Pyrrolidinyl peptide nucleic acid with α/β-peptide backbone

A conformationally constrained PNA with unusual hybridization properties

Chotima Vilaivan,[1] Choladda Srisuwannaket,[2] Cheeraporn Ananthanawat,[1] Chaturong Suparpprom,[3] Junji Kawakami,[4,5] Yoshie Yamaguchi,[4,5] Yuko Tanaka[4,5] and Tirayut Vilaivan[1,*]

[1]Organic Synthesis Research Unit; Department of Chemistry; Faculty of Science; Chulalongkorn University; Patumwan, Bangkok, Thailand; [2]Department of Chemistry; Faculty of Science;
King Mongkut's University of Technology Thonburi; Thungkru, Bangkok, Thailand; [3]Department of Chemistry and Center of Excellence for Innovation in Chemistry; Faculty of Science; Naresuan University; Muang, Phitsanulok Thailand; [4]Department of Nanobiochemistry; FIRST; Konan University
[5]Frontier Institute for Biomolecular Engineering Research (FIBER); Konan University; Chuo-ku, Kobe, Japan

We describe herein a new conformationally constrained analog of PNA carrying an alternating α/β amino acid backbone consisting of (2'R,4'R)-nucleobase-subtituted proline and (1S,2S)-2-aminocyclopentanecarboxylic acid (acpcPNA). The acpcPNA has been synthesized and evaluated for DNA, RNA and self-pairing properties by thermal denaturation experiments. It can form antiparallel hybrids with complementary DNA with high affinity and sequence specificity. Unlike other PNA systems, the thermal stability of acpcPNA·DNA hybrid is largely independent of G+C contents, and is generally higher than that of acpcPNA·RNA hybrid with the same sequence. Thermodynamic parameters analysis suggest that the A·T base pairs in the acpcPNA·DNA hybrids are enthalpically stabilized over G·C pairs. The acpcPNA also shows a hitherto unreported behavior, namely the inability to form self-pairing hybrids. These unusual properties should make the new acpcPNA a potentially useful candidate for various applications including microarray probes and antigene agents.

Introduction

Almost 20 years ago, Nielsen et al. demonstrated an unusually high affinity as well as sequence specificity in recognition of DNA and RNA by a new class of DNA analog called peptide nucleic acid (PNA).[1-3] In many aspects, PNA behaves just like a structural mimic of natural nucleic acids.[4] However, PNA also displays many unique characteristics not found in DNA/RNA or analogs that derived from partial modification of the natural sugar-phosphate backbone.[5] The electrostatically neutral backbone of PNA contributes to the enhanced stability of PNA·DNA hybrids, which is also relatively independent of salt concentrations In addition to binding to single stranded nucleic acid targets, PNA can bind, either by a triplex formation or strand invasion, to double stranded DNA[7,8] as well as RNA targets.[9] The unnatural backbone of PNA makes it stable towards degradation by nucleases and proteases.[10] These properties make PNA a potential candidate for many applications ranging from nucleic acid detection probes[11,12] to therapeutic agents via antisense or antigene applications.[13,14]

Until recently, the original PNA system derived from an achiral backbone of N-2-aminoethylglycine (now generally referred to as aegPNA) has been the most widely studied. Although a number of new PNA systems have been developed in the past decades, few of them offer comparable or better hybridization properties found in the original aegPNA.[15-21] It has been argued that the binding properties should be improved if one could restrict the conformational flexibility of the PNA backbone, for example, by locking the PNA backbone into cyclic structures[15-18] or by adding one or more substituents at a specific position of the aegPNA backbone.[19-21] In addition to improving binding affinity and specificity, conformational restriction should offer a possibility to selectively recognize different types of nucleic acids. While aegPNA has been shown to bind marginally more strongly to RNA over DNA, the selectivity towards RNA has been improved by incorporating one or more cyclohexane rings into the PNA backbone.[16,17] Kinetic selectivity in RNA recognition over DNA has been observed in some pyrrolidinyl PNA variants,[22,23] although this behavior seems to be highly sequence-dependent.[24,25] In a much fewer instances, preference for DNA binding over RNA has also been reported.[26]

Recently, oligomers with α/β peptide backbone were recognized as a potential foldamer system due to their well defined helical conformation.[27-29] We have recently developed a new class of pyrrolidinyl PNA consisting of an alternate sequence
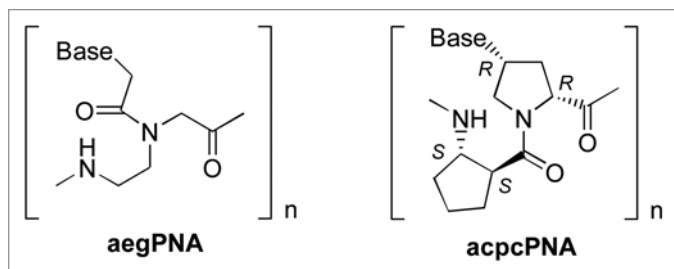
Figure 1. Structures of aegPNA and the pyrrolidinyl PNA carrying 2-amino-cyclopen-tanecarboxylic acid spacer (acpcPNA); B = nucleobase (A, C, G or T).

of nucleobase-modified proline and five-membered ring cyclic β-amino acids.[30-33] These pyrrolidinyl PNA retained the basic property of nucleic acid mimics, i.e., the ability to bind to their complementary nucleic acid targets in a sequence-specific fashion. The specificity towards DNA target is in fact considerably better than the original aegPNA system.[34] Applications of these pyrrolidinyl PNA as probes for DNA sequence determination have been successfully demonstrated Furthermore, our preliminary studies in a few representative sequences revealed additional unique features of these pyrrolidinyl PNA including the high directional specificity (parallel/antiparallel), the preference for binding to DNA over RNA, and the inability of two complementary PNA sequences to self-hybridize.[33] Nevertheless, from the limited data available, it is difficult to conclude whether these properties are general or limited to certain sequences as observed in many other cases.[24,25] Herein we describe a comprehensive study of DNA, RNA and self-pairing properties of the pyrrolidinyl PNA system with a backbone consisting of alternating nucleobase-modified proline with a (2'R,4'R)-configuration and (1S,2S)-2-aminocyclopentanecarboxylic acid (acpcPNA) (**Fig. 1**). The results clearly indicate that the high antiparallel selectivity, the lack of self-pairing, and the preference for binding to DNA over RNA are general properties of acpcPNA over a range of sequences with different G+C contents. They also further revealed an important new observation that the thermal stability of the acpcPNA·DNA hybrid is much less dependent on the G+C content than the corresponding acpcPNA·RNA hybrid.

