy of DNA melting alone, the ligand binding affinity and stoichiometry, the ligand binding enthalpy, and the ligand concentration.

Circular Dichroism Studies. CD spectroscopy was used to probe the binding of polyamide **2**−**7** in the minor groove of double-stranded DNA. CD assays were conducted by titrating each ligand to DNA solutions comprised of the DNA sequences tested in the thermal denaturation studies (one cognate and three control DNA sequences). In all cases, a fixed DNA concentration of 9 μM was used as were the ratios of polyamides (1, 2, 3, 4, 5, 6, and 8 molar equivalents) titrated into the solution. The results given in Figure 3 show that both Hx polyamides **2** and **3** bind effectively to their respective cognate sequences 5′-AAATTT-3′ and 5′-ATCGAT-3′ as shown by the strong DNA-induced ligand bands at ∼330 nm along with the appearance of a clear isodichroic point in the CD overlaid spectra. These results are corroborated by the aforementioned ΔT_M values for **2** and **3** and their respective cognate sequences (Table 1). Furthermore, HxIP (**3**) gave no induced CD band when titrated to its noncognate sequences 5′-AAATTT-3′ and 5′-ACGCGT-3′. A weak induced CD band was observed for the binding of **3** with the other noncognate sequence 5′-ACATGT-3′. Interestingly, the non-Hx tetramer PPIP **6** gave comparable CD results to its Hx counterpart **4**. The non-Hx tetramer **6** (PPIP) also shows the weakest induced CD. Consistent with thermal denaturation studies, the results suggest that the Hx moiety may be binding with higher affinity than that of two consecutive pyrrole moieties. On the other hand, HxPP (**2**) produced induced CD bands when titrated to the noncognates 5′-ACATGT-3′ and 5′-ATCGAT-3′ (Figure 3). However, this is more or less in correlation with its counterpart PPPP (**5**), which demonstrates binding to these noncognate sequences (Figure 3). In contrast to **2** and **3**, HxPI (**4**) shows a moderate DNA-induced band upon binding to its cognate 5′-ACATGT-3′ although a ΔT_M value of 19 °C was observed (Table 1). For the noncognates 5′-AAATTT-3′, 5′-ACGCGT-3′, and 5′-ATCGAT-3′, **4** appears to be binding in a differential fashion to the non-Hx tetramer PPPI (**7**) as

shown by the two CD spectra marked by an asterisk in Figure 3. This suggests a mole ratio of ligand/DNA of >33. In the complex, the induced CD spectra changed drastically indicating a significant change in the conformation of the DNA, possibly due to formation of a higher ordered structure.[30,31] Despite this unexpected binding behavior of **4**, the thermal denaturation data indicate that **4** binds with higher specificity to cognate 5′-ACATGT-3′ than **7**. In summary, comparison of the Hx-substituted molecules with the control tetramers suggests there is no loss of binding affinity or specificity upon incorporation of the Hx moiety into these polyamides, in turn, indicating that these molecules are reasonable analogues of pyrrole/imidazole polyamides. In addition, this data also suggest that the Hx moiety can act as two consecutive pyrrole (PP) heterocyclic moieties and f-P units[20c,f] in accordance with the aforementioned pairing rules.

