



Supporting Information

The P(III)-Amidite Based Synthesis of Stable Isotope Labeled mRNA-Cap-Structures Enables their Sensitive Quantitation from Brain Tissue

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Supporting Information – Experimental details

Table of Content:

| | | |
|-----|---|----|
| 1 | Synthesis of cap nucleotides | 3 |
| 1.1 | General remarks | 3 |
| 1.2 | Synthesis of c-PyPA (01)..... | 7 |
| 1.3 | Synthesis of methylated adenosine derivatives | 8 |
| 1.4 | Synthesis of diphosphate monoesters | 11 |
| 1.5 | Synthesis of guanosine P-amidite part | 31 |
| 1.6 | Synthesis of cap nucleotides | 48 |
| 2 | LC-MS/MS analytics | 68 |
| 2.1 | Preparation of standard solutions | 68 |
| 2.2 | LC-MS/MS method development..... | 68 |
| 2.3 | Cell and tissue extraction and RNA isolation | 74 |
| 2.4 | LC-MS/MS analysis of cell and tissue samples | 76 |
| 3 | Supporting references | 77 |

Abbreviations:

| | |
|-------------------|---|
| AZT | Azidothymidine |
| DCI | 4,5-Dicyanoimidazole |
| DMEM | Dulbeccos Modified Eagle Medium |
| DMF | Dimethylformamide |
| DMSO | Dimethyl sulfoxide |
| EDTA | Ethylene diamine tetraacetic acid |
| Et ₂ O | Diethylether |
| ETT | 5-(Ethylthio)-1 <i>H</i> -tetrazole |
| Fm | Fluorenylmethyl |
| <i>m</i> CPBA | <i>meta</i> -Chloroperoxybenzoic acid |
| MeCN | Acetonitrile |
| <i>m/z</i> | Mass-to-charge ratio |
| HPLC | High-performance liquid chromatography |
| HRMS | High resolution mass spectrometry |
| IDL | Instrument detection limit |
| LC-MS/MS | Liquid chromatography coupled with tandem mass spectrometry |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometer |
| PP _i | Inorganic Pyrophosphate |
| QQQ | Triple quadrupole |
| RP-MPLC | Reverse phase medium pressure liquid chromatography |
| SAX | Strong anion exchange |
| SILIS | Stable isotope-labeled internal standard |
| SIM | Single-ion monitoring |
| S/N | Signal-to-noise ratio |
| TBA | Tetrabutylammonium |
| TBHP | <i>tert</i> -Butyl hydroperoxide |
| TMS | Trimethylsilyl |
| UHP | Hydrogen peroxide - urea |

1 Synthesis of cap nucleotides

1.1 General remarks

Some reactions were performed under exclusion of oxygen and moisture in oven-dried glassware under dry nitrogen or argon-atmosphere, named as inert-conditions. Reagents were purchased from commercial suppliers and used as received unless noted otherwise. The reported yields are isolated products unless noted otherwise. Crude yields are given from the isolated product without correcting them by side-products, visible from NMR analysis.

Solvents

Solvents were purchased in analytical grade and used without further purification.

Oxidation and hydrolysis sensitive reactions were performed with dry solvents which were stored under an argon-atmosphere.

Diethyl ether (Et₂O), **dichloromethane** (DCM) and **tetrahydrofuran** (THF) were purified using *Braun Solvent Purification System 800* and stored under argon-atmosphere.

Acetonitrile (MeCN) was purchased from Acros (*Acetonitril, 99.9%, Extra dry over Molecular Sieve, AcroSeal™*) and used without further manipulation.

N,N-Dimethylformamide (DMF) was purchased from Sigma-Aldrich (*N,N-Dimethylformamide, anhydrous, 99.8%*).

N,N-Diisopropylethylamine (DIPEA) was distilled under argon-atmosphere and was stored over activated molecular sieve (3 Å) and argon-atmosphere.

Ribonuclease T2 (*RNase T2*) from *Aspergillus oryzae* (50ku) was purchased from Worthington Biochemical Corporation as lyophilized powder and dissolved in a storage buffer [glycerol/NaH₂PO₄ (10 mM, pH 6.8), 1:1]. The stock solution was stored at -20°C.

Reagents

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

Chromatography

Thin layer chromatography (TLC) was performed on *Merck* silica gel 60 F₂₅₄ plates (0.25 mm layer thickness, fluorescence indicator). Visualization was achieved by UV-light ($\lambda = 254$ nm) or with additional staining solutions:

- KMnO₄ (3.0 g), K₂CO₃ (20 g), aq. NaOH (5%, 5 mL), in ddH₂O (300 mL)

Flash chromatography (FCC) was carried out with silica gel 60 from *Macherey-Nagel* (0.04-0.063 mm, 230-400 mesh).

High pressure liquid chromatography (HPLC) was performed on the following devices:

Analytical HPLC-UV: Agilent 1100 (binary pump, autosampler, DAD) or Thermo Scientific Dionex UltiMate 3000 (binary pump, autosampler, DAD)

Method **1-1:** 100 mM aq. TEAA-buffer against MeCN

| | | | | | | | | | | |
|------------|---|---|----|----|----|----|----|----|----|----|
| Time [min] | 0 | 1 | 2 | 3 | 10 | 15 | 16 | 18 | 19 | 25 |
| % of MeCN | 0 | 0 | 10 | 10 | 40 | 40 | 75 | 75 | 0 | 0 |

Method **1-2:** 20mM aq. TEAA-buffer against MeCN

| | | | | | | |
|------------|---|---|----|----|----|----|
| Time [min] | 0 | 2 | 12 | 16 | 17 | 20 |
| % of MeCN | 5 | 5 | 95 | 95 | 5 | 5 |

Method **1-3:** 100mM aq. TEAA-buffer against MeCN

| | | | | | | | | | | | | |
|------------|---|---|----|----|----|----|----|----|----|----|------|----|
| Time [min] | 0 | 1 | 2 | 3 | 4 | 6 | 7 | 8 | 9 | 11 | 11.5 | 15 |
| % of MeCN | 2 | 2 | 10 | 10 | 15 | 15 | 20 | 20 | 70 | 70 | 2 | 2 |

Analytical HPLC-MS (LRMS): Thermo Scientific Dionex UltiMate 3000 (pump, autosampler, columnoven, DAD, Thermo Scientific MSQ Plus)

Method **2-1:** Water against MeCN; isocratic 10% 100mM TEAA-buffer

| | | | | | | |
|------------|---|---|----|------|----|----|
| Time [min] | 0 | 1 | 13 | 16.5 | 17 | 20 |
| % of MeCN | 0 | 0 | 75 | 75 | 0 | 0 |

Method **2-2:** Water against MeCN; isocratic 10% 100mM TEAA-buffer

| | | | | | | |
|------------|---|---|----|------|----|----|
| Time [min] | 0 | 1 | 13 | 16.5 | 17 | 20 |
| % of MeCN | 0 | 0 | 80 | 80 | 0 | 0 |

Preparative HPLC-UV: Knauer AZURA (binary pump, ASM 2.1L, DAD)

Method **3-1:** 100 mM aqueous TEAA-buffer against MeCN

| | | | | | | | | | |
|------------|---|-----|----|----|----|----|----|----|----|
| Time [min] | 0 | 1.5 | 7 | 11 | 13 | 15 | 19 | 20 | 25 |
| % of MeCN | 2 | 2 | 10 | 10 | 15 | 50 | 50 | 2 | 2 |

Method **3-2:** 50 mM aqueous TEAA-buffer against MeCN

| | | | | | | |
|------------|---|---|----|----|----|----|
| Time [min] | 0 | 3 | 20 | 25 | 26 | 30 |
| % of MeCN | 0 | 0 | 70 | 70 | 0 | 0 |

Anion exchange chromatography (AEX) was performed using ÄKTA pure™ system. Crude products were loaded as aqueous solutions and eluted by the following method:

Method **SAX-1:** MilliQ-water against aq. NH_4HCO_3 (1 M)

| | | | | | |
|--------------------|---|---|----|----|-----|
| Column volume [CV] | 0 | 2 | 10 | 11 | 13 |
| % of buffer | 0 | 0 | 40 | 40 | 100 |

Method **SAX-2:** MilliQ-water against aq. NH_4HCO_3 (1 M)

| | | | | | |
|--------------------|---|---|----|------|------|
| Column volume [CV] | 0 | 2 | 4 | 10,5 | 11,5 |
| % of buffer | 0 | 0 | 35 | 70 | 100 |

Medium pressure liquid chromatography (MPLC) was performed using PuriFlash® from interchim®.

For **RP-MPLC:** Crude products were dissolved in TEAA buffer (0.5 M, pH 7) and eluted by the following method:

Method **MPLC-1:** Water against MeCN; isocratic 15% 100mM TEAA-buffer

| | | | |
|--------------------|---|---|----|
| Column volume [CV] | 0 | 1 | 5 |
| % of MeCN | 0 | 0 | 15 |

Method **MPLC-2a:** 100 mM aqueous TEAA-buffer against MeCN

| | | | | | |
|--------------------|---|---|----|----|----|
| Column volume [CV] | 0 | 1 | 5 | 6 | 8 |
| % of MeCN | 0 | 0 | 40 | 75 | 75 |

Method **MPLC-2b**: 100 mM aqueous TEAA-buffer against MeCN

| | | | | | | | | |
|--------------------|---|---|----|----|----|----|----|----|
| Column volume [CV] | 0 | 1 | 4 | 5 | 6 | 7 | 12 | 13 |
| % of MeCN | 2 | 2 | 10 | 10 | 15 | 15 | 80 | 80 |

Method **MPLC-3**: Water against MeCN; isocratic 20% 100mM TEAA-buffer

| | | | | |
|--------------------|---|---|----|----|
| Column volume [CV] | 0 | 1 | 10 | 12 |
| % of MeCN | 0 | 0 | 75 | 75 |

Method **MPLC-4**: Water against MeCN; isocratic 10% 100mM TEAA-buffer

| | | | |
|--------------------|----|----|----|
| Column volume [CV] | 0 | 1 | 7 |
| % of MeCN | 15 | 15 | 90 |

Method **MPLC-5**: 100 mM aqueous TEAA-buffer against MeCN

| | | | | | |
|--------------------|---|---|----|----|----|
| Column volume [CV] | 0 | 1 | 6 | 8 | 11 |
| % of MeCN | 0 | 0 | 25 | 80 | 80 |

Method **MPLC-6**: Water against MeCN; isocratic 15% 100mM TEAA-buffer

| | | | | |
|--------------------|---|---|----|----|
| Column volume [CV] | 0 | 1 | 6 | 7 |
| % of MeCN | 0 | 0 | 25 | 25 |

For **silica-gel MPLC**: Crude products were dry loaded onto silica-gel 60 from *Carl Roth* (0.03-0.2 mm)

Method **MPLC-I**: MeOH against DCM

| | | | | | | |
|--------------------|---|---|---|---|----|----|
| Column volume [CV] | 0 | 1 | 3 | 4 | 7 | 12 |
| % of MeOH | 0 | 0 | 5 | 5 | 10 | 10 |

Method **MPLC-II**: MeOH against DCM

| | | | |
|--------------------|---|----|----|
| Column volume [CV] | 0 | 9 | 20 |
| % of MeOH | 0 | 30 | 30 |

Method **MPLC-III**: MeOH against DCM

| | | | | | | |
|--------------------|---|---|----|----|----|----|
| Column volume [CV] | 0 | 1 | 2 | 5 | 6 | 14 |
| % of MeOH | 0 | 0 | 10 | 10 | 20 | 20 |

Lyophilization

Lyophilization was performed using an *Alpha 2-4 LDplus* from *Christ* or a *VACO 5* from *Zirbus*.

Nuclear Magnetic Resonance (NMR)

NMR spectra were measured on spectrometers from *Bruker*:

Avance III HD 300 MHz, *Avance II 400 MHz* and *Avance III HD 500 MHz*.

The spectra were analyzed with the software *MestReNova 12.0.1*.

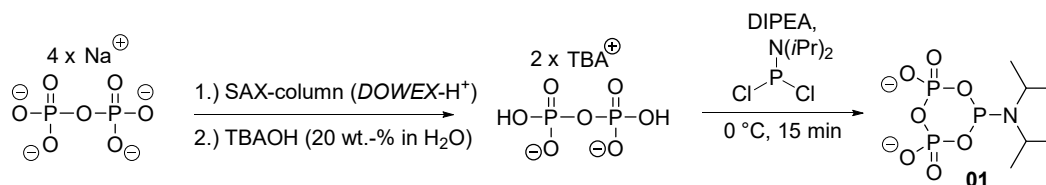
The chemical shift δ was given in part per million (ppm) and the coupling constant J in Hertz (Hz). The common abbreviations for characterization of signal multiplicity were used (s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sx, sextet; sept, septet; m, multiplet; m_c, centered multiplet; br., broad signal).

¹H-NMR and ¹³C-NMR spectra were referenced to an internal solvent signal standard as described in literature.^[1] ³¹P-NMR and ¹⁹F-NMR spectra were referenced to an external standard.

High resolution mass spectra (HRMS)

High resolution mass spectra were recorded in the analytical division of the university of Freiburg, institute of organic chemistry, using a *Thermo LCQ Advantage* (spray voltage: 2.5 – 4.5 kV, spray current: 5 μ A, ion transfer tube: 250 (150) °C, evaporation temp.: 50-400°C)

1.2 Synthesis of c-PyPA (01)



Tetrasodium pyrophosphate (7.2 mmol, 0.53 g, 1.0 eq.) was dissolved in water (30 mL) and passed through a H^+ -activated strong cation exchange column (*DOWEX-H⁺*-column, activation by acidification with aq. HCl (halfconc., 100 mL), then washed to neutral pH with dH₂O (250 mL) before applying the tetrasodium pyrophosphate). The ion exchange was monitored by pH value of the eluate; it was eluted until the pH value was neutral again, this marks the end of the elution of pyrophosphoric acid). TBAOH (20 wt.-% in water, 18.6 mL, 3.7 g, 14.4 mmol, 2.0 equiv.) was added into the eluate. Afterwards, this eluate was lyophilized overnight. The obtained waxy solid was transferred into a 100 mL flask and co-evaporated three times with dry MeCN (3 x 20 mL) on a rotatory evaporator. The residue was further dried under high vacuum for 6 h. Under inert conditions, the residue was then transferred into a 100 mL flask, containing a cross-shaped stirring bar and activated molecular sieve (3 Å), *via* dissolving it in dry MeCN (60 mL) to obtain a 0.12 M solution of (TBA)₂PP_i. (This solution was not used for at least 24 h, to guarantee residual water absorption into the molecular sieve).

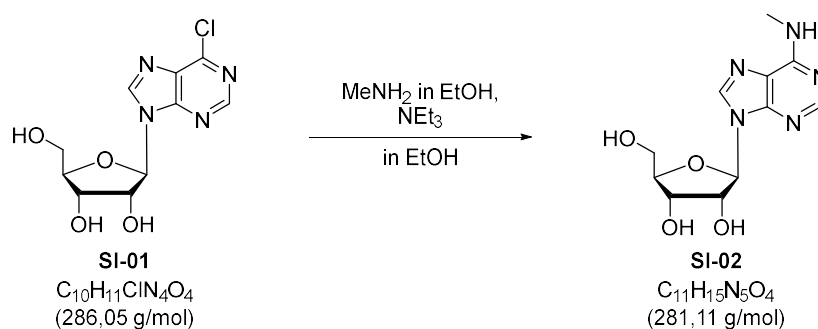
This (TBA)₂PP_i solution was then cooled to 0°C and dry *N,N*-diisopropylamine (DIPEA, 2.6 mL, 15.1 mmol, 2.1 eq) was added (rotation speed of the stirring bar maximum 100rpm to avoid crushing of the molecular sieve). Around 10 min later, dichloro-diisopropylaminophosphine (1.33 mL, 7.20 mmol, 1.0 eq.) was added dropwise within 2 min into the reaction mixture and it was stirred at 100 rpm at 0°C for further 15 min. Afterwards the solution was directly cooled and stored at –20°C until further use. An inert NMR of c-PyPA to check conversion and purity was taken after around 24 h.

Note: Do not let the c-PyPA solution stand for long without cooling, storage at –20°C prevents decomposition. Clean the septum with a paper towel before taking out c-PyPA to prevent condensate water entering *via* syringe.

³¹P{¹H}-NMR (122 MHz, CDCl₃, δ/ppm): 128.4 (t, *J* = 23.6 Hz, 1P), –19.9 (d, *J* = 23.6 Hz, 2P)

1.3 Synthesis of methylated adenosine derivatives

1.3.1 Synthesis of *N*⁶-methyladenosine (**SI-02**)



According to a reported procedure from I. B. YANACHKOV *et al.*^[2]

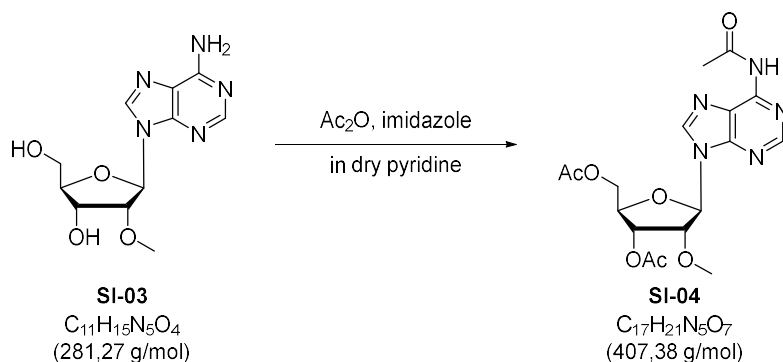
A solution of methylamine in EtOH (33wt%, 0.44 mL, 0.34 g, 3.6 mmol, 1.02 eq.), triethylamine (0.45 mL, 0.33 g, 3.3 mmol, 0.92 eq.) and 6-chloropurine riboside (**SI-01**, 1.0 g, 3.5 mmol, 1.0 eq.) in EtOH (50 mL) was stirred under reflux for 18 h. Afterwards, the solvent was removed under reduced pressure and the obtained residue was recrystallized from EtOH (30 mL). The title compound (**SI-02**, 0.73 g, 2.6 mmol, 74%) was isolated as colorless crystals.

Analytical data are consistent with those reported in the literature.^[3]

¹H-NMR (300 MHz, DMSO-*d*₆, δ /ppm): 8.34 (s, 1H), 8.23 (br. s, 1H), 7.80 (br. s, 1H), 5.89 (d, *J* = 6.2 Hz, 1H), 5.45 – 5.39 (m, 2H), 5.17 (d, *J* = 4.6 Hz, 1H), 4.67 – 4.54 (m, 1H), 4.15 (td, *J* = 4.8, 3.0 Hz, 1H), 3.99 – 3.93 (m, 1H), 3.68 (m, 1H), 3.56 (m, 1H), 2.97 (br. s, 3H).

Note: Recrystallization at 0°C for at least 4 h.

1.3.2 Synthesis of N-acetyl-2'-O-methyl-3',5'-diacetyl adenosine (SI-04)



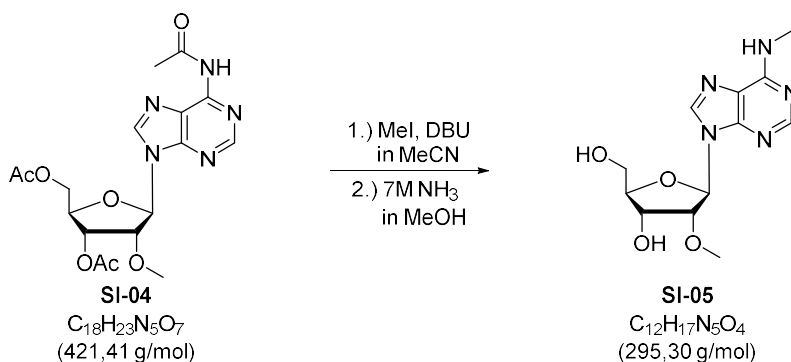
According to a reported procedure from V. I. TARAROV *et al.*^[4]

Ac₂O (1.4 mL, 1.5 g, 14 mmol, 8.0 eq.) was added to a mixture of 2'-OMe adenosine (**SI-03**, 0.5 g, 1.8 mmol, 1.0 eq.) in dry pyridine (4 mL) and it was stirred at r.t. overnight. Afterwards, the resulting solution was stirred at 60°C for 8 h. Next, EtOH was added and the solvent was removed under reduced pressure. Traces of pyridine were removed by co-evaporation with MeOH:EtOH (1:1, 2 x 15 mL) and EtOH (2 x 15 mL). The obtained colorless residue was dissolved in MeOH (5 mL) and after adding imidazole (95 mg, 1.4 mmol, 0.8 eq.) the mixture was stirred at r.t. for 5 h. Afterwards, EA (50 mL) was added and it was transferred into a separatory funnel. The organic phase was washed with brine (4 x 20 mL), dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was co-evaporated with DCM (2 x 5 mL) and the title compound (**SI-04**, 630 mg, 1.5 mmol, 87%) was obtained as a colorless foam.

Analytical data are consistent with those reported in the literature.^[5]

¹H-NMR (300 MHz, CDCl₃, δ/ppm): 8.68 (s, 1H), 8.67 (s, 1H), 8.16 (s, 1H), 6.10 (d, *J* = 5.1 Hz, 1H), 5.44 – 5.36 (m, 1H), 4.74 (t, *J* = 5.2 Hz, 1H), 4.51 – 4.31 (m, 3H), 3.43 (s, 3H), 2.64 (s, 3H), 2.19 (s, 3H), 2.13 (s, 3H).

1.3.3 Synthesis of *N*⁶-2'-*O*-diethyladenosine (**SI-05**)



Iodomethane (0.14 mL, 0.33 g, 2.3 mmol, 1.5 eq.) and DBU (0.46 mL, 0.47 g, 3.1 mmol, 2.0 eq.) were added into a solution of *N*-acetyl-2'-*O*-methyl-3',5'-diacetyl adenosine (**SI-04**, 630 mg, 1.5 mmol, 1.0 eq.) in MeCN (6 mL). It was stirred at r.t. for 10 h. The same amount of iodomethane and DBU was re-added, since reaction control *via* HPLC-MS revealed not complete conversion. After completeness, the reaction mixture was diluted with $CHCl_3$ (10 mL) and it was transferred into a separatory funnel. It was washed with aq. HCl (1.0 M, 10 mL), brine (3 x 20 mL) and it was dried over $MgSO_4$. The product was obtained as a colorless powder (590 mg). Half of it was dissolved in NH_3 in MeOH (7 N, 2.0 mL) and the mixture was stirred at r.t. for 48 h. Afterwards, it was diluted with dd H_2O and the mixture was lyophilized. The title compound (**SI-05**, 170 mg, 0.58 mmol, 95%) was isolated as colorless solid.

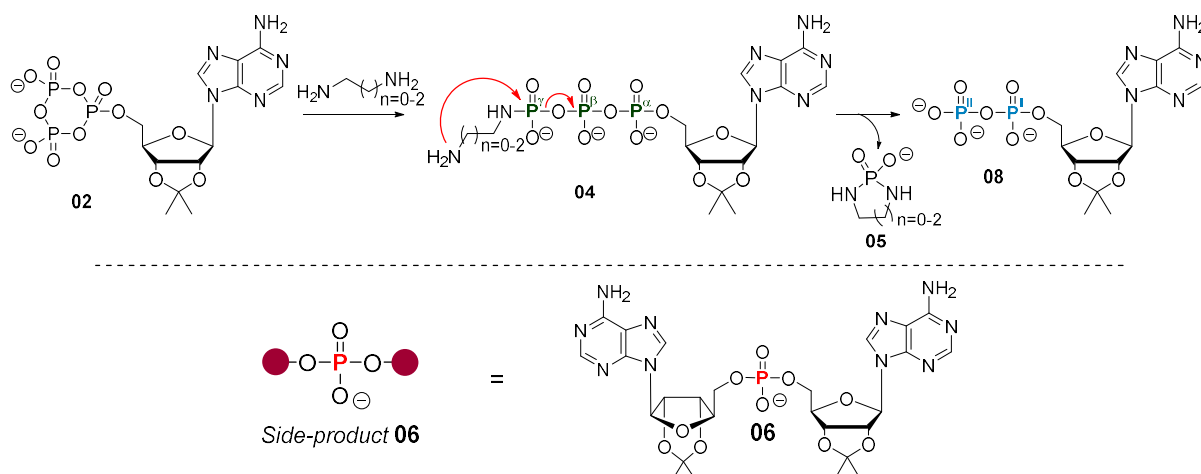
Analytical data are consistent with those reported in the literature.^[6]

¹H-NMR (300 MHz, $CDCl_3$, δ /ppm): 8.38 (s, 1H), 8.24 (s, 1H), 7.83 (s, 1H), 6.01 (d, J = 5.8 Hz, 1H), 5.42 (dd, J = 6.9, 4.7 Hz, 1H), 5.27 (d, J = 5.0 Hz, 1H), 4.43 – 4.27 (m, 2H), 3.99 (mc, 1H), 3.75 – 3.48 (m, 2H), 3.31 (s, 3H), 2.97 (s, 3H).

1.4 Synthesis of diphosphate monoesters

1.4.1 Mechanistic analysis for nucleoside diphosphate synthesis:

We had a closer look into the diphosphate synthesis *via* a cyclo-triphosphate intermediate, while using ethylenediamine for linearization. 2',3'-O-isopropyliden-adenosine was our model compound, as shown in Scheme SI-1.



Scheme SI-1: Top: Analysis of linearization and dephosphorylation, while applying different diamines. bottom: The molecular structure of side-product **06**.

Therefore, the reaction was tracked by $^{31}\text{P}\{^1\text{H}\}$ -NMR spectroscopy every 30 min over several hours. The amount of forming diphosphate was calculated by the integral relation between the linearized triphosphate **04** and the desired diphosphate **08** according following equation:

$$\text{Conversion to diphosphate} = \frac{\text{Integral} (P^I)}{[\text{Integral} (P^a) + \text{Integral}(P^I)]}$$

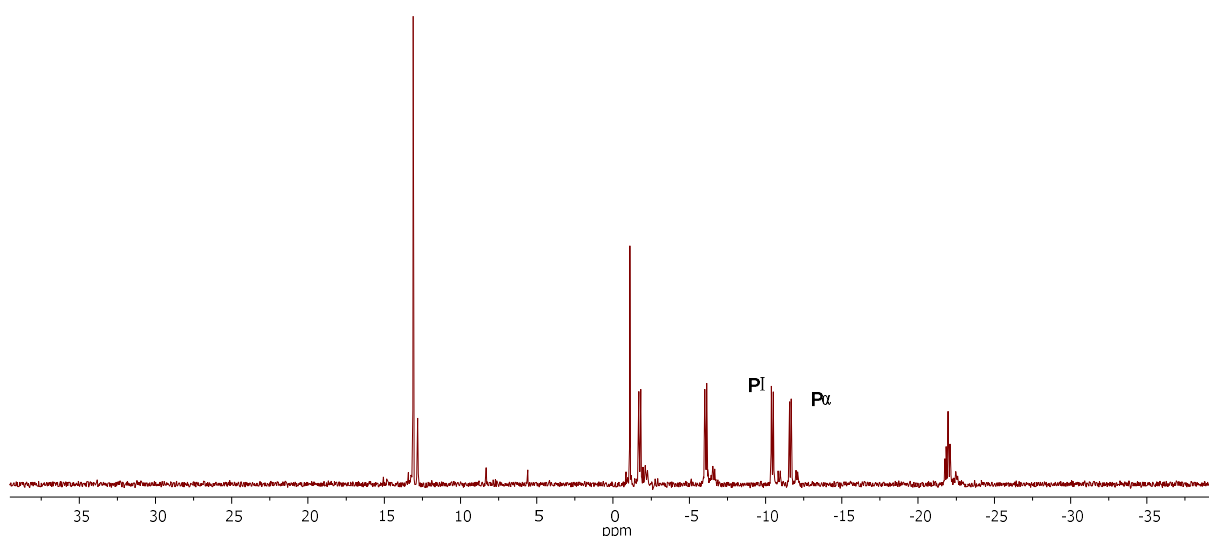


Figure SI-1: $^{31}\text{P}\{^1\text{H}\}$ -NMR spectra (162 MHz) measured in CD_3CN at 298 K.

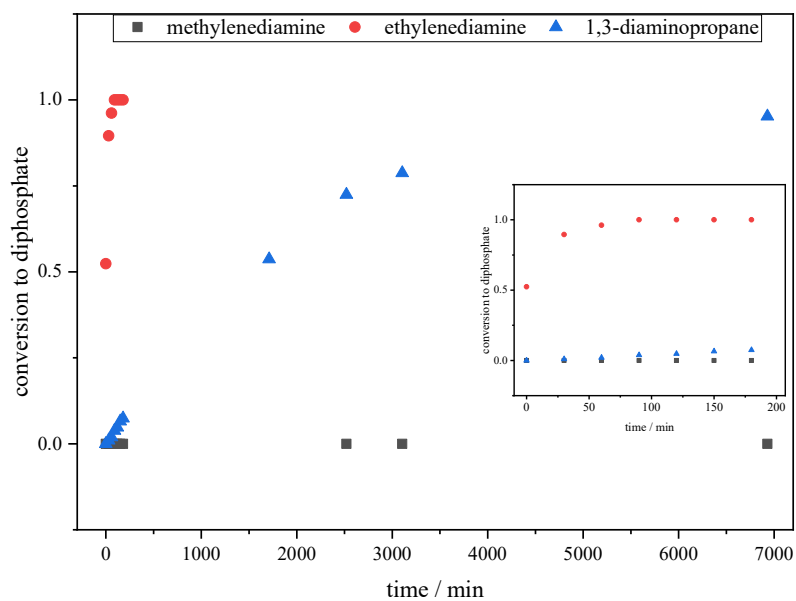


Figure SI-2: Reaction monitoring - Conversion from triphosphate **04** to diphosphate **08** over time for different diamines (20 eq.).

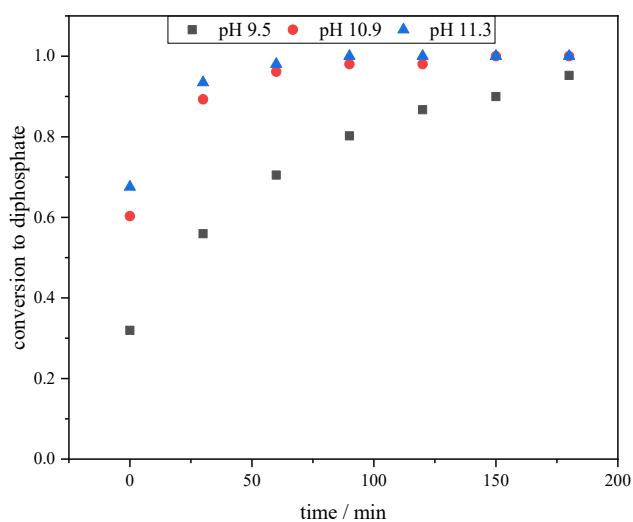


Figure SI-3: Reaction monitoring - Conversion from triphosphate **04** to diphosphate **08** over time for 20 eq. of diethylenediamine in reaction mixtures with different pH values.

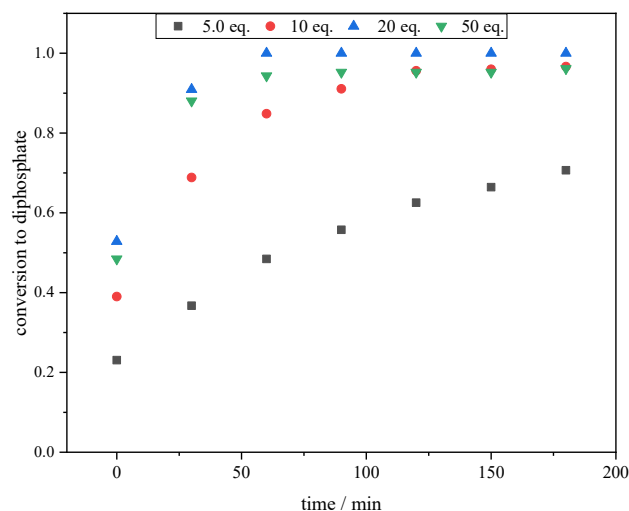


Figure SI-4: Reaction monitoring - Conversion from triphosphate **04** to diphosphate **08** over time for different equivalents of diethylenediamine.

Next, the conversion of the reaction and the formation of the monophosphoate diester (**06 = R-P-R**) was investigated by RP-HPLC.

A: Commercial 2',3'-O-isopropyliden-adenosine (1.0 eq.), *c*-PyPA (**1.0 eq.**), *m*CPBA (2.0 eq.) in dry MeCN. Afterwards addition in a mixture of ethylenediamine (20 eq.) : water (1:3).

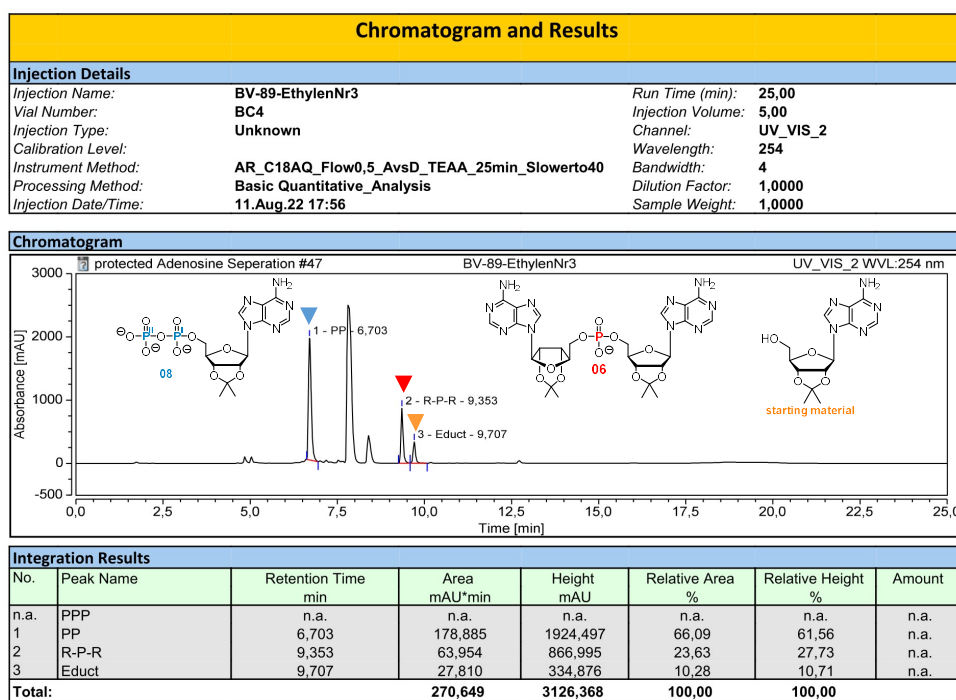
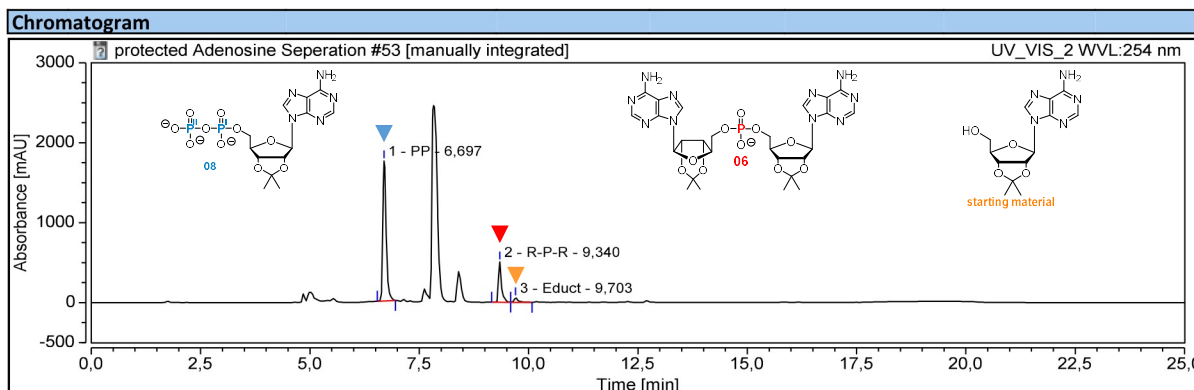


Figure SI-5: Reaction monitoring via RP-HPLC (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method 1-1, 0.5 mL/min. The two non-integrated peaks represent *m*CPBA and ETT.

B: Commercial 2',3'-O-isopropyliden-adenosine (1.0 eq.), *c*-PyPA (**1.2 eq.**), *m*CPBA (2.0 eq.) in dry MeCN. Afterwards addition in a mixture of ethylenediamine (20 eq.) : water (1:3).

Chromatogram and Results

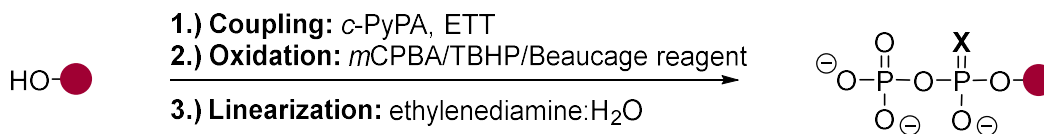
| Injection Details | | | |
|----------------------|---|-------------------|----------|
| Injection Name: | BV90-EthyleneNR3 | Run Time (min): | 25,00 |
| Vial Number: | BD4 | Injection Volume: | 5,00 |
| Injection Type: | Unknown | Channel: | UV_VIS_2 |
| Calibration Level: | | Wavelength: | 254 |
| Instrument Method: | AR_C18AQ_Flow0,5_AvsD_TEAA_25min_Slowerto40 | Bandwidth: | 4 |
| Processing Method: | Basic Quantitative Analysis | Dilution Factor: | 1,0000 |
| Injection Date/Time: | 12.Aug.22 17:50 | Sample Weight: | 1,0000 |



| Integration Results | | | | | | | |
|---------------------|-----------|-----------------------|-----------------|---------------|--------------------|----------------------|--------|
| No. | Peak Name | Retention Time min | Area mAU*min | Height mAU | Relative Area % | Relative Height % | Amount |
| n.a. | PPP | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| 1 | PP | 6,697 | 158,028 | 1751,004 | 77,31 | 75,80 | n.a. |
| 2 | R-P-R | 9,340 | 40,555 | 503,676 | 19,84 | 21,80 | n.a. |
| 3 | Educt | 9,703 | 5,837 | 55,448 | 2,86 | 2,40 | n.a. |
| Total: | | | 204,421 | 2310,128 | 100,00 | 100,00 | |

Figure SI-6: Reaction monitoring via RP-HPLC (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method 1-1, 0.5 mL/min. The two non-integrated peaks represent *m*CPBA and ETT.

1.4.2 Synthesis of nucleoside 5'-diphosphate (general procedure)

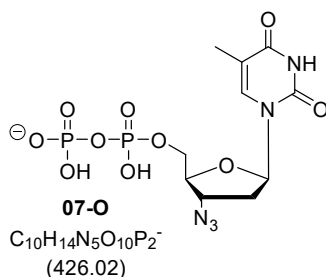


The following syntheses were all performed according to a general procedure under inert-conditions. Therefore, the used substrates and the acidic activator (DCI or ETT) were co-evaporated with dry MeCN (2 × 2 mL) in a pear shaped flask (except for volatile substrates as geraniol or isopentenol). Afterwards, the substrates were dissolved in dry DMF or dry MeCN (add as much as required for complete dissolution) and then *c*-PyPa (**01**, in DMF or MeCN) was added. The reaction mixture was stirred for 15 min at r.t. (complete conversion of the reaction can be monitored by ³¹P-NMR, shifting a triplet from 125 ppm to 100 ppm). The reaction mixture was cooled subsequently to 0°C and *m*CPBA/TBHP/Beaucage reagent was added followed by 10 min/90 min/75 min of stirring (complete conversion of the reaction can be monitored by ³¹P-NMR, shifting a triplet from 100 ppm to –20 ppm). The linearization was performed by adding the reaction mixture in a solution of ethylenediamine in water at 0°C and let it stir for at least 90 min and up to 3 h. (*It was stirred for 3 h to ensure complete conversion also in larger scales*). The isolation of the desired product as sodium salt was accomplished by adding the reaction mixture dropwise to a –20°C cold 0.5 M solution of NaClO₄ in acetone and the resulting precipitate was collected via centrifugation. After washing with acetone and drying *in vacuo* the crude product was obtained as a solid.

Exact amounts of reagents, further purification methods, specific yields and analytical data are reported in the individual procedures for each compound.

Note: The MPLC purification methods are indicated individually for each compound, method details can be found in general remarks. For each MPLC method one chromatogram is shown exemplary.

1.4.3 Synthesis of 3'-azidothymidine 5'-diphosphate (**07-O**):



c-PyPa (**01**, 0.12 M in MeCN, 5.6 mL, 0.60 mmol, 1.2 eq) was added to a solution of commercial AZT (134 mg, 0.50 mmol, 1.0 eq.) and DCI (120 mg, 1.0 mmol, 2.0 eq.) in dry MeCN (2 mL). After the addition of *m*CPBA (250 mg, 1.0 mmol, 2.0 eq.), the reaction mixture was added into a 0°C cold solution of ethylenediamine (0.67 mL, 0.60 g, 10 mmol, 20 eq.) in dH₂O (2.1 mL) and stirred at room temperature for 3 h. After precipitation with NaClO₄ in acetone, the resulting colorless solid was purified by SAX (column: *Q Sepharose*® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Repeated freeze drying of the product containing fractions afforded the title compound (**07-O**, 190 mg, 0.29 mmol, 57%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 2.3. This results in a molecular weight of 767 g/mol.

¹H-NMR (400 MHz, D₂O, δ/ppm): 7.78 (q, *J* = 1.2 Hz, 1H), 6.30 (t, *J* = 6.9 Hz, 1H), 4.60 (m_c, 1H), 4.27-4.17 (m, 3H), 2.54-2.50 (m, 2H), 1.95 (d, *J* = 1.2 Hz, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -10.86 (d, *J* = 21.0 Hz, 1P), -11.60 (d, *J* = 20.4 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 166.57, 151.71, 137.32, 111.83, 84.90, 83.05 (d, *J* = 9.2 Hz), 65.48 (d, *J* = 5.5 Hz), 60.94, 36.29, 11.63. **¹³C-Spectrum** was calibrated on CH₂ in TEA with 46.68 ppm.

HRMS (ESI⁻): *m/z* for C₁₀H₁₄O₁₀N₅P₂⁻: calcd. 426.0221 found 426.0215.

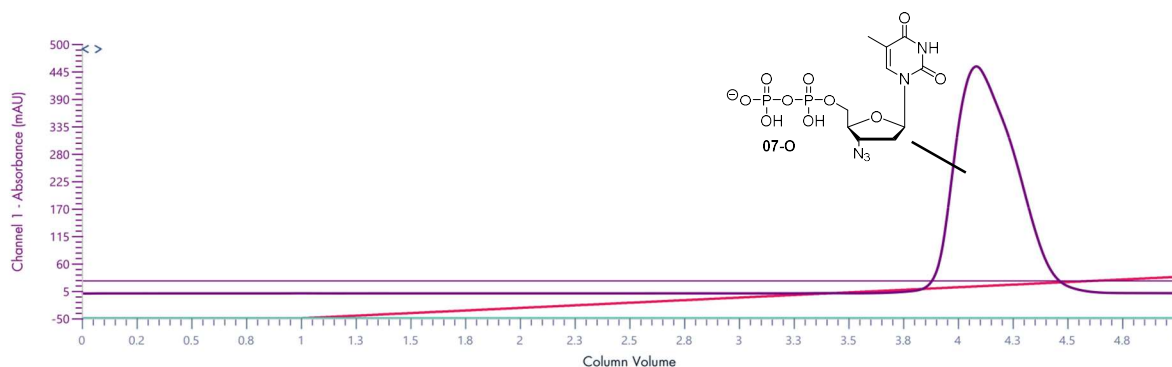
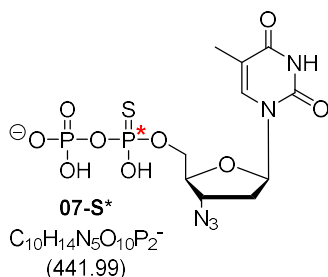


Figure SI-7: Purification of **07-O** via RP-HPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Gradient of %MeCN shown in red.

1.4.4 Synthesis of 3'-azidothymidine 5'- α -S-diphosphate (**07-S**):

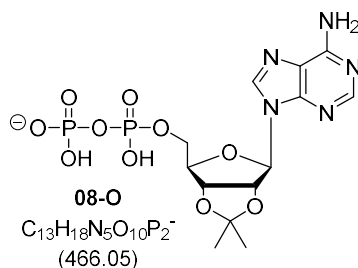


c-PyPa (**01**, 0.12 M in MeCN, 2.5 mL, 0.30 mmol, 1.2 eq) was added into solution of commercial AZT (67 mg, 0.25 mmol, 1.0 eq.) and DCI (59 mg, 0.50 mmol, 2.0 eq.) in dry MeCN (2.0 mL). After the addition of BEAUCAGE reagent (200 mg, 1.0 mmol, 4.0 eq.) at 0°C the solution was stirred for 75 min. The reaction mixture was added into a 0°C cold solution of ethylenediamine (0.33 mL, 0.30 g, 5 mmol, 20 eq.) in dH₂O (1.0 mL) and stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting yellow solid was purified by SAX (column: *Q Sepharose*[®] SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*[®] C18AQ, 30 μ m, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Repeated freeze drying of the product containing fraction afforded the title compound (**07-S***, 80 mg, 0.12 mmol, 50%) as a colorless solid and a mixture of two diastereomers (ratio: 0.7:1.0). The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 2.0. This results in a molecular weight of 650 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 7.82 (m_c, 1H), 6.31 (m_c, 1H), 4.65 – 4.60 (m, 1H), 4.32 – 4.22 (m, 3H), 2.58 – 2.46 (m, 2H), 1.98 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): 42.64 (d, J = 28.2 Hz, 1P), 42.46 (d, J = 28.0 Hz, 1P), -11.76 (d, J = 27.7 Hz, 1P), -11.77 (d, J = 28.3 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 166.57, 151.71, 137.36, 111.88, 84.87, 83.00 (d, J = 9.8 Hz), 82.93 (d, J = 9.9 Hz), 65.80 (d, J = 6.0 Hz), 65.56 (d, J = 6.0 Hz), 61.09, 60.90, 36.32, 36.26, 11.73. *The peaks marked in italic belong to the other diastereomer.* **¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.**

HRMS (ESI⁻): m/z for C₁₀H₁₄O₉N₅P₂S⁻: calcd. 441.9982 found 441.9990.

1.4.5 Synthesis of 2',3'-O-isopropylidene adenosine 5'-diphosphate (**08-O**):

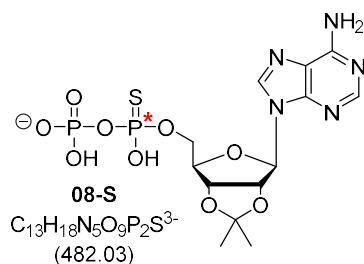


c-PyPa (**01**, 0.12 M in MeCN, 2.8 mL, 0.30 mmol, 1.2 eq) was added into a solution of 2',3'-O-isopropylidene adenosine (77 mg, 0.25 mmol, 1.0 eq.) and DCI (59 mg, 0.50 mmol, 2.0 eq.) in dry MeCN (2.0 mL). After the addition of *m*CBPA (120 mg, 0.5 mmol, 2.0 eq.) at 0°C the solution was stirred for 10 min. The reaction mixture was added into a 0°C cold solution of ethylenediamine (0.33 mL, 0.30 g, 5.0 mmol, 20 eq.) in dH₂O (1.0 mL) and stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting colorless solid was purified by SAX (column: *Q Sepharose*® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Repeated freeze drying of the product containing fraction afforded the title compound (**08-O**, 100 mg, 0.13 mmol, 54%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 2.5. This results in a molecular weight of 720 g/mol.

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.47 (s, 1H), 8.26 (s, 1H), 6.29 (d, *J* = 3.5 Hz, 1H), 5.43 (dd, *J* = 6.1, 3.5 Hz, 1H), 5.28 (dd, *J* = 6.1, 2.1 Hz, 1H), 4.71 – 4.67 (m, 1H), 4.24 – 4.13 (m, 2H), 1.71 (s, 3H), 1.48 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -9.94 (d, *J* = 20.6 Hz, 1P), -11.51 (d, *J* = 21.1 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.56, 152.82, 148.82, 140.13, 118.63, 114.97, 90.08, 84.62 (d, *J* = 9.4 Hz), 83.88, 81.40, 65.60 (d, *J* = 5.4 Hz), 26.18, 24.41. *¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI⁻): *m/z* for C₁₃H₁₈O₁₀N₅P₂⁻: calcd. 466.0523 found 466.0525.

1.4.6 Synthesis of 2',3'-O-isopropylidene adenosine 5'- α -S-diphosphate (**08-S**):

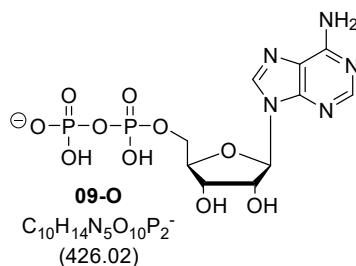


c-PyPa (**01**, 0.12 M in MeCN, 2.5 mL, 0.30 mmol, 1.2 eq) was added into a solution of 2',3'-O-isopropylidene adenosine (77 mg, 0.25 mmol, 1.0 eq.) and DCI (59 mg, 0.50 mmol, 2.0 eq.) in dry MeCN (2.0 mL). After the addition of BEAUCAGE reagent (100 mg, 0.5 mmol, 2.0 eq.) at 0°C the solution was stirred for 75 min. The reaction mixture was added into a 0°C cold solution of ethylenediamine (0.33 mL, 0.30 g, 5 mmol, 20 eq.) in dH₂O (1.0 mL) and stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting yellow solid was purified by SAX (column: *Q Sepharose*[®] SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*[®] C18AQ, 30 μ m, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Repeated freeze drying of the product containing fraction afforded the title compound (**08-S***, 74 mg, 0.11 mmol, 44%) as a colorless solid and a mixture of two diastereomers (ratio: 0.7:1.0). The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 2.0. This results in a molecular weight of 686 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.56 (s, 1H), 8.55 (s, 1H), 8.26 (s, 1H), 6.32 – 6.26 (m, 1H), 5.45 (m_c, 1H), 5.29 (m_c, 1H), 4.77 – 4.69 (m, 1H), 4.14 – 4.33 (m, 2H), 1.71 (s, 3H); 1.48 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): 42.7 (d, J = 27.2 Hz, 1P), 42.5 (d, J = 27.9 Hz, 1P), –11.5(d, J = 27.4 Hz, 1P), –11.5(d, J = 27.3 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 154.84, 151.84, 148.67, 140.65, 140.57, 118.58, 118.54, 114.82, 90.37, 90.34, 84.80 (d, J = 10.1 Hz), 84.72 (d, J = 9.9 Hz), 84.00, 81.60, 81.54, 65.90 (d, J = 6.2 Hz), 65.87 (d, J = 7.0 Hz), 26.16, 26.14, 24.38. *The peaks marked in italic belong to the other diastereomer. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI[–]): m/z for C₁₃H₁₈O₉N₅P₂S[–]: calcd. 482.0295 found 482.0303.

1.4.7 Synthesis of adenosine 5'-diphosphate (**09-O**):



c-PyPa (**01**, 0.12 M in MeCN, 4.2 mL, 0.50 mmol, 1.0 eq) was added into a solution of adenosine (130 mg, 0.50 mmol, 1.0 eq.) and DCI (120 mg, 1.0 mmol, 2.0 eq.) in dry DMF (2.0 mL). After the addition of *m*CBPA (250 mg, 1.0 mmol, 2.0 eq.) at 0°C the solution was stirred for 10 min. The reaction mixture was added into a 0°C cold solution of ethylenediamine (0.70 mL, 0.63 g, 10.0 mmol, 20 eq.) in dH₂O (2.1 mL) and stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting colorless solid was purified by SAX (column: *Q Sepharose*® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Repeated freeze drying of the product containing fraction afforded the title compound (**09-O**, 95 mg, 0.12 mmol, 24%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 3.6. This results in a molecular weight of 810 g/mol.

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.53 (d, *J* = 0.6 Hz, 1H), 8.26 (d, *J* = 0.6 Hz, 1H), 6.15 (d, *J* = 5.9 Hz, 1H), 4.79 (below the solvent peak, 1H), 4.58 (ddt, *J* = 5.2, 3.6, 0.5 Hz, 1H), 4.41 (m_c, 1H), 4.25 – 4.22 (m, 2H). The signal at 4.79 was indicated by ¹H, ¹³C-HSQC. **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): –10.37 (d, *J* = 21.1 Hz, 1P), –11.27 (d, *J* = 21.0 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.51, 152.72, 149.11, 139.96, 118.60, 86.83, 84.02 (d, *J* = 9.2 Hz), 74.33, 70.39, 65.02 (d, *J* = 5.4 Hz). ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.

HRMS (ESI[–]): *m/z* for C₁₀H₁₄O₁₀N₅P₂[–]: calcd. 426.0221 found 426.0215.

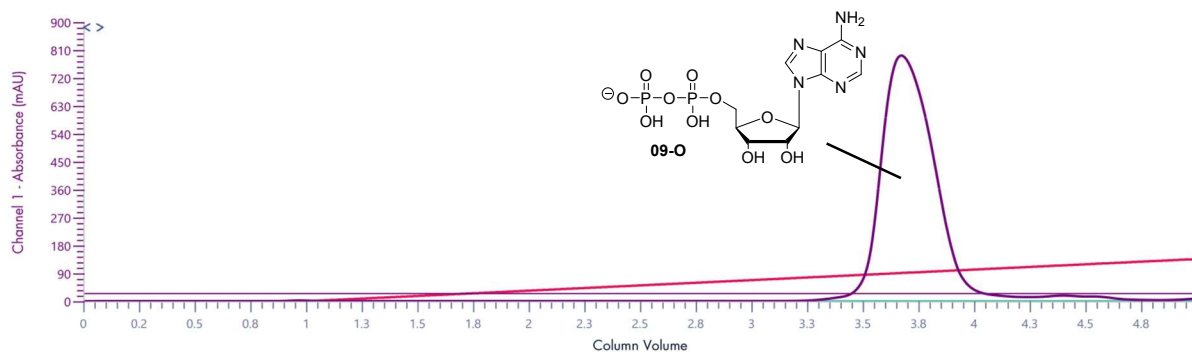
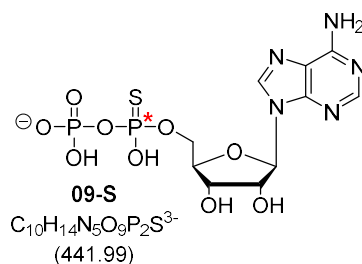


Figure SI- 8: Purification of **09-O** via RP-HPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm (purple); Method **MPLC-1**; 26 mL/min). Gradient of %MeCN shown in red.

1.4.8 Synthesis of adenosine 5'- α -S-diphosphate (**09-S**):



c-PyPa (**01**, 0.12 M in MeCN, 5.0 mL, 0.60 mmol, 1.2 eq) was added into a solution of adenosine (130 mg, 0.50 mmol, 1.0 eq.) and DCI (120 mg, 1.0 mmol, 2.0 eq.) in dry DMF (8.0 mL). After the addition of BEAUCAGE reagent (100 mg, 0.5 mmol, 2.0 eq.) at 0°C the solution was stirred for 75 min. The reaction mixture was added into a 0°C cold solution of ethylenediamine (0.33 mL, 0.30 g, 5 mmol, 20 eq.) in dH₂O (1.0 mL) and stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting yellow solid was purified by SAX (column: Q Sepharose® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*® C18AQ, 30 μ m, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Here, a separation of the diastereomers was possible and after repeated freeze drying of the title compound (**09-S**, 58 mg, 0.09 mmol, 18%; isomer A 8.0%, isomer B 10%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 2.0. This results in a molecular weight of 646 g/mol.

Isomer A: ¹H-NMR (400 MHz, D₂O, δ /ppm): 8.65 (s, 1H), 8.24 (s, 1H), 6.15 (d, J = 5.8 Hz, 1H), 4.79 (below the solvent peak, 1H), 4.59 (dd, d, J = 5.1, 3.5 Hz, 1H), 4.44 (m_c, 1H), 4.35 – 4.23 (m, 2H). The signal at 4.79 was indicated by ¹H,¹³C-HSQC. ³¹P-{¹H}-NMR (162 MHz, D₂O, δ /ppm): 42.94 (d, J = 28.5 Hz, 1P), -11.84 (d, J = 26.1 Hz, 1P). ¹³C-NMR (101 MHz, D₂O, δ /ppm): 155.36, 152.57, 149.04, 140.27, 118.51, 86.95, 83.95 (d, J = 9.7 Hz), 74.44, 70.56, 65.12 (d, J = 6.3 Hz). ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm. **Isomer B:** ¹H-NMR (400 MHz, D₂O, δ /ppm): 8.61 (s, 1H), 8.24 (s, 1H), 6.14 (d, J = 5.8 Hz, 1H), 4.79 (below the solvent peak, 1H), 4.59 (dd, d, J = 5.1, 3.5 Hz, 1H), 4.46 – 4.42 (m 1H), 4.36 – 4.26 (m, 2H). The signal at 4.79 was indicated by ¹H,¹³C-HSQC. ³¹P-{¹H}-NMR (162 MHz, D₂O, δ /ppm): 42.73 (d, J = 28.4 Hz, 1P), -10.82 (d, J = 28.2 Hz, 1P). ¹³C-NMR (101 MHz, D₂O, δ /ppm): 155.36, 152.58, 149.03, 140.16, 118.50, 86.92, 83.94 (d, J = 9.7 Hz), 74.44, 70.56, 65.28 (d, J = 6.0 Hz). ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm. **HRMS** (ESI⁻): m/z for C₁₀H₁₄O₉N₅P₂S⁻: calcd. 441.9993 found 442.0001.

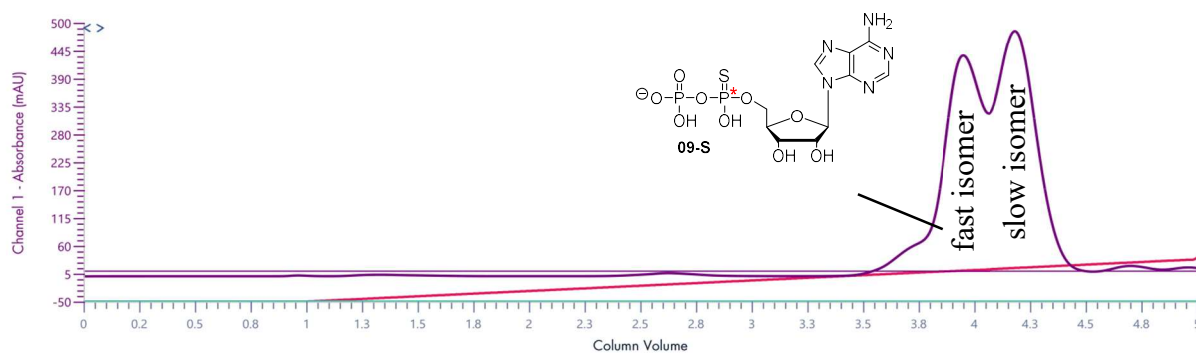
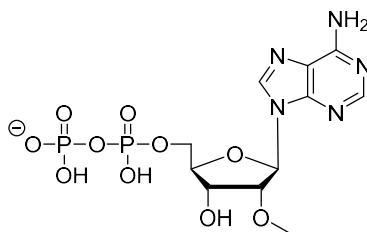


Figure SI-9: Purification of **09-S** via RP-HPLC (column: interchim® C18AQ, 30 μ m, F0040; λ = 254 nm (purple); Method **MPLC-1**; 26 mL/min). Gradient of %MeCN shown in red.

1.4.9 Synthesis of 2'-O-methyladenosine 5'-diphosphate (**10**):



10

$C_{11}H_{17}N_5O_{10}P_2$
(440.04)

c-PyPa (**01**, 0.12 M in MeCN, 5.4 mL, 0.60 mmol, 1.2 eq.) was added to a solution of 2'-O-methyladenosine (140 mg, 0.50 mmol, 1.0 eq.) and DCI (120 mg, 1.0 mmol, 2.0 eq.) in dry DMF (4.0 mL). After the addition of *m*CPBA (250 mg, 1.0 mmol, 2.0 eq.) the reaction mixture was added into a 0°C cold solution of ethylenediamine (0.67 mL, 600 mg, 10 mmol, 20 eq.) in dH₂O (2.0 mL) and it was stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, a yellow oil was received, which was washed with cold acetone followed by Et₂O. The resulting colorless solid was purified by SAX (column: Q Sepharose® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (divided into 5 runs, column: *interchim*® C18HP, 15 μm, F0012; λ = 254 nm; Method **MPLC-2a**; 15 mL/min). Repeated freeze drying of the product containing fractions afforded the title compound (**10**, 185 mg, 0.24 mmol, 47%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 3.2. This results in a molecular weight of 767 g/mol.

¹H-NMR: (400 MHz, D₂O, δ/ppm): 8.56 (s, 1H), 8.28 (s, 1H), 6.21 (d, *J* = 5.9 Hz, 1H), 4.77 (*below the solvent peak*, 1H), 4.52 (dd, *J* = 5.9, 5.1 Hz, 1H), 4.43 – 4.37 (m, 1H), 4.29 – 4.16 (m, 2H), 3.48 (s, 3H). *The signal at 4.77 was indicated by ¹H, ¹³C-HSQC.* **³¹P{¹H}-NMR:** (162 MHz, D₂O, δ/ppm): –9.37 (d, *J* = 21.1 Hz, 1P), –11.22 (d, *J* = 21.1 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.67, 152.94, 149.05, 140.00, 118.61, 85.28, 84.55 (d, *J* = 9.1 Hz), 83.15, 68.81, 64.83 (d, *J* = 5.5 Hz), 58.17. *¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI[–]) *m/z* for [C₁₁H₁₆N₅O₁₀P₂][–]: calcd. 440.0378 found 440.0379.

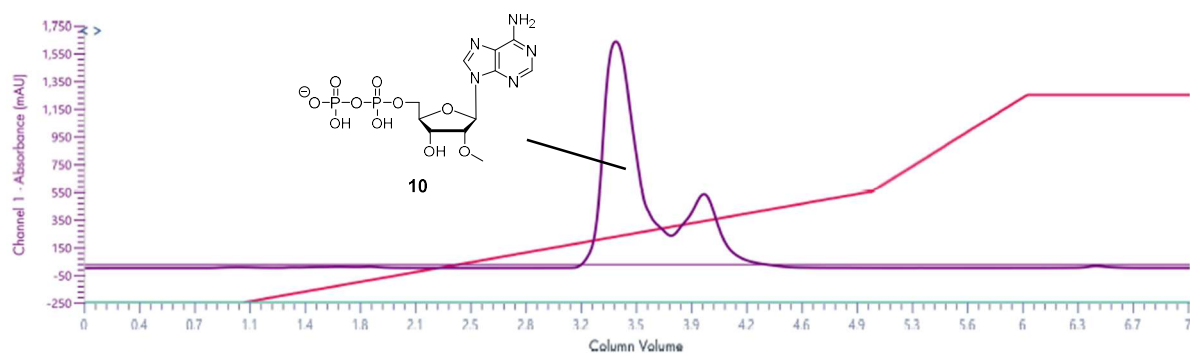
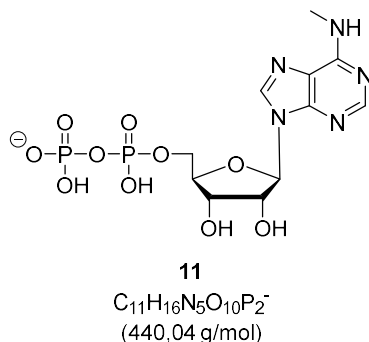


Figure SI-10: Purification of **10** via RP-HPLC (column: interchim® C18HP, 15 µm, F0012; λ = 254 nm (purple); Method **MPLC-2a**; 15 mL/min). Gradient of %MeCN shown in red

1.4.10 Synthesis of *N*⁶-methyladenosine 5'-diphosphate (**11**):

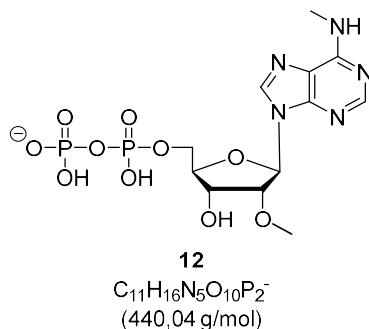


c-PyPa (**01**, 0.12 M in MeCN, 2.8 mL, 0.30 mmol, 1.0 eq.) was added to a solution of *N*⁶-methyladenosine (**SI-02**, 84 mg, 0.30 mmol, 1.0 eq.) and DCI (71 mg, 0.60 mmol, 2.0 eq.) in dry DMF (1.5 mL). After the addition of *m*CPBA (150 mg, 0.60 mmol, 2.0 eq.) the reaction mixture was added into a 0°C cold solution of ethylenediamine (0.40 mL, 0.36 g, 6.0 mmol, 20 eq.) in dH₂O (1.2 mL) and it was stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, a yellow oil was received, which was washed with cold acetone followed by Et₂O. The resulting colorless solid was purified by SAX (column: Q Sepharose® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm; Method **MPLC-1**; 26 mL/min). Repeated freeze drying of the product containing fractions afforded the title compound (**11**, 62 mg, 0.075 mmol, 25%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 3.8. This results in a molecular weight of 830 g/mol.

¹H-NMR: (400 MHz, D₂O, δ/ppm): 8.49 (s, 1H), 8.28 (s, 1H), 6.15 (d, *J* = 5.8 Hz, 1H), 4.76 (*below the solvent peak*, 1H), 4.60 (dd, *J* = 5.2, 3.7 Hz, 1H), 4.41 (dt, *J* = 5.6, 2.7 Hz, 1H), 4.22 (mc, 2H), 3.12 (s, 3H). *The signal at 4.76 was indicated by ¹H,¹³C-HSQC.* **³¹P{¹H}-NMR:** (162 MHz, D₂O, δ/ppm): -9.30 (d, *J* = 22.5 Hz), -11.18 (d, *J* = 21.1 Hz). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.24, 152.95, 147.93, 139.22, 118.97, 86.77, 83.97 (d, *J* = 9.2 Hz), 74.31, 70.26, 64.83 (d, *J* = 5.4 Hz), 27.39. *The peaks marked in italic were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI⁻) *m/z* for [C₁₁H₁₆N₅O₁₀P₂]⁻: calcd. 440.0378 found 440.0374.

1.4.11 Synthesis of 2'-O-methyl-N⁶-methyladenosine 5'-diphosphate (**12**):



c-PyPa (**01**, 0.12 M in MeCN, 1.6 mL, 0.20 mmol, 1.2 eq.) was added to a solution of 2'-O-methyl-N⁶-methyladenosine (**SI-05**, 50 mg, 0.17 mmol, 1.0 eq.) and DCI (40 mg, 0.34 mmol, 2.0 eq.) in dry MeCN (1.5 mL). After the addition of *m*CPBA (84 mg, 0.34 mmol, 2.0 eq.) the reaction mixture was added into a 0°C cold solution of ethylenediamine (0.24 mL, 0.22 g, 3.6 mmol, 20 eq.) in dH₂O (0.9 mL) and it was stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, a yellow oil was received, which was washed with cold acetone followed by Et₂O. The resulting colorless solid was purified by SAX (column: *Q Sepharose*® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (divided into 5 runs, column: *interchim*® C18HP, 15 μm, F0012; λ = 254 nm; Method **MPLC-2b**; 15 mL/min). Repeated freeze drying of the product containing fractions afforded the title compound (**12**, 49 mg, 0.09 mmol, 51%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 2.1. This results in a molecular weight of 543.1 g/mol.

¹H-NMR: (400 MHz, D₂O, δ/ppm): 8.50 (s, 1H), 8.28 (s, 1H), 6.20 (d, *J* = 6.0 Hz, 1H), 4.75 (dd, *J* = 5.3, 3.0 Hz, 1H), 4.52 (t, *J* = 5.4 Hz, 1H), 4.41 (mc, 1H), 4.28-4.18 (m, 2H), 3.49 (s, 3H), 3.12 (br. s, 3H). **³¹P{¹H}-NMR:** (162 MHz, D₂O, δ/ppm): -9.90 (d, *J* = 21.2 Hz), -11.27 (d, *J* = 21.0 Hz). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.21, 152.98, *147.86*, 139.25, 118.92, 85.19, 84.53 (d, *J* = 9.0 Hz), 83.14, 68.96, 64.92 (d, *J* = 5.5 Hz), 58.17, *27.44*. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI⁻) *m/z* for [C₁₂H₁₈N₅O₁₀P₂]⁻: calcd. 454.0534 found 454.0529.

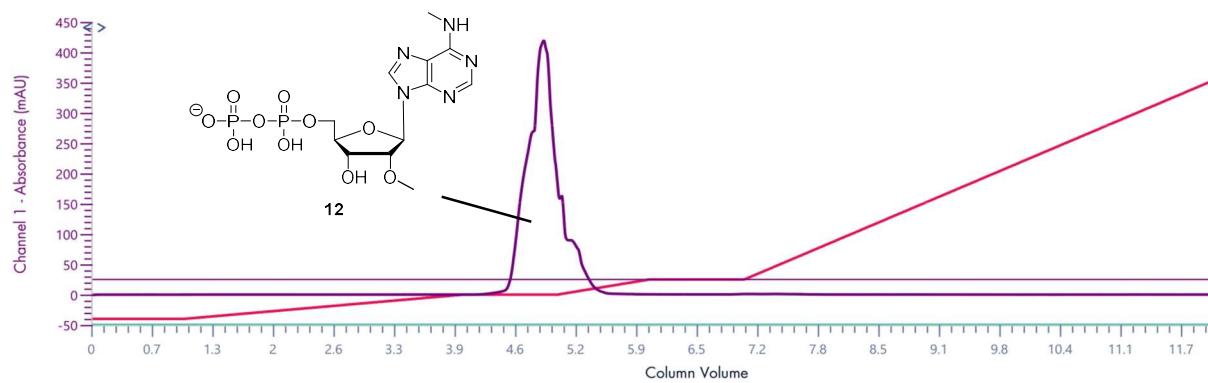
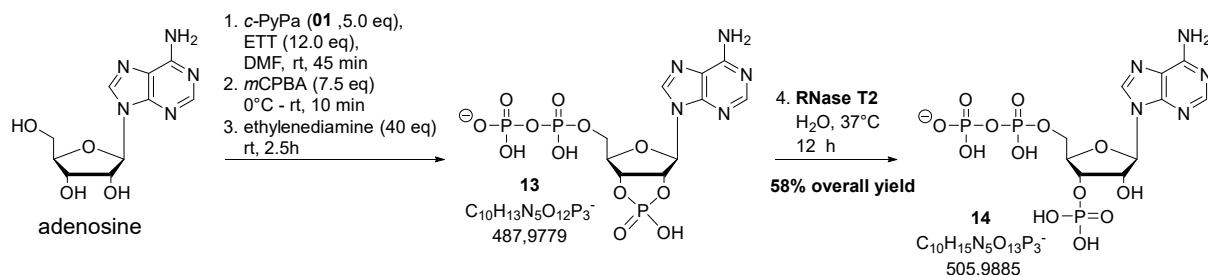


Figure SI-11: Purification of **12** via RP-HPLC (column: interchim® C18HP, 15 μ m, F0012; λ = 254 nm (purple); Method **MPLC-2b**; 15 mL/min). Gradient of %MeCN shown in red.

1.4.12 Synthesis of adenosine 3'-phosphate 5'-diphosphate, ppAp (**14**):

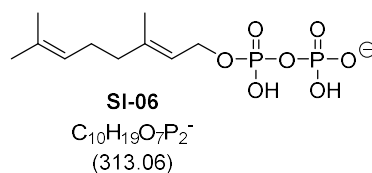


Adenosine (100 mg, 0.40 mmol, 1.0 eq) and ETT (584 mg, 4.80 mmol, 12.0 eq) were coevaporated together with dry MeCN (2 × 1.0 mL) in a flame dried 25 mL pear shaped flask. The residue was dissolved in dry DMF (6.0 mL). Then c-PyPA (0.4 M in DMF, 5.0 mL, 2.0 mmol, 5.0 eq) was added dropwise within 5 min, and the resulting solution was stirred at r.t. for 45 min. The solution was cooled to 0°C, and *m*CPBA (77%, 629 mg, 1.4 mmol, 7.5 eq) was added within 5 min in portions. The mixture was stirred at 0°C for 10 min. To the reaction mixture was added diethylamine (1.0 mL, 7.5 mmol, 40.0 eq) and the resulting solution was stirred at r.t. for 2.5 h, before precipitation by the addition of Et₂O (40 mL). The resulting solid was dissolved in H₂O (15 mL), and RNase T2 (200 u) was added. The solution was incubated at 37°C for 12 h. Afterwards the solution was directly purified by SAX (column: *Q Sepharose*® SK 16; λ = 254 nm; Method **SAX-2**; 5 mL/min). Repeated lyophilization of the product containing fractions afforded the title compound (**14**, 135 mg, 0.23 mmol, 58 %, 4xNH₄ as counter ion → 578 g/mol) as white solid.

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.55 (s, 1H), 8.26 (m_c, 1H), 6.19 – 6.17 (m, 1H), 4.88 – 4.86 (m, 2H), 4.61 (m_c, 1H), 4.28 – 4.26 (m, 2H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): 1.39 (s, 1P), -9.43 (d, *J* = 20.3 Hz, 1P), -10.98 (d, *J* = 20.3 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 154.93, 152.03, 149.10, 140.06, 118.54, 86.52, 83.35 (dd, *J* = 9.1, 3.9 Hz), 73.92 (d, *J* = 5.0 Hz), 73.78 (d, *J* = 4.8 Hz), 65.02 (d, *J* = 5.4 Hz).

HRMS (ESI⁻) *m/z* for [C₁₀H₁₅N₅O₁₃P₃]⁻: calcd. 505.9885 found 505.9884.

1.4.13 Synthesis of geranyl diphosphate (SI-06):



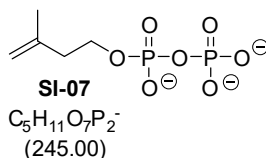
c-PyPa (**01**, 0.12 M in MeCN, 6.6 mL, 0.78 mmol, 1.2 eq.) was added to a solution of commercial geraniol (0.11 mL, 100 mg, 0.65 mmol, 1.0 eq.) and DCI (150 mg, 1.3 mmol, 2.0 eq.) in dry MeCN (1.0 mL). After the addition of *tert*-butyl hydroperoxide (5 M in decane, 0.26 mL, 1.3 mmol, 2.0 eq.) the reaction mixture was stirred at r.t. for 90 min. Afterwards, the solution was added into a 0°C cold solution of ethylenediamine (0.87 mL, 0.78 g, 13 mmol, 20 eq.) in dH₂O (2.6 mL) and it was stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting colorless solid was purified by SAX (column: Q Sepharose® SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized once and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*® C18AQ, 30 μm, F0040; λ = 254 nm; Method **MPLC-3**; 26 mL/min). The product containing fractions were lyophilized once, dissolved in a minimum of water and isolated by precipitation with NaClO₄ in acetone to obtain the title compound (**SI-06**, 101 mg, purity 97%*, 0.26 mmol, 40%) as a colorless solid.

Note: We observed significant degradation of the product, when it was repeated lyophilized. Hence, we isolated the product as sodium salt through final precipitation. *Some acetone remained in the product. 380 g/mol was applied for yield determination, corresponding to the 2x Na⁺ salt.

¹H-NMR: (400 MHz, D₂O, δ/ppm): 5.49 (tq, *J* = 7.1, 1.3 Hz, 1H), 5.24 (ddp, *J* = 6.9, 5.8, 1.5 Hz, 1H), 4.51 (t, *J* = 6.7 Hz, 2H), 2.22 – 2.08 (m, 4H), 1.75 (d, *J* = 1.3 Hz, 3H), 1.72 (t, *J* = 1.3 Hz, 3H), 1.69 – 1.61 (m, 3H). **³¹P{¹H}-NMR:** (162 MHz, D₂O, δ/ppm): –8.36 (d, *J* = 21.2 Hz, 1P), –10.42 (d, *J* = 21.1 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 142.79, 133.73, 124.14, 119.77 (d, *J* = 8.5 Hz), 62.71 (d, *J* = 5.5 Hz), 25.58, 24.81, 16.95, 15.57.

HRMS (ESI[–]) *m/z* for [C₁₀H₁₉O₇P₂][–]: calcd. 313.0611 found 313.0611.

1.4.14 Synthesis of isopentenyl diphosphate (SI-07); not optimized:



c-PyPa (**01**, 0.12 M in MeCN, 5.0 mL, 0.60 mmol, 1.2 eq.) was added to a solution of commercial 3-methylbut-3-en-1-ol (51 μL , 43 mg, 0.50 mmol, 1.0 eq.) and DCl (120 mg, 1.0 mmol, 2.0 eq.) in dry MeCN (1.0 mL). After the addition of *m*CPBA (0.25 mg, 1.0 mmol, 2.0 eq.) the reaction mixture was added into a 0°C cold solution of ethylenediamine (0.67 mL, 0.60 g, 10 mmol, 20 eq.) in dH₂O (2.0 mL) and it was stirred at r.t. for 3 h. After precipitation with NaClO₄ in acetone, the resulting solid was purified by SAX (column: *Q Sepharose*[®] SK 16; λ = 254 nm; Method **SAX-1**; 5 mL/min). The product containing fractions were lyophilized several times and the obtained solid was dissolved in 0.5 M TEAA buffer and further purified by RP-MPLC (column: *interchim*[®] C18AQ, 15 μm , F0012; ELSD; Method **MPLC-3**; 15 mL/min). Repeated freeze drying of the product containing fractions afforded the title compound (**SI-07**, 33 mg, 0.08 mmol, 16%) as a colorless solid. The exact amount of triethylammonium counterions per diphosphate molecule, calculated from ¹H-NMR, was found to be 1.7. This results in a molecular weight of 418 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 4.87 – 4.86 (m, 1H), 4.84 – 4.82 (m, 1H), 4.09 (m_c, 2H), 2.40 (t, *J* = 6.6 Hz, 2H), 1.77 (m_c, 3H). ³¹P{¹H}-NMR (162 MHz, D₂O, δ /ppm): –11.2 (s, 2P). ¹³C{¹H}-NMR (101 MHz, D₂O, δ /ppm): 143.44, 111.75, 64.96 (t, *J* = 3.1 Hz), 37.79 (t, *J* = 3.8 Hz), 21.64.

HRMS (ESI[–]): *m/z* for C₅H₁₁O₇P₂[–]: calcd. 244.9985, found 244.9985.

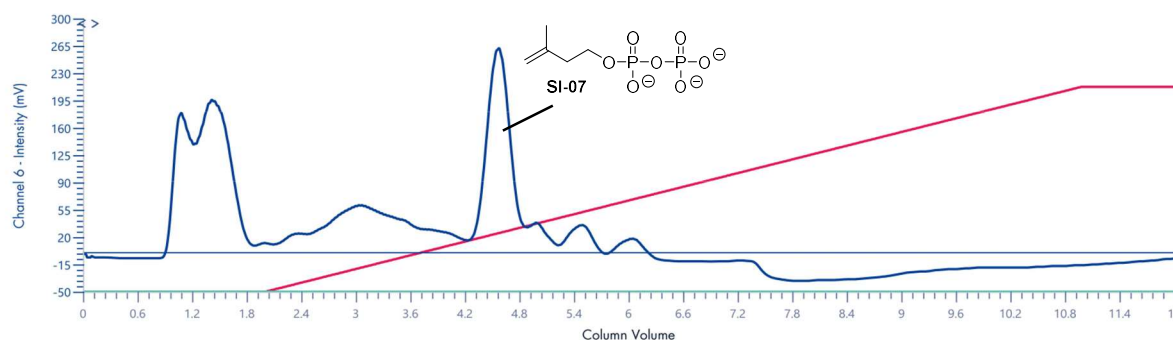
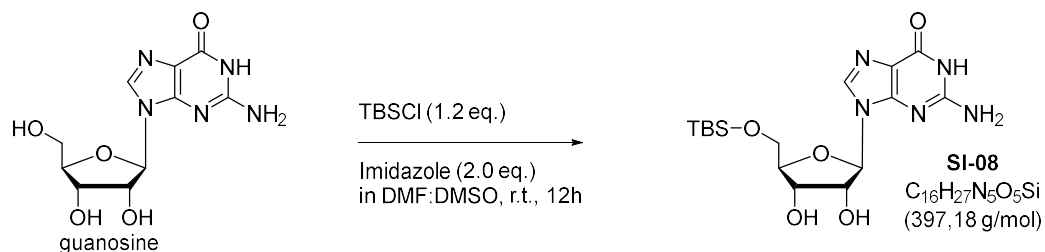


Figure SI-12: Purification of **SI-07** via RP-HPLC (column: *interchim*[®] C18AQ, 15 μm , F0012; ELSD (blue); Method **MPLC-3**; 15 mL/min). Gradient of %MeCN shown in red.

1.5 Synthesis of guanosine P-amidite part

1.5.1 Synthesis of 5'-TBS guanosine (SI-08)



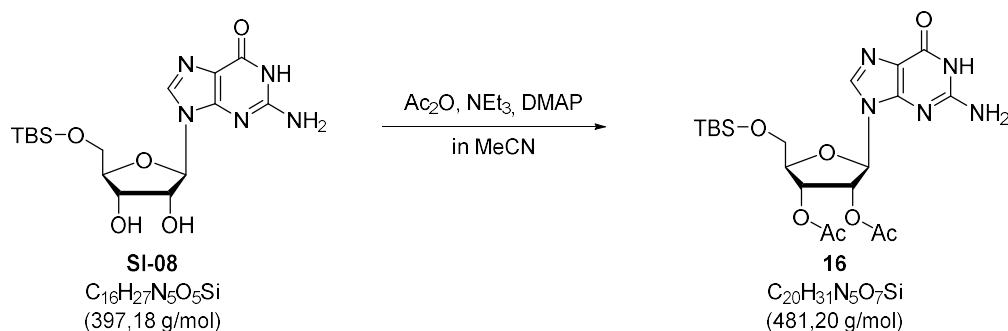
TBSCl (12.7 g, 84.8 mmol, 1.2 eq.) and imidazole (9.62 g, 141 mmol, 2.0 eq.) were added into a solution of commercial guanosine (20 g, 70.2 mmol, 1.0 eq.) in DMF (60 mL) and DMSO (100 mL). The reaction mixture was stirred overnight. Next, the reaction mixture was poured into ice-cold dH₂O (1200 mL) and it was left for 1 h before the formed precipitate was collected *via* Büchner funnel. Afterwards, this still wet solid was recrystallized from MeOH (ca. 740 mL) and the colorless crystals were isolated *via* Büchner funnel after 1 h chilling on ice. The title compound (**SI-08**, 19.9 g, 50 mmol, 71%) were obtained as colorless crystals.

Analytical data are consistent with those reported in the literature.^[7]

R_f = 0.16 (silica gel, DCM:MeOH 90:10)

¹H-NMR (300 MHz, DMSO-d₆, δ /ppm): 10.60 (br. s, 1H), 7.84 (s, 1H), 6.47 (br. s, 2H), 5.70 (d, J = 5.5 Hz, 1H), 5.46 (d, J = 5.8 Hz, 1H), 5.14 (d, J = 5.1 Hz, 1H), 4.33 (q, J = 5.4 Hz, 1H), 4.08 (q, J = 4.8 Hz, 1H), 3.89 (q, J = 3.9 Hz, 1H), 3.80 (dd, J = 11.4, 3.7 Hz, 1H), 3.71 (dd, J = 11.4, 4.2 Hz, 1H), 0.88 (s, 9H), 0.05 (s, 6H).

1.5.2 Synthesis of 2',3'-diacetoxy 5'-TBS guanosine (**16**)



Ac₂O (4.2 mL, 4.5 g, 44 mmol, 2.2 eq) was added into a suspension of 5'-TBS guanosine (**SI-08**, 8.0 g, 20 mmol, 1.0 eq.), NEt₃ (11 mL, 8.2 g, 82 mmol, 4.0 eq.) and DMAP (62 mg, 2.5mol%) in MeCN (300 mL) and it was stirred at r.t. for 3 h becoming a clear solution. Half of the solvent was removed under reduced pressure before the mixture was poured into a solution of ice-cold dH₂O (600 mL) and aq. sat. NH₄Cl (60 mL). The obtained precipitation was isolated, after chilling for 1 h, *via* Büchner funnel. The filter cake was washed with cold water. The title compound (**16**, 9.9 g, 20 mmol, quant.) was isolated as colorless solid.

Note: Add Ac₂O very quickly after adding NEt₃. Otherwise, the reaction contains impurities.

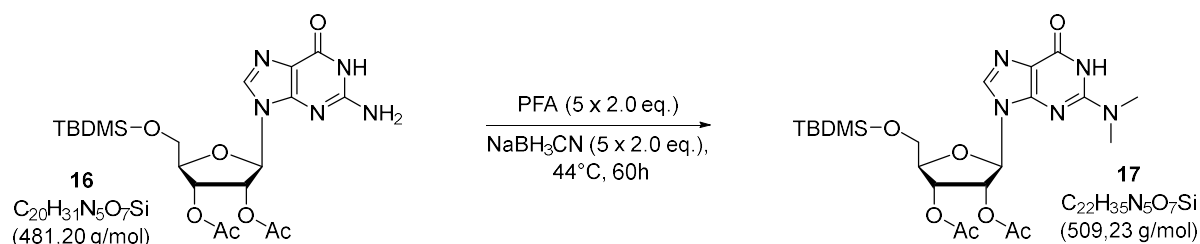
R_f = 0.51 (silica gel, DCM:MeOH 90:10)

¹H-NMR (400 MHz, DMSO-d₆, δ/ppm): 10.69 (br. s, 1H), 7.84 (s, 1H), 6.55 (br. s, 2H), 5.98 (d, *J* = 7.1 Hz, 1H), 5.68 (dd, *J* = 7.0, 5.5 Hz, 1H), 5.42 (dd, *J* = 5.5, 2.5 Hz, 1H), 4.21 (td, *J* = 3.6, 2.6 Hz, 1H), 3.89 – 3.80 (m, 2H), 2.11 (s, 3H), 2.00 (s, 3H), 0.88 (s, 9H), 0.08 (d, *J* = 1.1 Hz, 6H). **¹³C-NMR** (101 MHz, DMSO-d₆, δ/ppm): 169.46, 169.22, 156.60, 154.01, 151.32, 134.36, 116.56, 83.38, 82.84, 72.72, 70.90, 62.87, 25.73, 20.43, 20.14, 17.95, –5.56, –5.60.

HRMS (ESI⁺) *m/z* for [C₂₀H₃₂N₅O₇Si]⁺: calcd. 482.2066 found 482.2068.

HPLC-UV: *t*_R = 14.08 min (Bischoff ProntoSIL® C₁₈-AQ, 3 μm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min).

1.5.3 Synthesis of 2',3'-diacetoxy 5'-TBS N^{2,2}-dimethylguanosine (**17**)



Paraformaldehyde (5 × 190 mg, 6.2 mmol, 2.0 eq.) and NaBH₃CN (5 × 400 mg, 6.2 mmol, 2.0 eq.) were added 5-times over a time period of 60 h (10-14 h between the additions) into a 44°C warm solution of 2',3'-diacetoxy 5'-TBS guanosine (**16**, 1.5 g, 3.1 mmol, 1.0 eq.) in acetic acid (30 mL). Afterwards, the reaction mixture was slowly poured into a mixture of aq. sat. Na₂CO₃:H₂O:DCM (6:1:1, 400mL) and it was stirred for 15 min. The organic layer was separated, and the aq. layer was extracted with DCM (2 × 100 mL). The combined organic layers were dried over Na₂SO₄ and the solvent was removed under reduced pressure. The residue was split into three parts and each part was purified by silica-gel MPLC (column: *interchim*® SI-HP, 30µm, F0040; λ = 254 nm; Method **MPLC-I**; 26 mL/min). The title compound (**17**, 1.01 g, 1.96 mmol, 63%) was obtained as a white foam.

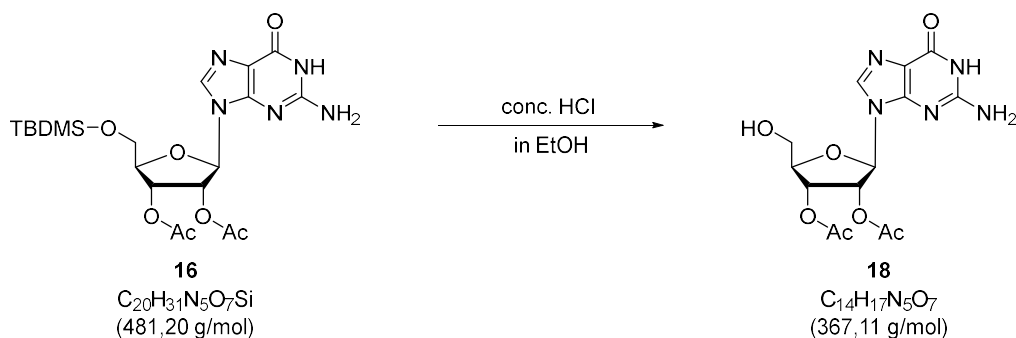
R_f = 0.41 (silica gel, DCM:MeOH 95:05)

¹H-NMR (400 MHz, DMSO-d₆, δ/ppm): 10.77 (br. s, 1H), 7.83 (s, 1H), 6.01 (d, *J* = 4.9 Hz, 1H), 5.97 (dd, *J* = 5.6 Hz, 1H), 5.58 (t, *J* = 5.4 Hz, 1H), 4.16 (dt, *J* = 5.2, 4.1 Hz, 1H), 3.82 (mc, 2H), 3.08 (s, 6H), 2.09 (s, 3H), 2.04 (s, 3H), 0.82 (s, 9H), 0.01 (s, 3H), -0.01 (s, 3H). **¹³C-NMR** (101 MHz, DMSO-d₆, δ/ppm): 169.40, 169.30, 157.22, 153.08, 150.32, 136.61, 116.35, 85.60, 81.62, 72.10, 70.00, 62.37, 37.87, 25.62, 20.35, 20.23, 17.91, -5.59, -5.65.

HRMS (ESI⁺) *m/z* for [C₂₂H₃₄N₅O₇Si]⁺: calcd. 508.2233 found 508.2226.

HPLC-UV: *t_R* = 12.55 min (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min).

1.5.4 Synthesis of 2',3'-diacetoxy guanosine (**18**)



Conc. HCl (38% in H_2O , 4.4 mL) was added into a solution of 2',3'-diacetoxy 5'-TBS guanosine (**16**, 3.6 g, 7.6 mmol, 1.0 eq.) in EtOH (100 mL) at $0^\circ C$ and it was stirred at $0^\circ C$ for 3 h. The completeness of the reaction was verified by LC-MS (Bischoff ProntoSIL[®] C₁₈-AQ, 3 μm , 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min). Afterwards Et₂O (13 mL for 1 mL EtOH) was added dropwise over a period of 1 h. The obtained suspension was store at $-20^\circ C$ overnight before the solid was isolated *via* Büchner filtration. The obtained solid was once washed with cold acetone (ca. 30 mL). The title compound (**18**, 2.8 g, 7.6 mmol, quant.) was obtained as colorless solid.

