A Comparison of H-Pin and Hairpin Polyamide Motifs for the Recognition of the Minor Groove of DNA

William A. Greenberg, Eldon E. Baird, and Peter B. Dervan*

Abstract: In order to compare strategies for covalent linkage of pyrrole-imidazole (Py-Im) polyamide subunits, equilibrium association constants (K_a) were determined for a series of polyamides coupled either C–N with a γ-aminobutyric acid linker (hairpin motif) or linked across a central Py/Py pair through a tetramethylene spacer (H-pin motif). Compared to the well-characterized hairpin motif, the H-pin motif represents a unique and relatively unexplored approach for increasing the affinity and the specificity of 2:1 polyamide–DNA complexes. Three H-pin polyamides containing six or 10 aromatic amino acid residues were synthesized by solid-phase methods using a Boc-protected bispyrrole monomer combined with bi-directional synthesis. The DNA-binding properties of six-ring and 10-ring H-pin polyamides were analyzed by quantitative DNase I footprint titration on a DNA fragment containing five or seven base pair match and mismatch sequences, respectively. The homodimeric H-Pin (Im**Py**Py-β-Dp)$_2$C$_4$ (site of the tetramethylene linker indicated in bold type) binds to the seven base pair match sequence 5′-TGTCA-3′ with $K_a = 9.3 \times 10^6\,M^{-1}$ and 9.4-fold specificity relative to the single base mismatch sequence 5′-TGT**T**A-3′ ($K_a = 9.9 \times 10^5\,M^{-1}$). The heterodimeric H-Pin (Im-**Py**Py-β-Dp)C$_4$(AcPy**Py**Py-β-Dp) binds a 5′-TGTTA-3′ match sequence with $K_a = 2.0 \times 10^6\,M^{-1}$ and 3.5-fold specificity versus the single base mismatch sequence 5′-TGT**C**A-3′ ($K_a = 5.7 \times 10^5\,M^{-1}$). The 10-ring H-pin (ImPy**Py**Py-Py-β-Dp)$_2$C$_4$ binds to the seven base pair match sequence 5′-TGTAACA-3′ with $K_a = 4.4 \times 10^8\,M^{-1}$ and 28-fold specificity versus the single base pair mismatch sequence 5′-TG**G**AACA-3′ ($K_a = 1.6 \times 10^7\,M^{-1}$). We find that H-pin polyamides bind with four- to 180-fold enhanced affinity and two- to 10-fold enhanced specificity relative to unlinked analogues, but with 25- to 150-fold reduced affinity and approximately one- to 20-fold reduced specificity compared to the corresponding hairpin polyamides. These results indicate that H-pin polyamides represent a viable motif for the recognition of predetermined sequences in the DNA minor groove; however, DNA-binding properties appear inferior to the corresponding hairpins.

Keywords: binding affinity · hydrogen bonds · molecular recognition · solid-phase synthesis · DNA recognition

Introduction

Efforts over the last several years have led to the development of 2:1 antiparallel pyrrole–imidazole polyamide–DNA complexes for sequence-specific recognition in the minor groove of DNA.[1–10] DNA sequence specificity is dictated by a linear combination of side-by-side pairings of aromatic pyrrole (Py) and imidazole (Im) amino acids. An Im on one ligand complemented by Py on the second (Im/Py pairing) recognizes a G·C base pair, while a Py/Im pairing recognizes C·G.[1] A Py/Py pair is degenerate for an A·T or T·A base pair.[1, 2] The validity of the polyamide pairing rules as a model for the design of ligands that recognize predetermined sequences is supported by a wide variety of polyamide structural motifs which have been characterized by footprinting, affinity cleaving, 2-D NMR, and X-ray methods.[1–9]

Covalently linking polyamide homodimers and heterodimers within the 2:1 motif has led to the development of designed ligands with enhanced affinity and sequence specificity.[7–8] The first approach for the covalent linkage of 2:1 polyamides entailed connection across a central Py/Py pair through the ring nitrogen atoms that point out of the minor groove (Figure 1a).[7] The nonlinear and hence nonconvergent synthesis of this class of polyamides, which we call the H-pin motif, made elaboration to larger, mixed sequence systems difficult. A polyamide hairpin motif with γ-aminobutyric acid (γ) serving as a turn-specific internal guide residue provided a more synthetically accessible method for linking polyamide subunits (Figure 1b).[8] Furthermore, the development of solid-phase methods for the linear synthesis

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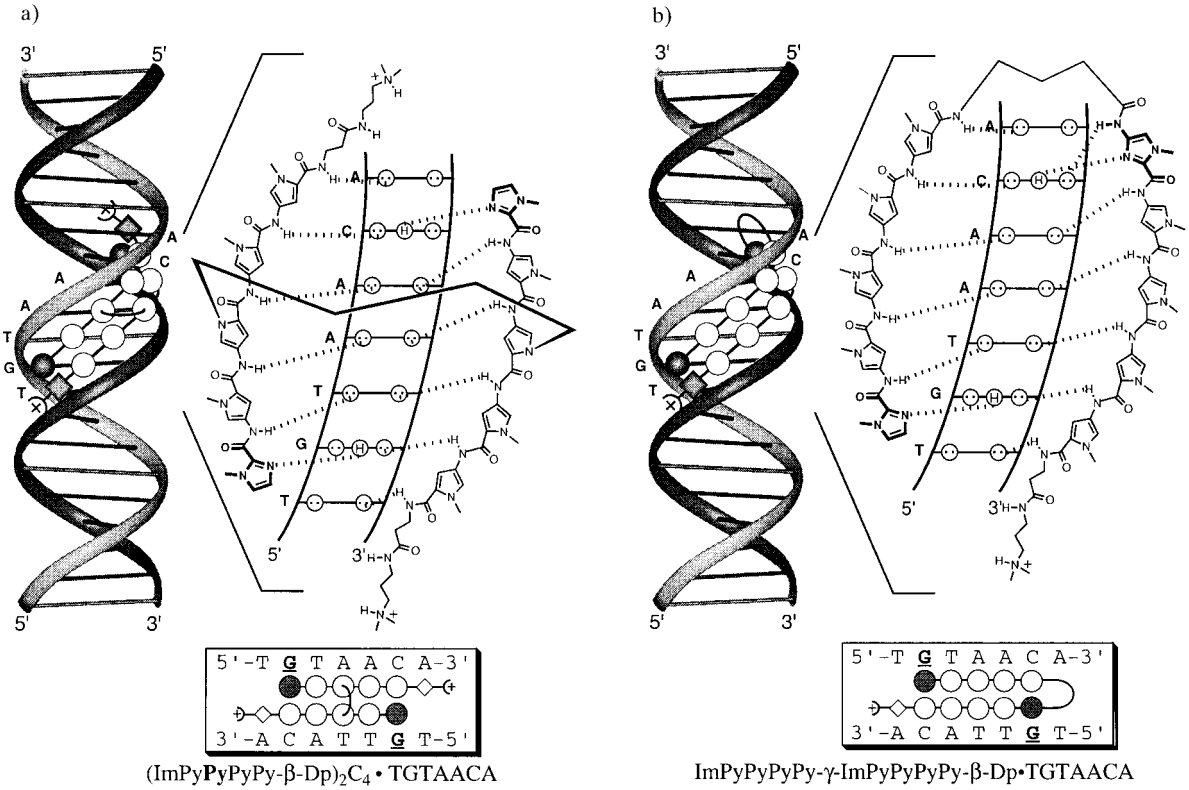


Figure 1. Models of the 2:1 polyamide:DNA complex formed with the minor groove of double stranded B-form DNA and a) the 10-ring H-pin polyamide (ImPy**Py**PyPy-β-Dp)$_2$C$_4$ or b) the 10-ring hairpin polyamide ImPyPyPyPy-γ-ImPyPyPyPyPy-β-Dp. Left: Ribbon model for the complex formed between the match sequence 5′-TGTAACA-3′ and the corresponding 10-ring polyamides. *N*-methylimidazole carboxamides are represented by filled circles, *N*-methylpyrrole carboxamides are represented by empty circles, and β-alanine amino acids are represented by squares. Right: Hydrogen-bonding model for the complex formed between the 5′-TGTAACA-3′ sequence and the corresponding polyamides. 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. Below each hydrogen-bonding model are the ball and stick representations of the polyamide binding. The amino acids are represented as in the ribbon model.

of hairpin polyamides facilitated the development of a broad scope of hairpin ligands[10] as well as applications of cell-permeable eight-ring hairpins for gene-specific regulation of transcription.[11] It remained to be determined if the H-pin polyamide motif might offer similar undiscovered properties.

