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# Nucleosides and Nucleotides

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A Practical Synthesis of N1-Methyl-2'deoxy- $\psi$ -uridine ( $\psi$ -Thymidine) and Its Incorporation into G-Rich Triple Helix Forming Oligonucleotides

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# A PRACTICAL SYNTHESIS OF N1-METHYL-2'-DEOXY-ψ-URIDINE (ψ-THYMIDINE) AND ITS INCORPORATION INTO G-RICH TRIPLE HELIX FORMING OLIGONUCLEOTIDES

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**ABSTRACT:** A convenient synthesis of N1-methyl-2'-deoxy- $\psi$ -uridine ( $\psi$ -thymidine,  $\psi$ T, <u>7a</u>) has been accomplished in good yield. The structural conformation of <u>7a</u> was derived by 2D NMR and 1D NOE experiments. The nucleoside <u>7a</u> has been incorporated into G-rich triplex forming oligonucleotides (TFOs) by solid-support, phosphoramidite method. The triplex forming capabilities of the modified TFOs (**S4**, **S5** and **S6**) containing  $\psi$ T has been evaluated in antiparallel motif with a target duplex (duplex-31) 5'd(CTGAGACCGGGAAGGAGGAGGAAGGAGGCCAGTGAC)3'-5'd(GACTCT-GGCCCTTCCTCCTTCCCGGTCACTG)3' (**D1**) at pH 7.6. The triplex formation of modified homopyrimidine-oligomers (**S1**, **S2** and **S3**) has also been studied in parallel motif with a duplex-10 (A<sub>10</sub>:T<sub>10</sub>) at pH 7.0.

In recent years the development of the modified sequence specific agents to modulate gene expression has created great opportunities for therapy of genetic, viral and malignant diseases.<sup>1-3</sup> The use of short oligonucleotides for control of gene expression could become a new type of gene therapy.<sup>4,5</sup> It has been demonstrated that oligonucleotides selectively inhibit gene expression through interference with posttranscriptional events such as splicing and translation.<sup>3,6-9</sup> It has been shown<sup>10-16</sup> that under suitable conditions, short oligonucleotides will bind in a sequence-specific manner to the major groove of the duplex DNA and form a local three stranded structure or triplex. Triplexes are formed in two different modes, parallel and antiparallel. In parallel binding mode, triple helix formation takes place when T (thymidine) in the third strand binds to AT base pairs (T:AT) and protonated 2'-deoxycytidine (C<sup>+</sup>) in the third strand binds to GC

base pairs  $(C^+:GC)^{10,17-20}$  through Hoogsteen hydrogen bonding. A major drawback in this mode is that protonation of the third strand C (at N3) is necessary to bind N7 of G (2'-deoxyguanosine) in the duplex, optimally requires a pH of 5-6, which is well below physiological range. In antiparallel motif, T or A (2'deoxyadenosine) in the third strand binds to AT base pairs and G binds to GC base pairs (G:GC) (*Figure 1*) through reverse Hoogsteen hydrogen bonding. All the bases involved in hydrogen bonding in antiparallel motif are in their normal, uncharged tautomeric forms, thus pH is not a factor in triplex formation.<sup>9,14</sup> Under certain conditions, G and T of third strand bind with high affinity and specificity to the duplex targets with an equilibrium association constant of 10<sup>-10</sup> M or less.

Despite certain favorable properties of TFOs in antiparallel motif (ap TFOs), there are several limitations that must be overcome. First, triplex formation ideally requires a homopurine:homopyrimidine target duplex where the ideal duplex site consists entirely of G and A in one strand and paired to its Watson-Crick complementary base in the other. This limits the number of biologically interesting DNA sequences that can be targeted.

A second limitation of apTFOs is that high affinity binding requires G-rich target sequences. It was found empirically that at least 60-65% G residues be present in one strand of the intended target to achieve stable binding.<sup>9</sup> This further limits the ability to target biologically relevant sequences.

Based on the available data on triplex structure, it is possible to make modifications to the third strand that will overcome the above limitations. The requirement for G-rich targets indicates that T:AT triplets are relatively unstable in antiparallel motif. This may be due in part to the known preference of T for forming parallel triplexes.<sup>8,10</sup> This problem may be overcome by replacing T in the third strand with a modified T analog having similar pKa which could bind to AT base pairs. As a part of our ongoing program to design suitable modified T analogs<sup>21,22</sup> which could provide better stability to T:AT triplets in antiparallel motif in G-rich TFOs, we have now synthesised N1-methyl-2'-deoxy- $\psi$ -uridine (**7a**). The nucleoside **7a** is isosteric as well as isoelectric to thymidine and both are 1,5-disubstituted uracils with identical pKa. N1-Methyl-2'-deoxy- $\psi$ -uridine ( $\psi$ T, **7a**) was incorporated into G-rich triplex forming oligonucleotides (TFOs) employing solid-support, phosphoramidite methodology. The binding abilities of these TFOs (**S4**, **S5** and **S6**) have been evaluated with the target duplex (**D1**) in antiparallel motif at pH 7.6. The triplex forming capabilities of modified homo-



Figure 1. Reverse Hoogsteen triplet interactions for (I) Thymine (T) with AT base pairs; (II)  $\psi$ -Thymine ( $\psi$ T) with AT base pairs; (III) Cytosine (C<sup>+</sup>) with GC base pairs; (IV) Guanine (G) with GC base pairs. dR signifies 2'-deoxyribose.

pyrimidine-oligomers (S1, S2 and S3) containing  $\psi$ T residues at different sites has also been studied in parallel motif with a short duplex (A<sub>10</sub>:T<sub>10</sub>) at pH 7.0.

The third strand T residues in both parallel and antiparallel motifs are normally in "anti" glycosidic conformation. Generally, the different conformation model can be reliably described by two-state models "syn" and "anti"; N/S type sugar puckering<sup>23</sup>. Some of the nucleosides manifest either "syn" or "anti" conformation around the glycosidic bond in the solid state.<sup>24</sup> The nucleosides having the "anti" conformation around the glycosidic bond is the preferred conformation in the third strand TFO to form stable triple helices in both parallel and antiparallel motifs. The  $\psi$ T (*T*a), a C-nucleoside , may reliably have either "syn" or "anti" conformation (*Figure 2*). 1D <sup>1</sup>H Nuclear Overhauser Enhancement spectroscopy (NOE) technique has been widely used to find qualitative or semiqualitative conformation<sup>25</sup> of nucleosides and nucleotides in solution. We also report here the conformation of  $\psi$ T in solution which we accomplished by 1D NOE experiments.



