Recognition in the Minor Groove of DNA at 5'-(A,T)GCGC(A,T)-3' by a Four Ring Tripeptide Dimer. Reversal of the Specificity of the Natural Product Distamycin

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Received November 28, 1994[®]

Abstract: The tripeptide ImPImP containing alternating imidazole and pyrrole carboxamides specifically binds the designated six base pair site 5'-d(A,T)GCGC(A,T)-3' in the minor groove of DNA. Quantitative footprint titration experiments demonstrate that ImPImP binds the sites 5'-AGCGCT-3' and 5'-TGCGCA-3' with apparent first order binding affinities of 3.8×10^5 M^{-1} and 3.6×10^5 M^{-1}, respectively (25 mM tris acetate, 10 mM NaCl, pH 7.0 and 22 °C). Affinity cleaving experiments with ImPImP-EDTA•Fe reveals equal cleavage on both sides of the 5'-(A,T)-GCGC(A,T)-3' site, consistent with a side-by-side antiparallel arrangement of the four ring peptides in the minor groove. This reversal of specificity of the natural product distamycin which prefers to bind pure A,T sequences underscores the utility of 2:1 peptide–DNA models for the design of ligands for sequence-specific recognition in the minor groove of DNA. By extending these peptides to four ring systems, a new lower limit of six base pair binding by 2:1 peptide–DNA complexes has been defined.

The natural products netropsin (N) and distamycin A (D) are crescent shaped di- and tripeptides, respectively, that bind in the minor groove of DNA at sites of four or five successive A,T base pairs (bp).[1-3] The structures of a number of peptide–DNA complexes have been determined by X-ray diffraction[4] and NMR spectroscopy,[5] and the thermodynamic profiles have been studied for these complexes.[6] This work suggests that favorable electrostatic interactions and extensive van der Waals contacts between the peptide and the floor and walls of the minor groove contribute to complex stability. The carboxamide NH's of the peptides participate in bifurcated hydrogen bonds with adenine N3 and thymidine O2 atoms on the floor of the minor groove. The aromatic hydrogens of the *N*-methylpyrrole rings are set too deeply in the minor groove to allow room for the guanine 2-amino group of a G,C base pair, affording binding specificity for A,T-rich sequences. Although this model has

aided in the design of oligopeptides for recognition of longer tracts of A,T-rich DNA,[7] efforts based on a 1:1 peptide–DNA model to design peptides capable of binding sequences containing both A,T and G,C base pairs have met with limited success.[8,9]

2:1 Peptide–DNA Complexes. The observation that distamycin (at 2–4 mM) is capable of binding in the minor groove of 5'-AAATT-3' as a side-by-side dimer required consideration of 2:1 complexes for recognition in the minor groove of DNA.[10] This explained why the nonnatural peptides pyridine-2-carboxamidenetropsin (2-PyN) and 1-methylimidazole-2-carboxamidenetropsin (2-ImN) bind the unanticipated sequence 5'-(A,T)G(A,T)C(A,T)-3' (Figure 1).[11] This example of specific recognition of sequences *containing both A•T and G•C base pairs* suggests that the 2:1 motif may serve as a new model for the design of peptide analogs for specific recognition of other sequences (Figure 1).[11]

The 2:1 models differ significantly from that of 1:1 models in that each ligand of the dimer interacts with one of the DNA strands in the minor groove. The imidazole nitrogen of each ligand hydrogen bonds specifically with one guanine amino group. The side-by-side combination of one imidazole ring on one ligand and a pyrrole ring on the second ligand recognizes

® Abstract published in *Advance ACS Abstracts*, March 15, 1995.

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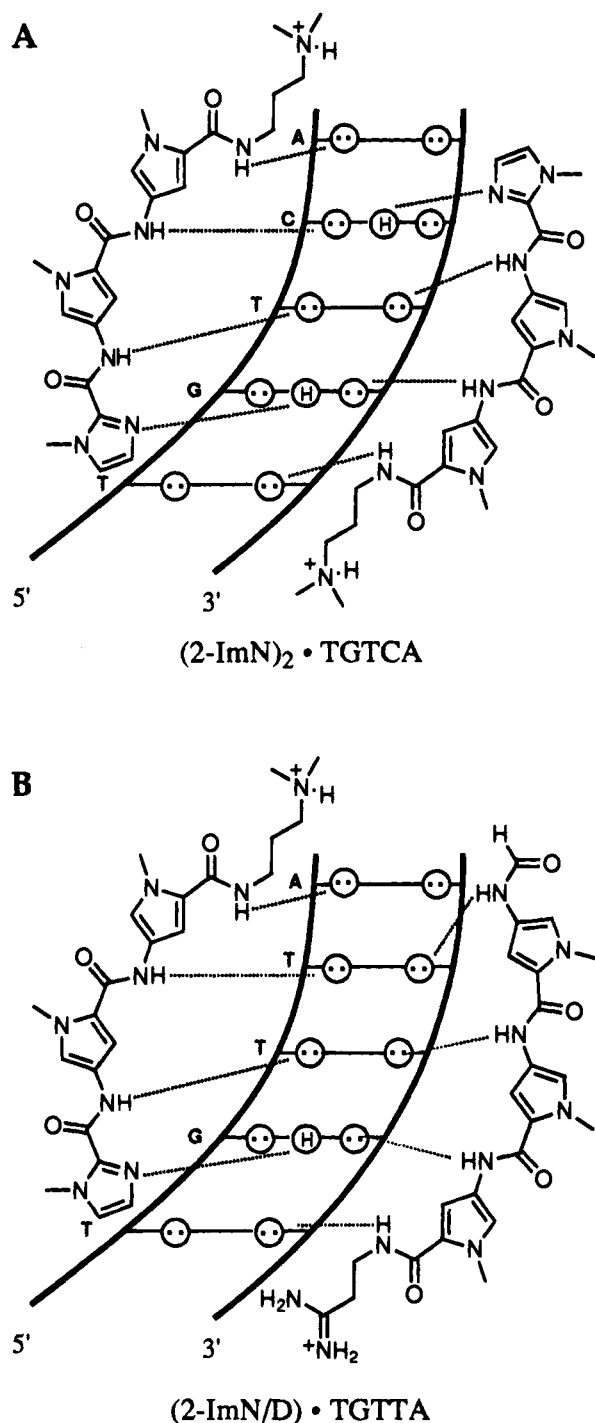
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A

(2-ImN)₂ • TGTCA

B

(2-ImN/D) • TGTTA

Figure 1. Binding models (2:1) for the complexes formed between (a) 2-ImN with a 5′-TGTCA-3′ sequence[11] and (b) 2-ImN and D with a 5′-TGTTA-3′ sequence.[12] Circles with dots represent lone pairs of N3 of purines and O2 of pyrimidines and circles containing an H represent the 2-amino group of guanine. Putative hydrogen bonds are illustrated by dashed lines.

