Sequence Selectivity of 3-Hydroxypyrrole/Pyrrole Ring Pairings in the DNA Minor Groove

Adam R. Urbach, Jason W. Szewczyk, Sarah White, James M. Turner, Eldon E. Baird, and Peter B. Dervan*

Contribution from the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California, 91125

Received August 23, 1999

Abstract: Hairpin polyamides containing the aromatic amino acids 3-hydroxypyrrole (Hp), pyrrole (Py), and imidazole (Im) are capable of discriminating all four Watson−Crick base pairs in the DNA minor groove according to a set of pairing rules. Equilibrium association constants for four eight-ring hairpins containing all four pairings of Hp and Py at a single common position (ImIm**X**Py-γ-Im**Y**PyPy-β-Dp, where **X**/**Y** is Py/Py, Py/Hp, Hp/Py, and Hp/Hp) were determined at four DNA sites, 5′-TGG**T**CA-3′, 5′-TGG**A**CA-3′, 5′-TGG**C**CA-3′, and 5′-TGG**G**CA-3′, to study the relative binding affinities of the 16 possible complexes. The protected 3-hydroxypyrrole amino acid building block, 3-methoxypyrrole, is prepared on a 50 g scale, and the solid-phase synthesis of hydroxypyrrole−imidazole−pyrrole polyamides is described. Quantitative DNase I footprint titrations demonstrate that a Py/Py pair is partially degenerate for A•T and T•A, but disfavors G•C and C•G base pairs by 53- and 17-fold, respectively. An Hp/Py pair placed opposite T•A binds at least 20-fold more tightly than when placed opposite A•T, G•C, and C•G base pairs. The Py/Hp pair selectively binds A•T with 11-fold higher affinity over T•A and with ≥30-fold selectivity relative to G•C and C•G. An Hp/Hp pairing is disfavored opposite all four base pairs, potentially limiting certain slipped motifs available to unlinked dimers in the minor groove. This study serves to guide the design of second-generation polyamides for DNA recognition.

Introduction

Cell permeable small molecules that target predetermined DNA sequences offer a chemical approach for the regulation of specific genes.[1] Hairpin polyamides containing the three aromatic amino acids 3-hydroxypyrrole (Hp), imidazole (Im), and pyrrole (Py) are synthetic ligands that bind to specified nucleotide 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. An antiparallel pairing of Im opposite Py (Im/Py pair) distinguishes G•C from C•G and both of these from A•T and T•A base pairs.[4] A Py/Py pair binds both A•T and T•A in preference to G•C and C•G.[4,5] It has been shown recently that an Hp/Py pair prefers T•A over A•T.[6a,c] From high-resolution X-ray diffraction data, it appears that the discrimination of T•A from A•T by an Hp/Py pair arises from two discrete mechanisms: (i) shape selection of an asymmetric cleft on the

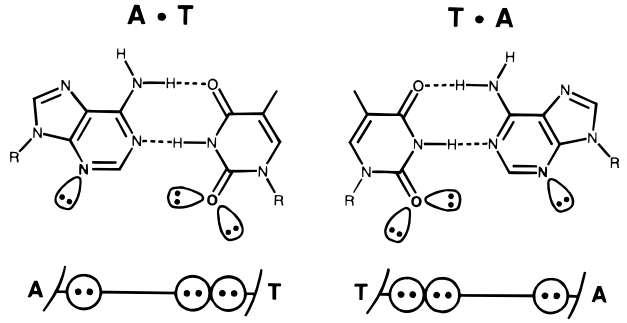


Figure 1. (Top) Structures for the A•T and T•A base pairs. Note the orbital asymmetry of the lone pair electrons in the minor groove (shown as ovals). (Bottom) Schematic representations of the orbital asymmetry of the A•T and T•A bases in the minor groove. A lone circle with two dots represents the lone pair of the N3 of adenine. Two touching circles with two dots represent the two lone pairs of the O2 of thymine.

(1) (a) Gottesfield, J. M.; Nealy, L.; Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1997**, *387*, 202−205. (b) Dickenson, L. A.; Guzilia, P.; Trauger, J. W.; Baird, E. E.; Mosier, D. M.; Gottesfeld, J. M.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12890−12895.

(2) (a) Mrksich, M.; Parks, M. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 7983−7988. (b) Parks, M. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1996**, *118*, 6147−6152. (c) Pilch, D. S.; Poklar, N. A.; Gelfand, C. A.; Law, S. M.; Breslauer, K. J.; Baird, E. E.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8306−8311. (d) de Clairac, R. P. L.; Geierstanger, B. H.; Mrksich, M.; Dervan, P. B.; Wemmer, D. E. *J. Am. Chem. Soc.* **1997**, *119*, 7909−7916. (e) Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 1113−1120. (f) Pilch, D. S.; Poklar, N. A.; Baird, E. E.; Dervan, P. B.; Breslauer, K. J. *Biochemistry* **1999**, *38*, 2143-2151.

(3) (a) Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1996**, *382*, 559−561. (b) Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 6953−6961. (c) Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Angew. Chem., Int. Ed.* **1998**, *37*, 1421−1423. (d) Turner, J. M.; Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 6219−6226. (e) Herman, D. M.; Baird, E. E.; Dervan, P. B. *Chem. Eur. J.* **1999**, *5*, 975−983.

(4) For specificity of Im/Py pairings see: (a) Wade, W. S.; Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 8783−8794. (b) Mrksich, M.; Wade, W. S.; Dwyer, T. J.; Geierstanger, B. H.; Wemmer, D. E.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7586−7590. (c) Wade, W. S.; Mrksich, M.; Dervan, P. B. *Biochemistry* **1993**, *32*, 11385−11389. (d) Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 2572−2576. (e) Geierstanger, B. H.; Mrksich, M.; Dervan, P. B.; Wemmer, D. E. *Science* **1994**, *266*, 646−650. (f) White, S.; Baird, E. E.; Dervan, P. B. *Chem. Biol.* **1997**, *4*, 569−578. (g) Kielkopf, C. L.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Nature Struct. Biol.* **1998**, *5*, 104−109.

(5) For Py/Py pairing see: (a) Pelton, J. G.; Wemmer, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5723−5727. (b) Pelton, J. G.; Wemmer, D. E. *J. Am. Chem. Soc.* **1990**, *112*, 1393−1399. (c) Chen, X.; Ramakrishnan, B.; Rao, S. T.; Sundaralingham, M. *Nature Struct. Biol.* **1994**, *1*, 169−175. (d) White, S.; Baird, E. E.; Dervan, P. B. *Biochemistry* **1996**, *35*, 12532−12537.

