Tricyclo-DNA: A Phosphodiester-Backbone Based DNA Analog Exhibiting Strong Complementary Base-Pairing Properties

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Due to their selective complementary base-association phenomena and their enhanced resistance against enzymatic degradation, DNA analogs attract considerable attention as tools in molecular biology and potential drugs in medicinal chemistry. Sequence selective complexation of single stranded RNA or double stranded DNA thereby constitutes the molecular prerequisites for inhibition of gene expression on the level of translation or transcription.[1−3] Oligonucleotide-like pairing systems might also be useful in materials science and nanotechnology,[4] e.g., as molecular scaffolds for the precise spatial positioning of metal clusters[5] and nanocrystals[6] or as optical or numerical functional units in computer technology.[7] The development of new oligonucleotide analogs displaying well defined and predictable association patterns with high complex stabilities are therefore of considerable interest.

In order to investigate the effect of structural preorganisation of DNA single strands on their pairing properties we recently developed the DNA analog bicyclo-DNA, which deviates from natural DNA by an ethylene unit introduced between the 3′ and 5′ centers of the deoxynucleoside units[8,9] (Figure 1). We found that bicyclo-DNA, in contrast to natural DNA, constitutes a very stable pairing system with a strong preference for the Hoogsteen and reversed Hoogsteen association mode[10] over the Watson−Crick association mode.[11] The enhanced complex stability can at least in part be attributed to the restricted conformational space of the bicyclonucleoside units, while the preference for Hoogsteen- and reversed Hoogsteen association has its origin in an alteration of the backbone torsion angle γ by ca. +100° compared to natural DNA.

In order to explore the effect of further conformational restriction on association mode and affinity we developed the analog tricyclo-DNA (Figure 1). The building blocks of tricyclo-DNA deviate from the bicyclonucleosides by an additional cyclopropyl unit introduced into position 5′ and 6′ of the carbocyclic ring. This leads to an additional conformational stabilization of the carbocyclic five-membered ring and to a partial correction of the torsional angle γ toward the anticlinal (ac) range. Here we present the synthesis and pairing properties

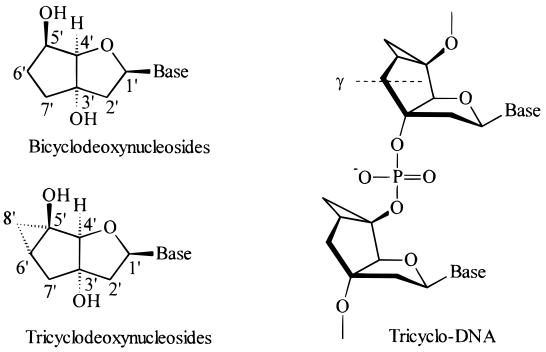
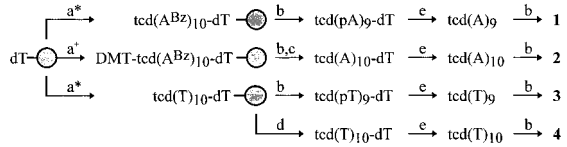


Figure 1. Structures of the bicyclo- and tricyclodeoxynucleoside as well as tricyclo-DNA.



Sequence		(M-H)⁻ calc.	(M-H)⁻ found
1, tcd(pA)$_8$	tcd(pApApApApApApApA)	2827.2	2830.1
2, tcd(pA)$_9$	tcd(pApApApApApApApApA)	3178.3	3182.1
3, tcd(pT)$_8$	tcd(pTpTpTpTpTpTpT)	2755.0	2752.8
4, tcd(pT)$_9$	tcd(pTpTpTpTpTpTpTpT)	3097.2	3094.2

Figure 2. Synthesis of tricyclodeoxy-oligonucleotides. Reaction conditions: (a) standard phosphoramidite oligonucleotide synthesis (coupling time 6 min, * trityl-off mode, $^+$ trityl-on mode); (b) concentrated NH_3, 55 °C, 15 h; (c) 400 μL of CH_3COOH, 100 μL of H_2O, room temperature, 20 min; (d) concentrated NH_3, room temperature, 2 h; (e) snake venom phosphodiesterase (3 mU/O.D.^{260}), alkaline phosphatase (100 mU/O.D.^{260}), 0.15 M NaCl 10 mM Tris·HCl, pH 7, 37 °C, 5 h.

of the four tricyclodeoxyoligonucleotides **1**−**4** containing the bases thymine and adenine.