As a minimum first step to evaluate the scope and limitations of the H-pin polyamide motif, we report the extension of solid-phase methodology to the synthesis of H-pins through the use of a bispyrrole monomer building block and a bi-directional synthetic strategy. Two six-ring and a 10-ring H-pin polyamide, (Im**Py**Py-β-Dp)$_2$C$_4$ (**1**), (Im**Py**Py-β-Dp)C$_4$(AcPy**Py**Py-β-Dp) (**2**), and (ImPy**Py**PyPy-β-Dp)$_2$C$_4$ (**6**) (site of tetramethylene linker indicated in bold type) were synthesized and their DNA binding properties analyzed on separate DNA fragments containing five and seven base pair match and mismatch sites. As a control, the corresponding unlinked homodimers ImPyPy-β-Dp (**5**), and ImPyPyPyPy-β-Dp (**8**), as well as the hairpin analogues ImPyPy-γ-ImPyPy-β-Dp (**3**),[8d] ImPyPy-γ-PyPyPy-β-Dp (**4**),[2d, 8b] and ImPyPyPy-Py-γ-ImPyPyPyPy-β-Dp (**7**)[8j] were also analyzed on the same DNA fragments (Figures 2 and 3). We report here on the DNA binding affinities and relative sequence selectivity of these eight polyamides, as determined by quantitative DNase I footprint titrations.

Results and Discussion

Polyamide synthesis: Synthesis of H-pin polyamides by solid phase methodology required the preparation of a suitably protected bispyrrole monomer unit, **12** (Scheme 1). The tetramethylene linker was chosen on the basis of previous linker length optimization studies in which linkers of three to six methylenes had been compared.[7a] The synthesis is amenable to large scale preparation, as only one column chromatography run is required. The synthesis of a homodimeric H-pin polyamide is illustrated for (Im**Py**Py-β-Dp)$_2$C$_4$, (**1**) (Scheme 2). H$_2$N-Py-β-Pam-resin was prepared in three steps from Boc-β-Pam-resin by means of previously reported Boc-chemistry solid-phase synthesis protocols.[10] Bis-OBt ester **15** was generated in situ (DCC, HOBt) from **12**, then coupled to the resin (DMF, DIEA) to provide (Boc-**Py**-OBt)C$_4$(Boc-**Py**Py-β-Pam-resin). This intermediate provides a resin-bound Py-OBt ester with the amino terminus of both chains Boc-protected. The C-terminal end of the polyamide was completed by coupling the appropriate amine (DMF, DIEA, 37 °C), in this case H$_2$N-Py-β-Dp **16**, to the resin-bound polyamide to provide (Boc-**Py**Py-β-Dp)C$_4$(Boc-**Py**Py-β-Pam-resin). The N-terminal end of each chain was completed by simultaneous removal of the Boc groups (TFA,

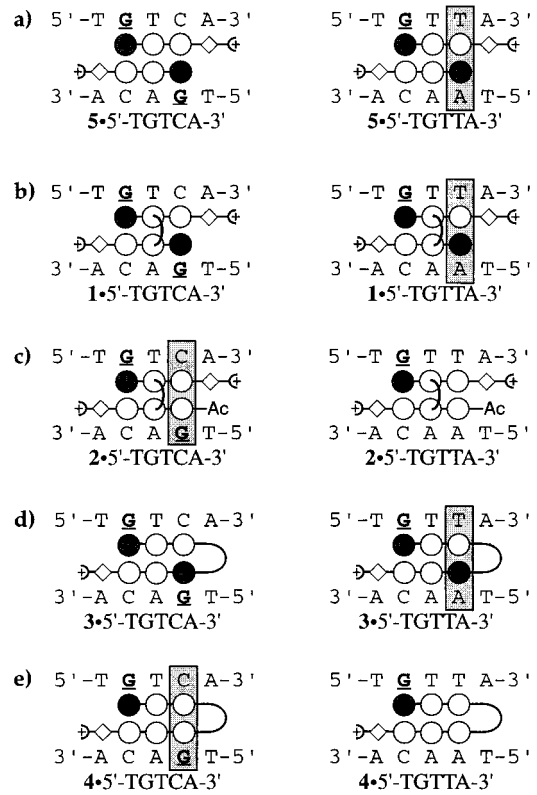
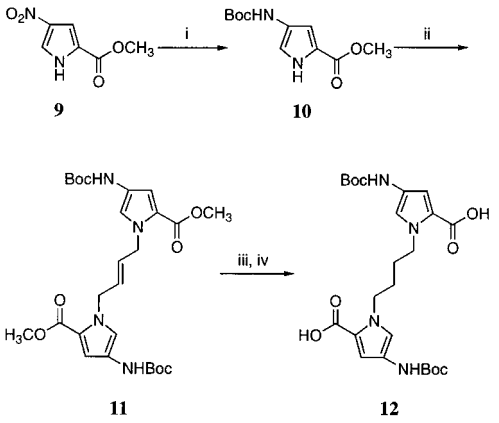


Figure 2. Schematic ball-and-stick models of six-ring H-pins. a) (Im**Py**Py-β-Dp)$_2$C$_4$ (**1**) and b) (Im**Py**Py-β-Dp)C$_4$(AcPy**Py**Py-β-Dp) (**2**); six-ring hairpin polyamides: c) ImPyPy-γ-ImPyPy-β-Dp (**3**) and d) ImPyPy-γ-PyPyPy-β-Dp (**4**); and three-ring homodimers ImPyPy-β-Dp (**5**) bound at (left) a 5′-TGTCA-3′ site and (right) a 5′-TGTTA-3′ site. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively. Diamonds represent the β-alanine residue. Ring pairing–DNA mismatch interactions are boxed.



Scheme 1. Synthesis of Boc-protected bispyrrole monomer **12**. Reaction conditions: i) H_2 (500 psi), 10% Pd/C, Boc_2O, DIEA, DMF; ii) 1,4-dibromo-(*E*)-2-butene (0.55 equiv), K_2CO_3, acetone, 56 °C; iii) H_2 (150 psi), 10% Pd/C, EtOAc; iv) 1 M KOH, 3:1 MeOH/H_2O, 45 °C.

DCM, PhSH) followed by acylation with activated imidazole-2-carboxylic acid (HBTU, DIEA) to provide (Im**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-resin). A single step aminolysis of the resin ester linkage with *N,N*-[(dimethylamino)propyl]amine (55 °C, 18 h) was then used to cleave the polyamide from the solid support. Polyamide **1** was subsequently isolated from the cleavage reaction by reversed-phase HPLC. Convergent solid phase synthetic methodology gave homodimeric six-ring H-pin **1** in eight steps and homodimeric 10-ring H-pin **6** in 11 steps.

The synthesis of a heterodimeric H-pin polyamide is illustrated for (Im**Py**Py-β-Dp)C$_4$(AcPy**Py**Py-β-Dp) (**2**) (Scheme 3). The synthesis proceeded as for the homodimer up to the step of extending the N-termini of the two chains,

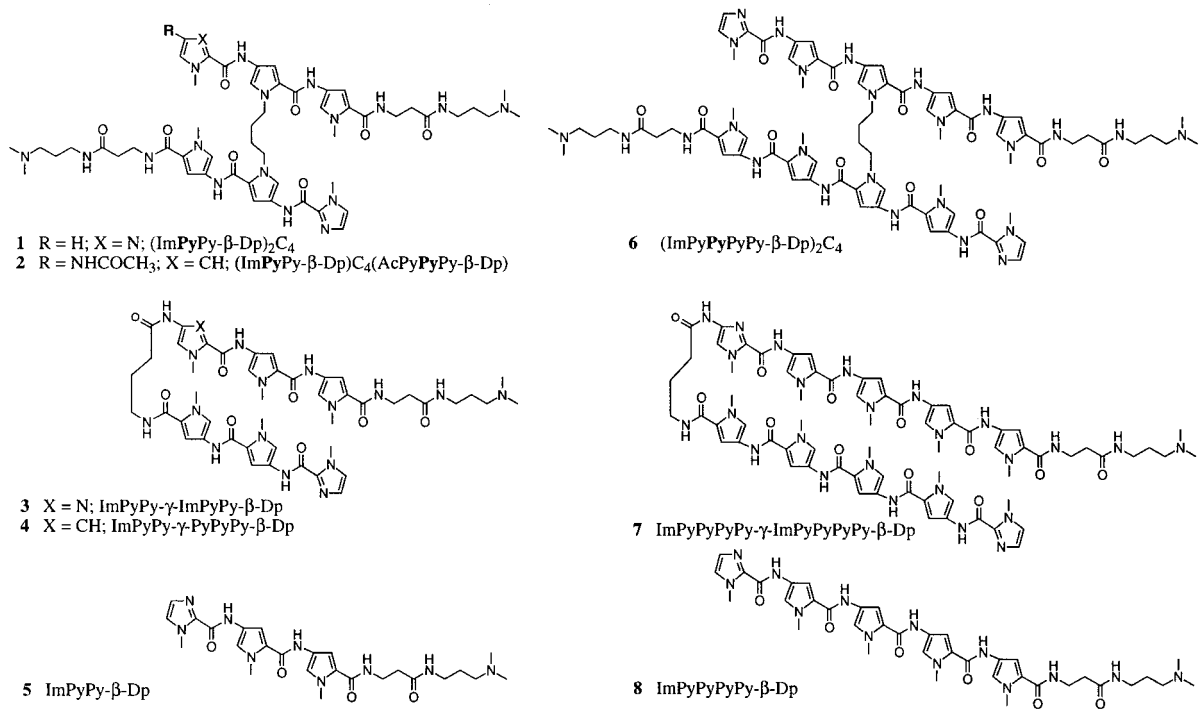


Figure 3. Structures of the six-ring H-pin polyamides **1** and **2**, six-ring hairpin polyamides **3** and **4**, unlinked three-ring polyamide **5**, 10-ring H-pin polyamide **6**, 10-ring hairpin polyamide **7**, and unlinked five-ring polyamide **8**.

