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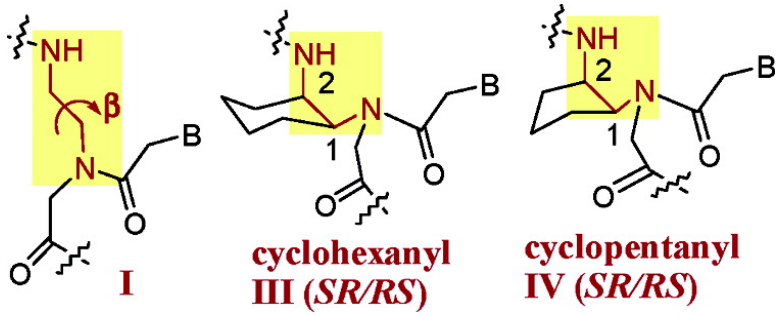

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(*SR/RS*)-Cyclohexanyl PNAs: Conformationally Preorganized PNA Analogues with Unprecedented Preference for Duplex Formation with RNA

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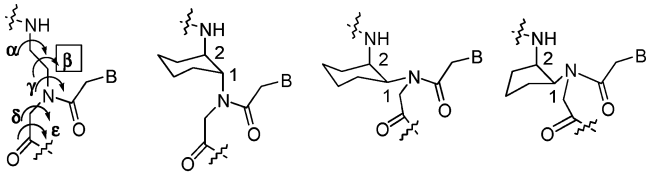
Peptide nucleic acid (PNA **I**) is a neutral DNA mimic derived by replacement of the anionic phosphate−sugar backbone with *N*-(1-aminoethyl)glycine units carrying A, G, C, and T nucleobases via methylene carbonyl linkages.[1] Polypyrimidine PNA forms PNA_2:DNA/RNA triplex, and mixed purine−pyrimidine PNA forms a duplex with complementary DNA/RNA strands.[2] Due to the high binding strength of derived complexes and biostability, PNA has a great potential for development as an antisense agent.[3] However, it has drawbacks, such as low cell penetration, similar affinity to DNA/RNA for complementary binding, and unlike other DNA mimics, it forms hybrids in both parallel (*p*) and antiparallel (*ap*) orientations. These would lead to undesired effects on the specificity for target sequences. Some of the PNA deficiencies have been addressed by rational chemical modifications.[4] However, a sufficiently important problem from an application perspective that has not been adequately dealt with is that of nondiscrimination of identical DNA and RNA sequences.



A comparison of the available X-ray and NMR structural data (Table 1) reveals that the preferred values for the backbone dihedral angle β in PNA_2:DNA triplex[5] and PNA:RNA[6] duplex are in the range of 60−70°, while that for PNA:DNA duplex is about 140°.[7] It occurred to us that it may be possible to rationally impart DNA/RNA duplex binding selectivity by tuning the dihedral angle β of the flexible ethylene part of the PNA backbone (**I**) through suitable chemical modifications. It was reported in the literature that 1,2-*trans*-(*RR*/*SS*)-disubstituted cyclohexyl PNA (**II**)[8] in *aeg*-PNA backbone destabilized complexes with both DNA and RNA.[8] From molecular modeling studies, it was suggested that cyclohexyl 1,2-substituents are in a diaxial disposition, corresponding to a dihedral angle β of ∼180°.[8] This is incompatible with the geometric requirements to form either PNA:DNA or PNA:RNA complexes (Table 1). Recently, *trans*-(*SS*)-cyclopentane-T in the *aeg*-PNA-T oligomer and mixmer sequence was shown to stabilize the PNA:DNA complex.[9]

