Contents

Abbreviations

APS		Ammonium persulfate
TBA	:	Tetrabutylammonium
$CyNH^+$	:	Cyclohexylammonium
ETT	:	5-(Ethylthio)-1*H*-tetrazole
*m*CPBA	:	*meta*-Chloroperbenzoic acid
EAA	:	Ethyl acetate
Et_2O	:	Diethyl ether
TEA	:	Triethylamine
DIPEA	:	Diisopropylethylamine
MeCN	:	Acetonitrile
DMF	:	Dimethyl formamide
PPi	:	Pyrophosphate
TripolyP	:	Tripolyphosphate
PCPi	:	Methylene diphosphate
APS	:	Ammonium persulfate
calcd	:	calculated
d	:	diameter
DBU	:	1,8-Diazabicyclo[5.4.0]undec-7-ene
DEACM	:	7-(diethylamino)-4-(hydroxymethyl)-coumarine
DMF	:	Dimethylformamide
DMSO	:	Dimethyl sulfoxide
EDTA	:	Ethylenediaminetetraacetic acid
Et_2O	:	Diethyl ether
EtOAc	:	Ethyl acetate
ETT	:	5-(Ethylthio)-1*H*-tetrazole
eq.	:	equivalents
FmOH	:	Fluorenylmethanol
RP-HPLC	:	Reverse phase high-pressure liquid chromatography
HRMS	:	High resolution mass spectrometry
Hz	:	Hertz
kV	:	Kilovolt
µA	:	Microampere
*m*CPBA	:	*meta*-Chloroperoxybenzoic acid
MeCN	:	Acetonitrile
MeOH	:	Methanol
MHz	:	Megahertz
min	:	minutes
NEt_3	:	Triethylamine
OGD		Orange G dye
PAGE		Polyacrylamide gel electrophoresis
pGp	:	Guanosine-3',5'-bisphosphate
pGpp	:	Guanosine-3'-diphosphate-5'-phosphate
ppGp	:	Guanosine-3'-phohsphate-5'-diphosphate
ppGpp	:	Guanosine-3',5'-bis(diphosphate)
pppGpp	:	Guanosine-3'-diphosphate-5'-triphosphate
Rnase	:	Ribonuclease
RP-MPLC	:	Reverse phase – medium pressure liquid chromatography
S	:	seconds
TBA	:	Tetrabutylammonium
TEAA	:	Triethylammonium acetate
TEMED	:	Tetramethylethylenediamine
UV	:	ultra-violet
vac.	:	vacuum

Synthetic methods & analyses

General remarks

Reactions were carried out using flame-dried glassware under an atmosphere of dry N_2 and magnetically stirred, unless noted otherwise. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless steel cannula.

Reagents were purchased from commercial suppliers (Acros, Aldrich, Fluka, TCI) and used without further purification, unless noted otherwise.

DBU (1-8-diazabicyclo[5.4.0]undec-7-en) was distilled under high vacuum and stored over molecular sieves before usage.

Solvents were obtained in analytical grade and used as received for extractions, precipitation and solid washing.

Dry DMF, DMSO and MeCN for reactions were purchased in a dry form from Sigma and stored over molecular sieves as well as under an atmosphere of dry N_2.

Dry Et_2O for reactions was purified by filtration and dried by passage over activated anhydrous neutral A-2 alumina (MBraun solvent purification system) under an atmosphere of dry N_2.

Ribonuclease T2 from *Aspergillus oryzae* (50 ku) was purchased from Worthington Biochemical Corporation as lyophilized powder and dissolved in a storage buffer [glycerol / NaH_2PO_4 (10 mM, pH 6.8), 1 / 1]. The stock solution was stored at -20 °C.

Deuterated solvents for NMR and reactions were obtained from Armar Chemicals, Switzerland and euriso-top, Germany, in the indicated purity grade and used as received for NMR spectroscopy.

Strong ion-exchange chromatography was performed using an automated Äkta® – system. Q-Sepharose was purchased from Aldrich. Buffer solutions were produced manually using milliQ H_2O.

Preparative RP-MPLC was performed using an automated Interchim® - system. The AQ- solid phase was purchased from Interchim.

Lyophilizations were done with Christ Freeze Dryer Alpha 1-4 LDplus and Christ Freeze Dryer Alpha 1-2 LDplus.

UV irradiation was performed using a Rayonet RPR-100 photochemical reactor.

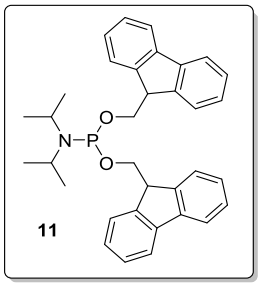
^{1}H-NMR spectra were recorded on Bruker 300 MHz spectrometers, Bruker 400 MHz (with cryoprobe) and Bruker 500 MHz spectrometers in the indicated deuterated solvent. Data are reported as follows: chemical shift (δ, ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br. s, broad signal), coupling constant(s) (*J*, Hz), integration. All signals were referenced to the internal solvent signal as standard (D_2O, δ 4.70; DMF-d7, δ 2.94).

^{13}C{^{1}H}-NMR spectra were recorded with ^{1}H-decoupling on Bruker 126 MHz, Bruker 101 MHz (with cryoprobe) spectrometers at 298 K in the indicated deuterated solvent.

^{31}P{^{1}H}-NMR spectra and ^{31}P-NMR spectra were recorded with ^{1}H-decoupling or ^{1}H coupling, respectively, on Bruker 202 MHz , 162 MHz (with cryoprobe) and Bruker 122 MHz spectrometers in the indicated deuterated solvent. All signals were referenced to an internal standard (PPP).

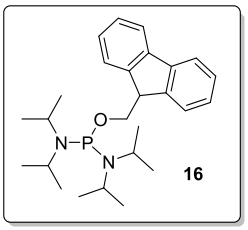
Mass spectra were recorded by C. Warth (Mass spectrometry service of the University of Freiburg) on a Thermo LCQ Advantage [spray voltage: 2.5 – 4.0 kV, spray current: 5 µA, ion transfer tube: 250 (150) °C, evaporation temperature: 50 – 400 °C.

Synthesis of $(FmO)_2P\text{-}N(iPr)_2$ (11)



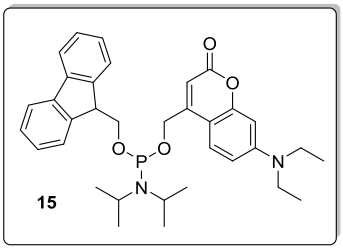
The compound was synthesized in two steps starting from PCl_3 as reported previously. Analytical data were identical to literature.[1] The compound was stored at −20 °C.

Synthesis of $(FmO)P\text{-}[N(iPr)_2]_2$ (16)



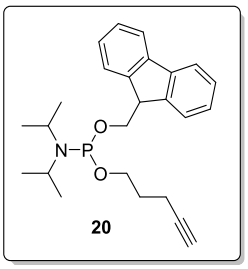
The compound was synthesized as reported previously. Crystallization from pentane increased the purity and delivered single crystals. Analytical data were identical to literature.[2] The compound was stored at −20 °C under light exclusion.

Synthesis of $(DEACMO)(FmO)P\text{-}N(iPr)_2$ (15)



The compound was synthesized as reported previously. Analytical data were identical to literature.[3] The compound was stored at −20 °C.

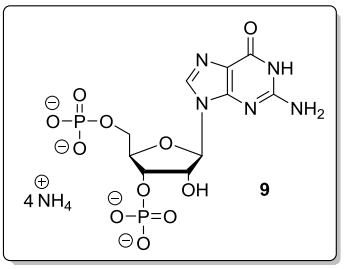
Synthesis of (PentynylO)(FmO)P-N(*i*Pr)$_2$ (20)



P-Diamidite **16** (1.00 g, 2.44 mmol) was coevaporated with dry MeCN (5 ml), dissolved in dry THF (10 ml) and cooled to 0 °C. ETT stock solution (100 mg/ml in MeCN, 3.18 ml, 2.44 mmol, 1.0 eq.) was added slowly, followed by pent-4-ynol stock solution (1 M in MeCN, 2.44 ml, 2.44 mmol, 1.0 eq.). The reaction was allowed to reach room temperature after 10 min. Dry Et_2O (10 ml) was added and the formed precipitate was filtered off. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (pent./EtOAc, 95:5, SiO_2 was treated with 10% NEt_3). After drying under high vac. for several hours, the product was obtained as sticky, colorless oil (580 mg, 1.41 µmol, 61%). The compound was stored at −20 °C.

^{1}H-NMR (300 MHz, $CDCl_3$, δ/ppm): 7.80 – 7.71 (m, 2H), 7.74 – 7.60 (m, 2H), 7.44 – 7.35 (m, 2H), 7.30 (dddd, *J* = 7.4, 7.4, 3.1, 1.3 Hz, 2H), 4.21 (dd, *J* = 7.3 Hz, 1H), 4.01 (ddd, *J* = 9.9, 6.6 Hz, 1H), 3.85 – 3.54 (m, 5H), 2.31 (ddd, *J* = 7.1, 2.6, 2.6 Hz, 2H), 1.94 (dd, *J* = 2.7 Hz, 1H), 1.83 (dddd, *J* = 7.1, 2H), 1.19 (d, *J* = 6.8 Hz, 6H), 1.16 (d, *J* = 6.8 Hz, 6H). **^{13}C{^{1}H}-NMR** (101 MHz $CDCl_3$, δ/ppm): 145.1, 144.8, 141.5, 141.4, 127.5, 127.4, 126.9, 126.9, 125.6, 125.3, 119.9, 119.8, 77.3, 68.54, 66.2 (d, *J* = 17.6 Hz), 62.0 (d, *J* = 17.4 Hz), 49.3 (d, *J* = 7.7 Hz), 43.1 (d, *J* = 12.5 Hz), 30.3 (d, *J* = 7.2 Hz), 24.8 (d, *J* = 3.3 Hz), 24.7 (d, *J* = 3.2 Hz), 15.3. **^{31}P{^{1}H}-NMR** (122 MHz, $CDCl_3$, δ/ppm): 146.2. **HRMS** (ESI) m/z for $C_{25}H_{33}NO_2P$ $[M+H]^+$: calcd 410.2243, found 4410.2243. **R_f** (SiO_2, pent/EtOAc, 95:5): 0.78.

Synthesis of guanosine-3',5'-bisphosphate (pGp, 9)



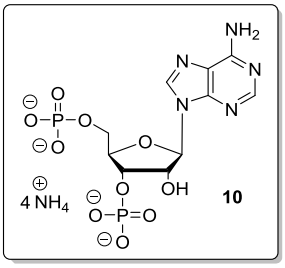
Guanosine dihydrate (**14**, 1.00 g, 3.13 mmol) was coevaporated using dry MeCN (3 x 15 ml). Afterwards, freshly distilled $P_2Cl_4O_2$ (4.77 mL, 34.5 mmol, 8.68 g, 11 eq.) was added in one portion at −35 °C. The solution was warmed to 0 °C and stirred for 3 h at 0 °C. Afterwards, overstoichiometric $P_2Cl_4O_2$ was removed by washing with Et_2O (−40 °C, 3 x 15 ml), using a syringe under N_2 atmosphere. The resulting solid was quenched by fast addition of $NaHCO_3$-buffer (-2 °C, 1.0 M, 60 ml) at −40 °C under vigorous stirring (additional stirring with a spatula is recommended). The mixture was warmed to room temperature and H_2O (500 ml) was added. Afterwards, ribonuclease T2 (1.0 ku) was added and the resulting solution was stirred for 12 h at 37 °C. H_2O (2.0 l) was added and the mixture was applied to an ion-exchange column (Q-Sepharose®, h = 17 cm, d = 3 cm). The column was rinsed using H_2O (1.0 l) and NH_4HCO_3 – buffer (150 mM, 1.0 l) before eluting pGp (**9**) with NH_4HCO_3 – buffer

(250 – 300 mM). Product containing fractions were combined and lyophilized. The product (1.39 g, 2.72 mmol, 87%) was isolated as white solid.

Tetraammonium pGp was converted into its TBA – salt (1.5 – 2.0 eq.) by ion exchange on Dowex 50WX8 (H^+) followed by the addition of TBA(OH) and lyophilization.

^{1}H-NMR (500 MHz, D_2O, δ/ppm): 8.05 (s, 1H), 5.90 (d, *J* = 5.9 Hz, 1H), 4.80 – 4.73 (m, 2H), 4.51 – 4.40 (m, 1H), 4.11 – 4.01 (m, 2H). **^{13}C{^{1}H}-NMR** (126 MHz, D_2O, δ/ppm): 159.0, 154.0, 151.9, 137.7, 116.3, 86.6, 83.3 (dd, *J* = 8.5, 4.1 Hz), 73.7 (d, *J* = 4.8 Hz), 73.2 (d, *J* = 4.5 Hz), 64.3 (d, *J* = 4.9 Hz). **^{31}P{^{1}H}NMR** (202 MHz, D_2O, δ/ppm): 0.83 (s, 1P), 0.54 (s, 1P). **HRMS** (ESI) m/z for $C_{10}H_{14}N_5O_{11}P_2$ $[M-H]^-$: calcd 442.0171, found 442.0172.

Synthesis of adenosine-3',5'-bisphosphate (pAp, 10)

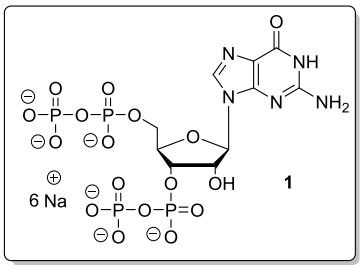


Adenosine (209 mg, 784 µmol) was coevaporated using dry MeCN (3 x 15 ml). Afterwards freshly distilled $P_2Cl_4O_2$ (1.25 mL, 2.28 g, 9.04 mmol, 11.5 eq., −20 °C) was added in one portion at −35 °C. The solution was warmed to 0 °C and stirred for 3 h at 0 °C. Afterwards, overstoichiometric $P_2Cl_4O_2$ was removed by washing with Et_2O (3 × 10 mL, −40 °C), using a syringe under N_2 atmosphere. The resulting solid was quenched by fast addition of $NaHCO_3$-buffer (−2 °C, 620 mM, 30 mL) at −40 °C under vigorous stirring (additional stirring with a spatula is recommended). The mixture was warmed to room temperature and H_2O (270 ml) was added. Afterwards, ribonuclease T2 (300 ku) was added and the resulting solution was stirred for 19 h at 37 °C. H_2O (740 ml) was added and the mixture was applied to an ion-exchange column (Q-Sepharose®, h = 17 cm, d = 3 cm). The column was rinsed using H_2O (500 ml) and NH_4HCO_3 – buffer (150 mM, 500 ml) before eluting pAp (**10**) with NH_4HCO_3 – buffer (250 – 300 mM). Product containing fractions were combined and lyophilized. The product (178 mg, 359 µmol, 46%) was isolated as white solid.

Tetraammonium pAp was converted into its TBA – salt (1.5 – 2.0 eq.) by ion exchange on Dowex 50WX8 (H+) followed by the addition of TBA(OH) and lyophilization.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.45 (s, 1H), 8.19 (s, 1H), 6.11 (d, *J* = 5.6 Hz, 1H), 4.79 – 4.75 (m, 2H), 4.50 (br. s, 1H), 4.11 – 4.07 (m, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 155.4, 152.6, 149.1, 139.9, 118.6, 86.7, 83.4 (dd, *J* = 8.6, 4.1 Hz), 73.9 (d, *J* = 4.5 Hz), 73.6 (d, *J* = 4.8 Hz), 64.2 (d, *J* = 5.0 Hz). **^{31}P{^{1}H}-NMR** (162 MHz, D_2O, δ/ppm): 0.89, 0.60. **HRMS (ESI)** for $C_{10}H_{14}O_{10}N_5P_2$: calcd 426.0221, found 426.0223.

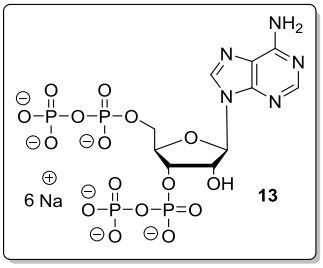
Synthesis of guanosine-3',5'-bis(diphosphate) (ppGpp, 1)



To a solution of pGp x 1.78 TBA (57.0 mg, 65.4 µmol) in dry DMF (1.0 ml) was added ETT (42.5 mg, 327 µmol, 5.0 eq.). Afterwards a solution of **11** (114 mg, 196 µmol, 3.0 eq.) in dry DMF (2.0 ml) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (53.2 mg, 216 µmol, 3.3 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Afterwards distilled DBU (300 µl) was added at 0 °C. The solution was stirred for 5 min at 0 °C and 25 min at room temperature before precipitation was induced by the addition of Et_2O (40 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 10 ml) and dried over high vac. The crude product was purified by strong ion-exchange chromatography (Q-Sepharose) using an Äkta-system and $NaClO_4$ – buffer (100 mM). The product containing fractions were precipitated using an 8-fold volume of $NaClO_4$ – solution (−20 °C, 500 mM in acetone). The resulting solid was washed with acetone (−20 °C, 3 x 10 ml) and dried over high vac. for 2 h. The product (37.5 mg, 51.0 µmol, 78%) was isolated as white solid.

Gram-scale: To a solution of pGp x 1.88 TBA (2.07 g, 2.32 mmol) in dry DMF (30 ml) was added ETT (1.51 g, 11.6 mmol, 5.0 eq.). Afterwards, a solution of $(FmO)_2P$-$(NiPr_2)$ (93%, 2.66 g, 5.11 mmol, 2.2 eq.) in dry DMF (30 ml) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 1.14 g, 5.11 mmol, 2.2 eq.) was added in four portions. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Afterwards distilled DBU (300 µl) was added at 0 °C. The solution was stirred for 5 min at 0 °C and 25 min at room temperature before precipitation was induced by addition of Et_2O (250 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 50 ml) and dried over high vac. The crude product was purified by strong ion-exchange chromatography (Q-Sepharose) using an Äkta-system and $NaClO_4$ – buffer (100 mM). The product containing fractions were precipitated using the 6-fold volume of $NaClO_4$ – solution (−20 °C, 500 mM in acetone). The resulting precipitate was redissolved in water (7 ml) and precipitated again using acetone (−20 °C, 40 ml). The resulting solid was washed with acetone (−20 °C, 3 x 20 ml) and dried over high vac. for 2 h. The product (1.15 g, 1.56 mmol, 67 %) was isolated as white solid.
^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.06 (s, 1H), 5.92 (d, *J* = 6.4 Hz, 1H), 4.88 (ddd, *J* = 8.5, 5.1, 3.1 Hz, 1H), 4.79 (dd, *J* = 5.5 Hz, 1H), 4.48 – 4.44 (m, 1H), 4.20 – 4.09 (m, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 159.1, 154.0, 151.9, 137.9, 116.3, 86.8, 83.4 (dd, *J* = 9.2, 4.3 Hz), 74.8 (d, *J* = 5.2 Hz), 73.1 (d, *J* = 4.4 Hz), 65.1 (d, *J* = 5.4 Hz). **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): –5.81 (d, *J* = 22.3 Hz), –6.55 (d, *J* = 21.3 Hz), –10.63 (d, *J* = 21.3 Hz), -10.83 (d, *J* = 22.3 Hz). **HRMS** (ESI) m/z for $C_{10}H_{15}N_5O_{17}P_4$ $[M\text{-}H_2]^{2-}$: calcd 300.4712, found 300.4711.

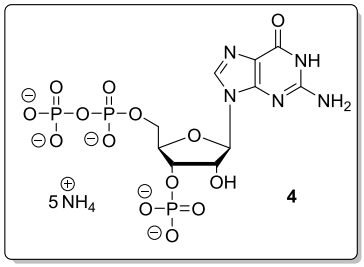
Synthesis of adenosine-3',5'-bis(diphosphate) (ppApp, 13)



To a solution of pAp x 2.33 TBA (39.9 mg, 40.3 µmol) in dry DMF (500 µl) was added ETT (19.1 mg, 164 µmol, 4.1 eq.). Afterwards, a solution of **11** (95%, 54.9 mg, 100 µmol, 2.5 eq.) in dry DMF (500 µl) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 19.6mg, 87.6 µmol, 2.2 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0°C. Afterwards, dry DMF (3.0 mL) and distilled DBU (225 µL µl) were added at 0 °C. The solution was stirred for 5 min at 0 °C and 25 min at room temperature before precipitation was induced by addition of Et_2O (25 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 20 ml) and dried over high vac. The crude product was purified by strong ion-exchange chromatography (Q-Sepharose) using an Äkta-system and $NaClO_4$ – buffer (100 mM). The product containing fractions were precipitated using an 8-fold volume of $NaClO_4$ – solution (−20 °C, 500 mM in acetone). The resulting solid was washed with acetone (−20 °C, 3 x 10 ml) and dried over high vac for 2 h. The product (16.6 mg, 23.1 µmol, 57%)) was isolated as white solid.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.48 (s, 1H), 8.19 (s, 1H), 6.12 (d, *J* = 6.5 Hz, 1H), 4.90 (ddd, *J* = 8.4, 4.9, 2.7 Hz, 1H), 4.79 (ddd, *J* = 5.0, 1.1 Hz, 1H), 4.55 – 4.52 (m, 1H), 4.19 – 4.13 (m, 2H). **^{31}P{^{1}H}-NMR** (162 MHz, D_2O, δ/ppm): −6.16 (d, *J* = 22.2 Hz, 1P), −7.32 (br. s, 1P), −10.79 (d, *J* = 20.2 Hz, 1P), −10.92 (d, *J* = 21.8 Hz, 1P). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 155.7, 152.9, 149.3, 140.1, 118.7, 86.6, 83.6 (ddd, *J* = 9.2, 4.2, 0.0 Hz), 74.9 (d, *J* = 5.3 Hz), 73.7 (d, *J* = 4.7 Hz), 65.0 (d, *J* = 5.2 Hz). **HRMS (ESI)** for $C_{10}H_{15}O_{16}N_5P_4$: calcd 292.4738, found 292.4738.

Synthesis of guanosine-3'-phosphate-5'-diphosphate (ppGp, 4)



To a solution of pGp x 1.78 TBA (70.0 mg, 80.3 µmol) in dry DMF (1.0 ml) was added ETT (52.2 mg, 402 µmol, 5.0 eq.). Afterwards, a solution of **11** (140 mg, 241 µmol, 3.0 eq.) in DMF (2.0 ml) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 65.3 mg, 265 µmol, 3.3 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Afterwards, distilled DBU (300 µl) was added at 0 °C. The solution was stirred for 5 min at 0 °C and 25 min at room temperature before precipitation was induced by the addition of Et_2O (40 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 15 ml) and dried over high vac.

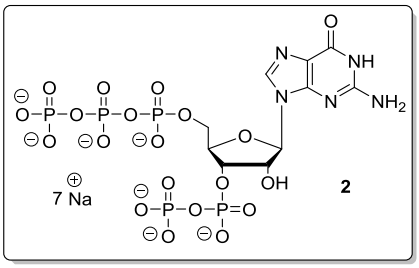
The intermediate product was dissolved in H_2O (20 ml) and the solution was acidified to pH 5.5 using HCl-solution. Subsequently ribonuclease *T2* (500 u) was added and the solution was stirred for 24 h at 37 °C. Afterwards the solution was directly applied to a strong ion-exchange column (Q-Sepharose). The product **4** was eluted using NH_4HCO_3 – buffer (350 mM). Lyophilization of the product containing fractions afforded the desired product (41.0 mg, 67.4 µmol, 84 %) as white solid.

When scaling up the reaction to >400 µmol the yield decreased to 73%.

Pentaammonium ppGp was converted into its TBA – salt (1.5 – 2.0 eq.) by ion exchange on Dowex 50WX8 (H+) followed by the addition of TBA(OH) and lyophilization.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.07 (s, 1H), 5.91 (d, *J* = 6.7 Hz, 1H), 4.89 – 4.74 (m, 2H), 4.54 – 4.43 (m, 1H), 4.22 – 4.11 (m, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 176.3, 159.0, 154.0, 137.8, 116.3, 86.5, 83.5 (dd, *J* = 9.4, 4.2 Hz), 73.7 (d, *J* = 4.9 Hz), 73.1 (d, *J* = 4.4 Hz), 65.1 (d, *J* = 5.3 Hz). **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): 1.35, -9.97 (d, *J* = 21.0 Hz), -11.18 (d, *J* = 20.8 Hz). **HRMS** (ESI) m/z for $C_{10}H_{15}N_5O_{14}P_3$ $[M\text{-}H]^-$: calcd 521.9834, found 521.9832.

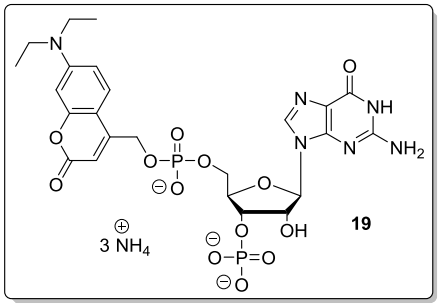
Synthesis of guanosine-3'-diphosphate-5'-triphosphate (pppGpp, 2)



To a solution of ppGp x 3.0 TBA (137 mg, 110 µmol) in dry DMF (1.0 ml) was added ETT (85.9 mg, 661 µmol, 6.0 eq.). Afterwards, a solution of **11** (215 mg, 330 µmol, 3.0 eq.) in DMF (2.0 ml) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 89.5 mg, 363 µmol, 3.3 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Afterwards, distilled DBU (300 µl) was added at 0 °C. The solution was stirred for 5 min at 0 °C and 25 min at room temperature before precipitation was induced by addition of Et_2O (40 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 10 ml) and dried over high vac. The crude product was purified by strong ion-exchange chromatography (Q-Sepharose) using an Äkta-system and $NaClO_4$ – buffer (100 mM). The product containing fractions were precipitated using an 8-fold volume of $NaClO_4$ – solution (−20 °C, 500 mM in acetone). The resulting solid was washed with acetone (−20 °C, 3 x 10 ml) and dried over high vac for 2 h. The product (**2**) (59.3 mg, 70.8 µmol, 64%) was isolated as white solid.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.06 (s, 1H), 5.92 (d, *J* = 6.5 Hz, 1H), 4.88 (ddd, *J* = 8.4, 5.0, 2.9 Hz, 1H), 4.82 – 4.76 (m, 1H), 4.51 – 4.44 (m, 1H), 4.24 – 4.14 (m, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 159.1, 154.0, 152.0, 138.0, 116.3, 86.9, 83.3 (dd, *J* = 9.1, 4.3 Hz), 74.9 (d, *J* = 5.1 Hz), 73.1 (d, *J* = 4.2 Hz), 65.5 (d, *J* = 5.2 Hz). **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): -6.21 (d, *J* = 21.9 Hz), -7.37 (d, *J* = 16.0 Hz), -10.85 (d, *J* = 13.9 Hz), -10.97 (d, *J* = 10.0 Hz), -21.77 (dd, *J* = 18.9 Hz). **HRMS** (ESI) m/z for $C_{10}H_{16}N_5O_{20}P_5$ $[M\text{-}H_2]^{2-}$: calcd 340.4544, found 340.4545.

Synthesis of 5'-DEACM–pGp (19)



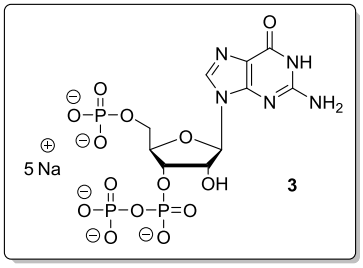
Guanosine dihydrate (**14**, 100 mg, 313 µmol), ETT (172 mg, 1.25 mmol, 4.0 eq.) and **15** (296 mg, 532 µmol, 1.7 eq.) were coevaporated separately with dry MeCN (each 3 x 3 ml). Afterwards, a solution of ETT in DMSO (600 µl) was added to guanosine and the resulting solution was cooled to 3 °C. A solution of **15** in DMF (1.3 ml) was cooled to 0 °C and added. The resulting solution was stirred for 1 h at 0 °C. Afterwards, **16** (174 mg, 408 µmol, 1.3 eq.) was added and the solution was stirred for 45 min at room temperature. Afterwards, the solution was cooled to -20 °C and TBHP (6 M in decanes, 172 µl, 1.03 mmol, 3.3 eq.) was added dropwise. The solution was warmed to room temperature and stirred for 1 h. Afterwards, piperidine (200 µl) was added and the solution was stirred for 30 min at room temperature. Precipitation was induced by the addition of Et_2O (10 ml). The precipitate was separated by centrifugation and washed with Et_2O (3 x 5 ml) and dried over high vac. The solid was dissolved in H_2O (10 ml) and ribonuclease T2 (500 u) was added. The solution was incubated for 12 h at 37 °C. The crude product was purified by strong ion-exchange chromatography using an Äkta – system (NH_4HCO_3 – buffer, VIS detection at 700 nm). The product containing fractions were identified by HPLC, combined and lyophilized. The product (118 mg, 163 µmol, 52%) was isolated as yellow solid.

Ammonium DEACM-pGp (**19**) was converted into its TBA – salt (2.2 eq.) by dissolution in H_2O and addition of defined amounts of TBA(OH) followed by lyophilization.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 7.70 (s, 1H), 6.74 (d, *J* = 9.0 Hz, 1H), 6.35 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.26 (d, *J* = 2.5 Hz, 1H), 5.98 (s, 1H), 5.68 (d, *J* = 4.6 Hz, 1H), 4.91 (dd, *J* = 15.9, 5.0 Hz, 1H), 4.45 – 4.36 (m, 1H), 4.22 – 4.09 (m, 2H), 3.30 (q, *J* = 7.1 Hz, 4H), 1.10 (t, *J* = 7.0 Hz, 6H).* **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 165.4, 158.0, 154.8, 153.8, 153.7, 153.3, 151.1, 151.0, 137.3, 123.6, 116.2, 109.5, 104.8, 102.4, 96.7, 87.1, 82.7 (dd, *J* = 8.5, 7.8 Hz), 73.0 (d, *J* = 4.9 Hz), 72.5 (d, *J* = 4.2 Hz), 65.8 – 65.6 (m), 62.6 – 62.4 (m), 44.4, 11.8. **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): 0.22, -0.32. **HRMS** (ESI) m/z for $C_{24}H_{30}N_6O_{13}P_2$ $[M+Na]^+$: calcd 695.1238, found 695.1245.

*three multiplets are hidden underneath the DHO-peak.

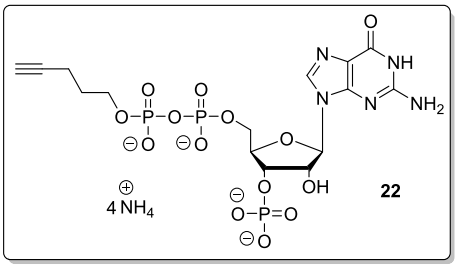
Synthesis of guanosine-3'-diphosphate-5'-phosphate (pGpp, 3)



To a solution of 5'-DEACM-pGp x 2.25 TBA (120 mg, 98.6 µmol) in dry DMF (750 µl) was added ETT (51.2 mg, 394 µmol, 4.0 eq.). Afterwards, a solution of **11** (121 mg, 197 µmol, 2.0 eq.) in DMF (750 µl) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 51.0 mg, 207 µmol, 2.1 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Afterwards, distilled DBU (200 µl) was added at 0 °C. The solution was stirred for 5 min at 0 °C and 25 min at room temperature before precipitation was induced by addition of Et_2O (10 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 10 ml) and dried over high vac. The solid was dissolved in H_2O/MeCN (4/1, 220 ml) and irradiated for 90 min in total using an UV-reactor. Each 10 min the sample was cooled to 10 °C. Afterwards, the solution was extracted with Et_2O (3 x 50 ml) and the aqueous layer was lyophilized. The resulting crude product was purified by automated preparative HPLC (Interchim) using an AQ-column (20% TEAA buffer (100 mM), 4 % MeCN). The product containing fractions were combined and 80% of the solvent was lyophilized. The residue was precipitated using an 8-fold volume of $NaClO_4$-solution (−20 °C, 500 mM in acetone). The precipitate was washed with acetone (−20 °C, 3 x 10 ml) and dried over high vac for 3 h. The product (**3**, 34.0 mg, 53.7 µmol, 55%) was isolated as white solid.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.13 (s, 1H), 5.91 (d, *J* = 6.3 Hz, 1H), 4.89 – 4.82 (m, 1H), 4.81 – 4.76 (m, 1H), 4.44 – 4.39 (m, 1H), 3.98 – 3.87 (m, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 159.3, 154.1, 151.9, 138.0, 116.2, 86.8, 84.1 (dd, *J* = 9.3 Hz), 75.1 (d, *J* = 5.3 Hz), 73.4 (d, *J* = 4.3 Hz), 63.8 (d, *J* = 4.3 Hz). **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): 3.7, –5.7 (d, *J* = 23.5 Hz), –10.8 (d, *J* = 22.1 Hz). **HRMS** (ESI) m/z for $C_{10}H_{15}N_5O_{14}P_3$ $[M-H]^-$: calcd 521.9834, found 521.9835.

Synthesis of 5'-pentynyl-ppGp (22)



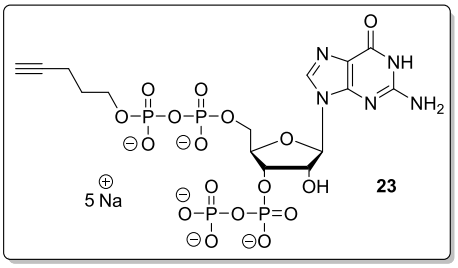
To a solution of pGp x 1.77 TBA (106 mg, 122 µmol) in dry DMF (500 µl) was added ETT (79.3 mg, 610 µmol, 5.0 eq.). Afterwards, a solution of mixed P-Amidite **21** (92%, 119 mg, 268 µmol, 2.2 eq.) in DMF (1.0 ml) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 59.8 mg, 268 µmol, 2.2 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Afterwards, precipitation was induced by addition of Et_2O (10 ml). The precipitate

was separated by centrifugation, washed with Et_2O (3 x 5 ml) and dried over high vac. The solid was dissolved in MeOH (3.0 ml) and the solution was stirred for 1 h at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in DMF (1.0 ml) before piperidine (100 µl) was added. The solution was stirred for 30 min at room temperature and precipitation was induced by the addition of Et_2O (10 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 5 ml) and dried over high vac. The solid was dissolved in H_2O (5.0 ml) and ribonuclease T2 (300 u) was added. The resulting solution was stirred for 12 h at 37 °C and afterwards applied to an automated strong ion-exchange (Q-Sepharose) column. The product was eluted using NH_4HCO_3 – buffer (600 mM). Product containing fractions were combined and lyophilized. The product (**23**, 49.0 mg, 74.6 µmol, 61%) was isolated as white solid.

Ammonium pentynyl-ppGp (**23**) was converted into its TBA – salt (2.1 eq.) by dissolution in H_2O and addition of defined amounts of TBA(OH) followed by lyophilization.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.08 (s, 1H), 5.91 (d, *J* = 6.8 Hz, 1H), 4.87 (ddd, *J* = 6.6, 5.1, 1.2 Hz, 1H), 4.78 (ddd, *J* = 8.0, 5.2, 2.7 Hz, 1H), 4.52 – 4.46 (m, 1H), 4.24 – 4.11 (m, 2H), 3.87 (ddd, *J* = 6.5 Hz, 2H), 2.19 (dd, *J* = 2.7 Hz, 1H), 2.16 – 2.10 (m, 2H), 1.66 (dddd, *J* = 6.8 Hz, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 159.0, 154.0, 152.0, 137.8, 116.3, 86.4, 84.9, 83.3 (dd, *J* = 9.3, 3.4 Hz), 74.2 (d, *J* = 5.1 Hz), 72.8 (d, *J* = 5.1 Hz), 69.2, 65.3 (d, *J* = 5.6 Hz), 65.2 (d, *J* = 6.1 Hz), 28.8 (d, *J* = 7.3 Hz), 14.1. **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): -0.09 (s, 1P), -10.95 (d, *J* = 21.2 Hz, 1P), -11.56 (d, *J* = 21.3 Hz, 1P).**HRMS** (ESI) m/z for $C_{15}H_{23}N_5O_{14}P_3$ $[M+H]^+$: calcd 590.0449, found 590.0448.

Synthesis of 5'-pentynyl-ppGpp (23)

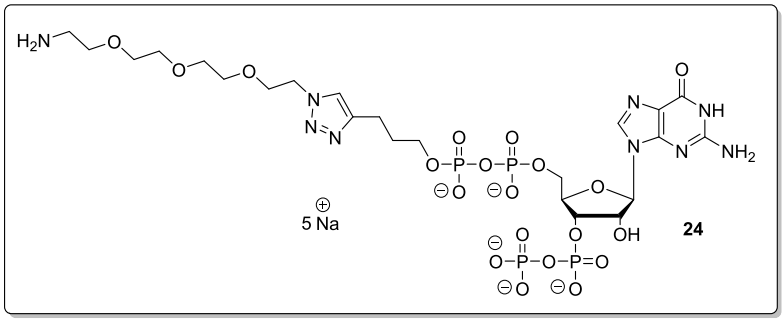


To a solution of pentynyl-ppGp x 2.1 TBA (54.0 mg, 49.4 µmol) in dry DMF (500 µl) was added ETT (19.3 mg, 148 µmol, 3.0 eq.). Afterwards, a solution of **11** (94%, 35.6 mg, 64.2 µmol, 1.3 eq.) in DMF (1.0 ml) was added in one portion and the resulting mixture was stirred for 15 min at room temperature. Afterwards, the solution was cooled to −20 °C and *m*CPBA (77%, 14.3 mg, 64.2 µmol, 1.3 eq.) was added. The solution was stirred for 5 min at −20 °C and for 10 min at 0 °C. Subsequently, DBU (100 µl) was added at 0 °C and the reaction mixture was stirred for 5 min at 0 °C and for 25 min at rt. Precipitation was induced by the addition of Et_2O (10 ml). The precipitate was separated by centrifugation, washed with Et_2O (3 x 5 ml) and dried over high vac. The crude product was purified by strong ion-exchange chromatography (Q-Sepharose) using an Äkta-system and $NaClO_4$ – buffer (120 mM). The product containing fractions were precipitated using an 8-fold volume of $NaClO_4$ – solution (−20 °C, 500 mM in acetone). The resulting solid was separated by centrifugation, washed with acetone (−20 °C, 3 x 10 ml) and dried over high vac for 2 h. The product (**23**, 22.1 mg, 28.4 µmol, 57%) was isolated as white solid.

^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.07 (s, 1H), 5.93 (d, *J* = 6.1 Hz, 1H), 4.91 (ddd, *J* = 8.7, 5.2, 3.4 Hz, 1H), 4.86 – 4.80 (m, 0H), 4.45 (br. s, *J* = 4.6, 2.5 Hz, 1H), 4.23 – 4.11 (m, 2H), 3.82 (ddd, *J* = 6.4 Hz, 2H), 2.18 (dd, *J* = 2.7 Hz, 1H), 2.13 – 2.04 (m, 2H), 1.61 (dddd, *J* = 6.8 Hz, 2H). **^{13}C{^{1}H}-NMR** (101 MHz, D_2O, δ/ppm): 159.2, 154.0, 152.0,

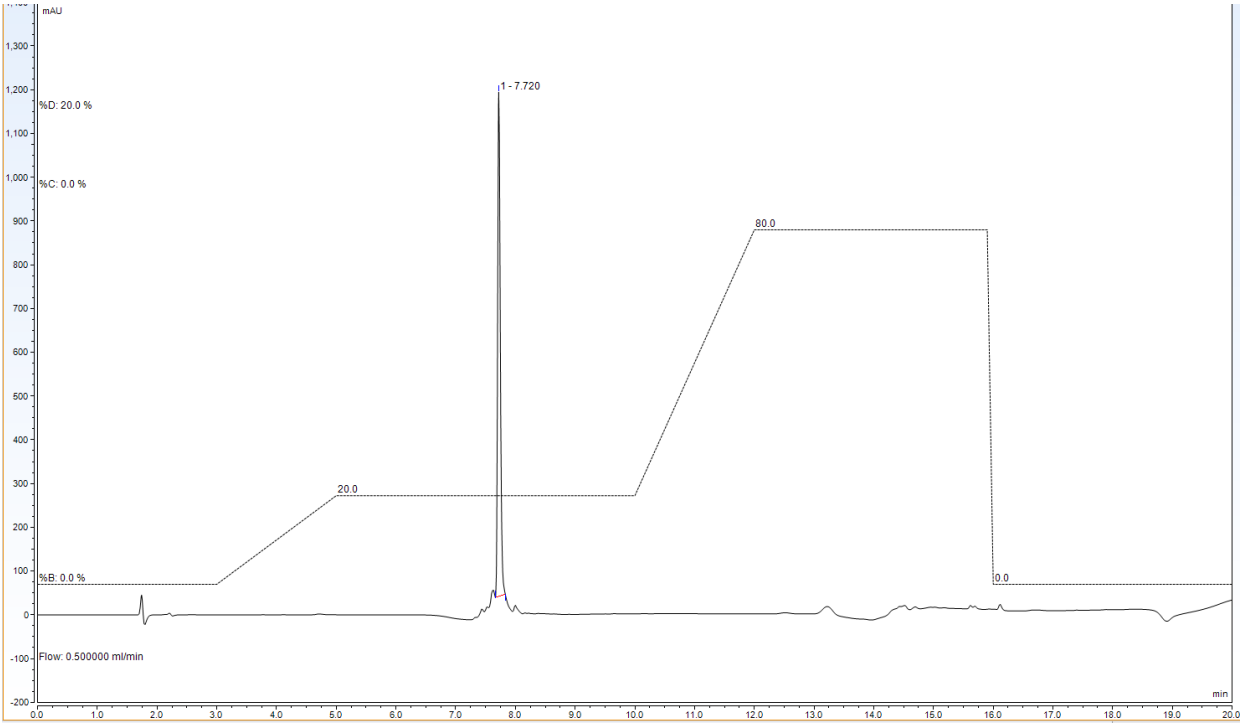
138.0, 116.4, 87.0, 84.9, 83.2 (t, *J* = 9.3, 8.8 Hz), 74.7 (d, *J* = 5.3 Hz), 72.9 (d, *J* = 3.9 Hz), 69.1, 65.6 (d, *J* = 5.6 Hz), 65.1 (d, *J* = 6.0 Hz), 28.8 (d, *J* = 7.3 Hz), 14.1 (d, *J* = 3.1 Hz). **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): –5.67 (d, *J* = 22.7 Hz, 1P), –10.88 (d, *J* = 21.5 Hz 2P), –11.51 (d, *J* = 20.4 Hz, 1P).
HRMS (ESI) m/z for $C_{15}H_{24}N_5O_{17}P_4$ $[M+H]^+$: calcd 670.0112, found 670.0110.

Synthesis of 5'-Amino-PEG-ppGpp (24) by click reaction



The sodium salt of pentynyl-ppGpp (**23**, 10.0 mg, 12.8 µmol) and 1-Amino-11-azido-3,6,9-trioxaundecane (3.80 µl, 4.19 mg, 19.2 µmol, 1.5 eq.) were dissolved in TEAA – buffer (1.20 ml, 200 mM). The solution was degassed for 10 min using argon. Afterwards sodium ascorbate (4.57 mg, 23.1 µmol, 1.8 eq.) and $CuSO_4$ x 5 H_2O (1.12 mg, 4.49 µmol, 0.35 eq.) were added and the resulting solution was stirred for 3 h under argon atmosphere. The crude product was precipitated by addition of NaClO4 – solution (20 ml, −20 °C, 500 mM in acetone) and isolated by centrifugation. Complete turnover was observed according to NMR-analysis. The crude product was purified by strong ion-exchange chromatography (Q-Sepharose) using an Äkta-system and $NaClO_4$ – buffer (120 mM). The product containing fractions were precipitated using an 8-fold volume of $NaClO_4$ – solution (−20 °C, 500 mM in acetone). The resulting solid was separated by centrifugation, washed with acetone (−20 °C, 3 x 3 ml) and dried over high vac for 2 h. The product (**24**, 6.70 mg, 6.72 µmol, 53%) was isolated as white solid.
^{1}H-NMR (400 MHz, D_2O, δ/ppm): 8.03 (s, 1H), 7.62 (s, 1H), 5.88 (s, 1H), 4.85 (s, 2H), 4.46 (s, 3H), 4.28 – 4.07 (m, 2H), 3.95 – 3.74 (m, 4H), 3.72 – 3.34 (m, 10H), 3.10 (s, 2H), 2.51 (s, 2H), 1.71 (s, 2H). **^{31}P{1H}-NMR** (162 MHz, D_2O, δ/ppm): –5.81 (br. s, 1P), –10.96 (br. s, 2P), –11.54 (br. s, 1P). **HRMS** (ESI) m/z for $C_{23}H_{39}N_9O_{20}P_4$ $[M-H_2]^{2-}$: calcd 442.5636, found 442.5634.

The product purity of compound **24** was determined by HPLC-UV (Dionex Ultimate 3000, C18_AQ-column, TEAA – buffer/MeCN gradient, WVL: 254 nm):



PAGE Analysis of Magic Spot Nucleotides

Polyacrylamide gel electrophoresis (PAGE) was carried out on a Hoefer SE660 Tall Standard Dual Cooled Vertical Unit. The PAGE procedure was conducted based on the general procedure as described by Losito *et al*.[4] Buffers and solutions for gel electrophoresis were prepared as follows:

Table 1: Description of buffers and solutions for PAGE.

10 × Tris/Borate/EDTA (TBE) buffer, pH 8.3	0.89 M Tris-HCl, 0.89 M boric acid, 20 mM EDTA
1 × Orange G dye (OGD), pH 7.0	10 mM Tris-HCl, 1 mM EDTA, 30% (w/v) glycerol, 0.1% (w/v) Orange G
Staining solution	0.1% (w/v) toluidine blue, 20% (w/v) MeOH, 2% (w/v) glycerol
De-staining solution	20% (w/v) MeOH, 2% (w/v) glycerol

During pre-run and run, the lower buffer chamber was filled with 6 L of pre-chilled 1 × TBE buffer (4 °C) and the buffer was stirred. A recirculating cooler was used for chilling the buffer. Sample loading was performed with gel-loading pipet tips. Samples were prepared by diluting stock solutions of ppGpp (**1**), pppGpp (**2**), ppApp (**13**), pGpp (**3**) and ppGp (**4**) with milli-Q H_2O to the desired concentration (all stock solutions were prepared in milli-Q H_2O).

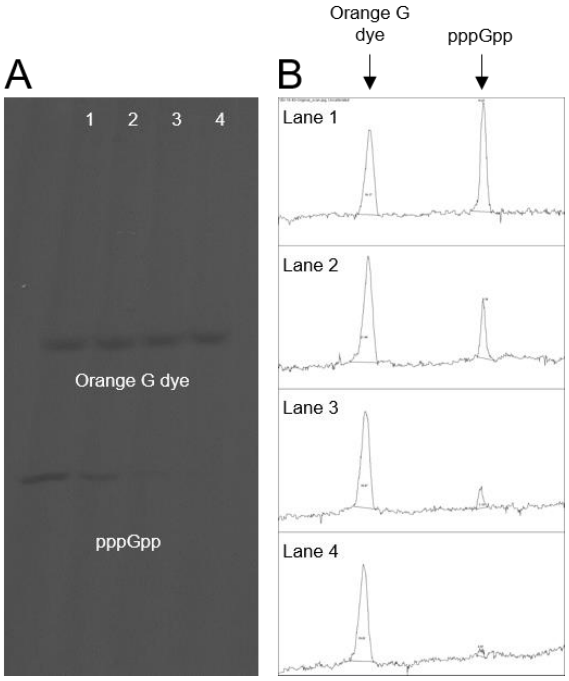
Electrophoretic separation of MSN

1. The gel sandwich was assembled using glass plates (24 × 18 cm) and spacers (1 cm wide, 1.0 mm thick).
2. Gel preparation (~ 40 mL/gel): 35.8 % (w/v) acrylamide:bis-acrylamide 19:1 (33.9 mL, 3030 Roth), 10.0 % (v/v) 10 × TBE buffer (3.8 mL) and 0.05 % (w/v) ammonium persulfate (APS; 200 µL of 10 % APS in milli-Q H_2O) were stirred for 2 min at 0 °C. 0.05 % (v/v) tetramethylethylenediamine (TEMED; 20 µL) was added and the solution was stirred for 1 min. The mixture was poured between the pre-casted glass-plates and a 15 lane comb was inserted. The solution was allowed to polymerize for 25-30 min at room temperature.
3. After polymerization, gels were pre-run at 4 °C in 1 × TBE buffer for 30 min at 300 V.
4. Samples (22 µL volume per sample) were prepared and 1 × Orange G dye (7 µL) was added to all samples prior to loading onto the gel.
5. Wells were washed with 1 × TBE buffer by using a syringe and needle to remove any precipitates and non-polymerized gel debris. The gel was then loaded leaving 2-3 wells empty on each side.
6. Gels were run at 4 °C in 1 × TBE buffer for 20 h at 500 V.
7. After the run, the gel apparatus was disassembled. One glass plate was removed leaving the gel on the other glass plate.
8. Workup: Gels were stained for 30 min with staining solution and then de-stained for 1.5 h with de-staining solution. The de-staining solution was replaced once during the entire procedure.
9. The gels were scanned with a photo scanner (Canon CanoScan 8800F).

10. Finally, the image manipulation program Gimp 2.8.22 was used to convert the images to greyscale and to adjust contrast and brightness.

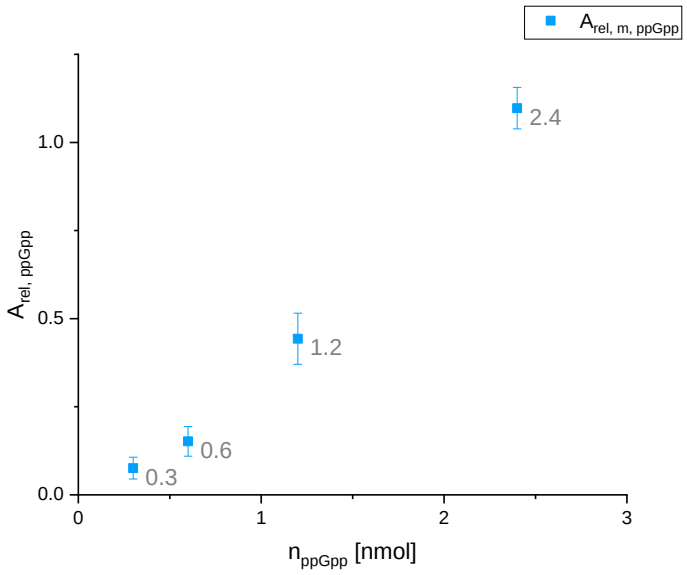
Quantification of ppGpp and pppGpp via electrophoretic densitometry

Standard solutions with different amounts of ppGpp (2.4 nmol, 1.2 nmol, 0.6 nmol, 0.3 nmol) and pppGpp (1.2 nmol, 0.6 nmol, 0.3 nmol, 0.15 nmol) were run on gel as described above.[4] Every experiment was conducted in triplicate. For comparing the band densities of the standards, 8-bit copies of the gel images were analysed by using ImageJ. Peak areas were measured by generating lane profile plots followed by drawing lines to enclose peaks of interest. Supporting Figure 1 illustrates the gel analysis procedure with ImageJ.[5]

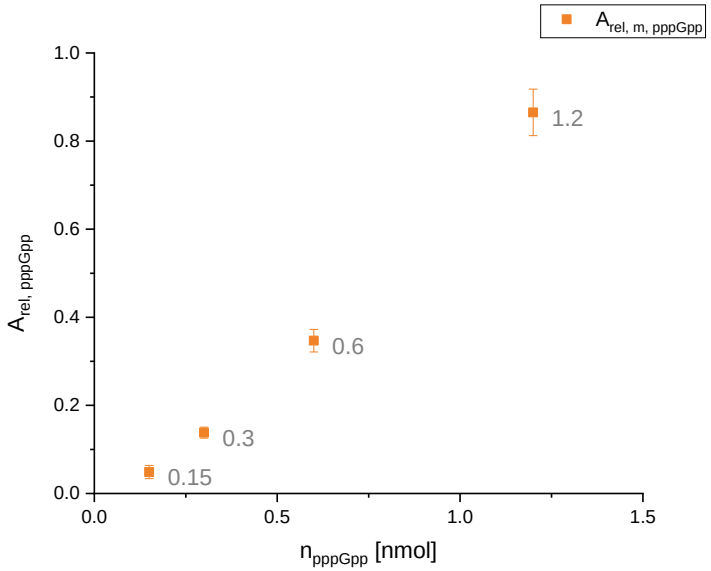


Supporting Figure 1: (A) 8-bit image of electrophoretically separated pppGpp after staining with toluidine blue. Lane 1: pppGpp (1.2 nmol), Lane 2: pppGpp (0.6 nmol). Lane 3: pppGpp (0.3 nmol). Lane 4: pppGpp (0.15 nmol). (B) Corresponding densitometric gel image analysis obtained via ImageJ. This experiment is in the following referred to as Experiment 2 b).