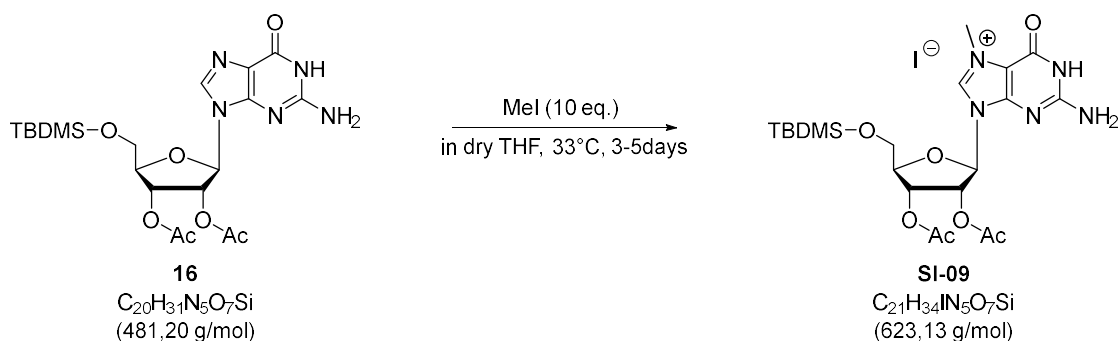
R_f = 0.3 (silica gel, DCM:MeOH 90:10)

¹H-NMR (300 MHz, DMSO- d_6 , δ /ppm): 11.36 (s, 1H), 8.64 (s, 1H), 7.04 (br. s, 2H), 6.04 (d, J = 6.6 Hz, 1H), 5.71 (dd, J = 6.5, 5.4 Hz, 1H), 5.45 (dd, J = 5.5, 2.8 Hz, 1H), 4.23 (q, J = 3.3 Hz, 1H), 3.67 (mc, 2H), 2.11 (s, 3H), 2.01 (s, 3H). **¹³C-NMR** (101 MHz, DMSO- d_6 , δ /ppm): 169.55, 169.21, 156.59, 153.94, 151.22, 135.19, 116.63, 83.61, 83.59, 72.74, 71.40, 61.02, 20.47, 20.16.

HRMS (ESI⁻) m/z for $[C_{14}H_{16}N_5O_7]^-$: calcd. 366.1055 found 366.1054.

HPLC-MS: t_R = 8.66 min (Bischoff ProntoSIL[®] C₁₈-AQ, 3 μm , 3.0 x 150 mm; λ = 272 nm; Method **2-13**, 0.5 mL/min).

1.5.5 Synthesis of 2',3'-diacetoxy 5'-TBS N⁷-methylguanosine iodide (**SI-09**)



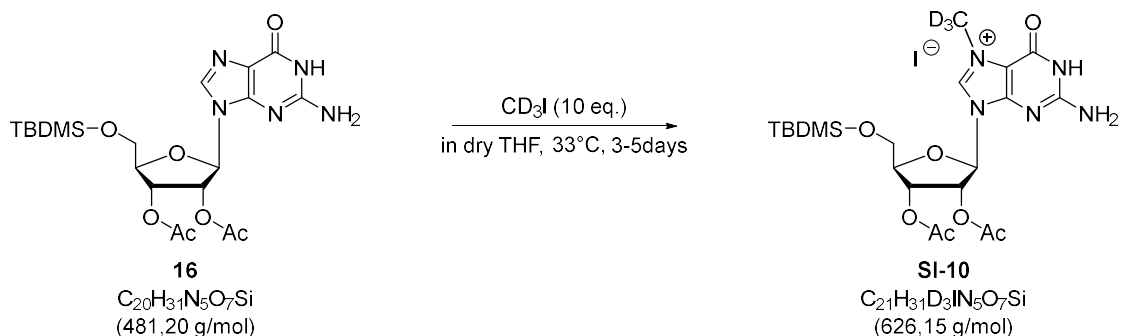
Iodomethane (1.9 mL, 4.4 g, 31 mmol, 10 eq.) was added into a solution of 2',3'-diacetoxy 5'-TBS guanosine (**16**, 1.5 g, 3.1 mmol, 1.0 eq.) in dry THF (27 mL) and it was stirred at 33°C for 4 days. The completeness of the reaction was verified by LC-MS (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min). If substrate was left, more Iodomethane was added until completeness was achieved. Afterwards, the obtained slurry was poured into ice-cold Et₂O (250 mL) and it was stirred at 0°C for 30 min. The obtained precipitate was isolated *via* Büchner filtration, and the filter cake was washed with cold Et₂O (2 x 15 mL). The title compound (**SI-09**, 1.8 g, 3.0 mmol, 94%) was obtained as bright yellow solid.

¹H-NMR (500 MHz, DMSO-d₆, δ/ppm): 11.75 (s, 1H), 9.27 (s, 1H), 7.18 (br. s, 2H), 6.15 (d, *J* = 4.8 Hz, 1H), 5.72 (m_c, 1H), 5.45 (dd, *J* = 5.6, 4.1 Hz, 1H), 4.37 (q, *J* = 4.1 Hz, 1H), 4.02 (s, 3H), 3.91 (m_c, 2H), 2.10 (s, 3H), 2.09 (s, 3H), 0.83 (s, 9H), 0.05 (d, *J* = 1.8 Hz, 6H). **¹³C-NMR** (126 MHz, DMSO-d₆, δ/ppm): 169.23, 169.11, 155.61, 153.27, 148.98, 136.27, 107.66, 87.29, 84.23, 73.46, 70.58, 62.63, 35.80, 25.62, 20.33, 20.22, 17.90, -5.58, -5.61.

HRMS (ESI⁺) *m/z* for [C₂₁H₃₄N₅O₇Si]⁺: calcd. 496.2222 found 496.2227.

HPLC-MS: *t_R* = 13.71 min (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min).

1.5.6 Synthesis of 2',3'-diacetoxy 5'-TBS *N*⁷-methyl-*d*₃-guanosine iodide (**SI-10**)



Iodomethane-*d*₃ (1.9 mL, 4.4 g, 31 mmol, 10 eq.) was added into a solution of 2',3'-diacetoxy 5'-TBS guanosine (**16**, 1.5 g, 3.1 mmol, 1.0 eq.) in dry THF (27 mL) and it was stirred at 33°C for 4 days. The completeness of the reaction was verified by LC-MS (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min). If substrate was left, more Iodomethane-*d*₃ was added till completeness was achieved. Afterwards, the obtained slurry was poured into ice-cold Et₂O (250 mL) and it was stirred at 0°C for 30 min. The obtained precipitate was isolated *via* Büchner filtration, and the filter cake was washed with cold Et₂O (2 × 15 mL). The title compound (**SI-10**, 1.9 g, 3.0 mmol, quant.) was obtained as bright yellow solid.

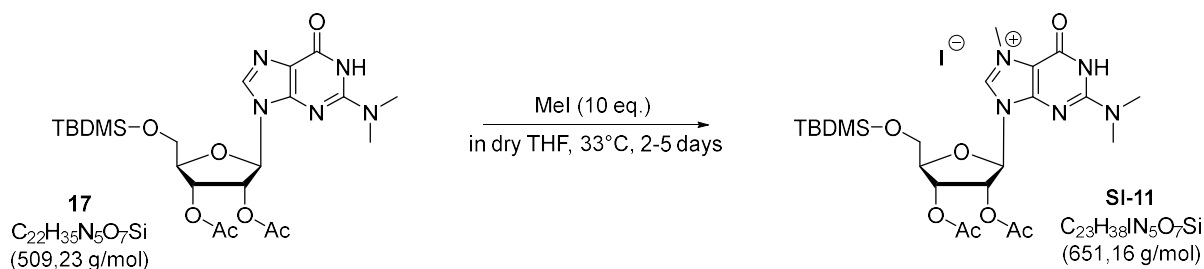
¹H-NMR (500 MHz, DMSO-*d*₆, δ/ppm): 11.75 (br. s, 1H), 9.27 (s, 1H), 7.18 (br. s, 2H), 6.15 (d, *J* = 4.8 Hz, 1H), 5.72 (dd, *J* = 5.6, 4.8 Hz, 1H), 5.45 (dd, *J* = 5.6, 4.1 Hz, 1H), 4.37 (q, *J* = 4.1 Hz, 1H), 3.91 (mc, 2H), 2.10 (s, 3H), 2.09 (s, 3H), 0.83 (s, 9H), 0.05 (s, 6H).

¹³C-NMR (126 MHz, DMSO-*d*₆, δ/ppm): 169.23, 169.11, 155.62, 153.28, 148.98, 136.27, 107.66, 87.30, 84.23, 73.46, 70.59, 62.63, 25.62, 20.33, 20.22, 17.90, -5.58, -5.61.

HRMS (ESI⁺) *m/z* for [C₂₁H₃₁D₃N₅O₇Si]⁺: calcd. 499.2410 found 499.2412.

HPLC-MS: *t*_R = 13.71 min (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min).

1.5.7 Synthesis of 2',3'-diacetoxy 5'-TBS N^{2,2,7}-trimethylguanosine iodide (**SI-11**)



Iodomethane (0.37 mL, 850 mg, 6.0 mmol, 10 eq.) was added into a solution of 2',3'-diacetoxy 5'-TBS N^{2,2}-dimethylguanosine (**17**, 300 mg, 0.60 mmol, 1.0 eq.) in dry THF (6 mL) and it was stirred at 33°C for 2 days. The completeness of the reaction was verified by HPLC-UV (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min). If substrate was left, more Iodomethane was added till completeness was achieved. Afterwards, the reaction mixture was purified by silica-gel MPLC (column: *interchim*® SI-HP, 30µm, F0012; λ = 254 nm; Method **MPLC-II**; 15 mL/min). The title compound (**SI-11**, 270 mg, 0.41 mmol, 69%) was obtained as colorless foam.

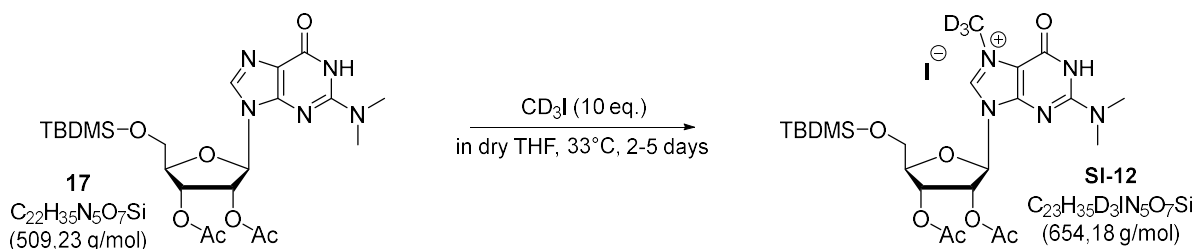
R_f = 0.33 (silica gel, DCM:MeOH 90:10)

¹H-NMR (400 MHz, DMSO-d₆, δ/ppm): 11.68 (br. s, 1H), 9.23 (d, *J* = 0.9 Hz, 1H), 6.20 (d, *J* = 3.2 Hz, 1H), 5.97 (dd, *J* = 5.8, 3.2 Hz, 1H), 5.57 (t, *J* = 6.0 Hz, 1H), 4.34 - 4.31 (m, 1H), 4.03 (d, *J* = 0.8 Hz, 3H), 3.92 (dd, *J* = 11.7, 3.8 Hz, 1H), 3.83 (dd, *J* = 11.6, 4.3 Hz, 1H), 3.12 (s, 6H), 2.10 (s, 3H), 2.07 (s, 3H), 0.80 (s, 9H), 0.00 (d, *J* = 5.7 Hz, 6H). **¹³C-NMR** (101 MHz, DMSO-d₆, δ/ppm): 169.26, 169.11, 155.12, 154.94, 148.21, 136.94, 107.02, 88.55, 82.98, 72.84, 69.68, 62.18, 38.01, 35.68, 25.53, 20.26, 20.22, 17.88, -5.65, -5.69.

HRMS (ESI⁺) *m/z* for [C₂₃H₃₈N₅O₇Si]⁺: calcd. 524.2535 found 524.2540.

HPLC-UV: *t_R* = 12.07 min (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min).

1.5.8 Synthesis of 2',3'-diacetoxy 5'-TBS N^{2,2,7}-trimethyl-⁷d₃-guanosine iodide (SI-12)



Iodomethane- d_3 (0.37 mL, 850 mg, 6.0 mmol, 10 eq.) was added into a solution of 2',3'-diacetoxy 5'-TBS N^{2,2}-dimethylguanosine (**17**, 300 mg, 0.60 mmol, 1.0 eq.) in dry THF (6 mL) and it was stirred at 33°C for 2 days. The completeness of the reaction was verified by HPLC-UV (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min). If substrate was left, more Iodomethane was added till completeness was achieved. Afterwards, the reaction mixture was purified by silica-gel MPLC (column: *interchim*® SI-HP, 30µm, F0012; λ = 254 nm; Method **MPLC-II**; 15 mL/min). The title compound (**SI-12**, 286 mg, 0.44 mmol, 73%) was obtained as colorless foam.

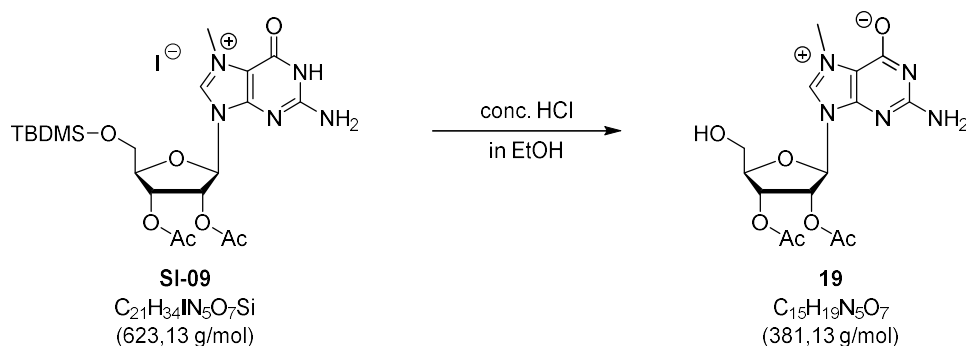
R_f = 0.33 (silica gel, DCM:MeOH 90:10)

¹H-NMR (500 MHz, DMSO- d_6 , δ/ppm): 11.69 (br. s, 1H), 9.25 (d, *J* = 0.7 Hz, 1H), 6.21 (d, *J* = 3.2 Hz, 1H), 5.96 (dd, *J* = 5.8, 3.2 Hz, 1H), 5.57 (t, *J* = 6.0 Hz, 1H), 4.34 – 4.31 (m, 1H), 3.92 (dd, *J* = 11.6, 3.8 Hz, 1H), 3.83 (dd, *J* = 11.6, 4.3 Hz, 1H), 3.12 (s, 6H), 2.10 (s, 3H), 2.07 (s, 3H), 0.80 (s, 9H), 0.00 (d, *J* = 7.2 Hz, 6H). **¹³C-NMR** (126 MHz, DMSO- d_6 , δ/ppm): 169.26, 169.11, 154.86, 154.69, 148.19, 137.08, 106.97, 88.57, 82.99, 72.85, 69.67, 62.17, 38.04, 35.07, 25.52, 20.26, 20.22, 17.87, -5.65, -5.70.

HRMS (ESI⁺) *m/z* for [C₂₃H₃₅D₃N₅O₇Si]⁺: calcd. 527.2723 found 527.2728.

HPLC-UV: *t_R* = 12.07 min (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min).

1.5.9 Synthesis of 2',3'-diacetoxy N⁷-methylguanosine (**19**)



Conc. HCl (38% in H₂O, 1.5 mL pro 1 g substrate) was added dropwise into a solution of 2',3'-diacetoxy 5'-TBS N⁷-methylguanosine iodide (**SI-09**, 0.89 g, 1.4 mmol, 1.0 eq.) in EtOH (12 mL pro 1 g substrate) and it was stirred at 0°C for 1 h. Reaction control *via* LC-MS (Bischoff ProntoSIL® C₁₈-AQ, 3 μm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min) revealed full conversion. Afterwards, the obtained slurry was added into four falcon tubes containing Et₂O:THF (3:1; 4 x 45 mL) and the precipitate, after chilling for 1 h at -20°C, was isolated *via* centrifugation. The obtained brownish solid was washed twice with Et₂O (each time 40 mL) and dried under high vacuum. The title compound (**19**, 0.56 g, 1.4 mmol, quant.) was obtained as a yellow solid.

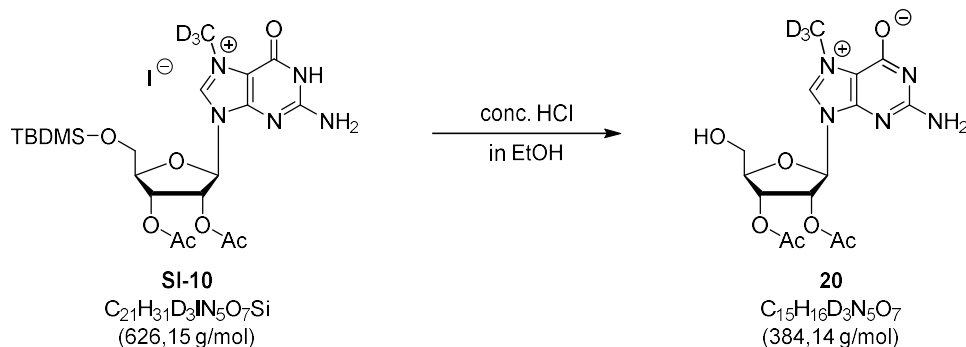
R_f = 0.27 (silica gel, DCM:MeOH 90:10)

¹H-NMR (500 MHz, DMSO-d₆, δ/ppm): 12.00 (s, 1H), 9.55 (s, 1H), 7.51 (br. s, 2H), 6.12 (d, *J* = 5.8 Hz, 1H), 5.67 (t, *J* = 5.6 Hz, 1H), 5.57 (br. s, 1H), 5.47 (dd, *J* = 5.4, 3.1 Hz, 1H), 4.33 (q, *J* = 3.1 Hz, 1H), 4.04 (d, *J* = 0.8 Hz, 3H), 3.71 (mc, 2H), 2.10 (s, 3H), 2.05 (s, 3H). **¹³C-NMR** (126 MHz, DMSO-d₆, δ/ppm): 169.37, 169.10, 155.98, 153.10, 149.15, 136.30, 107.43, 86.11, 84.98, 73.86, 71.03, 60.31, 35.78, 20.42, 20.20.

HRMS (ESI⁺) *m/z* for [C₁₅H₂₀N₅O₇]⁺: calcd. 382.1357 found 382.1364.

HPLC-MS: *t_R* = 8.01 min (Bischoff ProntoSIL® C₁₈-AQ, 3 μm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min).

1.5.10 Synthesis of 2',3'-diacetoxy N⁷-methyl-d₃-guanosine (**20**)



Conc. HCl (38% in H₂O, 1.5 mL pro 1 g substrate) was added dropwise into a solution of 2',3'-diacetoxy 5'-TBS N⁷-methyl-d₃-guanosine iodide (**SI-10**, 0.89 g, 1.4 mmol, 1.0 eq.) in EtOH (12 mL pro 1 g substrate) and it was stirred at 0°C for 1 h. Reaction control *via* LC-MS (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min) revealed full conversion. Afterwards, the obtained slurry was added into four falcon tubes containing Et₂O:THF (3:1; 4 x 45 mL) and the precipitate, after chilling for 1 h at -20°C, was isolated *via* centrifugation. The obtained brownish solid was washed twice with Et₂O (each time 40 mL) and dried under high vacuum. The title compound (**20**, 0.55 g, 1.4 mmol, quant.) was obtained as a yellow solid.

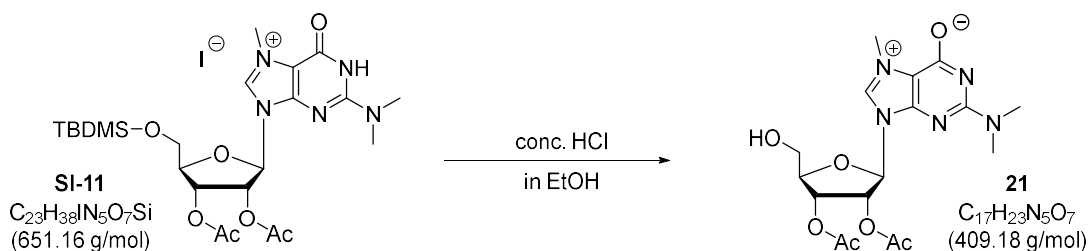
R_f = 0.27 (silica gel, DCM:MeOH 90:10)

¹H-NMR (500 MHz, DMSO-d₆, δ/ppm): 12.02 (s, 1H), 9.58 (s, 1H), 7.53 (br. s, 2H), 6.12 (d, *J* = 5.8 Hz, 1H), 5.67 (t, *J* = 5.6 Hz, 1H), 5.59 (br. s, 1H), 5.46 (dd, *J* = 5.4, 3.2 Hz, 1H), 4.32 (q, *J* = 3.1 Hz, 1H), 3.70 (m_c, 2H), 2.10 (s, 3H), 2.05 (s, 3H). **¹³C-NMR** (126 MHz, DMSO-d₆, δ/ppm): 169.39, 169.12, 156.01, 153.09, 149.15, 136.30, 107.44, 86.13, 85.00, 73.89, 71.06, 60.30, 35.16 (m), 20.43, 20.21.

HRMS (ESI⁺) *m/z* for [C₁₅H₁₇D₃N₅O₇]⁺: calcd. 385.1546 found 385.1547.

HPLC-MS: *t_R* = 8.01 min (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method **2-1**, 0.5 mL/min).

1.5.11 Synthesis of 2',3'-diacetoxy N^{2,2,7}-trimethylguanosine (21)



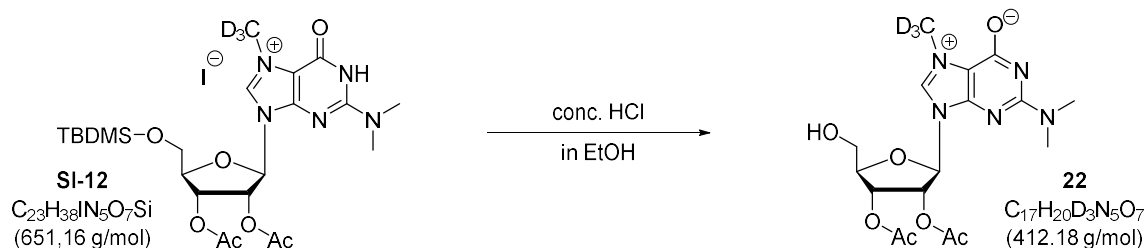
Conc. HCl (38% in H₂O, 1.5 mL pro 1 g substrate) was added dropwise into a solution of 2',3'-diacetoxy 5'-TBS N^{2,2,7}-trimethylguanosine iodide (**SI-11**, 0.25 g, 0.38 mmol, 1.0 eq.) in EtOH (12 mL pro 1 g substrate) and it was stirred at 0°C for 1 h. Reaction control *via* HPLC-UV (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min) revealed full conversion. Afterwards, the crude product was isolated by precipitation into –20°C cold Et₂O (2 x 40 mL). After centrifugation, the obtained brown oil was dissolved in acetone (2 mL) and precipitated again in –20°C cold Et₂O (2 x 40 mL). The now obtained brownish solid was purified by RP-MPLC (column: *interchim*® C18HP, 30 µm, F0012; λ = 254 nm; Method **MPLC-4**; 15 mL/min). The title compound (**21**, 120 mg, 0.29 mmol, 76%) was obtained as a colorless foam.

¹H-NMR (400 MHz, DMSO-*d*₆, δ/ppm): 8.94 (s, 1H), 6.11 (d, *J* = 4.6 Hz, 1H), 6.04 (dd, *J* = 5.7, 4.6 Hz, 1H), 5.58 (t, *J* = 5.3 Hz, 1H), 5.21 (br. s, 1H), 4.22 (q, *J* = 4.3 Hz, 1H), 4.02 (s, 3H), 3.68 (m_c, 2H), 3.00 (s, 6H), 2.09 (s, 3H), 2.06 (s, 3H). **¹³C-NMR** (101 MHz, DMSO-*d*₆, δ/ppm): 169.42, 169.14, 162.56, 162.31, 149.16, 132.46, 108.15, 86.95, 83.51, 72.44, 70.31, 60.63, 37.06, 35.07, 20.36, 20.21.

HRMS (ESI⁺) *m/z* for [C₁₇H₂₄N₅O₇]⁺: calcd. 410.1670 found 410.1673.

HPLC-UV: *t*_R = 8.28 min (Waters Symmetry® C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min).

1.5.12 Synthesis of 2',3'-diacetoxy $N^{2,2,7}$ -trimethyl- d_3 -guanosine (**22**)



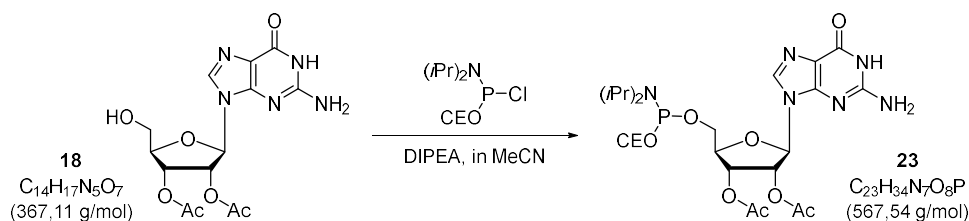
Conc. HCl (38% in H_2O , 1.5 mL pro 1 g substrate) was added dropwise into a solution of 2',3'-diacetoxy 5'-TBS $N^{2,2,7}$ -trimethyl- d_3 -guanosine iodide (**SI-12**, 0.25 g, 0.38 mmol, 1.0 eq.) in EtOH (12 mL pro 1 g substrate) and it was stirred at $0^\circ C$ for 1 h. Reaction control *via* HPLC-UV (Waters Symmetry[®] C₁₈, 5 μm , 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min) revealed full conversion. Afterwards, the crude product was isolated by precipitation into $-20^\circ C$ cold Et₂O (2 x 40 mL). After centrifugation, the obtained brown oil was dissolved in acetone (2 mL) and precipitated again in $-20^\circ C$ cold Et₂O (2 x 40 mL). The now obtained brownish solid was purified by RP-MPLC (column: *interchim*[®] C18HP, 30 μm , F0012; λ = 254 nm; Method **MPLC-4**; 15 mL/min). The title compound (**22**, 55 mg, 0.13 mmol, 34%) was obtained as a colorless foam.

¹H-NMR (400 MHz, DMSO- d_6 , δ /ppm): 8.94 (s, 1H), 6.12 (d, J = 4.6 Hz, 1H), 6.04 (m, 1H), 5.58 (t, J = 5.2 Hz, 1H), 4.23 (q, J = 4.4 Hz, 1H), 3.67 (m, 2H), 3.00 (s, 6H), 2.09 (s, 3H), 2.06 (s, 3H). **¹³C-NMR** (101 MHz, DMSO- d_6 , δ /ppm): 169.43, 169.15, 162.55, 162.31, 149.17, 132.49, 108.14, 86.97, 83.52, 72.45, 70.31, 60.63, 37.07, 20.37, 20.22.

HRMS (ESI⁺) m/z for $[C_{17}H_{21}D_3N_5O_7]^+$: calcd. 413.1859 found 413.1861.

HPLC-UV: t_R = 8.28 min (Waters Symmetry[®] C₁₈, 5 μm , 3.9 x 150 mm; λ = 254 nm; Method **1-2**, 1 mL/min).

1.5.13 Synthesis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-guanosine (**23**)



CEO(*i*Pr)₂NPCl (0.27 mL, 0.29 g, 1.2 mmol, 1.5 eq.) was added into a solution of 2',3'-diacetoxy guanosine (**18**, 0.30 g, 0.82 mmol, 1.0 eq.) and DIPEA (0.36 mL, 0.28 g, 2.1 mmol, 2.6 eq.) in dry DCM (11 mL). After it was stirred at r.t. for 3.5 h reaction control *via* TLC revealed full conversion. The reaction mixture was transferred into a separatory funnel with DCM. The organic layer was once washed with sat. aq. NaHCO₃, dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (deactivated silica gel, dry load, DCM:MeOH = 90:10 + 1% NEt₃) and the title compound (**23**, 0.41 g in 75% purity*, 0.53 mmol, 65%) was obtained as a colorless solid.

Note: Two diastereomers are obtained.

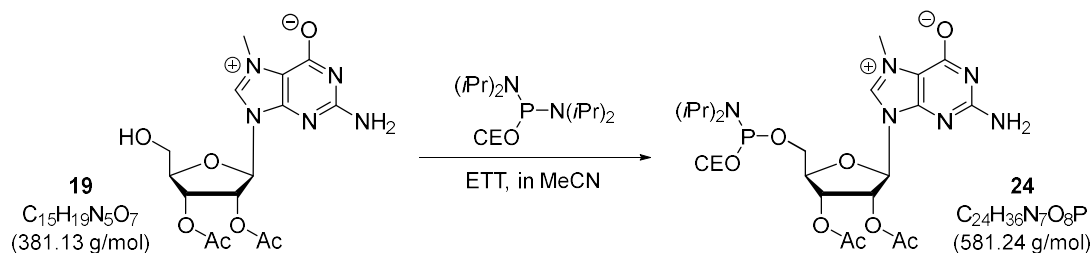
*It contained HNEt₃Cl. However, this helped to solidify the P-amidite. This impurity has no impact on the following coupling reaction.

R_f = 0.38 (silica gel, DCM:MeOH 90:10 + 1% NEt₃)

¹H-NMR (400 MHz, CDCl₃, δ/ppm): 7.90, 7.88 (s, 1H), 6.33 (br. s, 2H), 6.08 (m_c, 1H), 5.80 (m_c, 1H), 5.60 (m_c, 1H), 4.33 (m_c, 1H), 4.00 – 3.75 (m, 4H), 3.68 – 3.53 (m, 2H), 2.69 – 2.66 (m, 2H), 2.14, 2.13 (s, 3H), 2.04, 2.03 (s, 3H), 1.23 – 1.13 (m, 12H). **³¹P-{¹H}-NMR** (162 MHz, CDCl₃, δ/ppm): 149.46 (s, 1P), 149.09 (s, 1P). **¹³C-NMR** (101 MHz, CDCl₃, δ/ppm): δ 170.00, 169.91, 169.55, 169.47, 159.30, 159.26, 154.55, 154.48, 152.20, 152.06, 135.73, 135.47, 117.92, 117.88, 117.57, 117.47, 84.67, 84.24, 82.99 (d, *J* = 9.0 Hz), 82.67 (d, *J* = 9.2 Hz), 73.39, 73.25, 72.11, 71.85, 63.19 (d, *J* = 17.2 Hz), 62.98 (d, *J* = 15.6 Hz), 58.92 (d, *J* = 13.2 Hz), 58.71 (d, *J* = 12.9 Hz), 43.40 (d, *J* = 3.0 Hz), 43.27 (d, *J* = 3.0 Hz), 24.85 (d, *J* = 2.5 Hz), 24.78 (d, *J* = 2.5 Hz), 20.84, 20.81, 20.56, 20.55 (d, *J* = 4.7 Hz), 20.50, 20.48 (d, *J* = 4.6 Hz). The peaks marked in italic correspond to the other diastereomer.

HRMS (ESI⁻) *m/z* for [C₂₃H₃₃N₇O₈P]⁻: calcd. 566.2134 found 566.2126.

1.5.14 Synthesis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N⁷-methylguanosine (24**)**



CEO[(iPr)₂N]₂P (0.52 mL, 0.49 g, 1.5 mmol, 1.4 eq.) was added into a 0°C cold solution of 2',3'-diacetoxy N⁷-methylguanosine (**19**, 0.40 g, 1.0 mmol, 1.0 eq.) in dry DMF (8 mL) and it was stirred for 10 min. Afterwards, ETT (as stock solution in MeCN 125 mg/mL, 1.6 mL, 0.20 g, 1.3 mmol, 1.2 eq.) was added and reaction control *via* ³¹P{¹H}-NMR revealed full conversion after it was stirred for 4 h at 0°C. Next, the reaction mixture was diluted with DCM (30 mL), it was transferred into a separatory funnel and was washed once with aq. sat. NaHCO₃ (30 mL). The aqueous layer was once extracted with DCM (30 mL), the combined organic layers were dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (deactivated silica gel, dry load, DCM:MeOH = 95:5 + 3% NEt₃) and the title compound (**24**, 0.41 g, 0.71 mmol, 71%) was obtained as a colorless foam.

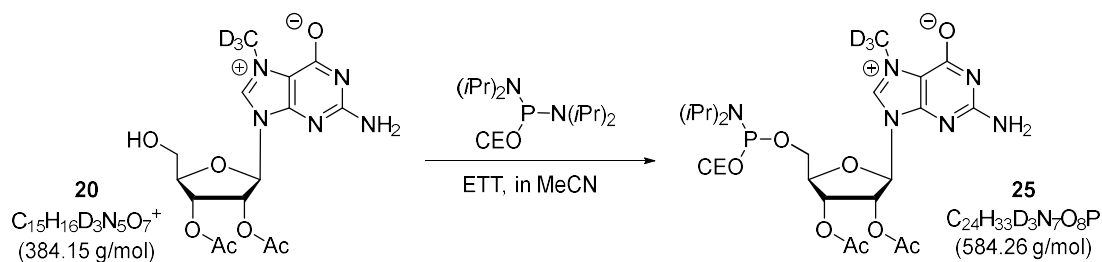
Note: Two diastereomers are obtained.

R_f = 0.58 (deactivated silica gel, DCM:MeOH 90:10 + 1% NEt₃)

¹H-NMR (400 MHz, CDCl₃, δ/ppm): 9.58, 9.28 (br. s, 1H), 6.28, 6.22 (d, *J* = 5.7 Hz, 1H), 5.98, 5.94 (t, *J* = 5.7 Hz, 1H), 5.72, 5.66 (m_c, 1H), 4.42 – 4.39 (m, 1H), 4.17 (s, 3H), 4.06 – 3.76 (m, 4H), 3.62 – 3.50 (m, 2H), 2.73 – 2.58 (m, 2H), 2.14, 2.13 (s, 3H), 2.09, 2.09 (s, 3H), 1.21 – 1.12 (m, 12H). **³¹P-{¹H}-NMR** (162 MHz, CDCl₃, δ/ppm): 150.14 (s, 1P), 148.75 (s, 1P). **¹³C-NMR** (101 MHz, CDCl₃, δ/ppm): 169.88, 169.77, 169.66, 169.55, 150.59, 150.19, 118.38, 118.11, 87.49, 86.32, 83.92, 83.16 (d, *J* = 8.8 Hz), 73.68, 73.48, 71.71, 71.20, 62.96, 62.54 (d, *J* = 17.0 Hz), 58.93, 58.55 (d, *J* = 21.6 Hz), 43.45, 43.33, 36.28, 36.24, 24.89 – 24.65 (m), 20.77 – 20.53 (m). *The peaks marked in italic belong to the other diastereomer.*

HRMS (ESI[−]) *m/z* for [C₂₄H₃₇N₇O₈P][−]: calcd. 582.2436 found 582.2441.

1.5.15 Synthesis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N⁷-methyl-d₃-guanosine (25)



CEO[(iPr)₂N]₂P (0.32 mL, 0.30 mg, 0.90 mmol, 1.4 eq.) was added into a 0°C cold solution of 2',3'-diacetoxy N⁷-methyl-d₃-guanosine (**20**, 0.25 g, 0.65 mmol, 1.0 eq.) in dry DMF (5 mL) and it was stirred for 10 min. Afterwards, ETT (as stock solution in MeCN 125 mg/mL, 0.80 mL, 0.10 g, 0.7 mmol, 1.2 eq.) was added and reaction control *via* ³¹P{¹H}-NMR revealed full conversion after it was stirred at r.t. for 1 h. Next, the reaction mixture was diluted with DCM (15 mL), it was transferred into a separatory funnel and was washed once with aq. sat. NaHCO₃ (15 mL). The aqueous layer was once extracted with DCM (15 mL), the combined organic layers were dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (deactivated silica gel, dry load, DCM:MeOH = 95:5 + 3% NEt₃) and the title compound (**25**, 0.26 g*, Purity: 85%, 0.38 mmol, 59%) was obtained as a colorless foam.

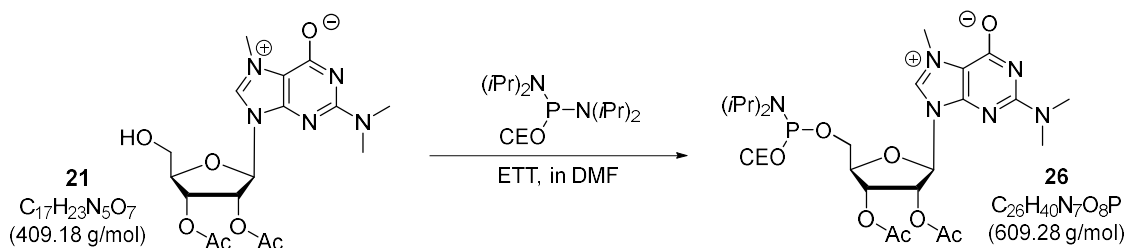
Note: Two diastereomers are obtained. *It contained some NEt₃. This impurity has no impact on the following coupling reaction

R_f = 0.58 (deactivated silica gel, DCM:MeOH 90:10 + 1% NEt₃)

¹H-NMR (300 MHz, CDCl₃, δ/ppm): 9.67, 9.35 (br. s, 1H), 6.29, 6.22 (d, *J* = 5.9 Hz, 1H), 5.97, 5.93 (t, *J* = 5.6 Hz, 1H), 5.71, 5.65 (m, 1H), 4.42 – 4.38 (m, 1H), 4.04 – 3.78 (m, 4H), 3.64 – 3.51 (m, 2H), 2.74 – 2.60 (m, 2H), 2.15, 2.10 (s, 3H), 2.09, 2.09 (s, 3H), 1.23 – 1.14 (m, 12H). **³¹P-{¹H}-NMR** (162 MHz, CDCl₃, δ/ppm): 150.20 (s, 1P), 148.72 (s, 1P).

HRMS (ESI⁻) *m/z* for [C₂₄H₃₄D₃N₇O₈P]⁻: calcd. 585.2624 found 585.2628.

1.5.16 Synthesis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N^{2,2,7}-trimethylguanosine (**26**)

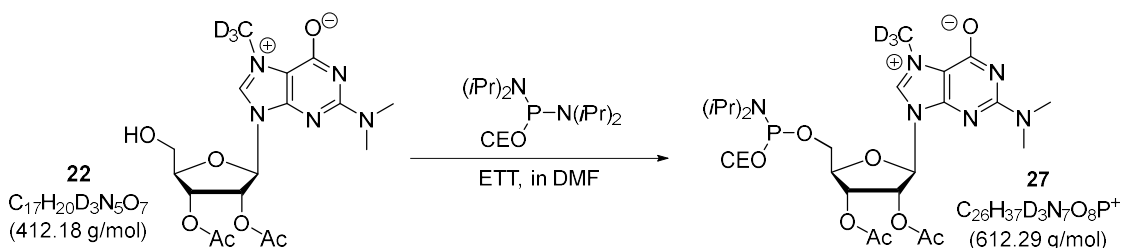


CEO[(iPr)₂N]₂P (82 μ L, 78 mg, 0.26 mmol, 1.4 eq.) was added into a 0°C cold solution of 2',3'-diacetoxy N^{2,2,7}-trimethylguanosine (**21**, 90 mg, 0.22 mmol, 1.0 eq.) in dry DMF (2 mL) and it was stirred for 10 min. Afterwards, ETT (as stock solution in MeCN 125 mg/mL, 0.30 mL, 37 mg, 0.27 mmol, 1.4 eq.) was added and reaction control *via* ³¹P{¹H}-NMR revealed full conversion after it was stirred at r.t. for 1 h. Next, the reaction mixture was diluted with DCM (5 mL), it was transferred into a separatory funnel and was washed once with aq. sat. NaHCO₃ (5 mL). The aqueous layer was once extracted with DCM (5 mL), the combined organic layers were dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by silica-gel MPLC* (column: *interchim*[®] SI-HP, 30 μ m, F0012; λ = 254 nm; Method **MPLC-III**; 15 mL/min) and the title compound (**26**, 100 mg**, purity 85%, 0.14 mmol, 64%) was obtained as a colorless foam.

Note: Two diastereomers are obtained. *Use DCM + 2% NEt₃ to equilibrate the column on the MPLC but only use DCM without NEt₃ during the run. **It contained NEt₃. This impurity has no impact on the following coupling reaction.

¹H-NMR (400 MHz, CDCl₃, δ /ppm): 8.62, 8.58 (d, *J* = 0.70 Hz, 1H), 6.29, 6.17 (d, *J* = 5.8 Hz, 1H), 5.92, 5.87 (t, *J* = 5.7 Hz, 1H), 5.62, 5.58 (dd, *J* = 5.8, 4.8 Hz, 1H), 4.41-4.38 (m, 1H), 4.20, 4.19 (d, *J* = 0.70 Hz, 3H), 4.03 – 3.74 (m, 4H), 3.64 – 3.50 (m, 2H), 3.13, 3.12 (s, 6H), 2.72 – 2.63 (m, 2H), 2.15, 2.11 (s, 3H), 2.07, 2.07 (s, 3H), 1.22 – 1.10 (m, 12H). **³¹P-{¹H}-NMR** (162 MHz, CDCl₃, δ /ppm): 150.42 (s, 1P), 148.61 (s, 1P). **¹³C-NMR** (101 MHz, CDCl₃, δ /ppm): 169.87, 169.67, 169.51, 169.31, 163.79, 163.77, 163.55, 163.44, 150.81, 150.32, 129.50, 129.04, 118.18, 117.92, 108.82, 108.64, 87.31, 85.72, 83.55, 82.86 (d, *J* = 9.3 Hz), 73.83, 73.80, 71.55, 70.82, 62.88, 62.59 (d, *J* = 16.7 Hz), 58.94, 58.48 (d, *J* = 21.3 Hz), 43.47, 43.34, 37.70, 37.66, 36.14, 36.11, 24.84, 24.80 (d, *J* = 7.3 Hz), 24.80, 24.65 (d, *J* = 7.3 Hz), 20.77, 20.69, 20.68, 20.62 (d, *J* = 5.3 Hz), 20.51. *The peaks marked in italic belong to the other diastereomer.* **HRMS** (ESI⁺) *m/z* for [C₂₆H₄₁N₇O₈P]⁺: calcd. 610.2749 found 610.2748.

1.5.17 Synthesis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N^{2,2,7}-trimethyl-⁷d₃-guanosine (27**)**



CEO[(iPr)₂N]₂P (80 μ L, 76 μ g, 0.25 mmol, 1.2 eq.) was added into a 0°C cold solution of 2',3'-diacetoxy N^{2,2,7}-trimethyl-⁷d₃-guanosine (**22**, 87 mg, 0.21 mmol, 1.0 eq.) in dry DMF and it was stirred for 10 min. Afterwards, ETT (as stock solution in MeCN 125 mg/mL, 0.27 mL, 33 mg, 0.25 mmol, 1.2 eq.) was added and reaction control *via* ³¹P{¹H}-NMR revealed full conversion after it was stirred at r.t. for 1 h. Next, the reaction mixture was diluted with DCM (5 mL), it was transferred into a separatory funnel and was washed once with aq. sat. NaHCO₃ (5 mL). The aqueous layer was once extracted with DCM (5 mL), the combined organic layers were dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by silica-gel MPLC* (column: *interchim*[®] SI-HP, 30 μ m, F0012; λ = 254 nm; Method **MPLC-III**; 15 mL/min) and the title compound (**27**, 70 mg**, Purity 86%, 0.10 mmol, 48%) was obtained as a colorless foam.

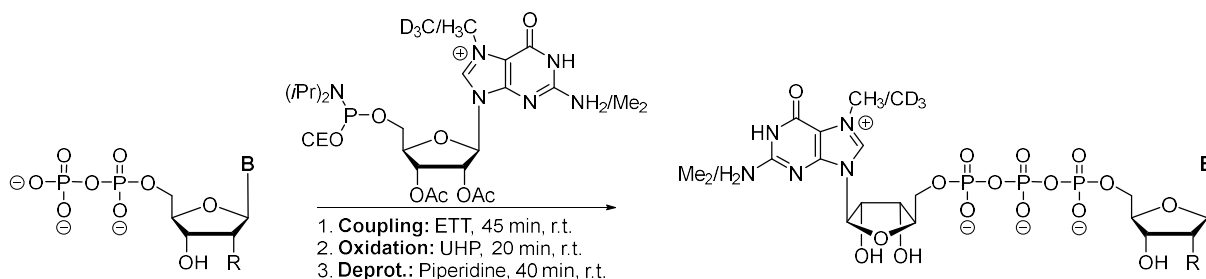
Note: Two diastereomers are obtained. *Use DCM + 2% NEt₃ to equilibrate the column on the MPLC but only use DCM without NEt₃ during the run. **It contained NEt₃. This impurity has no impact on the following coupling reaction.

¹H-NMR (400 MHz, CDCl₃, δ /ppm): 8.54 – 8.39 (m, 1H), 6.30, 6.17 (d, *J* = 5.5 Hz, 1H), 5.90 – 5.84 (m, 1H), 5.61 – 5.57 (m, 1H), 4.42– 4.39 (m, 1H), 4.03 – 3.76 (m, 4H), 3.64 – 3.51 (m, 2H), 3.15, 3.13 (s, 6H), 2.77 – 2.60 (m, 2H), 2.16, 2.13 (s, 3H), 2.08, 2.08 (s, 3H), 1.23 – 1.13 (m, 12H). **³¹P-{¹H}-NMR** (162 MHz, CDCl₃, δ /ppm): 150.48(s, 1P), 148.58 (s, 1P). **¹³C-NMR** (101 MHz, CDCl₃, δ /ppm): 169.90, 169.70, 169.53, 169.31, 163.81, 163.78, 163.61, 163.51, 150.88, 150.39, 128.90, 128.69, 118.19, 117.92, 108.87, 108.67, 87.16, 85.56, 83.66, 82.99 (d, *J* = 9.4 Hz), 73.99, 73.88, 71.63, 70.88, 62.93, 62.60 (d, *J* = 16.8 Hz), 58.97, 58.46 (d, *J* = 22.1 Hz), 43.49, 43.36, 37.69, 37.66, 35.77 (m_c), 24.91 – 24.64 (m), 20.79 – 20.51 (m). *The peaks marked in italic belong to the other diastereomer.*

HRMS (ESI⁺) *m/z* for [C₂₆H₃₈D₃N₇O₈P]⁺: calcd. 613.2937 found 613.2935.

1.6 Synthesis of cap nucleotides

1.6.1 General procedure for guanosine methylated cap nucleotides



The following syntheses were all performed according to a general procedure and were performed under inert conditions. Therefore, the nucleoside 5'-diphosphate and ETT were co-evaporated with dry MeCN (2 mL) in a pear-shaped flask. The solid was dissolved in dry DMF and guanosine P-amidite, as solution in dry DMF, was added. The reaction mixture was stirred for 45 min at r.t. Afterwards, UHP was added at 0°C and the reaction mixture was stirred at r.t. for 20 min. An aliquot of the reaction mixture was analyzed *via* HPLC-UV to determine the conversion. The reaction mixture was diluted with aq. TEAA-solution (0.1 M) and directly purified *via* RP-MPLC (column: *interchim*® C18AQ, 30 µm, F0025; λ = 254 nm; Method **MPLC-5**; 15 mL/min). The product containing fractions were combined and once evaporated under reduced pressure (20 mbar, 37 °C water bath temp.) for 40 min. The residue was diluted with dH₂O (ca. 40 mL) and then lyophilized overnight (See Note). The obtained wax was again dissolved in distilled water and lyophilized (two-times) to obtain a colorless solid. Afterwards, a final deprotection was performed by dissolving the solid in dry MeOH (obtain a solution of 10 mg/mL) and adding piperidine (10 µL for each mg of solid). The mixture was shaken at 1000 rpm, at 25 °C for 45 min. Afterwards, the piperidine was quenched by adding 1% formic acid in MeOH. The whole mixture was diluted with aq. TEAA (100 mM) and it was directly purified *via* prep-HPLC (column: waters xBridge® C₁₈, 5 µm, 19 x 250 mm; λ = 255-265 nm; Method **3-1**, 15 mL/min). The product containing fractions were combined and once evaporated under reduced pressure (20 mbar, 37 °C water bath temp.) for 40 min. The residue was diluted with distilled water (ca. 40 mL) and then lyophilized overnight. The obtained wax was again dissolved in distilled water and lyophilized (two-times) to obtain a colorless solid.

Exact amounts of reagents, specific yields and analytical data are reported in the individual procedures for each compound.

For compound **(CD₃)⁷GpppA_m** all chromatograms are shown as an example (analytical HPLC, MPLC, Prep-HPLC and CE-MS).

Note: The β-cyanoethyl group on the phosphate was cleaved during the lyophilization process after the MPLC, as proven by LC-MS:

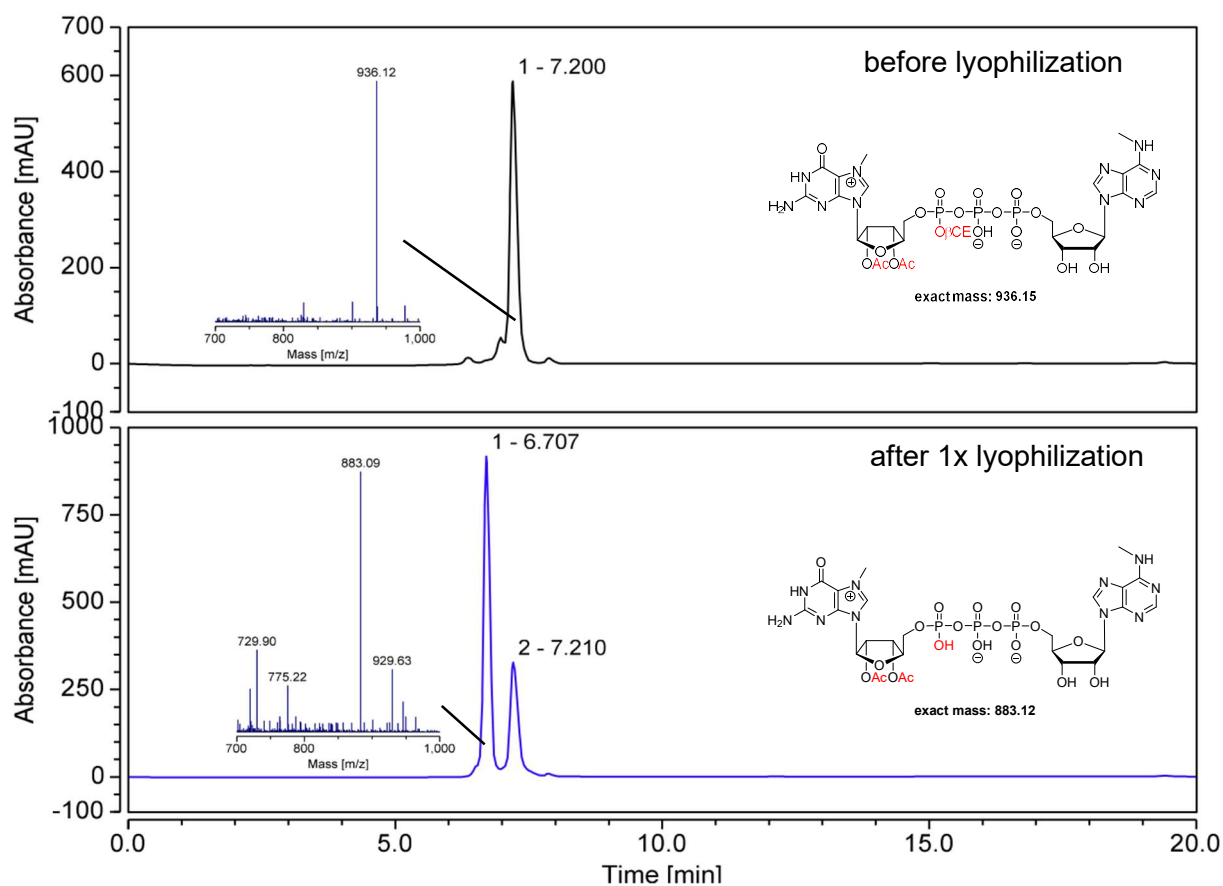
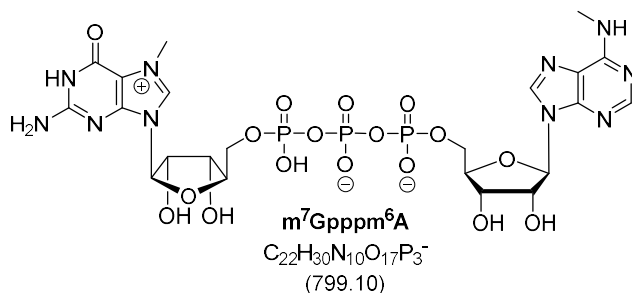


Figure SI-13: LC-MS control before lyophilization (top) and after 1-time lyophilization (bottom) (Bischoff ProntoSIL® C₁₈-AQ, 3 µm, 3.0 x 150 mm; λ = 272 nm; Method 2-2, 0.5 mL/min). This clearly proves the removal of the β-cyanoethyl group during lyophilization.

1.6.2 Synthesis of 1-5'-(7-methylguanosine) 3-5'-(6-methyl)-adenosine triphosphate (*m*⁷Gpppm⁶A):



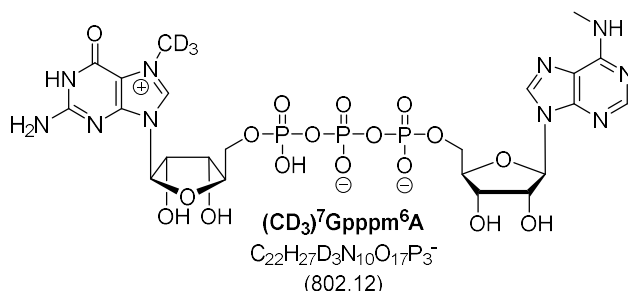
7-Methylguanosine P-amidite (**24**, 21 mg, 35 μ mol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(6-methyl)-adenosine diphosphate (**11**, 19 mg, 22 μ mol, 1.0 eq.) and ETT (6.9 mg, 55 μ mol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (5.3 mg, 55 μ mol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying, the protected intermediate was isolated as colorless solid (18.6 mg). 40% of the solid (7.6 mg) was dissolved in dry MeOH (0.9 mL) and piperidine (90 μ L) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound (***m*⁷Gpppm⁶A**, 5.1 mg, 4.6 μ mol, 52%*) was obtained as a colorless solid. 3.1 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 1111 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.39 (s, 1H), 8.18 (s, 1H), 6.03 (d, *J* = 6.2 Hz, 1H), 5.83 (d, *J* = 3.2 Hz, 1H), 4.70 (dd, *J* = 5.3 Hz, 5.9 Hz, 1H), 4.51 (dd, *J* = 3.2 Hz, 5.0 Hz, 1H), 4.45 – 4.28 (m, 8H), 3.97 (s, 3H), 3.09 (br, s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): -11.59 (d, *J* = 19.6, 1P), -11.72 (d, *J* = 19.6, 1P), -23.26 (t, *J* = 19.6 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 154.83, 152.97, 148.99, *147.50*, 138.85, 133.53, *118.67*, 108.65, 88.88, 86.31, 83.97 (d, *J* = 9.2 Hz), 83.25 (d, *J* = 9.2 Hz), 74.89, 74.49, 70.52, 68.75, 65.44 (d, *J* = 5.9 Hz), 64.22 (d, *J* = 5.9 Hz), 35.81, *27.48*. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) *m/z* for [C₂₂H₃₀N₁₀O₁₇P₃]⁻: calcd. 799.1009 found 799.1020.

1.6.3 Synthesis of 1-5'-(7-methyl-d₃-guanosine) 3-5'-(6-methyl)-adenosine triphosphate ((CD₃)⁷Gpppm⁶A):



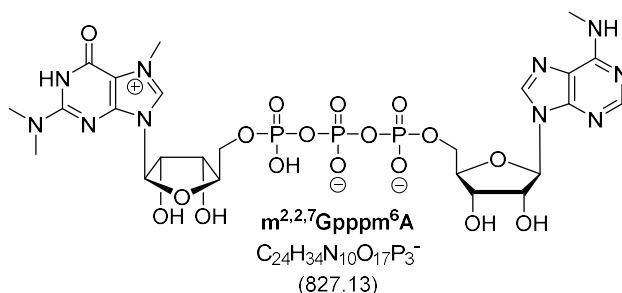
7-Methyl-d₃-guanosine P-amidite (**25**, 21 mg, 35 μmol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(6-methyl)-adenosine diphosphate (**11**, 19 mg, 22 μmol, 1.0 eq.) and ETT (6.9 mg, 55 μmol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (5.3 mg, 55 μmol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (32.1 mg). A third of the solid (11.6 mg) was dissolved in dry MeOH (1.0 mL) and piperidine (100 μL) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound ((CD₃)⁷Gpppm⁶A, 4.1 mg, 4.3 μmol, 54%*) was obtained as a colorless solid. 1.55 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 960.2 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.39 (s, 1H), 8.21 (s, 1H), 6.03 (d, *J* = 6.3 Hz, 1H), 5.88 (d, *J* = 3.4 Hz, 1H), 4.70 (dd, *J* = 6.3, 5.2 Hz, 1H), 4.52 (dd, *J* = 5.0, 3.1 Hz, 2H), 4.43 – 4.39 (m, 4H), 4.35 – 4.26 (m, 3H), 3.11 (br. s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -11.59 (d, *J* = 19.5 Hz, 1P), -11.65 (d, *J* = 19.5 Hz, 1P), -23.21 (t, *J* = 19.5 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.73, 154.88, 152.98, 149.03, *147.68*, 138.93, *118.47*, 107.67, 89.37, 86.28, 84.00 (d, *J* = 8.6 Hz), 83.72 (d, *J* = 9.3 Hz), 74.90, 74.40, 70.47, 68.96, 65.51 (d, *J* = 5.8 Hz), 64.25 (d, *J* = 5.3 Hz), *27.30*. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) *m/z* for [C₂₂H₂₆D₃N₁₀O₁₇P₃]⁻: calcd. 802.1197 found 802.1206.

1.6.4 Synthesis of 1-5'-(2-dimethyl-7-methylguanosine) 3-5'-(6-methyl)-adenosine triphosphate ($m^{2,2,7}$ Gpppm⁶A):



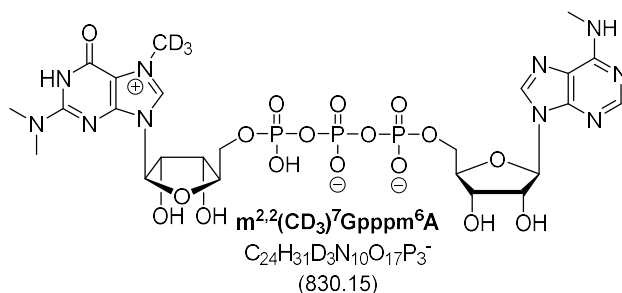
2-Dimethyl-7-methylguanosine P-amidite (**26**, 21 mg, 34 μ mol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(6-methyl)-adenosine diphosphate (**11**, 19 mg, 22 μ mol, 1.0 eq.) and ETT (6.9 mg, 55 μ mol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (5.3 mg, 55 μ mol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (18.5 mg). A third of the solid (6.2 mg) was dissolved in dry MeOH (0.6 mL) and piperidine (60 μ L) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound ($m^{2,2,7}$ Gpppm⁶A, 4.0 mg, 4.0 μ mol, 55%*) was obtained as a colorless solid. 1.65 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 995.3 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.34 (s, 1H), 8.20 (s, 1H), 5.97 (d, J = 5.9 Hz, 1H), 5.89 (d, J = 3.4 Hz, 1H), 4.59 (t, J = 5.5 Hz, 1H), 4.52 (mc, 1H), 4.47 (mc, 1H), 4.44 – 4.28 (m, 7H), 4.03 (s, 3H), 3.16 (s, 6H), 3.09 (br. s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): -11.61 (d, J = 19.1 Hz, 2P), -23.22 (t, J = 19.4 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 154.87, 153.79, 152.81, 149.05, *146.88*, 138.83, *118.04*, 106.13, 89.11, 86.54, 83.81 (d, J = 8.5 Hz), 83.57 (d, J = 9.3 Hz), 74.85, 74.61, 70.24, 68.97, 65.43 (d, J = 5.5 Hz), 64.32 (d, J = 5.2 Hz), 37.51, 36.03, 27.34. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) m/z for [C₂₄H₃₄N₁₀O₁₇P₃]⁻: calcd. 827.1322 found 827.1322.

1.6.5 Synthesis of 1-5'-(2-dimethyl-7-methyl-d₃-guanosine) 3-5'-(6-methyl)-adenosine triphosphate (*m*^{2,2}(CD₃)⁷Gpppm⁶A):



2-Dimethyl-7-methyl-d₃-guanosine P-amidite (**27**, 22 mg, 35 μmol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(6-methyl)-adenosine diphosphate (**11**, 18 mg, 22 μmol, 1.0 eq.) and ETT (6.9 mg, 55 μmol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (5.3 mg, 55 μmol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (21 mg).

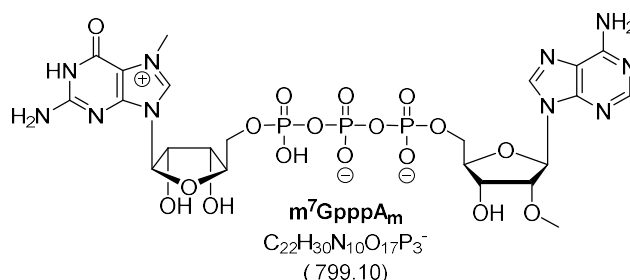
Half of the solid (10 mg) was dissolved in dry MeOH (0.9 mL) and piperidine (90 μL) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound (***m*^{2,2}(CD₃)⁷Gpppm⁶A**, 6.6 mg, 6.6 μmol, 58%*) was obtained as a colorless solid. 1.65 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 995.3 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.33 (s, 1H), 8.19 (s, 1H), 5.97 (d, *J* = 5.7 Hz, 1H), 5.89 (d, *J* = 3.5 Hz, 1H), 4.59 (t, *J* = 5.4 Hz, 1H), 4.52 (m_c, 1H), 4.47 (m_c, 1H), 4.44 – 4.24 (m, 7H), 3.15 (s, 6H), 3.08 (br. s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -11.63 (d, *J* = 19.4 Hz, 1P), -11.70 (d, *J* = 19.4, 1P), -23.23 (t, *J* = 19.4 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 154.85, 154.03, 152.81, 149.09, 147.11, 138.81, 135.45, 118.32, 106.19, 89.08, 86.55, 83.80 (d, *J* = 8.7 Hz), 83.55 (d, *J* = 9.3 Hz), 74.84, 74.62, 70.24, 68.96, 65.42 (d, *J* = 5.6 Hz), 64.31 (d, *J* = 5.4 Hz), 37.48, 27.31. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation via capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) *m/z* for [C₂₄H₃₁D₃N₁₀O₁₇P₃]⁻: calcd. 830.1510 found 830.1506.

1.6.6 Synthesis of 1-5'-(7-methylguanosine) 3-5'-(2'-methyl)-adenosine triphosphate (*m*⁷GpppA_m):



7-Methylguanosine P-amidite (**24**, 25 mg, 39 μ mol, 1.5 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl)-adenosine diphosphate (**10**, 22 mg, 26 μ mol, 1.0 eq.) and ETT (9.8 mg, 78 μ mol, 3.0 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (6.4 mg, 68 μ mol, 2.6 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (14 mg).

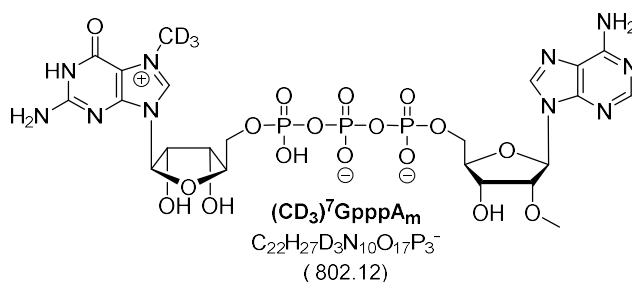
Half of the solid (6.5 mg) was dissolved in dry MeOH (0.7 mL) and piperidine (70 μ L) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound (***m*⁷GpppA_m**, 3.3 mg, 3.4 μ mol, 26%*) was obtained as a colorless solid. 1.72 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 974.2 g/mol. *Extrapolated for deprotection of all material.

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.47 (s, 1H), 8.25 (s, 1H), 6.11 (d, *J* = 5.8 Hz, 1H), 5.91 (d, *J* = 3.8 Hz, 1H), 4.69 – 4.66 (m, 1H), 4.59 – 4.56 (m, 1H), 4.47 (t, *J* = 5.0 Hz, 1H), 4.42 – 4.26 (m, 7H), 4.07 (s, 3H), 3.46 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): –11.56 (d, *J* = 19.4 Hz), –11.62 (d, *J* = 19.4 Hz), –23.21 (t, *J* = 19.4 Hz). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 155.36, 154.66, 154.59, 151.82, 149.20, 148.54, 140.04, 135.87, 118.26, 107.81, 89.36, 85.25, 84.27 (d, *J* = 8.7 Hz), 84.04 (d, *J* = 9.0 Hz), 83.09, 74.87, 69.27, 68.67, 65.25 (d, *J* = 5.9 Hz), 64.38 (d, *J* = 6.0 Hz), 58.14, 36.03. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation via capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI[–]) *m/z* for [C₂₂H₂₉N₁₀O₁₇P₃][–]: calcd. 799.1008 found 799.1015.

1.6.7 Synthesis of 1-5'-(7-methyl-d₃-guanosine) 3-5'-(2'-methyl)-adenosine triphosphate ((CD₃)⁷GpppA_m):



7-Methyl-d₃-guanosine P-amidite (**25**, 31 mg, 53 μmol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl)-adenosine diphosphate (**10**, 24 mg, 31 μmol, 1.0 eq.) and ETT (10 mg, 78 μmol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (7.3 mg, 78 μmol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (34.5 mg). A third of the solid (11.5 mg) was dissolved in dry MeOH (1.0 mL) and piperidine (100 μL) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound ((CD₃)⁷GpppA_m, 5.5 mg, 5.6 μmol, 54%*) was obtained as a colorless solid. 1.72 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 977.7 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.43 (s, 1H), 8.22 (s, 1H), 6.09 (d, *J* = 5.9 Hz, 1H), 5.90 (d, *J* = 3.8 Hz, 1H), 4.67 (dd, *J* = 5.2, 3.7 Hz, 1H), 4.56 (dd, *J* = 4.9, 3.8 Hz, 1H), 4.46 (t, *J* = 5.0 Hz, 1H), 4.44 – 4.24 (m, 7H), 3.45 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -11.58 (d, *J* = 19.5, 1P), -11.60 (d, *J* = 19.5, 1P), -23.24 (t, *J* = 19.6 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.53, 155.42, 154.79, 152.91, 149.16, 148.62, 139.67, 136.22, 118.24, 107.77, 89.33, 85.07, 84.23 (d, *J* = 8.6 Hz), 84.02 (d, *J* = 9.3 Hz), 83.03, 74.88, 69.27, 68.68, 65.31 (d, *J* = 5.6 Hz), 64.41 (d, *J* = 5.4 Hz), 58.13. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) m/z for [C₂₂H₂₇D₃N₁₀O₁₇P₃]⁻: calcd. 802.1197 found 802.1203.

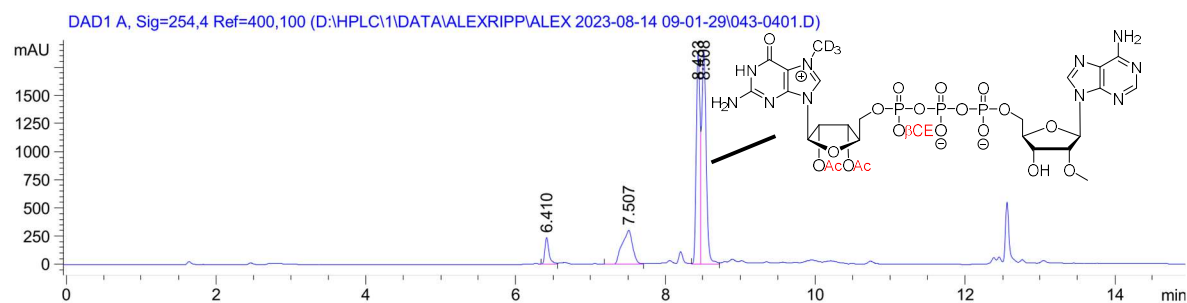


Figure SI-14: Reaction monitoring via RP-HPLC (waters xBridge™ C₁₈, 5 μm, 3.9 x 150 mm; λ = 254 nm; Method 1-3, 0.5 mL/min) after the oxidation step.

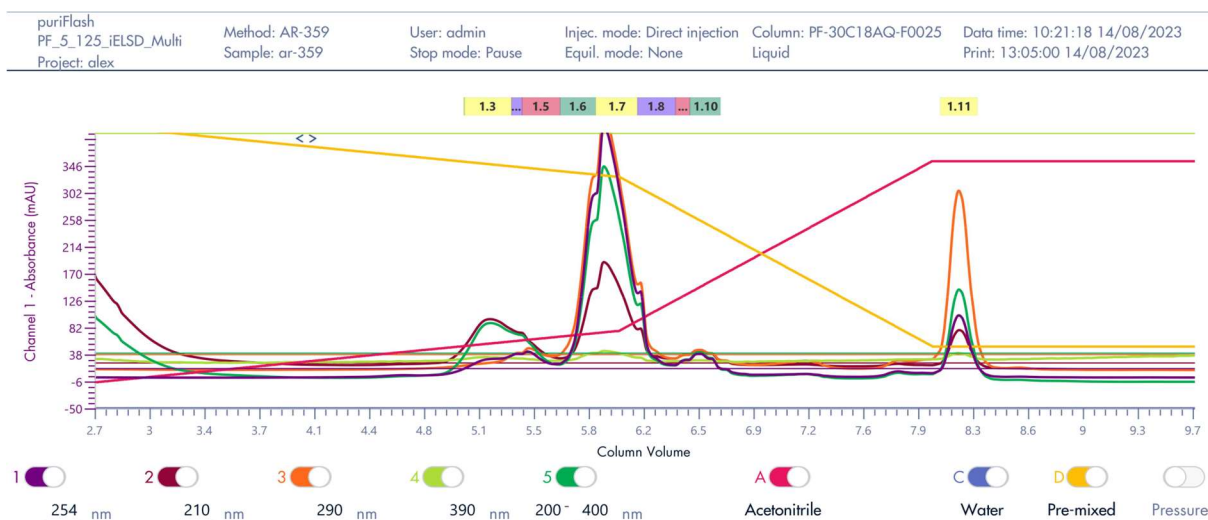


Figure SI-15: Isolation of the fully protected cap structure via RP-MPLC (column: interchim® C18AQ, 30 μm, F0025; λ = 254 nm; Method MPLC-5; 15 mL/min).

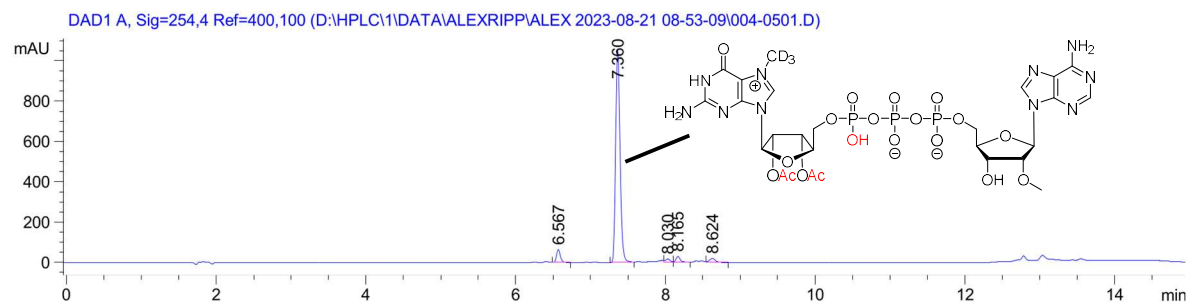


Figure SI-16: Reaction monitoring via RP-HPLC (waters xBridge™ C₁₈, 5 μm, 3.9 x 150 mm; λ = 254 nm; Method 1-3, 0.5 mL/min). Cap nucleotide is still protected with 2'- and 3'-OAc group, b-cyanoethyl group remove during lyophilization. This is before the final deprotection takes place.

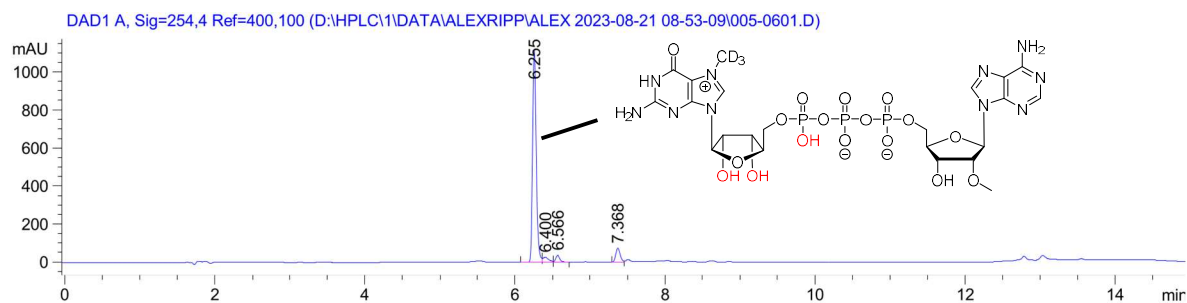
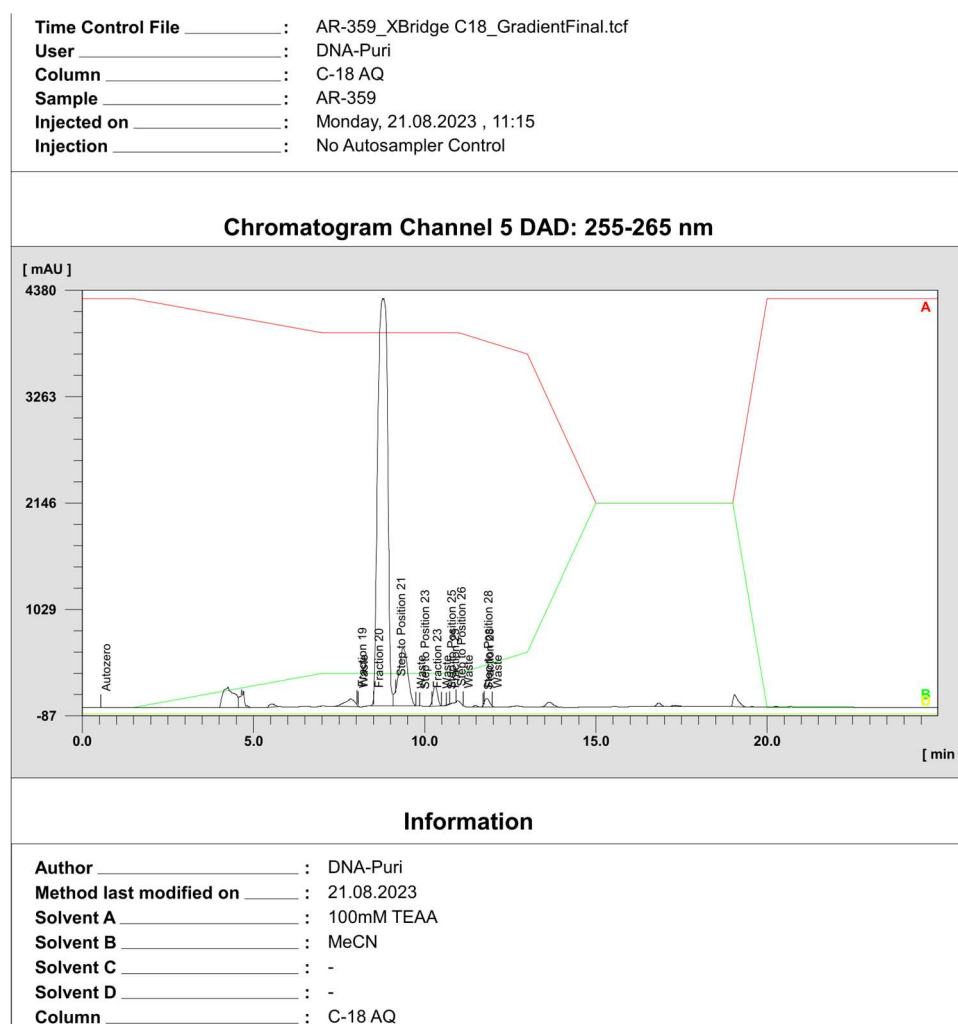


Figure SI-17: Reaction monitoring via RP-HPLC (waters xBridge™ C₁₈, 5 µm, 3.9 x 150 mm; λ = 254 nm; Method 1-3, 0.5 mL/min) after 40 min of deprotection (piperidine in MeOH), but before Prep-HPLC. **Note:** Analysis sample added to a solution of 1% formic acid in MeOH, before injection into RP-HPLC.



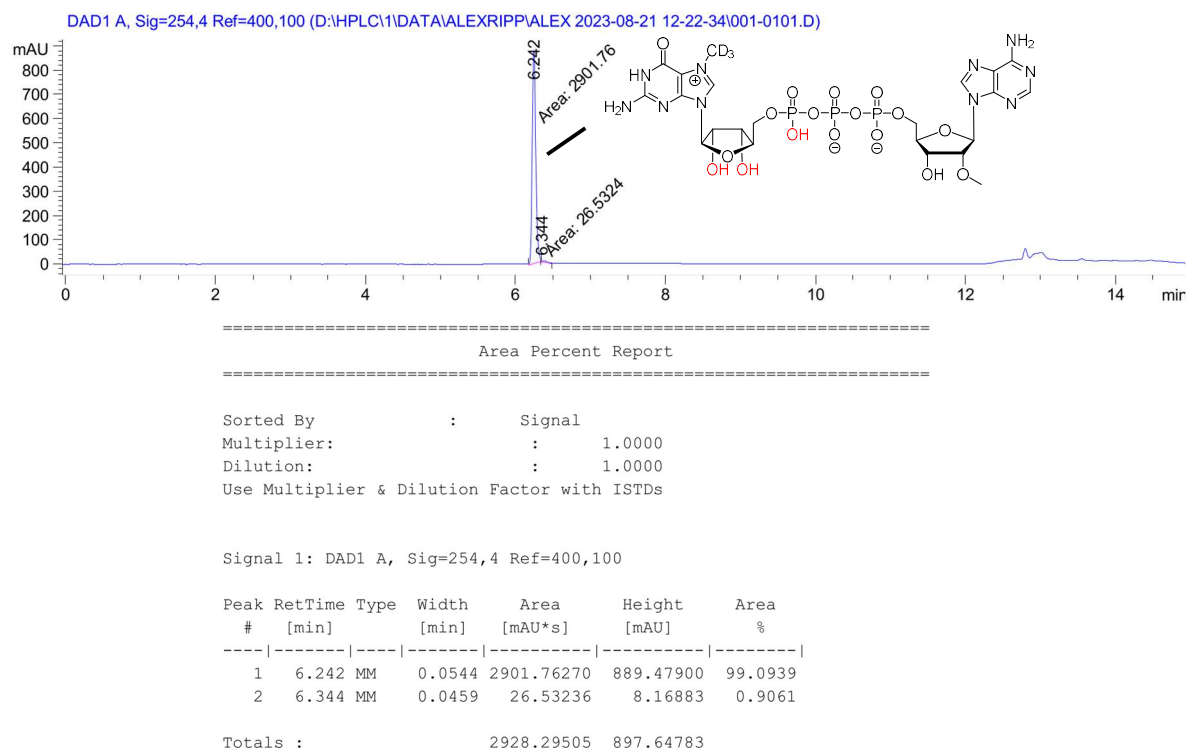
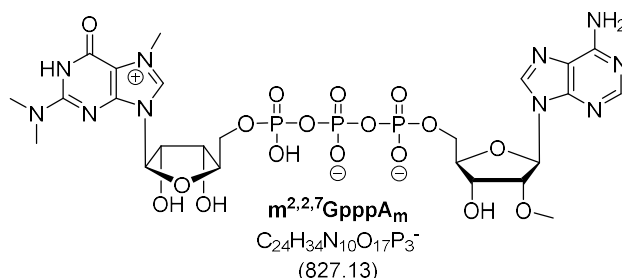


Figure SI-19: RP-HPLC (waters xBridge™ C₁₈, 5 μm, 3.9 x 150 mm; λ = 254 nm; Method 1-3, 0.5 mL/min) analysis of the final cap nucleotide.

1.6.8 Synthesis of 1-5'-(2-dimethyl-7-methylguanosine) 3-5'-(2'-methyl)-adenosine triphosphate ($m^{2,2,7}$ GpppA_m):



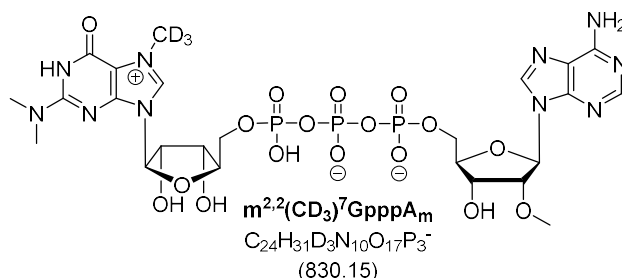
2-Dimethyl-7-methylguanosine P-amidite (**26**, 31 mg, 53 μ mol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl)-adenosine diphosphate (**10**, 24 mg, 31 μ mol, 1.0 eq.) and ETT (10 mg, 78 μ mol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (7.3 mg, 78 μ mol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (28.5 mg). A third of the solid (9.7 mg) was dissolved in dry MeOH (0.9 mL) and piperidine (90 μ L) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound ($m^{2,2,7}$ GpppA_m, 6.3 mg, 6.3 μ mol, 60%*) was obtained as a colorless solid. 1.76 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 1006.3 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.36 (s, 1H), 8.20 (s, 1H), 6.02 (d, J = 5.6 Hz, 1H), 5.91 (d, J = 3.8 Hz, 1H), 4.60 (dd, J = 5.2, 4.0 Hz, 1H), 4.55 (dd, J = 4.5, 3.8 Hz, 1H), 4.46 – 4.24 (m, 8H), 4.07 (s, 3H), 3.42 (s, 3H), 3.16 (s, 6H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): -11.65 (d, J = 19.6, 1P), -11.59 (d, J = 19.6, 1P), -23.27 (t, J = 19.6 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 155.35, 155.09, 153.76, 152.81, 149.17, 148.38, 139.46, 135.88, 118.15, 106.09, 88.95, 85.12, 84.06 (d, J = 8.4 Hz), 83.82 (d, J = 9.1 Hz), 83.30, 74.88, 69.26, 68.57, 65.31 (d, J = 5.6 Hz), 64.53 (d, J = 5.5 Hz), 58.14, 37.51, 36.02. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) m/z for [C₂₄H₃₄N₁₀O₁₇P₃]⁻: calcd. 827.1322 found 827.1333.

1.6.9 Synthesis of 1-5'-(2-dimethyl-7-methyl-d₃-guanosine) 3-5'-(2'-methyl)-adenosine triphosphate (*m*^{2,2}(CD₃)⁷GpppA_m):



2-Dimethyl-7-methyl-d₃-guanosine P-amidite (**27**, 31 mg, 53 μmol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl)-adenosine diphosphate (**10**, 24 mg, 31 μmol, 1.0 eq.) and ETT (10mg, 78 μmol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (7.3 mg, 78 μmol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (25.1 mg).

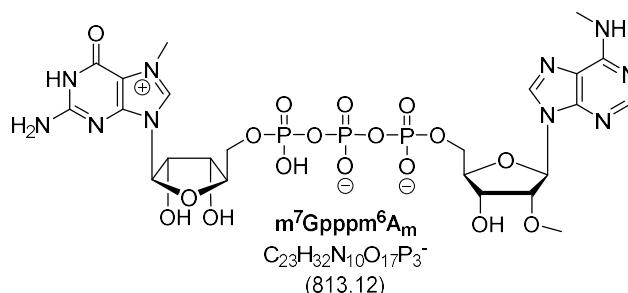
A third of the solid (8.7 mg) was dissolved in dry MeOH (0.8 mL) and piperidine (80 μL) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound (*m*^{2,2}(CD₃)⁷GpppA_m, 5.5 mg, 5.4 μmol, 50%*) was obtained as a colorless solid. 1.8 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 1013.8 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.36 (s, 1H), 8.20 (s, 1H), 6.03 (d, *J* = 5.6 Hz, 1H), 5.91 (d, *J* = 3.8 Hz, 1H), 4.60 (dd, *J* = 5.3, 3.9 Hz, 1H), 4.55 (t, *J* = 4.1 Hz, 1H), 4.46 – 4.24 (m, 8H), 3.42 (s, 3H), 3.16 (s, 6H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -11.63 (d, *J* = 19.6, 1P), -11.58 (d, *J* = 19.5, 1P), -23.26 (t, *J* = 19.5 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.35, 155.06, 153.74, 152.81, 149.17, 148.38, 139.46, 136.30, 118.15, 106.08, 88.95, 85.13, 84.05 (d, *J* = 8.4 Hz), 83.82 (d, *J* = 9.1 Hz), 83.30, 74.88, 69.27, 68.57, 65.31 (d, *J* = 5.8 Hz), 64.53 (d, *J* = 5.4 Hz), 58.14, 37.51. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) *m/z* for [C₂₄H₃₁D₃N₁₀O₁₇P₃]⁻: calcd. 830.1510 found 830.1509.

1.6.10 Synthesis of 1-5'-(7-methylguanosine) 3-5'-(2'-methyl-6-methyl)-adenosine triphosphate ($m^7Gpppm^6A_m$):



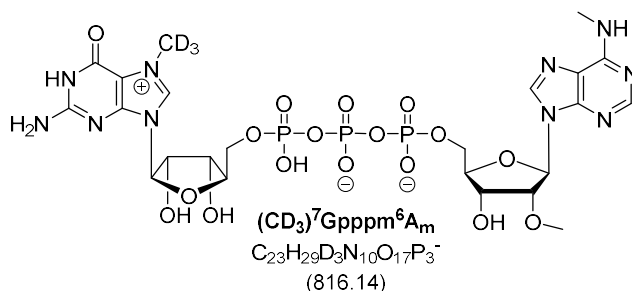
7-Methylguanosine P-amidite (**24**, 17 mg, 29 μ mol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl-6-methyl)-adenosine diphosphate (**12**, 11 mg, 17 μ mol, 1.0 eq.) and ETT (5.6 mg, 43 μ mol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (4.1 mg, 43 μ mol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (13.9 mg). Half of the solid (6.7 mg) was dissolved in dry MeOH (0.7 mL) and piperidine (70 μ L) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound ($m^7Gpppm^6A_m$, 3.4 mg, 3.5 μ mol, 42%*) was obtained as a colorless solid. 1.5 eq. TEA per triphosphate were found by 1H -NMR, resulting in a molecular weight of 964.6 g/mol. *Extrapolated for deprotection of all material.

1H -NMR (400 MHz, D_2O , δ /ppm): 8.38 (s, 1H), 8.22 (s, 1H), 6.08 (d, J = 6.1 Hz, 1H), 5.88 (d, J = 3.8 Hz, 1H), 4.66 (dd, J = 5.2, 3.6 Hz, 1H), 4.54 (dd, J = 4.8, 3.8 Hz, 1H), 4.48 – 4.21 (m, 8H), 4.03 (s, 3H), 3.45 (s, 3H), 3.12 (br. s, 3H). **^{31}P -{ 1H }-NMR** (162 MHz, D_2O , δ /ppm): –11.58 (d, J = 19.7 Hz, 1P), –11.64 (d, J = 19.7 Hz, 1P), –23.27 (t, J = 19.6 Hz, 1P). **^{13}C -NMR** (101 MHz, D_2O , δ /ppm): 155.36, 154.81, 154.49, 152.82, 149.09, 147.41, 139.03, 136.24, 118.50, 107.64, 89.35, 84.92, 84.20 (d, J = 8.6 Hz), 84.03 (d, J = 9.2 Hz), 82.94, 74.91, 69.28, 68.65, 65.33 (d, J = 5.6 Hz), 64.45 (d, J = 5.7 Hz), 58.11, 36.01, 27.09. The peaks, marked in *italic*, were assigned by HMBC, since they gave very broad peaks in the ^{13}C -spectrum. ^{13}C -Spectrum was calibrated on CH_2 in TEA with 46.68 ppm.

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH_4OAc pH 9.75 adjusted with NH_4OH , method: according to D. Qiu.^[8]

Detection (ESI^-) m/z for $[C_{23}H_{32}O_{17}N_{10}P_3]^-$: calcd. 813.1165 found 813.1168.

1.6.11 Synthesis of 1-5'-(7-methyl-d₃-guanosine) 3-5'-(2'-methyl-6-methyl)-adenosine triphosphate ((CD₃)⁷Gpppm⁶A_m):



7-Methyl-d₃-guanosine P-amidite (**25**, 17 mg, 29 μmol, 1.7 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl-6-methyl)-adenosine diphosphate (**12**, 14 mg, 17 μmol, 1.0 eq.) and ETT (5.6 mg, 43 μmol, 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (4.1 mg, 43 μmol, 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (16 mg).

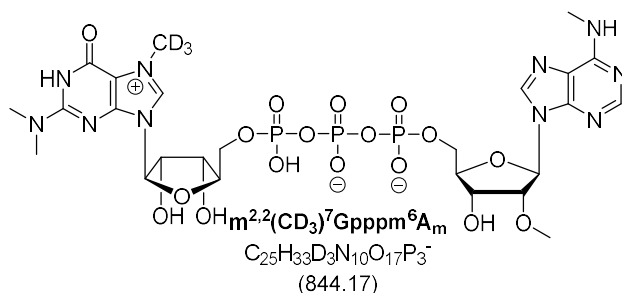
Half of the solid (8.2 mg) was dissolved in dry MeOH (0.8 mL) and piperidine (80 μL) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound ((CD₃)⁷Gpppm⁶A_m, 4.7 mg, 4.7 μmol, 54%*) was obtained as a colorless solid. 1.8 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 998.7 g/mol. **Extrapolated for deprotection of all material.*

¹H-NMR (400 MHz, D₂O, δ/ppm): 8.38 (s, 1H), 8.22 (s, 1H), 6.08 (d, *J* = 6.0 Hz, 1H), 5.88 (d, *J* = 3.8 Hz, 1H), 4.66 (dd, *J* = 5.2, 3.7 Hz, 1H), 4.54 (mc, 1H), 4.47 – 4.23 (m, 8H), 3.44 (s, 3H), 3.13 (br. s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ/ppm): -11.58 (d, *J* = 19.6 Hz, 1P), -11.64 (d, *J* = 19.6 Hz, 1P), -23.27 (t, *J* = 19.6 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ/ppm): 155.46, 154.92, 154.64, 152.96, 149.09, *146.83*, 138.98, *135.52*, *118.04*, 107.65, 89.33, 84.89, 84.20 (d, *J* = 8.6 Hz), 84.03 (d, *J* = 9.1 Hz), 82.94, 74.92, 69.28, 68.65, 65.34 (d, *J* = 5.4 Hz), 64.46 (d, *J* = 5.6 Hz), 58.11, 27.39. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI⁻) *m/z* for [C₂₃H₂₈D₃N₁₀O₁₇P₃]⁻: calcd. 816.1353 found 816.1355.

1.6.12 Synthesis of 1-5'-(2-dimethyl-7-methyl-d₃-guanosine) 3-5'-(2'-methyl-6-methyl)-adenosine triphosphate (*m*^{2,2}(CD₃)⁷Gpppm⁶A_m):



2-Dimethyl-7-methyl-d₃-guanosine P-amidite (**27**, 20 mg, 33 μmol , 1.9 eq.) was added, dissolved in 0.5 mL of dry DMF, to a solution of 5'-(2'-methyl-6-methyl)-adenosine diphosphate (**12**, 13 mg, 17 μmol , 1.0 eq.) and ETT (5.6mg, 43 μmol , 2.5 eq.) in dry DMF (0.5 mL). After 45 min of stirring at r.t., UHP (4.1 mg, 43 μmol , 2.5 eq.) was added at 0°C and it was stirred at r.t. for 20 min. After dilution with aq. TEAA solution, RP-MPLC and repeated freeze-drying the protected intermediate was isolated as colorless solid (15.5 mg).

