A Dual-App Nucleoside Probe Provides Structural Insights into the Human Telomeric Overhang in Live Cells

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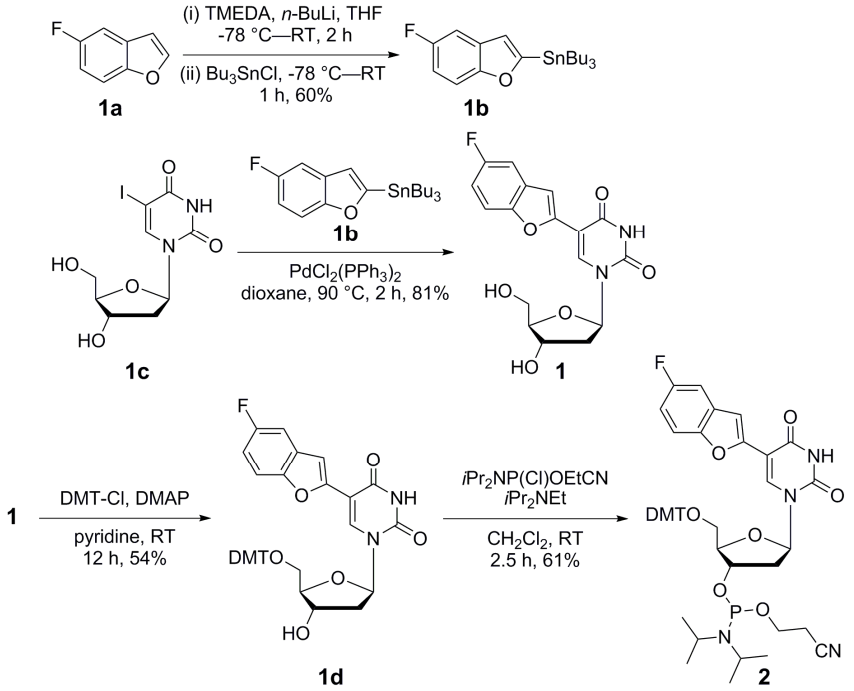
Supporting Information

1. Materials. *N,N,N′,N′*-tetramethylethylenediamine, *n*-butyllithium, tributyltin chloride, *bis*(triphenylphosphine)-palladium(II) dichloride, 5-iodo-2′-deoxyuridine, 4,4′-dimethoxytrityl chloride, *N,N*-diisopropylethylamine, pyridostatin trifluoroacetate salt, BRACO19 hydrochloride, progesterone, PEG 200, Ficoll 400 and all reagents (Bio-Ultra grade) used for buffer preparation were purchased from Sigma-Aldrich. 5-fluorobenzofuran (**1a**) was synthesized following a previously reported procedure[S1]. 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite was purchased from Alfa Aesar. *N*-benzoyl-protected dA, *N*,*N*-dimethylformamide-protected dG, *N*-acetyl-protected dC and dT phosphoramidite substrates for solid-phase synthesis were procured from Proligo Reagents. Solid supports for DNA synthesis were obtained from Glen Research. All other reagents, required for solid-phase oligonucleotide (ON) synthesis were purchased either from ChemGenes Corporation or from Sigma-Aldrich. DNA ONs **6**, **7**, **10** and **11** were purchased from Integrated DNA Technology, and purified by denaturing polyacrylamide gel electrophoresis (PAGE). ONs were eluted and desalted using Sep-Pak Classic C18 cartridges (Waters Corporation). Amicon Ultra (0.5 mL, 3K) centrifugal filters were obtained from Merck Millipore. Autoclaved Millipore water was used in all biophysical analysis.

2. Instruments. Modified ONs were synthesized on a ABI applied Biosystems 392 DNA/RNA synthesizer. Mass measurements were conducted either on an Applied Biosystems 4800 Plus MALDI TOF/TOF analyzer or on a Water Synapt G2 High Definition mass spectrometers. HPLC analysis was done using Agilent Technologies 1260 Infinity HPLC. Reverse-phase flash chromatographic (C18 RediSepRf column) purifications were carried out using Teledyne ISCO, Combi Flash Rf. Absorption spectra were recorded on a Shimadzu UV-2600 spectrophotometer. Steady-state and time-resolved fluorescence spectra

were recorded on a TCSPC Fluorolog-3 fluorescence spectrometer (Horiba Jobin Yvon). UV-thermal melting analysis of ONs was carried out on Cary 300 Bio UV-Vis spectrophotometer. CD analysis was performed on a JASCO J-815 CD spectrometer. NMR spectra of small molecules were recorded on a Bruker AVANCE III HD ASCEND 400 MHz spectrometer and processed using Mnova software from Mestrelab Research. NMR spectra of ONs were recorded on a Bruker AVANCE III HD ASCEND 600 MHz spectrometer equipped with BB(F) Double Channel Probe and processed using Bruker TopSpin Software. Microinjection of oocytes was performed by using a NARISHIGE micromanipulator equipped with a manual oocyte microinjection pipette (Drummond Scientific Company).

3. Synthesis of 5-fluorobenzofuran-2′-deoxyuridine 1 and corresponding phosphoramidite 2



Scheme S1. Synthesis of 5-fluorobenzofuran-2′-deoxyuridine **1** and corresponding phosphoramidite **2**. TMEDA: *N,N,N′,N′*-tetramethylethylenediamine; THF: tetrahydrofuran; DMT-Cl = 4,4'-dimethoxytrityl chloride, DMAP = 4-dimethylaminopyridine.

Tributyl(5-fluorobenzofuran-2-yl)stannane (1b): 5-Fluorobenzofuran **1a**[S1] (0.91 g, 6.7 mmol, 1.0 equiv) was dissolved in dry THF (30 mL) and was cooled at -78 °C. TMEDA (0.93 g, 8.0 mmol, 1.2 equiv) was added to the reaction mixture and the mixture was allowed

to stir at -78 °C for 30 min. Subsequently, *n*-BuLi (4.0 mL of 2 M solution in hexane, 8.0 mmol, 1.2 equiv) was added dropwise and the reaction mixture was allowed to stir for 1 h under N_2 atmosphere. The mixture was brought to room temperature over a period of 1 h and again cooled to -78 °C. Bu_3SnCl (2.17 mL, 8.0 mmol, 1.2 equiv) was added dropwise to the reaction mixture and stirred for 1 h. Afterwards, the reaction was quenched with ammonium chloride solution (50 mL) and extracted two times with diethyl ether (2 × 50 mL). The organic layer was dried over sodium sulphate and evaporated to give an oily residue. The residue was purified by silica gel flash column chromatography (hexane) to afford clear oil (1.7 g, 60%). TLC (5% EtOAc in hexane); R_f = 0.67; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.42–7.38 (m, 1H), 7.19 (dd, J_1 = 8.6 Hz, J_2 = 2.6 Hz, 1H), 6.93 (td, J_1 = 9.0 Hz, J_2 = 2.7 Hz, 1H), 6.86–6.84 (m, 1H), 1.63–1.55 (m, 6H), 1.40–1.31 (m, 6H), 1.20–1.11 (m, 6H), 0.90 (t, J = 7.4 Hz, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 168.2, 160.3, 157.9, 155.1, 129.0, 128.9, 118.2, 118.2, 111.4, 111.3, 111.1, 110.9, 105.7, 105.5, 29.1, 27.3, 13.8, 10.3; ^{19}F NMR (376.6 MHz, $CDCl_3$): δ (ppm) -123.70; MALDI-TOF: m/z Calculated for $C_{20}H_{31}FNaOSn$ $[M+Na]^+$ = 449.13, found = 449.14.

5-Fluorobenzofuran-2′-deoxyuridine (1): A mixture of 5-iodo-2′-deoxyuridine **1c** (0.7 g, 1.98 mmol, 1.0 equiv) and *bis*(triphenylphosphine)-palladium(II) dichloride (0.07 g, 0.10 mmol, 0.05 equiv) was dissolved in degassed anhydrous dioxane (30 mL) and tributyl(5-fluorobenzofuran-2-yl)stannane **1b** (2.5 g, 5.90 mmol, 3.0 equiv) was added. The mixture was heated at 90 °C for 2 h under N_2 atmosphere and filtered through Celite pad. The Celite pad was washed with methanol (2 × 15 mL). The filtrate was evaporated and the resulting residue was purified by reverse-phase column chromatography (C18 Redi*Sep*R_f column, 60% MeOH in H_2O) to afford product **1** as white solid (0.57 g, 81%). TLC (15% MeOH in CH_2Cl_2); R_f = 0.48; ^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 11.78 (s, 1H), 8.79 (s, 1H), 7.56 (dd, J_1 = 9.0 Hz, J_2 = 4.2 Hz, 1H), 7.43 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, 1H), 7.33 (br, 1H), 7.11 (td, J_1 = 9.2 Hz, J_2 = 2.8 Hz, 1H), 6.22 (t, J = 6.4 Hz, 1H), 5.30 (d, J = 4 Hz, 1H), 5.26–5.24 (m, 1H), 4.36–4.30 (m, 1H), 3.89–3.87 (m, 1H), 3.75–3.65 (m, 2H), 2.25–2.22 (m, 2H); ^{13}C NMR (100 MHz, d_6-DMSO): δ (ppm) 160.3, 159.8, 157.5, 151.1, 149.4, 149.3, 137.6, 129.9, 129.8, 111.7, 111.6, 111.3, 106.6, 106.3, 104.3, 103.9, 103.9, 87.6, 85.1, 70.0, 60.8, 40.5; ^{19}F NMR (376.6 MHz, d_6-DMSO): δ (ppm) -121.77; HRMS: m/z Calculated for $C_{17}H_{15}FN_2NaO_6$ $[M+Na]^+$ = 385.0812, found = 385.0806; λ_{max} (H_2O) = 267, 275 and 322 nm, ε_{267} = 12.6 x 10^3

M^{-1} cm^{-1}, ε_{275} = 13.31 x 10^3 M^{-1} cm^{-1}, ε_{322} = 17.11 x 10^3 M^{-1} cm^{-1}, ε_{260} = 10.31 x 10^3 M^{-1} cm^{-1}.

