Design of an Intercalating Linker Leading to the First Efficiently 5′,5′-Linked Alternate-Strand *Hoogsteen* Triplex with High Stability and Specificity

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This is the first report describing the design, synthesis, and incorporation of an intercalating linker leading to an efficiently 5′,5′-linked alternate strand *Hoogsteen* triplex. Using molecular modeling, the 5′,5′ problem was solved in a rather simple way. The two relatively distant 5′-ends have been connected with a molecule that provides rigidity to the structure, while being flexible enough to allow stacking upon all four strands. The synthesis of the core of the designed molecule was conducted by *Sonogashira* coupling of an appropriately substituted iodobenzene with 1,3-diethynylbenzene to give a conjugated system with three benzene rings interconnected with triple bonds. For the alternate-strand triplex obtained with the intercalating linker, the stability obtained is higher than that of the corresponding homotriplex of equivalent total length and with the same nucleotides. This is deduced from a 5° higher melting temperature of the alternating triplex. The sensitivity towards mismatches in the alternate-strand triplex is in the same range ($\Delta T_m = -13°$ to $-19°$) as those observed for homotriplexes.

1. Introduction. – In recent years, there has been growing interest to explore the increasing knowledge of the human genome in the design of new therapeutics. Attempts to target either DNA or mRNA with modified oligonucleotides have been made, and the efforts have led to several drug candidates [1][2], which all are *antisense* drugs that target mRNA. So far, there have been no *antigene* drugs targeting DNA to make it into clinical trials [3]. In spite of lacking success, the antigene strategy is still the most logical approach to generate new therapeutics because each diploid cell contains only two targets.

In the antigene strategy, an oligodeoxynucleotide (ODN) binds to the major groove of double helical DNA forming a triple helical structure (triplex). The triplex-forming oligodeoxynucleotide (TFO) consists of either pyrimidine nucleotides or purine nucleotides. When the TFO consists of pyrimidine nucleotides, the TFO binds to the target DNA strand through *Hoogsteen* Py·PuPy base triplets (T·AT and C^+·GC) in a parallel orientation compared to the purine strand of the DNA duplex [4][5]. When the TFO consists of purine nucleotides, the TFO is able to bind to the target DNA strand through reverse-*Hoogsteen* Pu·PuPy base triplets (A·AT and G·GC) in an antiparallel orientation [6][7]. In both cases, the target sequence needs to contain a purine tract of a considerable length both to ensure specificity of the TFO and to obtain a stable triplex. This considerably limits the number of targets available to the antigene

[1]) A research center funded by *The Danish National Research Foundation* for studies on nucleic acid chemical biology.

strategy. One method to increase the number of targets is the alternate strand triplex formation (*Fig. 1*). When the target DNA sequence consists of two adjacent purine tracts on opposite strands, it is possible to design a TFO, with inverted polarity and a suitable linker capable of binding to both purine tracts. As outlined in *Fig. 1*, in this approach, two different types of linkers are needed to meet the requirements of the parallel *Hoogsteen* triplexes, whereas another set of linkers has to be constructed for the reverse-*Hoogsteen* triplexes.

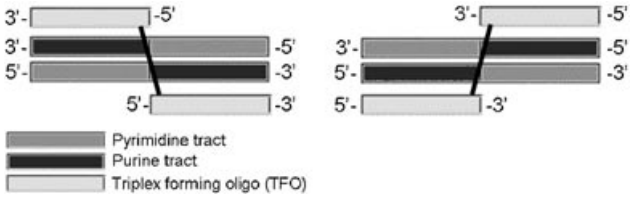


Fig. 1. *Two possibilities for parallel alternate-strand* Hoogsteen *triplex formation*

In this study, we have focused on finding a linker for the 5′,5′ linkage for the parallel alternate-strand triplex. There have been several successful designs of 3′,3′ linkers for the alternate-strand *Hoogsteen* triplex [8–13], but, until now, no satisfactory linker for the 5′,5′ linkage has been developed. The best result obtained so far was obtained by *Ueno et al*. [14] but their design showed only limited target specificity. The lack of a good *5′,5′* linker has limited the number of possible targets. A suitable 5′,5′ linker would substantially increase the number of possible targets. When combined with a 3′,3′ linker, it will be possible to design a TFO that is able to switch back and forth between several adjacent purine tracts on alternate strands.

