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Synthesis and nucleic acid binding studies of novel pyrrolidinyl PNA carrying an *N*-amino-*N*-methylglycine spacer

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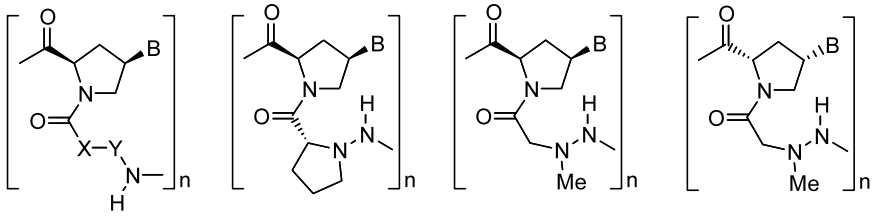
Abstract—Two novel pyrrolidinyl peptide nucleic acids comprising alternating sequences of thymine-modified D- or L-proline and an *N*-amino-*N*-methylglycine spacer were synthesized using solid-phase methodology. UV and CD titrations together with a gel-binding shift assay revealed that neither of the homothymine PNA decamers bind to their complementary DNA or RNA. This was considered to be due to an unfavorable secondary structure which could not be alleviated by the presence of the positively charged protonated amine in the PNA backbone. © 2003 Elsevier Science Ltd. All rights reserved.

Peptide nucleic acid (PNA) is a DNA analogue with a polyamide backbone which was shown to possess strong nucleic acid binding properties.[1] Recently there has been much interest in conformationally rigid analogues of PNA since in principle they should bind more strongly with nucleic acids according to the concept of pre-organization.[2] We have recently synthesized and studied the interactions of a conformationally constrained PNA based on an alternating sequence of (2*R*,4*R*)-4-thyminylproline and β-amino acid spacers **1** with complementary DNA and RNA.[3,4] It was evident that the stereochemistry of the spacer amino acid is critical for successful binding. Furthermore, we have discovered that PNA **2** bearing the aminopyrrolidine-2*R*-carboxylic acid spacer (D-Apc) shows an unusual preference for binding to its complementary DNA over RNA.[4] From the limited data available, it was not entirely clear whether a structural factor or the presence of a positive charge on the PNA strand at physiological pH, or both, were responsible for the unusual tight binding properties of the PNA **2**.

To investigate further the effect of structure and positive charge on the spacer, we designed a novel PNA **3** bearing a flexible *N*-amino-*N*-methylglycine spacer. This is expected to decrease the binding affinity of **3** compared to **2** due to the higher entropy loss on binding. On the other hand, the more flexible spacer would allow **3** to adopt some conformations not available to **2**, which may result in a PNA with different binding characteristics. Indeed, related PNA systems bearing a positively charged and conformationally



1 **2** **3** **4**

Keywords: nucleic acids; PNA; DNA; RNA; β-amino acids.

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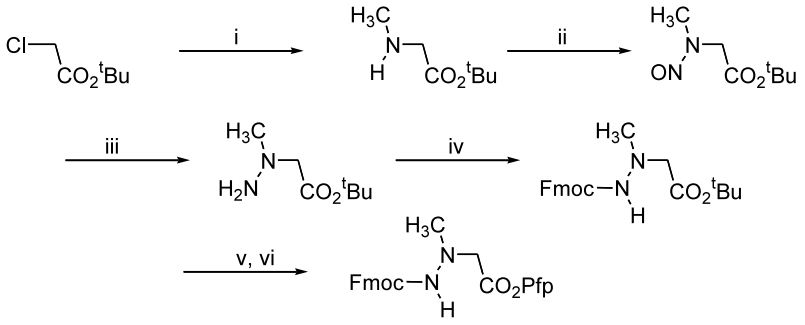


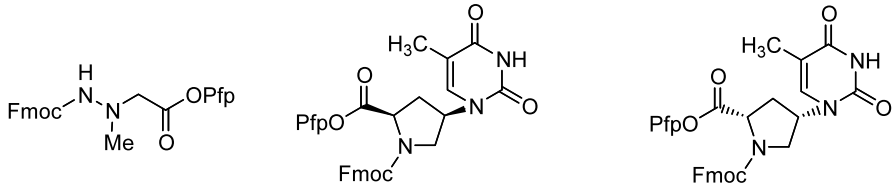
Scheme 1. *Reagents and conditions*: (i) excess 40% aq. $MeNH_2$, KI (1 equiv.) (91%); (ii) $NaNO_2$, 3 M H_2SO_4 0°C (87%); (iii) Zn, 50% HOAc 0°C (60%); (iv) FmocCl, DIEA; CH_2Cl_2 (55%); (v) TFA; CH_2Cl_2; (vi) $PfpOCOCF_3$, DIEA; CH_2Cl_2 (68%, two steps).

flexible aminoethyl spacer showed good binding to RNA and DNA.[5] Since different stereochemistries on the thyminylproline unit can have a dramatic effect on the binding characteristics of the PNA,[5,6] the PNA **4** was also included in our target molecules.

