nature chemistry

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41557-019-0225-x

In the format provided by the authors and unedited.

Prebiotic phosphorylation of 2-thiouridine provides either nucleotides or DNA building blocks via photoreduction

Jianfeng Xu[1], Nicholas J. Green[1], Clémentine Gibard[2], Ramanarayanan Krishnamurthy[2]* and John D. Sutherland[1]*

[1]MRC Laboratory of Molecular Biology, Cambridge, UK. [2]Department of Chemistry, The Scripps Research Institute, La Jolla, CA, USA.
*e-mail: rkrishna@scripps.edu; johns@mrc-lmb.cam.ac.uk

Prebiotic Phosphorylation of 2-Thiouridine Provides Either Nucleotides or DNA Building Blocks *via* Photoreduction

Jianfeng Xu[1], Nicholas J. Green[1], Clémentine Gibard[2], Ramanarayanan Krishnamurthy[2*], John D. Sutherland[1]*

[1]MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge CB2 0QH, UK. *e-mail: johns@mrc-lmb.cam.ac.uk
[2]Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA. *e-mail:rkrishna@scripps.edu

Abstract: Breakthroughs in the study of the origin of life have demonstrated the likely prebiotic existence of some of the building blocks essential to biology and therefore its development. Until prebiotic synthesis demonstrates their viability, missing building blocks are inferred to be products of primitive biosynthesis, which can stretch the limits of plausibility. Herein, syntheses under prebiotic conditions of 2'-deoxy-2-thiouridine and thence 2'-deoxyadenosine and 2-deoxyribose are reported. 2'-Deoxy-2-thiouridine is produced by photoreduction of 2,2'-anhydro-2-thiouridine, in turn formed by phosphorylation of 2-thiouridine, an intermediate of prebiotic RNA synthesis. 2'-Deoxy-2-thiouridine is an effective deoxyribosylating agent and may have functioned as such in either abiotic or proto-enzyme-catalysed pathways to DNA, as demonstrated by its conversion to 2'-deoxyadenosine by reaction with adenine, and 2-deoxyribose by hydrolysis. An alternative prebiotic phosphorylation of 2-thiouridine leads to its 5'-phosphate, showing that proposals in which 2-thiouridine was a key component of early RNA sequences are within the bounds of synthetic credibility.

General methods

Reagents and deuterated solvents used for reactions were purchased from Sigma-Aldrich or Acros Organics and were used without further purification. All photochemical reactions were carried out in Norell Suprasil quartz NMR tubes purchased from Sigma-Aldrich using Hg lamps with principal emission at 254 nm in a Rayonet photochemical chamber reactor RPR-200, acquired from The Southern New England Ultraviolet Company. Further details on the particular photochemical chamber set up, including emission spectra and detailed measurements of photon flux, are available in the literature.[1] A sample removed from the photochemical reactor after 3 hours had an internal temperature of 35 °C. A Mettler Toledo SevenEasy pH Meter S20 was used to monitor the pH, and deoxygenation of solution was achieved by sparging anhydrous argon through the solution for 15-20 min. A preparative Varian Prostar HPLC System was used for the reverse phase high-pressure liquid chromatography (RP-HPLC) linked with an Atlantis T3 C18 Prep Column OBD 10 μm (19× 250 mm). An Analytical HPLC was used to monitor the reaction, using a Thermofisher Ultimate 3000 UPLC and Waters Atlantis T3 C18 column (5 μm, 4.6 mm ×150 mm). All unknown compounds in the reaction mixtures were confirmed by spiking experiments with authentic compounds either purchased from Sigma-Aldrich or synthesized in house using conventional synthetic chemistry. DAP was synthesized according to our previous report.[2] ^{1}H and ^{13}C NMR spectra were acquired using a Bruker Ultrashield 400 Plus operating at 400.1 MHz and 100.6 MHz respectively. Samples consisting of H_2O/D_2O mixtures were analyzed using HOD suppression to collect ^{1}H NMR data. Chemical shifts (δ) are shown in ppm. The conversion yields were determined by relative integrations of the signals using a known amount of sodium formate or pentaerythritol as internal reference in the ^{1}H NMR spectrum. Coupling constants (J) are given in Hertz and the notations s, d, m represent the multiplicities singlet, doublet, and multiplet signal. Mass spectra were recorded with an Agilent Technologies 6130 Quadrupole LC-MS using positive and negative Electron Spray Ionisation. The accurate mass spectra were recorded with a Waters Vion IMS QTOF Ion Mobility Quadrupole Time-of-flight Mass spectrometer using positive ESI.

Experimental procedures

Phosphorylation of 2-thiouridine 8 in hot formamide.

10 mg of 2-thiouridine **8** (0.04 mmol), 5 mg of $NH_4H_2PO_4$ (0.04 mmol) and 23 mg of urea (0.40 mmol) were mixed in 0.20 ml of formamide. The mixture was heated at 100 °C for 20 hs. The solvent was removed under high vacuum. The dry residue was dissolved in D_2O, to which a 30 ul of 50 mM pentaerythritol was added to quantify the yields of products (24% yield for **14**) by ^{1}H NMR spectrum. The same reaction was repeated at the higher scale (20 mg of **8**) to obtain 5 mg (20 % yield) of 2-thiouridine-5'-phosphate **14** after HPLC purification for NMR characterization and spiking experiments. ^{1}H NMR (400 MHz, D_2O): δ 8.13 (1H, d, J = 8.1 Hz, H6), 6.58 (1H, d, J = 3.1 Hz, H1'), 6.16 (1H, d, J = 8.1 Hz, H5), 4.36 (1H, m, H2'), 4.25 (2H, m, H3' and H4'), 4.19, (1H, dd, J_1 = 11.8 Hz, J_2 = 4.5 Hz, H5'), 4.05 (1H, J_1 = 11.8 Hz, J_2 = 5.0 Hz, H5"); ^{13}C NMR (101 MHz, D_2O = 1:9): δ 176.0 (C2), 162.7 (C4), 141.9 (C6), 106.8 (C5), 93.1 (C1'), 82.9 (C4', d, J_{P-C} = 8.9 Hz), 74.6 (C2'), 68.5 (C3'), 63.1 (C5', d, J_{P-C} = 4.9 Hz); ^{31}P NMR (162 MHz, D_2O = 1:9): 0.29 (broad s); ESI-LCMS (neg. m/z): 338.9 $[M-1]^-$.

Phosphorylation of 2-thiouridine 8 in urea melt.

10 mg of 2-thiouridine **8** (0.04 mmol), 23 mg of urea (0.40 mmol), 15 mg of NH_4HCO_3 (0.19 mmol), 21 mg of NH_4Cl (0.40 mmol) and 12 mg of Na_2HPO_4 (0.08 mmol) were mixed in 2 ml of H_2O in a round bottom flask. The mixture was dried down under vacuum and heated at 100 °C in the dry state for 3 days. The dry residue was dissolved in D_2O and transferred to an NMR tube to record the ^{1}H and ^{13}P NMR spectra of the crude reaction. To obtain pure samples of 2,2'-anhydro-2-thiouridine-3'-phosphate **15b** and 2,2'-anhydro-2-thiouridine-5'-phosphate **15c** for NMR characterization, the reaction was repeated and the crude was applied to a preparative reverse phase HPLC. Acetonitrile in 25 mM triethylammonium bicarbonate buffer was used as the eluent. 0.5 mg of pure 2,2'-anhydro-2-thiouridine-3'-phosphate **15b** and 0.3 mg of pure 2,2'-anhydro-2-thiouridine-5'-phosphate **15c** were obtained as triethylammonium salt after purification.

2,2'-anhydro-2-thiouridine-3'-phosphate **15b**: ^{1}H NMR (400 MHz, D_2O): δ 7.89 (1H, d, J = 7.6 Hz, H6), 6.52 (1H, d, J = 7.3 Hz, H1'), 6.18 (1H, d, J = 7.6 Hz, H5), 4.70 (overlapped with H_2O, H3'), 4.64 (1H, dd, J_1 = 7.6 Hz, J_2 = 2.2 Hz, H2'), 4.37 (1H, m, H4'), 3.70 (1H, dd, J_1 = 12.7 Hz, J_2 = 3.7 Hz, H5'), 3.60 (1H, dd, J_1 = 12.7 Hz, J_2 = 6.2 Hz, H5"); ^{13}C NMR (101 MHz, D_2O = 1:9): δ 173.0 (C4), 168.3 (C2), 141.1 (C6), 108.9 (C5), 96.8 (C1'), 88.1 (C4', d, J_{P-C} = 6.6 Hz), 80.8 (C3', d, J_{P-C} = 4.8 Hz), 61.0 (C5'), 52.1 (C2', d, J_{P-C} = 3.0 Hz); ^{31}P NMR (162 MHz, D_2O = 1:9): 1.64 (d, J_{H-P} = 7.8 Hz); ESI-LCMS (neg. m/z): 320.9 $[M-1]^-$.

2,2'-anhydro-2-thiouridine-5'-phosphate **15c**: ^{1}H NMR (400 MHz, D_2O): δ 7.90 (1H, d, J = 7.6 Hz, H6), 6.44 (1H, d, J = 7.0 Hz, H1'), 6.20 (1H, d, J = 7.6 Hz, H5), 4.55 (t, J = 4.4 Hz, H3'), 4.42 (1H, dd, J_1 = 7.0 Hz, J_2 = 3.8 Hz, H2'), 4.30 (1H, m, H4'), 3.80 (2H, m, H5'); ^{13}C NMR (101 MHz, D_2O = 1:9): δ 173.0 (C4), 167.5 (C2), 141.1 (C6), 109.1 (C5), 96.2 (C1'), 86.6 (C4', d, J_{P-C} = 7.5 Hz), 78.0 (C3'), 63.5 (C5', d, J_{P-C} = 4.2 Hz), 52.0 (C2'); ^{31}P NMR (162 MHz, D_2O = 1:9): 3.83 (t, J_{H-P} = 6.7 Hz); ESI-LCMS (neg. m/z): 320.9 $[M-1]^-$.

Phosphorylation of 2-thiouridine 8 with DAP.

26 mg of 2-thiouridine **8** (0.1 mmol), 59 mg of DAP (0.5 mmol), 34 mg of imidazole (0.5 mmol) and couple drop of water were mixed in an Eppendorf tube. The moist-paste was kept at room temperature for 1 month. The reaction progress was followed by dissolving an aliquot of the crude-paste in D_2O, adjusting the pH to 7.0 by 4 M HCl and recording the ^{1}H, ^{13}C and ^{31}P NMR spectra. 59 mg of DAP was added when fully consumed.

Phosphorylation of 2-thiocyidine 5 in urea melt.

5 mg of 2-thiocytidine **5** (0.02 mmol), 11.5 mg of urea (0.20 mmol), 75 mg of NH_4HCO_3 (0.10 mmol), 10.5 mg of NH_4Cl (0.20 mmol) and 6 mg of Na_2HPO_4 (0.04 mmol) were mixed in 2 ml of H_2O in a round bottom flask. The mixture was dried down under vacuum and heated at 100 °C in the dry state for 3 days. The dry residue was dissolved in D_2O and transferred to an NMR tube to record the ^{1}H and ^{13}P NMR spectra of the crude reaction.

Phosphorylation of 2-thiocyidine 5 with DAP.

26 mg of 2-thiocytidine **5** (0.1 mmol), 24 mg of DAP (0.2 mmol), 8.3 mg of 2-aminoimidazole (0.1 mmol) and 1 mL of water were mixed in an Eppendorf tube and the pH was adjusted at 4.0 by addition of 4 M HCl. The solution was then evaporated at 60°C for 2 hours to form a moist-paste. The reaction was followed by dissolving an aliquot of the crude-paste in D_2O, adjusting the pH to 7 by 4 M HCl and recording the ^{1}H, ^{13}C and ^{31}P NMR spectra.

Dephosphorylation of nucleotide mono-phosphates.

The nucleotides were dissolved in 1 ml of H_2O (containing 10% of D_2O), after which 6 mg of NaCl and 2 mg of $MgCl_2$ were added. The solution was adjusted to pH 8.0 with 0.10 M NaOH. 7 ul of Alkaline phosphatase (20 units/µl, from Roche Diagnostics) was added, and the solution was incubated at 37°C for 14 hours. The solution was transferred to an NMR tube to record the ^{1}H and ^{13}P NMR spectra.

Synthesis of 2,2'-anhydro-2-thiouridine 15a.

200 mg of 2-thiouridine **8** (0.77 mmol), 190 mg of diphenyl carbonate (0.88 mmol) and 4 mg of $NaHCO_3$ (0.05 mmol) were dissolved in 2ml of anhydrous DMF. The mixture was heated at 150°C for 40 min. The solvent was removed under vacuum. The residue was dissolved in H_2O, then applied to reverse phase HPLC to obtain 130 mg (0.54 mmol, 70% yield) of 2,2'-anhydro-2-thiouridine **15a**. ^{1}H NMR (400 MHz, D_2O): δ 7.87 (1H, d, J = 7.6 Hz, H6), 6.49 (1H, d, J = 7.1 Hz, H1'), 6.18 (1H, d, J = 7.6 Hz, H5), 4.48 (1H, t, J = 3.3 Hz, H3'), 4.43 (1H, dd, J_1 = 7.1 Hz, J_2 = 3.1 Hz, H2'), 4.25 (1H, m, H4'), 3.67 (1H, dd, J_1 = 12.5 Hz, J_2 = 4.1 Hz, H5'), 3.55 (1H, dd, J_1 = 12.5 Hz, J_2 = 6.3 Hz, H5''); ^{13}C NMR (101 MHz, D_2O = 1:9): δ 172.8 (C4), 167.5 (C2), 141.0 (C6), 109.0 (C5), 96.8 (C1'), 88.5 (C4'),

78.4 (C3'), 61.1 (C5'), 52.5 (C2'). ESI-HRMS (pos. m/z) $[M+Na]^+$ calcd for $C_9H_{10}N_2NaO_4S$ 265.0259; found 265.0250.

Synthesis of 2,2'-anhydro-2-thiocytidine 21a.

2-thiocytidine **5** (100 mg, 0.39 mmol) was co-evaporated with dry DMF (5 ml) twice, then suspended in 2 ml of anhydrous acetonitrile together with 225 ul (1.54 mmol) of 2-acetoxyisobutyryl chloride. After 3 h's stirring at room temperature, the solution became clear, then the solvent was removed under vacuum. The residue was treated with 3 ml of 0.18 N methanolic hydrogen chloride at room temperature for 3 days. The solvent was removed, then the residue was partitioned between H_2O (5 ml) and ether (5 ml). The aqueous layer was concentrated, then applied to reverse phase HPLC to obtain 65 mg (0.27 mmol, 70% yield) of 2,2'-anhydro-2-thiocytidine **21a**. ^{1}H NMR (400 MHz, D_2O): δ 8.06 (1H, d, J = 7.5 Hz, H6), 6.60 (2H, m, H1' and H5), 4.51 (2H, m, H2' and H3'), 4.30 (1H, m, H4'), 3.67 (1H, dd, J_1 = 12.7 Hz, J_2 = 3.6 Hz, H5'), 3.57 (1H, dd, J_1 = 12.7 Hz, J_2 = 5,6 Hz, H5"); ^{13}C NMR (101 MHz, D_2O = 1:9): δ 168.2 (C2), 164.4 (C4), 142.0 (C6), 103.3 (C5), 97.5 (C1'), 88.7 (C4'), 78.4 (C3'), 60.7 (C5'), 52.7 (C2'). ESI-LCMS (pos. m/z): 242.1 $[M+1]^+$.

Photoreduction of 2,2'-anhydro-2-thiouridine 15a.

1.8 mg of 2,2'-anhydro-2-thiouridine **15a** (0.007 mmol) was mixed with 8.0 mg (0.086 mmol) of $NaSH.xH_2O$ (containing 60% NaSH) in 0.5 mL of H_2O (containing 10% of D_2O). The pH of mixture was adjusted to 7 with 1M HCl. The mixture was transferred to a Suprasil quartz NMR tube (sample dimensions 40 mm high, 4.2 mm diameter), which was irradiated at 254 nm in the *Rayonet* photochemical chamber reactor RPR-200 for 3 h. A sample removed from the photochemical reactor after 3 hours had an internal temperature of 35 °C. The sample was taken out from the reactor at intervals to record ^{1}H NMR spectra. At the end of the reaction, 27 µl of 50 mM pentaerythritol solution in H_2O was added to the mixture to enable quantification of the reaction products in ^{1}H NMR spectra. The same conditions were also applied to **15b** and **15c**. The same photoreduction condition was also applied to intermediates **1**, **3**, **6**, **7**, **8**, **9** to test their stabilities. (Supplementary Fig. 68-73)

Hydrolysis and Half-Life Determinations of 2'-deoxy-2-thiouridine 11.

0.45 mL of an aqueous solution containing buffer (300 mM) and nucleoside (18.2 mM) was added to 0.05 mL of a standard solution of pentaerythritol (2.76 mM in D_2O) in an NMR tube. The two buffers employed were sodium phosphate (pH 7.0) and sodium acetate (pH 4.1), and a third set of reactions set up using unbuffered water. An initial ^{1}H NMR spectrum (delay time 6 s) was recorded, and then the tube was incubated in an oil bath at 60 °C and periodically removed and resubmitted to NMR analysis. The degradation of nucleoside and formation of deoxyribose and free nucelobase was measured by integration against the internal standard, pentaerythritol. The identities of free nucleobase and pentaerythitol were confirmed at the completion of the experiment by spiking the NMR tube with authentic standards. Plotting of the natural logarithms of nucleoside concentration against time yielded

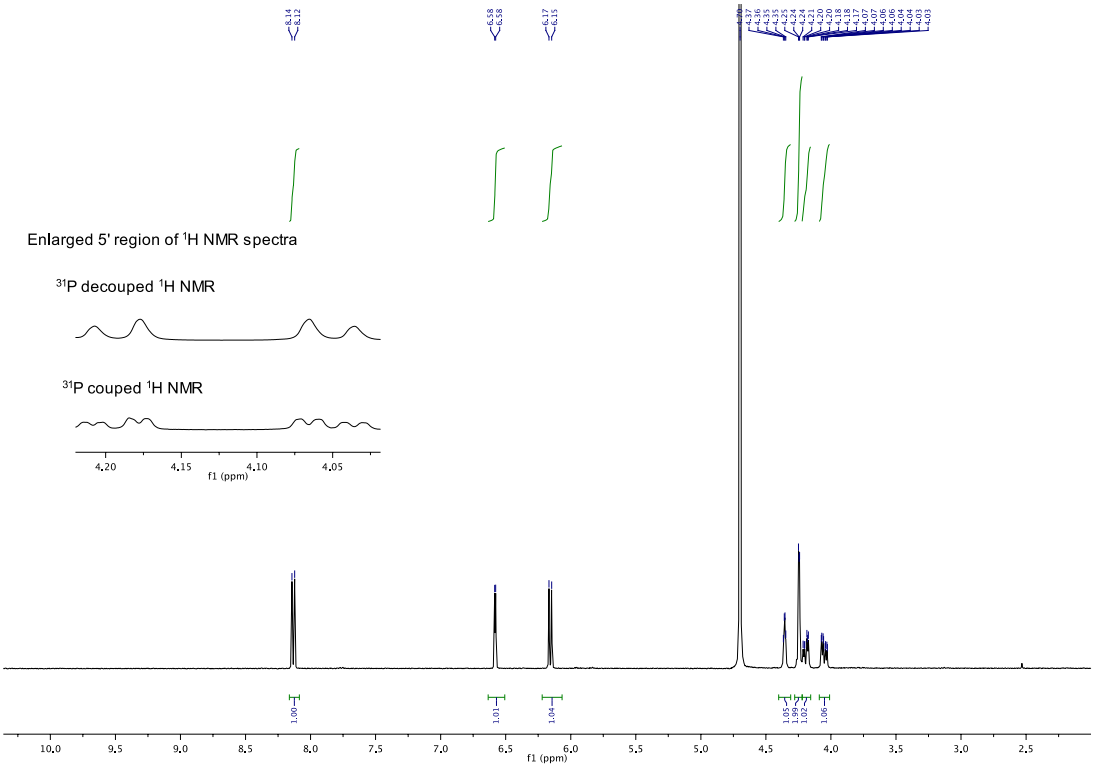
first order half-lives at pH 7.0 and 4.1 for 2'-deoxy-2-thiouridine **11** of 31 and 32 hours, respectively. In unbuffered solution, the half-life was calculated at 28 hours.
Yields: Unbuffered: 2'-Deoxyribose: 91%, thiouracil: 97%; at pH 7.0: 2'-Deoxyribose 84%, thiouracil: 95%; at pH 4.1: 2'-Deoxyribose 84%, thiouracil: 87%

Solid-state glycosylation of adenine 33 with 2'-deoxy-2-thiouridine 11 to form β-deoxyadenosine β-13.

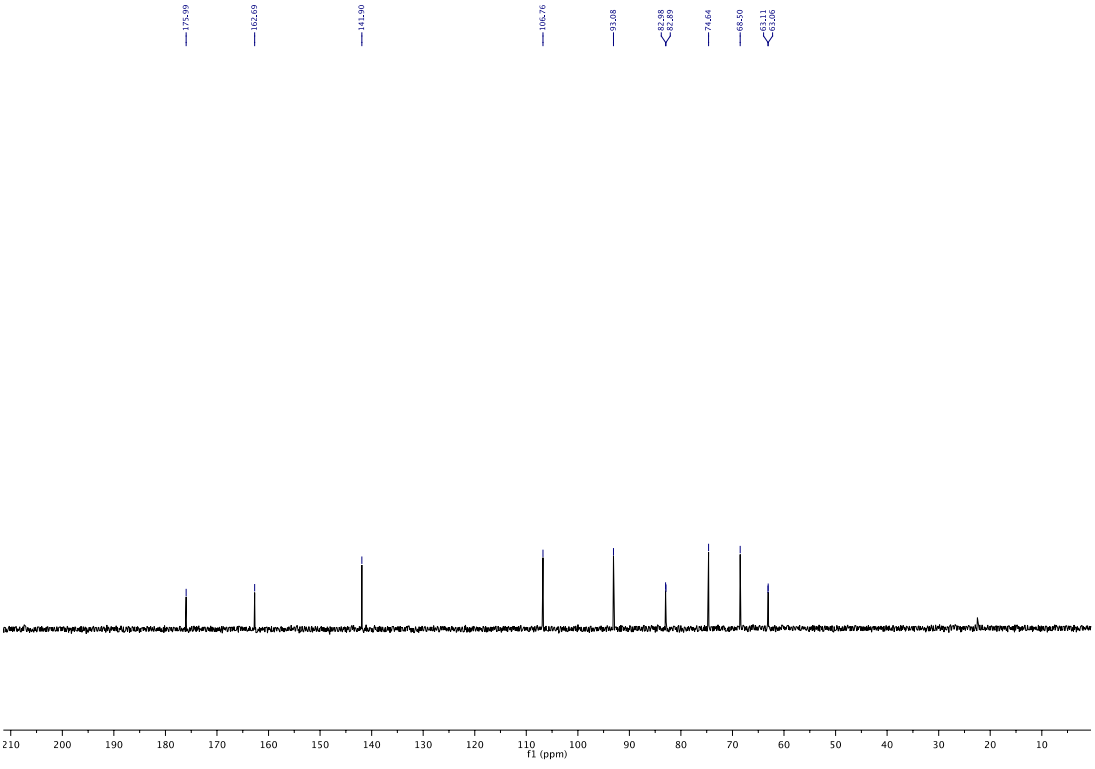
0.10 mL of a standard solution (40 mM in MeOH) of 2'-deoxy-2-thiouridine **11** (0.98 mg, 4.0 umol, 1.0 equiv.) was evaporated in a 5mL round-bottom flask, leaving **11** as a white solid distributed on the flask. Adenine **33** (2.7 mg, 20 umol, 5.0 equiv.), 0.40 M pH 7 phosphate buffer (20 uL, 2.0 equiv. of phosphate) and milliQ-filtered water (1.0 mL) were added and the suspension was sonicated until all the 2'-deoxy-2-thiouridine had been dissolved (the adenine remains insoluble). The pH at this stage was measured at 7.3. This mixture was then concentrated on a rotary evaporator at 45°C and approximately 5 mbar until the solvent had been evaporated, leaving a white deposit on the inside of the flask. The flask was then immersed in an oil bath at 100 °C. After 31 hours, the material was dissolved in 2mL of milliQ-filtered H_2O, and the pH was measured at 4.9. The solvent was evaporated and then the mixture was dissolved in D_2O, showing the presence of mainly 2-thiouracil **19**, and small quantities of both α- and β-deoxyadenosine **α / β-13**, with some leftover 2'-deoxy-2-thiouridine **11**. The identities of these products were confirmed by spiking experiments with authentic samples, but the yields could not be obtained by NMR due to overlapping peaks. Development of an analytical HPLC method (see below) gave conversions after 48 hours of 72%, 6%, 4% and 15% for **19**, **α-13**, **β-13**, and **11** respectively. Analytical HPLC was used to monitor the reaction, using a Thermofisher Ultimate 3000 UPLC and Waters Atlantis T3 C18 column (5 μm, 4.6 mm × 150 mm). UV absorption calibration curves for standards of 2'-deoxy-2-thiouridine **11**, thiouracil **19**, and β-2'-deoxyadenosine **β-13** were collected. α-2'-deoxyadenosine **α-13** was assumed to have a similar absorption curve to β-2'-deoxyadenosine **β-13**. The glycosylation experiment was repeated and periodically, the flask was removed from the oil bath, 2.00 mL of H_2O added, and an aliquot removed. The reaction was resumed by evaporating the water and heating again. This aliquot was loaded into the HPLC autosampler for precision injection (5.0 μL) and analysis. UV absorption for known peaks (verified by spiking) was recorded and calibration curves used to determine relative amounts. Recovery was calibrated to aliquots from time zero at which the yield of starting material 2'-deoxy-2-thiouridine **11** was set to 100%.

NMR, UV spectra, HPLC traces and UV absorption calibration curves et al.

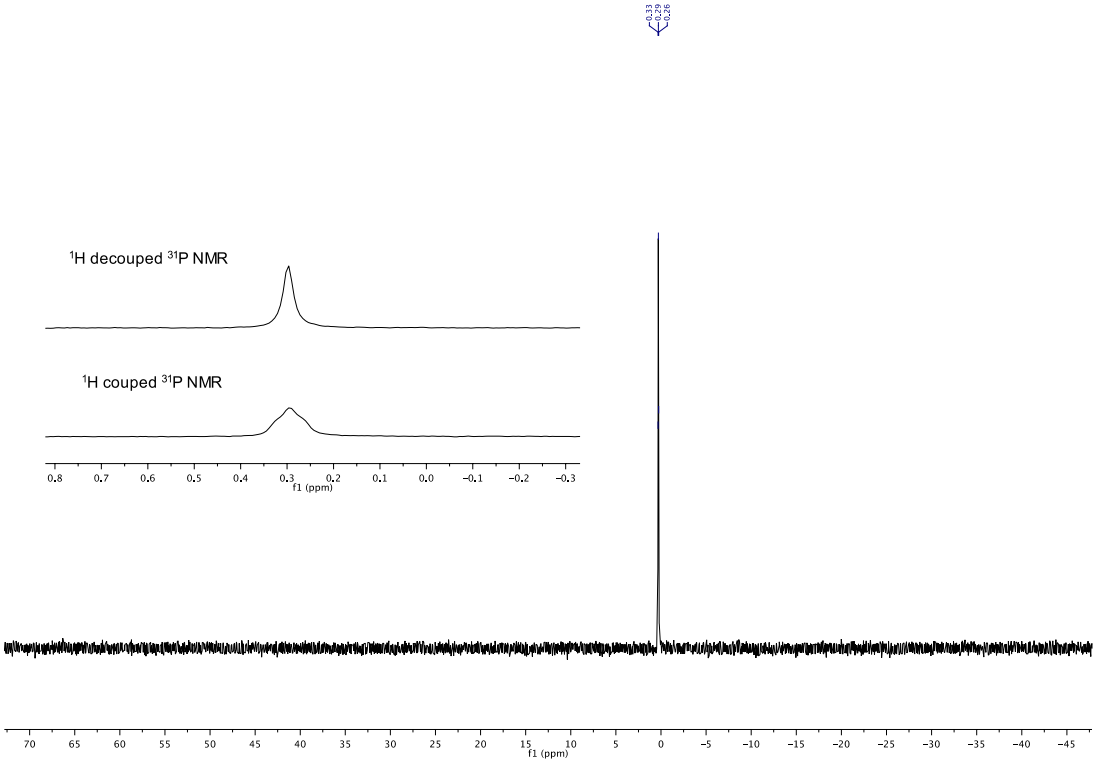
Supplementary Figure 1. ^{1}H NMR spectrum of 2-thiouridine-5'-phosphate 14 in D_2O.