G•C, whereas a pyrrole—imidazole pair targets a C•G base pair. A pyrrole—pyrrole combination is partially degenerate and binds to either A•T or T•A base pairs. Based on this new 2:1 model, it was demonstrated that the two different peptides distamycin and imidazole—pyrrole—pyrrole (2-ImN), bind the sequence 5′-TGTTA-3′ as a side-by-side antiparallel heterodimer (Figure 1).[12] In a related study, it has been shown by NMR that
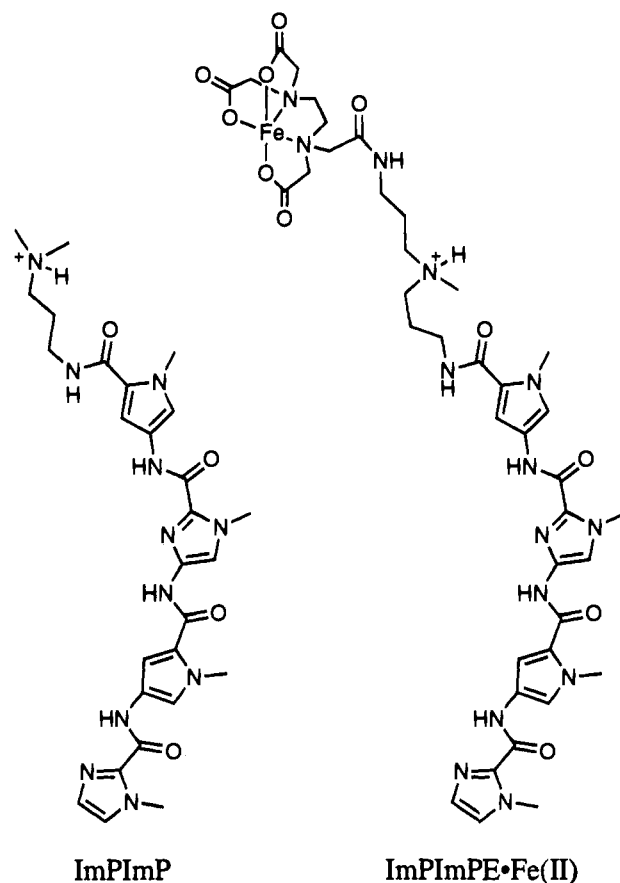
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ImPImP   ImPImPE•Fe(II)

Figure 2. Synthetic peptide ImPImP and affinity cleaving analog ImPImPE·Fe(II).

distamycin and a pyrrole—imidazole—pyrrole peptide bind a 5′-AAGTT-3′ site as a side-by-side heterodimer.[13]

Based on this new understanding, ImPImP was designed to recognize a family of six base pair sites 5′-(A,T)GCGC(A,T)-3′ containing a four base pair G•C rich core as a side-by-side antiparallel dimer in the minor groove (Figure 2). Each imidazole N3 of two side-by-side peptides is expected to form a specific hydrogen bond to unique guanine amino groups on the floor of the minor groove. Consideration of the geometry of the 2:1 peptide—DNA complex suggests that the DNA site containing four alternating GC base pairs would have the proper disposition of four guanine amino groups on the floor of the minor groove for recognition by the ImPImP dimer. We report here the eight-step synthesis of ImPImP and the affinity cleaving analog ImPImPE•Fe(II) (Figure 2). The DNA footprinting and affinity cleaving studies with these peptides reveal that reversal of the natural specificity of distamycin has been achieved.

Results

Synthesis. The synthetic methodology for ImPImP and ImPImPE is similar to that previously described for related analogs (Figure 3).[3b,11a] Coupling of 1-methyl-4-amino-2-(carboxamidopropyl-3-dimethylamino)pyrrole with 4-nitro-1-methylimidazole-2-carboxylic acid (DCC, HOBt) affords the imidazole—pyrrole derivative **4a**. Reduction of the nitroimidazole (300 psi H₂, Pd/C) and coupling with 4-nitro-1-methylpyrrole-2-acyl chloride yields **5a**. Further reduction of the nitropyrrole and coupling with 1-methylimidazole-2-carboxylic acid (DCC, HOBt) provides ImPImP. The affinity cleaving analog ImPImPE was synthesized in similar fashion,

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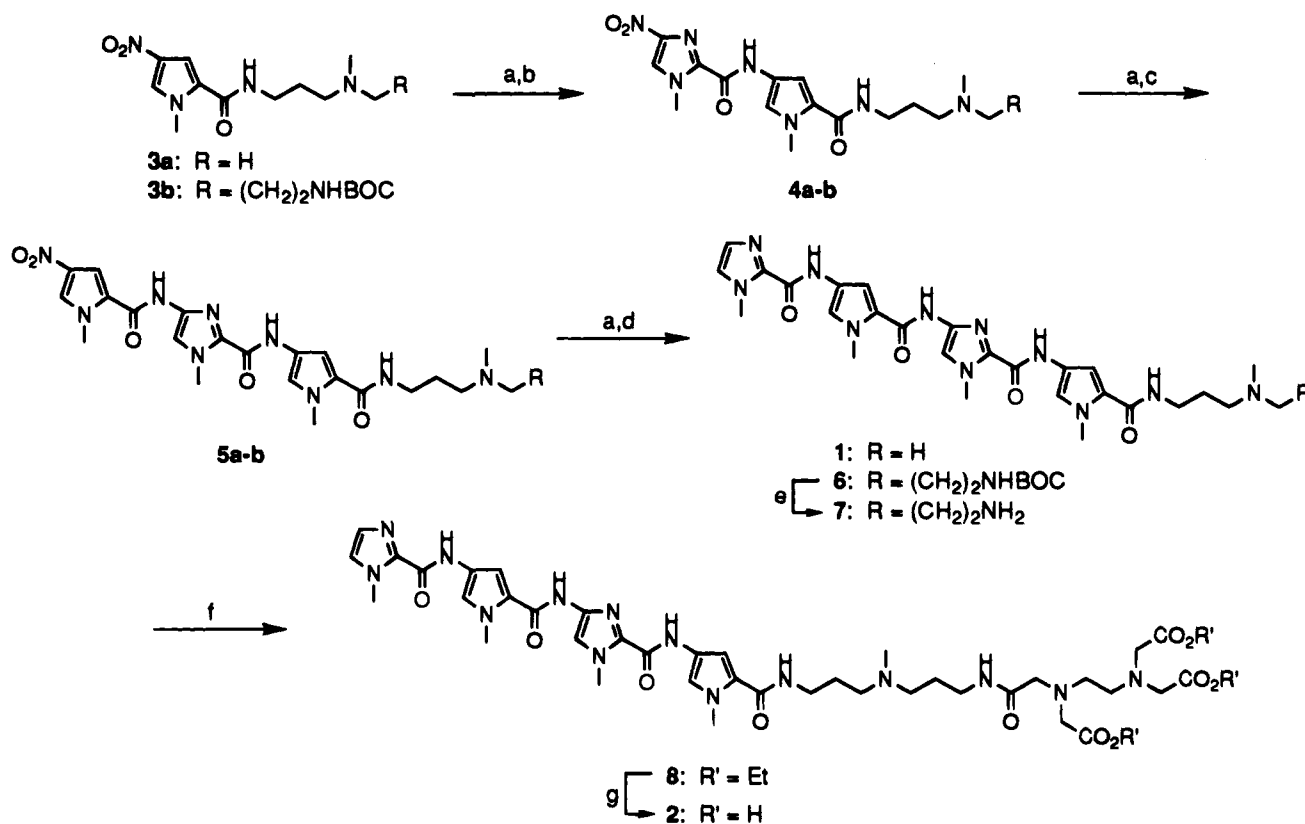
Figure 3. Synthetic scheme for ImPImP and ImPImPE: (a) 300 psi H_2, 10% Pd/C; (b) 4-nitro-1-methylimidazole-2-carboxylic acid, DCC, HOBt; (c) 4-nitro-1-methylpyrrole-2-carboxylic acid, $SOCl_2$; (d) 1-methylimidazole-2-carboxylic acid, DCC, HOBt; (e) 20% TFA/CH_2Cl_2; (f) (EtO_2-CCH_2)_2N(CH_2)_2N(CH_2CO_2Et)(CH_2CO_2H), DCC, HOBt; (g) LiOH, EtOH, H_2O.

with elaboration of nitropyrrole **3b** to the *tert*-butoxycarbonyl-amine **6**. Deprotection (TFA, CH_2Cl_2) and coupling of the primary amine with triethyl ethylenediaminetriacetate acetic acid (DCC, HOBt) gave the triethyl ester of ImPImPE. Saponification of this material (LiOH, EtOH, H_2O) afforded ImPImPE (Figure 3).

