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(2*S*,5*R*/2*R*,5*S*)-Aminoethylpipecolyl *aepip*-*aeg*PNA chimera: synthesis and duplex/triplex stability

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Abstract—This article reports the design and facile synthesis of novel chiral six-membered PNA analogues (2*S*,5*R*/2*R*,5*S*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid, *aepip*PNA **IV** that upon incorporation into standard *aeg*PNA sequences effected stabilization of complexes with complementary target DNA. Substitution of *aeg*PNA unit by the designed monomer at the C-terminus was more effective than substitution at N-terminus. The stabilizing behaviour improved with degree of substitution and was found to be dependent on their relative positions in the sequence. The six-membered piperidine ring in the design may freeze the rigid chair conformations and the relative stereochemistry of the substituents may in effect direct the complex formation with DNA/RNA by sequence-specific nucleobase recognition. In the present *aepip*PNA analogues, the L-*trans* stereochemical disposition of the substituents seems to lead to the favorable pre-organization of the PNA oligomers for complex formation with DNA. The results reported here further expand the repertoire of cyclic PNA analogues.

1. Introduction

Peptide nucleic acids (*aeg*PNA), a new class of DNA (**I**) (Fig. 1) mimics invented a decade ago are gaining importance as novel potential antigene and antisense agents in the field of medicinal chemistry. In *aeg*PNA (**II**), the charged sugar-phosphate backbone of DNA is replaced by a neutral and achiral polyamide backbone consisting of *N*-(2-aminoethyl)glycyl units.[1] The nucleobases are attached to this backbone through a rigid tertiary acetamide linker group and PNA binding to the target DNA/RNA sequences occurs with high sequence specificity and affinity.[2] In spite of its resistance to cellular enzymes such as nucleases and proteases, the major limitations confounding its application are ambiguity in orientational selectivity of binding, poor solubility in aqueous media and inefficient cellular uptake.[3,4] The (2-aminoethyl)-glycyl fused segment constituting the *aeg*PNA backbone is highly flexible and slowly reorganizes to the energetically preferred conformation for complex formation with DNA/RNA. Preorganizing the *aeg*PNA backbone into hybridization competent conformations should have entropic advantages. Our efforts[5–7] and those of others[8] to improve the properties of *aeg*PNA by optimal tuning of the PNA backbone to bind the complementary nucleic acids through a pre-organization strategy has resulted in a number of five-membered pyrrolidinyl PNA analogues. The configuration of the pyrrolidine ring and the mode of attachment of the nucleobase to the ring were found to be primarily responsible for the observed effects on the binding efficiency of chimeric pyrrolidinyl-*aeg*PNAs.[5,8] The five-membered pyrrolidine ring in *aep*PNA **III** is probably conformationally quite flexible, and the selection between parallel/antiparallel modes of binding to complementary DNA, although better than *aeg*PNA, was not

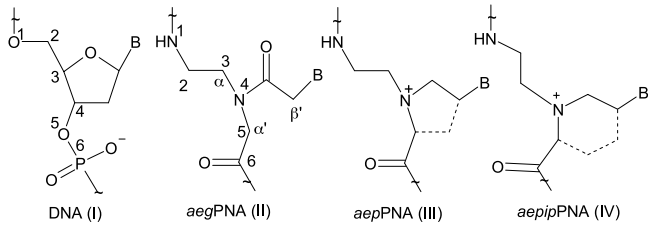

Figure 1. Structure of DNA, PNA, and modified PNAs.

Keywords: Peptide nucleic acids; Pipecolic acid PNA; *aepip*PNA.
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comparable with DNA itself.[5] Also, the binding efficiency was found to be nucleobase-dependent.[6] The fairly rigid six-membered ring structures as in hexose[9] and hexitol[10] nucleic acids have shown excellent section of parallel/antiparallel modes of binding to DNA. This has triggered interest in six-membered PNA analogs,[11] although with some initial misgivings.[12]

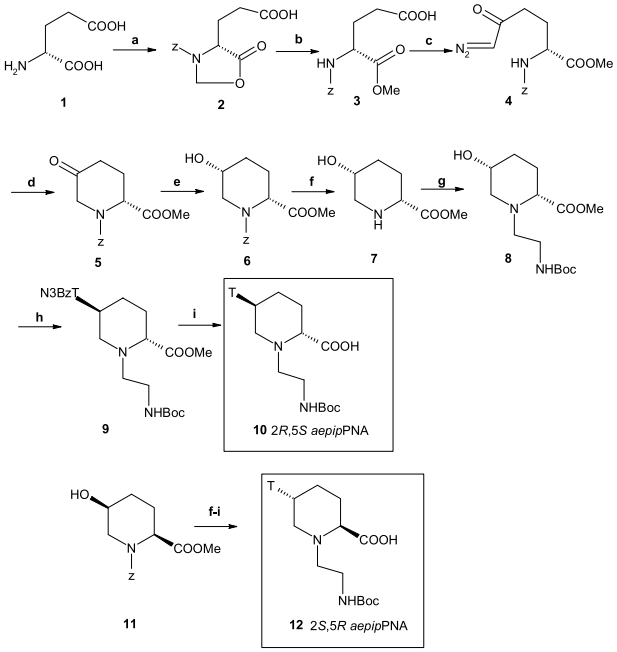
Recently, we reported the synthesis of chimeric PNAs, in which one of the isomers (2*S*,5*R*) of aminoethylpipecolyl PNA, *aepip*PNA **IV**,[13] was introduced at pre-determined sites of the oligomers. This six-membered homologue of *aep*PNA is derived by bridging the α′-C atom of the glycyl unit and the β′-C atom of linker to nucleobase in PNA with an ethylene bridge instead of a methylene bridge in **III**. The nucleobase in **IV** is directly attached to the piperidine ring at the C5 position, without altering the net number of backbone atoms connecting two successive nucleobases. In this article, we report the detailed synthesis and characterization of its enantiomer (2*R*,5*S*) from D-glutamic acid. The synthesis of chimeric, chiral (*aeg-aepip*PNA backbone) triplex forming (polypyrimidine) and duplex forming (mixed purine-pyrimidine) PNA sequences and their hybridization properties with the complementary DNA are presented.