Results and Discussion

Effect of stereochemistry on DNA binding. The backbone of the pyrrolidinyl PNA described in this study consists of an alternating nucleobase-modified proline and 2-aminocyclopentanecarboxylic acid, each of which carries two stereogenic centers. As a result, there are 16 possible stereoisomers of the pyrrolidinyl PNA. It is highly probable that the configuration of the monomers will exert significant influences on the ability of the PNA backbone to adopt the correct geometry required for binding with the nucleic acid targets. To systematically determine the effect of stereochemistry on the DNA binding properties, the spacer and the proline parts were optimized separately. Firstly, four diastereomers of pentathymidine (T$_5$) pyrrolidinyl PNA with different stereochemistry at the 2-aminocyclopentanecarboxylic acid

(acpc) part, namely (1S,2S)-, (1S,2R)-, (1R,2R)- and (1R,2S)-, but with the same (2'R,4'R)-configuration of the pyrrolidine part were synthesized. This particular configuration of the pyrrolidine part was chosen as the starting point because of its stereochemical similarity to natural nucleosides (assuming that the 2' and 4' positions in the pyrrolidine ring are equivalent to the 4' and 1' positions in natural nucleosides, respectively). Our previous results have also shown that conformationally restricted PNA derived from the (2'R,4'R)-pyrrolidine monomer can exhibit DNA and RNA binding properties.[30,31] Melting experiments of the hybrid formed between each of the four T$_5$ pyrrolidinyl PNA diastereomers and poly(dA) at 1:1 ratio of T and A nucleotides were carried out to evaluate the effect of the stereochemistry on the aminocyclopentanecarboxylic acid moiety to DNA binding. The thermal denaturation experiment (**Fig. 2A**) revealed that only the pyrrolidinyl PNA with a combination of (1S,2S)-acpc and (2'R,4'R)-pyrrolidine can form hybrids that are sufficiently stable to exhibit observable melting behavior above room temperature (T_m = 35.5°C). Accordingly, the (1S,2S)-acpc spacer was chosen for studying the effect of the pyrrolidine part. Three additional pyrrolidinyl PNAs with the same T$_5$ sequence but different stereochemistry at the pyrrolidine ring, i.e., (2'S,4'S), (2'R,4'S) and (2'S,4'R), all of which carry the same (1S,2S)-acpc spacer, were synthesized. The UV melting results suggested that the acpcPNA with (2'R,4'S) configurations on the proline ring could also form a stable hybrid with poly(dA) (T_m = 36.5°C) (**Fig. 2B**). These results, together with our earlier reported UV titration data[32] indicate that only the combination of (1S,2S)-acpc and (2'R,4'R)- or (2'R,4'S)-pyrrolidine could allow optimal binding with DNA. This paper will focus on the binding properties of the pyrrolidinyl PNA with (2'R,4'R)-proline and (1S,2S)-acpc backbone (**Fig. 1**), which will be simply referred as acpcPNA from this point onward. Synthesis and hybridization properties of the other pyrrolidinyl PNA diastereomer with (2'R,4'S)-pyrrolidine/ (1S,2S)-acpc backbone that also exhibits DNA binding properties are the subject of a separated investigation.

Hybridization studies of homothymine and homoadenine acpcPNA. Thermal denaturation experiments of the hybrids formed between the homothymine acpcPNA (**T5**, **T7**, **T9** and **T10**) and their complementary DNA (dA$_5$, dA$_7$, dA$_9$ and dA$_{10}$, respectively) were then carried out (**Fig. 3**). While the relatively short acpcPNA **T5** binds to poly(dA) giving a hybrid with a T_m value of 35.5°C (**Fig. 2A**), it does not show any melting when hybridized with dA$_5$. Evidently the hybrid formed between the short acpcPNA and DNA was too unstable. However, as observed with aegPNA, the thermal stability of the hybrid increased with the number of base pairs (T_m for 5-, 7-, 9- and 10-mer were <20, 55.5, 77.0 and >85°C, respectively). The T_m of the hybrid between the acpcPNA **T10** and DNA was so high that the melting was not yet complete at 90°C. These T_m figures are considerably higher than the (PNA)$_2$·DNA triplexes formed from homothymine aegPNA with comparable length (T_m for 6-, 8- and 10-mer were 31, 52 and 72°C, respectively).[37] This dramatic increase in the melting temperature as the number of base increases was further confirmed by variable temperature CD experiments (**Sup. Figs. 34 and 35**). In all cases the CD melting
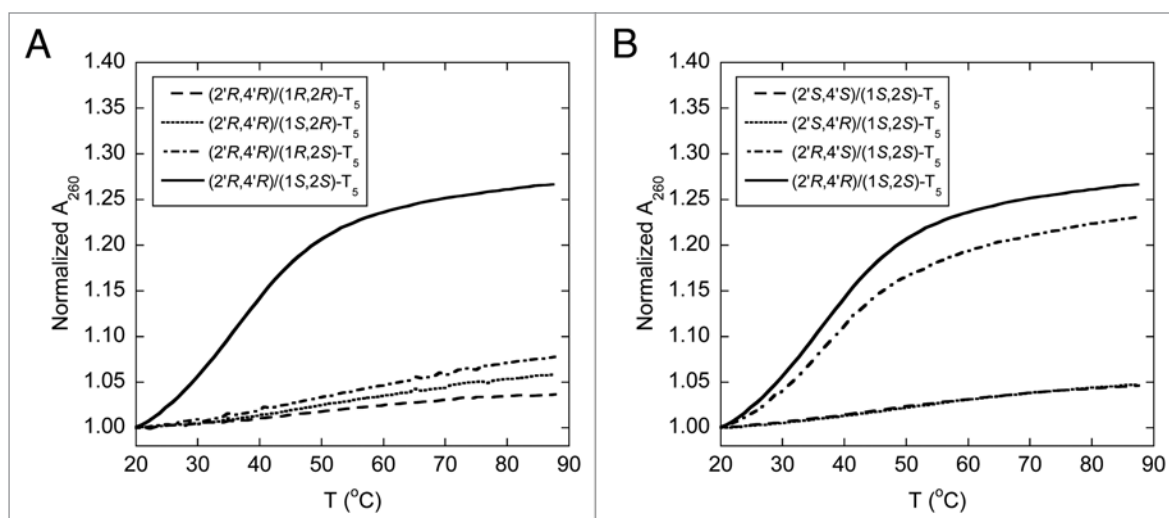
Figure 2. T_m curves of the hybrids between poly(dA) and T_5 pyrrolidinyl PNA with different stereochemistry on the aminocyclopentanecarboxylic acid part (A) and the proline part (B). Conditions: 10.0 μM PNA (by nucleotides), 10.0 μM DNA (by nucleotides), 10 mM sodium phosphate buffer pH 7.0, 0 mM NaCl, heating rate 1.0°C/min.