Figure 2. "Syn" and "Anti" conformation of (7a)

Results and Discussion: Matsuda et al.<sup>26</sup> reported the synthesis of N1methyl-2'-deoxy-w-uridine (wT, 7a) via reduction of N1-methyl-5'-O-trityl-2',3'-O-(thiocarbonyl)-w-uridine with n-Bu<sub>3</sub>SnH. This method gives a mixture of N1methyl-5'-O-trityl-2',3'-dideoxy-glycero-pent-2'-enofuranosyl-w-uridine, N1-methyl-5'-O-trityl-2'-deoxy-w-uridine and N1-methyl-5'-O-trityl-3'-deoxy-w-uridine, from which the separation of the desired N1-methyl-5'-O-trityl-2'-deoxy-wuridine was rather cumbersome. An alternate approach for the synthesis of (7a) was reported by Chu et al.27 via the reductive removal of 2'-chloro substituent from 2'-chloro-2'-deoxy-w-uridine, but this method has several drawbacks and suffers due to low yield of the desired product (7a). We have accomplished a very practical method for the synthesis of (7a) via a route as shown in Scheme 1. Acylation<sup>28</sup> of pseudouridine ( $\psi$ U) with Ac<sub>2</sub>O in dry DMF in the presence of DMAP at -25 °C gave 2',3',5'-tri-O-acetyl derivative (2). The acetylation at -25 °C is very selective and we neither observed acetylation at N3 position nor the formation of any other undesired acetyl side products. The methylation<sup>29</sup> of ( $\underline{2}$ ) with CH<sub>3</sub>I, followed by deacetylation with NH<sub>3</sub>/MeOH gave N1-methyl-wuridine (3b) in 90% yield. The 2'-deoxygenation of (3b) was accomplished via the intermediate N1-methyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-w-uridine synthesized by simultaneous protection<sup>30</sup> of 5'- and 3'-hydroxyl groups of (3b) with 1,3-dichloro1,1,3,3-tetraisopropyl disiloxane. Treatment of (3b) with TIPS-Cl afforded the tetraisopropyldisilyl derivative (4), which was converted into 2'thioimidazole ester (5) by reaction with 1,1'-thiocarbonyldiimidazole (TCDI) in dry DMF.

The reduction of (5) with *n*-Bu<sub>3</sub>SnH in dry toluene afforded *N*1-methyl-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)- $\psi$ -uridine (6), in a 82% yield.



(i) (CH<sub>3</sub>CO)<sub>2</sub>O/DMF/ -35 °C, (ii) BSA/CH<sub>3</sub>I/CH<sub>2</sub>Cl<sub>2</sub>, (iii) MeOH/NH<sub>3</sub>, (iv) TIPS-CI/Py,
(v) TCDI/DMF, (vi) *n*-Bu<sub>3</sub>SnH/AIBN/toluene, (vii) *n*-Bu<sub>4</sub>NF/THF, (viii) DMT-Cl /py,
(ix) *i*PrNP(Cl)OCH<sub>2</sub>CH<sub>2</sub>CN/*i*Pr<sub>2</sub>NEt/CH<sub>2</sub>Cl<sub>2</sub>

DCHEME 1	S	ch	e	n	1e	1
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Desilylation of <u>6</u> with *n*-Bu<sub>4</sub>NF and purification of the reaction product by flash silica gel chromatography yielded  $\psi T$  (<u>7a</u>) in 85% yield.

The anomeric configuration of (**7a**) was confirmed by <sup>1</sup>H NMR. The anomeric proton resonates at  $\delta$  4.83 as doublet of doublet,  $J_{1',2'} = 5.68$  Hz,  $J_{1',2''} = 7.28$  Hz,  $J_{1',6} < 0.6$  Hz and is in full agreement with the proven<sup>27</sup> configuration of  $\psi$ T (**7a**). The detail assignment of anomeric proton as well as other protons was further confirmed by HETCOR spectrum. From HETCOR spectrum (*Figure 3*), the peak at  $\delta$  34.90 coupled with singlet at 3.24 ppm and assigned as N1C, N1CH<sub>3</sub>. The peak at  $\delta$  40.70 coupled with multiplet peaks at 1.81 ppm and 2.05 ppm and was assigned C<sub>2'</sub>, H<sub>2'</sub> and H<sub>2''</sub>, respectively. The peaks at  $\delta$  62.09, 71.91 and 87.02 coupled with peak at 3.45 ppm, peak at 3.71 ppm and peak at 4.13 ppm and was assigned as C<sub>5'</sub>, H<sub>5'</sub>, H<sub>5''</sub>; C<sub>3'</sub>, H<sub>3'</sub> and C<sub>1'</sub>, H<sub>1'</sub>, respectively. The initial assignemnt and evidence about the presence of CH<sub>3</sub> group at N1 position of  $\psi$ T (**7a**), we for



Figure 3. HETCOR spectrum of  $\psi T$  (7a) in DMSO-d6 at 313 K. For details see experimental section.

the first time resolved by 1D NOE difference study. Since the presence of the CH<sub>3</sub> group at N1 position of  $\psi$ **T** (**7a**) is very important for Hoogsteen type of binding with a DNA duplex in parallel and antiparallel motif, we confirmed the position by 1D NOE experiment. The irradiation of C6 proton of N1-methyl- $\psi$ -uridine (**3b**) at 7.70 ppm led to a 6.28% enhancement of the resonance at 3.24 ppm confirming that this signal was due to the presence of CH<sub>3</sub> group at N1 position. Further, the irradiation of N1-methyl proton at 3.24 ppm of  $\psi$ **T** (**7a**) gave 6.54% enhancement of the resonance at 7.59 ppm (*Figure 4*) which further confirmed that this signal is due to the presence of CH<sub>3</sub> group at N1 position.

