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Synthesis of 4(*S*)-(*N*-Boc-amino)-2(*S*/*R*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-acetic acid: a novel cyclic PNA with constrained flexibility

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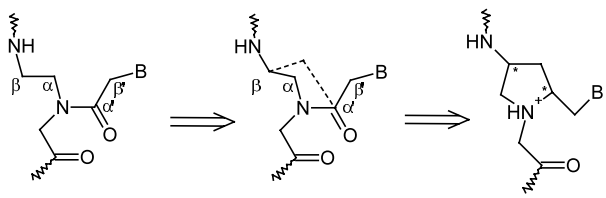
Abstract—Pyrrolidyl PNA has emerged as a result of our efforts to achieve optimum fine-tuning of the aminoethylglycyl PNA structure in binding to complementary nucleic acids. The two chiral centers in each unit can give rise to four diastereoisomers, leading to a library of PNA monomers and oligomers. The pyrrolidyl ring nitrogen atom is partially charged at physiological pH, leading to cationic PNA that also results in increased water-solubility. The constraint introduced by the pyrrolidyl ring could lead to pre-organization of the PNA structure and thus pose entropic advantages in binding to complementary nucleic acids. We report herein the synthesis of 4(*S*)-(*N*-Boc-amino)-2(*S*/*R*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-acetic acids, their site-specific incorporation into PNA oligomers and their preliminary DNA binding properties. © 2002 Published by Elsevier Science Ltd.

The very favorable binding affinity and specificity of aminoethylglycyl (*aeg*PNA, **I**)[1] for complementary DNA/RNA has sparked off the quest for *aeg*PNA analogues that would further improve their applicability by enhancing water-solubility and permeability across cell membranes.[2] Our own efforts in this direction have resulted in de novo syntheses of chiral and positively charged PNA derivatives based on the five-membered pyrrolidine ring system.[3] The pyrrolidine nucleus with two chiral centers can give rise to four diastereomers and various combinations of C2/C4 substituents, leading to a library of modified PNA monomers and oligomers.

The direct attachment of the nucleobase to the pyrrolidine ring in aminoethylprolyl (*aep*) PNA[4] and pyrrolidine PNA[5] was found to give rise to nucleobase-dependent DNA/RNA binding properties. Further, the preferences for parallel/antiparallel directionality of binding was also found to be controlled by the nucleobase.[4b] These attributes, contrary to natural DNA, are perhaps an outcome of differential pyrrolidine ring puckers (effect of substituents, charge) and/or *syn–anti* conformations adopted by the nucleobases directly attached to the pyrrolidine rings. The role of the backbone stereochemistry of the pyrrolidine rings is not yet well understood and much work is needed to decipher the effect of chiral, charged PNA backbones to achieve optimum tuning of their DNA binding properties.

Herein, we propose to introduce constraint in PNA by introducing a methylene bridge between the α′-carbon atom of the nucleobase linker and the β-carbon atom of the aminoethyl segment of aminoethylglycyl (*aeg*) PNA. This compensates the restricted rotation of the tertiary amide linker in *aeg*PNA and introduces a positive charge in the backbone. Such a ring-constraint in the backbone could lead to pre-organization of the PNA structure and impose entropic advantages for binding complementary nucleic acids. The flexibility of the nucleobase attachment as in *aeg*PNA is retained since the primary carbon atom employed for nucleobase attachment is not a part of the pyrrolidine ring. The constraint instituted in this way creates two stereocenters per PNA unit, causing multiple chirality in oligomers. Such modulation of the backbone stereochemistry through these centers could lead to structures with inherent conformational preferences, independent of the individual nucleobases.



Keywords: pyrrolidyl PNA; conformational preorganization; chiral cationic PNA.

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We report herein the syntheses of new chiral PNA monomers, 4(*S*)-(*N*-Boc-amino)-2(*S*/*R*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-acetic acids, their site-specific incorporation into PNA oligomers and the interesting preliminary results of their DNA binding.