Footprinting. Footprinting with methidiumpropyl-EDTA·Fe (MPE·Fe(II)) on the 3'- and 5'-^{32}P end-labeled 381 base pair *Eco*R 1/*Bam*H 1 restriction fragment from plasmid pBR322 (25 mM tris acetate, 10 mM NaCl, pH 7.0 and 22 °C) reveals that the peptide, at 20 μM concentration, binds specifically the three six base pair sites, 5'-TGCGCA-3', 5'-AGCGCT-3' and 5'-AACGCA-3' (Figures 4 and 5).[3] The energetics of association of ImPImP for these three sites were then determined by quantitative DNase I footprint titration experiments.[14] ImPImP binds the sites 5'-AGCGCT-3' and 5'-TGCGCA-3' with apparent first order binding affinities of 3.8 × 10^5 M^{-1} and 3.6 × 10^5 M^{-1}, respectively. The single base pair mismatch site 5'-AACGCA-3' bound with threefold lower affinity.

Affinity Cleaving. Cleavage experiments on the 381 base pair restriction fragment (25 mM tris acetate, 10 mM NaCl, pH 7.0 and 22 °C) reveal cleavage patterns which are shifted to the 3'-side of the binding site, consistent with binding in the minor groove (Figures 4 and 5).[2] ImPImPE·Fe(II) affords cleavage of *equal intensity flanking both ends* of the 5'-TGCGCA-3' site. The cleavage pattern at the 5'-AACGCA-3' and 5'-AGCGCT-3' sites are similar. In addition, the affinity cleaving experiments identify a 5'-AGCGCA-3' binding site near

the top of the gel (Figure 4). These cleavage data are consistent with a side-by-side antiparallel 2:1 peptide−DNA complex (Figure 6).

Discussion

Footprinting and affinity cleaving experiments demonstrate sequence-specific binding to the four six base pair sites 5'-AGCGCT-3', 5'-TGCGCA-3', 5'-AGCGCA-3', and 5'-AACGCA-3' by the peptide ImPImP (Figure 6). Affinity cleaving experiments with ImPImPE are consistent with the peptide binding as an antiparallel dimer in the minor groove at these sites. In collaboration with the Wemmer group, we have characterized directly by two-dimensional NMR the complex (ImPImP)$_2$·5'-TGCGCA-3'.[15] The peptide binds as a side-by-side antiparallel dimer with positive cooperativity in the minor groove. Energy minimization of the complex with constraints from NOESY experiments [15] affords a model wherein specific hydrogen bonds are formed between the four imidazole nitrogens of the peptides and the four guanine amino group on the floor of the minor groove (Figure 6).

The complex formed upon binding of ImPImP to the 5'-AACGCA-3' site contains a single hydrogen bond mismatch between the peptides and the floor of the minor groove (Figure 6). The preference for A,T base pairs in the first and sixth positions of the binding site may be due to favorable hydrogen bonds between the bases and the terminal pyrrolecarboxamides of the peptides. Alternatively, GC base pairs may be disfavored due to a lack of hydrogen bond acceptors on the peptides. In either case, the peptides appear not to discriminate between AT and TA base pairs. In contrast, the peptides *discriminate between GC and CG base pairs*, as evidenced by a lack of

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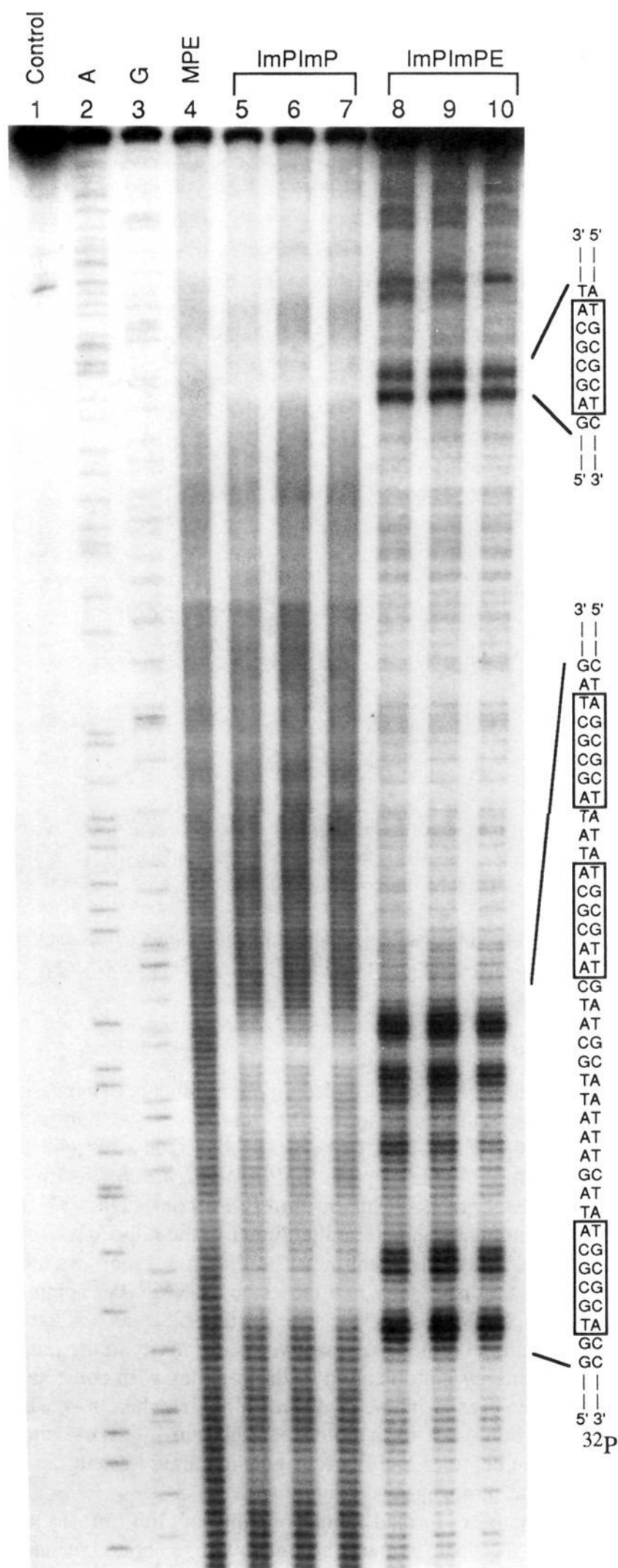
Figure 4. MPE·Fe(II) footprinting of ImPImP and specific cleavage by ImPImPE. Autoradiogram of a 6% denaturing polyacrylamide gel. All reactions contain 25 mM tris acetate (pH 7.0), 10 mM NaCl, 5 mM DTT, 100 μM bp calf thymus DNA, and 3'-³²P end labeled 381 bp restriction fragment. Lane 1, intact DNA; lane 2, A reaction; lane 3, Maxam–Gilbert G reaction; lane 4, MPE·Fe(II) standard; lane 5, 50 μM ImPImP; lane 6, 20 μM ImPImP; lane 7, 10 μM ImPImP; lane 8, 50 μM ImPImPE·Fe(II); lane 9, 20 μM ImPImPE·Fe(II); lane 10, 10 μM ImPImPE·Fe(II). Lanes 4–7 contain 5 μM MPE·Fe(II).

binding to the 5'-TGGCGT-3' and 5'-TCGGCA-3' sites on this restriction fragment. These observations provide guidelines for

predicting affinities and specificities of side-by-side peptides for other DNA sites. It is likely that targeting GC rich sequences by the 2:1 peptide model will afford the highest specificity.

