Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Aptamer-based proximity labeling guides covalent RNA modification

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Content

1. General remarks

All reagents were purchased from commercial suppliers SIGMA-ALDRICH, THERMOFISCHER, NEW ENGLAND BIOLABS, CARL ROTH and JENA BIOSCIENCE. Synthetic DNA oligonucleotides were purchased from INTEGRATED DNA TECHNOLOGIES. Oligonucleotide concentrations were determined using a NanoDrop-1000 spectrometer (PEQLAB). Polymerase chain reactions and labeling experiments were performed using a BIO-RAD T100 Thermal Cycler or an PTC-100® Programmable Thermal Controller (MJ RESEARCH). For all experiments, deionized water purified with a Milli-Q Synthesis A10 Water Purification system (MERCK) was used.

Denaturing polyacrylamide gel electrophoresis (PAGE) gels (8.3 M urea) were performed using acrylamide/bisacrylamide stock solution (Rotiphorese® Gel 40, 19:1) purchased from CARL ROTH. Electrophoresis was performed in TBE buffer (100 mM Tris-Borate (pH 8.3), 2 mM EDTA) and preparative gels were run at 25 W and sequencing gels at 45 W, respectively. Polyacrylamide gels were visualized using Typhoon FLA 9500 (GE HEALTHCARE) and where applicable storage phosphor screens (GE HEALTHCARE) were used. Obtained images were analyzed and quantified using the ImageQuant software (MOLECULAR DYNAMICS).

Liquid chromatography–mass spectrometry (LC-MS) experiments were performed on an AGILENT 1200 Series HPLC system equipped with a diode array detector coupled to a BRUKER micrOTOF QII-ESI system. Measurements were conducted on a PHENOMENEX Synergi 2.5 µm Fusion-RP 100 Å (100 x 2 mm) column with a constant flow rate of 0.3 ml/min using mixtures of an ammonium acetate buffer (5 mM, pH = 5.5) and acetonitrile as eluents.

All fluorescence measurements were performed with a JASCO FP-6500 spectrofluorometer equipped with a Peltier temperature controller in a 15 µl quartz cuvette at 25 °C.

1.1. In vitro transcription of RNAs

Double stranded DNA templates were prepared by polymerase chain reaction (PCR) using Taq polymerase (lab prepared stock) and primers listed in Table S1. To reduce non-templated nucleotide addition on the 3' end of the transcribed RNA, reverse primers with two C-2' methoxy modified nucleotides at the 5' termini were used for most experiments (see Table 2).[1] *In vitro* transcriptions (IVT) were set up using 1 µM dsDNA template and 50 ng/µl T7 RNA polymerase (lab prepared stock) in transcription buffer (40 mM Tris-HCl (pH 8.1), 1 mM Spermidine, 22 mM $MgCl_2$, 0.01% Triton X-100, 5% DMSO) supplemented with 2 mM ATP, GTP, CTP and the desired uridine triphosphate derivative (**UTP**, **vUTP**, **taUTP**, **cpUTP**, **norUTP**). For radiolabeled RNAs 0.4 µCi/µl ^{32}P α-ATP was spiked into the transcription mixture. The reaction was incubated at 37 °C for 4 hours and treated with 50U/ml DNAse

I (ROCHE) at 37 °C for 30 min. Next, the reaction was stopped by the addition one volume of gel loading buffer (100 mM Tris-Borate (pH 8.3), 2 mM EDTA in 90% formamide containg bromphenol blue) and purified by gel electrophoresis (10% denaturing polyacrylamide gel). After electrophoresis, bands were visualized via UV shadowing or phosphor imaging. Product bands were excised and eluted in 0.3 M NaOAc (pH 5.5) at 19 °C overnight. The filtered RNA solution was isopropanol precipitated and the obtained pellet was dissolved in water. The samples were desalted using 10k Amicon Ultra centrifugal filters (MERCK) and dissolved in water.

Table S1: Primer sequences (5' -> 3'). Overlapping primer sequence is underlined.

Primer	Sequence
RhoBAST_fwd	TCTAATACGACTCACTATAGGAACCTCCGCGAAAGCGGTGAAGG
RhoBAST_rev	**mGmG**AACCTGAGGCGGTTAACCTTGCGCCTCTCCTTCACCGCTTTCG
inactive_RhoBAST_fwd	TCTAATACGACTCACTATAGGAACCTCCGCGAAAGCGGTAGAAAC
inactive_RhoBAST_rev	**mGmG**AACCCGAGGCGGCTAATTTGCCTCCTCGTTTCTACCGCTTTC
elongated_inactive_RhoBAST_fwd	TCTAATACGACTCACTATAGGGCCCGGAACCTCCGCGAAAGCGGTAGAAAC
elongated_inactive_RhoBAST_rev	**mGmG**GCCCGGAACCCGAGGCGGCTAATTTGCCTCCTCGTTTCTACCGCTTTC
RhoBAST_U38U39_fwd	TCTAATACGACTCACTATAGGCGCCGCCGCGAAAGCGGGGAAGG
RhoBAST_U38U39_rev	GGCGCCGGGGGCGGTTAACCTTGCGCCCCGCCTTCCCCGCTTTCG
RhoBAST_U38_fwd	TCTAATACGACTCACTATAGGGCCCGCCGCGAAAGCGGGGAAGG
RhoBAST_U38_rev	GGGCCCGGCGGCGGTTGACCTTGCGCCGCGCCTTCCCCGCTTTCG
RhoBAST_U39_fwd	TCTAATACGACTCACTATAGGGCCCGCCGCGAAAGCGGGGAAGG
RhoBAST_U39_rev	GGGCCCGGCGGCGGTTAGCCTTGCGCCGCGCCTTCCCCGCTTTCG
39mer_fwd	TCTAATACGACTCACTATAGGGGCCCGGATAGCTCAGTCGG
39mer_rev	**mCmC**GCGGCCGCTGCTCTACCGACTGAGCTATCC

Table S2: DNA templates for IVT reactions (5' -> 3'). T7 promotor region is underlined.

DNA template	Sequence
RhoBAST	TCTAATACGACTCACTATAGGAACCTCCGCGAAAGCGGTGAAGGAGAGGCGCAAGGTTAACCGCCTCAGGTTCC
inactive RhoBAST	TCTAATACGACTCACTATAGGAACCTCCGCGAAAGCGGTAGAAACGAGGAGGCAAATTAGCCGCCTCGGGTTCC
elongated inactive RhoBAST	TCTAATACGACTCACTATAGGGCCCGGAACCTCCGCGAAAGCGGTAGAAACGAGGAGGCAAATTAGCCGCCTCGGGTTCCGGGCCC

RhoBAST_U38U39	TCTAATACGACTCACTATAGGCGCCGCCGCGAAAGCGGGGA AGGCGGGGCGCAAGGTTAACCGCCCCCGGCGCC
RhoBAST_U38	TCTAATACGACTCACTATAGGGCCCGCCGCGAAAGCGGGGA AGGCGCGGCGCAAGGTCAACCGCCGCCGGGCCC
RhoBAST_U39	TCTAATACGACTCACTATAGGGCCCGCCGCGAAAGCGGGGA AGGCGCGGCGCAAGGCTAACCGCCGCCGGGCCC
39mer	TCTAATACGACTCACTATAGGGGCCCGGATAGCTCAGTCGG TAGAGCAGCGGCCGCGG

1.2. Fluorescence turn-on

To ensure optimal folding of the respective RNA, all RNA samples were folded freshly prior to each labeling experiment. RNA stock solutions in water were diluted to the desired concentration and incubated at 75 °C for 2 min. Next, the solution was cooled down to 25 °C in 10 min and reaction buffer (20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 125 mM KCl) was added. Samples were incubated for additional 5 min at 25 °C before addition of the desired dyes (stock solutions in DMSO). The final DMSO concentration was kept below 0.5%. As excitation/emission wavelengths, the corresponding maxima of the RNA*dye complexes were used.

1.3. Binding affinities

Dissociation constants (K_D) were determined by measuring the fluorescence enhancement depending on the RNA concentration using a fixed probe concentration (20 nM). All measurements were performed using freshly folded RNA in reaction buffer (20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 125 mM KCl) supplemented with 0.05% Tween 20 at 25°C. The obtained curve was fitted using the following equation:

$$F = F_0 + \frac{(F_\infty - F_0) \times \{(K_D + P_0 + [Apt]) - \sqrt{([Apt] - P_0)^2 + K_D \times (K_D + 2[Apt] + 2P_0)}\}}{2P_0} \qquad (1)$$

where F is the fluorescence at any RNA concentration, F_0 is the fluorescence of the free probe, P_0 is the initial probe concentration, F_∞ is the maximum fluorescence of the RNA/probe complex, $[Apt]$ is the concentration of used RNA and K_D is the dissociation constant.[2]

1.4. LC-MS analysis of modified RNAs

RNA transcripts (4 µg) were digested to nucleosides using the Nucleoside Digestion Mix (NEW ENGLAND BIOLABS). Samples were diluted with water to a total volume 150 µl and filtered using 10k Amicon Ultra centrifugal filters (MERCK). The filter was washed twice with 100 µl water and the obtained combined filtrates were concentrated using a concentrator 5301 (EPPENDORF). Samples were dissolved in water and 1 volume ammonium acetate buffer (5 mM, pH = 5.5) was added prior to analysis *via* LC-MS. The applied LC method is shown in Table S3.

Table S3: LC method of analysis of digested RNA samples using mixtures of an ammonium acetate buffer (5 mM, pH = 5.5) and acetonitrile with a constant flow rate of 0.3 ml/min.

Time [min]	Acetonitrile [%]
0	0
20	10
30	30

1.5. IEDDA reactions with modified RNAs

For labeling reactions freshly folded RNA (see 1.2) and dye stocks in DMSO were used. All experiments were performed in reaction buffer (20 mM Hepes (pH 7.4), 1 mM MgCl2, 125 mM KCl) and the final DMSO concentration was kept below 0.5%. The mixtures were incubated at 37 °C and the reactions were stopped by the addition of one volume of gel loading buffer (100 mM Tris-Borate (pH 8.3), 2 mM EDTA in 90% formamide containg bromphenol blue) containing 1 mM (1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-OH).

For kinetic measurements, aliquots were taken at desired time points, added into one volume of gel loading buffer containing 1mM BCN-OH and stored at -20 °C prior gel electrophoresis. Reaction samples containing 1 mM TMR were treated with BCN-OH (250 µM) and ethanol precipitated using glycogen. Resulting pellets where washed twice with 70% ethanol (500µL) and dissolved in water and gel loading buffer (1:1).

1.6. Labeling of total RNA

Cloning of pET-RhoBAST and pET-inactive-RhoBAST was achieved *via* PCR using the pET-GFP plasmid (Addgene, plasmid #29663, *E. coli* prep.) as template and the primer listed in Table S4. After PCR, the pET-GFP plasmid was digested using DpnI (THERMOFISCHER) and the linear PCR product was phosphorylated (PNK, THERMOFISCHER) and self-ligated (T4 DNA Ligase, THERMOFISCHER).

Table S4: Primer sequences (5' -> 3') used for cloning of pET plasmids.

Primer	Sequence
pET_RhoBAST_fwd	AGGCGCAAGGTTAACCGCCTCAGGTTCCCTAGCATAACCCC TTGGGGCC
pET_RhoBAST_rev	CTCCTTCACCGCTTTCGCGGAGGTTCCTATAGTGAGTCGTA TTAATTTCGCGGGATC
pET_inactive_RhoBAST_fwd	AGGAGGCAAATTAGCCGCCTCGGGTTCCCTAGCATAACCCC TTGGGGCC
pET_inactive_RhoBAST_rev	CGTTTCTACCGCTTTCGCGGAGGTTCCTATAGTGAGTCGTA TTAATTTCGCGGGATC

Table S5: Sequences of used pET plasmids. T7 promotor region is underlined, inserted aptamer is highlighted in bold and T7 terminator is shown in italic.

Primer	Sequence
pET_RhoBAST	TAATACGACTCACTATA**GGAACCTCCGCGAAAGCGGTGAAG GAGAGGCGCAAGGTTAACCGCCTCAGGTTCC***CTAGCATAAC CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG*
pET_inactive_RhoBAST	TAATACGACTCACTATA**GGAACCTCCGCGAAAGCGGTAGAA ACGAGGAGGCAAATTAGCCGCCTCGGGTTCC***CTAGCATAAC CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG*

Competent BL21 Star™ (DE3) *E. coli* cells were transformed with pET-RhoBAST and pET-inactive-RhoBAST and stroked on a LB-agar plate containing Kanamycin (30 µg/mL). Next, single colonies were picked and grown in 5 mL M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.5 g/L NH_4Cl) supplemented with trace elements (50 mg/L EDTA, 8.3 mg/L $FeCl_3$ x 6 H_2O, 0.84 mg/L $ZnCl_2$, 0.13 mg/L $CuCl_2$ x 2 H_2O, 0.10 mg/L $CoCl_2$ x 6 H_2O, 0.10 mg/L H_3BO_3, 16 µg/L $MnCl_2$ x 6 H_2O), glucose (0.4% w/v) and Kanamycin (30 µg/mL) at 37 °C overnight with shaking at 150 rpm. The overnight cultures were used as starter cultures and diluted with supplemented M9 medium to an OD_{600} of 0.05 (20 mL) and vU (2 mM) was added. For negative controls, the corresponding amount of DMSO was added. Cultures were incubated at 37 °C with shaking at 150 rpm and induced with IPTG (1 mM) at an OD_{600} of 0.3 and harvested at an OD_{600} of 0.7. For this purpose, 2 mL of 5% phenol in ethanol was added and the cells were pelleted for 10 min at 4000g (4 °C). The supernatant was discarded, the pellet was suspended in preheated (65 °C) 20 mL lysis solution (1% SDS, 2 mM EDTA) and incubated at 95 °C for 3 min. Next, 20 mL of preheated (65 °C) aqueous phenol was added and the samples were incubated at 65 °C for 10 min while samples were inverted once per minute. To separate phases, mixtures were centrifuged at 8000 g at room temperature. After successful phase separation, the aqueous layer was extracted four times with 20 mL phenol/chloroform/isoamyl alcohol (P/C/I). The aqueous phase containing was transferred to new tube and the total RNA was precipitated by the addition of 0.1 volume 3 M NaOAc (pH 5.5) and 1 volume isopropanol. After incubation overnight at -20 °C, total RNA was pelleted at 11000 g for 90 min (4 °C). The obtained pellets were dissolved in 500 µL water and extracted three times with 500 µL diethyl ether. Remaining ether traces were removed upon centrifugation under vacuum for 15 min. Next, remaining DNA was digested using 0.2U/µL DNAse I (ROCHE) in 1X DNase buffer (ROCHE) for 30 min at 37 °C followed by P/C/I (600 µl, three times) and ether (600 µ, three times) extraction. Remaining ether traces were removed upon centrifugation under vacuum for 15 min and total RNA was precipitated by the addition of 0.1 volume 3 M NaOAc (pH 5.5) and 1 volume isopropanol. After incubation over night at -20 °C, total RNA was pelleted at 11000 g for 90 min (4 °C). The pellet was dissolved in water and PAGE purified as mentioned in **1.1**.

Labeling of isolated total RNA was performed as described for *in vitro* transcribed RNAs (see **1.5**)**.** Briefly, freshly folded total RNA (4 µg/µL) in reaction buffer was incubated with TMR-Tz (50 µM) at 37 °C for 20 h. To quench the labeling reaction, samples were treated with BCN-OH (250 µM) and ethanol precipitated using glycogen. Resulting pellets were washed twice with 70% ethanol and dissolved in water and gel loading buffer (1:1).

2. Supporting Figures

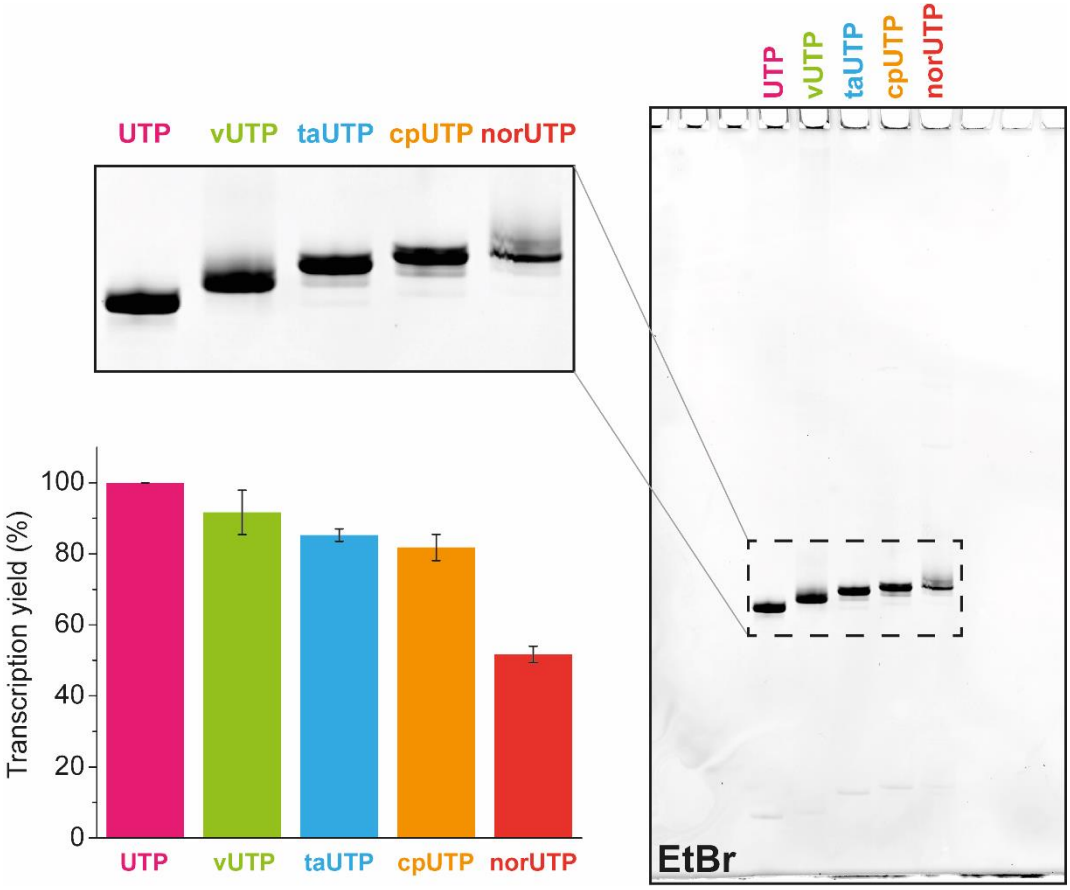


Figure S1: Denaturing urea PAGE gel (20%) of *in vitro* transcription reactions using unmodified and modified uridine triphosphates (**UTP**, **vUTP**, **taUTP**, **cpUTP**, **norUTP**) in combination with T7 RNAP. RNA was visualized using ethidium bromide staining (EtBr) and the full-length. In the graph relative yield of the full-length product of *in vitro* transcribed RhoBAST is shown.

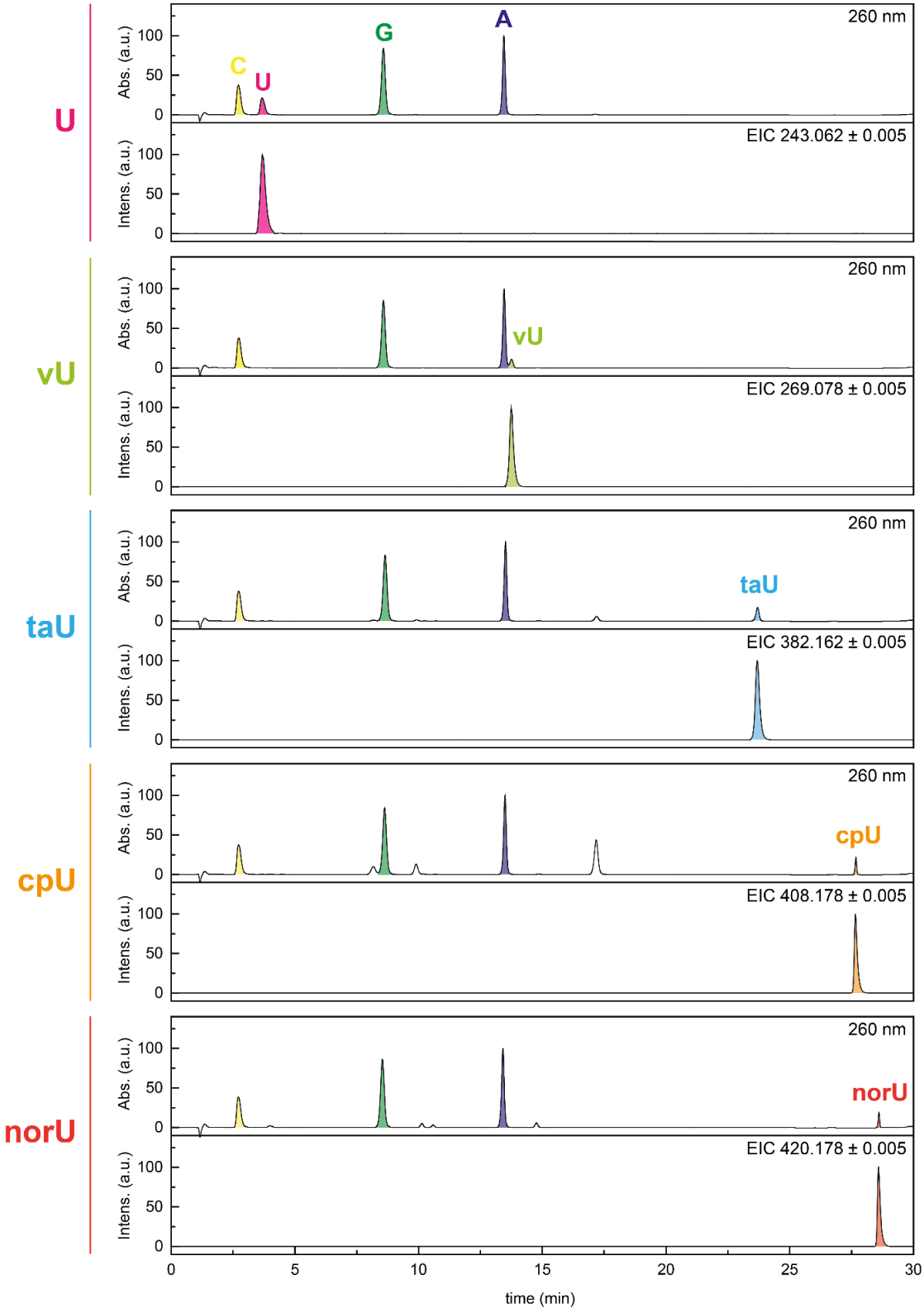


Figure S2: LC-MS chromatograms of nucleoside mixtures obtained from enzymatic digestion of RhoBAST. For each digested RNA, the UV chromatogram (260 nm) and extracted ion chromatogram (EIC) of the corresponding incorporated uridine derivative (**U**, **vU, taU, cpU, norU**; $[M-H]^-$) is shown. Additional LC-MS chromatograms and high-resolution mass spectra of incorporated uridine derivatives can be found in the Appendix (A1-A5).

