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Alternative Heterocycles for DNA Recognition: An *N*-Methylpyrazole/*N*-Methylpyrrole Pair Specifies for A·T/T·A Base Pairs

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Abstract—Side-by-side pairs of three five-membered rings, *N*-methylpyrrole (Py), *N*-methylimidazole (Im), and *N*-methylhydroxypyrrole (Hp), have been demonstrated to distinguish each of the four Watson–Crick base pairs in the minor groove of DNA. However, not all DNA sequences targeted by these pairing rules achieve affinities and specificities comparable to DNA binding proteins. We have initiated a search for new heterocycles which can expand the sequence repetoire currently available. Two heterocyclic aromatic amino acids, *N*-methylpyrazole (Pz) and 4-methylthiazole (Th), were incorporated into a single position of an eight-ring polyamide of sequence ImIm**X**Py-γ-Im**Py**PyPy-β-Dp to examine the modulation of affinity and specificity for DNA binding by a Pz/Py pair and/or a Th/Py pair. The **X/Py** pairings Pz/Py and Th/Py were evaluated by quantitative DNase I footprint titrations on a DNA fragment with the four sites 5′-TGGNCA-3′ (N=T, A, G, C). The Pz/Py pair binds T·A and A·T with similar affinity to a Py/Py pair but with improved specificity, disfavoring both G·C and C·G by about 100-fold. The Th/Py pair binds poorly to all four Watson–Crick base pairs. These results demonstrate that in some instances new heterocyclic aromatic amino acid pairs can be incorporated into imidazole–pyrrole polyamides to mimic the DNA specificity of Py/Py pairs which may be relevant as biological criteria in animal studies become important.

Introduction

Small molecules that target specific predetermined DNA sequences have the potential to control gene expression.[1–3] Polyamides containing the three aromatic amino acids 3-hydroxypyrrole (Hp), imidazole (Im) and pyrrole (Py) are synthetic ligands that have an affinity and specificity for DNA comparable to naturally occurring DNA binding proteins.[4–6] Recently, eight-ring hairpin polyamides have been found to regulate transcription and permeate a variety of cell types in culture.[1,2] DNA recognition depends on the side-by-side amino acid pairings oriented N→C with respect to the 5′→3′ direction of the DNA helix in the minor groove.[4–6] Antiparallel pairing of Im opposite Py (Im/Py pair) distinguishes G·C from C·G and both of these from A·T/T·A base pairs.[5,6] A Py/Py pair binds both A·T and T·A in preference to G·C/C·G.[5,6] The discrimination of T·A from A·T using Hp/Py pairs completes the four base pair (bp) code.[7–9]

Given the sequence dependent microstructure of the DNA helix,[10–16] it is significant that a simple recognition code can be developed.[17–20] In both published and unpublished work, over a hundred pyrrole–imidazole polyamides have been synthesized which recognize predetermined sequences. However, within this group, a number of DNA sequences have emerged as difficult targets for pyrrole–imidazole polyamides to bind with subnanomolar affinity. Sequence dependent DNA structural features such as intrinsic minor groove width, minor groove flexibility, and inherent DNA curvature may reduce polyamide binding at certain sites.[10–16] Since molecular composition could enhance affinity at difficult sequences, the discovery of new monomer subunits that provide polyamides with affinities and specificities comparable to naturally occurring DNA binding proteins remains a high priority.

The substitution of pyrrole C3-H with N or C3-OH to generate imidazole or hydroxypyrrole, respectively, clearly demonstrates the impact of atomic substitutions, in polyamide monomer units, on DNA recognition.[5–9] These substitutions also highlight the importance of the 3-position as a recognition element in the floor of the

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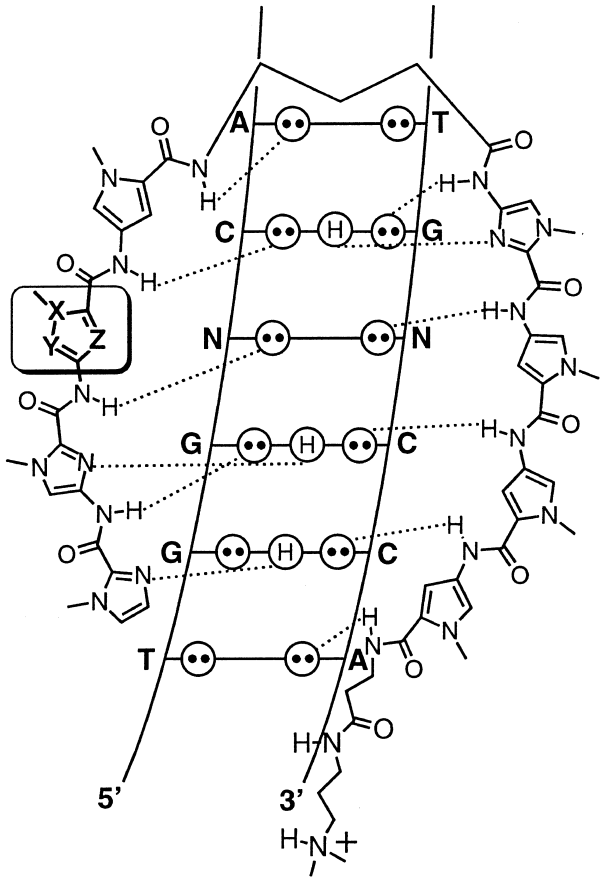
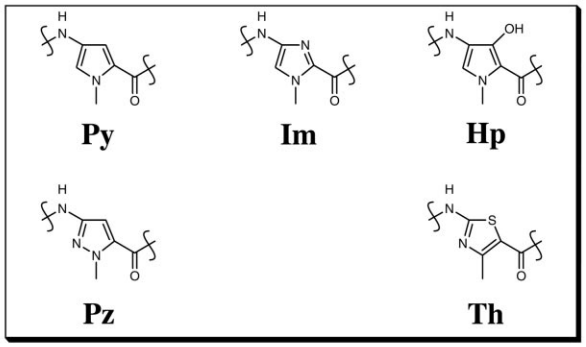
minor groove. The aromatic amino acid monomers of polyamides are also amenable to modification at the 1- and 5-positions of the pyrrole–imidazole rings. Substitutions at the 1-position have been investigated,[5] but until now, substitutions at the pyrrole 5-position have not been evaluated. Replacement of the C5-H of *N*-methylpyrrole with N generates a new heterocyclic amino acid subunit, *N*-methylpyrazole (Pz). Pyrazole was thought to be of interest in light of altered ring electronics, possible interactions between the newly introduced nitrogen and the polyamide backbone amide oxygen, differing cell-permeation properties or degradation pathways in living animals. Additionally, a previously reported heterocyclic aromatic amino acid, 4-methylthiazole (Th), which substitutes an S for C3-H, N for C5-H and C for N1 relative to pyrrole, was added to this study for comparison (Figs 1 and 2). When incorporated into three ring distamycin analogues, the 4-methylthiazole (Th) monomer,[21] which places a sulfur atom into the floor of the minor groove, conferred A/T preference over G/C.[21,22] Thiazole (Th) has been proposed in the literature as an A-specific recognition element since the bulkiness of the sulfur atom was expected to sterically clash opposite the O2 of thymine and therefore be better accommodated opposite an adenine base.[23,24] However, it has been shown that the asymmetric cleft in the minor groove of A/T pairs preferentially places the bulky C3-OH group of Hp opposite T rather than A, where there is a steric destabilization with the C2-H of adenine.[7,8] Since an additional hydrogen bond is also made between Hp and T, it remains quite interesting to



	Z	Y	X
Py	C-H	C-H	N
Hp	C-OH	C-H	N
Im	N	C-H	N
Pz	C-H	N	N

Figure 1. Binding model for the 1:1 complexes formed between the DNA and ImIm**X**Py-γ-ImPyPyPy-β-Dp. Circles with dots represent lone pairs of N3 of purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogen of guanine. N is represented as A or T but can be A, T, G, or C. Putative hydrogen bonds are illustrated by dotted lines. The atomic variations (at positions X, Y, and Z) between the pyrrole, hydroxypyrrole, imidazole, pyrazole and thiazole monomers are also shown.



