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Cyanuryl peptide nucleic acid: synthesis and DNA complexation properties

Raman Vysabhattar [a], K. N. Ganesh [a,b,*]

[a] Division of Organic Chemistry (Synthesis), National Chemical Laboratory, Pune 411 008, India
[b] Indian Institute of Science Education and Research, 900, NCL Innovation Park, Dr. Homi Bhabha Road, Pune 411 008, India

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Abstract

The synthesis of cyanuryl PNA monomer (*Cya*PNA) **6** was achieved by direct N-monoalkylation of cyanuric acid with *N*-(2-Boc-aminoethyl)-*N*′-(bromoacetyl)glycyl ethyl ester **4**. Compound **6** was incorporated as a T-mimic into PNA oligomers and biophysical studies on their triplexes/duplex complexes with complementary DNA oligomers indicated unusual stabilization of PNA:DNA hybrids when the cyanuryl unit was located in the middle of the PNA oligomer.

Keywords: PNA; Cyanuryl PNA

Peptide nucleic acids[1] (Fig. 1A) are a new class of DNA (Fig. 1B) mimics that have emerged as potential antigene/antisense agents in the field of nucleic acid-based therapeutics. The chiral and negatively charged sugar-phosphate backbone of DNA is replaced in PNA by an achiral, neutral backbone composed of *N*-(2-aminoethyl)glycyl (aeg) units.[1] The nucleobases are attached to the backbone through tertiary acetamide linkers, and PNA binding to the target DNA/RNA sequences occurs with high sequence specificity and affinity.[2] PNA binds to complementary DNA and RNA to form duplexes via Watson–Crick (WC) base pairs and triplexes through combined WC and Hoogsteen (HG) hydrogen bonding. PNA–DNA/RNA hybrids exhibit greater thermal stability than analogous DNA:DNA and DNA:RNA complexes.[3] Because of this attractive feature and stability to proteases and nucleases, PNAs are of great interest in medicinal chemistry, with potential for development as gene-targeted drugs and as reagents in molecular biology and diagnostics.[4]

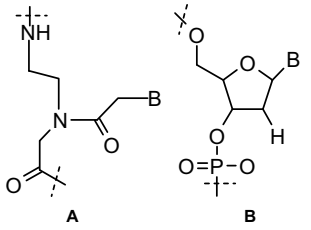

Fig. 1. Structures of (A) PNA and (B) DNA.

Nucleobases are attached to the PNA backbone via tertiary amide bonds leading to *syn* and *anti* conformers (Fig. 2) which are rotameric isomers as a result of the high energy barrier for their interconversion. These two non-interconvertible rotamers may have consequences on the

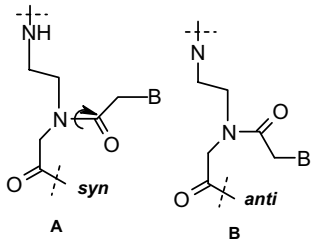

Fig. 2. The rotameric structures of aeg PNA (A) *syn* and (B) *anti* forms.

* Corresponding author. Tel.: +91 20 25898021; fax: +91 20 2589 8022.
E-mail address: kn.ganesh@iiserpune.ac.in (K. N. Ganesh).

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orientation of attached nucleobases, thereby affecting the strengths of their hydrogen bonding in complementation. The X-ray crystal structure of PNA showed a predominance of the *syn* conformation in the solid state,[5] whereas it exhibited both *syn* and *anti* conformers in solution.[6] In principle, it is possible to have different rotamers for each of the bases within the same PNA strand and the ultimate specificity and binding strength of a PNA oligomer is determined by the statistical predominance of identical, favorable rotamers leading to productive base pairing.

We hypothesized that a modified nucleobase with equal propensity for hydrogen bonding from each face would statistically double the probability of efficient hydrogen bonding, thereby negating the effects of non-productive rotamers. Several unnatural bases forming stable DNA triplexes have been reported,[7a,b] and recently, base modified PNAs have been described as well.[7c] Cyanuric acid (*Cya*) (Fig. 3A) is a symmetric six-membered cyclic imide with alternate arrangement of hydrogen bond donors and acceptors with the ability to form hydrogen bonds from both sides of the heterocyclic ring.[8] The base pairing potential of cyanuric acid and its *N*-alkyl analog with hydrogen bonding complements such as melamine[9] (Fig. 3C) and *N*-ethyladenine[10] (Fig. 3D) in monomeric form in the solid state have been well studied.