10.1021/ja9930667 CCC: $18.00
Published on Web 12/01/1999

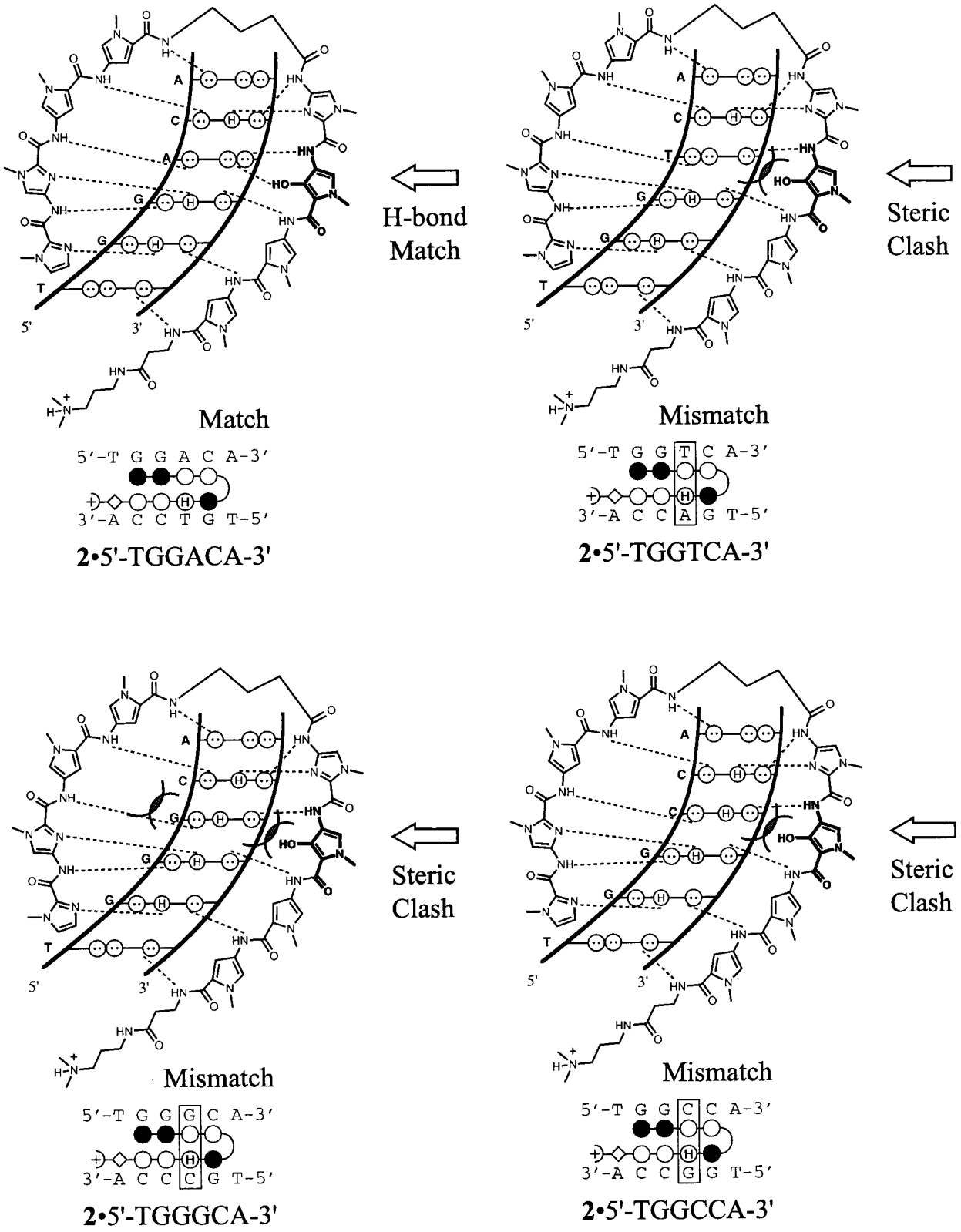


Figure 2. Hydrogen bonding model of the 1:1 polyamide:DNA complexes formed between the eight-ring hairpin polyamide ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp **2** with the four sites: 5′-TGG**A**CA-3′, 5′-TGG**T**CA-3′, 5′-TGG**G**CA-3′, and 5′-TGG**C**CA-3′. A circle with two dots represents the lone pair of N3 of purines and the O2 of cytosine. Two touching circles with dots represent the two lone pairs of the O2 of thymine. Circles containing an H represent the exocyclic amino hydrogen of guanine. Putative hydrogen bonds are illustrated by dotted lines. For the schematic binding model, imidazole and pyrrole rings are represented as shaded and unshaded circles, respectively, and a circle containing an H represents 3-hydroxypyrrole. The β-alanine residue is represented as an unshaded diamond. A steric clash between the DNA and polyamide is indicated by overlapped half circles.

floor of the minor groove formed by the O2 of thymine and C2−H of adenine, and (ii) formation of two specific hydrogen bonds between the 3-hydroxyl and 4-carboxamido groups of Hp with the two lone pairs on the O2 of thymine (Figures 1 and 2).[6b] The sequence selectivity of the Hp/Py pair toward all four Watson−Crick base pairs has not yet been fully docu-

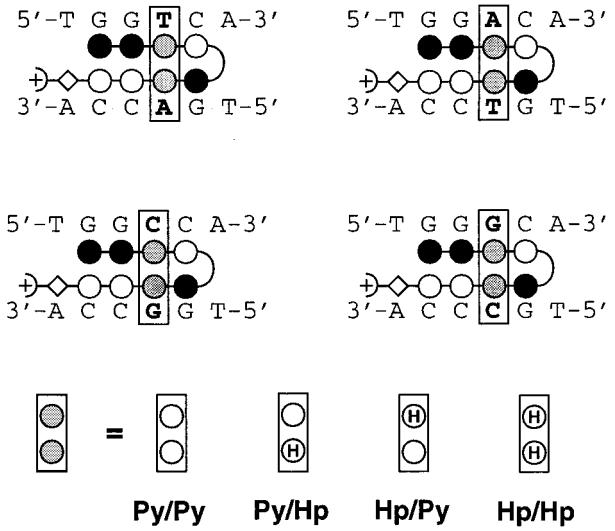


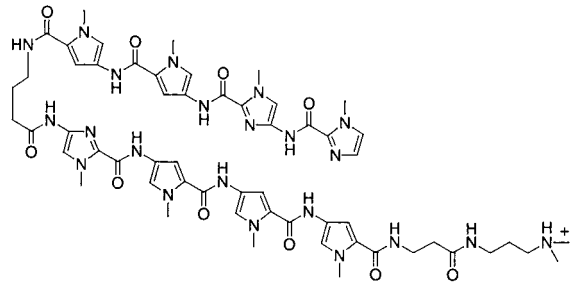
Figure 3. Schematic showing the 16 possible complexes formed by placement of the four binary combinations of 3-hydroxypyrrole (Hp) and pyrrole (Py) opposite the four Watson−Crick base pairs.

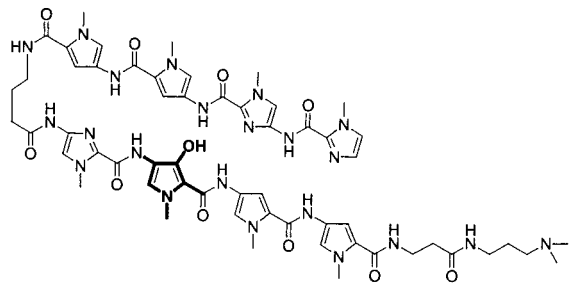
mented. We describe here the affinity of the Hp/Py pair for T•A, A•T, C•G, and G•C base pairs, confirming that the Hp/Py pair discriminates T•A from not only A•T but also C•G and G•C. In addition, we detail the synthesis of the protected Hp monomer (Boc-Op-acid, **8**) and the incorporation of this monomer into polyamides by machine-assisted solid-phase protocols.

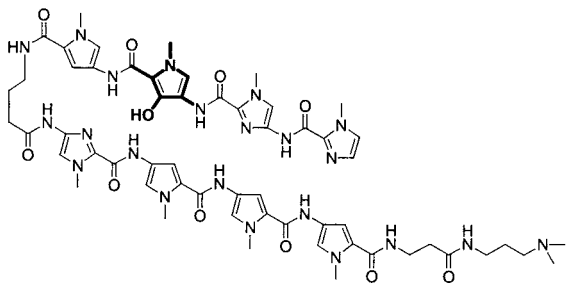
According to these pairing rules, a polyamide with the sequence ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp (β: β-alanine; Dp: dimethylaminopropylamide) would be expected to target the 6-base-pair sites 5′-TGG**T**CA-3′ (T•A) and 5′-TGG**A**CA-3′ (A•T) with approximately equal affinity and the sites 5′-TGG**C**CA-3′ (C•G) and 5′-TGG**G**CA-3′ (G•C) with much lower affinity. Selective substitution of one or two hydroxyl groups within the single Py/Py pair creates all four possible ring pairings (Py/Py, Py/Hp, Hp/Py, Hp/Hp) at a common position (Figure 3). An appropriate plasmid containing these four six-base-pair recognition sites was designed to place each of the four aromatic ring pairs opposite each of the four Watson−Crick base pairs (T•A, A•T, G•C, C•G), allowing the comparison of relative binding affinities for the 16 possible complexes. The four eight-ring polyamides, ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp **1** (Py/Py), ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp **2** (Py/Hp), ImIm**Hp**Py-γ-Im**Py**PyPy-β-Dp **3** (Hp/Py), and ImIm**Hp**Py-γ-Im**Hp**PyPy-β-Dp **4** (Hp/Hp), each representing a unique pair at a common position, were synthesized for this study by solid-phase methods (Figure 4).[7]

Results

Monomer synthesis. The 3-*O*-methyl-*N*−Boc-protected hydroxypyrrole amino acid (Boc-Op-acid) was synthesized in five steps from ethyl 4-carboxy-3-hydroxy-1-methylpyrrole-2-carboxylate **5** (Figure 5). The procedures for **5** can be scaled to provide 500 g quantities.[8] The 4-carboxylic acid of **5** is then converted to a 4-benzyl carbamate group using DPPA (TEA, CH_3CN, reflux, 5 h), followed by the addition of benzyl alcohol (reflux, 17 h) to provide ethyl 4-[(benzyloxycarbonyl)amino]-3-hydroxy-1-methylpyrrole-2-carboxylate **6**. The 3-hydroxyl group of **6** was protected as the methyl ether via alkylation with methyl iodide (anhydrous K_2CO_3, DMAP, 22 °C, 12 h) to give ethyl 4-[(benzyloxycarbonyl)amino]-3-methoxy-1-methylpyrrole-2-carboxylate. Next, the 4-benzyl carbamate protecting group of **6** was converted to *tert*-butyl carbamate by reduction and in situ reaction of the resulting 4-amino group with Boc


(**1**) ImImPyPy-γ-ImPyPyPy-β-Dp


(**2**) ImImPyPy-γ-ImHpPyPy-β-Dp


(**3**) ImImHpPy-γ-ImPyPyPy-β-Dp

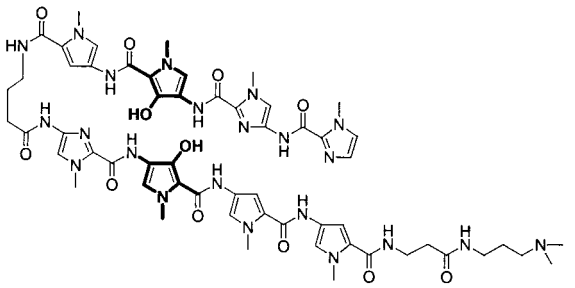

(**4**) ImImHpPy-γ-ImHpPyPy-β-Dp

Figure 4. Structures of the eight-ring hairpin hydroxypyrrole−imidazole−pyrrole polyamides **1**−**4**.

