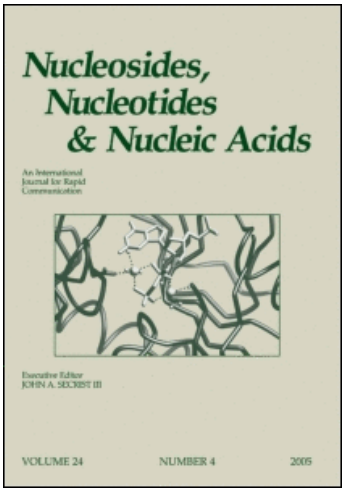

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Enhancement and Inhibition by 2′-O-Hydroxyethyl Residues of Gene Targeting Mediated by Triple Helix Forming Oligonucleotides

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ABSTRACT

Reagents that recognize and bind specific genomic sequences in living mammalian cells would have great potential for genetic manipulation, including gene knockout, strain construction, and gene therapy. Triple helix forming oligonucleotides (TFOs) bind specific sequences via the major groove, but pyrimidine motif TFOs are limited by their poor activity under physiological conditions. Base and sugar analogues that overcome many of these limitations have been described. In particular, 2′-O-modifications influence sugar pucker and third strand conformation, and have been important to the development of bioactive TFOs. Here we have analyzed the impact of 2′-O-hydroxyethyl (2′-HE) substitutions, in combination with other 2′ modifications. We prepared modified TFOs conjugated to psoralen and measured targeting activity in a gene knockout assay in cultured hamster

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cells. We find that 2′-HE residues enhance the bioactivity of TFOs containing 2′-O-methyl (2′-OMe) modifications, but reduce the bioactivity of TFOs containing, in addition, 2′-O-aminoethyl (2′-AE) residues.

Key Words: Triple helix; Oligonucleotides; Ribose analogue; Bioactivity.

INTRODUCTION

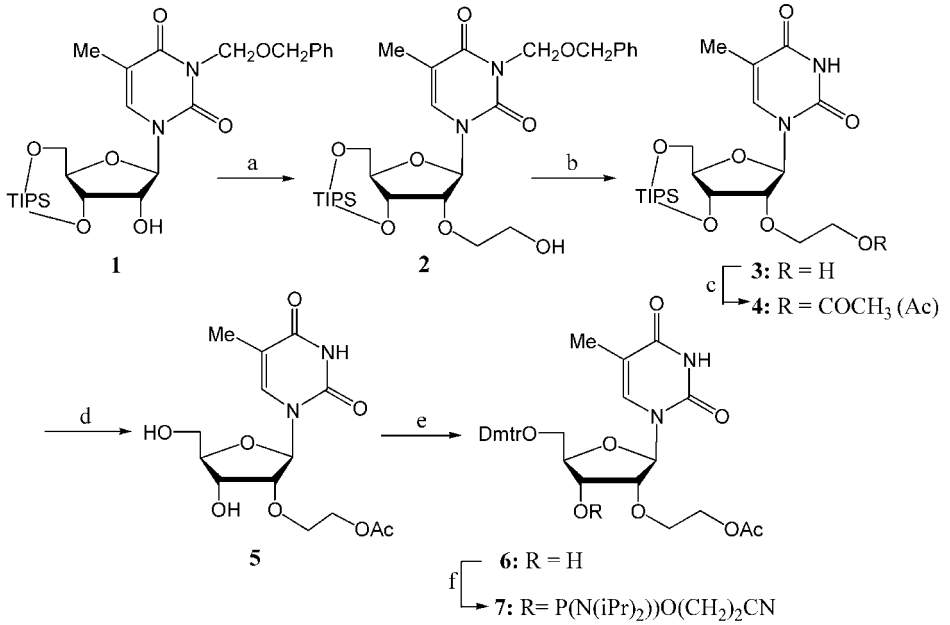
The DNA triple helix has been a focus of study for many years[1–3] in part because triple helix forming oligonucleotides (TFOs) have the potential for gene targeting applications in living cells.[4–6] The most stable triplexes are formed in the major groove of polypurine:polypyrimidine sequences by third strands consisting of either purines or pyrimidines, depending on the target sequence. Specificity and stabilization are established by hydrogen bonds between the third strand bases and the purines of the duplex.[7,8] Biological applications of pyrimidine motif deoxyribonucleotide TFOs are severely limited because of their poor binding at physiologically relevant pH (due to the need for cytosine protonation) and Mg^{++} concentration (required to suppress phosphate charge repulsion).[9,10] In addition, structural distortions imposed on both third strand and duplex by triplex formation also limit activity.[11] However sugar and base modifications have been developed which appear to overcome these barriers. 5-methylcystosine in place of cytosine permits triplex formation at neutral pH.[12,13]. The use of ribose analogues in TFOs (such as 2′-O-methyl, 2′-OMe) [14–16] affords considerable stability, in part because of the stabilization of the C3′-endo sugar pucker and the attendant preorganization of the TFO into a conformation requiring relatively little distortion of both the third strand and the duplex upon triplex formation.[11,17] The 2′-O-(2-aminoethyl)(2′-AE) modification has a similar influence on sugar pucker and also permits triplex formation at low levels of Mg^{++}. It has been shown to adopt the gauche + conformation which brings the amino group close to the phosphate of the preceding purine of the duplex strand.[17,18] Psoralen linked TFOs have been used in a variety of applications, including triplex targeted crosslinking and mutagenesis.[19–21] We have shown that psoralen linked TFOs containing all these modifications are active in a gene knockout assay in living mammalian cells.[22,23]

