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Hybridization Properties of Aromatic Peptide Nucleic Acids: A Novel Class of Oligonucleotide Analogues

Lee D. Fader[†] and Youla S. Tsantrizos*[,†,‡]

Departments of Chemistry, McGill University, Montreal, Quebec, Canada H3A 2K6, and Boehringer Ingelheim (Canada) Ltd., Laval, Quebec, Canada H7S 2G5

ytsantrizos@lav.boehringer-ingelheim.com

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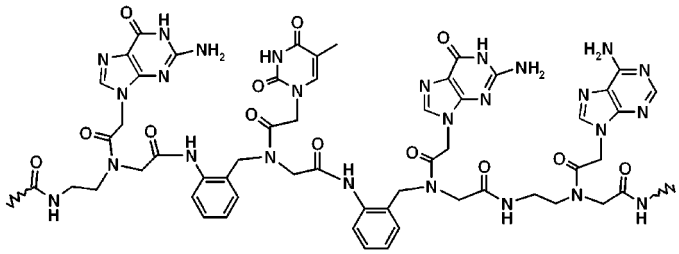
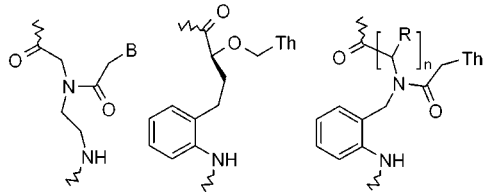
ABSTRACT



The synthesis of APNA-PNA chimeras containing all four DNA bases will be described. The hybridization properties of APNA-PNA chimeras with DNA and RNA are consistent with plausible intramolecular interactions that could preorganize the oligomers in a duplex or triplex structure with complementary DNA or RNA.

Peptide nucleic acids (PNAs, **I**) have great potential as antisense therapeutics and are known to hybridize with complementary DNA and RNA with remarkably high affinity and base-pairing selectivity.[1] Early results involving in vitro



I **II** **III** n =1,2
R = H,Me

cell-free systems or cellular microinjection of PNA oligomers were encouraging;[2] however, their therapeutic utility is compromised by their inefficient cellular uptake and lack of a therapeutically practical delivery system. In an attempt to improve the physicochemical properties of PNAs, a number of structural analogues have been synthesized.[3] Unfortunately, improved cell membrane permeability has not yet been reported.

From our own studies in this area, we recently reported the synthesis of the first analogues having an aromatic moiety as an integral part of the peptidic backbone and we termed these *aromatic peptide nucleic acids* (APNA, **II**[4] and **III**[5]).

[†] McGill University.
[‡] Boehringer Ingelheim (Canada) Ltd.

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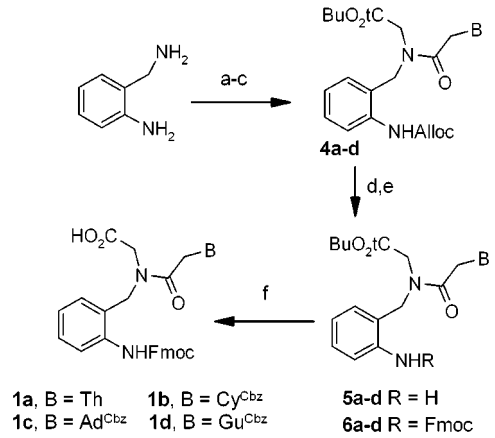
A key interest in the design of APNAs was to investigate the likelihood of forming intramolecular π-staking, or dipole−quadrapole interactions, along the backbone that could help in the preorganization of these oligomers and, consequently, favor duplex or triplex formation with complementary DNA or RNA. Moreover, we anticipate that the increased lipophilicity of these analogues would aid their cell membrane permeability.

More recently, we demonstrated that PNA hexamers which incorporated monomers of general structure **III** formed stable triplex structures (albeit less stable than the corresponding PNA homopolymers) most probably through Watson−Crick and Höogsteen base pairing.[5] In this Letter, we describe the synthesis of APNA monomers **1a**−**d** containing all four natural DNA bases, an optimized procedure for their oligomerization into decamers, and preliminary hybridization properties of their APNA-PNA chimeras with DNA and RNA.