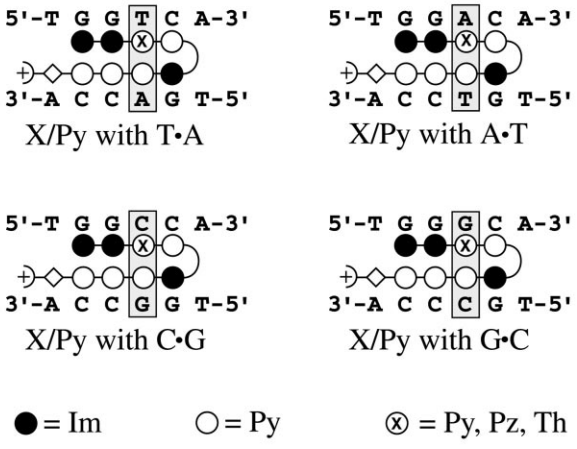
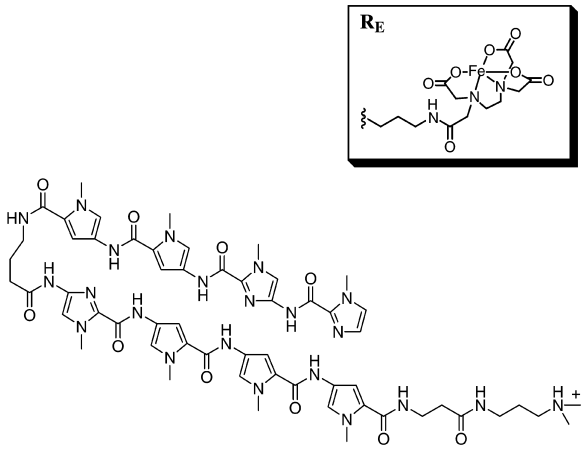


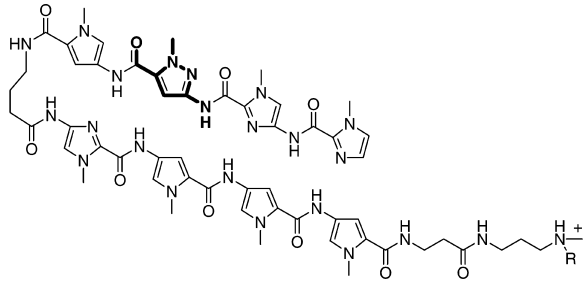
Figure 2. Monomer units incorporated into hairpin polyamides. Ball and stick polyamide–DNA binding models are also shown. Shaded circles denote Im while nonshaded circles represent Py. Nonshaded circles containing an X represent Py, Pz or Th monomer units. Nonshaded diamonds denote the β-residue. γ-Aminobutyric acid (γ) and dimethylaminopropylamide (Dp) are depicted as a curved line and a plus sign, respectively.

investigate the discrimination of A·T/T·A base pairs by purely steric means using the Th/Py pairing within the context of our hairpin motif.

To investigate these new pairings, three polyamides, ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp (**1**), ImIm**Pz**Py-γ-Im**Py**-PyPy-β-Dp (**2**), and ImIm**Th**Py-γ-Im**Py**PyPy-β-Dp (**3**), were synthesized by solid-phase methods[25] to contain either a side-by-side pairing of two pyrrole rings (Py/Py), a pyrazole and pyrrole pairing (Pz/Py), or a thiazole and pyrrole pairing (Th/Py), respectively. The corresponding EDTA analogue ImImPzPy-γ-ImPyPyPy-β-Dp-EDTA (**2E**) was also constructed to confirm the orientation and stoichiometry of this hairpin at each binding site (Fig. 3). We report here the DNA binding affinity and sequence selectivity of the previously reported[7,26] control polyamide ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp (**1**) in relation to the new polyamides ImIm**Pz**Py-γ-Im**Py**PyPy-β-Dp (**2**), and ImIm**Th**Py-γ-Im**Py**PyPy-β-Dp (**3**) for the 6 bp sites, 5′-TGGTCA-3′, 5′-TGGACA-3′, 5′-TGGGCA-3′, and 5′-TGGCCA-3′, thereby evaluating the three pairings Py/Py, Pz/Py and Th/Py, respectively, against all four Watson–Crick base pairs. Three separate techniques are used to characterize the DNA binding properties of the newly designed polyamides: DNase I footprinting, methidiumpropyl-EDTA·Fe(II) (MPE·Fe(II)) footprinting, and affinity cleavage. Quantitative DNase I footprinting titrations allow determination of equilibrium association constants (K_a) of the polyamides for respective match and mismatch sites.[27–29] Information about precise binding site size is gained from MPE·Fe(II) footprinting,[30,31] while affinity cleavage studies confirm the binding orientation and stoichiometry of the hairpin–DNA complex.[17,32]

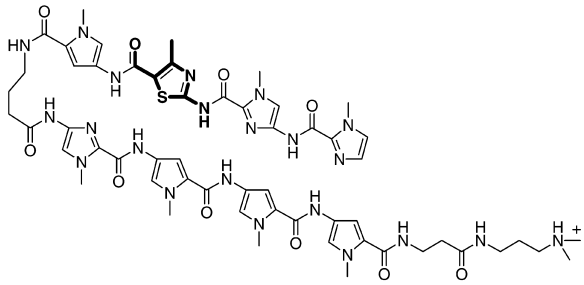


1 ImIm**Py**Py-γ-ImPyPyPy-β-Dp



2 R=CH_3 ImIm**Pz**Py-γ-ImPyPyPy-β-Dp

2E R= R_E ImIm**Pz**Py-γ-ImPyPyPy-β-Dp-EDTA·Fe(II)



3 ImIm**Th**Py-γ-ImPyPyPy-β-Dp

Figure 3. Structures of the eight-ring hairpin polyamides **1**, **2**, **3** and the corresponding Fe(II)·EDTA affinity cleavage derivative **2E** as synthesized by solid-phase methods.

Results

Monomer synthesis

Ethyl-3-amino-1-methylpyrazole-5-carboxylate (**4**) and ethyl-2-amino-4-methylthiazole-5-carboxylate (**6**) were prepared according to published procedures[33–35] on multi-gram scale. These amino ethyl esters were then Boc-protected and the ethyl ester hydrolyzed to produce 3-[(*tert*-butoxycarbonyl) amino]-1-methylpyrazole-5-carboxylic acid (**5**) (Boc-Pz-OH) and 2-[(*tert*-butoxycarbonyl) amino]-4-methylthiazole-5-carboxylic acid (**7**) (Boc-Th-OH) respectively (Fig. 4).