The Fmoc-protected *N*-amino-*N*-methylglycine monomer **5** was synthesized from readily available starting materials as shown in Scheme 1.[7] The pentafluorophenyl-activated Fmoc-protected thymine building blocks **6** and **7** were synthesized as previously described.[3,8] The target sequence for both **3** and **4** was H-T_{10}-LysNH_2, i.e. B = thymine. The lysinamide was included at the C-termini of both PNA for comparison with previous PNA in this series.[9] The PNA syntheses were carried out following our previously reported protocol.[10] The acid-sensitive Rink-amide Novasyn TGR resin was used as the solid support. The lysine, nucleoamino acids and spacer were introduced as their pentafluorophenyl esters (4 equiv.) in the presence of 1-hydroxy-7-azabenzotriazole (HOAt) in DMF (120 min, single coupling). UV monitoring of the dibenzofulvene–piperidine adduct suggested that the efficiency of each coupling step was greater than 95%, nevertheless, capping (Ac_2O/DIEA) was routinely performed at every step. The overall coupling efficiency was determined to be 48% for (**3**-T_{10}) and 58% for (**4**-T_{10}). After the synthesis of the entire sequence was completed, the PNA's were released from the resin by treatment with trifluoroacetic acid followed by ether precipitation and were purified by reverse phase HPLC. The purified PNAs were homogeneous by reverse phase HPLC (**3**-T_{10}, $t_R = 28.5$ min; **4**-T_{10}, $t_R = 25.5$ min, column: C-18, 4 μ particle size, acetonitrile:water gradient 10:90–90:10 over 20 min, hold time 5 min, flow rate 0.5 mL/min) and showed the expected molecular weight (MALDI TOF, **3**-T_{10} calcd for M^+, 3216.42, found 3218.06; **4**-T_{10}, calcd for M^+, 3216.42 found 3216.40).

Binding of the PNA **3**-T_{10} and **4**-T_{10} to polydeoxyadenylic acid (complementary DNA) and polyadenylic acid (complementary RNA) were studied by UV titration (20°C, 10 mM sodium phosphate buffer, pH 7.0). If the PNA and DNA can form a stable hybrid at or above the temperature at which the titration was carried out, a non-linear increment in absorbance at 260 nm would be expected due to hypochromism, with a discontinuity at the equivalence point. According to such criteria, no binding between **3**-T_{10} or **4**-T_{10} and complementary DNA was observed as the absorbance showed a near-perfect linearity with the observed and calculated absorbance in good agreement. Likewise, the UV-titration experiment indicated that **3**-T_{10} and **4**-T_{10} did not bind to their complementary RNA. The lack of binding was also supported by polyacrylamide gel electrophoresis of **3**-T_{10} in the presence of fluorescent-labeled $(dA)_{10}$, the only fluorescent band observed migrating at the same rate as the unhybridized fluorescent-labeled $(dA)_{10}$.

Finally, CD experiments confirmed the lack of binding between **3**-T_{10}/poly(dA), **3**-T_{10}/poly(rA), **4**-T_{10}/poly(dA) and **4**-T_{10}/poly(rA). Interestingly, the single stranded PNA **3**-T_{10} showed strong CD signals with an ellipticity maximum at 280 nm and a minimum at 225 nm while **4**-T_{10} showed a CD spectrum which is almost the mirror image of **3**-T_{10} (Fig. 1).[11] This suggests the presence of a stable secondary structure in both PNA as found in polymers of β-amino acids.[12] Nevertheless, in all cases the CD spectra of a 1:1 mixture of the PNA and poly(rA) or poly(dA) were identical to the sum of each component even at a temperature as low as 10°C (Fig. 2). In addition, a plot of ellipticity at different ratios of PNA:DNA in CD-titration experiments did not show any discontinuity, confirming the lack of binding.



