Exploring Hoogsteen and Reversed-Hoogsteen Duplex and Triplex Formation with Tricyclo-DNA Purine Sequences

Dorte Renneberg and Christian J. Leumann*[a]

The duplex- and triplex-formation properties of the tricyclo-DNA purine decamer 5′p-gagaaggaaa-3′ as a single strand or as part of a hairpin duplex with corresponding parallel and antiparallel pyrimidine DNA and RNA complements, as well as with antiparallel purine DNA and RNA complements, were investigated by UV melting curve analysis, circular dichroism spectroscopy, and gel mobility shift experiments. It was found that tricyclo-DNA forms very stable duplexes with the pyrimidine RNA and DNA complements not only in the Watson–Crick pairing mode, but also in the Hoogsteen one. Below pH 6.0, the tc-DNA/DNA and tc-DNA/RNA Hoogsteen duplexes were found to be more stable than the corresponding Watson–Crick DNA duplexes. Triplexes of the hairpin structure with parallel pyrimidine complements revealed even stronger Hoogsteen pairing relative to the duplexes, presumably due to structural preorganization phenomena. Triplex formation with antiparallel pyrimidine and purine third strands (reversed-Hoogsteen motif) could not be observed and seem to be unstable.

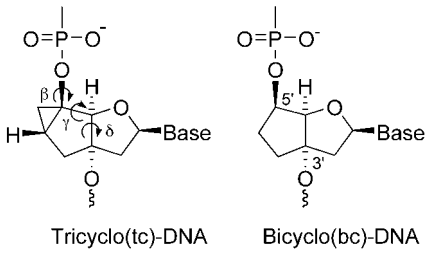
Introduction

The higher structural order of DNA and RNA is characterized by a substantial degree of polymorphism. As well as the classical Watson–Crick base-paired duplexes,[1] parallel-oriented duplexes,[2] antiparallel Hoogsteen duplexes,[3] triple helices,[4] and quadruplexes[5–8] are also known, and all display distinct and unique base-pairing patterns. The best-characterized nonstandard nucleic acid structures are the triple helices,[9] which can be classified into two structural motifs. In the pyrimidine motif, a homopyrimidine third strand binds in the major groove of a DNA duplex in an orientation parallel to the purine strand of the target duplex through Hoogsteen hydrogen bonding. In the purine motif, a purine-rich third strand binds in antiparallel fashion to the purine strand of the target duplex through reversed-Hoogsteen hydrogen bonding.

In the context of antisense research, a wide variety of oligonucleotide analogues have been synthesized and investigated in the past. Among those, the class of sugar-modified oligonucleotide analogues has emerged not only as a source of powerful antisense oligonucleotides, but also as a pool with which to study the interrelation between nucleoside structure and supramolecular association behavior of derived oligonucleotides.[10] Contributions from our laboratory include the analogues bicyclo(bc)-DNA and tricyclo(tc)-DNA (Scheme 1).

Bicyclo-DNA contains an additional ethylene bridge fused to the C(3′) and C(5′) centers of a deoxyribose unit. Structurally, this additional five-membered ring constrains the furanose unit in the C(1′)-*exo* (south, S) conformation and orients torsion angle γ into the anticlinal range. As a consequence, this DNA analogue shows an intrinsic energetic preference for the Hoogsteen and reversed-Hoogsteen pairing modes in sequence contexts in which this is permitted (homopurine/homopyrimidine sequences).[11,12]

Tc-DNA deviates structurally from bc-DNA by an additional cyclopropane unit. According to molecular-dynamics simulations, this constrains the furanose unit preferentially into the O(3′)-*endo* (north, N) conformation, while torsion angle γ maintains the anticlinal orientation as in bc-DNA. A further consequence of the cyclopropane ring seems to be a change of torsion angle β from *trans* to *gauche*. Relative to DNA, tc-DNA exhibits increased stability and base-pairing selectivity in the Watson–Crick pairing mode when complexed to complementary ssDNA and RNA.[13–15] Not surprisingly, tc-DNA thus performs very well in cellular antisense experiments.[16,17]



Scheme 1. *Structural representations of tricyclo- and bicyclo-DNA, with indications of relevant backbone torsion angles. The centers C(5′) and C(3′) are assigned as in DNA.*

In our efforts to explore the molecular association properties of tc-DNA comprehensively we report here on the Hoogsteen and reversed-Hoogsteen duplex and triplex formation behavior of tc-DNA purine sequences, by the use of UV melting, CD spectroscopy, and gel mobility shift experiments as the analytical tools.

