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Functionalized head-to-head hairpin polyamides: Synthesis, double-stranded DNA-binding activity and affinity

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Abstract—A series of 4 functionalized head-to-head-linked hairpin oligo(*N*-methylpyrrole) carboxamides with different linkers have been synthesized. Their ability to bind double-stranded DNA and sequence specificity were compared and the apparent K_d values of their DNA complexes were determined. These compounds, particularly those with iminodiacetic linkers, revealed a high affinity for DNA ($K_d = 4.5–4.8 \times 10^{-9}$ M) and sequence specific recognition of 9–10 base pairs.

1. Introduction

Hairpin minor groove binders (MGBs) composed of *N*-methylpyrrole (Py), *N*-methylimidazole (Im), 3-hydroxy-*N*-methylpyrrole, and β-alanine carboxamides show strong and sequence-specific interaction with the DNA minor groove (for review, see[1]). Dervan and coworkers have demonstrated a code of recognition of hairpin polyamides.[2] Many modifications of MGB have been already done to expand the range of recognizing units and to increase their DNA affinity, sequence specificity, and gene regulation properties.[3–6] Recently, we synthesized conjugates of minor groove binders and oligonucleotides having two head-to-head polycarboxamides attached to the same terminal phosphate (Fig. 1).[7–11] To our surprise, the conjugates formed a strong complex with a target double-stranded DNA containing A/T-tracts even when the triplex could not exist (at neutral, slightly basic pH, and high temperatures).[10,11] Since under these conditions (close to physiological) the complex stability was provided only by MGB part of the conjugate, we decided to replace triplex-forming oligonucleotide (TFO) by a functional group in order to create a possibility for attachment of other molecules with

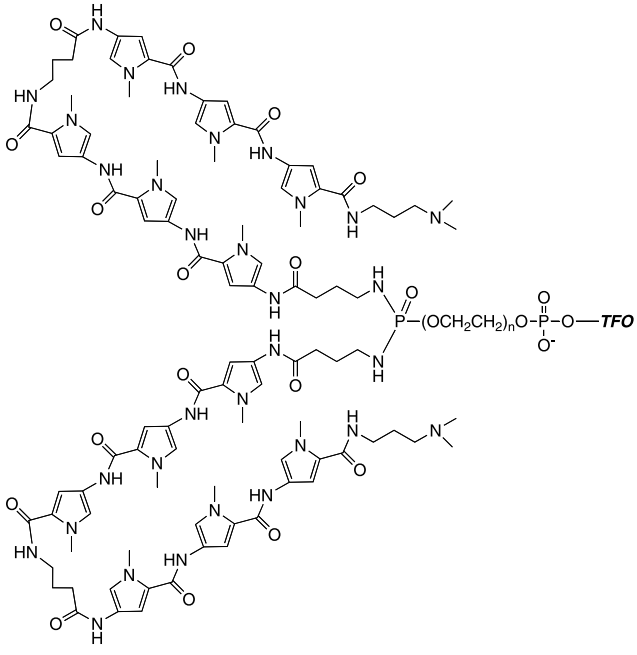

Figure 1. Two hairpin hexa(*N*-methylpyrrolecarboxamides) $H_2N\gamma(Py)_3\gamma(Py)_3Dp$ attached 'head-to head' to the terminal phosphate of a triplex-forming oligonucleotide (TFO), Dp, dimethylaminopropylamino group; γ,γ-aminobutyric acid residue, $n = 0$; 3 or 6.[9–11]

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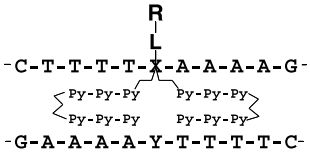


Figure 2. Principle of dsDNA recognition by 'head-to-head' bis-MGB conjugates: Py = *N*-methylpyrrolecarboxamide, L = linker, R = functional group (in our case Boc-NH-).

DNA modifying activity. We joined N-termini of two standard hairpin hexa(*N*-methylpyrrole) carboxamides (each containing two blocks of 3 monomers connected by γ-aminobutyric acid residue) in head-to-head orientation, similarly as shown in Figure 1.

