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ScienceDirect
Bioorganic & Medicinal Chemistry 14 (2006) 8539–8549
Bioorganic & Medicinal Chemistry

Exploring the limits of benzimidazole DNA-binding oligomers for the hypoxia inducible factor (HIF) site

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Received 18 July 2006; revised 19 August 2006; accepted 22 August 2006
Available online 12 September 2006

Abstract—The vascular endothelial growth factor (VEGF) and its receptors have been implicated as key-factors in tumor angiogenesis and are major targets in cancer therapy. New oligomers which mimic the architecture of DNA-binding polyamides have been designed to target the hypoxia inducible factor (HIF-1α) binding site on the promoter of VEGF gene. These oligomers incorporate an increasing number of six–five fused rings such as hydroxybenzimidazole–imidazole, benzimidazole–pyrrole, benzimidazole–chlorothiophene, and imidazopyridine–pyrrole, and bind the VEGF hypoxia response element (HRE) 5′-TACGT-3′ with high affinity and selectivity.

1. Introduction

DNA-binding polyamides that can access protein–DNA interfaces in the nucleus of living cells have emerged as promising gene modulators.[1] Since many diseases such as cancer result from aberrant gene expression,[2] programmable cell permeable DNA-binding small molecules might someday form the basis of transcription therapy.[1,3] Based on the architecture of the natural products netropsin and distamycin A,[4] additional sequence-specific recognition arises from the pairing of three different five-membered heterocyclic amino acids, pyrrole (Py), imidazole (Im), and hydroxypyrrole (Hp).[5,6] The ImPy pair distinguishes G·C from C·G, T·A, and A·T. Im presents a lone pair of electrons to the DNA minor groove and can accept a hydrogen bond from the exocyclic amine of guanine.[6a,b] The HpPy pair distinguishes T·A from A·T, G·C, and C·G.[4–7] Hp projects an exocyclic OH group toward the minor groove floor that is sterically accommodated in the cleft of the T·A base pair, preferring to lie over T not A.[4] These pairing rules have proven useful for programmed recognition of a variety of DNA sequences.[6c,d] These first generation molecules led us to explore whether other novel heterocycles could be discovered that can be programmed for a broad repertoire of DNA-sequence.[8–11] We recently introduced[10–12] the benzimidazole architecture as a new platform for the DNA-minor groove recognition elements. The six–five bicyclic-ring structure, with a smaller degree of curvature than five-membered ring polyamides, preserves the same atomic connectivity along the recognition edge. Selective base pair recognition can be achieved by introduction of heteroatoms and substituents on the six-membered ring (Figs. 1a, b, and c). We demonstrated that the imidazopyridine–pyrrole (IpPy) pair mimics the ImPy pair and distinguishes G·C from C·G, T·A, and A·T while the hydroxybenzimidazole–pyrrole pair (HzPy) mimics the HpPy pair and distinguishes T·A from A·T, G·C and, C·G.[11,12]

The next step is the evaluation of the benzimidazole architecture as a new tool to target specific transcription factor binding sites relevant to biological systems. We recently reported the down-regulation of the angiogenetic vascular endothelial growth factor (VEGF) by a DNA-binding fluorescein–polyamide conjugate in cell culture.[1] Under hypoxic conditions, the production of VEGF is up-regulated when the hypoxia inducible factor (HIF-1) dimerizes with its constitutively expressed partner, the aryl hydrocarbon receptor nuclear translocator (ARNT), and binds to the hypoxia response element (HRE) at the sequence 5′-TACGTG-3′ on the promoter of the VEGF gene. Designed to bind to the HRE at the HIF-1α site, the polyamide–fluorescein

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doi:10.1016/j.bmc.2006.08.028

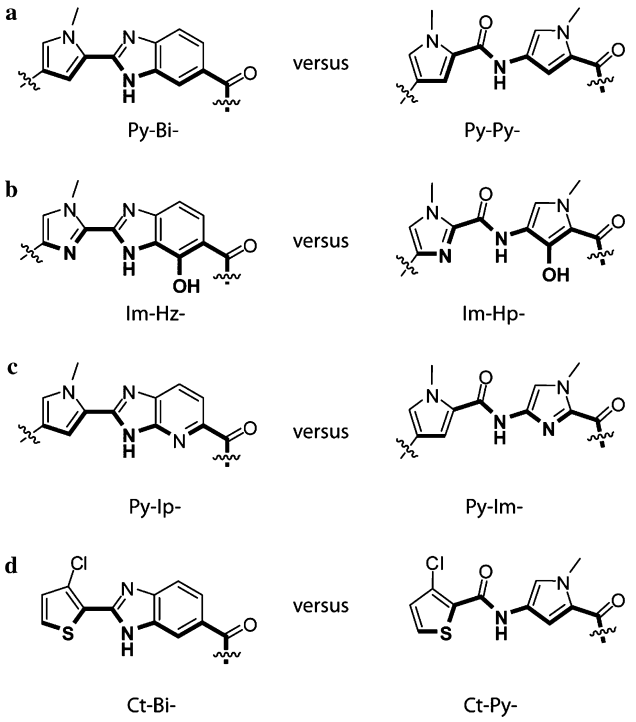


Figure 1. Structures of dimers (a) pyrrole–benzimidazole (Py-Bi), (b) imidazole–hydroxybenz–imidazole (ImHz), (c) pyrrole–imidazopyridine (Py-Ip), and (d) chlorothiophene–benzimidazole in comparison with their respective five-membered ring systems. Hydrogen-bonding surfaces to the DNA minor-groove floor are bolded.

conjugate **1** (Fig. 2) localizes in the nucleus of living cells, prevents HIF-1α from binding to the HRE, and down-regulates the production of VEGF in HeLa cells.[1] These results evidenced that polyamides can access transcription factor interfaces in the nucleus and affect the expression of an endogenous gene in living cells. From a medicinal chemistry point of view, a broader repertoire of structures targeting the HRE would be useful as our artificial transcription factor program moves from cell culture to small animal studies. We wish to investigate the potential of hairpin oligomers containing benzimidazoles to down-regulate the expression of endogenous genes such as VEGF. A minimum first step is to design programmable oligomers that would bind the hypoxia response element (HRE) binding site with good affinity and specificity.

We report here the synthesis and the DNA-binding properties of oligomers incorporating an increasing number of six–five fused rings and the evaluation of their ability to target the HIF-1α binding site within the sequence 5′-TACGT-3′, a sequence containing all four Watson–Crick base pairs (Fig. 3). The hydroxybenzimidazole–imidazole (HzIm), imidazopyridine–pyrrole (IpPy), benzimidazole–pyrrole (BiPy), and benzimidazole–chlorothiophene (BiCt) dimers were incorporated at various positions of the hairpin **2** resulting in oligomers **3**–**7**. From previous studies,[9–12] we expect the HzIm, IpPy, and BiPy dimers to target with good affinity and specificity their match site. The BiCt moiety is introduced as a new mimic of the chlorothiophene–pyrrole[13] (Fig. 1d). Quantitative DNase I footprinting titration experiments[14] were used to determine the binding affinities and specificities against single base pair mismatch sites of oligomers **3**–**7** (Figs. 5–7) as compared to their parent polyamide **2**.

We found that in oligomers **3**–**6** the six–five fused rings are effective mimics of their respective five-membered ring systems and that these oligomers target the HIF-binding site without loss of affinity as compared to parent hairpin polyamide **2**. As anticipated, the HzPy and the HzBi pairs are more specific than the PyPy pair and are able to distinguish T·A from AcdotT base pairs. The IpPy pair is able to distinguish G·C from C·G base pairs. The BiCt dimer effectively mimics CtPy. Oligomer **7**, which does not contain any traditional pyrrole– or imidazole–carboxamides, does not bind specific DNA sequences and evidences for the limitations of the benzimidazole platform. The results of this study also showed that oligomer **3** would be the best candidate for biological studies, being even more specific than hairpin **2**.