Errors on loading volumes were avoided by dividing the peak area of interest by the corresponding area of the Orange G dye A_{OGD} (quotients are hereafter referred to as relative peak areas A_{rel}). A graphical summary was created using OriginPro (Version 2018b) and is shown in Supporting Figure 2 (ppGpp) and Supporting Figure 3 (pppGpp).



Supporting Figure 2: *Plot of the mean $A_{rel,\ m}$ of ppGpp samples correlated to the corresponding loading amounts.*



Supporting Figure 3: *Plot of the mean $A_{rel,\ m}$ of pppGpp obtained via ImageJ versus the corresponding loading amounts.*

The experimental data as well as the mathematical equations used for calculating mean and error values are listed below. All gels and corresponding densitometric gel image analyses are shown in the Supporting Figures 4-8.

$$A_{rel,\ m} = \frac{1}{n}\sum_{i=1}^{n} A_{rel,\ i} \qquad \textit{(Equation 1)}$$

$A_{rel,\ i}$ = *measured value*
$A_{rel,\ m}$ = *mean value*
n = *number of measurements*

$$s = \sqrt{\frac{1}{1-n}\sum_{i=1}^{n}(A_{rel,\ i} - A_{rel,\ m})^2} \qquad \textit{(Equation 2)}$$

s = *standard deviation*

$$s_m = \frac{s}{\sqrt{n}} \qquad \textit{(Equation 3)}$$

s_m= *standard deviation of the mean value*

Experimental data for ppGpp

Supporting Table 2: *Peak areas (A) of different ppGpp standards and OGD on gel obtained via ImagJ analysis.*

Experiment	A_{ppGpp} *(2.4 nmol)*	A_{ppGpp} *(1.2 nmol)*	A_{ppGpp} *(0.6 nmol)*	A_{ppGpp} *(0.3 nmol)*
1 a)	8877	3458	1065	346
2 a)	14891	7110	2621	1588
3 a)	12177	4058	1245	526
Experiment	A_{OGD} *(2.4 nmol)*	A_{OGD} *(1.2 nmol)*	A_{OGD} *(0.6nmol)*	A_{OGD} *(0.3 nmol)*
1 a)	8313	7861	8798	7465
2 a)	12301	12472	11157	11527
3 a)	12016	12737	12564	12102

Supporting Table 3: *Relative peak areas* A_{rel} *of different ppGpp standards on gel as quotient of* A_{ppGpp} and A_{OGD}.

Experiment	$A_{rel,\ ppGpp}$ *(2.4 nmol)*	$A_{rel,\ ppGpp}$ *(1.2 nmol)*	$A_{rel,\ ppGpp}$ *(0.6 nmol)*	$A_{rel,\ ppGpp}$ *(0.3 nmol)*
1 a)	1.06787	0.43995	0.12099	0.04641
2 a)	1.21060	0.57010	0.23493	0.13775
3 a)	1.01341	0.31862	0.09909	0.04344

Supporting Table 4: *Mean values of the relative peak areas* $A_{rel,\ m}$ *of different ppGpp standards calculated according to Equation 1.*

$A_{rel,\ m,\ ppGpp}$ *(2.4 nmol)*	$A_{rel,\ m,\ ppGpp}$ *(1.2 nmol)*	$A_{rel,\ m,\ ppGpp}$ *(0.6 nmol)*	$A_{rel,\ m,\ ppGpp}$ *(0.3 nmol)*
1.09730	0.44289	0.15167	0.07586

Supporting Table 5: *Standard deviations of mean values* $s_{m,\ ppGpp}$ *(calculated according to Equation 2 and Equation 3).*

$s_{m,\ ppGpp}$ *(2.4 nmol)*	$s_{m,\ ppGpp}$ *(1.2 nmol)*	$s_{m,\ ppGpp}$ *(0.6 nmol)*	$s_{m,\ ppGpp}$ *(0.3 nmol)*
0.05879	0.07261	0.04211	0.03095

Experimental data for pppGpp:

Supporting Table 5: *Peak areas (A) of different ppGpp standards and OGD on gel obtained via ImagJ analysis.*

Experiment	A_{pppGpp} *(1.2 nmol)*	A_{pppGpp} *(0.6 nmol)*	A_{pppGpp} *(0.3 nmol)*	A_{pppGpp} *(0.15 nmol)*
1 b)	11501	4891	1752	694
2 b)	8254	3483	1200	368
3 b)	9733	4325	1981	792
Experiment	A_{OGD} *(1.2 nmol)*	A_{OGD} *(0.6 nmol)*	A_{OGD} *(0.3 nmol)*	A_{OGD} *(0.15 nmol)*
1 b)	12038	13450	12350	13440
2 b)	8857	11744	10447	10419
3 b)	12374	11369	12558	13356

Supporting Table 6: *Relative peak areas* A_{rel} *of different pppGpp standards on gel as quotient of* A_{pppGpp} and A_{OGD}.

Experiment	$A_{rel,\ pppGpp}$ *(1.2 nmol)*	$A_{rel,\ pppGpp}$ *(0.6 nmol)*	$A_{rel,\ pppGpp}$ *(0.3 nmol)*	$A_{rel,\ pppGpp}$ *(0.15 nmol)*
1 b)	0.95538	0.36368	0.14184	0.05165
2 b)	0.93195	0.29658	0.11483	0.03527
3 b)	0.78655	0.38040	0.15775	0.05929

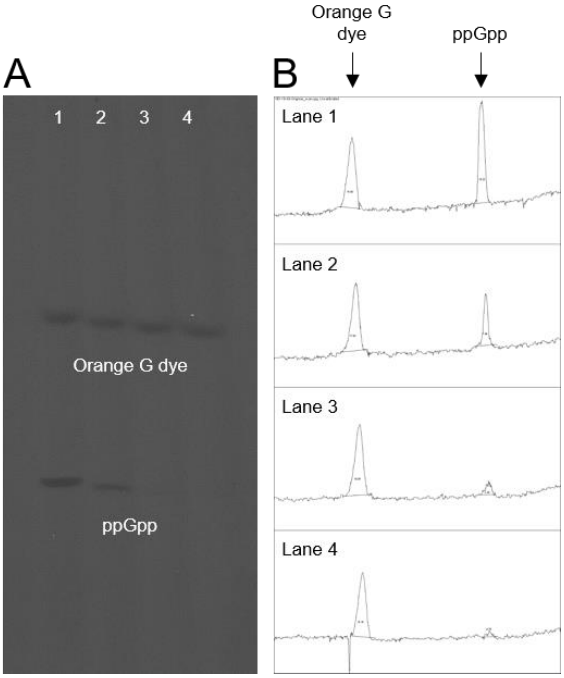
Supporting Table 7: *Mean values of the relative peak areas* $A_{rel,\ m}$ *of different pppGpp standards calculated according to Equation 1.*

$A_{rel,\ m,\ pppGpp}$ *(1.2 nmol)*	$A_{rel,\ m,\ pppGpp}$ *(0.6 nmol)*	$A_{rel,\ m,\ pppGpp}$ *(0.3 nmol)*	$A_{rel,\ m,\ pppGpp}$ *(0.15 nmol)*
0.89129	0.34689	0.13814	0.04874

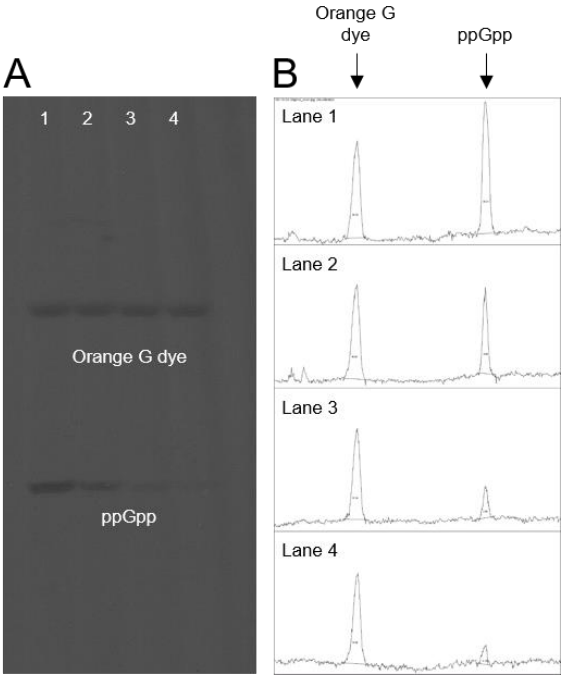
Supporting Table 8: *Standard deviations of mean values* $s_{m,\ pppGpp}$ *(calculated according to Equation 2 and Equation 3).*

$s_{m,\ pppGpp}$ *(1.2 nmol)*	$s_{m,\ pppGpp}$ *(0.6 nmol)*	$s_{m,\ pppGpp}$ *(0.3 nmol)*	$s_{m,\ pppGpp}$ *(0.15 nmol)*
0.05281	0.02562	0.01253	0.01508

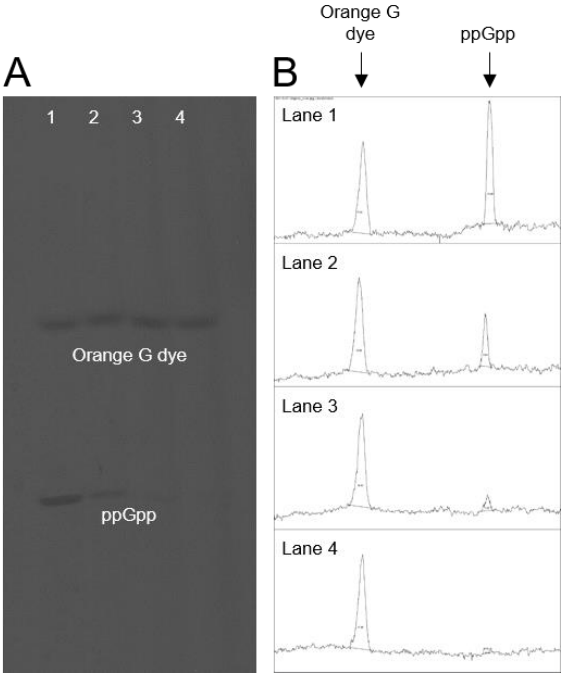
Gel images and corresponding densitometric analyses for ppGpp



Supporting Figure 4: Experiment 1 a). (A) 8-bit image of electrophoretically separated ppGpp after staining with toluidine blue. Lane 1: ppGpp (2.4 nmol). Lane 2: ppGpp (1.2 nmol). Lane 3: ppGpp (0.6 nmol). Lane 4: ppGpp (0.3 nmol). (B) Corresponding densitometric gel image analysis obtained via ImageJ.

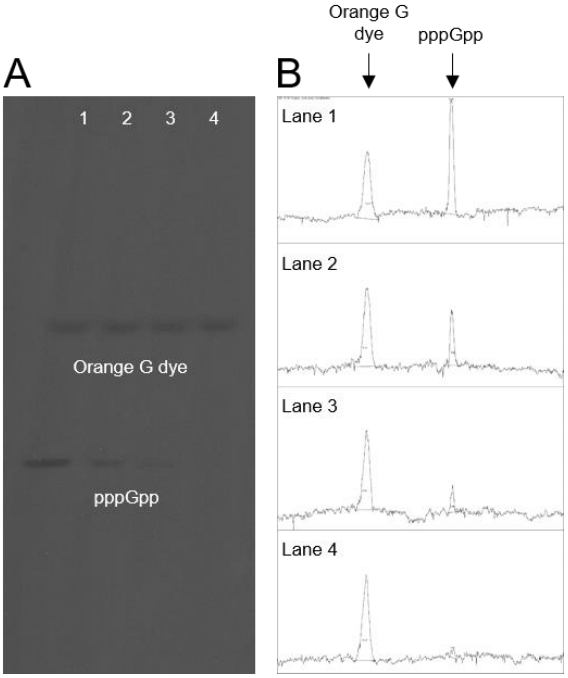


Supporting Figure 5: Experiment 2 a). (A) 8-bit image of electrophoretically separated ppGpp after staining with toluidine blue. Lane 1: ppGpp (2.4 nmol). Lane 2: ppGpp (1.2 nmol). Lane 3: ppGpp (0.6 nmol). Lane 4: ppGpp (0.3 nmol). (B) Corresponding densitometric gel image analysis obtained via ImageJ.

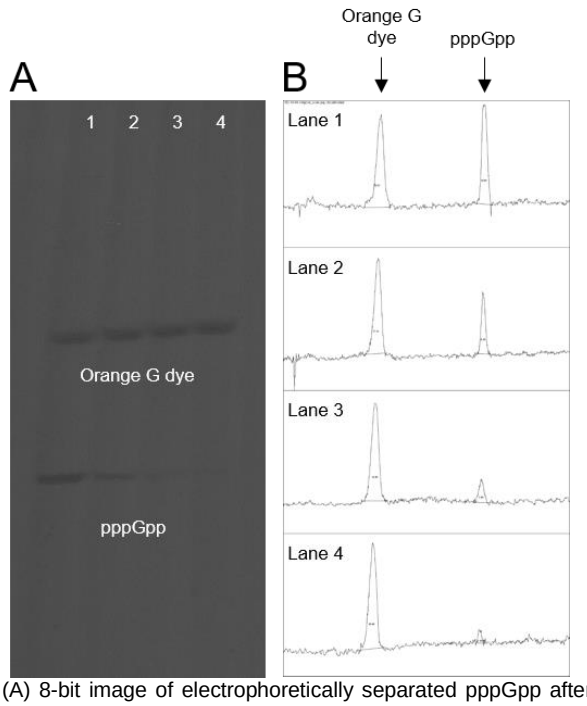


Supporting Figure 6: Experiment 3 a). (A) 8-bit image of electrophoretically separated ppGpp after staining with toluidine blue. Lane 1: ppGpp (2.4 nmol). Lane 2: ppGpp (1.2 nmol). Lane 3: ppGpp (0.6 nmol). Lane 4: ppGpp (0.3 nmol). (B) Corresponding densitometric gel image analysis obtained via ImageJ.

Gel images and corresponding densitometric analyses for pppGpp



Supporting Figure 7: Experiment 1 b). (A) 8-bit image of electrophoretically separated pppGpp after staining with toluidine blue. Lane 1: pppGpp (1.2 nmol). Lane 2: pppGpp (0.6 nmol). Lane 3: pppGpp (0.3 nmol). Lane 4: pppGpp (0.15 nmol). (B) Corresponding densitometric gel image analysis obtained via ImageJ.



Supporting Figure 8: Experiment 3 b). (A) 8-bit image of electrophoretically separated pppGpp after staining with toluidine blue. Lane 1: pppGpp (1.2 nmol). Lane 2: pppGpp (0.6 nmol). Lane 3: pppGpp (0.3 nmol). Lane 4: pppGpp (0.15 nmol). (B) Corresponding densitometric gel image analysis obtained via ImageJ.

References

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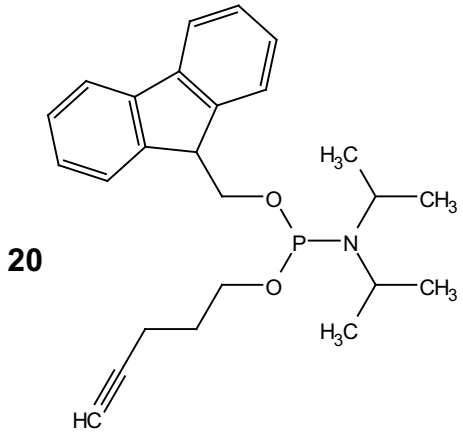
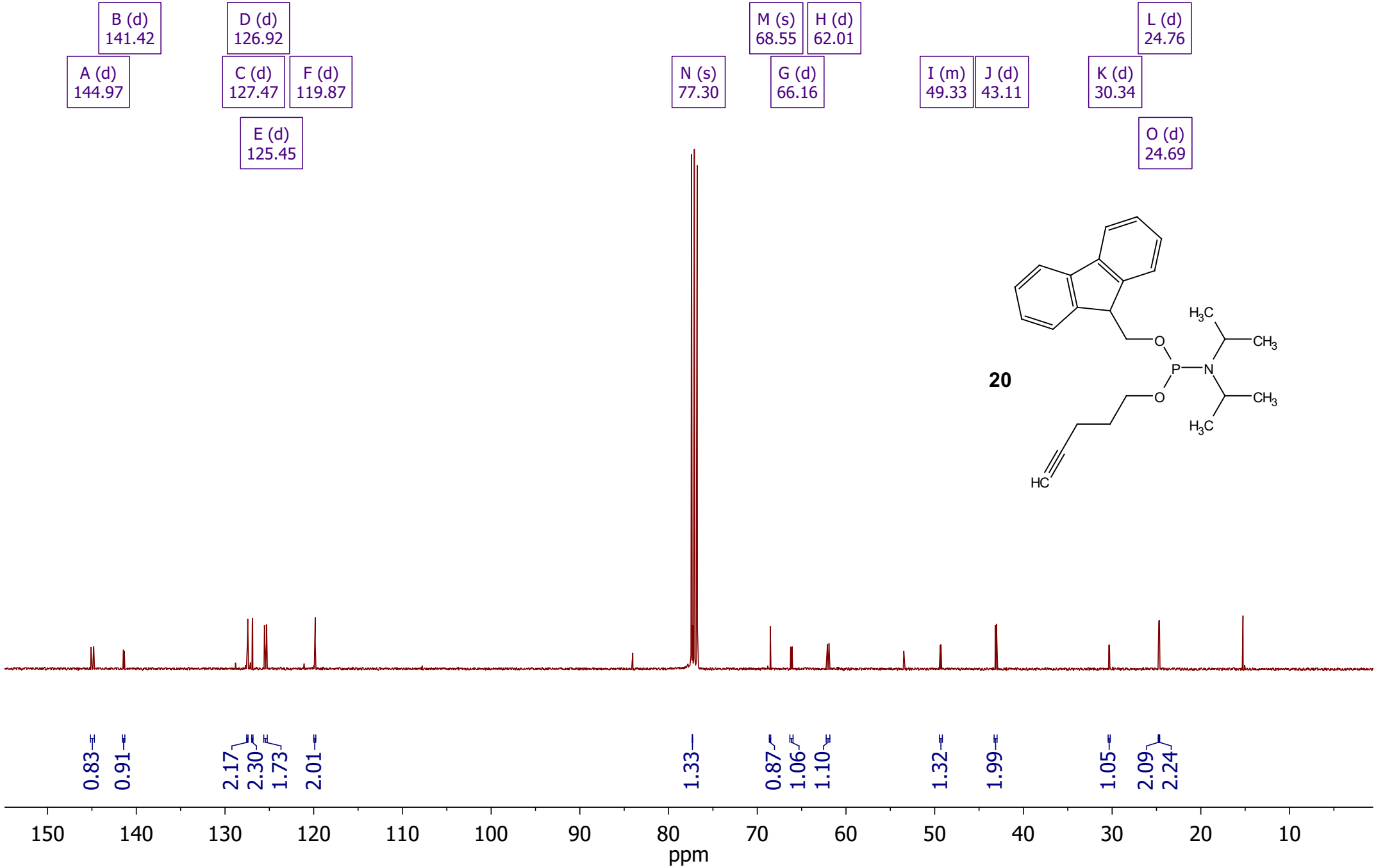
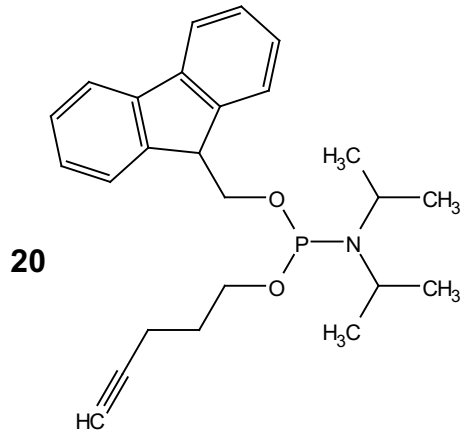
Compound 20 (pentynylO)(FmO)P-NiPr$_2$, ^{1}H - NMR (CDCl$_3$, 400 MHz)



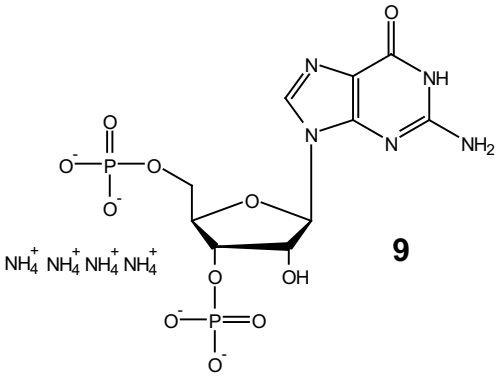
Compound 20 (pentynylO)(FmO)P-NiPr$_2$, $^{13}C[^1H]$ - NMR ($CDCl_3$, 101 MHz)



Compound 20 (pentynylO)(Fm)P-NiPr$_2$, $^{31}P[^1H]$ - NMR ($CDCl_3$, 162 MHz)

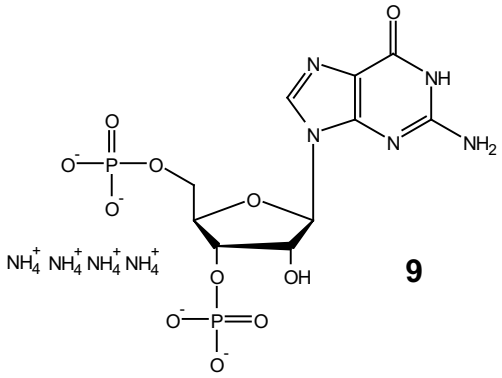


Compound 9 (pGp), ^{1}H - NMR (D_2O, 500 MHz)



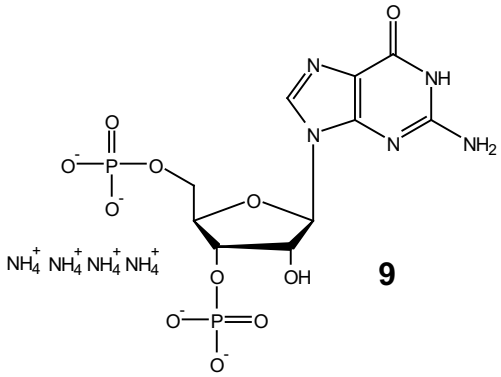
9

Compound 9 (pGp), $^{31}P[^{1}H]$ - NMR (D_2O, 202 MHz)



9

Compound 9 (pGp), $^{13}C[^{1}H]$ - NMR (D_2O, 121 MHz)



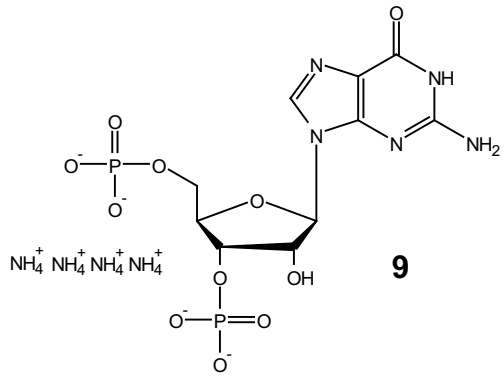
9

B (s) 153.97
A (s) 159.01
C (s) 151.85
D (s) 137.66
E (s) 116.28
G (dd) 83.32
F (s) 86.64
J (d) 64.29
H (d) 73.69
I (d) 73.21

260 250 240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20

[ppm]

Compound 9 (pGp), $^{13}C[^{1}H]$ - NMR (D_2O, 121 MHz), detailed



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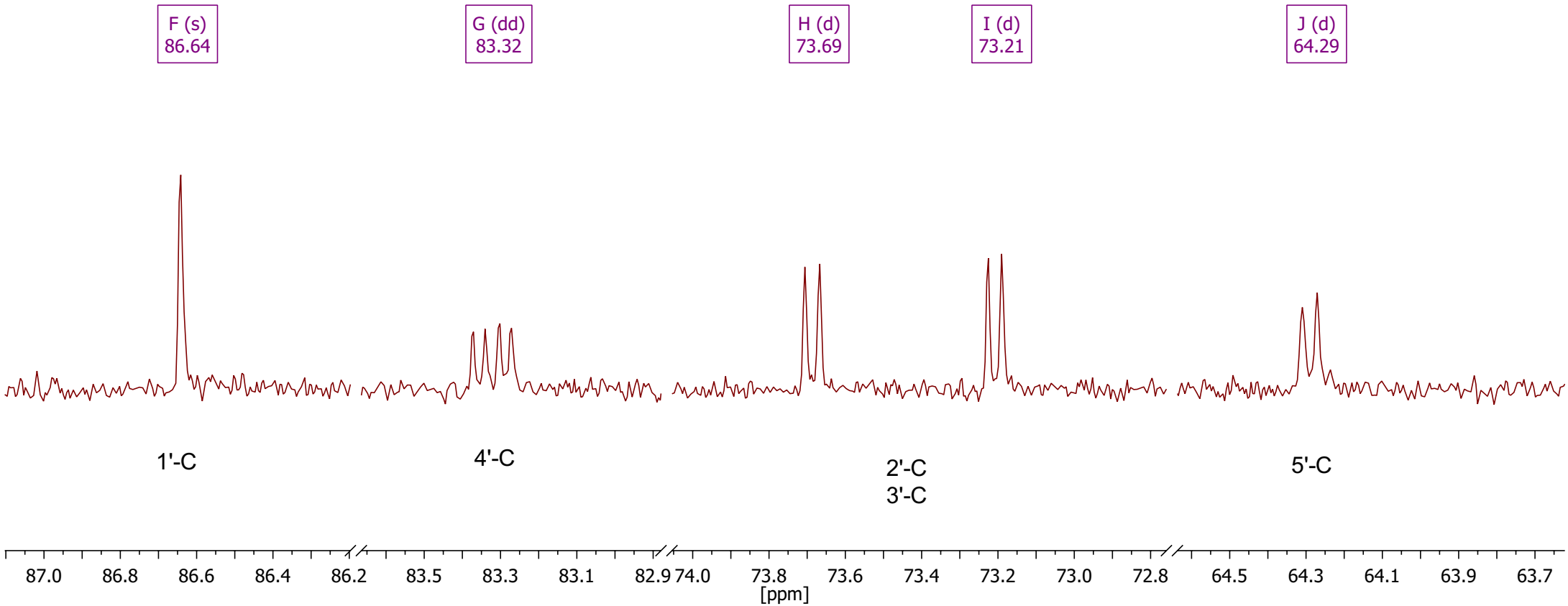
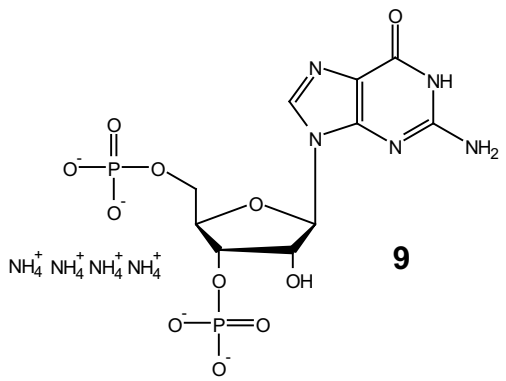


Compound 9 (pGp), DQF-COSY



9

1'-H
2'-H
3'-H
4'-H
5'-H2

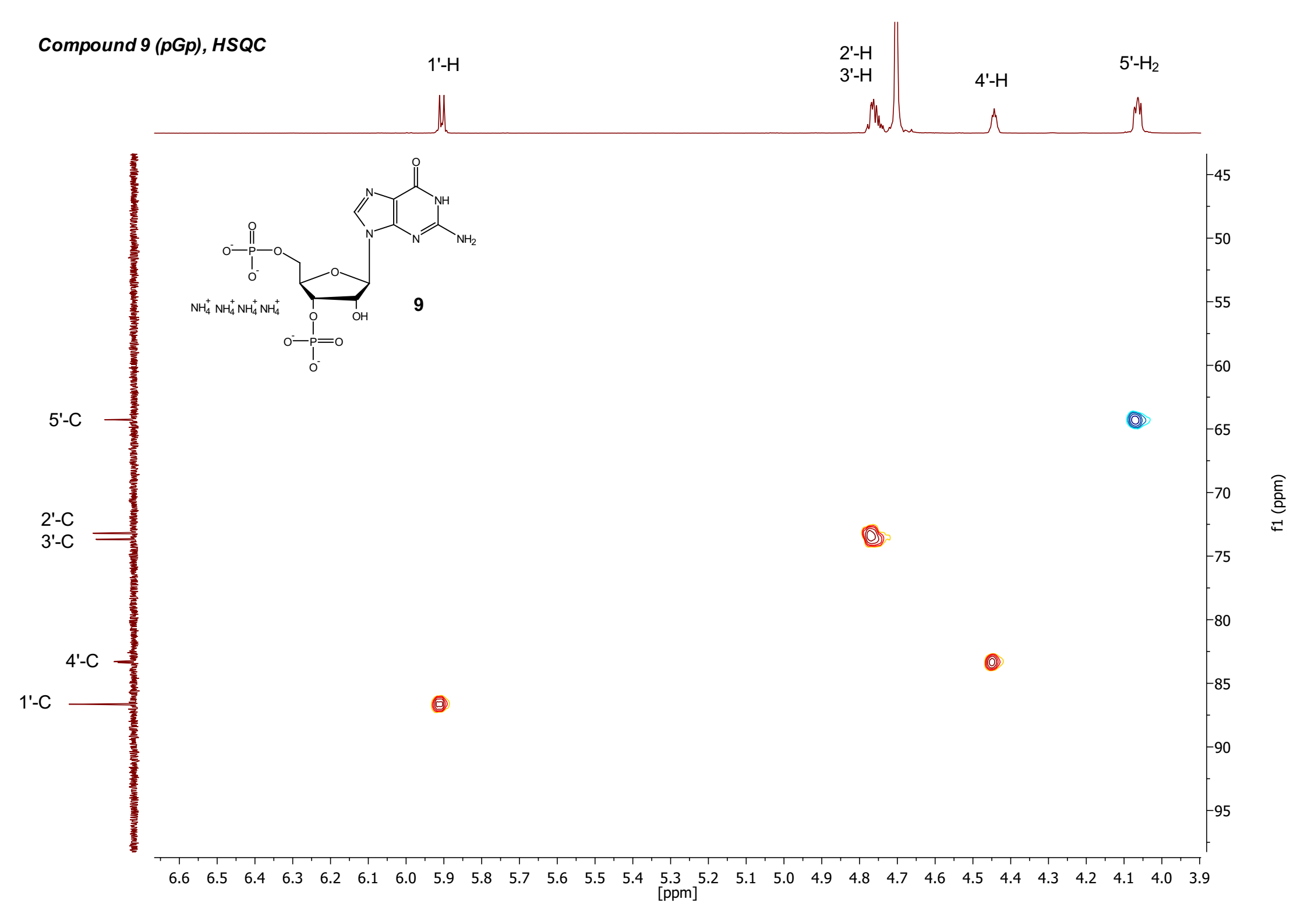
f1 (ppm)

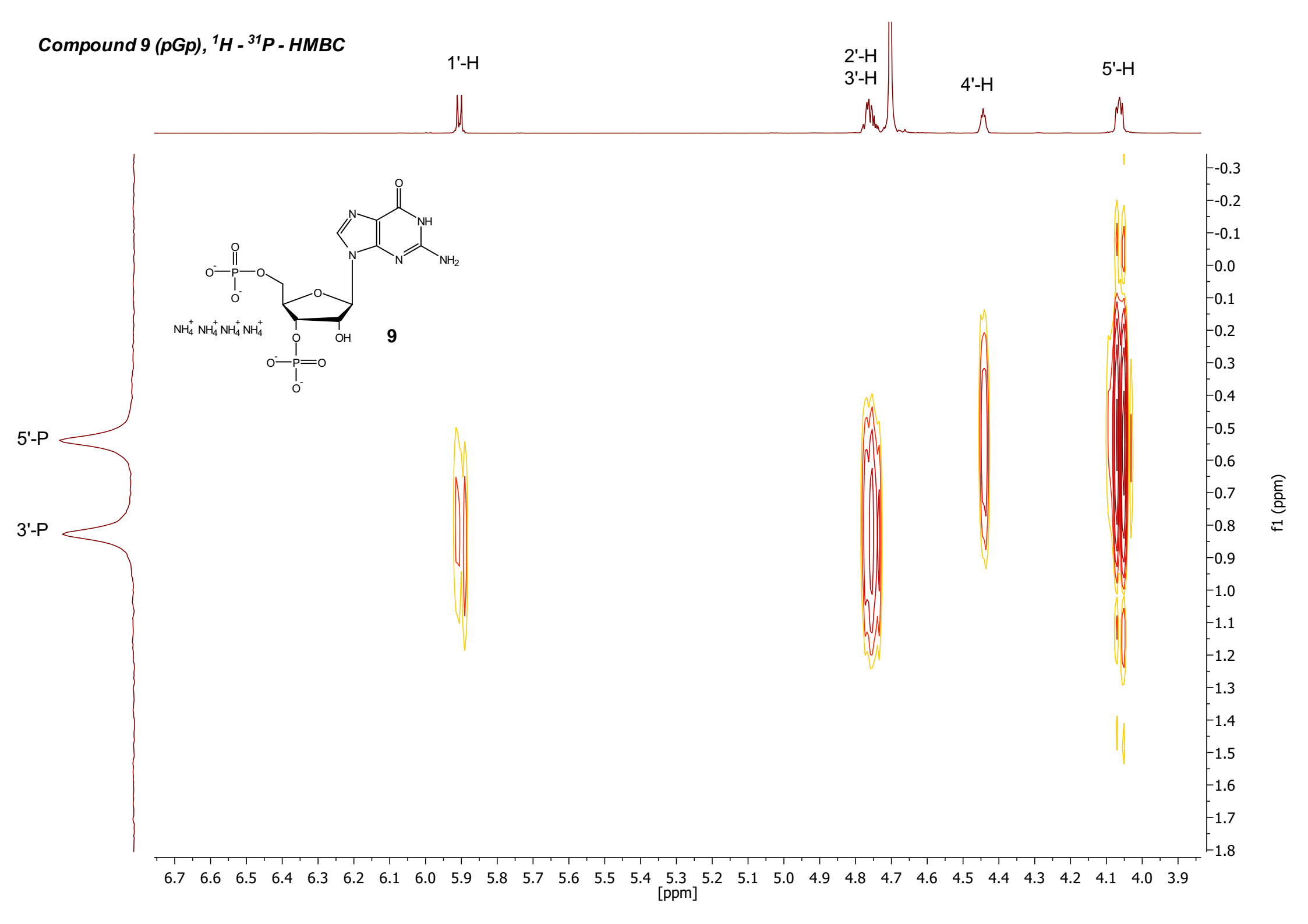
3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4

6.4 6.3 6.2 6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8

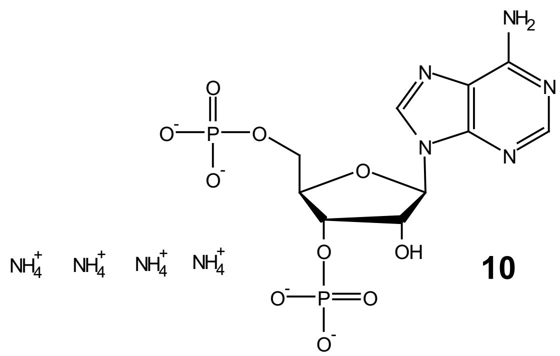
[ppm]

Compound 9 (pGp), HSQC




Compound 9 (pGp), ^{1}H - ^{31}P - HMBC
1'-H
2'-H
3'-H
4'-H
5'-H
5'-P
3'-P
9
[ppm]
f1 (ppm)

Compound 10 (pAp - NH_4), ^1H - NMR (D_2O, 400 MHz)



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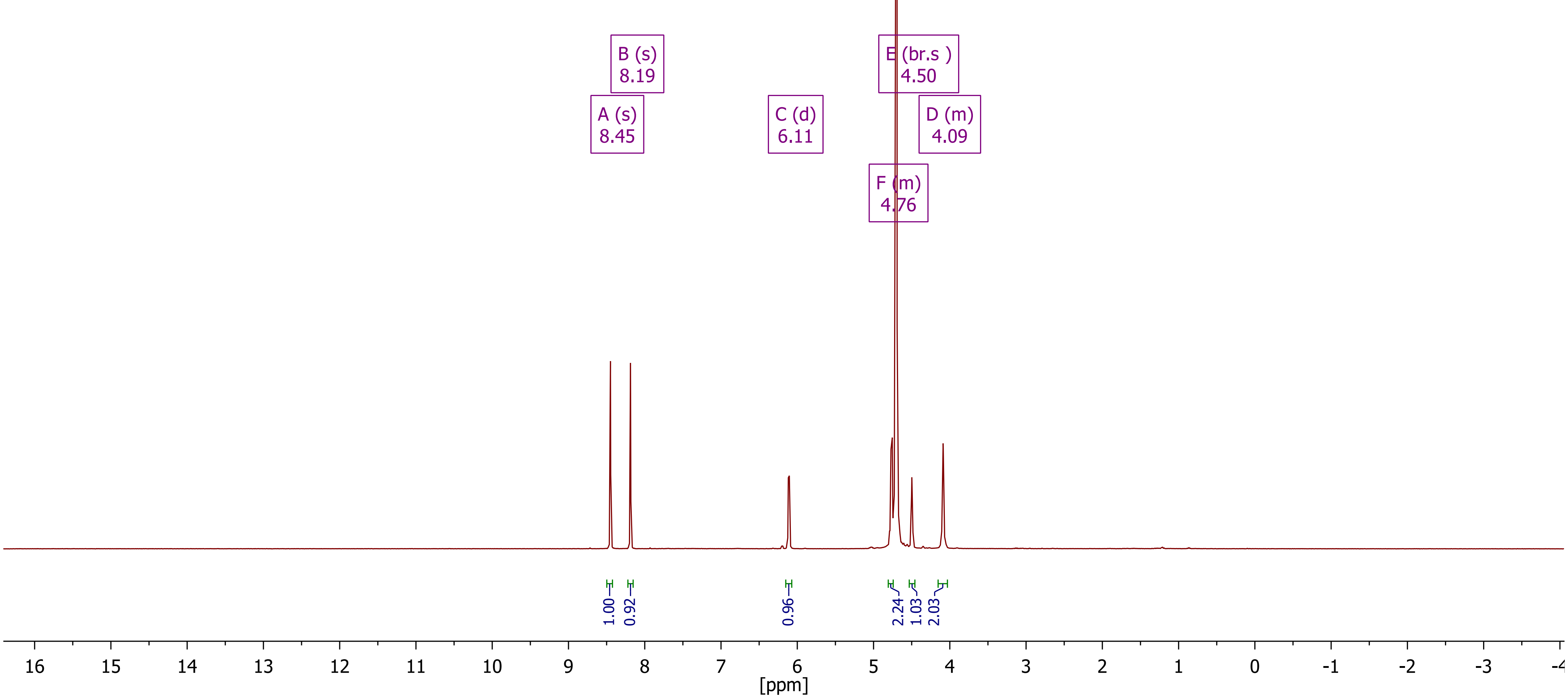
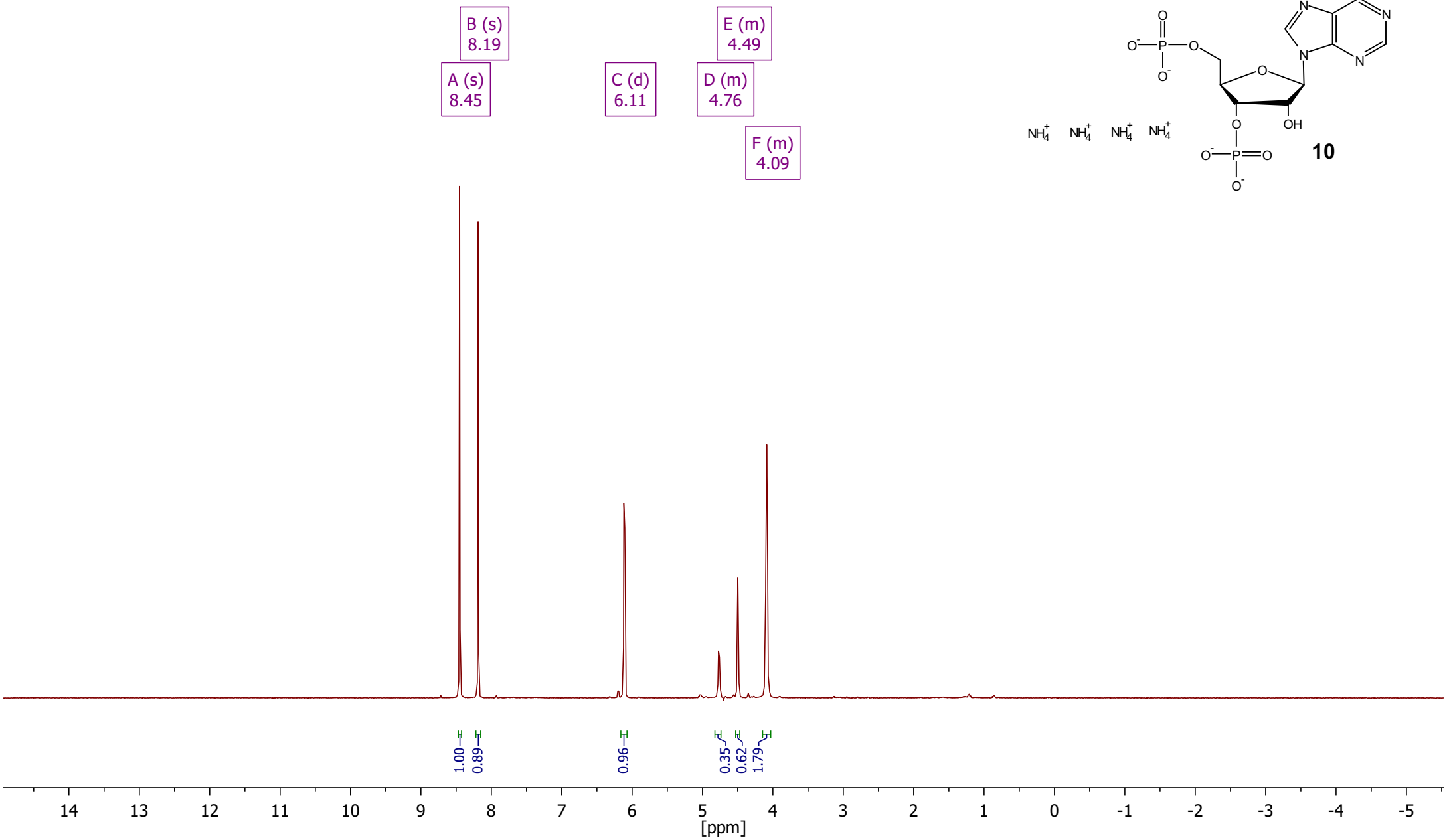
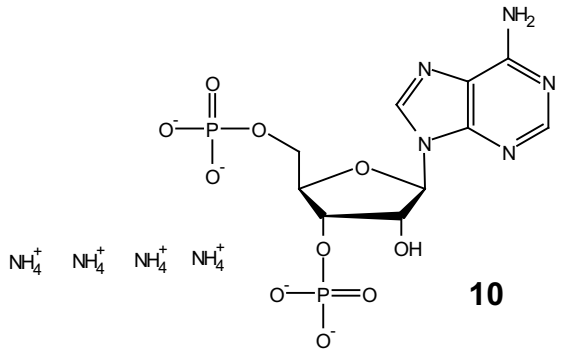


Compound 10 (pAp - NH_4), ^1H - NMR (D_2O, 400 MHz, water suppression)



10

Compound 10 (pAp - NH_4), $^{31}P[^1H]$ - NMR (D_2O, 162 MHz)



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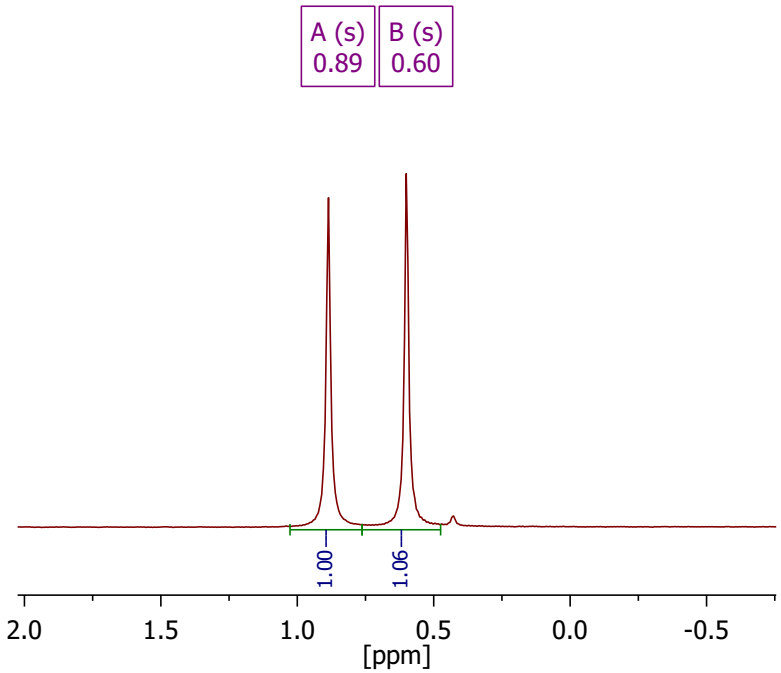
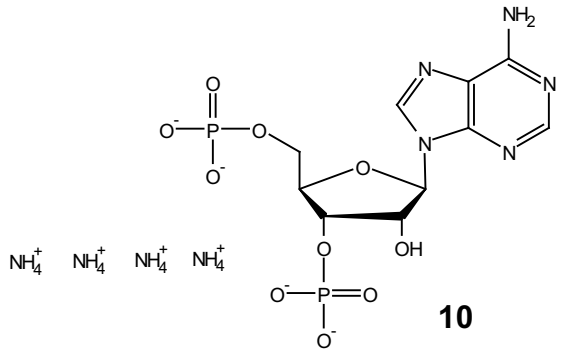


Compound 10 (pAp - NH_4), $^{13}C[^1H]$ - NMR (D_2O, 101 MHz)



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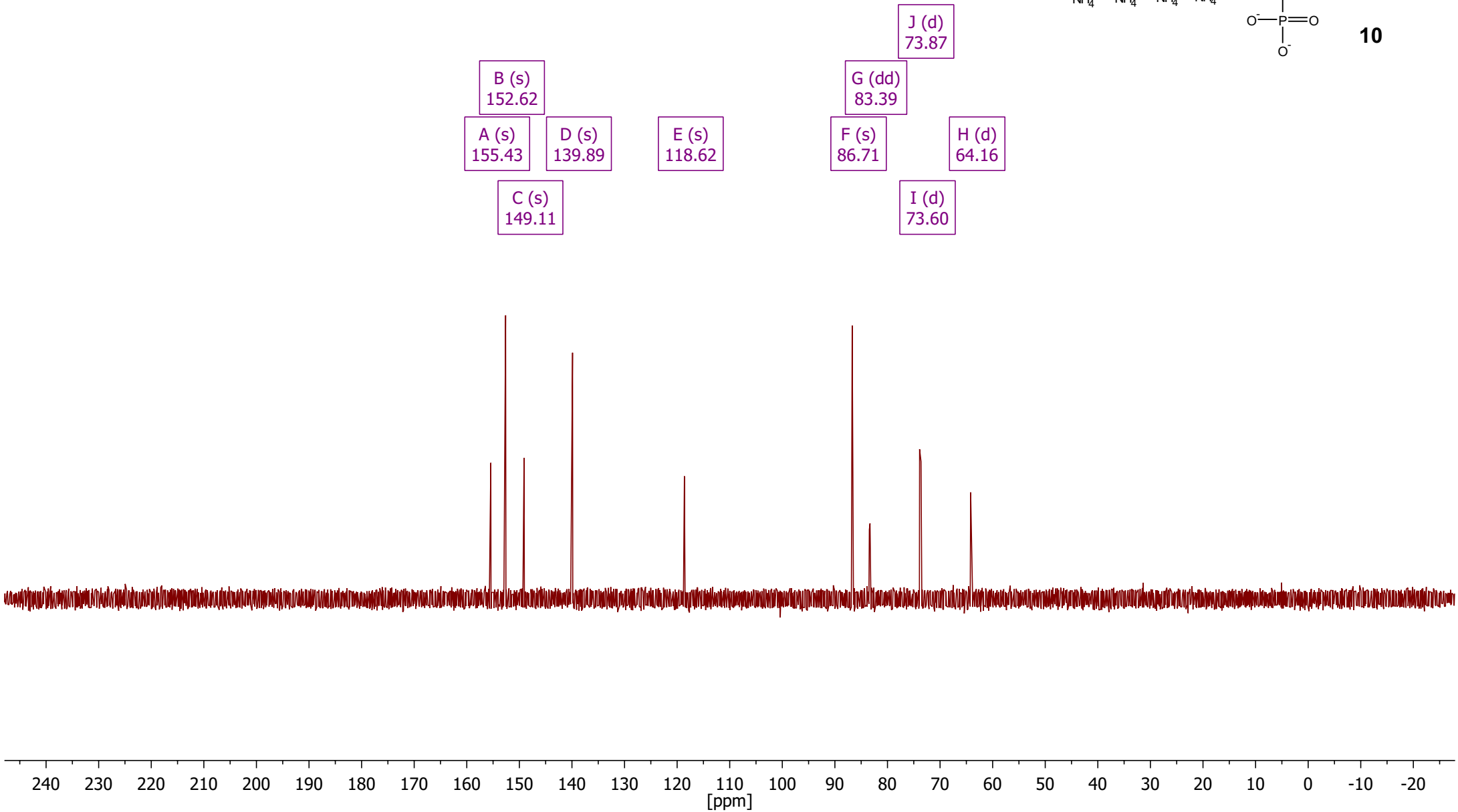
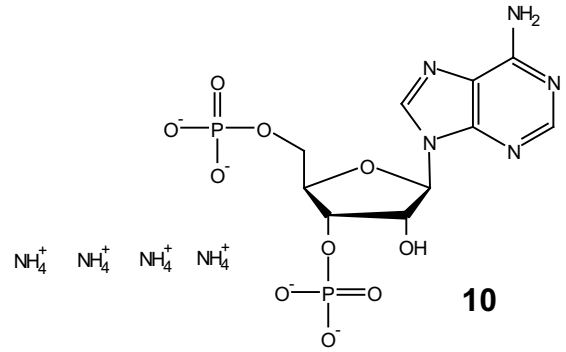