Implications for the Design of Minor Groove Binding Ligands. The 2:1 motif has guided the design of peptides for recognition of several different sequences: 5'-TGTCA-3', 5'-TGTTA-3', 5'-AAGTT-3', and 5'-TGCGCA-3'.[11–13,15] Binding of ImPImP to a pure four base pair G,C-core sequence represents reversal of the A,T specificity of the natural products distamycin and netropsin. Although we anticipate that other sequences may be targeted by matching pyrrole- and imidazolecarboxamides of designed peptides to ensembles of hydrogen bond acceptors and donors on the floor of the minor groove, undoubtedly there will be sequence composition limitations which remain to be defined. Finally, we find it interesting that the highly specific four ring peptide has such modest overall affinity ($\sim 4 \times 10^5$ M^{-1}) for the 5'-GCGC-3' sites. Perhaps there is an energetic price for forming four specific hydrogen bonds between the four imidazole acceptors on the peptide dimer and the four guanine amino donors protruding from the floor of the minor groove. It is likely that the ImPImP dimer does not sit as deeply in the minor groove diminishing energetically favorable van der Waals contact with the walls of the double helix. Covalently linking these dimers as hairpins or cyclics may provide a family of *higher affinity* ligands for sequence-specific recognition of these and other G·C rich DNA sites.[16]

Experimental Section

¹H NMR and ¹³C NMR spectra were recorded on a General Electric-QE 300 NMR spectrometer in CDCl₃ or CD₃OD, with chemical shifts reported in parts per million relative to tetramethyl silane or residual CHD₂OD, respectively. IR spectra were recorded on a Perkin-Elmer FTIR spectrometer. High-resolution mass spectra were recorded using fast atom bombardment (FAB) techniques at the Mass Spectrometry Laboratory at the University of California, Riverside. Reactions were executed under an inert argon atmosphere. Reagent grade chemicals were used as received unless otherwise noted. Tetrahydrofuran (THF) was distilled under nitrogen from sodium/benzophenone ketyl. Dichloromethane (CH₂Cl₂) and triethylamine were distilled under nitrogen from powdered calcium hydride. Dimethylformamide (DMF) was purchased as an anhydrous solvent from Aldrich. Flash chromatography was carried out using EM science Kieselgel 60 (230–400) mesh.[17] Thin-layer chromatography was performed on EM Reagents silica gel plates (0.5 mm thickness). All compounds were visualized with short-wave ultraviolet light. Reversed phase chromatography was performed on a Pharmacia FPLC system with a ProRPC 10/10 column and a solvent system of acetonitrile/100 mM triethylammonium acetate (0–40%, 40 min linear gradient). The desired fractions were pooled and concentrated *in vacuo*. The residue was desalted by dissolving in 5% ammonium hydroxide in methanol, concentrating, and passing through a short column of silica gel (1% ammonium hydroxide in methanol).

***N*-Methyl-4-(*N*-methyl-4-nitroimidazole-2-carboxamido)-2-(carboxamidopropyl-3-dimethylamino)pyrrole (4a).** To a slurry of 4-nitro-1-methylimidazole-2-carboxylic acid (300 mg, 1.75 mmol) in tetrahydrofuran (4.0 mL) was added oxalyl chloride (2.0 mL, 22.9 mmol), and the resulting solution was heated at reflux for 1.5 h. The solution was cooled and excess solvent was removed *in vacuo*, and the residue was dissolved in dimethylformamide (5.0 mL) *N*-Methyl-4-amino-2-(carboxamidopropyl)-3-(dimethylamino)pyrrole (0.400 g, 1.79 mmol) was added, and the reaction mixture was allowed to stir at room temperature for 4 h. Solvent was removed *in vacuo*, and the residue was purified by flash column chromatography (MeOH) to afford **4a**: yield 63% (414 mg); ¹H NMR (CD₃OD) δ 8.21 (s, 1 H), 7.27 (d, 1 H, J = 1.8 Hz), 6.82 (d, 1 H, J = 1.9 Hz), 4.12 (s, 3 H), 3.86 (s, 3

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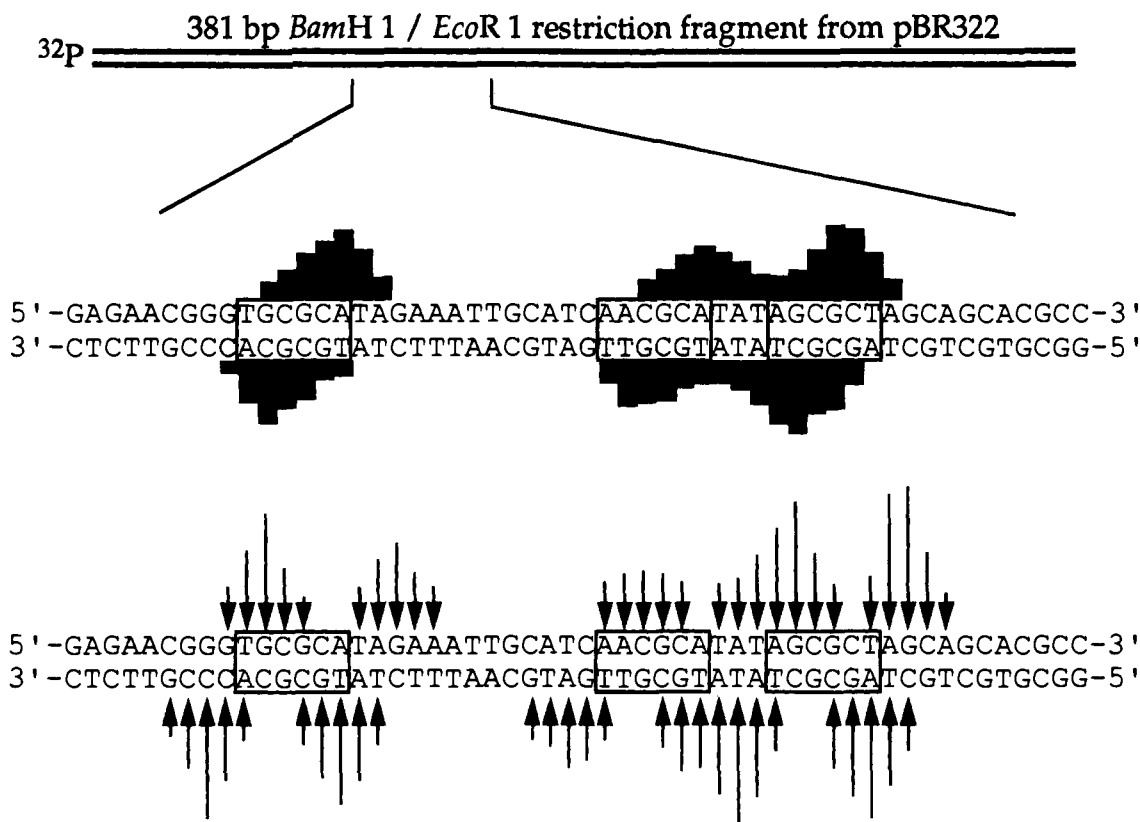
Figure 5. Histograms of cleavage protection (footprinting) and affinity cleaving data. (Top) Illustration of the 381 bp restriction fragment with the position of the sequence indicated. (Middle) MPE·Fe(II) protection pattern for ImPImP at 20 μM concentration. Bar heights are proportional to the relative protection from cleavage at each band. Boxes represent equilibrium binding sites determined by the published model.[2] (Bottom) Cleavage of the 381 bp restriction fragment by ImPImPE·Fe(II) at 20 μM concentration. Arrows are proportional to the integrated densities of the cleavage bands. Data for the bottom strand are shown in Figure 5.

