Chapter 9

Minor Groove Recognition of T,A Base Pairs by Benzothiophene/Pyrrole Pairs

The text of this chapter was taken in part from a manuscript coauthored with Carey F Hsu and Professor Peter B. Dervan (Caltech)

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Abstract.

Polyamides are designed oligomers that can bind to DNA in a sequence-specific manner with affinities comparable to those of natural DNA-binding proteins. Recently, an N-terminal 3-chloro-6-fluorobenzothiophene polyamide bound in the minor groove of DNA was co-crystallized at 1.1 Å resolution as an antiparallel dimer. In addition, antimicrobial screenings for this polyamide family and the development of N-terminal 3-chlorothiophene as a T-specific recognition element have been disclosed. These results inspired us to measure the affinities and specificities of N-terminal 3-substituted benzothiophene hairpin polyamides by DNase I footprint titration experiments. While the four polyamides bound with high affinity, none showed specificity for A•T over T•A or vice versa. This observation suggests that N-terminal 3-substituted benzothiophene polyamides prefer to adopt the rotational conformation that directs the sulfur atom towards the floor of the minor groove, a conclusion that is consistent with the recently solved crystal structure.

Introduction.

Hairpin polyamides constructed from *N*-methylpyrrole (Py), *N*-methylimidazole (Im), and *N*-methylhydroxypyrrole (Hp) amino acids comprise a class of synthetic ligands that bind within the minor groove of DNA in a sequence-specific manner.[2, 3] Inspired by the natural products netropsin and distamycin A,[4, 5] these programmed molecules recognize the Watson-Crick base pairs according to a series of pairing rules, where paired aromatic heterocycles on opposite sides of the hairpin polyamide are able to discriminate one Watson-Crick base pair from the other three combinations. The Py/Py pair recognizes A•T and T•A over C•G and G•C, whereas the Im/Py pair prefers G•C.[6, 7] The exocyclic amine of guanine presents steric hindrance to the C3-H of Py, while the N3 of Im accommodates the amine and accepts one of its hydrogen bonds. The Hp/Py pair discriminates T•A over A•T due to the steric fit of the hydroxy group protruding into the minor groove, thus completing the pairing rules.[6, 8] X-ray crystallography studies have provided confirmation of polyamide-DNA binding structure.[6-8]

The observation that Hp is unstable in the presence of acid or free radicals has necessitated the development of new T-specific recognition elements. This search has yielded two key advancements: 6-5 fused bicyclic ring systems[9-11] and N-terminal 3-substituted thiophene residues.[12, 13] Replacement of the Hp heterocycle with a phenol ring and incorporation of the carboxamide carbonyl as part of the imidazole moiety produces the hydroxybenzimidazole (Hz) fused aromatic ring. Linking the C2 of Py with the C2 of Hz completes the Py-Hz dimeric amino acid, which presents the same recognition face to the minor groove of DNA as Py and Hp connected N→C via an amide bond. This new heterocyclic ring pair is an excellent solution at internal positions on the

hairpin polyamide, as it remains stable under a wide range of conditions and, perhaps more importantly, its affinity and specificity for T•A over A•T are comparable to those observed for Py-Hp.[11, 14, 15]

Recognition of the minor groove at the N-terminal position of the hairpin polyamide has proven to be a difficult problem, since the heterocycle placed here is free to adopt either of two rotational conformations.[12, 16] Py/Py pairs at the terminal position exhibit decreased selectivity for A,T over C,G than Py/Py pairs placed at internal positions, likely due to this conformational freedom. The use of a 3-chlorothiophene (or 3-methoxythiophene) residue at the N-terminus not only restores the preference for A,T over C,G, but also creates a polyamide that is specific for T•A over the other three Watson-Crick base pairs.[12] The N-terminal 3-chlorothiophene polyamide exhibits a 3-fold preference for T•A over A•T and a 40-fold preference for C,G. DNase I footprint titration experiments coupled with *ab initio* molecular modeling calculations suggest that the syn rotamer of the 3-chlorothiophene moiety dominates in aqueous equilibrium with DNA, pointing the chlorine atom into the minor groove (Figure 9.1). This model suggests that a steric clash discourages binding across C•G or G•C and the ability of chlorine to fit next to the smaller thymine is the source of the selectivity for T•A over A•T.