Compound 10 (pAp - NH_4), $^{13}C[^1H]$ - NMR (D_2O, 101 MHz), detailed



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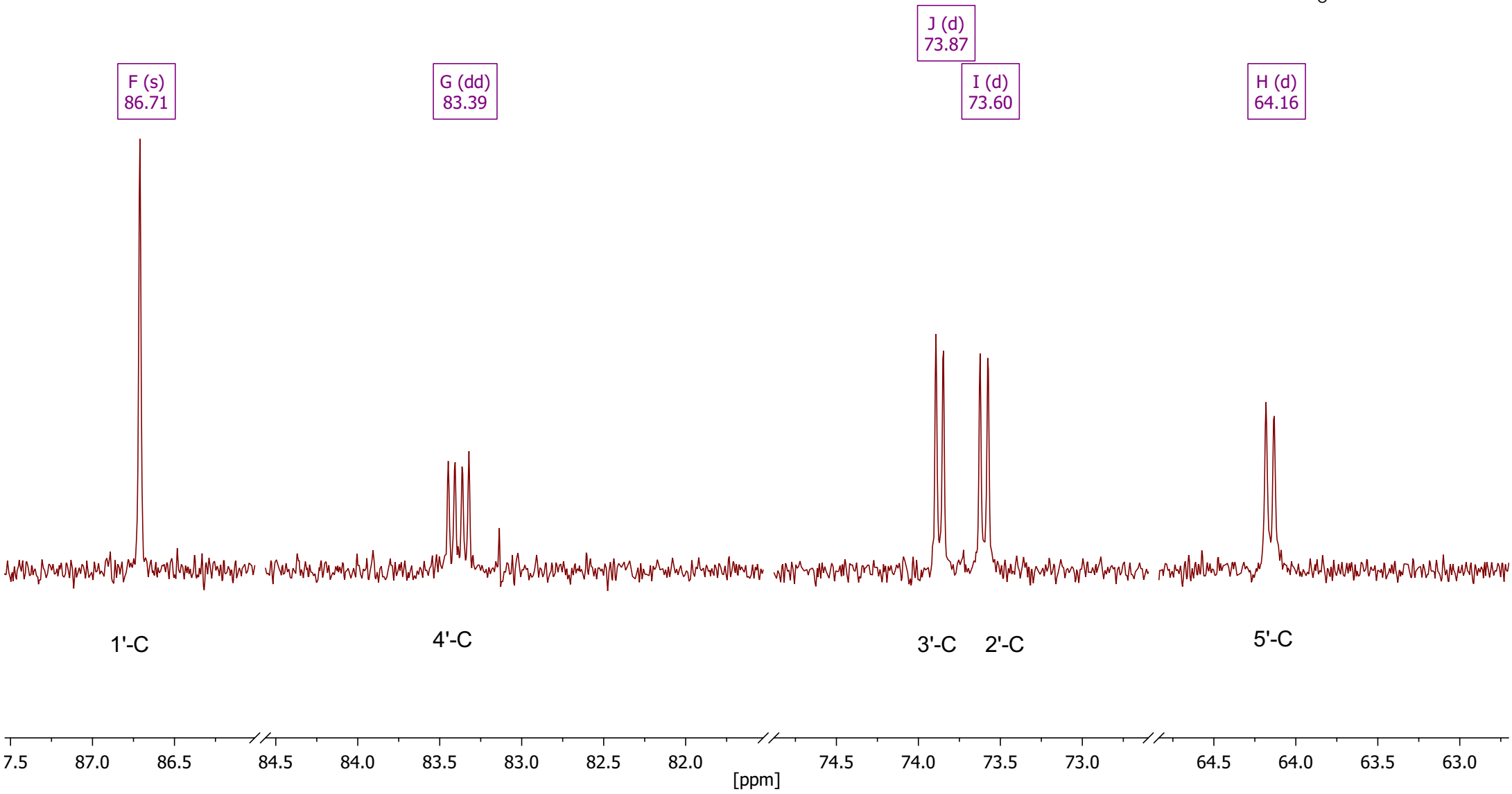
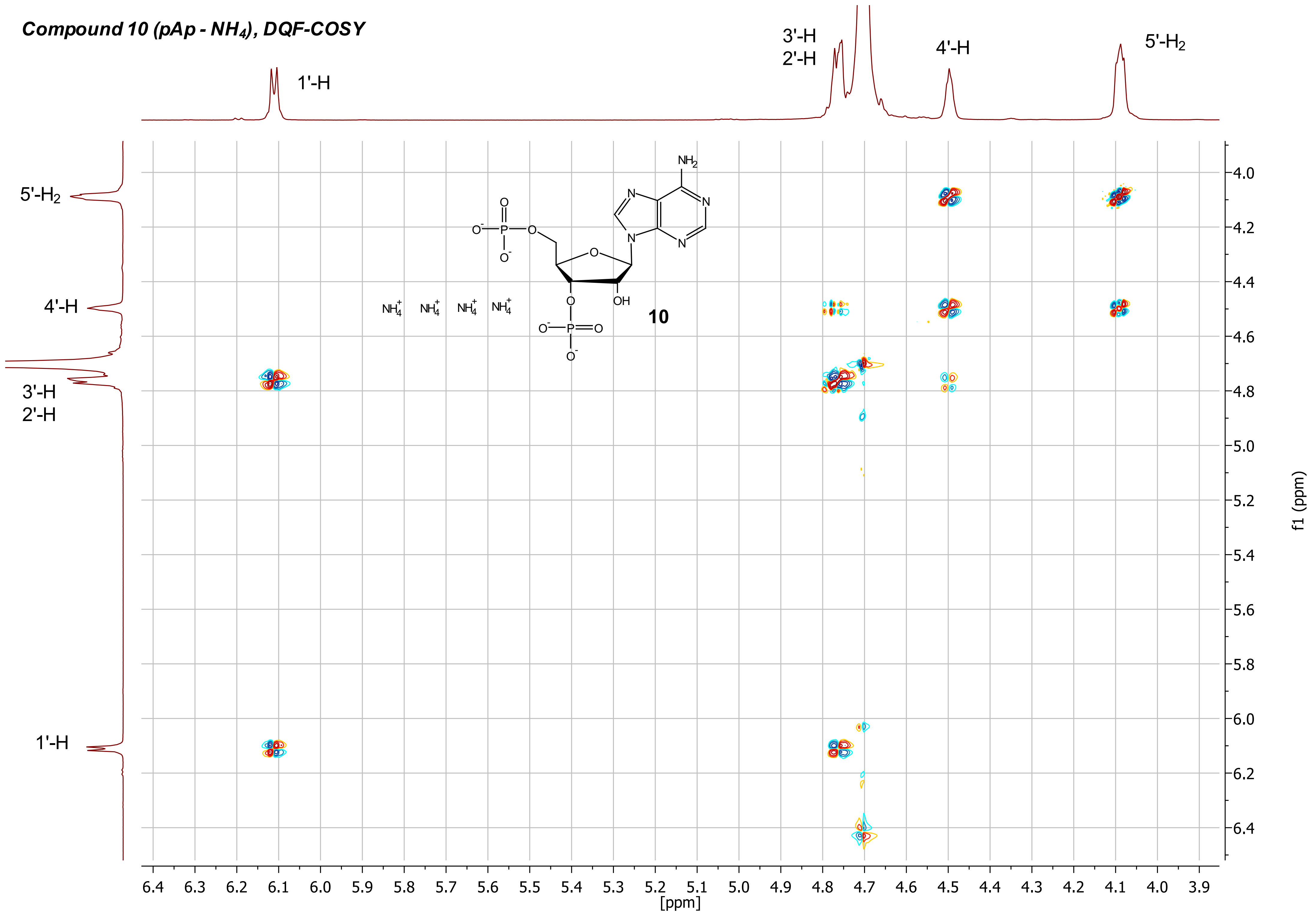
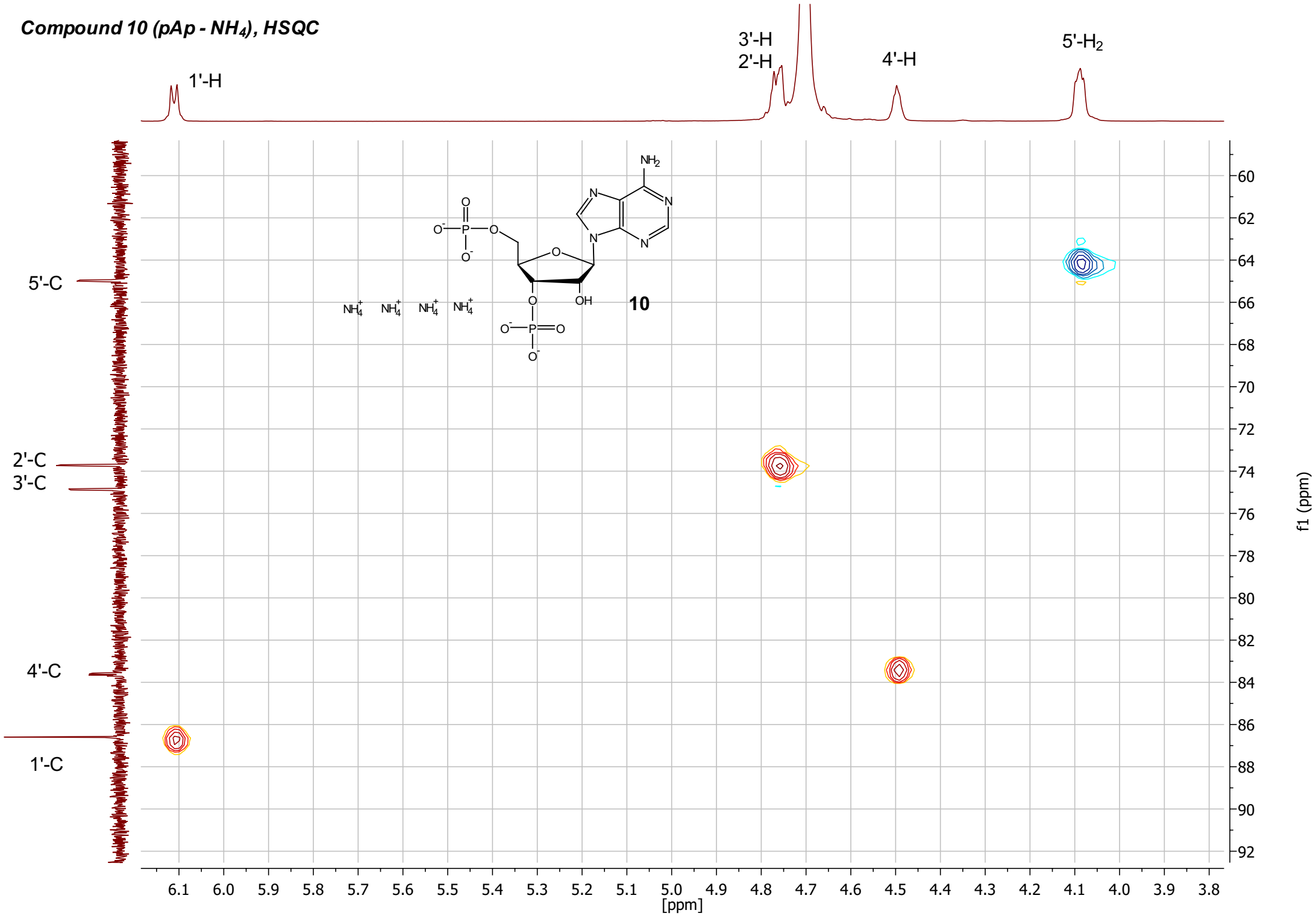
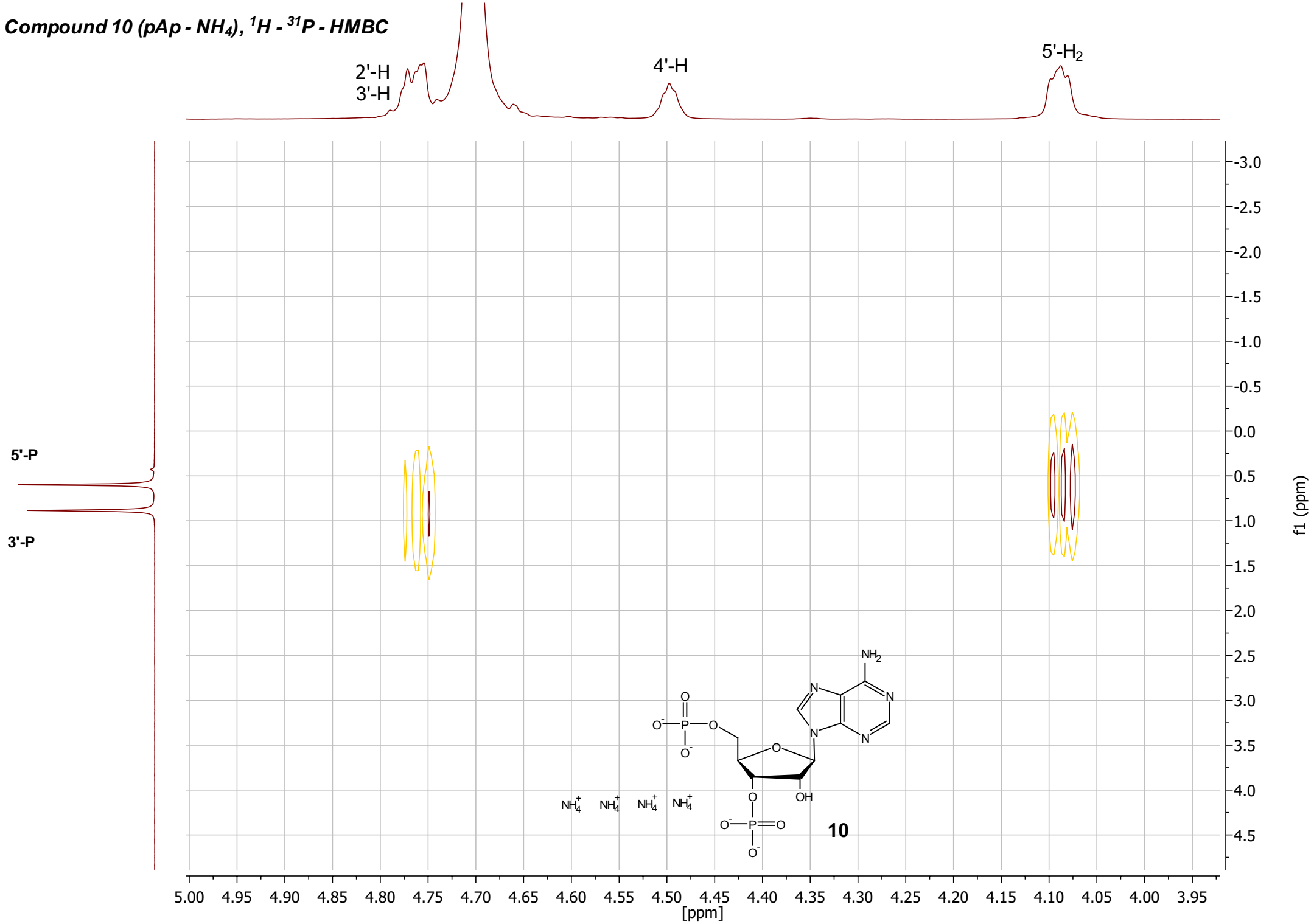


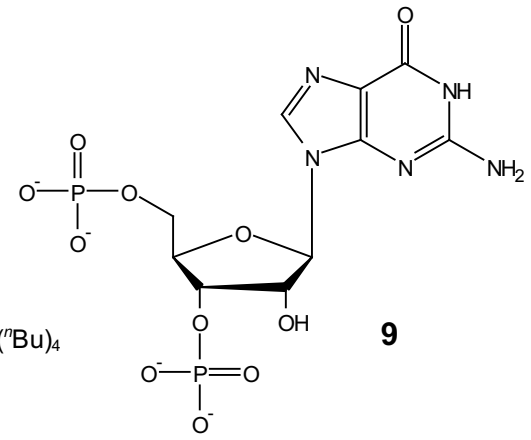
Compound 10 (pAp - NH_4), DQF-COSY




Compound 10 (pAp - NH4), HSQC
1'-H
3'-H
2'-H
4'-H
5'-H2
5'-C
2'-C
3'-C
4'-C
1'-C
NH2
OH
10
6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8
[ppm]
60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92
f1 (ppm)


Compound 10 (pAp - NH4), 1H - 31P - HMBC
2'-H
3'-H
4'-H
5'-H2
5'-P
3'-P
NH2
OH
10
f1 (ppm)
[ppm]

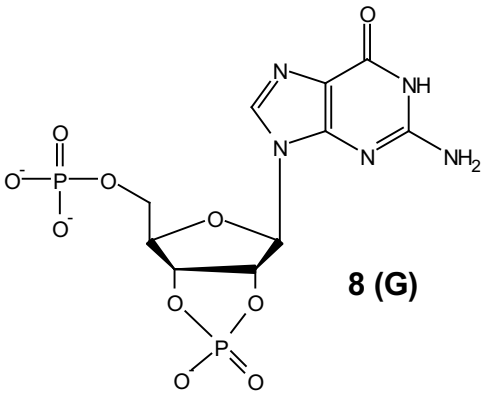
Compound 9 (pGp - TBA), ^{1}H - NMR (D_2O, 300 MHz)



1.93 x $^+N(^nBu)_4$

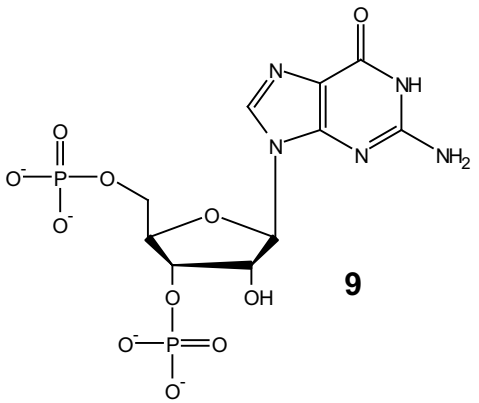
9

Reaction monitoring compound 9, $^{31}P[^{1}H]$ - NMR (D_2O, 121 MHz)



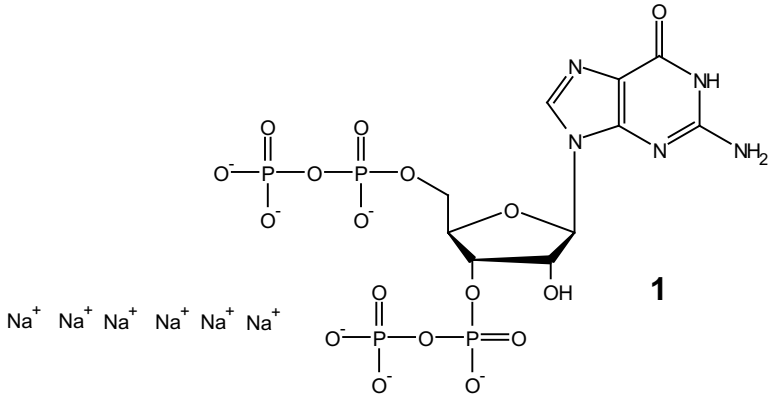
8 (G)

Rnase T2
37°C, 20 h



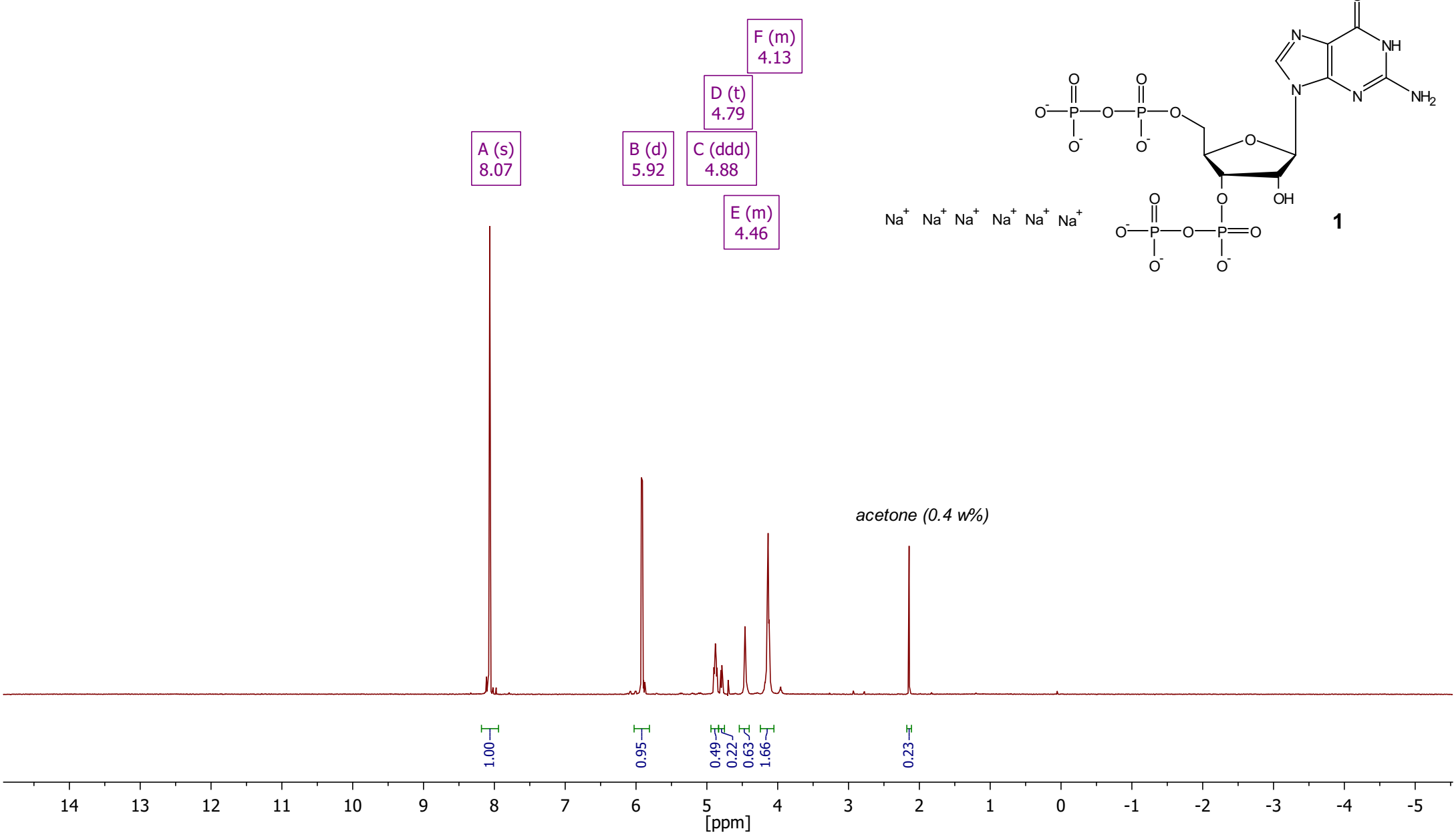
9

Compound 1 (ppGpp - Na), ^1H - NMR (D_2O, 400 MHz)

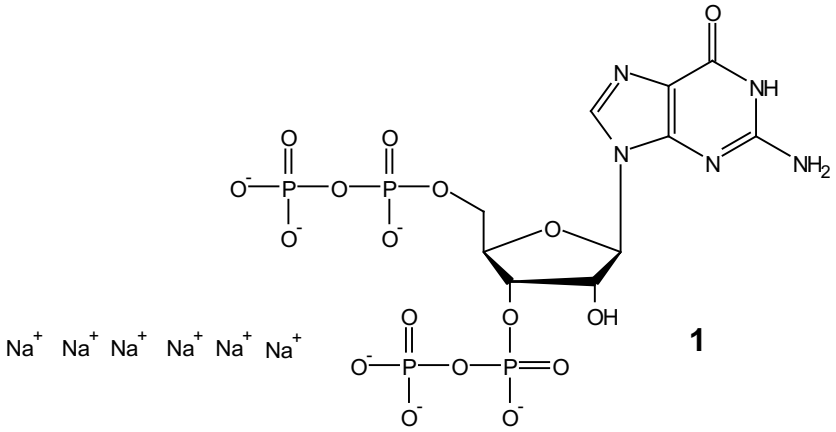


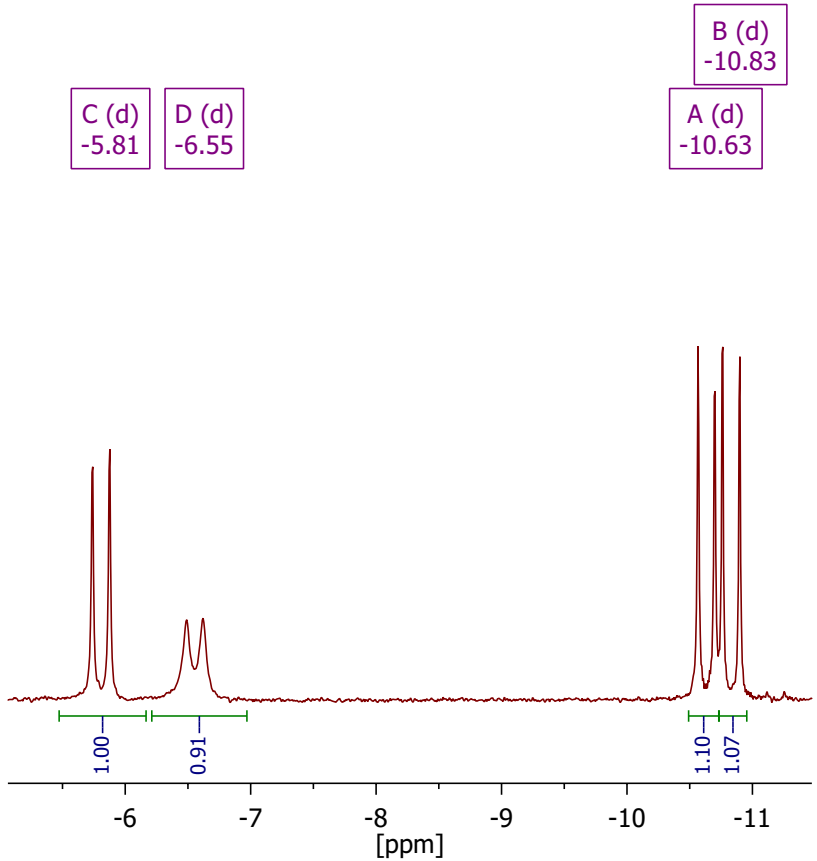
1

Compound 1 (ppGpp - Na), ^1H - NMR (D_2O, 400 MHz, water suppression)

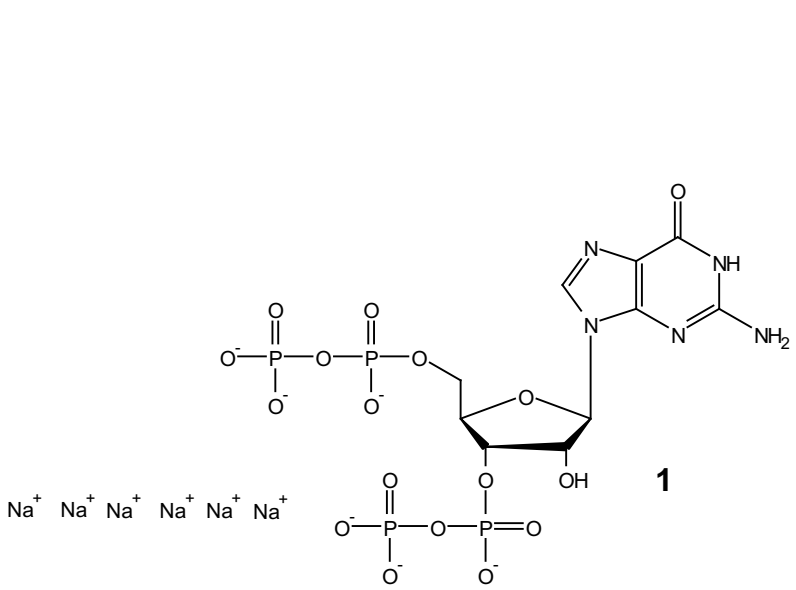


Compound 1 (ppGpp - Na), $^{31}P[^{1}H]$ - NMR (D_2O, 162 MHz)

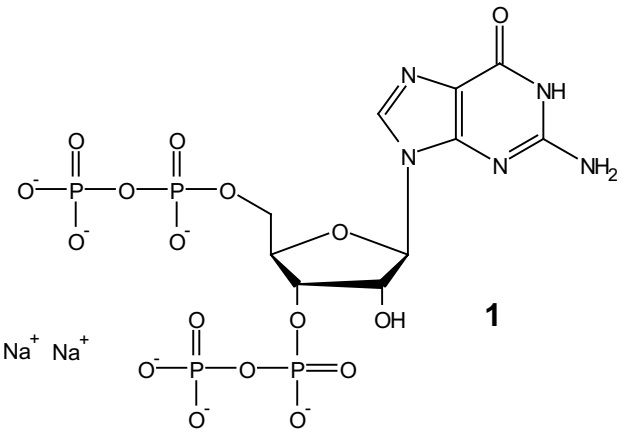


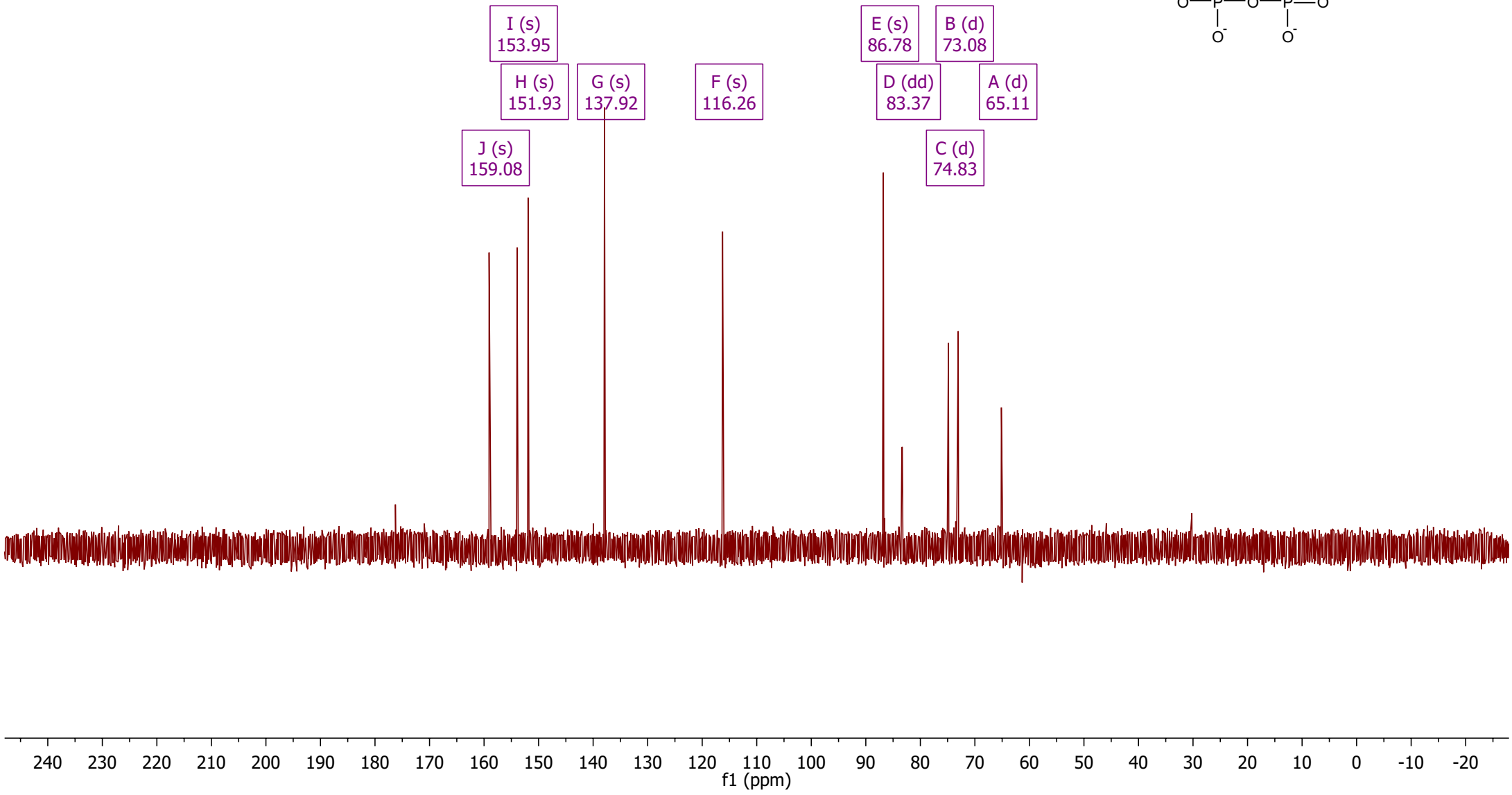


Compound 1 (ppGpp - Na), ^{31}P - NMR (D_2O, 162 MHz)

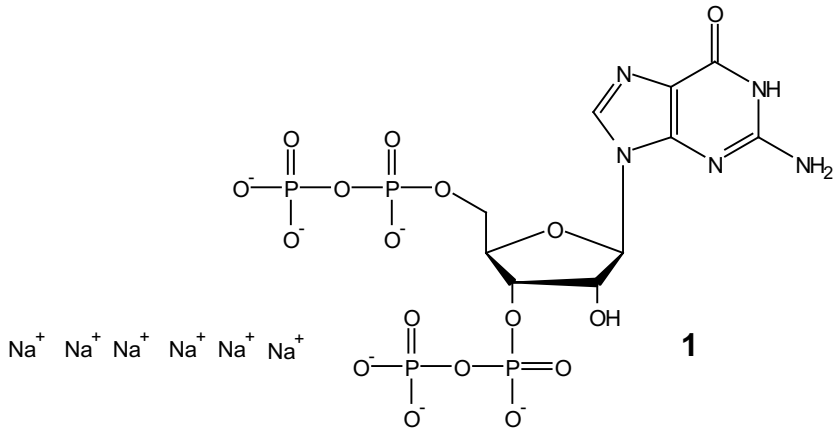


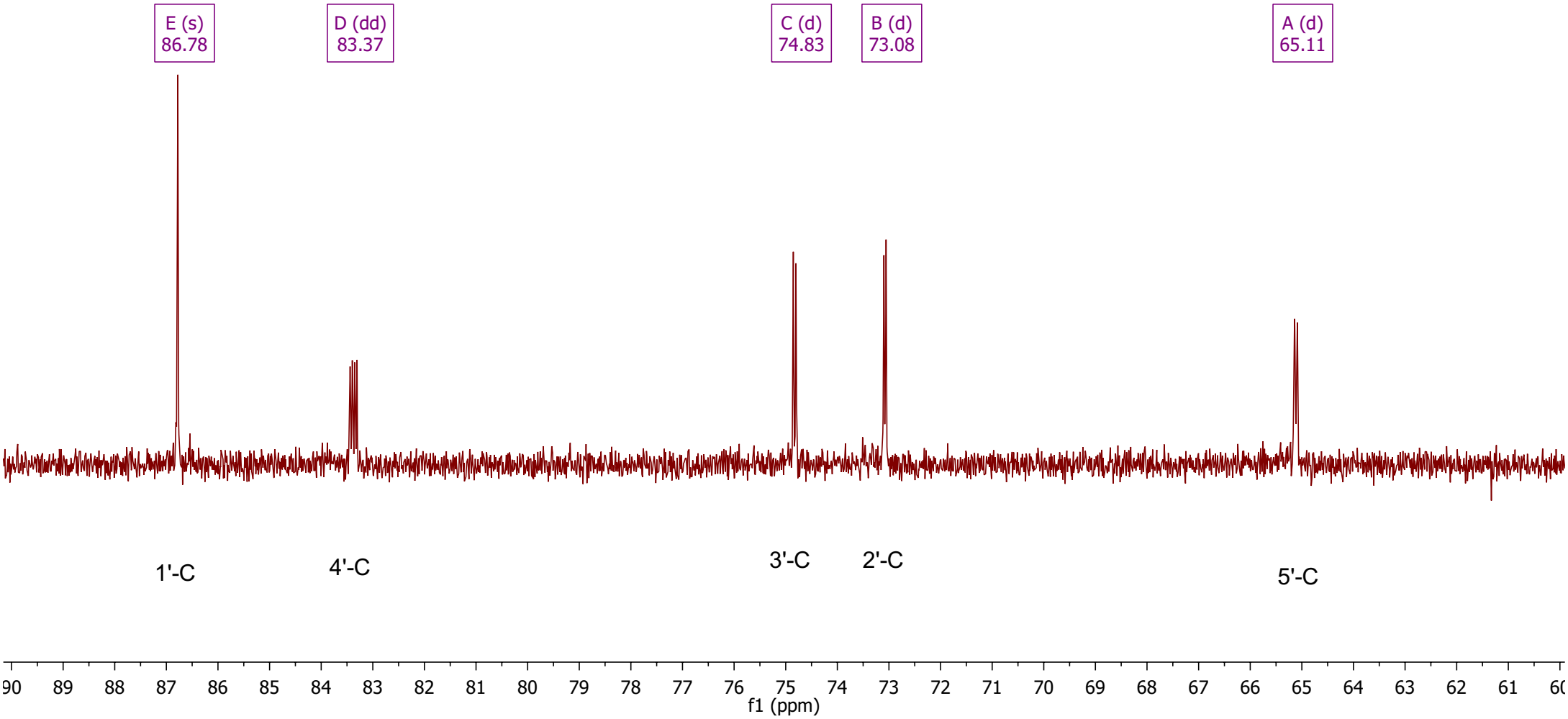
Compound 1 (ppGpp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz)

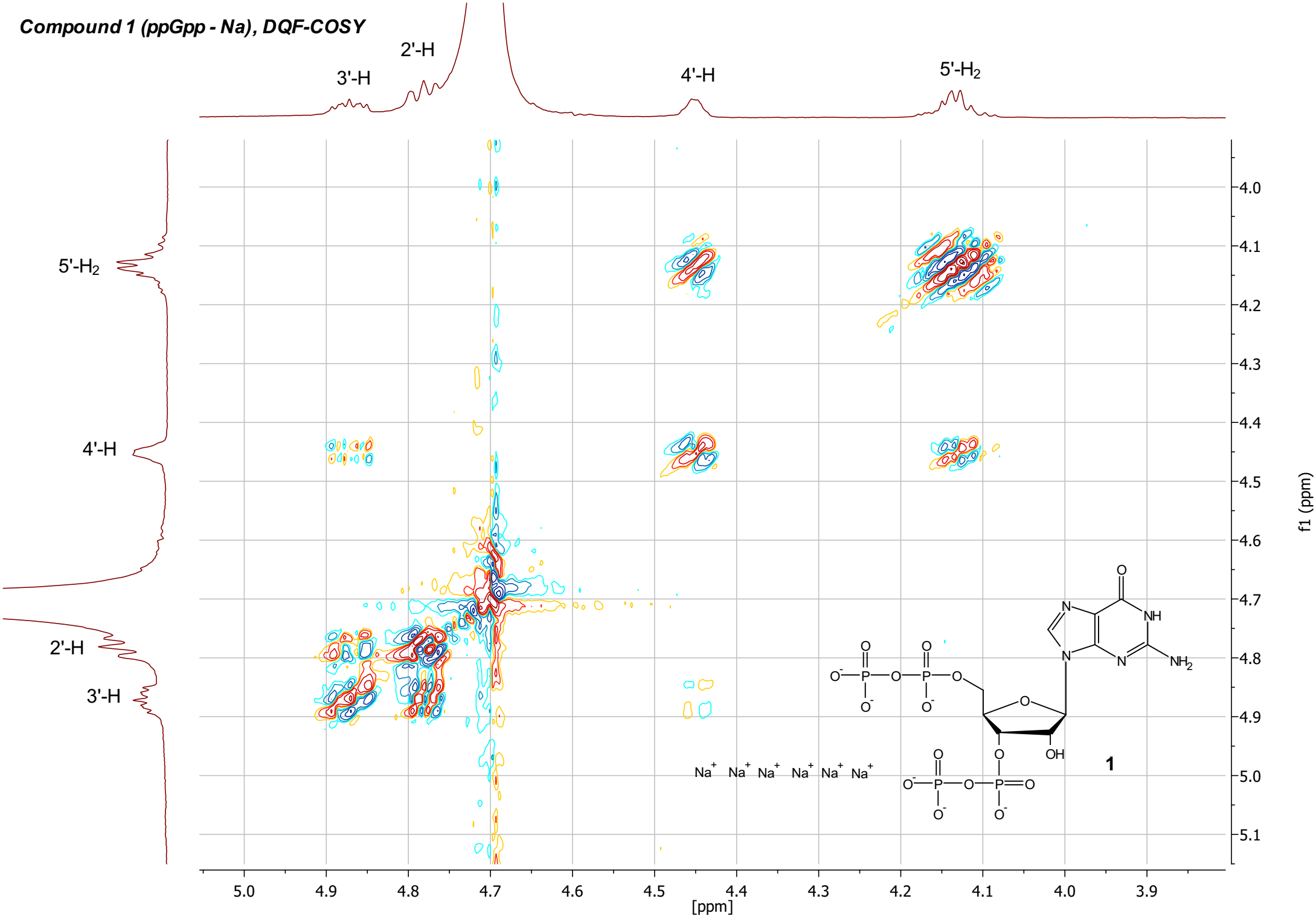


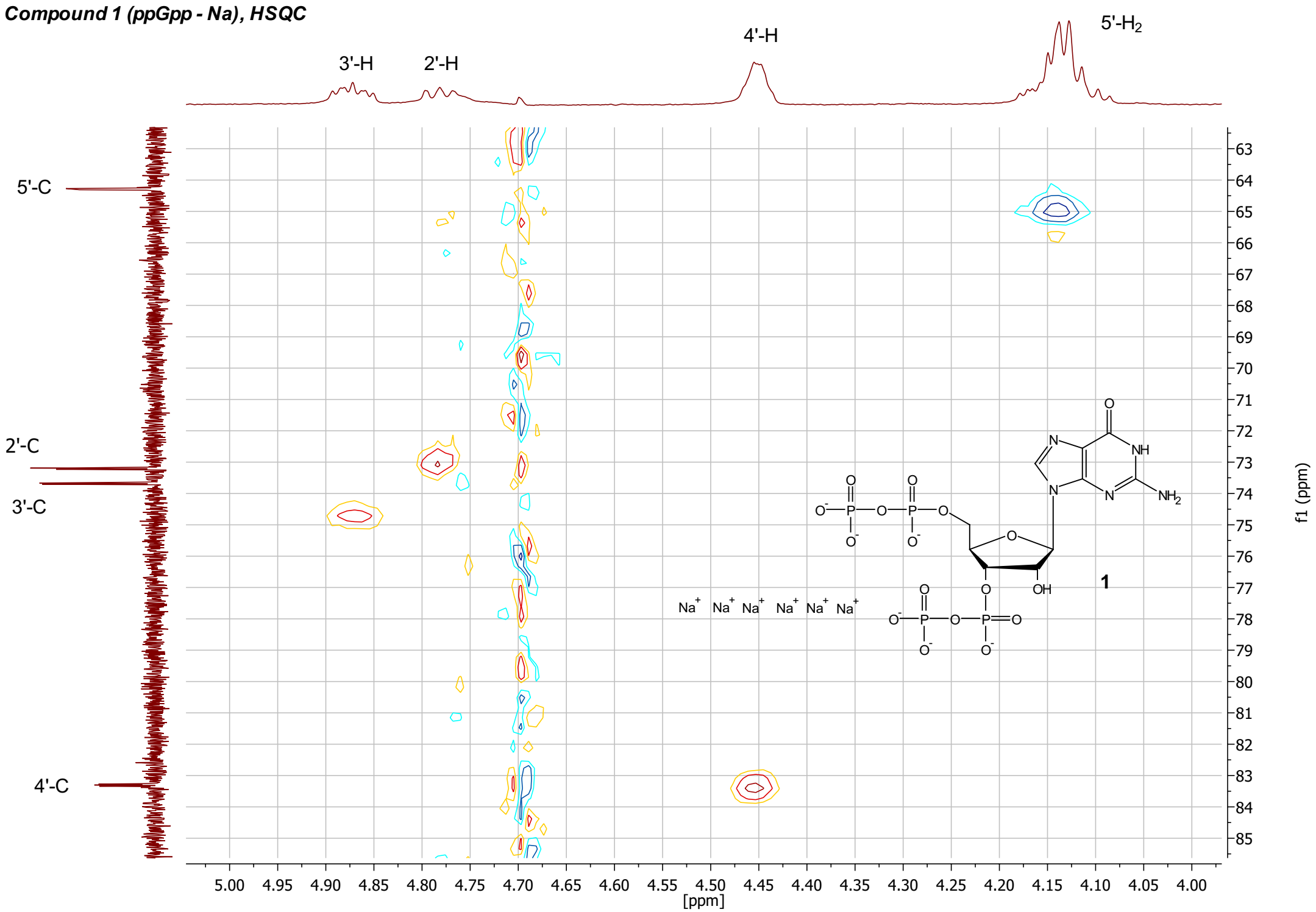


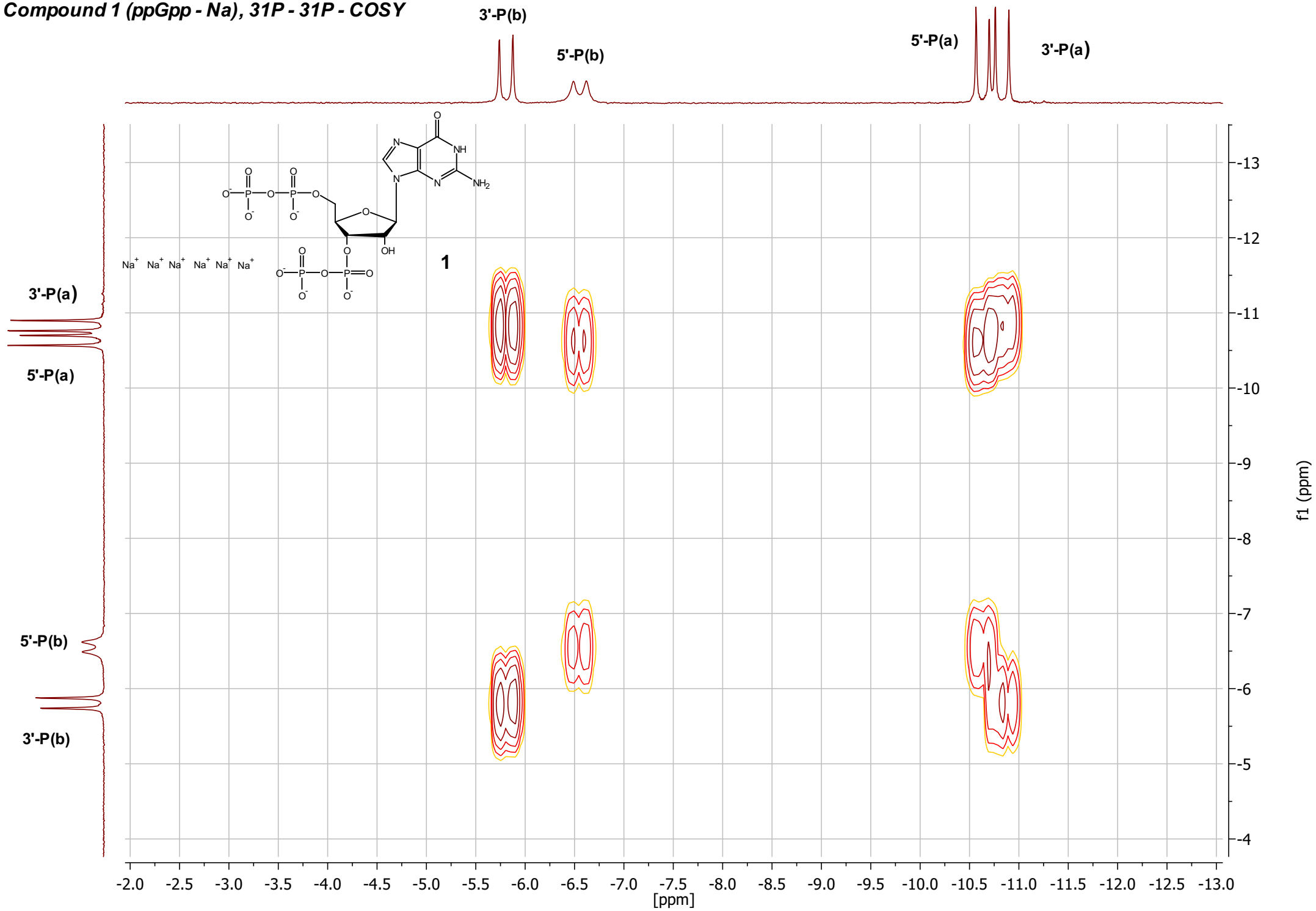
Compound 1 (ppGpp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz), detailed

