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Novel diamino imidazole and pyrrole-containing polyamides: Synthesis and DNA binding studies of mono- and diamino-phenyl-ImPy∗Im polyamides designed to target 5′-ACGCGT-3′

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ABSTRACT

Pyrrole- and imidazole-containing polyamides are widely investigated as DNA sequence selective binding agents that have potential use as gene control agents. The key challenges that must be overcome to realize this goal is the development of polyamides with low molar mass so the molecules can readily diffuse into cells and concentrate in the nucleus. In addition, the molecules must have appreciable water solubility, bind DNA sequence specifically, and with high affinity. It is on this basis that the orthogonally positioned diamino/dicationic polyamide Ph-ImPy∗Im **5** was designed to target the sequence 5′-ACGCGT-3′. Py∗ denotes the pyrrole unit that contains a N-substituted aminopropyl pendant group. The DNA binding properties of diamino polyamide **5** were determined using a number of techniques including CD, ΔT_M, DNase I footprinting, SPR and ITC studies. The effects of the second amino moiety in Py∗ on DNA binding affinity over its monoamino counterpart Ph-ImPyIm **3** were assessed by conducting DNA binding studies of **3** in parallel with **5**. The results confirmed the minor groove binding and selectivity of both polyamides for the cognate sequence 5′-ACGCGT-3′. The diamino/dicationic polyamide **5** showed enhanced binding affinity and higher solubility in aqueous media over its monoamino/monocationic counterpart Ph-ImPyIm **3**. The binding constant of **5**, determined from SPR studies, was found to be $1.5 \times 10^7\ M^{-1}$, which is ~3 times higher than that for its monoamino analog **3** ($4.8 \times 10^6\ M^{-1}$). The affinity of **5** is now approaching that of the parent compound f-ImPyIm **1** and its diamino equivalent **4**. The advantages of the design of diamino polyamide **5** over **1** and **4** are its sequence specificity and the ease of synthesis compared to the N-terminus pyrrole analog **2**.

1. Introduction

Pyrrole- and imidazole-containing analogs of distamycin are polyamides that bind in the minor-groove of DNA in a stacked fashion at specific DNA sequences.[1] They are potentially useful for targeting and modulating the expression of genes, including those associated with cancer cell growth.[2–4] Formamido(f)-Imidazole(Im)-Pyrrole(Py)-Imidazole(Im) (**1**, Fig. 1) is one such polyamide (PA) molecule that selectively binds 5′-ACGCGT-3′ sequence in the minor-groove of DNA in a 2:1 fashion (PA/DNA), with a binding affinity of $1.9 \times 10^8\ M^{-1}$.[5] The 5′-ACGCGT-3′ sequence is significant because it occurs in the core sequence of the *Mlu1* cell-cycle box (MCB) transcriptional element found in the promoter of the human DBf4 (huDbf4 or ASK, activator of S-phase kinase) gene. Dbf4 is the regulatory subunit of Cdc7 (cyclin dependent 7) kinase, and high levels of this kinase have been implicated for the development of various cancers.[6] The formamido group at the N-terminus of such polyamides has been shown to influence the binding of these compounds by forcing them to stack in a 'staggered', rather than an 'overlapped' motif.[7]

Polyamides in which the formamido group is replaced with a pyrrole or aryl groups at the N-terminus are known to bind in the 'overlapped' fashion; however, with diminished binding affinity but increased sequence selectivity.[8] The non-formamido polyamide (**2**, Fig. 1), which contains a pyrrole moiety at the N-terminus was synthesized while keeping the C-terminus dimethylamino moiety and heterocyclic core (-ImPyIm-) constant.[8] It was found to retain its preference for binding to 5′-ACGCGT-3′, but with enhanced sequence specificity albeit with a 25-fold lower binding affinity ($7.1 \times 10^6\ M^{-1}$) compared to f-ImPyIm (**1**) ($1.9 \times 10^8\ M^{-1}$).[8] This is not surprising since the formamido group is known to confer enhanced binding affinity.[7a,9] With regard to enhanced

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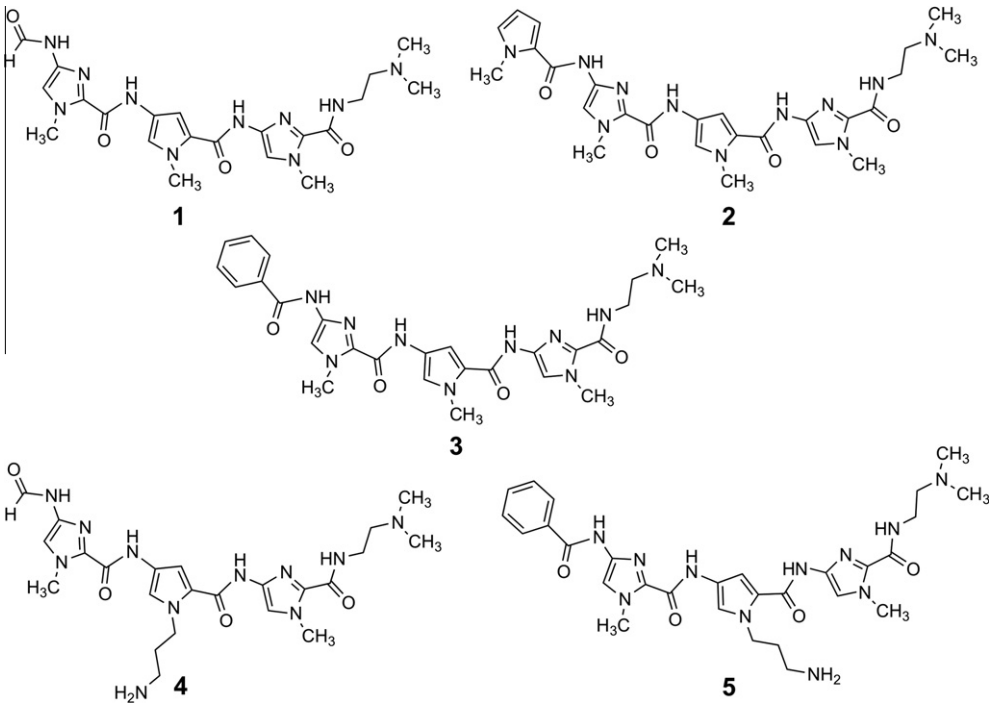


Figure 1. Structures of f-ImPyIm (**1**), tetraamide polyamide PyImPyIm (**2**), monoamino polyamide, Ph-ImPyIm (**3**), an orthogonally positioned diamino polyamide f-ImPy∗Im (**4**) and an orthogonally positioned diamino polyamide Ph-ImPy∗Im (**5**), Py∗ represents the *N*-(3-aminopropyl)-4-amidopyrrole-2-carboxamido moiety.