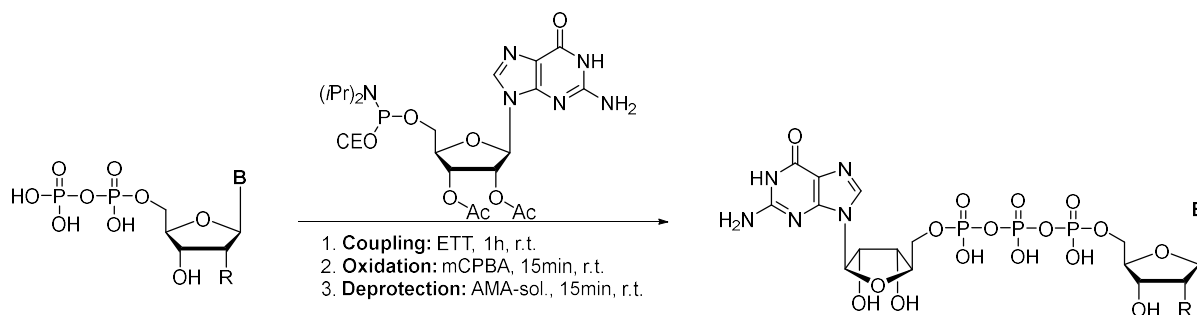
Half of the solid (7.2 mg) was dissolved in dry MeOH (0.7 mL) and piperidine (70 μL) was added. After shaking for 40 min at r.t., 1% formic acid in MeOH (1.0 mL) was added and the mixture was poured into aq. TEAA solution (8 mL). After prep-HPLC and repeated freeze drying the title compound (*m*^{2,2}(CD₃)⁷Gpppm⁶A_m, 4.0 mg, 3.9 μmol , 49%) was obtained as a colorless solid. 1.7 eq. TEA per triphosphate were found by ¹H-NMR, resulting in a molecular weight of 1015.9 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.32 (s, 1H), 8.20 (s, 1H), 6.02 (d, *J* = 5.6 Hz, 1H), 5.88 (d, *J* = 3.7 Hz, 1H), 4.60 (m_c, 1H), 4.54 (t, *J* = 3.9 Hz, 1H), 4.49 – 4.20 (m, 8H), 3.43 (s, 3H), 3.15 (s, 6H), 3.11 (br. s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): –11.64 (d, *J* = 19.4, 1P), –11.57 (d, *J* = 19.4, 1P), –23.26 (t, *J* = 19.4 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 154.90, *153.85*, 152.83, 149.10, *147.10*, 138.83, 135.92, *118.55*, 106.12, 89.01, 85.06, 84.02 (d, *J* = 8.6 Hz), 83.81 (d, *J* = 9.0 Hz), 83.27, 74.87, 69.25, 68.57, 65.28 (d, *J* = 5.6 Hz), 64.52 (d, *J* = 5.4 Hz), 58.14, 37.49, 27.36. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

CE-MS: Separation *via* capillary: 100 cm, bare fused silica, activated with 1.0 M aq. NaOH. Buffer: 35 mM NH₄OAc pH 9.75 adjusted with NH₄OH, method: according to D. Qiu.^[8]

Detection (ESI[–]) *m/z* for [C₂₅H₃₃D₃N₁₀O₁₇P₃][–]: calcd. 844.1667 found 844.1675.

1.6.13 General procedure for guanosine cap nucleotides

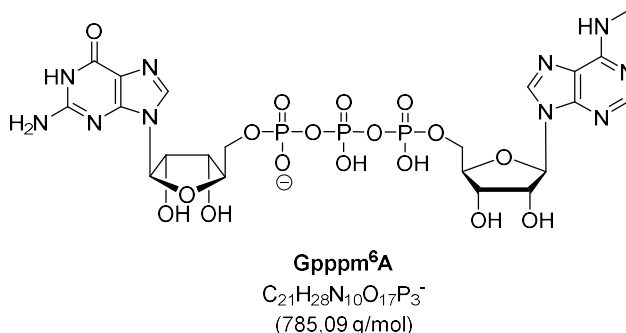


The following syntheses were all performed according to a general procedure under inert-conditions. Therefore, the used nucleoside 5'-diphosphate and the acidic activator ETT were co-evaporated with dry MeCN (2×2 mL) in a pear shaped flask. Afterwards, the solids were dissolved in dry DMF (add as much as is required for complete dissolving) and guanosine P-amidite was added. The reaction mixture was stirred for 30 min at r.t.. Afterwards, *m*CPBA was added and the reaction mixture was stirred at r.t. for 30 min. The protected triphosphate was isolated by pouring the reaction mixture into -20°C cold Et_2O and the resulting colorless precipitate was collected via centrifugation. After washing with Et_2O and drying *in vacuo* the desired product was obtained as a colorless solid. The crude material was purified by RP-MPLC (column: *interchim*® C18AQ, 30 μm , F0040; Method **MPLC-6**; Flow rate: 26 mL/min). Repeated freeze drying of the product containing fractions afforded the 2',3'-OAc protected intermediate.

Afterwards, final deprotection was performed by dissolving the solid in H_2O (obtain a solution of 10 mg/mL) and adding AMA-solution (50vol%; 1:1, v:v, 30% ammonium hydroxide and 40% aqueous methylamine). The reaction mixture was shaken on a thermocycler at r.t. for 15 min. The solution was diluted by 10-fold with dd H_2O and it was lyophilized. The obtained colorless solid was purified by prep-HPLC (*Bischoff ProntoSIL*® C₁₈-AQ, 5 μm , 250 x 20 mm; $\lambda = 255\text{-}265$ nm; Method **3-2**, 15 mL/min). Repeated freeze drying of the product containing fractions afforded the desired product as a colorless solid.

Exact amounts of reagents, further purification methods, specific yields and analytical data are reported in the individual procedures for each compound.

1.6.14 Synthesis of 1-5'-guanosine 3-5'-(6-methyl)-adenosine triphosphate (Gpppm⁶A):



Guanosine P-amidite (**23**, 21 mg, 37 μ mol, 1.6 eq.) was added, dissolved in 0.42 mL of dry DMF, to a solution of N⁶-methyadenosine 5'-diphosphate (**11**, 15 mg, 23 μ mol, 1.0 eq.) and ETT (18 mg, 138 μ mol, 6.0 eq.) in dry DMF (1.5 mL). After 30 min of stirring at r.t., *m*CPBA (14 mg, 81 μ mol, 3.5 eq.) was added and it was stirred at r.t. for another 30 min. After precipitation, RP-MPLC purification, and repeated freeze-drying the protected intermediate was obtained as a colorless solid (42 mg).

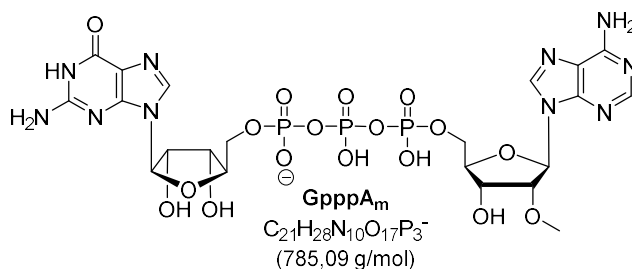
After AMA-deprotection, prep-HPLC, and repeated freeze drying the title compound (**Gpppm⁶A**, 5.5 mg, 5.0 μ mol, 22%) was obtained as a colorless solid.

The exact amount of triethylammonium counterions per triphosphate molecule, calculated from ¹H-NMR, was found to be 3.1. This results in a molecular weight of 1101.3 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.34 (s, 1H), 8.18 (s, 1H), 7.93 (s, 1H), 6.07 (d, *J* = 4.5 Hz, 1H), 5.81 (d, *J* = 5.1 Hz, 1H), 4.72 – 4.64 (m, 2H), 4.54 (t, *J* = 4.9 Hz, 1H), 4.51 (t, *J* = 4.8 Hz, 1H), 4.41 – 4.20 (m, 6H), 3.08 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): -11.61 (d, *J* = 19.8 Hz), -23.16 (t, *J* = 19.5 Hz). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 158.16, 154.53, 153.53, 152.56, 151.01, *147.00*, 138.67, 136.90, *118.42*, 115.60, 87.37, 87.08, 83.12 (d, *J* = 9.4 Hz), 82.97 (d, *J* = 9.4 Hz), 74.80, 74.03, 69.85, 69.54, 64.71 (d, *J* = 5.3 Hz), 64.58 (d, *J* = 5.5 Hz), *27.39*. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI⁻) *m/z* for [C₂₁H₂₇N₁₀O₁₇P₃]²⁻: calcd. 392.0390 found 392.0387.

1.6.15 Synthesis of 1-5'-guanosine 3-(2'-O-methoxy)5'-adenosine triphosphate (GpppA_m):



Guanosine P-amidite (**23**, 50 mg, 88 μ mol, 1.5 eq.) was added, dissolved in 1 mL of dry DMF, to a solution of 2'-O-methyladenosine 5'-diphosphate (**10**, 38 mg, 59 μ mol, 1.0 eq.) and ETT (38 mg, 300 μ mol, 5.0 eq.) in dry DMF (1.5 mL). After 30 min of stirring at r.t., *m*CPBA (35 mg, 140 μ mol, 2.3 eq.) was added and it was stirred at r.t. for 30 min. After precipitation, RP-MPLC purification, and repeated freeze-drying, the protected intermediate was obtained as a colorless solid (30 mg).

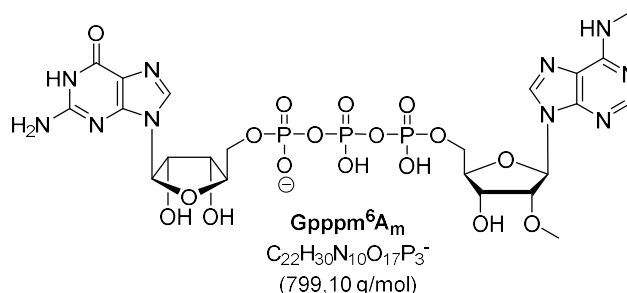
After AMA-deprotection, prep-HPLC, and repeated freeze drying the title compound (**GpppA_m**, 19.5 mg, 17.7 μ mol, 30%) was obtained as a colorless solid.

The exact amount of triethylammonium counterions per triphosphate molecule, calculated from ¹H-NMR, was found to be 3.1. This results in a molecular weight of 1101.3 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.38 (s, 1H), 8.19 (s, 1H), 8.01 (s, 1H), 6.12 (d, *J* = 4.4 Hz, 1H), 5.82 (d, *J* = 5.5 Hz, 1H), 4.72 – 4.68 (m_c, 1H), 4.63 (m_c, 1H), 4.51 (dd, *J* = 5.0, 3.9 Hz, 1H), 4.35 – 4.25 (m, 7H), 3.56 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): -9.15 (d, *J* = 19.8 Hz), -20.68 (t, *J* = 19.5 Hz). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 158.31, 155.03, 153.57, 152.45, 151.21, 148.34, 139.45, 137.16, 118.22, 115.77, 87.03, 85.63, 83.56, 83.36 (d, *J* = 6.3 Hz), 83.27 (d, *J* = 6.6 Hz), 73.93, 70.04, 68.13, 64.90 (d, *J* = 5.5 Hz), 64.42 (d, *J* = 5.2 Hz), 58.36. *¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI⁻) *m/z* for [C₂₁H₂₇N₁₀O₁₇P₃]²⁻: calcd. 392.0390 found 392.0390.

1.6.16 Synthesis of 1-5'-guanosine 3-(2'-O-methoxy)5'-(6-methyl)-adenosine triphosphate (Gpppm⁶A_m):



Guanosine P-amidite (**23**, 30 mg, 54 μ mol, 1.5 eq.) was added, to a solution of 2'-O-methyl-N⁶-methyladenosine 5'-diphosphate (**12**, 27 mg, 36 μ mol, 1.0 eq.) and ETT (28 mg, 215 μ mol, 6.0 eq.) in dry DMF:DMF- d_7 (2.5 mL). After 30 min of stirring at r.t., *m*CPBA (18 mg, 105 μ mol, 3.0 eq.) was added and it was stirred at r.t. for 30 min. After precipitation, RP-MPLC purification, and repeated freeze-drying the protected intermediate was obtained as a colorless solid (20 mg).

After AMA-deprotection, prep-HPLC, and repeated freeze drying the title compound (**Gpppm⁶A_m**, 15 mg, 13 μ mol, 36%) was obtained as a colorless solid.

The exact amount of triethylammonium counterions per triphosphate molecule, calculated from ¹H-NMR, was found to be 3.3. This results in a molecular weight of 1105.1 g/mol.

¹H-NMR (400 MHz, D₂O, δ /ppm): 8.32 (s, 1H), 8.18 (s, 1H), 7.96 (s, 1H), 6.12 (d, *J* = 4.1 Hz, 1H), 5.80 (d, *J* = 5.4 Hz, 1H), 4.68 (t, *J* = 5.2 Hz, 1H), 4.63 (m_c, 1H), 4.50 (dd, *J* = 5.0, 3.9 Hz, 1H), 4.40 – 4.19 (m, 7H), 3.58 (s, 3H), 3.10 (s, 3H). **³¹P-{¹H}-NMR** (162 MHz, D₂O, δ /ppm): -11.69 (d, *J* = 19.5 Hz, 2P), -23.22 (t, *J* = 19.6 Hz, 1P). **¹³C-NMR** (101 MHz, D₂O, δ /ppm): 158.22, 154.66, 153.51, 152.64, 151.10, 146.94, 138.65, 137.02, 118.47, 115.72, 87.06, 85.58, 83.23 (d, *J* = 9.1 Hz), 83.10 (d, *J* = 9.6 Hz), 73.91, 69.97, 68.05, 64.83 (d, *J* = 5.4 Hz), 64.33 (d, *J* = 5.5 Hz), 58.39, 27.51. *The peaks, marked in italic, were assigned by HMBC, since they gave very broad peaks in the ¹³C-spectrum. ¹³C-Spectrum was calibrated on CH₂ in TEA with 46.68 ppm.*

HRMS (ESI⁻) *m/z* for [C₂₂H₂₉N₁₀O₁₇P₃]²⁻: calcd. 399.0468 found 399.0464.

2 LC-MS/MS analytics

2.1 Preparation of standard solutions

GpppA and m⁷GppA were purchased from NEB. Gpppm⁶A, GpppAm, Gpppm⁶Am, m⁷Gpppm⁶A, m⁷GpppAm, and m⁷Gpppm⁶Am were synthesized as described in the results part. The cap standards were dissolved in nuclease-free water. The absorbance at 260 nm was measured using a Nanodrop 2000 (Thermo Scientific) to calculate the concentration based on published extinction coefficients.^[9] Standard solutions were prepared containing a mixture of the cap dinucleotides with same concentrations.

2.2 LC-MS/MS method development

An Agilent 1260 Infinity system equipped with a diode array detector in combination with an Agilent 6470 Triple Quadrupole (QQQ) mass spectrometer with an electrospray ion source was used.

2.2.1 MS parameters

The optimization process started by determining the analyte specific mass parameters, which includes the mass-to-charge ratio (m/z) of precursor and product ions, the fragmentor voltage and the collision energy. Therefore, 10 pmol of each pure standards were subjected to LC-MS/MS analysis.

The mobile phase consisted constantly of 90% of solvent A (20 mM ammonium acetate (Sigma-Aldrich), pH 6, adjusted with glacial acid (VWR)) and 10% of solvent B (acetonitrile LC/MS grade, VWR). A flow rate of 0.35 mL/min and a column temperature of 35°C were applied.

The MS was operated in full scan mode to detect precursor ions, which was followed by a further optimization step to test different fragmentor voltages by operating in single-ion monitoring (SIM) mode to yield maximum abundance.

In a next step, a product ion scan was performed, using the already determined precursor ion m/z and optimized fragmentor voltage for the first quadrupole, while different collision energies were applied in the collision cell. The most abundant resulting product ions with their respective collision energies were selected in an automated manner using the Optimizer Software (MassHunter Workstation Optimizer 10.0, Agilent Technologies). The resulting mass transitions, fragmentor voltages and collision energies that led to the highest abundant signals were selected for following LC-MS/MS methods and are listed in Table SI-1. Initially, the source parameters were adopted from nucleoside analysis^[10] and were further optimized for cap analysis as last step of the LC-MS/MS optimization process.

Table SI-1. Mass spectrometer settings for cap dinucleotides. The parameters of the isotope-labeled standards D₃C-m⁷Gpppm⁶A, D₃C-m⁷GpppAm, and D₃C-m⁷Gpppm⁶Am are listed as labeled precursor/product mass-to-charge ratios (m/z).

| Compound Name | Precursor Ion [m/z] | Product Ion [m/z] | Fragmentor voltage [V] | Collision Energy [V] | (D ₃ C) ⁷ G-labeled precursor [m/z] | (D ₃ C) ⁷ G-labeled product [m/z] |
|--------------------------------------|---------------------|-------------------|------------------------|----------------------|---|---|
| GpppA | 773 | 136 | 145 | 66 | | |
| Gpppm ⁶ A | 787 | 150 | 155 | 82 | | |
| GpppAm | 787 | 136 | 180 | 58 | | |
| Gpppm ⁶ Am | 801 | 150 | 165 | 82 | | |
| m ⁷ GpppA | 787 | 136 | 130 | 82 | | |
| m ⁷ Gpppm ⁶ A | 801 | 150 | 175 | 90 | 804 | 150 |
| m ⁷ GpppAm | 801 | 136 | 165 | 62 | 804 | 136 |
| m ⁷ Gpppm ⁶ Am | 815 | 150 | 165 | 74 | 818 | 150 |

2.2.2 HPLC optimization

The HPLC method was initially based on the method reported by Muthmann et al.,^[11] which focusses on the analytics of synthetic mRNA with GpppG and m⁷GpppG as relevant cap dinucleotides and was adapted and optimized for our equipment and our purposes. Different mobile phases (ion strength of ammonium acetate and pH value) and column temperatures were tested step-by-step and evaluated regarding signal amplitude, peak width and symmetry, as well as separation to find optimal parameters. An overview of the HPLC and MS parameters for each optimizing step are listed in Table SI-2.

10 fmol to 1 pmol standard solution containing a mixture of the cap dinucleotides were injected into the LC-MS. The mobile phase consisted of solvent A: 5, 10, or 20 mM ammonium acetate (Sigma-Aldrich), (pH 6 or 7, adjusted with acetic acid (VWR) and ammonium hydroxide (Honeywell Riedel-de Haen)) and solvent B: acetonitrile (VWR). For chromatographic separation, a Poroshell 120EC-C18, 3 x 150 mm, 2.7 μM (Agilent Technologies) was used at a column temperature of 15, 20, 25, or 35°C. The cap standards were chromatographically separated with a flow rate of 0.35 mL/min, applying different gradients (Table SI-2 and SI-3). The MS was operated in MRM (multiple reaction monitoring) mode using the previously optimized MS parameters (Table SI-1) and the source parameters as described in Table M4. Data analysis was performed by using the MassHunter software (MassHunter Qualitative Analysis 10.0, Agilent Technologies).

2.2.2.1 Buffer ion strength

1 pmol of a mixture of cap dinucleotides was injected to the LC-MS. The measurements were performed using three different mobile phases with solvent A consisting of 5, 10, or 20 mM ammonium acetate, each at pH 6.

2.2.2.2 Buffer pH

Analysing the concentration of 5 mM ammonium acetate as solvent A, the two different pH values pH 6 and pH 7 were tested. To investigate peak symmetry, 1 pmol of a mixture of cap dinucleotides was injected to the LC-MS. Tailing factors were calculated with the following formula:^[12]

$$A_s = W_{0.05h} / 2f$$

with $W_{0.05h}$ = peak width at 5% of peak maxima and f = distance from the leading edge of the peak to the peak maximum measured at 5% of the maximum peak height from the baseline. 10 fmol of a mixture of cap dinucleotides was injected to analyze signal-to-noise ratios (S/N).

2.2.2.3 Column temperature

Temperatures of 15°C, 20°C, 25°C and 35°C were studied. 1 pmol of a mixture of cap dinucleotides in injection volumes of 1 µL or 50 µL were injected to the LC-MS.

2.2.3 Ion source parameters

1 pmol of a mixture of cap dinucleotides was injected and the improved HPLC method was applied. The parameters drying gas flow and temperature, capillary voltage, nozzle voltage, sheath gas flow and temperature and nebulizer pressure were optimized successively. This was automated by the Source Optimizer Software (MassHunter Source Optimizer 10.0). The final parameters were selected according to the signal strength in agreement with all analyzed cap standards. A comparative measurement between the non-optimized source parameters and the optimized parameters (Table SI-4) was carried out.

The investigated parameters during LC-MS/MS method optimization are summarized in Figure SI-20

Table SI-2. Overview of HPLC and ion source parameters of each optimization step. The investigated parameters are highlighted in bold.

| Optimized parameter | Column | Flow rate | Mobile phase A | | Mobile phase B | Column temperature [°C] | Gradient (Table M3) | Source parameters (Table M4) |
|----------------------|---|-------------|-------------------------------------|----------------|----------------|---------------------------|---------------------|----------------------------------|
| | | | Ammonium acetate concentration [mM] | pH | | | | |
| Non-optimized method | | | 20 | 6 | | 20 | 1 | Non-optimized |
| Buffer ion strength | | | 5, 10, and 20 | 6 | | 20 | 1 | Non-optimized |
| Buffer pH | Poroshell 120EC-C18, 3 x 150 mm, 2.7 µM | 0.35 mL/min | 5 | 6 and 7 | Aceto-nitrile | 20 | 1 | Non-optimized |
| Column temperature | | | 5 | 7 | | 15, 20, 25, and 35 | 2 | Non-optimized |
| Source optimization | | | 5 | 7 | | 15 | 2 | Non-optimized / Optimized |
| Final method | | | 5 | 7 | | 15 | 3 | Optimized |

Table SI-3. Chromatographic elution gradients

| Gradient 1 | | | Gradient 2 | | | Gradient 3 | | |
|------------|-------|-------|------------|-------|-------|------------|-------|-------|
| Time [min] | A [%] | B [%] | Time [min] | A [%] | B [%] | Time [min] | A [%] | B [%] |
| 0 | 98 | 2 | 0 | 100 | 0 | 0 | 100 | 0 |
| 1 | 98 | 2 | 0.1 | 100 | 0 | 0.1 | 100 | 0 |
| 6 | 85 | 15 | 0.11 | 99 | 1 | 0.11 | 98 | 2 |
| 7 | 50 | 50 | 1.5 | 99 | 1 | 2 | 98 | 2 |
| 7.5 | 50 | 50 | 2 | 98 | 2 | 4 | 96 | 4 |
| 8.5 | 98 | 2 | 4.5 | 98 | 2 | 6 | 85 | 15 |
| 15 | 98 | 2 | 5 | 96 | 4 | 8 | 70 | 30 |
| | | | 6 | 96 | 4 | 10 | 10 | 90 |
| | | | 8 | 70 | 30 | 10.5 | 10 | 90 |
| | | | 9.5 | 10 | 90 | 11.5 | 100 | 0 |
| | | | 10.5 | 10 | 90 | 19 | 100 | 0 |
| | | | 11.5 | 100 | 0 | | | |
| | | | 20 | 100 | 0 | | | |

Table SI-4. Mass spectrometer ion source settings

| | Non-optimized parameters | Optimized parameters |
|------------------------|--------------------------|----------------------|
| Gas temperature | 350°C | 350°C |
| Gas flow | 8 L/min | 4 L/min |
| Nebulizer | 50 psi | 60 psi |
| Sheath gas temperature | 350°C | 350°C |
| Sheath gas flow | 12 L/min | 11 L/min |
| Capillary voltage | 3000 V | 2000 V |
| Nozzle voltage | 0 V | 0 V |

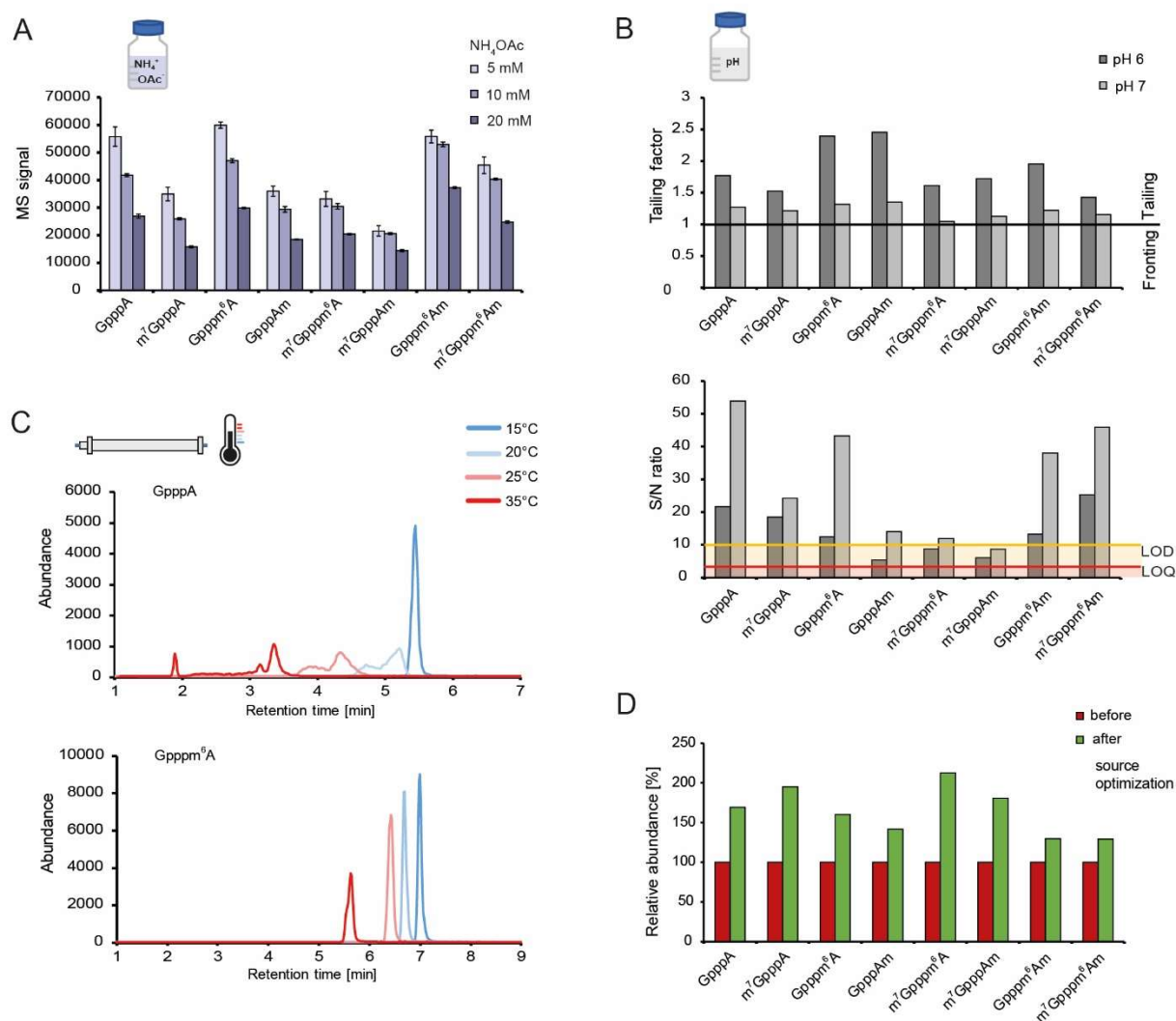


Figure SI-20: Impact of chromatographic optimization on signal amplitude, peak width and symmetry for the following cap nucleotides: GpppA, m⁷GpppA, Gpppm⁶A, GpppAm, m⁷Gpppm⁶A, m⁷GpppAm, Gpppm⁶Am, and m⁷Gpppm⁶Am. A.) MS intensities of the cap nucleotides for different amounts of ammonium acetate in the mobile phase. The measurement was performed in triplicates with injection amounts of 1 pmol per cap nucleotide. From 20 mM to 5 mM there was a gain in abundance of between 1.6-2.3. To achieve the most sensitive method feasible, it was decided to continue with 5 mM ammonium acetate. B.) Comparison of mobile phases with 5 mM ammonium acetate pH 6 and pH 7 regarding the calculated tailing factors. 1 pmol of each cap nucleotide was injected. Low tailing (Tailing factor > 1) was observed for all peaks. pH 7 showed advantages over pH 6 with values even closer to the ideal Gaussian distribution of 1 (depicted with a black line). The lower graph shows signal-to-noise (S/N) ratios for injections of 10 fmol per cap nucleotide compared for pH 6 and 7. The limit of detection (LOD) is defined as S/N < 10 and is highlighted in yellow, the limit of quantification (LOQ) with S/N < 3 is marked in red. The S/N ratios are increased at pH 7 for all cap nucleotides leading to only one value (m⁷GpppAm) below the LOQ, while there are three cap modifications below the LOQ at pH 6. Higher pH values did not show reproducible improvements. Therefore, it was agreed to proceed with pH 7.0. C.) Depiction of detected MS signals for GpppA and Gpppm⁶A at different column temperatures for injection volumes of 50 µL. Only the earliest eluting cap standard GpppA showed peak splitting at high injection volumes. This effect could be reduced by lowering the temperature. Since no major differences were observed in abundance and peak separation, it was therefore concluded to set the column temperature at 15°C. D.) Relative MS intensities of the cap nucleotides before (red) and after (green) ion source optimization. The starting parameters are set to 100%. The comparative measurement between the non-optimized source parameters and the optimized parameters resulted in abundance increase factors between 1.3 and 2.1.

2.2.4 Instrument detection limits

The instrument detection limits (IDLs) of the different cap analogs were determined for the non-optimized HPLC-MS method compared to the final method as described by technical guidelines from Agilent Technologies^[13]. Briefly, a suitable amount of analyte within a S/N ratio range of 5-10 is injected several

times in succession. The relative standard deviation of signal strength is used to calculate the IDL. 12 consecutive injections were performed using 5 fmol of the standard dinucleotide mixture applying the non-optimized method, while the concentration of 2 fmol was chosen for the final method. The LC-MS/MS parameters are listed in Table SI-5.

Table SI-5. Instrument detection limits.

| | IDL of non-optimized method [fmol] | IDL of final improved method [fmol] | Ratio non-optimized method/ final improved method |
|--------------------------------------|------------------------------------|-------------------------------------|---|
| GpppA | 1.9 | 0.9 | 2.1 |
| m ⁷ GpppA | 2.9 | 0.9 | 3.2 |
| Gpppm ⁶ A | 2.7 | 0.9 | 3.0 |
| GpppAm | 3.6 | 1.2 | 3.0 |
| Gpppm ⁶ Am | 3.2 | 1.0 | 3.2 |
| m ⁷ Gpppm ⁶ A | 3.7 | 1.1 | 3.4 |
| m ⁷ GpppAm | 2.4 | 1.0 | 2.4 |
| m ⁷ Gpppm ⁶ Am | 2.5 | 1.0 | 2.5 |

2.2.5 Method validation

A sample with known modification content was composed of a defined amount of cap dinucleotides (400 fmol) and 2 µg total HEK RNA. The sample was spiked with a stable isotope-labeled internal standard (SILIS) cap mixture containing 400 fmol D₃C-m⁷Gpppm⁶A, D₃C-m⁷GpppAm and D₃C-m⁷Gpppm⁶Am and digested to nucleoside level while leaving the cap intact by using 0.6 U nuclease P1 from *P. citrinum* (Sigma-Aldrich) in a reaction buffer containing 0.2 mM ZnCl₂ and 20 mM ammonium acetate (Merck) (pH 5.5) in an 1 h incubation at 37°C. Dephosphorylation reaction was carried out by adding 1 U FastAP (Thermo Scientific) and a reaction buffer containing 5 mM Tris (pH 8) (Invitrogen) and 1 mM MgCl₂ (Sigma-Aldrich). This mixture was incubated for another 1 h at 37°C. The digested mixture was spiked with 5 ng of digested ¹³C-labeled nucleosides from *S.cerevisiae*, adjusted to an specified volume with digestion buffer and subjected to LC-MS/MS. The final LC-MS method (Table SI-6) was applied.

Table SI-6. Detected cap composition of HEK total RNA spiked with 400 fmol of each considered cap nucleotide analyzed with and without labeled internal standard. Deviation from true value was calculated as follows: Deviation from true value = $[(\text{amount measured} - \text{true value}) / \text{true value} * 100\%]$.

| | With ISTD | | Without ISTD | |
|-------------------------------------|----------------|---------------------------|----------------|---------------------------|
| | Average [fmol] | Deviation from true value | Average [fmol] | Deviation from true value |
| GpppA | 428 | 7.0% | 471 | 17.7% |
| m ⁷ GpppA | 380 | 4.9% | 418 | 4.5% |
| Gpppm ⁶ A | 399 | 0.3% | 439 | 9.7% |
| GpppAm | 385 | 3.9% | 423 | 5.7% |
| Gpppm ⁶ Am | 414 | 3.6% | 456 | 14.0% |
| m ⁷ Gpppm ⁶ A | 383 | 7.0% | 422 | 5.5% |
| Average | | 4.0% | | 9.5% |

The MS was operated in MRM mode. The optimized MS parameters for the detection of cap dinucleotides are listed in Table SI-2). Absolute quantification was performed as described in Thüning et al.^[14] Briefly, external calibration dilutions were spiked with 400 fmol SILIS cap mixture, adjusted to the volume of the sample with digestion buffer and injected to LC-MS to cover a range of 10 – 1000 fmol cap dinucleotides. The resulting standard curve was used to calculate the amounts of cap dinucleotides in the sample. This was performed with and without taking SILIS correction into account. For cap dinucleotides for which no structurally identical SILIS was available, D₃C-m⁷Gpppm⁶A was used (Figure SI-20).

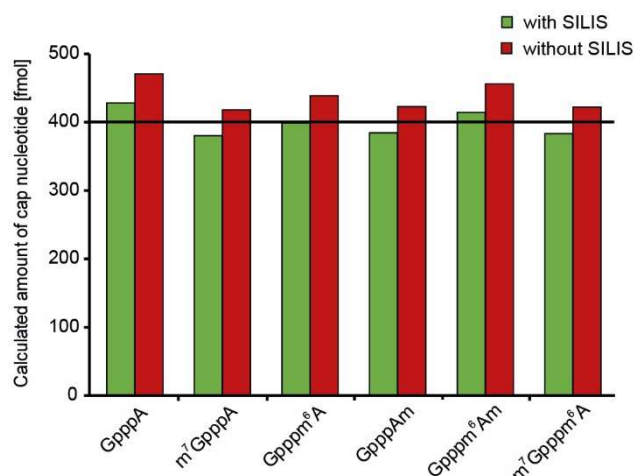


Figure SI-21. Detected cap composition of 2 µg HEK total RNA spiked with 400 fmol of each cap nucleotide comparing the analysis with (green) and without (red) stable isotope labeled internal standards (SILIS). The true value of 400 fmol is depicted with a black line. In 2 µg HEK total RNA without addition of cap nucleotides the respective cap nucleotides were not detectable.

2.3 Cell and tissue extraction and RNA isolation

2.3.1 HEK cells

HEK293-cells (#ACC 305, DSMZ) were cultivated at 37°C and 5 vol-% CO₂ in DMEM cell culture medium (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 10 U/mL Penicillin-Streptomycin (Thermo Fisher Scientific). For separation, the cells

were first washed with phosphate buffer (Thermo Fisher Scientific) and then detached by incubation in Trypsin-EDTA (Thermo Fisher Scientific).

For RNA extraction, detached cells resuspended in DMEM were first centrifuged at 400 xg for 5 min (Hettich GmbH & Co. KG), followed by discarding the supernatant and washing the pellet once with phosphate buffer. Next, the cell pellet was resuspended in 1 mL TRI Reagent (Sigma Aldrich) and stored at – 20°C.

2.3.2 Mouse tissue

Two male B6.129S mice (B6.129S-Trpc6tm1Lbi/Mmjax) were used at an age of 39 – 41 weeks. For housing conditions see Brandscheid et al.^[15] All experimental procedures were carried out in accordance with the European Communities Council Directive regarding care and use of animals for experimental procedures and was approved by local authorities (LUA-Rhineland-Palatinate). The mice were sacrificed by decapitation under isoflurane anesthesia. The brain was removed and brain areas (hippocampus, cortex, and cerebellum) were dissected, snap-frozen in liquid nitrogen and stored at – 80°C.

2.3.3 Total RNA isolation from cells or tissue

Total RNA from cell pellet or tissue was extracted with TRI Reagent (Sigma-Aldrich). The cell pellet was suspended in 1 mL TRI Reagent per 5×10^6 cells. The mouse brain tissues were suspended in 5 mL TRI Reagent, thoroughly mixed and crushed with a pestle, except hippocampus tissue which was suspended and mixed in only 1 mL TRI Reagent. This was followed by addition of 200 μ L chloroform (Honeywell Riedel-de Haen) per mL TRI Reagent, incubating at room temperature for 2 min, and centrifugation (13000 xg, 4°C, 15 min). The upper aqueous phase was transferred in a new tube and the chloroform extraction was repeated. The upper aqueous phase was again transferred in a new tube and 0.5 mL 2-propanol (Carl Roth) per mL TRI Reagent was added as well as 1 μ L Glycogen (Thermo Fisher Scientific). Following incubation for 10 min at room temperature, another round of centrifugation (13000 xg, 4°C, 30 min) was performed, the supernatant was removed and 1 mL 75% ethanol (Thermo Fisher Scientific) was added for a final centrifugation step. Afterwards, the supernatant was carefully removed, the RNA pellet was airdried and reconstituted in nuclease-free water. RNA concentration and integrity were determined using UV-VIS spectrometer Nanodrop 2000 (Thermo Fisher Scientific) and Agilent TapeStation 4200 (Agilent Technologies).

2.3.4 Isolation of polyA-tailed RNA

Purification of polyA-tailed RNA from total RNA was performed according to the manufacturer's instructions for Dynabeads (Thermo Scientific) with some modifications. Briefly, 100 μ L prewashed oligo d(T)25 magnetic beads (NEB) per 60 μ g total RNA were used. RNA was bound to the beads in binding buffer (20 mM Tris-HCl (Carl Roth), pH 7.5, 1.0 M LiCl (Carl Roth), 2 mM EDTA (Thermo Scientific)) for 5 min by rotating at room temperature. The tube was placed on a magnet and the supernatant was carefully removed. Two washing steps with washing buffer (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA) were performed. The RNA was eluted in 100 μ L nuclease-free water by heating for 2 min at 70°C. The tube was placed on a magnet and the supernatant containing the polyA RNA was transferred

to a new tube. A second round of purification was performed. The polyA RNA was purified and concentrated by using the RNA Clean&Concentrator 5 Kit (Zymo Research) according to the manufacturer's protocol.

2.4 LC-MS/MS analysis of cell and tissue samples

Up to 31.25 µg of polyA RNA from HEK cells and 1 – 3 µg of polyA RNA from mouse brain tissue were prepared for LC-MS/MS analysis. Each sample was spiked with a SILIS cap mixture containing 500 fmol D₃C-m⁷Gpppm⁶A, D₃C-m⁷GpppAm and D₃C-m⁷Gpppm⁶Am and digested to nucleoside level while leaving the cap intact as described in 2.2.5. Method validation. Following digestion, the samples were mixed with 5 ng of ¹³C stable isotope-labeled nucleosides from *S. cerevisiae*, adjusted to a specified volume with digestion buffer and subjected to LC-MS/MS analysis.

The final LC-MS method (Table SI-2) was applied. The four main nucleosides cytidine, uridine, guanosine, and adenosine were detected photometrically at 254 nm by a diode array detector. The MS was operated in MRM mode. In addition to the investigated cap dinucleotides (MS parameters in Table M1), selected nucleosides (m⁶A, Ψ, m^{6,6}A, m⁵C) were also analyzed (MS parameters in Table SI-7). Absolute quantification was performed as described in Thüring et al.^[14] Briefly, external calibration dilutions for cap dinucleotides and for nucleosides were prepared to cover a range of 2 – 1000 fmol cap dinucleotides and a range of 2 – 12000 fmol nucleosides. Additionally, the dilutions were spiked with 500 fmol SILIS cap mixture and 5 ng ¹³C labeled nucleosides from *S. cerevisiae*, adjusted to the volume of the samples with digestion buffer and injected to LC-MS. The resulting standard curves were used to calculate the amounts of cap dinucleotides and modified nucleosides in the sample by taking SILIS correction into account. For cap dinucleotides lacking an available structurally identical SILIS, D₃C-m⁷Gpppm⁶A was used. For mouse brain samples, the amount of cap dinucleotides was normalized to the amount of injected RNA in terms of cap dinucleotide per 1000 nucleotides. Therefore, an additional standard curve of 1 – 500 pmol of main nucleosides was measured.

Table SI-7. Mass spectrometer settings for modified nucleosides. The parameters of the isotope-labeled standards ¹³C-Ψ, ¹³C-m⁵C, ¹³C-m⁶A, and ¹³C-m^{6,6}A are listed as labeled precursor/product mass-to-charge ratios (m/z).

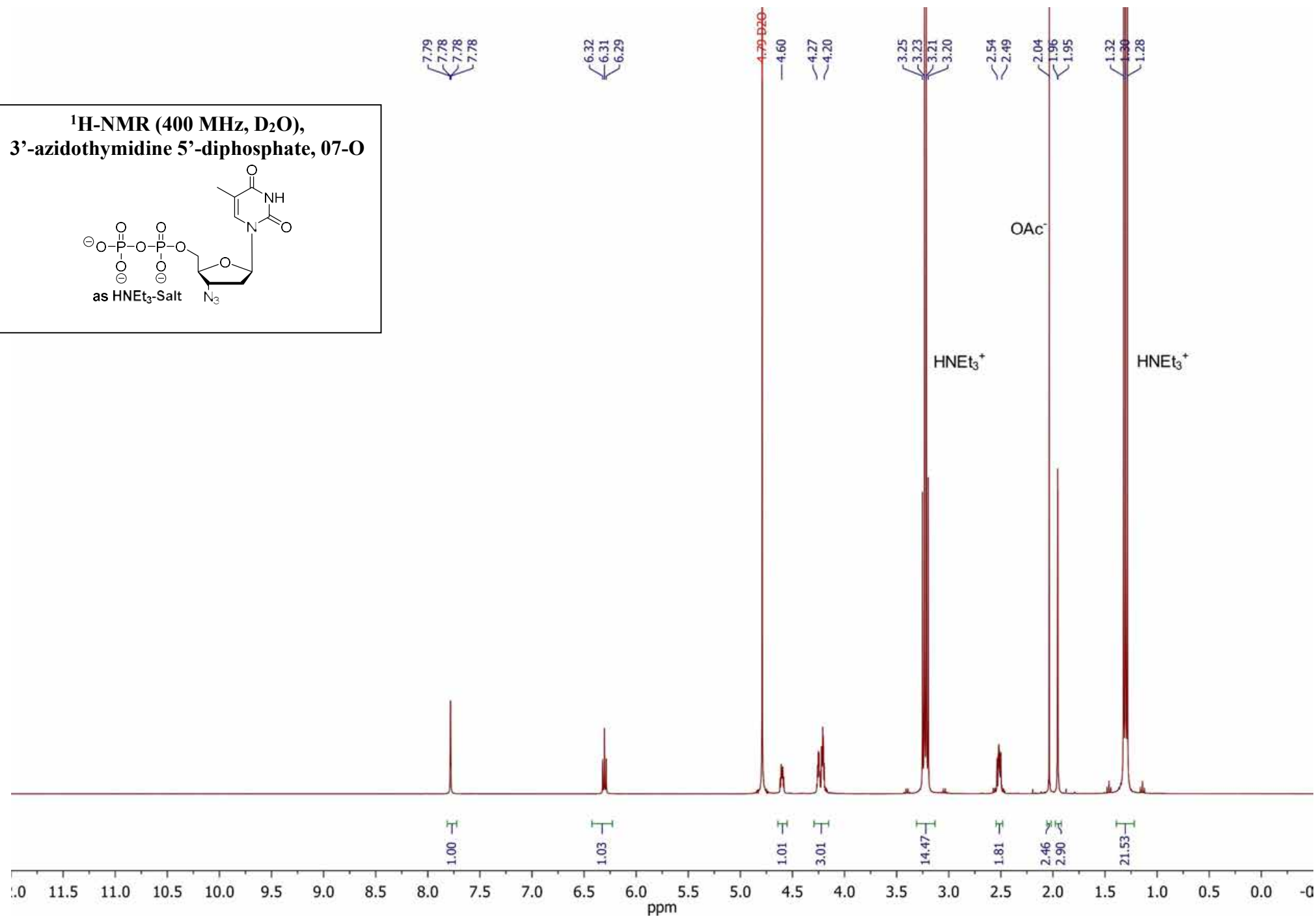
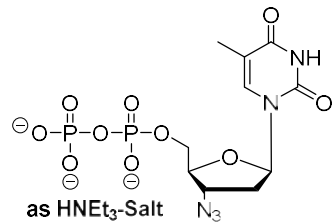
| Compound Name | Precursor Ion [m/z] | Product Ion [m/z] | Fragmentor voltage [V] | Collision Energy [V] | ¹³ C-labeled precursor [m/z] | ¹³ C-labeled product [m/z] |
|--------------------|---------------------|-------------------|------------------------|----------------------|---|---------------------------------------|
| Ψ | 245 | 209 | 85 | 9 | 254 | 218 |
| m ⁵ C | 258 | 126 | 75 | 13 | 268 | 131 |
| m ⁶ A | 282 | 150 | 110 | 21 | 293 | 156 |
| m ^{6,6} A | 296 | 164 | 115 | 21 | 308 | 171 |

3 Supporting references

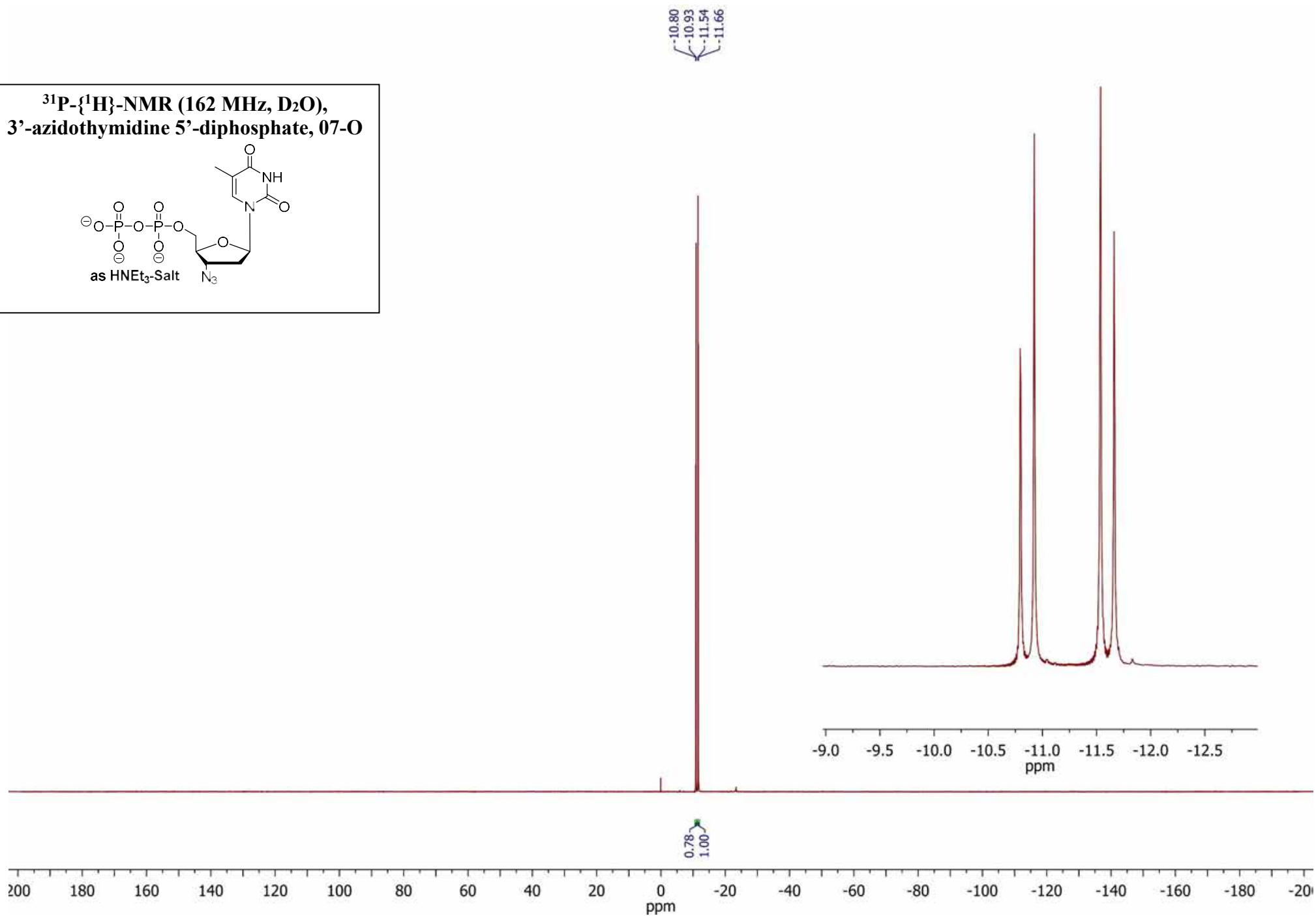
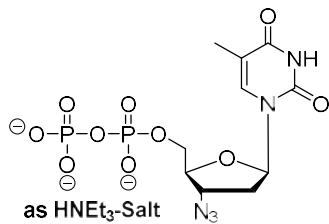
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Supporting Info – NMR results

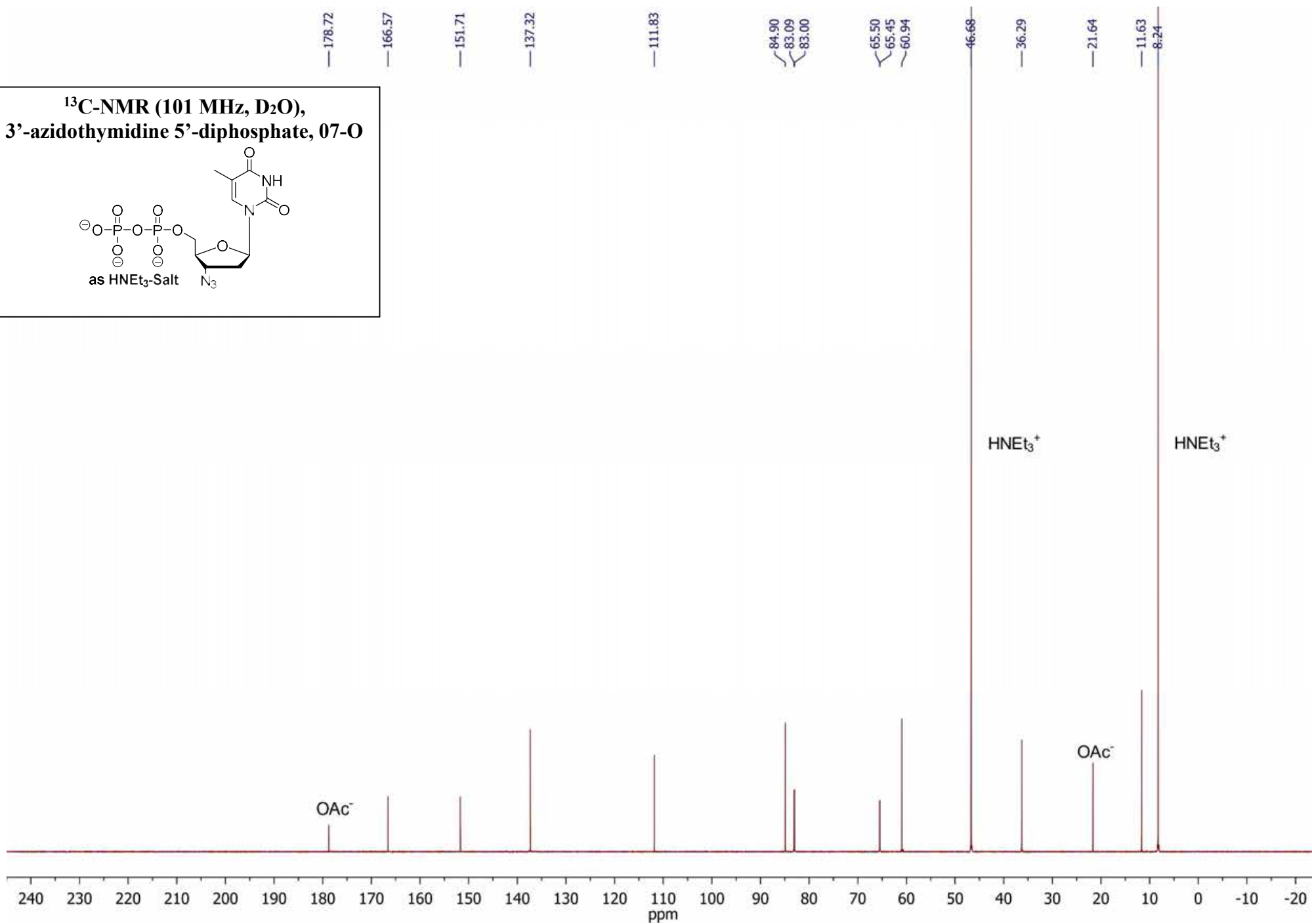
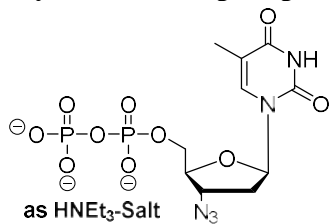
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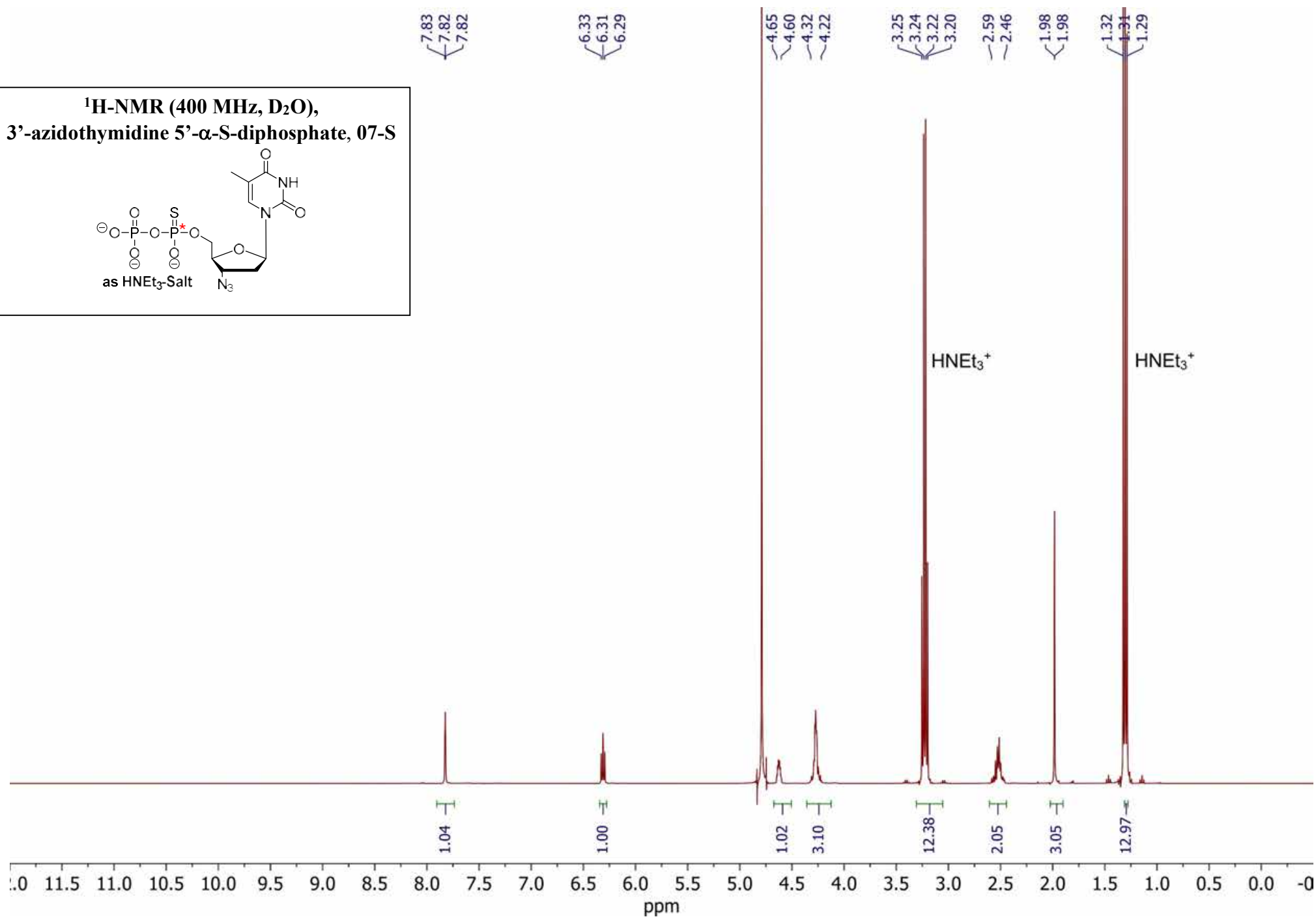
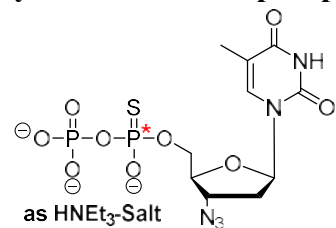
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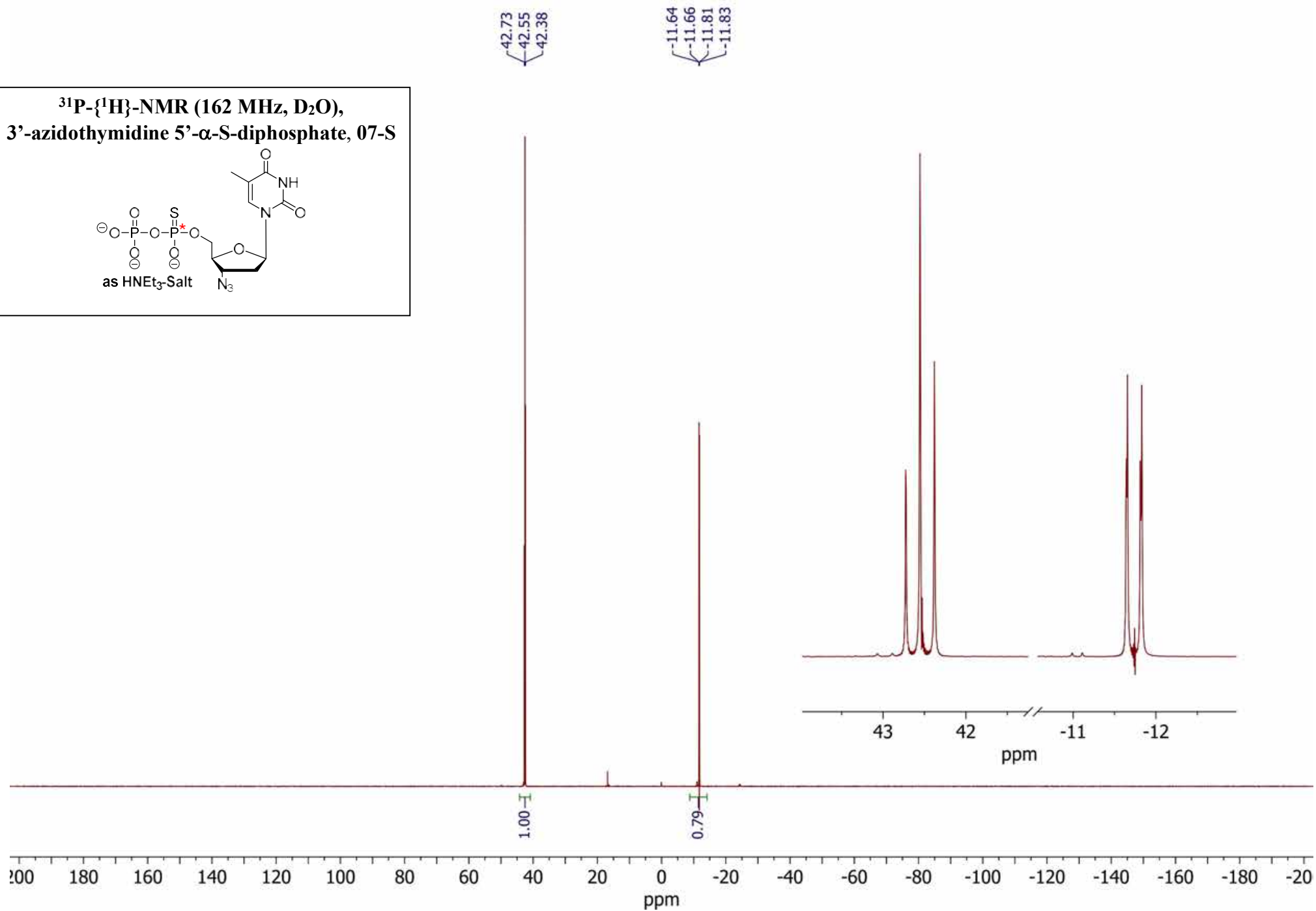
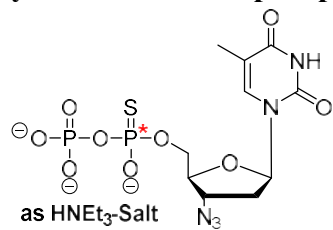
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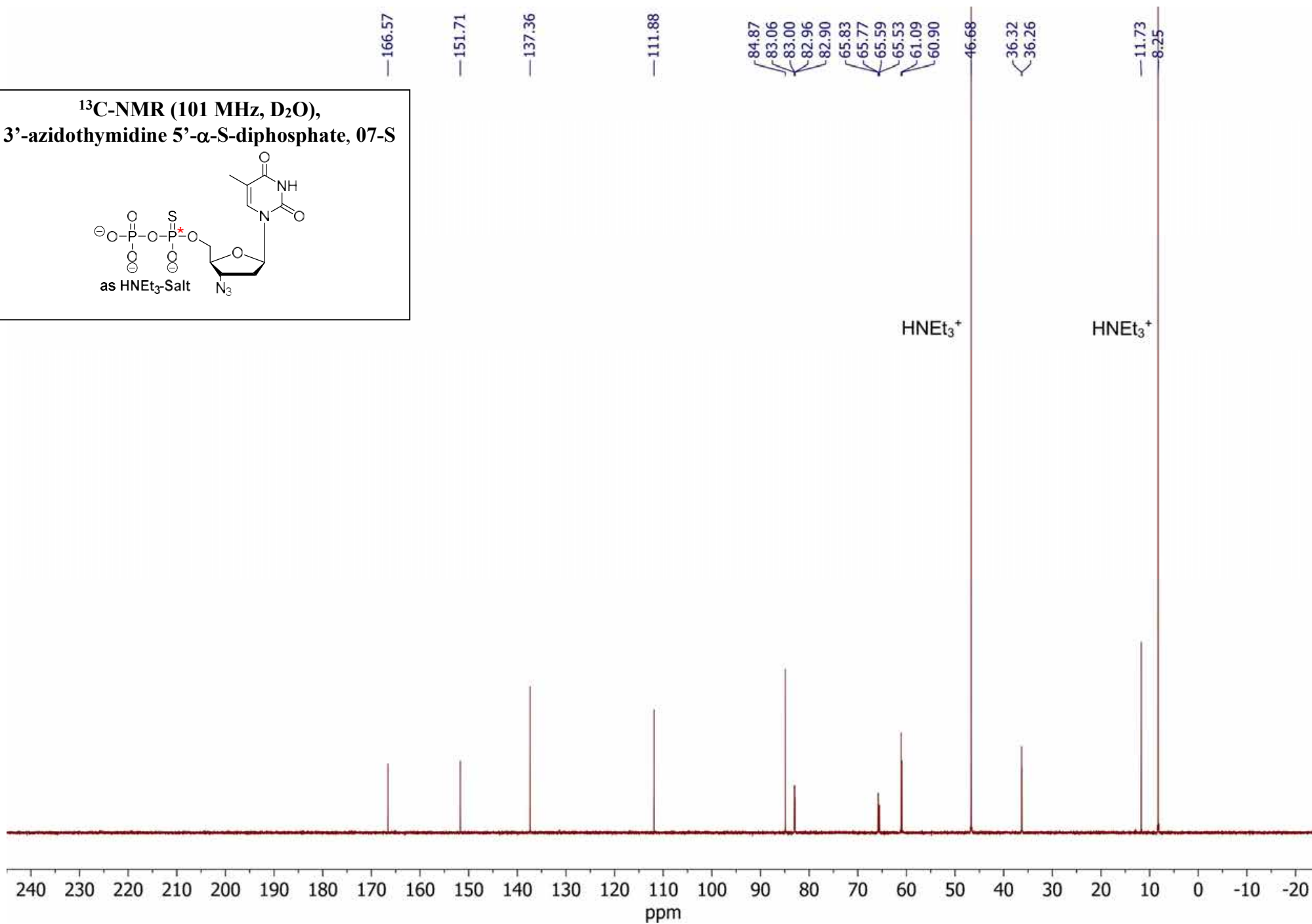
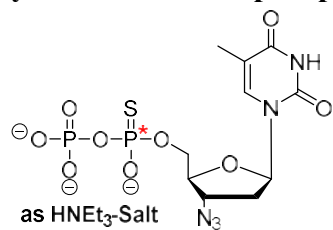
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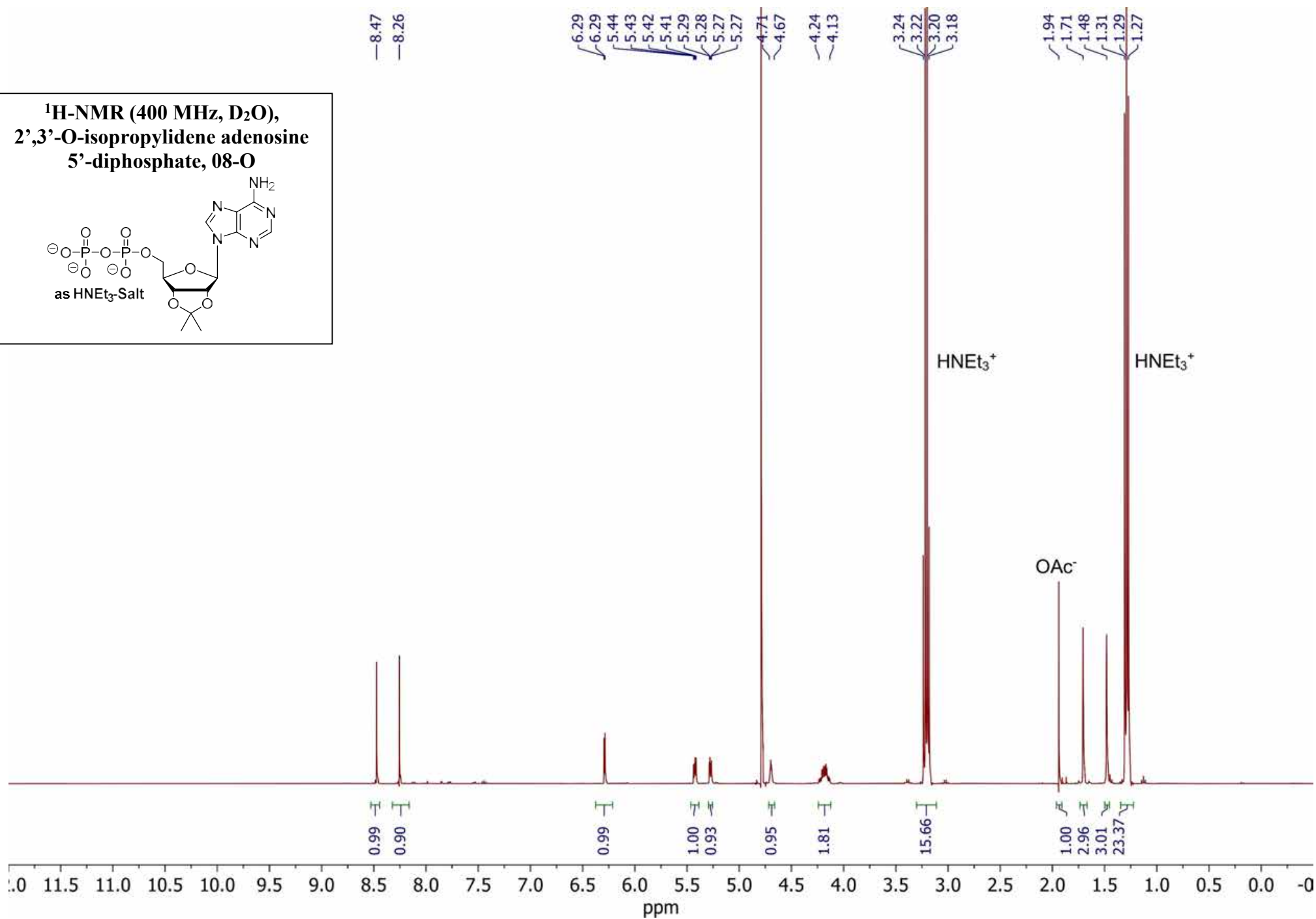
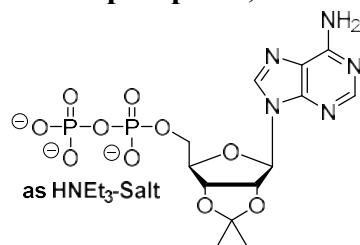
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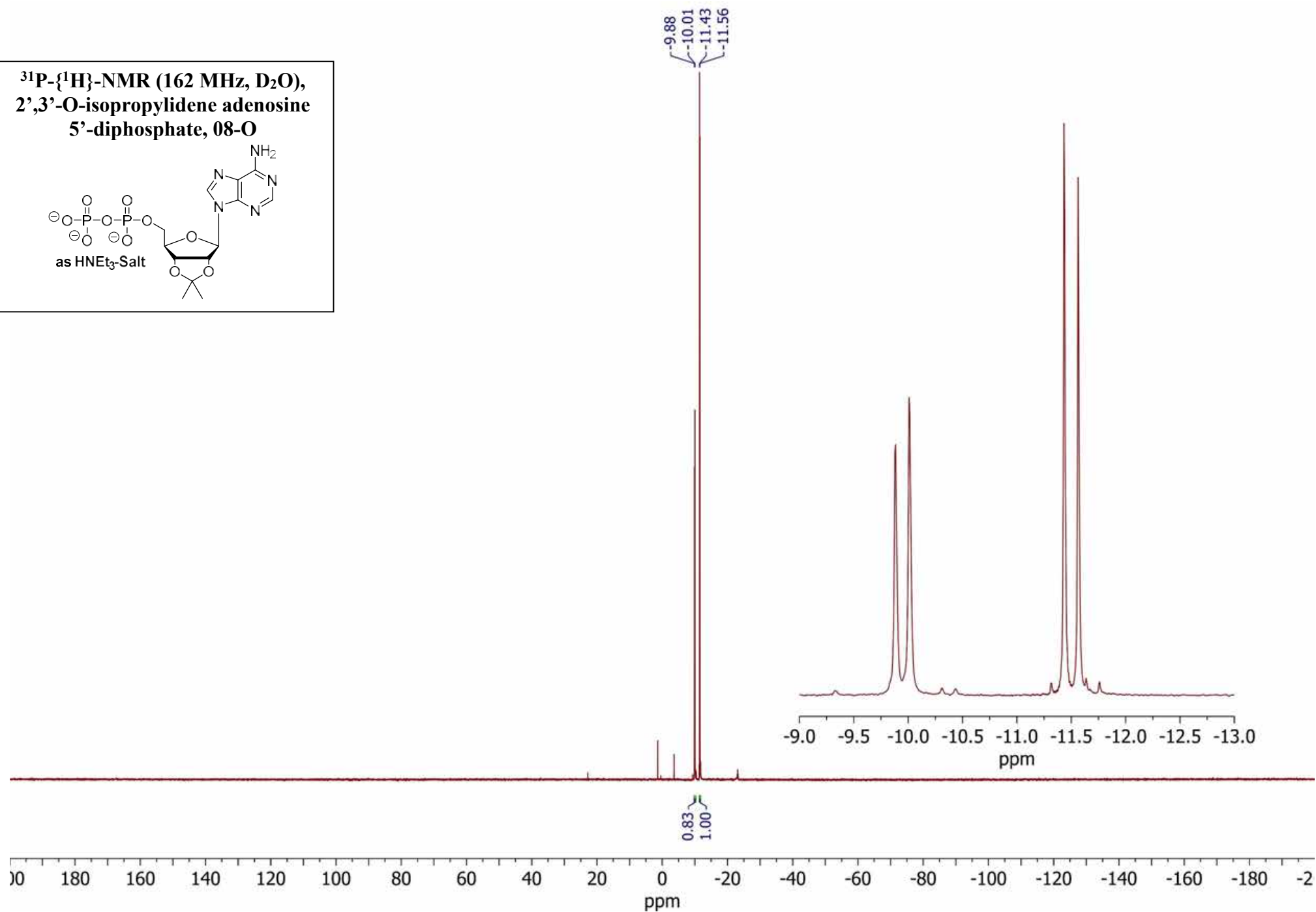
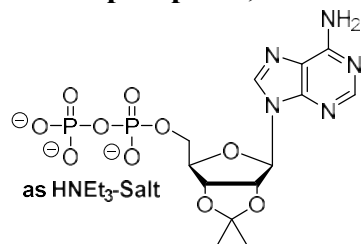
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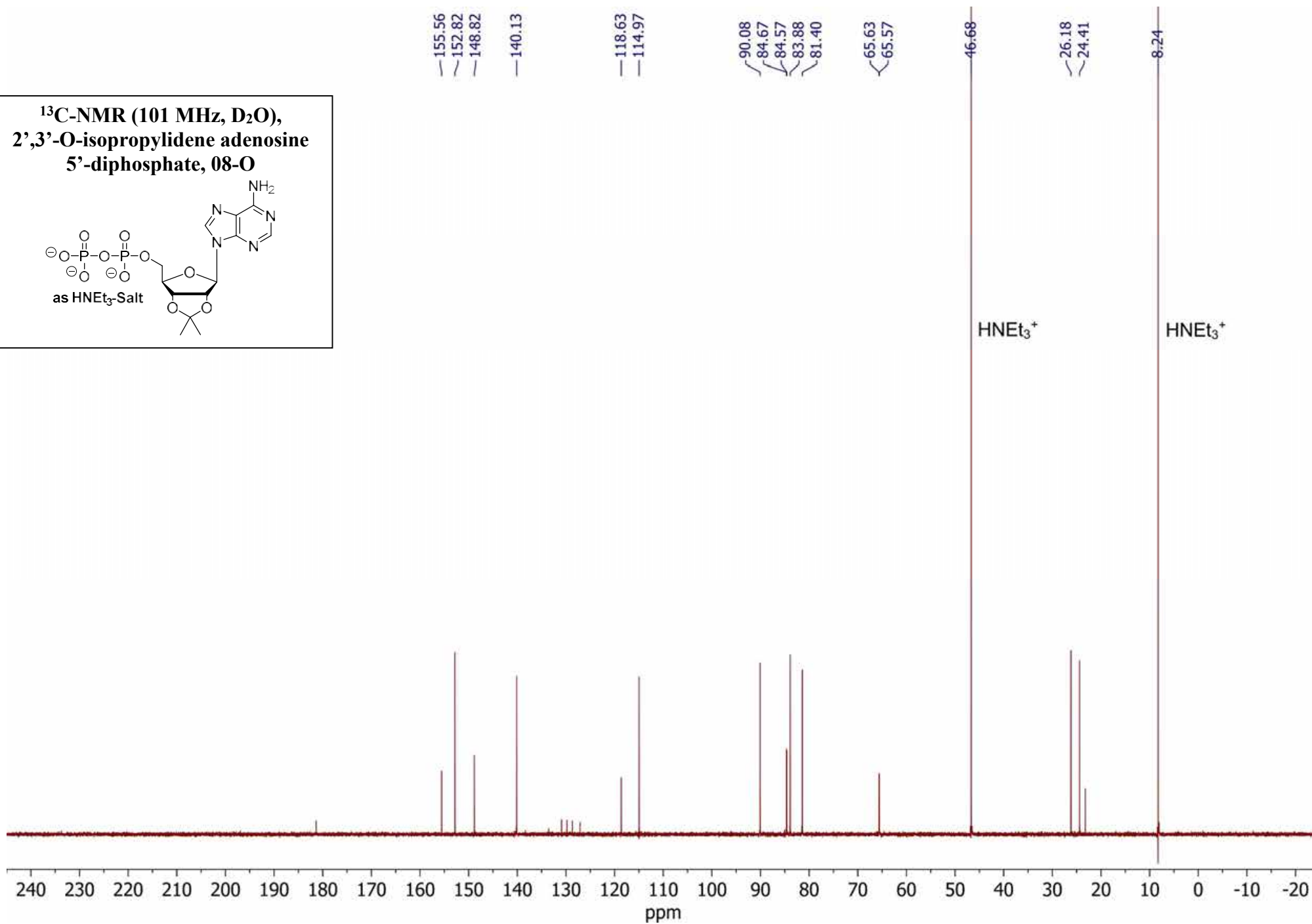
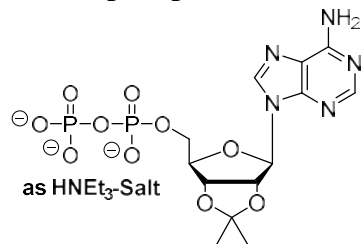
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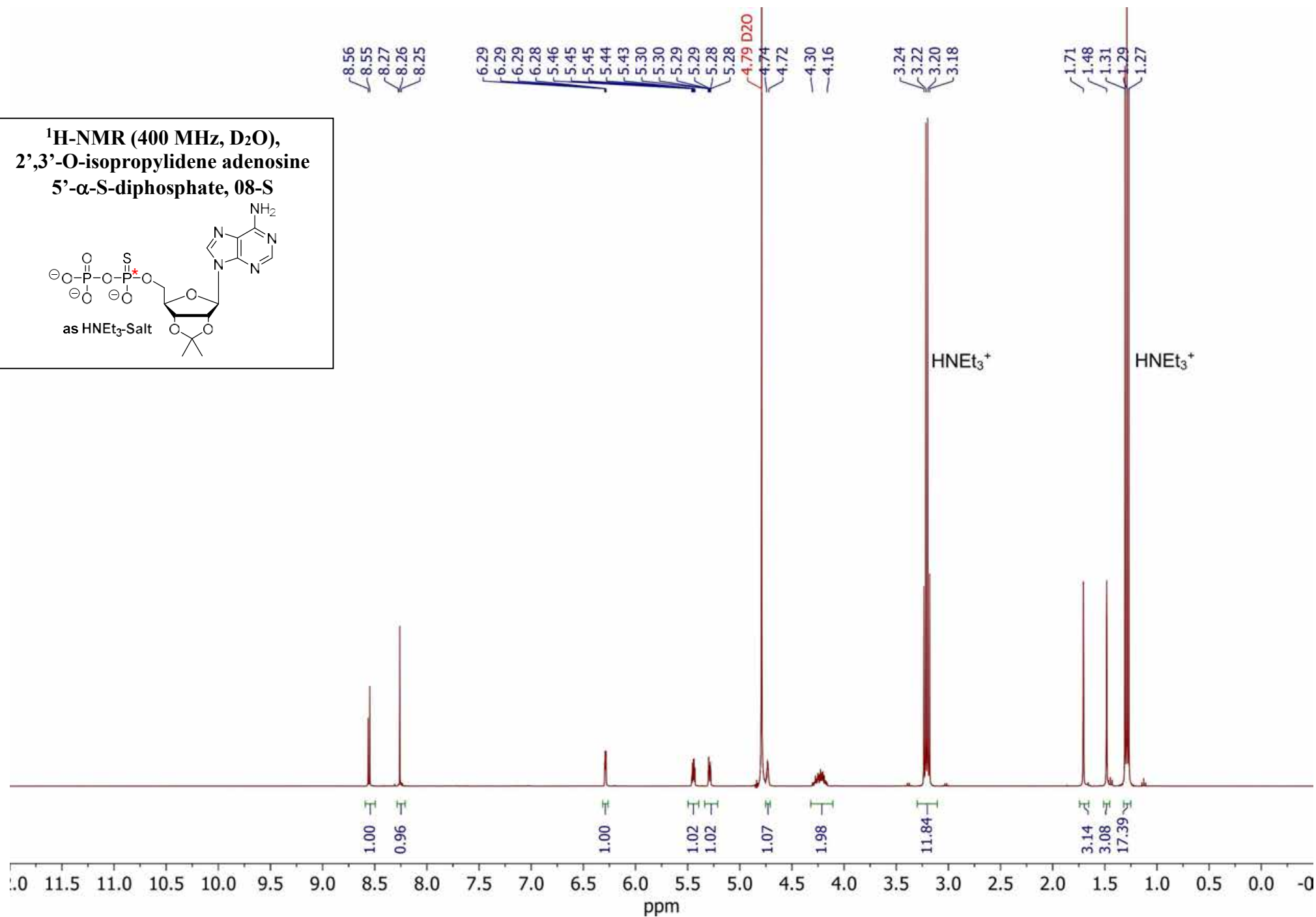
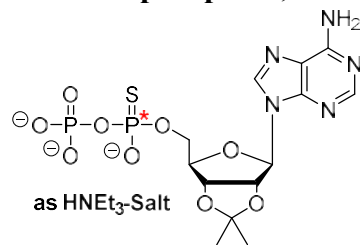
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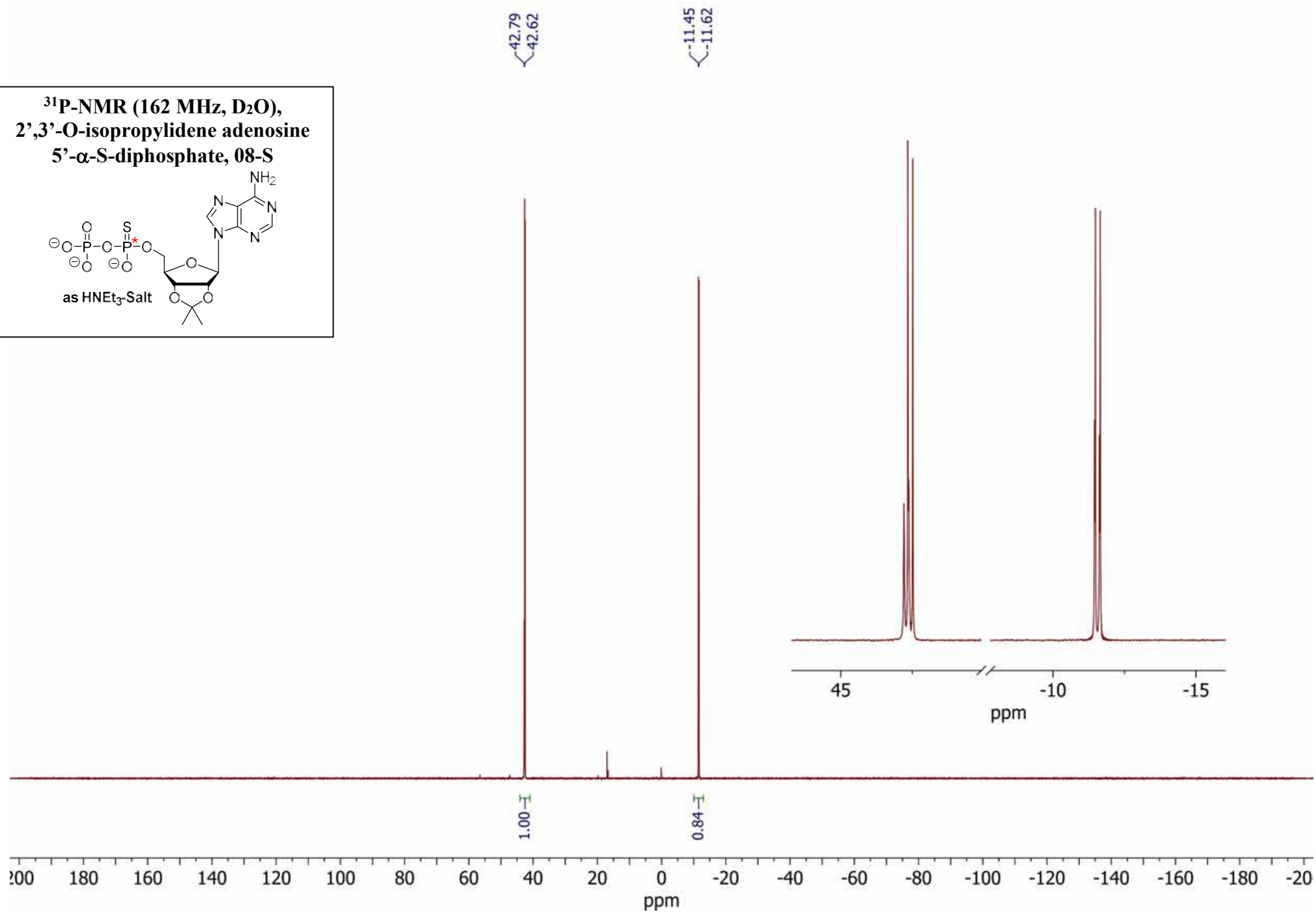
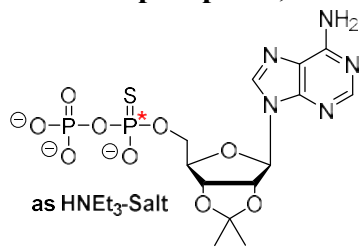
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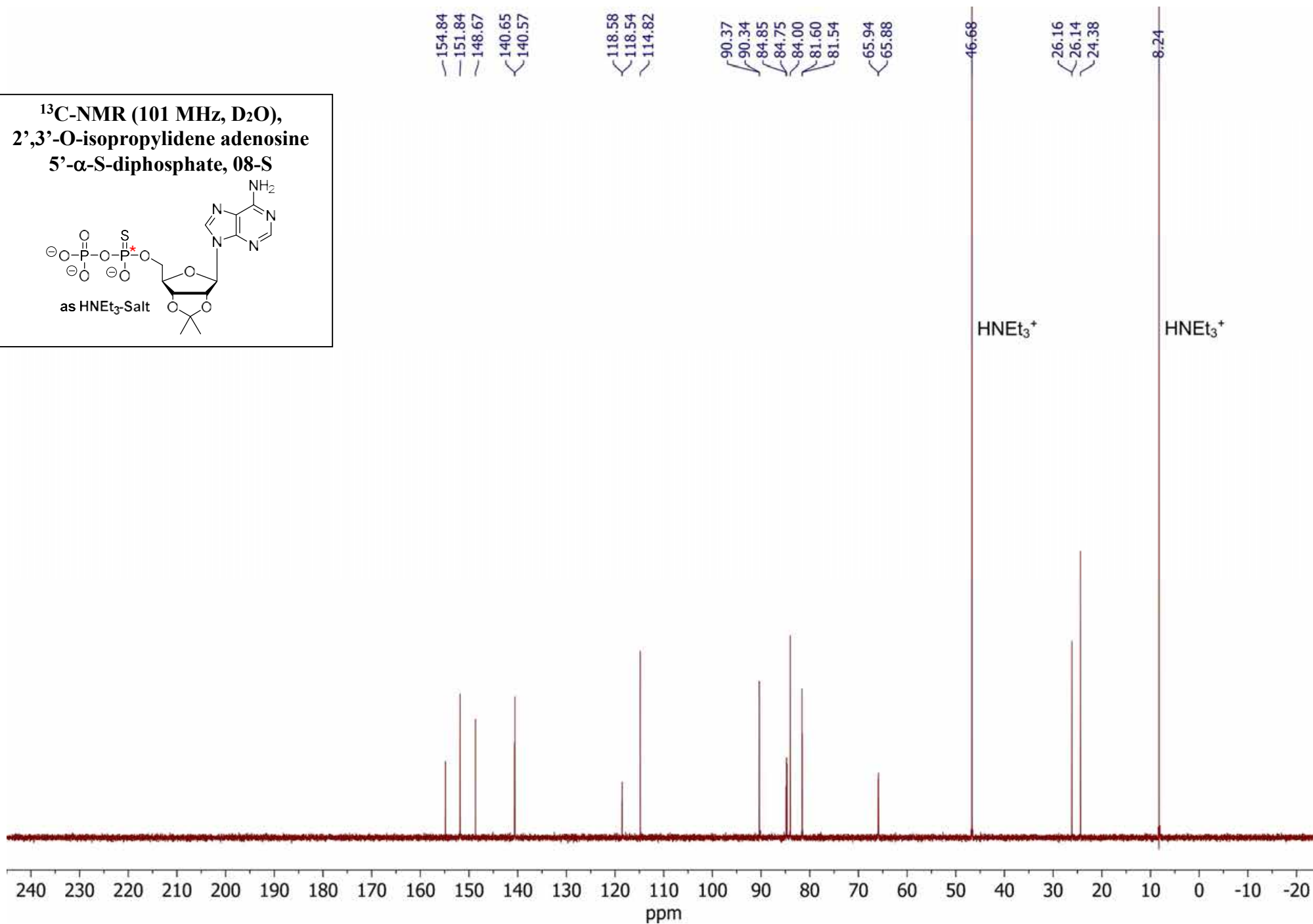
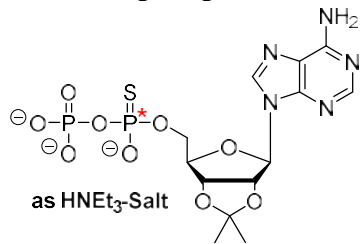
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5'- α -S-diphosphate, 08-S**

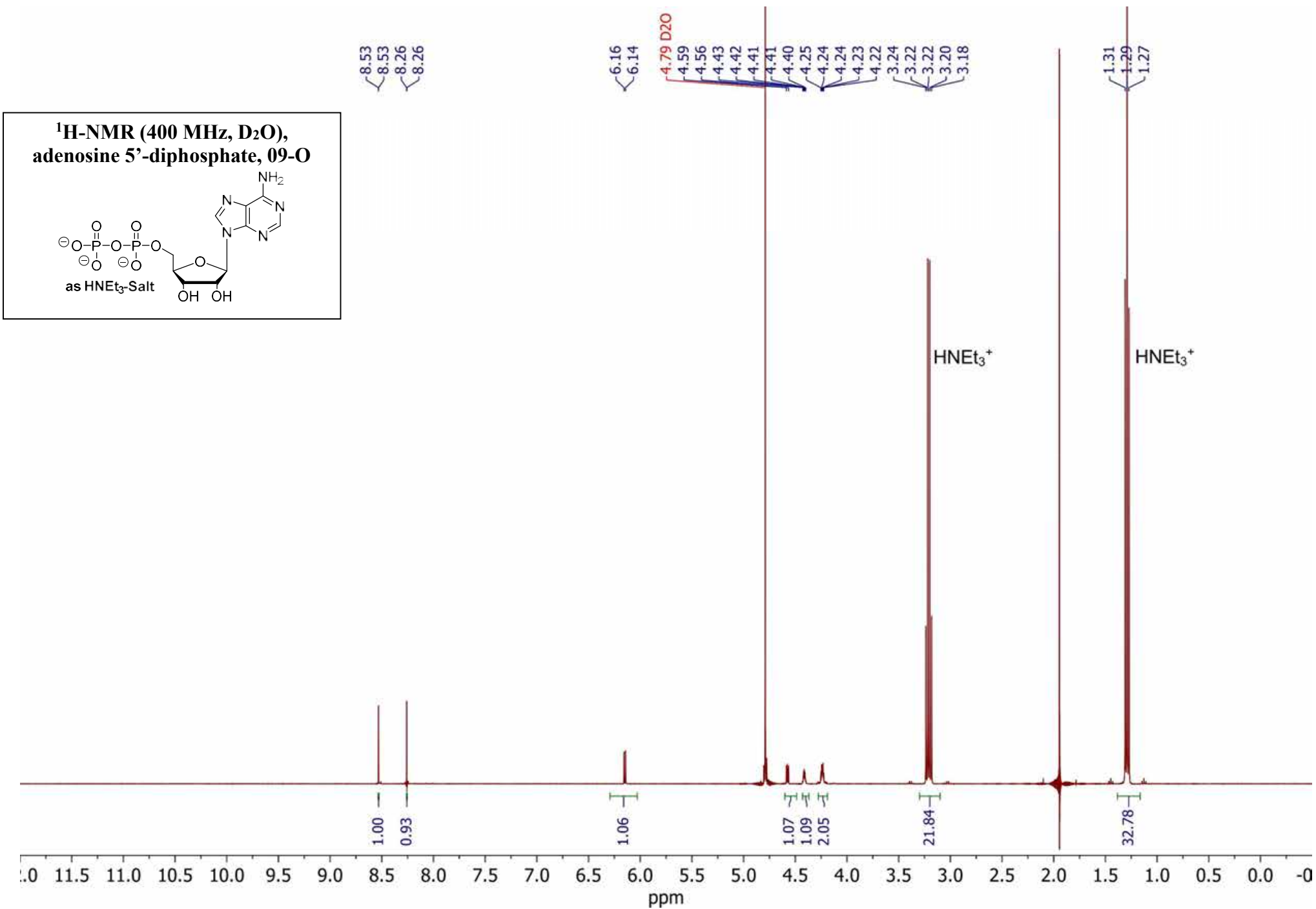
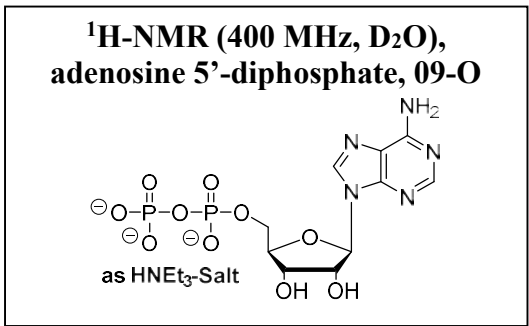


