

Supporting Information for Nucleoside Tetra- and Pentaphosphates Prepared Using a Tetraphosphorylation Reagent are Potent Inhibitors of Ribonuclease A

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1 General Considerations

All chemical syntheses were performed in a Vacuum Atmospheres model MO-40M glovebox under an inert atmosphere of purified nitrogen unless otherwise noted. All solvents used in the glovebox were obtained anhydrous and oxygen-free by the method of Grubbs,¹

bubble degassing with argon and purification through columns of alumina and Q5 purchased from SG Water. HPLC grade water was purchased from VWR and used as received. $[\text{PPN}]_2[\text{P}_4\text{O}_{11}]$,² $[\text{PPN}]_2[\text{P}_4\text{O}_{12}\text{H}_2]$,² $[\text{TBA}]_2\text{AMP}$,³ $[\text{TBA}]_2\text{UMP}$,³ and hexaguani-dinium tetraphosphate⁴ were synthesized according to literature procedures. All other reagents were purchased and used as received. Deuterated solvents were purchased from Cambridge Isotope Labs and used as received. NMR spectra were obtained at ambient temperature (ca. 25 °C) on a Bruker Avance 400 instrument. ^1H and ^{13}C NMR spectra were referenced internally to residual solvent signals. ^{31}P NMR spectra were referenced internally to the PPN signal. Electrospray ionization mass spectra (ESI-MS(−)) were acquired on a Micromass Q-TOF ESI spectrometer. Samples were prepared in acetonitrile at an approximate concentration of 20 ng/ μL , and a source temperature of 100 °C and desolvation gas temperature of 150 °C were used. HPLC was performed with Waters 515 HPLC pumps coupled to a Waters 2487 UV absorbance detector and a Macherey-Nagel VP 250/21 Nucleosil 100-5 C18 column. HPLC purifications were carried out with a gradient method from 100% A, 0% B to 50% A, 50% B (A = 95% water, 5% acetonitrile, 50 mM triethylammonium acetate; B = 5% water, 95% acetonitrile, 50 mM triethylammonium acetate) with a 12 mL/min flow rate and monitored by absorbance at 262 nm, in analogy to literature conditions.⁵ Ribonuclease A was purchased from Sigma-Aldrich (product R6513) and was used without further purification. Assays were performed as described previously with a 6-FAM-dArUdAdA-6-TAMRA substrate⁶ obtained from Integrated DNA Technologies and used without further purification.

2 Chemical Synthesis

2.1 Synthesis of $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$

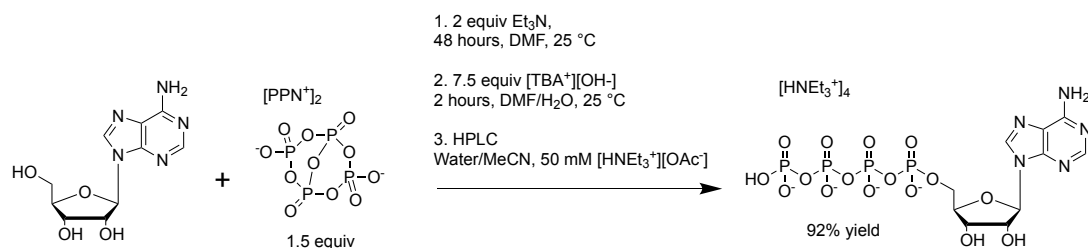


Figure S1: Synthesis of $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$

In the glovebox, a stock solution of adenosine in DMF at a concentration of 0.017 g/mL was stored over activated 4 Å sieves for at least 24 hours. Then, $[\text{PPN}]_2[\text{P}_4\text{O}_{11}]$ (0.084 g, 0.061 mmol) was dissolved in 1 mL of DMF. To this solution was added triethylamine (0.011 mL, 0.082 mmol) followed by the DMF solution of adenosine (0.63 mL, 0.041 mmol). This solution was then stirred at ca. 25 °C for 48 hours. The mixture was then removed from the box and a solution of 40% aqueous tetrabutylammonium hydroxide (0.20 g, 0.31 mmol)

was added. The mixture was then stirred for 2 hours, after which all volatile materials were removed under vacuum on a Schlenk line, giving an oily solid. To this solid was added 2 mL of high purity water. The resulting suspension was stirred for 15 minutes. The resulting solution was filtered through an Acrodisc 0.2 μm wwPTFE syringe filter and frozen until HPLC purification. Prior to HPLC purification, the sample was thawed and again filtered through an Acrodisc 0.2 μm wwPTFE syringe filter. The sample was then purified by HPLC according to the conditions given in the General Considerations above. Fractions containing the desired product were pooled and all volatile materials were removed under vacuum at 40 °C on a Schlenk line to give an oil. The resulting oil was redissolved in water and all volatile materials were again removed at 40 °C under reduced pressure. Redissolution in water and removal of all volatile materials under reduced pressure at 40 °C was repeated twice more to remove all volatile triethylammonium acetate buffer, giving the product as a colorless solid (0.037 g, 0.038 mmol, 92% yield).

^1H NMR (D_2O , 400 MHz) δ 8.59 (s, 1H), 8.33 (s, 1H), 6.14 (d, 1H), 4.60 (m, 2H), 4.41 (m, 1H), 4.26 (m, 2H), 3.19 (q, 24H), 1.27 (t, 36H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (D_2O , 100.6 MHz) δ 153.3 (s), 149.8 (s), 140.9 (s), 87.0 (s), 84.35 (d), 74.4 (s), 70.4 (s), 65.25 (d), 46.6 (s), 8.2 (s).

$^{31}\text{P}\{^1\text{H}\}$ NMR (D_2O , 162.0 MHz) δ -10.9 (d, J_{PP} = 18.2 Hz), -11.6 (d, J_{PP} = 18.5 Hz), -23.3 (m, J_{PP} = 18.2, 18.5).

ESI-MS(-) of $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$ in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, found 586.02 m/z (calc'd 585.95 m/z) for $\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{16}\text{P}_4^-$

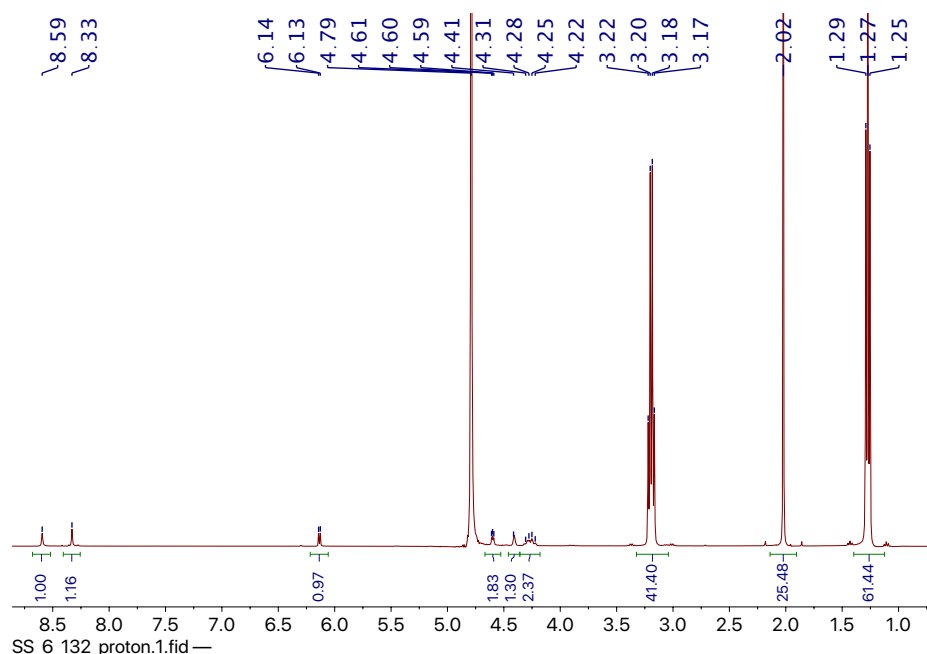


Figure S2: ^1H NMR spectrum of $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$ (D_2O , 400 MHz) with approximately three equivalents of residual triethylammonium acetate buffer

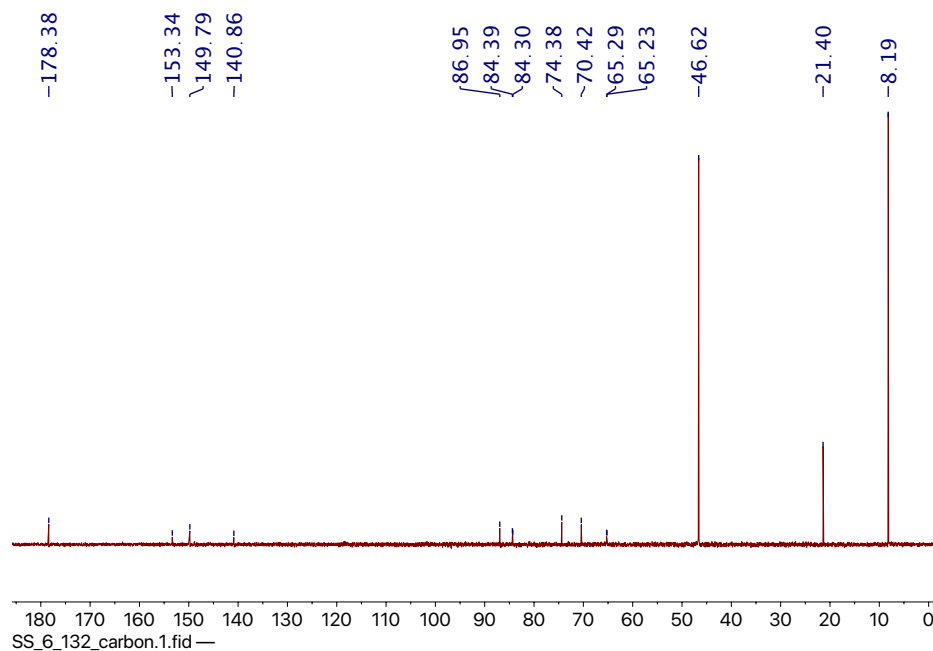


Figure S3: ^{13}C NMR spectrum of $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$ (D_2O , 100.6 MHz) with approximately three equivalents of residual triethylammonium acetate buffer

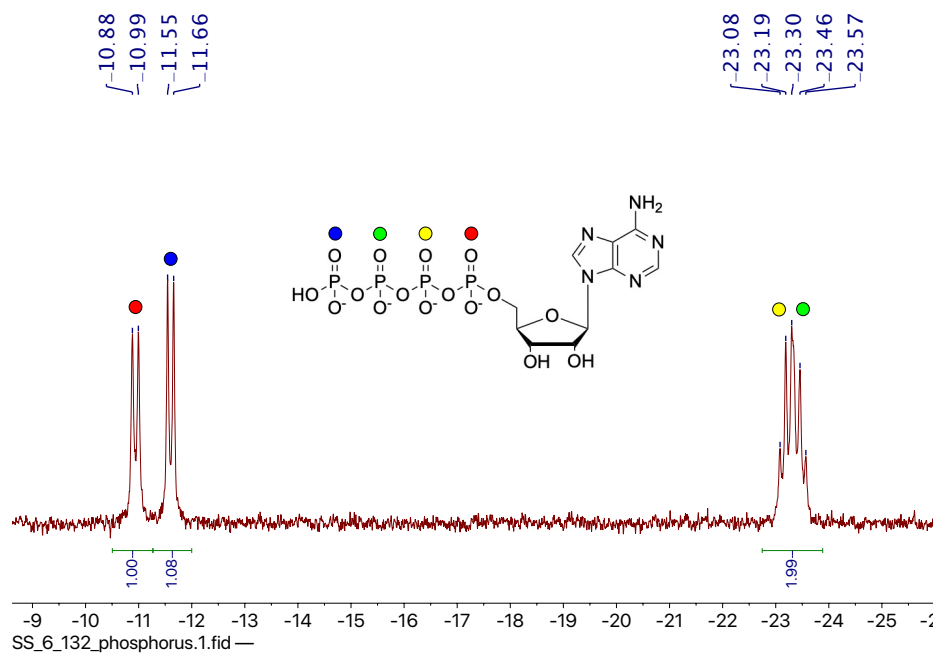


Figure S4: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$ (D_2O , 162.0 MHz), no other resonances were observed in the 200 to -200 ppm spectral window