sequence selectivity, whilst f-ImPyIm (**1**) gave a binding affinity of 2.2×10^5 and 5.3×10^4 M^{-1} for 5′-ACCGGT-3′ and 5′-AAATTT-3′, respectively,[5] PyImPyIm (**2**) did not produce binding to these two sequences at the same concentrations.[8] It is apparent from these results that tetraamide-based structures, such as **2**, are excellent templates for the design of novel DNA sequence specific agents for biological activity, yet having acceptable binding affinity. However, in our hands the synthesis of PyImPyIm (**2**), with a pyrrole moiety at the N-terminus, was cumbersome owing to susceptibility of *N*-methylpyrrole-2-carbonyl chloride to undergo polymerization during the reaction.[10] Our approach to overcome this limitation is to synthesize a novel polyamide Ph-ImPyIm (**3**, Fig. 1) by incorporating a phenyl moiety, in place of pyrrole, in tetraamide **2**. We envisaged that incorporation of a phenyl group, instead of pyrrole, at the *N*-terminus would simplify the process due to the higher stability and ready availability of inexpensive benzoyl chloride. It was also anticipated that the phenyl component of such a polyamide would behave similarly to a non-formamido, N-terminal pyrrole unit (Py), thus binding to A/T base pairs. In addition, benzamides have been reported to function in a similar manner to pyrrole-2-carboxamides in recognizing AT-sequences of DNA.[11,12] To confirm this hypothesis, a hitherto unreported novel polyamide, Ph-ImPyIm (**3**) was synthesized and evaluated for its DNA binding properties.

As part of a systematic study within the authors' laboratory our goal is to develop MCB-targeted polyamides which can be synthesized readily yet possess excellent sequence specificity, stronger binding affinity, high solubility in biological media and enhanced cell penetration and nuclear localization properties. The strategy undertaken in our laboratory to increase the solubility of polyamides is inclusion of an additional amine/ammonium group in the polyamide structure without compromising sequence selectivity. In this strategy, the authors are aware of earlier reports indicating that imidazole-containing polyamides with multiple cationic groups have affinity for A/T rich sequences due to attraction to the negative molecular electrostatic potential in the minor groove of A/T rich sequences.[13,14] Hence, caution must be exercised to develop multiamino polyamides. In this regard, Bruice and Satz have reported that polyamides containing a pyrrole-N1-alkyl spermine/spermidine group target A/T rich sequences and bind with high affinity.[15] These polyamides effectively inhibit the binding of transcription factors to dsDNA compared to other minor groove binders lacking an alkyl-multiamino side chain. It was suggested that upon binding, the additional cationic groups were attracted to the negatively charged phosphodiester groups of DNA. The presence of a second amino group (cationic at physiological pH) into the polyamide design should allow greater penetration in cells due to an increase in solubility of the compounds in biological media. Encouraged by these precedents, an orthogonally positioned diamino polyamide f-ImPy∗Im (**4**) was synthesized in the author's laboratory.[16] Investigation of DNA binding properties revealed that **4** had a binding constant that is 4 times higher than that of its monoamino/monocationic counterpart f-ImPyIm (**1**) (5.4×10^7 M^{-1}). The sequence specificity of **4** was found to be comparable to that of **1**. Incorporation of the propylamino group in polyamide backbone of **4** also offered a significant benefit over its monocationic triamide **1** in terms of enhanced water solubility of its corresponding HCl salt. Hence, to exploit the benefits of having a second positively charged group in the form of an orthogonally positioned alkylammonium side chain, the diamino or potentially dicationic Ph-ImPy∗Im (**5**, Fig. 1) was designed and synthesized, in which Py∗ represents the *N*-(2-aminopropyl)pyrrole moiety. A comparative study of DNA recognition properties of diamino containing polyamide **5** with that of monoamino containing polyamide **3** is presented in Figure 2.

2. Results and discussion

2.1. Synthesis

The monoamino Ph-ImPyIm **3** and the orthogonally positioned diamino/dicationic polyamide **5** were synthesized according to the

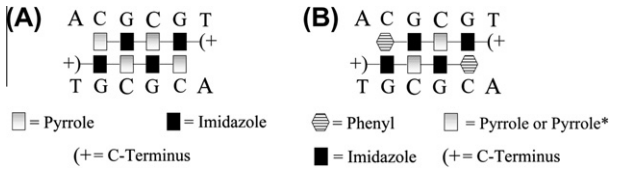


Figure 2. Schematic of PyImPyIm (**4**) (**A**) and phenyl containing polyamides Ph-ImPyIm (**3**) and Ph-ImPy∗Im (**5**) (**B**) binding to the cognate DNA sequence 5′-ACGCGT-3′.

approach depicted in Schemes 1 and 2, respectively. Hydrogenation of ethyl 1-methyl-4-nitroimidazol-2-carboxylate[17] **6** with 5% palladium on charcoal in ethanol followed by coupling of the resulting amine with benzoyl chloride in dry DCM in the presence of dry triethylamine gave ethyl 4-benzamido-1-methylimidazol-2-carboxylate **7** in 96% yield. The ester **7** was hydrolyzed with sodium hydroxide in a methanol/water (1:1) mixture at reflux to obtain the corresponding carboxylic acid 4-benzamido-1-methylimidazole-2-carboxylic acid **8** which was coupled to amino-pyrrole-imidazole diamide **9**[18] using PyBOP and diisopropylethylamine in DMF at room temperature. The reaction afforded the monoamino Ph-ImPyIm (**3**) in 38% yield. Similar coupling of the acid **8** with amino containing diamide **10**[16] yielded chloro compound **11**, which upon treatment with dry ammonia in methanol at 80 °C furnished the desired diamino/dicationic polyamide **5** in 62% yield.