5-Fluorobenzofuran-modified 5′-*O*-DMT-2′-deoxyuridine (1d): A mixture of 5-fluorobenzofuran modified deoxyuridine **1** (0.30 g, 0.84 mmol, 1.0 equiv), DMAP (0.010 g, 0.084 mmol, 0.1 equiv), DMT-Cl (0.37 g, 1.1 mmol, 1.3 equiv) and anhydrous pyridine (10 mL) was stirred at room temperature for 12 h under N_2 atmosphere. Then pyridine was evaporated under vacuum and the resulting residue was purified by silica gel column chromatography (1% MeOH in CH_2Cl_2 containing 0.5% Et_3N) to afford compound **1d** as an off white foam (0.3 g, 54%). TLC (5% MeOH in CH_2Cl_2 containing few drops of Et_3N); R_f = 0.4; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.51 (s, 1H), 7.50 (br, 1H), 7.49 (br, 1H), 7.38–7.35 (m, 5H), 7.22–7.18 (m, 2H), 7.13–7.09 (m, 2H), 6.72 (d, J = 8.4 Hz, 4H), 6.65–6.59 (m, 1H), 6.43 (t, J = 6.6 Hz, 1H), 5.99 (dd, J_1 = 8.8 Hz, J_2 = 4 Hz, 1H), 4.51–4.49 (m, 1H), 4.10–4.09 (m, 1H), 3.65–3.61 (m, 7H), 3.31 (dd, J_1 = 10.8 Hz, J_2 = 2.8 Hz, 1H) , 2.55–2.50 (m, 1H), 2.41–2.34 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 160.4, 160.0, 158.7, 158.1, 149.8, 149.4, 149.3, 144.6, 135.9, 135.7, 135.6, 130.2, 130.2, 128.3, 128.0, 127.1, 113.3, 113.3, 111.9, 111.7, 111.5, 111.4, 106.7, 106.5, 106.2, 105.9, 105.9, 87.0, 86.5, 85.7, 72.1, 63.3, 55.3, 41.6; ^{19}F NMR (376.6 MHz, $CDCl_3$): δ (ppm) -122.32; HRMS: m/z Calculated for $C_{38}H_{33}FN_2NaO_8$ $[M+Na]^+$ = 687.2119, found = 687.2119.

5-Fluobenzofuran modified 2′-deoxyuridine phosphoramidite substrate (2): To a solution of **1d** (0.2 g, 0.3 mmol, 1.0 equiv) in anhydrous dichloromethane (2.7 mL) was added DIPEA (0.26 mL, 1.5 mmol, 5 equiv) and stirred for 10 min. To this solution 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.10 mL, 0.45 mmol, 1.5 equiv) was added and the mixture was stirred for 2.5 h. Subsequently, the reaction mixture was evaporated to dryness and the residue was redissolved in ethyl acetate (20 mL). The organic layer was washed with 5% sodium bicarbonate solution (20 mL) and brine solution (20 mL) successively, dried over sodium sulphate and evaporated to dryness. The residue was purified by silica gel column chromatography (40% EtOAc in hexane containing 0.5% Et_3N) to afford the product **2** as a white foam (0.16 g, 61%). TLC (hexane:EtOAc= 50:50 containing 1% Et_3N); R_f = 0.56, 0.68 for two diastereomers; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.59 (s, 1H), 7.52–7.51 (m, 2H), 7.40–7.37 (m, 5H), 7.22–7.18 (m, 2H), 7.12–7.07 (m, 2H), 6.72–6.70 (m, 4H), 6.58–6.53 (m, 1H), 6.43–6.39 (m, 1H), 5.77 (dd, J_1 = 8.8 Hz, J_2 = 4 Hz, 1H), 4.61–4.56 (m, 1H), 4.23–4.22 (m, 1H), 3.69 (dd, J_1 = 10.8 Hz, J_2 = 2.4 Hz, 1H), 3.65 (s, 3H),

3.64 (s, 3H), 3.62–3.52 (m, 5H), 3.24 (dd, J_1 = 10.6 Hz, J_2 = 2.6 Hz, 1H), 2.60–2.54 (m, 1H), 2.41–2.38 (m, 2H), 1.17–1.14 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 160.4, 160.1, 158.7, 158.1, 149.7, 149.4, 149.2, 144.6, 136.0, 135.7, 135.7, 130.3, 130.2, 129.8, 129.7, 128.4, 128.0, 127.1, 117.5, 113.3, 113.2, 111.8, 111.5, 111.5, 111.4, 106.7, 106.3, 106.1, 105.9, 105.8, 86.8, 86.2, 86.2, 85.7, 73.2, 73.1, 62.8, 58.4, 58.2, 55.3, 43.5, 43.4, 40.9, 40.8, 24.8, 24.7, 24.7, 24.6, 20.3, 20.3; ^{31}P NMR (162 MHz, $CDCl_3$): δ (ppm) 149.23; ^{19}F NMR (376.6 MHz, $CDCl_3$): δ (ppm) -122.48; HRMS: m/z Calculated for $C_{47}H_{51}FN_4O_9P$ $[M+H]^+$ = 865.3378, found = 865.3379.

4. Photophysical analysis of 5-fluorobenzofuran-modified nucleoside analog 1 in different solvents.

UV absorption and steady-state fluorescence studies: UV absorption spectrum of nucleoside **1** (25 μM) in various solvents was recorded on a Shimadzu UV-2600 spectrophotometer in quartz cuvette (Hellma, path length 1 cm). Each sample contained 2.5% of DMSO and measurements were performed in triplicate. For steady-state fluorescence study, nucleoside **1** (5 μM) was excited in different solvents at their respective lowest energy absorption maximum (Table 1). Fluorescence experiments were performed in triplicate in micro fluorescence cuvette (Hellma, path length 1 cm) on a Fluorolog-3 spectrophotometer. Fluorescence samples contained 0.5% of DMSO. Anisotropy values (r) of nucleoside **1** in different solvents were determined by analysing the data using software provided with the instrument. Anisotropy measurements were performed in triplicate and each anisotropy value was an average of 10 successive measurements.

Time-resolved fluorescence study: Time-resolved fluorescence study was performed on a TCSPC instrument (Horiba Jobin Yvon, Fluorolog-3). Nucleoside **1** (5 μM) in water, ethylene glycol and glycerol was excited by using 339 nm LED source (IBH, UK, NanoLED-339L). In dioxane and methanol nucleoside **1** (400 μM) was excited by using 375 nm diode laser source (IBH, UK, NanoLED-375L). Fluorescence signal at respective emission maxima was collected. All studies were done in triplicate and lifetimes were calculated by fitting the decay profile using IBH DAS6 software. The χ^2 value for all the curve fits was found to be nearly one.

Quantum yield: Quantum yield of nucleoside **1** in different solvents was determined relative to 2-aminopurine as the standard. Following equation was used to calculate the quantum yield[S2].

$$\Phi_{F(x)} = (A_s/A_x)\,(F_x/F_s)\,(n_x/n_s)^2\,\Phi_{F(s)}$$

Where s is the standard, x is the modified nucleoside, A is the absorbance at excitation wavelength, F is the area under the emission curve, n is the refractive index of the solvent, and Φ_F is the quantum yield. Quantum yield of 2-aminopurine in water is 0.68. Quantum yield of fluorescent H-Telo DNA ONs and corresponding duplexes was determined relative to the quantum yield of nucleosides **1**. Quantum yield of nucleoside **1** in water is 0.11.

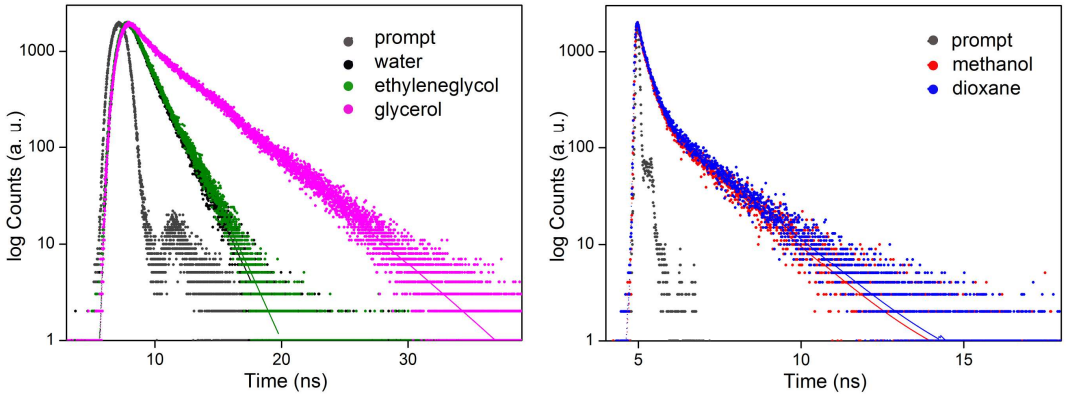


Figure S1. Excited-state decay profile of nucleoside **1** in solvents of different polarity and viscosity. Instrument response (prompt) is shown in gray dots. Curve fits are shown in solid lines.

