

## Clickable UTP Analog for the Posttranscriptional Chemical Labeling and Imaging of RNA

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### Electronic Supplementary Information

Contents	Page
<b>1. Materials</b>	S2
<b>2. Instrumentation</b>	S2
<b>3. Synthesis</b>	S3
<b>Scheme S1.</b> Synthesis of azide substrates <b>5</b> and <b>9</b> for CuAAC reactions	S3
<b>Fig. S1</b> Phosphorimage of transcription products obtained from reactions with alkyne- modified UTP <b>3</b>	S5
<b>Fig. S2</b> PAGE of <i>in vitro</i> transcription reaction performed in presence of template <b>T2</b>	S6
<b>Fig. S3</b> MALDI-TOF mass spectrum of alkyne-modified oligoribonucleotide transcripts <b>4</b>	S7
<b>Table S1</b> Yield and mass data of alkyne-modified RNA transcript <b>4</b> and <b>10</b>	S7
<b>4. Enzymatic digestion of RNA ON 4</b>	S7
<b>Fig. S4</b> HPLC chromatogram of enzymatic digestion of RNA ON <b>4</b>	S8
<b>Fig S5</b> UV-thermal melting of control unmodified duplex and alkyne-modified duplex	S9
<b>5. In vitro transcription with plasmid DNA template in the presence of 3</b>	S9
<b>Fig. S6</b> HPLC chromatogram of ribonucleoside products obtained from enzymatic digestion reactions of longer modified RNA transcripts containing <b>2</b>	S10
<b>6. Reverse transcription of alkyne-modified RNA transcripts containing 2</b>	S10
<b>Fig. S7</b> PCR amplified template sequence from pEGFPC1 vector used in transcription reactions	S12
<b>Fig. S8</b> Representative sequence alignment (BLAST) of PCR amplified DNA products	S12
<b>Table S2.</b> Yield and mass data of posttranscriptionally functionalized RNA ONs <b>4</b> and <b>10</b>	S13
<b>Fig. S9</b> Posttranscriptional chemical modification of EU-modified RNA ON <b>10</b> by CuAAC reaction	S14
<b>Fig. S10</b> Posttranscriptional chemical modification of 59mer ODU-RNA by CuAAC reaction using Alexa594-azide <b>6</b>	S15
<b>Fig. S11</b> Incorporation of ODU <b>2</b> into cellular RNA transcripts as detected by CuAAC reactions using Alexa594-azide <b>6</b> .	S15
<b>Fig. S12</b> Quantification plots showing percentage of labeled nuclei by click reaction	S16

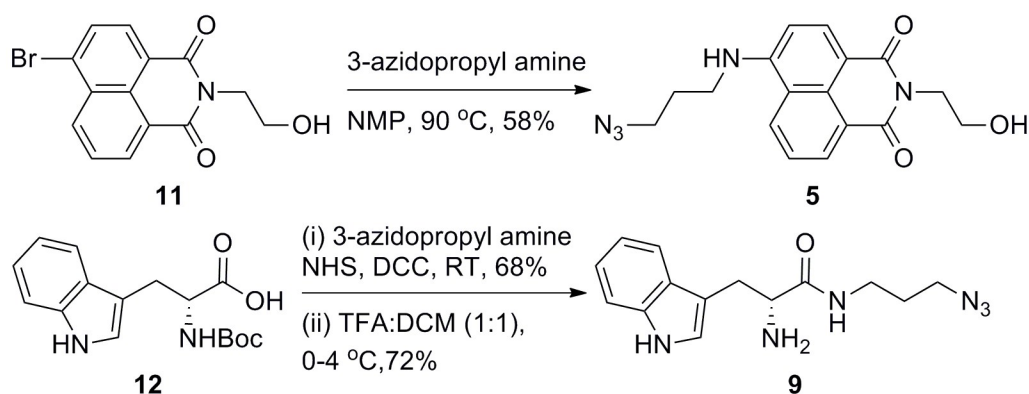
with Alexa594-azide <b>6</b> .	
<b>7. Anti-nucleolin staining</b>	S17
<b>Fig. S13</b> Images of HeLa cells transfected with <b>3</b> showing intense staining in nucleoli.	S18
<b>8. MTT assay in the presence of ODUTP 3</b>	S19
<b>Fig. S14</b> Plot showing the cell viability of HeLa cells in the presence of ODUTP <b>3</b>	S19
<b>Fig. S15</b> Effect of incubation of ODUTP in the absence ( <b>A</b> ) and presence ( <b>B</b> ) of hydroxyurea for extended periods (6–24 h)	S20
<b>Fig. S16</b> Confocal images of RNases A treatment experiment.	S21
<b>9. Raman analysis of alkyne-modified nucleosides EU and ODU</b>	S21
<b>10. NMR spectra</b>	S22
<b>11. References</b>	S26

**1. Materials.** Uridine, 5-iodouridine, tetrakis(triphenylphosphine) palladium (0), 1,7-octadiyne, copper(I) iodide, formaldehyde, 4-bromo-1,8-naphthalic anhydride, sodium azide, sodium ascorbate, CuSO<sub>4</sub>, biotin, DEAE Sephadex A-25 resin, triton X-100, actinomycin D, hydroxyurea and snake venom phosphodiesterase I were obtained from Sigma-Aldrich. POCl<sub>3</sub> was purchased from Across Organics and freshly distilled prior to use. Fluorescent dyes Alexa594-azide **6**, and calf intestinal alkaline phosphatase (CIP) were acquired from Invitrogen. Biotin azide (**7**) and sugar azide (**8**) were synthesized by following the procedure reported earlier.<sup>S1,S2</sup> Radiolabeled  $\alpha$ -<sup>32</sup>P ATP (2000 Ci/mmol) was purchased from the Board of Radiation and Isotope Technology, Government of India. T7 RNA polymerase, ribonuclease inhibitor (RiboLock), NTPs, RNase A and RNase T1 were obtained from Fermentas Life Science. Dulbecco's Modified Eagle Medium (DMEM), Opti-MEM, fetal bovine serum (FBS) and PBS were purchased from Gibco. Transecting agent DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate) was purchased from Roche. DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc., purified by gel electrophoresis under denaturing condition and desalted using Sep-Pak Classic C18 cartridges (Waters Corporation). Chemicals for preparing all buffer solutions were purchased from Sigma-Aldrich (BioUltra grade). Autoclaved water was used in all biochemical reactions and HPLC analysis.

**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, MicroMass ESI-TOF and Water Synapt G2 High Definition mass spectrometer. Absorption

spectra were recorded on PerkinElmer, Lambda 45 UV-Vis and Shimadzu UV-2600 spectrophotometers. Reversed-phase flash chromatographic (C18 RediSepRf column) purifications were carried out using Teledyne ISCO, Combi Flash Rf. RP-HPLC analysis were performed using Agilent Technologies 1260 Infinity and Dionex ICS 3000. UV-thermal melting analysis was performed using Cary 300Bio UV-Vis spectrophotometer. Phosphor images were taken on a Typhoon Trio+, GE-Healthcare phosphor imager. The cell images have been acquired by Zeiss Apotome upright microscope and Zeiss LSM710 Confocal Laser Scanning Microscope with oil immersion using 40X and 63X lenses. The channels used for acquiring images are DAPI filter ( $\lambda_{\text{ex}} = 350 \text{ nm}$  and  $\lambda_{\text{em}} = 470 \text{ nm}$ ), Alexa594 filter ( $\lambda_{\text{ex}} = 590 \text{ nm}$  and  $\lambda_{\text{em}} = 617 \text{ nm}$ ), Alexa488 filter ( $\lambda_{\text{ex}} = 495 \text{ nm}$  and  $\lambda_{\text{em}} = 519 \text{ nm}$ ). The Zen 2012 software has been used for image acquisition and z stacking. Z dimension has been captured in step sizes of  $0.114 \mu\text{m}$  to check existence of nuclear staining in all steps throughout the nucleus of cells and also to check the localization of nucleoli situated at different planes. Z dimension has been reconstructed into 3D image by Java ImageJ software. This software has been also used to split multichannel images and also for counting number of stained vs. unstained nuclei. The brightness/contrast of an image has not been modified exceeding the Gaussian values for the line graph through a nuclei.

### 3. Synthesis of azide substrates for CuAAC reaction with alkyne-modified RNA transcripts



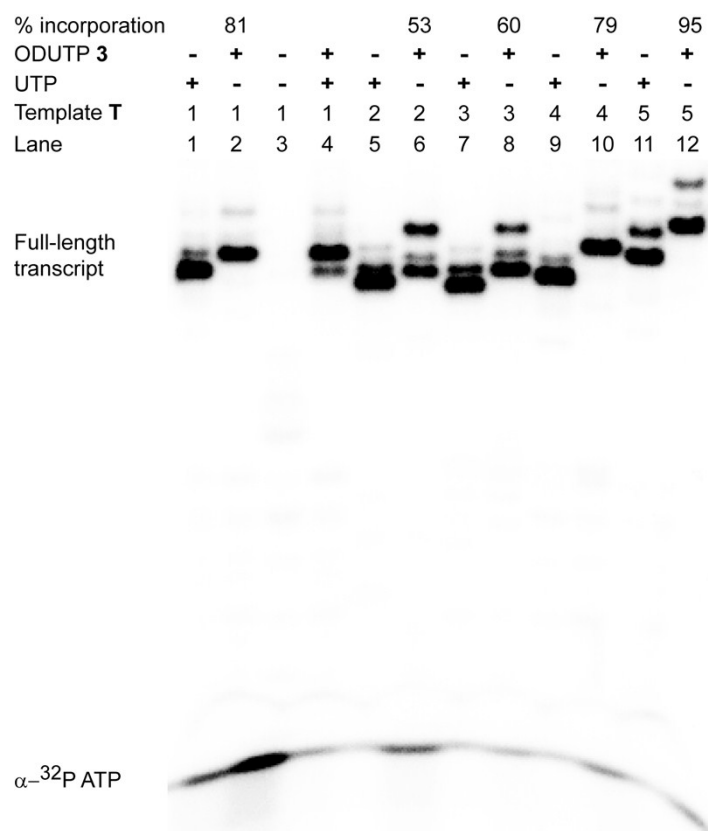
**Scheme S1.** Synthesis of azide substrates **5** and **9**. Azide **5** containing a Lucifer chromophore is a useful fluorescent probe. Azide **9** can provide amino acid-like functionality to RNA. NMP = *N*-methyl-2-pyrrolidone, NHS = *N*-hydroxysuccinimide, DCC = *N,N'*-dicyclohexylcarbodiimide.