Supplementary Figure 2. ^{13}C NMR spectrum of 2-thiouridine-5'-phosphate 14 in D_2O.

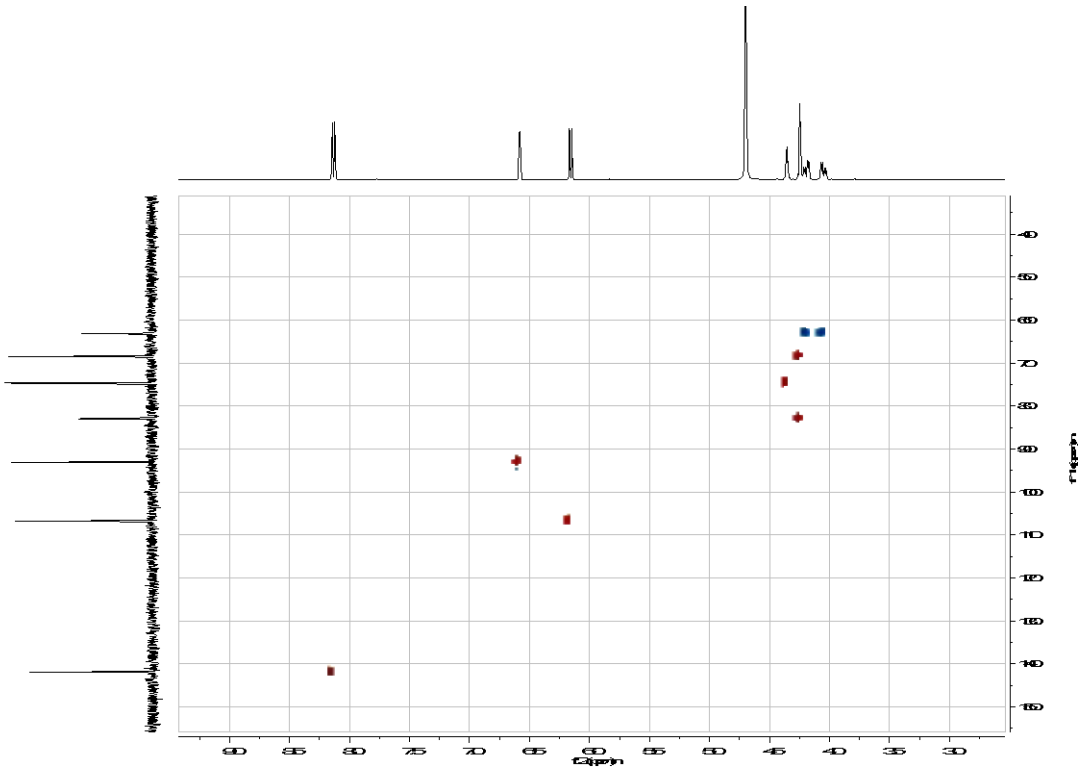


Supplementary Figure 3. ^{31}P NMR spectrum of 2-thiouridine-5'-phosphate 14 in D_2O.

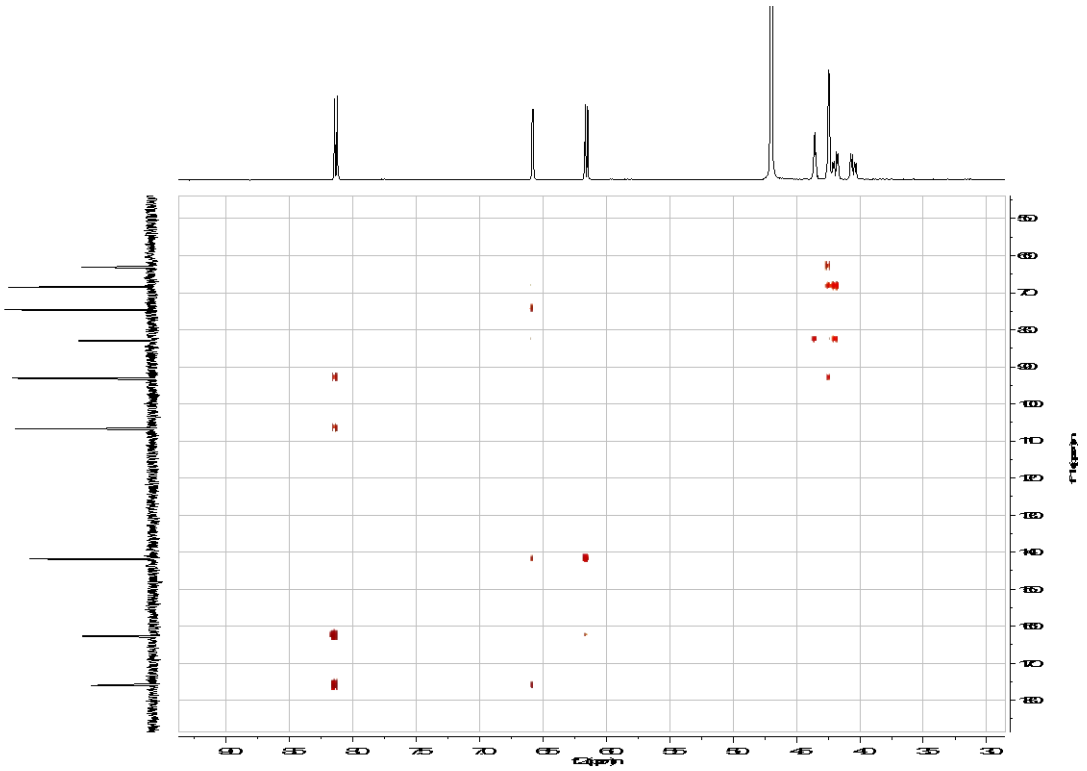


Supplementary Figure 4. COSY NMR spectrum of 2-thiouridine-5'-phosphate 14 in D_2O.

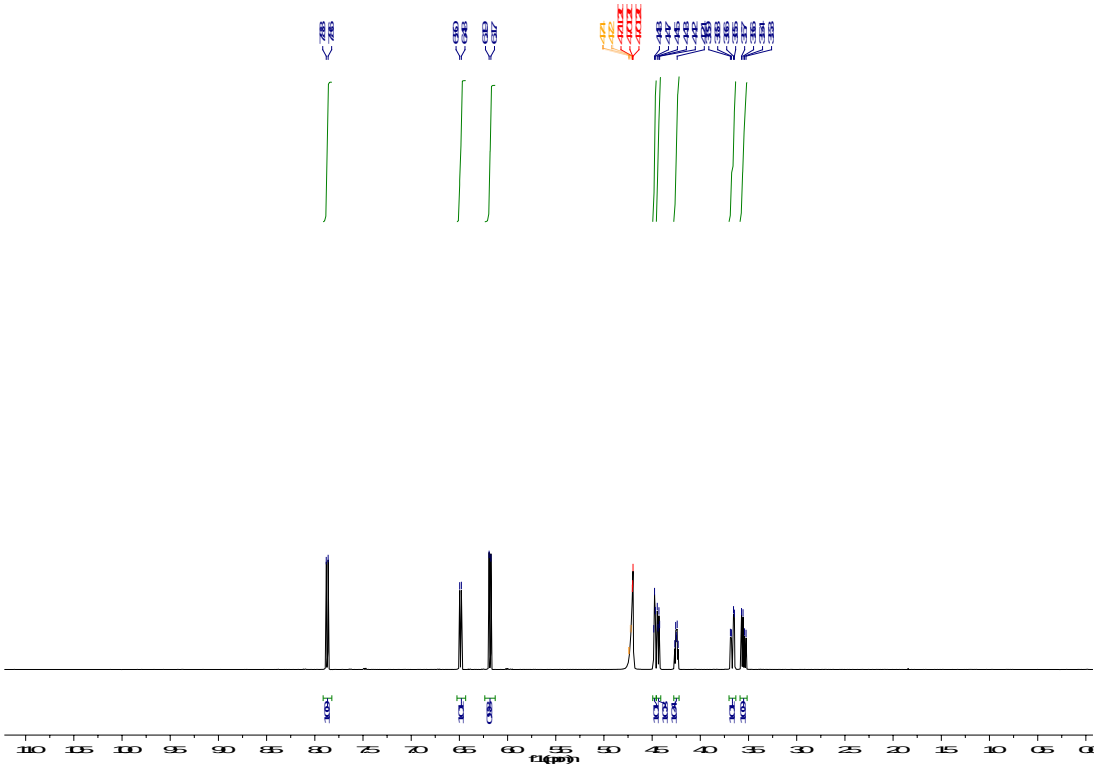
Supplementary Figure 5. HSQC NMR spectrum of 2-thiouridine-5'-phosphate 14 in D_2O.



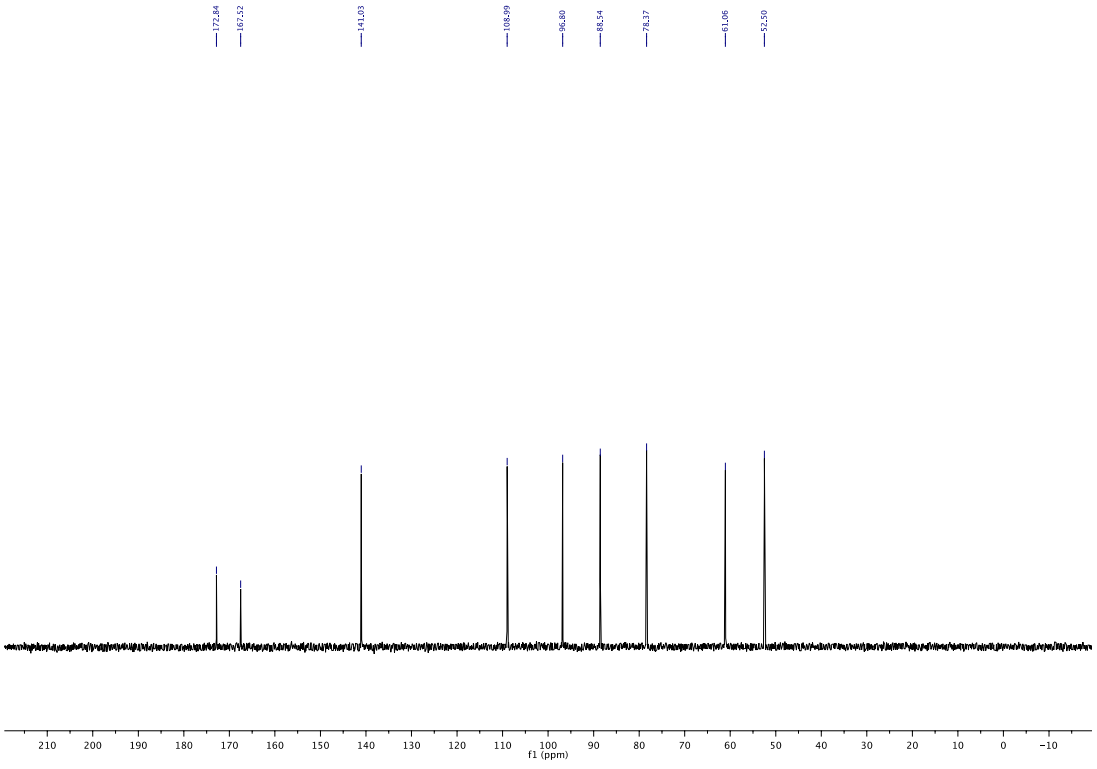
Supplementary Figure 6. HMBC NMR spectrum of 2-thiouridine-5'-phosphate 14 in D_2O.



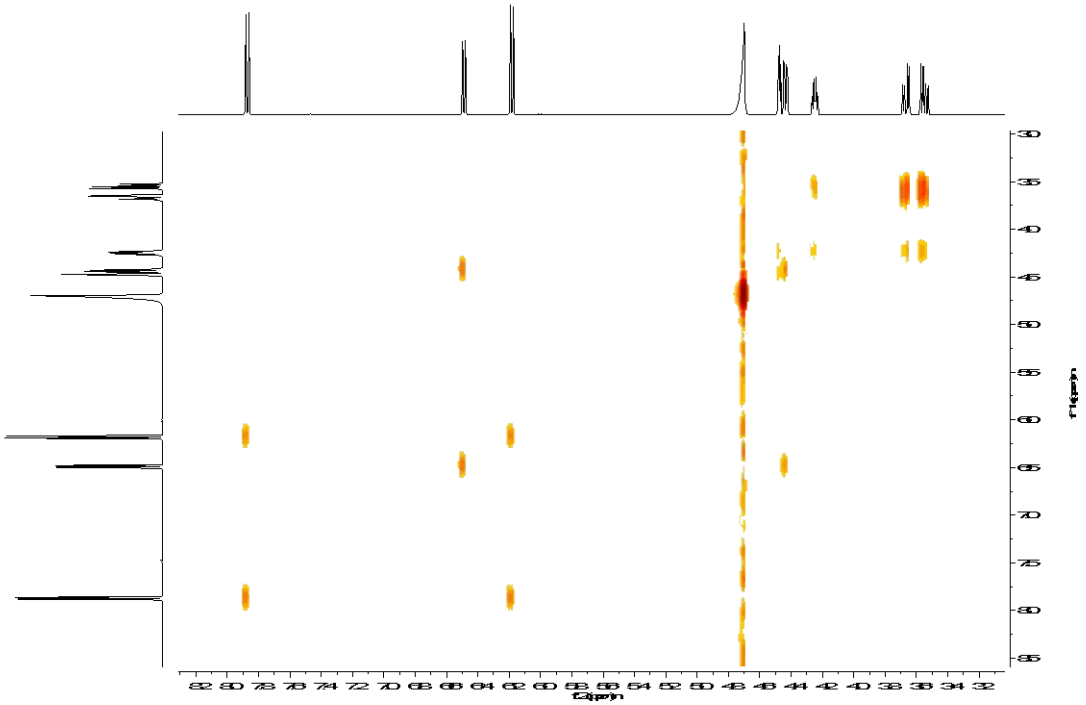
Supplementary Figure 7. ^{1}H NMR spectrum of 2,2'-anhydro-2-thiouridine 15a in D_2O.



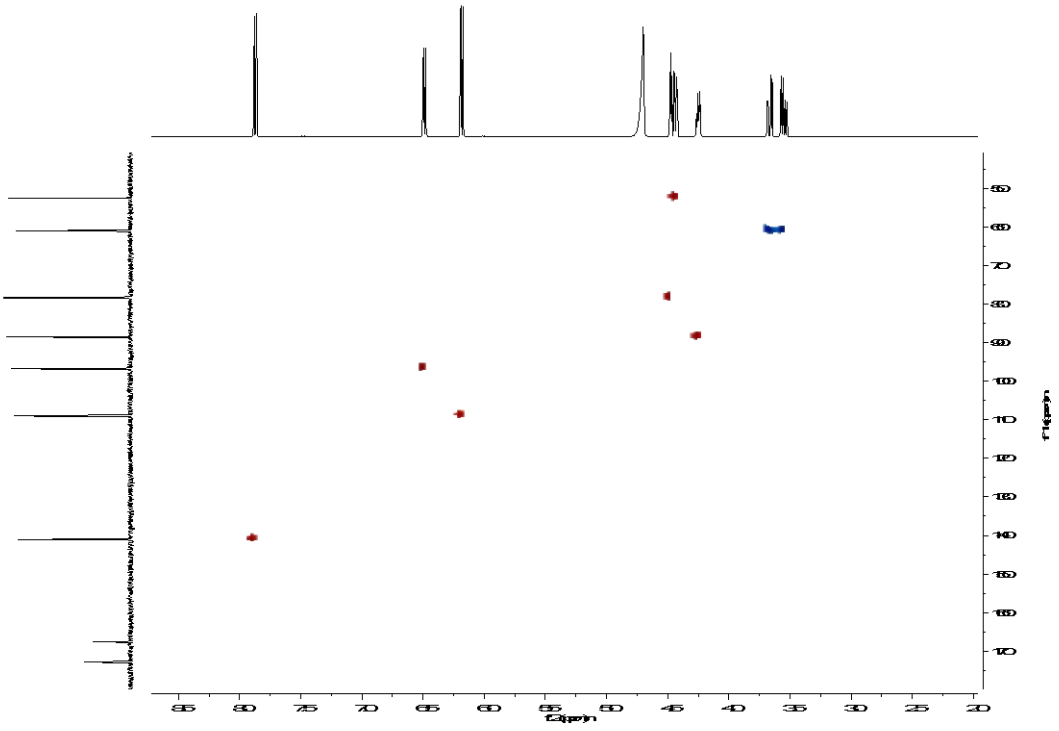
Supplementary Figure 8. ^{13}C NMR spectrum of 2,2'-anhydro-2-thiouridine 15a in D_2O.



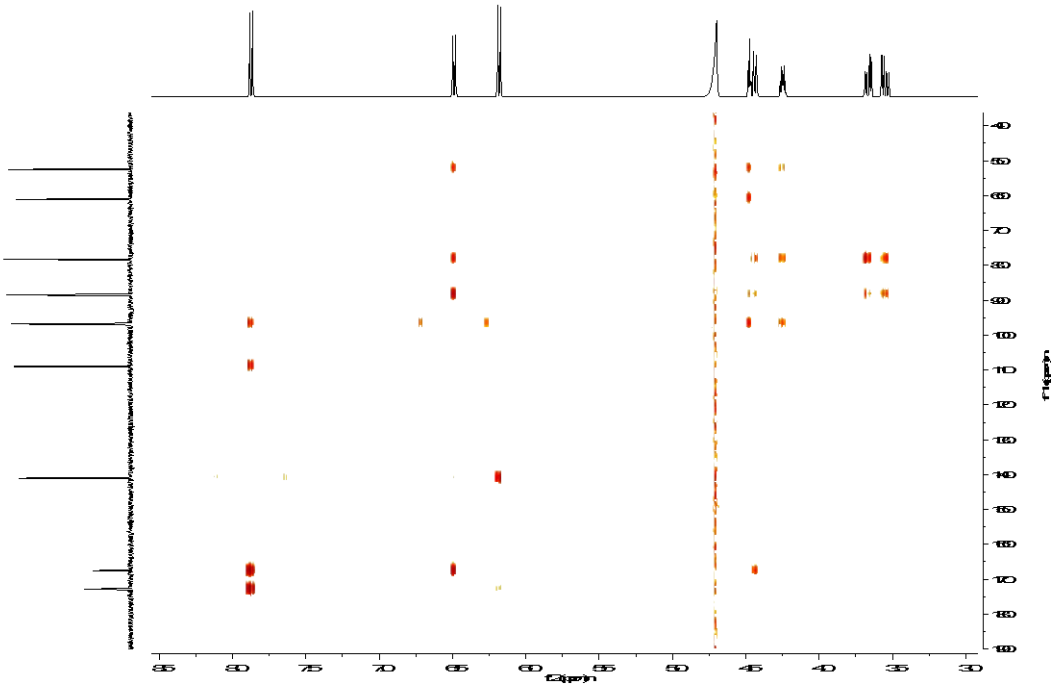
Supplementary Figure 9. COSY NMR spectrum of 2,2'-anhydro-2-thiouridine 15a in D_2O.



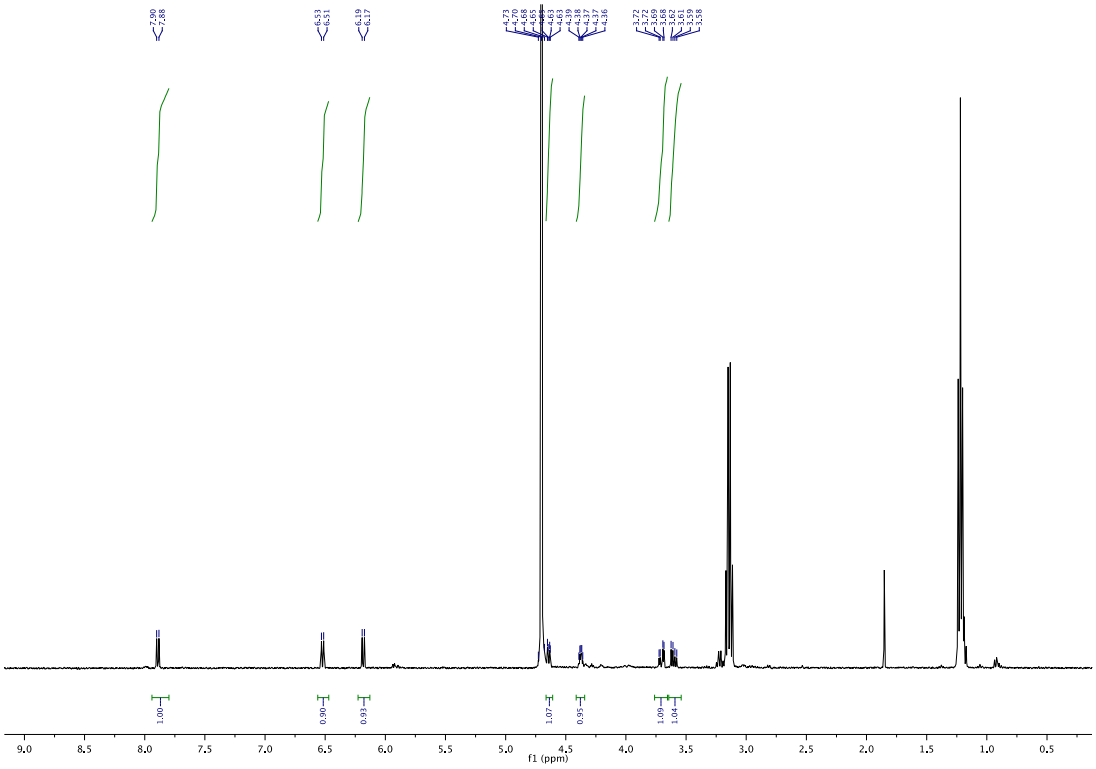
Supplementary Figure 10. HSQC NMR spectrum of 2,2'-anhydro-2-thiouridine 15a in D_2O.



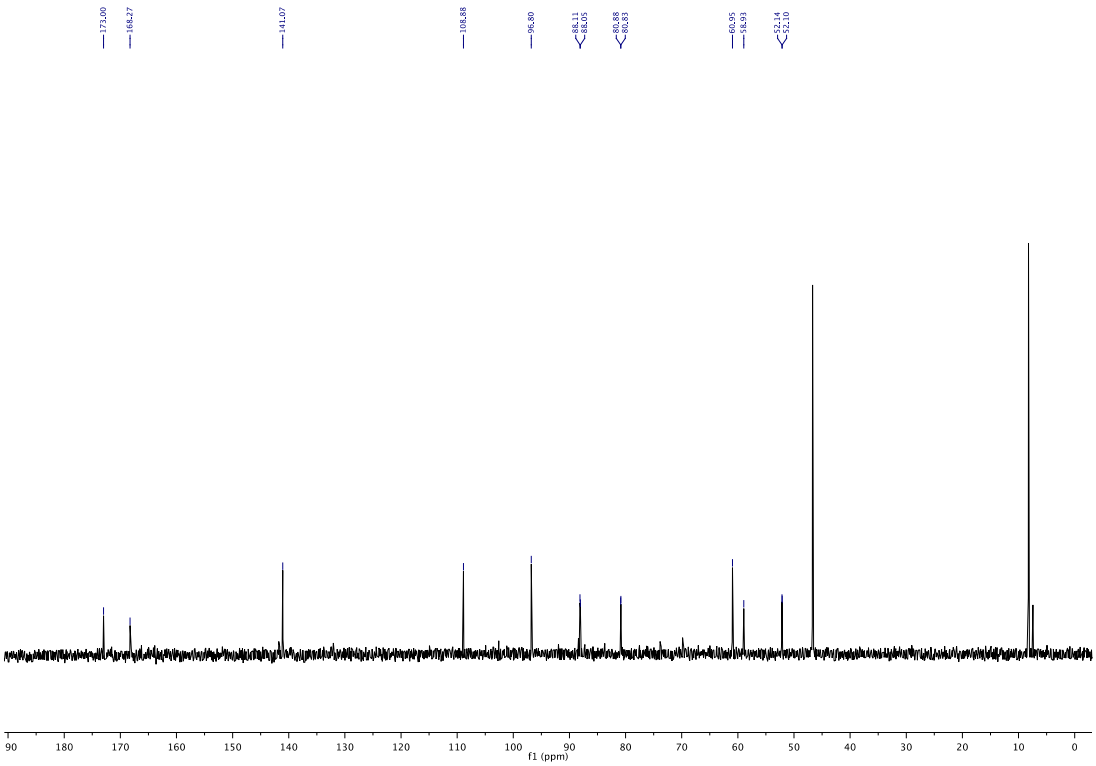
Supplementary Figure 11. HMBC NMR spectrum of 2,2'-anhydro-2-thiouridine 15a in D_2O.



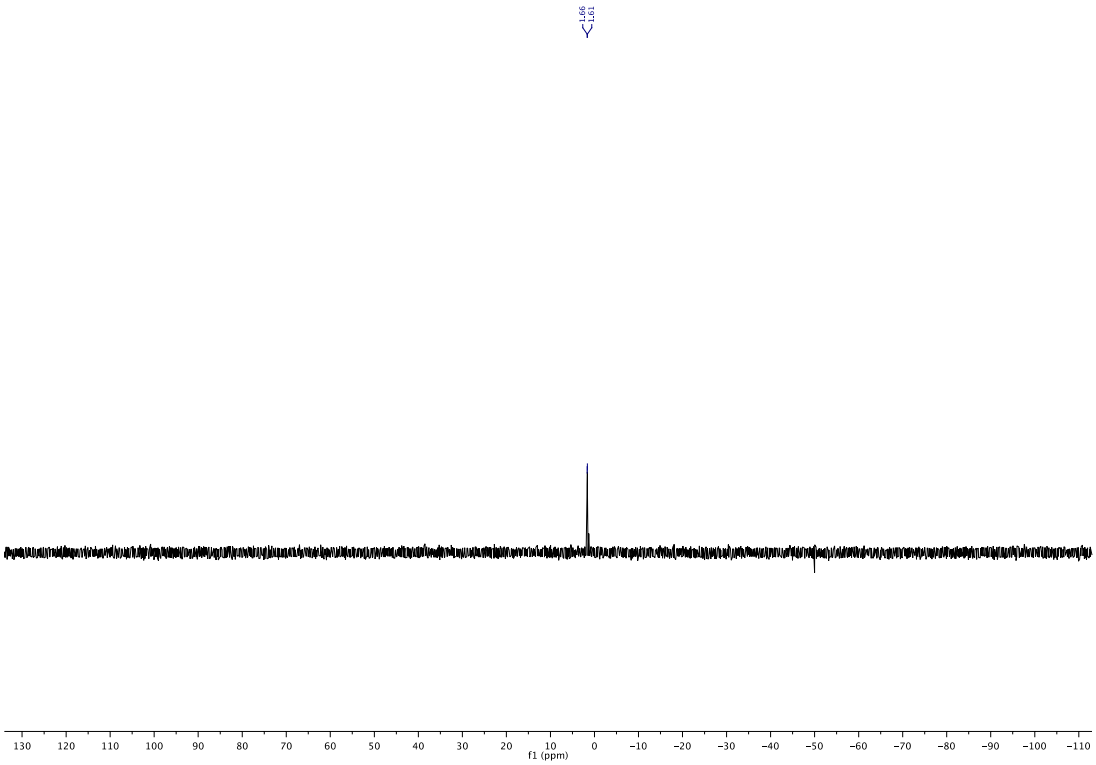
Supplementary Figure 12. ^1H NMR spectrum of 2,2'-anhydro-2-thiouridine-3'-phosphate 15b (as triethylammonium salt) in D_2O.



