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Alternative Heterocycles for DNA Recognition: A 3-Pyrazole/Pyrrole Pair Specifies for G·C Base Pairs

Zheng-Yun J. Zhan and Peter B. Dervan*

The Beckman Institute, California Institute of Technology, Pasadena, CA 91125, USA

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Abstract—Synthetic ligands comprising three aromatic amino acids, pyrrole (Py), imidazole (Im), and hydroxypyrrole (Hp), specifically recognize predetermined sequences as side-by-side pairs in the minor groove of DNA. To expand the repertoire of aromatic rings that may be utilized for minor groove recognition, three five-membered heterocyclic rings, 3-pyrazolecarboxylic acid (3-Pz), 4-pyrazolecarboxylic acid (4-Pz), and furan-2-carboxylic acid (Fr), were examined at the N-terminus of eight-ring hairpin polyamide ligands. The DNA binding properties of 3-Pz, 4-Pz, and Fr each paired with Py were studied by quantitative DNase I footprinting titrations on a 283 bp DNA restriction fragment containing four 6-bp binding sites 5′-ATNCCTAA-3′ (N = G, C, A, or T; 6-bp polyamide binding site is underlined). The pair 3-Pz/Py has increased binding affinity and sequence specificity for G·C bp compared with Im/Py.

Introduction

Cell-permeable small molecules which bind predetermined DNA sequences have the potential to control gene expression.[1] Polyamides containing the three aromatic amino acids pyrrole (Py), imidazole (Im), and hydroxypyrrole (Hp) are synthetic ligands which bind in the minor groove with affinity and specificity comparable to DNA binding proteins.[2] DNA sequence recognition depends on side-by-side amino acid pairings oriented N–C with respect to the 5′–3′ direction of the DNA duplex in the minor groove.[3–6] Antiparallel pairs of Im opposite Py (Im/Py) distinguishes G·C bp from C·G bp and both of these from A·T/T·A base pairs.[3–6] A Py/Py pair binds both A·T and T·A base pairs in preference to G·C/C·G base pairs.[7] The discrimination of T·A from A·T using Hp/Py pairs completes the four base pair code.[8,9]

Eight-ring hairpin polyamides have been found to regulate transcription in cell culture.[1,10] A key issue for specific inhibition of protein–DNA complexes is optimizing the affinity and sequence specificity of the DNA binding polyamides necessary for targeting single sites within single promoters in gigabase size genomes.[10] Therefore, in parallel to our ongoing gene inhibition cell culture studies, it remains a high priority within our chemistry program to explore the possibility of new ring pairing schemes which afford enhanced affinity and specificity for DNA over the original pairing code. We report here our effort to replace the terminal hairpin Im/Py pair which codes for G·C base pairs.

New aromatic pairs

One way to enhance the binding affinity and specificity is to increase the hydrogen-bond strength and/or electrostatic interactions selectively between the polyamide rings and the edges of the four Watson–Crick base pairs in the minor groove. For example, the specificity of Im for G is related to the fact that the nitrogen lone pair of Im points to the floor of the minor groove and forms a specific hydrogen bond with the exocyclic NH of G.[4,5] Other five-membered heterocycles such as 3-pyrazole carboxamide (3-Pz) or furan-2-carboxamide (Fr) could similarly present a heteroatom toward the floor of the minor groove. In this report, we examine three new monomers X (X = 3-pyrazolecarboxylic acid (3-Pz); 4-pyrazolecarboxylic acid (4-Pz), and furan-2-carboxylic acid (Fr)) incorporated into the N-terminus of an eight ring hairpin to investigate the affinity and specificity for a G·C base pair paired with a Py ring (X/Py pair, Fig. 1). Four eight-ring polyamides X-PyPyPy-(*R*)$H_2N\gamma$-PyIm ImPy-β-Dp (**1**: X = Im; **2**: X = 3-Pz; **3**: X = 4-Pz; **4**: X = Fr; (*R*)$H_2N\gamma$ = (*R*)-α,γ-diaminobutyric acid; β = β-alanine, and Dp = *N*,*N*-dimethylaminopropylamine, Fig. 2) were synthesized to study the relative affinities and specificities at four respective match and single base pair

*Corresponding author. Tel.: +626-356-6002; fax: +626-683-8753; e-mail: dervan@caltech.edu

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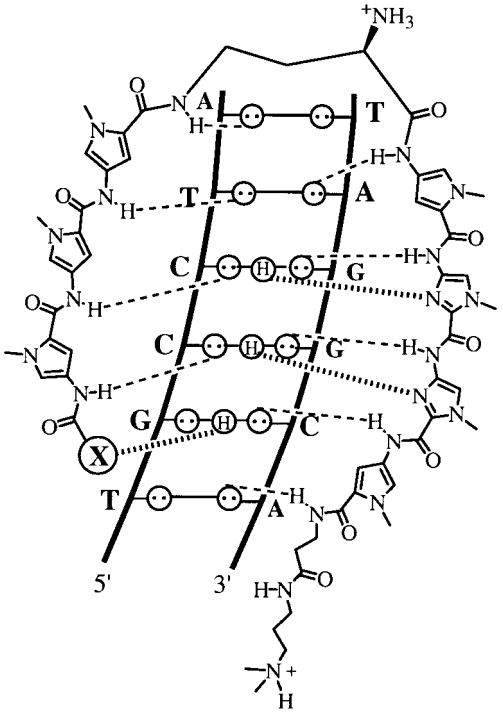

