Recognition of a 5′-(A,T)GGG(A,T)$_2$-3′ Sequence in the Minor Groove of DNA by an Eight-Ring Hairpin Polyamide

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Abstract: The use of pyrrole−imidazole polyamides for the recognition of core 5′-GGG-3′ sequences in the minor groove of double stranded DNA is described. Two hairpin pyrrole−imidazole polyamides, ImImIm-γ-PyPyPy-β-Dp and ImImImPy-γ-PyPyPyPy-β-Dp (Im = *N*-methylimidazole-2-carboxamide, Py = *N*-methylpyrrole-2-carboxamide, β = β-alanine, γ = γ-aminobutyric acid, and Dp = ((dimethylamino)propyl)amide), as well as the corresponding EDTA affinity cleaving derivatives, were synthesized and their DNA binding properties analyzed. Quantitative DNase I footprint titrations demonstrate that ImImIm-γ-PyPyPy-β-Dp binds the formal match sequence 5′-AGGGA-3′ with an equilibrium association constant of $K_a = 5 \times 10^6$ M^{-1} (10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.0 and 22 °C). ImImImPy-γ-PyPyPyPy-β-Dp binds the same site, 5′-AGGGAA-3′, approximately two orders of magnitude more tightly than the six ring polyamide, with an equilibrium association constant of $K_a = 4 \times 10^8$ M^{-1}. The eight-ring hairpin polyamide demonstrates greater specifity for single base pair mismatches than does the six ring hairpin. Polyamides with an EDTA·Fe(II) moiety at the carboxy terminus confirm that each hairpin binds in a single orientation. The high affinity recognition of a 5′-GGG-3′ core sequence by an eight ring polyamide containing three contiguous imidazole amino acids demonstrates the versatility of pyrrole−imidazole polyamides and broadens the sequence repertoire for DNA recognition.

Introduction

Pyrrole−imidazole polyamide−DNA complexes afford a general method for the design of non-natural molecules for sequence-specific recognition in the minor groove of DNA.[1−5] Polyamides containing *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) carboxamides bind to the minor groove as side-by-side antiparallel dimers and are capable of specific recognition of sequences containing G,C base pairs, where the N3 of each imidazole forms a hydrogen bond with a single guanine exocyclic amino group.[1] The side-by-side pairing of an imidazole ring from one polyamide with a pyrrole ring from the second polyamide recognizes a G·C base pair, while a pyrrole−imidazole combination recognizes a C·G base pair.[1] Finally, a pyrrole−pyrrole pair recognizes either an A·T or T·A base pair.[1,2] By employing the 2:1 model, specific recognition of the sequences 5′-(A,T)G(A,T)C(A,T)-3′, 5′-(A,T)G(A,T)$_3$-3′, 5′-(A,T)$_2$G(A,T)$_2$-3′, and 5′-(A,T)GCGC(A,T)-3′ has been achieved.[1−5]

Covalent head-to-tail linkage of two polyamides by γ-aminobutyric acid (γ) to form a "hairpin" polyamide results in both increased affinity and specificity, as compared to the unlinked polyamides.[6] For instance, the 1:1 hairpin motif has been used to recognize 5′-(A,T)G(A,T)$_3$-3′ by ImPyPy-γ-PyPyPy-Dp with approximately 300-fold greater affinity than the unlinked polyamides, ImPyPy and PyPyPy.[6] A C-terminal β-alanine residue increases both affinity and specificity and facilitates solid phase synthesis, as recently demonstrated with ImPyPy-γ-PyPyPy-β-Dp.[7,8] Furthermore, a sequence containing two contiguous G·C base pairs, 5′-(A,T)GG(A,T)$_2$-3′, has been recognized by ImImPy-γ-PyPyPy-β-Dp.[9]

To further expand the sequence repertoire available with the hairpin motif, two polyamides containing three contiguous imidazole rings, ImImIm-γ-PyPyPy-β-Dp (**1**) and ImImImPy-γ-PyPyPyPy-β-Dp (**2**), and the corresponding affinity cleaving analogs, ImImIm-γ-PyPyPy-β-Dp-EDTA (**1-E**) and ImImImPy-γ-PyPyPyPy-β-Dp-EDTA (**2-E**), were synthesized using solid phase synthetic protocols (Figures 1 and 2).[7] Specific hydrogen bonds are expected to form between each imidazole N3 and one of the three individual guanine 2-amino groups on the floor of the minor groove (Figure 1). The eight-ring hairpin polyamide, with a pyrrole between the C-terminal imidazole and the γ-linker, was synthesized to examine whether the positioning of the final imidazole immediately adjacent to the turn would adversely affect binding affinity or specificity. We report here the affinities and relative specificities of these tris-imidazole polyamides as determined by three separate techniques: MPE·Fe(II) footprinting,[10] DNase I footprinting,[11] and affinity cleaving.[12] Information about binding site size is gained from MPE·Fe(II) footprinting, while quantitative DNase I footprint titrations

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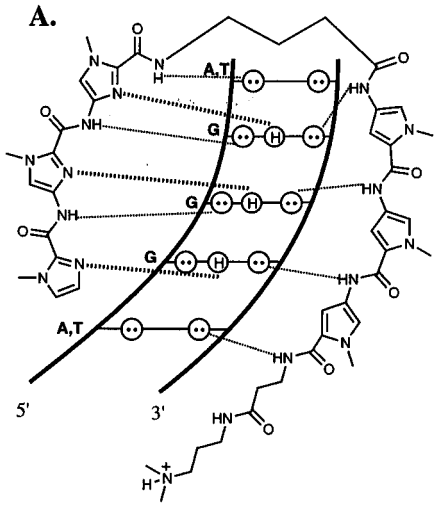
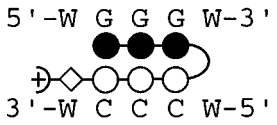
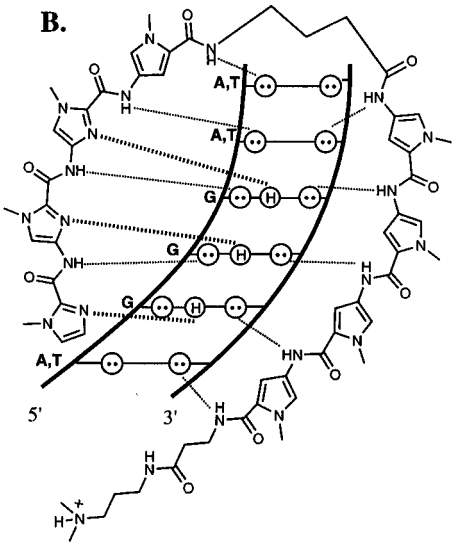


ImImIm-γ-PyPyPy-β-Dp •
(A,T)GGG(A,T)





ImImImPy-γ-PyPyPyPy-β-Dp •
(A,T)GGG(A,T)(A,T)

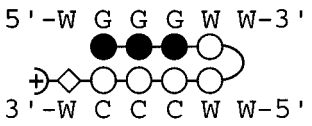


Figure 1. Binding model for the complexes formed between the DNA and either the six-ring hairpin polyamide ImImIm-γ-PyPyPy-β-Dp (A) or the eight-ring hairpin polyamide ImImImPy-γ-PyPyPyPy-β-Dp (B). Circles with dots represent lone pairs of N3 of purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogen of guanine. Putative hydrogen bonds are illustrated by dotted lines. Ball and stick models are also shown. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively. Nonshaded diamonds represent the β-alanine residue. W represents either an A or T base.

allow the determination of equilibrium association constants (K_a) for the polyamides to a variety of match and single base pair mismatch sequences. Affinity cleavage studies confirm the binding orientation and stoichiometry of the 1:1 hairpin:DNA complex.