2. Results and Discussion. – 2.1. *Design of an Intercalating 5′,5′ Linker*. *Fig. 2* shows a DNA duplex with two TFOs recognizing two purine tracts on opposite strands. The model has been minimized with the AMBER* force field [15][16] in MacroModel 8.0.

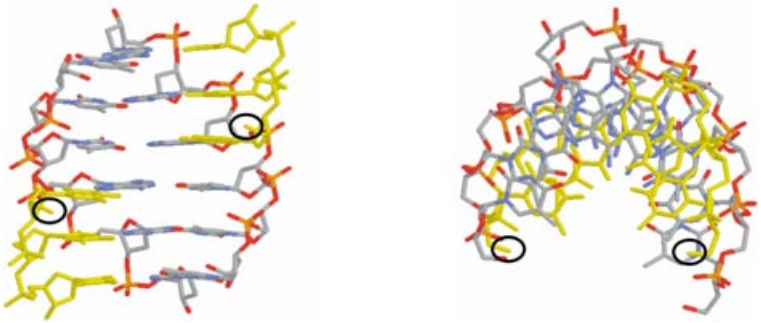


Fig. 2. *A duplex structure with two different TFOs* (yellow) *bound to purine tracts on opposite strands.* The circles mark the two 5′-ends to be connected by a linker.

Based on this model, a linkage between the two 5′-ends of the two TFOs has been developed. A conjugated system is designed with three benzene rings interconnected with triple bonds that allow the aromatic rings to intercalate with the duplex and with

both the TFOs. Only an additional short flexible linker at both ends is then required to make the correct length and the formation of a 5′,5′-linked alternate-strand triplex possible. To verify the design of the intercalating linker, a conformational search for the 5′,5′-linked triplex structure was performed as described in *Exper. Part*. The obtained structure (*Fig. 3*) of the designed 5′,5′ linker fulfilled the expectations, it being flexible enough to allow stacking interaction with the surrounding nucleobases and stiff enough to ensure a favorable entropy effect for the alternate-strand triplex formation.

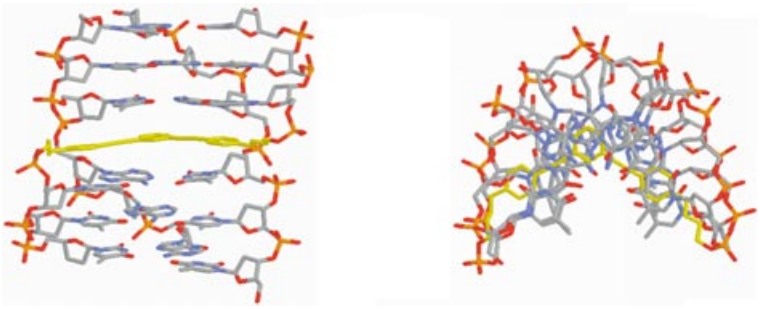
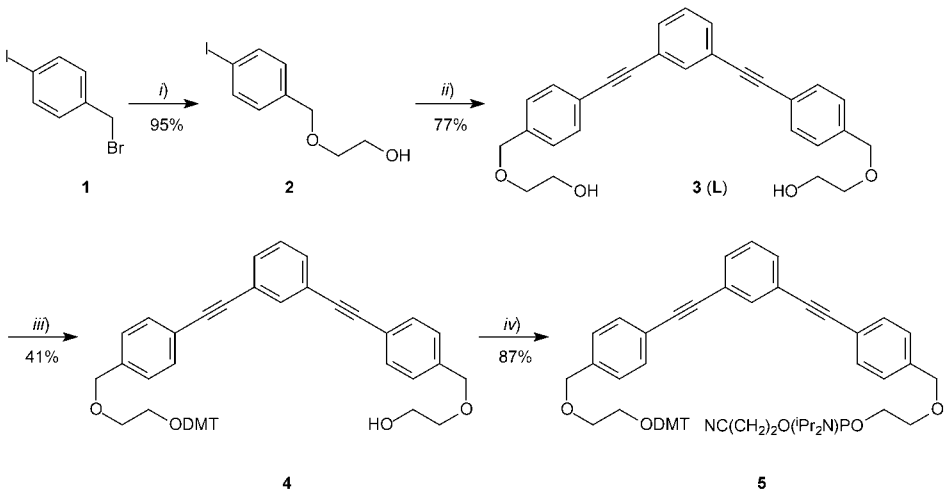