Figure 1. (top) Binding models for the complex formed between polyamide X-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (**1**, **2**, **3**, or **4**, respectively) and 5′-AT**G**CCAAT-3′. Circles with dots represent lone pairs of N3 of purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogen of guanine. Putative hydrogen bonds are illustrated by dotted lines; (bottom) ball and stick models are also shown. Shaded and nonshaded circles denote imidazole and pyrrole carboxamides, respectively. Non-shaded diamonds represent the β-alanine residue. Circle containing an X represents four kinds of different capping monomers, respectively.

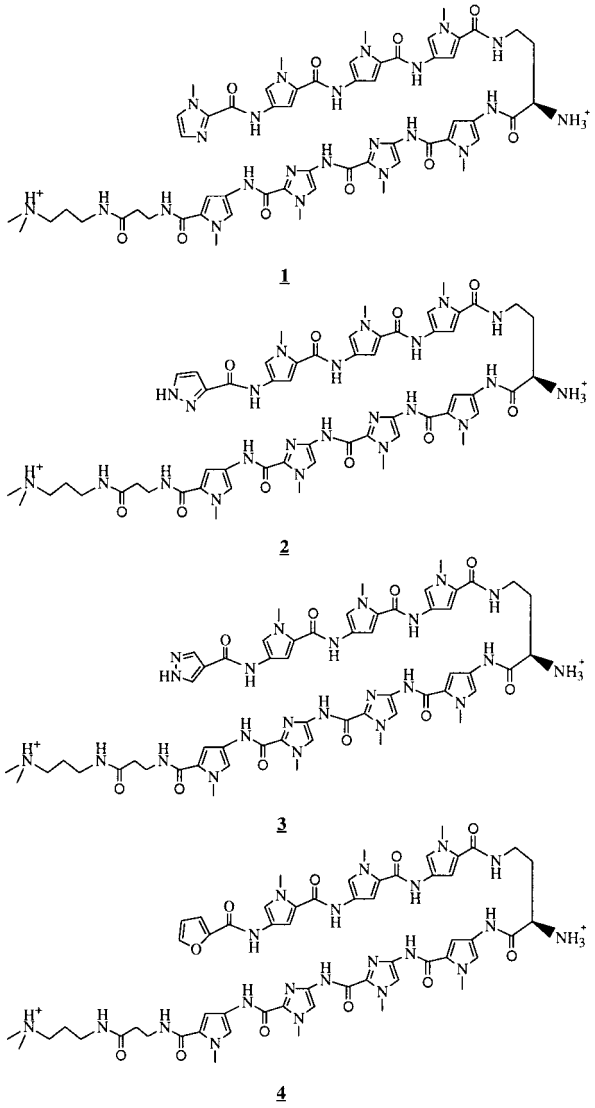

Figure 2. Structures of four eight-ring hairpin polyamides with four different monomers, respectively, **Im**-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (**1**), **3-Pz**-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (**2**), **4-Pz**-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (**3**), and **Fr**-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (**4**).

mismatch binding sites 5′-TNCCTA-3′ (N = T, G, C, or A, respectively; Fig. 3). Here we report that a (3-Pz)/Py pair specifies for a G·C bp with high affinity and specificity.

Results and Discussion

The three monomer carboxylic acids (3-Pz (**7**); 4-Pz (**8**), and Fr (**9**)) are commercially available. In addition, 3-pyrazolecarboxylic acid (**7**) can also be obtained by oxidation of 3-methylpyrazole with $KMnO_4$.[11] The four polyamides were synthesized by solid phase methods by coupling each of the monomers (**6**–**9**) at the N-terminus of H_2N-PyPyPy-(*R*)H_2Nγ-PyImImPy-β-Pam Resin (**5**) to afford Im-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (**1**), 3-Pz-PyPyPy-(*R*)H_2Nγ-PyImImPy-β-Dp (**2**), 4-Pz-PyPyPy-(*R*)H_2Nγ-PyImImPy-β-Dp (**3**), and Fr-PyPyPy-(*R*)H_2Nγ-PyImImPy-β-Dp (**4**).[12] All four polyamides were purified by preparatory HPLC and characterized by NMR and MS.

Quantitative DNase I footprinting titration

To determine the equilibrium association constants (K_a) of polyamides **1**–**4**, quantitative DNase I footprinting titrations (10 mM Tris–HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH = 7.0, and 22 °C) were performed to analyze the affinities and specificities for each polyamide on a 3′-^{32}P-labeled restriction fragment containing four binding sites which vary at a single position 5′-TNCCTA-3′ where N = T, G, C, A (Fig. 5). Footprinting results reveal that four polyamides bind at four match and mismatch sites with different affinities and specificities (Fig. 6 and Table 1).