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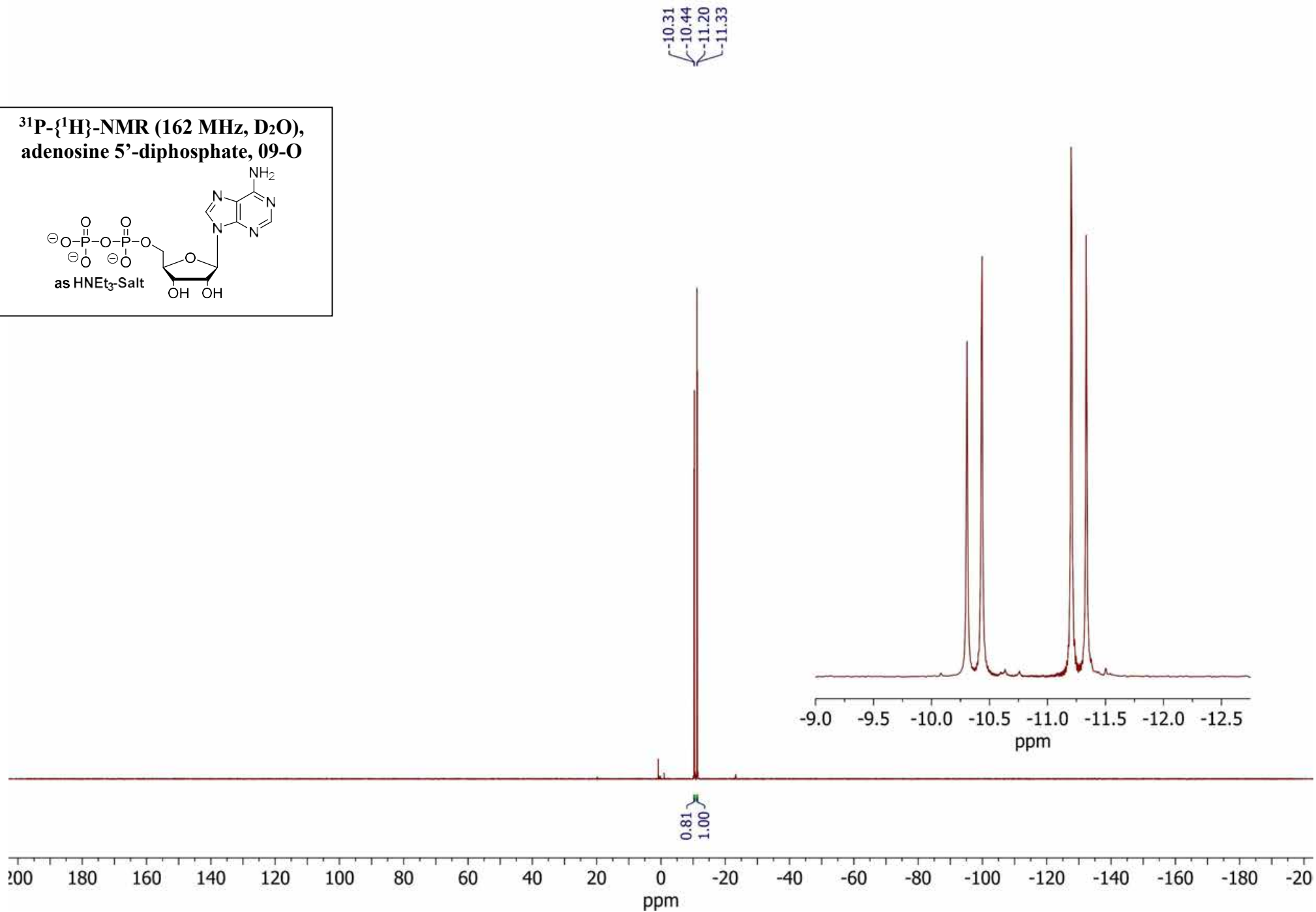
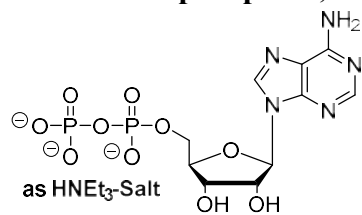


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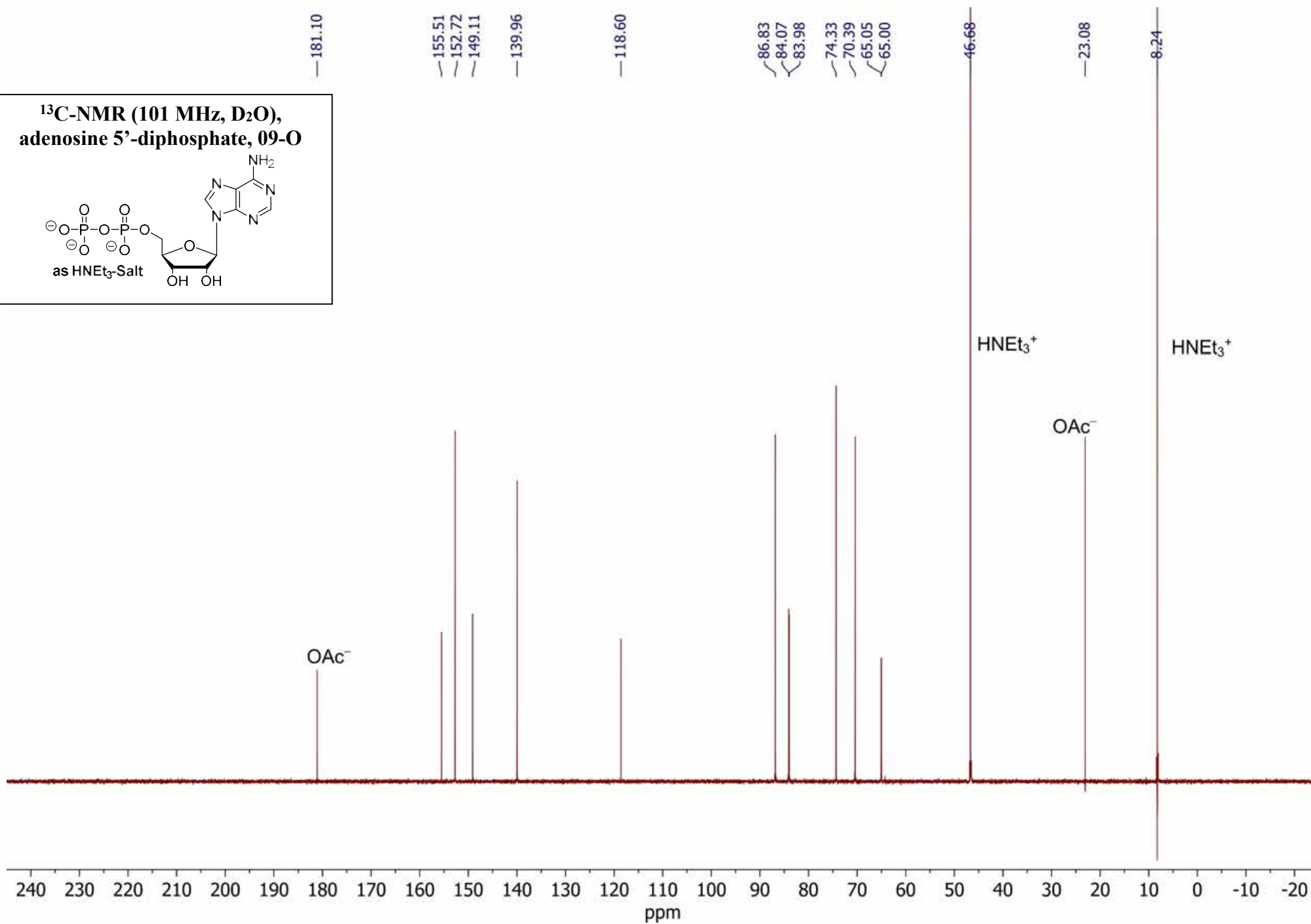
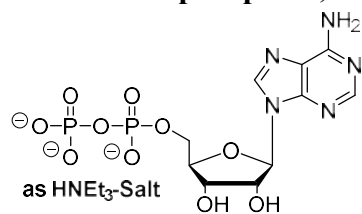
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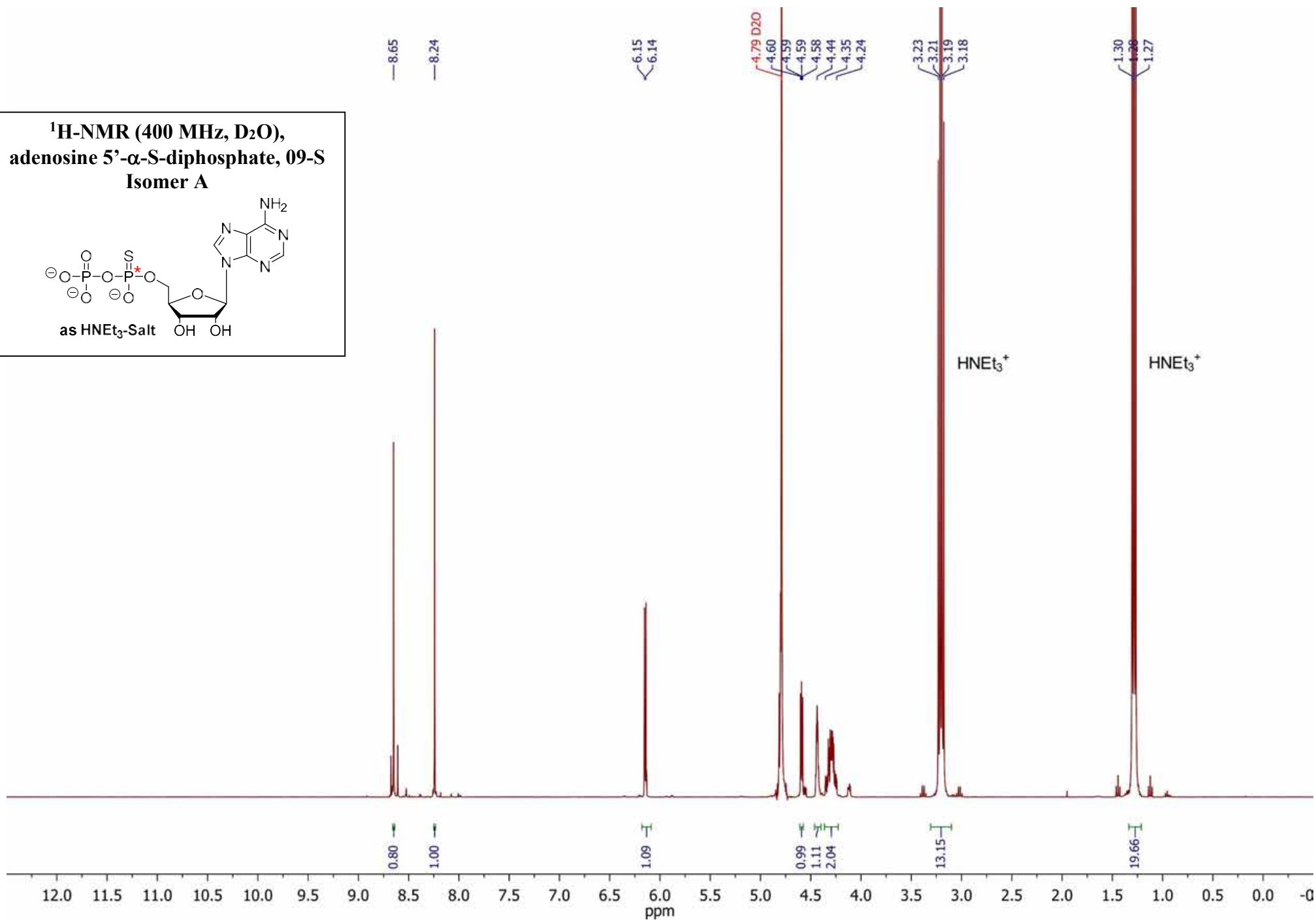
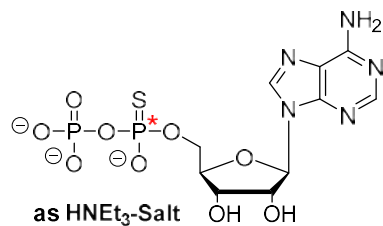
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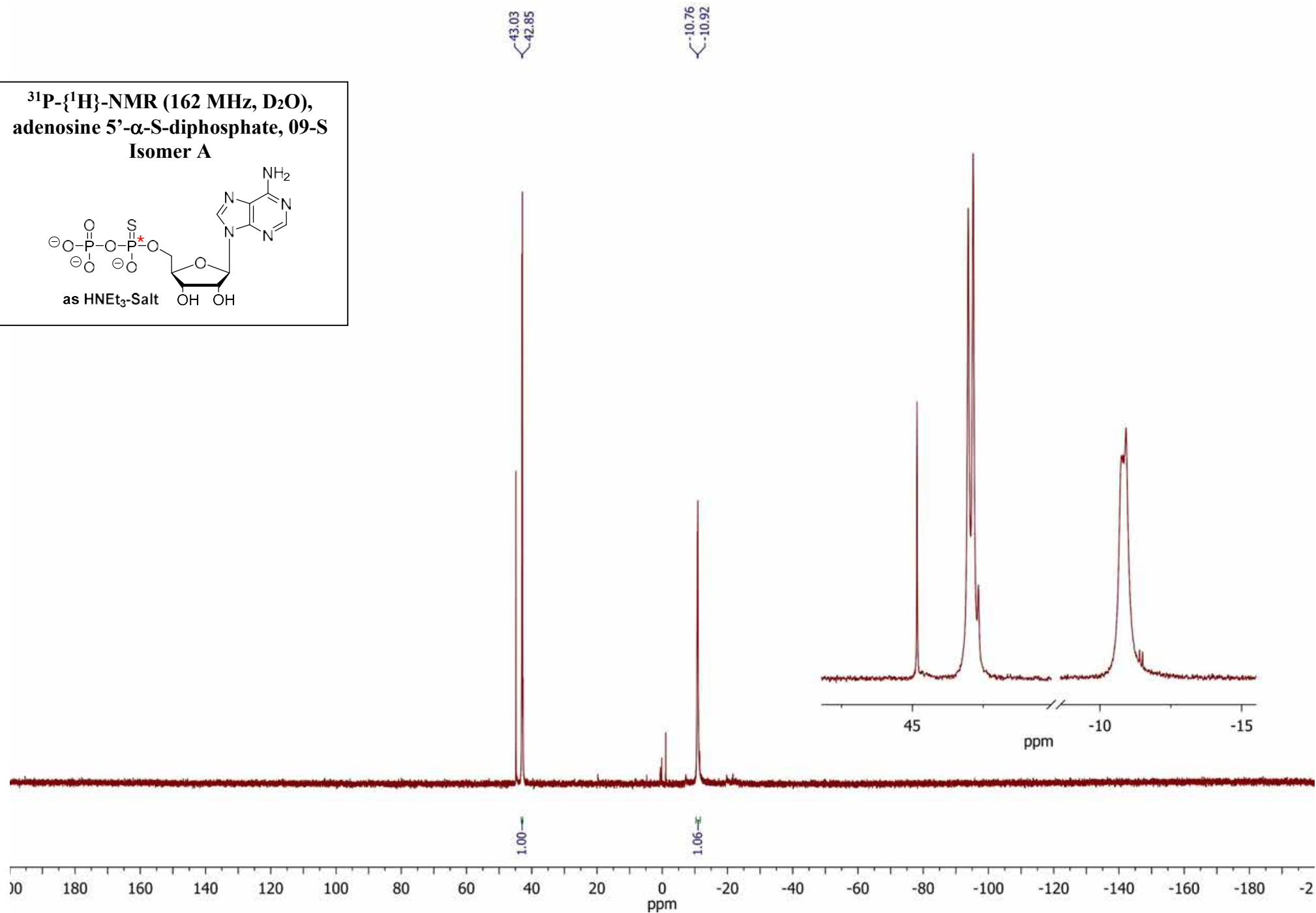
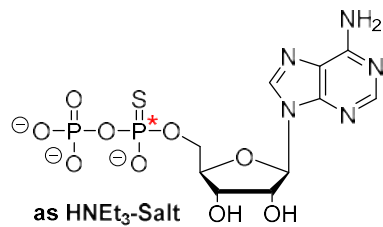
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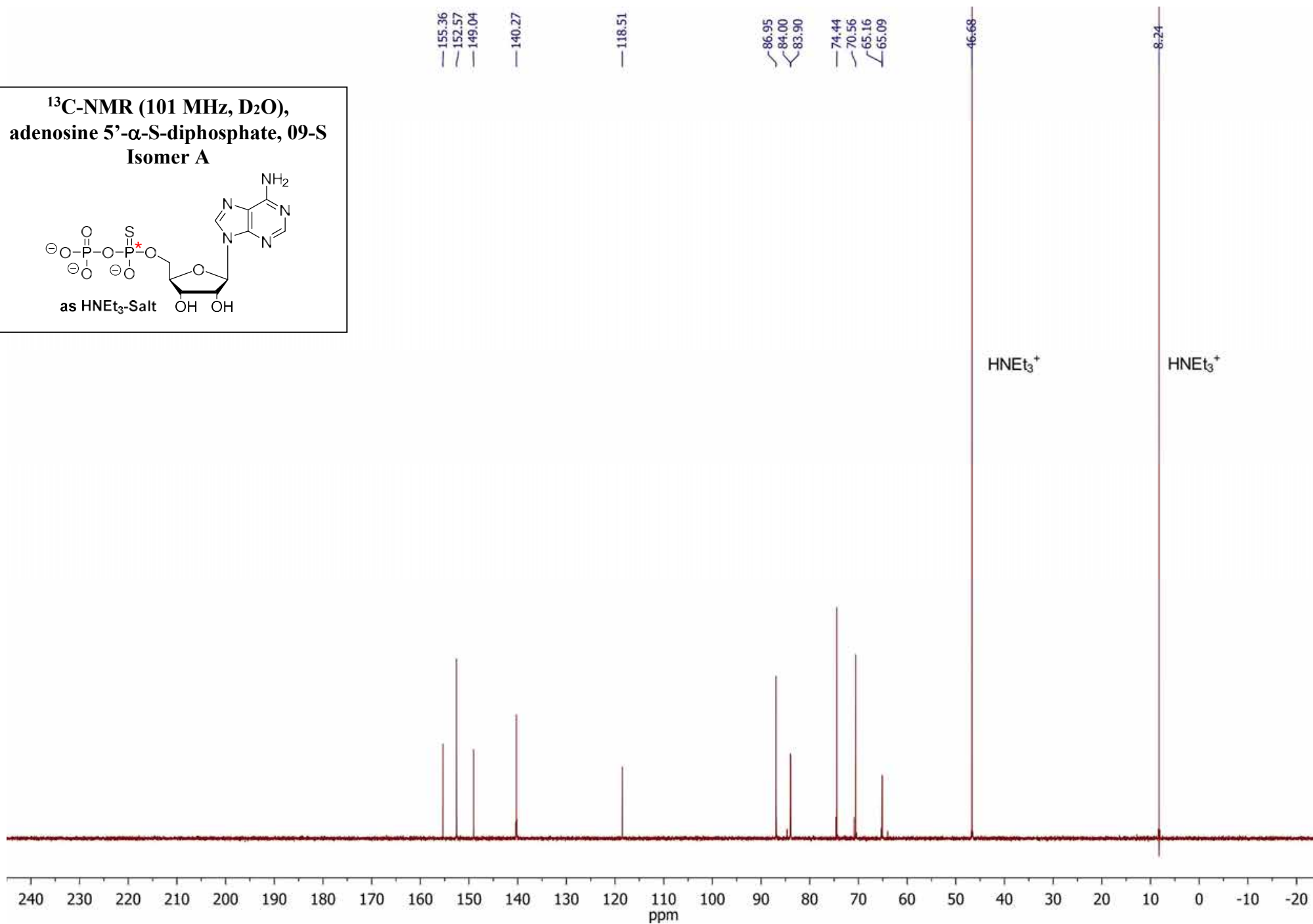
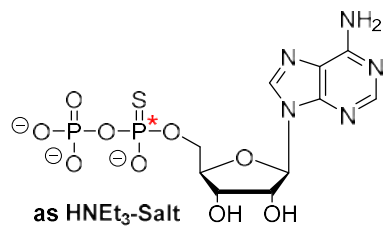
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Isomer A**



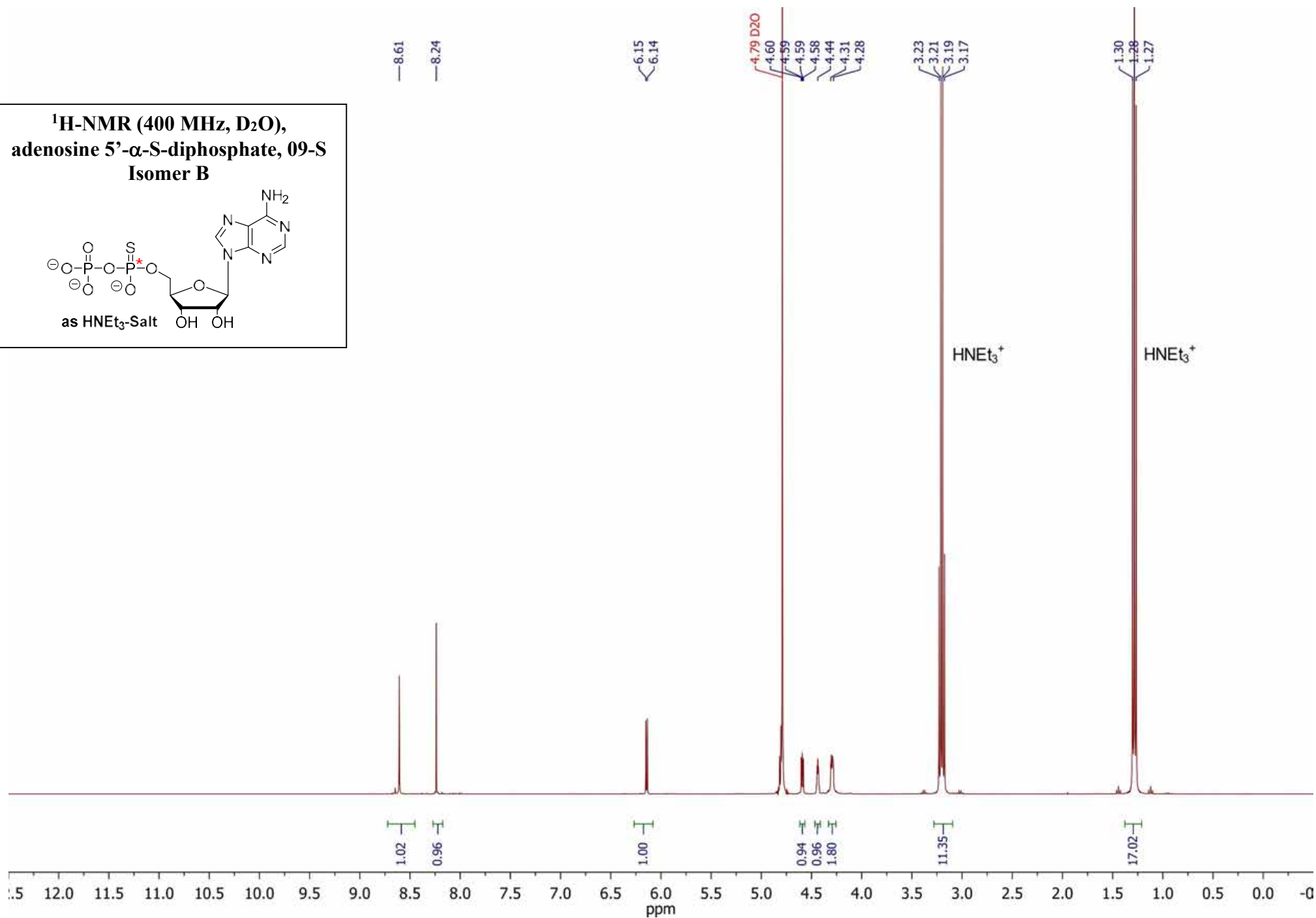
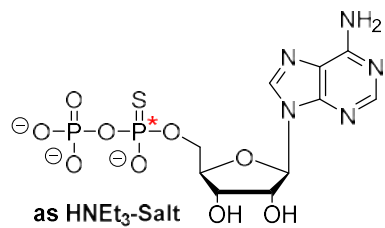
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Isomer A**



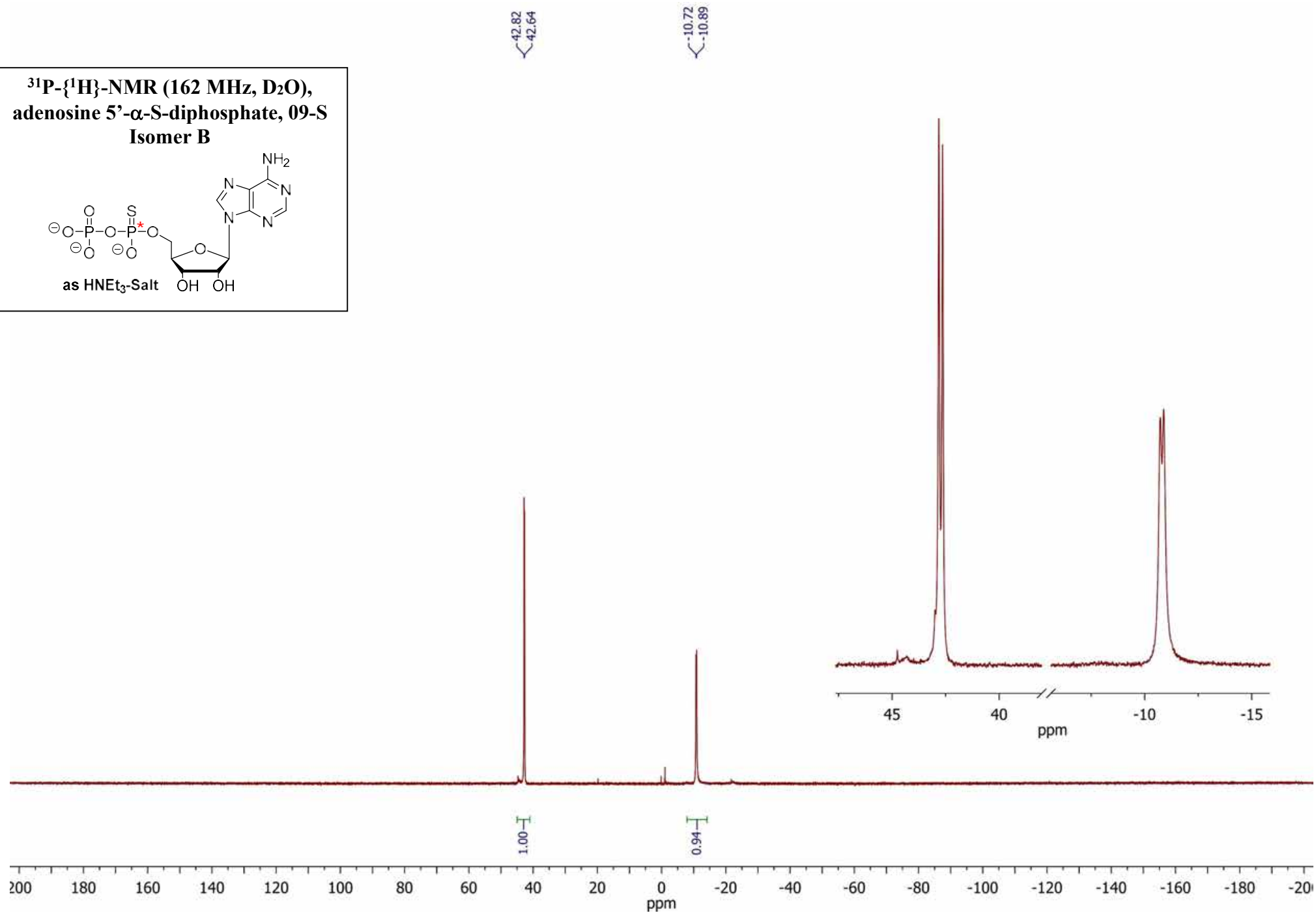
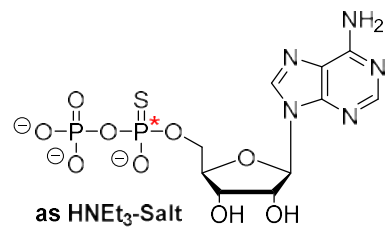
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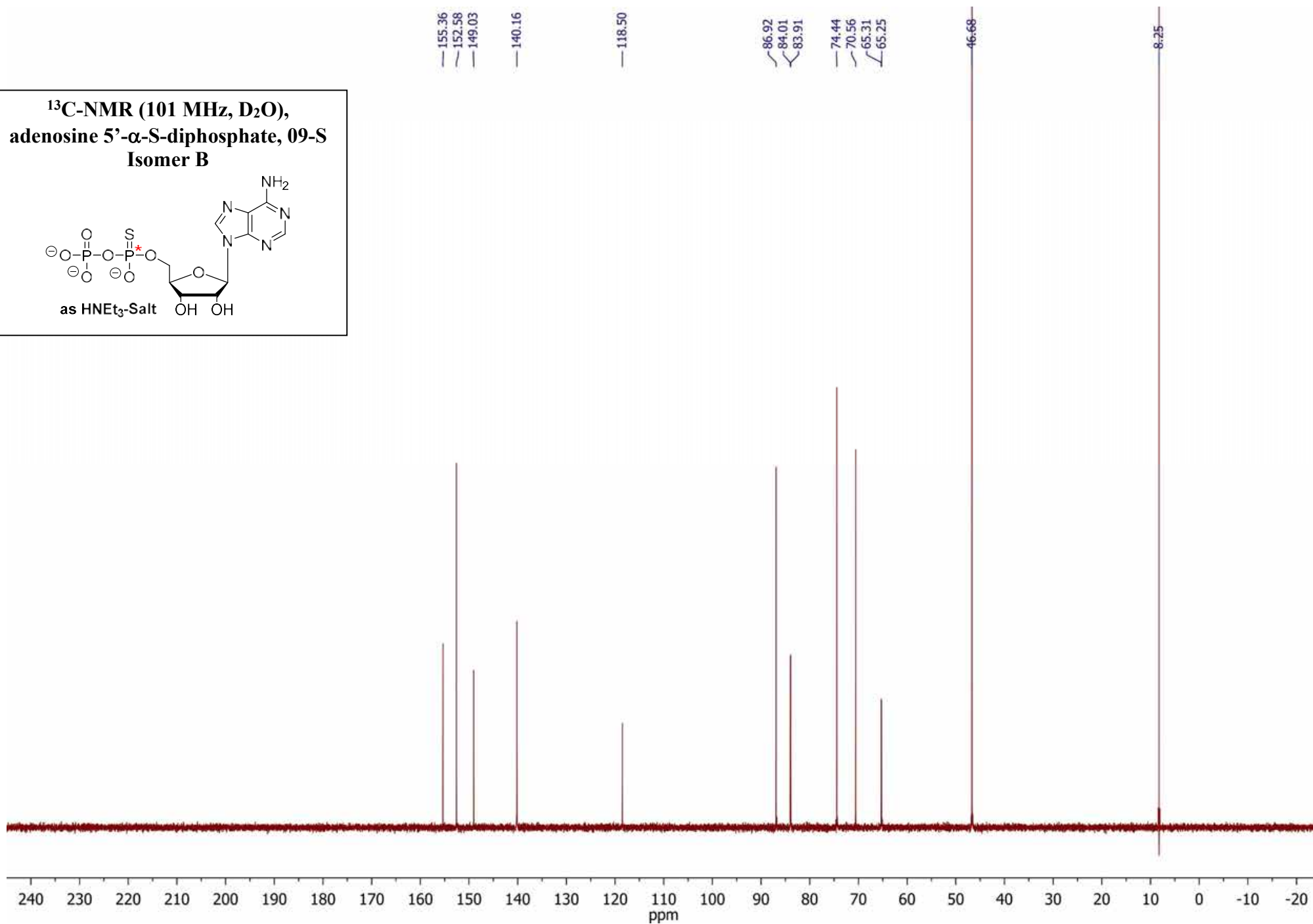
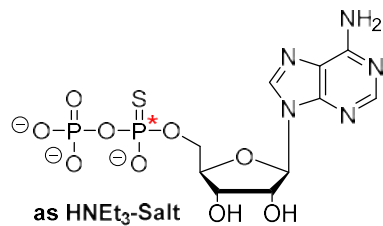
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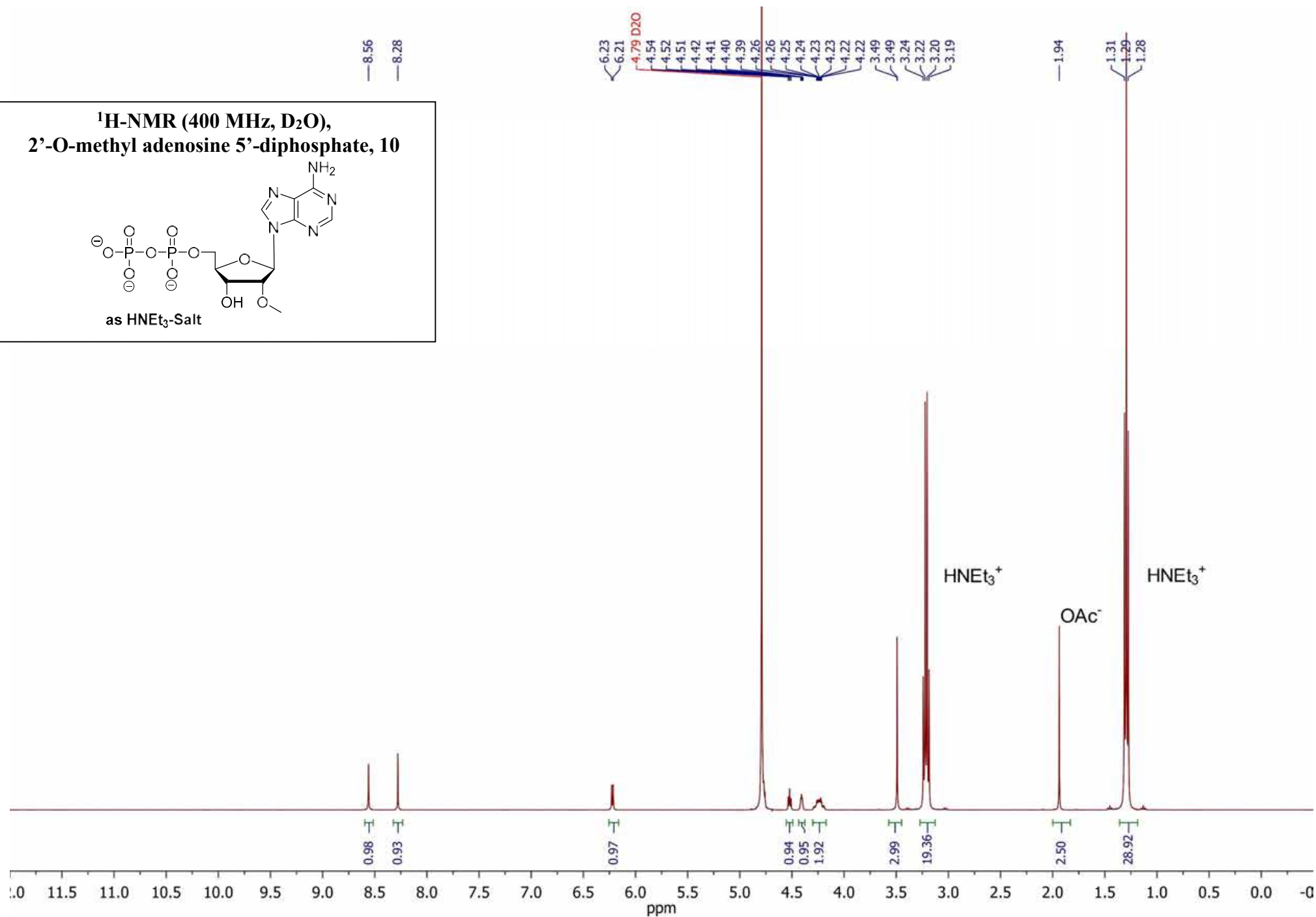
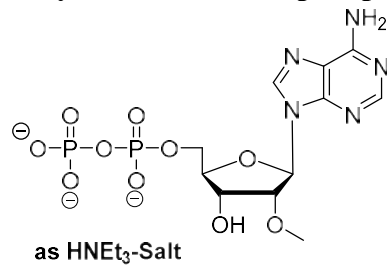
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Isomer B**



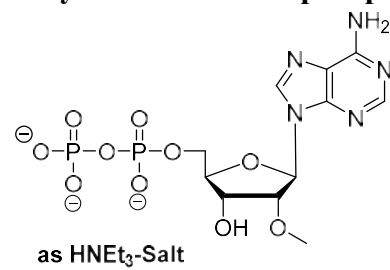
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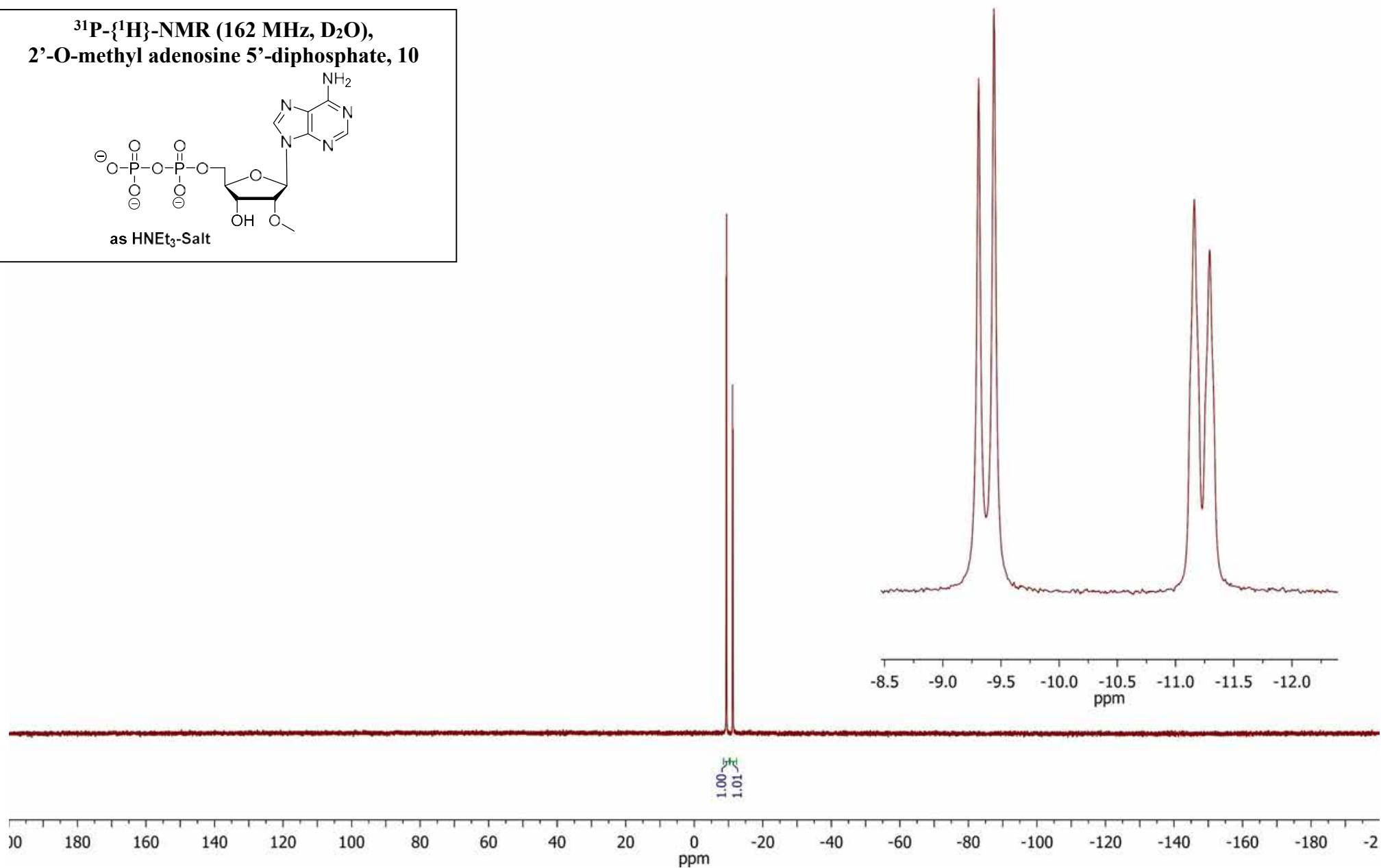
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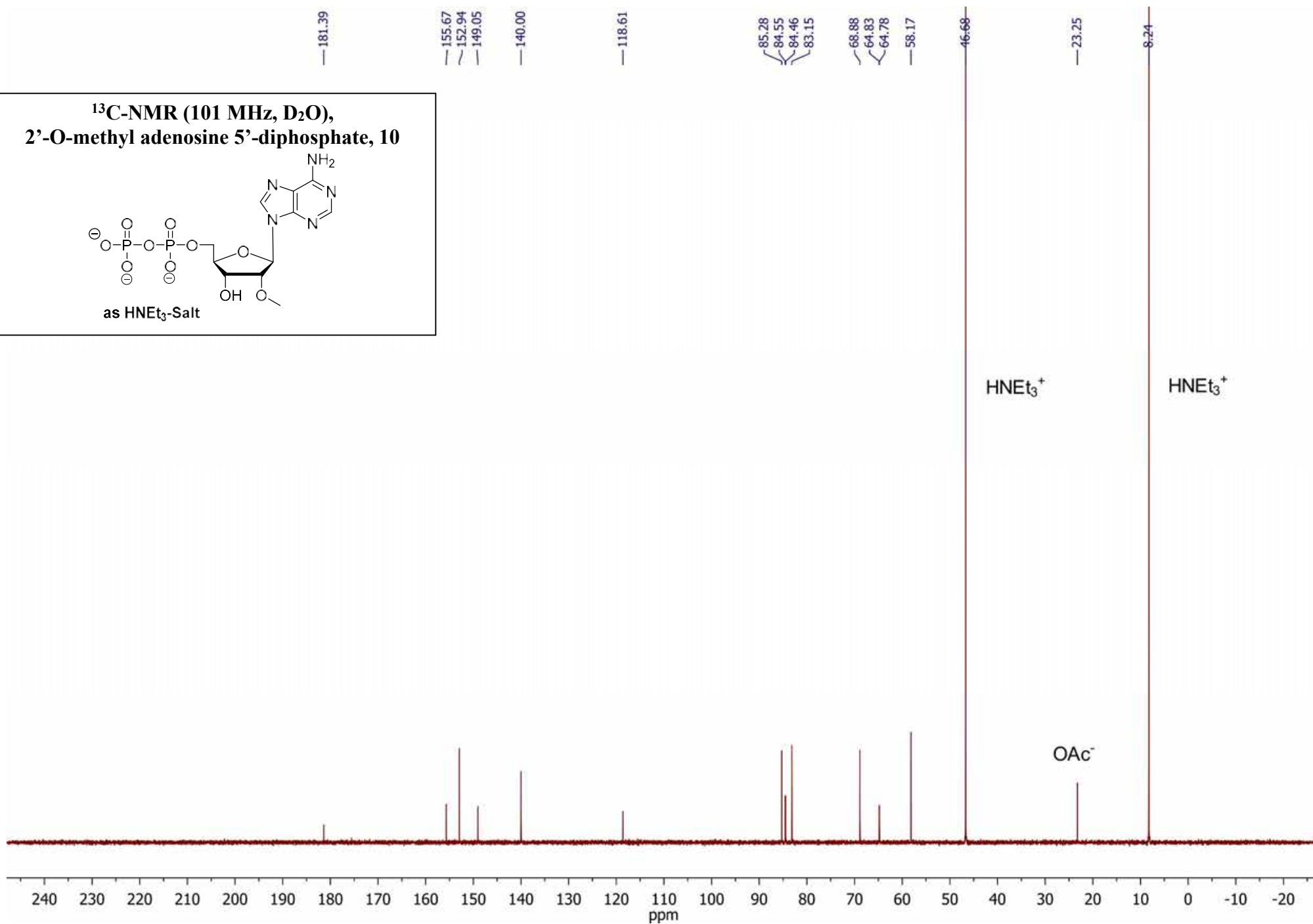
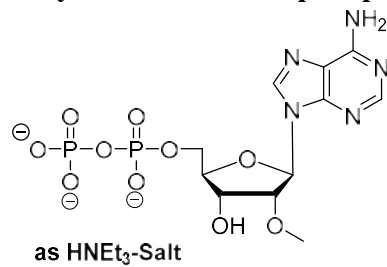
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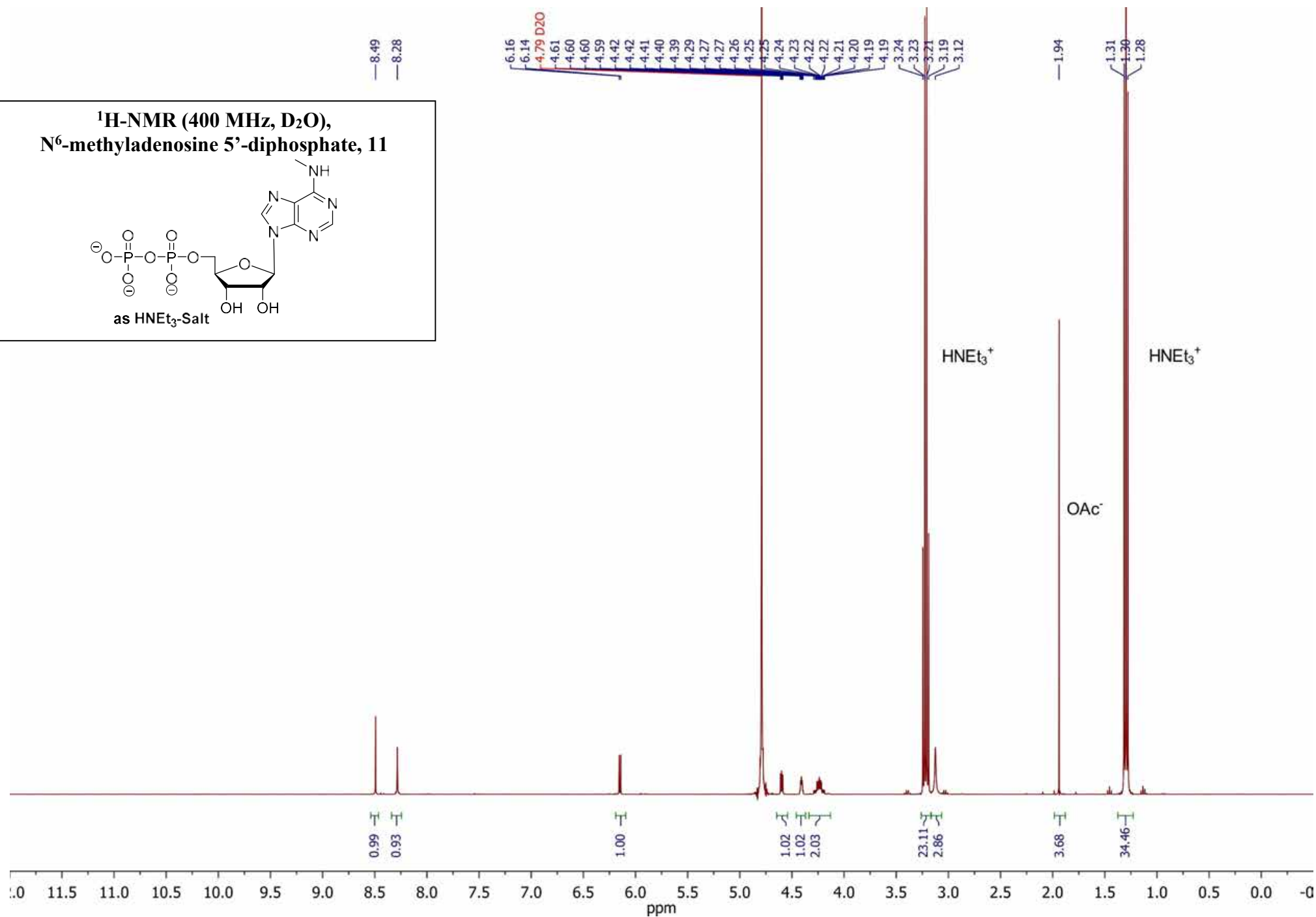
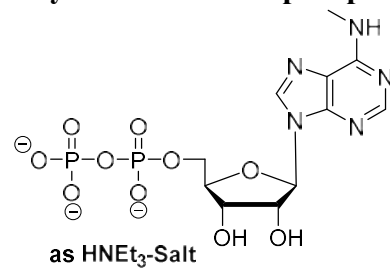
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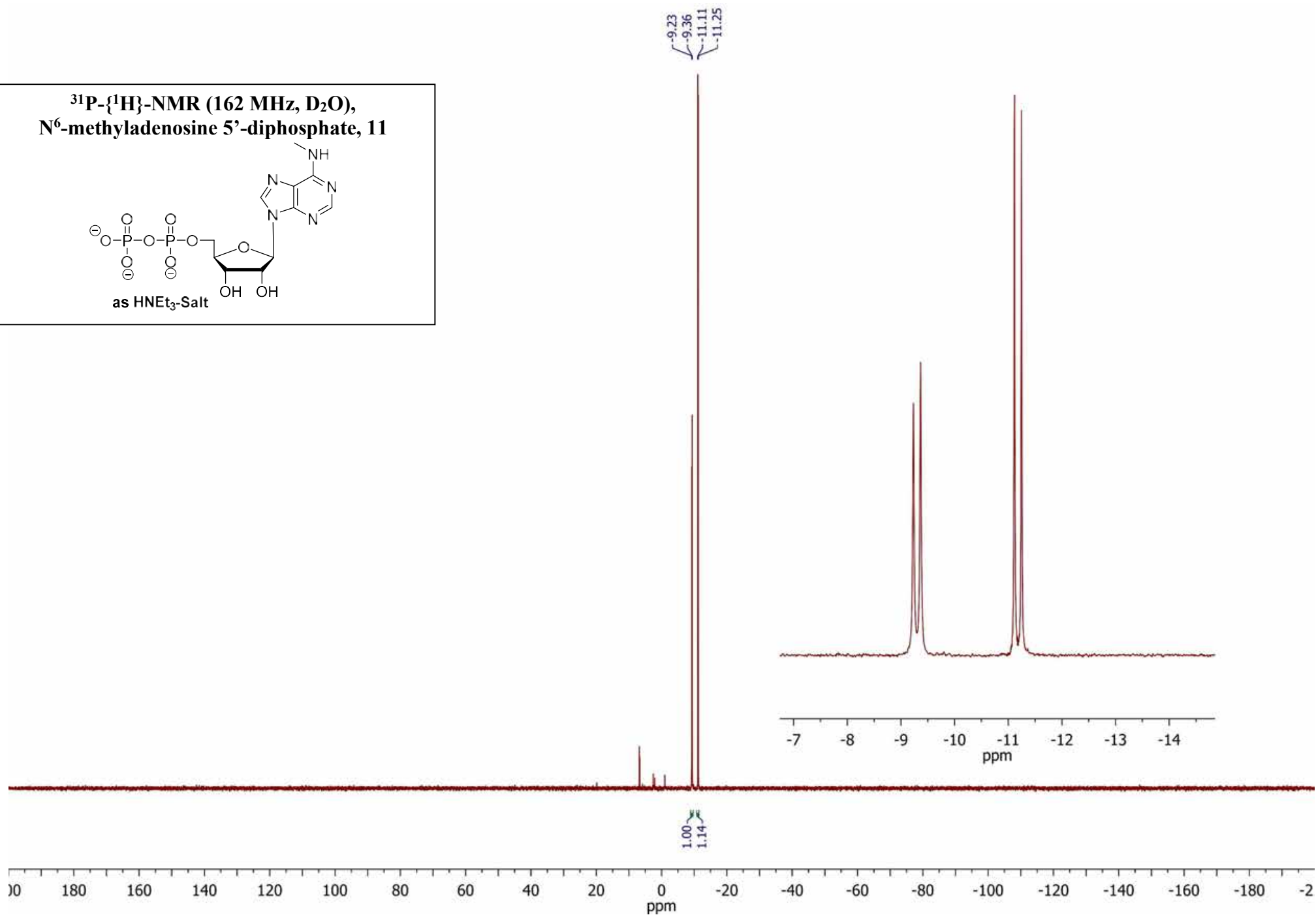
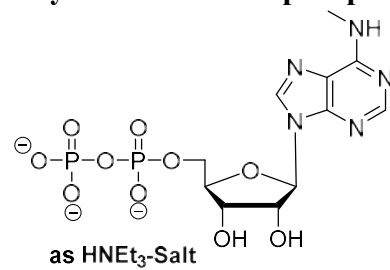
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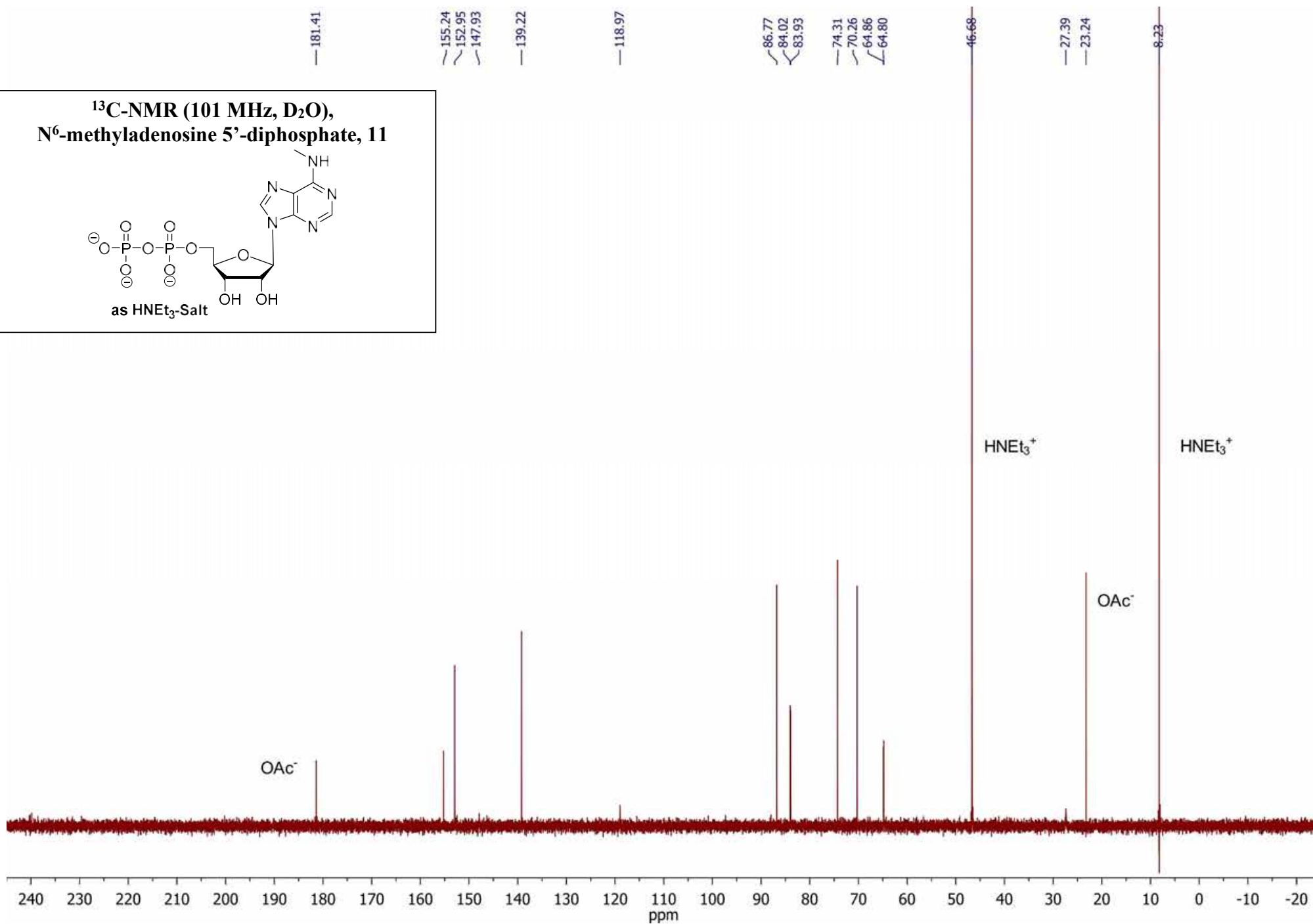
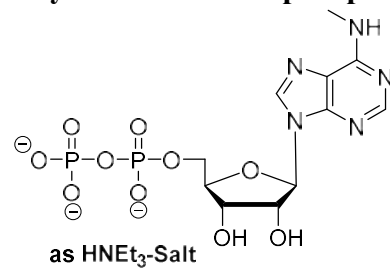
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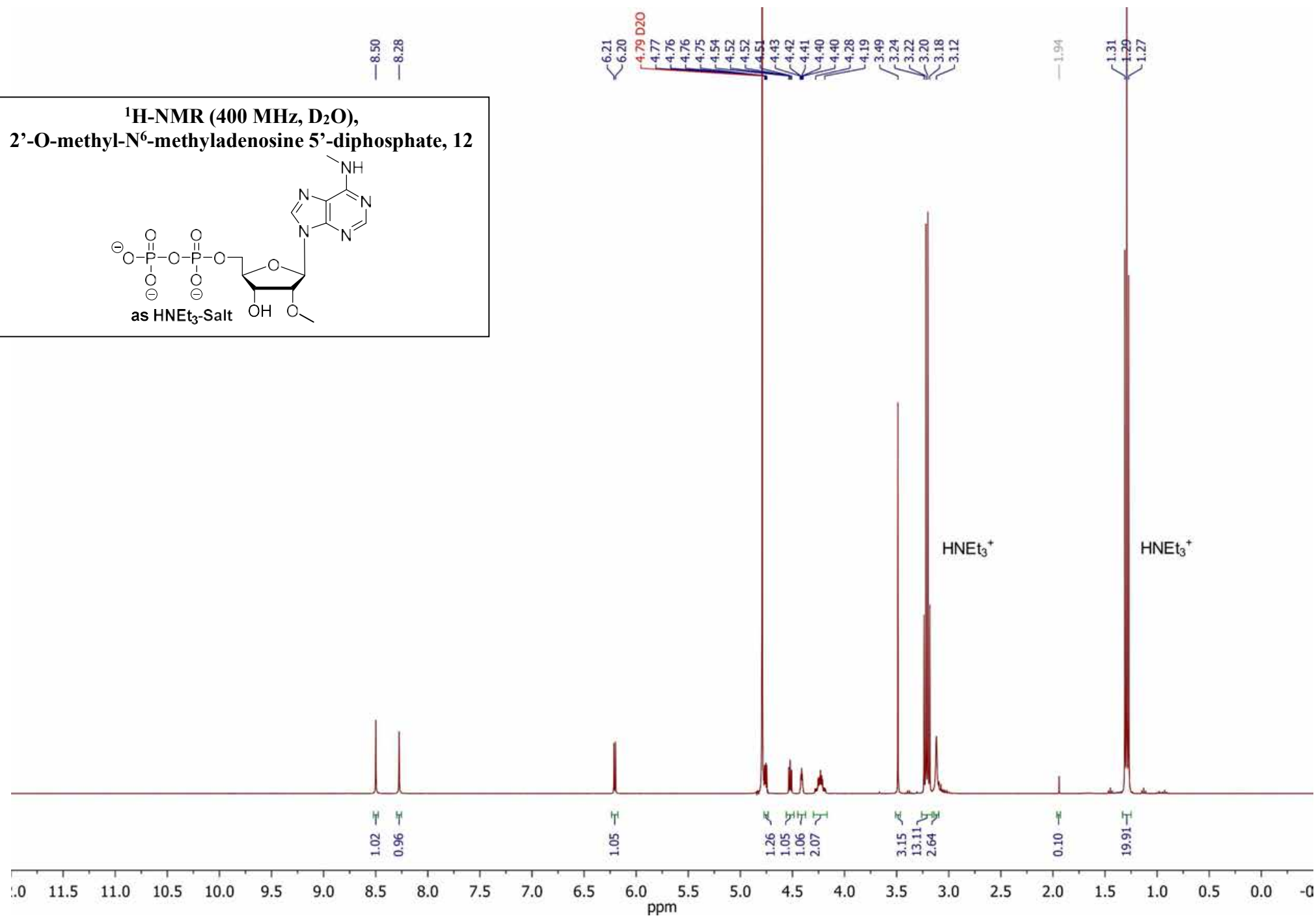
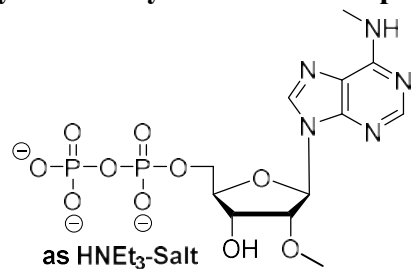
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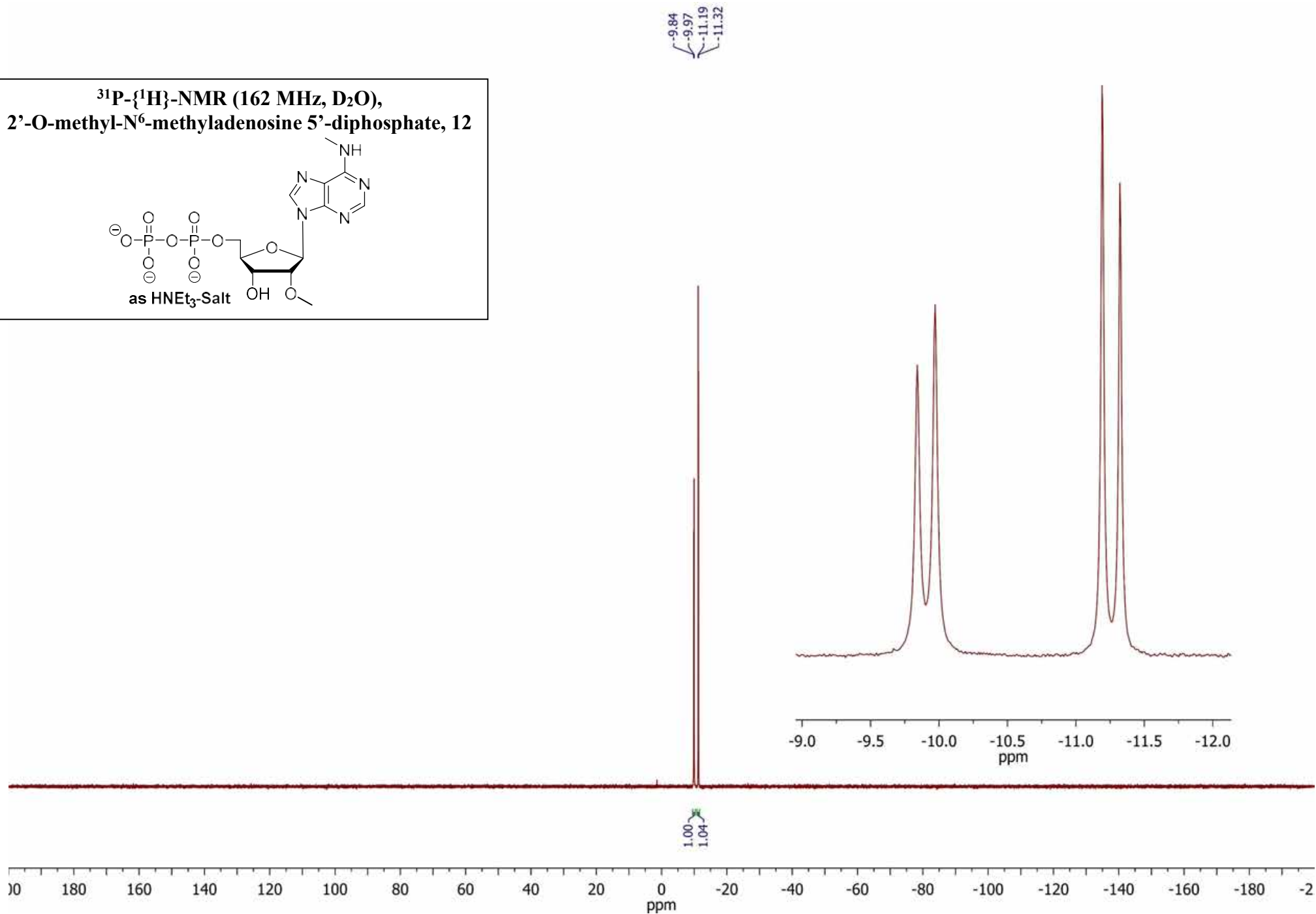
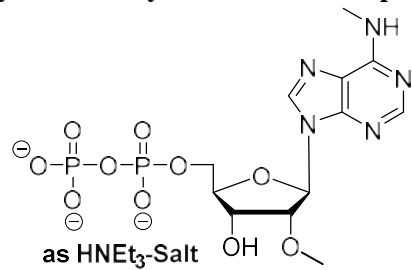
**^{13}C -NMR (101 MHz, D_2O),
 N^6 -methyladenosine 5'-diphosphate, 11**



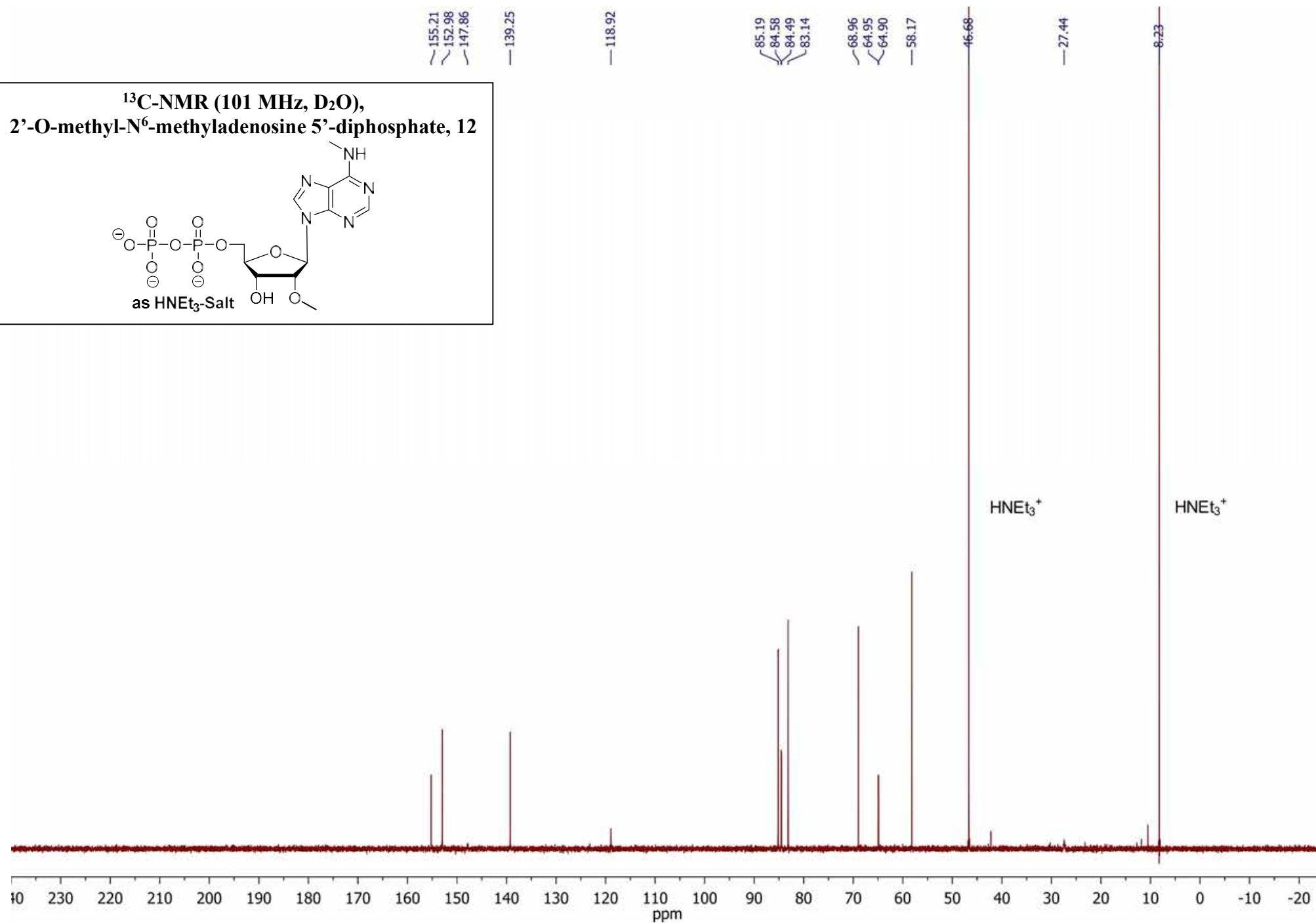
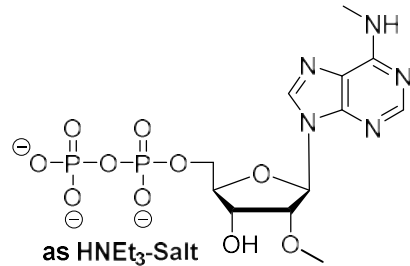
**¹H-NMR (400 MHz, D₂O),
2'-O-methyl-N⁶-methyladenosine 5'-diphosphate, 12**



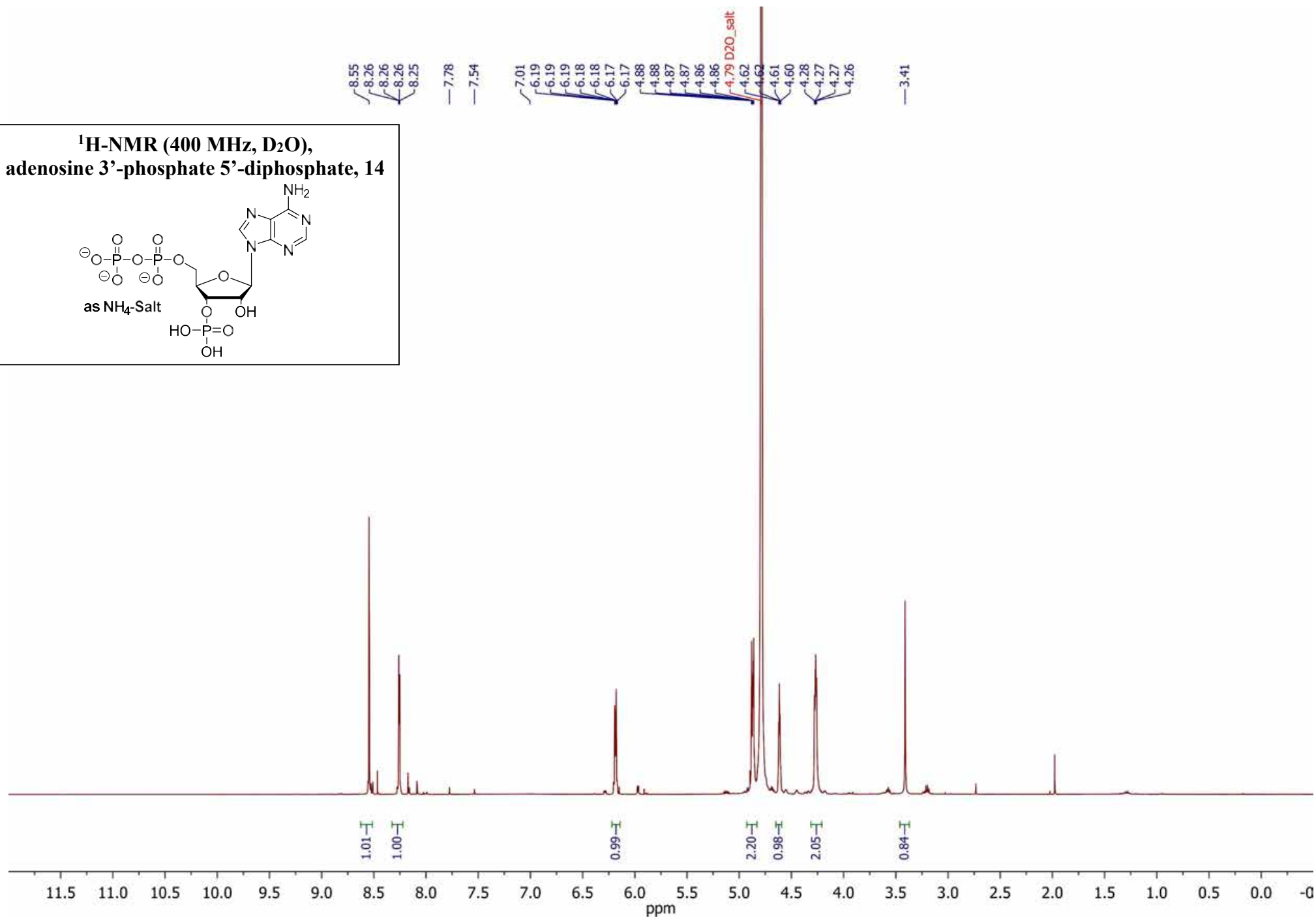
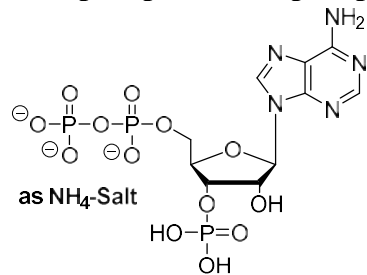
**^{31}P - $\{^1\text{H}\}$ -NMR (162 MHz, D_2O),
2'-O-methyl-N⁶-methyadenosine 5'-diphosphate, 12**



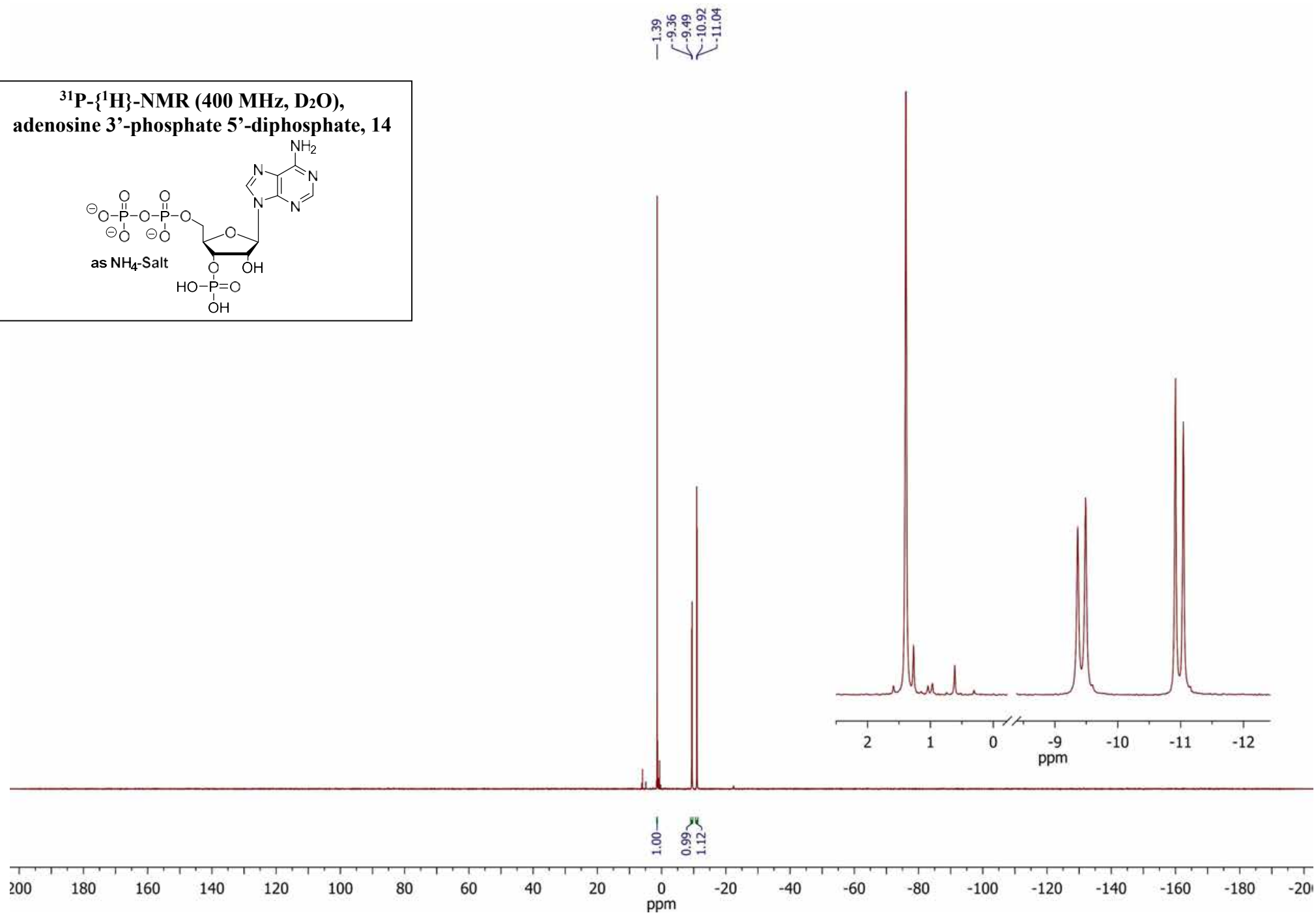
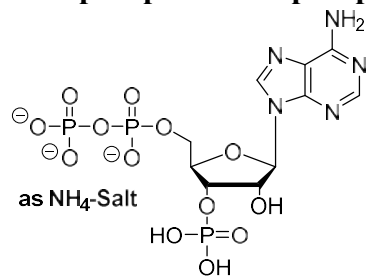
**^{13}C -NMR (101 MHz, D_2O),
2'-O-methyl-N⁶-methyladenosine 5'-diphosphate, 12**



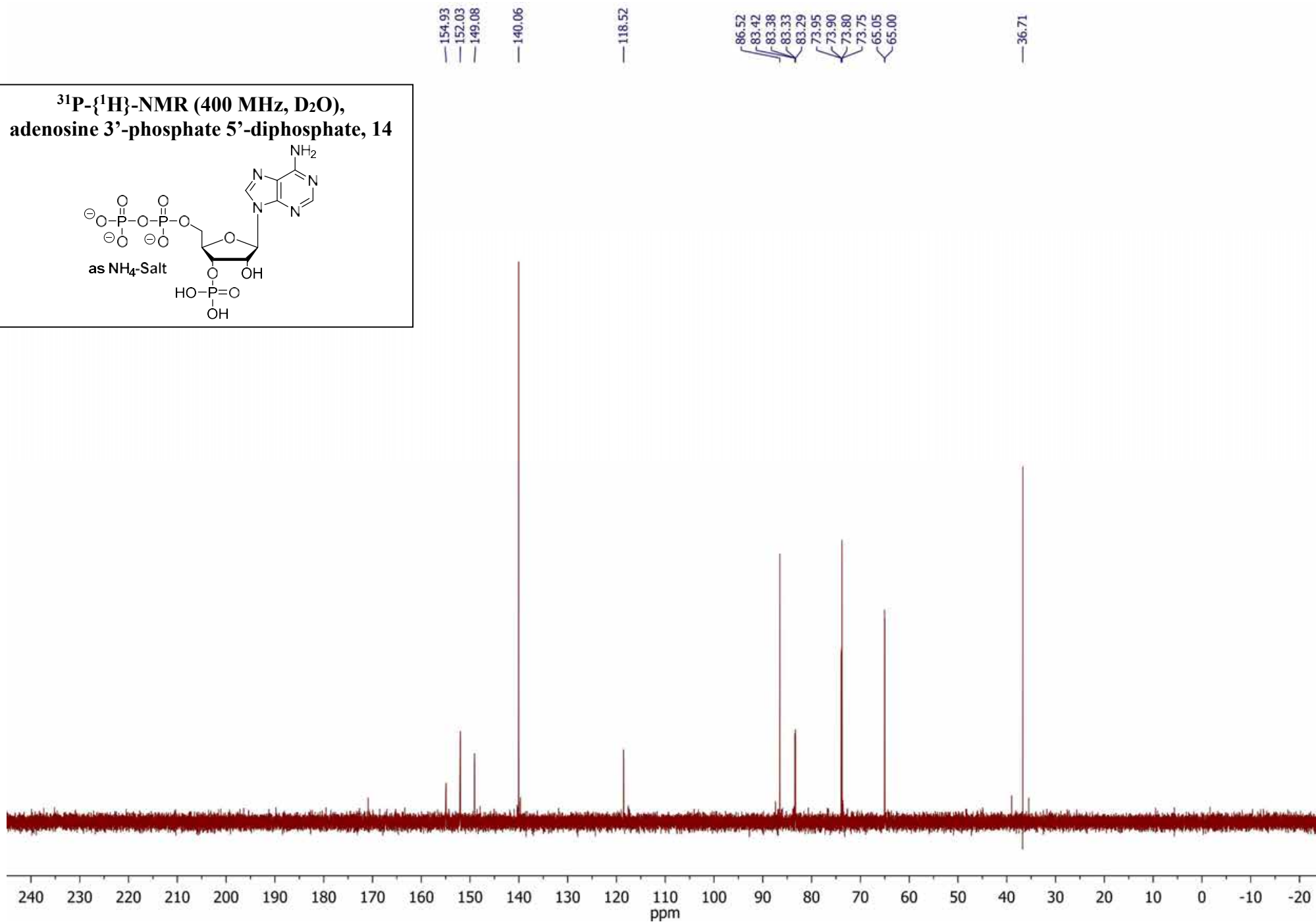
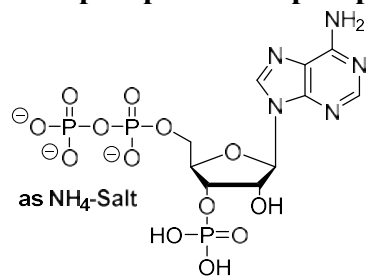
**¹H-NMR (400 MHz, D₂O),
adenosine 3'-phosphate 5'-diphosphate, 14**



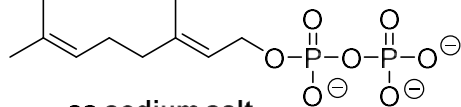
**$^{31}\text{P}\{-^1\text{H}\}$ -NMR (400 MHz, D_2O),
adenosine 3'-phosphate 5'-diphosphate, 14**



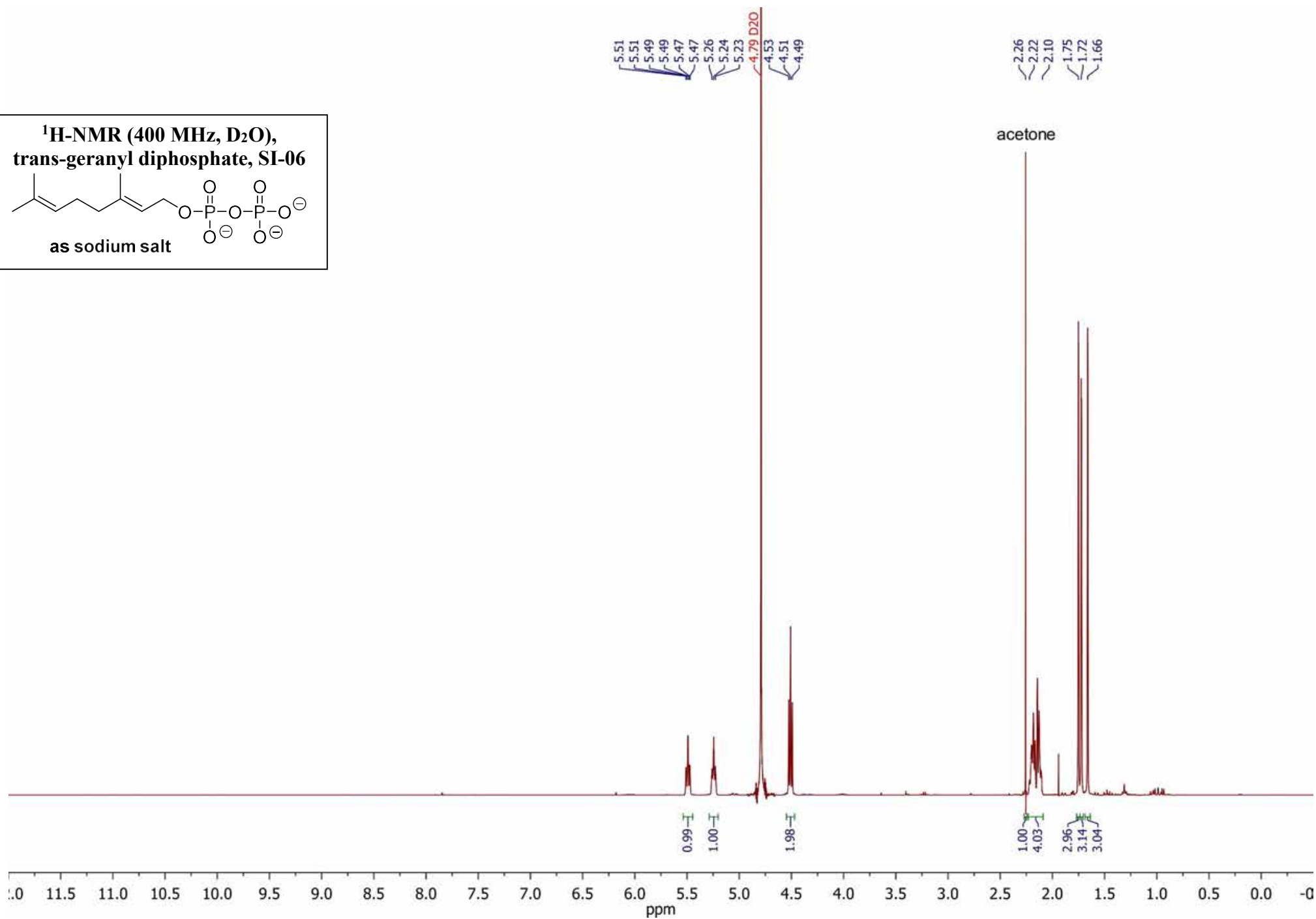
**^{31}P - $\{^1\text{H}\}$ -NMR (400 MHz, D_2O),
adenosine 3'-phosphate 5'-diphosphate, 14**



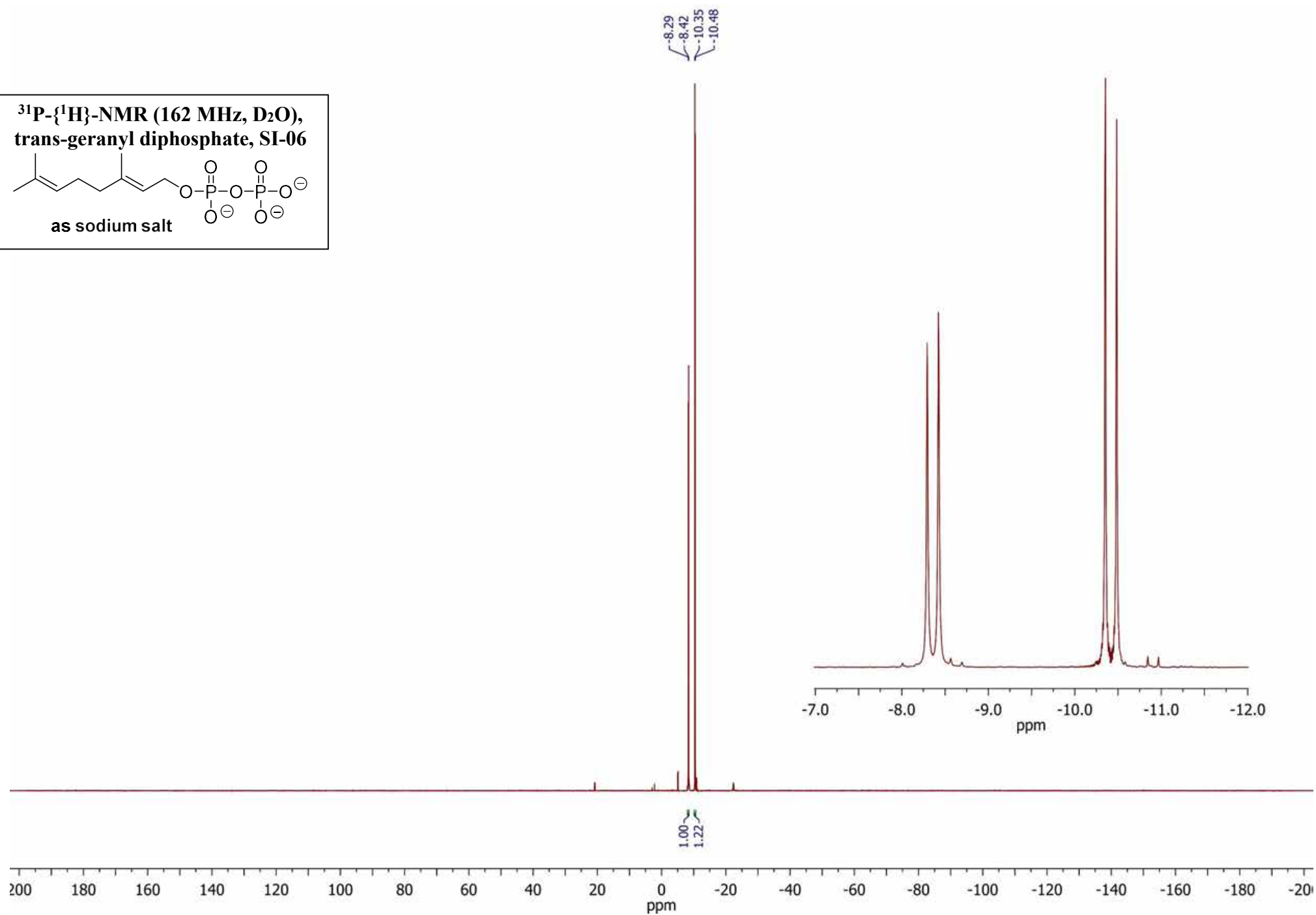
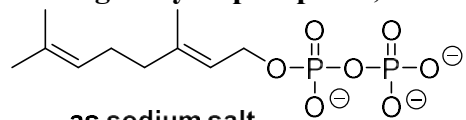
**¹H-NMR (400 MHz, D₂O),
trans-geranyl diphosphate, SI-06**