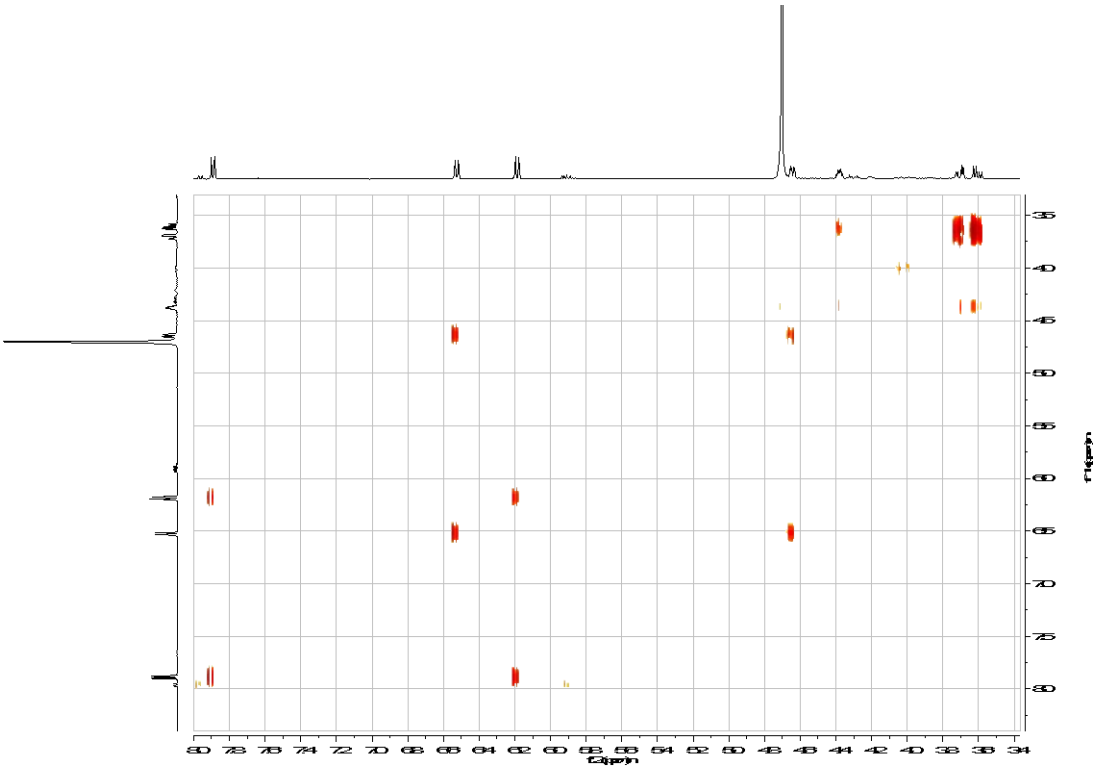
Supplementary Figure 13. ^{13}C NMR spectrum of 2,2'-anhydro-2-thiouridine-3'-phosphate 15b (as triethylammonium salt) in D_2O.



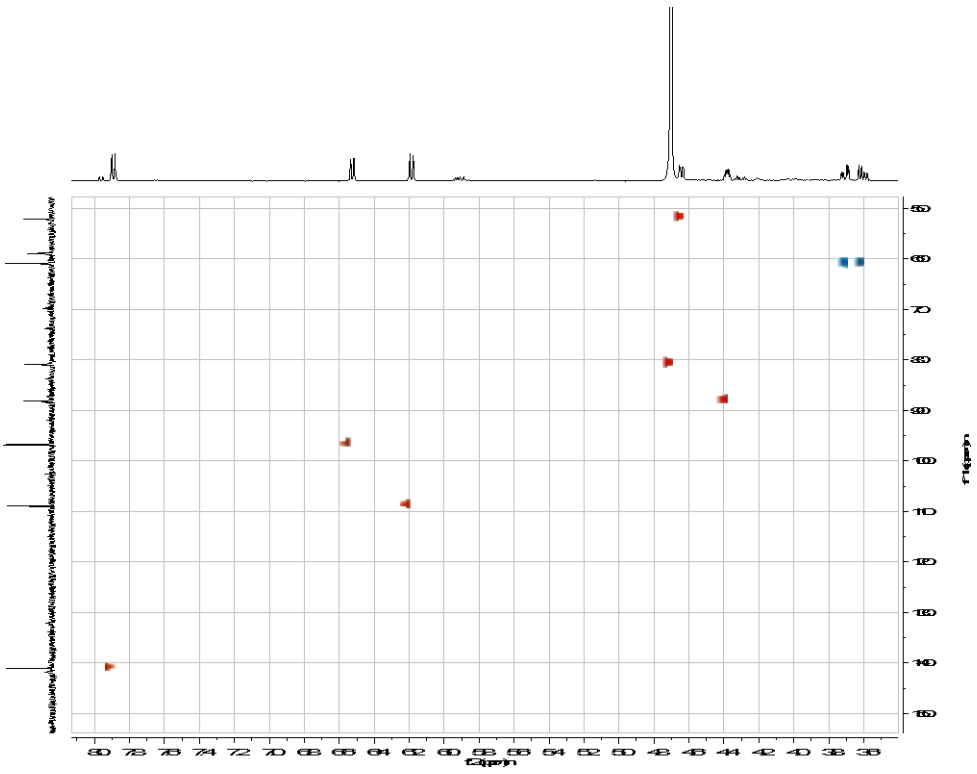
Supplementary Figure 14. ^{31}P NMR spectrum of 2,2'-anhydro-2-thiouridine-3'-phosphate 15b (as triethylammonium salt) in D_2O.



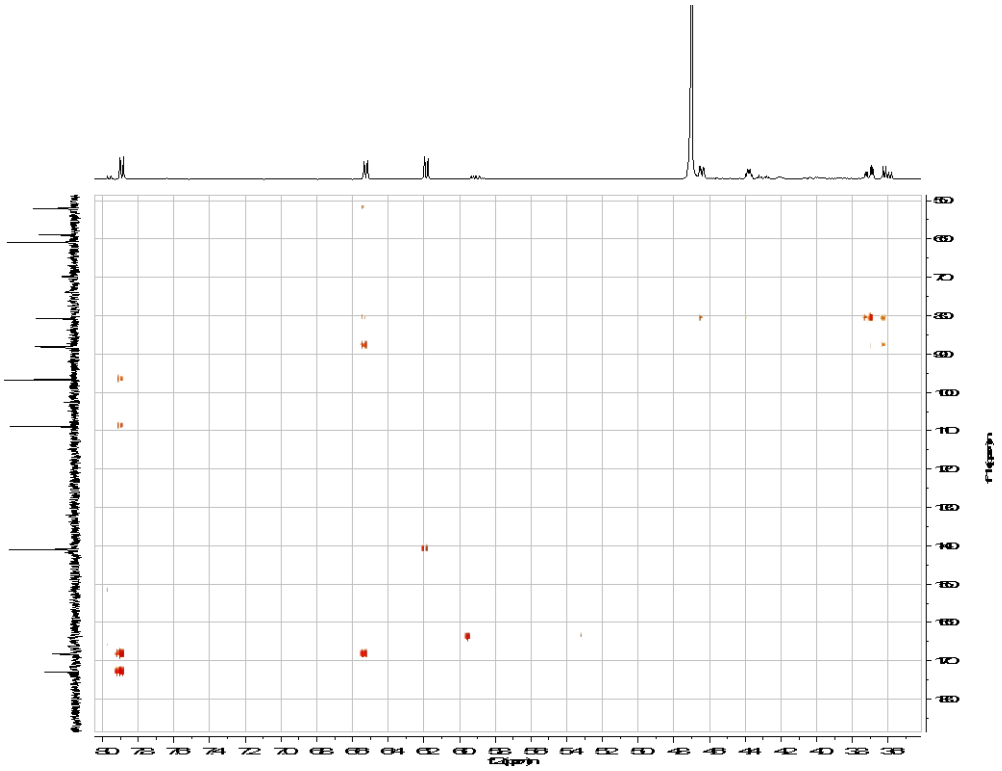
Supplementary Figure 15. COSY NMR spectrum of 2,2'-anhydro-2-thiouridine-3'-phosphate 15b (as triethylammonium salt) in D_2O.



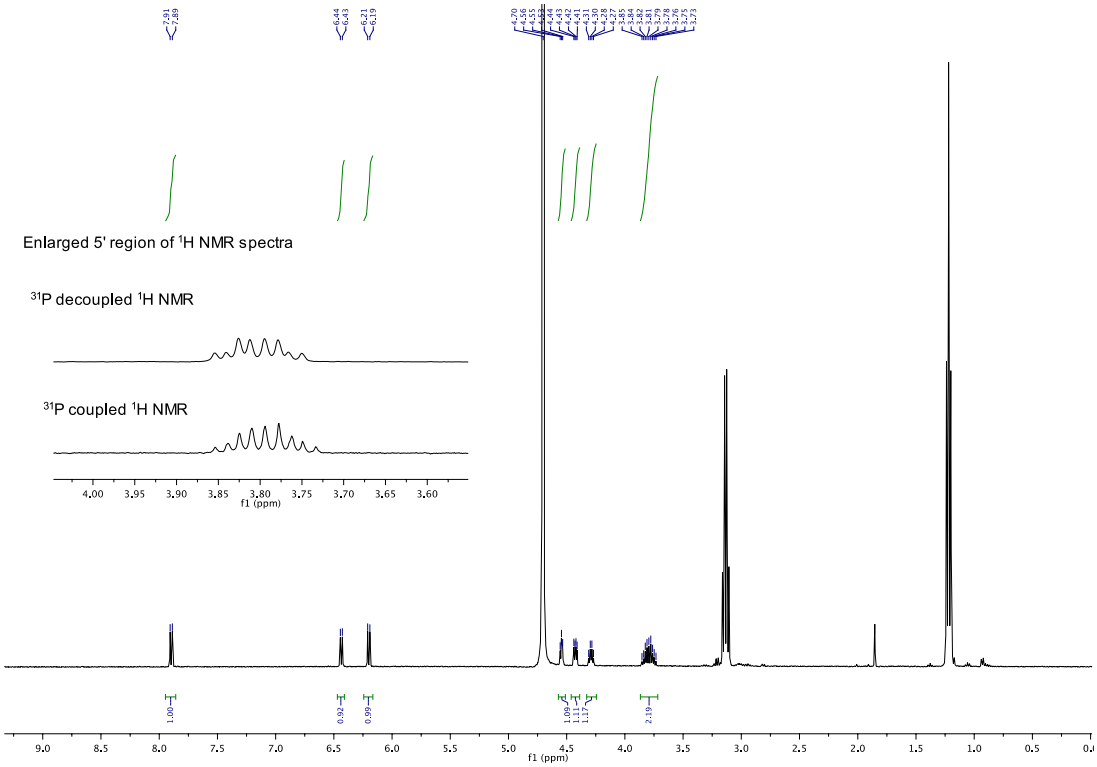
Supplementary Figure 16. HSQC NMR spectrum of 2,2'-anhydro-2-thiouridine-3'-phosphate 15b (as triethylammonium salt) in D_2O.



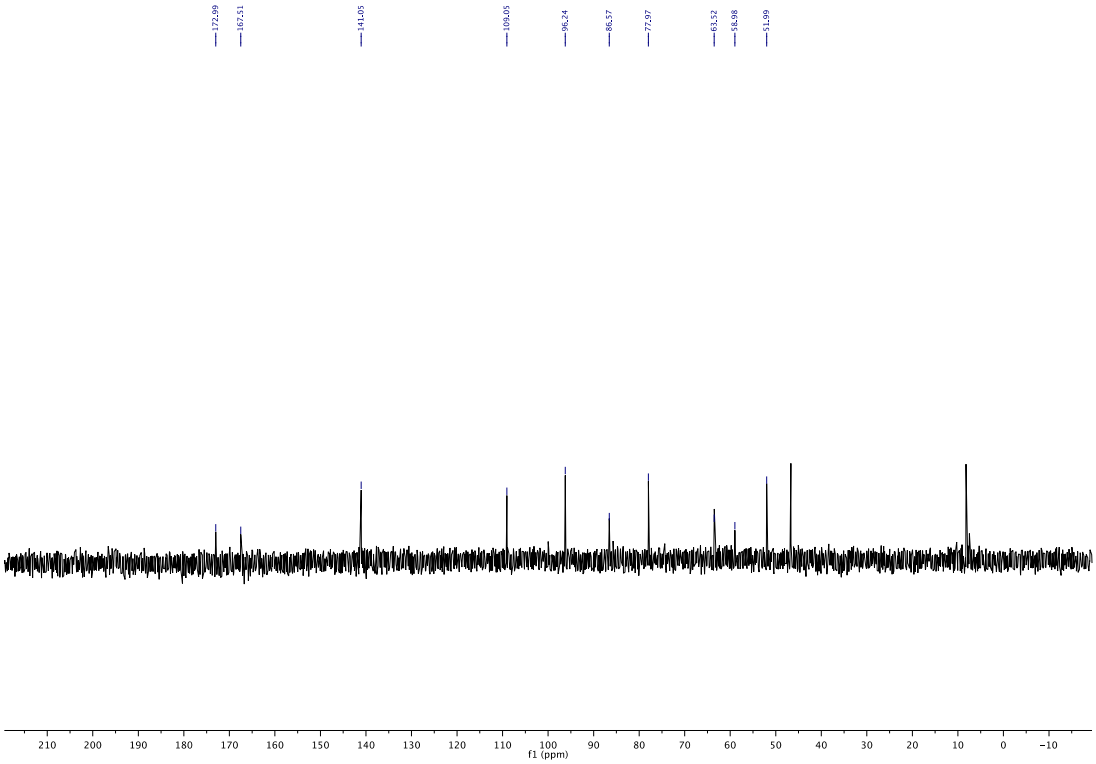
Supplementary Figure 17. HMBC NMR spectrum of 2,2'-anhydro-2-thiouridine-3'-phosphate 15b (as triethylammonium salt) in D_2O.



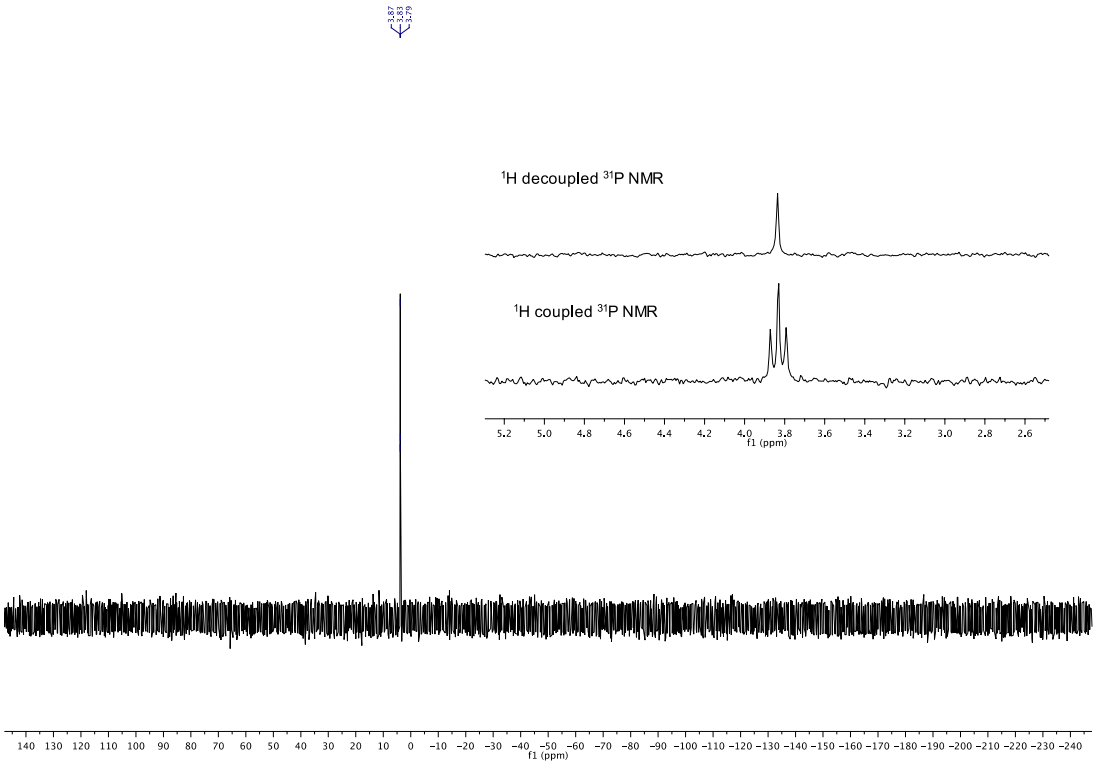
Supplementary Figure 18. ^1H NMR spectrum of 2,2'-anhydro-2-thiouridine-5'-phosphate 15c (as triethylammonium salt) in D_2O.



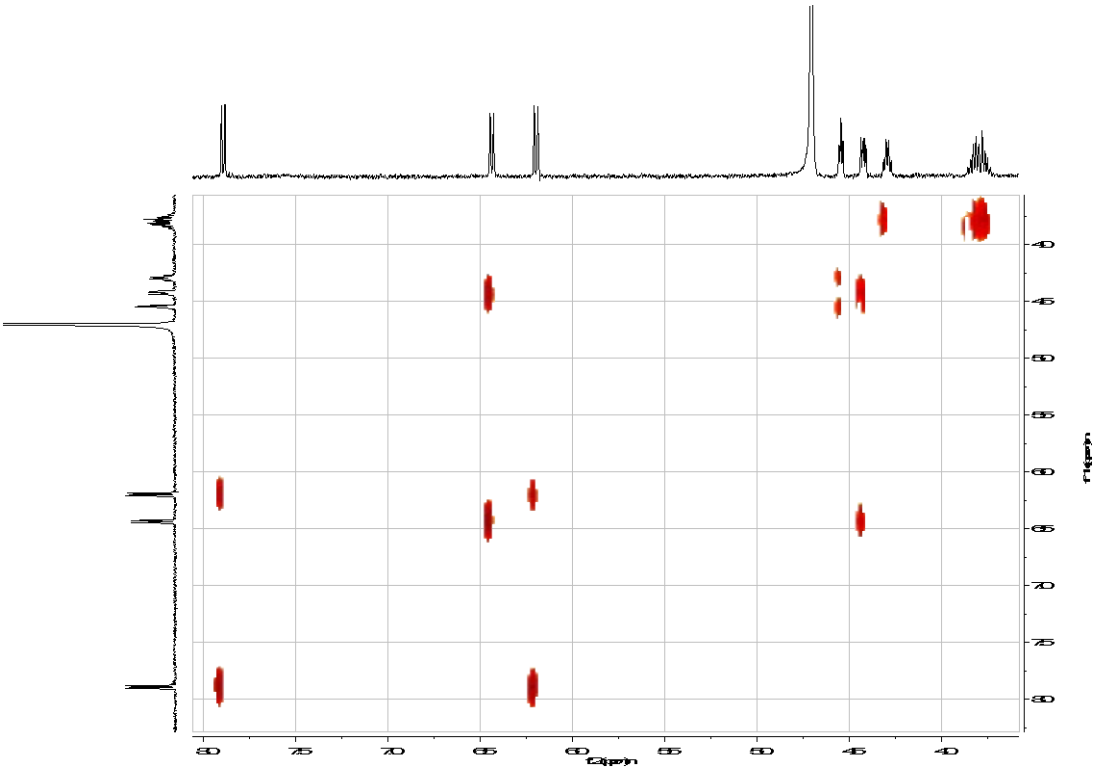
Supplementary Figure 19. ^{13}C NMR spectrum of 2,2'-anhydro-2-thiouridine-5'-phosphate 15c (as triethylammonium salt) in D_2O.



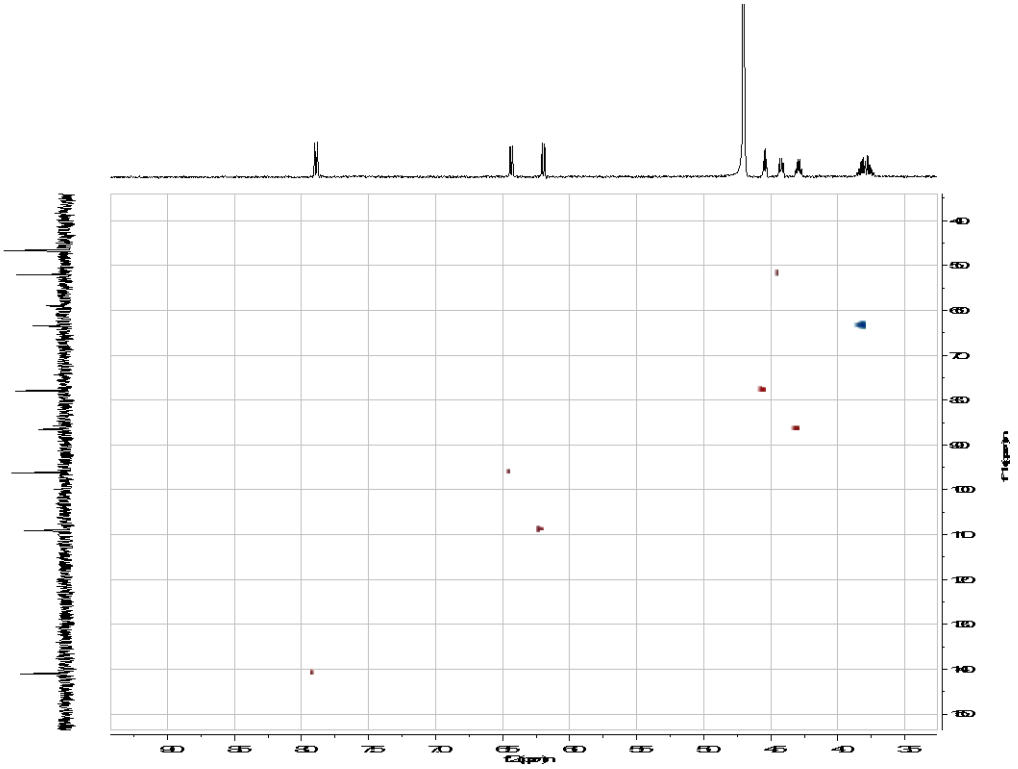
Supplementary Figure 20. ^{31}P NMR spectrum of 2,2'-anhydro-2-thiouridine-5'-phosphate 15c (as triethylammonium salt) in D_2O.



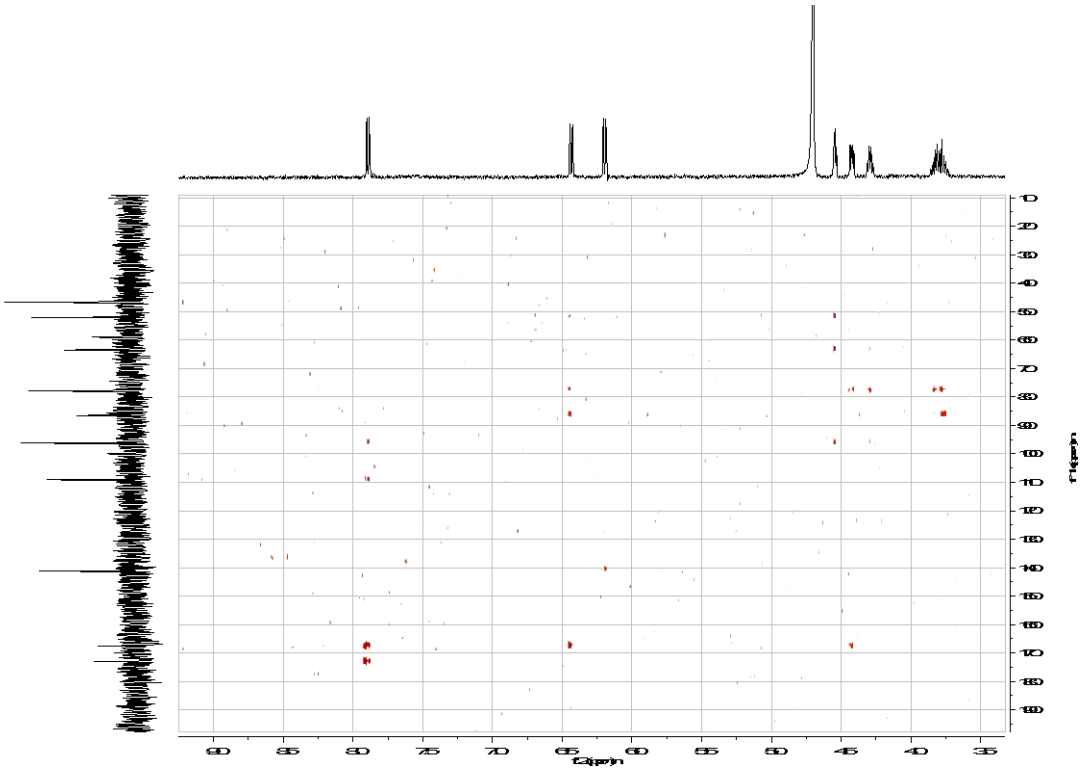
Supplementary Figure 21. COSY spectrum of 2,2'-anhydro-2-thiouridine-5'-phosphate 15c (as triethylammonium salt) in D_2O.



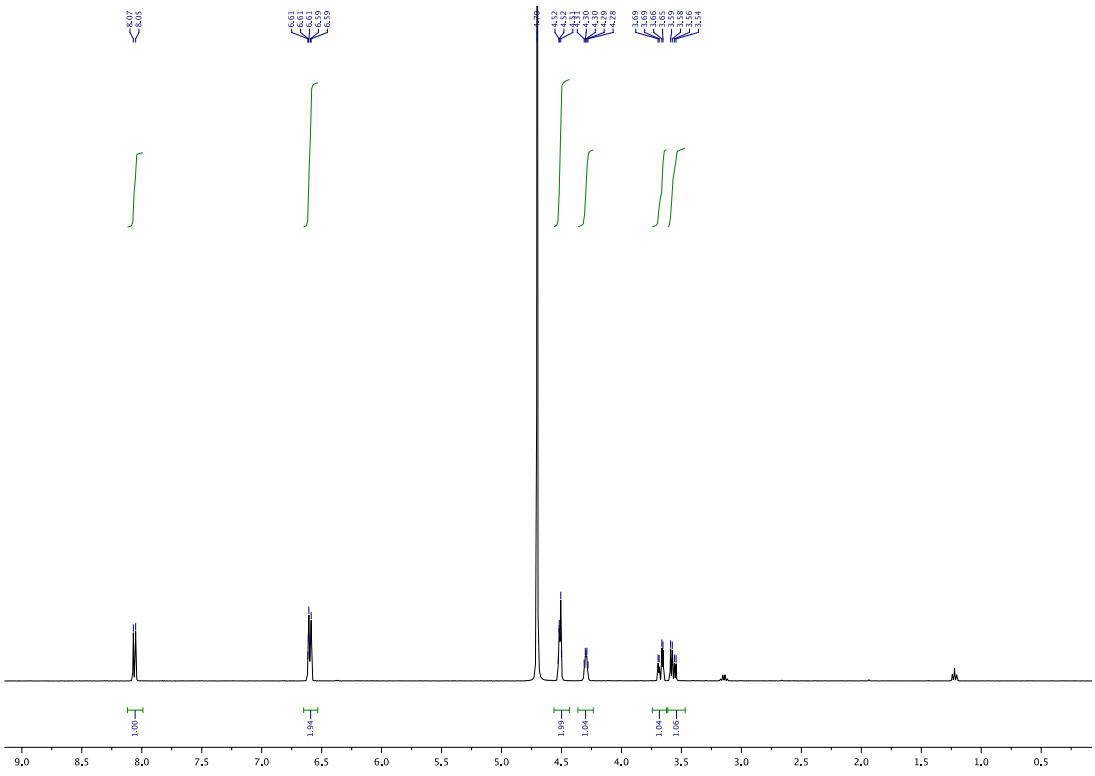
Supplementary Figure 22. HSQC spectrum of 2,2'-anhydro-2-thiouridine-5'-phosphate 15c (as triethylammonium salt) in D_2O.



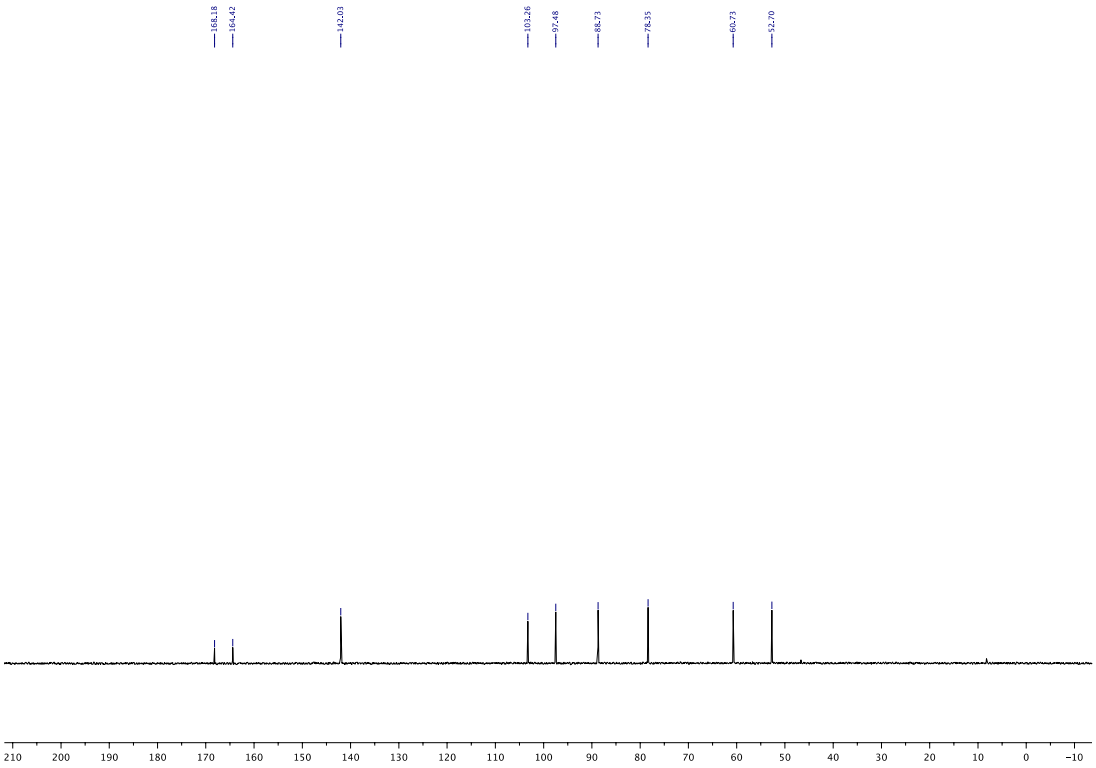
Supplementary Figure 23. HMBC spectrum of 2,2'-anhydro-2-thiouridine-5'-phosphate 15c (as triethylammonium salt) in D_2O.



