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Aminoethylprolyl Peptide Nucleic Acids (aepPNA): Chiral PNA Analogues That Form Highly Stable DNA:aepPNA$_2$ Triplexes

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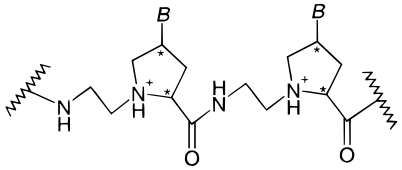
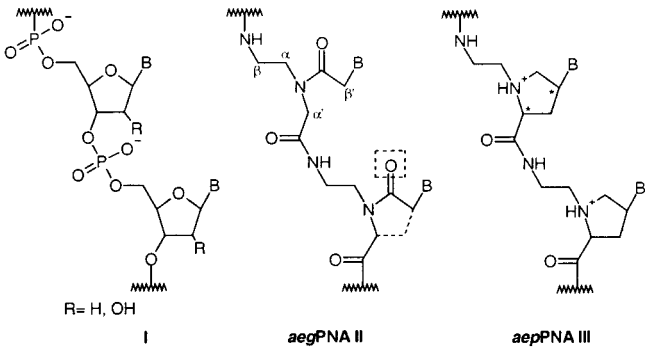
ABSTRACT



The replacement of the glycyl component in the peptide nucleic acid (PNA) backbone by a prolyl unit bearing a nucleobase leads to the aminoethylprolyl (aep) PNAs, which are chiral and cationic. The homooligomeric aepPNA binds to complementary DNA sequences with high affinity and sequence specificity, forming highly stable triplexes.

One of the most prominent outcomes of the search for DNA/RNA (I) analogues as effective antigene/antisense agents is the emergence of peptide nucleic acids (PNAs, II) in which



the charged sugar−phosphate backbone of DNA is replaced by a neutral and achiral polyamide backbone consisting of *N*-(2-aminoethyl)glycine units.[1] In PNAs, the nucleobases are attached to the backbone through a conformationally 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.[1d,3] Attempts to resolve the orientational ambiguity focus on introduction of chirality to PNA by linking chiral amino acids,[4] peptides,[5] and oligonucle-

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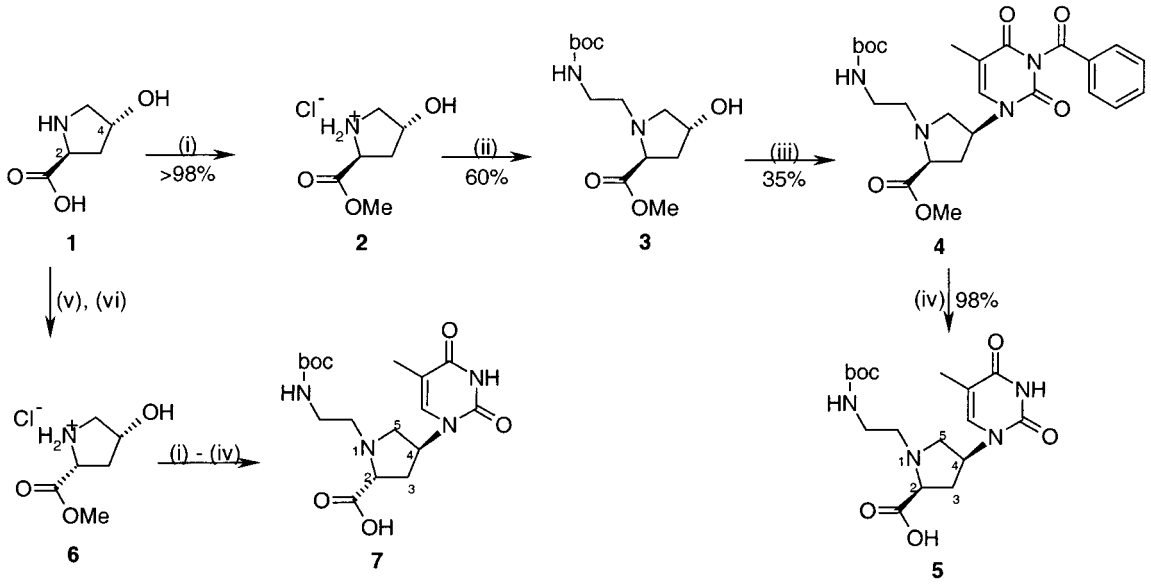
Scheme 1