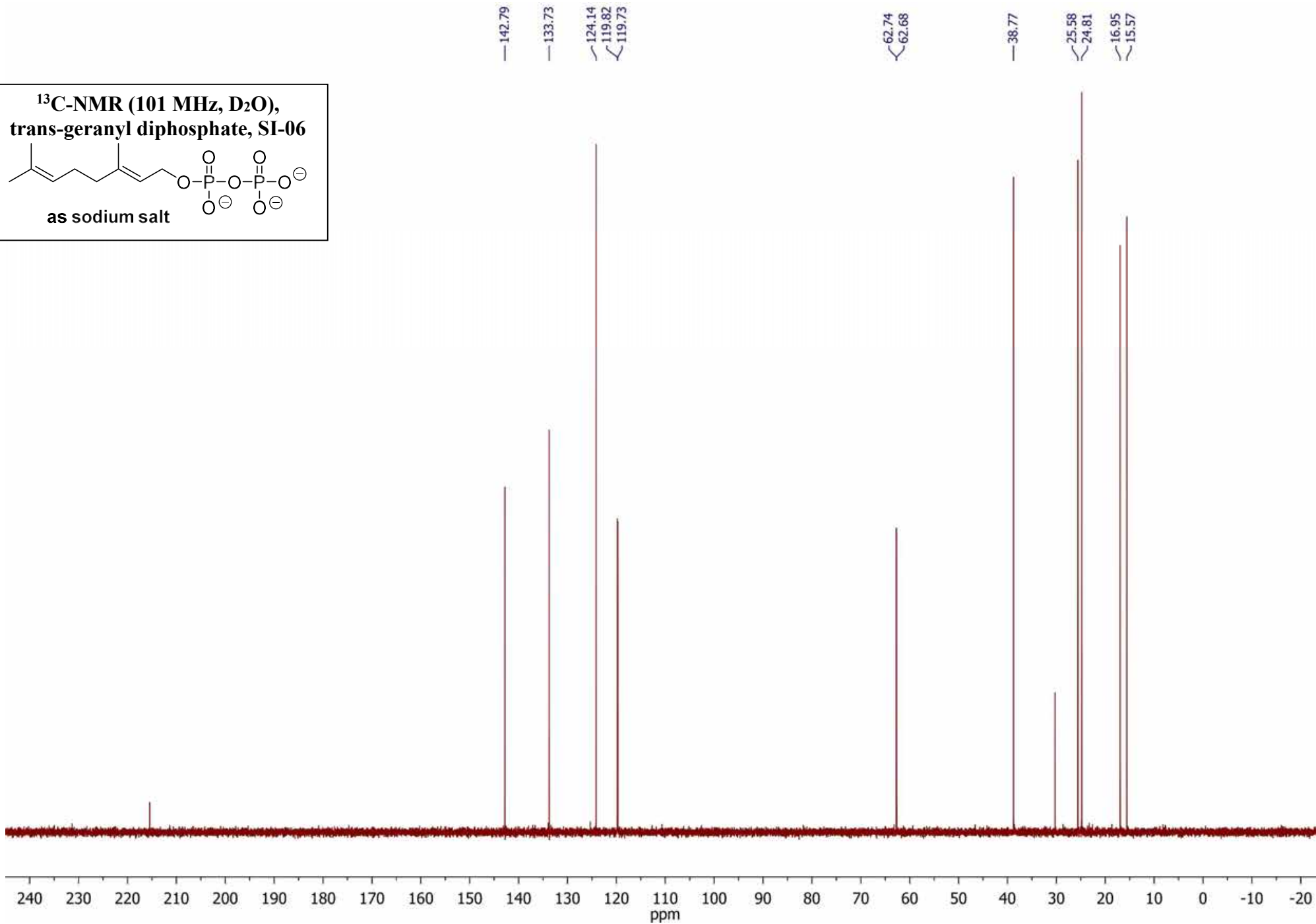
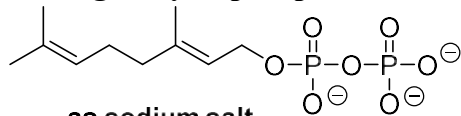
as sodium salt



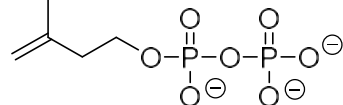
**$^{31}\text{P}\{-^1\text{H}\}$ -NMR (162 MHz, D_2O),
trans-geranyl diphosphate, SI-06**



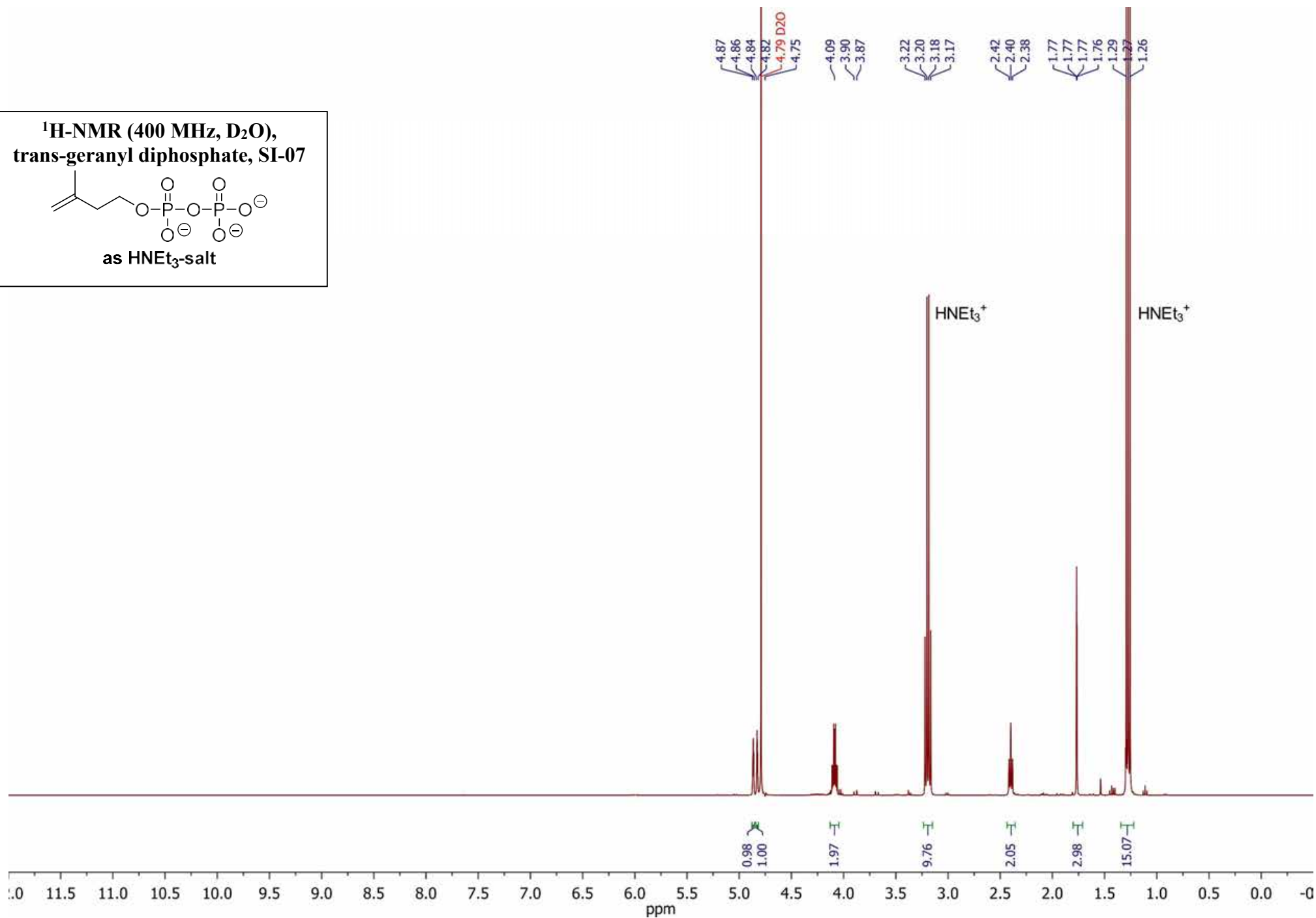
**^{13}C -NMR (101 MHz, D_2O),
trans-geranyl diphosphate, SI-06**



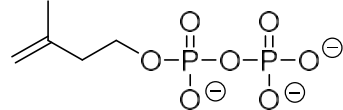
**^1H -NMR (400 MHz, D_2O),
trans-geranyl diphosphate, SI-07**



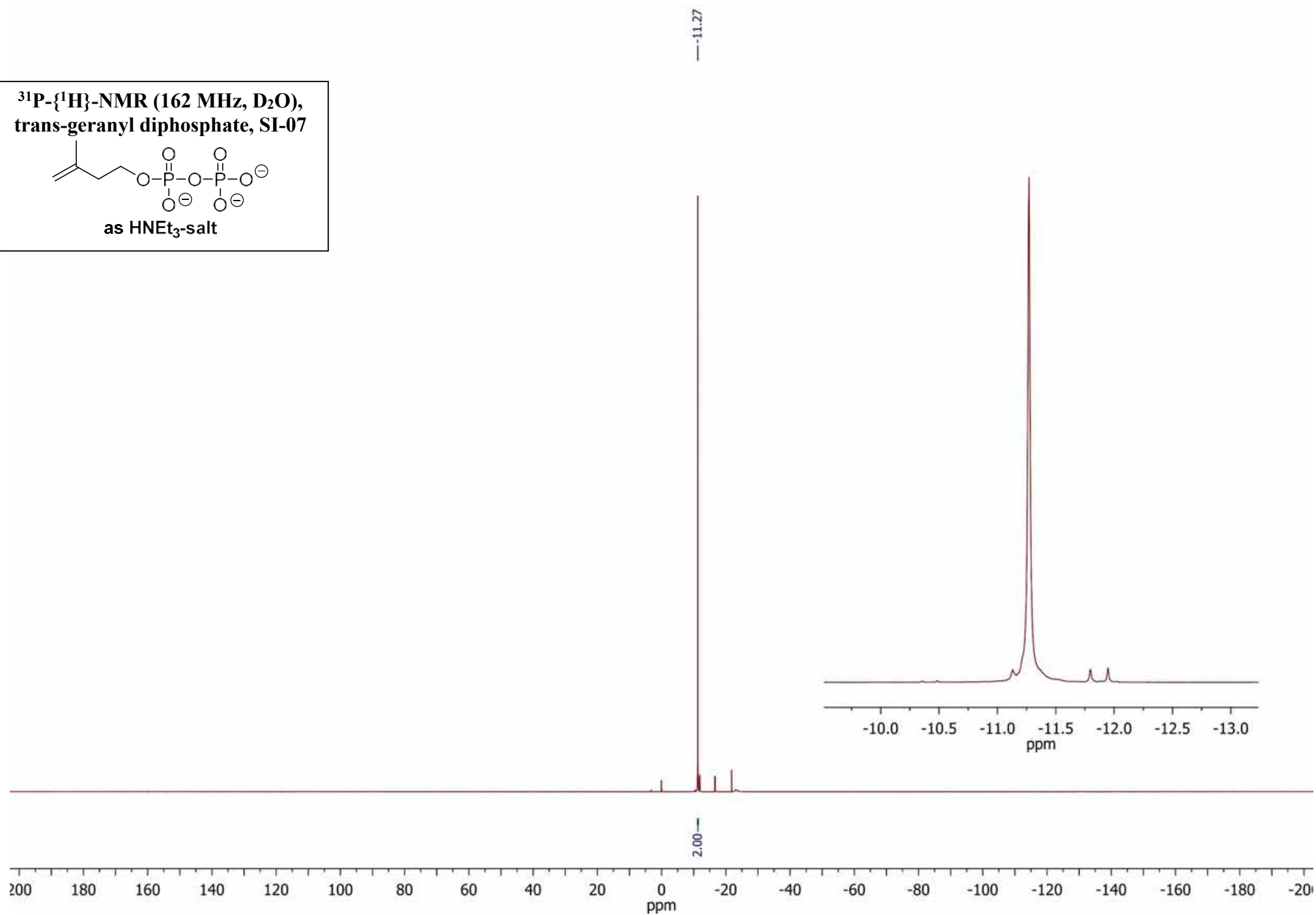
as HNEt_3 -salt



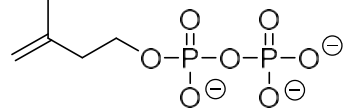
**$^{31}\text{P}\{-^1\text{H}\}$ -NMR (162 MHz, D_2O),
trans-geranyl diphosphate, SI-07**



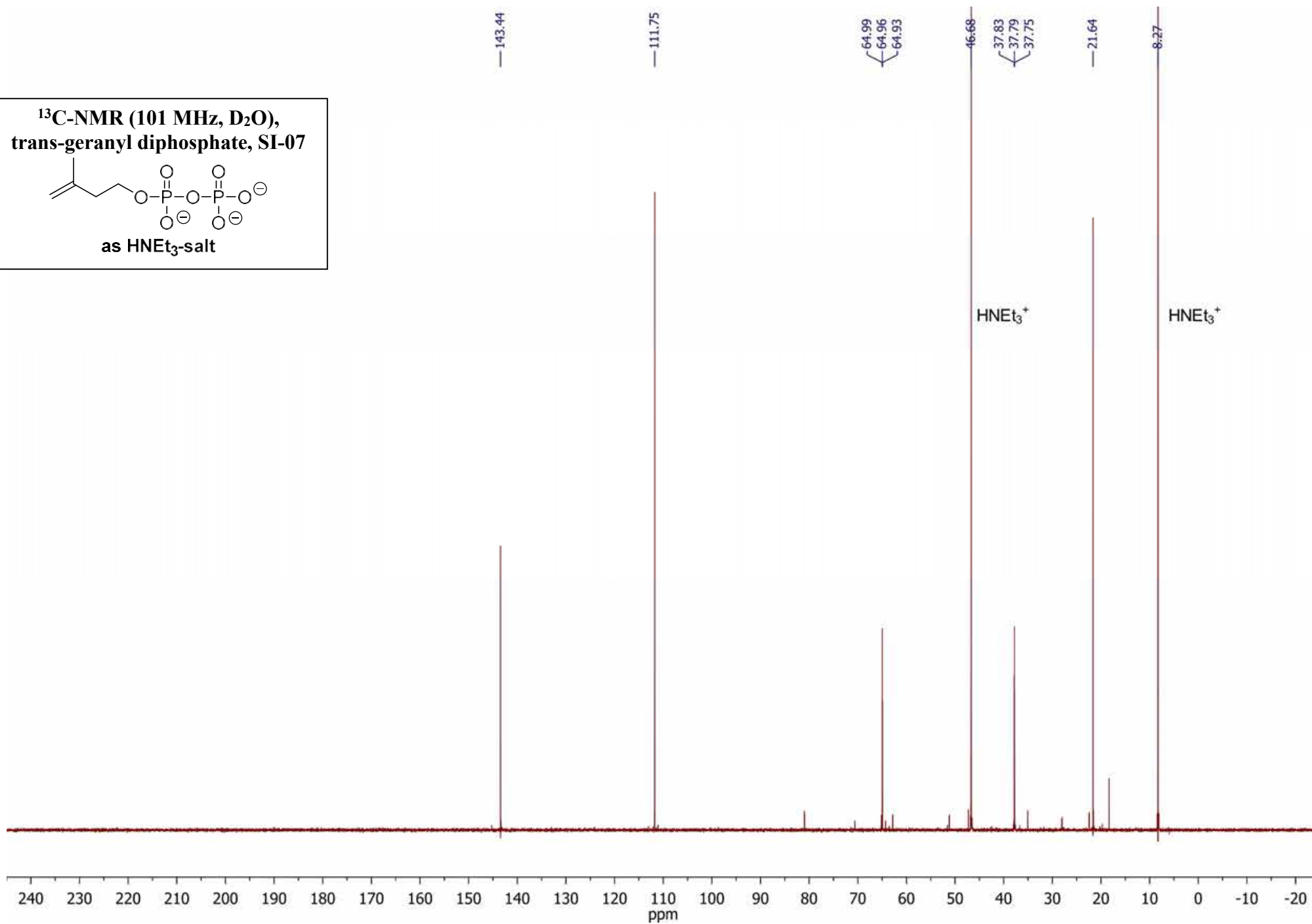
as HNEt_3 -salt



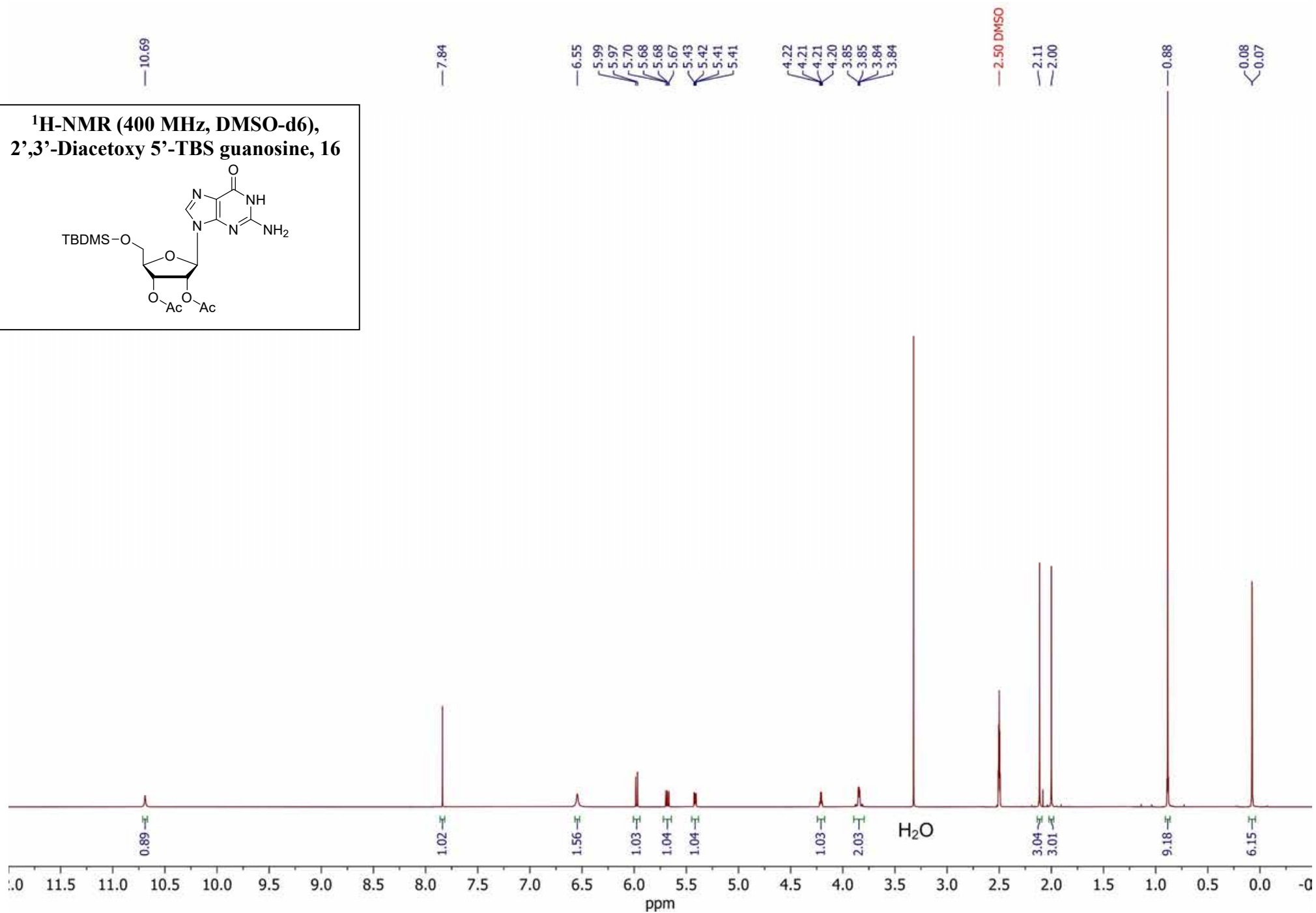
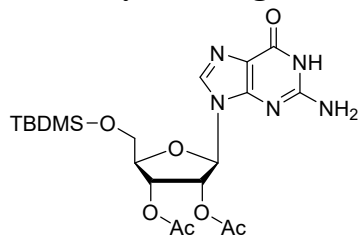
**^{13}C -NMR (101 MHz, D_2O),
trans-geranyl diphosphate, SI-07**



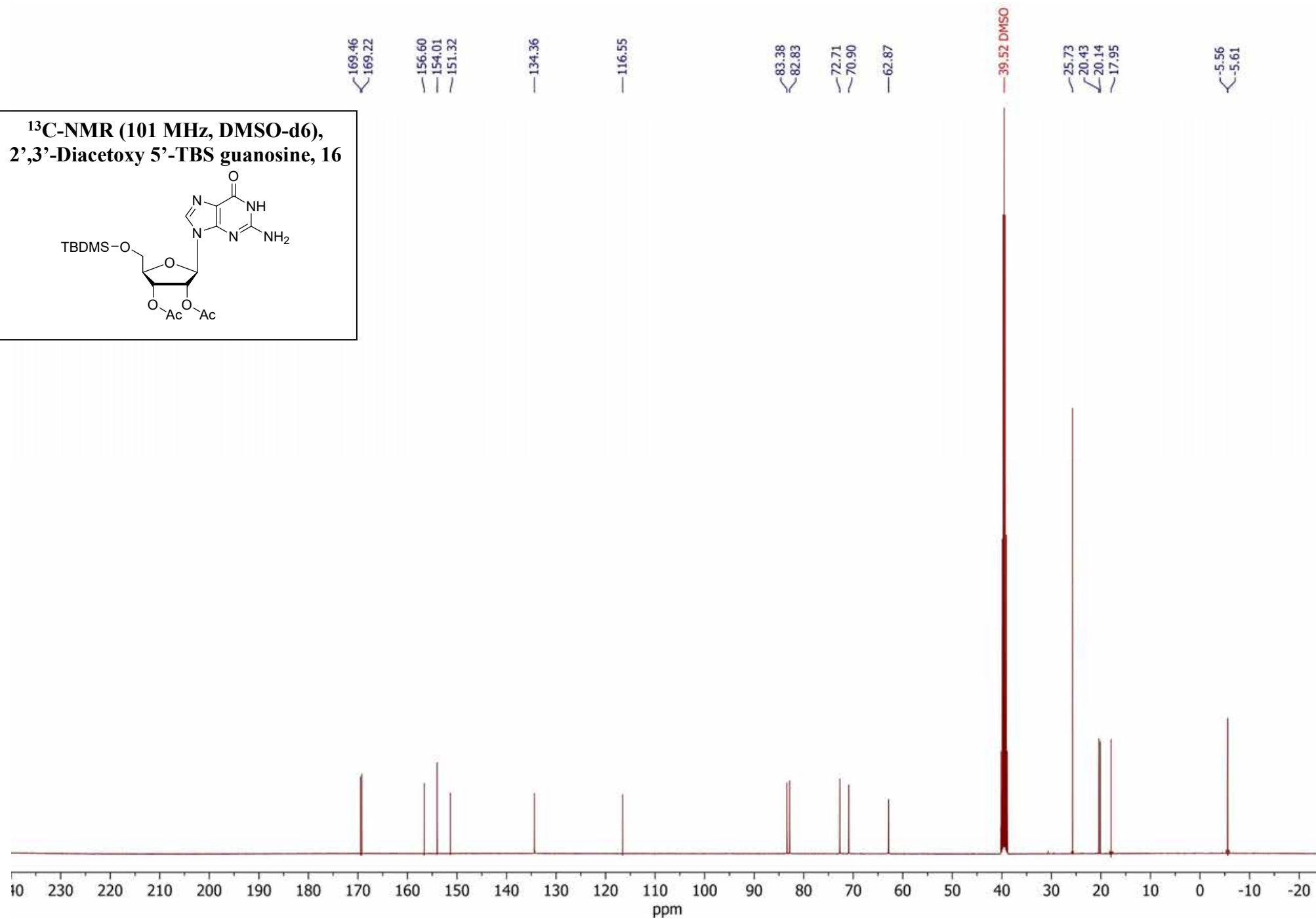
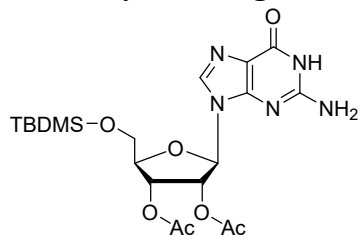
as HNEt_3 -salt



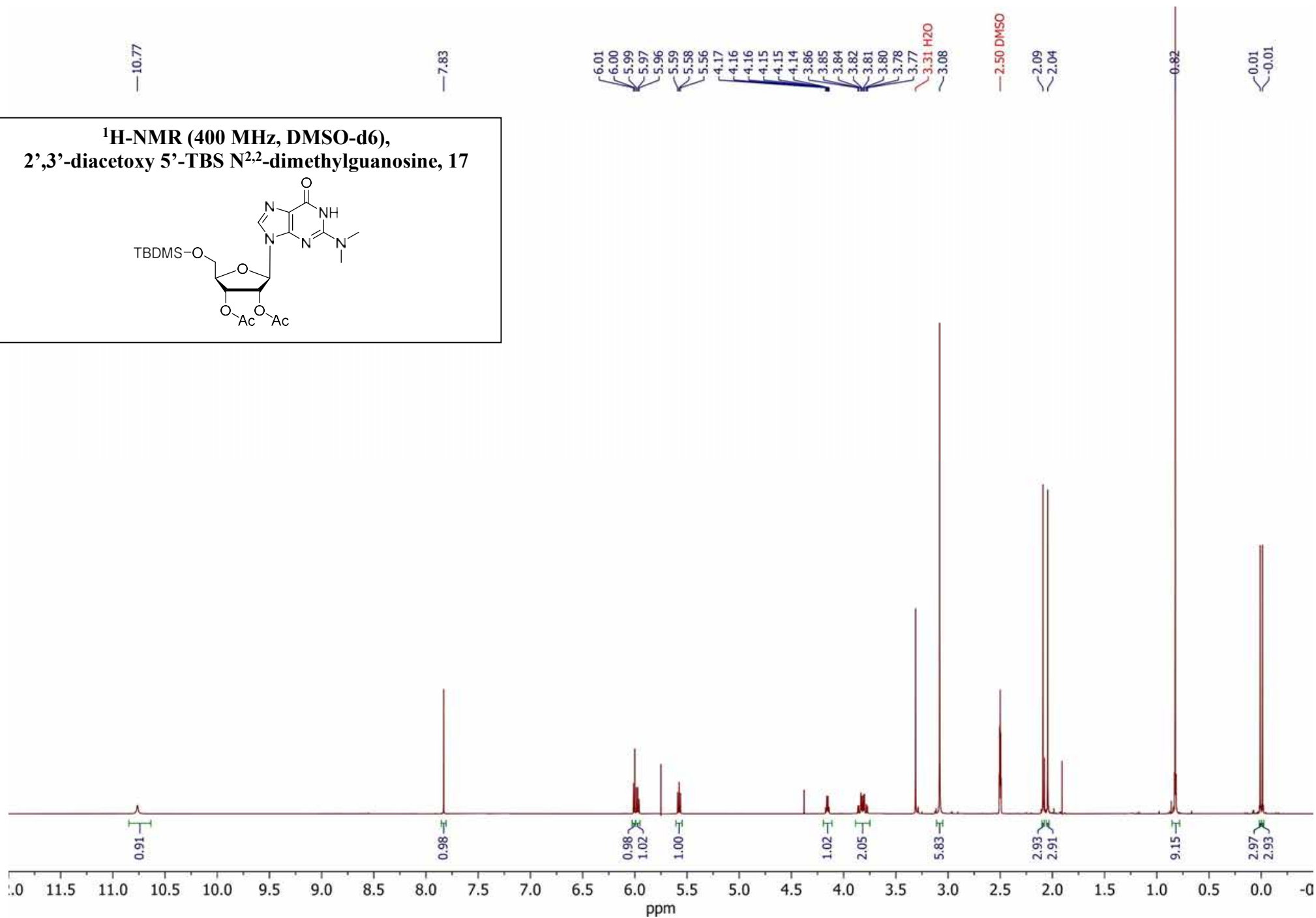
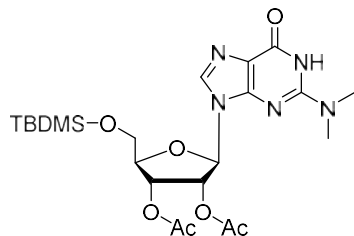
**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-Diacetoxy 5'-TBS guanosine, 16**

CC(=O)OC[C@H]1O[C@H](C[C@@H]2[C@@H](OC(=O)C)[C@H](OC(=O)C)O2)[C@H](C3=NC4=C(N)N=CN=C3N4)C[C@H](OC(C)(C)C)O1

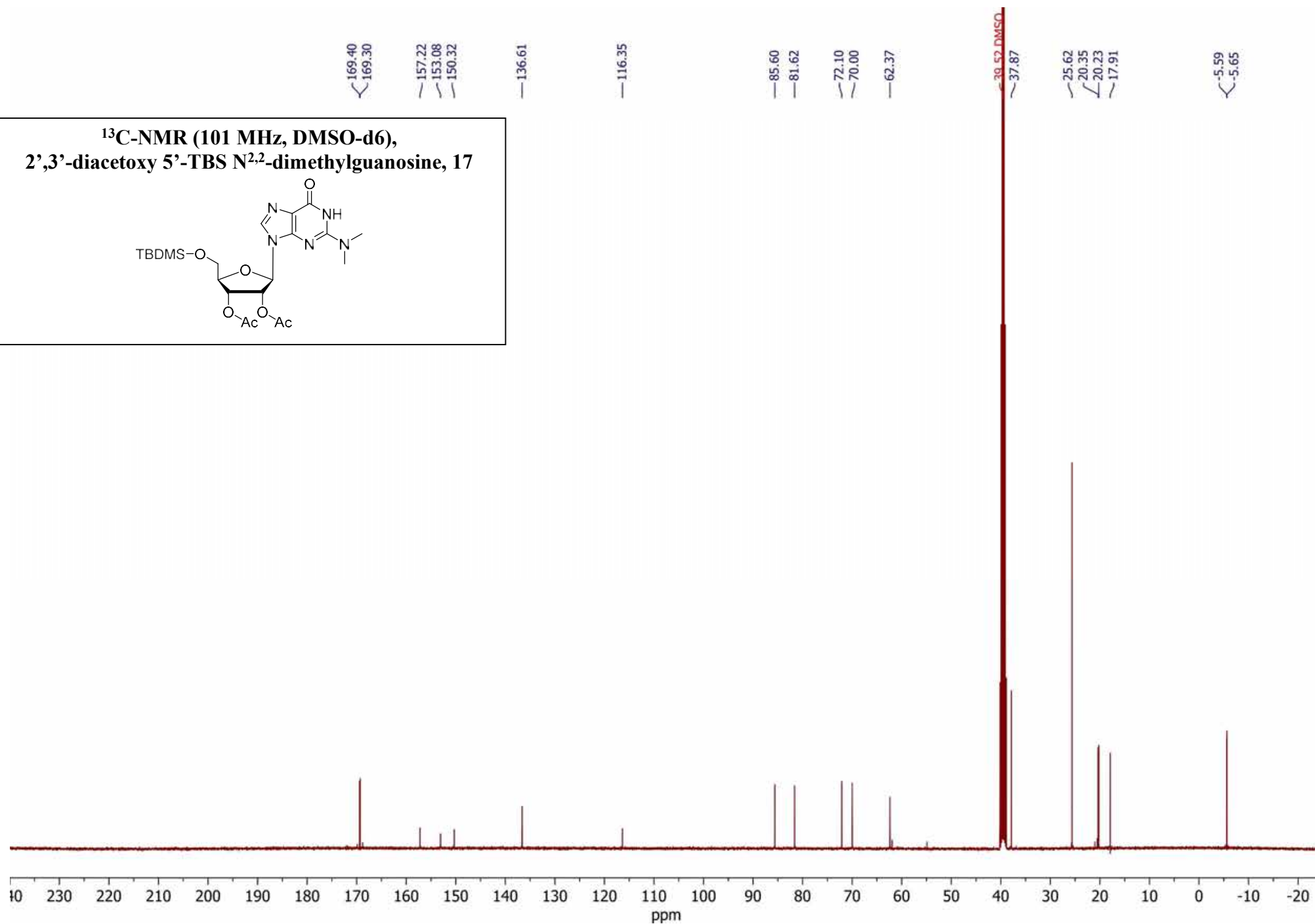
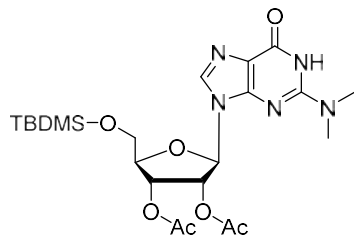
**^{13}C -NMR (101 MHz, DMSO- d_6),
2',3'-Diacetoxy 5'-TBS guanosine, 16**



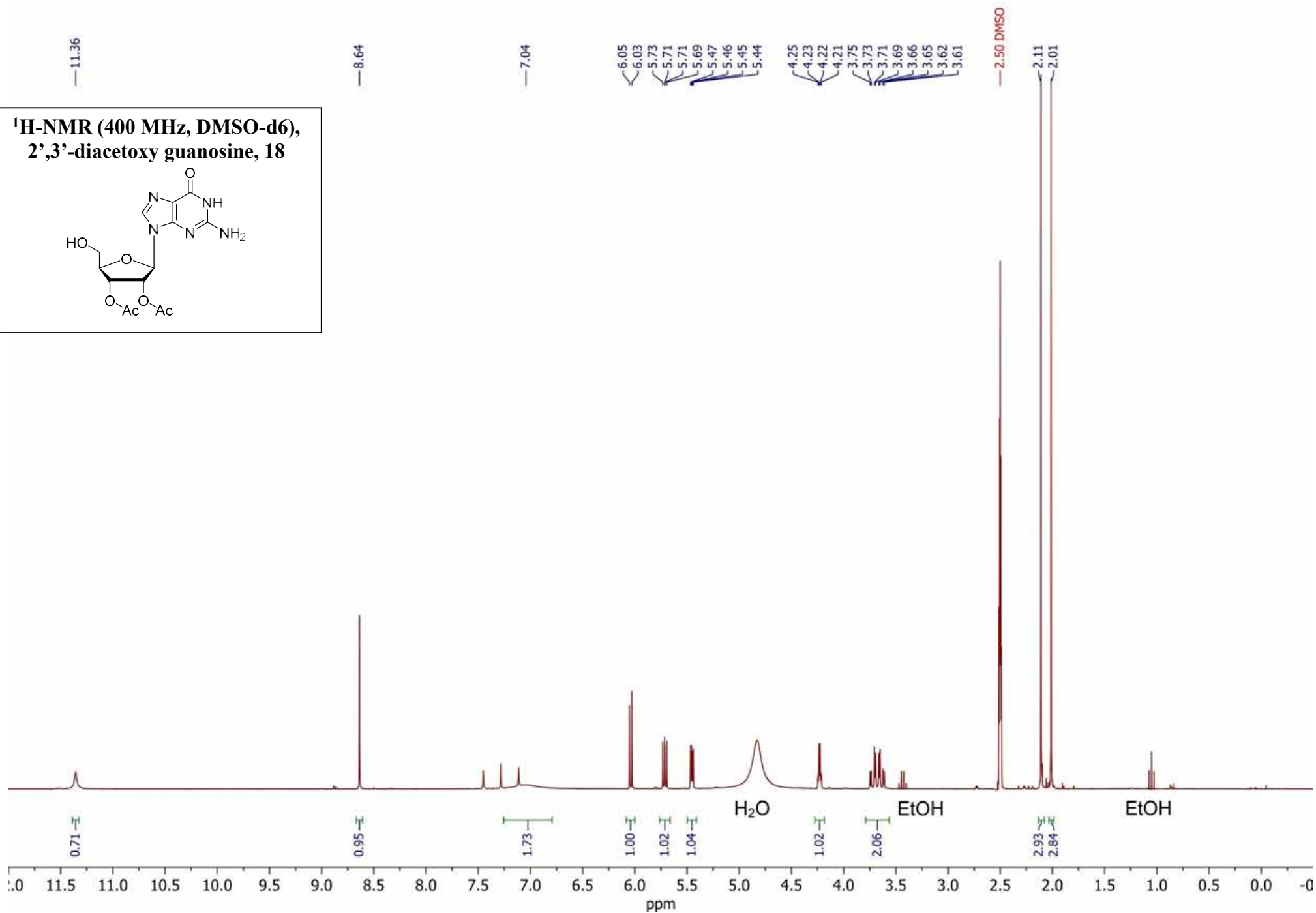
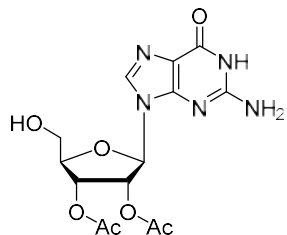
**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-diacetoxy 5'-TBS N^{2,2}-dimethylguanosine, 17**



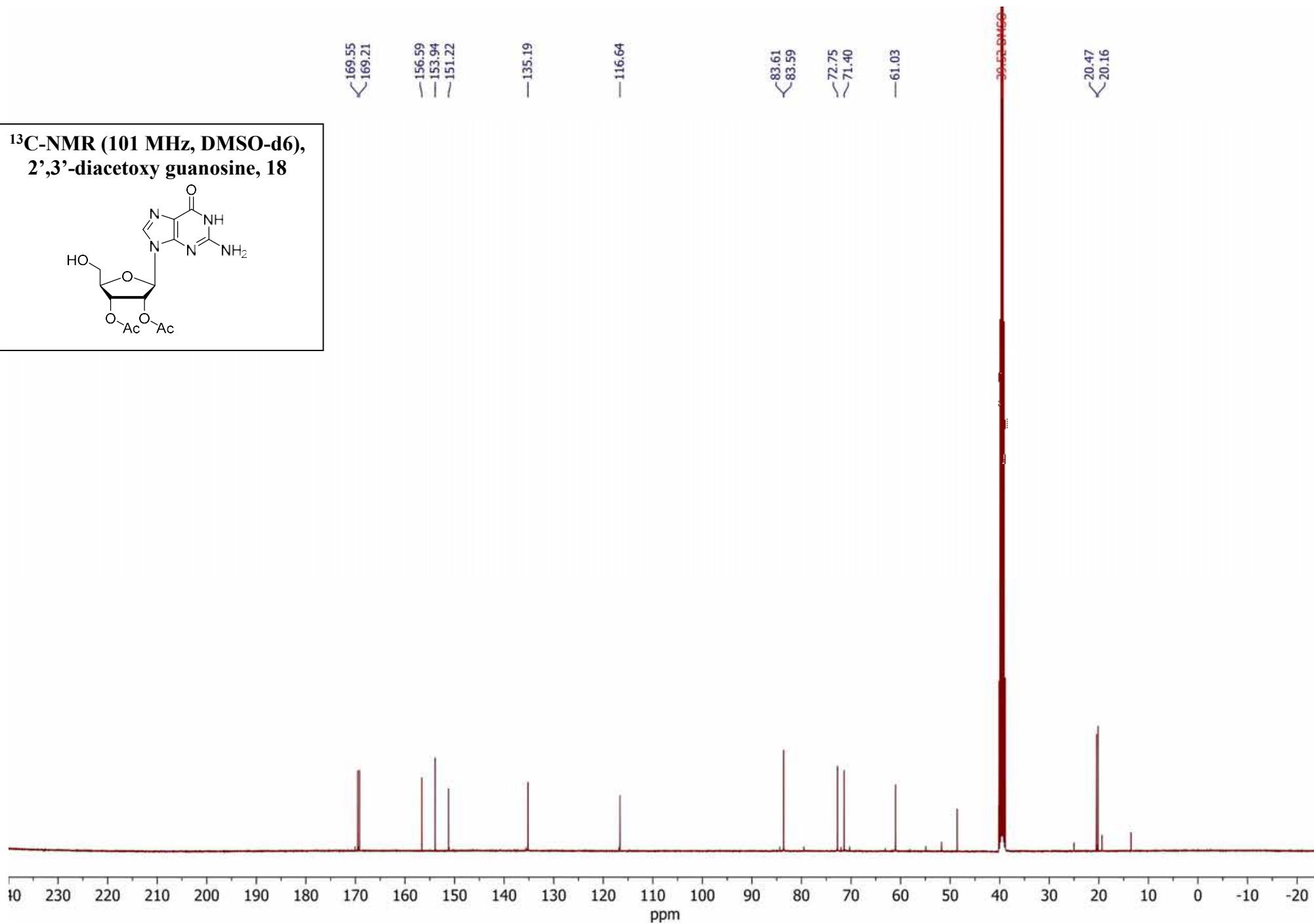
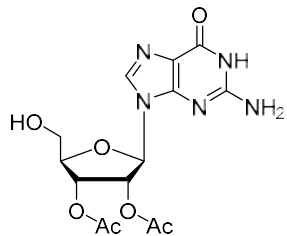
**^{13}C -NMR (101 MHz, DMSO- d_6),
2',3'-diacetoxy 5'-TBS N^{2,2}-dimethylguanosine, 17**



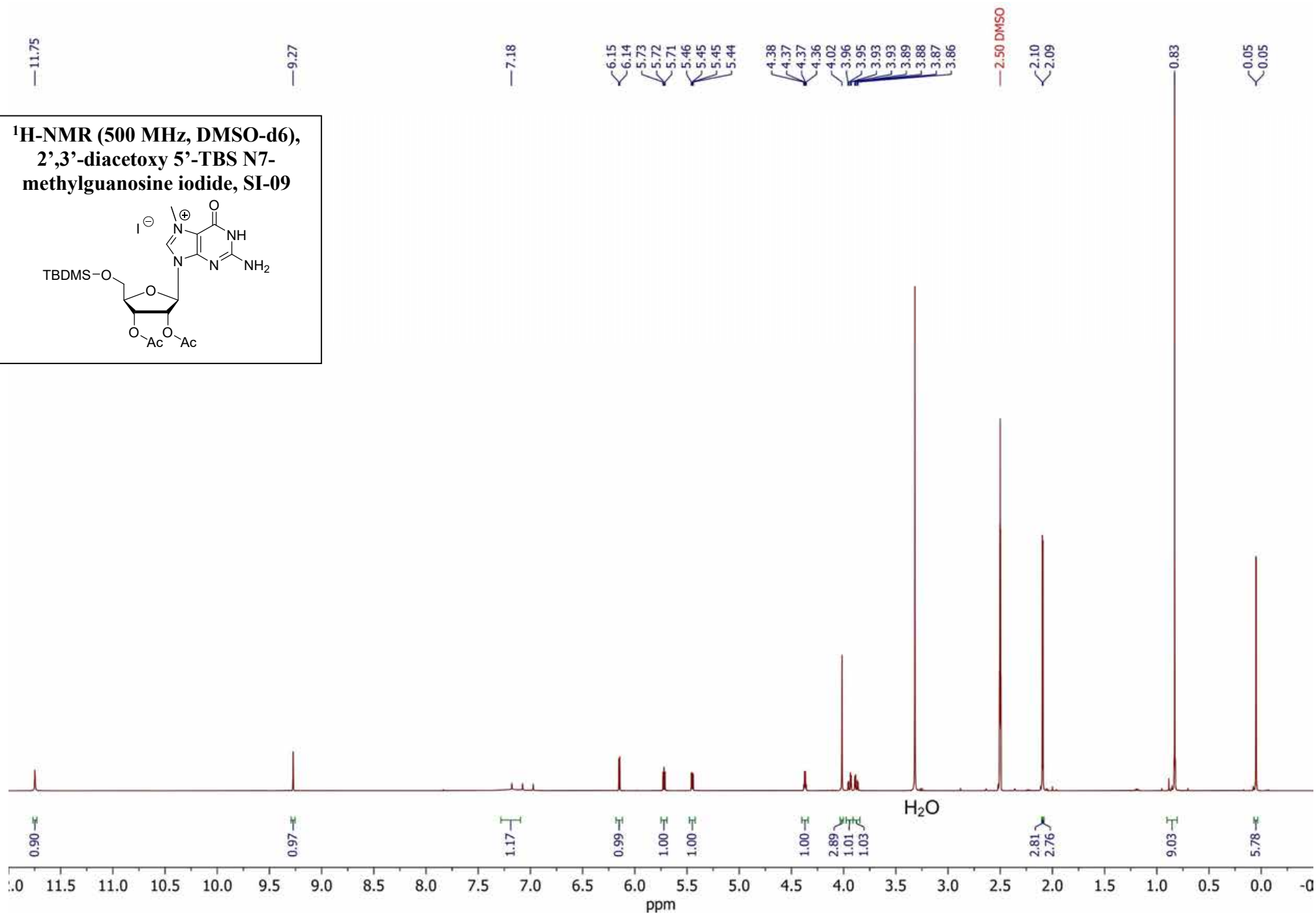
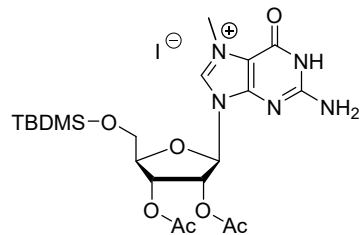
**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-diacetoxy guanosine, 18**



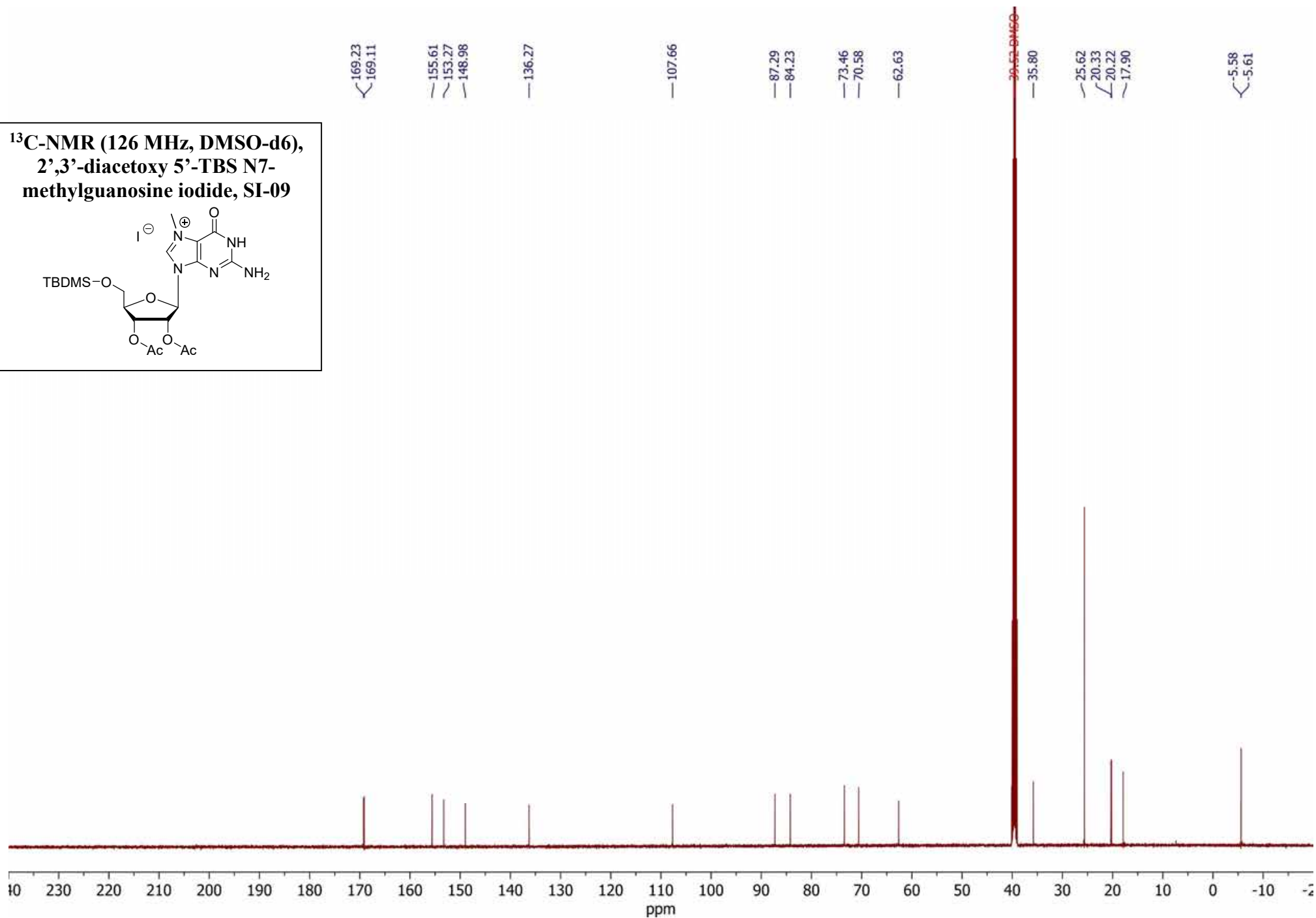
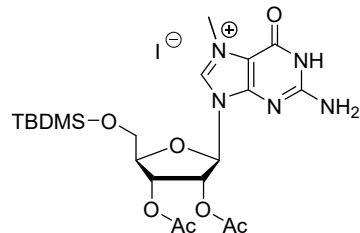
**^{13}C -NMR (101 MHz, DMSO- d_6),
2',3'-diacetoxy guanosine, 18**



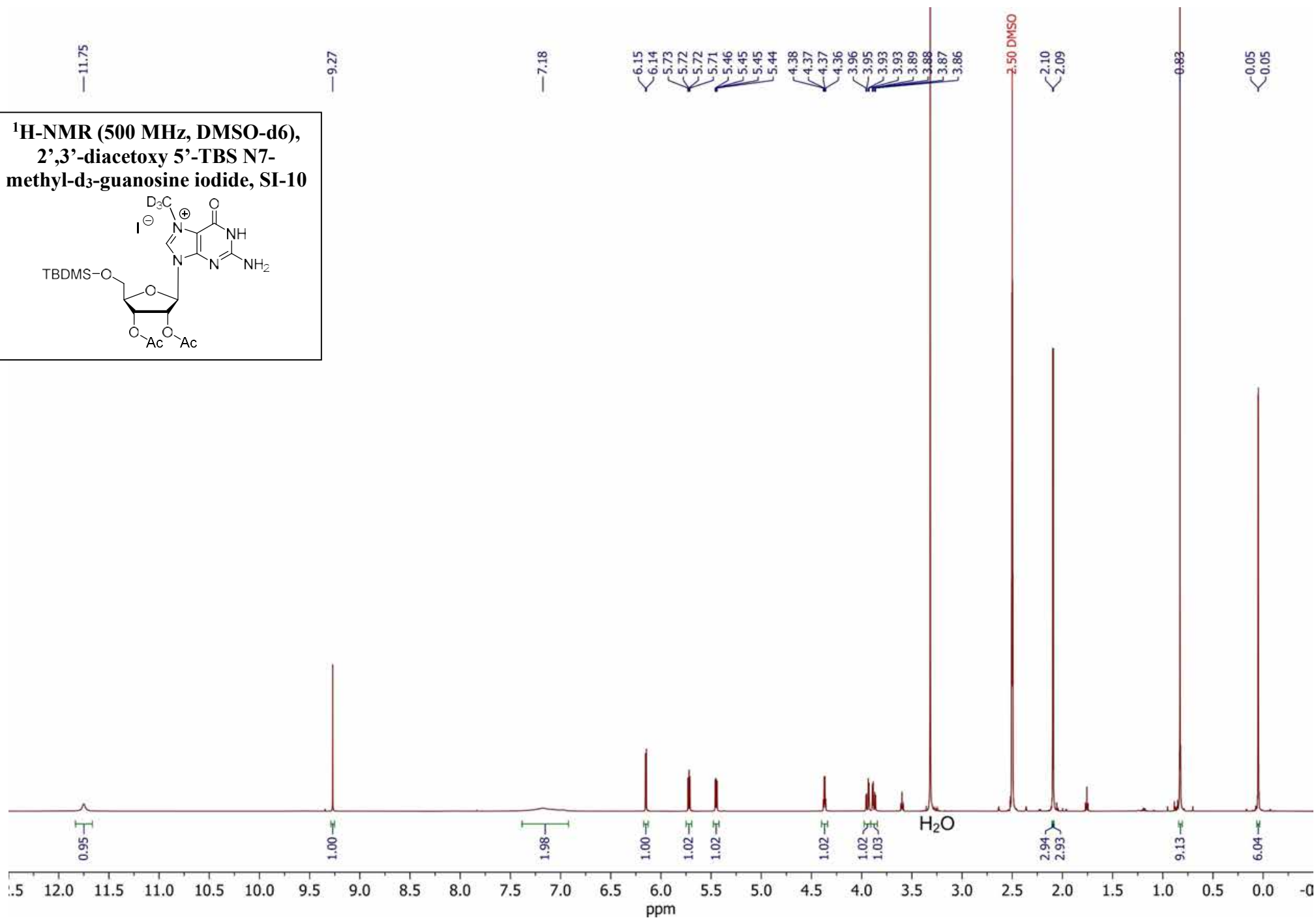
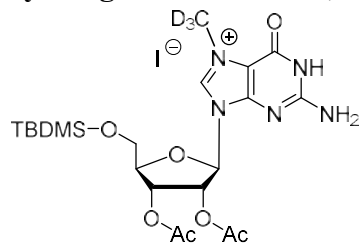
**¹H-NMR (500 MHz, DMSO-d₆),
2',3'-diacetoxy 5'-TBS N7-
methylguanosine iodide, SI-09**



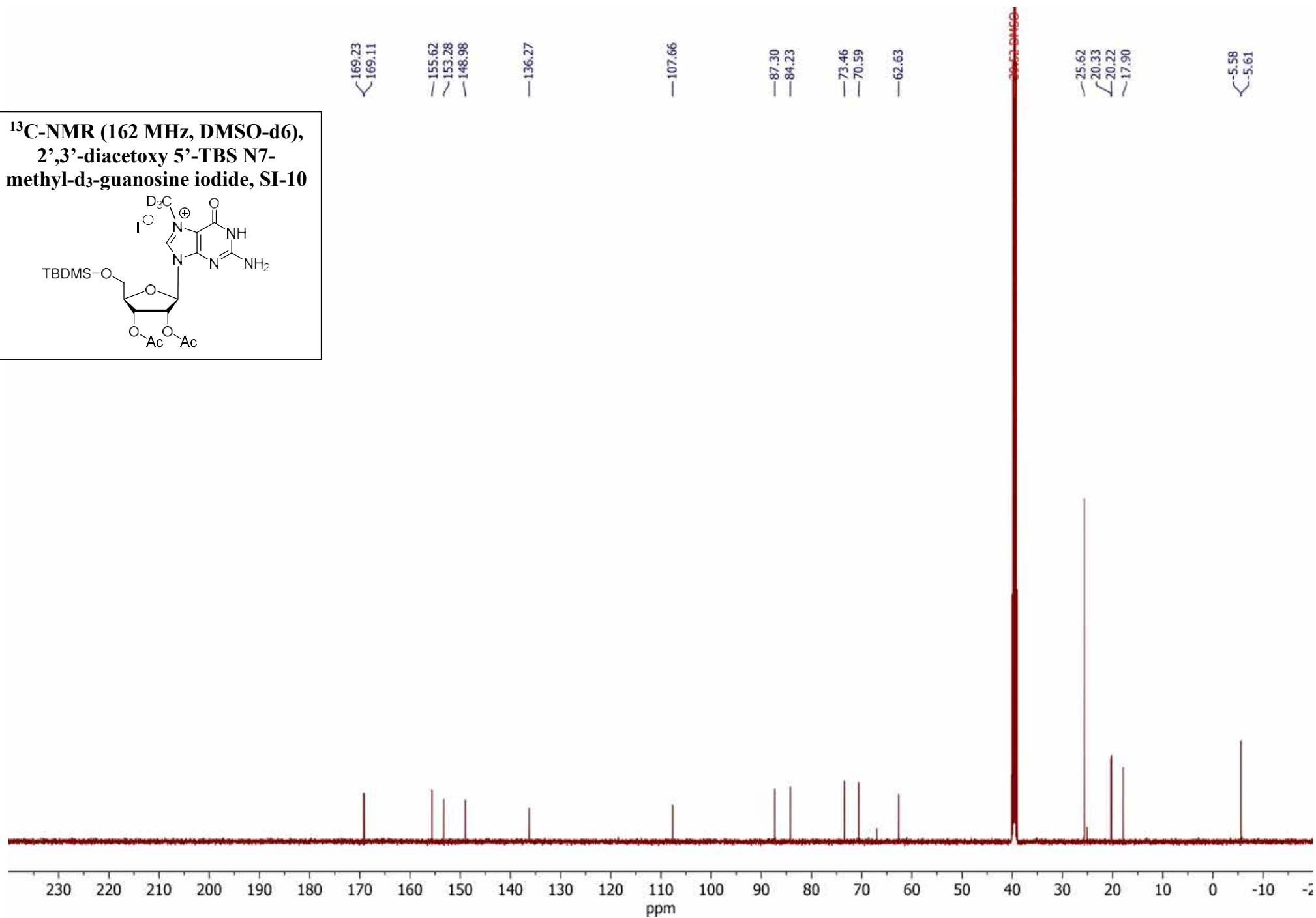
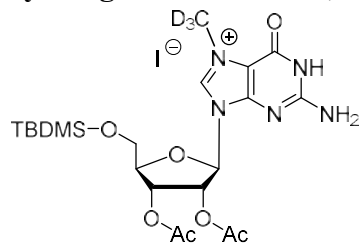
**^{13}C -NMR (126 MHz, DMSO- d_6),
2',3'-diacetoxy 5'-TBS N7-
methylguanosine iodide, SI-09**



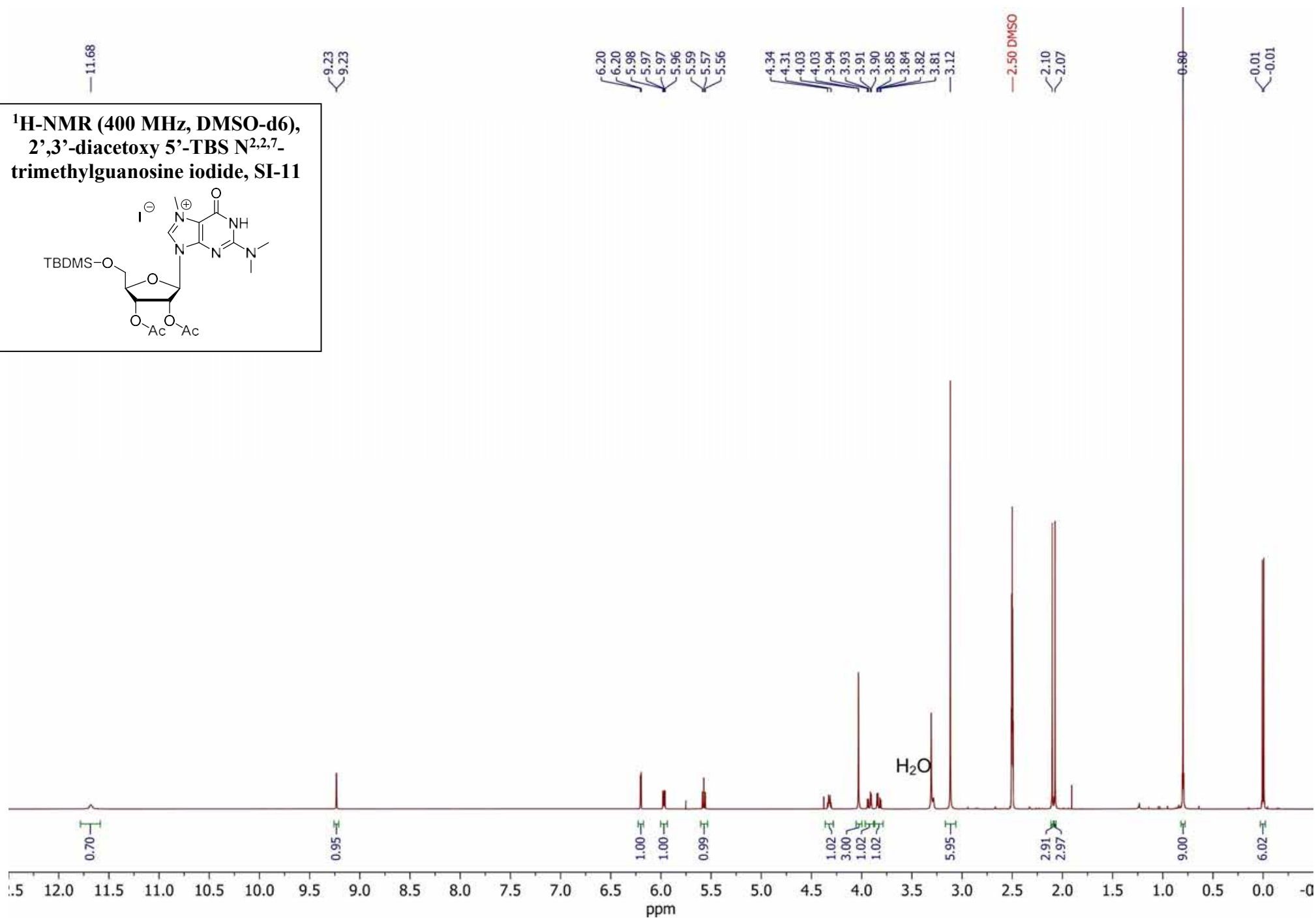
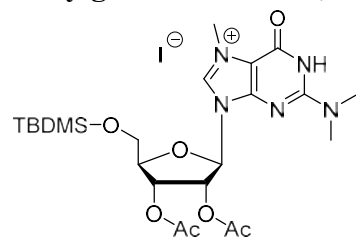
**¹H-NMR (500 MHz, DMSO-d₆),
2',3'-diacetoxy 5'-TBS N7-
methyl-d₃-guanosine iodide, SI-10**



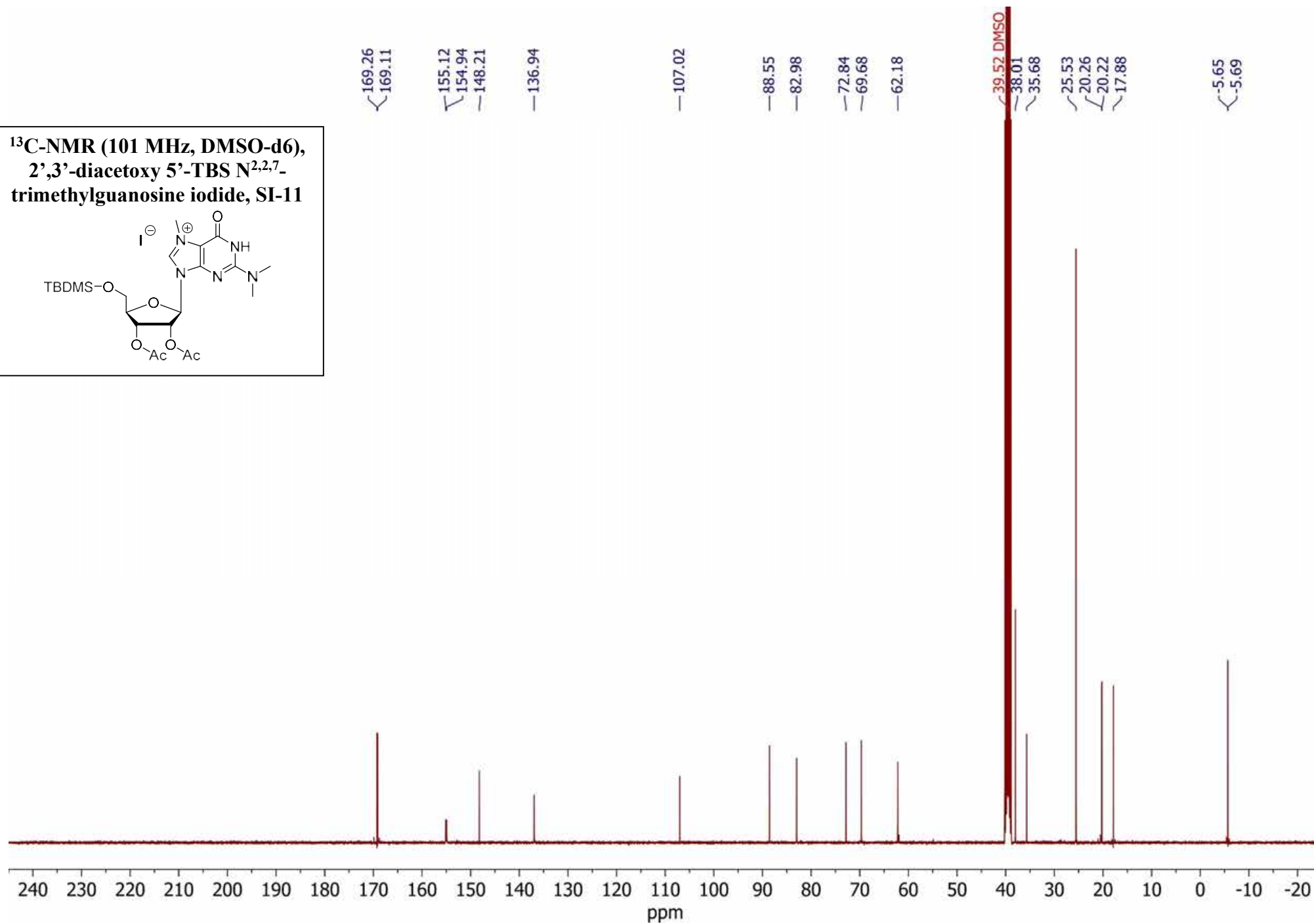
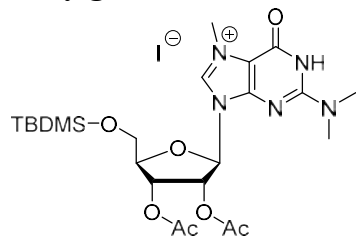
**^{13}C -NMR (162 MHz, DMSO- d_6),
2',3'-diacetoxy 5'-TBS N7-
methyl- d_3 -guanosine iodide, SI-10**

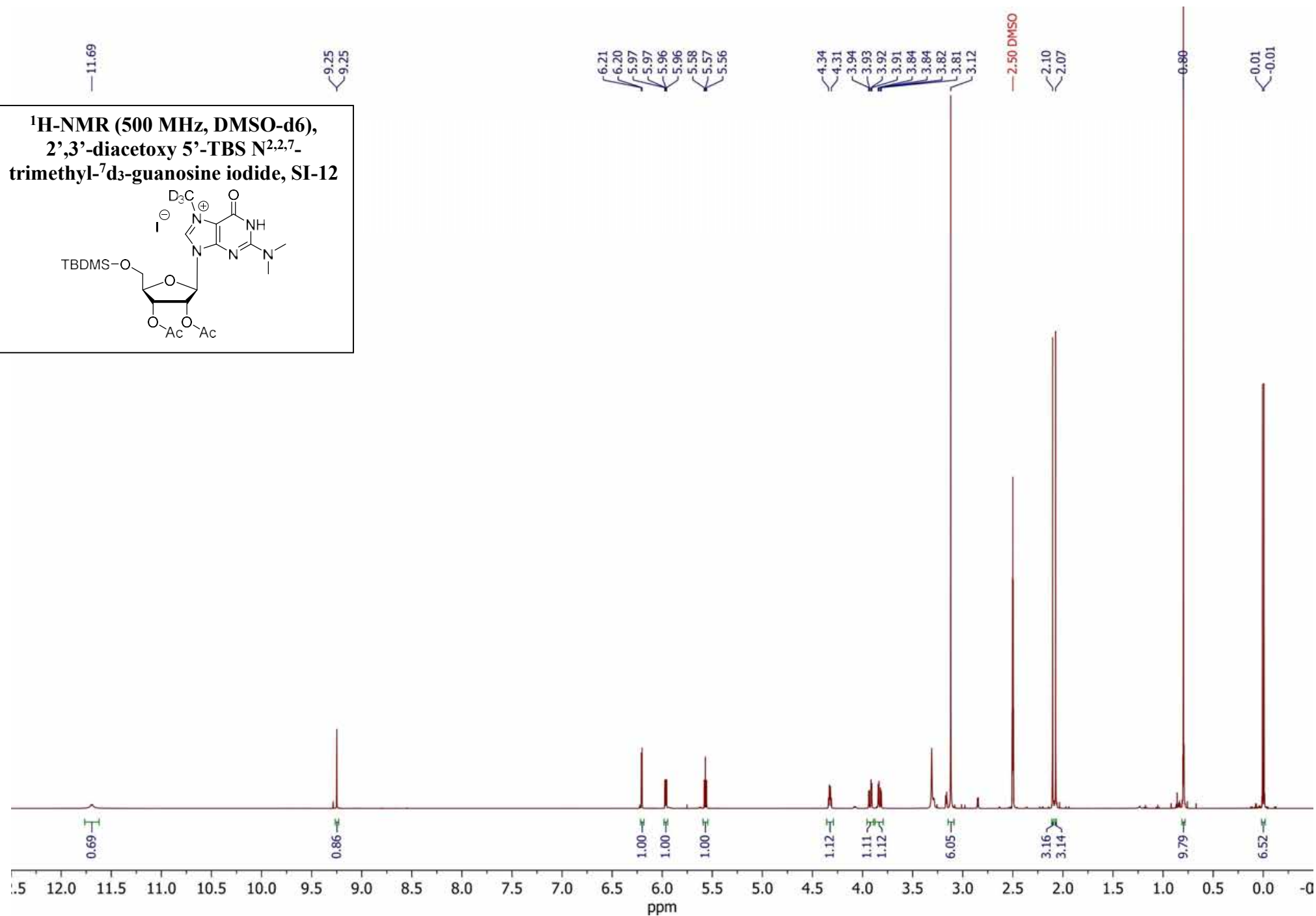


**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-diacetoxy 5'-TBS N^{2,2,7}-
trimethylguanosine iodide, SI-11**

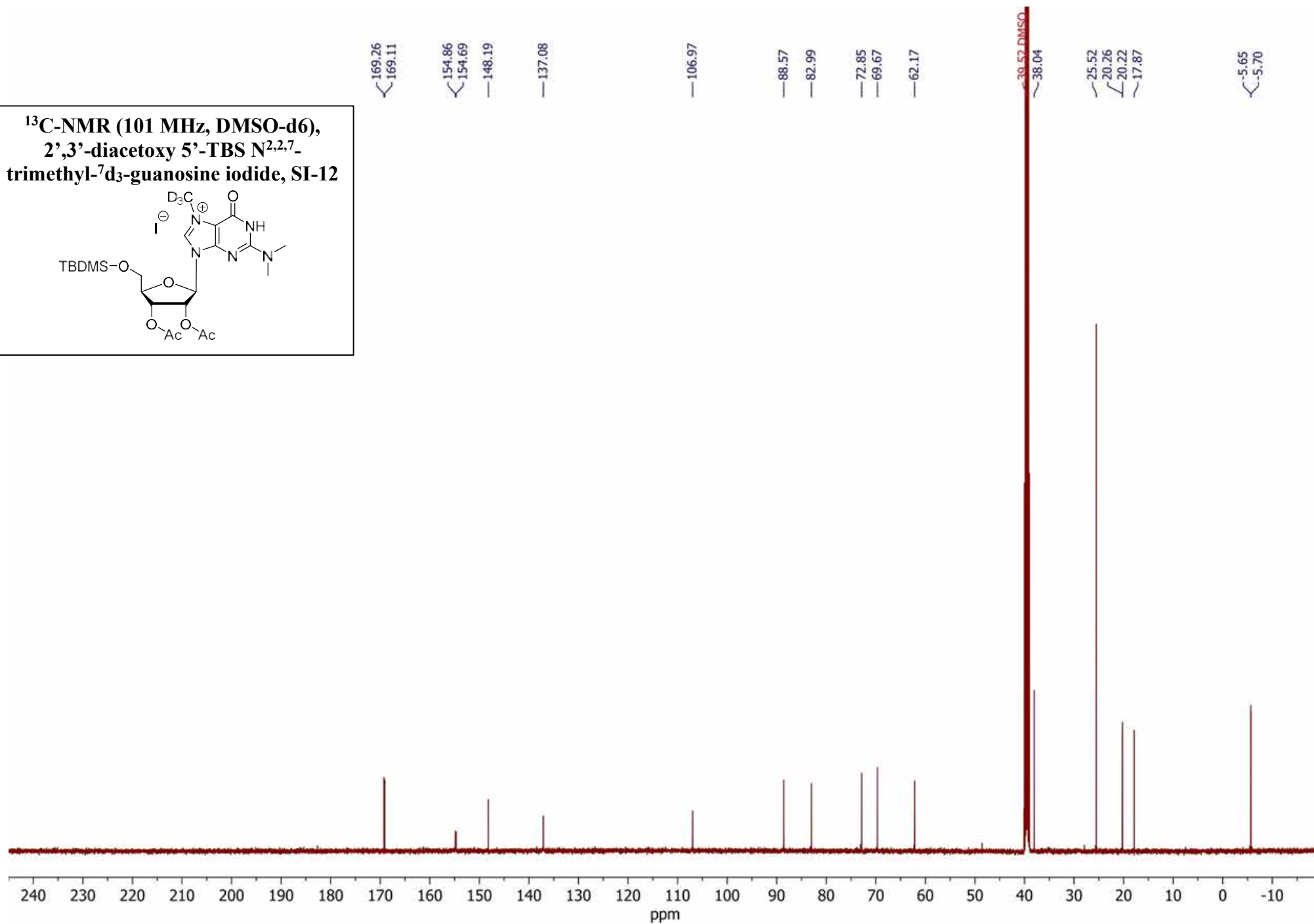
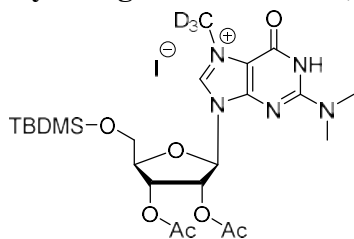


**^{13}C -NMR (101 MHz, DMSO- d_6),
2',3'-diacetoxy 5'-TBS N^{2,2,7}-
trimethylguanosine iodide, SI-11**

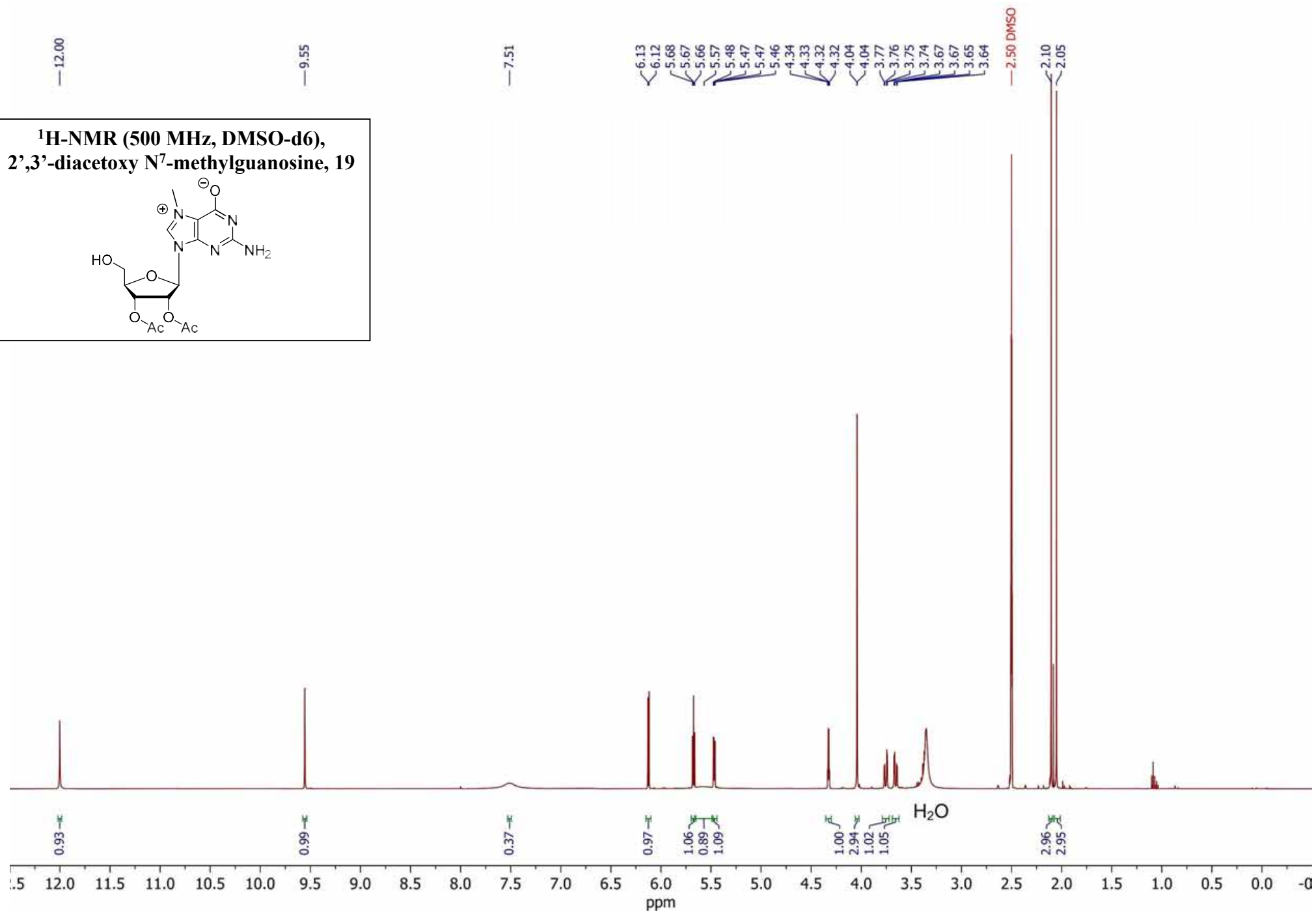
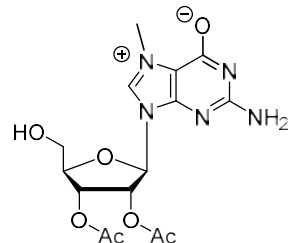




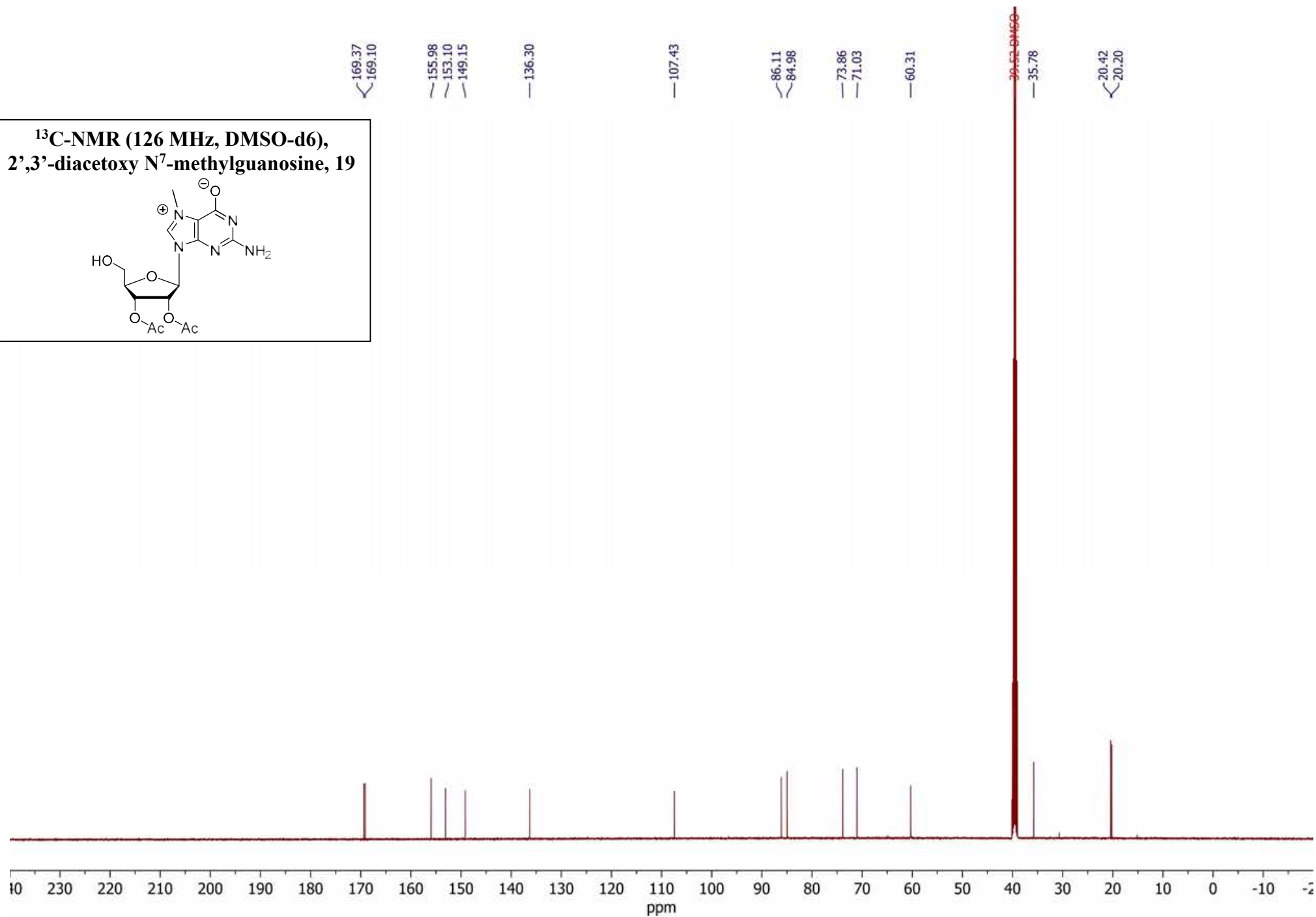
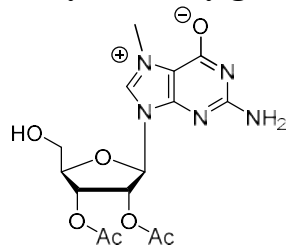
**^{13}C -NMR (101 MHz, DMSO- d_6),
2',3'-diacetoxy 5'-TBS N^{2,2,7}-
trimethyl- $^7\text{d}_3$ -guanosine iodide, SI-12**

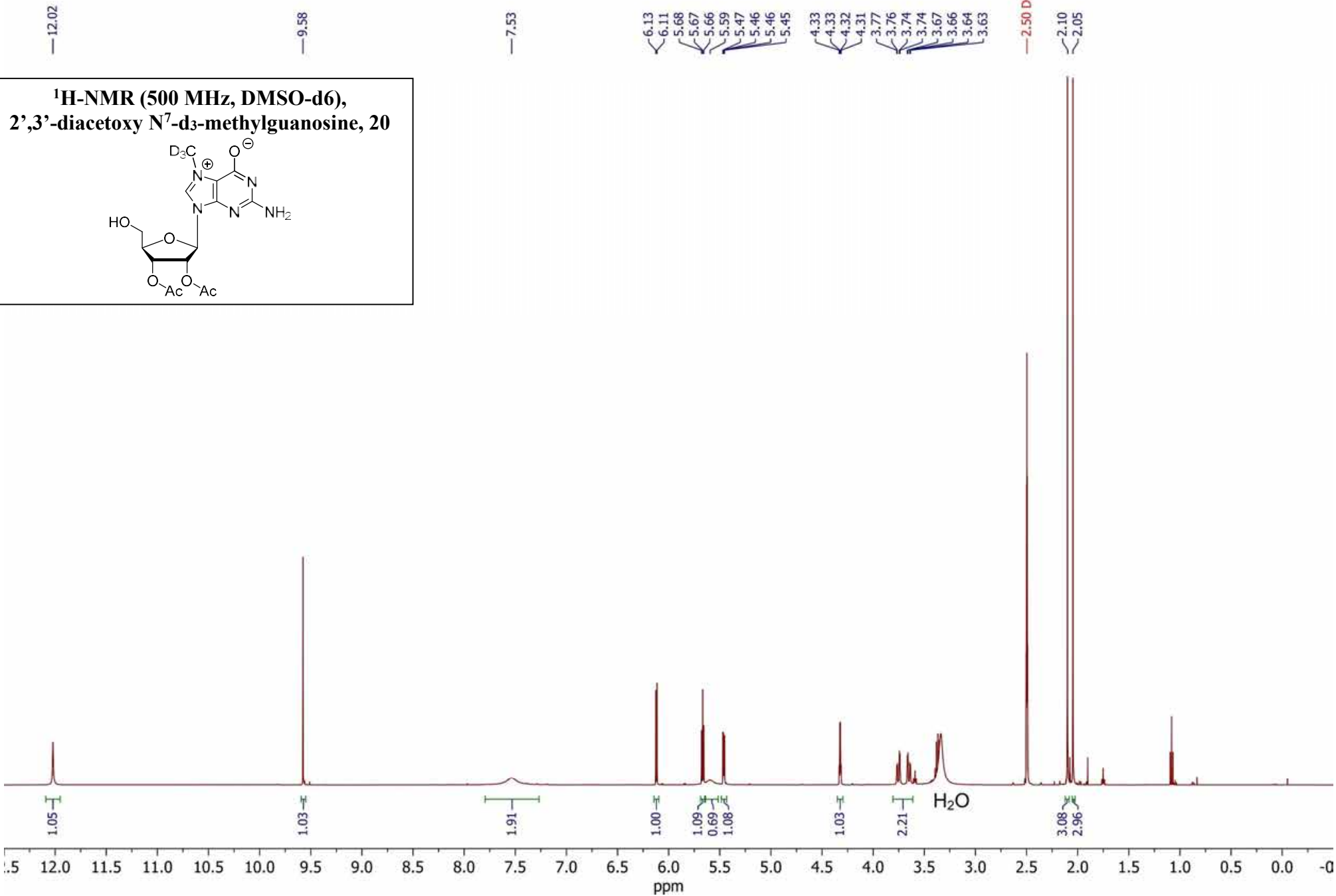


**¹H-NMR (500 MHz, DMSO-d₆),
2',3'-diacetoxy N⁷-methylguanosine, 19**

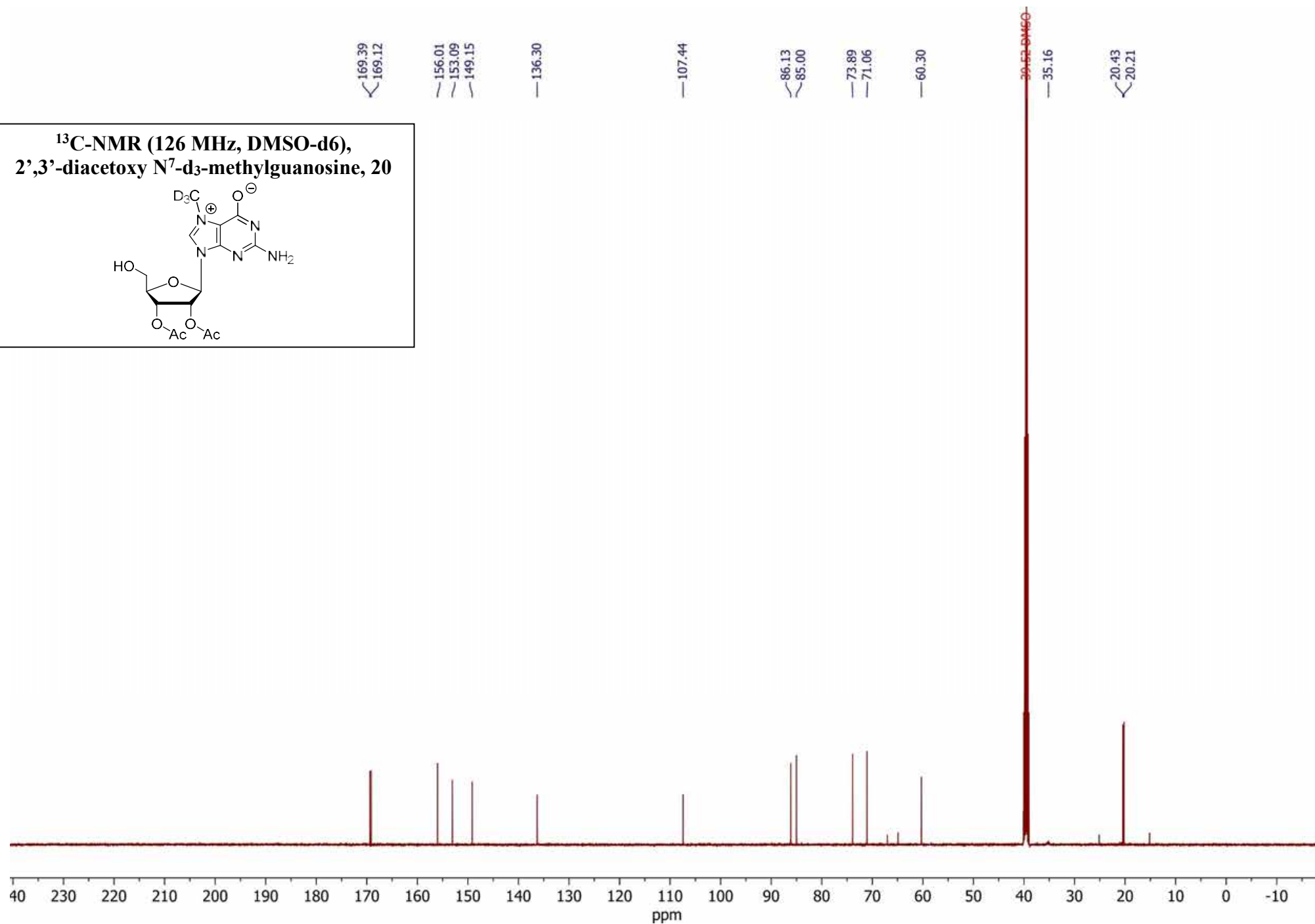
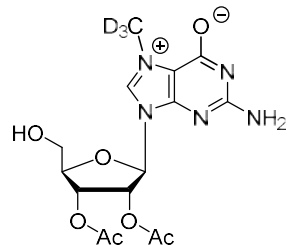


**^{13}C -NMR (126 MHz, DMSO- d_6),
2',3'-diacetoxy N⁷-methylguanosine, 19**

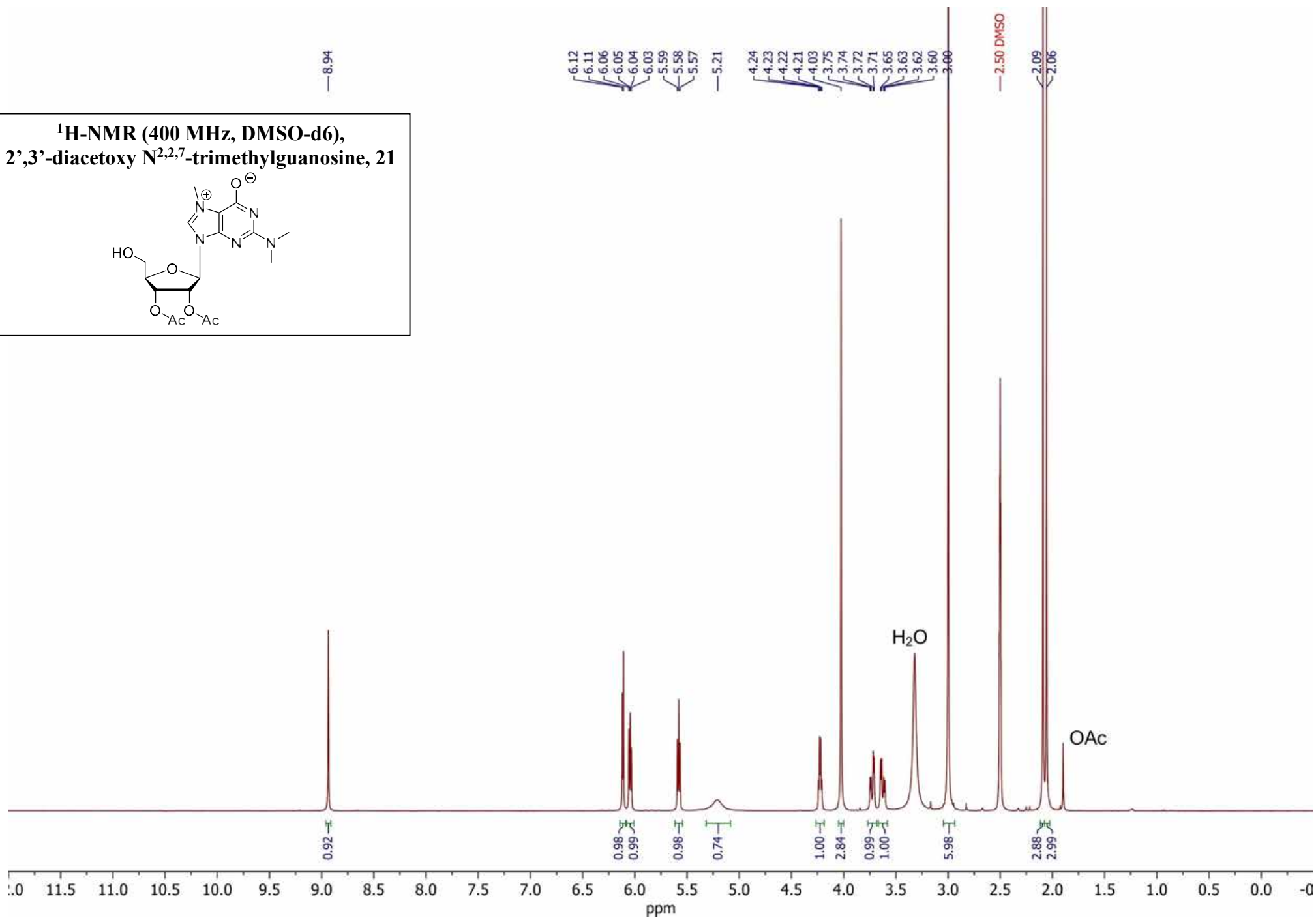
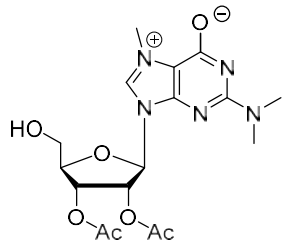