N-Methylcyanuric acid (MCA) (Fig. 3B) with close structural similarity to uracil forms hydrogen bonded complementary pairs with N^9-ethyladenine (EA),[10,11] as observed by X-ray crystallography. The interesting base pairing potential of cyanuric acid along with the biological properties of cyanuryl nucleotides[12] inspired us to synthesize *Cya*PNA oligomers containing cyanuryl units in place of thymine at specific sites and examine their DNA complexation properties. The work presented here describes the synthesis and characterization of sequence specific cyanuryl PNA oligomers and a study of their hybridization properties with complementary DNA oligomers.

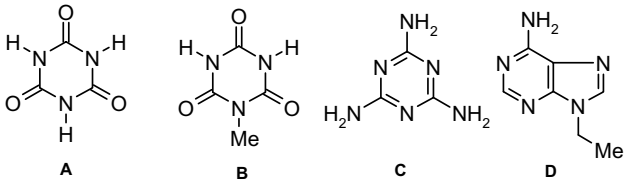
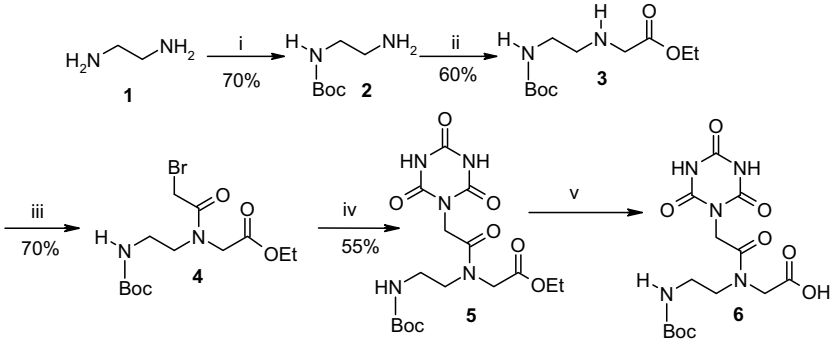


Fig. 3. Structures of (A) cyanuric acid, (B) methyl cyanuric acid, (C) melamine, and (D) N^9-ethyladenine.

Synthesis of CyaPNA monomer: The synthesis of *Cya*PNA monomer **6** through cyanuric acid ring construction had been reported earlier by us (Scheme 1).[13] However, poor yields prompted us to search for alternative methods to prepare **6**. Instead of the ring closure approach, we attempted N-alkylation of the commercially available cyanuric acid to obtain directly the target *Cya*PNA monomer **6**. N-Monoalkylation of cyanuric acid with ethyl *N*-(2-Boc-aminoethyl)-*N*-(1-bromoacetyl) glycinate **4** was attempted in DMSO and K_2CO_3 (Scheme 1). Compound **4** was prepared in three steps from ethylenediamine according to known procedures.[14,15] The precursor *N*-*Boc*-aminoethylglycine **3** was treated with bromoacetyl chloride in DCM in the presence of triethylamine to yield glycinate **4**.

N-Alkylation of cyanuric acid with the bromoacetyl compound **4** was carried out in DMSO at >70 °C where cyanuric acid is more soluble in DMSO. The reaction went to completion in 5 h to give the PNA ester **5** (Scheme 1). Hydrolysis of ester **5** was accomplished with LiOH, followed by neutralization with acid to yield the desired cyanuryl PNA acid monomer **6**. The structural integrity of monomer **6** and all other intermediate compounds was established by ^1H and ^{13}C NMR spectroscopic analysis and confirmed by mass spectrometry. Compound **6** was used for coupling with a solid support to synthesize the *Cya*PNA oligomers.

PNA oligomers **8**–**13** containing *Cya*PNA units at specific sites in place of thymine were assembled by solid-phase peptide synthesis on MBHA resin functionalized with N^{γ}-(benzyloxycarbonyl)-N^{α}-Boc-lysine. The unmodified PNA-T oligomer **7** and mixed base oligomer PNA **13** were synthesized for control studies (Table 1). The chimeric PNA oligomers containing *Cya*PNA units in different positions were designed to examine the effect of distance between the two sites of modifications on the stability of the derived *Cya*PNA:DNA complexes. The PNA



Scheme 1. Reagents and conditions: (i) $(Boc)_2O$, THF; (ii) Ethyl bromoacetate, Et_3N, acetonitrile; (iii) Bromoacetyl chloride, Et_3N, DCM; (iv) Cyanuric acid, K_2CO_3, DMSO; (v) 2 N LiOH, methanol.

