Articles

Hydroxybenzamide/Pyrrole Pair Distinguishes T·A from A·T Base Pairs in the Minor Groove of DNA

Ulf Ellervik, Clay C. C. Wang, and Peter B. Dervan*

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

Received May 16, 2000

Abstract: A new aromatic pair, 2-hydroxy-6-methoxybenzamide/1-methylpyrrole at the terminal position of hairpin polyamides has been designed for distinguishing T·A from A·T base pairs and both from G·C/C·G in the minor groove of DNA. Four eight-ring hairpin polyamides with benzamide (Bz), 2-hydroxybenzamide (Hb-**1**), 2-hydroxy-6-methylbenzamide (Hb-**2**), and 2-hydroxy-6-methoxybenzamide (Hb-**3**) at the N-terminal position were synthesized. The equilibrium association constants (K_a) were determined at four DNA sites which differ at a single common position, 5′-T**N**TACA-3′ (N = T, A, G, C). Quantitative DNase I footprint titration experiments reveal that (Hb-**3**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**4**) bound the sequences 5′-T**T**TACA-3′ and 5′-T**A**TACA-3′ with high affinity; $K_a = 2.6 \times 10^{10}$ M^{-1} and $K_a = 8.4 \times 10^{9}$ M^{-1}, respectively, a 3-fold specificity for T vs A was found. Importantly, the sequences 5′-T**G**TACA-3′ and 5′-T**C**TACA-3′ are bound with 50 and 200 times lower affinity, revealing an overall specificity of Hb-**3**/Py of T > A ≫ G > C. These results expand the repertoire of sequences targetable by hairpin polyamides.

Introduction

The ability to distinguish T·A from A·T base pairs by synthetic ligands which bind in the minor groove of DNA is an essential milestone for targeting specific A/T rich sequences within promoters for gene regulation studies.[1,2] We have shown that hydroxypyrrole/pyrrole (Hp/Py) pairs when placed *internal* in a hairpin distinguish T·A from A·T.[2] However, this has not been demonstrated for the terminal position in hairpin polyamides where "end effects" would potentially alter molecular interactions necessary for specificity.[3] For example, the substituent at the 4-position of the N-terminal five-membered ring is flanked in the minor groove by β-alanine at the C-terminus. To evaluate hairpin polyamides capable of distinguishing T·A/A·T base pairs at the terminal position, a series of hairpins with 3-hydroxy-1-methylpyrrole substituted at the 4-position by H, formamide, acetamide (Hp-**1**, Hp-**2**, Hp-**3**) were synthesized (Figure 1). From quantitative footprint titrations we find that polyamides with a terminal pyrrole containing a 3-hydroxyl and (i) a hydrogen in the 4-position showed no selectivity for T·A over A·T, (ii) an acetamide at the 4-position showed 3-fold specificity for T·A over A·T but diminished affinity, and (iii) a formamide at the 4-position showed higher affinity but no selectivity (see Supporting Information). One could imagine that unfavorable conformations at the terminal position due to rotation of the terminal Hp to form an intramolecular hydrogen bond between the 3-hydroxyl hydrogen and the carboxamide oxygen would orient the key hydroxyl recognition element away from the minor groove (Figure 2). This suggests that Hp/Py pairs may be limited within the hairpin motif to *internal* positions for T·A discrimination and there is a need for the design of new aromatic pairs at the terminal position for distinguishing T·A from A·T.

During the early development of minor groove binders, it was shown that six-membered aromatic rings such as 2-pyridine, as well as the five-membered ring imidazole Im, when paired with Py could target G·C base pairs.[4] The question arises whether 2-hydroxybenzene (Hb) at the N-terminal position of hairpin polyamides could act as a mimic of the 2-hydroxypyrrole (Hp) with improved affinity and specificity for T. We describe here a series of eight-ring polyamide hairpins with the general sequence **X**PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp and the N-terminal position X = benzamide (Bz), 2-hydroxybenzamide (Hb-**1**), 2-hydroxy-6-methylbenzamide (Hb-**2**), and 2-hydroxy-6-methoxybenzamide (Hb-**3**). Since Py is in the eighth position, the terminal pairs in the hairpin conformation are Hb-**1**/Py, Hb-**2**/Py, and Hb-**3**/Py (Figure 3). For controls, the parent benzamide without the hydroxy is included. Four eight-ring hairpin polyamides differing in the terminal position were synthesized by solid-phase methods (Figure 4). The plasmid pCW15 was designed to contain four six-base-pair recognition sites 5-T-**N**TACA-3′ (where N = T,A,G,C) which differ at a single common position allowing for comparison of the affinities

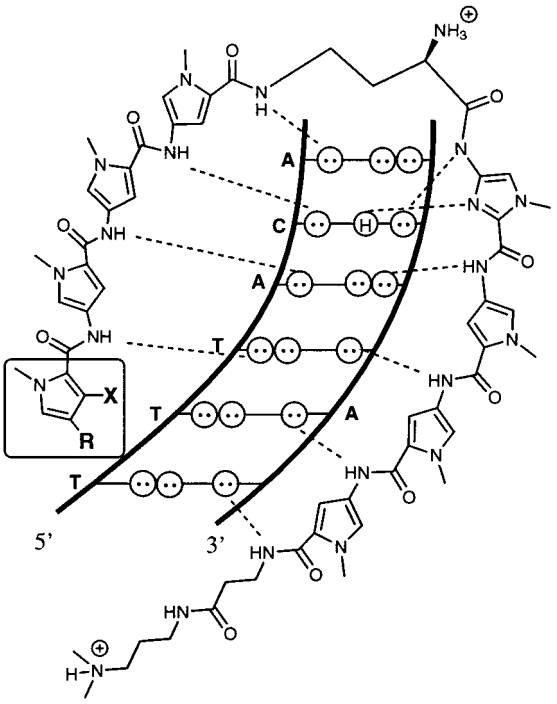
(1) (a) Dervan, P. B.; Bürli, R. W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 688−693. (b) Praseuth, D.; Guieysse, A. L.; Helene, C. *Biochim. Biophys. Acta* **1999**, *1489*, 181−206. (c) Bailly, C.; Chaires, J. B. *Bioconjugate Chem.* **1998**, *9*, 513−538.

(2) (a) Kielkopf, C. L.; White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *Science (Washington, D.C.)* **1998**, *282*, 111−115. (b) Kielkopf, C. L.; Bremer, R. B.; White, S.; Szewczyk, J. W.; Turner, J. M.; Baird, E. E.; Dervan, P. B.; Rees, D. C. *J. Mol. Biol.* **2000**, *295*, 557−567.

(3) Hawkins, C. A.; de Clairac, R. P.; Dominey, R. N.; Baird, E. E.; White, S.; Dervan, P. B.; Wemmer, D. E. *J. Am. Chem. Soc.* **2000**, *122*, 5235−5243.

(4) Wade, W. S.; Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 8783−8794.