[a] *Dr. D. Renneberg, Prof. Dr. C. J. Leumann*
Department of Chemistry and Biochemistry, University of Bern
Freiestraße 3, 3012 Bern (Switzerland)
Fax: (+41) 31-6313422
E-mail: leumann@ioc.unibe.ch

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Results and Discussion

A purine tc-DNA decamer and a corresponding hairpin containing the same asymmetric purine sequence were designed and synthesized in order to study their duplex and triplex formation properties with complementary pyrimidine and purine DNA and RNA oligomers. The oligonucleotides used in the present work are shown in Table 1. Figure 1 gives an overview of the different pairing motifs that were investigated.

Table 1. *Oligonucleotides prepared and used in this study.*

Oligomer	Sequence 5′→3′[a]	ESI-MS[b] calcd	ESI-MS[b] found
tc1	p-**gagaaggaaa**	3593.6	3594.1
tc2	TTTCCTTCTC-$(EG)_6$-**gagaaggaaa**	6839.4	6839.0
dna1	GAGAAGGAAA	3133.1	3133.6
dna2	TTTCCTTCTC-$(EG)_6$-GAGAAGGAAA	6458.0	6458.6
dna3	TTTCCTTCTC	2918.8	2919.6
dna4	CTCTTCCTTT	2918.8	2919.5
dna5	AAAGGAAGAG	3133.1	3133.3
rna1	*UUUCCUUCUC*	2994.8	2995.0
rna2	*CUCUUCCUUU*	2994.8	2995.1
rna3	*AAAGGAAGAG*	3293.1	3293.2

[a] Bold lowercase: tricyclo-DNA; uppercase: DNA; italic uppercase: RNA; $(EG)_6$: hexa(ethylene glycol) linker; p: 5′-phosphate. [b] Molecular masses were recorded in the negative ion mode and are reported as $[M-H]^-$.

In pyrimidine-motif triplexes, the tc-DNA was part of the duplex target and was hybridized to a parallel and antiparallel oriented pyrimidine strand (Figure 1, motifs A and B). In the purine motif, tc-DNA was either part of the target duplex and hybridized to an antiparallel purine strand (Figure 1, motif C) or was used as a third strand designed to bind to the duplex target in the antiparallel orientation (Figure 1, motif D).

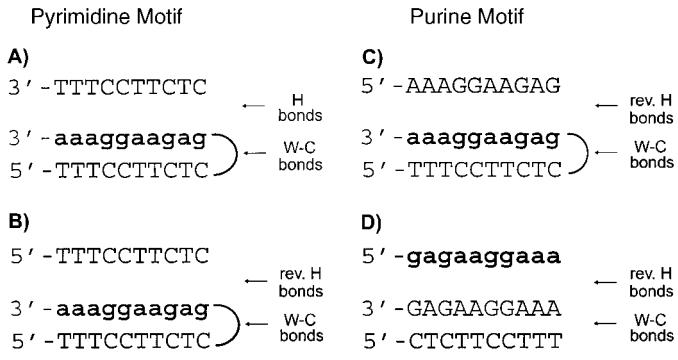

Figure 1. *Simplified illustration of the different triplex pairing motifs investigated. Duplex target: bold and lowercase letters, tc-DNA; uppercase letters, DNA; half-circle, hexa(ethylene glycol) linker. Third strands: DNA or RNA (U for T). H: Hoogsteen; rev. H: reverse-Hoogsteen; W–C: Watson–Crick pairing modes.*

Duplex formation and stability

In initial experiments, we were interested in the preferred pairing mode of the tc-oligonucleotide in duplex formation. In order to determine its hybridization properties we investigated the pairing of tc1 (5′-phosphorylated for reasons of chemical stability[14]) with its parallel and antiparallel DNA and RNA complements and compared it with that of natural DNA. UV melting curves exhibited cooperative and monophasic transitions in all cases. Self-association of the purine strand could be excluded (data not shown). All melting temperatures are summarized in Table 2, and the corresponding melting curves of the parallel (Hoogsteen) duplexes are depicted in Figure 2.