Fig. 3. *Structure obtained from the conformational search of the alternate-strand triplex with the intercalating linker* (**L**) *in yellow*

2.2. *Synthesis of the Intercalating 5′,5′ Linker* (*Scheme*). The synthesis of the phosphoramidite **5** was straightforward starting from commercially available 4-iodobenzyl bromide (**1**). Reaction with ethylene glycol and NaH yielded the alcohol **2** in excellent yield. The subsequent *Sonogashira* coupling [17] [18] of compound **2** with

Scheme



i) Ethylene glycol, NaH, 5 min, reflux. *ii*) 1,3-Ethynylbenzene, $Pd(PPh_3)_2Cl_2$, CuI, EtN^iPr_2, THF, 23 h, r.t. *iii*) DMT-Cl, Et_3N, CH_2Cl_2, 15 h, r.t. *iv*) $NC(CH_2)_2OP(N^iPr_2)_2$, diisopropylammonium tetrazolide, Et_3N, MeCN, 3 h, r.t.

the commercially available 1,3-diethynylbenzene afforded the core structure **3** of the intercalating linker. The intercalating linker was mono-protected with 4,4′-dimethoxytrityl chloride (DMT-Cl) in 41% overall yield from **3**. The low yield is not a result of low reactivity of the starting diol **3**, but rather a result of very poor selectivity between mono- and diprotected products, which, however, were easy to separate by chromatography. Attempts to optimize the reaction by varying the temperature and/or the amount DMT-Cl were unsuccessful. The phosphoramidite **5** was synthesized in 87% yield by reaction with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite.

2.3. *Effect of the Intercalating Linker on Triplex Stability.* To investigate the possible effect of intercalating the linker into a duplex, it was attached as a dangling end to a TFO. The thermal stability was measured of the so-formed TFO **I** against a 26-mer duplex containing the target sequence of **I** (*Table 1*).

Table 1. *Thermal-Stability Studies of Triplexes Comparing an Unmodified TFO with a TFO with the Intercalating Linker* **L** *Attached to the 5′-End and with a 5′,5′-Linked* (*via* **L**) *TFO Forming an Alternate-Strand Triplex*. Bold face marks the binding region of the TFOs

Entry	TFO	Target DNA 3′-TATGTATGGG**GAAAGAAA**TTCTTCTT-5′ 5′-ATACATACCCCTTTCTTT**AAGAAGAA**-3′	
		pH 5 T_m [°C]	pH 6 T_m [°C]
1	3′-CTTTCTTT-5′ + 5′-TTCTTCTT-3′	19	< 5
2	3′-CTTTCTTT-5′-L (**I**)	37	25
3	3′-CTTTCTTT-5′-L-5′-TTCTTCTT-3′ (**II**)	> 60	49
		3′-TATGTATGGG**GAAAGAAAAAGAAGAA**-5′ 5′-ATACATACCCCTTTCTTTTTCTTCTT-3′	
		pH 5 T_m [°C]	pH 6 T_m [°C]
4	3′-CTTTCTTTTTCTTCTT-5′	> 60	44

Comparison of the stability of the triplex formed with **I** with the one formed with the original TFO without the intercalating linker showed that the intercalating linker resulted in an increase in melting temperatures of 18° and > 20° at pH 5 and 6, respectively. In the latter case, the unmodified octamer TFO did not form a triplex above 5°. The very high stabilization showed that the intercalating linker was, indeed, able to stabilize the triplex structure.