Results

Synthesis of Polyamides. The polyamides ImImIm-γ-PyPyPy-β-Dp (**1**) and ImImImPy-γ-PyPyPyPy-β-Dp (**2**) were synthesized in a stepwise manner from Boc-β-alanine-Pam resin using recently described Boc-chemistry protocols in 14 and 18 steps, respectively.[7] The polyamides were then cleaved by a single step aminolysis reaction with ((dimethylamino)propyl)-amine and subsequently purified by HPLC chromatography. The syntheses of the polyamides, ImImImPy-γ-PyPyPyPy-β-Dp (**2**), ImImImPy-γ-PyPyPyPy-β-Dp-NH_2 (**2-NH$_2$**), and ImImImPy-γ-PyPyPyPy-β-Dp-EDTA (**2-E**), are outlined (Figure 3). For the synthesis of the EDTA analog, a sample of resin is treated with 3,3′-diamino-*N*-methyldipropylamine (55 °C) and then purified by preparatory HPLC to provide **2-NH$_2$**. The polyamide ImImImPy-γ-PyPyPyPy-β-Dp-NH_2 (**2-NH$_2$**) provides a free aliphatic primary amine group suitable for modification. This polyamide-amine is then treated with an excess of the dianhydride of EDTA (DMSO/NMP, DIEA, 55 °C) and the remaining anhydride hydrolyzed (0.1 M NaOH, 55 °C). The EDTA modified polyamide is then isolated by preparatory HPLC.

Identification of Binding Site Size and Orientation by MPE·Fe(II) Footprinting and Affinity Cleaving. MPE·Fe(II) footprinting on the 3′- and 5′-^{32}P end-labeled 274 base pair *Eco*RI/*Pvu*II restriction fragment from plasmid pSES1 (25 mM Tris−acetate, 10 mM NaCl, 100 μM calf thymus DNA, pH 7.0 and 22 °C) reveals that the polyamides, each at 10 μM concentration, are binding to the four designed sites, 5′-AGGGA(A)-3′, 5′-AGGCA(A)-3′, 5′-TGGGT(C)-3′, and 5′-TGGGC(T)-3′ (Figures 4 and 5). No footprinting is seen with either compound at the single base pair mismatch site, 5′-AGGAA(A)-3′ (Figure 4, quantitated data not shown). The footprinting patterns for the six-ring polyamide are consistent with five base pair binding sites, while the patterns for the eight-ring polyamide are indicative of six base pair binding sites. Affinity cleavage experiments on the same restriction fragment (25 mM Tris−acetate, 200 mM NaCl, 50 μg/mL glycogen, pH 7.0 and 22 °C) by the six-ring and eight-ring EDTA·Fe(II) analogs reveal cleavage patterns that are 3′-shifted and appear on only one side of each binding site (Figures 4 and 5). ImImIm-γ-PyPyPy-β-Dp-EDTA·Fe(II), at 1 μM, and ImImImPy-γ-PyPyPyPy-β-Dp-EDTA·Fe(II), at 100 nM, show cleavage patterns that demonstrate recognition of the same binding sites identified by MPE·Fe(II) footprinting. No carrier DNA was used in these experiments, and thus the relative cleavage intensities indicate that the six-ring polyamide binds most strongly to the two match sites 5′-AGGGA-3′ and 5′-TGGGT-3′, followed by the end mismatch 5′-TGGGC-3′. The core mismatch 5′-AGGCA-3′, with little appreciable cleavage at 1 μM concentration, is bound more weakly. Similarly, the eight-ring polyamide binds most strongly at 100 nM to 5′-AGGGAA-3′, much less strongly to 5′-TGGGTC-3′, and not significantly to 5′-TGGGCT-3′ and 5′-AGGCAA-3′.

Determination of Binding Affinities by Quantitative DNase I Footprinting. Quantitative DNase I footprint titration experiments (10 mM Tris−HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.0 and 22 °C) were performed to determine

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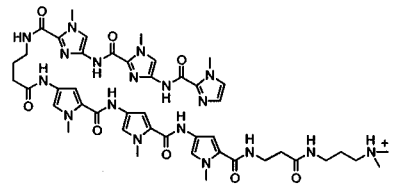
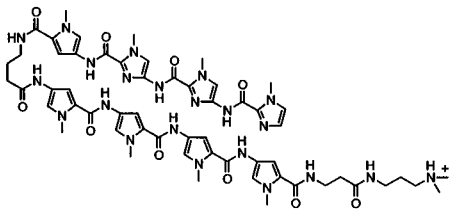


1 ImImIm-γ-PyPyPy-β-Dp



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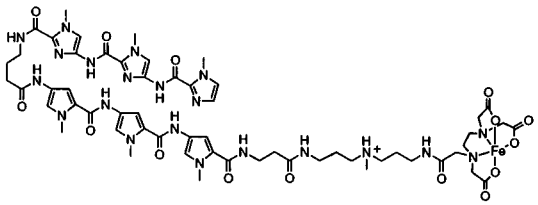
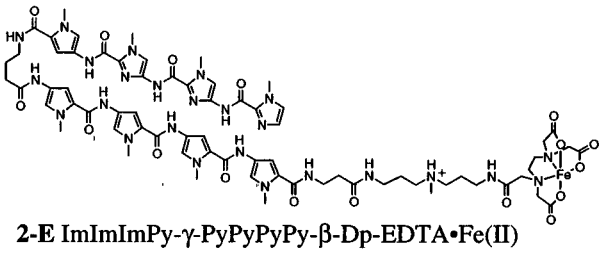


1-E ImImIm-γ-PyPyPy-β-Dp-EDTA•Fe(II)



2-E ImImImPy-γ-PyPyPyPy-β-Dp-EDTA•Fe(II)

Figure 2. Structures of the tris-imidazole polyamides and their EDTA derivatives synthesized using solid-phase methodology.[7]

the equilibrium association constants of the polyamides for the four bound sites (Table 1).[11] ImImIm-γ-PyPyPy-β-Dp binds the two match sites 5′-AGGGA-3′ and 5′-TGGGT-3′ with equilibrium association constants of $K_a = 4.6 \times 10^6$ and 7.6×10^6 M^{-1}, respectively (Figures 6−8). The sequence 5′-TGGGC-3′, which has a mismatch in the non-core final position (an "end" mismatch), is bound with 6-fold lower affinity than the best match, while the core mismatch sequence 5′-AGGCA-3′ is bound with 9-fold lower affinity. ImImImPy-γ-PyPyPyPy-β-Dp binds the match site 5′-AGGGAA-3′ with an equilibrium association constant of $K_a = 3.7 \times 10^8$ M^{-1}. The end mismatch 5′-TGGGTC-3′ is bound with 26-fold lower affinity, and the two core-mismatches, 5′-AGGCAA-3′ and 5′-TGGGCT-3′, are bound with 130-fold and 220-fold lower affinity, respectively. Neither polyamide shows any appreciable binding to the core mismatch 5′-AGGAA(A)-3′ (data not shown).

Discussion

The two hairpin polyamides recognize the targeted 5′-AGGGA(A)-3′ sequence, as determined by MPE•Fe(II) footprinting and affinity cleaving, demonstrating specific recognition by polyamides of sequences containing *three contiguous* G•C base pairs, 5′-GGG-3′. Affinity cleavage data indicate that each polyamide is binding in the minor groove in a single orientation, consistent with the hairpin binding model (Figures 1 and 5). The relative intensities of the cleavage patterns are consistent with quantitative DNase I footprint titration results.