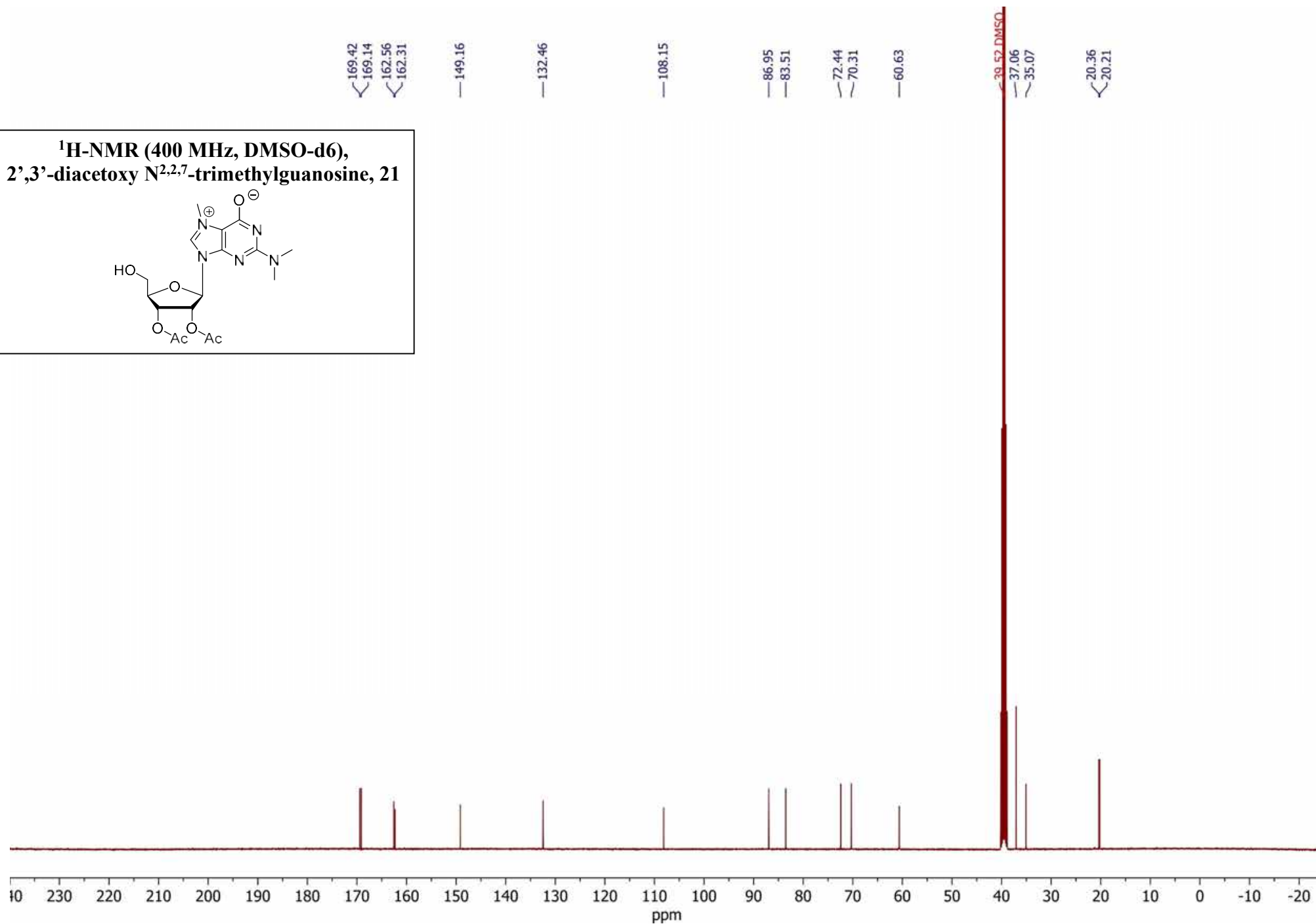
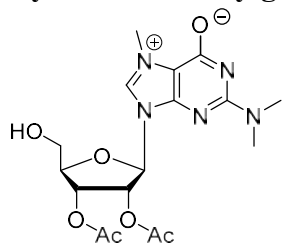
**^{13}C -NMR (126 MHz, DMSO- d_6),
2',3'-diacetoxy N^7 - d_3 -methylguanosine, 20**



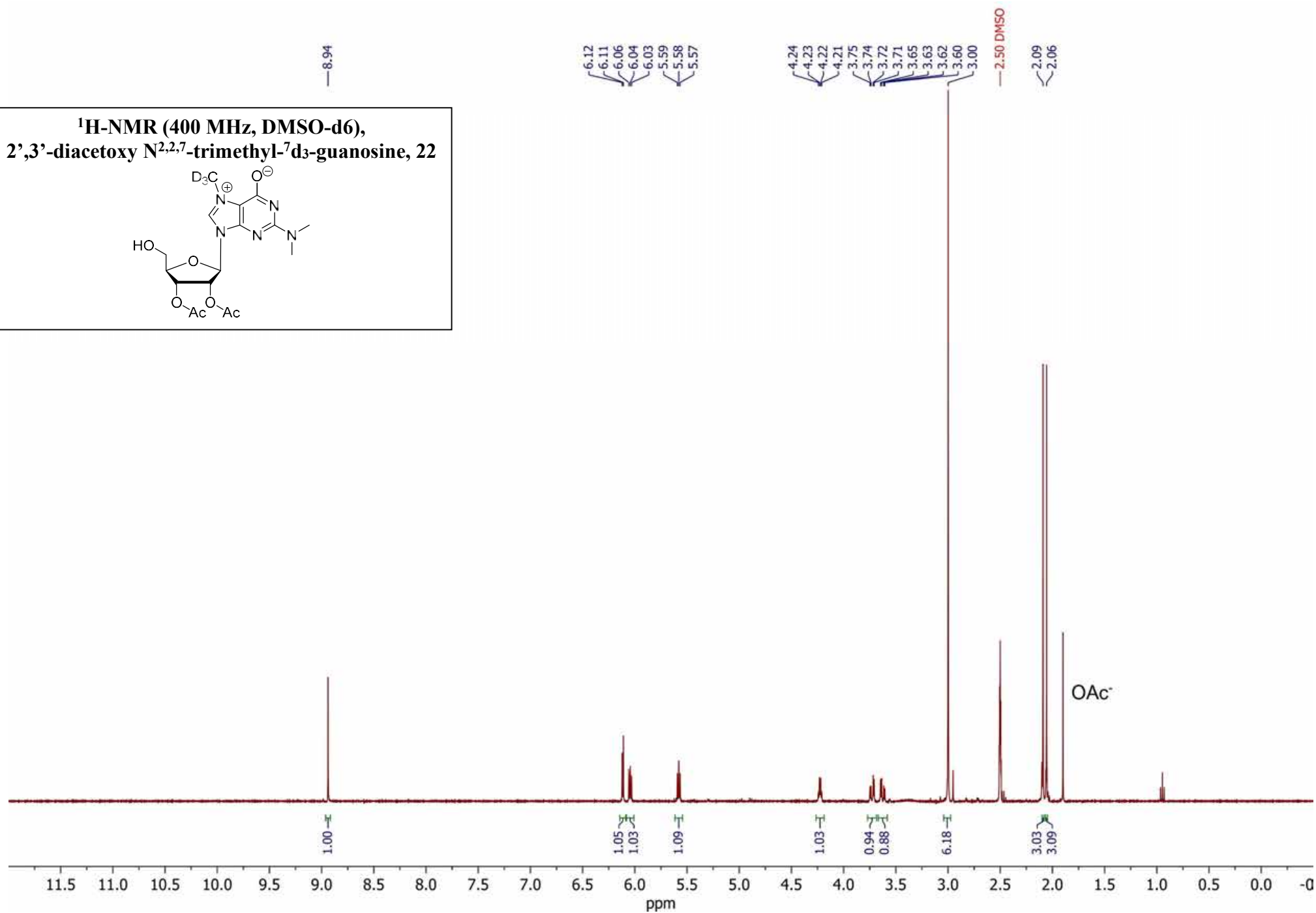
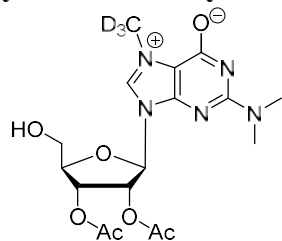
**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-diacetoxy N^{2,2,7}-trimethylguanosine, 21**



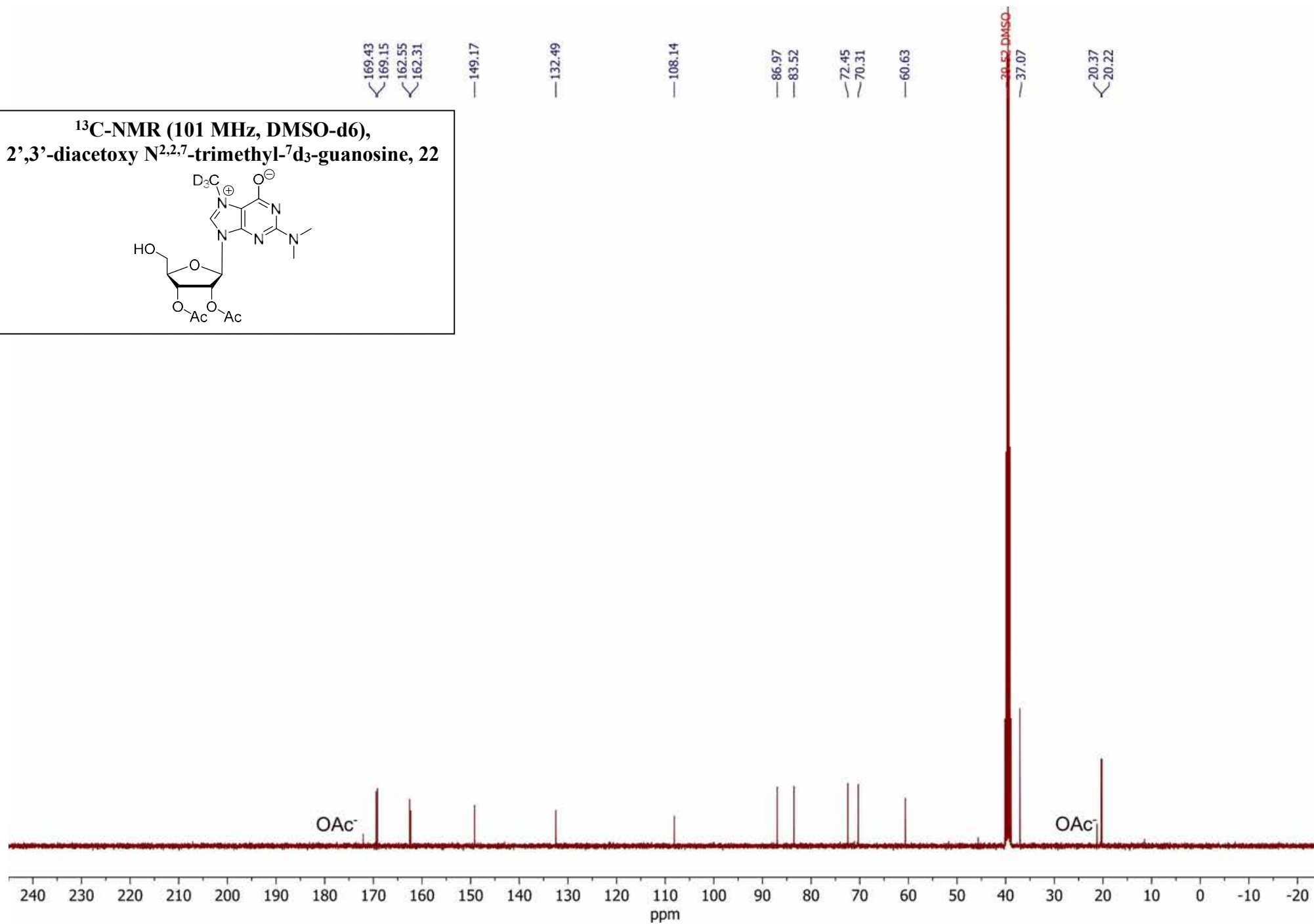
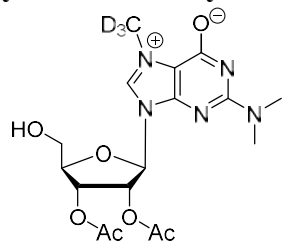
**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-diacetoxy N^{2,2,7}-trimethylguanosine, 21**



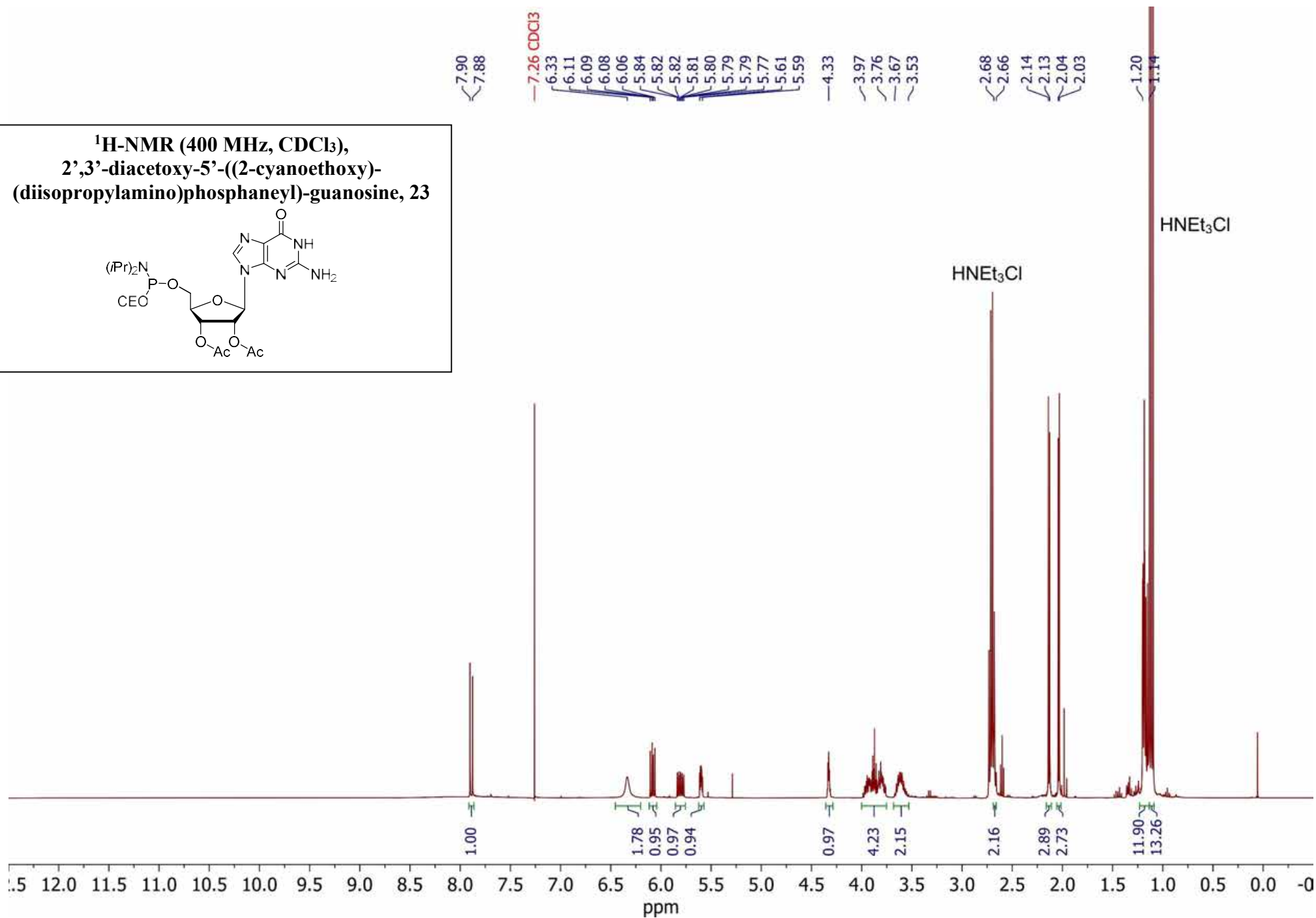
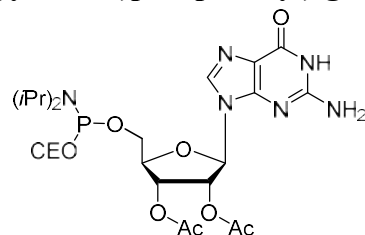
**¹H-NMR (400 MHz, DMSO-d₆),
2',3'-diacetoxy N^{2,2,7}-trimethyl-⁷d₃-guanosine, 22**



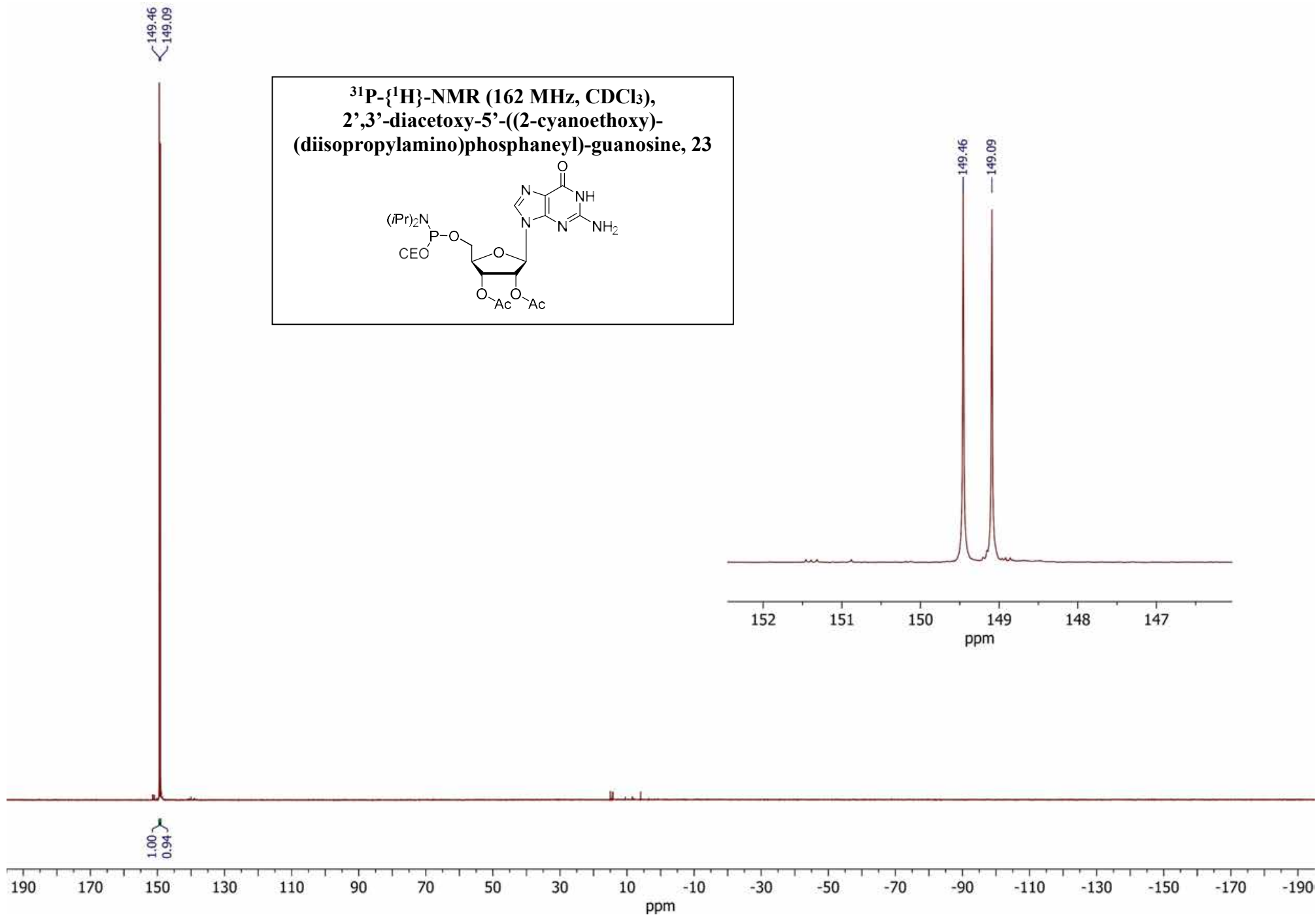
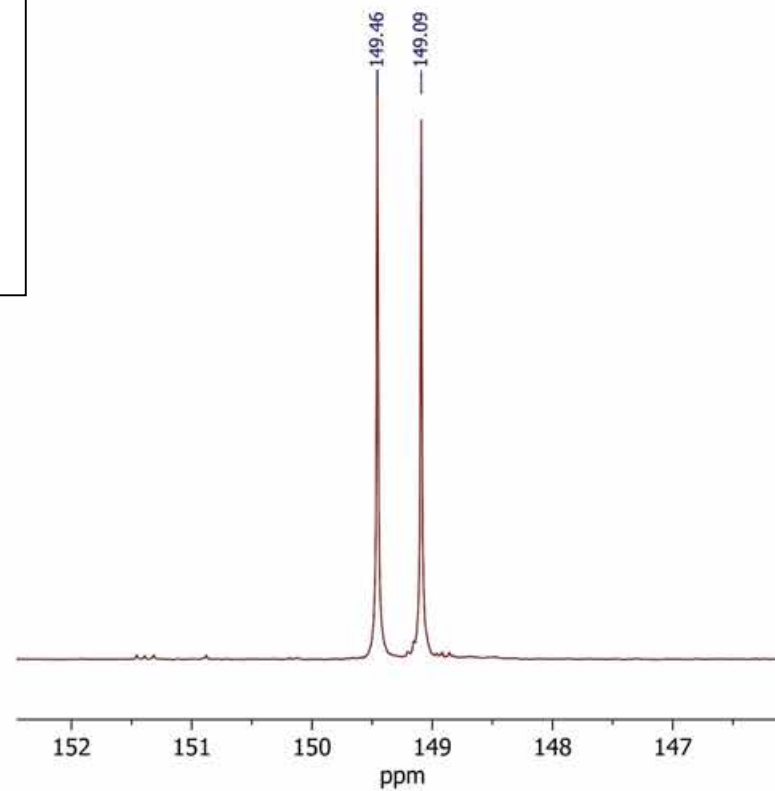
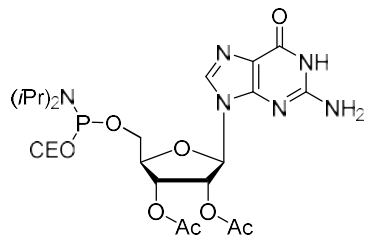
**^{13}C -NMR (101 MHz, DMSO- d_6),
2',3'-diacetoxy N^{2,2,7}-trimethyl- $^7\text{d}_3$ -guanosine, 22**



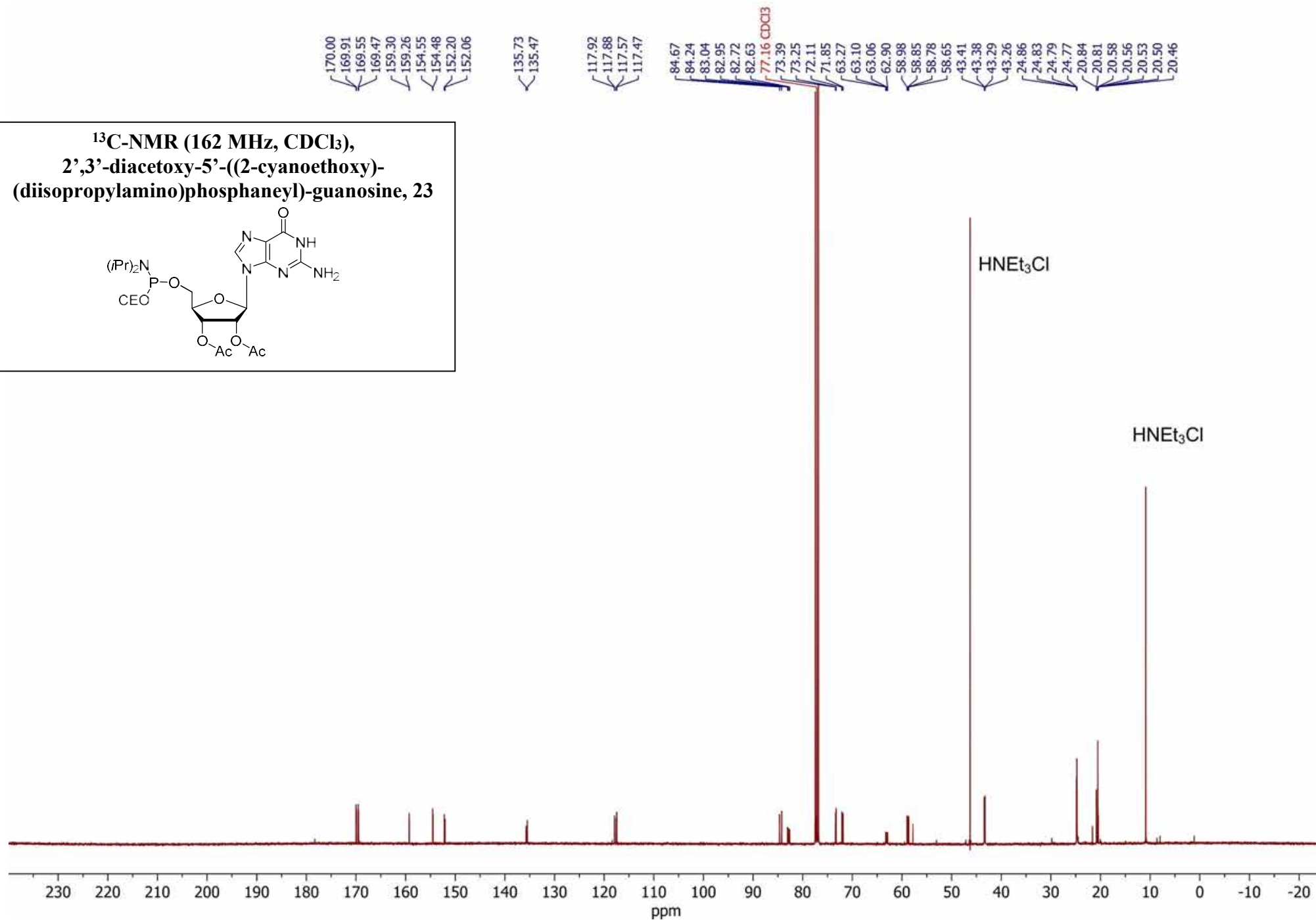
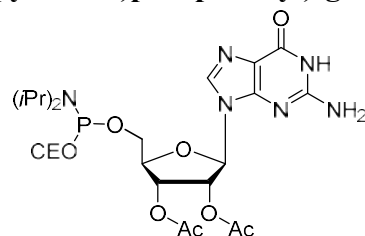
**¹H-NMR (400 MHz, CDCl₃),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphaneyl)-guanosine, 23**



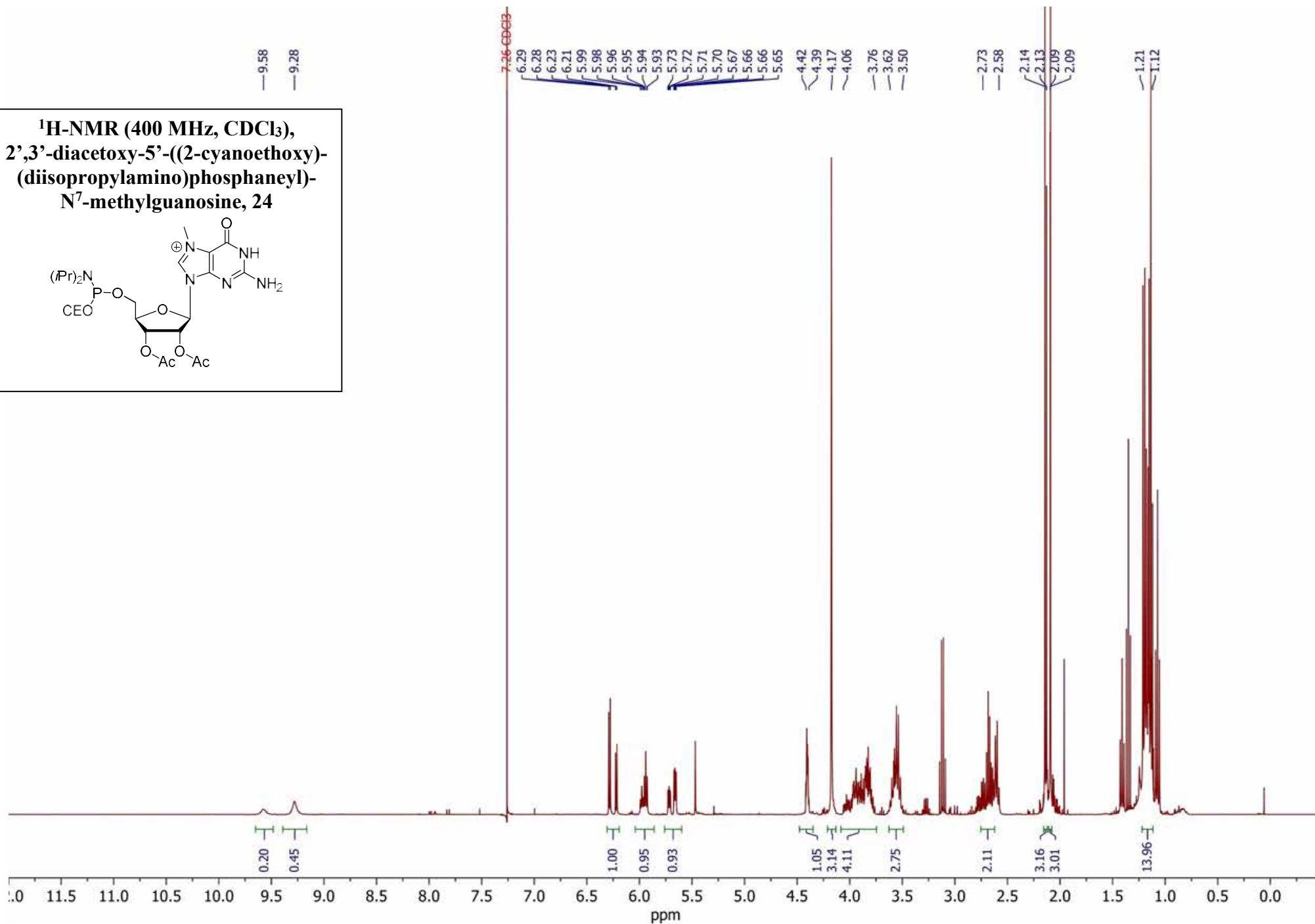
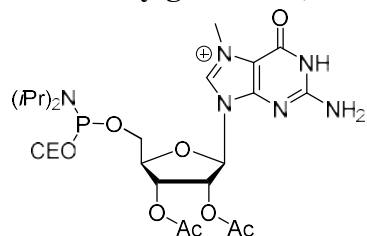
**^{31}P - $\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphanyl)-guanosine, 23**



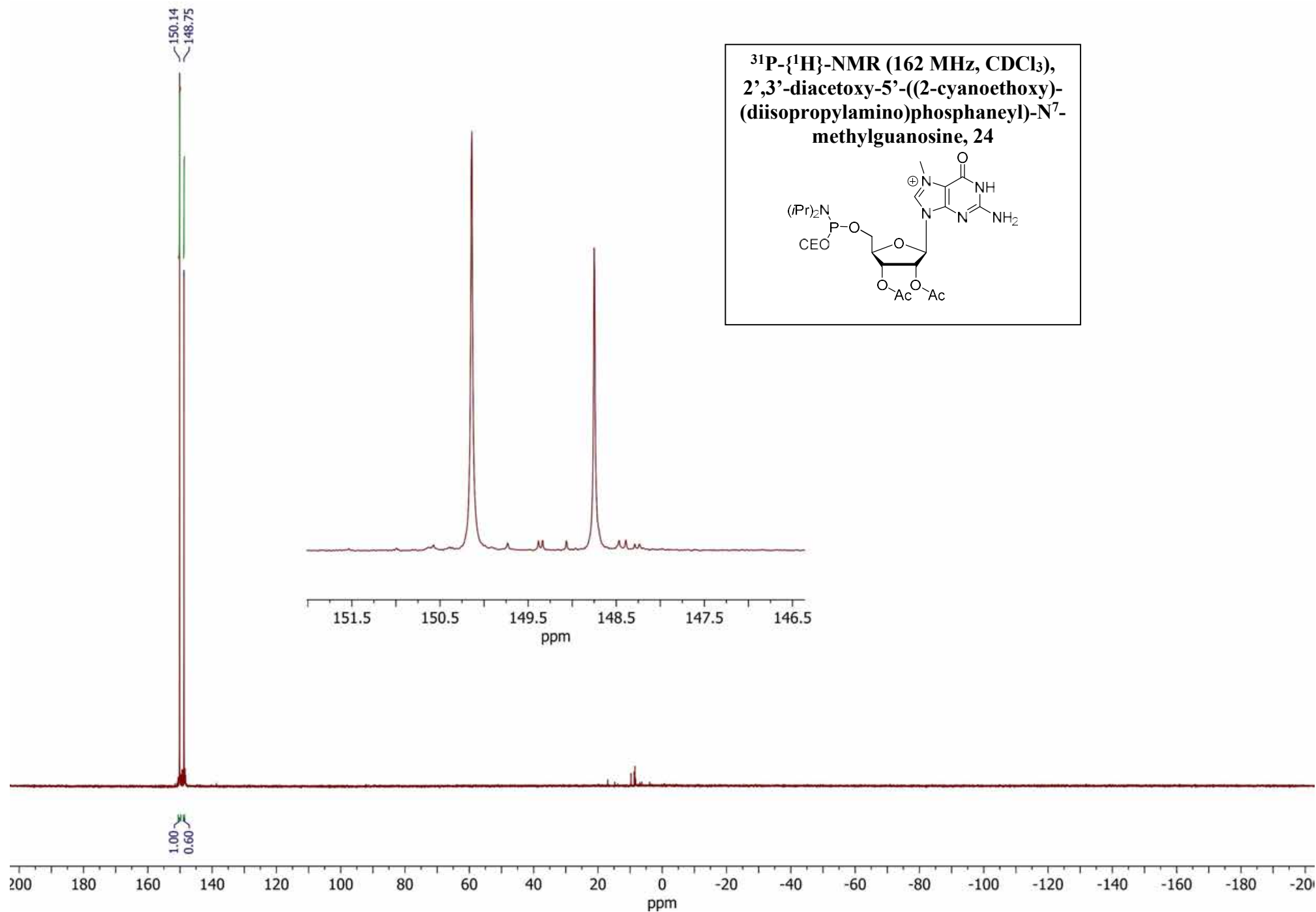
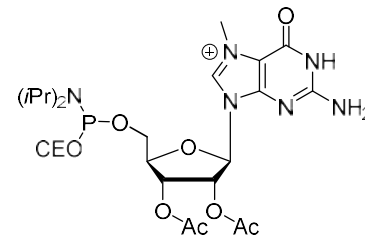
**^{13}C -NMR (162 MHz, CDCl_3),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphaneyl)-guanosine, 23**



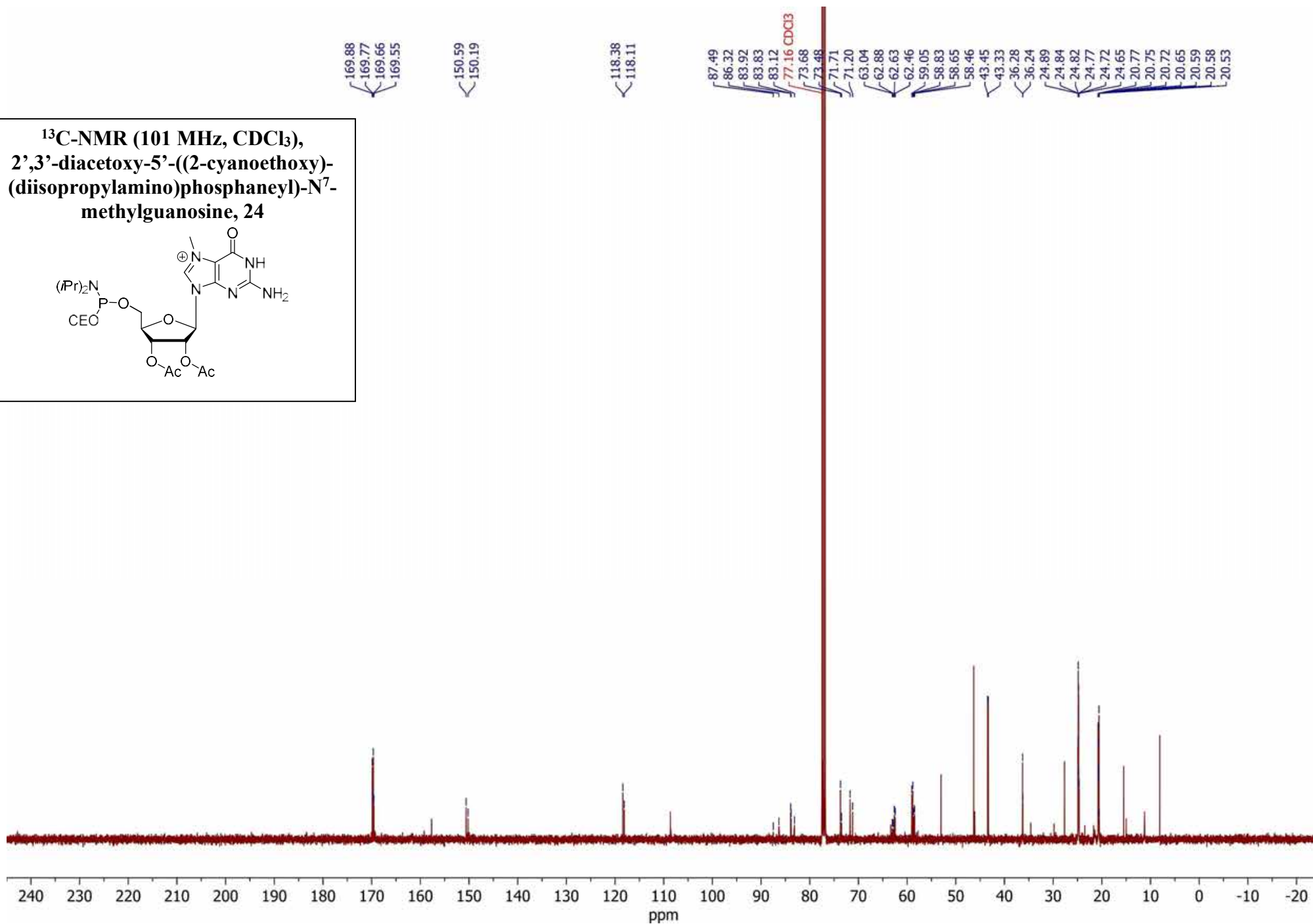
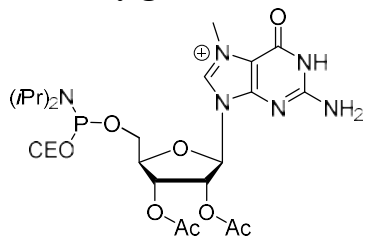
**¹H-NMR (400 MHz, CDCl₃),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphanyl)-
N⁷-methylguanosine, 24**



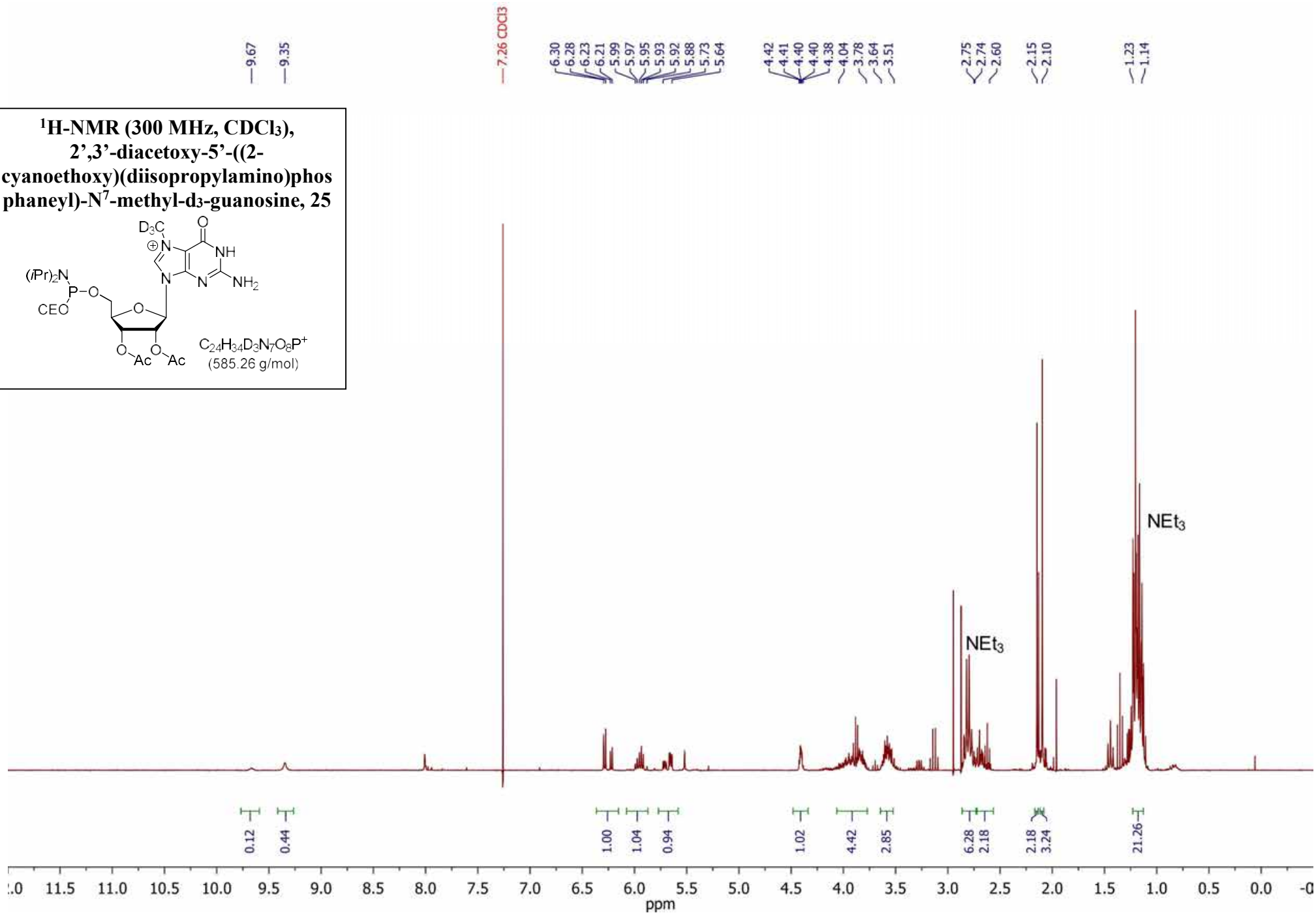
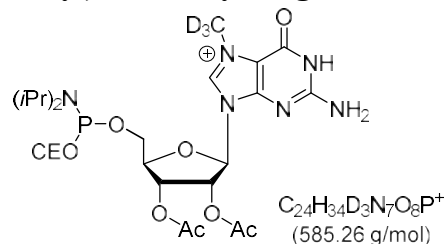
**^{31}P - $\{^1\text{H}\}$ -NMR (162 MHz, CDCl_3),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphaneyl)-N⁷-
methylguanosine, 24**

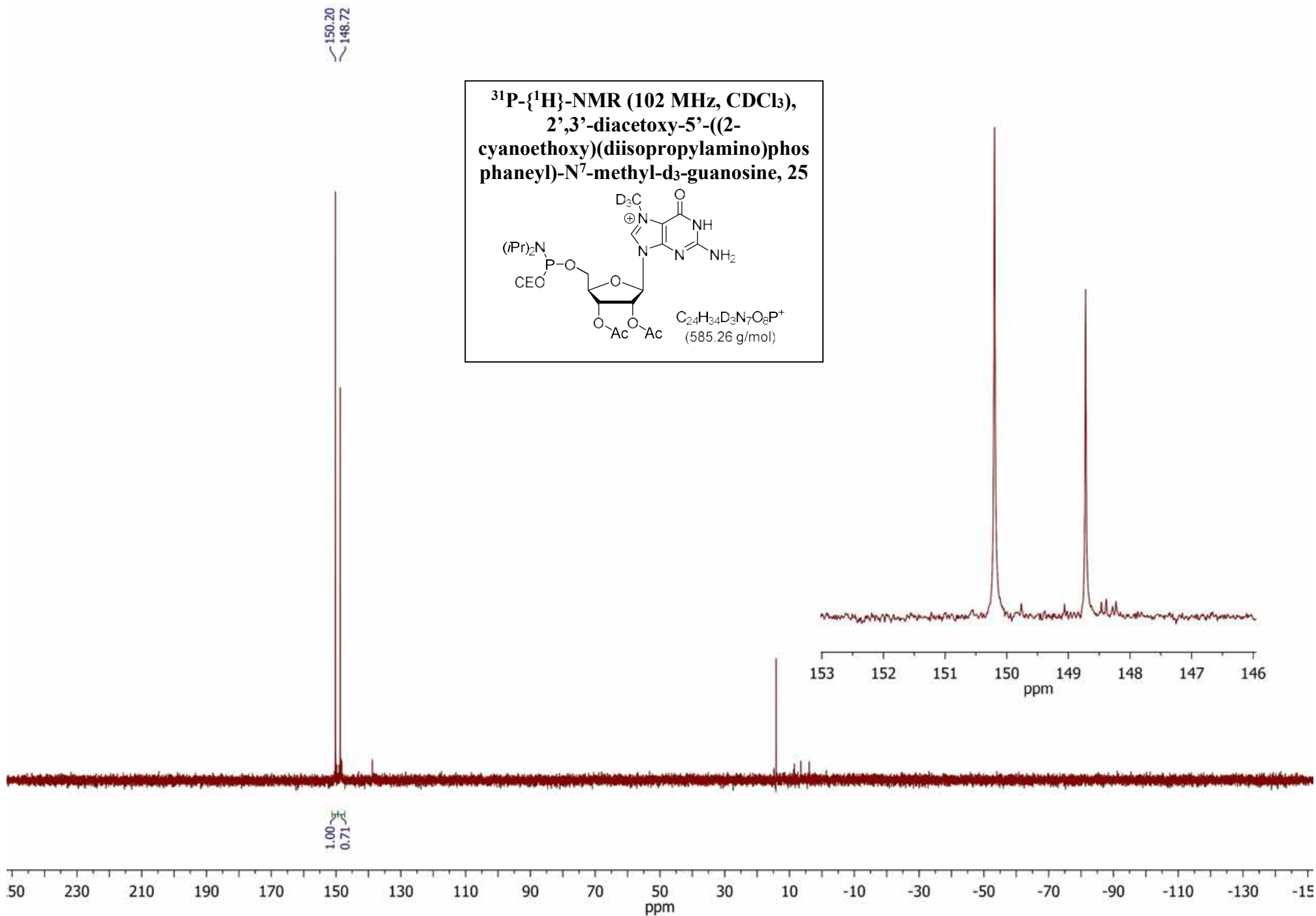


**^{13}C -NMR (101 MHz, CDCl_3),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphaneyl)-N⁷-
methylguanosine, 24**

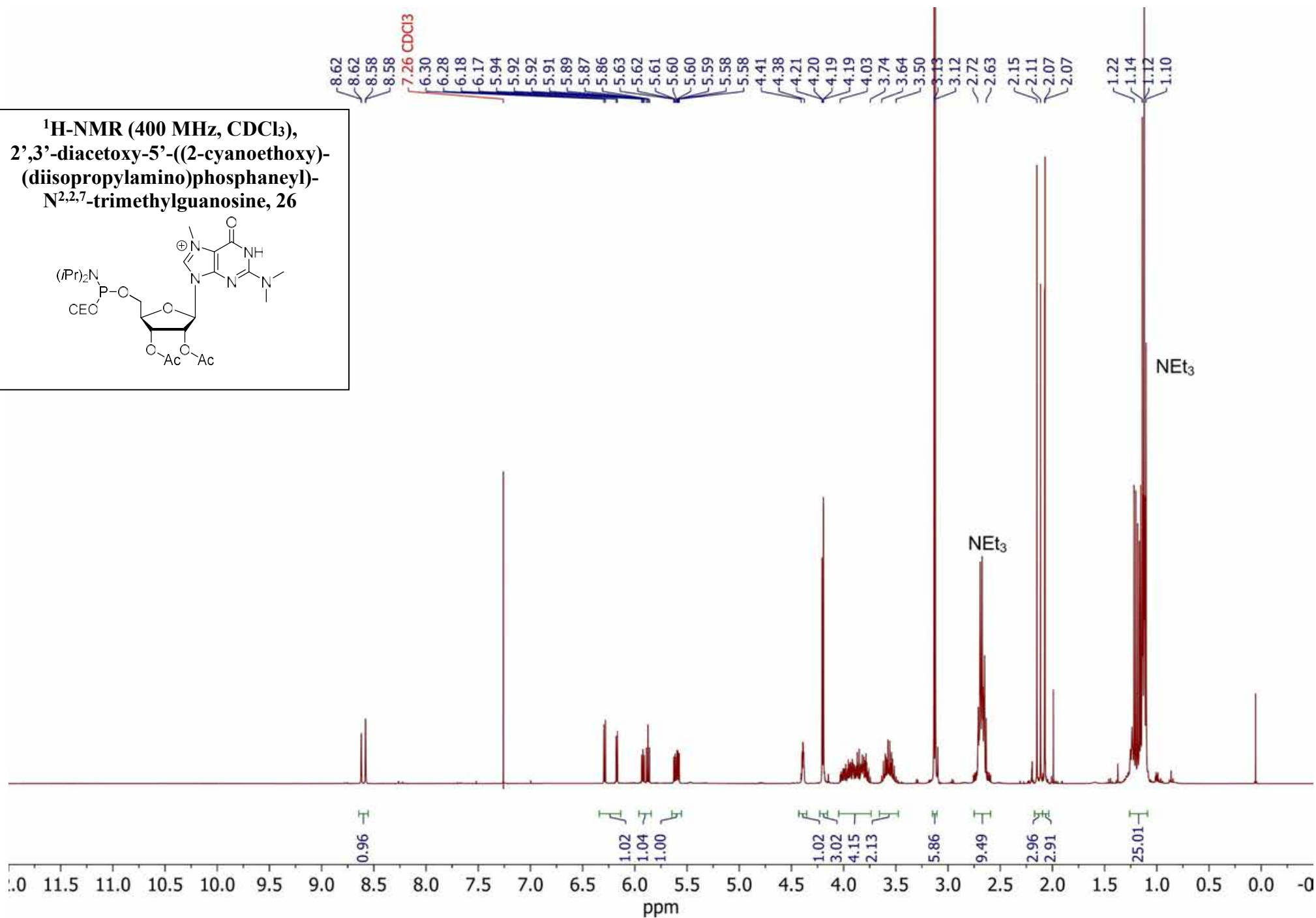
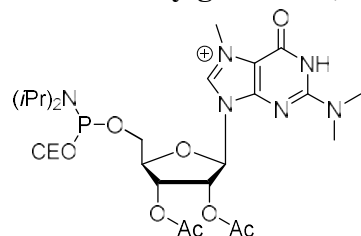


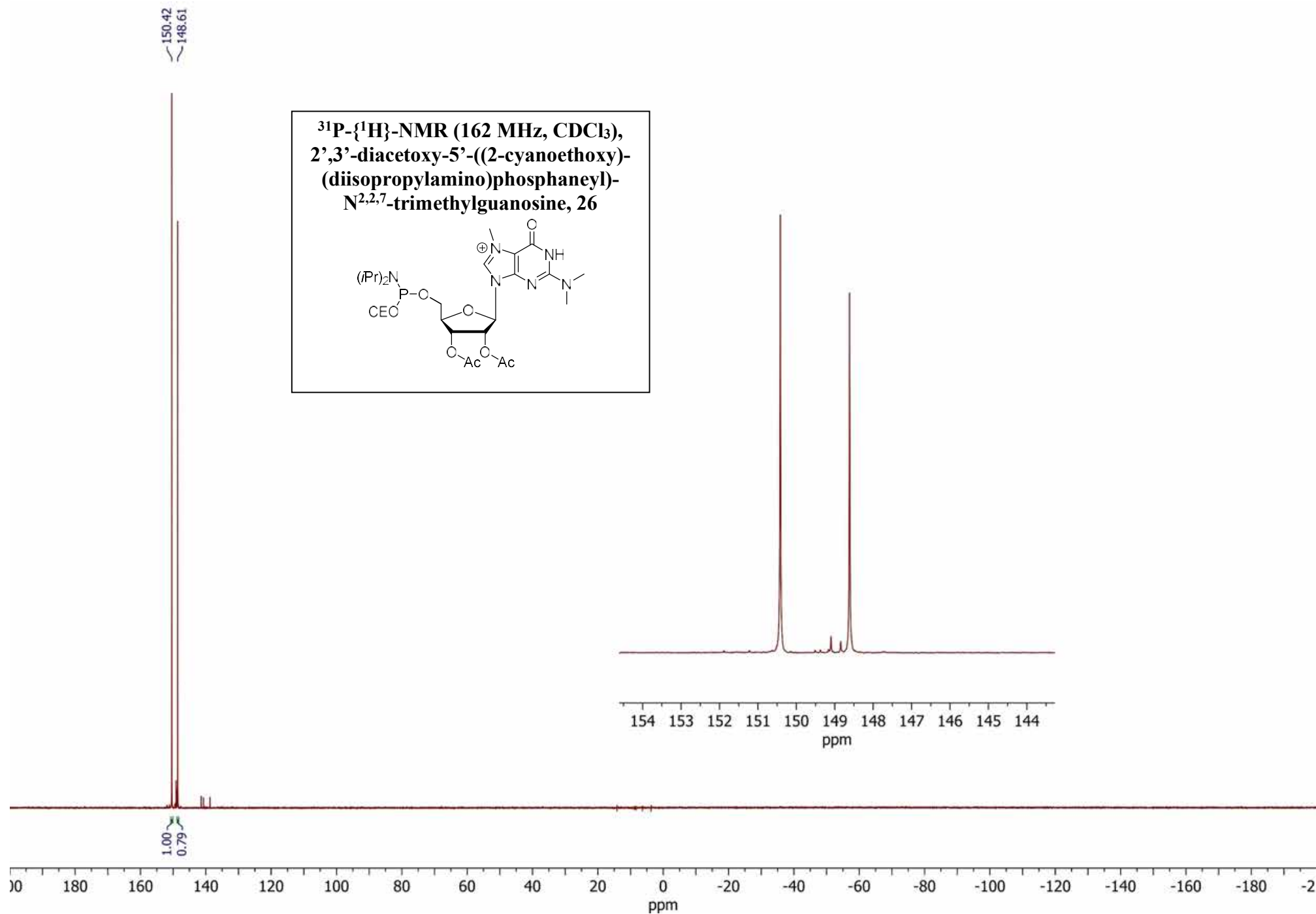
**¹H-NMR (300 MHz, CDCl₃),
2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phos-
phaneyl)-N⁷-methyl-d₃-guanosine, 25**



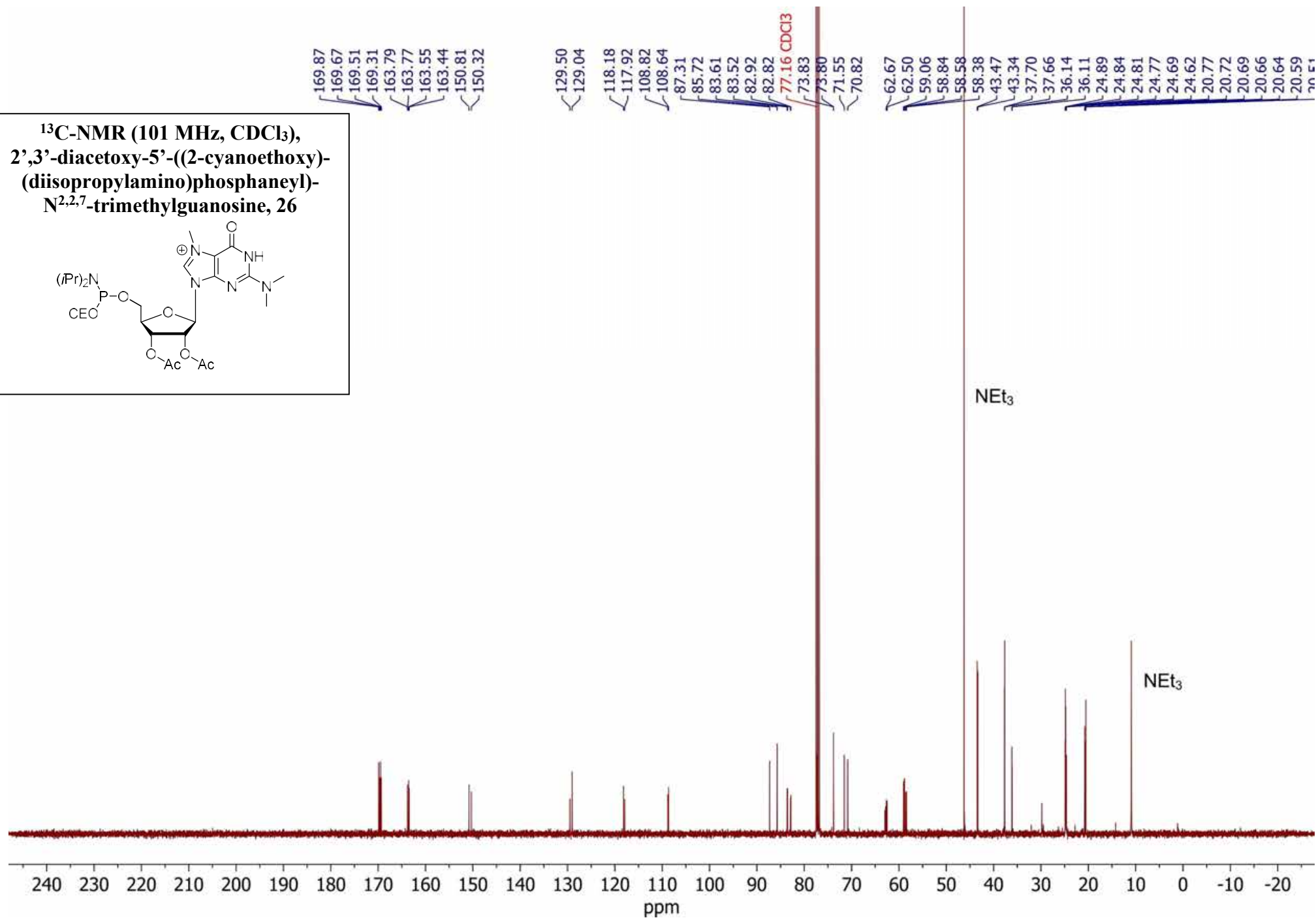
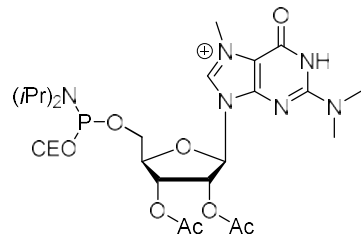


**¹H-NMR (400 MHz, CDCl₃),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphaneyl)-
N^{2,2,7}-trimethylguanosine, 26**

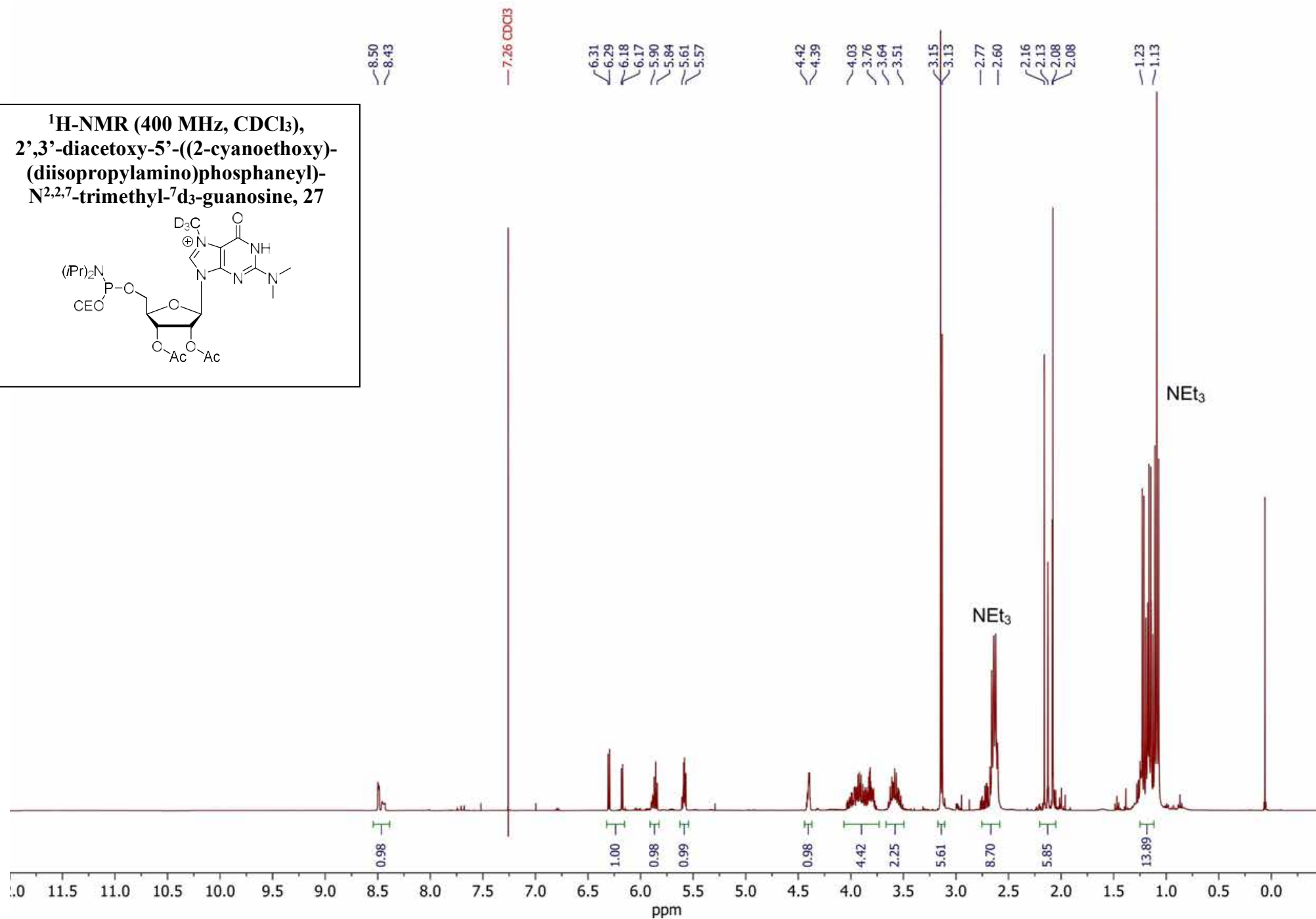
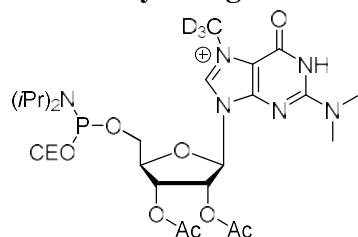


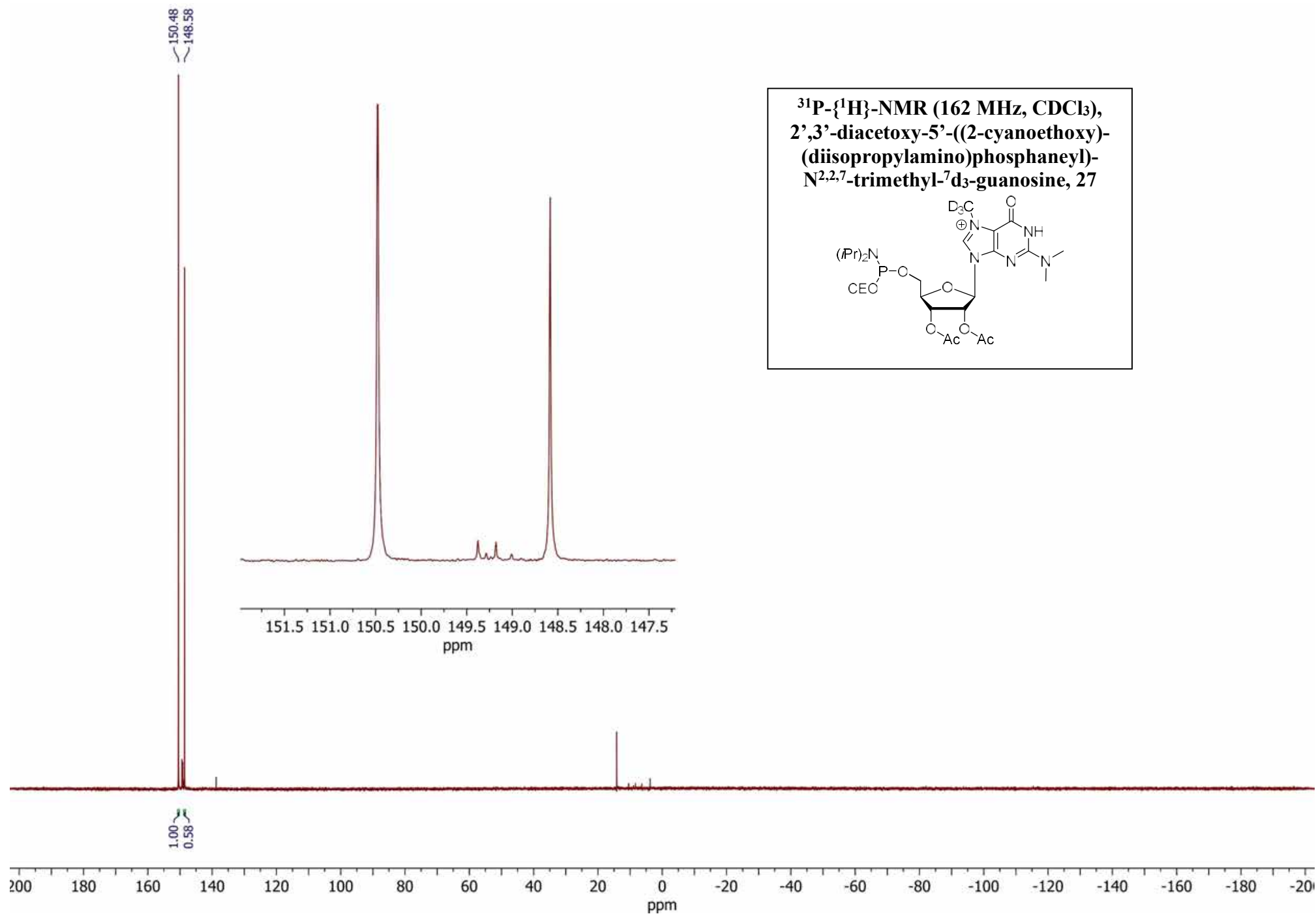


**^{13}C -NMR (101 MHz, CDCl_3),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphaneyl)-
 $\text{N}^{2,2,7}$ -trimethylguanosine, 26**

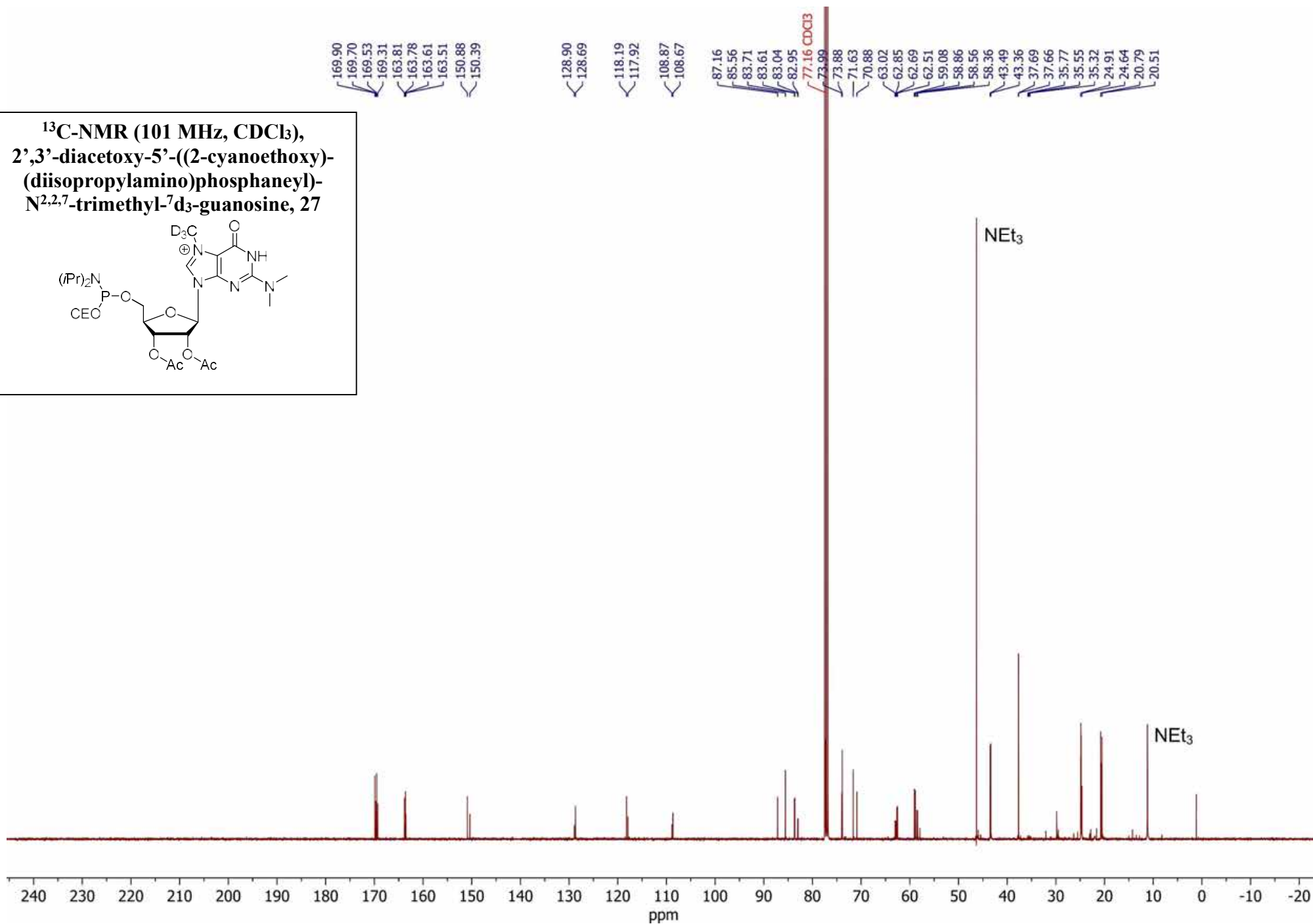
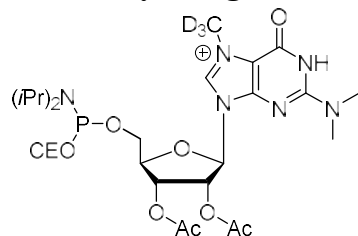


**¹H-NMR (400 MHz, CDCl₃),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphanyl)-
N^{2,2,7}-trimethyl-⁷d₃-guanosine, 27**



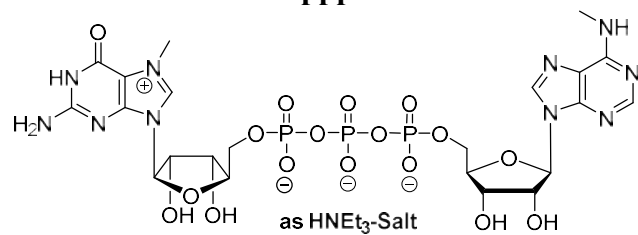


**^{13}C -NMR (101 MHz, CDCl_3),
2',3'-diacetoxy-5'-((2-cyanoethoxy)-
(diisopropylamino)phosphanyl)-
 $\text{N}^{2,2,7}$ -trimethyl- $^7\text{d}_3$ -guanosine, 27**



— 8.39
— 8.18

**¹H-NMR (400 MHz, D₂O),
m⁷Gpppm⁶A**



6.03
6.02
5.84
5.83
4.79 D2O
4.71
4.70
4.70
4.69
4.53
4.52
4.51
4.45
4.28
3.97
3.25
3.23
3.21
3.20
3.09
1.94
1.32
1.30
1.28

HNEt₃⁺

OAc⁻

HNEt₃⁺

1.01
0.88

1.00
0.99

1.16
0.98
8.05

3.02

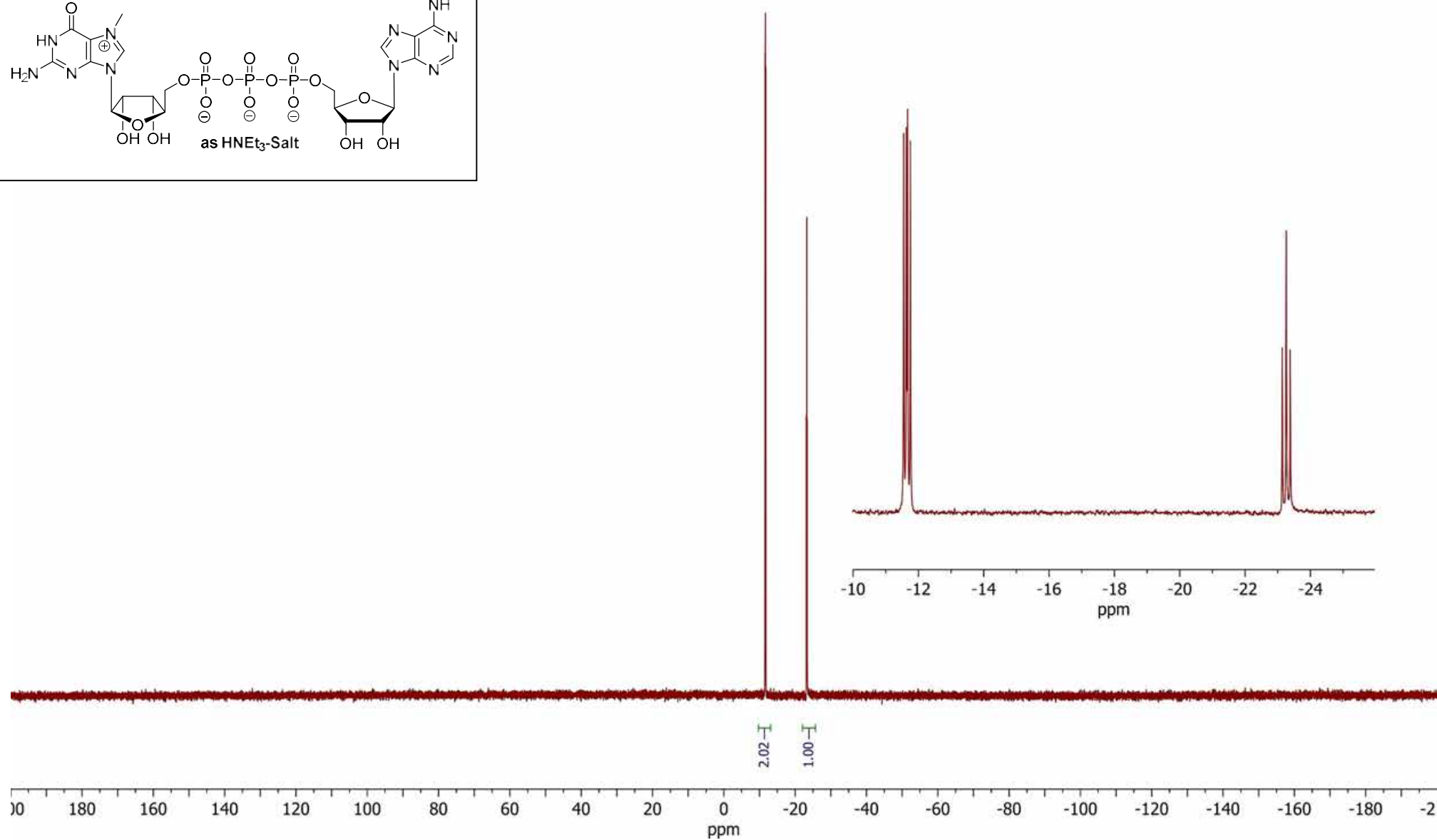
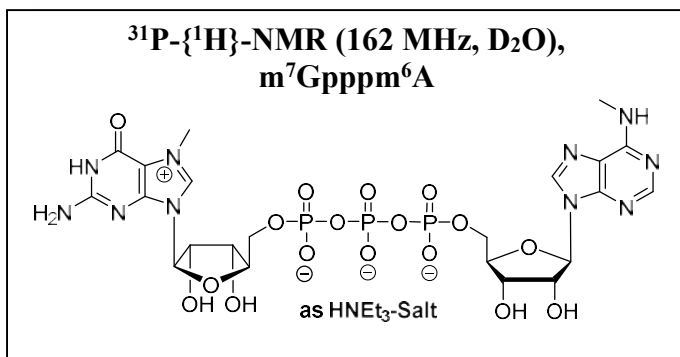
46.75
3.37

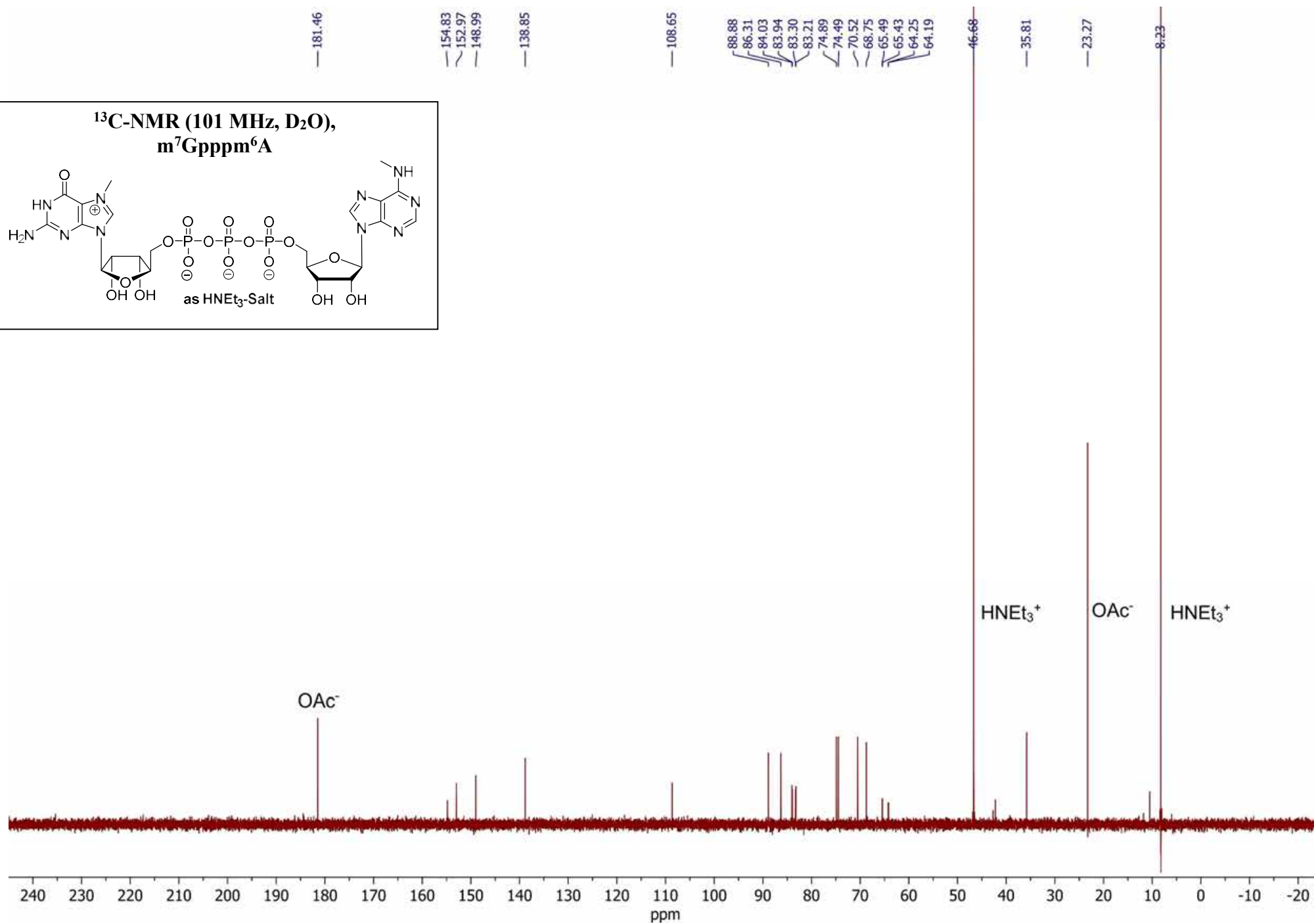
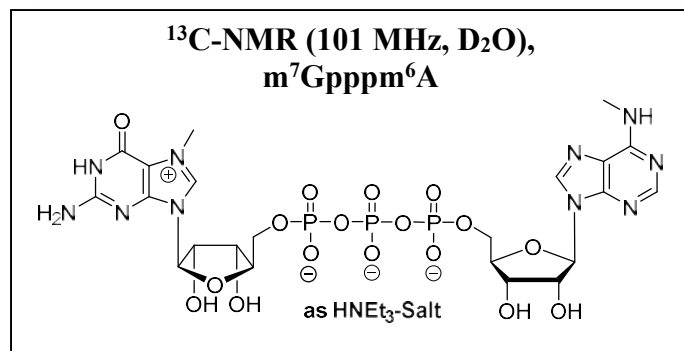
13.69

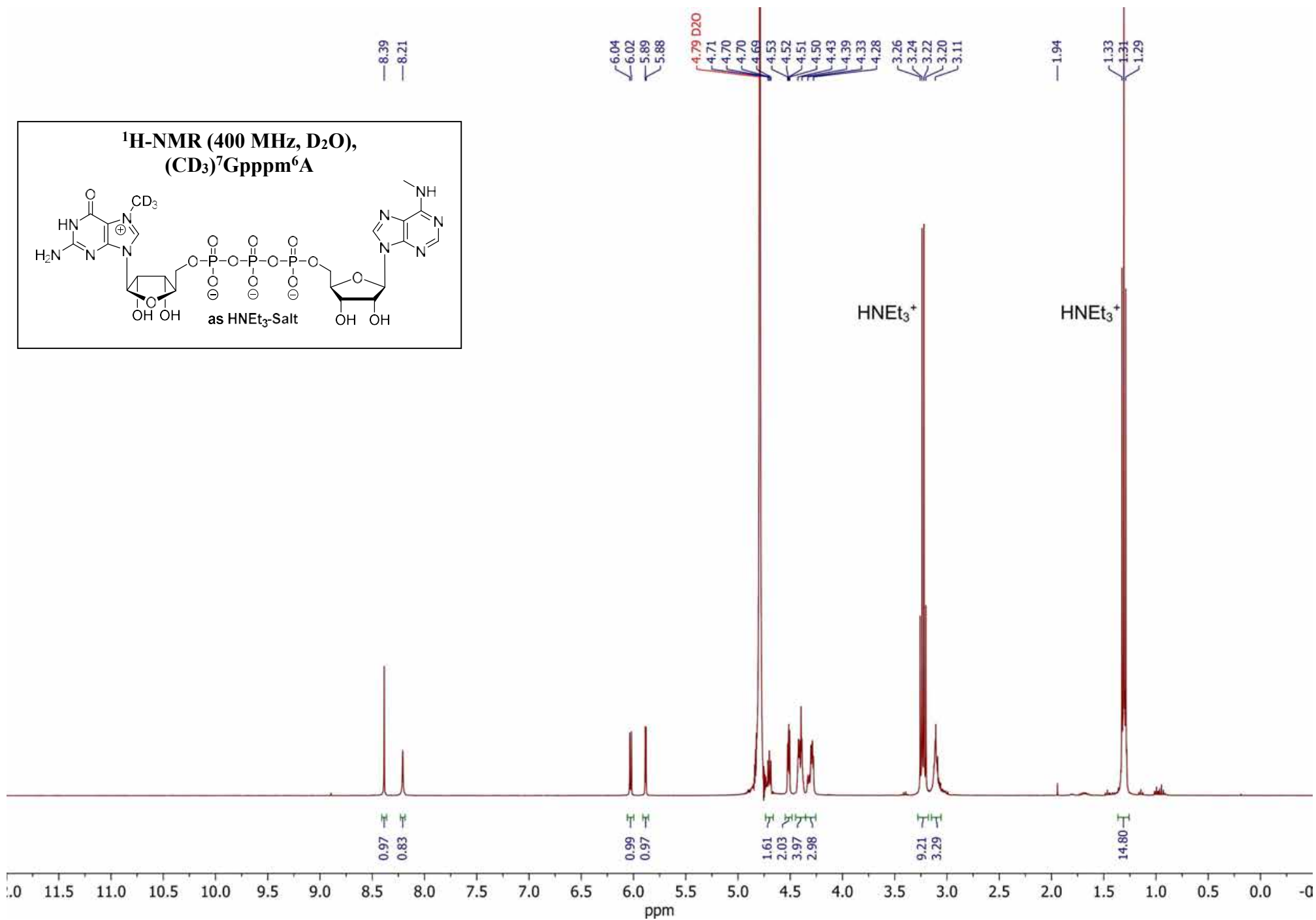
70.86

ppm

-11.55
-11.63
-11.67
-11.76
-23.14
-23.26
-23.38

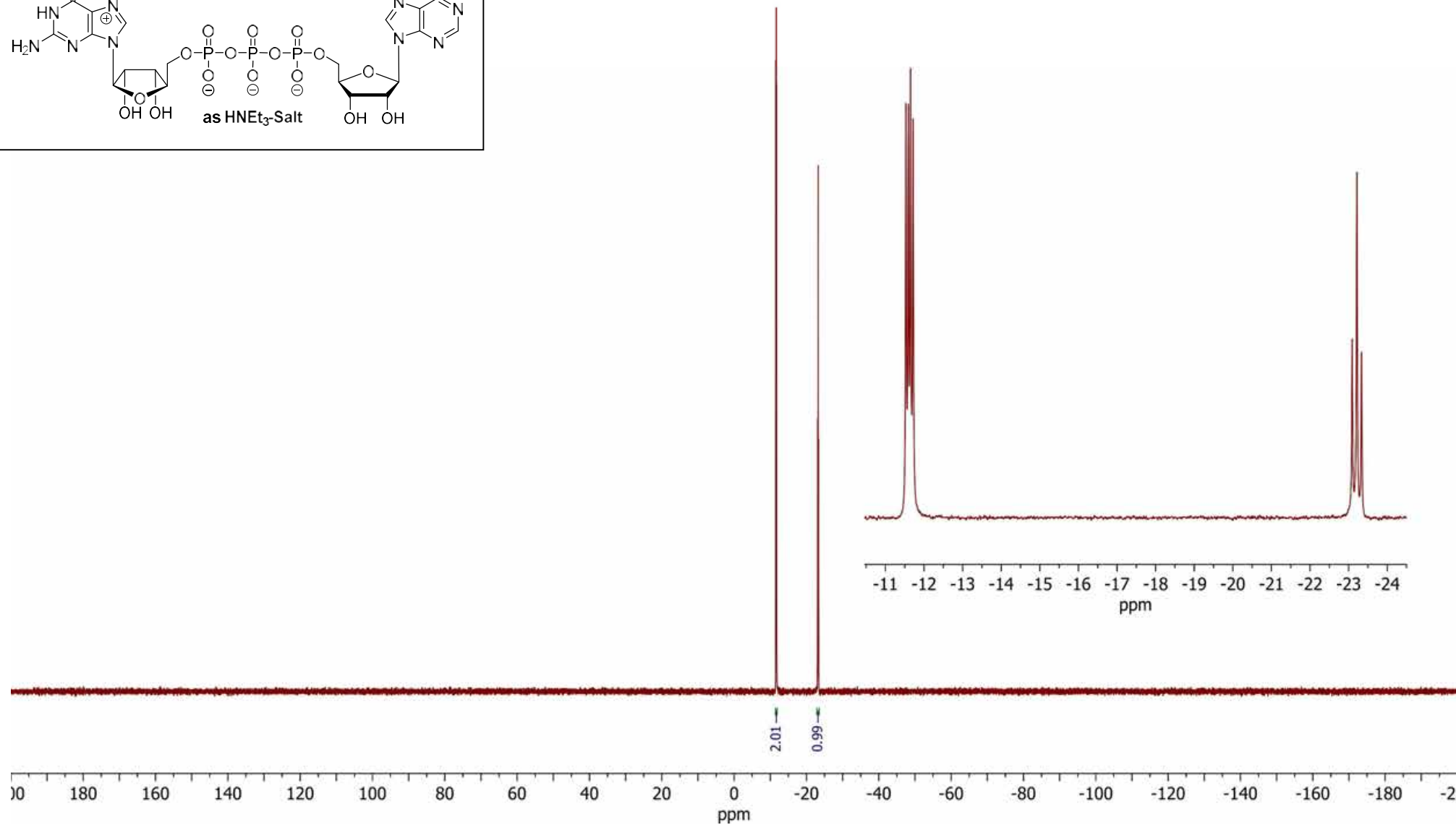
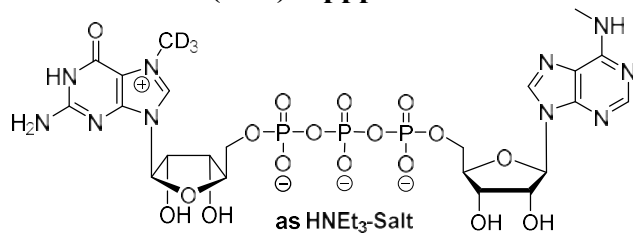






-11.53
-11.59
-11.65
-11.72
-23.09
-23.21
-23.33

**^{31}P - $\{^1\text{H}\}$ -NMR (162 MHz, D_2O),
(CD_3) $^7\text{Gpppm}^6\text{A}$**



155.73
154.88
152.98
149.03

138.93

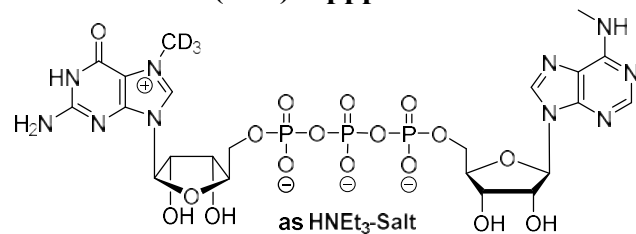
107.67

89.37
86.28
84.04
83.96
83.77
83.68
74.90
74.40
70.47
68.96
65.54
65.48
64.28
64.23