Table 2. *Melting temperatures (T_m) of the duplexes in the Watson–Crick and Hoogsteen pairing modes.[a]*

		Watson–Crick		Hoogsteen	
	pH	dna3	rna1	dna4	rna2
dna1	5.5	24.6	≈11	≈8	~10
	6.0	25.9	≈12	<5	<10
tc1	5.5	52.7	58.7	31.0	34.3
	6.0	53.3	59.1	25.9	29.6

[a] T_m values are given in °C (260 nm) and were measured in NaOAc (100 mM), EDTA (1 mM), $c = 1.5$ μM.

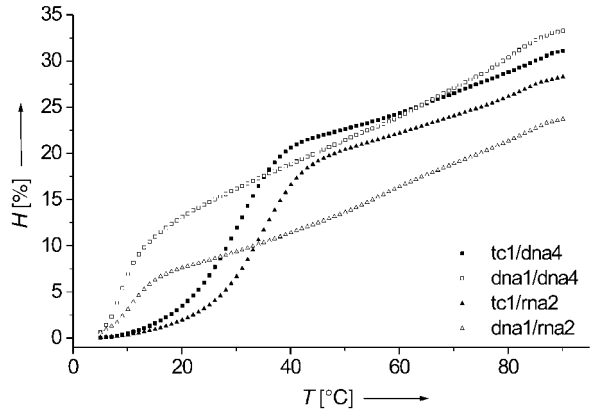

Figure 2. *UV melting curves (260 nm) of the Hoogsteen duplexes tc1/dna4 (solid squares) and tc1/rna2 (solid triangles), dna1/dna4 (open squares), and dna1/rna2 (open triangles) in NaOAc (100 mM), EDTA (1 mM), 1.5 μM, pH 5.5.*

The most stable of all investigated bimolecular duplexes was found to be the antiparallel (Watson–Crick) duplex tc1/rna1, with a T_m of 58.7 °C. Compared to the corresponding DNA/RNA duplex, with a T_m of ≈11 °C, this represents an increase in stability of +48 °C (+4.8 °C per base-pair (bp)). The corresponding DNA hybrid tc1/dna3 showed a T_m of 52.7 °C, thus revealing a higher thermal stability of $\Delta T_m = +28$ °C (+2.8 °C bp^{-1}) than for dna1/dna3, for which a T_m of 24.6 °C was found. The fact that the DNA/RNA duplex is less stable than the pure DNA duplex is in line with earlier observations on homopurine/

homopyrimidine systems.[18] Within the experimental error, the melting temperatures were, as expected, pH-independent, excluding duplexes of the reversed-Hoogsteen type in all cases.

In the parallel (Hoogsteen) duplex regime we found a T_m of 31.0 °C at pH 5.5 for the hybrid tc1/dna4, revealing a remarkable decrease in stability of almost −22 °C in relation to the Watson–Crick-paired tc1/dna3. At pH 6.0 a decrease of the T_m to 25.9 °C was observed, reflecting the pH dependency of the Hoogsteen complexes due to the required protonation of cytosine (C^+). A slightly more stable duplex ($T_m = 34.3$ °C at pH 5.5 and $T_m = 29.6$ °C at pH 6.0) was formed by pairing of tc1 to the parallel RNA complement rna2. The tc-DNA/RNA Hoogsteen hybrid is also inferior to the Watson–Crick tc1/rna1 duplex in thermal stability in this case, by −24 °C (pH 5.5). The Watson–Crick motif is thus by far the most stable of all investigated pairing motifs of tc-DNA. Interestingly, at pH < 6.0 the tc-DNA/DNA Hoogsteen duplex is still of higher thermal stability than the Watson–Crick duplex dna1/dna3. The CD spectra of the tc-DNA Hoogsteen systems are somewhat different from standard Watson–Crick duplexes and seem to be strongly influenced by the nature of the counter-strand (DNA or RNA). The tc1/rna2 hybrid exhibits a positive maximum at 268 nm while the tc1/dna4 complex shows a less intense positive maximum at 277 nm (Figure 3 A).