Oligonucleotide synthesis (Figure 2) was performed on a DNA synthesizer in a 1.3 μmol scale using the standard cyanoethyl phosphoramidite building blocks of the corresponding tricyclodeoxynucleosides.[12] For synthetic ease natural thymidine bound to CPG solid support was used as the starter unit. Chain elongation was performed with a standard synthesis cycle to which no changes, except a prolongation of the coupling time to 6 min were necessary. In 10 coupling cycles proceeding with 90−99% yield each (trityl assay) the solid-phase bound oligomers were obtained. Subsequent treatment with concentrated NH_3 led besides deprotection and cleavage from the solid support to elimination of the 5′-terminal tricyclodeoxynucleoside units (except for the case of tcd(A)$_{10}$-dT that was prepared in the trityl-on mode).[13] Treatment of the purified oligonucleotides with a cocktail of snake venom phosphodiesterase (SVP) and alkaline phosphatase removed the natural thymidine residues at the 3′-termini and where present the phosphate group at the 5′-end.[14] Subsequent retreatment with concentrated NH_3 (55 °C, 15 h) led again to the removal of the tricyclonucleoside

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(13) The elimination of the 5′-terminal tricyclo-nucleoside unit, displaying a free tertiary 5′-hydroxyl group, most probably arises from a thermally induced, base catalyzed rearrangement of the cyclopropanol unit to a ring-enlarged ketone, followed by a base catalyzed β-elimination of the 3′-phosphate substituent, leaving behind a 5′ phosphorylated oligonucleotide shortened by one nucleoside unit. Subsequent degradation of the remaining oligomer is hindered by the presence of the 5′-terminal phosphomonoester function. Base catalyzed cyclopropanol rearrangements to ketones in protic solvents are well precedented in the literature; e.g., Conia, J. M. *Pure Appl. Chem.* **1975**, *43*, 317−326.
(14) All oligonucleotide intermediates mentioned in Figure 2 as well as **1**−**4** were isolated by reversed phase- or DEAE-HPLC in analogy to[9] and characterized by MALDI-ToF mass spectrometry.

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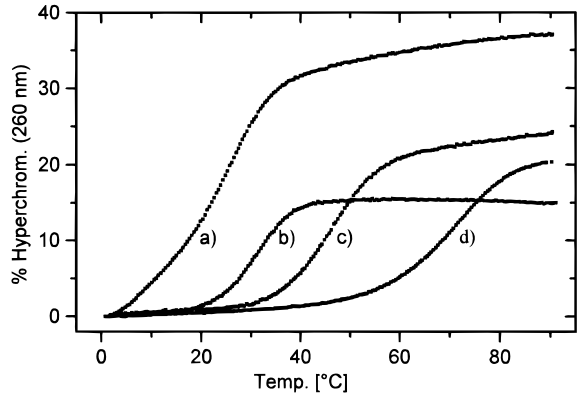

Figure 3. UV-melting curves (260 nm) of (a) $d(T_9)\cdot d(A_9)$, (b) **4**·$d(A_9)$, (c) $d(T_9)$·**2**, and (d) **4**·**2** in 10 mM NaH_2PO_4, pH 7.0, 1 m NaCl, duplex concentration = 4.6 μM (melting experiments with RNA: total base-pair concentration = 41−43 μM).

Table 1. T_m Data [°C] from UV-Melting Curves (260 nm) in 10 mM NaH_2PO_4, 1 M NaCl, pH 7.0[a]

	tcd(pA)$_8$ (**1**)	tcd(pA)$_9$ (**2**)	d(A)$_8$	d(A)$_9$	poly(A)
tcd(pT)$_8$ (**3**)	**61**		24		39
tcd(pT)$_9$ (**4**)		**72**		31	44
d(T)$_8$	40		**14.5**		28
d(T)$_9$		48		**24**	32
poly(U)	66	73	52	53	**76**

[a] Total base-pair concentration 41−43 μM.

unit at the 5′-end yielding the sequences **1**−**4**, that were purified by HPLC and identified by MALDI-ToF mass spectrometry (Figure 2). The sequences **1**−**4** proved to be completely chemically stable under the conditions used for UV-melting curve analysis as determined by HPLC and mass spectometry.

The complementary pairing properties of the tricyclo-DNA sequences **1**−**4** with itself and with the natural (non-5′-phosphorylated) DNA sequences d(T)$_9$, d(T)$_8$, d(A)$_9$, d(A)$_8$ and the RNA polymers poly(A) and poly(U) were investigated by UV-melting curve analysis. Representative melting curves are depicted in Figure 3, and the corresponding melting temperatures (T_m) are listed in Table 1.