profiles were in good agreement with those obtained from UV melting studies. The melting process is completely reversible and shows very little hysteresis (**Sup. Fig. 36**), suggesting fast kinetics of the hybridization. The single transition observed in the melting curves, together with the data from CD and UV titration experiments (**Sup. Figs. 37 and 39**) suggested that 1:1 hybrids were formed between these homothymine acpcPNA and DNA. The formation of only acpcPNA·DNA duplexes with homopyrimidine sequences is in sharp contrast to the behavior of aegPNA and many other PNA systems whereby the formation of (PNA)$_2$·DNA triplexes was usually observed.[8,37,38] Steric bulkiness of the present acpcPNA backbone could be one important factor that make the binding of the third PNA strand to the PNA·DNA duplex initially formed difficult.

The melting behavior of the hybrid between homoadenine acpcPNA **A9** and its complementary DNA is somewhat more complex than the corresponding homothymine sequence. When the DNA component (dT$_9$) was present in excess, the melting proceeded in two steps (**Fig. 4A**). The first transition took place just above room temperature (~34°C) whereas the other appeared at a much higher temperature (>80°C). Such double-transition melting curve is explained by the formation of a (DNA)$_2$·PNA triplex. The triplex could form by Hoogsteen base pairing between the T base of the second strand of DNA to the originally formed Watson-Crick pA·dT pair. When the acpcPNA was present in excess, only the transition at the higher temperature remained, suggesting the presence of only the acpcPNA·DNA duplex in the solution (**Fig. 4A**). The triplex and duplex melting showed an interesting opposite salt-dependency. When the salt concentration was increased, the T_m of the triplex melting increased markedly. This could be explained by the screening effect of salt on the electrostatically repulsive interaction between the duplex and the third DNA strand. On the other hand, the T_m of the duplex melting slightly decreased at high salt concentrations, which is in accordance with the results observed in aegPNA·DNA hybrids,
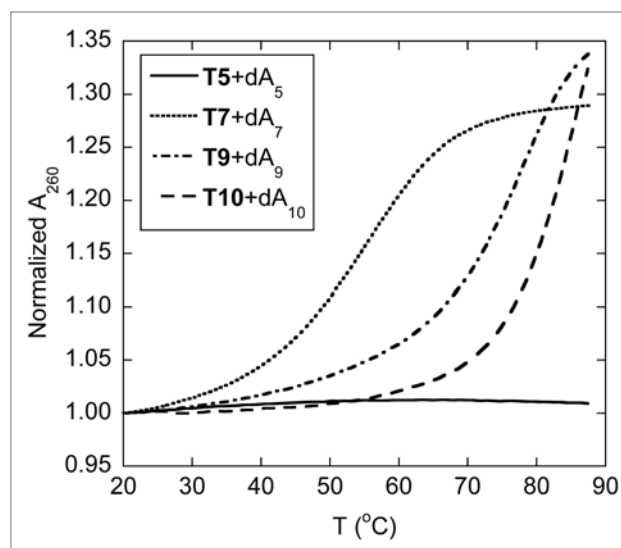

Figure 3. T_m curves of homothymine acpcPNA·DNA hybrids with different length. Conditions: 1.0 μM PNA, 1.0 μM DNA, 10 mM sodium phosphate buffer pH 7.0, 0 mM NaCl, heating rate 1.0°C/min.

and a similar explanation based on counterion release upon duplex formation should be applicable here.[6] At sufficiently high salt concentrations (~500 mM or higher), the two transitions merged to give just one melting curve, indicating a simultaneous dissociation of the triplex to the three single strand components at the same temperature.