2.2. DNase I footprinting studies

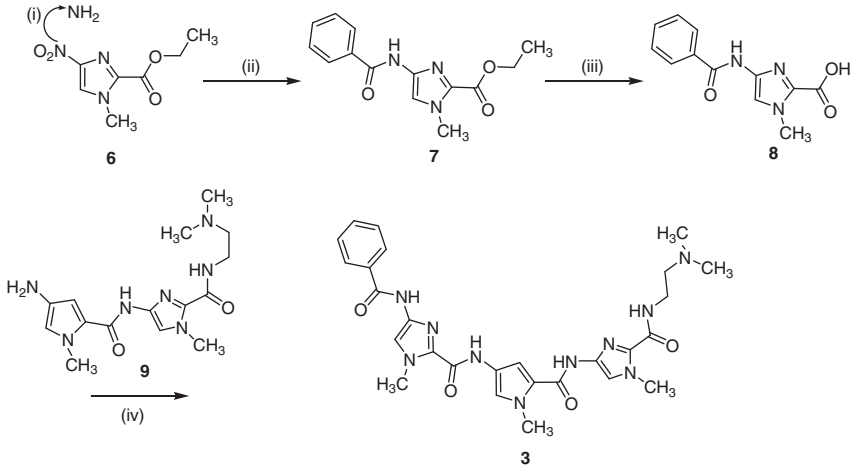
The sequence specificity of the monoamino polyamide **3** and its diamino counterpart **5** was investigated by DNase I footprinting studies[19] using a 125 bp 5′-[32P]-radiolabeled engineered DNA fragment containing the cognate sequence 5′-ACGCGT-3′, and the non-cognate 5′-ACCGGT-3′, 5′-ACACGT-3′ and 5′-AGCGCT-3′. The autoradiogram depicted in Figure 3A shows that the experiment with Ph-ImPyIm (**3**) produced a footprint at the 5′-ACGCGT-3′ site at 20 μM, and no other footprints were apparent even at 100 μM. The results for diamino polyamide **5** demonstrated superior binding affinity compared to its monoamino counterpart **3**. The footprint for 5′-ACGCGT-3′ for diamino polyamide **5** emerged at 0.5 μM (Fig. 3B), which is about 10-fold lower than its formamido parent compound f-ImPyIm (**1**).[5] This is an exciting discovery because having the second amino group made the molecule easier to dissolve in aqueous solution; it has an increased binding affinity, and its binding constant for 5′-ACGCGT-3′ compared to Ph-ImPyIm (**3**) is increased by a factor of about 3. In comparison to f-ImPyIm (**1**), the binding constant of diamino polyamide Ph-ImPy∗Im (**5**) was only 10-fold lower. Overall, the gain in binding affinity of compound **5** over **3** was achieved without compromising on sequence selectivity.

2.3. Thermal denaturation studies

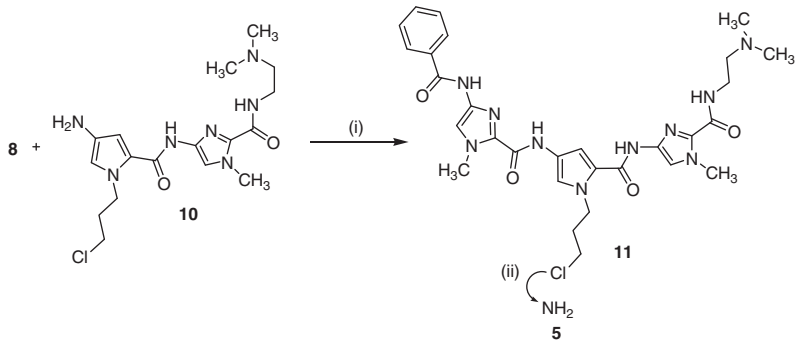
Thermal denaturation experiments were performed to further examine the binding of polyamides **3** and **5** using following DNA sequences; 5′-ACGCGT-3′, 5′-AAATTT-3′ (5′-A_3T_3-3′), and 5′-ACACTT-3′. These thermal denaturation experiments provided an indication on the preferred binding of the monoamino polyamide **3** along with its diamino/dicationic counterpart **5** to the DNA by measuring their ability to stabilize the DNA duplex upon heating. 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. In each case, 1.0 μM of DNA and 3.0 μM of polyamide were used. Comparable to its parent polyamide f-ImPyIm (**1**),[5] the monoamino polyamide **3** showed good binding interaction with the cognate sequence 5′-ACGCGT-3′ by giving a ΔT_M of 9 °C. It did not stabilize the other DNA sequences used in this study as demonstrated by the ΔT_M values expressed in Table 1. These results indicate that the monoamino polyamide **3** possesses selectivity for its cognate DNA sequence 5′-ACGCGT-3′. For the diamino Ph-ImPy∗Im, the ΔT_M value for binding to its cognate sequence 5′-ACGCGT-3′ is remarkable at >25 °C. It is able to stabilize the DNA significantly greater than either its monocationic counterpart **3** or its parent f-ImPyIm (**1**)[5], both of which gave a ΔT_M value of 9 °C.

2.4. Circular dichroism studies

CD spectroscopy was used to probe the binding of polyamides **3** and **5** in the minor groove of double-stranded DNA. These experiments were conducted by titrating each polyamide with DNA solutions comprised of the DNA sequences tested in the thermal denaturation studies. 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. Both polyamides **3** and **5** bind effectively to their cognate sequence 5′-ACGCGT-3′as can be seen from Figure 4. The presence of a strong and positive induced CD band at ~330 nm in CD spectra shown



Scheme 1. Reagents and conditions: (i) H_2, 5% Pd/C, ethanol, rt, 12 h; (ii) benzoyl chloride, TEA, DCM, rt, 12 h; (iii) NaOH, CH_3OH:H_2O (1:1), reflux, 45 min; (iv) PyBOP, DMF, DIPEA, rt, 3 days.



Scheme 2. Reagents and conditions: (i) PyBOP, DMF, DIPEA, rt, 3 days; (ii) dry NH_3, methanol, 80 °C, 12 h.