10.1021/ja001692u CCC: $19.00
Published on Web 09/15/2000



X	R =		
H	Py-**1**	Py-**2**	Py-**3**
OH	Hp-**1**	Hp-**2**	Hp-**3**

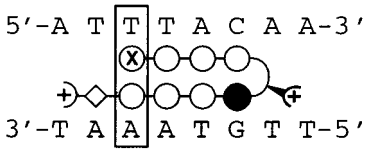


Figure 1. Hydrogen-bonding model of the polyamide/DNA complex between eight-ring hairpin polyamides XPyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (X = substituted Py or Hp) with 5′T**T**TACA-3′ sites. A circle with two dots represents the lone pairs of N3 of purines and O2 of cytosine. Two touching 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.

between different terminal pairs and the four Watson Crick base pairs in the minor groove of DNA (Figure 5).

Results

Polyamide Synthesis. Boc-PyPyPy-(R)$^{Fmoc}\gamma$-ImPyPyPy-β-PAM-resin was synthesized in a stepwise manner from Boc-β-alanine-PAM resin (0.55 mmol/g) using solid-phase methodology[5] in 16 steps (Figure 6). Sterically hindered acids such as Hb-**2** and Hb-**3** were coupled as acid chlorides. A sample of the resin was then cleaved by aminolysis with ((dimethyl)-amino)propylamine (55 °C, 16 h) and purified by reversed-phase HPLC to provide BzPyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**1**). Methyl-protected polyamides **2**−**4** were subsequently deprotected by treatment with sodium ethanethiolate in DMF (100 °C, 1 h) and purified by reversed-phase HPLC to afford (Hb-**1**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**2**), (Hb-**2**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**3**), and (Hb-**3**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**4**) (Figure 4).

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

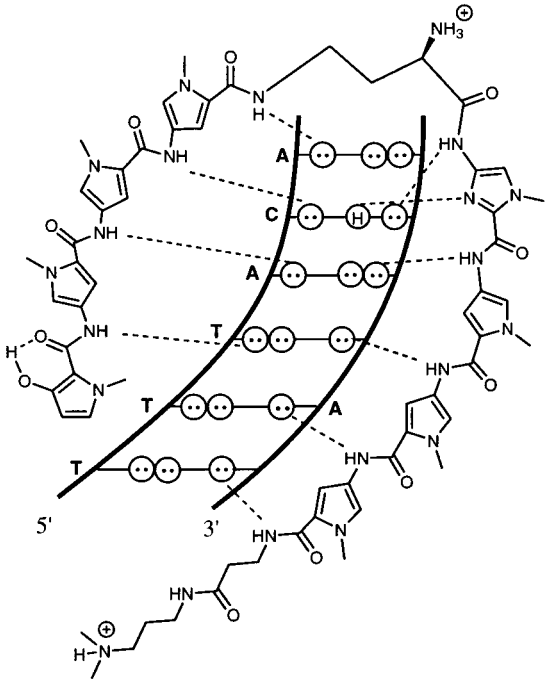


Figure 2. Binding model for the complex formed between polyamide (Hp-**1**)PyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp and a 5′-TTTACA-3′ sequence.

Quantitative DNase I Footprinting Titrations. Quantitative DNase I footprint titrations on the 3′-^{32}P-end-labeled 284 bp pCW15 *Eco*RI/*Pvu*II restriction fragment (10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.0, 22 °C) (Figure 7) reveal that BzPyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**1**) binds the 5′-T**T**TACA-3′and 5′-T**A**TACA-3′ sites with comparable equilibration association constants, $K_a = 1.9 \times 10^{11}$ M^{-1} and $K_a = 1.2 \times 10^{11}$ M^{-1}, respectively. Polyamide **1** binds the two sites 5′-T**G**TACA-3′ and 5′-T**C**TACA-3′ with 5- and 10-fold lower affinity, respectively, revealing that the Bz/Py pair is specific for both T·A and A·T over G·C and C·G.

Three polyamides containing 2-hydroxybenzamides at the N-terminal position, (Hb-**1**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**2**), (Hb-**2**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**3**), (Hb-**3**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**4**), were tested for their ability to discriminate 5′-T**T**TACA-3′ vs 5′-T**A**TACA-3′. (Hb-**1**)-PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**2**), containing a single hydroxyl, bound both 5′-T**T**TACA-3′ and 5′-T**A**TACA-3′ with the same affinity ($K_a = 1.9 \times 10^{10}$ M^{-1}). (Table 1)

(Hb-**2**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**3**), with a 2-hydroxyl and a 6-methyl substitution also bound the two sites with equal affinity. Discrimination of 5′-T**T**TACA-3′ from 5′-T**A**TACA-3′ was achieved by replacing the 6-methyl group in polyamide **5** with 6-methoxy. (Hb-**3**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**4**) bound 5′-T**T**TACA-3′ with an affinity of $K_a = 1.9 \times 10^{10}$ M^{-1} and 5′-T**A**TACA-3′ with 3-fold lower affinity. Importantly, polyamide **4** bound to 5′-T**G**TACA-3′ and to 5′-T**C**TACA-3′ with 55 and 200 times lower affinity, respectively.

Discussion

BzPyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (**1**) demonstrates that a simple benzene ring is able to pair with a 1-methylpyrrole ring (Bz/Py) to distinguish A·T/T·A base pairs from G·C/C·G base pairs in the minor groove of DNA. The Bz/Py pair is a higher affinity binder than the Py/Py pair at the terminal position. Interestingly, the Hb-**1**/Py pair with a single hydroxyl group did not discriminate T from A. The hydroxyl group is likely in

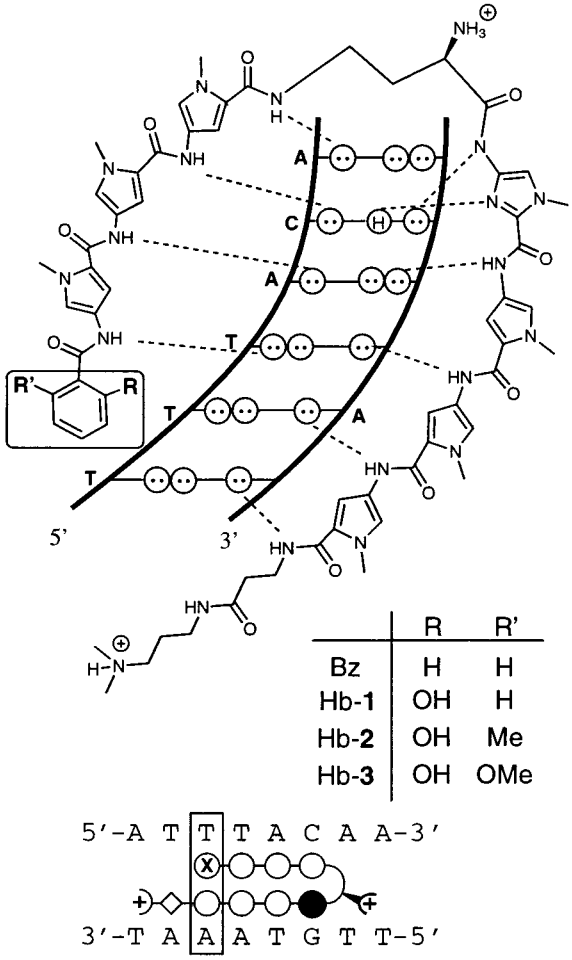


Figure 3. Hydrogen-bonding model of the hairpin polyamide/DNA containing substituted benzamides with the 5′T**T**TACA-site. A circle with two dots represents the lone pairs of N3 of purines and O2 of cytosine. Two touching circles with dots represent the two lone pairs of the O2 of thymine. Circles containing an H represent the exocyclic amino hydrogen of guanine. Putative hydrogen bonds are illustrated by dotted lines.

