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Synthesis and properties of triplex-forming oligonucleotides containing 2′-*O*-(2-methoxyethyl)-5-(3-aminoprop-1-ynyl)-uridine

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ABSTRACT

2′-*O*-(2-Methoxyethyl)-5-(3-aminoprop-1-ynyl)-uridine phosphoramidite (MEPU) has been synthesized from D-ribose and 5-iodouracil and incorporated into triplex-forming oligonucleotides (TFOs) by automated solid-phase oligonucleotide synthesis. The TFOs gave very high triplex stability with their target duplexes as measured by ultraviolet/fluorescence melting and DNase I footprinting. The incorporation of MEPU into TFOs renders them resistant to degradation by serum nucleases.

1. Introduction

Triplex-forming oligonucleotides (TFOs)[1] have been used as sequence-selective agents for gene-knockout and mutation correction, hence the synthesis of chemically modified TFOs with improved biological activity is potentially important.[2–8] TFOs bind to DNA duplexes to form triplexes by binding in the major groove of the double strand. In the more stable (parallel) version of DNA triple helices, the third strand runs in the same direction as the duplex purine-rich strand to which it binds. The TFO binds to the exposed edges of the base pairs and makes sequence-specific contacts to produce C^+.GC and T.AT triplets. The three-stranded structure is usually less stable than the underlying duplex, and has a requirement for low pH. This is because protonation of cytosine N3 ($pK_a \sim 4.5$) is necessary for formation of a stable C^+.GC triplet.[9,10] Protonated cytosine bases and analogues contribute disproportionally to the stability of parallel triplexes, so to increase triplex stability and restore the balance between the stability of C.GC and T.AT triplets, a number of thymidine analogues have been synthesized for incorporation into TFOs. One successful stabilization strategy has been to incorporate positively charged groups into the TFO to provide favorable interactions with the negatively charged backbone of the target duplex.[11–13] Modification of the 2′-position of the ribose sugar in the TFO is particularly desirable as this greatly reduces degradation of the TFO by nuclease enzymes in vivo. Because of this stabilizing effect, oligonucleotides modified at the 2′-position are of importance in other biological applications, for example as antisense agents or siRNA analogues. Several 2′-*O*-modified nucleoside monomers have been incorporated into TFOs and the effects of the modifications quantified.[14]

The objective of this study was to synthesize a novel analogue of thymidine, 2′-*O*-(2-methoxyethyl)-5-(3-aminoprop-1-ynyl)-uridine (MEPU), designed to stabilize T.AT triplets in triple helices

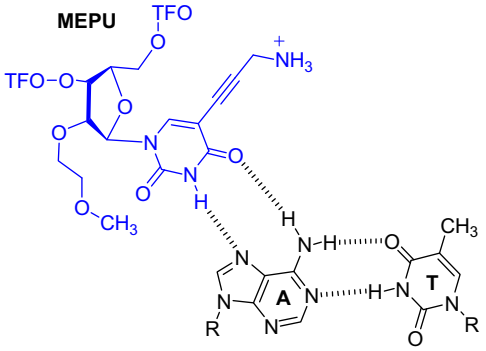

Figure 1. The MEPU-A.T triplet. TFO = triplex-forming oligonucleotide backbone, R = deoxyribose sugar. TFO in blue, duplex in black.

Abbreviations: MEPU, 2′-O-(2-methoxyethyl)-5-(3-aminoprop-1-ynyl)-uridine; TFOs, triplex-forming oligonucleotides; T_m, melting (dissociation) temperature; FCS, fetal calf serum; CEE, *Caenorhabditis elegans* extract.

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(Fig. 1). The amino group is protonated at physiological pH, thus providing a favorable electrostatic interaction with the DNA phosphodiester backbone, the alkyne further stabilizes the triplex through base stacking interactions and the 2′-substituent is designed to confer in vivo stability.[13,15,16]

2. Results and discussion

2.1. Synthesis of 1-*O*-methyl-3,5-di-*O*-benzyl-α-D-ribofuranoside

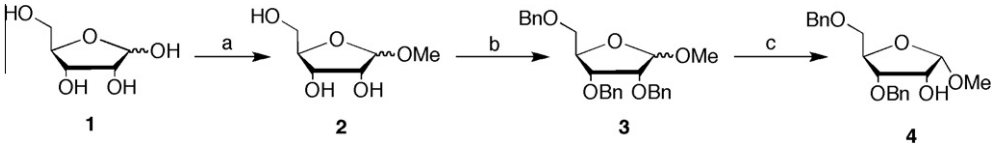
1-*O*-Methyl-3,5-di-*O*-benzyl-α-D-ribofuranoside (**4**) was prepared by minor modifications to the published methods (Scheme 1).[17,18] Firstly, D-ribose was reacted with anhydrous MeOH in the presence of Dowex-50 H^+ resin to produce 1-methyl-D-ribofuranoside **2** in quantitative yield. Benzylation of **2** with benzyl bromide in the presence of NaH gave the protected glycoside **3** (91% yield for two steps). Benzyl protection was chosen because of its stability under the reaction conditions and subsequent ease of removal by hydrogenation. The 2′-*O*-benzyl group of 1-*O*-methyl-2,3,5-tri-*O*-benzyl-D-ribose **3** was selectively deprotected with $SnCl_4$ in DCM to give dibenzyl sugar **4** in 86% yield. The 2′-OH of **4** was then functionalised with a *p*-tolouyl group to facilitate crystallization of compound **14** (Fig. 2). The X-ray structure of **14** confirmed the selectivity of debenzylation of **3** and the α-configuration at the anomeric center. Compound **4** is a potentially useful intermediate in the synthesis of various 2′-*O*-modified nucleosides as it has a single free hydroxyl group which can be readily alkylated prior to coupling the sugar with the appropriate nucleobase.

2.2. Synthesis of 2′-*O*-(2-methoxyethyl)-5-(3-trifluoroacetamidoprop-1-ynyl)-uridine phosphoramidite and incorporation into triplex-forming oligonucleotides

The synthesis of the desired phosphoramidite **13** is illustrated in Scheme 2. To prepare the required 2′-*O*-(2-methoxyethyl) ribose moiety, the 2′-OH group of compound **4** was reacted with bromomethylethyl ether in the presence of NaH in DMF to give compound **5** in 94% yield. This was heated in the presence of palladium hydroxide on carbon in ethanol to yield **6** which was protected at the 3′- and 5′-positions with benzoyl chloride in pyridine to give protected sugar **7** (95% yield for two steps). The benzyl groups on 3 and 5-positions of the sugar had to be removed and replaced with benzoyl before coupling to 5-iodouracil because reduction of the $C^5{=}C^6$ bond of the pyrimidine ring occurs under the conditions of catalytic hydrogenation required for debenzylation. Compound **7** was reacted with acetic anhydride and catalytic conc. sulfuric acid in acetic acid to afford activated sugar **8** (α- and β-anomers, 85% yield) after which 5-iodouracil was coupled to **8** to yield nucleoside **9** exclusively in the desired β-configuration (80% yield). The selectivity is probably due to steric hindrance from the side chain at the 2′-position preventing approach of the uracil base from below the sugar ring.[19] The benzoyl groups on the 5′- and 3′-positions of **9** were removed by reaction with NaOMe in anhydrous MeOH to generate **10** which was protected at the 5′-position with 4,4′-dimethoxytrityl chloride in 98% yield. In order to confirm its stereochemistry, intermediate **10** was also synthesized from uridine (Supplementary data, S1), from which identical spectroscopic properties were obtained. Continuing the synthesis of **13**, the iodine atom on the 5-position of **11** was substituted by 3-trifluoroacetamidoprop-1-yne using Sonogashira chemistry. This strategy allows for the introduction of other functional groups at the 5-position of the pyrimidine base. Finally compound **12** was phosphitylated with 2-cyanoethyl-*N*, *N*-diisopropylchlorophosphine to give phosphoramidite **13** in 91% yield. The overall yield of **13** was 22.4% from D-ribose.