1.1. Chemical syntheses of *aepip*PNA monomers

The syntheses of the (2*R*,5*S*) and (2*S*,5*R*) 1-(*N*-Boc-aminoethyl)-5-(*N*3-benzoylthymin-1-yl)pipecolic acid methyl ester was achieved in 10 steps starting from the D-glutamic acid **1** and the naturally occurring L-glutamic acid, respectively (Scheme 1). The selective protection of the α-amino group in D-glutamic acid **1** was achieved via formation of the oxazolidinone **2** followed by ring opening with sodium methoxide to yield the α-ester **3** in 82% yield. This, upon treatment with ethyl chloroformate, gave the corresponding mixed anhydride that on reaction with diazomethane generated the diazoketone **4** in 65% overall yield from **3**. The direct conversion of diazoketone **4** to the protected 5-oxopipecolic acid **5** was achieved by ring closure carbene insertion into the N–H bond using rhodium (II) acetate as catalyst. Finally, stereospecific reduction with sodium borohydride gave the *cis*-5-hydroxy-D-pipecolic acid ester **6**, which was *N*1-deprotected to give **7**. *N*1-Alkylation of the piperdine ring in the pipecolic acid methyl ester **7** with *N*-Boc-aminoethyl mesylate[6] afforded the (1-*N*-Boc-aminoethyl)pipecolic acid ester **8**. The replacement of the 5*R*-hydroxyl function in **8** with *N*3-benzoylthymine under Mitsunobu reaction conditions yielded the (2*R*,5*S*)-5-(*N*3-benzoylthymin-1-yl) pipecolate ester **9**, accompanied by inversion of stereochemistry at C5. The simultaneous hydrolysis of the methyl ester and removal of N3-benzoyl protecting group of thymine was achieved by treatment with 1 M sodium hydroxide in aqueous methanol to obtain (2*R*,5*S*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid **10** as the desired monomer. Synthesis of the enantiomeric (2*S*,5*R*) **12** was accomplished[13] starting from *cis*-5*S*-hydroxy-2*S*-*N*1-benzyloxycarbonyl pipecolate methyl ester **11** obtained from L-glutamic acid according to Bailey et al.[14,15] The structural integrity of the *aepip*PNA monomers **10** and **12** was confirmed by spectral analysis (^{1}H, ^{13}C NMR and mass spectrometry) and optical rotation with opposite signs as shown in Section 4.

1.2. Solid phase synthesis of *aeg-aepip*PNA oligomers

PNA oligomers containing the *aepip*PNA units were assembled by solid-phase peptide synthesis on Merrifield resin derivatized with *N*-Boc-β-alanine. The *aepip*PNA monomers **12** and **10** were suitably incorporated into the PNA octamer sequence H-T$_8$-NHCH$_2$CH$_2$COOH at



Scheme 1. Synthesis of *aepip*PNA monomers. a. (i) Benzyloxy carbonyl chloride, $NaHCO_3$ (94%) (ii) $(CH_2O)_n$, TsOH, benzene, reflux (82%); b. NaOMe, MeOH (93%); c. (i) EtOCOCl, Et_3N, THF (ii) CH_2N_2, Et_2O (65%); d. $[Rh(OAc)_2]_2$, benzene, reflux (52%); e. $NaBH_4$, MeOH (93%); f. H_2/Pd–C, 60 psi (92%); g. BOC-NH-$(CH_2)_2$OMs, DIPEA, ACN:DMF (37%); h. *N*3-BzT, DIAD, PPh_3, THF (32%); i. 1 M NaOH, MeOH/water (95%).

predefined positions to yield the modified *aeg*PNAs **13**–**14** and **18**–**21**, respectively. The modified monomer (2*S*,5*R*) **12** was also incorporated into a mixed base homopyrimidine sequence **15**. For control studies, the unmodified *aeg*PNA sequences H-T_8-$NHCH_2CH_2$-COOH **16** and H-TTCTCTTT-$NHCH_2CH_2$-COOH **17** were synthesized by similar procedures. The oligomers after solid phase assembly were cleaved from the support by treatment with TFA-TFMSA[16] to yield the corresponding PNAs carrying β-alanine at the carboxy terminus. PNAs (**13**–**26**) were purified by FPLC on a PepRPC column, and the purity of the oligomers was rechecked by HPLC on RPC-18 column and these were characterized by MALDI-TOF mass spectrometry.[17]