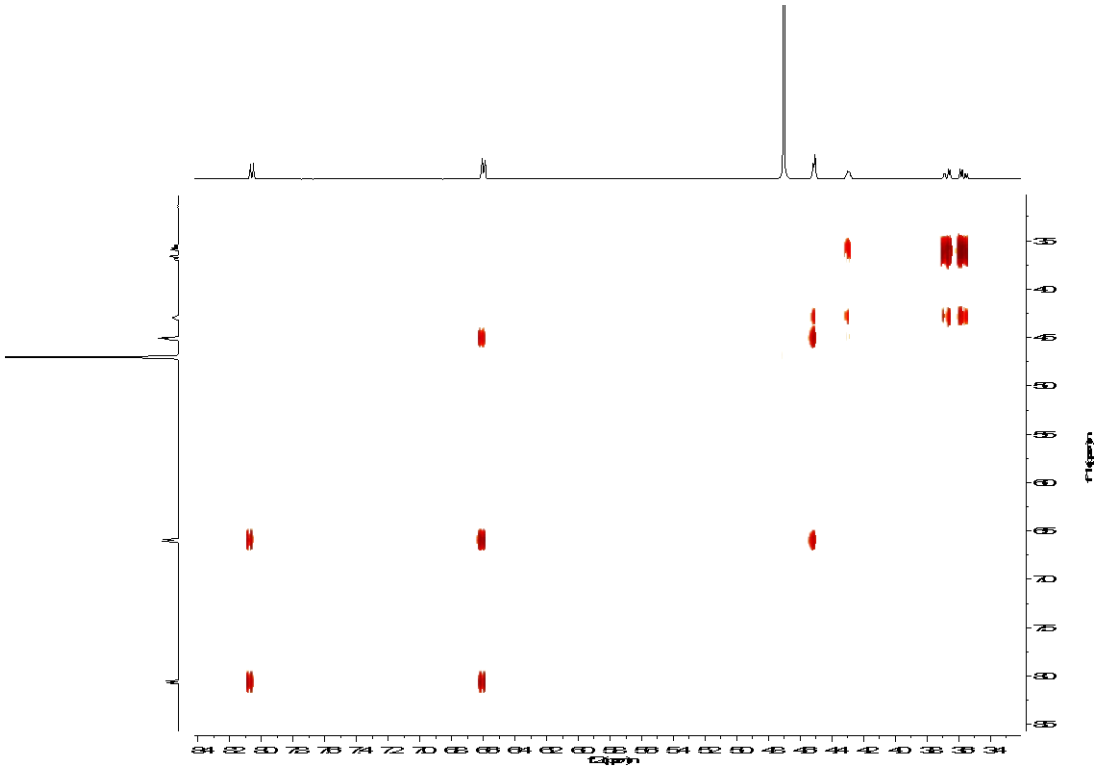
Supplementary Figure 24. ^1H NMR spectrum of 2,2'-anhydro-2-thiocytidine 21a in D_2O.



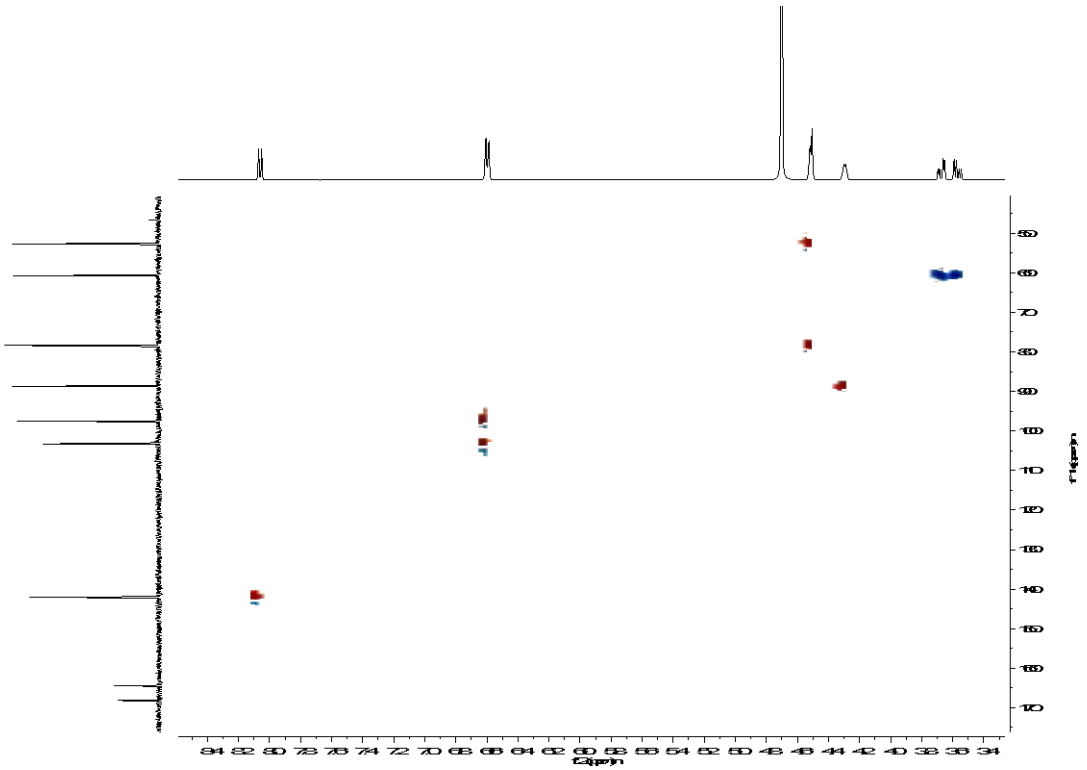
Supplementary Figure 25. ^{13}C NMR spectrum of 2,2'-anhydro-2-thiocytidine 21a in D_2O.



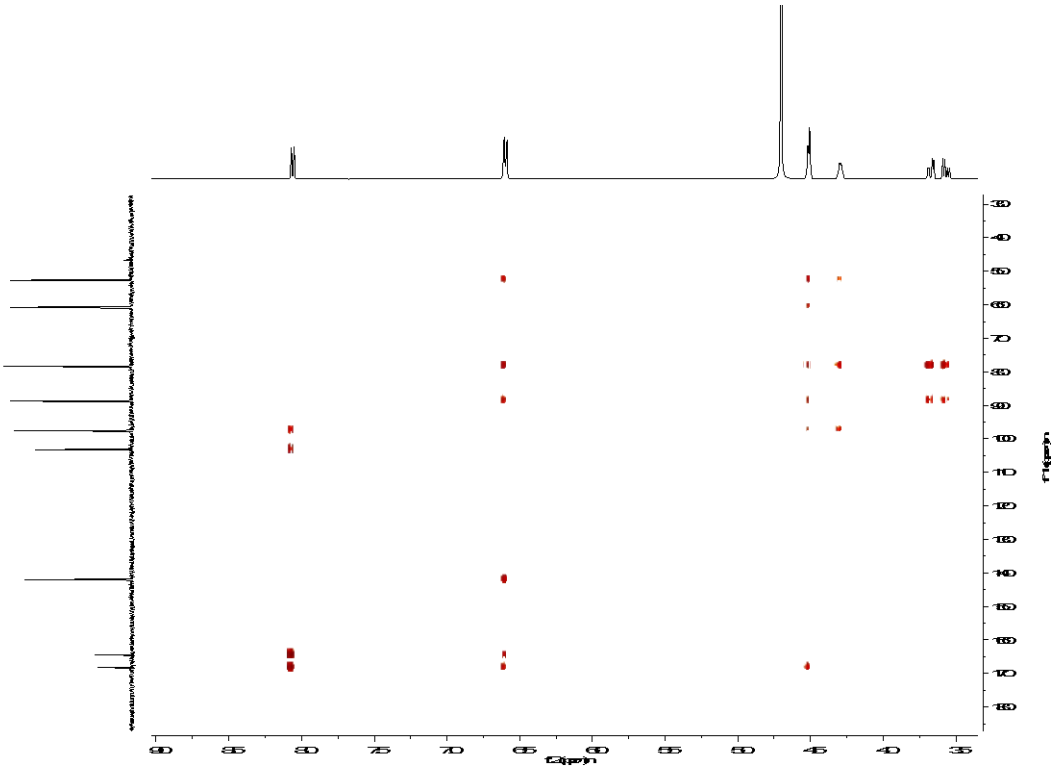
Supplementary Figure 26. COSY NMR spectrum of 2,2'-anhydro-2-thiocytidine 21a in D_2O.



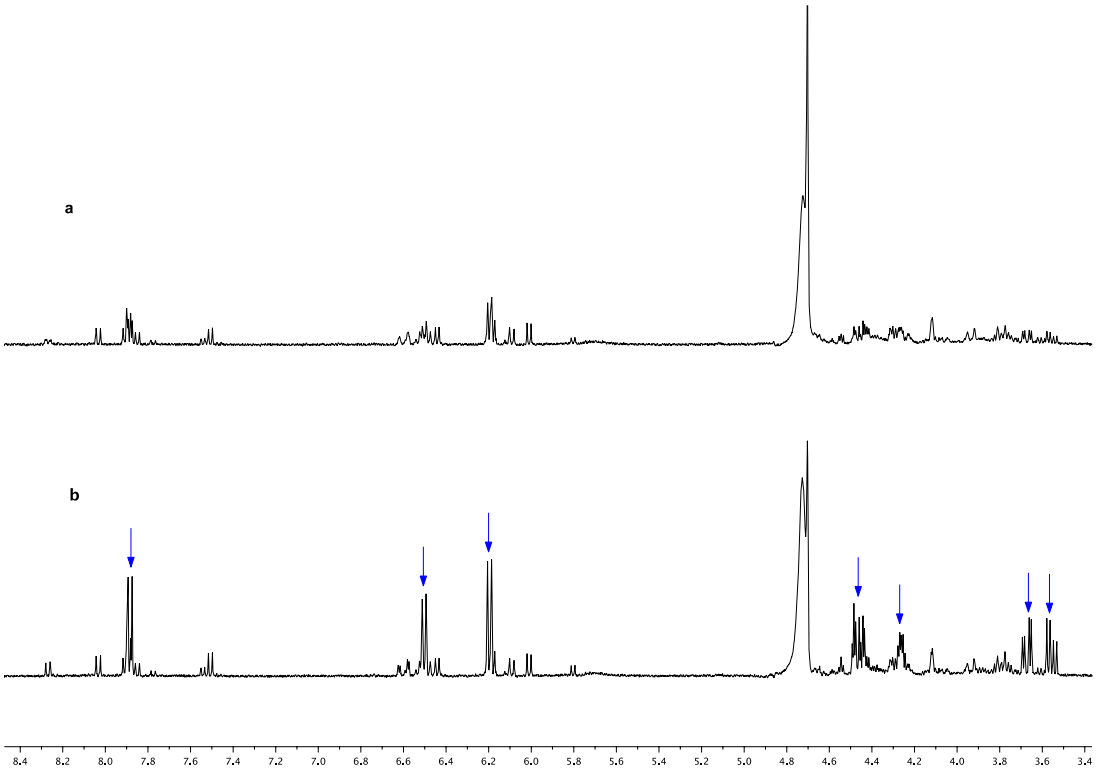
Supplementary Figure 27. HSQC NMR spectrum of 2,2'-anhydro-2-thiocytidine 21a in D_2O.



Supplementary Figure 28. HMBC NMR spectrum of 2,2'-anhydro-2-thiocytidine 21a in D_2O.

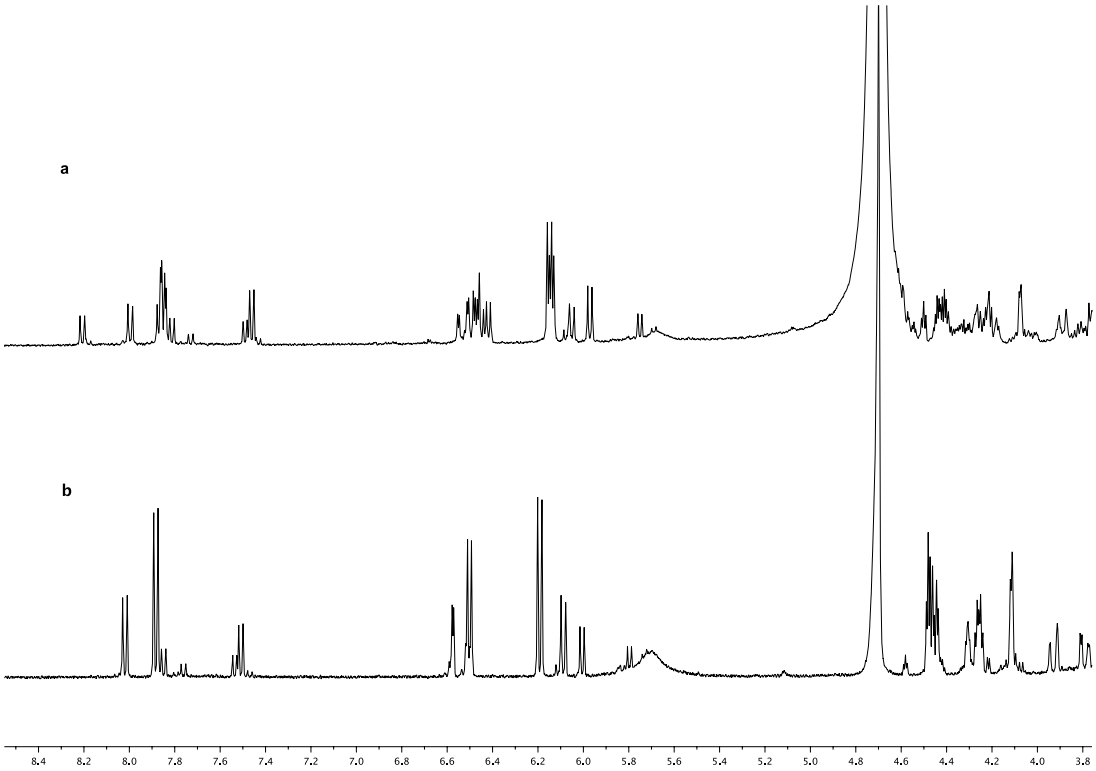


Supplementary Figure 29. ^{1}H NMR spectra of the spiking experiments for phosphorylation of 2-thiouridine 8 in semi-molten urea before dephosphorylation.



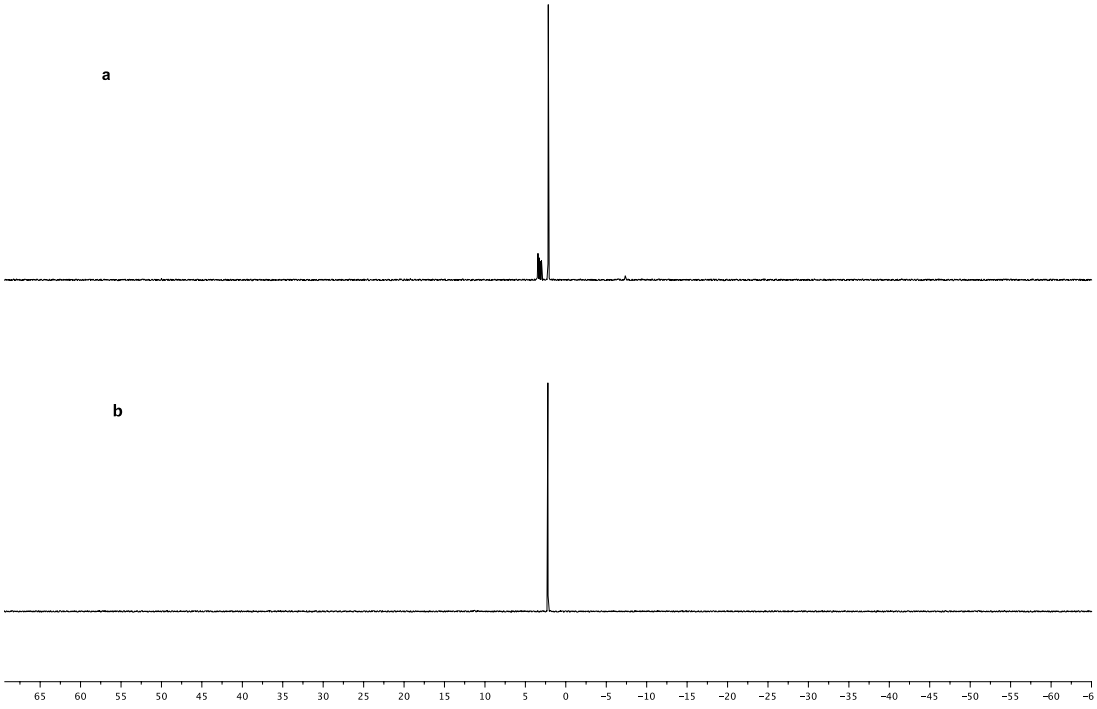
a) ^{1}H NMR spectrum of the reaction mixture after phosphorylation; **b)** as a), spiked with 2,2'-anhydro-2-thiouridine **15a**. Signals from the spiking compounds are indicated with blue arrows, as shown above.

Supplementary Figure 30. Stacked ^{1}H NMR spectra of phosphorylation reaction of 2-thiouridine 8 in semi-molten urea before and after treatment with alkaline phosphatase.



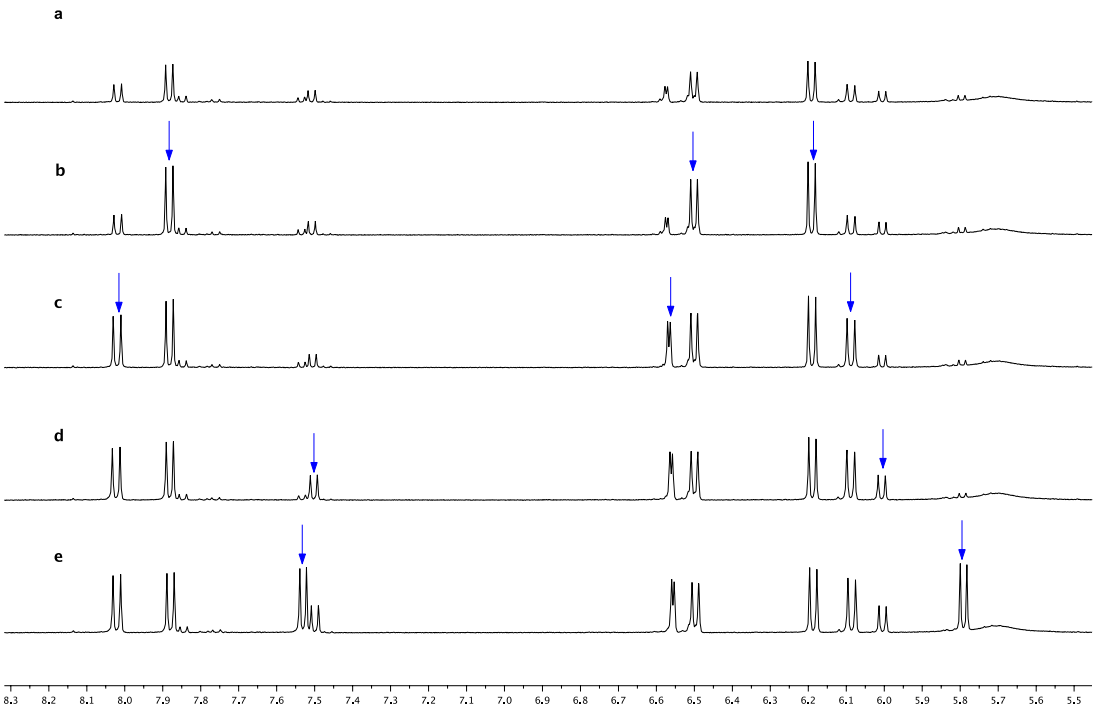
a) ^{1}H NMR spectrum of the phosphorylation mixture; **b)** ^{1}H NMR spectrum of the mixture after treatment with alkaline phosphatase.

Supplementary Figure 31. Stacked ^{31}P NMR spectra of phosphorylation reaction of 2-thiouridine 8 in semi-molten urea before and after treatment with alkaline phosphatase.



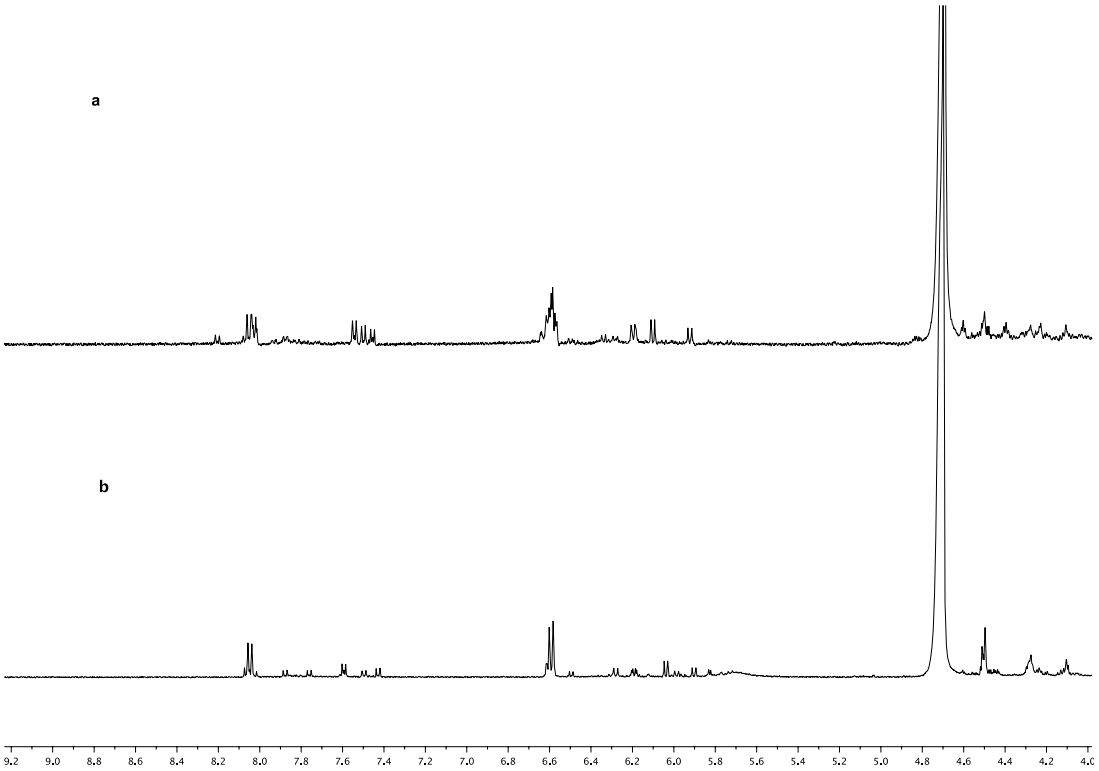
a) ^{31}P NMR spectrum of the phosphorylation mixture; **b)** ^{31}P NMR spectrum of the mixture after treatment with alkaline phosphatase.

Supplementary Figure 32. ^{1}H NMR spectra of spiking experiments for the phosphorylation of 2-thiouridine 8 in semi-molten urea after dephosphorylation with enzyme.



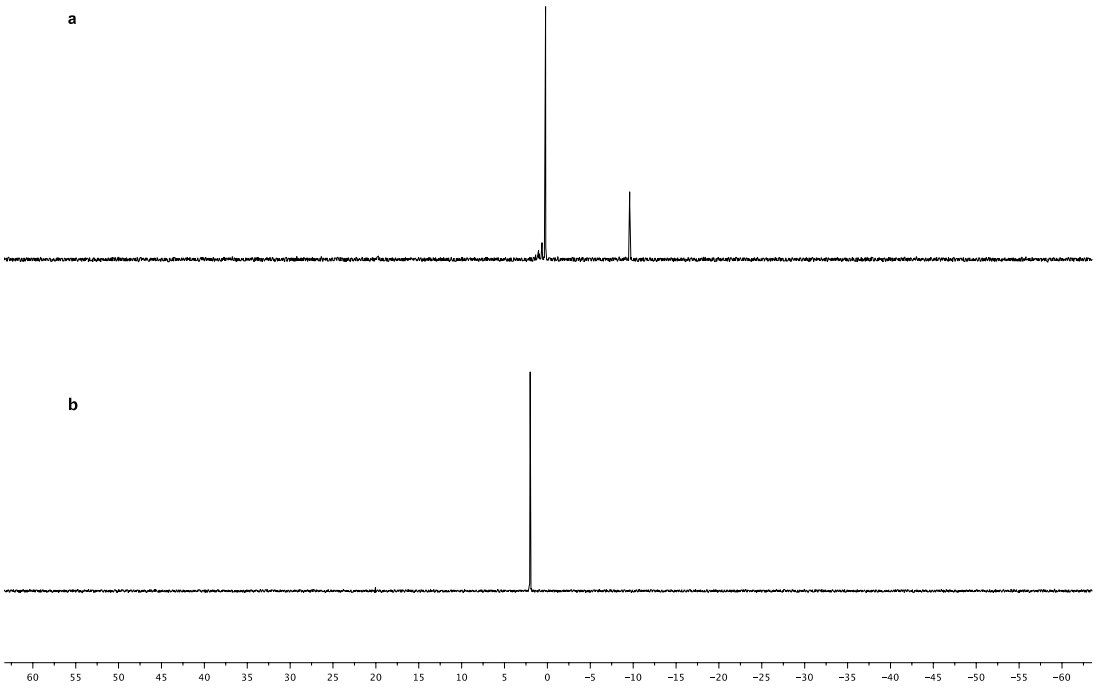
a) ^{1}H NMR spectrum of the crude mixture after dephosphorylation; **b)** as a), spiked with 2,2'-anhydro-2-thiouridine **15a**; **c)** as b), spiked with 2-thiouridine **8**; **d)** as c), spiked with 2-thiouracile **19**; **e)** as d), spiked with isocytosine **20**. Signals from the spiking compounds are indicated with blue arrows, as shown above.

Supplementary Figure 33. Stacked ^{1}H NMR spectra of phosphorylation reaction of 2-thiocytidine 5 in semi-molten urea before and after treatment with alkaline phosphatase.



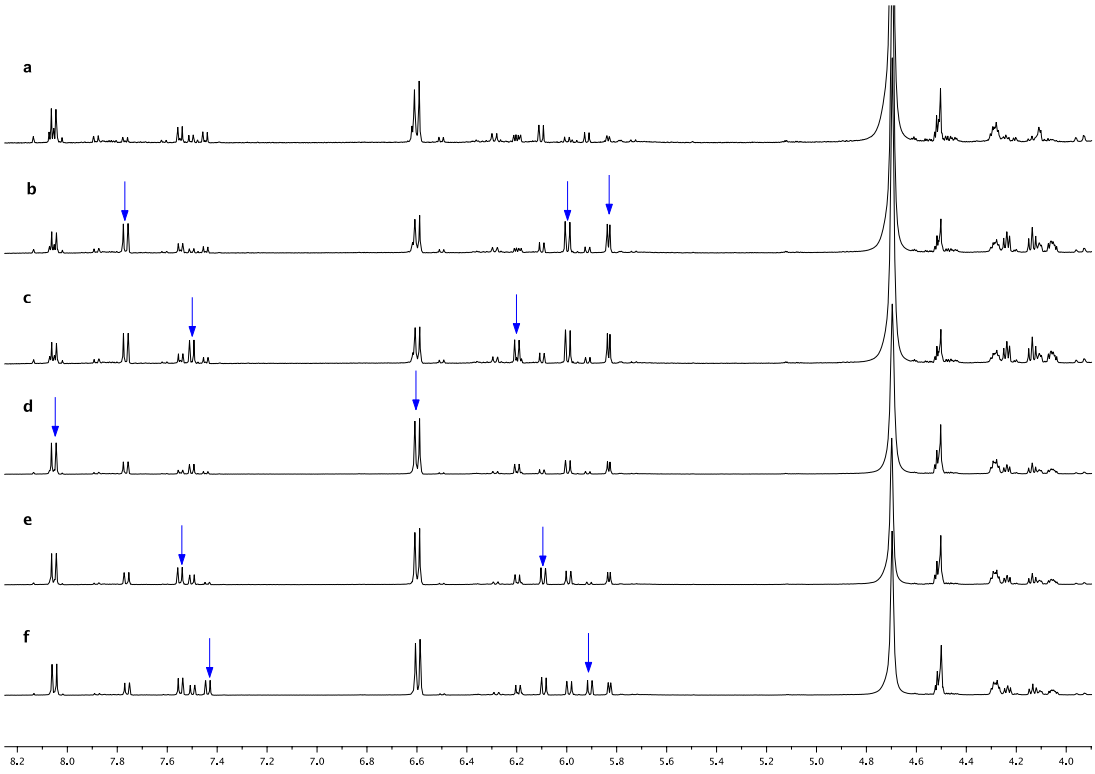
a) ^{1}H NMR spectrum of the phosphorylation mixture; **b)** ^{1}H NMR spectrum of the mixture after treatment with alkaline phosphatase.