5 **6** **7**

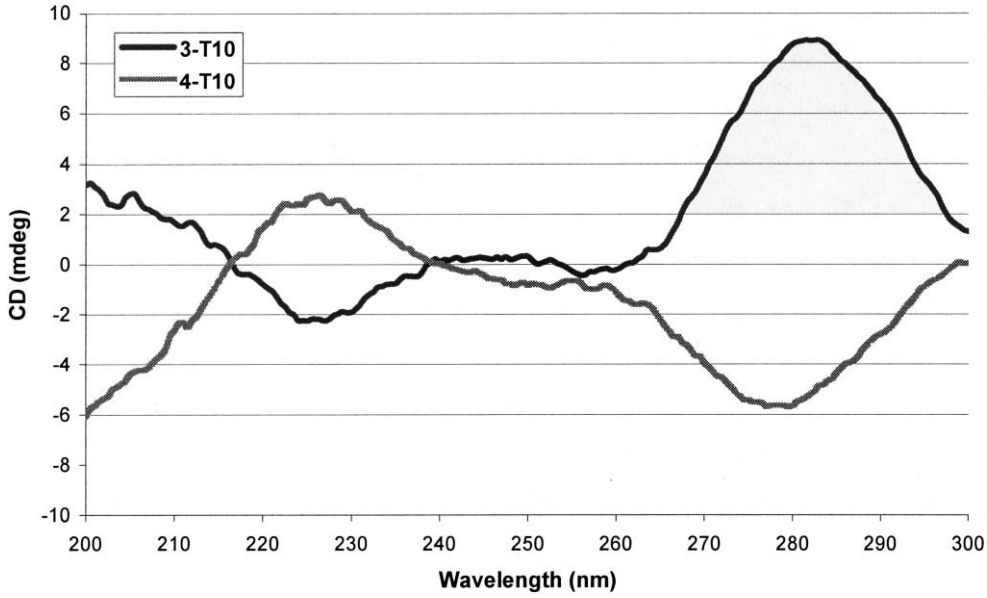


Figure 1. CD spectra of single stranded PNA **3**-T_{10} and **4**-T_{10}. Conditions: concentration of PNA strand = 1 μM, 10 mM sodium phosphate pH 7.0, 20°C.

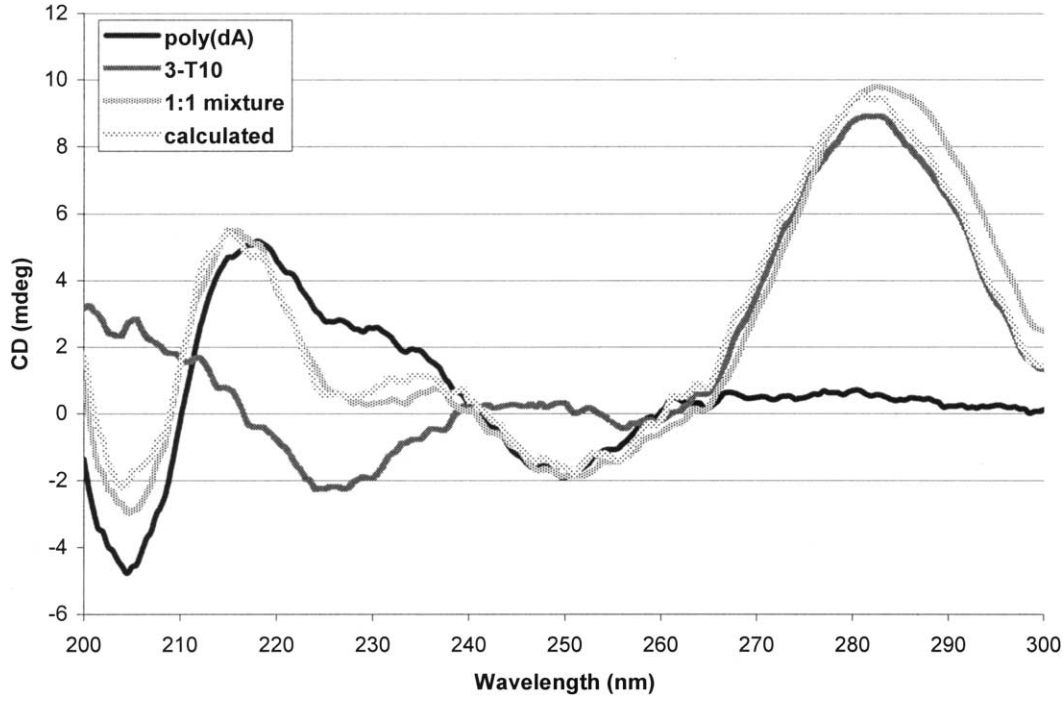


Figure 2. CD spectra of single-stranded poly(dA), **3**-T_{10} and their 1:1 mixture compared with the calculated spectra. Conditions: concentration of PNA = DNA = 10 μM nucleotides, 10 mM sodium phosphate pH 7.0, 20°C.

The binding experiments indicate that the PNA **3**-T_{10} and **4**-T_{10} were unable to form stable hybrids with poly(dA) or poly(rA). This is in sharp contrast to the PNA **2**-T_{10} which forms a very stable 1:1 PNA–DNA hybrid with a T_m of 80°C (10 mM sodium phosphate buffer, pH 7.0) and a less stable hybrid with RNA (T_m 32°C).[4] The results suggested that the presence of a positively charged protonated amine on the PNA strand is probably not the major contributor to the unusual nucleic acid binding properties of PNA **2**. Consequently, the presence of the structurally rigid aminopyrrolidine carboxylic acid spacer appeared to be the more important factor. The fact that single stranded PNA **3**-T_{10} and **4**-T_{10} show strong CD spectra (Fig. 1) indicates that the PNA's adopt secondary structures which are not favorable for binding to complementary DNA or RNA, the need for structural-reorganization rendering the binding process thermodynamically unfavorable.

In conclusion, we have successfully synthesized two novel pyrrolidinyl PNAs carrying the *N*-amino-*N*-methylglycine spacer. The T_{10} sequence of both PNAs did not hybridize with poly(dA) and poly(rA) under the conditions studied. The lack of binding provides valuable new information on the structural requirements for the PNA–nucleic acids interaction which is important in designing further PNA systems with improved binding properties.

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