Synthesis, Photophysical Characterization and Enzymatic Incorporation of a Microenvironment-Sensitive Fluorescent Uridine Analog

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Supporting Information

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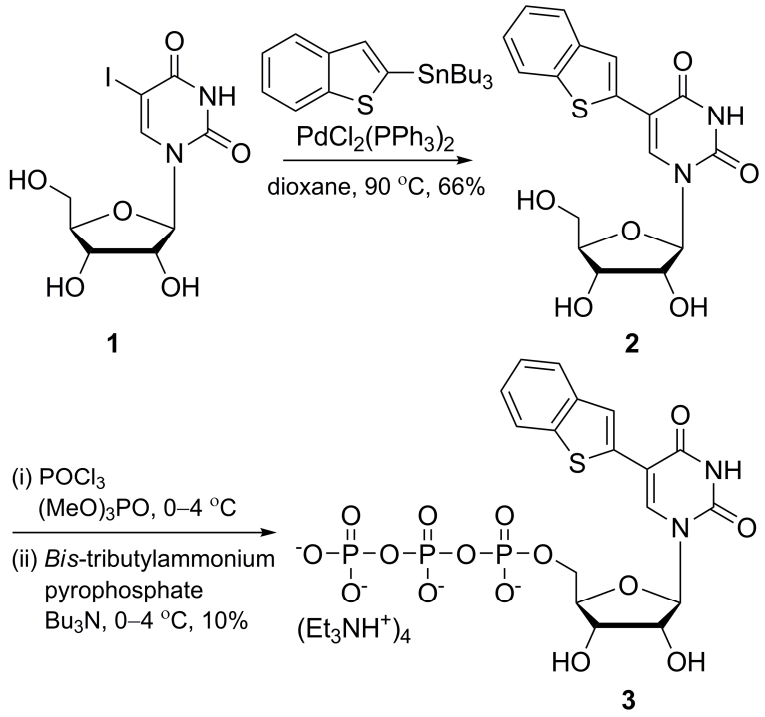
1. Materials: Unless otherwise mentioned, materials obtained from commercial suppliers were used without further purification. 5-iodouridine, 2-(tributylstannyl)benzo[*b*]thiophene and *bis*(triphenylphosphine)-palladium(II) chloride were obtained from Sigma-Aldrich. DNA oligonucleotides were either purchased from Integrated DNA Technologies, Inc. or from Sigma-Aldrich. Oligonucleotides were purified by gel electrophoresis under denaturing condition and desalted on Sep-Pak Classic C18 cartridges (Waters Corporation). Custom synthesized RNA oligonucleotides purchased from Dharmacon RNAi Technologies were deprotected according to the supplier's procedure, PAGE-purified and desalted on Sep-Pak Classic C18 cartridges. T7 RNA polymerase, ribonuclease inhibitor (RiboLock) and NTPs were obtained from Fermentas Life Science. Radiolabeled α-^{32}P ATP (2000 Ci/mmol) was obtained from the Board of Radiation and Isotope Technology, Government of India. Chemicals for preparing buffer solutions were purchased from Sigma-Aldrich (BioUltra grade). Autoclaved water was used in all biochemical reactions and fluorescence measurements.

2. Instrumentation: NMR spectra were recorded on a 400 MHz Jeol ECS-400. All MALDI-MS measurements were recorded on an Applied Biosystems 4800 Plus MALDI TOF/TOF analyzer. Absorption spectra were recorded on a PerkinElmer, Lambda 45 UV-Vis spectrophotometer. UV-thermal melting analyses of oligonucleotides were performed on a Cary 300Bio UV-Vis spectrophotometer. Steady State and time-resolved fluorescence experiments were carried out in a micro fluorescence cuvette (Hellma, path length 1.0 cm) on a TCSPC instrument (Horiba Jobin Yvon, Fluorolog-3). Reversed-phase flash chromatographic (C18 Redi*SepRf* column) purifications were carried out using Teledyne ISCO, Combi Flash Rf.

3. Synthesis:



Scheme 1. Synthesis of benzo[*b*]thiophene-conjugated uridine **2** and its corresponding ribonucleoside triphosphate **3**

Benzo[*b*]thiophene-conjugated uridine 2: To a suspension of 5-iodouridine (0.200 g, 0.54 mmol, 1 equiv) and *bis*(triphenylphosphine)-palladium(II) chloride (0.007 g, 0.01 mmol, 0.02 equiv) in degassed anhydrous dioxane (15 ml) was added 2-(tributylstannyl)benzo[*b*]thiophene (0.571 g, 1.34 mmol, 2.5 equiv). The reaction mixture was heated at 90 °C for 2 hr, cooled, filtered through celite pad. Celite pad was washed with hot dioxane (3 x 10 ml). The solvent was

evaporated and the residue was purified by flash chromatography using a C18 reverse phase column (C18 Redi*SepRf* 43 g column, 10–60% acetonitrile in water for 30 min) to afford the product as off-white solid (0.133 g, 66%). TLC (CH_2Cl_2:MeOH = 85:15) *Rf* = 0.53; ^{1}H NMR (400 MHz, *d*$_6$-DMSO): δ (ppm) 11.79 (br, 1H), 8.83 (s, 1H), 7.90 (d, *J* = 8.24 Hz, 1H), 7.79 (d, *J* = 6.80 Hz, 1H), 7.78 (s, 1H), 7.37–7.28 (m, 2H), 5.85 (d, *J* = 4.0 Hz, 1H), 5.54 (t, *J* = 4.4 Hz, 1H), 5.51 (d, *J* = 5.2 Hz, 1H), 5.12 (d, *J* = 5.6 Hz, 1H), 4.16 (dd, *J* = 9.2 Hz, *J* = 4.8 Hz, 1H), 4.10 (dd, *J* = 5.2 Hz, *J* = 10.4 Hz, 1 H), 3.96–3.94 (m, 1H), 3.84–3.80 (m, 1H), 3.70–3.67 (m, 1H); ^{13}C NMR (100 MHz, *d6*-DMSO): δ (ppm) 161.4, 149.5, 139.1, 138.9, 137.6, 135.2, 124.4, 124.0, 123.1, 122.0, 119.0, 107.8, 89.0, 84.5, 74.4, 69.1, 60.0; MALDI-TOF MS (m/z): Calculated for $C_{17}H_{16}N_2O_6S$ [M] = 376.073, found $[M+K]^+$ = 414.931; λ_{max} (H_2O) = 274 and 318 nm, ε_{274} = 11760 $M^{-1}cm^{-1}$, ε_{318} = 9347 $M^{-1}cm^{-1}$, ε_{260} = 9040 $M^{-1}cm^{-1}$.