The formation of a triplex structure between **A9** and dT$_9$ was also confirmed by UV titration and circular dichroism (CD) spectroscopy. The UV titration revealed two inflection points at acpcPNA:DNA ratio of 1:1 and 1:2 respectively (**Sup. Fig. 38**). The CD spectrum of single-stranded acpcPNA **A9** (**Fig. 4B**) shows strong signals in the region of nucleobase absorption around 265 nm. This suggests that the single stranded
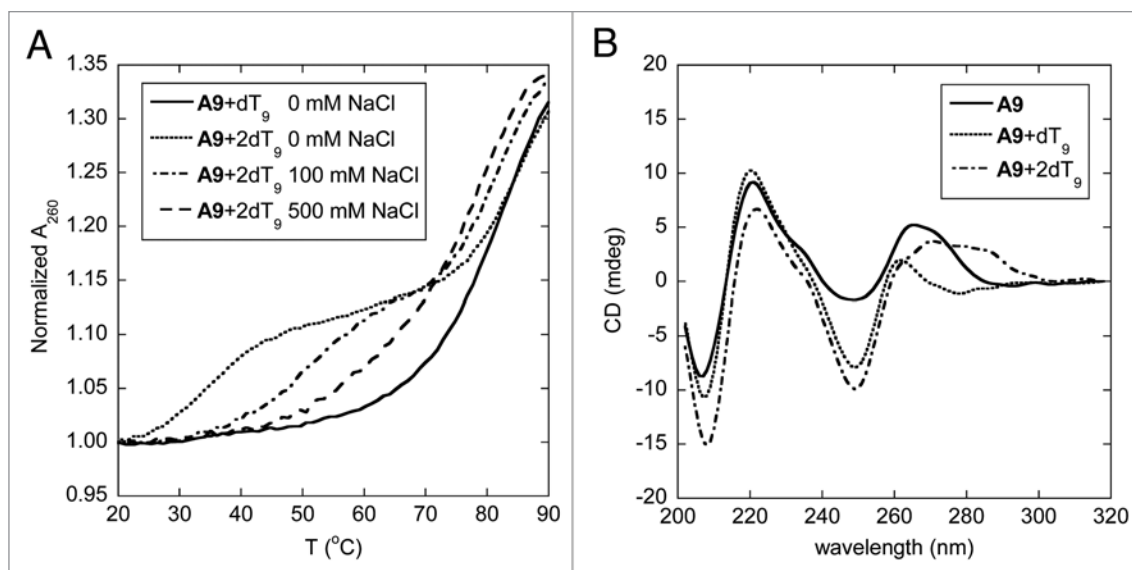
Figure 4. T_m curves (A) and CD spectra (B) of homoadenine acpcPNA·DNA hybrids at different ratio. Conditions: (A) 1.0 μM acpcPNA, 10 mM sodium phosphate buffer pH 7.0, 0–500 mM NaCl, heating rate 1.0°C/min; (B) 2.0 μM acpcPNA, 10 mM sodium phosphate buffer pH 7.0, 0 mM NaCl, 25°C.

homoadenine acpcPNA exists in a well-defined helical conformation, allowing effective stacking of the nucleobases. Addition of one equivalent of dT_9 to this acpcPNA solution caused a substantial spectral change in both position and magnitude of the CD bands in the nucleobase absorption region. The positive CD bands at 265 nm in the original single stranded acpcPNA shifted to a shorter wavelength of 260 nm in the duplex. This is also accompanied by an increase in the intensity of the negative CD band at 250 nm. The CD spectrum of this $A9·dT_9$ is remarkably similar to the CD spectrum of the $T9·dA_9$ duplex reported earlier.[32] Upon addition of the second equivalent of the DNA, a distinctive change was again observed in the nucleobase absorption region. The measured CD spectrum is clearly different from the sum of the CD spectra of each component, therefore it can be concluded that yet another species—most likely to be the triplex—is formed at 2:1 DNA:PNA ratio. In all cases, very little changes of the CD signal were observed in the peptide backbone region suggesting that there is little conformational change of the acpcPNA backbone upon hybrid formation.

Despite the extremely high stability of the homothymine and homoadenine acpcPNA hybrids with their complementary DNA, T_m data of mismatched acpcPNA·DNA hybrids (**Table 2**) revealed that the specificity is not compromised. It can be clearly seen that the base T in the acpcPNA **T9** can recognize only the base A in DNA, and the base A in the acpcPNA **A9** can recognize only the base T in DNA. Introduction of a mismatched base in the DNA strand caused a significant destabilization of the mismatched duplex as shown by the large decrease of T_m (ΔT_m) ranging from -23.4 to -37.8°C. The T_m decreases per mismatch in these cases are considerably larger than those typically observed in aegPNA (~10–15°C per mismatched base).[4]

Hybridization studies of mixed-base acpcPNA—directional preference and base pairing specificity. We have previously

shown that the mixed sequence PNA decamer **M10a** preferred hybridization with DNA in antiparallel orientation.[33] To ascertain that the antiparallel directional preference is a general behavior of the present acpcPNA system, additional T_m experiments were performed with nine different 10mer mixed-base PNA sequences (**Table 3**). Although the stability of acpcPNA·DNA hybrids is not expected to be very much dependent to the ionic strength as in the case of DNA·DNA hybrids, the melting studies were performed in 10 mM sodium phosphate buffer both in the absence and presence of NaCl (100 mM) conditions. All antiparallel hybrids showed single-transition melting curves with T_m in the range of 57.0–65.5°C (at 0 mM NaCl) and 52.8–61.0°C (at 100 mM NaCl). The slightly lower T_m values at high salt conditions compared to the low salt conditions (~4–5°C) are consistent with the behavior of aegPNA.[6] On the other hand, no sigmoidal transtitions could be observed when parallel DNA were present under both low salt and high salt conditions in all cases. This indicates that the parallel hybrids would have T_m values of less than 20°C for all nine sequences tested. The T_m difference by more than 30°C suggests that the antiparallel PNA·DNA hybrids are much more stable than the corresponding parallel hybrids. This property is in sharp contrast to the original aegPNA which could form both antiparallel and parallel hybrids with DNA, although the antiparallel hybrid was slightly more stable (ΔT_m antiparallel vs. parallel hybrids ~13°C for a 10mer mixed base aegPNA with the same sequence as **M10a**).[4] It has been observed that a modification of the aegPNA backbone with a short stretch of chiral monomer ("chiral box") could greatly enhance the antiparallel preference by destabilization of the parallel hybrid.[39] It appears that the presence of stereoregular backbone in these chiral PNAs allows binding to DNA in only one direction, thus enhancing the orientation selectivity relative to the achiral backbone of aegPNA.