Polyamide synthesis

The polyamide resin ImImPyPy-γ-ImPyPyPy-β-Pam-resin was synthesized as previously described.[7,26] The

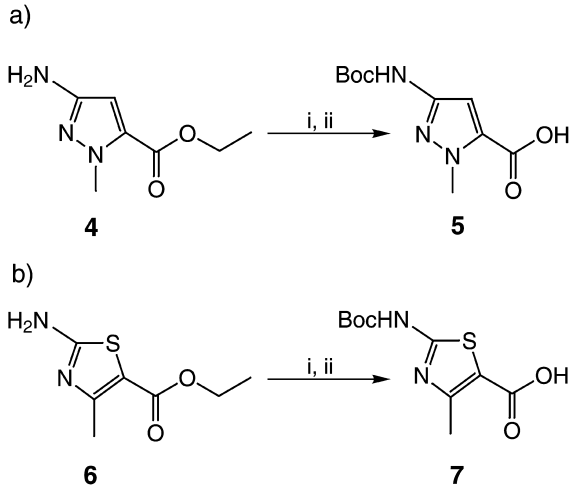


Figure 4. (a) Synthesis of Boc-Pz-OH (**5**). (i) Boc_2O, DIEA, DMF, (ii) 1 M NaOH; (b) Synthesis of Boc-Th-OH (**7**). (i) Boc_2O, DIEA, DMF, (ii) 1 M KOH.

polyamide resins ImImPzPy-γ-ImPyPyPy-β-Pam-resin and ImImThPy-γ-ImPyPyPy-β-Pam-resin were synthesized using previously described Boc-chemistry manual synthesis protocols.[25] Both Boc-Pz-OH and Boc-Th-OH monomers were activated with HBTU/DIEA and coupled for 20 h at 37 °C before termination with acetic anhydride. After Boc-deprotection of the resin-bound polyamide chain, the Boc-Im-OH was activated with

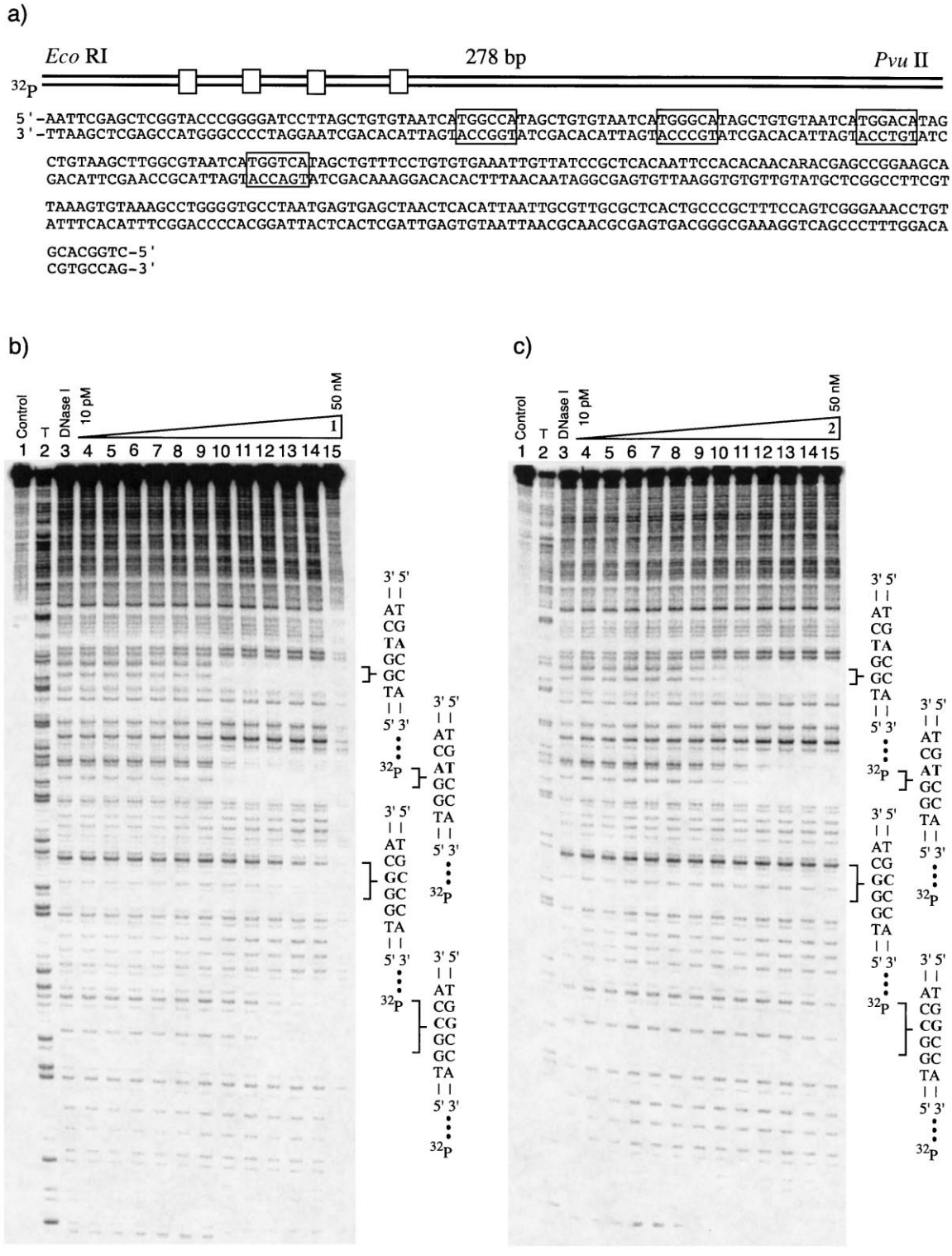


Figure 5. (a) Illustration and complete sequence of the 278 bp pDHN1 *Eco*RI/*Pvu*II restriction fragment. The four designed binding sites that were analyzed in quantitative footprint titration experiments are indicated with a box surrounding each of the 6 bp sites. Quantitative DNase I footprint titration experiment with (b) ImImPyPy-γ-ImPyPyPy-β-Dp (**1**) and (c) ImImPzPy-γ-ImPyPyPy-β-Dp (**2**) on the 3′-end labeled 278 bp restriction fragment: lane 1, intact DNA; lane 2, T reaction; lane 3, DNase I standard; lanes 4–15, DNase I digestion products in the presence of 10 pM, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 5 nM, 10 nM, 20 nM and 50 nM polyamide respectively. All four 6 bp binding sites 5′-TGGTCA-3′, 5′-TGGACA-3′, 5′-TGGGCA-3′ and 5′-TGGCCA-3′ were analyzed and are shown on the right side of the autoradiogram. All reactions contain 15 kcpm restriction fragment, 10 mM Tris·HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$ and 5 mM $CaCl_2$.

HBTU/DIEA and coupled onto NH_2-PzPy-γ-ImPy-PyPy-β-Pam-resin and NH_2-ThPy-γ-ImPyPyPy-β-Pam-resin at 37 °C for 24 and 40 h, respectively. The terminal imidazole was incorporated as previously published.[25] A single-step aminolysis, using dimethylaminopropylamine (55 °C, 18 h), cleaved the resin ester linkage of all three polyamides from their solid supports. The resin cleavage products were purified by reversed phase HPLC to give ImImPyPy-γ-ImPyPyPy-β-Dp (**1**), ImImPzPy-γ-ImPyPyPy-β-Dp (**2**), and ImImThPy-γ-ImPyPyPy-β-Dp (**3**). The polyamide ImImPzPy-γ-ImPyPyPy-β-Dp-EDTA (**2E**) was prepared by aminolysis of the corresponding resin with 3,3′-diamino-*N*-methyldipropylamine (55 °C, 18 h) followed by reversed phase HPLC purifications which afforded a free amine group at the C-terminus for postsynthetic modification. The polyamide-amine ImImPzPy-γ-ImPyPyPy-β-Dp-NH_2 (**2-NH_2**) was treated with an excess of EDTA-dianhydride (DMSO/NMP, DIEA, 55 °C, 15 min), the remaining anhydride hydrolyzed (0.1 N NaOH, 55 °C, 10 min) and the EDTA-modified polyamide purified by reversed phase HPLC.