We also examined the thermal stabilities of the Hoogsteen duplexes in the DNA series (dna1/dna4 and dna1/rna2). These duplexes were found to be significantly less stable, with T_ms of ≈8 °C and 10 °C at pH 5.5, respectively. Increasing the pH to 6.0 further reduced duplex stability with DNA and RNA to virtual non-existence in the temperature range investigated. Comparison with the tc-DNA Hoogsteen hybrids showed a difference in stability of about +24 °C (+2.4 °C bp^{-1}) and is thus in the same range as found for the tc-DNA Watson–Crick duplexes (with the exception of the DNA/RNA hybrid dna1/rna1, which is even less stable). These results show that tc-DNA purine strands stabilize duplexes in both the Watson–Crick and the Hoogsteen motif equally well.

The CD spectrum of the Hoogsteen duplex dna1/dna4 shows a characteristic low-intensity positive maximum at 280 nm and a negative minimum around 212 nm. In contrast, the dna1/rna2 hybrid exhibits a hypsochromic shift of the positive maximum (270 nm) with higher intensity, suggesting more A-type character in this case.[19] Noteworthy are the large differences in intensity of the broad band with its center at about 225 nm. These are absent in the tc-DNA Hoogsteen duplexes. Thus this CD spectral information reveals distinct differences in secondary structures between the two systems as a function of the nature of the counter-strand.

As expected, the two intramolecular (Watson–Crick) hairpin duplexes tc2 and dna2, with the same sequence composition as the corresponding bimolecular duplexes, were found to be of much higher thermal stabilities. While the hairpin dna2 shows a T_m of 68.6 °C, for tc2 we observed a melting temperature of 90 °C or more. Once again, tricyclo-DNA stabilizes by more than 2.2 °C bp^{-1} relative to DNA, even in an intramolecular arrangement.

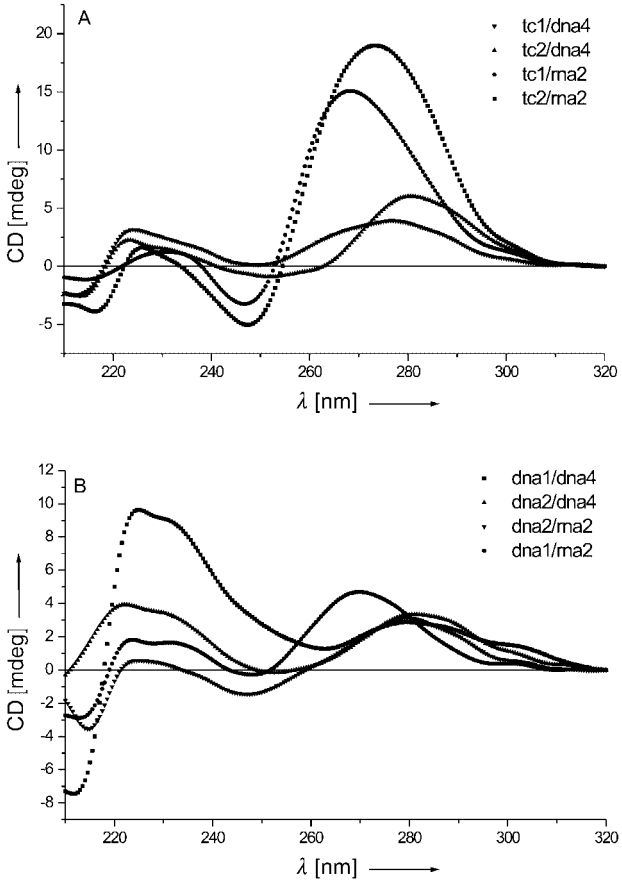

Figure 3. *CD traces of duplexes and triplexes containing tc-DNA, DNA, and RNA: A) tc-DNA-containing complexes; B) DNA reference complexes in NaOAc (100 mM), EDTA (1 mM), 1.5 μM, pH 5.5 (3–10 °C).*