The modified *aeg*-*aepip*PNA oligomers **13**, **14**, **18**–**21** permit the study of the positional effects on PNA_2:DNA triplex stability, while the mixed base PNA sequences **25**–**26** allow explicit testing of the relative stereochemical effects of (2*S*,5*R*) and (2*R*,5*S*) *aepip*PNA units on duplex formation. The complementary DNA oligomer **22** having CG/GC lock at the end to prevent slippage, complementary DNA for mixed homopyrimidine sequence **23**, and a mismatched sequence **24** and DNA **27**–**28** for constituting the duplexes were synthesized on an automated DNA synthesiser using standard phosphoramidite chemistry,[18] followed by ammonia deprotection. These were purified by gel filtration and their purities checked by HPLC.

The pK_a of the piperidine ring nitrogen of the *aepip*PNA monomer was determined by acid–base titration and found to be 6.76, not very much different from 6.72 for the pyrrolidine nitrogen in *aep*PNA.[5] The constituted PNA oligomers are thus expected to be partially protonated under physiological conditions. No precipitation was observed in samples of *aepip*PNA even after prolonged storage, suggesting improved solubility of *aepip*PNA oligomers.

2. Results and discussion

2.1. UV-T_m studies on PNA_2–DNA triplexes

The polypyrimidine PNA oligomers (**13**–**21**, Tables 1 and 2) are homopyrimidine sequences that are well known to form DNA:PNA_2 triplexes.[19] The DNA:*aeg*-*aepip*PNA stoichiometry in these complexes was found to be 1:2 from mixing curves (Job's plot) generated from CD ellipticity data at 260 nm. Hence, all complementation studies were performed with 1:2 stoichiometries of DNA and *aeg*/*aepip*PNA (Fig. 2).

Table 2. UV-T_m (°C) of DNA:PNA_2 complexes[a]

Entry		PNA	T_m
1	**18**	H-T T T T **t** T T T-(β-Ala)-OH	44 (10.9)
2	**19**	H-T T T T T **t** T **t**-(β-Ala)-OH	52 (26.7)
3	**20**	H-T T T **t** T T T **t**-(β-Ala)-OH	51 (25.2)
4	**21**	H-T **t** T T T T T **t**-(β-Ala)-OH	49 (14.3)

[a] DNA: **22**, 5′-GCAAAAAAAACG-3′; **23**, 5′-AAAGAGAA-3′; **24**, 5′-GCAAAATAAACG-3′; T=*aeg*-PNA; **t**=*aepip*PNA; Buffer: 10 mM sodium phosphate, pH 7.30. T_m values are accurate to (±)0.5 °C. Experiments were repeated at least three times and the T_m values were obtained from the peaks in the first derivative plots. Values in parentheses represent % hyperchromicity.

The thermal stabilities of PNA_2:DNA complexes were studied by temperature dependent UV absorbance measurements. The temperature-percent hyperchromicity first derivative plots for DNA:PNA_2 triplexes indicated a single transition (Figs. 3 and 4B), characteristic of both PNA strands dissociating simultaneously from DNA in a single step. The T_m values (entries 1 and 2, Table 1) indicate that the *aepip*PNA oligomers **13** and **14** having single modification of either stereomer at N or C-terminus exhibited stabilization compared to the unmodified PNA T_8 homooligomer (**16**). While a (2*S*,5*R*) *aepip* unit at the N-terminus has better stability of its complex with the complementary DNA **22** compared to (2*R*,5*S*), the situation was reversed for corresponding modification at C-terminus; the (2*R*,5*S*) oligomer forming the much more stable hybrid compared to that of the (2*S*,5*R*) oligomers (entry 2). As the percent hyperchromicity of this particular transition was found to be very low, the complex formation was confirmed by a CD

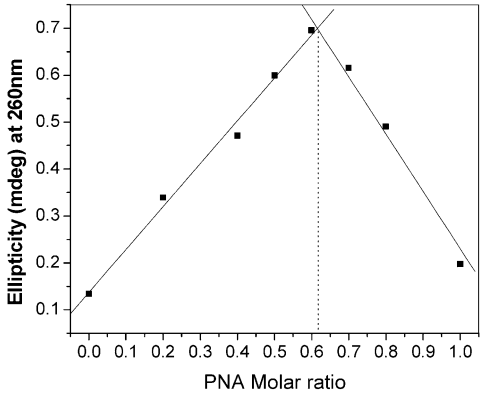


Figure 2. A CD mixing curve (Job's plot) of complex **14**:**22**(2*R*,5*S*).