46.68

8.22

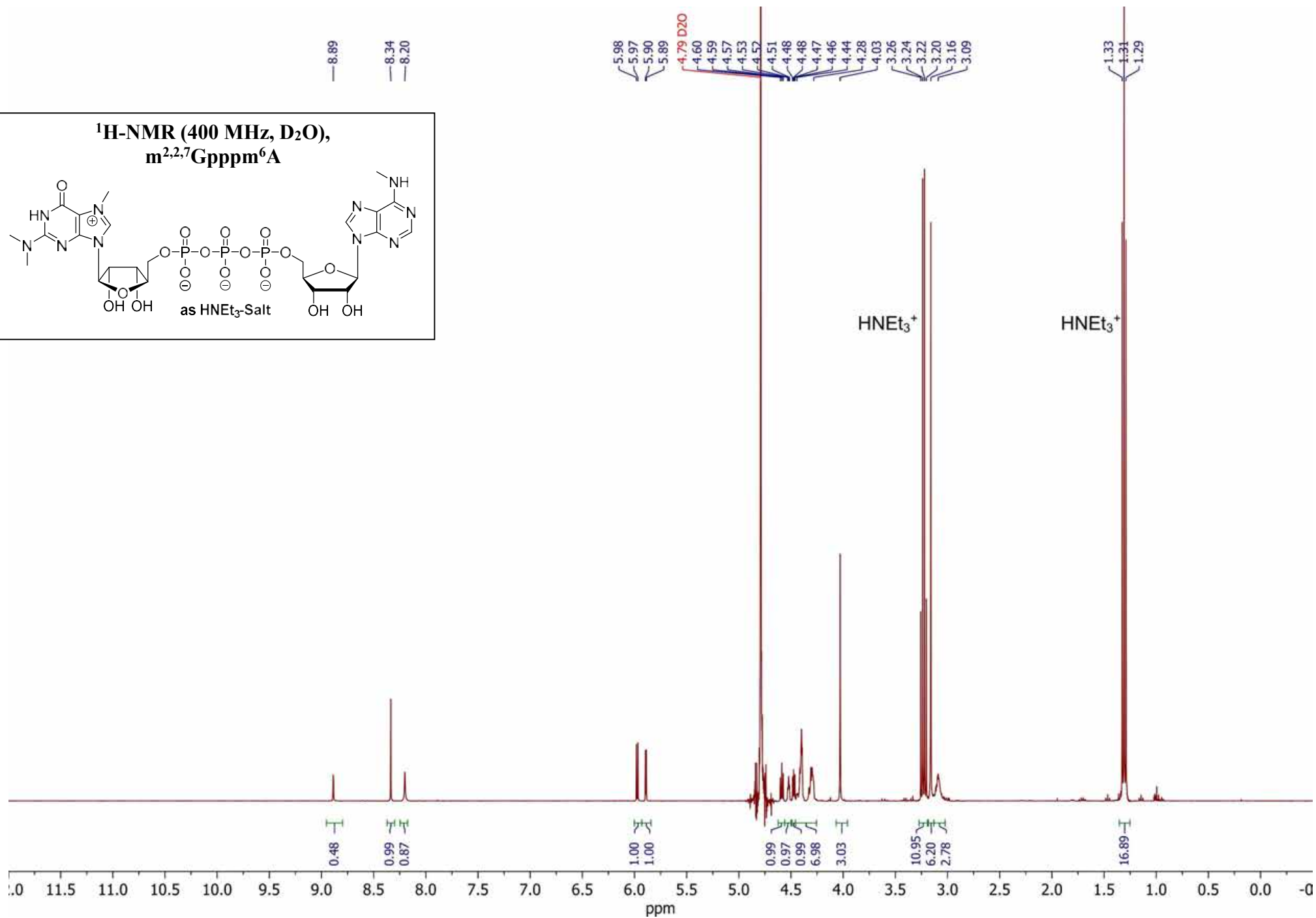
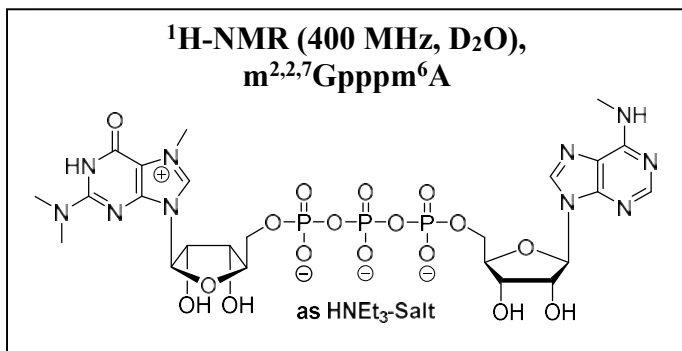
**^{13}C -NMR (101 MHz, D_2O),
(CD_3) $^7\text{Gpppm}^6\text{A}$**



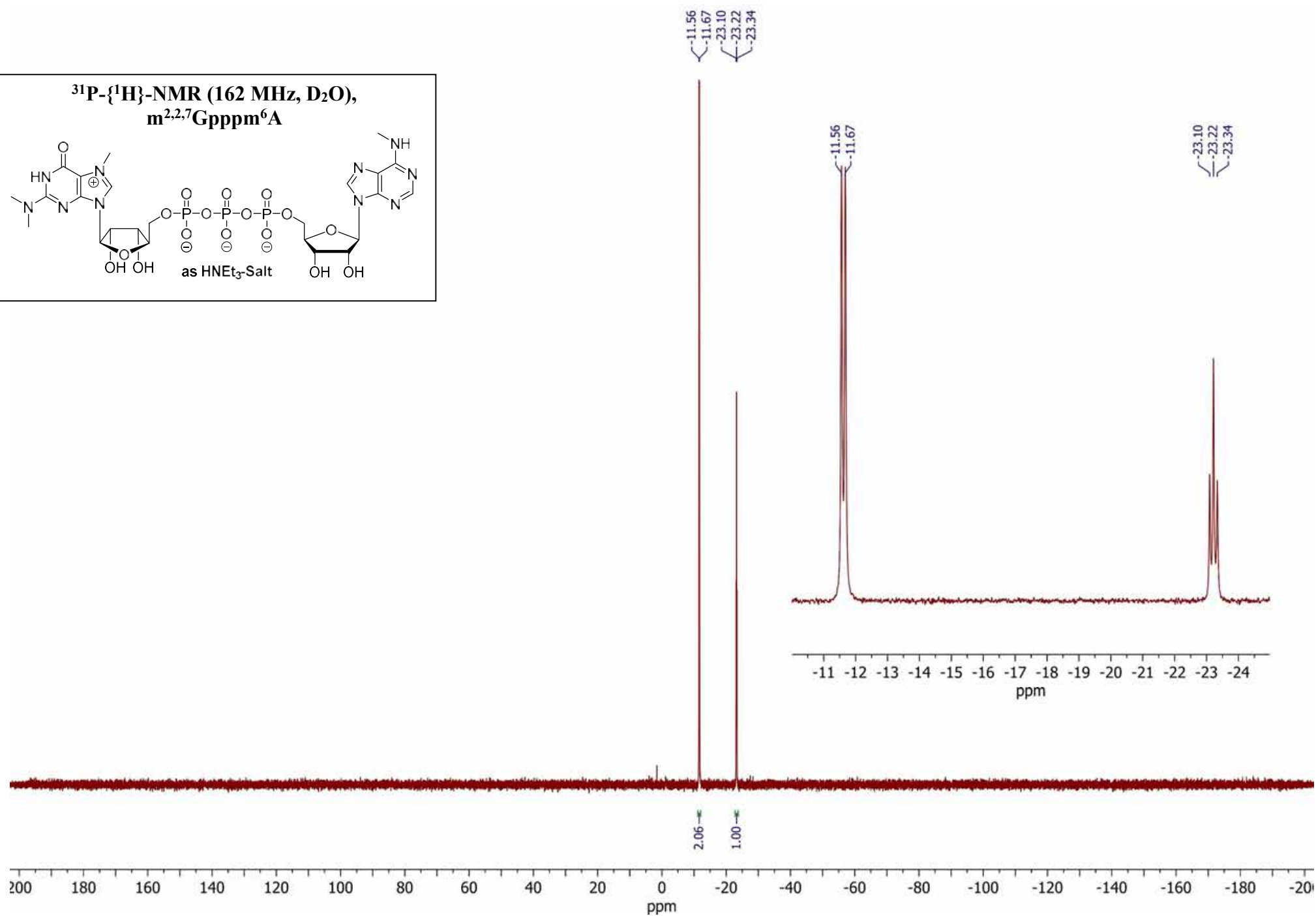
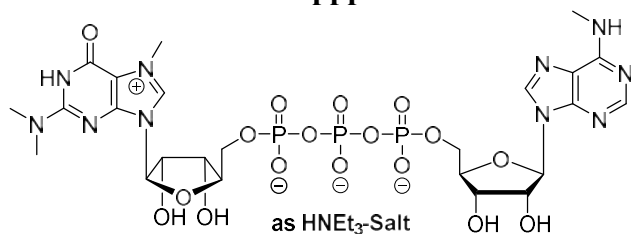
HNEt_3^+

HNEt_3^+

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



**$^{31}\text{P}\{-^1\text{H}\}$ -NMR (162 MHz, D_2O),
 $\text{m}^{2,2,7}\text{Gpppm}^6\text{A}$**



154.87
153.79
152.81
149.05

138.83

106.13

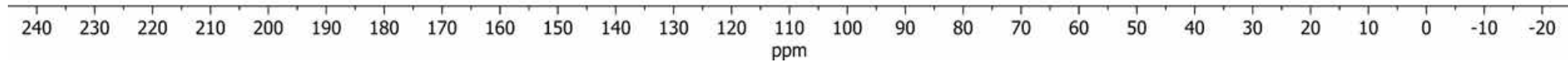
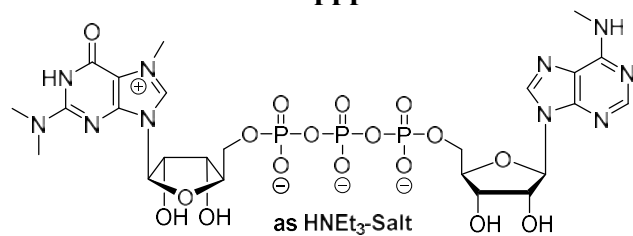
89.11
86.54
83.86
83.77
83.62
83.53
74.85
74.61
70.24
68.97
65.46
65.41
64.34
64.29

46.68

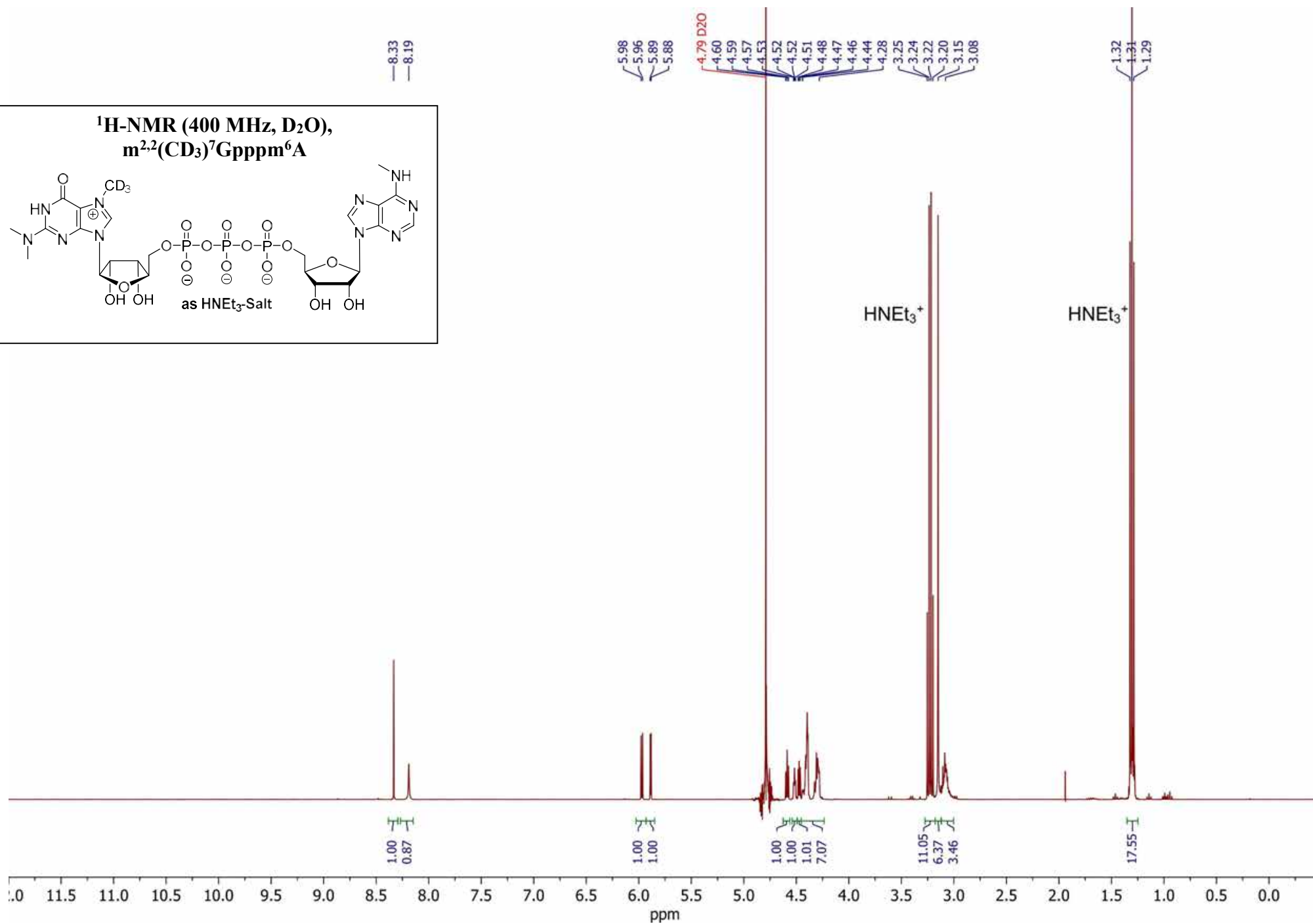
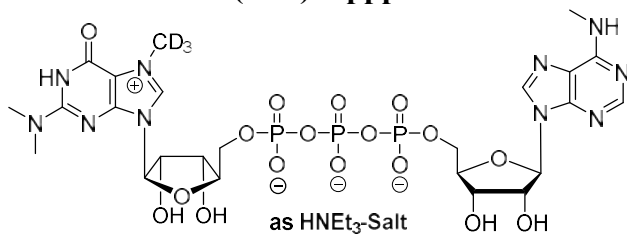
37.51
36.03

8.22

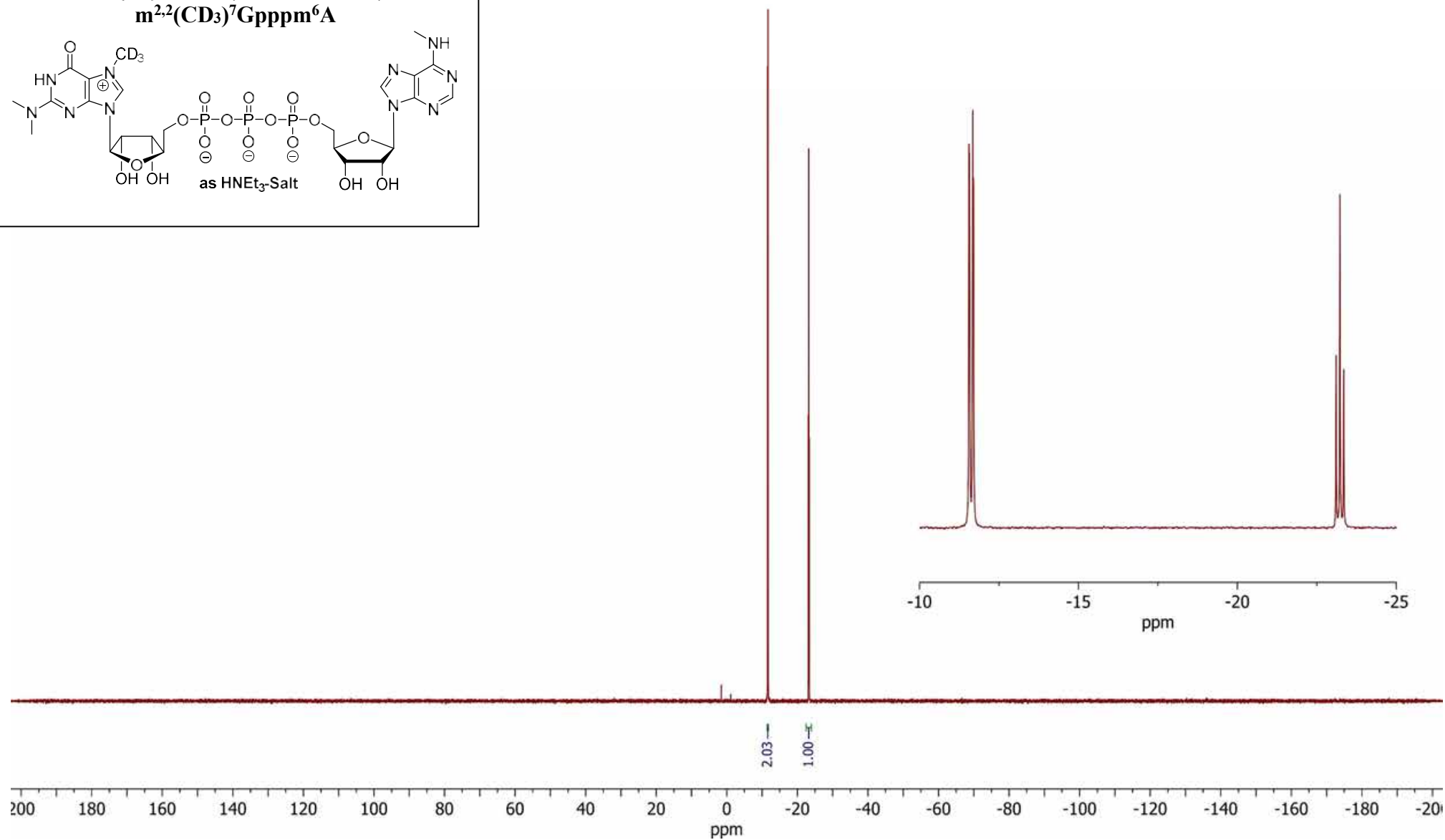
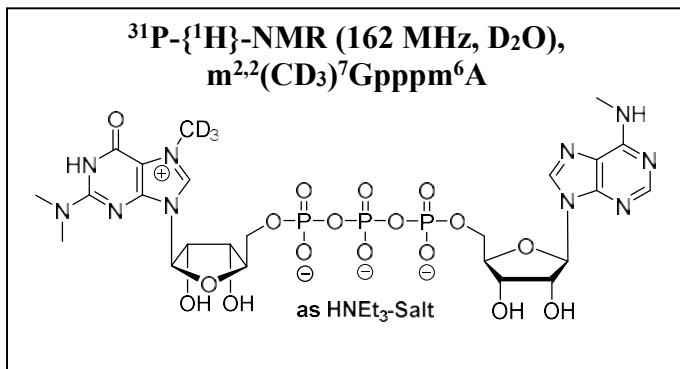
**¹³C-NMR (101MHz, D₂O),
m^{2,2,7}Gpppm⁶A**



**¹H-NMR (400 MHz, D₂O),
m^{2,2}(CD₃)⁷Gpppm⁶A**



-11.55
-11.57
-11.67
-11.70
-23.11
-23.23
-23.35



154.85
152.81
149.09

138.81

106.19

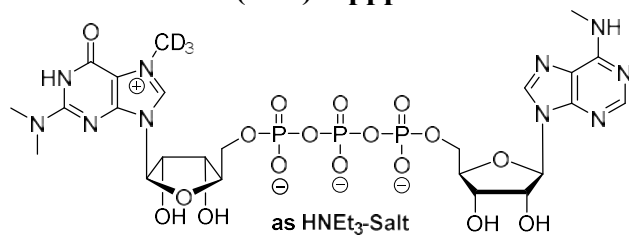
89.08
86.55
83.85
83.76
83.60
83.50
74.84
74.62
70.24
68.96
65.45
65.39
64.34
64.29

46.68

37.48

8.22

^{13}C -NMR (101 MHz, D_2O),
 $\text{m}^{2,2}(\text{CD}_3)^7\text{Gpppm}^6\text{A}$

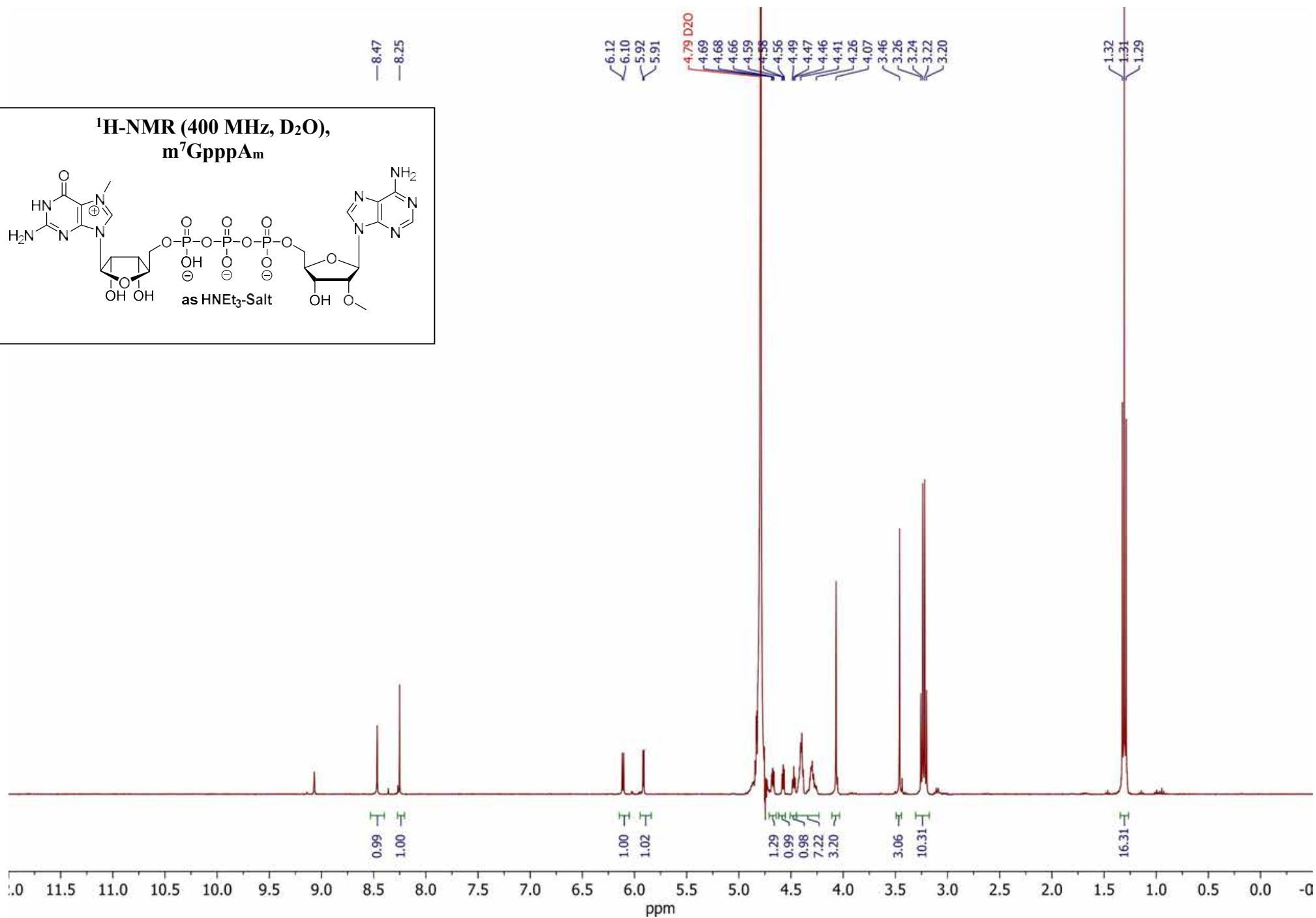
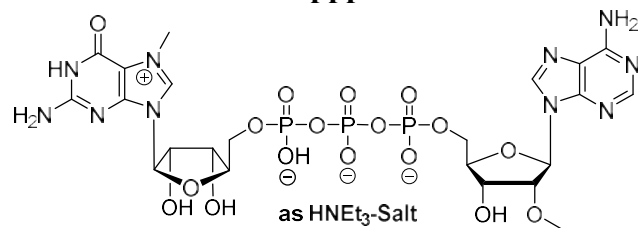


HNEt_3^+

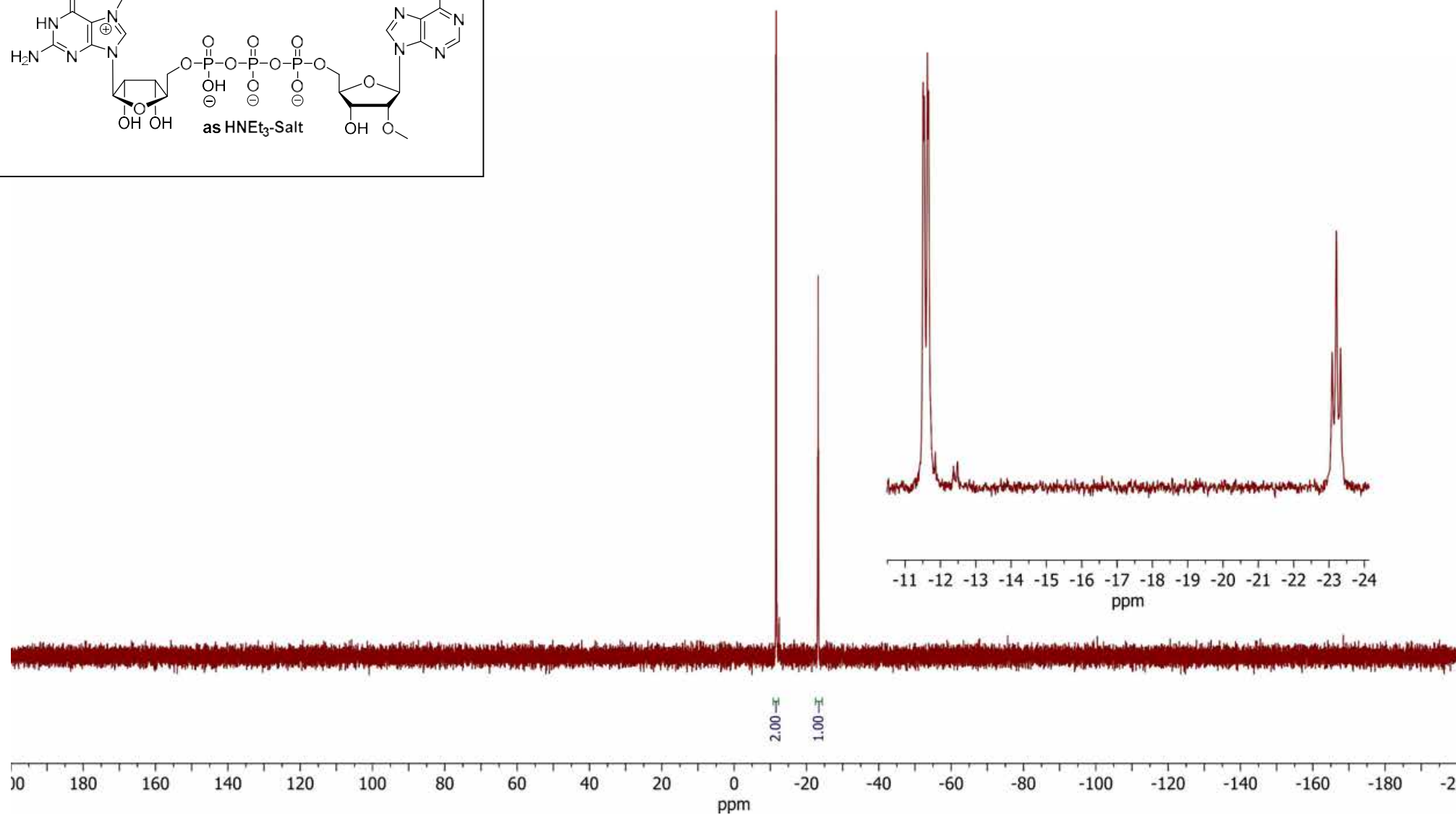
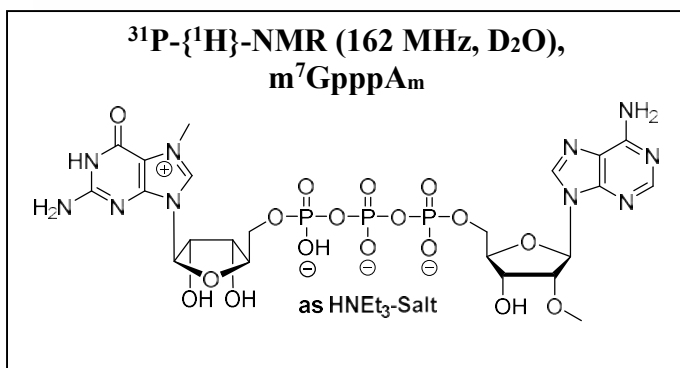
HNEt_3^+

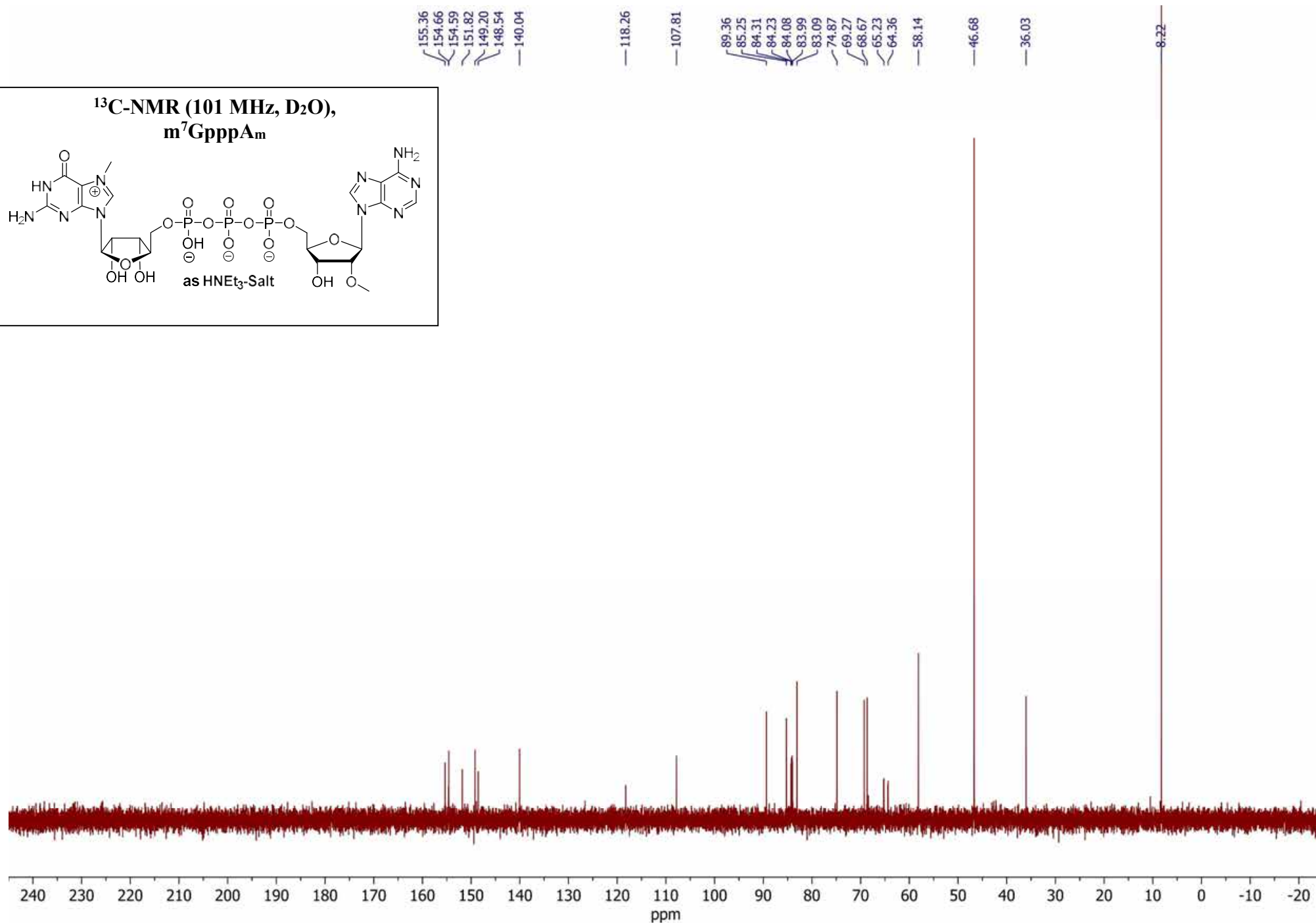
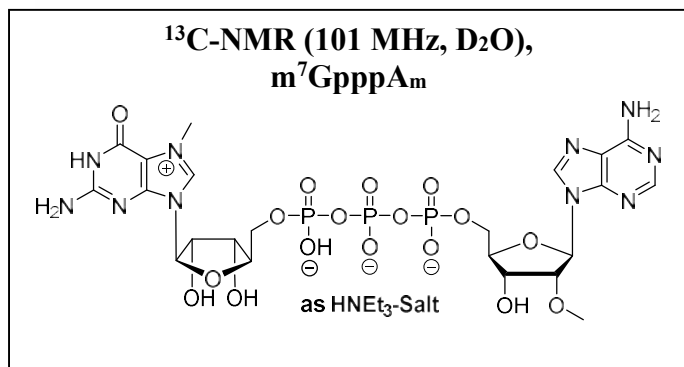
240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
ppm

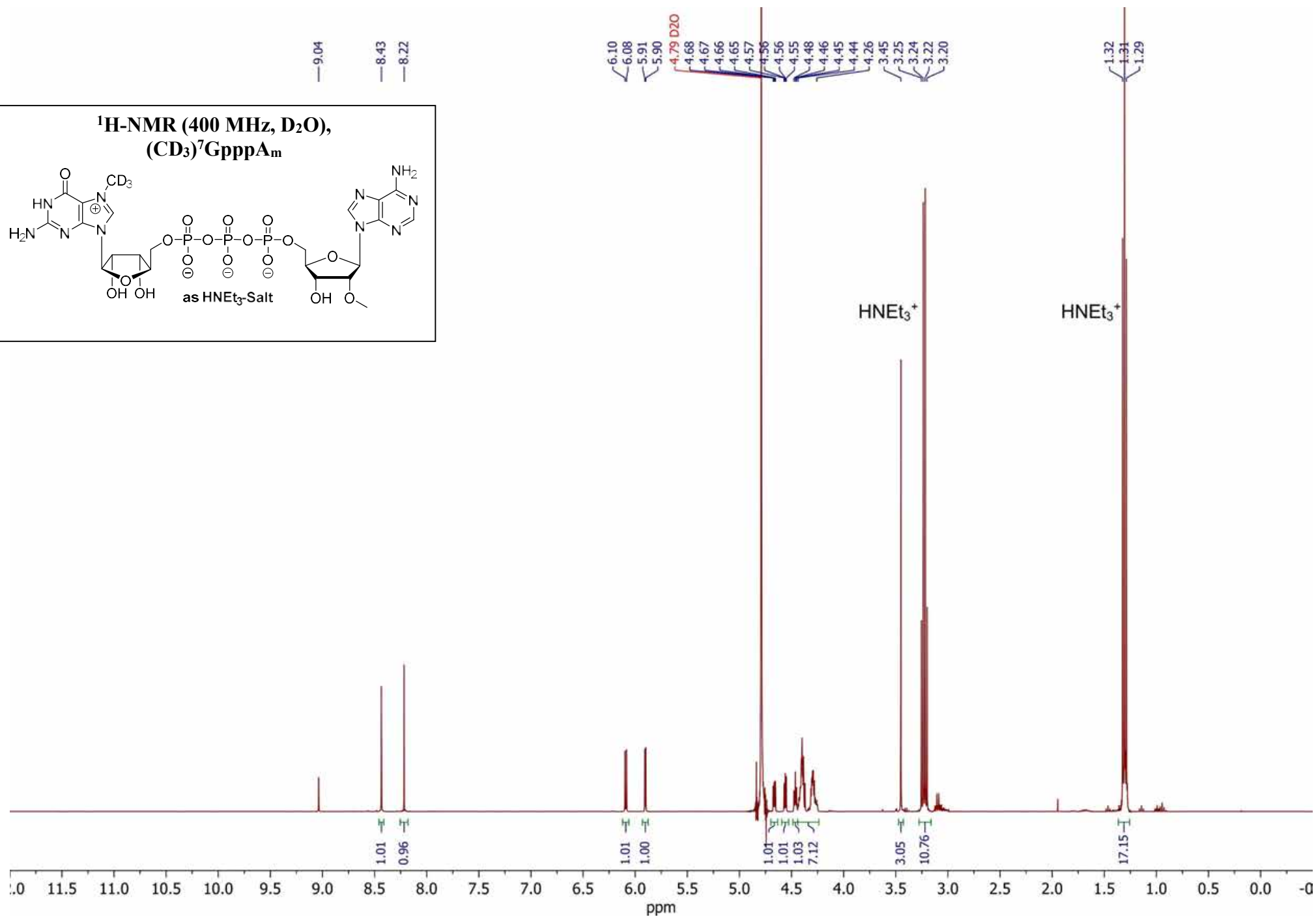
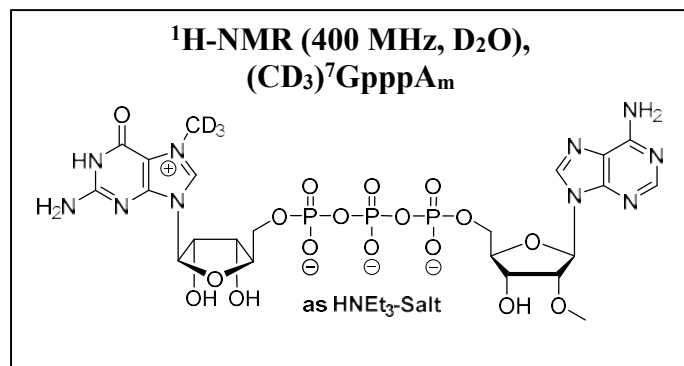
**¹H-NMR (400 MHz, D₂O),
m⁷GpppA_m**

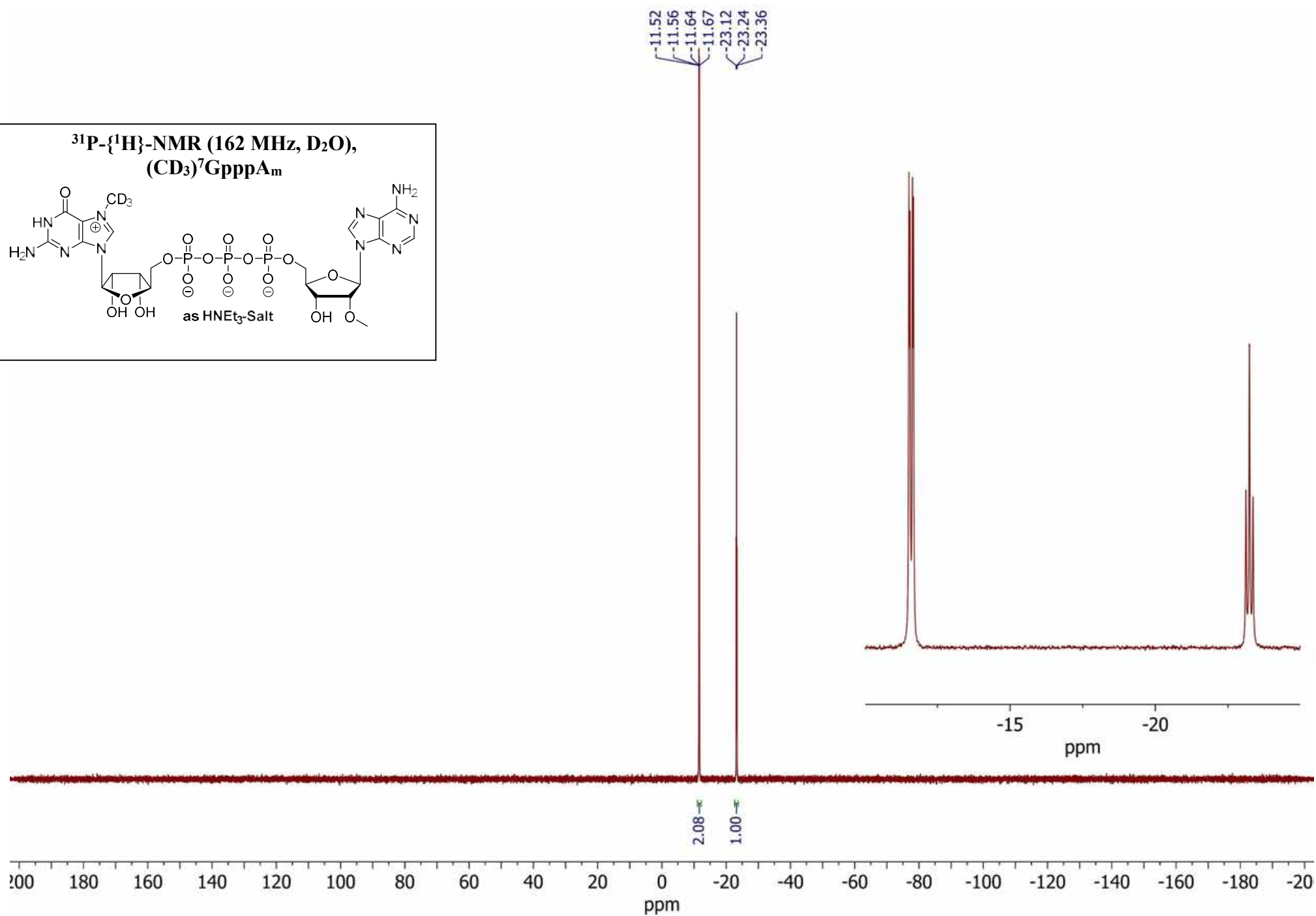
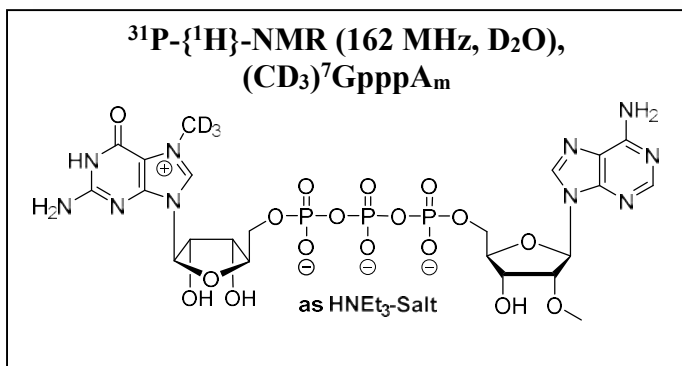


³¹P-¹H-NMR (162 MHz, D₂O),
^m7GpppA_m









155.53
155.42
154.79
152.91
149.16
148.62
— 139.67

— 118.24

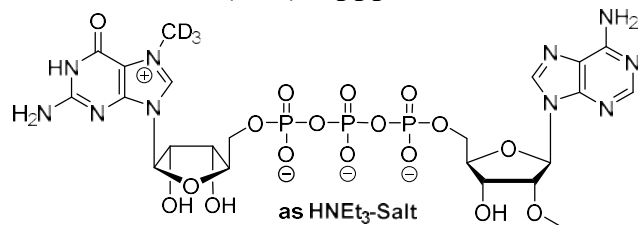
— 107.77

89.33
85.07
84.27
84.18
84.06
83.97
83.03
74.88
69.27
68.68
65.34
65.28
64.44
64.39
— 58.13

— 46.68

8.22

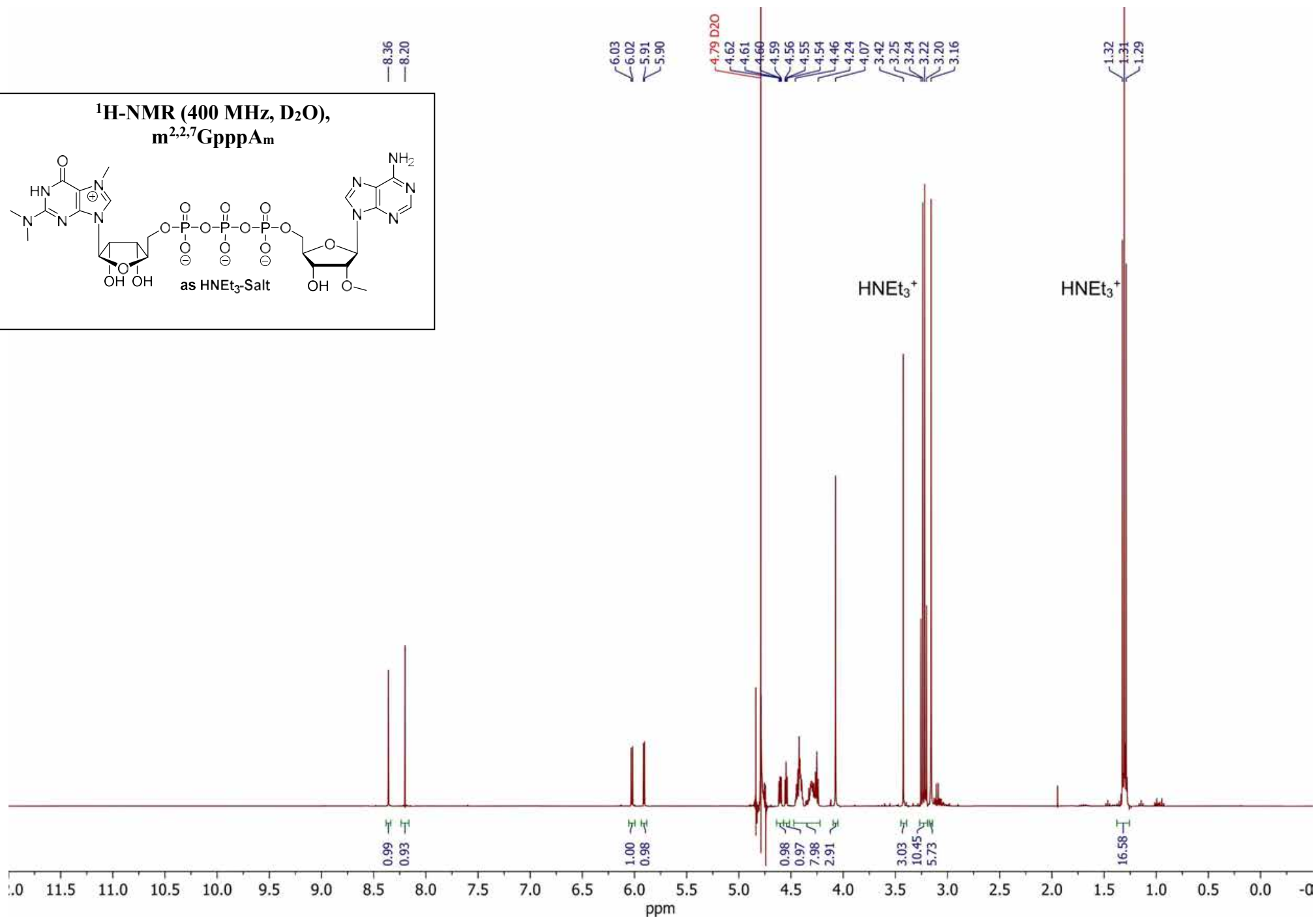
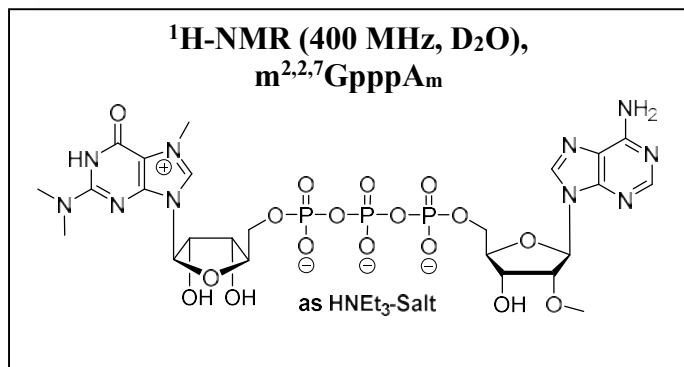
**^{13}C -NMR (101 MHz, D_2O),
(CD_3) $^7\text{GpppA}_m$**

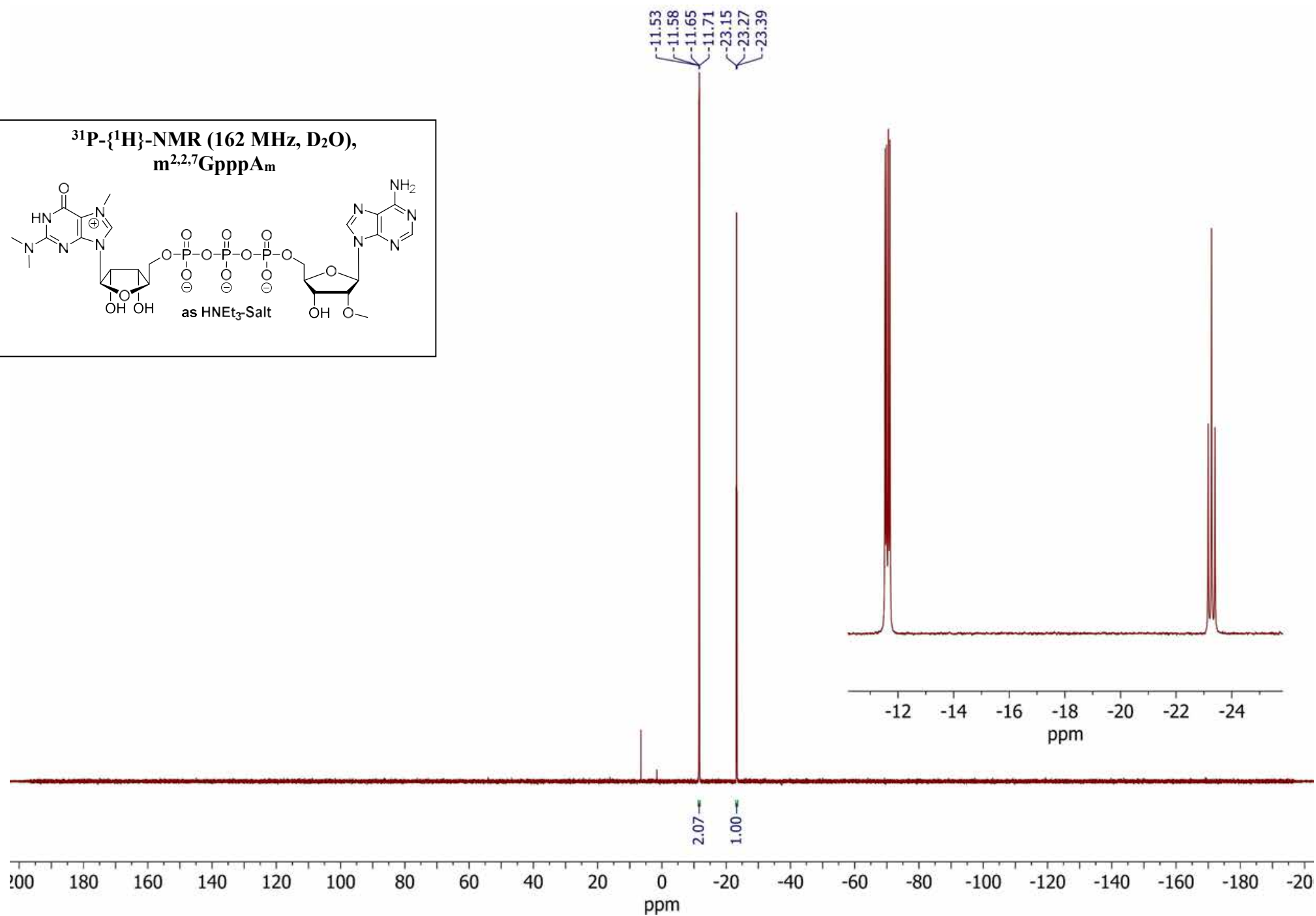
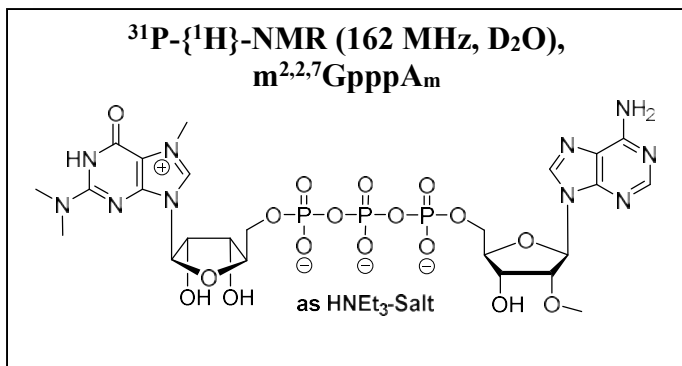


HNEt_3^+

HNEt_3^+

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





155.35
155.09
153.76
152.81
149.17
148.38
— 139.46

— 118.15

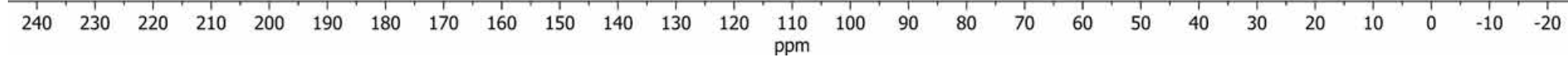
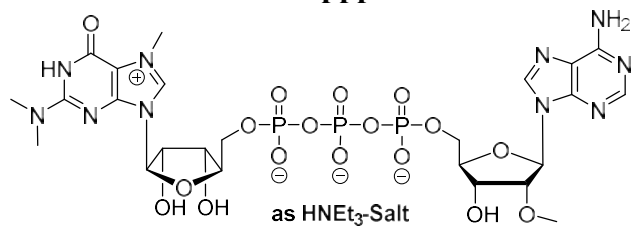
— 106.09

88.95
85.12
84.10
84.02
83.86
83.77
83.30
74.88
69.26
68.57
65.34
65.28
64.56
64.50
— 58.14

46.68
42.71
42.24
37.51
36.02

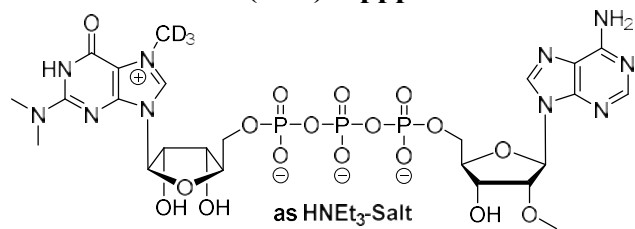
19.11
18.21
11.79
10.51
10.47
8.22

**¹³C-NMR (101 MHz, D₂O),
m^{2,2,7}GpppA_m**



— 8.36
— 8.20

**¹H-NMR (400 MHz, D₂O),
m^{2,2}(CD₃)⁷GpppAm**



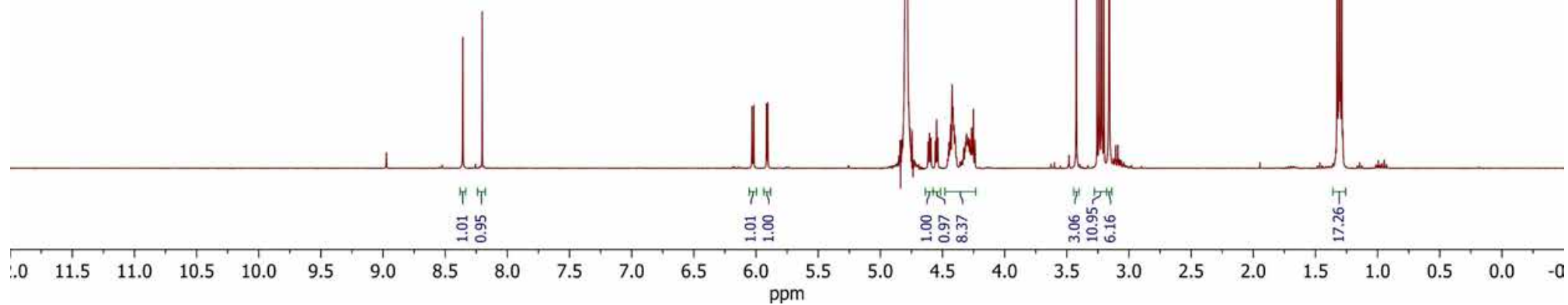
6.03
6.02
5.92
5.91

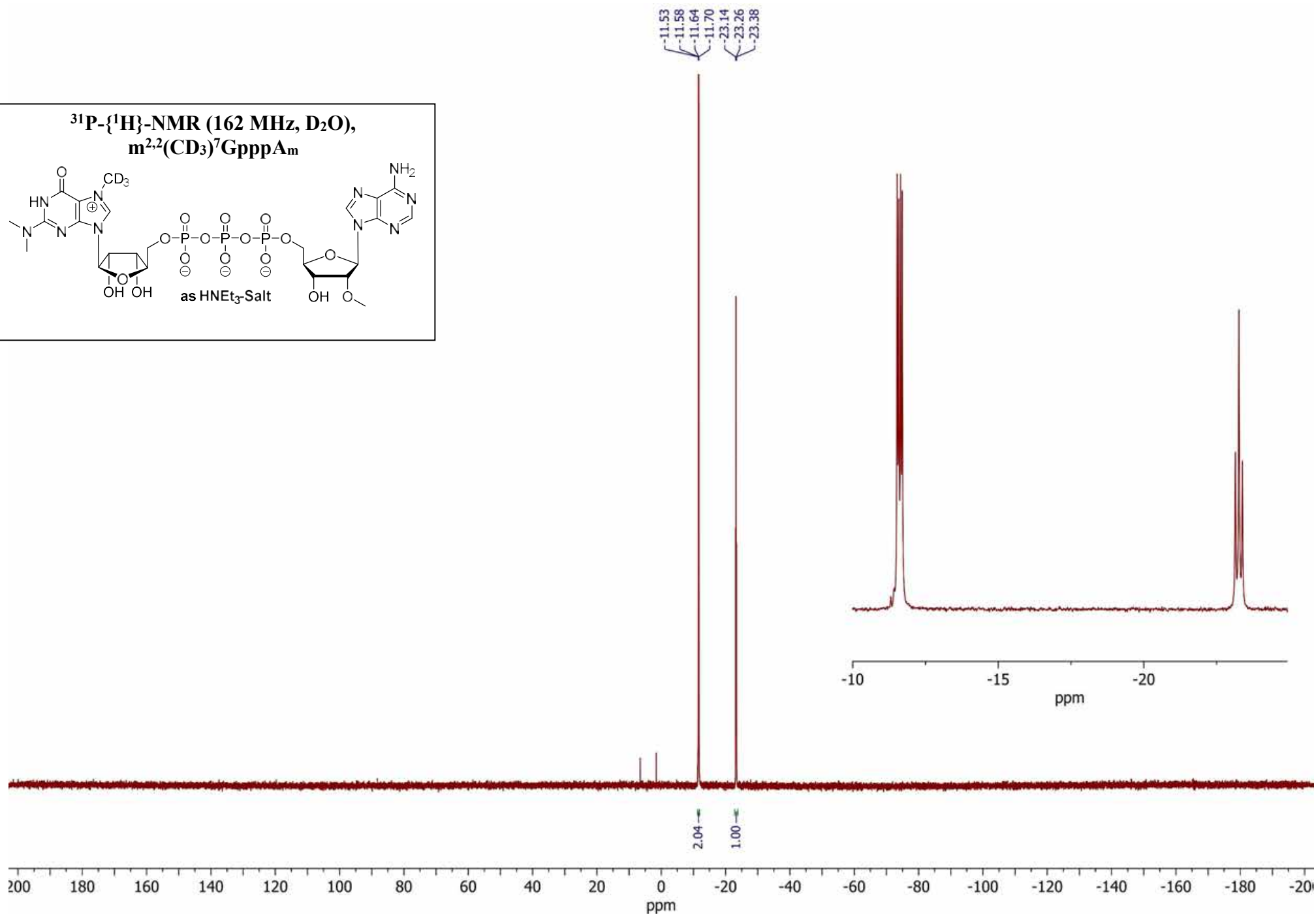
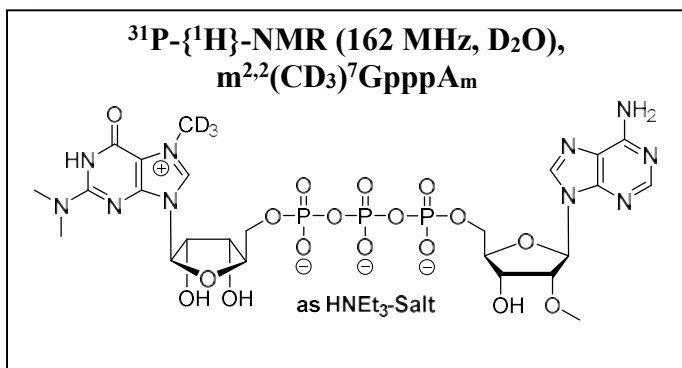
4.79 D₂O
4.62
4.61
4.60
4.59
4.56
4.55
4.54
4.46
4.24
3.42
3.26
3.24
3.22
3.20
3.16

1.33
1.31
1.29

HNEt₃⁺

HNEt₃⁺





155.35
155.06
153.74
152.81
149.17
148.38
— 139.46

— 118.15

— 106.08

88.95
85.13
84.10
84.01
83.86
83.77
83.30

74.88

69.27

68.57

65.34

65.28

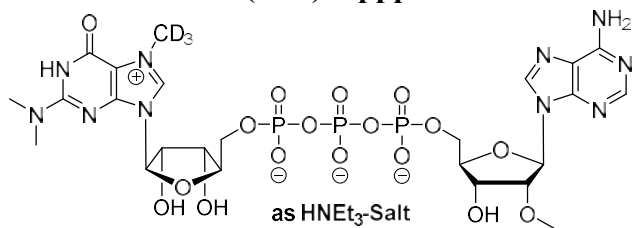
64.56
64.50
— 58.14

— 46.68

— 37.51

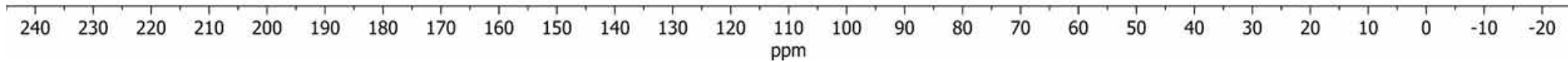
8.22

**^{31}P - $\{^1\text{H}\}$ -NMR (101 MHz, D_2O),
 $\text{m}^{2,2}(\text{CD}_3)^7\text{GpppAm}$**



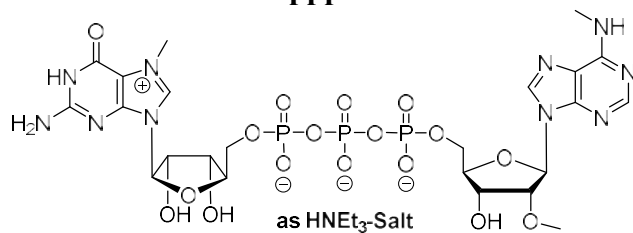
HNEt_3^+

HNEt_3^+



—8.38
—8.22

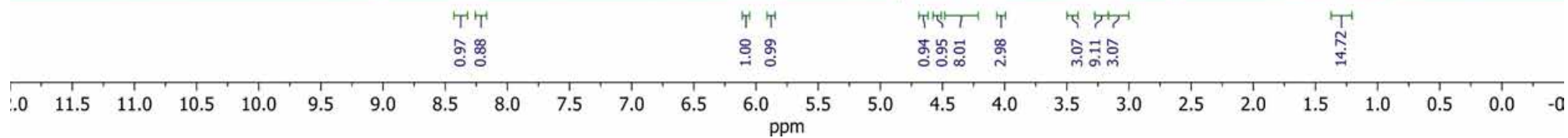
**¹H-NMR (400 MHz, D₂O),
m⁷Gpppm⁶A_m**

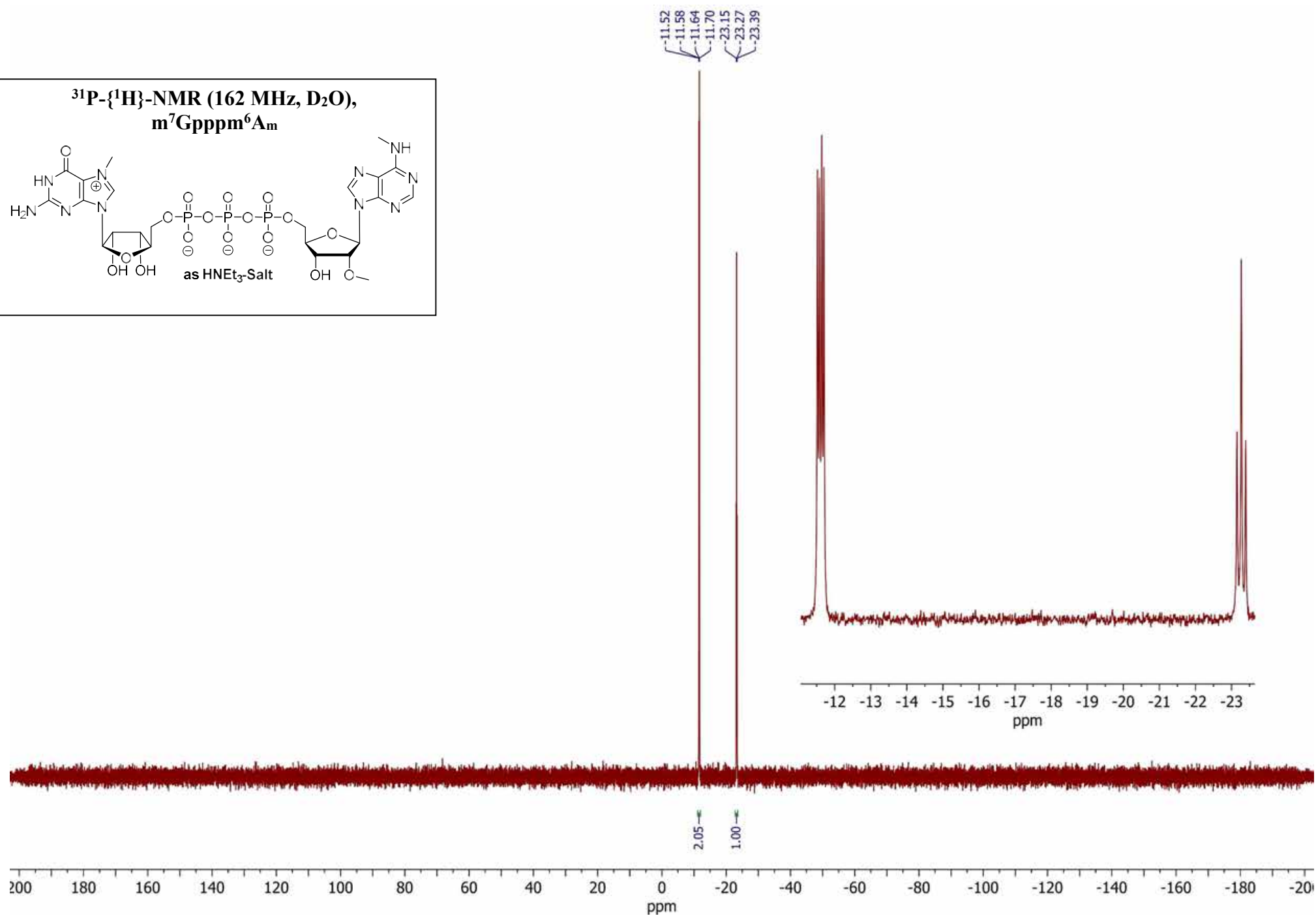
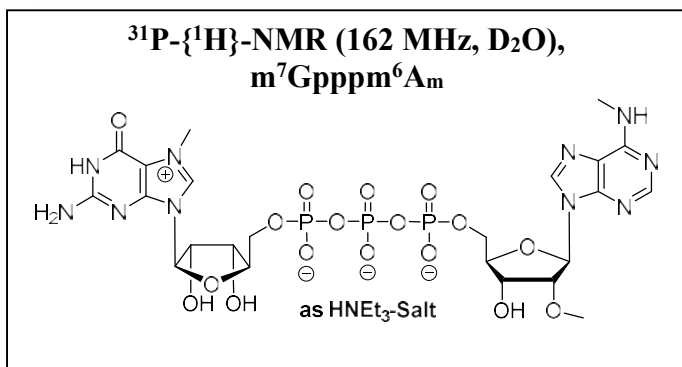


6.09
6.07
5.88
5.87
4.79 D2O
4.67
4.66
4.66
4.65
4.55
4.54
4.54
4.53
4.45
4.26
4.03
3.45
3.25
3.24
3.22
3.20
3.12
1.32
1.31
1.29

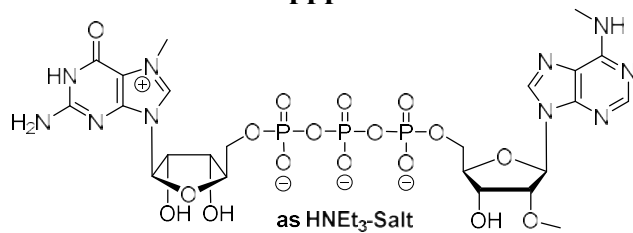
HNEt₃⁺

HNEt₃⁺





**^{13}C -NMR (162 MHz, D_2O),
 $\text{m}^7\text{Gpppm}^6\text{A}_\text{m}$**



155.36
154.81
154.49
152.82
149.09
— 139.03

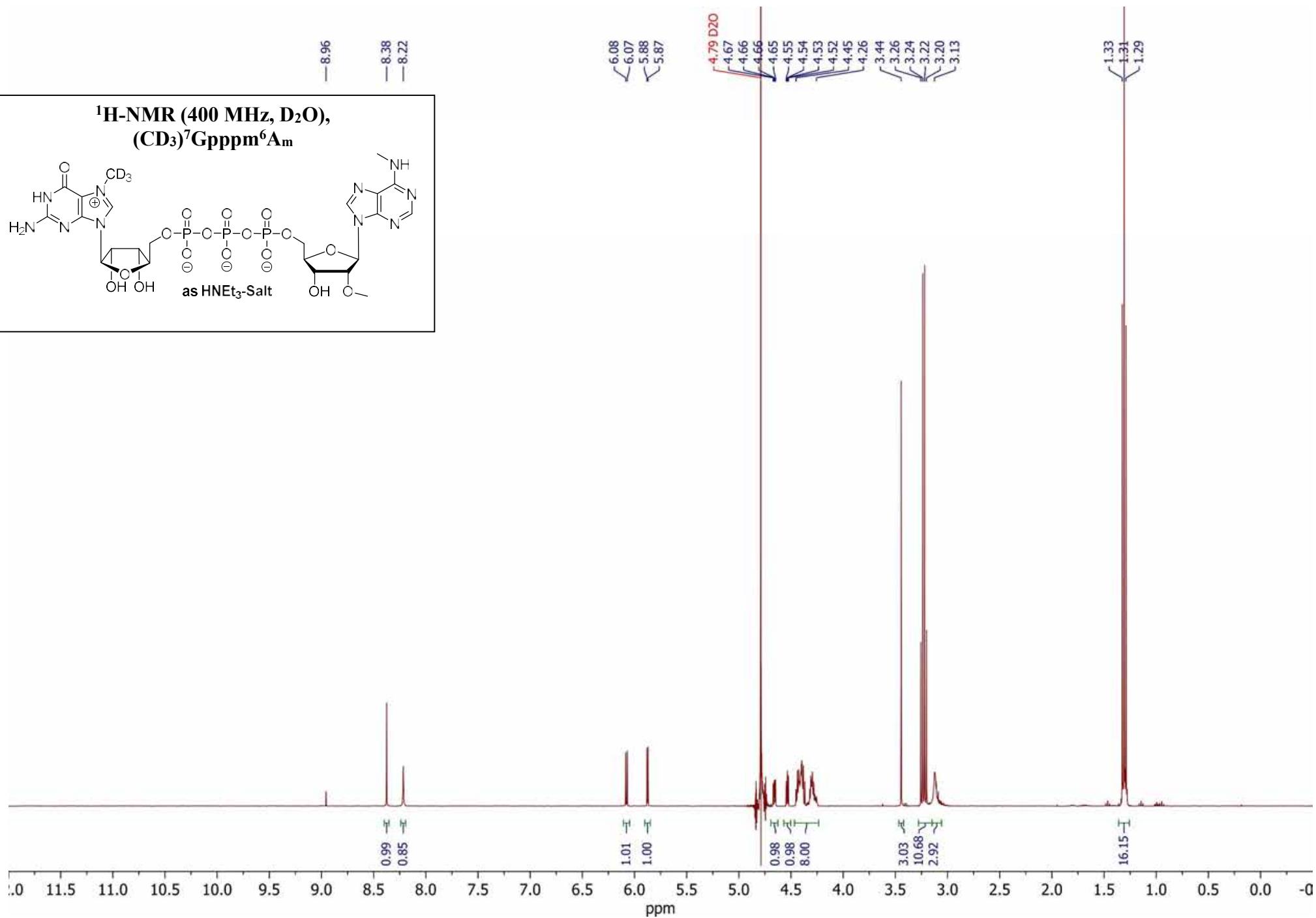
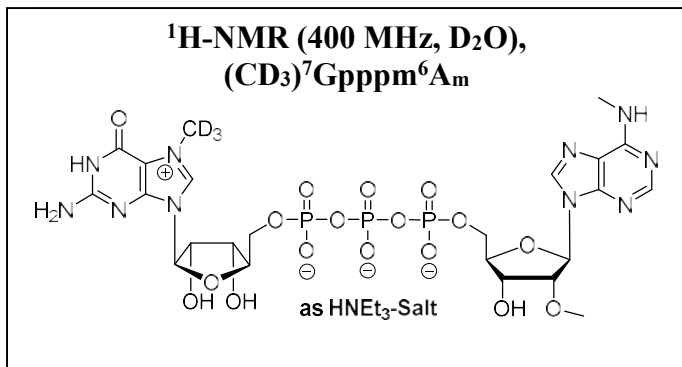
— 107.64
89.35
84.92
84.25
84.16
84.08
83.98
82.94
74.91
69.28
68.65
65.36
65.30
64.48
64.42
— 58.11

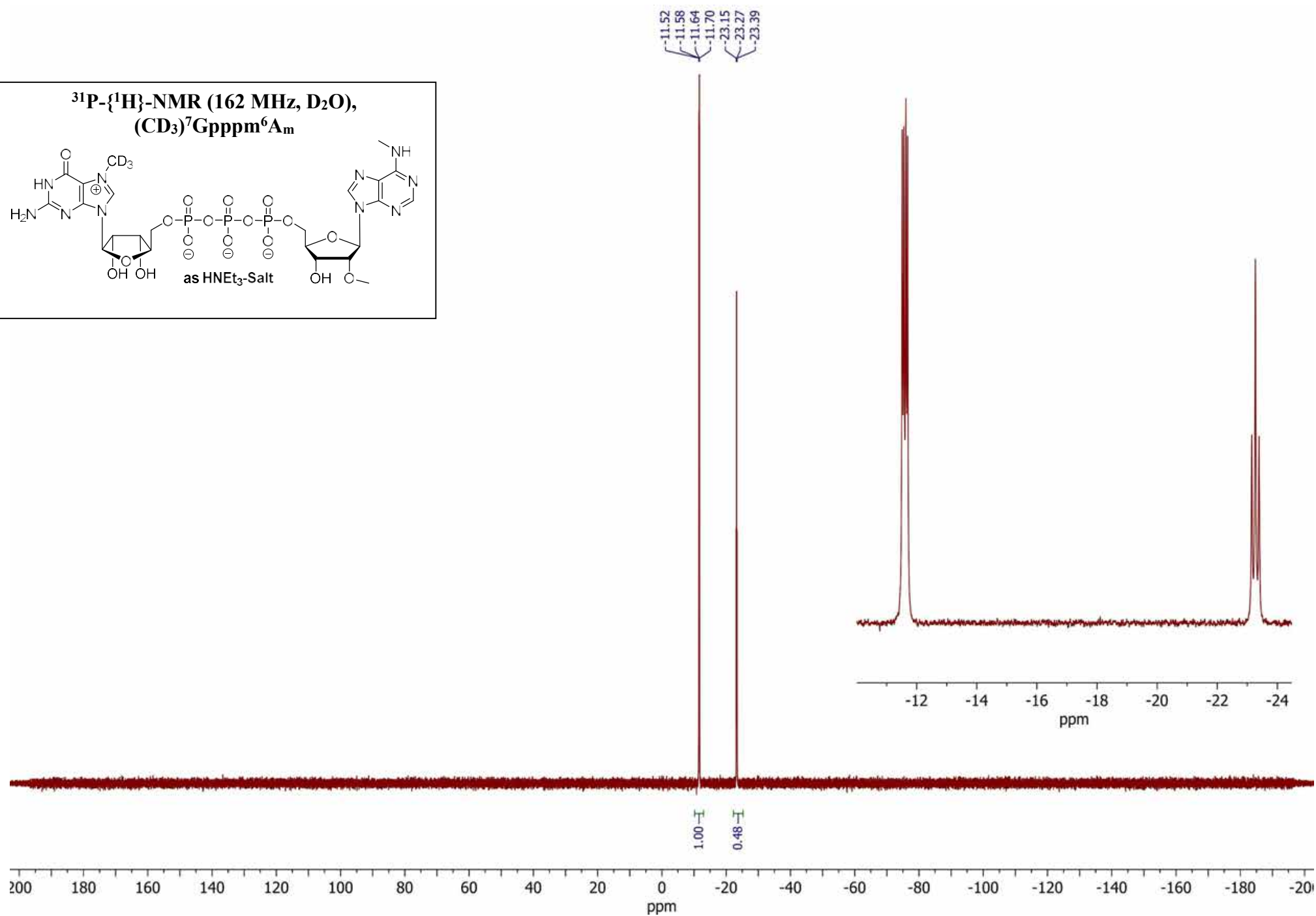
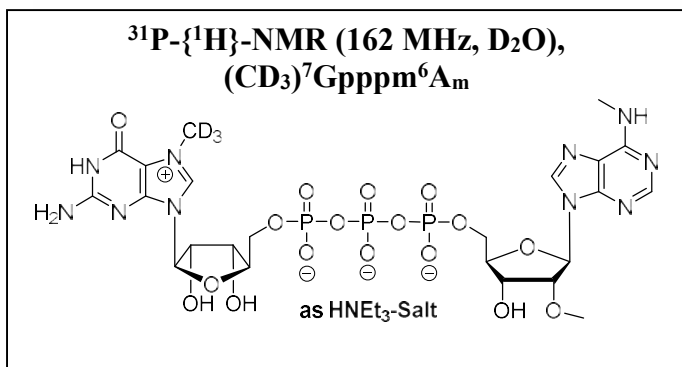
— 46.68

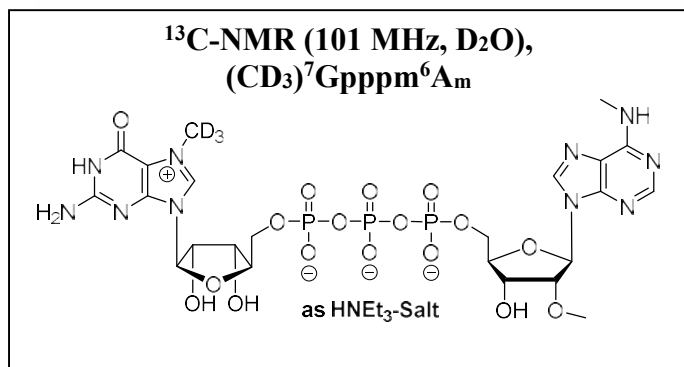
— 36.01

— 8.22

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







155.46
154.92
154.64
152.96
149.09
— 138.98

— 107.65
89.33
84.89
84.24
84.15
84.07
83.98
82.94
74.92
69.28
68.65
65.37
65.31
64.49
64.43
— 58.11

— 46.68

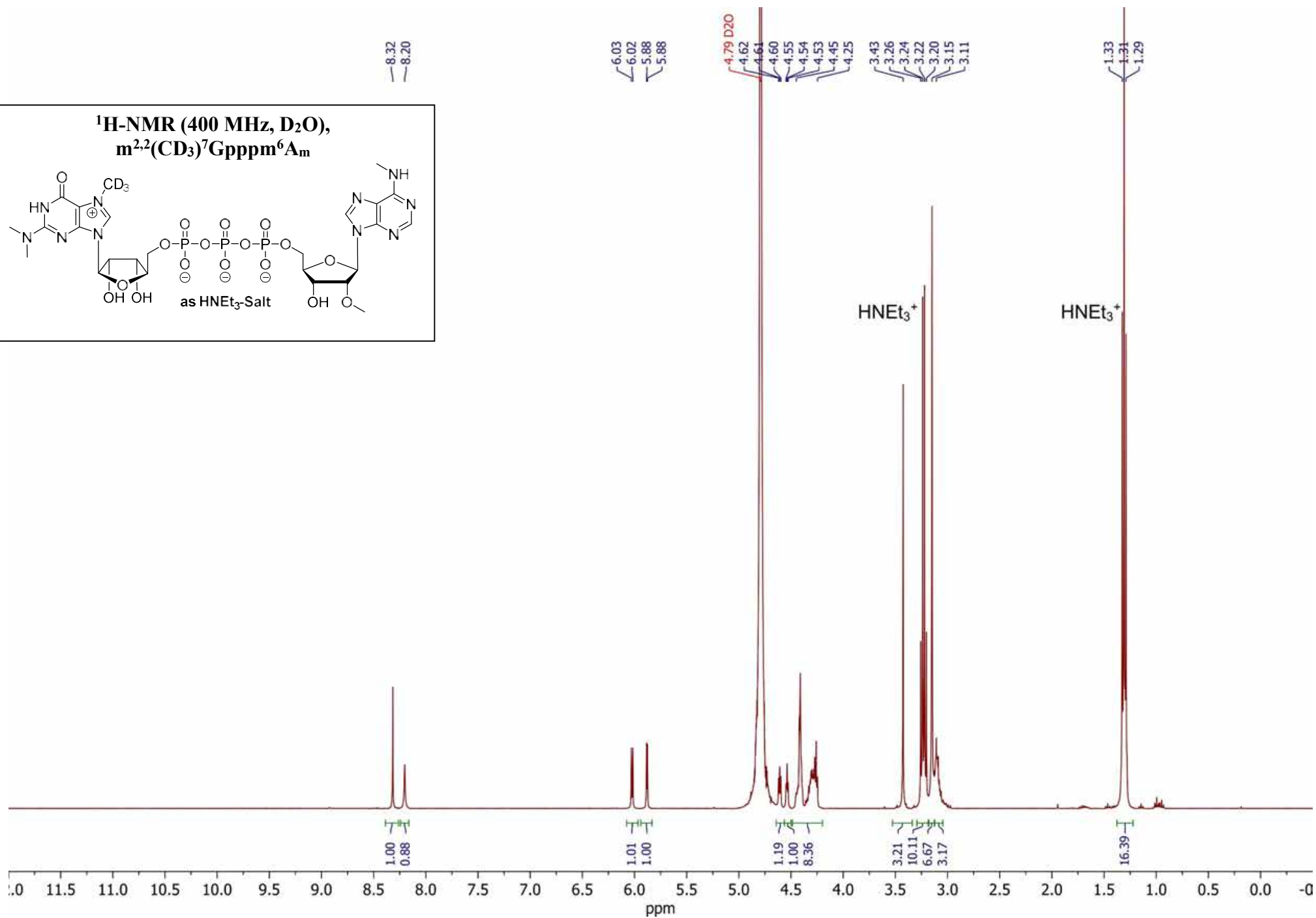
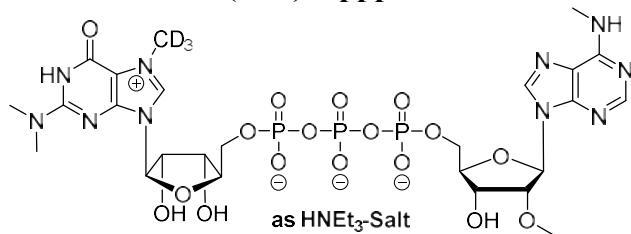
8.22

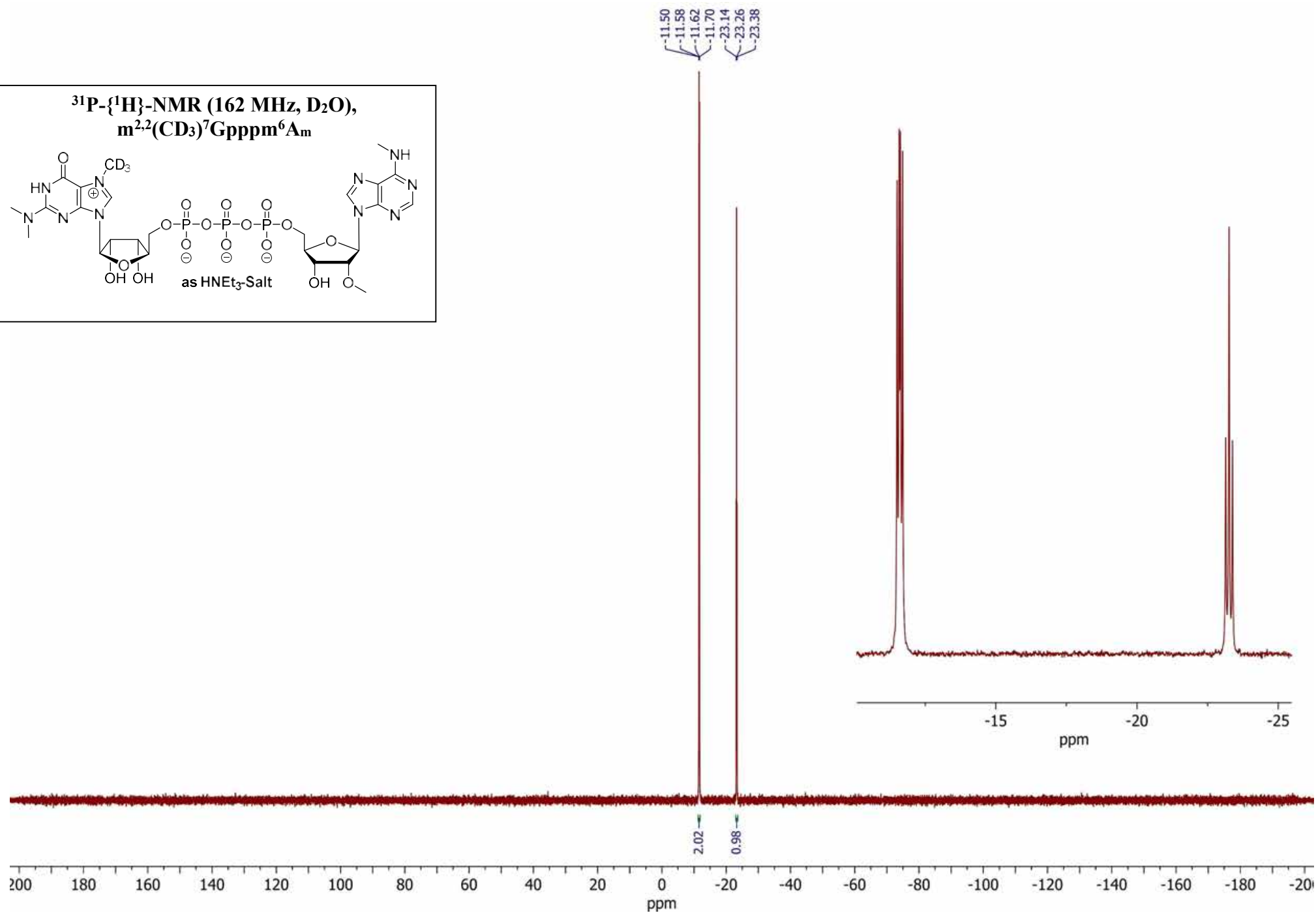
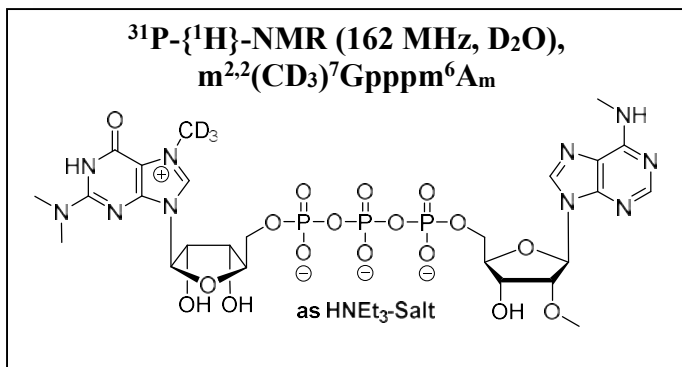
HNEt_3^+

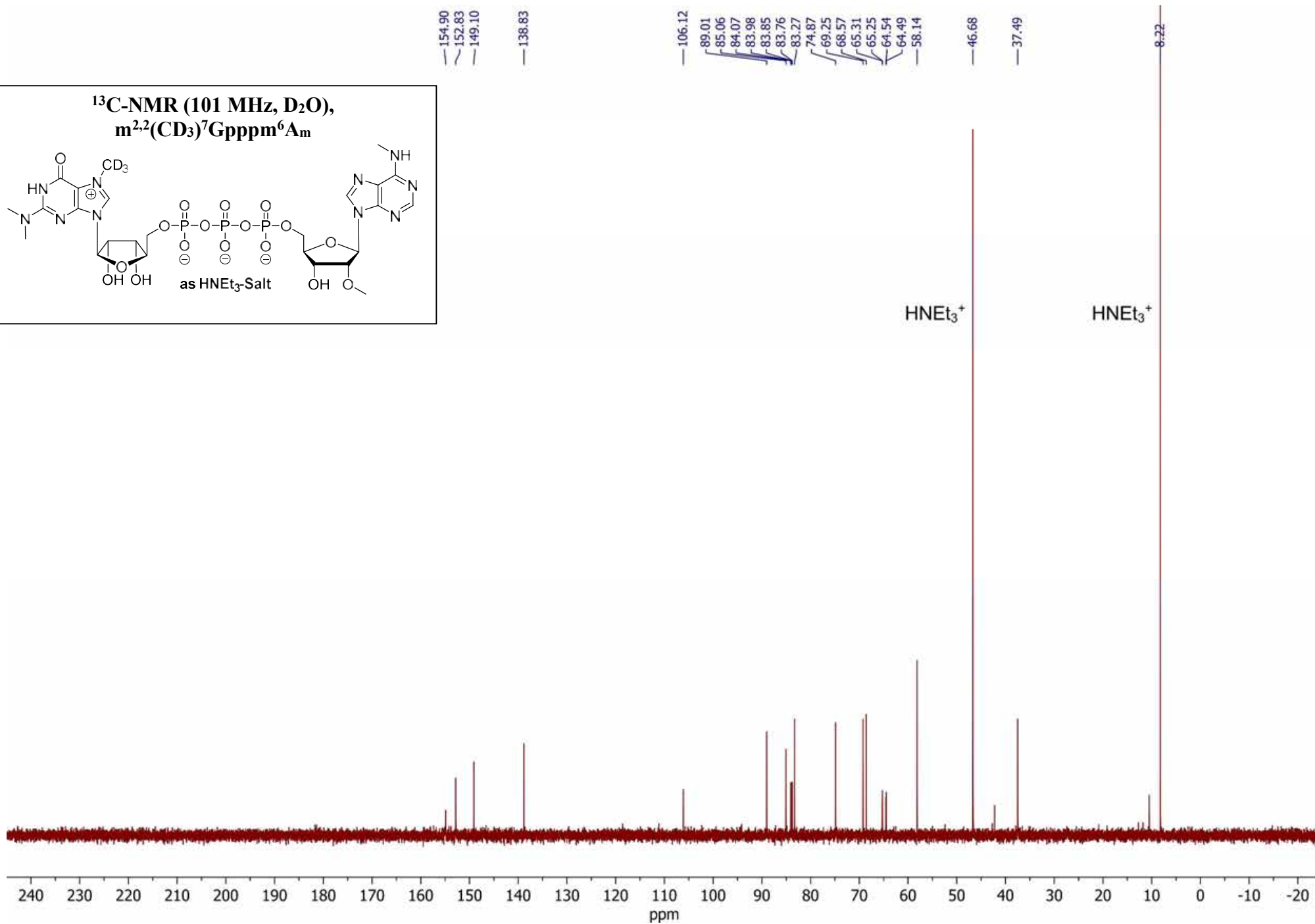
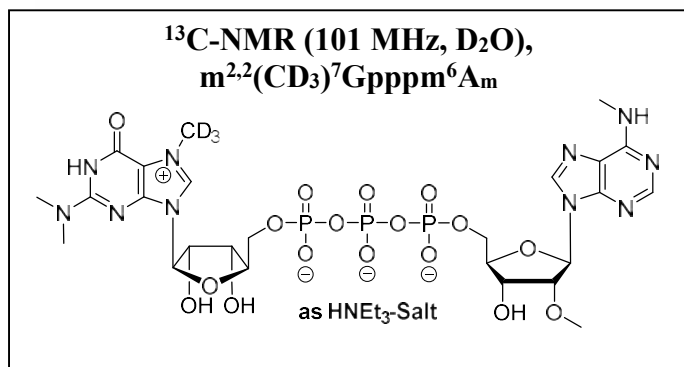
HNEt_3^+

