Metabolic RNA labeling in non-engineered cells following spontaneous uptake of fluorescent nucleoside phosphate analogues

Supplementary Data:

Metabolic RNA labeling in non-engineered cells following spontaneous uptake of fluorescent nucleoside phosphate analogues

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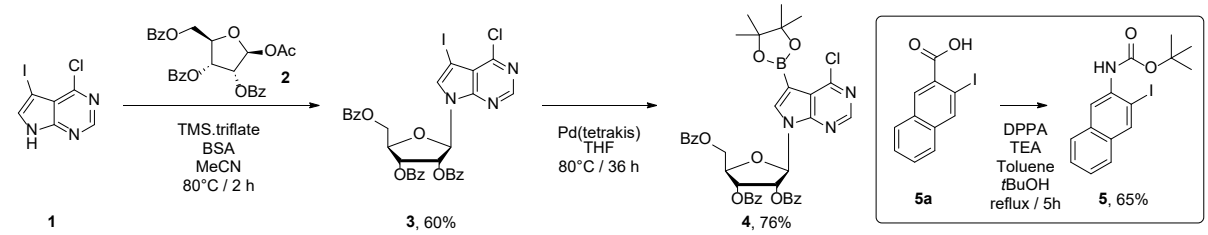
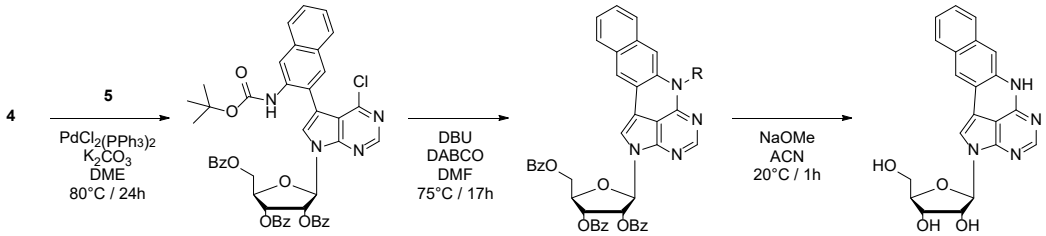
Contents

1. pA ribonucleoside triphosphate synthesis

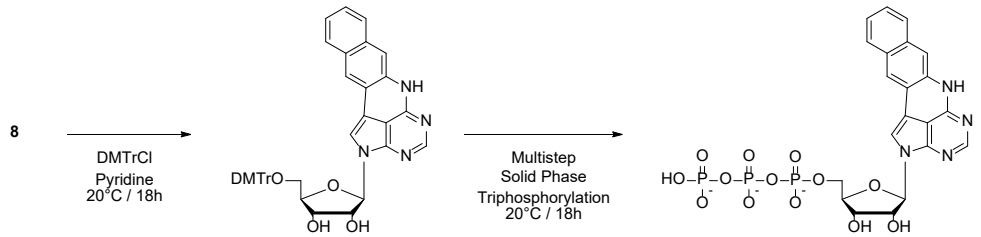
1.1 Synthetic scheme



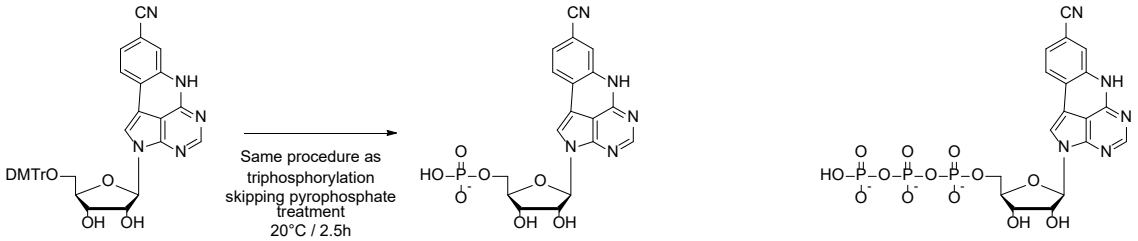
1 **3**, 60% **4**, 76% **5a** **5**, 65%



4 **6**, 75% **7a**, R=H **7b**, R=Boc **8**, 17% over 2 steps



8 **9**, 23% **10**, 60%



11, Nucleic Acids Res., 2020, 48, 7640-7652 **12**, 40% **13**, PCCP, 2023, 25(30), 20218-20224

1.2 Experimental procedures

All reactions were performed in flame-dried or oven-dried glassware under a nitrogen atmosphere unless otherwise noted. Reagents were purchased from various chemical vendors and either used as received or purified according to standard techniques. The following reagents used for the triphosphorylation were bought from Sigma-Aldrich: DCA deblock for ÄKTA, CAP A for ÄKTA, CAP B1 and B2 for ÄKTA, BTT Activator. All solvents used for reactions were HPLC-grade and purchased dry. Microwave reactions were performed with a Biotage Initiator using single mode microwave irradiation with temperature and pressure control and with fixed hold time on. Reactions were monitored by TLC on silica gel plates analyzed under UV (254 nm), and by UPLC-MS (ESI/UV), using a Waters Acquity system equipped with either an Acquity UPLC HSS C18 column (1.8 µm, length 50 mm, ID 2.1 mm) running a gradient of water-MeCN (95:5) to water-MeCN (5:95), with the water eluent containing 1% formic acid (pH 3) or an Acquity UPLC BEH C18 column (1.7µm, length 50 mm, ID 2.1 mm) running a gradient of water-MeCN (95:5) to water-MeCN (5:95), with the water eluent containing 1% ammonium hydroxide (pH 10). Flash chromatography was performed by automated column chromatography using pre-packed silica columns. HPLC purification was performed with ammonia as modifier on a preparative HPLC system with an Xbridge C18 column (10 µm, 250 × 50 mm). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz system equipped with a CryoProbe. ^{31}P NMR spectra were recorded at 300 K on a Bruker 500 MHz system. All shifts are recorded in ppm relative to the deuterated solvent: $CDCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C), DMSO-d_6 (2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C) or D_2O.

(2R,3R,4R,5R)-2-((Benzoyloxy)methyl)-5-(4-chloro-5-iodo-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrofuran-3,4-diyl dibenzoate *(3)*: 4-chloro-5-iodo-7H-pyrrolo[2,3-d]pyrimidine (**1**, 20 g, 71.6 mmol) was dissolved in MeCN (480 mL). trimethylsilyl (E)-N-(trimethylsilyl)acetimidate (19.5 mL, 78.7 mmol) was added dropwise. The mixture was stirred at RT for 20 min. (2S,3R,4R,5R)-2-acetoxy-5-((benzoyloxy)methyl)tetrahydrofuran-3,4-diyl dibenzoate (**2**, 46.9 g, 93.0 mmol) was added in one portion, followed by dropwise addition of trimethylsilyl trifluoromethanesulfonate (15.2 mL, 78.7 mmol). The reaction mixture was stirred at 80 °C for 2 h. The reaction mixture was allowed to cool to RT and diluted with EtOAc (200 mL). The organic phase was washed with aq. satd. $NaHCO_3$ (100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4, filtered, concentrated *in vacuo*, absorbed onto Celite and purified by flash column chromatography (Hept:EtOAc 90:10 to 70:30, KP-Sil 330 g) to yield **3** (31.0 g, 60%) as a white solid.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.09 – 8.13 (m, 2H), 7.99 (dd, *J* = 8.3, 1.2 Hz, 2H), 7.92 (dd, *J* = 8.4, 1.2 Hz, 2H), 7.49 – 7.64 (m, 6H), 7.39 (dt, *J* = 22.8, 7.8, 7.8 Hz, 4H), 6.67 (d, *J* = 5.4 Hz, 1H), 6.09 – 6.17 (m, 2H), 4.90 (dd, *J* = 12.3, 3.1 Hz, 1H), 4.80 (q, *J* = 3.5, 3.5, 3.4 Hz, 1H), 4.68 (dd, *J* = 12.3, 3.6 Hz, 1H).

^{13}C NMR (125 MHz, $CDCl_3$) δ 166.2, 165.5, 165.2, 153.3, 151.4, 151.1, 133.94, 133.91, 133.7, 132.1, 129.98, 129.96, 129.8, 129.4, 128.9, 128.8, 128.71, 128.66, 128.5, 117.9, 86.9, 80.8, 74.3, 71.6, 63.6, 53.8.

HRMS (ESI-TOF) m/z calcd for $C_{32}H_{23}ClIN_3O_7$ [M + H]+ : 724.0347, found: 724.0384.

(2R,3R,4R,5R)-2-((Benzoyloxy)methyl)-5-(4-chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrofuran-3,4-diyl dibenzoate *(4)*: Compound **3** (31 g, 42.8 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.99 g, 0.86 mmol) were dissolved in THF (360 mL) to which triethylamine (59 mL, 428

mmol) was added. The reaction mixture was cooled to -78°C and stirred for 5 min before 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (9.3 mL, 64.2 mmol) was added dropwise to the mixture. The reaction mixture was then allowed to warm to RT and successively heated to 80 °C for 36 h. The reaction mixture was allowed to cool to RT, concentrated *in vacuo*, absorbed onto Celite and purified by flash-chromatography (KP-Sil, 330 g, Hept:EtOAc, 95:5 to 70:30 to yield the target compound (**4**, 23.5 g, 76 %) as a white solid.

^{1}H NMR (500 MHz, DMSO) δ 8.58 (s, 1H), 8.33 (s, 1H), 7.98 (ddd, *J* = 11.8, 8.3, 1.2 Hz, 4H), 7.83 (dd, *J* = 8.3, 1.2 Hz, 2H), 7.59 – 7.69 (m, 3H), 7.50 (dt, *J* = 9.8, 8.1, 8.1 Hz, 4H), 7.39 – 7.43 (m, 2H), 6.76 (d, *J* = 5.4 Hz, 1H), 6.35 – 6.39 (m, 1H), 6.14 – 6.18 (m, 1H), 4.79 – 4.89 (m, 2H), 4.69 (dd, *J* = 12.1, 5.0 Hz, 1H), 1.28 (s, 12H).

^{13}C NMR (126 MHz, DMSO) δ 165.4, 164.7, 164.4, 152.8, 152.5, 150.9, 138.7, 133.94, 133.87, 133.5, 129.4, 129.3, 129.23, 129.17, 128.75, 128.72, 128.71, 128.6, 128.2, 119.8, 86.7, 83.6, 79.3, 73.3, 70.8, 63.5, 54.9, 24.5.

HRMS (ESI-TOF) m/z calcd for $C_{38}H_{35}BClN_3O_9$ [M + H]+ : 724.2233, found: 724.2245.

***tert*-Butyl (3-Iodonaphthalen-2-yl)carbamate *(5)*:** A oven-dried 3-necked 1L round-bottom flask equipped with a magnetic stir bar was charged with 3-iodo-2-naphthoic acid (**5a**, 24.8 g, 83.2 mmol) dissolved in toluene (320 mL) and triethylamine (14 mL, 100.0 mmol) was added. The flask was fitted with a reflux condenser and an addition funnel. The reaction was heated to reflux and diphenyl phosphorazidate (21.6 mL, 100.0 mmol) in toluene (80 mL) was added dropwise to the reaction mixture over a total period of 60 min. The rate of addition was kept to 1 drop every 2-5 s and bubbles was observed after 5 min of addition. The addition funnel was rinsed with additional toluene (20 mL). After 15 min of stirring at reflux the bubble formation stopped. After an additional 15 min the addition funnel was charged with 2-methylpropan-2-ol (40 mL, 416.0 mmol) in toluene (60 mL), which was cautiously (*note*: the formed intermediate is extremely reactive and must be handled with care) added dropwise to the reaction mixture at reflux. The reaction was stirred at reflux for an additional 3 h. The reaction mixture was allowed to cool to RT, transferred to a separatory funnel and the material was washed sequentially with water (3x500 mL), aq. satd. $NaHCO_3$ (3x250 mL) followed by brine (1x500 mL). The resulting orange solution was dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield **5** (20.0 g, 65 %) as a beige solid.

^{1}H NMR (500 MHz, DMSO) δ 8.63 (s, 1H), 8.52 (s, 1H), 7.94 (s, 1H), 7.86 (dd, *J* = 14.9, 8.1 Hz, 2H), 7.46 – 7.54 (m, 2H), 1.48 (s, 9H).

^{13}C NMR (126 MHz, DMSO) δ 153.4, 138.1, 136.1, 132.7, 132.2, 127.3, 126.8, 126.4, 126.1, 123.5, 96.7, 79.2, 28.1.

HRMS (ESI-TOF) m/z calcd for $C_{15}H_{16}INO_2$ [M + H]+ : 370.0304, found: 370.0295.

(2R,3R,4R,5R)-2-((Benzoyloxy)methyl)-5-(5-(3-((tert-butoxycarbonyl)amino)naphthalen-2-yl)-4-chloro-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrofuran-3,4-diyl dibenzoate *(6)*: Compound **4** (22 g, 30.4 mmol), tert-butyl (3-iodonaphthalen-2-yl)carbamate (**5**, 10.7 g, 29.0 mmol), bis(triphenylphosphine)palladium(II) dichloride (1.02 g, 1.45 mmol), potassium carbonate (10.0 g, 72.4 mmol) was dissolved in DME (300 ml) and the reaction was stirred at 80 °C for 24 h. The reaction mixture was concentrated *in vacuo*, absorbed on Celite and purified by flash-chromatography (KP-Sil 330 g, Hept:EtOAc 90:10 to 40:60) to yield **6** (18.2 g, 75 %) as a yellow solid.

^{1}H NMR (500 MHz, DMSO) δ 8.64 (s, 1H), 8.40 (s, 1H), 8.15 (s, 1H), 8.10 (s, 1H), 7.99 (d, *J* = 7.3 Hz, 2H), 7.88 – 7.94 (m, 5H), 7.84 (d, *J* = 6.6 Hz, 2H), 7.63 – 7.68 (m, 2H), 7.57 (t, *J* =

7.3, 7.3 Hz, 1H), 7.4 – 7.53 (m, 8H), 6.87 (d, *J* = 4.7 Hz, 1H), 6.39 (t, *J* = 5.1, 5.1 Hz, 1H), 6.23 (t, *J* = 5.9, 5.9 Hz, 1H), 4.91 (q, *J* = 5.1, 5.0, 5.0 Hz, 1H), 4.82 (dd, *J* = 12.1, 3.5 Hz, 1H), 4.71 (dd, *J* = 12.1, 5.0 Hz, 1H), 1.27 (s, 9H).

^{13}C NMR (125 MHz, DMSO) δ 165.4, 164.6, 164.5, 153.2, 151.5, 151.3, 150.5, 135.6, 134.0, 133.9, 133.5, 133.1, 130.6, 129.5, 129.4, 129.3, 129.2, 129.17, 128.9, 128.8, 128.7, 128.6, 128.5, 128.3, 127.4, 127.0, 126.5, 125.5, 125.1, 116.6, 112.9, 86.6, 78.8, 73.8, 31.2, 27.9, 22.1, 13.9.

HRMS (ESI-TOF) m/z calcd for $C_{47}H_{39}ClN_4O_9$ [M + H]+ : 839.2484, found: 839.2485.

(2R,3R,4S,5R)-2-(2,3,5,6-Tetraazacyclopenta[de]tetracen-2(6H)-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol *(8)*: Compound **6** (7 g, 8.3 mmol) was dissolved in DMF (18 mL) to which 1,4-diazabicyclo[2.2.2]octane (7.5 mL, 16.7 mmol) and 2,3,4,6,7,8,9,10-octahydropyrimido[1,2-a]azepine (2.5 mL, 16.7 mmol) was added. The reaction mixture was stirred at 75 °C for 17 h. The reaction mixture was allowed to cool to RT and was subsequently co-evaporated with toluene (5x15 mL). The crude product was purified by flash chromatography (KP-Sil 100 g, DCM:MeOH 100:0 to 95:5) which yielded a mixture of products consisting of Boc- and de-Boc protected product (**7a** and **7b**, 3.1 g). The material obtained was used in the next step without further purification.

The mixture of compound **7a** and **7b** (2.85 g) was dissolved in MeCN (32 mL) and sodium methanolate (3.9 mL, 21.3 mmol) was added. The reaction mixture was stirred at RT for 1 h. The reaction mixture was concentrated *in vacuo*, absorbed onto Celite and purified by flash chromatography (KP-Sil 25 g, DCM:MeOH 100:0 to 90:10) to yield **8** (0.55 g, 17 % over two steps) as a white solid.