The experiments described here provide a second look at the N-terminal thiophene family of hairpin polyamides, drawing upon 3-substituted benzothiophene residues to extend the series. Previously, this scaffold has been incorporated into polyamides at the N-terminus, yielding molecules that were shown to have strong antimicrobial properties.[17] In this lead optimization study, derivatives of the tripyrrole

natural product distamycin A were examined in an in vitro antibacterial screen, yielding the N-terminal 3-chlorothiophene derivative as a strong candidate. Further elucidation of this novel end cap revealed the derivatives containing an N-terminal 3-chloro-6-fluorobenzothiophene and a C-terminal ethyl morpholine, which exhibited excellent potency and no adverse effects when tested in mice.[18, 19]

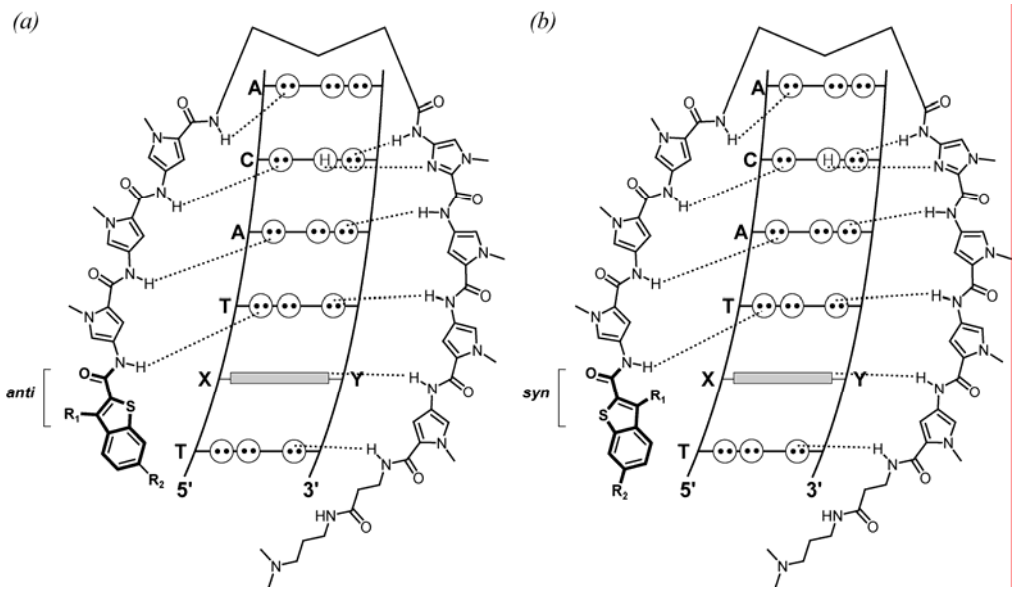

Figure 9.1. Proposed binding models for hairpin polyamides with 5'-TXTACA-3' site. A circle enclosing two dots represents a lone pair on N3 of purines or O2 of pyrimidines. A circle containing an H represents the exocyclic amine of guanine. Putative hydrogen bonds are indicated by dashed lines; (a) N-terminal residue drawn in "sulfur down" anti conformation; (b) N-terminal residue drawn in "sulfur up" syn conformation.

Furthermore, Kielkopf and coworkers recently solved the crystal structure of a polyamide bound to DNA at 1.1 Å resolution. In this case, the molecule was a dipyrrole with an N-terminal 3-chloro-6-fluorobenzothiophene and a C-terminal ethyl morpholine. The polyamide was co-crystallized in 2:1 stoichiometry as an antiparallel dimer with a 5'-CCAITACTGG-3' oligomer. This crystal structure indicated that the N-terminal 3-chloro-6-fluorobenzothiophene polyamide was bound to the 5'-AITACT-3' tract with the