2.3. TFO design, synthesis, and triplex melting studies

Standard solid-phase phosphoramidite methods were used to prepare a series of modified oligonucleotides (Fig. 3) which were used in ultraviolet melting experiments to determine triplex stability. TFO-1 was synthesized as a control without any modifications at thymidine, TFO-2 consists of six 2′-*O*-(2-methoxyethyl)-5-(3-aminoprop-1-ynyl)-uridine (MEPU) units distributed throughout the sequence, while TFO-3 contains two clusters of the modified nucleoside, one of two clusters with two MEPU modifications and the other with four. Theoretically, when clustered, the ability of the 2′-*O*-modified nucleoside to stabilize triplexes might be decreased due to steric hindrance and the high concen-



Scheme 1. Reagents and conditions: (a) Dowex-50 (H^+), methanol, 0 °C, 24 h; (b) benzyl bromide (6.1 equiv), sodium hydride (6.1 equiv), DMF, rt, 14 h, 91% for two steps; (c) 1 M $SnCl_4$/DCM solution (1 equiv), DCM, 0 °C, 24 h, 86%.

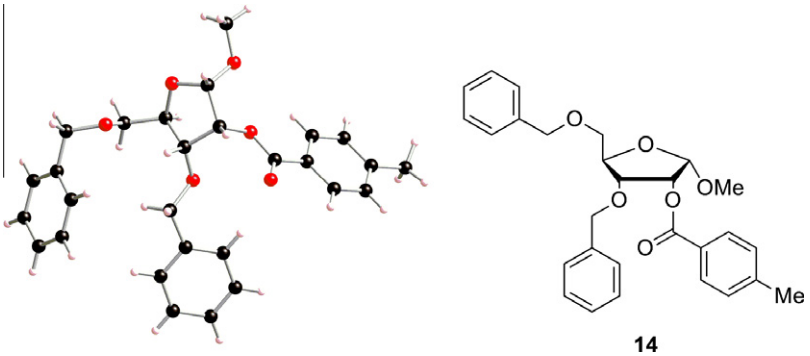
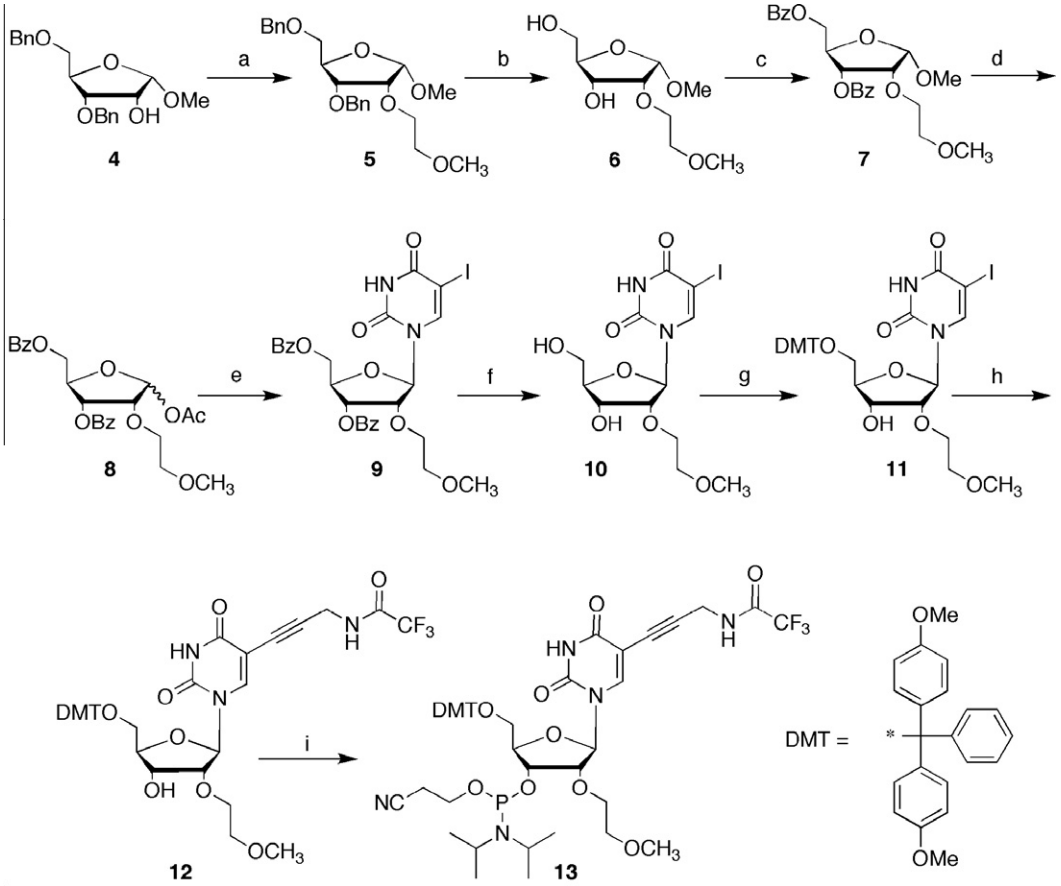


Figure 2. The X-ray crystal structure (left) and the chemical structure (right) of 1-*O*-methyl-2-(*p*-tolouyl)-3,5-di-*O*-benzyl-D-α-ribofuranoside (**14**).



Scheme 2. Reagents and conditions: (a) NaH (3.4 equiv), anhydrous DMF, 0 °C, 1 h; $BrCH_2CH_2OMe$ (6.2 equiv), rt, 8 h, 94%; (b) $Pd(OH)_2$ 20% on carbon (0.1 equiv), H_2, anhydrous EtOH, 50 °C, 3 h; (c) PhCOCl (2.6 equiv), anhydrous pyridine, rt, overnight, 95% for two steps; (d) AcOAc/AcOH (1:1), 1% concd sulfuric acid, rt, 5 h, 85%; (e) 5-iodouracil (1.2 equiv), *N*,*O*-bis(trimethylsilyl)acetamide (5.5 equiv), anhydrous CH_3CN, rt, overnight; **8**, $SnCl_4$ (1.2 equiv), anhydrous CH_3CN, 50 °C, 16 h, 80%; (f) NaOMe (2.8 equiv), anhydrous MeOH, rt, 1.5 h, 68%; (g) DMTCl (1.2 equiv), anhydrous pyridine, rt, 18 h, 98%; (h), 3-trifluoroacetamidoprop-1-yne (1.2 equiv), CuI (0.8 equiv), $(Ph_3P)_4Pd(0)$ (0.07 equiv), Et_3N (10 equiv), anhydrous DMF, rt, overnight, 78%; (i) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphine (1.4 equiv), DIPEA (5 equiv), anhydrous DCM, rt, 3 h, 91%.

```
5'-2T55TT5T5TTTTTT5TT    TFO-1

5'-2X55TX5T5XTXTXT5XT    TFO-2

5'-2T55XX5T5TXXXXT5TT    TFO-3

5'-GTG6TAGGAAGAGAAAAAAGAACTGGTH)
3'-CACAATCCTTCTCTTTTTTCTTGACCAH
```

Figure 3. Oligonucleotide sequences used in ultraviolet and fluorescence melting experiments. In each case the third strand is shown in bold and the duplex target is boxed: H = hexaethylene glycol linker, 2 = dabcyl, 5 = 5-methyldeoxycytidine, 6 = fluorescein dT, X = 5-aminopropynyl-2′-*O*-methoxyethyluridine. Modifications 2 and 6 enable fluorescence melting experiments to be carried out.

Table 1
T_m values (°C) of TFOs and hairpin target measured by UV melting and fluorescence melting (in parentheses)

	pH 6.2	pH 6.6	pH 7.0	pH 7.5	pH 8.0
TFO-1	44.3 (43.9)	38.5 (37.5)	33.3 (—)	34.1	—
TFO-2	71.9 (71.0)	64.6 (62.7)	57.0 (53.2)	43.8	32.5
TFO-3	71.9 (69.8)	62.5 (60.9)	54.5 (50.5)	40.6	29.9

All experiments were performed in 10 mM sodium phosphate (pH 6.2, 6.6, 7.0, 7.5 or 8.0), containing 200 mM NaCl. The concentration of TFOs: target duplex were 3.0 μM:1.0 μM for uv and 2.5 μM:0.25 μM for fluorescence melting. The fluorescence experiments were carried out with very slow melting to eliminate hysteresis, whereas the ultraviolet melting experiments were carried at heating rates typically used in the literature.

tration of mutually repulsive adjacent positive charges in the third strand. In contrast, with the six 2′-*O*-modified nucleosides dispersed, the steric hindrance and concentration of charge would be avoided and triplex-stabilizing capacity maximized.