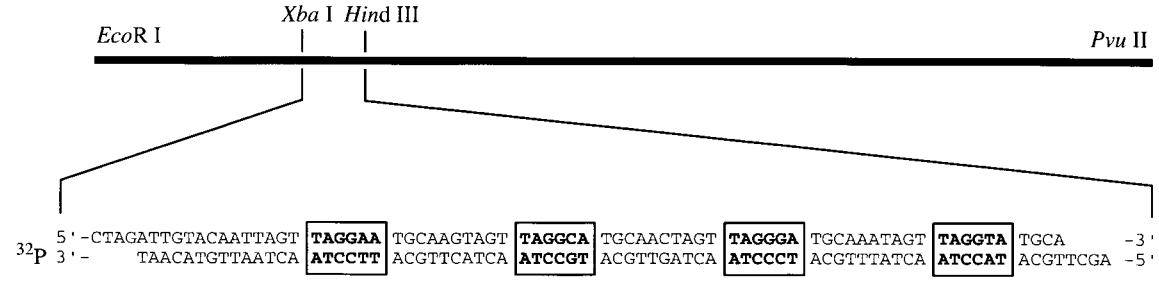

Figure 3. Illustration of the *Eco*R I/*Pvu* II restriction fragment with the *Xba* I/*Hin*d III insertion sites indicated. Four six-base pair binding sites which differ at a single common position are shown in four boxes.

For the recognition of G·C bp by an X/Py pairing, quantitative DNase I footprinting titrations indicate that the binding affinity decreases in the order: 3-Pz/Py > Fr/Py~Im/Py > 4-Pz/Py. Remarkably, the binding affinity of furan-capped polyamide Fr-PyPyPy-(*R*) H_2Nγ-PyImImPy-β-Dp for G·C bp ($K_a = 5.3\times10^9$ M^{-1}) is comparable to that of an Im-capped polyamide Im-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp ($K_a = 3.6\times10^9$ M^{-1}), which suggests that the oxygen atom 'O' at the 1-position of the furan ring may form a hydrogen bond with the H-bond donor 'N–H' of 2-exocyclic amino group of guanine. For the two 3-Pz/Py and 4-Pz/Py pairs, the 3-Pz/Py pair has the best affinity and specificity for a G·C bp ($K_a = 1.7\times10^{11}$ M^{-1}). In contrast, the 4-Pz/Py binds both T·A and A·T in preference to G·C/C·G and mimics a Py/Py pair.

Conclusion

The 3-Pz/Py pair mimics the Im/Py pair but with enhanced affinity and specificity. The sequence recognition for G·C bp likely results from the formation of a specific hydrogen bond between the exocyclic amino group of guanine and the N2 of the 3-pyrazole ring. The specificity of 3-Pz/Py pair for G·C bp is 53–650-fold greater relative to that of the other three base pairs, C·G, A·T, and T·A. This can be compared with the specificity of an Im/Py pair for G·C bp, which is about 12–120-fold preference to the other three base pairs. Whether the benefits of enhanced affinity and specificity will be realized in gene inhibition cell culture studies remains to be seen and will be reported in due course.

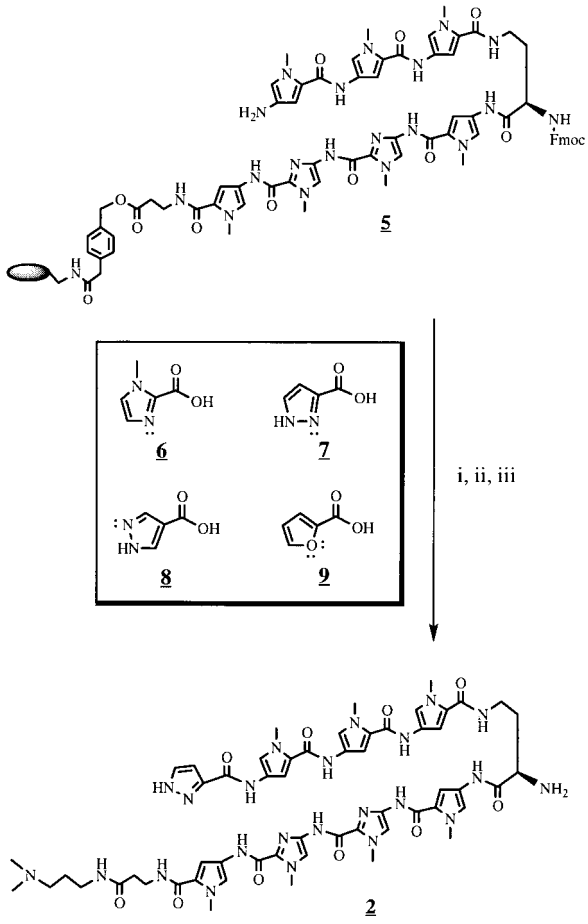

Figure 4. (box) Four monomers at N-terminus: 2-imidazolecarboxylic acid (**6**), 3-pyrazolecarboxylic acid (**7**), 4-pyrazolecarboxylic acid (**8**), and furan-2-carboxylic acid (**9**). (i) 3-Pz acid **1** (HBTU, DIEA, DMF); (ii) 80% piperdine:DMF (22 °C, 45 min); (iii) *N*,*N*-dimethylaminopropylamine (Dp), 22 °C, 15 h.