5. Solid phase DNA synthesis: 5-fluorobenzofuran-modified H-Telo DNA ONs **3**–**5, 8** and **9** were synthesized (1 µmole scale, 1000 Å CPG solid support) by a standard ON synthesis protocol using phosphoramidite **2**[S3]. In DNA synthesis, the solid support after final detritylation step was treated with 30% aqueous ammonium hydroxide solution for 16 h at 55 °C and 30 min at 70 °C. The solution was evaporated to dryness using speed vac and the residue was purified by denaturing PAGE (20% gel). The band corresponding to the full-length modified ON product was identified by UV shadowing, which was cut and transferred to a Poly-Prep column (Bio-Rad). The gel pieces were crushed with a sterile glass rod, and the ON was extracted using ammonium acetate buffer (0.5 M, 3 mL) for 12 h and desalted using Sep-Pak classic C18 cartridges (Waters). The purity of ONs **3**–**5**, **8** and **9** was confirmed by RP-HPLC and characterized by MALDI TOF mass analysis (see Figure **S2**, Figure S3 and Table S1).

6. MALDI-TOF mass analysis of ONs: A mixture of 2 μL of the modified ON (~200 μM), 4 μL of a 9:1 solution of 3-hydroxypicolinic acid and ammonium citrate buffer (100 mM, pH 9) and 2 μL of an internal DNA standard (100 μM) was desalted using an ion-exchange resin (Dowex 50W-X8, 100-200 mesh, ammonium form). The sample was incubated at 55 °C for 10 min and cooled to RT. The sample was then spotted on a MALDI plate, air dried and subjected to mass analysis. The resultant spectra were calibrated relative to the internal DNA standard (Figure S3 and Table S1).

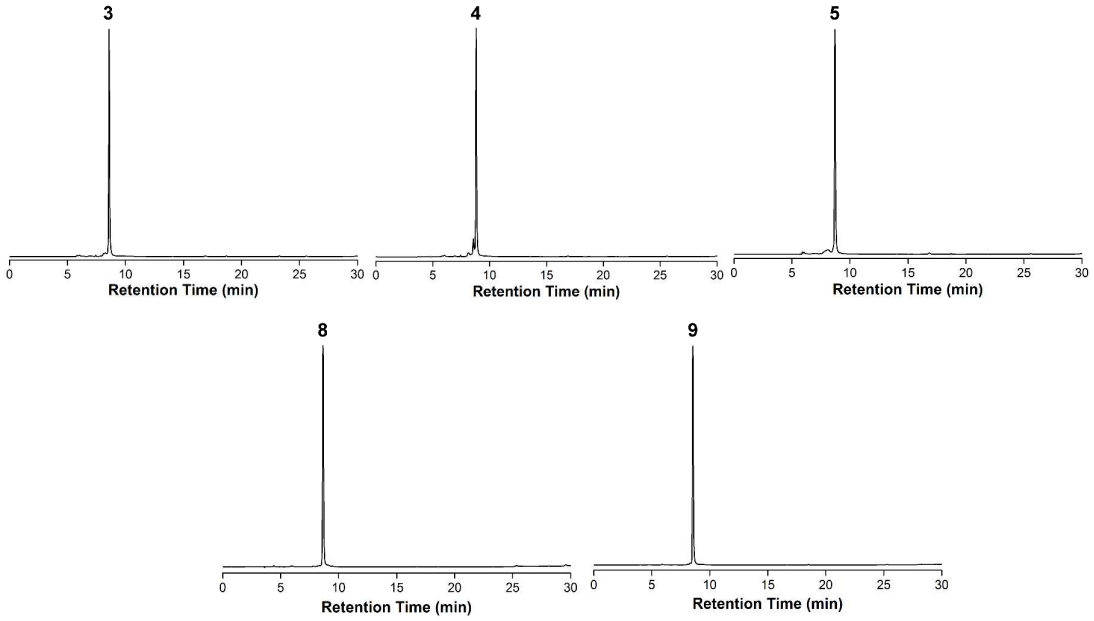


Figure S2. HPLC chromatogram of PAGE purified ONs **3**–**5**, **8** and **9** at 260 nm. Mobile phase A = 50 mM triethylammonium acetate buffer (pH 7.5), mobile phase B = acetonitrile. Flow rate = 1 mL/min. Gradient = 0–100% B in 30 min. HPLC analysis was performed using a Luna C18 column (250 x 4.6 mm, 5 micron).

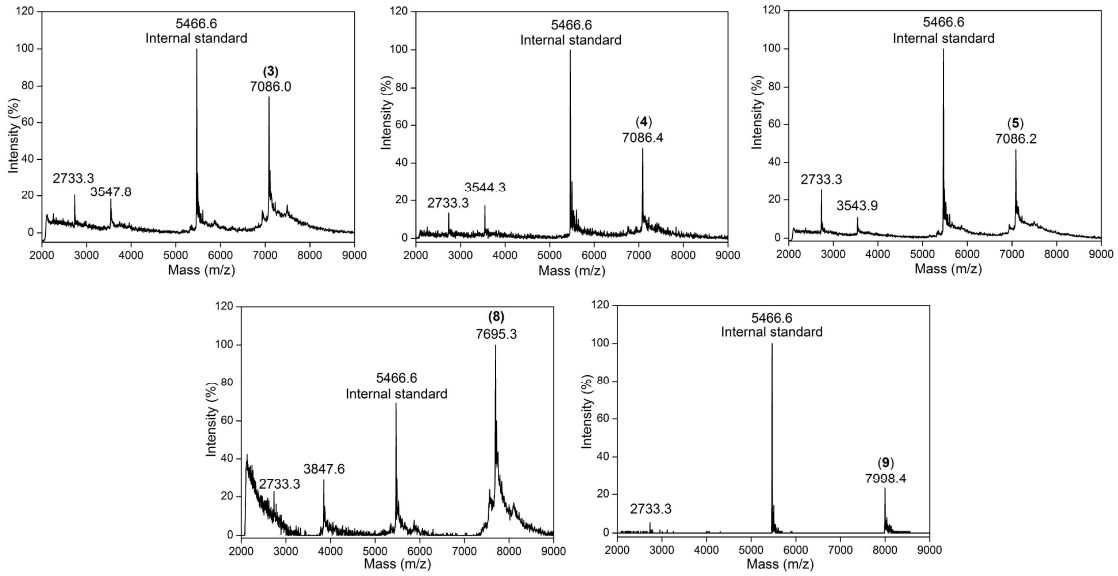


Figure S3. MALDI-TOF spectrum of modified H-Telo DNA ONs calibrated relative to the +1 and +2 ions of an internal 18-mer DNA ON standard (m/z for +1 and +2 ions are 5466.6 and 2733.3, respectively). See Table S1 for details.

Table S1. Molar extinction coefficient and mass of modified H-Telo DNA ONs.

H-Telo DNA ON	$\varepsilon_{260}{}^{a}$ ($M^{-1}cm^{-1}$)	Calculated mass	Observed mass
3	230.4 x 10^3	7086.6	7086.0
4	230.4 x 10^3	7086.6	7086.4
5	230.4 x 10^3	7086.6	7086.2
8	246.2 x 10^3	7695.0	7695.3
9	255.0 x 10^3	7999.2	7998.4

[a]Molar absorption coefficient ε_{260} of the modified ONs was determined by using OligoAnalyzer 3.1, The extinction coefficient of nucleoside **1** (ε_{260} = 10.31 x 10^3 $M^{-1}cm^{-1}$) was used in the place of thymidine.

7. Circular dichroism (CD) studies. Modified and unmodified ONs (8 μM) were heated at 90 °C for 3 min in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl, or in potassium phosphate buffer (10 mM, pH 7.0) containing 100 mM KCl or in intraoocyte buffer (25 mM HEPES (pH = 7.5), 10.5 mM NaCl, 110 mM KCl, 130 nM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EDTA). The samples were allowed to come to room temperature over a period of 2 h and were kept at 4 °C for 30 min before recording the spectrum. CD spectra were recorded from 200 to 350 nm in a quartz cuvette (Starna Scientific, path length 5 mm)

on a J-815 CD spectropolarimeter (Jasco, USA) using 1 nm bandwidth at 20 °C. Each CD profile is an average of three scans collected at a scan speed of 100 nm min^{-1}. CD measurements were performed in duplicate and all spectra were corrected using an appropriate blank solution in the absence of ONs.

8. Thermal melting analysis of DNA ONs: Modified and unmodified DNA ONs (1 μM) were annealed similarly like CD samples and thermal melting analysis was performed using Cary 300 Bio UV-Vis spectrophotometer. The temperature was increased from 20 °C to 90 °C at 1 °C/min and the absorbance was measured every 1 °C interval at 295 nm.