Benzo[*b*]thiophene-conjugated uridine triphosphate 3: To an ice cold solution of ribonucleoside **2** (0.090 g, 0.24 mmol, 1 equiv) in trimethyl phosphate (1 ml) was added freshly distilled $POCl_3$ (60 μL, 0.64 mmol, 2.7 equiv). The solution was stirred for 34 h at 0–4 °C. TLC revealed only partial conversion of the ribonucleoside into the product. A solution of *bis*-tributylammonium pyrophosphate[S1] (0.5 M in DMF, 2.7 ml, 5.7 equiv) and tributylamine (0.63 ml, 2.64 mmol, 11 equiv) was rapidly added under ice-cold condition. The reaction was quenched after 30 min with 1 M triethylammonium bicarbonate buffer (TEAB, pH 7.5, 18 ml) and was extracted with ethyl acetate (20 ml). The aqueous layer was evaporated and the residue was purified first on DEAE sephadex-A25 anion exchange column (10 mM–1M TEAB buffer, pH 7.5) followed by reverse-phase flash column chromatography (C18 Redi*SepRf*, 0–40% acetonitrile in 100 mM triethylammonium acetate buffer, pH 7.2, 40 min). Appropriate fractions were lyophilized to afford the desired triphosphate product **3** as a tetratriethylammonium salt (24 mg, 10%). ^{1}H NMR (400 MHz, D_2O): δ (ppm) 8.17 (s, 1H), 7.88 (d, *J* = 7.6 Hz, 1H), 7.86 (s, 1H), 7.74 (s, 1H), 7.40–7.31 (m, 2H), 5.92 (d, *J* = 2.8 Hz, 1H), 4.45 (br, 2H), 4.30–4.27 (m, 3H); ^{13}C NMR (100 MHz, D_2O): δ (ppm) 162.5, 150.2, 139.1, 138.9, 136.8, 133.1, 124.7, 124.6, 123.8, 121.9, 121, 109.4, 89.1, 83.4, 73.7, 69.8, 58.6; ^{31}P NMR (162 MHz, D_2O): δ (ppm) -8.87 (br, P_γ), -10.98 (br, P_α), -22.04 (br, P_β); MALDI-TOF MS negative mode (m/z): Calculated for $C_{17}H_{19}N_2O_{15}P_3S$ [M] = 615.972, found $[M-H]^-$ = 615.035.

4. Photophysical characterization of ribonucleoside 2:

Steady-state fluorescence of ribonucleoside 2 in various solvents: Ribonucleoside **2** (5 μM) in water, methanol, acetonitrile and dioxane was excited at respective lowest energy absorption maximum with an excitation slit width of 4 nm and emission slit width of 6 nm, and the emission profile in each solvent was recorded. All solutions contained 0.5% DMSO. Fluorescence experiments were performed in triplicate in a micro fluorescence cell (Hellma, path length 1.0 cm) on a Horiba Jobin Yvon, Fluorolog-3.

Time-resolved fluorescence measurements: Lifetimes of **2** (5 μM) were determined under the same conditions as mentioned above using TCSPC instrument (Horiba Jobin Yvon, Fluorolog-3). Fluorescently modified ribonucleoside **2** was excited using 339 nm LED source (IBH, UK, NanoLED-339L) with a band pass of 6 nm and fluorescence signal at respective emission maximum was collected. Lifetime measurements were performed in duplicate and decay profiles were analyzed using IBH DAS6 analysis software. Fluorescence intensity decay profiles in all solvents were found to be biexponential with χ^2 (goodness of fit) values very close to unity.

Radiative and non-radiative decay rate constants: Radiative (k_r) and nonradiative decay (k_{nr}) rate constants of **2** in different solvents were determined from their respective quantum yield (Φ) and average lifetime (τ) using following equations.[S2]

$$k_r = \Phi/\tau$$

$$1/\tau = k_r + k_{nr}$$

5. Quantum yield determination: Quantum yield of ribonucleoside **2** in different solvents relative to 2-aminopurine standard was determined using the following equation (Du, H.; Fuh, R. A.; Li, J.; Corkan, A.; Lindsey, J. S. *Photochem. and Photobiol.,* **1998**, *68*, 141–142).

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

Where s is the standard, x is the ribonucleoside, 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 (Lavabre, D.; Fery-Forgues, S. *J. Chem. Educ.*, **1999**, *76*, 1260–1264).

Table S1. Lifetimes of fluorescent ribonucleoside **2** in various solvents

Sample	λ_{em} (nm)	τ_1[a] (ns)	τ_2[a] (ns)	τ_{Ave}[b] (ns)
Water	458	0.97 (0.95)	2.45 (0.05)	1.04
Methanol	446	0.33 (0.72)	1.27 (0.28)	0.60
Acetonitrile	443	0.25 (0.60)	0.87 (0.40)	0.49
Dioxane	435	0.20 (0.77)	1.40 (0.23)	0.45

[a]Relative amplitude is given in parenthesis. [b]Standard deviations for τ_{ave} in solvents are $\leq$ 0.02 ns.

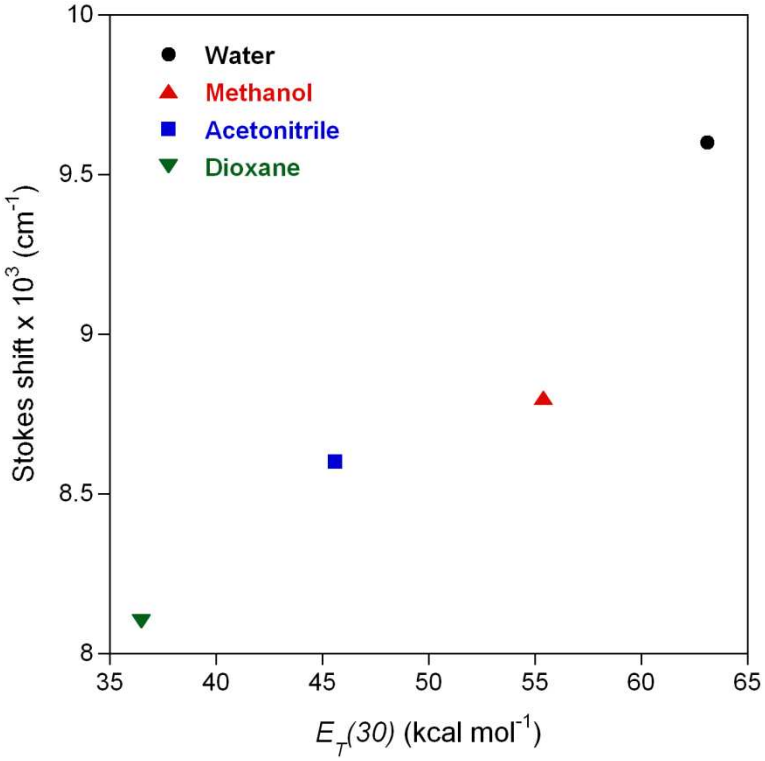


Figure S1. A plot of Stokes shift vs. $E_T(30)$, microscopic solvent polarity parameter showing good linear correlation.