H), 3.22 (m, 2 H), 2.45 (t, 2 H, $J = 7.6$ Hz), 2.30 (s, 6 H), 1.78 (qn, 2 H, $J = 7.5$ Hz); IR (thin film) 3385 (m), 2950 (w), 1644 (m), 1634 (m), 1538 (s), 1463 (m), 1454 (m), 1386 (w), 1308 (m); FABMS *m/e* 378.1890 (M + H, 378.1879 calcd for $C_{16}H_{23}N_7O_4$).

N-Methyl-4-(N-methyl-4-(N-methyl-4-nitropyrrole-2-carboxamido)-imidazole-2-carboxamido)-2-(carboxamidopropyl-3-dimethylamino)-pyrrole (5a). To a flask equipped with a reflux condensor was added 4-nitro-1-methylpyrrole-2-carboxylic acid (105 mg, 0.618 mmol) and thionyl chloride (5.0 mL, 68.5 mmol), and the mixture was heated at 85 °C for 3 h. The solution was allowed to cool, and excess reagent was removed *in vacuo*. The residue was dissolved in dimethylformamide (3.0 mL) and cooled to 0 °C. Separately, to a solution of **4a** (90 mg, 0.239 mmol) in dimethylformamide (3.5 mL) was added Pd/C catalyst (10%, 35 mg), and the mixture was hydrogenated in a Parr bomb apparatus (300 psi H₂) for 2 h. The catalyst was removed by filtration through Celite, and the filtrate was immediately added to the acid chloride and allowed to warm to room temperature and stir 8 h. Methanol (1.0 mL) was added, and all solvents were removed *in vacuo*. Flash column chromatography of the residue (methanol) was followed by reversed phase chromatography to afford **5a** as a yellow powder (46 mg, 39%): ¹H NMR (CD₃OD) δ 7.82 (d, 1 H, $J = 1.8$ Hz), 7.41 (d, 1 H, $J = 1.9$ Hz), 7.37 (s, 1 H), 7.17 (d, 1 H, $J = 1.9$ Hz), 6.77 (d, 1 H, $J = 1.9$ Hz), 4.00 (s, 3 H), 3.98 (s, 3 H), 3.85 (s, 1 H), 3.34 (m, 2 H), 2.47 (t, 2 H, $J = 7.7$ Hz), 2.32 (s, 6 H), 1.79 (q, 2 H, $J = 7.5$ Hz); ¹³C NMR (CD₃OD) δ 164.2, 159.3, 157.7, 137.4, 136.2, 135.6, 129.1, 127.0, 124.8, 122.5, 120.2, 115.8, 109.2, 105.6, 58.1, 45.2, 38.1, 36.0, 28.1; IR (thin film) 3405 (m), 2949 (w), 1654 (m), 1637 (m), 1546 (s), 1499 (m), 1466 (m), 1438 (m), 1311 (m), 1120 (w); FABMS *m/e* 500.2370 (M + H, 500.2357 calcd for $C_{22}H_{30}N_9O_5$).

N-Methyl-4-(N-methyl-4-(N-methyl-4-(N-methylimidazole-2-car-boxamido)pyrrole-2-carboxamido)imidazole-2-carboxamido)-2-(car-boxamidopropyl-3-dimethylamino)pyrrole (1). To a solution of 1-methylimidazole-2-carboxylic acid (33 mg, 0.297 mmol) and N-hydroxybenzotriazole hydrate (41 mg, 0.30 mmol) in dimethylforma-mide (0.60 mL) was added a solution of 1,3-dicyclohexylcarbodiimide

(62 mg, 0.30 mmol) in methylene chloride (0.60 mL), and the mixture was allowed to stir for 90 min. Separately, to a solution of **5a** (20 mg, 0.040 mmol) in dimethylformamide (2.5 mL) was added Pd/C catalyst (10%, 10 mg), and the mixture was hydrogenated in a Parr bomb apparatus (300 psi H₂) for 90 min. The catalyst was removed by filtration through Celite, and the filtrate was immediately added to the activated acid and allowed to stir 2.5 h. Methanol (1.0 mL) was added, and all solvents were removed *in vacuo*. Flash column chromatography of the residue (0.5% ammonium hydroxide in methanol) was followed by reversed phase chromatography to afford **1** as a yellow powder (10 mg, 43%): ¹H NMR (CD₃OD) δ 7.43 (s, 1 H), 7.35 (d, 1 H, $J = 1.8$ Hz), 7.25 (d, 1 H, $J = 1.1$ Hz), 7.23 (d, 1 H, $J = 1.8$ Hz), 7.05 (d, 1 H, $J = 1.1$ Hz), 7.01 (d, 1 H, $J = 1.9$ Hz), 6.82 (d, 1 H, $J = 1.9$ Hz), 4.06 (s, 3 H), 4.05 (s, 3 H), 3.94 (s, 3 H), 3.88 (s, 3 H), 3.31 (m, 2 H), 2.48 (t, 2 H, $J = 7.7$ Hz), 2.32 (s, 6 H), 1.79 (q, 2 H, $J = 7.6$ Hz); IR (thin film) 3285 (m), 2956 (w), 1654 (m), 1542 (s), 1466 (m), 1426 (m), 1406 (m), 1250 (w), 1123 (w); UV (H₂O) λ_max (ε) 252 (26 900), 312 (44 400) nm; FABMS *m/e* 578.2952 (M + H, 578.2985 calcd for $C_{27}H_{36}N_{11}O_4$).

