

PyrrolidinyI Peptide Nucleic Acid Homologues: Effect of Ring Size on Hybridization Properties

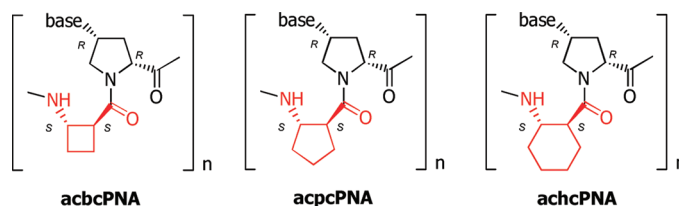
Woraluk Mansawat,[†] Chotima Vilaivan,[†] Árpád Balázs,[‡] David J. Aitken,[‡] and Tirayut Vilaivan^{*†}

Organic Synthesis Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Road, Patumwan, Bangkok 10330, Thailand, and Laboratoire de Synthèse Organique & Méthodologie, ICMO – CNRS UMR 8182, Université Paris-Sud, 15 rue Georges Clemenceau, 91405 Orsay cedex, France

vtirayut@chula.ac.th

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ABSTRACT



The effect of ring size of four- to six-membered cyclic β -amino acid on the hybridization properties of pyrrolidinyI peptide nucleic acid with an alternating $\alpha\beta$ peptide backbone is reported. The cyclobutane derivatives (acbcPNA) show the highest T_m and excellent specificity with cDNA and RNA.

Nucleic acids form higher order structures in a predictable fashion as a result of the highly specific Watson–Crick type base pairing. This property is essential for the function of nucleic acids in the storage and transfer of genetic information in all living organisms. It is also the basis of various applications, ranging from biomedical sciences through biotechnologies to material sciences.¹ In recent years, there has been considerable interest in the design of alternative nucleic acid recognition systems that may offer advantages over DNA or RNA in terms of chemical stability, binding affinities, and/or specificities toward different types of nucleic acids. Peptide nucleic acid (PNA) is a class of nucleic acid analogues in which a peptide-like backbone replaces the deoxyribose-phosphate backbone of DNA. In addition to being able to recognize its cDNA/RNA with high affinity and sequence specificity, the electrostatically neutral nonphosphate backbone of PNA gives rise to a number of unique properties not observed in other classes of DNA analogues,

such as complete resistance to nucleases and the relative insensitivity of PNA·DNA hybrids to ionic strength.² Conformational restriction of the *N*-aminoethylglycyl backbone of the original PNA (now known as aegPNA), for example, by incorporation of a cyclic moiety or one or more substituents along the PNA backbone, can provide PNA with an improved binding affinity as well as specificity.^{3–5}

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[†] Chulalongkorn University.

[‡] Université Paris-Sud.

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Inspired by the discovery that oligomers of cyclic β -amino acids can adopt well-defined helical conformations,^{6,7} we previously developed a series of pyrrolidiny PNA with an α/β peptide backbone consisting of an alternating sequence of nucleobase-modified proline and cyclic β -amino acid “spacer” such as 1-aminopyrrolidinecarboxylic acid (dapcPNA)⁸ or 2-aminocyclopentane-carboxylic acid (acpcPNA).⁹ These pyrrolidiny PNAs bind to DNA with higher affinity and sequence specificity compared to aegPNA.¹⁰ They also exhibited some unusual features, including the preference for binding to DNA over RNA as well as the inability to form self-hybrids. The failure of pyrrolidiny PNA carrying acyclic spacers to recognize DNA/RNA targets¹¹ suggested that the cyclic five-membered ring spacer locks the conformation of the PNA into a form that is close to optimal for DNA binding. The work carried out so far has been limited to a five-membered ring spacer, and in order to gain further insight into the structural properties of these pyrrolidiny PNA, an investigation of the effect of the ring size of the spacer part was warranted. Modification of the ring size should change both the rigidity and the torsional angle (θ) of the NH–C2–C1–CO which is an integral part of the PNA backbone⁷ and could in turn significantly affect the hybridization properties of the resulting PNA.

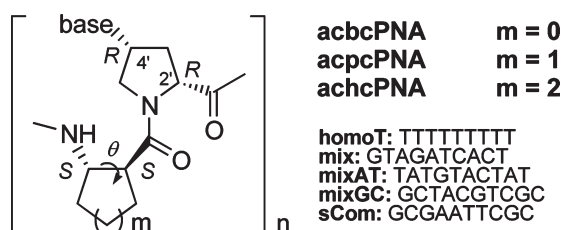


Figure 1. Structures and sequences of PNA.