^{1}H NMR (500 MHz, DMSO) δ 10.90 (s, 1H), 8.19 (s, 1H), 8.15 (s, 1H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.72 (d, *J* = 8.1 Hz, 1H), 7.70 (s, 1H), 7.55 (s, 1H), 7.33 – 7.43 (m, 2H), 5.95 (d, *J* = 6.4 Hz, 1H), 5.40 (d, *J* = 6.5 Hz, 1H), 5.18 (d, *J* = 4.5 Hz, 1H), 4.55 (q, *J* = 6.3, 6.3, 6.3 Hz, 1H), 4.15 (q, *J* = 4.6, 4.6, 4.6 Hz, 1H), 3.97 (q, *J* = 3.8, 3.8, 3.8 Hz, 1H), 3.68 (d, *J* = 12.0 Hz, 1H), 3.55 – 3.61 (m, 1H), 2.54 (s, 1H).

^{13}C NMR (126 MHz, DMSO) δ 155.2, 153.6, 147.2, 137.4, 132.6, 129.6, 129.2, 128.5, 127.2, 126.6, 126.3, 124.7, 122.4, 120.3, 113.4, 113.3, 110.4, 106.7, 88.5, 85.6, 74.1, 70.9, 61.9

HRMS (ESI-TOF) m/z calcd for $C_{21}H_{18}N_4O_4$ [M + H]+ : 391.1406, found: 391.1405.

(2R,3R,4S,5R)-2-(2,3,5,6-tetraazacyclopenta[de]tetracen-2(6H)-yl)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3,4-diol *(9)*: Compound **8** (150 mg, 0.38 mmol) was co-evaporated with pyridine (25 mL) thrice before pyridine (3 mL) was added, and the flask was placed in an ice bath (0 °C) to which DMTr-Cl (143 mg, 0.42 mmol) was added in one portion. The reaction mixture was stirred for 5 min before being returned to RT and stirred for 18 h. MeOH (3 mL) was added and the reaction mixture was extracted with EtOAc (50 mL), washed with water (20 mL) and brine (10 mL). The organic layers were dried over $MgSO_4$, concentrated *in vacuo*, absorbed onto Celite and purified by flash chromatography (KP-Sil 25 g, flushed with 2% Et_3N in DCM prior to use, EtOAc in DCM: 0 to 50%) to yield **9** (60 mg, 23 %) as a light brown solid.

^{1}H NMR (500 MHz, DMSO-d_6) δ 10.90 (bs, 1H), 8.19 (s, 1H), 7.74 (s, 1H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.66 (d, *J* = 9.9 Hz, 2H), 7.52 (s, 1H), 7.40 (d, *J* = 7.5 Hz, 3H), 7.33 – 7.39 (m, 2H), 7.28 (t, *J* = 8.3 Hz, 6H), 7.20 (t, *J* = 7.3 Hz, 1H), 6.84 (dd, *J* = 8.9, 6.9 Hz, 4H), 6.03 (d, *J* = 4.5 Hz, 1H), 5.57 (bs, 1H), 4.67 (t, *J* = 4.8 Hz, 1H), 4.39 (t, *J* = 5.0 Hz, 1H), 4.07 (q, *J* = 4.4 Hz, 1H), 3.66 (s, 3H), 3.65 (s, 3H), 3.22 (m, 2H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 158.1, 158.0, 155.5, 153.6, 147.6, 144.8, 137.6, 135.7, 135.6, 132.7, 129.8, 129.7, 129.5, 127.9, 127.8, 127.1, 126.7, 126.6, 126.4, 124.7, 122.2, 120.3, 113.3, 113.2, 112.8, 110.7, 106.5, 88.4, 85.6, 82.8, 74.2, 70.6, 63.7, 55.0, 54.9.

HRMS (ESI-TOF) m/z calcd for $C_{42}H_{36}N_4O_6$ [M+H]+: 693.2713, found: 693.2729.

((2R,3S,4R,5R)-5-(2,3,5,6-tetraazacyclopenta[de]tetracen-2(6H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl hydrogen triphosphate *(10)*: Reaction was performed following procedure described by Baladi *et al.*[1] using CPG-bound pA nucleoside (**9**, 400 mg, 18 umol/g loading, 0.0072 mmol). Briefly, steps were performed as following:

a. 5'-DMT removal: the support was washed with a flow of DCA deblock until the filtrate was colorless, whereafter it was washed with ACN (5 x 5 mL).

b. Coupling: *N*,*N*-diisopropyl-4*H*-benzo[*d*][1,3,2]dioxaphosphinin-2-amine (345 mg, 1.36 mmol) was dissolved in 5 mL ACN and reacted portion wise with the support (3 equally charged couplings with reaction times 60 s, 60 s, and 90 s, respectively). To each coupling, BTT activator (2.4 mL) was also added. The support was subsequently washed with ACN (3 x 5 mL).

c. Oxidation: Pyridine/Water/Iodine (9/1/12.7 v/v/w, 5 mL) for 45 s, followed by ACN wash (3 x 5 mL) and drying of the support in an argon flow.

d. Triphosphorylation: Two injections of bis(tetrabutylammonium) dihydrogen diphosphate (0.5 M, 5 ml) were carried out and allowed to react for 15 min and 18 hours, respectively. The support was subsequently rinsed with DMF (5 mL), water (3 x 5 mL), ACN (5 mL) and then dried in an argon flow.

e. Cleavage and Purification: Cleavage of the triphosphate was done with AMA (50/50 v/v mix of 23% aq. NH4OH and 40% aq. methylamine, 5 mL) for 2 h at RT. After 2 h, the AMA filtrate was purged in a round-bottom flask and the support was rinsed 3 times with 23% aq. NH4OH solution. After freeze-drying the mixture, purification by HPLC (XBridge BEH C18 OBD, 5 μm 10x250 mm column, 25 mL/min, A: dibutylammonium acetate 50 mM pH7 in 95% water and 5% ACN, B: ACN, 15%–45% B over 10 min followed by 100% B over 2 min) was performed to yield pATP (2.7 mg, 60 %, as determined by UV absorption) as a white solid (dibutylammonium salt). The salt form could subsequently be exchanged to ammonium using standard phase HPLC in ammonium carbonate or ammonium ion-exchange columns.

^{1}H NMR (500 MHz, D_2O, 25°C) δ 4.37 (s, 2H), 4.44 (s, 1H), 4.67 (d, J = 4.2 Hz, 2H), 5.88 (d, J = 5.9 Hz, 1H), 6.89 (s, 1H), 7.24 (z, 3H), 7.34–7.42 (m, 2H), 7.55 (s, 1H), 7.70 (s, 1H).

^{13}C NMR (126 MHz, D_2O) δ 134.3, 131.9, 129.5, 127.2, 126.7, 126.5, 125.3, 122.7, 118.2, 114.3, 112.3, 105.0, 86.8, 83.4, 74.7, 70.7, 65.6.

^{31}P NMR (202 MHz, D_2O, 25°C) δ -22.58 (J = 20.1), -11.08 (J = 19.9), -8.72.

HRMS (ESI-TOF) m/z calcd for $C_{21}H_{21}N_4O_{13}P_3$ [M+H]+: 631.0396, found: 631.0416.

((2R,3S,4R,5R)-5-(8-cyano-2,3,5,6-tetraazaaceanthrylen-2(6H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl dihydrogen phosphate (*12*): 2CNqAMP was synthesized using a similar method than pATP **10**, starting from the corresponding 2CNqA

nucleoside, but replacing step d with a simple water wash to hydrolyze the cyclic phosphoramidite intermediate. Compound **12** was isolated as a yellow solid (2 mg, 40 %).

^{1}H NMR (500 MHz, D_2O) 4.04 – 4.2 (m, 2H), 4.36 (s, 1H), 4.53 (t, *J* = 4.2 Hz, 1H), 4.72 (t, *J* = 5.7 Hz, 1H), 5.99 (d, *J* = 6.0 Hz, 1H), 6.76 (s, 1H), 6.87 (d, *J* = 8.0 Hz, 1H), 7.15 (d, *J* = 7.9 Hz, 1H), 7.38 (s, 1H), 7.81 (s, 1H).

^{31}P NMR (202 MHz, D_2O) 1.75. MS (ESI+ , MeOH) m/z: 446.1 [M+H]+.

((2R,3S,4R,5R)-5-(8-cyano-2,3,5,6-tetraazaaceanthrylen-2(6H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl hydrogen triphosphate (*13*): 2CNqATP was synthesized as previously described by our group.[5] An additional ^{13}C NMR experiment was recorded to strengthen characterization data.

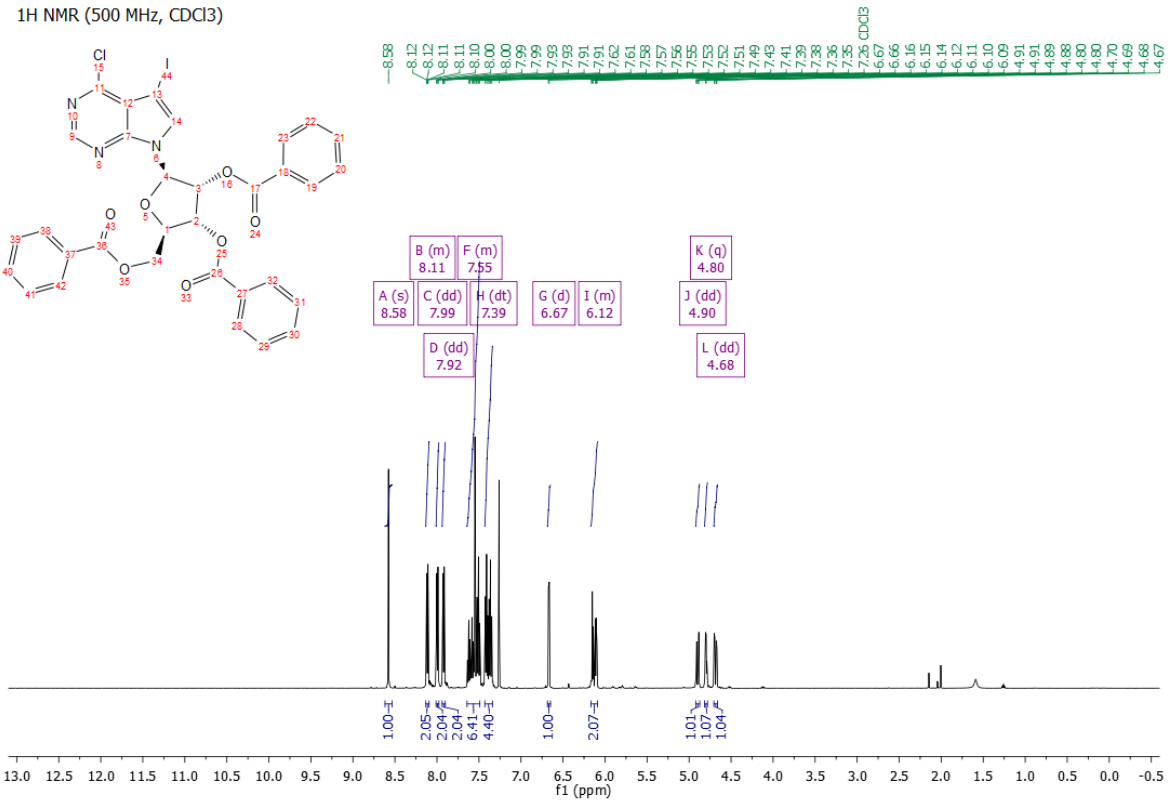
^{1}H NMR (500 MHz, D_2O) δ 7.88 (s, 1H), 7.47 (s, 1H), 7.34 (d, J = 7.9 Hz, 1H), 6.99 (d, J = 7.8 Hz, 1H), 6.83 (s, 1H), 6.04 (d, J = 5.8 Hz, 1H), 4.76 (s, 1H), 4.61 (s, 1H), 4.39 (s, 1H), 4.31 (s, 2H).

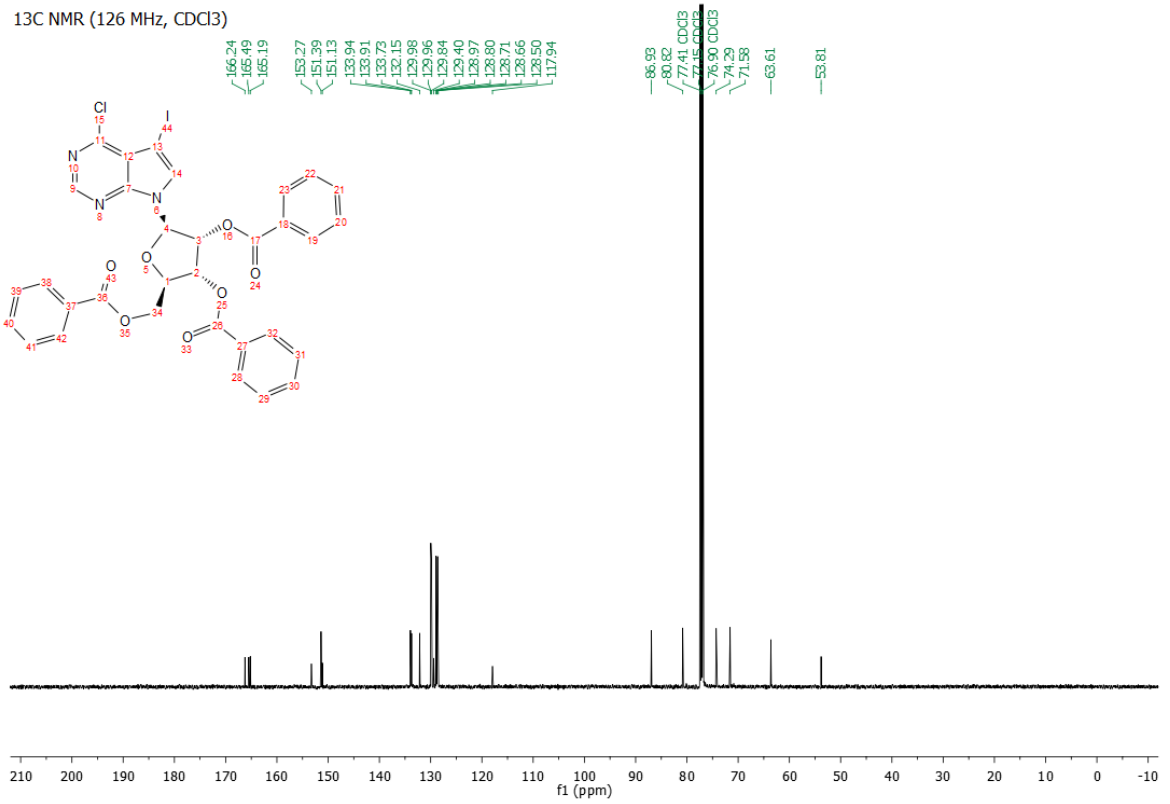
^{31}P NMR (202 MHz, D_2O) δ -22.48 (bs, 1P), -11.19 (d, J=19.9 Hz, 1P), -9.25 (bs, 1P).

^{13}C NMR (126 MHz, D_2O) δ 154.3, 152.2, 146.1, 137.6, 126.9, 125.1, 123.7, 120.9, 119.1, 111.7, 111.2, 109.1, 107.7, 86.7, 83.6, 83.5, 74.6, 70.6.