Table 1
MALDI-TOF spectral analysis of PNA oligomers

Entry	Sequence	Mass (calcd)	Mass[a] observed
PNA **7**	H-TTTTTTTT-Lys-NH_2	2273.93	2275.24
PNA **8**	H-*Cya*TTTTTTT-Lys-NH_2	2298.91[b]	2300.64[b]
PNA **9**	H-TTTTTTT*Cya*-Lys-NH_2	2276.91	2276.62
PNA **10**	H-TTTT*Cya*TTT-Lys-NH_2	2276.91	2279.77
PNA **11**	H-*Cya*TTT*Cya*TTT-Lys-NH_2	2201.88[b]	2301.56[b]
PNA **12**	H-GTAGATCACT-Lys-NH_2	2855.24	2858.46
PNA **13**	H-G*Cya*AGA*Cya*CAC*Cya*-Lys-NH_2	2864.32	2868.17

T = thyminyl PNA; *Cya* = cyanuryl PNA.
[a] =HRMS.
[b] $=(M^+ + Na)^+$.

oligomers were cleaved from the solid support using trifluoromethanesulfonic acid (TFMSA) in the presence of trifluoroacetic acid (TFA)[16] to yield PNAs carrying lysine at the C-terminus. All PNAs **7**–**13** were purified by HPLC on an RPC-18 column. The purity of the oligomers was confirmed by HPLC using a RPC-18 column and they were characterized by MALDI-TOF mass spectrometry (Table 1). The mixed sequences containing both purines and pyrimidines PNA **12** and PNA **13** were synthesized to check the duplex stability and discrimination between parallel and antiparallel DNA. The results of cyanuryl PNA **13** were compared with the results of control PNA **12**.

The complementary DNA oligomers for triplex studies (DNA **14**, GCAAAAAAAACG and DNA **15**, GCAAAACAAACG) having a CG/GC lock at the ends to prevent slippage and DNA oligomers for the duplex studies (DNA **16**, AGTGATCTAC and DNA **17**, CATCTAGTGA) were synthesized on an automated DNA synthesizer using standard phosphoramidite chemistry,[17] and purified by C18 RP HPLC.

UV–T_m studies on CyaPNA$_2$:DNA triplexes: The PNA homopyrimidine sequences with complementary homopurine DNA sequences are well known to form PNA_2:DNA triplexes.[18] The stoichiometry of the *Cya*PNA:DNA complexes was found to be 2:1 by UV-mixing curves (recorded at 260 nm) (Fig. 4).[18] Keeping the total concentration constant, a series of mixtures of PNA and DNA of different relative molar equivalents of PNA and DNA were prepared and the UV absorbance of each mixture was recorded at 260 nm. The net UV absorbance steadily decreased due to hypochromic effects upon complexation as the PNA concentration increased, until all the strands present were fully involved in complex formation. The absorbance then increased upon the addition of excess PNA. The stoichiometry of *Cya*PNA:DNA complexation was derived from the minimum in the plot of absorbance with respect to mole fraction (Fig. 4) and found to be 2:1.

The annealed PNA_2:DNA triplexes were subjected to temperature-dependent UV absorbance measurements. The normalized absorbance versus temperature plots derived from these experiments exhibited a single sigmoidal transition, and the maxima in first derivative plots (Fig. 5) indicate the melting temperature T_m (Table 2).[18] The control PNA_2:DNA triplex derived from unmodified aeg PNA-T_8 (Table 2, entry 1) had a T_m of 44.6 °C, which was increased by 23.3 °C when a *Cya*PNA unit was incorporated at the C-terminus (Table 2, entry 3). However, the *Cya*PNA substitution at the N-terminus destabilized the PNA_2:DNA triplex by 10.4 °C (Table 2, entry 2).