Triplex formation in the pyrimidine motif

In the presence of a complementary Hoogsteen strand (Figure 1, motif A) stable triplexes are formed in all cases. However, as can be seen from Table 3, which presents T_m data of third strand dissociation, remarkable differences were observed. Illustrative melting curves at pH 6.0 are shown in Figure 4.

Dissociation of dna4 from dna2 occurs at 17.2 °C (pH 6.0). Study of the same dissociation with dna1/dna3 as a bimolecular duplex target showed that melting of dna4 occurs at

Table 3. *Melting temperatures (T_ms) of Hoogsteen and reversed-Hoogsteen third strand melting in the pyrimidine motif.*[a]

		Hoogsteen strand		rev.-Hoogsteen strand	
Duplex target	pH	dna4	rna2	dna3	rna1
dna2[b]	5.5	23.2	32.6	n.d.[c]	n.d.[c]
	6.0	17.2	22.3	n.d.[c]	n.d.[c]
tc2[b]	5.5	48.6	49.1	n.d.[c,d]	n.d.[c]
	6.0	36.6	39.1	n.d.[c]	n.d.[c,d]

[a] T_m values for triplex melting are given in °C (260 nm) and were measured in NaOAc (100 mM), EDTA (1 mM), $c = 1.5$ μM. [b] T_m of the hairpin dna2 = 68.6 °C and of tc1 > 90 °C. [c] n.d. = no T_m detected. [d] Melting curves were also measured at 300 nm.

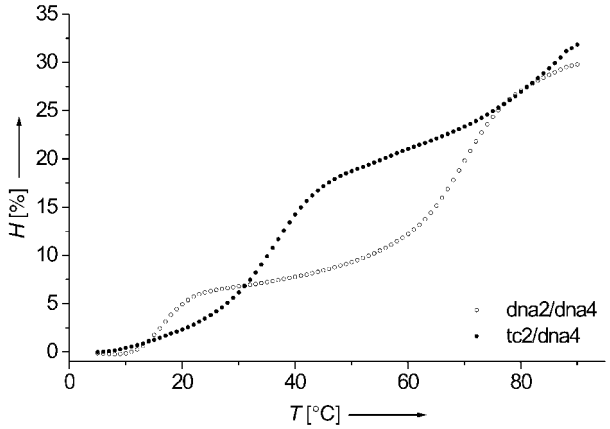

Figure 4. *UV melting curves (260 nm) of dna2/dna4 (open circles), tc2/dna4 (solid circles) in NaOAc (100 mM), EDTA (1 mM), 1.5 μM, pH 6.0.*

17.8 °C and so is—within the error limit—identical in both systems. The loop structure therefore has no influence on third strand melting. With rna2 as third strand, stable triplexes with $T_m = 32.6$ °C (pH 5.5) and $T_m = 22.3$ °C (pH 6.0) were measured. As expected, the triplex with RNA as third strand is of higher thermal stability than that with DNA.[18,20]

The same experiments with tc2 revealed dramatically higher T_ms for third strand melting. Interestingly, the melting/annealing curves involving tc2 as a duplex target in all cases showed smaller hysteresis than for dna2, indicating faster association kinetics (data not shown). When tc2 was hybridized to dna4, a clear third strand melting event at 48.6 °C (pH 5.5) was observed. Comparison of the triplex stability with the natural DNA reference reveals a difference in T_m of +25.4 °C in favor of the tc-DNA target, corresponding to +2.5 °C per base triplet. Interestingly, with rna2 as the Hoogsteen strand the triplexes are only marginally more stable (by ca. 1–2 °C) than with dna4, which contrasts with the data obtained with the DNA target dna2.