Table 1. UV-T_m (°C) of DNA:PNA_2 complexes[a]

Entry		PNA	PNA_2:DNA	UV-T_m (°C)	
				2*S*,5*R*	2*R*,5*S*
1	**13**	H-**t** T T T T T T T-(β-Ala)-OH	**13**:**22**	43 (23.4)	36 (8.0)
2	**14**	H-T T T T T T T **t**-(β-Ala)-OH	**14**:**22**	48 (19.2)	76 (2.3)
			14:**24**	24 (3.7)	23 (3.3)
3	**15**	H-**t** T C T C T T T-(β-Ala)-OH	**15**:**23**	60 (26.7)	56 (26.6)
4	**16**	H-T T T T T T T T-(β-Ala)-OH	**16**:**22**	43 (18.3)	
5	**17**	H-T T C T C T T T-(β-Ala)-OH	**17**:**23**	51 (18.5)	

[a] DNA: **22**, 5′-GCAAAAAAAACG-3′; **23**, 5′-AAAGAGAA-3′; **24**, 5′-GCAAAATAAACG-3′; T=*aeg*-PNA; **t**=*aepip*PNA; Buffer: 10 mM sodium phosphate, pH 7.30. T_m values are accurate to (±)0.5 °C. Experiments were repeated at least three times and the T_m values were obtained from the peaks in the first derivative plots. Values in parentheses represent % hyperchromicity.

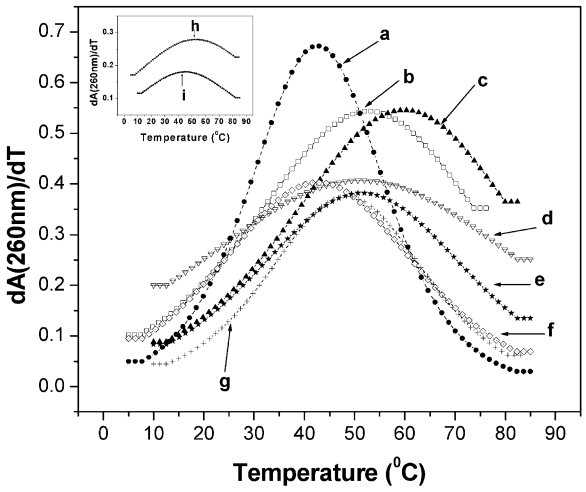


Figure 3. UV-melting first derivative curves of PNA_2:DNA (2*S*,5*R*) complexes. a. **13**:**22**; b. **19**:**22**; c. **15**:**23**; d. **20**:**22**; e. **17**:**23**; f. **16**:**22**; g. **21**:**22**. Inset: h. **14**:**22**, i. **18**:**22**.

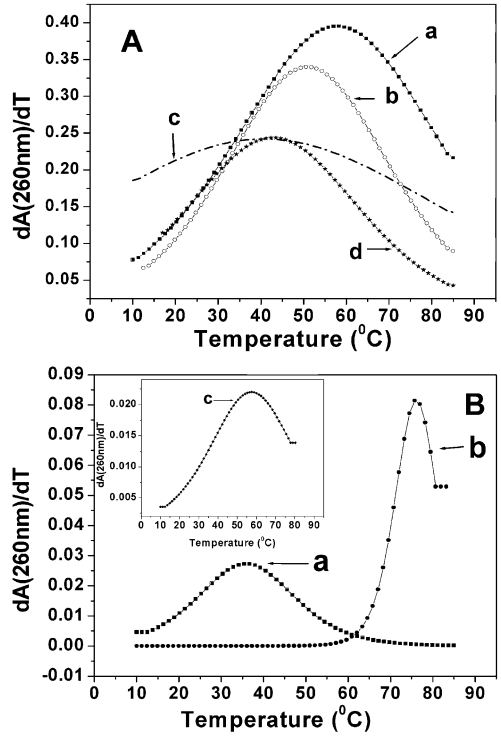


Figure 4. UV-melting first derivative curves. (A) (2*S*,5*R*) PNA:DNA duplexes. a. **25**:**27**; b. **26**:**27**; c. **25**:**28**; d. **26**:**28**. (B) (2*R*,5*S*) PNA_2:DNA triplexes. a. **13**:**22**; b. **14**:**22**; inset c. **15**:**22**.

mixing curve (Job's plot) and was found to be 2:1 PNA_2:DNA complex. The complex formation **14**:**22** was further confirmed by the introduction of single mismatched base in **14**:**24**. The mismatched complex **14**:**24** was destabilized by a larger extent (ΔT_m −53 °C) for (2*R*,5*S*) *aepip* stereochemistry and by $\Delta T_m \sim -24$ °C for (2*S*,5*R*) *aepip* stereochemistry. The control mismatch complex with unmodified PNA (**16**:**24**) showed a linear increase in absorbance without any sigmoidal transition.

The stability of the DNA complexes of *aeg-aepip*-PNA oligomers with mixed pyrimidine base sequence and N-terminus modifications (**15**:**23**) was higher by 5 °C for (2*R*,5*S*) and 9 °C for (2*S*,5*R*) as compared to that of the control complex **17**:**23** (entries 3 and 5 Table 1). The percent hyperchromicity was enhanced compared to the control complex in these transitions when chiral unit is at the N-terminus. Only in the case of the complex **14**:**22** (2*R*,5*S*) and **18**:**22** (2*S*,5*R*),(Table 2) where the *aepip* unit is in the center of the sequence, percent hyperchromicity accompanying the melting was found to be low.

A single (2*S*,5*R*) *aepip* modification in the middle of the sequence did not affect the stability of the DNA hybrid (Table 2, entry 1). Increasing the number of *aepip*PNA modifications further enhanced the T_m (Table 2, entry 2–4). PNA oligomers with one *aepip* modification at C-terminus and a second *aepip* unit at the third (**19**), fifth (**20**) or seventh (**21**) base positions, respectively, were used to study the relative positional effects of the modifications. A synergistic stabilizing effect was observed with a second modified *aepip* unit in all the cases (**19**:**22**, **20**:**22** and **21**:**22**). The maximum benefit per additional unit was observed (ΔT_m + 4 °C) when the second *aepip*PNA unit was separated by one base (**19**:**22**). (Table 2).