Melting studies show that as the pH increases, the stability of all the triplexes is reduced markedly, largely due to destabilization of the ^{Me}C.GC triplets, and to a lesser extent as a result of deprotonation of the 5-aminopropynyl group of MEPU (Table 1). It is evident that MEPU greatly enhances triplex stability relative to thymidine. Figure 3A shows UV melting studies at pH 6.6 (melting curves at pH 6.2, 7.0, 7.5, and 8.0 are shown in Supplementary data Fig. S1). At pH 7.0, compared to thymidine, there is on average a 3.9 °C increase in T_m per MEPU residue. This is greater than most previously studied 2′-*O*-modified ribothymidine analogues such as 2′-*O*-(aminoethyl)ribothymidine (+3.5 °C per modification),[20] 2′-*O*-methyl-5-propynyluridine (+3.2 °C), and 2′-*O*-methyl-5-(3-amino-1-prop-1-ynyl)uridine (+3.7 °C).[14,21–23] Stabilization due to MEPU is good, but it is not as effective as 2′-*O*-(aminoethoxy)-5-(3-aminoprop-1-ynyl)uridine (+5.2 °C per modification).[24] However, the latter is more difficult to synthesize. The positioning of the MEPU monomer in the TFO has a small influence on triplex stability; T_m values for TFO-2 are always slightly higher than the corresponding values for TFO-3 (Fig. 4A), which confirms that

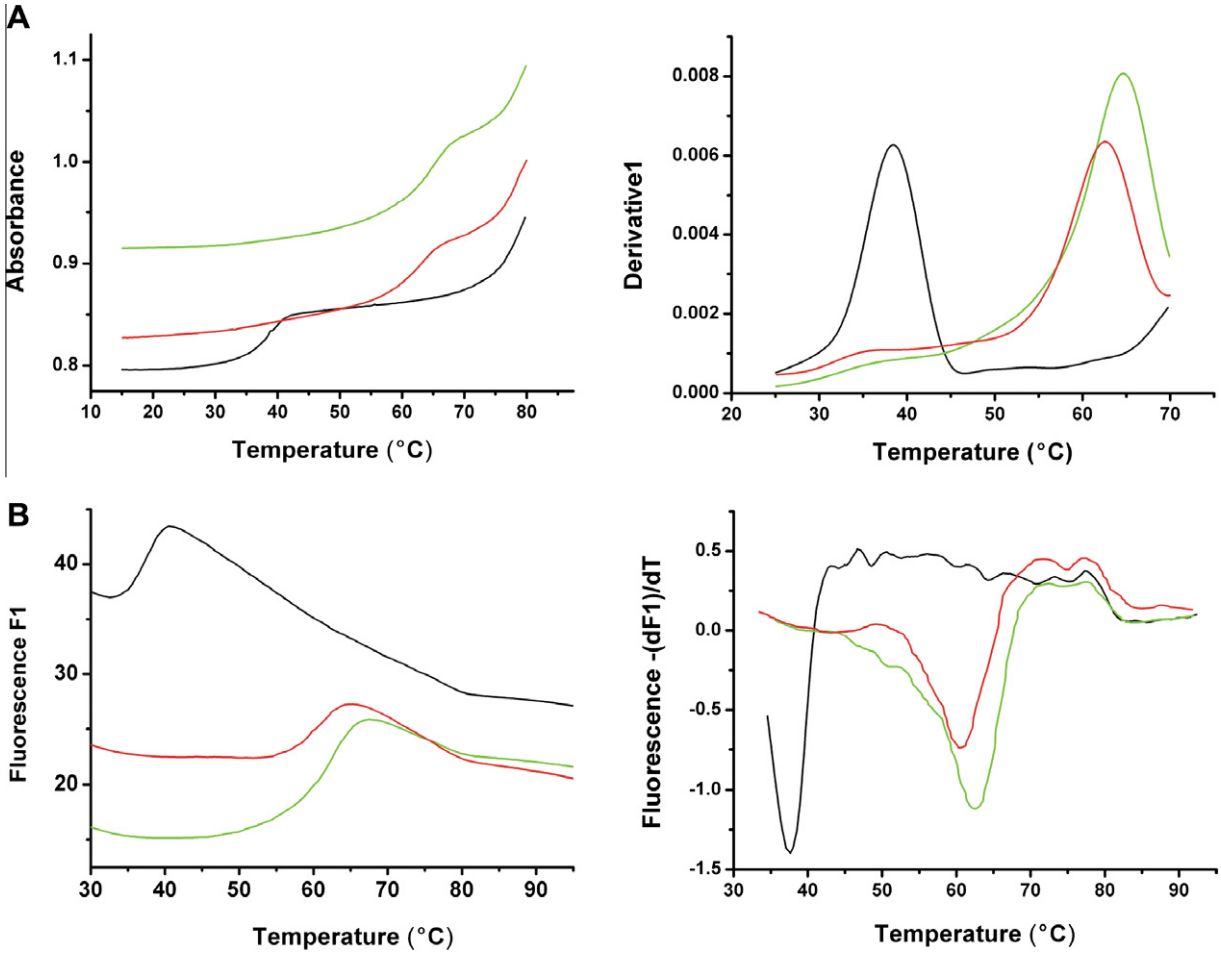


Figure 4. Melting curves (left) and derivatives (right) of TFO-1 (black), TFO-2 (green), TFO-3 (red) with target hairpin duplex at pH 6.6. (A) UV melting. (B) Fluorescence melting. Experiments were performed in 10 mM sodium phosphate pH 6.6 containing 200 mM NaCl. The concentration of TFOs: target duplex were 3.0 μM:1.0 μM for UV melting and 2.5 μM:0.25 μM for fluorescence melting.

dispersed modified bases are slightly more effective than clustered ones.[9,10] The high stability of triplexes containing MEPU was confirmed by fluorescence melting experiments (Table 1, T_m values in parentheses). Figure 4B shows fluorescence melting studies at pH 6.6 (melting curves and derivatives at pH 6.2 and 7.0 are shown in Supplementary data Fig. S3).[25] The fluorescence experiments were carried out with very slow melting to eliminate hysteresis and ensure that the systems are under thermodynamic control, whereas the ultraviolet melting experiments were carried at heating rates typically used in the literature. This difference in heating rate explains the small differences between UV and fluorescence melting temperatures at higher pH. The above ultraviolet and fluorescence melting experiments confirm that MEPU is a potentially useful triplex-stabilizing monomer for in vivo applications.

2.4. DNase I footprinting

We confirmed the ability of oligonucleotides containing MEPU to form stable triplex helices by DNase I footprinting. For these experiments we prepared a target template based on the promoter region of the *unc-22* gene from *Caenorhabditis elegans*, containing the target site GGAAGGAAACAAAA and examined the interaction with 5′-FAM-CCXTCCXTXMXXXT where C is 5-methylcytosine, X is MEPU and M is an *N*-methylpyrrolopyrimidine monomer designed for recognizing CG base pairs.[26] The DNase I footprint is shown in the left hand panel of Figure 5 and reveals the presence of a clear footprint at the intended target site.

2.5. Biological stability

The footprinting TFO, and a control oligonucleotide in which X = thymidine, were incubated in either fetal calf serum (FCS) or an extract prepared from *C. elegans* (CEE), Samples were taken at various times and the products were separated on denaturing polyacrylamide gels. The results are shown in the right hand panels of Figure 5. The control oligonucleotide was rapidly degraded (especially in FCS) while the oligonucleotide containing MEPU remained largely intact, even after 20 h incubation at 37 °C. This confirms the stabilizing effect of the 2-methoxyethyl group.

