Peptide Nucleic Acids with a Conformationally Constrained Chiral Cyclohexyl-Derived Backbone

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Abstract: Peptide nucleic acid (PNA) is an achiral nucleic acid mimic with a backbone consisting of partly flexible aminoethyl glycine units. By replacing the aminoethyl portion of the backbone by an amino cyclohexyl moiety, either in the (*S*,*S*) or the (*R*,*R*) configuration, we have synthesized conformationally constrained PNA residues. PNA oligomers containing (*S*,*S*)-cyclohexyl residues were able to form hybrid complexes with DNA or RNA, with little effect on the thermal stability ($\Delta T_m = \pm 1\,^{\circ}C$ per (*S*,*S*) unit, depending on their number and the sequence). In contrast, incorporation of the (*R*,*R*) isomer resulted in a drastic decrease in the stability of the PNA–DNA (or RNA) complex ($\Delta T_m = -8\,^{\circ}C$ per (*R*,*R*) unit). In PNA–PNA duplexes, however, the (*R*,*R*)- and (*S*,*S*)-cyclohexyl residues only exerted a minor effect on the stability, and the complexes formed with the two isomers are of opposite handedness, as evidenced from circular dichroism spectroscopy. In some cases the introduction of a single (*S*,*S*) residue in a PNA 15-mer improves its sequence specificity for DNA or RNA. From the thermal stabilities and molecular modeling based on the solution structure of a PNA–DNA duplex determined by NMR techniques, we conclude that the right-handed helix can accommodate the (*S*,*S*) isomer more easily than the (*R*,*R*) isomer. Thermodynamic measurements of ΔH and ΔS upon PNA–DNA duplex formation show that the introduction of an (*S*,*S*)-cyclohexyl unit in the PNA does indeed decrease the entropy loss, indicating a more conformationally constrained structure. However, the more favorable entropic contribution is balanced by a reduced enthalpic gain, indicating that the structure constrained by the cyclohexyl group is not so well suited for DNA hybridization.

Keywords
DNA recognition · helical structures · nucleotides · peptide nucleic acid · thermal stability

Introduction

Peptide nucleic acid (PNA) is a DNA mimic with a pseudopeptide backbone composed of *N*-(2-aminoethyl)glycine units to which the nucleobases are attached by methylene carbonyl linkers (Figure 1).[1–6] PNA hybridizes to complementary oligonucleotides with a specificity that is often superior to that of DNA, and the duplexes formed have a higher thermal stability (generally 1 °C per base pair) than the corresponding DNA–DNA and RNA–DNA duplexes.[7] Thus PNAs—apart from their significance as DNA mimics in bioorganic chemistry—are also of great interest in medicinal chemistry for developing gene-targeted (antisense and antigene) drugs.[8–11]

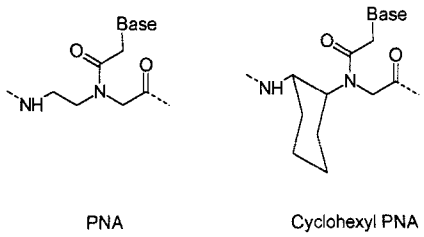

Figure 1. Structures of PNA and cyclohexyl PNA.

PNA–oligonucleotide duplex formation is enthalpically driven and is accompanied by a significant decrease in the entropy as two flexible single strands hybridize to a more rigid and structured duplex.[7, 12] The free energy gain of duplex formation may be increased by reducing the entropy loss. This may be accomplished by means of oligonucleotide analogues that are more rigid in their single-stranded state, provided that their preferred conformation is sufficiently close to that found in the duplex. Such an approach using bicyclic DNA analogues[13–15] has previously been reported and has been partially successful with cycloriboformacetal derivatives in triplex-forming oligonucleotides.[15] In PNA the ethyl portion of the backbone con-

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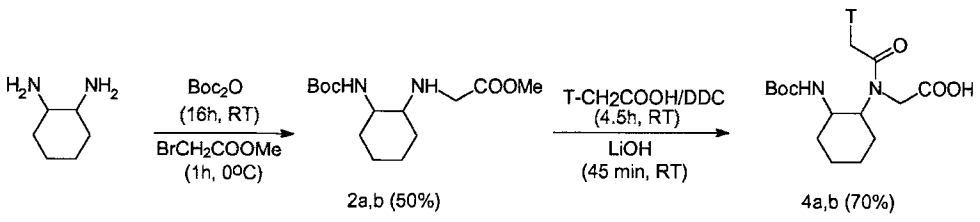
tributes much of its flexibility, and is thus an attractive target for structural constraints.

We here report on the synthesis and hybridization properties of PNAs that are conformationally constrained by a cyclohexyl ring that replaces the ethyl moiety of the backbone (Figure 1). The introduction of cyclohexyl units with (*S*,*S*) configuration into PNAs has only minor effects on the stability of the PNA–DNA duplex. In some cases it confers improved sequence discrimination, as concluded from thermal stability, calorimetric, and circular dichroism (CD) measurements. Conversely, PNAs containing cyclohexyl residues with (*R*,*R*) configuration hybridize less efficiently to the complementary oligonucleotides.

Results

Synthesis of cyclohexyl monomers and oligomerization: The cyclohexyl PNA monomers were prepared from (1,2)-diaminocyclohexane, commercially available in the (*R*,*R*) and (*S*,*S*) enantiomeric forms, and essentially following the procedures previously employed with other PNA monomers.[16–18]

Monoprotection of the 1,2-diaminocyclohexane with di-*t*-butylpyrocarbonate (Boc_2O), *N*-alkylation with methyl bromoacetate, and chromatography on silica gave the chiral backbone **4** (Scheme 1). Coupling of *N*-1-carboxymethylthymine

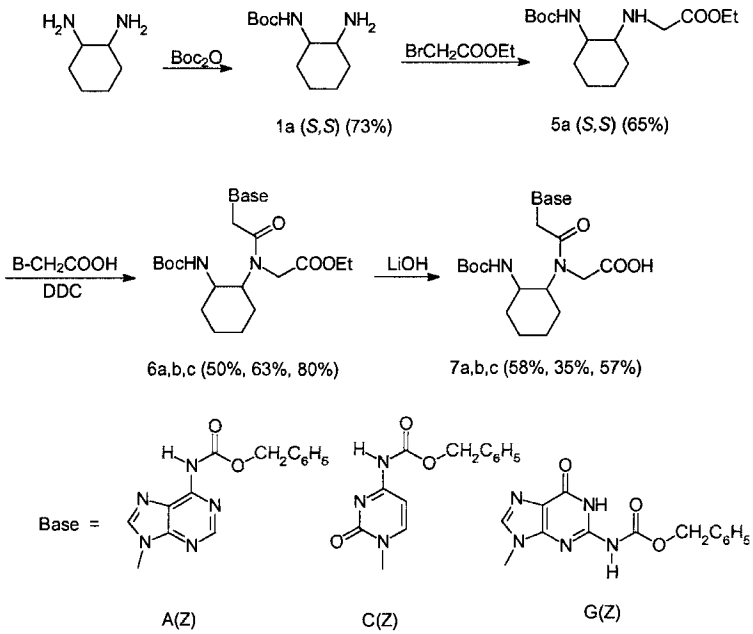


Scheme 1. Synthesis of cyclohexyl–T PNA monomer (T = thymine).