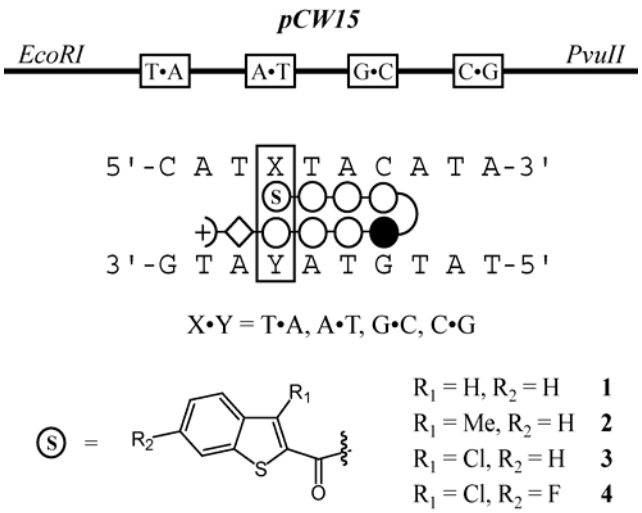

Figure 9.2. (top) pCW15 plasmid design; (bottom) ball and stick model of hairpin polyamides varying the N-terminal residue. Imidazole and pyrrole are shown as filled and non-filled circles, respectively; β-alanine is shown as a diamond; the dimethylaminopropylamide tail is shown as a half-circle with a plus; the γ-aminobutyric acid turn residue is shown as a semicircle connecting the two subunits; and the N-terminal benzothiophene residue is shown as a circle containing an S.

sulfur atom of the benzothiophene ring directed into the minor groove. This remarkable result spurred our interest in exploring how the DNA recognition properties of N-terminal benzothiophene polyamides differ from those of the N-terminal thiophene polyamides that were previously studied. Specifically, we wanted to use DNase I footprint titration experiments to examine how benzothiophene/pyrrole pairs at the N-terminal position of a polyamide affect the affinity and specificity of these compounds and compare the data with the recent crystal structure and footprinting results (Figure 9.2).

Results.

Polyamide synthesis. The Boc-protected intermediate resin R1 was synthesized using previously described solid-phase peptide synthesis methods (Figure 9.3).[20] R1 was deprotected with 80% TFA in dichloromethane to yield the free amine. The 3-substituted benzothiophene-2-carboxylic acid, which had been pre-activated with HBTU, was coupled to the N-terminus of the polyamide at room temperature for 18 h. The

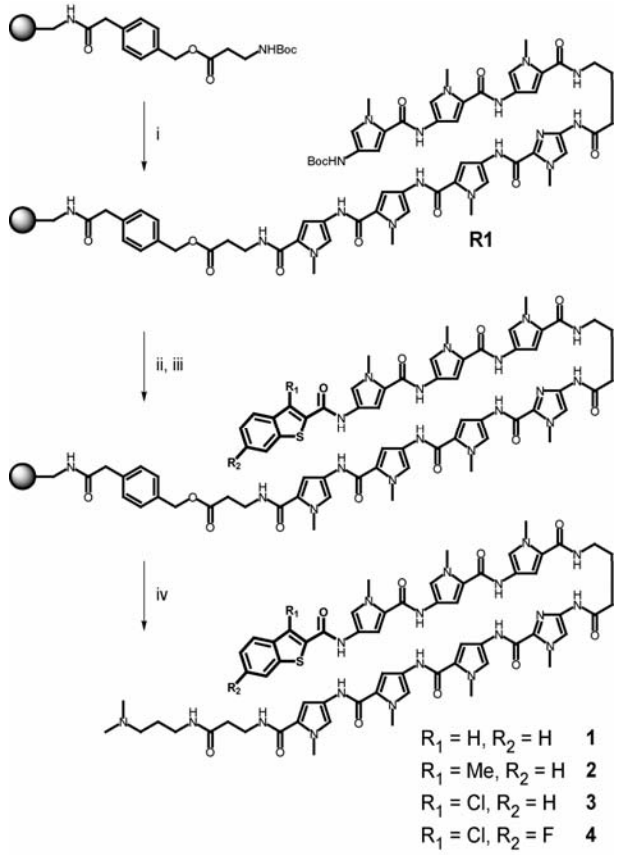

Figure 9.3. Synthesis of hairpin polyamides: (i) Synthesis of polyamide resin by standard solid-phase techniques; (ii) TFA, CH_2Cl_2; (iii) 3-R_1-6-R_2-benzothiophene-2-CO_2H, HBTU, DIEA, DMF; (iv) Dp, 80°C.

compound was cleaved from the support resin with 3-(dimethylamino)propylamine (Dp) at 80°C for 2 h to yield the crude final product, which was purified by preparatory reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry.