**Synthesis of 4-(3-azidopropyl) amino)-N-(2-hydroxyethyl)-1, 8-naphthalimide (5).** A mixture of **11**<sup>S3</sup> (0.210 g, 0.66 mmol, 1.0 equiv) and 3-azidopropyl amine (0.275 g, 2.72 mmol, 4.1 equiv)

in NMP (15 mL) was heated at 90 °C for 12 h. Reaction mixture was diluted by adding 50 mL of dichloromethane and washed with ice cold water (2×25 mL). Organic layer was dried over sodium sulphate and evaporated, and the resulting crude product was purified by silica gel column chromatography (0–10% MeOH in CHCl<sub>3</sub>) to yield bright yellow solid product **5** (0.13 g, 58%). TLC (CHCl<sub>3</sub>:MeOH = 90:10); *R<sub>f</sub>* = 0.22; <sup>1</sup>H NMR (400 MHz, *d*<sub>6</sub>-DMSO): δ (ppm) 8.67 (d, *J* = 8.0 Hz, 1H), 8.40 (d, *J* = 6.8 Hz, 1H), 8.24 (d, *J* = 8.2 Hz, 1H), 7.89–7.58 (m, 2H), 6.77 (d, *J* = 8.2 Hz, 1H), 4.79 (s, 1H), 4.11 (s, 2H), 3.62–3.32 (m, 6H), 2.03–1.88 (m, 2H); <sup>13</sup>C NMR (100 MHz, *d*<sub>6</sub>-DMSO): δ (ppm) 163.9, 163.0, 150.4, 134.2, 130.6, 129.4, 128.6, 124.3, 122.0, 120.2, 108.0, 103.8, 58.0, 48.6, 41.4, 27.2.; HRMS: (*m/z*) Calculated for C<sub>17</sub>H<sub>18</sub>N<sub>5</sub>O<sub>3</sub> [M+H]<sup>+</sup> = 340.1404, found = 340.1410.

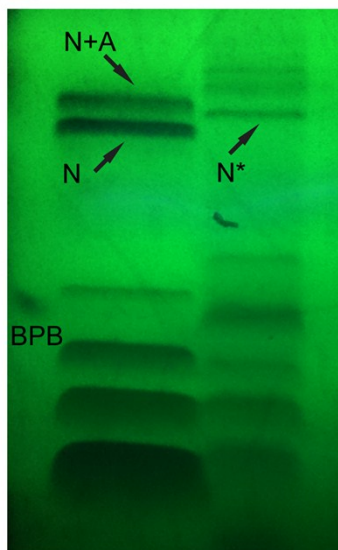
**Synthesis of *N*-(3-azidopropyl)-tryptophan (**9**).** *N*-Boc-protected tryptophan **12** (0.251 g, 0.82 mmol, 1.0 equiv), 3-azidopropyl amine (0.166 g, 1.64 mmol, 2.0 equiv) and *N*-hydroxysuccinimide (0.141 g, 1.23 mmol, 1.5 equiv) were dissolved in anhydrous DMF (1 mL). Resultant reaction mixture was stirred at 0 °C for 15 min and DCC (0.168 g, 0.82 mmol, 1.0 equiv) was added and stirred at RT for 24 h. Reaction mixture was diluted with dichloromethane (20 mL) and filtered through celite pad. Celite pad was washed with dichloromethane (10 mL). The organic layer was washed with saturated sodium carbonate (20 mL) followed by 1M HCl solution (20 mL). Organic layer was dried over sodium sulphate and the solvent was evaporated. The resulting crude product was purified by column chromatography (0–5% MeOH in CHCl<sub>3</sub>) to yield Boc-protected azide derivative as a white solid. This product was dissolved in TFA/DCM (1:1, 5 mL) and stirred for 30 min; solvent was evaporated completely and the residue was suspended in saturated sodium carbonate (100 mL). The product was extracted in ethyl acetate (3×25 mL). The organic extract was dried over sodium sulphate and evaporated, and the resulting crude product was purified by column chromatography (0–10% MeOH in CHCl<sub>3</sub>) to yield product **9** as a pale yellow viscous oil (0.053 g, 72%). TLC (CHCl<sub>3</sub>:MeOH = 90:10); *R<sub>f</sub>* = 0.30; <sup>1</sup>H NMR (400 MHz, *d*<sub>6</sub>-DMSO): δ (ppm) 10.89 (s, 1H), 8.03 (s, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.14 (s, 1H), 7.06 (t, *J* = 7.5 Hz, 1H), 6.97 (t, *J* = 7.4 Hz, 1H), 3.36 (t, *J* = 7.0 Hz, 2H), 3.19 (t, *J* = 6.8 Hz, 2H), 3.09 (t, *J* = 6.4 Hz, 2H), 2.84 (dd, *J* = 14.0, *J* = 7.6 Hz, 1H), 1.60–1.51 (m, 2H), 1.23 (s, 2H); <sup>13</sup>C NMR (100 MHz, *d*<sub>6</sub>-DMSO): δ (ppm) 172.4,

136.2, 127.3, 124.1, 120.9, 118.5, 118.3, 111.4, 109.3, 54.6, 52.0, 48.2, 35.8, 28.2; MALDI-TOF (m/z)  $C_{14}H_{18}N_6ONa$   $[M+Na]^+ = 309.14$ , found = 309.21.



**Fig. S1** Phosphor image of PAGE-resolved transcription products obtained from reactions with templates **T1–T5** in the presence of UTP and or alkyne-modified UTP **3**. % formation of modified full-length transcript is given relative to the amount of full-length transcript (considered as 100%) formed in the presence of natural NTPs.

ODUTP <b>3</b>	-	-	+
UTP	-	+	-
Template <b>T</b>	-	<b>2</b>	<b>2</b>
Lane	1	2	3



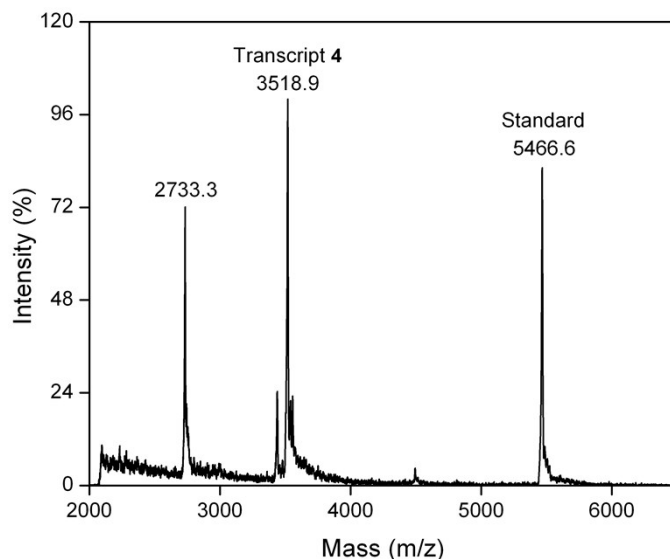
**Fig. S2** PAGE of transcription reaction products obtained using template **T2** and in the presence of ATP, GTP, CTP and UTP or ODUTP **3**. The gel was UV-shadowed. Control transcription reaction with UTP produced two intense bands corresponding to full-length transcript (N) and full-length transcript containing additional adenosine residue (N+A). Reaction in the presence of ODUTP produced desired full-length modified RNA (N\*). Other bands heavier than the full-length modified transcript were formed in trace amounts and could not be further characterized. Other abortive sequences were also observed.

MALDI-TOF MS data:

Control full-length unmodified RNA (N): calcd. mass = 3414.9; observed mass = 3415.6

Control RNA (N+A): calcd. mass = 3744.1; observed mass = 3745.4

Full-length ODU modified RNA (N\*): calcd. mass = 3519.2; observed mass = 3520.2



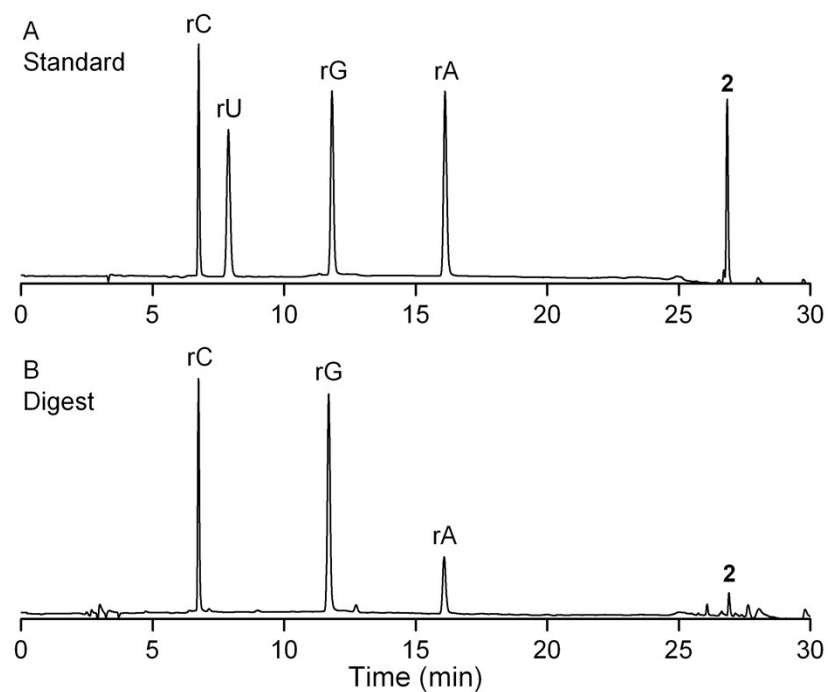
**Fig. S3** MALDI-TOF mass spectrum of ODU-labeled RNA transcript **4** calibrated relative to the +1 and +2 ion of an internal 18-mer DNA oligonucleotide standard ( $m/z$  for +1 and +2 ion are 5466.6 and 2733.3, respectively). Calcd. for **4**: 3519.2 [M]; found: 3518.9. 3-hydroxypicolinic acid was used as the matrix.

**Table S1** Extinction coefficient and MALDI-TOF mass data of ODU- and EU-labeled RNA transcripts obtained from large-scale transcription reactions with template **T1**.

RNA Transcript	$\epsilon_{260}$ ( $M^{-1}cm^{-1}$ )	Isolated yield (nmol) <sup>a</sup>	Calcd. mass	Observed mass
<b>4</b>	84680	15	3519.2	3518.9
<b>10</b>	85040	18	3438.9	3439.4

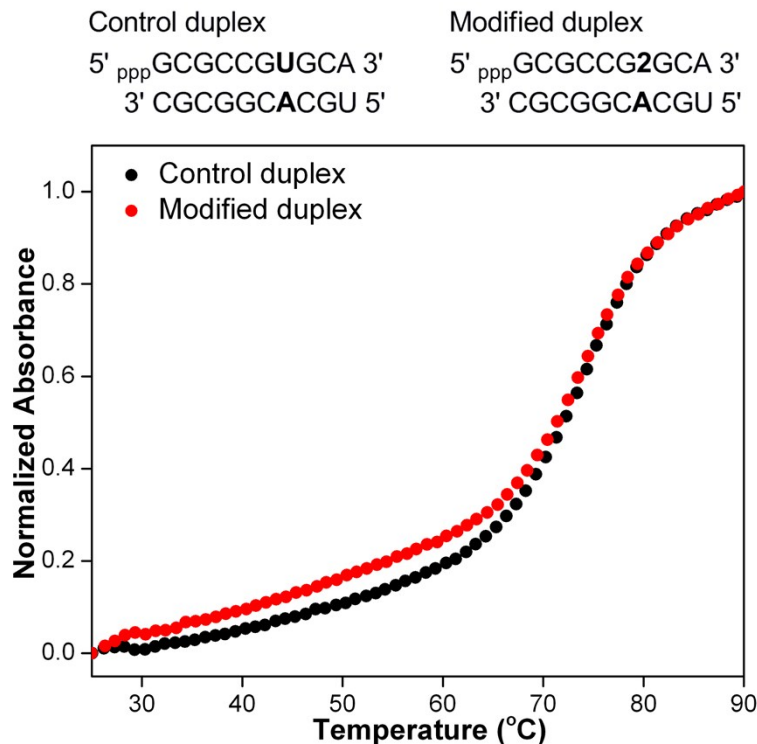
<sup>a</sup>Yield from large-scale transcription reactions (250  $\mu$ L volume reaction).