2.4. *Alternate-Strand Triplex Formation* via *the Intercalating 5′,5′ Linker*. The TFO **II** (*Table 1*) was synthesized with two regions of opposing orientations connected at the 5′-ends to the intercalating linker. The two regions matched two sequences on the alternating purine strands of the 26-mer target duplex. Thermal-stability measurements of the triplex between **II** and the target duplex showed additional increases in melting temperatures of > 23° and 24° at pH 5 and 6, respectively, when compared to the melting temperatures obtained from **I** (*Table 1*). In this case, the full effect could not be estimated at pH 5 because of melting of the target duplex. The high additional gain in stability is best explained as a result of an alternate-strand triplex formation. To investigate the stability of the alternate-strand triplex compared to a traditional triplex,

the melting temperature was measured of a triplex between a 16-mer TFO having the same T/C ratio as **II** and a 26-mer target duplex. The melting temperature of the homotriplex was 44° at pH 6, which is 5° lower than that observed for the alternate-strand triplex. One should expect lower stability of an alternate triplex, because of interruption of the intrastrand stacking. However, with the 5′,5′ linkage, the loss in stability due to intrastrand interruption is more than compensated by stacking onto the TFO regions and by intercalation into the duplex.

2.5. *Mismatch Investigations of 5′,5′-Linked Alternate-Strand Triplex*. It is known that the thermal stability of a homotriplex decreases dramatically (>10°) in case of a mismatch between the TFO and the target duplex [19]. Therefore, it was of interest to investigate whether this specificity is maintained for the 5′,5′-linked alternate-strand triplex using the TFO **II**.

It was anticipated that the intercalating linker would make the largest conformational changes of the TFO in close proximity to the linker. For this reason, the thermal stabilities of alternate-strand triplexes with mismatches between the duplex and the TFO at the position next to the 5′,5′ linkage were measured. The mismatch studies showed that the alternate-strand triplex is very sensitive to a mismatch with a decrease in the melting temperature in the range of 13° to 19° at pH 6 (*Table 2*). Finally, the stability of an alternate-strand triplex between **II** and a duplex where the two target purine tracts were separated by an additional nucleotide was measured. In this case, the linker is not able to intercalate as efficiently as in the previous cases, although the nucleobases of the TFO are fully matched with the target duplex. The combination resulted in a decrease in melting temperature of 7° at pH 6, which is smaller than that observed for a simple mismatch next to the linker.

Table 2. *Thermal-Stability Studies of Mismatched Alternate-Strand Triple Helices.* Bold face marks the binding regions of the TFOs and underlined base pairs mark mismatches of the TFO.

Entry	Target DNA	TFO 3′-CTTTCTTT-5′-L-5′-TTCTTCTT-3′ (**II**)	
		pH 6	
		T_m [°C]	ΔT_m [°C]
1	3′-TATGTATGGG**GAAAGAAA**TTCTTCTT-5′ 5′-ATACATACCCCTTTCTTT**AAGAAGAA**-3′	49	–
2	3′-TATGTATGGG**GAAAGAA**G<u>T</u>TCTTCTT-5′ 5′-ATACATACCCCTTTCTT<u>C</u>**AAGAAGAA**-3′	32	– 17
3	3′-TATGTATGGG**GAAAGAA**C<u>T</u>TCTTCTT-5′ 5′-ATACATACCCCTTTCTT<u>G</u>**AAGAAGAA**-3′	30	– 19
4	3′-TATGTATGGG**GAAAGAA**<u>T</u>TTCTTCTT-5′ 5′-ATACATACCCCTTTCTT<u>A</u>**AAGAAGAA**-3′	36	– 13
5	3′-TATGTATGGG**GAAAGAAA**CTTCTTCTT-5′ 5′-ATACATACCCCTTTCTTTG**AAGAAGAA**-3′	42	– 7

3. Conclusions. – We have designed and synthesized the first efficient linker for 5′,5′-linked alternate-strand triplex formation. The linker was designed so that it would be able to stabilize the triplex by intercalation. Thermal-stability studies showed high stability of this new *5′,5′*-linked alternate-strand triplex. The alternate-strand triplex showed stability even higher than that of a homotriplex of the same length. The

additional stability compared to a traditional triplex is most likely due to intercalation and stacking.