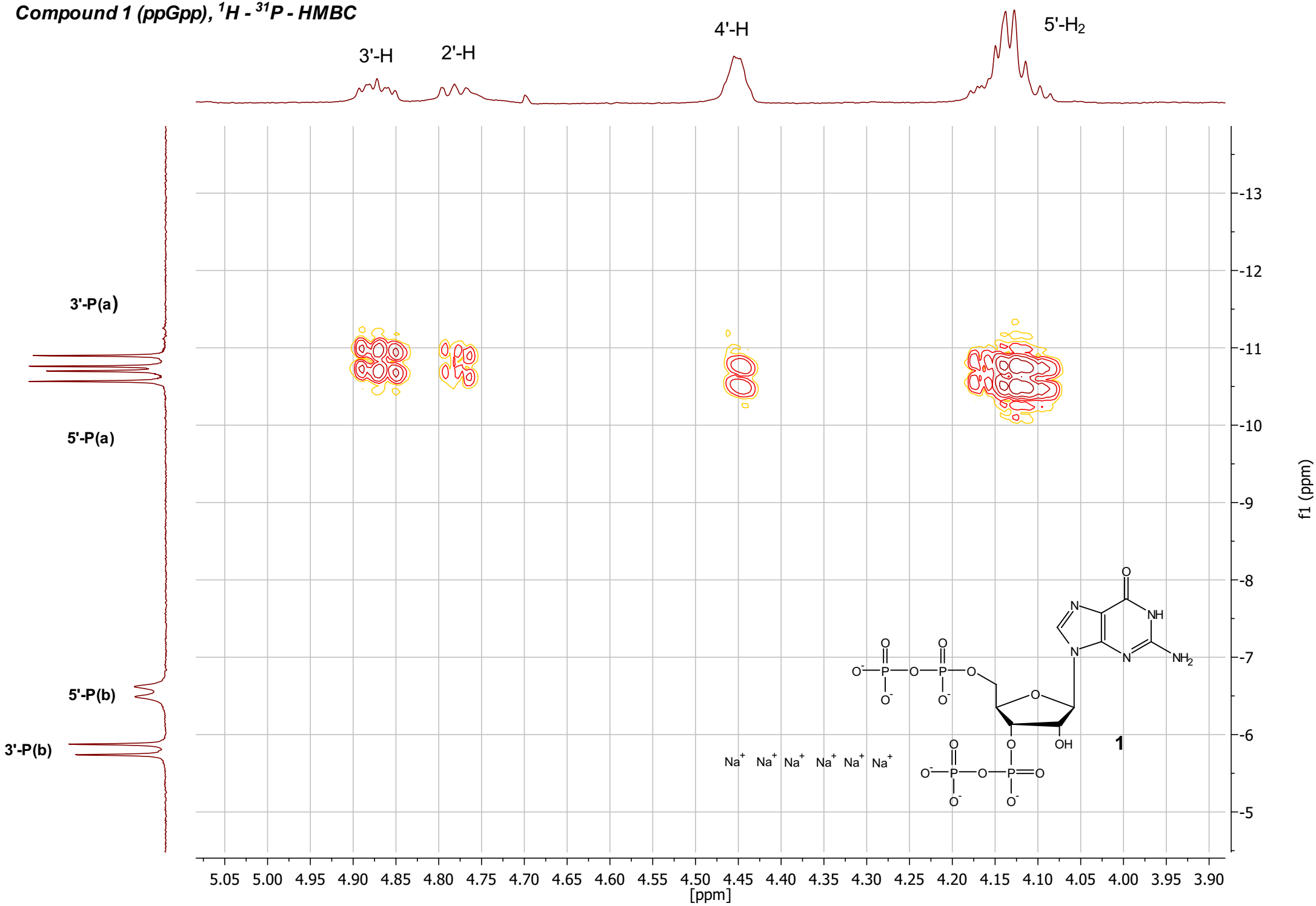


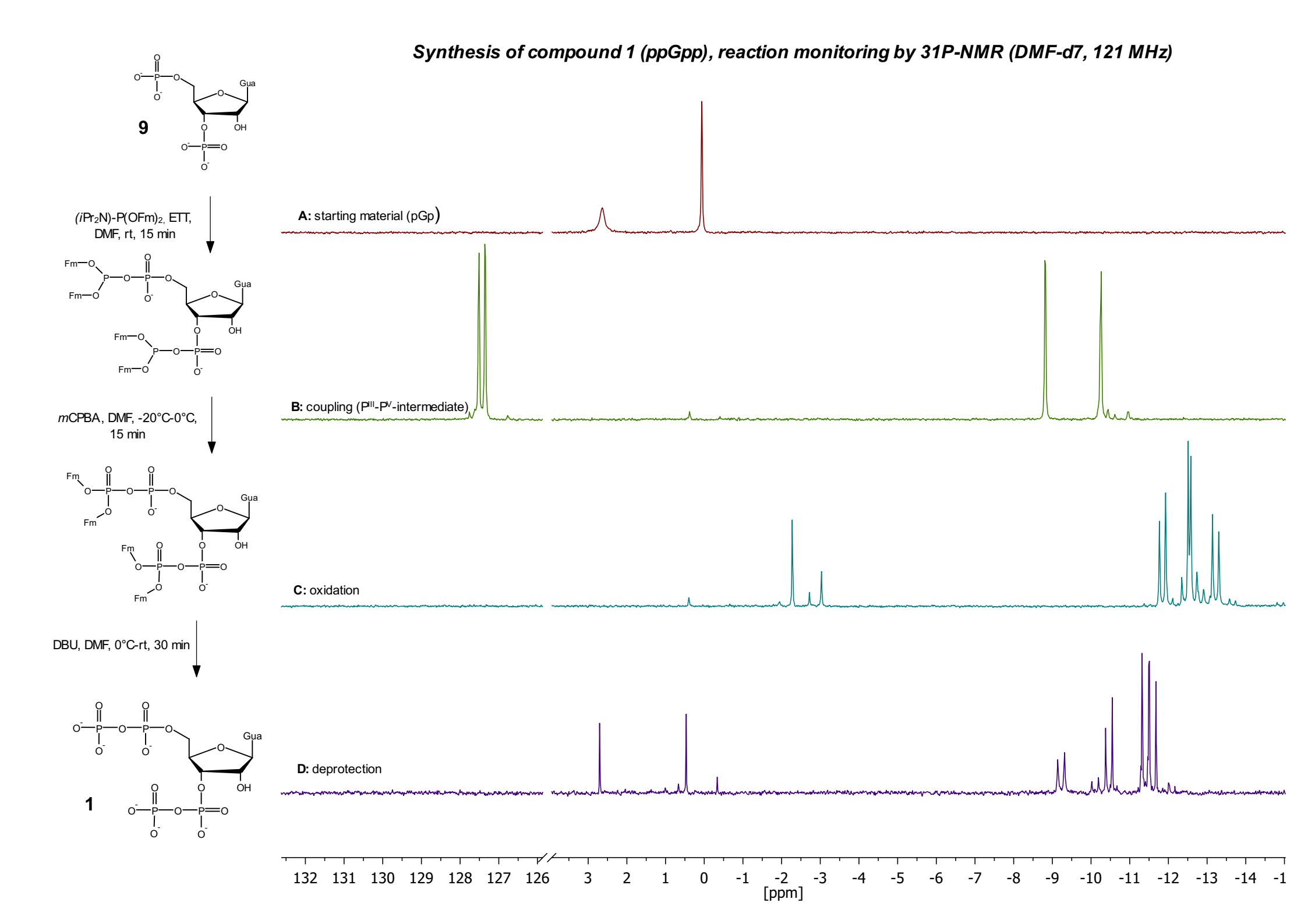



Compound 1 (ppGpp - Na), DQF-COSY
3'-H
2'-H
4'-H
5'-H2
5'-H2
4'-H
2'-H
3'-H
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
5.0
5.1
f1 (ppm)
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
4.0
3.9
[ppm]
Na+ Na+ Na+ Na+ Na+ Na+
1

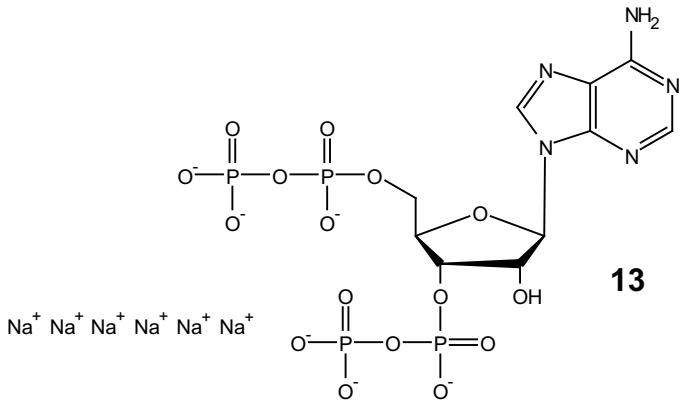

Compound 1 (ppGpp - Na), HSQC
3'-H
2'-H
4'-H
5'-H2
5'-C
2'-C
3'-C
4'-C
Na+ Na+ Na+ Na+ Na+ Na+
1
[ppm]
f1 (ppm)


Compound 1 (ppGpp - Na), 31P - 31P - COSY
3'-P(b)
5'-P(b)
5'-P(a)
3'-P(a)
1
Na+ Na+ Na+ Na+ Na+ Na+
3'-P(a)
5'-P(a)
5'-P(b)
3'-P(b)
-2.0 -2.5 -3.0 -3.5 -4.0 -4.5 -5.0 -5.5 -6.0 -6.5 -7.0 -7.5 -8.0 -8.5 -9.0 -9.5 -10.0 -10.5 -11.0 -11.5 -12.0 -12.5 -13.0
[ppm]
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
f1 (ppm)


Compound 1 (ppGpp), ^{1}H - ^{31}P - HMBC
3'-H
2'-H
4'-H
5'-H2
3'-P(a)
5'-P(a)
5'-P(b)
3'-P(b)
f1 (ppm)
[ppm]
1


Synthesis of compound 1 (ppGpp), reaction monitoring by 31P-NMR (DMF-d7, 121 MHz)
9
(iPr2N)-P(OFm)2, ETT,
DMF, rt, 15 min
mCPBA, DMF, -20°C-0°C,
15 min
DBU, DMF, 0°C-rt, 30 min
1
A: starting material (pGp)
B: coupling (PIII-PV-intermediate)
C: oxidation
D: deprotection
132 131 130 129 128 127 126 3 2 1 0 -1 -2 -3 -4 -5 -6 -7 -8 -9 -10 -11 -12 -13 -14 -1
[ppm]

Compound 13 (ppApp - Na), ^{1}H - NMR (D_2O, 400 MHz)



13

B (s) 8.19

A (s) 8.48

C (d) 6.12

G (ddd) 4.90

E (m) 4.53

D (m) 4.15

F (ddd) 4.79

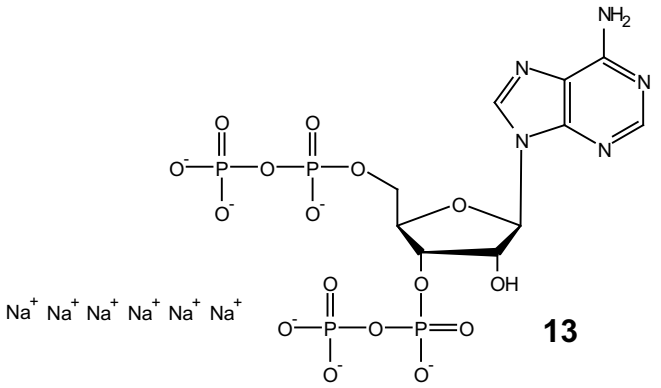
1.4 % acetone

1.00 0.89 0.98 0.88 0.85 0.93 1.93 1.24

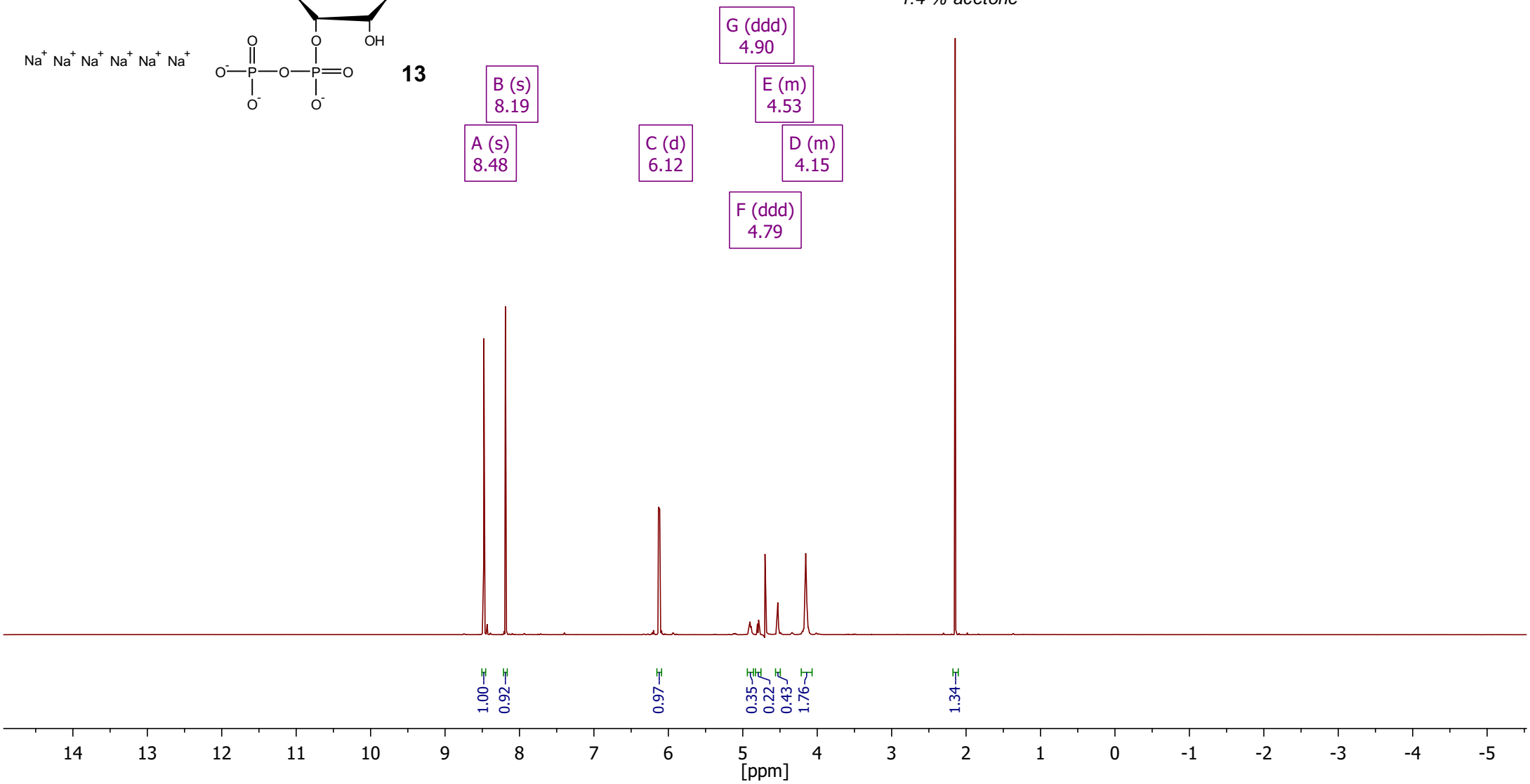
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3 -4

[ppm]

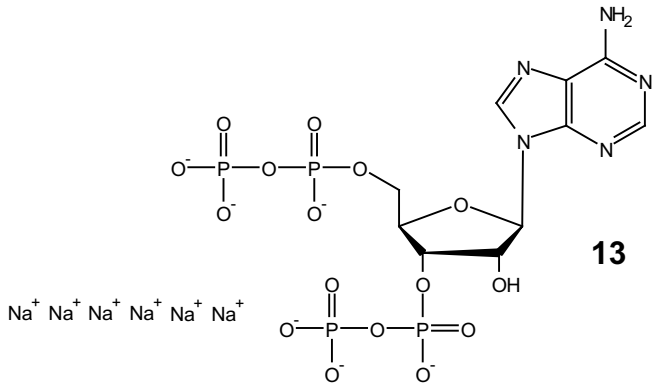
Compound 13 (ppApp - Na), ^{1}H - NMR (D_2O, 400 MHz, water suppression)



13



Compound 13 (ppApp - Na), $^{31}P[^{1}H]$ - NMR (D_2O, 162 MHz)



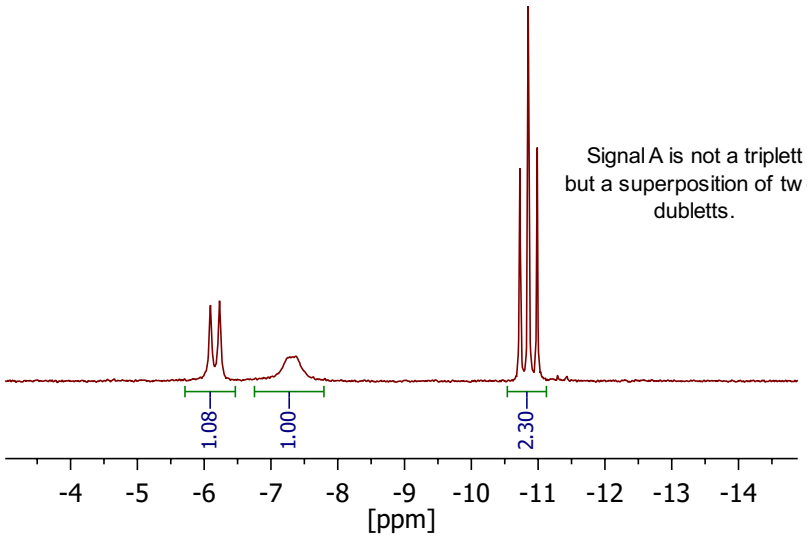
13

C (br. s)
-7.32

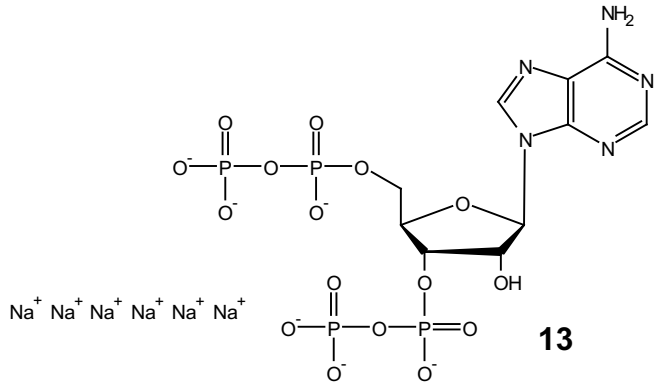
B (d)
-6.16

A (t)
-10.85

Signal A is not a triplett but a superposition of two dubletts.



Compound 13 (ppApp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz), detailed



13

B (s) 149.32

A (s) 152.88

C (s) 140.05

E (s) 118.65

I (d) 73.72

F (s) 86.59

H (d) 74.86

D (s) 155.66

G (ddd) 83.62

J (d) 64.97

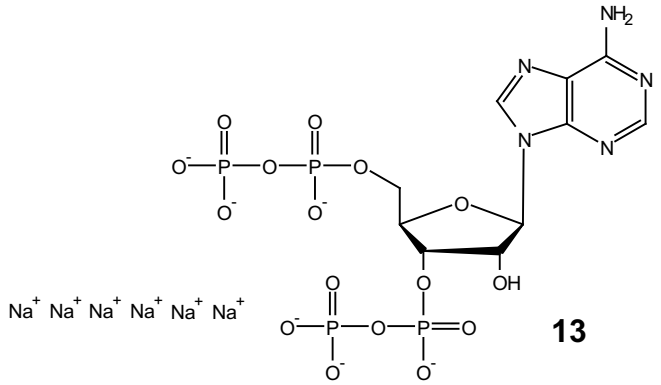
acetone

acetone

240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20

[ppm]

Compound 13 (ppApp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz), detailed



13

F (s) 86.59

G (ddd) 83.62

H (d) 74.86

I (d) 73.72

J (d) 64.97

1'-C

4'-C

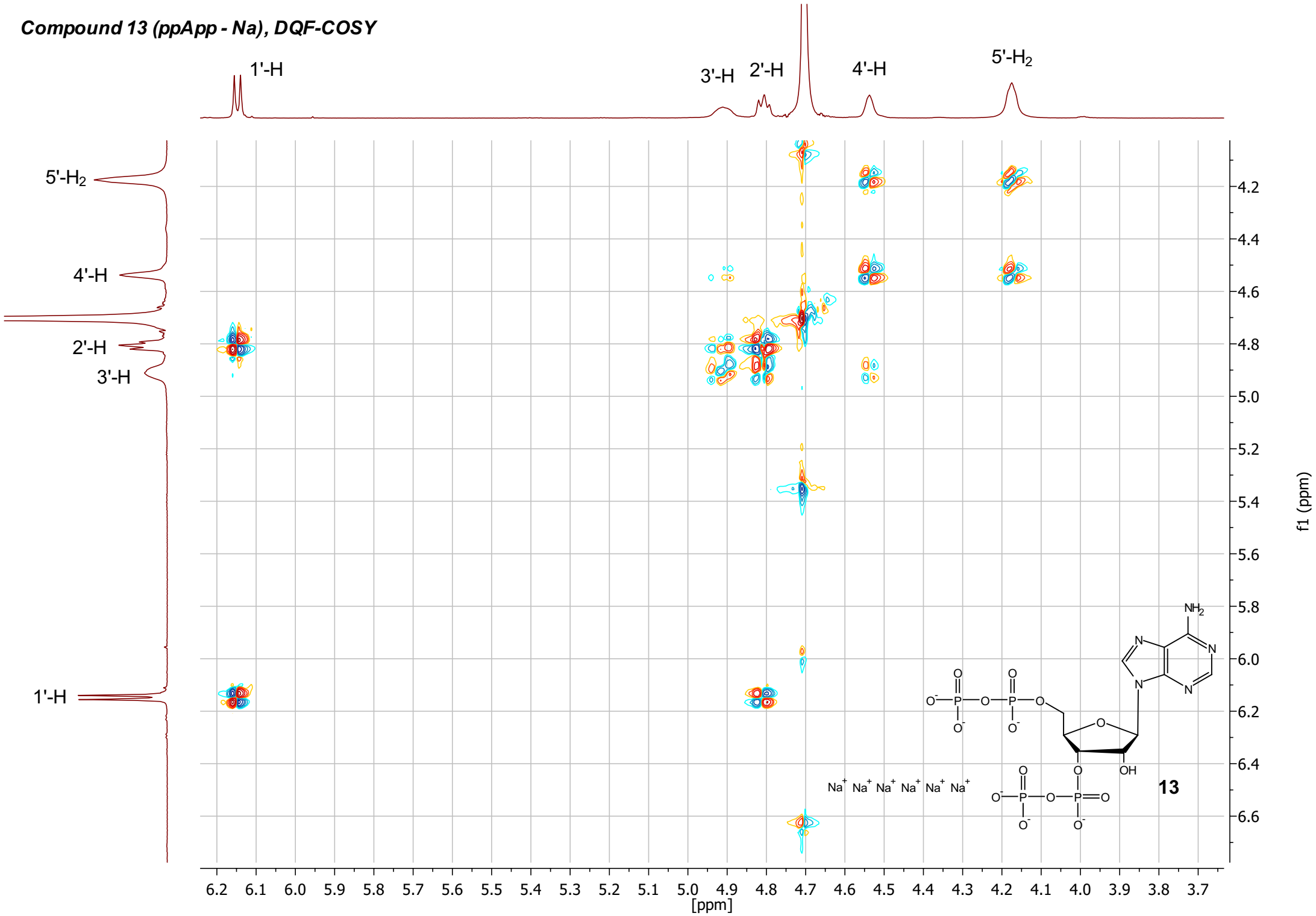
3'-C

2'-C

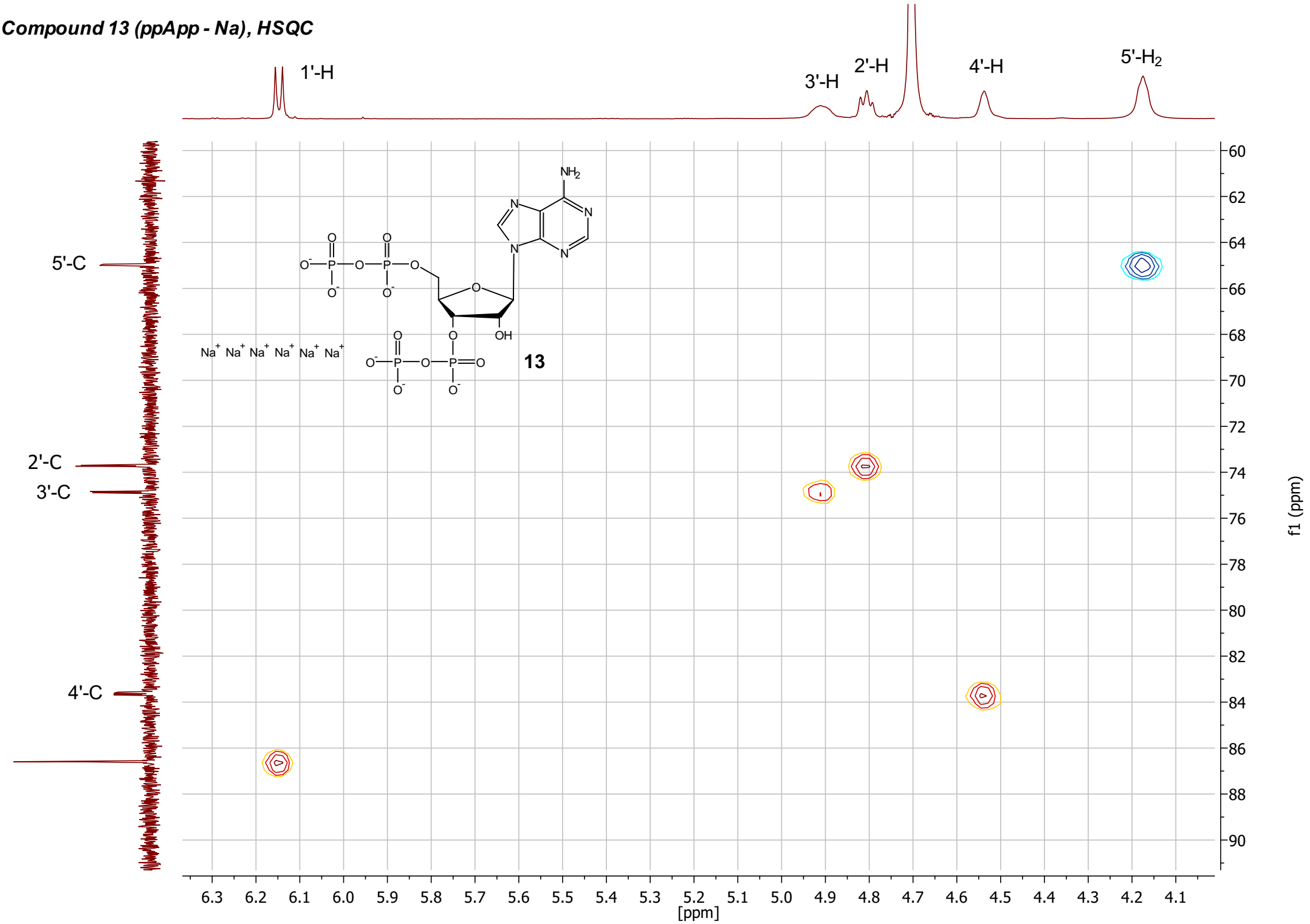
5'-C

89 88 87 86 85 84 83 82 81 80 79 78 77 76 75 74 73 72 71 70 69 68 67 66 65 64 63

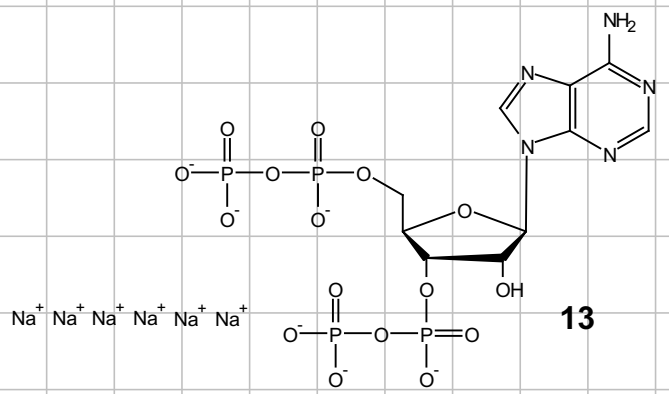
[ppm]


Compound 13 (ppApp - Na), DQF-COSY
1'-H
3'-H
2'-H
4'-H
5'-H2
5'-H2
4'-H
2'-H
3'-H
1'-H
6.2 6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7
[ppm]
4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0 6.2 6.4 6.6
f1 (ppm)
Na+ Na+ Na+ Na+ Na+ Na+
13

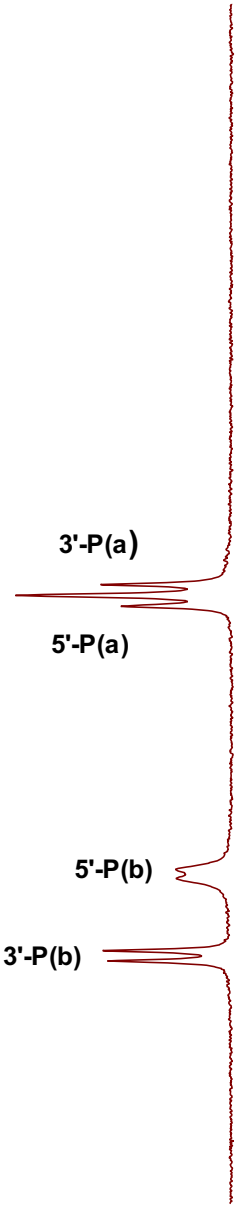
Compound 13 (ppApp - Na), HSQC

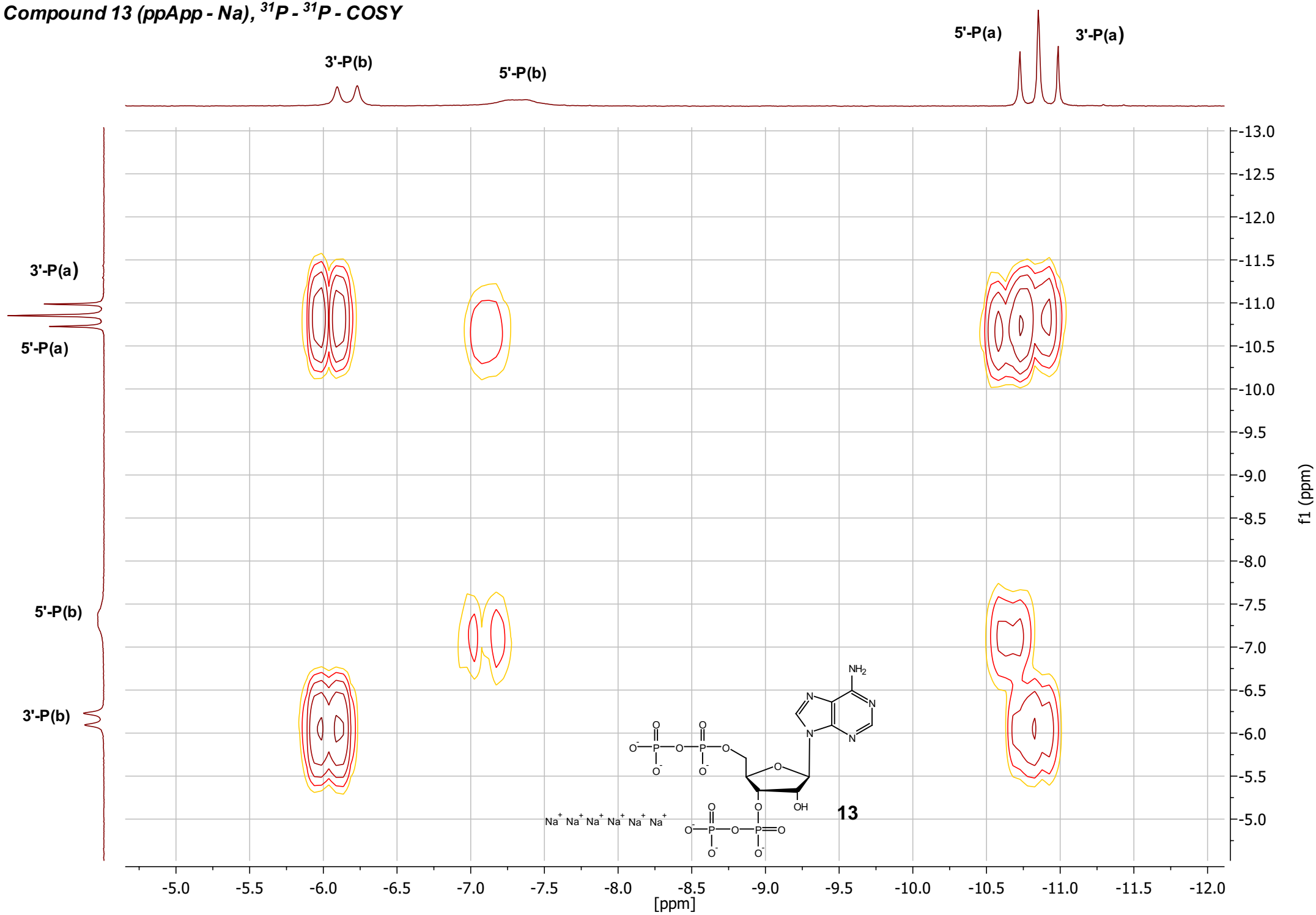


Compound 13 (ppApp - Na), ^{1}H - ^{31}P - HMBC

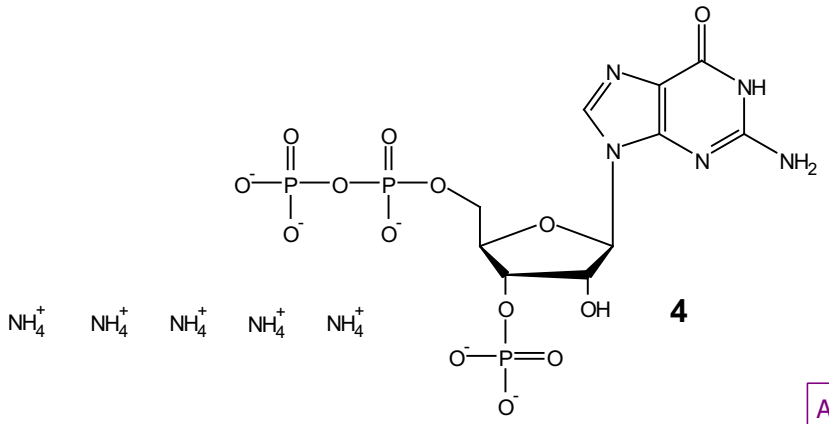


13




Compound 13 (ppApp - Na), 31P - 31P - COSY
3'-P(b)
5'-P(b)
5'-P(a)
3'-P(a)
3'-P(a)
5'-P(a)
5'-P(b)
3'-P(b)
13
[ppm]
f1 (ppm)

Compound 4 (ppGp - NH_4), ^1H - NMR (D_2O, 400 MHz)



NH_4^+ NH_4^+ NH_4^+ NH_4^+ NH_4^+

4

A (s) 8.07

B (d) 5.91

C (m) 4.80

D (m) 4.48

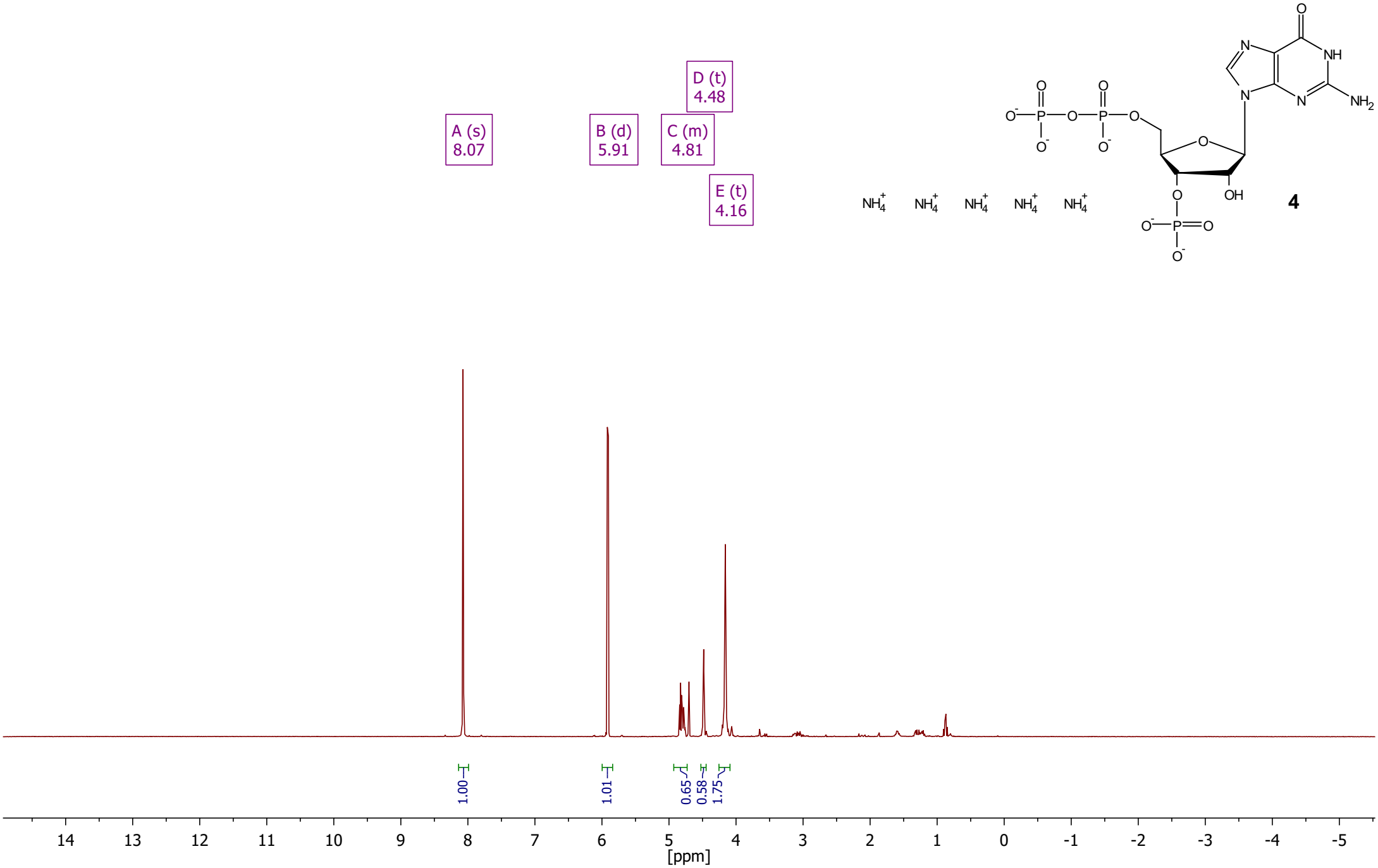
E (m) 4.16

1.00 0.96 2.46 1.03 1.82

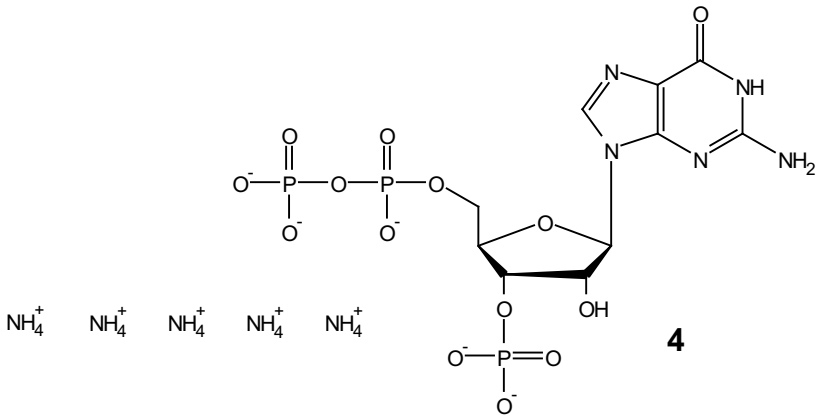
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3 -4

[ppm]

Compound 4 (ppGp - NH_4), ^1H - NMR (D_2O, 400 MHz, water suppression)

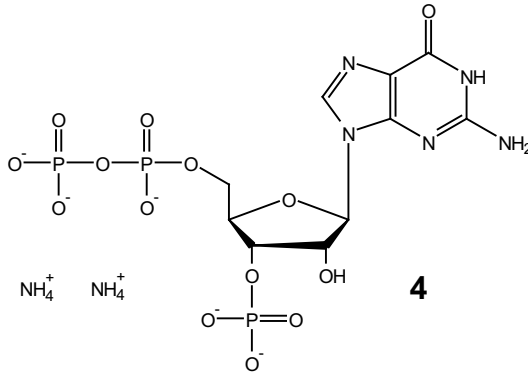


Compound 4 (ppGp - NH_4), ^{31}P-[^1H] - NMR (D_2O, 162 MHz)



4

Compound 4 (pGpp - NH_4), $^{13}C[^1H]$ - NMR (D_2O, 101 MHz)



I (s)
176.26

F (s)
159.04

E (s)
153.95

G (s)
137.80

H (s)
116.27

J (s)
86.48

B (d)
73.09

D (dd)
83.46

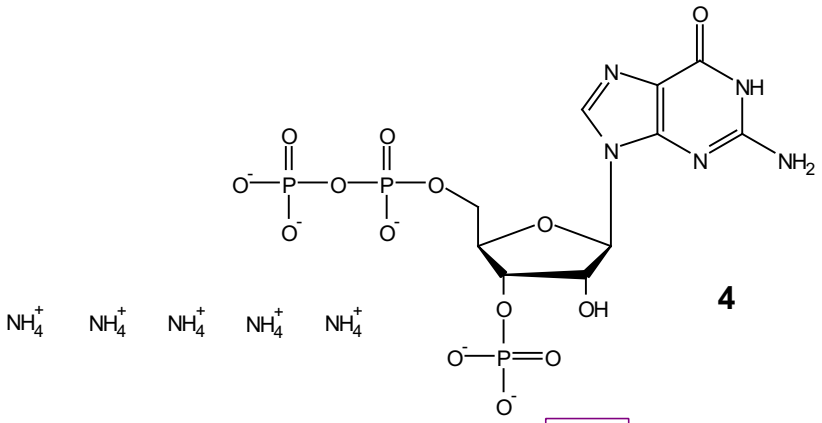
A (d)
65.14

C (d)
73.72

240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20

f1 (ppm)

Compound 4 (pGpp - NH_4), $^{13}C[^1H]$ - NMR (D_2O, 101 MHz), detailed



J (s)
86.48

D (dd)
83.46

C (d)
73.72

B (d)
73.09

A (d)
65.14

1'-C

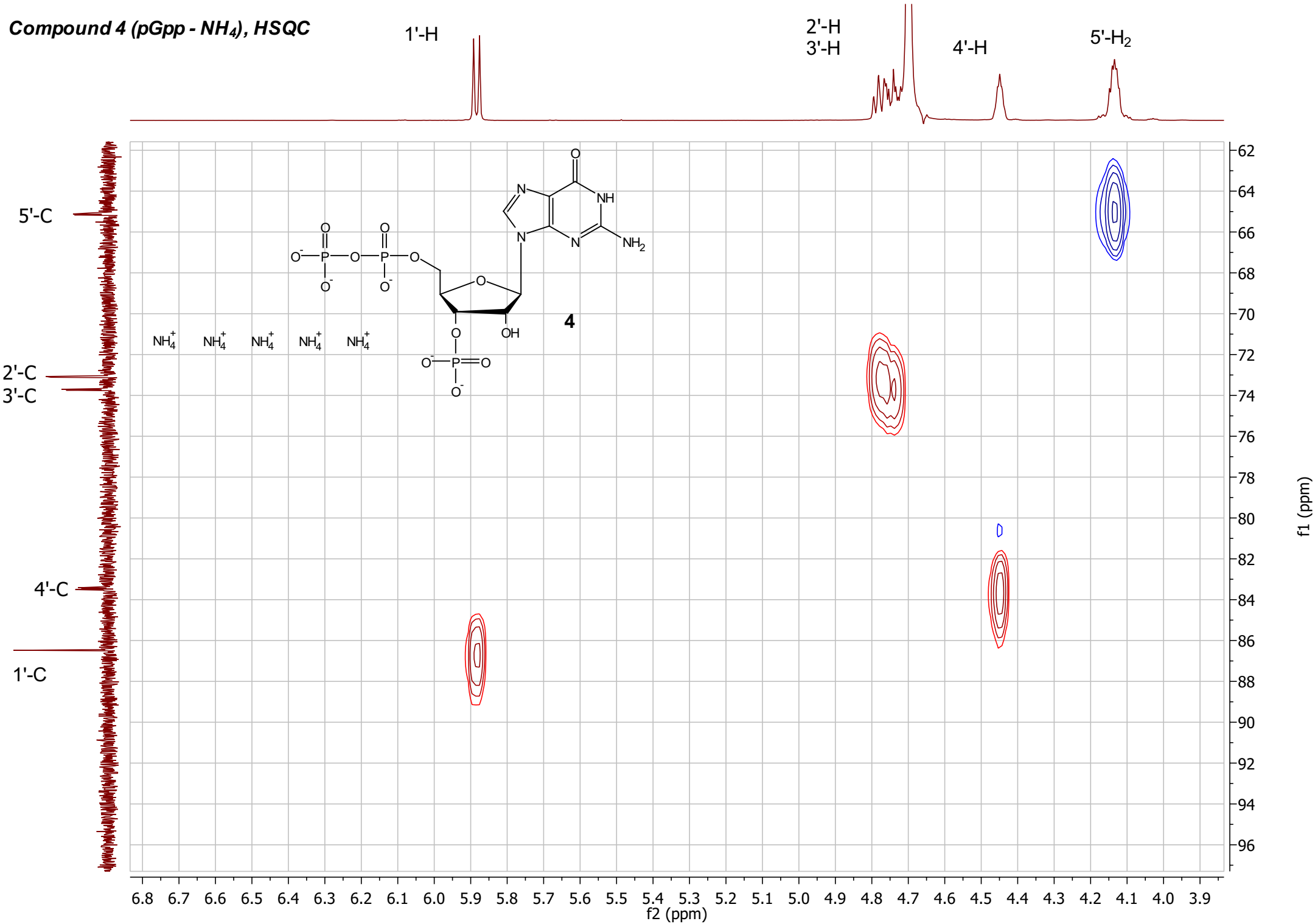
4'-C

2'-C
3'-C

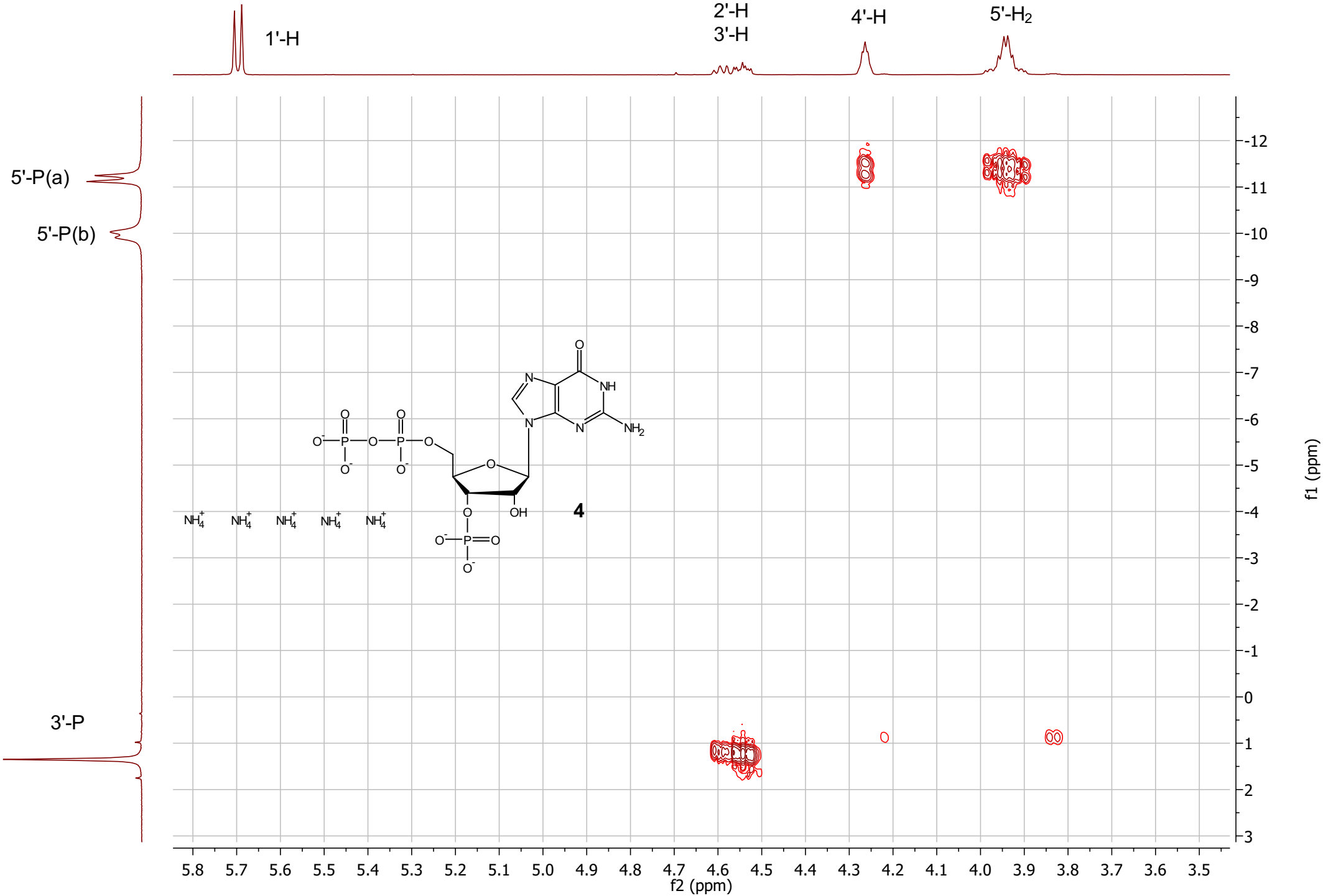
5'-C

88 87 86 85 84 83 82 81 80 79 78 77 76 75 74 73 72 71 70 69 68 67 66 65 64 63

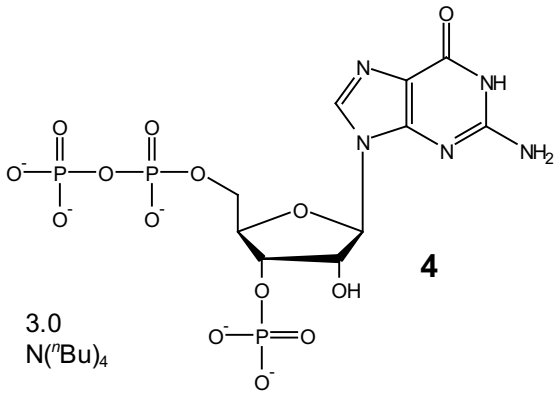
f1 (ppm)


Compound 4 (pGpp - NH4), HSQC
1'-H
2'-H
3'-H
4'-H
5'-H2
5'-C
2'-C
3'-C
4'-C
1'-C
4
NH4+ NH4+ NH4+ NH4+ NH4+
f2 (ppm)
f1 (ppm)

Compound 4 (pGpp - NH_4), 31P - ^{1}H - HMBC (D_2O, water suppression)



Compound 4 (ppGp - TBA), ^{1}H - NMR (D_2O, 300 MHz)



4

3.0
$N(^nBu)_4$

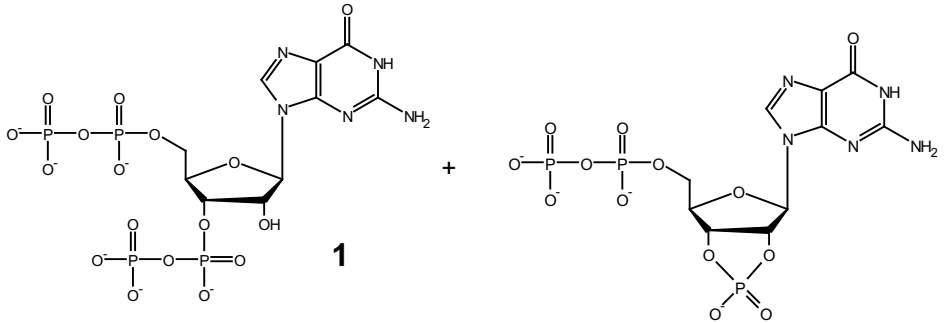
A (s) 8.07
B (d) 5.91
C (m) 4.78
D (m) 4.49
E (m) 4.16
F (m) 3.13
G (m) 1.58
H (tq) 1.30
I (t) 0.88

1.00 0.95 2.72 1.04 2.00 24.10 24.14 24.57 36.14

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3
[ppm]

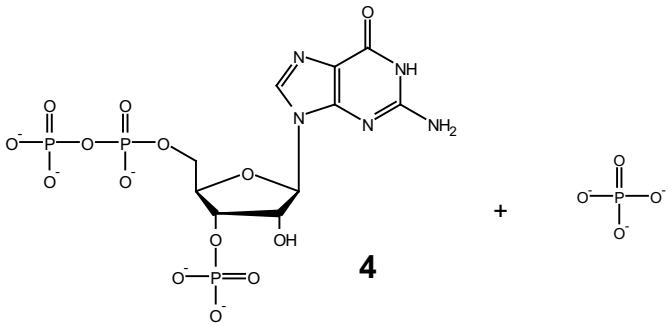
10000 9000 8000 7000 6000 5000 4000 3000 2000 1000 0

Synthesis of compound 4 (pGpp), reaction monitoring by ^{31}P-NMR (D_2O, 121 MHz)