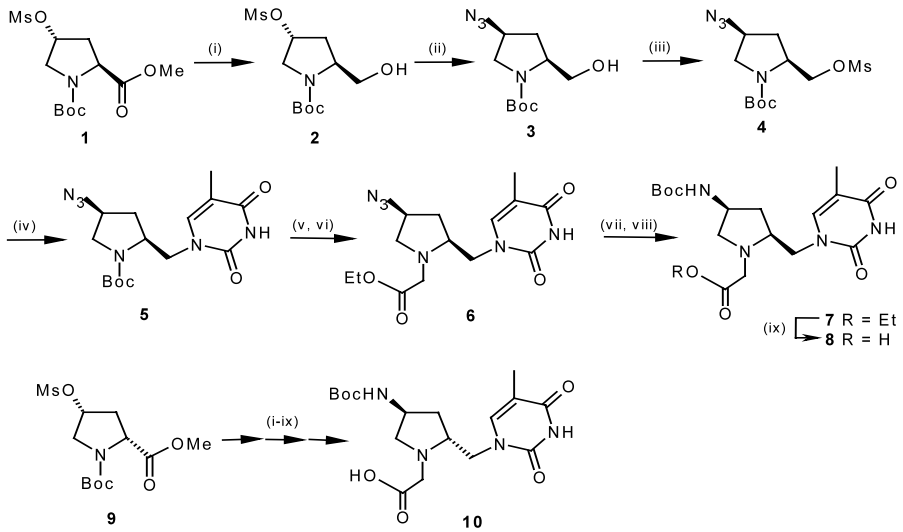
Chemical synthesis of pyrrolidyl PNA monomers and oligomers

The (2*S*,4*S*) monomer **8** was synthesized from the naturally occurring 4(*R*)-hydroxy-2(*S*)-proline by following the steps indicated in Scheme 1. The ester function in protected 4(*R*)-*O*-mesyl-2(*S*)-proline **1** was reduced to give the prolinol **2** by in situ generated lithium borohydride using an equimolar mixture of sodium borohydride and lithium chloride.[6] S_N2 displacement of the 4(*R*)-*O*-mesyl group by treatment with sodium azide yielded the 4(*S*)-azide **3**. The primary hydroxy function in **3** was mesylated by treatment with mesyl chloride in pyridine to give **4**. *N*1-Alkylation of thymine was effected by reaction with **4** in the presence of K_2CO_3 and 18-crown-6 in DMF to give the *N*1-thyminylmethyl derivative **5**. The pyrrolidine ring nitrogen in **5** was deprotected prior to alkylation with ethyl bromoacetate in the presence of K_2CO_3 to yield **6**. The azido function in **6** was then reduced by catalytic hydrogenation and the resultant primary amine was subsequently protected as its *t*-butyl carbamate **7**. The ethyl ester **7** was hydrolyzed using sodium hydroxide in aqueous methanol to give the free acid **8**, the monomer block for building the oligomers. The 4(*S*)-(*N*-Boc-amino)-2(*R*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-acetic acid **10** was prepared from 1-(*N*-Boc)-4(*R*)-*O*-mesyl-2(*R*)-proline methyl ester[7,3] **9** following the same steps as for the 2(*S*) isomer. The structural integrity of the pyrrolidyl PNA monomers **8** and **10** was confirmed by NMR and mass spectroscopy.[8]

To evaluate the effect of these PNA monomers on the structural perturbations in oligomeric PNA, the two monomers were individually incorporated into *aeg*PNA sequences that were assembled on Merrifield resin derivatized with *N*-Boc-β-alanine (0.29 mequiv./g resin) by solid-phase synthesis. The pyrrolidyl PNA unit was introduced into a PNA octamer, H-T_8-$NHCH_2$-CH_2COOH to yield the pyrrolidyl PNAs **11**.