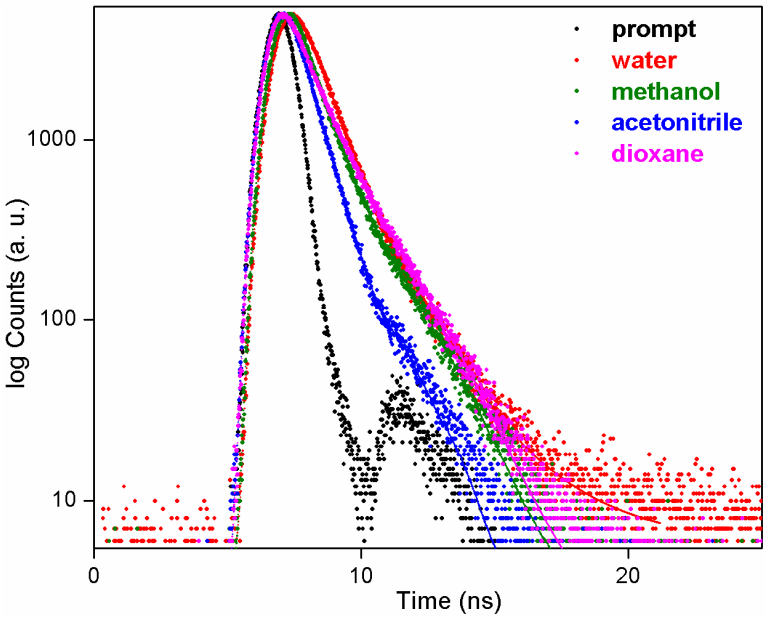


Figure S2. Excited state decay profile of ribonucleoside **2** (5 μM) in various solvents. Laser profile is shown in black (prompt). Curve fits are shown in solid lines. For experimental details see section 4.

6. Enzymatic incorporation of ribonucleoside triphosphate 3:

Transcription reactions with α-^{32}P ATP: Promoter-template duplexes were assembled by heating a 1:1 concentration (final 5 μM) of deoxyoligonucleotide templates (**T1**–**T5**) and an 18-mer T7 RNA polymerase consensus promoter deoxyoligonucleotide sequence in TE buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.8) at 90 °C for 3 min and cooling the solution slowly to room temperature. The duplexes were then placed on crushed ice for 20 min and stored at -40 °C. Transcription reactions were performed in 40 mM Tris-HCl buffer (pH 7.9) containing 250 nM annealed templates, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM dithiothreitol (DTT), 2 mM spermidine, 1 U/μL RNase inhibitor (RiboLock), 1 mM GTP, 1 mM CTP, 1 mM UTP and or 1 mM modified UTP **3**, 20 μM ATP, 5 μCi α-^{32}P ATP and 3 U/μL T7 RNA polymerase in a total volume of 20 μL. After 3.5 h at 37 °C, reactions were quenched by adding 20 μL of the loading buffer (7 M urea in 10 mM Tris-HCl, 100 mM EDTA, 0.05% bromophenol blue, pH 8), heated to 75 °C for 3 min and cooled on an ice bath. The samples (4 μL) were loaded onto a sequencing 18% denaturing polyacrylamide gel. The products on the gel were exposed to X-ray film (1–2 h) and the exposed film was developed, fixed and dried. The bands were then quantified using the software (GeneTools from Syngene) to determine the relative transcription efficiencies. Percentage incorporation of modified ribonucleoside triphosphate **3** is

reported with respect to transcription efficiency in the presence of natural NTPs. All reactions were performed in duplicate and the standard deviations were ± 6%.

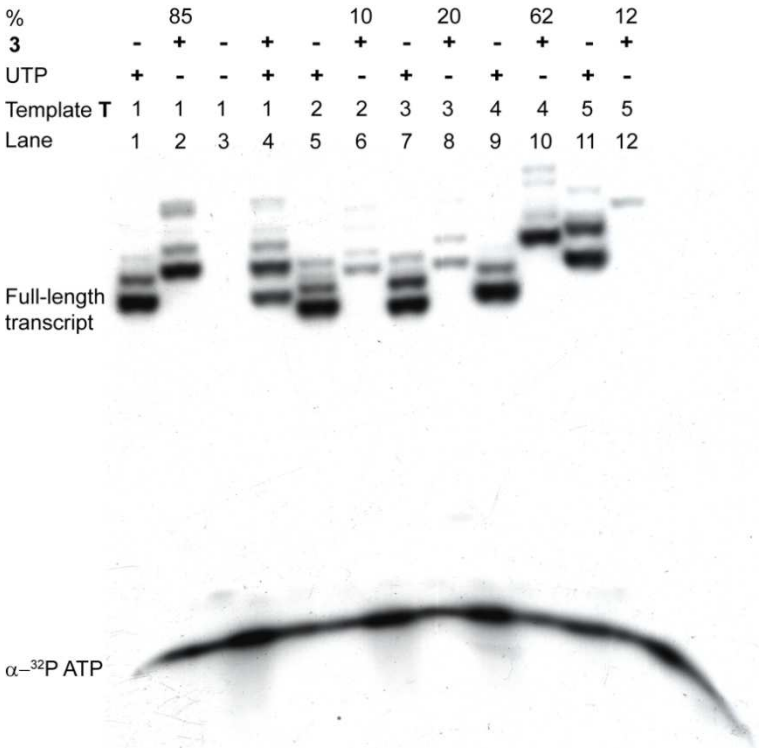


Figure S3. In vitro transcription reactions with templates **T1–T5** in the presence of UTP and modified ribonucleoside triphosphate **3**. Percentage incorporation of **3** is reported with respect to transcription efficiency in the presence of natural NTPs. All reactions were performed in duplicate and the standard deviations were ± 6%.

Large-scale transcription reactions: Large-scale transcription reactions using template **T1** were performed in a 250 µL reaction volume under similar conditions to isolate oligoribonucleotides **4** and **5** for further characterization and fluorescence studies. The reaction contained 2 mM GTP, 2 mM CTP, 2 mM ATP, 2 mM UTP or 2 mM modified UTP **3**, 20 mM $MgCl_2$, 0.4 U/µL RNase inhibitor (RiboLock), 300 nM annealed template and 800 units T7 RNA polymerase. After incubation for 12 h at 37 °C, the precipitated salt of pyrophosphate was removed by centrifugation. The reaction volume was reduced approximately to 1/3 by Speed Vac, and 50 µL of loading buffer was added. The mixture was heated at 75 °C for 3 min and cooled on the ice bath. The sample was loaded onto a preparative 20% denaturing polyacrylamide gel. The gel was UV shadowed; appropriate band was excised, extracted with

0.3 M sodium acetate and desalted using Sep-Pak classic C18 cartridge. Typical yields of the transcripts are 14–15 nmol. Transcript **4**, ε_{260} = 91000 $M^{-1}cm^{-1}$; transcript **5**, ε_{260} = 90340 $M^{-1}cm^{-1}$.

7. MALDI-TOF MS measurements: Molecular weight of RNA transcript **5** was determined using Applied Biosystems 4800 Plus MALDI TOF/TOF analyzer. 1 µL of a ~150 µM stock solution of the transcript was combined with 1 µL of 100 mM ammonium citrate buffer (pH 9), 1 µL of a 100 µM DNA standard (18-mer) and 4 µL of saturated 3-hydroxypiccolinic acid solution. The samples were desalted with an ionexchange resin (Dowex 50W-X8, 100-200 mesh, ammonium form) and spotted on the MALDI plate, and were air dried. The resulting spectra were calibrated relative to an internal 18-mer DNA standard.