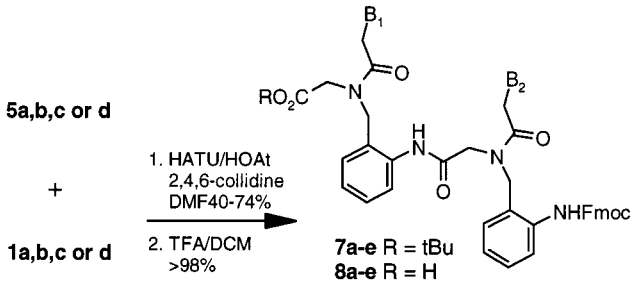
Synthesis of APNA Monomers and Their Incorporation into PNA-APNA Chimeras. Methodology that allows for the efficient preparation of Fmoc-protected APNA monomers was adapted from our original synthesis of this class of molecules (Scheme 1, see Supporting Information for synthetic details).[5,6] To ensure high overall yields and purity of the desired APNA homopolymers or APNA-PNA chimeras, APNA dimers were first synthesized in solution before their oligomerization on solid support. Solution phase couplings and deprotections of the aniline monomers **5** with the free acids **1** were followed by deprotection to give the dimers **8a**−**e** (Scheme 2). The subsequent incorporation of dimers **8a**−**e** into the APNA-PNA chimeras **10**−**12** and **14**−**16** was achieved by automated solid-phase synthesis on MBHA resin (Table 1). The efficiency of coupling at each condensation step was monitored by spectrophotometric analysis of the liberated piperidine−dibenzofulvene adduct.[7] A protocol involving preactivation of each APNA dimer with HATU/HOAt/2,4,6-collidine, followed by reaction with the aniline moiety of the resin-bound oligomer (6 h), led to a coupling

Scheme 1[a]



[a] Conditions: (a) (i) Boc_2O/THF, (ii) AllocCl, pyr/DCM, (iii) HCl/dioxane, 88%, three steps; (b) $BrCH_2CO_2tBu$, DIPEA/DMF, 64−71%; (c) BCH_2CO_2H, HATU, DIPEA/DMF, 76−97%; (d) Bu_3SnH, $Pd(PPh_3)_4$, H_2O/DCM, 84−86%; (e) FmocCl, Na_2CO_3, aq dioxane, 81−89%; (f) TFA/DCM, >97%.

Scheme 2



Compound	B_1	B_2	%yield
7a	T	T	58
7b	A^{Cbz}	C^{Cbz}	70
7c	T	A^{Cbz}	74
7d	G^{Cbz}	A^{Cbz}	40
7e	T	G^{Cbz}	49

Table 1. List of Sequences Used in Hybridization Studies[a]

PNA	sequence
9	Ac-ATCATTCTCT-Lys-NH_2
10	Ac-ATCAT-$\mathbf{T}_\alpha$-CTCT-Lys-NH_2
11	Ac-ATCA-$\mathbf{T}_\alpha\mathbf{T}_\alpha$-CTCT-Lys-$NH_2$
12	H-$\mathbf{A}_\alpha\mathbf{T}_\alpha\mathbf{C}_\alpha\mathbf{A}_\alpha$-TTCTCT-Lys-$NH_2$
13	H-GTAGATCACT-Lys-NH_2
14	H-GTAGA-$\mathbf{T}_\alpha$-CACT-Lys-NH_2
15	H-GTAG-$\mathbf{A}_\alpha\mathbf{T}_\alpha$-CACT-Lys-$NH_2$
16	H-$\mathbf{G}_\alpha\mathbf{T}_\alpha\mathbf{A}_\alpha\mathbf{G}_\alpha$-ATCACT-Lys-$NH_2$
17	H-ATCACT-Lys-NH_2
18	5′-dAGAGAATGAT-3′ (antiparallel)[b]
19	5′-rAGAGAAUGAU-3′ (antiparallel)
20	5′-dAGTGATCTAC-3′ (aniparallel)
21	5′-dCATCTAGTGA-3′ (parallel)
22	5′-rAGUGAUCUAC-3′(antiparallel)
23	5′-rCAUCUAGUGA-3′ (parallel)
24	5′-dAGTGA-**A**-CTAC-3′(antiparallel)
25	5′-dAGTG-**T**-TCTAC-3′ (antiparallel)
26	5′-dAGTGATC-A-AC-3′ (antiparallel)

