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Chimeric peptide nucleic acids incorporating (2*S*,5*R*)-aminoethyl pipecolyl units: synthesis and DNA binding studies ☆

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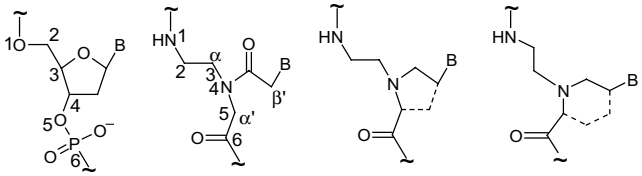
Abstract—The design and facile synthesis of a novel chiral six-membered PNA analogue (2*S*,5*R*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid, *aepip*PNA, that upon incorporation into PNA sequences effected stabilization of complexes with target complementary DNA. This is the first example where a six membered-PNA is shown to be capable of forming stable complexes with DNA and further expands the repertoire of cyclic PNA analogues.

Peptide nucleic acids (PNA **II**), a new class of DNA (**I**) mimics invented a decade ago, is emerging rapidly as novel, potential antigene, and antisense agents, in the field of medicinal chemistry. In PNA, a neutral and achiral polyamide backbone consisting of *N*-(2-aminoethyl)glycyl, *aeg* units[1] replaces the charged sugar-phosphate backbone of DNA. The nucleobases are attached to the backbone through a rigid tertiary acetamide linker and PNA binding to the target DNA/RNA sequences occurs with high sequence specificity and affinity.[2] In spite of the resistance of PNAs to cellular enzymes such as nucleases and proteases, the major limitations complicating the therapeutic application of PNAs are ambiguity in orientational selectivity of binding, poor solubility in aqueous media, and inefficient cellular uptake.[3,4] Our efforts[5] and those of others[6] to improve the properties of *aeg*PNA and achieve fine-tuning of an aminoethylglycyl, *aeg*PNA backbone to bind to the complementary nucleic acids through a pre-organization strategy, has led to a number of five-membered pyrrolidinyl PNA analogues. We have previously reported chiral aminoethylprolyl, *aep*PNA(**III**), designed by linking the glycyl α′-carbon in the *aeg*PNA to the acetamido β′-carbon via a methylene bridge.[5] In *aep*PNA, the nucleobase is directly attached to a pyrrolidine ring instead of being linked to the backbone via an acetamido moiety. This PNA analogue with positively charged tertiary amine in the backbone is conformationally constrained due to the five-membered pyrrolidine ring and has significantly improved solubility accompanied by affinity and selectivity in DNA:PNA binding, without compromising the base-pairing specificity.[5] The stability of the duplexes was found to be dependent upon the stereochemistry of the pyrrolidine ring and also the nucleobase.[5]



In this communication, we report the synthesis and preliminary evaluation of a six-membered homologue of *aep*PNA, the chiral aminoethylpipecolylPNA, *aepip*PNA (**IV**). This is a result of bridging the α′-C atom of the glycyl unit and the β′-C atom of linker to a nucleobase in PNA with an ethylene bridge instead of a methylene bridge of *aep*PNA to generate a six-membered piperidine ring **IV**. Although the ethylene bridge might be expected to be more flexible compared to the methylene bridge, chair/boat conformations in six-membered rings are rigidly locked structures in contrast to the relatively flexible five-membered rings. It will be

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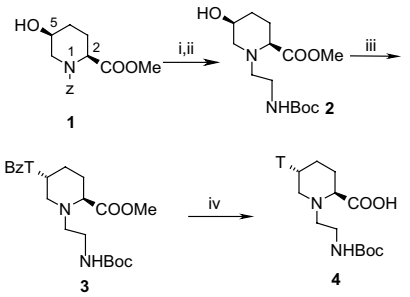
interesting to study the DNA binding ability of the resulting PNA sequences in order to understand the contributing structural factors to assist evolution of analogues with optimum properties. Herein, we report the synthesis of one of the four possible stereoisomers, (2*S*,5*R*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid **4**, its site-specific incorporation into various PNA oligomers by solid-phase synthesis and hybridization properties with complementary DNA sequences.