(T-CH_2COOH) with dicyclohexyl carbodiimide (DCC) or 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH) followed by alkaline hydrolysis yielded the desired monomer **4** in 35% overall yield (Scheme 1). The Boc-protected monomer gave a single peak in HPLC, indicating that no epimerization had occurred during the synthesis.

The benzoyloxy- ((Z)-)protected monomers of adenine, cytosine, and guanine were prepared from the (Z)-protected nucleobase acetic acid, which was coupled to the cyclohexyl backbone with DCC/DhbtOH in an essentially analogous manner to the thymine monomer (Scheme 2).

The oligomerization was performed on a (4-methylbenzhydryl)amine resin (initial loading 0.1 meq g^{-1}) with *O*-(benzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethyl amine (DIEA) in DMF/pyridine as the coupling reagent. The free PNAs were cleaved from the resin with the low/high TFMSA (trifluoromethane sulfonic acid) procedure,[19] purified by HPLC, and characterized by mass spectrometry (MALDI-TOF- and/or FAB-MS).



Scheme 2. Synthesis of cyclohexyl–A, G, and C PNA monomers.

Triplex formation and stability: We synthesized all-thymine PNA decamers containing one (*S*,*S*)- or one (*R*,*R*)-cyclohexyl unit (PNA **1** and PNA **2**, respectively), which hybridize to the complementary DNA strand d(A_{10}) forming PNA_2–DNA triplexes. The thermal stabilities (T_m) of these complexes decreased drastically for triplexes containing the (*R*,*R*)-cyclohexyl isomer ($\Delta T_m = 19$ °C), as compared to a regular PNA strand (PNA **3**, see Table 1). Only a slight decrease in T_m (1.5 °C) was observed for the (*S*,*S*)-cyclohexyl isomer. The thermal stabilities of complexes between the (*R*,*R*)- or (*S*,*S*)-cyclohexyl PNAs **1** and **2** and DNA strands containing a single mismatch [d(A_4CA_5)] were also measured. The results indicate that triplex-forming PNAs containing a single (*R*,*R*)- or (*S*,*S*)-cyclohexyl residue exhibit improved discrimination against a single mismatch compared to the regular aminoethylglycine PNA. In addition, the stabilities of complexes between PNA **1** and PNA **2** and the complementary RNA strand were measured and found to follow the same pattern as the PNA_2–DNA triplexes; the (*S*,*S*)-containing PNA shows higher stability than the (*R*,*R*) isomer (Table 1).

Circular dichroism (CD) spectra of complexes between PNA **1** and PNA **2** and d(A_{10}) are nearly identical (Figure 2), and are also very similar to the CD spectrum of the unmodified (PNA–T_{10})$_2$–d(A_{10}) triplex.[20] These observations indicate that the overall structures of the triplexes with PNA **1** and PNA **2** are very similar to each other as well as to the regular PNA_2–DNA triplex.

Duplex formation and stability: For studies of duplexes modified by cyclohexyl units, we synthesized 10-mer PNA strands of

Table 1. Thermal stabilities (T_m [°C]) of PNA–DNA, PNA–RNA, and PNA–PNA complexes.

Strand 1	Strand 2 [a]	Triplex	Antiparallel duplex	Parallel duplex	Mismatch	RNA	RNA mismatch	PNA
H-TTTTT$_{SS}$TTTTT-Lys-NH$_2$ (**1**)	**I**	70.0	–	–	59.5	75.5	–	75.5
H-TTTTT$_{RR}$TTTTT-Lys-NH$_2$ (**2**)	**I**	52.5	–	–	40	56	–	72.0
H-TTTTTTTTTT-Lys-NH$_2$ (**3**)	**I**	71.5	–	–	66	81	–	76.0
H-GT$_{SS}$AGAT$_{SS}$CACT$_{SS}$-Lys-NH$_2$ (**4**)	**II**	–	51 [b]	41 [b]	27.5 [b]	54	40	64.0
H-GT$_{RR}$AGAT$_{RR}$CACT$_{RR}$-Lys-NH$_2$ (**5**)	**II**	–	33.5 [b]	[c]	[c]	33	32	66.0
H-GTAGATCACT-Lys-NH$_2$ (**6**)	**II**	–	55 [b]	38 [b]	34.5 [b]	55.5	46	68.5
H-(GTAGATCACT)$_{SS}$-Lys-NH$_2$ (**7**)	**II**	–	41	[c]	[c]	37	[c]	47.0
H-TGTACGT$_{SS}$CACAACTA-Lys-NH$_2$ (**8**)	**III**	–	69	57	57	73	61	83.0
H-TGTACGTCACAACTA-Lys-NH$_2$ (**9**)	**III**	–	68.5	54	56	72.5	56	76.5

[a] **I** = 5′d(CGCA$_{10}$CGC) or 5′d(CGCA$_4$*C*A$_5$CGC); **II** = 5′d(AGTGATCTAC) or 5′d(AGTG*G*TCTAC); **III** = 5′d(TAGTTGTGACGTACA) or 5′d(TAGTTGTG-*C*CGTACA) (mismatches in *italics*). [b] Measured by CD in order to avoid interference from thermal transitions of the single-stranded PNAs. [c] A well-defined transition assignable to this complex could not be identified.

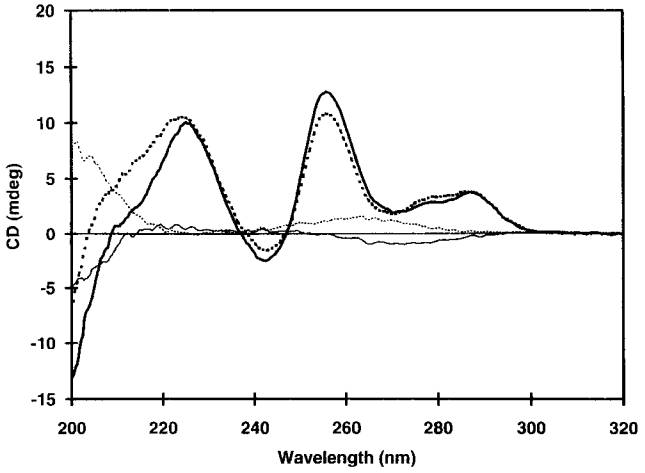


Figure 2. Circular dichroism (CD) spectra of PNA **1** (thick solid curve) and PNA **2** (thick broken curve) in complex with d(A$_{10}$). The CD spectra of the single-stranded PNA **1** (thin solid curve) and PNA **2** (thin broken curve) are also shown.