The NOE experiment also allowed us to assign the H2' ( $\delta$  1.81 ppm) and H2" ( $\delta$  2.05 ppm) protons of the  $\psi$ T (**<u>7a</u>**), *Table 1*. The lower field signal at 2.05



Figure 4. The NOE difference spectrum of (7a) in DMSO-d<sub>6</sub> at 313 K. The low power irradiation frequency was cycled every  $\mu$ sec.

ppm (H2") was enhanced by 5.2% on irradiation of the anomeric proton (H1'). The higher field signal at 1.81 ppm (H2') was not enhanced. Irradiation of the C6 proton at 7.59 ppm led to a 7.2 % enhancement of the resonance at 1.81 ppm, further confirming that this signal was due to H2'. There was significantly smaller NOE effect between H6 proton and H1' proton (2.1%). With this information, we assigned the coupling constants as shown in *Table 1* and confirmed that majority population of  $\psi$ T (*Ta*) exist as "*anti*" conformer in DMSO-d<sub>6</sub> solution (*Figure 2*).

The incorporation of  $\psi$ T (7a) into oligonucleotides was done by Watanabe et al.<sup>31</sup> via H-phosphonate method. Recently, Ono et al.<sup>32,33</sup> reported the incorporation of 2'-O-methyl- $\psi$ -isocytidine via phosphoramidite method. The automated synthesis of oligonucleotides by phosphoramidite method<sup>34,35</sup> is generally superior to H-phosphonate method. Piccirili et al.<sup>36</sup> incorporated  $\psi$ T (7a) into oligonucleotides via phosphoramidite and used methoxy phosphate protecting group. It has been found<sup>37</sup> that incorporation of C-nucleoside analogues into G-rich oligonucleotides via phosphoramidite method by using cyanoethoxy phosphate protecting group increased the coupling efficiency. We



Table 1. wT (7a) NMR data (Coupling constants)

synthesized the phosphoramidite of  $\psi T$  (8) by using cyanoethoxy phosphate protecting group. The phosphoramidite (8) was synthesised *via* 5'-O-DMT derivative (7b). Tritylation of (7a) with 4,4'-dimethoxytrityl chloride in dry pyridine afforded the 5'-O-trityl derivative (7b) in a 88% yield. The phosphitylation of (7b) with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in dry CH<sub>2</sub>Cl<sub>2</sub> in the presence of *N*,*N*-diisopropylchlorophosphoramidite in dry CH<sub>2</sub>Cl<sub>2</sub> in the presence of *N*,*N*-diisopropylethylamine gave *N*1-methyl-5'-{*O*-[(4,4'-dimethoxy-trityl)-2-deoxy- $\psi$ -uridine]}-3'-[2-cyanoethyl bis--(1-methylethyl)]phosphoramidite (8) in a 85% yield.

The phosphoramidite ( $\underline{8}$ ) was successfully incorporated into homooligomers (TFOs) and G-rich TFOs by solid support methodology in an automated DNA synthesizer. The sequence of TFOs (**S1**, **S2**, **S3**, **S4**, **S5** and **S6**) containing  $\psi$ T has been listed in *Table 2*. These TFOs have been synthesised on 1 µmol scale and were purified by ion-exchange chromatography.<sup>38</sup> The purity of homooligomers and G-rich TFOs was determined by analytical HPLC (Q-Sepharose ion-exchange column) and by labeling with <sup>32</sup>P-ATP using polynucleotide kinase followed by analysis on a 20% denaturing polyacrylamide gel.<sup>39</sup>

No.	Oligonucleotide Sequence (TFOs)	Stepwise Coupling Yield (%)			
S1	5'-TTTT <b>VT</b> TT <b>VT</b> TT-3'	98.5			
S2	5 ' - TY <b>T</b> TTY <b>T</b> Y <b>T</b> Y <b>T</b> T-3 '	98.6			
<b>S</b> 3	5 ' -ψτψτψτψτψτψτψτψτψ	98.7			
54	5 '-GGGTTGG <b>ψT</b> GGTTGG-3 '	98.5			
<b>S</b> 5	5 ' -GGG <b>ψTψT</b> GGGGGG <b>ψTψT</b> GGG-3 '	97.8			
<b>S</b> 6	5 ' -GGG <b>WTWT</b> GGW <b>T</b> GGW <b>TWT</b> GGG-3 '	98.4			
The second s					

Table 2

The nucleoside composition of all the modified TFOs containing  $\psi$ T was determined by digestion with P1 nuclease/bacterial alkaline phosphatase. A mixture of standard solutions of three deoxynucleosides, thymidine (T), 2'-deoxyguanosine (G) and  $\psi$ -thymidine ( $\psi$ T) was analysed by a C<sub>18</sub> reversed phase HPLC column using 0.05 M KH<sub>2</sub>PO<sub>4</sub> buffer and CH<sub>3</sub>CN with flow rate of 2.0 mL/min, which gave the order of elution as  $\psi$ T, G and T. The relative amounts of each of the deoxynucleosides in digestion mixtures were estimated from the peak areas. Observed relative ratios of the nucleosides ( $\psi$ T, G and T) were in agreement with the calculated ratios.

The antiparallel triplex formation of the G-rich TFOs (**S4**, **S5** and **S6**) was assayed by the gel mobility shift method<sup>11,16</sup> at pH 7.5 with the target duplex 5'-d(CTGAGACCGGGAAGGAGGAAGGGCCAGTGAC)3'-5'd(GTCACTGGCCC-TTCCTCCTTCCCGGTCTCAG)3'. Samples containing trace concentrations of radiolabelled duplex and increasing concentration of TFOs were incubated in 20 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 10% sucrose at 37 °C for 18-22 h. The expectation of electrophoretic analysis would be that the oligomer GGGTTGGTGGTTGGG (control) will bind to the target duplex in antiparallel motif to form triple helix consisting of G:GC and T:AT base triplets, and with TFOs (**S4**, **S5** and **S6**) to form G:GC and  $\psi$ T:AT base triplets. The apparent association constant (K<sub>d</sub>) for antiparallel triplex formation was estimated as equivalent to the TFO concentration required to bind 50% of the labelled duplex.<sup>11</sup> The K<sub>d</sub> values of the TFOs (**S4**, **S5** and **S6**) and the control oligomer (**S**) are listed in *Table 3*.

