Articles

Recognition of the Minor Groove of DNA by Hairpin Polyamides Containing α-Substituted-β-Amino Acids

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Abstract: Incorporation of the flexible amino acid β-alanine (β) into hairpin polyamides composed of *N*-methylpyrrole (Py) and *N*-methylimidazole (Im) amino acids is required for binding to DNA sequences longer than seven base pairs with high affinity and sequence selectivity. Pairing the α-substituted-β-amino acids (*S*)-isoserine (^{S}Is), (*R*)-isoserine (^{R}Is), β-aminoalanine (Aa), and α-fluoro-β-alanine (Fb) side-by-side with β in hairpin polyamides alters DNA binding affinity and selectivity relative to the parent polyamide containing a β/β pairing. Quantitative DNase I footprinting titration studies on a restriction fragment containing the sequences 5′-TGCNGTA-3′ (N = A, T, G, and C) show that the polyamide ImPy^{S}IsImPy-γ-PyPyβImPy-β-Dp (^{S}Is/β pairing) binds to N = T ($K_a = 4.5 \times 10^9$ M^{-1}) in preference to N = A ($K_a = 6.2 \times 10^8$ M^{-1}). This result stands in contrast to the essentially degenerate binding of the parent ImPyβImPy-γ-PyPyβImPy-β-Dp (β/β pairing) to N = T and N = A, and to the slight preference of ImPyβImPy-γ-PyPy^{S}IsImPy-β-Dp (β/^{S}Is pairing) to N = A over N = T. Additionally, this study reveals that incorporation of ^{R}Is, Aa, and Fb into polyamides significantly reduces binding affinity. Therefore, DNA binding in the minor groove is sensitive to the stereochemistry, steric bulk, and electronics of the substituent at the α-position of β-amino acids in hairpin polyamides containing β/β pairs.

Introduction

Small molecules which permeate cells and bind to predictable sequences of DNA can potentially control the expression of specific genes.[1] Polyamides containing *N*-methylpyrrole (Py), *N*-methylimidazole (Im), and *N*-methyl-3-hydroxypyrrole (Hp) amino acids are synthetic ligands that have an affinity and sequence specificity for DNA which rival naturally occurring DNA binding proteins.[2,3] DNA recognition depends on side-by-side amino acid pairings in the minor groove.[2−8] Antiparallel pairing of imidazole opposite pyrrole (Im/Py) selectively recognizes a G·C base pair, while a Py/Im combination recognizes a C·G base pair.[4] A Py/Py pair binds with equal affinity to A·T and T·A and in preference to G·C and C·G.[4,5] However, the unsymmetrical Hp/Py pair distinguishes T·A from A·T.[3] Covalently linking the antiparallel polyamide subunits into a hairpin structure in the N → C orientation with γ-aminobutyric acid (γ)[7] increases both binding affinity[2,7] and, through the suppression of slipped binding motifs,[8] sequence selectivity.

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An analysis of an X-ray crystal structure of a polyamide/DNA complex[4f] reveals that the curvature of polyamides comprised solely of Py, Im, and Hp rings does not perfectly match the curvature of B-form DNA, creating an upper limit of 4−5 contiguous aromatic rings for minor groove binding.[8a] Incorporation of the flexible amino acid β-alanine (β) into polyamides relaxes the curvature, allowing for selective recognition of significantly longer sequences of DNA.[8c−f,9] The β/β pair binds to A·T and T·A in preference to C·G and G·C[8c−f] but, like the Py/Py pair does not distinguish A·T from T·A. Since the β/β pair will be a pivotal component in polyamides designed to bind DNA sequences longer than 7 base pairs the question arises as to whether the A·T/T·A degeneracy of the β/β pair can be broken.

This work addresses the feasibility of altering the molecular recognition properties of the β/β pair through the incorporation of functionalized β-amino acids. For reasons of chemical stability functionalization was limited to the α-position of β-alanine. An examination of a model of a DNA/polyamide complex containing a β/β pair indicates that the hydrogens at the α-carbon reside in a sterically hindered environment. Additionally, the environment around each enantiotopic hydrogen at the α-position is quite different, with the *pro-R* hydrogen projecting toward the wall of the minor groove and the *pro-S* hydrogen projecting toward the floor of the minor groove. Therefore binding to DNA should be affected by both the size of the substituent and the stereochemistry of the α-carbon. To test this, the α-substituted β-amino acids (*S*)-isoserine (^{S}Is), (*R*)-isoserine (^{R}Is), (*S*)-α-fluoro-β-alanine (Fb), and (*S*)-β-aminoalanine (Aa) have been incorporated into polyamides and the binding of these polyamides to DNA has been studied (Figure 1). These substituted β-amino acids differ from β not only in size but in hydrogen bonding ability and polarity, offering the possibility for altered molecular recognition properties.

Because hairpin polyamides keep the side-by-side pairing units *in register* and disfavor slipped motifs seen in unlinked homo- and heterodimers, the relative binding affinities of the four unsymmetrical aliphatic pairs $\beta/^S$Is, $\beta/^R$Is, β/Fb, and β/Aa were characterized within the context of a 2β2 hairpin motif. Five polyamides which differ at a single β-alanine position were synthesized by solid-phase methodology (Figure 2).[10] The binding affinities of polyamides ImPyβImPy-γ-PyPyβImPyβDp (**1**), ImPy^{S}IsImPy-γ-PyPyβImPyβDp (**2**), ImPy^{R}IsImPy-γ-PyPyβImPyβDp (**3**), ImPyβImPy-γ-PyPy^{S}IsImPyβDp (**4**), ImPyAaImPy-γ-PyPyβImPyβDp (**5**), and ImPyFbImPy-γ-PyPy-βImPyβDp (**6**) (Dp = dimethylaminopropylamine) to the seven base pair sequence 5′-TGC<u>N</u>GTA-3′ (Figure 1), where <u>N</u> = A, T, G, or C, have been studied by quantitative DNase I footprinting.[11] The binding of polyamide **2** and its Fe(II)/EDTA analogue **2E** to their target sequences has also been studied by MPE·Fe(II)[12] footprinting and affinity cleaving.[13] MPE·Fe(II) footprinting affords information about the exact size and location of the binding sites, and affinity cleaving studies determine the binding orientation and stoichiometry of the polyamide/DNA complex. Quantitative DNase I footprinting titrations provide equilibrium association constants (K_a) for the polyamides with match and mismatch binding sites.

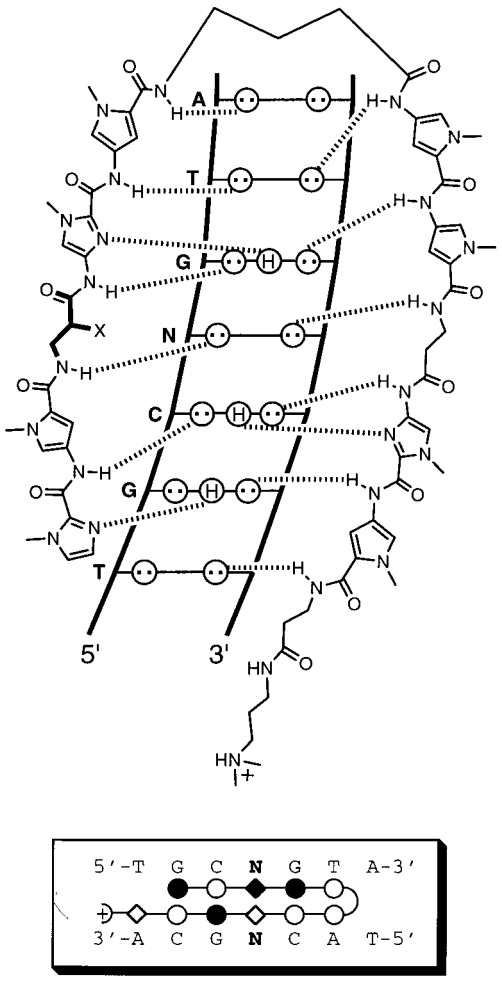

Figure 1. Binding model for the complexes formed between the DNA target sequences and ImPyAbImPy-γ-PyPyβImPy-β-Dp (Ab = α-substituted-β-amino acid). Circles with dots represent lone pairs of N3 of purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogen of guanine. Putative hydrogen bonds are illustrated by dotted lines. A ball-and-stick model is also shown. Shaded and nonshaded circles represent imidazole and pyrrole carboxamides, respectively. Nonshaded diamonds represent β-alanine, and the shaded diamond represents the α-substituted-β-amino acid.