mixed purine/pyrimidine content including three thymines with either the (*S*,*S*)- or the (*R*,*R*)-cyclohexyl backbone (PNAs **4** and **5**, respectively). Measurements of the thermal stability of duplexes formed between these PNAs and complementary DNA strands showed that the complex with the (*S*,*S*)-modified PNA is more stable than the corresponding (*R*,*R*)-PNA complex (cf. Table 1). Furthermore, the PNA strand containing (*S*,*S*)-cyclohexyl thymines exhibited improved discrimination against a single (T–G) mismatch compared to the regular PNA (PNA **6**). Like the regular PNA **6**, the PNA **4** modified with (*S*,*S*)-cyclohexyl units bound considerably better to a DNA target in the antiparallel rather than in the parallel orientation. However, the discrimination between antiparallel and parallel duplexes was less efficient when the PNA strand included (*S*,*S*)-cyclohexyl residues (PNA **4**, $\Delta T_m = 10\,^{\circ}C$) than without (PNA **6**, $\Delta T_m = 17\,^{\circ}C$). This difference in behavior may be due to the superior ability of the unmodified PNA to adapt to the DNA strand structure, resulting in a more favorable enthalpic contribution to duplex formation (see the calorimetry section). The parallel duplex, on the other hand, may enthalpically favor the (*S*,*S*)-modified PNA strand.

We also prepared a PNA 10-mer of the same sequence with an all (*S*,*S*)-cyclohexyl unit backbone (PNA **7**). The PNA–DNA duplex formed with this oligomer had a lower T_m than that formed with PNA **4** containing only three (*S*,*S*) residues, or with the unmodified PNA **6** (41 °C, 51 °C, and 55 °C, respectively). The PNA **7**–DNA duplex was, however, considerably more stable than the PNA **5**–DNA complex that contained three (*R*,*R*) residues ($T_m = 33.5\,^{\circ}C$). The T_m of a regular DNA–DNA duplex of the same sequence is 33.5 °C, illustrating the destabilizing effect of electrostatic repulsion for duplex stability; only the structurally poorly suited sequence PNA **5** showed as low a T_m as the regular DNA duplex of the same sequence.

Hybrid complexes of the PNAs **4**, **5**, **7**, and **8** with RNA and PNA were also studied (Table 1). The relative stabilities of these complexes were similar to those of the corresponding DNA complexes, and generally followed the order: PNA–PNA > PNA–RNA > PNA–DNA.[7] It should be noted, however, that PNA **4** (*S*,*S*) and PNA **5** (*R*,*R*) bound equally well to the complementary achiral PNA strand, indicating that these form identical helical structures which are mirror images (vide infra). Interestingly, the presence of one (*S*,*S*) residue (PNA **8**) increased the stability of the 15-mer sequence, whereas three (*S*,*S*) residues lowered the stability of the 10-mer PNA–PNA duplex. This observation either reflects sequence context effects, or suggests that the presence of several (*S*,*S*) residues may disturb the structure, while a single (*S*,*S*)-cyclohexyl unit is more easily accommodated in the duplex. One (*S*,*S*) unit may be entropically favorable, owing to significant preorganization of the duplex. The binding of PNA **4** and PNA **5** to RNA correlates well with their binding to DNA; that is, the (*S*,*S*) form exhibits higher duplex stability than the (*R*,*R*) form.

In order to further explore the sequence-discriminating properties of PNA containing a (*S*,*S*)-cyclohexyl unit, we used a 15-mer PNA with a (*S*,*S*)-cyclohexyl thymine residue close to the center of the sequence (PNA **8**, Table 1) and measured the stability effects of mismatched base pairs directly on either side of the cyclohexyl-modified portion of the backbone, that is, opposite PNA G6 or T7. The results, presented in Table 2, show no clear trend in the sequence discrimination of the PNA thymine containing the (*S*,*S*)-cyclohexyl backbone, but in general, and especially when hybridized to RNA, the discrimination appears to be less efficient than with the unmodified PNA. The cyclohexyl PNA guanine, however, showed improved discrimination towards G–G and G–T mismatches with RNA.

Calorimetry: The enthalpic contribution to the duplex stability was measured by isothermal titration calorimetry experiments

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Table 2. Effects on thermal stability (ΔT_m [°C]) of single mismatches opposite PNA G 6 or T 7 in a PNA–DNA duplex. Strand 1 [a]: H-5′-TGTAC**GT**$_x$CACAACTA; strand 2: 3′-ACATG**XY**GTGTTGAT-5′.

Strand 1	Mismatch: Strand 2:	T–C		T–G		T–T		G–A		G–G		G–T	
		DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
regular PNA (**9**)		12.5	16.5	10	9.8	10	12.8	17	18	14.5	16.8	8	11.8
cyclohexyl PNA (**8**)		12	12	11	10	8.5	8.5	16.5	15	13.5	20	11.5	16
DNA		11	–	5	–	9.5	–	13	–	10.5	–	9	–

[a] T_x designates the position of any (*S*,*S*)-cyclohexyl-modified PNA thymine residue.

Table 3. Thermodynamic parameters for PNA–DNA duplex formation obtained from isothermal titration calorimetry experiments performed at 20 °C. The sequence of the DNA strand was d(AGTGATCTAC) in all cases.

PNA	K (M^{-1})	ΔG (kJ mol^{-1})	δH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
H-GT$_{SS}$AGAT$_{SS}$CACT$_{SS}$-Lys-NH$_2$ (**4**)	$6.7 \cdot 10^7$	−44.0	−127	−280
H-GT$_{RR}$AGAT$_{RR}$CACT$_{RR}$-Lys-NH$_2$ (**5**)	$5.0 \cdot 10^4$	−26.3	(−216) [a]	(−650) [a]
H-GTAGATCACT-Lys-NH$_2$ (**6**)	$5.8 \cdot 10^7$	−43.5	−153	−375

[a] The accuracy of these values is not very good due to the relative instability of the complex.

of the hybridization of PNA decamers containing (*R*,*R*) or (*S*,*S*) residues and their complementary DNA decamers. By applying van't Hoff analysis to the binding isotherms, recorded as reaction enthalpies, the free energy and entropy loss of the interstrand reactions were calculated. Hybridization of PNA **4** containing three (*S*,*S*) units resulted in a smaller loss of entropy, but also a less favorable enthalpic change than did the regular PNA **6** (see Table 3). Thus, there was no net gain in duplex stability over the unmodified sequence, as can be seen from the similar equilibrium constants (K) and their similar melting temperatures (Table 1). Therefore, the gain in entropy is essentially lost in enthalpy, probably due to the (*S*,*S*) unit being structurally less well suited than the regular PNA for hybridization with DNA. With PNA **5**, which contained three (*R*,*R*) residues, a larger entropic loss and a more favorable enthalpic gain was indicated compared to regular PNA **6**. However, due to weak binding (PNA **5** binds almost 10^3-fold less efficiently to the complementary DNA than does PNA **4** or PNA **6**), the thermodynamic data measured for the (*R*,*R*) isomer are much less accurate than the other values given. The values of K, ΔH, and ΔS obtained from calorimeteric measurements correlate well qualitatively with values calculated independently from thermal melting experiments monitored by CD.