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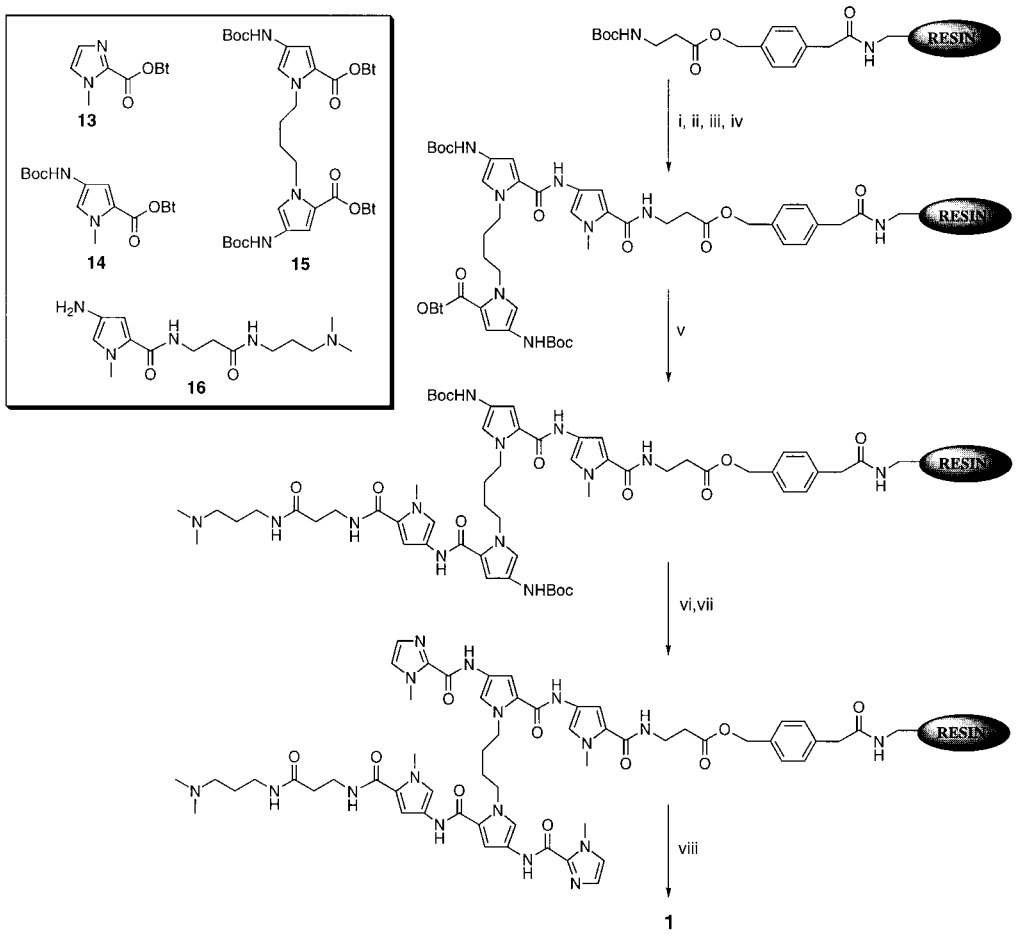
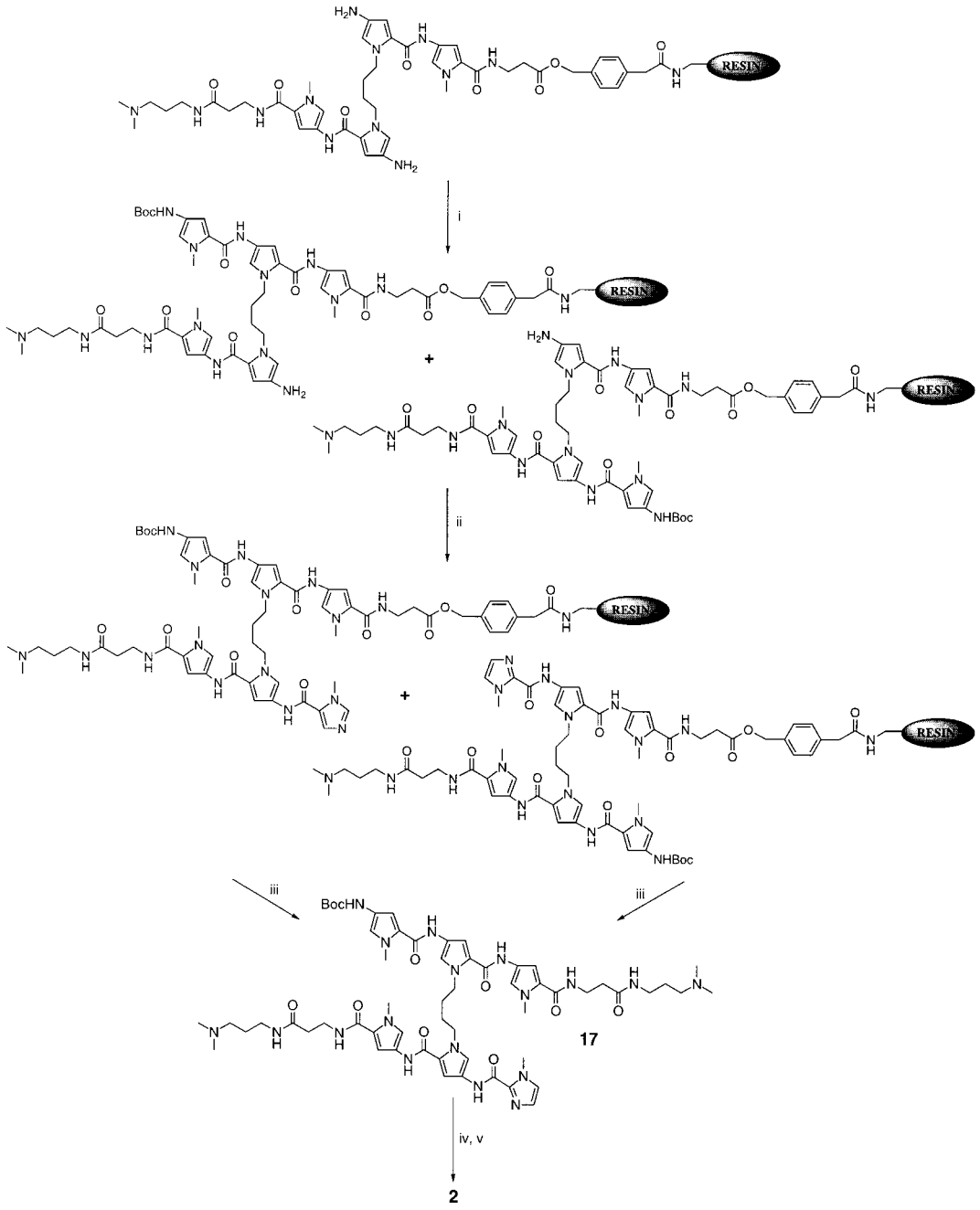


Scheme 2. Solid-phase synthesis of homodimeric H-pin (Im**Py**Py-β-Dp)$_2$C$_4$. (**1**). Inset: Pyrrole and imidazole monomers for polyamide synthesis; activated imidazole-2-carboxylic acid **13**[1a, 10], Boc-Pyrrole-OBt ester **14**, Boc-bis(pyrrole)-OBt ester **15**, and H_2N-Py-β-Dp **16**. Reaction conditions: i) 80% TFA/DCM, 0.4 M PhSH; ii) Boc-Py-OBt, DIEA, DMF, (acetic anhydride after 60 min); iii) 80% TFA/DCM, 0.4 M PhSH; iv) Boc-bis(pyrrole)-OBt ester **15**, DIEA, DMF, 37 °C; v) H_2N-Py-β-Dp **16**, DMF, DIEA, 45 °C; *N,N*-[(dimethylamino)propyl]amine, 22 °C after 10 h; vi) 80% TFA/DCM, 0.4 M PhSH; vii) imidazole-2-carboxylic acid (HBTU/DIEA); viii) *N,N*-[(dimethylamino)propyl]amine, 55 °C.