Synthesis of aepipPNA monomer and oligomers. The synthesis of *cis*-5(*S*)-hydroxy-2(*S*)-*N*1-benzyloxycarbonyl pipecolic acid methyl ester **1** from naturally occurring L-glutamic acid was accomplished according to the procedure of Bailey et al.[7,8] The deprotection of *N*1 in **1** followed by alkylation of the piperidine ring nitrogen with *N*-Boc-aminoethyl mesylate[6] afforded 1-(*N*-Boc-aminoethyl)pipecolyl methyl ester derivative **2**. Displacement of the C5-(*S*)-hydroxyl function with *N*3-benzoylthymine (BzT) under Mitsunobu reaction conditions yielded the (2*S*,5*R*)-5-(*N*3-benzoylthymin-1-yl) pipecolic ester derivative **3** with inversion of configuration at C-5. The product, even after repeated purification by column chromatography, was contaminated with a small amount of diisopropyl hydrazide. The hydrolysis of the ester function and *N*3-benzoyl deprotection of thymine in **3** was simultaneously effected, by treatment with sodium hydroxide in aqueous methanol to obtain (2*S*,5*R*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid **4**, the desired *aepip*PNA monomer for PNA synthesis (Scheme 1).

The structural integrity of all the new compounds was confirmed by ^{1}H,^{13}C NMR spectroscopic analysis and mass spectrometry.

PNA oligomers **5**–**11** containing *aepip*PNA units were assembled by solid-phase peptide synthesis on Merrifield resin derivatized with *N*-Boc-β-alanine. The *aepip*PNA monomer **4** was incorporated into the PNA octamer sequence H–T_8–$NHCH_2CH_2COOH$ at predefined positions to yield the modified *aeg*PNAs **5**–**11** (Table 1). The chimeric PNA oligomers containing *aepip*PNA were designed to test the effect of modifications at C/N terminals and the distance between two modifications on the stability of the derived PNA:DNA complexes. The unmodified *aeg*PNA sequences H–T_8–



Scheme 1. Synthesis of *aepip*PNA monomer (i) H_2/Pd–C, 60 psi (92%); (ii) Boc-NH–$(CH_2)_2$OMs, DIPEA, acetonitrile–DMF (37%); (iii) BzT, DIAD, PPh_3, THF (32%); (iv) NaOH, MeOH–water (95%).

Table 1. UV–T_m (°C) of DNA:PNA_2 complexes

PNA	PNA_2:DNA	UV–T_m °C
5, H–**t** T T T T T T T–(β-Ala)–OH	**5**:**14**	43 (23.4)
	5:**16**	19 (8.9)
6, H–T T T T T T T **t**–(β-Ala)–OH	**6**:**14**	48 (19.2)
	6:**16**	24 (3.8)
7, H–T T T T **t** T T T–(β-Ala)–OH	**7**:**14**	44 (10.9)
8, H–T T T T T **t** T **t**–(β-Ala)–OH	**8**:**14**	52 (26.7)
9, H–T T T **t** T T T **t**–(β-Ala)–OH	**9**:**14**	51 (25.2)
10, H–T **t** T T T T T **t**–(β-Ala)–OH	**10**:**14**	49 (14.3)
11, H–**t** T C T C T T T–(β-Ala)–OH	**11**:**15**	60 (26.7)
12, H–T T T T T T T T–(β-Ala)–OH	**12**:**14**	43 (18.3)
13, H–T T C T C T T T–(β-Ala)–OH	**13**:**15**	51 (18.5)

T = *aeg*PNA; **t** = *aepip*PNA; DNA: **14**, 5′–GCAAAAAAAACG–3′; **15**, 5′–AAAGAGAA–3′; **16**, 5′–GCAAAATAAACG–3′; buffer: 10 mM sodium phosphate, pH 7.30. The T_m values are accurate to (±) 0.5 °C. Experiments were repeated at least thrice and the T_m values were obtained from the peaks in the first derivative plots. Values in parentheses represents % hyperchromicity.

