Electronic Supplementary Material (ESI) for Chemical Communications

Posttranscriptional Chemical Functionalization of Azide-Modified Oligoribonucleotides by Bioorthogonal Click and Staudinger Reactions

Harita Rao, Anupam A. Sawant, Arun A. Tanpure, Seergazhi G. Srivatsan*

Indian Institute of Science Education and Research, Sai Trinity Building, Pashan, Pune 411021, India

Supplementary Information

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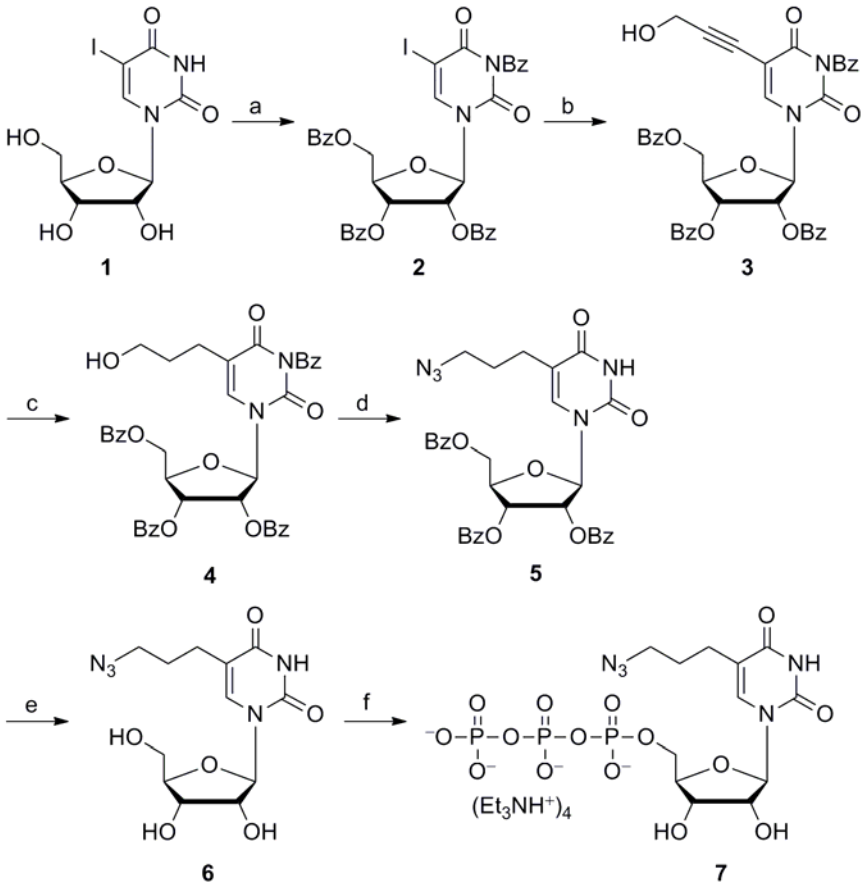
1. Materials: 5-iodouridine, 10% Pd/C, sodium azide, tetrakis(triphenylphosphine)palladium(0) and biotin were obtained from Sigma-Aldrich. Benzoyl Chloride was purchased from Merck-India. MsCl was obtained from Avra Synthesis, India. $POCl_3$ was purchased from Acros Organics, and was distilled before use. Triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt (TPPTS) was obtained from Alpha Aesar. Biotin-alkyne substrate **15** was synthesis by reacting biotin with propargyl amine in the presence of EDC.HCl.[S1] The water-soluble CuI stabilizing ligand, tris-(3-hydroxypropyltriazolylmethyl)amine (THPTA) was synthesized by following a literature report.[S2] DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. Oligonucleotides were purified by gel electrophoresis under denaturing condition and desalted using Sep-Pak Classic C18 cartridges (Waters Corporation). T7 RNA polymerase, ribonuclease inhibitor (RiboLock), NTPs, RNase A and RNase T1 were obtained from Fermentas Life Science. Calf intestinal alkaline phosphatase (CIP) and snake venom phosphodiesterase I was procured from Invitrogen and Sigma-Aldrich, respectively. Radiolabeled α-^{32}P ATP (2000 Ci/mmol) was purchased 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 HPLC analyses.

2. Instrumentation: NMR spectra were recorded on a 400 MHz Jeol ECS-400. Mass measurements were recorded on Applied Biosystems 4800 Plus MALDI TOF/TOF analyzer and MicroMass ESI-TOF mass spectrometer. Absorption spectra were recorded on a PerkinElmer, Lambda 45 UV-Vis spectrophotometer. Reversed-phase (RP) flash chromatographic (C18 Redi*SepRf* column) purifications were carried out using Teledyne ISCO, Combi Flash Rf. RP-

HPLC analyses were performed using Dionex ICS 3000. Phosphorimages were recorded on a Typhoon Trio+, GE-Healthcare phosphorimager. Steady State 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).

3. Synthesis



Scheme S1. Synthesis of azide-modified ribonucleoside **6** and triphosphate **7**. Reagents and conditions: (a) benzoyl chloride, DMAP, pyridine, RT, 97%. (b) propargyl alcohol, $Pd(PPh_3)_4$, CuI, Et_3N, DMF, RT, 74%. (c) Pd/C, H_2, MeOH, RT, 83%. (d) (i) MsCl, Et_3N, CH_2Cl_2, 0 °C; (ii) NaN_3, DMF, 40 °C, 87%. (e) sodium methoxide, MeOH, 60 °C, 52%. (f) (i) $POCl_3$, $(MeO)_3PO$, ~4 °C; (ii) tributylammonium pyrophosphate, Bu_3N, ~4 °C, 26%.

***N*^{3},2′,3′,5′-*O*-Tetrabenzoyl-5-iodouridine (2)[S3]:** Benzoyl chloride (15.3 mL, 131.9 mmol, 15 equiv) was added to a solution of 5-iodouridine **1** (3.26 gm, 8.81 mmol, 1 equiv) and DMAP

(0.115 gm, 0.94 mmol, 0.1 equiv) in anhydrous pyridine (75 mL). The reaction mixture was stirred at room temperature (RT) for 18 h. Solvent was evaporated, and the residue was coevaporated twice with toluene. The resulting syrup was dissolved in ethyl acetate (200 mL) and washed with water, saturated bicarbonate followed by brine. The organic extract was dried over sodium sulphate and evaporated. The resulting residue was purified by silica gel column chromatography to afford the product **2** as pale yellow solid (6.70 g, 97%). TLC (pet. ether:EtOAc = 70:30) R_f = 0.37; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.15 (d, J = 7.6 Hz, 2H), 8.00 (d, J = 7.2 Hz, 2H), 7.99 (s, 1H), 7.89 (t, J = 8.0 Hz, 4H), 7.65–7.57 (m, 3H), 7.52 (t, J = 7.6 Hz, 3H), 7.43–7.39 (m, 4H), 7.32 (t, J = 7.8 Hz, 2H), 6.38 (d, J = 6.4 Hz, 1H), 5.91 (dd, J = 6.0, 3.2 Hz, 1H), 5.76 (t, J = 6.2 Hz, 1H), 4.83 (dd, J = 13.8, 3.7 Hz, 1H), 4.77–4.72 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 167.3, 166.2, 165.7, 165.4, 158.6, 149.2, 143.7, 135.4, 134.0, 133.9, 130.9, 130.7, 130.0, 130.0, 129.9, 129.3, 129.2, 129.1, 128.8, 128.7, 128.6, 128.2, 87.8, 81.3, 74.2, 71.6, 69.6, 63.9; HRMS: (m/z): Calculated for $C_{37}H_{27}IN_2O_{10}Na$ $[M+Na]^+$ = 809.0608, found = 809.0605.

***N*^{3},2′,3′,5′-*O*-Tetrabenzoyl-5-(3-hydroxypropynl) uridine (3):** Perbenzoylated iodouridine **2** (5.11 g, 6.5 mmol, 1 equiv), CuI (0.124 g, 0.65 mmol, 0.1 equiv), tetrakis(triphenylphosphine)palladium(0) (0.376 g, 0.33 mmol, 0.05 equiv) and Et_3N (1.9 mL, 13.63 mmol, 2.1 equiv) were dissolved in degassed anhydrous DMF (25 ml). Propargyl alcohol (0.76 mL, 13 mmol, 2 equiv) was added to the above solution. The reaction mixture was stirred at RT for 4 h in dark. Solvent was evaporated and the resulting syrup was dissolved in ethyl acetate (300 mL), and washed with water, saturated bicarbonate and brine. The organic extract was dried over sodium sulphate and evaporated. The residue was purified by silica gel column chromatography to afford the product **3** as pale yellow solid (3.44 g, 74%). TLC (pet. ether:EtOAc = 50:50) R_f = 0.60; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.14–8.11 (m, 2H), 8.00 (d, J = 7.2 Hz, 2H), 7.92–7.85 (m, 5H), 7.63–7.56 (m, 3H), 7.53–7.48 (m, 3H), 7.43–7.39 (m, 4H), 7.30 ($_{app}$t, J = 7.8 Hz, 2H), 6.35 (d, J = 6.0 Hz, 1H), 5.90 (dd, J = 6.6, 3.4 Hz, 1H), 5.78 (t, J = 6.0 Hz, 1H), 4.80–4.70 (m, 3H), 4.21 (d, J = 2.4 Hz, 2H), 2.33 (t, J = 5.2 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 167.4, 166.2, 165.6, 165.4, 160.5, 148.4, 142.0, 135.4, 134.0, 133.8, 131.0, 130.7, 130.0, 129.9, 129.8, 129.3, 129.1, 129.0, 128.7, 128.6, 128.2, 101.0, 93.4, 88.3,

81.1, 75.4, 74.3, 71.5, 64.0, 51.3; HRMS: (m/z): Calculated for $C_{40}H_{30}N_2O_{11}Na$ $[M+Na]^+$ = 737.1747, found = 737.1746.