DNA binding energetics. Quantitative DNase I footprint titration experiments (10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 7.0, 22°C) were performed on the 284 bp, 5'-^{32}P-end-labeled PCR product of plasmid pCW15 (Figure 9.4 & 9.5).[16] The plasmid was designed to contain four potential binding sites of 5'-ATXTACA-3', where X = T, A, G, C. The energetics of polyamide binding in the minor groove of DNA can be calculated from the Hill equation isotherms following DNase I cleavage and gel separation of the fragments. The equilibrium association constants determined in this way provide a quantitative measure of polyamide affinity at a given DNA binding site (Table 9.1). Comparing these constants across the four potential

binding sites allows a relative measure of specificity for each base pair at the targeted position.

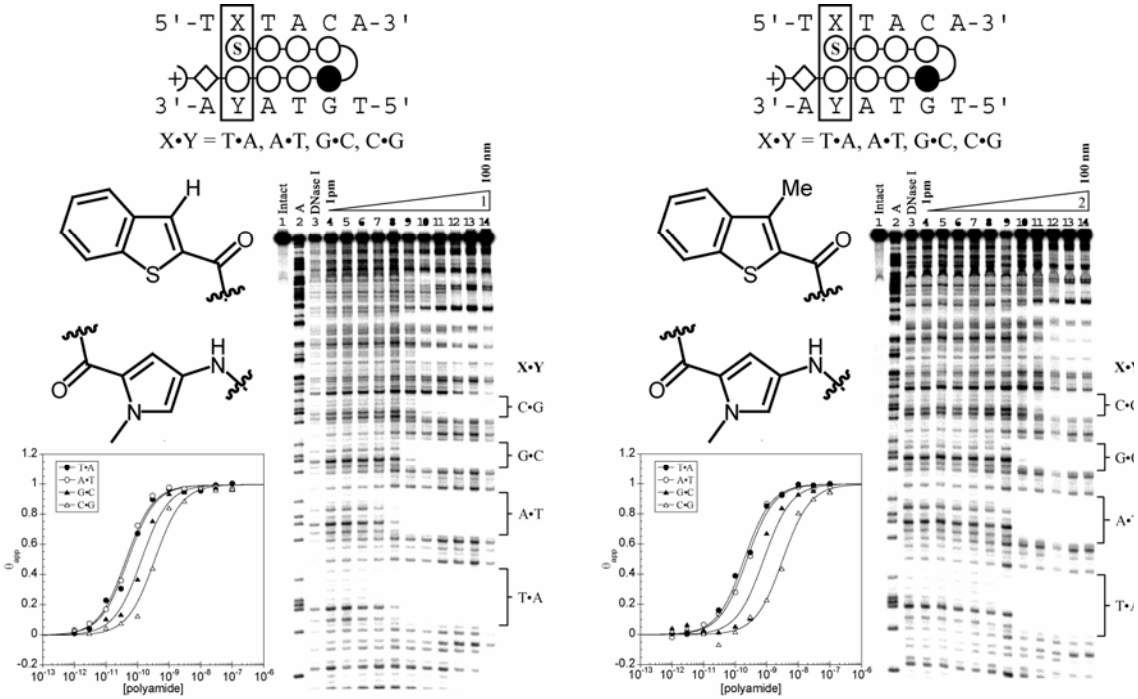

Figure 9.4. Quantitative DNase I footprint titration experiments for polyamides **1** and **2**, respectively, on the 284 bp, 5'-^{32}P-end-labeled PCR product of plasmid pCW15: lane 1, intact DNA; lane 2, A reaction; lane 3, DNase I standard; lanes 4-14, 1 pM. 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM polyamide, respectively. Each footprinting gel is accompanied by the following: (top left) chemical structure of the N-terminal 3-substituted benzothiophene/pyrrole pair of interest and (bottom left) binding isotherms for the four designed sites. θ_{norm} values were obtained according to published methods.[1] A binding model for the hairpin motif is shown centered at the top as a dot model with the polyamide bound to its target DNA sequence. Imidazole and pyrrole are shown as filled and non-filled circles, respectively; β-alanine is shown as a diamond; the dimethylaminopropylamide tail is shown as a half-circle with a plus; the γ-aminobutyric acid turn residue is shown as a semicircle connecting the two subunits; and the N-terminal benzothiophene residue is shown as a circle containing an S.