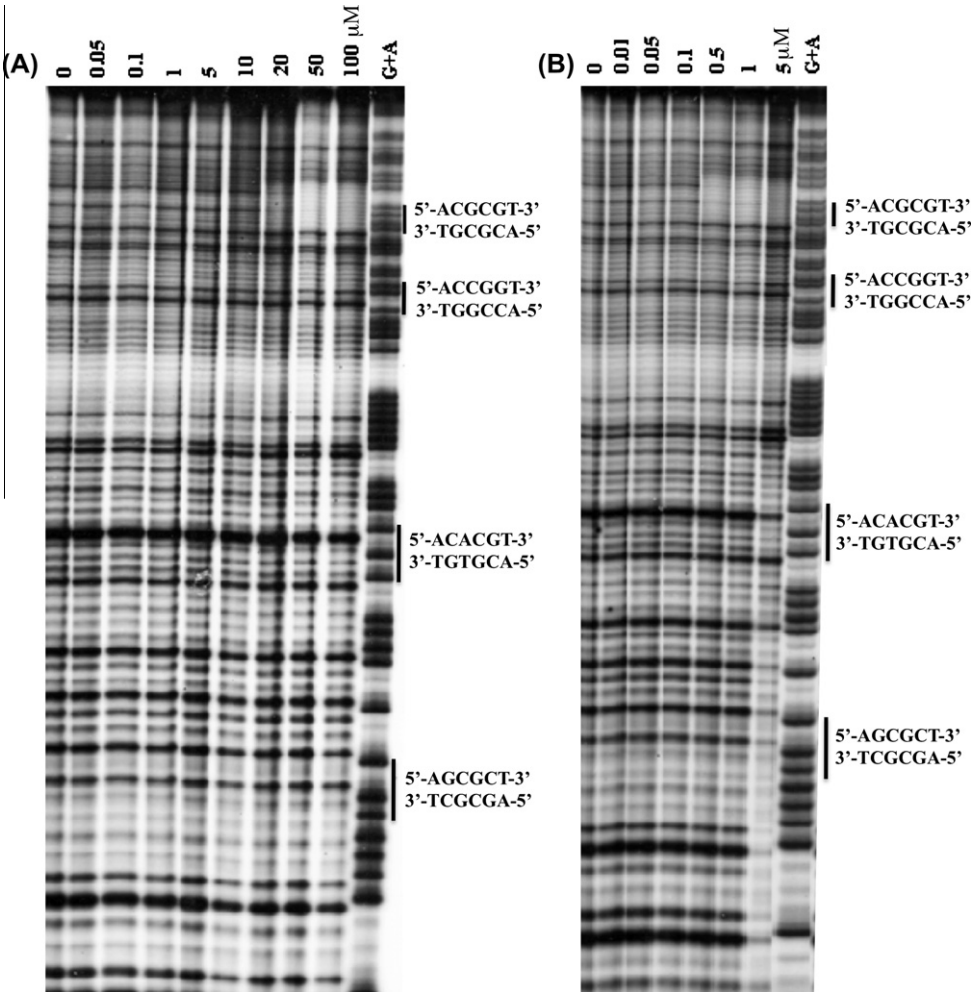


Figure 3. DNase I footprinting of monoamino polyamide Ph-ImPyIm (**3**) (A) and diamino polyamide Ph-ImPy∗Im (**5**) (B).

Table 1
Thermal denaturation data and SPR binding constants for polyamides **3**, **5** and **1**

Polyamide	Footprinting at cognate sequence (μM)	ΔT_M (°C)		
		ACGCGT	AAATTT	ACACTT
3	20	9	0	0
5	0.5	>25	7.6	0
1[a]	0.05	9	1	ND[b]

[a] Data taken from Ref. 5
[b] Not determined.

in Figure 4 suggests minor grove binding for polyamides **3** and **5**. The distinct isodichroic point at ~310 nm provides evidence that both **3** and **5** bind to the DNA sequence by a single mechanism, presumably by interacting in the minor groove as a stacked dimer. These results also indicate that an increase of one positive charge does not significantly affect sequence selectivity. The results also show that polyamide **5** could bind to its non-cognate sequence at the concentrations used in CD experiments and exhibit some tolerance for flanking base pair on either side of the central GC core.

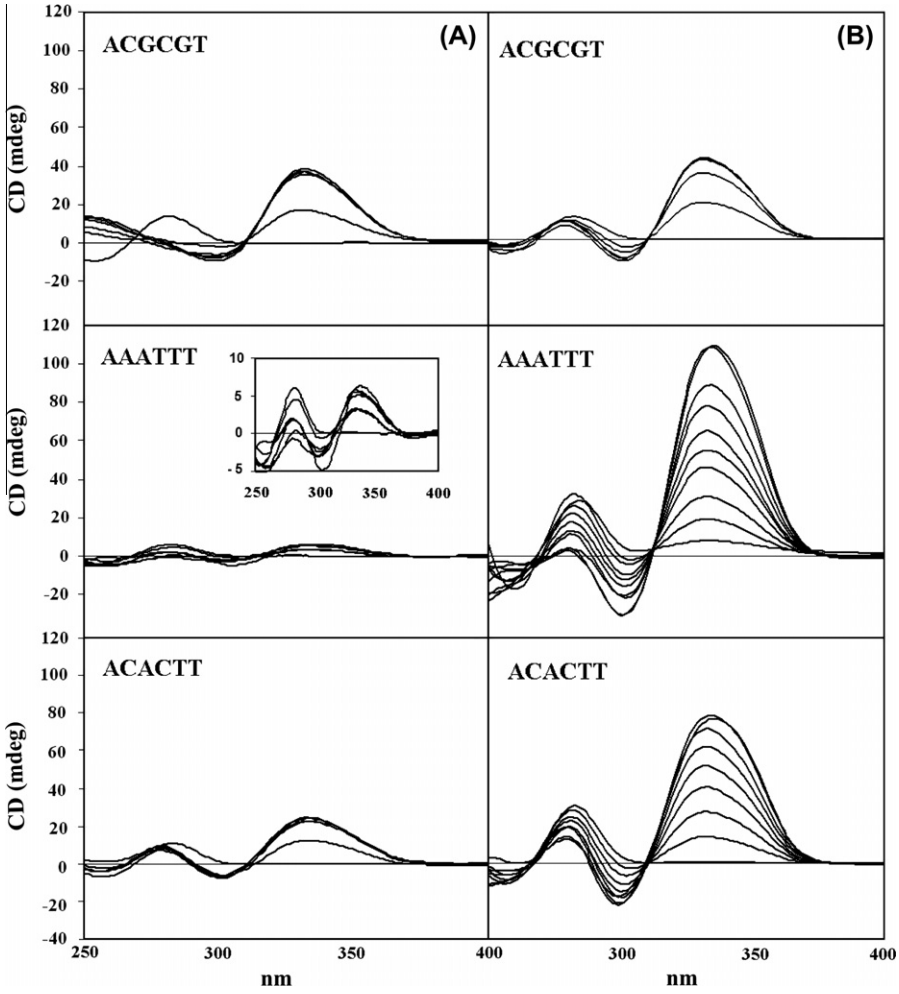


Figure 4. CD data for Ph-ImPyIm (**3**) (A) and its diamino counterpart **5** (B) with 5′-ACGCGT-3′, 5′-AAATTT-3′ and 5′-ACACTT-3′. Data were obtained using $PO_4$5 buffer.

These results are corroborated by the aforementioned ΔT_M values for **3** and **5** (Table 1).