Quantitative DNase I footprint titrations reveal that ImImIm-γ-PyPyPy-β-Dp binds the designed match sites 5′-AGGGA-3′ and 5′-TGGGT-3′ with equilibrium association constants of $K_a = 5 \times 10^6$ and 8×10^6 M^{-1}, respectively. For comparison, the analogous six-ring hairpins containing only one and two contiguous imidazoles, ImPyPy-γ-PyPyPy-β-Dp and ImImPy-γ-PyPyPy-β-Dp, bind their respective match sites with affinities of $K_a \approx 10^8$ M^{-1}.[8,9] The addition of the third imidazole reduces binding affinity significantly, perhaps due to the inability of the polyamide to sit deeply in the sterically crowded minor groove, decreasing energetically favorable van der Waals contacts. Examination of the eight-ring hairpin, ImImImPy-γ-PyPyPyPy-β-Dp, reveals a dramatically increased affinity, the match site 5′-AGGGAA-3′ being bound with an equilibrium association constant of $K_a = 4 \times 10^8$ M^{-1}. The 80-fold increase in affinity in expanding from a three-ring to a four-ring hairpin polyamide mirrors the 66-fold enhancement of ImPyPyPy-Dp over ImPyPy-Dp that was observed in the 2:1 homodimeric polyamide:DNA motif.[13]

In addition to the observation that the eight-ring ImImImPy-γ-PyPyPyPy-β-Dp binds with higher affinity than the six ring ImImIm-γ-PyPyPy-β-Dp, the enhanced specificity is perhaps more significant. The six-ring hairpin polyamide binds 5′-TGGGC-3′, an end mismatch site, with 6-fold lower affinity compared to 5′-TGGGT-3′ (the highest affinity match), while the eight-ring hairpin polyamide binds its end mismatch site 5′-TGGGTC-3′ with 26-fold lower affinity compared to its match site 5′-AGGGAA-3′. Similarly, 5′-AGGCA-3′, a site containing a single base pair core mismatch, is bound by the six-ring system with 9-fold lower affinity, while the two core mismatch sites for the eight-ring system, 5′-AGGCAA-3′ and 5′-TGGGCT-3′, are recognized with 130-fold and 220-fold lower affinity, respectively. For both polyamides, binding of a site with a core single base pair mismatch results in a greater energetic penalty than binding of a site with single base pair mismatch in the end position. The increased specificity of ImImImPy-γ-PyPyPyPy-β-Dp over ImImIm-γ-PyPyPy-β-Dp may indicate that an imidazole placed immediately to the N-terminal side of the γ turn does not form as strong a hydrogen bond as in other positions.

Implications for the Design of Minor Groove Binding Molecules. Pyrrole−imidazole polyamides have been used to recognize a variety of target sequences containing A•T and G•C base pairs.[1,2,4,5,9] By recognizing sequences containing *three* contiguous G•C base pairs, 5′-(A,T)GGG(A,T)-3′ and 5′-(A,T)-GGG(A,T)$_2$-3′, this work expands the sequence-composition repertoire targetable by the hairpin polyamide motif. Both affinity and specificity for a G,C rich sequence are increased by the use of an eight-ring hairpin polyamide. This ability to enlarge the sequence repertoire, combined with rapid solid-phase synthesis, brings us one step closer to a universal approach for the recognition of any desired DNA sequence by strictly chemical methods.

Experimental Section

Dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 0.2 mmol/g of Boc-β-alanine−(4-carboxamidomethyl) benzyl ester−copoly(styrene−divinylbenzene) resin (Boc-β-Pam-Resin) were purchased from Peptides International. *N,N*-Diiso-

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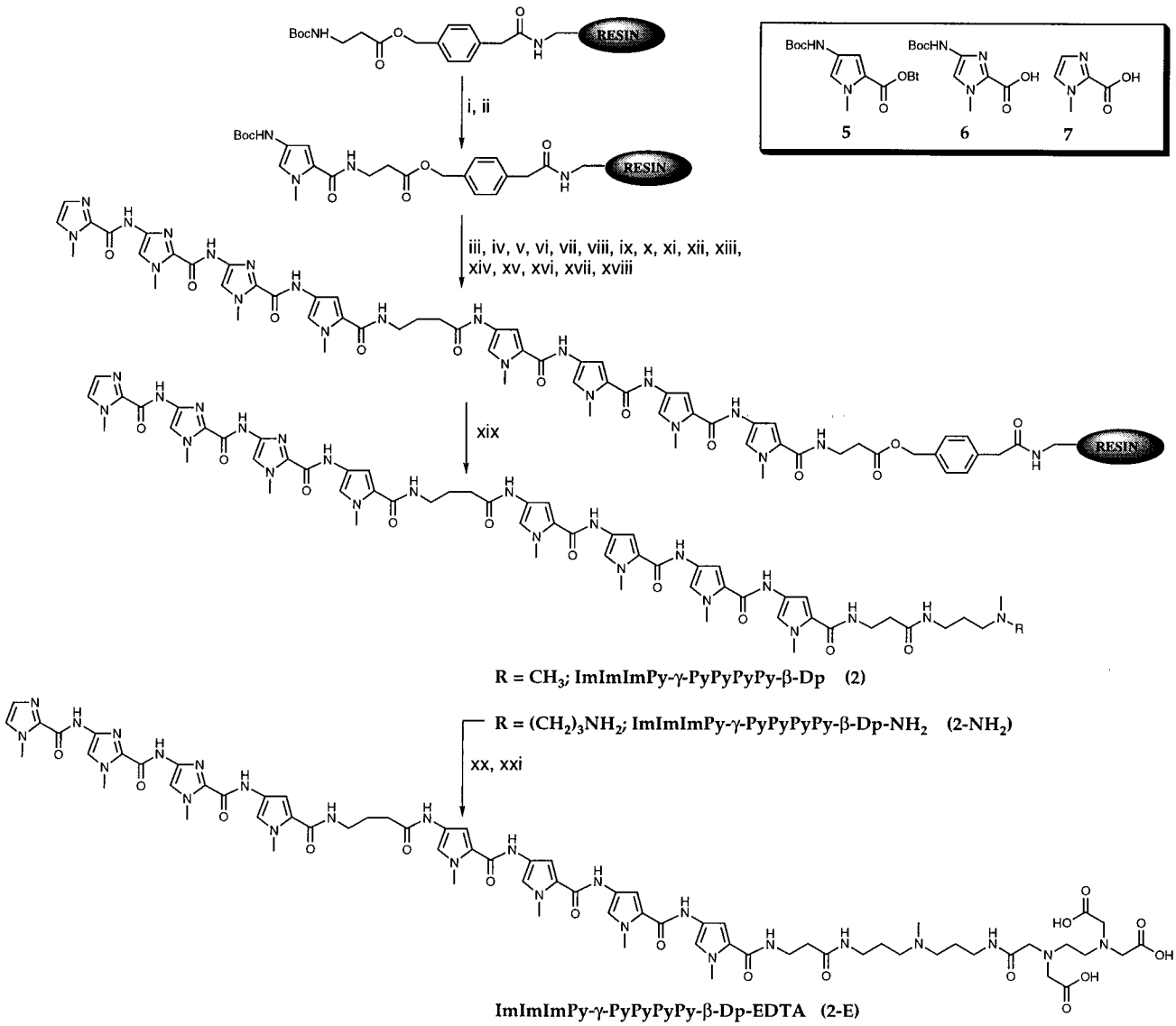