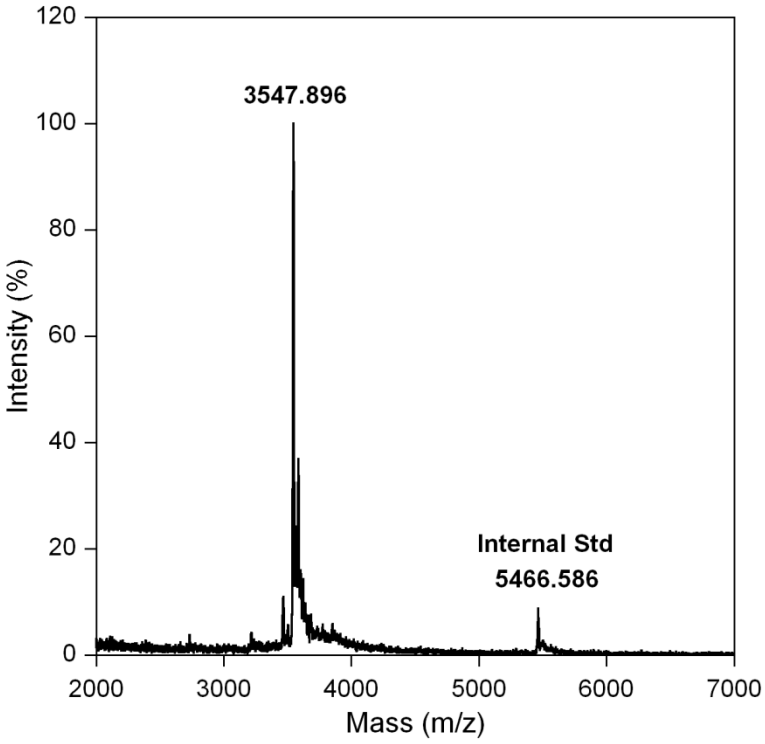


Figure S4. MALDI-TOF MS spectrum of the modified RNA transcript **5** calibrated relative to the +1 ion of an internal 18-mer DNA oligonucleotide standard (m/z: 5466.586). Calculated mass [M] = 3547.075; observed mass = 3547.896.

8. Photophysical characterization of transcript 5:

Steady-state fluorescence of benzo[*b*]thiophene-modified duplexes: Oligoribonucleotide **5** (10 μM) was annealed to custom DNA and RNA oligonucleotides (**6–10**, 11 μM) by heating a 1:1.1 mixture of the oligonucleotides in 20 mM cacodylate buffer (pH 7.1, 500 mM NaCl, 0.5 mM EDTA) at 90 °C for 3 min and cooling the samples slowly to room temperature, followed by incubating the solutions in crushed ice. Samples were diluted to give a final concentration of 1 μM (with respect to **5**) in cacodylate buffer. Fluorescently modified duplex constructs were excited at 320 nm with an excitation slit width of 10 nm and emission slit width of 12 nm. Fluorescence experiments were performed at 18 °C in a micro fluorescence cell (Hellma, path length 1.0 cm) on a Horiba Jobin Yvon, Fluorolog-3.

Time-resolved fluorescence measurements: Lifetimes of the duplexes (2 μM) were determined under the same conditions as mentioned above using TCSPC instrument (Horiba Jobin Yvon, Fluorolog-3). Fluorescently modified oligoribonucleotides were excited using 339 nm LED source (IBH, UK, NanoLED-339L) with a band pass of 10 nm and fluorescence signal at respective emission maximum was collected. Experiments were performed in duplicate and decay profiles were analyzed using IBH DAS6 analysis software. Fluorescence intensity decay profiles for all oligonucleotide constructs were found to be biexponential with χ^2 (goodness of fit) values very close to unity.

Table S2. Steady-state and time-resolved fluorescence spectroscopy measurements of benzo[*b*]thiophene-conjugated ribonucleoside **2** and oligoribonucleotide constructs.[a]

Sample	λ_{em} (nm)	Φ	τ_1 (ns)	τ_2 (ns)	τ_{Ave} (ns)	k_r/k_{nr}
2	475	0.027 ± 0.0003	1.12	-	1.12 ± 0.004	0.028
5	482	0.010 ± 0.002	0.67 (0.77)	3.03 (0.23)	1.21 ± 0.005	0.010
5•6	470	0.011 ± 0.003	0.65 (0.86)	3.11 (0.14)	1.00 ± 0.03	0.011
5•7	488	ND	0.77 (0.76)	3.41 (0.24)	1.41 ± 0.03	ND
5•8	482	ND	0.72 (0.49)	4.15 (0.51)	2.46 ± 0.12	ND
5•9	476	ND	0.96 (0.60)	4.14 (0.40)	2.23 ± 0.05	ND
5•10	463	0.013 ± 0.002	0.63 (0.86)	3.13 (0.14)	0.98 ± 0.05	0.013

[a]For conditions, see section 8. Relative amplitude is given in parenthesis. ND = not determined.

9. Quenching Studies and Stern-Volmer plot: Quenching of ribonucleoside fluorescence by NMPs was performed by adding aliquots of concentrated nucleotide solution (40 mM) to the ribonucleoside **2**, where both solutions contained equal concentration of the ribonucleoside (5 μM). All solutions were prepared in 20 mM cacodylate buffer containing 0.5% DMSO (100 mM NaCl, 0.5 mM EDTA, pH 7.1). The ribonucleoside was excited at 320 nm with an excitation slit width of 4 nm and emission slit width of 6 nm, and changes in fluorescence was monitored at the emission maximum, 475 nm.

Stern-Volmer plot was obtained by plotting F_0/F vs. concentration of the quencher (NMPs) and was fit using the following equation.

$$F_0/F = 1 + K_{sv}[\text{NMPs}]$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quenchers (NMPs) respectively, and K_{sv} is the quenching constant.

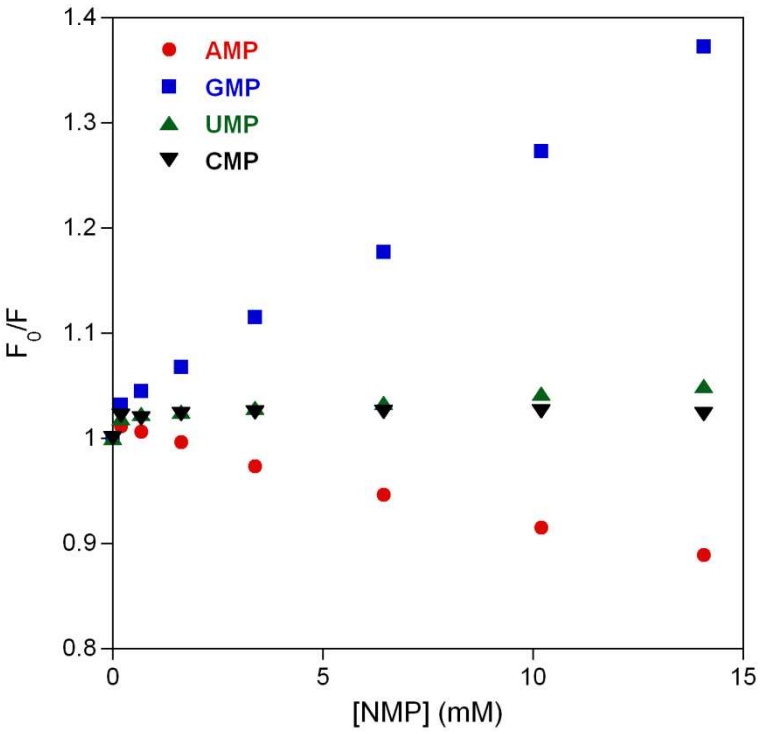


Figure S5. Steady-state Stern-Volmer plot for the titration of ribonucleoside **2** with NMPs. UMP and CMP did not show any quenching. GMP shows very marginal quenching with a K_{sv} of 24.9 ± 2.4 μM^{-1}. Interesting, AMP shows fluorescence enhancement with a very small negative K_{sv} of -8.7 ± 0.9 μM^{-1}. Taken together, collisional quenching of ribonucleoside's fluorescence by NMPs is practically insignificant.[S2] Therefore, diffusion controlled collisional interaction does not significantly contribute to the observed fluorescence quenching of ribonucleoside **2** within oligoribonucleotides.