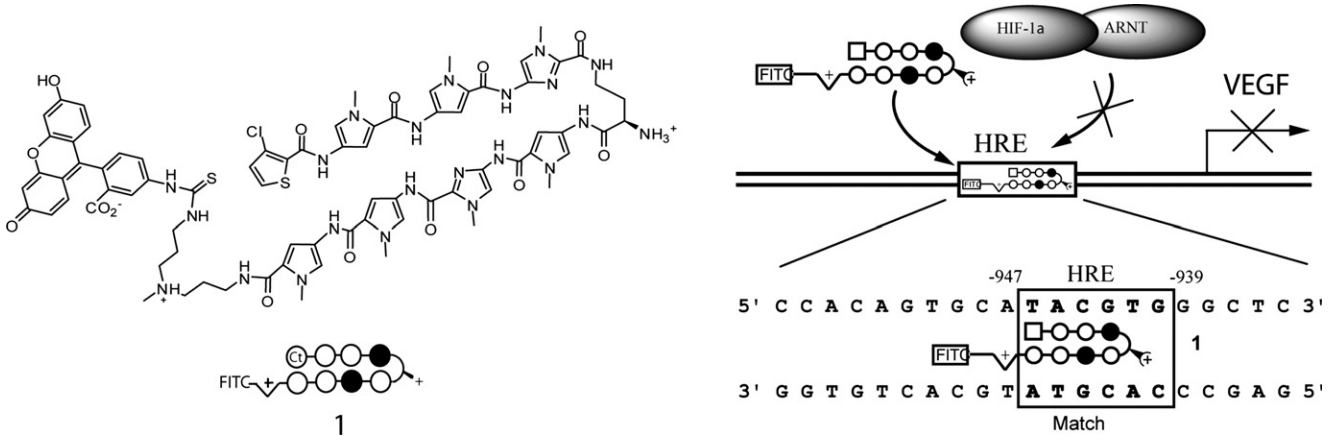


Figure 2. Structure of the fluorescein–polyamide conjugate **1** that was shown to down-regulate the expression of VEGF under hypoxic conditions[12] and map of the VEGF promoter with the HRE site. Fluorescein–polyamide conjugate **1** prevents HIF-1α/ARNT from binding to the HRE.

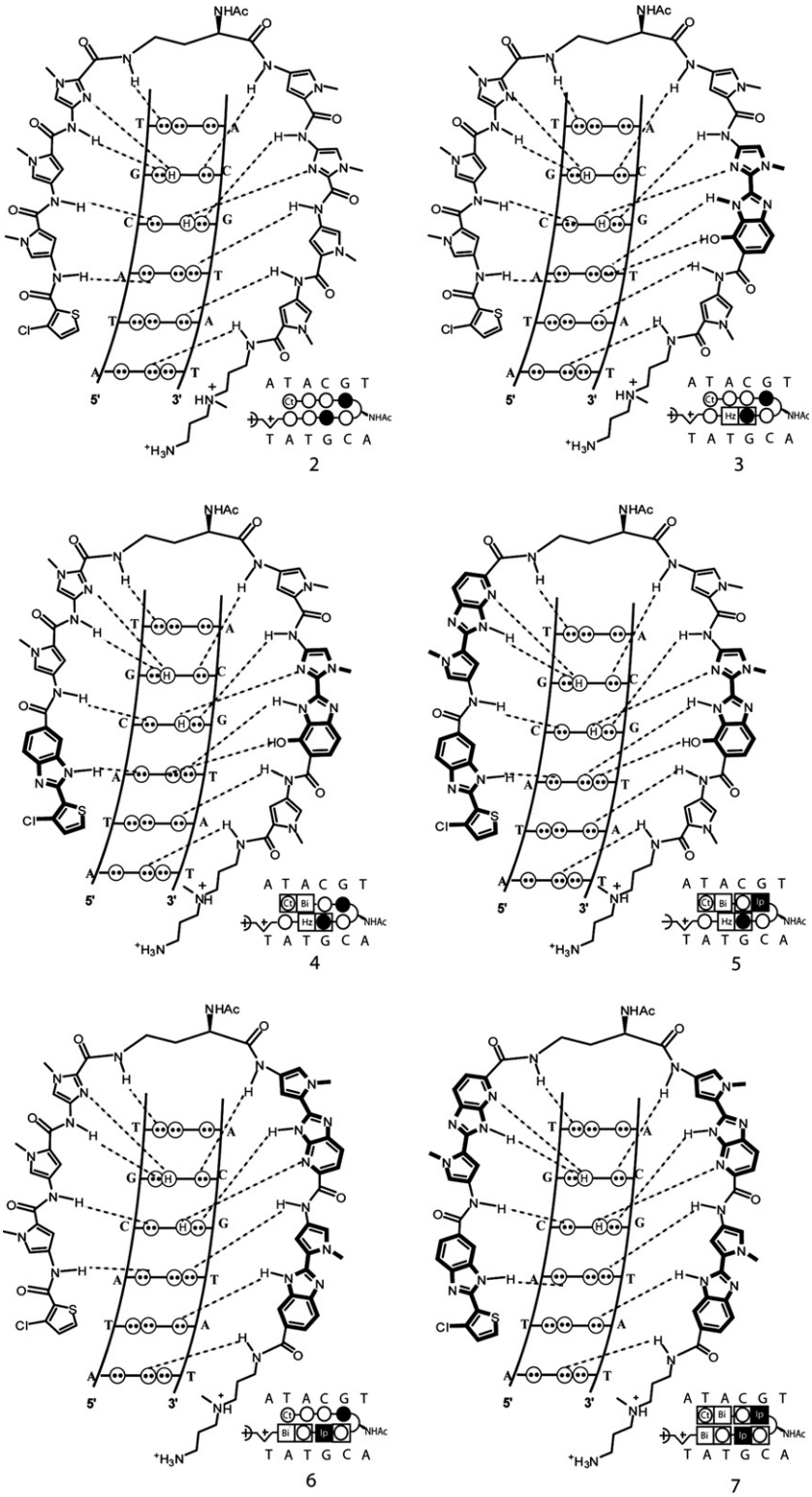
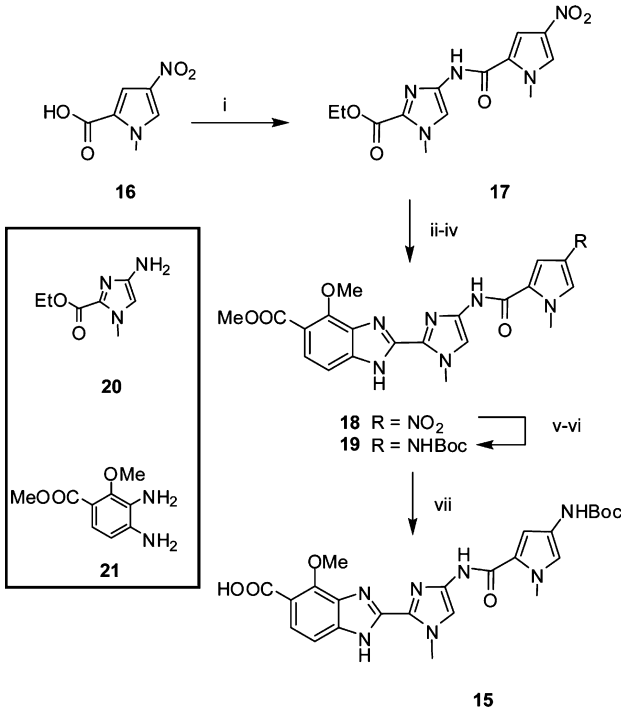


Figure 3. Structures, ball-and-stick models of the parent eight-ring haipin polyamide **2** and the benzimidazole-containing polyamides **3**–**7** and postulated hydrogen-bonding models of the 1:1 hairpin polyamides–DNA complexes formed between CtPyPyIm-(*R*)-$^{AcHN}\gamma$-PyImPyPy (**2**), CtPyPyIm-(*R*)-$^{AcHN}\gamma$-PyImHzPy (**3**), CtBiPyIm-(*R*)-$^{AcHN}\gamma$-PyImHzPy (**4**), CtBiPyIp-(*R*)-$^{AcHN}\gamma$-PyImHzPy (**5**), CtPyPyIm-(*R*)-$^{AcHN}\gamma$-PyIpPyBi (**6**), CtBiPyIp-(*R*)-$^{AcHN}\gamma$-PyIpPyBi (**7**) and the HIF binding site on the HRE promoter (5′-ATACGT-3′). Circles with two dots represents the lone pairs of the N(3) of purines and O(2) of pyrimidines. Circles containing 'H' represent the N(2) of G. Putative hydrogen bonds are represented by dotted lines. Below each hydrogen-bonding model are the ball-and-stick representations of polyamides. Imidazoles and pyrroles are shown as filled and non-filled circles, respectively; chlorothiophene rings are annotated with a 'Ct' in the ring. (*R*)-Diamino butyric acid (DABA) is shown as a semicircle connecting the two subunits. Two touching squares represent benzimidazole (Bi)–pyrrole, hydroxybenzimidazole (Hz)–imidazole, imidazopyridine (Ip)–pyrrole, and benzimidazole–chlorothiophene constructs.