(i)$SOCl_2$ (1.1eq.),CH_3OH (ii) Boc.NH$(CH_2)_2$Br (1.5eq.), CH_3CN (iii) N3-Bz-Thymine (1 eq.), DIAD (1.2eq.), PPh_3 (1.2eq.), THF (iv)1N NaOH (CH_3OH:H_2O, 1:1), 8h, r.t. (v) Ac_2O, AcOH (vi) HCl

otides[6] to the PNA or by using chiral amino acids in the backbone itself.[7] Bridging the β-C of the ethylenediamine moiety of PNAs and the glycyl α'-carbon with a methylene group generates a five-membered pyrrolidine ring which imparts structural rigidity in the PNA backbone with simultaneous introduction of chirality.[8] This has marginally improved the affinity and selectivity in DNA:PNA_2 binding in a desired manner. Conjugation of spermine to the C-terminus rendered the PNA cationic and improved its aqueous solubility.[9] Linking of the nucleobase to the polyamide backbone via a flexible ethylene linker instead of an acetamide linker creates a positive charge in the PNA backbone at the expense of the conformational rigidity of the tertiary amide linkage.[10] Although this improved the aqueous solubility of the PNA, the detrimental effect on the stability of DNA:PNA_2 complexes stressed the importance of rigid preorganization of the PNA structure for effective binding to ss/ds DNA.

In this Letter, we introduce the new chiral aminoethylprolyl (aep) PNA backbone (III), designed by linking the glycyl α'-carbon of the PNA with the acetamido β'-carbon via a methylene bridge. This modification is aimed at tuning the rigidity and flexibility of the PNA backbone, with concomitant introduction of a positive charge on the tertiary nitrogen of proline. This has potential conformational advantages over a similar modification reported earlier,[11] which has the nitrogen of the pyrrolidine ring in the backbone in the tertiary amide form and, hence, is structurally too rigid. The nucleobase in III is directly attached to the pyrrolidine ring at the C4 position, without changing the net number of atoms connecting two successive nucleobases. Further, the two chiral centers at C2 and C4 offer an opportunity to study the stereochemical implications on the interactions of chiral PNA with ss/ds DNA/RNA. The synthesis of two of the four possible stereoisomers, (2*S*/*R*,4*S*)-1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline, and their site-specific incorporation into various PNA oligomers by solid-phase synthesis and hybridization properties with complementary DNA is reported.

Chemical Syntheses of aepPNA Monomer and Oligomers. The syntheses of the (2*S*,4*S*) and (2*R*,4*S*) isomers of 1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline were achieved in four steps starting from the naturally occurring (2*S*,4*R*)-4-hydroxyproline (**1**) as indicated in Scheme 1. The N-alkylation of the pyrrolidine ring in *trans*-4-hydroxy-L-proline methyl ester **2** by *N*-Boc-aminoethyl bromide afforded the 1-(*N*-Boc-aminoethyl)proline derivative **3**. The replacement of the C4 hydroxyl function with N3-benzoylthymine under Mitsunobu reaction conditions[12] then yielded the (2*S*,4*S*)-4-(N3-benzoylthymin-1-yl)proline derivative **4** with inverted stereochemistry at C4. The hydrolysis of the methyl ester and N3-benzoyl protecting group of thymine was achieved in a single step, by employing sodium hydroxide (1 N) in aqueous methanol to provide (2*S*,4*S*)-1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline (**5**) as the desired monomer. Starting from (2*R*,4*S*)-4-hydroxyproline **6**[8a] and by following the same

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steps (i to iv), the (2*R*,4*S*)-1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline isomer (**7**) was prepared as shown in Scheme 1. The structural integrity of the aepPNA monomers **5** and **7** was confirmed by spectral data (NMR and mass spectroscopy).

PNA oligomers containing the (2*S*/*R*,4*S*)-1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline units were assembled by solid-phase peptide synthesis on Merrifield resin derivatized with *N*-Boc-β-alanine (0.29 mequiv/g of resin). The aepPNA unit was incorporated into a PNA octamer, H-T_8-$NHCH_2$-CH_2COOH at predetermined positions to yield the aepPNAs **8**−**11**. The unmodified aegPNA sequence H-T_8-$NHCH_2CH_2$-COOH **12** was also synthesized for control studies. The oligomers were cleaved from the solid support by treatment with trifluoroacetic acid−trifluoromethanesulfonic acid[14] to yield the corresponding sequences carrying β-alanine at their carboxy termini. These were purified by FPLC on a PepRPC column. No precipitation was observed in samples of aepPNA even after prolonged storage. The purity of the oligomers was rechecked by HPLC on a RPC-18 column and characterized by MALDI-TOF mass spectrometry (PNA **8**: M_{obsd} = 2218; M_{calcd} = 2217.17. PNA **9**: M_{obsd} = 2219; M_{calcd} = 2215.19. PNA **10**: M_{obsd} = 2212; M_{calcd} = 2212). The DNA oligomers **13** and **14** were synthesized on a Pharmacia Gene Assembler Plus synthesizer by employing standard phosphoramidite chemistry,[15] followed by ammonia deprotection. These were purified by gel filtration and their purities checked by HPLC.