Our finding that sugar modifications influence TFO bioactivity led us to examine the contribution of the 2′-O-hydroxyethyl modification (2′-HE). This side chain is also likely to assume a gauche + conformation with the hydroxyl interacting with the same phosphate in the duplex as the 2′-2′-AE.[17] However, in contrast to the 2′-AE, it is not positively charged under physiological conditions. Thus the 2′-HE modification allowed us to distinguish the relative influence of the side chain conformation vs. protonation on TFO bioactivity. Here we describe an improved synthesis of 2′-HE thymidine (T_{HE}) and its unexpected effects on targeted mutagenesis by pso-TFOs. Our results suggest that biochemical measurements of TFO activity in vitro may not reliably predict TFO activity in vivo.

RESULTS AND DISCUSSION

N-3 & 3′,5′-*O*-protected-2′-*O*-(2-hydroxyethyl) ribothymidine **1** was obtained from ribothymidine following published procedures (Sch. 1).[18] Hydrogenolytic cleavage of the benzyloxymethyl group followed by subsequent protection of the –OH group as an acetyl ester afforded **4**. Simultaneous deprotection of the 3′ & 5′-*O*-silyl group using tetrabutylammonium fluoride (TBAF) led to compound **5** which was then transformed into the required phosphoroamidite **7** using standard techniques. All compounds were characterized by NMR and mass spectroscopy. The advantage of this protocol over the existing method for this class of compounds[18] is that the target compound **7** could be obtained in fewer steps starting from protected ribothymidine with ~45% overall yield and could be scaled to a multi-gram synthesis.

The TFO bioassay was based on mutagenesis of the genomic target sequence following the introduction of targeted psoralen crosslinks. Consequently, preparation of TFOs linked to intact psoralen was crucial. However, psoralen is inactivated by conventional deprotection schemes employing ammonium hydroxide at 55°C.[22] After some investigation we found that 2–5 hr incubation in 33% methylamine in absolute ethanol at room temperature afforded efficient deprotection and cleavage from the CPG without loss of psoralen activity, as measured in plasmid restriction



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Scheme 1. Reagents & Conditions: a) Ref.[18] b) Pd/C-H_2, MeOH, 12 hrs., 90% c) Ac_2O, Py, RT, 12 hrs., 74% d) TBAF, THF, RT, ~15 mins., 100% e,f) Ref.[18]. Abbreviation: TIPS = 1,1,3,3-Tetraisopropyl-1,3-disiloxane, Py = Pyridine, THF = Tetrahydrofuran, TBAF = Tetra-*n*-butylammonium fluoride, Dmtr = 4,4′-Dimethoxytriphenylmethyl.