The T_m of the complementary hybrid formed between the acpcPNA **M10a** and its complementary DNA was 53.3°C at

100 mM NaCl and 10 mM sodium phosphate. This figure is slightly higher than the corresponding aegPNA system with identical sequence (48.9°C) under comparable conditions.[15] To determine the base pairing specificity, the acpcPNA **M10a** was hybridized, one by one, with twelve other DNA probes carrying a mismatched base between the positions 4 to 7 of the sequence. The results, which are summarized in **Table 4**, indicated that the acpcPNA·DNA hybridization strictly follows the Watson-Crick base pairing rules and is highly specific. As observed in the case of homothymine or homoadenine acpcPNA hybrids with DNA, the presence of one mismatched base in the DNA part caused a substantial decrease in T_m values (-22.3 to -29.5°C) of the mixed sequence acpcPNA·DNA hybrids. With few exceptions, the pyrimidine-pyrimidine mismatched pairs tend to be more stable than the purine-purine mismatched pairs. The potentially wobble G·T pairs[40] showed a similar drop in T_m to other mismatched pairs. It is important to note that the T_m values of all single mismatched hybrids of acpcPNA are smaller than the corresponding mismatched hybrids of aegPNA. As a result, the differences in thermal stability between complementary and mismatched hybrids of the present acpcPNA system are significantly greater than those of the original aegPNA system (ΔT_m = -12.9 to -16.9°C for aegPNA/DNA with the same sequences).[15] The enhanced directional selectivity and discrimination of single base mismatched target of acpcPNA over aegPNA have also been independently verified by surface plasmon resonance (SPR) experiments in a different sequence.[34] Thus it can be concluded that the more rigid acpcPNA backbone improves both the binding affinity and the specificity for single base mismatches discrimination towards DNA targets.

Hybridization studies of mixed-base PNA—comparison between DNA and RNA. We have previously noted that the acpcPNA·DNA hybrids are more thermally stable than the corresponding acpcPNA·RNA hybrids in a few representative sequences.[33] In the present study, a larger pool of acpcPNA and RNA sequences were included to obtain a clearer picture. Comparison between the results shown in **Table 5** and **Table 3** confirmed our previous observation that the acpcPNA·DNA hybrids are more stable than acpcPNA·RNA hybrids with identical sequences in all cases. The results also unexpectedly revealed an interesting relationship between T_m and G+C content, which can be observed more clearly in **Figure 5**. The T_m values of the antiparallel acpcPNA·RNA hybrids appears to show relationships to the %G+C content in the same way as normal DNA/RNA duplexes, i.e., duplexes with greater %G+C content possess higher T_m values. The nine antiparallel acpcPNA·RNA hybrids included in this study showed T_m values between 32.6 and 51.2°C (T_m difference = 18.6°C). On the other hand, the T_m values for the corresponding antiparallel acpcPNA·DNA hybrids span a much smaller temperature range between 52.8 and 61.0°C (T_m difference = 8.2°C) and do not show obvious correlations with %G+C content. It has been earlier noted that aegPNA·DNA duplexes derived from purine-rich aegPNA were much more stable than those derived from pyrimidine-rich aegPNA with identical G+C content.[41] Such purine-stabilizing effect should be minimal here since all acpcPNA sequences used in this study contain a
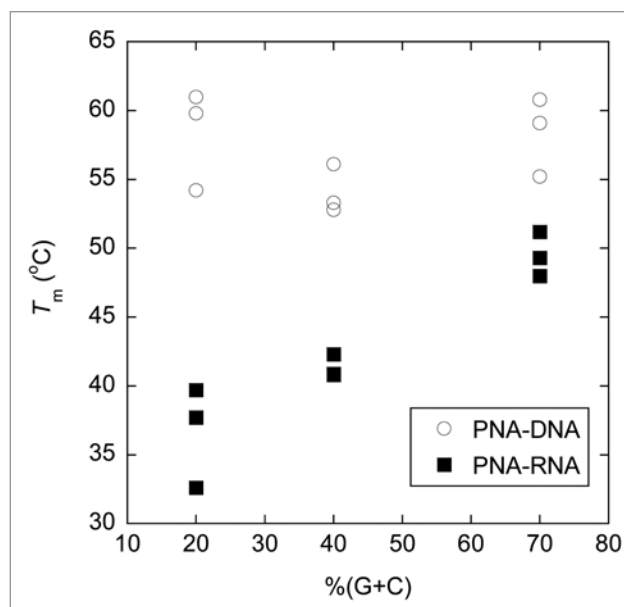

Figure 5. A plot showing relationship between G+C content and thermal stability of acpcPNA·DNA and acpcPNA·RNA hybrids. Conditions: 1.0 µM acpcPNA, 1.0 µM DNA, 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, heating rate 1.0°C/min. T_m values are accurate to within ±0.5°C.

similar purine content of 40–60%. Furthermore, no correlation could be observed between thermal stability and purine content of the acpcPNA·DNA and acpcPNA·RNA hybrids used in this study and in other additional sequences.[42] As shown in **Figure 5**, the difference in the stability between acpcPNA·DNA and acpcPNA·RNA hybrids is more pronounced at lower G+C contents than at higher ones. It is also interesting to note that the formation of parallel acpcPNA·RNA hybrids could be observed, especially when the G+C content is high. Nevertheless, the T_m values of these parallel acpcPNA·RNA hybrids are much lower than the corresponding antiparallel hybrids. Hence it can be concluded that (1) the acpcPNA binds more strongly to DNA over RNA, but the difference in thermal stability is dependent on the G+C content and (2) although the antiparallel binding mode is preferred in acpcPNA·RNA hybrids, the selectivity between the antiparallel/parallel binding modes is lower than the corresponding acpcPNA·DNA hybrids.