SPR and ITC Studies. The binding constants (K_{eq}) of the Hx-containing compounds were determined using a surface plasmon resonance (SPR) biosensor assay. The studies were conducted using similar DNA sequences as those used in the DNA melting and CD titration studies. The sensorgrams recorded from the SPR experiments for the Hx compounds are given in Figure 4. According to the sensorgram in Figure 4A, it is evident that Hx-PP (**2**) showed preference for 5′-AAATTT-3′ (K_{eq} = 1 × 10^6 M^{-1}, Table 1) and avoided a G/C-containing DNA sequence, 5′-AATGAT-3′. It would not even tolerate a single G/C mismatched sequence. Similarly, the sensorgram for Hx-IP **3**, given in Figure 4B, demonstrated a preference for its cognate 5′-ATCGAT-3′ (K_{eq} = 4 × 10^6 M^{-1}) and avoided a mismatched sequence, 5′-AATGAT-3′. HxPI (**4**) showed a preference for its cognate 5′-ACATGT-3′ (K_{eq} = 7 × 10^6 M^{-1}) as exhibited by the slow off-rates in the sensorgram (Figure 4C). The binding constant of compounds **2** and **3** were ascertained from fitting the binding data to a two-site model and the values are reported in Table 1. In comparison, the nonfluorescing non-formamido tetraamides **5**−**7** gave lower binding constants in the 10^5 M^{-1} range (Table 1), in agreement with the biophysical data discussed previously. Likewise, the reported binding constants for formamido-triamides **8**−**10**[20c,f] were also lower that their Hx-counterparts but higher than the non-formamido tetraamides. This finding is significant because thus far structural modifications of the *N*-terminus formamido group in polyamides have resulted in lowering affinity. The Hx group stands alone as a heterocyclic moiety that imparts higher binding affinity to the cognate sequence compared to the formamido-pyrrole (f-P) unit.

In further thermodynamic studies on the Hx-containing compounds, polyamides **2**−**4** were titrated to their cognate DNA sequences and monitored by isothermal titration calorimetry (ITC) studies at 25 °C (data not shown).[25] In comparison, titration of distamycin to 5′-AAATTT-3′, done under identical conditions gave a negative enthalpy, with a ΔH comparable to the reported value.[28] The results provided evidence that the enthalpy of binding for all three compounds **2**−**4** were essentially zero, suggesting the interactions were driven by entropy. Using the binding constants determined from SPR studies, the free energies of binding were calculated from the equation ($\Delta G = -RT \ln K_{eq}$, R = 1.987 cal mol^{-1} K^{-1}, and T = 298 K). The ΔG for polyamide **2** for 5′-AAATTT-3′, **3** for 5′-ATCGAT-3′, **4** for 5′-ACATGT-3′ are 8.2, 8.8, and 9.3 kcal mol^{-1}.

Emission Studies. Fluorescence emission studies were performed on **2** and **3** to confirm the pattern of induced fluorescence of the Hx fluorophore upon binding to dsDNA. 20 μM of DNA was used in these studies and the ratios of polyamides titrated were 1, 2, 3, 4, 5, and 6 mol equiv. The Hx-compound was excited

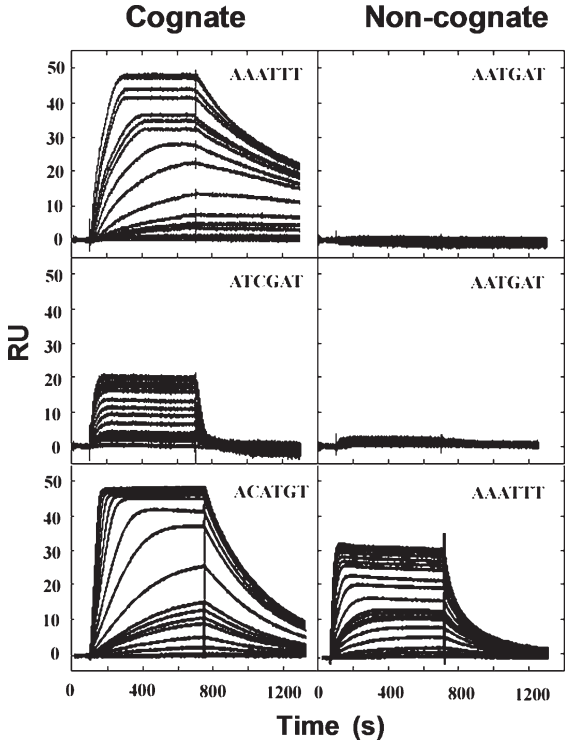

Figure 4. Representative SPR sensorgrams for the interaction of (top) Hx-PP, (middle) Hx-IP, and (bottom) Hx-PI with cognate and non-cognate DNA sequences. The compound concentrations from bottom to top are 0−1 μM in all plots and the experiments were performed as described in the Experimental Section.