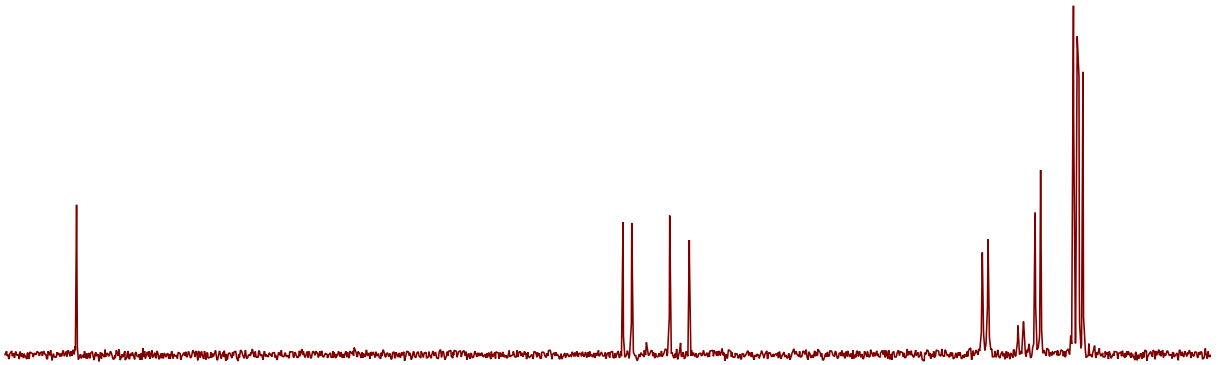


1 + **impurity**

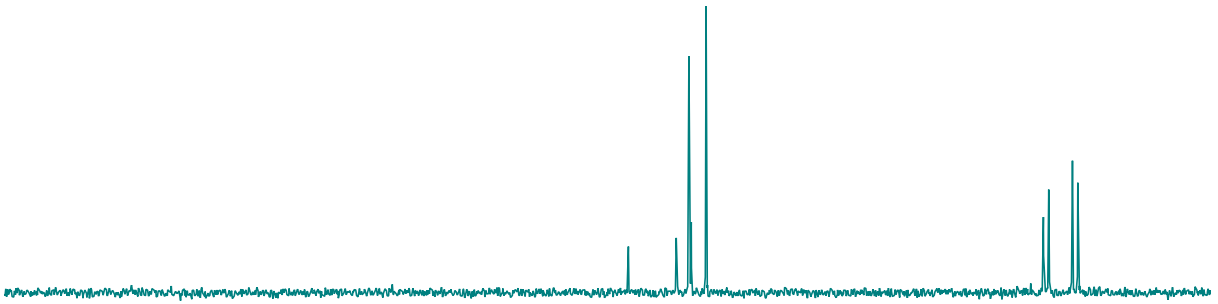
Rnase T2, pH 5.5, 37°C



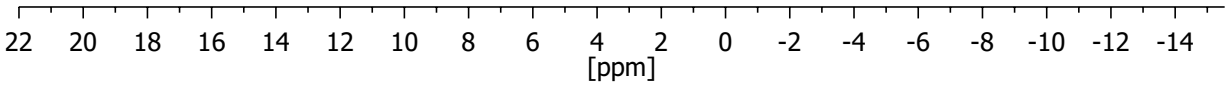
4 +



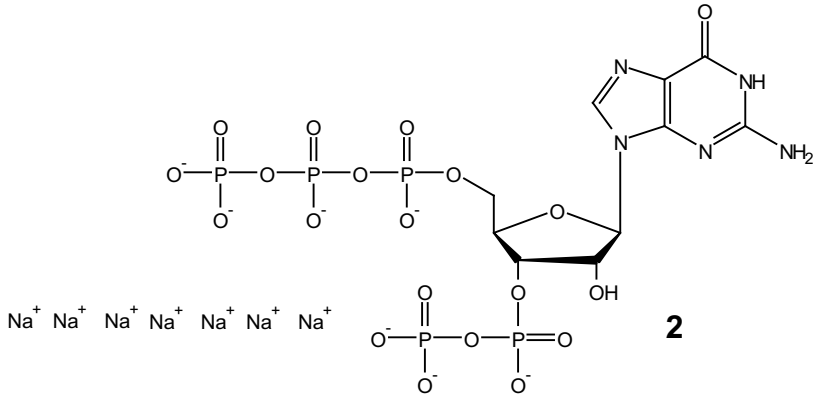
A: ppGpp crude product



B: ppGp crude product

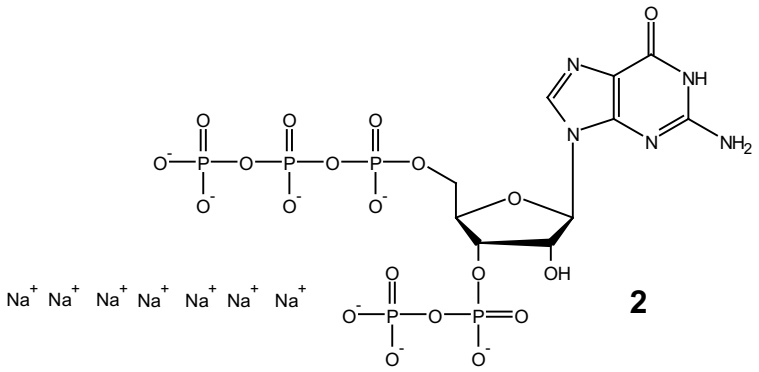


Compound 2 (pppGpp - Na), ^{1}H - NMR (D_2O, 400 MHz)

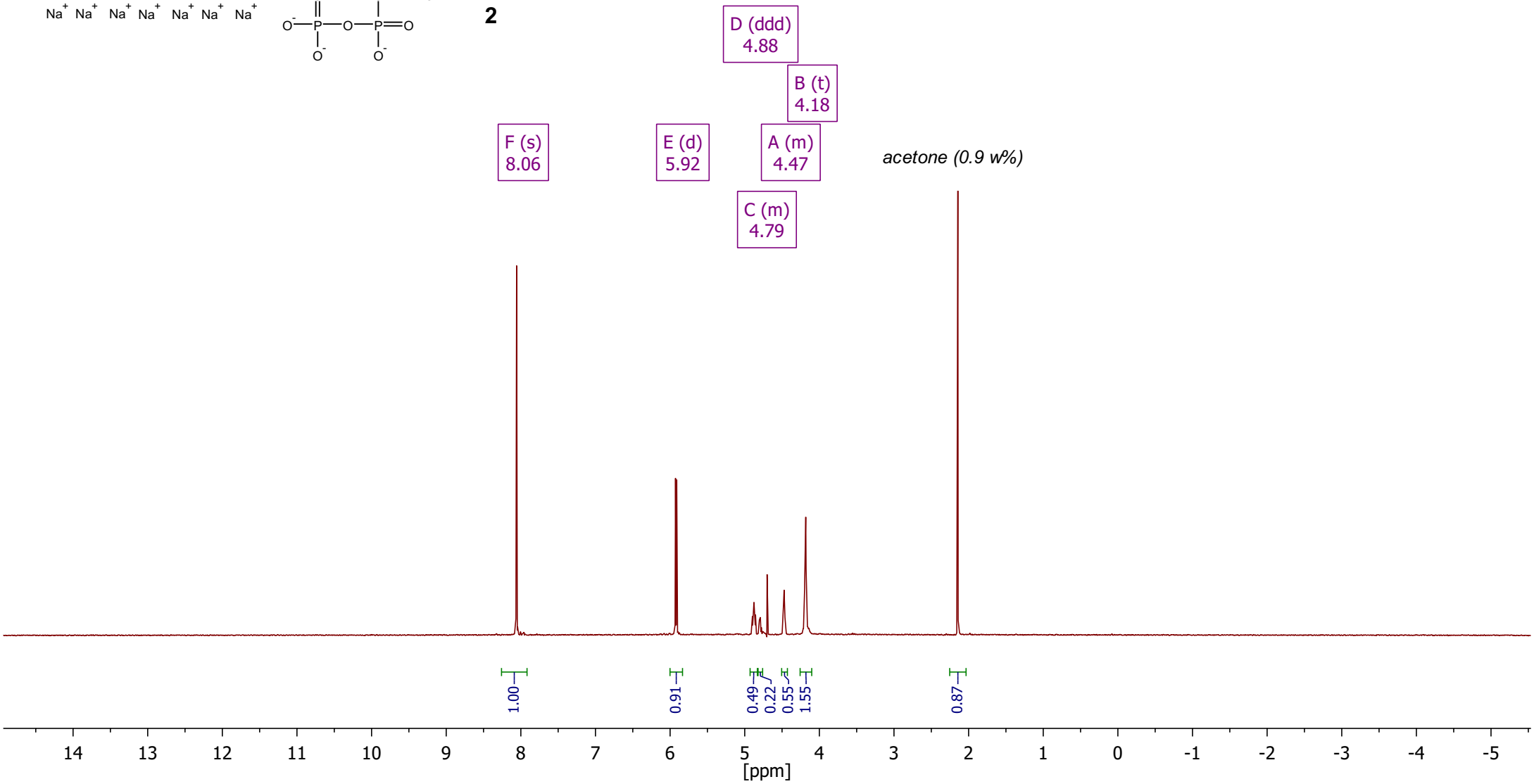


2

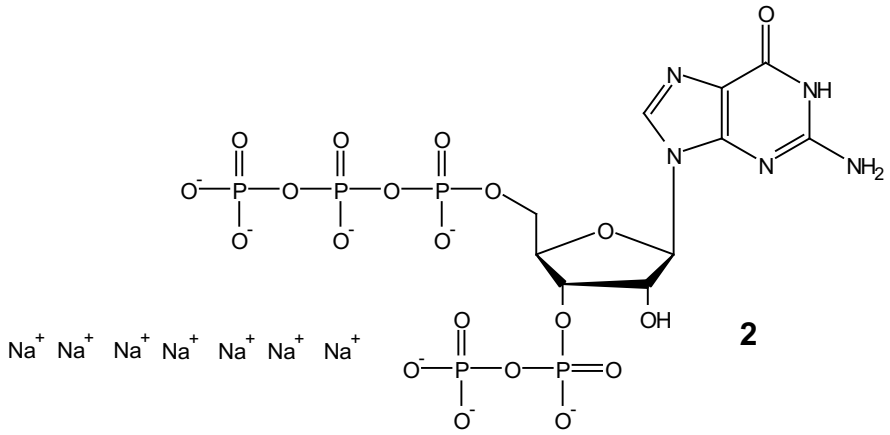
Compound 2 (pppGpp - Na), ^{1}H - NMR (D_2O, 400 MHz, water suppression)



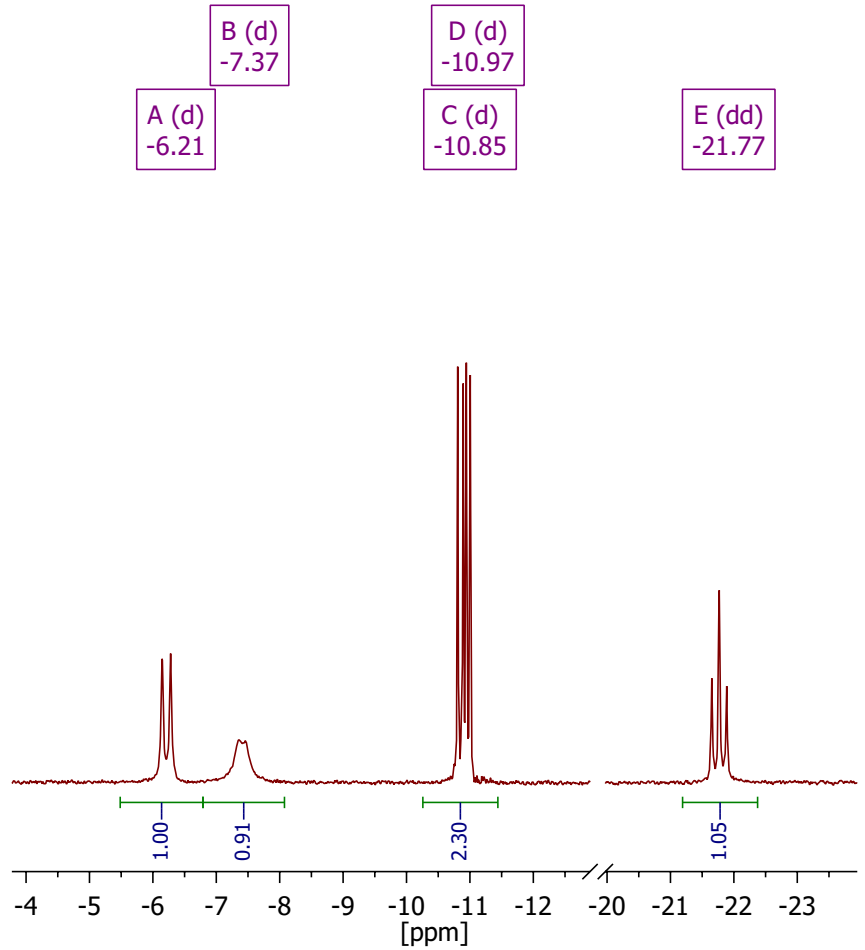
2



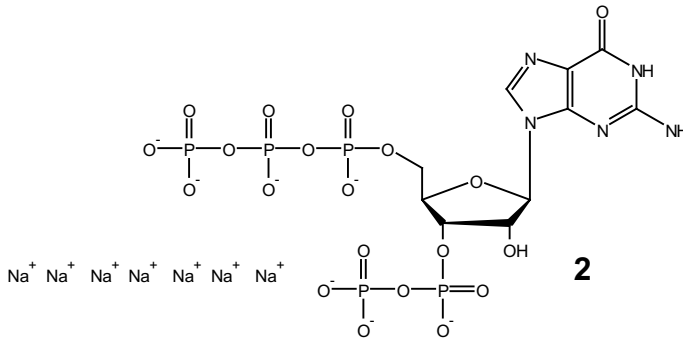
Compound 2 (pppGpp - Na), ^{31}P - [^{1}H] - NMR (D_2O, 162 MHz)



2



Compound 2 (pppGpp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz)



2

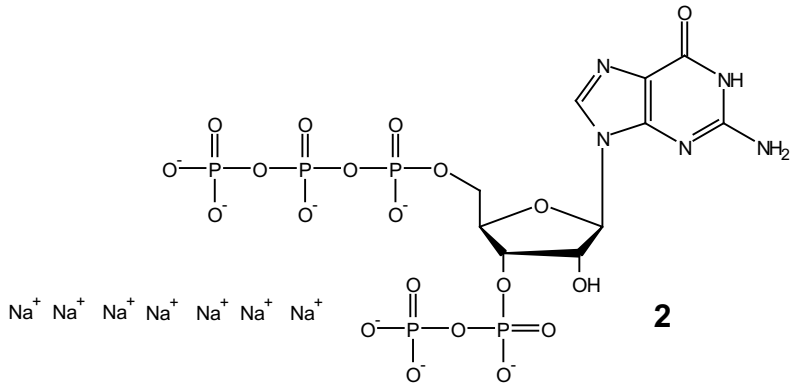
I (s) 153.97
H (s) 151.97
G (s) 138.03
F (s) 116.34
J (s) 159.14
E (s) 86.88
B (d) 73.08
D (dd) 83.34
A (d) 65.53
C (d) 74.87

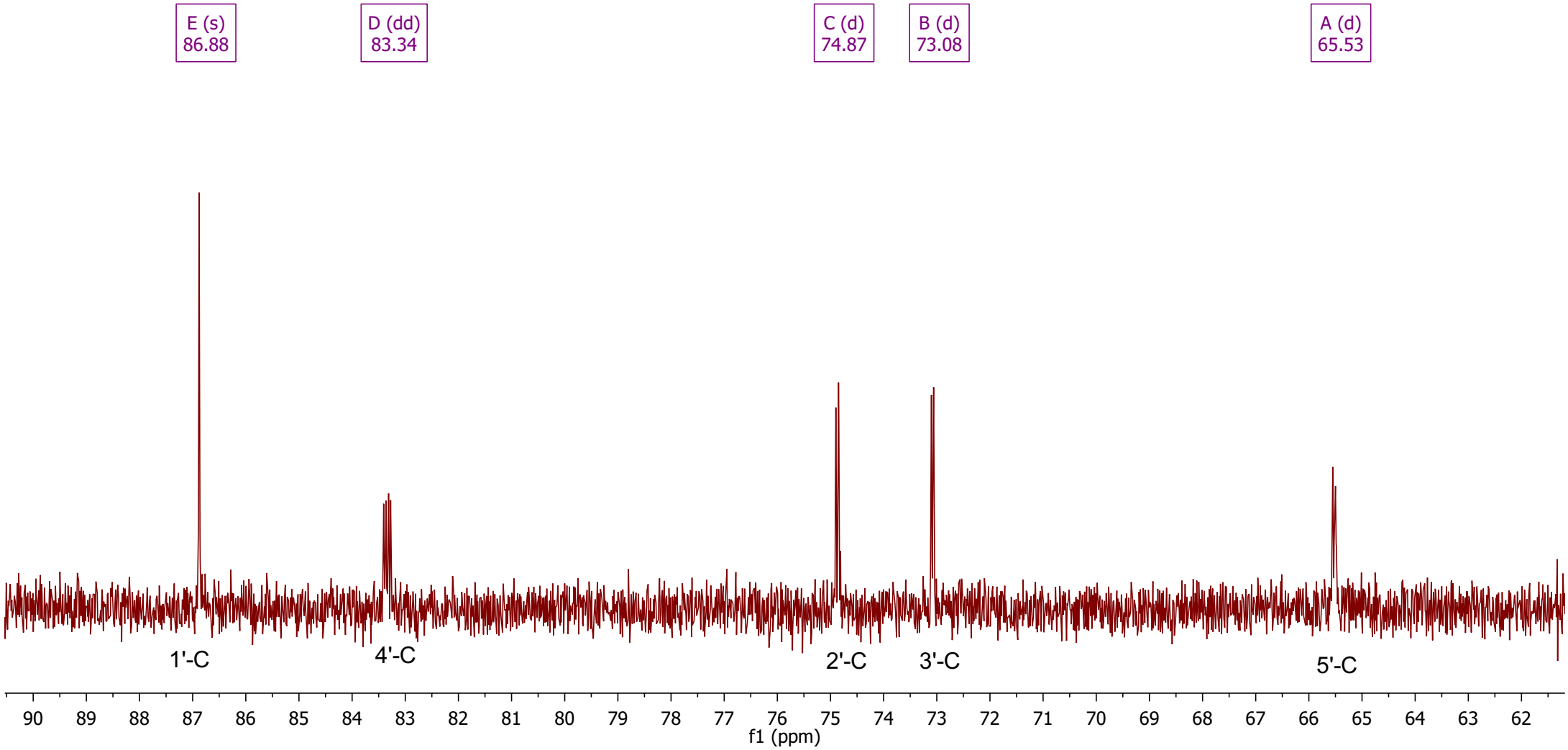
acetone (0.9 w%)

240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20

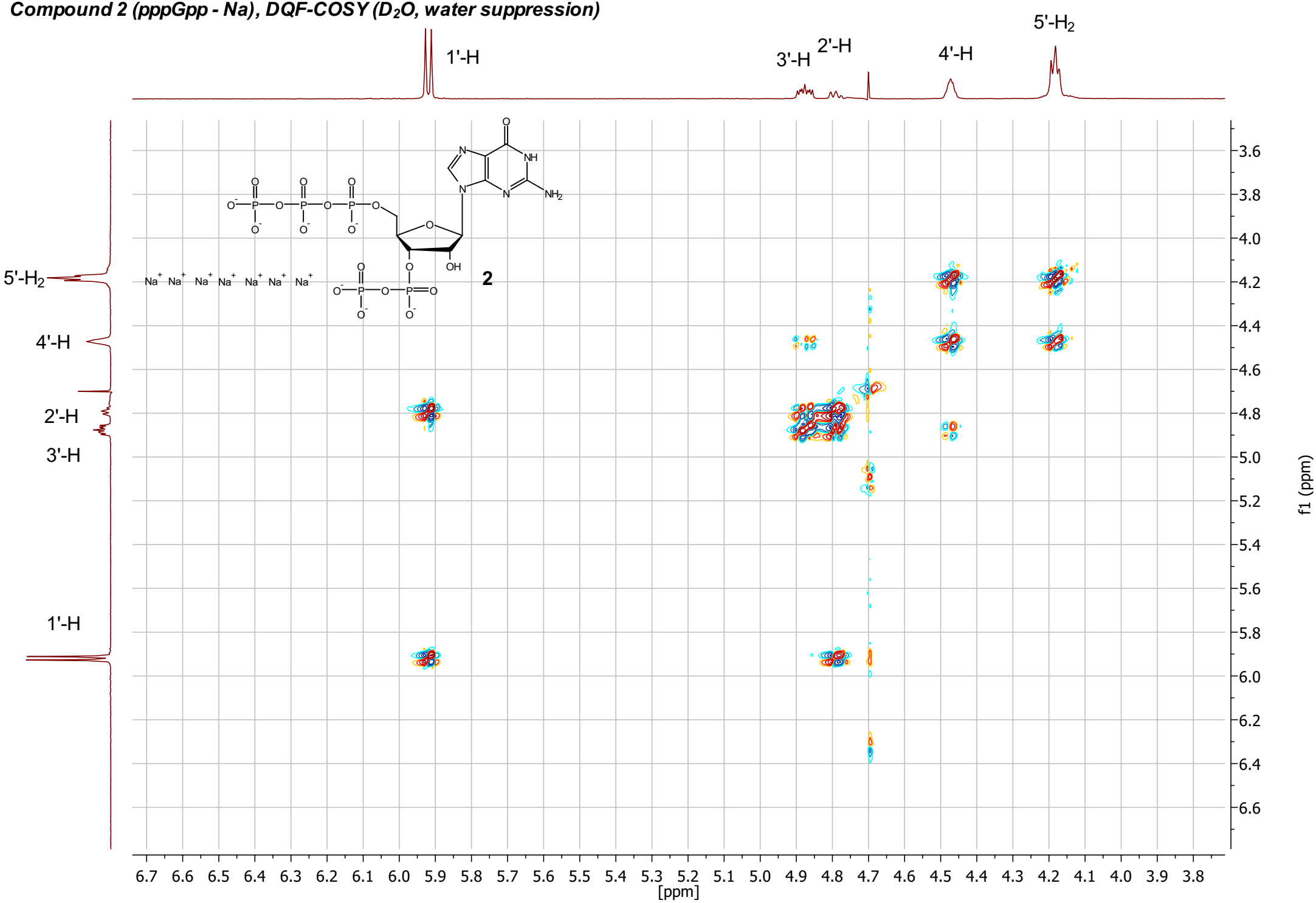
f1 (ppm)

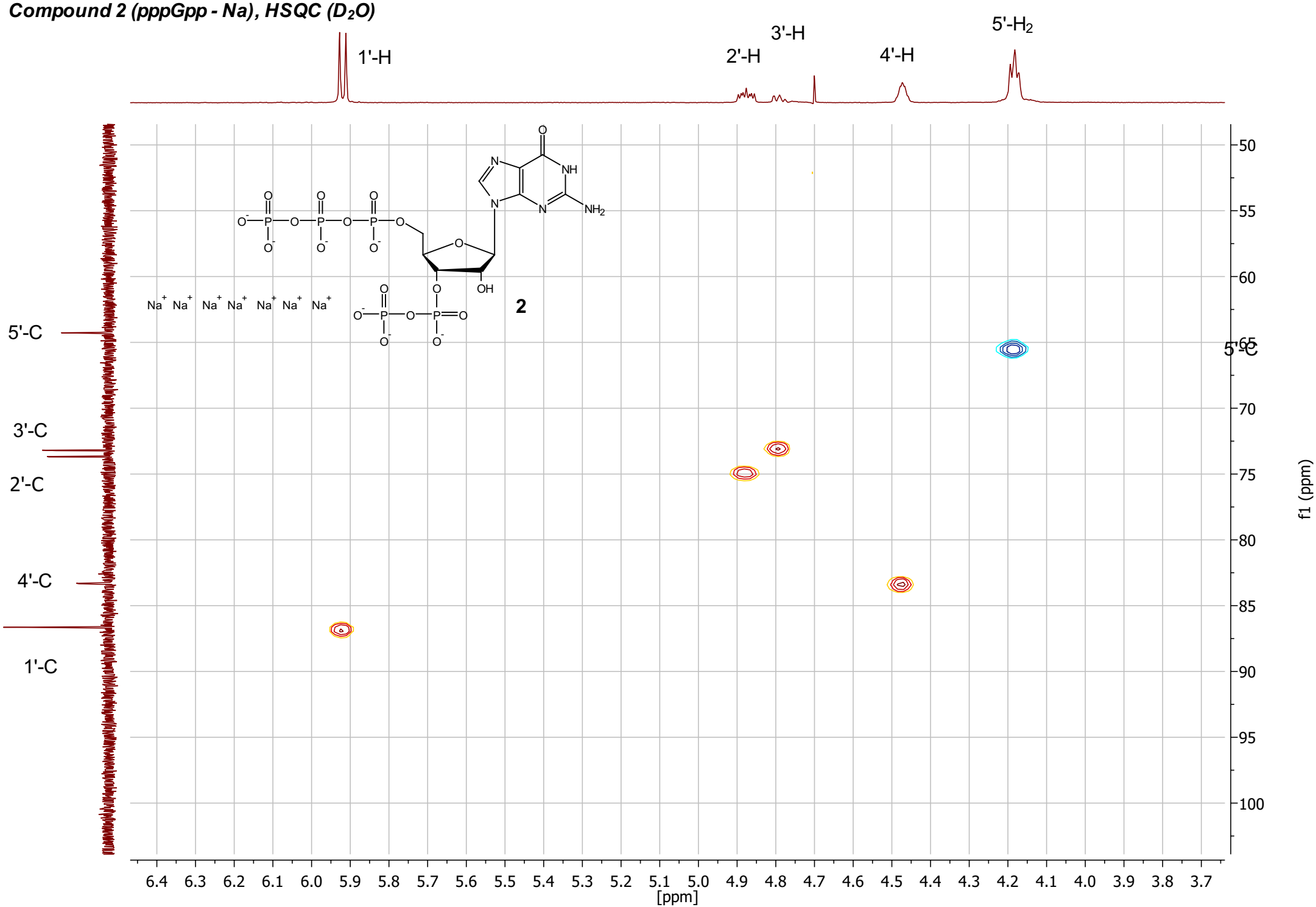
Compound 2 (pppGpp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz), detailed

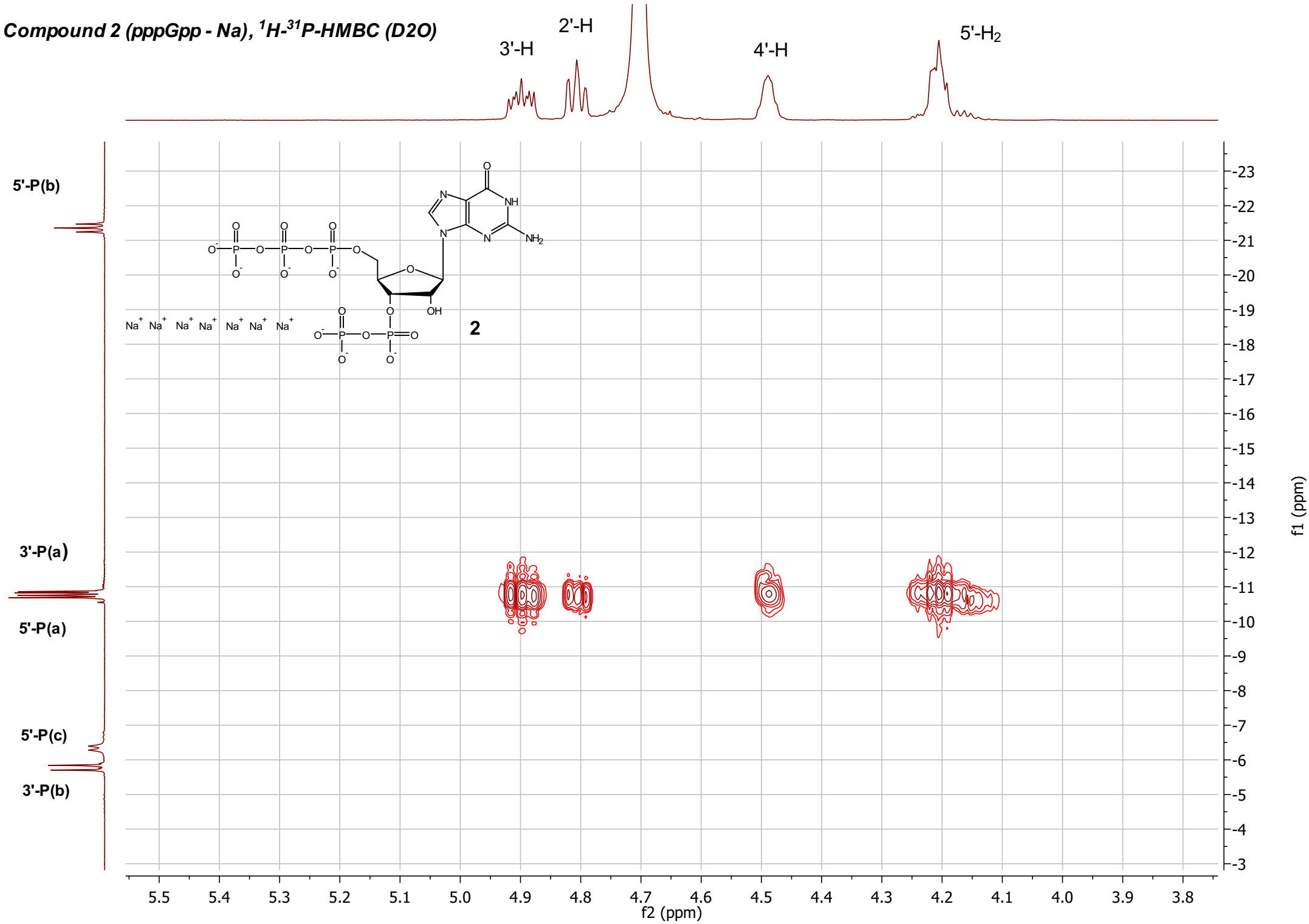


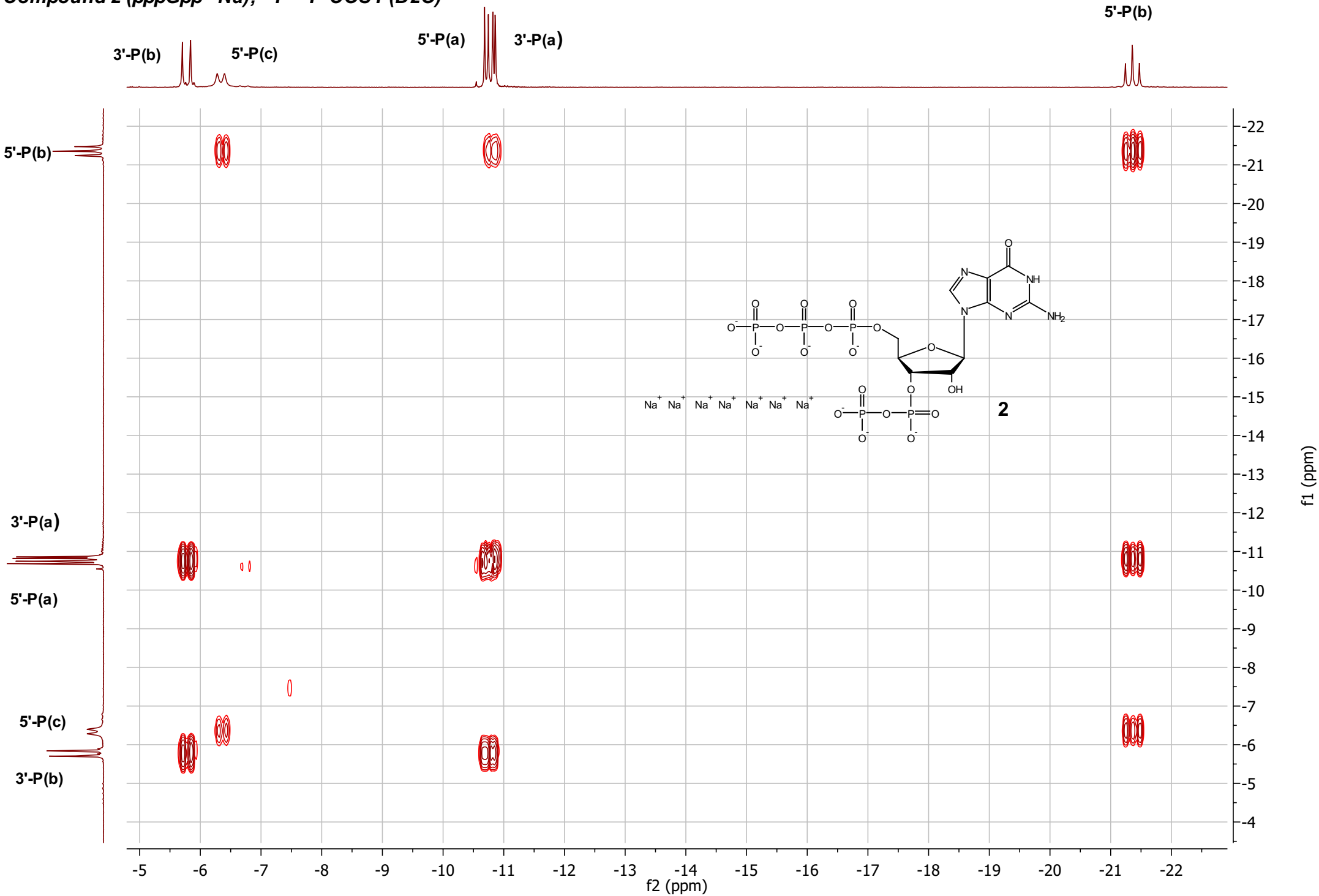


Compound 2 (pppGpp - Na), DQF-COSY (D_2O, water suppression)

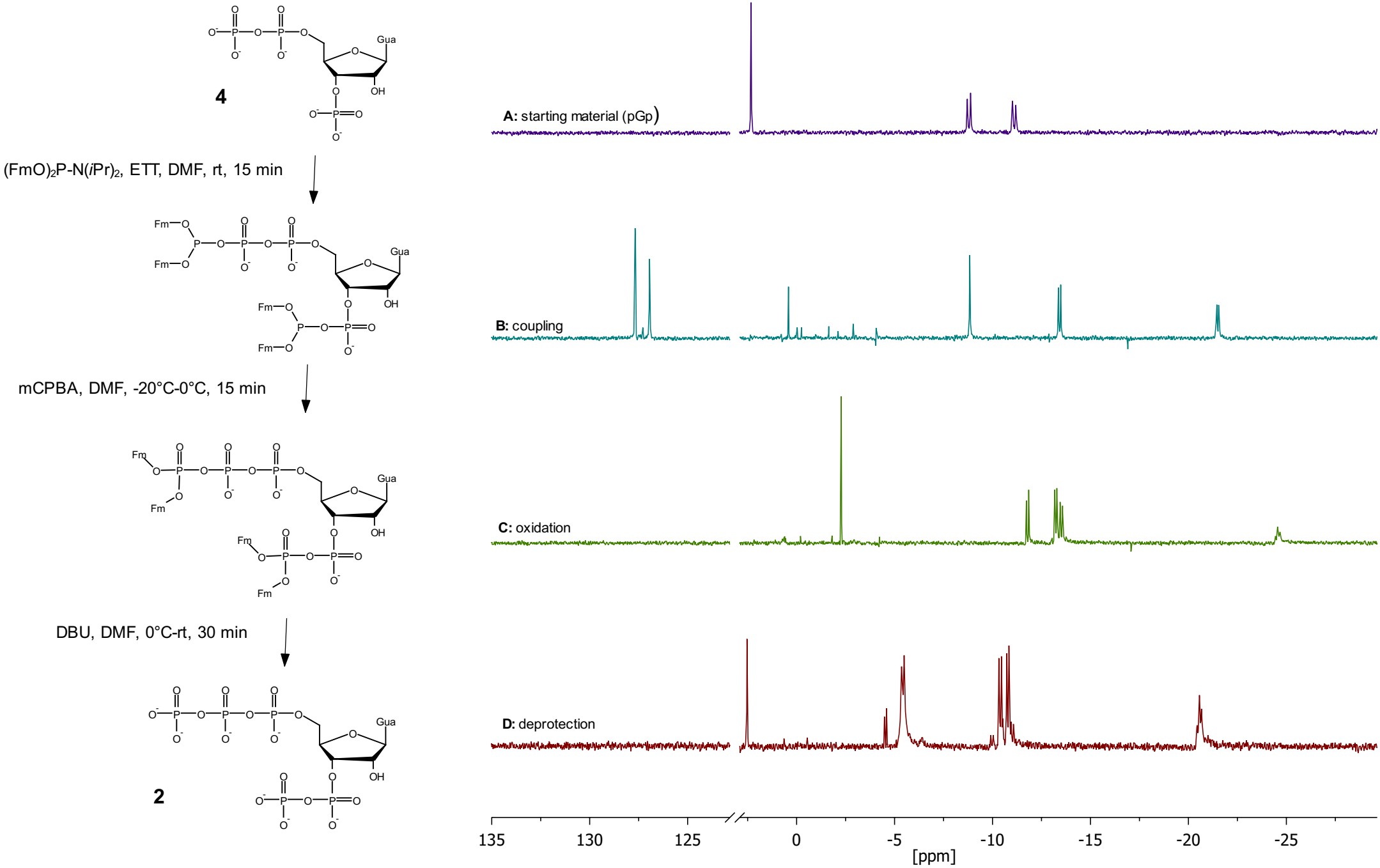



Compound 2 (pppGpp - Na), HSQC (D2O)
1'-H
2'-H
3'-H
4'-H
5'-H2
5'-C
3'-C
2'-C
4'-C
1'-C
2
Na+ Na+ Na+ Na+ Na+ Na+ Na+
6.4 6.3 6.2 6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7
[ppm]
50 55 60 65 70 75 80 85 90 95 100
f1 (ppm)

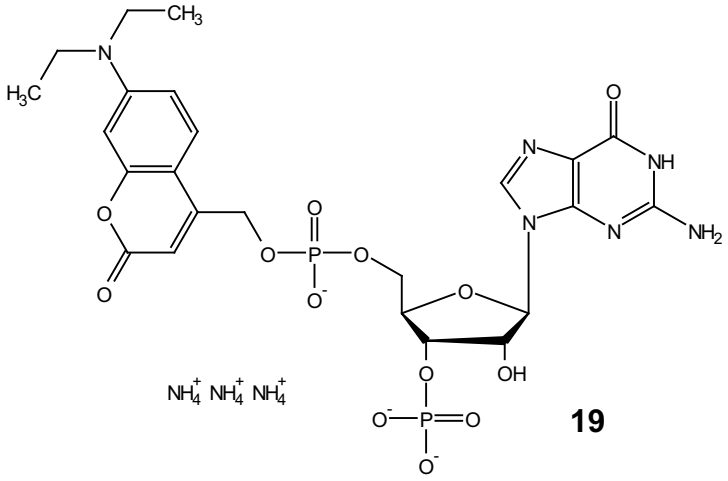

Compound 2 (pppGpp - Na), ^{1}H-^{31}P-HMBC (D2O)
3'-H
2'-H
4'-H
5'-H2
5'-P(b)
3'-P(a)
5'-P(a)
5'-P(c)
3'-P(b)
2
f2 (ppm)
f1 (ppm)


Compound 2 (pppGpp - Na), 31P-31P-COSY (D2O)
3'-P(b)
5'-P(c)
5'-P(a)
3'-P(a)
5'-P(b)
5'-P(b)
3'-P(a)
5'-P(a)
5'-P(c)
3'-P(b)
2
f2 (ppm)
f1 (ppm)

Synthesis of compound 2 (pppGpp), reaction monitoring by ^{31}P-NMR (DMF and D_2O, 162 MHz)

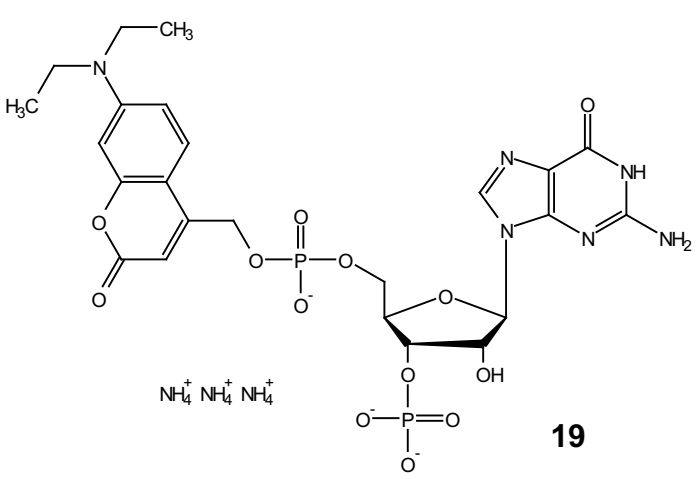


Compound 19 (DEACM-pGp - NH_4), ^1H - NMR (D_2O, 400 MHz)



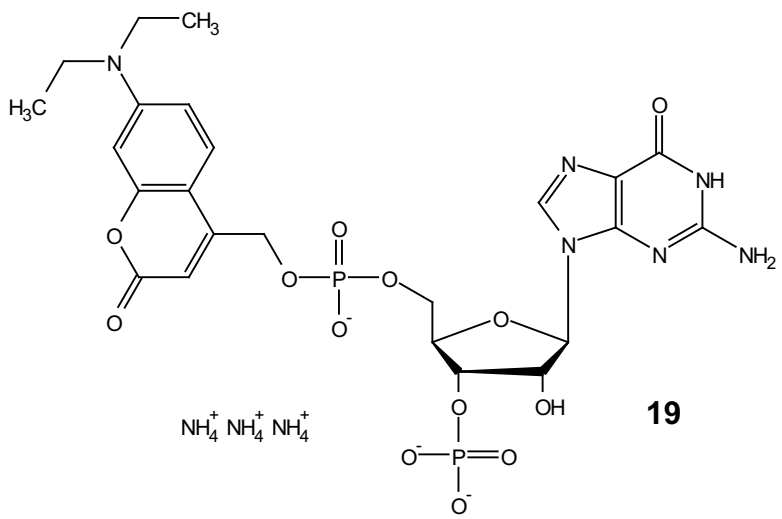
19

Compound 19 (DEACM-pGp - NH_4), ^{1}H - NMR (D_2O, 400 MHz, water suppression)

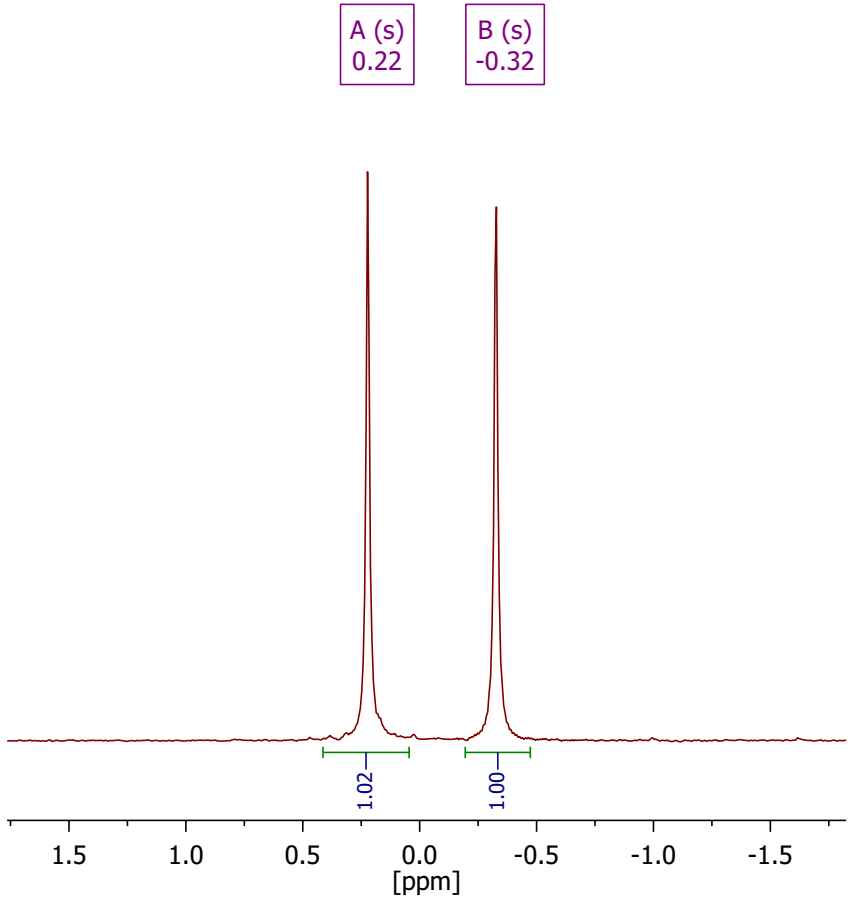


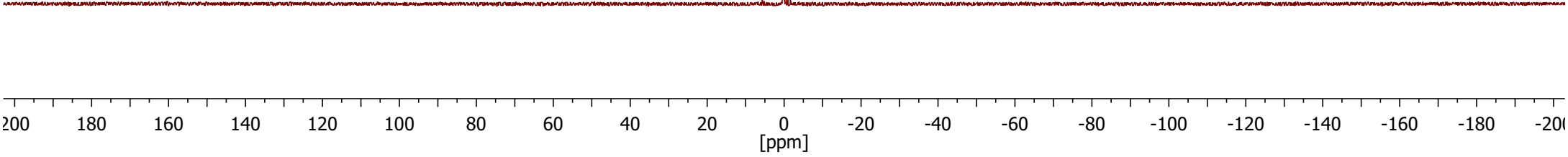
19

Compound 19 (DEACM-pGp - NH_4), ^{31}P [^1H] - NMR (D_2O, 162 MHz)

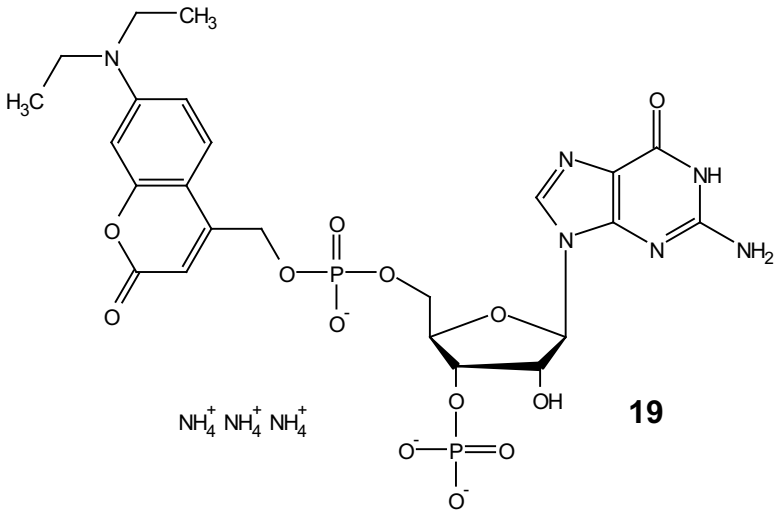


19



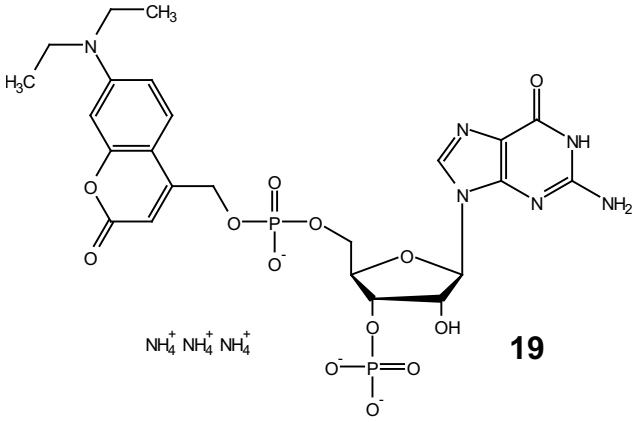


Compound 19 (DEACM-pGp - NH_4), ^{31}P - NMR (D_2O, 162 MHz)