(6) For Hp/Py pairings see: (a) White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B. *Nature* **1998**, *391*, 468−471. (b) Kielkopf, C. L.; White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Science* **1998**, *282*, 111−115. (c) White, S.; Turner, J. M.; Szewczyk, J. W.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 260−261.

(7) Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1996**, *118*, 6141−6146.

(8) Momose, T.; Tamaka, T.; Yokota, T.; Nagamoto, N.; Yamada, K. *Chem. Pharm. Bull.* **1978**, *26*, 2224−2232.

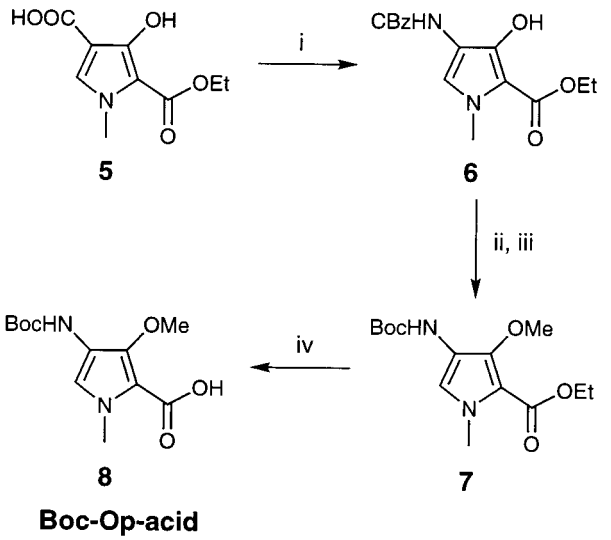


Figure 5. (i) (a) DPPA, TEA, CH_3CN, reflux 5 h; (b) benzyl alcohol, reflux 17 h. (ii) MeI, DMAP, K_2CO_3, acetone, 22 °C, 22 h. (iii) 10% Pd/C, H_2, Boc_2O, DIEA, DMF, 22 °C, 2 h. (iv) 1 M NaOH (aqueous), EtOH, 22 °C, 4 days. (CBz = benzyloxycarbonyl, Boc = *t*-butoxycarbonyl).

anhydride (H_2, Pd/C, DIEA, 2 h) to provide ethyl 4-[(*tert*-butoxycarbonyl)amino]-3-methoxy-1-methylpyrrole-2-carboxylate **7**. Finally, the 2-ethyl ester of the Boc-protected 3-methoxypyrrole **7** was hydrolyzed (1 M NaOH, 22 °C, 4 days), affording 4-[(*tert*-butoxycarbonyl)amino]-3-methoxy-1-methylpyrrole-2-carboxylic acid **8** (Boc-Op-acid) for use in solid-phase protocols.

Polyamide Synthesis. Four polyamide resins, ImImPyPy-γ-ImPyPyPy-β-Pam-resin, ImImPyPy-γ-ImOpPyPy-β-Pam-resin, ImImOpPy-γ-ImPyPyPy-β-Pam-resin, and ImImOpPy-γ-ImOpPyPy-β-Pam-resin, were synthesized in 16 steps from commercially available Boc-β-alanine-Pam-resin (1 g of resin, 0.2 mmol/g of substitution) using previously described Boc-chemistry machine-assissted protocols (Figure 6).[7] Hydroxypyrrole amino acid residues were introduced as orthogonally protected 3-methoxypyrrole derivatives. In machine-assisted synthetic protocols, Boc-Op acid **8** was incorporated by placing the amino acid and an equivalent of HBTU in a machine synthesis cartridge. Upon automated delivery of DMF and DIEA activation occurs. A single-step aminolysis of the resin ester linkage was used to cleave the polyamide from the solid support. A sample of resin was treated with dimethylaminoproplyamine (55 °C, 18 h), affording **1** and the methoxy-protected polyamides, **2-Me**, **3-Me**, and **4-Me**. Resin cleavage products were purified by reversed phase HPLC to give ImImPyPy-γ-ImPyPyPy-β-Dp **1**, ImImPyPy-γ-ImOpPyPy-β-Dp **2-Me**, ImImOpPy-γ-ImPyPyPy-β-Dp **3-Me**, and ImImOpPy-γ-ImOpPyPy-β-Dp **4-Me**. Polyamides containing 3-methoxypyrrole were subsequently deprotected by treatment with sodium thiophenoxide in DMF (100 °C, 2 h) and purified by reversed phase HPLC to provide ImImPyPy-γ-ImHpPyPy-β-Dp **2**, ImImHpPy-γ-ImPyPyPy-β-Dp **3**, and ImImHpPy-γ-ImHpPyPy-β-Dp **4**. The eight-ring hairpin polyamide products are soluble in aqueous solution at concentrations $\leq$10 mM.

Quantitative DNase I footprint titrations. To directly compare polyamide binding at all four target sites on the DNA (5′-TGG**T**CA-3′, 5′-TGG**A**CA-3′, 5′-TGG**C**CA-3′, and 5′-TGG**G**CA-3′), the plasmid pAU2 was constructed which contains these sites and an additional 5′-TGG**T**CA-3′ site to serve as an internal control (Figure 7). Quantitative DNase I footprint titrations[9] (Figures 8−10) were performed to determine the equilibrium association constants (K_a) of each eight-ring hairpin polyamide (Table 1). The *Eco*RI/*Pvu*II restriction fragment derived from plasmid pAU2 was bound by polyamide **1** with decreasing affinity for sites 5′-TGG**T**CA-3′ $\approx$ 5′-TGG**A**CA-3′ > 5′-TGG**C**CA-3′ > 5′-TGG**G**CA-3′. Polyamide **2** bound these sites with relative affinities 5′-TGG**A**CA-3′ > 5′-TGG**T**CA-3′ > 5′-TGG**C**CA-3′ $\approx$ 5′-TGG**G**CA-3′. Polyamide **3** bound 5′-TGG**T**CA-3′ > 5′-TGG**A**CA-3′ $\approx$ 5′-TGG**C**CA-3′ $\approx$ 5′-TGG**G**CA-3′. Polyamide **4** bound nonspecifically to DNA at concentrations $\leq$100 nM. These results indicate sensitivity to a single atomic substitution at the 3-position of the *N*-methyl pyrrole rings within each pair.

Discussion

The Py/Py pair. The polyamide ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp **1** (Py/Py pair bold-faced) binds opposite both T•A (5′-TGG**T**CA-3′) and A•T (5′-TGG**A**CA-3′) with 2-fold selectivity for T•A. However, placement opposite a G•C (5′-TGG**G**CA-3′) or C•G (5′-TGG**C**CA-3′) base pair decreases affinity by 53 and 17-fold, respectively. The discrimination of A•T and T•A from G•C and G•C base pairs by Py/Py is likely due to the exocyclic amine groups of guanine which present a steric hindrance to deep polyamide binding in the minor groove.[10] The Py/Py pair provides a baseline from which to evaluate hydroxypyrrole ring pairings.

The Hp/Py pair. Among the four ligands ImIm**Hp**Py-γ-Im**Py**PyPy-β-Dp **3** (Hp/Py pair bold-faced) bound the 5′-TGG**T**CA-3′ site with the highest specificity. The 5′-TGG**A**CA-3′, 5′-TGG**C**CA-3′, and 5′-TGG**G**CA-3′ mismatch sites are bound with $\geq$20-fold lower affinity, indicating that Hp/Py is the optimal ring pairing for recogntion of a T•A base pair. The polyamide ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp **2** (Py/Hp pair bold-faced) bound with 11-fold enhanced affinity opposite an A•T base pair relative to a T•A base pair. Placement of a Py/Hp pair opposite G•C or C•G disfavors binding by over 30-fold. The observed selectivity demonstrates that a Py/Hp pairing efficiently discriminates A•T from the other three Watson−Crick base pairs, thus forming the second hydroxypyrrole ring pairing which breaks the polyamide A•T/T•A degeneracy in the minor groove.[5d]

The Hp/Hp pair. As a control, the polyamide ImIm**Hp**Py-γ-Im**Hp**PyPy-β-Dp **4** (Hp/Hp pairing bold-faced) was examined. Polyamide **4** bound nonspecifically to DNA at concentrations $\geq$100 nM. The observed reduction in binding affinity by a potential steric clash of the 3-hydroxyl group with the floor of the minor groove supports the notion of shape selective recognition of an asymmetric cleft in the minor groove.[6b]

Conclusions. A pairing code has been developed to correlate DNA sequence with polyamide sequence composition. The results described herein demonstrate the sequence specificity of the four binary combinations of hydroxypyrrole and pyrrole (Table 2). The Hp/Py pair recognizes T•A but is disfavored when placed opposite A•T, C•G, and G•C. An Hp/Hp pair is disfavored with all four base pairs, breaking any potential degeneracy for recognition by preventing unlinked polyamide dimers from binding in certain slipped motifs.[11] These results

(9) (a) Brenowitz, M.; Senear, D. F.; Shea, M. A.; Ackers, G. K. *Methods Enzymol.* **1986**, *130*, 132−181. (b) Brenowitz, M.; Senear, D. F.; Shea, M. A.; Ackers, G. K. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8462−8466. (c) Senear, D. F.; Brenowitz, M.; Shea, M. A.; Ackers, G. K. *Biochemistry* **1986**, *25*, 7344−7354.