Figure 3. (Box) Pyrrole and imidazole monomers for synthesis of all compounds described here: Boc-pyrrole-OBt ester **5**,[7] Boc-imidazole-acid **6**,[7] and imidazole-2-carboxylic acid **7**.[1a] Solid-phase synthetic scheme for ImImImPy-γ-PyPyPyPy-β-Dp, ImImImPy-γ-PyPyPyPy-β-Dp-NH_2, and ImImImPy-γ-PyPyPyPy-β-Dp-EDTA prepared from commercially available Boc-β-alanine-Pam-resin (0.2 mmol/g): (i) 80% TFA/DCM, 0.4 M PhSH; (ii) BocPy-OBt, DIEA, DMF; (iii) 80% TFA/DCM, 0.4 M PhSH; (iv) BocPy-OBt, DIEA, DMF; (v) 80% TFA/DCM, 0.4 M PhSH; (vi) BocPy-OBt, DIEA, DMF; (vii) 80% TFA/DCM, 0.4 M PhSH; (viii) BocPy-OBt, DIEA, DMF; (ix) 80% TFA/DCM, 0.4 M PhSH; (x) Boc-γ-aminobutyric acid (HBTU, DIEA), DMF; (xi) 80% TFA/DCM, 0.4 M PhSH;3233 (xii) BocPy-OBt, DIEA, DMF; (xiii) 80% TFA/DCM, 0.4 M PhSH; (xiv) BocIm-OBt (DCC/HOBt), DIEA, DMF; (xv) 80% TFA/DCM, 0.4 M PhSH; (xvi) BocIm-OBt (DCC/HOBt), DIEA, DMF; (xvii) 80% TFA/DCM, 0.4 M PhSH; (xviii) imidazole-2-carboxylic acid (HBTU/DIEA); (xix) ((*N*,*N*-dimethylamino)propyl)amine or 3,3′-diamino-*N*-methyldipropylamine, 55 °C; (xx) EDTA-dianhydride, DMSO/NMP, DIEA, 55 °C; (xxi) 0.1 M NaOH.

propylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), DMSO/NMP, acetic anhydride (Ac_2O), and 0.0002 M potassium cyanide/pyridine were purchased from Applied Biosystems. Boc-γ-aminobutyric acid was from NOVA Biochem, dichloromethane (DCM) and triethylamine (TEA) were reagent grade from EM, thiophenol (PhSH), ((dimethylamino)propyl)amine was from Aldrich, trifluoroacetic acid (TFA) was from Halocarbon, phenol was from Fisher, and ninhydrin was from Pierce. All reagents were used without further purification.

Quik-Sep polypropylene disposable filters were purchased from Isolab Inc. and were used for filtration of DCU. Disposable polypropylene filters were also used for washing resin for ninhydrin and picric acid tests and for filtering predissolved amino acids into reaction vessels. A shaker for manual solid-phase synthesis was obtained from St. John Associates, Inc. Screw-cap glass peptide synthesis reaction vessels (5 and 20 mL) with a #2 sintered glass frit were made as described by Kent.[14] ^{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 Institiute of Technology. HPLC analysis was performed on either a HP 1090M analytical HPLC or 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 reverse-phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 × 100 mm, 100 μm C18 column equipped with a guard, 0.1% (wt/v) TFA, 0.25% acetonitrile/min. The 18MΩ water was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered.

ImImIm-γ-PyPyPy-β-Dp (1). The product was synthesized by manual solid-phase protocols[7] and recovered as a white powder (2.4 mg, 4% recovery). UV λ_{max} 312 (48 500); ^{1}H NMR (DMSO-d_6) δ

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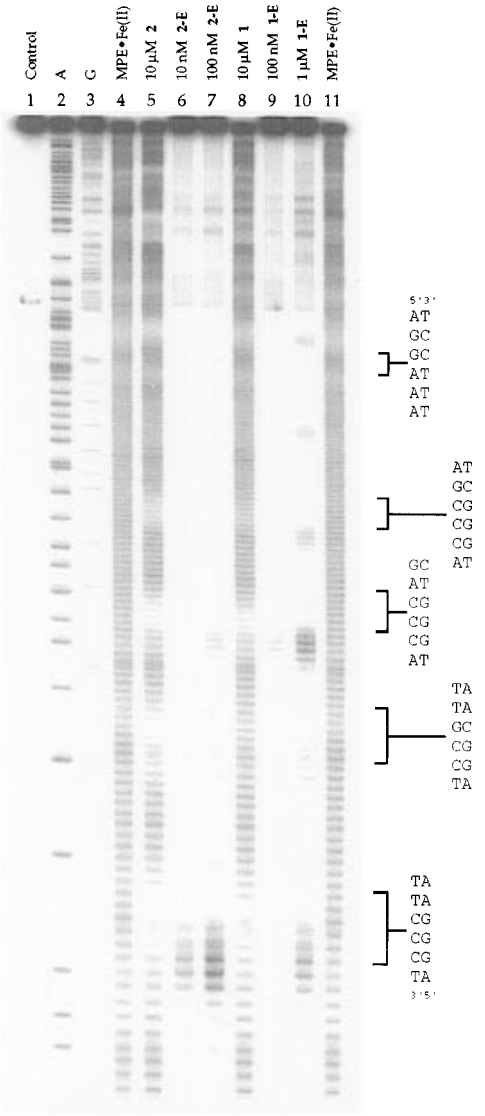

Figure 4. MPE·Fe(II) footprinting and affinity cleavage experiments on a 3′-^{32}P-labeled 274 bp *Eco*RI/*Pvu*II restriction fragment from plasmid pSES1. The 5′-AGGGA(A)-3′, 5′-AGGCA(A)-3′, 5′-TGGGT-(C)-3′, 5′-TGGGC(T)-3′, and 5′-AGGAA(A)-3′ sites are shown on the right side of the autoradiogram. Lane 1, intact DNA; lane 2, A reaction; lane 3, G reaction; lanes 4 and 11, MPE·Fe(II) standard; lane 5, 10 μM ImImImPy-γ-PyPyPyPy-β-Dp; lanes 6 and 7, 10 nM and 100 nM ImImImPy-γ-PyPyPyPy-β-Dp-EDTA·Fe(II); lane 8, 10 μM ImImIm-γ-PyPyPy-β-Dp; lanes 9 and 10, 100 nM and 1 μM ImImIm-γ-PyPyPy-β-Dp-EDTA·Fe(II). All lanes contain 15 kcpm 3′-radiolabeled DNA. The control and MPE·Fe(II) lanes (1, 4, 5, 8, and 11) contain 25 mM Tris−acetate buffer (pH 7.0), 10 mM NaCl, and 100 μM/base pair calf thymus DNA. The affinity cleavage lanes (6, 7, 9, and 10) contain 25 mM Tris−acetate buffer (pH 7.0), 200 mM NaCl, and 50 μg/mL glycogen.

10.09 (s, 1 H), 9.89 (s, 1 H), 9.88 (s, 1 H), 9.83 (s, 1 H), 9.57 (s, 1 H), 9.19 (br s, 1 H), 8.36 (t, 1 H, J = 5.6 Hz), 8.03 (m, 2 H), 7.64 (s, 1 H), 7.51 (s, 1 H), 7.45 (s, 1 H), 7.20 (d, 1 H, J = 1.0 Hz), 7.15 (d, 1 H, J = 2.0 Hz), 7.14 (s, 1 H), 7.08 (s, 1 H), 7.04 (s, 1 H), 6.87 (d, 2 H, J = 2.2 Hz), 4.01 (s, 3 H), 3.99 (s, 3 H), 3.95 (s, 3 H), 3.82 (s, 3 H), 3.82 (s, 3 H), 3.79 (s, 3 H), 3.37 (q, 2 H, J = 5.8 Hz), 3.26 (q, 2 H, J = 6.1 Hz), 3.10 (q, 2 H, J = 6.1 Hz), 2.99 (m, 2 H), 2.73 (d, 6 H, J = 4.8 Hz), 2.34 (t, 2 H, J = 7.2 Hz), 2.27 (t, 2 H, J = 7.3 Hz), 1.79 (m, 4 H); MALDI-TOF-MS, 980.1 (980.1 calcd for M + H).