2. Results and discussion

2.1. Monomer syntheses

The syntheses of oligomers **3**–**7** require the preparation of the *N*-Boc-*O*-methyl protected hydroxybenzimidazole–imidazole[12] (Boc–ImHzOMe–OH) **8**, *N*-Boc-protected benzimidazole–pyrrole (Boc-PyBi-OH)[11] **9**, and *N*-Boc- protected imidazo[4,5-*b*]pyridine–pyrrole[11] (Boc-PyIp-OH) **10** amino acids in addition to the traditional five-membered rings building blocks **11**–**14** (Scheme 3). These compounds were prepared following procedures reported earlier.[11,12] However, attempts to synthesize polyamides **3**–**5** from Boc–ImHzOMe–OH **8** failed to provide the desired molecules, presumably because the coupling of the immobilized imidazole amine with the next pyrrole acid failed on resin. The synthesis of polyamides **3**–**5** therefore required the synthesis of the *O*-methyl-hydroxybenzimidazole–imidazole–pyrrole building block **15** in solution prior to solid-phase synthesis (Scheme 1). HBTU activation of the known pyrrole carboxylic acid **16**[15] followed by coupling to the imidazole amine **22**[15] in DMF yielded **17** (48%). The ethyl ester of **17** was hydrolyzed with sodium hydroxide in ethanol and the resulting acid was coupled to diamine **21**[11] using HBTU in a DIEA–DMF mixture to afford **18** after cyclo-dehydration in acetic acid at 90 °C (61%, 3 steps). Reduction of the nitro group of **18** with Pd/C in the presence of H_2 and in situ Boc protection of the free amine intermediate yielded **19** (98%). Final hydrolysis of the methyl ester of **19** with sodium hydroxide in dioxane–H_2O gave the desired *N*-Boc-*O*-methyl protected hydroxybenzimidazole–imidazole–pyrrole (Boc-PyImHzOMe–OH) **15** in 96% yield.



Scheme 1. Synthesis of the Boc-protected benzimidazole amino acid **15**. Reagents: (i) HBTU, **20**, DIEA, DMF; (ii) NaOH, EtOH; (iii) HBTU, **21**, DIEA, DMF; (iv) AcOH; (v) H_2, Pd/C, DMF; (vi) Boc_2O, DIEA, DMF; (vii) NaOH, MeOH.

Additionally, the synthesis of polyamides **4**, **5**, and **7** required the synthesis of the benzimidazole–chlorothiophene building block **22** (CtBi–OH). Condensation of chlorothiophene carboxylic acid **14**[16] with the commercially available diamine **23** (HBTU coupling and cyclodehydration in acetic acid) followed by hydrolysis of the methyl ester (sodium hydroxide in dioxane–H_2O) provided **22** (70%, 3 steps, Scheme 2).

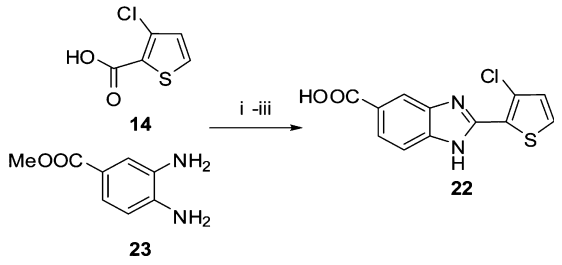
2.2. Polyamide synthesis

Hairpin polyamides were synthesized manually on oxime resin (polyamides **2**–**5**) and from the 4-hydroxyphenylsulfanylmethyl polystyrene resin[17] (polyamides **6** and **7**) in a stepwise fashion according to established solid-phase protocols[11,15] using Boc-protected amino acids **9**–**13** and **15** as well as 4-chloro-thiophene-2 carboxylic acid **14** (Scheme 3). Couplings were achieved using pre-activated monomers (Boc-Py-OBt) or by HBTU activation in a DIEA and DMF mixture. Coupling times ran from 3 to 24 h at 25 °C. Deprotection of the growing oligomer was accomplished using 20% TFA/DCM.

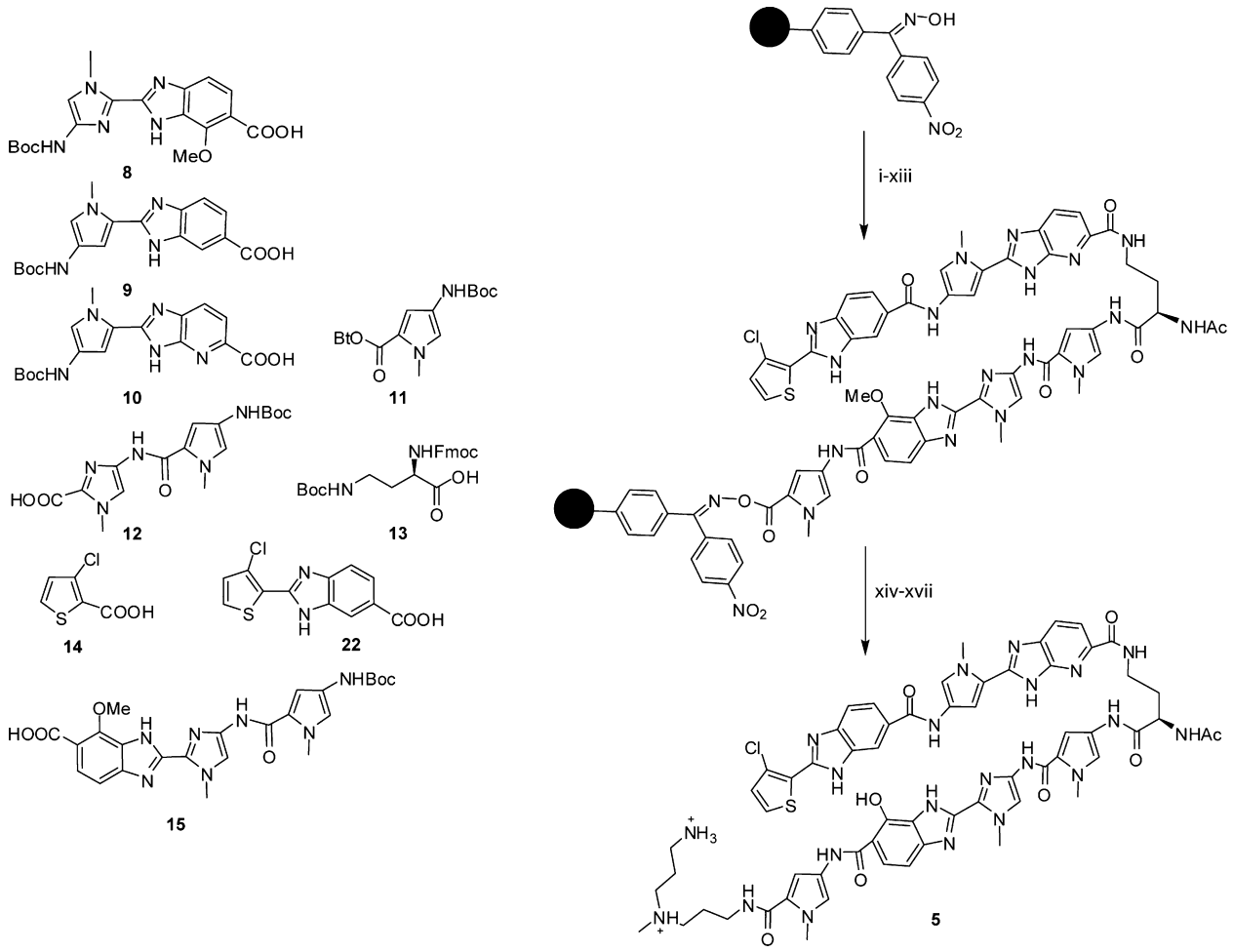
Polyamides were cleaved from the resin by treatment with neat 3,3′-diamino-*N*-methyl-dipropylamine at 80 °C for 2 h and purified by preparatory reverse-phase HPLC to yield CtPyPyIm-(*R*)-$^{AcHN}\gamma$-PyImPyPy (**2**), CtPyPyIm-(*R*)-$^{AcHN}\gamma$-PyIpPyBi (**6**), and CtBiPyIp-(*R*)-$^{AcHN}\gamma$-PyIpPyBi (**7**). Treatment with thiophenol and NaH in DMF (80 °C, 2 h) accomplished the *O*-methyl deprotection to provide the free hydroxy derivatives (**3**–**5**) and after a second HPLC purification yielded CtPyPyIm- (*R*)-$^{AcHN}\gamma$-PyImHzPy (**3**), CtBiPyIm-(*R*)-$^{AcHN}\gamma$-PyImHzPy (**4**), and CtBiPyIp-(*R*)-$^{AcHN}\gamma$-PyImHzPy (**5**).

