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Stereochemical Control of the DNA Binding Affinity, Sequence-Specificity, and Orientation-Preference of Chiral Hairpin Polyamides in the Minor Groove.

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Experimental Section

Dicyclohexylcarbodiimide (DCC), Hydroxybenzotriazole (HOBt), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate (HBTU) and 0.2 mmol/gram Boc-β-alanine-(-4-carboxamidomethyl)-benzyl-ester-copoly(styrene-divinylbenzene) resin (Boc-β-Pam-Resin) were purchased from Peptides International. (*R*)-2-Fmoc-4-Boc-diaminobutyric acid, (*S*)-2-Fmoc-4-Boc-diaminobutyric acid, and (*R*)-2-amino-4-Boc-diaminobutyric acid were from Bachem. *N*,*N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), DMSO/NMP, Acetic anhydride (Ac_2O), and 0.0002 M potassium cyanide/pyridine were purchased from Applied Biosystems. Dichloromethane (DCM) and triethylamine (TEA) were reagent grade from EM, thiophenol (PhSH), dimethylaminopropylamine (Dp), were from Aldrich, trifluoroacetic acid (TFA) Biograde 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. A shaker for manual solid phase synthesis was obtained from St. John Associates, Inc. HPLC analysis was performed on either a HP 1090M analytical HPLC or a Beckman Gold system using a RAINEN C_{18}, Microsorb MV, 5μm, 300 x 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. 18MΩ water was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered.

Enzymes were purchased from Boehringer-Mannheim and used with their supplied buffers. Deoxyadenosine and thymidine 5′-[α-^{32}P] triphosphates were obtained from Amersham, and deoxyadenosine 5′-[γ-^{32}P]triphosphate was purchased from I.C.N. 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.

Preparation of 3′- and 5′-End-Labeled Restriction Fragments. The plasmid pMM5 was linearized with *Eco*RI and *Bsr*BI, then treated with the Sequenase enzyme, deoxyadenosine 5′-[α-^{32}P]triphosphate and thymidine 5′-

[α-^{32}P]triphosphate for 3′ labeling. Alternatively, pMM5 was 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 *Bsr*BI. The labeled fragment (3′ or 5′) was loaded onto a 6% non-denaturing polyacrylamide gel, and the desired 135 base pair band was visualized by autoradiography and isolated. Chemical sequencing reactions were performed according to published methods.[20]

MPE•Fe(II) Footprinting. All reactions were carried out in a volume of 40 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were: 25 mM Tris-acetate buffer (pH 7.0), 10 mM NaCl, 100 μM/base pair calf thymus DNA, and 30 kcpm 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 4 hours. 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 5 minutes. 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 6 min, and a 5 μL sample (~ 15 kcpm) was immediately loaded onto an 8% denaturing polyacrylamide gel (5% crosslink, 7 M urea) at 2000 V.

Affinity Cleaving. All reactions were carried out in a volume of 40 μL. A polyamide stock solution or water (for reference lanes) was added to an assay buffer where the final concentrations were: 25 mM Tris-acetate buffer (pH 7.0), 20 mM NaCl, 100 μM/base pair calf thymus DNA, and 20 kcpm 3′- or 5′-radiolabeled DNA. The solutions were allowed to equilibrate for 8 hours. 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 minutes. 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 gel (5% crosslink, 7 M urea) at 2000 V.

DNase I Footprinting. 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 hours 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 6 min, and immediately loaded onto an 8% denaturing polyacrylamide gel (5% crosslink, 7 M urea) at 2000 V for 1 hour. The gels were dried under vacuum at 80 °C, then quantitated using storage phosphor technology.

Equilibrium association constants were determined as previously described.[9a] The data were analyzed by performing volume integrations of the 5′-TGTTA-3′ and 5′-TGACA-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:

$$\theta_{app} = 1 - \frac{I_{tot}/I_{ref}}{I_{tot}^{\circ}/I_{ref}^{\circ}} \tag{1}$$

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 fit to a Langmuir binding isotherm (eq 2, n=1 for polyamides **1-3**, n=2 for polyamides **4** and **5**) by minimizing the difference between θ_{app} and θ_{fit}, using the modified Hill equation:

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

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 PhosphorImager was used to obtain all data from the storage screens. The data were analyzed by performing volume integrations of all bands using the ImageQuant v. 3.2.