(10) Kopka, M. L.; Yoon, C.; Goodsell, D.; Pjura, P.; Dickerson, R. E. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1376−1380.

(11) (a) Trauger, J. W.; Baird, E. E.; Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1996**, *118*, 6160−6166. (b) Geierstanger, B. H.; Mrksich M.; Dervan, P. B.; Wemmer, D. E. *Nature Struct. Biol.* **1996**, *3*, 321−324.

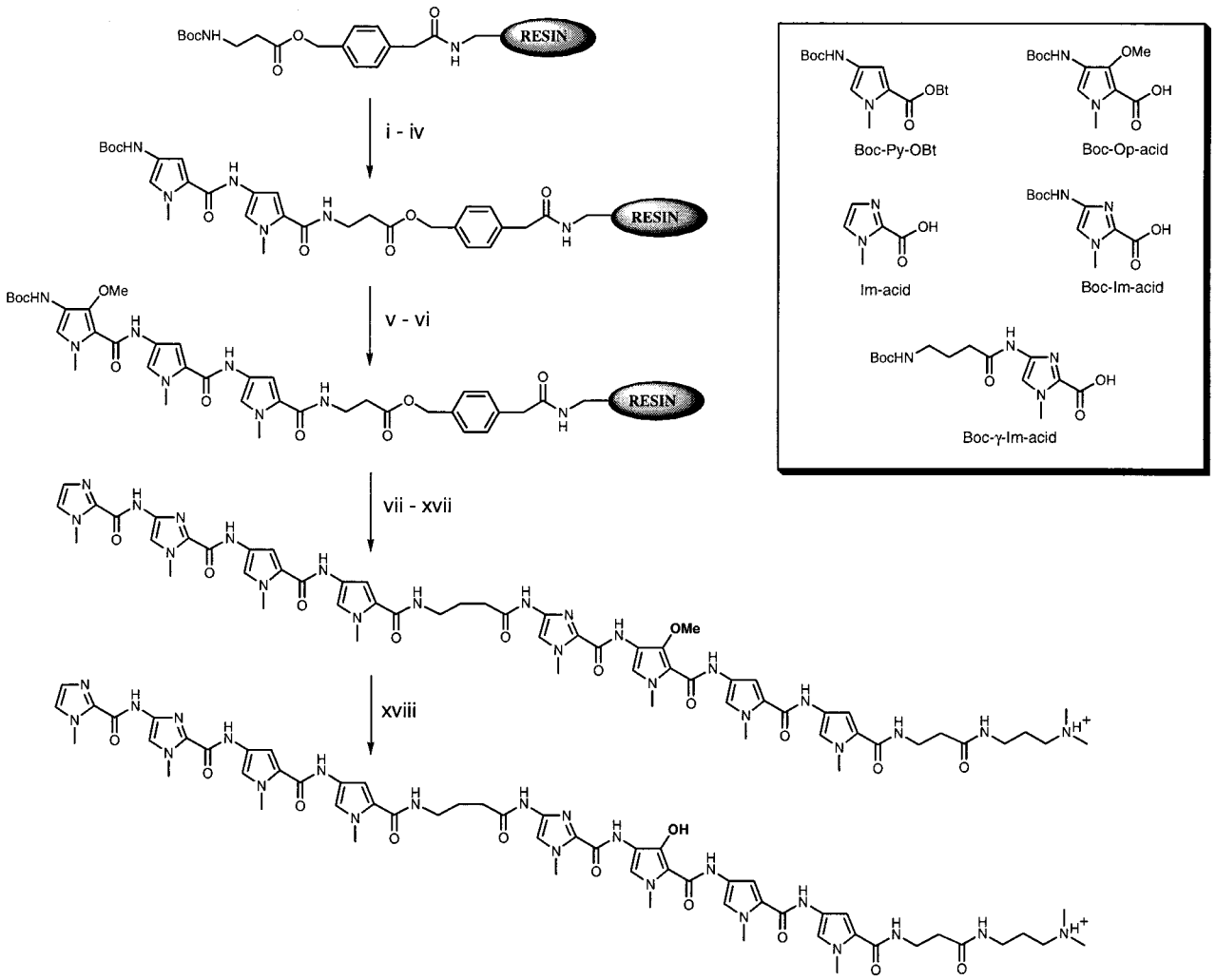


Figure 6. Solid-phase synthetic scheme for ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp **2** starting from commercially available Boc-β-Pam-resin: (i) 80% TFA/DCM, 0.4 M PhSH; (ii) Boc-Py-OBt, DIEA, DMF; (iii) 80% TFA/DCM, 0.4 M PhSH; (iv) Boc-Py-OBt, DIEA, DMF; (v) 80% TFA/DCM, 0.4 M PhSH; (vi) Boc-Op-acid, HBTU, DIEA, 45 min; (vii) 80% TFA/DCM, 0.4 M PhSH, 20 min; (viii) Boc-γ-Im-acid, HBTU, DIEA; (ix) 80% TFA/DCM, 0.4 M PhSH; (x) Boc-Py-OBt, DIEA, DMF; (xi) 80% TFA/DCM, 0.4 M PhSH; (xii) Boc-Py-OBt, DIEA, DMF; (xiii) 80% TFA/DCM, 0.4 M PhSH; (xiv) Boc-Im-acid, HBTU, DIEA; (xv) 80% TFA/DCM, 0.4 M PhSH; (xvi) Im-acid, HBTU, DIEA; (xvii) *N*,*N*-dimethylaminopropylamine, 55 °C; (xviii) sodium thiophenoxide, DMF, 100 °C, 2 h. (Inset) Monomers for solid-phase synthesis.

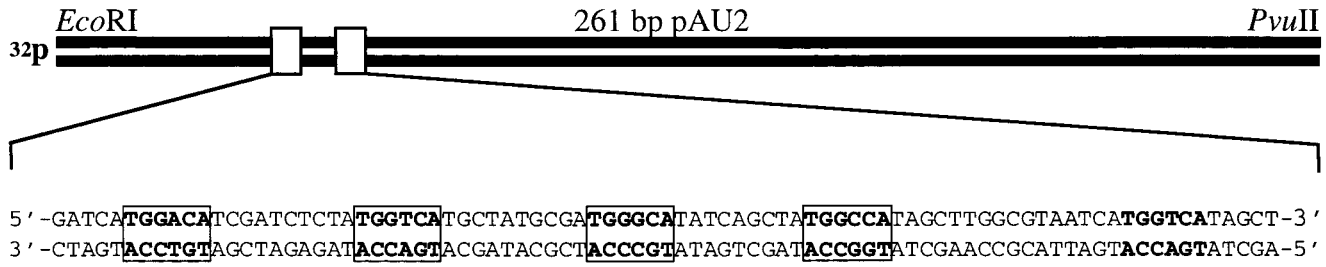


Figure 7. 261 base pair *Eco*RI/*Pvu*II restriction fragment derived from plasmid pAU2. The targeted six-base-pair recognition sites are shown in boxes.

will guide second generation design of hydroxypyrrole−imidazole−pyrrole polyamides.

Experimental Section

Materials. Dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt) and 2-(1H−benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Peptides International. *N*,*N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), acetic anhydride (Ac_2O), and 0.0002 M potassium cyanide/pyridine were purchased from Applied Biosystems. Boc-γ-aminobutyric acid and 0.6 mmol/gram Boc-β-alanine-(4-carboxamidomethyl)-benzyl-ester-copoly(styrene-divinylbenzene) resin (Boc-β-Pam-resin) were from NOVA Biochem. Dichloromethane (DCM) and triethylamine (TEA) were reagent grade from EM. Diphenylphosphorylazide (DPPA), methyl iodide, dimethylaminopyridine (DMAP), thiophenol (PhSH), and dimethylaminopropylamine were 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. 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