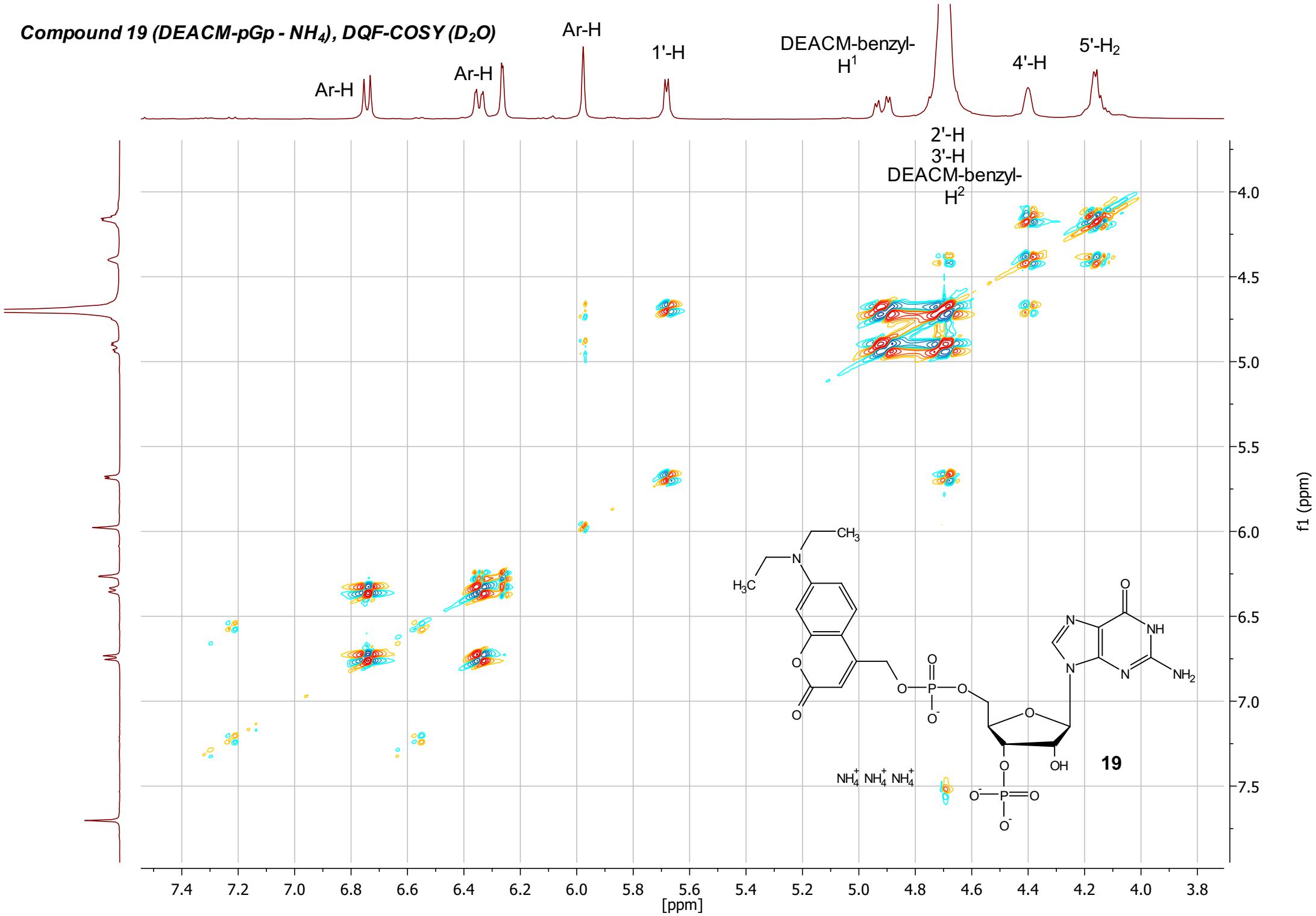


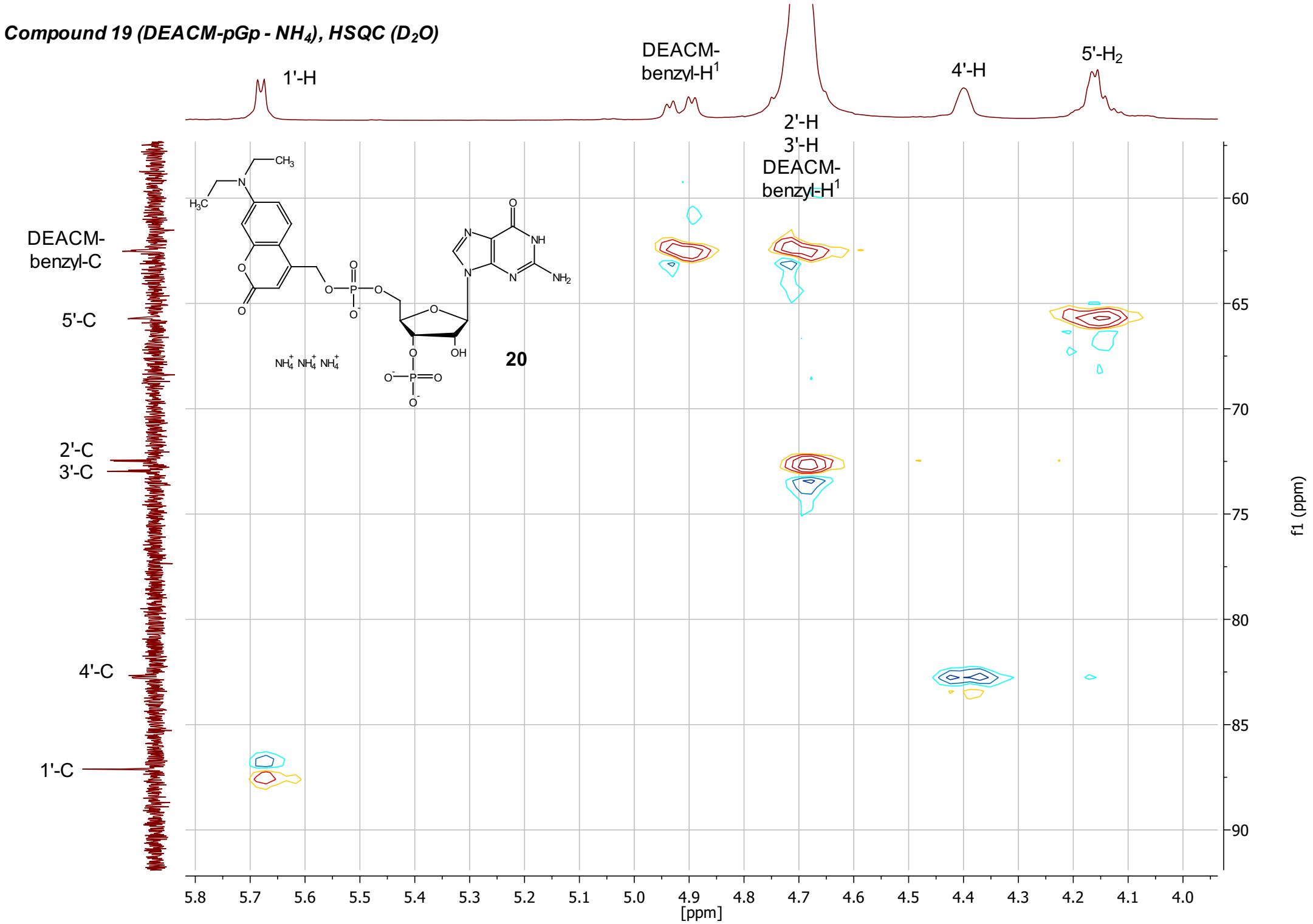
19

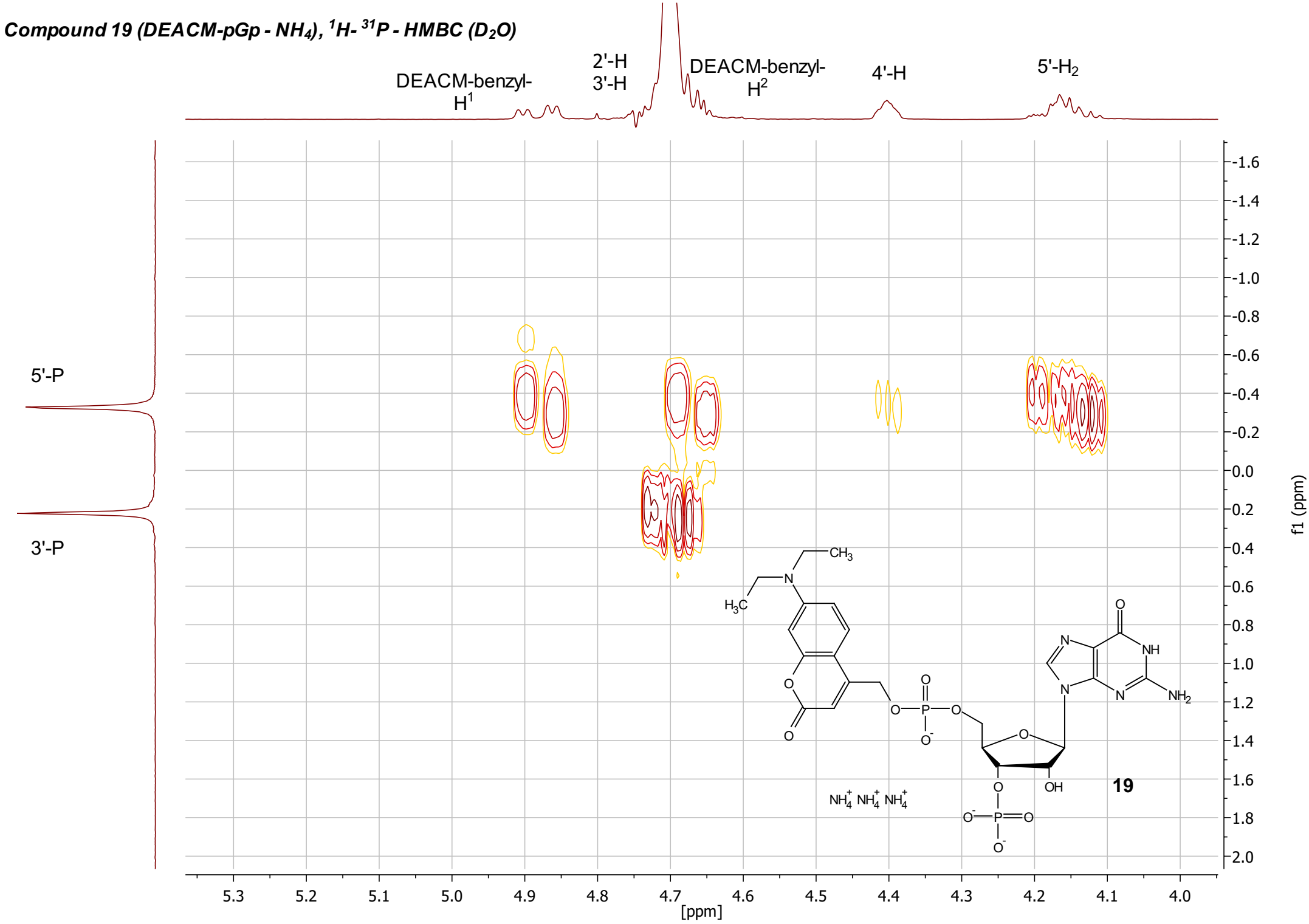
Compound 19 (DEACM - pGp), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz)



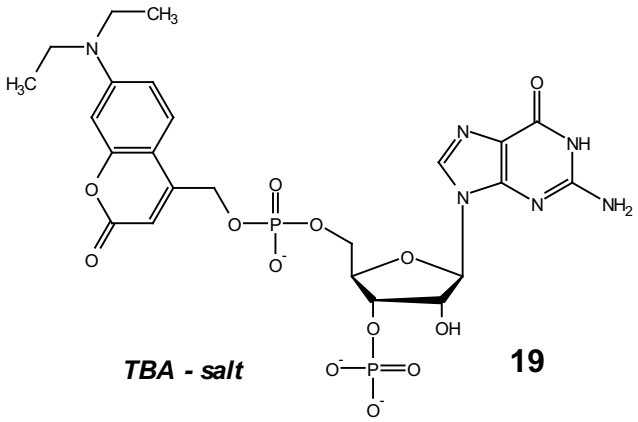
19


Compound 19 (DEACM-pGp - NH4), DQF-COSY (D2O)
Ar-H
Ar-H
Ar-H
1'-H
DEACM-benzyl-H1
4'-H
5'-H2
2'-H
3'-H
DEACM-benzyl-H2
19
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
[ppm]
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
f1 (ppm)


Compound 19 (DEACM-pGp - NH4), HSQC (D2O)
1'-H
DEACM-benzyl-H1
2'-H
3'-H
DEACM-benzyl-H1
4'-H
5'-H2
DEACM-benzyl-C
5'-C
2'-C
3'-C
4'-C
1'-C
20
[ppm]
f1 (ppm)


Compound 19 (DEACM-pGp - NH4), 1H- 31P - HMBC (D2O)
DEACM-benzyl-H1
2'-H
3'-H
DEACM-benzyl-H2
4'-H
5'-H2
5'-P
3'-P
f1 (ppm)
[ppm]
19

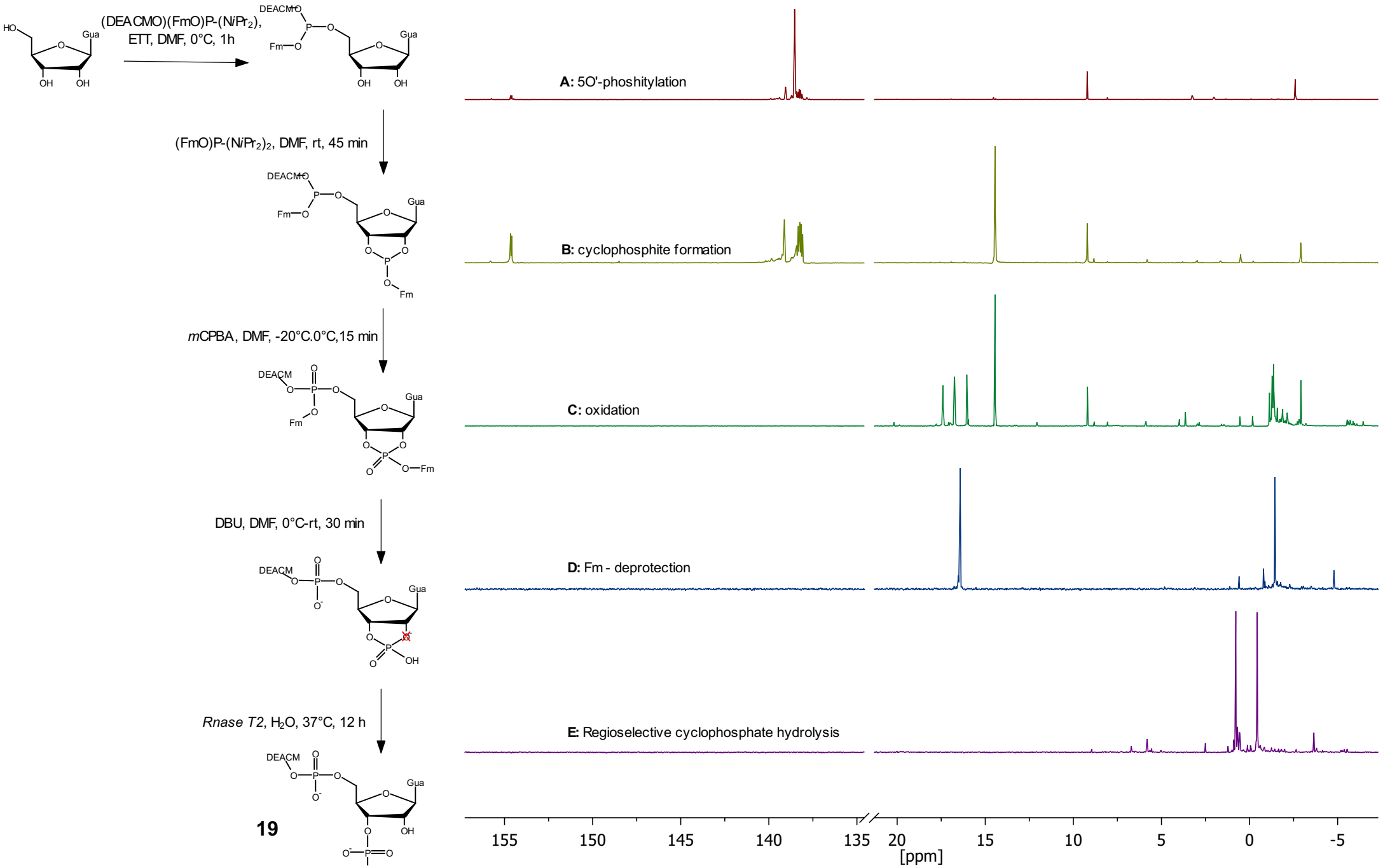
Compound 19 (DEACM-pGp - TBA), ^{1}H - NMR (D_2O, 400 MHz, water suppression)



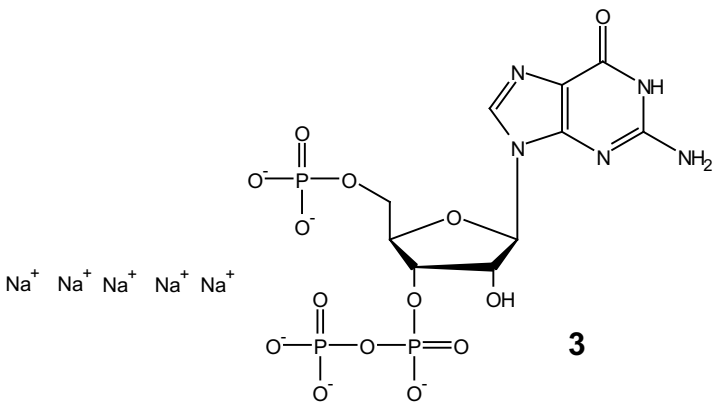
TBA - salt

19

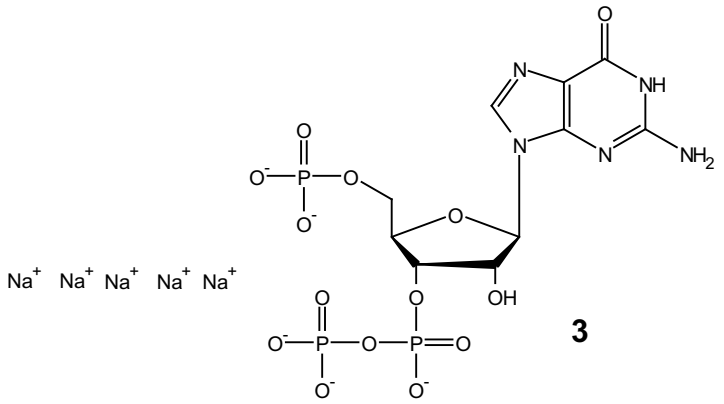
Synthesis of compound 19 (DEACM - pGp), reaction monitoring by ^{31}P-NMR (DMF and D_2O, 162 MHz)



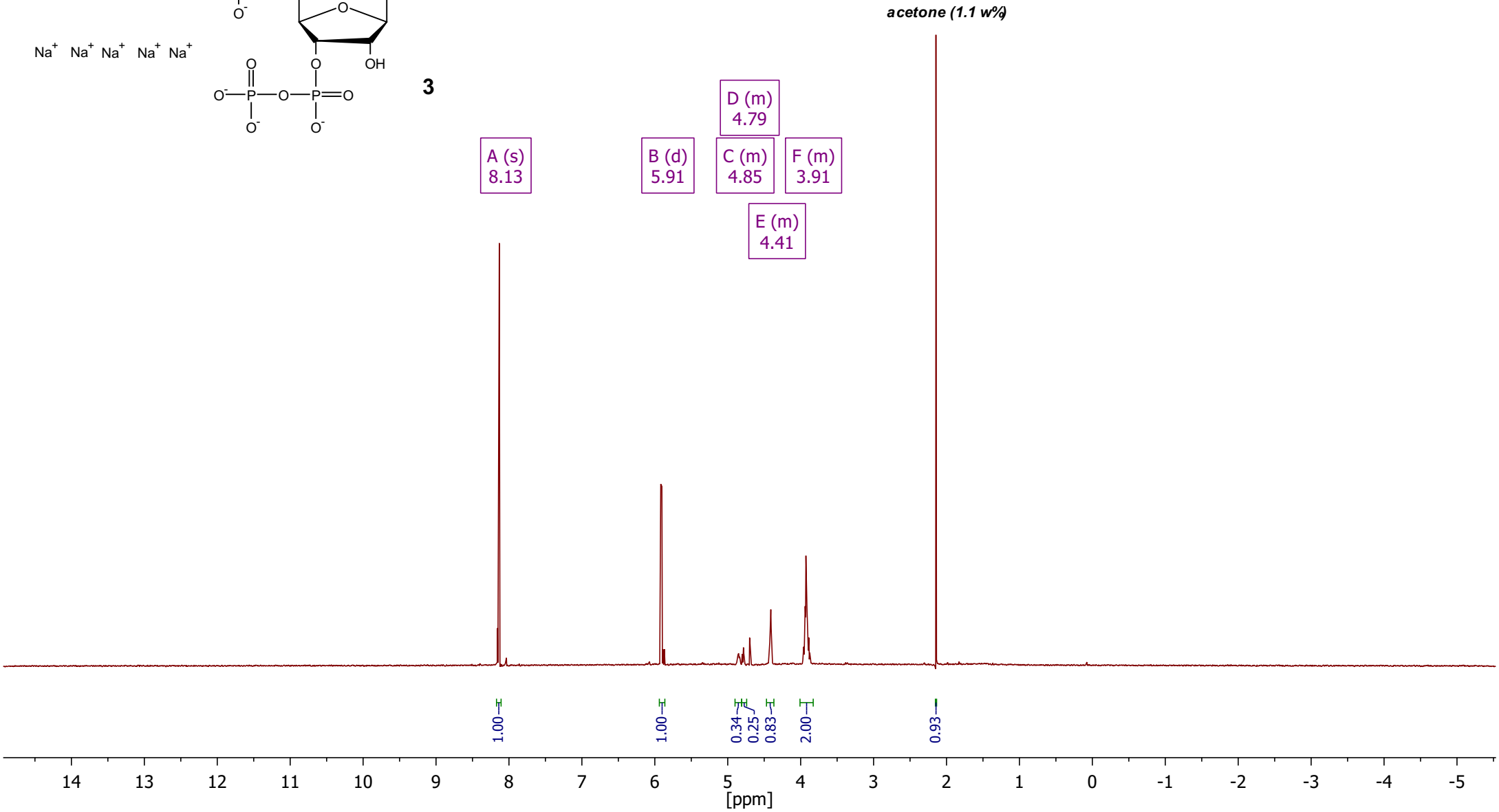
Compound 3 (pGpp - Na), ^1H - NMR (D_2O, 400 MHz)



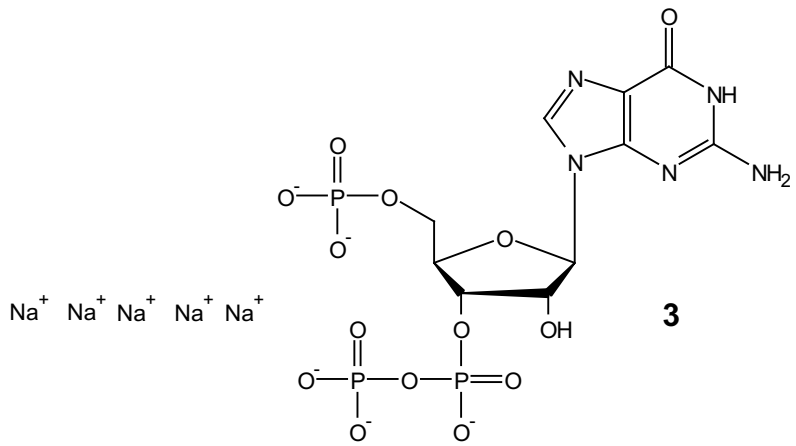
Compound 3 (pGpp - Na), ^1H - NMR (D_2O, 400 MHz, water suppression)



3

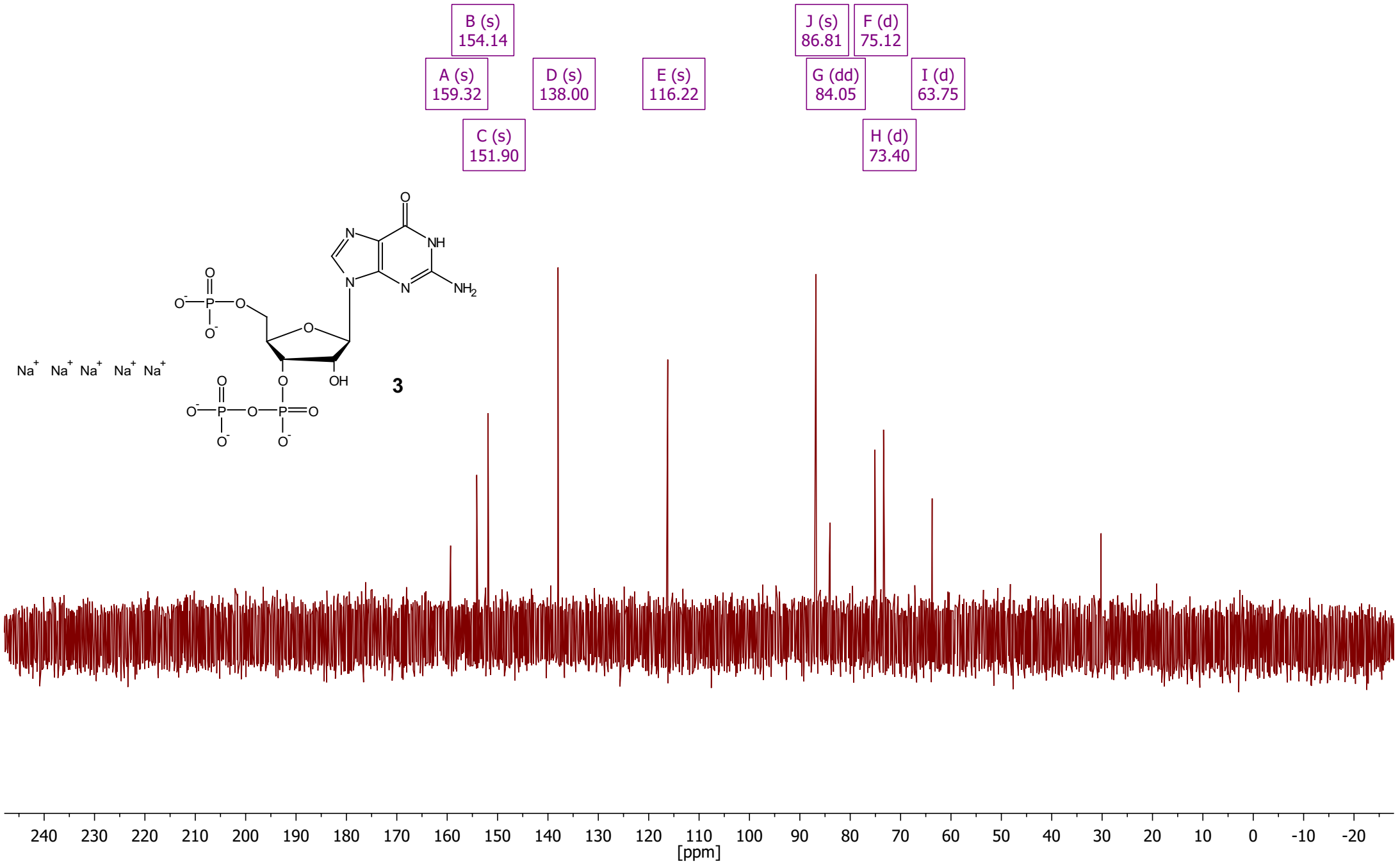


Compound 3 (pGpp - Na), $^{31}P[^{1}H]$ - NMR (D_2O, 162 MHz)

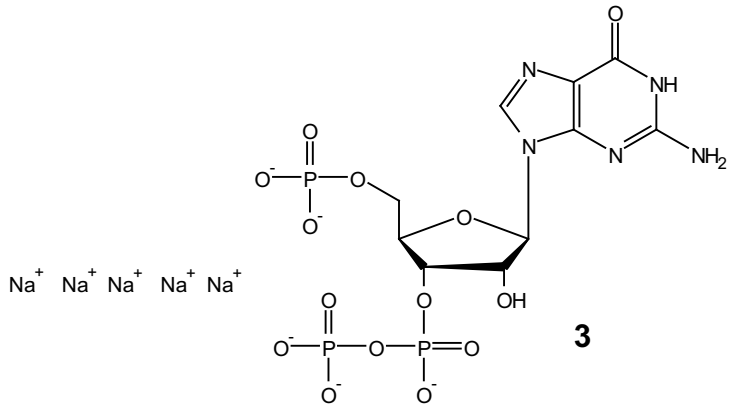


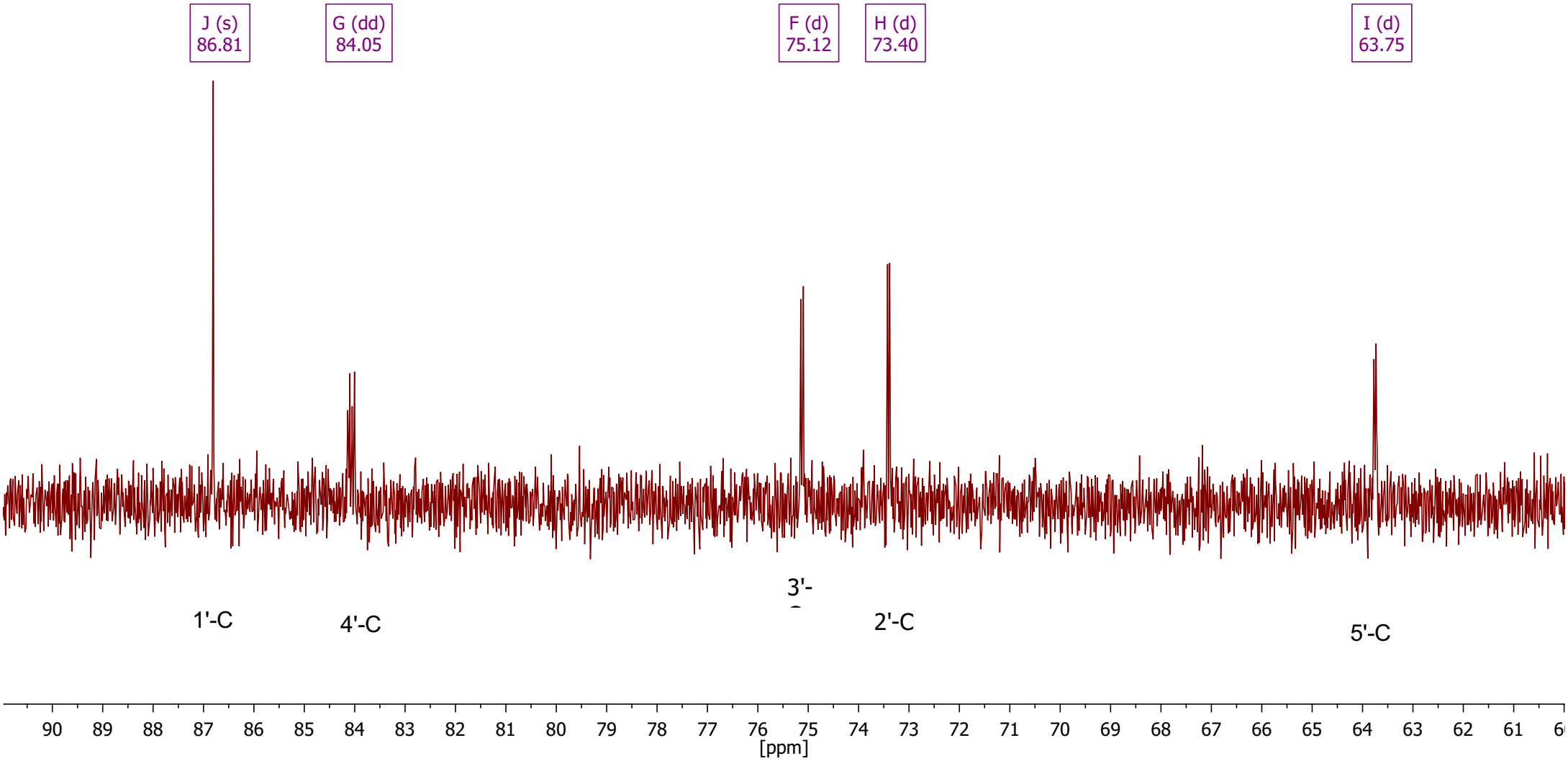
3

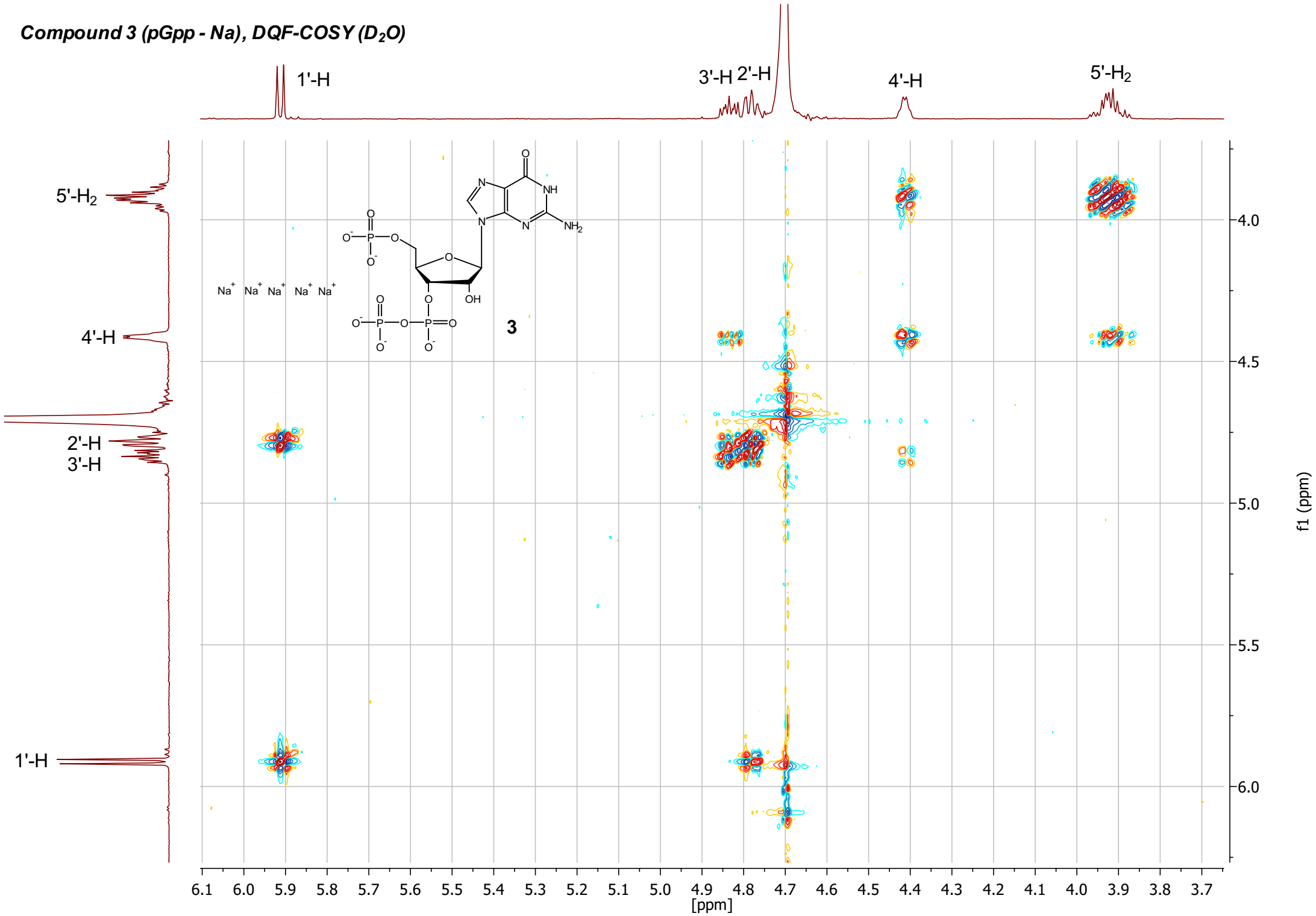
Compound 3 (pGpp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz)

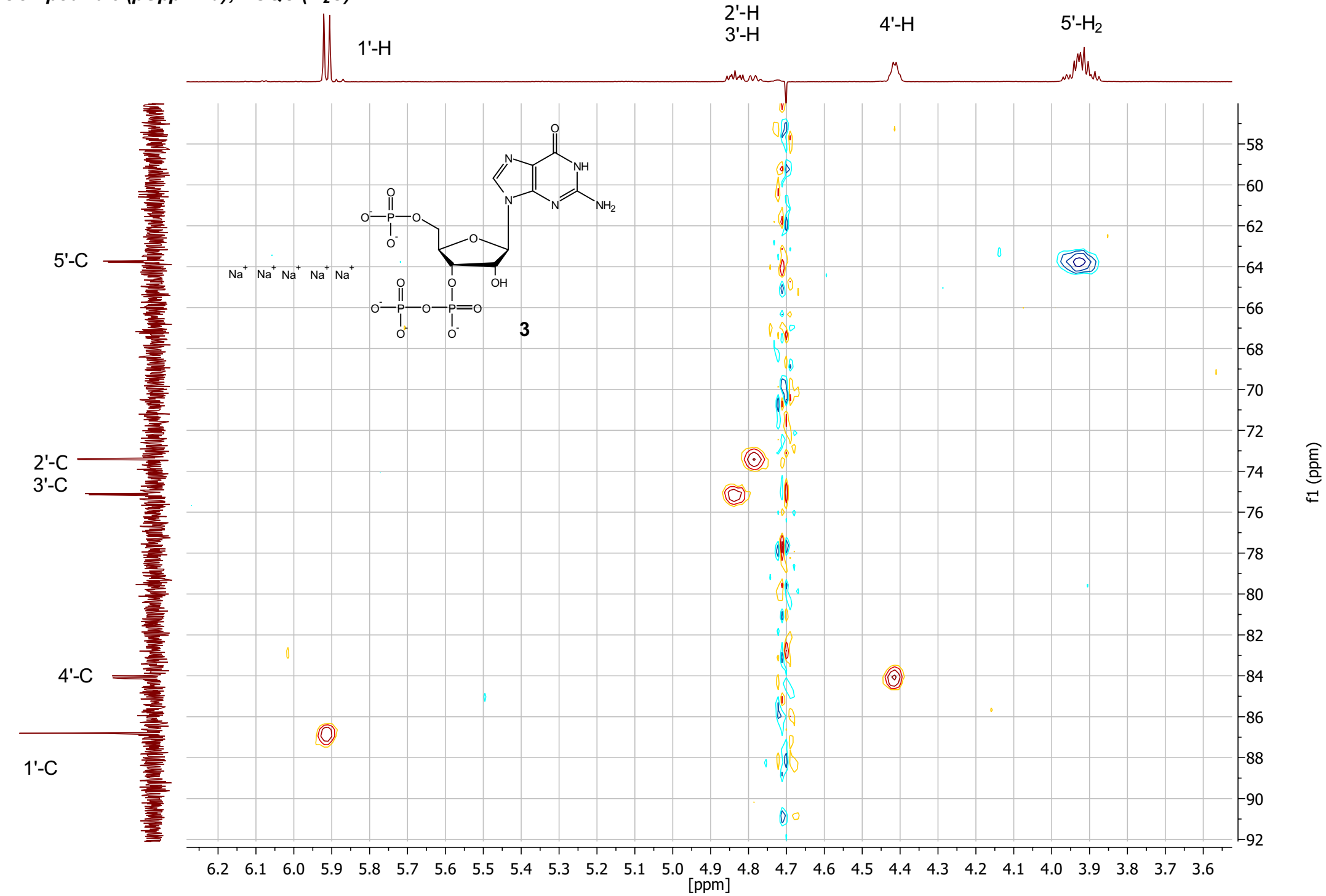


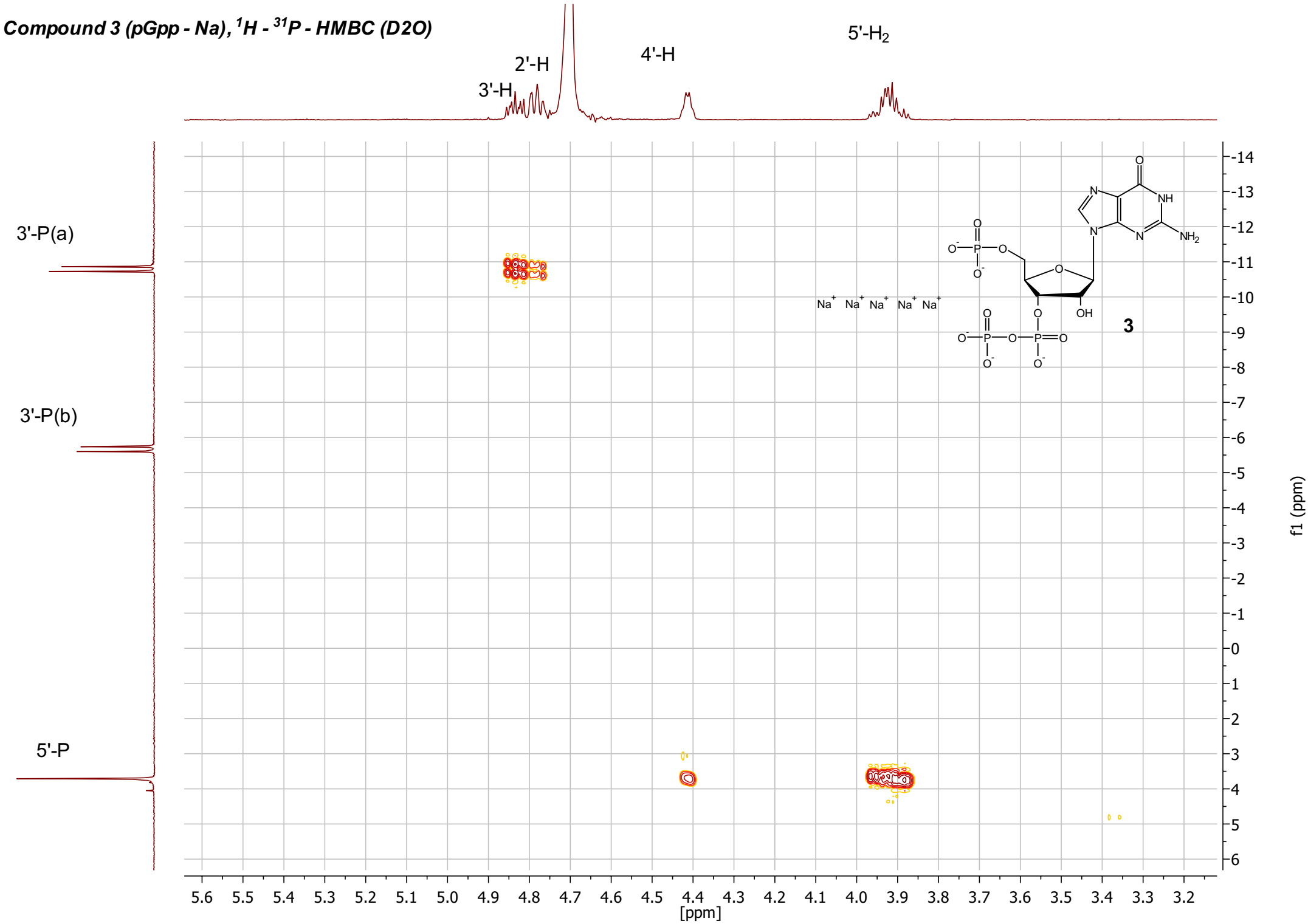
Compound 3 (pGpp - Na), $^{13}C[^{1}H]$ - NMR (D_2O, 101 MHz), detailed

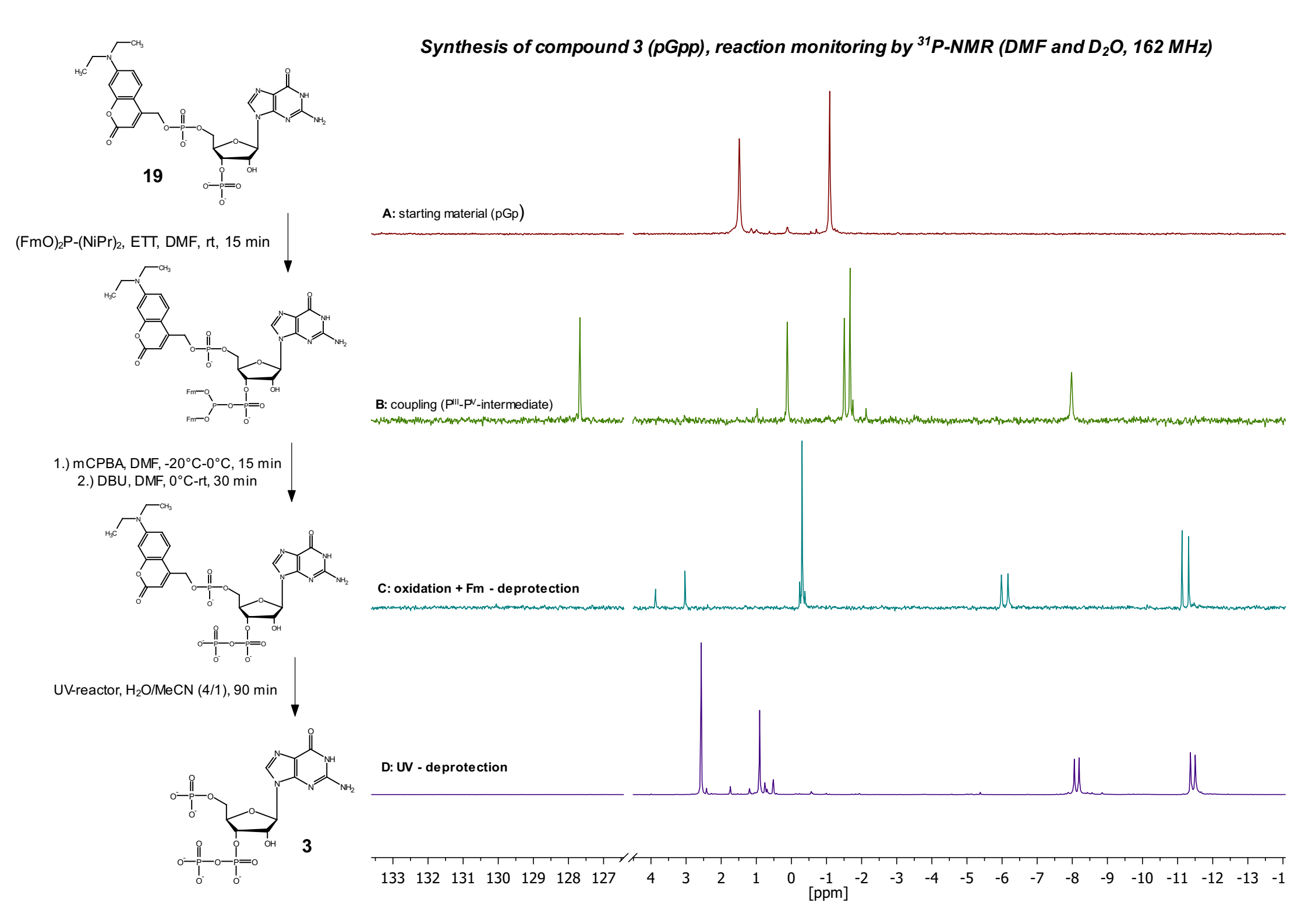




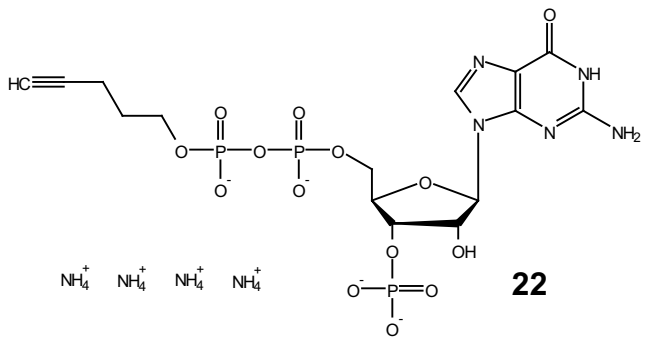

Compound 3 (pGpp - Na), DQF-COSY (D2O)
1'-H
3'-H
2'-H
4'-H
5'-H2
5'-H2
4'-H
2'-H
3'-H
1'-H
Na+ Na+ Na+ Na+ Na+
3
6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7
[ppm]
4.0
4.5
5.0
5.5
6.0
f1 (ppm)


Compound 3 (pGpp - Na), HSQC (D2O)
1'-H
2'-H
3'-H
4'-H
5'-H2
5'-C
2'-C
3'-C
4'-C
1'-C
Na+ Na+ Na+ Na+ Na+
3
6.2 6.1 6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6
[ppm]
58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92
f1 (ppm)


Compound 3 (pGpp - Na), ^{1}H - ^{31}P - HMBC (D2O)
3'-H
2'-H
4'-H
5'-H2
3'-P(a)
3'-P(b)
5'-P
Na+ Na+ Na+ Na+ Na+
OH
3
5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2
[ppm]
f1 (ppm)


Synthesis of compound 3 (pGpp), reaction monitoring by 31P-NMR (DMF and D2O, 162 MHz)
19
(FmO)2P-(NiPr)2, ETT, DMF, rt, 15 min
1.) mCPBA, DMF, -20°C-0°C, 15 min
2.) DBU, DMF, 0°C-rt, 30 min
UV-reactor, H2O/MeCN (4/1), 90 min
3
A: starting material (pGp)
B: coupling (PIII-PV-intermediate)
C: oxidation + Fm - deprotection
D: UV - deprotection
133 132 131 130 129 128 127 4 3 2 1 0 -1 -2 -3 -4 -5 -6 -7 -8 -9 -10 -11 -12 -13 -1
[ppm]

Compound 22 (5' - pentynyl - ppGp), ^{1}H - NMR (D_2O, 400 MHz)



22

D (s) 8.08

E (d) 5.91

J (ddd) 4.78

G (m) 4.17

I (ddd) 4.87

F (q) 3.87

H (m) 4.48

B (m) 2.13

A (dd) 2.19

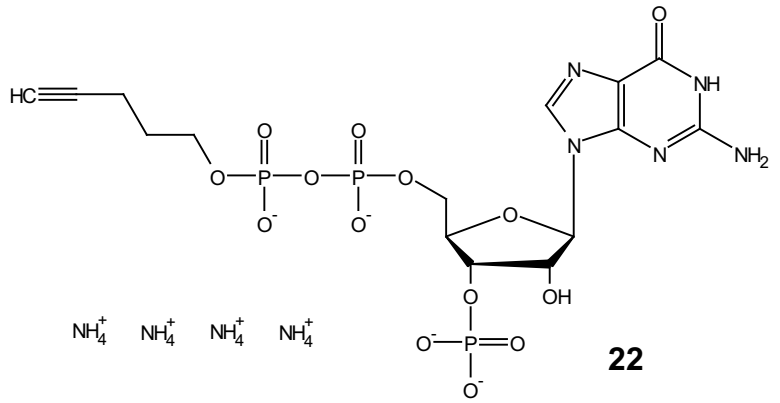
C (dddd) 1.66

1.00 1.01 1.28 2.09 1.05 2.06 1.99 0.90 1.88 2.01

16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3 -4

[ppm]

Compound 22 (5' - pentynyl - ppGp), ^{1}H [^{31}P] - NMR (D_2O, 400 MHz)



22

C (dd) 4.88

D (dd) 4.78

E (ddd) 4.48

F (m) 4.15

G (dd) 3.87

1.37

1.78

1.00

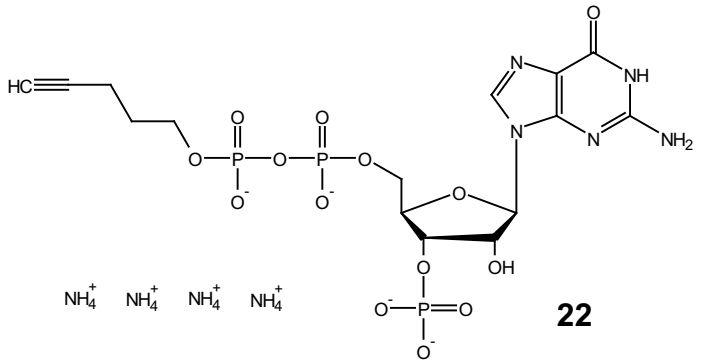
1.97

1.86

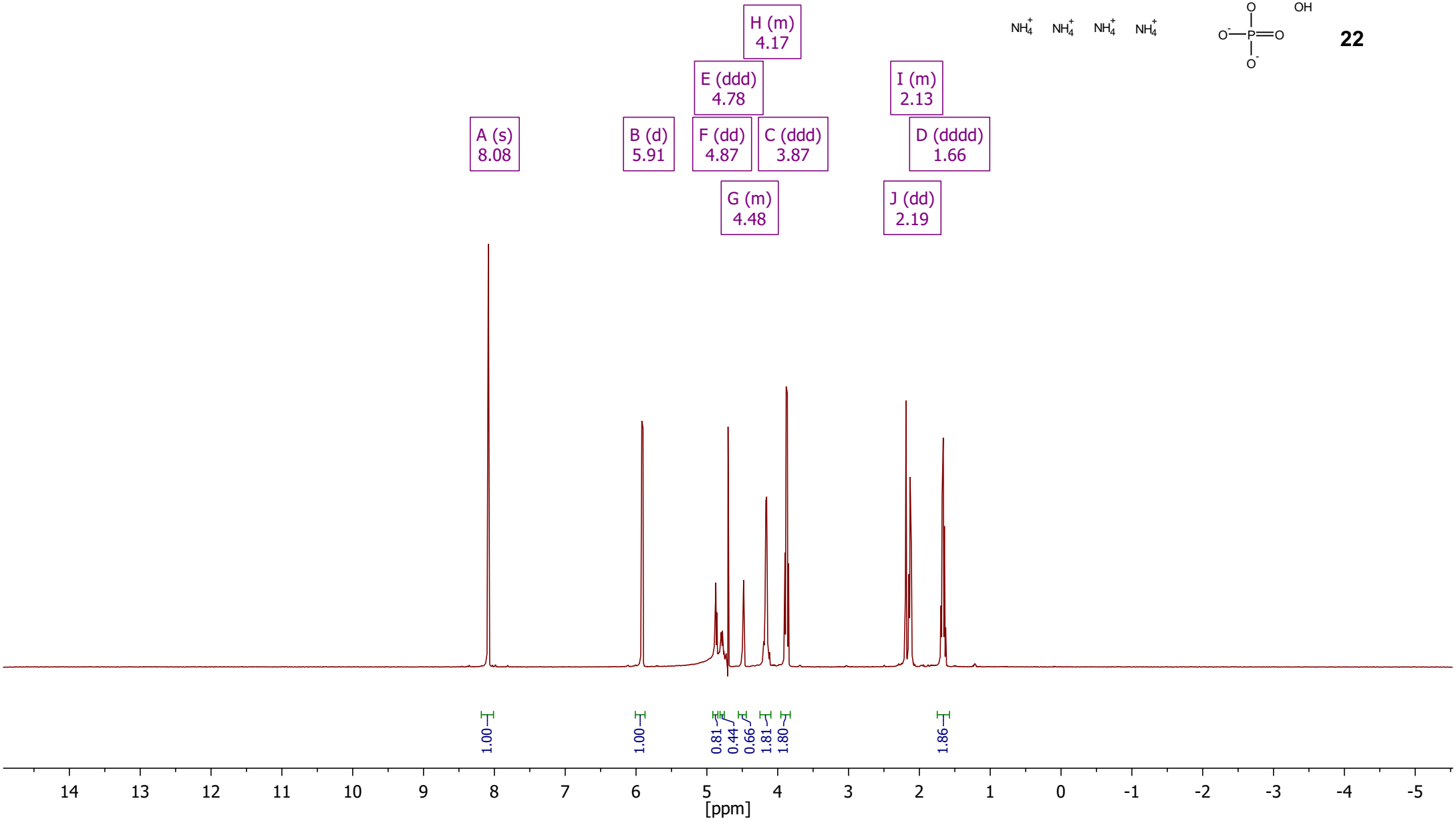
5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6

[ppm]

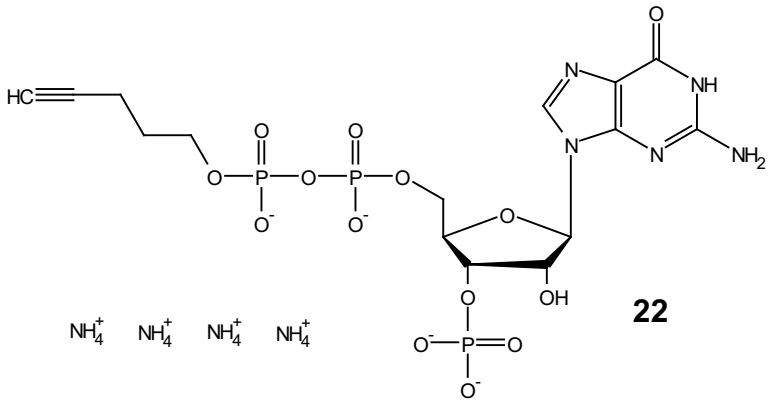
Compound 22 (5' - pentynyl - ppGp), ^{1}H - NMR (D_2O, 400 MHz, water suppression)