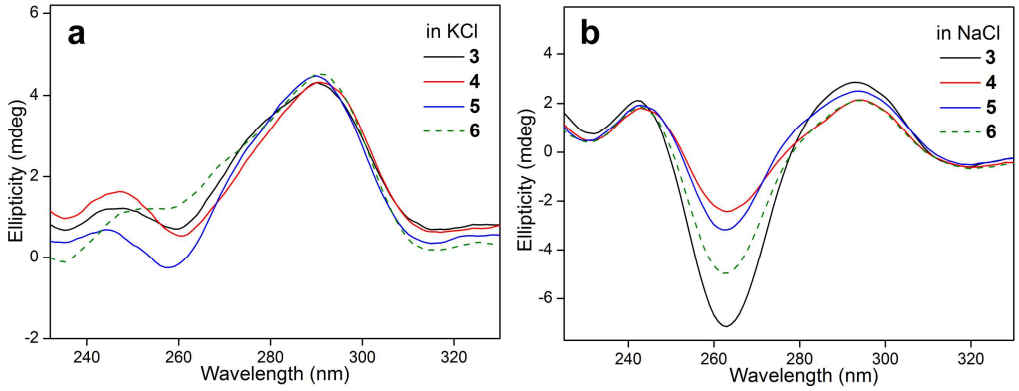


Figure S4. CD spectrum of 5-fluorobenzofuran-modified H-Telo DNA ONs **3**–**5** and unmodified control H-Telo DNA ON **6**. (**a**) In potassium phosphate buffer (10 mM, pH 7.0) containing 100 mM KCl and (**b**) in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl.

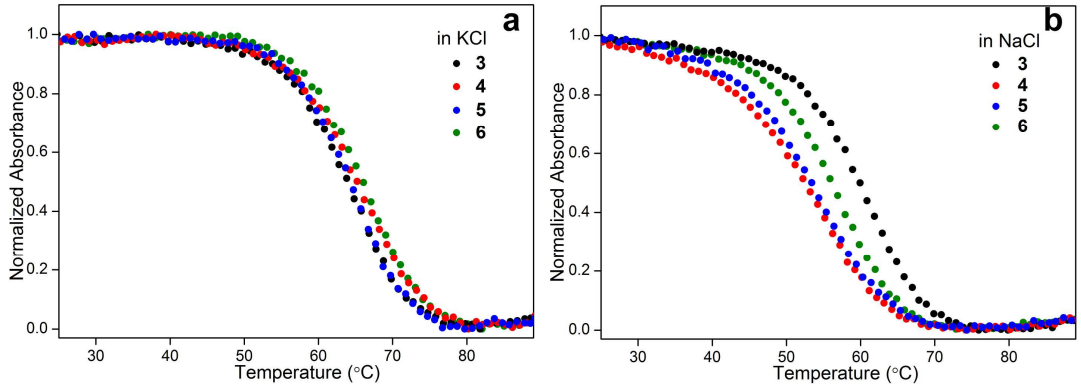


Figure S5. UV-thermal melting profile of 5-fluorobenzofuran-modified H-Telo DNA ONs **3**–**5** and unmodified H-Telo DNA ON **6.** (**a**) In potassium phosphate buffer (10 mM, pH 7.0) containing 100 mM KCl and (**b**) in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl. For T_m values see Table S2.

Table S2. T_m values of modified (**3**–**5**) and control unmodified (**6**) H-Telo DNA ONs.

H-Telo DNA ON	T_m (°C) in KCl	T_m (°C) in NaCl
3	66.5 ± 0.4	61.5 ± 0.4
4	65.8 ± 0.4	55.5 ± 0.5
5	65.5 ± 0.7	55.0 ± 0.4
control ON **6**	66.5 ± 0.7	57.8 ± 0.4

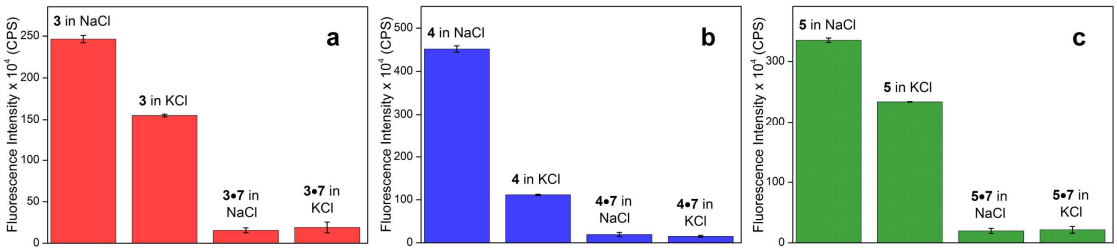


Figure S6. Bar diagram of fluorescence intensity of H-Telo DNA ONs (**a**) **3** and corresponding duplex **3•7**, (**b**) **4** and corresponding duplex **4•7** and (**c**) **5** and corresponding duplex **5•7** in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl or in potassium phosphate buffer (10 mM, pH 7.0) containing 100 mM KCl at respective emission maximum (Table 2). Samples (0.5 μM) were excited at 330 nm with an excitation and emission slit width of 4 and 6 nm, respectively.

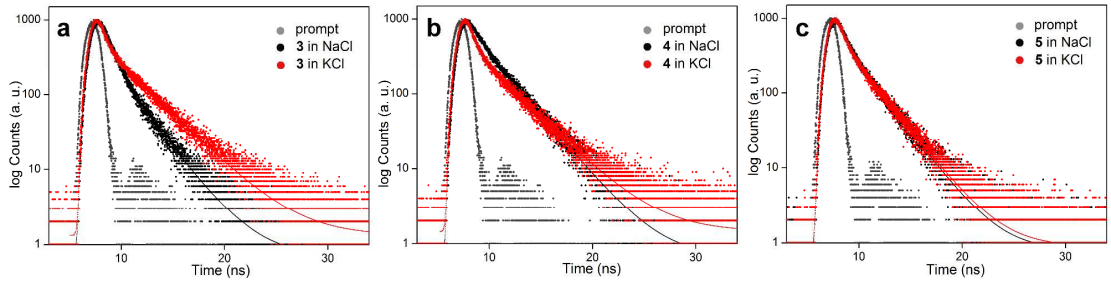


Figure S7. Time-resolved fluorescence spectrum of H-Telo DNA ONs (0.5 μM) (**a**) **3** (**b**) **4** and (**c**) **5** in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl or in potassium phosphate buffer (10 mM, pH 7.0) containing 100 mM KCl. Instrument response is shown in gray dots. Curve fits are shown in solid lines.

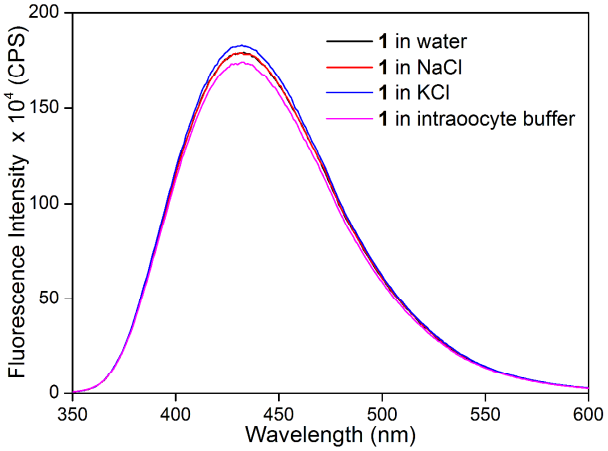


Figure S8. Fluorescence spectra of nucleoside **1** (5 μM) in water, sodium phosphate buffer containing 100 mM NaCl, potassium phosphate buffer containing 100 mM KCl and intraoocyte buffer (25 mM HEPES (pH = 7.5), 10.5 mM NaCl, 110 mM KCl, 130 nM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EDTA). Changes in ionic conditions did not affect the fluorescence profile of the free nucleoside probe.

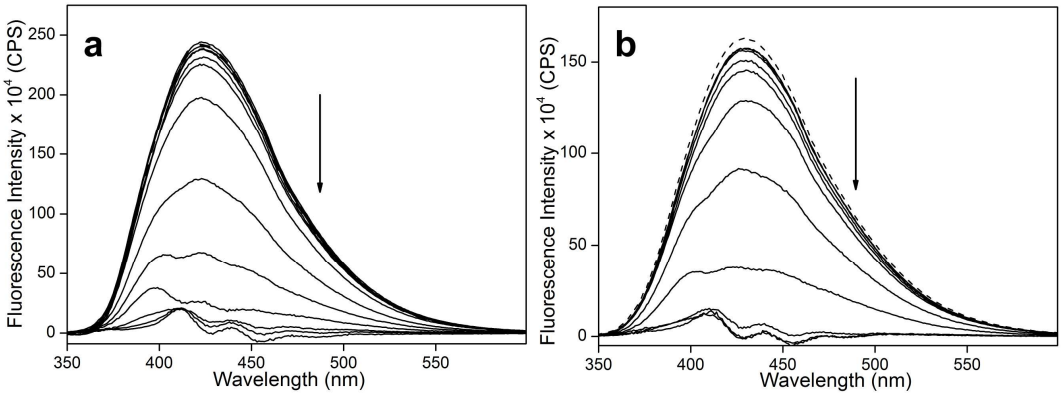


Figure S9. Emission spectra for the titration of labeled H-Telo DNA ON **3** (0.5 μM) with increasing concentration of BRACO19 in (**a**) sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl and (**b**) potassium phosphate buffer (10 mM, pH 7.0) containing 100 mM KCl. Samples were excited at 330 nm with an excitation and emission slit width of 4 nm and 6 nm, respectively. The dashed line represents the spectrum of ON **3** without BRACO19.