Figure Captions:

Figure S1. MPE•Fe(II) footprinting experiments on a 3′-^{32}P-labeled 135 bp restriction fragment. 5′-TGTTA-3′, 5′-TGTCA-3, and 5′-ACATT-3′ sites are shown on the right side of the autoradiogram. Lane 1, A reaction; lane 2, G reaction; lane 3 MPE•Fe(II) standard; lane 8, intact DNA; lanes 4-7, 1.25 μM, 2.5 μM, 5 μM, and 10 μM polyamide. (a) ImPyPy-$(R)^{H_2N}\gamma$-PyPyPy-β-Dp **1-*R***; (b) ImPyPy-$(R)^{Ac}\gamma$-PyPyPy-β-Dp **3-*R*** (c); ImPyPy-$(S)^{H_2N}\gamma$-PyPyPy-β-Dp **3-*S***; (d) ImPyPy-$(S)^{Ac}\gamma$-PyPyPy-β-Dp **3-*S***. All lanes contain 15 kcpm 3′-radiolabeled DNA, 25 mM Tris-acetate buffer (pH 7.0), 10 mM NaCl, and 100 μM/base pair calf thymus DNA.

Figure S2. Affinity cleaving experiments on the 3′-^{32}P-labeled 135 bp restriction fragment. 5′-TGTTA-3′, 5′-TGTCA-3, and 5′-ACATT-3′ sites are shown on the right side of the autoradiograms as appropriate. Lane 1, A reaction; lane 2, G reaction; lane 8, intact DNA; lanes 3-7, 110 nM, 330 nM, 1.0 μM, and 3.3 μM, and 10 μM polyamide. (a) ImPyPy-$(R)^{H_2N}\gamma$-PyPyPy-β-Dp-EDTA•Fe(II) (**4-*R*•Fe(II)**); (b) ImPyPy-$(R)^{EDTA \cdot Fe(II)}\gamma$-PyPyPy-β-Dp (**5-*R*•Fe(II)**); (c) ImPyPy-$(S)^{H_2N}\gamma$-PyPyPy-β-Dp-EDTA•Fe(II) (**4-*S*•Fe(II)**); (d) ImPyPy-$(S)^{EDTAFe(II)}\gamma$-PyPyPy-β-Dp (**5-*S*•Fe(II)**). All lanes contain 15 kcpm 3′-radiolabeled DNA, 25 mM Tris-acetate buffer (pH 7.0), 10 mM NaCl, and 100 μM/base pair calf thymus DNA.

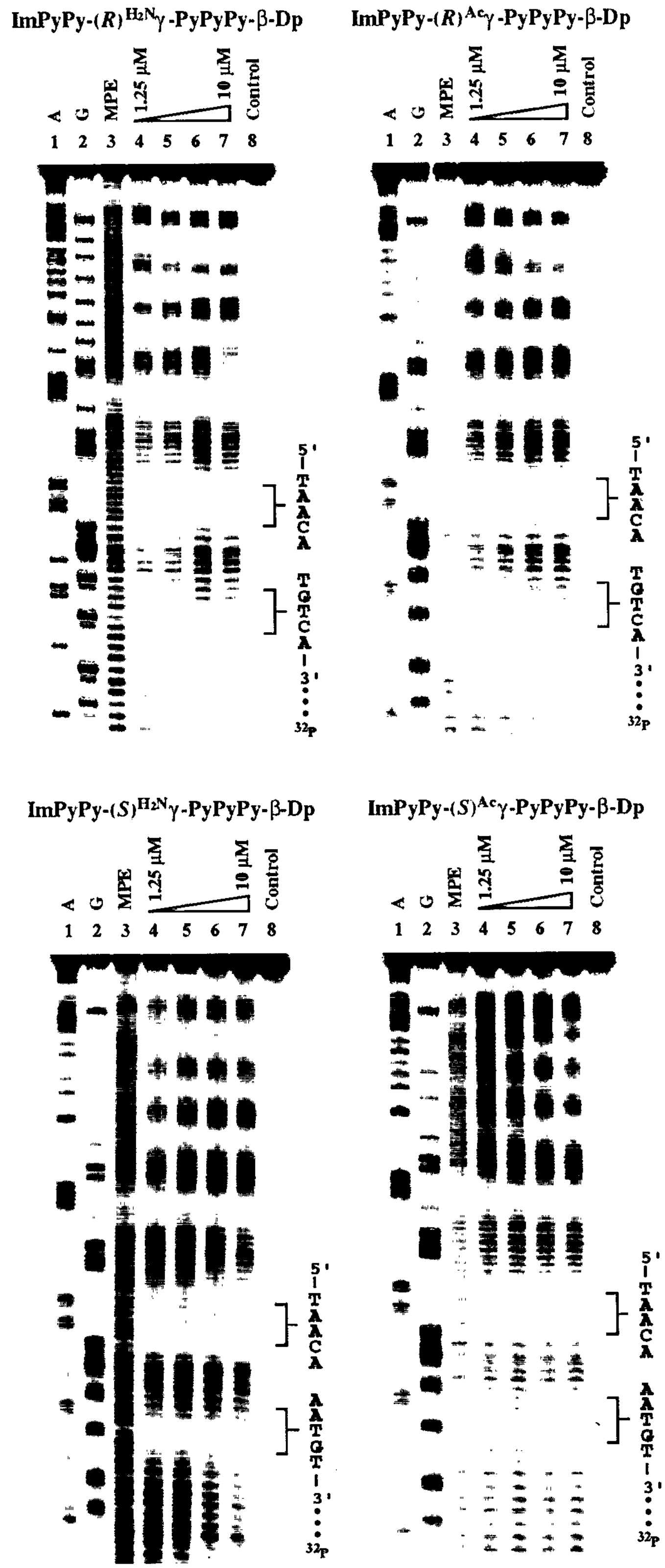
ImPyPy-(R)H2Nγ-PyPyPy-β-Dp
ImPyPy-(R)Acγ-PyPyPy-β-Dp
ImPyPy-(S)H2Nγ-PyPyPy-β-Dp
ImPyPy-(S)Acγ-PyPyPy-β-Dp
A
G
MPE
1.25 μM
10 μM
Control
1 2 3 4 5 6 7 8
5'-TAACA TGTCA-3'
5'-TAACA AATGT-3'
32P