which are different in this case. In order to differentiate the almost identical polyamide chains, it was necessary to determine the conditions for partial acylation of the aromatic amines with BocPy-OBt (**14**). The half-life for coupling Boc-Py-OBt (1 mmol) in DMF (2 mL) and DIEA (1 mL) to resin-bound H_2N-Py was determined to be four minutes by picric acid titration of free amine groups at one minute intervals.[13a] Picric acid titration was used because the aromatic amines are not reactive in the quantitative ninhydrin test.[10, 13] Partial acylation was then performed with Boc-Py-OBt (**14**) (1 mmol) in DMF (2 mL) and DIEA (1 mL) and the reaction terminated after four minutes by washing with an excess of DMF. With the two chains now differentiated, it was possible to complete the synthesis by acylating with imidazole-2-carboxylic acid (HBTU, DIEA). Partial coupling should provide an approximately 1:1:1:1 statistical mixture of (Im**Py**Py-β-Dp)C$_4$(Boc-Py**Py**Py-β-Pam-resin), (Boc-Py**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-resin), (Im**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-resin), and (Boc-Py**Py**Py)C$_4$(BocPy**Py**Py-β-Pam-Resin). Because the C-terminal portion of each chain is the same, (Im**Py**Py-β-Dp)C$_4$(Boc-Py**Py**Py-β-Pam-resin) and (Boc-Py-**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-resin) both provide (BocPy-**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Dp) (**17**) upon cleavage from the resin with *N,N*-[(dimethylamino)propyl]amine (Scheme 3). This requirement for symmetry at either the C-terminal or N-terminal side of the linker limits the range of heterodimeric H-pin polyamides that can be prepared by this route. The N-terminal Boc-protected polyamide was cleaved from the resin and purified by reversed-phase HPLC, utilizing the hydrophobicity of the Boc protecting group to separate **17** from the two major side products, (Im**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Dp), which lacks a Boc group and (Boc-Py**Py**Py-β-Dp)C$_4$(BocPy-**Py**Py-β-Dp) which has two Boc protecting groups. Polyamide **2** was then prepared by removal of the N-terminal Boc group (TFA), followed by acetylation with acetic anhydride (DMF, DIEA) and purification by reversed-phase HPLC.

Binding affinities and specificities: Affinities and specificities of H-pin polyamides were determined by quantitative DNase I footprint titration[12] on 3′-^{32}P labeled 135 base pair[8a] or 252 base pair[6a] DNA restriction fragments (10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.0, 22 °C). Analysis of the footprints (Figure 4) and binding isotherms generated from the titrations (Figures 5 and 6) indicates that the H-pin polyamides bind to their designated sites as 1:1 complexes, as expected for covalent dimers binding according



Scheme 3. Solid-phase synthesis of heterodimeric H-pin (Im**Py**Py-β-Dp)C_4(AcPy**Py**Py-β-Dp) starting from (H_2N-**Py**Py-β-Dp)C_4(H_2N-**Py**Py-β-Pam-resin). Reaction conditions: i) 0.4 M Boc-Py-OBt, 2:1 DMF/DIEA, 4 min, 22 °C; ii) imidazole-2-carboxylic acid, HBTU, DIEA, DMF; iii) *N,N*-[(dimethylamino)-propyl]amine, 55 °C; iv) TFA; v) Ac_2O, DIEA, DMF.

to the side-by-side 2:1 polyamide-DNA model. The equilibrium association constants obtained for H-pin polyamides were compared to those found on the same restriction fragments for the analogous polyamide hairpins (Tables 1). Apparent monomeric association constants were also determined for unlinked polyamides **5** and **8**.

Comparison of the equilibrium association constants of (Im**Py**Py-β-Dp)$_2C_4$ (**1**) for its match 5′-TGTCA-3′ (K_a = 9.3 ($\pm$1.6) $\times$ 10^6 M^{-1}) and mismatch 5′-TGT**T**A-3′ (K_a = 9.9 ($\pm$2.7) $\times$ 10^5 M^{-1}) sites reveals approximately 10-fold specificity. The affinity of H-pin **1** for the match site is at least 186-fold greater than that of its unlinked analogue ImPyPy-β-Dp ($K_a \leq 1 \times 10^5$ M^{-1}). The analogous hairpin, ImPyPy-γ-ImPyPy-β-Dp (**3**), binds the 5′-TGTCA-3′ match (K_a = 2.0 ($\pm$0.3) $\times$ 10^8 M^{-1}) and the 5′-TGT**T**A-3′ mismatch (K_a = 7.0 ($\pm$2.9) $\times$ 10^6 M^{-1}) site with 20-fold stronger affinity and threefold enhanced specificity relative to H-pin **1**. The heterodimeric H-pin (Im**Py**Py-β-Dp)C_4(AcPy**Py**Py-β-Dp) (**2**) binds its match site 5′-TGTTA-3′ (K_a = 2.0 ($\pm$0.9) $\times$ 10^6 M^{-1}) and single base pair mismatch site 5′-TGT**C**A-3′ (K_a = 5.7 ($\pm$1.3) $\times$

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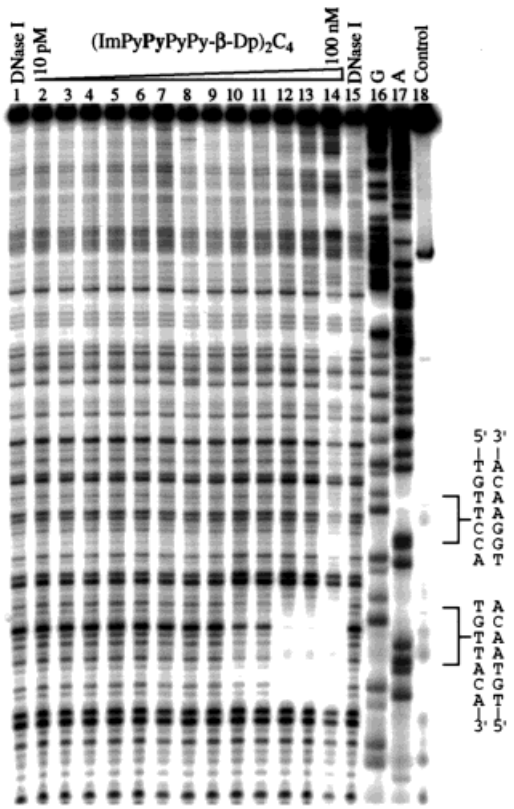


Figure 4. Storage phosphor autoradiogram of a 8% denaturing polyacrylamide gel used to separate fragments generated by DNase I digestion in a quantitative footprint titration experiment with (ImPy**Py**PyPy-β-Dp)$_2$C$_4$ (**3**) on the 3′-^{32}P-labeled 252 base pair *Eco* RI/*Pvu* II restriction fragment from plasmid pJK7. Lanes 1 and 15: DNase I digestion products in the absence of polyamide. Lanes 2–14: DNase I digestion products obtained in the presence of 10 pM, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 pM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM polyamide. Lanes 16 and 17: G and A sequencing lanes. Lane 18: intact DNA. The targeted binding sites are indicated on the right side of the autoradiograms. All reactions contain 15 kcpm restriction fragment, 10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

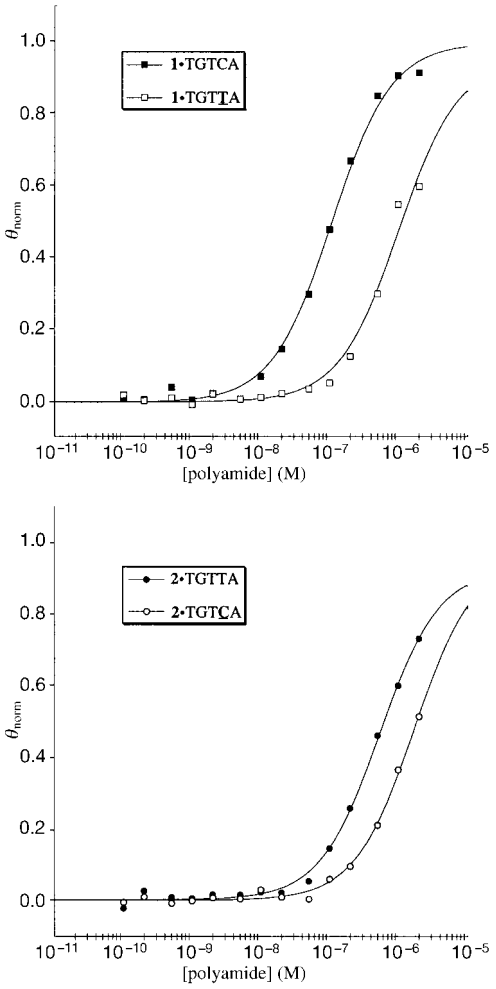


Figure 5. Data obtained from quantitative DNase I footprint titration experiments on six-ring H-pin polyamides (Im**Py**Py-β-Dp)$_2$C$_4$ (top) and (Im**Py**Py-β-Dp)C$_4$(AcPy**Py**Py-β-Dp) (bottom). The (θ_{norm}, $[L]_{tot}$) data points were obtained as described in the Experimental Section, and each is the average value obtained from three experiments.

$10^5\,M^{-1}$) with slightly lower affinity and reduced specificity compared to the homodimeric H-pin **1**. This may reflect a detrimental contribution from the N-terminal acetyl group.[8b] Again, the analogous hairpin ImPyPy-γ-PyPyPy-β-Dp (**4**) is superior to the H-pin in affinity ($K_a = 2.9\,(\pm 0.5) \times 10^8\,M^{-1}$) at the 5′-TGTTA-3′ match site and specificity (60-fold) versus the 5′-TGT**C**A-3′ ($K_a = 4.8\ (\pm 1.1) \times 10^6\,M^{-1}$) single base mismatch site.