***N*³,2′,3′,5′-*O*-Tetrabenzoyl-5-(3-hydroxypropyl) uridine (4):** To an ice cold solution of **3** (2.516 g, 3.52 mmol, 1 equiv) in dry MeOH (100 mL) was added 10% Pd/C (1.132 g) in portions. The reaction mixture was stirred at RT under H_2 atmosphere for 18 h, and filtered through celite pad. The celite pad was washed with methanol (20 ml x 3). Solvent was evaporated, and the residue was purified by silica gel column chromatography to afford the product **4** as pale yellow solid (2.09 g, 83%). TLC (pet. ether:EtOAc = 20:80) R_f = 0.76; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.15 (d, *J* = 7.6 Hz, 2H), 8.00 (d, *J* = 7.6 Hz, 2H), 7.89 (t, *J* = 6.4 Hz, 4H), 7.65–7.50 (m, 6H), 7.41 (t, *J* = 7.4 Hz, 4H), 7.34–7.31 (m, 3H), 6.42 (d, *J* = 6.8 Hz, 1H), 5.93 (dd, *J* = 5.2, 3.2 Hz, 1H), 5.78 (t, *J* = 6.0 Hz, 1H), 4.89 (d, *J* = 12.0 Hz, 1H), 4.72–4.66 (m, 2H), 3.50 (t, *J* = 5.6 Hz, 2H), 2.28–2.14 (m, 2H), 2.04 (br, 1H), 1.60–1.52 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 168.4, 166.2, 165.7, 165.5, 162.8, 149.4, 135.7, 135.2, 134.1, 134.0, 134.0, 131.4, 130.6, 130.1, 130.0, 129.8, 129.3, 129.2, 129.1, 128.8, 128.7, 128.3, 115.7, 87.2, 80.9, 73.6, 71.6, 64.0, 61.0, 31.5, 23.0; HRMS: (m/z): Calculated for $C_{40}H_{34}N_2O_{11}Na$ $[M+Na]^+$ = 741.2060, found = 741.2067.

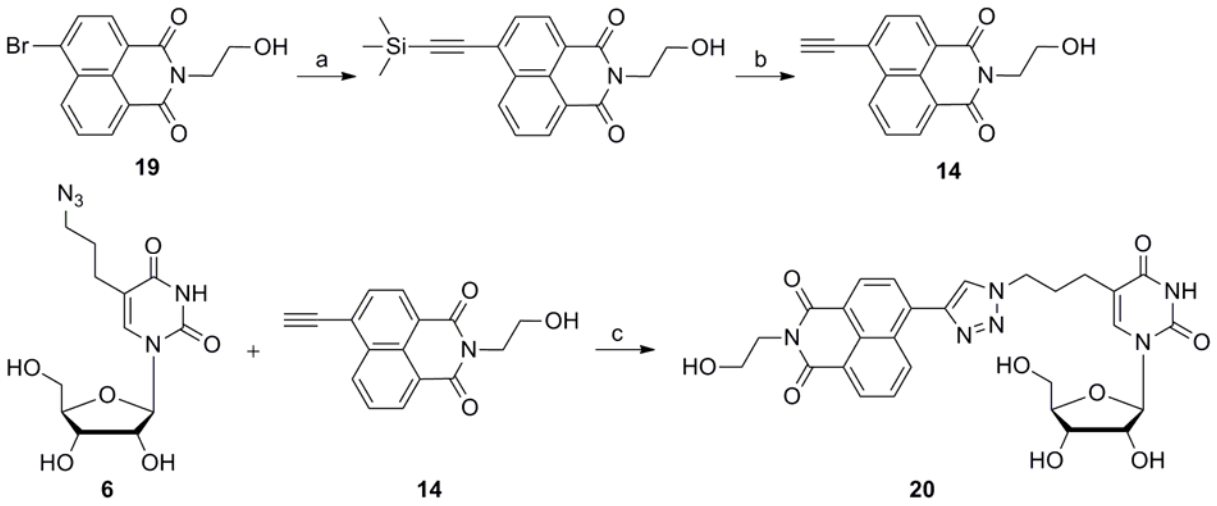
2′,3′,5′-*O*-Tribenzoyl-5-(3-azidopropyl) uridine (5): To an ice cold solution of **4** (2.01 g, 2.80 mmol, 1 equiv) in dry chloroform (30 mL) was added triethylamine (1.17 mL, 8.4 mmol, 3 equiv). Mesyl chloride (0.65 mL, 8.4 mmol, 3 equiv) was then added to the above solution over a period of 15 minutes. The reaction was stirred for 2 h in ice bath. To the reaction mixture was added saturated solution of ammonium chloride, and extracted in ethyl acetate (3 x 20 mL). The organic extract was dried over sodium sulphate and evaporated. The crude mesylated product (2.62 g) and NaN_3 (1.38 g, 21.37 mmol) were dissolved in dry DMF (25 mL). The reaction mixture was stirred at 40 °C for 6 h. Solvent was evaporated, and the resulting syrup was dissolved in ethyl acetate (100 mL), washed with water, saturated bicarbonate solution followed by brine. After drying over sodium sulphate the organic extract was evaporated. The residue was purified by silica gel column chromatography to afford the product **5** as pale yellow solid (1.55 g, 87%). TLC (Pet. ether:EtOAc = 50:50) R_f = 0.61; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.76 (br, 1H), 8.15 (dd, *J* = 8.2, 1 Hz, 2H), 8.00 (dd, *J* = 8.2, 1.4 Hz, 2H), 7.95 (dd, *J* =

8.2, 1 Hz, 2H), 7.66–7.50 (m, 5H), 7.44–7.34 (m , 4H), 7.19 (s, 1H), 6.41 (d, *J* = 6.5 Hz, 1H), 5.93 (dd, *J* = 5.8, 3.4 Hz, 1H), 5.78 (t, *J* = 6.4 Hz, 1H), 4.88 (dd, *J* = 12.2, 5.2 Hz, 1H), 4.71 (dd, *J* = 6.2, 3.4 Hz, 1H), 4.65 (dd, *J* = 12.0, 3.6 Hz, 1H), 3.13 (t, *J* = 6.6 Hz, 2H), 2.17–2.02 (m, 2H), 1.63–1.56 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 166.1, 165.5, 165.5, 163.0, 150.3, 135.8, 134.0, 133.9, 133.9, 130.1, 130.0, 129.8, 129.3, 129.0, 128.7, 128.7, 128.4, 114.9, 87.2, 80.8, 73.4, 71.6, 64.2, 50.6, 27.5, 24.4; MALDI-TOF MS (m/z): Calculated for $C_{33}H_{29}N_5O_9K$ $[M+K]^+$ = 678.16, found = 678.14.

5-(3-azidopropyl) uridine (6): To a flask containing compound **5** (1.50 g, 2.35 mmol, 1 equiv) was added sodium methoxide in dry methanol (0.5 M in MeOH, 200 mL) slowly. The reaction mixture was stirred for 30 min at 60 °C and was cooled to RT. The pH was adjusted to ~7 by adding 1N HCl. The reaction mixture was evaporated and the residue was purified by silica gel column chromatography to afford the product **6** as white solid (0.400 g, 52%). TLC (CH_2Cl_2:MeOH = 85:15) R_f = 0.78; ^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 11.32 (s, 1H), 7.77 (s, 1H), 5.77 (d, *J* = 5.2 Hz, 1H), 5.36 (d, *J* = 5.6 Hz, 1H), 5.13 (t, *J* = 5.2 Hz, 1H), 5.07 (d, *J* = 5.2 Hz, 1H), 4.04 (dd, *J* = 10.8, 5.2 Hz, 1H), 3.97 (dd, *J* = 9.4, 5.0 Hz, 1H), 3.83 (dd, *J* = 7.2, 3.2 Hz, 1H), 3.67–3.62 (m, 1H), 3.57–3.52 (m, 1H), 3.35–3.33 (m, 2H), 2.32–2.19 (m, 2H), 1.73–1.65 (m, 2H) ; ^{13}C NMR (100 MHz, d_6-DMSO): δ (ppm) 163.4, 150.6, 136.8, 112.4, 87.7, 84.7, 73.5, 69.8, 60.8, 50.2, 27.2, 23.7; HRMS: (m/z): Calculated for $C_{12}H_{17}N_5O_6Na$ $[M+Na]^+$ = 350.1077, found = 350.1078; ε_{260} = 8833 $M^{-1}cm^{-1}$.