In this context, we designed the *cis*-1,2 equatorial-axial (*SR*/*RS*)-cyclohexyl PNA (**III**) with β of ∼65°, and the derived *aeg-ch*-PNA chimera formed both PNA_2:DNA and PNA_2:RNA triplexes.[10] Substituted cyclohexyl ring is inherently rigid, locked up in either of the two chair conformations, and a relatively flexible system would be a cyclopentyl ring,[11] endowed with a puckering option. We made a *cis*-(*SR*/*RS*)-*cp*-PNA (**IV**) monomer, which had the dihedral angle β of ∼25°, as seen from its crystal structure.[12a] The derived *cp*-PNA-T_8 homooligomer formed highly stable PNA_2:DNA/RNA triplexes, but without much DNA/RNA discrimination.[12b]

Table 1. Dihedral Angles in PNA and PNA:DNA/RNA Complexes

	PNA_2:DNA[5]	PNA:RNA[6]	PNA:DNA[7]	*ch*-PNA (1*S*,2*R*)[a]	*ch*-PNA (1*R*,2*S*)[a]	*cp*-PNA (1*S*,2*R*)[a]	*cp*-PNA (1*R*,2*S*)[a]
α	−103	170	105	128	−129	84	−84
β	73	67	141	−63	66	−24	25
γ	70	79	78	76	−78	86	−86
δ	93	84	139	119	−119	90	−90

[a] Values for monomers.[9,12]

In this paper, we report the remarkable hitherto unseen DNA/RNA duplex discrimination (ΔT_m > 30−50 °C) when *ch*-PNA-T units are installed into the *aeg*-PNA backbone in a mixed base sequence, in contrast to the *cp*-PNA-T analogues. This strongly validates our design motifs based on tuning the dihedral angle β to achieve DNA/RNA duplex selectivity.

The N-protected enantiomeric (1*S*,2*R*) and (1*R*,2*S*) cyclohexyl-T and cyclopentyl-T monomers[10,12] were individually introduced into *aeg*-PNA mixed decamer **1** at three thymine positions by solid-phase synthesis on MBHA resin functionalized by L-lysine linker, according to standard procedures. Cleavage of PNAs from the resin with TFA-TFMSA was followed by purification using RP HPLC to yield the oligomers **1**−**5**, which were characterized by mass spectral data. The T_m values of various PNAs hybridized with cDNA/RNA for parallel and antiparallel binding were determined from temperature-dependent UV absorbance (Figure 1) or CD plots

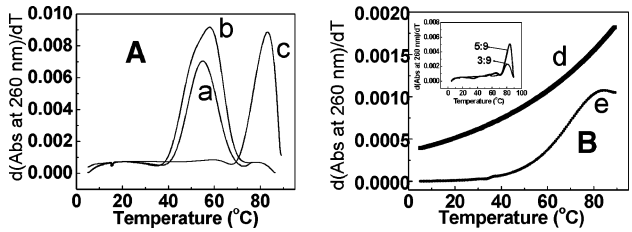


Figure 1. First derivative UV−T_m curves of *ap*-PNA:RNA duplexes (a) **1**:**7**, (b) **2**:**7**, (c) **4**:**7**, (d) **3**:**7**, and (e) **5**:**7**. Inset in B shows derivative UV−T_m of parallel PNA:RNA duplexes **3**:**9** and **5**:**9**.

(Supporting Information) and are summarized in Table 2.

Data in Table 2 show that the reference *aeg*-PNA **1** forms *ap*-duplexes with complementary DNA **6** and RNA **7** with equal stability (entry 1). Compared to the T_m of *aeg*-PNA:DNA duplex, the T_m values of *ch*-PNA:DNA duplexes (entries 2 and 3) are lower (ΔT_m = −30 °C for *SR* and −20 °C for *RS*), and those of *cp*-PNA:DNA duplexes (entries 4 and 5) are higher (ΔT_m = +22 °C for *SR* and +24 °C for *RS*). The thermal stability of *ch*-PNA/*cp*-PNA duplexes with complementary DNA/RNA oligonucleotides is stereochemistry dependent with the T_m of *RS* duplexes more than with that of *SR* duplexes. *ch*-PNAs **2** and **3**, having low binding affinity with *ap*-DNA **6**, were ineffective in forming parallel duplexes with DNA **8**.