The 10-ring homodimeric H-pin (ImPy**Py**PyPy-β-Dp)$_2$C$_4$ (**6**) binds its designated match 5′-TGTAACA-3′ ($K_a = 4.4\,(\pm 1.4) \times 10^8\,M^{-1}$) site with slightly higher affinity ($\approx$ fourfold) and discriminates the single base pair mismatch 5′-TG**G**AACA-3′ ($K_a = 1.6\ (\pm 0.5) \times 10^7\,M^{-1}$) site with twofold higher specificity than the unlinked ImPyPyPyPy-β-Dp (**8**) (Table 2). Binding isotherms are consistent in each case with 1:1 H-pin:DNA and 2:1 unlinked dimer:DNA complex formation (Figure 6). The 10-ring hairpin ImPyPyPyPy-γ-ImPyPyPyPy-β-Dp (**7**)[8j] binds more tightly ($K_a = 1.2\,(\pm 0.2) \times 10^{10}\,M^{-1}$) to the 5′-TGTAACA-3′ match site than 10-ring H-pin (**6**), although in this case the H-pin is more specific than the hairpin (28-fold vs. 18-fold).

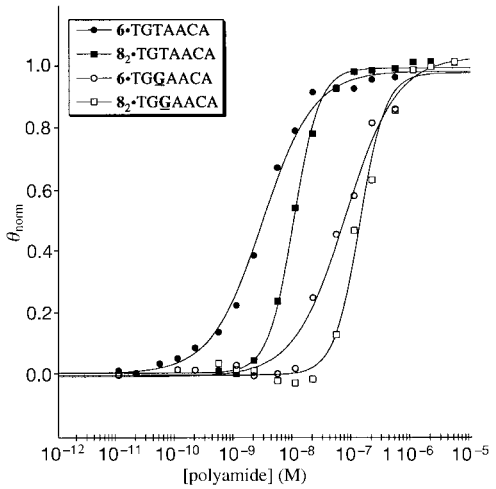
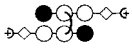
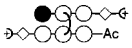
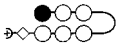
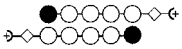
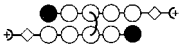
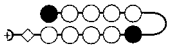


Figure 6. Data obtained from quantitative DNase I footprint titration experiments on the 10-ring H-pin polyamide (ImPy**Py**PyPy-β-Dp)$_2$C$_4$ (circles) and the unlinked analogue ImPyPyPyPy-β-Dp (squares). The (θ_{norm}, $[L]_{tot}$) data points were obtained as described in the Experimental Section and each is the average value obtained from three experiments.

Table 1. Association constants [M^{-1}] for polyamides **1**–**8**.[a–d]

Polyamide		5′-TGTCA-3′	5′-TGTTA-3′	Specificity
5		$K_a \leq 1 \times 10^5$	$K_a \leq 1 \times 10^5$	n/d
1		$K_a = 9.3 \times 10^6$	$K_a = 9.9 \times 10^5$	9[e]
3		$K_a = 2.0 \times 10^8$	$K_a = 7.0 \times 10^6$	29[e]
2		$K_a = 5.7 \times 10^5$	$K_a = 2.0 \times 10^6$	3.5[f]
4		$K_a = 4.8 \times 10^6$	$K_a = 2.9 \times 10^8$	60[f]
		5′-TGTAACA-3′	5′-TGGAACA-3′	
8		$K_a = 1.2 \times 10^8$	$K_a = 8.8 \times 10^6$	14[g]
6		$K_a = 4.4 \times 10^8$	$K_a = 1.6 \times 10^7$	28[g]
7		$K_a = 1.2 \times 10^{10}$	$K_a = 6.8 \times 10^8$	18[g]

[a] The reported equilibrium association constants are the mean values obtained from three DNase I footprint titration experiments. [b] The assays were carried out at 22 °C, pH 7.0 in the presence of 10 mM Tris · HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$. [c] Apparent monomeric association constants were determined for polyamide homodimers.[21] [d] Equilibrium association constants for polyamide **4** are from reference [8b], those for polyamide **7** are from reference [8j]. [e] Calculated as K_a(5′-TGTCA-3′/K_a(5′-TGTTA-3′). [f] Calculated as K_a(5′-TGTTA-3′)/K_a(5′-TGTCA-3′). [g] Calculated as K_a(5′-TGTAACA-3′/K_a(5′-TGGAACA-3′).

Conclusions

We have developed a methodology for the solid-phase synthesis of H-pin polyamides, a class of covalently linked polyamide dimers for the sequence-specific recognition of the minor groove of DNA. A series of H-pin polyamides showed increased affinity and specificity toward their designated sites when compared with the corresponding unlinked subunits. However, the H-pin polyamides are inferior to the hairpin analogues. H-pin polyamides may still prove useful as a complement to, or in conjunction with, other polyamide motifs such as the hairpin[8] and extended[6] motifs in the design of nonnatural ligands for the sequence-specific recognition of DNA.

Experimental Section

General: Dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), 2-(1 H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and Boc-β-alanine(-4-carboxamidomethyl)-benzyl-ester-copoly(styrene-divinylbenzene) resin (Boc-β-Pam-Resin, 0.2 mmol g^{-1}) were purchased from Peptides International. *N,N*-Diisopropylethylamine (DIEA), *N,N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), acetic anhydride (Ac_2O), and potassium cyanide/pyridine (0.0002 M) were purchased from Applied Biosystems. Dichloromethane (DCM) and triethylamine (TEA) were reagent grade from EM; thiophenol (PhSH) and *N,N*-[(dimethylamino)propyl]amine (Dp) were from Aldrich; trifluoroacetic acid (TFA) was Biograde from Halocarbon; K_2CO_3 was from Mallinckrodt; phenol from Fisher; and ninhydrin 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 grade 2 sintered glass frit were made as described by Kent.[15] NMR spectra were recorded on a GE QE300 instrument operating at 300 MHz (^{1}H) and 75 MHz (^{13}C). Spectra were recorded in [D_6]DMSO with chemical shifts reported in ppm relative to residual [D_5]DMSO. UV spectra were measured on a Hewlett-Packard 8452A diode array spectrophotometer. High-resolution mass spectra were obtained at the Mass Spectrometry Laboratory at the University of California, Riverside. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was carried out at the Protein and Peptide Microanalytical Facility at the California Institute of Technology. Thin-layer chromatography was performed on silica gel 60 F_{254} precoated plates, and chromatographic separations were performed with EM silica gel 60 (230–400 mesh). HPLC analysis was performed on either a HP 1090M analytical HPLC or a Beckman Gold system with a RAINEN C_{18}, Microsorb MV, 5 μm, 300 × 4.6 mm reversed-phasecolumn in 0.1 % (wt/v) TFA with acetonitrile as the eluent and a flow rate of 1.0 mL min^{-1}, gradient elution 1.25 % acetonitrile min^{-1}. Preparatory reverse phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 × 100 mm, 100 μm C_{18} column equipped with a guard, 0.1 % (wt/v) TFA, 0.25 % acetonitrile min^{-1}. 18 MΩ water was obtained from a Millipore MilliQ water purification system, and all buffers were sent through 0.2 μm filters.

Methyl 4-[(*tert*-butoxycarbonyl)amino]-pyrrole-2-carboxylate (**10**): Methyl 4-nitropyrrole-2-carboxylate[16] (**9**, 10.0 g, 58.8 mmol) was dissolved in DMF (50 mL) and DIEA (12.5 mL). The solution was purged with Ar for 5 min, then 10 % Pd/C (1.8 g) and di-*tert*-butyl dicarbonate (13.0 g, 59.5 mmol) were added. The mixture was stirred vigorously under H_2 (500 psi) for 2.5 h. Pd/C was removed by filtration, DMF was removed under reduced pressure, and the product was purified by silica gel chromatography (hexanes/diethyl ether with a gradient of 3:1 to 2:3) to provide **10** (8.23 g, 58 %) as a white solid. $R_f = 0.4$ (hexanes/diethyl ether 2:3); ^{1}H NMR (300 MHz, [D_6]DMSO, 20 °C): $\delta = 11.55$ (s, 1 H; Py NH), 9.10 (s, 1 H; Boc NH), 6.93 (s, 1 H; CH), 6.57 (s, 1 H; CH), 3.70 (s, 3 H; OCH_3), 1.41 (s, 9 H; $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): $\delta =$ 161.3, 153.2, 113.2, 109.8, 106.7, 102.6, 80.2, 51.7, 28.4; MS: *m/z* = calcd. for $C_{11}H_{16}N_2O_4$ 240.1110, found 240.1111.