5-(3-azidopropyl) uridine-5′-triphosphate (7): To an ice cold solution of azide-modified ribonucleoside **6** (0.063 g, 0.19 mmol, 1 equiv) in trimethyl phosphate (1.2 mL) was added freshly distilled $POCl_3$ (36 μL, 0.38 mmol, 2.5 equiv). The solution was stirred for 24 h at ~4 °C. A solution of *bis*-tributylammonium pyrophosphate[S4] (0.5 M in DMF, 2 mL, 5.2 equiv) and tributylamine (0.51 mL, 2.11 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, 15 mL) and was washed 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–1 M TEAB buffer, pH 7.5) followed by reversed-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 **7** as a tetratriethylammonium salt (57 mg, 26%); ^{1}H NMR (400 MHz, D_2O): δ (ppm) 7.76 (s, 1H), 5.96 (d, *J* = 5.2 Hz, 1H), 4.41–4.38 (m, 2H), 4.24–4.17 (m , 3H), 3.34 (t, *J* = 6.8 Hz, 2H), 2.45–2.39 (m, 2H), 1.87–1.74 (m, 2H) ; ^{13}C NMR (100 MHz, D_2O): δ (ppm); 165.9, 151.9, 137.8, 114.8, 87.8, 83.6 (d, *J* = 9.3 Hz), 73.4, 69.9, 65.2, 50.5, 27.0, 23.7 ; ^{31}P NMR (162 MHz, D_2O): δ (ppm) -9.30 (br, P_γ), -11.17 (br, P_α), -22.45 (br, P_β); ; MALDI-TOF MS (m/z) negative mode: Calculated for $C_{12}H_{20}N_5O_{15}P_3$ [M] = 567.02, found $[M-H]^-$ = 566.12.



Scheme S2. Synthesis of alkyne substrate **14** and click product **20**. Reagents and conditions: (a) CuI, $Pd(PPh_3)_4$, TMS-acetylene, *i*Pr_2NEt, THF, RT, 89%. (b) 1M TBAF in THF, MeOH, RT, 46%. (c) $CuSO_4$, sodium ascorbate, DMSO, *t*BuOH, H_2O, RT, 61%.

4-Ethynyl-*N*-(2-hydroxyethyl)-1,8-naphthalimide (14): To a mixture of compound **19**[S5] (0.811 g, 2.53 mmol, 1.0 equiv), $Pd(PPh_3)_4$ (0.291 g, 0.25 mmol, 0.1 equiv) and CuI (0.092 g, 0.48 mmol, 0.2 equiv) in dry THF (25 mL) was added trimethylsilylacetylene (1.8 mL, 12.74 mmol, 5.0 equiv) and *N*,*N*-diisopropylethylamine (1.5 mL) under nitrogen atmosphere. The reaction mixture was stirred overnight at RT. The reaction mixture was diluted with ethyl acetate (100 mL), washed with saturated NH_4Cl and brine solutions. The organic extract was dried over Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel column chromatography to afford the coupled product (0.764 g, 89%). To a solution of the coupled product (0.516 g, 1.53 mmol) in dry MeOH (26 mL) was added 1 M tetrabutylammonium

fluoride solution in THF (5.5 mL). The deprotection was performed at 60 °C for 30 min. The reaction mixture was concentrated, dissolved in ethyl acetate (50 mL), and washed with water and brine. The organic extract was evaporated and the residue was purified by silica gel column chromatography to afford the alkyne substrate **14** as pale yellow solid (0.187 g, 46%). TLC (pet. ether:EtOAc = 50:50) R_f = 0.42; ^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 8.62 (dd, J = 8.4, 0.8 Hz, 1H), 8.55 (d, J = 7.2 Hz, 1H), 8.44 (d, J = 7.6 Hz, 1H), 8.04 (d, J = 7.6 Hz, 1H), 7.98 ($_{app}$t, J = 8.0 Hz, 1H), 5.11 (s, 1H), 4.82 (t, J = 6.0 Hz, 1H), 4.15 (t, J = 6.4 Hz, 2H), 3.62 ($_{app}$q, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz, d_6-DMSO): δ (ppm) 163.3, 163.1, 131.7, 131.5, 131.2, 129.8, 128.4, 127.4, 125.4, 122.9, 122.6, 90.4, 80.1, 57.8, 42.0; MALDI-TOF MS (m/z): Calculated for $C_{16}H_{11}NaNO_3$ $[M+Na]^+$ = 288.06, found = 288.07; λ_{max} (H_2O) = 354 and 368 nm, ε_{354} = 16160 $M^{-1}cm^{-1}$, ε_{368} = 14390 $M^{-1}cm^{-1}$; λ_{em}(H_2O) = 417 nm.

Fluorescent click product (20): Ribonucleoside **6** (0.071 g, 0.22 mmol, 1.0 equiv), $CuSO_4$ (0.108 g, 0.43 mmol, 2.0 equiv) and sodium ascorbate (0.172 g, 0.87 mmol, 4.0 equiv) were taken in degassed water (2.8 mL). To the above mixture was added a solution of alkyne-modified naphthalimide **14** (0.129 g, 0.49 mmol, 2.3 equiv) in degassed DMSO-*t*BuOH solution (3:1, 3.5 mL). The reaction mixture was stirred at RT for 5 h. The reaction mixture was filtered through celite pad and washed extensively with methanol. Volatile solvents were evaporated and the residue was purified by silica gel flash column chromatography to afford the click product **20** as pale yellow solid (0.078 g, 61%). TLC (CH_2Cl_2:MeOH = 85:15) R_f = 0.67; ^{1}H NMR (400 MHz, d_6-DMSO): δ (ppm) 11.35 (s, 1H), 9.14 (dd, J = 8.4, 1.2 Hz, 1H), 8.87 (s, 1H), 8.56–8.53 (m, 2H), 8.13 (d, J = 7.6 Hz, 1H), 7.92 (dd, J = 8.6, 7.4 Hz, 1H), 7.81 (s, 1H), 5.77 (d, J = 5.2 Hz, 1H), 5.37 (br, 1H), 5.10 (br, 1H), 4.83 (br, 1H), 4.51 (t, J = 7.0 Hz, 2H), 4.16 (t, J = 6.6 Hz, 2H), 4.06 (t, J = 5.0 Hz, 1H), 3.99 (t, J = 4.6 Hz, 1H), 3.82 (dd, J = 7.2, 3.2 Hz, 1H), 3.67–3.62 (m, 3H), 3.55 (dd, J = 12.2, 3.0 Hz, 1H), 3.16 (s, 1H), 2.35–2.25 (m, 2H), 2.17–2.10 (m, 2H); MALDI-TOF MS (m/z): Calculated for $C_{28}H_{28}KN_6O_9$ $[M+K]^+$ = 631.16, found = 631.22; λ_{max}(H_2O) = 264 and 362 nm, ε_{264} = 11020 $M^{-1}cm^{-1}$, ε_{362} = 15240 $M^{-1}cm^{-1}$, ε_{260} = 10880 $M^{-1}cm^{-1}$; λ_{em}(H_2O) = 460 nm.

4. Enzymatic incorporation of azide-modified UTP 7:

Transcription reactions with α-^{32}P ATP: The promoter-template duplexes (5 μM) were assembled by heating T7 RNA polymerase consensus promoter DNA sequence and DNA template (**S1**–**S5)** in TE buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.8) at 90 °C for 3 min. The solution was allowed to come to room temperature slowly and then placed on an ice bath for 20 min, and stored at -40 °C. The transcription reactions were carried out at 37 °C in 40 mM Tris-HCl buffer (pH 7.9) containing 250 nM annealed promoter-template duplexes, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM of dithiothreitol (DTT), 2 mM spermidine, 1 U/μL RNase inhibitor (Riboblock), 1 mM GTP, CTP, UTP and or modified UTP **7**, 20 μM ATP, 5 μCi α-^{32}P ATP and 3 U/μL T7 RNA in a total volume of 20 μL. The reaction was quenched after 3.5 h by adding 20 μL of loading buffer (7 M urea in 10 mM Tris-HCl, 100 mM EDTA, 0.05% bromophenol blue, pH 8), heated for 3 min at 75 °C followed by cooling the samples on an ice bath. The samples (4 μL) were loaded on a sequencing 18% denaturing polyacrylamide gel and electrophoresed. The radioactive bands were phosphorimaged and then quantified using the GeneTools software from Syngene to determine the relative transcription efficiencies. Percentage incorporation of azide-modified ribonucleoside triphosphate **7** has been reported with respect to transcription efficiency in the presence of natural NTPs. All reactions were performed in duplicate and the errors in yields were ≤ 6%.