N-Methyl-4-nitro-2-(carboxamido-(3-tert-butoxycarbonylamino-3'-amino-N-methyldipropylamino)pyrrole (3b). To a flask equipped with a reflux condensor was added 4-nitro-1-methylpyrrole-2-carboxylic acid (2.00 g, 11.8 mmol) and thionyl chloride (10.0 mL, 137 mmol), and the mixture was heated at 85 °C for 3 h. The solution was allowed to cool, and excess reagent was removed *in vacuo*. The acid chloride was dissolved in methylene chloride (10.0 mL) and cooled to 0 °C, and a solution of 3-(tert-butyloxycarbonylamino)-3'-amino-N-methyl-dipropylamine (2.60 g, 10.61 mmol) in methylene chloride (10.0 mL) was added. The resulting solution was allowed to stir 12 h followed by addition of methanol (5.0 mL) followed by stirring 30 min. Solvents were removed *in vacuo* and the residue was purified by flash column chromatography (20% methanol in methylene chloride) to afford the protected amine in 93% yield (3.90 g): ¹H NMR (DMSO-d_6) δ 8.40 (t, 2 H), 8.11 (d, 1 H), 7.39 (d, 1 H), 6.77 (d, 1 H), 3.89 (s, 3 H), 3.19
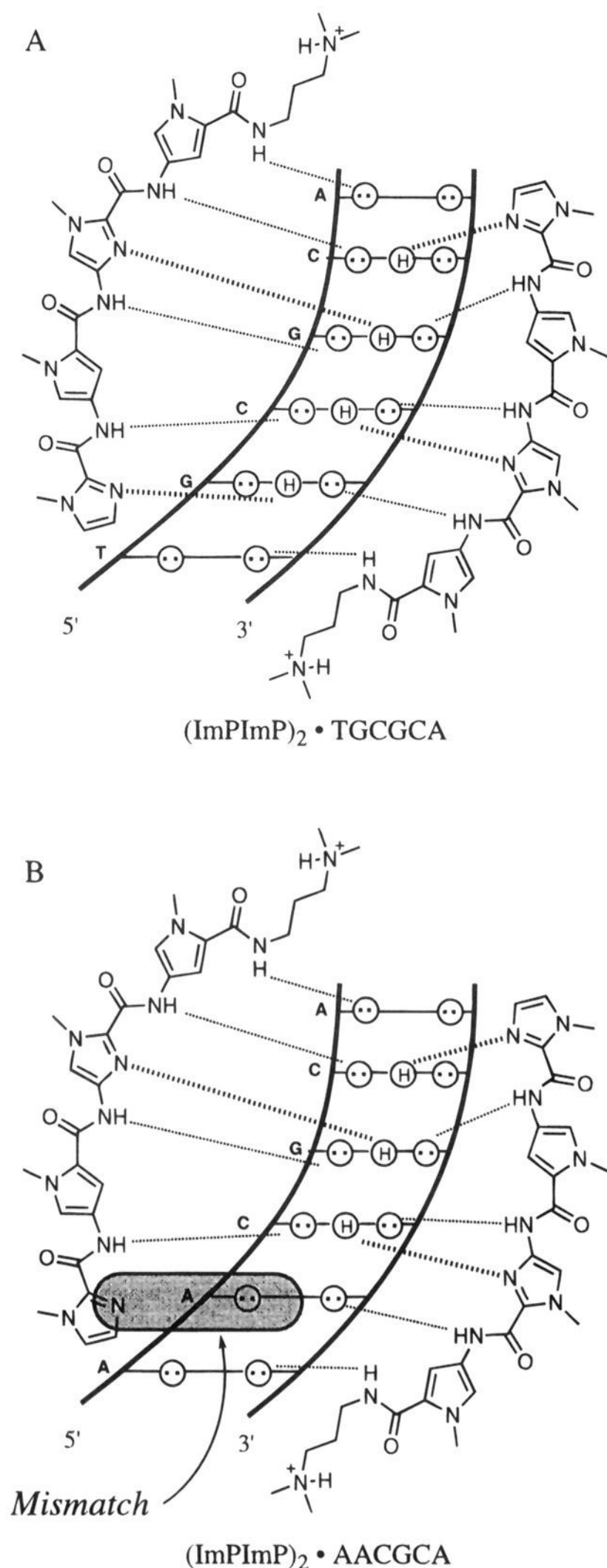
A

(ImPImP)$_2$ • TGCGCA

B

Mismatch

(ImPImP)$_2$ • AACGCA

Figure 6. (A) Model for the complex formed between ImPImP with the 5′-TGCGCA-3′ site. (B) Model for the complex formed between ImPImP with the 5′-AACGCA-3′ site. The absence of a hydrogen bond donor on the floor of the minor groove for interaction with the imidazole N3 atom of the peptide results in a single hydrogen bond mismatch (illustrated with shading).

(m, 2 H), 2.92 (m, 2 H), 2.28 (t, 2 H), 2,24 (t, 2 H), 2.10 (s, 3 H), 1.59 (m, 2 H), 1.48 (m, 2 H), 1.36 (s, 9 H); IR (thin film) 3317 (m), 2975 (m), 1698 (s), 1652 (s), 1530 (s), 1505 (m), 1418 (m), 1312 (s), 1272 (m), 1169 (m); FABMS *m/e* 398.2403 (M + H, 398.2411 calcd for C$_{18}$H$_{32}$N$_5$O$_5$).

***N*-Methyl-4-(*N*-methyl-4-nitroimidazole-2-carboxamido)-2-(carboxamido-(3-*tert*-butoxycarbonylamino-3′-amino-*N*-methyldipropylamino)pyrrole (4b).** To a slurry of 4-nitro-1-methylimidazole-2-carboxylic acid (400 mg, 2.33 mmol) in tetrahydrofuran (5.0 mL) was added oxalyl chloride (2.5 mL, 28.7 mmol), and the resulting solution was heated at reflux for 2 h. The solution was cooled, excess solvent was removed *in vacuo*, and the residue was dissolved in dimethylformamide (5.0 mL) and cooled to 0 °C. Separately, to a solution of nitropyrrole **3b** (1.00 g, 2.52 mmol) in dimethylformamide (10.0 mL) was added Pd/C catalyst (10%, 450 mg), and the mixture was hydrogenated in a Parr bomb apparatus (500 psi H$_2$) for 6 h. The catalyst was removed by filtration through Celite, and the filtrate was immediately added to the acid chloride and allowed to warm to room temperature and stir 24 h. Following addition of methanol (1.0 mL), solvent was removed *in vacuo*, and the residue was purified by flash column chromatography (15% methanol in methylene chloride) to afford **2** (260 mg, 20%): ^{1}H NMR (CD$_3$OD) δ 8.17 (s, 1 H), 7.23 (d, 1 H, *J* = 1.8 Hz), 6.83 (d, 1 H, *J* = 1.8 Hz), 4.10 (s, 3 H), 3.85 (s, 3 H), 3.35 (q, 2 H, *J* = 6.0 Hz), 3.10 (t, 2 H, *J* = 6.6 Hz), 2.80 (m, 4 H), 2.55 (s, 3 H), 1.89 (m, 2 H), 1.79 (m, 2 H), 1.40 (s, 9 H); ^{13}C NMR (CD$_3$OD) δ 164.1, 158.5, 156.1, 146.1, 138.6, 126.6, 124.3, 122.2, 120.3, 105.6, 80.0, 55.8, 55.7, 41.3, 38.9, 37.8, 37.3, 37.0, 28.7, 27.1, 26.8; IR (thin film) 3333 (m), 2966 (w), 1682 (s), 1638 (m), 1540, (m), 1456 (m), 1387 (w), 1308 (m), 1168 (w); FABMS *m/e* 521.2836 (M + H, 521.2830 calcd for C$_{23}$H$_{37}$N$_8$O$_6$).