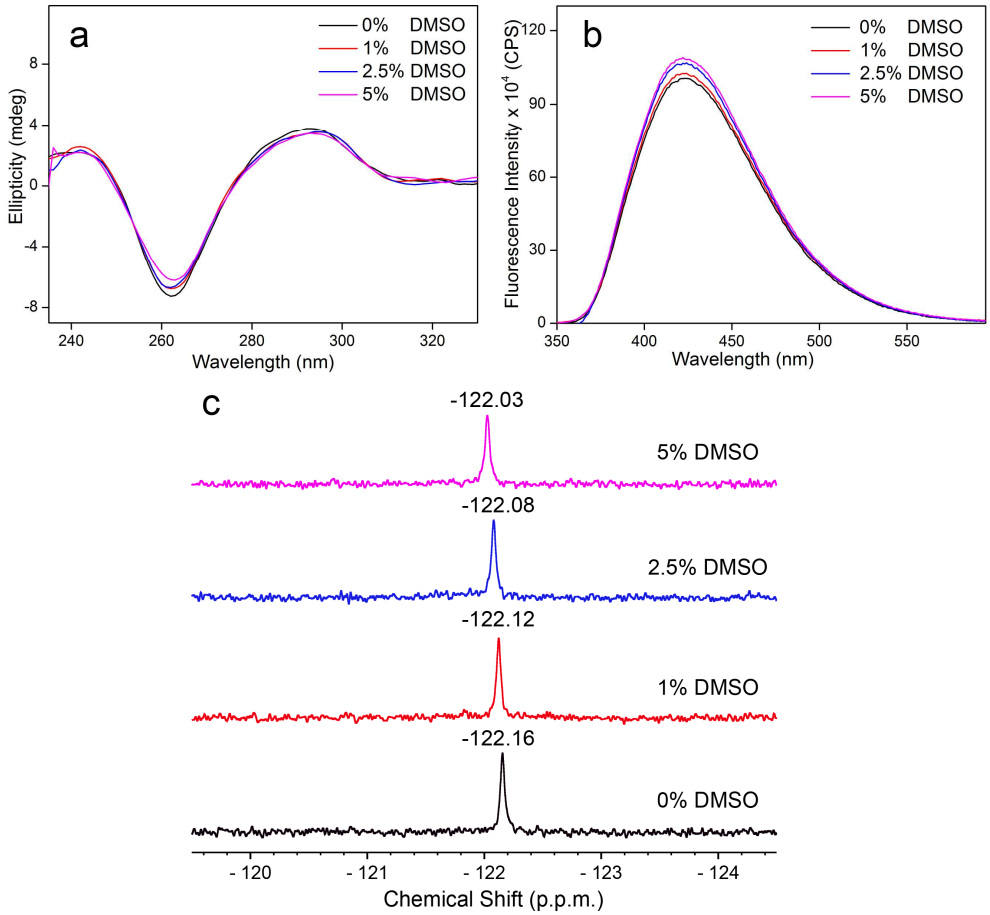


Figure S10. CD (8 μM), fluorescence (0.5 μM) and ^{19}F NMR spectra (100 μM) of ON **3** in sodium phosphate buffer containing 100 mM NaCl with increasing percentage of DMSO.

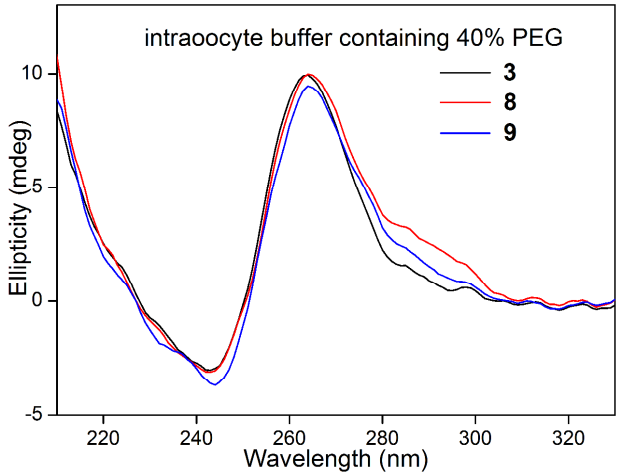


Figure S11. CD spectra of 5-fluorobenzofuran modified H-Telo DNA **3**, **8** and **9** (8 μM) in intraoocyte buffer (25 mM HEPES, pH = 7.5, 10.5 mM NaCl, 110 mM KCl, 130 nM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EDTA) containing 40% PEG. In presence of PEG all three modified ONs adopted a parallel GQ conformation.

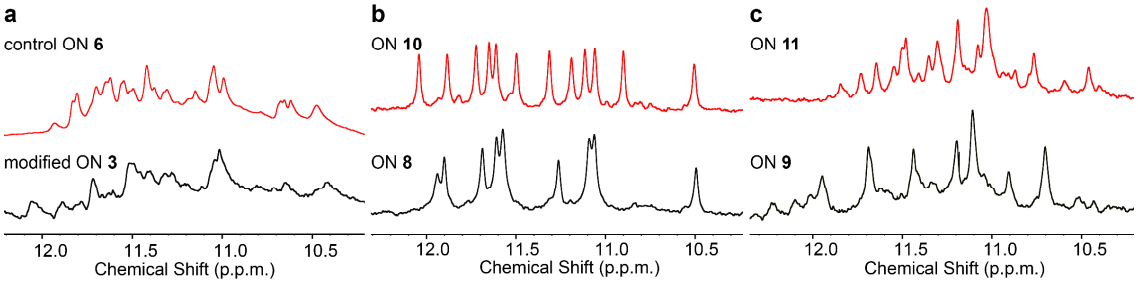


Figure S12. ^{1}H NMR spectrum of 5-fluorobenzofuran-labeled (black) and control unmodified (red) H-Telo DNA ONs in intraoocyte buffer conditions. All samples (100 µM) contained 20% D_2O and all spectra were recorded at 18 °C.

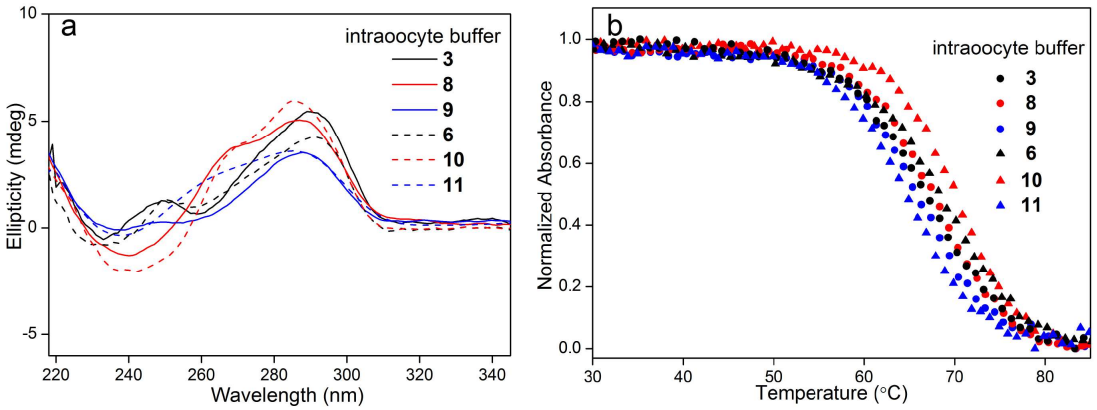


Figure S13. CD spectra of 5-fluorobenzofuran-modified H-Telo DNA ONs **3**, **8** and **9** (8 µM) and respective unmodified H-Telo DNA ONs **6**, **10** and **11** in intraoocyte buffer at 20 °C. (b) UV-thermal melting profile of above ONs (1 µM) in intraoocyte buffer. For T_m values see Table S3.

Table S3. T_m values of modified H-Telo DNA ONs (**3**, **8** and **9)** and corresponding unmodified H-Telo DNA ONs (**6**, **10** and **11)** in intraoocyte buffer.

Modified H-Telo DNA ON	T_m (°C)	Control H-Telo DNA ON	T_m (°C)
3	68.1 ± 0.6	**6**	69.7 ± 0.5
8	68.1 ± 0.4	**10**	70.9 ± 0.1
9	66.7 ± 0.5	**11**	66.4 ± 0.9

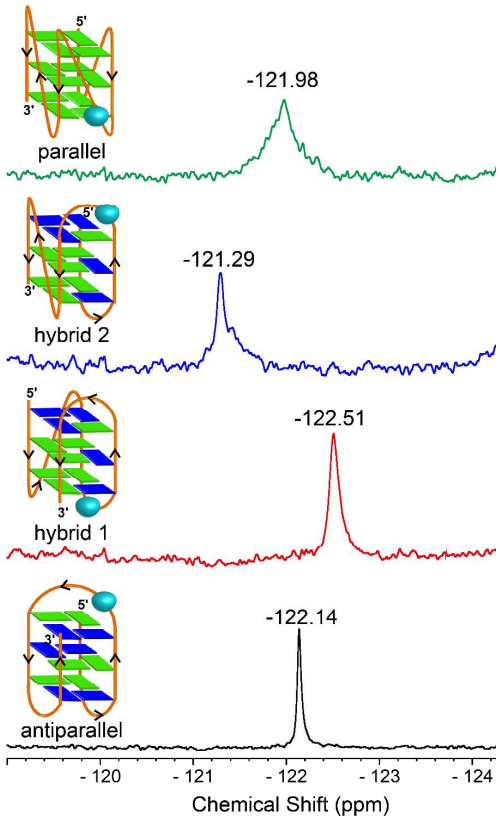


Figure S14. ^{19}F NMR signature for different GQ topologies of H-Telo DNA ON repeats.