Large-scale transcription reactions: Large-scale transcription reactions were performed using DNA template **S1**. Each reaction (250 μL) was performed in the presence of 2 mM GTP, CTP, ATP, modified UTP **7**, 20 mM $MgCl_2$, 0.4 U/μL RNase inhibitor (Riboblock), 300 nM annealed template and 800 units T7 RNA polymerase. After incubating for 12 h at 37 °C, the reaction volume was reduced to 1/3 by speed vac. 50 μL of the loading buffer was added and the sample was loaded on a preparative 20% denaturing polyacrylamide gel. The gel was UV shadowed; appropriate band was cut out, extracted with 0.3 M sodium acetate and desalted using Sep-Pak classic C18 cartridge. Under these conditions, transcription reactions afforded near 20 nmoles of the transcript **9** (ε_{260} = 90133 $M^{-1}cm^{-1}$).

5. MALDI-TOF MS analysis of transcripts: 2 μL of ~200 μM transcript, 1 μL of 100 mM ammonium acetate buffer (pH 9), 2 μL of 100 μM DNA standard and 4 μL of matrix (saturated 3-hydropiccolinic acid) were mixed together. The sample was desalted using ion-exchange resin (Dowex 50W-X8, 100-200 mesh, ammonium form) and analyzed using MALDI-TOF mass spectrometry. The resulting spectrum was calibrated relative to an internal DNA oligonucleotide standard.

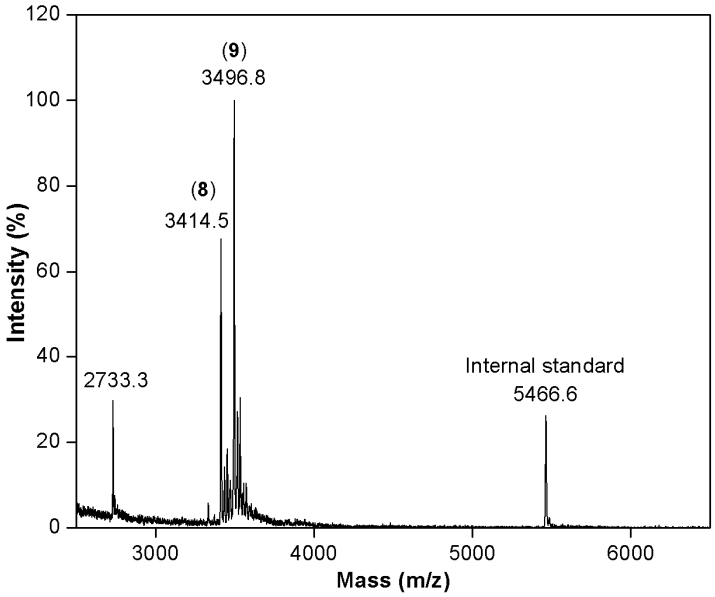


Figure S1. MALDI-TOF mass spectrum of oligoribonucleotide transcripts isolated from a large-scale transcription reaction with template **S1** and in the presence of equimolar concentrations of UTP and modified UTP **7**. The spectrum is calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard (m/z for +1 and +2 ion are 5466.6 and 2733.3, respectively). The mass spectrum shows the presence of both unmodified (**8**) and modified (**9**) oligoribonucleotide transcripts. Calcd. for **8**: 3414.9 [M]; found: 3414.5; Calcd. for **9**: 3498.0 [M]; found: 3496.8.

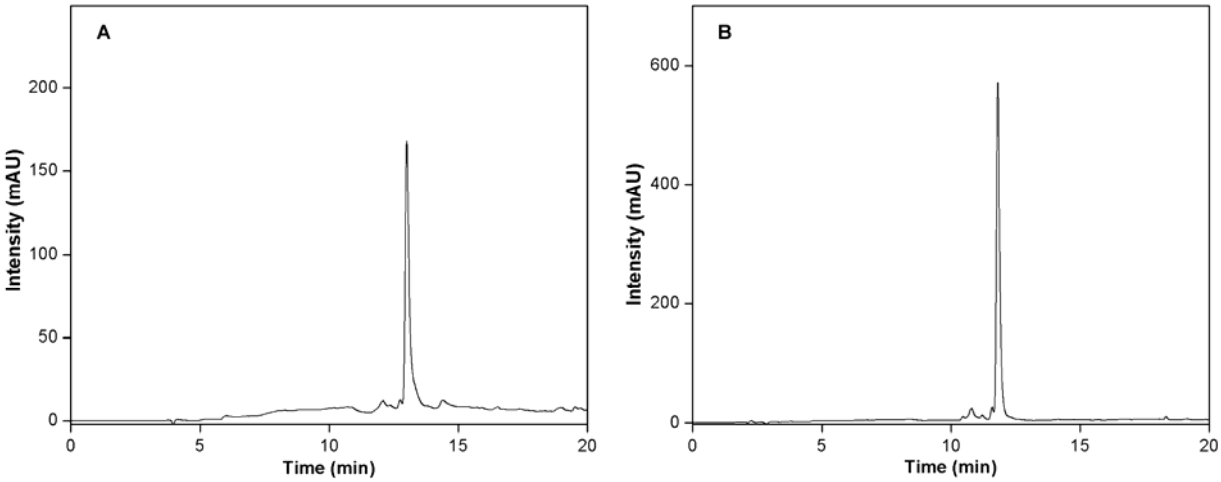


Figure S2. (**A**) RP-HPLC profile of transcript **9** at 260 nm. (**B**) RP-HPLC profile of the 5′-dephosphorylated transcript **9** at 260 nm. Dephosphorylation was performed by incubating the transcript with calf intestinal alkaline phosphatase for 60 min. Mobile phase A: 50 mM triethylammonium acetate buffer (pH 7.5), mobile phase B: acetonitrile. Flow rate: 1 mL/min. Gradient: 0−10% B in 10 min and 10−100% B in 20 min.

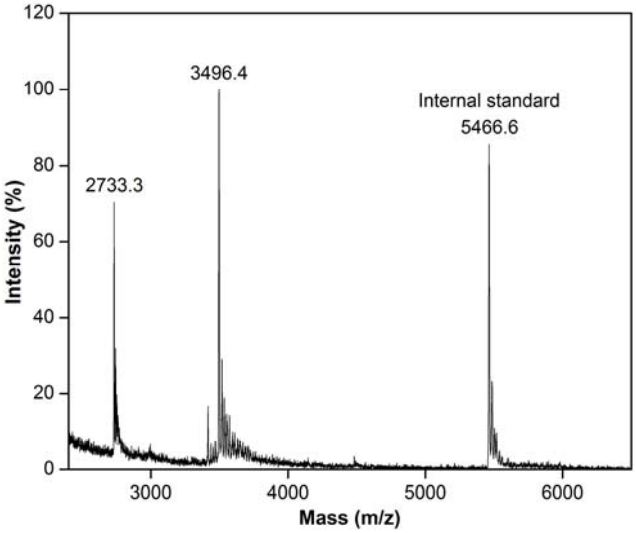


Figure S3. MALDI-TOF mass spectrum of azide-modified oligoribonucleotide transcript **9** calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard (m/z for +1 and +2 ion are 5466.6 and 2733.3, respectively). Calcd. for **9**: 3498.0 [M]; found: 3496.4.

6. Enzymatic digestion of oligoribonucleotide 9: ~4 nmol of the modified oligoribonucleotide transcript **9** was treated with snake venom phosphodiesterase I (0.01 U), calf intestinal alkaline phosphatase (10 µL , 1 U/µL), and RNase A (0.25 µg) in a total volume of 100 µL in 50 mM Tris-HCl buffer (pH 8.5, 40 mM $MgCl_2$, 0.1 mM EDTA) for 12 h at 37 °C. After this period, RNase T1 (0.2 U/µL) was added, and the sample was incubated for another 4 h at 37 °C. The ribonucleoside mixture obtained from the digest was analyzed by reversed-phase HPLC using Phenomenex-Luna C18 column (250 x 4.6 mm, 5 micron) at 260 nm. Mobile phase A: 50 mM triethylammonium acetate buffer (pH 7.5), mobile phase B: acetonitrile. Flow rate: 1 mL/min. Gradient: 0−10% B in 20 min and 10−100% B in 10 min.

