Abstract: Motifs for covalent linkage of side-by-side complexes of pyrrole—imidazole (Py–Im) polyamides in the DNA minor groove provide for small molecules that specifically recognize predetermined sequences with subnanomolar affinity. Polyamide subunits linked by a turn-specific γ-aminobutyric acid (γ) residue form hairpin polyamide structures. Selective amino-substitution of the prochiral α-position of the γ-residue relocates the cationic charge from the hairpin C terminator. Here we report the synthesis of pyrrole resin as well as a solid-phase strategy for the preparation of cycle polyamides. The DNA binding properties of two eight-ring cycle polyamides were analyzed on a DNA restriction fragment containing six base pair match and mismatch binding sites. Quantitative footprint titrations demonstrate that a cycle polyamide of sequence composition cyclo-(γ-ImPyPyPy-(R)H[2]γ-ImPyPyPy-) binds a 5′-AGTACT-3′ site with an equilibrium association constant $K_a = 7.6 \times 10^{10}$ M$^{-1}$, a 3600-fold enhancement relative to the unlinked homodimer (ImPyPyPy-(β-Dp))25′-AGTACT-3′, and an 8-fold enhancement relative to hairpin analogue ImPyPyPy-(R)H[2]γ-ImPyPyPy-C3=OH5′-AGTACT-3′. Replacement of a single nitrogen atom with a C–H (Im–py) regulates affinity and specificity of the cycle polyamide by 2 orders of magnitude. The results presented here suggest that addition of a chiral γ-turn combined with placement of a second γ-turn within the hairpin structure provides a cycle polyamide motif with favorable DNA binding properties.

Introduction

Small molecules that target predetermined DNA sequences have the potential to control gene expression.1 Polyamides containing the three aromatic amino acids 3-hydroxypyrrole (Hp), imidazole (Im), and pyrrole (Py) are synthetic ligands that bind to predetermined DNA sequences with subnanomolar affinity.2,3 DNA recognition depends on a code of side-by-side amino acid pairings oriented N–C with respect to the 5′–3′ direction of the DNA helix in the minor groove.2,4–9 An antiparallel pairing of Im opposite Py (Im/Py pair) distinguishes G–C from C–G and both of these from A–T/A–T base pairs.4 A Py/Py pair binds both A–T and T–A in preference to G–C/C–G.4,5 The discrimination of T–A from A–T using Hp/Py pairs completes the four base pair (bp) code.5 The linker amino acid γ-aminobutyric acid (γ) connects polyamide subunits C–N in a “hairpin motif”, and these ligands bind to predetermined target sites with > 100-fold enhanced affinity relative to dimers.2,9 In both published and unpublished work, eight-ring hairpin polyamides have been found to regulate transcription and permeate a variety of cell types in culture.1 Because topology could


potentially regulate cell-permeation properties, discovery of new motifs for covalent linkage that provide polyamides with affinities and specificities comparable to naturally occurring DNA binding proteins remains a high priority.

**Design of Cycle Polyamides.** In a formal sense, addition of a second γ-turn at the C and N termini of a hairpin polyamide allows covalent closure to form a cycle. An initial report described a six-ring (γ-3-γ-3-γ) cycle polyamide which bound to a five base pair DNA sequence with higher affinity than a corresponding hairpin polyamide; however, sequence specificity versus mismatch DNA sequences was extremely poor (∼3-fold), compared to 40-fold observed for the hairpin. It was initially thought that the cycle restricted polyamide flexibility, limiting the available conformers to prevent formation of a specific recognition complex. It remained to be determined if γ-turn cycle polyamides could be designed that have comparable DNA binding properties to hairpin-polyamides.

Because of the labor intensive solution-phase cycle polyamide synthesis and the initial discouraging thermodynamics with regard to sequence specificity, the cycle polyamides have not been investigated further until this report. We describe here a pyrrole resin which enables cycle polyamide linear precursors to be synthesized by solid-phase methods, reducing the synthetic effort from weeks to days. Two eight-ring (γ-4-γ-4-γ) cycle polyamides have been prepared for the studies described here. Two key design elements from the original six-ring cycle have been altered. First, the number of ring pairings increases from three to four. Polyamide–DNA binding affinity is predicted to increase as the number of consecutive ring pairings increases from three to four. In addition, the charge has been moved from a Py-N-methyl group to a γ-turn. Although the detailed effects of the placement of charge remain to be determined, substitution of the prochiral α-position of the γ-turn residue to provide (R)-2,4-diaminobutyric acid has been previously found to yield chiral linked hairpins with enhanced DNA binding sequence specificity and orientation preference.