CD spectroscopy: The results from CD spectroscopy are fully consistent with the T_m and calorimetry data. The CD spectrum of the (*S*,*S*)-PNA **4**–DNA duplex (Figure 3) is nearly identical to that of the PNA **6**–DNA duplex, indicating similar helical structures. In contrast, the spectrum of the (*R*,*R*)-PNA **5**–DNA complex is profoundly different, suggesting that the structure of this duplex is less regular.

CD spectra of the PNA–PNA complexes with PNA **4** and PNA **5** are presented in Figure 4. Since the aminoethylglycine backbone is achiral, PNAs made solely with this backbone exhibit no CD signal.[21, 22] However, single-stranded PNAs containing the chiral (*S*,*S*)- or (*R*,*R*)-thymine units have weak but distinct CD spectra, which are nearly mirror images of each other (because of the presence of the terminal (L)-lysine, perfect symmetry is not expected). Duplexes formed between PNA con-

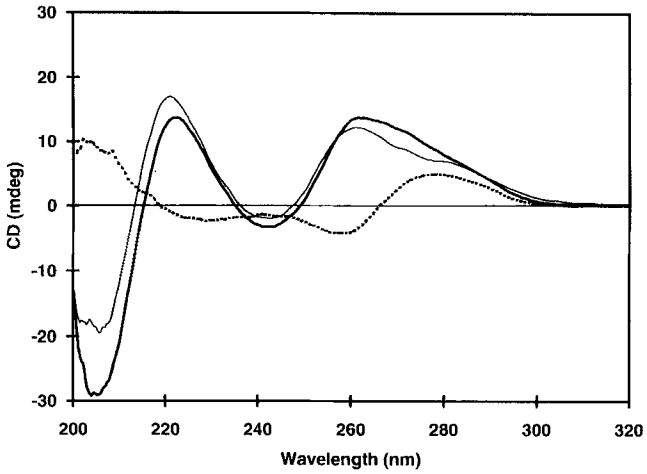

Figure 3. CD spectra of PNA **4** (thick solid curve), PNA **5** (thick broken curve), and PNA **6** (thin solid curve) complexed with complementary antiparallel oligodeoxyribonucleotide 5′-d(AGTGATCTAC).

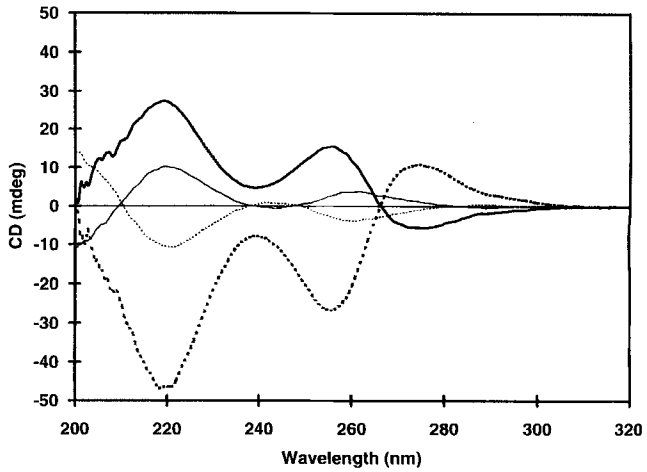

Figure 4. CD spectra of PNA **4** (thin solid curve) and PNA **5** (thin broken curve) and their complexes with the complementary antiparallel PNA H-AGTGATCTAC-Lys-NH$_2$; PNA **4**–PNA (thick solid curve) and PNA **5**–PNA (thick broken curve).

taining (*R*,*R*)- or (*S*,*S*)-cyclohexyl units and its nonchiral complementary PNA strand gave strong CD signals, suggesting that the complexes adopt helical structures of stacked basepairs. These CD spectra are close to mirror images, indicating opposite helical handedness for the (*R*,*R*) and (*S*,*S*) complexes.

The CD spectrum of the PNA **5**–PNA complex ((*R*,*R*) configuration) is essentially identical to that of the PNA **6**–PNA duplex (same sequence with only a C-terminal (L)-lysine providing chirality). It has been proposed, based on quantum mechanical calculations and its similarities to the corresponding DNA–DNA duplex, that the PNA **6**–PNA complex is a right-handed helix.[22] It is surprising that the PNA **5**–PNA duplex appears to prefer a right-handed conformation, because it forms relatively unstable right-handed complexes with DNA. However, given the substantial structural differences between PNA–PNA[23] and PNA–DNA[24, 25] duplexes recently discovered, the tentative assignment of the PNA 6–PNA duplex as right-handed[22] may well be incorrect since this conclusion assumed a B-like helix for the PNA duplex. In fact the present results argue in favour of the PNA 6–PNA duplex being left-handed.

Structure modeling: The pronounced differences in duplex stability between DNA and PNAs containing the (*S*,*S*)- or the (*R*,*R*)-cyclohexyl backbone units are most likely to be related to the structure; therefore we performed molecular modeling experiments. The structure of the PNA–DNA octamer duplex H-GCTATGTC-NH_2·d(GACATAGC), previously determined by NMR techniques,[24] was taken as the initial model after replacing the three thymine residues of the PNA strand by either (*R*,*R*)- or (*S*,*S*)-cyclohexyl backbone units. Molecular dynamics simulations were performed on the model structures (keeping the DNA strand essentially fixed) followed by energy minimization. This resulted in markedly different structures for the (*R*,*R*)- and (*S*,*S*)-PNA models. The cyclohexyl ring in all three modified residues of the (*S*,*S*) model allows the backbone to adopt a conformation roughly similar to that of the unmodified structure (Figure 5). The bulky cyclohexyl rings are consistently located on the periphery of the helix and cause only limited steric interference with the thymine bases and the backbone and base of the preceding residue. The (*R*,*R*) model, on the other hand, shows considerable changes in the backbone conformation, particularly around the cyclohexyl region (backbone torsion angles β and γ). The cyclohexyl rings are positioned in the major groove, where they appear to pry apart the stacking between the thymine and the preceding base. Thus, the (*R*,*R*) backbone seems to be poorly suited to fit in a right-handed helix without significantly disturbing the structure.

These modeling results qualitatively agree with the experimental T_m and CD results on the various PNA–DNA duplexes. In the model, the (*S*,*S*)-cyclohexyl backbone requires little structural change to be accommodated and it does not significantly affect the stability (similar T_m) or disturb the base stacking (similar CD spectra). In contrast, the model with the (*R*,*R*)-cyclohexyl units appears to introduce more severe distortions in the PNA strand structure. The considerable differences in the CD spectra of the oligomer duplexes containing (*R*,*R*) units support the idea of drastic changes in the base pair stacking around the modified residues (although some direct contributions of the chiral (*R*,*R*) backbone cannot be ruled out).