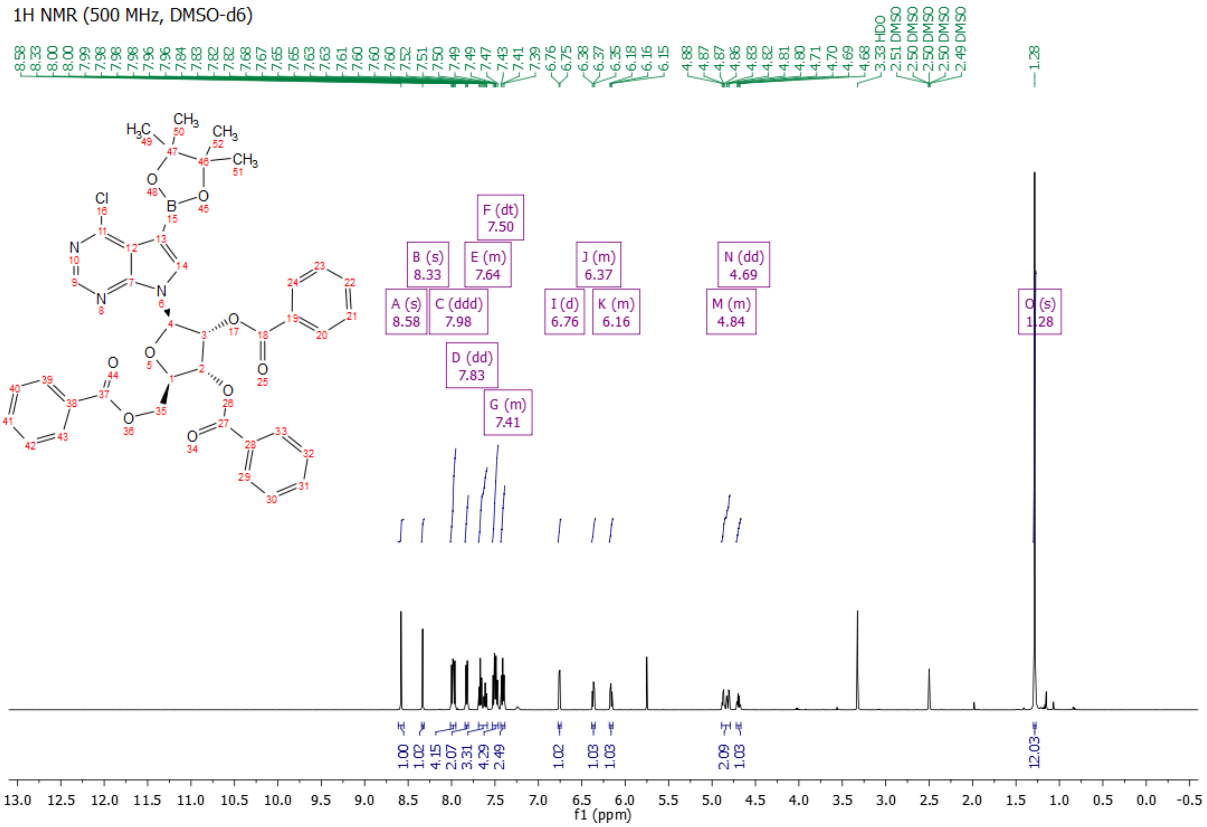
1.3 Spectral data

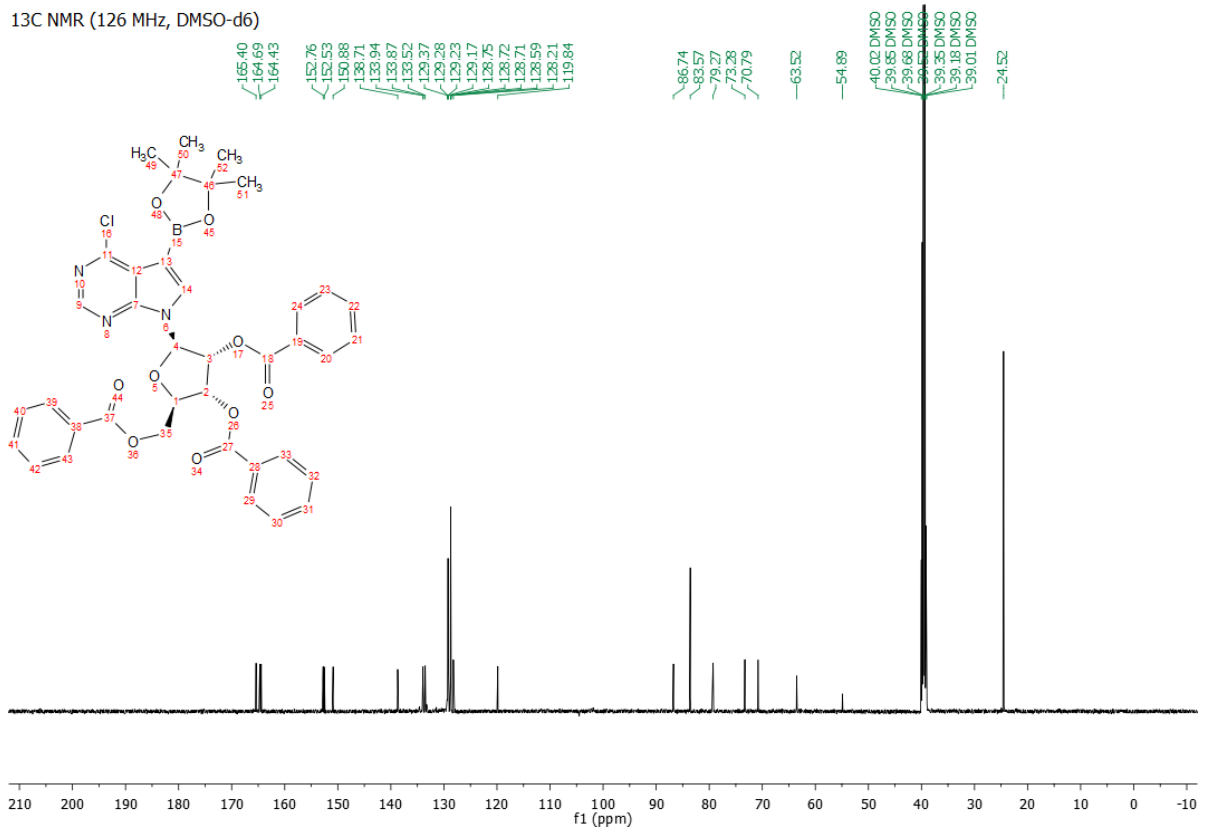
(2R,3R,4R,5R)-2-((benzoyloxy)methyl)-5-(4-chloro-5-iodo-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrofuran-3,4-diyl dibenzoate *(3)*



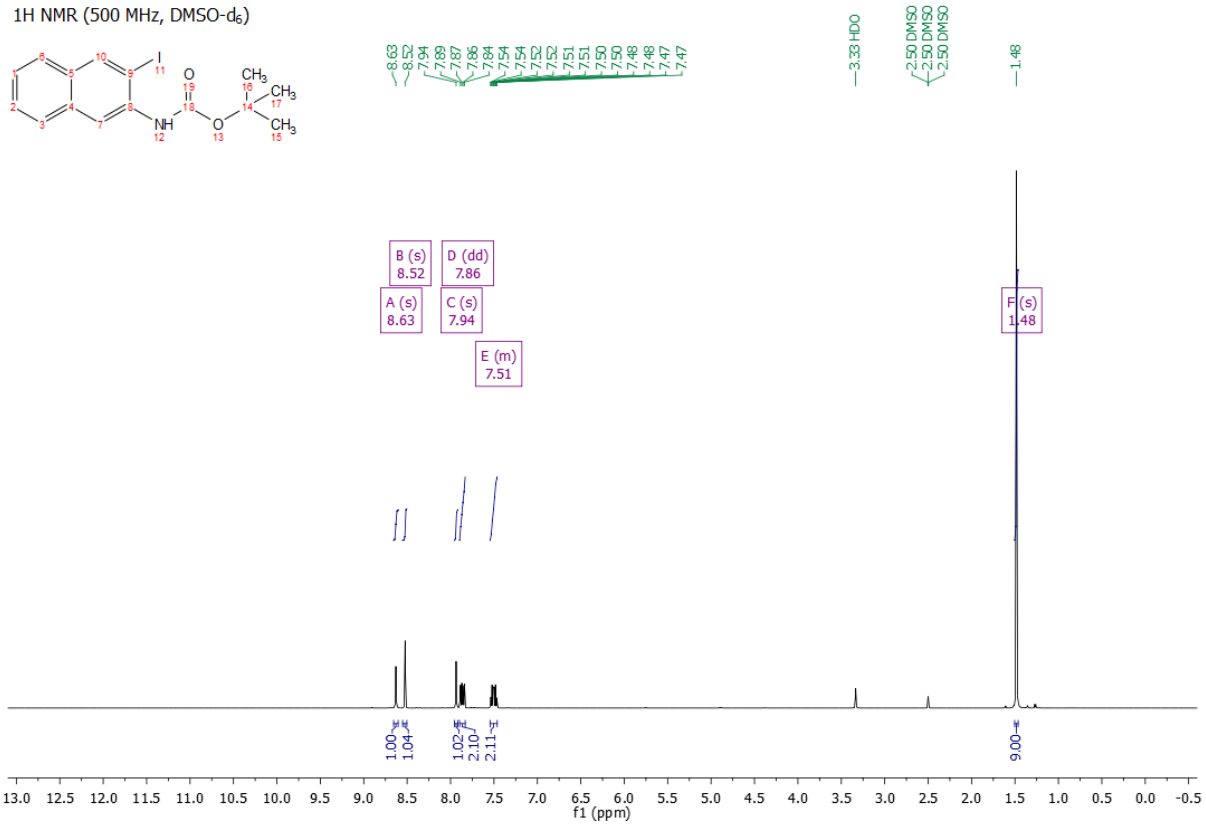


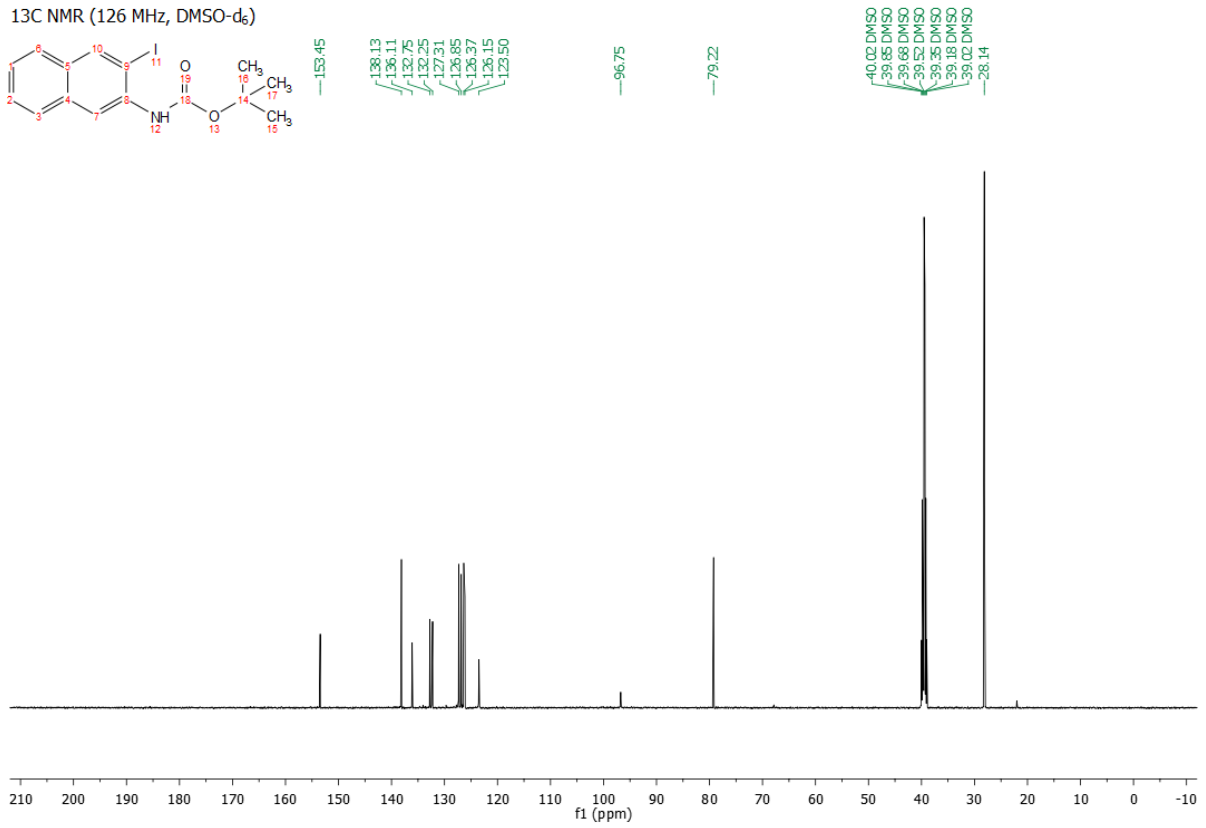
(2R,3R,4R,5R)-2-((benzoyloxy)methyl)-5-(4-chloro-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrofuran-3,4-diyl dibenzoate *(4)*



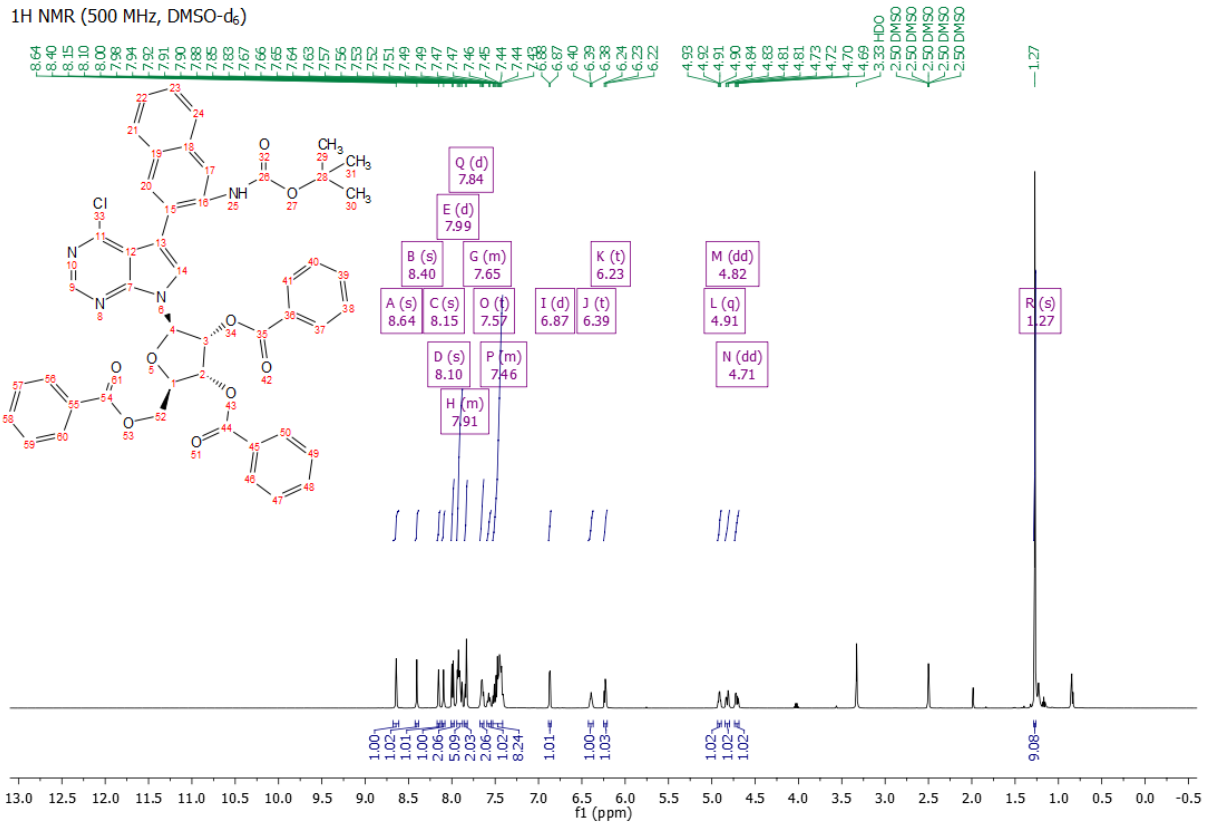


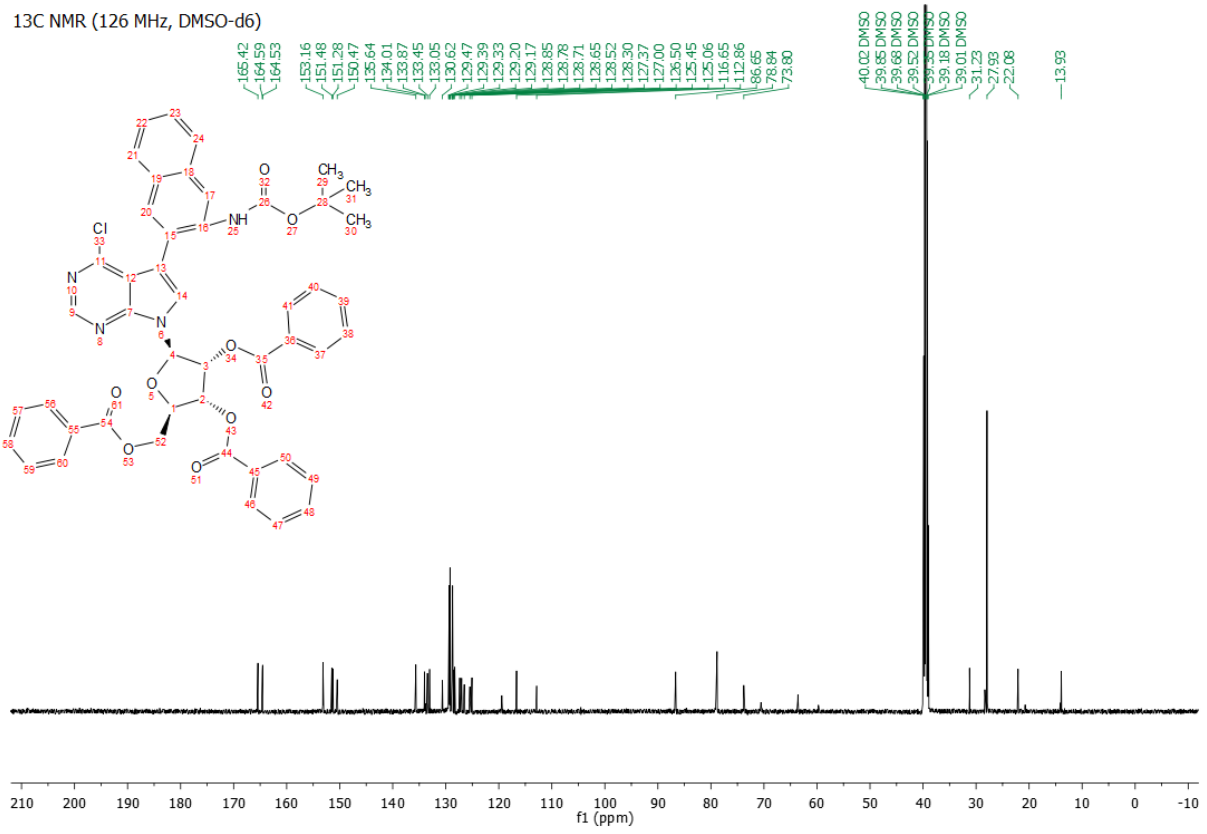
tert-butyl (3-iodonaphthalen-2-yl)carbamate *(5)*