Experimental

Materials

Boc-β-alanine-(-4-carboxamidomethyl)-benzyl-ester-co-poly(styrene-divinylbenzene) resin (Boc-β-Pam-Resin), dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and Boc-β-alanine were purchased from Peptides International. (*R*)-2-Fmoc-4-Boc-diaminobutyric acid was from Bachem. *N*,*N*-Diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), acetic anhydride (Ac_2O), and 0.0002 M potassium cyanide/pyridine were purchased from Applied Biosystems. Boc-γ-aminobutyric acid was from NOVA Biochem, dichloromethane (DCM) and triethylamine (TEA) were reagent grade from EM, thiophenol (PhSH), *N*,*N*-dimethylaminopropylamine (Dp), trichloroacetyl chloride, *N*-methylpyrrole, *N*-methylimidazole, and picric acid from Aldrich, trifluoroacetic acid (TFA) from Halocarbon, phenol from Fisher, and ninhydrin from Pierce. All reagents were used without further purification.

Quik-Sep polypropylene disposable filters were purchased from Isolab Inc. ^{1}H NMR spectra were recorded on a 300 MHz General Electric-QE NMR and 500 MHz

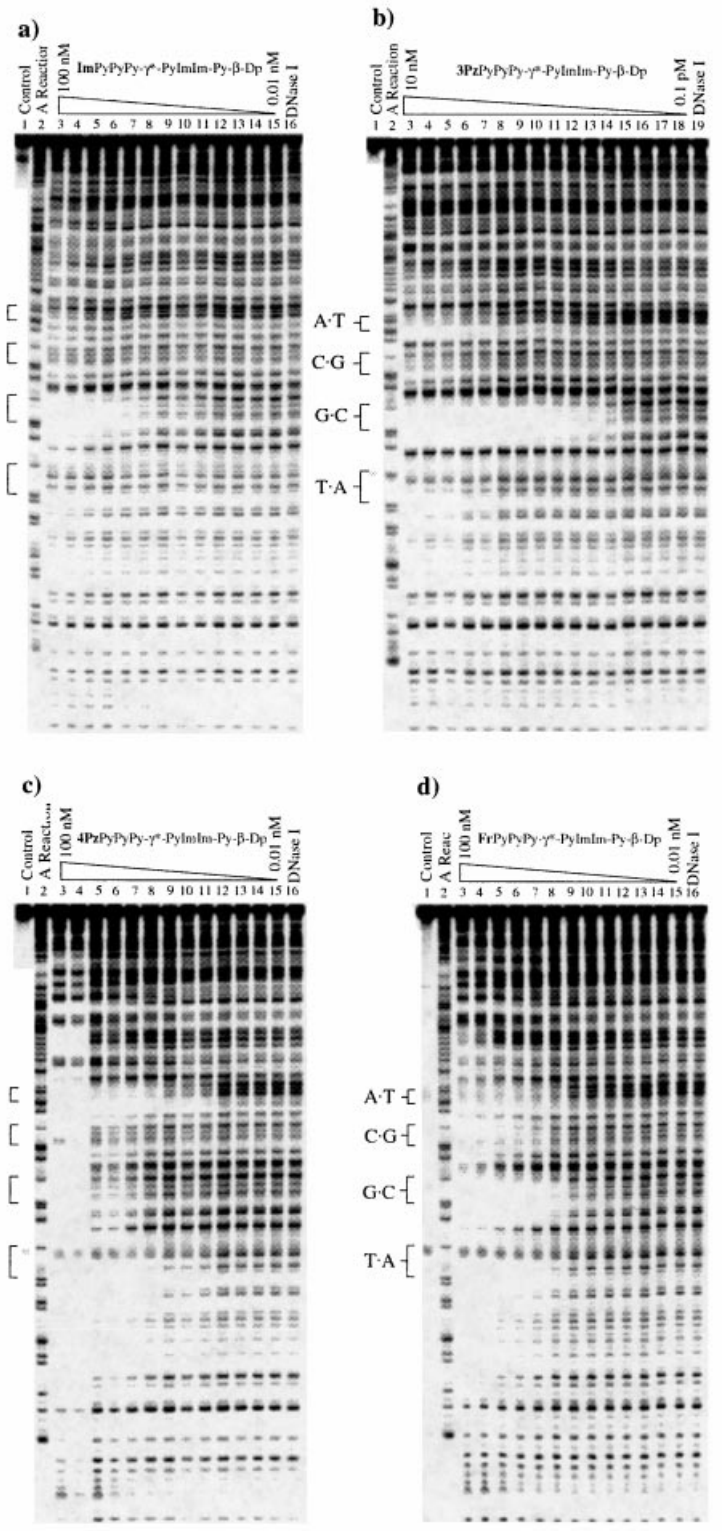

Figure 5. Quantitative DNase I footprint titration experiment with polyamides **1**, **2**, **3**, or **4** on the *Eco*R I/*Pvu* II restriction fragments derived from plasmid pZYJZ-3. γ* = (*R*)-α,γ-diaminobutyric acid in this figure; (a) lane 1, intact DNA; lane 2, A reaction; lanes 3–15, 100, 50, 20, 10, 5, 2 and 1 nm, 500, 200, 100, 50, 20 and 10 pM (**1**); lane 16, DNase I standard; (b) lane 1, intact DNA; lane 2, A reaction; lanes 3–18, 10, 5, 2 and 1 nm, 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 pM (**2**); lane 19, DNase I standard; (c) lane 1, intact DNA; lane 2, A reaction; lanes 3–15, 100, 50, 20, 10, 5, 2 and 1 nm, 500, 200, 100, 50, 20 and 10 pM (**3**); lane 16, DNase I standard; (d) lane 1, intact DNA; lane 2, A reaction; lanes 3–15, 100, 50, 20, 10, 5, 2 and 1 nm, 500, 200, 100, 50, 20 and 10 pM (**4**); lane 16, DNase I standard. Four binding sites 5′-TNCCTA-3′ (N = A, C, G, and T, respectively) for each polyamide are shown on the left side of the autoradiograms. All reactions contain 15 kcpm restriction fragment, 10 mM Tris–HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$ and 5 mM $CaCl_2$.