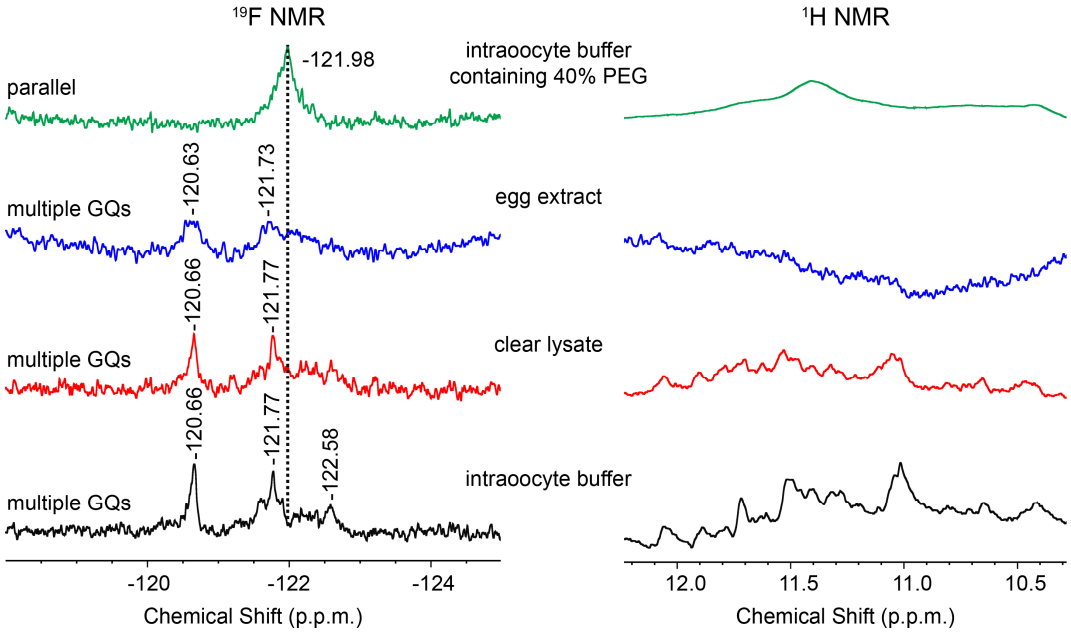


Figure S15. ^{19}F and ^{1}H NMR spectra of H-Telo DNA ON **3** in intraoocyte buffer, oocyte clear lysate, egg extract and intraoocyte buffer containing 40% PEG at 18 °C.

9. ON leakage study by ^{19}F NMR and fluorescence: To check the leakage of microinjected ONs during the course of NMR analysis (~10 h), the extracellular buffer was isolated and ^{19}F NMR was recorded. No signal was observed in ^{19}F NMR spectrum (Figure S16a).

The leakage of ON was also tested by fluorescence analysis. 3.6 mM stock of ON **9** was injected into two different sets of 30 oocytes. Oocytes were incubated for 1 h in Ori Ca^{2+} buffer and thoroughly washed with the same buffer. One set of 30 injected oocytes were transferred to an Eppendorf tube loaded with 150 µL of Ori Ca^{2+} buffer containing 10% Ficoll. Oocytes were crushed, heat denatured and centrifuged as before to obtain a clear lysate. Final volume of the clear lysate was adjusted to 500 µL with Ori Ca^{2+} buffer containing 10% Ficoll. This lysate sample was considered as a control (zero time) containing 100% of the labeled H-Telo ON **9** (sample A). Another set of 30 injected oocytes was prepared in a similar fashion and incubated in 150 µL of Ori Ca^{2+} buffer containing 10% Ficoll for 10 h. After this period, the extracellular buffer was removed and the volume was adjusted to 500 µL with Ori Ca^{2+} buffer containing 10% Ficoll (sample B). Further, the oocytes from this set were carefully washed with Ori Ca^{2+} buffer containing 10% Ficoll (4 x 200 µL). Oocytes were crushed, heat denatured and centrifuged as before to obtain a clear lysate (sample C). Samples A, B and C were excited at 330 nm with an excitation and emission slit width of 4 nm and 6 nm, respectively. Appropriate buffer/lysate was used for blank correction. By comparing the area under the fluorescence curve of lysate obtained after 0 h of incubation (sample A) and 10 h incubation (sample C), it was found that only 6% of the ON sample leaked out of the oocytes into the extracellular buffer during the course of NMR acquisition time. Similarly, comparing sample A and B nearly 10% leakage was estimated.

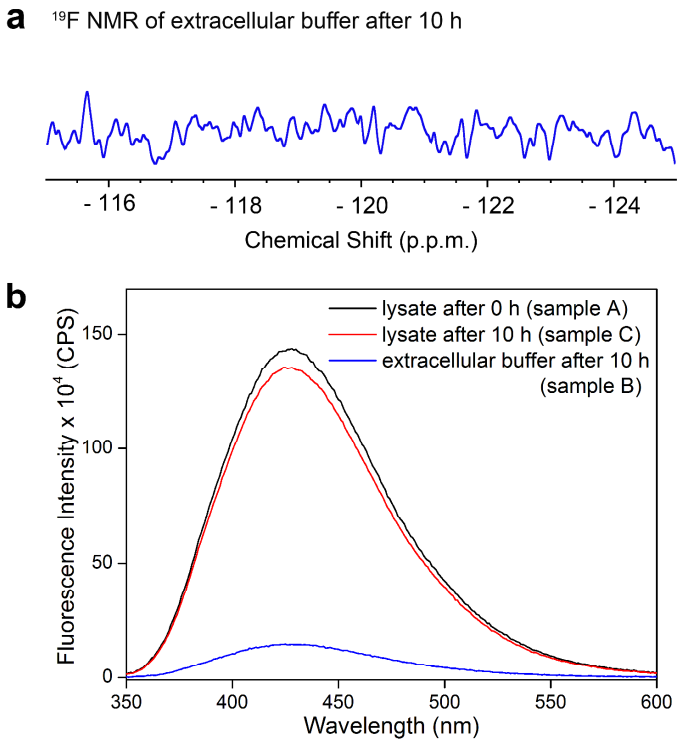


Figure S16. (**a**) ^{19}F NMR spectrum of extracellular buffer after 10 h of in-cell NMR analysis. (**b**) Fluorescence leakage assay: Emission spectrum of lysates obtained after 0 h (sample A) and 10 h of incubation after microinjection of ON **9** (sample C) and extracellular buffer after 10 h of incubation (sample B). Samples were excited at 330 nm with excitation and emission slit width 4 nm and 6 nm, respectively. See above section for details.

10. HPLC and mass analysis of egg extract and oocytes after NMR analysis: The egg extract containing H-Telo DNA ON **9** after NMR analysis was heated at 95 °C and centrifuged at 20000 g for 10 min to obtain clear lysate. Oocytes microinjected with ON **9** after NMR experiment were crushed and the lystae was prepared as before. Both the samples were concentrated using Amicon Ultra 3K centrifugal filter and analyzed by HPLC. Appropriate fraction corresponding to ON **9** was isolated and further subjected to mass analysis. See Figure S17 and Table S4.