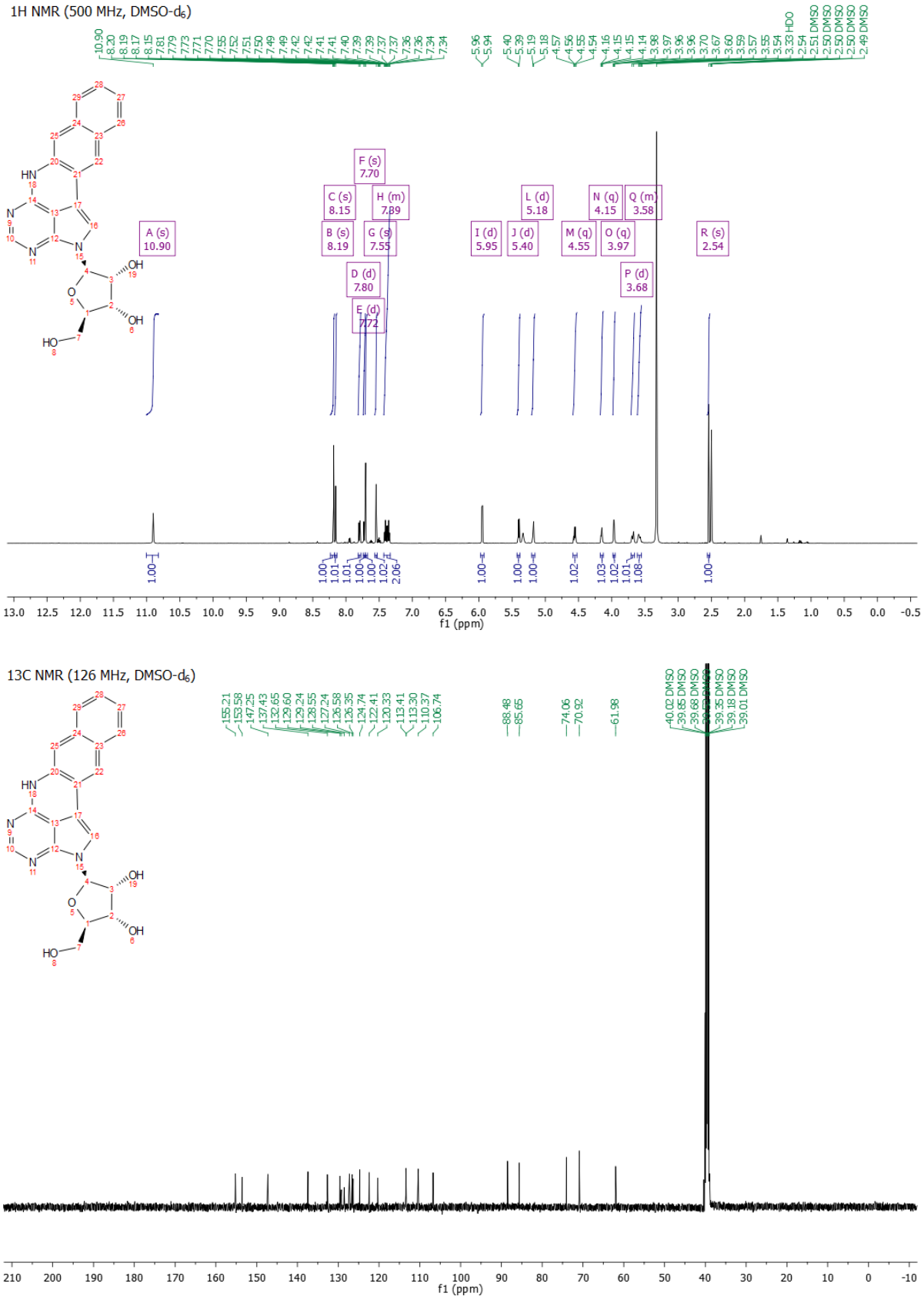


(2R,3R,4R,5R)-2-((benzoyloxy)methyl)-5-(5-(3-((tert-butoxycarbonyl)amino)naphthalen-2-yl)-4-chloro-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrofuran-3,4-diyl dibenzoate *(6)*

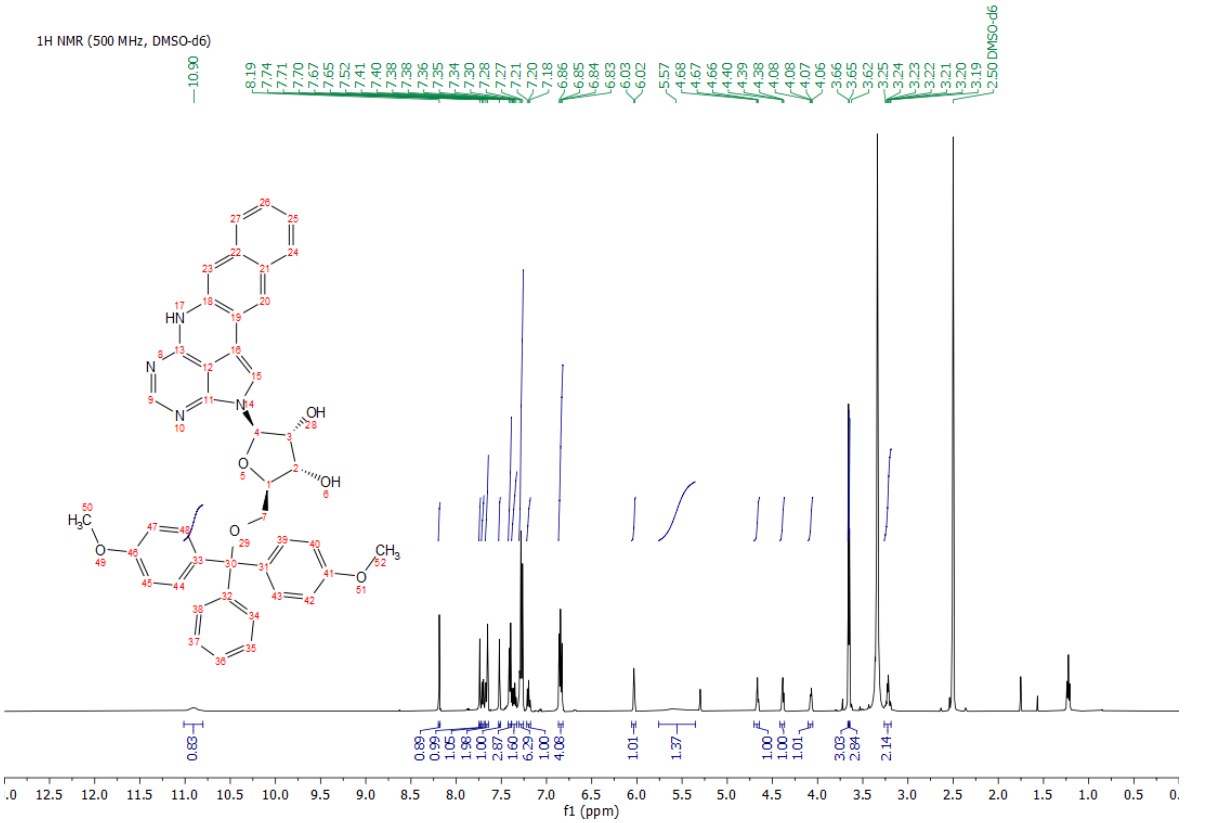


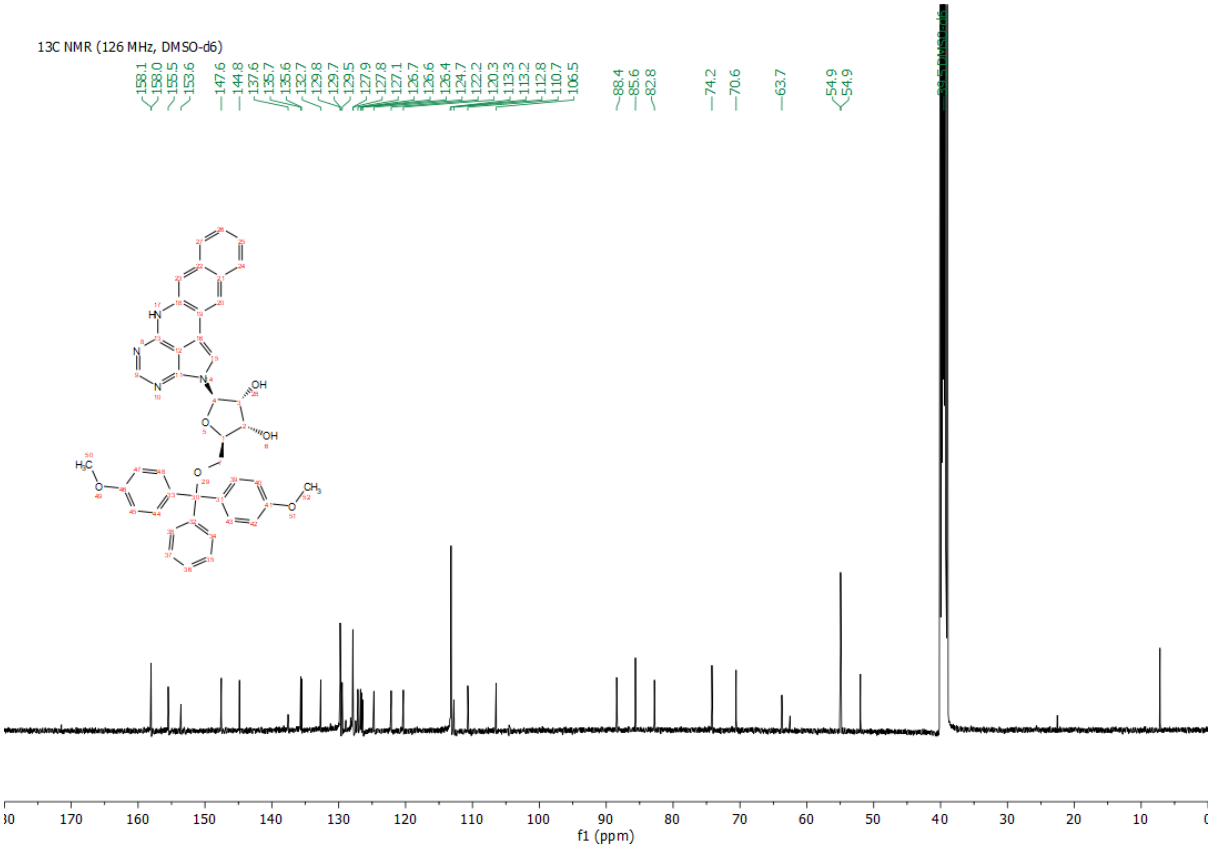


(2R,3R,4S,5R)-2-(2,3,5,6-tetraazacyclopenta[de]tetracen-2(6H)-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol *(8)*

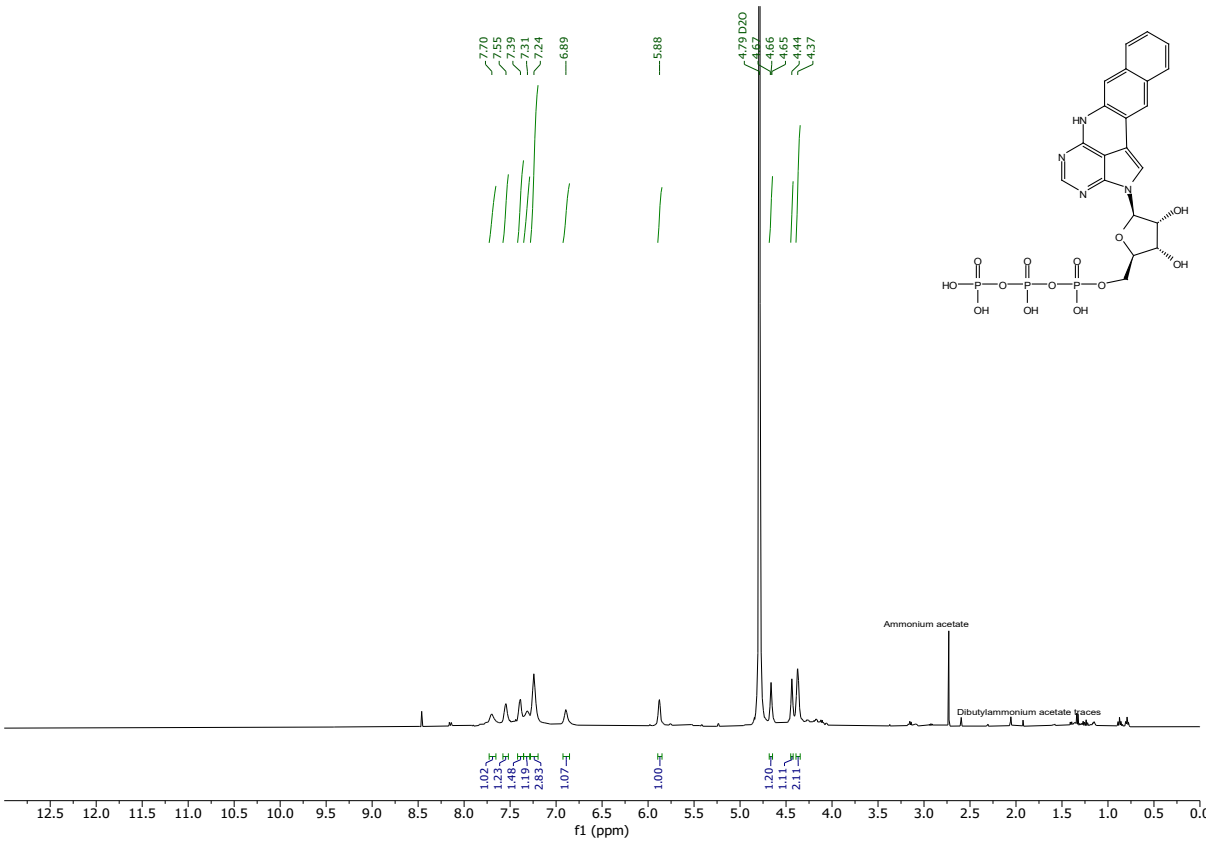


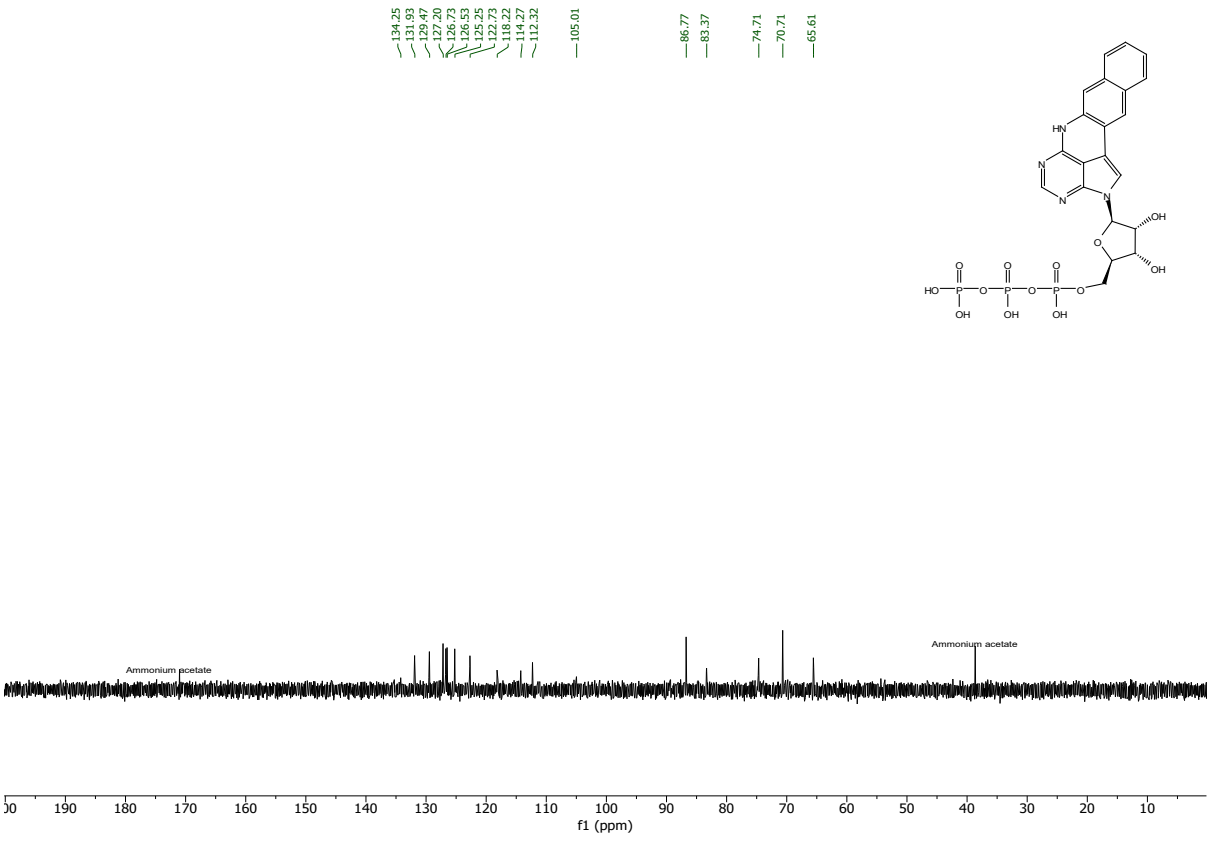
(2R,3R,4S,5R)-2-(2,3,5,6-tetraazacyclopenta[de]tetracen-2(6H)-yl)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3,4-diol *(9)*