First, using the phosphate activation method,[12,13] we synthesized phosphoramidate and phosphonamidate bis-conjugates. Then, to expand the structural repertoire, two iminodiacetic acid derivatives were synthesized, similar to those described by Boger et al. for linear ligands,[14] but containing two hairpin MGB blocks, a linker and a possibility for further functionalization. The concept of this construction is demonstrated in Figure 2.

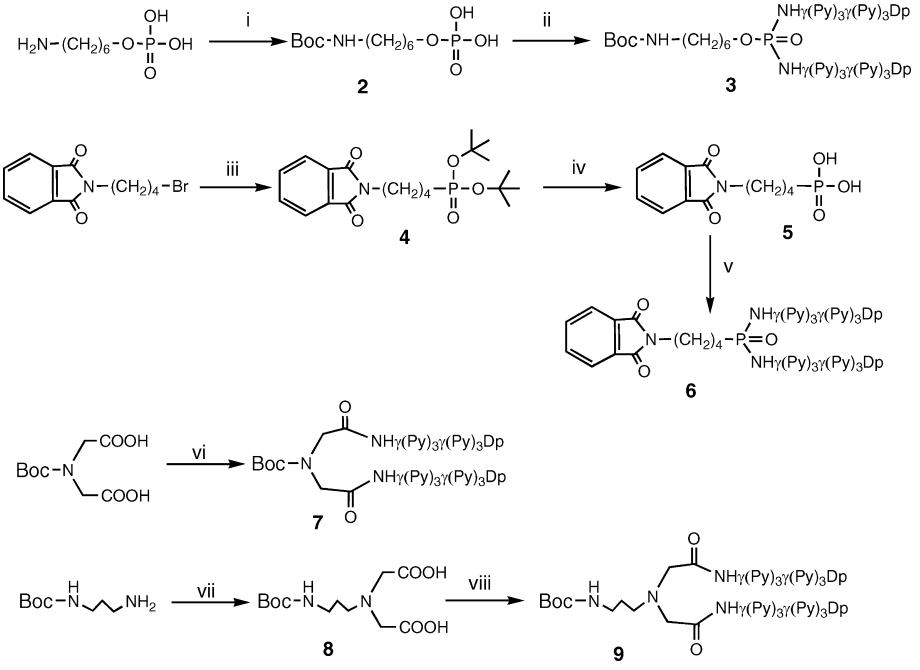
Taking into account A/T specificity of oligo(*N*-methylpyrrolecarboxamides), we studied their DNA-binding properties. The iminodiacetic acid derivatives are stable and provide high affinity to A/T-rich tracts of dsDNA recognizing more than 9 base pairs on the target.

2. Synthesis

Minor groove binders were synthesized according to Sinyakov et al.[15,16] Their structure and purity were approved by UV-spectrophotometry, TLC, NMR, and mass-spectrometry. The routes of synthesis of the conjugates are shown in Scheme 1.

The phosphoramidate compound **(3)** was obtained from the 6-aminohexylphosphate. After N-Boc protection of the free amine (quantitative yield), the phosphate terminal group was activated 15 min at room temperature by 4-dimethylaminopyridine (DMAP) (10 eq.), 2,2′-dipyridyl disulfide $(PyS)_2$ (10 eq.), and triphenylphosphine (PPh_3) (10 eq.) in fresh distilled pyridine. Then 2.5 eq. of MGB in DMF was added. The reaction mixture was kept for 48 h at room temperature to provide directly the *bis*-substituted compound **(3)** (35% yield).

The second compound **(6)** was synthesized from the 4-phtalimidoaminobutylphosphonate **(5)**.[17] Potassium salt of di-*tert*-butylphosphite was formed by reacting di-*tert*-butylphosphite (2 eq.) with potassium bis(trimethylsilyl) amide (2 eq.) in toluene for 30 min at −10 °C. The product then reacted with 4-bromobutylphtalimide 8 h under reflux (47% yield). The *tert*-butyl protective groups were eliminated with a solution of 1 M HCl in 50% methanol, 4 h at room temperature (90% yield). The phosphonate activation and MGB coupling (31% yield) have been done exactly as in case of phosphate derivative.