All sequences **1**−**4** showed no cooperative melting behavior in the temperature interval 0−90 °C without complement. Therefore self-pairing can be excluded in all cases. Within the tricyclo-DNA series, however, strong complementary duplex formation was observed. Under high salt conditions (1 M NaCl, pH 7.0) the T_m of the nonamer duplex **2**·**4** is higher by 48 °C and that of **1**·**3** by 46 °C compared to the natural duplexes of the same length.[15] The T_m of the nonamer duplex **2**·**4** of 72 °C thus equals that of the strong triplex formed between d(A)$_{10}$ and the peptide nucleic acid complement PNA-(T)$_{10}$.[16] The adenine- (**1**, **2**) and thymine-containing tricyclo-DNA sequences (**3**, **4**) also form remarkably stable duplexes with the corresponding natural complementary sequences. The differences in T_m-values, relative to the reference duplexes are 24 and 25.5 °C for the pair **2**·d(T)$_9$ and **1**·d(T)$_8$, respectively, as well as 9 and 7 °C for the pairs **3**·d(A)$_8$ and **4**·d(A)$_9$, respectively. The

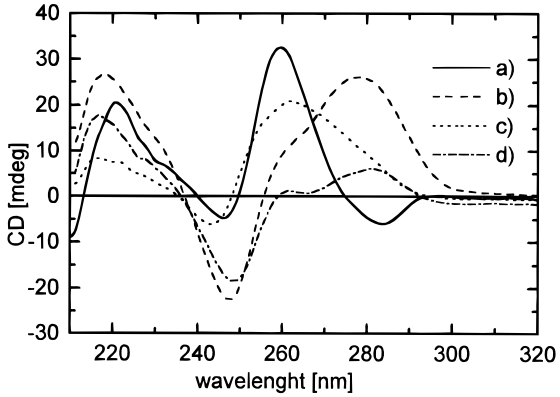

Figure 4. CD spectra of the duplexes **4**·**2** (a), **4**·d(A$_9$) (b), **2**·d(T$_9$) (c), and d(T$_9$)·d(A$_9$) (d) in 10 mM NaH_2PO_4, pH 7.0, 0.15 M NaCl, T = 20 °C (a), 8 °C (b−d). Concentrations as for Figure 3.

complexes of **1**−**4** with RNA (poly(A) and poly(U)) are again dramatically more stable than those of the natural DNA-octa- and nonamers. The stability of the complex **2**·poly(U) (T_m = 73 °C) almost equals that poly(A)·poly(U) (T_m = 76 °C) under comparable buffer conditions.

The CD-spectrum of **2**·**4** shows distinct differences to that of d(A)$_9$·d(T)$_9$ (Figure 4) but is largely similar to that of the corresponding bicyclo-DNA sequences.[9] This implies that tricyclo-DNA purine sequences, in analogy to bicyclo-DNA, prefer the Hoogsteen and/or reversed Hoogsteen and not the Watson−Crick base-pairing mode.

The sequences **1**−**4** described here are inert against the action of the 3′-exonuclease SVP. Under the conditions used for the removal of the natural 3′-terminal thymidine unit (Figure 2), no products arising from cleavage of other phosphodiester linkages could be observed according to HPLC and mass spectrometric analysis. These properties, together with the higher hydrophobicity of tricyclo-DNA compared to DNA are of interest in connection with potential biological applications.

In conclusion we have shown here that (i) tricyclo-DNA, despite the sterically strained, double-tertiary phosphodiester functions, can efficiently be synthesized using the classical phosphoramidite chemistry; (ii) 5′-end phosphorylated tricyclo-DNA sequences are chemically stable in neutral aqueous solution at elevated temperatures; (iii) tricyclo-DNA is completely stable against the nucleolytic enzyme SVP. Within homobasic sequences, tricyclo-DNA so far constitutes the most stable A−T base pairing system entirely based on a charged phosphodiester backbone.[17] A more detailed analysis of the pairing properties as well as the question whether tricyclo-DNA can undergo strand invasion processes in a similar manner as PNA[16] is currently under investigation.

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Supporting Information Available: A listing of UV-melting curves of 1:1 and 1:2 mixtures of **2** and **4** at 260 and 284 nm (Figure 5) as well as a nondenaturing polyacrylamide gel shift assay of 1:1 and 1:2 mixtures of **2** and **4** (Figure 6) (2 pages). See any masthead page for ordering and Internet access instructions.

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(15) UV-melting curves (260 nm) of stoichiometric 1:1 mixtures in the case of **2** and **4** display only one transition, whereas 1:2 mixtures show two melting transitions with the T_m's of 38 and 72 °C, respectively, the one at lower temperature being invisible at 284 nm (Figure 5, Supporting Information). From this it can be concluded that 1:1 mixtures (in contrast to PNA) form duplexes that do not disproportionate into triplex structures and free single strands. T·A−T triplexes are formed given the correct stoichiometric ratio of single strands. Further proof for nondisproportionation comes from a gel retardation assay (Figure 6, Supporting Information) in which no excess purine strand was observable at 1:1 stoichiometry of **2** and **4**.

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