3. Conclusions

2′-*O*-(2-Methoxyethyl)-5-(3-aminoprop-1-ynyl)-uridine phosphoramidite (MEPU) has been synthesized from D-ribose and 5-iodouracil. The phosphoramidite monomer has been incorporated into triplex-forming oligonucleotides which were shown to produce very stable triplexes as measured by ultraviolet and fluorescence melting studies and DNase I footprinting. The TFOs are also more stable than natural counterparts to enzymatic degradation in serum extracts. The synthetic strategy presented here could be

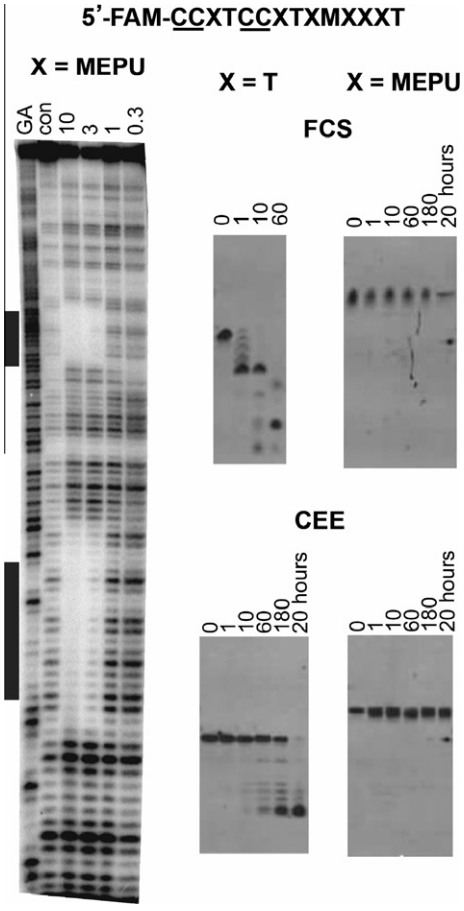


Figure 5. *Left hand panel*: DNase I footprinting in the presence of various concentrations of TFO 5′-FAM-CCXTCCXTXMXXXT (X = MEPU, C = 5-methylcytosine, M = CG recognition monomer. The oligonucleotide concentration (μM) is shown at the top of each gel lane. The track labeled 'GA' is a marker specific for the purines. The locations of the two target sites are indicated by the bars. The upper site shows the purine-containing strand of the target, while the lower site reveals the pyrimidine-containing strand. *Right hand panels*: stability of oligonucleotides (X = T or MEPU) in fetal calf serum (FCS) or *C. elegans* extract (CEE). Samples were removed from the mixture at the times (min) as indicated at the top of each gel lane and separated on 15% polyacrylamide gels containing 8 M urea. Note that the MEPU-containing oligonucleotide has a lower mobility as a result of the additional positive charges.

adapted for the synthesis of other ribonucleoside analogues with substituents on the 2′-position of the sugar.

4. Experimental

4.1. General experimental

All reagents used were purchased from Aldrich, Fluka or Lancaster and used without purification. DNA phosphoramidite monomers, solid supports and additional reagents were purchased from Link Technologies Ltd, Glen Research or Applied Biosystems Ltd. Dichloromethane (DCM), acetonitrile (CH_3CN), *N*,*N*-diisopropylethylamine (DIPEA), triethylamine (Et_3N), and pyridine were distilled over calcium hydride. All reactions were carried out under an argon atmosphere using glassware that had been dried at 120 °C overnight. Column chromatography was carried out under pressure using Fisher scientific DAVSIL 60A 30–70 μ silica. Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F_{254} (0.22 mm thickness, aluminum backed). Compounds were visualized at 254 nm or stained with 10% sulfuric acid in EtOH. ^{1}H NMR spectra were measured at 300 MHz or 400 MHz on a Bruker AV 300 or a Bruker DPX 400 spectrometer. ^{13}C NMR spectra were measured at 75 MHz or 100 MHz on the same spectrometers. Chemical shifts are given in ppm and *J* values are given in Hertz. All assignments for ^{1}H NMR and ^{13}C NMR have been confirmed by H–H COSY, HMQC, and HMBC. ^{31}P NMR spectra were recorded on a Bruker AV 300 spectrometer at 121 MHz. $CDCl_3$ and DMSO-d_6 were used as solvents. The IR spectra were obtained on ThermoNicolet 380 FT-IR spectrometer with Smart Orbit Goldengate attachment. Low resolution mass spectra were recorded in acetonitrile using the electrospray technique on a Fisons VG platform instrument. High resolution mass spectra were recorded in acetonitrile or methanol using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. HPLC grade of MeOH or CH_3CN were used as solvents.

4.1.1. 1-*O*-Methyl-2,3,5-*O*-tri-benzyl-D-ribofuranoside (3)

A solution of D-ribose (5.53 g, 36.8 mmol) in anhydrous MeOH (50 mL) was cooled to 0 °C, Dowex-50w (H^+) was added and the mixture was stirred at 0 °C for 24 h. After filtration, the solvent was removed in vacuo and the residue was dried under high vacuum overnight before dissolving in anhydrous DMF (70 mL). Benzyl bromide (26.75 mL, 225 mmol) was added and the reaction mixture cooled to 0 °C before adding NaH in portions (60% dispersion in mineral oil, 9.0 g, 225 mmol). The reaction mixture was left to stir at room temperature for 14 h after which water (5 mL) was added. The mixture was evaporated to dryness then portioned between DCM (300 mL) and water (150 mL). The organic layer was washed with diluted hydrochloric acid (0.5%, 150 mL × 2), saturated sodium bicarbonate (150 mL), water (150 mL), and brine (150 mL). After drying over sodium sulfate, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (23% ethyl acetate in petroleum ether) to give the title product as an amber oil (14.59 g, β:α = 3:1, 91%). R_f β-anomer: 0.49; α-anomer: 0.30 (ethyl acetate/hexane, 1:2). β-anomer: ^{1}H NMR: (300 MHz, $CDCl_3$) δ ppm 7.38–7.13 (m, 15H, Ar-*H*), 4.84 (s, 1H, C*H*^{1}), 4.60 (d, *J* = 12.10 Hz, 1H, C*H*H), 4.53 (d, *J* = 12.10 Hz, 1H, CH*H*), 4.52 (d, *J* = 12.16 Hz, 1H, C*H*H), 4.48 (m, 1H, CH*H*), 4.47 (d, *J* = 12.14 Hz, 1H, C*H*H); 4.40 (d, *J* = 11.90 Hz, 1H, CH*H*), 4.26 (m, 1H, C*H*), 3.94 (dd, *J* = 6.99, 4.73 Hz, 1H, C*H*), 3.76 (d, *J* = 4.55 Hz, 1H, C*H*^{2}), 3.54 (dd, *J* = 10.60, 3.76 Hz, 1H, C*H*H^5), 3.43 (dd, *J* = 10.60, 5.78 Hz, 1H; CH*H*^{5}), 3.22 (s, 3H, C*H*$_3$). ^{13}C NMR: (75 MHz, $CDCl_3$) δ ppm 138.3 (*C*-Ar), 137.8 (*C*-Ar), 128.4 (CH-Ar), 128.3 (CH-Ar), 128.0 (CH-Ar), 127.9 (CH-Ar), 127.8 (CH-Ar), 127.6 (CH-Ar), 127.5 (CH-Ar), 106.3 (*C*H^1), 80.5 (*C*H), 79.6 (*C*H^2), 78.4 (*C*H), 73.2 (*C*H$_2$), 72.4, 72.3 (*C*H$_2$, *C*H$_2$), 71.3 (*C*H$_2^5$), 55.0 (*C*H$_3$). IR (neat): 3062, 3029, 2911, 1605, 1496, 1453, 1360, 1205, 1103, 1065, 1026, 946, 734, 696 cm^{-1}. *m*/*z* (%) LRMS [ES$^+$, MeCN]: 452.3 ([M+NH$_4$]$^+$, 100%), 891.6 ([2 M+Na]$^+$, 30%). HRMS [ES$^+$]: $C_{27}H_{30}O_5Na$ requires 457.1985 found 457.1979. α-anomer: ^{1}H NMR: (300 MHz, $CDCl_3$) δ ppm 7.25–7.10 (15H, m, C*H*-Ar), 4.91 (d, *J* = 4.10 Hz, 1H, C*H*^{1}), 4.75–4.58 (m, 4H, C*H*$_2$), 4.52 (d, *J* = 12.11 Hz, 1H, C*H*H), 4.45 (d, *J* = 12.10 Hz, 1H, CH*H*), 4.30–4.25 (m, 1H, C*H*), 3.88–3.78 (m, 2H, C*H*), 3.39 (s, 3H, C*H*$_3$), 3.45 (dd, *J* = 10.44, 4.11 Hz, 1H, C*H*H^5), 3.38 (dd, *J* = 10.43, 4.19 Hz, 1H; CH*H*^{5}). ^{13}C NMR: (75 MHz, $CDCl_3$) δ ppm 138.3 (*C*-Ar), 138.0 (*C*-Ar), 137.8 (*C*-Ar), 128.4 (*C*H-Ar), 128.3 (*C*H-Ar), 128.0 (*C*H-Ar), 127.8 (*C*H-Ar), 127.7 (*C*H-Ar), 102.5 (*C*H^1), 82.1 (*C*H), 77.8 (*C*H), 75.0 (*C*H), 73.5 (*C*H$_2$), 72.5 (*C*H$_2$), 72.3 (*C*H$_2$), 70.2 (*C*H$_2^5$), 55.6 (*C*H$_3$). IR (neat): 3062, 3029, 2910, 1605, 1496, 1453, 1360, 1205, 1104, 1026, 911, 853, 734, 696 cm^{-1}. *m*/*z* (%) LRMS [ES$^+$, MeCN]: 457.3 ([M+Na]$^+$, 70%), 891.7 ([2 M+Na]$^+$, 100%). HRMS [ES$^+$]: $C_{27}H_{30}O_5Na$ requires 457.1985 found 457.1988.