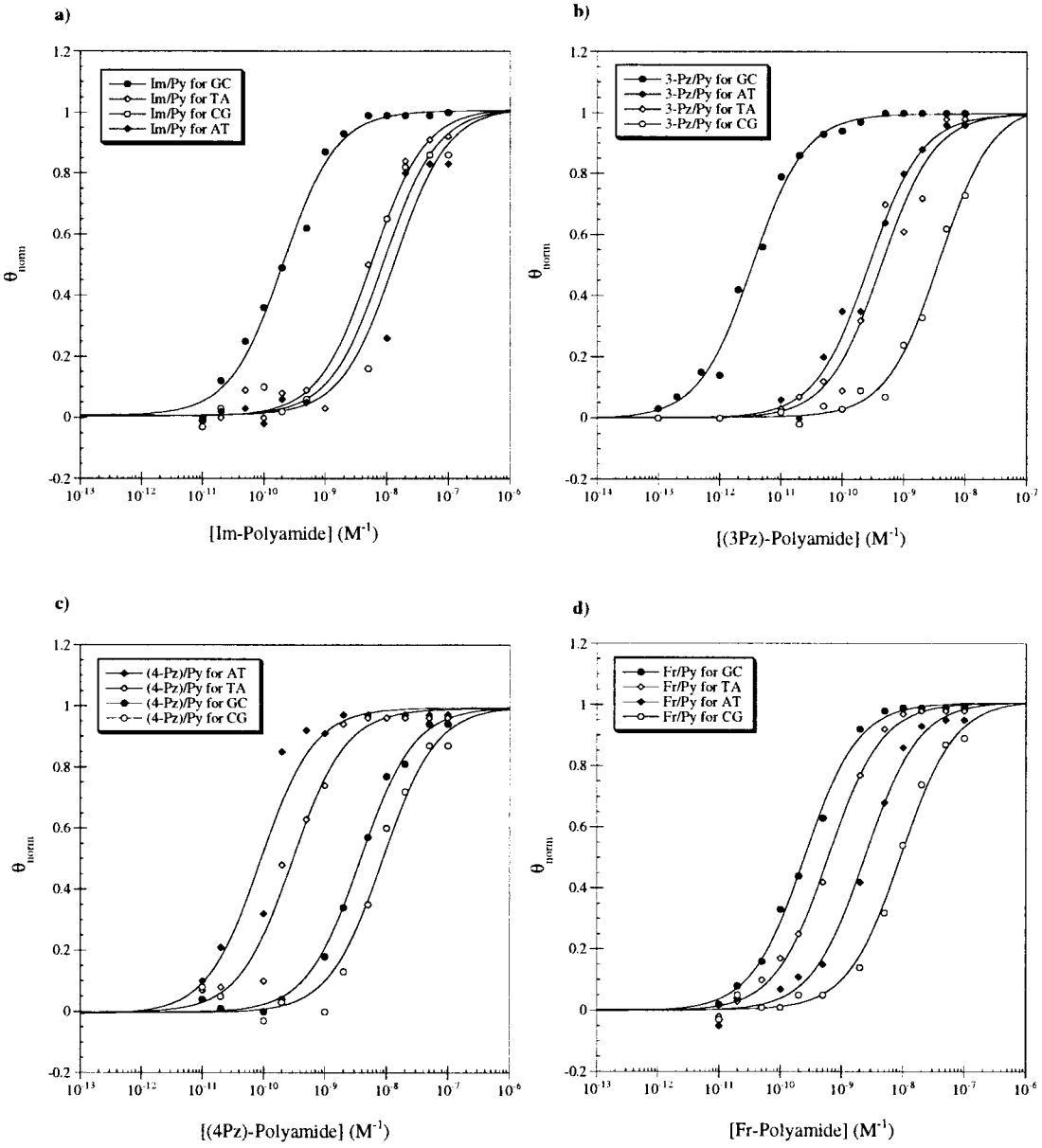

Figure 6. Binding isotherms from the quantitative DNase I footprinting titration experiments for **X**-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp at four sites 5′-TNCCTA-3′ (N = A, C, G, and T, respectively); (a) X = 3-Pz; (b) X = Fr; (c) X = 4-Pz; and (d) X = Im, where (◆) X/Py for A·T bp; (◇) X/Py for T·A bp; (●) X/Py for G·C bp; (○) X/Py for C·G bp. Θ_{norm} values were obtained according to published methods. The solid lines are best fit Langmuir binding titration isotherms obtained by a nonlinear least-squares algorithm.

Bruker NMR spectrometers. 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. HPLC analysis was performed on either an HP 1090 analytical using a RAINEN C-18, Microsorb MV, 5 μm, 300×4.6 mm reversed phase column in 0.1% (wt/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min, gradient elution 1.25% acetonitrile/min. Preparatory reverse phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25×100 mm, 100 μm C-18 column equipped with a guard, 0.1% (wt/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.

H_3^+N-PyPyPy-(*R*)^{Fmoc}γ-PyImImPy-β-Pam-resin (5).[12] Boc-β-alanine-Pam-Resin (1.0 g, 0.55 mmol/g) is placed in a 20 mL glass reaction vessel, shaken in 10 mL DMF for 5 min and the reaction vessel drained. The resin was washed with DCM (10 mL, 2×30 s) and the Boc group was removed with 80% TFA/DCM/0.5 M PhSH (10 mL, 20 min). The resin was washed with DCM (10 mL, 2×30 s) followed by DMF (10 mL, 1×30 s). A resin sample (~5 mg) was taken for