***N*-Methyl-4-(*N*-methyl-4-(*N*-methyl-4-nitropyrrole-2-carboxamido)imidazole-2-carboxamido)-2-(carboxamido-(3-*tert*-butoxycarbonylamino-3′-amino-*N*-methyldipropylamino)pyrrole (5b).** To a flask equipped with a reflux condensor was added 4-nitro-1-methylpyrrole-2-carboxylic acid (170 mg, 1.18 mmol) and thionyl chloride (5.0 mL 68.5 mmol), and the mixture was heated at 85 °C for 2 h. The solution was allowed to cool, excess reagent was removed *in vacuo*, and the residue was dissolved in dimethylformamide (2.0 mL) and cooled to 0 °C. Separately, to a solution of **4b** (180 mg, 0.346 mmol) in dimethylformamide (5.0 mL) was added Pd/C catalyst (10%, 90 mg), and the mixture was hydrogenated in a Parr bomb apparatus (450 psi H$_2$) for 3 h. The catalyst was removed by filtration through Celite, and the filtrate was immediately added to the acid chloride and allowed to warm to room temperature and stir 24 h. Methanol (1.0 mL) was added, and all solvents were removed *in vacuo*. Flash column chromatography of the residue (methanol) provided **5b** as a yellow powder (105 mg, 47%): ^{1}H NMR (CD$_3$OD) δ 7.80 (d, 1 H, *J* = 1.9 Hz), 7.40 (d, 1 H, *J* = 1.9 Hz), 7.35 (s, 1 H), 7.15 (d, 1 H, *J* = 1.9 Hz), 6.76 (d, 1 H, *J* = 1.9 Hz), 3.99 (s, 3 H), 3.96 (s, 3 H), 3.84 (s, 3 H), 3.31 (m, 2 H), 3.05 (t, 2 H, *J* = 6.8 Hz), 2.42 (m, 4 H), 2.23 (s, 3 H), 1.75 (m, 2 H), 1.65 (m, 2 H), 1.39 (s, 9 H); ^{13}C NMR (CD$_3$OD) δ 163.9, 159.1, 158.4, 157.5, 137.3, 136.1, 135.5, 129.0, 126.9, 124.8, 122.5, 120.0, 115.7, 109.2, 105.5, 79.9, 56.5, 56.2, 42.3, 39.7, 38.8, 38.2, 36.9, 36.0, 28.8, 28.2, 27.8; IR (thin film) 3329 (m), 2941 (w), 1654 (s), 1540, (s), 1466 (m), 1366 (w), 1314 (m), 1166 (w), 1116 (w); FABMS *m/e* 643.3316 (M + H, 643.3308 calcd for C$_{29}$H$_{43}$N$_{10}$O$_7$).

***N*-Methyl-4-(*N*-methyl-4-(*N*-methyl-4-(*N*-methylimidazole-2-carboxamido)pyrrole-2-carboxamido)imidazole-2-carboxamido)-2-carboxamido-(3-*tert*-butoxycarbonylamino-3′-amino-*N*-methyldipropylamino)pyrrole (6b).** To a solution of 1-methylimidazole-2-carboxylic acid (46 mg, 0.414 mmol) and *N*-hydroxybenzotriazole hydrate (57 mg, 0.42 mmol) in dimethylformamide (0.84 mL) was added a solution of 1,3-dicyclohexylcarbodiimide (87 mg, 0.42 mmol) in methylene chloride (0.84 mL.), and the mixture was allowed to stir for 1 h. Separately, to a solution of **5b** (50 mg, 0.078 mmol) in dimethylformamide (2.0 mL) was added Pd/C catalyst (10%, 23 mg), and the mixture was hydrogenated in a Parr bomb apparatus (350 psi H$_2$) for 2 h. The catalyst was removed by filtration through Celite, and the filtrate was immediately added to the activated acid and allowed to stir 3 h. Methanol (1.0 mL) was added, and all solvents were removed *in vacuo*. Flash column chromatography of the residue (methanol) was followed by reversed phase chromatography to afford **6** as a yellow powder (18 mg, 32%): ^{1}H NMR (CD$_3$OD) δ 7.43 (s, 1 H), 7.35 (d, 1 H, *J* = 1.8 Hz), 7.24 (d, 1 H, *J* = 1.0 Hz), 7.23 (d, 1 H, *J* = 1.9 Hz), 7.05 (d, 1 H, *J* = 1.0 Hz), 7.00 (d, 1 H, *J* = 1.9 Hz), 6.86 (d, 1 H, *J* = 1.9 Hz), 4.06 (s, 3 H), 4.05 (s, 1 H), 3.94 (s, 3 H), 3.88 (s, 3 H), 3.35 (m, 2 H), 3.12 (t, 2 H, *J* = 6.8 Hz), 2.76 (m, 4 H), 2.52

(s, 3 H), 1.80 (m, 2 H), 1.75 (m, 2 H), 1.41 (s, 9 H); IR (thin film) 3300 (m), 2966 (w), 1655 (s), 1578 (m), 1542 (s), 1466 (m), 1406 (w), 1250 (w), 1168 (w), 1123 (w); FABMS *m/e* 721.3898 (M + H, 721.3901 calcd for $C_{34}H_{49}N_{12}O_6$).

N-Methyl-4-(N-methyl-4-(N-methyl-4-(N-methylimidazole-2-carboxamido)pyrrole-2-carboxamido)imidazole-2-carboxamido)-2-(carboxamido-(3-amino-3′-amino-N-methyldipropylamino)pyrrole (7). To a solution of protected amine **6** (11 mg, 0.015 mmol) in methylene chloride (2.0 mL) was added trifluoroacetic acid (0.5 mL), and the resulting mixture was allowed to stir 20 min. The ammonium salt was precipitated by the addition of hexanes (40 mL) and stirring for 20 min. The oil was dissolved in 1% ammonium hydroxide in methanol (50 mL), and solvent was removed *in vacuo*. The product was purified by flash column chromatography (6% ammonium hydroxide in methanol) to afford the primary amine **7** (8 mg, 86%): ¹H NMR (CD₃-OD) δ 7.43 (s, 1 H), 7.35 (d, 1 H, *J* = 1.9 Hz), 7.25 (d, 1 H, *J* = 1.0 Hz), 7.22 (d, 1 H, *J* = 1.9 Hz), 7.05 (d, 1 H, *J* = 1.0 Hz), 7.00 (d, 1 H, *J* = 1.9 Hz), 6.83 (d, 1 H, *J* = 1.9 Hz), 4.06 (s, 3 H), 4.05 (s, 1 H), 3.94 (s, 3 H), 3.88 (s, 3 H), 3.34 (m, 2 H), 2.75 (t, 2 H, *J* = 7.0 Hz), 2.47 (m, 4 H), 2.26 (s, 3 H), 1.82−1.67 (m, 4 H); IR (thin film) 3333 (m), 2954 (w), 1653 (m), 1541 (s), 1472 (m), 1431 (m), 1254 (w), 1208 (w), 1123 (w); FABMS *m/e* 621.3374 (M + H, 621.3386 calcd for $C_{29}H_{41}N_{12}O_4$).

ImPImPEDTA(OEt)₃ (8). To a solution of EDTA triethyl ester (165 mg, 0.439 mmol) and *N*-hydroxysuccinimide (53 mg, 0.461 mmol) in 1,4-dioxane (2.0 mL) was added a solution of 1,3-dicyclohexylcarbodiimide (96 mg, 0.465 mmol) in methylene chloride (0.93 mL), and the solution was allowed to stir 90 min. A solution of primary amine **7** (7.0 mg, 0.0113 mmol) in dimethylformamide (1.5 mL) was added and allowed to stir 8 h. Methanol (0.5 mL) was added, the mixture was filtered through Celite, and solvent was removed *in vacuo*. Flash column chromatography (gradient 0−1% ammonium hydroxide in methanol) afforded the triester **8** (7.0 mg, 63%): ¹H NMR (CD₃OD) δ 7.43 (s, 1 H), 7.35 (d, 1 H, *J* = 1.9 Hz), 7.25 (d, 1 H, *J* = 1.1 Hz), 7.23 (d, 1 H, *J* = 1.8 Hz), 7.05 (d, 1 H, *J* = 1.1 Hz), 7.02 (d, 1 H, *J* = 1.9 Hz), 6.86 (d, 1 H, *J* = 1.9 Hz), 4.15−4.08 (m, 6 H), 4.06 (s, 3 H), 4.05 (s, 3 H), 3.94 (s, 3 H), 3.88 (s, 3 H), 3.62 (s, 2 H), 3.59 (s, 4 H), 3.51 (s, 2 H), 3.30 (m, 4 H), 2.83−2.70 (m, 8 H), 2.49 (s, 3 H), 1.91−1.83 (m, 4 H), 1.26−1.19 (m, 9 H); IR (thin film) 3385 (m), 2951 (w), 1734 (s), 1654 (s), 1591 (m), 1542 (s), 1458 (m), 1420 (m), 1209 (s), 1125 (w); FABMS *m/e* 979.5114 (M + H, 979.5081 calcd for $C_{45}H_{67}N_{14}O_{11}$).