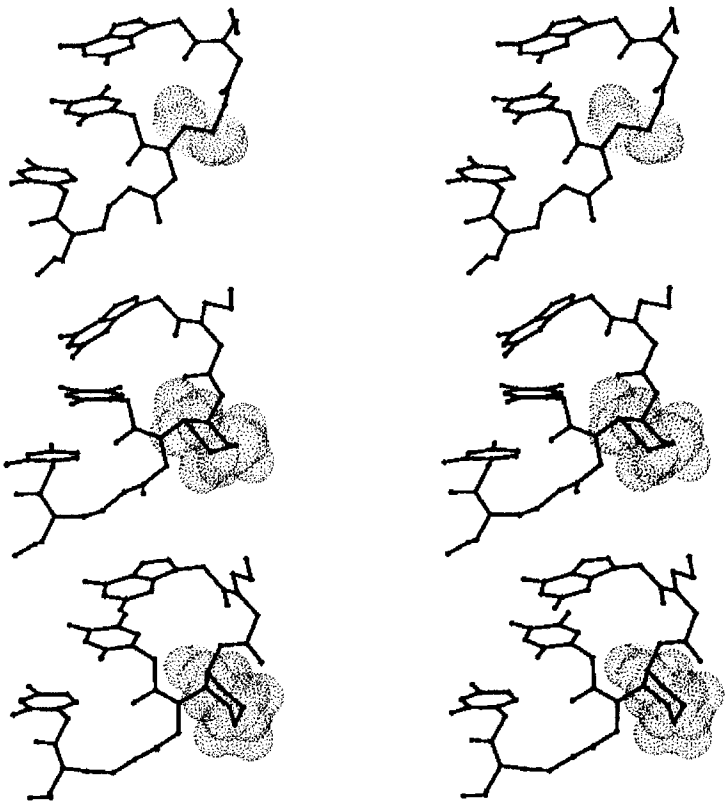

Figure 5. Stereoview model of PNA–DNA octamer duplex H-GCTATGTC-NH_2·d(GACATAGC) containing three cyclohexyl-modified PNA thymine residues. $CT_{ss}G$ portion of PNA strand shown. Regular (top), (*R*,*R*) (center), and (*S*,*S*) PNA (bottom).

Conclusions

Peptide nucleic acid with the backbone conformationally constrained by cyclohexyl modifications in the (*S*,*S*) configuration are well suited to form complexes with DNA or RNA, whereas their (*R*,*R*)-cyclohexyl isomers are not. PNAs with (*S*,*S*)-modified residues cause changes in T_m of only about 1 °C per unit (relative to regular PNA, and depending on the sequence). In contrast, incorporation of (*R*,*R*)-cyclohexyl units dramatically decreases the thermal stability of the PNA–DNA (RNA) complex ($\Delta T_m = -8$ °C per (*R*,*R*) residue). Molecular modeling experiments, based on the solution structure of a PNA–DNA duplex determined by NMR methods, suggest that the (*S*,*S*) isomer is more easily accommodated in a right-handed hybrid duplex with DNA than is the (*R*,*R*) isomer. The PNA–DNA hybrid formation is accompanied by a smaller loss of entropy for the PNA strand modified with (*S*,*S*)-cyclohexyl units than for the regular PNA. However, the improved entropic effect is approximately outweighed by a lowered enthalpic gain. This suggests that the reduced conformational flexibility of the (*S*,*S*)-cyclohexyl residues causes some prestructuring of the PNA strand, but that the constrained PNA strand is less well suited for DNA hybridization than is the regular PNA backbone, and therefore no significant net gain in hybrid stability is observed. These results indicate that chemical constraint of the conformational freedom in the PNA backbone may indeed lead to improved hybridization potency.

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Experimental Section

Abbreviations: DCC: dicyclohexyl carbodiimide; DCU: dicyclohexyl urea; DhbtOH: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; DIEA: diisopropylethyl amine; DMF: dimethyl formamide; DMSO: dimethyl sulfoxide; HBTU: *O*-(benzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TFMSA: trifluoromethane sulfonic acid; THF: tetrahydrofuran; (Z): benzoyloxy.

(1*S*,2*S*)-1-(*N*-*t*-butyloxycarbonylamino)-2-aminocyclohexane [(*S*,*S*)-**1a**]: To a cooled solution of (1*S*,2*S*)-diaminocyclohexane (5 mL, 41.6 mmol) in CH_2Cl_2 (25 mL) was added a solution of di-*t*-butyl dicarbonate (3.03 g, 13.9 mmol) in CH_2Cl_2 (25 mL) over a period of 30 min. The reaction mixture was stirred overnight at RT. Water (20 mL) and CH_2Cl_2 (25 mL) were added in order to dissolve the precipitate. After separation of the two phases, the organic phase was concentrated under reduced pressure, and the residue was dissolved in ether (25 mL) and water (25 mL). The mixture was acidified to pH 5 with 4 M HCl, and the bis-protected diamine was extracted with ether (3 × 25 mL). The aqueous phase was adjusted to pH 10.5 with 2 M NaOH and extracted with AcOEt (6 × 30 mL). The organic phase was then dried over sodium sulfate, filtered, and evaporated under reduced pressure to yield (*S*,*S*)-**1a** (2.3 g, 73% based on Boc_2O). M.p. 109–111 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.0–1.3 (m, 4H, 2CH_2 cycl), 1.45 (s, 9H, *t*Boc), 1.6 (m, 2H, CH_2 cycl), 1.85 (m, 2H, CH_2 cycl), 2.4 (dt, 1H, CHN), 2.9 (m, 1H, CHN), 6.6 (m, 1H, NH carbamate); ^{13}C NMR ([D$_6$]DMSO): δ = 155.6 (carbamate), 77.4 (*t*Boc), 57.0, 53.8, 34.4, 32.2, 25.8, 24.8 (C cycl), 28.4 (*t*Boc)

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl]-glycine methyl ester** [(*S*,*S*)-**2a**]: To a cooled suspension of (*S*,*S*)-**1a** (2 g, 9.35 mmol) and potassium carbonate (3.87 g, 28.05 mmol) in DMF (15 mL) was added a solution of methyl bromoacetate (0.9 mL, 9.35 mol) in DMF (5 mL) over 5 min. After 1 h at 0 °C the salts were filtered off and washed with DMF and CH_2Cl_2. The filtrate was evaporated under reduced pressure and the residue was purified by chromatography on silica gel (eluent AcOEt). Yield: 1.9 g (70%). M.p. 68–70 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.0–1.3 (m, 4H, 2CH_2 cycl), 1.45 (s, 9H, *t*Boc), 1.4–2.0 (m, 5H, 2CH_2 cycl + NH), 2.3 (dt, 1H, CHN), 3.1 (m, 1H, CHN), 3.4 (dt, 2H, CH_2COO), 3.7 (s, 3H, $COOCH_3$), 6.7 (m, 1H, NH carbamate); ^{13}C NMR ([D$_6$]DMSO): δ = 172.9 (ester), 155.5 (carbamate), 77.5 (*t*Boc), 59.7, 47.6 (CH_2COOCH_3), 53.7, 51.4, 32.1, 31.1, 24.6, 24.2 (C cycl), 28.3 (*t*Boc); MS (FAB$^+$): 287.0 [*M* + 1].