Results and Discussion

Monomer Synthesis. The protected isoserine monomer (*S*)-**9** was synthesized through a variation of the route developed by Swindell and co-workers (Scheme 1).[14] (*S*)-glycidyl *p*-methoxyphenyl ether was prepared in enantiomerically pure form through a hydrolytic kinetic resolution[15] of the racemic epoxide **7**. The epoxide was opened with NaN_3 and the resulting azido alcohol was protected as its benzyl ether (**8**). The azide was reduced with Ph_3P and H_2O[16] and, in the same step, converted to a Boc-carbamate. The *p*-methoxyphenyl ether was removed with ceric

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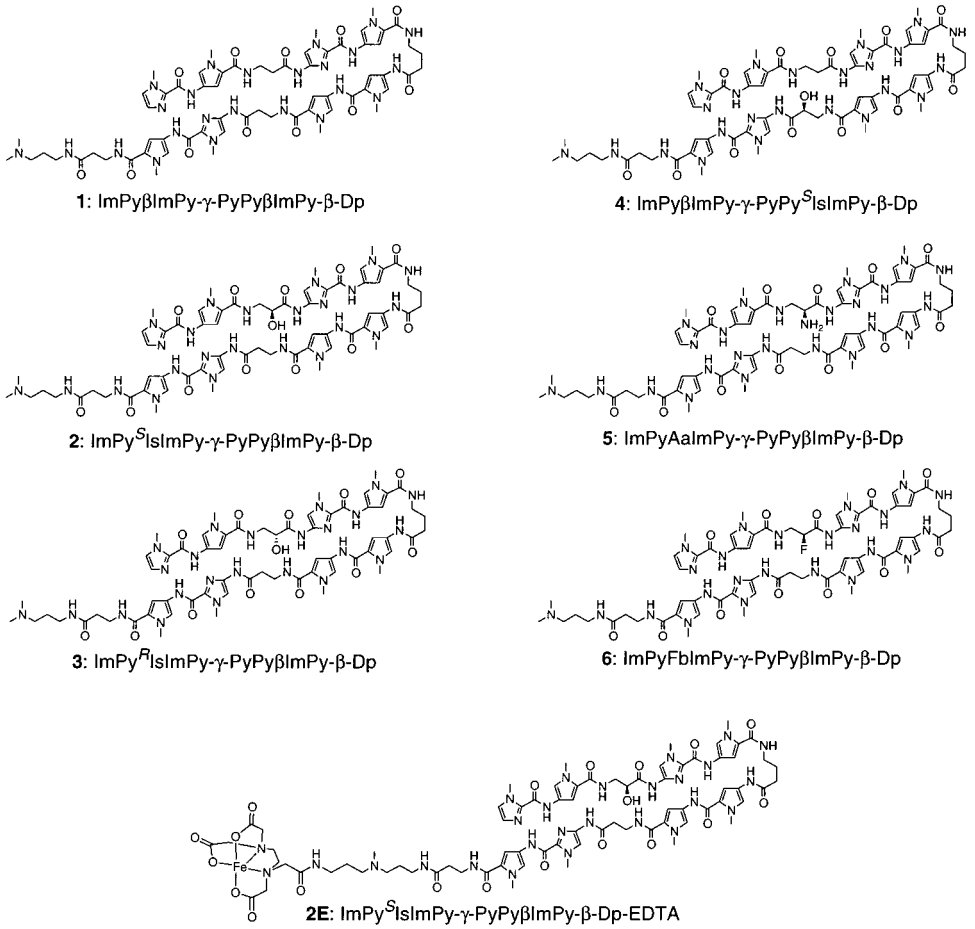
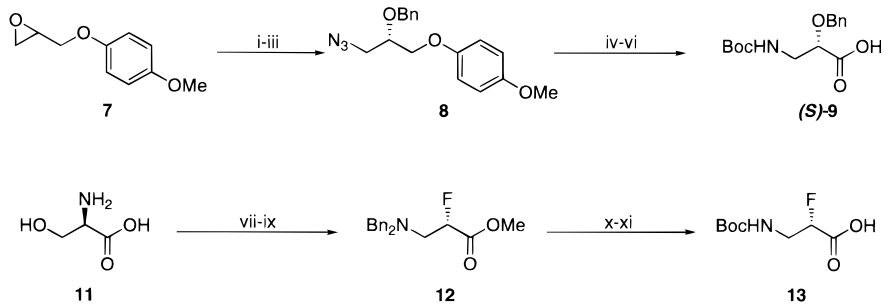


Figure 2. Polyamides synthesized for this study.

Scheme 1[a]



[a] (i) (*R*,*R*)-*N*,*N*′-bis(3, 5-di-*tert*-butylsalicylidene)-1,2-cyclohexanediaminocobalt(II), H_2O, *p*-dioxane. (ii) NaN_3, NH_4Cl, 95% EtOH. (iii) NaH, DMF, then BnBr. (iv) Ph_3P, THF, then H_2O, then $(Boc)_2O$, *i*-Pr_2NEt. (v) CAN, $NaHCO_3$, CH_3CN, H_2O. (vi) PDC, DMF. (vii) BnCl, KOH, EtOH, H_2O. (viii) Me_3SiCHN_2, MeOH, CH_2Cl_2. (ix) Et_2NSF_3, THF. (x) NH_4O_2CH, 10% Pd/C, MeOH, then $(Boc)_2O$, *i*-Pr_2NEt, *p*-dioxane. (xi) KOH, THF, H_2O.

ammonium nitrate,[17] and the primary alcohol was oxidized to carboxylic acid **9** with PDC in DMF.[18] The *R*-enantiomer of **9** (*R*-**9**) was prepared in an identical manner except that the opposite enantiomer of the Co(II)·salen catalyst was employed for the hydrolytic kinetic resolution of **7**. The products of this route were shown to have enantiomeric excesses of >98% based on an ^1H NMR analysis of the α-methylbenzyl amide derivatives of (*S*)-**9** and (*R*)-**9**. Orthogonally protected *β*-aminoalanine **10** (Scheme 2) was prepared from Boc-L-serine as reported by Roy and Imperiali.[19]

Boc-protected α-fluoro-*β*-alanine (**13**) was prepared through a variation of the sequence developed by Young and co-workers (Scheme 1).[20] (*R*)-serine (**11**) was converted to its *N*,*N*-dibenzylamine by treatment with BnCl and NaOH, and the product of this reaction was esterified by adding $TMSCHN_2$. The resulting dibenzylamino alcohol was converted to fluoride **12** with migration of the amino group and inversion of stereochemistry through the action of Et_2NSF_3. The benzyl groups were removed with hydrogenolysis, and the crude amine was directly protected as a Boc-carbamate. Carboxylic acid **13** was formed by saponification of the resulting methyl ester with