ImImImPy-γ-PyPyPyPy-β-Dp (2). The product was synthesized by manual solid-phase protocols[7] and recovered as a white powder (7.6 mg, 11% recovery). UV λ_{max} 248 (42 000), 312 (48 500); ^{1}H NMR (DMSO-d_6) δ 10.32 (s, 1 H), 10.13 (s, 1 H), 9.93 (s, 1 H), 9.90 (s, 1 H), 9.89 (s, 1 H), 9.84 (s, 1 H), 9.59 (s, 1 H), 9.23 (br s, 1 H), 8.09 (t, 1 H, J = 5.3 Hz), 8.04 (m, 2 H), 7.65 (s, 1 H), 7.57 (s, 1 H), 7.46 (d, 1 H, J = 0.6 Hz), 7.22 (m, 3 H), 7.16 (s, 2 H), 7.09 (d, 1 H, J = 0.8 Hz), 7.06 (d, 2 H, J = 1.1 Hz), 7.00 (d, 1 H, J = 1.7), 6.88 (d, 1 H, J = 1.8), 6.87 (d, 1 H, J = 1.8 Hz), 4.02 (s, 3 H), 4.00 (s, 3 H), 3.99 (s, 3 H), 3.84 (s, 3 H), 3.83 (s, 3 H), 3.83 (s, 3 H), 3.80 (s, 3 H), 3.79 (s, 3 H), 3.37 (q, 2 H, J = 6.2 Hz), 3.21 (q, 2 H, J = 6.4 Hz), 3.10 (q, 2 H, J = 6.2 Hz), 3.00 (m, 2 H), 2.73 (d, 6 H, J = 4.9 Hz), 2.34 (t, 2 H, J = 7.2 Hz), 2.28 (t, 2 H, J = 7.0 Hz), 1.76 (m, 4 H); MALDI-TOF-MS, 1225.9 (1224.3 calcd for M + H).

ImImIm-γ-PyPyPy-β-Dp-NH$_2$ (1-NH$_2$). A sample of machine-synthesized resin (350 mg, 0.17 mmol/g[15]) was placed in a 20 mL glass scintillation vial and treated with 2 mL of 3,3′-diamino-*N*-methyldipropylamine at 55 °C for 18 h. The resin was removed by filtration through a disposable propylene filter, and the resulting solution was dissolved in water to a total volume of 8 mL and purified directly by preparatory reversed-phase HPLC to provide ImImIm-γ-PyPyPy-β-Dp-NH$_2$ (28 mg, 41% recovery) as a white powder. ^{1}H NMR (DMSO-d_6) δ 10.14 (s, 1 H), 9.89 (s, 1 H), 9.88 (s, 1 H), 9.83 (s, 1 H), 9.6 (br s, 1 H), 9.59 (s, 1 H), 8.36 (t, 1 H, J = 5.5 Hz), 8.09 (t, 1 H, J = 5.0 Hz), 8.03 (t, 1 H, J = 5.0 Hz), 7.9 (br s, 3 H), 7.63 (s, 1 H), 7.50 (s, 1 H), 7.44 (s, 1 H), 7.19 (d, 1 H, J = 1.2 Hz), 7.13 (m, 2 H), 7.08 (d, 1 H, J = 1.3 Hz), 7.02 (d, 1 H, J = 1.2 Hz), 6.85 (m, 2 H), 4.01 (s, 3 H), 3.99 (s, 3 H), 3.97 (m, 6 H), 3.80 (s, 3 H), 3.77 (s, 3 H), 3.34 (q, 2 H, J = 5.3 Hz), 3.23 (q, 2 H, J = 6.0 Hz), 3.05 (m, 6 H), 2.83 (q, 2 H, J = 5.0 Hz), 2.70 (d, 3 H, J = 4.0 Hz), 2.32 (t, 2 H, J = 6.9 Hz), 2.25 (t, 2 H, J = 6.9 Hz), 1.90 (m, 2 H), 1.77 (m, 4 H); MALDI-TOF-MS, 1022.8 (1023.1 calcd for M + H).

ImImImPy-γ-PyPyPyPy-β-Dp-NH$_2$ (2-NH$_2$). A sample of machine-synthesized resin (350 mg, 0.16 mmol/g[15]) was placed in a 20 mL glass scintillation vial and treated with 2 mL of 3,3′-diamino-*N*-methyldipropylamine at 55 °C for 18 h. The resin was removed by filtration through a disposable propylene filter, and the resulting solution was dissolved in water to a total volume of 8 mL and purified directly by preparatory reversed-phase HPLC to provide ImImImPy-γ-PyPyPyPy-β-Dp-NH$_2$ (31 mg, 40% recovery) as a white powder. ^{1}H NMR (DMSO-d_6) δ 10.37 (s, 1 H), 10.16 (s, 1 H), 9.95 (s, 1 H), 9.93 (s, 1 H), 9.91 (s, 1 H), 9.86 (s, 1 H), 9.49 (br s, 1 H), 9.47 (s, 1 H), 8.12 (m, 3 H), 8.0 (br s, 3 H), 7.65 (s, 1 H), 7.57 (s, 1 H), 7.46 (s, 1 H), 7.20 (m, 3 H), 7.16 (m, 2 H), 7.09 (d, 1 H, J = 1.5 Hz), 7.05 (m, 2 H), 7.00 (d, 1 H, J = 1.6 Hz), 6.88 (m, 2 H), 4.01 (s, 3 H), 3.99 (s, 3 H), 3.98 (s, 3 H), 3.83 (s, 3 H), 3.82 (s, 3 H), 3.81 (s, 3 H), 3.79 (s, 3 H), 3.78 (s, 3 H), 3.36 (q, 2 H, J = 5.3 Hz), 3.21−3.05 (m, 8 H), 2.85 (q, 2 H, J = 4.9 Hz), 2.71 (d, 3 H, J = 4.4 Hz), 2.34 (t, 2 H, J = 5.9 Hz), 2.26 (t, 2 H, J = 5.9 Hz), 1.85 (quintet, J = 5.7 Hz), 1.72 (m, 4 H); MALDI-TOF-MS, 1267.1 (1267.4 calcd for M + H).

ImImIm-γ-PyPyPy-β-Dp-EDTA (1-E). 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 ImImIm-γ-PyPyPy-β-Dp-NH$_2$ (**1-NH$_2$**) (8.0 mg, 7 μmol) and dissolved in 750 μL of DMSO. The mixture was heated at 55 °C for 25 min, treated with 3 mL of 0.1 M NaOH, and heated at 55 °C for 10 min. TFA (0.1%) was added to adjust the total volume to 8 mL and the solution was purified directly by preparatory HPLC chromatography to provide **1-E** as a white powder (3.3 mg, 30% recovery). ^{1}H NMR (DMSO-d_6) δ 10.14 (s, 1 H), 9.90 (s, 1 H), 9.89 (s, 1 H), 9.85 (s, 1 H), 9.58 (s, 1 H), 9.3 (br s, 1 H), 8.40 (m, 2 H), 8.02 (m, 2 H), 7.65 (s, 1 H), 7.51 (s, 1 H), 7.45 (s, 1 H), 7.20 (d, 1 H, J = 1.5 Hz), 7.15 (m, 2 H), 7.08 (d, 1 H, J = 1.1 Hz), 7.04 (d, 1 H, J = 1.5 Hz), 6.86 (m, 2 H), 4.00 (s, 3 H), 3.98 (s, 3 H), 3.94 (s, 3 H), 3.87 (m, 4 H), 3.82 (s, 3 H), 3.81 (s, 3 H), 3.78 (s, 3 H), 3.72 (m, 4 H), 3.4−3.0 (m, 16 H), 2.71 (d, 3 H, J = 4.2 Hz), 2.33 (t, 2 H, J = 5.1 Hz), 2.25 (t, 2 H, J = 5.9 Hz), 1.75 (m, 6 H); MALDI-TOF-MS, 1298.4 (1298.3 calcd for M + H).