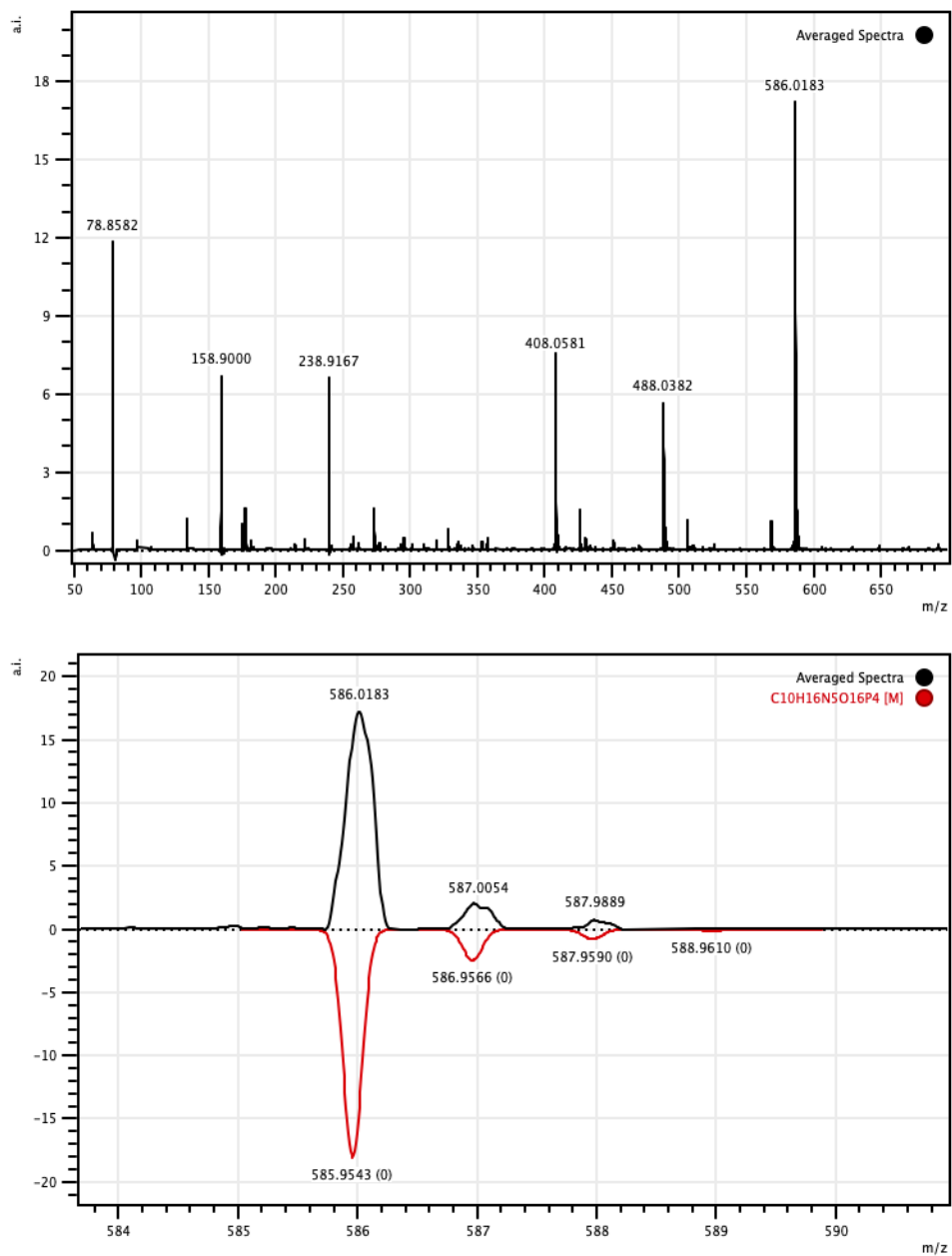


Figure S5: ESI-MS(–) of $[HNEt_3]_4[Hp_4A]$ in CH_3CN/H_2O with zoomed in portion showing the molecular ion ($H_4P_4A^-$, top) and calculated isotope pattern ($C_{10}H_{16}N_5O_{16}P_4^-$, bottom)

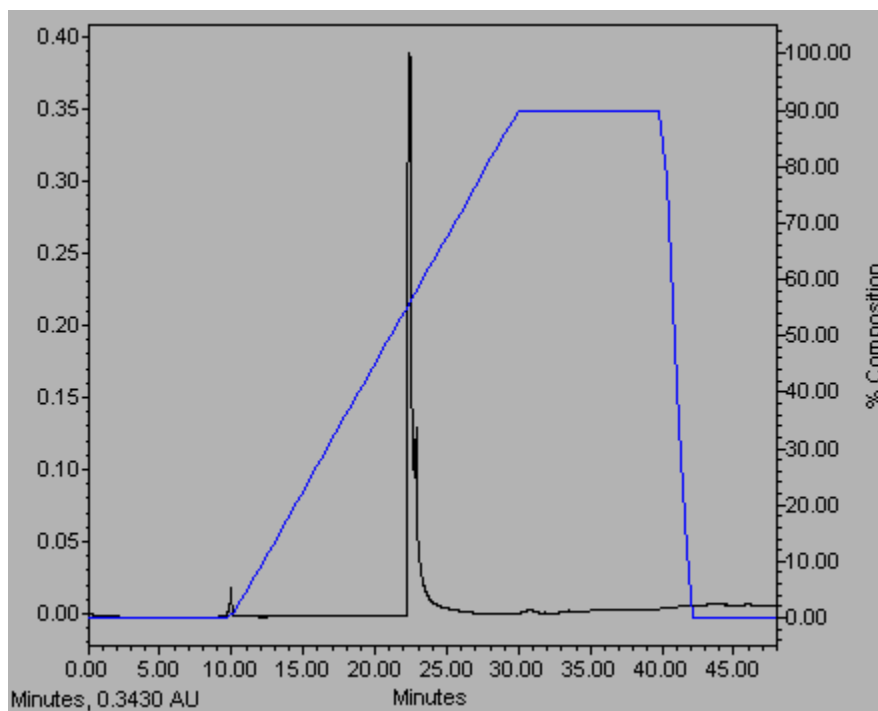


Figure S6: HPLC trace of purified $[\text{HNEt}_3]_4[\text{Hp}_4\text{A}]$. The species eluted at 9 min is a ghost peak due to a minor impurity in solvent A. The product peak integrates to 98.4%

2.2 Synthesis of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$

Method A:

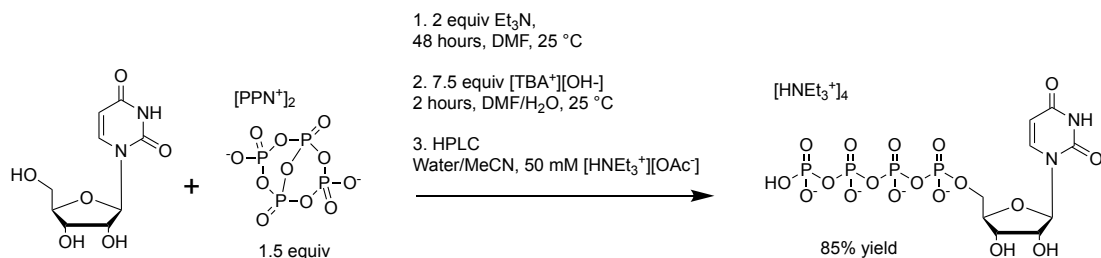


Figure S7: Synthesis of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$ (Method A)

In the glovebox, a stock solution of uridine in DMF at a concentration of 0.12 g/mL was stored over activated 4 Å sieves for at least 24 hours. Then, $[\text{PPN}]_2[\text{P}_4\text{O}_{11}]$ (0.084 g, 0.061 mmol) was dissolved in 1 mL of DMF. To this solution was added triethylamine (0.011 mL, 0.080 mmol) followed by the DMF solution of uridine (0.081 mL, 0.040 mmol). This solution was then stirred at ca. 25 °C for 48 hours. The mixture was then removed from the box and a solution of 40% aqueous tetrabutylammonium hydroxide (0.19 g, 0.30 mmol)

was added. The mixture was then stirred for 2 hours, after which all volatile materials were removed under reduced pressure on a Schlenk line, giving an oily solid. To this solid was added 2 mL of high purity water. The resulting suspension was stirred for 15 minutes. The resulting solution was filtered through an Acrodisc 0.2 μm wwPTFE syringe filter and frozen until HPLC purification. Prior to HPLC purification, the sample was thawed and again filtered through an Acrodisc 0.2 μm wwPTFE syringe filter. The sample was then purified by HPLC according to the conditions given in the General Considerations above. Fractions containing the desired product were pooled and solvent was evaporated at 40 °C under reduced pressure on a Schlenk line to give an oil. The resulting oil was redissolved in water and all volatile materials were removed at 40 °C under reduced pressure. Redissolution in water and removal of all volatile material at 40 °C was repeated twice more to remove all volatile triethylammonium acetate buffer, giving the product as a colorless solid (0.033 g, 0.034 mmol, 85% yield).

Method B:

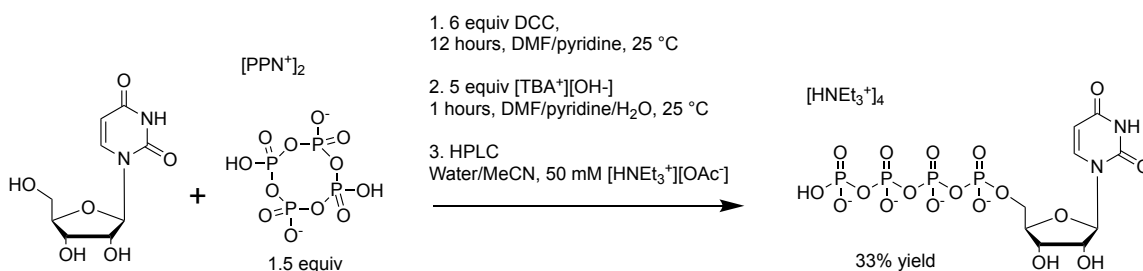


Figure S8: Synthesis of [HNEt₃]₄[Hp₄U] (Method B)

Under an anhydrous nitrogen atmosphere one Schlenk flask was charged with dry DMF (3 mL), and another Schlenk flask was charged with dry pyridine (3 mL). To the flask containing DMF was added [PPN]₂[P₄O₁₂H₂] (0.18 g, 0.13 mmol) and dicyclohexylcarbodiimide (DCC, 0.054 g, 0.26 mmol) against a flow of nitrogen. To the flask containing pyridine was added uridine (0.022 g, 0.088 mmol) and DCC (0.054 g, 0.26 mmol) against a flow of nitrogen. The two mixtures were stirred for 30 minutes during which time dicyclohexylurea (DCU) formed as a precipitate. The pyridine solution was then transferred under an inert nitrogen atmosphere to a Schlenk flask containing the DMF solution. This mixture was then stirred for 12 hours at ambient temperature. The mixture was then exposed to air and [TBA][OH] (40% in water, 0.29 g, 0.44 mmol) in water (2 mL) was added. The mixture was then stirred for 1 hour, after which all volatile materials were removed under reduced pressure on a Schlenk line, giving an oily solid. To this solid was added 2 mL of HPLC grade water. The resulting suspension was stirred for 15 minutes. The resulting solution was filtered through an Acrodisc 0.2 μm wwPTFE syringe filter and frozen until HPLC purification. Prior to HPLC purification, the sample was thawed and again filtered through an Acrodisc 0.2 μm wwPTFE syringe filter. The sample was then purified by HPLC according to the conditions given in the General Considerations above. Fractions containing the desired product were pooled and solvent was evaporated at 40 °C under reduced pressure on a Schlenk line to give an oil. The resulting oil was redissolved in water and all volatile materials were removed at 40 °C under reduced pressure. Redissolution in water and removal of all volatile material

at 40 °C was repeated twice more to remove all volatile triethylammonium acetate buffer, giving the product as a colorless solid (0.029 g, 0.029 mmol, 33% yield).

^1H NMR (D_2O , 400 MHz) δ 7.98 (d, 1H), 6.02 (d, 1H), 5.99 (d, 1H), 4.43 (m, 2H), 4.27 (m, 2H), 3.21 (q, 24H), 1.29 (t, 36H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (D_2O , 100.6 MHz) δ 166.2 (s), 151.9 (s), 141.7 (s), 102.8 (s), 87.9 (s), 83.5 (d), 73.6 (s), 69.8 (s), 65.1 (d), 46.6 (s), 34.5 (s), 22.3 (s), 8.2 (s).

$^{31}\text{P}\{^1\text{H}\}$ NMR (D_2O , 162.0 MHz) δ -10.9 (d, $J_{\text{PP}} = 19.2$ Hz), -11.7 (d, $J_{\text{PP}} = 18.8$ Hz), -23.4 (m, $J_{\text{PP}} = 19.2, 18.8$).

ESI-MS(-) of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$ in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, found 563.02 m/z (calc'd 562.93 m/z) for $\text{C}_9\text{H}_{15}\text{N}_2\text{O}_{18}\text{P}_4^-$

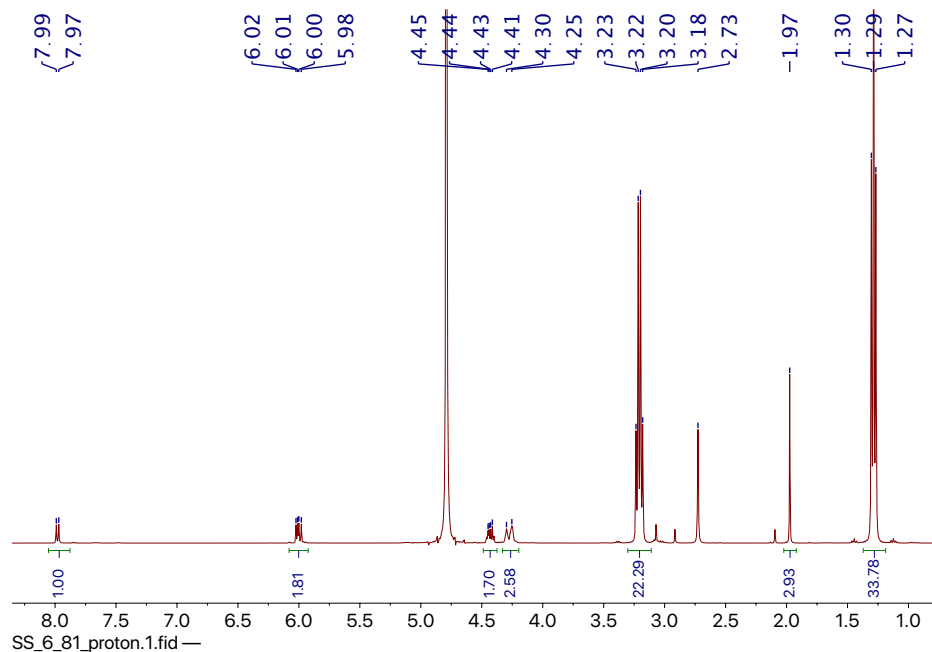


Figure S9: ^1H NMR spectrum of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$ (D_2O , 400 MHz)