Oligonucleotides	Sequence	Apparent		
(TFOs)		Kd		
<b>S4</b>	5 ' -GGGTTGG <b>ψT</b> GGTTGGG-3 '	1X10 <sup>-6</sup> M		
S5	5 ' -GGG <b>\T\TGGGGG\T\TGGGG-3</b> '	1X10 <sup>-6</sup> M		
<b>S</b> 6	5 ' -GGG <b>\T\T</b> GG\ <b>T</b> GG\ <b>T</b> GG\ <b>T\G</b> GG-3 '	1X10 <sup>-6</sup> M		
Control Oligo	(S) 3'-GGGTTGGTGGTTGGG-5'	>1X10 <sup>-6</sup> M		
	• • • • • • • • • • • • • • • • •			
Target Duplex	5 ' - CTGAGACCGGGAAGGAGGAAGGGCCAGTGA	TGAGACCGGGAAGGAGGAAGGGCCAGTGAC-3 '		
(D1)	3 ' -GACTCTGGCCCTTCCTCCTCCCGGTCACTG-5 '			

Table 3. The apparant Kd values of TFOs (S4, S5 and S6) with duplex (D1).

The preliminary data ( $K_d$  values) indicated that the modified TFOs are not better triple helix substrates and the substitution of T with  $\psi$ T did not improve the stability of triple helices.

Parallel triplex formation was assayed by UV melting experiments. Triplex melting experiments were performed with 10-mer homopyrimidine-oligomer TFOs (S1, S2 and S3) which contains  $\psi$ T at various sites. A DNA melting experiment was conducted by diluting  $T_{10}$  (thymidine 10-mer) (1.62  $\mu$ L of a 7.98 x 10<sup>-4</sup> M solution) and A<sub>10</sub> (2'-deoxyadenosine 10-mer) (2.65  $\mu$ L of a 6.45 x 10<sup>-4</sup> M solution) in buffer (40 mM sodium cacodylate, 80 mM MgCl<sub>2</sub>, 4.0 M NaCl) (pH 7.0). The melting experiment of duplex-10, 5'-d(TTTTTTTTTT)3'-5'd(AAAAAAAAA)3' (A<sub>10</sub>:T<sub>10</sub>) was repeated three times to obtain consistent melting profiles. When  $T_{10}$  homopyrimidine-oligomer (control) and  $(A_{10}:T_{10})$ were mixed in high salt buffer at pH 7.0, two distinct melting transitions were observed. The first transition at 16.6 °C corresponds to the melting of triplex  $T_{10}$ : A<sub>10</sub>:  $T_{10}$  and the second transition at 33.99 °C represents melting of duplex  $A_{10}$ :T<sub>10</sub>. The mixture of TFO (S1) and duplex ( $A_{10}$ :T<sub>10</sub>) were mixed in the same buffer at pH 7.0, two distinct transitions were observed. The lower transition at 9.2 °C indicates melting transition of  $\psi T_{10}$ : A<sub>10</sub>:T<sub>10</sub>. The higher teperature 33.89 °C corresponds to the melting transition of the duplex A<sub>10</sub>:T<sub>10</sub>. We also observed the triplex melting transition ( $T_m = 6.7$  °C) with the TFO (S2) and duplex (A<sub>10</sub>:T<sub>10</sub>) but lower than (S1). We observed that substitution of 4Ts with 4wTs decreased the melting transition  $(T_m)$  about 3 °C indicating that  $\psi T$  did not increase the

stability of triple helices. We also observed that the replacement of all 10Ts with 10 $\psi$ Ts gave low triplex melting transition (T<sub>m</sub> = -7.5 °C) and indicates clearly that  $\psi$ T did not improve the stability of triple helices in comparison to T. The gradual decrease in the triplex melting transition with increasing numbers of  $\psi$ T residues indicates that  $\psi$ T is not a better substitute for T.

In summary, a practical synthesis of  $\psi T$  has been accomplished and successfully incorporated into G-rich triplex forming oligonucleotides (TFOs) via phosphoramidite methodology. 1D <sup>1</sup>H NOE data indicated that majority population of  $\psi T$  (7a) exists as a "anti" conformer in DMSO-d<sub>6</sub> solution. The binding results indicated that the replacement of T with  $\psi T$  did not improve the stability of triple helices in both antiparallel and parallel motifs. In parallel motif, the gradual decrease in the triplex melting transition (T<sub>m</sub>) with increasing number of  $\psi$ T residues further indicated that minor modification of T with  $\psi$ T influencing the stability of triple helices. In  $\psi T$  the double bond in the pyrimidine ring is located on the carbon of C-C glycosidic linkage which might have affected the neighboring base stacking interaction in anti-parallel and parallel motifs and may be the reason in decreasing the stability of the triple helices. However, Paul Ts'o et al<sup>40</sup> reported recently that 2'-deoxy- $\psi$ -uridine, when incorporated into the second strand of a duplex gave better stability of triplex A:yUC as compared to 2'deoxyuridine triplex A:UC. Furthermore, the pyrimidine C-nucleoside analogues if incorporated<sup>40</sup> with adenosine and guanosine in a second strand with Watson-Crick complementarity, the naturally occurring target nucleic acid may serve as the first strand of a triplex.

#### **EXPERIMENTAL**

Melting points (uncorrected) were determined in a Thomas-Hoover capillary melting-point apparatus. Elemental analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ. The presence of water as indicated by elemental analysis was verified by <sup>1</sup>H NMR spectroscopy. Thin layer chromatography (TLC) was performed on aluminum plates coated (0.2 mm) with silica gel 60F<sub>254</sub> (EM Science). Silica gel (EM Science, 230-400 mesh) was used for flash column chromatography. All solvents used were reagent grade and the solvent mixtures are in volumes. Detection of nucleoside components on TLC was by uv light, and with 10% H<sub>2</sub>SO<sub>4</sub> in MeOH spray followed by heating. Evaporations were conducted under diminished pressure with the bath temperature below 30 °C. Infrared (IR in KBr) spectra were recorded with a Perkin-Elmer 1420 IR spectrophotometer and ultraviolet spectra (UV) were recorded on a Hewlett-Packard 8452 diode array spectrophotometer.