**4. Enzymatic digestion of RNA ON 4.** ~4 nmol of the alkyne-modified RNA transcript **4** was treated with snake venom phosphodiesterase I (0.01 U), calf intestinal alkaline phosphatase (10  $\mu$ L, 1 U/ $\mu$ L), and RNase A (0.25  $\mu$ g) in a total volume of 100  $\mu$ L in 50 mM Tris-HCl buffer (pH 8.5, 40 mM  $MgCl_2$ , 0.1 mM EDTA) and incubated for 12 h at 37 °C. RNase T1 (0.2 U/ $\mu$ L) was then added, and the samples were incubated for another 4 h at 37 °C. The ribonucleoside mixture obtained from the digest was analyzed by RP-HPLC using Phenomenex-Luna C18 column (250 x 4.6 mm, 5 micron) at 260 nm.



**Fig. S4** HPLC chromatogram of ribonucleoside products obtained from an enzymatic digestion of RNA transcript **4** at 260 nm. (A) Standard mix of natural ribonucleosides and ODU **2**. (B) RNA ON **4** digests. Mobile phase A: 100 mM TEAA (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. Mass analysis of HPLC fraction corresponding to ribonucleosides also confirmed the integrity of the ribonucleosides (data not shown).





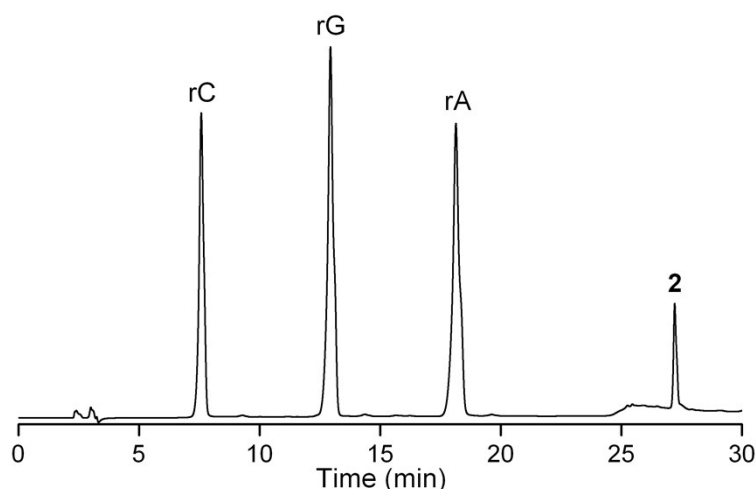
**Fig. S5** UV-thermal melting of control unmodified duplex (black, 1  $\mu$ M) and alkyne-modified duplex (red, 1  $\mu$ M) in 20 mM cacodylate buffer (pH 7.1, 500 mM NaCl, 0.5 mM EDTA). Duplexes were formed by heating a 1:1 mixture of the ONs at 90 °C for 3 min and cooling the solutions slowly to room temperature.  $T_m$  for control duplex was  $76.0 \pm 0.7$  °C and modified duplexes was  $75.4 \pm 0.5$  °C.

### 5. In vitro transcription with linearized plasmid DNA templates in the presence of ODUTP

3. Linearized plasmid DNA templates were obtained by single restriction digestion of DNA plasmids (pDisplay, ZiFiinpDisplay, eGFPinpDisplay)<sup>S4</sup> as follows: 100  $\mu$ L of digestion reactions were carried out in the presence of 1x CutSmart® Buffer (10  $\mu$ L, 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100  $\mu$ g/mL BSA, pH 7.9) containing 10  $\mu$ g of plasmid DNA. To each reaction solution 5  $\mu$ L of restriction enzyme XhoI (10 units/ $\mu$ L) was added and was incubated at 37 °C for 3 h. Linearized plasmid DNA product from respective reaction was then purified using GenElute™ PCR Clean-Up Kit (Sigma Aldrich; Catalog No. NA1020) by following the manufacturer's instructions. Linearized plasmid DNAs pDisplay, ZiFiinpDisplay and eGFPinpDisplay serve as templates for the synthesis of 400 bp, 600 bp and 1.1 kb RNA transcripts, respectively.

The transcription reactions were carried out in 40 mM Tris-HCl buffer (pH 7.9) containing 500 ng of the linearized plasmid DNA template, 1 mM GTP, CTP, ATP,

UTP/ODUTP **3**, 10 mM MgCl<sub>2</sub>, 10 mM NaCl, 10 mM of dithiothreitol (DTT), 2 mM spermidine, 1 U/μL RNase inhibitor (Riboblock) and 40 units T7 RNA polymerase in a total volume of 25 μL. Samples were incubated at 37 °C for 3 h. LiCl (7.5 M, 12.5 μL) was added and the reaction solutions were kept at -20 °C for 1 h followed by centrifugation (12000 rpm) at 4 °C for 15 min. The RNA pellet formed from each reaction was washed with 70% aqueous ethanol, dried and dissolved in 20 μL of nuclease free water. Control and modified transcripts obtained after transcription reactions were resolved and detected by 1% agarose gel containing SYBR® Safe (Invitrogen, catalog no. S33102). Further, the presence of ODU (**2**) label in RNA transcript was confirmed by enzymatic digestion followed by HPLC analysis of ribonucleoside products obtained from the digestion reaction (See section 4 for experimental details and Fig. S6).



**Fig. S6** HPLC chromatogram of ribonucleoside products obtained from enzymatic digestion reactions of RNA transcripts, which were prepared by *in vitro* transcription reactions with linearized EGFP<sub>in</sub>Display plasmid template and in the presence of ODUTP **3**. The presence of modified ribonucleoside **2** in RNA transcript was confirmed by comparing the retention times with standard ribonucleoside mix as before.

**6. Reverse transcription of ODU-modified RNA transcript.** Standard PCR conditions with Platinum Pfx polymerase (Invitrogen) were used to amplify the EGFP gene from pEGFPC1 vector (Clontech) (forward primer: 5' GGACCGA AATTAATACGACTCACTATAGGGGTCTATATAAGCAGAGCTGGTTTG 3' and reverse primer: 5' CAGGTTTCAGGGGGAGGTGTG 3', Fig. S7). PCR amplified DNA product purified by phenol-chloroform extraction was used as the template for transcription reactions in the

presence of UTP and ODUTP **3** as described above. Trace amounts of DNA template present in the transcription products were removed by DNase treatment. The DNase reaction was carried out in Q1 reaction buffer (40 mM Tris-HCl, 100 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, pH 8.0) containing 500 ng of unmodified (control) or ODU-modified RNA transcripts, and 1 unit Q1 RNase free DNase (Promega) in a total volume of 10  $\mu$ L. The reaction mixture was incubated at 37 °C for 3 h. The reaction was stopped by adding 1  $\mu$ L stop solution (20 mM EGTA pH 8.0) and followed by incubation at 65 °C for 10 min. An aliquot of this reaction was used as a negative control (–RT control) for the PCR after cDNA preparation.

The reverse transcription reaction was carried out in the reaction buffer (Promega, ImProm-II™ 5X reaction buffer, M289A) containing 4  $\mu$ L of DNase treated unmodified or modified RNA transcript, 0.5  $\mu$ M reverse primer (5' GCGCGCAGATCTCTTGTA CAGCTCGTCCATGCC 3'), 1 mM MgCl<sub>2</sub>, 1 mM dNTPs, reverse transcriptase (Promega, ImProm-II™ reverse transcriptase, M314A) in a total volume of 10  $\mu$ L. (reaction conditions: 25 °C for 5 min, 42 °C for 60 min, 70 °C for 15 min).

**PCR amplification of cDNA.** The PCR reaction was set up in PCR amplification buffer (Invitrogen, Pfx amplification buffer, Part # 52806) containing 2  $\mu$ L of cDNA, 0.25  $\mu$ M forward primer (5' GCGCGCGTCGACATGGTGAGCAAGGGCGAGGAG 3') and reverse primer (5' GCGCGCAGATCTCTTGTA CAGCTCGTCCATGCC 3'), 0.5 mM dNTPs, 1 mM MgSO<sub>4</sub>, 0.25 units Platinum® Pfx DNA polymerase (50 mM Tris-HCl, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, pH 8.0) in a total volume of 100  $\mu$ L. [PCR conditions: denaturation at 94 °C for 5 min, 35 cycles (denature: 94 °C for 0.5 min, anneal: 60 °C for 0.5 min, extend: 72 °C for 1 min), final extension at 72 °C for 10 min]. PCR product was purified by phenol-chloroform extraction method. PCR product was sequenced by dideoxy sequencing method (1<sup>st</sup> Base, Asia) using PCR amplified DNA (40 ng/ $\mu$ L, 10  $\mu$ L) and reverse primer (10 pmol/ $\mu$ L, 10 $\mu$ L, Fig. S8).

5'GGACCGAAATTAATACGACTCACTATAGGGGTCTATATAAGCAGAGCTGGTTTAGTAACCG  
 TCAGATCCGCTAGCGCTACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTACCC  
 GGGGTGGTGGCCATCCTGGTTCGAGCTGGACGGCGACGTAACGGCCACAAGTTCAGCGTGT  
 CCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCAC  
 CGGCAAGCTGCCCCTGCCCTGGCCACCCTCGTGACCACCCTGACCTACGGCGTGACGTGCT  
 TCAGCCGCTACCCCGACCACATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCCGAAGGC  
 TACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG  
 TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGA  
 GGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTACAACAGCCACAACGTCTATATC  
 ATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGG  
 ACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGGCAGCGCCCCGT  
 GCTGCTGCCCAGCAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAG  
 AAGCGCGATCACATGGTCTGCTGGAGTTCGTGACCGCCCGGGATCACTCTCGGCATGG  
ACGAGCTGTACAAGTCCGGACTCAGATCTCGAGCTCAAGCTTTCGAATTCTGCAGTCGACGG  
 TACCGCGGGCCCGGGATCCACCGGATCTAGATAACTGATCATAATCAGCCATAACCACATTTG  
 TAGAGGTTTTACTTGCTTTAAAAAACCTCCACACCTCCCCCTGAACCTG 3'

**Fig. S7** PCR amplified template sequence from pEGFPC1 vector (Clontech, #6084-1) used in transcription reactions. T7 promoter sequence is underlined. Primer site for reverse transcription is denoted in red. Regions highlighted in yellow are primer sites for PCR amplification of cDNA. See section 6 for details.