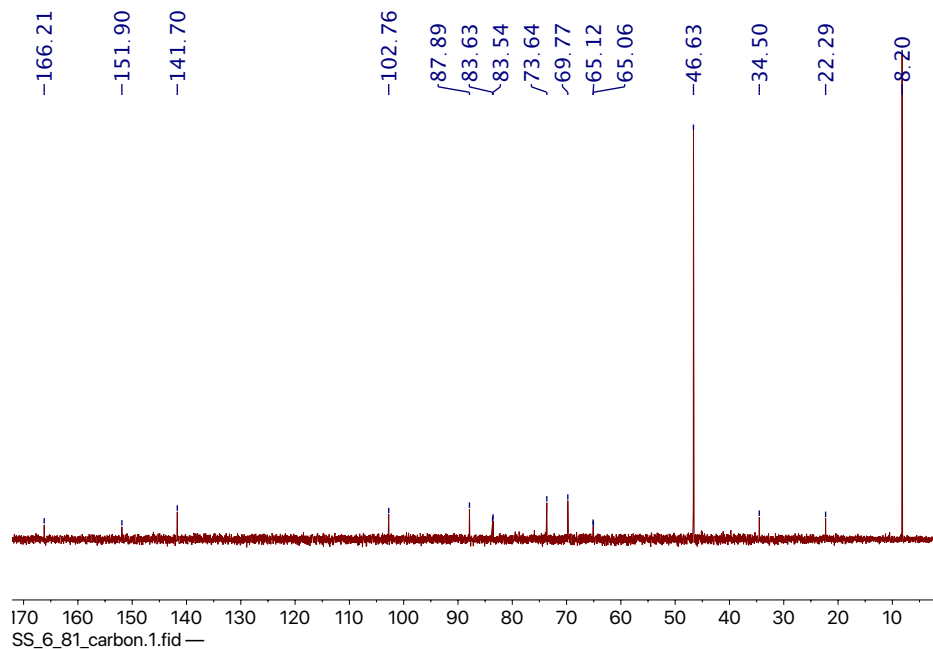


Figure S10: ^{13}C NMR spectrum of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$ (D_2O , 100.6 MHz)

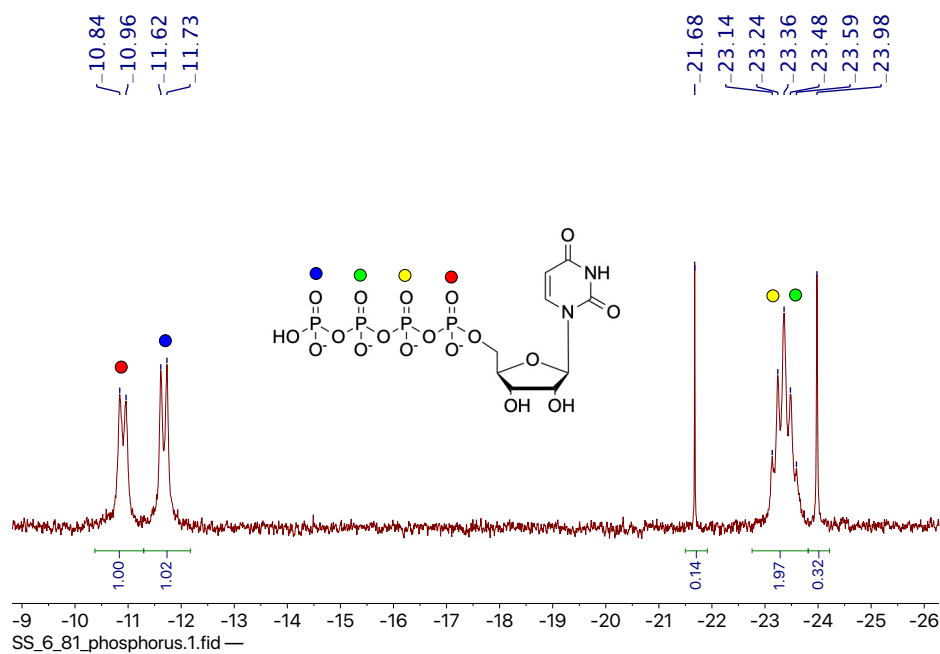


Figure S11: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$ (D_2O , 162.0 MHz) with residual trimetaphosphate (-21.7 ppm) and tetrametaphosphate (-24.0 ppm) signals, no other resonances were observed in the spectral window 200 ppm to -200 ppm

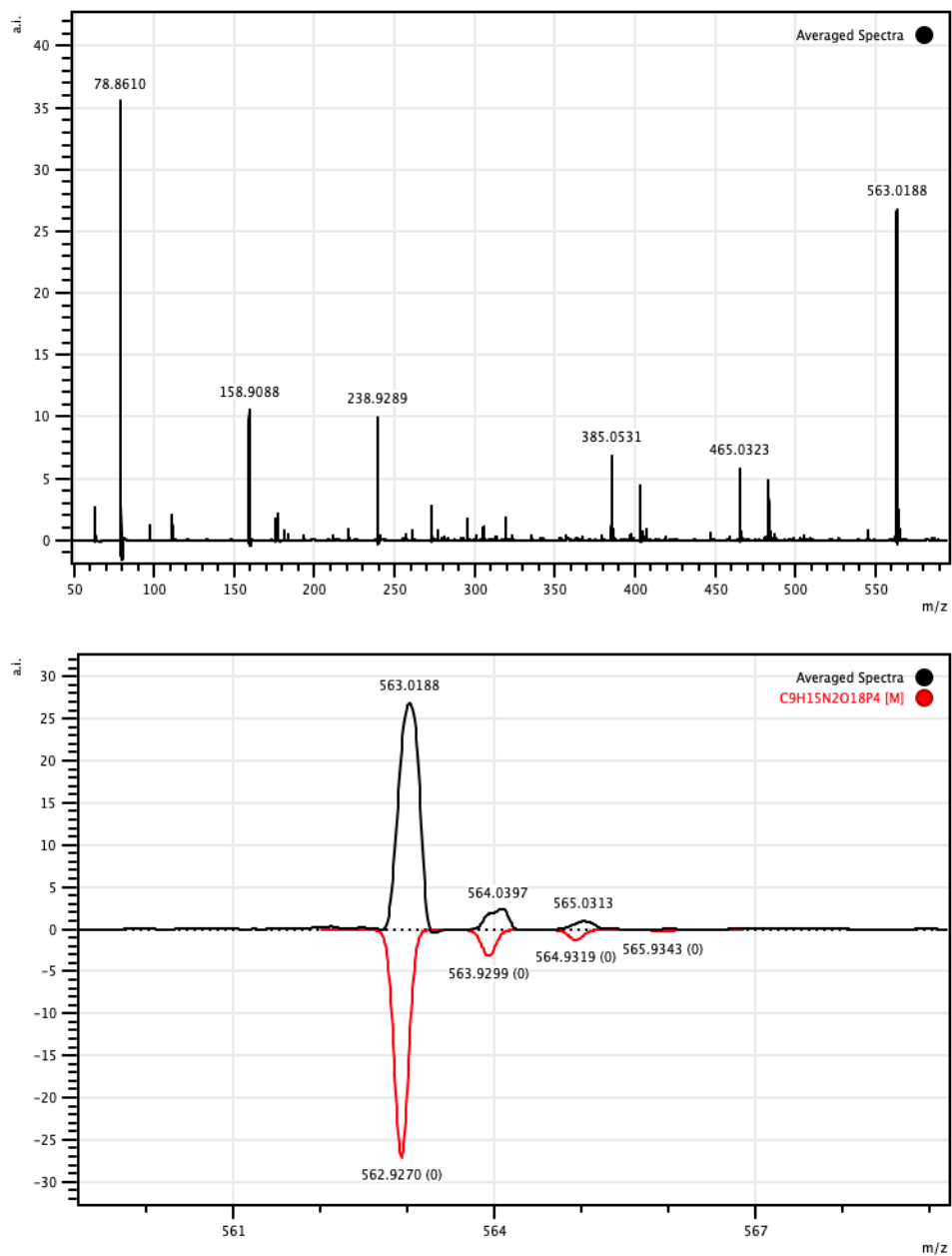


Figure S12: ESI-MS(–) of $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$ in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ with zoomed in portion showing the molecular ion ($\text{H}_4\text{P}_4\text{U}^-$, top) and calculated isotope pattern ($\text{C}_9\text{H}_{15}\text{N}_2\text{O}_{18}\text{P}_4^-$, bottom)

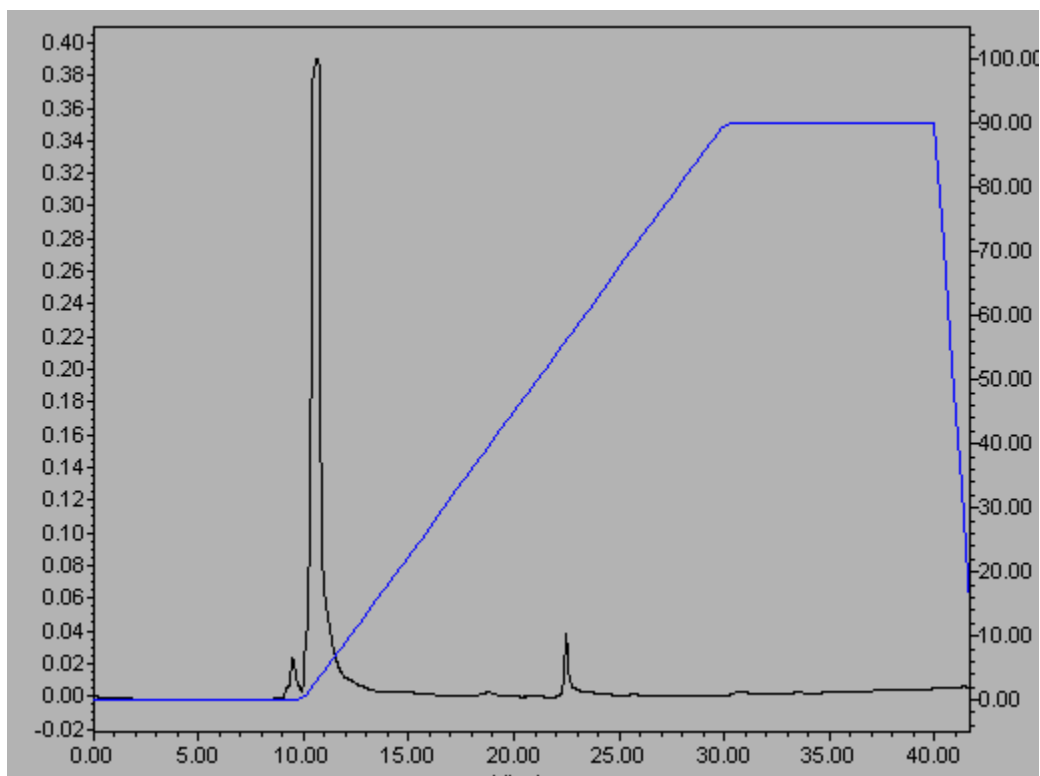


Figure S13: HPLC trace of purified $[\text{HNEt}_3]_4[\text{Hp}_4\text{U}]$. The species eluted at 9 min is a ghost peak due to a minor impurity in solvent A. The species eluted at 23 min may correspond to UMP or U formed during prolonged storage of this compound. The product peak integrate to 93.2%

2.3 Synthesis of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pA}]$

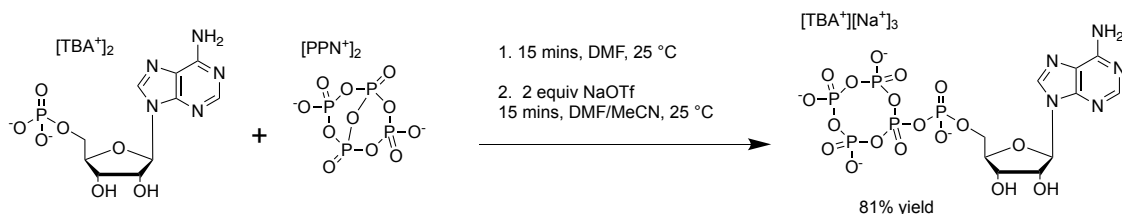


Figure S14: Synthesis of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pA}]$

A solution of $[\text{TBA}]_2[\text{AMP}]^3$ in DMF at a concentration of 0.10 g/mL was stored over activated 4 Å sieves for at least 24 hours in the glovebox. $[\text{PPN}]_2[\text{P}_4\text{O}_{11}]$ (0.14 g, 0.10 mmol) was then dissolved in 1 mL of DMF. To this was added the DMF solution of $[\text{TBA}]_2[\text{AMP}]$ (0.85 mL, 0.10 mmol), and the mixture was stirred for 15 minutes. To this solution was then added a solution of sodium triflate (0.35 g, 0.20 mmol) dissolved in 1 mL of acetonitrile, inducing precipitation. The resulting mixture was stirred for 15 minutes. The reaction

vessel was then removed from the glovebox and all volatile materials were removed under reduced pressure on a Schlenk line at ca. 25 °C, giving an oily solid. This solid was then suspended in 2 mL of high purity water and the resulting suspension was stirred for 15 minutes. The resulting solution was filtered through an Acrodisc 0.2 μm wwPTFE syringe filter and lyophilized, giving the product as a colorless solid (0.079 g, 0.082 mmol, 81% yield). ^1H NMR integration reveals the product contains one equivalent of tetrabutylammonium counterion per equivalent of nucleoside with three protons or sodium counterions to provide charge balance. Above 0 °C, the product decomposes to a mixture of tetrametaphosphate, adenosine monophosphate, and adenosine pentaphosphate over the course of several hours, preventing further purification.

^1H NMR (D_2O , 400 MHz) δ 8.59 (s, 1H), 8.33 (s, 1H), 6.14 (d, 1H), 4.60 (m, 2H), 4.41 (m, 1H), 4.26 (m, 2H), 3.21 (m, 8H), 1.65 (tt, 8H), 1.37 (tq, H), 0.96 (t, 12H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (D_2O , 100.6 MHz) δ 150.4 (s), 148.3 (s), 141.9 (s), 87.7 (s), 84.0 (d), 74.6 (s), 70.3 (s), 65.9 (d), 36.9 (s), 23.1 (s), 19.1 (s), 12.8 (s).

