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Pyrrolidine Nucleic Acids: DNA/PNA Oligomers with 2-Hydroxy/Aminomethyl-4-(thymin-1-yl)pyrrolidine-*N*-acetic acid

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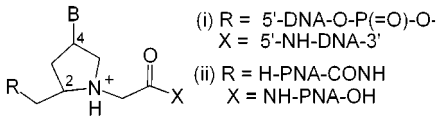
ABSTRACT



Synthesis of pyrrolidine-based chiral positively charged DNA analogues is reported. The synthesis of (2*S*,4*S*) and (2*R*,4*R*) thymin-1-ylpyrrolidine-*N*-acetic acid, its site specific incorporation in PNA:DNA chimera and PNA, and the study of their binding properties with complementary DNA/RNA sequences is presented.

Cat-anionic oligonucleotide analogues[1] along with PNA:DNA chimera[2] are gaining increasing attention in the current literature because of their favorable binding, aqueous solubility, and cellular permeation properties for potential therapeutic applications. The higher affinity of duplexes derived from cat-anionic DNA and positively charged PNA[3] is probably due to electrostatic complexation leading to fast on-rates of hybridization. Introduction of chirality and conformational restraint in the achiral aminoethylglycyl PNA with the intention of preorganizing the PNA structure compatible for binding with target DNA/RNA and to discriminate the parallel/antiparallel binding modes[4] has been partially successful. In our recent efforts,[5] we simultaneously introduced chirality, constrained flexibility, and positive charge in the PNA backbone by linking the α-carbon of the glycyl backbone to the *β*-carbon of the side chain (Figure 1, path **a**) to result in an aminoethylglycyl PNA (*aep*PNA) that formed highly stable PNA:DNA complexes. Extending the logic of constrained backbone,[4b,c,5] we herein report the

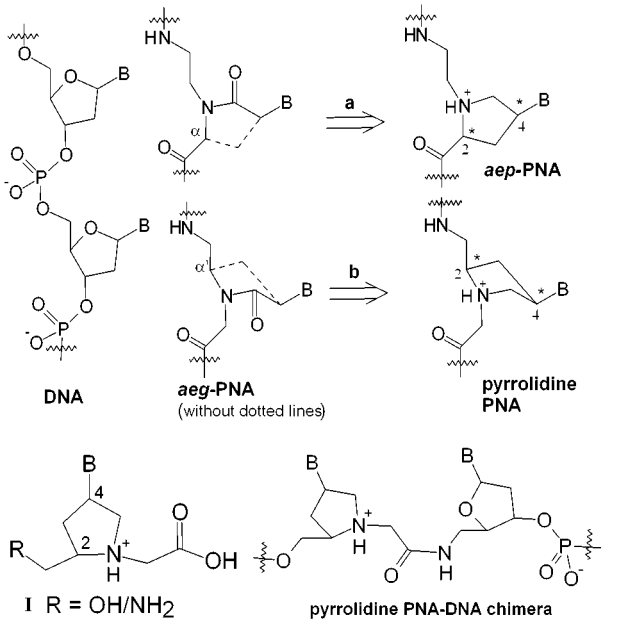


Figure 1. Structures of DNA, PNA, and their analogues. B = adenine, cytosine, guanine, or thymine.

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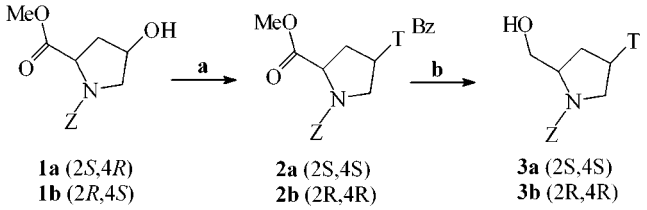
linking of the α′-carbon of the ethylenediamine backbone segment to the β-carbon of the side chain, generating the isomeric cationic, chiral PNA (Figure 1, path **b**). Further, among the several DNA backbone modifications, the ideal amide linkage[6] (3′-CH_2CONH-5′) presumably organizes the sugar into a high N-pucker, resulting in a stable A-type duplex formation. Introduction of a positively charged pyrrolidine ring carrying the $HN^+-CH_2-CO-NH$-5′ linkage for replacement of the sugar phosphate backbone in DNA (Figure 1) may therefore be expected to have cumulative advantages, leading to novel pyrrolidine PNA:DNA chimeric antisense constructs. The monomeric unit 2-hydroxy/aminomethyl-4-thymin-1-ylpyrrolidine-*N*-acetic acid **I** containing a positively charged tertiary nitrogen has dual utility. It is compatible for introduction in both PNA and DNA (in the form of dimeric unit **5c**) depending upon the nature of the C2-substituent (hydroxymethyl or aminomethyl) on the pyrrolidine ring. In this Letter, we report the synthesis and site-specific incorporation of **I** (R = NH_2) and **5c** into PNA/DNA oligomers and the evaluation of their binding properties with target DNA sequences.