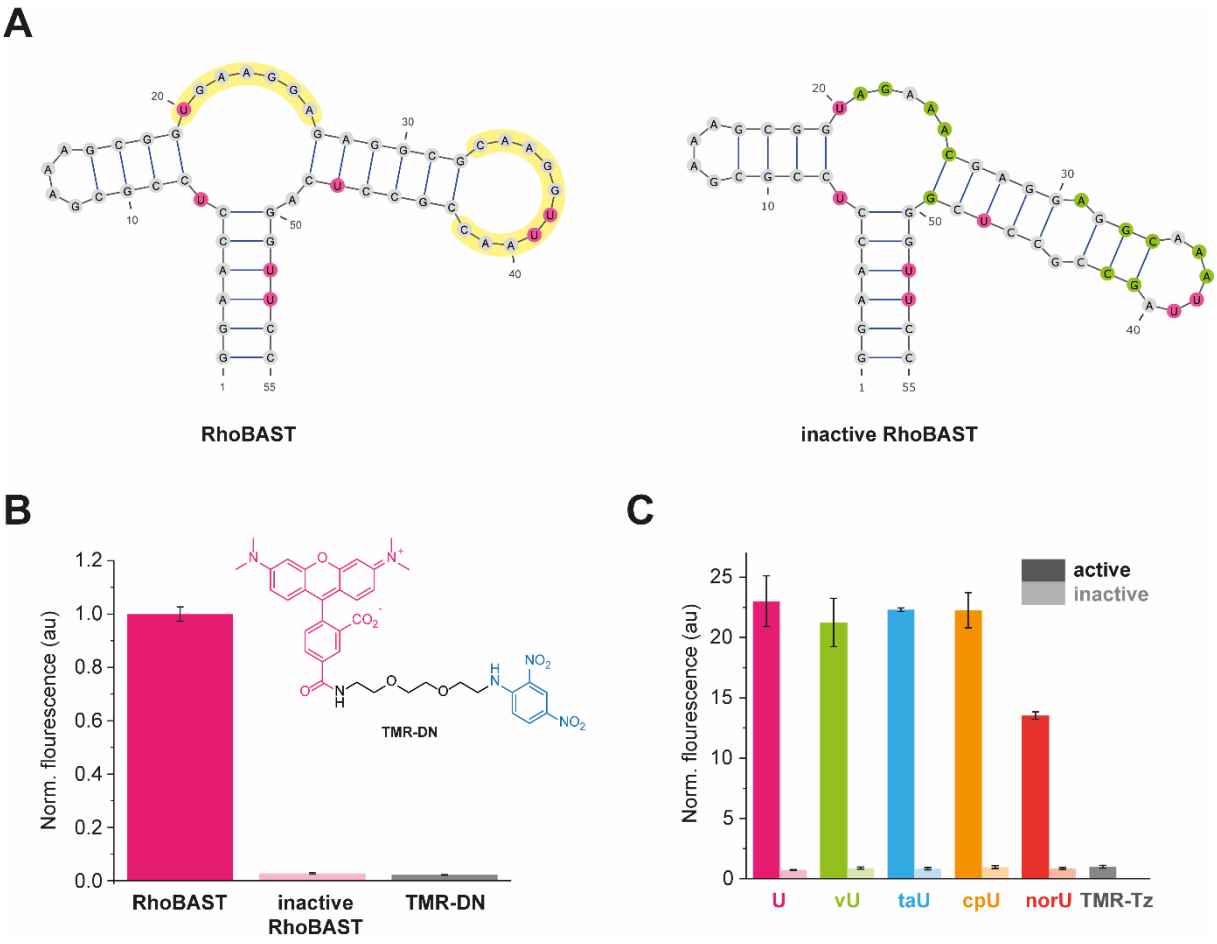
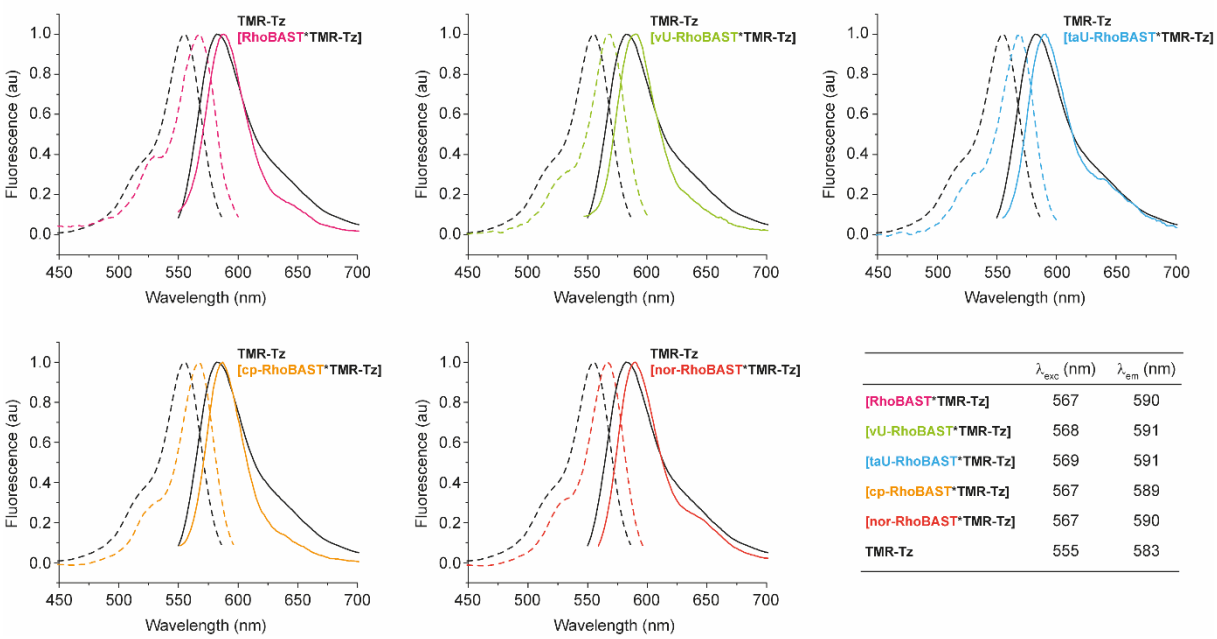


Figure S3: Design of the inactive RhoBAST mutant. (A) *In silico* predicted secondary structure of the RhoBAST aptamer and its inactive mutant. Highly conserved regions of the RhoBAST aptamer are highlighted in yellow.[3, 4] Inserted mutations are highlighted in green. The mutant was designed to contain the same number of uridines (highlighted in magenta) at the same positions and embedded in the same secondary structure motifs (helix, loops) as in the active aptamer. (B) Fluorescence increase of active and inactive RhoBAST aptamer (1 µM) after incubation with **TMR-DN** (10 nM) in reaction buffer at 25 °C. Binding of **TMR-DN** to the RhoBAST aptamer leads to a disruption of the quenching and consequently to a significant fluorescent increase, which can serve as readout in binding assays.[2, 3] (C) Fluorescence turn-on of active and inactive RhoBAST aptamers (2 µM) with **TMR-Tz** (20 nM).



	λ_{exc} (nm)	λ_{em} (nm)
[RhoBAST*TMR-Tz]	567	590
[vU-RhoBAST*TMR-Tz]	568	591
[taU-RhoBAST*TMR-Tz]	569	591
[cp-RhoBAST*TMR-Tz]	567	589
[nor-RhoBAST*TMR-Tz]	567	590
TMR-Tz	555	583

Figure S4: Normalized excitation and emission spectra of **TMR-Tz** (20 nM) in the absence and presence of RhoBAST aptamer (5 µM) in reaction buffer at 25°C.

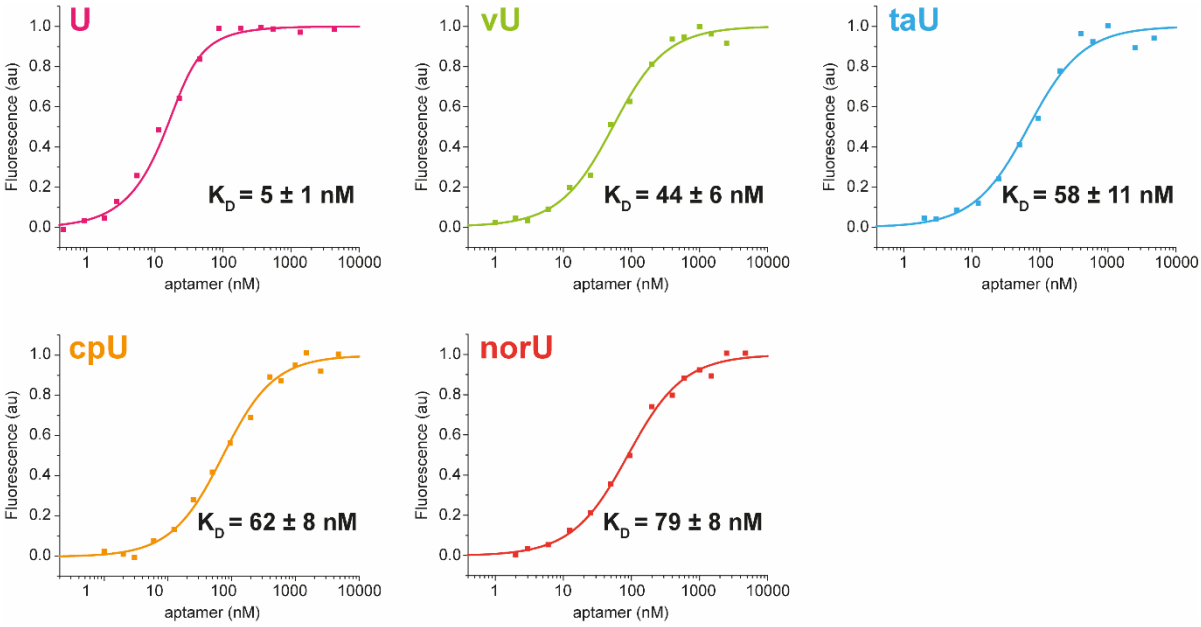


Figure S5: Titration curves of unmodified and modified RhoBAST aptamers with a fixed **TMR-Tz** (20 nM) concentration at 25 °C. Dissociation constants (K_D) of the corresponding complexes were calculated according to equation 1 (see 1.3).

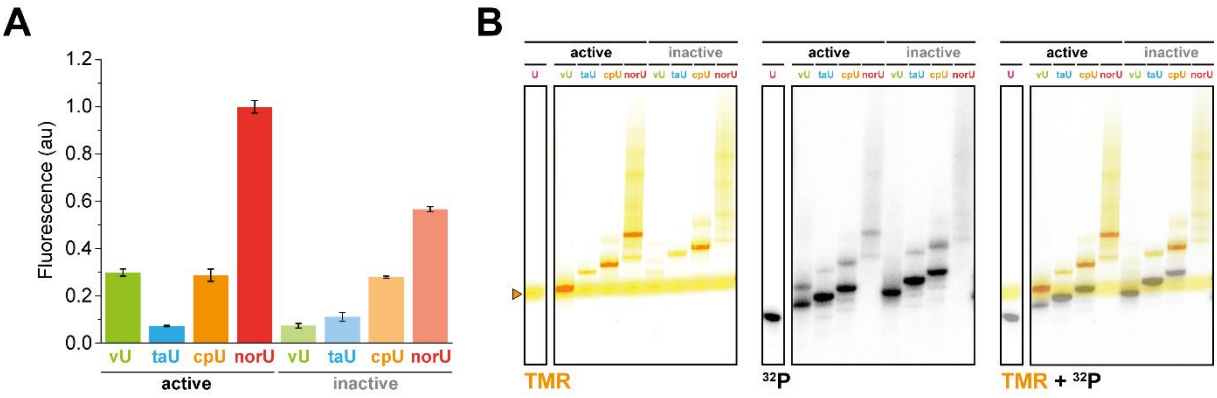


Figure S6: IEDDA reactions of dienophile-modified RNA (1 µM) with an excess of **TMR-Tz** (5 µM) at 37 °C for 20 h. (A) Integration of fluorescence bands for the corresponding modified RhoBAST RNA. (B) Denaturing urea PAGE gel (20%) of IEDDA reaction. A degradation product of the **TMR-Tz** dye is indicated with an orange triangle. For quantification, the corresponding fluorescence signal was subtracted from the sample bands.

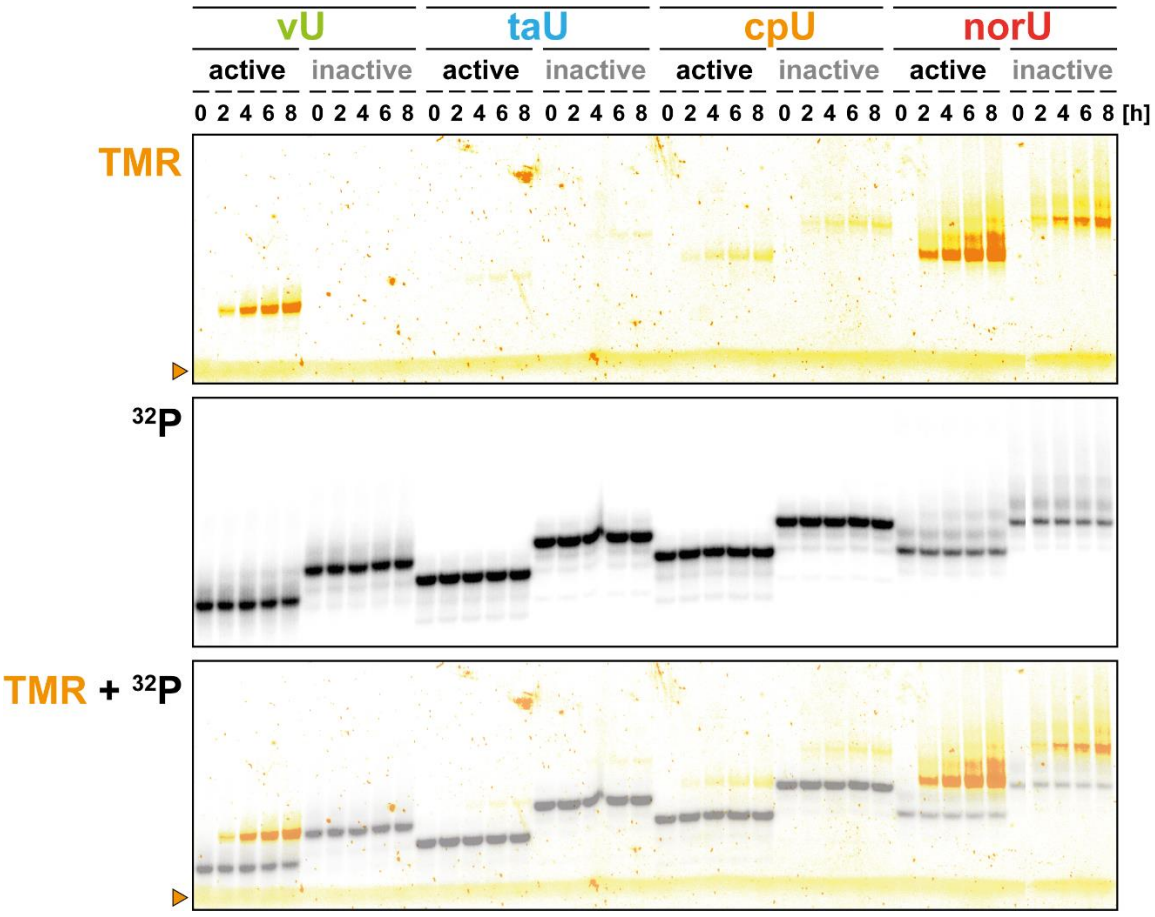


Figure S7: Kinetic measurements of IEDDA reactions of modified RNA (1 µM) with **TMR-Tz** (1 µM) at 37°C up to 8 h. The degradation product of **TMR-Tz** dye is indicated with an orange triangle.

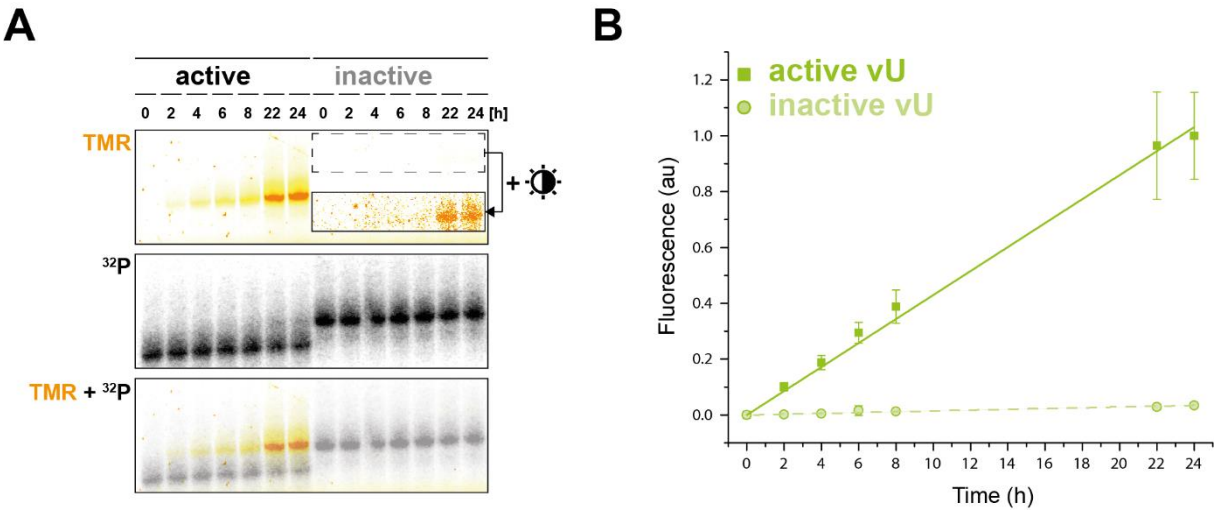


Figure S8: Kinetic measurements of IEDDA reactions of **vU**-modified active and inactive RhoBAST. (A) Denaturing PAGE gel (20%) of labeling of active and inactive RhoBAST aptamer (1 µM) and **TMR-Tz** (1 µM) including excerpt with increased contrast to visualize labeled inactive RhoBAST RNA. (B) Analysis of labeling kinetics of IEDDA reaction in (A) based on fluorescence increase.

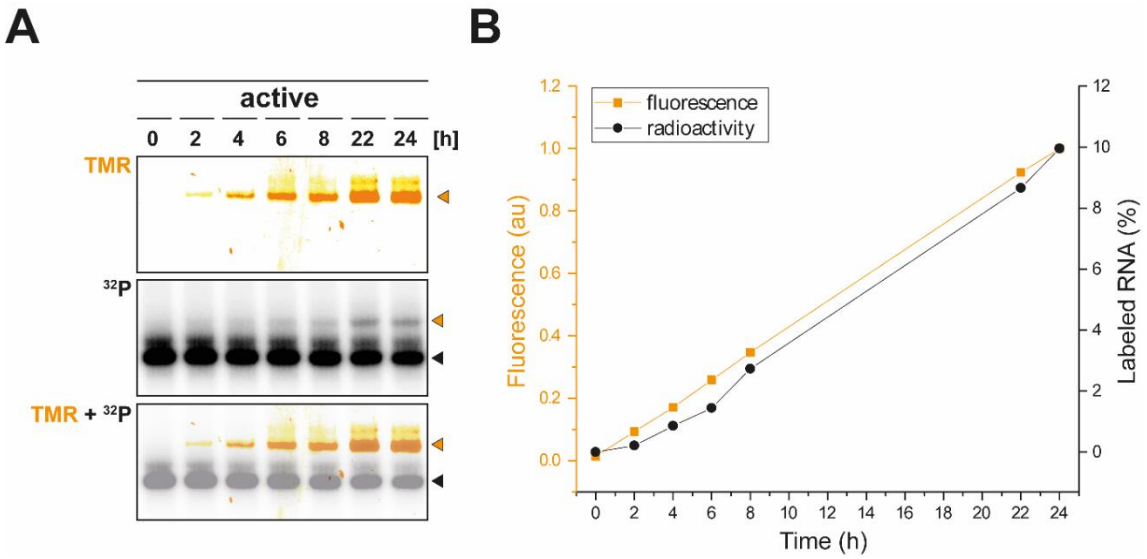


Figure S9: Kinetic measurements and quantification of IEDDA reactions of **vU**-modified active RhoBAST. (A) Denaturing urea PAGE gel (20%) analysis of the IEDDA reaction between **vU**-modified active RhoBAST (1 µM) with **TMR-Tz** (1 µM) at 37°C up to 24 h. The unlabeled RNA fraction is highlighted with a black and the labeled fraction with an orange triangle. (B) Quantification of labeled RNA based on integration of the radioactive signal of shifted RNA bands.

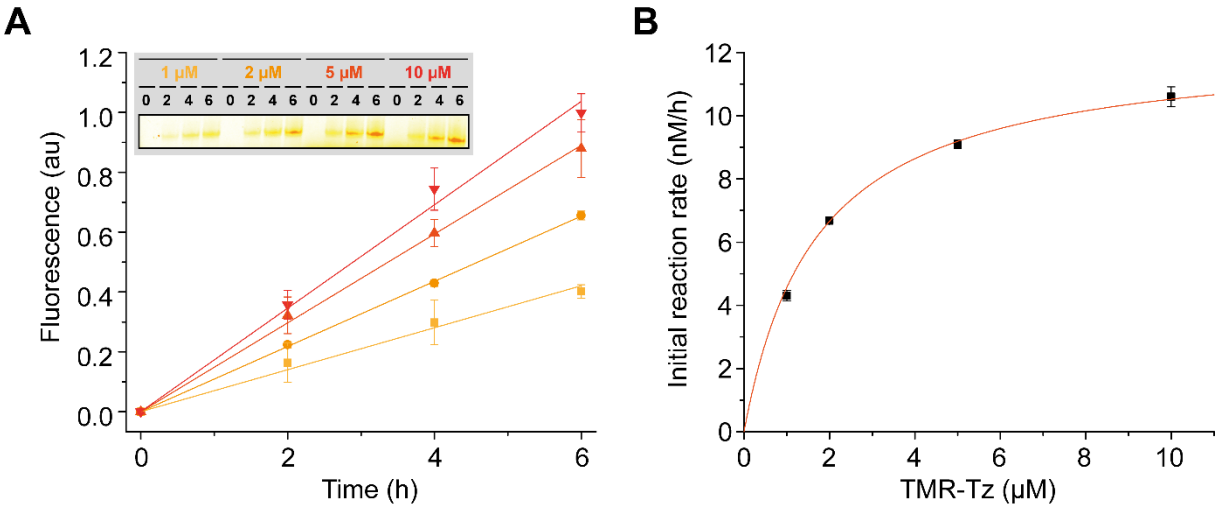


Figure S10: Substrate concentration dependency of proximity-induced IEDDA reaction. (A) Kinetic measurement of the initial rate of the IEDDA labeling reaction of **vU**-modified active RhoBAST (1 µM) with 1, 2, 5 and 10 µM **TMR-Tz** at 37°C. (B) Plot of the initial reaction rate of the proximity-induced IEDDA as a function of substrate concentration.