Polyamide **1**, with an unsubstituted N-terminal benzothiophene ring, has the highest equilibrium association constant of 2×10^{10} M^{-1} for the A•T and T•A binding sites. **1** exhibits 3-fold specificity for A,T over the G•C binding site and 10-fold specificity over C•G. When a 3-methyl group is added to the benzothiophene system in **2**, binding affinity is lowered by 4- to 5-fold for each of the four sites.

In polyamide **3**, substitution of a chlorine atom for the methyl group partially restores the binding affinity, as an association constant of 1×10^{10} M^{-1} is calculated for A•T and T•A. As compared to **1**, polyamide **3** shows similar 3-fold specificity for A,T over G•C but greater 30-fold specificity over C•G. Addition of a 6-fluoro substituent to yield polyamide **4** gives association constants for T•A and A•T that are within statistical error of each other, indicating no specificity for one binding site over the other. The

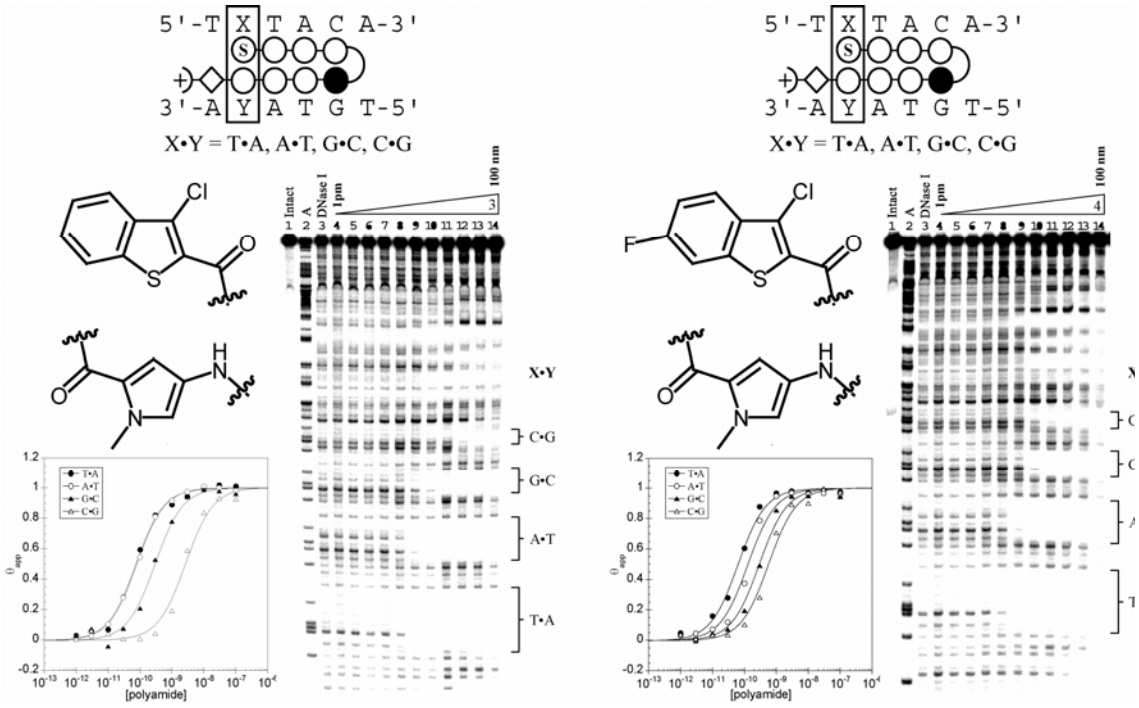

Figure 9.5. Quantitative DNase I footprint titration experiments for polyamides **3** and **4**, respectively, on the 284 bp, 5'-^{32}P-end-labeled PCR product of plasmid pCW15: lane 1, intact DNA; lane 2, A reaction; lane 3, DNase I standard; lanes 4-14, 1 pM. 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM polyamide, respectively. Each footprinting gel is accompanied by the following: (top left) chemical structure of the N-terminal 3-substituted benzothiophene/pyrrole pair of interest and (bottom left) binding isotherms for the four designed sites. θ_{norm} values were obtained according to published methods.[1] A binding model for the hairpin motif is shown centered at the top as a dot model with the polyamide bound to its target DNA sequence. Imidazole and pyrrole are shown as filled and non-filled circles, respectively; β-alanine is shown as a diamond; the dimethylaminopropylamide tail is shown as a half-circle with a plus; the γ-aminobutyric acid turn residue is shown as a semicircle connecting the two subunits; and the N-terminal benzothiophene residue is shown as a circle containing an S.

specificity profile for **4** is otherwise very similar to that for **1**, albeit with a small decrease in overall binding affinity.