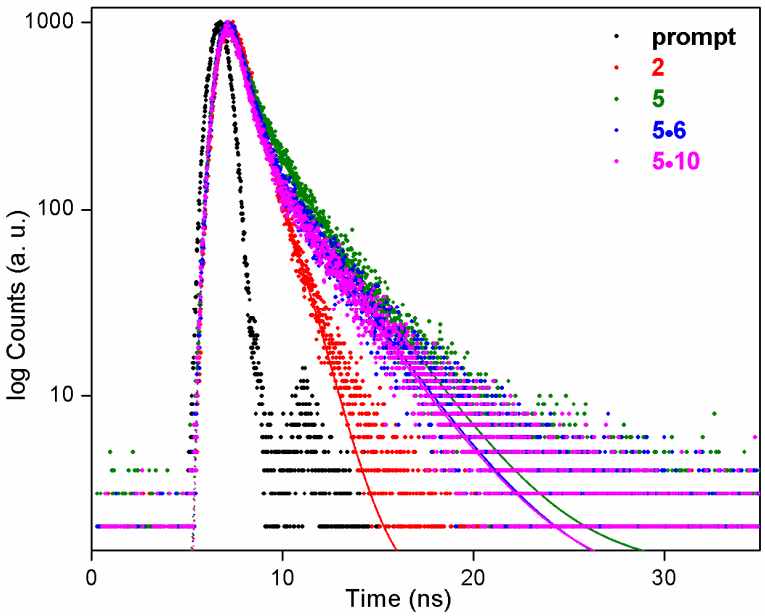


Figure S6. Excited state decay profile of ribonucleoside **2**, RNA transcript **5** and duplexes **5•6** and **5•10**. Curve fits are shown in solid lines. Laser profile is shown in black (prompt).

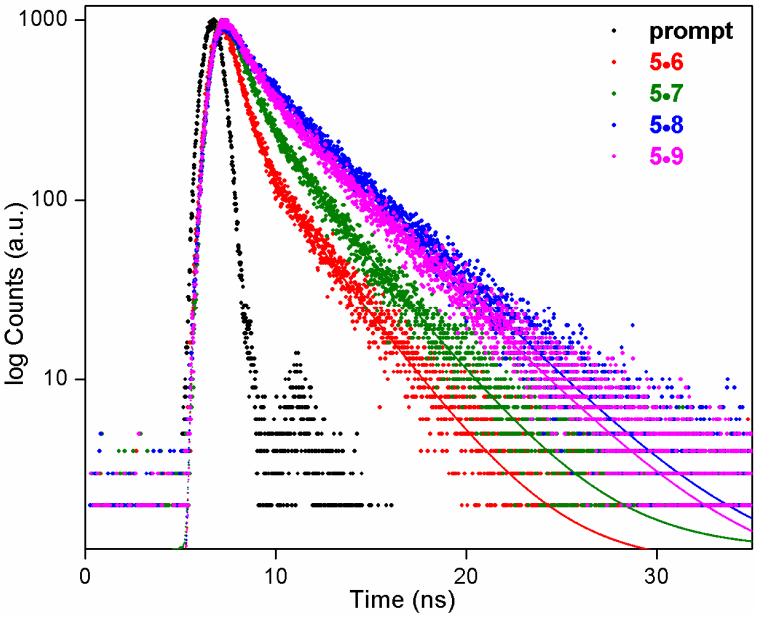


Figure S7. Excited state decay profile of duplexes **5•6**, **5•7**, **5•8** and **5•9**. Curve fits are shown in solid lines. Laser profile is shown in black (prompt).

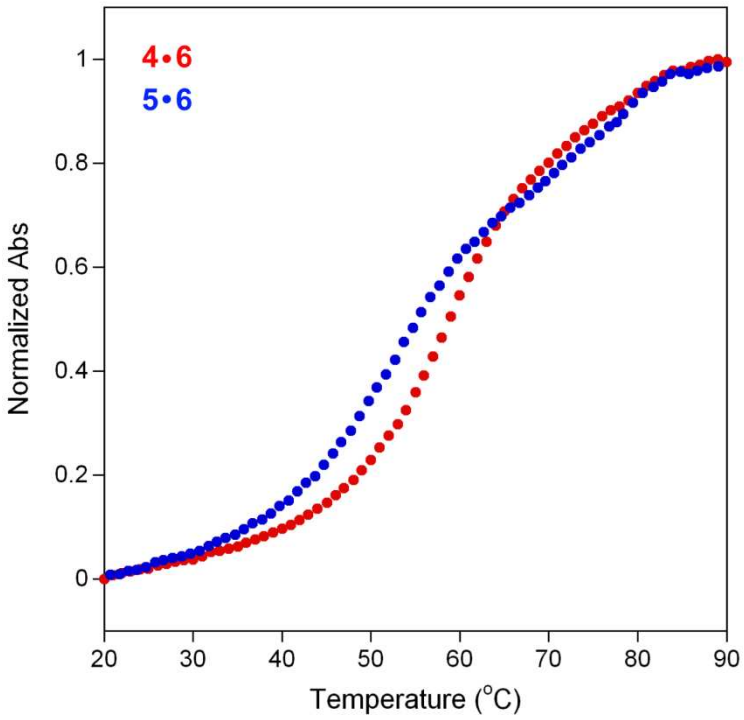


Figure S8. UV-thermal melting of control unmodified duplex **4•6** (red, 1 μM) and fluorescently-modified duplex **5•6** (blue, 1 μM) in 20 mM cacodylate buffer (pH 7.1, 500 mM NaCl, 0.5 mM EDTA). Duplexes were hybridized by heating a 1:1 mixture of the oligonucleotides at 90 °C for 3 min and cooling the solutions slowly to room temperature. Samples were placed in crushed ice for at least 30 min before analysis. T_m for duplexes **4•6** and **5•6** are 57.7 ± 0.9 °C and 54.4 ± 1.1 °C, respectively.