In the case of PNA:RNA duplexes, with the exception of *SR*-*ch*-PNA **2** (entry 2), other analogues, **3**−**5** (entries 3−5), had a large stabilizing effect (ΔT_m > 30 °C) over that of control *aeg*-

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Table 2. T_m (°C) of PNA:DNA and PNA:RNA Duplexes[a]

entry	PNA (N → C)	DNA **6**(**8**)	RNA **7**(**9**)	$\Delta T_{m(RNA-DNA)ap}$
1	*aeg*-GTAGATCACT, **1**	55[b]	55.4	0.4
2	*ch*-G$\mathbf{t}_{SR}$AGA$\mathbf{t}_{SR}$CAC$\mathbf{t}_{SR}$, **2**	25[b] (nb)	58 (nb)	33
3	*ch*-G$\mathbf{t}_{RS}$AGA$\mathbf{t}_{RS}$CAC$\mathbf{t}_{RS}$, **3**	35[b] (nb)	>85 (80)	>50
4	*cp*-G$\mathbf{t}_{SR}$AGA$\mathbf{t}_{SR}$CAC$\mathbf{t}_{SR}$, **4**	77.1 (nb)	84 (71)	6. 9
5	*cp*-G$\mathbf{t}_{RS}$AGA$\mathbf{t}_{RS}$CAC$\mathbf{t}_{RS}$, **5**	78.8 (71)	>85 (84)	>6. 2

[a] All values are an average of at least three experiments and are accurate to within ±0.5 °C. Buffer: sodium phosphate (10 mM), pH 7.0, with 100 mM NaCl and 0.1 mM EDTA. [b] Measured by CD to avoid interference from thermal transitions of single-stranded PNAs.[8] A, T, G, and C are *aeg*-PNA bases, $\mathbf{T}_{SR/RS}$ *ch*-PNA-T (**2**, **3**); $\mathbf{t}_{SR/RS}$ *cp*-PNA-T (**4**, **5**); DNA **6**, 5′-AGTGATCTAC-3′(*ap*); RNA **7**, 5′-AGUGAUCUAC-3′(*ap*); DNA **8**, 5′-CATCTAGTGA-3′(*p*); RNA **9**, 5′-CAUCUAGUGA-3′(*p*). Values in parentheses are T_m of parallel duplexes; nb, no binding.

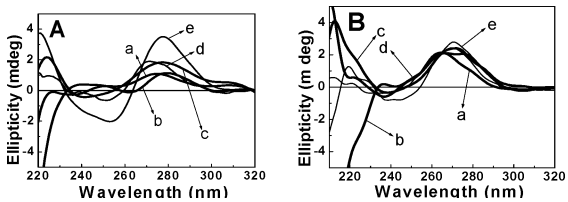

Figure 2. CD profiles: (A) PNA:DNA **6** and (B) PNA:RNA **7** duplexes. (a) **1**, (b) **2**, (c) **3**, (d) **4**, and (e) **5**. Buffer: sodium phosphate (10 mM), pH 7.0, with 100 mM NaCl and 0.1 mM EDTA.

PNA (entry 1). PNA:RNA antiparallel duplexes (**3:7**, **5:7**, entries 3 and 5) having T_m values >85 °C did not disassociate completely, whereas parallel duplexes (**3:9**, **5:9**) with a lower stability showed complete melting (Figure 1B, inset). The most important feature of the data in Table 2 is that *SR*/*RS*-*ch*-PNAs destabilize the DNA duplex but enormously stabilize the RNA duplex, while *SR/RS*-*cp*-PNAs remarkably stabilize both RNA and DNA complexes. In the process, the *ch*-PNAs induce remarkable differences in duplex stabilities among their DNA and RNA complexes, with ΔT_m(RNA/DNA) = +33 °C for *SR*-*ch*-PNA **2** and +50 °C for *RS*-*ch*-PNA **3**. This is a highly significant, exceptional binding selectivity of PNA to RNA over the same DNA sequence.