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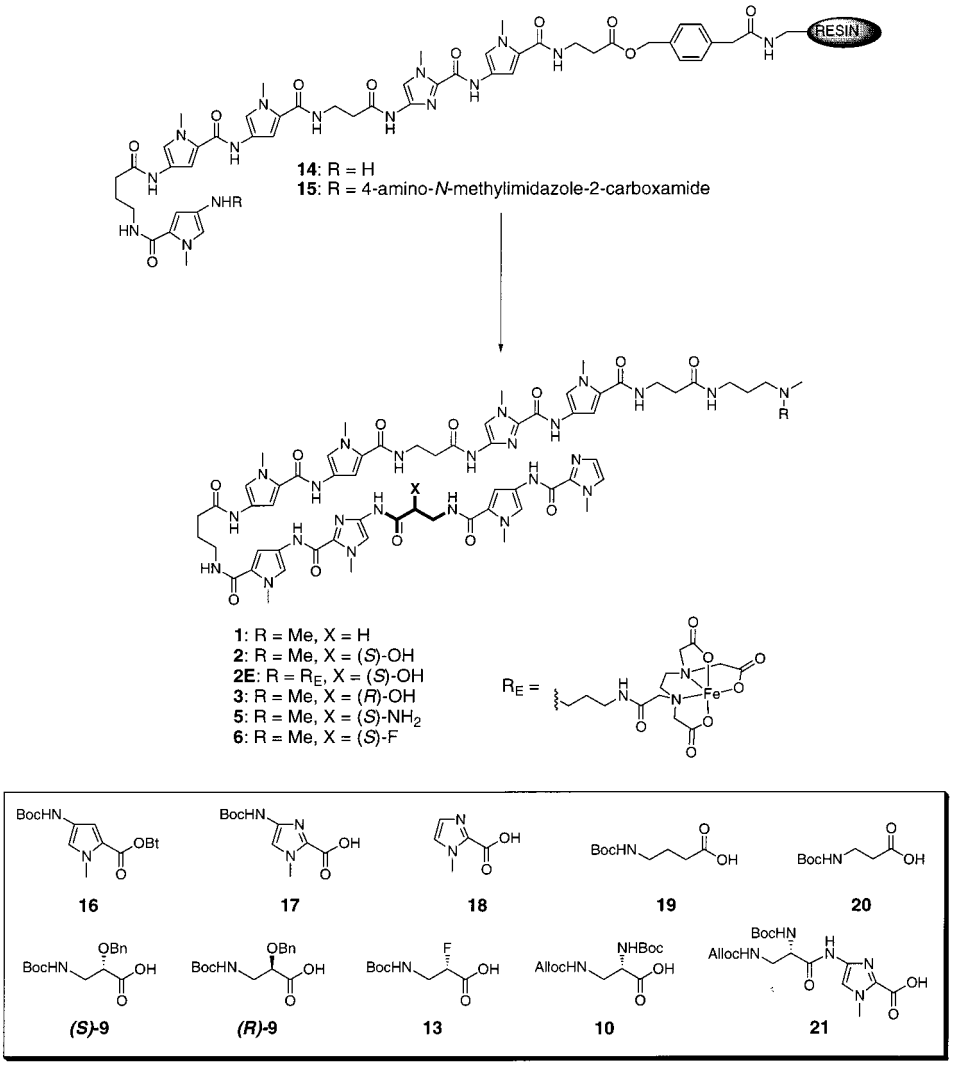
Scheme 2: Solid-Phase Synthesis of Polyamides[a]



[a] For details see text and Experimental Section.

aqueous NaOH. The enantiomeric purity of **13** was shown to be >98% through an ^{19}F NMR analysis of its α-methylbenzyl amide.

Polyamide Synthesis. Polyamides **1**−**6** were prepared using standard solid-phase synthesis starting with Boc-β-alanine Pam-resin using the monomers shown in Scheme 2. Resins **14** and **15** were prepared according to established protocols.[10] For polyamide **1** coupling proceeded smoothly from **15** using Boc-β-alanine and HBTU.[10,21] The synthesis of **1** was completed according to standard protocols.[10] Coupling of (*S*)-**9** and *(R)*-**9** to **15** was also accomplished with HBTU. The synthesis of the resins bearing the benzyl ethers of the polyamides was completed according to established procedures. The resins were cleaved with Dp and the benzyl ethers were removed with TMSBr and PhSMe in anhydrous TFA[22] to provide **2** and **3**. The resin containing **9** was also cleaved with 3,3′-diamino-*N*-methyldipropylamine. After removal of the benzyl ether the polyamide was coupled with EDTA dianhydride to provide **2E**.

Monomer **10** did not couple well to the imidazole amine of **15**. Therefore, dimer **21** was synthesized in solution from **10**.[10] HBTU-mediated coupling of **21** to resin **14** resulted in efficient incorporation of the Aa module into the polyamide. Alloc-protecting group removal was achieved with Pd_2dba_3·$CHCl_3$, Ph_3P, and $BuNH_2$·HCO_2H in THF.[23] The resulting amine was coupled to Fmoc-Py[24] with HBTU and *i*-Pr_2NEt. After deprotection of the Fmoc group with piperidine the resin was capped with *N*-methylimidazole carboxylic acid and HBTU, the Boc group was removed under standard conditions, and the polyamide was cleaved from the resin with Dp to provide **5**.

Coupling of **13** with **15** was accomplished with an HBTU coupling, and the synthesis of **6** was completed with standard conditions.

The synthesis of **4** (not shown in Scheme 2) required the coupling of ImPy-β-Pam-resin with **9**. Standard conditions were employed to complete the synthesis of the benzyl ether of **4**, which was converted to **4** according to the method used for the synthesis of **2** and **3**.

Quantitative DNase I Footprinting. Equilibrium association constants (K_a) between polyamides **1**−**6** and the 3′-^{32}P-end-labeled 294 bp restriction fragment from the cloned plasmid pSES 19-1 were determined with quantitative DNaseI foot-

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Table 1. Effects of Stereochemistry on Equilibrium Association Constants (M^{-1})[a,b]

polyamide	pairing	K_a(T)[c]	K_a(A)[d]	specificity[e]
1	β/β	1.6×10^{10} (0.5)	1.4×10^{10} (0.3)	1.1
2	^{S}Is/β	4.5×10^{9} (1.3)	6.2×10^{8} (2.3)	7.2
3	^{R}Is/β	$<3 \times 10^{8}$	$<1 \times 10^{8}$	-
4	$\beta/^{S}$Is	1.6×10^{9} (0.3)	3.2×10^{9} (0.3)	0.5

[a] Values reported for polyamides **1**, **2**, and **4** are the mean values from at least three DNase I footprinting titration experiments. Due to non-specific ligand binding exact values for K_a could not be determined for polyamide **3**. Numbers in parentheses are the standard deviations for each value. [b] The assays were performed at 22 °C at pH 7.0 in the presence of 10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$. [c] K_a(T) is the equilibrium association constant for the sequence 5′-TGCTGTA-3′. [d] K_a(A) is the equilibrium association constant for the sequence 5′-TGCAGTA-3′. [e] Specificity is defined as K_a(T)/K_a(A).

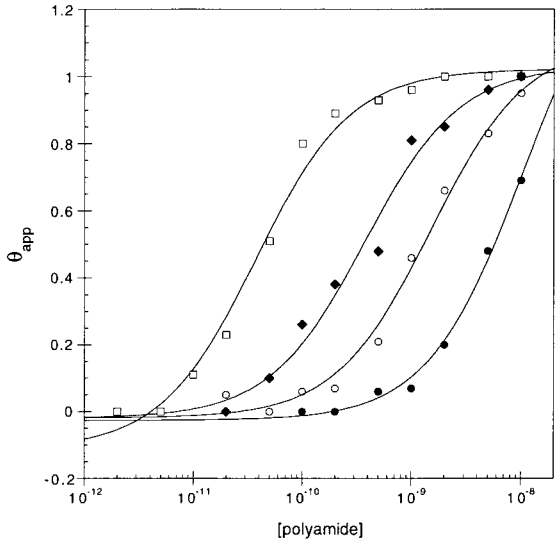

Figure 3. Binding isotherms for the DNase I footprinting titrations of polyamides **1**−**4** at the 5′-TGCTGTA-3′ site of the 292 base pair 3′-^{32}P-labeled restriction fragment from the plasmid pSES 19-1. Open squares represent polyamide **1**, filled diamonds represent polyamide **2**, filled circles represent polyamide **3**, and open circles represent polyamide **4**.

printing titrations (10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.0, 22 °C). This restriction fragment contains the four possible 5′-TGCNGTA-3′ binding sites with identical intervening sequences, providing binding affinity information for the functionalized β-amino acid/β couples against all four base pairs in a single experiment.