Download [Graphics](#)

Sequence ID: lc|14833 Length: 712 Number of Matches: 1

Range 1: 1 to 712 [Graphics](#) ▼ Next Match ▲ Previous Match

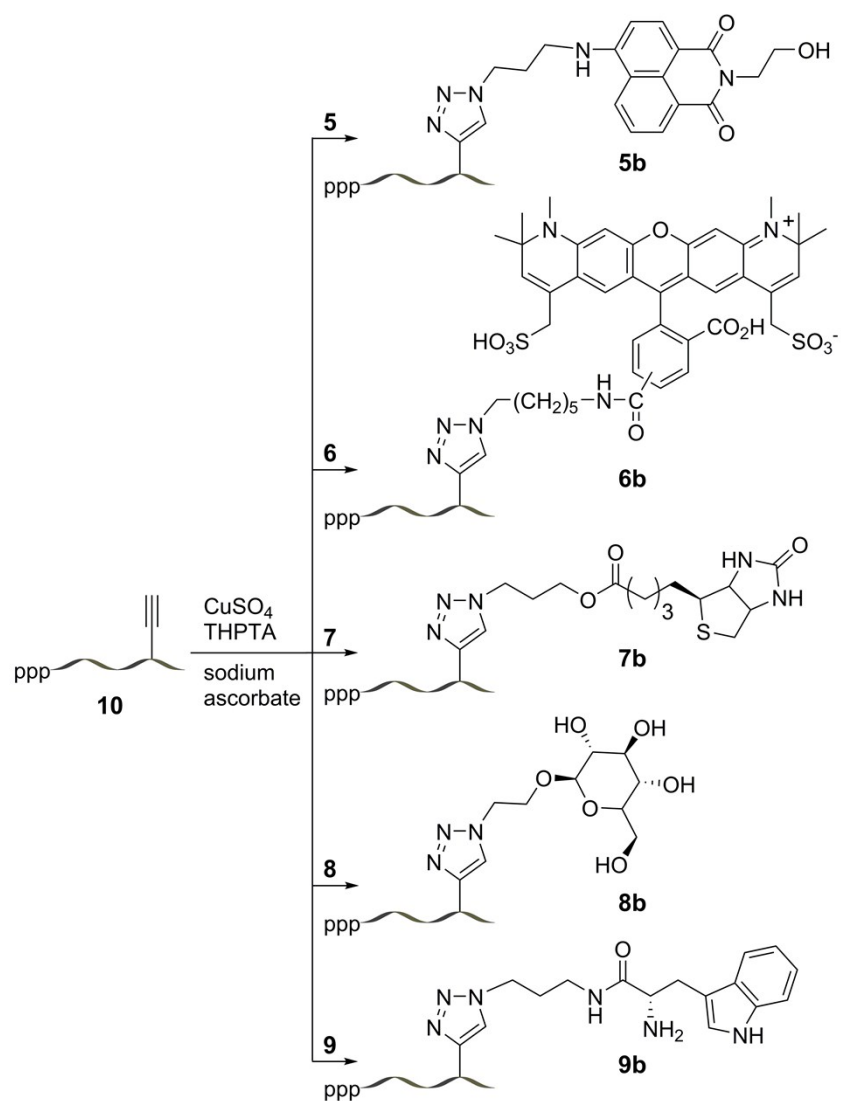
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Sbjct 1		GCGTGTCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCT	60	
Query 238	GCACCACCGGCAAGCTGCCCCTGGCCACCCTCGTGACCACCCTGACCTACGGCG	297		
Sbjct 61		GCACCACCGGCAAGCTGCCCCTGGCCACCCTCGTGACCACCCTGACCTACGGCG	120	
Query 298	TGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAGACTTCTTCAAGTCCGCCA	357		
Sbjct 121		TGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAGACTTCTTCAAGTCCGCCA	180	
Query 358	TGCCCCAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGA	417		
Sbjct 181		TGCCCCAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGA	240	
Query 418	CCCGCGCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA	477		
Sbjct 241		CCCGCGCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA	300	

**Fig. S8** Representative sequence alignment (BLAST)<sup>S5</sup> of PCR amplified DNA products obtained from cDNA, which was in turn obtained by reverse transcribing ODU-containing RNA transcript. The sequencing data of PCR product matched well with the template DNA sequence used for the incorporation of ODUTP into RNA by *in vitro* transcription reaction. See section 6 for details.

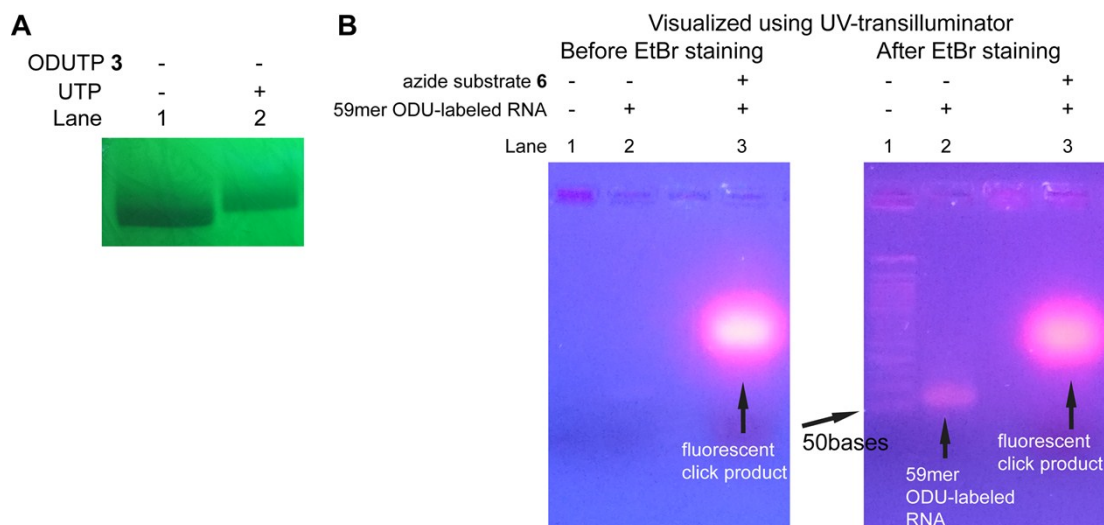
**Table S2** Yield and mass data of click products obtained by posttranscriptional chemical modification of ODU- (**4**) and EU-labeled (**10**) RNA transcripts.

entry	alkyne-RNA ON	azide substrate	click product	yield (nmol)	isolated yield <sup>a</sup> %	MALDI-TOF mass analysis of click products (m/z)	
						calculated mass	observed mass
1	<b>4</b>	<b>5</b>	<b>5a</b>	7.8	65	3858.6	3858.3
2	<b>4</b>	<b>6</b>	<b>6a</b>	8.2	68	4367.0	4368.3
3	<b>4</b>	<b>7</b>	<b>7a</b>	6.3	52	3846.8	3846.4
4	<b>4</b>	<b>8</b>	<b>8a</b>	4	33	3768.8	3768.2
5	<b>4</b>	<b>9</b>	<b>9a</b>	4	33	3806.1	3805.3
6	<b>10</b>	<b>5</b>	<b>5b</b>	6	50	3778.3	3778.5
7	<b>10</b>	<b>6</b>	<b>6b</b>	7	58	4286.9	4286.2
8	<b>10</b>	<b>7</b>	<b>7b</b>	3	25	3766.8	3766.5
9	<b>10</b>	<b>8</b>	<b>8b</b>	3	25	3688.2	3688.2
10	<b>10</b>	<b>9</b>	<b>9b</b>	4.5	38	3725.3	3725.7

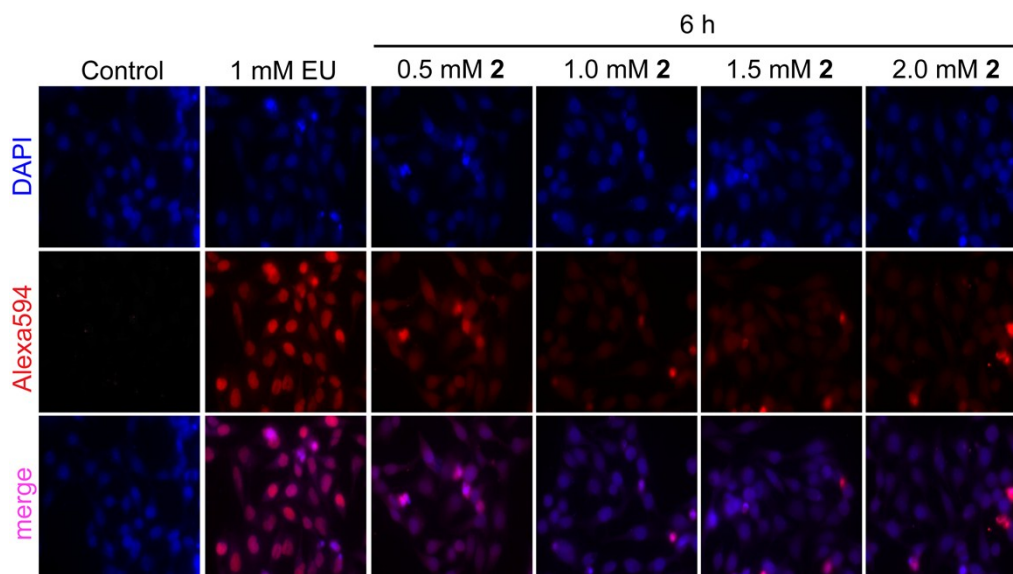
<sup>a</sup>All reactions were performed on 12 nmol scale of alkyne-modified RNA ONs. Yields reported are with respect to the RNA products isolated after PAGE purification.



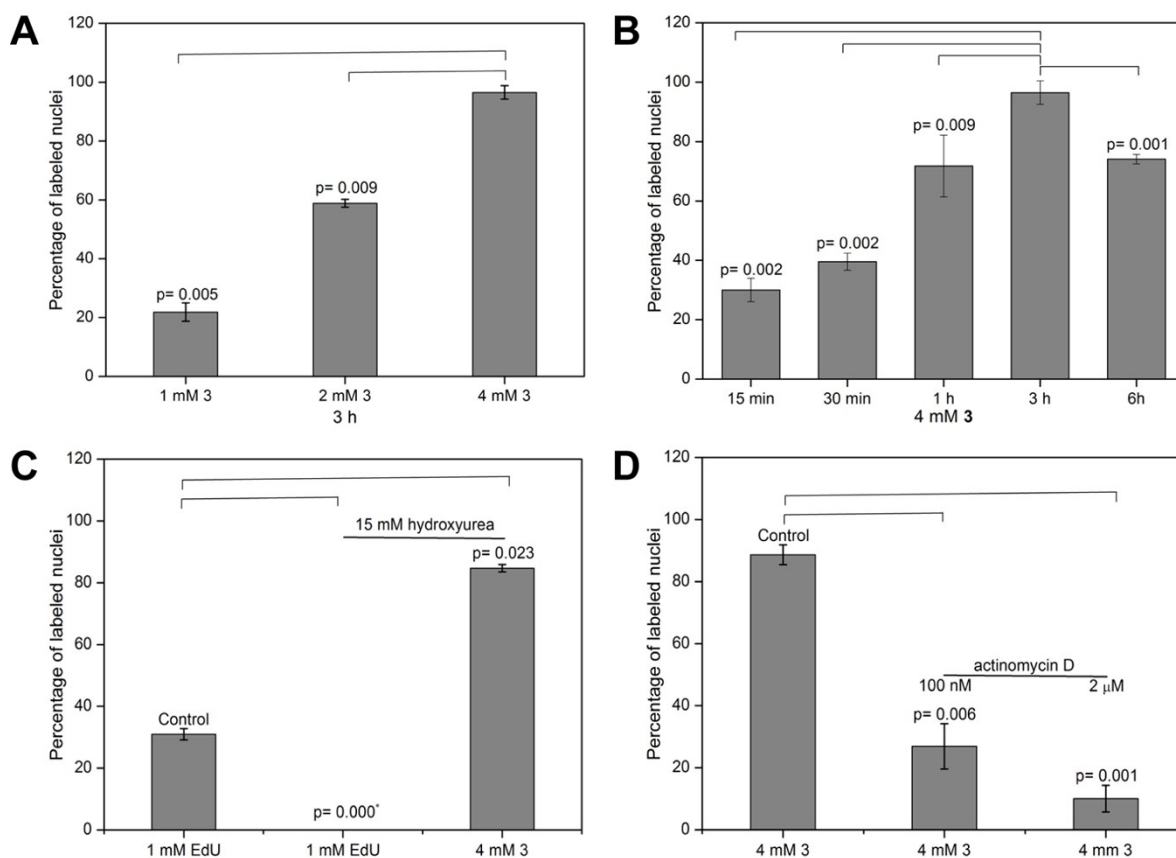
**Fig. S9** Posttranscriptional chemical functionalization of EU-labeled RNA transcript **10** with azide substrates **5–9** by using CuAAC reaction.