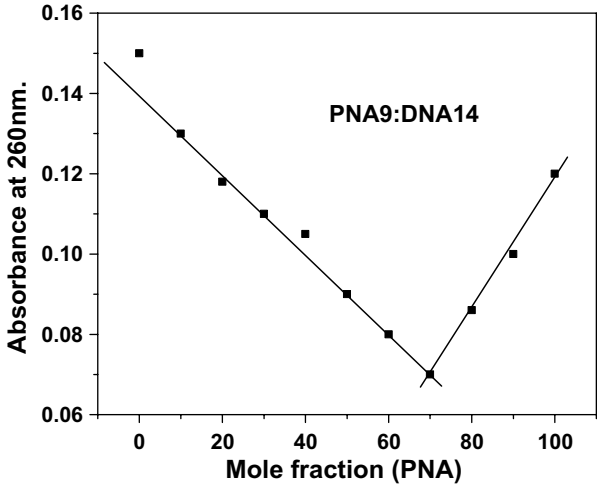


Fig. 4. UV-Job plot corresponding to the PNA **9**:DNA **14** mixtures in the molar ratios of 100:0, 80:20, 60:40, 50:50, 40:60, 20:80, 0:100 (buffer, 10 mM sodium phosphate pH 7.2, 100 mM NaCl), UV absorbance at 260 nm.

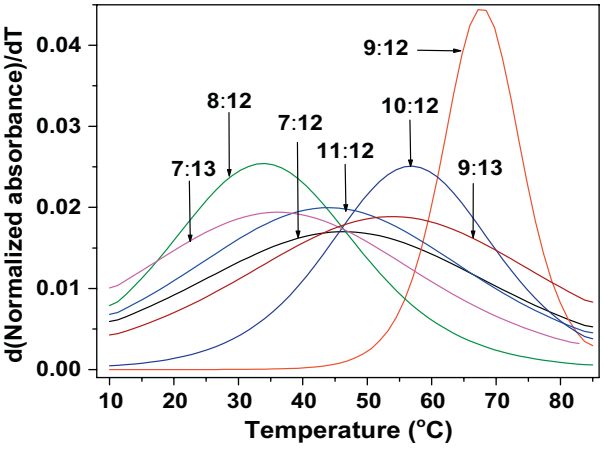


Fig. 5. UV–T_m first derivative curves of PNA_2:DNA triplexes.

Table 2
UV-melting temperatures of *Cya*PNA:DNA **14** triplexes

Entry	Sequence	T_m (°C)	ΔT_m (°C)
1	PNA **7**, H-TTTTTTTT-Lys-NH_2	44.6	—
2	PNA **8**, H-*Cya*TTTTTTT-Lys-NH_2	34.2	−10.4
3	PNA **9**, H-TTTTTTT*Cya*-Lys-NH_2	67.9	+23.3
4	PNA **10**, H-TTTT*Cya*TTT-Lys-NH_2	56.7	+12.1
5	PNA **11**, H-*Cya*TTT*Cya*TTT-Lys-NH_2	44.2	−0.4

T = Thyminyl PNA, *Cya* = cyanuryl PNA, T_m = melting temperature (measured in the buffer 10 mM sodium phosphate, 10 mM NaCl, pH 7.0).

Interestingly, substitution of a *Cya*PNA unit in the middle of the sequence stabilized the triplex by 12.1 °C (Table 2, entry 4). PNA **11** (Table 2, entry 5) with two simultaneous modifications at the N-terminus and middle of the sequence formed a triplex with stability as good as that from unmodified PNA **7**, without much further stabilization. The stabilization by the center modification seems to negate the destabilization effects of N-terminus modification.

The sequence specificity of *Cya*PNA hybridization was examined through T_m measurements with single mismatch-containing DNA oligomer (DNA **15**) (Table 3, entries 2 and 4). The control sequence PNA **7** formed a triplex with single mismatch sequence (DNA **13**) with a T_m lower by 9.6 °C (Table 3, entry 2), while cyanuryl PNA **9** exhibited destabilization by 13.2 °C. Thus *Cya*PNAs show better sequence discrimination (Table 3, entry 4).

The effect of incorporation of a *Cya*PNA unit in place of a thyminyl PNA unit in mixed purine-pyrimidine sequence PNA **12** was studied to examine the consequence of substitution on duplex stability. In PNA **13**, all three thyminyl PNA units in PNA **12** were replaced with a *Cya*PNA monomer and annealed with corresponding DNA **16** and DNA **17** to generate antiparallel and parallel duplexes, respectively. The establishment of stable duplexes was indicated by sigmoidal transitions in the UV–T_m curves and confirmed by peaks in the first derivative plots (Fig. 6).