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl]-*N*-(thymin-1-ylacetyl)-glycine methyl ester** [(*S*,*S*)-**3**]: DCC (1.08 g, 5.24 mmol) was added to a solution of (*S*,*S*)-**2a** (1.5 g, 5.24 mmol), thymine acetic acid (0.96 g, 5.22 mmol), and DhbtOH (0.85 g, 5.2 mmol) in DMF (15 mL) and CH_2Cl_2 (15 mL). After 4.5 h at RT, DCU was filtered off and washed with CH_2Cl_2 (100 mL). The filtrate was washed with 1 M $NaHCO_3$ (3 × 40 mL), 1 M $KHSO_4$ (2 × 40 mL) and H_2O (40 mL). The organic phase was dried over sodium sulfate and filtered. Petroleum ether (100 mL) was added and after 48 h at 0 °C, (*S*,*S*)-**3a** was collected by filtration. Yield: 1.7 g (72%). M.p. 205–207 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.2–2.0 (m, CH_2 cycl), 1.45 (s, *t*Boc), 1.9 (CH_3 thymine), 3.7 (s, $COOCH_3$), 3.7 (dd, CH_2COO), 4.8 (dd, CH_2-T), 6.95 (m, NH carbamate), 7.2 (s, H–C=C–Me), 11.35 (s, NH imide); ^{13}C NMR ([D$_6$]DMSO): δ = 169.7, 167.1, 164.3, 154.9, 150.9 (C=O), 141.5, 108.2 (C=C), 77.9 (*t*Boc), 59.8, 47.5 (CH_2COOCH_3), 53.7, 51.4, 32.1, 31.1, 24.6, 24.2 (C cycl), 28.2 (*t*Boc), 11.9 (CH_3 thymine); MS (FAB$^+$): 453.3 [*M* + 1], 353.3 [*M* + 1 − *t*Boc].

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl]-*N*-(thymin-1-ylacetyl)-glycine** [(*S*,*S*)-**4a**]: The monomer ester (*S*,*S*)-**3a** (1.5 g, 3.3 mmol) was suspended in THF (15 mL), and a solution of 0.5 M LiOH (15 mL, 7.5 mmol) was added together with water (5 mL). After 45 min at RT, water (10 mL) was added, and the mixture was washed with AcOEt (2 × 10 mL). The aqueous phase was acidified to pH 3 and extracted with AcOEt (4 × 120 mL). The organic phase was dried over sodium sulfate and evaporated under reduced pressure. Yield: 1.36 g (94%). ^{1}H NMR ([D$_6$]DMSO): δ = 1.2–2.0 (m, CH_2 cycl), 1.45 (s, *t*Boc), 1.9 (CH_3 thymine), 3.9 (dd, CH_2COO), 4.8 (dd, CH_2-T), 6.95 (m, NH carbamate), 7.2 (s, H–C=C–Me), 11.35 (s, NH imide), 12.4 (m, COOH); ^{13}C NMR ([D$_6$]DMSO): δ = 170.3, 166.8, 164.3, 155.0, 150.9 (C=O), 141.5, 108.1 (C=C), 77.9, 28.2 (*t*Boc), 59.7, 49.9, 47.9, 44.2, 32.1, 29.7, 24.4, 24.3 (C cycl + 2CH_2), 11.9 (CH_3 thymine); MS (FAB$^+$): 439.2 [*M* + 1], 339.1 [*M* + 1 − *t*Boc].

(1*R*,2*R*)-1-(*N*-t-butyloxycarbonylamino)-2-aminocyclohexane [(*R*,*R*)-**1b**]: To a cooled solution of (1*R*,2*R*)-(−)-*trans*-1,2-diaminocyclohexane (5 mL, 41.6 mmol) in CH_2Cl_2 (25 mL) was added a solution of di-*t*-butyl dicarbonate (3.03 g, 13.9 mmol) in CH_2Cl_2 (25 mL) over a period of 30 min. The reaction mixture was stirred overnight at RT. Water (20 mL) and CH_2Cl_2 (25 mL) were added in order to dissolve the precipitate. After separation, the organic phase was concentrated under reduced pressure and the residue dissolved in ether (25 mL) and water (25 mL). The mixture was acidified to pH 5 with 4 M HCl, and the bis-protected diamine was extracted with ether (3 × 25 mL). The aqueous phase was adjusted to pH = 10.5 with 2 M NaOH and extracted with AcOEt (6 × 30 mL). The organic phase was dried over sodium sulfate, filtered, and evaporated under reduced pressure to yield (*R*,*R*)-**1b** (2 g, 69.5% based on Boc_2O). M.p. 109–111 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.0–1.3 (m, 4H, 2CH_2 cycl), 1.45 (s, 9H, *t*Boc), 1.6 (m, 2H, CH_2 cycl), 1.85 (m, 2H, CH_2 cycl), 2.4 (dt, 1H, CHN), 2.9 (m, 1H, CHN), 6.6 (m, 1H, NH carbamate); ^{13}C NMR ([D$_6$]DMSO): δ = 155.6 (carbamate), 77.4 (*t*Boc), 57.0, 53.8, 34.4, 32.2, 25.8, 24.8 (C cycl), 28.4 (*t*Boc)

***N*-[(2*R*)-Boc-aminocyclohex-(1*R*)-yl]-glycine methyl ester** [(*R*,*R*)-**2b**]: To a cooled suspension of (*R*,*R*)-**1b** (2 g, 9.35 mmol) and potassium carbonate (3.87 g, 28.05 mmol) in DMF (15 mL) was added a solution of methyl bromoacetate (0.9 mL, 9.35 mol) in DMF (5 mL) over a period of 5 min. After 1 h at 0 °C the salts were filtered off and washed with DMF (15 mL) and CH_2Cl_2 (15 mL). The filtrate was evaporated under reduced pressure and the residue was purified by chromatography on silica gel (eluent AcOEt). Yield: 1.99 g (74%). M.p. 68–70 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.0–1.3 (m, 4H, 2CH_2 cycl), 1.45 (s, 9H, *t*Boc), 1.4–2.0 (m, 5H, 2CH_2 cycl + NH), 2.3 (dt, 1H, CHN), 3.1 (m, 1H, CHN), 3.4 (dt, 2H, CH_2COO), 3.7 (s, 3H, $COOCH_3$), 6.7 (m, 1H, NH carbamate); ^{13}C NMR ([D$_6$]DMSO): δ = 172.9 (ester), 155.5 (carbamate), 77.5 (*t*Boc), 59.7, 47.6 (CH_2COOCH_3), 53.7, 51.4, 32.1, 31.1, 24.6, 24.2 (C cycl), 28.3 (*t*Boc); MS (FAB$^+$): 287.0 [*M* + 1].