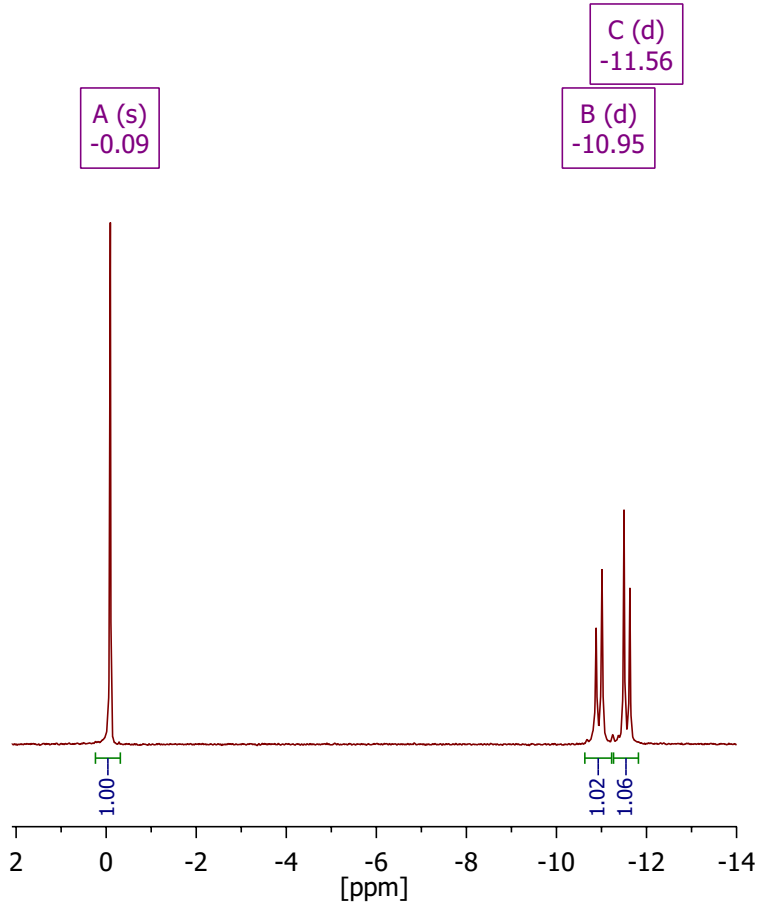
22



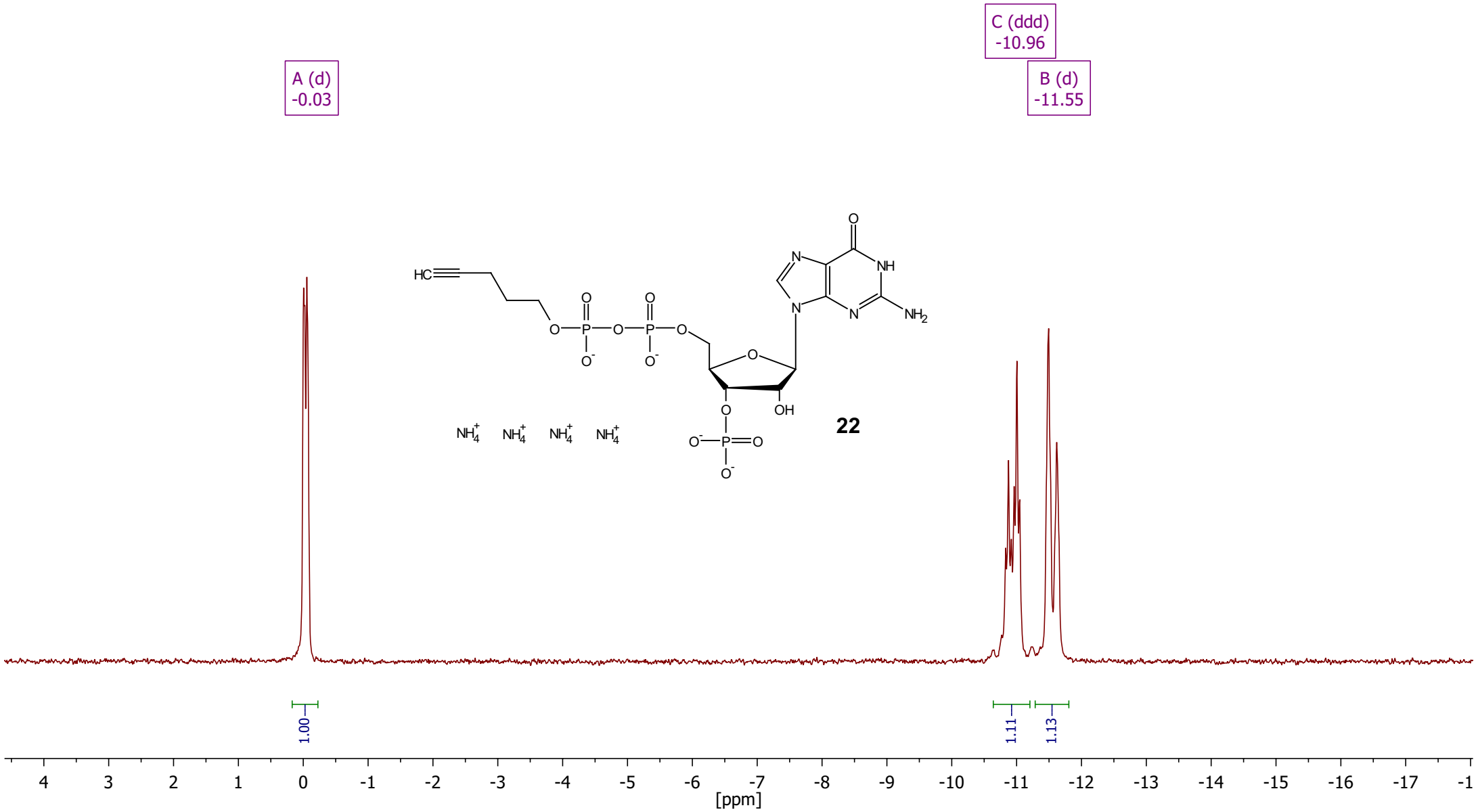
Compound 22 (5' - pentynyl - ppGp), ^{31}P [^{1}H] - NMR (D_2O, 162 MHz)



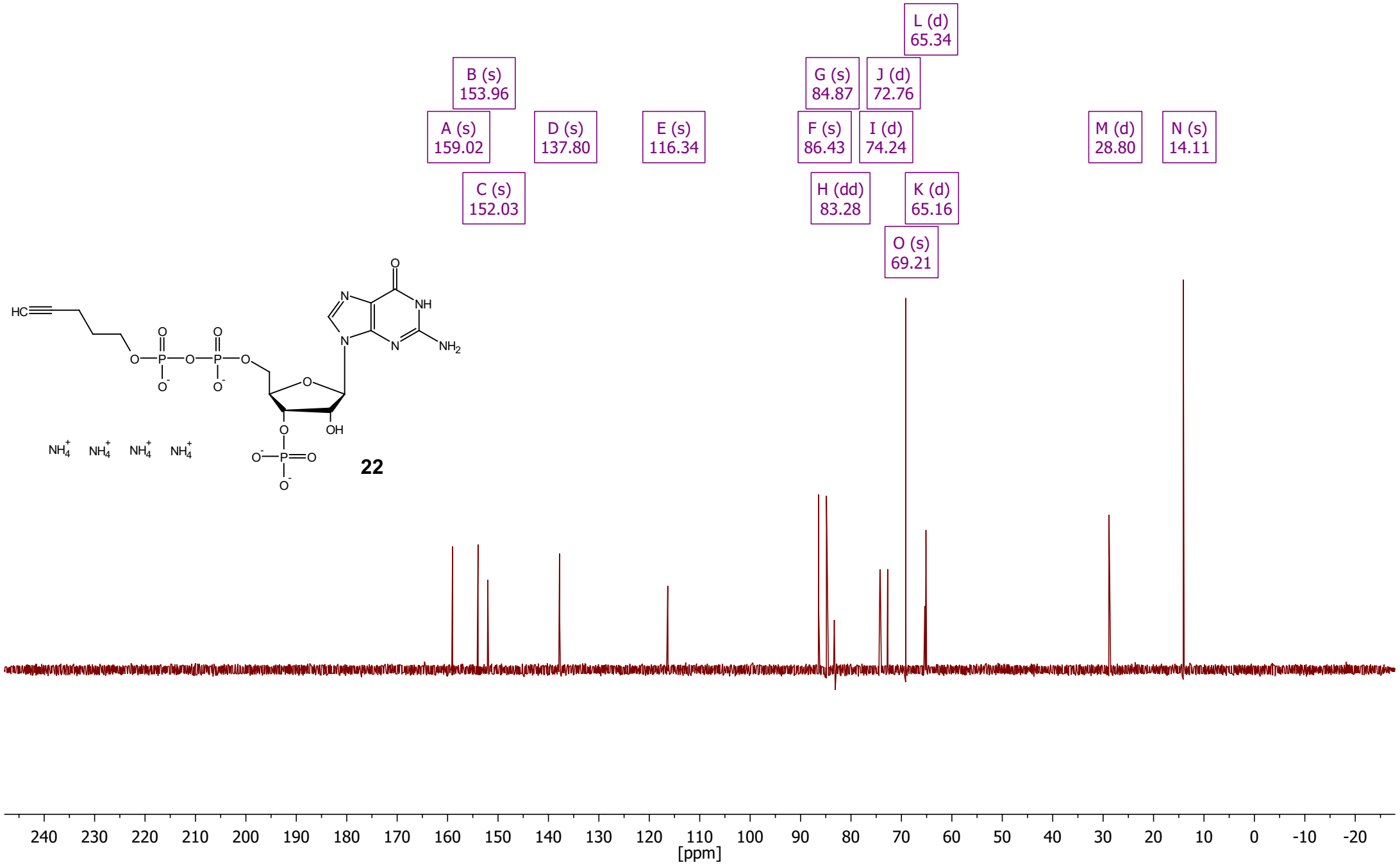
22



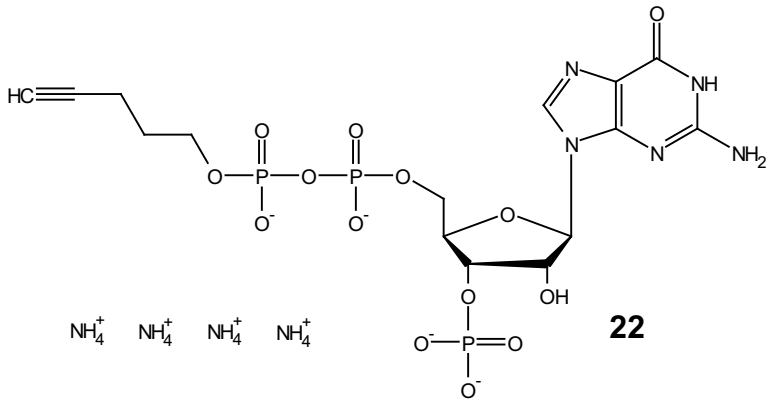
Compound 22 (5' - pentynyl - ppGp), ^{31}P - NMR (D_2O, 162 MHz, detailed)

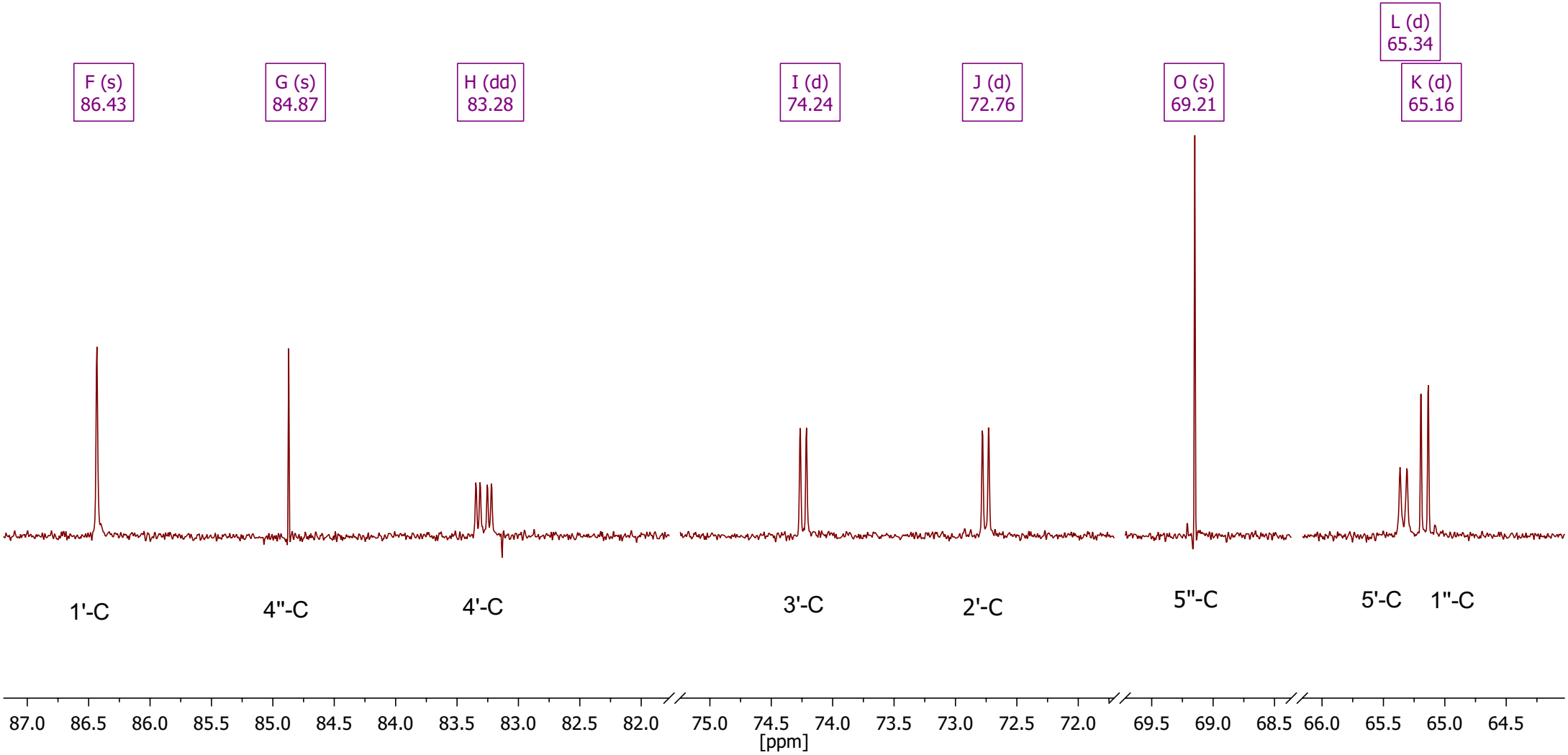


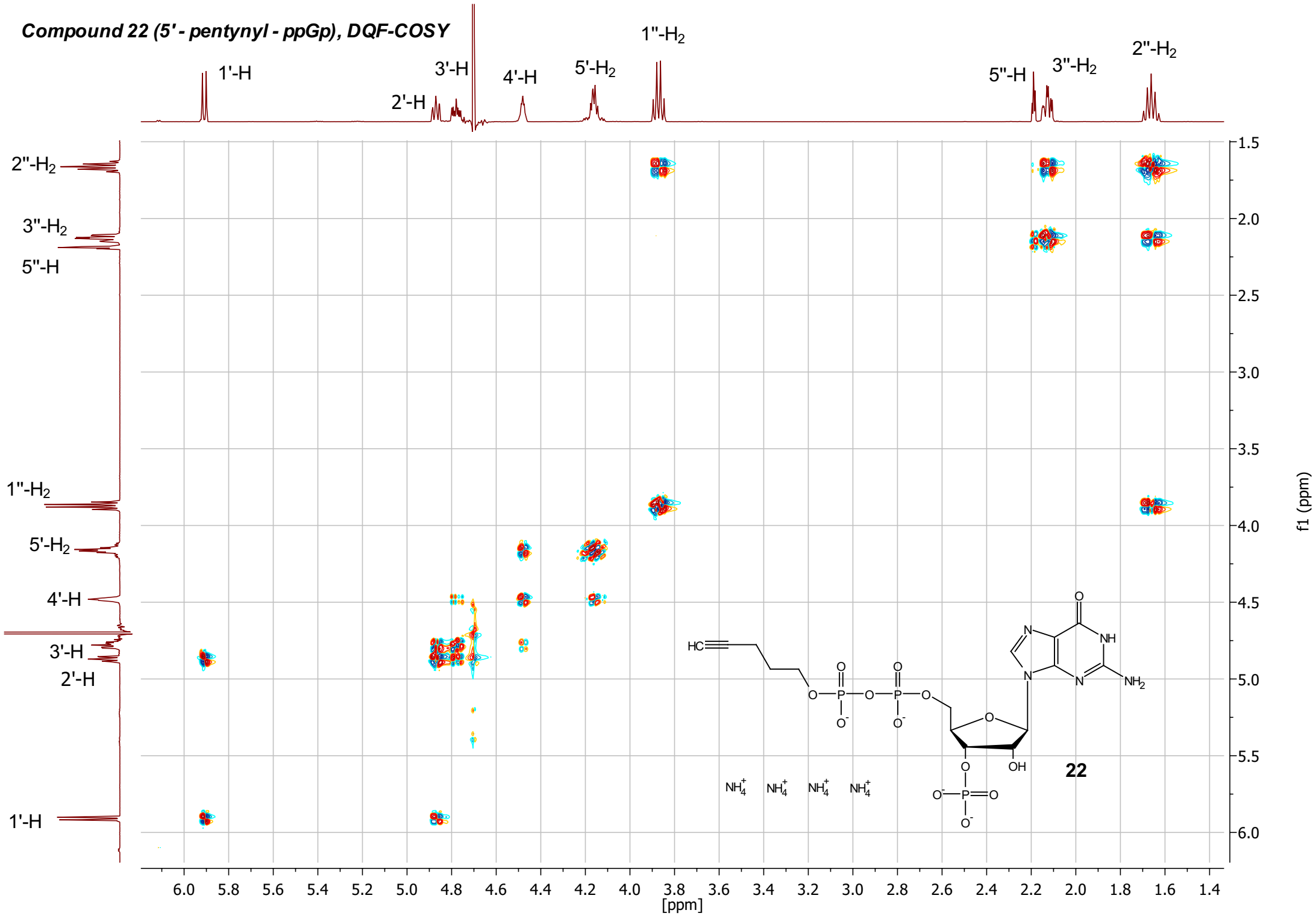
Compound 22 (5' - pentynyl - ppGp), ^{13}C - NMR (D_2O, 101 MHz)

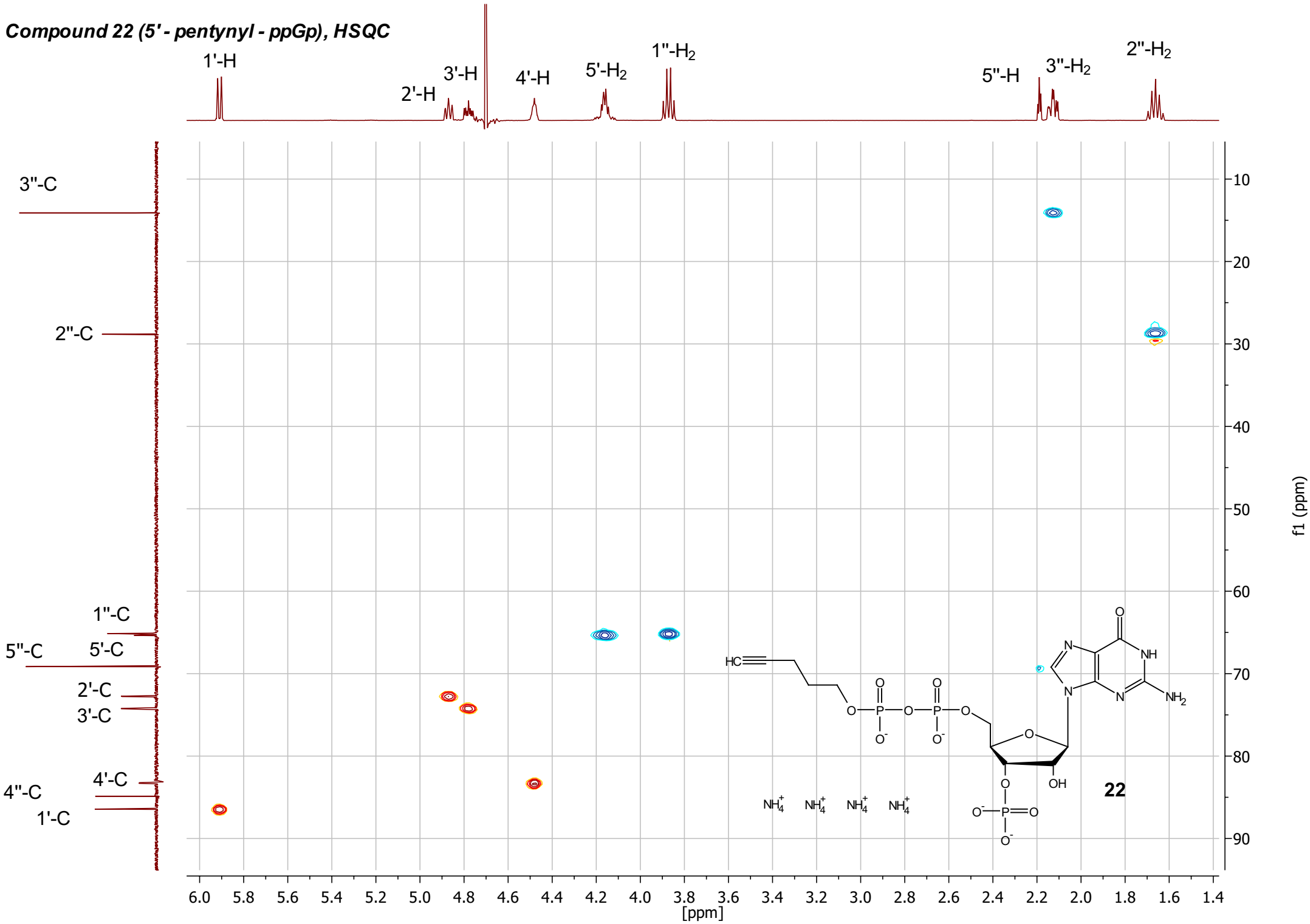


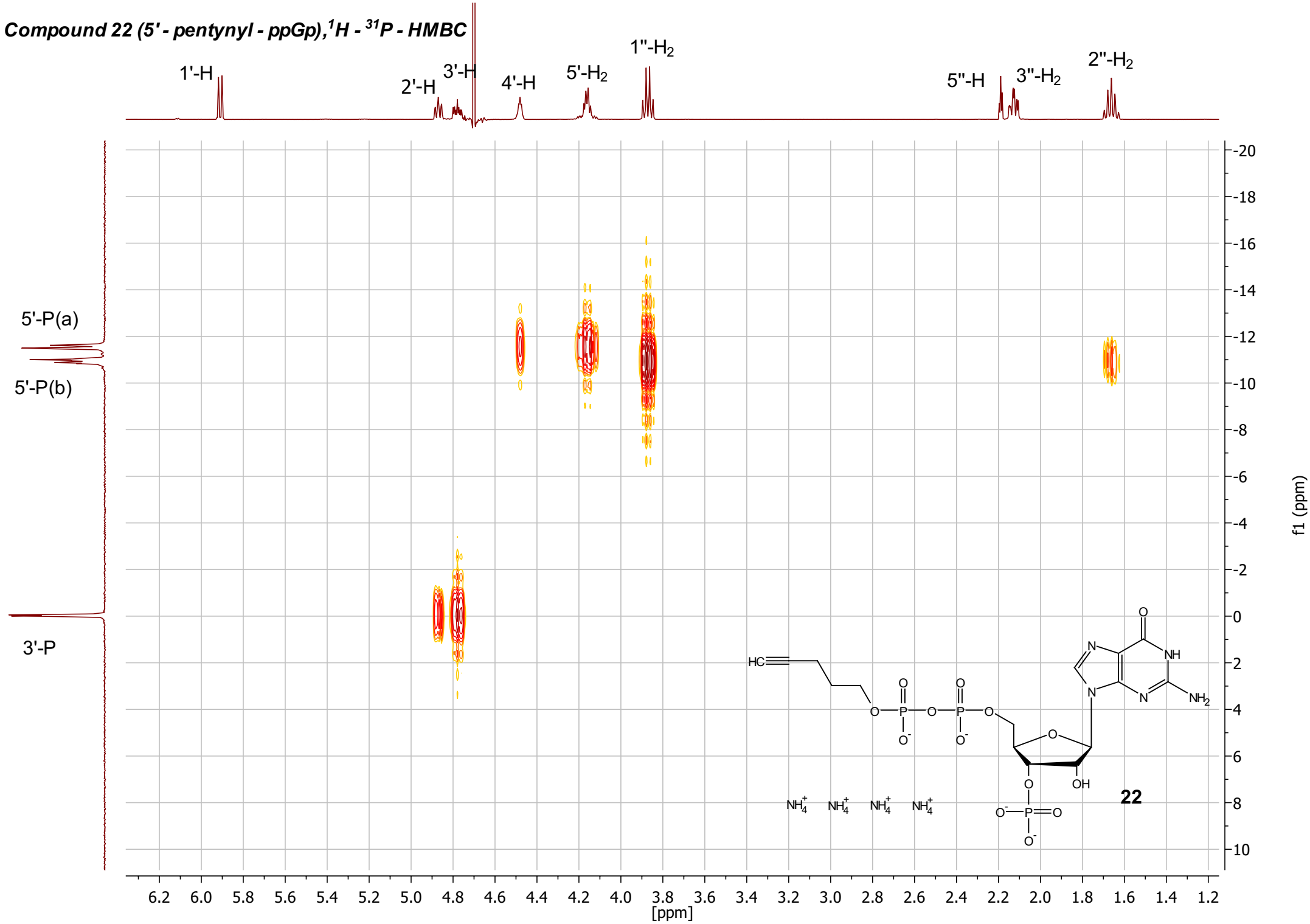
Compound 22 (5' - pentynyl - ppGp), ^{13}C - NMR (D_2O, 101 MHz, detailed)





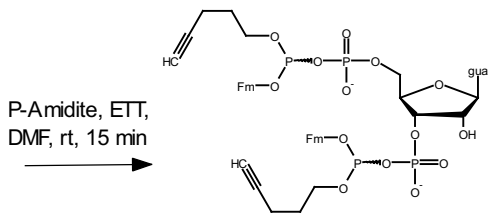

Compound 22 (5' - pentynyl - ppGp), DQF-COSY
1'-H
2'-H
3'-H
4'-H
5'-H2
1"-H2
5"-H
3"-H2
2"-H2
f1 (ppm)
[ppm]
22


Compound 22 (5' - pentynyl - ppGp), HSQC
1'-H
2'-H
3'-H
4'-H
5'-H2
1"-H2
5"-H
3"-H2
2"-H2
3"-C
2"-C
1"-C
5"-C
5'-C
2'-C
3'-C
4'-C
4"-C
1'-C
6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4
[ppm]
10 20 30 40 50 60 70 80 90
f1 (ppm)
22


Compound 22 (5' - pentynyl - ppGp), ^{1}H - ^{31}P - HMBC
1'-H
2'-H
3'-H
4'-H
5'-H$_2$
1"-H$_2$
5"-H
3"-H$_2$
2"-H$_2$
5'-P(a)
5'-P(b)
3'-P
f1 (ppm)
[ppm]
22

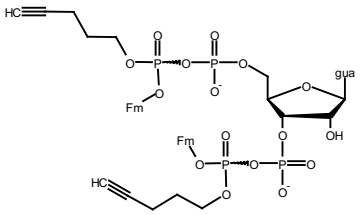
Synthesis of compound 22 (5' - pentynyl - ppGp), reaction monitoring by ^{31}P-NMR (DMF and D_2O, 162 MHz)

9

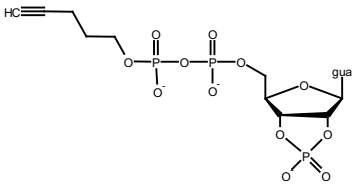


P-Amidite, ETT,
DMF, rt, 15 min

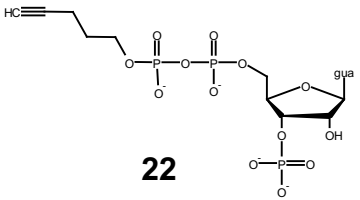
mCPBA, DMF, -20°C-0°C, 15 min



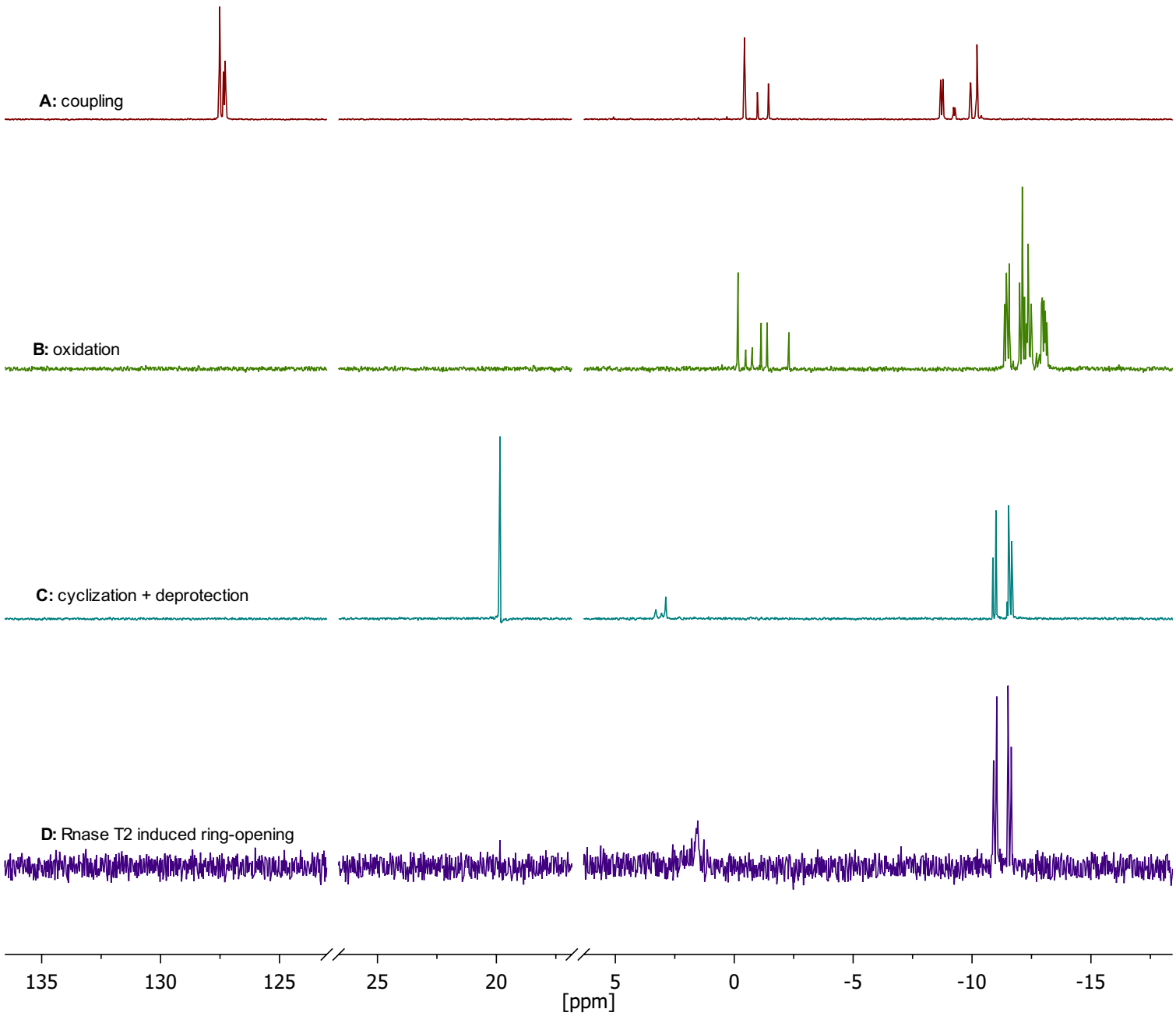
1.) MeOH, rt, 90 min
2.) piperidine, DMF, 0°C-rt, 30 min



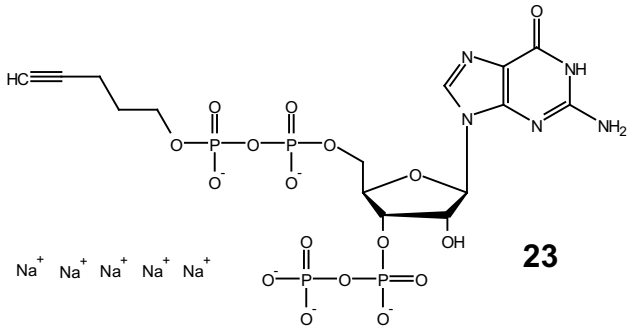
Rnase T2, H_2O, 37°C, 12h



22

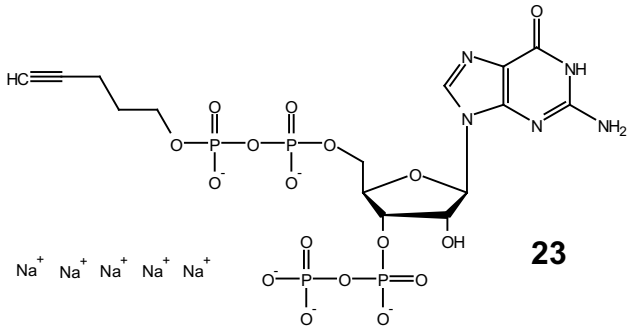


Compound 23 (5' - pentynyl - ppGpp), ^{1}H - NMR (D_2O, 400 MHz)



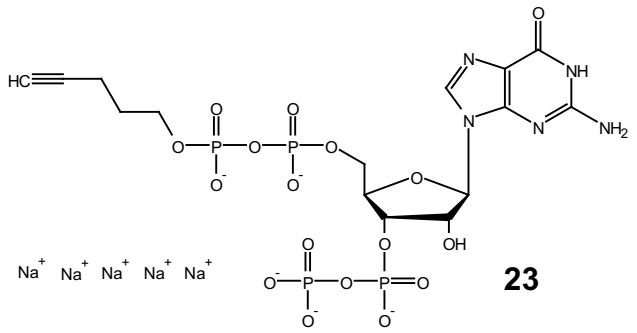
23

Compound 23 (5' - pentynyl - ppGpp), ^{1}H - NMR (D_2O, 400 MHz, water suppression)



23

Compound 23 (5' - pentynyl - ppGpp), ^{1}H [^{31}P] - NMR (D_2O, 400 MHz)



23

D (d)
4.83

C (dd)
4.91

E (d)
4.46

F (m)
4.17

G (dd)
3.82

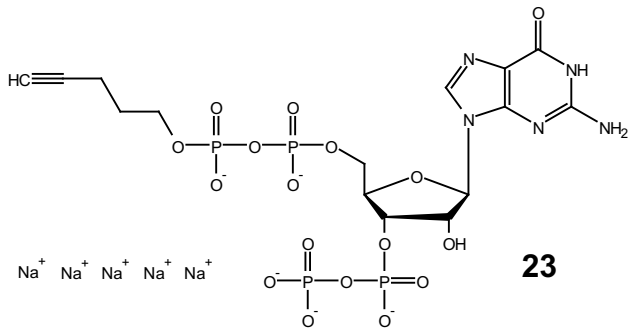
AB - signal

1.43 1.87 1.12 2.23 2.09

5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2

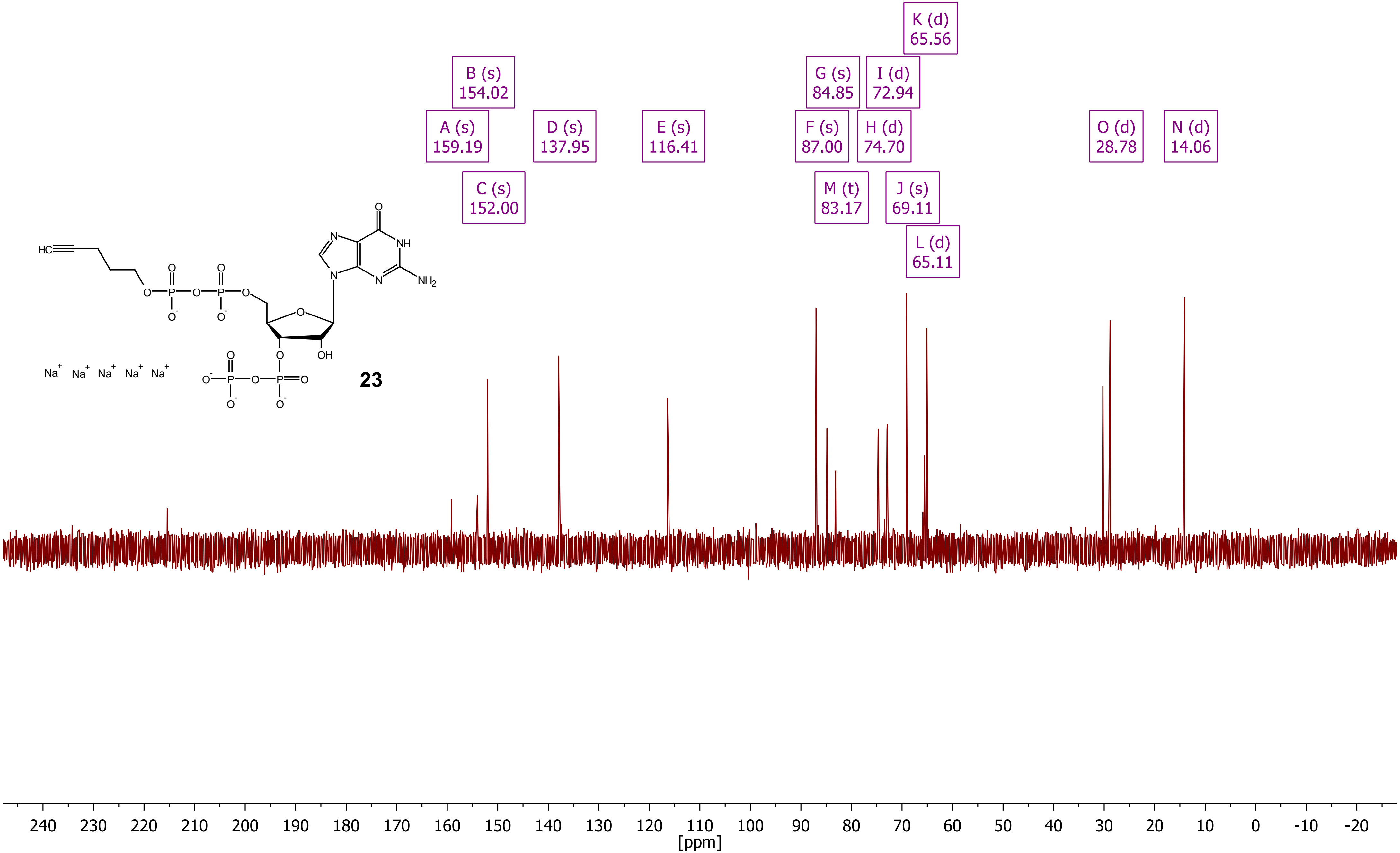
[ppm]

Compound 23 (5' - pentynyl - ppGpp), ^{31}P [^{1}H] - NMR (D_2O, 162 MHz)

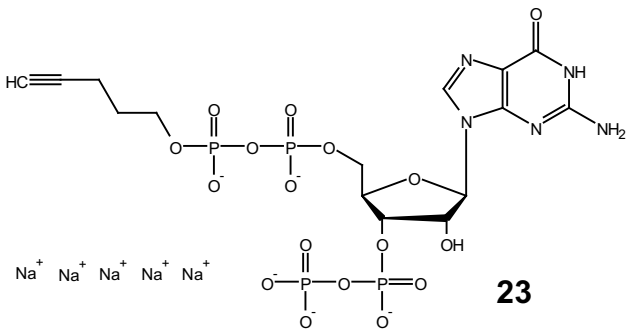


23

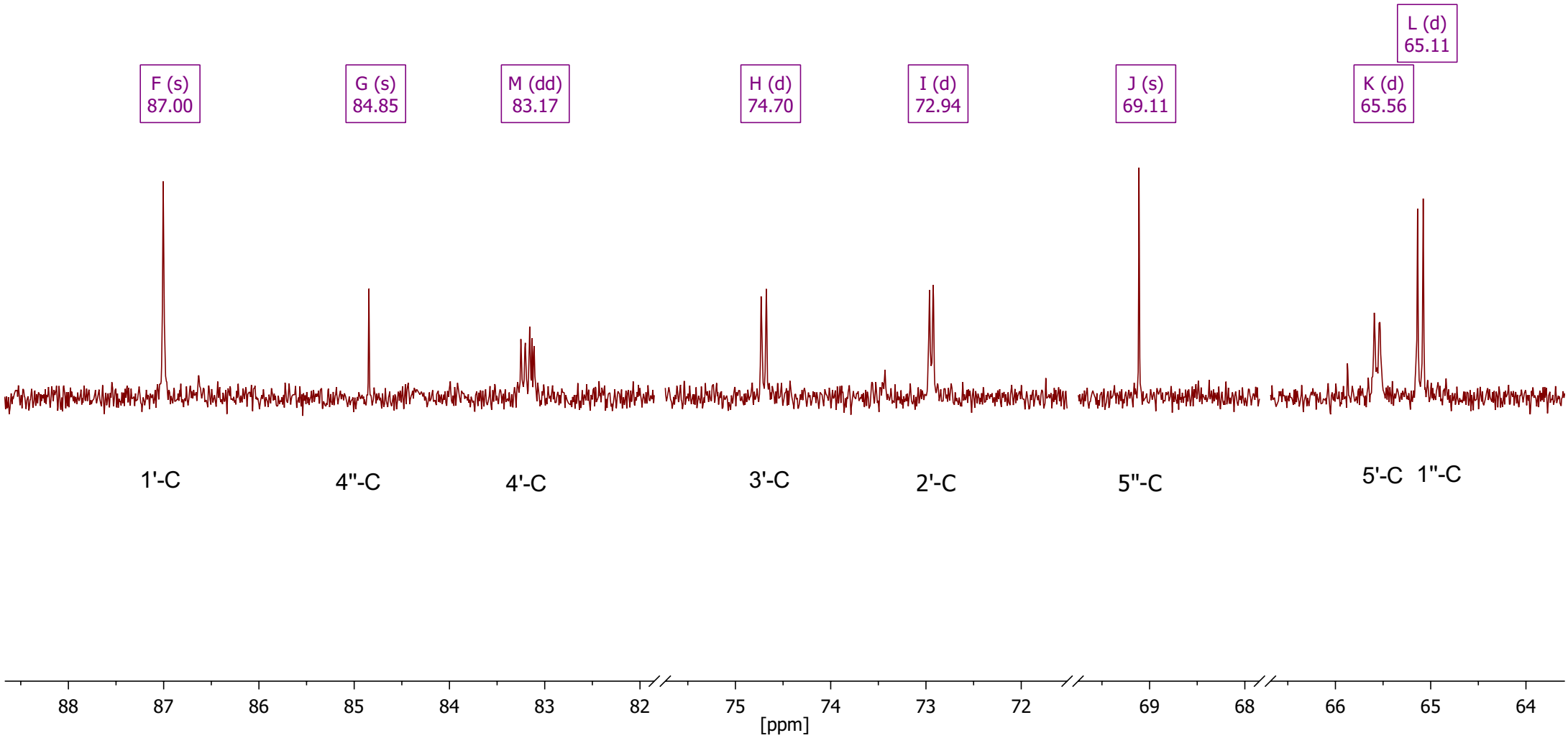
Compound 23 (5' - pentynyl - ppGpp), ^{13}C [^{1}H] - NMR (D_2O, 101 MHz)

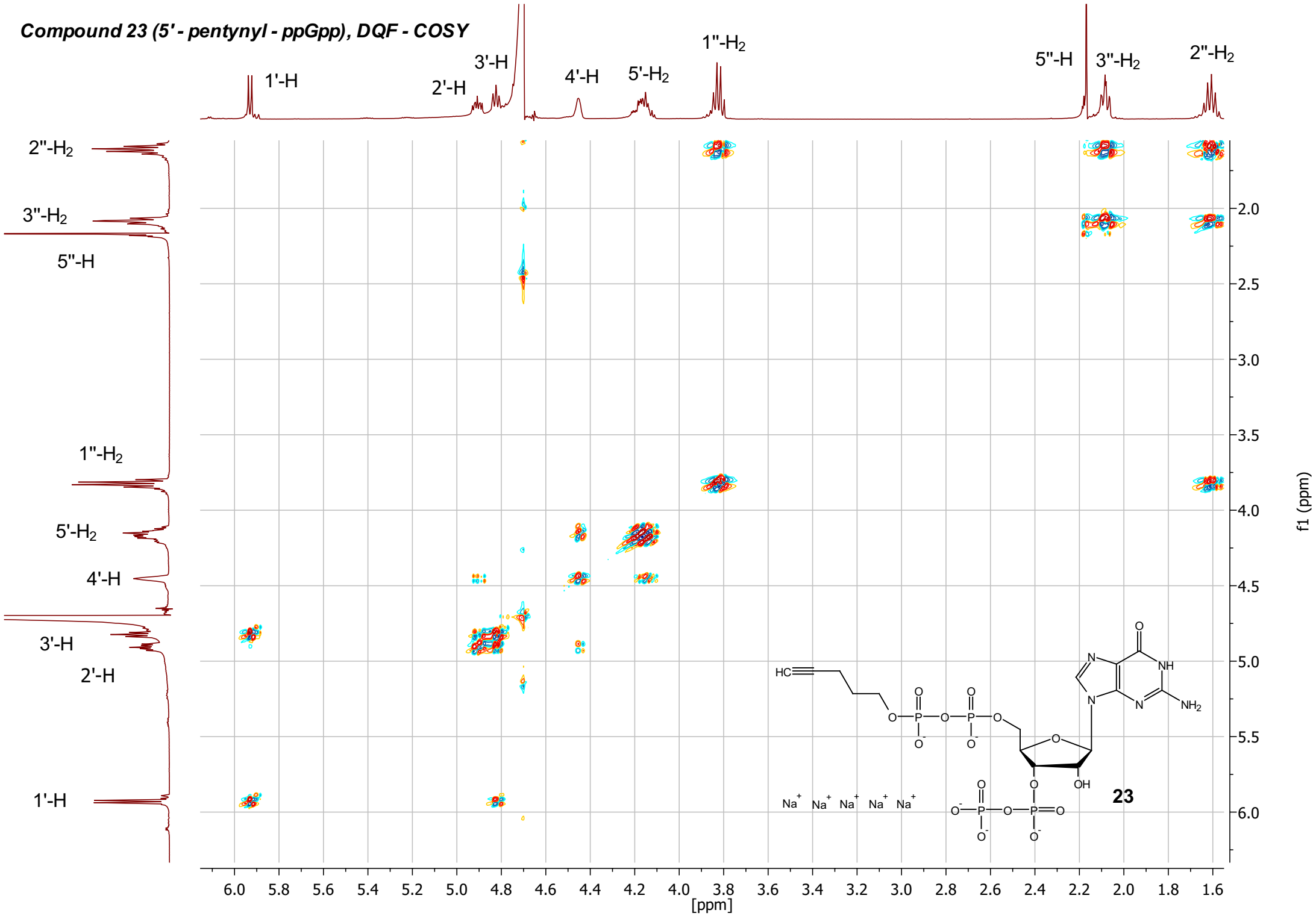


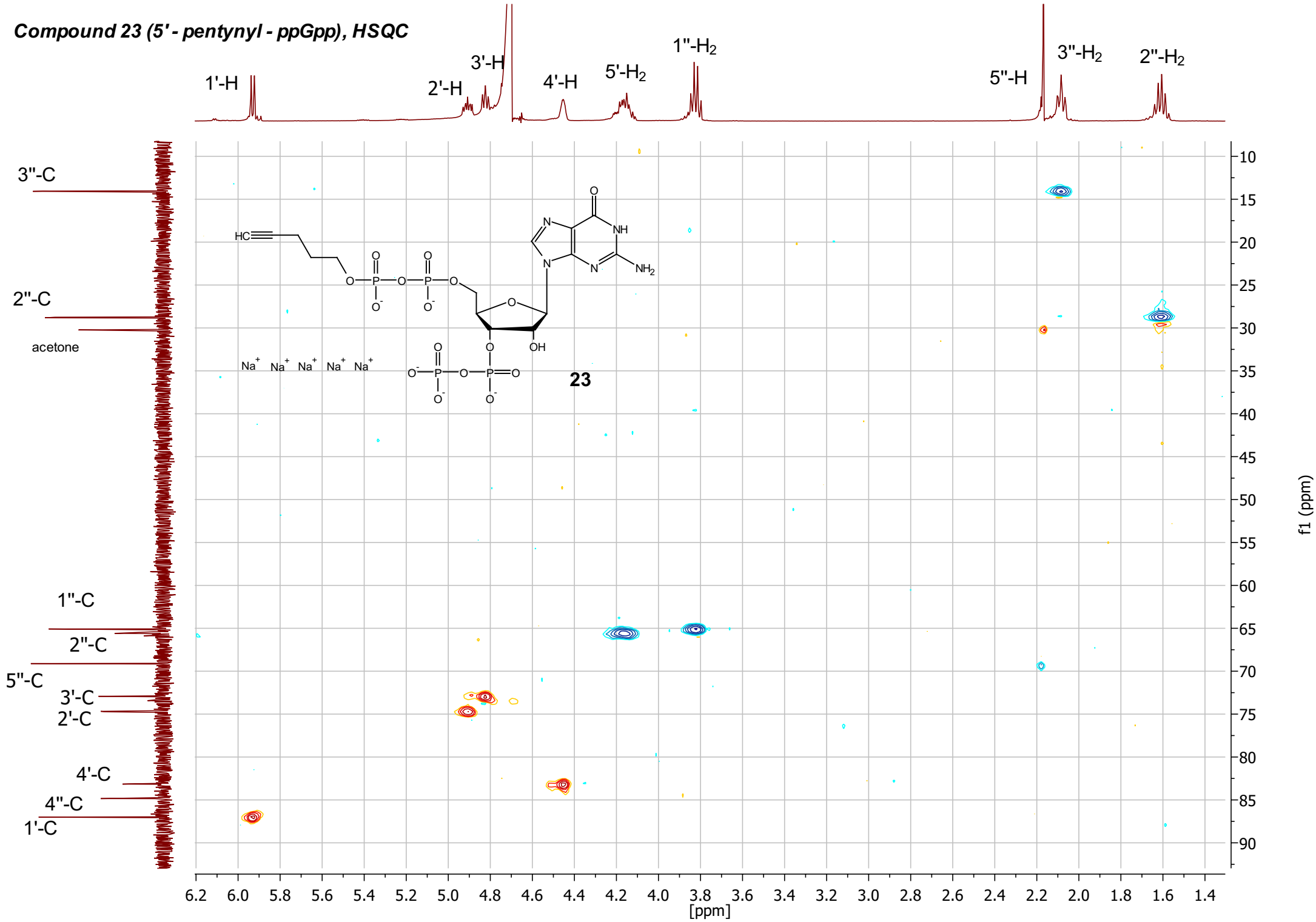
Compound 23 (5' - pentynyl - ppGpp), ^{13}C [^{1}H] - NMR (D_2O, 101 MHz, detailed)