Nuclear magnetic resonance spectra were recorded on a Bruker wide bore AM-400 FT-NMR spectrometer operating at a field strength of 9.4 T (400.14 MHz for <sup>1</sup>H, 100.62 MHz for <sup>13</sup>C, and 161.98 MHz for <sup>31</sup>P). The chemical shift values ( $\delta$ ) are expressed in ppm (parts per million). <sup>1</sup>H chemical shift is expressed relative to tetramethylsilane (TMS) as the internal standard, <sup>31</sup>P chemical shift is relative to 85% polyphosphoric acid as external standard. The DMSO-d6 multiplet (39.5 ppm) served as the reference for <sup>13</sup>C chemical shifts. The digital resolution employed for <sup>1</sup>H and <sup>13</sup>C were 0.66 Hz/pt and 0.65 Hz/pt, respectively, in the 1D spectra (key: s = singlet, d = doublet, t = triplet, dd =doublet of doublet, q = quartet, m = multiplet,  $\psi t = pseudo triplet$ , refers to a doublet of doublet that has the appearence of triplet, br = broad). Data processing was performed with ASPECT 3000 equipped with an array processor and all spectra were run at 313K. The Heteronuclear (13C, 1H) correlation spectrum (HETCOR) was performed in the absolute value mode using standard pulse sequence. The 1D <sup>1</sup>H NOE difference spectroscopy was performed using a 20 ppm spectral window with 64 K data points.

**2',3',5'-Tri-O-acetyl-\psi-uridine (2)**. To a solution of  $\psi$ -uridine<sup>41</sup> (**1**, 1.0 g, 4.09 mmol) in anhydrous *N*,*N*-dimethylformamide (60 mL) at -30 °C was added 4-dimethylaminopyridine (DMAP, 0.20 g, 1.63 mmol) followed by acetic anhydride (2 mL, 19.6 mmol) portionwise over a period of 15 min. The reaction mixture was stirred at -30 °C for 3 h and then the temperature was raised to room temperature. The reaction was quenched with MeOH (2 mL) and the solvents were evaporated to dryness. The residue was co-evaporated with MeOH (2 x 10 mL), dissolved in CH<sub>2</sub>Cl<sub>2</sub> (60 mL), washed with H<sub>2</sub>O (50 mL), the organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and concentrated. The residual product was purified on flash silica gel column (3 x 25 cm) using CH<sub>2</sub>Cl<sub>2</sub>:EtOAc (7:3) as the eluent to afford 1.45 g (95%) of **2**; IR v<sub>max</sub> 1725 (C=O); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  2.01 (br s, 9 H, 3CH<sub>3</sub>CO), 4.14 (m, 2 H, C<sub>5</sub>·H<sub>2</sub>), 4.31 (tt, 1 H, C<sub>4</sub>·H), 4.68 (d, J = 4.32 Hz, 1 H, C<sub>3</sub>·H), 5.25 (q, 1 H, C<sub>2</sub>·H), 5.38 (t, J<sub>1</sub>',<sub>2'</sub> = 5.2 Hz, 1 H, C<sub>1</sub>·H), 7.44 (s, 1 H, C<sub>6</sub>H), 11.15 (br s, 2 H, NH). Anal. Calcd for C<sub>15</sub>H<sub>18</sub>N<sub>2</sub>O<sub>9</sub>: C, 47.49; H, 5.05; N, 7.39. Found: C, 47.41; H, 5.15; N, 7.29.

**N1-Methyl-2',3',5'-tri-O-acetyl-\psi-uridine (3a)**. To a solution of 2',3',5'-tri-O-acetyl- $\psi$ -uridine (2, 10.0 g, 27.02 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (250 mL) was added *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (11.85 g, 58.25 mmol) and the reaction mixture was stirred at room temperature for 1 h. To this mixture, CH<sub>3</sub>I (2.15 mL, 33.77 mmol) was added and heated under reflux for 5 days. The reaction mixture was evaporated, and the residue was co-evaporated with toluene (2 x 20 mL). The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (400 mL), washed with 1% NaHCO<sub>3</sub> solution (50 mL), dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to dryness. The residual product was purified by flash silica gel column (6 x 35 cm) using CH<sub>2</sub>Cl<sub>2</sub>;EtOAc (8:2) as the eluent to give 9.56 g (92%) of **3a** as white foam; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  2.14 (br s, 9 H, 3CH<sub>3</sub>CO), 3.26 (s, 3 H, NCH<sub>3</sub>), 4.17 (m, 2 H, C<sub>5</sub>·H<sub>2</sub>), 4.31 (dd, 1 H, C<sub>4</sub>·H), 4.68 (d, J = 5.2 Hz, 1 H, C<sub>3</sub>·H), 5.28 (t, 1 H, C<sub>2</sub>·H), 5.40 (t, J<sub>1</sub>',<sup>2</sup> = 5.12 Hz, 1 H, C<sub>1</sub>·H), 7.67 (s, 1 H, C<sub>6</sub>H), 11.15 (br s, 1 H, NH). Anal. Calcd for C<sub>16</sub>H<sub>20</sub>N<sub>2</sub>O<sub>9</sub>·0.25 H<sub>2</sub>O: C, 49.42; H, 5.31; N, 7.20. Found: C, 49.45; H, 5.48; N, 6.91.