The mixed purine-pyrimidine *aeg-aepip*PNA (**25**) and *aeg*PNA (**26**) oligomers were synthesized to examine the orientational selectivity in binding to DNA. The UV-T_m profiles of complexes of PNAs **25** and **26** with DNA sequences **27** and **28** designed to bind in antiparallel and parallel orientations, respectively, is shown in Fig. 4 and values given in Table 3. In both PNAs, the antiparallel duplex was more stable than the parallel duplex. However, the modified *aeg-aepip*PNA **25** (2*S*,5*R*) stabilized the antiparallel duplex (DNA **27**) by 17 °C compared to the 8 °C by *aeg*PNA over the parallel duplex. The complex formation was confirmed by a CD mixing curve (Job's plot) and was found to be a 1:1 PNA:DNA complex (Supplementary material).

Table 3. UV-T_m (°C) of DNA:PNA (2*S*,5*R*) duplexes[a]

	PNA	PNA:DNA	UV-T_m (°C)
25	H- A T G **t** T C T C T T T-(β-Ala)-OH (*ap*)	**25**:**27**	57.8 (20.2)
	(*p*)	**25**:**28**	40.8 (15.0)
26	H- A T G T T C T C T T T-(β-Ala)-OH (*ap*)	**26**:**27**	51.2 (16.2)
	(*p*)	**26**:**28**	43.0 (11.3)

[a] T, C, A, G=*aeg*-PNA; **t**=*aepip*PNA; DNA: **27**, 5′-AAAGAGAACAT-3′; **28**, 3′-TACAAGAGAAA-5′; Buffer: 10 mM sodium phosphate, pH 7.30. T_m values are accurate to (±)0.5 °C. Experiments were repeated at least three times and the T_m values were obtained from the peaks in the first derivative plots. Values in parentheses represent % hyperchromicity.

The PNA_2:DNA triplexes and PNA:DNA duplexes are expected to differ in the base stacking patterns, and this should be reflected in their circular dichroism (CD) spectra. The CD profile for single stranded *aepip*PNA **13** (2*S*,5*R*) and (2*R*,5*S*) were observed as mirror images of each other (Supplementary data). Figure 5A shows the CD profiles of *aeg*PNA and selected *aeg-aepip*PNA (2*S*,5*R*) triplexes while Figure 5B shows the CD profiles of *aeg*PNA and *aeg-aepip*PNA (2*S*,5*R*) duplexes. The positive double hump profile seen in 250–265 nm region is characteristic of polyT.polyA.polyT triplexes. The duplexes show a different CD profile compared to triplexes and the overall CD patterns suggest that incorporation of chiral pipecolic units does not very much alter the base stacking.

Thus overall, the substitution of the six-membered *aepip*PNA monomer in both enantiomeric (2*S*,5*R*) and (2*R*,5*S*) forms into the *aeg*PNA backbone increased the T_m of the derived complexes with DNA. This is interesting since in an earlier study[12] it had been remarked that the six-membered piperidine rings are unlikely to stabilize the derived PNA structures for complex formation. In the present *aepip*PNA analogues the stereochemical dispositions of substituents seem to lead to a favorable pre-organization of PNA backbone for the formation of stable triplexes with DNA. In this context, work on *aepip*PNAs derived from other nucleobases and on the duplex and triplex stability is under progress.

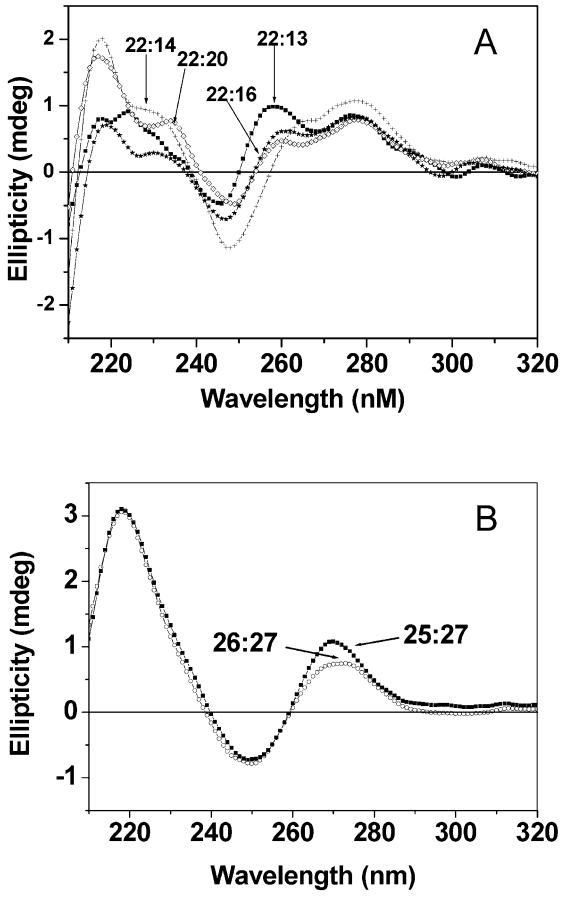


Figure 5. CD spectra of *aeg-aepip*PNA:DNA complexes. A. (2*S*,5*R*) triplexes. B. (2*S*,5*R*) duplexes.