Most other PNA systems reported to date bind more strongly to RNA than DNA, and many attempts have been made to further enhance the RNA binding with a view to antisense and other applications involving RNA targets.[16,17,22,23] It is interesting to note that while the original aegPNA showed a preference for binding to RNA over DNA, substitution at the γ-position of the PNA backbone by a methyl group increased the stability of the DNA hybrids considerably more than the corresponding RNA hybrid, leading to a lower discrimination between DNA and RNA.[19] The present acpcPNA system and its aza-analog described previously by our group,[31] as well as a chimeric (aeg-pyrrolidine)PNA developed by Kumar,[26] are among a few examples of PNA that show a preference for DNA binding.[23] Although the structural basis

Table 1. Sequences and characterization data of acpcPNA used in this study

Entry	PNA	PNA sequence[a] (N → C)	m/z (calcd for [M+H]+)	m/z (found)
1	**T5**	TTT TT	1848.9	1848.8
2	**T7**	TTT TTT T	2513.2	2513.5
3	**T9**	TTT TTT TTT	3177.5	3177.6
4	**T10**	TTT TTT TTT T	3467.6	3468.8
5	**A9**	AAA AAA AAA	3258.6	3258.7
6	**M10a**	GTA GAT CAC T	3556.7	3555.9
7	**M10b**	GCT ACG TCG C	3533.7	3533.9
8	**M10c**	TAT GTA CTA T	3546.7	3546.9
9	**M10d**	AGT GAT CTA C	3556.7	3556.0
10	**M10e**	CAT CTA GTG A	3556.7	3556.5
11	**M10f**	GCG ACG TAG C	3582.7	3582.6
12	**M10g**	CGA TGC AGC G	3582.7	3582.6
13	**M10h**	ATA GTA CAT A	3564.7	3564.6
14	**M10i**	ATA CAT GAT A	3564.7	3564.5

[a]All sequences carried a C-terminal lysinamide and were N-acetylated.
[b]MALDI-TOF, linear mode, CCA matrix.

Table 2. T_m data for the hybrids between acpcPNA **T9** and **A9** with complementary and single mismatched DNA

Entry	PNA	DNA		T_m (°C)[a,b]	ΔT_m (°C)[c]
1	**T9**	dAAAAXAAAA	X = A	76.8	-
2			X = T	47.6	-29.2
3			X = C	47.7	-29.1
4			X = G	39.0	-37.8
5	**A9**	dTTTTYTTTT	Y = A	54.6	-25.3
6			Y = T	79.9	-
7			Y = C	56.5	-23.4
8			Y = G	52.7	-27.2

[a]Conditions: 1.0 μM acpcPNA, 1.0 μM DNA, 10 mM sodium phosphate buffer pH 7.0, 0 mM NaCl, heating rate 1.0°C/min. T_m values are accurate to within ±0.5°C. [b]Data for **T9** (entries 1–4) were taken from reference 33. [c]T_m (mismatched) - T_m (complementary).

Table 3. T_m data for the binding between mixed base acpcPNA and DNA in antiparallel and parallel directions[a]

Entry	PNA	T_m (antiparallel DNA) (°C)		T_m (parallel DNA) (°C)	
		0 mM NaCl	100 mM NaCl	0 mM NaCl	100 mM NaCl
1	**M10a**	57.0[b]	53.3[c]	<20[b]	<20[c]
2	**M10b**	60.4	54.5	<20	<20
3	**M10c**	59.6	54.2	<20	<20
4	**M10d**	57.2	52.8	<20	<20
5	**M10e**	60.6	56.1	<20	<20
6	**M10f**	65.2	60.8	<20	<20
7	**M10g**	65.1	59.1	<20	<20
8	**M10h**	65.5	61.0	<20	<20
9	**M10i**	65.3	59.8	<20	<20

[a]Conditions: 1.0 μM acpcPNA, 1.0 μM DNA, 10 mM sodium phosphate buffer pH 7.0 at 0 or 100 mM NaCl concentrations, heating rate 1.0°C/min. T_m values are accurate to within ±0.5°C. [b]Data taken from reference 33. [c]Data taken from reference 36.

attempt to understand this unusual behavior, the melting curves of DNA and RNA hybrids of the acpcPNA **M10a–M10c** having different G+C contents between 20–70% were analyzed by non-linear curve fitting[44] and by van't Hoff analysis[45] to extract the thermodynamic parameters (**Table 6**). The data suggested that despite the large enthalpy gain for G·C rich acpcPNA hybrids compared to A·T rich ones, the entropy terms are more negative (less favorable). This is true for both acpcPNA·DNA (entry 2) and acpcPNA·RNA hybrids (entry 5). In order to achieve the required geometry for the usual three hydrogen bonding pattern and/or maximum stacking of the G·C pairs, the structure of the base pairs must be distorted to some extent, resulting in a large entropy loss. On the other hand, the enthalpy term of the A·T rich acpcPNA·DNA hybrids (entry 3) were relatively large compared to the corresponding acpcPNA·RNA hybrid (entry 6), while the entropy terms were similar. Since Watson-Crick A·T pairs can form only two hydrogen bonds compared to three in G·C pairs, hydrogen bonding cannot be the main contributor to the enthalpy term. We propose that base stacking must be the main factor to stabilize the A·T pair relative to the G·C pairs in the acpcPNA·DNA hybrids. The stacking-hydrogen bonding compensation[46-48] might explain why the difference of the enthalpy change between G·C and A·T pairs is rather small. The same stabilization effects of A·T pair are absent in acpcPNA·RNA hybrids, perhaps due to the more limited conformation space available in acpcPNA·RNA hybrids, which may keep the spatial geometry from achieving maximum stacking. Additional structural studies, e.g., by X-ray or NMR are required to understand this behavior in details.

Evidences for the absence of self-hybridization in acpcPNA. In addition to the preference for binding to DNA over RNA, the acpcPNA system also displayed another rather unusual property, namely the inability of two complementary PNA strands to form self-pairing hybrids. This behavior has been previously demonstrated for acpcPNA with the sequence **M10a** and its antiparallel and parallel complementary acpcPNA sequences

of this preference is not available at present, it can be speculated that the preferential binding to DNA over RNA may be due to the more limited conformations space available in RNA duplexes compared to DNA duplexes as a result of the additional 2'-OH group present in RNA. Upon binding to RNA, it is likely that the acpcPNA could not adopt the same conformation as when it is bound to DNA. Works in this area, including molecular dynamics simulations[43] and NMR spectroscopic studies are in progress.