10. Gel mobility shift experiments using ^{32}P-labeled oligoribonucleotide 5:

^{32}P-labeled oligoribonucleotide **5** was synthesized by transcription reaction using α-^{32}P ATP in the presence of template **T1**. Transcription reaction was performed in 40 mM Tris-HCl buffer (pH 7.9) containing 300 nM annealed template **T1**, 15 mM $MgCl_2$, 10 mM NaCl, 10 mM dithiothreitol (DTT), 2 mM spermidine, 0.4 U/μL RNase inhibitor (RiboLock), 0.4 mM GTP, 0.4 mM CTP, 1 mM modified UTP **3**, 40 μM ATP, 25 μCi α-^{32}P ATP and 3.2 U/μL T7 RNA polymerase in a total volume of 50 μL. After 6 h at 37 °C, reactions were quenched by adding 25 μL of the loading buffer, heated to 75 °C for 3 min and cooled on an ice bath. The sample was loaded onto a preparative 20% denaturing polyacrylamide gel. The radioactive band corresponding to the full-length RNA transcript **5** was excised and extracted with 0.3 M sodium acetate, and finally desalted using Sep-Pak classic C18 cartridge.

Non-radiolabeled transcript RNA **5** was doped with ^{32}P-labeled **5**, and was annealed to custom DNA and RNA oligonucleotides (**6–10**) under the same conditions used in the

fluorescence experiments. To each sample was added 5 μL of non-denaturing loading buffer (10 mM Tris-HCl, pH 8, 500 mM NaCl, 30% sucrose, 10% glycerol, 0.05% bromophenol blue), and samples (6 μL) were loaded onto an analytical 18% non-denaturing polyacrylamide gel containing 500 mM NaCl. The gel was electrophoresed in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA) containing 500 mM NaCl at ~20 °C (150 V). The buffer was recirculated using peristaltic pump. The products on the gel were exposed to X-ray film (3 h), and the exposed film was developed, fixed and dried.

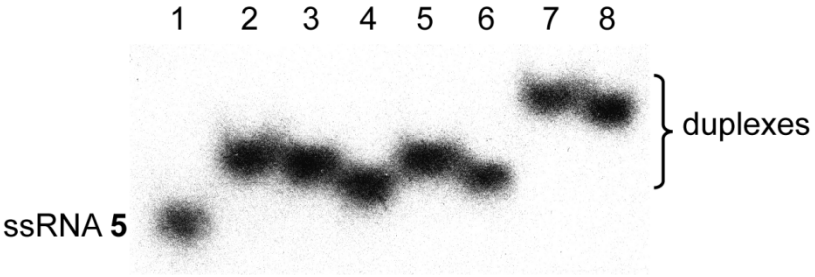
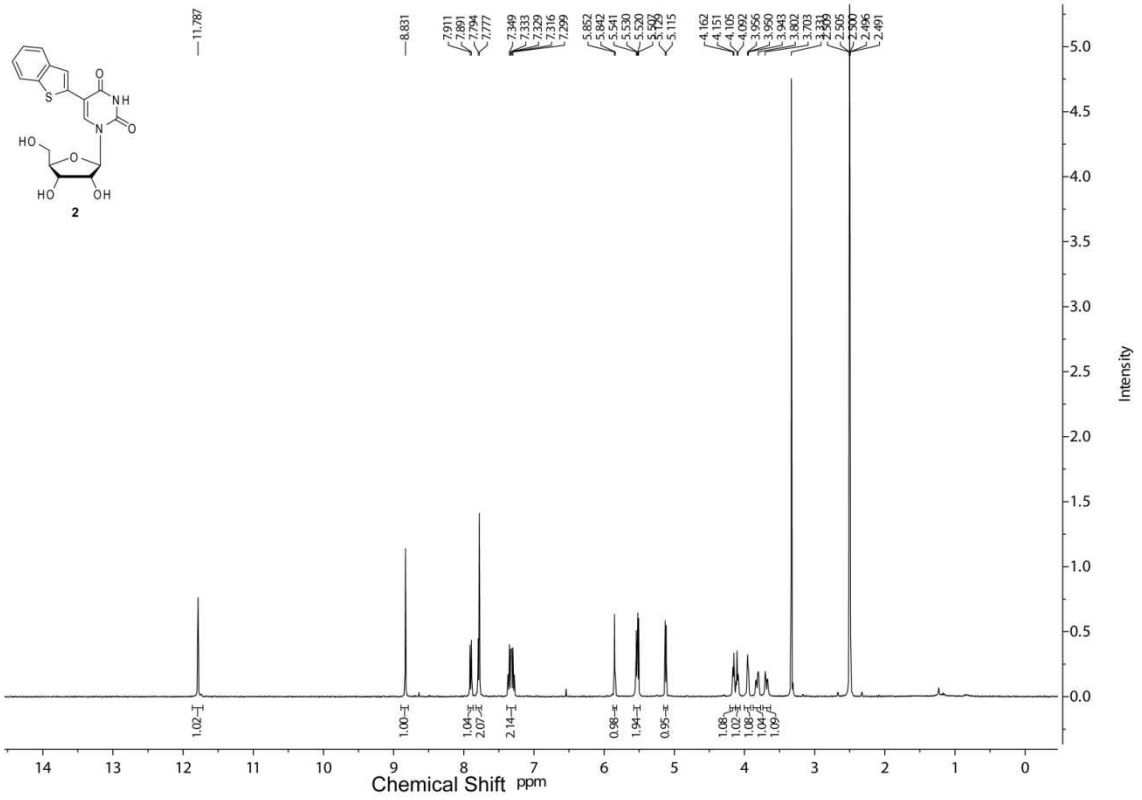
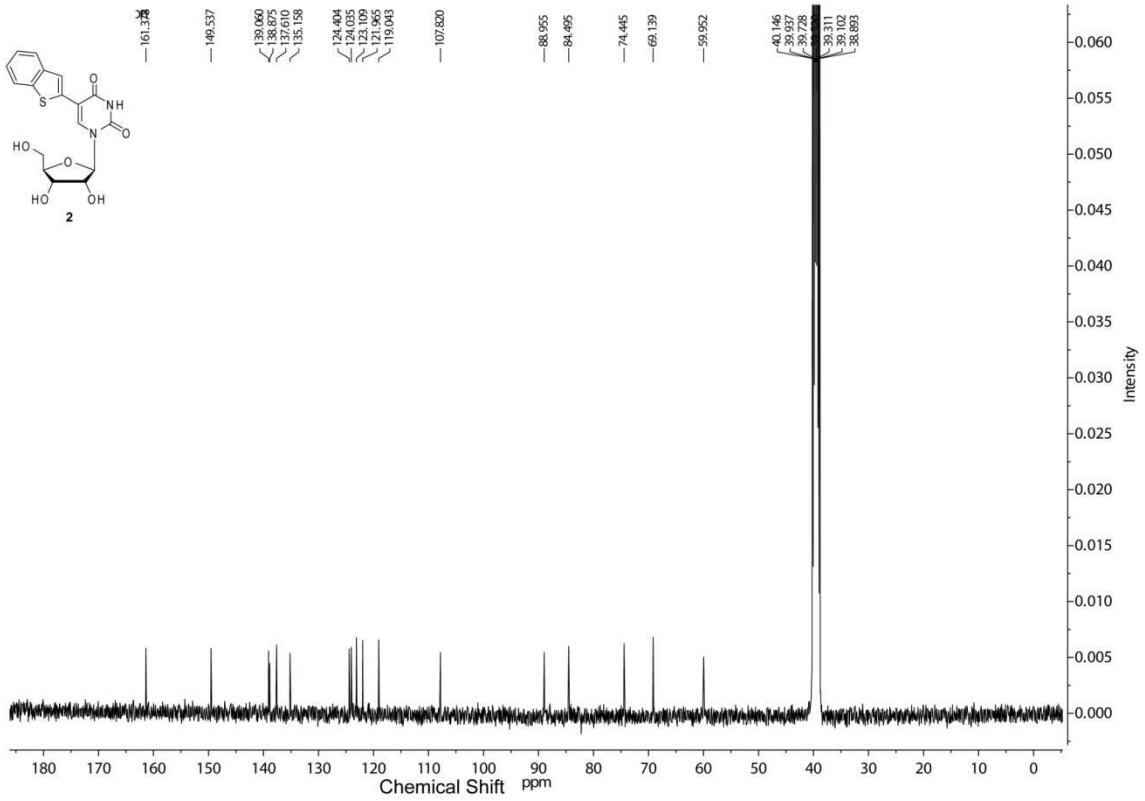