Here we report the DNA binding affinities and sequence specificities of the eight-ring cycle polyamides, cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-β-Dp) (1) and cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-) (4) that differ by a single amino acid substitution (underlined), for their respective six base pair match sites, 5′-AGTACT-3′ and 5′-AGTATT-3′, which differ by a single base pair (underlined). In control experiments, the binding affinity and sequence specificity of the ImPyPyPy-β-Dp (1) dimer, and a hairpin analogue ImPyPyPy-(R)H2Nγ-ImPyPyPy-C3−OH (2) were also studied (see Figure 2). An EDTA

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DNA Binding Properties of Cycle Polyamide Motifs

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Figure 2. Structures of polyamides ImPyPyPy-β-Dp (1), ImPyPyPy-(R)<sub>1</sub>ImPyPyPy-β-Dp (2), ImPyPyPy-(R)<sub>1</sub>ImPyPyPy-OH (3), cyclo-(γ-ImPyPyPy-(R)<sub>1</sub>ImPyPyPy) (3) and cyclo-(γ-ImPyPyPy-(R)<sub>1</sub>ImPyPyPy) (4) as synthesized by solid-phase methods.

Figure 3. Synthesis of Boc-Py-PAM resin (7). (i) K$_2$CO$_3$, DMF, (ii) Zn/AscOOH (iii) DCC/HOBt, DMF; (iv) aminomethylated-polystyrene; DIEA.

Results and Discussion

Resin Synthesis. The Py-Pam ester (5) was prepared according to the published procedures of Merrifield, with Boc-Py acid substituted for the standard Boc protected α-amino acid.

formate was added (500 mg, 8 h) and the reaction mixture purified by reversed phase HPLC to provide H₂N-ç-ImPyPyPy-(R) Boc ç-ImPyPyPy-COOH (12) and H₂N-ç-ImPyPyPy-(R) Boc ç-ImPyPyPy-COOH (13). Cyclization of H₂N-ç-ImPyPyPy-(R) Boc ç-ImPyPyPy-COOH (13) and H₂N-ç-ImPyPyPy-(R) Boc ç-ImPyPyPy-COOH (12) was achieved with DPPA and potassium
carbonate, as described previously. The Boc-protecting group was then removed in situ by treatment with neat TFA to yield the cyclic compounds cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-) (3) and cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-) (4) after subsequent purification by reversed phase HPLC. The cycle polyamides are obtained with similar yield and purity and have similar solubility as their hairpin counterparts. Binding Site Size. MPE-Fe(II) footprinting on 3'- or 5'-32P end-labeled 229 base pair restriction fragments reveals that each cycle polyamide, at 10 nM concentration, binds to its designated six base pair match sites (25 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) (Figure 5). The polyamide cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-) (3) which contains an Im/Py and a Py/Im pair, protects the designated six base pair match sites (25 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) (Figure 5). The polyamide cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-) (3) which contains an Im/Py and a Py/Im pair, protects the cognate 5'-AGTATT-3' site. Binding of the single base pair mismatch site 5'-AGTATT-3' and at the 5'- and 3'- sides of the 5'-AGTATT-3' match site (Figure 6c), at the 5'- and 3'- sides of the 5'-AGTACT-3' mismatch site corresponding to the EDTA-Fe(II) moiety placement off the (R)H2Nγ-turn residue.