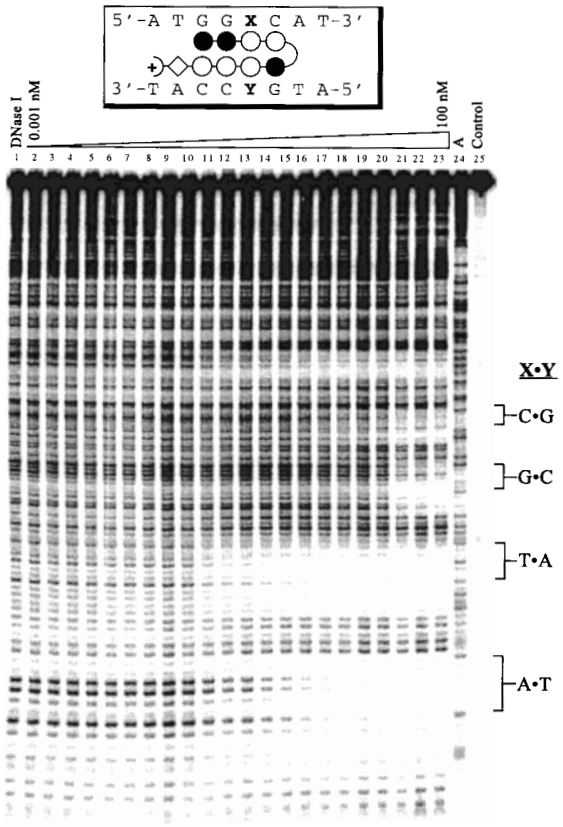


Figure 8. Quantitative DNase I footprint titration experiment with ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp **(1)** on the 3′-end-labeled 261-bp *Eco*RI/*Pvu*II restriction fragment derived from pAU2: lane 1, DNase I standard; lanes 2−23, 1.0, 2.0, 5.0, 10, 15, 25, 40, 65, 100, 150, 250, 400, and 650 pM and 1.0, 1.5, 2.5, 4.0, 6.5, 10, 20, 50, and 100 nM **1**, respectively; lane 24, A reaction; lane 25, intact DNA. Ball and stick models are shown with shaded and nonshaded circles indicating imidazole and pyrrole carboxamides, respectively; a nonshaded diamond represents β-alanine; the u-turn indicates γ-aminobutyric acid. The analyzed six-base-pair binding site locations are designated in brackets with their respective unique base pairs indicated.

sintered glass frit were made as described by Kent.[12] ^{1}H NMR spectra were recorded on a General Electric-QE NMR spectrometer at 300 MHz in DMSO-d_6, with chemical shifts reported in parts per million relative to residual solvent. UV spectra were measured in water on a Hewlett-Packard model 8452A diode array spectrophotometer. Matrix-assisted, laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was performed at the Protein and Peptide Microanalytical Facility at the California Institute of Technology. High-resolution FAB mass spectra were recorded at the Mass Spectroscopy Laboratory at the University of California Riverside. DNA sequencing was perfomed at the Sequence/Structure Analysis Facility (SAF) at the California Institute of Technology. HPLC analysis was performed on either a HP 1090M analytical HPLC or a Beckman Gold system using a Rainin C_{18}, Microsorb MV, 5 μm, 300 × 4.6 mm reversed phase column in 0.1% (wt/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min, gradient elution 1.25% acetonitrile/min. Preparatory reversed phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 × 100 mm, 100 μm C18 column equipped with a guard, 0.1% (wt/v) TFA, 0.25% acetonitrile/min. Water (18MΩ) was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered.

Monomer Synthesis. Ethyl 4-[(Benzyloxycarbonyl)amino]-3-hydroxy-1-methylpyrrole-2-carboxylate (6). Pyrrole acid **5** (125 g, 587 mmol) was slurried in 587 mL dry acetonitrile under argon. TEA (59.4 g, 587 mmol, 81.9 mL) was added, followed by DPPA (161.5 g, 587 mmol, 126 mL).[13] The mixture was refluxed for 5 h, followed by addition of benzyl alcohol (562 mL, 5.0 mol), and reflux continued for 17 h. The solution was allowed to cool, and the volatiles were removed in vacuo. The black tar was partitioned between 1:1 water: ethyl acetate, and the layers were separated. The aqueous phase was acidified to pH = 3 and extracted with ethyl acetate. Organic extracts were combined, washed with 0.1 N H_2SO_4, dried with $MgSO_4$, and concentrated in vacuo to yield a dark red tar. The residue was then triturated with diethyl ether (10 times), decanted, and dried over $MgSO_4$, and the solvent was removed in vacuo to give a crude solid. The residue was absorbed onto silca, loaded in 19:1 hexanes:ethyl acetate, and chromatagraphed with 4:1 hexanes:ethyl acetate resulting in a white solid. The solid was redissolved in acetone, filtered, and dried in vacuo to provide **6** as a white solid (98.7 g, 311 mmol, 53%). TLC on silica gel (4:1 hexanes/ethyl acetate) R_f 0.19; ^{1}H NMR (DMSO-d_6) δ 8.73 (s, 1H), 8.31 (s, 1H), 7.31 (m, 5H), 6.96 (s, 1H), 5.08 (s, 2H), 4.21 (q, 2H, J = 7.1 Hz), 3.66 (s, 3H), 1.25 (t, 3H, J = 7.1 Hz); FAB-MS *m/e* 319.163 (M + H 319.122 calcd for $C_{16}H_{18}N_2O_5$).

Ethyl 4-[(*tert*-Butoxycarbonyl)amino]-3-methoxy-1-methylpyrrole-2-carboxylate (7). Hydroxypyrrole **6** (98.7 g, 311 mmol) was dissolved in 810 mL acetone. Anhydrous K_2CO_3 (86.0 g, 622 mmol) was added and the solution allowed to stir for 15 min. Methyl iodide (44.1 g, 311 mmol, 19.4 mL) was then added followed by DMAP (3.5 g, 28.6 mmol). Progress of the reaction was monitored by TLC with additional methyl iodide (0.25 eq) added every 2 h, as needed, until 100% conversion. The solid K_2CO_3 was removed by filtration, and 1 L of water was added. Volatiles were removed in vacuo, and the solution was made acidic with addition of 1 N H_2SO_4 to pH = 3. The aqueous layer was extracted with diethyl ether. The ether extractions were combined, washed with 10% H_2SO_4, dried over $MgSO_4$, and concentrated in vacuo to give a white solid sufficiently pure to be used without further purification. The solid was redissolved in DMF (280 mL) and DIEA (60 g, 464 mmol, 81 mL). Boc anhydride (67.9 g, 311 mmol) and 10% Pd/C (3.5 g) were added, and the solution was stirred under hydrogen for 2.1 h. The slurry was filtered through Celite which was rinsed with methanol. Water (1 L) was added to the solution, and volatiles were removed in vacuo. The aqueous layer was extracted with diethyl ether. The organic layers were combined, washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo to provide **7** as a white solid (65.8 g, 221 mmol, 71%). TLC (4:1 hexanes/ethyl acetate) R_f 0.21; ^{1}H NMR (DMSO-d_6) δ 8.43 (s, 1H), 7.03 (s, 1H), 4.19 (q, 2H, J = 7.1 Hz), 3.70 (s, 3H), 3.67 (s, 3H), 1.42 (s, 9H), 1.26 (t, 3H, J = 7.1); FAB-MS *m/e* 299.161 (M + H 299.153 calcd for $C_{14}H_{22}N_2O_5$).

4-[(*tert*-Butoxycarbonyl)amino]-3-methoxy-1-methylpyrrole-2-carboxylic Acid (8). The ester **7** (65.8 g, 221 mmol) was dissolved in 221 mL of ethanol. NaOH (aqeuous, 2 M, 221 mL) was added and the solution stirred for 4 days at room temperature. Additional water (1 L) was added and the ethanol removed in vacuo. The aqueous solution was extracted with diethyl ether to remove unreacted starting material, acidified with sulfuric acid to pH = 2−3, and re-extracted with diethyl ether. The extractions were combined and dried over $MgSO_4$, and the solvent was removed in vacuo to yield **8** as a fine white powder (54.3 g, 201 mmol, 91%). ^{1}H NMR (DMSO-d_6) δ 12.14 (s, 1H), 8.37 (s, 1H), 6.98 (s, 1H), 3.69 (s, 3H), 3.66 (s, 3H), 1.42 (s, 9H); FAB-MS *m/e* 293.112 (M + Na 293.112 calcd for $C_{12}H_{17}N_2O_5Na$).

Solid-Phase Synthesis. Resin Substitution. Resin substitution can be calculated as $L_{new}(\text{mmol/g}) = L_{old}/(1 + L_{old}(W_{new} - W_{old}) \times 10^{-3})$, where L is the loading (mmol of amine per g of resin), and W is the weight (gmol^{-1}) of the growing polyamide attached to the resin.[14]

ImImPyPy-γ-ImOpPyPy-β-Dp (2-Me). ImImPyPy-γ-ImOpPyPy-β-Pam-resin was synthesized in a stepwise fashion by machine-assisted solid-phase methods from Boc-β-Pam-resin (0.66 mmol/g).[7] 3-Methoxypyrrole-Boc amino acid (Boc-Op acid) was incorporated by placing the amino acid (0.5 mmol) and HBTU (0.5 mmol) in a machine synthesis cartridge. Upon automated delivery of DMF (2 mL) and DIEA (1 mL), activation occurs, and coupling continues for 45 min.

(12) Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957−989.

(13) Ninomiya, K.; Shioiri, T.; Yamaoa, S. *Tetrahedron* **1974**, 30, 2151−2157.

(14) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513−520.