**Fig. S10** (A) UV-shadow (260 nm) of PAGE-resolved 59mer control unmodified (lane 1) and ODU-modified (lane 2) RNA transcripts. ODU-modified RNA runs slower than control unmodified RNA. The ODU-labeled RNA band was cut, purified and subjected to click reaction with Alexa594-azide **6**. (B) CuAAC reaction between 59mer ODU-labeled RNA and Alexa594-azide **6**. The clicked product was resolved by Agarose gel and visualized by using UV-transilluminator before and after ethidium bromide (EtBr) staining. The fluorescent clicked product (lane 3) which runs slower than 59mer ODU-labeled RNA (lane 2) could be clearly visualized by UV-transilluminator even before EtBr staining. 50 bases ladder (lane 1) was used. See experimental section for details.



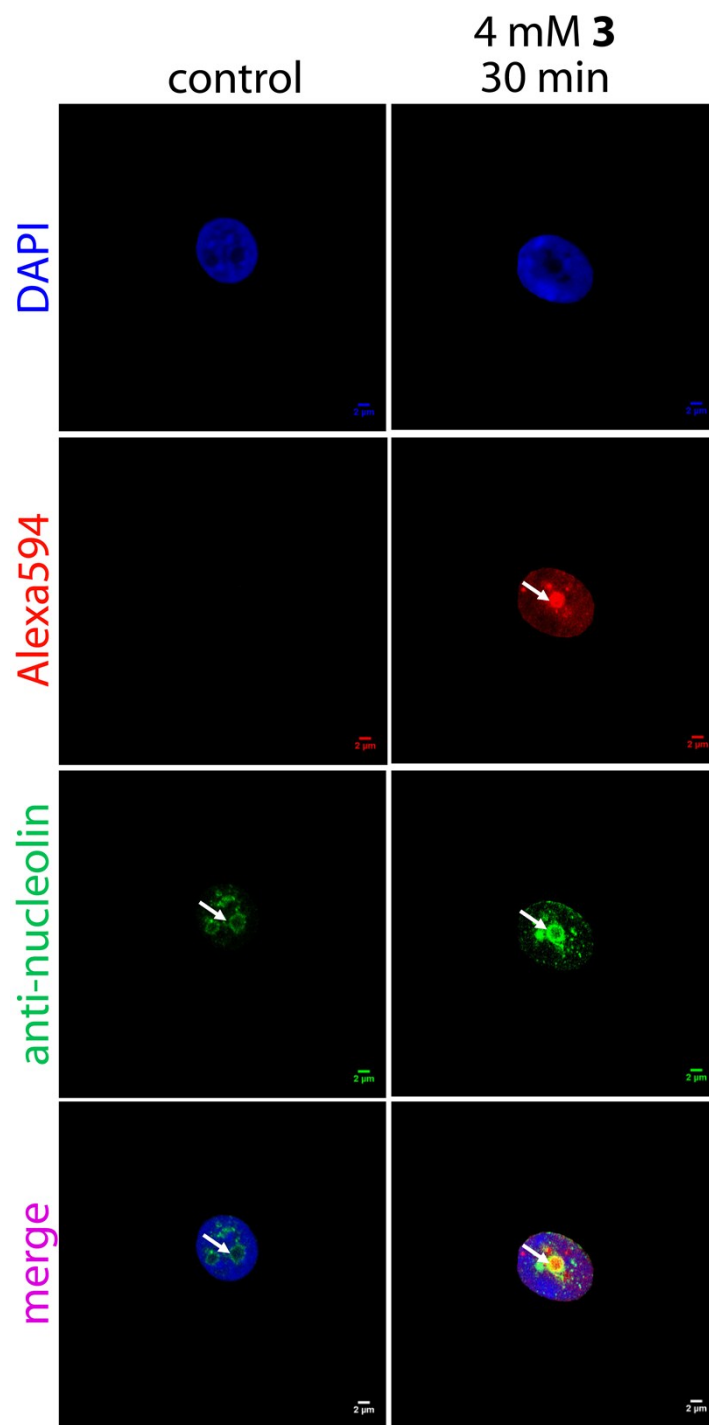
**Fig. S11** ODU (**2**) ribonucleoside is not effectively incorporated into cellular RNA by the ribonucleoside salvage pathway. HeLa cells in culture were incubated with ODU **2** (0.5–2.0 mM) for 6 h. The cells were then fixed, permeabilized and ODU labeling was detected by performing CuAAC reaction with Alexa594-azide **6**. Fluorescence signal from the cells was very low indicating that the modified ODU is not incorporated into RNA. In a positive control experiment EU (column 2) was effectively incorporated into cellular RNA under similar conditions (EU staining pattern was similar to the literature report).<sup>S6</sup>



**Fig. S12** Quantification plots showing percentage of fluorescently labeled nuclei by click reaction with Alexa594-azide **6**. **(A)** Cells transfected with increasing concentration of ODUTP **3** showed dose-dependent increase in number of nuclei showing fluorescence. **(B)** Cells transfected with 4 mM of ODUTP as a function of incubation time showed time-dependent increase in number of cells showing nuclear staining up to 3 h. Longer incubation time showed detectable decrease in staining. **(C)** Inhibition of DNA synthesis in presence of hydroxyurea does not affect the number of labeled nuclei with Alexa594-azide **6**. **(D)** Treatment with variable concentration of actinomycin D (RNA polymerases inhibitor) significantly decreased the number of cells showing nuclear staining by click reaction using Alexa594-azide **6**. In all statistical analysis the total numbers of cells labeled with Alexa594-azide was counted against the total number of cells stained with DAPI and percentage was calculated. Standard deviation was calculated from three independent experiments and probability value (p) was calculated by t-test and only a value of  $p < 0.05$  was considered throughout the analysis. See experimental section for details.

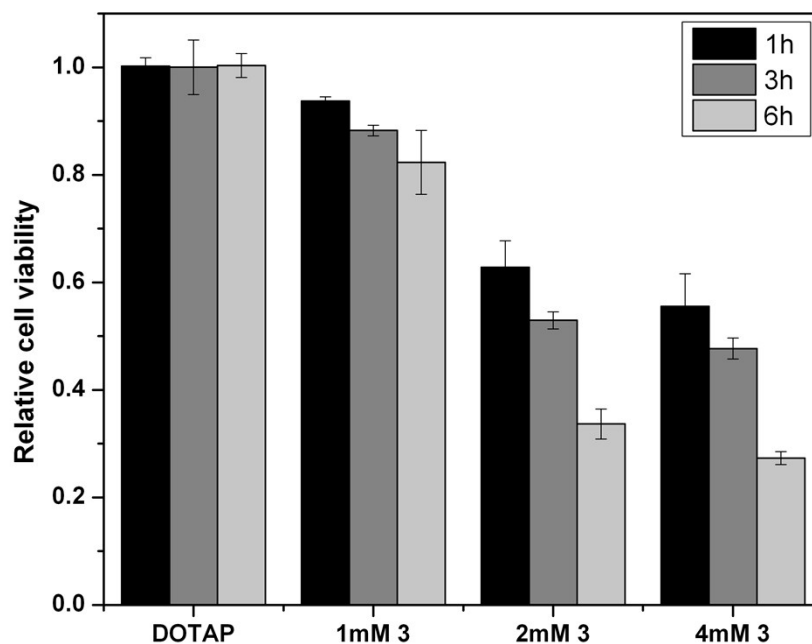


**7. Anti-nucleolin staining:** To perform anti-nucleolin staining cells were first transfected with ODUTP **3** (4 mM) for 3 h and stained by using CuAAC reaction with alexa594-azide **6** as before. Primary antibody staining was performed in closed moist chamber with 50  $\mu$ L of anti-nucleolin (ab-22758, containing 5% BSA in 1X PBS) taken on parafilm. Over this solution click-stained coverslip was inverted and incubated overnight at 4 °C. After incubation, cover slip was washed twice with 1X PBS and followed by secondary antibody staining in presence of AF-488 anti-rabbit (A-11034, Life technologies). Secondary antibody staining was performed in closed moist chamber using 50  $\mu$ L of AF-488 anti rabbit antibody dilution in 1X PBS containing 5% BSA. The coverslip was inverted over this solution and incubated overnight at 4 °C. After secondary antibody staining coverslip was washed twice with 1X PBS and DAPI staining was performed and cells were imaged as before. In a control experiment, anti-nucleolin staining was performed on cells which were not transfected with ODUTP.