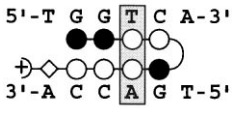
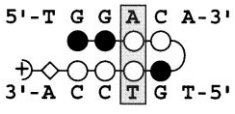
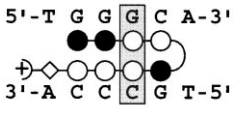
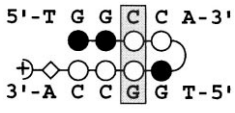
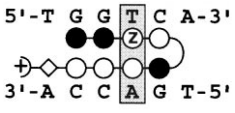
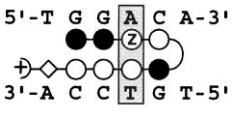
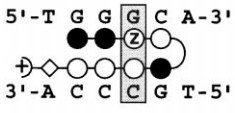
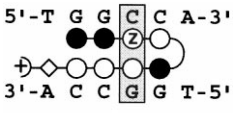
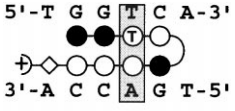
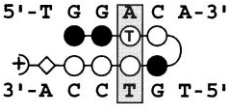
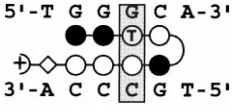
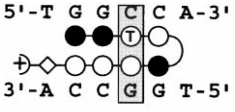
Binding energetics

Quantitative DNase I footprinting titration experiments[27–29] (10 mM Tris–HCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.0, 22 °C) were performed on a 3′-^{32}P end labeled 278 bp *Eco*RI/*Pvu*II restriction fragment from the plasmid pDHN1 to determine the equilibrium association constants for polyamides **1**, **2** and **3** at the four designed sites (Fig. 5 and Table 1). On this restriction fragment, ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp (**1**) bound the two match sites 5′-TGGTCA-3′ and 5′-TGGACA-3′ with equilibrium association constants of $K_a = 4.7\ (\pm 0.7) \times 10^9\ M^{-1}$ and $K_a = 3.1\ (\pm 0.4) \times 10^9\ M^{-1}$, respectively. The mismatch sequences 5′-TGGGCA-3′ and 5′-TGGCCA-3′ are bound with at least 15-fold lower affinity ($K_a = 2.2\ (\pm 0.6) \times 10^8\ M^{-1}$ and $K_a = 2.5\ (\pm 0.9) \times 10^8\ M^{-1}$, respectively). ImIm**Pz**Py-γ-Im**Py**-PyPy-β-Dp (**2**) bound the two sites 5′-TGGTCA-3′ and 5′-TGGACA-3′ with equilibrium association constants of $K_a = 2.0\ (\pm 0.5) \times 10^9\ M^{-1}$ and $K_a = 1.0\ (\pm 0.3) \times 10^9\ M^{-1}$, respectively. No binding of polyamide **2** is observed within the concentration range of these DNase I footprinting titration experiments for the two sites 5′-TGGGCA-3′ and 5′-TGGCCA-3′ ($K_a \leq 2.0 \times 10^7\ M^{-1}$ at both sites). Polyamide **3** containing a Th/Py pairing did not show any binding ($K_a \leq 2.0 \times 10^7\ M^{-1}$ at all sites), at concentrations up to 50 nM, for any site. At concentrations greater than 50 nM, polyamide **3** exhibited non specific binding for multiple DNA sites. While polyamide **2** bound its match sites with binding isotherms consistent with binding in a 1:1 polyamide–DNA complex (Fig. 6), further characterization was necessary to assure similar behavior to pure imidazole–pyrrole polyamides.

Binding site size

MPE•Fe(II) footprinting[30,31] (20 mM HEPES buffer (pH 7.3), 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, 0.5 μM MPE•Fe(II), and 22 °C) on 3′- and 5′-^{32}P end labeled *Eco*RI/*Pvu*II 278 bp restriction fragments from the plasmid pDHN1 reveals that ImIm**Pz**Py-γ-Im**Py**PyPy-β-Dp (**2**), at 50 nM concentration, binds to and protects its designated six base pair match sites 5′-TGGTCA-3′ and 5′-TGGACA-3′. Binding at the single base pair mismatch sites 5′-TGGGCA-3′ and 5′-

Table 1. Equilibrium association constants[a] (M^{-1})

Polyamide	5′-caTGGTCAta-3′	5′-caTGGACAta-3′	5′-caTGGGCAta-3′	5′caTGGCCAta-3′
Py/Py				
	$K_a = 4.7 \times 10^9$	$K_a = 3.1 \times 10^9$	$K_a = 2.2 \times 10^8$	$K_a = 2.5 \times 10^8$
Pz/Py				
	$K_a = 2.0 \times 10^9$	$K_a = 1.0 \times 10^9$	$K_a \leq 2 \times 10^7$	$K_a \leq 2 \times 10^7$
Th/Py				
	$K_a \leq 2 \times 10^7$	$K_a \leq 2 \times 10^7$	$K_a \leq 2 \times 10^7$	$K_a \leq 2 \times 10^7$

[a]The reported equilibrium association constants are the mean values obtained from three DNase I footprint titration experiments. Assays were carried out in the presence of 10 mM Tris–HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ at pH 7.0 and 22°C. The new monomer pyrazole is indicated with an open circle containing a Z (Ⓩ) while thiazole is represented as an open circle with T inside (Ⓣ).

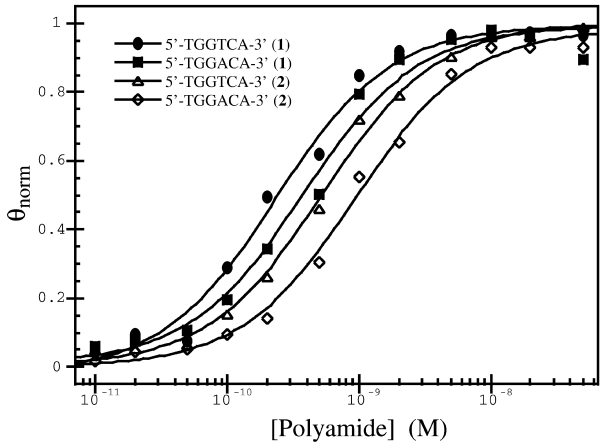


Figure 6. Data from quantitative DNase I footprint titration experiments for ImImPyPy-γ-ImPyPyPy-β-Dp (**1**) and ImImPzPy-γ-ImPyPyPy-β-Dp (**2**) binding to the two match sites 5′-TGGTCA-3′ and 5′-TGGACA-3′. θ_{norm} points were obtained using storage phosphor autoradiography and processed by standard methods.[26,36] The data for the binding of polyamide **1** to 5′-TGGTCA-3′ is indicated by filled circles, of polyamide **1** to 5′-TGGACA-3′ by filled squares, of polyamide **2** to 5′-TGGTCA-3′ by open triangles, and of polyamide **2** to 5′-TGGACA-3′ by open diamonds. Each data point shows the average value obtained from three footprinting experiments. The solid curves are best-fit Langmuir binding titration isotherms obtained from nonlinear least squares algorithm where $n=1$ as previously described.[26,36]

TGGCCA-3′ could not be seen at the 50 nM concentration. The sizes of the asymmetrically 3′-shifted footprint cleavage patterns are consistent with a hairpin polyamide binding at six base pair site in the minor groove of DNA as observed for purely imidazole–pyrrole hairpin polyamides (Figs 7a and 8).