Discussion.

The equilibrium association constant at each potential binding site for polyamide **1** provides a baseline figure for the N-terminal benzothiophene scaffold and also permits comparison with the previously studied thiophene series. In general, **1** has a higher affinity than the corresponding thiophene/pyrrole pair, suggesting that benzothiophene binds more tightly to DNA than thiophene, perhaps due to the hydrophobic benzene ring that can be shielded from aqueous solvation when placed in the minor groove. The unsubstituted bicylic system generally prefers A,T over C,G, but there is no energetic preference for A•T over T•A or vice versa. Interestingly, benzothiophene is much more tolerant when placed next to the sterically demanding exocyclic amine of guanine, as the association constant of **1** across G•C is 16-fold higher than that of the thiophene/pyrrole pair.[12] **1** displays a lack of specificity across the binding sites that is consistent with observations of the unsubstituted thiophene ring, resulting from the similar recognition faces in the two rotamers. The syn and anti conformations present a hydrogen atom and the lone pairs of sulfur, respectively, to the minor groove of DNA. Neither moiety has the steric or electronic bulk to show appreciable preference for a single Watson-Crick base pair.

Polyamide **2** has a 3-methyl group added to the benzothiophene skeleton, yielding a nearly uniform decrease in binding affinity across the four binding sites when compared with **1**. This implies that the rotational equilibrium favors the anti conformation with the

sulfur atom directed into the minor groove because the specificity profile across the four Watson-Crick base pairs is the same as that for **1**. If the methyl group were pointed towards the minor groove face of the nucleotides, it would be expected that some sort of specificity would be observed on the basis of sterics alone. Most importantly, for polyamide **2**, the A•T and T•A binding sites have the same association constant. The drop in overall affinity from **1** may be explained by the energetic penalty that **2** incurs by exposing the hydrophobic methyl group to aqueous solution.

The 3-methyl group is replaced by a 3-chloro substituent in polyamide **3**, yielding a compound that shows similar binding affinities for the A•T and T•A sites. Again, this suggests that the anti conformation is energetically preferred, as the electron cloud of the sulfur atom is able to accommodate both A and T equally, as well as the exocyclic amine of guanine. In contrast, previous work on the thiophene system indicated that, for the N-terminal 3-chloro thiophene residue, T•A was favored by 3-fold over A•T. Furthermore, T•A showed 40-fold specificity over G•C and 60-fold specificity over C•G, leading to the conclusion that the syn rotamer was energetically preferred, placing the chlorine atom in the minor groove of DNA.[12] The behavior of the 3-chloro-benzothiophene for C•G is somewhat surprising, as the drop in affinity is significantly more than for any of the other polyamides in the series.

Polyamide **4** contains an N-terminal 3-chloro-6-fluorobenzothiophene ring, the same end cap that was used in the polyamide that Kielkopf and coworkers recently co-crystallized with DNA. In fact, the three aromatic rings of the polyamide in the crystal structure have the same substitution pattern and are in the same order as **4**. There is no preference for T•A over A•T or vice versa, as these two figures are within statistical

error. T•A exhibits 5-fold specificity over G•C and 10-fold specificity over C•G.

Table 9.1. Equilibrium association constants: K_a [M^{-1}][a]

Polyamide	R_1	R_2	T•A	A•T	G•C	C•G
1	H	H	$1.7\ (\pm0.3)\times10^{10}$	$2.1\ (\pm0.3)\times10^{10}$	$7.2\ (\pm1.5)\times10^{9}$	$2.3\ (\pm0.5)\times10^{9}$
2	Me	H	$4.2\ (\pm0.6)\times10^{9}$	$4.0\ (\pm0.5)\times10^{9}$	$1.7\ (\pm0.5)\times10^{9}$	$4.4\ (\pm0.6)\times10^{8}$
3	Cl	H	$1.2\ (\pm0.1)\times10^{10}$	$1.1\ (\pm0.1)\times10^{10}$	$3.6\ (\pm0.6)\times10^{9}$	$3.5\ (\pm0.6)\times10^{8}$
4	Cl	F	$1.5\ (\pm0.3)\times10^{10}$	$9.5\ (\pm1.7)\times10^{9}$	$3.0\ (\pm0.6)\times10^{9}$	$1.7\ (\pm0.3)\times10^{9}$

[a] Values reported are the mean values from at least three DNase I footprint titration experiments, with the standard deviation given in parentheses. Assays were performed at 22°C in a buffer of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$ at pH 7.0.