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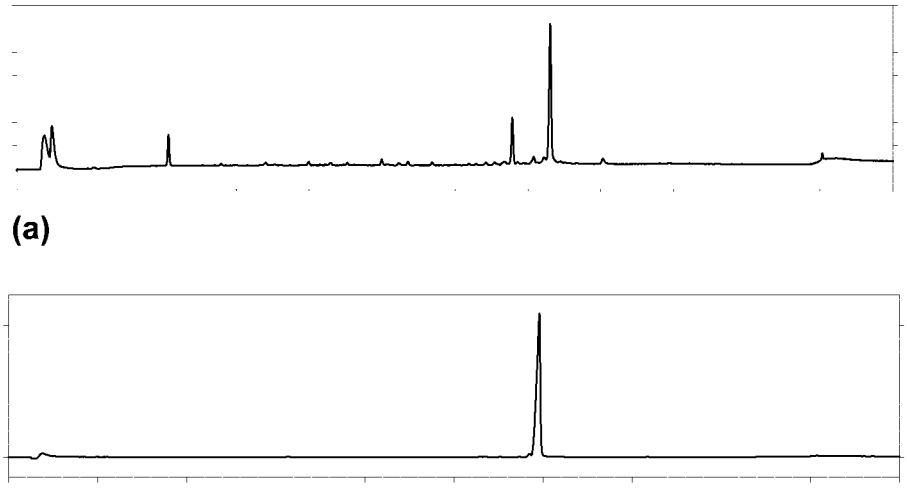

Figure 1. HPLC profile of psoralen linked TFO (HE-03) containing three 2′-HE residues after 33% methylamine treatment (a) and after purification (b).

protection assays.[22] Following deprotection the psoralen linked oligonucleotide was purified by ion exchange HPLC (Fig. 1).

We prepared pso-TFOs designed to target a sequence in the Chinese Hamster Ovary (CHO) hypoxanthine phosphoribosyl transferase gene (*Hprt*). 5-methylcytosine was used in place of cytosine in all TFOs. The TFOs contained various combinations of 2′-OMe and 2′-HE residues as shown in Fig. 2. We examined the effect of clustered as well as dispersed 2′-HE substitutions. Interestingly, the oligonucleotide with complete replacement of all thymidines with 2′-HE derivatives proved to be difficult to manipulate and bound the target sequence poorly, and was not further characterized (not shown). The triplexes formed by the TFOs were characterized by Tm analysis as described previously.[23] The TFO containing only 2′-OMe substitutions (PS-01) had a Tm at pH 7.0 of 64°C. We have shown previously that these TFOs have single transition melting profiles, with the triplex Tm higher than that of the underlying duplex.[23] The heating and cooling profiles for the duplex were superimposable. However it was not possible to perform the same heating/cooling analysis for the triplexes because triplex formation occurs necessarily after duplex formation, and all triplexes were more stable than the underlying duplex. Thus triplex formation could not take place until after the duplex had reformed, which occurred at lower temperature than the triplex Tm. Replacement of some of the 2′-OMe residues with 3, 5, or 6, 2′-HE residues resulted in a moderate increase in Tm (Fig. 2).

TFO affinity was measured in a restriction protection assay described previously.[22] TFOs at various concentrations were incubated with a plasmid containing the target sequence with a restriction enzyme (*XbaI*) recognition sequence located at the psoralen crosslink site. The incubations were for at least 24 hr after

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HPRT Target Sequence

Intron 4 Exon 5

5′ TTTCTCTTTTTTCT TCTAGa atgt..

3′ AAAGAGAAAAAAGA AGATCt taca..