2.3. DNA affinity and sequence specificity

Quantitative DNase-I footprinting titration experiments[14] (10 mM Tris–HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$, PH 7.0, 22 °C, equilibration time: 12–14 h) were performed on a ^{32}P-5′-labeled *Eco*RI/*Puv*II restriction fragment of plasmid AV1 (Fig. 4). This restriction fragment contains 4 six-base pair binding sites: site I, 5′-ATACGT-3′, regarded as a match site for polyamides **2**–**7**, in addition to site II,



Scheme 2. Synthesis of the benzimidazole–chlorothiophene cap **22**. Reagents: (i) HBTU, DIEA, DMF; (ii) AcOH; (iii) NaOH, MeOH.



Scheme 3. Representative solid-phase synthesis of polyamide **5** along with a table of the amino acid building blocks used. Reagents and conditions: (i) Boc-Py-OBt **11**, DIEA, DMF; (ii) Ac_2O, DIEA, DMF; (iii) 20% TFA/DCM; (iv) Boc-Py-Im-HzOMe-OH **15**, HBTU, DIEA, DMF; (v) Ac_2O, DIEA, DMF; (vi) 20% TFA/DCM; (vii) Boc-(R)-$H_2N\gamma$-OH **13**, HBTU, DIEA, DMF; (viii) Ac_2O, DIEA, DMF; (ix) 20% TFA/DCM; (x) Boc-Py-Ip-OH **10**, HBTU, DIEA, DMF; (xi) Ac_2O, DIEA, DMF; (xii) 20% TFA/DCM; (xiii) Ct-Bi-OH **22**, HBTU, DIEA, DMF; (xiv) 3,3′-diamino-*N*-methyl-dipropylamine, 80 °C 2 h; (xv) preparative HPLC; (xvi) thiophenol, NaH, DMF; (xvii) preparative HPLC. Amino acids Boc-Py-Bi-OH **9**, Boc-Py-Im-OH **12** as well as 4-chloro-thiophene-2 carboxylic acid **14** were used in the synthesis of polyamides **2**–**7** (see supporting information).

5′-AT**T**CGT-3′, site III, 5′-ATAC**C**T-3′, and site IV, 5′-A**A**ACGT-3′ regarded as single base pair mismatch sites.

2.4. Affinity

All polyamides synthesized for this study, with the exception of **7**, bind the 5′-ATACGT-3′ site with high affinity, ranging from $K_a = 5.9 \times 10^8\ M^{-1}$ to $K_a = 2.3 \times 10^{10}\ M^{-1}$ and evidence that the incorporation of the benzimidazoles structures at selected sites (polyamide **3**), and even at multiple site (oligomers **4**–**6**), of the hairpin resulted in no significant loss of affinity as compared to the parent polyamide **2** that binds the 5′-ATACGT-3′ site with an affinity of $K_a = 5.1 \times 10^9\ M^{-1}$.

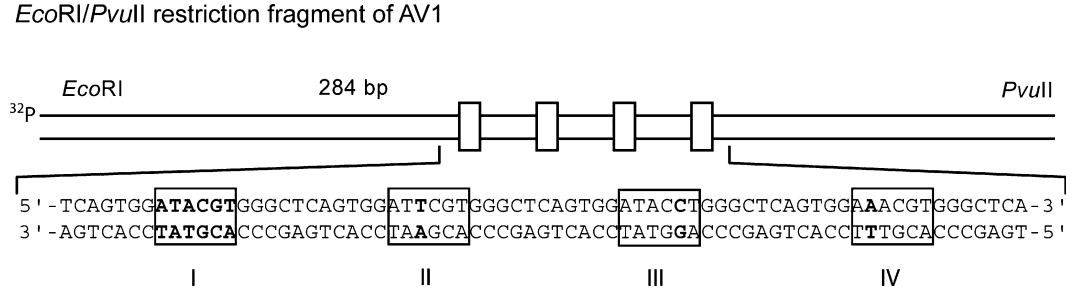


Figure 4. Illustration of the *Eco*RI/*Puv*II restriction fragment derived from plasmid AV1. The four designed binding sites that were analyzed in quantitative footprint titrations are indicated with a box surrounding each of the 6 bp sites.

2.5. Specificity. The HzPy and the HzBi pairs

As anticipated,[12] the HzPy pair can discriminate T·A from A·T base pairs. The polyamide **3** (Fig. 5) shows a 10-fold specificity for the 5′-ATACGT-3′ match site ($K_a = 3.1 \times 10^9\ M^{-1}$) versus the 5′-AT**T**CGT-3′ ($K_a = 4.0 \times 10^8\ M^{-1}$) site. In oligomers **4** and **5** the hydroxybenzimidazole Hz is paired against a benzimidazole Bi to discriminate between the T·A and A·T sites. Specificity of these HzBi pairs for the 5′-ATACGT-3′ site versus the 5′-AT**T**CGT-3′ site, however, is only of a 3- to 5-fold higher than the PyPy pair (Table 1, Fig. 6).

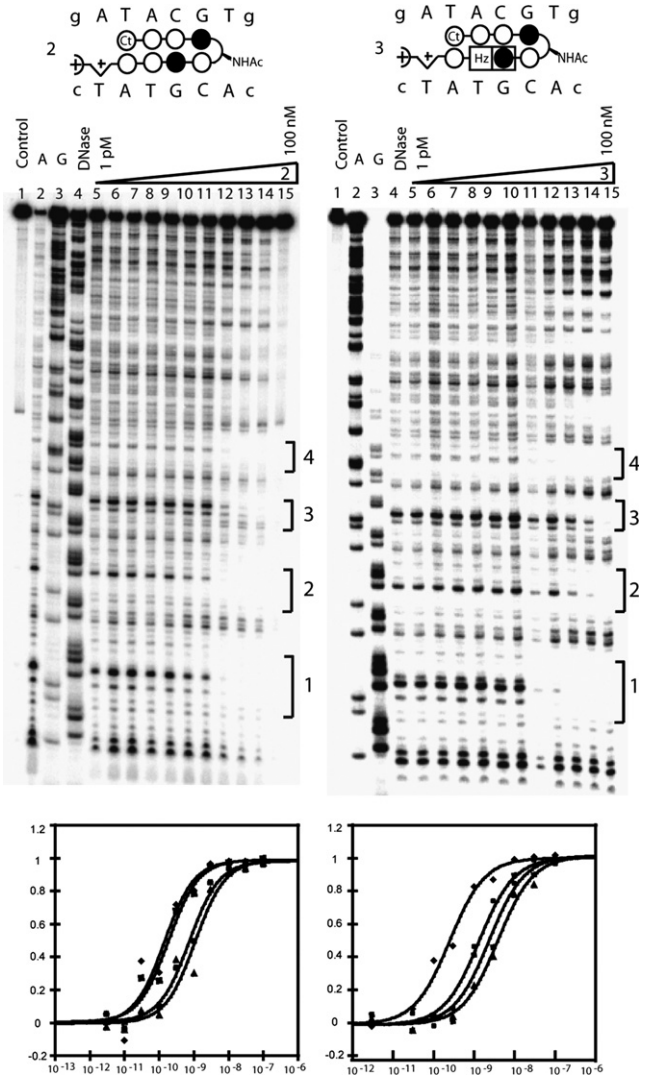


Figure 5. Quantitative DNase I footprinting experiments in the hairpin motif for polyamides **2** and **3** on the 278 bp, 5′-end-labeled PCR product of plasmid AV1: lane 1, intact DNA; lane 2, A reaction; lane 3, G reaction; lane 4, DNase I standard; lanes 5–15, 1 pM, 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, and 100 nM polyamide, respectively. Each footprinting gel is accompanied by the following: (top) 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; and (bottom) binding isotherms for the four designed sites. θ_{norm} values were obtained according to published methods.[15]