However, this specificity profile is nearly identical to the unsubstituted polyamide **1**, suggesting that this energetic bias does not result from the steric effects of the 3-substituent in the minor groove. The similarity of the binding of all four polyamides **1**-**4** at the A•T and T•A sites indicates that the sulfur atom is projected into the minor groove of DNA. Thus, it appears that polyamide **4** adopts the same rotational conformation in aqueous solution as in crystal form.

Conclusion.

The benzothiophene derivatives were initially examined within the context of an antibacterial lead optimization study.[18, 19] The binding mode of this heterocyclic system was characterized by Kielkopf et al., showing a complex with the endocyclic sulfur atom of the benzothiophene bicycle presented to the DNA minor groove. To examine the binding energetics of this heterocycle, and compare it to an analogous N-terminal thiophene cap system, we conducted a series of DNase I footprinting titration experiments. X-ray structural data are collaborated by the thermodynamic titrations,

which indicate that the benzothiophen system shows no evident T vs. A specificity and moderate A,T over G,C specificity. While it seems clear that within the context of the benzothiophene system, sulfur is presented to the DNA minor groove, it is still uncertain which rotomer is favored in the 5-membered chlorothiophene (Ct) system.[12] Confirmation of these binding models awaits NMR structural elucidation in aqueous solution.

Experimental

General.

N,*N*-Dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIEA), 3-(dimethylamino)propylamine (Dp), benzo[b]thiophene-2-carboxylic acid, and 3-chloro-benzo[b]thiophene-2-carboxylic acid were purchased from Aldrich. 3-Methyl-benzo[b]thiophene-2-carboxylic acid was purchased from Avocado. 3-Chloro-6-fluoro-benzo[b]thiophene-2-carboxylic acid was purchased from Oakwood. [(*tert*-Butoxy)carbonyl]-*β*-alanine-(4-carbonylaminomethyl)-benzyl-ester-copoly(styrene-divinylbenzene)resin (Boc-*β*-PAM-resin), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and Boc-*β*-alanine were purchased from NOVA Biochem. Trifluoroacetic acid (TFA) was purchased from Halocarbon. All other solvents were reagent-grade from EMD Chemicals. Oligonucleotide inserts were synthesized by the Biopolymer Synthesis Center at the California Institute of Technology. Precoated silica gel plates $60F_{254}$ for TLC were purchased from J. T. Baker. Silica gel 60 (40 μm) for column chromatography was purchased from Merck. Glycogen (20 mg/mL), dNTPs (PCR nucleotide mix), Taq DNA polymerase, calf thymus DNA

(sonicated, deproteinized), and DNase I (7500 units/mL, FPLC pure), and all other enzymes, unless otherwise stated, were purchased from Roche. Adenosine [γ-^{32}P] triphosphate was purchased from MP Biomedicals. Tris-HCl, DTT, RNase-free H_2O, and 0.5M EDTA were purchased from United States Biochemical. Calcium chloride, potassium chloride, and magnesium chloride were purchased from Fluka. Tris-borate EDTA was purchased from GIBCO. Bromophenol blue was purchased from Acros. All reagents were used without further purification.

NMR spectra were recorded on a Varian spectrometer at 300 MHz in DMSO-d_6 or $CDCl_3$, with chemical shifts reported in parts per million relative to residual solvent. UV spectra were measured on a Hewlett-Packard model 8452A diode-array spectrophotometer. Matrix-assisted, laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was conducted at the Protein and Peptide Microanalytical Facility at the California Institute of Technology.

Polyamide synthesis.