3. Conclusion

In summary, this article reports the design and synthesis of novel six-membered pipecolic acid derived PNA analogues (2*S*,5*R* and 2*R*,5*S*)-1-(*N*-Boc-aminoethyl)-5-(thymin-1-yl)pipecolic acid. The homopyrimidine-*aeg*PNA backbone comprising these units effect stabilization of the resulting triplexes with complementary DNA strands depending upon stereochemistry and position of the modified unit. A single modified 2*S*,5*R* *aepip*PNA unit in the center of a mixed purine-pyrimidine duplex forming oligomer discriminates the parallel versus antiparallel DNA sequence much better than the unmodified *aeg*PNA. The results reported here further expand the repertoire of cyclic PNA analogues to six-membered series and future work is focused on studying the properties of other nucleobases.

4. Experimental

4.1. General

The chemicals used were of laboratory or analytical grade and the solvents used were purified according to the literature procedures.[20] The reactions were monitored by TLC and usual work-up implies sequential washing of the organic extract with water and brine followed by drying over anhydrous sodium sulfate and evaporation under vacuum. Column chromatography was performed for purification of compounds on silica gel (60–120 mesh). TLCs were carried out on pre-coated silica gel GF_{254} aluminium sheets. TLCs were performed using dichloromethane–methanol or petroleum ether–ethyl acetate solvent systems for most compounds. Free acids were chromatographed by TLC using a solvent system of methanol/acetic acid/water in the proportion 9:1:1. The compounds were visualized with UV light and/or by spraying with Ninhydrin reagent subsequent to Boc-deprotection (exposing to HCl vapors) and heating. The DNA oligomers were synthesized on CPG solid support by β-cyanoethyl phosphoramidite chemistry followed by ammonia treatment[21] and their purities checked by HPLC prior to use. *aeg*PNA monomers were synthesized according to literature procedures.[22]

4.1.1. 5-(*S*/*R*)-Hydroxy-*N*-benzyloxycarbonyl-2-(*S*/*R*)-pipecolic acid methyl ester (6). A cooled (0 °C) solution of 5-oxo-*N*-benzyloxycarbonyl-2-(*S*/*R*)-pipecolic acid methyl ester **5** (3.2 g, 10.8 mmol) in methanol (50 mL) was treated with sodium borohydride (0.62 g, 16.3 mmol). After stirring for 2 h, the reaction mixture was concentrated in vacuo and the residue was dissolved in ethyl acetate (100 mL). The organic solution was washed with 10% ammonium chloride solution, brine, dried over sodium sulfate and concentrated. The resulting residue was chromatographed to give colourless oil **6** (3.0 g, 93%).[10]

4.1.2. 5-(*S*)-Hydroxy-*N*-benzyloxycarbonyl-2-(*S*)-pipecolic acid methyl ester. ^{1}H NMR ($CDCl_3$) δ: 7.35 (s, 5H), 5.14 (s, 2H), 4.90 and 4.78 (br d, $J=4.4$ Hz, 1H), 4.30–4.10 (m, 1H), 3.73–3.55 (m, 4H), 2.89–2.72 (m, 1H), 2.35–2.28 (m, 1H) 2.00–1.66 (m, 3H), 1.35–1.15 (m, 1H): $[\alpha]_{589}^{25} = -17.8$ (*c* 0.9, CH_3OH) (lit.[14] not reported).

4.1.3. 5-(*R*)-Hydroxy-*N*-benzyloxycarbonyl-2-(*R*)-pipecolic acid methyl ester. ^{1}H NMR ($CDCl_3$) δ: 7.35 (s, 5H), 5.15 (s, 2H), 4.89–4.77 (m, 1H), 4.27–4.21 (m, 1H), 3.73–3.65 (m, 4H), 2.85–2.71 (m, 1H), 2.48–2.26 (m, 2H) 1.98–1.73 (m, 2H): $[\alpha]_{D\ 589}^{25} = +15.38$ (*c* 0.26, CH_3OH).

4.1.4. 5-(*S*)-Hydroxy-2-(*S*)-pipecolic acid methyl ester (7). The N-deprotection of methyl ester **6** (2.20 g, 7.8 mmol) was done under hydrogenation over Pd–C (10%) to obtain **7** (1.10 g, 92%). ^{1}H NMR ($CDCl_3$) δ: 3.83–3.81 (m, 1H), 3.74 (s, 3H), 3.40–3.33 (t, $J=6.6$ Hz, 1H), 3.08–3.00 (dd, $J=2.4$, 12 Hz, 1H) 2.88–2.80 (dd, $J=2$, 12.2 Hz, 1H), 1.90–1.83 (m, 3H), 1.74–1.61 (m, 1H).