The specificity of the alternate-strand triplex was shown to be in the same range as for a traditional triplex in the case of a single mismatch. However, when the two purine tracts on alternate strands were separated by a single nucleotide the selectivity was reduced to a difference of 7° in thermal stability.

We thank *Kirsten Østergaard* for her help with ODN synthesis.

Experimental Part

General. TLC: TLC plates *60 F254* (*Merck*), visualization by UV light (254 nm). Column chromatography (CC): silica-gel-packed column (silica gel *60*, 0.040 ± 0.063 mm, *Merck*). NMR Spectra: *Varian Gemini-2000* spectrometer (^{1}H: 300 MHz, ^{13}C: 75.5 MHz, ^{31}P: 121.5 MHz); δ values in ppm relative to Me_4Si as internal standard (^{1}H-NMR); for ^{13}C-NMR: $CDCl_3$ (δ 77.0). MALDI-MS: FTICR-MS ion spectrometer.

1. *2-[(4-Iodobenzyl)oxy]ethanol* (**2**). The reaction was carried out under N_2. NaH (60% in mineral oil, 2.61 g, 0.065 mol) was added in portions to ethylene glycol (25 ml) at 0°. After addition, the mixture was allowed to come to r.t. while stirring for 30 min. *4-Iodobenzyl bromide* (**1**; 3.88 g, 0.013 mol) was added, and the mixture was heated with a heat gun until a clear soln. was obtained. The mixture was poured into H_2O (300 ml) and extracted with Et_2O (3 × 100 ml). The combined org. phases were washed with brine (100 ml), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by CC (silica gel; AcOEt/petroleum ether 1:1 (*v*/*v*) to yield **2** (3.46 g, 95%). Clear oil. ^{1}H-NMR ($CDCl_3$): 2.30 (br. *s*, OH); 3.57 (*t*, *J* = 4.5, OC*H*$_2$CH$_2$OH); 3.74 (*m*, C*H*$_2$OH); 4.49 (*s*, PhC*H*$_2$O); 7.08 (*d*, *J* = 8.1, 2 arom. H); 7.67 (*d*, *J* = 8.1, 2 arom. H). ^{13}C-NMR ($CDCl_3$): 61.7 (CH_2OH); 71.5 (O*C*H$_2$CH$_2$OH); 72.5 (Ph*C*H$_2$O); 93.2, 129.5, 137.4, 137.6 (arom. C).

2. *2,2'-[1,3-Phenylenebis(2,1-ethynediyl-4,1-phenylenemethyleneoxy)bis[ethanol]* (**3**). 1,3-Diethynylbenzene (0.69 g, 5.5 mmol), **2** (3.06 g, 11 mmol), $Pd(PPh_3)_2Cl_2$ (86 mg, 0.12 mmol), and CuI (86 mg, 0.45 mmol) were suspended in 40 ml of dry THF under N_2. Et*N*^{i}Pr$_2$ (3.25 ml) was added, and the mixture was stirred at r.t. for 23 h, H_2O (50 ml) was added, and the mixture was extracted with AcOEt (3 × 25 ml). The combined org. phases were washed with brine (2 × 25 ml), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by CC (silica gel; AcOEt/petroleum ether 3:1 (*v*/*v*)) to afford **3** (1.80 g, 77%). White solid. M.p. 125° – 127°. ^{1}H-NMR ($CDCl_3$): 2.15 (*m*, 2 OH); 3.61 (*t*, *J* = 4.5, 2 OC*H*$_2$CH$_2$OH); 3.77 (*m*, 2 C*H*$_2$OH); 4.57 (*s*, 2 PhC*H*$_2$); 7.30 – 7.71 (*m*, 12 arom. H). ^{13}C-NMR ($CDCl_3$): 61.9 (CH_2OH); 71.5 (*C*H$_2$CH$_2$OH); 72.8 (Ph*C*H$_2$O); 88.6, 89.8 (C≡C); 122.4, 123.6, 127.6, 128.5, 131.3, 131.7, 134.6, 138.4 (arom. C). HR-MALDI-MS: 449.1732 ($C_{28}H_{26}NaO_4^+$, $[M + Na]^+$; calc. 449.1723).