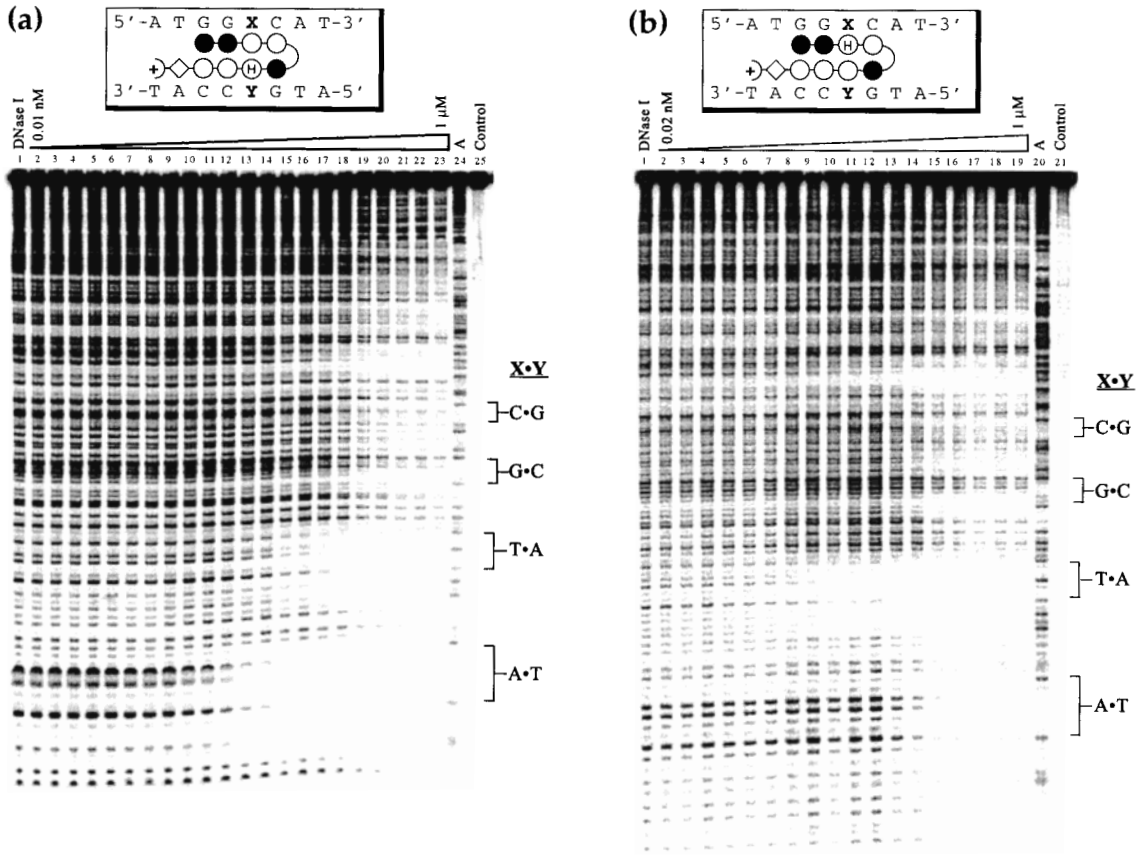


Figure 9. Quantitative DNase I footprint titration experiments with (a) ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp **(2)**, and (b) ImIm**Hp**Py-γ-Im**Py**PyPy-β-Dp **(3)** on the 3′-end-labeled 261-bp *Eco*RI/*Pvu*II restriction fragment derived from pAU2: (a) lane 1, DNase I standard; lanes 2−23, 10, 20, 50, 100, 150, 250, 400, and 650 pM and 1.0, 1.5, 2.5, 4.0, 6.5, 10, 15, 25, 40, 65, 100, 200, and 500 nM **2**, respectively; lane 24, A reaction; lane 25, intact DNA; (b) lane 1, DNase I standard; lanes 2−19, 20, 50, 100, 150, 250, 400, and 650 pM and 1.0, 1.5, 2.5, 4.0, 15, 40, 6.5, 100, 200, 500, and 1000 nM **3**, respectively; lane 20, A reaction; lane 21, intact DNA. Ball and stick models are shown with shaded and nonshaded circles indicating imidazole and pyrrole carboxamides, respectively; circles containing an H represent 3-hydroxypyrrole carboxamide; a nonshaded diamond represents β-alanine; the u-turn indicates γ-aminobutyric acid. The analyzed six-base-pair binding site locations are designated in brackets with their respective unique base pairs indicated.

A sample of ImImPyPy-γ-ImOpPyPy-β-Pam-resin (400 mg, 0.40 mmol/gram) was placed in a glass 20 mL peptide synthesis vessel, treated with neat dimethylaminopropylamine (2 mL), and heated at 55 °C with periodic agitation for 16 h. The reaction mixture was then filtered to remove resin, 0.1% (wt/v) aqueous TFA added (6 mL), and the resulting solution purified by reversed phase HPLC. ImImPyPy-γ-ImOpPyPy-β-Dp was recovered upon lyophilization of the appropriate fractions as a white powder (101 mg, 50% recovery). UV (H_2O) λ_{max} 246, 316 (66 000); ^{1}H NMR (DMSO-d_6) δ 10.47 (s, 1 H), 10.36 (s, 1 H), 9.94 (s, 1 H), 9.87 (s, 1 H), 9.78 (s, 1 H), 9.23 (s, 1 H), 8.99 (s, 1 H), 8.07 (m, 3 H), 7.57 (s, 1 H), 7.51 (s, 1 H), 7.46 (s, 1 H), 7.28 (d, 1 H), 7.27 (d, 1 H), 7.22 (d, 1 H), 7.17 (d, 1 H), 7.15 (m, 2 H), 7.08 (s, 1 H), 7.03 (d, 1 H), 6.88 (m, 2 H), 4.00 (s, 6 H), 3.96 (s, 3 H), 3.84 (s, 6 H), 3.82 (m, 3 H), 3.80 (s, 9 H), 3.39 (q, 2 H, J = 6.0 Hz), 3.17 (q, 2 H, J = 5.6 Hz), 3.14 (q, 2 H, J = 6.2 Hz), 3.03 (m, 2 H, J = 5.5 Hz), 2.74 (s, 3 H), 2.72 (s, 3 H), 2.33 (m, 4 H), 1.75 (m, 4H); MALDI-TOF-MS (monoisotopic), 1253.5 (1253.6 calcd for $C_{58}H_{72}N_{22}O_{11}$).

ImImPyPy-γ-ImHpPyPy-β-Dp (2). To remove the methoxy-protecting group, a sample of ImImPyPy-γ-ImOpPyPy-β-Dp (5 mg, 3.9 μmol) was treated with sodium thiophenoxide at 100 °C for 2 h. DMF (1.0 mL) and thiophenol (0.5 mL) were placed in a (13 × 100 mm) disposable Pyrex screw cap culture tube. A 60% dispersion of sodium hydride in mineral oil (100 mg) was slowly added. Upon completion of the sodium hydride addition, ImImPyPy-γ-ImOpPyPy-β-Dp (5 mg) dissolved in DMF (0.5 mL) was added. The solution was agitated and heated to 100 °C for 2 h. Upon completion of the reaction, the reaction mixture was cooled to 0 °C and 7 mL of a 20% (wt/v) aqueous solution of trifluoroacetic acid added. The aqueous layer was separated from the resulting biphasic solution. To remove the last trace of thiophenol, the aqueous layer was extracted three times with ethyl acetate. (Note that complete removal of thiophenol is essential because polyamides appear to degrade in the presence of trace thiophenol impurity.) The deprotected polyamide was purified by reversed phase preparatory HPLC. ImImPyPy-γ-ImHpPyPy-β-Dp is recovered upon lyophilization of the appropriate fractions as a white powder (3.2 mg, 66% recovery). UV (H_2O) λ_{max} 246, 312 (66 000); ^{1}H NMR (DMSO-d_6) δ 10.37 (s, 1 H), 10.34 (s, 1 H), 10.27 (s, 1 H), 9.95 (s, 1 H), 9.81 (s, 1 H), 9.77 (s, 1 H), 9.70 (s, 1 H), 9.12 (s, 1 H), 8.05 (br s, 3 H), 7.57 (s, 1 H), 7.51 (s, 1 H), 7.47 (s, 1 H), 7.30 (d, 1 H), 7.28 (s, 1 H), 7.24 (s, 1 H), 7.17 (d, 1 H), 7.15 (d, 2 H), 7.09 (s, 1 H), 7.00 (s, 1 H), 6.90 (s, 1 H), 6.86 (s, 1 H), 4.00 (s, 6 H), 3.95 (s, 3 H), 3.84 (s, 6 H), 3.79 (s, 3 H), 3.68 (s, 6 H), 3.40 (q, 2 H, J = 5.8 Hz), 3.20 (q, 2 H, J = 5.6 Hz), 3.13 (q, 2 H, J = 5.9 Hz), 3.00 (m, 2 H, J = 5.4 Hz), 2.74 (s, 3 H,), 2.72 (s, 3 H), 2.34 (m, 4 H), 1.76 (m, 4H); MALDI-TOF-MS (monoisotopic), 1239.6 (1239.6 calcd for $C_{57}H_{71}N_{22}O_{11}$).