$^{31}\text{P}\{^1\text{H}\}$ NMR (D_2O , 162.0 MHz) δ -12.0 (J_{PP} = 15.5 Hz), -24.7 (J_{PP} = 26.1 Hz), -25.6 (J_{PP} = 26.1, 22.3 Hz), -36.9 (J_{PP} = 22.3, 15.5 Hz)

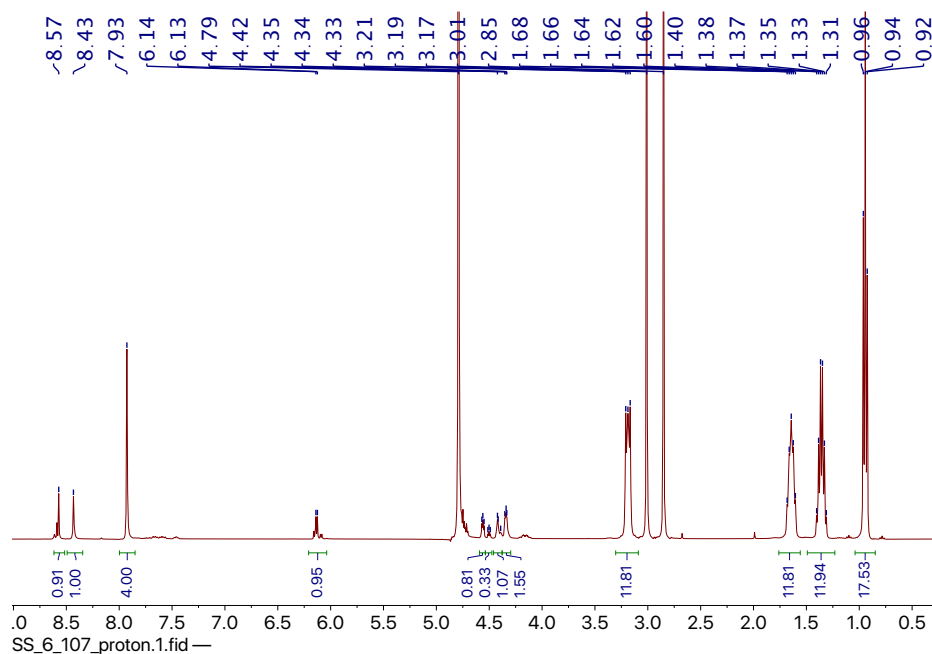


Figure S15: ^1H NMR spectrum of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pA}]$ (D_2O , 400 MHz)

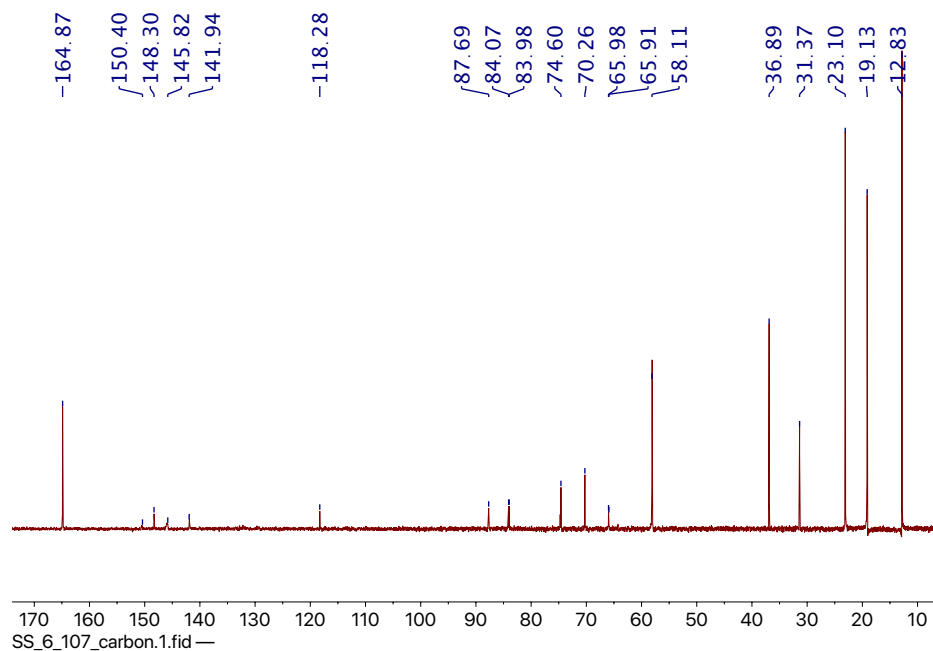


Figure S16: ^{13}C NMR spectrum of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pA}]$ (D_2O , 100.6 MHz)

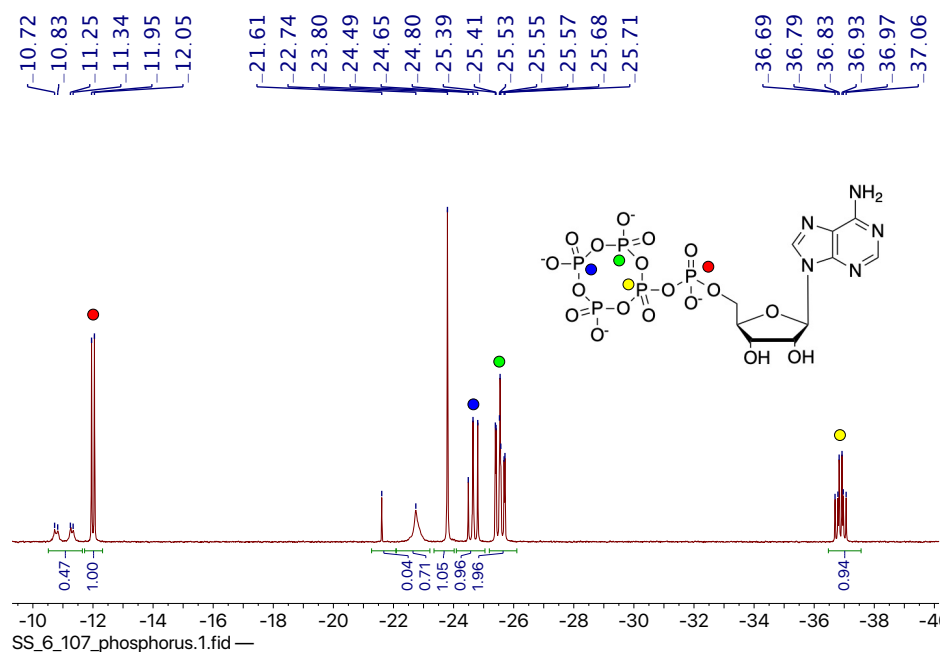


Figure S17: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pA}]$ (D_2O , 162.0 MHz), the other resonances correspond to trimetaphosphate, tetrametaphosphates and p_5A due to the slow hydrolysis of cp_4pA , no other resonances were observed in the spectral window 200 ppm to -200 ppm

2.4 Synthesis of [TBA][Na]₃[cp₄pU]

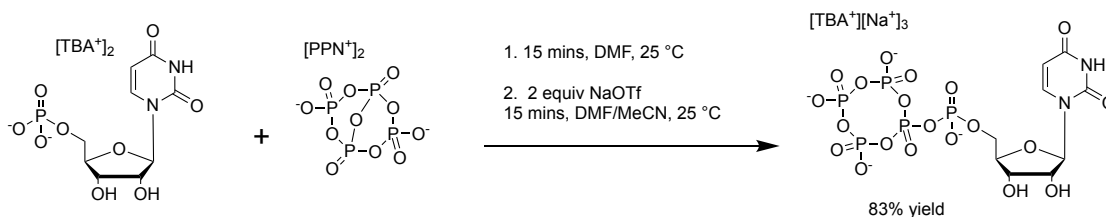


Figure S18: Synthesis of [TBA][Na]₃[cp₄pU]

A solution of [TBA]₂[UMP]³ in DMF at a concentration of 0.045 g/mL was stored over activated 4 Å sieves for at least 24 hours in the glovebox. [PPN]₂[P₄O₁₁] (0.069 g, 0.050 mmol) was then dissolved in 1 mL of DMF. To this was added the DMF solution of [TBA]₂[UMP] (0.90 mL, 0.050), and the mixture was stirred for 15 minutes. To this solution was then added a solution of sodium triflate (0.017 g, 0.10 mmol) dissolved in 1 mL of acetonitrile, inducing precipitation. The resulting mixture was stirred for 15 minutes. The reaction vessel was then removed from the glovebox and all volatile materials were removed under reduced pressure on a Schlenk line at ca. 25 °C, giving an oily solid. This solid was then suspended in 2 mL of high purity water and the resulting suspension was stirred for 15 minutes. The resulting solution was filtered through an Acrodisc 0.2 μm wwPTFE syringe filter and lyophilized, giving the product as a colorless solid (0.039 g, 0.042 mmol, 83% yield). ¹H NMR integration reveals the product contains one equivalent of tetrabutylammonium counterion per equivalent of nucleoside with three protons or sodium counterions to provide charge balance. Above 0 °C, the product decomposes to a mixture of tetrametaphosphate, uridine monophosphate, and uridine pentaphosphate over the course of several hours, preventing further purification.

¹H NMR (D₂O, 400 MHz) δ 7.92 (d, 1H), 6.02 (d, 1H), 6.00 (d, 1H), 4.40 (m, 2H), 4.32 (m, 2H), 3.21 (m, 8H), 1.65 (tt, 8H), 1.37 (tq, H), 0.96 (t, 12H).

¹³C{¹H} NMR (D₂O, 100.6 MHz) δ 166.2 (s), 151.9 (s), 141.4 (s), 102.8 (s), 88.0 (s), 83.2 (d), 73.6 (s), 69.7 (s), 65.9 (s), 58.1 (s), 23.1 (s), 19.1 (s), 12.8 (s).

³¹P{¹H} NMR (D₂O, 162.0 MHz) δ −12.04 (*J*_{PP} = 15.6 Hz), −24.7 (*J*_{PP} = 25.6 Hz), −25.6 (*J*_{PP} = 25.6, 22.7 Hz), −37.1 (*J*_{PP} = 22.7, 15.7 Hz).

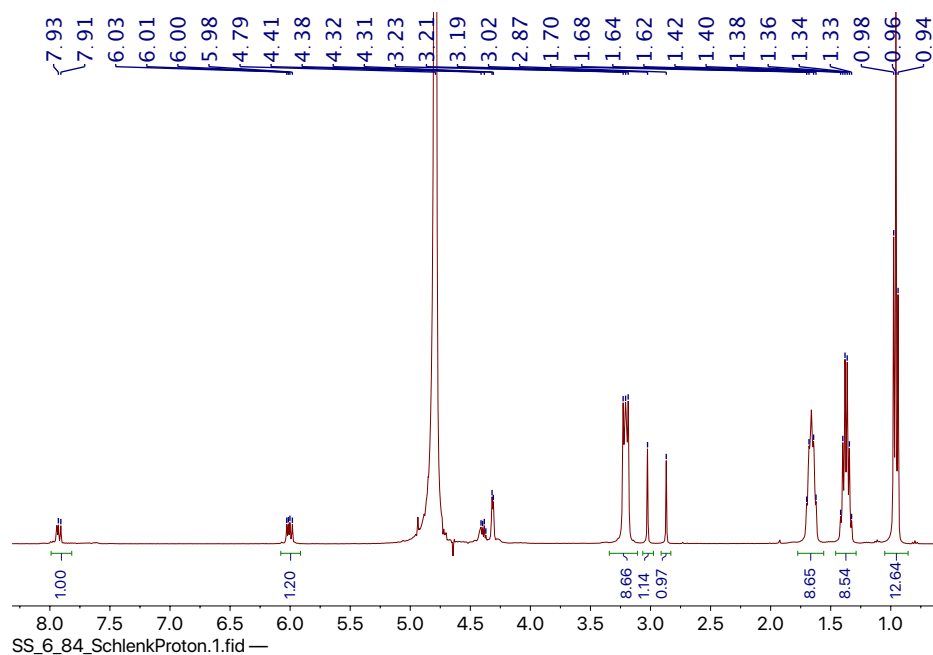


Figure S19: ^1H NMR spectrum of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pU}]$ (D_2O , 400 MHz)

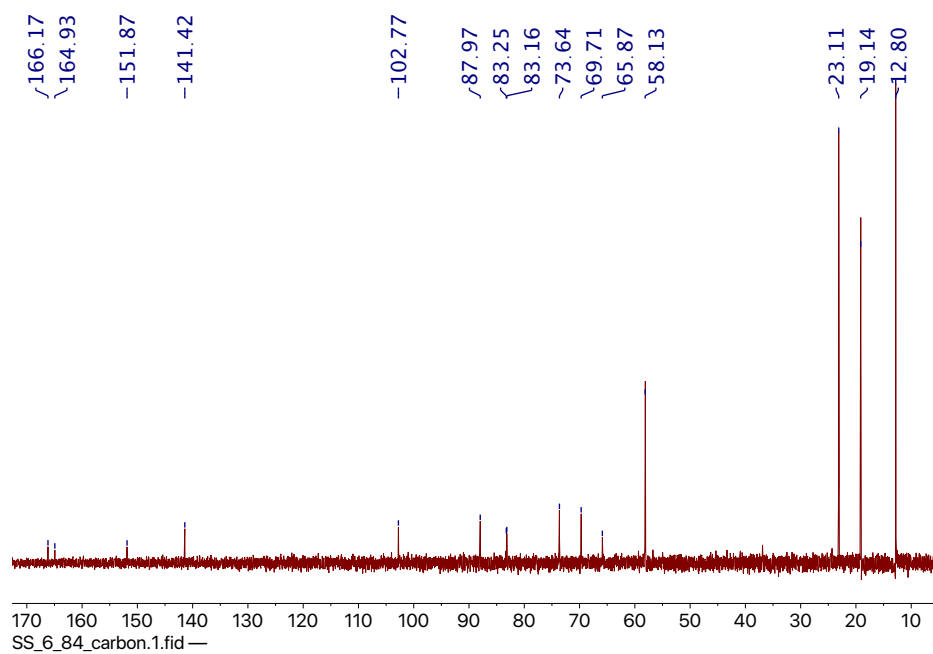


Figure S20: ^{13}C NMR spectrum of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pU}]$ (D_2O , 100.6 MHz)