The *aeg*PNA sequence H-T_8-(β-alanine)-COOH **12** was synthesized for comparative studies. The oligomers were cleaved from the support using trifluoroacetic acid–trifluoromethanesulphonic acid[9] to yield the PNA oligomers carrying β-alanine at their carboxy termini. These were purified by FPLC on a C8-PepRPC column.† The purity of the oligomers was rechecked by HPLC on an RPC-C18 column and characterized by MALDI-TOF mass spectrometry.

The DNA oligomers **13** and **14** were synthesized on a Pharmacia Gene Assembler Plus synthesizer by employing standard phosphoramidite chemistry[10] followed by ammonia deprotection. These were purified by gel filtration and their purities determined to be ≥98% by HPLC.



Scheme 1. Synthesis of 4(*S*)-(*N*-Boc-amino)-2(*S*/*R*)-(thymin-1-ylmethyl)pyrrolidine-*N*-1-acetic acid monomers. (i) LiCl:$NaBH_4$ 1:1 THF:EtOH 3:4, 98%; (ii) NaN_3, DMF, 98%; (iii) MsCl, pyridine, 45%; (iv) thymine, K_2CO_3, 18-crown-6, DMF, 75°C, 45%; (v) 50% TFA/CH_2Cl_2; (vi) $BrCH_2COOEt$, K_2CO_3, CH_3CN, 70%; (vii) H_2, Pd–C, MeOH; (viii) Boc-N_3, dioxane, H_2O, 62%; (ix) 1N NaOH.

† No precipitation was observed in samples of pyrrolidyl PNAs even after prolonged storage, indicative of their superior water-solubility in comparison to *aeg*PNA. The pK_a of the pyrrolidine ring nitrogen atom in the monomeric unit was determined to be ≈6.8, indicating that it is at least partially protonated at physiological pH. This cationic nature was probably the reason for the better water-solubility of the oligomers.

PNA sequences
11 H-T T T **t** T T T T-(β-Ala)-COOH
12 H-T T T T T T T T-(β-Ala)-COOH
DNA sequences
13 5′-G C A A A A A A A A C G-3′
14 5′-G C A A A A T A A A A C G-3′
t = pyrrolidyl unit 2*S* (**8**); **t** = 2*R* (**10**)

The homopyrimidine PNA sequences used are well-known to form PNA_2:DNA triplexes[11] as confirmed by UV titration experiments. Hence, all complementation studies were performed using a 2:1 stoichiometry of PNA:DNA. The plots of the percent hyperchromicity versus temperature were sigmoidal, with a single transition, characteristic of PNA_2:DNA triplexes, where both the PNA strands dissociate from DNA simultaneously in a single step.[11] The T_m values were determined from the peaks in the first derivative plots (Fig. 1). From the UV-T_m results presented in Table 1, it can be seen that the T_m of 43°C for the control *aeg*PNA_2:DNA complex (**12**:**13**) is shifted to 59°C ($\Delta T_m = +16$°C) for PNA **11** containing the (2*R*,4*S*) pyrrolidyl unit. The triplex melting temperature is much lower ($\Delta T_m = -14$°C) for PNA oligomer **11** containing the (2*S*,4*S*) pyrrolidyl unit. The DNA **14** having a single mismatch shows no co-operative transition when complexed with (2*S*,4*S*) pyrrolidine PNA or with *aeg*PNA **12**. However, the (2*R*,4*S*) pyrrolidine PNA **11** complexes with mismatched DNA and shows a destabilization of ~20°C as compared to the fully matched sequence. While *aeg*PNA:DNA hybrids exhibited a sharp transition, the transitions of DNA hybrids from modified PNA were relatively broad for reasons not yet understood. Normally, modifications in the middle of the *aeg*PNA sequences destabilize the complex and it is significant that stabilization is seen in the present case for the (2*R*,4*S*) stereochemistry.