Effects of Stereochemistry on Binding. The binding affinities of polyamides **1**−**4** were studied in order to determine the effects of introducing a hydroxyl group and altering the stereochemistry at the α-position of the β-amino acid (Table 1 and Figure 3). As expected polyamide **1**, containing a β/β pairing, bound with essentially equal affinity to the sequences 5′-TGCTGTA-3′ and 5′-TGCAGTA-3′.[25] Virtually no binding of this polyamide (or of **2**−**6)** was observed at the sequences 5′-TGCCGTA-3′ and 5′-TGCGGTA-3′. Incorporation of the ^{S}Is/β pair (polyamide **2**), however, demonstrated a preference for binding to 5′-TGCTGTA-3′ over 5′-TGCAGTA-3′. Inversion of the stereochemistry of the α-carbon in the isoserine residue (polyamide **3**, ^{R}Is/β pairing) resulted in a substantial loss of

(25) DNase I footprinting gels of polyamides **1**, **4**, **5**, and **6** with the 3′-^{32}P-labeled restriction fragment from pSES 19-1, the MPE footprinting gel of **2** with the 5′-^{32}P-labeled restriction fragment, and the affinity cleaving gel of **2E** with the 5′-^{32}P-labeled restriction fragment are included in the Supporting Information.

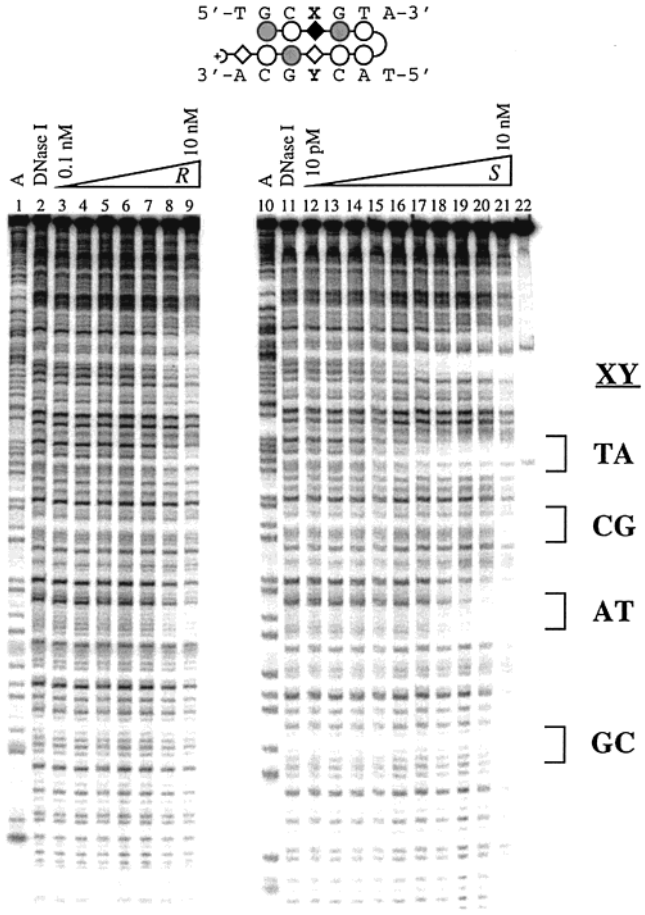

Figure 4. Quantitative DNase I footprinting titration with ImPy^{R}IsImPy-γ-PyPyβImPy-β-Dp (**3**, left) and ImPy^{S}IsImPy-γ-PyPyβImPy-β-Dp (**2**, right) on the 292-bp restriction fragment from the plasmid pSES 19-1: lane 1, A reaction; lane 2, DNase I control; lanes 3−9, 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 5 nM, and 10 nM **3**; lane 10, A reaction; lane 11, DNase I control; lanes 12−21, 10 pM, 20 pM, 50 pM, 100 pM, 200 pM, 500 pM, 1 nM, 2 nM, 5 nM, and 10 nM **2**; lane 22, intact DNA. The four putative binding sites are shown on the right of the autoradiograms. All reactions contain a 15 kcpm restriction fragment, 10 mM Tris·HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

binding affinity at all sites (Figure 4). Precise determinations of equilibrium association constants for **3** were impossible due to significant nonspecific ligand binding at concentrations required to observe a footprint. Noteworthy in these experiments was that polyamide **4**, which contains a $\beta/^{S}$Is pairing, binds to the sequence 5′-TGCAGTA-3′ preferentially with respect to 5′-TGCTGTA-3′.[25] Although the preference is modest, this result demonstrates that the T·A/A·T binding degeneracy normally observed with the β/β pairing can be altered in a predictable manner through the incorporation of either the ^{S}Is/β or $\beta/^{S}$Is pairings. The association constants of **1**, **2**, and **4** reveal that, although the introduction of a hydroxyl group destabilizes binding to both the T and A sites, the destabilization is greater at the A site than at the T site.

Effects of Substituent on Binding. The binding affinities of polyamides **2**, **5**, and **6**, were compared to evaluate the effects of changing the substituent of the α-substituted-β-amino acid (Table 2). Polyamide **5**, containing the Aa/β pairing and polyamide **6**, containing the Fb/β pairing, showed remarkably weak binding to the A·T and T·A sequences.

MPE·Fe(II) Footprinting and Affinity Cleavage. MPE footprinting studies were performed on polyamide **2** and affinity

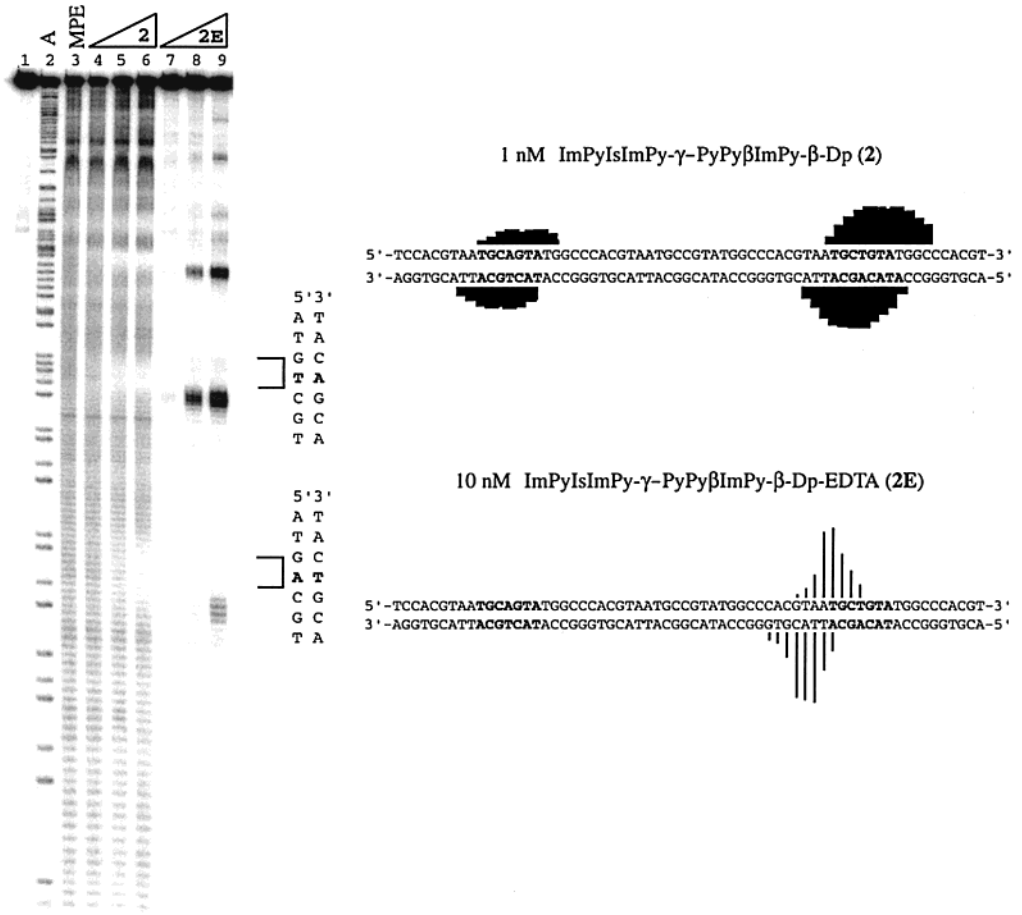

Figure 5. (a) MPE·Fe(II) footprinting titration of **2** and affinity cleaving study of **2E** on the 292-bp 3′-^{32}P-labeled restriction fragment from the plasmid pSES 19-1: lane 1, intact DNA; lane 2, A reaction; lane 3, MPE control; lanes 4−6, 100 pM, 1 nM, and 10 nM **2**; lanes 7−9, 100 pM, 1 nM, and 10 nM **2E**. The sequences 5′-TGCTGTA-3′ and 5′-TGCAGTA-3′ are shown at the right of the autoradiogram. All reactions contain a 15 kcpm restriction fragment, 20 mM HEPES buffer (pH 7.0), and 10 mM NaCl. (b) Results from MPE·Fe(II) footprinting titration of **2**. Bold sequences represent binding sites determined by the published model. Bar heights are proportional to the relative protection from cleavage at each band. (c) Results from the affinity cleaving study of **2E**. Bold sequences represent binding sites determined by the published model. Line heights are proportional to the relative cleavage at each band.