1,4-Bis-[methyl-*N,N*′-4-[(*tert*-butoxycarbonyl)amino]-pyrrolyl-2-carboxylate]-(*E*)-2-butene (**11**): Pyrrole **10** (2.66 g, 11.1 mmol) was dissolved in acetone (60 mL). Anhydrous K_2CO_3 (5.3 g, 38.3 mmol) was added, followed by 1,4-dibromo-2-butene (predominantly *trans*) (1.30 g, 6.10 mmol, 0.55 equiv). The mixture was refluxed for total of 40 h, after 15 h the KBr generated was filtered and fresh K_2CO_3 (5.0 g) was added. After removal of the salts by filtration, acetone was removed under reduced pressure and the mixture taken up in 2:1 hexanes/diethyl ether, from which **11** precipitated as a white solid (1.85 g, 64 %): $R_f = 0.3$ (hexanes/diethyl ether 2:3); ^{1}H NMR (300 MHz, [D_6]DMSO, 20 °C): $\delta = 9.12$ (s, 2 H; NH), 7.07 (s, 2 H; arom CH), 6.58 (s, 2 H; arom CH), 5.56 (m, 2 H; aliph CH), 4.77 (m, 4 H; NCH_2), 3.64 (s, 6 H; OCH_3), 1.39 (s, 18 H; $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): $\delta = 161.2$, 153.3, 129.2, 122.7, 118.8, 107.9, 102.6, 80.3, 51.2, 49.9, 28.4; MS: *m/z* = calcd. for $C_{26}H_{37}N_4O_8$ 533.2611, found 533.2620.

1,4-Bis-[methyl-*N,N*′-4-[(*tert*-butoxycarbonyl)amino]-pyrrolyl-2-carboxylate]-butane: To bispyrrole **11** (2.30 g, 4.32 mmol) dissolved in ethyl acetate (30 mL) was added Pd/C (500 mg), and the mixture then stirred under H_2 (150 psi) for 2 h. Filtration and removal of ethyl acetate under reduced pressure yielded 1,4-bis-[methyl-*N,N*′-4-[(*tert*-butoxycarbonyl)amino]-pyrrolyl-2-carboxylate]-butane as a white solid (2.12 g, 92 %): $R_f =$ 0.3 (hexanes/diethyl ether 2:3); ^{1}H NMR (300 MHz, [D_6]DMSO, 20 °C): $\delta = 9.14$ (s, 2 H; NH), 7.12 (s, 2 H; CH), 6.59 (s, 2 H; CH), 4.18 (m, 4 H; NCH_2), 3.68 (s, 6 H; NCH_3), 1.53 (m, 4 H; CH_2), 1.41 (s, 18 H; $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): $\delta = 161.3$, 153.2, 122.3, 118.9, 107.9, 102.6, 80.2, 51.2, 48.6, 28.4, 28.2; MS: *m/z* = calcd. for $C_{26}H_{39}N_4O_8$ 535.2768, found 535.2778.

1,4-Bis-[*N,N*′-4-[(*tert*-butoxycarbonyl)amino]-pyrrolyl-2-carboxylic acid]-butane (**12**): To a solution of 1,4-bis-[methyl-*N,N*′-4-[(*tert*-butoxycarbonyl)-amino]-pyrrolyl-2-carboxylate]-butane (2.05 g, 3.83 mmol) dissolved in methanol (15 mL) was added aqueous KOH (5 mL, 4 M). The mixture was stirred at 45 °C for 20 h, during which time the cloudy suspension became clear. The solution was acidified to pH 2 with aqueous HCl, resulting in a precipitate. Methanol was removed under reduced pressure and the product extracted into ethyl acetate (40 mL). After drying in vacuo **12** was precipitated from 1:1 hexanes/diethyl ether as an off-white powder

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(1.90 g, 97%): $R_f = 0.1$ (diethyl ether); ^{1}H NMR (300 MHz, [D$_6$]DMSO, 20 °C): $\delta = 12.1$ (br s, 2H; COOH), 9.02 (s, 2H; NH), 7.01 (s, 2H; CH), 6.53 (s, 2H; CH), 4.18 (m, 4H; NCH_2), 1.54 (m, 4H; CH_2), 1.41 (s, 18H; $C(CH_3)_3$); ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): $\delta = 162.8$, 153.3, 122.7, 118.7, 109.1, 102.6, 79.1, 48.1, 28.4, 28.2; MS: m/z = calcd. for $C_{24}H_{34}N_4O_8$ 506.2377, found 506.2367.

Activation of bispyrrole acid 12: 1,4-Bis-[*N*,*N*′-4-[(*tert*-butoxycarbonyl)-amino]-pyrrolyl-2-carboxylic acid]-butane (**12**, 253 mg, 0.5 mmol) and HOBt (135 mg, 1 mmol) were dissolved in DMF (2 mL). DCC (202 mg, 1 mmol) was then added and the solution periodically agitated for 30 min. DCU was removed by filtration through a disposable polypropylene filter and the bis-OBt ester **15** used without further purification.

H_2N-Py-β-Dp (**16**): Boc-β-alanine-Pam-Resin (3 g, 0.2 mmol) was placed in a 20 mL glass reaction vessel, shaken in DMF for 5 min, and the reaction vessel drained. The resin was washed with DCM (2 × 30 s) and the Boc group removed with 80% TFA/DCM/PhSH (0.5 M) (1 × 30 s, 1 × 20 min). The reaction vessel was then drained and the resin washed with DCM (2 × 30 s) followed by DMF (2 × 30 s) The vessel was drained completely and Boc-Py-OBt (**14**) (714 mg, 2 mmol) dissolved in DMF (4 mL) added, followed by DIEA (2 mL). The reaction vessel was shaken vigorously to make a slurry. After 60 min acetic anhydride (1 mL) was added to the reaction vessel. After 5 min the resin was washed sequentially with an excess of DMF, DCM, MeOH, and ethyl ether and then dried in vacuo to provide the Boc-Py-β-Pam-resin. A sample of BocPy-β-Pam-resin (2 g, 0.2 mmol g^{-1}) was treated with *N*,*N*-[(dimethylamino)propyl]amine (2 mL) and heated (55 °C) with periodic agitation for 16 h. The reaction mixture was then filtered to remove resin, 0.1% (wt/v) TFA added (6 mL), and the resulting solution purified by reversed-phase HPLCto provide Boc-Py-β-Dp as a brown oil upon lyophilization of the appropriate fractions (130 mg, 66% recovery); ^{1}H NMR (300 MHz, [D$_6$]DMSO, 20 °C): $\delta = 9.5$ (br s, 1H; CF_3COOH); 9.12 (s, 1H; arom NH); 8.00 (t, 1H, aliph NH), 7.94 (t, 1H, aliph NH); 6.88 (d, 1H, $J = 1.6$ Hz; CH); 6.56 (d, 1H, $J = 1.6$ Hz; CH); 3.71 (s, 6H; NCH_3); 3.33 (q, 2H, $J = 5.6$ Hz; CH_2); 3.01 (m, 4H; CH_2); 2.71 (d, 6H; $J = 4.7$ Hz; $N(CH_3)_2$); 2.31 (m, 2H; CH_2); 1.71 (quintet, 2H, $J = 5.4$ Hz; CH_2); 1.36 (s, 9H; $C(CH_3)_3$); MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{19}H_{33}N_5O_4$: 396.3; found 396.3. Boc-Py-β-Dp was then treated with neat TFA (10 mL). After 30 min, excess TFA was removed in vacuo, the resulting H_2N-Py-β-Dp (**16**) dissolved in DMF (2 mL) and DIEA (1 mL), and then used for coupling without subsequent purification.