4.1.2. 1-*O*-Methyl-3,5-di-*O*-benzyl-α-D-ribofuranoside (4)

A solution of **3** (14.60 g, 33.6 mmol) in anhydrous DCM (200 mL) was cooled to 0 °C before $SnCl_4$/DCM solution (1 M, 33.6 mL, 33.6 mmol) was added dropwise into the mixture. The solution was stirred at 0 °C for 24 h then water (10 mL) was added.

After addition of DCM (200 mL), the organic layer was washed with water (150 mL × 3), diluted hydrochloric acid (0.5%, 150 mL × 2), saturated sodium bicarbonate (150 mL), water (150 mL), and brine (150 mL), then dried over anhydrous sodium sulfate. After filtration, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (23%, ethyl acetate in petroleum ether) to give the product as an amber oil (10.0 g, 86%). R_f 0.33 (ethyl acetate/hexane, 1:2) ^{1}H NMR: (300 MHz, $CDCl_3$) δ ppm 7.49–7.22 (m, 10H, C*H*-Ar), 4.91 (d, *J* = 4.63 Hz, 1H, CH^1), 4.75 (d, *J* = 12.38 Hz, 1H, C*H*H), 4.60 (d, *J* = 12.37 Hz, 1H; CH*H*), 4.54 (d, *J* = 12.11 Hz, 1H, C*H*H), 4.47 (d, *J* = 12.11 Hz, 1H; CH*H*), 4.21–4.09 (m, 2H, C*H*), 3.81 (dd, *J* = 7.10, 3.19 Hz, 1H, CH^3), 3.50 (s, 3H, CH_3), 3.47 (dd, *J* = 10.45, 4.11 Hz, 1H, C*H*H^5), 3.39 (dd, *J* = 10.39, 4.26 Hz, 1H; CHH^5), 2.97 (d, *J* = 11.16 Hz, 1H, O*H*). ^{13}C NMR: (75 MHz, $CDCl_3$) δ ppm 137.9 (*C*-Ar), 137.8 (*C*-Ar), 128.4 (*C*H-Ar), 128.0 (*C*H-Ar), 127.9 (*C*H-Ar), 127.7 (*C*H-Ar), 103.0 (CH^1), 82.0 (*C*H), 76.3 (CH^3), 73.5 (CH_2), 73.0 (CH_2), 71.8 (*C*H), 70.0 (CH_2^5), 55.7 (CH_3). IR (neat): 3334, 3030, 2913, 1496, 1453, 1412, 1361, 1271, 1187, 1090, 1025, 850, 737, 697 cm^{-1}. *m*/*z* (%) LRMS [ES$^+$, MeCN]: 367.2 ([M+Na]$^+$, 100%), 711.5 ([2 M+Na]$^+$, 65%). HRMS [ES$^+$]: $C_{20}H_{24}O_5Na$ requires 367.1516 found 367.1514.

4.1.3. 1-*O*-Methyl-2-*O*-(2-methoxyethyl)-3,5-di-*O*-benzyl-α-D-ribofuranoside (5)

A solution of **4** (6.11 g, 17.7 mmol) in anhydrous DMF (60 mL) was cooled to 0 °C and NaH was added (60% dispersion in mineral oil, 2.40 g, 60.0 mmol). The mixture was stirred at 0 °C for 1 h then 2-bromoethyl methyl ether was added dropwise (10.40 mL, 110.6 mmol). The suspension was stirred at room temperature for 8 h, after which water (5 mL) was added. After the solvent was removed in vacuo, the residue was dissolved in DCM (300 mL) and washed with water (100 mL × 3), diluted hydrochloric acid (0.5%, 150 mL), saturated sodium bicarbonate (150 mL), water (150 mL) and brine (150 mL). After drying over anhydrous sodium sulfate, the DCM was evaporated in vacuo. The residue was purified by silica gel column chromatography (55% ethyl acetate in petroleum ether) to give the product as a pale yellow oil (6.74 g, 94%). R_f 0.22 (ethyl acetate/hexane, 1:1). ^{1}H NMR: (300 MHz, $CDCl_3$) δ ppm 7.39–7.11 (m, 10H, C*H*-Ar), 4.91 (d, *J* = 3.73 Hz, 1H, CH^1), 4.63 (d, *J* = 12.72 Hz, 1H, C*H*H), 4.51 (d, *J* = 12.71 Hz, 1H; CH*H*), 4.45 (d, *J* = 12.13 Hz, 1H, C*H*H), 4.38 (d, *J* = 12.13 Hz, 1H; CH*H*), 4.16 (dd, *J* = 6.80, 4.08 Hz, 1H, CH^4), 3.82–3.74 (m, 2H, CH^3, CH^2), 3.66–3.59 (m, 2H, CH_2), 3.56–3.50 (m, 2H, CH_2), 3.39 (s, 3H, CH_3), 3.41–3.27 (m, 2H, CH_2^5), 3.30 (s, 3H, CH_3). ^{13}C NMR: (75 MHz, $CDCl_3$) δ ppm 138.4 (*C*-Ar), 138.0 (*C*-Ar), 128.4 (*C*H-Ar), 128.2 (*C*H-Ar), 127.7 (*C*H-Ar), 127.6 (*C*H-Ar), 102.4 (*C*H^1), 82.0 (*C*H^4), 79.7 (*C*H), 75.0 (*C*H), 73.5 (CH_2), 72.3 (CH_2), 72.1 (CH_2), 70.2 (CH_2^5), 70.1 (CH_2), 59.1 (CH_3), 55.4 (CH_3). IR (neat): 3029, 2911, 1604, 1496, 1453, 1243, 1106, 1025, 852, 736, 697 cm^{-1}. *m*/*z* (%) LRMS [ES$^+$, MeCN]: *m*/*z* 425.3 ([M+Na]$^+$, 100%), 827.6 ([2 M+Na]$^+$, 45%). HRMS [ES$^+$]: $C_{23}H_{30}O_6Na$ requires 425.1935 found 425.1942.

4.1.4. 1-*O*-Methyl-2-*O*-(2-methoxyethyl)-α-D-ribofuranoside (6)

Palladium hydroxide (1.30 g, 3.0 mmol, 20% on activated carbon) was added to a solution of **5** (13.28 g, 33.0 mmol) in anhydrous EtOH (75 mL). The suspension was stirred under a hydrogen atmosphere at 50 °C for 3 h. After filtration, the solvent was removed in vacuo and the residue was used in next step without any further purification. R_f 0.11 (ethyl acetate). *m*/*z* (%) LRMS [ES$^+$, MeCN]: 245.2 ([M+Na]$^+$, 100%).