Scheme 1. Reagents and conditions: (i) Boc_2O (2.2 eq.), K_2CO_3 (1.5 eq.), H_2O/iPrOH, 12 h, room temperature, (ii) DMAP (10 eq.), $(PyS)_2$ (10 eq.), PPh_3 (10 eq.), $H_2N\gamma(Py)_3\gamma(Py)_3Dp$ ((**1**), 2.5 eq.), fresh distilled pyridine, 30 h room temperature, (iii) $K^+P^-O(OtBu)_2^-$ (2 eq.), toluene, 8 h, reflux (iv) HCl 2 M, MeOH, 4 h, room temperature, (v) DMAP (10 eq.), $(Py)S_2$ (10 eq.), PPh_3 (10 eq.), $H_2N\gamma(Py)_3\gamma(Py)_3Dp$ ((**1**), 2.5 eq.), fresh pyridine, 30 h, room temperature, (vi) HATU (2.5 eq.), DIPEA (4 eq.), $H_2N\gamma(Py)_3\gamma(Py)_3Dp$ ((**1**), 2.5 eq.), dry DMF, 4 h, room temperature, (vii) ICH_2COOH (2.1 eq.), K_2CO_3 (5 eq.), acetonitrile, 18 h, 45 °C (viii) HATU (2.5 eq.), DIPEA (4 eq.), $H_2N\gamma$ $(Py)_3\gamma(Py)_3Dp$ (**1**), (2.5 eq.), 4 h, room temperature Dp, dimethylaminopropylamino group; γ, γ-aminobutyric acid residue.

Direct coupling of Boc-iminodiacetic acid with two minor groove binders gave yield to **(7)**. The carboxyl groups of Boc-aminodiacetic acid were activated by *O*-7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate (HATU) (2.5 eq.) and DMAP (2 eq.) in the presence of *N*,*N*-diisopropylethylamine (DIPEA) (4eq.) for 5 min at room temperature. Then MGBs (4 eq.) were added and the reaction mixture was incubated for 4 h at room temperature to obtain **(7)** (52% yield).

The last compound **(9)** was prepared from the Boc-aminopropanamine. Iodoacetic acid (2.1eq.) reacted with Boc-aminopropanamine in acetonitrile for 18 h at 45 °C in the presence of K_2CO_3 (5eq.) to afford *N*-Boc-aminopropyliminodiacetic acid **(8)** (58% yield). Free acid groups were activated by HATU and condensed with MGB as it was described previously (59% yield).

It must be noted that in case of substances **(7)** and **(9)**, the reaction could be stopped on the stage of attachment of only one oligo(*N*-methylpyrrolecarboxamide). This opens the way for synthesis of asymmetric bis-conjugates by following attachment of second different oligocarboxamide. Removal of Boc- or phtalimide protective groups generates a nucleophilic amino group for further functionalization.

All the bis-conjugates were purified by HPLC,[18] and their structure and purity were approved by mass spectrometry (only one peak corresponding to desired mass was observed in the mass spectra).[19]

3. DNA-binding properties

As a target for studying DNA-binding properties, we used the polypurine tract (PPT) of human immunodefficiency provirus (29 complementary base pairs, Fig. 3) that contains a long A/T-rich region.[20]

Then the target recognition region was mutated in order to vary its size and sequence without change in the total fragment length. Complex formation was followed by non-denaturing gel electrophoresis with fluorescent or ^{32}P-labeled DNA target fragment and various concentrations of conjugates. Figure 4 demonstrates one example of such gel and experimental conditions. Apparent dissociation constants were then calculated.[11]

First, we have compared the affinity of the different conjugates for the native HIV DNA fragment. The results are summarized in Table 1.

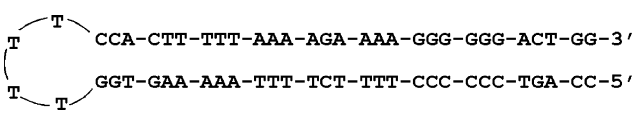


Figure 3. Double-stranded target DNA hairpin (polypurine tract of HIV provirus DNA, 29 complementary base pairs connected by a loop of 4 T).

