DNA Recognition

DOI: 10.1002/anie.200601127

High Thermal Stability of 5′-5′-Linked Alternate Hoogsteen Triplexes at Physiological pH**

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Sequence-specific recognition, targeting, treatment, and alteration of double-stranded DNA (dsDNA) are topics of considerable interest for the study of mutated or recombined genes.[1] The formation of triple helixes through binding of a single-stranded triplex-forming oligonucleotide (TFO) to a homopurine sequence of dsDNA through major-groove interactions is one of the approaches to generate novel therapeutics on the dsDNA level. The design of a third strand that will bind to the dsDNA under physiological conditions is still challenging. In the parallel binding motif, the sensitivity of C^+·G-C Hoogsteen base pairs to pH must be overcome.[2] The formation of stable secondary structures, such as quadruplexes,[2,3] and the instability of triplexes in the presence of K^+ and Na^+ ions are major limitations for purine-containing TFOs.[2,4] Another limitation for the generation of a TFO is its length, which is supposed to be in the range of 15 to 30 base pairs to ensure target-specificity and binding affinity.[5]

To increase the number of targets, alternate-strand TFOs have been designed, which consist of two sequences with inverted polarity that are linked to each other through an appropriately chosen linker to ensure binding to two adjacent purine tracts on opposite strands of the dsDNA.[6] Most of the sequences have been designed in a 3′-3′ fashion,[7] and only a few examples have been published for the 5′-5′ linkage.[8] A phosphodiester,[6a,7] a short unpaired oligonucleotide sequence,[9] and alkyl chains[10] are usually used as the linkers. However, such linkers do not ensure the stability of the final TFOs under physiological conditions. Attachment of an intercalator at adjacent positions of the alternate-strand TFOs has been used to increase the stability.[11] Recently, we designed an intercalating 5′-5′ linker **1** (Scheme 1) to connect two 8-mer sequences with inverted polarity to generate a Hoogsteen-type triplex, with a considerable increase in the thermal stability.[8b] For further biological evaluation of the alternate-strand TFO containing the bridge **1**, we chose the extensively studied HIV-1 sequence[12] (D1,

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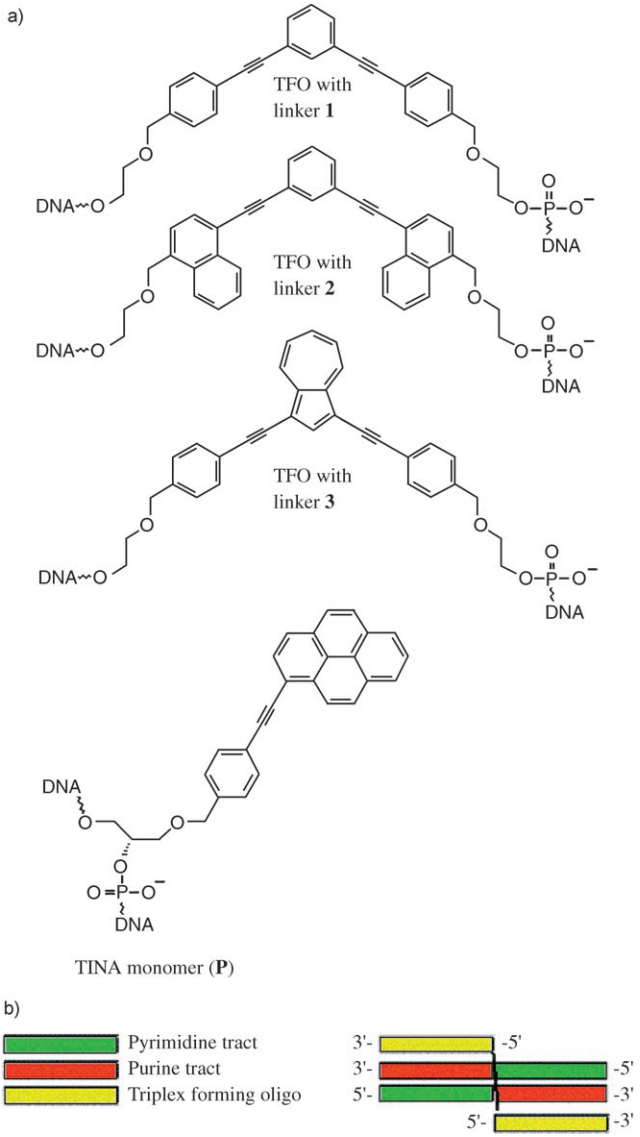
[**] The Nucleic Acid Center is funded by The Danish National Research Foundation for studies on nucleic acid chemical biology.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 1. a) Structures of the monomer of twisted intercalating nucleic acids (**P**, TINA) and linkers for alternate strand TFOs (**1**–**3**). b) Representation of the 5′-5′ alternate-strand triplex concept.