Binding Energetics. Quantitative DNase I footprint titrations (10 mM Tris·HCl, 10 mM KCl, 10 mM MgCl2, 5 mM CaCl2, pH 7.0, and 22 °C) were performed to determine the equilibrium association constants (Kd) for the respective binding interactions. For synthesis of the EDTA analogue, cyclo-(γ-ImPyPyPy-(R)H2Nγ-ImPyPyPy-) (4) was treated with an excess of EDTA-dianhydride (DMSO/NMP, DIEA, 55 °C, 15 min) and the remaining anhydride was hydrolyzed (0.1 M NaOH, 55 °C, 10 min). Cyclo-(γ-ImPyPyPy-(R)EDTAγ-ImPyPyPy-) (4-E-Fe(II)) was then isolated by reversed phase HPLC (Figure 6a). Affinity cleavage experiments were performed on the same 3'- or 5'-32P end-labeled 229 base pair DNA restriction fragment from the plasmid pJ78 (20 mM HEPES buffer (pH 7.3), 200 mM NaCl, 50 μg/mL glycogen, 5 mM DTT, 1 μM Fe(II), pH 7.0 and 22 °C). The observed cleavage pattern for cyclo-(γ-ImPyPyPy-(R)EDTAγ-ImPyPyPy-) (4-E-Fe(II)) (Figure 6b and c) are 3'-shifted, consistent with minor groove occupancy. In the presence of 100 nM 4-E-Fe(II), a major cleavage locus proximal to the 3'-side of the 5'-AGTATT-3' match sequence is revealed, consistent with formation of an oriented 1:1 cycle polyamide-DNA complex. At the same ligand concentration, minor cleavage loci located 3' and 5' adjacent to the single base pair mismatch 5'-AGTATT-3' site appear, consistent with dual binding orientations at this symmetrical binding site. The cyclo-polyamide binding model is further supported by the location of cleavage loci at the 5'-side of the 5'-AGTATT-3' match site (Figure 6c), and at the 5'- and 3'- sides of the 5'-AGTACT-3' mismatch site corresponding to the EDTA-Fe(II) moiety placement off the (R)H2Nγ-turn residue.
and mismatch sites with binding isotherms consistent with 2:1 dimer formation.²² Hairpin polyamide (2) and cycle polyamides (3) and (4) bind their respective match and mismatch sequences with binding isotherms consistent with binding in a 1:1 polyamide-DNA complex. ³³ Polyamides bind the 5′-AGTATT-3′ sequence with decreasing affinity; match cycle (4) > mismatch cycle (3) > mismatch hairpin (2) > mismatch dimer (1).

Table 1. Association Constants (M⁻¹) for Polyamides 1–4⁺dling

<table>
<thead>
<tr>
<th>Polyamide</th>
<th>Motif</th>
<th>5′-AGTACT-3′</th>
<th>5′-AGTATT-3′</th>
<th>Specificity</th>
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<tr>
<td>1</td>
<td>dimer</td>
<td>Kₐ = 2.1 × 10⁷</td>
<td>Kₐ = 1.4 × 10⁶</td>
<td>15</td>
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<tr>
<td>2</td>
<td>hairpin</td>
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<td>Kₐ = 7.4 × 10⁸</td>
<td>Kₐ = 1.3 × 10⁹</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>cycle</td>
<td>Kₐ = 4.2 × 10⁹</td>
<td>Kₐ = 3.1 × 10⁹</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* The reported equilibrium association constants are the mean values obtained from three DNase I footprint titration experiments. The assays were carried out at 22 °C, pH 7.0 in the presence of 10 mM Tris·HCl, 10 mM KCl, 10 mM MgCl₂, and 5 mM CaCl₂. Cartoon monomer association constants were determined for polyamide homodimers.²³ Specificity calculated as Kₐ(5′-AGTACT-3′)/Kₐ(5′-AGTATT-3′).

Figure 6. (a) Synthesis of 4-E-Fe(II): (i) EDTA dianhydride (DMSO/NMP, DIEA, 55 °C, 15 min); (ii) 0.1 M NaOH (55 °C, 10 min). (b) Affinity cleavage pattern for cyclo-(γ-ImPyPyPyPy-(R)EDTAγ-ImPyPyPyPy) (4-E-Fe(II)) at 100 nM concentration depicting a single binding orientation at the 5′-AGTATT-3′ match site and no orientational preference at the 5′-AGTACT-3′ mismatch site. (c) Ball-and-stick model of 4-E-Fe(II)-5′AGTATT-3′ complex. Bar heights are proportional to the relative cleavage intensities at each base pair. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively. The boxed Fe denotes the EDTA-Fe(II) cleavage moiety. See Supporting Information for autoradiograms.

Figure 7. Quantitative DNase I footprint titration experiments with (a) cyclo-(γ-ImPyPyPyPy-(R)EDTAγ-ImPyPyPyPy) (3) and (b) cyclo-(γ-ImPyPyPyPy-(R)EDTAγ-ImPyPyPyPy) (4) on the 3′-end labeled 229 base pair restriction fragment: lane 1, intact; lane 2, A reaction; lane 3, DNase I standard; lanes 4–14, 5 pM, 10 pM, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 5 nM, and 10 nM. The 5′-AGTACT-3′ and 5′-AGTATT-3′ sites were analyzed and are shown on the right side of the autoradiogram. Additional sites not analyzed were 5′-TGTAAA-3′, 5′-TGTGCT-3′, and 5′-TTAAGT-3′. All reactions contain 20 kcpm restriction fragment, 10 mM Tris·HCl (pH 7.0), 10 mM KCl, 10 mM MgCl₂, and 5 mM CaCl₂.