$NHCH_2CH_2$–COOH **12** and the mixed homopyrimidine H–TTCTCTTT–$NHCH_2CH_2$–COOH **13** were also synthesized for control studies. The oligomers were cleaved from the solid support by treatment with TFA-TFMSA[9] to yield the corresponding PNAs carrying β-alanine at the carboxy terminus. All PNAs (**5**–**13**) were purified by FPLC on a PepRPC column. The purity of the oligomers was rechecked by HPLC on an RPC-18 column and these were characterized by MALDI-TOF mass spectrometry.[10] The complementary DNA oligomers **14** having a CG/GC lock at the ends to prevent slippage, the mixed homopyrimidine sequence **15** and a mismatched sequence **16** were synthesized on an automated DNA synthesizer using standard phosphoramidite chemistry[11] and purified by C_{18} RPHPLC. The pK_a of the piperidine ring nitrogen of the *aepip*PNA monomer as determined by pH-titration was found to be 6.76, similar to that of pyrrolidine nitrogen in *aep*PNA[5] and may be partially protonated under the experimental conditions. No precipitation was observed in samples of *aepip*PNA even after prolonged storage.

UV–T_m studies on DNA:PNA_2 triplexes. The PNA sequences used here are homopyrimidine sequences that are known to form DNA:PNA_2 triplexes.[12] The DNA:PNA stoichiometry in these complexes was found to be 1:2 as studied by mixing curves (Job's plot) generated from both UV absorbance at 260 nm and CD ellipticity data.[12] The annealed PNA_2:DNA complexes were subjected to temperature dependent UV absorbance measurements. The percent hyperchromicity versus temperature plots derived from these experiments exhibited a single sigmoidal transition as observed in the first derivative plots (Fig. 1). The T_m results (Table 1) indicate that compared to unmodified *aeg*PNA T_8 oligomer **12**, the modified homothymine chimeric PNA oligomers containing *aepip*PNA (**5**–**10**) stabilized corresponding DNA(**14**):PNA_2 triplexes. Modification at C-terminus in sequence **6** stabilized the complex whereas with a single modification at the N-terminus and internal positions, as in **5** and **7**, respectively, stability remained unaffected. Increasing the number of modifications from the C-terminus end further enhanced the

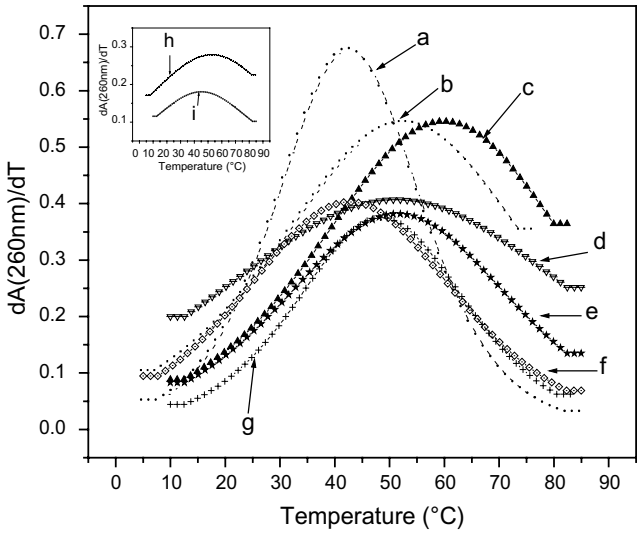


Figure 1. UV–T_m first derivative curves of DNA:PNA_2 complexes. (a) **14**:**5**; (b) **14**:**8**, (c) **15**:**11**, (d) **14**:**9**, (e) **15**:**13**, (f) **14**:**12**, (g) **14**:**10**. Inset: (h) **14**:**6**, (i) **14**:**7**.