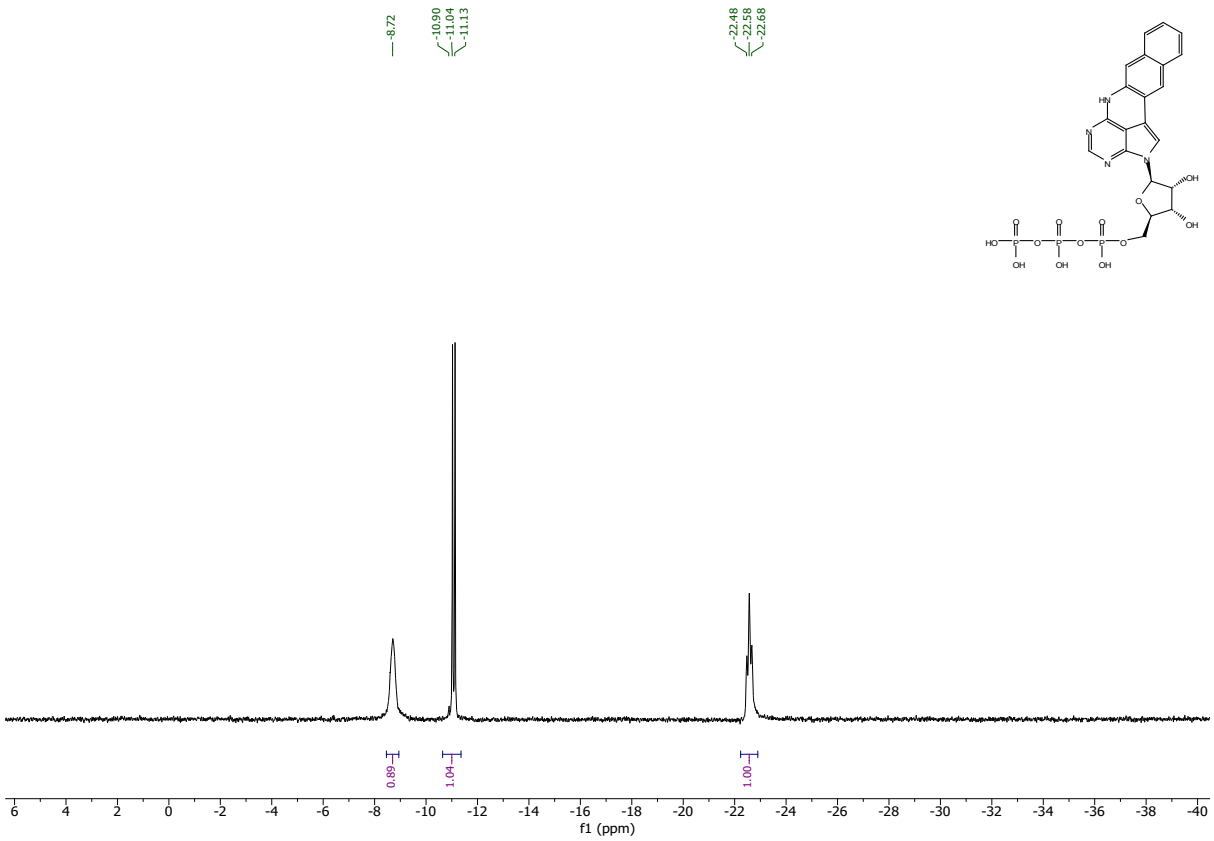




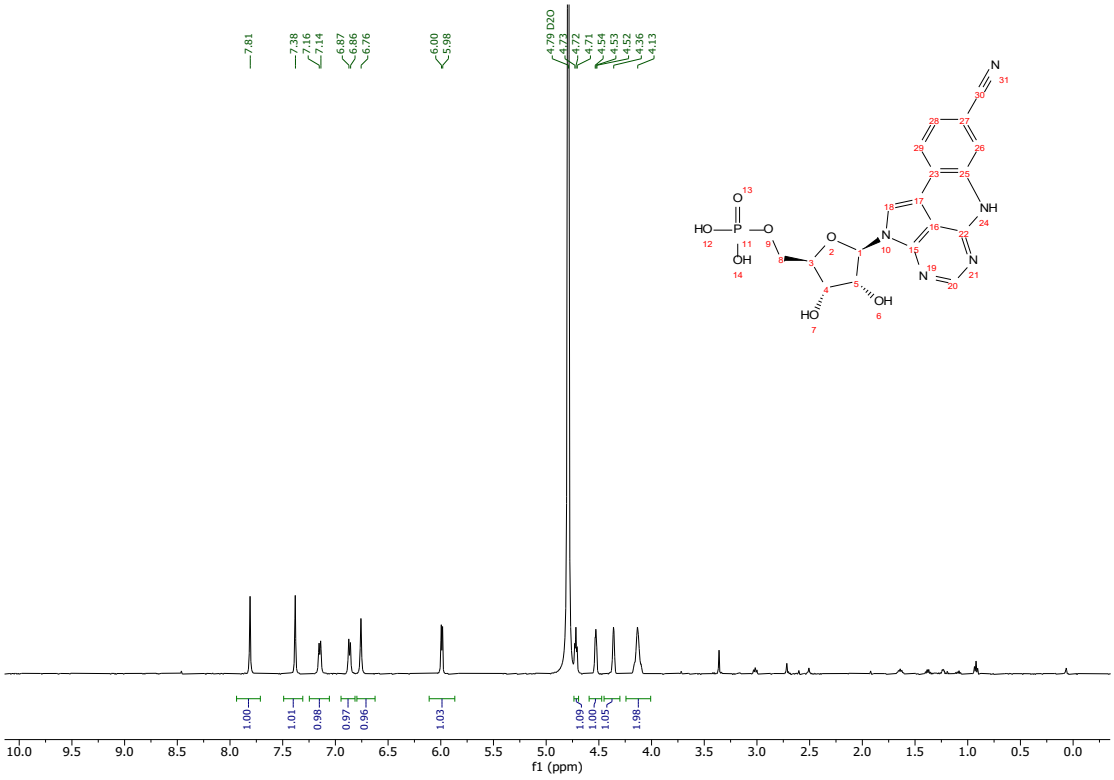
((2R,3S,4R,5R)-5-(2,3,5,6-tetraazacyclopenta[de]tetracen-2(6H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl hydrogen triphosphate *(10)*

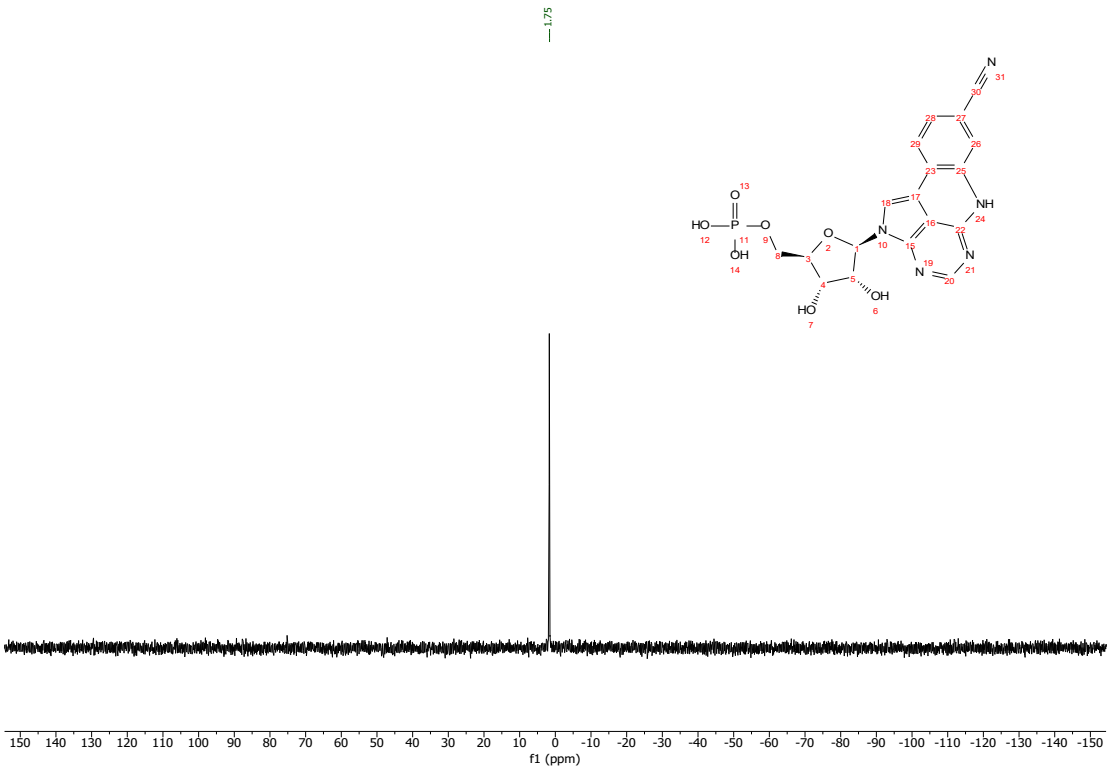


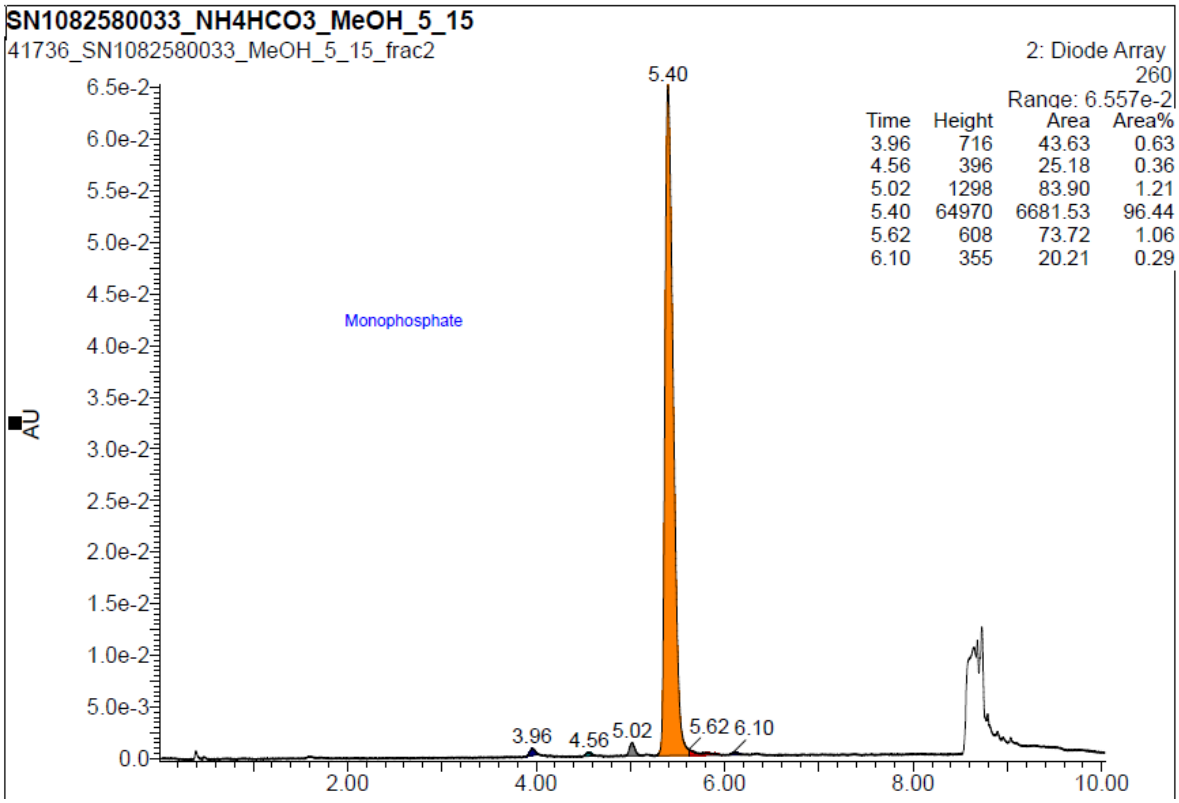




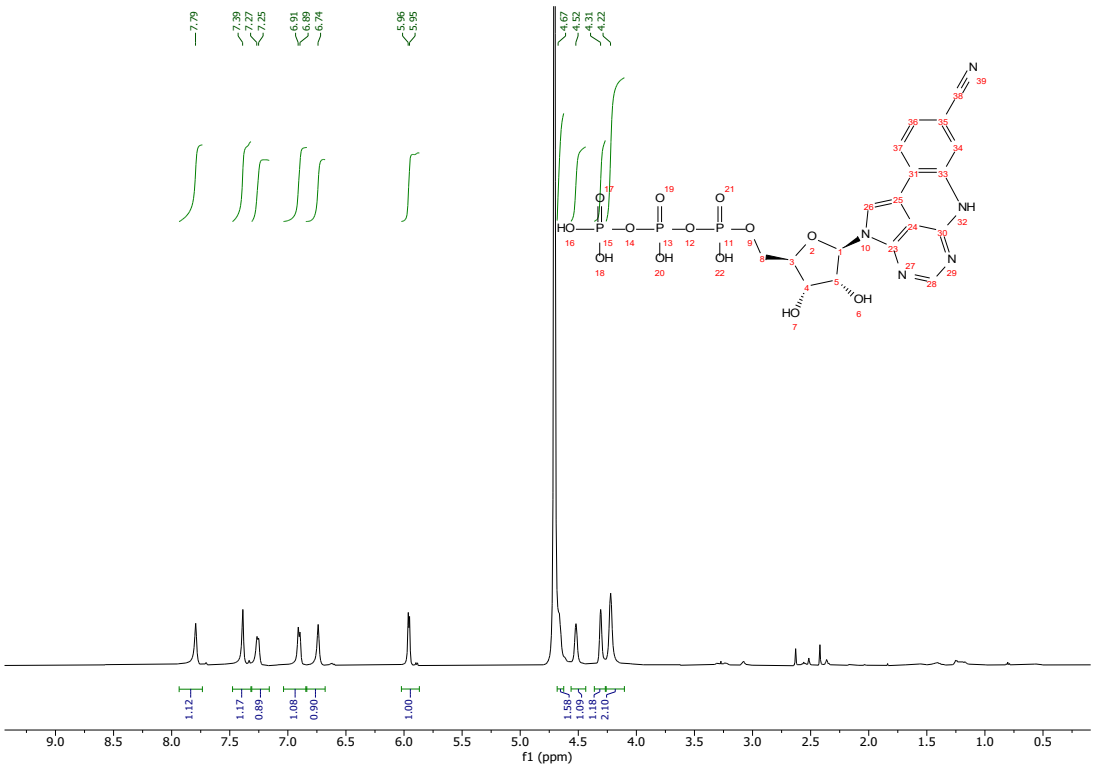
((2R,3S,4R,5R)-5-(8-cyano-2,3,5,6-tetraazaaceanthrylen-2(6H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl dihydrogen phosphate (*12*)