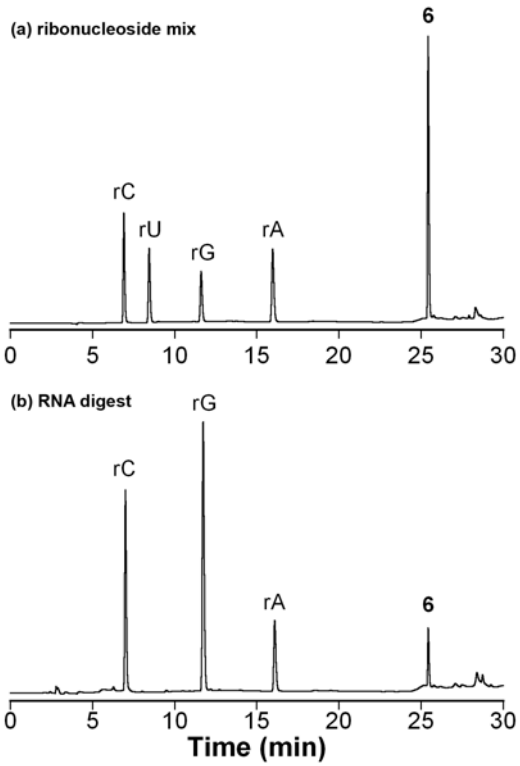


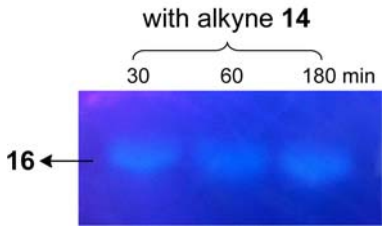
Figure S4. HPLC chromatogram of ribonucleoside products obtained from an enzymatic digestion of oligoribonucleotide **9** at 260 nm. (a) Natural ribonucleosides and azide-modified ribonucleoside **6** mix. (b) Oligoribonucleotide **9** digest.

Table S1. MALDI-TOF mass analysis of HPLC fractions of oligoribonucleotide **9** digest.

HPLC fractions of the digest	Calcd. for	Found
rC	$C_9H_{13}N_3O_5Na$: 266.2 $[M+Na]^+$	265.9
rG	$C_{10}H_{13}N_5O_5$: 283.2 $[M]^+$	283.0
rA	$C_{10}H_{13}N_5O_4$: 267.1 $[M]^+$	267.2
6	$C_{12}H_{17}N_5O_6$: 327.3 $[M]^+$	327.3

7. Click reaction on azide-modified oligoribonucleotide 9 with alkyne substrates 14 and 15:

A premixed aqueous solution of the ligand (2 µL, 94 mM), $CuSO_4$ (2 µL, 47 mM) and sodium ascorbate (2 µL, 94 mM) was added to a solution of oligoribonucleotide **9** in water (14 µL, 0.54 mM, 7.6 nmol). Degassed water was used to prepare the above stock solutions. The alkyne substrate (5 µL, 7.5 mM) dissolved in degassed DMSO was then added to the above solution and mixed well. Total volume of the reaction was 25 µL, and contained 20% DMSO. The reaction mixture was incubated at 37 °C, and aliquots of reaction mixture (8 µL) at 30, 60 and 180 min were dephosphorylated by adding 5 µL of Tris-HCl buffer (50 mM, 0.10 mM EDTA, pH 8.5) and calf intestinal alkaline phosphatase (2 µL, 1 U/µL) to remove the 5'-triphosphate. After incubating at 37 °C for 1.5 h, PAGE loading buffer (5 µL) was added, and the samples were loaded on an analytical 20% denaturing polyacrylamide gel and electrophoresed. The bands corresponding to the products were visualized by UV-shadowing method (short wave UV, 254 nm and long wave UV, 365 nm). Under this condition, a reaction with 4-ethynyl-1,8-naphthalimide derivative **14** resulted in the formation of the fluorescent click product **16** with no apparent degradation of the oligoribonucleotides. However, a reaction with alkyne **15** gave the desired oligoribonucleotide click product as well as an unknown side product. Surprisingly, when the dephosphorylation step was avoided, the biotinylated click product was obtained as the major product (see below for experimental details).



UV-shadow (long wave UV, 365 nm) of the bands corresponding to the fluorescent click product **16**

Large-scale click reaction with naphthalimide-alkyne 14: A premixed aqueous solution of the ligand (4 µL, 94 mM), $CuSO_4$ (4 µL, 47 mM) and sodium ascorbate (8 µL, 47 mM) was added to a solution of oligoribonucleotide **9** in water (24 µL, 0.64 mM, 15.4 nmol). Degassed water was used to prepare the above stock solutions. The alkyne substrate **14** (10 µL, 7.5 mM) dissolved in degassed DMSO was then added to the above solution and mixed well. Total volume of the reaction was 50 µL, and contained 20% DMSO. The reaction mixture was incubated at 37 °C for 1 h, and was dephosphorylated by adding 40 µL of Tris-HCl buffer (50 mM, 0.10 mM EDTA, pH 8.5) and CIP (10 µL, 1 U/µL). After incubating at 37 °C for 1.5 h, PAGE loading buffer (25 µL) was added, and the samples were loaded on a preparative 20% denaturing polyacrylamide gel and electrophoresed. The gel was UV shadowed; appropriate band was cut out, extracted with 0.3 M sodium acetate and desalted using Sep-Pak classic C18 cartridge. The yield of the click product **16** after PAGE purification was nearly 5 nmol.

Click reaction with biotin-alkyne 15: A premixed aqueous solution of the ligand (2 µL, 94 mM), $CuSO_4$ (2 µL, 47 mM) and sodium ascorbate (2 µL, 94 mM) was added to oligoribonucleotide **9** in water (14 µL, 0.50 mM, 7.0 nmol). Degassed water was used to prepare the above stock solutions. The alkyne substrate (5 µL, 7.5 mM) dissolved in degassed DMSO was then added to the above solution and mixed well. Total volume of the reaction was 25 µL, and contained 20% DMSO. The reaction mixture was incubated at 37 °C. Aliquots of reaction mixture (8 µL) at 30, 60 and 180 min were mixed with loading buffer (7 µL), and loaded on an analytical 20% denaturing polyacrylamide gel and electrophoresed. The products of the reactions were visualized by UV-shadowing method (see figure S7). At 30 min reaction time point the biotinylated click product is the major product.

Large-scale click reaction with biotin-alkyne 15: Large-scale reaction was performed with transcript **9** (15 nmol) under similar conditions in a total volume of 50 µL for 30 min. Loading buffer (25 µL) was added, and the click product was purified by PAGE under denaturing condition. The yield of the click product **17** after PAGE purification was 4.6 nmol.

8. Fluorescence spectra of click products 16 and 20: Fluorescence experiments were performed in a micro fluorescence cuvette (Hellma, path length 1.0 cm) on a Horiba Jobin Yvon, Fluorolog-3. Emission profile of the oligoribonucleotide click product **16** (2 µM) was obtained

by exciting the sample at 362 nm with an excitation and emission slit width of 6 nm and 9 nm, respectively. Emission profile of the click product **20** (2 μM) was obtained by exciting the sample at 362 nm with an excitation and emission slit width of 3 nm and 4 nm, respectively (see Figure 4).

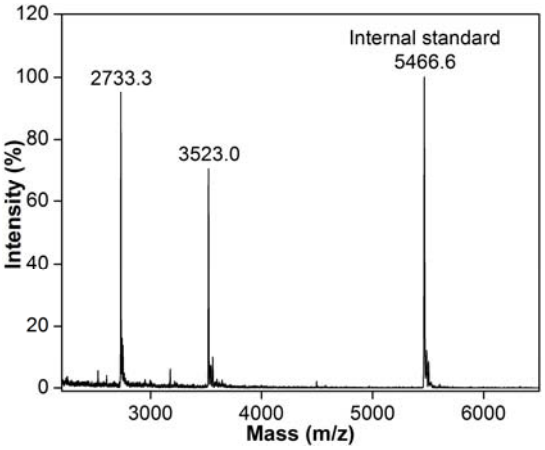


Figure S5. MALDI-TOF mass spectrum of click product **16** calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard (m/z for +1 and +2 ion are 5466.6 and 2733.3, respectively). Calcd. for **16**: 3523.3 [M]; found: 3523.0.