Synthesis of Dimeric Block for Chimeric DNA:PNA. N3-Benzoylthymine was alkylated at N1 with N-protected proline methyl esters[5] **1a** (2*S*,4*R*) or **1b** (2*R*,4*S*) (Scheme 1)

Scheme 1[a]

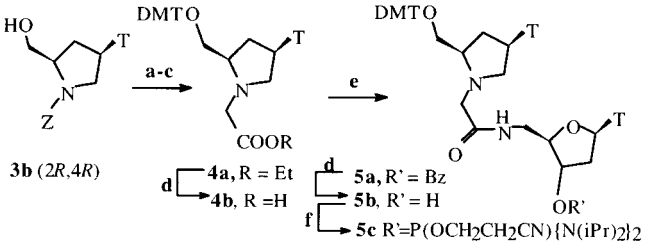


1a (2*S*,4*R*) **1b** (2*R*,4*S*) **2a** (2S,4S) **2b** (2R,4R) **3a** (2S,4S) **3b** (2R,4R)

[a] (**a**) N3-Benzoylthymine, DEAD, PPh_3, benzene, 68%; (**b**) $LiBH_4$, THF, 92%.

via Mitsunobu reaction at C4 to yield **2a** and **2b**, respectively. Subsequent reduction with excess $LiBH_4$ afforded the alcohol **3a**/**3b** with simultaneous removal of the N-3 benzoyl of thymine. The pyrrolidine derivative **3b** (2*R*,4*R*) with stereochemistry equivalent to that of the natural nucleoside was used to synthesize chimeric dimer block **5c** suitable for solid-phase DNA synthesis (Scheme 2). The primary hydroxyl function in **3b** was protected as the dimethoxytrityl ether followed by deprotection of the ring nitrogen by hydrogenation over Pd−C and subsequent alkylation with ethyl

Scheme 2[a]

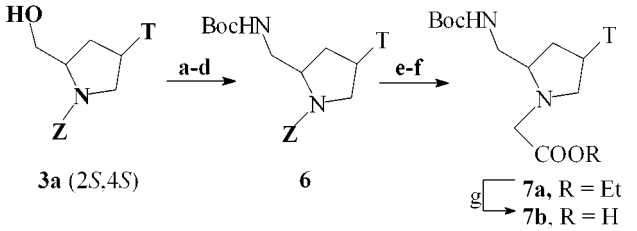


[a] (**a**) DMTCl, pyridine, 70%; (**b**) H_2 Pd/C, MeOH−ethyl acetate, 98%; (**c**) $BrCH_2COOEt$, CH_3CN, KF/Celite, 67%; (**d**) aqueous NaOH, MeOH, 90%; (**e**) TBTU, HOBt, 3′-*O*-benzoyl-5′-amino-5′-deoxythymidine, 42%; (**f**) tetrazole, $P(OCH_2CH_2CN)\{N(^iPr)_2\}_2$, CH_3CN, 80%.

bromoacetate to give the DMT ester **4a**. Hydrolysis of the ester with methanolic NaOH to give the acid **4b** and condensation with 3′-*O*-benzoyl-5′-amino-5′-deoxythymidine in the presence of TBTU−HOBt yielded the dimer **5a**. Alkaline deprotection of the 3′-*O*-benzoyl in **5a** to **5b**, followed by 3′-*O*-phosphitylation, gave the protected chimeric dimer phosphoramidite **5c** for site-specific incorporation into oligonucleotides by automated solid-phase synthesis. All the synthesized compounds were characterized by ^1H and ^{13}C NMR and mass spectral analysis.[7]

Synthesis of 2-Aminomethyl Monomer I for PNA. The monomer **7b** needed for insertion into PNA oligomers was synthesized from **3a** (Scheme 3) by sequential tosylation of

Scheme 3[a]



3a (2*S*,4*S*) **6**

[a] (**a**) TsCl, pyridine, 66%; (**b**) NaN_3, DMF, 70 °C, 98%; (**c**) Raney-Ni, H_2, 95%; (**d**) Boc N_3/Et_3N, DMSO, 50 °C, 90%; (**e**) Pd/C, H_2, 98%; (**f**) $BrCH_2COOEt$, CH_3CN, KF/Celite, 60 °C, 56%; (**g**) aqueous NaOH, MeOH, 95%.