		Tm(°C)	K_D
PS-01	3′ TTTCTCTTTTTTCTTCT-Pso	64	380±40
HE-01	TTTCTCTTTTTTCT$_{HE}$T$_{HE}$CT$_{HE}$-Pso	65	314±38
HE-05	TTTCTCTTTTT$_{HE}$T$_{HE}$CT$_{HE}$TC$_{HE}$T$_{HE}$-Pso	65	220±33
HE-06	TT$_{HE}$TCT$_{HE}$CTT$_{HE}$TTT$_{HE}$TCT$_{HE}$TCT$_{HE}$-Pso	65.6	205±29
AE-06	TT$_{AE}$T$_{AE}$C$_{AE}$TCTTTTTTCTTCT-Pso	71.5	140±21
HEAE-01	TT$_{AE}$T$_{AE}$C$_{AE}$TCTTTTT$_{HE}$TCTTCT-Pso	73	103±14
HEAE-02	TT$_{AE}$T$_{AE}$C$_{AE}$TCTTTTT$_{HE}$TCTTCT$_{HE}$-Pso	73	98±11
HEAE-03	TT$_{AE}$T$_{AE}$C$_{AE}$TCTTTTTTCT$_{HE}$T$_{HE}$CT$_{HE}$-Pso	72.4	100±12

Figure 2. Schematic of Intron4(caps)/Exon5(small letters) target sequence in the Chinese Hamster *Hprt* gene. The psoralen crosslink site (TA:AT) is indicated in enlarged letters. The recognition sequence for *XbaI*, including the crosslink site, is separated by spaces from the adjacent sequences. The sequence and composition of each TFO is indicated. The psoralen is at the 5′ end of all TFOs and would crosslink the T residues shown enlarged. All residues were 2′-O-methyl unless indicated as 2′-hydroxyethoxy (HE) or 2′-aminoethoxy (AE). C = ^{5Me}C. The Tm values of the triplexes, and the K_D values (in 1 mM $MgCl_2$) of the TFOs are listed.

which time the psoralen was photoactivated to crosslink the bound TFOs to the duplex. Only those TFOs bound at the time of photoactivation would be crosslinked. Then the plasmids were digested with *XbaI*. Plasmids with crosslinks were protected from digestion and the extent of protection was monitored by agarose gel electrophoresis. These determinations showed that the PS-01 TFO had a K_D of 380 nM while the 2′-HE TFOs had K_D values in the range of about 200-300 nM, measured in buffer containing 1 mM $MgCl_2$, pH 7.0. These results were consistent with the earlier report on the enhanced stability of triplexes formed by 2′-HE TFOs.[18] We chose to use 1 mM $MgCl_2$ because the more conventional 10 mM $MgCl_2$ is considered much higher than the intracellular concentration of unbound Mg^{++} (discussed in Ref.[23]).

The bioactivity of the pso-TFOs was measured in the *Hprt* knockout assay.[22] Briefly, TFOs were introduced into cultured CHO cells by electroporation, followed by exposure to long wave UV (UVA) light to photoactivate the psoralen. The cells were passaged and incubated in thioguanine selection medium. Cells that have inactivating mutations in the *Hprt* gene survive selection and form colonies while those that express functional enzyme are killed. The results (Fig. 3a) showed that the TFOs containing both 2′-HE and 2′-OMe residues were as much as 5–6 fold more active than PS-01 (2′-OMe only). Interestingly the TFO with the greatest number of 2′-HE residues had somewhat lower activity than the others. In previous work we have shown that the mutations were deletions of the triplex target and adjacent sequence.[22] Control experiments showed that there were no mutations in the *Aprt*