(15) Resin substitution has been corrected for the weight of the polyamide chain. The change in substitution during a specific coupling or for the entire synthesis can be calculated as L_{new}(mmol/g) = $L_{old}/(1 + L_{old}(W_{new} - W_{old}) \times 10^{-3})$, where L is the loading (mmol of amine per gram of resin), and W is the weight (g mol^{-1}) of the growing polyamide attached to the resin. See: Barlos, K.; Chatzi, O.; Gatos, D.; Stravropoulos, G. *Int. J. Peptide Protein Res.* **1991**, *37*, 513.

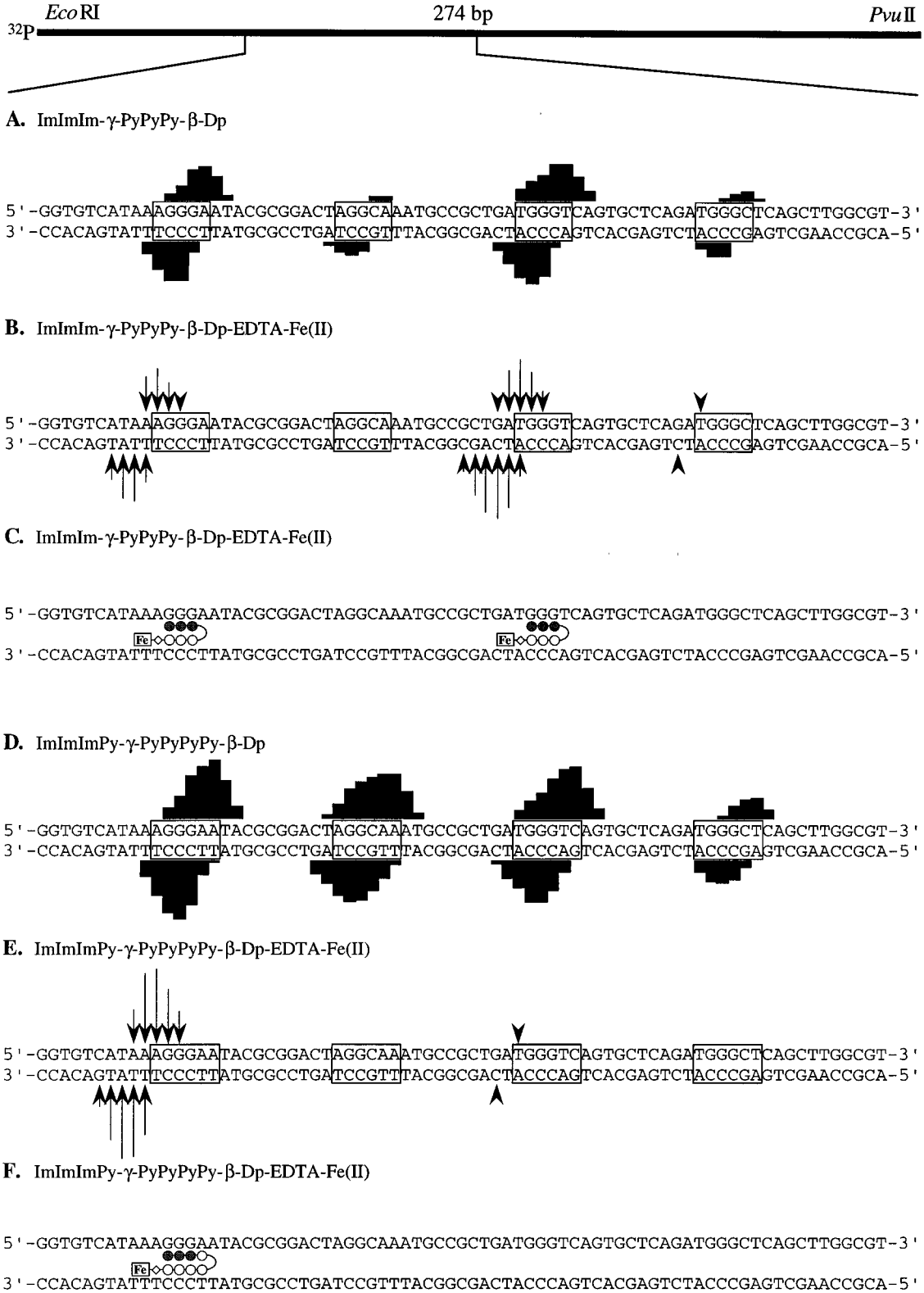

Figure 5. Results from MPE·Fe(II) footprinting of ImImIm-γ-PyPyPy-β-Dp and ImImImPy-γ-PyPyPyPy-β-Dp and affinity cleavage of ImImIm-γ-PyPyPy-β-Dp-EDTA·Fe(II) and ImImImPy-γ-PyPyPyPy-β-Dp-EDTA·Fe(II). (Top) Illustration of the 274 bp restriction fragment with the position of the sequence indicated. Boxes represent equilibrium binding sites determined by the published model. Only sites that were quantitated by DNase I footprint titrations are boxed. (A and D): MPE·Fe(II) protection patterns for polyamides at 10 μM concentration. Bar heights are proportional to the relative protection from cleavage at each band. (B and E): Affinity cleavage patterns of ImImIm-γ-PyPyPy-β-Dp-EDTA·Fe(II) at 1 μM and of ImImImPy-γ-PyPyPyPy-β-Dp-EDTA·Fe(II) at 100 nM, respectively. Arrow heights are proportional to the relative cleavage intensities at each base pair. (C and F): Ball and stick binding models for the single orientation binding to formal match sequences by the six-ring and eight-ring EDTA·Fe(II) analogs, respectively. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively. Nonshaded diamonds represent the β-alanine residue. The boxed **Fe** denotes the EDTA·Fe(II) cleavage moiety.

ImImImPy-γ-PyPyPyPy-β-Dp-EDTA (2-E). Compound **2-E** was prepared as described for compound **1-E** (yield 3.8 mg, 40%). ^{1}H NMR (DMSO-d_6) δ 10.34 (s, 1 H), 10.11 (s, 1 H), 9.92 (s, 1 H), 9.90 (s, 1 H), 9.89 (s, 1 H), 9.84 (s, 1 H), 9.57 (s, 1 H), 8.42 (m, 1 H), 8.03 (m, 3 H), 7.64 (s, 1 H), 7.56 (s, 1 H), 7.44 (s, 1 H), 7.20 (m, 3 H), 7.15 (m, 2 H), 7.07 (d, 1 H, J = 1.6 Hz), 7.05 (m, 2 H), 6.99 (d, 1 H, J = 1.6

Table 1. Equilibrium Association Constants (M^{-1})[a,b,c]

	match site		end mismatch	core mismatches
polyamide	5′-AGGGA-3′	5′-TGGGT-3′	5′-TGGG**C**-3′	5′-AGG**C**A-3′
ImImIm-γ-PyPyPy-β-Dp	4.6×10^6 (0.3)	7.6×10^6 (0.5)	1.3×10^6 (0.3)	8.6×10^5 (0.4)

	match site	end mismatch	core mismatches	
polyamide	5′-AGGGAA-3′	5′-TGGGT**C**-3′	5′-TGGG**C**T-3′	5′-AGG**C**AA-3′
ImImImPy-γ-PyPyPyPy-β-Dp	3.7×10^8 (0.8)	1.4×10^7 (0.5)	1.7×10^6 (0.3)	2.9×10^6 (0.3)

[a] Values reported are the mean values measured from a minimum of three DNase I footprint titration experiments, with the standard deviation for each data set indicated in parentheses. [b] The assays were performed at 22 °C at pH 7.0 in the presence of 10 mM Tris−HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$. [c] Base pairs that are in bold represent formal mismatches.