We now report on the behavior of two new PNA manifolds, which incorporate 2-aminocyclobutane-carboxylic acid (ACBC) or 2-aminocyclohexane-carboxylic acid (ACHC) spacers, referred to here as acbcPNA and achcPNA respectively, and compare them with acpcPNA

which contains a 2-aminocyclopentane-carboxylic (ACPC) spacer (Figure 1). Since the (1*S*,2*S*) configuration was previously found to be optimal for the ACPC spacer,⁹ the same (1*S*,2*S*) configuration was a logical starting point for the new ring systems. Thus, the Fmoc-derivative of (1*S*,2*S*)-ACBC¹² was prepared, along with the known Fmoc-(1*S*,2*S*)-ACHC,¹³ and each of these derivatives was combined with the (4'*R*)-nucleobase-modified (2'*R*)-proline employing the Fmoc solid-phase peptide synthesis previously developed¹⁴ to give eight new pyrrolidiny PNA sequences as shown in Table S1, Supporting Information. All of these pyrrolidiny PNA possess the same (2'*R*,4'*R*) configuration at the proline part except for *epi*-acbcPNA-sCom. This latter PNA, with a (2'*R*,4'*S*) configuration, was synthesized in order to investigate the self-pairing abilities not observed in the (2'*R*,4'*R*) epimer of acpcPNA.¹⁵ The PNA was purified by HPLC (>90% purity), and the identities confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. It is noteworthy that the intrinsically labile ACBC¹⁶ is compatible with the basic conditions required during Fmoc group removal and nucleobase side chain deprotection procedures.

Table 1. Thermal Stabilities of DNA and RNA Hybrids of Homologous Pyrrolidiny PNA^a

entry	PNA	apDNA	pDNA	apRNA	pRNA
1	acbcPNA-homoT	73.8 (72.5)	—	n.d.	n.d.
2	acbcPNA-mixAT	63.2 (54.2)	<20 (<20)	50.8 (32.6)	25.8 (<20)
3	acbcPNA-mix	66.1 (53.3)	<20 (<20)	58.2 (42.3)	31.7 (<20)
4	acbcPNA-mixGC	69.8 (54.5)	34.1 (<20)	62.9 (48.0)	48.6 (32.9)
5	achcPNA-homoT	<20 (72.5)	—	n.d.	n.d.
6	achcPNA-mix	<20 (53.3)	<20 (<20)	<20 (42.3)	<20 (<20)

^a T_m values of the corresponding acpcPNA hybrids under identical conditions⁹ are shown in parentheses, and ap and p are antiparallel and parallel, respectively. Conditions: 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, and [PNA] = [DNA] = 1 μ M. T_m values are accurate to within ± 0.5 °C.

The hybridization properties of **acbcPNA-homoT** and **achcPNA-homoT** with cDNA (dA₉) were investigated by thermal denaturation experiments (monitored by UV–vis at 260 nm) (Table 1, entries 1 and 5). A well-defined thermal denaturation curve was observed only with **acbcPNA-homoT**. The melting temperature (T_m) of

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73.8 °C is comparable to that of acpcPNA with the same sequence (72.5 °C). Circular dichroism (CD) experiments also clearly confirmed that the **acbcPNA-homoT**, but not **acbcPNA-homoT**, could form a stable hybrid with dA₉ (Figure S9, Supporting Information). The melting curve of the hybrid between **acbcPNA-homoT** and dA₉, as monitored by CD spectroscopy, was in good agreement with that obtained from UV–vis experiments (Figure 2).

Although homopyrimidine PNAs generally form (PNA)₂·DNA triplexes by Watson–Crick/Hoogsteen base pairing,¹⁷ UV and CD titrations of **acbcPNA-homoT** and dA₉ (Figure S10, Supporting Information) clearly showed that only the 1:1 duplex was formed. The lack of triplex formation is analogous to acpcPNA, which may be ascribed to the steric repulsion of the bulky acpcPNA/acbcPNA backbone that destabilizes such triplexes.⁹

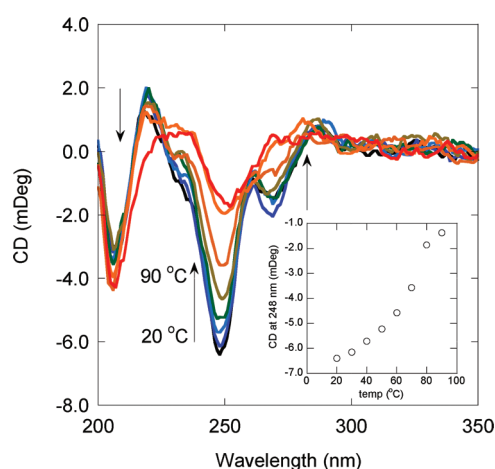


Figure 2. CD spectra of hybrid of **acbcPNA-homoT** and dA₉ at different temperatures (a) and change of CD signal at 248 nm as a function of temperature (b). Conditions: 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, and [PNA] = [DNA] = 1 μM.