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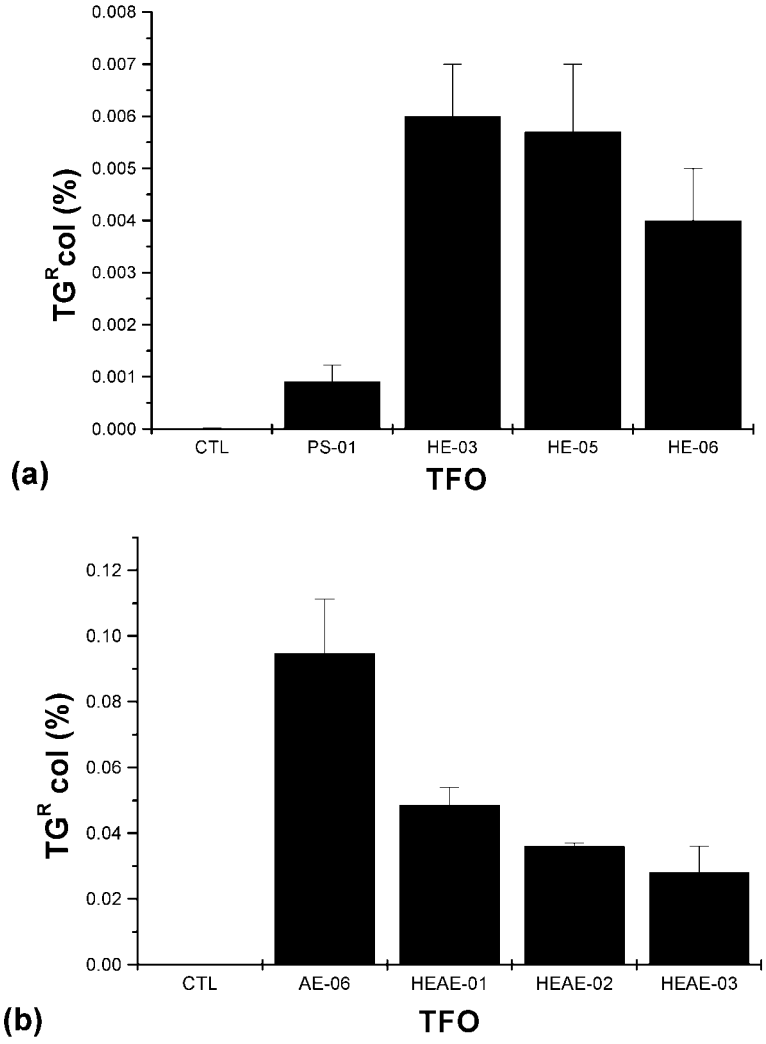

Figure 3. Thioguanine resistant colonies recovered after treatment of CHO cells with pso-TFOs. Cells were electroporated with 4 μM TFO, incubated for 3 hr, and then exposed to UVA light (3 min). After passage for 1 week to allow levels of preexisting enzyme to decline the cells were incubated in 15 μM thioguanine for 7 d. Resistant colonies were stained and counted. (a) Cells treated with pso TFOs containing 2′-OMe and HE substitutions. (b) Cells treated with pso TFOs containing 2′-OMe, HE and AE substitutions.

(adeninephosphoribosyl transferase) gene. This gene has triplex target sites that are similar, but not identical to the *Hprt* target. Thus the TFOs were target specific, at least at the level of the gene.

Although the data suggested that the 2′-HE modification did enhance TFO bioactivity, the level of targeted mutagenesis was relatively low, particularly in the light of our recent work with TFOs containing 2′-AE residues. We have found

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that TFOs with a patch of three 2′-AE residues are approximately 100 fold more bioactive than PS-01, and so 15–25 fold more active than the 2′-HE TFOs shown in Fig. 2.[23] While both modifications have a similar effect on sugar pucker and side chain conformation, only the 2′-AE substitution is protonated. We conclude that the sugar pucker and side chain conformation may be necessary but are not sufficient for bioactivity, and that the positive charge of the 2′-AE makes a major contribution to function in vivo.