Binding orientation and stoichiometry

Affinity cleavage experiments[17,32] were performed on 3′- and 5′-^{32}P end labeled *Eco*RI/*Pvu*II 278 bp restriction fragments from the plasmid pDHN1 (20 mM HEPES buffer, pH 7.3, 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, 1 μM Fe(II), and 22 °C). Affinity cleavage experiments were performed with a carboxy terminus EDTA·Fe(II) modified analogue **2E** of polyamide **2**. The observed cleavage patterns for ImIm**Pz**Py-γ-Im**Py**-PyPy-β-Dp-EDTA (**2E**) are 3′-shifted, consistent with minor groove occupancy (Figs 7b and 8). A single cleavage locus proximal to the 5′-side of both the 5′-TGGTCA-3′ and 5′-TGGACA-3′ binding sites at 5 nM is consistent with a single orientation 1:1 polyamide–DNA complex.

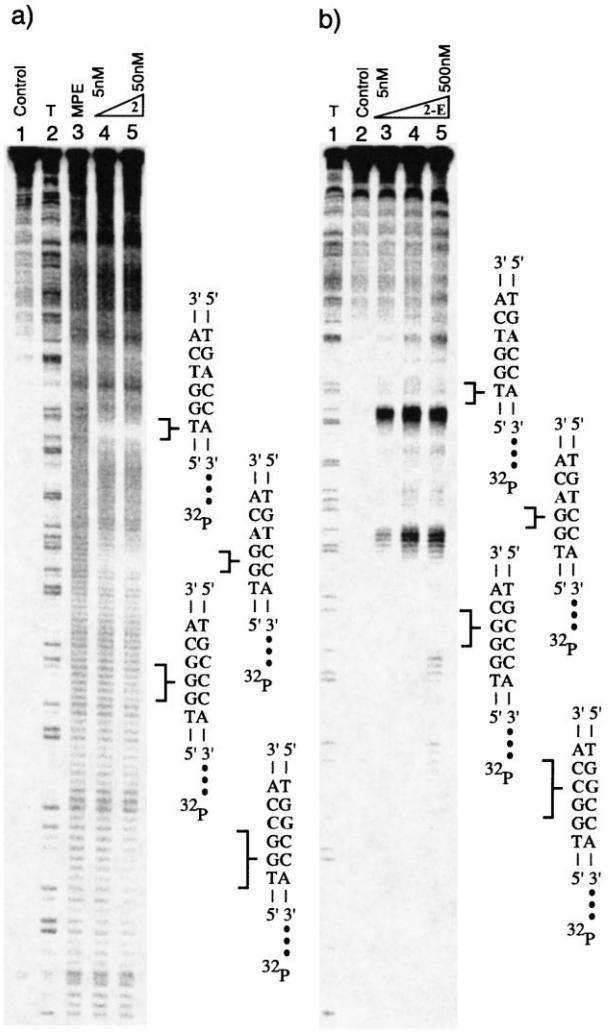


Figure 7. (a) MPE·Fe(II) footprinting experiment on the 3′-^{32}P end labeled 278 bp *Eco*RI/*Pvu*II restriction fragment from plasmid pDHN1. The targeted 5′-TGGTCA-3′, 5′-TGGACA-3′, 5′-TGGGCA-3′, and 5′-TGGCCA-3′ sites are shown on the right side of the autoradiogram. Lane 1, intact DNA; lane 2, T reaction; lane 3, MPE·Fe(II) standard in the absence of any polyamide; lanes 4 and 5, MPE·Fe(II) cleavage products in the presence of 5 nM and 50 nM ImImPzPy-γ-ImPyPyPy-β-Dp (**2**), respectively. All lanes contain 15 kcpm 3′-radiolabeled DNA, 20 mM HEPES buffer (pH 7.3), 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, and 0.5 μM MPE·Fe(II). (b) Affinity cleavage experiment on the 3′-^{32}P end labeled 278 bp *Eco*RI/*Pvu*II restriction fragment from plasmid pDHN1. The targeted 5′-TGGTCA-3′, 5′-TGGACA-3′, 5′-TGGGCA-3′, and 5′-TGGCCA-3′ sites are shown on the right side of the autoradiogram. Lane 1, T reaction; lane 2, intact DNA; lanes 3–5, affinity cleavage products in the presence of 5 nM, 50 nM, and 500 nM ImImPzPy-γ-ImPyPyPy-β-Dp-EDTA (**2E**), respectively. All lanes contain 15 kcpm 3′-radiolabeled DNA, 20 mM HEPES buffer (pH 7.3), 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, and 1.0 μM Fe(II).

Discussion

The Pz/Py pair

Quantitative DNase I footprinting reveals that the incorporation of a pyrazole monomer into a hairpin polyamide results in a 100-fold increase in specificity for the match sites 5′-TGGTCA-3′ and 5′-TGGACA-3′ over the mismatch sites 5′-TGGGCA-3′ and 5′-TGGCCA-3′ due to significantly lower mismatch affinities. The purely imidazole–pyrrole polyamide **1** only exhibits a 20-fold greater specificity for its match sites. Although a Pz/Py pairing exhibits slightly lower affinity for A·T/T·A base pairs than a Py/Py pairing, polyamide **2** continues to bind to DNA with an affinity and specificity comparable to DNA binding proteins. The 2.3-fold decrease in affinity of the Pz/Py pairing relative to a Py/Py pairing may arise from some electrostatic repulsion between the N2 of pyrazole and the amide oxygen of the polyamide backbone (Fig. 9a). MPE·Fe(II) footprinting

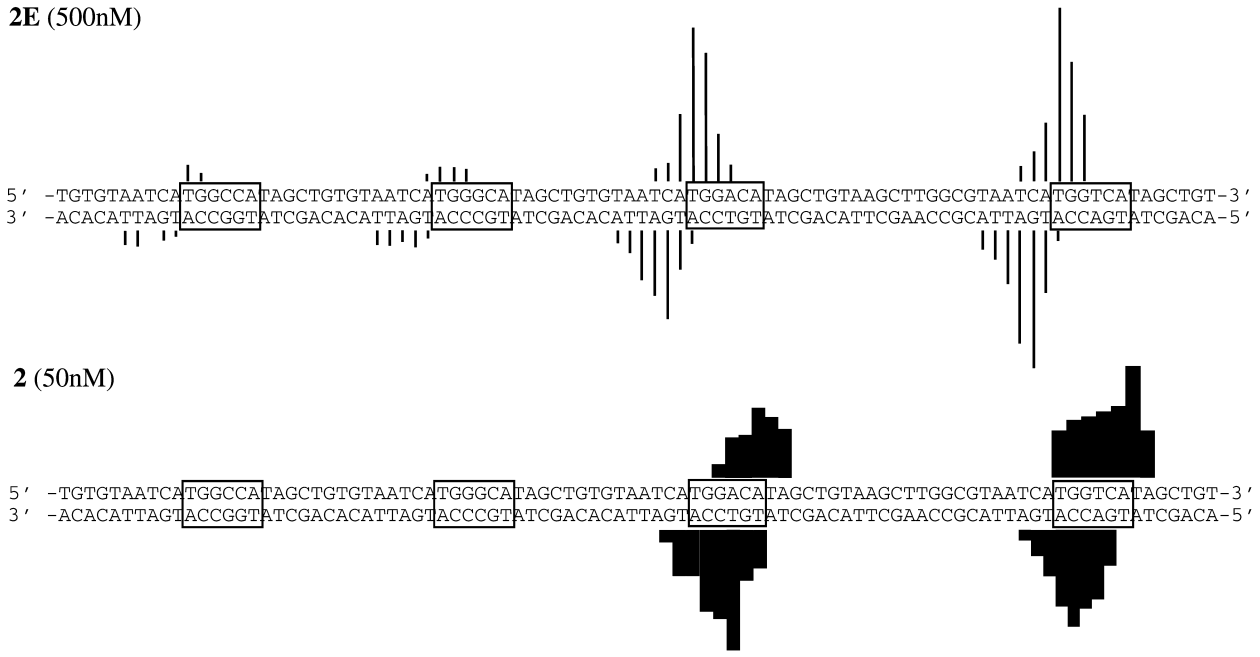


Figure 8. (Top) Affinity cleavage patterns for ImImPzPy-γ-ImPyPyPy-β-Dp-EDTA (**2E**) at 500 nM. (Bottom) MPE•Fe(II) protection patterns for ImImPzPy-γ-ImPyPyPy-β-Dp (**2**) at 50 nM.