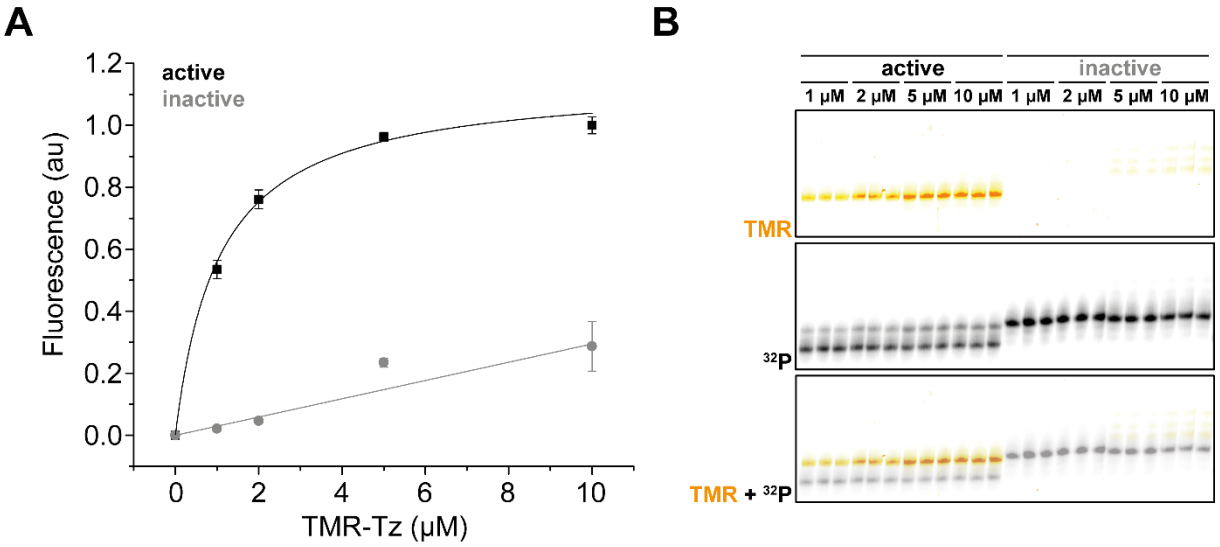


Figure S11: Comparison of substrate concentration dependency of the labeling yield of proximity-induced and non-proximity-induced reaction. (A) Integrated fluorescence of the product band formed in the reaction between **vU**-modified active and inactive RhoBAST RNAs (1 µM) with **TMR-Tz** (1,2,5 or 10 µM) at 37°C for 20 h. (B) Denaturing urea PAGE gel (20%) of the labeling reactions.

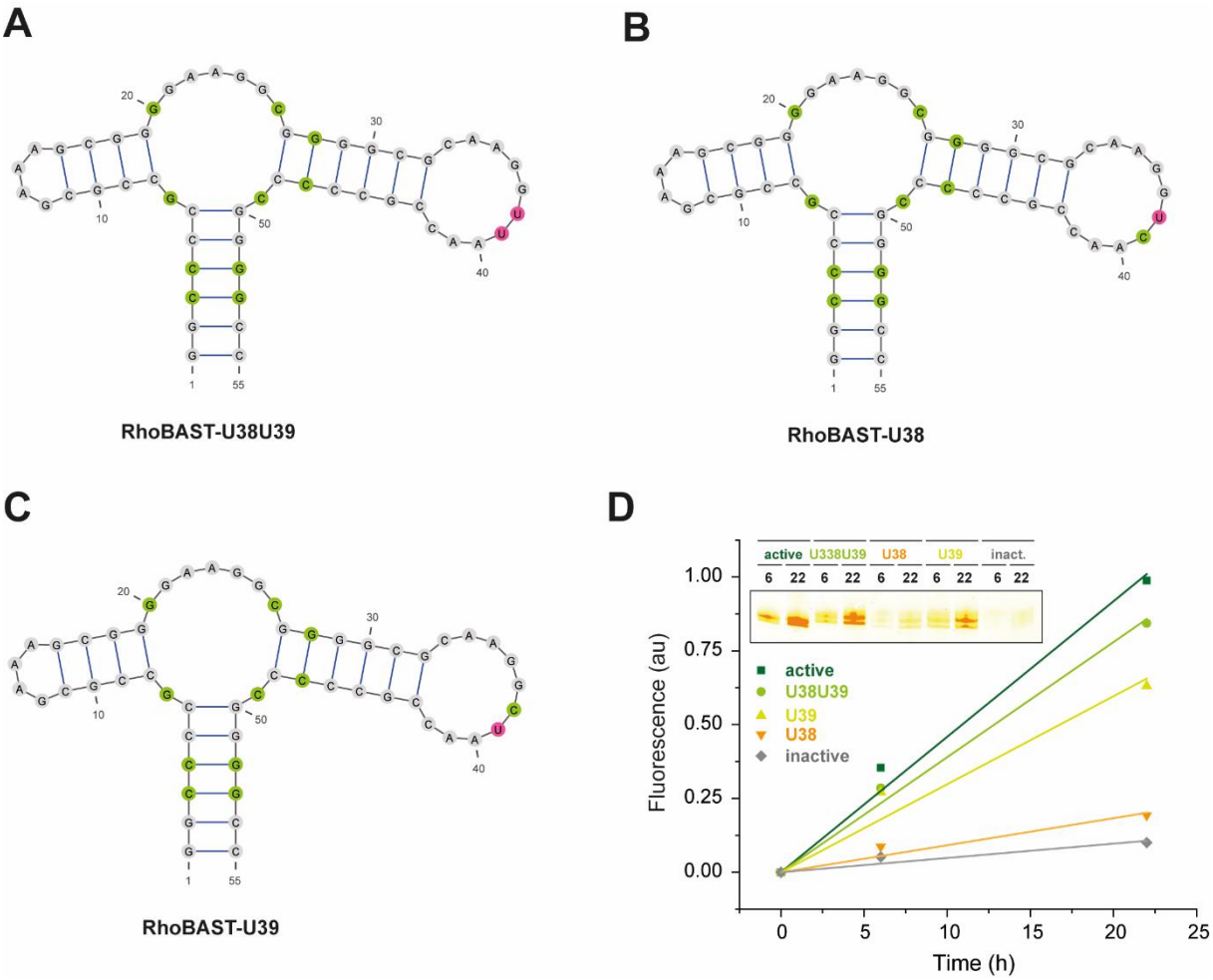


Figure S12: Mutation analysis of RhoBAST. *In silico* predicted secondary structure of the RhoBAST mutants U38U39 (A), U38 (B) and U39 (C). Inserted mutations are highlighted in green and remaining uridines in magenta. (D) Integrated fluorescence of the product band formed in the IEDDA reaction between **vU**-modified RhoBAST mutants (1 µM) with **TMR-Tz** (1 µM) at 37°C and denaturing urea PAGE gel (20%, TMR fluorescence) of the corresponding labeling reaction.

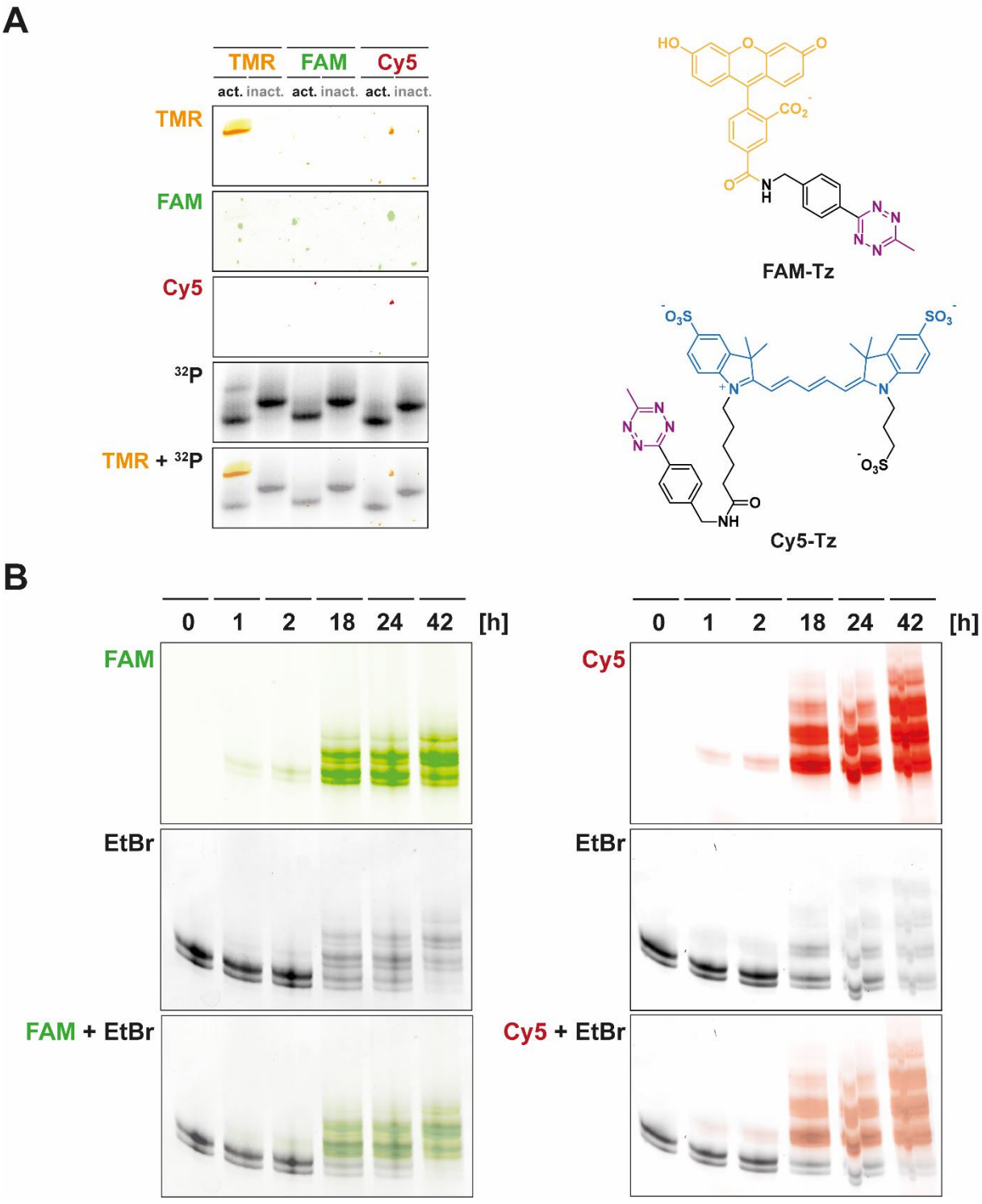


Figure S13: (A) IEDDA reaction of **vU**-modified RhoBAST RNAs (1 µM) with different dye-tetrazine derivatives (1 µM) at 37 °C for 20 h. Chemical structures of Fluorescein (FAM) and cyanine (Cy5) based tetrazine probes purchased from JENA BIOSIENCE. (B) Denaturing urea PAGE gel (20%) of the labeling reactions of 5 µM **vU**-RhoBAST and 100 µM **FAM-Tz** or **Cy5-Tz**.

3. Chemical synthesis

3.1. General experimental information for chemical synthesis

All chemicals were purchased from commercial suppliers like SIGMA-ALDRICH, ABCR, ACROS, TCI and ALFA AESER and used without further purification. Reactions requiring the exclusion of moisture and/or oxygen were carried out under argon atmosphere using standard Schlenk techniques.

TLC was performed on Polygram® SIL G/UV_{254} TLC plates purchased from MACHERY-NAGEL. If not otherwise mentioned, visualization of spots was carried out by fluorescence quenching with 254 nm UV light. Purification of crude products by normal phase column chromatography was performed using silica gel (high-quality, pore size 60 Å, 40-63 µm particle size) purchased from SIGMA-ALDRICH. Reverse phase and anion exchange column chromatography was performed using a INTERCHIM PuriFlash 420 purification system (flow rate: 8 ml/min) equipped with TELOS C18 Flash columns (23g) or self-prepared DEAE-Sephadex A-25 anion exchange columns, respectively.

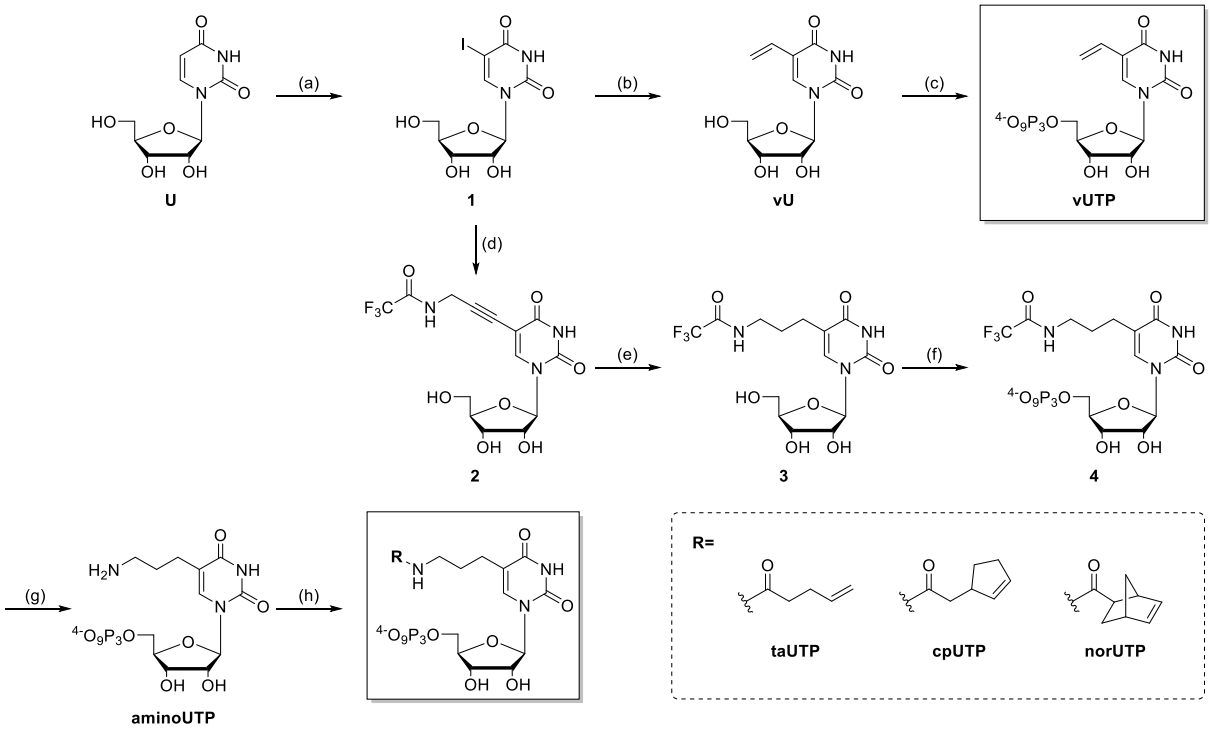
Preparative reverse phase High Performance Liquid Chromatography (HPLC) was performed on an AGILENT 1100 Series HPLC system equipped with a diode array detector using a PHENOMENEX Luna 5 µm C-18(2) 100 Å (250 x 21.2 mm) column. Purification was performed with a constant flow rate of 5 ml/min using mixtures of buffer A (100 mM NEt_3/HOAc (pH = 7.0) in water) and buffer B (100 mM NEt_3/HOAc (pH = 7.0) in 80% acetonitrile) as solvent system.

Nuclear Magnetic Resonance Spectroscopy (NMR) spectra were recorded using a VARIAN Mercury Plus 300 or VARIAN Mercury Plus 500 system (300 MHz and 500 MHz as basic transmitter frequency for ^{1}H measurements at 300 K). Deuterated solvents were purchased from EURISO-TOP. Chemical shifts (δ) are given in ppm with respect to tetramethylsilane and coupling constants (*J)* in Hz. Recorded ^{1}H and ^{13}C spectra were referenced to the protio impurity or the ^{13}C signal of the deuterated solvent. Abbreviations used for observed multiplicities are s for singlet, d for doublet, t for triplet, q for quartet, quin for quintet, m for multiplet. ^{13}C measurements were recorded as APT spectra. Signal assignments are based on APT, COSY, HSQC and HMBC spectra.

Mass spectra were obtained utilizing a BRUKER micrOTOF QII-ESI system and high resolution mass (HR-MS) measurements were performed using sodium formiate as internal calibrant. Reported mass values refer to the isotopic peak with the highest intensity.

UV-Vis absorption spectra were recorded on a NanoDrop-1000 spectrometer system purchased from PEQLAB and extinction coefficients of the HPLC purified nucleoside triphosphates were determined in water.

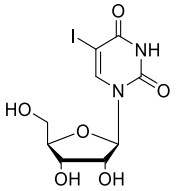
3.2. Synthetic overview



Scheme S1: Synthesis of modified uridine triphosphates. (a) I_2, HNO_3, $CHCl_3$; (b) (vinyl)(*n*-Bu)$_3$Sn, $Pd_2(dba)_3$, P(furyl)$_3$, DMF; (c) $POCl_3$, proton sponge, TMP, (*n*-$Bu_3NH)_2H_2P_2O_7$, *n*-Bu_3N; (d) TFA-propagyl amine, $Pd(PPh_3)_4$, CuI, NEt_3, DMF; (e) H_2, PtO_2, MeOH; (f) $POCl_3$, proton sponge, TMP, (*n*-$Bu_3NH)_2H_2P_2O_7$, *n*-Bu_3N; (g) NH_3(aq); (h) NHS-ester, $Na_2B_4O_7$ buffer, DMF.

3.3. Synthetic procedures

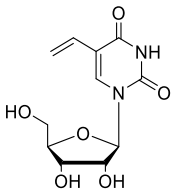
5-Iodouridine (**1**)



Uridine (20.0 g, 81.9 mmol, 1.00 eq) and iodine beads (22.9 g, 90.1 mmol, 1.10 eq) were dissolved in a mixture of $CHCl_3$ (120 mL) and 1M HNO_3 (200 mL) and the reaction was heated at reflux for 5 h. The reaction mixture was cooled down to room temperature and the obtained crystals were collected by filtration and washed with $CHCl_3$ (3x 100 mL). After drying in vacuum overnight product was obtained as a white solid (14.2 g, 47%). Recorded spectrum is in accordance with literature.[5]

^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.67 (s, 1H), 8.47 (s, 1H), 5.72 (d, *J* = 4.4 Hz, 1H), 5.40 (d, *J* = 5.1 Hz, 1H), 5.24 (t, *J* = 4.2 Hz, 1H), 5.05 (d, *J* = 4.8 Hz, 1H), 4.06 – 4.00 (m, 1H), 4.00 – 3.94 (m, 1H), 3.89 – 3.83 (m, 1H), 3.72 – 3.53 (m, 2H) ppm.

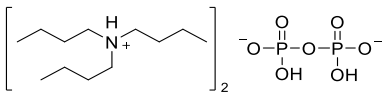
5-Vinyluridine (**vU**)



First, 5-Iodouridine (1.00 g, 2.70 mmol, 1.00 eq), Pd_2dba_3, (124 mg, 135 μmol, 0.05 eq) and $P(furyl)_3$ (75.3 mg, 324 μmol, 0.12 eq) were dissolved in degassed anhydrous DMF (20 mL) under argon atmosphere. Next, tributyl(vinyl)tin (1.07 g, 3.38 mmol, 1.25 eq.) was added dropwise and the reaction was stirred for 4 h at 60 °C. The mixture was filtered through Celite and the pad was washed with MeOH (3x with 50 mL). The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (MeOH/$CHCl_3$ = 9:1) to afford the product as a white solid (573 mg, 79%). Recorded spectrum is in accordance with literature.[6]

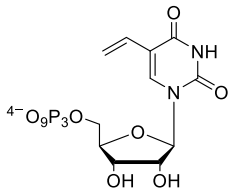
^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.42 (s, 1H), 8.20 (s, 1 H), 6.37 (dd, *J* = 17.9, 11.4 Hz, 1H), 5.91 (dd, *J*=17.7, 2.2 Hz, 1H), 5.78 (d, *J* = 4.5 Hz, 1H), 5.40 (d, *J* = 5.4 Hz, 1H), 5.23 (t, *J* = 5.0 Hz, 1H), 5.15 - 5.09 (m, 1H), 5.06 (d, *J* = 5.3 Hz, 1H), 4.10 - 4.04 (m, 1H), 3.96 - 4.04 (m, 1H), 3.89 - 3.82 (m, 1H), 3.75 - 3.53 (m, 2 H) ppm.

Bis(tri-buthyl ammonium) pyrophosphate[7]



Sodium pyrophosphate decahydrate (3.34 g, 7.50 mmol, 1.00 eq) was dissolved in 75 mL of water under stirring. Then 21 g Dowex 50X8 (20-50 mesh, H^+ form) was added and the suspension was stirred for further 20 min. Next, the suspension was filtered and the filtrate was directly added into an ice cold solution of ethanol (30 mL) and tri-*n*-buthylamine (3.57 mL, 15 mmol, 2.00 eq). The resin on the filter was washed with water until the filtrate showed a pH of 7. The solvent was removed under reduced pressure (temperature below 35 °C) and coevaporated three times with ethanol and three times with dry DMF. The residue was dissolved in dry DMF (15 ml) in order to obtain a 0.5 M bis(tri-*n*-buthylammonium) pyrophosphate solution, which was stored over molecular sieves and under an argon atmosphere at 4 °C.