2.5. Biosensor-surface plasmon resonance studies

To obtain a more accurate measure of binding affinity and sequence selectivity plus to probe the stoichiometry of binding, polyamides **3** and **5** were subjected to experiments using the surface plasmon resonance (SPR)-biosensor method.[20] The experiments were conducted using cognate DNA sequence 5′-ACGCGT-3′ and non-cognate sequences 5′-AAGCTA-3′ and 5′-AAATTT-3′. The sensorgrams recorded from the SPR experiments for **3** and **5** binding to 5′-ACGCGT-3′ are given in Figure 5. According the sensorgram pattern displayed in Figure 5B, it is clearly evident that the monoamino polyamide **3** binds strongly to its cognate sequence 5′-ACGCGT-3′ as a cooperative dimer ($n \sim 2$) with a binding constant (K_{eq}) $4.8 \times 10^6\ M^{-1}$. The sensorgram shown in Figure 5A displays a similar slow observed kinetics pattern suggesting that the diamino polyamide **5** also binds strongly to the cognate sequence 5′-ACGCGT-3′ as a cooperative dimer ($n \sim 2.2$). The binding constant (K_{eq}) of **5** to its cognate sequence was found to be 1.5×10^7, which is almost threefold higher than that of **3**. This result is consistent with the results recorded for the DNase I footprinting experiments. Both polyamides **3** and **5** also bind to their non-cognate DNA sequence 5′-AAGCTA-3′, but with a lower affinity (Table 2). The monoamino Ph-ImPyIm (**3**) binds to 5′-AAGCTA-3′ as a dimer in a non-cooperative manner with binding constant (K_{eq}) $2.5 \times 10^5\ M^{-1}$, while its diamino counterpart **5** binds to same DNA sequence also as a dimer but in a slightly cooperative manner with higher binding constant (K_{eq}) $2.1 \times 10^6\ M^{-1}$. Binding to 5′-AAGCTA-3′ is not surprising since the stacked dimer of both polyamides **3** and **5** contain the 'ImPy' central pairing, which the authors have reported to give the most favorable binding to the 'GC' core sequence.[5] However, none of the polyamides showed any binding affinity to their non-cognate DNA sequence 5′-AAATTT-3′ under these conditions. This is not surprising since all previously reported f-ImPyIm analogs[5,16] did not show any binding to the 5′-AAATTT-3′ (data not shown), indicating GC-sequence preference by Im/Py pairs. Furthermore, the enhanced binding of **5** over **3** is evidently a result of a slightly slower or "flatter" off-rate as revealed by the SPR sensorgrams for dicationic polyamide **5** than those for the monoamino analog **3**. To further elaborate on the sequence selectivity of non-formamido polyamides, such as the phenyl-containing polyamides **3** and **5**, the SPR data given in Table 2 were compared to those previously reported for f-ImPyIm (**1**). It is evident that whilst polyamides **3** and **5** did not show any binding to 5′-AAATTT-3′, f-ImPyIm (**1**) was reported to bind and it gave a binding constant of $5.3 \times 10^4\ M^{-1}$, albeit it was almost 4-orders of magnitude lower than the binding of compound **1** to its cognate 5′-ACGCGT-3′. This observation provided evidence for enhanced sequence selectivity of the phenyl-containing polyamides **3** and **5** compared to their formamido counterpart **1**.

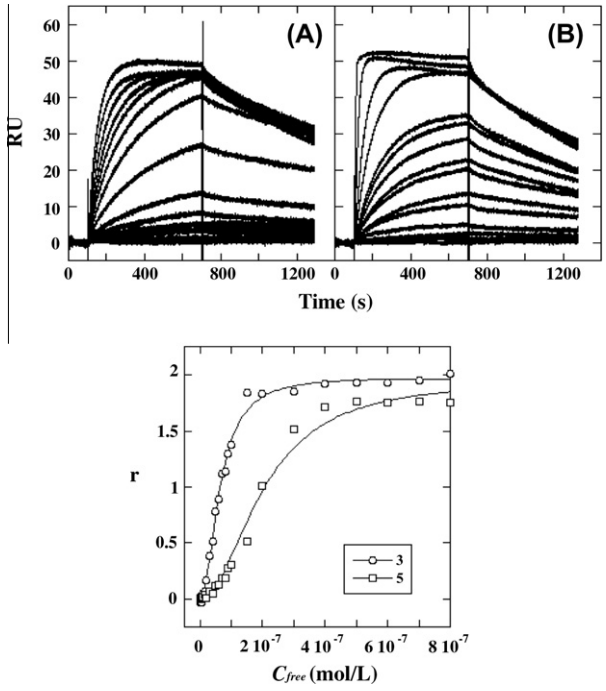


Figure 5. SPR sensorgrams (A) and (B) showing binding of **3** and **5** to 5′-ACGCGT-3′, respectively, at concentration range from 0 to 0.8 μM. (C) Plot of r versus concentration including best fit lines.

Table 2
Binding constants and thermodynamic profiles of polyamides **3**, **5** and **1** to DNA sequences 5′-ACGCGT-3′, 5′-AAGCTA-3′ and 5′-AAATTT-3′

Polyamide	DNA sequences		
	ACGCGT[a]	AAGCTA[a]	AAATTT[a]
3	$K_1 = 6.1 \times 10^4$ M^{-1} $K_2 = 3.7 \times 10^8$ M^{-1} $K_{eq} = 4.8 \times 10^6$ M^{-1} $\Delta G = -9.1$ kcal/mol $\Delta H = -2.4$ kcal/mol $T\Delta S = 6.7$ kcal/mol	$K_1 = 2.3 \times 10^5$ M^{-1} $K_2 = 2.8 \times 10^5$ M^{-1} $K_{eq} = 2.5 \times 10^5$ M^{-1} $\Delta G = -7.4$ kcal/mol $\Delta H = -2.2$ kcal/mol $T\Delta S = 5.2$ kcal/mol	NR[b]
5	$K_1 = 8.7 \times 10^5$ M^{-1} $K_2 = 2.7 \times 10^8$ M^{-1} $K_{eq} = 1.5 \times 10^7$ M^{-1} $\Delta G = -9.8$ kcal/mol $\Delta H = -2.9$ kcal/mol $T\Delta S = 6.9$ kcal/mol	$K_1 = 2.9 \times 10^5$ M^{-1} $K_2 = 1.2 \times 10^7$ M^{-1} $K_{eq} = 2.1 \times 10^6$ M^{-1} $\Delta G = -8.6$ kcal/mol $\Delta H = -2.2$ kcal/mol $T\Delta S = 6.4$ kcal/mol	NR[b]
1[c]	$K_1 = 1.9 \times 10^8$ M^{-1} $\Delta G = -11.3$ kcal/mol $\Delta H = -7.7$ kcal/mol $T\Delta S = 3.6$ kcal/mol	ND[d]	$K_1 = 5.3 \times 10^4$ M^{-1}

[a] Binding constants were measured by SPR.
[b] No response detected.
[c] Data taken from Ref. 5
[d] Not determined.