4.1.5. 1-*O*-Methyl-2-*O*-(2-methoxyethyl)-3,5-di-*O*-benzoyl-α-D-ribofuranoside (7)

Benzoyl chloride (10.0 mL, 86.1 mmol) was added dropwise to a solution of the crude product **6** (7.80 g) in anhydrous pyridine (70 mL). The mixture was stirred at room temperature overnight. After removal of the solvent in vacuo the residue was dissolved in DCM (200 mL) and washed with water (150 mL × 2), diluted hydrochloric acid (0.5%, 150 mL), saturated sodium bicarbonate (150 mL), water (150 mL), and brine (150 mL). After drying over anhydrous sodium sulfate, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (40% ethyl acetate in hexane) to give the product as a colorless oil (13.50 g, 95% for two steps). R_f 0.64 (ethyl acetate/hexane, 3:1). ^{1}H NMR: (400 MHz, $CDCl_3$) δ ppm 8.03 (dd, *J* = 8.35, 1.29 Hz, 2H, C*H*-Ar), 7.97 (dd, *J* = 8.32, 1.22 Hz, 2H, C*H*-Ar), 7.52–7.46 (m, 2H, C*H*-Ar), 7.40–7.33 (m, 4H, C*H*-Ar), 5.44 (dd, *J* = 6.73, 2.93 Hz, 1H, CH^3), 5.03 (d, *J* = 4.37 Hz, 1H, CH^1), 4.58 (dd, *J* = 13.12, 4.76 Hz, 1H, C*H*H^5), 4.52–4.45 (m, 2H, CHH^5, CH^4), 4.12 (dd, *J* = 6.73, 4.40 Hz, 1H, CH^2), 3.72–3.64 (m, 2H, CH_2), 3.45 (s, 3H, CH_3), 3.48–3.36 (m, 2H, CH_2), 3.17 (s, 3H, CH_3). ^{13}C NMR: (100 MHz, $CDCl_3$) δ ppm 166.2 (*C*=O), 166.0 (*C*=O), 133.1, 129.9, 129.7, 129.5, 128.3, 128.2 (*C*H-Ar, *C*-Ar), 102.8 (CH^1), 80.7 (CH^4), 79.7 (CH^2), 72.8 (CH_2), 71.3 (CH_2), 71.0 (CH^3), 64.8 (CH_2^5), 59.3 (CH_3), 55.9 (CH_3). IR (neat): 2927, 1716, 1601, 1584, 1491, 1451, 1375, 1266, 1176, 1111, 1067, 1024, 953, 855, 708, 687 cm^{-1}. *m*/*z* (%) LRMS [ES$^+$, MeCN]: 453.3 ([M+Na]$^+$, 90%), 883.7 ([2 M+Na]$^+$, 100%). HRMS [ES$^+$]: $C_{23}H_{26}O_8Na$ requires 453.1520 found 453.1518.

4.1.6. 1-*O*-Acetyl-2-*O*-(2-methoxyethyl)-3,5-di-*O*-benzoyl-D-ribofuranoside (8)

Concentrated sulfuric acid (0.3 mL) was added with care to a solution of **7** (3.00 g, 6.97 mmol) in acetic anhydride and acetic acid (1:1, 30 mL). The solution was stirred at room temperature for 5 h, after which water (30 mL) was added, followed by sodium bicarbonate (solid) to adjust the pH to 7. The mixture was extracted with DCM (50 mL × 2) and the organic layer was washed with water (30 mL × 2) and brine (30 mL). After drying over anhydrous sodium sulfate, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (50% ethyl acetate in hexane) to give the title product as a colorless oil (2.71 g, 85%). R_f 0.80 (ethyl acetate/hexane, 3:1). *m*/*z* (%) LRMS [ES$^+$, MeCN]: 481.3 ([M+Na]$^+$, 90%), 939.7 ([2 M+Na]$^+$, 100%). HRMS [ES$^+$]: $C_{24}H_{26}O_9Na$ requires 481.1469 found 481.1470.

4.1.7. 2′-*O*-(2-Methoxyethyl)-3′,5′-di-*O*-benzoyl-5-iodouridine β-anomer (9)

5-Iodouracil (0.31 g, 1.30 mmol) was suspended in anhydrous CH_3CN (15 mL), *N*,*O*-bis(trimethylsilyl)acetamide (1.47 mL, 6.01 mmol) was added and the mixture was stirred at room temperature for overnight. The reaction mixture became colorless and was then cooled to 0 °C before a solution of **8** (0.50 g, 1.09 mmol) in anhydrous CH_3CN (5 mL) and $SnCl_4$ (0.15 mL, 1.28 mmol) were added dropwise. After 20 min, the reaction mixture was then heated to 50 °C and stirred for 16 h. DCM (100 mL) was added and the solution was washed with water (60 mL × 3), diluted hydrochloric acid (0.5%, 20 mL), saturated sodium bicarbonate (20 mL), water (20 mL), brine (20 mL) and dried over anhydrous sodium sulfate. After filtration, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (60% ethyl acetate in hexane) to afford the title product as a white foam (0.56 g, 80%). R_f 0.36 (ethyl acetate/hexane, 1:1). ^{1}H NMR: (400 MHz, DMSO-d_6) δ ppm 11.95 (s, 1H, NH^3), 8.28 (s, 1H, CH^6), 8.17–8.10 (m, 4H, C*H*-Ar), 7.84–7.75 (m, 2H, C*H*-Ar), 7.70–7.62 (m, 4H, C*H*-Ar), 6.02 (d, *J* = 5.45 Hz, 1H, C$H^{1'}$), 5.72 (dd, *J* = 5.65, 4.86 Hz, 1H, C*H*), 4.77–4.68 (m, 4H, C*H*, C*H*, C$H_2^{5'}$), 3.81–3.65 (m, 2H, CH_2), 3.46–3.40 (m, 2H, CH_2), 3.17 (s, 3H, CH_3). ^{13}C NMR: (100 MHz, DMSO-d_6) δ ppm 166.8 (*C*=O), 166.2 (*C*=O), 161.7 (*C*=O), 151.4 (*C*=O), 146.7 (CH^6), 134.9, 130.7, 130.6, 130.5, 130.1, 130.0 (*C*-Ar, *C*H-Ar), 89.2 (C$H^{1'}$), 79.5 (*C*H), 79.4 (*C*H), 72.0

(CH_2), 71.6 (CH), 70.4 (CH_2), 64.4 ($CH_2^{5'}$), 58.6 (CH_3). *m*/*z* (%) LRMS [ES^+, MeCN]: 659.4 ([M+Na]$^+$, 100%). HRMS [ES^+]: $C_{26}H_{25}IN_2O_9Na$ requires 659.0502 found 659.0491.

4.1.8. 2′-*O*-(2-Methoxyethyl)-5-iodouridine (10)

NaOMe (0.20 g, 3.70 mmol) was added to a solution of **9** (0.83 g, 1.31 mmol) in anhydrous MeOH (15 mL) and was stirred at room temperature for 1.5 h under argon. Dowex-50w (H^+) was added and the mixture was stirred for 15 min at room temperature. The resin was removed by filtration and washed extensively with MeOH. The filtrate was then reduced to dryness in vacuo and the residue was purified by silica gel column chromatography (10% MeOH in ethyl acetate) to give the title product as a white foam (0.38 g, 68%). R_f 0.52 (ethyl acetate/MeOH, 10:1). ^{1}H NMR: (400 MHz, DMSO-d_6) δ ppm 11.74 (s, 1H, N*H*^{3}), 8.57 (s, 1H, C*H*^{6}), 5.84 (d, *J* = 4.08 Hz, 1H, C*H*$^{1'}$), 5.34 (t, *J* = 4.60, 4.60 Hz, 1H, 5′-O*H*), 5.06 (d, *J* = 6.01 Hz, 1H, 3′-O*H*), 4.16 (m, 1H, C*H*$^{3'}$), 4.02 (t, *J* = 4.51, 4.51 Hz, 1H, C*H*$^{2'}$), 3.95–3.91 (m, 1H, C*H*$^{4'}$), 3.78–3.72 (m, 3H, CH_2, C*H*H$^{5'}$), 3.67–3.59 (m, 1H, CH*H*$^{5'}$), 3.53–3.49 (m, 2H, CH_2), 3.29 (s, 3H, CH_3). ^{13}C NMR: (100 MHz, DMSO-d_6) δ ppm 160.9 (*C*═O), 150.6 (*C*═O), 145.3 (*C*H^6), 87.2 (*C*H$^{1'}$), 85.2 (*C*H$^{4'}$), 82.3 (CH$^{2'}$), 71.7 (CH_2), 69.5 (CH_2), 68.4 (*C*H$^{3'}$), 60.2 ($CH_2^{5'}$), 58.6 (CH_3). *m*/*z* (%) LRMS [ES^+, MeCN]: 451.1 ([M+Na]$^+$, 100%), 879.4 ([2 M+Na]$^+$, 22%). HRMS [ES^+]: $C_{12}H_{17}IN_2O_7Na$ requires 450.9973 found 450.9972.