***N*-[(2*R*)-Boc-aminocyclohex-(1*R*)-yl]-*N*-(thymin-1-ylacetyl)-glycine, methyl ester** [(*R*,*R*)-**3b**]: To a solution of [(*R*,*R*)-**2b**] (1.5 g, 5.24 mmol), thymine acetic acid (0.96 g, 5.22 mmol), and DhbtOH (0.85 g, 5.2 mmol) in DMF (20 mL) and CH_2Cl_2 (15 mL) was added DCC (1.08 g, 5.24 mmol). After 4.5 h at RT, DCU was filtered off and washed with CH_2Cl_2 (100 mL). The filtrate was washed with 1 M $NaHCO_3$ (3 × 40 mL), 1 M $KHSO_4$ (2 × 40 mL), H_2O (40 mL). The organic phase was dried over sodium sulfate and filtered. Petroleum ether (100 ml) was added and after 48 h at 0 °C, (*R*,*R*)-**3b** was collected by filtration. Yield: 1.77 g (74%). M.p. 205–207 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.2–2.0 (m, CH_2 cycl), 1.45 (s, *t*Boc), 1.9 (CH_3 thymine), 3.7 (s, $COOCH_3$), 3.7 (dd, CH_2COO), 4.8 (dd, CH_2-T), 6.95 (m, NH carbamate), 7.2 (s, H–C=C–Me), 11.35 (s, NH imide); ^{13}C NMR ([D$_6$]DMSO): δ = 169.7, 167.1, 164.3, 154.9, 150.9 (C=O), 141.5, 108.2 (C=C), 77.9 (*t*Boc), 59.8, 47.5 (CH_2COOCH_3), 53.7, 51.4, 32.1, 31.1, 24.6, 24.2 (C cycl), 28.2 (*t*Boc), 11.9 (CH_3 thymine); MS (FAB$^+$): 453.3 [*M* + 1], 353.3 [*M* + 1 − *t*Boc].

***N*-[(2*R*)-Boc-aminocyclohex-(1*R*)-yl]-*N*-(thymin-1-ylacetyl)-glycine** [(*R*,*R*)-**4b**]: The monomer ester (*R*,*R*)-**3b** (1.5 g, 3.3 mmol) was suspended in THF (15 mL), and a solution of 0.5 M LiOH (15 mL, 7.5 mmol) was added as well as water (5 mL). After 45 min at RT, water (30 mL) was added and the mixture was washed with CH_2Cl_2 (3 × 30 mL). The aqueous phase was acidified to pH 2.5–3 and extracted with AcOEt (6 × 120 mL). The organic phase was dried over sodium sulfate and evaporated under reduced pressure. Yield: 1.38 g (95%). ^{1}H NMR ([D$_6$]DMSO): δ = 1.2–2.0 (m, CH_2 cycl), 1.45 (s, *t*Boc), 1.9 (CH_3 thymine), 3.9 (dd, CH_2COO), 4.8 (dd, CH_2-T), 6.95 (m, NH carbamate), 7.2 (s, H–C=C–Me), 11.35 (s, NH imide), 12.4 (m, COOH); ^{13}C NMR ([D$_6$]DMSO): δ = 170.3, 166.8, 164.3, 155.0, 150.9 (C=O), 141.5, 108.1 (C=C), 77.9, 28.2 (*t*Boc), 59.7, 49.9, 47.9, 44.2, 32.1, 29.7, 24.4, 24.3 (C cycl + 2CH_2), 11.9 (CH_3 thymine); MS (FAB$^+$): 439.2 [*M* + 1], 339.1 [*M* + 1 − *t*Boc].

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl]-glycine ethyl ester** [(*S*,*S*)-**5a**]: A solution of ethyl bromoacetate (1.9 mL, 17.3 mL) in DMF (10 mL) was added to a cooled suspension of (*S*,*S*)-**1a** (3.7 g, 17.3 mmol) and potassium carbonate (7.2 g, 51.9 mmol) in DMF (30 mL) over a period of 5 min. After 1 h at 0 °C and 1 h at RT the salts were filtered off and washed with DMF (30 mL) and CH_2Cl_2 (30 mL). The filtrate was evaporated under reduced pressure and the residue was purified by chromatography on silica gel (eluent AcOEt). Yield: 3.4 g (65%). ^{1}H NMR ([D$_6$]DMSO): δ = 1.0–2.5 (m, CH cycl + NH), 1.2 (t,

3H, CH_3), 1.45 (s, 9H, *t*Boc), 3.4 (q, 2H, NCH_2COO), 4.2 (q, 2H, $COOCH_2$), 6.8 (m, 1H, NH carbamate); ^{13}C NMR ([D$_6$]DMSO): δ = 172.3 (ester), 155.4 (carbamate), 77.5 (*t*Boc), 60.0, 59.6, 53.7, 47.8, 32.1, 31.1, 24.6, 24.2 (C cycl + N–CH_2–CO + $COOCH_2$)), 28.3 (*t*Boc), 14.1 (CH_3); MS (FAB$^+$): 301.2 [M+1].

Coupling of the (Z)-protected nucleobases adenine, guanine, and cytosine to the backbone: DCC (0.34 g, 1.66 mmol) was added to a solution of (*S*,*S*)-**2** (0.5 g, 1.66 mmol), (Z)-protected nucleobase acetic acid (1.66 mmol), and DhbtOH (0.27 g, 1.66 mmol) in DMF (7 mL) and CH_2Cl_2 (5 mL). After 4 h at RT, DCU was filtered off and washed with CH_2Cl_2 (30 mL). The filtrate was washed with 1 M $NaHCO_3$ (3 × 10 mL), 1 M $KHSO_4$ (2 × 10 mL) and water (2 × 10 ml). The organic phase was dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel for the adenine and cytosine monomers (eluent AcoEt, yield: 50% and 63% respectively) or by precipitation from ether for the guanine monomer (yield: 80%).

Hydrolysis of the monomer esters: The monomer ester from the preceding reaction was suspended in THF, and a 0.5 M LiOH solution (5 mL, 2.5 mmol) was added. After 1 h at RT, water (10 mL) was added, and the mixture was extracted with CH_2Cl_2 (4 × 10 mL). The aqueous phase was acidified to pH 3. The adenine monomer precipitated and was collected by filtration (yield: 58%). The cytosine and guanine monomers were extracted with CH_2Cl_2 (4 × 10 mL). Yield: 35% for the cytosine monomer and 57% for the guanine monomer. The monomers gave a single HPLC peak and were used for the PNA oligomerization without further purification.