ImPImPE (2). To a solution of triethyl ester **8** (6.0 mg, 0.0061 mmol) in ethanol (0.7 mL) was added 0.5 M LiOH (0.7 mL), and the solution was allowed to stir 15 h. HCl (1 N, 3 mL) was added, and solvent was removed *in vacuo*. Purification by reversed phase chromatography afforded ImPyImPyE (2.0 mg, 37 %): ¹H NMR (CD₃-OD) δ 7.44 (s, 1 H), 7.36 (d, 1 H, *J* = 1.9 Hz), 7.28 (d, 1 H, *J* = 1.9 Hz), 7.24 (d, 1 H, *J* = 1.0 Hz), 7.06 (d, 1 H, *J* = 1.9 Hz), 7.05 (d, 1 H, *J* = 1.1 Hz), 6.94 (d, 1 H, *J* = 1.9 Hz), 4.06 (s, 3 H), 4.05 (s, 3 H), 3.95 (s, 3 H), 3.89 (s, 3 H), 3.84 (s, 4 H), 3.30 (m, 4 H), 2.85 (s, 4 H), 2.15−1.96 (m, 4 H), 1.95 (s, 3 H); IR (thin film) 3385 (m), 2956 (w), 1644 (s), 1591 (m), 1540 (s), 1469 (m), 1404 (m), 1251 (w), 1208 (s), 1123 (w); FABMS *m/e* 895.4175 (M + H, 895.4193 calcd for $C_{39}H_{55}N_{14}O_{11}$).

DNA Reagents and Materials. Doubly distilled water was further purified through the Milli Q filtration system from Millipore. Sonicated, deproteinized calf thymus DNA was purchased from Pharmacia. Plasmid pBR322 was obtained from Boehringer-Mannheim. Enzymes were obtained from Boehringer-Mannheim or New England Biolabs and used with the buffers supplied. Deoxyadenosine 5′-[α-³²P]-triphosphate and adenosine 5′-[γ-³²P] triphosphate were obtained from Amersham. Storage phosphor technology autoradiography was performed using a Molecular Dynamics 400S Phosphorimager and ImageQuant software. The 381 base pair 3′- and 5′-end labeled *Eco*R I/*Bam*H I restriction fragments from plasmid pBR322 were prepared and purified as previously described.[3b] Chemical sequencing reactions were performed according to published methods.[18,19] Standard tech-

niques were employed for DNA manipulations.[20] All other reagents and materials were used as received.

MPE·Fe(II) Footprinting and Affinity Cleaving.[21] A 50 μM MPE·Fe(II) solution was prepared by mixing 100 μL of a 100 μM MPE solution with 100 μL of a freshly prepared 100 μM ferrous ammonium sulfate solution. A 100 μM ImPImPE·Fe(II) solution was prepared by mixing 10 μL of a 1 mM ImPImP solution with 10 μL of a 1 mM ferrous ammonium sulfate solution and diluting to 100 μL. Solutions were prepared containing 1 μL/tube 20× pH 7.0 tris acetate buffer, 2 μL/tube 1 mM-bp calf thymus DNA, 1 μL/tube 200 mM sodium chloride, labeled restriction fragment, and water to make 14 μL/tube total solution. Two microliters of a 10× solution of the peptide was added, and the tubes were incubated for 20 min at 22 °C. To the footprinting reactions was added 2 μL of the 50 μM MPE·Fe(II) solution and incubated 5 min. Cleavage was initiated by the addition of 2 μL of a freshly prepared 50 mM DTT solution. Final concentrations were 25 mM tris acetate (pH 7.0), 10 mM sodium chloride, 100 μM-bp DNA, 5 μM MPE·Fe(II), and 5 mM DTT in 20 μL volume. The reactions were incubated at 22 °C for 25 min, ethanol precipitated, resuspended in 100 mM tris-borate-EDTA/80% formamide loading buffer, and electrophoresed on 6% denaturing polyacrylamide gels (5% cross-link, 7 M urea) at 1500 V for 3−4 h. The gels were dried and quantitated using storage phosphor technology.

Quantitative DNase I Footprint Titration Experiments.[21] Apparent first order binding constants were determined by DNase footprint titrations as previously described.[11c,16a] The ligands (200 to 100 nM) were added to solutions of radiolabeled restriction fragment (20 000 cpm), tris acetate (10 mM, pH 7.0), KCl (10 mM), MgCl₂ (10 mM), and CaCl₂ (5 mM) and incubated for 30 min at 22 °C. Footprinting reactions were initiated by the addition of 1 μL a stock solution of DNase I (10 units/mL) containing 1 mM dithiothreitol and allowed to proceed for 3 min at 22 °C. The reactions were stopped by addition of a 3 M ammonium acetate solution containing 250 mM EDTA and ethanol precipitated. The reactions were resuspended in 100 mM tris-borate−EDTA/80% formamide loading buffer and electrophoresed on 6% polyacrylamide denaturing gels (5% cross-link, 7 M urea) at 1000 V for 3−4 h.

The data were analyzed by performing volume integrations of the 5′-TGCGCA-3′, 5′-AGCGCT-3′, and 5′-AACGCA-3′ target sites and a 5′-AGCA-3′ reference site. The apparent DNA target site saturation, θ_{app}, was calculated for each concentration of peptide using the following equation

$$\theta_{app} = 1 - \frac{I_{tot}/I_{ref}}{I_{tot}°/I_{ref}°} \qquad (1)$$

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 peptide has been added. At higher concentrations of peptide (>20 μM), the reference sites become partially protected, resulting in low θ_{app} values. For these data points, the reference value was determined from the amount of radioactivity loaded per lane, using the mean value for all data points from lanes with <20 μM peptide. The ([L]$_{tot}$, θ_{app}) data points were fit to a general Hill model by minimizing the difference between θ_{app} and θ_{fit}, using the

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(21) Two different footprinting reagents, MPE·Fe and DNase I, are used to characterize the specificity of the synthetic peptides. One reagent, MPE·Fe(II), is highly nonspecific and has the virtue of revealing quite precisely the binding *location, site size,* and *sequence*. In these experiments it is convenient to add carrier calf thymus DNA. For characterization of the energetics of binding at each of these specific sites we utilize the enzyme DNase I and the method of Ackers−Brenowitz for quantitative footprint titration methods.[14] In these quantitative titration experiments, *no carrier calf thymus DNA is added* in order not to change the concentration of total ligand added (see also ref 16d).

modified Hill equation

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

where $[\text{L}]_{\text{tot}}$ corresponds to the total peptide concentration, K_a corresponds to the apparent monomeric 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) running on a Macintosh IIfx computer with K_a, θ_{max}, θ_{min}, and n as the adjustable parameters. Consistent with cooperative dimeric binding by the peptides, n was in the range 1.8–2.0. We note explicitly that treatment of the data in this manner does not represent an attempt to model a binding mechanism. Rather, we have chosen to compare values of the apparent first order association constant, because this parameter represents the concentration of peptide at which the binding site is half-saturated.

Quantitation by Storage Phosphor Technology Autoradiography. Photostimulable storage phosphor imaging 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 15–20 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. 3.0 software running on an AST Premium 386/33 computer.

JA9438472