2.6. The IpPy pair

Parent polyamide **2** shows a clear 10-fold specificity for G·C ($K_a = 5.1 \times 10^9\ M^{-1}$) versus the C·G ($K_a = 5.3 \times 10^8\ M^{-1}$, Table 1, Fig. 5). The imidazo[4,5-*b*]pyridine **Ip** containing polyamide **5** binds the 5′-ATACGT-3′ match site with an affinity of $K_a = 1.6 \times 10^9\ M^{-1}$ a 3-fold lower affinity than the parent compound **2** and also discriminates the C·G site by a factor of 9 (Table 1, Fig. 6). Within this sequence context, the replacement of the ImPy pair by the IpPy pair was more efficient in terms of specificity than expected from previous results.[18]

2.7. The CtPy and CtBi pairs

In polyamides **2** and **3**, the CtPy pair shows a 5- to 6-fold specificity for T·A versus A·T base pairs. The oligomers **4** and **5**, which contain the chlorothiophene moiety fused to the benzimidazole structure, show a somewhat lower specificity for T·A versus A·T base pairs (2- to 3-fold, Fig. 6, Table 1). These results are in agreement with previous studies,[13] in which the chlorothiophene cap showed a 3-fold specificity for T·A versus A·T base pairs. The CtBi pair shows lower sequence specificity: oligomer **6** binds the 5′-A**A**ACGT-3′ site ($K_a = 1.5 \times 10^{10}\ M^{-1}$) as well as the 5′-ATACGT-3′ ($K_a = 2.3 \times 10^{10}\ M^{-1}$) site (Table 1, Fig. 7).

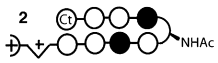
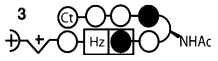
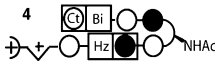
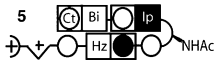
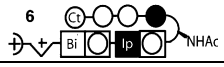
2.8. Design of an oligomer for 5′-ATACGT-3′

The synthesis of oligomer **7** containing 4 six-five fused ring structures was achieved via the stepwise addition of Boc-amino acid dimers in the same manner as previously described for polyamide syntheses. Quantitative DNase-I footprinting titration experiments of this oligomer on the plasmid AV1 (Fig. 4) revealed that **7** binds DNA non-specifically at high concentrations (Fig. 7). The oligomer's large hydrophobic surface may account for this effect. This result is not general for this class of compounds as previous studies[19] have shown that a compound consisting exclusively of six–five fused ring systems and minimal carboxamide linkages still demonstrated high levels of specificity (6-fold for a single base pair mismatch and 30-fold for a double base pair mismatch) and excellent binding affinity ($K_a \approx 10^{10}\ M^{-1}$).

3. Conclusion

The hairpin polyamides designed for this study, containing the HzIm, IpPy, CtBi, and BiPy dimers, are able to target the 5′-TACGT-3′ sequence with good affinity. The dimer CtBi mimics CtPy and represents a new recognition element for the minor groove of DNA. The hairpins **3**, **4**, and **5** target their match with good affinity as compared to the parent hairpin polyamide **2**, while the HzPy and HzBi pairs demonstrate increased specificity for a single base pair mismatch site as compared to **2**. These hairpins are therefore promising candidates for biological studies. Hairpin **6** lacks sufficient specificity to be used on a biological assay. The effect of the oligomers **3**–**5** on the regulation of VEGF in cancer cells is ongoing and will be reported in due course.

Table 1. Equilibrium association constants K_a [M^{-1}] and specificities for polyamides **1–6** on the AV1 plasmid [a,b]

Oligomer	5′-aTACGt-3′	5′-aTTCGt-3′	5′-aTACCt-3′	5′-aAACGt-3′
2 	$5.1(\pm2.0)\times10^{9}$	$3.7(\pm1.5)\times10^{9}$	$5.3(\pm4.0)\times10^{8}$	$1.1(\pm0.4)\times10^{9}$
3 	$3.1(\pm0.5)\times10^{9}$	$4.0(\pm0.4)\times10^{8}$	$3.9(\pm0.8)\times10^{8}$	$6.9(\pm0.3)\times10^{8}$
4 	$5.9(\pm0.3)\times10^{8}$	$1.8(\pm0.5)\times10^{8}$	—	$2.8(\pm0.2)\times10^{8}$
5 	$1.6(\pm0.3)\times10^{9}$	$4.5(\pm0.3)\times10^{8}$	$1.7(\pm0.7)\times10^{8}$	$8.1(\pm0.8)\times10^{8}$
6 	$2.3(\pm0.3)\times10^{10}$	$1.8(\pm0.1)\times10^{10}$	$1.2(\pm0.1)\times10^{10}$	$1.5(\pm0.1)\times10^{10}$

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

4. Experimental

4.1. General

N,*N*-Dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIEA), thiophenol, 3,3-diamino-*N*-methyldipropylamine, and sodium hydride were purchased from Aldrich. Oxime (0.48 mmol/g) and 4-hydroxyphenylsulfanylmethyl polystyrene resins were purchased from Novabiochem. 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and (*R*)-1-(9*H*-fluoren-9-yl)-11,11-dimethyl-3,9-dioxo-2,10-dioxa-4,8-diazadodecane-5- carboxylic acid (**13**) were purchased from Peptide International. Trifluoroacetic acid (TFA) was purchased from Halocarbon. Precoated silica gel plates 60 F_{254} for TLC and silica gel 60 (40 μm) for flash chromatography were from Merck.

Oligonucleotide inserts were synthesized by the Biopolymer Synthesis Center at the California Institute of Technology. Glycogen (20 mg/mL), dNTPs (PCR nucleotide mix), and all enzymes, unless otherwise stated, were purchased from Boehringer–Mannheim. pUC19 was purchased from New England Biolaboratorys, and deoxyadenosine [γ-^{32}P]triphosphate was provided by ICN. Calf thymus DNA (sonicated, deproteinized) and DNase I (7500 U/mL, FPLC pure) were from Amersham Pharmacia. AmpliTaq DNA polymerase was from Perkin-Elmer and was used with the provided buffers. Tris–HCl, DTT, RNase-free water, and 0.5 M EDTA were from United States Biochemical. Ethanol (200 proof) was from Equistar. Calcium chloride, potassium chloride, and magnesium chloride were purchased from Fluka. Pre-mixed Tris–borate–EDTA (Gel-Mate, used for gel running buffer) was from Gibco. Bromophenol blue and xylene cyanol were from Acros. All reagents were used without further purification. ^{1}H NMR and ^{13}C spectra were recorded on a Varian Mercury 300 MHz 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. Mass Spectra were recorded on the following spectrometer at the Mass Spectroscopy Laboratory at the California Institute of Technology: Matrix-Assisted, Laser Desorption/Ionization Time Of Flight (MALDI-TOF) on a Voyager DE-PRO from Applied Biosystems, high-resolution Fast Atom Bombardement (FAB) on a JEOL JMS-600H double focusing high-resolution magnetic sector, and Electrospray Injection (ESI) LCQ ion trap on a LCQ classic, Thermofinnigan.