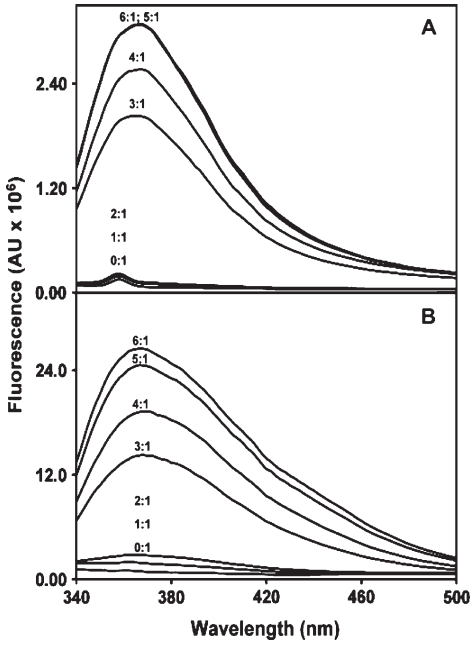

Figure 5. Emission studies on **2** (A) and **3** (B). Aliquots of polyamide solutions were titrated into the 20 μM solution of DNA in $PO_4$0 buffer at ratios of 1, 2, 3, 4, 5, and 6 mol equiv of polyamides relative to DNA. A ratio of 0:1 denotes DNA alone. HxPP (**2**) was titrated to its cognate sequence 5′-AAATTT-3′ and HxIP (**3**) was titrated to its cognate sequence 5′-ATCGAT-3′.

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at 322 nm, based on results from UV−vis spectroscopy of the Hx-polyamide compound. Figure 5 illustrates that upon binding to their cognate sequences, both Hx-PP and Hx-IP showed strong fluorescence with a λ_{max} of 370 nm when bound to their respective 5′-AAATTT-3′ and 5′-ATCGAT-3′ cognate sequence. This behavior of enhancement of emission is consistent with the binding of Hoechst 33258 itself[32] as well as DNA intercalating agents such as ethidium bromide.[33] The enhanced emission can be attributed to the reduction in interaction between the photoexcited bound Hx fluorophore and solvent.[34]

DNase I and Micrococcal Nuclease Footprinting Studies. Footprinting experiments were performed on **2**−**7** using a 131 bp 5′-[^{32}P]-radiolabeled DNA fragments containing the following sequences: (a) 5′-AAATTT-3′; (b) 5′-TTGCAA-3′; (c) 5′-TCTAGA-3′ (an alternative cognate for **4**); (d) 5′-ACTAGT-3′ (another alternative cognate for **4**); and (e) 5′-TCTTGA-3′. Autoradiogram A of a micrococcal nuclease footprinting gel (Figure 6A) shows that HxPP (**2**) and PPPP (**5**) exhibit sequence specificity for 5′-AAATTT-3′. Micrococcal nuclease was used rather than DNase I in this case because of the poor