In order to check whether the differences in triplex stability are related to changes in conformation we performed a CD analysis (Figure 3). In the case of the DNA reference system the CD spectra of the DNA (dna2/dna4) and RNA (dna2/rna2) triplexes deviate only marginally (Figure 3B), as expected.[21] The higher ellipticities below 260 nm in the all-DNA triplex most probably arise from the partially denatured state of the system at the given temperature. This suggests that only small structural variation exists within the two systems. The picture is somewhat different for tc-DNA triplexes (Figure 3A). While the triplex with the RNA third strand (tc2/rna2) exhibits an intense positive maximum, similar to the Hoogsteen duplex tc1/rna2, the triplex with the DNA third strand (tc2/dna4) is more comparable to the Watson–Crick duplex, although accompanied by a shift of the positive band to 278 nm. Thus, it is possible that differences in the overall conformations of the DNA and RNA triplexes are going along with compensational effects in the thermal stabilities of the tc2/rna2 and tc2/dna4 triplexes.

Comparison of the thermal stabilities of the Hoogsteen duplexes (Table 2) and the Hoogsteen triplexes (Table 3) revealed an interesting feature. We found an increase in affinity of about 15–20 °C for dna4 and rna2 binding to the double-stranded targets tc2 and dna2, as compared to binding to the single-stranded targets dna1 and tc1. This increase in T_m is also observed in the termolecular system tc1/dna3/dna4 (T_m of third strand melting 49.2 °C) and is thus independent of the hairpin loop structure of the target. A higher thermal stability upon binding to a double-stranded target would be expected to be electrostatically disfavored due to the higher number of negative charges in the complex. However, the entropic benefit of a single strand binding to a conformationally preorganized target as compared to a random coiled single strand seems to overcompensate for the unfavorable electrostatics.

We were also interested in whether stable pyrimidine triplexes are formed with a third strand in the antiparallel orientation, thus binding in the reversed-Hoogsteen mode (Figure 1, motif B). Neither with dna2 nor with tc2 as targets were we able to observe triplex formation with the reversed-Hoogsteen strands dna3 and rna1 (Table 3). In order to confirm the non-existence of triplexes we also recorded UV melting curves at 300 nm in the cases of tc2/dna3 at pH 5.5 and tc2/rna1 at pH 6.0. At this wavelength a hyperchromic effect due to protonation of the base cytosine should be observable.[22] No transitions in the 0–90 °C temperature range were detected under these conditions either, definitely ruling out the existence of reversed-Hoogsteen triplex structures in this sequence motif.

Triplex formation in the purine motif

In another set of experiments, we used tc1 in the target duplex and either dna5 or rna3 as third strand (Figure 1, motif C). Alternatively, we also investigated the ternary mixtures of duplexes dna4/dna5 and rna2/rna3 with the third strands dna1 and tc1 (Figure 1, motif D). In none of the investigated systems were triplexes formed, and only one melting transition originating from the melting of the Watson–Crick duplex was observed. In order to verify this result we also examined triplex formation by gel shift experiments. Again, no signs of triplex formation could be found in the presence or in the absence of 10 mM $MgCl_2$, conditions known for promoting triplex formation[23,24] (gels not shown). We thus conclude that, at least in this sequence context, tc-DNA does not form triplexes according to the purine motif.

We have identified two potential reasons why no triplex formation occurs in these cases. In the purine motif the base triples are not isomorphic. It is highly possible that the conformationally constrained nature of the tc-DNA backbone does not allow for easy structural adjustments of the sugar–phosphate backbone in order to compensate for the non-isomorphism of the base-triplets. Alternatively, it is known that RNA purine strands are ineffective in forming triplexes in the purine motif.[25] Given that tc-DNA is a structural RNA analogue (CD spectroscopic evidence) it may not form triplexes for the very same reason as RNA.