Table 2. Effects of Substituent on Equilibrium Association Constants (M^{-1})[a,b]

polyamide	pairing	K_a(T)[c]	K_a(A)[d]	specificity[e]
1	β/β	1.6×10^{10} (0.5)	1.4×10^{10} (0.3)	1.1
2	^{S}Is/β	4.5×10^{9} (1.3)	6.2×10^{8} (2.3)	7.2
5	Aa/β	1.8×10^{8} (0.1)	1.1×10^{8} (0.2)	1.6
6	Fb/β	4.9×10^{8} (1.0)	3.4×10^{8} (0.2)	1.4

[a] Values are the mean values from at least three DNase I footprinting titration experiments. Numbers in parentheses are the standard deviations for each value. [b] The assays were performed at 22 °C at pH 7.0 in the presence of 10 mM Tris·HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$. [c] K_a(T) is the equilibrium association constant for the sequence 5′-TGCTGTA-3′. [d] K_a(A) is the equilibrium association constant for the sequence 5′-TGCAGTA-3′. [e] Specificity is defined as K_a(T)/K_a(A).

cleaving studies were performed on polyamide **2E** with both the 3′- and the 5′-^{32}P-end-labeled restriction fragment 294 bp restriction fragment from pSES 19-1 in order to confirm the exact location of the binding site and the binding orientation (Figure 5).[25] These experiments demonstrated that the incorporation of an ^{S}Is residue into a hairpin polyamide alters only binding affinity and selectivity and not the expected binding site or orientation.

Basis for the Substituent Effects. A recent NMR study of the complex of a polyamide containing a β/β pairing in the minor groove of DNA[9] offers insight into the origin of the results of this study. Introducing a substituent to the *pro-R* α-position of β causes a steric clash with the minor groove and, thereby, diminishes binding affinity. When introduced to the *pro-S* α-position of β a small substituent, such as a hydroxyl group, can fit into the floor of the minor groove without a significant energetic cost. The cleft of the T·A base pair accommodates the hydroxyl group of ^{S}Is, whereas H2 of adenine in the A·T base pair introduces an unfavorable interaction with the hydroxyl group. Additionally, the possibility of a second hydrogen bond to O2 of T exists when ^{S}Is is placed opposite T. This explanation for the binding selectivity of the ^{S}Is/β pairing is consistent with the conclusions drawn from an X-ray crystal structure the complex of a polyamide containing an Hp/Py pairing in the minor groove.[3b] The amino group of Aa, which is expected to be protonated under the conditions of the assay, interacts unfavorably with the 3′-base. The poor binding of polyamide **6** is somewhat curious given the small size of fluorine and indicates that the electron density of fluorine could possibly introduce an electrostatic repulsion with the electron-rich minor groove.

Implications for the Design of Minor Groove Binding Molecules. These results demonstrate that the incorporation of α-substituted-β-amino acids into hairpin polyamides can significantly alter the DNA binding properties of these molecules relative to polyamides containing β-alanine. Binding is sensitive to the stereochemistry, steric bulk, and electronic properties of

the α-substituent. Polyamides with the ^{S}Is/β pairing show moderate selectivity for binding to T·A over A·T and suffer a modest loss in binding affinity compared to the parent polyamide containing a β/β pairing. This demonstrates that binding selectivity can be modulated through manipulation of the flexible linker unit. The effects of incorporating the ^{S}Is/β pair on cellular uptake and transcription inhibition remain to be studied and will be reported in due course.

Experimental Section

General. Dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Boc-β-alanine, Boc-γ-aminobutyric acid, and 0.2 mmol/g Boc-β-alanine-(4-carboxamidomethyl)-benzyl-ester-copoly-(styrene-divinylbenzene) resin (Boc-β-Pam-resin) were purchased from Peptides International. *N*,*N*-diisopropylethylamine, *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), and acetic anhydride were purchased from Applied Biosystems. Reagent grade dichloromethane and triethylamine were purchased from EM. Biograde trifluoroacetic acid (TFA) was purchased from Halocarbon. All other chemicals were purchased from Aldrich. All reagents were used without further purification. A shaker for manual solid-phase synthesis was obtained from Thermolyne. Screw-cap glass peptide synthesis reaction vessels (5 mL and 20 mL) with a no. 2 sintered glass frit were made as described by Kent.[26] ^{1}H NMR spectra were recorded on a General Electric-QE NMR 300 MHz spectrometer. Infrared spectra were recorded on a Perkin-Elmer Paragon 1000 FT-IR spectrometer. 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 1090M analytical HPLC or a Beckman Gold system using a RAINEN C18, 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. Preparatory reverse phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 × 100 mm, 100 μm C18 column equipped with a guard, 0.1% (wt/v) TFA, 0.25% acetonitrile/min. 18Ω water was obtained from a Millipore MilliQ water purification system, and all buffers were filtered through a 0.2 μm membrane. Polyamide **1** and resins **14** and **15** were prepared as previously described.[10]

(*S*)-Glycidyl *p*-Methoxyphenyl Ether ((*S*)-7). To (*R*, *R*)-*N*,*N*′-bis-(3, 5-di-*tert*-butylsalicylidene)-1, 2-cyclohexanediaminocobalt(II) (120 mg, 0.2 mmol) in toluene (2 mL) was added glacial HOAc (24 mg, 0.4 mmol). The mixture was stirred at room temperature for 1 h. The toluene was removed under reduced pressure. To the brown residue was added (±)-glycidyl *p*-methoxyphenyl ether (3.6 g, 20 mmol) and H_2O (210 mg, 12 mmol) in *p*-dioxane (2 mL). The reaction was stirred at room temperature for 18 h. The mixture was purified by flash chromatography (30% EtOAc in hexanes) to provide the enantiomerically pure epoxide (1.2 g, 33% recovery, 83% of the theoretical yield). $[\alpha]^{22}_{D} = +9.4°$ (*c* 0.16, acetone).

(2*S*)-1-Azido-3-(4-methoxyphenoxy)propan-2-ol.[14] To the glycidyl ether (4.5 g, 25 mmol) in 95% EtOH (250 mL) were added NaN_3 (8.2 g, 125 mmol) and NH_4Cl (6.7 g, 125 mmol). The mixture was stirred at room temperature for 14 h and was then filtered and concentrated. The residue was partitioned between EtOAc and H_2O. The organics were dried (Na_2SO_4), filtered, and concentrated. The residue was purified by flash chromatography (30% EtOAc in hexanes) to provide the azido alcohol (5.5 g, 98%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.50 (d, 1H, J = 5.0 Hz), 3.50 (m, 2H), 3.76 (s, 3H), 3.95 (m, 2H), 4.13 (m, 1H), 6.83 (s, 4H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 54.0, 56.3, 70.0, 70.4, 115.3, 115.5, 152.9, 154.9 ppm; IR (neat) ν_{max} 3432, 2934, 2102, 1508, 1230, 1043, 825 cm^{-1}.

(2*S*)-3-(4-Methoxyphenoxy)-2-(phenylmethoxy)propylazide (8). To NaH (60%, 1.0 g, 25 mmol) in DMF (30 mL) at 0 °C was added the azido alcohol (2.0 g, 8.3 mmol) in DMF (20 mL). The mixture was stirred at 0 °C for 45 min. To the resulting alkoxide was added BnBr (2.0 g, 12 mmol). The mixture was stirred for 3 h while slowly warming to room temperature. The reaction was quenched by adding H_2O, and the organic material was extracted into EtOAc. The EtOAc layer was washed with brine (4 × 50 mL) and was then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (16% EtOAc in hexanes) to provide the benzyl ether (2.2 g, 79%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.52 (t, 2H, J = 4.6 Hz), 3.79 (s, 3H), 3.98 (m, 3H), 4.75 (s, 2H), 6.85 (s, 4H), 7.39 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 51.7, 55.5, 67.7, 72.3, 76.2, 114.2, 115.2, 127.7, 128.3, 137.5, 152.3, 153.9 ppm; IR (neat) ν_{max} 3022, 2933, 2872, 2835, 2102, 1509, 1232, 1041, 825 cm^{-1}; Exact mass calcd for $C_{17}H_{19}N_3O_3$: 313.1426. Found: 313.1435 (EI).