1:1 polyamide–DNA complex.²² Polyamides bind the 5′-AGTACT-3′ site with decreasing affinity; match cycle (3) > match hairpin (2) > mismatch cycle (4) > match dimer (1). Polyamides bind the 5′-AGTATT-3′ sequence with decreasing affinity; match cycle (4) > mismatch cycle (3) > mismatch hairpin (2) > mismatch dimer (1). Covalent coupling of dimer (1) to form hairpin polyamide (2) results in a 428-fold increase in the DNA binding affinity and comparable DNA binding sequence specificity. It is interesting to compare hairpin polyamide (2), ImPyPyPyPy-(R)EDTAγ-ImPyPyPyPy-OH, to the previously reported hairpin ImPyPyPyPy-γ-ImPyPyPyPy-β-Dp.²⁴ Each hairpin contains eight aromatic rings and a single charge located either on the γ-turn or a C-terminal β-Dp group. Although hairpin polyamide (2) binds to DNA with affinity and specificity comparable to DNA
binding proteins, it does bind with 4-fold lower affinity and 5- lower sequence specificity than the previously described hairpin ImPyPyPy-q-ImPyPyPy-q-Dp. This probably results from loss of favorable interactions between the β-Dp group and A,T rich flanking sequences. Since cycle polyamides have no C-terminal β-Dp group, hairpin polyamide (2) is a more applicable control for the study described here.

On the basis of the pairing rules for polyamide–DNA complexes, the 5′-AGTACT-3′ and 5′-AGTATT-3′ sites represent “match” and “single base pair mismatch” sites for cycle-3, respectively, and “single base pair mismatch” and “match” sites for cycle-4, respectively. Cycle polyamide (3), cyclo(q-ImPyPyPy-(R)16H2Nq-ImPyPyPy), binds the six base pair 5′-AGTACT-3′ target sequence with an equivalence association constant, \( K_e = 7.6 \times 10^{10} \text{ M}^{-1} \), and 55-fold specificity over the single base pair mismatch 5′-AGTATT-3′ site (\( K_e = 1.3 \times 10^{9} \text{ M}^{-1} \)). These affinities represent a 3600-fold increase relative to dimer (1) and an 8-fold enhancement relative to hairpin polyamide (2). Furthermore, the affinity and specificity of (3) are comparable to those of the previously described hairpin ImPyPyPy-q-ImPyPyPy-q-Dp. The cycle polyamide (4), cyclo(q-ImPyPyPy-(R)16H2Nq-ImPyPyPy), which contains a single ImPy pyrrole preferentially binds the 5′-AGTATT-3′ match site (\( K_e = 3.1 \times 10^{10} \text{ M}^{-1} \)) versus the single base pair mismatch 5′-AGTACT-3′ (\( K_e = 4.2 \times 10^{9} \text{ M}^{-1} \)) with a 7-fold preference. Therefore, replacing a single pyrrole amino acid in cyclo(q-ImPyPyPy-(R)16H2Nq-ImPyPyPy) (4) with an imidazole residue in cyclo(q-ImPyPyPy-(R)16H2Nq-ImPyPyPy) (3), regulates cycle polyamide specificity and affinity by 2 orders of magnitude (see Figure 7).

Conclusions. A second generation cycle polyamide motif has been designed and characterized. Two eight-ring (γ-4-γ-4) cycle polyamides were found here to bind to DNA with affinity and specificity comparable to naturally occurring DNA binding proteins. The polyamide DNA binding affinity increases as expected as the number of consecutive ring pairings increases from 3 to 4.96 Important key design factors likely contributed to the improved specificity of the 8-ring cycles compared with the original design (Figure 8).10 Moving the charge to the γ-turn may enhance the cycle polyamide binding orientation preference and hence binding specificity. Determination of the exact molecular basis for the optimization of the cycle polyamides awaits further footprinting efforts as well as high resolution structure studies. However, it is clear from comparison of first and second generation cycles that polyamide design for DNA recognition can be continually optimized using affinity and specificity as two key criteria in parallel with continued investment in synthetic methodology.