Conclusion

Hoogsteen base-paired structures occur quite frequently in natural DNA and have been identified as playing roles in transcription, replication, and other cellular processes.[26] In this study we have shown that tc-DNA purine sequences are able to form remarkably stable Watson–Crick and Hoogsteen duplexes as well as triplexes with DNA and RNA in the parallel motif, while being unable to form triplexes in the purine motif. The increases in thermal stability in both the Watson–Crick and the Hoogsteen binding modes are in a similar range to those seen with natural DNA or RNA, of about 2–4 °C per modification. Thus, below pH 6.0, the decameric tc-DNA Hoogsteen duplex is more stable than the corresponding Watson–Crick DNA duplex. The relative stabilities of the different complexes studied here can be summarized and ranked as follows: W–C tc-DNA/RNA > W–C tc-DNA/DNA > W–C RNA/RNA > H tc-DNA/RNA > H tc-DNA/DNA > W–C DNA/DNA > W–C DNA/RNA > H DNA/RNA > H DNA/DNA.

It is evident that the energetically preferred pairing mode of tc-DNA at neutral or slightly acidic pH is the Watson–Crick mode. This is different from the case of bicyclo-DNA, in which the Hoogsteen binding mode is preferred over the Watson–Crick mode.[11,12] Thus, a seemingly small structural change—the presence or absence of the three-membered ring—coincides with a remarkably large change in the base-pairing preferences. Current structural understanding of the two systems suggests that the differences arise from the backbone torsion angles β and δ, which are antiperiplanar/anticlinal in bicyclo-DNA but gauche/synclinal in tricyclo-DNA.

In conclusion, tc-DNA purine strands display interesting Hoogsteen pairing properties that can surpass the Watson–Crick pairing properties of natural DNA. This may be of importance for applications of tc-DNA, as diagnostic probes, for example, or in antisense technology.

Experimental Section

Oligonucleotide synthesis and purification: Tc-oligonucleotides were prepared on the 1.3 μmol scale on a Pharmacia Gene Assembler Special™ DNA-synthesizer by use of the modified phosphoramidite chemistry procedure as reported previously.[15] The coupling time was set to 10 min, and 5-(ethylthio)-1*H*-tetrazole (0.25 M in CH_3CN) was used as activator. The oligomers tc1 and tc2 were assembled on a universal solid support (CT-Gen, San Jose, CA), and for all other oligonucleotides the standard CPG-solid supports from Glen Research were used. The hexa(ethylene glycol) linker was also purchased from Glen Research (spacer phosphoramidite 18). Deprotection and cleavage from the solid support was performed under standard conditions except for the use of a longer deprotection time (concentrated NH_3, 60 h, 55–65 °C). Crude oligonucleotides were purified by DEAE ion-exchange HPLC and/or reversed-phase HPLC. Oligodeoxyribonucleotides and oligoribonucleotides (2′O-TBDMS protection) were prepared by standard methods. The nucleotide compositions of the oligonucleotides were confirmed by ESI-TOF-MS.

UV melting curves: The UV melting curves were recorded at 260 and 300 nm on a Varian Cary-100 UV/Vis spectrophotometer. A heating-cooling-heating cycle in the 5→90 °C temperature range with a gradient of 0.5 °C min^{-1} was applied. T_m values were defined as the maximum of the first derivative of the melting curves (heating ramp) and were analyzed by use of the Origin 5.0 software package. In case of the triplex to duplex transition, the heating to cooling profiles were not superimposable, thus reflecting slow third strand association. This behavior was not observed in the case of the duplex to single strand or coil transitions.

Circular dichroism: CD spectra were recorded on a Jasco J-715 spectropolarimeter with a Jasco PFO-350S temperature controller over the 210–320 nm wavelength range. Samples were placed in a quartz cell (10 mm path length) thermostated at 10 °C (3 °C in the case of dna1/dna4). The concentration of the oligonucleotide complexes was 1.5 μM, in NaOAc (100 mM), EDTA (1 mM), pH 5.5. Each spectrum represents the average of three measurements.

Acknowledgements

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Keywords: DNA recognition · DNA triple-helix · Hoogsteen pairing · oligonucleotides · Watson–Crick pairing

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