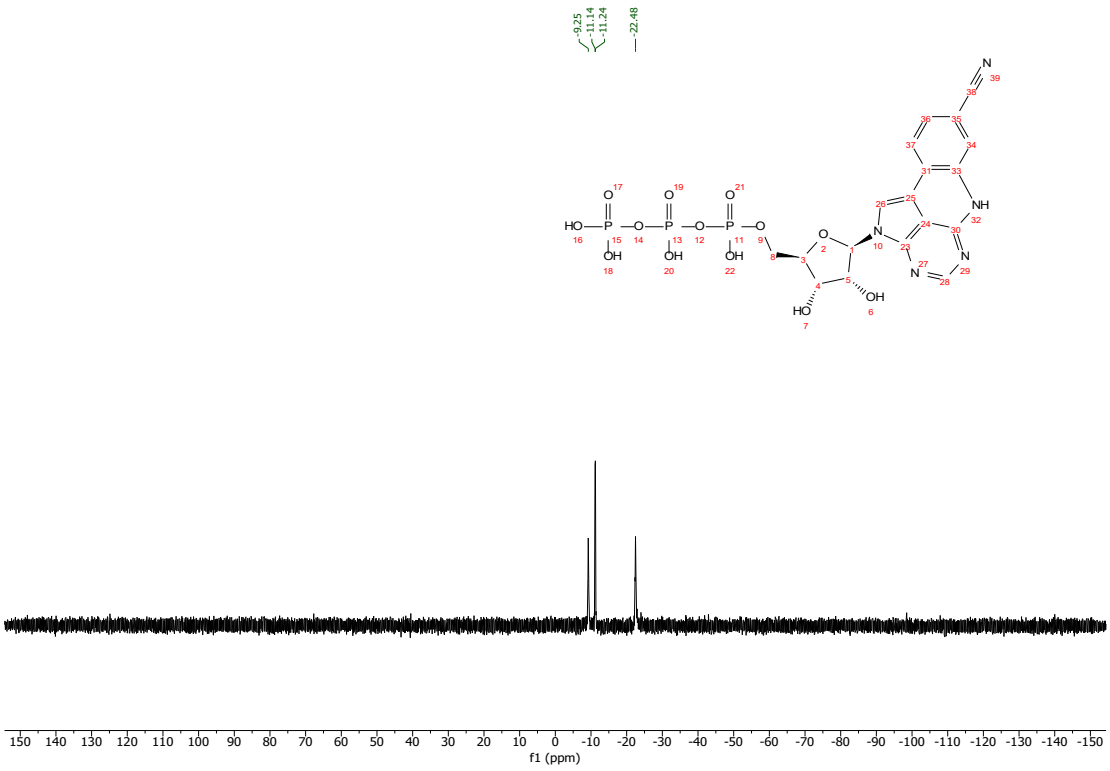


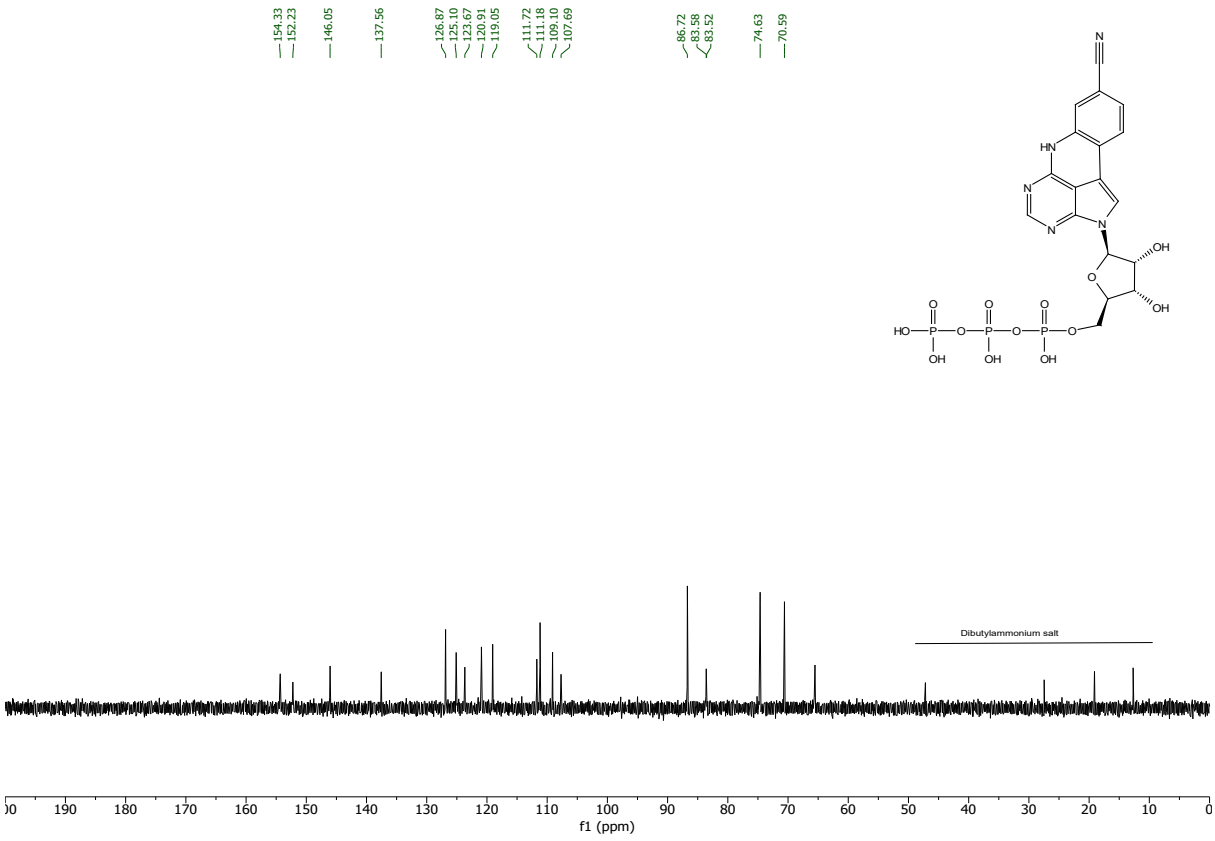


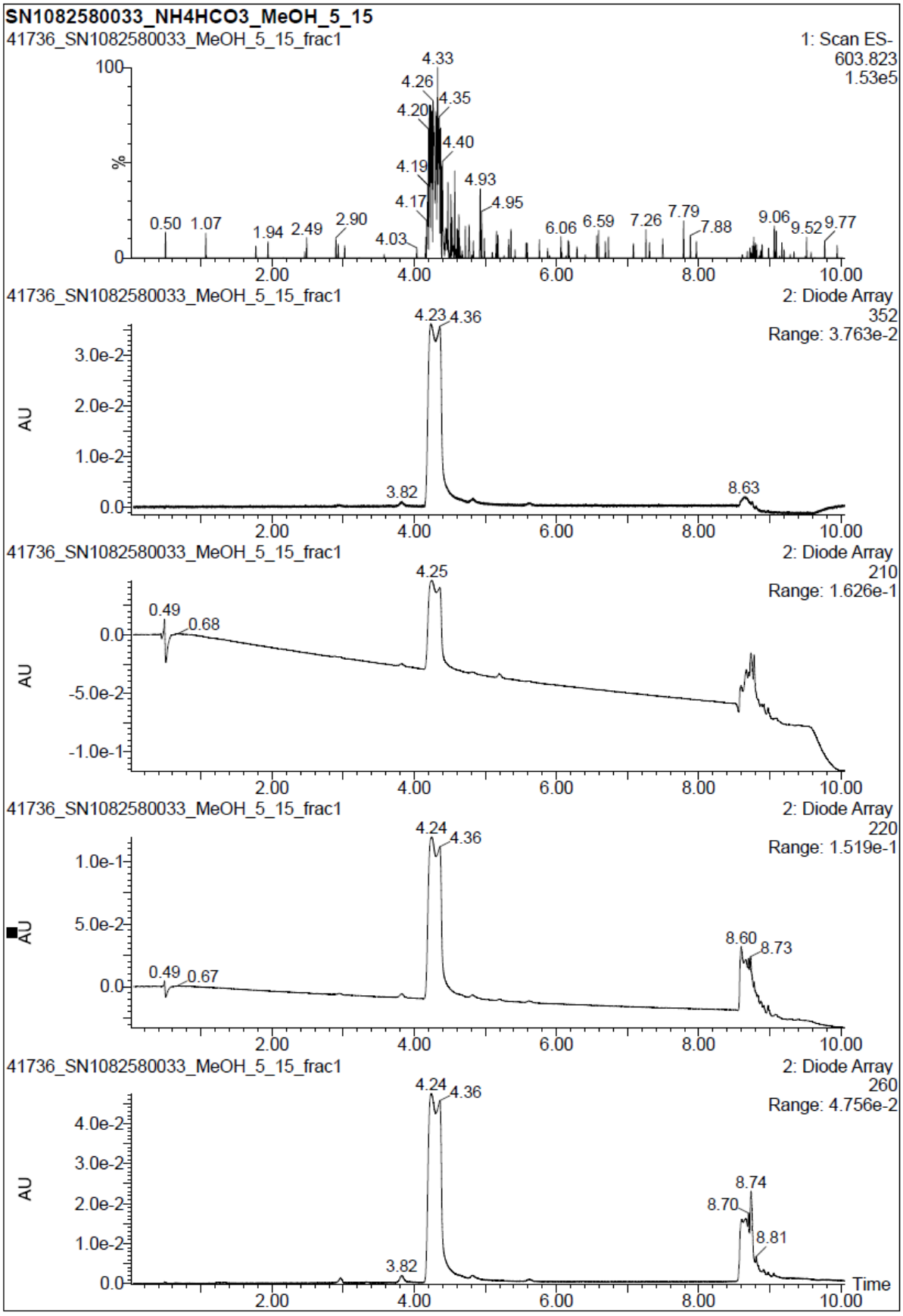


((2R,3S,4R,5R)-5-(8-cyano-2,3,5,6-tetraazaaceanthrylen-2(6H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl hydrogen triphosphate (*13*)









1.4 UV-vis and fluorescence spectroscopy

Supplementary Table S1. Photophysical characteristics of the FBA-TPs used in this study, recorded at room temperature (*ca.* 22 °C). For buffer conditions, see materials and methods section or the indicated references. ε_{260}: molar absorptivity at 260 nm; Φ_F: fluorescence quantum yield referenced to quinine sulphate in 0.5 M H_2SO_4; $\lambda_{abs,max}$: wavelength of absorption maximum; $\lambda_{em,max}$: wavelength of emission maximum; $\varepsilon_{max,\ FBA}$ x Φ_F: brightness at the absorption maximum; $\varepsilon_{max,\ FBA}$ x Φ_F: brightness at 405 nm which is the excitation wavelength used for the microscopy and flow cytometry experiments in this study; τ: fluorescence lifetime

FBA-TP	ε_{260} **(M^{-1} cm^{-1})**	Φ_F **(%)**	$\lambda_{abs,\ max}$ **(nm)**	$\lambda_{em,\ max}$ **(nm)**	$\varepsilon_{max,\ FBA}$ x Φ_F **(M^{-1} cm^{-1})**	$\varepsilon_{405,\ FBA}$ x Φ_F **(M^{-1} cm^{-1})**	**τ (ns)**
tC^{O}TP	11000[2]	27	360	453	2700	417	3.4
2CNqATP	14600[3]	48[5]	352[5]	471[5]	4900	504	9.9[5]
pATP	22300[4]	49	386	420	6000	909	6.0

2. Analysis of nucleoli under ActD exposure

Supplementary Table S2. Analysis of nucleoli area under ActD exposure using 2CNqATP uptake as readout. Three images per condition (ActD treated or 2CNqATP only, *i.e.* control) were used for analysis. Using the fluorescent channel and the transmission image nuclei were identified and nucleoli inside them were used for analysis. Using the polygon selection tool in ImageJ nucleoli were outlined and measured. Visualization of this data is presented in the main Figure 2D.

ActinomycinD treated		**Control**	
#nucleolus	**Area [µm^2]**	**#nucleolus**	**Area [µm^2]**
1	5.858	1	27.624
2	1.967	2	15.560
3	1.381	3	11.553
4	3.640	4	13.983
5	1.506	5	6.053
6	5.670	6	8.611
7	3.965	7	8.910
8	5.542	8	6.480
9	4.604	9	6.096
10	2.558	10	7.929
11	1.108	11	32.678
12	1.918	12	17.532
13	6.569	13	9.331
14	4.142	14	7.615
15	3.724	15	16.862
16	4.059	16	9.080
17	2.720	17	2.803
18	2.092	18	11.883
19	8.034	19	16.779
20	4.059	20	6.109
21	1.841	21	5.942
22	3.389	22	5.983
23	1.967	23	4.644
24	5.607	24	4.477
25	4.937	25	24.268
26	2.008	26	9.373
27	4.937	27	5.732
28	1.716	28	4.142
29	1.046	29	9.875
30	2.720	30	13.975
Mean	**3.509**	**Mean**	**11.063**
Standard Deviation	**1.781**	**Standard Deviation**	**7.029**
	***t* test : p = 3.13872E-06**		

3. Pulse-Chase Experimental Setup

Supplementary Table S3. Tested conditions using ActD and $NaAsO_2$. Cells were first exposed to 2.5 µM 2CNqATP over three hours for maximum fluorescent signal. Hereafter, cells were washed once with PBS and exposed to indicated concentrations of ActD or $NaAsO_2$. Effects were observed under confocal laser scanning microscope over time. Conditions used for data presented in the main text are highlighted in bold.