T_m. Sequences **8**, **9**, and **10** were constructed with 2 *aepip* units, one at the C-terminus and the second *aepip* unit at third, fifth, and seventh base positions, respectively, to study the positional effect of the modified units with respect to each other. A synergistic stabilizing effect was observed with the second modified *aepip* unit in all the cases (**8**:**14**, **9**:**14**, and **10**:**14**). The maximum benefit per additional unit was observed ($\Delta T_m + 4\,°C$) when the second *aepip*PNA unit was placed nearer to the C-terminal unit (**8**:**14**). The complexes of the modified PNAs (**5**, **6**) with DNA **16** (Table 1) having a single mismatch in the center of the sequence, were destabilized with a $\Delta T_m \sim -24\,°C$ (data not shown). However, the complex of control PNA **12** with mismatch DNA **16**, only shows linear increase in absorbance and does not show a sigmoidal transition. The stability of the mixed C/T base *aepip*-PNA:DNA complexes containing N-terminus substitution (**11**:**15**) was higher by 9 °C compared to that of the control complex **13**:**15** when the orientation of the DNA strand was antiparallel with respect to both PNA strands. This result contrasts with the homothymine oligomers where the relative orientation of the DNA strand remains ambiguous.[5c] The percent hyperchromicity accompanying the melting of DNA:*aepip*-PNA complexes was also enhanced compared to the control complex suggesting a better stacking of bases induced by the incorporation of the *aepip*PNA modification. Only in the case of the complex **7**:**14** where the *aepip* modification is in the center of the sequence, was the percent hyperchromicity accompanying the melting found to be lower than the control. The formation of stable complexes with the *aepip*PNA was further confirmed by a nondenaturing gel shift assay (Fig. 2) in which the DNA band exhibited characteristic retardation upon formation of the complex with PNAs. The ssPNAs alone moved much more slowly compared to the complexes.

In summary, the substitution of the six-membered *aepip*PNA monomer into the *aeg*PNA backbone increased the T_m of the derived complexes with DNA. This

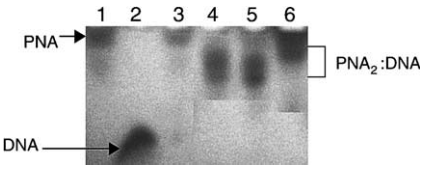


Figure 2. PNA:DNA complexation by gel shift assay: lane 1, ssPNA **12** (aeg-T_8); lane 2, ssDNA **14**; lane 3, ssPNA **6**; lane 4, PNA **6**+ DNA **14**; lane 5, PNA **12** (aeg-T_8)+DNA **14**; lane 6, PNA **8**+ DNA **14**.

is interesting since from an earlier study[13] based on piperidines with a different substitution pattern, it was suggested that six-membered rings are unlikely to stabilize the derived PNA structures for complex formation with DNA. In the present *aepip*PNA analogues the stereochemical dispositions of substituents seem to lead to a favorable pre-organization of PNA for the formation of stable DNA triplexes. In this context, work is in progress in our laboratory to examine the implications on the duplex and triplex stability of *aepip*PNAs constituted from other diastereomers and nucleobases.

Acknowledgements

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10. PNA **5**: $M_{obsd} = 2232$; $M_{calcd} = 2231.17$, PNA **6**: $M_{obsd} = 2232$; $M_{calcd} = 2231.17$, PNA **7**: $M_{obsd} = 2232$; $M_{calcd} = 2231.17$, PNA **8**: $M_{obsd} = 2243$; $M_{calcd} = 2244.23$, PNA **9**: $M_{obsd} = 2243$; $M_{calcd} = 2244.23$; PNA **10**: $M_{obsd} = 2243$; $M_{calcd} = 2244.23$; PNA **11**: $M_{obsd} = 2233$; $M_{calcd} = 2201.14$, PNA **12**: $M_{obsd} = 2220$; $M_{calcd} = 2219.11$, PNA **13**: $M_{obsd} = 2189$; $M_{calcd} = 2189.10$.
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