5-Vinyluridine-5'-triphosphate (**vUTP**)



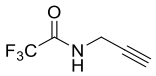
Freshly distilled $POCl_3$ (52.0 μL, 405 μmol, 1.50 eq) was slowly added to 5-vinyluridine (100 mg, 370 μmol, 1.00 eq) and proton sponge (95.2 mg, 444 μmol, 1.20 eq) in trimethylphosphate (1.67 mL) at 0°C under argon atmosphere. The solution was stirred for 1.5 h at 0 °C. Bis-tributylammonium pyrophosphate (0.5 M in DMF, 3.60 mL, 1.85 mmol, 5.00 eq) and tributylamine (707 μL, 2.96 mmol, 8.00 eq) was rapidly added to the solution at 0 °C. After stirring for 1 h at 0 °C, the reaction was quenched with 0.1 M triethylammonium bicarbonate buffer (TEAB, pH 7.5, 20 mL) and the reaction mixture was stirred for an additional hour at 0 °C. The solvent was removed under reduced pressure and the residue was purified using a DEAE sephadex-A25 anion exchange column (0.1 M → 1.3 M TEAB) and was then further purified by preparative reverse phase HPLC (3 → 18% B in 50 min, R_t = 38.3 min). Desired fractions were lyophilized to obtain the product as triethylammonium salt (30.5 mg, 9%). Recorded spectra are in accordance with literature.[6]

^{1}H NMR (500 MHz, D_2O): δ = 7.99 (s, 1H), 6.54 (dd, *J* = 17.7, 11.6 Hz, 1H), 6.06 – 5.97 (m, 2H), 5.34 (d, *J* = 11.5 Hz, 1H), 4.48 – 4.44 (m, 2H), 4.34 – 4.22 (m, 3H) ppm.

^{31}P NMR (202 MHz, D_2O) δ = -10.94 (d, *J* = 19.1 Hz P_γ), -11.87 (d, *J* = 20.6 Hz, P_α), -23.32 (t, *J* = 20.0 Hz, P_β) ppm.

MS (HR-ESI, neg): meas. m/z = 508.9749, calc. 508.9769 for $C_{11}H_{16}N_2O_{15}P_3$ $[M-H]^-$.

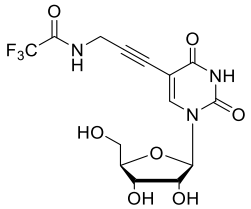
N-propargyl trifluoroacetamide



Propargylamine (7.50 g, 136 mmol, 1.00 eq) and ethyl trifluoroacetate (25.1 g, 177 mmol, 1.30 eq) were dissolved in 250 mL MeOH. The mixture was stirred for 18 h at room temperature and the solvent was removed under reduced pressure. Next, saturated sodium bicarbonate solution (100 mL) was added to the residue and the aqueous phase was extracted with CH_2Cl_2 (3x with 50 mL). The combined organic layers were dried over magnesium sulfate, filtered and the solvent was removed under reduced pressure to afford the product as brown oil (16.7 g, 81%). The recorded spectrum is in accordance with literature.[8]

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.59 (bs, 1H), 4.16 (dd, *J* = 5.2, 2.5 Hz, 2H), 2.34 (t, *J* = 2.6 Hz, 1H) ppm.

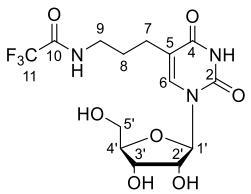
5-(trifluoroacetamidopropynyl)uridine (**2**)



First, 5-iodouridine (2.00 g, 5.40 mmol, 1.00 eq) was dissolved in dry DMF (25 mL) and CuI (205 mg, 1.08 mmol, 0.20 eq), Et_3N (1.51 mL, 10.8 mmol, 2.00 eq), *N*-propargyl trifluoroacetamide (2.45 g, 16.2 mmol, 3.00 eq), and $Pd(PPh_3)_4$ (624 mg, 540 µmol, 0.10 eq) were added under argon atmosphere. The reaction mixture was stirred at room temperature for 18 h. The solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 9:1) to afford the product as orange crystals (1.72 g, 81%). The recorded spectrum is accordance with literature.[9]

^{1}H NMR (300 MHz, CD_3OD) δ = 8.39 (s, 1H), 5.88 (d, *J* = 3.8 Hz, 1H), 4.27 (s, 2H), 4.18 – 4.14 (m, 2H), 4.04 – 4.00 (m, 1H), 3.87 (dd, *J* = 12.8, 2.0 Hz, 1H), 3.74 (dd, *J* = 12.2, 2.8 Hz, 1H) ppm.

5-(trifluoroacetamidopropyl)uridine (**3**)



5-(trifluoroacetamidopropynyl)uridine (546 mg, 1.39 mmol, 1.00 eq) was dissolved in dry MeOH (130 mL) and PtO_2 (63.1 mg, 278 μmol, 0.20 eq) was added under argon atmosphere. The argon atmosphere was exchanged by a hydrogen atmosphere and the mixture was stirred for 3 h at room temperature. The catalyst was removed by filtration through a Celite pad, which was washed with methanol (50 mL). The solvent was evaporated and the residue was purified by column chromatography (EtOAc/MeOH = 20:1) to afford the product as yellow solid (282 mg, 51%).

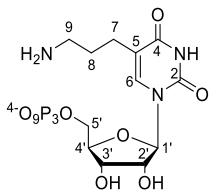
TLC (EtOAc:MeOH = 20:1): R_f = 0.44.

^{1}H NMR (500 MHz, CD_3OD): δ = 7.90 (s, 1H, 6-H), 5.91 (d, *J* = 4.6 Hz, 1H, H-1'), 4.24 - 4.20 (m, 1H, H-2'), 4.20 - 4.17 (m, 1H, H-3'), 4.03 - 3.99 (m, 1H, H-4'), 3.87 (dd, *J* = 12.3, 2.6 Hz, 1H, H-5'), 3.76 (dd, *J* = 12.2, 3.1 Hz, 1H, H-5'), 3.32 - 3.28 (m, 2H, H-9), 2.41 - 2.29 (m, 2H, H-7), 1.79 (quin, *J* = 7.2 Hz, 2H, H-8) ppm.

^{13}C{^{1}H} NMR (126 MHz, CD_3OD): δ = 166.1 (C-4), 159.1 (d, $^2J_{C\text{-}F}$ = 36.7 Hz, C-10), 152.6 (C-2), 139.2 (C-6), 117.7 (q, $^1J_{C\text{-}F}$ = 286.6 Hz, C-11), 114.6 (C-5), 90.8 (C-1'), 86.4 (C-4'), 75.7 (C-2'), 71.3 (C-3'), 62.4 (C-5'), 40.1 (C-9), 28.8 (C-8), 25.2 (C-7) ppm.

MS (HR-ESI, pos): meas. m/z = 420.0990, calc. 420.0989 for $C_{14}H_{18}F_3N_3NaO_7$ $[M+Na]^+$.

5-(aminopropyl)uridine-5’-triphosphate (**aminoUTP**)



Freshly distilled POCl3 (35.0 µL, 378 µmol, 1.50 eq) was slowly added to 5-(trifluoroacetamido-propyl)uridine (100 mg, 252 µmol, 1.00 eq), proton sponge (80.9 mg, 378 µmol, 1.50 eq), trimethylphosphate (1.14 mL) under ice-cold conditions. The solution was stirred for 1.5 h at 0 °C. Bis-tributylammonium pyrophosphate (0.5 M in DMF, 2.52 mL, 1.26 mmol, 5.00 eq) and tributylamine (481 µL, 2.02 mmol, 8.00 eq) was added to the solution at 0 °C. Next, the reaction mixture was stirred 1.5 h at 0 °C and subsequently quenched with 0.1 M triethylammonium bicarbonate buffer (TEAB, pH 7.5, 17 mL). The mixture was allowed to stirr for an additional hour at room temperature and concentrated aqueous ammonia solution (12.6 mL) was added. The reaction mixture was stirred at room temperature overnight. Then the solvent was removed under reduced pressure and the residue was dissolved in 0.1 M triethylammonium bicarbonate buffer (TEAB, pH 7.5, 8 mL). The crude mixture was purified by anion exchange chromatography on DEAE sephadex-A25 anion exchange column (0.1 M → 1.3 M TEAB) and was then further purified by preparative reverse phase flash column chromatography (2 → 8% B in 60 min, R_t = 28 min). The appropriate fraction was lyophilized to afford the triphosphate product as triethylammonium salt (50.1 mg, 21%).

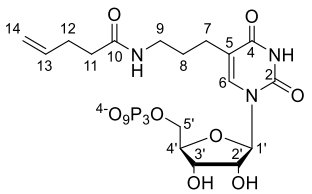
^{1}H NMR (500 MHz, D_2O): δ = 7.89 (s, 1H, H-6), 6.03 (d, *J* = 5.0 Hz, 1H, H-1’), 4.46 – 4.42 (m, 1H, H-3’), 4.41 – 4.37 (m, 1H, H-2’), 4.33 – 4.24 (m, 3H, H-4’, H-5’), 3.03 (t, *J* = 7.2 Hz, 2H, H-9), 2.53 – 2.45 (m, 2H, H-7), 1.95 - 1.89 (m, 2H, H-8) ppm.

^{13}C{^{1}H} NMR (126 MHz, D_2O): δ = 165.7 (C-4), 151.7 (C-2), 137.5 (C-6), 113.9 (C-5), 87.9 (C-1’), 83.3 (d, $^{3}J_{C\text{-}P}$ = 9.5 Hz C-4’), 73.9 (C-2’), 69.5 (C-3’), 64.8 (d, $^{2}J_{C\text{-}P}$ = 5.3 Hz, C-5’), 38.5 (C-9), 25.5 (C-8), 22.8 (C-7) ppm.

^{31}P{^{1}H} NMR (202 MHz, D_2O): δ = -10.81 (d, *J* = 19.1 Hz, P_γ), -11.63 (d, *J* = 19.8 Hz, P_α), -23.06 (t, *J* = 19.1 Hz, P_β) ppm.

MS (HR-ESI, neg): meas. m/z = 540.0192, calc. 540.0191 for $C_{12}H_{21}N_3O_{15}P_3$ $[M\text{-}H]^-$.

taUTP



AminoUTP (10 mg, 10.6 μmol, 1.00 eq) was dissolved in 1 mL sodium borate buffer (0.1 M, pH 9) and pent-4-enoic acid succinimidyl ester[10] (6.70 mg, 31.7 μmol, 3.00 eq) dissolved in 1 mL DMF was added. The reaction mixture was stirred overnight at room temperature and the crude reaction mixture was purified by reversed phase HPLC (10 → 50% B in 50 min, R_t = 24.9 min). Appropriate fractions were lyophilized to afford the product as triethylammonium salt (2.80 mg, 25%).

Note: Non-reacted **aminoUTP** could be recovered after HPLC purification and reused in further reactions.

^{1}H NMR (500 MHz, D_2O): δ = 7.78 (s, 1H, H-6), 6.02 (d, *J* = 5.5 Hz, 1H, H-1'), 5.91 – 5.83 (m, 1H, H-13), 5.12 – 5.02 (m, 2H, H-14), 4.47 – 4.43 (m, 2H, H-2', H-3'), 4.31 – 4.23 (m, 3H, H-4', H-5'), 2.47 – 2.40 (m, 2H, H-7), 2.38 – 2.33 (m, 4H, H-11, H-12), 1.77-1.72 (m, 2H, H-8) ppm.

Note: Signal for H-9 lies under signal of triethylammonium protons (3.22 ppm). H-9 signal could be detected and assigned in COSY (3.22 ppm, 1.75 ppm) and HSQC (3.22 ppm, 38.6 ppm) experiments.

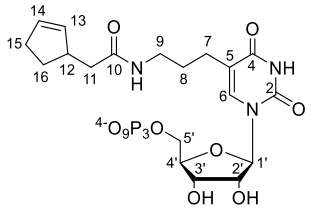
^{13}C{^{1}H} NMR (126 MHz, D_2O): δ = 176.0 (C-10), 165.8 (C-4), 151.8 (C-2), 137.4 (C-6), 137.1 (C-13), 115.5 (C-14), 115.0 (C-5), 87.8 (C-1'), 83.6 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, C-4'), 73.5 (C-2'), 69.9 (C-3'), 65.1 (d, $^2J_{C\text{-}P}$ = 3.1 Hz, C-5'), 38.6 (C-9), 35.0 (C-11), 29.4 (C-12), 27.1 (C-8), 23.6 (C-7) ppm.

^{31}P{^{1}H} NMR (202 MHz, D_2O): δ = -10.80 (d, *J* = 19.8 Hz, P_γ), -11.86 (d, *J* = 20.6 Hz, P_α), -23.34 (t, *J* = 20.2 Hz, P_β) ppm.

MS (HR-ESI, neg): meas. m/z = 622.0598, calc. 622.0610 for $C_{17}H_{27}N_3O_{16}P_3$ $[M\text{-}H]^-$.

UV (H_2O): ε_{268} = 6100 $M^{-1}cm^{-1}$.

cpUTP



AminoUTP (10 mg, 10.6 μmol, 1.00 eq) was dissolved in 1 mL sodium borate buffer (0.1 M, pH 9) and 2-(cyclopent-2-en-1-yl)acetic acid succinimidyl ester[10] (7.08 mg, 31.7 μmol, 3.00 eq) dissolved in 1 mL DMF was added. The reaction mixture was stirred overnight at room temperature and the crude reaction mixture was purified by reversed phase HPLC (10 → 50% B in 50 min, R_t = 31.4 min). Appropriate fractions were lyophilized to afford the product as triethylammonium salt (6.10 mg, 55%).

Note: Non-reacted **aminoUTP** could be recovered after HPLC purification and reused in further reactions.

^{1}H NMR (500 MHz, D_2O): δ = 7.79 (s, 1H, H-6), 6.01 (d, *J* = 5.4 Hz, 1H, H-1'), 5.87 – 5.85 (m, 1H, H-13), 5.73 –5.69 (m, 1H, H-14), 4.47 – 4.43 (m, 2H, H-2', H-3'), 4.30 – 4.22 (m, 3H, H-4', H-5'), 3.04 – 2.98 (m, 1H, H-12), 2.45 – 2.41 (m, 2H, H-7), 2.40 – 2.21 (m, 4H, H-11, H-15), 2.09 –2.02 (m, 1H, H-16), 1.79 – 1.74 (m, 2H, H-8), 1.51 – 1.44 (m, 1H, H-16) ppm.

Note: Signal for H-9 lies under signal of triethylammonium protons (3.23 ppm). H-9 signal could be detected and assigned in COSY (3.23 ppm, 1.77 ppm) and HSQC (3.23 ppm, 38.7 ppm) experiments.

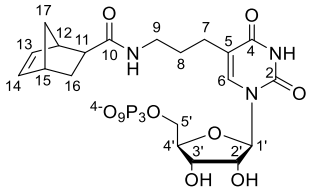
^{13}C{^{1}H} NMR (126 MHz, D_2O): δ = 175.8 (C-10), 165.8 (C-4), 151.8 (C-2), 137.5 (C-6), 133.6 (C-13), 132.1 (C-14), 115.02 (C-5), 87.8 (C-1'), 83.5 (d, $^3J_{C\text{-}P}$ = 9.1 Hz, C-4'), 73.4 (C-2'), 69.8 (C-3'), 65.1 (d, $^2J_{C\text{-}P}$ = 6.2 Hz, C-5'), 42.6 (C-12), 41.7 (C-11), 38.7 (C-9), 31.3 (C-15), 28.8 (C-16), 27.2 (C-8), 23.7 (C-7) ppm.

^{31}P{^{1}H} NMR (202 MHz, D_2O): δ = -10.32 (d, *J* = 19.8 Hz, P_γ), -11.84 (d, *J* = 20.6 Hz, P_α), -23.24 (t, *J* = 19.8 Hz, P_β) ppm.

MS (HR-ESI, neg): meas. m/z = 648.0751, calc. 648.0766 for $C_{19}H_{29}N_3O_{16}P_3$ $[M\text{-}H]^-$.

UV (H_2O): ε_{268} = 6200 $M^{-1}cm^{-1}$.

norUTP



AminoUTP (10 mg, 10.6 μmol, 1.00 eq) was dissolved in 1 mL sodium borate buffer (0.1 M, pH 9) and 5-norbornene-exo-2-carboxylic acid succinimidyl ester[10] (7.76 mg, 31.7 μmol, 3.00 eq) dissolved in 1 mL DMF was added. The reaction mixture was stirred overnight at room temperatureand the crude reaction mixture was purified by reversed phase HPLC (10 → 50% B in 50 min, R_t = 37.3 min). Appropriate fractions were lyophilized to afford the product as triethylammonium salt (10.0 mg, 89%).

^{1}H NMR (500 MHz, D_2O): δ = 7.75 (s, 1H, H-6), 6.17 (s, 2H, H-13, H-14), 5.97 (d, *J* = 4.5 Hz, 1H, H-1'), 4.46 – 4.38 (m, 2H, H-2', H-3'), 4.29 – 4.15 (m, 3H, H-4', H-5'), 2.92 – 2.84 (m, 2H, H-12, H-15), 2.47 – 2.33 (m, 2H, H-7), 2.17 – 2.11 (m, 1H, H-11), 1.79 – 1.70 (m, 2H, H-8), 1.70 – 1.61 (m, 1H, H-16), 1.46 (d, *J* = 8.3 Hz, 1H, H-17), 1.40 – 1.32 (m, 1H, H-16) ppm.

Note: Signal for H-9 lies under signal of triethylammonium protons (3.17 ppm). H-9 signal could be detected and assigned in COSY (3.20 ppm, 1.74 ppm) and HSQC (3.20 ppm, 38.9 ppm) experiments. Second Signal for H-17 lies under signal of triethylammonium protons (1.25 ppm). H-17 signal could be detected and assigned in COSY (1.29 ppm, 1.46 ppm) and HSQC (1.30 ppm, 45.9 ppm) experiments.

^{13}C{^{1}H} NMR (126 MHz, D_2O): δ = 179.0 (C-10), 165.8 (C-4), 151.8 (C-2), 138.1 (C-13), 137.4 (C-6), 136.3 (C-14), 115.1 (C-5), 87.8 (C-1'), 83.5 (d, $^3J_{C\text{-}P}$ = 6.3 Hz, C-4'), 73.4 (C-2'), 69.9 (C-3'), 65.2 (d, $^2J_{C\text{-}P}$ = 5.2 Hz, C-5'), 46.4* (C-12), 45.9* (17-C), 43.9* (C-11), 41.2 (C-15), 38.8 (C-9), 30.0* (C-16), 27.1 (C-8), 23.8 (C-7) ppm.

Note: For highlighted (*) carbon atoms slightly separated signals were observed for the two diastereomers (see excerpts of APT spectrum). For clarity only one signal is listed.

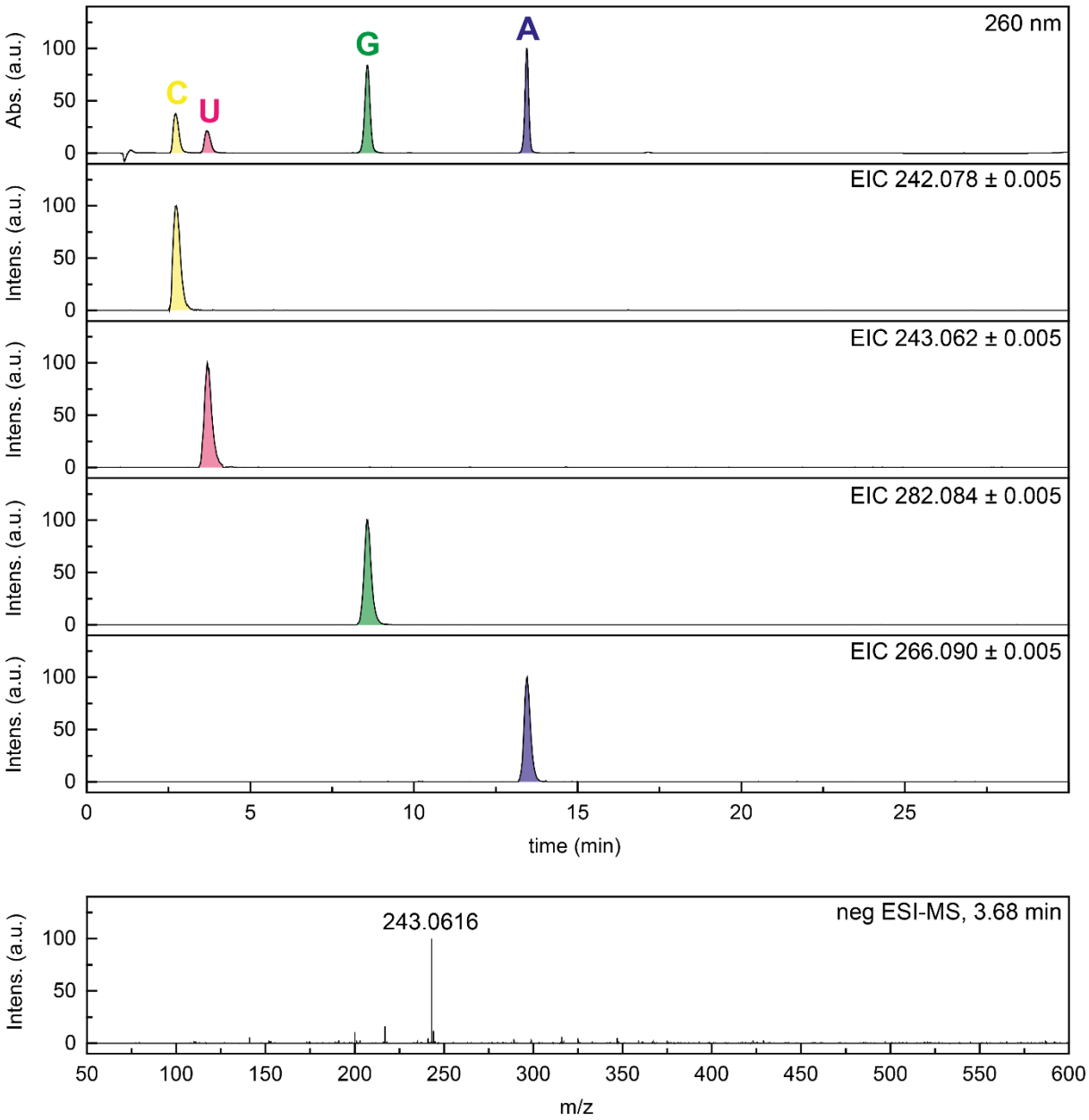
^{31}P{^{1}H} NMR (202 MHz, D_2O): δ = -10.53 (d, *J* = 18.3 Hz, P_γ), -11.84 (d, *J* = 21.4 Hz, P_α), -23.23 (t, *J* = 16.8 Hz, P_β) ppm.

MS (HR-ESI, neg): meas. m/z =660.0765, calc. 660.0766 for $C_{20}H_{29}N_3O_{16}P_3$ $[M\text{-}H]^-$.