Treatment	Concentration	Observation
ActD	1 µM	No visible effect after several hours.
	2 µM	After 3 h nucleoli visibly smaller.
	4 µM	After 1.5 h nucleoli become visibly smaller, cells start dying at around **3 h**.
$NaAsO_2$	0.5 µM	No visible effect after several hours.
	50 µM	Cells start dying at around **2 h**, nucleoli remain intact.
	200 µM	Cells die within one hour

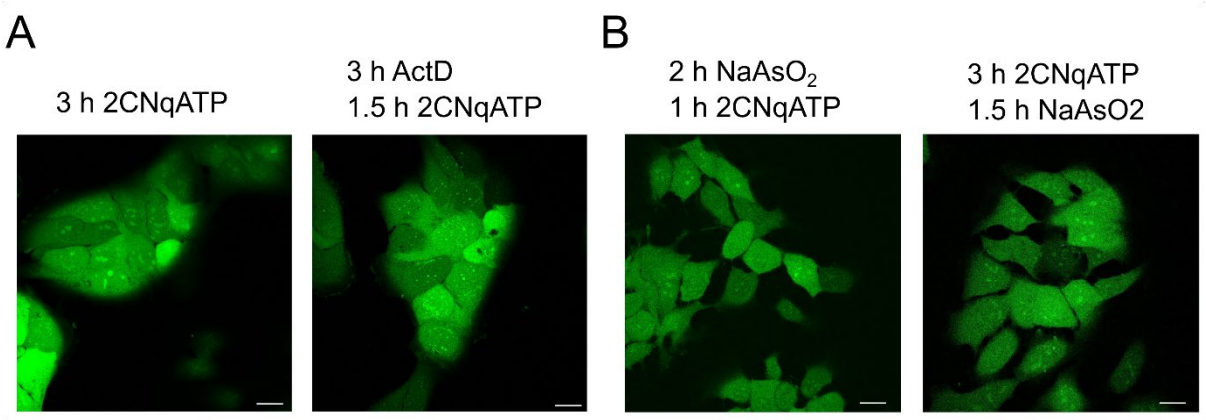
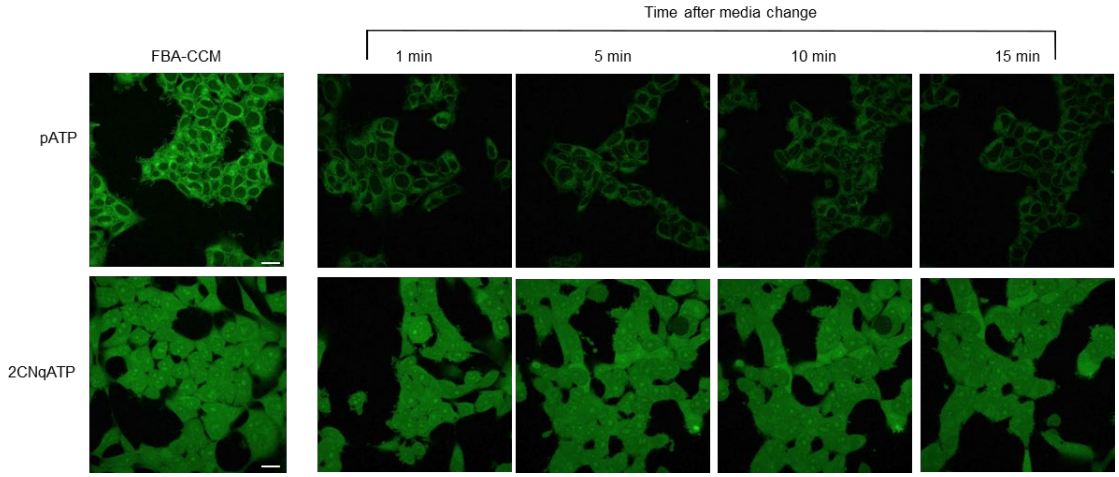


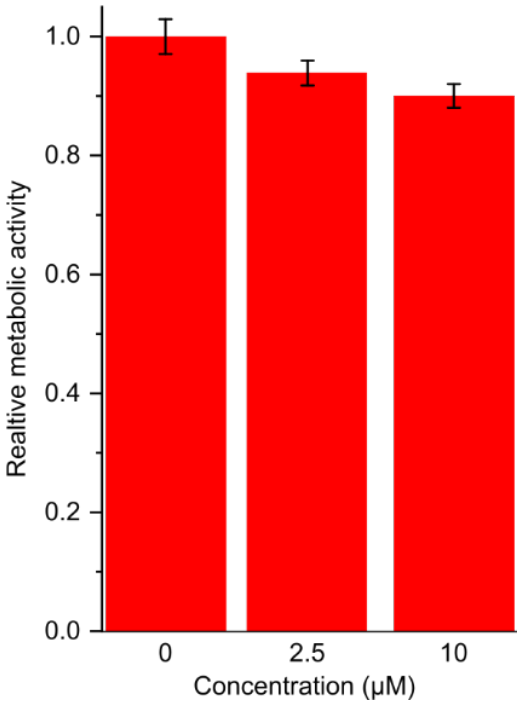
Figure S1. A Left: Huh-7 cells exposed to 2.5 µM 2CNqATP for comparison. Right: Huh-7 cells were first exposed to 4 µM ActD for 1.5 h. Then 2CNqATP was added while ActD was kept in the medium. Indicated times in the figure are total exposure times at imaging time. **B** Huh-7 cells exposed to 2CNqATP and $NaAsO_2$. Left: cells were first exposed to 50 µM $NaAsO_2$ for one hour and then 2.5 µM 2CNqATP was added. Right: Cells were first incubated with 2.5 µM 2CNqATP for three hours, then washed once with PBS and hereafter exposed to 50 µM $NaAsO_2$ for 1.5 h. Note that brightness and contrast of the images are adjusted for better visibility of nucleolar structure. Due to different 2CNqATP exposure times the signal intensity varied. Scale bars 20 µm.

4. Accumulation of pATP and 2CNqATP in SH-SY5Y cells before and after exchanging the FBA treatment solutions to fresh cell culture medium (CCM)



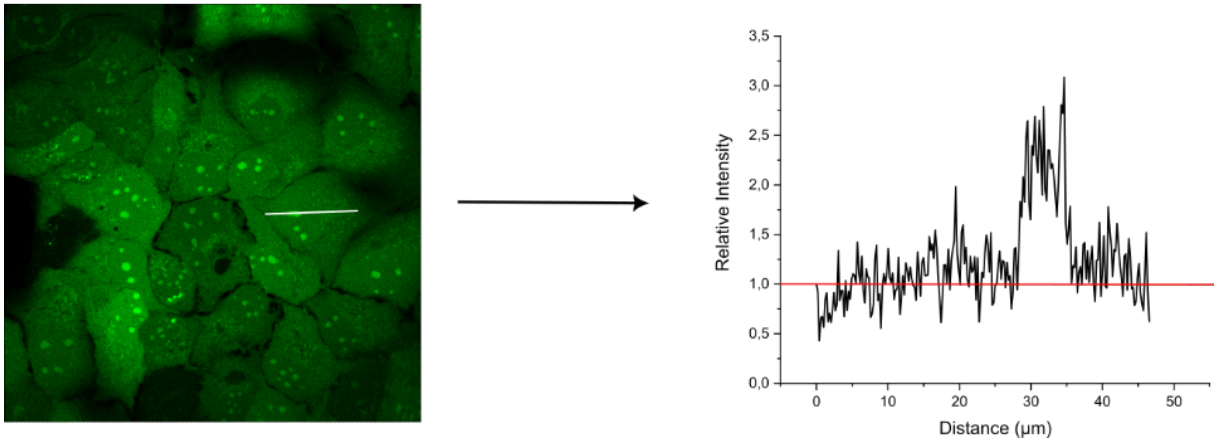
Supplementary Figure S2. Confocal microscopy images of SH-SY5Y cells before (24 h after exposure) and after the exchange of the 2.5 µM FBA treatment solution with normal CCM, showing the clearance as function of time. Scale bars represent 20 µm.

5. Cytotoxicity assessment of 2CNqAMP



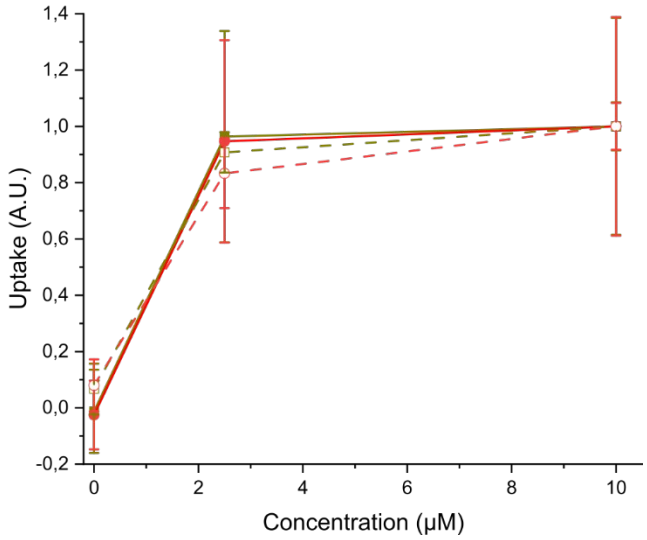
Supplementary Figure S3. Cytotoxicity of 2CNqAMP to Huh-7 cells determined using the AlamarBlue assay. Relative metabolic activity was referenced to that of untreated cells. All treatments were performed in triplicates in a single experiment. The data is presented as mean ± standard deviation.

6. Intensity profile analysis of 2CNqAMP subcellular distribution in Huh-7 cells



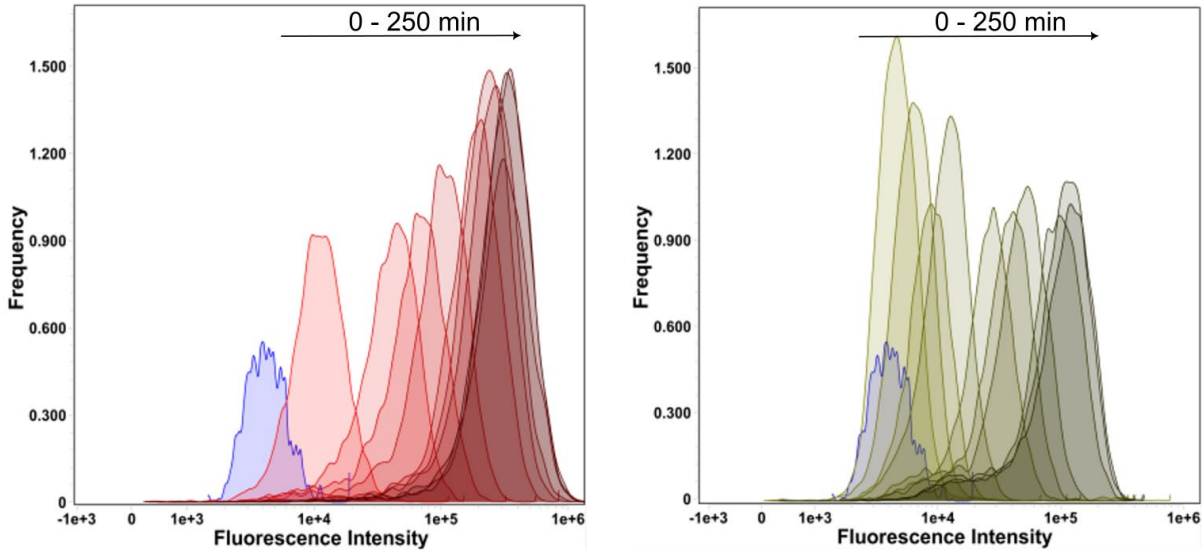
Supplementary Figure S4. Representative fluorescence intensity line profile of cellular 2CNqAMP intensity after 24 h of incubation of Huh-7 cells at 37 °C with 2.5 µM 2CNqAMP in the cell medium. The nucleoli display a 2.3 ± 0.1 times higher emission intensity compared to the nuclei and cytosol.

7. Comparison of flow cytometry readout with lysate readout using plate reader



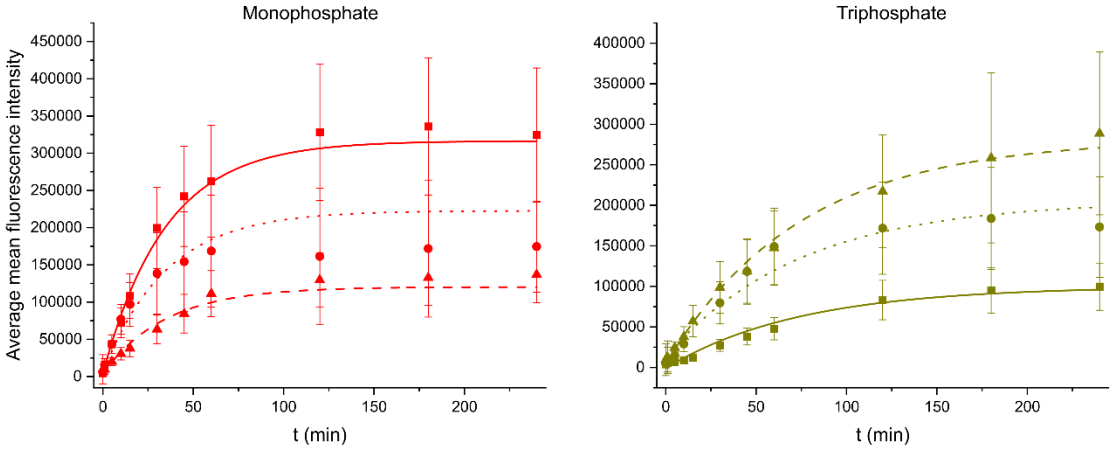
Supplementary Figure S5. Comparison between readout methods of the cellular uptake of 2CNqATP (yellow) and 2CNqAMP (red) at 37 °C at various concentration after 24 h. Filled circles with solid lines represent readout of flow cytometry readout of detached Huh-7 WT cells after exposure. Empty circles with dotted lines show readout of lysed cells using a plate reader. Lines were added to guide the eyes. Experiment was performed once including two exposures of each compound at each concentration (mean ± S.D.).

8. Flow cytometry histograms showing FBA uptake as function of time



Supplementary Figure S6. Representative flow cytometry histograms showing the distribution of cellular fluorescence intensity as function of incubation time for Huh-7 cells. The blue histograms show the background signal of unexposed Huh-7 cells. Left panel: Cells exposed to 2CNqAMP (2.5 µM) with lightest red directly after exposure ("0 min") and darkest red 250 min after exposure. Right panel: Cells exposed to 2CNqATP (2.5 µM) with lightest green directly after exposure ("0 min") and darkest green 250 min after exposure. Time interval was 5 min over 15 min, followed by an interval of 15 min to 60 min exposure time and finally 60 min interval until a total exposure time of 250 min was reached.

9. Fitting of the uptake kinetics

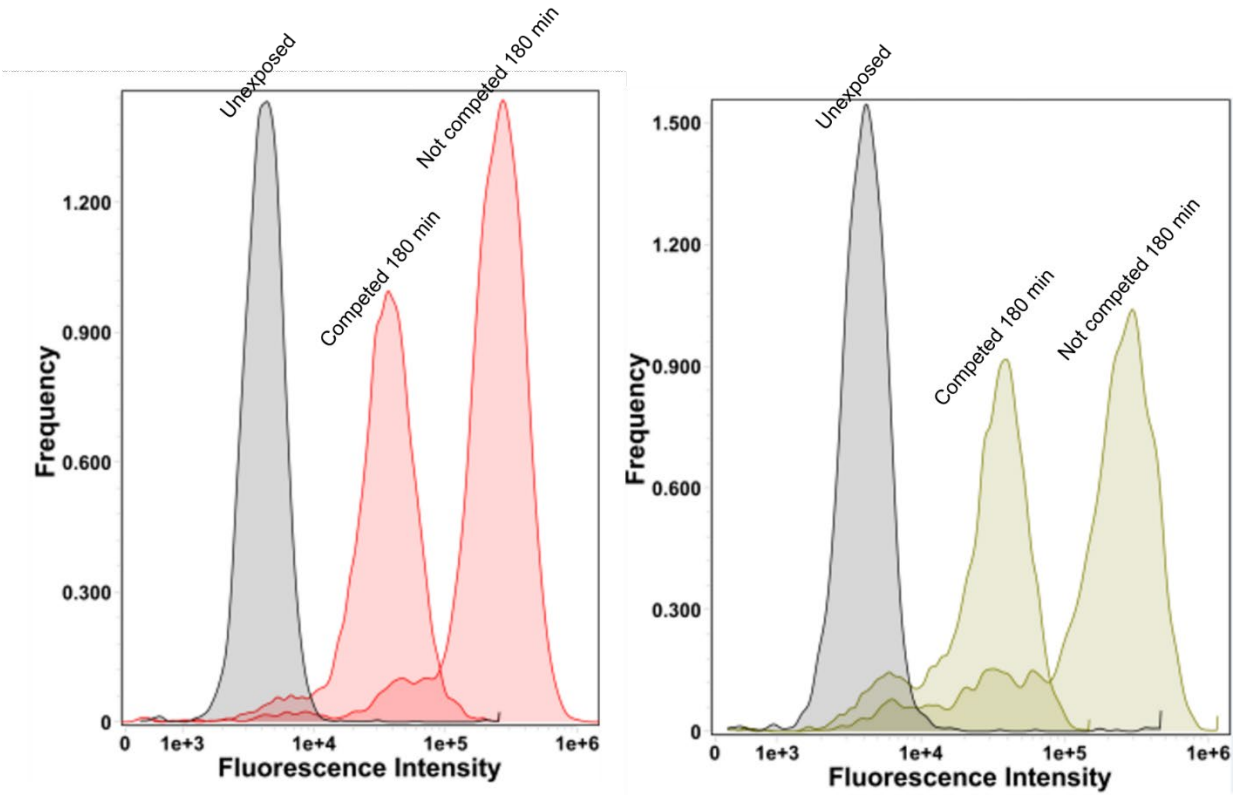


Supplementary Figure S7. Uptake kinetics of 2CNqAMP (left) and 2CNqATP (right) in Huh-7 cells at 37 °C measured using flow cytometry. Cells were exposed to 2.5 µM 2CNqAMP or 2CNqATP, respectively. Data is presented as mean fluorescence intensity (MFI) ± S.D. (n=3). Each experiment was done three times, indicated in triangles (dashed line), squares (solid line), and dots (dotted line). Lines represent the fitted mono-exponential curve as detailed in Supplementary Table S4.

Supplementary Table S4. Fitting details of the uptake kinetics presented in Supplementary Figure S7. Using OriginPro (Version 9.7.0.188) a mono-exponential fit (ExpDec1; y = A1*exp(-x/t1) + y0) in global data fit mode was performed using Levenberg Marquard iteration algorithm (iterations until fit converged, chi-square tolerance value 1E-9 was reached). The fit included all three replicates with instrumental weighting (square of the reciprocal of the error values) and a shared time constant *i.e.* shared for the triplicates during fitting procedure. The table shows the resulting fit values with the time constants highlighted in bold.