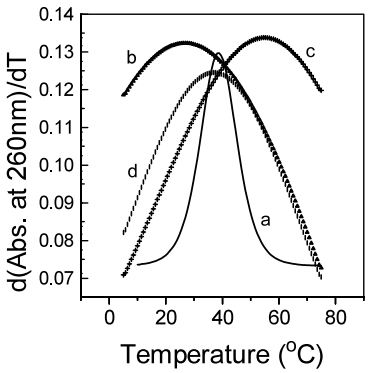


Figure 1. First derivatives of the percent hyperchromicity versus temperature plots of the PNA:DNA complexes **12**:**13** (**a**), **11** (2*S*,4*S*):**13** (**b**), **11** (2*R*,4*S*):**13** (**c**) and the mismatched complex **11** (2*R*,4*S*):**14** (**d**).

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

	PNA **11** H-T_3 **t** T_4-(β-Ala)-COOH		PNA **12** H-T_8-(β-Ala)-COOH
	(2*S*,4*S*)	(2*R*,4*S*)	Control
DNA **13**	28.4	59	43.0
DNA **14**	–	39	–

It is interesting to note from the T_m data that when the stereochemistry of the backbone containing the ethylenediamine unit is identical, the thermal stability is determined by the nature of the stereochemistry at C2 where the nucleobase is attached. It is possible that the regio- and stereo-constraint in **10** preorganizes the nucleobase containing the side-chain for a favorable hybridization event and may correspond to one of the rotameric forms of *aeg*PNA. The binding efficiency of the pyrrolidyl PNA oligomers seems to be dictated by the stereochemistry of the C2 center that is a part of the acetyl linker connecting the nucleobase in the control *aeg*PNA. This is in contrast to the behavior of *aep*PNA where no differences in binding were observed as a function of the stereochemistry at C2 which was in the backbone. Such a favorable binding of triplex forming oligomers to DNA by one of the isomers (2*S*/2*R*) was not observed in *aep*PNA[4a] where the stereochemistry of the nucleobase attachment to the pyrrolidine ring was 4*S* in both isomers.

In conclusion, we have reported a new positively charged conformationally constrained PNA monomer with high potential for selective binding to DNA. This is the first example of a constrained PNA exhibiting profound effects of the backbone geometry for preferential DNA binding. The syntheses of monomers bearing other nucleobases from the intermediate alcohol **3**, their incorporation into oligomers and binding studies with target DNA are currently under progress.