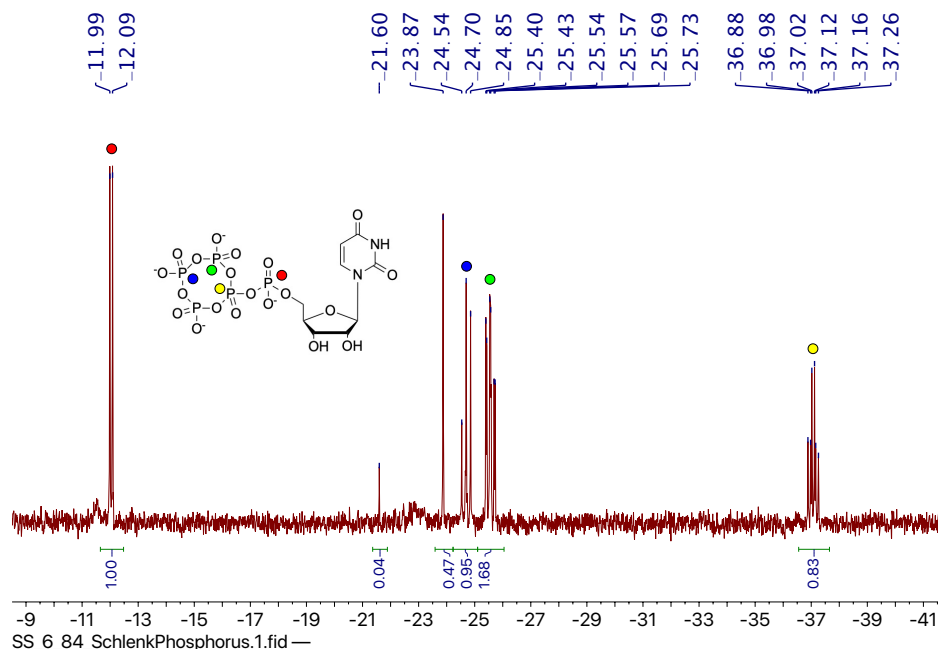


Figure S21: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{TBA}][\text{Na}]_3[\text{cp}_4\text{pU}]$ (D_2O , 162.0 MHz), the other resonances correspond to trimetaphosphate, tetrametaphosphates and p₅U due to the slow hydrolysis of cp₄pU, no other resonances were observed in the spectral window of 200 ppm to -200 ppm

2.5 Synthesis of $[\text{HNEt}_3]_5[\text{Hp}_5\text{A}]$

Method A:

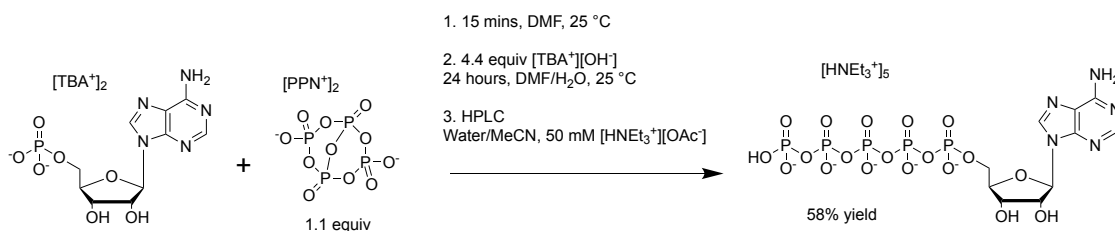


Figure S22: Synthesis of $[\text{HNEt}_3]_5[\text{Hp}_5\text{A}]$ (method A)

A solution of $[\text{TBA}]_2[\text{AMP}]^3$ in DMF at a concentration of 0.10 g/mL was stored over activated 4 Å sieves for at least 24 hours in the glovebox. $[\text{PPN}]_2[\text{P}_4\text{O}_{11}]$ (0.065 g, 0.047 mmol) was then dissolved in 1 mL of DMF. To this was added the DMF solution of $[\text{TBA}]_2[\text{AMP}]$ (0.36 mL, 0.043 mmol), and the mixture was stirred for 15 minutes. The reaction vessel was then removed from the glovebox and an aqueous 40% solution of tetrabutylammonium hydroxide (0.12 g, 0.19 mmol) was added. The resulting mixture was stirred for twenty four

hours after which all volatile materials were removed under reduced pressure on a Schlenk line at ca. 25 °C giving an oily solid. To this solid was added 2 mL of high purity water. The resulting suspension was stirred for 15 minutes. The resulting solution was filtered through a syringe filter and frozen until HPLC purification. Prior to HPLC purification, the sample was thawed and filtered through an Acrodisc 0.2 μm wwPTFE syringe filter. The sample was then purified by HPLC according to the conditions given in the General Considerations above. Fractions containing the desired product were pooled and all volatile materials were removed under reduced pressure on a Schlenk line at 40 °C to give an oil. The resulting oil was redissolved in water and volatile materials were removed at 40 °C again. Redissolution in water and evaporation of volatile materials at 40 °C was repeated twice more to remove all volatile triethylammonium acetate buffer, giving the product as a colorless solid (0.029 g, 0.025 mmol, 58% yield).

Method B:

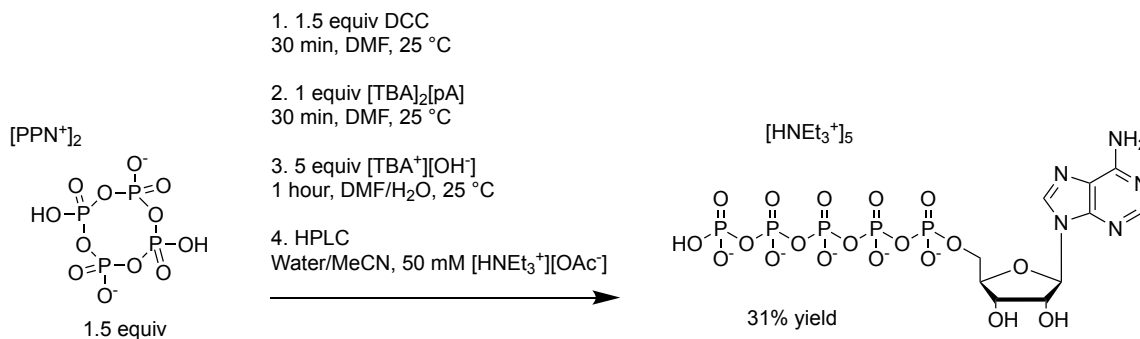


Figure S23: Synthesis of [HNEt₃]₅[Hp₅A] (method B)

Under an inert nitrogen atmosphere a Schlenk flask was charged with dry DMF (3 mL). Against a flow of nitrogen was added solid [PPN]₂[P₄O₁₂H₂] (0.12 g, 0.089 mmol) and DCC (0.019 g, 0.089 mmol). This mixture was stirred for 30 minutes during which time DCU formed as a precipitate. To this mixture was added solid [TBA]₂[pA] (0.051 g, 0.060 mmol). The resulting mixture was stirred for 30 minutes. The mixture was then exposed to air and a solution of [TBA][OH] (40% in water, 0.12 g, 0.19 mmol) in water (2 mL) was added. The resulting mixture was stirred for twenty four hours after which all volatile materials were removed under reduced pressure on a Schlenk line at ca. 25 °C giving an oily solid. To this solid was added 2 mL of high purity water. The resulting suspension was stirred for 15 minutes. The resulting solution was filtered through a syringe filter and frozen until HPLC purification. Prior to HPLC purification, the sample was thawed and filtered through an Acrodisc 0.2 μm wwPTFE syringe filter. The sample was then purified by HPLC according to the conditions given in the General Considerations above. Fractions containing the desired product were pooled and all volatile materials were removed under reduced pressure on a Schlenk line at 40 °C to give an oil. The resulting oil was redissolved in water and volatile materials were removed at 40 °C again. Redissolution in water and evaporation of volatile materials at 40 °C was repeated twice more to remove all volatile triethylammonium acetate buffer, giving the product as a colorless solid (0.022 g, 0.019 mmol, 31% yield).

¹H NMR (D₂O, 400 MHz) δ 8.58 (s, 1H), 8.30 (s, 1H), 6.10 (d, 1H), 4.59 (m, 2H), 4.38

(m, 1H), 4.23 (m, 2H), 3.17 (q, 30H), 1.25 (t, 40H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (D_2O , 100.6 MHz) δ 153.8 (s), 150.4 (s), 140.7 (s), 86.9 (s), 84.4 (d), 74.3 (s), 70.5 (s), 65.3 (d), 46.6 (s), 8.18 (s).

$^{31}\text{P}\{^1\text{H}\}$ NMR (D_2O , 162.0 MHz) δ -10.93 (d, $J_{\text{PP}} = 18.5$ Hz), -11.6 (d, $J_{\text{PP}} = 17.5$ Hz), -23.4 (m, $J_{\text{PP}} = 18.5, 17.5$ Hz).

ESI-MS(-) of $[\text{HNEt}_3]_4[\text{Hp}_5\text{A}]$ in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, found 665.64 m/z (calc'd 665.92 m/z) for $\text{C}_{10}\text{H}_{17}\text{N}_5\text{O}_{19}\text{P}_5^-$

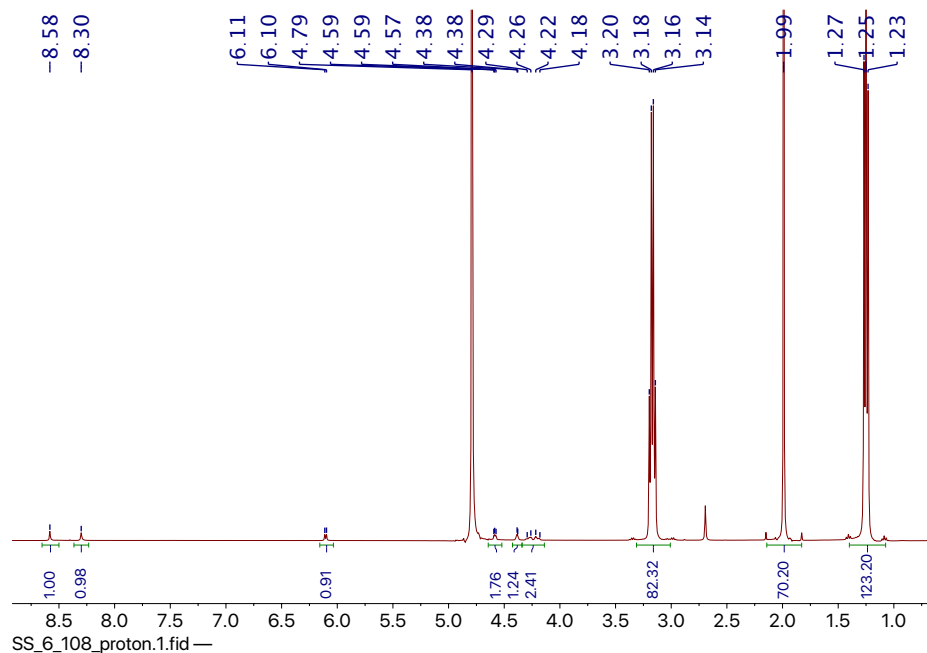


Figure S24: ^1H NMR spectrum of $[\text{HNEt}_3]_5[\text{Hp}_5\text{A}]$ (D_2O , 400 MHz) with approximately eight equivalents of residual triethylammonium acetate buffer

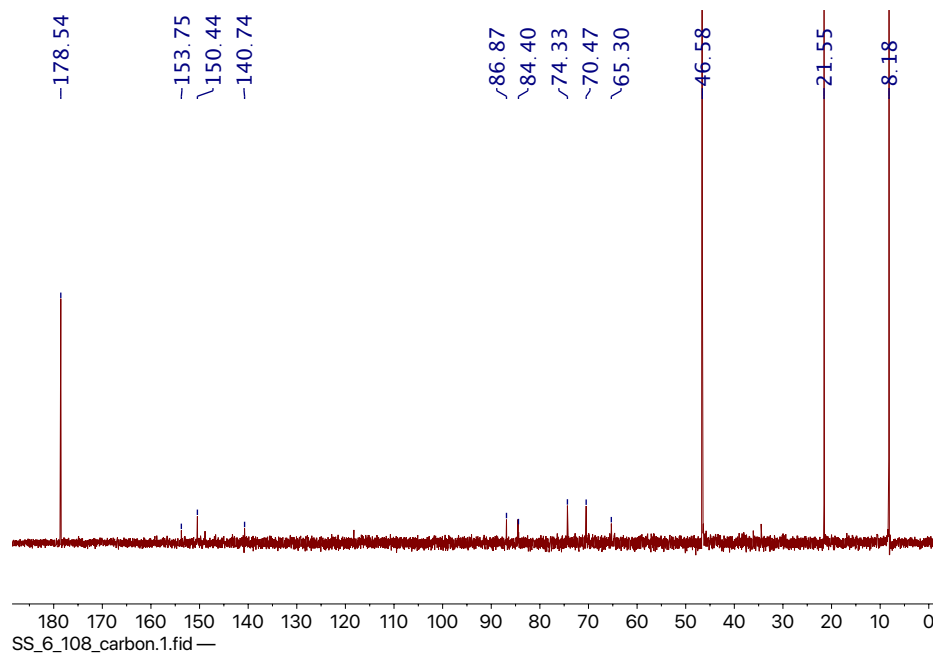


Figure S25: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{HNEt}_3]_5[\text{Hp}_5\text{A}]$ (D_2O , 100.6 MHz) with approximately eight equivalents of residual triethylammonium acetate buffer