The few-fold increase in activity of the 2′-HE TFOs, relative to PS-01 led us to examine the activity of TFOs containing a patch of 2′-AE residues and various amounts of 2′-HE and 2′-OMe residues. Accordingly we synthesized the HEAE pso-TFOs shown in Fig. 2. Tm analysis showed that these formed triplexes of similar stability, 8–9 degrees greater than PS-01. The mixed TFOs bound the duplex target with similar affinities (Fig. 2). Consequently we expected that TFOs carrying all three modifications would show equivalent or greater activity than the TFO with 2′-OMe and 2′-AE residues. However the mutation assay showed that the HEAE TFOs were actually less effective than the parent AE-06, with the activity inversely proportional to the 2′-HE content (Fig. 3b). In particular, the number and location of 2′-HE residues that had given greatest activity in the first series of experiments (HE-03) was the least active when combined with the 2′-AE modifications (HEAE-03).

Important work by a number of investigators has called attention to the contribution of third strand sugar pucker to triplex stability.[11,14,16,24,25] A concept that has emerged from these studies is that a consequence of the C3′-endo conformer is the preorganization of the third strand into a conformation compatible with triplex formation. This reduces entropic barriers and imposes minimal distortion on the underlying duplex.[26] Although this is generally recognized as advantageous, it has been noted that extensive use of "preorganizing" substitutions could result in too much constraint. This could limit the conformational adjustments required for triplex formation, and thus be counterproductive.[27] This may explain the results with the TFOs containing the 2′-HE residues in addition to the other two modifications. While they form stable triplexes in vitro measured under equilibrium binding conditions, they may be too constrained to permit efficient triplex formation in vivo. Indeed these data suggest that in vitro measurements of triplex stability may not be a reliable indicator of TFO activity in the nuclear environment of living cells. One of the challenges of the future will be the identification of biochemical assays that are predictive of TFO bioactivity. The development of TFOs with robust biological activity will require the analysis of TFOs with novel base and sugar modifications in quantative bioassays. The data presented here suggest that not all modifications, however effective in biochemical analyses, will improve biological activity.

EXPERIMENTAL

Anhydrous solvents (99.8%) were purchased from Aldrich and kept over 3 Å molecular sieves prior use. The reagents were purchased from Aldrich, Acros, or Fluka. Each reaction was carried out at least twice, first in small scale and then followed by scaling up. The chromatographic separations were performed on Fisher Scientific S734-1, 100–200 mesh silica gel using petroleum ether and ethyl acetate

(Mallinckrodt Baker Inc.) as eluent. Thin layer chromatography was performed on EM Science, Silica gel 60, F254 precoated plates. ^{1}H NMR spectra were recorded on Brucker AMX & Varian Unity Plus spectrophotometer at 300 & 400 MHz respectively using TMS as internal standard. ^{13}C NMR experiments were carried out on Varian Unity Plus at 100 MHz using the central peak of $CDCl_3$ as reference. Chemical shifts (δ) are reported in ppm. Mass analyses were done with Agilent Technologies 1100 LC/MSD and Applied Biosystems Voyager-DE Biospectrometry workstation at The Scripps Research Institute (La Jolla, CA).

2′-*O*-(2-Hydroxyethyl)-3′,5′-*O*-tetraisopropyl-di-silox-yl-N^3-benzyloxymethyl-5-methyl uridine (2). The synthesis of this compound was done starting from protected ribothymidine **1** following the literature procedure.[18] ^{1}H NMR ($CDCl_3$) δ: 7.57 (d, J = 1.2 Hz, 1H, H6), 7.41–7.24 (m, 5H, Ph), 5.72 (s, 1H, H1′), 5.56–5.48 (2d, J = 9.6 Hz, 2H, -N-C$\underline{H}_2$-O-), 4.75 (s, 2H, -OC$\underline{H}_2$Ph), 4.31–4.13, 4.03–3.90 & 3.76 (m, m & bs, 3H, 4H & 2H respectively, H2′, 3′, 4′, 5′, 5″, -OC$\underline{H}_2$C$\underline{H}_2$OH), 1.93 (s, 3H, 5-C$\underline{H}_3$), 1.14–1.05 (m, 28H, TIPS). MS (ESI): 665 ($M^+ + H$).