and affinity cleavage experiments demonstrate that ImImPzPy-γ-ImPyPyPy-β-Dp (**2**) binds its designated six base pair sites, in the minor groove of DNA, in a single orientation and as a 1:1 polyamide–DNA complex, consistent with the hairpin polyamide binding model.

The Th/Py pair

It has been proposed in the literature that the degeneracy of A•T/T•A recognition by polyamides could be overcome by using a thiazole (Th) monomer that would be sterically destabilized when placed opposite a thymine base.[23, 24] Thiazole was therefore proposed to be an A-specific recognition element.[23,24] Conversely, recent studies[7–9] on hydroxypyrrole (Hp) have shown that steric destabilization, arising from the bulky hydroxyl group, occurs only when Hp is located opposite adenine, resulting in the loss of DNA binding affinity. Since this steric destabilization is absent and an extra hydrogen bond is formed when hydroxylpyrrole is placed opposite thymine,[8] Hp is a T-specific recognition element. While the Hp structural data[8] reduce the plausibility of thiazole conferring A-specificity as proposed, it does allow for the possibility of thiazole being T-specific based solely on steric discrimination. Therefore, the singular steric discrimination by thiazole was interesting in light of the dual steric and hydrogen-bonding components of T/A differentiation by hydroxypyrrole (Hp). The quantitative DNase I footprinting titrations reveal that the binding affinity of an eight-ring hairpin polyamide is compromised by the incorporation of a single 4-methylthiazole monomer. It is puzzling that the 4-methylthiazole/*N*-methylpyrrole pair (Th/Py) has low affinity for both A•T and T•A. Perhaps different analogues of this ring system should be investigated further.

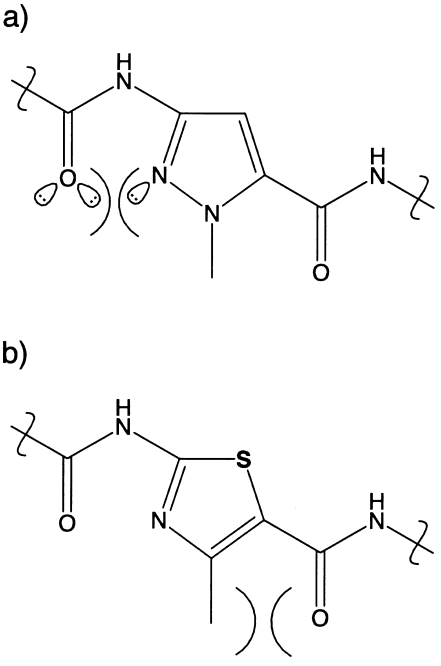


Figure 9. (a) Model for the electrostatic repulsion between the lone electron pairs on N2 of pyrazole and the lone electron pairs on the amide oxygen of the polyamide backbone. (b) Model for the possible steric clash between the 4-methyl of thiazole and the carbonyl of the proximal carboxamide.

For example, what is the role of the methyl substituent at the 4-position? With the change in both length and bond angle of the thiazole core,[21] one could imagine an energetically unfavorable steric clash between the 4-methyl and the carbonyl of the proximal carboxamide (Fig. 9b).

Implications for the design of minor groove binding polyamides

The results presented here reveal that the *N*-methylpyrazole ring might replace the *N*-methylpyrrole moiety for minor groove DNA recognition. The ability to generate alternative pairing schemes for the minor groove with new monomers could greatly expand the targetable sequence repertoire of hairpin polyamides (Table 2). As issues of biodistribution in animal studies become important, an expanded set of pairing rules such as Pz/Pz or Im/Pz would be of interest to examine in this series for biological applications.

Experimental

Materials

Dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 0.75 mmol/g Boc-β-alanine-(4-carboxamidomethyl)-benzyl-ester-copoly (styrene–divinylbenzene) resin (Boc-β-Pam-resin), and Boc-γ-aminobutyric acid were purchased from Peptides International. *N*,*N*-Diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), 1:1 dimethylsulfoxide: *N*-methylpyrrolidinone (DMSO/NMP), and acetic anhydride (Ac_2O) were purchased from Applied Biosystems. Dichloromethane (DCM) and triethylamine (TEA) were reagent grade from EM. Thiophenol (PhSH), dimethylaminopropylamine (Dp), and 3,3′diamino-*N*-methyldipropylamine (Dp-NH_2) and EDTA-dianhydride were purchased from Aldrich. Trifluoroacetic acid (TFA) Biograde was from Halocarbon. All reagents were used without further purification.

^{1}H NMR spectra were recorded on a General Electric–QE NMR spectrometer at 300 MHz in DMSO-d_6 with chemical shifts reported in parts per million relative to residual solvent. UV spectra were measured in water on a Hewlett–Packard Model 8452A diode array spectrophotometer. Matrix-assisted, laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) was performed at the Protein and Peptide Microanalytical Facility at the California Institute of Technology. High-resolution FAB mass spectra were recorded at the Mass Spectroscopy Laboratory at University of California, Riverside. DNA sequencing was performed at the Sequence/Structure Analysis Facility (SAF) at the California Institute of Technology. HPLC analysis was performed on a Beckman Gold System using a RAINEN C_{18}, Microsorb MV, 5 μm, 300×4.6 mm reversed phase column in 0.1% (wt/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min, gradient elution 1.25% acetonitrile/min. Preparatory reversed phase HPLC was performed on a Beckman HPLC with a Waters BondaPak 25×100 mm, 100 μm C_{18} column equipped with a guard, 0.1% (wt/v) TFA, 8.0 mL/min, 0.25% acetonitrile/min. 18 MΩ water was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered.

Table 2. Polyamide ring pairings

	T·A	A·T	G·C	C·G
Py/Py	+	+	−	−
Pz/Py	+	+	−	−
Th/Py	−	−	−	−

Monomer synthesis

3-[(*tert*-Butoxycarbonyl)amino]-1-methylpyrazole-5-carboxylic acid (5). The amino ethyl ester **4** (6.0 g, 35.5 mmol) was dissolved in 100 mL of DMF and DIEA (12.3 mL, 70.6 mmol). To the stirred solution di-*tert*-butyl-dicarbonate (15.4 g, 70.6 mmol) was added and allowed to react overnight. The solution was poured into water and extracted with diethyl ether. The organic layers were combined, dried with $MgSO_4$, and concentrated in vacuo to provide a white solid that was then dissolved in ethanol. 1 M NaOH was added and the solution heated at 45 °C for 1 h. The clear solution was diluted with water and the ethanol removed in vacuo. The solution was extracted with diethyl ether, the pH of the aqueous layer reduced to 3 with 10% (v/v) H_2SO_4 and the mixture extracted with ethyl acetate. The combined organic layers were dried with $MgSO_4$ and concentrated in vacuo to provide **5** as a white powder (3.24 g, 38% yield): ^{1}H NMR (DMSO-d_6) δ 13.32 (br s, 1H), 9.73 (s, 1H), 6.70 (s, 1H), 3.95 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 161.5, 153.7, 146.8, 133.8, 101.4, 80.2, 39.5, 29.0; FABMS *m*/*e* 264.0958 (M + Na, 264.0960 calcd for $C_{10}H_{15}N_3O_4$ + Na).