Table 1), which enabled the possibility for the design of alternate-strand triplexes consisting of nine base pairs from one side and five base pairs from another. However, a remarkable drop in the melting temperature (T_m) of the triplex for an alternate-strand TFO possessing linker **1** (ON1/D1, Table 1) from 42.5 °C to less than 5 °C was observed when the pH value of the solution was changed from 6.0 to 7.2. We saw also that the reference 9-mer sequence (ON9), which is the longest part of one of the alternate-strand TFO arms, was unable to bind to dsDNA (D1) at pH 7.2, and a T_m value of 16.0 °C was observed at pH 6.0. This was a result of the presence of a single 5-methylcytosine in the sequence, which is supposed to be less sensitive to pH than cytosine.[13] Increasing the length of one of the strands by three 5-methylcytosine groups in ON2 did not improve the T_m values at neutral pH. As the thermal stability of TFOs should be higher than or in the range of 37 °C to ensure the TFOs have biological activity, we decided to design new 5′-5′ alternate, intercalating linkers for Hoogsteen-type triplexes and also use recently discovered twisted intercalating nucleic acids (TINAs).[14] TINAs possess bulged insertions of (*R*)-1-*O*-[4-(1-pyrenylethynyl)phenylmethyl]glycerol (**P**, Scheme 1) in the middle of homopyrimidine sequences and lead to parallel triplexes with extraordinary thermal stability.[14] Herein, we present novel 5′-5′ alternate-strand Hoogsteen-type oligonucleotides, which can bind to dsDNA at pH 7.2 and possess intercalating linkers. We also report on their combinations with TINA monomer **P**.

We assumed that better π–π interactions with surrounding nucleobases within the complex of TFO/dsDNA and, therefore, increased thermal stabilities could be achieved by increasing the number of fused aromatic rings in the linker **1**. Triple bonds have been considered to be good connectors that provide structural rigidity, twisting ability,[8b,14] and improved intercalation.[15] The two peripheral benzene rings of **1** were replaced by naphthalene rings to give the linker **2** (Scheme 1). The central benzene ring of **1** was also substituted by 1,3-azulene to provide structure **3** (Scheme 1). Models of both linkers in triple helixes were minimized with the AMBER force field in MacroModel 8.0 and the results are presented in Figure 1.[16] As can be seen from these pictures, the naphthalene rings stacked with the nucleic bases of the alternate-strand TFO and they also partially covered purine bases involved in Hoogsteen base pairing (Figure 1 a,c). In contrast to this, the azulene unit was positioned in the middle of the dsDNA part of the triplex, just above the area of Watson–Crick base pairing, partially stacking with both duplex bases (Figure 1 b,d).

The dimethoxytrityl (DMT) protected phosphoramidites of linkers **2** and **3** needed for DNA synthesis were prepared from the corresponding aromatic diols, which were obtained in a Sonogashira Pd^0-catalyzed reaction from 1,3-diethynylbenzene and 2-(4-bromonaphthalen-1-ylmethoxy)ethanol in the case of **2**, and from 1,3-diiodoazulene and 2-(4-iodobenzyloxy)ethanol in the case of **3**.[17] Phosphoramidites were used for the synthesis of TFOs on a DNA synthesizer.[17] The thermal stability of the Hoogsteen-type triplexes was assessed by thermal denaturation experiments. The melting temperatures (T_m, °C) determined as the first derivatives of the melting curves at 260 nm are presented in Table 1. To be sure that the resulting alternate-strand triplexes bind to both purine-rich parts of the dsDNA we checked the thermal stability toward mismatched dsDNA (D2 and D3, Table 1). For comparison, **P** was singly and doubly inserted in a 9-mer (ON11 and ON12, respectively) at the same intercalation sites as in ON5 and ON7. In cases of the coincident melting of the triplexes and duplexes when using a **P** monomer, thermal denaturation was also monitored at 373 nm (T_m values in parentheses in Table 1).