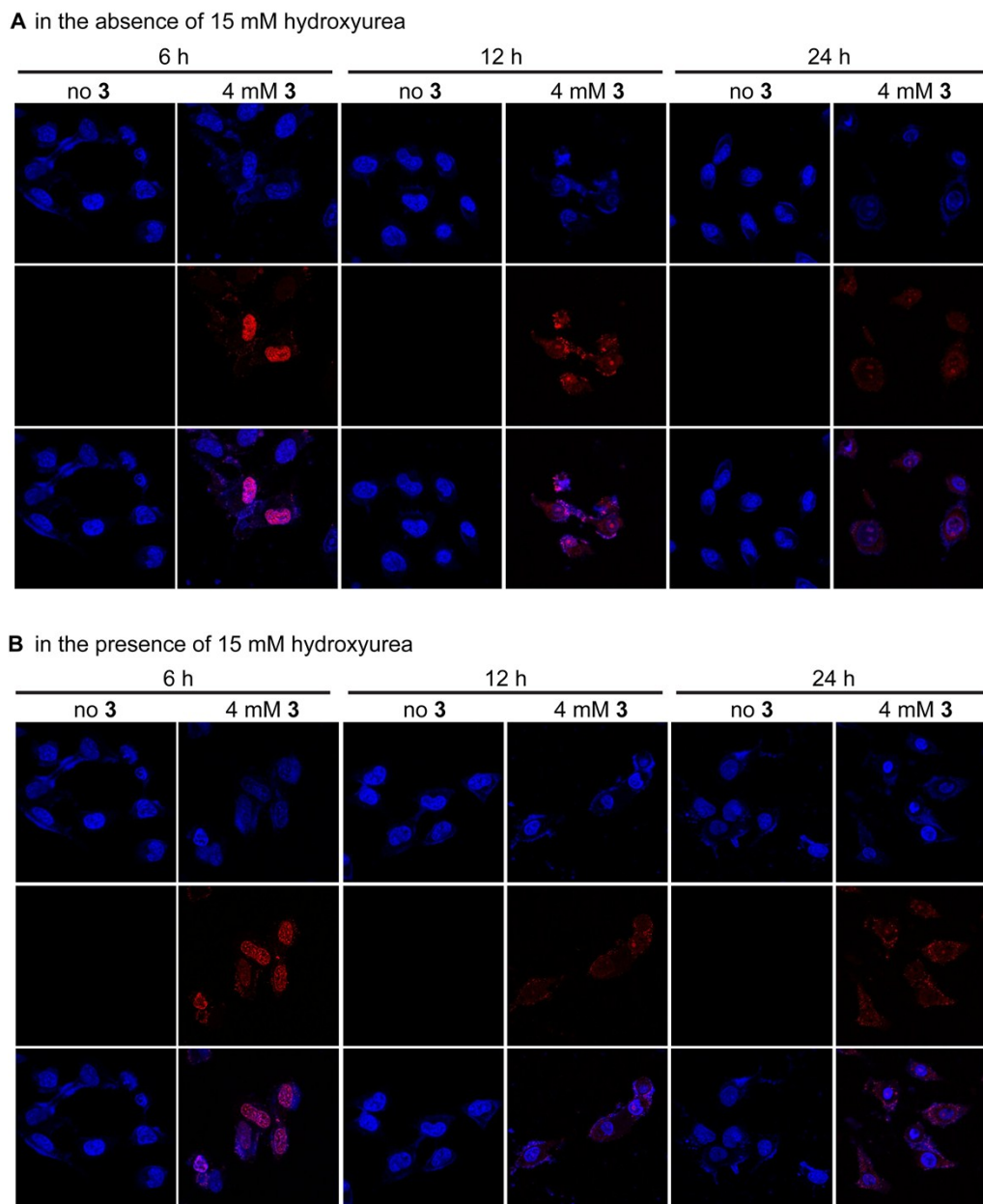


**Fig. S13** Confocal images (cross section) of HeLa cells transfected with ODUTP **3** showing intense staining in nucleoli (marked using an arrow). (Right column) Anti-nucleolin (green, Abcam, ab-22758), which targets nucleolin present at the periphery of nucleoli,<sup>S7</sup> was used to confirm the localization of ODU labeling in the nucleoli. Merging the signals obtained after click reaction (red) and anti-nucleolin (green) staining revealed concentrated ODU labeling within the nucleoli. (Left column) In a control experiment, anti-nucleolin staining was performed on cells, which were not transfected with ODUTP. See section 7 for experimental details.

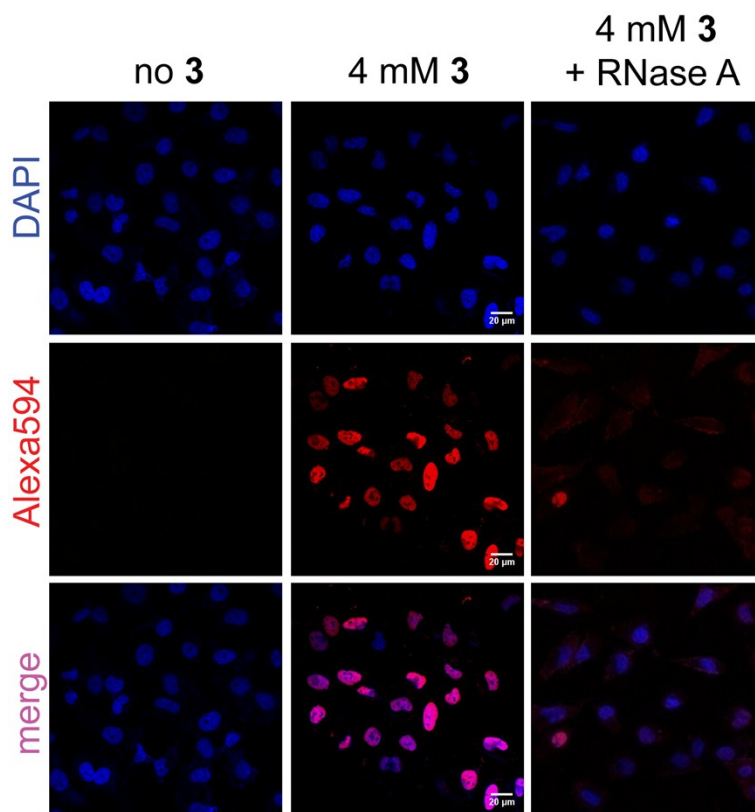
**8. MTT assay.** The cytotoxicity of ODUTP **3** on HeLa cells were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. For the experiment  $10^4$  cells per well were seeded in 96-well plate and incubated for 12–16 h at 37 °C. Cells were transfected with 100  $\mu$ L of transfection solution containing varying concentrations of ODUTP **3**. Three time points for ODUTP **3** were chosen for the MTT assay. The transfected cells were treated with 100  $\mu$ L of MTT solution (final conc. of 0.05 mg/mL to each well) at different time points and incubated in dark at 37 °C for 4 h. Formazon crystals were dissolved by adding 100  $\mu$ L of DMSO to each well and the absorbance was measured at 570 nm on a plate reader. The % cell viability was normalized to cells transfected with DOTAP alone as a control.



**Fig. S14** Plot showing the cell viability of HeLa cells in the presence of varying concentrations of ODUTP **3**. Cell viability in the presence of **3** is reported relative to cell viability in the presence DOTAP alone. See section 8 for details.



**Fig. S15** Effect of incubation of ODUTP in the absence (A) and presence (B) of hydroxyurea for extended periods (6–24 h). 0.1–0.2 million HeLa cells were seeded in a 12 well plate on glass cover slips for nearly 16 h before the experiment. Cells were transfected with transfection mix (300  $\mu$ L) containing 4 mM **3** in the absence and presence of 15 mM hydroxyurea. Cells were then incubated for time intervals of 6 h, 12 h and 24 h. Cells from each time point were washed, fixed, permeabilized and stained by click reaction using Alexa594-azide **6** as before. The cells were imaged using a confocal fluorescence microscope with an oil immersion using 63X objective. Although longer incubation times led to significant reduction in fluorescence signal the staining pattern in the absence and presence of hydroxyurea was found to be similar.

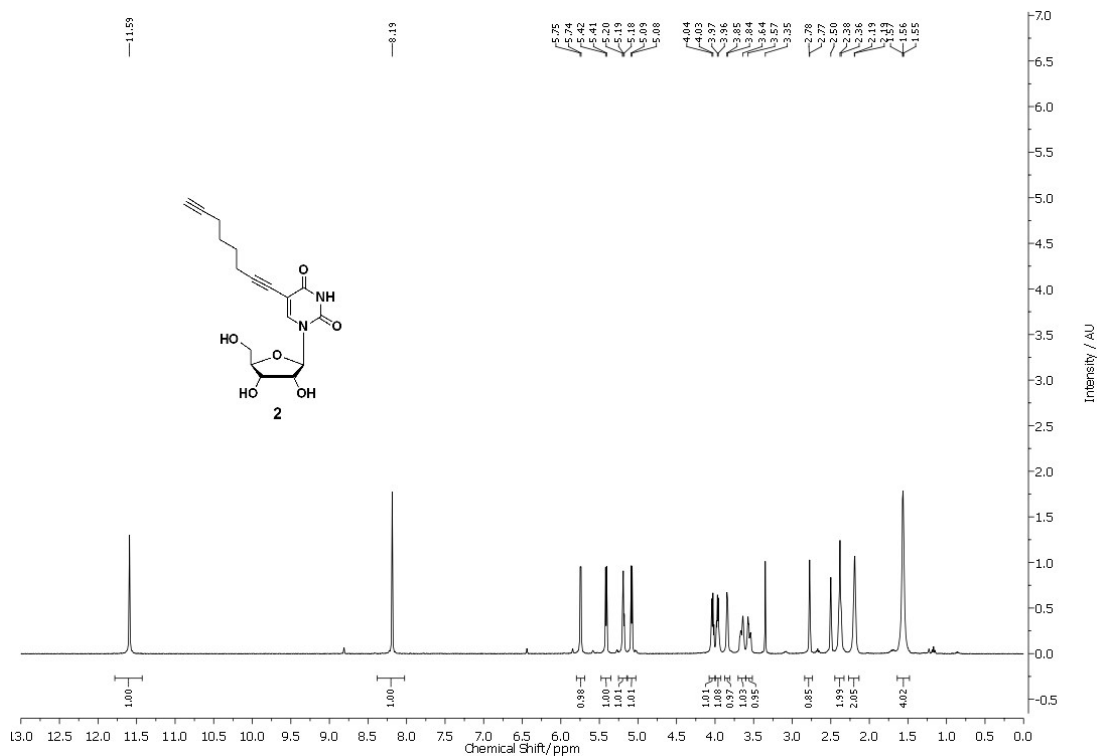


**Fig. S16** RNase treatment experiment reveals specific incorporation of ODUTP **3** into transcribing RNA in cells. HeLa cells were transfected with ODUTP **3** (4 mM) for 3 h. Fixed cells were then treated with RNase A (200  $\mu\text{g}/\text{mL}$ ). The cells were stained using Alexa594-azide **6** by click reaction and imaged as before. Drastically reduced fluorescence signal from the cells treated with RNase A revealed the selective labeling of ODU into RNA by endogenous RNA polymerases.