2-[(*tert*-Butoxycarbonyl)amino]-4-methylthiazole-5-carboxylic acid (7). The amino ethyl ester **6** (10 g, 53.5 mmol) and di-*tert*-butyl-dicarbonate (23.4 g, 107 mmol) were dissolved in 150 mL of DMF and DIEA (19 mL, 107 mmol). The stirred reaction was heated to 50 °C for 3 days. The reaction was allowed to cool to room temperature and diluted with 350 mL of water. The pH of the aqueous layer was reduced to 3 with 10% (v/v) H_2SO_4 and the mixture extracted with diethyl ether. The organic layers were combined, dried with $MgSO_4$, and concentrated in vacuo to provide a suspended solid. This solid was collected by vacuum filtration and washed with hexanes to provide a tan powder that was then dissolved in ethanol. 1 M KOH was added and the solution heated to 70 °C for 2 h. The mixture was poured into 250 mL of water and washed with diethyl ether, the pH of the aqueous layer reduced to 3 with 10% (v/v) H_2SO_4, and the milky white solution extracted with diethyl ether. The combined organic extracts were concentrated in vacuo to provide **7** as a white solid (7.1 g, 27.8 mmol, 52% yield): ^{1}H NMR (DMSO-d_6) δ 12.5 (br s, 1H), 11.8 (br s, 1H), 2.4 (s, 3H), 1.4 (s, 9H); ^{13}C NMR (DMSO-d_6) δ 164.6, 162.2, 156.8, 153.7, 115.9, 82.8, 28.8, 17.8; EIMS *m*/*e* 259.0751 (M + H, 259.0752 calcd for $C_{10}H_{15}N_2O_4S$).

ImImPzPy-γ-ImPyPyPy-β-Dp (2). ImImPzPy-γ-ImPyPyPy-β-Pam-resin was synthesized in a stepwise fashion by manual solid-phase protocols[25] from Boc-β-alanine-Pam-resin (1.0 g, 0.75 mmol/g). Boc-Pz-OH (241 mg, 1 mmol) and HBTU (360 mg, 0.95 mmol) were

combined in 2 mL of DMF. DIEA (1 mL) was then added and the reaction mixture allowed to stand for 5 min. The activated monomer was added to the reaction vessel, containing NH_2-Py-γ-ImPyPyPy-β-Pam-resin, and the coupling was allowed to proceed for 20 h at 37 °C before termination with acetic anhydride. After Boc-deprotection of the resin-bound polyamide chain, the Boc-Im-OH was activated with HBTU/DIEA as previously described and coupled onto NH_2-PzPy-γ-ImPyPyPy-β-Pam-resin at 37 °C for 24 h. The terminal imidazole was incorporated as previously published. A sample of ImImPzPy-γ-ImPyPyPy-β-Pam-resin (300 mg, 0.44 mmol/g) was placed in a 20 mL scintillation vial, 2 mL of dimethylaminopropylamine was added, and the mixture was allowed to stand at 55 °C for 18 h. Resin was removed through filtration through a disposable propylene filter, and the resulting solution diluted with water to a total volume of 8 mL and purified directly by reversed phase HPLC to provide ImImPzPy-γ-ImPyPyPy-β-Dp (**2**) (5.7 mg, 3.5% recovery) as a white powder upon lyophilization of the appropriate fractions. ^{1}H NMR (DMSO-d_6) δ 10.47 (s, 1H), 10.29 (s, 1H), 10.14 (s, 1H), 10.04 (s, 1H), 10.02 (s, 1H), 9.97 (s, 1H), 9.92 (s, 1H), 9.25 (br s, 1H), 8.1 (m, 3H), 7.68 (s, 1H), 7.48 (s, 2H), 7.43 (s, 1H), 7.28 (s, 1H), 7.2 (m, 2H), 7.18 (s, 2H), 7.11 (s, 1H), 7.08 (s, 1H), 6.96 (s, 1H), 6.89 (s, 1H), 4.06 (s, 3H), 4.04 (s, 3H), 4.02 (s, 3H), 3.97 (s, 3H), 3.87 (s, 3H), 3.85 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.4 (m, 2H), 3.2 (m, 2H), 3.1 (m, 2H), 3.0 (m, 2H), 2.7 (m, 6H), 2.4 (m, 4H), 1.8 (m, 4H); MALDI-TOF-MS (monoisotopic), 1224.63 (1224.57 calcd for M + H).

ImImPzPy-γ-ImPyPyPy-β-Dp-NH_2 (2-NH_2). A sample of ImImPzPy-γ-ImPyPyPy-β-Pam-resin (250 mg, 0.44 mmol/g) was placed in a 20 mL scintillation vial, 2 mL of 3,3′-diamino-*N*-methyldipropylamine was added, and the mixture was allowed to stand at 55 °C for 18 h. Resin was removed through filtration through a disposable propylene filter, and the resulting solution diluted with water to a total volume of 8 mL and purified directly by reversed phase HPLC to provide ImImPzPy-γ-ImPyPyPy-β-Dp-NH_2 (**2-NH_2**) (8.2 mg, 5.9% recovery) as a white powder upon lyophilization of the appropriate fractions. ^{1}H NMR (DMSO-d_6) δ 10.47 (s, 1H), 10.29 (s, 1H), 10.14 (s, 1H), 10.03 (s, 2H), 9.98 (s, 1H), 9.92 (s, 1H), 9.25 (br s, 1H), 8.1 (m, 3H), 7.82 (br s, 3H), 7.68 (s, 1H), 7.48 (s, 2H), 7.43 (s, 1H), 7.29 (s, 2H), 7.2 (m, 1H), 7.18 (s, 1H), 7.1 (m, 2H), 6.95 (s, 2H), 6.90 (s, 1H), 4.06 (s, 3H), 4.04 (s, 3H), 4.02 (s, 3H), 3.97 (s, 3H), 3.87 (s, 3H), 3.85 (s, 3H), 3.83 (s, 3H), 3.81 (s, 3H), 3.2–3.0 (m, 8H), 2.9 (m, 2H), 2.5 (m, 4H), 2.37 (br s, 4H), 2.0–1.7 (br m, 6H); MALDI-TOF-MS (monoisotopic), 1267.78 (1267.61 calcd for M + H).

ImImPzPy-γ-ImPyPyPy-β-Dp-EDTA (2E). EDTA-dianhydride (50 mg) was dissolved in 1 mL of DMSO/NMP solution and 1 mL of DIEA by heating at 55 °C for 5 min. The dianhydride solution was added to ImImPzPy-γ-ImPyPyPy-β-Dp-NH_2 (**2-NH_2**) (8.2 mg, 6.5 μmol) dissolved in 750 μL of DMSO. The mixture was heated to 55 °C for 25 min, treated with 3 mL of 0.1 N NaOH, and heated to 55 °C for 10 min. TFA (0.1%) was added to adjust the total volume to 8 mL, and the solution purified directly by preparatory reversed phase HPLC to provide **2E** as a white powder (3.6 mg, 36% recovery). MALDI-TOF-MS (monoisotopic), 1542.16 (1542.81 calcd for M + H).