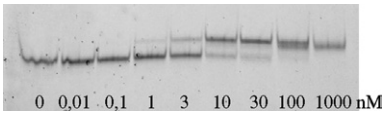


Figure 4. Gel shift studies of complex formation between conjugate **9** and target double-stranded HIV DNA fragment (Fig. 3) labeled by fluorescein at the 5′-terminus. The samples containing 1 nM DNA fragment and indicated concentrations of conjugate **9** in 0.05 M Hepes buffer, pH 7.3, were loaded on 20% non-denaturing polyacrylamide gel and electrophoresis was carried out at 5 W during 6 h.

Table 1. Apparent dissociation constants of conjugate–DNA complexes in 0.05 HEPES buffer, pH 7.3, at room temperature

Conjugate	K_d (nM)
(3)	18 (± 1)
(6)	34 (± 6)
(7)	4.3 (± 0.1)
(9)	4.8 (± 0.1)

The best binding affinity was demonstrated by the compounds with iminodiacetamide structures **(7)** and **(9)** (K_d = 4.3–4.8 nM). Phosphoramidate **(3)** and phosphonamidate **(6)** have lower affinity, 34 and 18 nM, respectively. The advantage of the amide bonds in **(7)** and **(9)** is its stability at acid pH compared to phosphoramide bond in **(3)** and **(6)**.[21] In addition, we noted that products **(3)** and **(6)** do not support heating up to 55 °C and degrade in thermal denaturation experiment in contrast to the conjugates **(7)** and especially **(9)**.

Compound **(9)** is the product of choice due to its better stability and affinity. Its other feature is the presence of an imino group which is able to be protonated within the linker and a possibility to generate a free nucleophilic amino group for further modifications after Boc-deprotection. However, we did not remove Boc group because we wanted to minimize non-specific contribution of the positive charge into ligand DNA affinity. Conservation of protected amino group eliminates additional non-specific electrostatic effects and imitates the presence of DNA-modifying chemical group. Hence we used **(9)** to determine its preferential recognition sequence in target DNA and the minimal size of the target recognition region using the mutated HIV fragments. The results are summarized in Table 2.

The complex formation was not observed even at 100 nM conjugate concentration when targets contained less than 7 contiguous A/T base pairs, in agreement with Dervan's rules. It also confirms our observation that when only one of two hairpins can be inserted into the DNA minor groove, the presence of the second one rather destabilizes complex.[22] From this data, we cannot surely exclude that two MGB moieties bind with a target sequence being in a linear parallel orientation rather than in that shown in Figure 2. However, there are 2 arguments supporting two-hairpin configuration. 7 A/T pairs could be replaced by 2 contiguous blocks of 3 A/T pairs spaced by one G/C base pair. Moreover, apparent dissociation constants are close for 9 or 10 A/T

Table 2. Apparent dissociation constants of conjugate **(9)** with different dsDNA targets at 25 °C and pH 7.2

Target	Sequence[a]	Apparent K_d (nM)
5A/T	-TTTTT- -AAAAA-	No complex[b]
6A/T	-TTTTTT- -AAAAAA-	No complex[b]
7A/T	-TTTTTTT- -AAAAAAA-	63 (± 4)
2*3A/T spaced by 1 G/C	-TTTGTTT- -AAACAAA-	56 (± 4)
8A/T	-TTTTTTTT- -AAAAAAAA-	94 (± 6)
9A/T	-TTTTTTTTT- -AAAAAAAAA-	25 (± 3)
2*4A/T spaced by 1 G/C	-TTTTGTTTT- -AAAACAAAA-	29 (± 5)
10AT	-TTTTTTTTTT- -AAAAAAAAAA-	21 (± 2)
2*4A/T spaced by 2 GC	-TTTTGGTTTT- -AAAACCAAAA-	34 (± 5)
5T/A +4A/T	-TTTTTAAAA- -AAAAATTTT-	6.2 (± 0.4)
Natural HIV sequence	-TTTTTAAAAGAAAA- -AAAAATTTTCTTTT-	4.8 (± 0.1)

[a] The indicated sequences were flanked by several G/C or alternate G/C–A/T pairs. The total fragment length was always 29 complementary base pairs.