the primary hydroxyl group, azidation with NaN_3, and conversion to amine by hydrogenation with Ra−Ni followed by protection as a *tert*-butylcarbamate to give **6**. The deprotection of the ring nitrogen by hydrogenation over Pd−C and subsequent alkylation with ethyl bromoacetate gave **7a** (2*S*,4*S*). The monomer[7] **7b** was obtained by hydrolysis with 1 M NaOH (aqueous methanol) and used for solid-phase peptide synthesis using Boc chemistry.

DNA:PNA Chimeras. The dimer block phosphoramidite **5c** was incorporated into oligonucleotide sequences **9**−**13** at the desired positions by automated DNA synthesis to yield the chimeric oligomers. The purity of all modified oligomers

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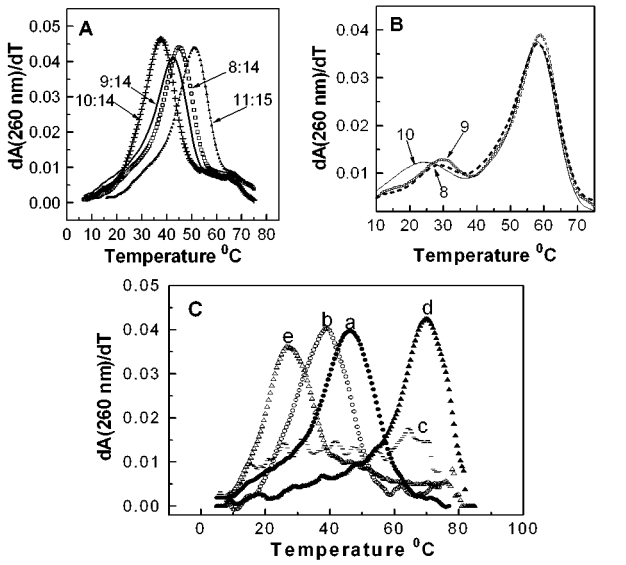

Figure 2. First-derivative normalized UV−T_m plots: **A**, PNA:DNA chimeric duplexes; **B**, triplexes derived from duplexes **17**:**18** and PNA:DNA chimeric third strands **8**−**10**; **C**, PNA:DNA/RNA triplexes (a) **19**:**20**, (b) **19**:**21**, (c) **19**:**22**, and (d) **21**:poly rA and (e) **22**:poly rA.

was established by RP-HPLC, and the compounds were characterized by mass spectra. The chimeric ODNs **9**−**10** and **11**−**13** were individually hybridized with the complementary DNA strands **14** and **15**, respectively, to obtain duplexes for UV−T_m measurements (Figure 2A), while duplexes **8**:**14** and **16**:**15** served as the control.

8 TTC TTC TTC TTT TCT TTT
9 TTC TTC TTC TTT TCT t*TT
10 TTC TTC TTC t*TT TCT TTT
11 CTT GTA CTt*TTC CGG TTT
12 CTT GTA Ct*T t*TC CGG TTT
13 CTT GTA CTt*TTC CGG t*TT
14 AAA AGA AAA GAA GAA GAA
15 AAA CCG GAA AAG TAC AAG
16 CTT GTA CTT TTC CGG TTT
17 TCC AAG AAG AAG AAA AGA AAA TAT
18 ATA TTT TCT TTT CTT CTT CTT GGA
19 GCAAAAAAAACG
PNA 20 H-TTTTTTTT-β-Ala-OH
PNA 21 H-TTTTTTTt-β-Ala-OH
PNA 22 H-TTTTtTTt-β-Ala-OH

T= *aeg*PNA monomer, **t** = pyrrolidine **T** monomer, **t*T** = pyrrolidineamide dimer block