As can be seen from the T_m data in Table 1, the use of linker **2** in the 5′-5′ alternate TFO (ON3) resulted in increased thermal stability ($\Delta T_{m(ON3/D1-ON1/D1)} = +6.5$ °C) at pH 6.0 of the triplex with matched dsDNA (D1) relative to that formed from the TFO with linker **1**. Moreover, a T_m value of 43.5 °C

Table 1: T_m [°C] data for third-strand melting, taken from UV/melting curves ($\lambda = 260$ nm).[a]

Entry	Sequence	D1 pH 6.0	D1 pH 7.2	D2 pH6.0	D3 pH 6.0
ON1	3′-TT TTT-5′-**1**–5′-TTT T**C**T TTT-3′	42.5	< 5.0	22.5	< 5.0
ON2	3′-TT TTT-5′-**1**–5′-TTT T**C**T TTT **CCC**-3′	44.5	< 5.0	28.5	30.0
ON3	3′-TT TTT-5′-**2**–5′-TTT T**C**T TTT-3′	49.0	43.5	26.0	29.5
ON4	3′-TT TTT-5′-**3**–5′-TTT T**C**T TTT-3′	35.5	< 5.0	20.5	< 5.0
ON5	3′-TT TTT-5′-**1**–5′-TTT T**CTP**TTT-3′	55.5 (55.0)[b]	50.5 (48.5)	44.5	< 5.0
ON6	5′-**P**-3′-TT TTT-5′-**1**–5′-TTT T**C**T TTT-3′	50.0 (50.0)	43.5 (43.5)	32.0	30.5
ON7	3′-TT TTT-5′-**2**–5′-TTT T**CTP**TTT-3′	60.0 (57.5)	50.0 (50.0)	42.5	46.0
ON8	5′-**P**-3′-TT TTT-5′-**2**–5′-TTT T**C**T TTT-3′	54.5 (49.0)	47.5	35.5	36.5
ON9	5′-TTT T**C**T TTT-3′	16.0	< 5.0	n.d.[c]	n.d.
ON10	5′-**2**–5′-TTT T**C**T TTT-3′	41.0	39.0	n.d.	n.d.
ON11	5′-TTT T**CTP**TTT-3′	42.0	35.0	n.d.	n.d.
ON12	5′-**P**TTT T**CTP**TTT-3′	65.5 (58.5)	65.5 (52.5)	50.0 (50.0)	n.d.

[a] $C = 1.5$ μM of ON1–12 and 1.0 μM of each strand of dsDNA (D1–3) in 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 6.0 and 7.2; duplex $T_m = 68.0$ (D1: 3′-GGT GAA AAA TTT TCT TTT CCC CCC TGA CC-5′/5′-CCA CTT TTT AAA AGA AAA GGG GGG ACT GG-3′, pH 6.0), 70.0 °C (D2: 3′-GGT GAA AAA *C*TT TCT TTT CCC CCC TGA CC-5′/5′-CCA CTT TTT *G*AA AGA AAA GGG GGG ACT GG-3′, pH 6.0), 70.0 °C (D3: 3′-GGT GACCAA TTT TCT TTT CCC CCC TGA CC-5′/5′-CCA CT*G*GTT AAA AGA AAA GGG GGG ACT GG-3′, pH 6.0) and 68.0 °C (D1, pH 7.2); bases in italics are mismatched to TFOs; **C** is 5-methylcytosine. [b] T_m values determined at 373 nm for **P**-containing sequences are given in parentheses in cases of overlaid triplex and duplex meltings. [c] n.d.–not determined.