4.2. Monomer synthesis

1-Methyl-4-nitro-1*H*-pyrrole-2-carboxylic acid[15] (**16**), 1-methyl-4-amino-1*H*-imidazole-2-carboxylic acid ethyl ester[15] (**20**), diamino-2-methoxy-benzoic acid methyl ester[11] (**21**), 4-chloro-thiophene-2 carboxylic acid[16] (**14**), 1*H*-benzo[*d*][1,2,3]triazol-1-yl-4-(*tert*-butoxycarbonylamino)-1-methyl-1*H*-pyrrole-2-carboxylate[15] (**11**), 2-(4-*tert*-butoxycarbonylamino-1-methyl-1*H*-pyrrol-2-yl)-3*H*-imidazo[4,5-*b*]pyridine-6-carboxylic acid[11] (**10**), 2-(4-(*tert*-butoxycarbonylamino)-1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid[10] (**9**), 4-(4-(*tert*-butoxycarbonylamino)-1-methyl-1*H*-pyrrole-2-carboxamido)-1-methyl-1*H*-imidazole-2-carboxylic acid[15] (**12**), and 2-(4-(*tert*-butoxycarbonylamino)-1-methyl-1*H*-imidazol-2-yl)-4-methoxy-1*H*-benzo[*d*]imidazole-5-carboxylic acid[11] (**8**) were synthesized according to previously published protocols.[10,11,15,16]

4.2.1. 1-Methyl-4-[(1-methyl-4-nitro-1*H*-pyrrole-2-carbonyl)-amino]-1*H*-pyrrole-2-carboxylic acid ethyl ester (17). Under argon, a solution of **16** (0.8 g, 4.7 mmol) in DMF was treated with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.7 g, 4.5 mmol) and DIEA (1.74 mL) and stirred for 10 min at room temperature. The imidazole amine **20** (0.8 g, 4.7 mmol) was added and the resulting mixture stirred for 12 h at 60 °C. The mixture was then cooled to room temperature and poured on ice. The resulting yellow precipitate was filtered, washed with water and methanol, and dried under vacuum to yield 0.74 g of **17** (48%) as a yellow powder. Data of **17**: R_f

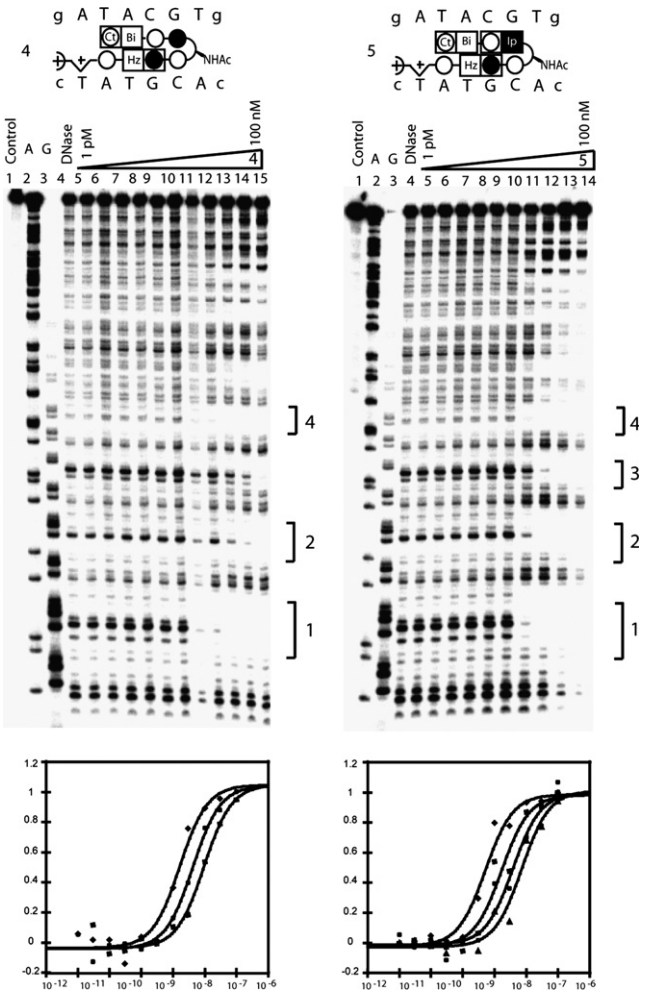


Figure 6. Quantitative DNase I footprinting experiments in the hairpin motif for polyamides **4** and **5** on the 278 bp, 5′-end-labeled PCR product of plasmid AV1: lane 1, intact DNA; lane 2, A reaction; lane 3, G reaction; lane 4, DNase I standard; lanes 5–15 (14), 1 pM, 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM (3 nM), 3 nM (10 nM), 10 nM (30 nM), 30 nM (100 nM), and 100 nM polyamide, respectively. Each footprinting gel is accompanied by the following: (top) 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; and (bottom) Binding isotherms for the four designed sites. θ_{norm} values were obtained according to published methods.

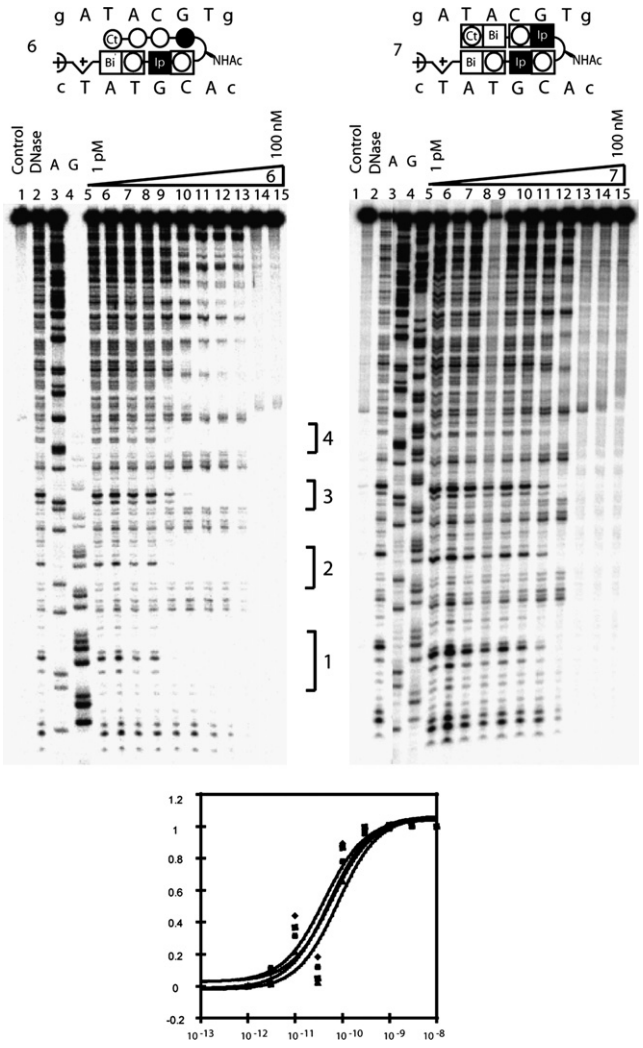


Figure 7. Quantitative DNase I footprinting experiments in the hairpin motif for polyamides **6** and **7** on the 278 bp, 5′-end-labeled PCR product of plasmid AV1: lane 1, intact DNA; lane 2, DNase I standard; lane 3, A reaction; lane 4, G reaction; lanes 5–15, 1 pM, 3 pM, 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, 30 nM, and 100 nM polyamide, respectively. Each footprinting gel is accompanied by the following: (top) 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; and (bottom) Binding isotherms for the four designed sites. θ_{norm} values were obtained according to published methods.

0.54 (EtOAc/hexane 4:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.28 (t, J = 7.2 Hz, 3H), 3.92 (s, 3H), 3.94 (s, 3H), 4.26 (q, J = 7.2 Hz, 2H), 7.67 (s, 1H), 7.80 (s, 1H), 8.18 (s, 1H), 11.17 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 14.74, 36.16, 38.43. 61.33, 109.43, 116.29, 125.88, 129.38, 131.77, 134.63, 137.79, 157.97, 159.10. MS (ESI) m/z (rel intensity): 322 (100).

4.2.2. 4-Methoxy-2-{1-methyl-4-[(1-methyl-4-nitro-1*H*-pyrrole-2-carbonyl)-amino]-1*H*-imidazol-2-yl}-3*H*-benzoimidazole-5-carboxylic acid methyl ester (18). A solution of **17** (0.7 g, 2.2 mmol) in EtOH (20 mL) and aq NaOH (2 M, 20 mL) was stirred for 12 h at room temperature, ethanol was evaporated under vacuum, and 1 N HCl was added dropwise to adjust the pH to 2–3, while a precipitate formed. The precipitate was filtered and dried under vacuum. The resulting acid (0.4 g, 1.5 mmol) was dissolved in DMF treated with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.5 g, 1.5 mmol) and DIEA (0.4 mL), and stirred for 10 min at room temperature, whereupon the diamine **21** (0.3 g, 1.5 mmol) was added. The mixture was stirred for 12 h at 60 °C, added dropwise to ice. The precipitate was filtered, washed with water, died under vacuum, suspended in AcOH (10 mL), and then heated to 90 °C for 12 h. The suspension was cooled to room temperature and Et_2O (20 mL) was added. The precipitate was filtered and rinsed with Et_2O to yield 0.6 g **18** (61%). Data of **18**: R_f 0.56 (EtOAc). ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.67 (s, 3H), 3.82 (s, 3H), 3.95 (s, 3H), 3.96 (s, 3H), 7.70-7.65 (m, 2H), 7.83 (s, 1H), 8.20 (s, 1H), 9.02 (s, 1H), 9.65 (s, 1H), 10.97

(s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 35.86, 38.39, 51.92, 61.98, 109.48, 110.54, 115.63, 126.04, 129.32, 131.60, 134.60, 134.80, 136.38, 151.68, 158.15, 158.50, 158.96, 165.73, 172.75. MS (ESI) *m*/*z* (rel intensity): 454.9 (50), 475.8 (100).