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl]-*N*-(adenine(Z)-1-ylacetyl)glycine monomer (7a)**: ^1H NMR ([D$_6$]DMSO): δ = 1.4 (s, 9H, *t*Boc), 1.2–2.2 (m, CH cyc), 3.9 (dd, 2H, CH_2COOH), 5.3 (s, 2H, $CH_2-C_6H_5$), 5.5 (dd, 2H, adenine-CH_2–CO), 7.0 (m, 1H, NH carbamate), 7.5 (m, 5H, C_6H_5), 8.2 (s, 1H, H adenine), 8.7 (s, 1H, H adenine), 10.8 (s, 1H, NH adenine), 12.0 (broad, 1H, COOH); ^{13}C NMR ([D$_6$]DMSO): δ = 24.4, 24.7, 29.7, 32.1, 44.3, 50.0, 60.1, 66.3 (C cyc, N–CH_2–COOH and adenine–CH_2–CO), 28.2, 77.9 (*t*Boc), 123.0, 127.8–128.4 (C_6H_5), 136.4, 144.8, 149.4, 151.4 (adenine), 152.6, 155.0 (carbamate), 166.4 (amide), 170.5 (acid); MS (FAB$^+$): 582.3 [M+1].

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl)-*N*-(cytosine(Z)-1-ylacetyl)glycine monomer (7b)**: ^1H NMR ([D$_6$]DMSO): δ = 1.4 (s, 9H, *t*Boc), 1.2–2.1 (m, CH cyc), 3.9 (dd, 2H, CH_2COOH), 5.0 (dd, 2H, cytosine–CH_2–CO), 5.3 (s, 2H, $CH_2-C_6H_5$), 6.9 (m, 1H, NH carbamate), 7.1 (d, 1H, H cytosine), 7.5 (m, 5H, C_6H_5), 7.8 (d, 1H, H cytosine), 10.5 12.0 (broad, 2H, NH cytosine and COOH); ^{13}C NMR ([D$_6$]DMSO): δ = 24.3, 24.7, 29.7, 32.1, 44.1, 49.5, 50.0, 59.8, 66.5 (C cyc, N–CH_2–COOH and cytosine–CH_2–CO), 28.2, 77.9 (*t*Boc), 123.0, 127.8–128.4 (C_6H_5), 150.1, 154.9 (carbamate), 162.9 (CO cytosine), 166.8 (amide), 170.5 (acid); MS (FAB$^+$): 558.2 [M+1].

***N*-[(2*S*)-Boc-aminocyclohex-(1*S*)-yl]-*N*-(guanine(Z)-1-ylacetyl)glycine monomer (7c)**: ^1H NMR ([D$_6$]DMSO): δ = 1.4 (s, 9H, *t*Boc), 1.2–2.0 (m, CH cyc), 3.9 (dd, 2H, CH_2COOH), 5.2 (dd, 2H, guanine–CH_2–CO), 5.3 (s, 2H, $CH_2-C_6H_5$), 7.1 (m, 1H, NH carbamate), 7.4–7.6 (m, 5H, C_6H_5), 7.8 (d, 1H, H guanine), 11.4, 11.6 (broad, 2H, NH guanine and COOH); ^{13}C NMR ([D$_6$]DMSO): δ = 24.4, 24.6, 29.6, 32.0, 43.9, 44.2, 50.1, 60.0, 67.2 (C cyc, NCH_2COOH and guanine–CH_2CO), 28.2, 77.9 (*t*Boc), 123.0, 127.8–128.4 (C_6H_5), 140.0, 147.2, 150.0 (C guanine), 154.9, 155.1 (carbamate), 160.1 (CO guanine), 166.4 (amide), 170.7 (acid); MS (FAB$^+$): 598.8 [M+1].

Incorporation of the cyclohexyl–PNA monomers into PNA oligomers: The oligomerization of the modified PNAs was performed by means of the standard procedure on a (4-methylbenzhydryl)amine resin (initial loading 0.1 meq g^{-1}) with HBTU/DIEA in DMF/pyridine as a coupling reagent.[2–5, 16] The free PNAs were cleaved from the resin with TFMSA, purified by HPLC, and characterized by MALDI-TOF mass spectrometry on a Kratos MALDI-II instrument (Table 4). The overall yields of purified material were typically 10–30%, depending on the quality of the raw product. Regular PNA oligomers were prepared as described previously.[19]

T_m measurements: Duplexes and triplexes were annealed by keeping the samples at 95 °C for 5 minutes followed by slow cooling to RT. Absorbance versus temperature profiles were obtained in 100 mM NaCl, 1 mM EDTA, 10 mM Na phosphate, pH 7.0 at 260 nm with a Gilford response II spectrophotometer scanning from 5 to 90 °C at a rate of 0.5 °C per minute. The concentrations of PNA and oligonucleotides were determined optically at 60 °C from the molar extinction coefficients of the four nucleosides.

Table 4. Mass spectral analysis of synthesized PNA.

PNA	Calculated mw	Measured mw
H-TTTTT$_{SS}$TTTTT-Lys-NH_2 (**1**)	2860	2862 [a]
H-TTTTT$_{RR}$TTTTT-Lys-NH_2 (**2**)	2860	2862 [a]
H-(TTTTTT)$_{SS}$-Lys-NH_2 (**10**)	2065	2067 [a]
H-GT$_{SS}$AGAT$_{SS}$CACT$_{SS}$-Lys-NH_2 (**4**)	3014	3016 [b]
H-(GTAGATCACT)$_{SS}$-Lys-NH_2 (**7**)	3014	3016 [b]
H-TGTACGT$_{SS}$CACAACTA-Lys-NH_2 (**8**)	3394	3396 [b]
H-TGTACGTCACAACTA-Lys-NH_2 (**9**)	4099	4101 [b]

[a] MS (FAB$^+$). [b] MALDI-TOF.

Isothermal titration calorimetry (ITC): A MicroCal ITC MC-2 system was used in conjunction with an electronically controlled circulating water bath keeping the temperature constant at 25 °C in all experiments. All samples used in ITC experiments were kept in a 5 mM sodium phosphate buffer at pH 7.0. The solution of one of the strands was placed in the cell (6 μM in a volume of 1.4 mL) and 100 μL of the solution of the other strand (0.14 mM) was placed in a syringe equipped with a paddle-shaped stirring needle rotating at 400 rpm. Typically 20 injections of 4 μL each at intervals of 5 minutes were made. The heat produced after each injection was measured and was taken as the enthalpy of the reaction (determined from the average of the 5–10 initial injections). The equilibrium constant was obtained by fitting these titration data to a binding isotherm within a 1:1 bonding model. The entropy change for the reaction was determined from the free energy change of the reaction (from the equilibrium constant) and the measured enthalpy change.

Circular dichroism spectroscopy: CD spectra were recorded on a Jasco model 720 spectropolarimeter equipped with a thermoelectrically controlled cell holder. Each spectrum shown is the average of at least eight scans, recorded at 20 °C with a 1 cm optical path length. The samples were kept in a 5 mM sodium phosphate buffer at pH 7.0.

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