ImImOpPy-γ-ImPyPyPy-β-Dp (3-Me). ImImOpPy-γ-ImPyPyPy-β-Pam-resin was synthesized in a stepwise fashion by machine-assisted solid-phase methods from Boc-β-Pam-resin (0.66 mmol/g) as described for ImImPyPy-γ-ImOpPyPy-β-Dp (**2-Me**). A sample of ImImOpPy-γ-ImPyPyPy-β-Pam-resin (400 mg, 0.40 mmol/gram) was placed in a glass 20 mL peptide synthesis vessel and treated with neat dimethylaminopropylamine (2 mL) and heated at 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. ImImOpPy-γ-ImPyPyPy-β-Dp was recovered upon lyophilization of the appropiate fractions as a white powder (97 mg, 49% recovery). UV (H_2O) λ_{max} 246, 316 (66 000); ^{1}H NMR (DMSO-

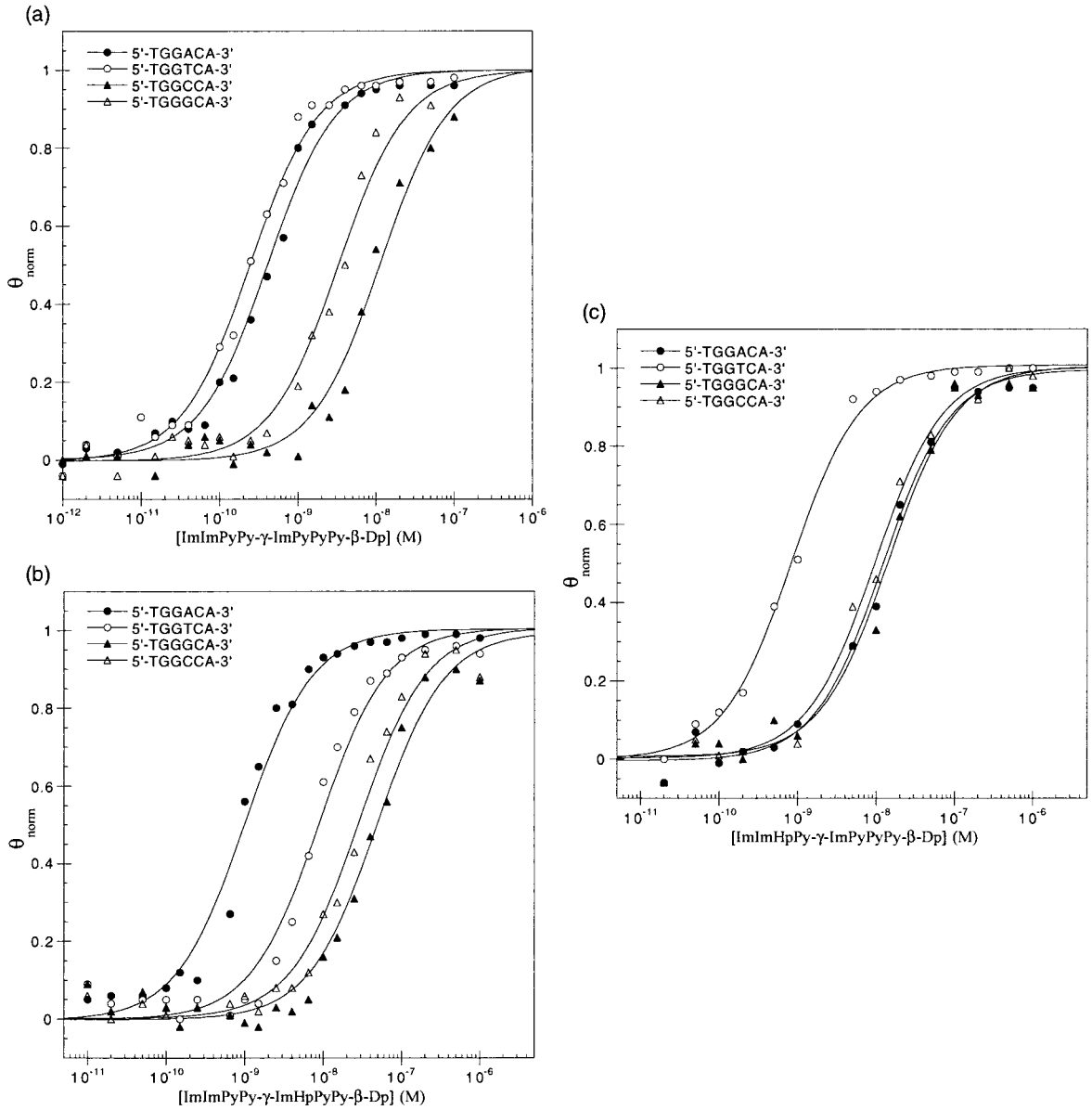


Figure 10. Binding isotherms from the DNase I quantitative footprint titration experiments for (a) ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp **1**, (b) ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp **2**, and (c) ImIm**Hp**Py-γ-Im**Py**PyPy-β-Dp **3**. θ_{norm} values were obtained according to published methods[2]. (●) data points for the 5′-TGG**A**CA-3′ site, (○) for 5′-TGG**T**CA-3′, (▲) for 5′-TGG**G**CA-3′, and (△) for 5′-TGG**C**CA-3′. The solid lines are best fit Langmuir binding titration isotherms obtained by a nonlinear least-squares algorithm.[3]

Table 1. Equilibrium Association Constants, K_a $(M^{-1})^{a,b}$

polyamide	pairing	5′-TGG**T**CA-3′	5′-TGG**A**CA-3′	5′-TGG**C**CA-3′	5′-TGG**G**CA-3′
ImIm**Py**Py-γ-Im**Py**PyPy-β-Dp	Py/Py	4.8 (±0.9) × 10^9	2.8 (±0.5) × 10^9	2.8 (±0.4) × 10^8	9.0 (±1.0) × 10^7
ImIm**Py**Py-γ-Im**Hp**PyPy-β-Dp	Py/Hp	9.8 (±0.9) × 10^7	1.1 (±0.2) × 10^9	3.3 (±1.0) × 10^7	2.5 (±0.3) × 10^7
ImIm**Hp**Py-γ-Im**Py**PyPy-β-Dp	Hp/Py	1.6 (±0.3) × 10^9	8.1 (±1.9) × 10^7	7.9 (±2.1) × 10^7	5.5 ±1.5) × 10^7

[a] Mean values reported were measured from at least three DNase I footprint titration experiments, with the standard deviation for each data set indicated in parentheses. [b] The binding reactions 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 $CaCl_2$.

d_6) δ 10.24 (s, 1 H), 10.14 (s, 1 H), 9.99 (s, 1 H), 9.94 (s, 1 H), 9.88 (s, 1 H), 9.4 (br s, 1 H), 9.25 (s, 1 H), 9.11 (s, 1 H), 8.05 (m, 3 H), 7.60 (s, 1 H), 7.46 (s, 1 H), 7.41 (s, 1 H), 7.23 (d, 1 H), 7.21 (d, 1 H), 7.19 (d, 1 H), 7.13 (m, 2 H), 7.11 (m, 2 H), 7.02 (d, 1 H), 6.83 (m, 2 H), 3.96 (s, 6 H), 3.90 (s, 3 H), 3.81 (m, 6 H), 3.79 (s, 3 H), 3.75 (d, 9 H), 3.33 (q, 2 H, J = 5.4 Hz), 3.15 (q, 2 H, J = 5.5 Hz), 3.08 (q, 2 H, J = 6.0 Hz), 2.96 (m, 2 H, J = 5.6 Hz), 2.70 (d, 6 H, J = 4.5 Hz), 2.32 (m, 4 H), 1.71 (m, 4 H); MALDI-TOF-MS (monoisotopic), 1253.5 (1253.6 calcd for $C_{58}H_{72}N_{22}O_{11}$).

ImImHpPy-γ-ImPyPyPy-β-Dp (3). A sample of ImImOpPy-γ-ImPyPyPy-β-Dp (5 mg, 3.9 μmol) was treated with sodium thiophenoxide and purified by reversed phase HPLC as described for ImImPyPy-γ-ImHpPyPy-β-Dp (**2**). ImImHpPy-γ-ImPyPyPy-β-Dp was

Table 2. Specificity of Py and Hp pairings[a]

pair	T•A	A•T	G•C	C•G
Py/Py	+	+	−	−
Hp/Py	+	−	−	−
Py/Hp	−	+	−	−
Hp/Hp	−	−	−	−

[a] Favored (+), disfavored (−).

recovered as a white powder upon lyophilization of the appropriate fractions (3.8 mg, 77% recovery). UV (H_2O) λ_{max} 246, 312 (66 000); ^{1}H NMR (DMSO-d_6) δ 10.34 (s, 1 H), 10.24 (s, 1 H), 10.00 (s, 2 H), 9.93 (s, 1 H), 9.87 (s, 1 H), 9.83 (s, 1 H), 9.4 (br s, 1 H), 9.04 (s, 1 H), 8.03 (m, 3 H), 7.58 (s, 1 H), 7.44 (s, 1 H), 7.42 (s, 1 H), 7.23 (s, 1 H), 7.20 (m, 3 H), 7.12 (m, 2 H), 7.05 (d, 1 H), 7.02 (d, 1 H), 6.83 (s, 1 H), 6.79 (s, 1 H), 3.96 (s, 6 H), 3.90 (s, 3 H), 3.81 (s, 6 H), 3.79 (s, 3 H), 3.75 (d, 6 H), 3.33 (q, 2 H, J = 5.4 Hz), 3.14 (q, 2 H, J = 5.4 Hz), 3.08 (q, 2 H, J = 6.1 Hz), 2.99 (m, 2 H, J = 5.4 Hz), 2.69 (d, 6 H, J = 4.2 Hz), 2.31 (m, 4 H), 1.72 (m, 4 H); MALDI-TOF-MS (monoisotopic), 1239.6 (1239.6 calcd for $C_{57}H_{71}N_{22}O_{11}$).