2.6. Isothermal titration calorimetry studies

ITC experiments were performed at 25 °C to probe the thermodynamics of DNA binding interactions of **3** and **5**. The monoamino polyamide **3** again demonstrated binding to the cognate sequence as evidenced by the exothermic enthalpy of binding of **3** to 5′-ACGCGT- 3′ (Fig. 6). Because of the very strong binding of these polyamides to the cognate DNA, a constant enthalpy was obtained below a mole ratio of 0.6 (Fig. 6) where essentially all of the added compound is bound to DNA. A linear fit and extrapolation back to 0.0 mole ratio gave a ΔH of $\sim -2.4 \pm 0.14$ kcal/mol. The similar binding interaction with 5′-ACGCGT-3′ was observed for diamino polyamide **5** as evident from the ITC data shown in Figure 6. A linear fit and extrapolation of heats back to 0.0 mole ratio yielded a ΔH of $\sim -2.9 \pm 0.2$ kcal/mol (Fig. 6). With these ΔH values and the ΔG for binding from SPR ($\Delta G = -RT$ ln K_{eq} where, R is 1.987 cal mol^{-1} K^{-1} and T is measured in K) the $T\Delta S$ values were calculated ($\Delta G = \Delta H - T\Delta S$). The results for 5′-ACGCGT-3′ reveal that the enhanced binding affinity, hence the free energy of binding, in the dicationic polyamide **5** over monocation **3** is driven more significantly by an enthalpy gain ($\Delta\Delta H \sim 0.5$ kcal/mol). In contrast, the gain in entropy was smaller ($\Delta T\Delta S \sim 0.2$ kcal/mol). The results suggest that introduction of a second amino moiety provides favorable thermodynamic interactions between the ammonium function and DNA, presumably via electrostatic or hydrogen bond interactions with the phosphate group in the DNA backbone.

An even more drastic difference is noted by comparing the thermodynamic data of polyamides **3** and **5** to their parent molecule f-ImPyIm (**1**).[5] The results given in Table 2 show that unlike the phenyl-containing polyamide whose binding was driven significantly by entropic means, the binding of f-ImPyIm (**1**) was driven primarily by enthalpy. This finding is intriguing and useful because it demonstrated that even a small change from *N*-formamido to an *N*-benzamide could produce dramatic changes in the thermodynamics of interactions. The results indicate that in our next step of molecular design to further increase the binding affinity of diamino compound **5**, the strategy must include elements that increase enthalpy. Such elements would include incorporating groups that would increase favorable intermolecular interactions.

3. Conclusion

The results from DNA binding studies show that having a second positively charged group in the form of an orthogonally positioned alkylammonium side chain on the polyamide backbone does not affect DNA sequence selectivity. In fact, the diamino polyamide Ph-ImPy∗Im (**5**) demonstrated an excellent level of specificity for the cognate 5′-ACGCGT-3′ sequence compared to its monoamino counterpart **3**. In addition, the binding affinity of **5** is enhanced by ~3-fold compared to its monocationic analog **3**; thus, effectively raising the binding affinity of **5** to 5′-ACGCGT-3′ to a level that is only 10-fold lower than its parent molecule f-ImPyIm (**1**). The enhanced binding affinity of **5** over **3** is possibly due to favorable electrostatic interaction between the negatively charged DNA phosphodiester groups and the ammonium species. The side chain of **5** also offers a significant benefit over its monocationic triamide **3** in terms of enhanced water solubility of the corresponding HCl salts as evidenced by the ease in dissolving polyamide **5** over **3**. Given its small size, it is our hypothesis that diamino polyamides such as **5** should readily be taken up by cells. Further studies to probe the ability of polyamides **3** and **5** to affect gene function are underway and results will be reported in due course.

4. Experimental

Solvents and organic reagents were purchased from Aldrich or Fisher, and in most cases were used without further purification. DCM (P_2O_5), and DMF (BaO) were distilled prior to use. Melting points (mp) were performed using a Mel-temp instrument and are uncorrected. Infrared (IR) spectra were recorded using a Perkin Elmer Paragon 500 FT-IR instrument as films on KBr disks. ^{1}H NMR spectra were obtained using a Varian Unity Inova 400 MHz instrument unless otherwise stated. Chemical shifts (δ) are reported at 20 °C in parts per million (ppm) downfield from internal tetramethylsilane (Me_4Si). High-resolution mass spectra (HRMS) and