Supplementary Figure 34. Stacked ^{31}P NMR spectra of phosphorylation reaction of 2-thiocytidine 5 in semi-molten urea before and after treatment with alkaline phosphatase.



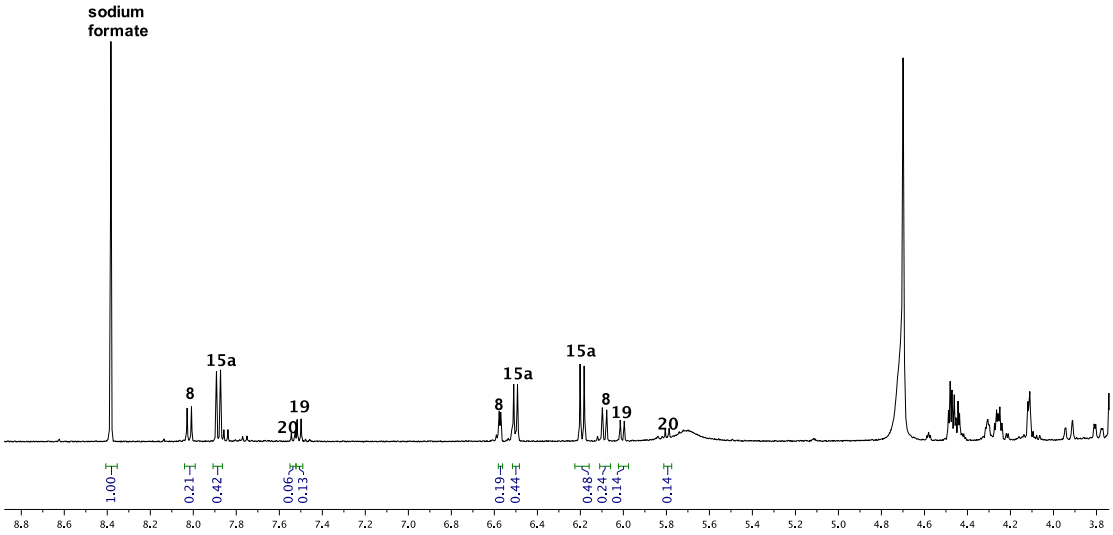
a) ^{31}P NMR spectrum of the phosphorylation mixture; **b)** ^{31}P NMR spectrum of the mixture after treatment with alkaline phosphatase.

Supplementary Figure 35. ^{1}H NMR spectra of spiking experiments for the phosphorylation of 2-thiocytidine 5 in semi-molten urea after dephosphorylation with enzyme.

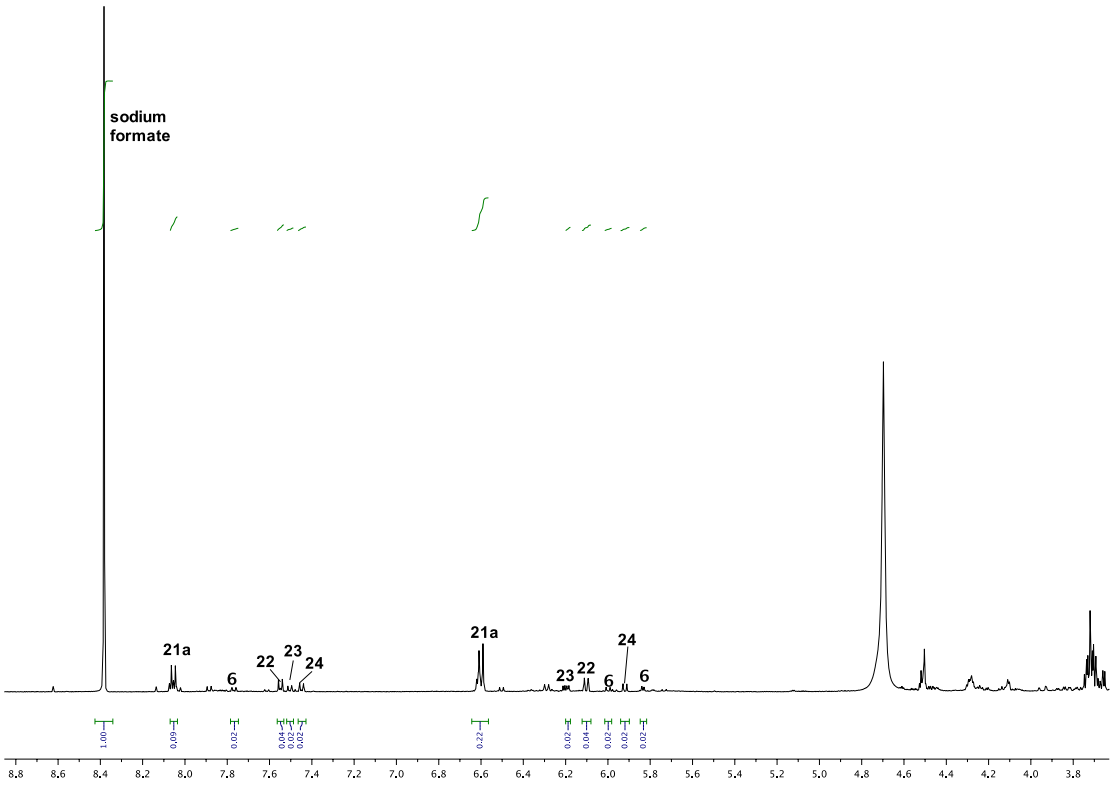


a) ^{1}H NMR spectrum of the crude mixture after dephosphorylation; **b)** as a), spiked with cytidine **6**; **c)** as b), spiked with 2-thiocytosine **23**; **d)** as c), spiked with 2,2'-anhydro-2-thiocytidine **21a**; **e)** as d), spiked with 2,4-diaminopyrimidine **22**. **f)** as e), spiked with cytosine **24**. Signals from the spiking compounds are indicated with blue arrows, as shown above.

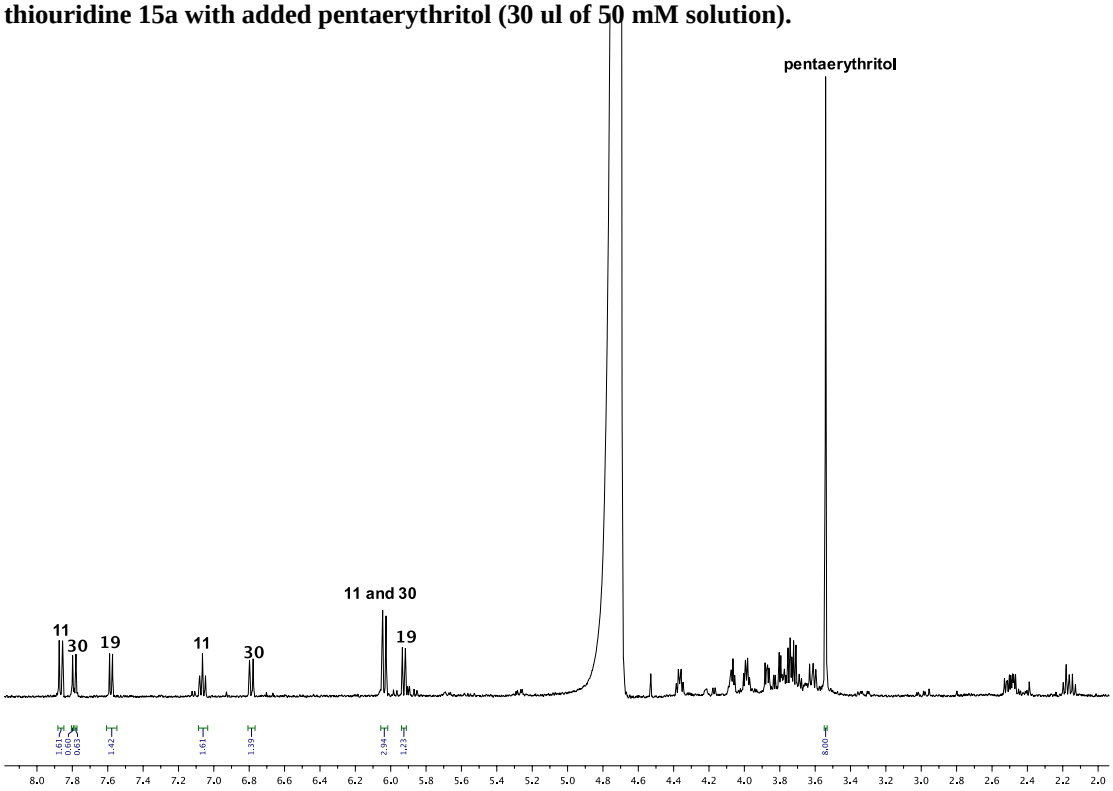
Supplementary Figure 36. ^{1}H NMR spectra of the phosphorylation mixture of 2-thiouridine 8 in semi-molten urea after dephosphorylation with added sodium formate (3 mg).



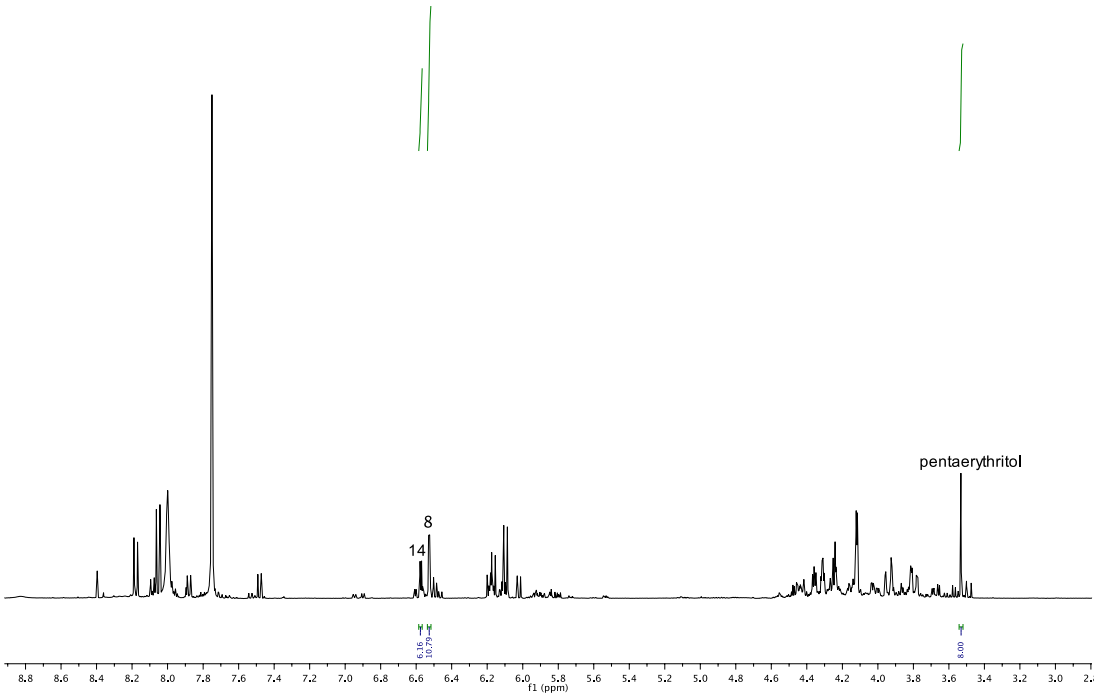
Supplementary Figure 37. ^{1}H NMR spectra of the phosphorylation mixture of 2-thocytidine 5 in semi-molten urea after dephosphorylation with added sodium formate (5 mg).



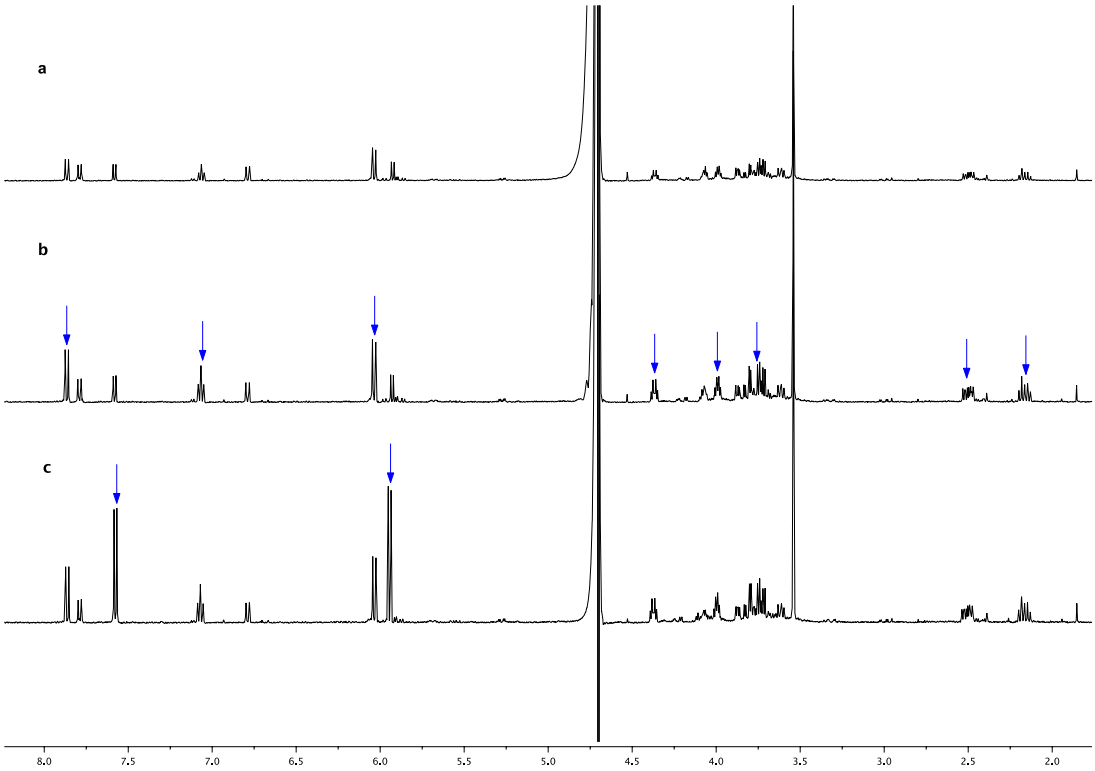
Supplementary Figure 38. ^{1}H NMR spectra of the photoreduction mixture of 2', 2-anhydro-2-thiouridine 15a with added pentaerythritol (30 ul of 50 mM solution).



Supplementary Figure 39. ^{1}H NMR spectra of the phosphorylation mixture of 2-thiouridine 8 in hot formamide with added pentaerythritol (30 ul of 50 mM solution).

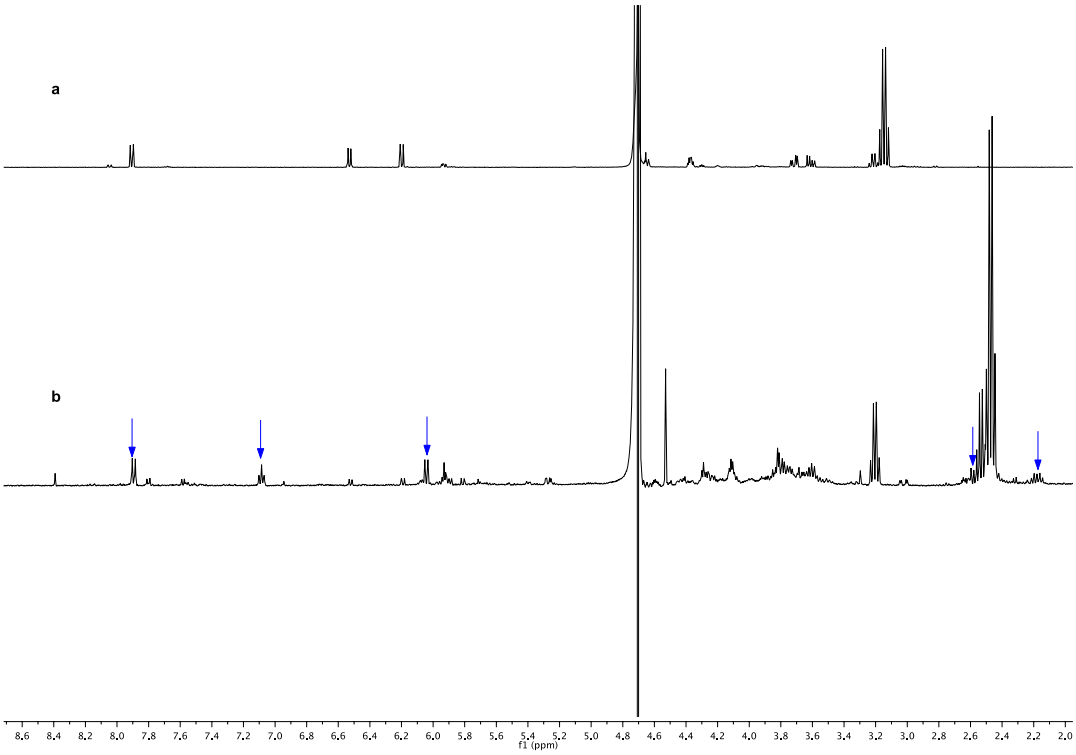


Supplementary Figure 40. ^{1}H NMR spectra of spiking experiments for the photoreduction of 2', 2-anhydro-2-thiouridine 15a.



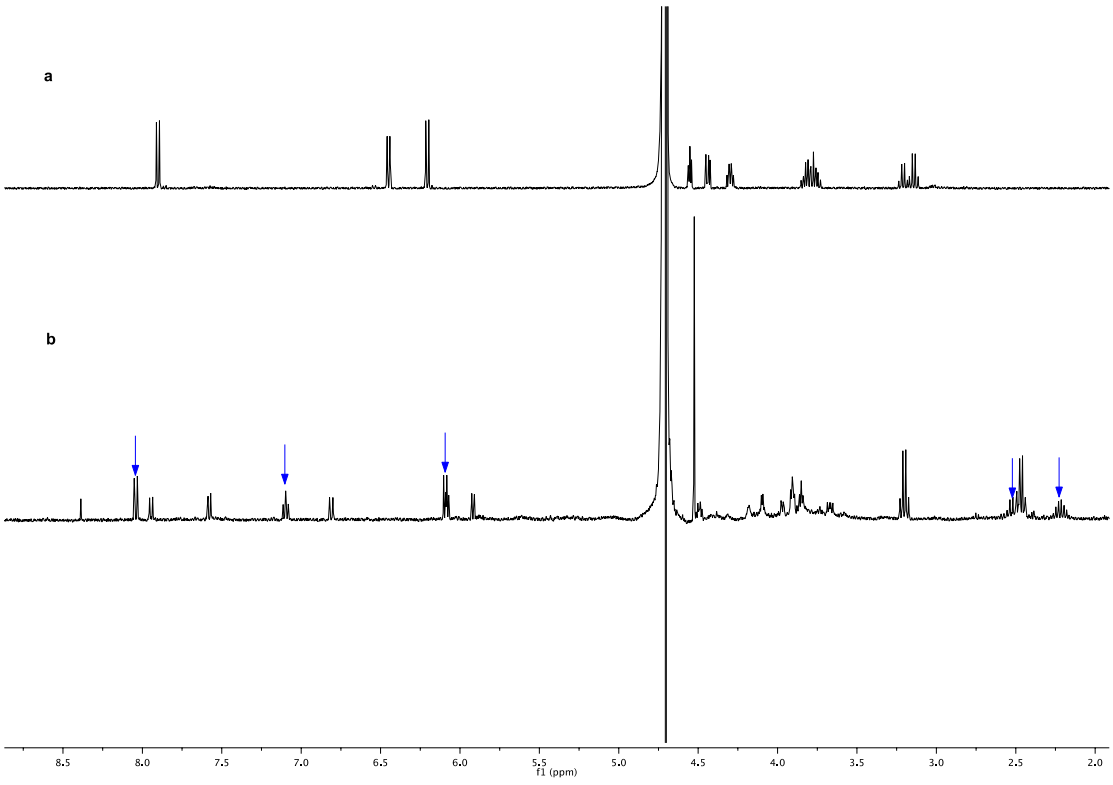
a) ^{1}H NMR spectrum of the crude mixture after photoredution of 2',2-anhydro-2-thiourine **15a**; **b)** as a), spiked with 2'-deoxy-2-thiouridine **11**; **c)** as b), spiked with 2-thiouracile **19**. Signals from the spiking compounds are indicated with blue arrows, as shown above.

Supplementary Figure 41. ^{1}H NMR spectra of the photoreduction of 2', 2-anhydro-2-thiouridine-3'-phosphate 15b.



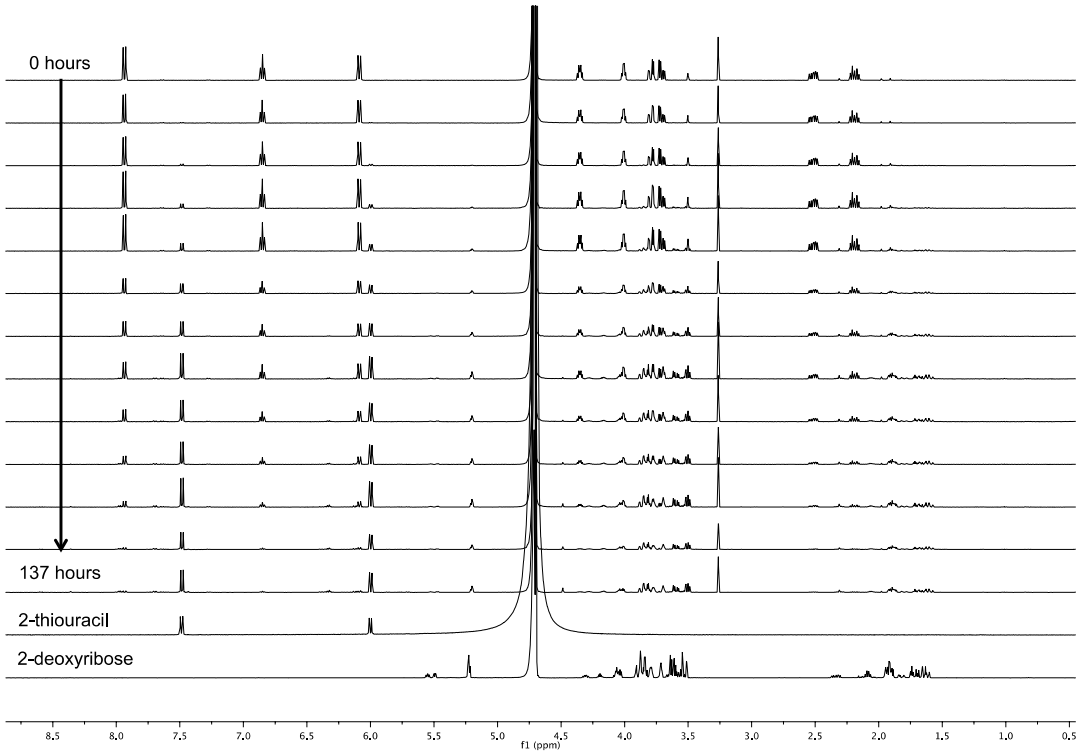
a) ^{1}H NMR spectrum of **15b**; **b)** ^{1}H NMR spectrum of photoreduction of **15b**. Signals from the deoxynucleotides are indicated with blue arrows, as shown above. The yield of deoxynucleotide was estimated at 55% based on the integration of ^{1}H NMR spectrum.

Supplementary Figure 42. ^{1}H NMR spectra of the photoreduction of 2', 2-anhydro-2-thiouridine-5'-phosphate 15c.

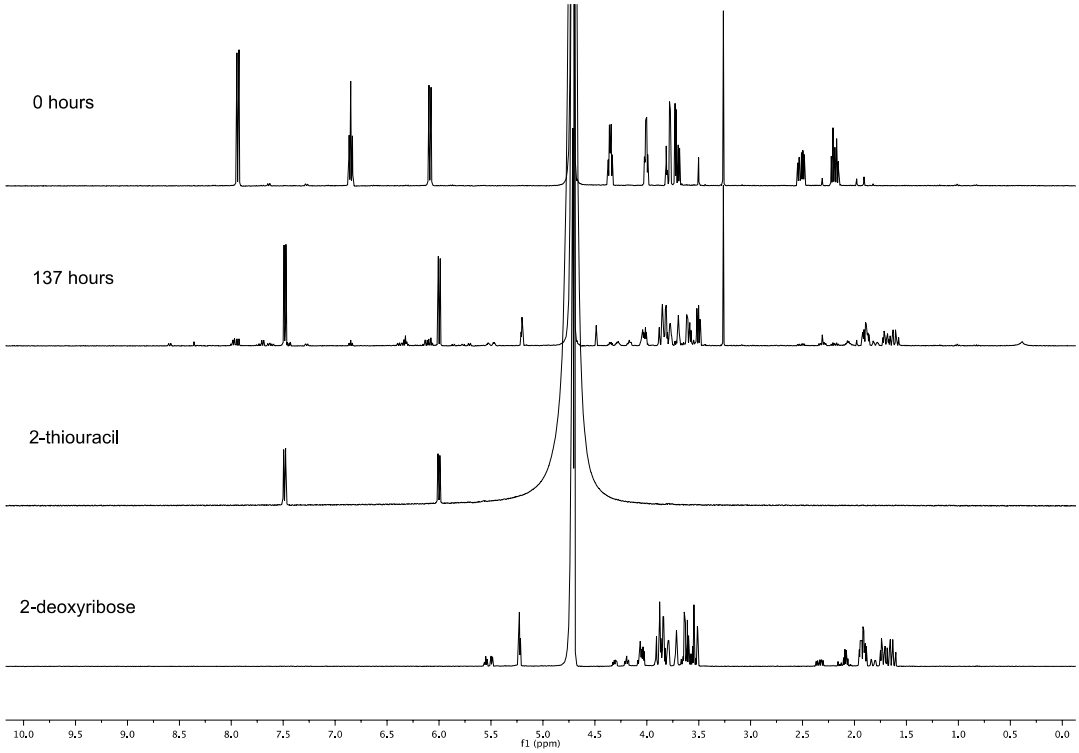


a) ^{1}H NMR spectrum of **15c**; **b)** ^{1}H NMR spectrum of photoreduction of **15c**. Signals from the deoxynucleotides are indicated with blue arrows, as shown above. The yield of deoxynucleotide was estimated at 41% based on the integration of ^{1}H NMR spectrum.