[a] APNA residues with subscript α and bold text, PNA residues in normal text. [b] Orientation defined by the convention introduced by Nielsen et al. See ref 10.

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(6) (a) For the synthesis of (Cbz)$GuCH_2CO_2H$, see: Coull, J. M.; Hodge, R. P. Patent WO, 95/17403, 1995. (b) For synthesis of (Cbz)$AdCH_2CO_2H$ and $ThCH_2CO_2H$, see: Thomson, S. A.; Josey, J. A.; Cadilla, R.; Gaul, M. D.; Hassman, C. F.; Luzzio, M. J.; Pipe, A. J.; Reed, K. L.; Ricca, D. J.; Wiethe, R. W.; Noble, S. A. *Tetrahedron* **1995**, *51(22)*, 6179−6194. (c) (Cbz)$CyCH_2CO_2H$ was synthesized using an independent method.

efficiency of >95% for each cycle. The PNA portions of PNA-APNA chimeras were synthesized from Fmoc/Cbz-protected monomers, prepared as previously described,[6b] followed by oligomerization on solid support using standard chain elongation reactions.[8] The oligomers were then cleaved from the resin and purified as previously described.[5]

Hybridization of (APNA-PNA)$_2$:DNA or (APNA-PNA)$_2$:RNA Triplexes. The hybridization properties of triplexes between the PNA decamer **9** and complementary DNA or RNA were examined by T_m measurements and compared to those of the APNA-PNA chimeras **10**−**12** (Table 2). The

Table 2. Summary of T_m Values for Complexes Formed between PNA **9**−**12** and DNA **18** or RNA **19**

	T_m[a] of PNA strand			
target strand	**9**	**10**	**11**	**12**
18	34	27	26	29
19	51	40	35	38

[a] PNA:DNA or PNA:RNA molar ratio was 1:1. Solutions were 4−5 μM in both PNA and DNA or RNA. Samples were heated from 5 to 95 °C and/or cooled from 90 to 5 °C at a rate of 0.5 °/min, and the absorbance at 260 nm was monitored as a function of temperature. T_m values are the maxima of the first derivative plots of the absorbance versus temperature data. Buffer conditions: 150 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH = 7.0.

hybridization affinities of all the oligomers with complementary antiparallel RNA strands were always greater than those observed with the complementary antiparallel DNA strands, consistent with the known properties of PNAs (Table 1).[1] Although the APNA-PNA chimeras **10**−**12** gave complexes with both DNA and RNA that were thermally less stable than those of the PNA decamer **9**, they were significantly more stable than the corresponding DNA:DNA and DNA:RNA complexes under the same conditions (data not shown). The destabilization observed with chimera **10** was consistent with our previous hybridization results for this class of monomer.[5] Moreover, since APNA-PNA decamer **12** was found to hybridize equally or more favorably than the APNA-PNA decamers **10** and **11**, it seemed that multiple insertions of APNA monomers into PNA oligomers was well tolerated. Job plots[9] confirmed a 2:1 stoichiometry of binding between the PNA strands, or the APNA-PNA chimeras, and the DNA or RNA oligomers. In addition, the T_m values showed a dependence on the pH of the solution, indicating that the complexes formed were most likely triplexes, presumably involving Watson−Crick and Höogsteen base pairing.