Acknowledgements

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8. NMR data of selected compounds of the 2(*S*) series and the 2(*R*) monomer: 1-(*N*-Boc)-4(*R*)-*O*-mesyl-pyrrolidine-2(*S*)-methyl alcohol **2**: ^{1}H NMR ($CDCl_3$) δ: 5.23 (br s, 1H), 4.10 (m, 1H), 3.30 (br m, 4H), 3.05 (s, 3H), 2.60 (m, 1H), 2.25 (m, 1H), 1.45 (s, 9H). $[\alpha]_D^{25} = -30.8$ ($c = 0.005$, MeOH).
1-(*N*-Boc)-4(*S*)-azido-pyrrolidine-2(*S*)-methyl alcohol **3**: ^{1}H NMR ($CDCl_3$) δ: 4.05 (m, 2H), 3.70 (m, 4H), 3.30 (dd, $J = 4.8$ Hz, 12.2 Hz, 1H), 2.30 (m, 1H), 1.70 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 156.0, 80.7, 66.4, 58.9, 58.0, 52.1, 33.8, 28.4.
1-(*N*-Boc)-4(*S*)-azido-2(*S*)-(methyl-*O*-mesyl)-pyrrolidine **4**: ^{1}H NMR ($CDCl_3$) δ: 4.45 (dd, $J = 4.8$ Hz, 9.7 Hz, 1H), 4.15 (br m, 3H), 3.65 (m, 1H), 3.35 (m, 1H), 3.03 (s, 3H), 2.30 (m, 1H), 2.13 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 153.7, 80.3, 68.9, 59.0, 54.8, 51.7, 36.8, 32.6, 28.0. $[\alpha]_D^{25} = -7.2$ ($c = 0.005$, MeOH).
1-(*N*-Boc)-4(*S*)-azido-2(*S*)-(thymin-1-ylmethyl)-pyrrolidine **5**: ^{1}H NMR ($CDCl_3$) δ: 9.50 (min) and 9.25 (maj) (s, 1H), 7.00 (s, 1H), 4.25 (m, 3H), 3.90–3.50 (br m, 2H), 3.40 (m, 1H), 2.25 (m, 1H), 2.05 (m, 1H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 164.5, 154.3, 151.4, 140.9, 110.1, 80.3, 59.9, 55.4, 52.1, 50.5, 33.8, 28.0, 12.0. $[M^+] = 350$. $[\alpha]_D^{25} = +98.4$ ($c = 0.005$, MeOH).
4(*S*)-Azido-2(*S*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-ethyl acetate **6**: ^{1}H NMR ($CDCl_3$) δ: 9.45 (s, 1H), 7.24 (s, 1H), 4.15 (m, 3H), 3.75 (m, 2H), 3.55–3.10 (m, 4H), 3.00 (dd, $J = 5.4$ Hz, 10.8 Hz, 1H), 2.35 (m, 1H), 1.90 (s, 3H), 1.75 (m, 1H), 1.25 (t, $J = 8.1$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 170.2, 164.6, 151.3, 142.1, 109.1, 60.2, 59.8, 57.6, 56.6, 53.6, 50.6, 34.2, 13.7, 11.7. $[M^+] = 382$. $[\alpha]_D^{25} = -276.86$ ($c = 0.005$, MeOH).
4(*S*)-(*N*-Boc-amino)-2(*S*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-ethyl acetate **7**: ^{1}H NMR ($CDCl_3$) δ: 9.30 (s, 1H), 7.32 (s, 1H), 5.00 (d, $J = 6$ Hz, 1H), 4.20 (q, $J = 5.4$ Hz, 2H), 4.10 (m, 2H), 3.55–2.85 (m, 4H), 2.75 (m, 1H), 2.30 (m, 1H), 1.95 (s, 3H), 1.60 (m, 1H), 1.45 (s, 9H), 1.30 (t, $J = 5.4$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 170.8, 164.7, 155.5, 151.8, 141.9, 110.2, 79.5, 61.2, 60.9, 60.0, 54.8, 49.9, 49.6, 35.9, 28.4, 14.3, 12.2. $[\alpha]_D^{25} = -4.4$ ($c = 0.005$, MeOH).
4-(*S*)-(*N*-Boc-amino)-2-(*R*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-acetic acid **8**: ^{1}H NMR ($CDCl_3$) δ: 7.60 (s, 1H), 4.40 (m, 1H), 4.20 (m, 3H), 3.75 (m, 2H), 3.50 (m, 1H), 3.30 (m, 1H), 2.65 (m, 1H), 1.90 (s, 4H), 1.49 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 171.1, 167.3, 158.3, 154.5, 114.0, 112.0, 82.4, 66.5, 61.3, 56.5, 47.9, 47.4, 32.7, 28.3, 12.1.
4-(*S*)-(*N*-Boc-amino)-2-(*R*)-(thymin-1-ylmethyl)-pyrrolidine-*N*-1-acetic acid **10**: ^{1}H NMR ($CDCl_3$) δ: 7.50 (s, 1H), 4.30 (m, 2H), 3.95 (m, 4H), 3.65 (m, 1H), 3.40 (m, 1H), 2.75 (m, 1H), 2.00 (m, 1H), 1.85 (s, 3H), 1.35 (s, 9H). ^{13}C NMR ($CDCl_3$) δ: 171.1, 167.3, 158.3, 154.5, 144.0, 112.0, 82.4, 66.5, 61.3, 57.0, 48.1, 47.4, 32.7, 28.3, 12.1.
MALDI-TOF: PNA **11** $M_{calcd} = 2217.17$, $M_{obsd} = 2219.9$ (M+H).
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