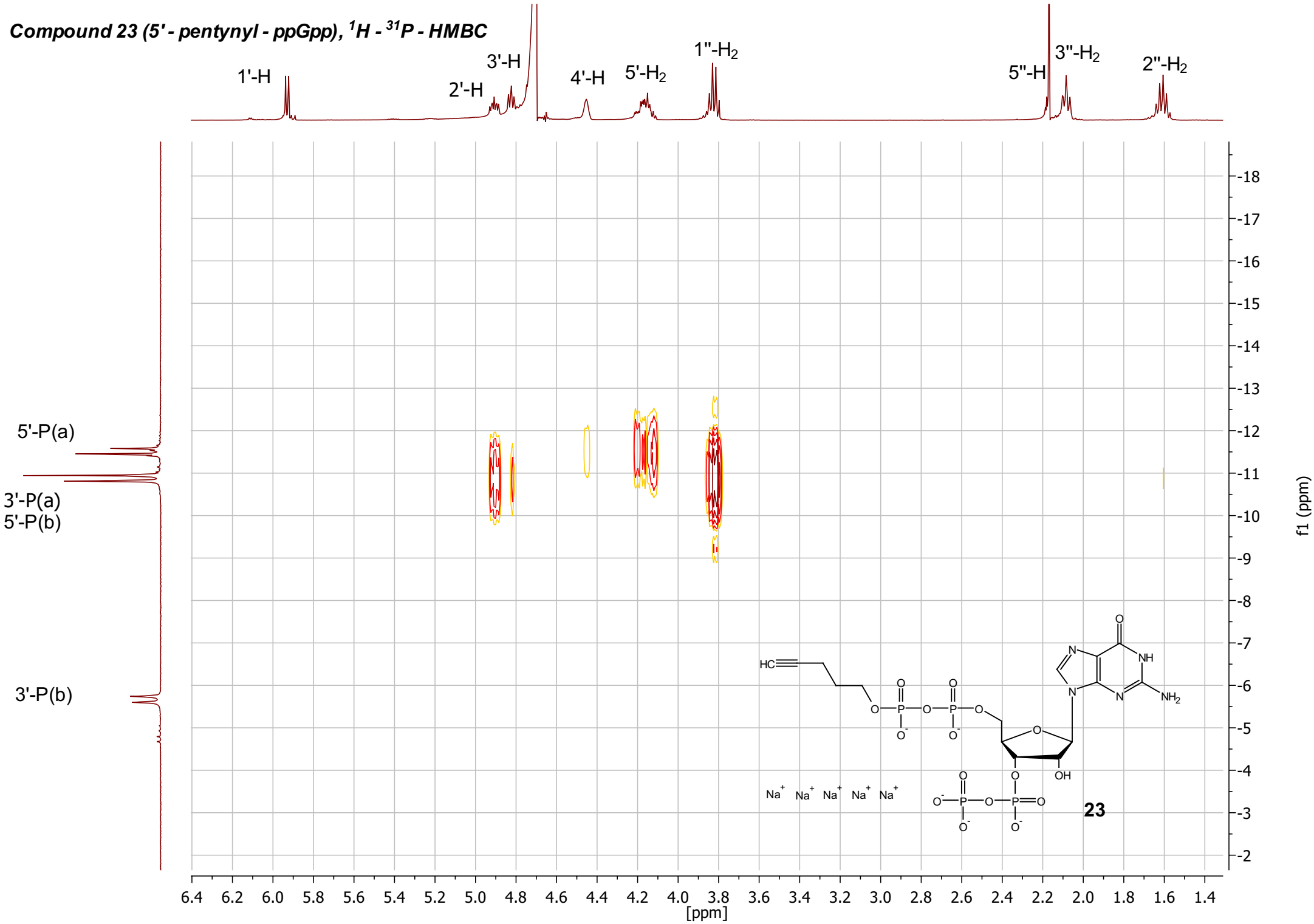


23

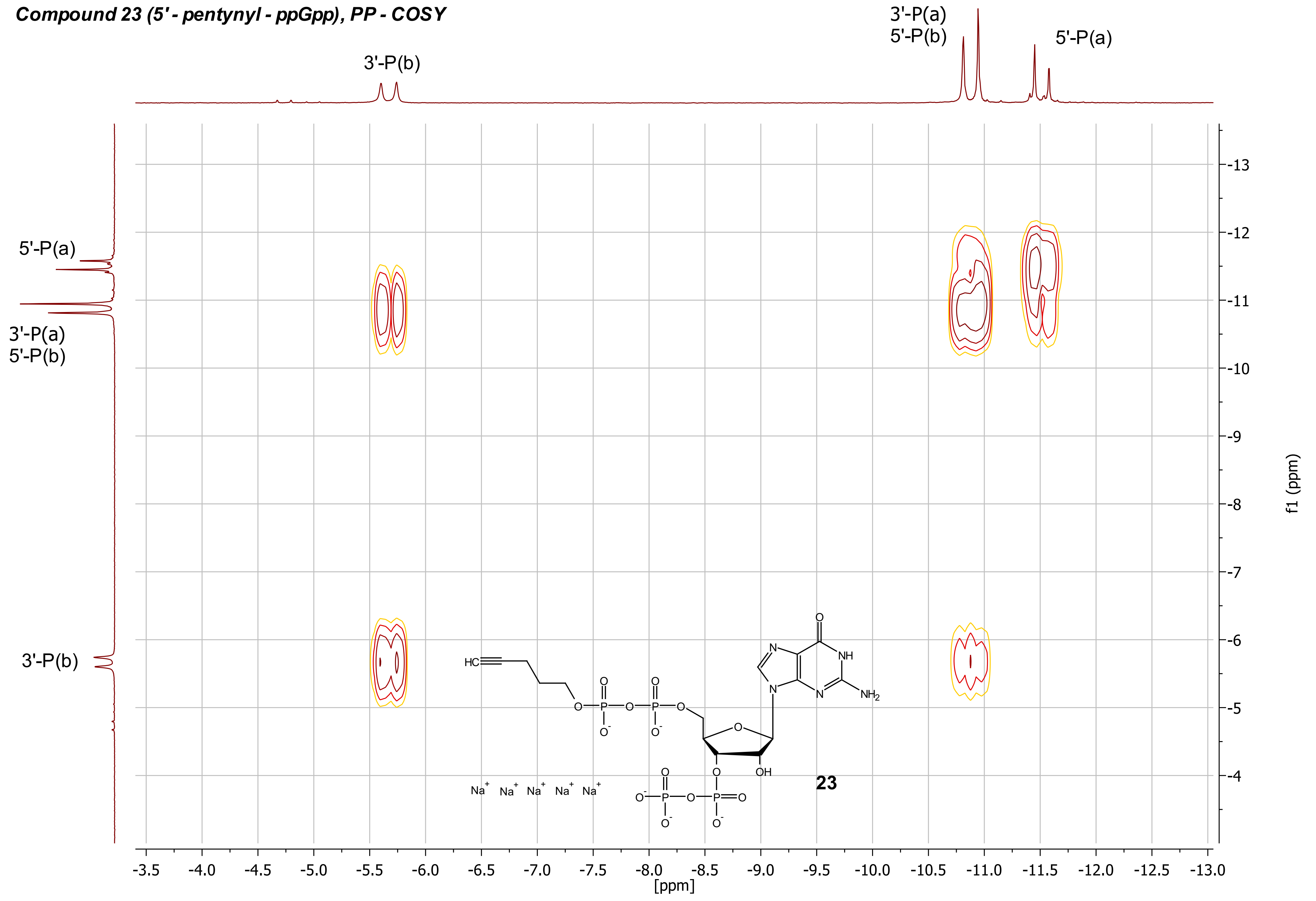



Compound 23 (5' - pentynyl - ppGpp), DQF - COSY
1'-H
2'-H
3'-H
4'-H
5'-H2
1"-H2
5"-H
3"-H2
2"-H2
f1 (ppm)
[ppm]
23

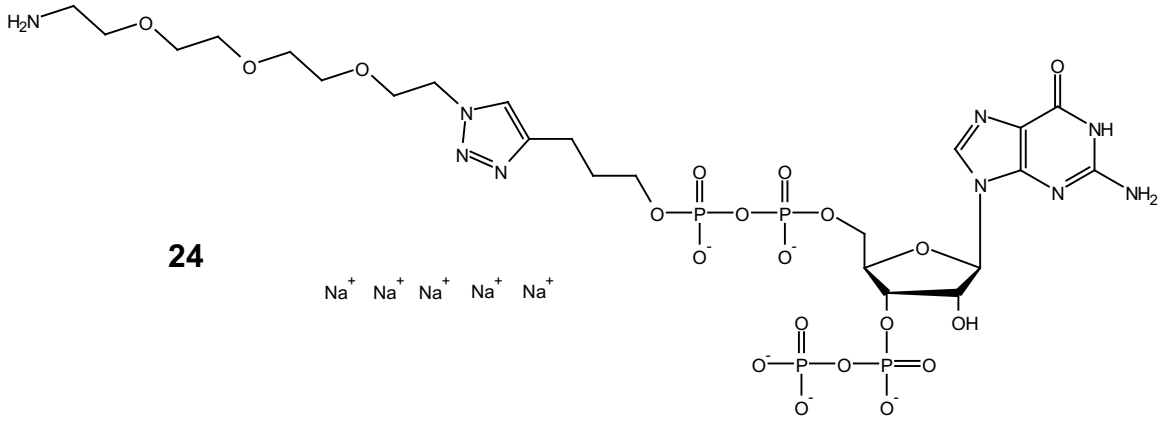

Compound 23 (5' - pentynyl - ppGpp), HSQC
1'-H
2'-H
3'-H
4'-H
5'-H2
1"-H2
5"-H
3"-H2
2"-H2
3"-C
2"-C
acetone
1"-C
2"-C
5"-C
3'-C
2'-C
4'-C
4"-C
1'-C
23
[ppm]
f1 (ppm)


Compound 23 (5' - pentynyl - ppGpp), ^{1}H - ^{31}P - HMBC
1'-H
2'-H
3'-H
4'-H
5'-H$_2$
1"-H$_2$
5"-H
3"-H$_2$
2"-H$_2$
5'-P(a)
3'-P(a)
5'-P(b)
3'-P(b)
f1 (ppm)
[ppm]
23

Compound 23 (5' - pentynyl - ppGpp), PP - COSY



Compound 24 (5'-Amino-PEG-ppGpp), ^{1}H - NMR (D_2O, 400 MHz)



24

acetone

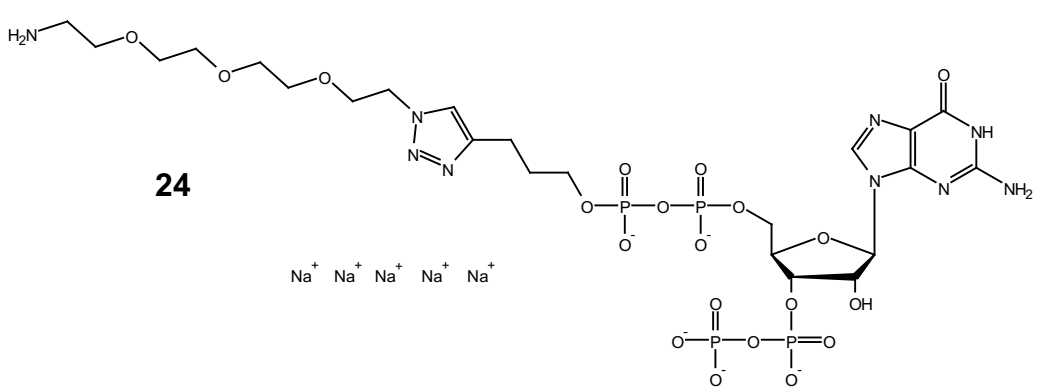
B (s) 7.62
A (s) 8.03
C (s) 5.88
E (s) 4.46
D (s) 4.85
H (m) 3.49
G (m) 3.83
J (s) 2.51
K (s) 1.71
F (m) 4.16
I (s) 3.10

1.04 1.00 1.11 2.75 2.22 4.68 12.10 1.92 2.18 2.28

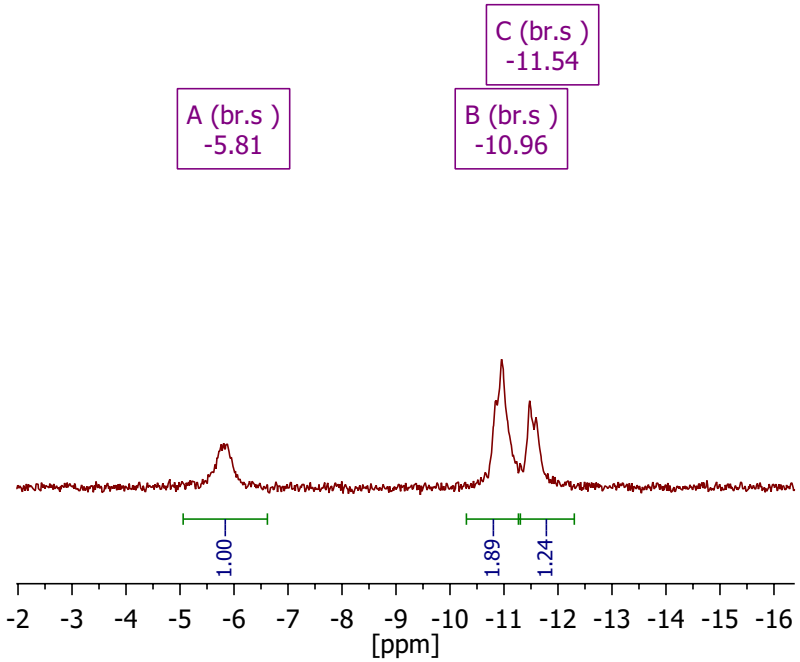
16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3 -4

[ppm]

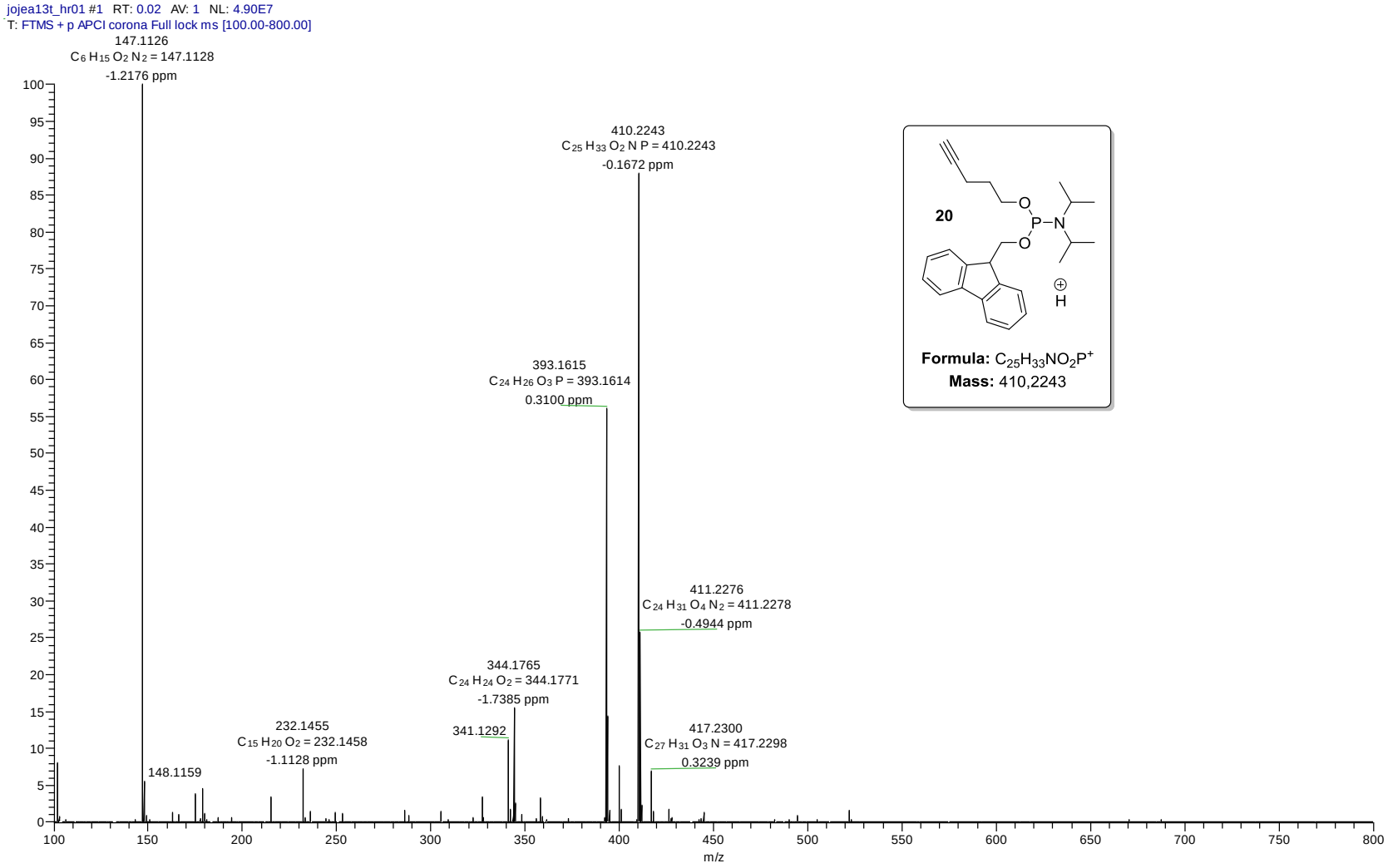
Compound 24 (5'-Amino-PEG-ppGpp), $^{31}P[^{1}H]$ - NMR (D_2O, 162 MHz)



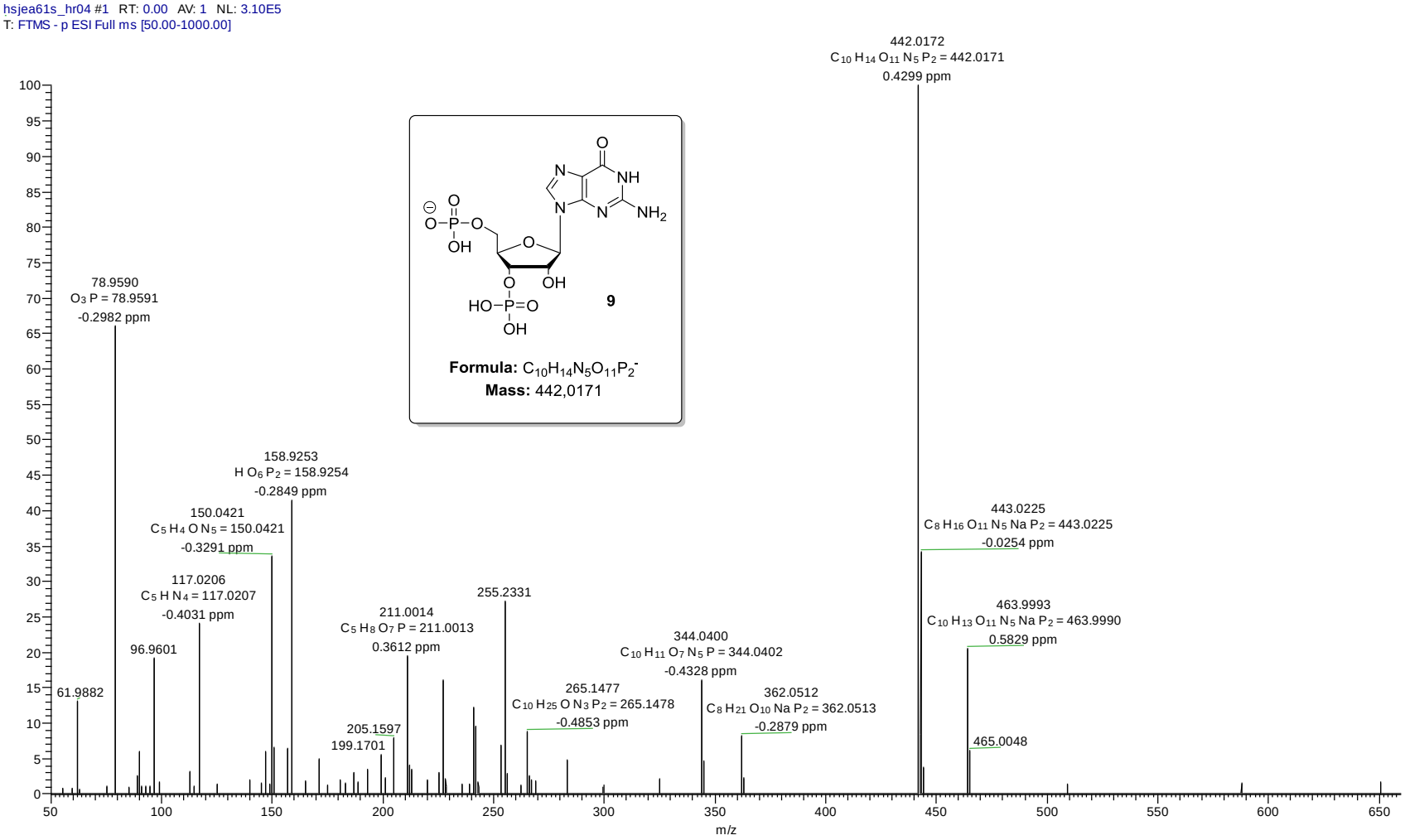
24



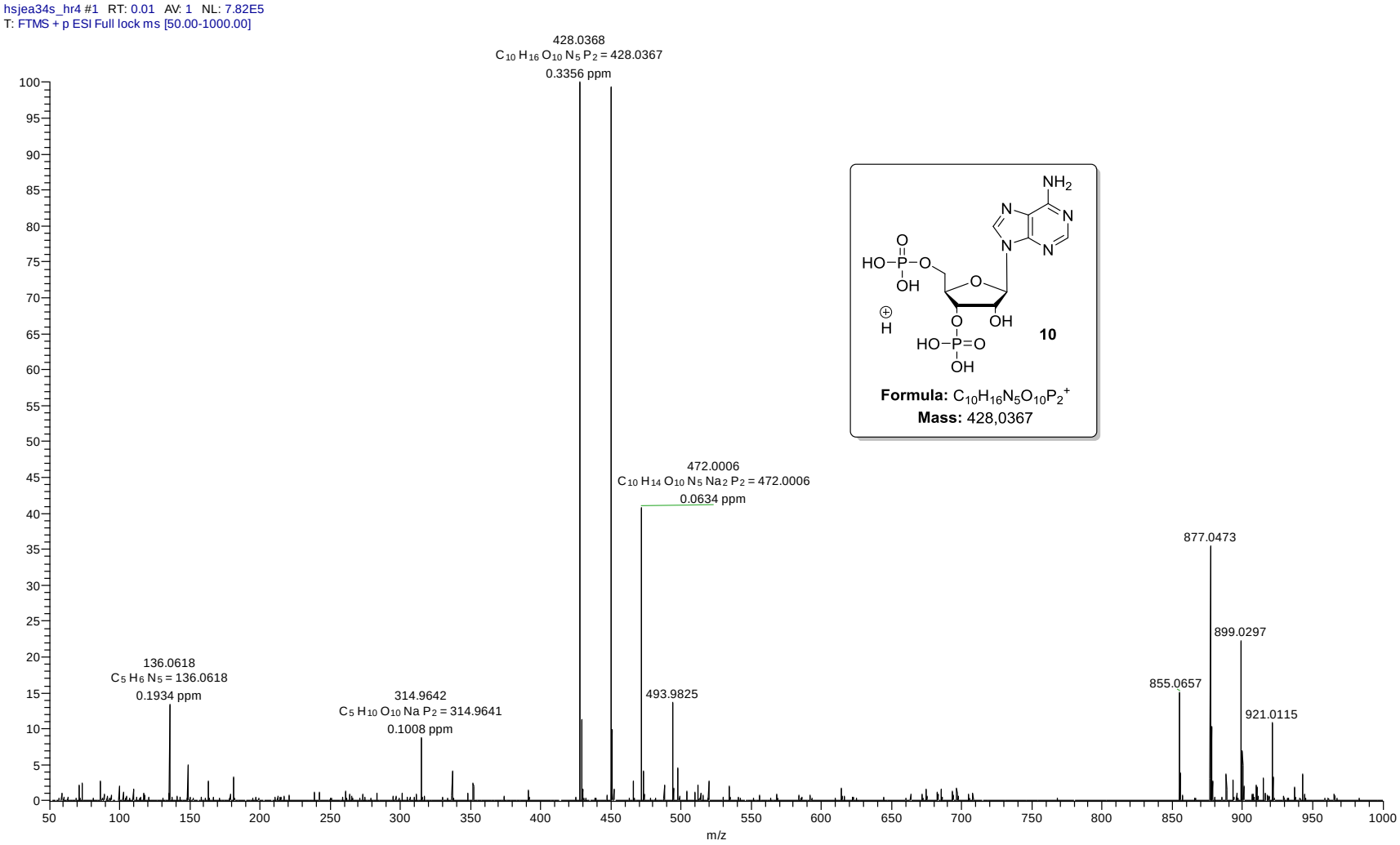
HRMS (ESI) Analysis of compound 20 (PentynO)(FmO)P-N(iPr)$_2$



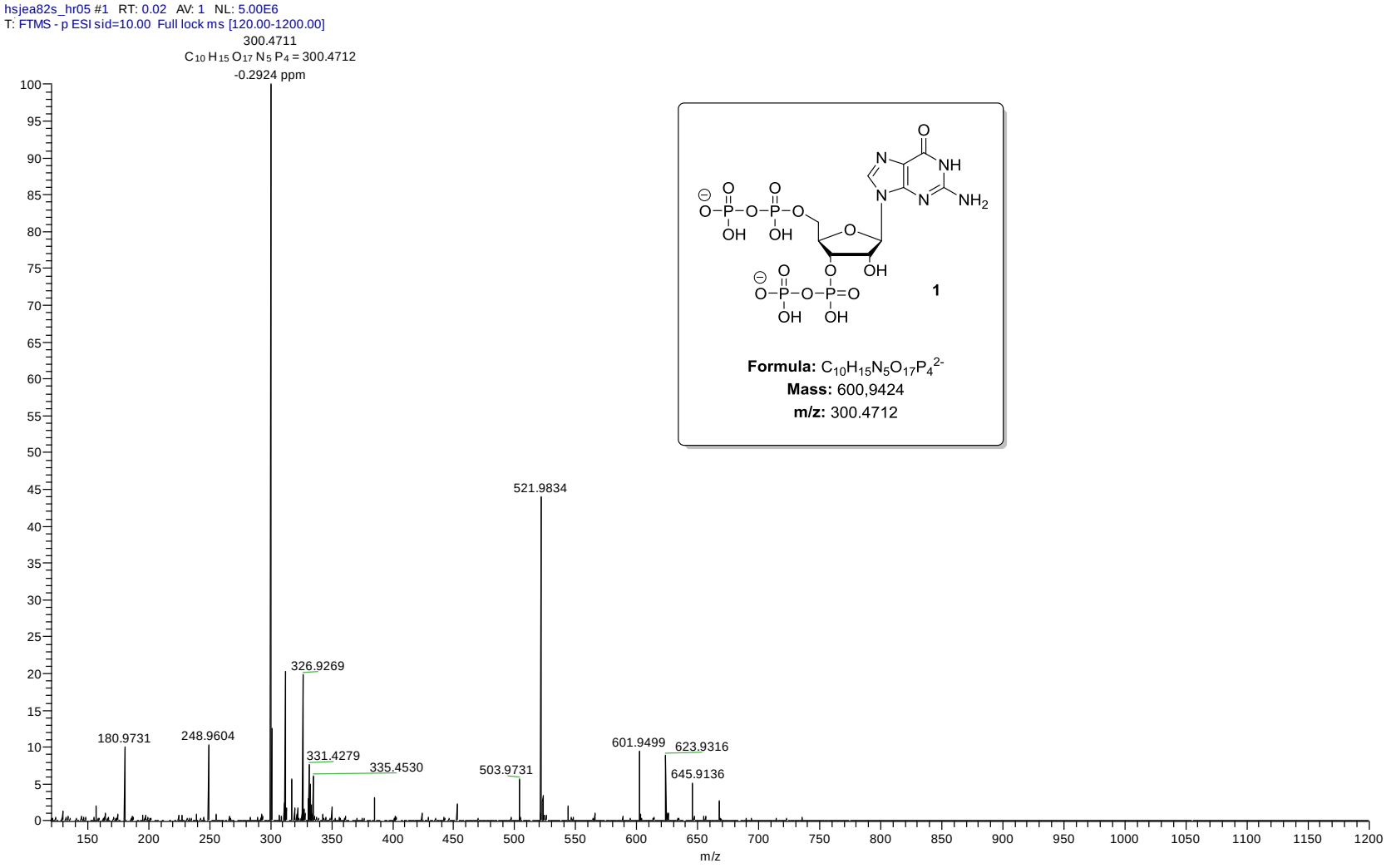
HRMS (ESI) Analysis of compound 9 (pGp)



HRMS (ESI) Analysis of compound 10 (pAp)

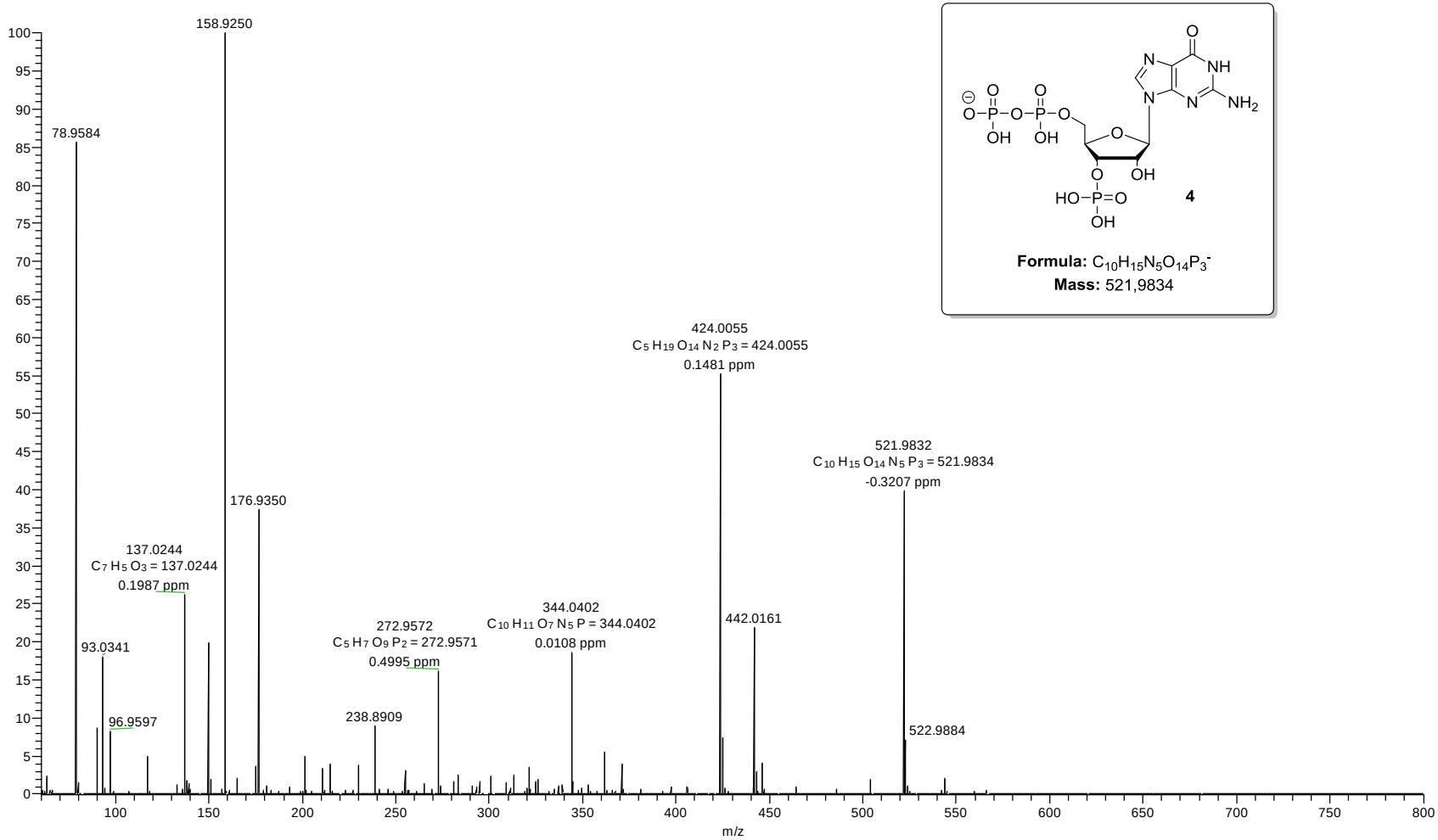


HRMS (ESI) Analysis of compound 1 (ppGpp)



HRMS (ESI) Analysis of compound 4 (ppGp)

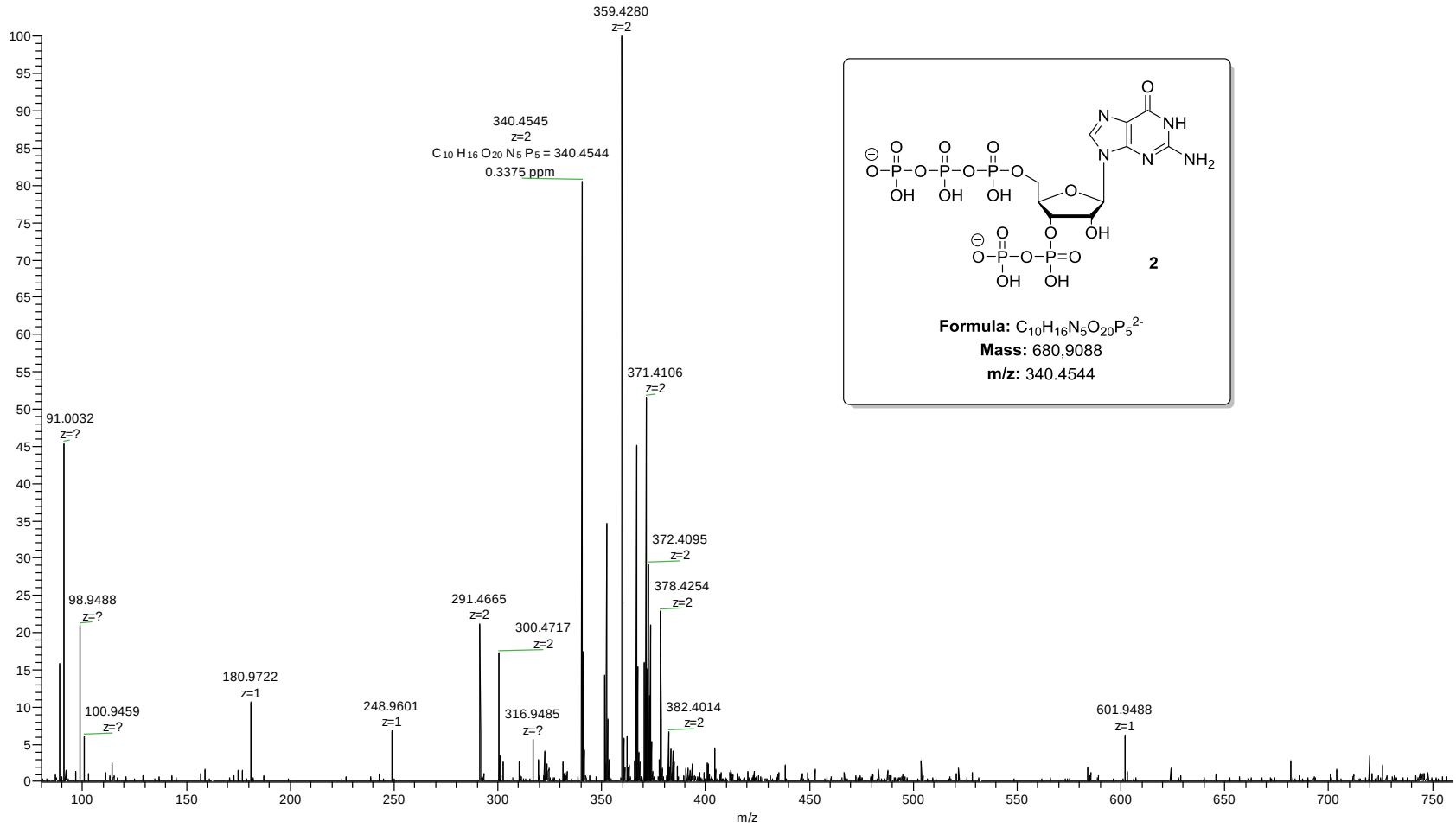
hsbra63s_hr04 #1 RT: 0.01 AV: 1 NL: 2.50E6
T: FTMS - p ESI Full ms [60.00-800.00]



Formula: $C_{10}H_{15}N_5O_{14}P_3^-$
Mass: 521,9834

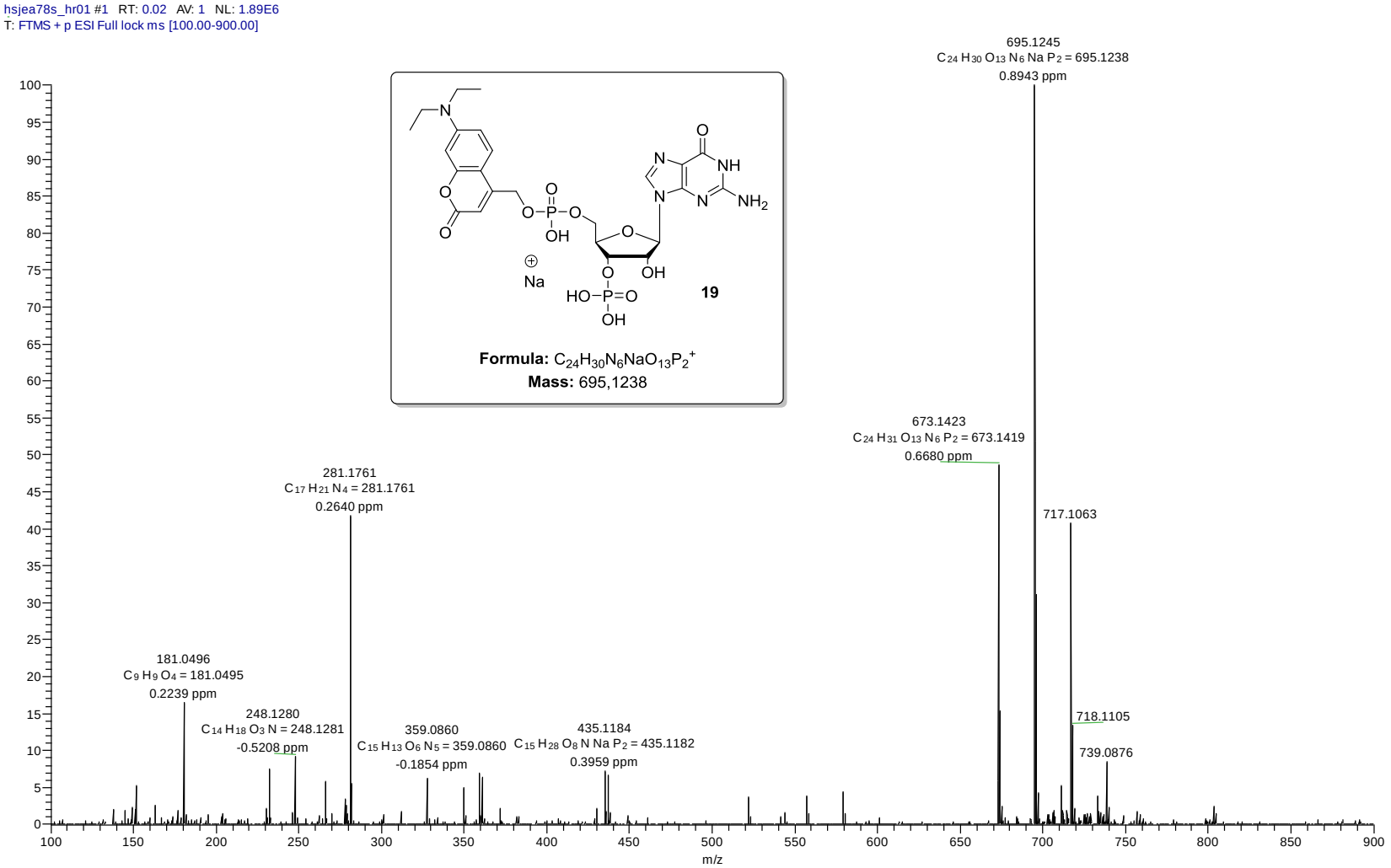
HRMS (ESI) Analysis of compound 2 (pppGpp)

hsjea67s_hr13 #1 RT: 0.00 AV: 1 NL: 6.26E6
T: FTMS - p ESI Full lock ms [80.00-1000.00]

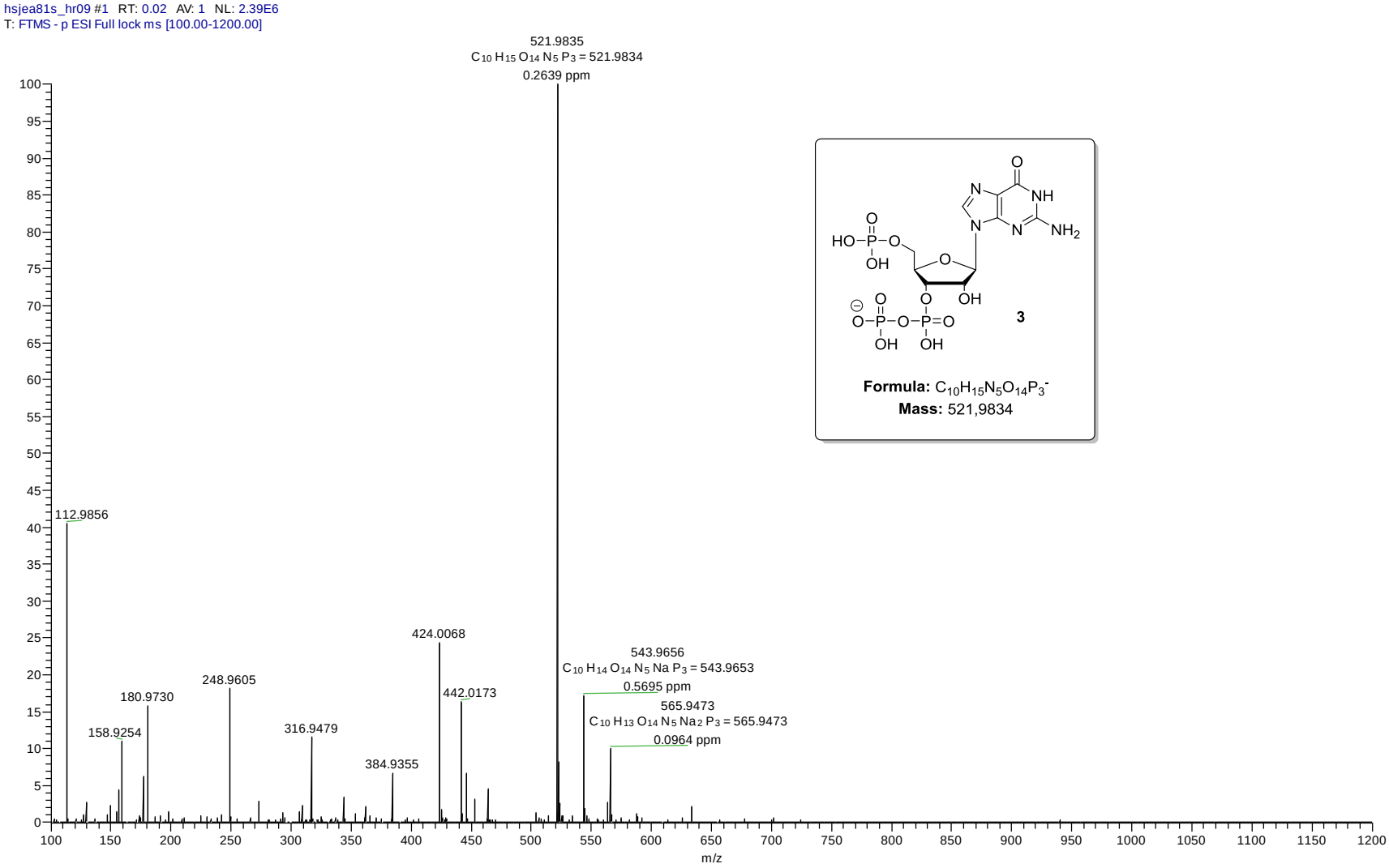


Formula: $C_{10}H_{16}N_5O_{20}P_5^{2-}$
Mass: 680,9088
m/z: 340.4544

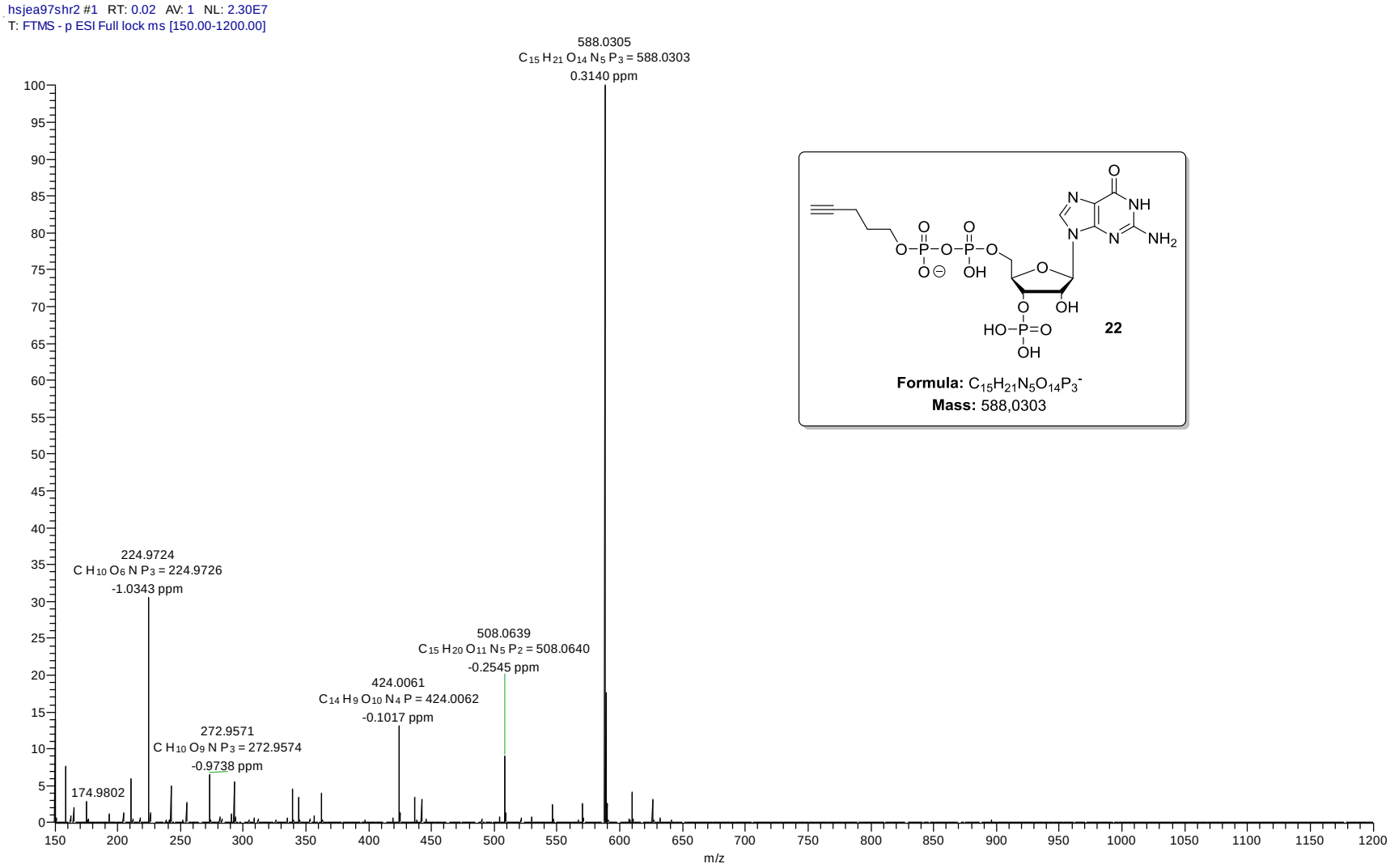
HRMS (ESI) Analysis of compound 19 (DEACM – pGp)



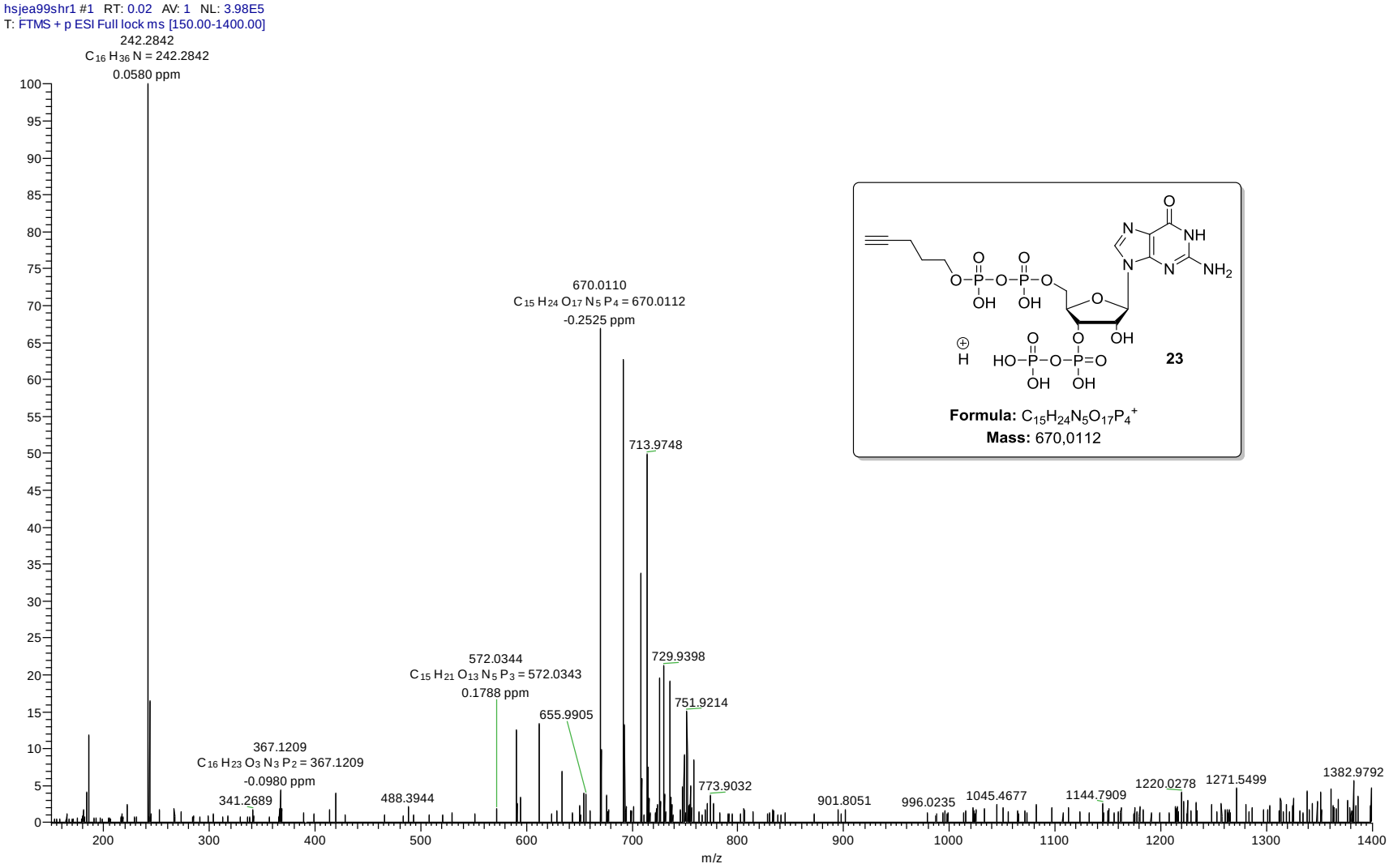
HRMS (ESI) Analysis of compound 3 (pGpp)



HRMS (ESI) Analysis of compound 22 (5'-pentynyl-ppGp)



HRMS (ESI) Analysis of compound 23 (5'-pentynyl-ppGpp)



HRMS (ESI) Analysis of compound 24 (5'-Amino-PEG-ppGpp)