Thermodynamic parameters of acpcPNA·DNA and acpcPNA·RNA hybridization. The extremely high stability of homoadenine/homothymine acpcPNA·DNA hybrids compared to the mixed base acpcPNA·DNA of comparable length, as well as the sequence-independent stability of acpcPNA·DNA, but not acpcPNA·RNA, hybrids are quite unexpected. In an

Table 4. Comparison of T_m data to show specificity of binding between mixed base acpcPNA **M10a** and complementary/single base mismatched DNA

Entry	DNA sequence (5' → 3')	T_m (°C)[a]	ΔT_m (°C)[b]	Note
1	AGT GAT CTA C	53.3[c]	-	complementary
2	AGT AAT CTA C	26.4	-26.9	pC·dA mismatch
3	AGT TAT CTA C	27.8	-25.5	pC·dT mismatch
4	AGT CAT CTA C	31.0	-22.3	pC·dC mismatch
5	AGT GGT CTA C	23.9[c]	-29.4	pT·dG mismatch
6	AGT GTT CTA C	29.4[c]	-23.9	pT·dT mismatch
7	AGT GCT CTA C	23.8[c]	-29.5	pT·dC mismatch
8	AGT GAG CTA C	26.5	-26.8	pA·dG mismatch
9	AGT GAA CTA C	28.4	-24.9	pA·dA mismatch
10	AGT GAC CTA C	28.8	-24.5	pA·dC mismatch
11	AGT GAT GTA C	26.4	-26.9	pG·dG mismatch
12	AGT GAT ATA C	24.2	-29.1	pG·dA mismatch
13	AGT GAT TTA C	28.5	-24.8	pG·dT mismatch

[a]Conditions: 1.0 μM acpcPNA, 1.0 μM DNA, 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, heating rate 1.0°C/min. T_m values are accurate to within ±0.5°C. [b]T_m (mismatched) - T_m (complementary). [c]Data taken from reference 36.

Table 5. T_m data for the binding between mixed base acpcPNA and RNA in antiparallel and parallel directions[a]

Entry	PNA	%G+C	T_m (antiparallel RNA) (°C)	T_m (parallel RNA) (°C)
1	**M10a**	40	42.3[b]	<20[b]
2	**M10b**	70	48.0	32.9
3	**M10c**	20	32.6	<20
4	**M10d**	40	40.8	not determined
5	**M10e**	40	40.9	not determined
6	**M10f**	70	49.3	30.2
7	**M10g**	70	51.2	28.5
8	**M10h**	20	39.7	not determined
9	**M10i**	20	37.7	not determined

[a]Conditions: 1.0 μM acpcPNA, 1.0 μM DNA, 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, heating rate 1.0°C/min. T_m values are accurate to within ±0.5°C. [b]Data taken from reference 36.

M10d and **M10e**.[33] In this work, we extend the study to four other acpcPNA·acpcPNA pairs to demonstrate that the non-self pairing behavior is general over a range of sequences with different G+C contents (**Table 7**). Indeed, no melting could be observed in all of the antiparallel hybrids indicating that these hybrids were not stable above 20°C. On the other hand, some rather unstable parallel hybrids could be observed for sequences with ≥40% G+C content (entries 2 and 4). The low stability of both the antiparallel and parallel acpcPNA·acpcPNA hybrids is in sharp contrast to the situation of aegPNA, whereby the PNA·PNA hybrids are very stable. In fact, the stability of aegPNA·aegPNA antiparallel hybrids is generally much higher than the corresponding aegPNA·DNA and aegPNA·RNA hybrids.[6] To the best of our knowledge this absence of self-pairing, despite the ability to cross-pair with both DNA and RNA,

has not been reported in other PNA systems. Interestingly, our recent investigation revealed that the epimeric acpcPNA with the (2'R,4'S)-configuration on the proline ring could form an antiparallel self-pairing hybrid.[36] Nevertheless, the self-pairing hybrid is considerably less stable than the corresponding acpcPNA·DNA hybrid. Although more studies are required to understand its structural basis, this unusual property is a highly desirable one. For example, the non-self-pairing acpcPNA may be useful for targeting dsDNA via a "double duplex invasion" mechanism whereby the two PNA strands designed to target two opposite DNA strands in the same region of the dsDNA target. The original aegPNA is not suitable for this purpose as the two complementary PNA strands binds to each other much more strongly than with their respective DNA targets.[49,50] Current strategies to use aegPNA for double duplex invasion involve modification of the aegPNA by modifying the nucleobases[49] and incorporation of multiple positive charges into the PNA backbone[50] to provide a so-called pseudocomplementary PNA that binds more strongly to DNA than to itself. The present acpcPNA system does not require any modification to achieve this property. The absence of self-pairing in acpcPNA also means that there is a low propensity for secondary structure formation by intra- or interstrand self-pairing, which are often problematic in designing DNA and aegPNA sequences for various applications such as probes for DNA sequence determination. On the other hand, this property restricts the use of acpcPNA in applications that require self-organization of the PNA strand such as molecular beacons with the classical stem-loop design.[51]

In conclusion, we have developed new conformationally constrained pyrrolidinyl PNA systems based on an alternating nucleobase-substituted proline and 2-aminocyclopentanecarboxylic acid backbone. The specific stereochemical requirements of both the proline and the 2-aminocyclopentanecarboxylic acid parts that allow effective binding to DNA have been identified. The PNA system with (2'R,4'R)-proline/(1S,2S)-2-aminocyclopentanecarboxylic acid backbone, which is named acpcPNA, binds to DNA only in antiparallel orientation with exceptionally high affinity and sequence specificity following the Watson-Crick base pairing rules. In addition, the stability of the acpcPNA·DNA hybrids is largely independent of the base

Table 6. Thermodynamic parameters of hybrids of acpcPNA with DNA and RNA[a]