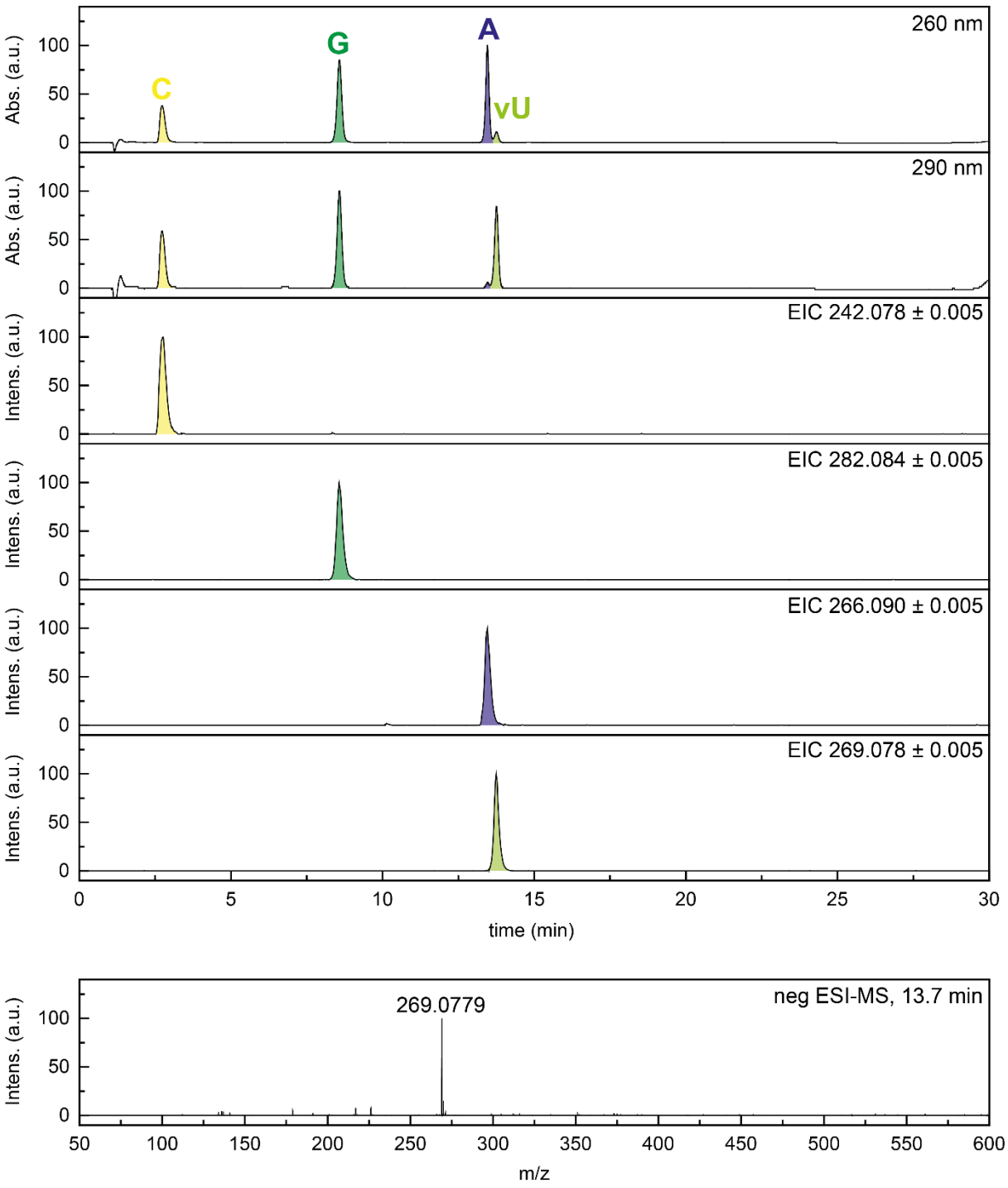
UV (H_2O): ε_{268} = 6000 $M^{-1}cm^{-1}$.

4. Appendix

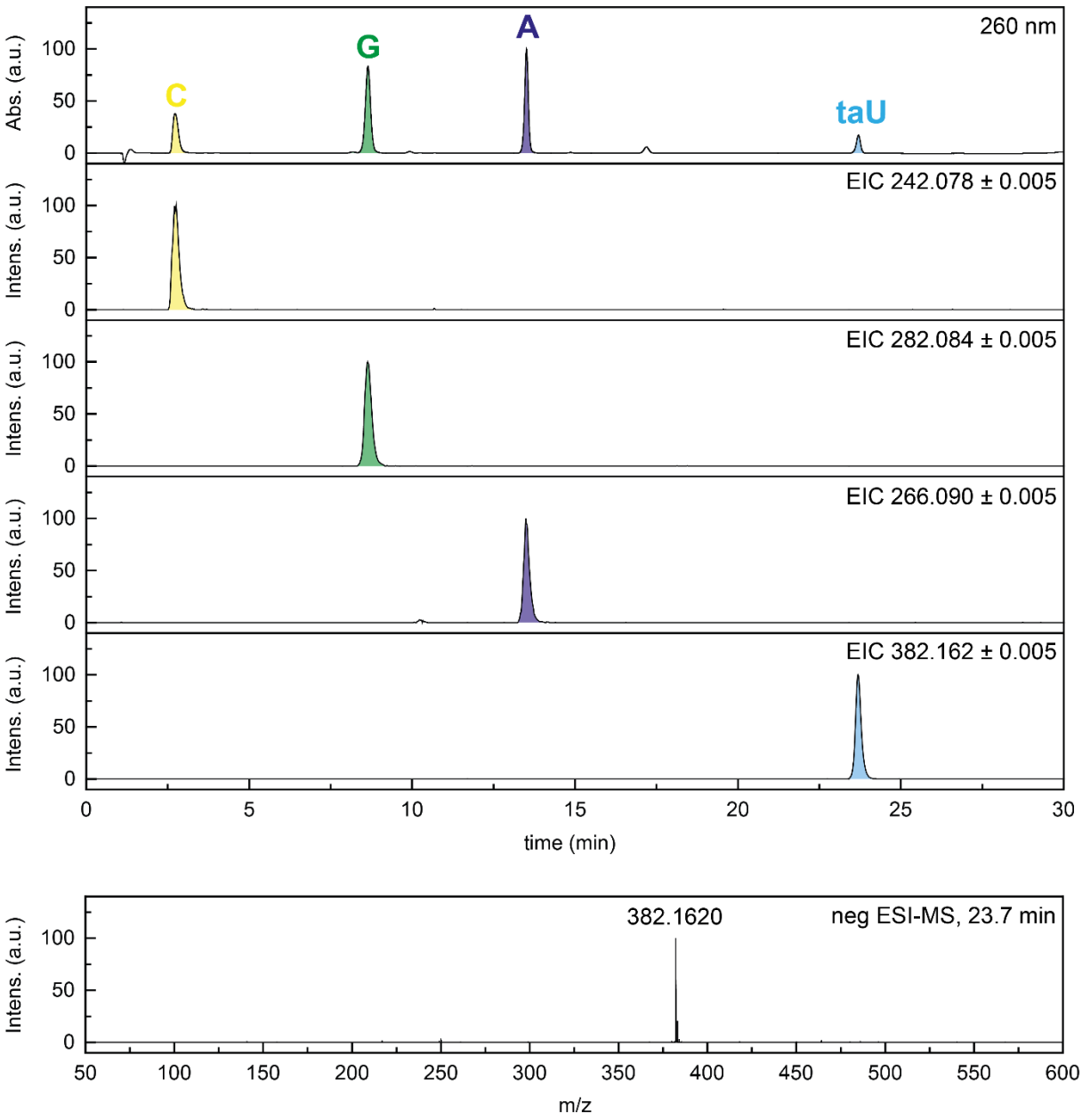
4.1. LC-MS



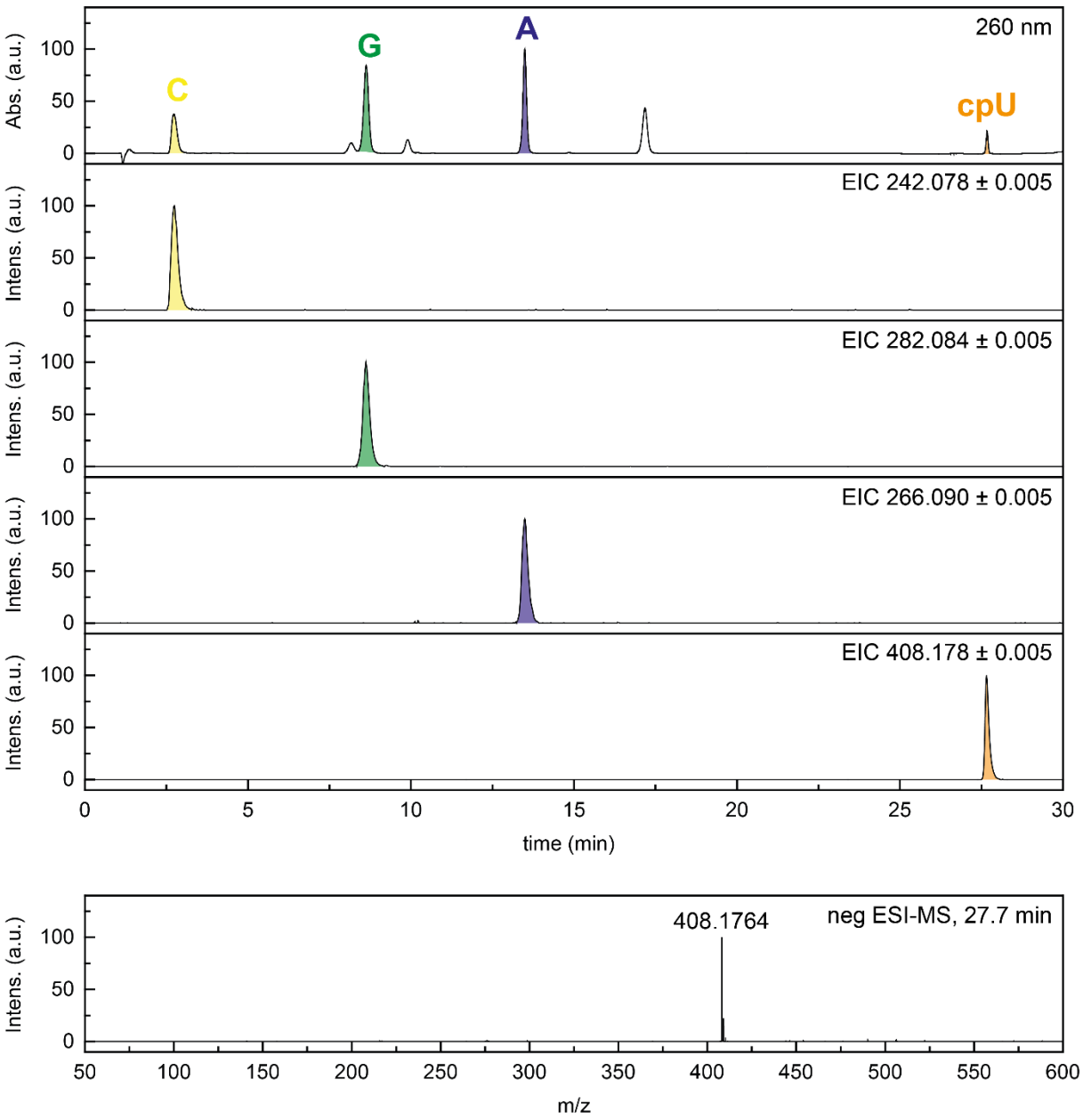
A1: LC-MS chromatogram of the nucleoside mixture obtained from enzymatic digestion of unmodified RhoBAST. The obtained UV chromatogram (260 nm), extracted ion chromatogram (EIC) of all nucleosides (C, U, G, A; $[M-H]^-$) and high resolution mass spectrum of uridine (calc. 243.0623 for $C_9H_{11}N_2O_6$ $[M-H]^-$) is shown.



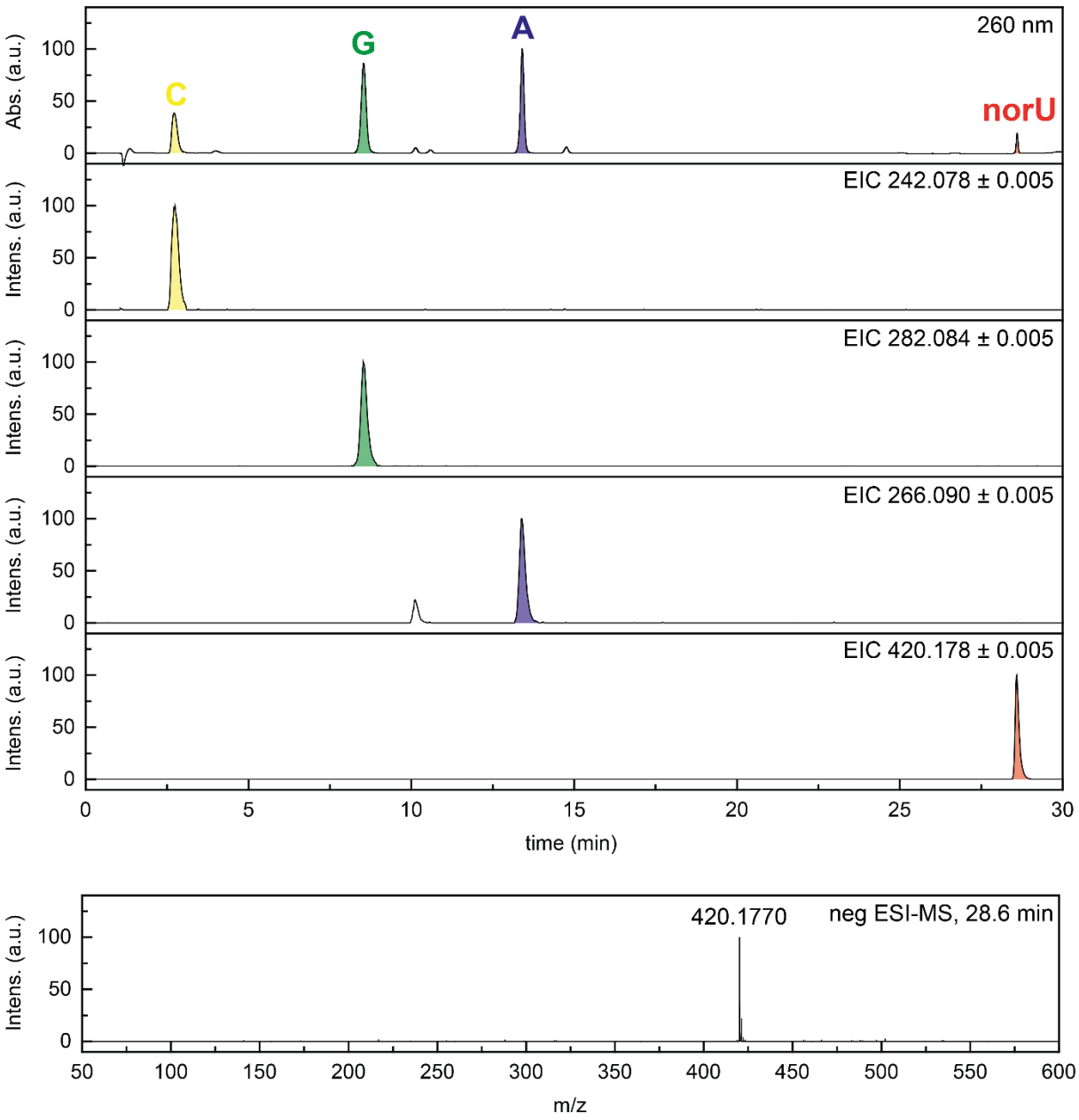
A2: LC-MS chromatogram of the nucleoside mixture obtained from enzymatic digestion of **vU** modified RhoBAST. The obtained UV chromatograms (260 nm, 290 nm), extracted ion chromatogram (EIC) of all nucleosides (C, G, A, **vU**; $[M-H]^-$) and high resolution mass spectrum of **vU** (calc. 269.0779 for $C_{11}H_{13}N_2O_6$ $[M-H]^-$) is shown.



A3: LC-MS chromatogram of the nucleoside mixture obtained from enzymatic digestion of **taU** modified RhoBAST. The obtained UV chromatogram (260 nm), extracted ion chromatogram (EIC) of all nucleosides (C, G, A, **taU**; $[M-H]^-$) and high resolution mass spectrum of **taU** (calc. 382.1620 for $C_{17}H_{24}N_3O_7$ $[M-H]^-$) is shown.

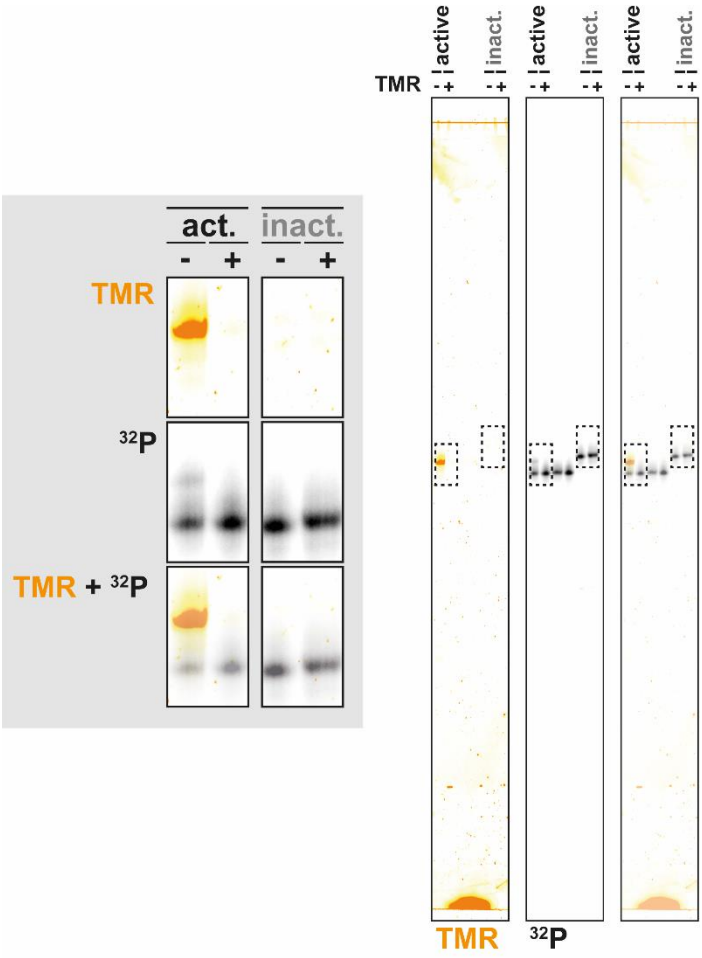


A4: LC-MS chromatogram of the nucleoside mixture obtained from enzymatic digestion of **cpU** modified RhoBAST. The obtained UV chromatogram (260 nm), extracted ion chromatogram (EIC) of all nucleosides (C, G, A, **cpU**; $[M-H]^-$) and high resolution mass spectrum of **cpU** (calc. 408.1776 for $C_{19}H_{26}N_3O_7$ $[M-H]^-$) is shown.

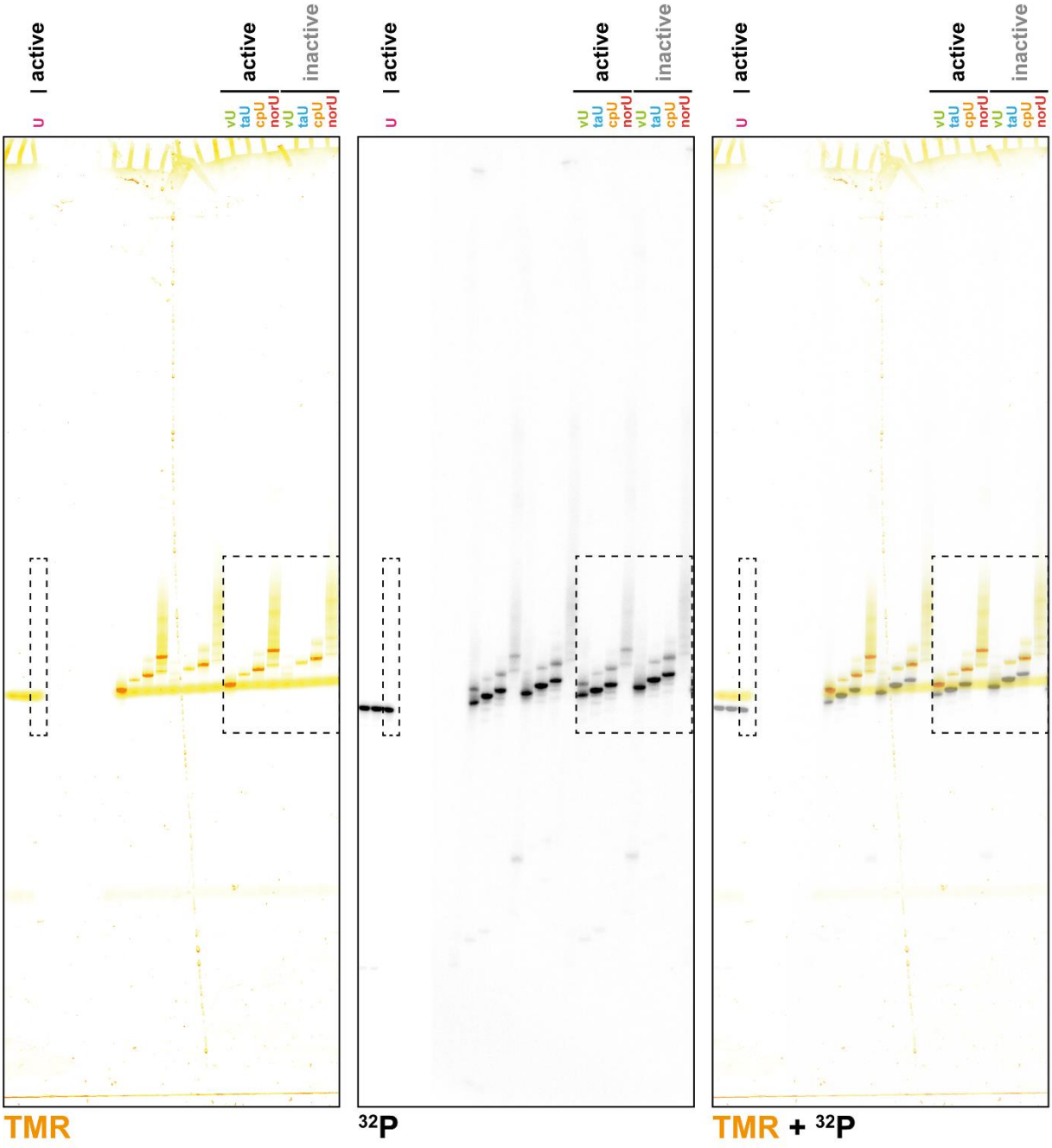


A5: LC-MS chromatogram of the nucleoside mixture obtained from enzymatic digestion of **norU** modified RhoBAST. The obtained UV chromatogram (260 nm), extracted ion chromatogram (EIC) of all nucleosides (C, G, A, **norU**; $[M-H]^-$) and high resolution mass spectrum of **norU** (calc. 420.1776 for $C_{19}H_{26}N_3O_7$ $[M-H]^-$) is shown.