4.1.9. 2′-*O*-(2-Methoxyethyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-iodouridine (11)

4,4′-Dimethoxytrityl chloride (0.14 g, 0.42 mmol) in anhydrous pyridine (3 mL) was added dropwise to a solution of **10** (0.15 g, 0.35 mmol) in anhydrous pyridine (5 mL). The reaction mixture was stirred at room temperature for 18 h then MeOH (5 mL) was added to quench the reaction. After removing the solvent in vacuo, the residue was purified by silica gel column chromatography (65% ethyl acetate in hexane with 2.5% pyridine) to give the title product as a white foam (0.250 g, 98%). R_f 0.41 (ethyl acetate/hexane, 3:1). ^{1}H NMR: (400 MHz, DMSO-d_6) δ ppm 11.83 (s, 1H, N*H*^{3}), 8.05 (s, 1H, C*H*^{6}), 7.43–7.25 (m, 9H, C*H*-Ar), 6.96 (d, *J* = 8.82 Hz, 4H, C*H*-Ar), 5.85 (d, *J* = 4.33 Hz, 1H, C*H*$^{1'}$), 5.15 (d, *J* = 5.81 Hz, 1H, 3′-O*H*), 4.27–4.15 (m, 2H, C*H*$^{3'}$, C*H*$^{2'}$), 4.06–4.00 (m, 1H, C*H*$^{4'}$), 3.80 (s, 6H, CH_3), 3.83–3.70 (m, 2H, CH_2), 3.46 (dd, *J* = 5.62, 3.93 Hz, 2H, CH_2), 3.29 (s, 3H, CH_3), 3.40–3.19 (m, 2H, $CH_2^{5'}$). ^{13}C NMR: (100 MHz, DMSO-d_6) δ ppm 160.9, 150.6 (C^4, C^2), 144.8 (*C*H^6), 130.2 (*C*H-Ar), 128.4 (*C*H-Ar), 128.1 (*C*H-Ar), 127.2 (*C*H-Ar), 124.4 (*C*H-Ar), 113.8 (*C*H-Ar), 87.8 (*C*H$^{1'}$), 83.7 (*C*H$^{4'}$), 81.4 (*C*H), 71.9 (CH_2), 69.7 (CH_2), 69.1 (*C*H), 63.7 ($CH_2^{5'}$), 58.7 (CH_3), 55.5 (CH_3). *m*/*z* (%) LRMS [ES^+, MeCN]: 753.4 ([M+Na]$^+$, 30%). HRMS [ES^+]: $C_{33}H_{35}IN_2O_9Na$ requires 753.1279 found 753.1261.

4.1.10. 2′-*O*-(2-Methoxyethyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-(3-trifluoroacetamidoprop-1-ynyl)-uridine (12)

Copper iodide (0.13 g, 0.68 mmol) was added to a solution of **11** (0.62 g, 0.84 mmol) in anhydrous DMF (15 mL) in a 50 mL round bottom flask, followed by *N*-propargyltrifluoroacetamide (0.15 g, 0.99 mmol) and distilled triethylamine (1.2 mL, 8.6 mmol). The flask was covered by aluminum foil to exclude light. After stirring at room temperature for 20 min, tetrakis(triphenylphosphine)-palladium (0) (0.07 g, 0.06 mmol) was added and the reaction was stirred under argon at room temperature overnight. The mixture was reduced to dryness in vacuo, the residue was purified by silica gel column chromatography (70% ethyl acetate in hexane with 2.5% pyridine) to afford the product as a white foam (0.50 g, 78%). R_f 0.21 (ethyl acetate/hexane, 3:1). ^{1}H NMR: (400 MHz, DMSO-d_6) δ ppm 11.78 (s, 1H, N*H*^{3}), 10.02 (t, *J* = 5.31, 5.31 Hz, 1H, N*H*), 7.99 (s, 1H, C*H*^{6}), 7.51–7.24 (m, 9H, C*H*-Ar), 6.98–6.92 (m, 4H, C*H*-Ar), 5.82 (d, *J* = 4.28 Hz, 1H, C*H*$^{1'}$), 5.18 (d, *J* = 6.38 Hz, 1H, 3′-O*H*), 4.29–4.23 (m, 1H, C*H*$^{3'}$), 4.21–4.17 (m, 1H, C*H*$^{2'}$), 4.08–4.02 (m, 3H, CH_2NH, C*H*$^{4'}$), 3.80 (s, 6H, CH_3), 3.81–3.73 (m, 2H, CH_2), 3.53 (t, *J* = 4.75, 4.75 Hz, 2H, CH_2), 3.41–3.37, 3.21–3.13 (m, 2H, $CH_2^{5'}$), 3.29 (s, 3H, CH_3). ^{13}C NMR: (100 MHz, DMSO-d_6) δ ppm 167.8 (*C*═O), 158.6 (*C*═O), 150.1 (*C*═O), 144.3 (*C*H^6), 130.2 (*C*H-Ar), 130.1 (*C*H-Ar), 128.4 (*C*H-Ar), 128.0 (*C*H-Ar), 127.1 (*C*H-Ar), 113.7 (*C*H-Ar), 88.2 (*C*H$^{1'}$), 83.6 (*C*H$^{4'}$), 81.4 (*C*H$^{2'}$), 71.8 (CH_2), 69.7 (CH_2), 69.1 (*C*H$^{3'}$), 63.5 ($CH_2^{5'}$), 58.6 (CH_3), 55.5 (CH_3), 29.8 (CH_2NH). *m*/*z* (%) LRMS [ES^+, MeCN]: 776.5 ([M+Na]$^+$, 100%). HRMS [ES^+]: $C_{38}H_{38}F_3N_3O_{10}Na$ requires 776.2402 found 776.2408.

4.1.11. 2′-*O*-(2-Methoxyethyl)-3′-*O*-(2-cyanoethyl(diisopropylamino)phosphanyl)-5′-*O*-(4,4′-dimethoxytrityl)-5-(3-trifluoroacetamidoprop-1-ynyl)-uridine (13)

N,*N*-Diisopropylethylamine (0.57 mL, 3.33 mmol) was added to a solution of **12** (0.50 g, 0.66 mmol) in anhydrous DCM (15 mL), followed by *N*,*N*-diisopropylethylamine (0.57 mL, 3.33 mmol) and 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphine (0.20 mL, 0.89 mmol). The mixture was stirred under argon for 3 h at room temperature then saturated potassium chloride and anhydrous DCM (50 mL) were added. The organic layer was separated under an argon atmosphere and the solvent was removed in vacuo. The residue was purified under an argon atmosphere by silica gel column chromatography (50% ethyl acetate in hexane with 2.5% triethylamine) to yield the product as a white foam (0.57 g, 91%). R_f 0.45, 0.57 (ethyl acetate/hexane, 3:1). ^{1}H NMR: (400 MHz, DMSO-d_6) δ ppm 11.89–11.67 (m, 1H, N*H*^{3}), 10.09–9.96 (m, 1H, N*H*), 8.06 (s, 1H, C*H*^{6}), 7.53–7.26 (m, 9H, C*H*-Ar), 6.98–6.91 (m, 4H, C*H*-Ar), 5.85–5.79 (m, 1H, C*H*$^{1'}$), 4.57–4.29 (m, 2H, C*H*$^{3'}$, C*H*$^{2'}$), 4.23–4.12 (m, 1H, C*H*$^{4'}$), 4.10–3.98 (m, 2H, CH_2NH), 3.79, 3.80 (s, 6H, CH_3), 3.92–3.46 (m, 2H, C*H*), 3.92–3.46 (m, 6H, CH_2), 3.45–3.38 (m, 1H, C*H*H$^{5'}$), 3.26–3.17 (m, 1H, CH*H*$^{5'}$), 3.28, 3.27 (s, 3H, CH_3), 2.89–2.62 (m, 2H, CH_2), 1.38–0.93 (m, 12H, CH_3). ^{13}C NMR: (100 MHz, DMSO-d_6) δ ppm 167.8 (*C*═O), 158.6 (*C*═O), 150.1 (*C*═O), 144.8 (*C*H^6), 130.2 (*C*H-Ar), 130.1 (*C*H-Ar), 128.4 (*C*H-Ar), 128.0 (*C*H-Ar), 127.1 (*C*H-Ar), 113.7 (*C*H-Ar), 89.2 (*C*H$^{1'}$), 83.6 (*C*H$^{4'}$), 80.3 (*C*H$^{2'}$), 71.9 (CH_2), 71.8 (CH_2), 70.5 (*C*H$^{3'}$), 63.8 ($CH_2^{5'}$), 58.9 (CH_2), 58.6 (CH_3), 55.5 (CH_3), 29.8 (CH_2NH), 24.8 (CH_3), 20.3 (CH_2). ^{31}P NMR (121 MHz, DMSO-d_6): δ ppm 150.28, 149.81. *m*/*z* (%) LRMS [ES^+, MeCN]: 976.8 ([M+Na]$^+$, 100%). HRMS [ES^+]: $C_{47}H_{55}F_3N_5O_{11}PNa$ requires 976.3480 found 976.3481.