the wrong conformation, oriented away from the floor of the minor groove and forming a hydrogen bond with the proximal carbonyl group. To force the hydroxyl group toward the minor groove of the DNA by an unfavorable intramolecular steric interaction, two 2-hydroxybenzamides substituted at the 6 position with methyl and methoxy were examined. Quantitative footprint titration reveals that T versus A discrimination can be achieved with the 6-methoxy but not the 6-methyl derivative. These results suggest that the 6-methyl substituent projecting to the floor of the helix is not large enough to prevent the hydroxyl group from hydrogen bonding to the carbonyl group. Presumably, the steric bulk of the 6-methoxy group favors the rotamer with the hydroxyl group oriented toward the minor groove (Figure 8). Polyamide **4** with the Hb-**3**/Py pair binds 5′-T**T**TACA-3′ and 5′-T**A**TACA-3′ with binding affinities of $K_a = 2.6 \times 10^{10}$ M^{-1} and $K_a = 8.4 \times 10^{9}$ M^{-1}, respectively. Although the 3-fold T versus A selectivity is similar to the Hp/Py pair, the overall affinity is significantly higher, and most importantly, the selectivity of A/T vs G/C improved. The new hydroxybenzamide/pyrrole pair (Hb/Py) is a step toward hairpin polyamides capable of differentiating pure A·T tract sequences important in gene regulation studies

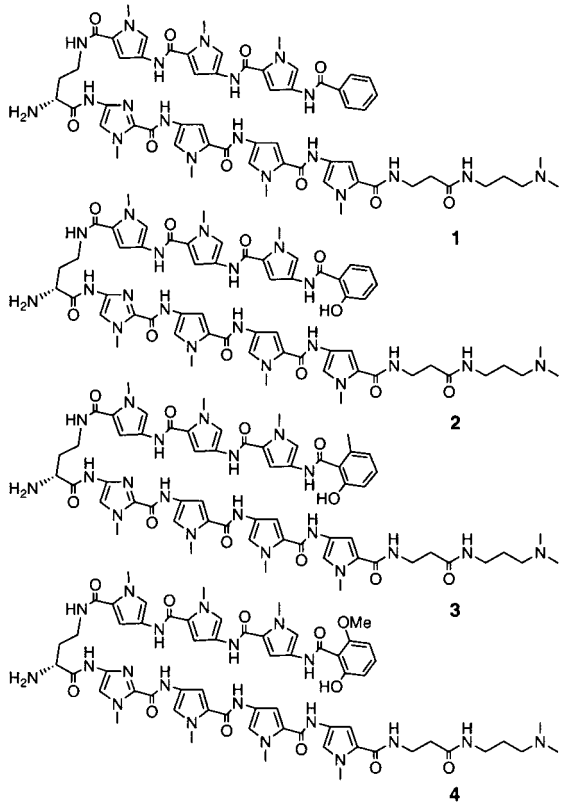


Figure 4. Structures of polyamides BzPyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**1**), (Hb-**1**)PyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**2**), (Hb-**2**)PyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**3**), (Hb-**3**)PyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**4**).

Experimental Section

Boc-β-alanine-(-4-carboxamidomethyl)-benzyl-ester-copoly(styrene-divinylbenzene) resin (Boc-β-PAM-Resin), *N*,*N*′-Dicyclohexylcarbodiimide (DCC), *N*-Hydroxybenzotriazole (HOBt), 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate (HBTU), and Boc-β-alanine were purchased from Peptides International. *N*,*N*-diisopropylethylamine (DIEA) and *N*,*N*-dimethylformamide (DMF) were purchased from Applied Biosystems. DMF was distilled under reduced pressure prior to synthesis. (*R*)-2-Fmoc-4-Boc-diaminobutyric acid was from Bachem, dichloromethane (DCM) was reagent grade from EM, thiophenol (PhSH) and dimethylaminopropylamine were from Aldrich, and trifluoroacetic acid (TFA) was from Halocarbon. ^{1}H NMR were recorded on a Varian Mercury 300 instrument. Chemical shifts are reported in parts-per-million downfield from the signal for Me_4Si, with reference to the solvent residual signal. UV spectra were measured on a Hewlett-Packard model 8452A diode array spectrophotometer. Matrix-assisted, laser desorption/ionization time-of-flight mass spectrometry was carried out at the Protein and Peptide Microanalytical Facility at the California Institute of Technology. HPLC analysis was performed on a Beckman Gold system using a RAINEN C_{18}, Microsorb MV, 5-μm, 300 × 4.6 mm reversed-phase column in 0.1% (w/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min, gradient elution 1.25% acetonitrile/min. Preparatory HPLC was carried out on a Beckman HPLC using a Waters DeltaPak 25 × 100 mm, 100-μm C_{18} column, 0.1% (w/v) TFA, 0.25% acetonitrile/min. 18MΩ water was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2-μm filtered. Reagent-grade chemicals were used unless otherwise stated.

Boc-PyPyPy-(R)$^{Fmoc}\gamma$-ImPyPyPy-β-PAM-resin. Boc-PyPyPy-(R)$^{F}{}_{moc}\gamma$-ImPyPyPy-β-PAM-resin (0.335 mmol/g) was synthesized in a stepwise fashion from 0.55 mmol/g Boc-β-PAM-resin by manual solid-phase methods.[5] (*R*)-2-Fmoc-4-Boc-diaminobutyric acid was incorporated as previously described for Boc-γ-aminobutyric acid.[5,6]

(6) Herman, D. M.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1998**, *120,* 1382−1391.

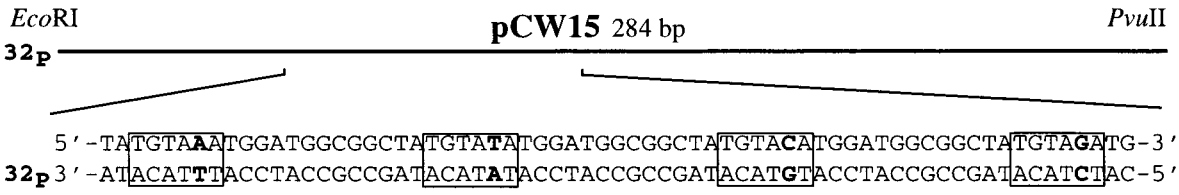


Figure 5. Two hundred eighty-four base pair *Eco*RI/*Pvu*II restriction fragment derived from plasmid pCW15. The targeted six-base-pair recognition sites are shown in boxes.