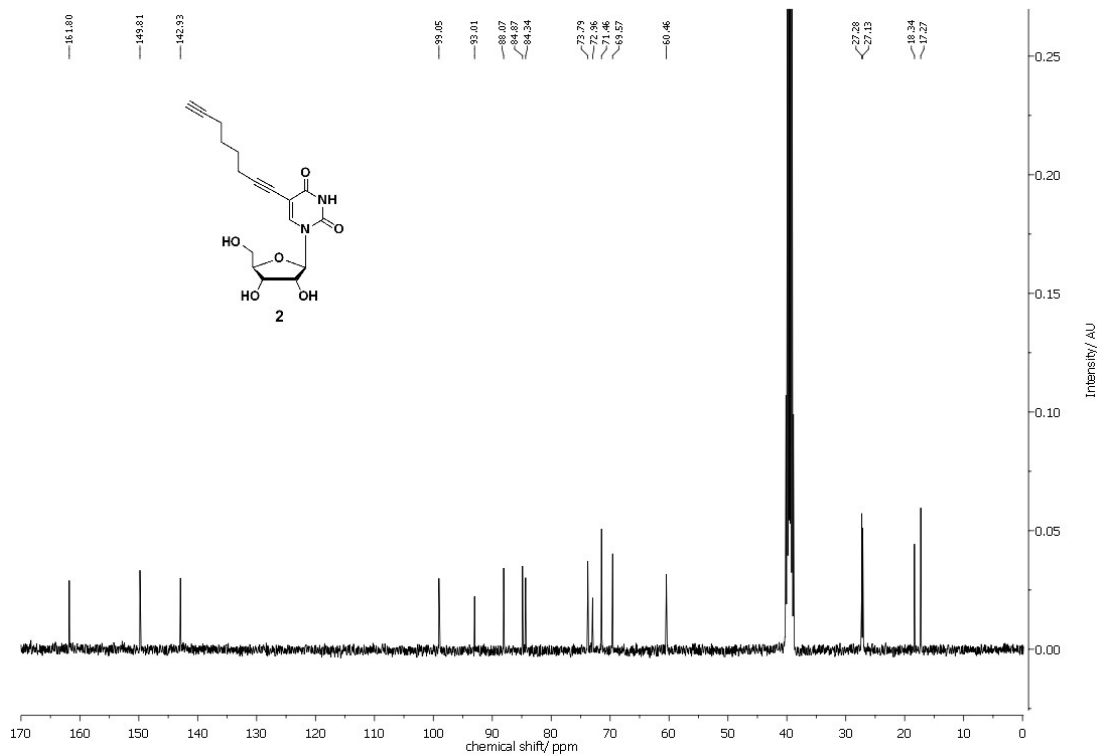
**9. Raman analysis of alkyne-modified nucleosides EU and ODU.** An aqueous solution (1 mM) of EU or ODU in 10% DMSO was drop casted on a quartz support and dried. The laser (633 nm) output with a power of 2  $\text{mW}/\mu\text{m}^2$  was focused on the sample using a 60 $\times$ /0.7 numerical aperture (NA) air immersion objective lens. The confocal slit width of the spectrograph was 200  $\mu\text{m}$  with grating 1800 grooves/mm. The spectrum was recorded on a LabRAM HR 800, HORIBA confocal Raman microscope with an acquisition time of 90 sec.

## 10. NMR spectra

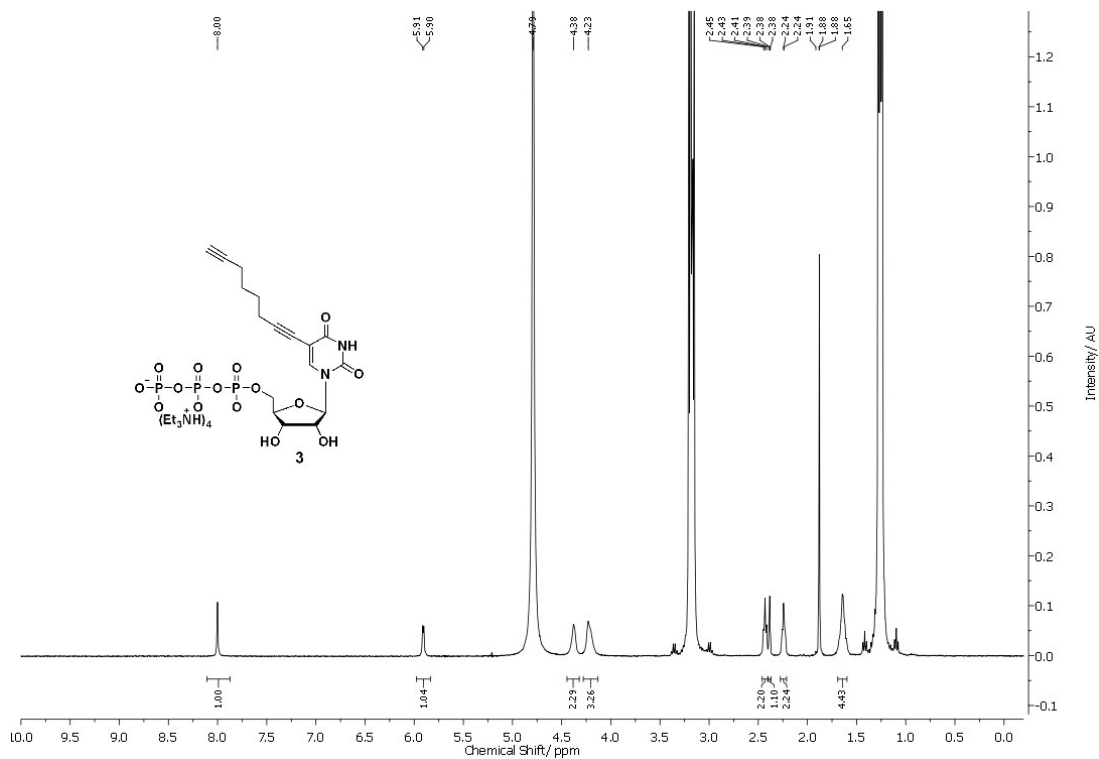
### $^1\text{H}$ -NMR of alkyne-modified ribonucleoside **2** in $d_6$ -DMSO



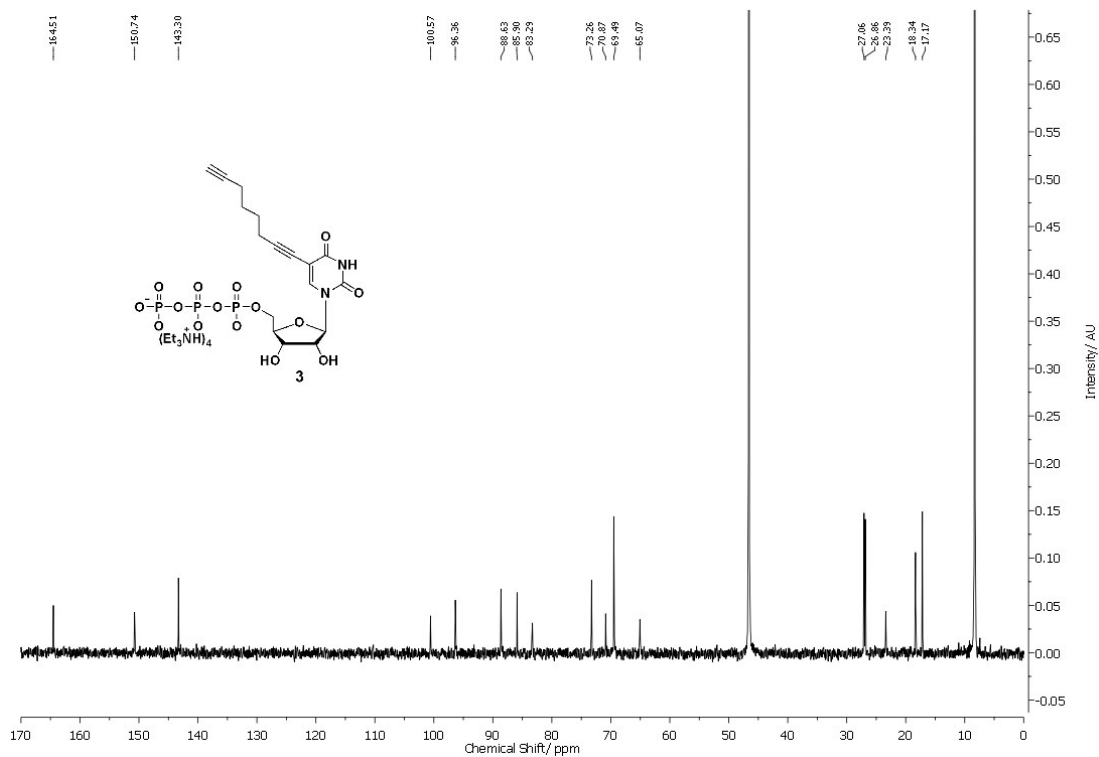
### $^{13}\text{C}$ -NMR of alkyne-modified ribonucleoside **2** in $d_6$ -DMSO



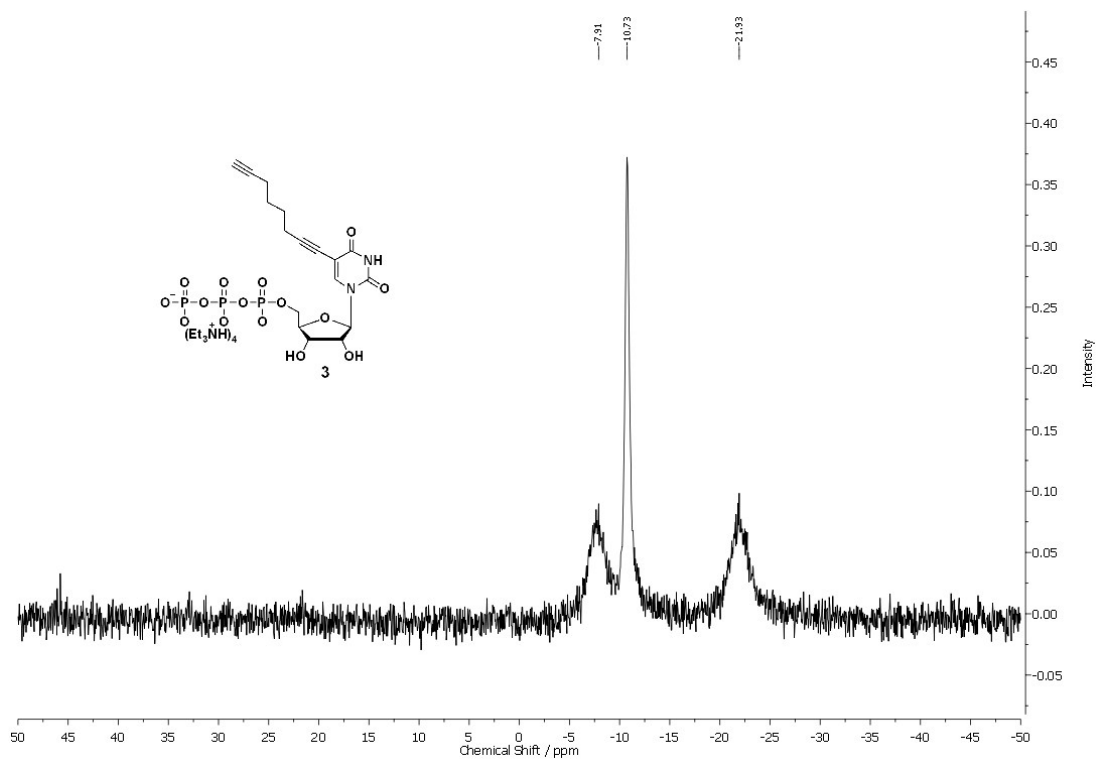
### $^1\text{H-NMR}$ of alkyne-modified ribonucleoside triphosphate **3** in $\text{D}_2\text{O}$