The duplex with polypyrimidine ODN **9** carrying the amide-linked pyrrolidine−sugar dimer at the 3′ terminus exhibited a 3 °C destabilization compared to the control duplex **8**:**14** (Table 1, entries 1 and 2) while the destabilization increased when the dimer was at the center as in **10**:**14** (−7 °C, entry 3). The duplex **11**:**15** with a single modification at the center in a mixed pyrimidine−purine sequence also showed a similar destabilization of 6 °C compared to the relevant control duplex **16**:**15** (Table 1, entries 4 and 7). The duplex **12**:**15** containing two modified units adjacent to each other in the center (Table 1, entry 5) was destabilized to a greater extent (<12 °C), while introduction of a second modified unit at the 3′ terminus as in **13**:**15** effected a lesser destabilization of 7 °C (Table 1, entry 6). The dimer **5c**, though configurationally equivalent to natural DNA, perhaps induces structural deviations in the duplexes of oligomers **9**−**13** resulting in their destabilization as compared to the control, unmodified duplexes. The polypyrimidine oligomers **8**, **9**, or **10** also form a third (Hoogsteen) strand of a triplex with the duplex **17**:**18** (Figure 2B). It was found that the stability of the triplex **9*17**:**18** with a chimeric oligomer having the pyrrolidine dimer **5c** at the 3′-end was similar to that of the corresponding unmodified triplex **8*17**:**18**. The triplex with a third strand carrying the dimer in the middle (**10*17**:**18)** exhibited a 4 °C destabilization (Table 1, entries 8, 9, and 10). This indicates that the modification is accommodated better at the 3′ terminus compared to the modification in the middle of the sequence.

Table 1. UV−T_m of DNA Duplexes and Triplexes[a]

entry	complex	T_m (°C)	ΔT_m (°C)
1	8:14	45	
2	9:14	42	−3
3	10:14	38	−7
4	11:15	51	−6
5	12:15	45	−12
6	13:15	50	−7
7	16:15	57	
8	8*17:18	29	
9	9*17:18	29	0
10	10*17:18	25	−4

[a] Buffers: (a) duplexes, 10 mM sodium phosphate, pH 7.0, 100 mM NaCl, 0.1 mM EDTA; (b) triplexes, 10 mM sodium phosphate, 200 mM NaCl, pH 7.0. All T_m values are accurate to ±0.5 °C and measured in four melting experiments.

Cationic and Chiral PNA. The monomer (2*S*,4*S*)-2-(*N*-Boc-aminomethyl)-4-thymin-1-ylpyrrolidine-*N*-acetic acid **7b** was used to prepare the homopyrimidine PNA oligomers **21** and **22** by standard PNA synthesis protocols[8] along with the control PNA **20**. The purity of the oligomers was rechecked by RP HPLC on a C18 column and characterized by MALDI-TOF mass spectrometry. No precipitation was

(7) **5b**. ^{1}H NMR in $CDCl_3$ 8.6 (NHCO), 7.62 (s, 1H, H6 Thy), 7.4−7.2 (m, 9H, DMT + 1H, NH Thy, + 1H, H6 Thy), 6.8−6.45 (d, 4H, DMT), 6.04 (t, 1H, H1′), 5.05 (bs, 1H, C4H), 4.25 (bs, 1H H3′), 3.85 (m, 1H, H4′), 3.75 (s, 6H, OMe), 3.7−3.5 (m, 2H, CH_2ODMT), 3.5−3.15 (m, 4H, H5, H5′, C5H, C5′H), 3.0−2.6 (m, 2H, CH2N), 2.4−2.2 (m, 3H, H2′, H2″, C3H), 2.1−1.9 (m, 1H, C3′H), 1.82 (s, 3H, Me Thy), 1.67 (s, 3H, 5-MeThy). ^{13}C NMR $CDCl_3$ + pyridine-d_5 δ 169.74 (CO amide), 163.86, 163.73 (2 × C4 Thy), 157.59 (C2 Thy), 148.72, 148.52 (2 × C6 Thy), 109.83 (C5), 85.26 (C1′), 84.47 (C4′), 70.73 (C3′), 62.78 (amide CH_2), 62.06 (C4), 58.27 (C6), 55.71 (C5), 54.03 (OMe), 51.62 (C2), 40.54 (C5), 38.88 (C2′), 34.42 (C3), 11.46 (Thy Me). FAB mass 831 (M + Na), calculated mass 808.3. **5c.** ^{31}P in $CDCl_3$, 149.50, 148.42. **7b.** ^{1}H NMR in D_2O δ 7.5 (s, 1H, Thy H6), 4.8 (m, 1H, H4), 4.1−3.55 (m, 2H, H5, H5′), 3.6−3.35 (m, 5H, CH_2-NH, CH_2CO, H2), 2.85−2.6 (m 1H, H3), 2.4−1.9 (m, 1H, H3′), 1.9 (s, 3H, Thy CH3), 1.49 (s, 9H, t-Boc). ^{13}C in D_2O δ 170.95 (CO acid), 167.53 (CO ThyC4), 159.22 (CO Boc), 144.19 (CO ThyC2), 144.19 (C6 Thy), 111.48 (C5 Thy), 82.55 (C-Boc), 68.29 (C4 Pro), 60.31 (CH2COO), 59.24 (C2 Pro), 56.04 (CH_2NH), 38.44 (C5 Pro), 32.7 (C3 Pro), 28.55 (Me Boc), 12.33 (Me Thy). FAB mass 383 (M^++ H), calculated mass 382.