4.2.3. 2-(4-{[4-(*tert*-Butoxycarbonylmethyl-amino)-1-methyl-1*H*- pyrrole-2-carbonyl]-amino}-1-methyl-1*H*-imidazol-2-yl)-4-methoxy-1*H*-benzoimidazole- 5-carboxylic acid methyl ester (19). A solution of **18** (150 mg, 0.33 mmol) in DMF (10 mL) was treated with Pd/C (10 wt%, 60 mg) and transferred to a Parr apparatus. The mixture was stirred for 5 h at room temperature under a hydrogen pressure of 5 atm, transferred to a round-bottomed flask, treated with DIEA and Boc_2O, and stirred under argon for 12 h at room temperature. The mixture was filtered through a pad of Celite, rinsed with methanol, and the solvents evaporated. FC (EtOAc/hexane) yielded 170 mg **19** (98%). Data of **19**: R_f 0.3 (EtOAc). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.43 (s, 9H), 3.78 (s, 3H), 3.81 (s, 3H), 4.14 (s, 3H), 4.34 (s, 3H), 6.86 (s, 1H), 6.98 (s, 1H), 7.18 (d, J = 8.7, 1H), 7.54 (d, J = 8.4, 1H), 7.55 (s, 1H), 9.09 (s, 1H), 10.20 (s, 1H). UV (CH_3CN/H_2O 1:1) λ_{max} 330 nm (17875). MS (ESI) *m*/*z* (rel intensity): 524.0 (20), 546.0 (100).

4.2.4. 2-(4-{[4-(*tert*-Butoxycarbonylmethyl-amino)-1-methyl-1*H*- pyrrole-2-carbonyl]-amino}-1-methyl-1*H*-imidazol-2-yl)-4-methoxy-1*H*-benzoimidazole- 5-carboxylic acid (15). The methyl ester **19** (170 mg, 0.32 mmol) was dissolved 1 M NaOH (1:1 dioxane/H_2O 5 mL), stirred for 10 h at room temperature, and heated to 60 °C for 2 h. The reaction mixture was cooled down to 0 °C and 1 N HCl was added dropwise to adjust the pH to 2–3, while a white precipitate formed. Separation of the product was accomplished by centrifugation and decanting of the supernatant. Lyophilization yielded 160 mg **15** (98%). Data of **15**: R_f 0.1 (EtOAc). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.44 (s, 9H), 3.81 (s, 3H), 4.14 (s, 3H), 4.34 (s, 3H), 6.87 (s, 1H), 6.98 (s, 1H), 7.18 (d, J = 8.7, 1H), 7.54 (d, J = 8.4, 1H), 7.55 (s, 1H), 8.6 (br s, 1H), 9.09 (s, 1H), 10.20 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 28.88, 35.87, 36.79, 61.81, 79.00, 101.66, 113.88, 114.56, 116.25, 118.82, 122.73, 123.13, 126.57, 137.70, 139.81, 142.73, 143.99, 151.84, 153.57, 159.40, 167.58, 168.29. HRMS (FAB) *m*/*z* calcd for $C_{24}H_{28}N_7O_6$, 510.2101. Found: 510.2108.

4.2.5. 4-Methoxy-2-{1-methyl-4-[(1-methyl-4-nitro-1*H*-pyrrole-2-carbonyl)-amino]-1*H*-imidazol-2-yl}-3*H*-benzoimidazole-5-carboxylic acid (22). The acid **14** (0.5 g, 3.1 mmol) was dissolved in DMF treated with 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.0 g, 3.0 mmol) and DIEA (3.2 mL), and stirred for 10 min at room temperature, whereupon the diamine **23** (0.5 g, 3.1 mmol) was added. The mixture was stirred for 12 h at 60 °C, cooled down to room temperature, and added dropwise to ice. The precipitate was filtered, washed with water, dry under vacuum, suspended in AcOH (20 mL), and heated to 90 °C for 12 h. The suspension was down cooled to room temperature and EtOAc was added. The organic phase was separated, washed with $NaHCO_3$ and brine, dried with $MgSO_4$, and the solvents evaporated. The resulting methyl ester was dissolved 1 M NaOH (1:1 dioxane/H_2O 5 mL), stirred for 10 h at room temperature, and heated at 60 °C for 2 h. The reaction mixture was cooled to 0 °C and 1 N HCl was added dropwise to adjust the pH to 2–3, which resulted in the formation of a white precipitate. Separation of the product was accomplished by centrifugation and decanting of the supernatant. Lyophilization yielded 0.6 g **22** (70%). Data of **22**: R_f 0.45 (EtOAc). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.29 (d, J = 5.4, 1H), 7.72 (dd, J = 8.4, 0.6, 1H), 7.88 (d, J = 8.7, 1H), 7.97 (d, J = 5.7, 1H), 8.24 (d, J = 0.6, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 115.46, 117.22, 123.15, 126.04, 126.81, 127.38, 130.28, 133.08, 135.85, 139.15, 145.20, 167.82. HRMS (FAB) *m*/*z* calcd for $C_{12}H_8N_2O_2S$, 278.9995. Found: 279.0004.

4.3. Polyamide synthesis

Polyamides were synthesized in a stepwise fashion from 0.48 mmol/g oxime resin (**1**–**5**) or from 4-hydroxyphenylsulfanylmethyl polystyrene resin[17] (1.7 mmol/g) (**6**–**7**) by manual solid-phase methods using Boc-protected amino acid building blocks as previously reported.[11,15] *N*-Boc deprotection of the immobilized polyamides-containing building blocks was accomplished using 20% TFA in CH_2Cl_2 for 23 min.

Extinction coefficients were calculated based on ε = 8690 cm L mol^{-1} per ring at 310 nm (**1**) and determined for polyamides **2**–**6**: ε = 33,000 cm L mol^{-1} at 321 nm (**2**), ε = 15,800 cm L mol^{-1} at 326 nm (**3**), ε = 23,100 cm L mol^{-1} at 336 nm (**4**), ε = 58,700 cm L mol^{-1} at 330 nm (**5**), ε = 60,924 cm L mol^{-1} at 342 nm (**6**).