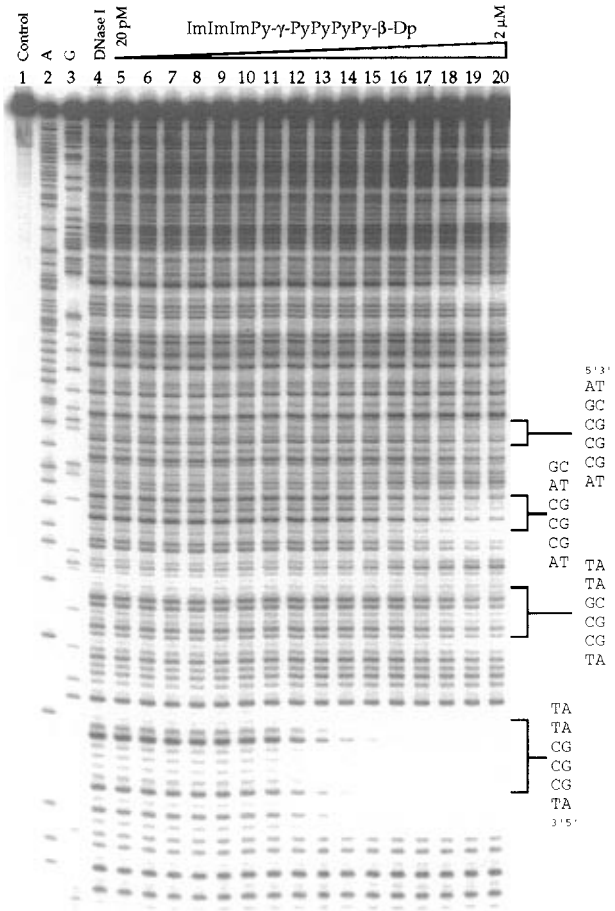

Figure 6. Quantitative DNase I footprint titration experiment with ImImImPy-γ-PyPyPyPy-β-Dp on the *Eco*RI/*Pvu*II restriction fragment from plasmid pSES1: lane 1, intact DNA; lane 2, A reaction; lane 3, G reaction; lane 4, DNase I standard; lanes 5−20, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 2 μM ImImImPy-γ-PyPyPyPy-β-Dp. The 5′-AGGGAA-3′, 5′-AGGCAA-3′, 5′-TGGGTC-3′, and 5′-TGGGCT-3′ sites that were analyzed are shown on the right side of the autoradiogram. All reactions contain 20 kcpm restriction fragment, 10 mM Tris·HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

Hz), 6.87 (m, 2 H), 4.00 (s, 3 H), 3.98 (s, 3 H), 3.97 (s, 3 H), 3.83 (m, 4 H), 3.82 (s, 6 H), 3.79 (s, 3 H), 3.78 (s, 6 H), 3.67 (m, 4 H), 3.4−3.0 (m, 16 H), 2.71 (d, 3 H, J = 4.2 Hz), 2.34 (t, 2 H, J = 5.4 Hz), 2.25 (t, 2 H, J = 5.9 Hz), 1.72 (m, 6 H); MALDI-TOF-MS, 1542.2 (1542.6 calcd for M + H).

DNA Reagents and Materials. Enzymes were purchased from Boehringer-Mannheim or New England Biolabs and were used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P]-triphosphates and deoxyadenosine 5′-[γ-^{32}P]triphosphate were obtained from Amersham. Purified water was obtained by filtering doubly-distilled water through the MilliQ filtration system from Millipore. Sonicated, deproteinized calf thymus DNA was acquired from Pharmacia. All other reagents and materials were used as received. All DNA manipulations were performed according to standard protocols.[16]

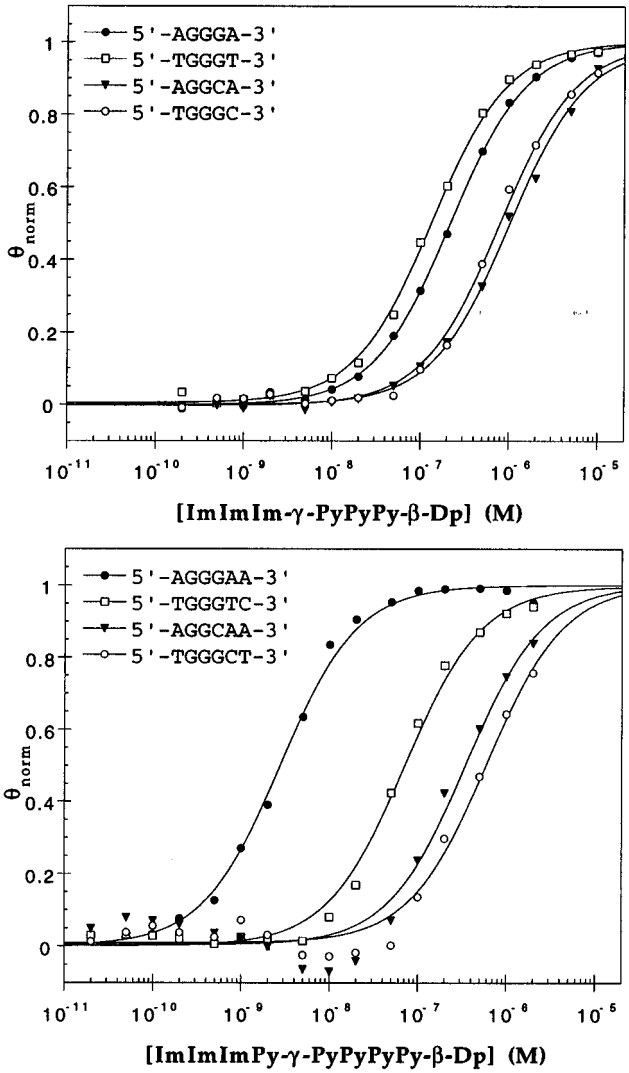

Figure 7. Data from the quantitative DNase I footprint titration experiments for the two polyamides, ImImIm-γ-PyPyPy-β-Dp (top) and ImImImPy-γ-PyPyPyPy-β-Dp (bottom), in complex with the designated sites. The θ_{norm} points were obtained using photostimulable storage phosphor autoradiography and processed as described in the Experimental Section. The data points for 5′-AGGGA(A)-3′, 5′-TGGGT(C)-3′, 5′-AGGCA(A)-3′, and 5′-TGGGC(T)-3′ sites are indicated by filled circles (●), open squares (□), filled inverted triangles (▼), and open circles (○), respectively. The solid curves are the best-fit Langmuir binding titration isotherms obtained from the nonlinear least-squares algorithm using eq 2.

Construction of Plasmid DNA. The plasmid pSES1 was constructed by hybridization of the inserts, 5′-GATCCGGTGTCATAAAGGGAATACGCGGACTAGGCAAATGCCGC-TGATGGGTCAGTGCTCAGATGGGCTC-3′ and 5′-AGCTGAGC-