The binding studies of a mixed sequence PNA with DNA and RNA in both antiparallel and parallel orientations were performed on three different acbcPNA sequences with various G+C contents (**acbcPNA-mixAT**, **acbcPNA-mix**, **acbcPNA-mixGC**) and one achcPNA sequence (**achcPNA-mix**) (Table 1 and Figure S11, Supporting Information). The results were compared with the corresponding T_m values from acpcPNA with identical sequences.⁹ Several important observations were made. First, the acbcPNA binds to DNA and RNA preferentially in an antiparallel direction, although it can also form quite stable parallel acbcPNA·RNA hybrids. Second, both acbcPNA·DNA and acbcPNA·RNA hybrids show higher thermal stabilities than the corresponding acpcPNA hybrids. The extent of T_m increase depends on G+C content, ranging from 9 °C (20% G+C) to 15 °C (70% G+C) in acbcPNA·DNA hybrids and from 15 °C

(70% G+C) to 18 °C (20% G+C) in acbcPNA·RNA hybrids. Third, the general preference for binding to DNA over RNA in the original acpcPNA was still observed in acbcPNA. Finally, the achcPNA with a mix sequence (**achcPNA-mix**) did not show any binding to cDNA or RNA in both antiparallel and parallel orientations.

The results from T_m experiments were also further confirmed by CD spectroscopy. The binding of **acbcPNA-mix** to antiparallel DNA, antiparallel RNA and parallel RNA resulted in a significant change in CD spectra compared to the sum of each component (Figure S12, Supporting Information). On the other hand, no such change was observed upon binding of **acbcPNA-mix** to parallel DNA as well as in all DNA and RNA hybrids with **achcPNA-mix** (Figure S13, Supporting Information).

Table 2. Thermal Stabilities of Mismatched DNA Hybrids of **acbcPNA-mix**

entry	DNA (5'→3')	T_m (°C) ^a	ΔT_m (°C) ^b
1	dAGTGATCTAC	66.1 (53.3)	—
2	dAGTAATCTAC	40.4 (26.4)	−25.7 (−26.9)
3	dAGTTATCTAC	40.6 (27.8)	−25.5 (−25.5)
4	dAGTCATCTAC	42.8 (31.0)	−23.3 (−22.3)
5	dAGTGGTCTAC	36.4 (23.9)	−29.4 (−29.5)
6	dAGTGTTCTAC	46.6 (29.4)	−19.5 (−23.9)
7	dAGTGCTCTAC	39.8 (23.8)	−26.3 (−29.5)
8	dAGTGAGCTAC	46.0 (26.5)	−20.1 (−26.8)
9	dAGTGAACCTAC	47.0 (28.4)	−19.1 (−24.9)
10	dAGTGACCTAC	45.1 (28.8)	−21.0 (−24.5)
11	dAGTGATGTAC	41.2 (26.4)	−24.9 (−26.9)
12	dAGTGATATAC	38.3 (24.2)	−27.8 (−29.1)
13	dAGTGATTAC	44.6 (28.5)	−21.5 (−24.8)

^a T_m values of the corresponding acpcPNA hybrids under identical conditions⁹ are shown in parentheses. Conditions: 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, and [PNA] = [DNA] = 1 μM. ^b T_m (complementary) − T_m (mismatched). T_m values are accurate to within ±0.5 °C.

Specificity is perhaps one of the most important aspects of any nucleic acid recognition systems. The T_m of hybrids between the PNA **acbcPNA-mix** and various DNA targets carrying a mismatched base at the positions 4–7 (Table 2) showed that the specificity (determined from the difference of T_m values between mismatched and cDNA hybrids) was comparable to acpcPNA. A T_m decrease of > 20 °C was observed in all but two cases (pT·dT and pA·dA mismatch, which showed ΔT_m of −19.5 and −19.1 °C, respectively). This level of mismatch discrimination is better than the corresponding DNA and the original aegPNA (typical ΔT_m −10 to −14 °C).²

Another unique property of acpcPNA is the inability to form self-pairing hybrids between two complementary acpcPNA sequences.⁹ This could lead to a number of useful applications in targeting DNA duplexes by a double duplex invasion mechanism that has been traditionally achieved with pseudocomplementary aegPNA bearing modified bases (2,4-diaminopurine/2-thiouracil).¹⁸