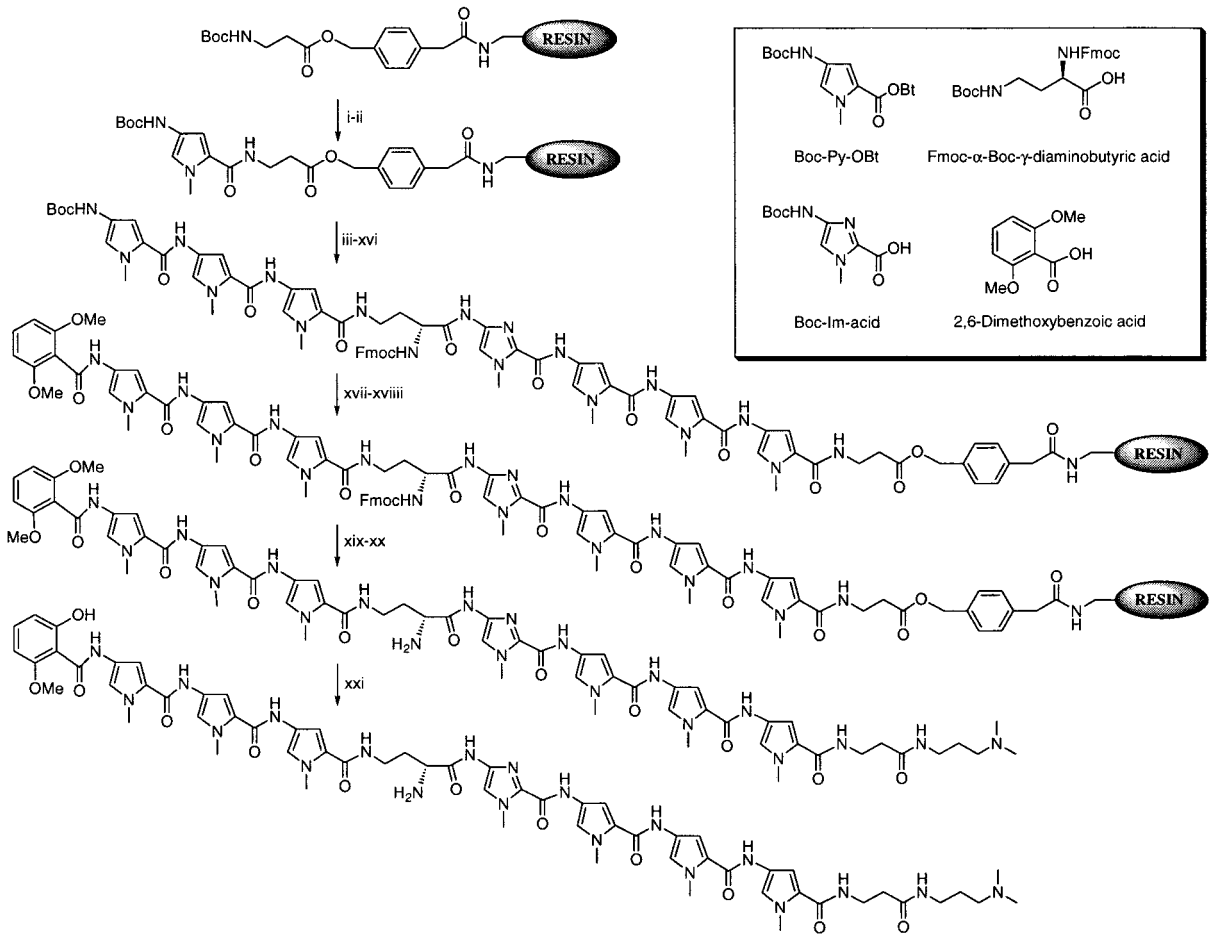


Figure 6. Solid-phase synthetic scheme for (Hb-**3**)PyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**4**) starting from commercially available Boc-β-PAM-resin: (i) 80% TFA/DCM, 0.4 M PhSH; (ii) Boc-Py-OBt, DIEA, DMF; (iii) 80% TFA/DCM, 0.4 M PhSH; (iv) Boc-Py-OBt, DIEA, DMF; (v) 80% TFA/DCM, 0.4 M PhSH; (vi) Boc-Py-OBt, DIEA, DMF; (vii) 80% TFA/DCM, 0.4 M PhSH; (viii) Boc-Im-acid, DCC, HOBt, DIEA, DMF; (ix) 80% TFA/DCM, 0.4 M PhSH; (x) Fmoc-α-Boc-γ-diaminobutyric acid, HBTU, 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-Py-OBt, DIEA, DMF; (xv) 80% TFA/DCM, 0.4 M PhSH; (xvi) Boc-Py-OBt, DIEA, DMF; (xvii) 80% TFA/DCM, 0.4 M PhSH; (xviii) 2,6-Dimethoxybenzoic acid, DMF, DCM, oxalyl chloride, DIEA, r.t. 3 h; (xix) Piperidine/DMF 3:1; (xx) (Dimethylamino)propylamine, 55 °C, 16 h;(xxi) NaH, EtSH, DMF, 100 °C, 1h.

Procedure for Cleavage from the Resin. After the coupling was completed, the resin was filtered off the reaction and washed with DMF (2 × 30 s). DMF (1 mL) and piperidine (3 mL) were added and the mixture shaken for 30 min at 22 °C. The resin was filtered off and washed with DMF (2 × 30 s), DCM (3 × 30 s), MeOH (1 × 30 s), Et_2O (1 × 30 s) and dried in vacuo. The resin was treated with (dimethylamino)propylamine (1 mL) with periodic agitation at 55 °C for 16 h. The reaction mixture was then filtered to remove the resin, TFA (0.1% (w/v), 7 mL) added, and the resulting solution purified by reversed-phase HPLC. The pure compounds were recovered as white powders upon lyophilization of the appropriate fractions.

Deprotection of Methyl-Protected Polyamides. To a slurry of NaH (80 mg, 60% in mineral oil) in DMF (0.50 mL) was added a solution of ethanethiol (0.32 mL) in DMF (0.50 mL), and the mixture was heated to 100 °C for 5 min in a sealed tube. The polyamide, dissolved in DMF (1.00 mL), was added to the ethanethiolate solution, and the mixture was heated to 100 °C for 1 h in a sealed tube and then cooled to 0 °C. The mixture was added to HOAc (3 mL), and all volatiles were removed (high vacuum, 40 °C). The residue was dissolved in CH_3CN (1 mL) and TFA (7 mL, 0.1% (w/v)) and purified by preparative HPLC.

BzPyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (1). Benzoic acid (70 mg, 0.57 mmol) was dissolved in DMF (1.0 mL), and HBTU (75 mg, 0.20 mmol) and DIEA (0.5 mL) were added. The mixture was allowed to stir for 10 min and added to the deprotected Boc-PyPyPy-(R)$^{Fmoc}\gamma$-ImPyPyPy-β-PAM-resin (100 mg). The mixture was shaken for 5.0 h at 22 °C, and the polyamide was cleaved from the resin according to the general procedure. Preparative HPLC gave **1** (16 mg, 39% isolated yield): UV λ_{max} (H_2O) 240, 312 (69500); ^{1}H NMR (DMSO-d_6) δ 11.10, 10.42, 10.20, 10.07, 10.04, 10.02, 9.97 (s, 1H each), 9.41 (bs, 1 H), 8.42 (bs, 2 H), 8.26 (t, 1 H, J = 5.7 Hz), 8.12 (q, 2 H, J = 5.6 Hz), 8.00 (dd, 2 H, J = 6.3, 1.5 Hz), 7.56−7.70 (m, 3 H), 7.40 (d, 1 H, J = 1.5 Hz), 7.33 (d, 1 H, J = 1.5 Hz), 7.30 (d, 1 H, J = 1.8 Hz), 7.28 (d, 1 H, J = 1.5 Hz), 7.26 (d, 1 H, J = 2.1 Hz), 7.25 (d, 1 H, J = 2.1

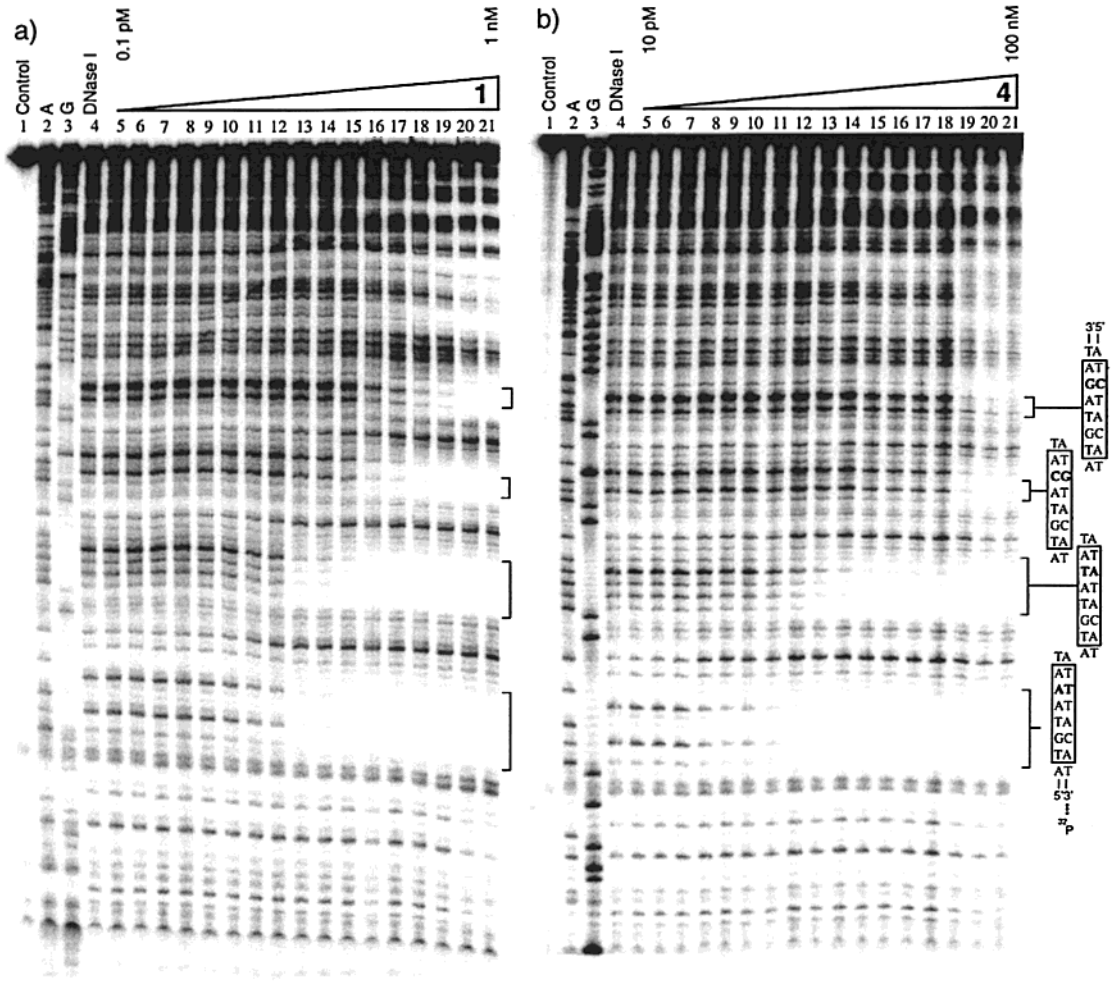


Figure 7. (a) Quantitative DNase I footprint titration experiment with BzPyPyPy-(R)$^{H}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**1**) on the 284 bp *Eco*RI/*Pvu*II restriction fragment from plasmid pCW15: lane 1, intact DNA; lane 2, A-specific reaction; lane 3, G-specific reaction; lane 4, DNase I standard; lanes 5−21, 0.1 pM, 0.2 pM, 0.5 pM, 1 pM, 2 pM, 5 pM, 10 pM, 15 pM, 25 pM, 40 pM, 65 pM, 100 pM, 150 pM, 250 pM, 450 pM, 650 pM, 1 nM BzPyPyPy-(R)$^{H}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**1**). The four sites 5′-T**T**TACA-3′, 5′-T**A**TACA-3′, 5′-T**G**TACA-3′, and 5′-T**C**TACA-3′ that were analyzed are shown on the right side of the gel. (b) Quantitative DNase I footprint titration experiment with (Hb-**3**)PyPyPy-(R)$^{H}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**4**) on the 284 bp *Eco*RI/*Pvu*II restriction fragment from plasmid pCW15: lane 1, intact DNA; lane 2, A-specific reaction; lane-3, G-specific reaction; lane 4, DNase I standard; lanes 5−21, 10 pM, 20 pM, 50 pM, 100 pM, 150 pM, 250 pM, 400 pM, 650 pM, 1 nM, 1.5 nM, 2.5 nM, 4 nM, 6.5 nM, 10 nM, 20 nM, 50 nM, 100 nM (Hb-**3**)PyPyPy-(R)$^{H}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp (**7**). The four sites 5′-T**T**TACA-3′, 5′-T**A**TACA-3′, 5′-T**G**TACA-3′, and 5′-T**C**TACA-3′ that were analyzed are shown on the right side of the gel.