(8) *Peptide Nucleic Acids protocols and applications*; Nielson, P. E., Egholm, M., Eds.; Horizon Scientific Press: Norfolk, England, 1999.

observed in the samples of modified PNAs **21** and **22** even after prolonged storage.

Homopyrimidine PNA sequences are known to form PNA_2:DNA triplexes with a homopurine DNA such as **19**. The PNA oligomers **20**−**22** were independently hybridized with the complementary DNA oligomer **19** and poly rA, with a PNA:DNA/RNA stoichiometry of 2:1 for T_m studies (Figure 2C). The DNA:PNA complex **19**:**21** having the modified unit **7b** at the C-terminus had a T_m 6° lower than that of the control duplex **19**:**20** (Table 2, entries 1 and 2).

Table 2. UV−T_m of PNA:DNA/poly rA[a]

entry	complex	T_m (°C)
1	**19**:**20**	46
2	**19**:**21**	40
3	**19**:**22**	
4	poly rA:**21**	70
5	poly rA:**22**	27

*All T_m values are accurate to ±0.5 °C and measured in four melting experiments. Buffers: entry 1−3, 10 mM sodium phosphate, pH 7.3; entries 4−5, as above with 100 mM NaCl.

The simultaneous presence of the modified unit in the center as well as at the C-terminus in the complex **19**:**22** largely affected the stability, and the absence of any temperature-dependent UV absorbance change suggested no complexation with the target DNA (curve **c**, Figure 2C).

The hybridization efficiency of modified PNAs with RNA was also examined by complexation with poly rA. The PNA **21** with a single modification had a T_m of 70 °C whereas the T_m of PNA **22** was much lower (27 °C). Under the buffer conditions employed, the control poly rA:**20** complex did not melt.

While this Letter was in preparation, three interesting reports concerning pyrrolidine PNA appeared in the literature.[9] These are related to our ideas on constrained flexibility in prolyl PNA[4b,c] and *aep*PNA.[5] As originally envisaged by us, the possibility of four diastereomers from the two chiral centers in the pyrrolidene ring, in conjunction with different combinations of ring substitution, leads to a number of regio- and stereocentric modifications. From our work and that of others, interesting and unforeseen aspects of prolyl/pyrrolidyl-PNA:DNA/RNA binding selectivity are now emerging. While cationic *aep*PNA in both (2*S*,4*S*) and (2*R*,4*S*) modifications exhibited high binding affinity to cDNA,[5] the corresponding (2*R*,4*R*) analogue was reported to bind preferentially to cRNA.[9b] In the present modification series, the (2*R*,4*R*) PNA analogues displayed improved binding affinity toward the complementary DNA and RNA.[9c] The (2*S*,4*S*) geometry of the pyrrolidine ring in PNA reported here does not seem to be structurally compatible in the PNA backbone, although it was very effective in forming DNA:*aep*PNA_2 triplexes.[5]

The (2*R*,4*R*) stereochemistry used for the pyrrolidine−sugar thymine dimer unit was expected to be compatible with DNA geometry, the nucleobase being *cis* to the aminomethyl segment of the backbone, as in DNA sugar. However, a positively charged pyrrolidine ring nitrogen might adapt a ring pucker, resulting in a nucleobase orientation detrimental to duplex formation with the complementary DNA. Further studies on the effective stereochemical preferences exerted by these units on the DNA/RNA binding properties are necessary.

In conclusion, the preliminary results reported here add to a growing stereooligomeric library of pyrrolidine PNAs that offer new insights into understanding PNA−DNA/RNA complexation. Such studies may eventually allow for rational design of potential antisense molecules that display a high discrimination in recognition of DNA/RNA. The binding results obtained so far from mixed backbone, homopyrimidine/purine sequences may have limitations in extrapolation to mixed sequence complexes. Pure modified backbone oligomers may bind DNA/RNA differently than the mixed backbone oligomers. The presence of a positive charge on pyrrolidine PNA may also favor cell permeation as seen in the case of cationic peptide−PNA conjugates.[10] Future work in these directions is in progress to delineate the binding preferences of different stereochemically divergent pyrrolidine PNA analogues to complementary DNA/RNA.

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