4.1.5. 1-(*N*-Boc-aminoethyl)-5-(*S*/*R*)-hydroxy-2-(*S*/*R*)-pipecolic acid methyl ester (8). To a cooled solution of 5-(*S*/*R*)-hydroxy-2-(*S*/*R*)-pipecolic acid methyl ester **7** (1.0 g, 6.3 mmol), DIPEA (2.7 mL, 15.7 mmol), DMAP (0.15 g, 1.3 mmol) in dry DMF/acetonitrile (1:1) (10 mL) was added with stirring for 15 min. *N*-Boc aminoethyl mesylate (1.50 g, 6.3 mmol) in DMF (3 mL) was then added and the reaction mixture was heated to 50 °C for 20 h. Evaporation of the solvent followed by column chromatography gave thick brown oil of **8** (0.7 g, 37%).

4.1.6. 1-(*N*-Boc-aminoethyl)-5-(*S*)-hydroxy-2-(*S*)-pipecolic acid methyl ester. ^{1}H NMR ($CDCl_3$) δ: 5.18 (br s, 1H), 3.93–3.82 (m, 1H), 3.73 (s, 3H), 3.54–3.50 (m, 3H), 3.05–2.99 (m, 1H) 2.75–2.50 (m, 3H), 1.90–1.64 (m, 4H), 1.45 (s, 9H): ^{13}C NMR ($CDCl_3$) δ: 175.1, 161.2, 79.2, 64.9, 62.8, 55.3, 55.1, 51.8, 40.6, 37.2, 29.1, 28.3: $[\alpha]_{D\ 589}^{25} = -10.7$ (*c* 0.56, CH_3OH). MS: (ESI) M_{calc}: 302.37, M_{obs}: 302.

4.1.7. 1-(*N*-Boc-aminoethyl)-5-(*R*)-hydroxy-2-(*R*)-pipecolic acid methyl ester. ^{1}H NMR ($CDCl_3$) δ: 5.34 (br s, 1H), 3.96–3.93 (m, 1H), 3.75 (s, 3H), 3.41–3.25 (m, 3H), 3.16–3.13 (m, 1H) 2.69–2.63 (m, 3H), 1.96–1.66 (m, 4H), 1.45 (s, 9H): ^{13}C NMR ($CDCl_3$) δ: 173.47, 156.26, 79.13, 65.67, 64.90, 55.69, 54.98, 51.55, 40.60, 37.70, and 28.43: $[\alpha]_{D\ 589}^{25} = +11.9$ (*c* 0.42, CH_3OH). MS: (ESI) M_{calc}: 302.37, M_{obs}: 302.

4.1.8. 1-(*N*-Boc-aminoethyl)-5-(*R*/*S*)-(*N*3-benzoylthymin-1-yl)-2-(*S*/*R*)-pipecolic acid methyl ester (9). To a stirred solution of 1-(*N*-Boc-aminoethyl)-5-(*S*/*R*)-hydroxy-2-(*S*/*R*)-pipecolic acid methyl ester **8** (0.55 g, 1.8 mmol), *N*3-benzoylthymine (0.84 g, 3.6 mmol) and triphenyl phosphine (0.95 g, 3.6 mmol) in dry THF (10 mL) at 0 °C, was added dropwise diethylazodicarboxylate (DIAD, 0.47 mL, 3.6 mmol). After completion of the reaction as indicated by TLC (24 h), the solvent was removed in vacuo and residue purified by silica gel column chromatography to get the pure product **9** (0.3 g, 32% yield).

4.1.9. 1-(*N*-Boc-aminoethyl)-5-(*R*)-(*N*3-benzoylthymin-1-yl)-2-(*S*)-pipecolic acid methyl ester. ^{1}H NMR ($CDCl_3$) δ: 7.92 (s, 1H), 7.88 (s, 1H), 7.62–7.44 (m, 4H) 5.28 (br s, 1H), 3.74 (s, 1H), 3.72 (s, 3H), 3.48–3.22 (m, 3H), 2.94–2.69 (m, 3H) 2.14–2.00 (m, 1H), 2.00 (s, 3H), 2.00–1.63 (m, 4H), 1.42 (s, 9H): ^{13}C NMR ($CDCl_3$) δ: 175.3, 169.0, 163.2, 156.0, 150.3, 142.5, 138.8, 131.6, 130.3, 129.1, 109.5, 79.5, 66.6, 64.7, 56.0, 52.2, 51.2, 39.7, 29.4, 28.4, 27.7, 12.2: $[\alpha]_{D\ 589}^{25} = +10.0$ (*c* 0.5, CH_3OH). MS: (ESI) M_{calc}: 514.58, M_{obs}: 514.

4.1.10. 1-(*N*-Boc-aminoethyl)-5-(*S*)-(*N*3-benzoylthymin-1-yl)-2-(*R*)-pipecolic acid methyl ester. ^{1}H NMR ($CDCl_3$) δ: 7.90–7.89 (m, 2H), 7.68–7.45 (m, 4H) 5.24 (br s, 1*H*), 3.75 (s, 1H), 3.72 (s, 3H), 3.46–3.22 (m, 3H), 3.00–2.68 (m, 3H) 2.16–2.00 (m, 1H), 2.00 (s, 3H), 1.96–1.67 (m, 4H), 1.43 (s, 9H): ^{13}C NMR ($CDCl_3$) δ: 175.55, 169.36, 163.50, 156.27, 150.57, 142.84, 135.13, 131.91, 130.62, 129.39, 109.76, 79.82, 66.87, 64.97, 56.26, 52.50, 51.51, 39.99, 29.67, 28.66, 28.02, 12.53: $[\alpha]_{D\ 589}^{25} = -12.0$ (*c* 0.25, CH_3OH). MS: (ESI) M_{calc}: 514.58, M_{obs}: 515.