Experimental Section

Dicyclohexylcarbodiimide (DCC), Hydroxybenzotriazole (HOBT), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate (HBTU); aminomethylated polystyrene, 4-(bromomethyl)phenylacetic acid phenyl ester, and 0.6 mmol/g Boc-β-alanine-(4-carboxamidomethyl)-benzyl-ester-copoly(styrene-divinylbenzene) resin (Boc-β-Pam resin) was purchased from Peptides International (0.2 mmol/gram) (R)-2-Fmoc-4-Boc-diaminobutyric acid, and (R)-2-amino-4-Boc-diaminobutyric acid were from Bachem. N,N-diosopropylhydroxylamine (DIEA), N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), DMSO/NMP, acetic anhydride (Ac2O), and 0.0002 M potassium cyanide/pyridine were purchased from Applied Biosystems. Dichloromethane (DCM) and triethylamine (TEA) were reagent grade from Aldrich, trifluoroacetic acid (TFA) Biograde was from Halocarbon, Dichloromethane (DCM) and triethylamine (TEA) were reagent grade from Aldrich, trifluoroacetic acid (TFA) Biograde was from Halocarbon, phenol was from Fisher, and ninyhydrin was from Pierce. All reagents were used without further purification. Quik-Sep polypropylene disposable filters were purchased from Isolab, Inc. A shaker for manual solid-phase synthesis was obtained from St. John Associates, Inc. Screw-cap glass peptide synthesis reaction vessels (5 mL and 20 mL) with a no. 2 sintered glass frit were made as described by Kent.20 1H NMR spectra were recorded on a General Electric QE NMR spectrometer at 300 MHz 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. Optical rotations were recorded on a JASCO DIP 1000 digital polarimeter. 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. HPLC analysis was performed on either a HP 1090M analytical HPLC or a Beckman Gold system using a RAINEN C18, Microsorb MV, 5μm, 300 x 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 DeltaPak 25 x 100 mm, 100 μm C18 column equipped with a guard, 0.1% (wt/v) TFA, 0.25% acetonitrile/min. Water (18G) was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered.

Resin Substitution. Resin substitution can be calculated as 
\[ W = L - (W_{text{resin}} - W_{text{dimer})} \times 10^3 \] (eq 2), where L is the loading (nmol of amine per gram of resin), and W is the weight (g/mol) of the growing polyamide attached to the resin.21

Control Hairpin ImPyPyPy-(R)16H2Nq-ImPyPyPy-C3-OH (2). ImPyPyPy-(R)16H2Nq-ImPyPyPy-β-Pam resin was synthesized in a stepwise fashion by machine-assisted solid-phase methods from Boc-β-alanine-Pam resin (0.6 mmol/g).13 (R)-2-Fmoc-4-Boc-diaminobutyric acid (0.7 mmol) was incorporated as previously described for Boc-γ-aminobutyric acid. ImPyPyPy-(R)16H2Nq-ImPyPyPy-β-Pam resin was placed in a glass 20-mL peptide synthesis vessel and treated with DMF (2 mL), and then with piperidine (8 mL) and agitated (22 °C, 30 min). ImPyPyPy-(R)16H2Nq-ImPyPyPy-β-Pam resin was isolated by filtration and washed sequentially with an excess of DMF, DCM, MeOH, and ethyl ether, and the amine resin was dried in vacuo. A sample of resin (200 mg 0.40 mmol/gram) was suspended in absolute ethanol (25 mL). LiBH4 (200 mg) was added and the mixture refluxed for 16 h. The reaction mixture was then filtered to remove resin, neat TFA was added (6 mL), and the resulting solution was concentrated in vacuo, resuspended in 0.1% (wt/v) TFA (8 mL), and purified twice by reversed phase HPLC to provide the trifluoroacetate salt of ImPyPyPy-(R)16H2Nq-ImPyPyPy-C3-OH (2) as a white powder upon lyophilization of the appropriate fractions: 2.5 mg; 3% recovery; 1H NMR (DMSO-d6) of 11.3 (s, 1 H), 10.47 (s, 1 H), 10.13 (9.97) (s, 2 H), 9.94 (s, 1 H), 9.90 (s, 1 H) 8.53 (br s, 3 H), 8.29 (m, 1 H), 8.01 (m, 1 H), 7.39 (m, 2 H), 7.32 (m, 2 H), 7.76 (m, 2 H), 7.22 (s, 1 H), 7.17 (m, 2 H), 7.08 (s, 2 H), 7.05 (m, 1 H), 7.02 (s, 2 H), 6.93 (s, 1 H), 6.84 (s, 1 H), 5.32 (m, 1 H), 3.98 (br s, 3 H), 3.85 (m, 3 H), 3.83 (m, 4 H), 3.80 (s, 3 H), 3.78 (s, 3 H), 2.73 (m, 2 H), 2.35 (m, 2 H), 1.95 (m, 4 H), 1.73 (m, 2 H), MALDI-TOF-MS (monoisotopic) [M + H] 1139.5 (1139.5 calcd for C53H63N20O10).