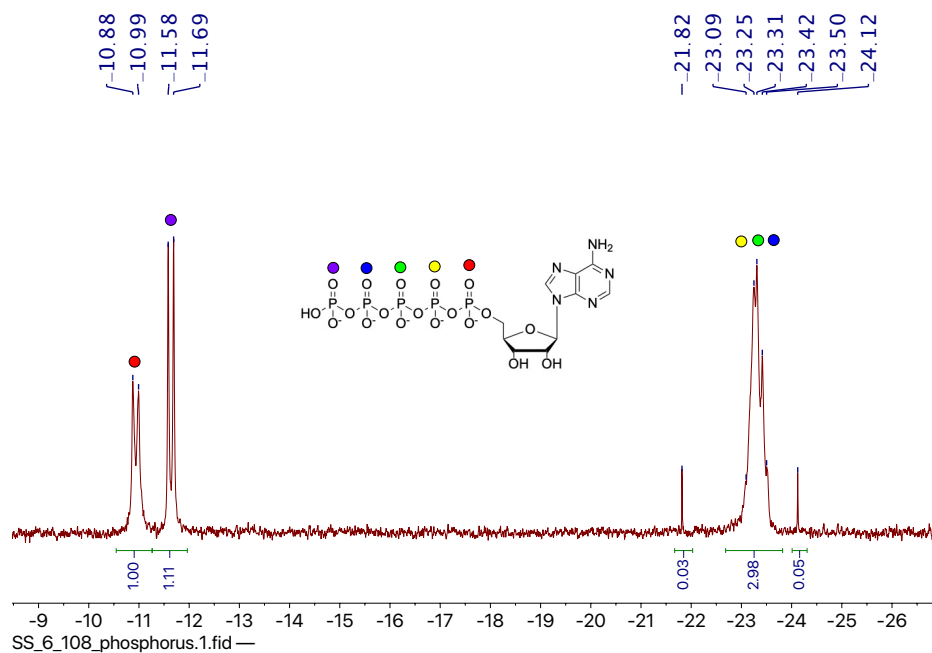


Figure S26: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{HNEt}_3]_5[\text{Hp}_5\text{A}]$ (D_2O , 162.0 MHz) with residual trimetaphosphate and tetrametaphosphate signals

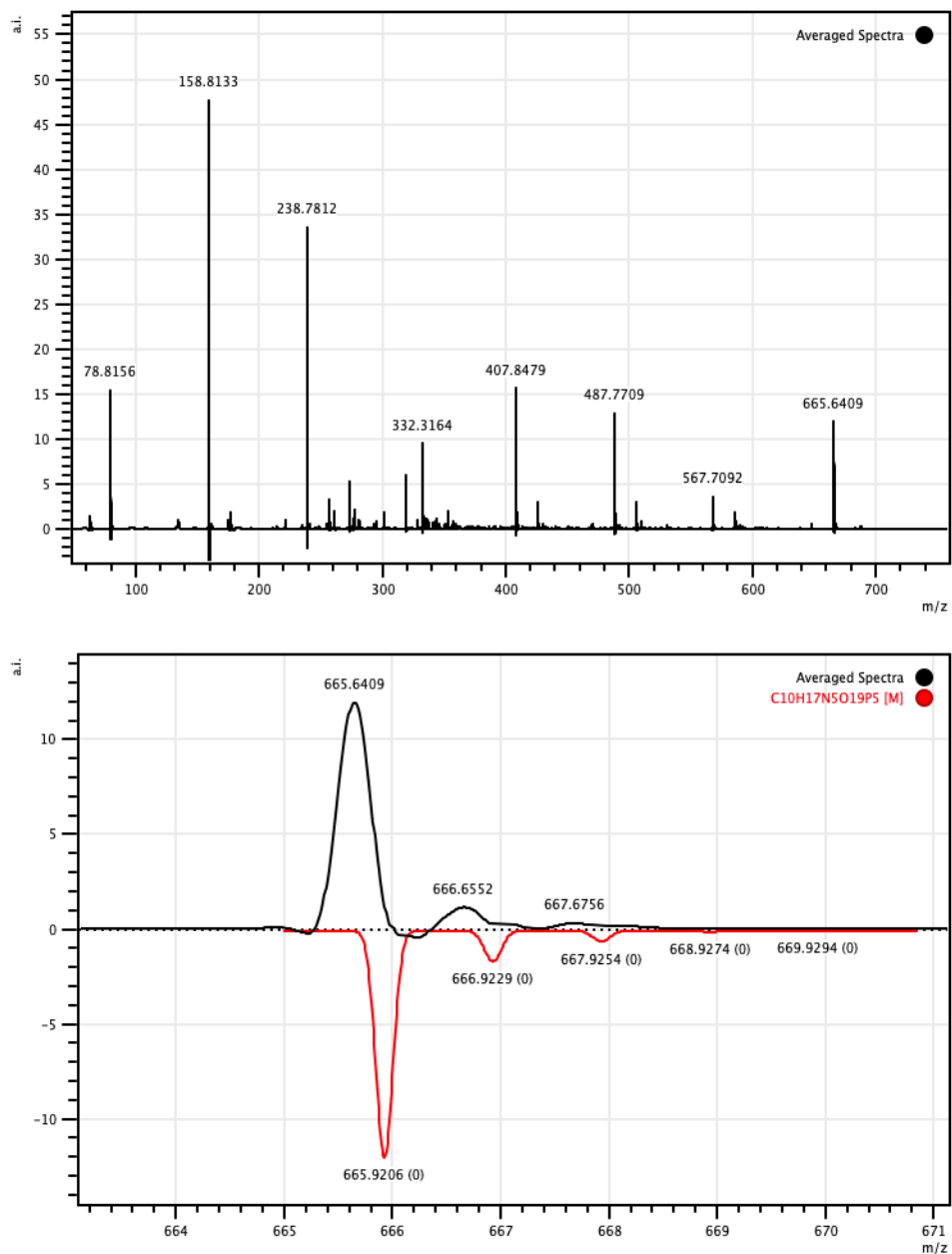


Figure S27: ESI-MS(–) of $[HNEt_3]_5[Hp_5A]$ in CH_3CN/H_2O with zoomed in portion showing the molecular ion ($H_5p_5A^-$, top) and calculated isotope pattern ($C_{10}H_{17}N_5O_{19}P_5^-$, bottom), the discrepancy between observed and calculated mass to charge ratio is attributed to poor calibration

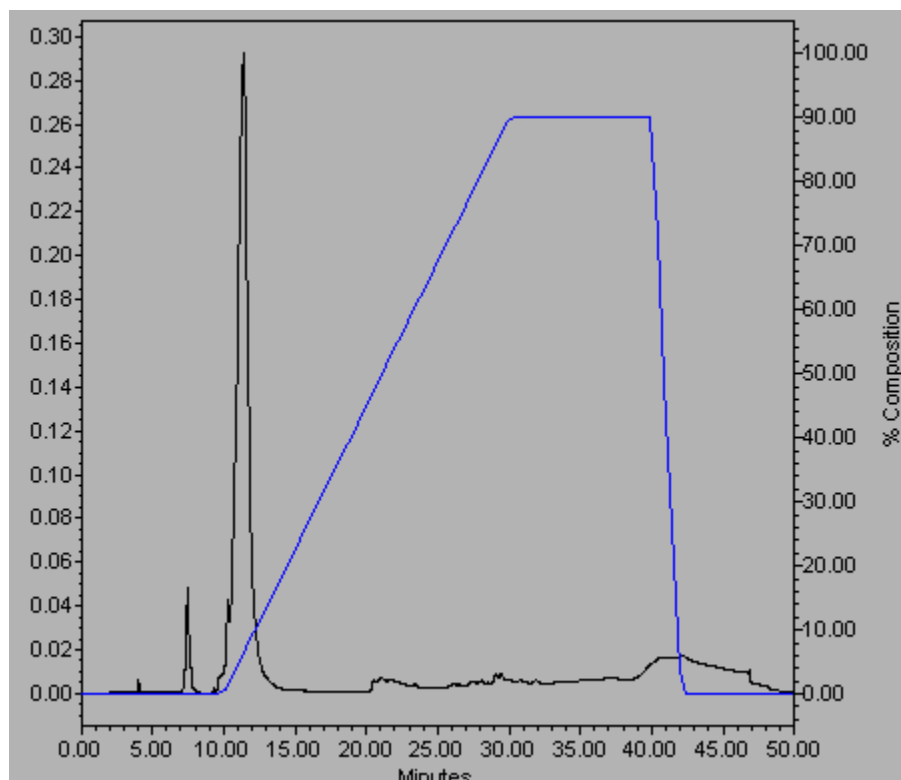


Figure S28: HPLC trace of purified $[\text{HNEt}_3]_5[\text{Hp}_5\text{A}]$. The species eluted at 9 min is a ghost peak due to a minor impurity in solvent A. The product peak integrates to 91.3%

2.6 Synthesis of $[\text{HNEt}_3]_5[\text{Hp}_5\text{U}]$

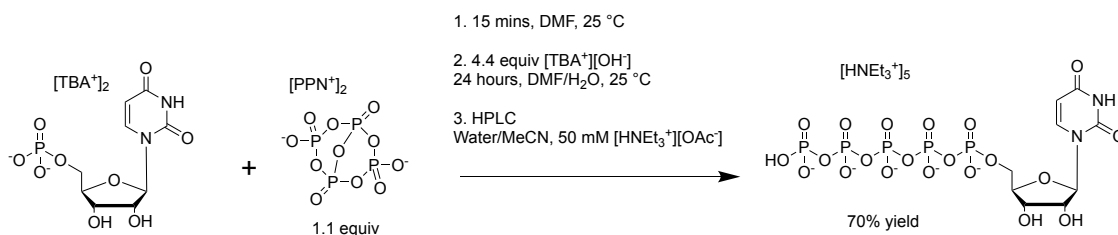


Figure S29: Synthesis of $[\text{HNEt}_3]_5[\text{Hp}_5\text{U}]$

A solution of $[\text{TBA}]_2[\text{UMP}]^3$ in DMF at a concentration of 0.10 g/mL was stored over activated 4 Å sieves for at least 24 hours in the glovebox. $[\text{PPN}]_2[\text{P}_4\text{O}_{11}]$ (0.062 g, 0.045 mmol) was then dissolved in 1 mL of DMF. To this was added the DMF solution of $[\text{TBA}]_2[\text{UMP}]$ (0.33 mL, 0.041 mmol), and the mixture was stirred for 15 minutes. The reaction vessel was then removed from the glovebox and an aqueous 40% solution of tetrabutylammonium hydroxide (0.12 g, 0.18 mmol) was added. The resulting mixture was stirred for twenty four hours after which all volatile materials were removed under reduced pressure on a Schlenk

line at ca. 25 °C giving an oily solid. To this solid was added 2 mL of high purity water. The resulting suspension was stirred for 15 minutes. The resulting solution was filtered through an Acrodisc 0.2 μm wwPTFE syringe filter and frozen until HPLC purification. Prior to HPLC purification, the sample was thawed and filtered through an Acrodisc 0.2 μm wwPTFE syringe filter. The sample was then purified by HPLC according to the conditions given in the General Considerations above. Fractions containing the desired product were pooled and all volatile materials were removed under reduced pressure on a Schlenk line at 40 °C to give an oil. The resulting oil was redissolved in water and all volatile materials were removed at 40 °C again. Redissolution in water and evaporation of volatile materials at 40 °C was repeated twice more to remove all volatiles triethylammonium acetate buffer, giving the product as a colorless solid (0.033 g, 0.029 mmol, 70% yield).

^1H NMR (D_2O , 400 MHz) δ 7.99 (d, 1H), 6.03 (d, 1H), 5.99 (d, 1H), 4.47 (m, 1H), 4.42 (m, 1H), 4.31 (m, 1H), 4.27 (m, 2H), 3.21 (q, 30H), 1.29 (t, 40H).

$^{13}\text{C}\{^1\text{H}\}$ NMR (D_2O , 100.6 MHz) δ 166.2 (s), 151.9 (s), 141.7 (s), 102.8 (s), 87.7 (s), 83.8 (d), 73.6 (s), 69.9 (s), 65.2 (d), 46.6 (s), 30.2 (s), 8.2 (s).

$^{31}\text{P}\{^1\text{H}\}$ NMR (D_2O , 162.0 MHz) δ -10.9 (d, $J_{\text{PP}} = 18.7$ Hz), -11.7 (d, $J_{\text{PP}} = 18.2$ Hz), -23.5 (m, $J_{\text{PP}} = 18.7, 18.2$ Hz).

ESI-MS(-) of $[\text{HNEt}_3]_4[\text{p}_5\text{UH}]$ in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, found 642.63 m/z (calc'd 642.89 m/z) for $\text{C}_9\text{H}_{16}\text{N}_2\text{O}_{21}\text{P}_5^-$

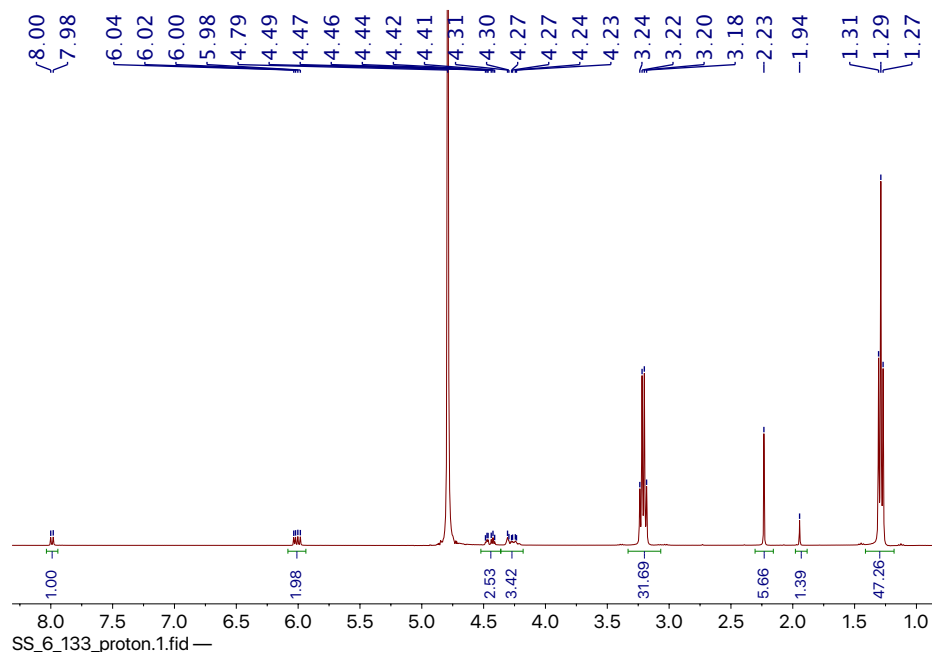


Figure S30: ^1H NMR spectrum of $[\text{HNEt}_3]_5[\text{Hp}_5\text{U}]$ (D_2O , 400 MHz)