***N*-[(2*S*)-3-(4-Methoxyphenoxy)-2-(phenylmethoxy)propyl](*tert*-butoxy)carboxamide.** To azide **8** (2.1 g, 6.7 mmol) in THF (50 mL) was added Ph_3P (2.1 g, 8.1 mmol). The mixture was stirred at room temperature for 1 h. To the mixture was added H_2O (2 mL). The mixture was stirred at room temperature for 2 h. To this solution were added Et_3N (2.0 mL, 1.4 g, 14 mmol) and $(Boc)_2O$ (1.8 g, 8.1 mmol). The mixture was stirred at room temperature for 1 h, then was partitioned between EtOAc and H_2O. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The residue was purified by flash chromatography (15% EtOAc in hexanes) to provide the carbamate (1.2 g, 46%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 9H), 3.33 (m, 1H), 3.48 (m, 1H), 3.75 (s, 3H), 3.85 (m, 1H), 3.98 (d, 2H, J = 5.1 Hz), 4.64 (d, 1H, J = 11.7 Hz) 4.73 (d, 1H, J = 11.7 Hz), 4.83 (br s, 1H), 6.82 (s, 4H), 7.30 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 28.9, 42.2, 56.3, 69.7, 72.9, 76.8, 115.2, 116.1, 128.4, 128.5, 129.0, 138.7, 153.3, 156.6 ppm; IR (neat) ν_{max} 3366, 2976, 2932, 1712, 1509, 1232, 1170, 1040 cm^{-1}; Exact mass calcd for $C_{22}H_{29}NO_5$: 387.2046. Found: 387.2042 (EI).

***N*-[(2*S*)-3-Hydroxy-2-(phenylmethoxy)propyl](*tert*-butoxy)carboxamide.** To the *p*-methoxyphenyl ether (1.2 g, 3.1 mmol) in CH_3CN (40 mL) and H_2O (10 mL) were added $NaHCO_3$ (2.1 g, 25 mmol) and ceric ammonium nitrate (4.5 g, 8.2 mmol). The mixture was stirred at room temperature for 15 min and then was diluted with EtOAc and washed with brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The residue was purified by flash chromatography (30% EtOAc in hexanes to 50% EtOAc/50% hexanes) to provide the alcohol (630 mg, 72%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.44 (s, 9H), 3.25 (m, 3H), 3.40 (dd, 1H, J = 3.3, 7.0 Hz), 3.57 (m, 2H), 4.58 (s, 2H), 4.95 (m, 1H), 7.36 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 28.1, 39.9, 60.8, 71.5, 76.4, 79.6, 127.5, 127.7, 128.3, 137.8, 156.8 ppm; IR (neat) ν_{max} 3359, 2976, 2932, 1691, 1513, 1366, 1252, 1170 cm^{-1}; Exact mass calcd for $C_{15}H_{23}NO_4$: 282.1627. Found: 282.1696 (EI).

(2*S*)-3-[(*tert*-Butoxy)carbonylamino]-2-(phenylmethoxy)propanoic Acid (9). To the alcohol (580 mg, 2.1 mmol) in DMF (15 mL) was added PDC (3.0 g, 8.0 mmol). The mixture was stirred at room temperature for 16 h and was then diluted with EtOAc and washed several times with brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The residue was purified by flash chromatography (100% EtOAc to 10% MeOH in EtOAc) to provide the carboxylic acid (230 mg, 38%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.39 (s, 9H), 3.44 (br m, 1H), 3.56 (br m, 1H), 4.03 (br s, 1H), 4.47 (d, 1H, J = 11.5 Hz), 4.76 (d, 1H, J = 11.5 Hz), 5.03 (br s, 1H), 7.30 (m, 5H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 28.9, 42.9, 73.0, 80.3, 128.6, 128.7, 129.0, 130.6, 137.7, 156.5, 174.2 ppm; IR (neat) ν_{max} 3352, 2978, 1715, 1515, 1367, 1252, 1168, 1122 cm^{-1}; $[\alpha]^{22}_{D} = -47.1°$ (*c* 0.31, acetone); Exact mass [M + H] calcd for $C_{16}H_{21}NO_5$: 296.1498. Found: 296.1499 (DCI).

Determination of the enantiomeric purity of *(S)*-9 and *(R)*-9. To ***(S)*-9** or ***(R)*-9** (50 mg, 0.17 mmol) in DMF (2.5 mL) were added HBTU (250 mg, 0.7 mmol) and *i*-Pr_2NEt (0.5 mL). The mixture was stirred at room temperature for 15 min. To the mixture was added (*S*)-α-methylbenzylamine (125 mg, 1.0 mmol). The mixture was stirred at room temperature for 5 h, then was diluted with EtOAc. The organic layer was washed successively with 10% aqueous citric acid and brine. The organic material was dried (Na_2SO_4), filtered, and concentrated. The residue was filtered through a short plug of silica gel (40% EtOAc in hexanes) to provide the amide (64 mg, 88%). The final products were analyzed by ^{1}H NMR (300 MHz, $CDCl_3$). The amide from ***(S)*-9**

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

showed a triplet ($J = 6.0$ Hz) at δ 3.89 ppm and the amide from *(R)*-**9** showed a triplet ($J = 6.0$ Hz) at δ 3.93 ppm. In both spectra no evidence of diastereomeric contamination (<3%) was observed.

Methyl (*2S*)-3-[(*tert*-Butoxy)carbonylamino]-2-fluoropropanoate. To **12**[20] (500 mg, 1.7 mmol) in MeOH (15 mL) were added 10% Pd/C (75 mg) and ammonium formate (1.5 g, 24 mmol). The mixture was immersed in a preheated oil bath (80 °C). The mixture was stirred at reflux for 1.75 h. The mixture was filtered over Celite and the filtrate was concentrated. The residue was swirled in EtOAc, and the solution was decanted away from the solids. The solution was concentrated to provide the amine. To the crude amine in *p*-dioxane (12 mL) were added Boc_2O (750 mg, 2.9 mmol) and *i*-Pr_2NEt (1 mL, 740 mg, 5.7 mmol). The mixture was stirred at room temperature for 2 h, then was concentrated. The residue was purified by flash chromatography (33% EtOAc in hexanes) to provide the protected amine (90 mg, 24%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.41 (s, 9H), 3.65 (m, 2H), 3.78 (s, 3H), 4.88 (m, 1.5H), 5.04 (m, 0.5H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 28.8, 42.7, 43.0, 53.1, 87.0, 89.4, 156.1, 168.7 ppm; IR (neat) ν_{max} 3376, 2979, 1766, 1715, 1520, 1368, 1251, 1170, 1109 cm^{-1}.

(*2S*)-3-[(*tert*-Butoxy)carbonylamino]-2-fluoropropanoic Acid (13). To Boc-α-fluoro-*β*-alanine methyl ester (90 mg, 0.4 mmol) in MeOH (5 mL) was added aqueous NaOH (1.0 M, 2 mL, 2 mmol). The mixture was stirred at room temperature for 1.5 h. The MeOH was removed under reduced pressure, and the aqueous solution was acidified to pH 1 with aqueous HCl (1 M). The mixture was extracted with EtOAc to provide the carboxylic acid. ^{1}H NMR (MeOH-d4, 300 MHz) δ 1.47 (s, 9H), 3.55 (m, 2H), 4.96 (ddd, 1H, $J = 49.0$, 6.6, 2.6 Hz) ppm; ^{13}C NMR (MeOH-d4, 75 MHz) δ 38.4, 52.7, 90.2, 97.5, 99.9, 167.9, 181.1, 181.4 ppm; IR (neat) ν_{max} 3369, 2990, 1740, 1698, 1525, 1254, 1160, 1104 cm^{-1}; $[\alpha]^{22}_D = -6.0°$ (*c* 0.23, acetone); Exact mass [M + H] calcd for $C_8H_{15}FNO_4$: 208.0985. Found: 208.0988 (DCI).