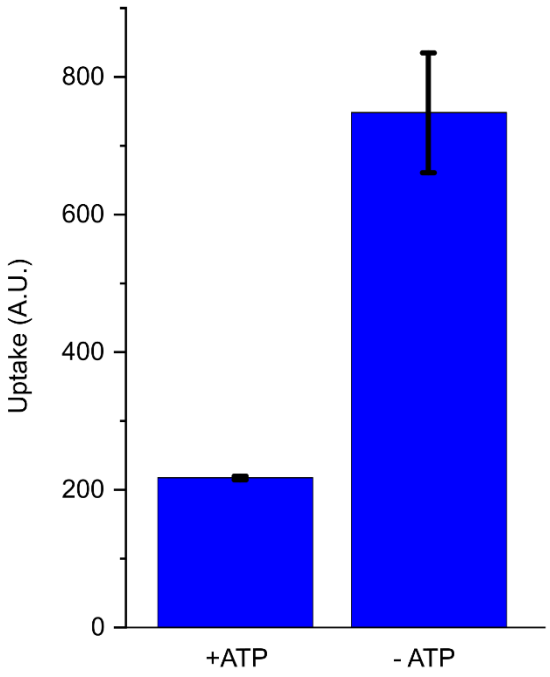
Results for fit of monophosphate (2CNqAMP) uptake				Results for fit of triphosphate (2CNqATP) uptake		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
y0	316497.39633 ± 18934.51137	222725.81616 ± 15126.43651	120373.67685 ± 7275.02599	205342.48712 ± 8935.19101	281824.20989 ± 10736.64207	99992.65641 ± 7489.47324
A1	-312155.39145 ± 18964.11055	-217355.75712 ± 15182.48138	-116344.62157 ± 8083.96919	-192318.37472 ± 11354.53877	-277360.3993 ± 11386.24771	-100985.11065 ± 10118.66503
t1	**34.89731** ± 3.0207	**34.89731** ± 3.0207	**34.89731** ± 3.0207	**74.21937** ± 7.27118	**74.21937** ± 7.27118	**74.21937** ± 7.27118

10. Uptake of 2CNqATP and 2CNqAMP in the presence of competitors



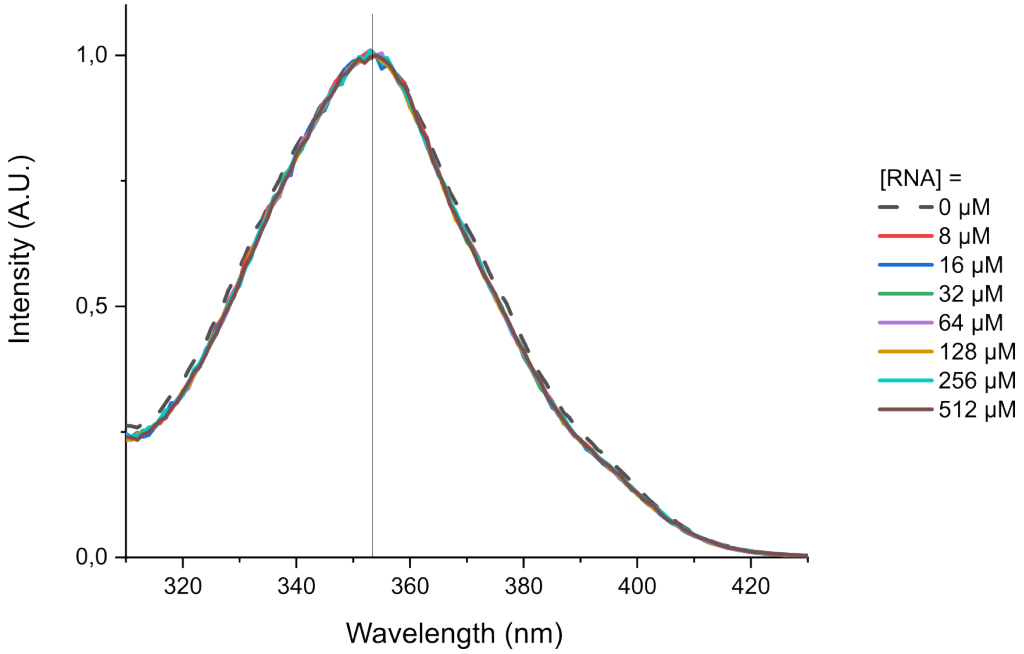
Supplementary Figure S8. Representative flow cytometry histograms of the uptake of 2CNqATP and 2CNqAMP into Huh-7 cells at 37 °C in presence of respectively ATP and AMP as competitors, corresponding to the mean cell intensity data shown in Figure 3f in the main text. The grey histograms show the background signal of the unexposed cells. Left: Cells exposed to 2.5 µM 2CNqAMP (red) for 3 h, with and without 625 µM AMP. Right: Cells exposed to 2.5 µM 2CNqATP (green) for 3 h, with and without 625 µM ATP.

11. Uptake of pATP in the presence of ATP as competitor



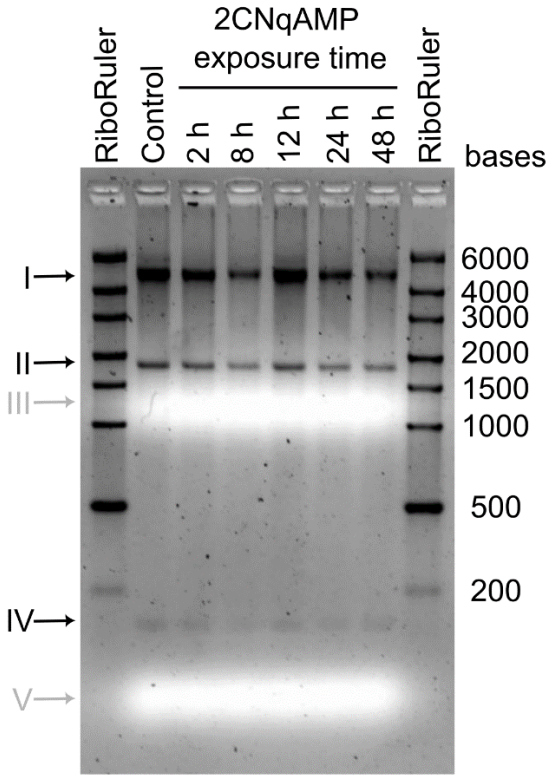
Supplementary Figure S9. Uptake of pATP in Huh-7 cells at 37 °C determined using flow cytometry. Cells were exposed to 2.5 µM pATP for 3 h, with and without 625 µM ATP. Data is presented as mean ± S.D. (n=2).

12. Titration of RNA to 2CNqAMP



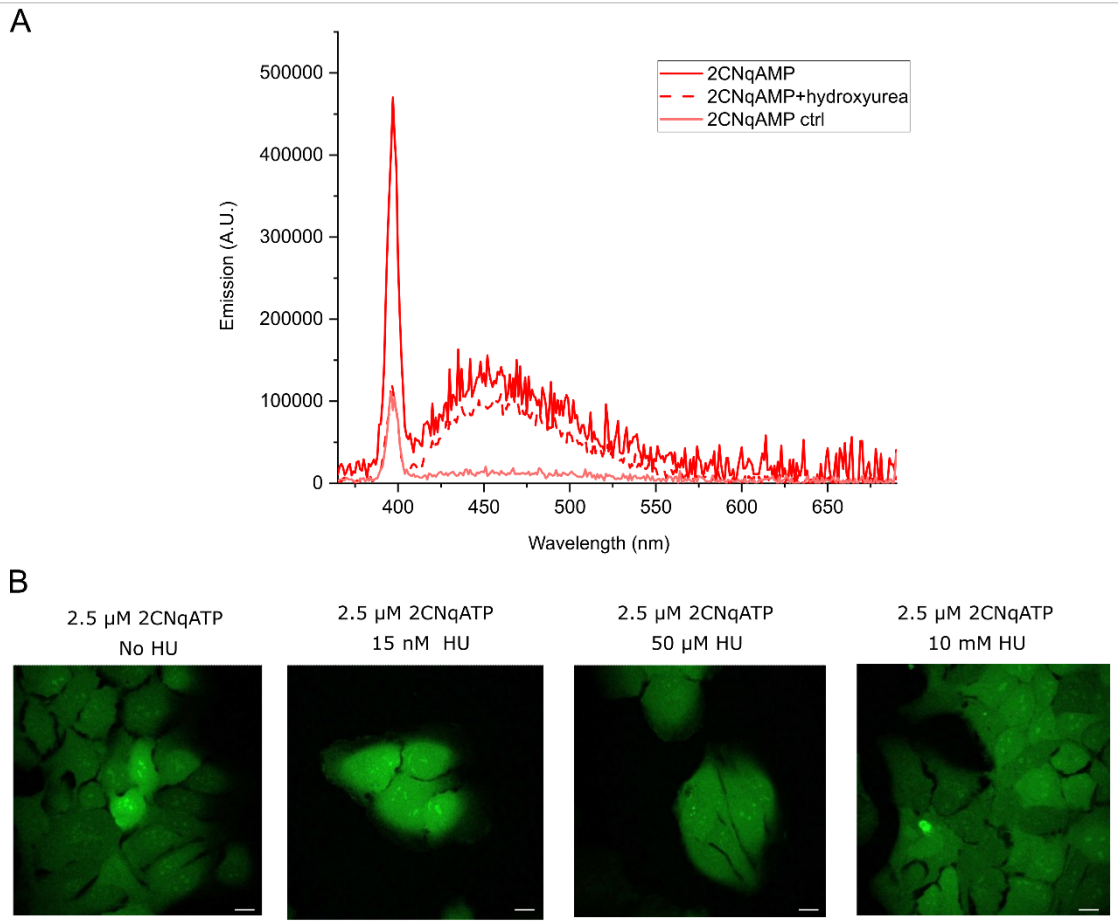
Supplementary Figure S10. Fluorescence excitation spectra of 2CNqAMP (10 µM) in the presence of increasing concentrations of unlabelled RNA. The excitation maxima are indicated by the vertical line at 352 nm.

13. Agarose gel of total RNA extracts of cells exposed to 2CNqAMP



Supplementary Figure S11. 2% agarose gel (%w/v) of total RNA extracts of Huh-7 cells exposed 2.5 µM 2CNqAMP over indicated time. For control 2CNqAMP was added to cell lysate before extraction. 500 ng RNA of each sample was loaded and SYBRsafe was added to the gels for detection. Indicated bands are 28S rRNA (I), 18 S rRNA (II), xylene cyanol dye present in the loading dye (grey III) small RNAs (IV), and bromophenol blue present in the loading dye (grey V). Ladder is RiboRuler High Range (Thermo Scientific). Gel was run for 1 h at 100 V.

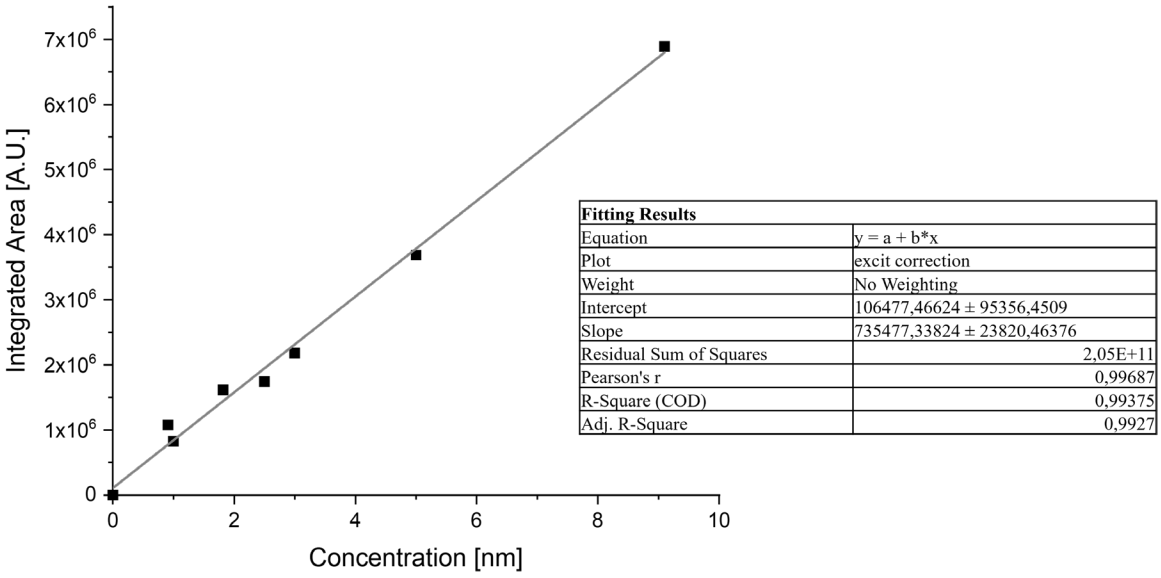
14. Images of cells exposed to 2CNqAMP and hydroxyurea



Supplementary Figure S12. A Representative emission spectra of RNA extracts from Huh-7 cells that had been exposed to 2.5 µM 2CNqAMP and 15 nM hydroxyurea for 24 h (dashed dark red line). For control, RNA extraction was done as shown in Figure 4a. **B** Microscopy images of Huh-7 cells exposed to 2.5 µM 2CNqATP and various concentrations of hydroxyurea (HU) after 4 h. Scale Bars 20 µm.

15. Standard curve for determination of 2CNqA concentration in extracted RNA

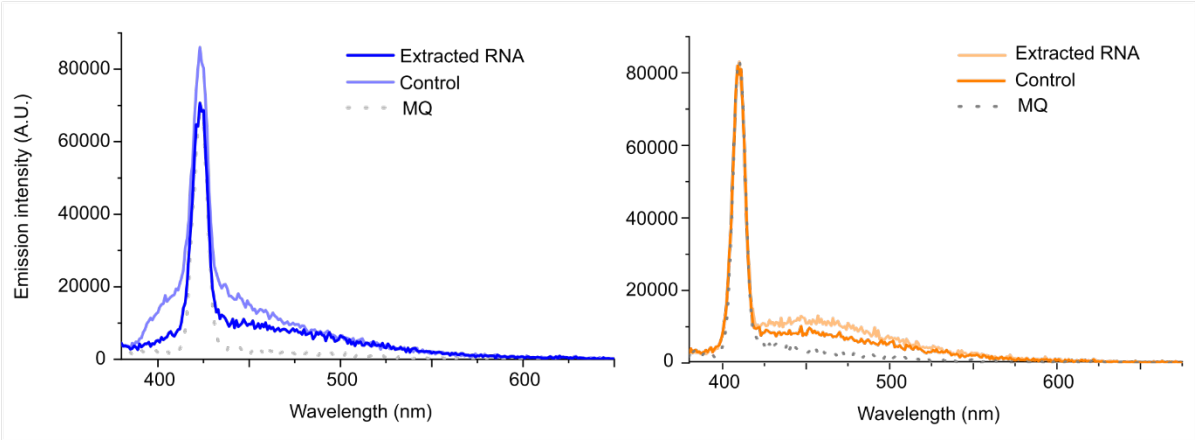
Concentration	Integrated Area	Corrected for QY	Corrected for Excitation
[nM]	[A.U.]	Factor 3.9	Factor 1.1
0	3,24E-06	8,30E-07	7,55E-07
0,91	4,61E+06	1,18E+06	1,07E+06
1	3,54E+06	9,06E+05	8,24E+05
1,82	6,92E+06	1,77E+06	1,61E+06
2,5	7,47E+06	1,92E+06	1,74E+06
3	9,36E+06	2,40E+06	2,18E+06
5	1,58E+07	4,05E+06	3,69E+06
9,1	2,96E+07	7,58E+06	6,89E+06



Fitting Results	
Equation	y = a + b*x
Plot	excit correction
Weight	No Weighting
Intercept	106477,46624 ± 95356,4509
Slope	735477,33824 ± 23820,46376
Residual Sum of Squares	2,05E+11
Pearson's r	0,99687
R-Square (COD)	0,99375
Adj. R-Square	0,9927

Supplementary Figure S13. Recorded values of a dilution series of 2CNqATP in milliQ and correction for change in quantum yield and excitation at emission wavelength (top table) and the resulting standard curve. Linear fit was applied with fitting results presented in table in the graph.

16. Emission spectra of RNA extracts from cells exposed to pATP or tC^{O}TP



Supplementary Figure S14. Representative emission spectra of RNA extracts from Huh-7 cells that had been exposed to 2.5 µM pATP (left, blue) or tC^{O}TP (right, orange) for 24 h. Controls, performed as indicated for 2CNqA in main Figure 4a, are shown in faint corresponding color. Water was recorded to confirm appearance of the Raman peak at the same wavelength as in the sample (grey dotted line).

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