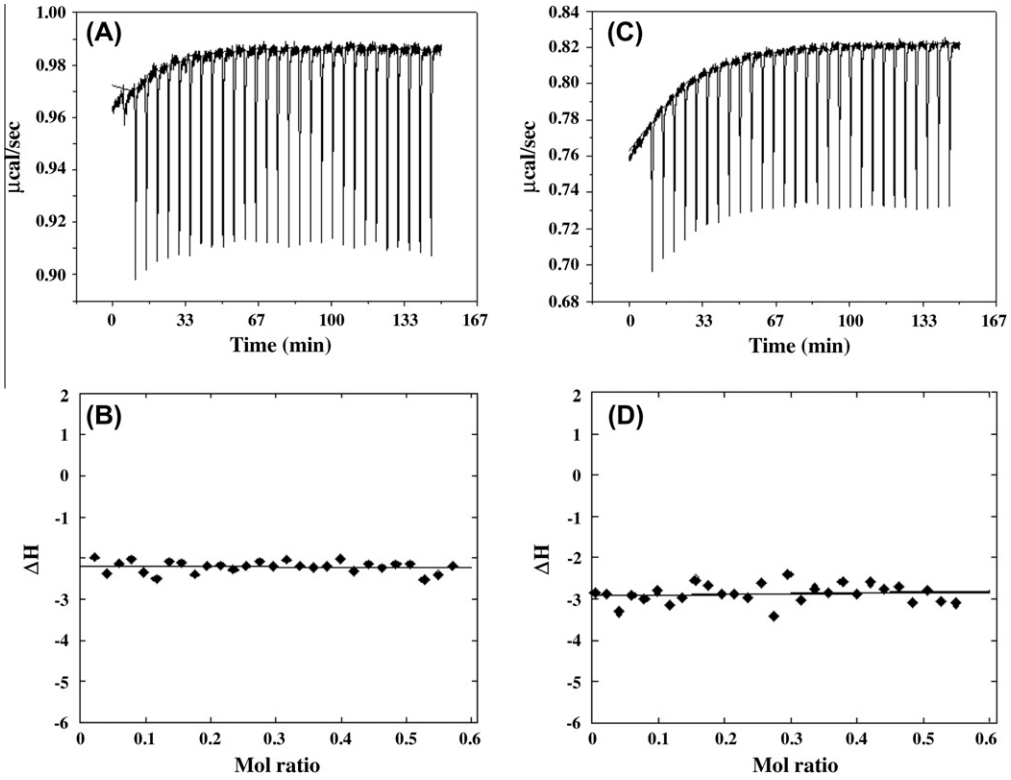


Figure 6. (A) Raw ITC data for the 'model free' titration of **3** into 5′-ACGCGT-3′. (B) The integrated heats for each injection of **3** used for the titration (red dots) and a linear fit (red line) of that data. (C) Raw ITC data for the 'model free' titration of **5** into 5′-ACGCGT-3′. (D) The integrated heats for each injection of **5** used for the titration (red dots) and a linear fit (red line) of that data. The titration consisted of 30–10 µL injections of 80 µM of **3** or **5** into 30 µM DNA. The titration was completed up to a mole ratio of ~0.6:1 (ligand/DNA). The linear fits were completed using KaleidaGraph 4.0.

low-resolution mass spectra (LRMS) were provided by the Mass Spectrometry Laboratory, University of South Carolina, Columbia. Reaction progress was assessed by thin-layer chromatography (TLC) using Merck silica gel (60 F_{254}) on aluminum plates unless otherwise stated. Visualization was achieved with UV light at 254 nm and/or 366 nm, I_2 vapor staining and ninhydrin spray.

4.1. Synthesis

4.1.1. Ethyl 4-benzamido-1-methylimidazole-2-carboxylate (7)

Ethyl 1-methyl-4-nitroimidazole-2-carboxylate[17] (**6**, 613 mg, 3.1 mmol) was hydrogenated over 5% palladium on charcoal in ethanol. The resulting amino compound was dried thoroughly under vacuum to remove traces of ethanol. It was then dissolved in dichloromethane (20.0 mL) and the solution was cooled to 0–5 °C. To the cold solution was added triethylamine (0.5 mL, 3.7 mmol) followed by benzoyl chloride (0.4 mL, 3.4 mmol). The reaction mixture was allowed to attain room temperature and then stirred for 12 h. Upon completion, the reaction was quenched with water (15.0 mL) and stirred for 15 min. The organic layer was separated and aqueous layer was washed with dichloromethane (2 × 10 mL). Combined organic layers were dried over sodium sulfate and evaporated to obtain a yellow oil. The crude product was purified by column chromatography using silica gel and 2% methanol in chloroform as the eluent system to obtain **7** as a yellow solid (808 mg, 96%), mp 120–125 °C, R_f: 0.76 (9.5:0.5 v/v, $CHCl_3$:CH_3OH); IR: υ 2976, 1712, 1663, 1554, 1444, 1123, 1019, 708 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.84 (s, 1H), 7.88–7.86 (d, 2H, J = 8.0 Hz), 7.69 (s, 1H), 7.56–7.47 (m, 3H), 4.40 (q, 2H, J = 8.0 Hz), 4.05 (s, 3H), 1.42 (t, 3H, J = 8.0 Hz); LRMS (ES^+) m/z = 274 ($[M+H]^+$, 100%).

4.1.2. 4-Benzamido-1-methylimidazole-2-carboxylic acid (8)

To the slurry of ethyl 4-benzamido-1-methylimidazole-2-carboxylate (**7**, 800 mg, 2.9 mmol) in methanol:water (1:1, 20.0 mL) was added aqueous sodium hydroxide solution (2.0 M, 1.8 mL). The reaction mixture was refluxed for 45 min and then distilled to remove methanol. It was then made acidic (pH 3–4) by adding dilute hydrochloric acid (6.0 M) to obtain 4-benzamido-1-methylimidazole-2-carboxylic acid (**8**) as a white solid which was filtered, washed with water and dried in vacuum at room temperature (664 mg, 85%), mp 156–158 °C, R_f: 0.24 (3:7 v/v, $CHCl_3$:CH_3OH); IR: υ 3445, 2254, 2127, 1661, 1033, 818, 753 cm^{-1}; ^1H NMR ($CDCl_3$) δ 11.05 (s, br, 1H), 8.04–8.06 (d, 2H, J = 8.0 Hz), 7.70 (s, 1H), 7.54–7.46 (m, 3H), 3.95 (s, 3H); LRMS (ES^+) m/z = 246 ($[M+H]^+$, 100%).

4.1.3. Monoamino Ph-ImPyIm polyamide (3)

4-Benzamido-1-methylimidazole-2-carboxylic acid (**8**, 107 mg, 0.44 mmol) was dissolved in DMF (2.0 mL). To the clear solution was added PyBOP (230 mg, 0.46 mmol) followed by diisopropylethylamine (DIPEA, 0.15 mL, 0.87 mmol). The reaction mixture was stirred at room temperature for 30 min and to it was added solution of **9**[18] (133 mg, 0.4 mmol) in DMF (1.0 mL). The reaction mixture was stirred for 3 days at room temperature under argon atmosphere and then poured on water (10.0 mL). It was extracted with chloroform (3 × 10.0 mL) and combined organic layers were dried over sodium sulfate and evaporated on rotary evaporator. The crude product was purified by column chromatography using silica gel and 10% methanol in chloroform to obtain monoamino Ph-ImPyIm polyamide (**3**) as a yellow solid (87 mg, 38%), mp 163–165 °C, R_f: 0.53 (1:1 v/v, $CHCl_3$:CH_3OH); IR: υ 2928, 1663, 1531, 1468, 1438, 1258, 1117, 907 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.83 (s, br, 1H), 8.48 (s, br, 1H), 8.09 (s, br, 1H), 7.93 (d, 2H, J = 8.0 Hz),

7.62 (s, 1H), 7.55 (s, 1H), 7.53–7.51 (m, 3H), 7.42 (s, 1H), 7.29 (s, 1H), 6.76 (s, 1H), 4.49 (q, 2H, J = 8.0 Hz), 4.12 (s, 3H), 4.03 (s, 3H), 3.98 (s, 3H), 2.55 (t, 2H, J = 8.0 Hz), 2.32 (s, 6H); LRMS (ES^+) m/z = 561 ($[M+H]^+$, 100%); HRMS $[M+H]^+$ calcd for m/z $C_{27}H_{32}N_{10}O_4$ 561.2691; found m/z 561.2686.