3. *2-({4-[(3-{[4-({2-[Bis(4-methoxyphenyl)(phenyl)methoxy]ethoxy}methyl)phenyl]ethynyl}phenyl)ethynyl]phenyl}methoxy)ethanol* (**4**). The diol **3** (1.16 g, 2.71 mmol) and dry Et_3N (454 µl, 3.26 mmol) were dissolved in dry CH_2Cl_2 (40 ml) under N_2, and DMT-Cl (1.103 g, 3.26 mmol) in CH_2Cl_2 (15 ml) was slowly added during 15 min. The mixture was stirred at r.t. for 15 h, and the solvent was removed *in vacuo*. The residue was purified by CC (silica gel; AcOEt/petroleum ether/Et_3N (50:50:1 (*v*/*v*/*v*)) to afford **4** (0.81 g, 41%). Clear oil. ^{1}H-NMR ($CDCl_3$): 2.08 (br. *s*, OH); 3.28 (*t*, *J* = 4.9, C*H*$_2$ODMT); 3.60 (*t*, *J* = 4.5, C*H*$_2$CH$_2$OH); 3.68 (*t*, *J* = 4.9, C*H*$_2$CH$_2$ODMT); 3.77 (br. *s*, 2 MeO, C*H*$_2$OH); 4.57 (*s*, PhC*H*$_2$O); 4.61 (*s*, PhC*H*$_2$O); 6.81 (*d*, *J* = 8.8, 4 H, DMT); 7.17 – 7.72 (*m*, 21 arom. H). ^{13}C-NMR ($CDCl_3$): 55.2 (2 MeO); 61.9 (CH_2OH); 63.2 (*C*H$_2$ODMT); 69.9 (*C*H$_2$CH$_2$ODMT); 71.5 (*C*H$_2$CH$_2$OH); 72.6 (Ph*C*H$_2$O); 72.8 (Ph*C*H$_2$O); 85.9 (Ar$_3$*C*); 88.4, 88.7, 89.7, 90.0 (2 C≡C); 113.0, 126.6, 127.3, 128.2, 130.0, 136.3, 145.0, 158.4 (DMT), 122.0, 122.4, 123.5, 123.6, 127.6, 127.7, 128.4, 131.19, 131.24, 131.6, 131.7, 134.6, 138.4, 139.2 (arom. C). HR-MALDI-MS: 751.3033 ($C_{49}H_{44}NaO_6^+$, $[M + Na]^+$; calc. 751.3030).

4. *Phosphoramidite* **5**. The alcohol **4** (0.33 g, 0.45 mmol) was dissolved under N_2 in dry MeCN (6 ml). Et_3N (124 µl, 0.90 mmol), 2-cyanoethyl *N*,*N*,*N*',*N*'-tetraisopropylphosphordiamidite (283 µl, 0.90 mmol), and diisopropylammonium tetrazolide (0.145 g, 0.85 mmol) were added, and the mixture was stirred at r.t. for 3 h. The product was purified by CC (silica gel; AcOEt/petroleum ether/Et_3N 25:75:1 (*v*/*v*/*v*)) to afford **5** (0.36 g, 87%). Yellow oil. ^{13}C-NMR ($CDCl_3$): 20.25, 20.34 (*C*H$_2$CN), 24.5, 24.57, 24.59, 24.7 (4 Me); 43.0, 43.1 (2 Me$_2$*C*H); 55.2 (2 MeO); 58.3, 58.5 (CH_2OP); 62.6, 62.8 (*C*H$_2$CH$_2$OP); 63.2 (*C*H$_2$ODMT); 69.9 (*C*H$_2$CH$_2$ODMT); 70.3, 70.4 (Ph*C*H$_2$O); 72.6 (Ph*C*H$_2$O); 85.9 (Ar$_3$*C*); 88.4, 88.5, 89.8, 89.9 (2 C≡C); 113.0, 126.6, 127.3, 128.2, 130.0, 136.3, 145.0, 158.4 (DMT); 122.0, 122.1, 123.58, 123.61, 127.5, 127.7, 128.4, 131.2, 131.6, 134.5, 138.8, 139.2 (arom. C). ^{31}P-NMR ($CDCl_3$): 149.5.