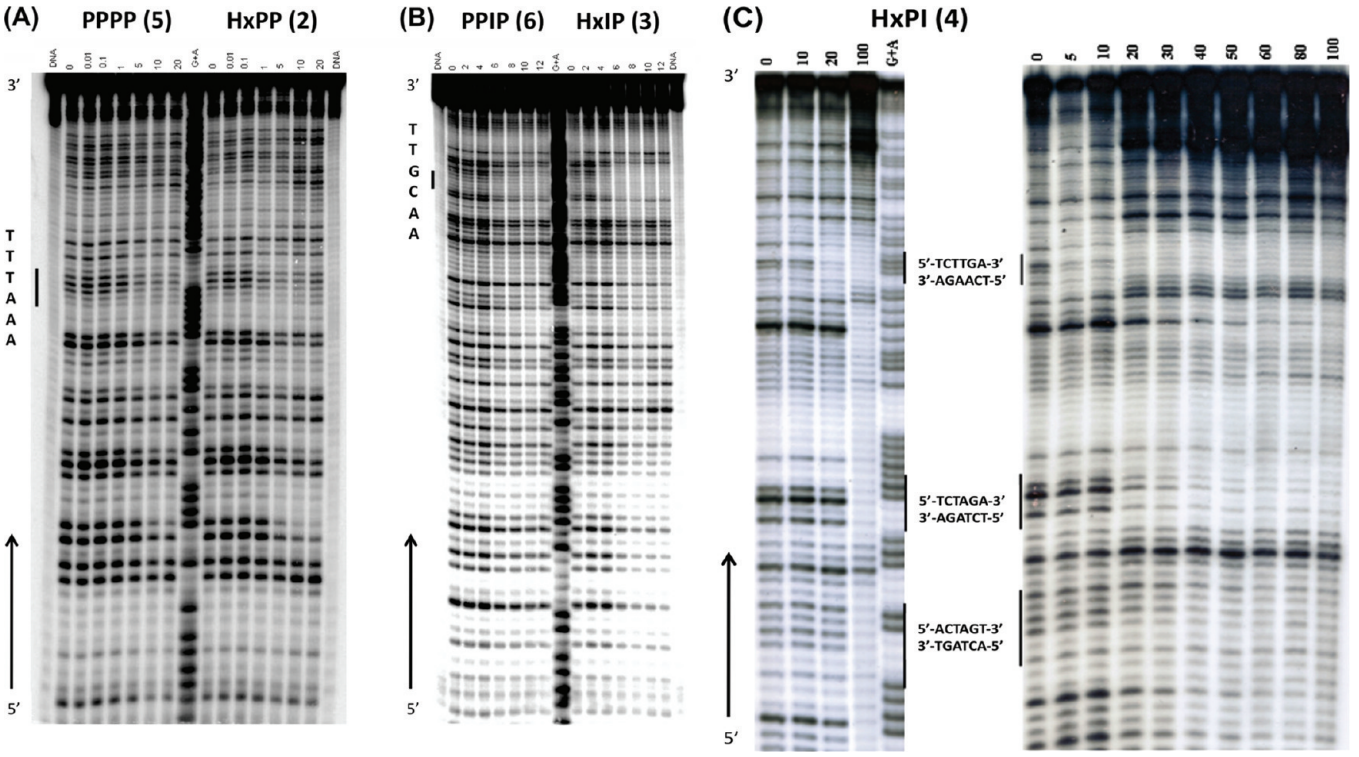

Figure 6. DNA footprinting using micrococcal nuclease (A) or DNase I (B, C) of compounds **2** and **5** (A), **3** and **6** (B) and **4** (C) using three different 131 bp 5′-^{32}P-radiolabeled DNA fragments containing the corresponding cognate sites as denoted on the autoradiograms. DNA is the undigested control, and G + A is the sequencing lane.

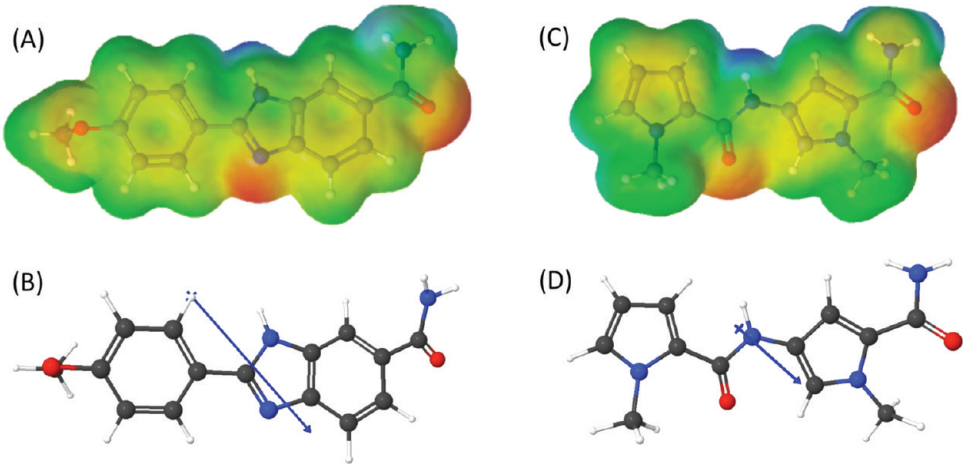

Figure 7. Molecular electrostatic surfaces of Hx-carboxamide (A) and PP-caboxamide (B), and dipole moments of Hx-carboxamide (C) and PP-caboxamide (D). The structures depicted as electrostatic surfaces or dipole moment images are identical including the hybridization of each atom in the molecules.