analysis. The vessel was drained completely and activated monomer added, followed by DIEA if necessary. The reaction vessel was shaken vigorously to make a slurry. The coupling reaction was allowed to proceed for 45–60 min at rt at the N-terminus of Py monomer, but for ~4 h at 37 °C at the N-terminus of Im monomer. The reaction vessel was then washed with DCM (10 mL), followed by DMF (10 mL). Following the developed procedure in 15 steps,

Table 1. Equilibrium constants (K_a, M^{-1})[a,b]

Polyyamides	Pairing	5′-a**TT**CCTAa-3′	5′-a**TG**CCTAa-3′	5′-a**TC**CCTAa-3′	5′-a**TA**CCTAa-3′
ImPyPyPy-γ*-PyImImPy-β-Dp, **1**	Im/Py	2.9×10^{8} (±1.1)	3.6×10^{9} (±0.7)	4.7×10^{7} (±0.9)	2.8×10^{7} (±1.2)
3Pz PyPyPy-γ*-PyImImPy-β-Dp, **2**	3-Pz/Py	2.1×10^{9} (±0.3)	1.7×10^{11} (±0.6)	2.6×10^{8} (±1.1)	3.2×10^{9} (±0.3)
4Pz PyPyPy-γ*-PyImImPy-β-Dp, **3**	4-Pz/Py	2.9×10^{9} (±0.5)	2.3×10^{8} (±0.3)	1.2×10^{8} (±0.7)	7.5×10^{9} (±0.3)
FrPyPyPy-γ*-PyImImPy-β-Dp, **4**	Fr/Py	1.7×10^{9} (±0.6)	5.3×10^{9} (±0.5)	1.8×10^{8} (±0.6)	3.9×10^{8} (±0.8)

[a]Values reported are the mean values measured from at least three DNase I footprint titration experiments, with the standard deviation for each data set indicated in parentheses.
[b]The assays were performed at 22 °C at pH 7.0 in the presence of 10 mM Tris–HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$. γ* = (*R*)-α,γ-diaminobutyric acid.

the polyamide H_3^+N-PyPyPy-(*R*)^{Fmoc}γ-PyImImPy-β-Pam-resin (**13**) was prepared for coupling with four different capping monomers, respectively (Fig. 4).