ImImOpPy-γ-ImOpPyPy-β-Dp (4-Me). ImImOpPy-γ-ImOpPyPy-β-Pam-resin was synthesized in a stepwise fashion by machine-assisted solid-phase methods from Boc-β-Pam-resin (0.66 mmol/g) as described for ImImPyPy-γ-ImOpPyPy-β-Dp (**2Me**). A sample of ImImOpPy-γ-ImOpPyPy-β-Pam-resin (400 mg, 0.40 mmol/gram) was placed in a glass 20 mL peptide synthesis vessel and treated with neat dimethylaminopropylamine (2 mL) and heated at 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. ImImOpPy-γ-ImOpPyPy-β-Dp was recovered upon lyophilization of the appropriate fractions as a white powder (97 mg, 49% recovery). UV (H_2O) λ_{max} 246, 316 (66 000); ^{1}H NMR (DMSO-d_6) δ 10.46 (s, 1 H), 10.00 (s, 1 H), 9.88 (s, 1 H), 9.30 (s, 1 H), 9.23 (s, 1 H), 9.13 (s, 1 H), 8.99 (s, 1 H), 8.06 (m, 3 H), 7.62 (s, 1 H), 7.50 (s, 1 H), 7.46 (s, 1 H), 7.28 (s, 1 H), 7.24 (d, 1 H), 7.22 (s, 1 H), 7.16 (s, 1 H), 7.15 (d, 2 H), 7.08 (d, 1 H), 7.03 (d, 1 H), 6.92 (s, 1H), 6.86 (s. 1 h), 3.99 (s, 9 H), 3.96 (s, 3 H), 3.85 (s, 6 H), 3.82 (s, 3 H), 3.79 (s, 9 H), 3.15 (q, 2 H, J = 7.5 Hz), 3.05 (q, 2 H, J = 6.2 Hz), 2.98 (q, 2 H, J = 4.2 Hz), 2.84 (m, 2 H, J = 4.2 Hz), 2.74 (s, 3 H), 2.73 (s, 3H), 2.34 (m, 4 H), 1.75 (m, 4 H); MALDI-TOF-MS (monoisotopic), 1282.61 (1282.59 calcd for $C_{59}H_{74}N_{22}O_{12}$).

ImImHpPy-γ-ImHpPyPy-β-Dp (4). A sample of ImImOpPy-γ-ImOpPyPy-β-Dp (5 mg, 3.9 μmol) was treated with sodium thiophenoxide and purified by reversed phase HPLC as described for ImImPyPy-γ-ImHpPyPy-β-Dp **2**. ImImHpPy-γ-ImHpPyPy-β-Dp is recovered as a white powder upon lyophilization of the appropriate fractions (3.8 mg, 77% recovery). UV (H_2O) λ_{max} 246, 312 (66 000); ^{1}H NMR (DMSO-d_6) δ 10.34 (s, 1 H), 10.24 (s, 1 H), 10.00 (s, 2 H), 9.93 (s, 1 H), 9.87 (s, 1 H), 9.83 (s, 1 H), 9.4 (br s, 1 H), 9.04 (s, 1 H), 8.03 (m, 3 H), 7.58 (s, 1 H), 7.44 (s, 1 H), 7.42 (s, 1 H), 7.23 (s, 1 H), 7.20 (m, 3 H), 7.12 (m, 2 H), 7.05 (d, 1 H), 7.02 (d, 1 H), 6.83 (s, 1 H), 6.79 (s, 1 H), 3.96 (s, 6 H), 3.90 (s, 3 H), 3.81 (s, 6 H), 3.79 (s, 3 H), 3.75 (d, 6 H), 3.33 (q, 2 H, J = 5.4 Hz), 3.14 (q, 2 H, J = 5.4 Hz), 3.08 (q, 2 H, J = 6.1 Hz), 2.99 (m, 2 H, J = 5.4 Hz), 2.69 (d, 6 H, J = 4.2 Hz), 2.31 (m, 4 H), 1.72 (m, 4 H); MALDI-TOF-MS (monoisotopic), 1254.57 (1254.55 calcd for $C_{57}H_{70}N_{22}O_{12}$).

DNA Reagents and Materials. Enzymes were purchased from Boehringer-Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P] triphosphates were obtained from Amersham. Calf thymus DNA (sonicated, deproteinized) and DNase I (7500 u/mL, FPLC pure) were obtained from Pharmacia. Tris-HCl, dithiothreitol (DTT), RNase-free water (used for all footprinting reactions), and 0.5 M EDTA were purchased from US Biochemicals. Xgal and IPTG were from ICN Biomedicals. Ampicillin trihydrate was acquired from Sigma. Ethanol (200 proof) was purchased from Equistar. Calcium chloride, potassium chloride, and magnesium chloride were from Fluka. Formamide and premixed tris-borate-EDTA (Gel-Mate) were from Gibco. Bromophenol blue was from Acros. All reagents were used without further purification. DNA manipulations were performed according to standard protocols.[15]

Construction of Plasmid DNA. The plasmid pAU2 was constructed by hybridization of the inserts, 5′-GATCATGGACATCGATCTC-TATGGTCATGCTATGCGATGGGCATATCAGCGTATGGCCAT-3′ and 5′-AGCTATGGCCATACGCTGATATGCCCATCGCATAGC-ATGACCATAGAGATCGATGTCCAT-3′. The hybridized insert was ligated into linear pUC19 *Bam*HI/*Hin*DIII plasmid using T4 DNA ligase. The resulting construct was transformed into Top10F′ OneShot competent cells from Invitrogen. Ampicillin-resistant white colonies were selected from 25 mL Luria-Bertani (LB) medium agar plates (containing 50 μg/mL ampicillin and treated with XGAL and IPTG). Large-scale plasmid purification was performed with Qiagen Maxi purification kits. Dideoxy sequencing was used to verify the presence of the insert. The concentration of the resulting plamid was determined at 260 nm using the relationship of 1 OD unit = 50 μg/mL.

Preparation of 3′-End-Labeled Restriction Fragments. The plasmid pAU2 was linearized with *Pvu*II and *Eco*RI restriction endonucleases and then treated with Klenow fragment, deoxyadenosine 5′-[α-^{32}P]triphosphate and thymidine 5′-[α-^{32}P]triphosphate for 3′-end-labeling. The labeled fragments were loaded onto a 7% non-denaturing preparatory polyacrylamide gel (5% cross-link), and the desired 261 bp fragment was visualized by autoradiography and isolated. Adenosine sequencing reaction was performed according to published methods.[16]

Quantitative DNase I Footprint Titrations.[9] All reactions were carried out in a volume of 400 μL. We note explicitly that no carrier DNA was used in these reactions until after DNase I cleavage. A polyamide stock solution (or water for reference and intact 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 25 kcpm 3′-radiolabeled DNA. The solutions were allowed to equilibrate for 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 1.13 u/mL) and allowed to proceed for 7 min at 22 °C. The reactions were stopped by adding 50 μL of a solution containing 2.25M NaCl, 150 mM EDTA, 0.6 mg/mL glycogen, and 30 μM base-pair calf thymus DNA, and then ethanol-precipitated (2.1 volumes). The cleavage products were washed with 75% ethanol, resuspended in 16 μL RNase-free water, lyophilized to dryness, and then resuspended in 100 mM Tris-borate-EDTA/80% formamide loading buffer (with bromophenol blue as dye), denatured at 90 °C for 10 min, and loaded directly onto a pre-run 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1 h. The gels were dried in vacuo at 80 °C and then exposed to a storage phosphor screen (Molecular Dynamics). Equilibrium association constants were determined as previously described.[3]

Acknowledgment. We are grateful to the National Institutes of Health (GM-27681) for research support, National Institutes of Health for a research service award to J.W.S and S.W., J. Edward Richter for an undergraduate fellowship to J.M.T., the National Science Foundation for a predoctoral fellowship to A.R.U., and the Howard Hughes Medical Institute for a predoctoral fellowship to E.E.B. We thank G. M. Hathaway for MALDI-TOF mass spectrometry.

JA9930667

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

(16) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823−7830.