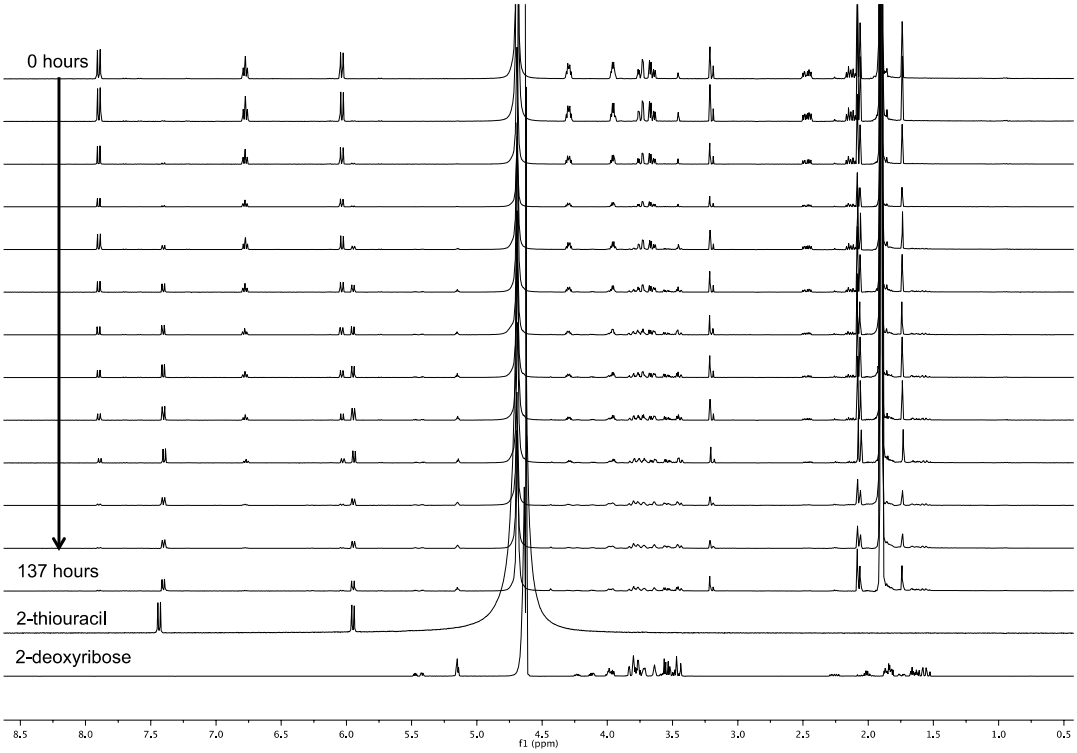
Supplementary Figure 43. ^{1}H NMR stack for hydrolysis of 2'-deoxy-2-thiouridine 11 in phosphate buffer (pH 7.0).



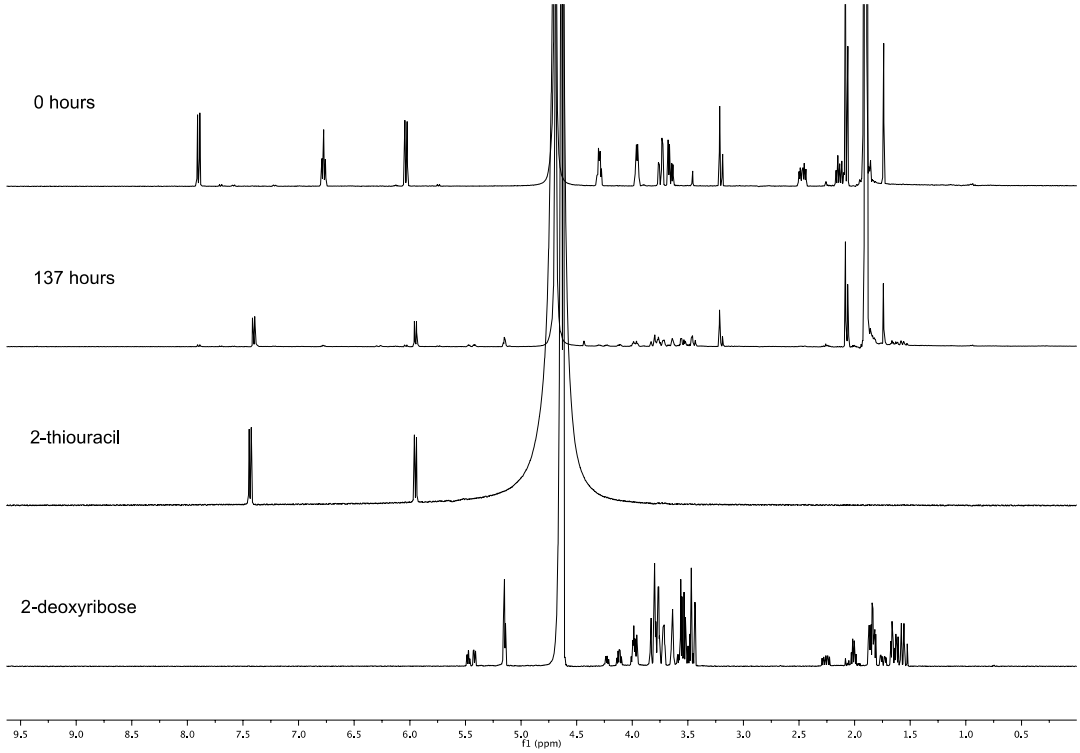
Supplementary Figure 44. Abbreviated ^{1}H NMR stack for hydrolysis of 2'-deoxy-2-thiouridine 11 in phosphate buffer (pH 7.0).



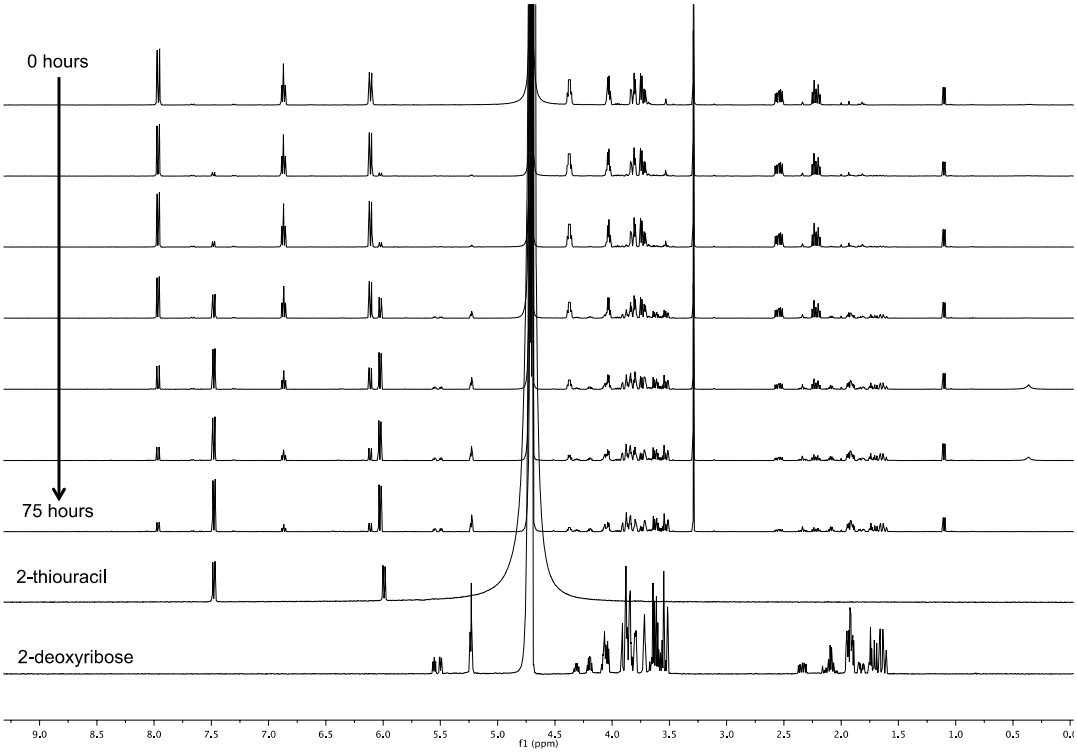
Supplementary Figure 45. ^{1}H NMR stack for hydrolysis of 2'-deoxy-2-thiouridine 11 in acetate buffer (pH 4.1).



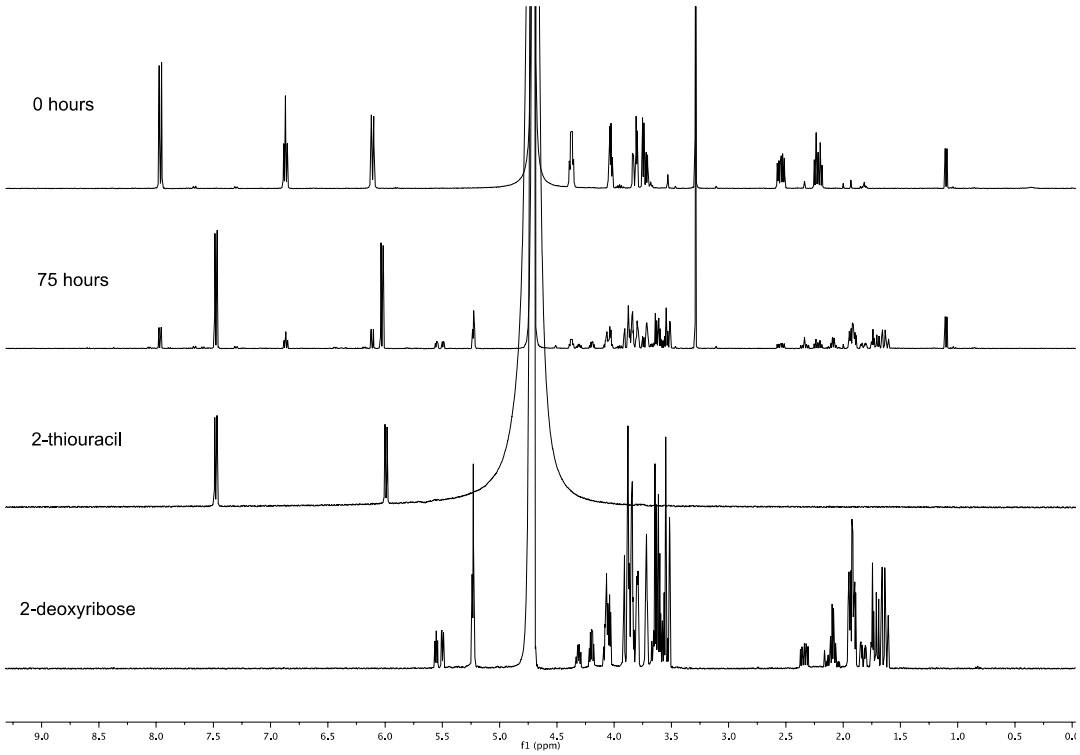
Supplementary Figure 46. Abbreviated ^{1}H NMR stack for hydrolysis of 2'-deoxy-2-thiouridine 11 in acetate buffer (pH 4.1).



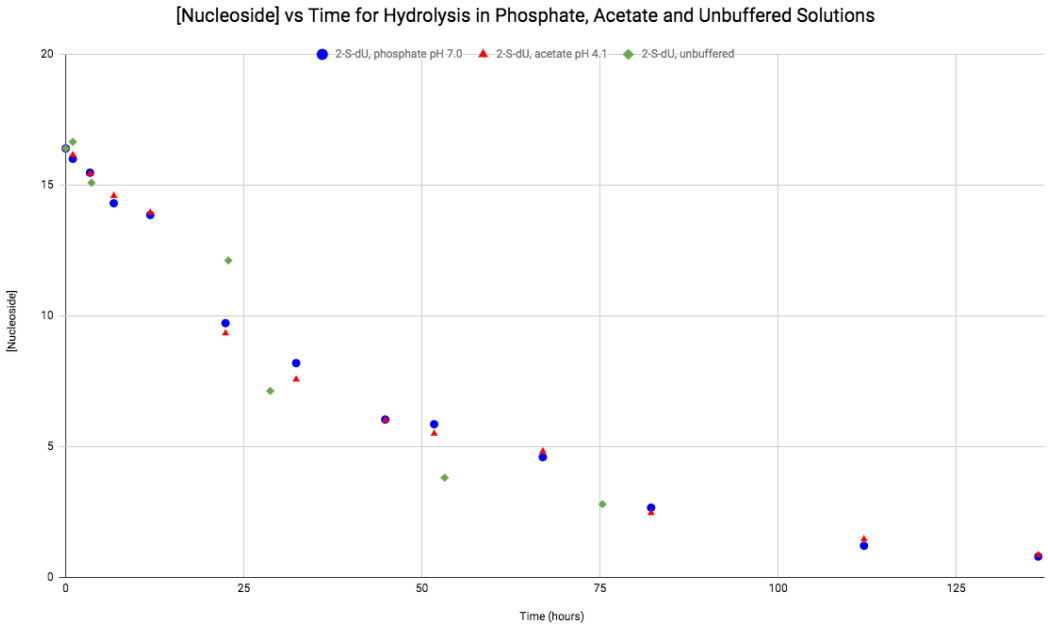
Supplementary Figure 47. ^{1}H NMR stack for hydrolysis of 2'-deoxy-2-thiouridine 11 in unbuffered solution.



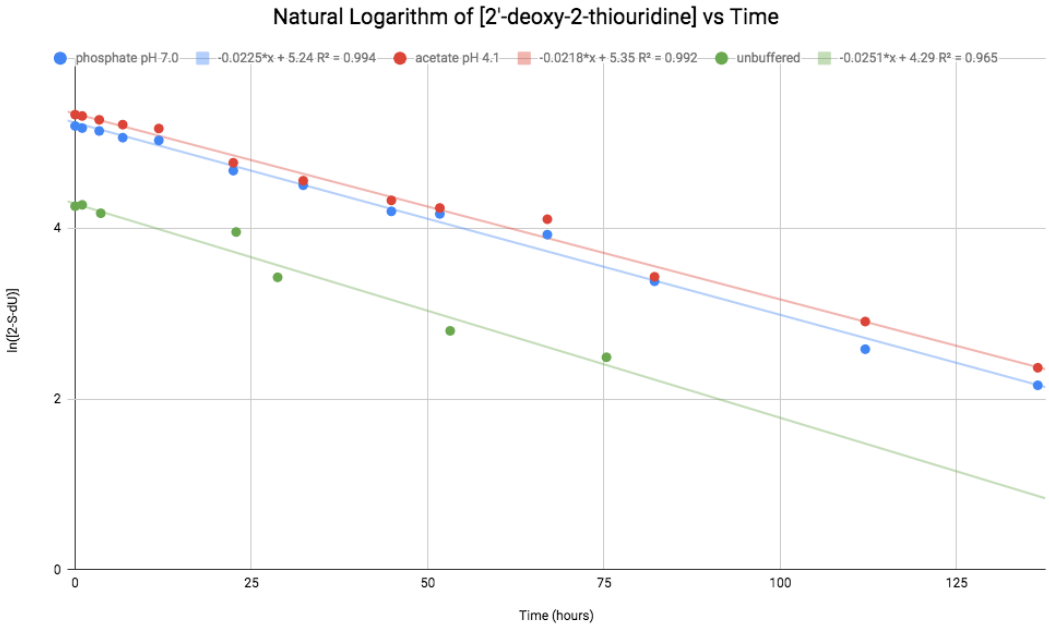
Supplementary Figure 48. Abbreviated ^{1}H NMR stack for hydrolysis of 2'-deoxy-2-thiouridine 11 in unbuffered solution.



Supplementary Figure 49. Hydrolysis curves for hydrolysis of 2'-deoxy-2-thiouridine 11.

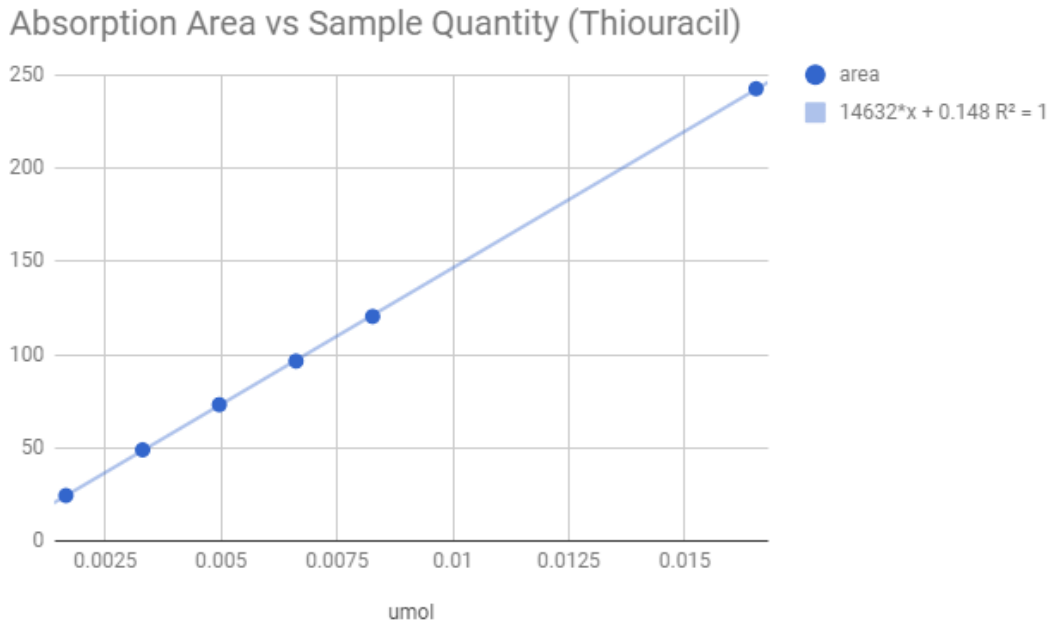


Supplementary Figure 50. Natural logarithm of 2'-deoxy-2-thiouridine 11 vs time plot for half-life determination.



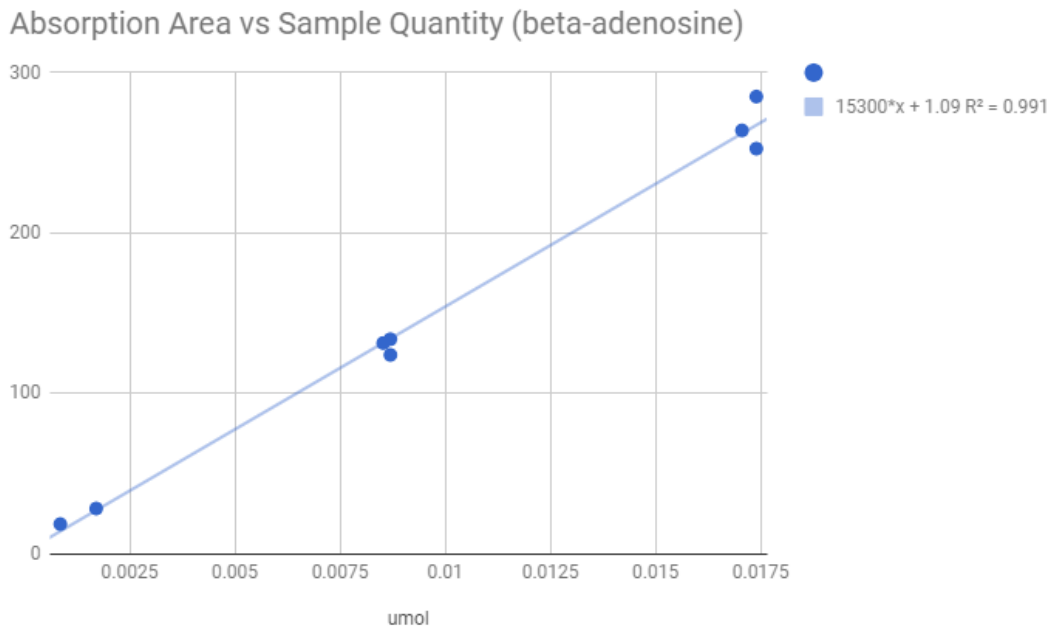
Half-lives: 31 hours (phosphate pH 7.0)
32 hours (acetate pH 4.1)
28 hours (unbuffered)

Supplementary Figure 51. UV absorption calibration curve of thiouracil 19.



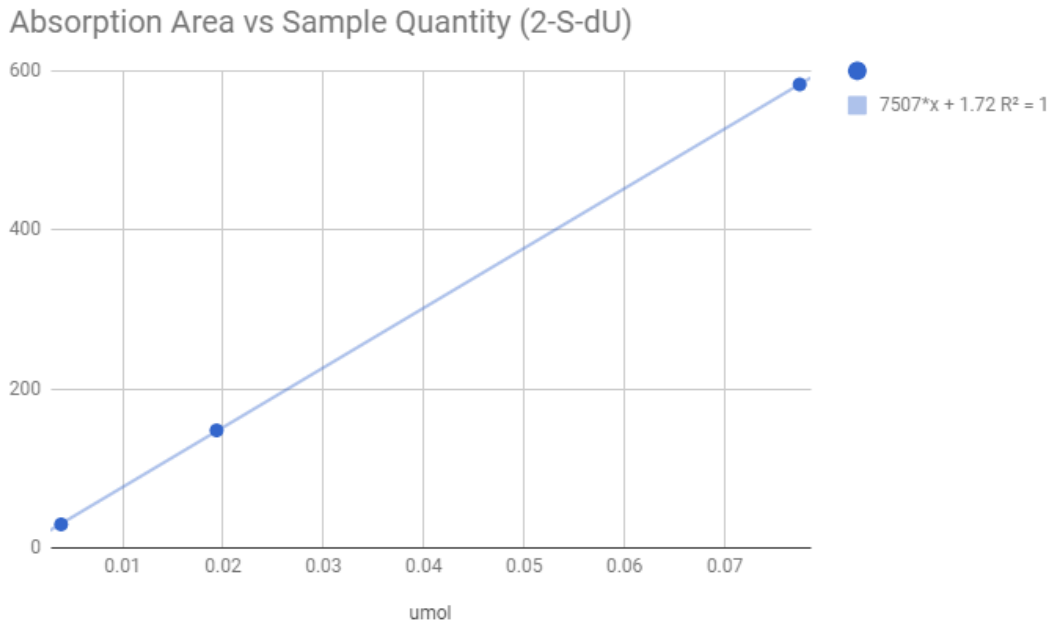
Response factor: 14632 units/umol, or 6.834E-05 umol per unit.

Supplementary Figure 52. UV absorption calibration curve of β-deoxyadenosine (β-13).



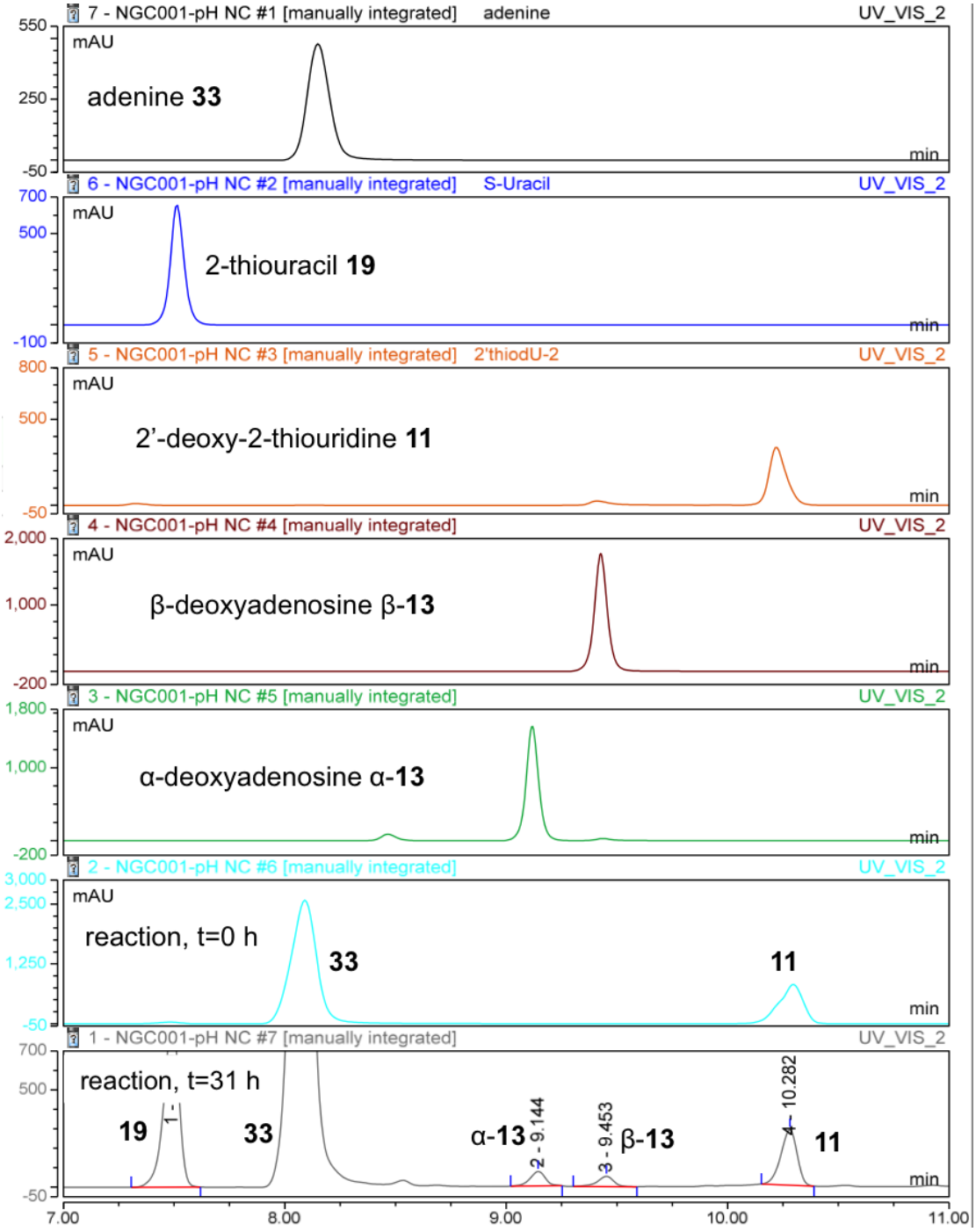
Response factor: 15121 units/umol, or 6.536E-05 umol per unit

Supplementary Figure 53. UV absorption calibration curve of 2'-deoxy-2-thiouridine 11.

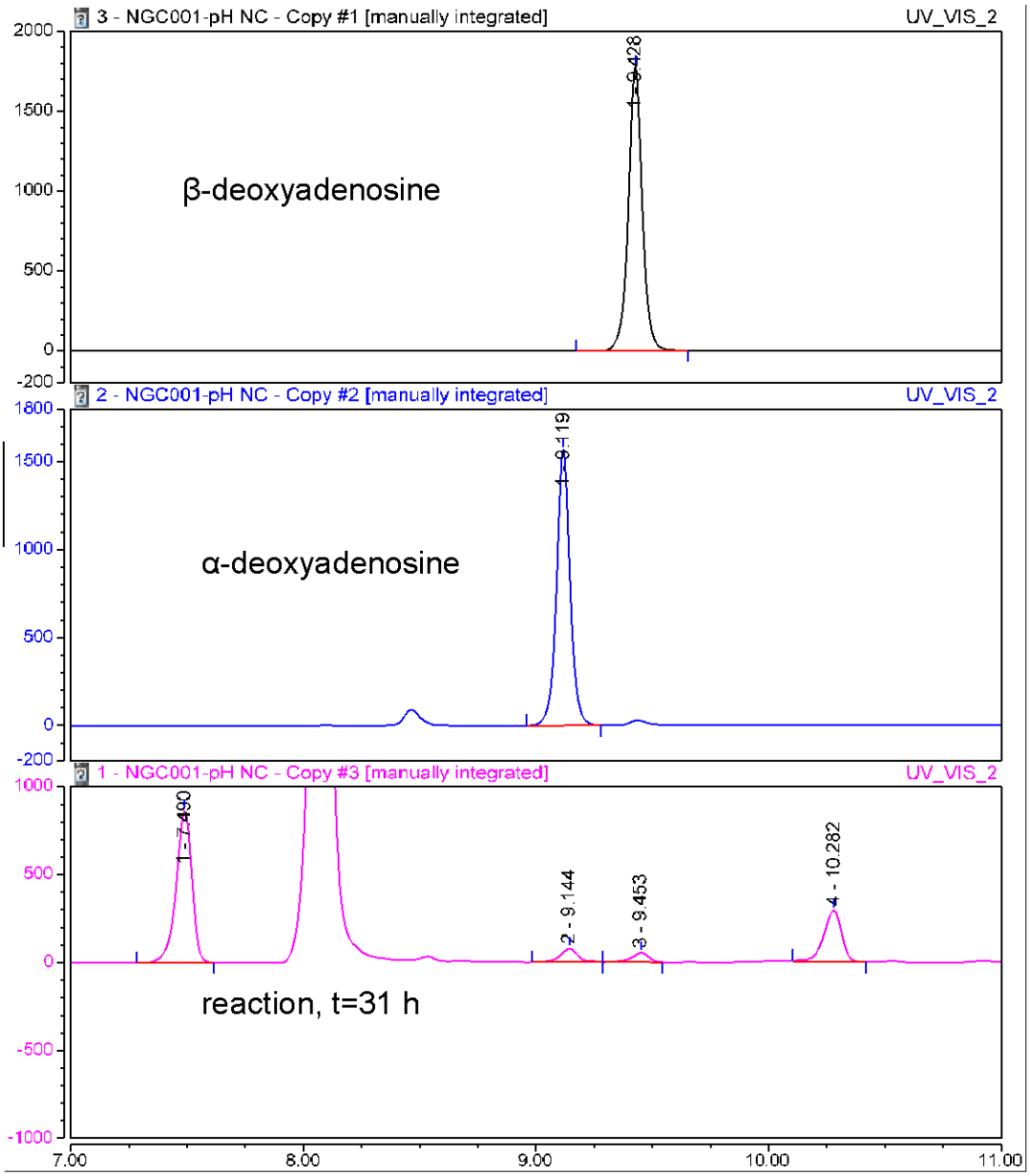


Response factor: 7507 units/umol, or 0.0001.332E-04 umol per unit

Supplementary Figure 54. Analytical HPLC chromatograms of reaction and standards for synthesis of deoxyadenosine 13.