Figure S9. Gel mobility shift experiments to determine the hybridization efficiency of duplexes made of fluorescent oligoribonucleotide **5** and complementary and mismatched DNA and RNA oligonucleotides. Lane 1, single stranded oligoribonucleotide **5**; Lanes 2–5 and 7, duplexes **5•6**, **5•8**, **5•7**, **5•9** and **5•10**, respectively. Lanes 6 and 8, duplexes made of **5** and complementary DNA and RNA oligonucleotides containing an abasic site opposite to the ribonucleoside **2**, respectively. Native gel retardation experiments clearly reveal complete hybridization.

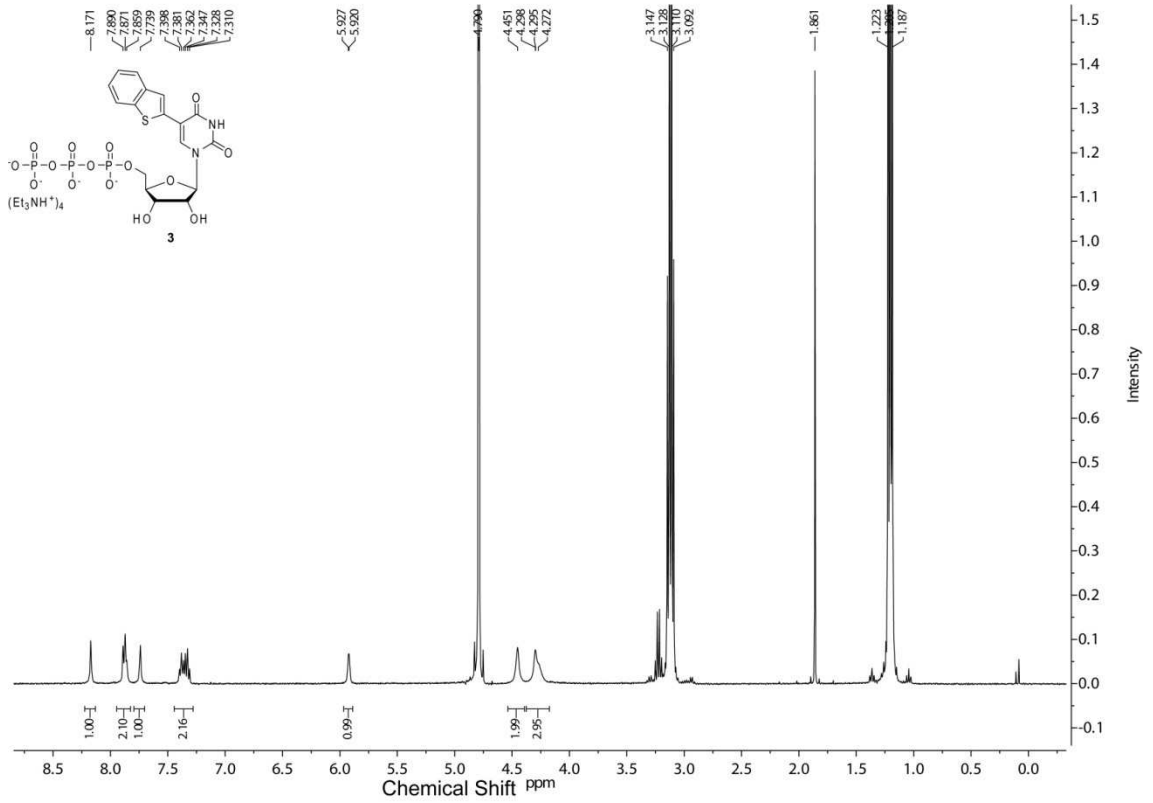
^{1}H NMR (400 MHz, *d*$_{6}$-DMSO)



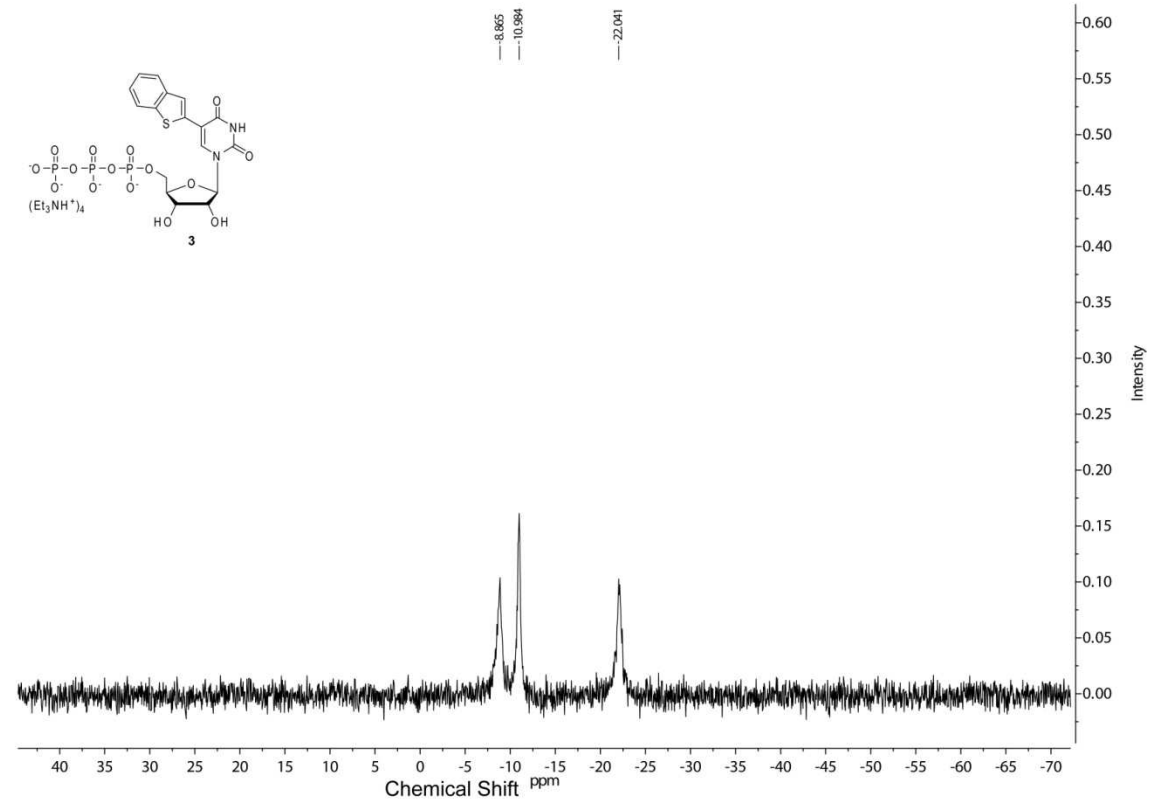
^{13}C NMR (100 MHz, *d6*-DMSO)