Figure 2 displays the CD profiles for all *ap*-PNA:DNA and PNA:RNA duplexes. The *ch*-PNA:DNA duplexes (Figure 2A, b and c) show CD profiles similar to that of *aeg*-PNA:DNA duplex (a), with a positive band at 277 nm and a low intensity negative band at 250 nm. In comparison, the CD profiles of *cp*-PNA:DNA duplexes (Figure 2A, d and e) show a higher intensity positive band at 275 nm and a negative band (250 nm). The CD profiles of *ch*/*cp*-PNA:RNA duplexes (Figure 2B, b−e) consisting of a high intensity positive band (260−270 nm) and a low intensity negative band (245 nm), like that of *aeg*-PNA:RNA duplex (Figure 2B, a), suggest a high degree of helical identity among the *aeg/ch/cp*-PNA:RNA duplexes. The CD features of *cp*-PNA:DNA duplexes are akin to their RNA complexes, implying that *cp*-PNA:DNA duplexes can adopt a more RNA-like structure with higher stability. The larger elliptic intensities seen in *ch*/*cp*-PNA:RNA duplexes are indicative of a better base stacking in these compared to *ch*-PNA:DNA duplexes.

Classical *aeg*-PNA being flexible can easily attain competent conformation to hybridize with DNA as well as RNA and, hence, has near equal binding affinity to both as measured by T_m. In (*SR/RS*)-*ch*-PNA, the monomer dihedral angle β matches the range (65°) found in the PNA:RNA duplex, rather than that in PNA:DNA duplex (141°). The derived *ch*-PNAs thus exhibit a higher affinity to RNA and destabilize the complex with DNA. (*SR/RS*)-*cp*-PNA with a lower dihedral angle β (25°) binds to both RNA and DNA with a higher avidity compared to those of *aeg*-PNA and *ch*-PNA, but lack the differentiating ability of *ch*-PNA. In the five-membered rings of *cp*-PNA and DNA, the flexible ring puckering allows better torsional adjustments to attain hybridization competent conformation. The inherently rigid six-membered ring of *ch*-PNAs forbids such structural adjustments and consequently destabilizes its binding with DNA. Further, in *ch/cp*-PNAs, the favorable conformational features of the monomer seem to be cooperatively transmitted to the oligomer level even in a chimera. Thus, the rigid (*SR/RS*)-*ch*-PNA imparts an unparalleled selectivity for binding to RNA over DNA for duplex formation (>30−50 °C), while the flexible (*SR/RS*)-*cp*-PNA binds both DNA and RNA with high affinity, but without any selectivity.

SR-*ch*-PNA binds RNA only in antiparallel mode but not in parallel mode. The analogous *RS* isomer formed parallel RNA hybrid with a T_m lower than that of the antiparallel hybrid. Such orientation effects were marginal for *cp*-PNA hybrids. The differences in binding of parallel and antiparallel hybrids suggest that conformational preorganization through the dihedral angle β, the cis geometry, and the chirality (*SR/RS*) act together to impart RNA binding selectivity for *ch*-PNA.

The present results for the strong preference of (*SR/RS*)-*ch*-PNA to bind RNA is in consonance with our strategy of adjusting dihedral angle β through chemical modifications to achieve structure-based selectivity in PNAs.[4b] This is perhaps the first report of any PNA analogue that overwhelmingly discriminates identical DNA/RNA sequences with a strong preference for RNA. The modification or conjugation of PNAs with cell-penetrating ligands may also prompt their nuclear entry, and induction of binding specificity for the DNA/RNA complement would be highly desirable to improve the selectivity and efficiency of nucleic acid-based drugs.[13] Further studies to examine the sequence dependence on RNA/DNA discrimination properties and control of binding selectivity are in progress.

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Supporting Information Available: The synthesis details, HPLC, and mass spectra of *ch*/*cp*-PNAs, UV, and CD data. This material is available free of charge via the Internet at http://pubs.acs.org.

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