Table 1. Equilibration Association Constants (M^{-1})[a]

polyamide	5′-T**T**TACA-3′	5′-T**A**TACA-3′	5′-T**G**TACA-3′	5′-T**C**TACA-3′
1	1.9 (±0.3) × 10^{11}	1.2 (±0.1) × 10^{11}	3.5 (±1.0) × 10^{10}	1.7 (±0.7) × 10^{10}
2	1.9 (±0.1) × 10^{10}	1.9 (±0.3) × 10^{10}	6.3 (±1.7) × 10^{9}	3.7 (±0.6) × 10^{9}
3	1.0 (±0.2) × 10^{10}	1.0 (±0.1) × 10^{10}	1.5 (±0.6) × 10^{9}	≤1.0 × 10^{8}
4	2.6 (±0.9) × 10^{10}	8.4 (±1.0) × 10^{9}	4.7 (±0.5) × 10^{8}	≤1.0 × 10^{8}

[a] Values reported are mean values from at least three DNase I footprint titration experiments, with the standard deviation for each data set indicated in parentheses. The assays were perfomed 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 $CaCl_2$.

Hz), 7.21 (d, 1 H, J = 2.1 Hz), 7.18 (d, 1 H, J = 1.8 Hz), 7.15 (d, 2 H, J = 1.5 Hz), 7.05 (d, 1 H, J = 2.1 Hz), 6.95 (d, 1 H, J = 1.5 Hz), 4.05, 3.94, 3.93, 3.92, 3.91, 3.88, 3.87 (s, 3 H each), 3.30−3.50 (m, 4 H), 3.17 (q, 2 H, J = 6.3 Hz), 3.06 (p, 2 H, J = 4.8 Hz), 2.81 (d, 6 H, J = 4.8 Hz), 2.42 (t, 2 H, J = 7.2 Hz), 2.00−2.10 (m, 2H), 1.81 (p, 2 H, J = 7.5 Hz); MALDI-TOF-MS calcd. for $C_{60}H_{72}N_{20}O_{10}$ (M + H): 1233.6. Found: 1233.7.

(Hb-1)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (2). 2-Methoxybenzoic acid (70 mg, 0.46 mmol) was dissolved in DMF (1.0 mL), and HBTU (75 mg, 0.20 mmol) and DIEA (0.5 mL) were added. The mixture was allowed to stir for 10 min and then was added to the deprotected Boc-PyPyPy-(R)$^{Fmoc}\gamma$-ImPyPyPy-β-PAM-resin (100 mg). The mixture was shaken for 4.5 h at 22 °C, and the polyamide was cleaved from the resin according to the general procedure. Preparative HPLC gave methyl-protected (Hb-**1**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (15 mg, 35% isolated yield): UV λ_{max} (H_2O) 240, 312 (69500); ^{1}H NMR (DMSO-d_6) δ 11.10, 10.19, 10.07 (s, 1 H each), 10.03 (s, 2 H), 10.01, 9.97 (s, 1 H each), 8.38−8.50 (bs, 2 H), 8.26 (t, 1 H, J = 6.0 Hz), 8.12 (q, 2 H, J = 6.0 Hz), 7.72 (d, 1 H, J = 1.8 Hz), 7.69 (d, 1 H, J = 1.8 Hz), 7.61 (s, 1 H), 7.58 (d, 1 H, J = 2.1 Hz), 7.55 (bs, 1 H), 7.52 (d, 1 H, J = 1.8 Hz), 7.38 (d, 1 H, J = 1.8 Hz), 7.33 (d, 1 H, J = 1.5 Hz), 7.29 (d, 1 H, J = 1.5 Hz), 7.28 (d, 1 H, J = 1.5 Hz), 7.25 (s, 2 H), 7.22 (s, 1 H), 7.10−7.20 (m, 4 H), 7.05 (d, 1 H, J = 1.5 Hz), 6.95 (d, 1 H, J = 1.5 Hz), 4.05, 3.96, 3.93, 3.92, 3.91,3.90, 3.88, 3.87 (s, 3 H each), 3.30−3.50 (m, 4 H), 3.17 (q, 2 H, J = 6.0 Hz), 3.07 (p, 2 H, J = 5.7 Hz), 2.80 (d, 6 H, J = 4.8 Hz), 2.42 (t, 2 H, J = 7.2 Hz), 2.06 (bs, 2

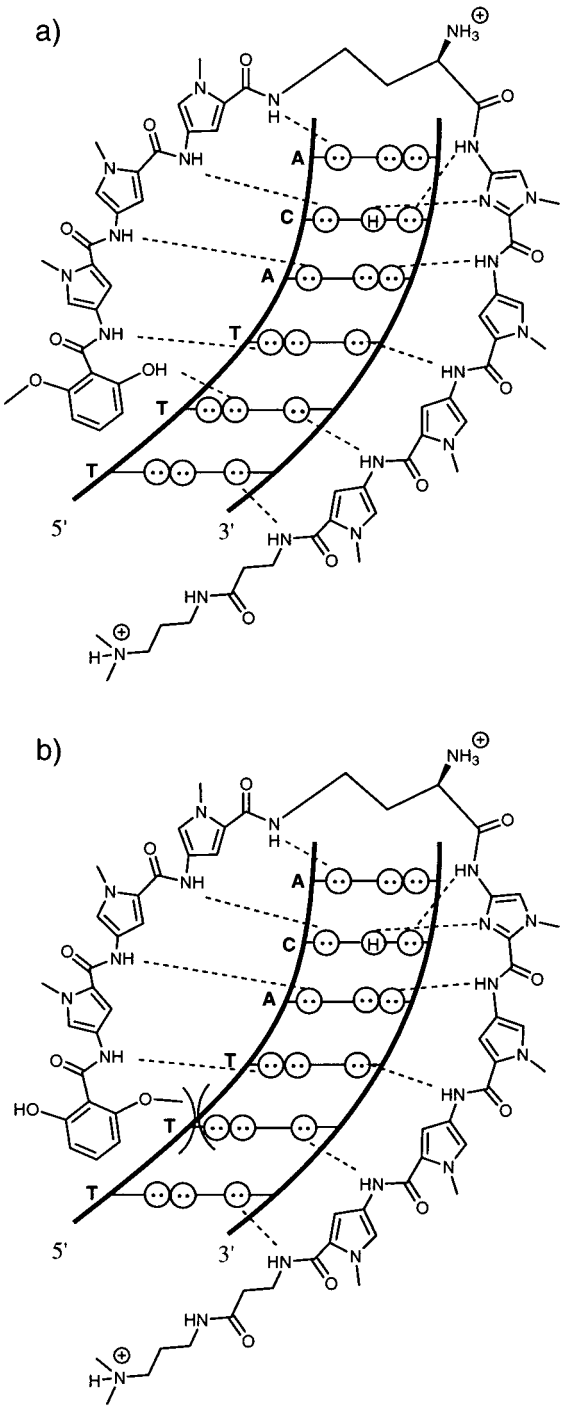


Figure 8. Binding models for two rotamers of (Hb-**3**)PyPyPy-(R)$^{H}{}_{2}{}^{N}\gamma$-ImPyPyPy-β-Dp and a 5′-TTTACA-3′ sequence.