Boc-Pyrrolyl-4-(oxymethyl)phenylacetic Acid Phenyl Ester (5). A solution of Boc-Py (6.9 g, 29 mmol), 4-(bromomethyl)phenylacetic acid phenyl ester (10 g, 29 mmol), and DIEA (7.2 mL, 41 mmol) in 60 mL of DMF were stirred at 50 °C for 6 h. The solution was cooled and partitioned between 400 mL of water and 400 mL of ethyl ether. The ether layer was washed sequentially (2 x 200 mL each) with 10% citric acid, brine, saturated NaHCO3, and brine. The organic phase was dried (sodium sulfate) and concentrated in vacuo.
The crude product was recrystallized from 3:1 ethyl acetate/hexanes to yield 5 as a fluffy white foam: 6.1 g, 42% yield; TLC (2:3 hexanes/ethyl acetate v/v) Rf 0.6 \( ^{1}H \) NMR (DMSO-d\( _{6} \)) \( \delta \) 7.07 (s, 1 H), 7.00 (d, 1 H, \( J = 7.6 \) Hz), 7.61 (t, 1 H, \( J = 7.6 \) Hz), 7.50 (t, 1 H, \( J = 7.5 \) Hz), 7.31 (m, 4 H), 7.06 (s, 1 H), 6.62 (s, 1 H), 5.47 (s, 2 H), 5.16 (s, 2 H), 3.80 (s, 2 H), 3.76 (s, 3 H), 1.42 (s, 9 H), 1.42 (s, 9 H). 

**Boc-Pyrrol-4-(oxymethyl)phenylacetic Acid (6)** Zinc dust was activated with 1 M HCl (aqueous) as described.\(^{17}\) Boc-Pyrrol-4-(oxymethyl)phenylacetic acid phenacyl ester (3 g, 5.9 mmol) was dissolved in 90 mL 4:1 acetic acid/water (v/v). Zinc dust (9.6 g, 147 mmol) was added and the reaction stirred for 18 h at room temperature. The zinc was removed by filtration and the reaction mixture partitioned between 200 mL of ethyl ether and 200 mL of water. The layers were separated, the aqueous layer was extracted (ethyl ether, 1 × 200 mL), and the combined ether layers were washed (water, 5 × 100 mL), dried (sodium sulfate), concentrated in vacuo, and azeotroped (benzene, 6 × 100 mL). The crude acid product was purified by flash chromatography (2:1 hexanes/ethyl acetate) to yield a yellow oil: 2.0 g, 86% recovery; \( ^{1}H \) NMR (DMSO-\( d_{6} \)) \( \delta \) 7.31 (m, 4 H), 7.06 (s, 1 H), 6.62 (s, 1 H), 5.47 (s, 2 H), 5.16 (s, 2 H), 3.80 (s, 2 H), 3.76 (s, 3 H), 3.22 (m, 2 H), 2.81 (m, 2 H), 2.39 (t, 2 H, \( J = 6.9 \) Hz), 1.83 (m, 4 H), 1.39 (s, 9 H), MALDI-TOF-MS (monoisotopic) [M + H] \( ^{1}H \) 1281.5 (1281.5 calcd for C\(_{65}\)H\(_{77}\)N\(_{22}\)O\(_{17}\)).