ImImThPy-γ-ImPyPyPy-β-Dp (3). ImImPzPy-γ-ImPyPyPy-β-Pam-resin was synthesized in a stepwise fashion by manual solid-phase protocols[25] from Boc-β-alanine-Pam-resin (1.0 g, 0.75 mmol/g). Boc-Th-OH (258 mg, 1 mmol) and HBTU (360 mg, 0.95 mmol) were combined in 2 mL of DMF. DIEA (1 mL) was then added and the reaction mixture allowed to stand for 5 min. The activated monomer was added to the reaction vessel, containing NH_2-Py-γ-ImPyPyPy-β-Pam-resin, and the coupling was allowed to proceed for 20 h at 37 °C before termination with acetic anhydride. After Boc-deprotection of the resin-bound polyamide chain, the Boc-Im-OH was activated with HBTU/DIEA as previously described and coupled onto NH_2-ThPy-γ-ImPyPyPy-β-Pam-resin at 37 °C for 40 h. The terminal imidazole was incorporated as previously published. A sample of resin (150 mg, 0.43 mmol/g) was placed in a 20 mL scintillation vial, 2 mL of dimethylaminopropylamine was added, and the mixture was allowed to stand at 55 °C for 18 h. Resin was removed by filtration through a disposable polypropylene filter, and the resulting solution diluted with water to a total volume of 8 mL and purified directly by reversed phase HPLC to provide ImImThPy-γ-ImPyPyPy-β-Dp (1.5 mg, 1.8% recovery) as a white powder upon lyophilization of the appropriate fractions. ^{1}H NMR (DMSO-d_6) δ 10.29 (s, 1H), 10.05 (s, 1H), 10.02 (s, 1H), 9.98 (s, 3H), 9.92 (s, 1H), 9.2 (br s, 1H), 8.1 (m, 3H), 7.78 (s, 1H), 7.49 (s, 2H), 7.32 (s, 1H), 7.28 (s, 1H), 7.26 (s, 1H), 7.24 (s, 2H), 7.18 (s, 1H), 7.10 (s, 1H), 6.9 (m, 2H), 3.99 (s, 3H), 3.97 (s, 3H), 3.91 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.7 (m, 9H), 3.4 (m, 2H), 3.2 (m, 2H), 3.1 (m, 2H), 3.0 (m, 2H), 2.7 (m, 6H), 2.4 (m, 4H), 1.8 (m, 4H); MALDI-TOF-MS (monoisotopic), 1241.67 (1241.53 calcd for M + H).

DNA reagents and materials

Enzymes were purchased from Boehringer–Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P] triphosphates were obtained from Dupont/NEN. Calf thymus DNA (sonicated, deproteinized), DNase I (7500 u/mL, FPLC pure), Tris–HCl, dithiothreitol (DTT), RNase-free water (used for all footprinting and affinity cleavage reactions), and 0.5 M EDTA were purchased from Amersham Pharmacia. XGal and IPTG were from ICN. Ampicillin trihydrate was acquired from Sigma. Absolute ethanol was purchased from Equistar. Calcium chloride, potassium chloride, and magnesium chloride were from Fluka. Formamide and pre-mixed Tris-borate–EDTA (Gel-Mate) were from Gibco. All reagents were used without further purification. DNA manipulations were performed according to standard protocols.[36]

Construction of plasmid DNA

The plasmid pDHN1 was prepared by hybridization of a complementary set of synthetic oligonucleotides: 5′-

GATCCTTAGCTGTGTAATCATGGCCATAGCTG TGTAATCATGGGCATAGCTGTGTAATCATGGA CATAGCTGTA-3′ and 5′-AGCTTACAGCTATGTC CATGATTACACAGCTATGCCCATGATTACACA GCTATGGCCATGATTACACAGCTAAG-3′. The hybridized insert was ligated into *Bam*HI/*Hin*dIII linearized pUC19 using T4 DNA ligase. *Epicurean coli* XL-1 Blue supercompetent cells were then transformed with the ligated plasmid, and plasmid DNA from ampicillin-resistant white colonies isolated using a Promega Maxi-Prep kit. The presence of the desired insert was determined by dideoxy sequencing.

Preparation of ^{32}P-end-labeled restriction fragments

Plasmid pDHN1 was linearized with *Eco*RI and *Pvu*II restriction enzymes, then treated with Sequenase, deoxyadenosine 5′-[α-^{32}P]triphosphate, and thymidine 5′-[α-^{32}P]triphosphate for 3′ labeling. The 5′ labeling of the desired restriction fragment was achieved through PCR amplification using the primers [γ-^{32}P]DHN1-A (5′-[γ-^{32}P]AATTCGAGCTCGGTACCCGGG-3′) and DHN1-B (5′-CTGGCACGACAGGTTTCCCGA-3′). The labeled fragment (3′ or 5′) was loaded onto a 7% nondenaturing polyacrylamide gel, and the desired 278 bp band was visualized by autoradiography and isolated.

DNase I footprinting

All reactions were carried out in a volume of 400 μL. We note explicitly that no carrier DNA was used in these reactions until after DNase I cleavage. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were 10 mM Tris–HCl buffer (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 15 kcpm 3′-radiolabeled DNA. The solutions were equilibrated for a minimum of 12 h at 22 °C. Cleavage was initiated by the addition of 10 μL of a DNase I stock solution (diluted with 1 mM DTT to give a stock concentration of 0.75 u/mL) and was allowed to proceed for 7 min at 22 °C. The reactions were stopped by adding 50 μL of a solution containing 2.25 M NaCl, 150 mM EDTA, 0.6 mg/mL glycogen, and 30 μM base pair calf thymus DNA and then ethanol-precipitated. The cleavage products were resuspended in 100 mM Tris-borate–EDTA/80% formamide loading buffer, denatured at 85 °C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1 h 45 min. The gels were dried under vacuum at 80 °C and then quantitated using storage phosphor technology. Equilibrium association constants were determined as previously described.[26–29]

MPE·Fe(II) footprinting

All reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were 20 mM HEPES buffer (pH 7.3), 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, and 0.5 μM MPE·Fe(II). Equilibration proceeded for 10 min after the addition of 40 μL of 5 μM MPE·Fe(II) (freshly made by combining equal volumes of 10 μM MPE and 10 μM $Fe(NH_4)_2(SO_4)_2$). The reactions were initiated with 10 μL of 200 mM DTT and cleavage allowed to proceed for 14 min. The reactions were stopped by ethanol precipitation, resuspended in 100 mM Tris-borate–EDTA/80% formamide loading buffer, denatured at 85 °C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1 h 45 min.

Affinity cleavage

All reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were 20 mM HEPES buffer (pH 7.3), 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, and 1.0 μM Fe(II). Equilibration proceeded for 20 min after the addition of 20 μL of 10 μM $Fe(NH_4)_2(SO_4)_2$. The reactions were initiated with 40 μL of 50 mM DTT and cleavage allowed to proceed for 30 min. The reactions were stopped by ethanol precipitation, resuspended in 100 mM Tris-borate–EDTA/80% formamide loading buffer, denatured at 85 °C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1 h 45 min.

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Supporting Information Available: DNase I footprinting experiments of polyamide **3** (PDF). See any current masthead page for ordering and Internet access instructions.

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