4.1.4. Ph-ImPy(C_3H_6Cl)Im (11)

4-Benzamido-1-methylimidazole-2-carboxylic acid (**8**, 126 mg, 0.5 mmol) was dissolved in DMF (2.0 mL). To the clear solution was added PyBOP (280 mg, 0.55 mmol) followed by diisopropylethylamine (DIPEA, 0.18 mL, 1.0 mmol). The reaction mixture was stirred at room temperature for 30 min and to it was added solution of **10**[16] (208 mg, 0.5 mmol) in DMF (1.0 mL). The reaction mixture was stirred for 3 days at room temperature under argon atmosphere and then poured on water (10.0 mL). It was extracted with chloroform (3 × 10.0 mL) and combined organic layers were dried over sodium sulfate and evaporated on rotary evaporator. The crude product was purified by column chromatography using silica gel and 15% methanol in chloroform to obtain Ph-ImPy(C_3H_6-Cl)Im (**11**) as a white solid (104 mg, 34%), mp 238-240 °C, R_f: 0.44 (1:1 v/v, $CHCl_3$:CH_3OH); IR: υ 3300, 1654, 1450, 1111, 1030, 930 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.87 (s, br, 1H), 8.49 (s, br, 1H), 8.25 (s, br, 1H), 8.05 (s, br, 1H), 7.94–7.92 (d, 2H, J = 8.0 Hz), 7.62 (s, 1H), 7.59–7.55 (m, 3H), 7.52 (s, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 4.53 (t, 2H, J = 8.0 Hz), 4.13 (s, 3H), 4.03 (s, 3H), 3.54-3.48 (m, 4H), 2.57 (t, 2H, J = 8.0 Hz), 2.33 (s, 6H), 2.31 (t, 2H, J = 8.0 Hz); LRMS (ES^+) m/z = 623 ($[M+H]^+$, 50%).

4.1.5. Diamino Ph-ImPy∗Im polyamide (5)

The solution of Ph-ImPy(C_3H_6Cl)Im (**11**, 10 mg, 0.016 mmol) in dry methanol (5.0 mL) was purged with dry ammonia for 6.0 h in a re-sealable tube. The tube was sealed tightly and heated at 80 °C for 12 h. After completion of reaction, the solution was evaporated on a rotary evaporator to dryness to obtain the crude product. Purification by column chromatography (silica, gradient, 0:100–100:0%v/v, $CHCl_3$/MeOH) gave the desired diamino polyamide **5** as a white solid (6.0 mg, 62%), mp 150–152 °C, R_f: 0.41 (15:5:1% v/v, $CHCl_3$:CH_3OH:NH_3); IR: υ 3421, 3359, 3310, 3080, 3000, 2973, 1651, 1541, 1471, 1405, 1257, 1188, 1084, 1012, 847 cm^{-1}; ^1H NMR (CD_3OD) δ 7.98 (d, 1H), 7.57–7.64 (m, 6H), 7.49 (s, 1H), 7.07 (s, 1H), 4.52 (t, J = 4.0 Hz, 2H), 4.13 (s, 3H), 4.05 (s, 3H), 3.52 (t, J = 4.0 Hz, 2H), 2.81 (t, J = 4.0 Hz, 2H), 2.60 (t, J = 8.0 Hz, 2H), 2.35 (s, 6H), 2.01 (t, J = 8.0 Hz, 2H), 2.11 (quint, J = 4.0 Hz, 2H); LRMS (ES^+) m/z (rel intensity) 601 ($[M+H]^+$, 90%), 301 (100%); HRMS $[M+H]^+$ calcd for $C_{29}H_{37}N_{11}O_4$ m/z 601.2635; found m/z 601.2634.

4.2. Biophysical

4.2.1. Buffers

Ten millimolar phosphate buffers were prepared at the following salt (NaCl) concentrations: 12.5 mM ($PO_4$0), 50 mM ($PO_4$5), 200 mM ($PO_4$20) with the addition of 1 mM EDTA, pH 6.2.

4.2.2. DNase I footprinting

A radiolabeled DNA fragment of 125 base pairs was generated by polymerase chain reaction as described previously.[21] The resulting labeled fragment was purified on a Bio-Gel P-6 column (Bio-Rad) followed by agarose gel electrophoresis and isolated using a Mermaid kit (MP biomedicals) according to the manufacturer's instructions. 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 resuspended 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. The gel was exposed overnight to Fuji medical X-ray film and developed on a Konica Medical Film Processor SRX-101A.

4.2.3. Thermal denaturation (TM)

Thermal denaturation data were obtained using the Cary 100 BioMelt (Varian) spectrophotometer with DNA (1 μM) in $PO_4$0 and the required compound (3 μM), using the procedure previously reported.[7a] Thermal melts were obtained for each compound using 5′-ACGCGT-3′, 5′-AAATTT-3′ and 5′-ACACTT-3′.

4.2.4. Circular dichroism (CD)

CD studies were performed using the Olis DSM20 instrument. Each run was performed over 400–220 nm wavelength range (180 increments) and an integration time of 1 s and the average of two scans were used for analysis. The required compound (500 μM in double distilled H_2O) was titrated in 1 molar equivalents into the required DNA (160 μL of 9 μM DNA), in $PO_4$5, until saturation was observed. Data analysis was performed as previously described.[7a] CD experiments were performed with 5′-ACGCGT-3′, 5′-AAATTT-3′ and 5′-ACACTT-3′.

4.2.5. Surface plasmon resonance (SPR)

SPR measurements were performed with a four-channel BIAcore T100 optical biosensor system (Biacore, GE Healthcare Inc.). 50-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-N0-[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 **3** 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 (sensor chip SA) as previously reported.[20a,b,22] 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,22]

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

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

4.2.6. Isothermal titration microcalorimetry (ITC)

ITC analysis was performed using a VP-ITC microcalorimeter (MicroCal). Compound 3 was dissolved in 0.01 M cacodylic acid (CCA) buffer and the instrument equilibrated to 25 °C. ITC experiments were conducted using the 'model-free' method as previously reported.[23] {insert B Chaires ref here (Biochem 2000, 39, 8439–8447)}. After an initial delay of 300 s, compound 3 (80 μM) was titrated, via 30 injections (10 μL for 20 s, repeated every 300 s), into 30 μM DNA (0.01 M CCA). The data were imported to Origin 7.0, which was used to integrate the area under the curve as a function of time. In order to normalize for nonspecific heat components, including heats of dilution, nonspecific heat component integrations were subtracted from the reaction integrations.[5] A linear fit of the normalized ΔH (y-axis) values versus mol ratio (x-axis) was done using KaleidaGraph 4.0. Extrapolation back to 0 mol ratio of this plot yields the enthalpy (ΔH) of the reaction. The DNA used in this experiment was 5′-ACGCGT-3′, 5′-AAATTT-3′, and 5′-ACCGGT-3′.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.010.

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