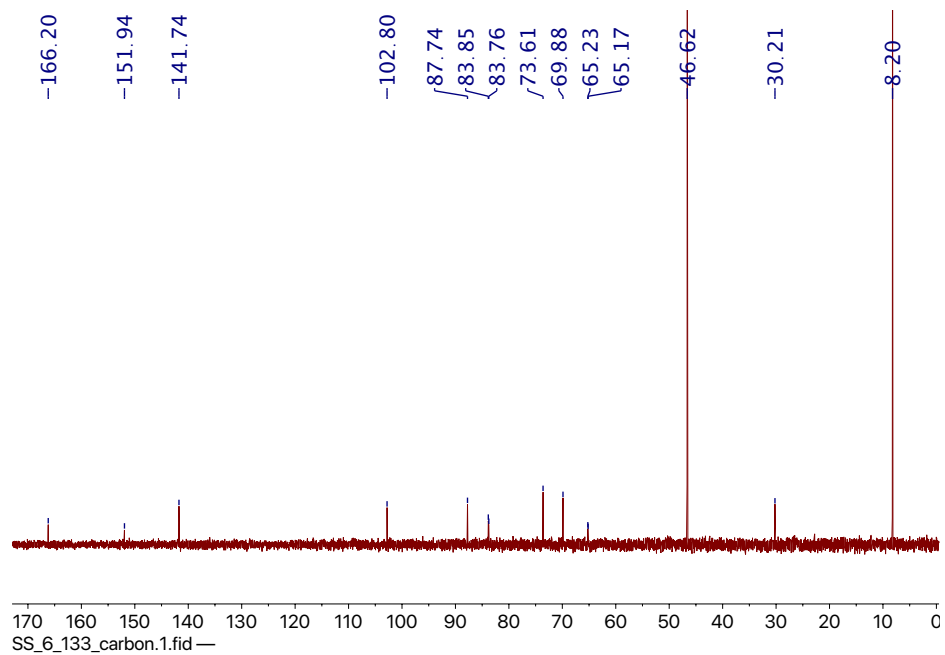


Figure S31: ^{13}C NMR spectrum of $[\text{HNEt}_3]_5[\text{Hp}_5\text{U}]$ (D_2O , 100.6 MHz)

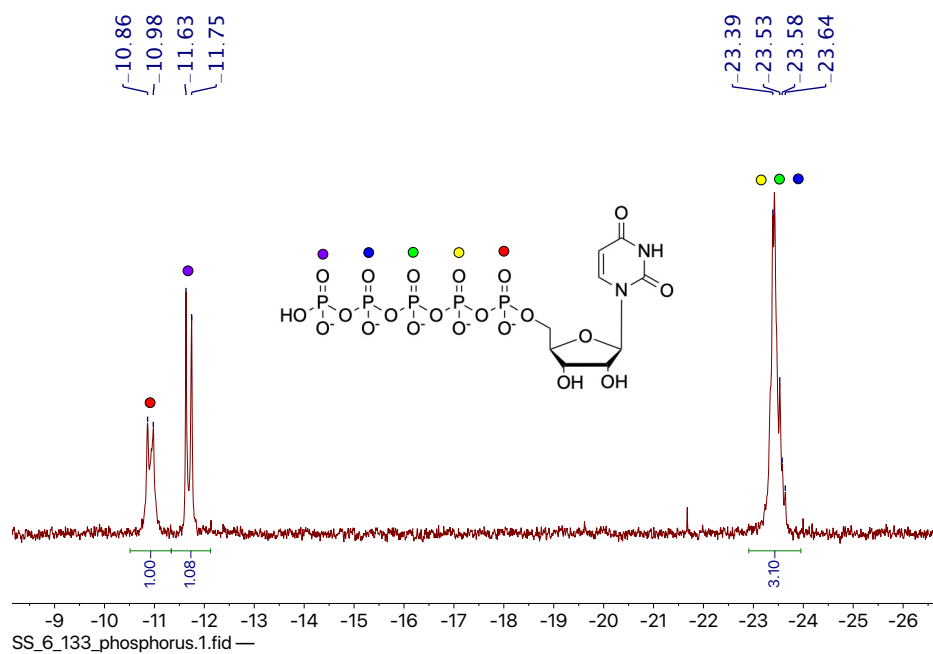


Figure S32: $^{31}\text{P}\{^1\text{H}\}$ NMR spectrum of $[\text{HNEt}_3]_5[\text{Hp}_5\text{U}]$ (D_2O , 162.0 MHz)

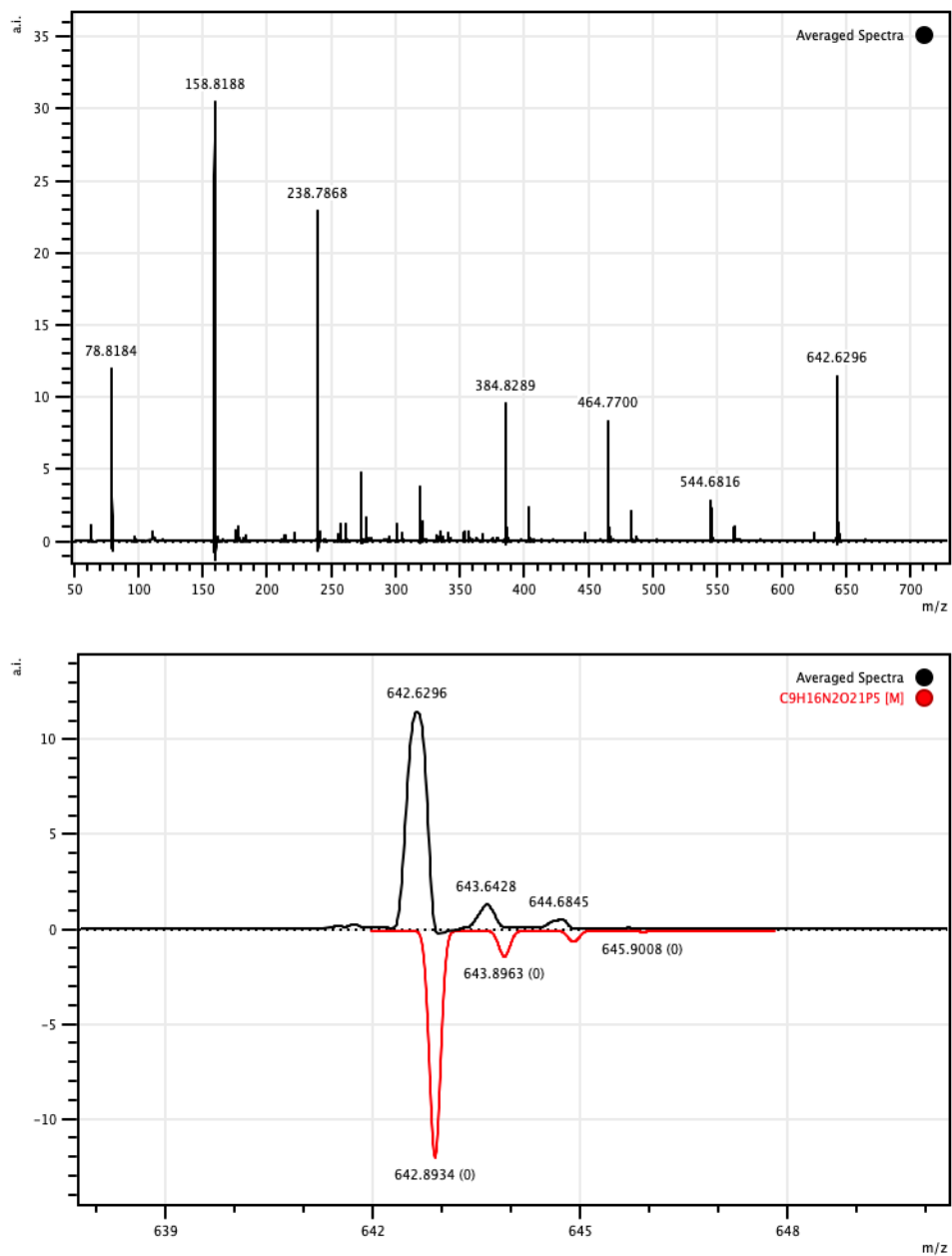


Figure S33: ESI-MS(–) of $[HNEt_3]_5[Hp_5U]$ in CH_3CN/H_2O with zoomed in portion showing the molecular ion ($H_5P_5U^-$, top) and calculated isotope pattern ($C_9H_{16}N_2O_{21}P_5^-$, bottom), the discrepancy between observed and calculated mass to charge ratio is attributed to poor calibration

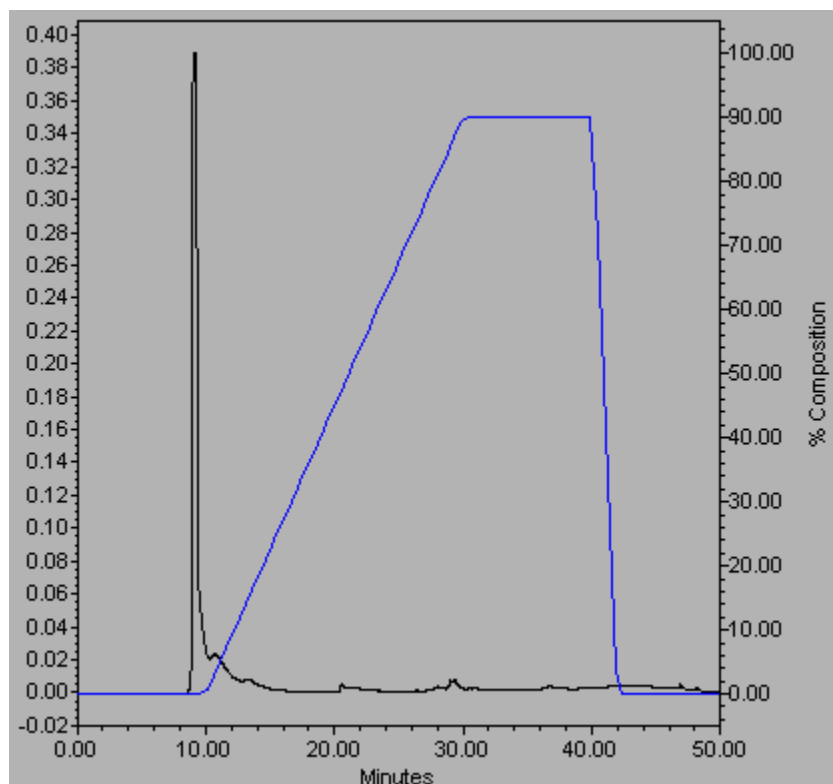


Figure S34: HPLC trace of purified $[\text{HNEt}_3]_5[\text{Hp}_5\text{U}]$. The product peak eluting at 9 min integrates to 88%. The shoulder peak eluting at 10 min integrates to 11% and may be due to dead volume or solvent cavitation rather than an impurity.

3 Inhibition Kinetics

Stock solutions of RNase A and substrate were made in assay buffer containing 50 mM MES-NaOH buffer (free of oligo(vinylsulfonate)), pH 6.0, containing NaCl (100 mM). A concentrated stock of RNase A was prepared at 20.0 mg/mL (1.46 mM) and diluted to a final concentration of 1 pM in assays. Substrate stocks were prepared at 40 μM and diluted to a final concentration of 80 nM in assays. Final reaction volumes were 200 μL . Prior to performing inhibition kinetic assays, the specificity constant ($k_{\text{cat}}/K_{\text{M}}$) of RNase A for the substrate was measured to be $2.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, in agreement with the previous report of $3.6 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$.⁶

Inhibition kinetics were measured for the cleavage of the 6-FAM-dArUdAdA-6-TAMRA substrate⁶ by RNase A. Cleavage of this substrate results in a turn-on of fluorescence. Assays were performed in a flat, black 96-well plate with a non-binding surface (Corning item 6505) using an M1000 plate reader from Tecan. Inhibition assays were performed by titrating a fixed concentration of enzyme with a range of inhibitor concentrations. Polyphosphate inhibitors were titrated using a 10-fold dilution series from 10^{-3} to 10^{-10} M, whereas nucleotide inhibitors were titrated using a 4-fold dilution series from 10^{-5} to 10^{-12} M. Inhibitor stocks were prepared by serial dilutions at a concentration of 2 \times , of which 100 μL was combined with 90 μL of a substrate stock. Fluorescence was measured prior to enzyme addition to

ensure low background. Despite treating the assay buffer with diethyl pyrocarbonate, using nuclease-free tips and tubes, and wiping the workspace and pipettes with a ribonuclease-inactivating spray, a few wells exhibited high background fluorescence and were omitted from the analysis. Each replicate of unique conditions was initiated simultaneously by addition of 10 μ L from a 20 \times stock (20 pM RNase A) delivered with a multichannel pipette. Reaction velocity measurements were performed in triplicate. Only a slight variation was observed in uninhibited velocities between different K_i determinations.

The enzyme concentration was selected to ensure that more than 10 points could be measured before the turnover of 10% of the substrate. Initial velocities were obtained by linear regression analysis using Prism 7 software from Graphpad. Initial velocities were fitted to a single-site binding equation by non-linear regression analysis using a user-defined equation in Prism 7:

$$\frac{\Delta F}{\Delta t_i} = \frac{\Delta F}{\Delta t_o} \left[1 - \frac{[\text{Inhibitor}]}{[\text{Inhibitor}] + K_i} \right] \quad (1)$$

No normalization or other data manipulation beyond fitting was performed. Initial velocities and the nonlinear fits of eq 1 can be found in Figure S29. The ensuing inhibition constants (K_i) are reported in Table 1.

A study of the dependence of salt concentration on inhibition of RNase A by p₅U was also performed. Assays were performed as described above but with a variable sodium chloride concentration of 100 mM, 150 mM, or 200 mM. Values of K_i were determined as described above and the data from this study are described in Table S1 and Figure S36.