Determination of the Enantiomeric Purity of 13. To ***(S)*-13** or *(R)*-**13** (9 mg, 0.044 mmol) in DMF (1.0 mL) were added HBTU (17 mg, 0.14 mmol) and *i*-Pr_2NEt (10 μL). The mixture was stirred at room temperature for 15 min. To the mixture was added (*S*)-α-methylbenzylamine (12 mg, 0.1 mmol). The mixture was stirred at room temperature for 5 h and then was diluted with EtOAc. The organic layer was washed successively with 10% aqueous citric acid and brine. The organic material was dried (Na_2SO_4), filtered, and concentrated. The residue was filtered through a short plug of silica gel (100% EtOAc) to provide the amide (10 mg, 73%). The final products were analyzed by ^{19}F NMR (470 MHz, $CDCl_3$). The amide from *(S)*-**13** showed a doublet of triplets ($J = 47$, 23 Hz) at δ −24.2 ppm and the amide from *(R)*-**13** showed a doublet of triplets ($J = 50$, 23 Hz) at δ −24.4 ppm. In both spectra no evidence of diastereomeric contamination (<3%) was observed.

ImPy^{S}IsImPy-*γ*-PyPy*β*ImPy-*β*-Dp (2). To *(S)*-**9** (400 mg, 1.4 mmol) in DMF (2 mL) were added HBTU (500 mg, 1.3 mmol) and *i*-Pr_2NEt (1 mL). The mixture was agitated for 30 s and was then allowed to stand for 5 min. The mixture was added to resin **15** (600 mg, 0.12 mmol) and then was agitated at 37 °C for 12 h. Completion of the synthesis of resin-bound benzylated **2** proceeded according to established protocols.[10] To the resin (500 mg, 0.1 mmol) was added 3-dimthylaminopropylamine (2 mL). The mixture was shaken at 37 °C for 10 h then was filtered. The residue was purified by reverse phase HPLC and was lyophilized to provide benzylated **2** (30 mg, 20% recovery). To benzylated **2** (28 mg, 0.02 mmol) in TFA (1 mL) at 0 °C were added thioanisole (300 mg, 2.4 mmol) and TMSBr (300 mg, 2.0 mmol). The mixture was stirred at 0 °C for 3 h. The residue was purified by reverse phase HPLC and was lyophilized to provide **2** (8 mg, 29% recovery). MALDI-TOF-MS (monoisotopic) 1381.7 (1381.7, calcd for $C_{63}H_{81}N_{24}O_{13}$); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.72 (m, 4H), 2.23 (m, 2H), 2.31 (m, 2H), 2.55 (m, 2H), 2.70 (s, 3H), 2.72 (s, 3H), 2.9−3.6 (m, 8H), 3.70 (s, 9H), 3.92 (s, 3H), 3.94 (s, 6H), 4.27 (m, 1H), 6.93 (s, 1H), 6.98 (s, 1H), 7.05 (s, 1H), 7.20 (s, 1H), 7.21 (s, 1H), 7.22 (s, 1H), 7.28 (s, 1H), 7.40 (s, 1H), 7.44 (s, 1H), 7.49 (s, 1H), 8.05 (m, 2H), 9.51 (s, 1H), 9.82 (s, 1H), 9.85 (s, 1H), 9.94 (s, 1H), 9.94 (s, 1H), 9.94 (s, 1H), 10.36 (s, 1H), 10.48 (s, 1H) ppm; $[\alpha]^{22}_D = -2.4°$ (*c* 0.02, H_2O).

ImPy^{S}IsImPy-*γ*-PyPy*β*ImPy-*β*-Dp-EDTA (2E). To the resin containing benzylated **2** (50 mg, 0.01 mmol) was added *N*,*N*,-bis(3-propylamino)-*N*-methylamine (1 mL). The mixture was shaken at 37 °C for 10 h and then was filtered. The residue was purified by reverse phase HPLC and was lyophilized. To the product of the cleavage in TFA (0.5 mL) at 0 °C were added thioanisole (100 mg, 0.8 mmol) and TMSBr (100 mg, 0.7 mmol). The mixture was stirred at 0 °C for 2 h and then was purified by reverse phase HPLC. The product was lyophilized and then was dissolved in DMSO (1 mL). To this solution was added EDTA dianhydride (50 mg, 0.2 mmol) in DMSO (0.5 mL) and NMP (0.5 mL). The mixture was shaken at 37 °C for 3 h. To the mixture was added aqueous NaOH (1 M, 1 mL). The mixture was shaken an additional 30 min at 37 °C. The mixture was purified by reverse phase HPLC to provide **2E** (0.2 mg, 1% recovery from resin). MALDI-TOF-MS (monoisotopic) 1698.8 (1698.8, calcd for $C_{75}H_{100}N_{27}O_{20}$).

ImPy^{R}IsImPy-*γ*-PyPy*β*ImPy-*β*-Dp (3). The preparation of **3** from **15** was conducted in a manner identical to that of the synthesis of **2**. MALDI-TOF-MS (monoisotopic) 1381.6 (1381.7, calcd for $C_{63}H_{81}N_{24}O_{13}$); $[\alpha]^{22}_D = +2.7°$ (*c* 0.04, H_2O).

ImPy*β*ImPy-*γ*-PyPy^{S}IsImPy-*β*-Dp (4). To *(S)*-**9** (400 mg, 1.4 mmol) in DMF (2 mL) were added HBTU (500 mg, 1.3 mmol) and *i*-Pr_2NEt (1 mL). The mixture was agitated for 30 s and then was allowed to stand for 5 min. The mixture was added to ImPy-*β*-Pam resin (700 mg, 0.14 mmol, prepared according to established protocols[10]) and the reaction was shaken at 37 °C for 10 h. The completion of the synthesis of benzylated **4** proceeded according to established protocols. To benzylated **4** (50 mg, 0.03 mmol) in TFA (1 mL) at 0 °C were added thioanisole (300 mg, 2.4 mmol) and TMSBr (300 mg, 2.0 mmol). The mixture was stirred at 0 °C for 3 h. The residue was purified by reverse phase HPLC and was lyophilized to provide **4** (15 mg, 9% recovery from resin). MALDI-TOF-MS (monoisotopic) 1381.9 (1381.7, calcd for $C_{63}H_{81}N_{24}O_{13}$); ^{1}H NMR (DMSO-d_6) δ 1.71 (m, 4H), 2.35 (m, 4H), 2.53 (m 2H), 2.68 (s, 6H), 2.8−3.5 (m, 8H), 3.70 (s, 3H), 3.74 (s, 6H), 3.75 (s, 9H), 3.89 (s, 3H), 3.93 (s, 3H), 4.23 (m, 1H), 6.92 (s, 1H), 7.03 (s, 1H), 7.16 (s, 1H), 7.19 (s, 1H), 7.37 (s, 1H), 7.41 (s, 1H), 7.45 (s, 1H), 8.03 (m, 2H), 9.43 (s, 1H), 9.81 (s, 1H), 9.89 (s, 1H), 10.19 (s, 1H), 10.27 (s, 1H), 10.43 (s, 1H) ppm; $[\alpha]^{22}_D = +6.8°$ (c 0.07, H_2O).