5. *Molecular Modeling*. The molecular modeling was performed with MacroModel 8.0 from *Schrödinger*. All calculations were conducted with the AMBER* force field [15][16] and the GB/SA water model [20]. The dynamics simulation was performed with stochastic dynamics [21], a SHAKE algorithm to constrain bonds to hydrogen [22], a time step of 1.5 fs, and a simulation temp. of 300 K. Simulation for 1 ns generated 500 structures, which were all minimized using a truncated *Newton* conjugate gradient method with a convergence threshold of 0.005 kJ/mol. The minimized structures were examined with XCluster from *Schrödinger*, and a representative structure for the lowest-energy cluster was selected. The starting structure was generated with Insight II v97.2 from MSI, followed by incorporation of the desired linkage between the two 5′-ends.

6. *Oligodeoxynucleotide Synthesis, Purification, and Thermal-Stability Studies.* Unmodified ODNs were purchased from *DNA Technology A/S*, DK-8000 Aarhus. The two modified TFOs, **I** and **II**, were synthesized on a 0.2-μmol scale on an *Expedite*™ *Nucleic Acid Synthesis System* model *8909* from *Applied Biosystems*. The phosphoramidite **5** was dissolved in dry MeCN, as a 0.1M soln., and used for oligo-synthesis under standard conditions for nucleotide couplings (2-min coupling). The coupling efficiency of the intercalating linker was *ca.* 90%. In the case of **II**, the change in polarity around the linker was accomplished by using 5′-phosphoramidites purchased from *ChemGenes Corporation*, Ashland MA, USA, instead of the usual 3′-phosphoramidites. The TFOs were synthesized with DMT-on and purified on a *Waters Prep LC 4000* HPLC with a *Waters Prep LC* controller, and a *Waters 2487 Dual λ* absorbance detector on a *Waters Xterra*™ *MS* C_{18} column. Buffer *A*, 950 ml of 0.1M NH_4HCO_3 and 50 ml of MeCN, pH 9.0; buffer *B*, 250 ml of 0.1M NH_4HCO_3 and 750 ml of MeCN, pH 9.0. Gradient, 5 min 100% *A*, linear gradient to 70% *B* in 30 min, 2 min 70% *B*, linear gradient to 100% *B* in 8 min and then 100% *A* in 15 min. t_R 46 and 38 min for **I** and **II**, resp. DMT was removed by treatment with 20 μl of H_2O and 80 μl of AcOH. Both modified TFOs were confirmed by MALDI-TOF-MS analysis on a *Voyager Elite Biospectrometry Research Station* from *PerSeptive Biosystems.*

The thermal stability studies were conducted on a *Perkin-Elmer* UV/VIS spectrometer *Lambda 2* with a *PTP-6* temp. programmer, using PETEMP rev. 5.1 software and PECSS software package v.4.3. The melting temp. were measured on a 1.5 μM scale in 1 ml of buffer consisting of 0.1M NaCl, 0.01M $MgCl_2$, and 0.02M sodium cacodylate, where the desired pH was obtained by addition of conc. HCl. The samples were initially heated to 80°, followed by cooling to 5° before the melting temp. were measured by increasing the temp. 2°/min from 5° to 85°.

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Received May 21, 2004