NaCl concentration (mM)	K_i (μ M)
100	0.068 ± 0.007
150	0.20 ± 0.01
200	0.66 ± 0.03

Table S1: Calculated values of K_i from NaCl concentration study of RNase A inhibition by p₅U

4 X-ray Crystallography

Ribonuclease A was dissolved in nuclease-free water at a concentration of 20 mg/mL. Crystals were grown as previously described by 2-fold dilution of hanging drops (4 μ L final) over a mother liquor of 20 mM sodium citrate buffer, pH 6.0, containing PEG 4000 (20% v/v).⁷ Crystals grew within 3 to 5 days to a size of roughly 0.5 \times 0.5 \times 0.05 mm. Stocks of ligands were prepared at 50 mM in mother liquor containing PEG 4000 (30% v/v). Crystals were soaked in with the ligand solution for 2-24 h. Crystals were cryo-protected with diluted soaking solution containing glycerol (20% v/v). Single-crystal X-ray diffraction experiments were performed with a Bruker D8 Venture Kappa Duo diffractometer equipped with a Bruker Photon2 CPAD detector using Cu K_α radiation ($\lambda = 1.54178$ Å). Data were indexed with APEX3 software, reduced with SAINT,⁸ and scaled and absorption corrected using SADABS.⁹

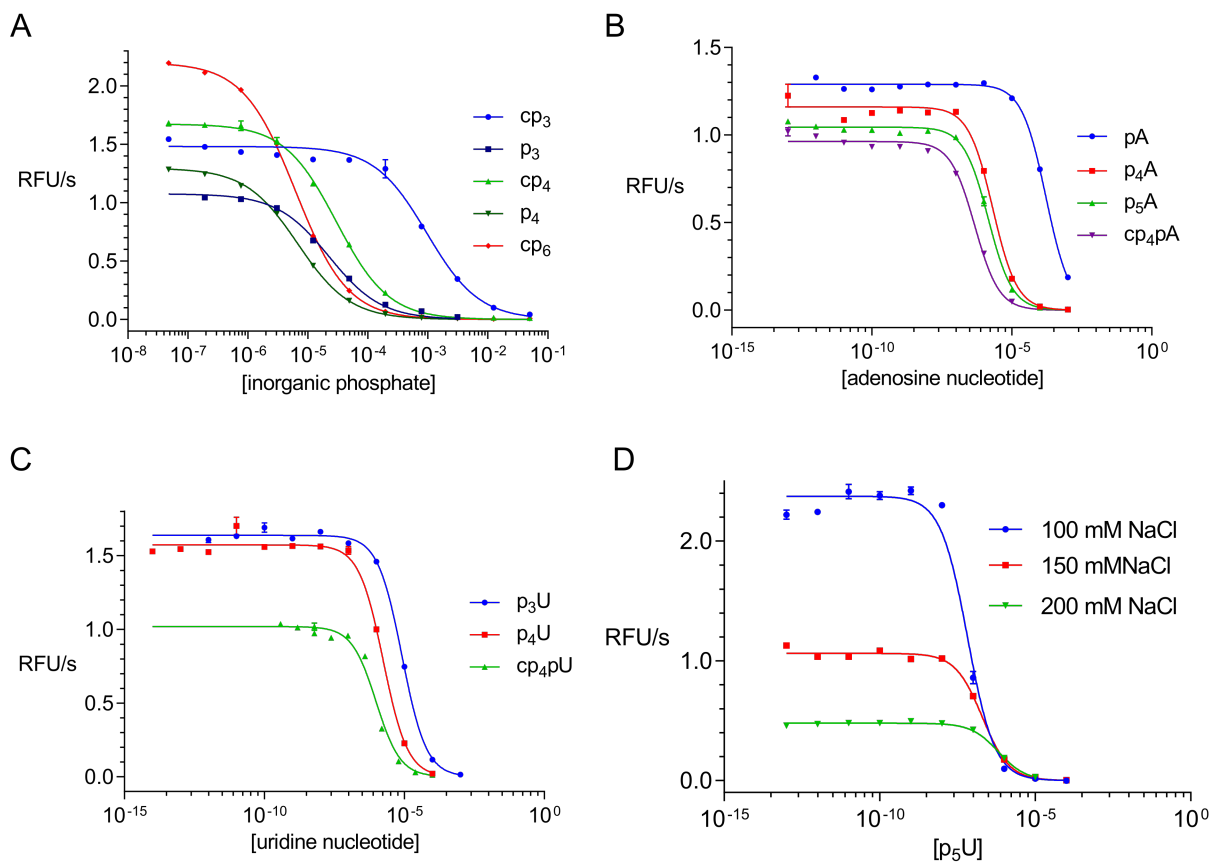


Figure S35: Graphs showing the effect of (A) polyphosphates, (B) adenosine oligophosphates, (C) uridine oligophosphates, and (D) salt-dependency of p_5U on catalysis by RNase A.

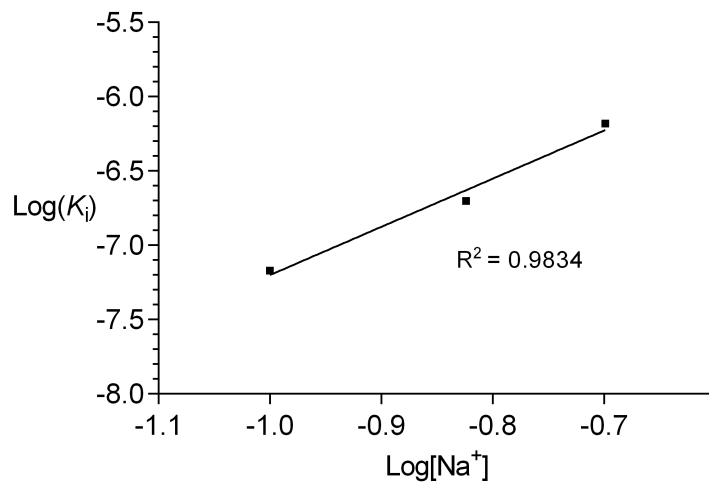


Figure S36: Quantitative analysis of salt dependence of p_5U inhibition of RNase A. A log-log plot was prepared using the data in Table S1 and the slope was determined to afford a $\delta \log K_d / \delta \log [\text{Na}^+]$ value of 3.2 by linear regression analysis.

RNase A-ligand structures were solved by molecular replacement using the Phaser program as implemented in PHENIX.¹⁰ The atomic coordinates of protein atoms from the apo structure of RNase A (1afu) with alternative conformations removed were used as a starting model.⁷ Refinement was performed with phenix.refine, and model building was conducted with COOT.^{10,11} After building a satisfactory apo structure, density consistent with inhibitors bound to the active site was found. Restraints for inhibitors were prepared with eLBOW in PHENIX and placed with COOT. Details of the X-ray data collection and model refinement are shown in Table S2 and densities from final structures are shown in Figure S37.

Ligand (PDB ID)	cp6i (6pvu)	psA (6pvv)	cp4pA (6pvw)	psU (6pvx)
Data collection				
Space group	C121	C121	C121	C121
<i>a</i> , <i>b</i> , <i>c</i> (Å)	100.35, 32.64, 72.61	99.79, 32.25, 71.95	100.64, 32.71, 72.68	100.54, 32.64, 72.17
α , β , γ (°)	90, 90.7, 90	90, 90.3, 90	90, 90.6, 90	90, 90.4, 90
Wavelength (Å)	1.54178	1.54178	1.54178	1.54178
Resolution (Å)	29.57–1.49 (1.55–1.49)	29.25–1.65 (1.71–1.65)	25.16–1.60 (1.66–1.60)	29.42–1.55 (1.61–1.55)
Total Reflections	205782 (14775)	344704 (8059)	311650 (20167)	413831 (21558)
Unique Reflections	38224 (3454)	27900 (2635)	31680 (3142)	34001 (2948)
Multiplicity	5.4 (4.3)	12.35 (3.06)	9.8 (6.4)	12.1 (7.3)
Completeness (%)	99.0 (89.8)	99.54 (95.85)	99.99 (100.00)	98.7 (87.1)
Mean <i>I</i> / σ (<i>I</i>)	20.5 (3.2)	18.2 (2.0)	18.5 (2.3)	25.4 (3.0)
Wilson <i>B</i> factor (Å ²)	13.58	17.01	16.51	14.68
<i>R</i> _{merge}	0.047 (0.410)	0.086 (0.503)	0.068 (0.490)	0.060 (0.552)
<i>R</i> _{meas}	0.052 (0.466)	0.090 (0.597)	0.071 (0.534)	0.062 (0.594)
<i>R</i> _{p.i.m.}	0.022 (0.217)	0.023 (0.313)	0.021 (0.208)	0.017 (0.215)
CC _{1/2}	0.999 (0.857)	0.999 (0.623)	0.999 (0.877)	1.000 (0.823)
Refinement				
Reflections				
Work set	38222 (3454)	27897 (2635)	31678 (3140)	33999 (2948)
Test set	1993 (178)	1849 (171)	1674 (167)	1983 (176)
<i>R</i> _{work}	0.2016 (0.2338)	0.1915 (0.2588)	0.1891 (0.2312)	0.1991 (0.2557)
<i>R</i> _{free}	0.2360 (0.2749)	0.2225 (0.2727)	0.2259 (0.2604)	0.2282 (0.2939)
R.m.s.d., bond lengths (Å)	0.006	0.008	0.012	0.006
R.m.s.d., bond angles (Å)	0.94	1.02	1.10	0.85
No. of atoms				
Total	2236	2216	2252	2275
Protein	1901	1907	1904	1921
Ligand	72	78	70	144
Water	263	231	278	210
Average <i>B</i> factor (Å ²)				
Overall	20.24	25.58	24.72	22.47
Protein	18.54	24.40	23.59	20.69
Ligand	51.29	40.34	31.30	38.27
Water	24.05	30.28	30.80	27.90
Ramachandran statistics				
Favored (%)	94.67	94.67	94.26	95.49
Allowed (%)	4.51	4.92	5.74	4.51
Outliers (%)	0.82	0.41	0.00	0.00

Table S2: Crystallographic data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

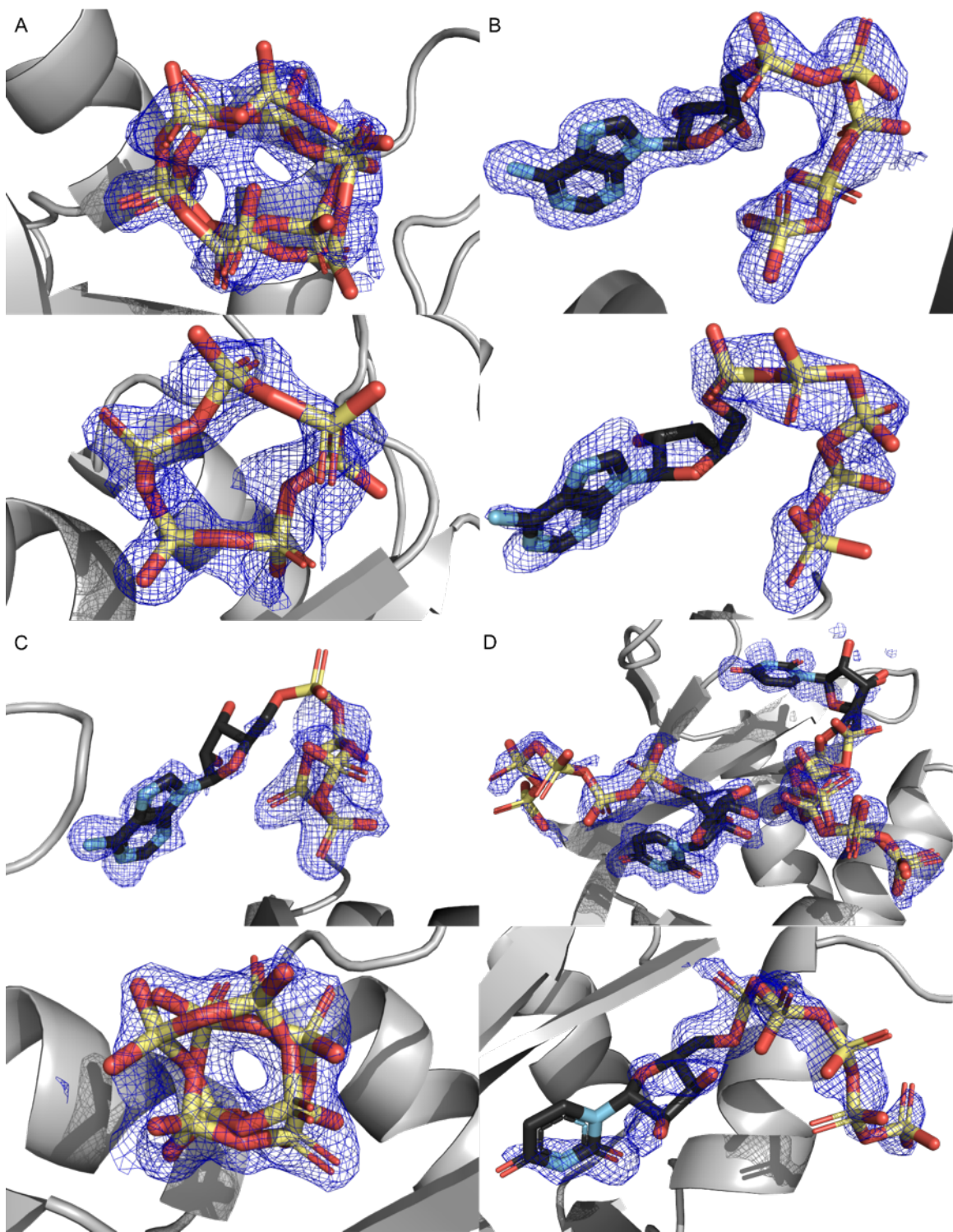


Figure S37: Densities of ligands bound to RNase A. Meshes are depicted of $2F_o - F_c$ maps contoured a 1σ for A. cp₆, B. p₅A, C. cp₄pA, and D. p₅U of both chain A (top) and chain B (bottom).

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