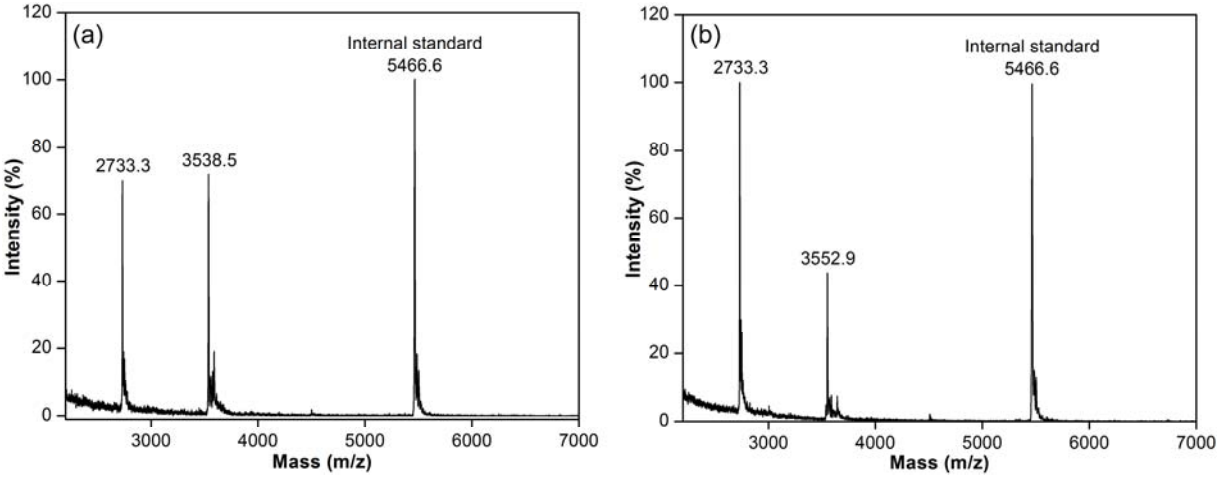


Figure S6: MALDI-TOF mass spectra of oligoribonucleotide products calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard (m/z for +1 and +2 ion are 5466.6 and 2733.3, respectively). (b) Calcd. for biotinylated click product **17**: 3539.4 [M]; found: 3538.5. (b) Unknown slower migrating oligoribonucleotide product showing a m/z value of 3552.9.

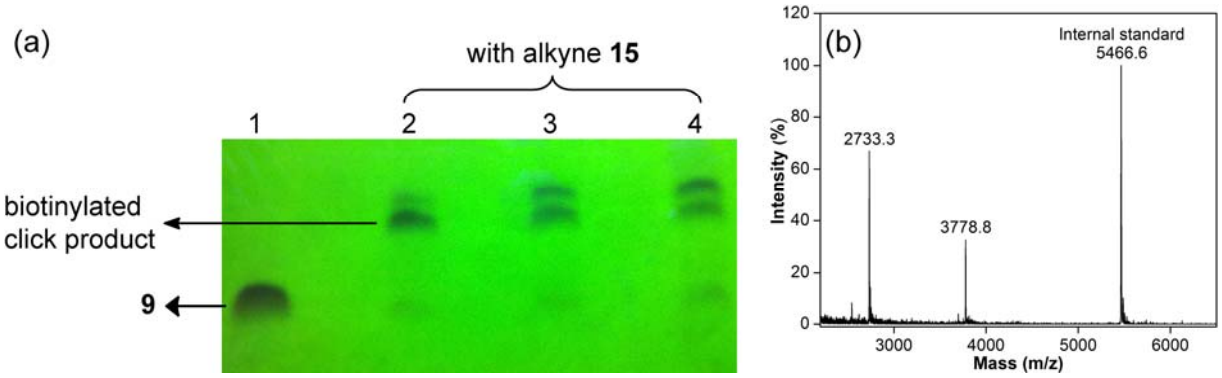


Figure S7. (a) UV-shadow (short wave UV, 254 nm) of polyacrylamide gel electrophoresis of oligoribonucleotide products obtained from a click reaction between azide-modified oligoribonucleotide **9** and biotin-alkyne **15**. Lane 1, oligoribonucleotide **9**. Lanes 2–4, aliquots of reaction mixture at 30, 60 and 180 min, respectively. Band corresponding to the biotinylated click product is indicated with an arrow.
(b) MALDI-TOF mass spectrum of biotinylated click product calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard (m/z for +1 and +2 ion are 5466.6 and 2733.3, respectively). Calcd. for **17** with 5′-triphosphate: 3779.4 [M]; found: 3778.8.

9. Staudinger reduction of azide-modified oligoribonucleotide 9: Reduction of azide-modified oligoribonucleotide **9** (200 μM, 10 nmoles) by TPPTS (5 mM) was carried out in cacodylate buffer (20 mM, 50 mM NaCl, pH 7.0) in a total volume of 50 μL at 55 °C for 15 h. To the above solution was added dephosphorylation buffer (46 μL, 25 mM Tris-HCl, 0.05 mM EDTA, pH 8.5) and CIP (4 μL , 1 U/μL), and incubated at 37 °C for 60 min. 20 μL of the loading buffer was added and the sample was loaded on a preparative 20% denaturing polyacrylamide gel. The gel was UV shadowed; appropriate band was cut out, extracted with 0.3 M sodium acetate and desalted using Sep-Pak classic C18 cartridge. Under these conditions reduction reactions afforded 4.8 nmoles of the amine-modified oligoribonucleotide product **18**. The formation of the reduced product **18** was confirmed by MALDI-TOF MS analysis.

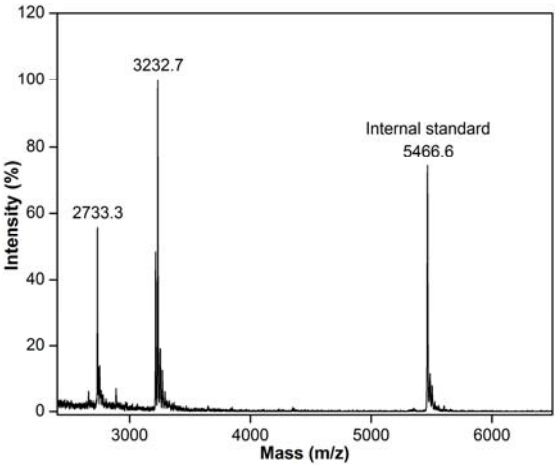
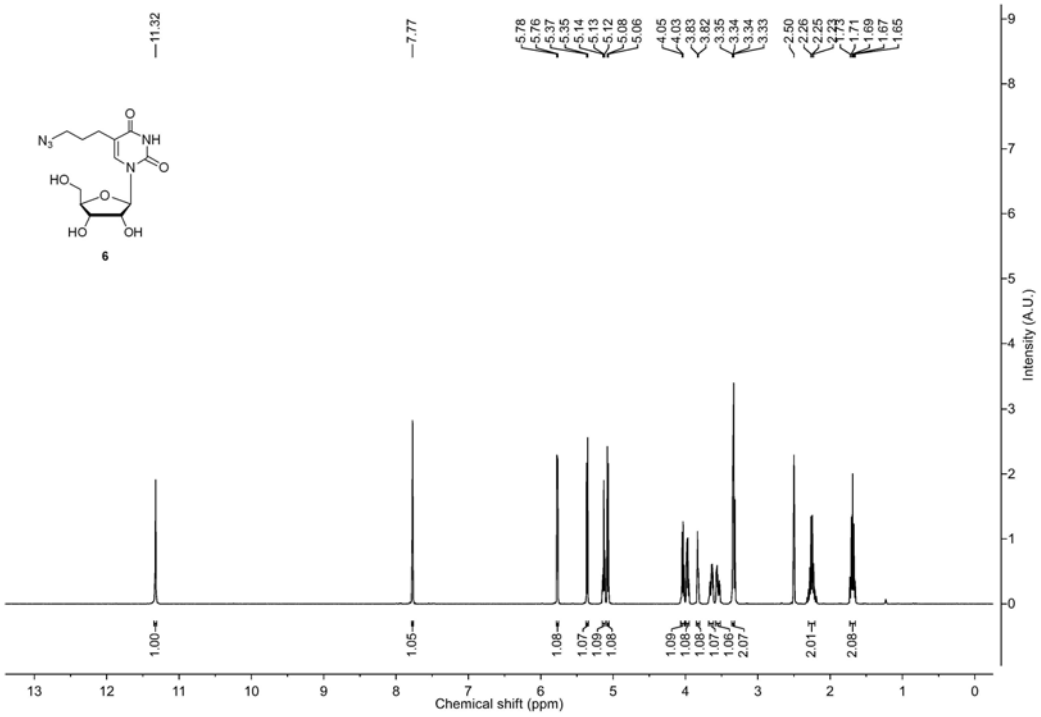
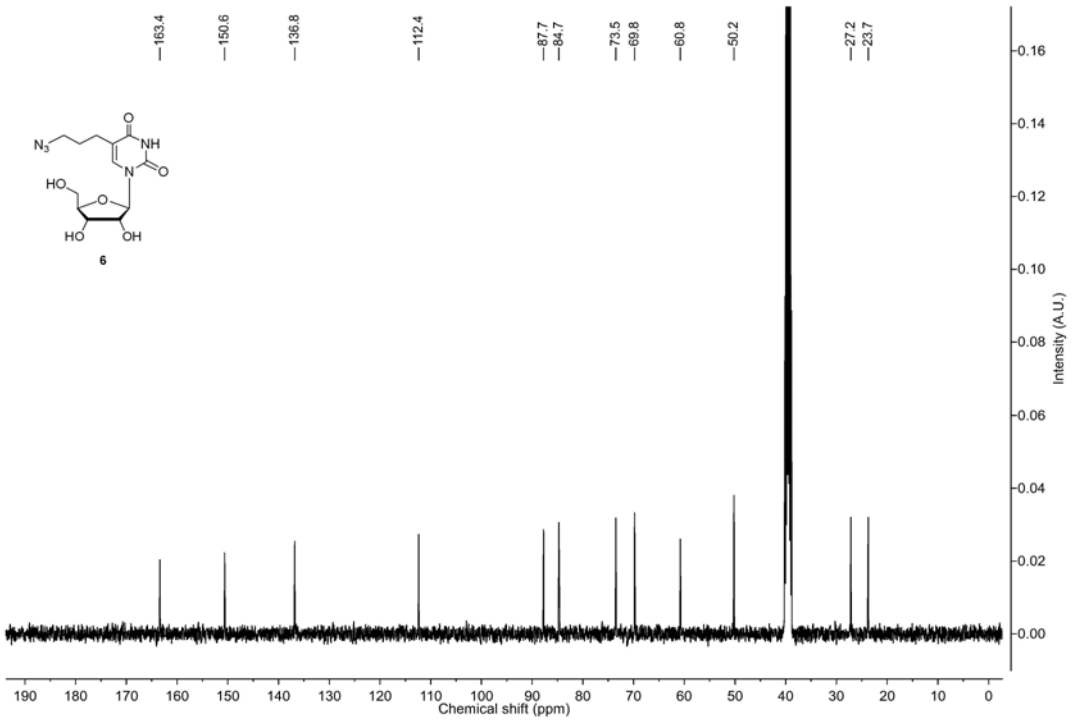