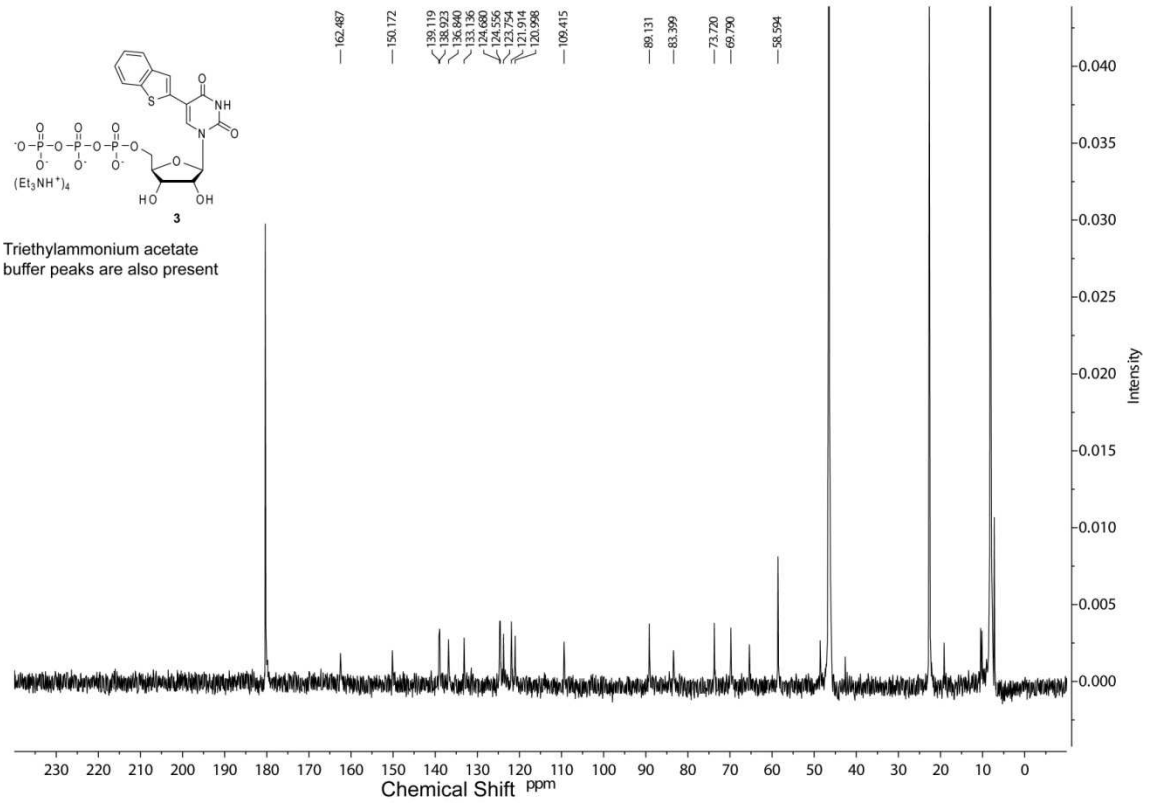
^{1}H NMR (400 MHz, D_2O)



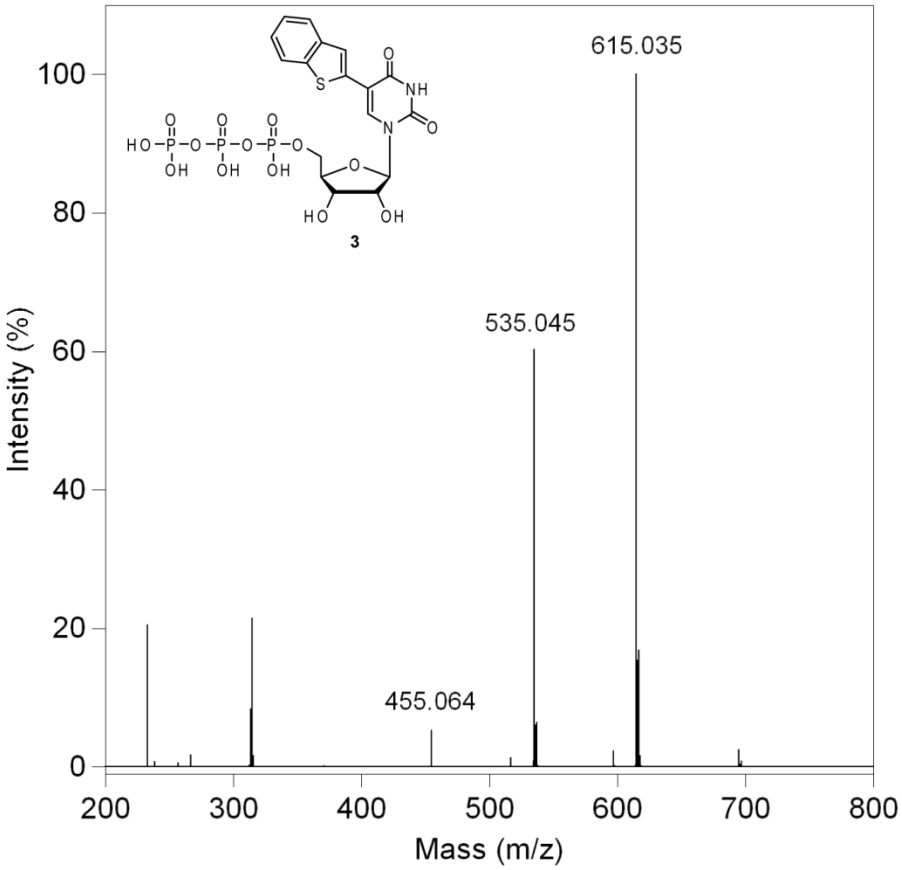
^{31}P NMR (162 MHz, D_2O)



^{13}C NMR (100 MHz, D_2O)



MALDI-TOF MS spectrum



MALDI-TOF MS spectrum of modified triphosphate **3** in negative mode showing m/z values for triphosphate, and fragmented di- and monophosphate. Calculated for $C_{17}H_{19}N_2O_{15}P_3S$ [M] = 615.972, found $[M-H]^-$ = 615.035.

12. References

S1. Moffatt, J. G. *Can. J. Chem.* **1964**, *42*, 599–604.

S2. J. R. Lakowicz. *Principles of Fluorescence Spectroscopy*, 3rd ed., Springer, New York (2006).