Hybridization of APNA-PNA/DNA or APNA-PNA/RNA Duplexes. Similar experiments were conducted with APNA-PNA chimeras designed to form duplex structures with DNA and RNA. A base sequence previously described by Nielsen and co-workers was chosen so that we could make direct comparisons to published data (i.e., oligomer **13**, Table 3).[10,11] In our hands, the PNA decamer **13** gave T_m values of

Table 3. Summary of T_m Values for Complexes Formed between PNA **13**−**17** and Oligonucleotides **20**−**26**

	T_m[a] of PNA strand				
target strand	**13**	**14**	**15**	**16**	**17**
20	54	40	30	43	24
21	39	34	26	28	<10
22	58	48	38	45	26
23	42	31	27	28	20
24	34[b]	27	nd	nd	nd
25	36	nd	17	nd	nd
26	44	nd	nd	38	nd

[a] Duplex concentration = 4−5 μM. T_m experiments were performed as described for data presented in Table 2. Buffer conditions: 100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA, pH = 7.0. [b] Mismatched base in compounds **24**−**26** is indicated in bold text (see Table 1).

54 °C (antiparallel, **13**:**21**) and 39 °C (parallel, **13**:**22**) with DNA (Table 3)[12] and slightly higher values with RNA (T_m = 58 °C for antiparallel, **13**:**23**) and (T_m = 42 °C for parallel, **13**:**24**). Surprisingly, the degree of destabilization observed upon introduction of a single APNA unit in the middle of an antiparallel PNA:DNA duplex (Table 3, **14**:**20**) was far greater than that observed for the PNA$_2$:DNA triplex (Table 2, **10**:**18**). However, the degree of destabilization observed with the parallel PNA:DNA, antiparallel PNA:RNA and parallel PNA:RNA duplexes was similar in magnitude to that observed with the corresponding triplexes reported in Table 2.

Subsequently, the effects of longer APNA inserts into the APNA-PNA chimeras were examined. Incorporation of two APNA units (decamer **15**) led to further destabilization of the complexes formed with both DNA (**15**:**20** and **15**:**21**) and RNA (**15**:**22** and **15**:**23**). However, chimeras **16** which is composed of four APNA units at the N-terminal of the decamer did not exhibit the same degree of destabilization per APNA insert as chimera **15** relative to the control oligomer **13**. These results further support the existence of some cooperative intraresidue interaction between adjacent APNA momoners that enhances the stability of the duplexes

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(9) Job, P. *Ann. Chim. (Paris)* **1928**, *9*, 113−203.

(10) (a) Hyrup, B.; Egholm, M.; Nielsen, P. E.; Wittung, P.; Nordén, B.; Buchardt, O. *J. Am. Chem. Soc.* **1994**, *116*, 7964. (b) Sforza, S.; Haaima, G.; Marchelli, R.; Nielsen, P. E. *Eur. J. Org. Chem.* **1999**, *197*, 7−204.

(11) In some cases the N-terminal of the oligomers are capped with an acetate unit in order to prevent acyl transfer of the last nucleobase-carboxymethyl moiety. However, this measure was not taken with chimeras **14**−**17** since the sequences studied in ref 13 were not capped in this way. For comments on the influence a positively charged N-terminal has on the stability of PNA:DNA complexes, see: Egholm, M.; Buchardt, O.; Nielsen, P. E.; Berg, R. H. *J. Am. Chem. Soc.* **1992**, *114*, 1895.

(12) The T_m value given for this PNA sequence in refs 10a,b was 50 °C in the case of the antiparallel complex.

formed. These results are also consistent with the hybridization data of the triplexes (Table 2). It should be noted that well-defined melting curves were obtained for all duplexes reported in Table 3. The melting curves observed in these experiments were distinctly different from those observed by measuring the UV of each corresponding single strand as a function of temperature. In cases where the T_m of the transition assigned to the melting of the PNA:DNA or PNA:RNA duplex was similar to an apparent transition of the single strand alone, genuine transitions corresponding to melting of the duplexes could be distinguished by recording the T_m experiments as a function of both increasing and decreasing temperature. The single strands showed pronounced hysteresis, whereas the duplexes did not, suggesting that the sigmoidal curves obtained with the PNA:DNA or PNA:RNA mixtures are due to the melting of the expected duplexes. Furthermore, the T_m values of the duplexes formed between the C-terminal PNA_6 segment of the APNA-PNA chimera **16** (i.e., hexamer **17**) with the same complementary DNA or RNA decamers were found to be significantly lower than those of decamer **16**. The latter results clearly prove the participation of the N-terminal $APNA_4$ segment of decamer **16** in the duplexes formed with its complementary natural oligonucleotides (Table 3).