N1-Methyl-ψ-Uridine (3b). Compound 3a (1.0g, 2.60 mmol) in MeOH/NH<sub>3</sub> (saturated at 0 °C, 40 mL) was stirred in a pressure bottle at room temperature for 24 h. The pressure bottle was opened carefully and the reaction mixture was evaporated to dryness. The residue was triturated with isopropanol (25 mL) to give a solid which was washed with ether and crystallized from EtOH to give 0.61 g (90%) of 3b; mp 182-184 °C [ Lit<sup>29</sup> 181-184 °C]; IR v<sub>max</sub> 3300-3400 (OH, NH), 1685 and 1700 (C=O) cm<sup>-1</sup>; UV  $\lambda_{max}$  nm (ε x 10<sup>-3</sup>): (pH 1) 270 (8.9), 212 (7.6); (pH 7) (MeOH) 268 (8.8), 212 (7.7); (pH 11) 270 (8.6), 212 (8.4); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 3.24 ( s, 3 H, NCH<sub>3</sub>), 3.5 (m, 2 H, C<sub>5</sub>·H<sub>2</sub>), 3.7 (m, 1 H, C<sub>4</sub>·H), 3.98 (q, 1 H, C<sub>3</sub>·H), 4.06 (t, 1 H, C<sub>2</sub>·H), 4.44 (d, 1 H, C<sub>3</sub>·OH), 4.67 (m, 2 H, C<sub>2</sub>·OH and C<sub>5</sub>· OH), 4.81 (d, J<sub>1</sub>·<sub>2</sub>· = 5.2 Hz, 1 H, C<sub>1</sub>·H), 7.70 (s, 1 H, C<sub>6</sub>H), 11.21 (br s, 1 H, NH). Anal. Calcd for C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>6</sub>: C, 46.51; H, 5.46; N, 10.84. Found: C, 46.24; H, 5.48; N, 10.73.

# N1-Methyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-ψ-uridine

(4). N1-Methyl- $\psi$ -uridine (3b, 2.0 g, 7.75 mmol) was co-evaporated with dry pyridine (3 x 25 mL) and dissolved in anhydrous pyridine (50 mL). To the solution was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.56 g, 8.13 mmol) at 0-5 °C. The mixture was stirred for 1 h at 0 °C and then for 4 h at ambient temperature under argon. The mixture was evaporated to dryness and co-evaporated with toluene (3 x 20 mL). The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (85 mL), washed with H<sub>2</sub>O (300 mL), followed by 5% solution of NaHCO<sub>3</sub>. After drying (Na<sub>2</sub>SO<sub>4</sub>) the solvent was evaporated to dryness. The residual product was purified by silica gel column (3 x 28 cm) using CH<sub>2</sub>Cl<sub>2</sub>:EtOAc (6:4) as the eluent to yield 3.65 g (94%) of 4; mp 101 °C; IR v<sub>max</sub> 3250-3450 (OH, NH), 2835

(NCH<sub>3</sub>), 1720 (C=O) cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.05 (m, 28 H, *i*-Pr), 3.19 (s, 3 H, NCH<sub>3</sub>), 3.9 (m, 2 H, C<sub>5</sub>'H<sub>2</sub>), 4.02 (m, 2 H, C<sub>3</sub>'H and C<sub>4</sub>'H), 4.2 (m, 1 H, C<sub>2</sub>'H), 4.5 (br s, 1 H, C<sub>2</sub>'OH), 4.72 (d, J<sub>1',2'</sub> = 4.32 Hz,1 H, C<sub>1</sub>'H), 7.43 (s, 1 H, C<sub>6</sub>H), 11.14 (br s, 1 H, NH). Anal. Calcd for C<sub>22</sub>H<sub>40</sub>N<sub>2</sub>O<sub>7</sub>Si<sub>2</sub>: C, 52.77; H, 8.05; N, 5.60. Found: C, 52.82; H, 8.11; N, 5.50

*N*1-Methyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-[(imidazol-1-yl)thiocarbonyl]-ψ-uridine (**5**). A mixture of *N*1-methyl-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-ψ-uridine (**4**, 1.0 g, 1.99 mmol), (thiocarbonyl)diimidazole (TCDI) (0.43 g, 2.4 mmol) and dry DMF (10 mL) was stirred at room temperature for 4.5 h. The reaction mixture was evaporated to dryness, the residue was dissolved in EtOAc (80 mL), and washed with H<sub>2</sub>O (150 mL). After drying (Na<sub>2</sub>SO<sub>4</sub>) the solvent was evaporated. The light yellow residue was purified by flash silica gel column (4 x 35 cm) using CH<sub>2</sub>Cl<sub>2</sub>:EtOAc (7:3) as the eluent to give 1.0 g (82%) of <u>5</u>; mp 125-127 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 1.06 (m, 28 H, *i*-Pr), 3.25 (s, 1 H, NCH<sub>3</sub>), 4.01 (m, 3 H, C<sub>4</sub>·H, C<sub>5</sub>·H<sub>2</sub>), 4.60 (t, 1 H, C<sub>3</sub>·H), 4.87 (s, 1 H, C<sub>1</sub>·H), 6.17 (t, 1 H, C<sub>2</sub>·H), 7.10 (s, 1 H, *imidazole*), 7.66 (s, 1 H, C<sub>6</sub>H), 7.85 (s, 1 H, *imidazole*), 8.52 (s, 1 H, *imidazole*), 11.40 (br s, 1 H, NH). Anal. Calcd for C<sub>26</sub>H<sub>42</sub>N<sub>4</sub>O<sub>7</sub>SSi<sub>2</sub>: C, 51.12; H, 6.93; N, 9.17; S, 5.25. Found: C, 51.23; H, 7.03; N, 9.03; S, 5.05.