PNA sequences

8	H-	T	T	T	T	T	T	T	t	-(β-Ala)-OH
9	H-	T	T	T	t	T	T	T	t	-(β-Ala)-OH
10	H-	T	t	T	t	T	t	T	t	-(β-Ala)-OH
11	H-	t	t	t	t	t	t	t	t	-(β-Ala)-OH
12	H-	T	T	T	T	T	T	T	T	-(β-Ala)-OH

T = *aeg*PNA-T, t = *aep*PNA -T

DNA sequences

13	5'-	G	C	A	A	A	A	A	A	A	A	C	G	-3'
14	5'-	G	C	A	A	A	T	A	A	A	A	C	G	-3'

The PNA sequences used here are homopyrimidines that are known to form DNA:PNA_2 triplexes.[4] UV mixing and titration experiments indicated a 1:2 binding stoichiometry (DNA:aepPNA$_2$) for PNA oligomers of both alternating aep-aeg units and homooligomers of aep units, as in the control aegPNA. Hence, all complementation studies were performed with 1:2 stoichiometry of DNA and aeg/aepPNA. The percent hyperchromicity−temperature plots derived from the UV melting data indicated a single transition (Figure 1A), characteristic of DNA:PNA_2 triplexes, in which both PNA strands dissociate from DNA simultaneously, in a single step.

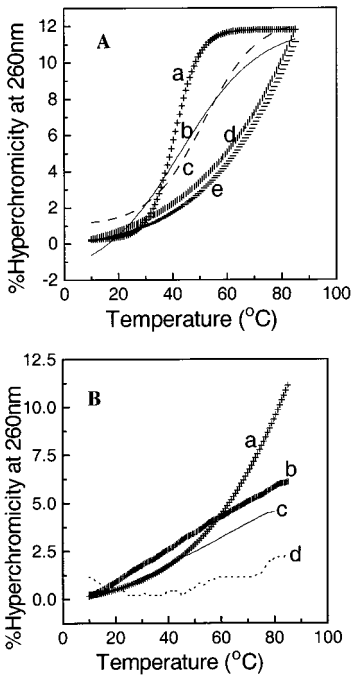

Figure 1. Melting profiles of DNA:PNA_2 complexes. **A**: for the 2*S* modification, (a) **13**:**12**, (b) **13**:**8**, (c) **13**:**9**, (d) **13**:**10**, (e) **13**:**11**. **B**: (a) **13**:**11**, (b) **14**:**11,** and for single strands, (c) **14**, (d) **11**.

The presence of a single 2*S*,4*S*/2*R*,4*S* aep unit at the C-terminus of the oligomer **8** has no detrimental effect on the stability of its complex with the complementary DNA (Table 1, entries 1 and 7). Increasing the number of aep units in the oligomer (two in the aep-aeg oligomer **9**, four in the alternating aep-aeg oligomer **10**, and eight in the aep homooligomer **11**) leads to a progressive increase in the stability of complexes with DNA (ΔT_m = +5−8 °C per aep unit, Table 1, entries 2−4), with incomplete melting for oligomers **10** and **11** even beyond 75 °C. When a single T−T mismatch is present in the middle of the sequence as in **14**:**10** and **14**:**11**, the DNA:PNA_2 complexes failed to show sigmoidal transitions in UV−melting curves, resulting in only a small linear increase in percent hyperchromicity (entries 5 and 6 and Figure 1B). Thus aepPNA units enormously stabilize DNA:PNA_2 triplexes but still retain the stringency

Table 1. UV−T_m (°C) of DNA:PNA_2 Complexes

Entry	DNA:PNA_2	2'*S*	2'*R*
1	**13:8**	44	42 (38)
2	**13:9**	51	54
3	**13:10**	>75	>75 (67)
4	**13:11**	>80	>80 (>74)
5	**14:10**	nd	nd
6	**14:11**	nd	nd
7	**13:12**	43 (38)	

*nd: not detected. Buffer: 10mM sodium phosphate, pH 7.4. Values in brackets: T_m(°C) with 100 mM NaCl. 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.