H), 1.80 (p, 2H, J = 8.7 Hz); MALDI-TOF-MS calcd. for $C_{61}H_{74}N_{20}O_{11}$ (M+H): 1263.6. Found: 1263.7. The methyl-protected (Hb-**1**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (3.1 mg, 2.5 μmol) was deprotected according to the general procedure to give **2** (0.5 mg, 16% isolated yield): UV λ_{max} (H_2O) 240, 312 (69500); ^{1}H NMR (DMSO-d_6) δ 12.25, 10.96, 10.43, 10.06, 9.92, 9.90, 9.88, 9.83 (s, 1 H each), 8.25 (bs, 3 H), 8.13 (t, 1 H, J = 5.9 Hz), 7.98 (q, 2 H, J = 6.0 Hz), 7.92 (dd, 1 H, J = 8.4, 1.8 Hz), 7.48 (s, 1 H), 7.37 (ddd, 1 H, J = 8.7, 7.2, 1.5 Hz), 7.28 (d, 1 H, J = 1.5 Hz), 7.20 (d, 1 H, J = 1.8 Hz), 7.17 (d, 1 H, J = 1.5 Hz), 7.14 (d, 1 H, J = 1.5 Hz), 7.11 (bs, 2 H), 7.08 (d, 1 H, J = 1.5 Hz), 7.07 (d, 1 H, J = 2.1 Hz), 7.02 (d, 2 H, J = 1.5 Hz), 6.85−6.95 (m, 3 H), 6.82 (d, 1 H, J = 1.5 Hz), 3.92, 3.82, 3.79, 3.78, 3.77, 3.75, 3.73 (s, 3 H each), 3.04 (q, 2 H, J = 6.6 Hz), 2.93 (bt, 2 H, J = 7.2 Hz), 2.68 (bs, 6 H), 2.28 (t, 2 H, J = 7.2 Hz), 1.92 (bq, 2 H, J = 7.7 Hz), 1.67 (p, 2 H, J = 7.8 Hz); MALDI-TOF-MS calcd. for $C_{60}H_{72}N_{20}O_{11}$ (M + H): 1249.6. Found: 1249.7.

(Hb-2)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (3). 2-Methoxy-6-methylbenzoic acid (26 mg, 0.16 mmol) was dissolved in DMF/DCM (1:1, 0.2 mL) and added to a mixture of DMF (0.1 mL) and oxalyl chloride (2M in DCM, 0.072 mL). The mixture was allowed to stir for 20 min, and DMF (0.3 mL) and DIEA (0.2 mL) were added. The mixture was then added to the deprotected Boc-PyPyPy-(R)$^{Fmoc}\gamma$-ImPyPyPy-β-PAM-resin (50 mg) and shaken for 4.0 h at 22 °C. The polyamide was cleaved from the resin according to the general procedure. Preparative HPLC afforded methyl-protected (Hb-**2**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (9 mg, 41% isolated yield): UV λ_{max} (H_2O) 240, 312 (69 500); ^{1}H NMR (DMSO-d_6) δ 10.27, 10.19 (s, 1 H each), 10.03 (s, 2 H), 10.00, 9.97 (s, 1 H each), 8.26 (t, 1 H, J = 6.0 Hz), 8.12 (q, 2 H, J = 5.1 Hz), 7.61 (s, 1 H), 7.30−7.40 (m, 3 H), 7.29 (d, 1 H, J = 1.5 Hz), 7.28 (d, 1 H, J = 2.4 Hz), 7.25 (bs, 2 H), 7.22 (d, 1 H, J = 1.8 Hz), 7.15 (bs, 2 H), 7.04, 7.03, 7.00, 6.96 (s, 1 H each), 6.95 (d, 1 H, J = 1.5 Hz), 6.93, 6.91 (s, 1 H each), 4.05 (2, 3 H), 3.92, 3.91 (s, 6 H each), 3.88, 3.87, 3.81 (s, 3 H each), 3.30−3.50 (m, 4 H), 3.17 (q, 2 H, J = 6.0 Hz), 3.05 (bt, 2 H, J = 7.6 Hz), 2.79 (s, 6H), 2.42 (t, 2 H, J = 7.2 Hz), 2.27 (s, 3 H), 2.06 (bs, 2H), 1.80 (p, 2 H, J = 8.1 Hz); MALDI-TOF-MS calcd. for $C_{62}H_{76}N_{20}O_{11}$ (M+H): 1277.6. Found: 1277.7. The methyl-protected (Hb-**1**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (3.1 mg, 2.4 μmol) was deprotected according to the general procedure to give **3** (1.4 mg, 48% isolated yield): UV λ_{max} (H_2O) 240, 312 (69500); ^{1}H NMR (DMSO-d_6) δ 10.19, 10.18, 10.03, 10.02, 10.00, 9.96, 9.63 (s, 1 H each), 8.25 (bt, 1 H, J = 5.8 Hz), 8.12 (q, 2 H, J = 3.4 Hz), 7.61 (s, 1 H), 7.32 (d, 1 H, J = 1.7 Hz), 7.31 (d, 1 H, J = 1.8 Hz), 7.29 (d, 1 H, J = 2.1 Hz), 7.28 (d, 1 H, J = 2.1 Hz), 7.24 (d, 2 H, J = 1.8 Hz), 7.22 (d, 1 H, J = 1.5 Hz), 7.10−7.20 (m, 3 H), 7.06 (d, 1 H, J = 2.1 Hz), 7.04 (d, 1 H, J = 1.8 Hz), 6.95 (d, 1 H, J = 1.5 Hz), 6.78 (d, 1 H, J = 8.1 Hz), 6.73 (d, 1 H, J = 7.2 Hz), 4.05 (s, 3 H), 3.92, 3.91 (s, 6 H each), 3.88, 3.87 (s, 3 H each), 3.17 (q, 2 H, J = 6.0 Hz), 3.04 (bs, 2 H), 2.78 (s, 6 H), 2.41 (t, 2 H, J = 6.9 Hz), 2.25 (s, 3 H), 2.05 (bs, 2 H), 1.79 (p, 2 H, J = 6.6 Hz); MALDI-TOF-MS calcd. for $C_{61}H_{74}N_{20}O_{11}$ (M+H): 1263.6. Found: 1263.7.