Figure S8. MALDI-TOF mass spectrum of the reduced oligoribonucleotide product **18** calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard (m/z for +1 and +2 ion are 5466.6 and 2733.3, respectively). Calcd. for **18**: 3232.1 [M]; found: 3232.7.

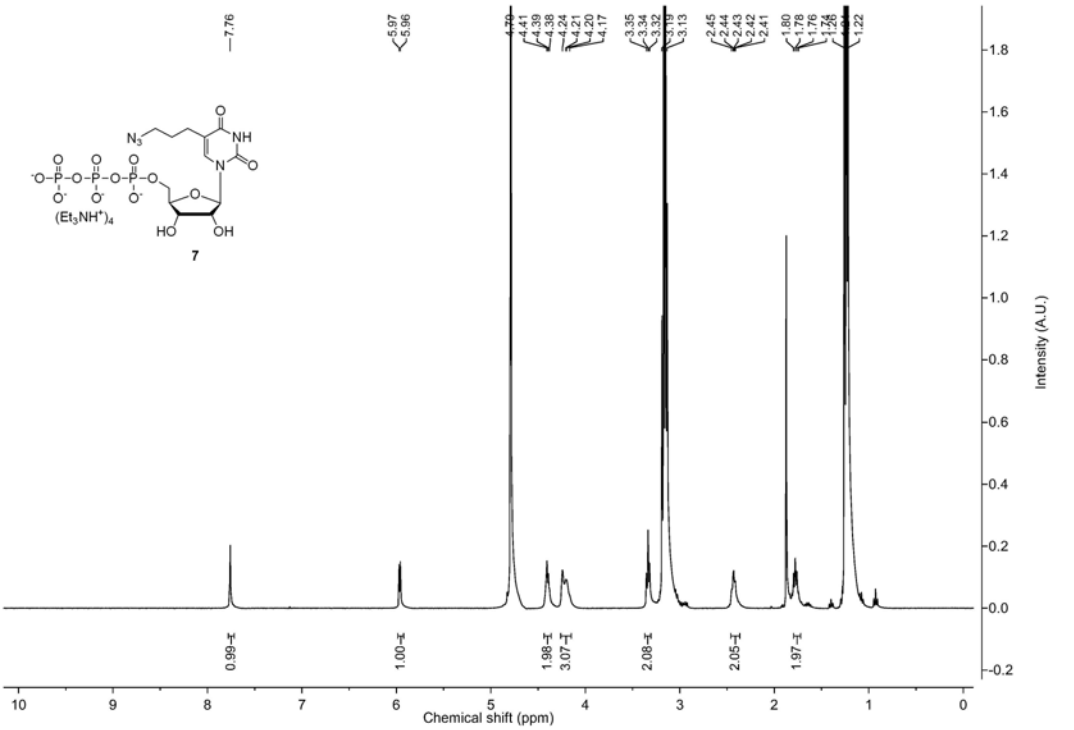
10. NMR and MS spectra



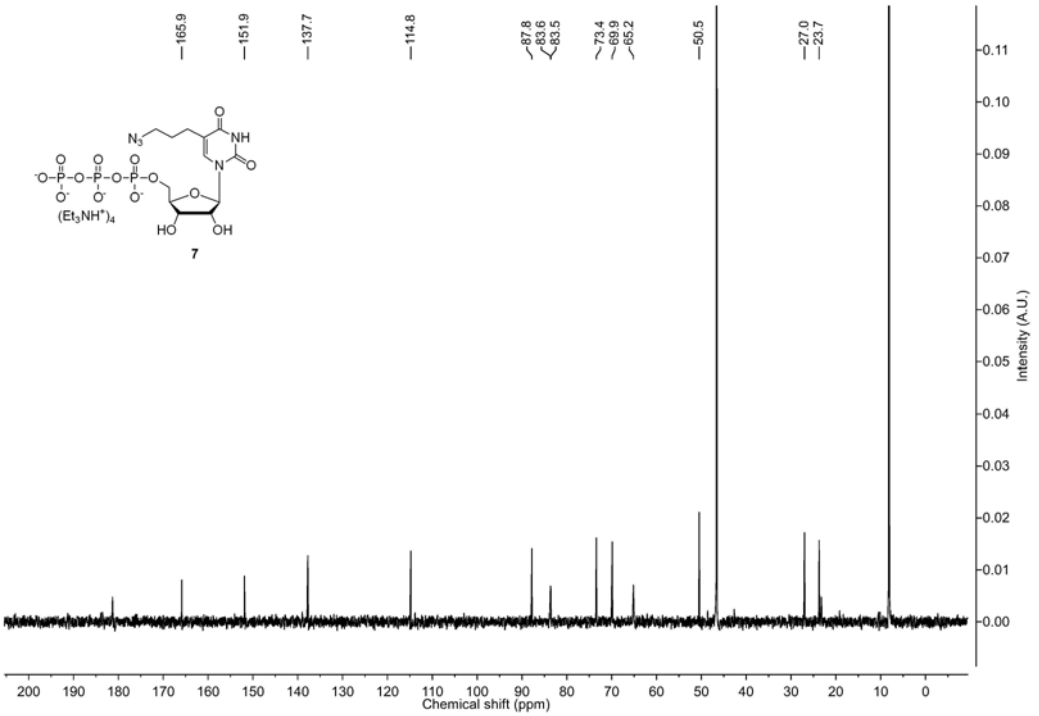
^{1}H-NMR of ribonucleoside **6** in d_6-DMSO



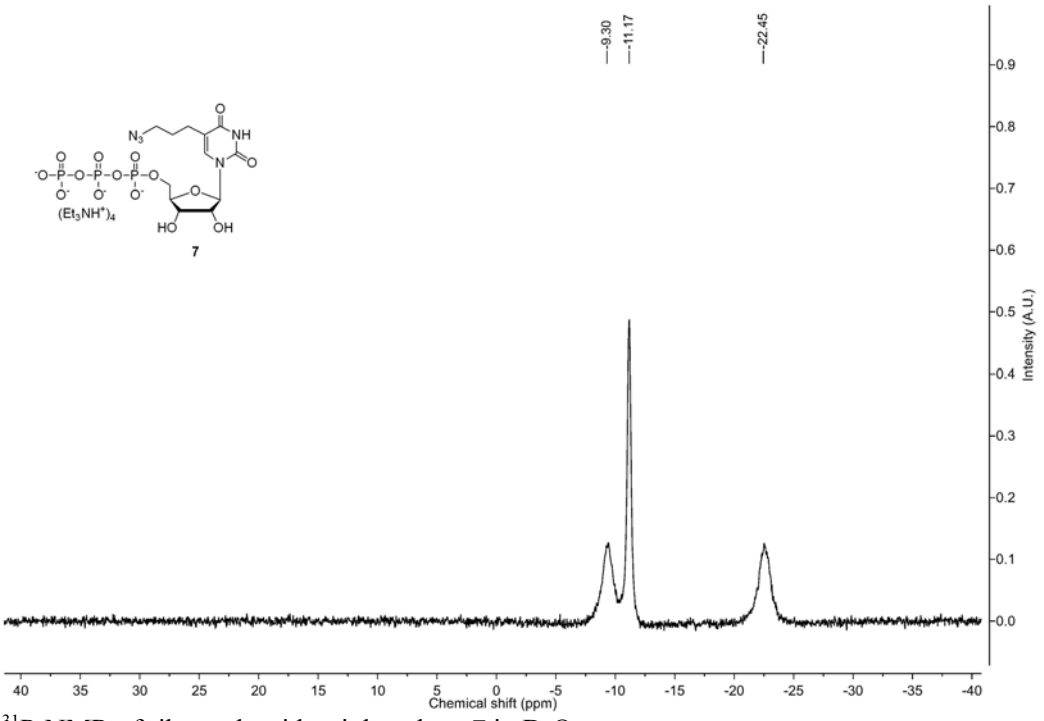
^{13}C-NMR of ribonucleoside **6** in d_6-DMSO