N1-Methyl-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)- $\psi$ uridine (6). A mixture of 2,2'-azobis(2-methylpropionitrile) (AIBN) (0.30 g, 1.83 mmol) and *n*-Bu<sub>3</sub>SnH (2.0 g, 6.87 mmol) and dry toluene (10 mL) was added dropwise over a period of 1 h to a refluxing solution of **5** ( 1.0 g, 1.63 mmol) in dry toluene (30 mL). The reaction mixture was refluxed for an additional 1 h and evaporated. The residual syrup was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) and loaded on the top of a pre-packed (CH<sub>2</sub>Cl<sub>2</sub>) silica gel column (3 x 28 cm). The column was eluted with CH<sub>2</sub>Cl<sub>2</sub>:EtOAc (8:2) and the appropriate homogeneous fractions were pooled and concentrated to give 0.65 g (82.2%) of **6** as a white foam; mp 95-96 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.06 (m, 28 H, *i*-Pr), 2.19 (m, 2 H, C<sub>2</sub>:H and C<sub>2</sub>:"H), 3.23 (d, J = 4.4 Hz, 3 H, NCH<sub>3</sub>), 3.66 (q, J4',3' = 3.2 Hz, 1 H, C<sub>4</sub>'H), 3.97 (m, 2 H, C<sub>5</sub>:H<sub>2</sub>), 4.45(t, J3',4' = 6.4 Hz, 1 H, C<sub>3</sub>:H), 4.79 (t, J1',2' = 6.88 Hz, J1',2'' = 6.91 Hz, 1 H, C<sub>1</sub>'H), 7.45 (s, 1 H, C<sub>6</sub>H), 11.23 (br s, 1 H, NH). Anal. Calcd for C<sub>22</sub>H<sub>40</sub>N<sub>2</sub>O<sub>6</sub>Si<sub>2</sub>: C, 54.51; H, 8.32; N, 5.78. Found: C, 54.13; H, 8.37; N, 5.59.

N1-Methyl-2'-deoxy-ψ-uridine (ψ-Thymidine, <u>7a</u>). To a solution of N1methyl-2'-deoxy-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-ψ-uridine (<u>6</u>, 1.75 g, 3.61 mmol) in dry THF (20 mL) was added dropwise a 1.0 M solution of *n*-Bu<sub>4</sub>NF in THF (10 mL) and the reaction mixture was stirred at room temperature for 1 h. The compound that precipitated was collected by filteration and purified on a flash silica gel column (3 x 28 cm) using CH<sub>2</sub>Cl<sub>2</sub>:MeOH (85:15) as the eluent to give a white powder which on crystallization from a mixture of MeOH and hexane gave 0.75 g (85%) of <u>7a</u>: mp 160-161 °C [Lit<sup>27</sup> mp 158-160 °C]; IR v<sub>max</sub> 3300-3400 (OH) cm<sup>-1</sup>; UV  $\lambda_{max}$  nm ( $\epsilon$  x 10<sup>-3</sup>): (pH 1) 270 (5.5), 212 (5.0); (pH 7) (MeOH) 268 (8.8), 212 (7.7); (pH 11) 270 (8.2), 212 (8.6); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.81 (m, 1 H, J<sub>2',3'</sub> = 5.84 Hz, C<sub>2'</sub>H), 2.05 (m, J<sub>2',2''</sub> = 12.8 Hz, 1 H, C<sub>2''</sub>H), 3.24 (s, 3 H, NCH<sub>3</sub>), 3.45 (m, J<sub>5',5''</sub> = 11.76 Hz, 2 H, C<sub>5</sub>:H<sub>2</sub>), 3.71 (q, J<sub>4',5'</sub> = 2.32 Hz, 1 H, C<sub>4</sub>:H), 4.13 (t, J<sub>3',4'</sub> = 4.36 Hz, 1 H, C<sub>3</sub>:H), 4.60 (s, 1 H, 3'OH), 4.83 (dd, J<sub>1',2'</sub> = 5.68 Hz, J<sub>1',2''</sub> = 7.28 Hz, 1 H, C<sub>1'</sub>H), 4.89 (d, J = 3.68 Hz, 1 H, C<sub>5</sub>:OH), 7.59 (s, 1 H, C<sub>6</sub>H), 11.16 (br s, 1H, NH). Anal. Calcd for C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>5</sub> : C, 49.58; H, 5.83; N, 11.56. Found: C, 49.48; H, 5.68; N, 11.35.

N1-Methyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxy-w-uridine (7b). Compound 7a (0.40 g, 1.65 mmol) was co-evaporated with dry pyridine ( $3 \times 25$  mL) and then dissolved in dry pyridine (15 mL). To this solution, 4,4'-dimethoxytrityl chloride (0.587 g, 1.73 mmol) and triethylamine (0.5 mL) were addded and the reaction mixture was stirred at room temperature for 4 h with the exclusion of moisture. The reaction was quenched by the addition of MeOH (3 mL) and the mixture was stirred for an additional 15 min. The mixture was evaporated and the residue was co-evaporated with toluene (3 x 20 mL) to remove the last traces of pyridine. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (150 mL), washed with 15% aqueous solution of NaHCO<sub>3</sub>, dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to dryness. The residue was purified by flash silica gel column (3 x 28 cm) using CH<sub>2</sub>Cl<sub>2</sub>:MeOH (98:2) as the eluent to give 0.76 g (84.5%) of <u>7b</u>; mp 98-101 °C; <sup>1</sup>H NMR (DMSOd6) δ 1.85 (m, 1 H, C<sub>2</sub>·H), 2.10 (m, 1 H, C<sub>2</sub>·H), 3.09 (m, 2 H, C<sub>5</sub>·H<sub>2</sub>), 3.11 (s, 1 H, NCH<sub>3</sub>), 3.73 (s, 6 H, 2OCH<sub>3</sub>), 3.84 (t, J<sub>4',5'</sub> = 4.45 Hz, J<sub>4',5''</sub> = 5,72 Hz, 1 H, C<sub>4'</sub>H), 4.16 (t, J = 2.81 Hz, 1 H, C<sub>3</sub>'H), 4.87 (dd,  $J_{1',2'} = 6.0$  Hz,  $J_{1',2''} = 6.34$  Hz, 1 H,  $C_{1'}H$ ), 5.02 (d, 1 H, C<sub>3</sub>OH), 6.67-7.42 (m, 13 H, DMT), 7.52 (s, 1 H, C<sub>6</sub>H), 11.24 (br s, 1 H, NH). Anal. Calcd for C31H32N2O7 H2O: C, 66.17; H, 6.08; N, 4.98. Found: C, 66.50; H, 5.94; N, 4.86.