(Hb-3)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (4). 2,6-Dimethoxybenzoic acid (28 mg, 0.16 mmol) was dissolved in DMF/DCM (1:1, 0.2 mL) and added to a mixture of DMF (0.1 mL) and oxalyl chloride (2M in DCM, 0.072 mL). The mixture was allowed to stir for 20 min, and DMF (0.3 mL) and DIEA (0.2 mL) were added. The mixture was added to the deprotected Boc-PyPyPy-(R)$^{Fmoc}\gamma$-ImPyPyPy-β-PAM-resin (50 mg) and shaken for 3.0 h at 22 °C. The polyamide was then cleaved from the resin according to the general procedure. Preparative HPLC gave methyl-protected (Hb-**3**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (10 mg, 48% isolated yield): UV λmax (H_2O) 240, 308 (69500); ^{1}H NMR (DMSO-d_6) δ 10.96, 10.06, 10.03, 9.90, 9.88, 9.87, 9.83 (s, 1 H each), 8.26 (bs, 3 H), 8.12 (t, 1 H, J = 6.2 Hz), 7.99 (q, 2 H, J = 5.7 Hz), 7.48 (s, 1 H), 7.27 (t, 2 H, J = 8.4 Hz), 7.20 (d, 1 H, J = 1.6 Hz), 7.16 (d, 1 H, J = 1.8 Hz), 7.14 (d, 1 H, J = 2.1 Hz), 7.13 (d, 1 H; J = 1.8 Hz), 7.11 (d, 1 H, J = 1.5 Hz), 7.08 (d, 1 H, J = 1.5 Hz), 7.02 (d, 1 H, J = 1.9 Hz), 7.01 (d, 1 H, J = 1.6 Hz), 6.90 (d, 1 H, J = 1.5 Hz), 6.89 (d, 1 H, J = 2.1 Hz), 6.82 (d, 1 H, J = 1.8 Hz), 6.66, 6.63 (s, 1 H each), 3.92, 3.79, 3.78 (s, 3 H each), 3.78 (s, 6 H), 3.75, 3.74 (s, 3 H each), 3.67 (s, 6 H), 3.04 (q, 2 H, J = 6.0 Hz), 2.94 (p, 2 H, J = 4.8 Hz), 2.68 (d, 6 H, J = 4.5 Hz), 2.28 (t, 2 H, J = 6.9 Hz), 1.94 (bs, 2 H), 1.67 (p, 2 H, J = 7.6 Hz); MALDI-TOF-MS calcd. for $C_{62}H_{76}N_{20}O_{12}$ (M+H): 1293.6; found: 1293.7. The methyl-protected (Hb-**3**)PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (2.7 mg, 2.1 μmol) was deprotected according to the general procedure to give **4** (1.9 mg, 42% isolated yield) together with (2,6-dihydroxybenzoyl)HN−PyPyPy-(R)$^{H}2^{N}\gamma$-ImPyPyPy-β-Dp (1.3 mg, 28% isolated yield): UV λ_{max} (H_2O) 240, 310 (69500); ^{1}H NMR (DMSO-d_6) δ 11.84, 10.08, 10.06, 9.89, 9.88, 9.83 (s, 1 H each), 8.25 (bs, 3 H), 8.12 (t, 1 H, J = 5.7 Hz), 7.98 (q, 2 H, J = 4.8 Hz), 7.48 (s, 1 H), 7.18−7.28 (m, 3 H), 7.16 (d, 1 H, J = 1.5 Hz), 7.14 (d, 1 H, J = 1.5 Hz), 7.11 (bs, 2 H), 7.08 (d, 1 H, J = 1.5 Hz), 7.02 (d, 2 H, J = 1.5 Hz), 7.01 (d, 1H, J = 1.8 Hz), 6.91 (d, 1 H, J = 1.5 Hz), 6.83 (d, 1 H, J = 1.8 Hz), 6.52 (d, 1H, J = 7.8 Hz), 6.47 (d, 1 H, J = 8.4 Hz), 3.92 (s, 3 H), 3.80 (s, 6 H), 3.79, 3.78, 3.77, 3.75, 3.73 (s, 3H each), 3.04 (q, 2 H, J = 6.0 Hz), 2.94 (p, 2 H, J = 4.8 Hz), 2.68 (d, 6 H, J = 4.2 Hz), 2.28 (t, 2 H, J = 7.5 Hz), 1.94 (bq, 2H, J = 8.4 Hz),

1.67 (p, 2 H, $J = 8.7$ Hz); MALDI-TOF-MS calcd. for $C_{61}H_{74}N_{20}O_{12}$ (M+H): 1279.6. Found: 1279.7.

Construction of Plasmid DNA. The plasmid pCW15 was constructed by hybridization of the inserts 5′-CTAGCGGCTATGTAA-ATGGATGGCGGCTATGTA TATGGATGGCGGCTATGTACAT-GGATGGCGGCTATGTAGATGGATTGCA-3′ and 5′-ATCCATC-TACATAGCCGCCATCCATGTACATAGCCGCCATCCATATA

CATAGCCGCCATCCATTTACATAGCC-3′. The hybridized insert was ligated into linearized pUC19 *Xba*I /*Pst*I plasmid using T4 DNA ligase. The resultant constructs were used to transform Top10F′ OneShot competent cells from Invitrogen. Ampicillin-resistant white colonies were selected from 25 mL of Luria-Bertani medium agar plates containing 50 μg/mL ampicillin and treated with XGAL and IPTG solutions. Large-scale plasmid purification was performed with Qiagen Maxi purification kits. Dideoxy sequencing was used to verify the presence of the desired insert.

Preparation of 3′- and 5′-End-Labeled Restriction Fragments. The plasmid pCW15 was linearized with *Eco*RI and *Pvu*II and then treated with Sequenase (version 2.0 from United States Biochemical), deoxyadenosine 5′-[$\alpha-^{32}$P]triphosphate, and thymidine 5′-[$\alpha-^{32}$P]-triphosphate for 3′ labeling. The 3′ labeled fragment was loaded onto a 7% nondenaturing polyacrylamide gel, and the desired 284 base pair band was visualized by autoradiography and isolated. Chemical sequencing reactions were performed according to published methods.[7]

Quantitative DNase I Footprinting. DNase I Footprinting reactions were carried out as previously described.[8] Photostimulable storage phosphorimaging plates (Kodak Storage Phosphor Screen S0230 obtained from Molecular Dynamics) were pressed flat against gel samples and exposed in the dark at 22 °C for 12−16 h. A Molecular Dynamics 400S PhosphorImager was used to obtain all data from the storage screens. The data were analyzed by performing volume integration of all bands using ImageQuant v. 3.2 software. All DNA manipulations were performed according to standard protocols.[9]

Acknowledgment. We are grateful to the National Institutes of Health (GM-27681) for research support, the Knut and Alice Wallenberg Foundation for a postdoctoral fellowship to U.E., and the National Institutes of Health for a research traineeship award (GM-08501) to C.C.C.W. We thank G. M. Hathaway for MALDI-TOF mass spectrometry.

Supporting Information Available: Details on the synthesis and quantitative DNase I footprinting titrations of eight-ring hairpin polyamides containing substituted pyrroles and hydroxypyrroles at the terminal position (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA001692U

(7) (a) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823−7830. (b) Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499−560.

(8) Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature (London)* **1996**, *382*, 559−561.

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