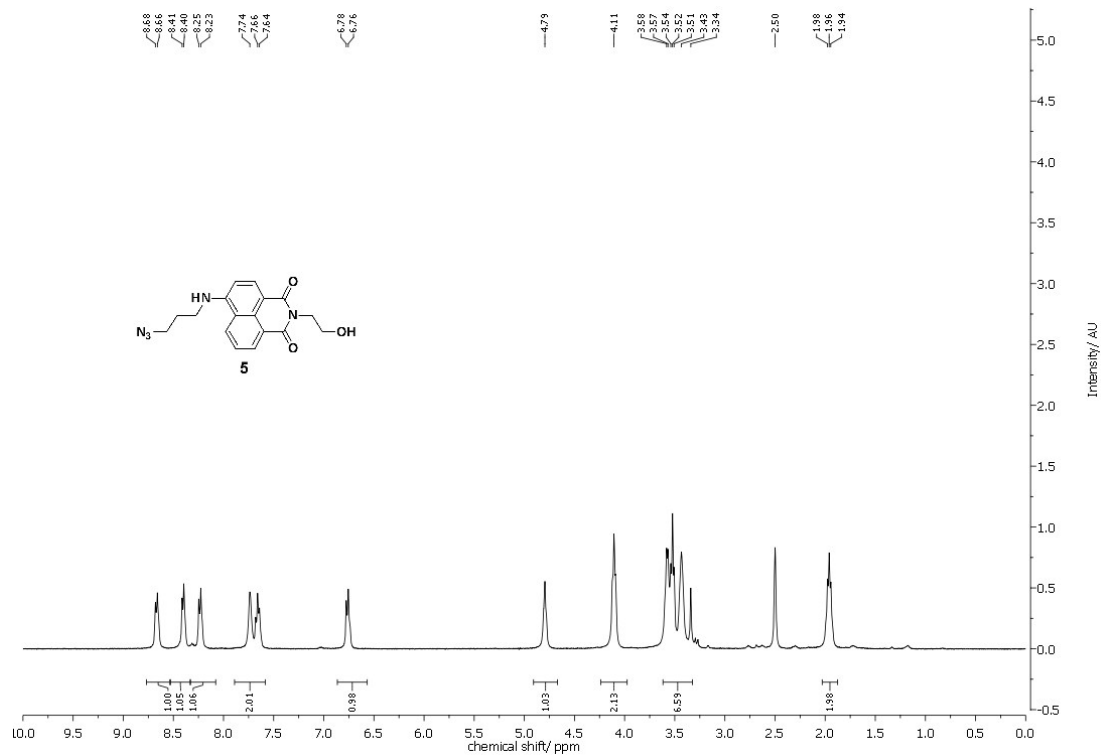
### $^{13}\text{C-NMR}$ of alkyne-modified ribonucleoside triphosphate **3** in $\text{D}_2\text{O}$



### $^{31}\text{P}$ -NMR of alkyne-modified ribonucleoside triphosphate **3** in $\text{D}_2\text{O}$

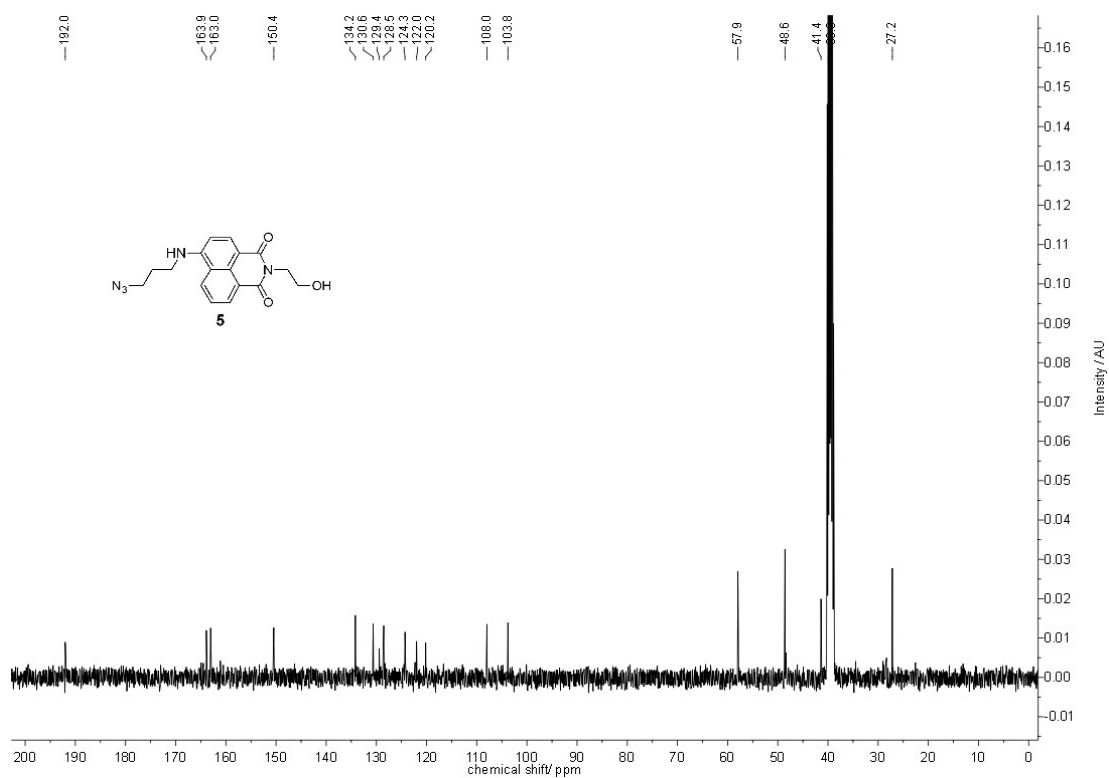


### $^1\text{H}$ -NMR of naphthalimide azide **5** in $d_6$ -DMSO

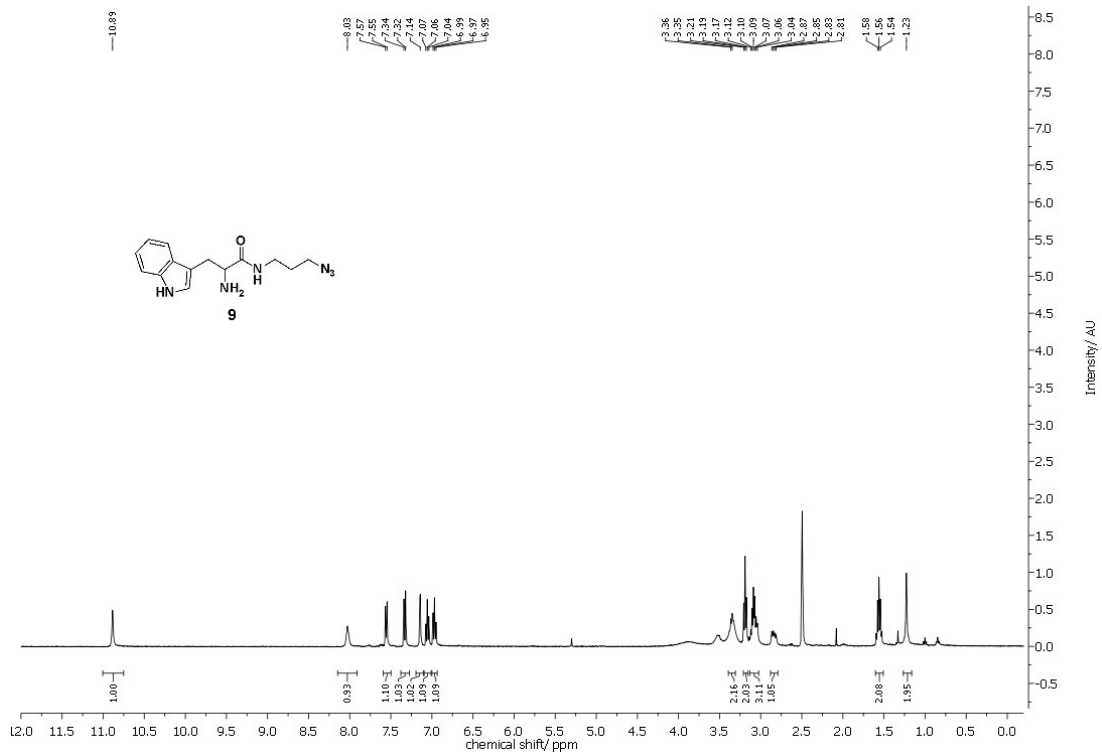




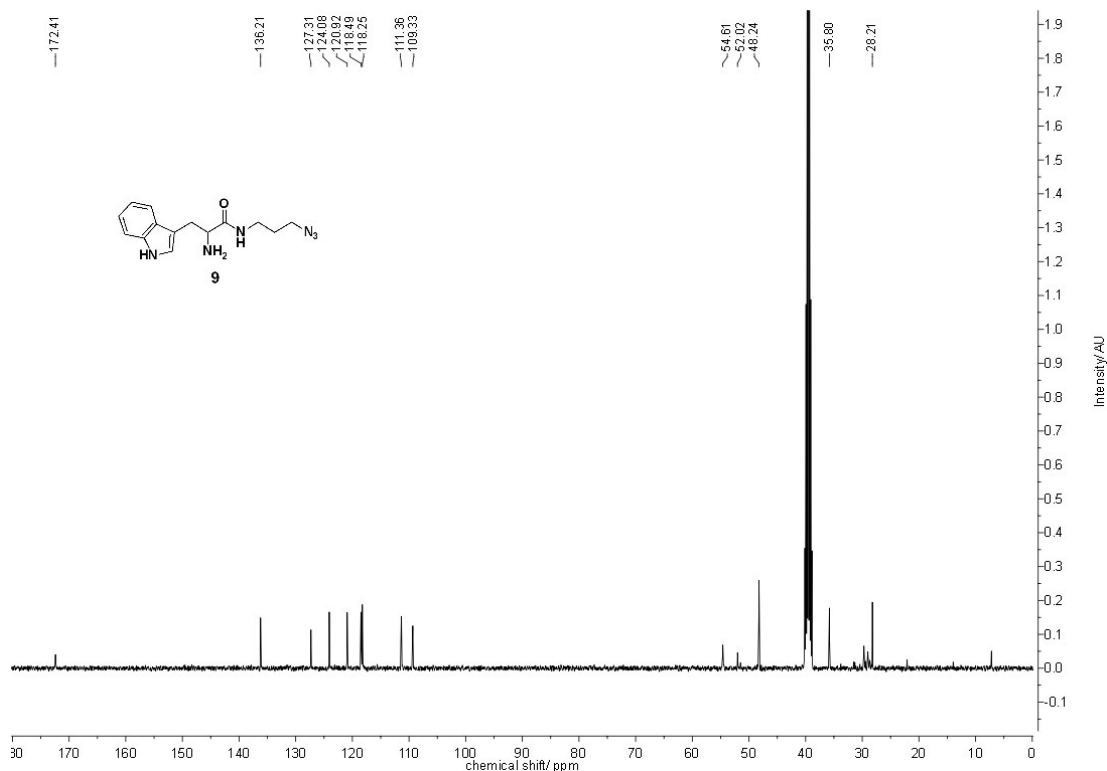
$^{13}\text{C}$ -NMR of naphthalimide azide **5** in  $d_6$ - DMSO



$^1\text{H}$ -NMR of tryptophan azide **9** in  $d_6$ - DMSO



### <sup>13</sup>C-NMR of tryptophan azide **9** in *d*<sub>6</sub>-DMSO



## 11. References

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