(ImPyPy-β-Dp)$_2$C$_4$ (**1**): A sample of Boc-Py-β-Pam-resin (1 g, 0.2 mmol g^{-1}) was treated with 80% TFA/DCM/0.5 M PhSH (1 × 30 s, 1 × 20 min). The resin was then washed with DCM (2 × 30 s) followed by DMF (2 × 30 s) The vessel was drained completely and Bis-Py-OBt (**15**) (0.5 mmol) dissolved in DMF (2 mL) was added, followed by DIEA (1 mL). The reaction vessel was shaken vigorously to make a slurry and then heated (37 °C) with constant agitation for 6 h. The resin was then washed with DMF (4 × 30 s). The vessel was drained completely and H_2N-Py-β-Dp (**16**) (0.26 mmol) dissolved in DMF (2 mL) and DIEA (1 mL) was added. The reaction vessel was shaken vigorously to make a slurry and then heated (45 °C) with constant agitation for 10 h. *N*,*N*-[(Dimethylamino)-propyl]amine was then added (300 μL) to cap any unconverted Py-OBt ester and the reaction shaken vigorously (22 °C) for 5 min. The resin was then washed with DMF (6 × 30 s) followed by DCM (2 × 30 s), and both Boc groups removed with 80% TFA/DCM/0.5 M PhSH, (1 × 30 s, 1 × 20 min). The resin was washed sequentially with an excess of DCM, MeOH, and ethyl ether and then dried in vacuo to provide (H_2N-**Py**Py-β-Dp)C$_4$(H_2N-**Py**Py-β-Pam-resin). A sample of (H_2N-**Py**Py-β-Dp)C$_4$(H_2N-**Py**Py-β-Pam-resin) (240 mg, 0.18 mmol g^{-1} substitution[17]) was treated with activated imidazole-2-carboxylic acid (**13**)[10] (200 mg) in DMF (2 mL) and DIEA (1 mL). After shaking the reaction vessel for 60 min (22 °C), the resin was washed sequentially with an excess of DCM, MeOH, and ethyl ether and then dried in vacuo to provide (Im**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-Resin). A sample of resin (240 mg, 0.18 mmol g^{-1} substitution) was treated with neat *N*,*N*-[(dimethylamino)propyl]amine (2 mL) and heated (55 °C) with periodic agitation for 16 h. The reaction mixture was then filtered to remove resin, 0.1% (wt/v) TFA (6 mL) was added and the resulting solution was purified by reversed-phase HPLC. (Im**Py**Py-β-Dp)$_2$C$_4$ (**1**) was recovered upon lyophilization of the appropriate fractions as a white powder (14 mg, 27% recovery). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 20 °C): $\delta = 10.45$ (s, 2H; arom NH), 9.91 (s, 2H; arom NH), 9.2 (br s, 2H; CF_3COOH), 8.02 (m, 4H; aliph NH), 7.38 (s, 2H; CH), 7.31 (s, 2H; CH), 7.16 (s, 2H; CH), 7.08 (d, 2H; $J = 1.2$ Hz; CH), 7.03 (s, 2H; CH), 6.81 (d, 2H; $J = 1.6$ Hz; CH), 4.25 (t, 4H; $J = 4.4$ Hz; linker CH_2), 3.94 (s, 6H; NCH_3), 3.75 (s, 6H; NCH_3), 3.33 (q, 4H; $J = 6.4$ Hz; CH_2), 3.04 (q, 4H; $J = 6.4$ Hz; CH_2), 2.95 (m, 4H; CH_2), 2.70 (d, 12H, $J = 4.7$ Hz; $N(CH_3)_2$), 2.30 (t, 4H, $J = 7.3$ Hz; CH_2), 1.70 (quintet, 4H, $J = 6.7$ Hz; CH_2), 1.60 (quintet, 4H; $J = 5.0$ Hz; linker CH_2). UV/Vis (H_2O): λ_{max} (ε) = 304 (50000 calculated from $\varepsilon = 8333$ ring[8f]), 246 nm; MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{52}H_{73}N_{18}O_8$ 1077.6, found 1077.4.

(ImPyPy-β-Dp)C$_4$(BocPyPyPy-β-Dp) (**17**): A sample of (H_2N-**Py**Py-β-Dp)C$_4$(H_2N-**Py**Py-β-Pam-resin) was treated with Boc-Py-OBt (**14**) (357 mg, 1 mmol) in DMF (2 mL) and DIEA (1 mL). The reaction vessel was shaken vigorously at 22 °C for 4 min, and then was washed with DMF (4 × 30 s). The reaction vessel was drained completely and then activated imidazole-2-carboxylic acid (**13**)[10] (200 mg) in DMF (2 mL) and DIEA (1 mL) added. The reaction vessel was shaken vigorously at 22 °C for 60 min. The resin was then washed sequentially with an excess of DMF, DCM, MeOH, and ethyl ether and then dried in vacuo. The resin consists of a mixture of (Im**Py**Py-β-Dp)C$_4$(Boc-Py**Py**Py-β-Pam-resin), (Boc-Py**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-resin), (Im**Py**Py-β-Dp)C$_4$(Im**Py**Py-β-Pam-resin), and (Boc-Py**Py**Py)C$_4$(BocPy**Py**Py-β-Pam-Resin). A sample of resin (240 mg, 0.18 mmol g^{-1} substitution) was treated with neat *N*,*N*-[(dimethylamino)propyl]amine (2 mL) and heated (55 °C) with periodic agitation for 16 h. The reaction mixture was then filtered to remove resin, 0.1% (wt/v) TFA added (6 mL), and the resulting solution purified by reversed-phase HPLC. (Im**Py**Py-β-Dp)C$_4$(BocPy**Py**Py-β-Dp) (**17**) is recovered as the major product upon lyophilization of the appropriate fractions to provide a white powder (6 mg, 10% recovery), ^{1}H NMR (300 MHz, [D$_6$]DMSO, 20 °C): $\delta = 10.44$ (s, 1H; arom NH), 9.92 (s, 1H; arom NH), 9.90 (s, 1H; arom NH), 9.85 (s, 1H; arom NH), 9.2 (br s, 2H; CF_3COOH), 9.09 (s, 1H; arom NH), 8.04 (m, 4H; aliph NH), 7.37 (s, 1H; CH), 7.31 (d, 1H; $J = 1.4$ Hz; CH), 7.23 (d, 1H; $J = 1.6$ Hz; CH), 7.16 (d, 1H; $J = 1.4$ Hz; CH), 7.09 (d, 1H; $J = 1.6$ Hz; CH), 7.01 (s, 1H; CH), 6.98 (d, 1H; $J = 1.6$ Hz; CH), 6.88 (d, 1H, $J = 1.4$ Hz; CH), 6.86 (d, 1H; $J = 1.7$ Hz; CH), 6.84 (d, 1H; $J = 1.4$ Hz; CH), 6.82 (d, 1H; $J = 1.5$ Hz), 6.79 (d, 1H, $J = 1.6$ Hz), 4.25 (m, 4H; linker CH_2), 3.94 (s, 3H; NCH_3), 3.75 (s, 15H; NCH_3), 3.66 (m, 4H; CH_2), 3.08 (m, 4H; CH_2), 2.92 (m, 4H; CH_2), 2.70 (m, 12H; $N(CH_3)_2$), 2.43 (m, 4H; CH_2), 1.71 (m, 4H; CH_2), 1.62 (m, 4H; linker CH_2), 1.42 (s, 9H; $C(CH_3)_3$); MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{58}H_{83}N_{18}O_{10}$ 1191.7; found 1191.7.

(ImPyPy-β-Dp)C$_4$(AcPyPyPy-β-Dp) (**18**): A sample of (Im**Py**Py-β-Dp)C$_4$(BocPy**Py**Py-β-Dp) (**17**, 4 mg, 3 μmol) was treated with neat TFA (10 mL) for 30 min (22 °C). TFA was removed in vacuo and DMF (1 mL) added to resuspend the polyamide. The polyamide-amine was treated with a solution of acetic anhydride (1 mL) and DIEA (1 mL) in DMF (1 mL) and heated (55 °C) with periodic agitation for 30 min. Residual acetic anhydride was hydrolyzed (0.1 M NaOH, 1 mL, 55 °C, 10 min); 0.1% (wt/v) TFA was added (6 mL) and the resulting solution was purified by reversed-phase HPLC to provide (Im**Py**Py-β-Dp)C$_4$(AcPy**Py**Py-β-Dp) as a white powder upon lyophilization of the appropriate fractions (2 mg, 50% recovery). UV/Vis (H_2O): λ_{max} (ε) = 302 (50000 calculated from $\varepsilon = 8333$ ring[8f]), 244 nm; MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{55}H_{77}N_{18}O_9$ 1133.6, found 1133.6.

ImPyPy-β-Dp (**5**): A sample of ImPyPy-β-Pam-resin[10] (240 mg, 0.19 mmol g^{-1}[17]) was treated with neat *N*,*N*-[(dimethylamino)propyl]-amine (2 mL) and heated (55 °C) with periodic agitation for 16 h. The reaction mixture was then filtered to remove resin, 0.1% (wt/v) TFA added (6 mL) and the resulting solution purified by reversed-phase HPLC. ImPyPy-β-Dp is recovered upon lyophilization of the appropriate fractions as a white powder (19 mg, 68% recovery). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 20 °C): $\delta = 10.48$ (s, 1H; arom NH), 9.88 (s, 1H; arom NH), 9.2 (br s, 1H; CF_3COOH), 8.03 (m, 2H; aliph NH), 7.39 (s, 1H; CH), 7.24 (d, 1H, $J = 1.$ Hz; CH), 7.13 (d, 1H, $J = 1.6$ Hz; CH), 7.10 (d, 1H, $J = 1.6$ Hz; CH), 7.06 (s, 1H; CH), 6.83 (s, 1H, $J = 1.5$ Hz), 3.94 (s, 3H; NCH_3), 3.79 (s, 3H; NCH_3), 3.75 (s, 3H; NCH_3), 3.33 (q, 2H, $J = 5.9$ Hz; CH_2), 3.08 (q, 2H, $J = 5.8$ Hz; CH_2), 2.96 (q, 2H, $J = 5.5$ Hz; CH_2), 2.69 (d, 6H; $J = 4.6$ Hz; $N(CH_3)_2$), 2.31 (t, 2H, $J = 7.0$ Hz; CH_2), 1.70 (quintet, 2H, $J = 5.0$ Hz; CH_2); UV/Vis (H_2O): λ_{max} (ε) = 302 (25,000 calculated from $\varepsilon = 8,333$ ring[8f]), 248 nm; MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{25}H_{36}N_9O_4$, 526.3; found 526.3.