The introduction of single-point mismatches in the complementary DNA strands (compounds **24**−**26**) opposite to APNA residues (chimeras **14**−**16**) led to the anticipated decrease in the T_m values of the corresponding duplexes as compared to the fully complementary duplexes (Table 3). These results strongly support that the APNA monomers participate in Watson−Crick interactions and contribute to the overall sequence-specific recognition and binding of decamers **14**, **15**, and **16** to complementary DNA and RNA. It is conceivable that the destabilization observed upon insertion of an APNA monomer into a PNA oligomer may be due to a conformational incompatibility and/or distance between units at the PNA_1-APNA-PNA_2 junctions; studies that address this questions are currently in progress.

Duplex formation between compounds **13**−**17** and oligonucleotides **20** and **22** was also confirmed using the molecular recognition technology developed by Armitage and coworkers.[13] It has been shown that cyanine dye 3-ethyl-2-[5-(3-ethyl-3*H*-benzothiazol-2-ylidene)penta-1,3-dienyl]benzothiazol-3-ium iodide (**DiSC$_2$(5)**) binds to the minor groove of duplexes and triplexes (composed in whole or in part of PNA oligomers) in a spontaneous and cooperative fashion as a highly ordered aggregate. The binding is accompanied by a hypsochromic shift of the main visible absorption band of the dye, allowing for convenient detection of PNA containing duplexes or triplexes (see Supporting Information for the absorption spectra of antiparallel[14] duplexes **13**:**20** and **13**:**22**, Figures S2a and S2b, respectively). Similar results were obtained with all the complexes formed between compounds **13**−**16** and oligonucleotides **20** and **22** (for example, see figures S2c and S2d for antiparallel duplexes **15**:**20** and **15**:**22**).[15]

Although the observed trend in the hybridization properties of the APNA-PNA chimeras **10**−**12** and **14**−**16** cannot be fully explained by our present data, it appears that continuous stretches of APNA monomers are well tolerated in both duplexes and triplexes. In an attempt to evaluate the binding properties of APNA homopolymers, and any plausible cooperative stabilization via intraresidue interactions, a decamer composed of only APNA units was synthesized. Unfortunately, this molecule was insoluble in aqueous solvents, prohibiting hybridization studies. The synthesis of more soluble APNA analogues, composed of heteroaromatic and substituted phenyl moieties along the backbone, is currently underway.

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Supporting Information Available: ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR spectra for compounds **3** and **1a**−**d**, ^{1}H NMR spectra for dimers **8a**−**e**, MS and HPLC data for oligomers **9**−**16**, and $\mathbf{T_m}$ (first derivative) curves for thermal denaturation of the complexes indicated in Tables 2 and 3. This material is available free of charge via the Internet at http//pubs.acs.org.

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(13) Smith, J. O.; Olson, D. A.; Armitage, B. A. *J. Am. Chem. Soc.* **1999**, *121*, 2686−2695.

(14) Only the antiparallel complexes were examined for binding to **DiSC$_2$(5)**. The authors of ref 13 have demonstrated that the dye binds with very low affinity to parallel PNA complexes.

(15) In a series of control experiments, the UV−vis spectra of compounds **13**−**17** in the presence of **DiSC2(5)** were recorded at 40 and then 7 °C, in the absence of complementary oligonucleotide **20** or **22**. No significant change in the spectra was observed, indicating that a (**DiSC$_2$(5)**)$_n$:ssAPNA-PNA aggregate was not responsible for the observed spectral changes.