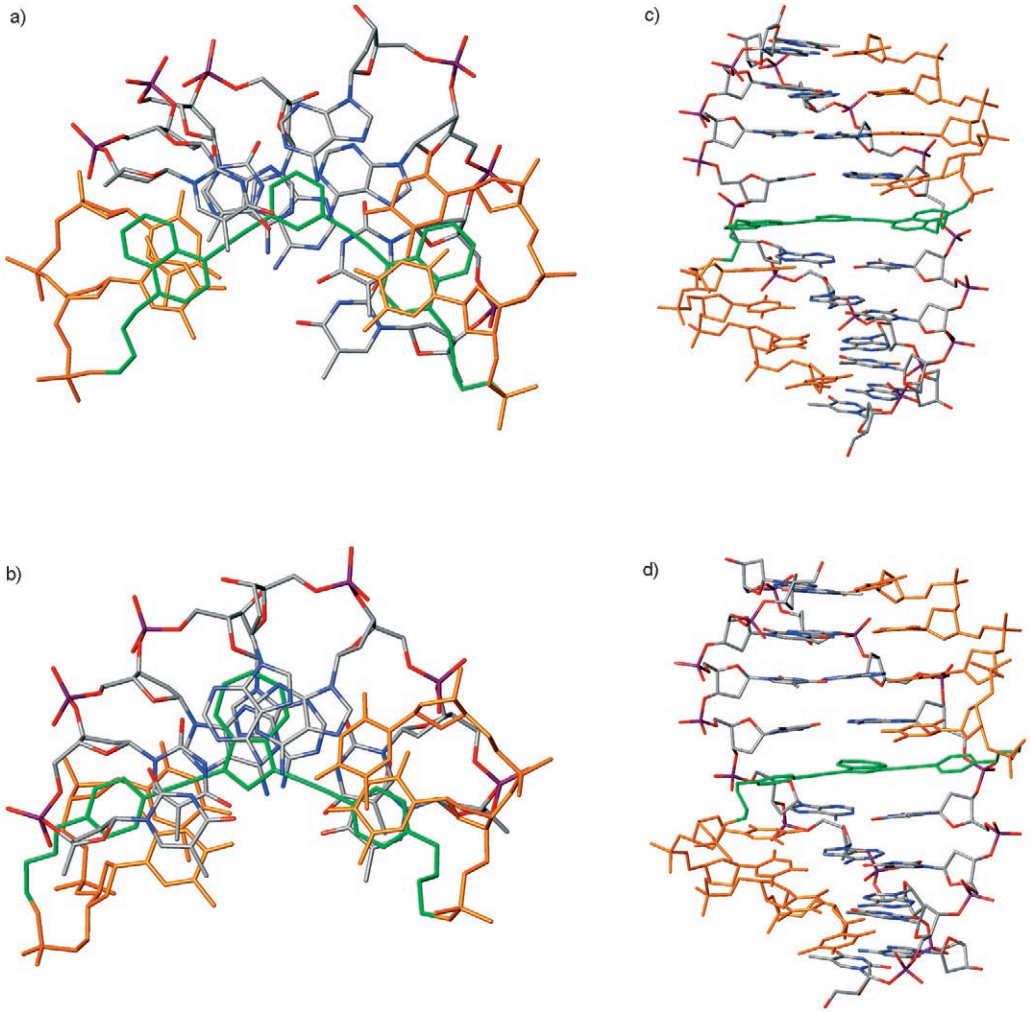


Figure 1. Structures obtained by molecular modeling studies of the triplexes with alternate-strand TFOs in yellow with intercalating linkers **2** (Figure 1 a,c) and **3** (Figure 1 b,d) in green. Top views of the structures are shown in Figure 1 a and b; side views are shown in Figure 1 c and d.

was detected for ON3/D1 at pH 7.2. In contrast, linker **3** (ON4) lowered the thermal stability of the triplex at pH 6.0 relative to those with **1** and **2**, and no triplex transition was observed at pH 7.2. In cases of binding to mismatched dsDNA (D2 and D3), the considerable drop in the triplex T_m value was observed with linkers **1**–**3** (ON1–ON4) at pH 6.0. The T_m value of the triplex with D1 also increased when the second, shortest arm of the alternate TFO was attached to the linker **2** (ON3 versus ON10). These experiments indicate that both arms of the synthesized alternate-strand TFOs bind to both purine-rich stretches of dsDNA (D1). It could be expected that T_m values for ON10/D1 at pH 6.0 and for the triplex formed between ON3 and the mismatched dsDNA (D3) should be similar. However, a difference of 11.5 °C in the T_m values was observed. We presume that this effect is a result of a different positioning of the intercalator **2** in the dsDNA part of the triplex. The bulky mismatched dangling end of ON3 may force the intercalator **2** into a less favorable position inside the duplex. Interestingly, the replacement of AT base pairing next to the intercalating linker in D1 by GC in D2 gave a large reduction in the T_m values with ON1–ON4, despite the non-interrupting purine sequence in the dsDNA. From these data we can conclude that it is advantageous to use an intercalating linker to connect two alternate strands of the TFO as it leads to a sequence-specific oligonucleotide.