**Cyclo-(\( ^{1}H \)\text{-ImPyPyPy-} (R)\text{-ImPyPyPy-}) (3)** The amine-polyamide 12 (2.8 mg, 2.0 mmol) was dissolved in DMF (7 mL), and treated with DPPA (12.5 \( \mu \)L) and K\(_{2}CO\(_{3}\) (100 mg) for 3 h. The reaction mixture was concentrated in vacuo, treated with TFA (3 mL, 1 h), and purified by reverse phase HPLC to provide the trifluoroacetate salt of cyclo-(\( ^{1}H \)\text{-ImPyPyPy-} (R)\text{-ImPyPyPy-}) (3). Cyclo-(\( ^{1}H \)\text{-ImPyPyPy-} (R)\text{-ImPyPyPy-}) was recovered as a white powder upon lyophilization of the appropriate fractions (1.0 mg, 38% recovery). MALDI-TOF-MS (monoisotopic) [M + H] \( ^{1}H \) 1164.5 (1164.5 calcd for C\(_{65}\)H\(_{77}\)N\(_{22}\)O\(_{17}\)).

**Cyclo-(\( ^{1}H \)\text{-ImPyPyPy-} (R)\text{-ImPyPyPy-}) (4)** The amine-polyamide 13 (7 mg, 5.0 mmol) was dissolved in DMF (7 mL), and treated with DPPA (12.5 \( \mu \)L) and K\(_{2}CO\(_{3}\) (100 mg) for 3 h. The reaction mixture was concentrated in vacuo, treated with TFA (3 mL, 1 h), diluted to 8 mL with 0.1% (wt/v) TFA, and purified by reversed phase HPLC to provide cyclo-(\( ^{1}H \)\text{-ImPyPyPy-} (R)\text{-ImPyPyPy-}) (4). Cyclo-(\( ^{1}H \)\text{-ImPyPyPy-} (R)\text{-ImPyPyPy-}) was recovered as a white powder upon lyophilization of the appropriate fractions: 0.25 mg, 18% recovery. MALDI-TOF-MS (monoisotopic) [M + H] \( ^{1}H \) 1163.4 (1163.5 calcd for C\(_{65}\)H\(_{77}\)N\(_{22}\)O\(_{17}\)).

**DNA Reagents and Materials.** Enzymes were purchased from Boehringer-Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5'-[\( ^{32}\)P]triphosphates were obtained from Amersham, and deoxyadenosine 5'-[\( ^{32}\)P]triphosphate was purchased from I. C. N. Sonicated, deproteinized calf thymus DNA was acquired from Pharmacia. RNase free water was obtained from USB and used for all footprinting reactions. All other reagents and materials were used as received. All DNA manipulations were performed according to standard protocols.\(^{22}\)

**Preparation of 3' and 5'-End-Labeled Restriction Fragments.** The plasmid pT7 was constructed as previously reported, pT7 was linearized with AffI and FspI restriction enzymes, then treated with the Sequenase enzyme, deoxyadenosine 5'-[\( ^{32}\)P]triphosphate, and thymidine 5'-[\( ^{32}\)P]triphosphate for 3' labeling. Alternatively, these plasmids were linearized with AffI, treated with calf alkaline phosphatase, and then 5'-labeled with T4 polynucleotide kinase and deoxyadenosine 5'-[\( ^{32}\)P]triphosphate. The 5'-labeled fragment was
then digested with FspI. The labeled fragment (3′ or 5′) was loaded onto a 6% nondenaturing polyacrylamide gel, and the desired 229 base pair band was visualized by autoradiography and isolated.

**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 7.0), 10 mM NaCl, 100 µM/base pair calf thymus DNA, and 30 kcpm 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 4 h. A fresh 50 µM MPE-Fe(II) solution was prepared from 100 µL of a 100 µM MPE solution and 100 µL of a 100 µM ferrous ammonium sulfate (Fe(NH₄)₂(SO₄)₆H₂O) solution. MPE-Fe(II) solution (5 µM) was added to the equilibrated DNA, and the reactions were allowed to equilibrate for 5 min. Cleavage was initiated by the addition of dithiothreitol (5 mM) and allowed to proceed for 14 min. Reactions were stopped by ethanol precipitation, resuspended in 100 mM Tris-borate-EDTA/80% formamide loading buffer, and denatured at 85 °C for 6 min, and a 5-µL sample (~15 kcpm) was immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V.

**Affinity Cleaving.** 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 10 mM Tris·HCl buffer (pH 7.0), 10 mM KCl, 10 mM MgCl₂, 5 mM CaCl₂, and 30 kcpm 3′-radiolabeled DNA. The solutions were allowed to equilibrate 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 1.875 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 6 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1 h. The gels were dried under vacuum at 80 °C and then quantitated using storage phosphor technology. Equilibrium association constants were determined as previously described.

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**Supporting Information Available:** Affinity-cleaving experiments of 4-E-Fe(III) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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