4.1.11. 1-(*N*-Boc-aminoethyl)-5-(*R*/*S*)-(thymin-1-yl)-2-(*S*/*R*)-pipecolic acid (10). To a solution of the methyl ester **9** (0.36 g, 0.7 mmol) in methanol (2 mL), was added aqueous 2 M NaOH (2 mL). The reaction mixture was further stirred overnight followed by neutralization with Dowex-50 H^+ resin, which was then filtered off. The filtrate was concentrated under vacuum and the residue was taken up in water. This was washed with ethyl acetate before concentrating it to dryness to obtain the product **10** (0.25 g, 95%) as white solid foam.

4.1.12. 1-(*N*-Boc-aminoethyl)-5-(*R*)-(thymin-1-yl)-2-(*S*)-pipecolic acid. ^{1}H NMR (D_2O) δ: 7.50 (s, 1H) 4.52–4.44 (m, 1H), 4.21–3.94 (m, 3H) 3.50–3.45 (m, 4H), 2.34–2.24 (m, 4H), 1.89 (s, 3H), 1.45 (s, 9H): $[\alpha]_{D\ 589}^{25} = +32.0$ (*c* 0.1, CH_3OH). MS: (ESI) M_{calc}: 396.45, M_{obs}: 396.

4.1.13. 1-(*N*-Boc-aminoethyl)-5-(*S*)-(thymin-1-yl)-2-(*R*)-pipecolic acid. ^{1}H NMR (D_2O) δ: 7.55 (1H) 4.55–4.48 (m, 1H), 4.26–3.94 (m, 3H) 3.54–3.42 (m, 4H), 2.50–2.32 (m, 3H), 1.81–1.72 (m, 1H), 1.96 (s, 3H), 1.51 (s, 9H): $[\alpha]_{D\ 589}^{25} = -34.0$ (*c* 0.1, CH_3OH). MS: (ESI) M_{calc}: 396.45, M_{obs}: 398.

4.2. Solid phase synthesis of the PNA oligomers on the solid support

The PNA oligomers were synthesized manually by solid phase peptide synthesis using the Boc-protection strategy and employing diisopropylcarbodiimide (DIPCDI) or *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′ tetramethyl-uronium hexafluorophosphate (HBTU) and 1-hydroxy-benzotriazole (HOBt) as the coupling agents. The solid support used was Merrifield resin derivatized with β-alanine (0.17 mequiv/g resin). The synthesis involved repetitive cycles, each comprising (i) deprotection of the N-protecting Boc-group using 50% trifluoroacetic acid (TFA) in CH_2Cl_2, (ii) neutralization of the TFA salt formed with DIPEA (5% solution in CH_2Cl_2, v/v) and (iii) coupling of the free amine with the free carboxylic acid group of the incoming monomer (4 equiv) in the presence of DIPCDI and HOBt, in DMF or NMP as the solvent. The deprotection of the *N*-Boc protecting group and the coupling reaction were monitored by Kaiser's test.[23] The coupling efficiencies were found to be >98%.

The PNA oligomers were cleaved from the solid support using TFA-TFMSA to yield oligomers with free carboxy terminus.[10] The resin-bound PNA oligomer (10 mg) was

stirred in an ice-bath with thioanisole (20 μL) and 1,2-ethanedithiol (8 μL) for 10 min. TFA (120 μL) was then added and the stirring was continued for another 10 min followed by TFMSA (16 μL) while cooling in an ice-bath and stirring for 2 h. The reaction mixture was filtered through a sintered funnel, the residue washed with TFA (3×2 mL) and the combined filtrate and washings were evaporated under vacuum. The residual pellet was re-dissolved in methanol (~0.1 mL) and re-precipitated by adding ether to obtain the crude PNA oligomer. This was desalted by gel filtration over Sephadex G25 and the purity of the PNA oligomer as checked by RP HPLC on a C18 column was found to be >90%.

4.3. UV-melting

The concentration of the PNA oligomers was calculated on the basis of the absorption at 260 nm, assuming the molar extinction coefficients of the nucleobases to be as in DNA, T, 8.8 cm^2/μmol; C, 7.3 cm^2/μmol; G, 11.7 cm^2/μmol and A, 15.4 cm^2/μmol. The PNA oligomers (**13–21**) and the appropriate complementary DNA oligonucleotide (**22/23/24**) were mixed together in a 2:1 molar ratio, while PNA oligomers (**25–26**) and the appropriate complementary DNA oligonucleotide (**27/28**) were mixed together in a 1:1 molar ratio in 0.01 M sodium phosphate buffer, pH 7.3 to get a final strand concentration of 1.5 and 2 μM, respectively The samples were annealed by heating at 85 °C for 1–2 min, followed by slow cooling to room temperature, kept at room temperature for ~30 min and then, refrigerated overnight. The samples were heated at a rate of 0.2 or 0.5 °C rise per minute and the absorbance at 260 nm was recorded at every minute. The percent hyperchromicity at 260 nm was plotted as a function of temperature and the melting temperature was deduced from the peak in the first derivative plots.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tet.2004.07.080

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