2′-*O*-(2-Hydroxyethyl)-3′,5′-*O*-tetraisopropyl-disiloxyl-5-methyl uridine (3). 35 mg of **2** was dissolved in dry methanol (0.35 mL) followed by the addition of 10 mg of Pd on carbon. A balloon filled with hydrogen gas was attached to the flask and the mixture was stirred at room temperature for 12 hrs. The reaction mixture was filtered over celite; the celite was washed with methanol and the combined filtrate and washings were concentrated under vacuum. The residue was dissolved in ethyl acetate, washed with saturated sodium bicarbonate and dried over magnesium sulfate. The solvent was removed and the product **3** (25 mg, 90%) was used in the next step without further purification. ^{1}H NMR ($CDCl_3$) δ: 9.07 (s, 1H, NH), 7.62 (s, 1H, H6), 5.74 (s, 1H, H1′), 4.31–3.95 (2m, 7H, H2′, H3′, H4′, -OCH_2CH_2OH), 3.77 (bs, 2H, H5′,5″), 3.05 (bs, 1H, -OH), 1.93 (s, 3H, -CH_3), 1.13–1.06 (m, 28H, TIPS). ^{13}C NMR($CDCl_3$) δ: 164.1 (C4), 150.4 (C2), 134.6 (C6), 110.4 (C5), 89.6 (C1′), 82.9 (C4′), 81.9 (C2′), 73.0 (C3′), 68.3 (C5′), 61.6 & 59.1 (-OCH_2-CH_2OH), 17.4–16.8 & 13.5, 12.8, 12.6, 12.5. MS (ESI): 545 ($M^+ + H$).

2′-*O*-(2-Acetoxyethyl)-3′,5′-*O*-tetraisopropyl-disiloxyl-5-methyl uridine (4). Compound **3** (150 mg, 0.27 mmol) was dissolved in dry pyridine (1.3 mL). Acetic anhydride (0.03 mL, 0.33 mmol) was added slowly and the mixture stirred for 4 hr at room temperature under nitrogen atmosphere. Saturated sodium bicarbonate was added and the solution was extracted with ethyl acetate. The extract was concentrated and the product was purified by column chromatography to give **4** (120 mg, 74%). ^{1}H NMR ($CDCl_3$) δ: 9.42 (s, 1H, NH), 7.61 (s, 1H, H6), 5.72 (s, 1H, H1′), 4.30–3.86 (m, 9H, H2′, H3′, H4′, H5′,5″, -OCH_2CH_2O-), 2.08 (s, 3H, -$COCH_3$), 1.95 (s, 3H, -CH_3), 1.12–0.98 (m, 28H, TIPS). ^{13}C NMR($CDCl_3$) δ: 171.0 (-COCH3), 164.2 (C4), 150.0 (C2), 135.0 (C6), 110.1 (C5), 89.1 (C1′), 82.6 (C4′), 81.5 (C2′), 69.0 (C3′), 68.3 (C5′), 63.5 & 59.2 (-OCH_2CH_2O-), 20.8, 17.4–16.8 & 13.4, 12.8, 12.7, 12.5. MS (ESI): 587 ($M^+ + H$).

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2′-*O*-(2-Acetoxyethyl)-5-methyl uridine (5). The nucleoside derivative **4** (100 mg, 0.17 mmol) was taken up in tetrahydrofuran and 1M tetrabutylammonium fluoride (TBAF) was added. The progress of the reaction was monitored by TLC. After the reaction was over, volatiles were removed under vacuum and the crude material was chromatographed on a silica gel column to afford **5** (60 mg, 100%). ^{1}H NMR ($CDCl_3$) δ: 7.63 (s, 1H, H6), 5.75 (d, J = 3.6 Hz, 1H, H1′), 4.40–3.99 (m, 7H, H2′, H3′, H4′, -OCH_2CH_2O-), 3.90–3.81 (m, 2H, H5′,5″) 2.09 (s, 3H, -$COCH_3$), 1.91 (s, 3H, -CH_3). ^{13}C NMR($CDCl_3$) δ: 172.8 (-$COCH_3$), 164.4 (C4), 152.3 (C2), 138.1 (C6), 111.3 (C5), 88.9 (C1′), 86.0 (C4′), 83.6 (C2′), 69.8 (C3′), 69.7 (C5′), 64.6 & 61.6 (-OCH_2CH_2O-), 20.7, 12.5. MS (ESI): 345 ($M^+ + H$).