Lower T_m values and a longer time was needed for the formation of a triplex with ON4 containing linker **3** than those required for alternate-strand triplex bridges **1** and **2** (ON1, ON3). Thus, for ON4, keeping the samples for 24 h at 4 °C prior to thermal denaturation was required for the observion of the triplex transition at 260 nm, while samples with TFOs possessing linkers **1** and **2** needed to stand for only 1 h at 5 °C in the same buffer solutions. We suppose that these properties are consequences of placing the azulene ring in linker **3** exactly in the middle of the dsDNA, in the zone where Watson–Crick hydrogen bonds are formed. On the other hand, as can be seen from molecular modeling studies (Figure 1 B), one of the benzene rings of linker **3** does not stack with the TFO bases, because of a difference in the geometry arising from the 1,3-substitution in the 5- and 6-membered rings. For the future design of 5′-5′ Hoogsteen alternate linkers, it can be concluded that structures with increased aromatic surface should cover the area of the TFO bases and purine residues of dsDNA and be connected through a 1,3-disubstituted benzene or similar ring to provide intercalation in the whole system. The importance of the proper intercalation and stacking properties of **2** can also be illustrated by comparison of the thermal stability of triplexes formed between ON1, ON3, and ON4 with D1 at pH 6.0 and 7.2. With ON1 and ON4, the drop in the T_m value was significant when the pH value was increased from 6.0 to 7.2. In contrast, a ΔT_m value of 5.5 °C was observed for the triplex ON3/D1. Double insertion of **P** in a 9-mer gave rise to an extremely stable triplex (ON12/D1) at pH 7.2. This finding is also an indication that the intercalating properties of 5′-5′ alternate linkers in Hoogsteen-type TFOs can be further improved by appropriately chosen modification of structures **1** and **2**. However, the mismatch sensitivity for ON12 was much lower than in the alternate-strand triplex.

To further increase the thermal stabilities of the alternate-strand triplexes at pH 7.2 we prepared several TFOs possessing a TINA monomer (ON5–ON8). The use of inverted thymidine derivatives (3′-O-DMT, 5′-phosphoramidites) in the synthesis of alternate-strand TFOs meant that monomer **P** was linked through secondary hydroxy groups at the 3′-end of the thymidine residue in both ON6 and ON8 at dangling ends (Table 1). Increased thermal stabilities of triplexes possessing **P** in either arm of the TFOs (ON5, ON6 and ON7, ON8) were observed relative to those with ON1 and ON3, respectively. Good discrimination ($\Delta T_m > 10.0$ °C) between matched and mismatched alternate triplexes with **P** insertions (ON5–ON8 with D2 and D3) was observed in all cases at pH 6.0. There is a quite large difference in the thermal stability of the mismatched triplexes ON5/D3 relative to the triplex of ON11/D1 at pH 6.0. This again reflects the increased sensitivity arising from the mismatched dangling end as discussed above. As can be seen, even a single insertion of **P** in the TFOs with linker **1** (ON5, ON6) led to T_m values over 37 °C at pH 7.2. It is interesting to note that at neutral pH the influence of **P** insertion on the thermal stability of the triplexes was larger for TFOs possessing linker **1** than linker **2**. The stabilizing effect coming from linkers **1** and **2** for oligonucleotides with **P** insertion can be illustrated by comparison of the thermal stability of the triplex formed between D1 and ON11 and those with ON5 and ON7 at pH 7.2. In both cases an increase in the T_m value of more than 13.5 °C was observed.

Despite the extraordinary high thermal stability of the triplex with non-alternate ON12 at pH 7.2, the use of alternate-strand TFOs with a single **P** insertion may still be preferred for a more target-specific oriented design, as high binding affinity of nucleic acids is often accompanied by a loss of target specificity.[18] In this regard, alternate-strand triplexes divide the TFO sequence into two or more parts and are proposed to be a new way to achieve specificity.

The triplex stabilities described herein are remarkable, and allows high T_m values to be reached by connection of short homopyrimidine stretches (for example, the 5-mer and 9-mer shown here), which are unable to bind separately to dsDNA at physiological pH values. More importantly, the final T_m values of the systems exceed 37 °C, which gives a possibility to extend the number of gene targets available for biological studies. This result highlights the importance of an appropriate design of intercalating systems for achieving the desired level of stacking interactions within the nucleic acids. Monomer **2** is the first example of a 5′-5′ alternate linker for Hoogsteen-type triplexes, which enables the targeting of dsDNA at pH 7.2. This approach gives a unique opportunity to develop triplex technology since the monomer insertion is compatible with the synthesis of numerous modified oligonucleotides.

Received: March 22, 2006
Revised: May 30, 2006
Published online: July 17, 2006

Keywords: DNA recognition · DNA structures · DNA triplex · helical structures · intercalation

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[16] More detailed information about the molecular modeling studies and pictures of the alternative positioning of linkers are given in the Supporting Information.

[17] The synthesis of DMT-protected phosphoramidites of linkers **2** and **3**, their incorporations into DNA, MALDI-TOF analysis of the final TFOs, and details about thermal stability measurements along with the representative first derivatives of the melting curves are described in the Supporting Information.

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