[b] The complex band was not detectable on the gel even at 100 nM conjugate concentration.

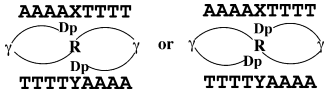


Figure 5. Hypothetical disposition of ligand **(9)** in DNA minor groove. Dp, dimethylaminopropylamine group; γ, γ-aminobutyric acid residue.

pairs and 2 blocks of 4 A/T pairs spaced by 1 or by 2 G/C pairs. A linker between them tolerates one or two degenerated base pairs in contrast to short γ-aminobutyric residue.

The other argument is that the natural HIV DNA fragment is the best target for molecule **(9)**. It seems that the disposition of A/T and T/A plays an important role in bis-MGB binding and the binding blocks must be symmetrical relatively to central base pair, so that $(T/A)_{3-5}$ sequence must follow by $(A/T)_{3-5}$ sequence, as it is shown in Figure 5. Thus, among the mutated fragments, maximal affinity (close to that of natural HIV sequence) was revealed for a fragment containing 5 A/T pairs followed by 4 T/A pairs.

Replacement of one or several *N*-methylpyrroles by *N*-methylimidazoles or other specific monomers[2,6] could make it possible to construct asymmetrical bis-conjugates that would be able to recognize mixed A/T:G/C sequences. Ensuing deprotection and functionalization of the primary amino group with chemically or photochemically active moieties will permit building of highly specific synthetic DNA-directed enzyme-like molecules.

Acknowledgments

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18. The products **(3)**, **(6)**, **(7)**, and **(9)** were purified by HPLC on a C-18 X-terra column (Waters, 7 μm, 7.8 × 300 mm) in a linear 0–80% water/acetonitrile gradient with 0.02 M ammonium acetate (products **(3)** and **(6)**), or 0.1% trifluoroacetic acid (products **(7)** and **(9)**). The flow rate was 2 ml/min.
19. Analytical data: **(2)** ES-MS: found: (M−H) 296.14; $C_{11}H_{24}NO_6P$ calculated: 297.13; 1H NMR (300 MHz, C_2D_6SO): 1.2 (m, 6H); 1.4 (s, 9H); 2.9 (m, 2H); 3.5 (q, 2H, J = 6.5 Hz); 6.7 (m, 1H); **(3)** ES-MS: found: 2270.55; $C_{109}H_{148}N_{33}O_{20}P$ calculated: 2270.13; **(4)** ES-MS: Found: (M+H) 396.15; $C_{20}H_{30}NO_5P$ calculated: 395.19; 1H NMR (300 MHz, C_2D_6SO): 1.3 (s, 18H); 1.4 (m, 4H); 1.7 (m, 2H); 3.5 (t, 2H, J = 6.8 Hz); 7.8–7.9; (m, 4H); **(5)** ES-MS: Found: (M−H) 283.22; $C_{12}H_{14}NO_5P$ calculated: 283.06; 1H NMR (300 MHz, C_2D_6SO): 1.4 (m, 4H); 1.7 (m, 2H); 3.6 (t, 2H, J = 6.8 Hz); 7.8–7.9 (m, 4H); **(6)** ES-MS:

Found: 2256.60; $C_{110}H_{138}N_{33}O_{19}P$ calculated: 2256.06; **(7)** ES-MS: Found: 2206.69; $C_{107}H_{139}N_{33}O_{20}$ calculated: 2206.09; **(8)** ES-MS: found: (M−H) 290.12; $C_{12}H_{22}N_2O_6$, calculated: 290.31; 1H NMR (300 MHz, C_2D_6SO): 1.3 (m, 9H); 1.4–1.5 (q, 2H; $J = 7.1$ Hz); 2.6–2.7 (t, 2H, $J = 7.3$ Hz); 2.9–3.0 (m, 2H); 3.2 (s, 4H); 6.7 (m, 1H); **(9)** ES-MS: found: 2264.21; $C_{110}H_{146}N_{34}O_{20}$ calculated: 2264.55.

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