4.2. Oligonucleotide synthesis purification and analysis

Oligonucleotide synthesis was carried out on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 0.2 μmol or 1.0 μmol phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous CH_3CN to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 25 s and this was extended to 6 min for all modified monomers. Stepwise coupling efficiencies and overall yields were determined by automated trityl cation conductivity monitoring and in all cases were >98.0%. The oligonucleotides attached to the synthesis columns were treated with 20% diethylamine in acetonitrile for 20 min then washed with acetonitrile (5 × 1 mL). This procedure removes cyanoethyl groups from the phosphotriesters and scavenges the resultant acrylonitrile, preventing cyanoethyl adducts being formed at the primary amines of MEPU. After this, cleavage of oligonucleotides from the solid support and deprotection were achieved by exposure to concentrated aqueous ammonia for 12 h at room temperature. Purification of oligonucleotides was carried out by reversed-phase HPLC on a Gilson system using a Brownlee Aquapore column (C8, 8 mm × 250 mm, 300 Å pore) with a gradient of CH_3CN in NH_4OAc increasing from 0% to 50% buffer B over 30 min with a flow rate of

4 mL/min (buffer A: 0.1 M NH_4OAc, pH 7.0; buffer B: 0.1 M NH_4OAc with 50% CH_3CN, pH 7.0). Elution of oligonucleotides was monitored by ultraviolet absorption at 298 nm (or 310 nm for TFOs containing MEPU). After HPLC purification, oligonucleotides were desalted using NAP-10 Sephadex columns (GE Healthcare) according to the manufacturer's instructions.

TFOs were analysed by negative mode electrospray MS on a Fisons VG platform spectrometer in water with triisopropylamine (0.02%). Hairpin duplexes were analysed by MALDI-TOF using a ThermoBioAnalysis Dynamo MALDI-TOF spectrometer in positive ion mode.[27]

4.3. Ultraviolet melting studies

To determine triplex melting temperatures (T_m), UV melting studies were carried out on a Varian Cary 400 scan UV–vis spectrophotometer using Hellma SUPRASIL synthetic quartz 10 mm path length cuvettes, monitoring at 280 nm with a DNA duplex concentration of 1.0 μM and a volume of 1.2 mL. Samples were prepared as follows: The third strand and the duplex were mixed in a 3:1 ratio in 2 mL Eppendorf tubes then lyophilized before suspending in 1.2 mL of the appropriate buffer solution (10 mM sodium phosphate, pH 6.2, 6.6, 7.0, 7.5 or 8.0 containing 200 mM NaCl). The samples were then filtered into the cuvettes with Kinesis regenerated cellulose 13 mm, 0.45 μm syringe filters. The UV melting protocol involved initial denaturation by heating to 80 °C at 10 °C/min followed by annealing by cooling to 15 °C at 0.5 °C/min, then maintaining at 15 °C for 20 min before starting the melting experiment which involved heating from 15 °C to 80 °C at 0.5 °C/min, holding at 80 °C for 2 min then cooling to 15 °C at 0.5 °C/min. Two successive melting curves were measured before fast annealing from 80 °C to 20 °C at 10 °C/min. T_m values were calculated using Cary Win UV thermal application software, taking an average of the two melting curves.

4.4. Fluorescence melting studies

Fluorescence melting studies were conducted on a Roche LightCycler 1.5 instrument with 10 mM phosphate buffer solution at pH 6.2, 6.6 or 7.0 containing 200 mM NaCl. Melting experiments were performed in a total reaction volume of 20 μL which contained 0.25 μM duplex and 2.5 μM third strand. These complexes were first denatured by rapidly heating to 95 °C at 20 °C/s and left to equilibrate for 5 min. They were then slowly cooled to 30 °C at 0.5 °C/s in 0.5 °C steps (each step held for 80 s) and held at 30 °C for a further 1 h. The complexes were heated again to 95 °C at 0.5 °C/s in 0.5 °C steps (each step held for 80 s). Samples were maintained at 95 °C for 5 min before annealing by cooling to 30 °C at the same slow rate as above. After they were held at 30 °C for another 1 h, these complexes were then melted again by heating to 95 °C at the same rate as the same steps. Two successive melting curves were recorded before fast annealing from 95 °C to 30 °C at 20 °C/s. Recordings were taken during both the melting steps as well as during annealing. The LightCycler has one excitation source (480 nm) and fluorescence emission was recorded at 525 nm. T_m values were calculated using Roche LightCycler software, taking an average of the two melting curves. This slow melting and annealing procedure was designed to eliminate hysteresis ands produce reliable melting temperatures under thermodynamic control.

4.5. DNase I footprinting

The target sequence for the DNase I footprinting experiments with the TFOs was prepared by ligating the sequence 5′-GATCAAAAACAAAGCTGGAAGGAAACAAAACAACTGTATGC (TFO target site underlined) into the BamHI site of pUC19. The resulting clone contained a dimer of this sequence with the two copies oriented in opposite directions, enabling simultaneous visualization of the pyrimidine (lower site) and purine (upper site) strands. The radiolabelled footprinting fragment was prepared by digesting the plasmid with EcoRI and HindIII and labeling the insert at the 3′-end of the EcoRI site with α-[^{32}P]-dATP using reverse transcriptase. DNase I digestion was performed as previously described.[28] Radiolabelled DNA (1.5 μL) was incubated overnight with 3 μL oligonucleotide (dissolved in 50 mM sodium acetate pH 5.0 containing 2.5 mM $MgCl_2$). Samples were digested with DNase I (0.01 units/mL, dissolved in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$) for 2 min before stopping the reaction by adding 4 μL of formamide containing 10 mM EDTA. The products of the reaction were separated on 10% polyacrylamide gels containing 8 M urea. The gel was fixed, dried and exposed to a phosphorimager screen overnight.

4.6. Serum stability studies

Oligonucleotides (2–4 μg) were incubated with FCS at 37 °C or *C. elegans* extract (2.75 mg/mL protein) at 20 °C in a total volume of 100 μL. For the *C. elegans* extract, adult worms were cultured[29] and washed off plates with M9 buffer (KH_2PO_4 22 mM, Na_2HPO_4 42 mM, NaCl 85 mM, $MgSO_4$ 1 mM), spun at 2000 rpm for 2 min, washed again to remove bacteria and spun as before. The worms were resuspended in 1.5 mL of M9 buffer, cooled on ice and sonicated on ice for a total of 2 min in 5 s bursts, separated by 10 s. This was then spun at 13,000 rpm for 2 min, the supernatant was carefully removed, and the protein concentration was determined from the absorbance at 280 nm. 15 μL samples were removed at various times and the reaction stopped by adding 7.5 μL of formamide containing 10 mM EDTA and bromophenol blue. The products of the reaction were separated on 15% polyacrylamide gels containing 8 M urea. The gels were visualized under UV light observing the fluorescence of the attached 5′-FAM.

4.7. Crystallographic data

Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC 770522. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

Data were collected on a Bruker Nonius KappaCCD with a Mo rotating anode generator; standard data collection and processing procedures were followed. Absolute structure was not determined experimentally but inferred from the synthetic procedure.

Crystal data for 1-*O*-methyl-2-(*p*-tolouyl)-3,5-di-*O*-benzyl-D-α-ribofuranoside: M = 462.52, Monoclinic, a = 10.5849(6), b = 4.9348(2), c = 22.8500(12) Å, β = 91.029(2)°, U = 1193.36(10) Å^3, T = 120(2) K, space group *P2(1)*, Z = 2, 9138 reflections measured, 2360 unique reflections (R_{int} = 0.0668). The final $R1$ values were 0.0480 ($I>2\sigma(I)$). The final $wR(F_2)$ values were 0.1016 ($I>2\sigma(I)$). The final R_1 values were 0.0676 (all data). The final $wR(F_2)$ values were 0.1108 (all data).

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Supplementary data

Supplementary data (alternative synthesis of 2′-*O*-(2-methoxyethyl)-5-iodouridine, UV and fluorescence melting curves and derivatives of TFO-1-3 and target hairpin duplex at different pH values) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.005.

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