4.4. Procedure for 3,3-diamino-*N*-methyl-dipropylamine cleavage from resin

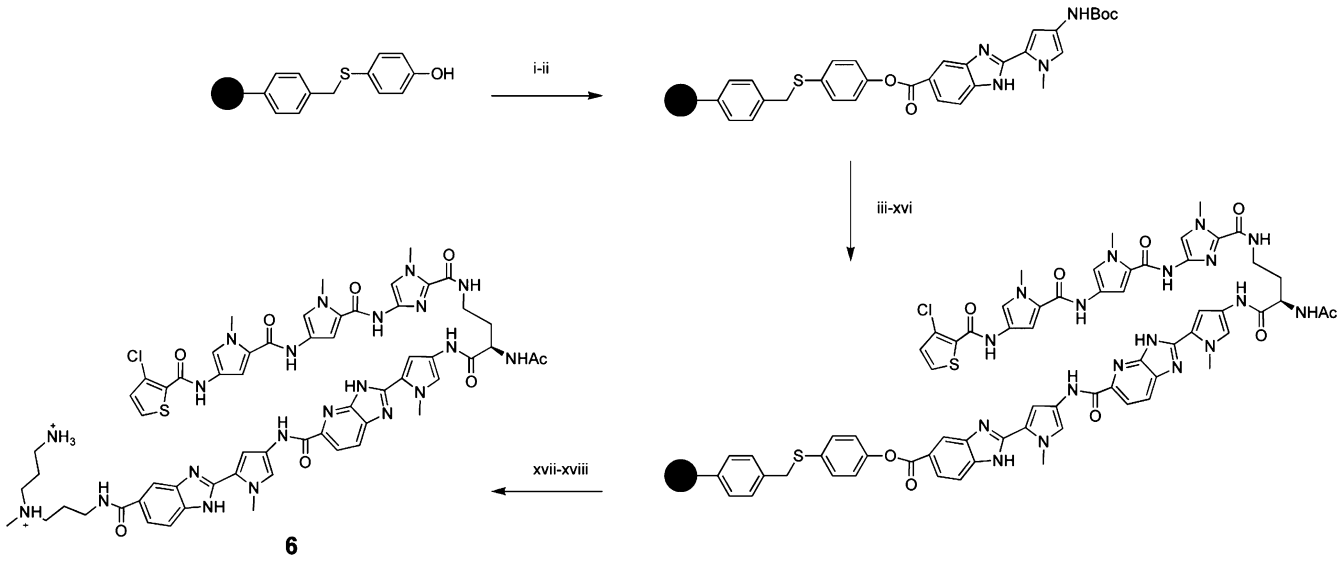
After completion of the synthesis of the polyamides the resin was washed with DMF, DCM, MeOH, and Et_2O, and dried under vacuum. The resin was treated with 3,3-diamino-*N*-methyl-dipropylamine (1 mL) under periodic agitation at 80 °C for 2 h, removed by filtration, and washed with DMF (1 mL). The filtrate was diluted with 0.1% TFA-H_2O and purified by reversed-phase HPLC to yield the pure polyamides.

4.5. CtPyPyIm-(*R*)-$H_2N\gamma$-PyImPyPy (2)

Starting from 100 mg of resin the title compound was synthesized. After HPLC purification, it was recovered upon lyophilization of the appropriate fraction as a colorless powder (0.8 mg). UV(H_2O) 248, 303. MALDI-TOF-MS: calcd for $C_{58}ClH_{71}N_{21}O_{10}SNa$, 1312.8. Found, 1312.7.

4.6. CtPyPyIm-(*R*)-$H_2N\gamma$-PyIpPyBi (6)

Starting from 90 mg of resin the title compound was synthesized. After HPLC purification, it was recovered upon lyophilization of the appropriate fraction as a



Scheme 4. Representative solid-phase synthesis of polyamide **6**. Reagents and conditions: (i) Boc-PyBi-OH **11**, HBTU, DIEA, DMF, 12 h; (ii) Ac_2O, DIEA, DMF; (iii) 20% TFA/DCM; (iv) Boc-Py-Ip-OH **12**, HBTU, DIEA, DMF; (v) Ac_2O, DIEA, DMF; (vi) 20% TFA/DCM; (vii) Boc-(*R*)-$H_2N\gamma$-OH **15**, HBTU, DIEA, DMF; (viii) Ac_2O, DIEA, DMF; (ix) 20% TFA/DCM; (x) Boc-Py-Im-OH **14**, HBTU, DIEA, DMF; (xi) Ac_2O, DIEA, DMF; (xii) 20% TFA/DCM; (xiii) Boc-Py-OBt **13**, DIEA, DMF; (xiv) Ac_2O, DIEA, DMF; (xv) 20% TFA/DCM; (xvi) Ct-OH **16**, HBTU, DIEA, DMF; (xvii) 3,3′-diamino-*N*-methyl-dipropylamine, 80 °C 2 h; (xviii) preparative HPLC.

colorless powder (0.7 mg). UV(H_2O) 243, 330. MALDI-TOF-MS: calcd for $C_{60}ClH_{68}N_{21}O_8S$, 1277.5. Found, 1277.3 (Scheme 4).

4.7. CtBiPyIp-(*R*)-$H_2N\gamma$-PyIpPyBi (7)

Starting from 30 mg of resin the title compound was synthesized. After HPLC purification, it was recovered upon lyophilization of the appropriate fraction as a colorless powder (0.6 mg). UV(H_2O) 251, 342. MALDI-TOF-MS: calcd for $C_{62}ClH_{63}N_{21}O_6S$, 1264.4. Found, 1264.1.

4.8. Deprotection of the *O*-methyl-protected polyamides

Following a previously reported procedure.[10] To a slurry of NaH (40 mg) in DMF (0.8 mL) was added thiophenol (0.3 mL) and the mixture was heated to 80 °C for 20 min. The polyamide (1 μmol) was dissolved in DMF (0.2 mL), added to the thiophenolate solution, and the mixture was heated to 80 °C for 2 h. After cooling to room temperature, 7 mL of 20 %TFA-H_2O was added and extracted with Et_2O. The organic phases were discarded and the aq solution purified by preparative reversed-phase HPLC to yield the pure polyamides.

4.9. CtPyPyIm-(*R*)-$H_2N\gamma$-PyImHzPy (3)

Starting from 90 mg of resin the title compound was synthesized. After HPLC purification, it was recovered upon lyophilization of the appropriate fraction as a colorless powder (0.9 mg). UV(H_2O) 247, 321. ESI-MS: calcd for $C_{59}ClH_{69}N_{21}O_{10}S$, 1298.4. Found, 1298.2.

4.10. CtBiPyIp-(*R*)-$H_2N\gamma$-PyImHzPy (4)

Starting from 90 mg of resin the title compound was synthesized. After HPLC purification, it was recovered upon lyophilization of the appropriate fraction as a colorless powder (2.7 mg). UV(H_2O) 245, 326. ESI-MS: calcd for $C_{60}ClH_{68}N_{21}O_9S$, 1293.4. Found, 1293.4.

4.11. CtBiPyIp-(*R*)-$H_2N\gamma$-PyImHzPy (5)

Starting from 90 mg of resin the title compound was synthesized. After HPLC purification, it was recovered upon lyophilization of the appropriate fraction as a colorless powder (0.7 mg). UV(H_2O) 251, 336. ESI-TOF-MS: calcd for $C_{61}ClH_{65}N_{21}O_8S$, 1286.5. Found, 1286.4.

4.12. Construction of plasmid DNA

The plasmid AV1 was constructed by hybridization of the two oligonucleotides 5′-GATCT CAGTG GATAC GTGGG CTCAG TGGAT TCGTG GGCTC AGTGG ATACC TGGGC TCAGT GGAAA CGTGG GCTCA-3′ and 5′-AGTCA CCTAT GCACC CGAGT CACCT AAGCA CCCGA GTCAC CTATG GACCC GAGTC ACCTT TGCAC CCGAG TTCGA-3′ followed by ligation into the pUC19 using T4 DNA ligase. The resultant plasmid was then used to transform *Escherichia coli* XL-1 Blue Supercompetent cells. Ampicillin-resistant white colonies were selected from 25 mL Luria–Bertani agar plates containing 50 mg/mL ampicillin, treated with XGAL and IPTG solutions, grown overnight at 37 °C, and cells harvested. Large-scale plasmid purification was performed using WizardPlus Midi Preps from Promega. The presence of the desired insert was determined by dideoxy sequencing.

4.13. Preparation of 5′-end labeled DNA restriction fragments[20]

Two 21-base-pair primer oligonucleotides, 5′-GAATT CGAGC TCGGT ACCCG G-3′ (forward) and 5′ CTGGC ACGA C AGGTT TCCCG AC-3′ (reverse), were constructed for PCR amplification. The forward

primer was radiolabeled using [γ-^{32}P]-dATP and polynucleotide kinase, followed by purification using Micro-Spin G-50 columns. The desired DNA segment was amplified as previously described.[14] The labeled fragment was loaded onto a 7% nondenaturing preparatory polyacrylamide gel (5% cross-link) and the desired 270-base pair band was visualized by autoradiography and isolated. Chemical sequencing reactions were performed according to published protocols.[21]

4.14. Quantitative DNase I footprint titrations

All reactions were carried out in a volume of 400 mL according to published protocols.[14] Quantitation by storage phosphor autoradiography and determination of association constants were done as previously described.[21]

Acknowledgments

We thank B. Boras for the synthesis of **3**. We thank The National Institutes of Health for grant support (GM 27681) and the Swiss National Science Foundation for a fellowship to A.V.

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