Polyamides were synthesized from intermediate resin (**R1**) that was prepared according to published protocols using Boc-β-alanine-PAM resin (50 mg, 0.59 mmol/g) and purified by preparatory reverse-phase HPLC according to published manual solid-phase protocols.[20] Products were characterized by MALDI-TOF mass spectrometry.

(1). Resin (**R1**) was treated with 80% TFA in dichloromethane and washed thoroughly. Benzo[b]thiophene-2-carboxylic acid (16 mg, 88.5 μmol) was incorporated by activation with HBTU (32 mg, 84 μmol), DIEA (23 mg, 31 μL, 177 μmol), and DMF (390 μL). After standing for 15 min at room temperature, the mixture was added to the

reaction vessel containing the deprotected resin **R1**. Coupling was allowed to proceed for 18 h by shaking at room temperature. After filtering and washing with DMF, the resin was cleaved with Dp (1 mL) at 80°C for 2 h. The crude product was purified by preparatory reverse-phase HPLC to afford (**1**) as a white solid upon lyophilization (0.2 mg, 0.4% recovery). MALDI-TOF-MS (monoisotopic) *m/z* 1273.91 (1274.54 calcd. for $[M + H]^+$ $C_{62}H_{72}N_{19}O_{10}S^+$).

(2). Resin (**R1**) was treated with 80% TFA in dichloromethane and washed thoroughly. 3-Methyl-benzo[b]thiophene-2-carboxylic acid (17 mg, 88.5 μmol) was incorporated by activation with HBTU (32 mg, 84 μmol), DIEA (23 mg, 31 μL, 177 μmol), and DMF (390 μL). After standing for 15 min at room temperature, the mixture was added to the reaction vessel containing the deprotected resin **R1**. Coupling was allowed to proceed for 18 h by shaking at room temperature. After filtering and washing with DMF, the resin was cleaved with Dp (1 mL) at 80°C for 2 h. The crude product was purified by preparatory reverse-phase HPLC to afford (**2**) as a white solid upon lyophilization (0.2 mg, 0.5% recovery). MALDI-TOF-MS (monoisotopic) *m/z* 1288.28 (1288.56 calcd. for $[M + H]^+$ $C_{63}H_{74}N_{19}O_{10}S^+$).

(3). Resin (**R1**) was treated with 80% TFA in dichloromethane and washed thoroughly. 3-chloro-benzo[b]thiophene-2-carboxylic acid (19 mg, 88.5 μmol) was incorporated by activation with HBTU (32 mg, 84 μmol), DIEA (23 mg, 31 μL, 177 μmol), and DMF (390 μL). After standing for 15 min at room temperature, the mixture was added to the reaction vessel containing the deprotected resin **R1**. Coupling was allowed to proceed for 18 h by shaking at room temperature. After filtering and washing with DMF, the resin was cleaved with Dp (1 mL) at 80°C for 2 h. The crude product was

purified by preparatory reverse-phase HPLC to afford (**3**) as a white solid upon lyophilization (0.2 mg, 0.5% recovery). MALDI-TOF-MS (monoisotopic) *m/z* 1308.06 (1308.50 calcd. for $[M + H]^+$ $C_{62}H_{71}ClN_{19}O_{10}S^+$).

(4). Resin (**R1**) was treated with 80% TFA in dichloromethane and washed thoroughly. 3-Chloro-6-fluoro-benzo[b]thiophene-2-carboxylic acid (20 mg, 88.5 μmol) was incorporated by activation with HBTU (32 mg, 84 μmol), DIEA (23 mg, 31 μL, 177 μmol), and DMF (390 μL). After standing for 15 min at room temperature, the mixture was added to the reaction vessel containing the deprotected resin **R1**. Coupling was allowed to proceed for 18 h by shaking at room temperature. After filtering and washing with DMF, the resin was cleaved with Dp (1 mL) at 80°C for 2 h. The crude product was purified by preparatory reverse-phase HPLC to afford (**4**) as a white solid upon lyophilization (0.2 mg, 0.6% recovery). MALDI-TOF-MS (monoisotopic) *m/z* 1326.52 (1326.49 calcd. for $[M + H]^+$ $C_{62}H_{72}N_{19}O_{10}S^+$).

Footprinting experiments.

Plasmid pCW15 was constructed and 5'-radiolabeled as previously described.[16] DNase I footprint titrations were performed according to standard protocols.[1]

Acknowledgments

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