Table 3
Mismatch studies of *Cya*PNA:DNA triplexes

Entry	PNA sequence	DNA	T_m (°C)	ΔT_m
1	PNA **7**, H-TTTTTTTT-Lys-NH_2	DNA **14**	44.6	—
2		DNA **15**	35.0	−9.6
3	PNA **9**, H-TTTTTTT*Cya*-Lys-NH_2	DNA **14**	67.9	—
4		DNA **15**	54.7	−13.2

T = Thyminyl PNA, *Cya* = cyanuryl PNA, T_m = melting temperature (measured in the buffer 10 mM sodium phosphate, 10 mM NaCl, pH 7.0).

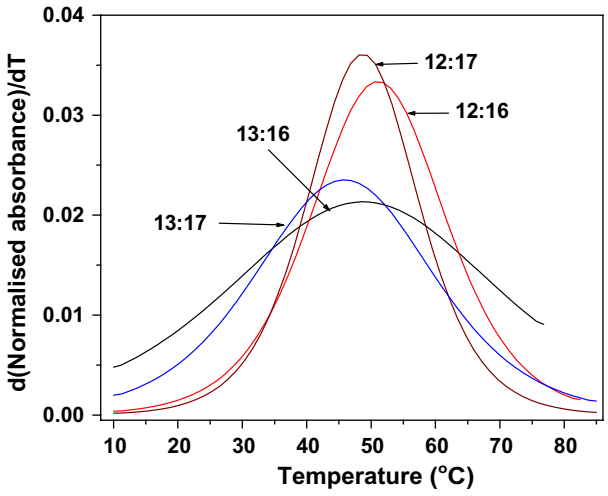


Fig. 6. UV–T_m first derivative curves of PNA:DNA duplexes.

Table 4
UV-melting temperatures of *Cya*PNA:DNA duplexes

Entry	Sequence	DNA	T_m	ΔT_m (ap-p)
1	PNA **12**, H-GTAGATCACT-Lys-NH_2	**16**	51.2	2.6
2		**17**	48.6	
3	PNA **13**, H-G*Cya*AGA*Cya*CAC*Cya*-Lys-NH_2	**16**	48.7	2.9
4		**17**	45.8	

T = Thyminyl PNA, *Cya* = cyanuryl PNA, T_m = melting temperature (measured in the buffer 10 mM sodium phosphate, 10 mM NaCl, pH 7.0).

PNA **12** showed a T_m of 51.2 °C (Table 4, entry 1) for the antiparallel duplex with DNA **16**, whereas the T_m of the corresponding parallel duplex with DNA **17** was 48.6 °C (Table 4, entry 2) which is lower by 2.6 °C. In comparison, the antiparallel duplex of *Cya*PNA **13** with DNA **16** exhibited a T_m of 48.7 °C (Table 4, entry 3) and the parallel duplex with DNA **17** showed a T_m of 45.8 °C (Table 4, entry 4). The *Cya*PNA:DNA duplexes thus showed destabilization by 2.5 °C compared to control PNA **12**, but with almost the same degree of discrimination (2.9 °C) among parallel and antiparallel duplexes as the control PNA **12**. Thus *Cya*PNA modification is well tolerated within the mixed PNA sequences for duplex formation. The insertion of *Cya*PNA units within the PNA oligomer does not alter the base stacking pattern as the CD profile of the derived triplexes was unaffected.

In conclusion, this work demonstrates that a cyanuryl PNA unit with the capacity to form hydrogen bonds from either side of the heterocyclic ring can act as a pyrimidine mimic and stabilizes remarkably the corresponding PNA_2:DNA triplexes. Normally, base modifications incorporated in the middle of sequences show destabilization of the derived triplexes and in this context, *Cya*PNA is interesting in showing the opposite behavior. This is perhaps due to the propensity of the cyanuryl unit to form H-bonds from both sides, irrespective of the rotameric form. The cyanuryl PNA also exhibits a better sequence discrimination of the derived triplexes than the unmodified PNA. The destabilization seen with *Cya*PNA at the N-terminus in triplexes arises perhaps from unfavorable base stacking at this end, in constrast to the C-terminus or in the middle. In the case of duplexes, even though *Cya*PNA **13** destabilized the antiparallel duplex with complementary DNA **16**, it is as good as the control PNA **12** in discriminating the parallel and antiparallel duplexes. A study of the hybridization properties of cyanuryl PNA with complementary RNA is in progress.

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