Fig S1

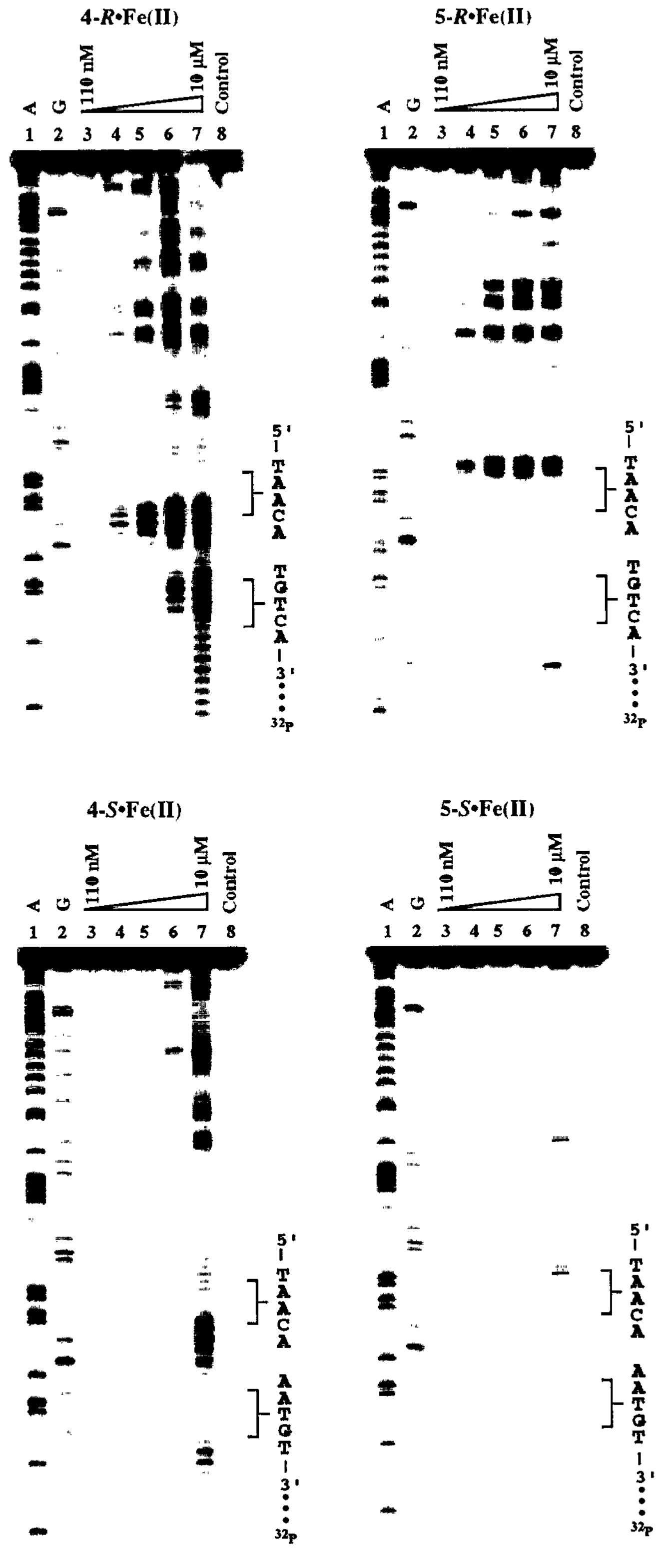
4-R•Fe(II)
5-R•Fe(II)
4-S•Fe(II)
5-S•Fe(II)
A
G
110 nM
10 μM
Control
1 2 3 4 5 6 7 8
5'
TAACA
TGTCA
AATGT
3'
32P

Fig S2