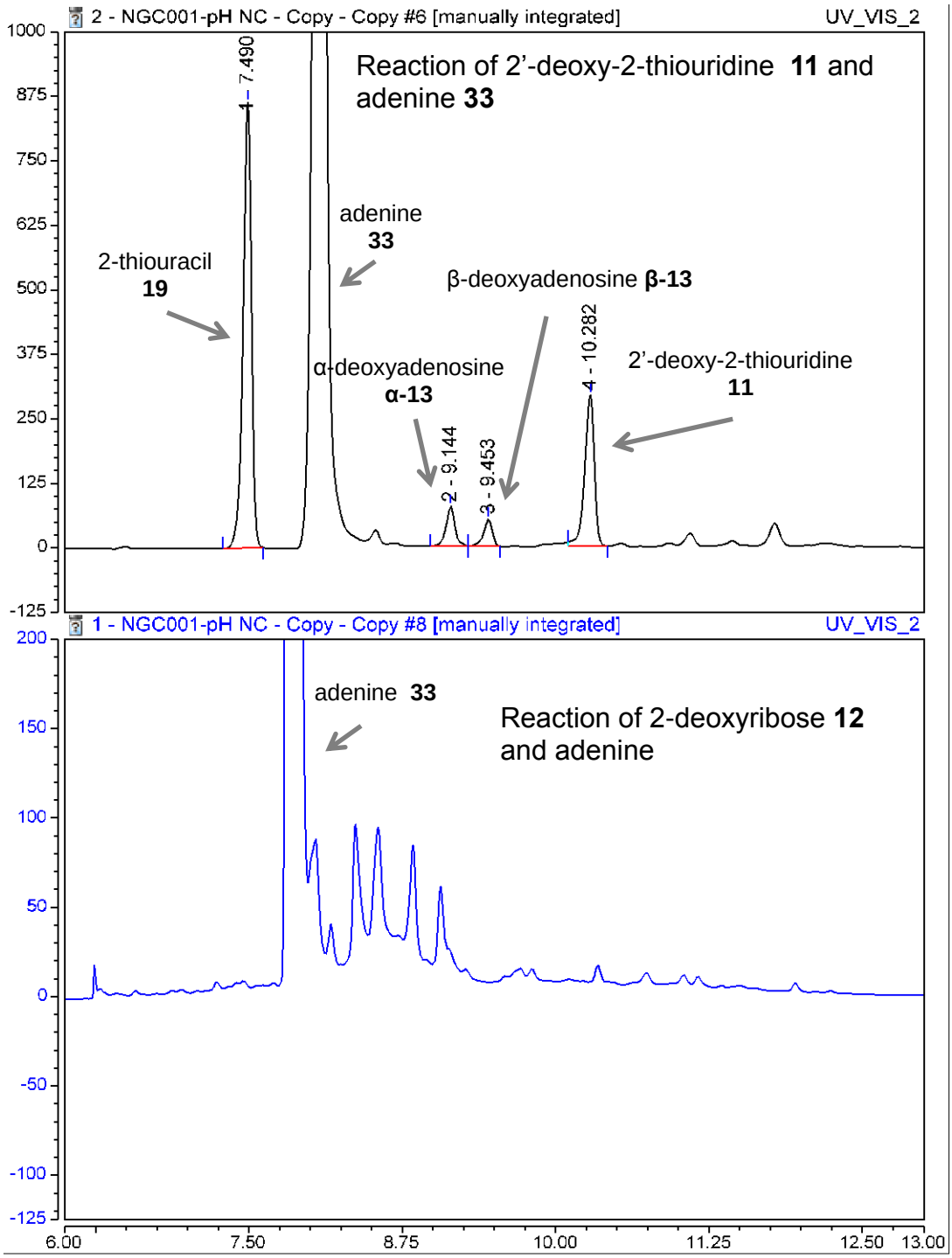


Supplementary Figure 55. Abbreviated analytical HPLC chromatograms of reaction and standards for synthesis of deoxyadenosine 13.

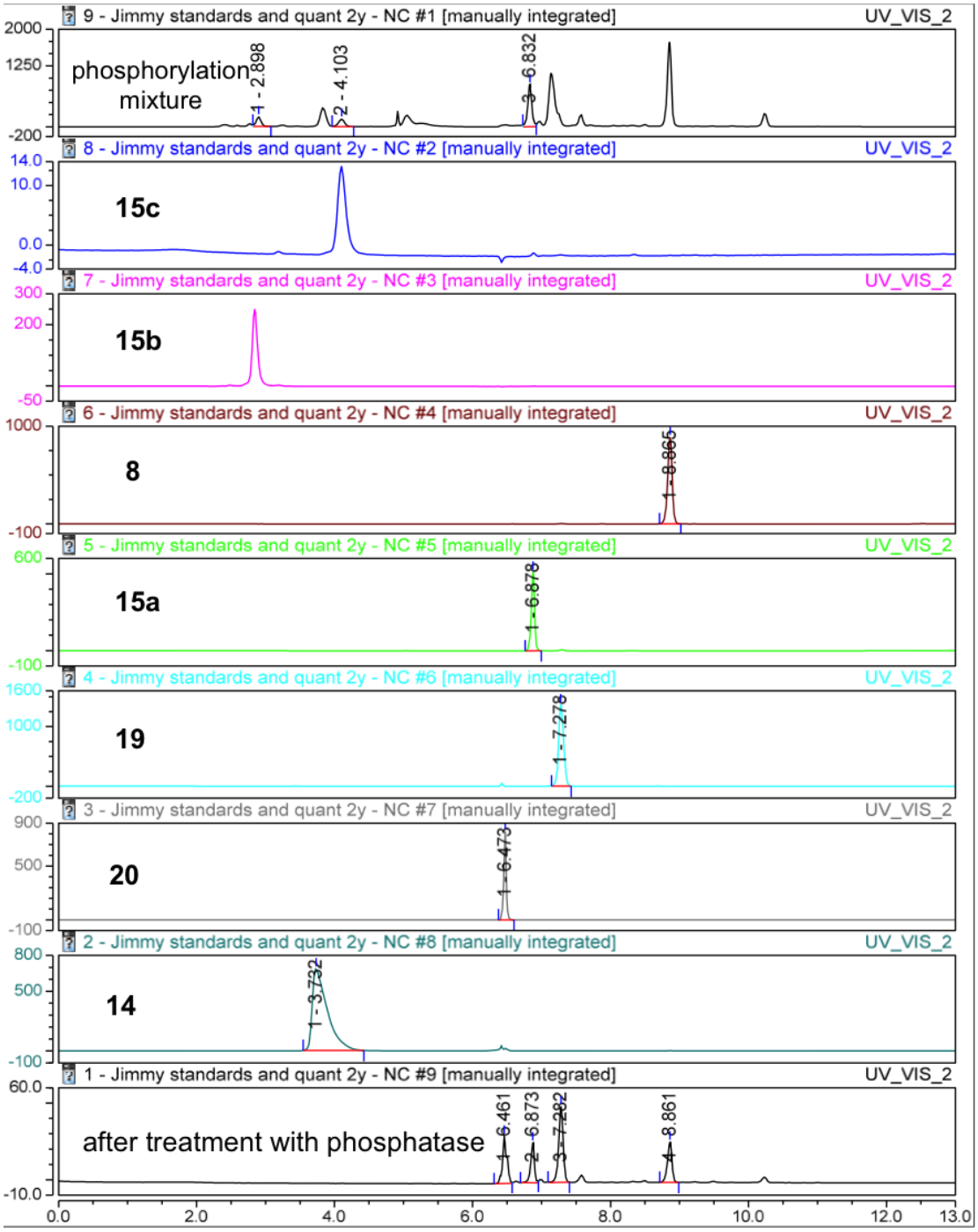


No.	Peak Name	etention Tin min	Area mAU*min	Height mAU	elative Are %	elative Heig %	Amount n.a.
1		7.490	68.404	863.163	64.61	67.20	n.a.
2		9.144	6.590	77.267	6.22	6.02	n.a.
3		9.453	4.342	52.102	4.10	4.06	n.a.
4		10.282	26.543	291.975	25.07	22.73	n.a.
Total:			**105.880**	**1284.506**	**100.00**	**100.00**	

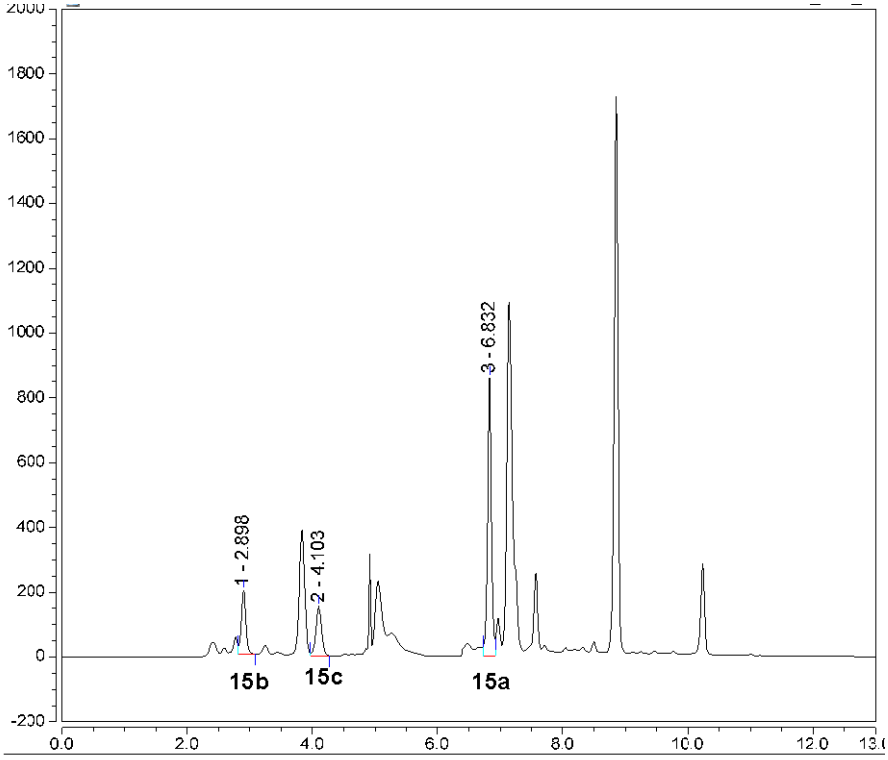
Supplementary Figure 56. Comparison of HPLC chromatograms between solid state reaction of 11 and adenine, and 12 and adenine.



Supplementary Figure 57. Analytical HPLC chromatograms for phosphorylation of 2-thiouridine 8 in semi-molten urea and purified standards.

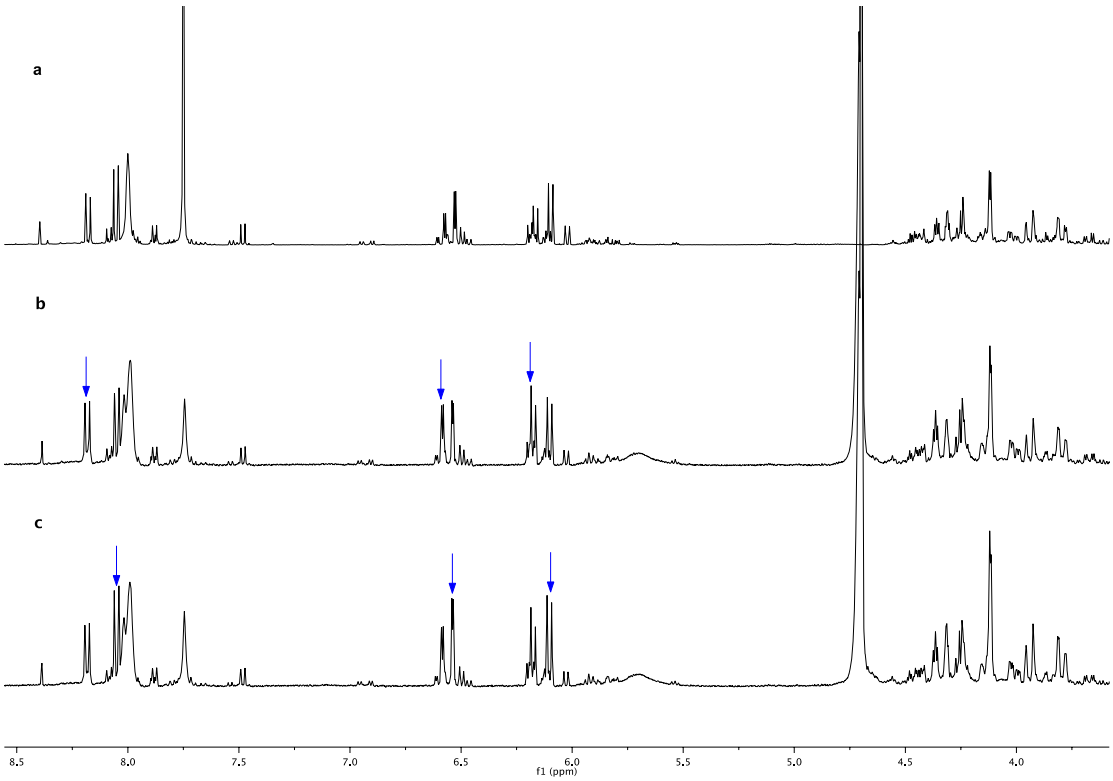


Supplementary Figure 58. Annotated analytical HPLC chromatogram for phosphorylation of 2-thiouridine 8 in semi-molten urea.



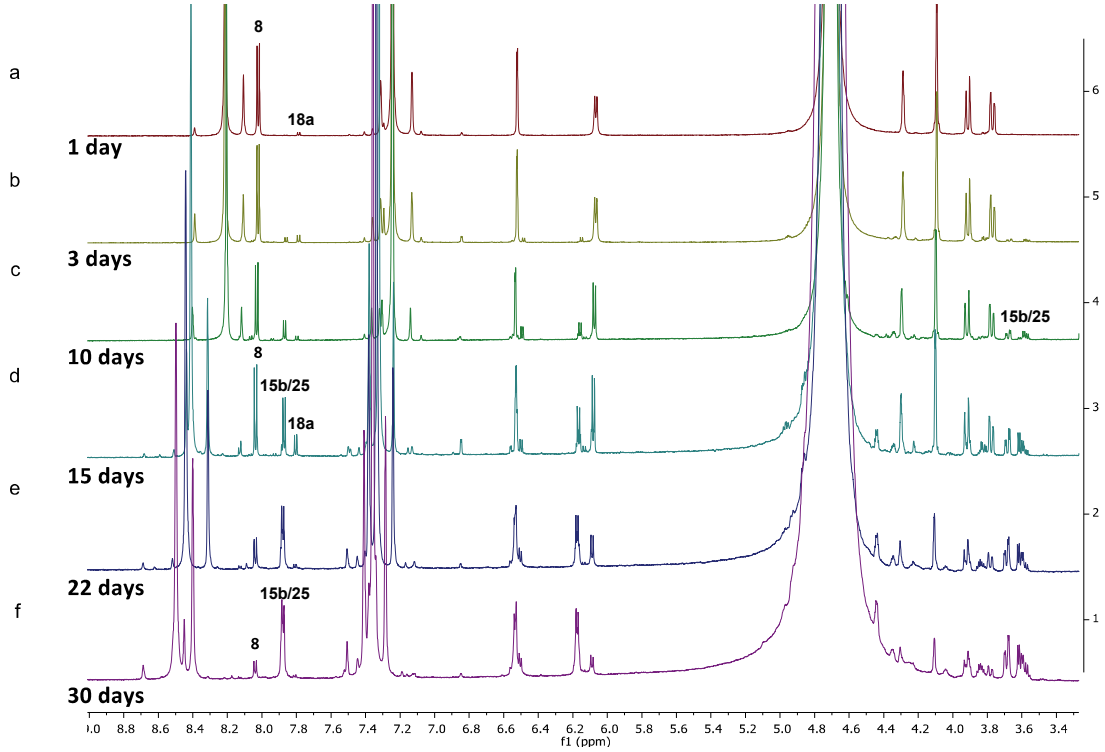
No.	Peak Name	etention Tin min	Area mAU*min	Height mAU	elative Are %	elative Heig %	Amount n.a.
1		2.898	16.371	196.554	17.80	16.27	n.a.
2		4.103	16.057	152.830	17.46	12.65	n.a.
3		6.832	59.538	858.764	64.74	71.08	n.a.
Total:			**91.966**	**1208.148**	**100.00**	**100.00**	

Supplementary Figure 59. ^{1}H NMR spectra of spiking experiments for phosphorylation of 2-thiouridine 8 in hot formamide.



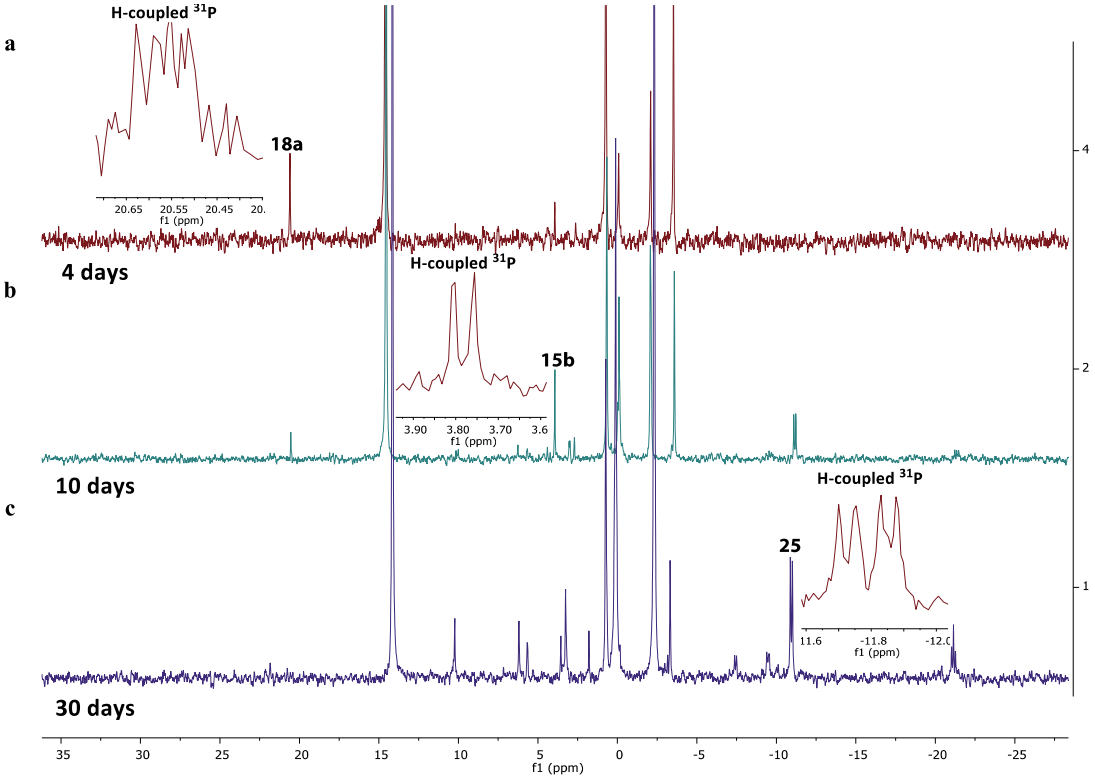
a) ^{1}H NMR spectrum of phosphorylation mixture; **b)** as a), spiked with 2-thiouridine-5'-phosphate **14**; **c)** as b), spiked with 2-thiouridine **8**. Signals from the spiking compounds are indicated with blue arrows, as shown above.

Supplementary Figure 60. ^{1}H NMR spectra of the phosphorylation of 2-thiouridine 8 with DAP and formation of 3'-(amido-di)phosphate of 2,2'-anhydro-2-thiouridine 15b/25 in D_2O.



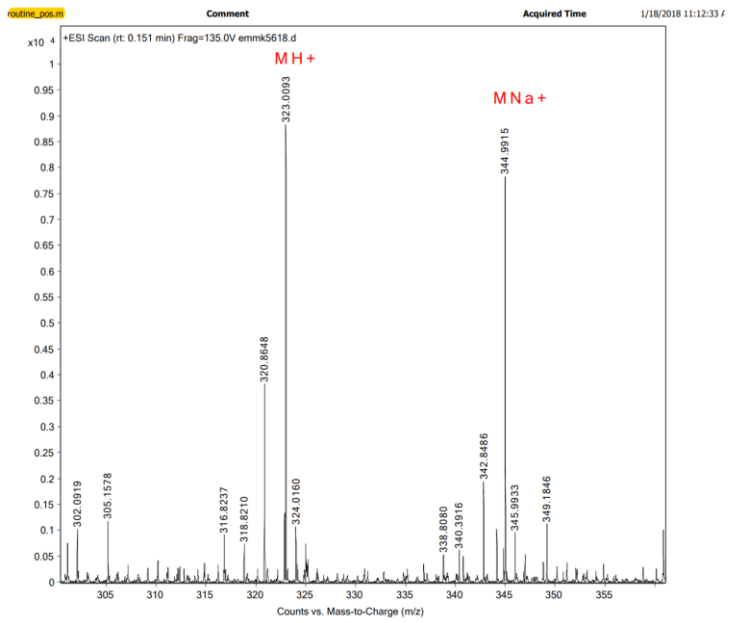
a) ^{1}H NMR spectrum of the phosphorylation mixture after 1 day; **b)** ^{1}H NMR spectrum of the phosphorylation mixture after 3 days; **c)** ^{1}H NMR spectrum of the phosphorylation mixture after 10 days; **d)** ^{1}H NMR spectrum of the phosphorylation mixture after 15 days; **e)** ^{1}H NMR spectrum of the phosphorylation mixture after 22 days; **f)** ^{1}H NMR spectrum of the phosphorylation mixture after 30 days. The spectra show that the formation of the cyclic phosphate **18a** is followed by the cyclisation leading to **15b** and then to **25**.

Supplementary Figure 61. ^{31}P NMR spectra of the phosphorylation of 2-thiouridine 8 with DAP and formation of 3'-(amido-di)phosphate of 2,2'-anhydro-2-thiouridine 15b/25 in D_2O.

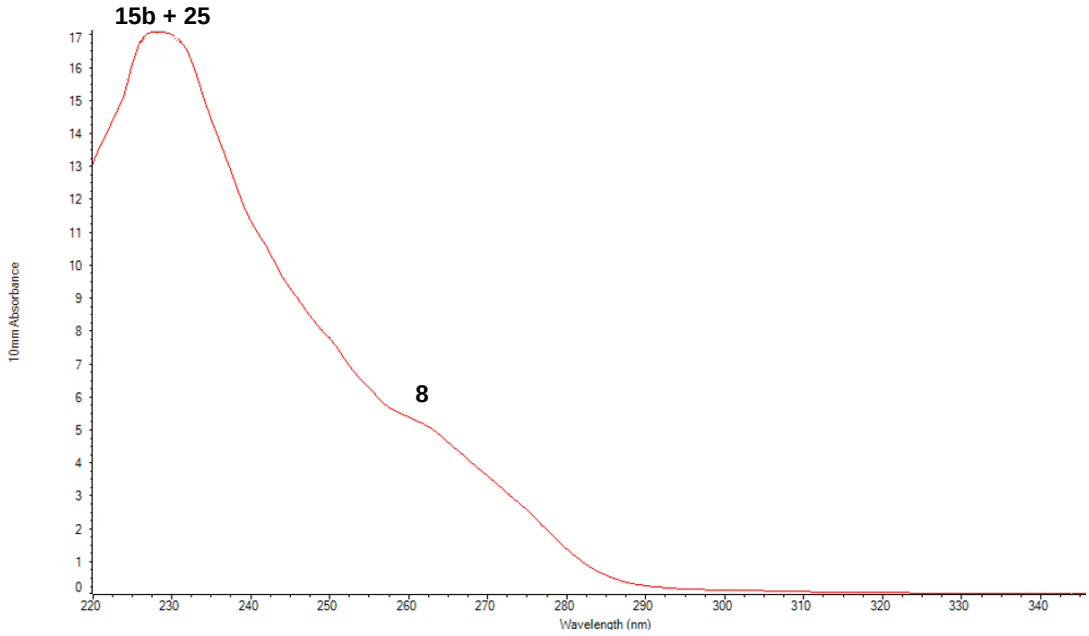


a) ^{31}P NMR spectrum of the phosphorylation mixture after 4 days; **b)** ^{31}P NMR spectrum of the phosphorylation mixture after 10 days; **c)** ^{31}P NMR spectrum of the phosphorylation mixture after 30 days. The spectra show that the formation of the cyclic phosphate **18a** is followed by the cyclization leading to **15b** and then to **25**.

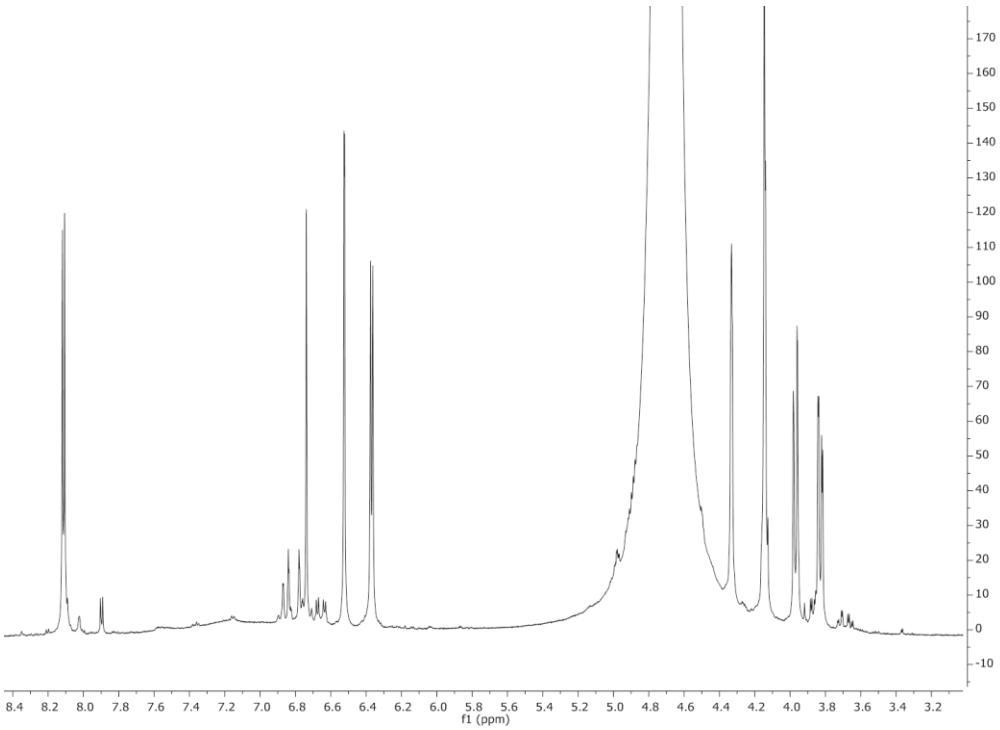
Supplementary Figure 62. High resolution Mass spectra of 15b obtained from DAP phosphorylation.



Supplementary Figure 63. UV-spectrum of the phosphorylation of 2-thiouridine 8 with DAP and formation of 3'-(amido-di)phosphate of 2,2'-anhydro-2-thiouridine 15b/25 in D_2O after 30 days.