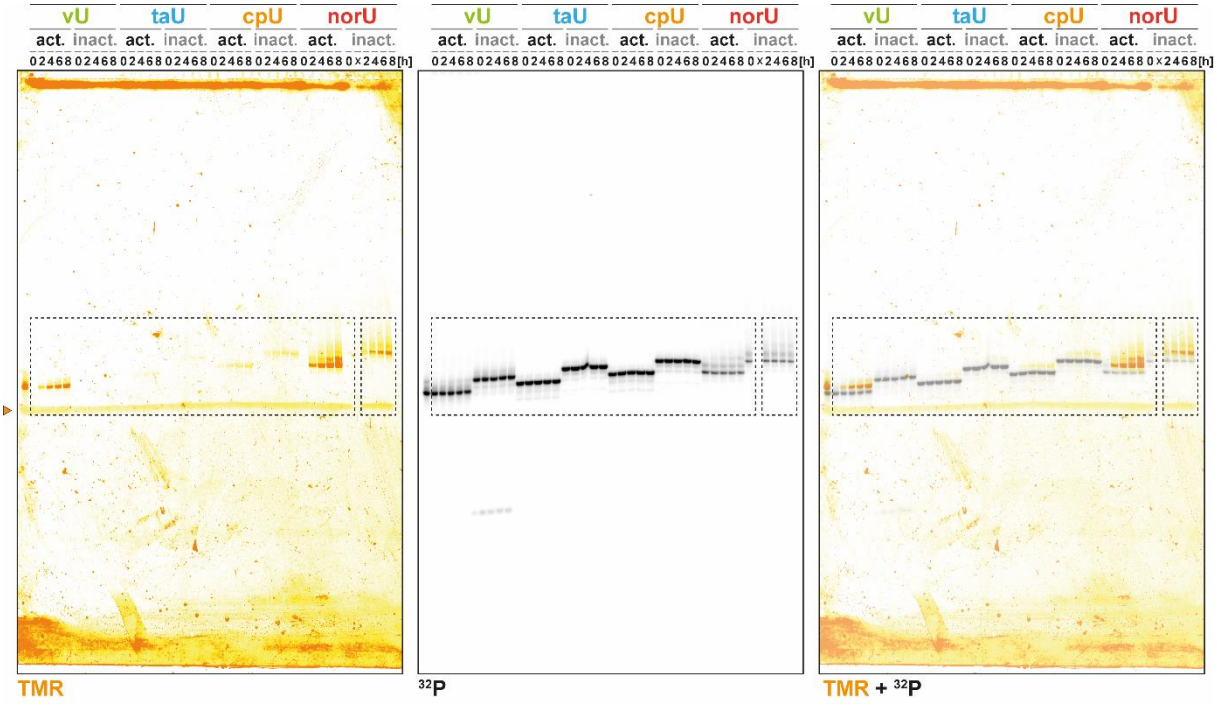
4.2. PAGE gels



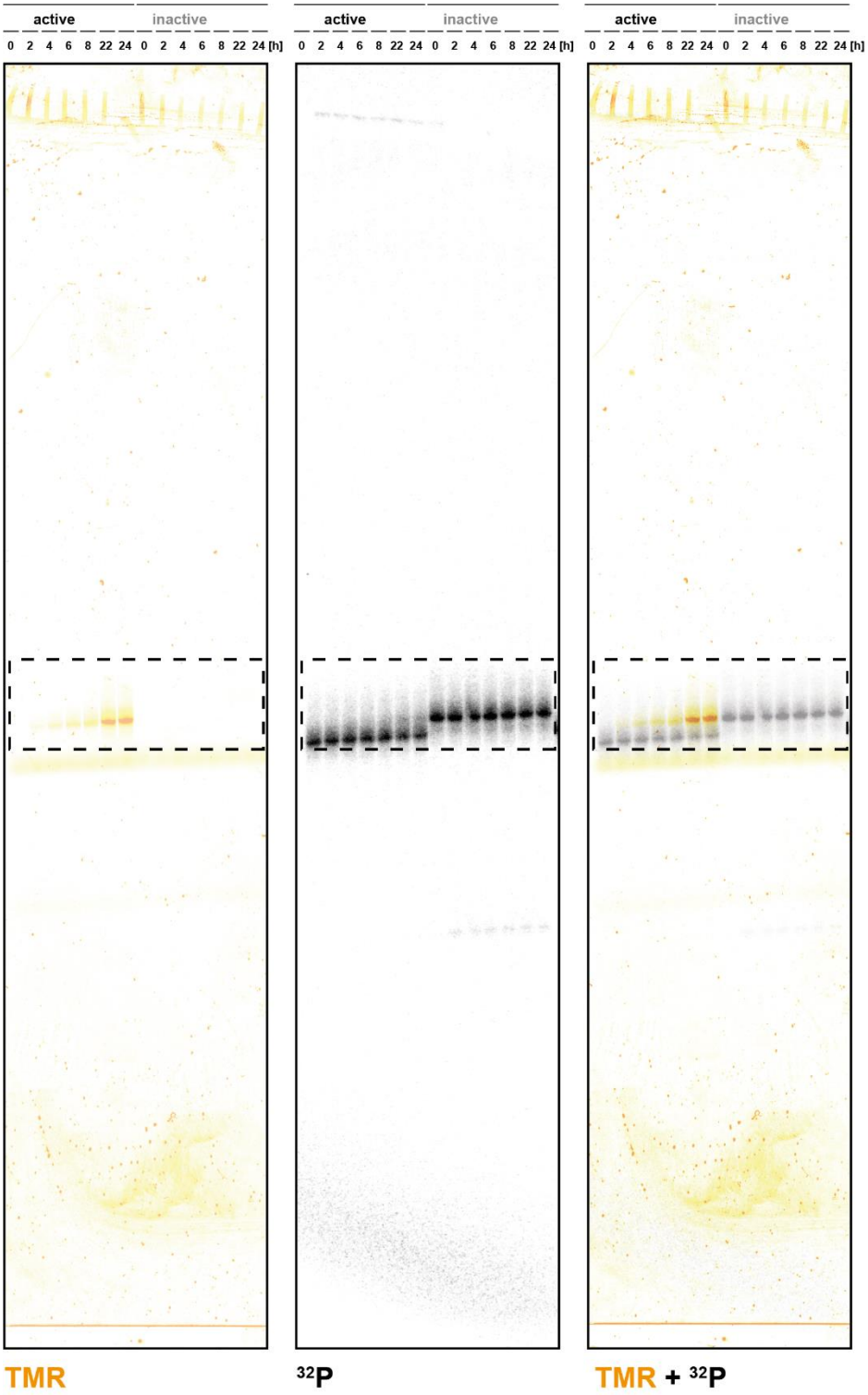
A6: Full gel corresponding to the section shown in Fig. 2B.



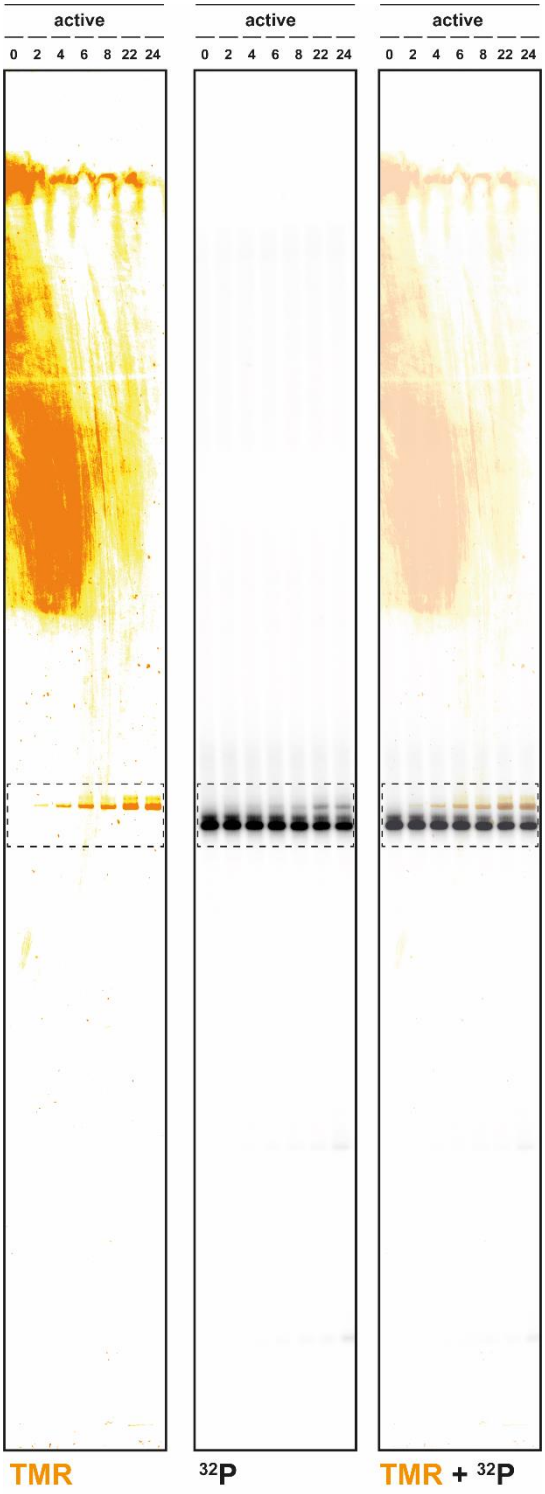
A7: Full gel corresponding to the section shown in Fig. S6.



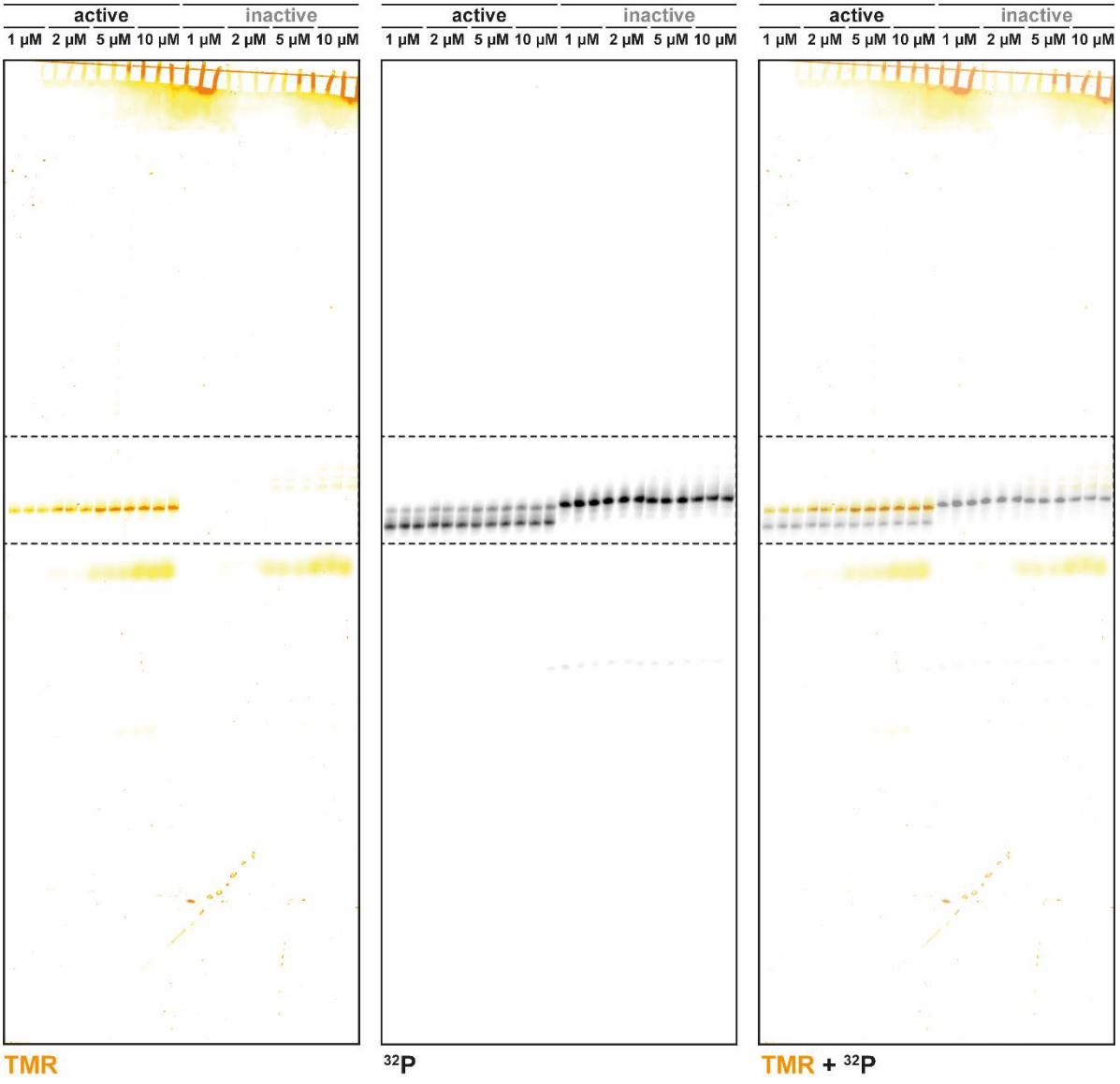
A8: Full gel corresponding to the section shown in Fig. S7.



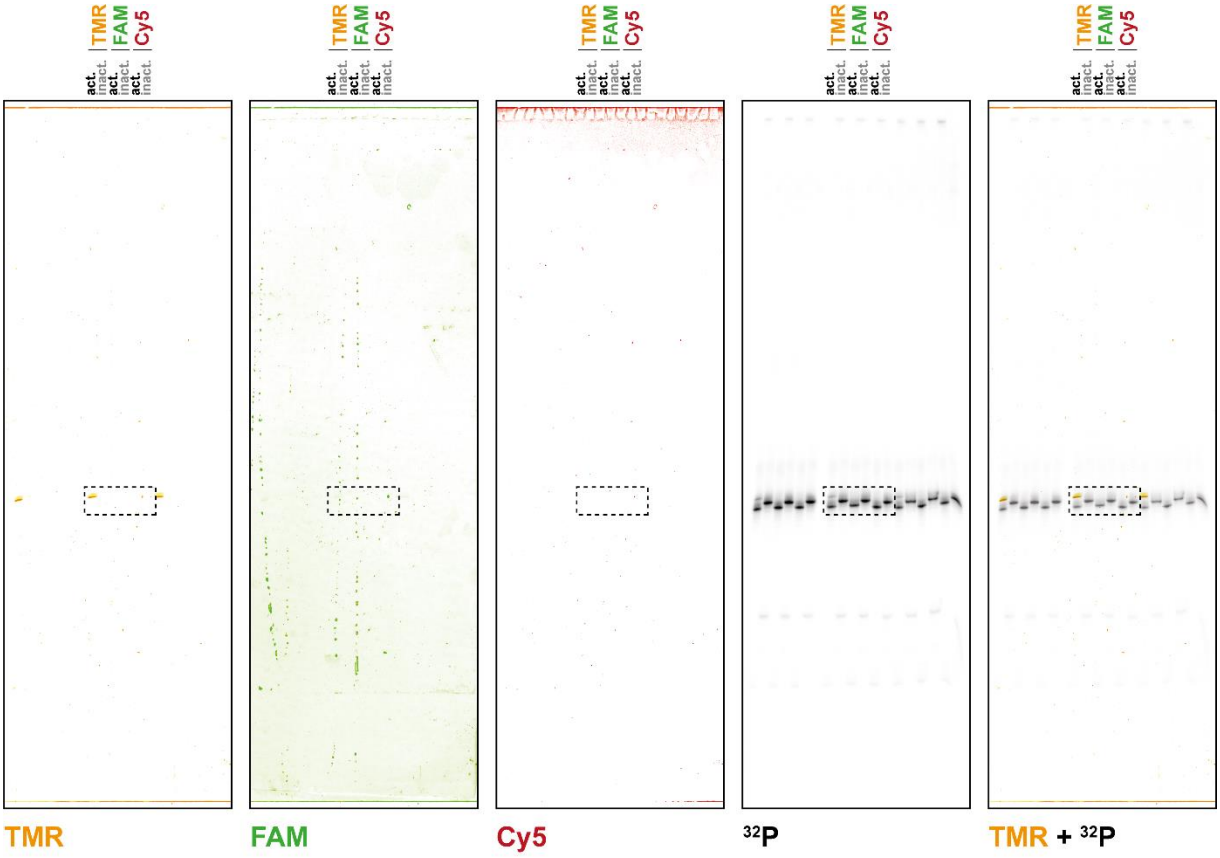
A9: Full gel corresponding to the section shown in Fig. S8.



A10: Full gel corresponding to the section shown in Fig. S9.



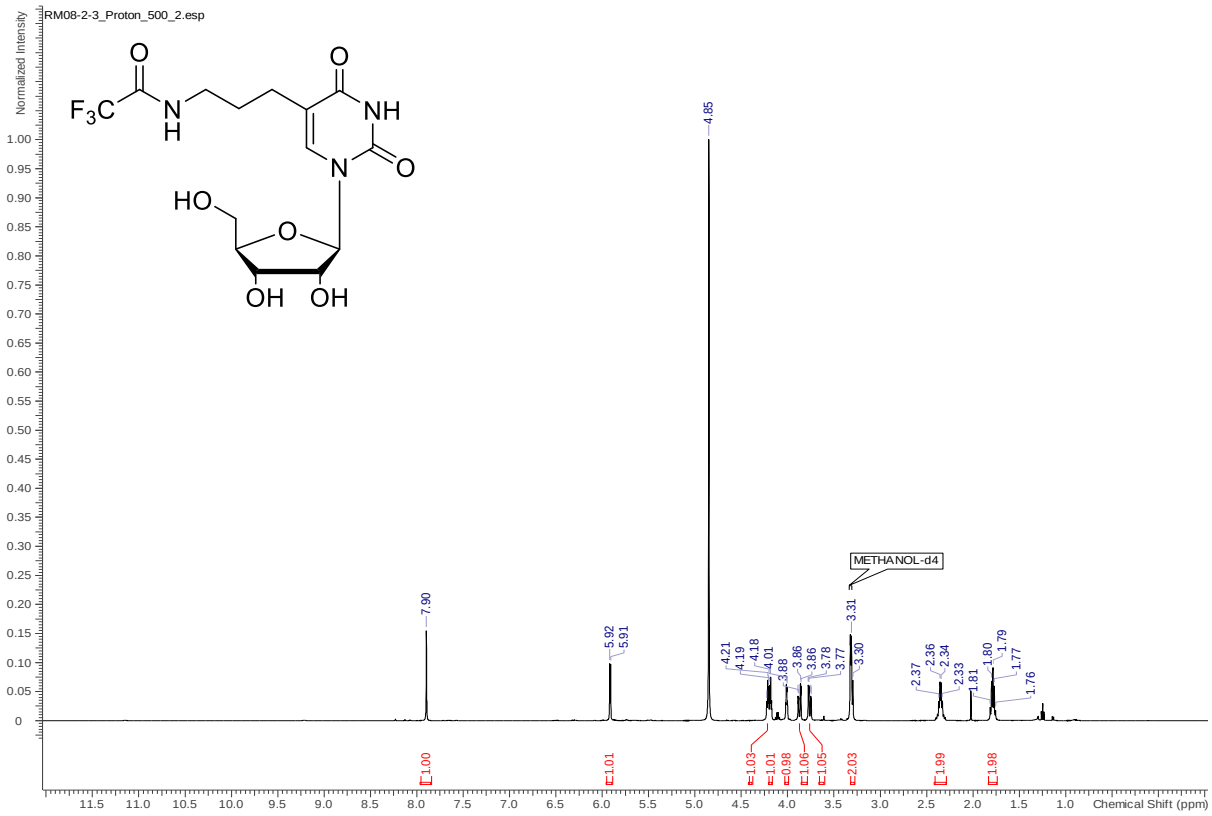
A11: Full gel corresponding to the section shown in Fig. S11.



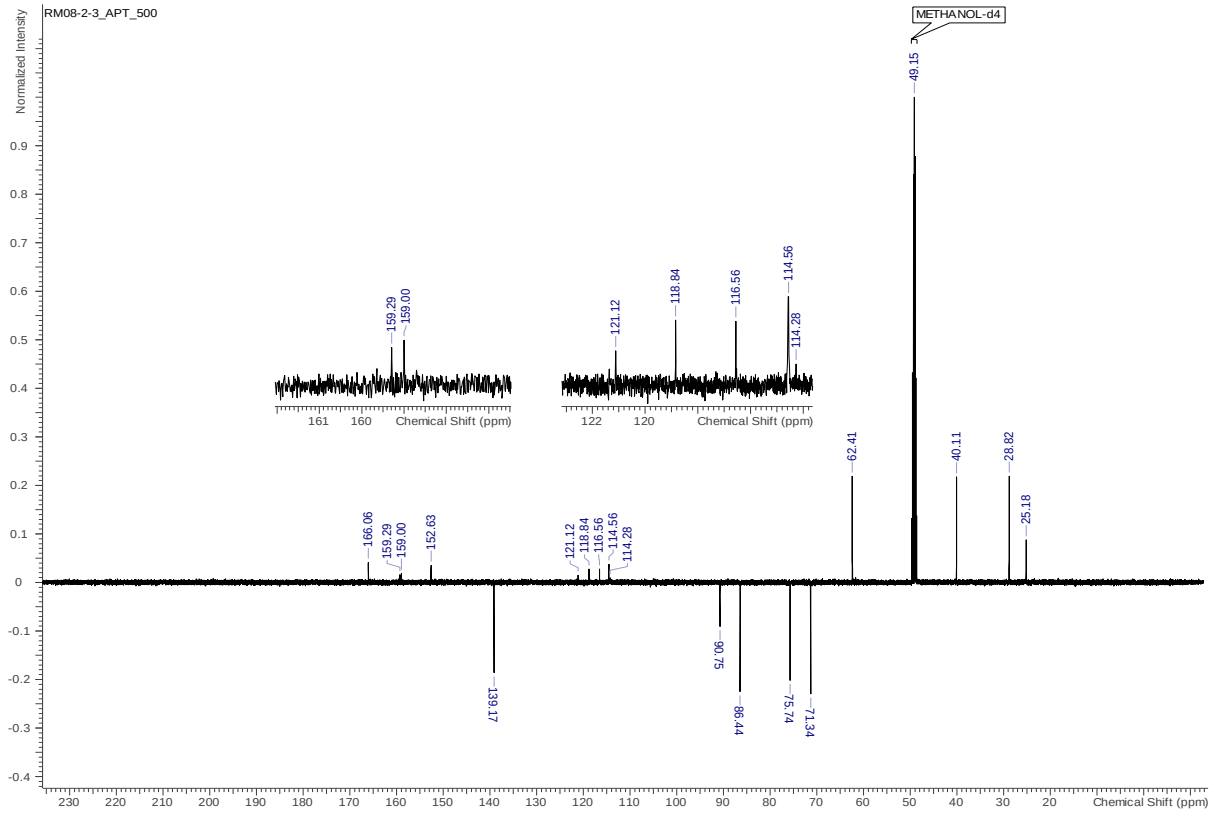
A12: Full gel corresponding to the section shown in Fig. S13.