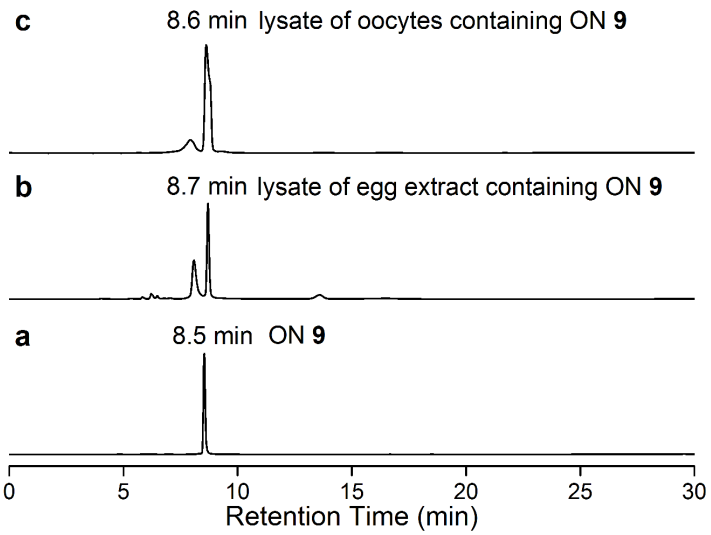


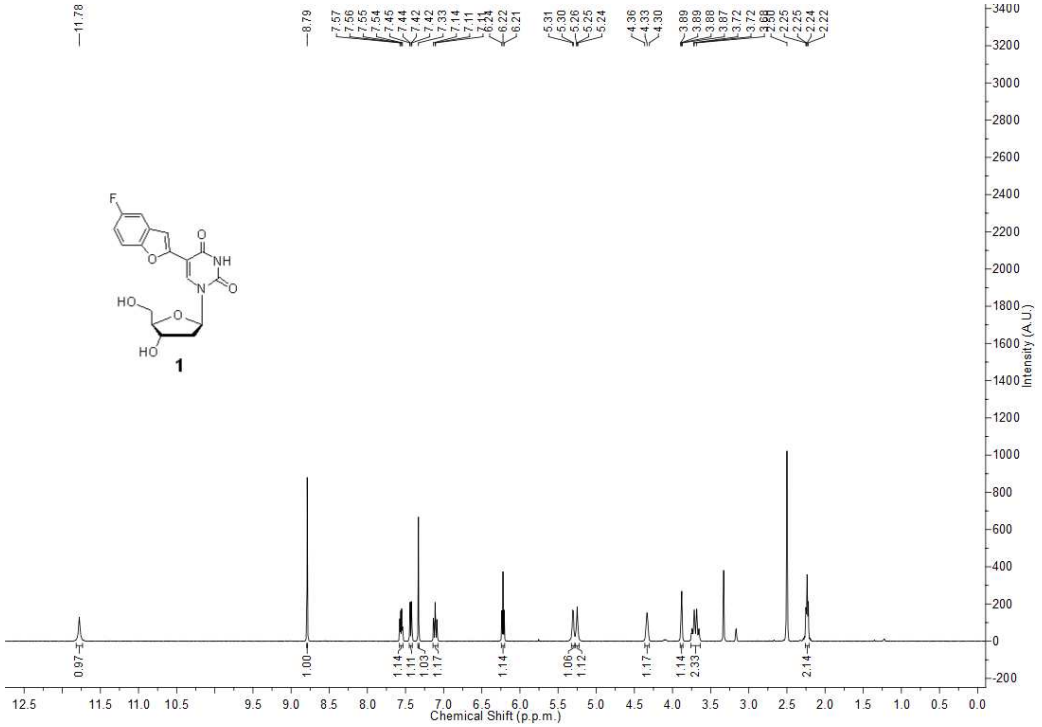
Figure S17. HPLC chromatogram of (**a**) neat ON **9**, (**b**) lysate of egg extract containing H-Telo DNA ON **9** after NMR analysis, (**c**) lysate of oocytes microinjected with H-Telo DNA ON **9** after NMR analysis. Mobile phase A = 50 mM triethylammonium acetate buffer (pH 7.5), mobile phase B = acetonitrile. Flow rate = 1 mL/min. Gradient = 0–100% B in 30 min. HPLC analysis was performed using Luna C18 column (250 x 4.6 mm, 5 micron).

Table S4. Mass analysis of HPLC fractions obtained from NMR samples of ON **9**.

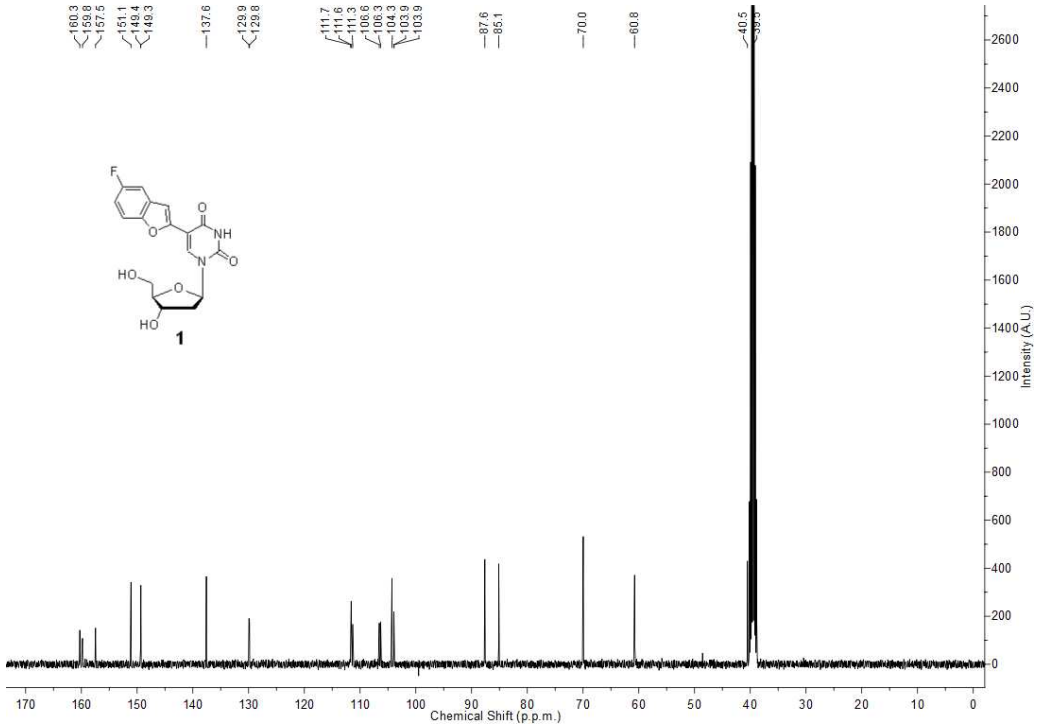
Sample	Calculated mass of ON **9**	Observed mass
lysate of egg extract containing H-Telo DNA ON **9**	7999.2	7999.4
lysate of oocytes microinjected with H-Telo DNA ON **9**	7999.2	7998.9

11. NMR spectra

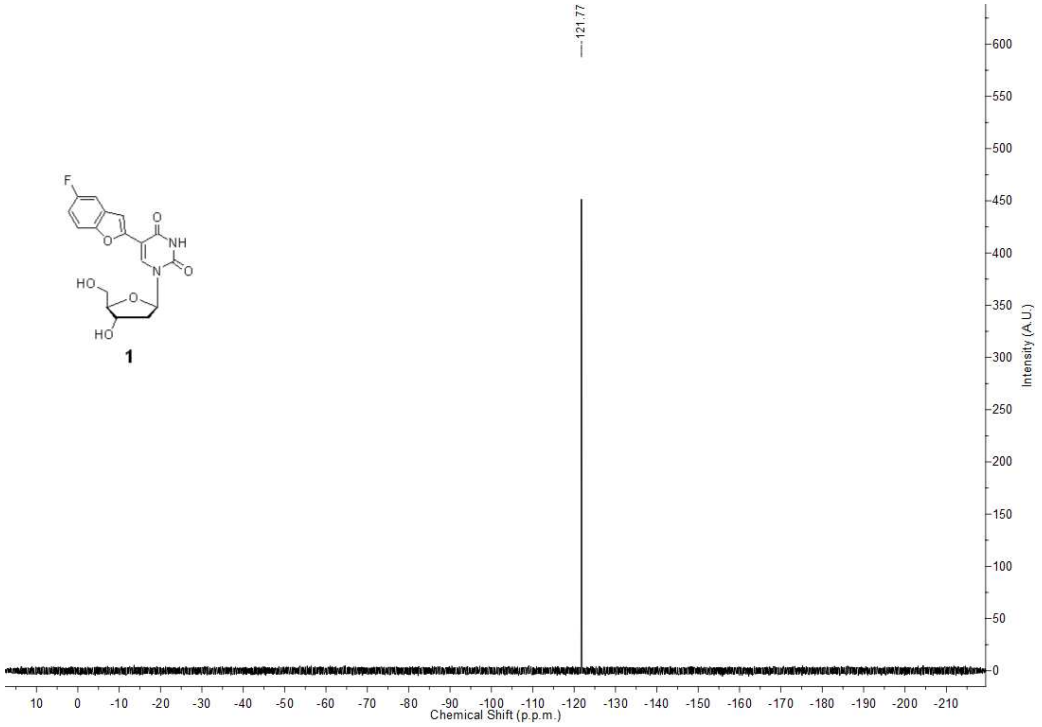
^{1}H NMR of **1** (400 MHz, d_6-DMSO)



^{13}C NMR of **1** (100 MHz, d_6-DMSO)

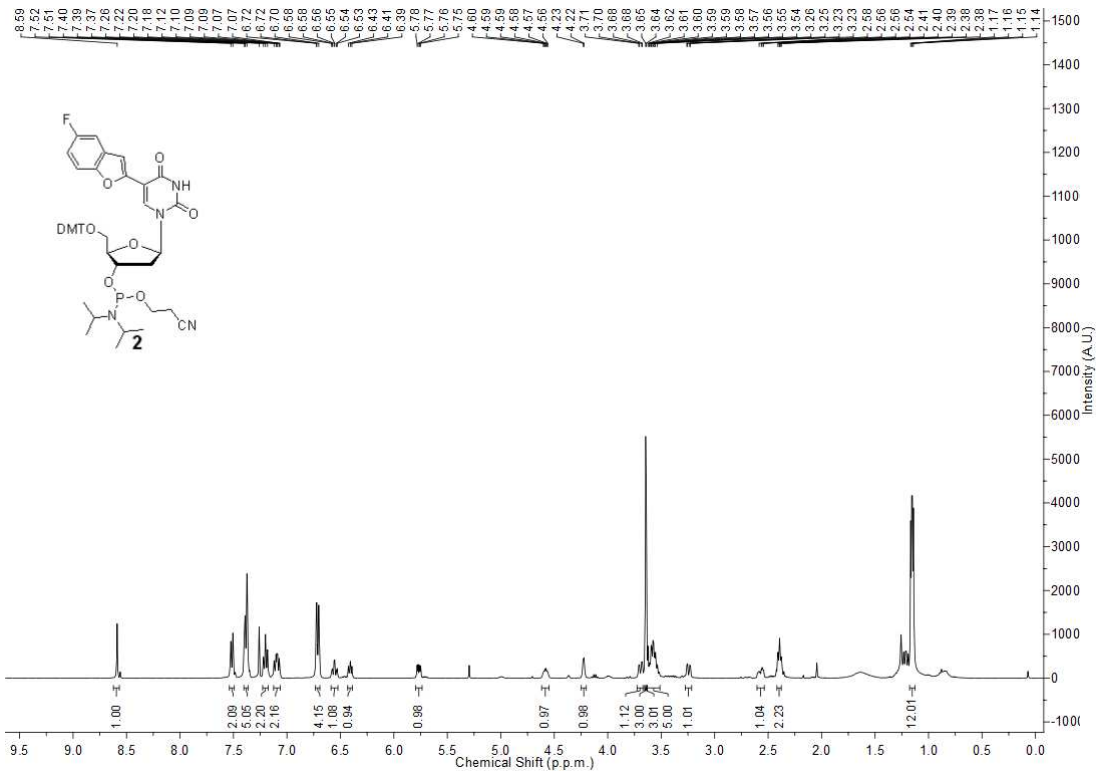


^{19}F NMR of **1** (376.6 MHz, d_6-DMSO)