Entry	PNA	Counterstrand[b]	%G+C	T_m (°C)[c]	$\Delta H°$ (kcal/mol)[c]	$\Delta S°$ (cal/mol·K)[c]	$\Delta G°_{298}$ (kcal/mol)[c]
1	**M10a**	DNA	40	53.1	-64.1 ± 1.0 (-64.1 ± 0.7)	-168 ± 3 (-167 ± 2)	-14.1 ± 0.1 (-14.1 ± 0.0)
2	**M10b**	DNA	70	56.4	-68.9 ± 1.9 (-69.0 ± 3)	-180 ± 6 (-180 ± 10)	-15.2 ± 0.3 (-15.2 ± 0.4)
3	**M10c**	DNA	20	53.8	-60.9 ± 1.0 (-61.7 ± 1.0)	-157 ± 3 (-160 ± 3)	-14.0 ± 0.0 (-14.1 ± 0.0)
4	**M10a**	RNA	40	42.8	-60.0 ± 0.2 (-60.1 ± 0.9)	-161 ± 0.5 (-161 ± 3)	-12.0 ± 0.1 (-12.0 ± 0.0)
5	**M10b**	RNA	70	48.1	-68.0 ± 2.6 (-67.9 ± 2.8)	-183 ± 8 (-183 ± 8)	-13.5 ± 0.3 (-13.5 ± 0.3)
6	**M10c**	RNA	20	33.1	-54.9 ± 3.1 (-54.3 ± 1.4)	-151 ± 10 (-148 ± 4)	-10.1 ± 0.3 (-10.0 ± 0.2)

[a]Conditions: 1.0 µM of each acpcPNA and DNA or RNA strand, 10 mM sodium phosphate buffer pH 7.0, 150 mM NaCl, heating rate 1.0°C/min. [b]Antiparallel complementary DNA or RNA. [c]T_m and thermodynamic parameters are obtained from curve fitting.[44] Those obtained from van't Hoff analysis[45] are shown in parentheses. All figures are average of at least three independent analyses. Errors are calculated based on standard deviation.

Table 7. T_m data for the binding between two complementary strands of acpcPNA in antiparallel and parallel directions

Entry	PNA pairs	%G+C	T_m (°C)[a]	Note
1	**M10a·M10d**	40	<20	antiparallel
2	**M10a·M10e**	40	24.2	parallel
3	**M10b·M10f**	70	<20	antiparallel
4	**M10b·M10g**	70	35.1	parallel
5	**M10c·M10h**	20	<20	antiparallel
6	**M10c·M10i**	20	<20	parallel

[a]Conditions: 1.0 µM of each acpcPNA strand, 10 mM sodium phosphate buffer pH 7.0, 100 mM NaCl, heating rate 1.0°C/min. T_m values are accurate to within ±0.5°C.

sequence. This particular property should render the acpcPNA system highly useful as probes in multiplex SNP analysis and microarray applications whereby several DNA targets are to be analyzed simultaneously.[35,49] The acpcPNA also binds to complementary RNA, albeit with lower hybrid stability and directional specificity compared to DNA. Consequently, the present acpcPNA system may be less useful for applications that require targeting RNA such as RNA detection or antisense applications. The difference in stability between acpcPNA·DNA and acpcPNA·RNA hybrids is more pronounced in A·T rich than G·C rich sequences, which could be explained by enthalpic stabilization of A·T pairs over G·C pairs in acpcPNA·DNA hybrids. Finally, the unique absence of self-pairing between two complementary strands of acpcPNA suggests that it should be a potential candidate for targeting double stranded DNA by double duplex invasion, which could be useful for antigene applications.

Materials and Methods

Materials and reagents. Oligonucleotides were obtained from Biodesign (Pathumthani, Thailand) and were used as received. The concentration of oligonucleotides and acpcPNAs was determined from the absorbance at 260 nm using the reported molar extinction coefficients at 260 nm (ε_{260}) for DNA monomers.[53]

PNA synthesis. Synthesis and characterization data of acpcPNA monomers have either been previously described [(2'R,4'R) and (2'R,4'S) diastereomers][33,36] or can be found in the supporting information [(2'S,4'S) and (2'S,4'R) diastereomers]. All acpcPNAs were synthesized manually from the corresponding

Fmoc-protected acpcPNA monomers and β-amino acids spacers by Fmoc-solid phase peptide synthesis on a Tentagel resin carrying the acid-labile Rink amide linker as described previously.[33] L-Lysinamide was incorporated at the C-termini of the acpcPNAs to improve water solubility and the PNA was end-capped by acetylation. The acpcPNAs were purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry. The sequences and MALDI-TOF analysis data of the synthesized PNA are shown in **Table 1**.

UV melting experiments. UV melting experiments were performed on a CARY 100 UV Spectrophotometer (Varian Inc., Australia) equipped with a Peltier temperature controller and a thermal analysis software. The sample for thermal denaturation measurement was prepared by mixing calculated amounts of stock oligonucleotide, PNA and buffer solutions together to give the final concentration of PNA = 1.0 µM and DNA = 1.0 µM, sodium phosphate buffer pH 7.0 (10 mM) and sodium chloride (0 or 100 mM). The final volumes were adjusted to 1.0 mL by addition of deionized water. The samples were transferred to a 10 mm quartz cell with Teflon stopper and equilibrated at the starting temperature for 10 min. The A_{260} was recorded in steps from 20 to 90°C in two heating and one cooling cycles (20-90-20-90) with a temperature increment of 1.0°C/min and a hold time of 10 min at the end of each cycle. The recorded temperature was corrected by a linear equation obtained from the relationship between the recorded temperature and actual temperature measured by a built-in temperature probe. Only the result taken from the last heating cycle was used. This was normalized by dividing the absorbance at each temperature by the initial absorbance. Thermodynamic parameters were obtained from melting curves by non-linear curve fitting[44] and by van't Hoff analysis.[45] The data obtained are average of at least three melting curves for each PNA samples at a concentration of PNA = DNA = 1.0 µM (c_t = 2.0 µM) in sodium phosphate buffer, pH 7.0 and 150 mM NaCl. The $\Delta G°$ at 298 K and T_m were calculated from the $\Delta H°$ and $\Delta S°$ values.

CD experiments. CD measurements were performed on a JASCO Model J-715 or J-815 Spectropolarimeter (JASCO,

Japan). The samples were prepared by mixing the calculated amounts of stock oligonucleotide and PNA solutions in an appropriate buffer in a quartz cell (path length = 10 mm). The spectra were measured at 25°C from 320 to 200 nm and averaged 4 times then subtracted from a spectrum of the buffer under the same conditions.

Note

Supplementary information for this article can be found at http://www.landesbioscience.com/journals/artificialdna/article/16340/

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