4.3. NMR spectra

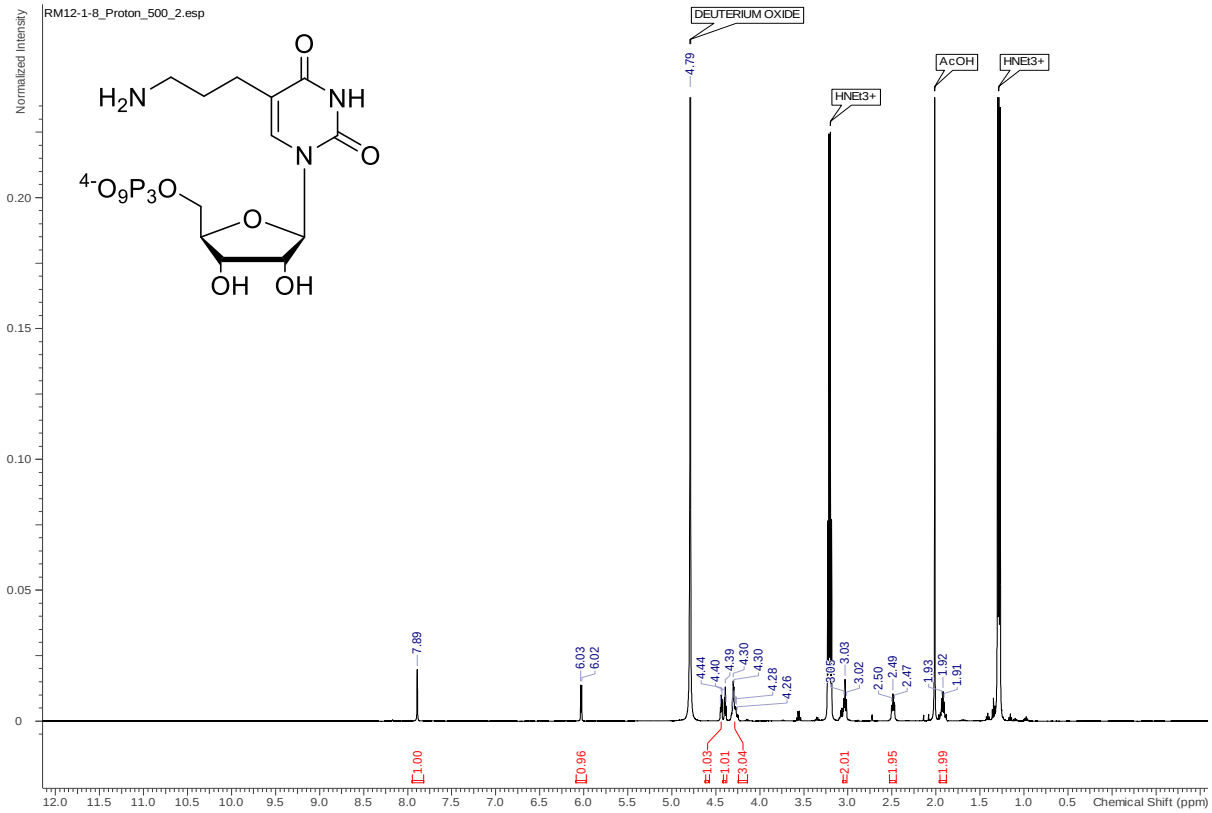
^{1}H-NMR of compound 3:



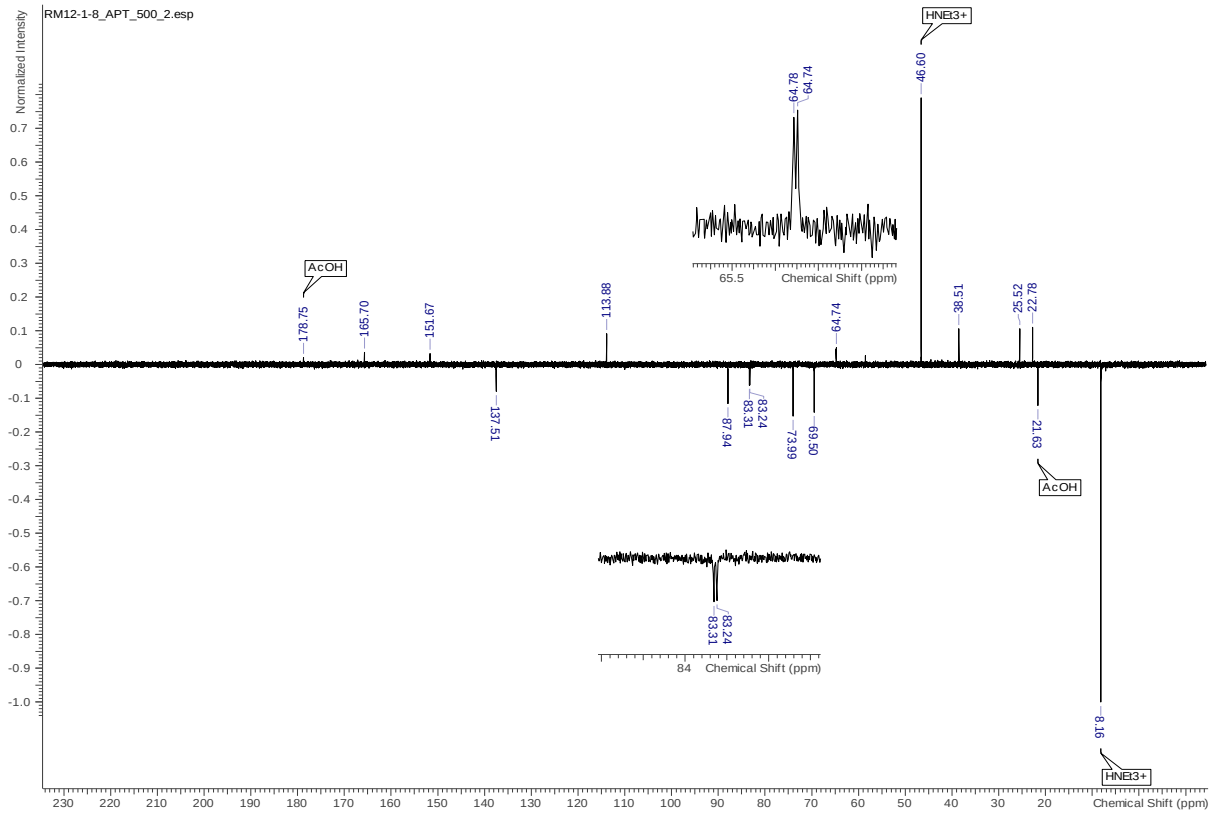
^{13}C-NMR of compound 3:



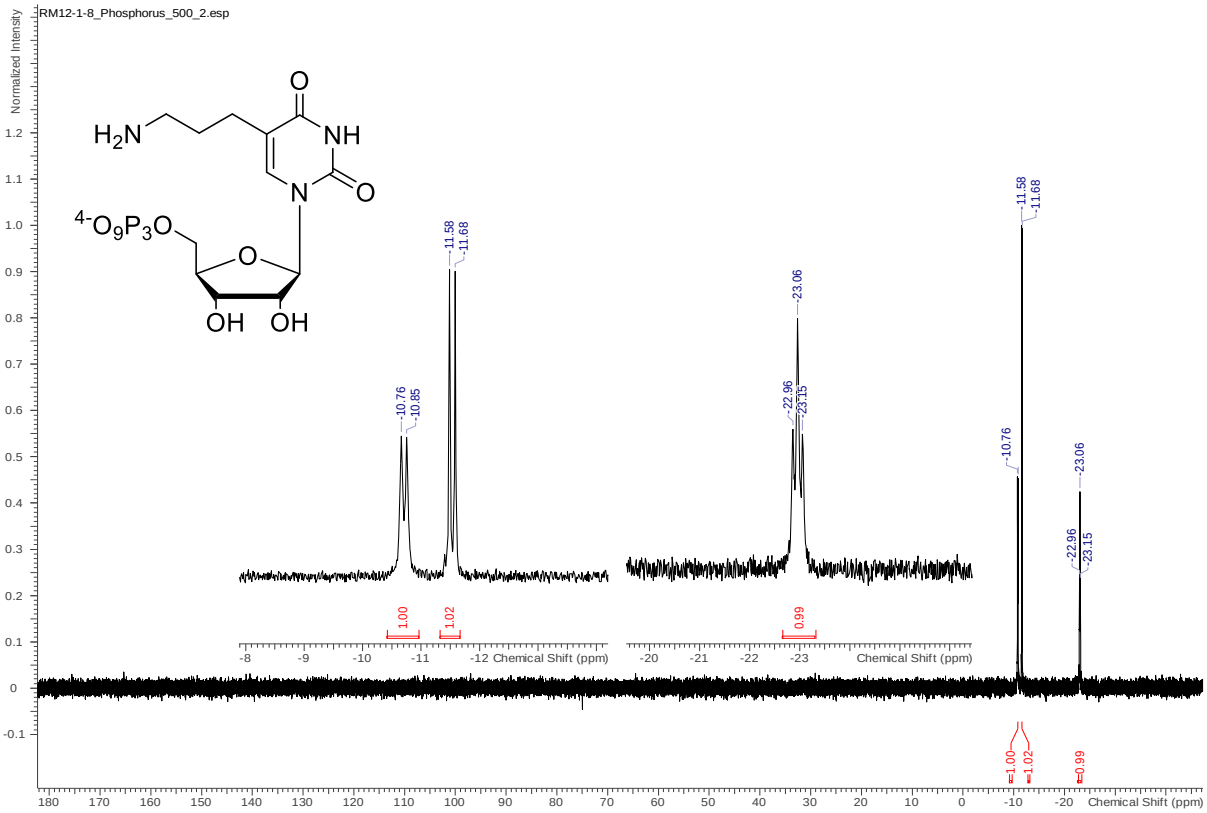
^{1}H-NMR of aminoUTP:



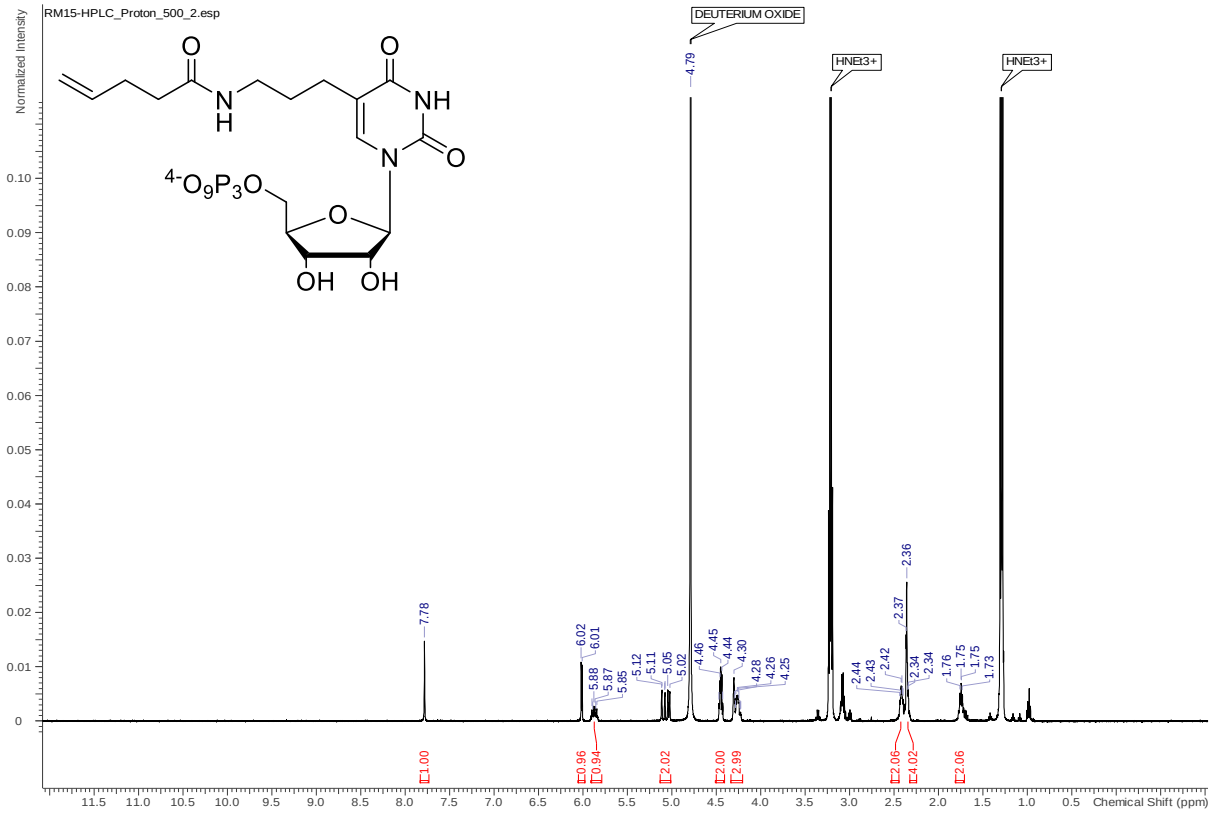
^{13}C-NMR of aminoUTP:



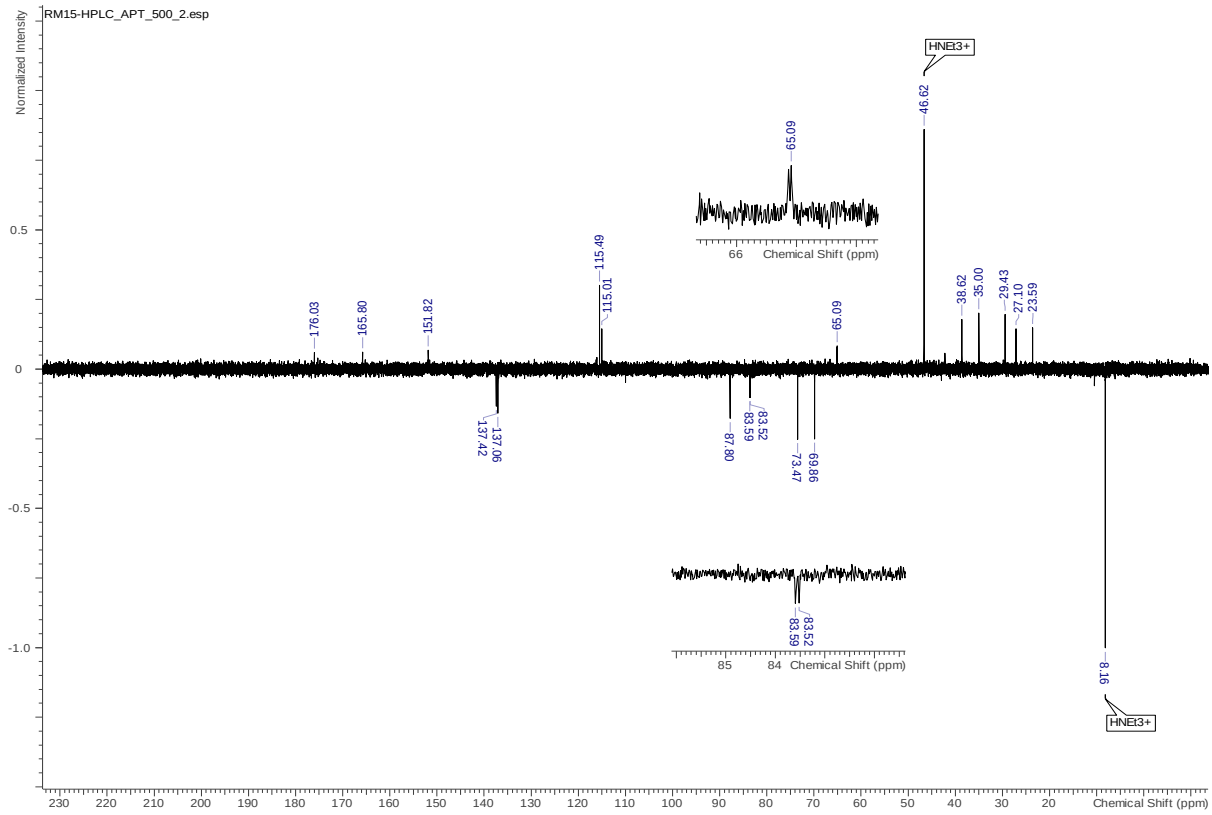
^{31}P-NMR of aminoUTP:



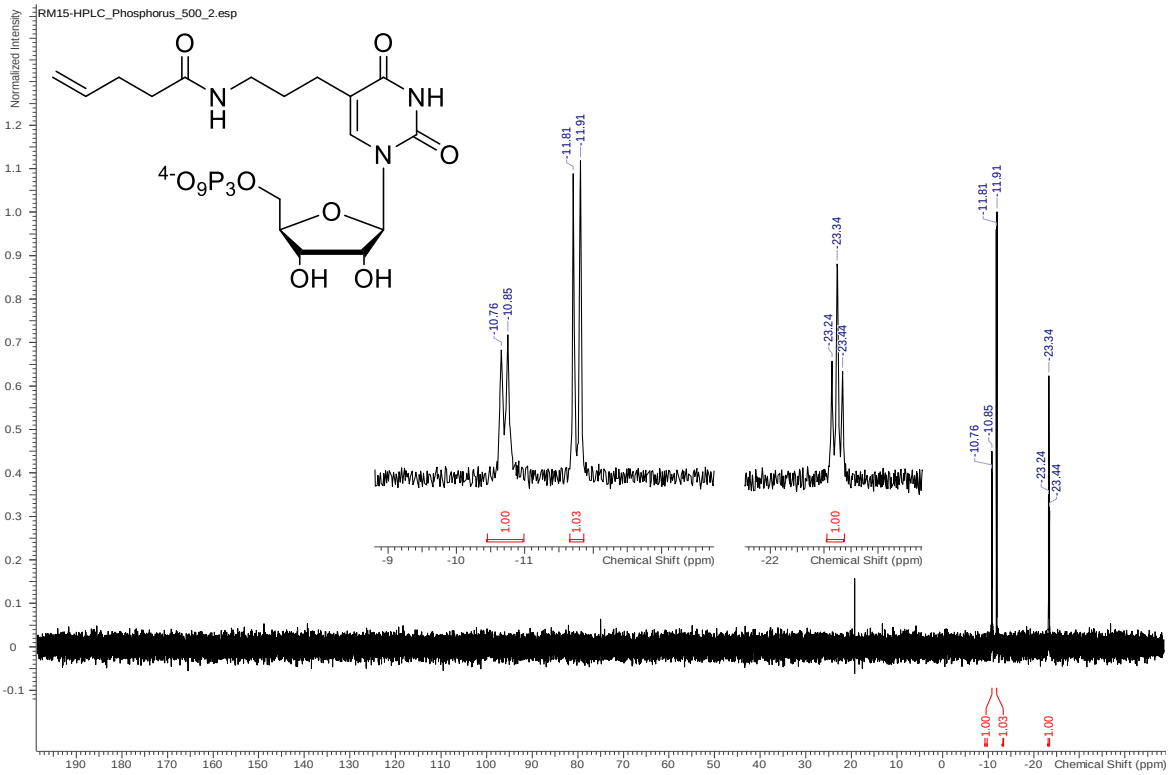
^{1}H-NMR of taUTP:



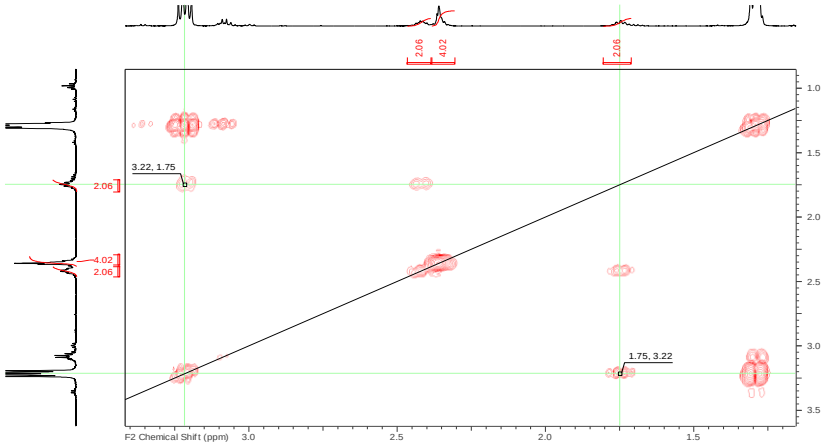
^{13}C-NMR of taUTP:



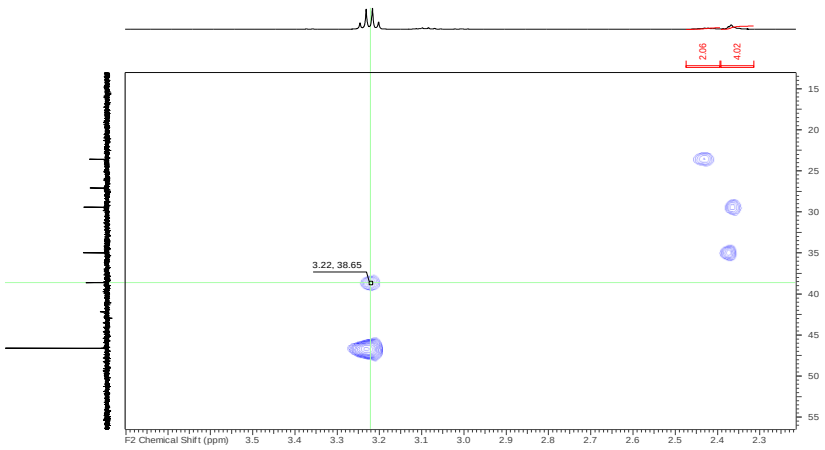
^{31}P-NMR of taUTP:



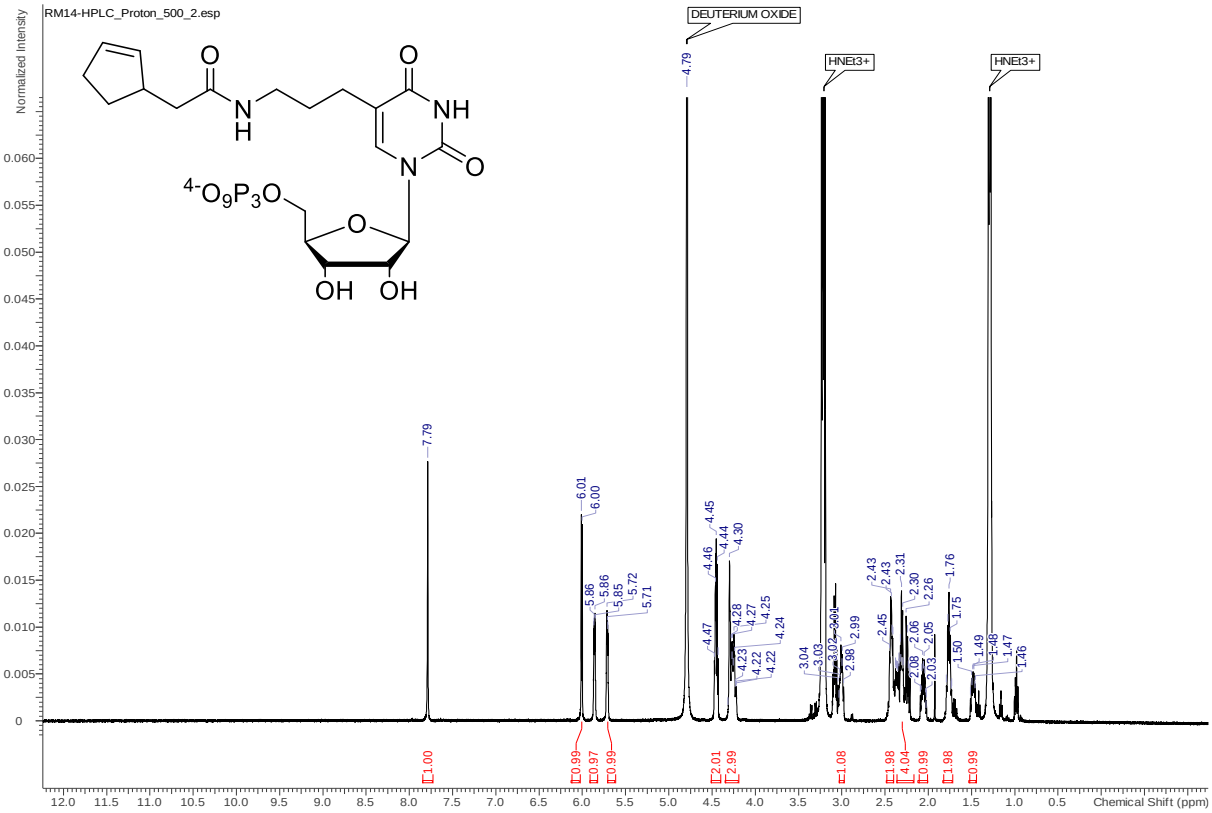
COSY of taUTP (excerpt):



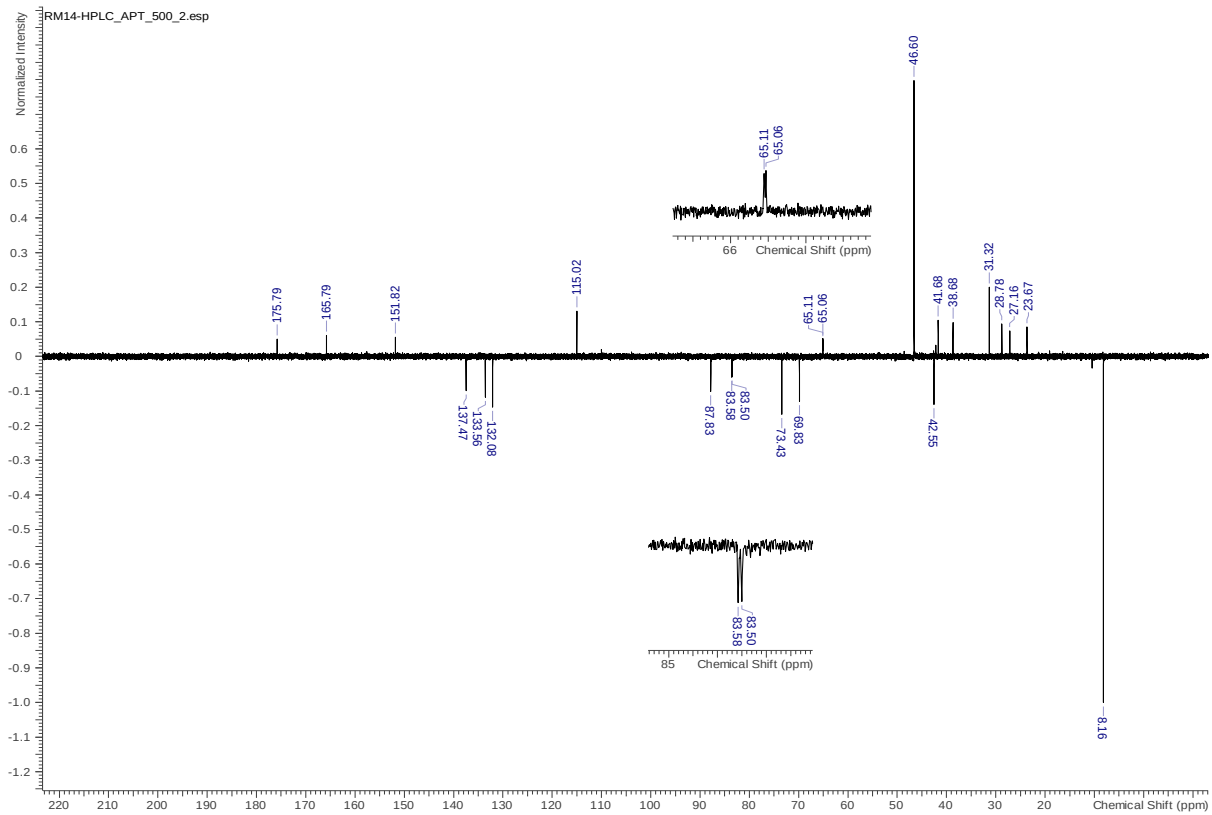
HSQC of taUTP (excerpt):



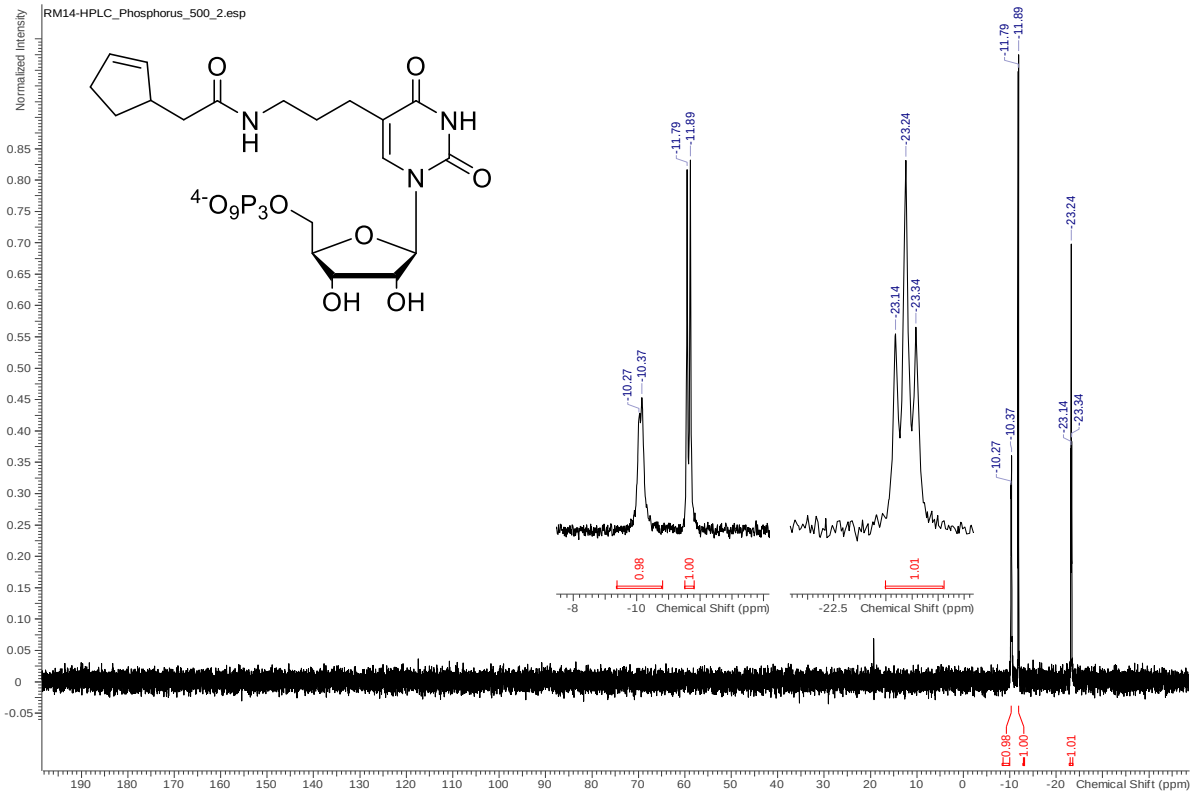
^{1}H-NMR of cpUTP:



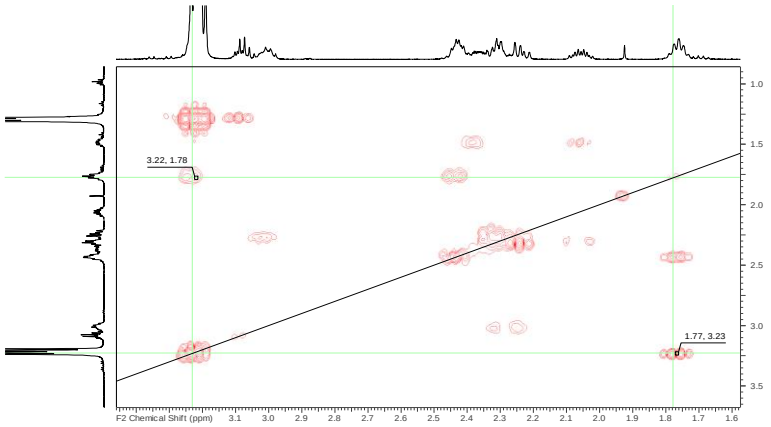
^{13}C-NMR of cpUTP:



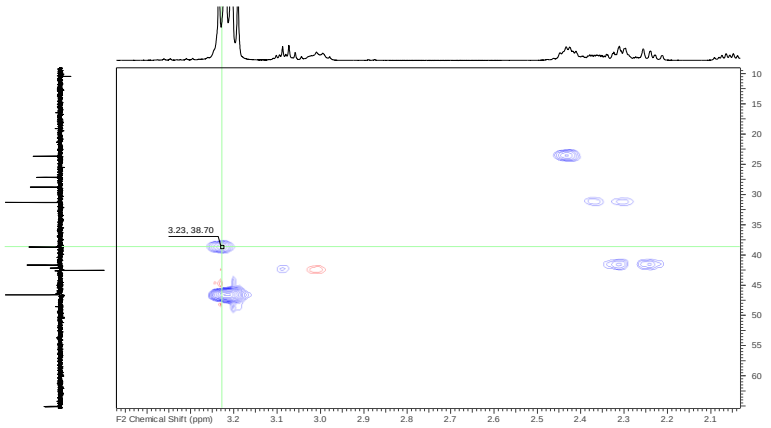
^{31}P-NMR of cpUTP:



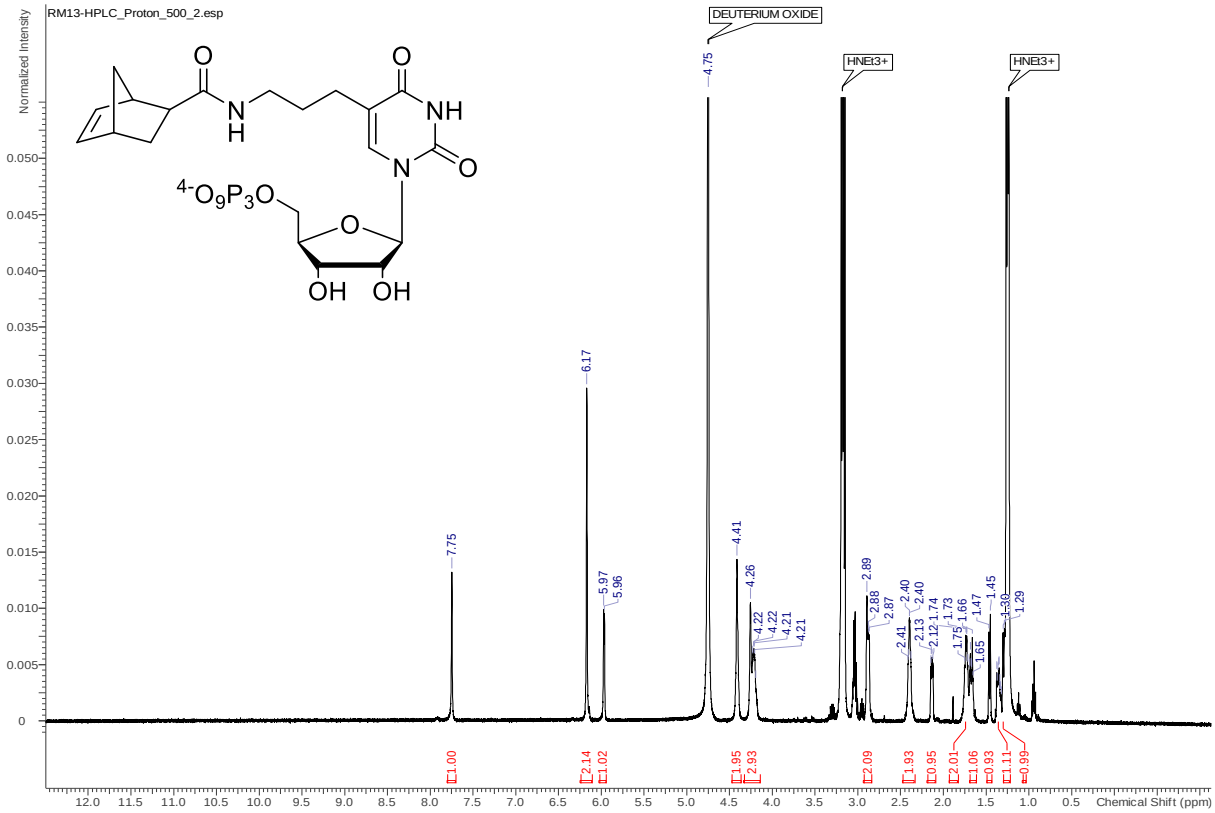
COSY of cpUTP (excerpt):



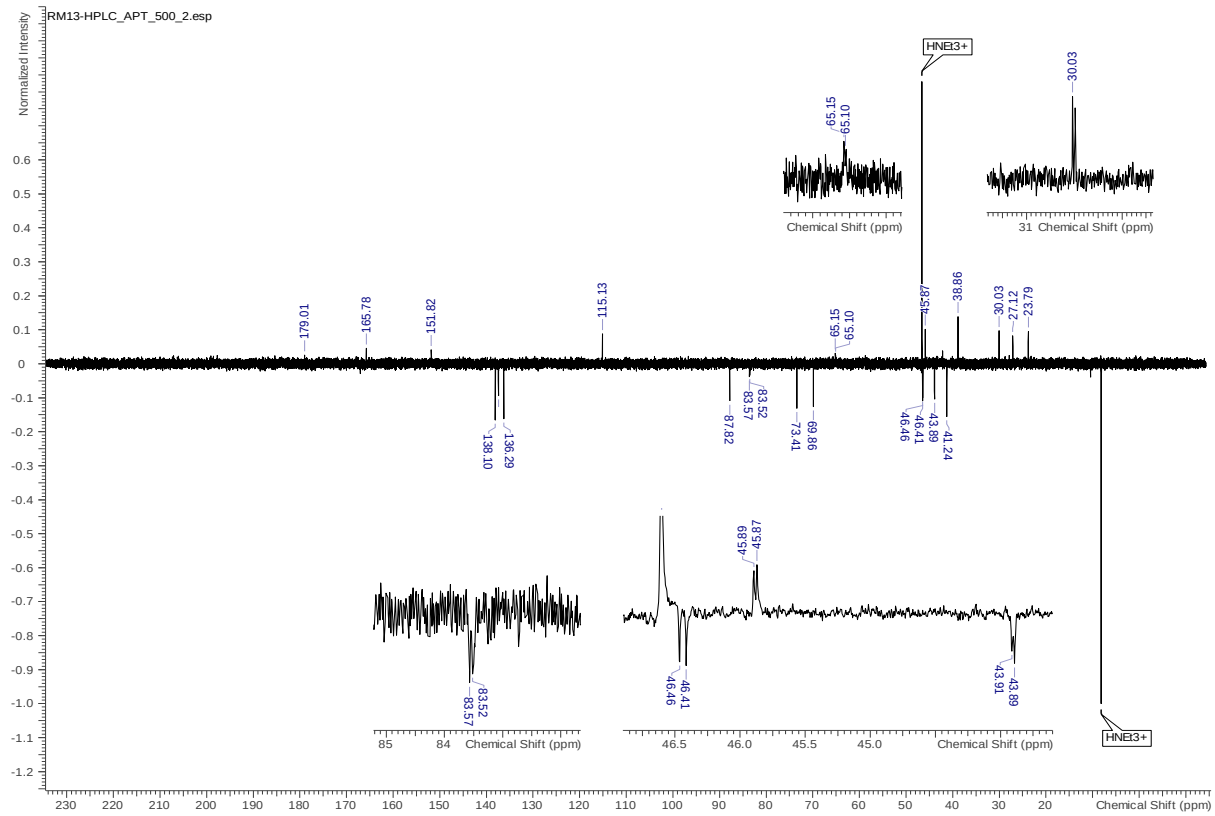
HSQC of cpUTP (excerpt):



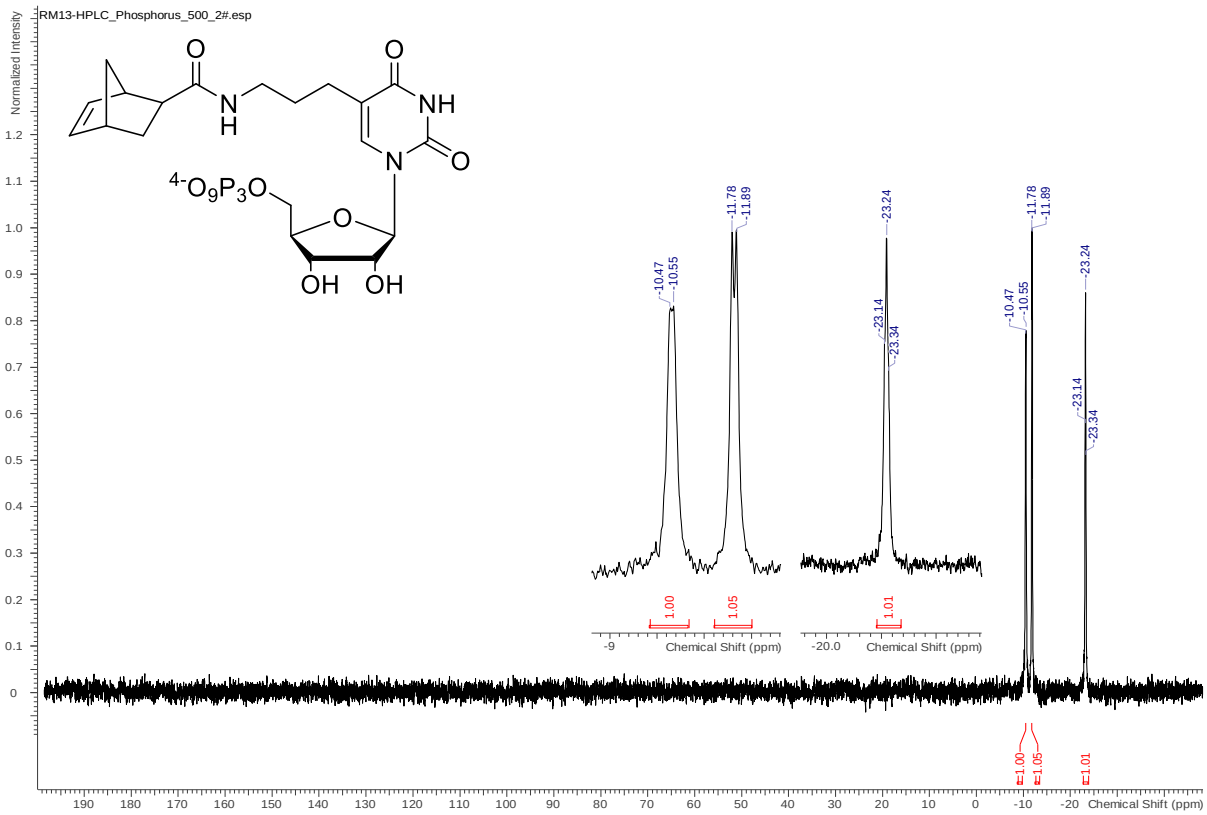
^{1}H-NMR of norUTP:



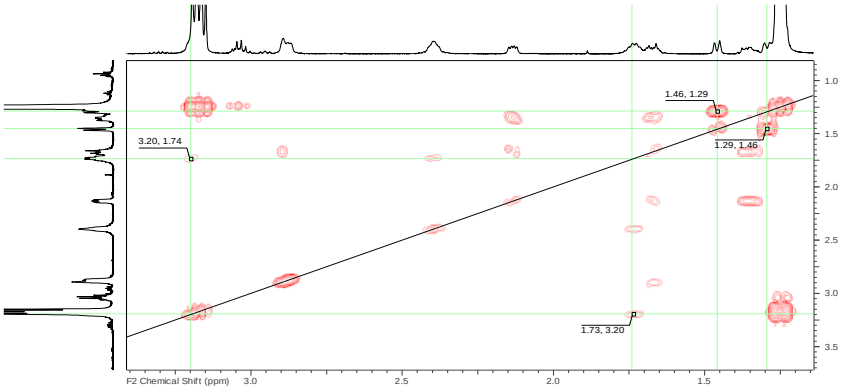
^{13}C-NMR of norUTP:



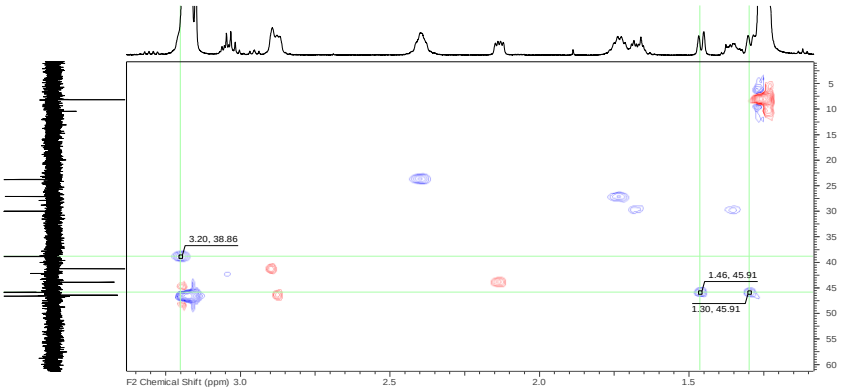
^{31}P-NMR of norUTP:



COSY of norUTP (excerpt):



HSQC of norUTP (excerpt):



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