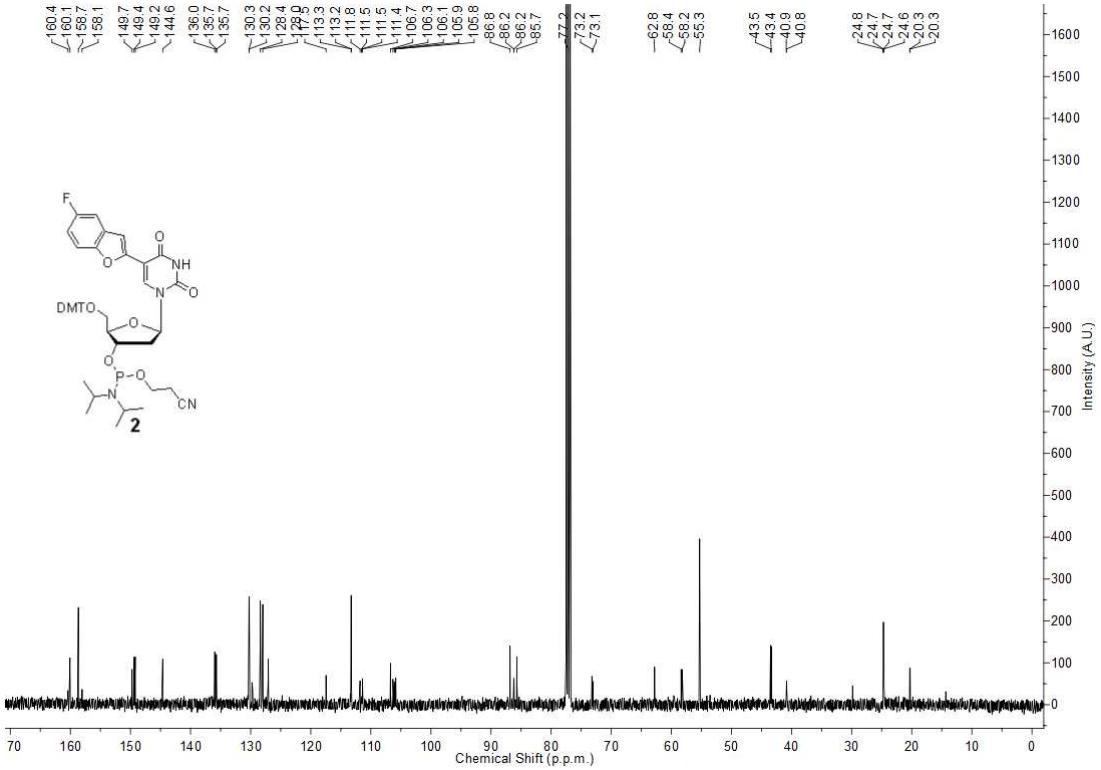
1

^{1}H NMR of **2** (400 MHz, $CDCl_3$)

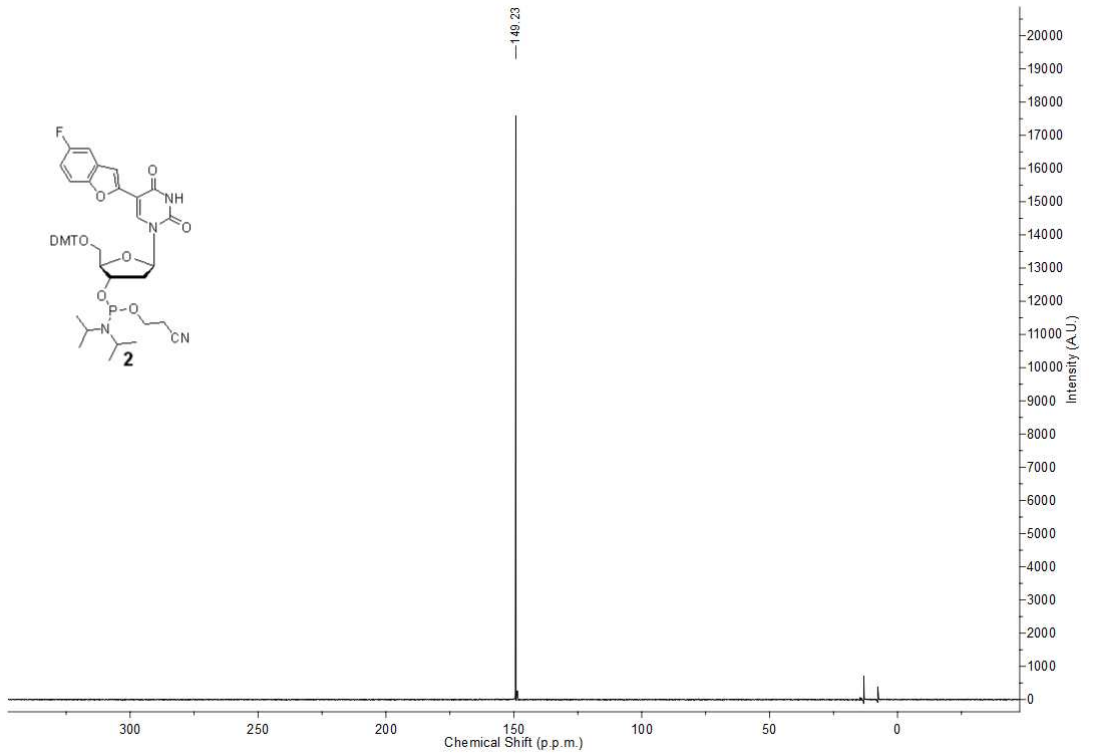


2

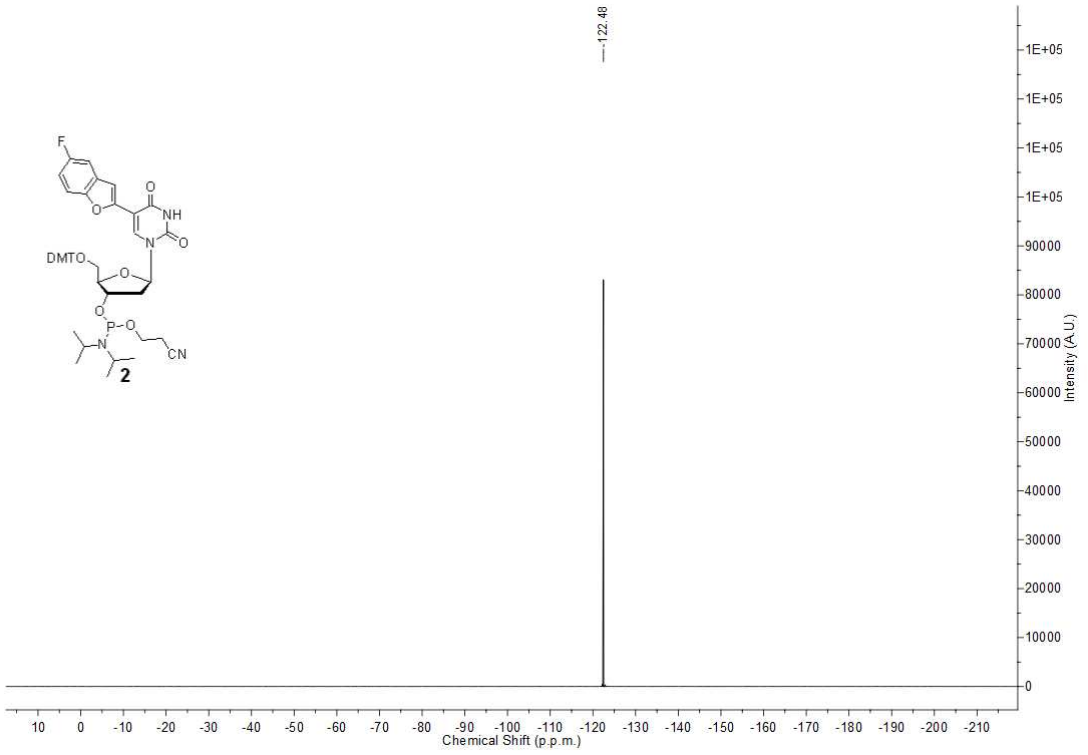
^{13}C NMR of **2** (100 MHz, $CDCl_3$)



^{31}P NMR of **2** (162 MHz, $CDCl_3$)



^{19}F NMR of **2** (376.6 MHz, $CDCl_3$)



12. References.

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S2. Lavabre, D.; Fery-Forgues, S. *J. Chem. Educ.* **1999**, *76*, 1260–1264.

S3. Tanpure, A. A.; Srivatsan, S. G. *ChemBioChem* **2012**, *13*, 2392–2399.