Compound **6** and phosphoramidite **7** were prepared as described in the literature.[18] The ^{1}H NMR spectroscopic data were similar to those published.

Synthesis of TFOs

5-Methyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-methyluridine-3′-*O*-(β-cyanoethyl-N,N-diisopropyl)phosphoramidite and 5-methyl-N4-acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-methylcytidine-3′-*O*-(β-cyanoethyl-N,N-diisopropyl)phosphoramidite were purchased from Chemgenes, MA and Glen Research, VA respectively. 5-methyl-5′-*O*-(4,4′-dimethoxytrityl) -2′-*O*-methyl-3′-*O*-succinamido-N6-hexanamido-N3-propyl uridine controlled pore glass (500 Å), CAP-A, CAP-B and acetonitrile as diluent were purchased from Chemgenes, MA. Deblocking reagent (3% trichloroacetic acid in dichloromethane) and the activator 5-ethylthio-1H-tetrazole were bought from Glen Research, VA. The oligonucleotides were synthesized on a Expedite 8909 synthesizer. Solutions of 0.05 M phosphoramidte were prepared in anhydrous acetonitrile. The coupling time was 360 s except for the psoralen phosphoramidite coupling, which was 90 s. TFOs were deprotected as described in RESULTS. The pso-TFOs were purified by HPLC (Shimadzu, LC-10ADVP) using an ion-exchange column (Dionex) and a gradient of 0.0–1.0 M sodium chloride in 0.1 M Tris, pH 7.8 to elute the column. The compositions of the TFOs were verified by MALDI-TOF.

Tm Analyses

Thermal denaturation and renaturation studies were carried out on a Varian CARY-3E spectrophotometer fitted with a thermoregulated sample compartment. The samples were heated at 0.4°C/min and the absorbance was monitored at 260 nm. The cell block was continuously purged with N_2 at low temperature to avoid moisture condensation. All samples (1 μM each strand) were prepared in a buffer containing 100 mM sodium chloride, 50 mM 3-(N-morpholino)propane sulfonic acid (MOPS) and 2.5 mM $MgCl_2$ at pH 7.0. The duplex strands were 5′TCAGAAGAAAAAAGAGAAA and 5′ TTTCTCTTTTTTCTTCTGA. The strands were mixed at room temperature and incubated for 24 h prior to an analysis. Reanealing of the duplex at the same temperature of melting was shown in control experiments. Each analysis was performed two or three times with errors of no more than 0.5°C.

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Cells and *Hprt* Mutagenesis

Chinese hamster ovary cells were grown in Dulbecco's modified Eagle's medium supplemented with penicillin and streptomycin and 10% fetal bovine serum. Prior to an experiment cells were cultured in HAT medium (10^{-4} M hypoxanthine, 5×10^{-6} M aminopterin, 10^{-5} M thymidine) for 1 week to remove pre-existing *Hprt*-deficient cells. Cells were suspended at 10^7/mL in complete medium and Pso-TFOs added to 5 μM. The cell/TFO mixture was then electroporated (Bio-Rad) at a setting of 110 volts, 960 microfarads, followed by incubation at room temperature for 3 h, and exposure in the Rayonet chamber to UVA light for 3 min at 1.8 J/cm^2. The cells were plated in complete medium for 8–10 days with 2–3 passages, and then placed in selective medium depleted of hypoxanthine and containing 20 μM thioguanine (200,000 cells/100-mm dish). Cells were also plated in selective medium without thioguanine to determine plating efficiency. After 10 days resistant colonies were counted.

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