(16) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

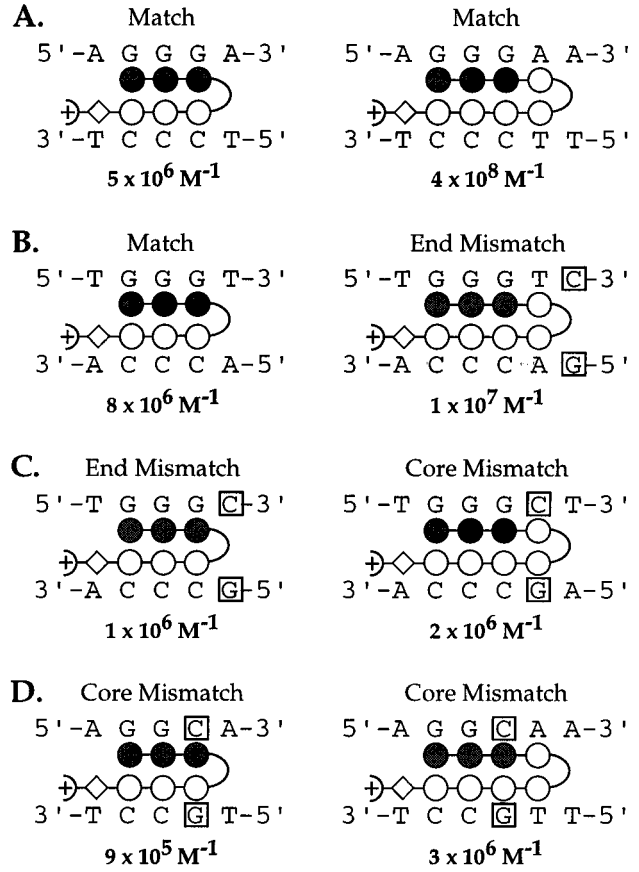

Figure 8. Ball and stick models of ImImIm-γ-PyPyPy-β-Dp (left) and ImImImPy-γ-PyPyPyPy-β-Dp (right) for each binding site, with the corresponding equilibrium association constants shown below each individual model. The binding sites shown are (a) 5′-AGGGA(A)-3′, (b) 5′-TGGGT(C)-3′, (c) 5′-TGGGC(T)-3′, and (d) 5′-AGGCA(A)-3′. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively. Nonshaded diamonds represent the β-alanine residue. Formally mismatched base pairs are boxed.

CCATCTGAGCACTGACCCATCAGCGGCATTTGCCTAGT-CCGCGTATTCCCTTTATGACACCG-3′. The hybridized insert was ligated into linearized pUC19 *Bam*HI/*Hind*III plasmid using T4 DNA ligase. The resultant constructs were used to transform Epicurian Coli XL-2 Blue competent cells from Stratagene. Ampicillin-resistant white colonies were selected from 25 mL of Luria-Bertani medium agar plates containing 50 μg/mL ampicillin and treated with XGAL and IPTG solutions. Large-scale plasmid purification was performed with Qiagen Maxi purification kits. Dideoxy sequencing was used to verify the presence of the desired insert. Concentration of the prepared plasmid was determined at 260 nm using the relationship of 1 OD unit = 50 μg/mL of duplex DNA.

Preparation of 3′- and 5′-End-Labeled Restriction Fragments. The plasmid pSES1 was linearized with *Eco*RI and then treated with either Klenow fragment, deoxyadenosine 5′-[α-^{32}P]triphosphate and thymidine 5′-[α-^{32}P]triphosphate for 3′ labeling, or calf alkaline phosphatase and then 5′ labeled with T4 polynucleotide kinase and deoxyadenosine 5′-[γ-^{32}P]triphosphate. The labeled fragment (3′ or 5′) was then digested with *Pvu*II and loaded onto a 5% non-denaturing polyacrylamide gel. The desired 274 base pair band was visualized by autoradiography and isolated. Chemical sequencing reactions were performed according to published methods.[17]

MPE·Fe(II) Footprinting. All reactions were carried out in a volume of 40 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were 25 mM Tris−acetate buffer (pH 7.0), 10 mM NaCl, 100 μM/base pair calf thymus DNA, and 15 kcpm 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 4 h. A fresh 50 μM MPE·Fe(II) solution was made from 100 μL of a 100 μM MPE solution and 100 μL of a 100 μM ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) solution. After the 4 h equilibration, MPE·Fe(II) solution (5 μM) was added, and the reactions were equilibrated 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, denatured at 85 °C for 5 min, placed on ice, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% crosslink, 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 25 mM Tris−acetate buffer (pH 7.0), 200 mM NaCl, 50 μg/mL of glycogen, and 15 kcpm 3′- or 5′-radiolabeled DNA. After the reactions were allowed to equilibrate for 4 h, ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$), 10 μM final concentration, was added. After another 15 min, cleavage was initiated by the addition of dithiothreitol (5 mM) and allowed to proceed for 12 min. Reactions were stopped by ethanol precipitation, resuspended in 100 mM tris-borate−EDTA/80% formamide loading buffer, denatured at 85 °C for 5 min, placed on ice, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V.

DNase I Footprinting. All reactions were carried out in a volume of 40 μL. We note explicitly that no carrier DNA was used in these reactions. 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 20 kcpm 3′-radiolabeled DNA. The solutions were allowed to equilibrate for a minimum of 4 h at 22 °C (the four-ring hairpin was allowed to equilibrate for up to 12 h with no noticeable affect on the data set). Cleavage was initiated by the addition of 4 μL of a DNase I stock solution (diluted with 1 mM DTT to give a stock concentration of 0.225 u/mL) and was allowed to proceed for 5 min at 22 °C. The reactions were stopped by the addition of 3 M sodium acetate solution containing 50 mM EDTA and then ethanol precipitated. The cleavage products were resuspended in 100 mM tris-borate−EDTA/80% formamide loading buffer, denatured at 85 °C for 5 min, placed on ice, 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, then quantitated using storage phosphor technology.

Equilibrium association constants were determined as previously described.[6,11] The data were analyzed by performing volume integrations of the 5′-AGGGA(A)-3′, 5′-TGGGT(C)-3′, 5′-TGGGC(T)-3′, and 5′-AGGGA(A)-3′ sites and a reference site. The apparent DNA target site saturation, θ_{app}, was calculated for each concentration of polyamide using the following equation:

$$\theta_{app} = 1 - \frac{I_{tot}/I_{ref}}{I_{tot}^{\circ}/I_{ref}^{\circ}} \qquad (1)$$

where I_{tot} and I_{ref} are the integrated volumes of the target and reference sites, respectively, and I_{tot}° and I_{ref}° correspond to those values for a DNase I control lane to which no polyamide has been added. The ($[L]_{tot}$, θ_{app}) data points were fit to a Langmuir binding isotherm (eq 2, $n = 1$) by minimizing the difference between θ_{app} and θ_{fit}, using the modified Hill equation:

$$\theta_{fit} = \theta_{min} + (\theta_{max} - \theta_{min}) \frac{K_a^n [L]_{tot}^n}{1 + K_a^n [L]_{tot}^n} \qquad (2)$$

where $[L]_{tot}$ corresponds to the total polyamide concentration, K_a corresponds to the apparent monomeric association constant, and θ_{min} and θ_{max} represent the experimentally determined site saturation values when the site is unoccupied or saturated, respectively. Data were fit using a nonlinear least-squares fitting procedure of KaleidaGraph software (version 2.1, Abelbeck software) with K_a, θ_{max}, and θ_{min} as the adjustable parameters. All acceptable fits had a correlation coefficient of $R > 0.97$. At least three sets of acceptable data were used in determining each association constant. All lanes from each gel were used unless visual inspection revealed a data point to be

(17) (a) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823. (b) Maxam, A. M.; Gilbert, W. S. *Methods Enzymol.* **1980**, *65*, 499.

obviously flawed relative to neighboring points. The data were normalized using the following equation:

$$\theta_{norm} = \frac{\theta_{app} - \theta_{min}}{\theta_{max} - \theta_{min}} \quad (3)$$

Quantitation by Storage Phosphor Technology Autoradiography. Photostimulable storage phosphorimaging plates (Kodak Storage Phosphor Screen S0230 obtained from Molecular Dynamics) were pressed flat against gel samples and exposed in the dark at 22 °C for 12−16 h. A Molecular Dynamics 400S PhosphorImager was used to obtain all data from the storage screens. The data were analyzed by performing volume integrations of all bands using the ImageQuant v. 3.2.

Acknowledgment. This paper is dedicated to Nelson Leonard on the occasion of his 80th birthday. We are grateful to the National Institutes of Health (GM-2768) for research support, the National Institutes of Health for a research service award to S.E.S., and the Howard Hughes Medical Institute for a predoctoral fellowship to E.E.B. We would like to thank G. M. Hathaway for MALDI-TOF mass spectrometry.

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