Im-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (1). The synthetic procedure is the same as in the synthesis of **5**. In a 5 mL glass reaction vessel, the amine group of **5** (0.25 g, 0.55 mmol/g) was capped by coupling with Im monomer (1-methylimidazole-2-carboxylic acid, 110 mg, 1 mmol; HBTU, 341 mg, 0.9 mmol; DMF, 1 mL; DIEA, 0.5 mL), followed by treatment with piperidine (20% in DMF, 5 mL, 45 min) to remove the Fmoc group. The polyamide mixture was cleaved from resin by heating the resin overnight at 55 °C in *N*,*N*-dimethylaminopropylamine (1 mL, 9 mmol) and was purified by C-18 reversed phase preparatory HPLC. The appropriate fractions were lyophilized to yield a white powder (24 mg, 14% recovery). ^{1}H NMR (DMSO-d_6) δ 10.48 (s, 1H); 10.26 (s, 1H); 9.97 (s, 1H); 9.94 (s, 1H); 7.65 (s, 1H); 7.59 (s, 1H); 7.40 (s, 1H); 7.35 (s, 1H); 7.29 (s, 1H); 7.23 (s, 1H); 7.20 (s, 1H); 7.17 (s, 1H); 7.16 (s, 1H); 7.09 (s, 1H); 7.05 (s, 1H); 7.04 (s, 1H); 7.03 (s, 1H); 6.99 (s, 1H); 4.02 (s, 3H); 4.00 (s, 3H); 3.99 (s, 3H); 3.88 (s, 3H); 3.85 (s, 6H); 3.83 (s, 3H); 3.80 (s, 3H); 3.10 (m, 2H); 3.00 (m, 2H); 2.74 (s, 6H); 2.19 (m, 4H); MALDI-TOF-MS (monoisotopic) [M + H$^+$] 1238.6 (1238.6 calcd for $C_{56}H_{70}N_{23}O_{10}$).

(3-Pz)-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (2). In a 5 mL glass reaction vessel, the amine group of **5** (0.25 g, 0.55 mmol/g) was capped by coupling with 3-Pz monomer (3-pyrazolecarboxylic acid, 112 mg, 1 mmol; HBTU, 341 mg, 0.9 mmol; DMF, 1 mL; DIEA, 0.5 mL), followed by treatment with piperidine (20% in DMF, 5 mL, 45 min) to remove the Fmoc group. The resin was washed with DMF (1×20 mL), DCM (1×20 mL), and MeOH (1×20 mL), then placed in a 20 mL glass scintillation vial. 1.0 mL (9 mmol) of *N*,*N*-dimethylaminopropylamine (Dp) was added to the vial, and the solution was kept at 22 °C for 15–20 h. The resin was removed by filtration through a disposable propylene filter and washed with 1 mL of DMF. The polyamide mixture was purified by C-18 reversed phase preparatory HPLC. The appropriate fractions were lyophilized to yield a white powder (19 mg, 11% recovery). ^{1}H NMR (DMSO-d_6) δ 10.48 (s, 1H); 10.26 (s, 1H); 10.12 (s, 1H); 9.96 (s, 1H); 9.94 (s, 1H); 8.05 (br s, 1H); 7.87 (s, 1H); 7.65 (s, 1H); 7.59 (s, 1H); 7.36 (s, 1H); 7.27 (s, 1H); 7.23 (s, 1H); 7.19 (d, 1H, J = 1.3 Hz); 7.15 (s, 1H); 7.13 (s, 1H); 7.09 (s, 1H); 7.03 (s, 1H); 6.99 (s, 1H); 6.72 (s, 1H); 4.02 (s, 3H); 4.00 (s, 3H); 3.88 (s, 6H); 3.85 (s, 3H); 3.83 (s, 3H); 3.80 (s, 3H); 3.11 (m, 2H); 3.01 (m, 2H); 2.74 (s, 6H); 1.73 (m, 2H); MALDI-TOF-MS (monoisotopic) [M + H$^+$] 1224.6 (1224.6 calcd for $C_{56}H_{69}N_{23}O_{10}$).

(4-Pz)-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (3). The synthetic procedure is the same as in the synthesis of **5**. In a 5 mL glass reaction vessel, the amine group of **5** (0.25 g, 0.55 mmol/g) was capped by coupling with activated 4-Pz monomer (4-pyrazolecarboxylic acid, 112 mg, 1 mmol; HBTU, 341 mg, 0.9 mmol; DMF, 1 mL; DIEA, 0.5 mL), followed by treatment with piperidine (20% in DMF, 5 mL, 45 min) to remove the Fmoc group. The polyamide mixture was cleaved from resin with *N*,*N*-dimethylaminopropylamine as described for the preparation of **2**, and was purified by C-18 reversed phase preparatory HPLC. The appropriate fractions lyophilized to yield a white powder (10 mg, 6% recovery). ^{1}H NMR (DMSO-d_6) δ 10.48 (s, 1H); 10.26 (s, 1H); 9.96 (s, 1H); 9.94 (s, 1H); 9.88 (s, 1H); 8.06 (br s, 2H); 7.65 (s, 1H); 7.59 (s, 1H); 7.35 (s, 1H); 7.23 (s, 1H); 7.20 (s, 1H); 7.15 (s, 1H); 7.08 (s, 1H); 7.04 (s, 2H); 7.02 (s, 1H); 6.99 (s, 1H); 4.02 (s, 3H); 4.00 (s, 3H); 3.88 (s, 3H); 3.86 (s, 6H); 3.85 (s, 3H); 3.83 (s, 3H); 3.80 (s, 3H); 3.11 (m, 2H); 3.01 (m, 2H); 2.77 (m, 6H); 1.99 (m, 4H); 1.74 (m, 2H); MALDI-TOF-MS (monoisotopic) [M + H$^+$] 1224.6 (1224.6 calcd for $C_{56}H_{70}N_{23}O_{10}$).