(13) (a) (2*S*,4*S*)-1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline **5**: ^{1}H NMR (D_2O) δ 7.4 (s, 1H), 4.1 (br m, 2H), 3.6 (br m, 4H), 3.4 (br m, 1H), 3.1 (br m, 2H), 2.4 (m, 1H), 1.8 (s, 3H), 1.4 (s, 9H). *m*/*e* = 382. $[\alpha]^{25}_D$ −34.5° (*c* = 0.12, CH_3OH). (b) (2R,4S)-1-(*N*-Boc-aminoethyl)-4-(thymin-1-yl)proline **7**: ^{1}H NMR (D_2O) δ 7.4 (s, 1H), 4.4 (t, 1H), 4.2 (m, 1H), 3.7−3.3 (br m, 6H), 2.9 (m, 1H), 2.6 (m, 1H), 1.9 (s, 3H), 1.4 (s, 9H). *m*/*e* = 382. $[\alpha]^{25}_D$ +43.6° (*c* = 0.12, CH_3OH).

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in terms of base mismatches. PNA single strands, when subjected to the same temperature program as the DNA:PNA_2 complexes, exhibited <3% change in absorbance (Figure 1B), thus ruling out any contribution from single-stranded ordering to the sigmoidal transition of the DNA:PNA_2 triplex. The tight binding of aepPNAs with complementary DNA is also seen in diagnostic gel mobility shift experiments, where even a single modification effected significant retardation.

To study the effect of pH on the stability of the DNA:$aepPNA_2$ complexes, the hybrids were constituted in 10 mM sodium phosphate buffers of different pHs in the range 6.0−7.4. No change in T_m ($\Delta T_m = \pm 1°$) was detected in the above pH range. DNA:PNA_2 complexes are known to be destabilized in the presence of salts, and the stability of complexes of DNA with positively charged ligands is strongly salt-dependent.[16] The presence of salt (100 mM) in the medium destabilized the higher substituted DNA:$aepPNA_2$ complexes **13**:**10** and **13**:**11** to a greater extent ($\Delta T_m \geq 8°$), compared to the complexes of control aegPNA **13**:**12** or monosubstituted aepPNA **13**:**8** ($\Delta T_m = 4-5°$). The higher salt dependence of T_m of DNA complexes with aepPNAs, both in alternating and homooligomers, is perhaps a consequence of the protonated proline ring N in aepPNA units, leading to electrostatic destabilization under increased ionic strengths. The pH−titration curve of **4** indicated a $pK_a \approx 6.5$ for the pyrrolidine nitrogen, substantiating its protonation status even at neutral pH. The large stabilization observed for the alternating aep-aeg oligomer (ΔT_m per aep unit $\approx +7-8°$; entry 3) is at least partly due to electrostatic interaction between the positively charged backbone of aepPNA and the negatively charged sugar−phosphate DNA backbone, and this is further amplified in aepPNA homooligomers, which are polycationic. The main cause of DNA:aepPNA recognition still appears to be the specific hydrogen bonding between the complementary nucleobases of PNA (aep/aeg) and DNA, since even a single mismatch in the middle of the sequence was found to inhibit DNA:aepPNA complexation, as is the case with control aegPNA. Both isomers studied here have the same spatial disposition of the attachment of the nucleobase to the proline ring at C4 equivalent to the nucleobase attachment at C1′ in DNA. Changing the stereochemistry at the other center, i.e., C2, located in the PNA backbone does not appreciably affect the stability of DNA:$aepPNA_2$ complexes. The structural changes caused by the difference in stereochemistry at C2 are probably accommodated due to the flexibility imparted to the backbone by the aminoethyl moiety linking the proline ring. Such flexibility is lost when the proline nitrogen is part of an amide moiety.[11] Molecular modeling studies are in progress to further examine these effects.

In summary, we have shown that new peptide nucleic acid analogues aepPNAs offer one of the best tunings between rigidity and flexibility of a PNA backbone with positive charge and show high sequence specific binding to target DNA sequences. The controlled flexibility in the backbone accommodates both 2(*S*/*R*) stereocenters with equal efficiency. The introduction of a positive charge in PNA also enhances its solubility in aqueous media. The synthesis of mixed oligo aepPNA sequences is currently underway to explore the potential for orientational selectivity of binding and efficient DNA duplex strand invasion by these aepPNA oligomers similar to that seen recently with other cationic PNAs.[17]

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Supporting Information Available: UV titration and mixing profiles indicating PNA−DNA binding stoichiometry, ^1H and ^{13}C NMR data for selected compounds **3**−**7**, MALDI-TOF mass spectrum of aepPNA oligomer **8**−**10**, and gel mobility shift data. This material is available free of charge via the Internet at http://pubs.acs.org.

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