H_2N-PyPy-β-Dp: Boc-PyPy-β-Dp was prepared as a white power as described for Boc-Py-β-Dp (130 mg, 66% recovery); ^{1}H NMR (300 MHz,

[D_6]DMSO, 20 °C): $\delta = 9.79$ (s, 1H; arom NH), 9.5 (br s, 1H; CF_3COOH), 9.07 (s, 1H; arom NH), 8.01 (m, 2H; aliph NH), 7.11 (s, 1H; CH), 6.84 (s, 1H; CH), 6.81 (s, 1H; CH), 6.78 (s, 1H; CH), 3.75 (s, 6H; NCH_3), 3.35 (q, 2H, $J = 5.9$ Hz; CH_2), 3.08 (q, 2H, $J = 5.5$ Hz; CH_2), 2.96 (q, 2H, $J = 5.8$ Hz; CH_2), 2.70 (d, 6H; $J = 4.6$ Hz; $N(CH_3)_2$), 2.31 (t, 2H, $J = 6.8$ Hz; CH_2), 1.68 (quintet, 2H, $J = 5.0$ Hz; CH_2), 1.41 (s, 9H; $C(CH_3)_3$); MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calc. for $C_{25}H_{40}N_7O_5$ 518.3; found 518.3. Boc-PyPy-β-Dp was then deprotected to provide H_2N-PyPy-β-Dp as described for **16.** H_2N-PyPy-β-Dp was dissolved in DMF (2 mL) and DIEA (1 mL) and used for coupling without subsequent purification.

(ImPyPyPyPy-β-Dp)$_2$C$_4$ (**6**): (ImPy**Py**PyPy-β-Dp)C_4(ImPy**Py**PyPy-β-Pam-Resin) was prepared as described for (Im**Py**Py-β-Dp)C_4(Im**Py**Py-β-Pam-Resin). A sample of (ImPy**Py**PyPy-β-Dp)C_4(ImPy**Py**PyPy-β-Pam-Resin) (240 mg, 0.17 mmol g^{-1}) was treated with *N,N*-[(dimethylamino)-propyl]amine (2 mL) and heated (55 °C) with periodic agitation for 16 h. The reaction mixture was then filtered to remove resin, 0.1% (wt/v) TFA added (6 mL) and the resulting solution purified by reversed-phase HPLC to provide (ImPyPyPyPy-β-Dp)$_2C_4$ (**6**) as a white powder upon lyophilization of the appropriate fractions (13 mg, 20% recovery). ^{1}H NMR (300 MHz, [D_6]DMSO, 20 °C): $\delta = 10.45$ (s, 2H; arom NH), 9.91 (s, 2H; arom NH), 9.89 (s, 4H; arom NH), 9.2 (br s, 2H; CF_3COOH), 8.02 (m, 4H; aliph NH), 7.38 (s, 2H; CH), 7.29 (m, 2H; CH), 7.22 (m, 2H; CH), 7.15 (m, 4H; CH), 7.05 (m, 4H; CH), 7.00 (m, 4H; CH), 6.85 (m, 2H; CH), 4.27 (m, 4H; linker CH_2), 3.97 (s, 6H; NCH_3), 3.82 (s, 12H; NCH_3), 3.82 (s, 6H; NCH_3), 3.76 (s, 6H; NCH_3), 3.35 (m, 4H; CH_2) 3.03 (m, 4H; CH_2), 2.92 (m, 4H; CH_2), 2.72 (d, 12H, $J = 4.6$ Hz; $N(CH_3)_2$), 2.35 (m, 4H; CH_2), 1.70 (m, 4H; CH_2), 1.60 (m, 4H; linker CH_2); UV/Vis (H_2O): λ_{max} (ε) = 308 (83 000 calculated from $\varepsilon = 8333$ ring[8f]), 246 nm; MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{76}H_{97}N_{26}O_{12}$ 1566.8; found 1566.9.

ImPyPyPyPy-β-Dp (**8**): ImPyPyPyPy-β-Pam-resin was prepared and cleaved (240 mg, 0.18 mmol g^{-1}) as described for **5**. (16 mg, 42% recovery). ^{1}H NMR (300 MHz, [D_6]DMSO, 20 °C): $\delta = 10.45$ (s, 1H; arom NH), 9.95 (s, 1H; arom NH), 9.93 (s, 1H; arom NH), 9.88 (s, 1H; arom NH), 9.2 (br s, 1H; CF_3COOH), 8.02 (m, 2H; aliph NH), 7.37 (s, 1H; CH), 7.26 (d, 1H, $J =$ 1.4 Hz; CH), 7.21 (d, 1H, $J = 1.5$ Hz; CH), 7.17 (d, 1H, $J = 1.6$ Hz; CH), 7.14 (d, 1H, $J = 1.6$ Hz; CH), 7.06 (m, 2H; CH), 7.02 (m, 2H; CH), 6.85 (d, 1H, $J = 1.6$ Hz; CH), 3.96 (s, 3H; NCH_3), 3.82 (s, 6H; NCH_3), 3.81 (s, 3H; NCH_3), 3.77 (s, 3H; NCH_3), 3.65 (m, 2H; CH_2) 3.23 (m, 2H; CH_2), 3.03 (m, 2H; CH_2), 2.72 (d, 6H, $J = 4.7$ Hz; $N(CH_3)_2$), 2.43 (t, 2H, $J = 6.7$ Hz; CH_2), 1.71 (quintet, 2H, $J = 6.6$ Hz; CH_2); UV/Vis (H_2O): λ_{max} (ε) = 306 (42,000 calculated from $\varepsilon = 8{,}333$ ring[8f]), 244 nm; MALDI-TOF-MS [$M^+ - H$] (monoisotopic): calcd. for $C_{37}H_{48}N_{13}O_6$ 770.4; found 770.4.

DNA reagents and materials: Enzymes were purchased from Boehringer-Mannheim and used with the provided buffers according to manufacturer's protocols. [α^{32}P] Thymidine-5′-triphosphate and [α^{32}P] deoxyadenosine-5′-triphosphate were obtained from Amersham. Sonicated, deproteinized calf thymus DNA and DNase I were acquired from Pharmacia. Sequenase (version 2.0) and RNase-free water were obtained from United States Biochemical. All other reagents and materials were used as received. All DNA manipulations were performed according to standard protocols.[18]

Preparation of ^{32}P end-labeled restriction fragments. The 135 base pair 3′-end labeled *Eco* RI/*Bsr* BI restriction fragment from plasmid pMM5 which contains the five base pair 5′-TGTTA-3′ and 5′-TGTCA-3′ sites was prepared and purified as previously described.[8a] The 252 base pair *Eco* RI/*Pvu* II restriction fragment from plasmid pJK7, which contains the seven base pair 5′-TGTTACA-3′ and 5′-TGGTTCA-3′ sites, was prepared and purified as previously described.[6a] Chemical sequencing reactions were performed according to published procedures.[19, 20]

Quantitative DNase I footprint titration. All reactions were performed in a total volume of 50 μL, with no calf thymus DNA present. A polyamide stock solution or H_2O (for reference lanes) was added to an assay buffer containing radiolabeled restriction fragment (15 000 cpm) affording final solution conditions of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.0, and polyamide over a range of concentrations. The solutions were allowed to equilibrate for 6 h at 22 °C. Footprinting reactions were initiated by addition of 5 μL of a DNase I solution (final concentration 0.10 units mL^{-1}) containing 1 mM dithiothreitol and allowed to proceed for 6 min at 22 °C. Reactions were stopped by addition of 12.5 μL of a solution containing 1.25 M NaCl, 100 mM EDTA, and 0.2 mg mL^{-1} glycogen, and ethanol precipitated. The reaction mixtures were resuspended in 1X TBE/80% formamide denaturing loading buffer; denatured by heating at 90 °C for 5 minutes; and separated by polyacrylamide gel electrophoresis on an 8% gel (5% crosslinking, 7 M urea) in 1X TBE at 2000 V. Gels were dried and exposed to a Molecular Dynamics storage phosphor screen.

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 16–20 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 version 3.2.

Quantitation and data analysis. Data from footprint titrations were analyzed by performing volume integration of rectangles encompassing footprint sites and a reference site at which DNase I reactivity was invariant over the range of the titration. Values were generated for site intensities (I_{site}) and reference intensity (I_{ref}). The apparent fractional occupancies (θ_{app}) of the sites was calculated with Equation (1).

$$\theta_{app} = 1 - \frac{I_{site}/I_{ref}}{I^{\circ}_{site}/I^{\circ}_{ref}} \quad (1)$$

where I°_{site} and I°_{ref} are the site and reference intensities, respectively, for a control lane in which no polyamide was added.

The ([L]$_{tot}$, θ_{app}) data points were fit to a general Hill equation [Eq. (2)] by minimizing the difference between θ_{app} and θ_{fit}:

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

where $[L]_{tot}$ is the total polyamide concentration, K_a is either the apparent monomeric association constant[21] for unlinked dimers or the equilibrium association constant for H-pin and hairpin polyamides, and θ_{min} and θ_{max} are the experimentally determined site saturation values when the site is unoccupied and saturated, respectively. The data was fitted using a nonlinear least squares fitting procedure with K_a, θ_{max}, and θ_{min} as adjustable parameters, and either $n = 1$ for H-pins and hairpins or $n = 2$ for unlinked polyamides. All acceptable fits had a correlation coefficient of $R > 0.97$. Binding isotherms were normalized with Equation (3)

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

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 obviously flawed relative to neighboring points.

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