Fr-PyPyPy-(*R*)^{H_2}Nγ-PyImImPy-β-Dp (4). The synthetic procedure is the same as in the synthesis of **5**. In a 5 mL glass reaction vessel, the amine group of **5** (0.25 g, 0.55 mmol/g) was capped by coupling with activated Fr monomer (furan-2-carboxylic acid, 112 mg, 1 mmol; HBTU, 341 mg, 0.9 mmol; DMF, 1 mL; DIEA, 0.5 mL), followed by treatment with piperidine (20% in DMF, 5 mL, 45 min) to remove the Fmoc group. The polyamide mixture was cleaved from resin with *N*,*N*-dimethylaminopropylamine as described for the preparation of **2**, and was purified by C-18 reversed phase preparatory HPLC. The appropriate fractions lyophilized to yield a white powder (14 mg, 8% recovery). ^{1}H NMR (DMSO-d_6) δ 10.63 (s, 1H); 10.48 (s, 1H); 10.33 (s, 2H); 10.26 (s, 1H); 9.99 (s, 1H); 9.94 (s, 1H); 9.41 (s, 1H); 7.90 (s, 1H); 7.65 (s 1H); 7.59 (s, 1H); 7.36 (s, 1H); 7.26 (s, 1H); 7.23 (d, 1H, J = 1.5 Hz); 7.21 (s, 1H); 7.19 (s, 1H); 7.16 (s, 1H); 7.08 (s, 1H); 7.04 (s, 1H); 7.03 (s, 1H); 6.99 (d, 1H, J = 1.4 Hz); 6.68 (br s, 2H); 4.02 (s, 3H); 4.00 (s, 3H); 3.88 (s, 3H); 3.86 (s, 3H); 3.85 (s, 3H); 3.83 (s, 3H); 3.80 (s, 3H); 3.10 (m, 2H); 3.00 (m, 2H); 2.75 (s, 6H); 1.74 (m, 2H); MALDI-TOF-MS (monoisotopic) [M + H$^+$] 1224.6 (1225.6 calcd for $C_{57}H_{70}N_{21}O_{11}$).

DNA reagents, materials, and 3′-[α-^{32}P]-labeled fragments. Enzymes were purchased from Boehringer-Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P] triphosphates were obtained from Amersham. Sonicated, deproteinized calf thymus DNA was acquired from Pharmacia. RNase free water was obtained from USB and used for all footprinting reactions. All other reagents and materials were used as received. All DNA manipulations were performed according to standard protocols.

The plasmid pZYJZ-3 was constructed by cloning two 76 bp complementary oligonucleotide strands into pUC19 plasmid with the *Xba* I/*Hin*d III enzyme digest (Fig. 3). pZYJZ-3 was linearized with *Eco*R I/*Pvu* II restriction enzymes, then treated with the Sequenase enzyme, deoxyadenosine 5′-[α-^{32}P]-triphosphate and thymidine 5′-[α-^{32}P]-triphosphate for 3′ labeling. The 3′-labeled fragments were loaded onto a 6% non-denaturing polyacrylamide gel, and the desired 283 bp band was visualized by autoradiography and isolated.

DNase I footprinting.[13–15] 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 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 30 kcpm 3′-radiolabeled DNA. The solutions were allowed to equilibrate for a minimum of 12 h at 22 °C. Cleavage was initiated by the addition of 10 μL of a DNase I stock solution (diluted with 1 mM DTT to give a stock concentration of 1.875 u/mL) and was allowed to proceed for 7 min at 22 °C. The reactions were stopped by adding 50 μL of a solution containing 2.25 M NaCl, 150 mM EDTA, 0.6 mg/mL glycogen, and 30 μM base-pair calf thymus DNA, and then precipitated with ethanol. The cleavage products were resuspended in 100 mM tris-borate-EDTA/80% formamide loading buffer, denatured at 85 °C for 6 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% crosslink, 7 M urea) at 2000 V. The gels were dried under vacuum at 80 °C, then quantitated using storage phosphor technology.

Equilibrium association constants were determined as previously described.[13–15] The data were analyzed by performing volume integrations of the 5′-ATNCCTAA-3′ sites and a reference site. The apparent DNA target site saturation, θ_{app}, was calculated for each concentration of polyamide using the following equation:

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

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

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

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

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

Quantitation by storage phosphor technology autoradiography. Photostimulable storage phosphorimaging plates (Kodak Storage Phosphor Screen S0230 obtained from Molecular Dynamics) were pressed flat against gel samples and exposed in the dark at 22 °C for 12–20 h. A Molecular Dynamics 400S Phosphor Imager was used to obtain all data from the storage screens. The data were analyzed by performing volume integrations of all bands using the ImageQuant v. 3.2.

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