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According to UV and CD melting studies, the PNA **acpcPNA-sCom** with a 10-mer self-complementary sequence showed no cooperative melting. On the other hand, **acbcPNA-sCom** showed a broad UV melting curve with apparent T_m of ca. 45 °C. At 20 °C, the same PNA showed a positive CD signal at 260 nm and a strong negative CD signal at 280 nm, which is distinctly different from the CD spectra of nonself-complementary acbcPNA (compare Figures S12 and S16, Supporting Information). These signals disappeared upon heating and reappeared upon cooling. The results suggest that **acbcPNA-sCom** can form a self-complementary hybrid. We also synthesized the (2'*R*,4'*S*) epimer of both acbcPNA and acpcPNA to compare the self-pairing behavior with the "normal" acpcPNA and acbcPNA. As shown in Table 3 and Figures S15 and S16, Supporting Information, the **epi-acbcPNA-sCom** self-pairing hybrid was considerably more stable than the corresponding **epi-acpcPNA-sCom** self-pairing hybrid. All self-complementary pyrrolidiny PNAs can form stable hybrids with DNA with T_m values in the range of 67 °C (acpcPNA) to 73–78 °C (acbcPNA). In all cases, the PNA·DNA hybrids are more stable than the PNA·PNA hybrids at the same total strands concentration. The difference between T_m of PNA·DNA and PNA·PNA hybrids is smallest in **epi-acbcPNA** (ΔT_m = 14 °C) and greatest in acpcPNA (ΔT_m > 46 °C).

Table 3. Thermal Stabilities of Self-Complementary and DNA Hybrids of acbcPNA and acpcPNA^a

entry	PNA	config	T_m self	T_m apDNA
1	acbcPNA-sCom	2' <i>R</i> ,4' <i>R</i>	45.7	77.7
2	acpcPNA-sCom	2' <i>R</i> ,4' <i>R</i>	<20	66.8
3	epi-acbcPNA-sCom	2' <i>R</i> ,4' <i>S</i>	59.3	73.3
4	epi-acpcPNA-sCom	2' <i>R</i> ,4' <i>S</i>	32.7	66.8

^a Conditions: 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, and [PNA] = 2 μ M (self-hybridization) or [PNA] = [DNA] = 1 μ M (PNA·DNA hybridization). T_m values are accurate to within ± 0.5 °C.

Since the absence of NH in proline residues does not allow the formation of regular 11- and 14/15-helices usually observed in α/β peptides,¹⁹ the preorganization into helical conformations of these α/β pyrrolidiny PNA should be governed by the limited conformational space available to the cyclic β -amino acid spacer rather than hydrogen bonding. Based on our previous molecular dynamics simulations,²⁰ the origin of stabilization of acbcPNA·DNA relative to acpcPNA·DNA hybrids

might be explained by a more favorable preorganization of acbcPNA as follows: Models of dpcPNA and acpcPNA showed the values of torsional angle NH–C2–C1–CO (θ) (Figure S17, Supporting Information) of the D-APC and (*S,S*)-ACPC spacers of $99.2 \pm 6.4^\circ$ and $99.2 \pm 3.1^\circ$ (averaged from 10 D-APC and 13 ACPC residues, respectively). A relatively large difference between these values compared to the corresponding θ figure from an X-ray structure of (*R,R*)-ACPC oligomer ($-85.9 \pm 12.2^\circ$; averaged from 6 residues)²¹ suggests that there must be a substantial conformational reorganization of the ACPC moiety to allow optimal binding to DNA. While the five-membered ring of ACPC is flexible enough to allow this, there is an entropic penalty associated with the conformational reorganization process. On the other hand, the torsional angle θ obtained from an X-ray structure of (*R,R*)-ACBC oligomer ($-95.5 \pm 3.7^\circ$; averaged from 8 residues)²² is closer to the optimal θ values shown above. Hence, the acbcPNA is better preorganized for binding to DNA when compared to acpcPNA. The torsional angle θ of ACHC obtained from an X-ray structure of (*S,S*)-ACHC hexamer was in the range of only 51.5 – 61.4° .²³ The large deviation from the optimal θ , together with the rigidity of the cyclohexane ring system which does not allow efficient conformational reorganization, should explain the inability of achcPNA to pair with DNA. The origin of the greater stabilization of acbcPNA·RNA compared to acpcPNA·RNA hybrids cannot be explained without further structural information, but the ability to bind strongly to RNA in addition to DNA clearly suggests potential of applications of acbcPNA involving RNA targets.

In conclusion, we have successfully synthesized homologues of acpcPNA with four-membered ring (acbcPNA) and six-membered ring (achcPNA) spacers. The replacement of ACPC with ACBC resulted in a new pyrrolidiny PNA with general binding properties similar to acpcPNA but with an increase of T_m with cDNA and RNA hybrids by 0.9–1.8 °C per modification without compromising the specificity. In contrast, the replacement of ACPC with ACHC resulted in total loss of binding to both DNA and RNA. The results are explained by a more favorable conformational preorganization of the ACBC residue compared to ACPC and ACHC.

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Supporting Information Available. Synthesis of Fmoc-(1*S*,2*S*)-ACBC-OH and NMR spectra, analytical HPLC and MALDI-TOF mass spectral data of PNA, and additional UV and CD spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

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