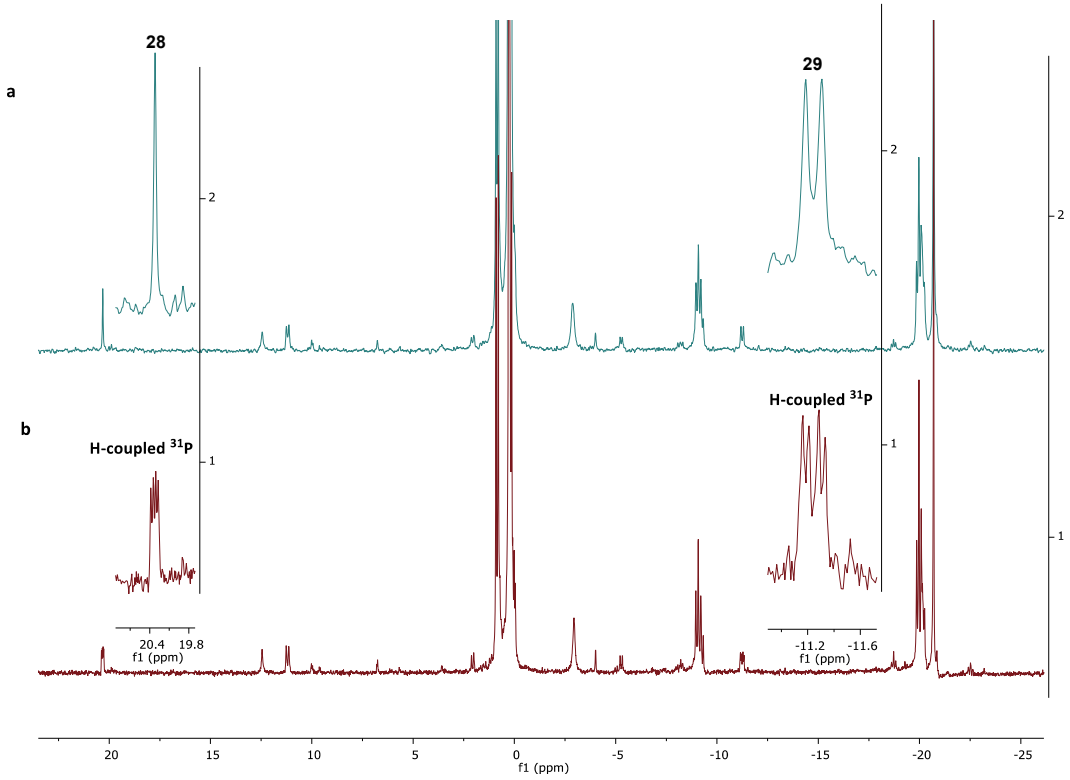


Supplementary Figure 64. ^{1}H NMR spectrum of the phosphorylation of 2-thiocytidine 5 with DAP.



The major signals of this spectrum represent the starting material 2-thiocytidine **5**. The cyclophosphate **28** and the 3'-amido-diphosphate of 2,2'-anhydro-2-thiocytidine **29** are superimposed.

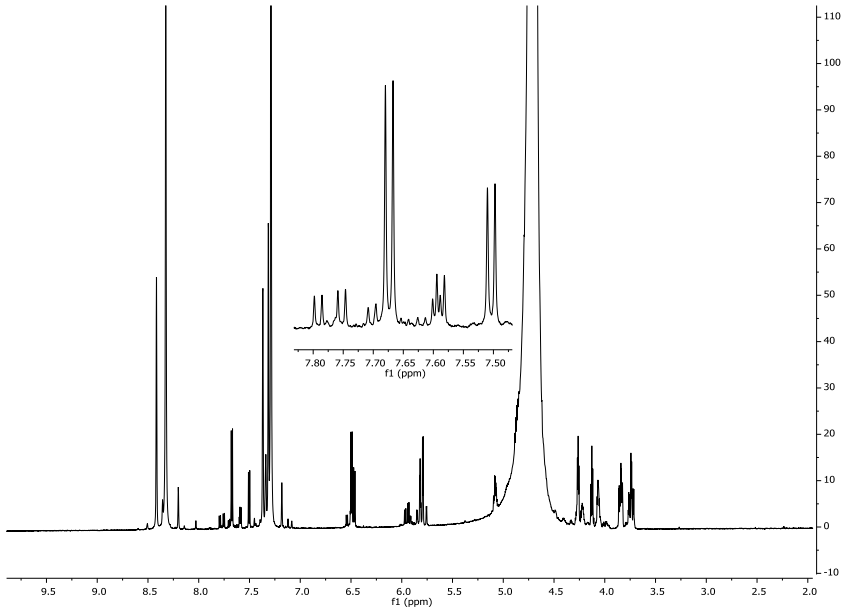
Supplementary Figure 65. ^{31}P NMR spectra of the phosphorylation of 2-thiocytidine 5 with DAP and formation of 28/29 in D_2O.



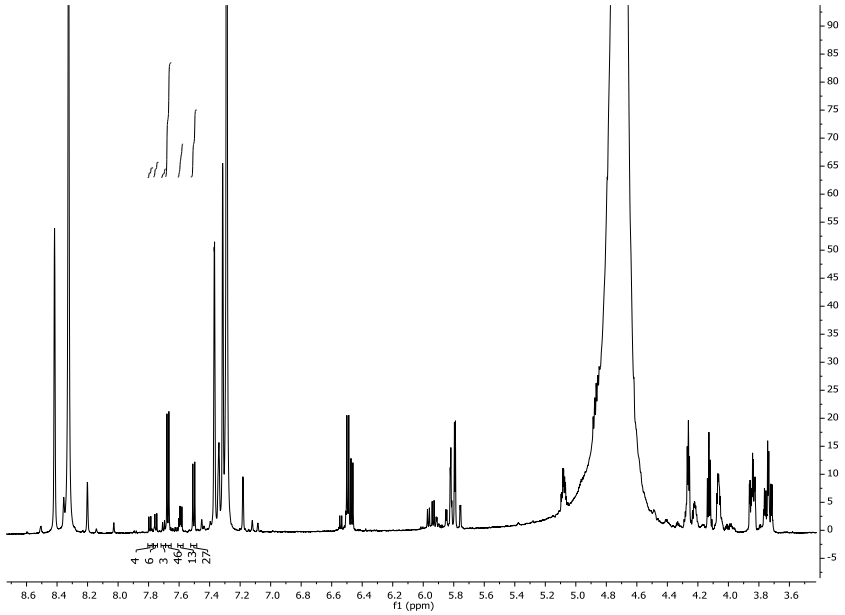
a) H-decoupled ^{31}P NMR spectrum of the phosphorylation mixture after a dry/wet cycle at pH 6; **b)** H-coupled ^{31}P NMR spectrum of the phosphorylation mixture after a dry/wet cycle at pH 6.

Supplementary Figure 66. ^{1}H NMR spectra of the phosphorylation of 4-thiouridine 26 with DAP.

a

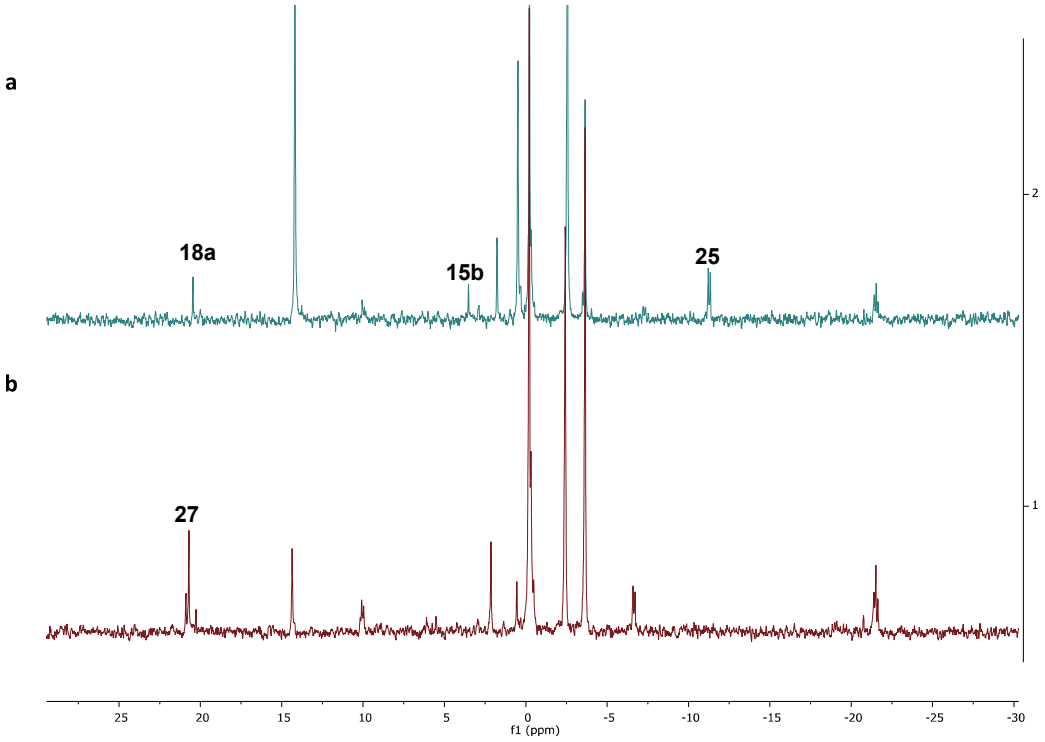


b



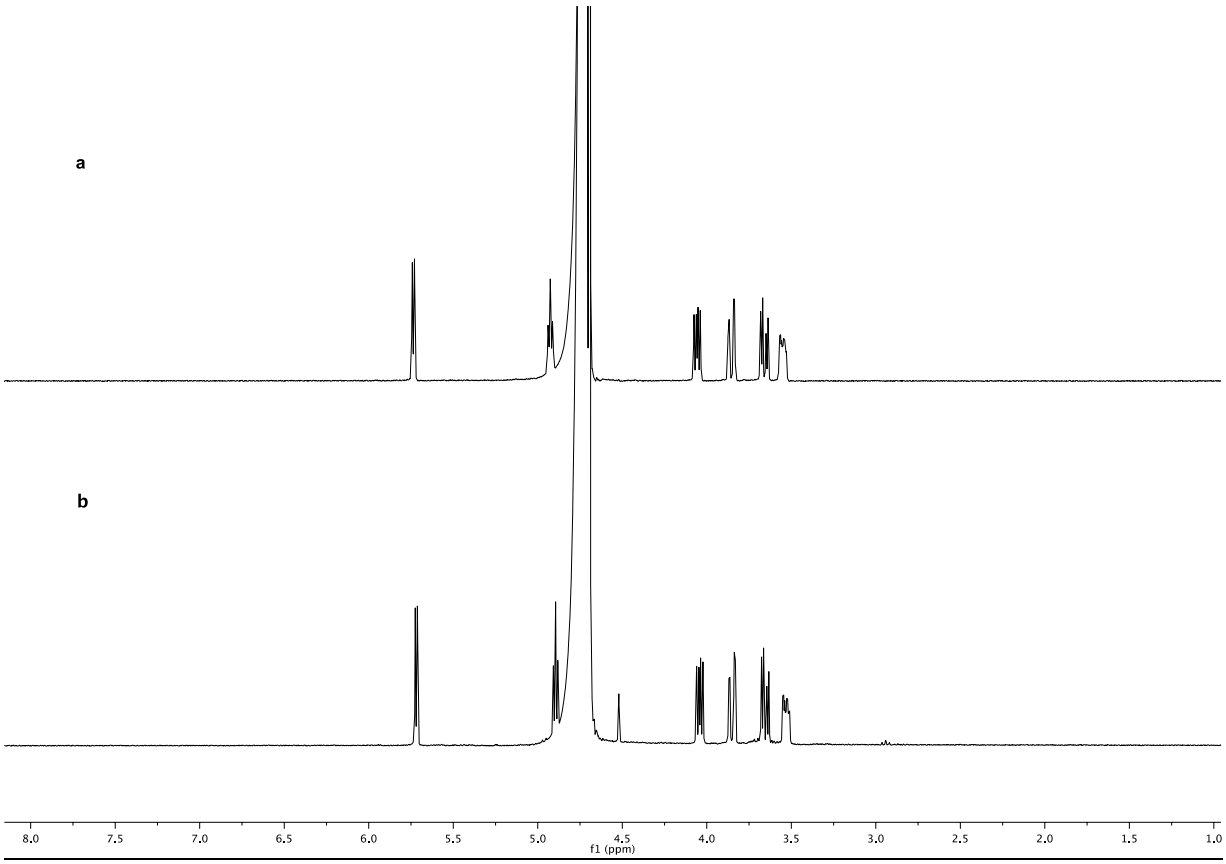
a) ^{1}H NMR spectrum of the phosphorylation mixture of 4-thiouridine **26** with DAP after 15 days with enlarged inserted spectrum; **b)** ^{1}H NMR spectrum of the phosphorylation mixture of 4-thiouridine **26** with DAP with integration for quantification.

Supplementary Figure 67. ^{31}P NMR spectra showing the comparison of the phosphorylation of 2-thiouridne 8 versus 4-thiouridine 26 with DAP.



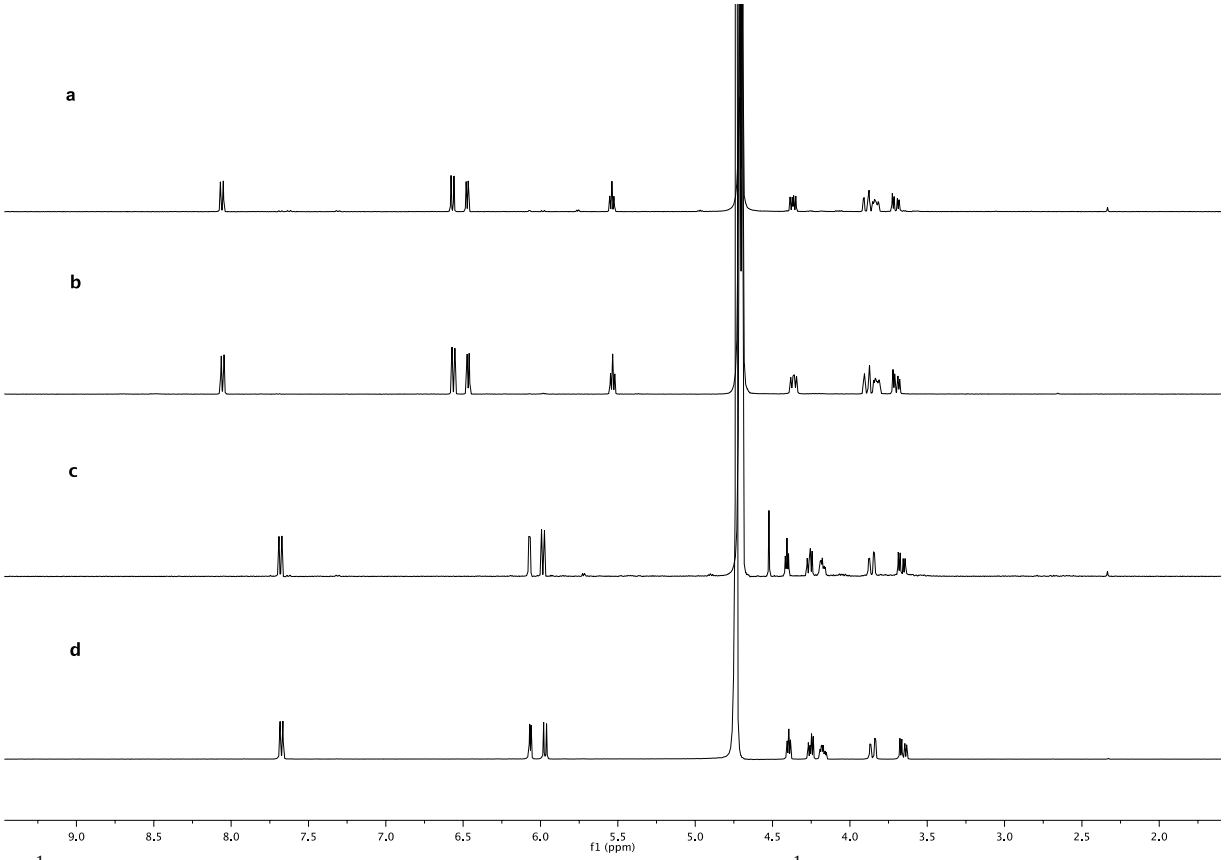
a) ^{31}P NMR spectrum of the phosphorylation mixture of 2-thiouridine **8** with DAP after 15 days; **b)** ^{31}P NMR spectrum of the phosphorylation mixture of 4-thiouridine **26** with DAP after 15 days.

Supplementary Figure 68. ^{1}H NMR spectra of 1 before and after irradiation.



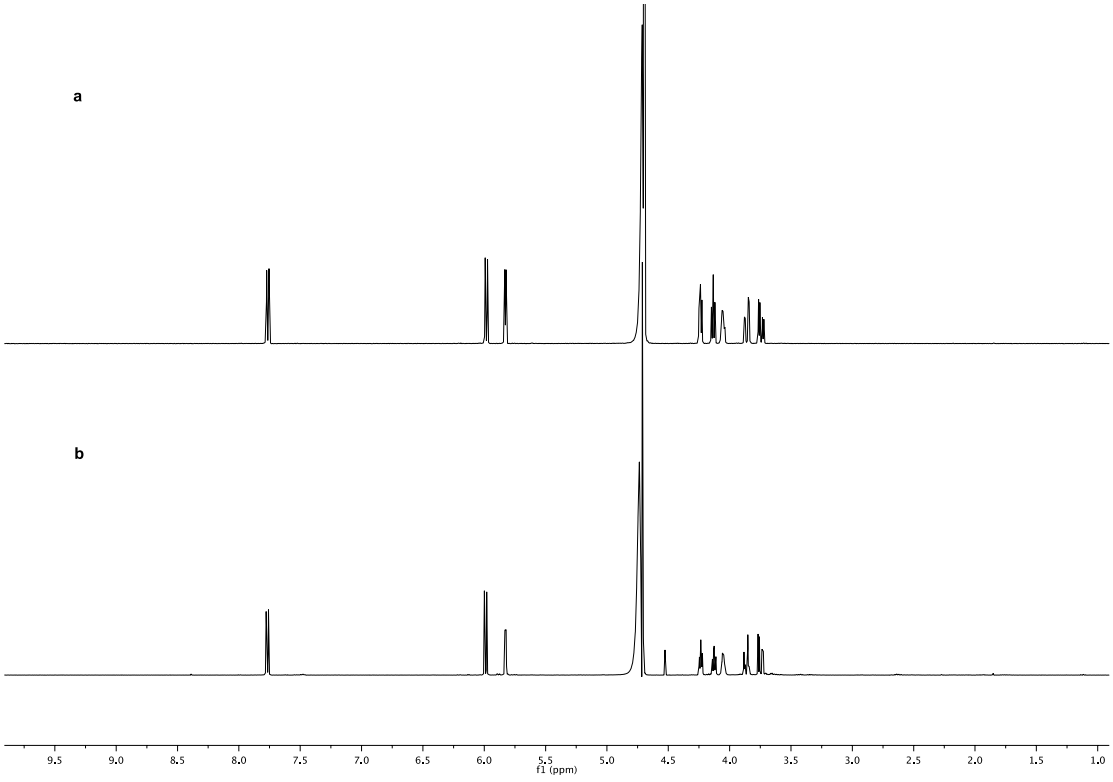
a) ^{1}H NMR spectrum of **1** before irradiation with hydrogen sulfide; **b)** ^{1}H NMR spectrum of **1** after irradiation with hydrogen sulfide for 3 hrs.

Supplementary Figure 69. ^{1}H NMR spectra of 3 before and after irradiation.



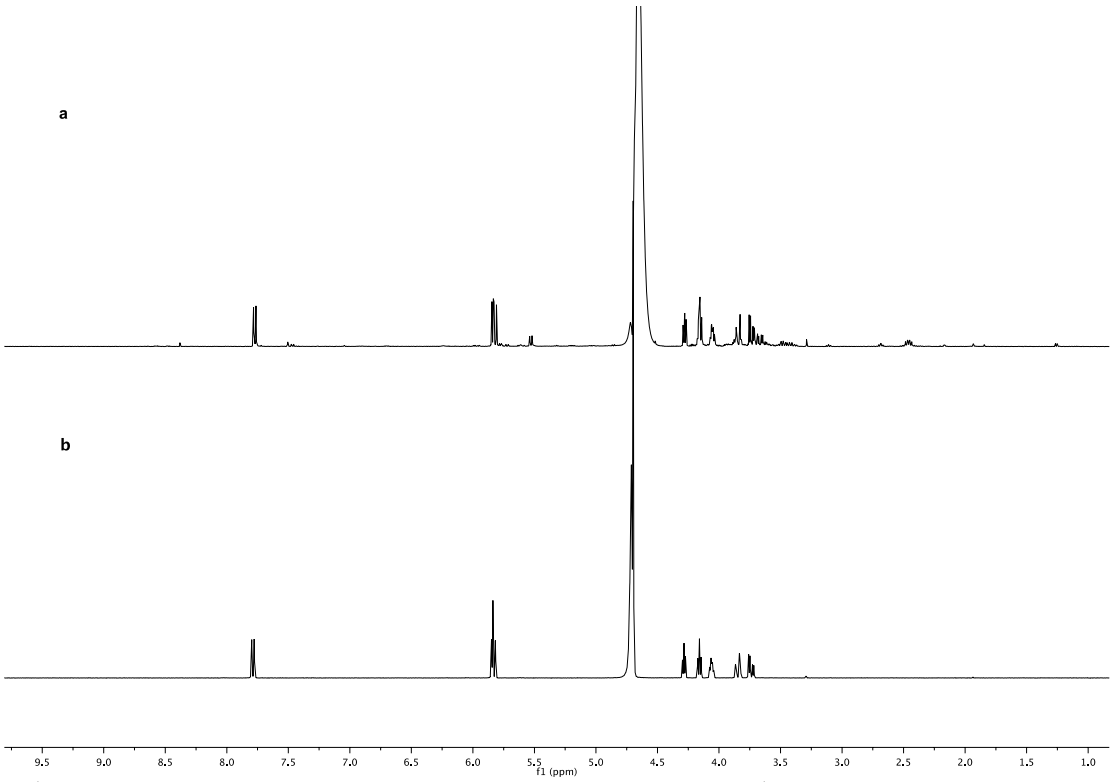
a) ^{1}H NMR spectrum of **3** before irradiation with hydrogen sulfide; **b)** ^{1}H NMR spectrum of **3** after irradiation without hydrogen sulfide for 3 hrs. **c)** ^{1}H NMR spectrum of **3** after irradiation with hydrogen sulfide for 3 hrs. (hydrolysed to α-cytidine**) d)** ^{1}H NMR spectrum of α-cytidine.

Supplementary Figure 70. ^{1}H NMR spectra of 6 before and after irradiation.



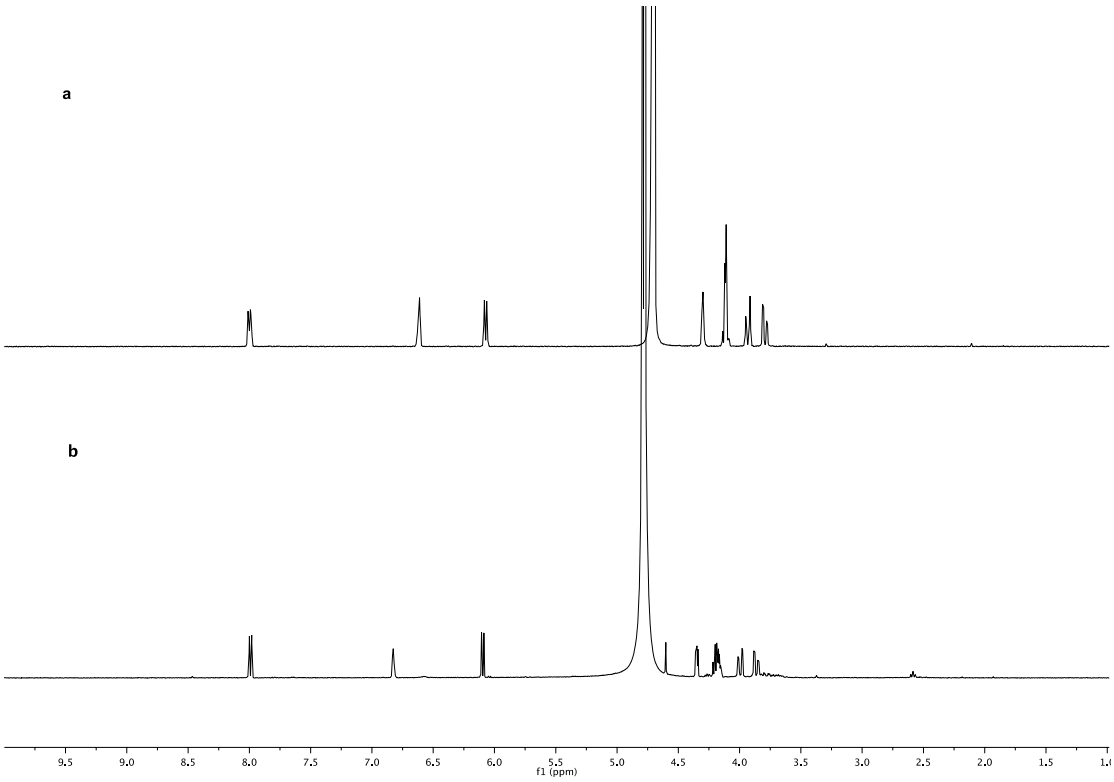
a) ^{1}H NMR spectrum of **6** before irradiation with hydrogen sulfide; **b)** ^{1}H NMR spectrum of **6** after irradiation with hydrogen sulfide for 3 hrs.

Supplementary Figure 71. ^{1}H NMR spectra of 7 before and after irradiation.



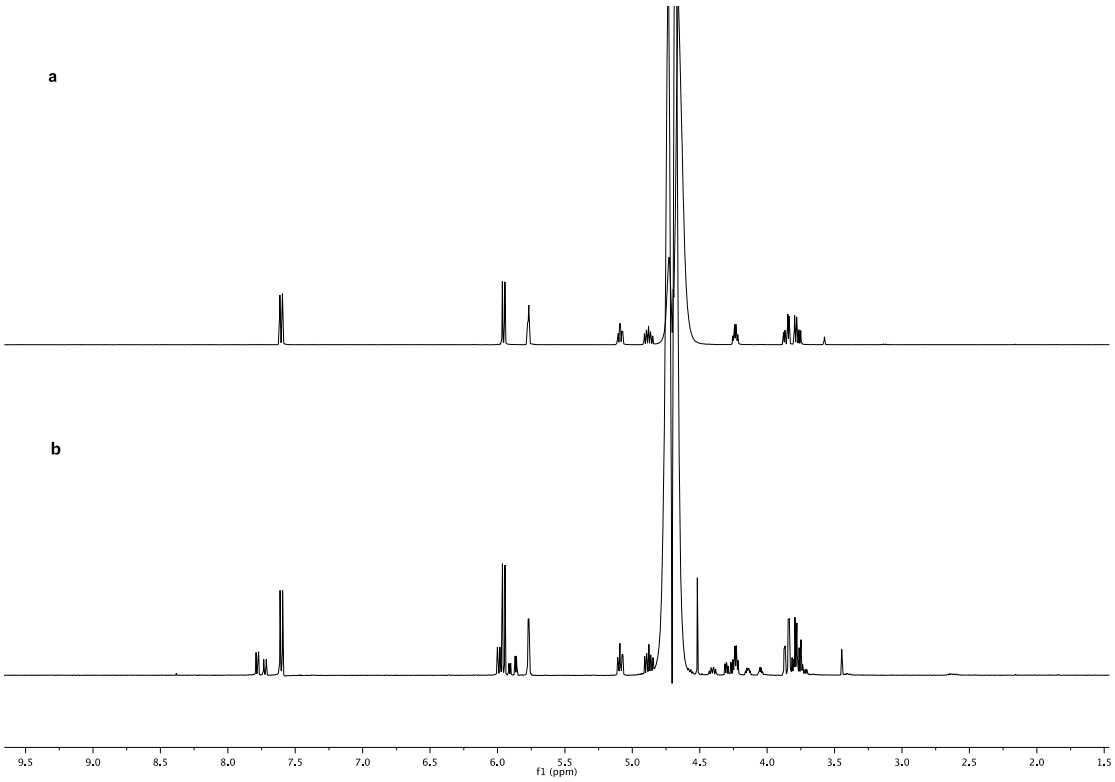
a) ^{1}H NMR spectrum of **7** before irradiation with hydrogen sulfide; **b)** ^{1}H NMR spectrum of **7** after irradiation with hydrogen sulfide for 3 hrs.

Supplementary Figure 72. ^{1}H NMR spectra of 8 before and after irradiation.



a) ^{1}H NMR spectrum of **8** before irradiation with hydrogen sulfide; **b)** ^{1}H NMR spectrum of **8** after irradiation with hydrogen sulfide for 3 hrs.

Supplementary Figure 73. ^{1}H NMR spectra of 9 before and after irradiation.



a) ^{1}H NMR spectrum of **9** before irradiation with hydrogen sulfide; **b)** ^{1}H NMR spectrum of **9** after irradiation with hydrogen sulfide for 3 hrs. (hydrolysed to cytidine-2'-phosphate and cytidine-3'-phosphate in 34% yield)

References

1. Rimmer, P. B. *et al.* The origin of RNA precursors on exoplanets. *Science Advances* **4**, doi:10.1126/sciadv.aar3302 (2018).

2. Gibard, C., Bhowmik, S., Karki, M., Kim, E. K. & Krishnamurthy, R. Phosphorylation, oligomerization and self-assembly in water under potential prebiotic conditions. *Nat. Chem.* **10**, 212-217 (2018).