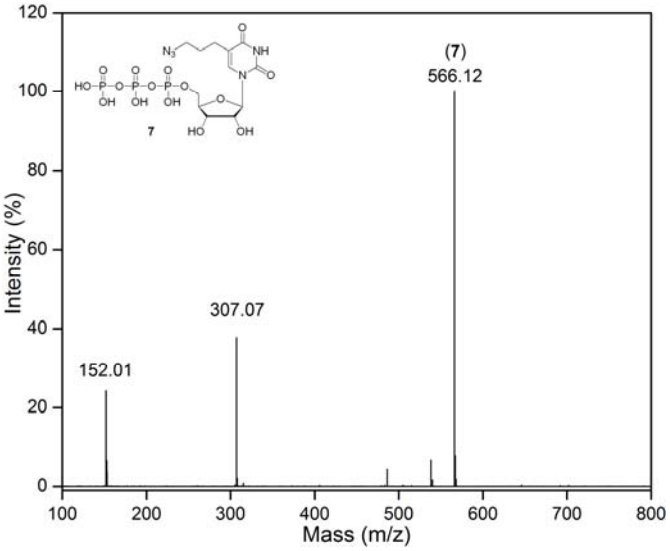
^{1}H-NMR of ribonucleoside triphosphate **7** in D_2O. Trace amounts of triethylammonium acetate buffer is also present.



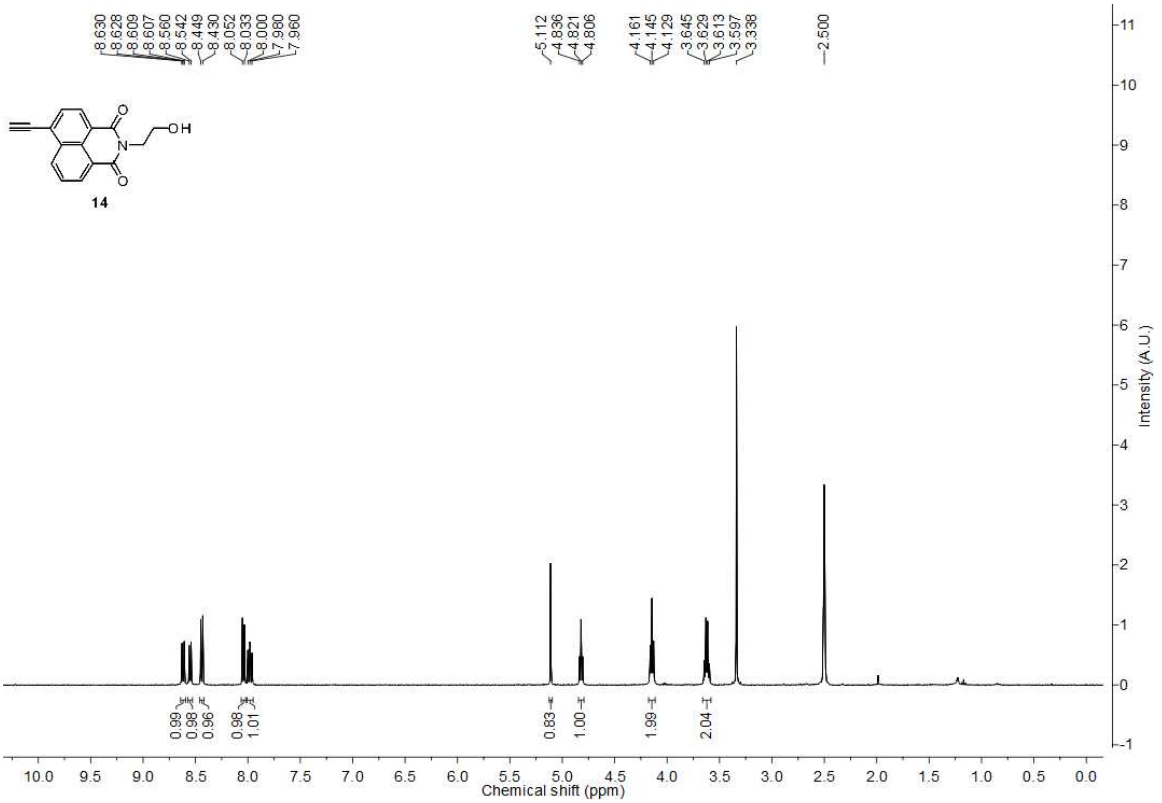
^{13}C-NMR of ribonucleoside triphosphate **7** in D_2O. Trace amounts of triethylammonium acetate buffer is also present.



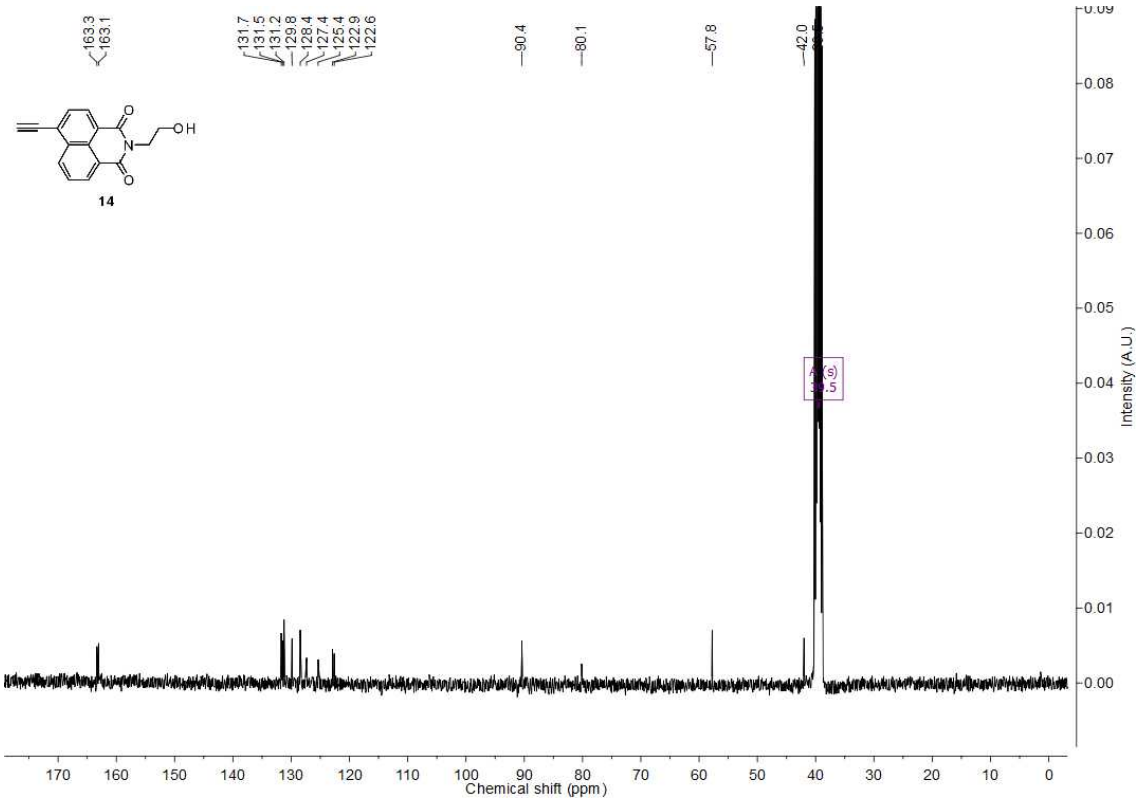
^{31}P-NMR of ribonucleoside triphosphate **7** in D_2O



MALDI-TOF MS spectrum of modified triphosphate **7** in negative mode. Calculated for $C_{12}H_{20}N_5O_{15}P_3$ [M] = 567.02, found $[M-H]^-$ = 566.12.



^{1}H-NMR of alkyne substrate **14** in d_6-DMSO



^{13}C-NMR of alkyne substrate **14** in d_6-DMSO

11. References:

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(S3) Argyropoulou, E. C.; Tsitabani, M.; Petrantonakis, G.; Terzis, A.; Raptopoulou, C. *Org. Biomol. Chem*. **2003**, *1*, 1382–1388.

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(S5) MacGregor, K. A.; McCluskey, A. *Tet. Lett*. **2011**, *52*, 767–769.