ImPyAaImPy-*γ*-PyPy*β*ImPy-*β*-Dp (5). To **10**[19] (250 mg, 0.87 mmol) in DMF (3 mL) at room temperature were added HOBt (140 mg, 1 mmol) and DCC (1.0 mL, 1 M in NMP, 1 mmol). The reaction was stirred at room temperature for 6 h. The mixture was filtered into a solution of ethyl 4-amino-1-methylimidazole-2-carboxylate[10] (320 mg, 1 mmol). To this solution was added *i*-Pr_2NEt (1 mL). The reaction was stirred at room temperature for 12 h and then was diluted with EtOAc and washed (4×) with brine. The organic material was dried ($MgSO_4$), filtered, and concentrated. The residue was dissolved in MeOH (5 mL). To this mixture was added aqueous NaOH (5 mL, 5 M, 5 mmol). The reaction was stirred at room temperature for 1 h and then was acidified to pH 1 with 10% aqueous HCl. The product was extracted into EtOAc and then was dried (Na_2SO_4), filtered, and concentrated to provide crude **21** (110 mg). To **21** (110 mg, 0.3 mmol) in DMF (2 mL) were added HBTU (200 mg, 0.6 mmol) and *i*-Pr_2NEt (0.8 mL). The mixture was agitated for 30 s and then was allowed to stand for 5 min. The mixture was added to resin **14** (200 mg, 0.04 mmol) and was shaken at 37 °C for 3 h. The solvent was drained from the vessel, and the resin was washed with THF. To the resin were added $Pd_2(dba)_3$ (120 mg), Ph_3P (500 mg), and $BuNH_2 \cdot HCO_2H$ in THF (10 mL). The mixture was shaken at room temperature for 2 h. The liquids were drained, and the resin was washed several times with a solution prepared from $Na_2S_2C(NEt_2)$ (1.0 g) and Et_3N (1.0 mL) in DMF (100 mL). To the resin were added Fmoc-pyrrole (250 mg), HBTU (320 mg), and *i*-Pr_2NEt (1 mL) in DMF (2 mL). The mixture was shaken at 37 °C for 1 h. The liquids were drained, and the Fmoc was removed by piperidine (20% in DMF, 30 min, room temperature). The synthesis was completed according to established protocols, except that the resin was washed with TFA before cleavage with Dp. To the resin was added dimethylaminopropylamine (2 mL). The mixture was shaken at 37 °C for 10 h and then was filtered. The residue was purified by reverse phase HPLC and was lyophilized to provide 5 (4 mg, 8% recovery). MALDI-TOF-MS (monoisotopic) 1380.7 (1380.7, calcd for $C_{63}H_{82}N_{25}O_{12}$); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 1.72 (m, 4H), 2.31 (m,

2H), 2.45 (m, 2H), 2.53 (m, 2H), 2.69 (s, 6H), 2.95 (m, 2H), 3.06 (m, 2H), 3.15 (m, 2H), 3.36 (m, 3H), 3.80 (s, 3H), 3.82 (s, 3H), 3.85 (s, 3H), 3.89 (s, 6H), 3.91 (s, 9H), 6.80 (s, 1H), 6.89 (s, 1H), 6.92 (s, 1H), 6.99 (s, 1H), 7.03 (s, 1H), 7.11 (s, 1H), 7.12 (s, 1H), 7.13 (s, 1H), 7.16 (s, 1H), 7.19 (s, 1H), 7.34 (s, 1H), 7.41 (s, 1H), 7.47 (s, 1H), 8.02 (m, 2H), 9.80 (s, 1H), 9.82 (s, 1H), 9.90 (s, 1H), 9.94 (s, 1H), 10.24 (s, 1H), 10.47 (s, 1H), 10.92 (s, 1H) ppm; $[\alpha]^{22}_D = -3.1°$ (*c* 0.06, H_2O).

ImPyFbImPy-γ-PyPyβImPy-β-Dp (6). To **13** (75 mg, 0.4 mmol) in DMF (1.5 mL) were added HBTU (180 mg, 0.5 mmol) and *i*-Pr_2-NEt (0.8 mL). The mixture was agitated for 30 s and was allowed to stand for 5 min. The mixture was added to resin **15** (100 mg, 0.02 mmol) and was shaken at 37 °C for 12 h. The completion of the synthesis proceeded according to standard protocols[10] to provide **6** (8 mg, 30% recovery). MALDI-TOF-MS (monoisotopic) 1383.6 (1838.7, calcd for $C_{63}H_{80}FN_{24}O_{12}$); ^{1}H NMR (DMSO-$d_6$) δ 1.72 (m, 4H), 2.5−3.3 (m, 12H), 2.75 (s, 3H), 2.78 (s, 3H), 3.75 (s, 9H), 3.88 (s, 6H), 3.92 (s, 6H), 3.93 (s, 3H), 6.80 (s, 1H), 6.89 (s, 1H), 6.93 (s, 1H), 6.99 (s, 1H), 7.11 (s, 1H), 7.13 (s, 1H), 7.16 (s, 1H), 7.22 (s, 1H), 7.34 (s, 1H), 7.41 (s, 1H), 7.48 (s, 1H), 8.03 (m, 2H), 9.80 (s, 1H), 9.82 (s, 1H), 9.91 (s, 1H), 10.02 (s, 1H), 10.24 (s, 1H), 10.40 (s, 1H), 10.50 (s, 1H) ppm; $[\alpha]^{22}_D = +1.4°$ (*c* 0.08, H_2O).

DNA Reagents and Materials. Enzymes were purchased from Boehringer-Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P]triphosphates, and deoxyadenosine 5′-[γ-^{32}P]triphosphate were purchased from New England Nuclear. 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 established protocols.[27]

Preparation of 3′- and 5′-End-Labeled Restriction Fragments. The plasmid pSES 19-1 was constructed according to standard protocols. pSES 19-1 was linearized with *Eco*RI and *Pvu*II restriction enzymes, then treated with the Klenow fragment, deoxyadenosine 5′-[α-^{32}P]triphosphate and thymidine 5′-[α-^{32}P]triphosphate for 3′ labeling. Alternatively, these plasmids were linearized with *Eco*RI, treated with calf alkaline phosphatase, and then 5′-labeled with T4 polynucleotide kinase and deoxyadenosine 5′-[γ-^{32}P]triphosphate. The 5′-labeled fragment was then digested with *PvuII*. The labeled fragment (3′ or 5′) was loaded onto a 7% non-denaturing polyacrylamide gel, and the desired 292 base pair band was visualized by autoradiography and isolated.

DNase I Footprinting.[11] All reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were: 10 mM Tris·HCl buffer (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 15 kcpm 3′-radiolabeled DNA. The solutions were allowed to equilibrate for 2−12 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 ethanol precipitated. The cleavage products were resuspended in 100 mM Tris-borate-EDTA/80% formamide loading buffer, denatured at 85 °C for 10 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% cross-link, 7 M urea) at 2000 V for 1.5 h. The gels were dried under vacuum at 80 °C, then quantified using storage phosphor technology. Equilibrium association constants were determined as previously described.[8]

MPE·Fe(II) Footprinting.[12] All reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were: 20 mM HEPES buffer (pH 7.0), 10 mM NaCl, and 15 kcpm 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 3 h. A fresh 50 μM MPE·Fe(II) solution was prepared from 100 μL of a 100 μM MPE solution and 100 μL of a 100 μM ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) solution. MPE·Fe(II) solution (5 μM) was added to the equilibrated DNA, and the reactions were allowed to equilibrate for 10 min. Cleavage was initiated by the addition of dithiothreitol (5 mM) and allowed to proceed for 14 min. Reactions were stopped by ethanol precipitation, resuspended in 100 mM Tris-borate-EDTA/80% formamide loading buffer, denatured at 85 °C for 10 min, and a 7 μL sample was immediately loaded onto an 8% denaturing polyacrylamide gel at 2000 V. The gels were dried under vacuum at 80 °C, then quantified using storage phosphor technology.

Affinity Cleaving.[13] All reactions were carried out in a volume of 400 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were: 20 mM HEPES buffer (pH 7.0), 20 mM NaCl, and 15 kcpm 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 3 h. A fresh solution of ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) (10 μM) was added to the equilibrated DNA, and the reactions were allowed to equilibrate for 15 min. Cleavage was initiated by the addition of dithiothreitol (10 mM) and allowed to proceed for 30 min. Reactions were stopped by ethanol precipitation, resuspended in 100 mM Tris-borate-EDTA/80% formamide loading buffer, denatured at 85 °C for 6 min, and the entire sample was immediately loaded onto an 8% denaturing polyacrylamide at 2000 V. The gels were dried under vacuum at 80 °C, then quantified using storage phosphor technology.

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Supporting Information Available: Quantitative DNase I footprinting gels of polyamides **1**, **4**, **5**, and **6** with the 3′-^{32}P-labeled restriction fragment from pSES 19-1, the MPE footprinting gel of **2** with the 5′-^{32}P-labeled restriction fragment, and the affinity cleaving gel of **2E** with the 5′-^{32}P-labeled restriction fragment (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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