N1-Methyl-5'-{O-[(4,4'-dimethoxytrityl)-2-deoxy- $\psi$ -uridine]}-3'-[2-cyanoethyl bis(1-methylethyl)]phosphoramidite (8). Compound <u>7b</u> (0.85 g, 1.56 mmol) was co-evaporated with dry toluene (2 x 20 mL), dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (15 mL) and was stirred under argon for 10 min. To this solution, *N*,*N*- diisopropylethylamine (0.905 g, 7.02 mmol) and 2-cyanoethyl-N,Ndiisopropylchlorophosphoramidite (0.26 g, 1.11 mmol) were added. The reaction mixture was stirred at ambient temperature for 45 min and was diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL). The solution was washed with 10% solution of NaHCO<sub>3</sub> (120 mL), dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to dryness. The residual syrup was dissolved in a mixture of 2% triethylamine in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) and loaded on the top of a pre-packed silica gel column (hexane:CH2Cl2:Et3N; 90:5:5). The column was eluted with CH<sub>2</sub>Cl<sub>2</sub>:EtOAc:Et<sub>3</sub>N (90:7:3). The appropriate homogeneous fractions were pooled and evaporated to dryness. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 mL) and added with stirring to hexane (-40 °C). The cloudy solution was evaporated to dryness to give 0.87 g (75%) of **§** as a white foam. <sup>1</sup>H NMR (CD<sub>3</sub>CN) δ 1.29 {m, 12 H, N[CH(CH<sub>3</sub>)<sub>2</sub>]<sub>2</sub>}, 1.88 (m, 2 H, NCH<sub>2</sub>), 1.93 (m, 1 H, C2'H), 2.21 (m, 1 H, C2"H), 3.17 (s, 3 H, NCH3), 3.35 (m, 2 H, OCH2), 3.58 (m, 2 H, C5'H2), 3.67 [m, 2 H, 2CH(CH3)2], 3.74 (s, 6 H, 2OCH3), 3.95 (q, 1 H, C4'H), 4.45 (m, 1 H, C<sub>3</sub>'H), 4.85 (dd, 1 H, C<sub>1</sub>'H), 6.83-7.51(m, 14 H, DMT and C<sub>6</sub>H), 11.21 (br s, 1 H, NH); <sup>31</sup>P NMR (CD<sub>3</sub>CN) δ 148.94. Anal. Calcd for C<sub>40</sub>H<sub>49</sub>N<sub>4</sub>O<sub>8</sub>P·0.75H<sub>2</sub>O: C. 63.39; H, 6.66; N, 7.41; P 4.09. Found: C, 63.28; H, 6.86; N, 7.61; P, 4.01.

Synthesis, Purification and Characterization of Oligonucleotides (TFOs): The oligonucleotides (TFOs) were synthesized on a Applied Biosystem DNA synthesizer (models 380B or 394) using phosphoramidite method on 1  $\mu$ mol scale. The phosphoramidite of T (thymidine) and G (2'-deoxyguanosine) were purchased from Chem. Implex International, Inc. The isobutyl-protected G was used for incorporation of G residues into TFOs. The concentration of the phosphoramidites used was 0.1 µM and the coupling time was increased to 900 seconds. The coupling efficiency was measured by UV spectrophotometric quantitation of released dimethoxytrityl cation at 498 nm on each synthesis cycle, which indicated that the average coupling efficiency of this synthon  $(\underline{8})$  is more than 98%. After the synthesis, the TFOs were cleaved from the solid-support by treatment with ethanolic ammonia at 56 °C for 15-18 h. The purification of oligonucleotides (TFOs) was done by HPLC using ion exchange Q-Sepharose (Pharmacia) column<sup>38</sup> and purified oligos were desalted by passage through a C<sub>18</sub> sep-pak (Waters) cartridge. The HPLC purification gave about 90% pure modified TFOs which was further purified by polyacrylamide gel. The purity of these TFOs was determined by labeling with <sup>32</sup>P-ATP using polynucleotide kinase followed by analysis on a 20% denaturing polyacrylamide gel.<sup>39</sup>

### PSEUDOTHYMIDINE INCORPORATION INTO OLIGONUCLEOTIDES

The nucleoside composition of the modified TFOs containing  $\psi$ T was determined by digestion with P1 nuclease/bacterial alkaline phosphatase. A mixture of standard solutions of three nucleosides,  $\psi$ T ( $\psi$ -thymidine), G (2'-deoxyguanosine) and T (thymidine) was analysed by C<sub>18</sub> reverse-phase HPLC column using 0.05 M KH<sub>2</sub>PO<sub>4</sub> buffer and CH<sub>3</sub>CN with a flow rate of 2.0 mL/min, which gave the order of elution as  $\psi$ T, G and T.

**Binding Experiments:** The antiparallel triplex formation of the TFOs was assayed by gel mobility shift method.<sup>11,16</sup> The TFOs containing trace concentrations of radiolabeled duplex and increasing concentrations of the TFOs were incubated in 20  $\mu$ M Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 10% sucrose, at 37 °C for 18-22 h and were electrophorased in 12% polyacrylamide gels buffered with 89 mM tris-borate, 10 mM MgCl<sub>2</sub>. Following electrophoresis, gels were dried and autoradiographed. The apparent association constant (K<sub>d</sub>) for antiparallel triplex formation was estimated as equivalent to the TFO concentration required to bind 50% of the labelled duplex.

The parallel triplex formation was assayed by UV-thermal denaturation in a GBC 916 scanning spectrophotometer and detection was at 260 nm. A duplex DNA melting experiment was conducted by diluting  $T_{10}$  (1.62 µL of a 7.98 x 10<sup>-4</sup> M solution) and  $A_{10}$  (2.65 µL of 6.45 x 10<sup>-4</sup> M solution) in milli Q water (370 µL) and buffer A: (40 mM sodium cacodylate, 80 mM MgCl<sub>2</sub>, 4.0 M NaCl; 125 µL) (pH 7.0). A reference blank was prepared with milli Q water (370 µL) and buffer A (125 µL). The GBC peltier unit containing the TFO and reference blank was cooled in a -20 °C freezer for 1 h, achieving a starting temperature approximately -5 °C. The peltier unit was heated at a rate of 0.5 °C/min up to 100 °C and then cooled to 25 °C at a rate of 0.5 °C/min. The duplex melting experiment was repeated three times to obtain consistent melting profiles.

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