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digestion of DNase I at AT-rich sequences. For **2**, a footprint begins to appear at 5′-AAATTT-3′ at 1 μM, while for **5**, a footprint starts to appear at 5 μM, which is consistent with the higher binding affinity of HxPP over PPPP observed in the thermal denaturation studies (Table 1). For HxIP (**3**) and PPIP (**6**), autoradiogram B (Figure 6B) shows the appearance of a footprint at 5′-TTGCAA-3′ at 4 μM for **3**, while a weak footprint appears at 6 μM for **6**, consistent with results from SPR studies indicating that HxIP (**3**) binds slightly more strongly than PPIP (**5**) at the 5′-TTCGAA-3′ site. For HxPI (**4**), autoradiogram C (Figure 6C) shows binding at 20 μM at the 5′-ACTAGT-3′ and 5′-TCTTGA-3′ cognate sites. The binding to the 5′-TCTAGA-3′ site occurs at a slightly higher concentration (30−40 μM) suggesting the ability of HxPI to discriminate an A/T base pair in the context of the respective sequences. Overall, these data indicate that the Hx moiety clearly behaves similarly to two consecutive pyrrole heterocyclic moieties, and this, in turn, is in accordance with the thermal denaturation and SPR data.

Molecular Modeling Studies. Electrostatic potential surfaces of each of the molecules studied and depicted in Figure 7A and B show distinct electropositive regions where the molecule binds to the minor groove. Qualitatively, the Hx-carboxamide surfaces resemble those of PP-carboxamide, and the dipole moments for both molecules are also in alignment (Figure 7C,D). These results provide a theoretical framework for supporting the resemblance of Hx in recognizing two consecutive A/T base pairs in the same manner as PP. It is worthy to note that the magnitude of dipole moment for Hx is significantly larger than that for PP (5.845 versus 2.813 Debye) suggesting a possible contribution toward the higher observed binding affinity of HxIP (**3**) for its cognate sequence over PPIP (**6**).

CONCLUSIONS

The results described in this study indicate that the Hx heterocyclic moiety is a novel DNA recognition element that targets A/T base pairs. Hx-polyamide compounds bind DNA in a highly sequence selective manner, and upon binding they strongly emit blue light upon excitation at 322 nm. The Hx moiety when incorporated into polyamides provides a direct means to measure polyamide binding in the minor groove of DNA in a test tube or in cells by fluorescence. In addition, the Hx moiety behaves similarly to a two consecutive pyrrole unit such that an Hx/PP pairing found in HxPP (**2**) recognizes two consecutive A/T base pairs. An Hx/IP pairing present in HxIP (**3**) recognizes an A/T.C//G.A/T and an Hx/PI pairing found in HxPI (**4**) recognizes C.A/T//A/T.G sequence, respectively. Overall, the Hx polyamides bind in a similar fashion to the nonfluorophore counterparts: non-formamido tetraamides **5**−**7** and formamido-triamides **8**−**10** but with a higher affinity for their cognate sequences. Given its simple molecular design and ease of synthesis, the Hx moiety represents a significant advancement in the polyamide field. Sequence targeted Hx polyamides such as those synthesized in this article provide a direct and simple way to monitor binding, without the incorporation of a bulky fluorescent tag. The application of such fluorophores can be vital in studying the use of these compounds in living systems.

ASSOCIATED CONTENT

Supporting Information. The synthesis and characterization of compounds **2**−**7** as well as representative DNA melt graphs for HxIP (**3**) are given in this section. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

(ΔT_M), thermal melts; (CD), circular dichroism; (ITC), isothermal titration calorimetry; (SPR), surface plasmon resonance

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