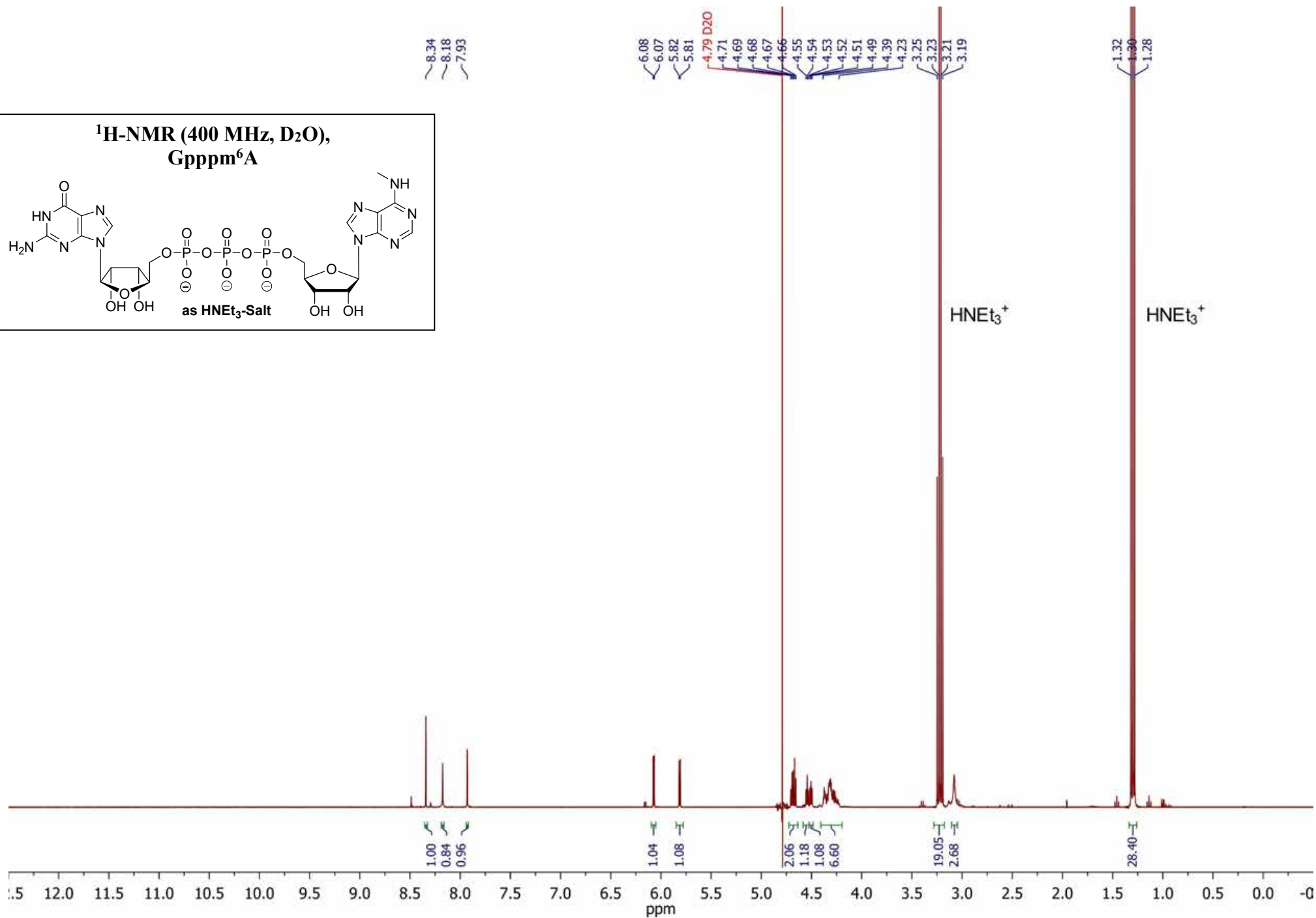
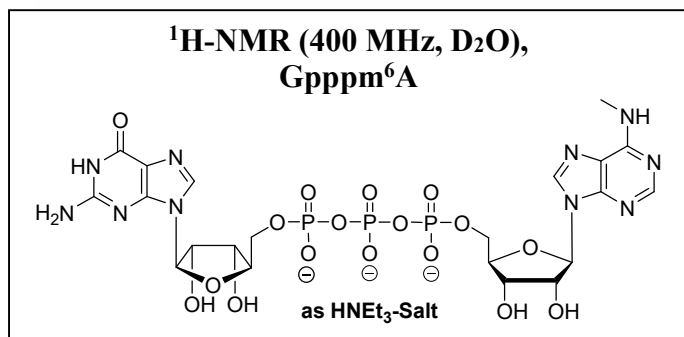
240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
ppm

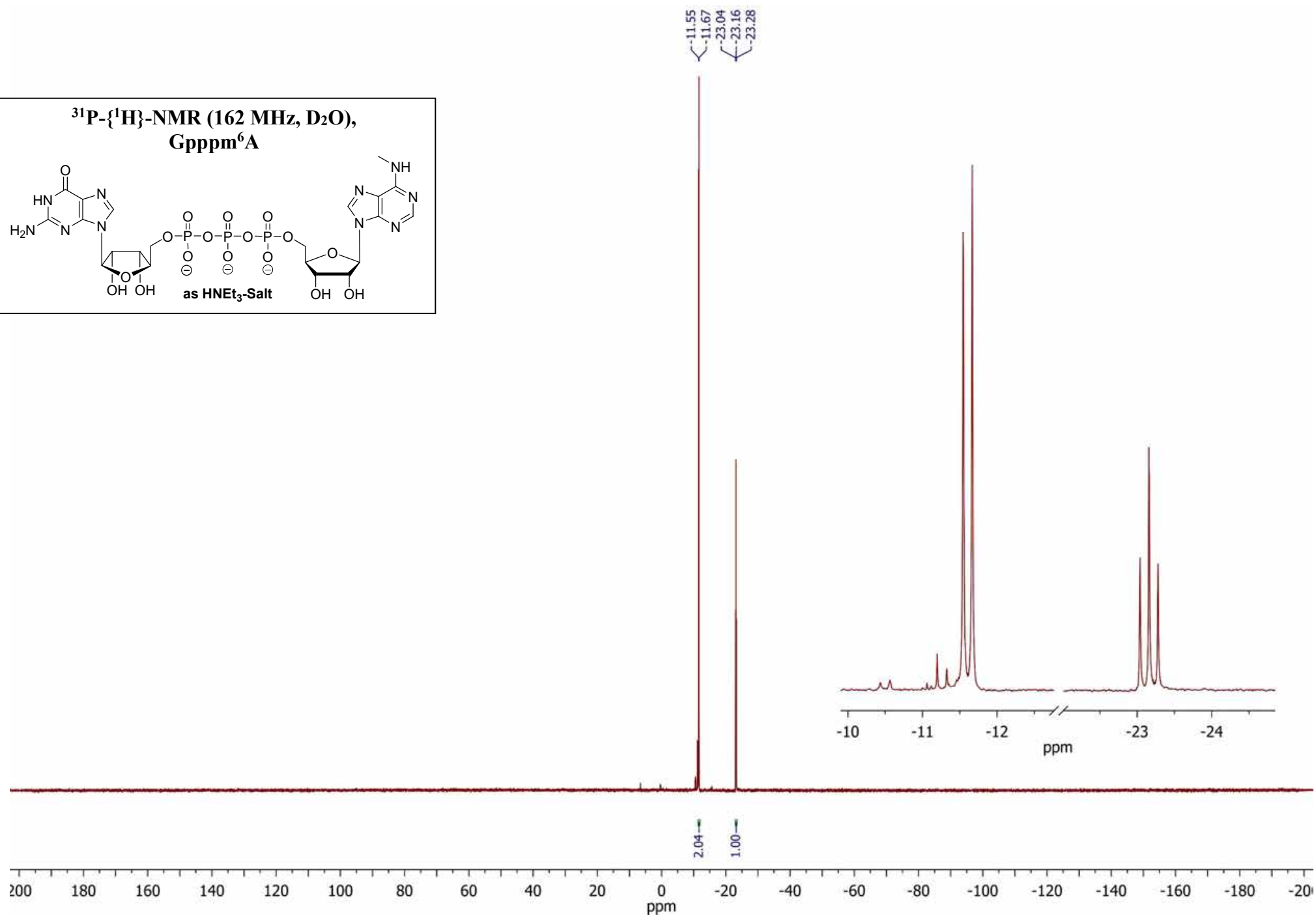
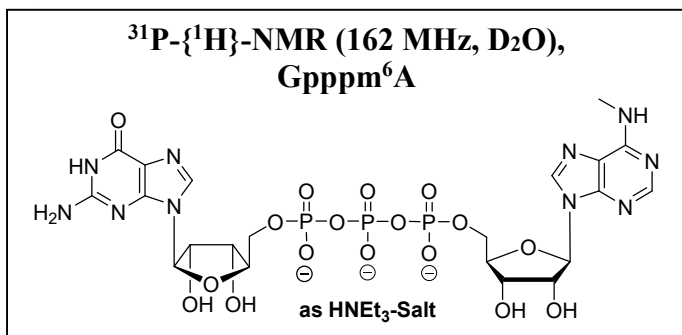
**^1H -NMR (400 MHz, D_2O),
 $\text{m}^{2,2}(\text{CD}_3)^7\text{Gpppm}^6\text{A}_\text{m}$**

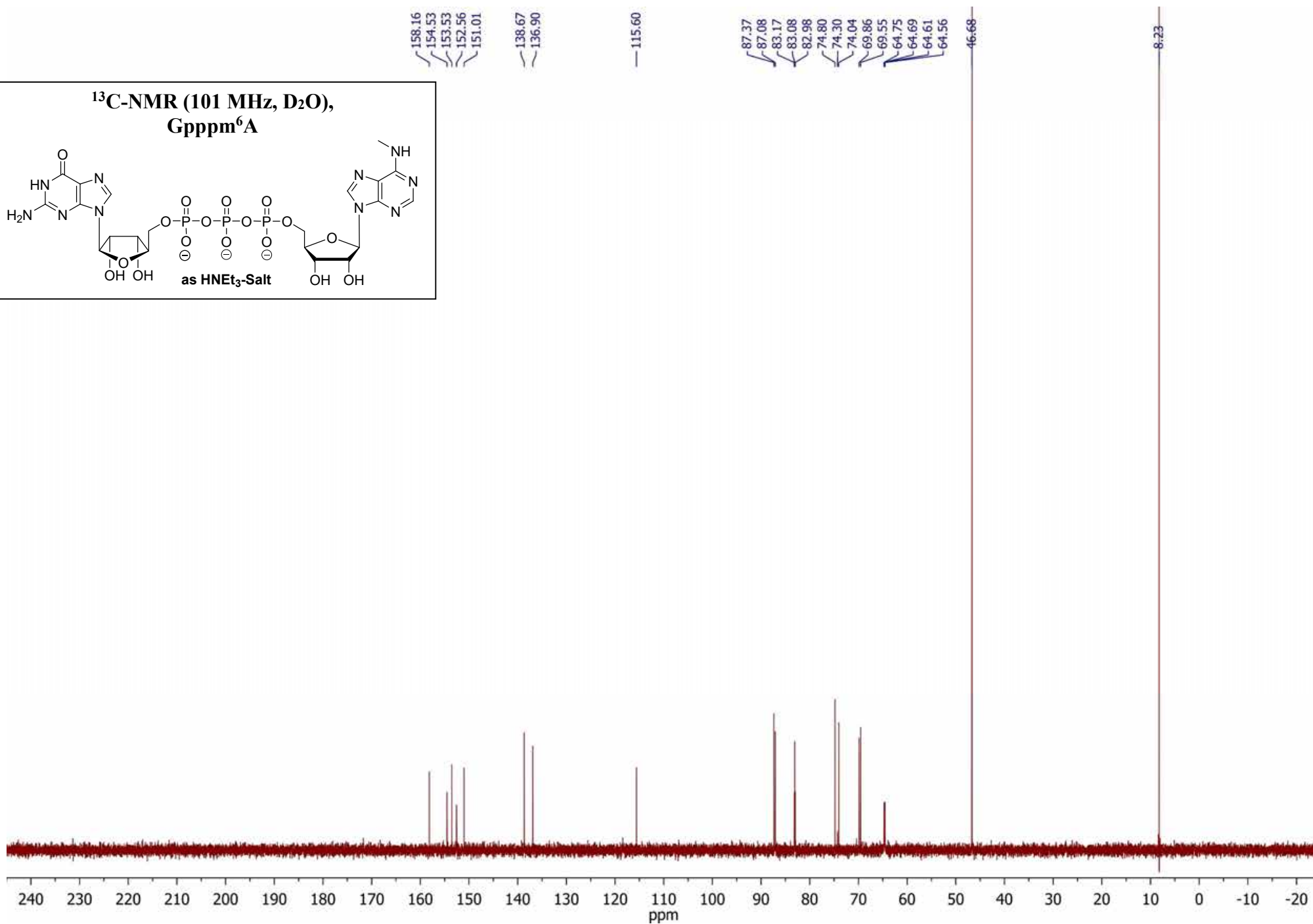
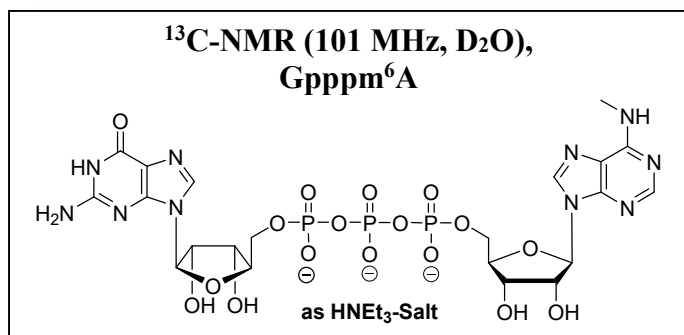


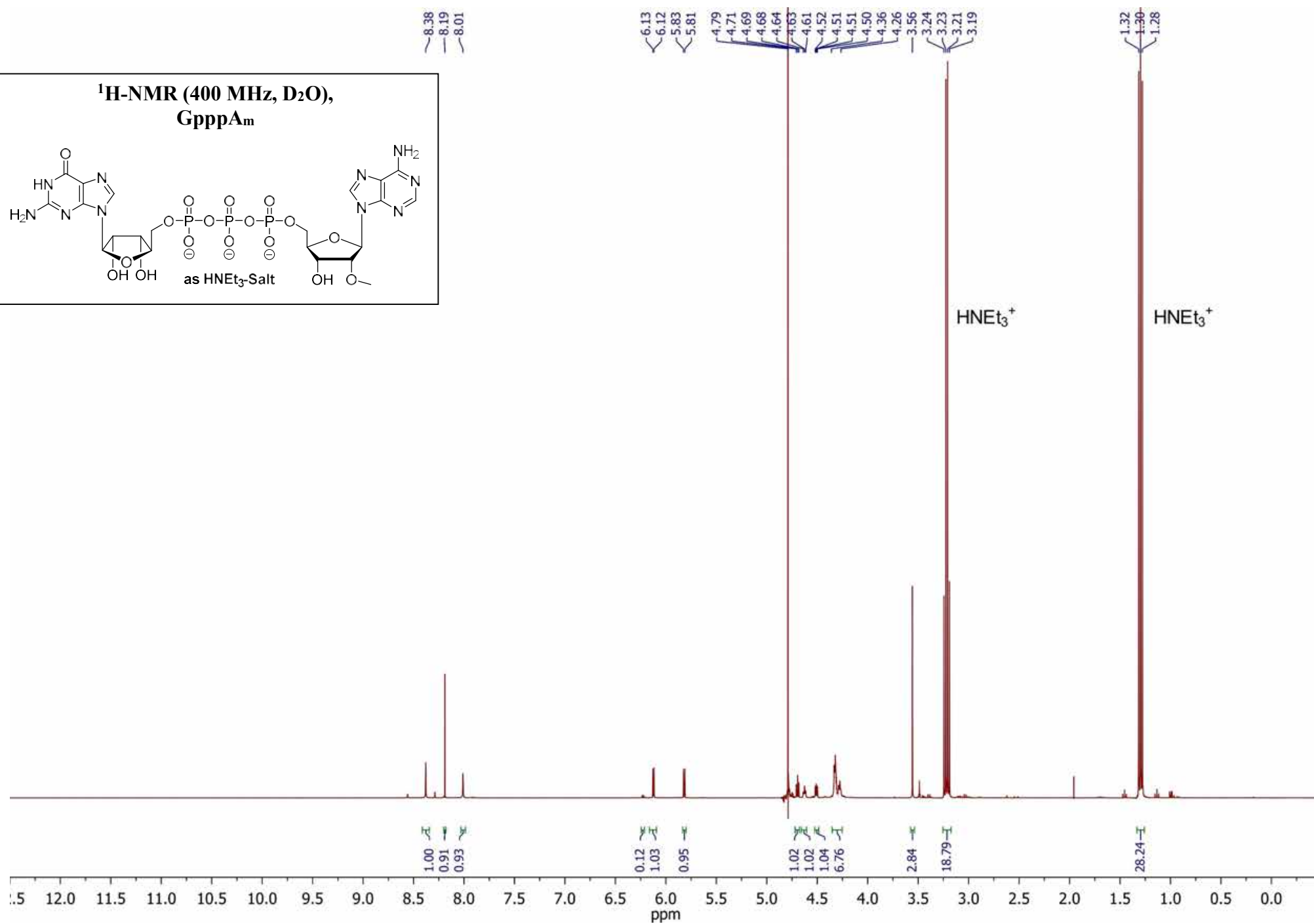
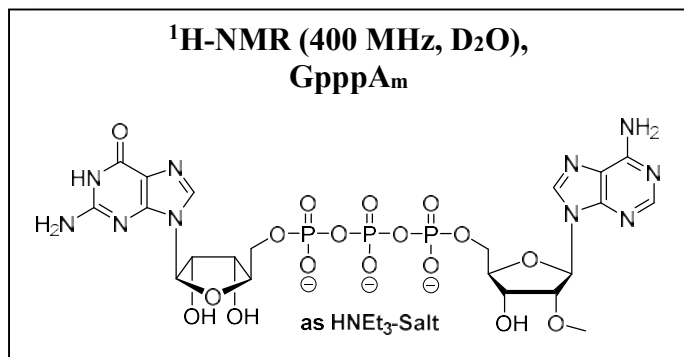






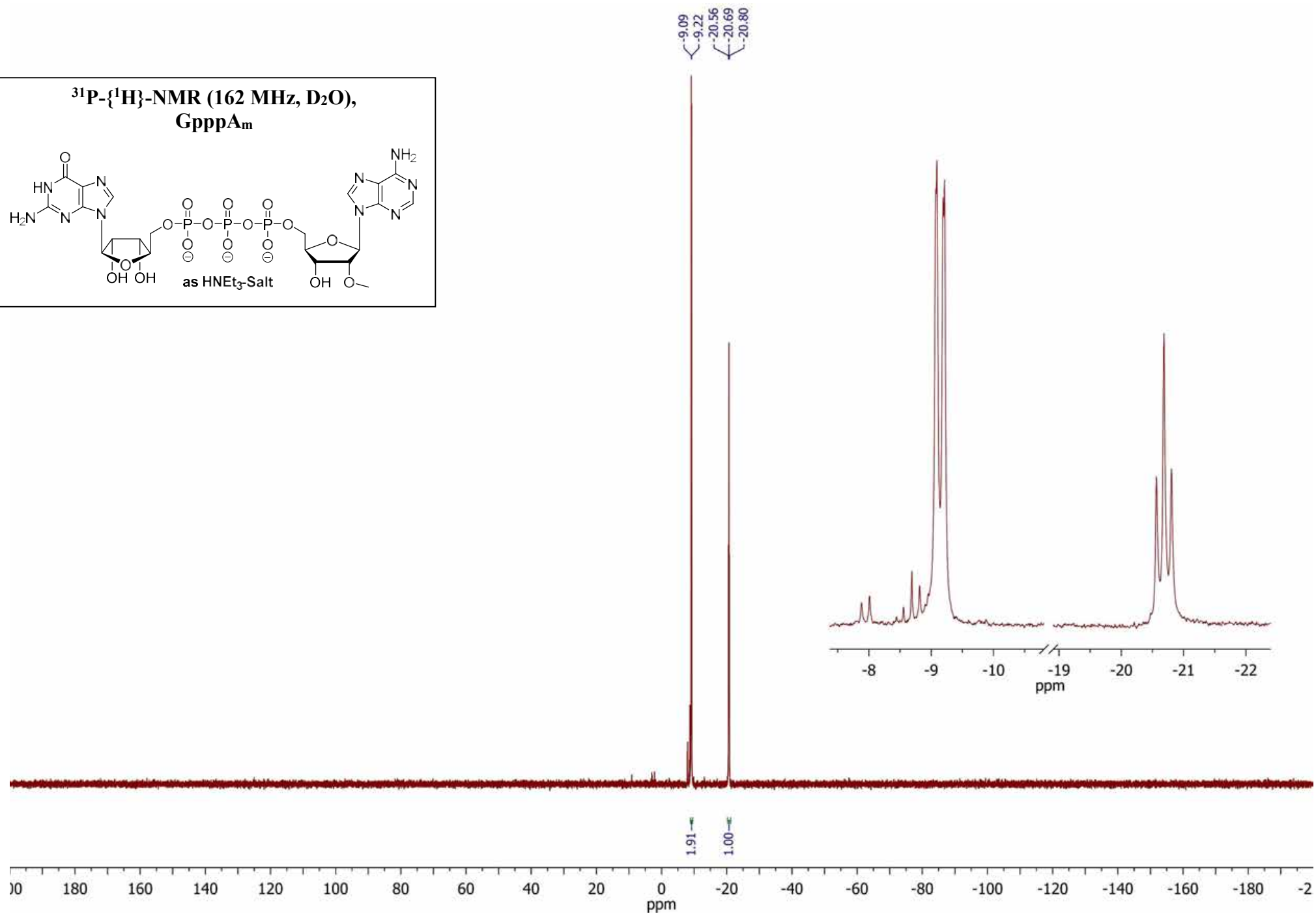
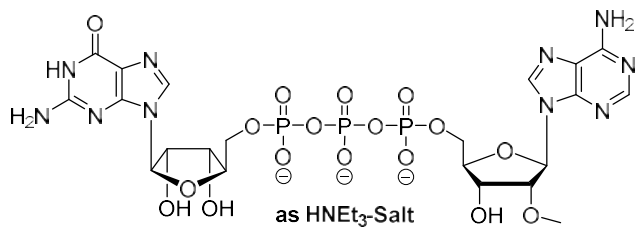




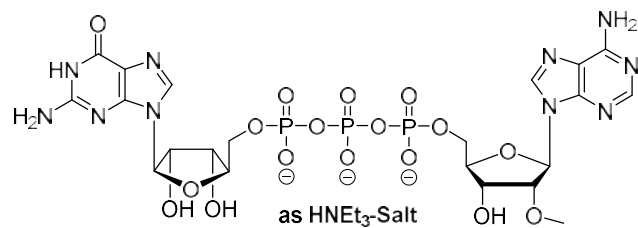


**^{31}P - $\{^1\text{H}\}$ -NMR (162 MHz, D_2O),
GpppA_m**

Chemical structure of GpppA_m (Guanosine-3'-phosphate-3'-phosphate-5'-phosphate-adenosine), showing the dinucleotide structure with a triphosphate group linking the 3' position of the deoxyribose sugar to the 5' position of the ribose sugar. The structure is labeled as HNEt₃-Salt.



**^{13}C -NMR (101 MHz, D_2O),
GpppA_m**



158.31
155.03
153.57
152.45
151.21
148.34

139.45
137.16

118.22
115.77

87.03
85.63
83.56
83.39
83.33
83.30
83.23
73.93
70.04
68.13
64.93
64.88
64.44
64.39
58.36

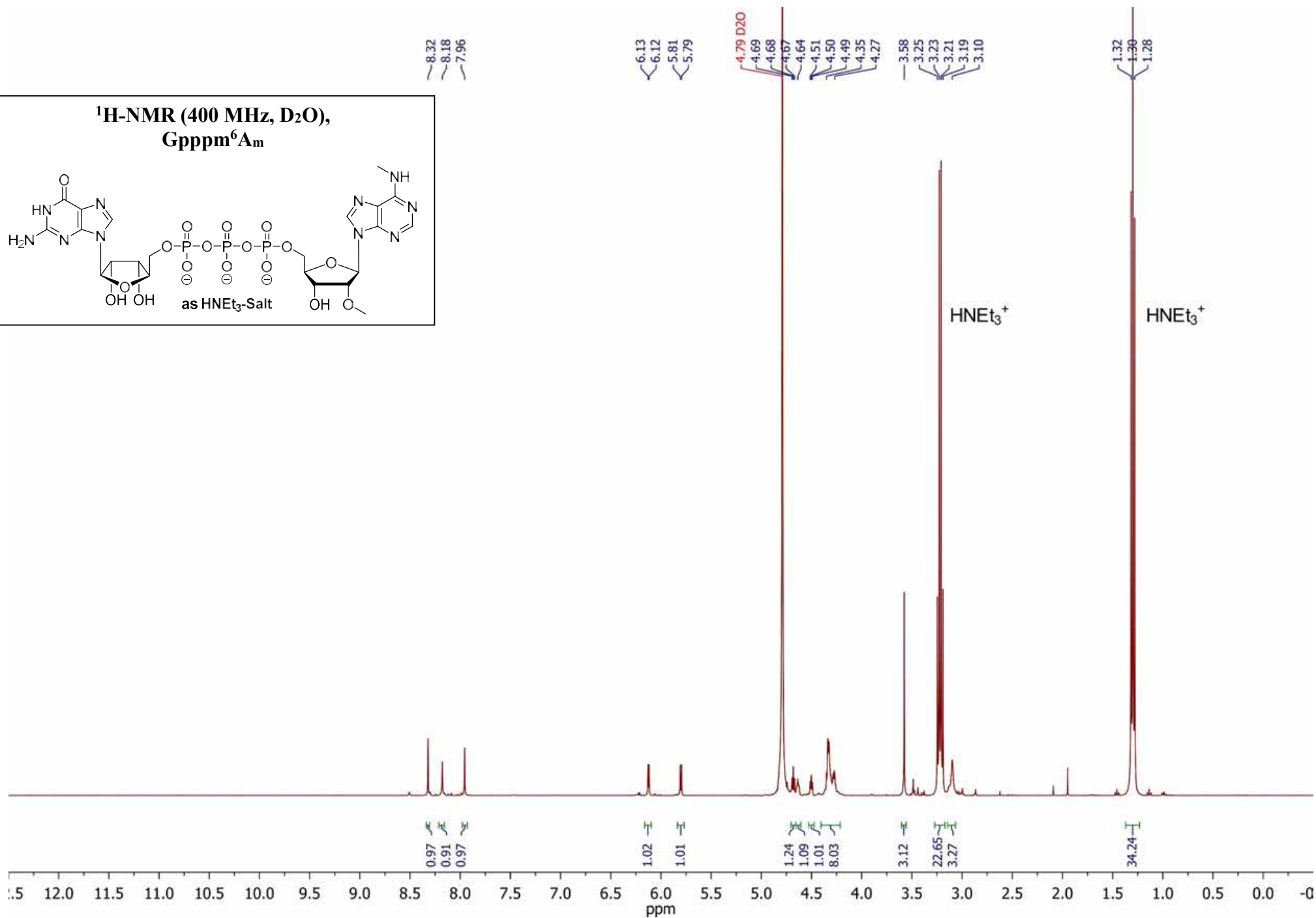
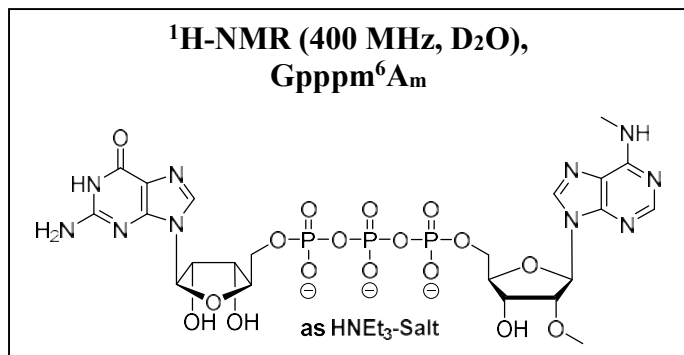
46.67

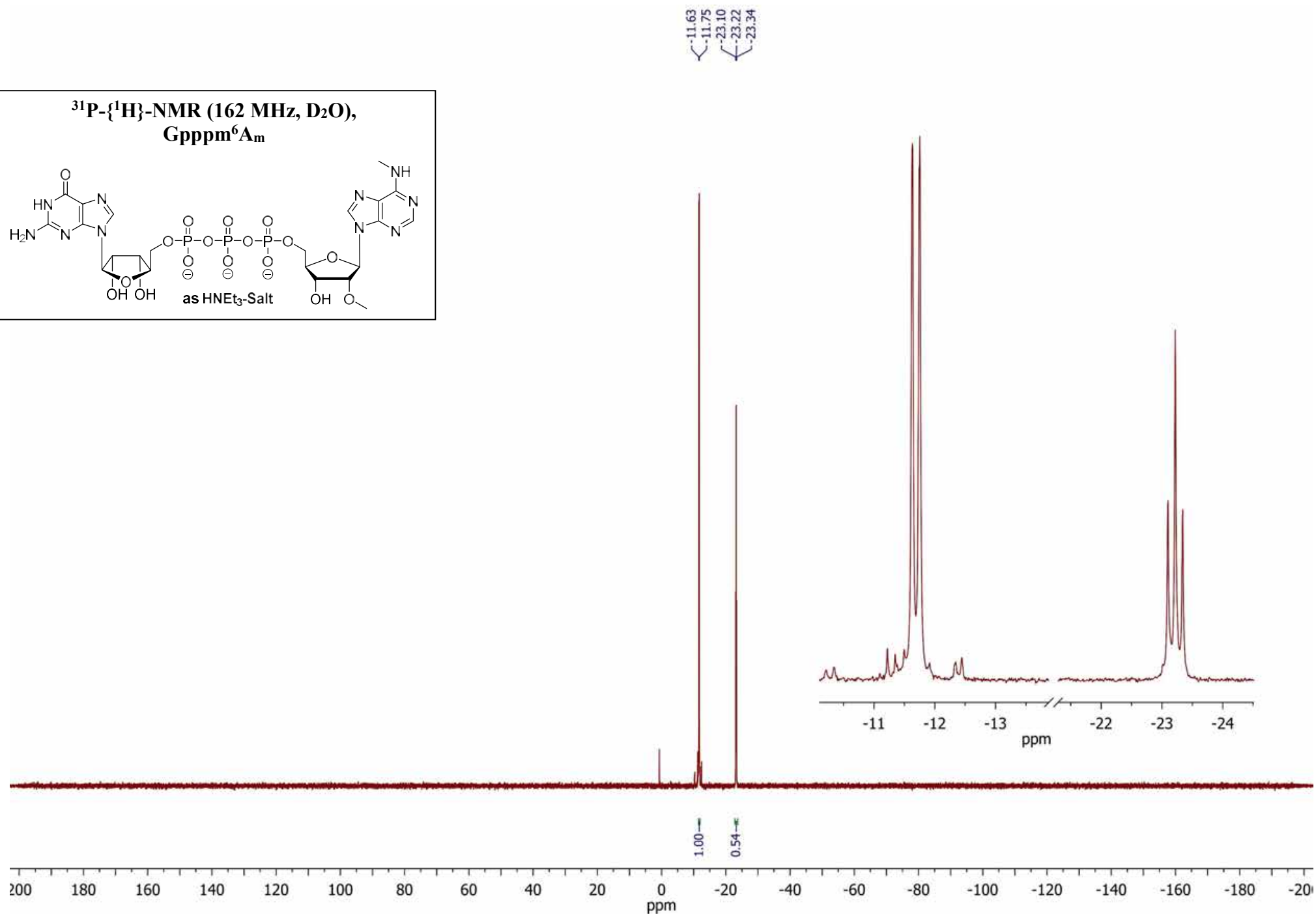
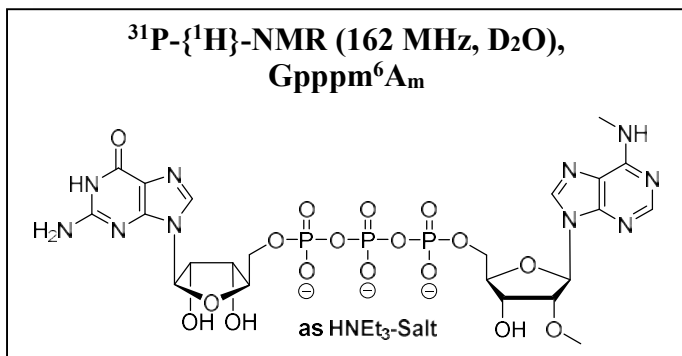
8.22

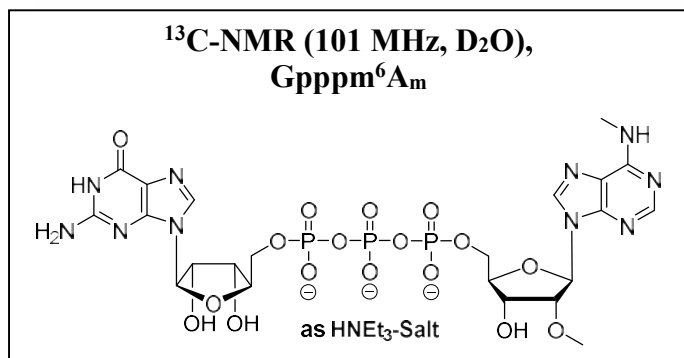
HNEt_3^+

HNEt_3^+

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







158.22
154.66
153.51
152.64
151.10

138.65
137.02

115.72

87.06
85.58
83.57
83.27
83.18
83.15
83.05
73.91
69.97
68.05
64.86
64.80
64.35
64.30
58.39

46.67

8.22

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

2 HRMS-Data:

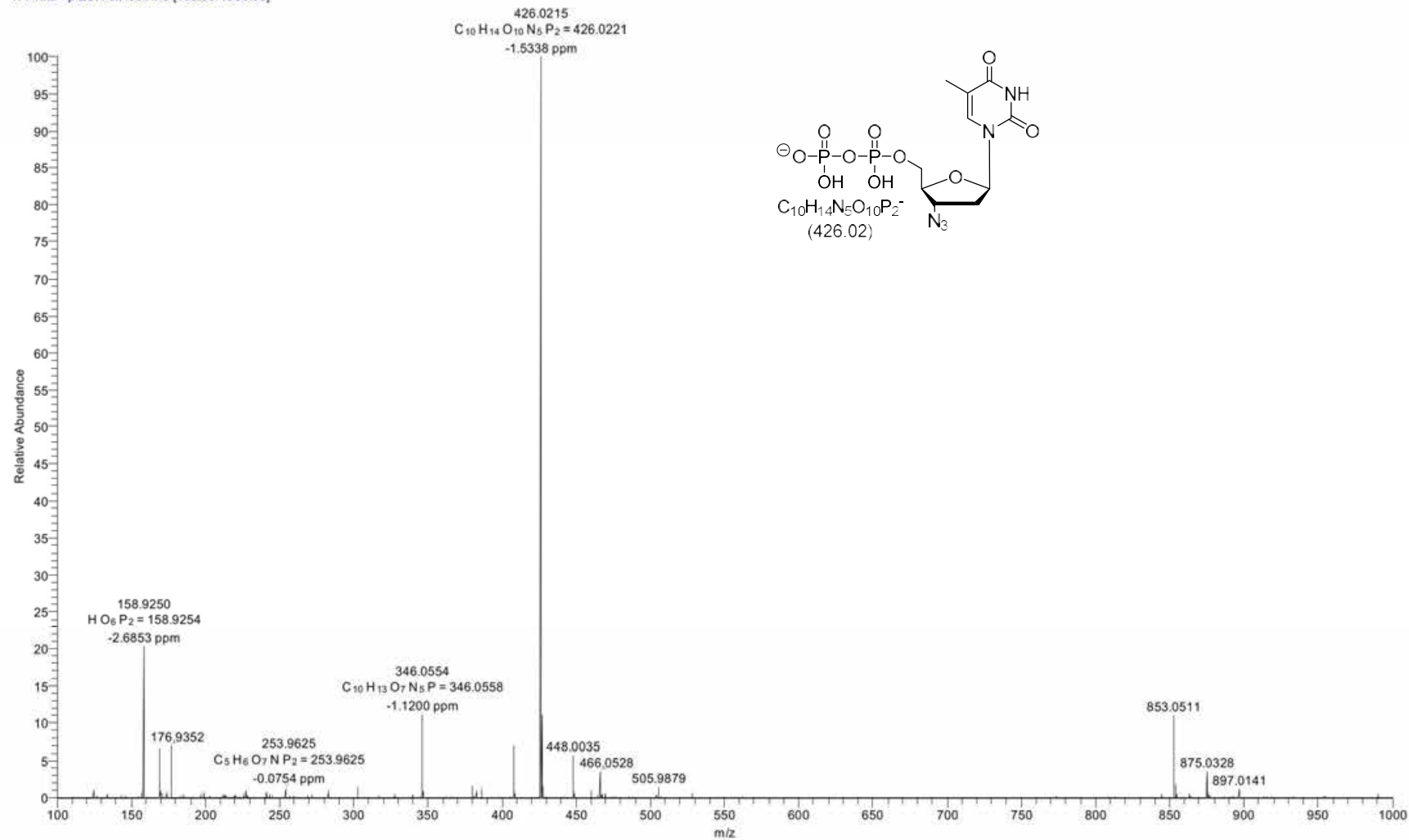
HRMS (ESI) analysis of 3'-azidothymidine 5'-diphosphate (07-O):

D:\data_2022\bjea22shr1

10/18/2022 11:45:16 AM

bv64

bjea22shr1 #1 RT: 0.02 AV: 1 NL: 7.84E6
T: FTMS - p ESI Full lock ms [100.00-1000.00]



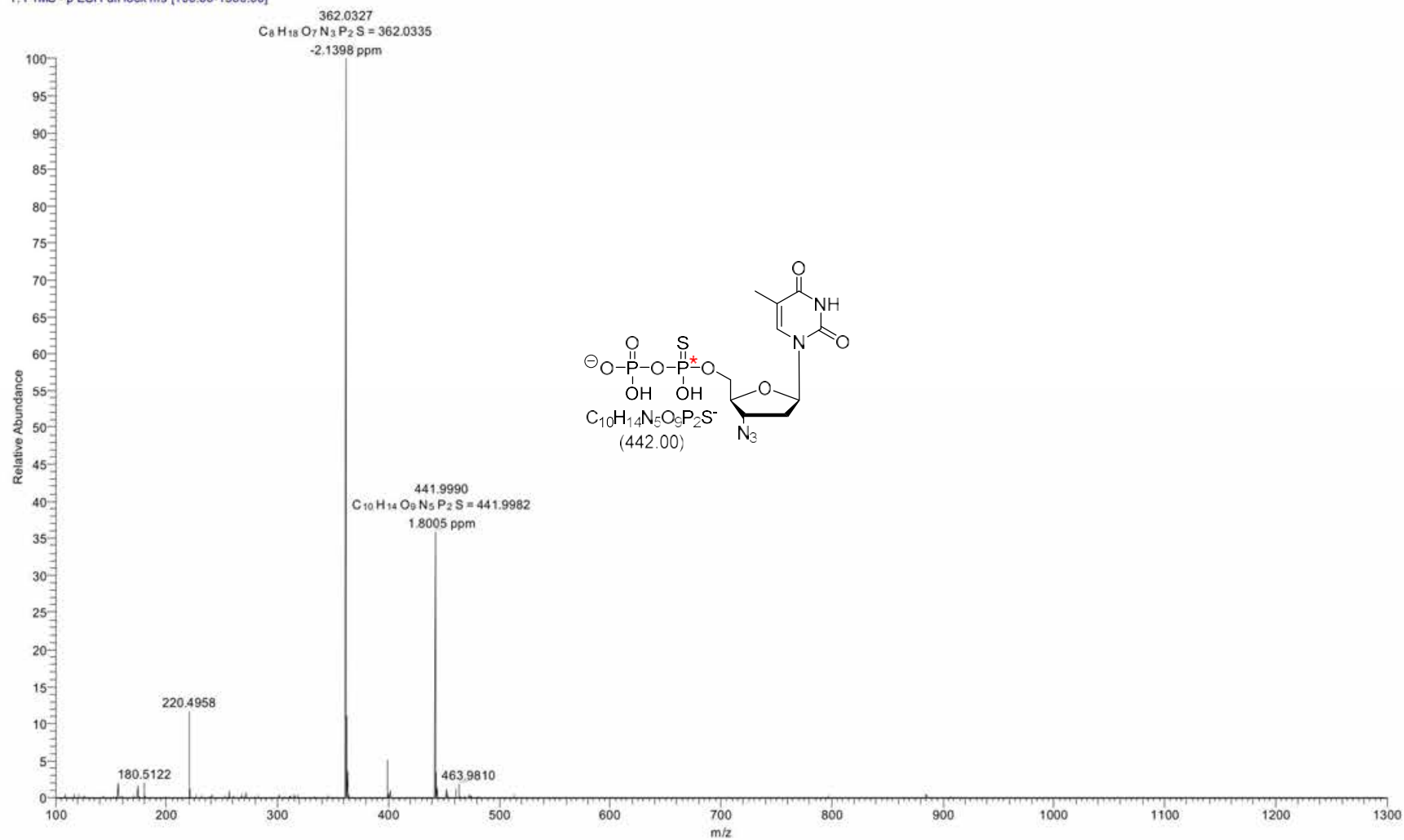
HRMS (ESI) analysis of 3'-azidothymidine 5'- α -S-diphosphate (07-O):

D:\data_2022\bjea25shr1

11/8/2022 12:22:13 PM

bv132

bjea25shr1 #1 RT: 0.02 AV: 1 NL: 2.52E8
T: FTMS - p ESI Full lock ms [100.00-1300.00]



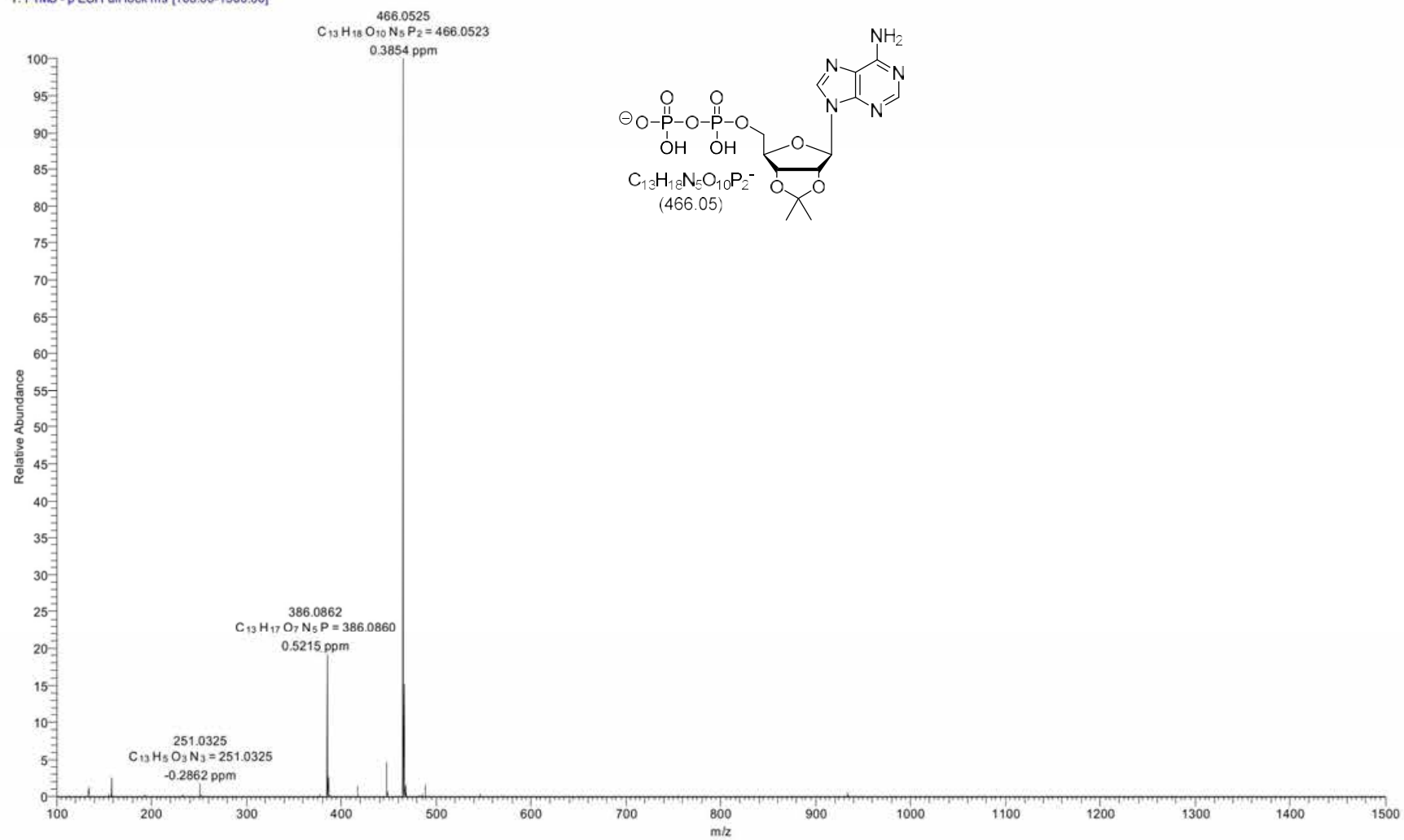
HRMS (ESI) analysis of 2',3'-O-isopropylidene adenosine 5'-diphosphate (08-O):

D:\data_2022\b\jea18shr1

10/14/2022 11:31:42 AM

bv107

b\jea18shr1 #1 RT: 0.02 AV: 1 NL: 3.16E8
T: FTMS - p ESI Full lock ms [100.00-1500.00]



HRMS (ESI) analysis of 2',3'-O-isopropylidene adenosine 5'- α -S-diphosphate (08-S):

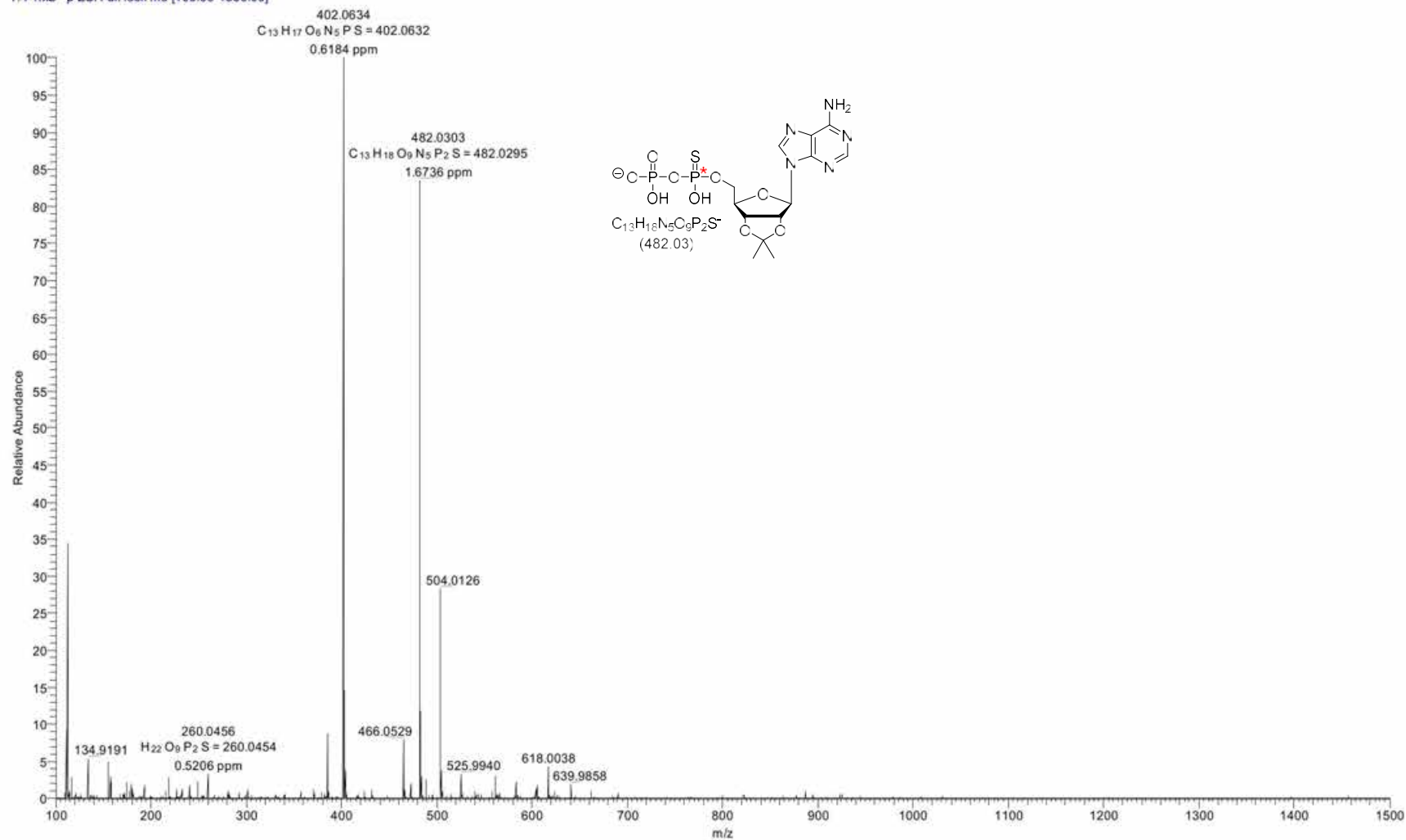
D:\data_2022\bjea19shr1

10/14/2022 11:40:30 AM

bv126

bjea19shr1 #1 RT: 0.02 AV: 1 NL: 2.48E7

T: FTMS - p ESI Full lock ms [100.00-1500.00]



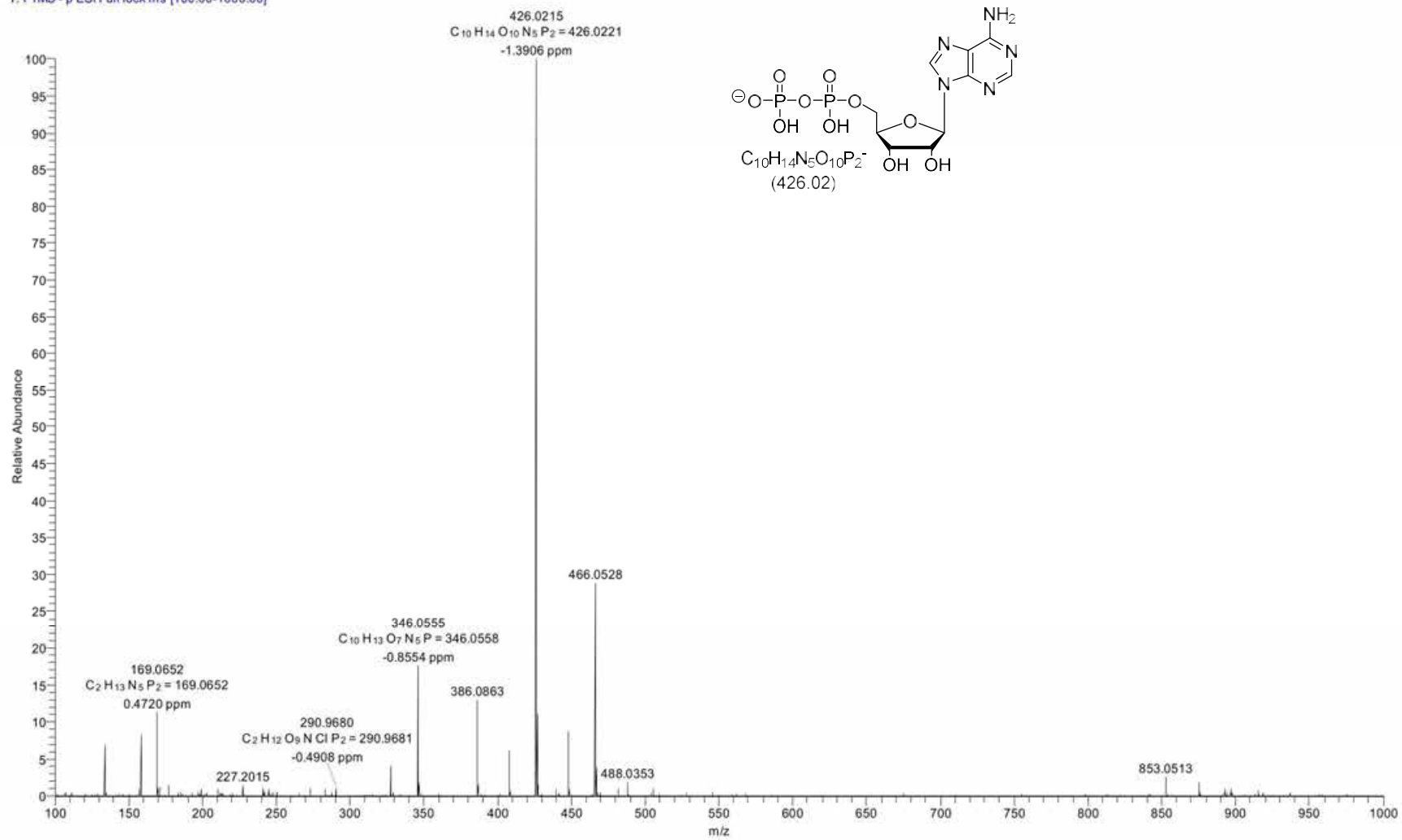
HRMS (ESI) analysis of adenosine 5'-diphosphate (09-O):

D:\data_2022\b\jea21shr1

10/18/2022 11:36:24 AM

bv63

b\jea21shr1 #1 RT: 0.02 AV: 1 NL: 6.18E6
T: FTMS - p ESI Full lock ms [100.00-1000.00]



HRMS (ESI) analysis of adenosine 5'- α -S-diphosphate (09-S):

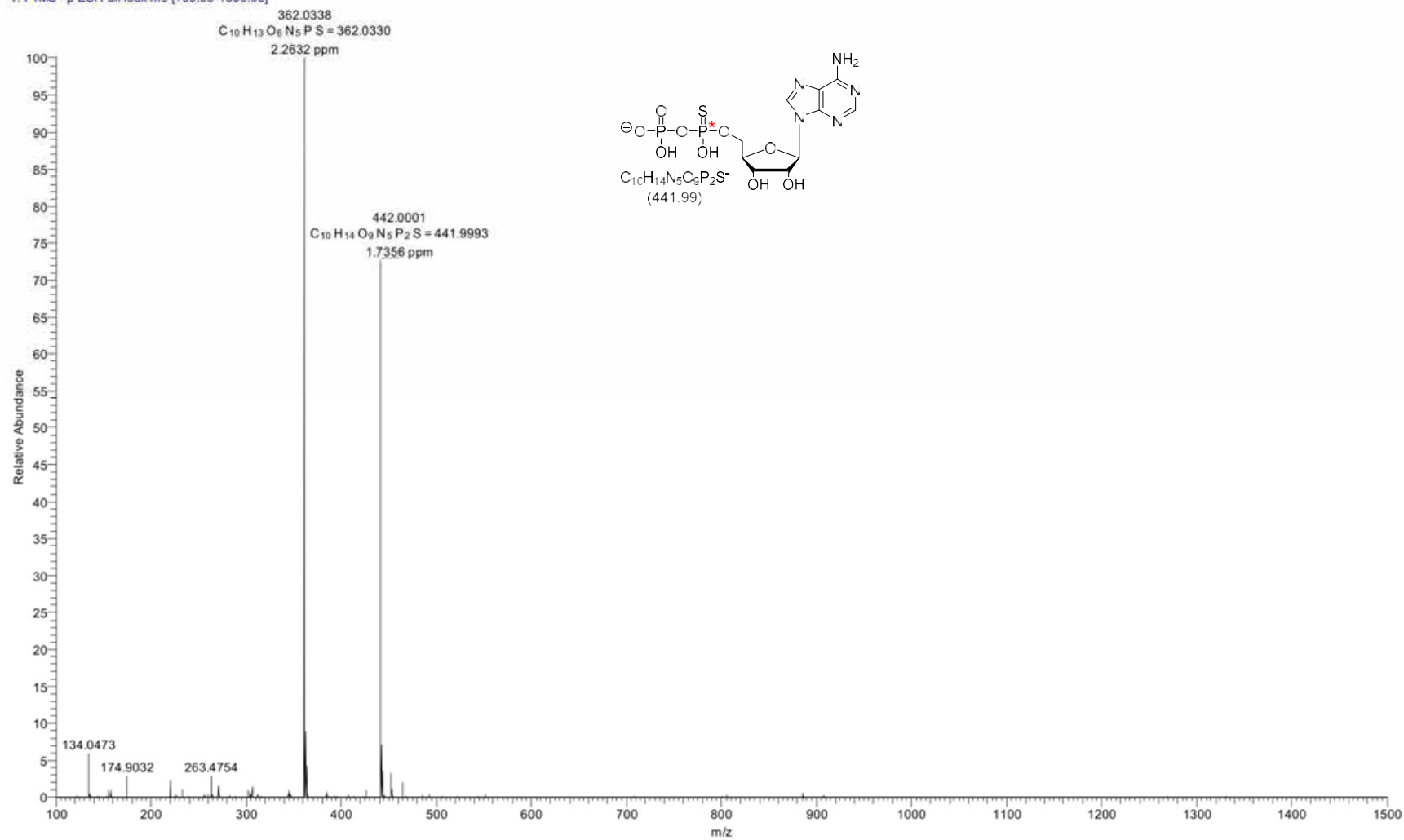
D:\data_2022\bjea32shr2

11/17/2022 11:27:44 AM

bv134a

bjea32shr2 #1 RT: 0.02 AV: 1 NL: 6.09E8

T: FTMS - p ESI Full lock ms [100.00-1500.00]



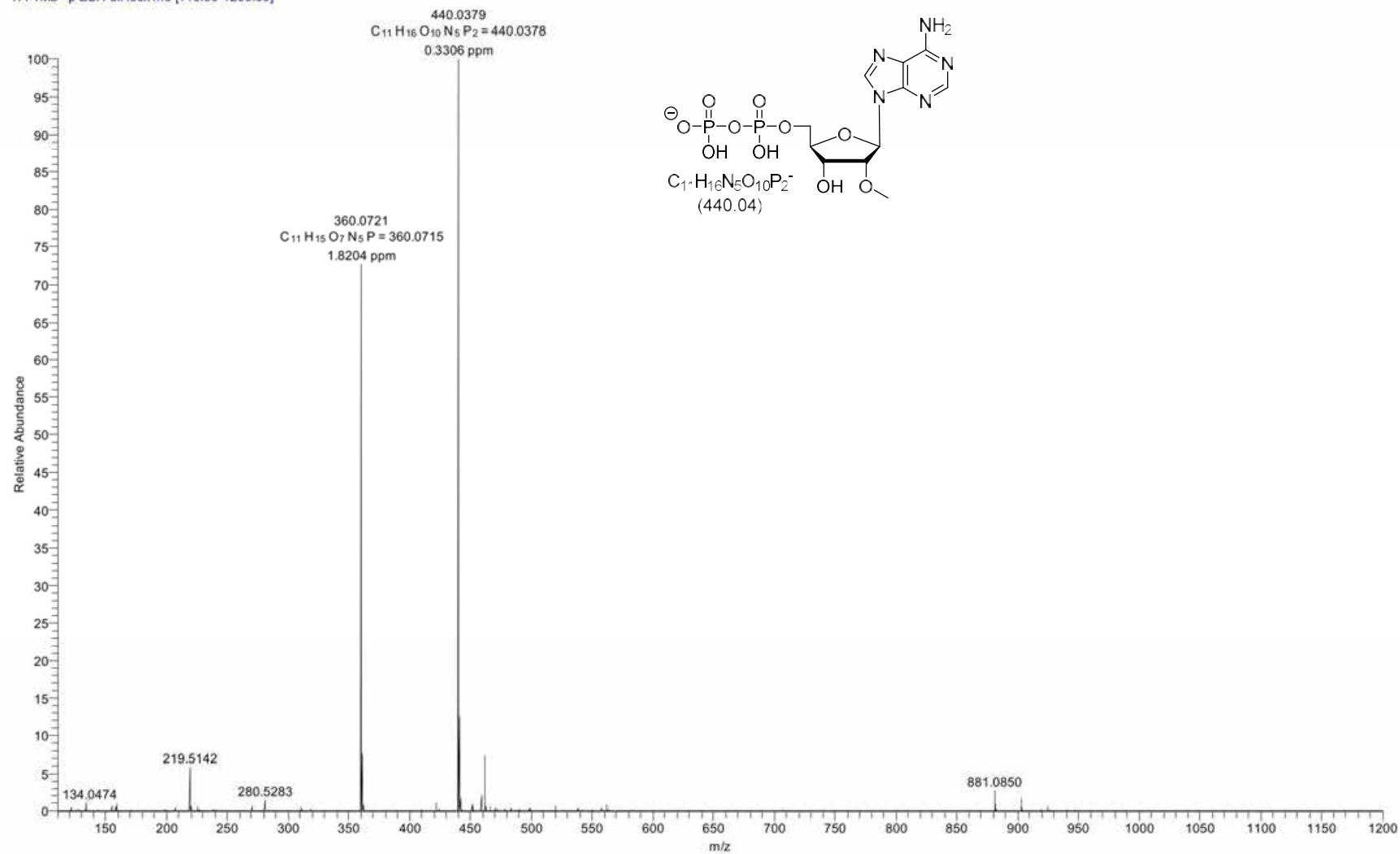
HRMS (ESI) analysis of 2'-O-methyladenosine 5'-diphosphate (10):

D:\data_2022\vpjeb14shr2

3/15/2022 4:24:56 PM

ar195

vpjeb14shr2 #1 RT: 0.02 AV: 1 NL: 5.53E7
T: FTMS - p ESI Full lock ms [110.00-1200.00]



HRMS (ESI) analysis of N⁶-methyladenosine 5'-diphosphate (11):

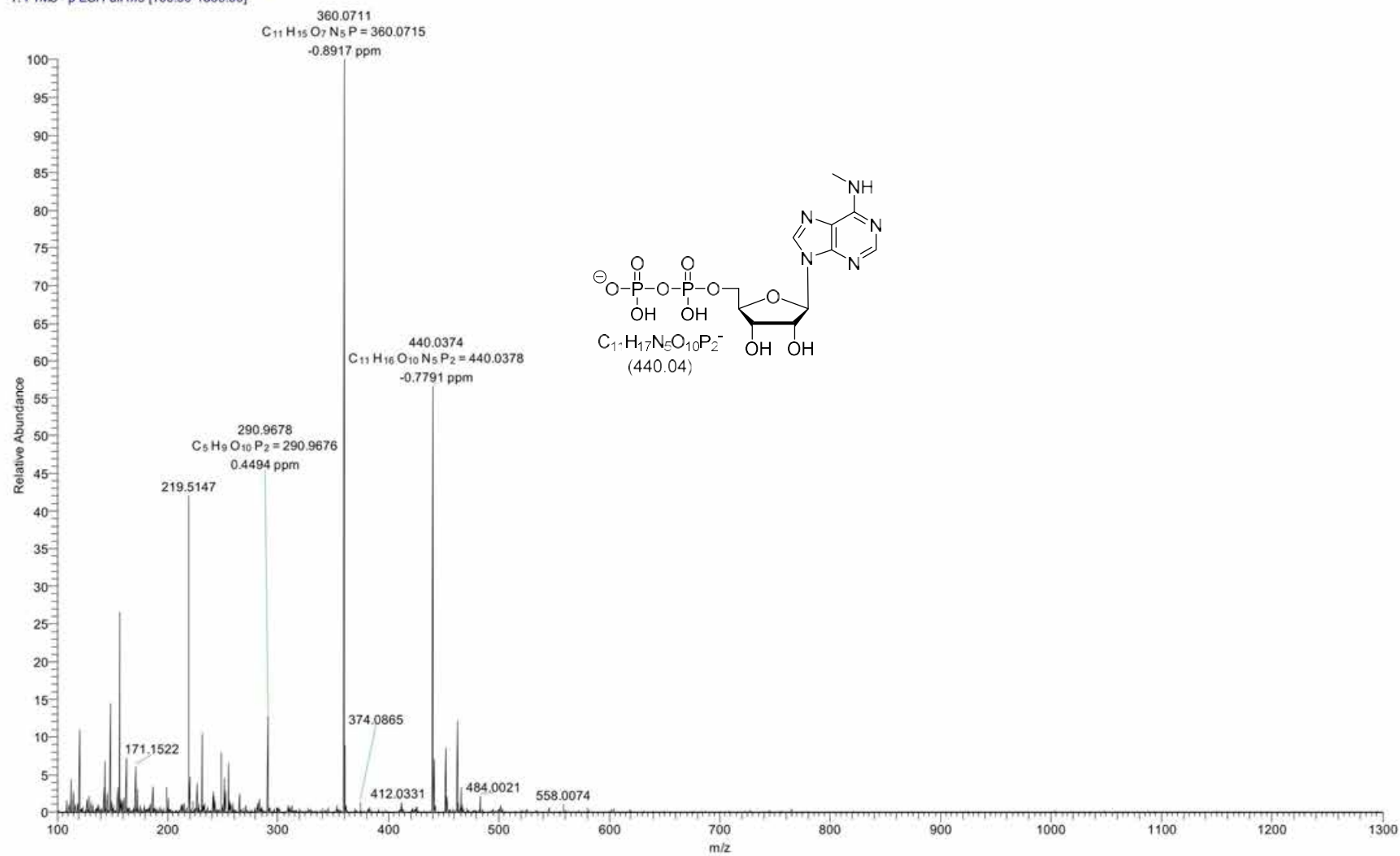
D:\data_2022\vpjeb38shr1

6/23/2022 9:23:14 AM

ar225

vpjeb38shr1 #1 RT: 0.02 AV: 1 NL: 2.69E6

T: FTMS - p ESI Full ms [100.00-1300.00]



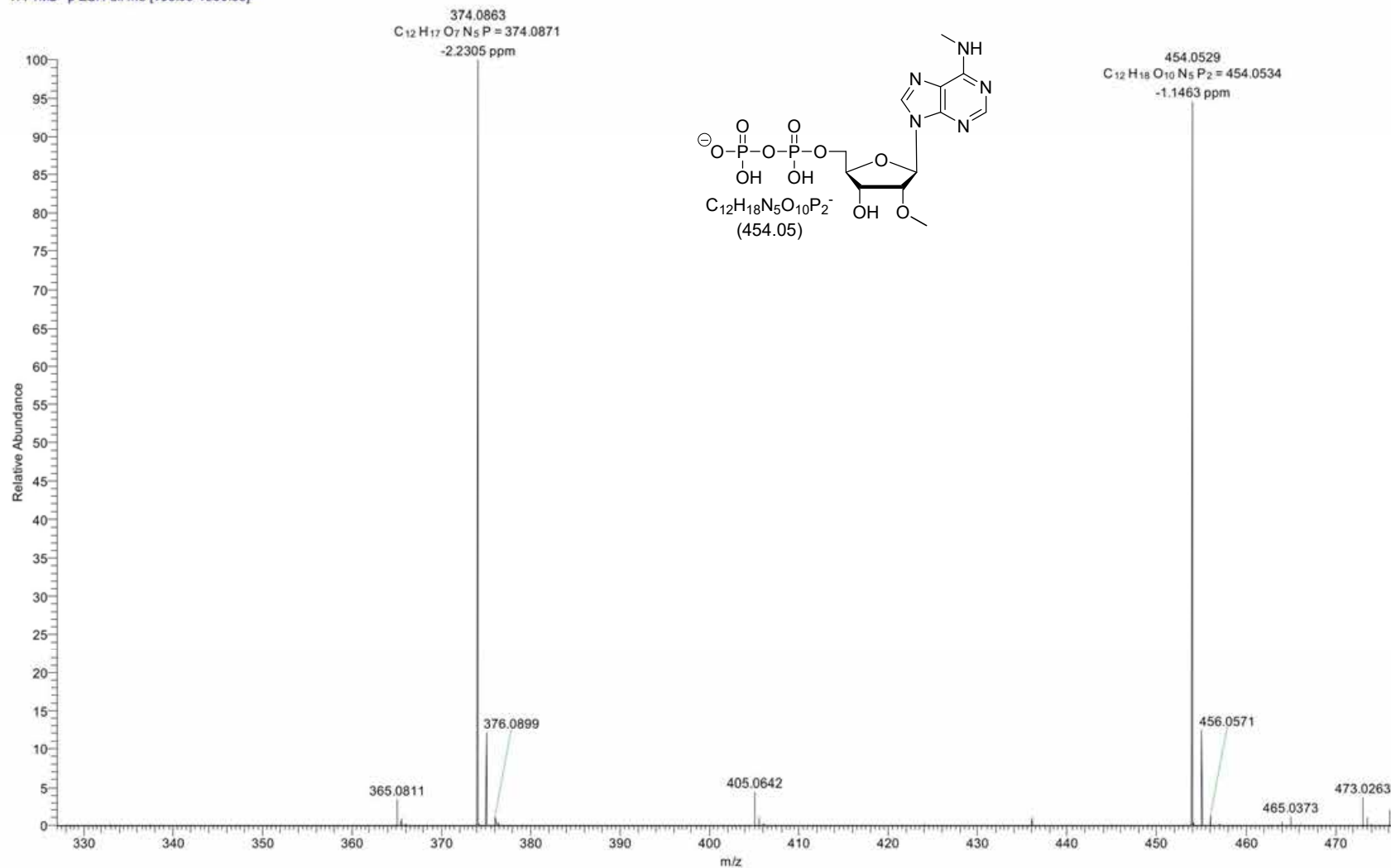
HRMS (ESI) analysis of 2'-O-methyl-N⁶-methyladenosine 5'-diphosphate (12):

D:\data_2022\vpjeb37shr1

6/20/2022 2:10:16 PM

ar236

rpjeb37shr1 #1 RT: 0.02 AV: 1 NL: 1.51E8
T: FTMS - p ESI Full ms [100.00-1000.00]



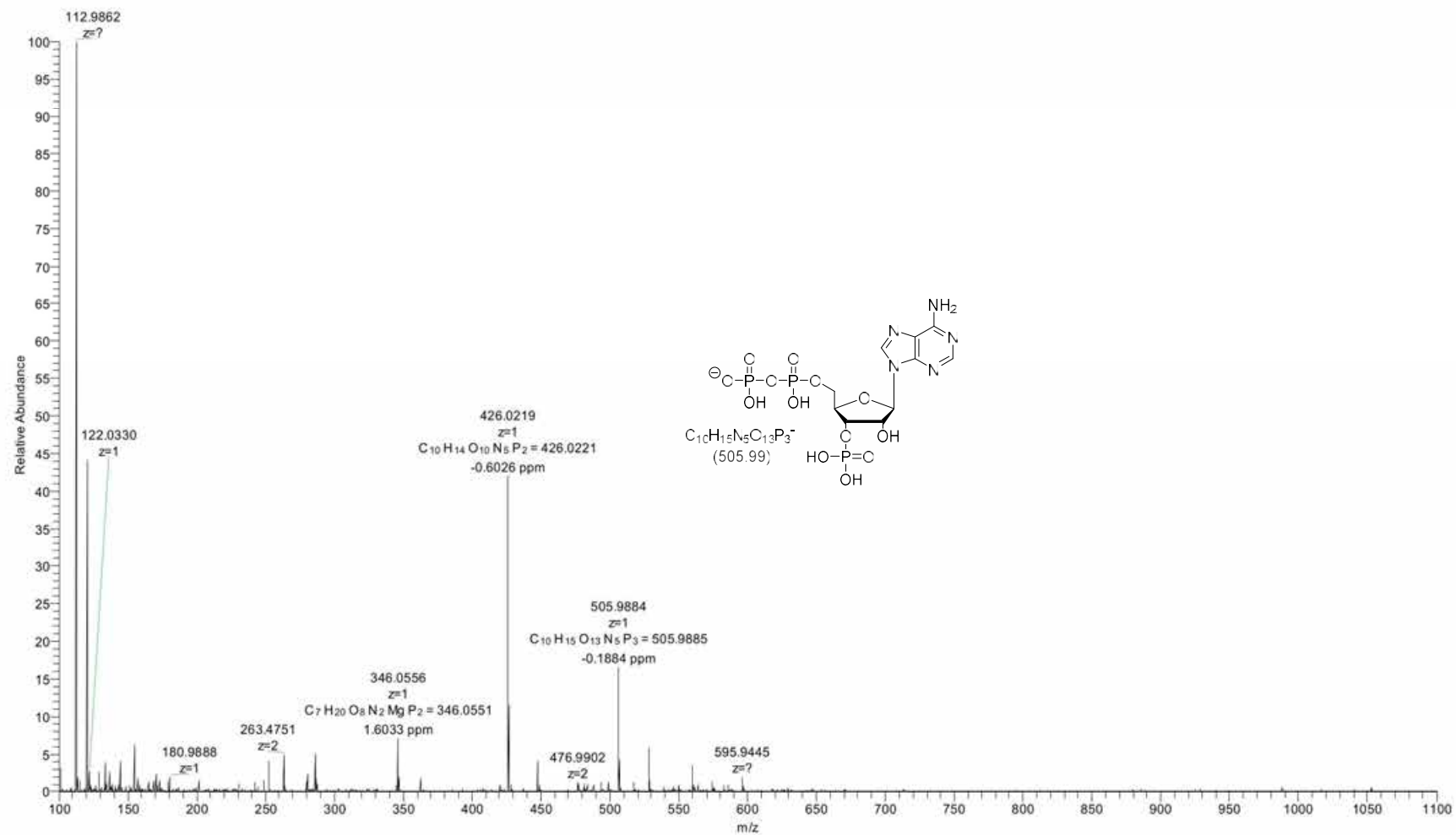
HRMS (ESI) analysis of adenosine 3'-phosphate 5'-diphosphate (14):

D:\data_2024\mpjea38shr1

1/3/2024 3:09:43 PM

ppAp

mpjea38shr1 #1 RT: 0.02 AV: 1 NL: 4.09E6
T: FTMS - p ESI Full lock ms [100.00-1100.00]



HRMS (ESI) analysis of *trans*-geranyl diphosphate (SI-06):

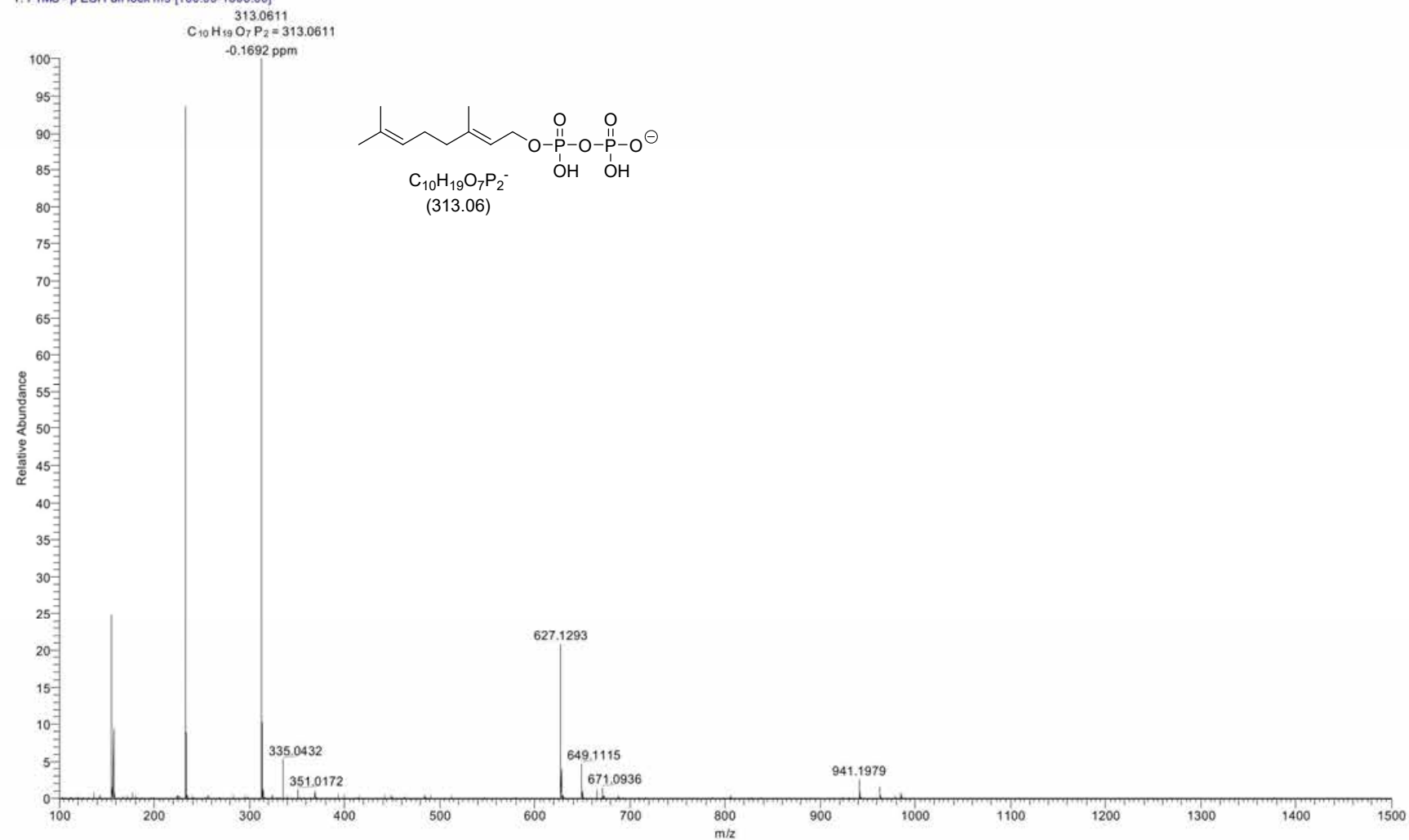
D:\data_2022\b\jea31shr1

11/17/2022 11:02:49 AM

bv36

b\jea31shr1 #1 RT: 0.02 AV: 1 NL: 4.60E8

T: FTMS - p ESI Full lock ms [100.00-1500.00]



HRMS (ESI) analysis of isopentenyl diphosphate (SI-07):

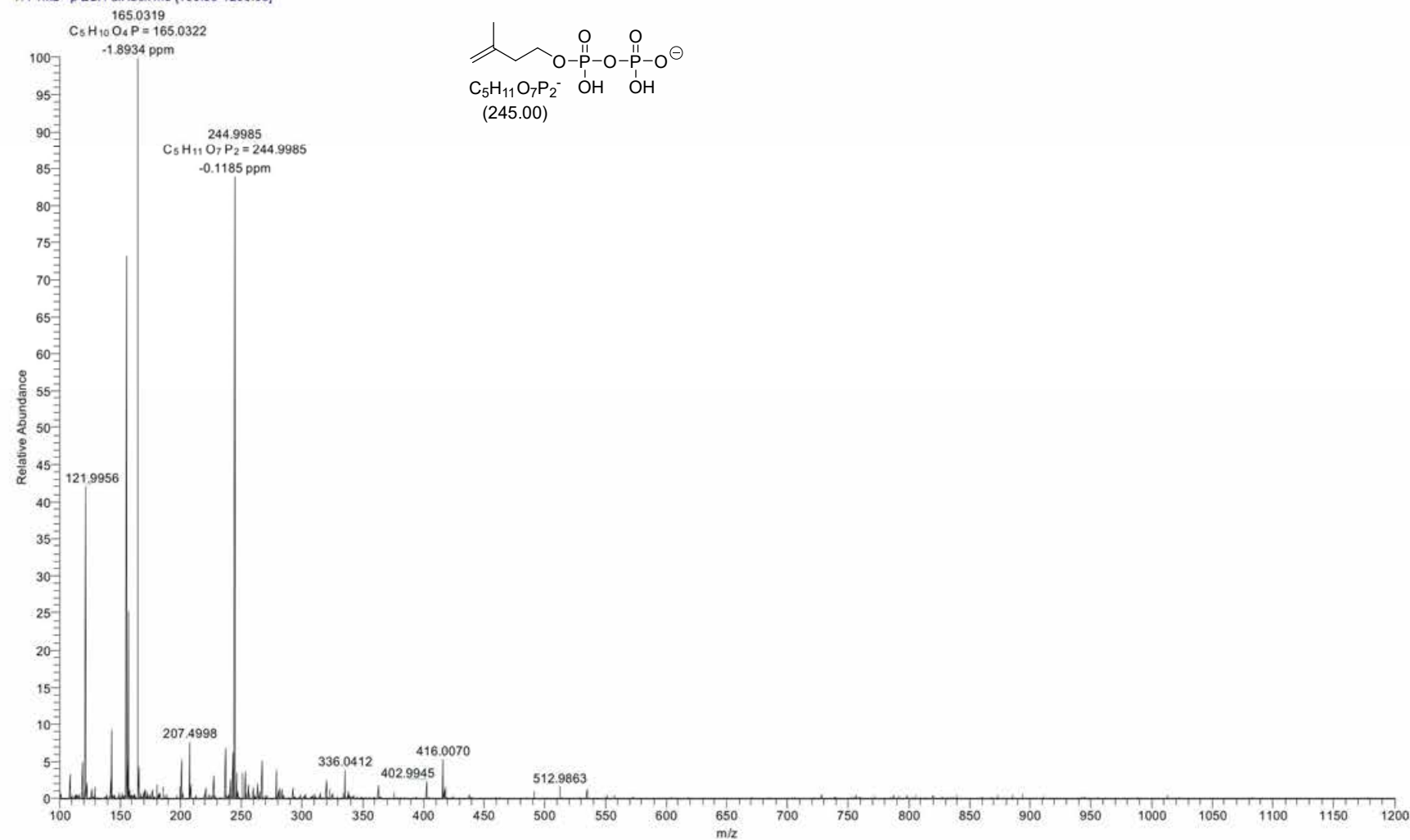
D:\data_2022\bjea27shr1

11/11/2022 11:38:05 AM

bv141

bjea27shr1 #1 RT: 0.02 AV: 1 NL: 3.89E7

T: FTMS - p ESI Full lock ms [100.00-1200.00]



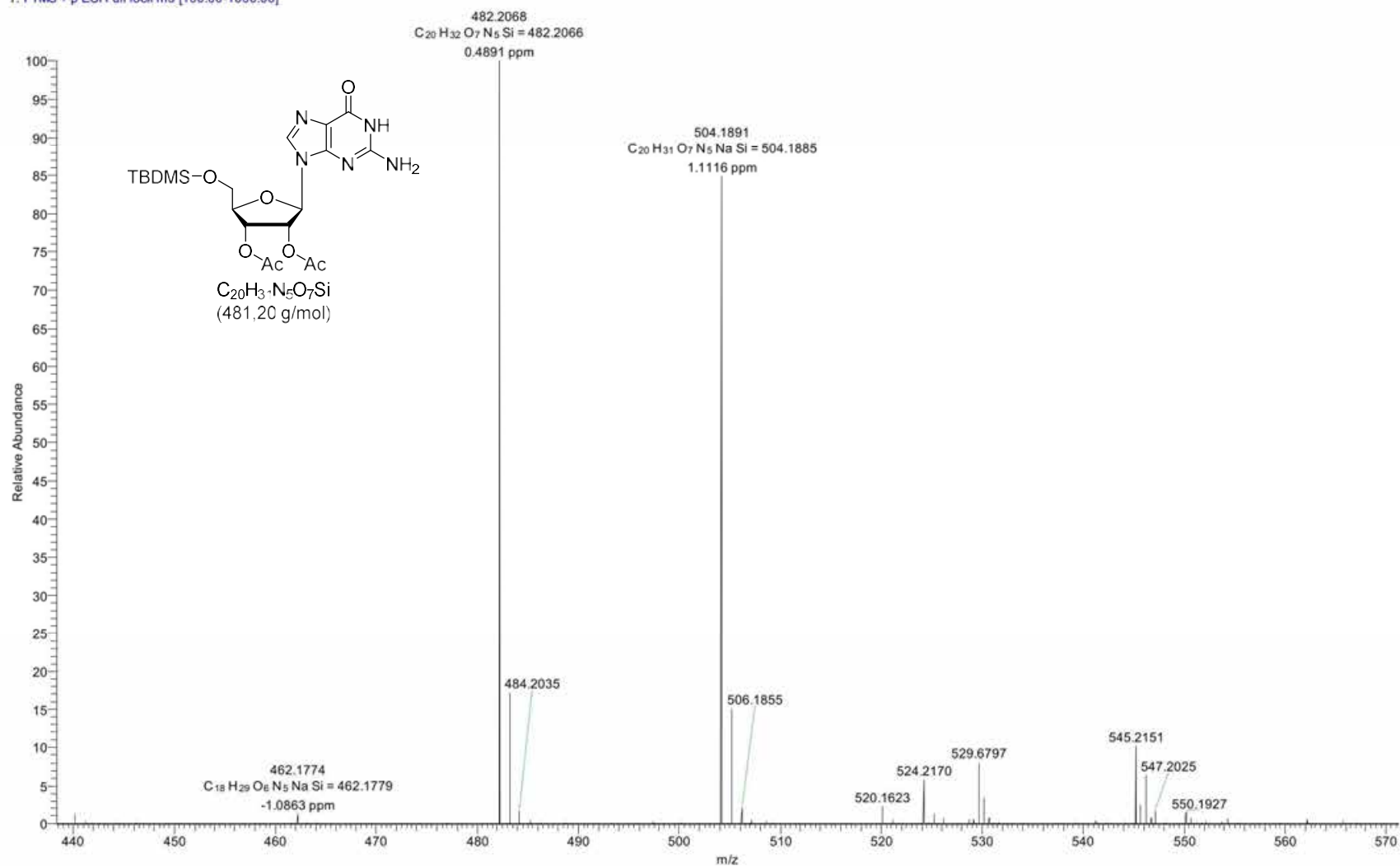
HRMS (ESI) analysis of 2',3'-diacetoxy 5'-TBS guanosine (16):

D:\data_2022\vpjeb09shr1

2/21/2022 8:32:38 AM

ar186

vpjeb09shr1 #1 RT: 0.02 AV: 1 NL: 5.55E6
T: FTMS + p ESI Full lock ms [100.00-1000.00]



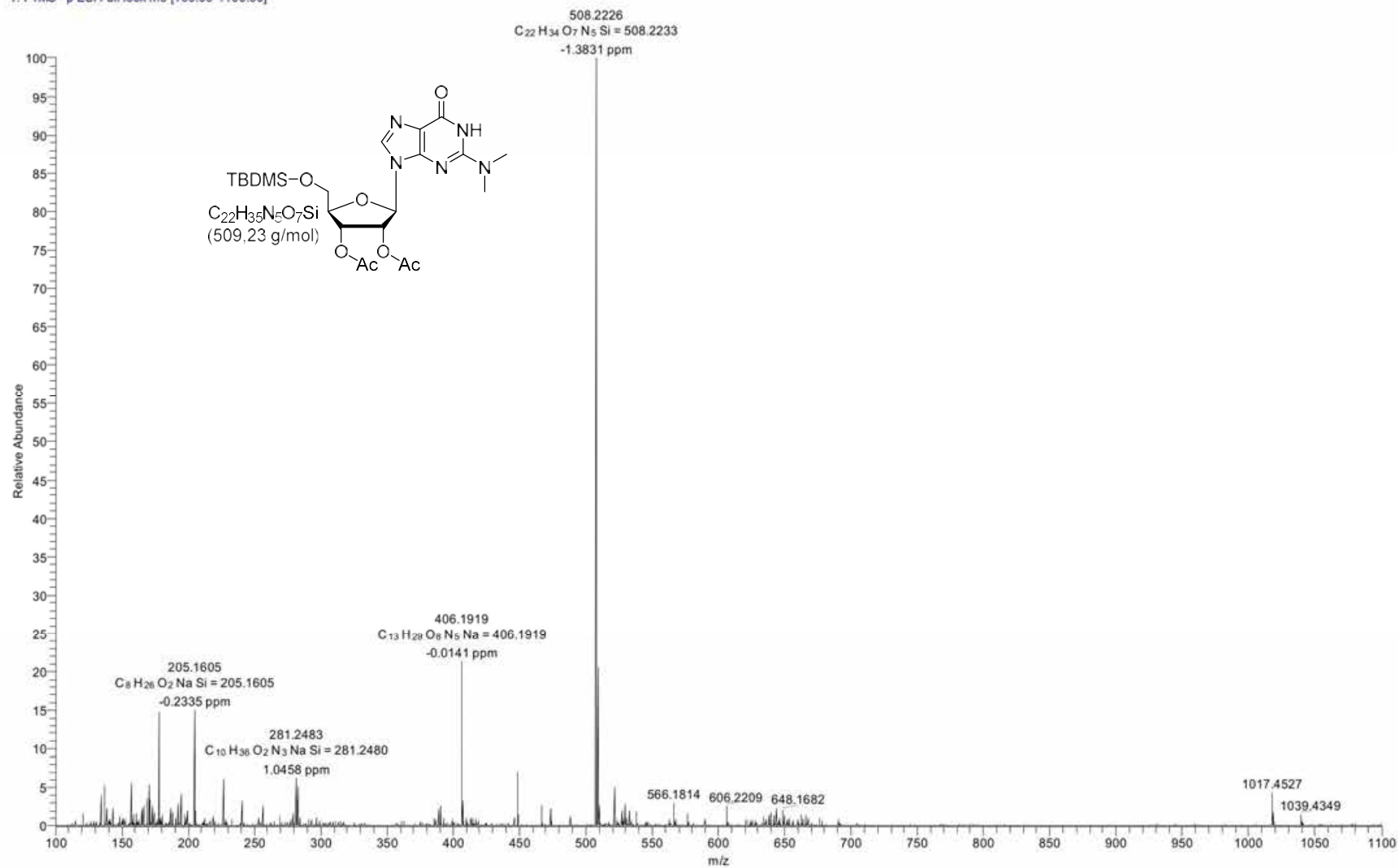
HRMS (ESI) analysis of 2',3'-diacetoxy 5'-TBS N^{2,2}-dimethylguanosine (17):

D:\data_2023\vpjeb72shr7

4/24/2023 2:37:20 PM

ar329f5

vpjeb72shr7 #1 RT: 0.02 AV: 1 NL: 1.90E6
T: FTMS - p ESI Full lock ms [100.00-1100.00]



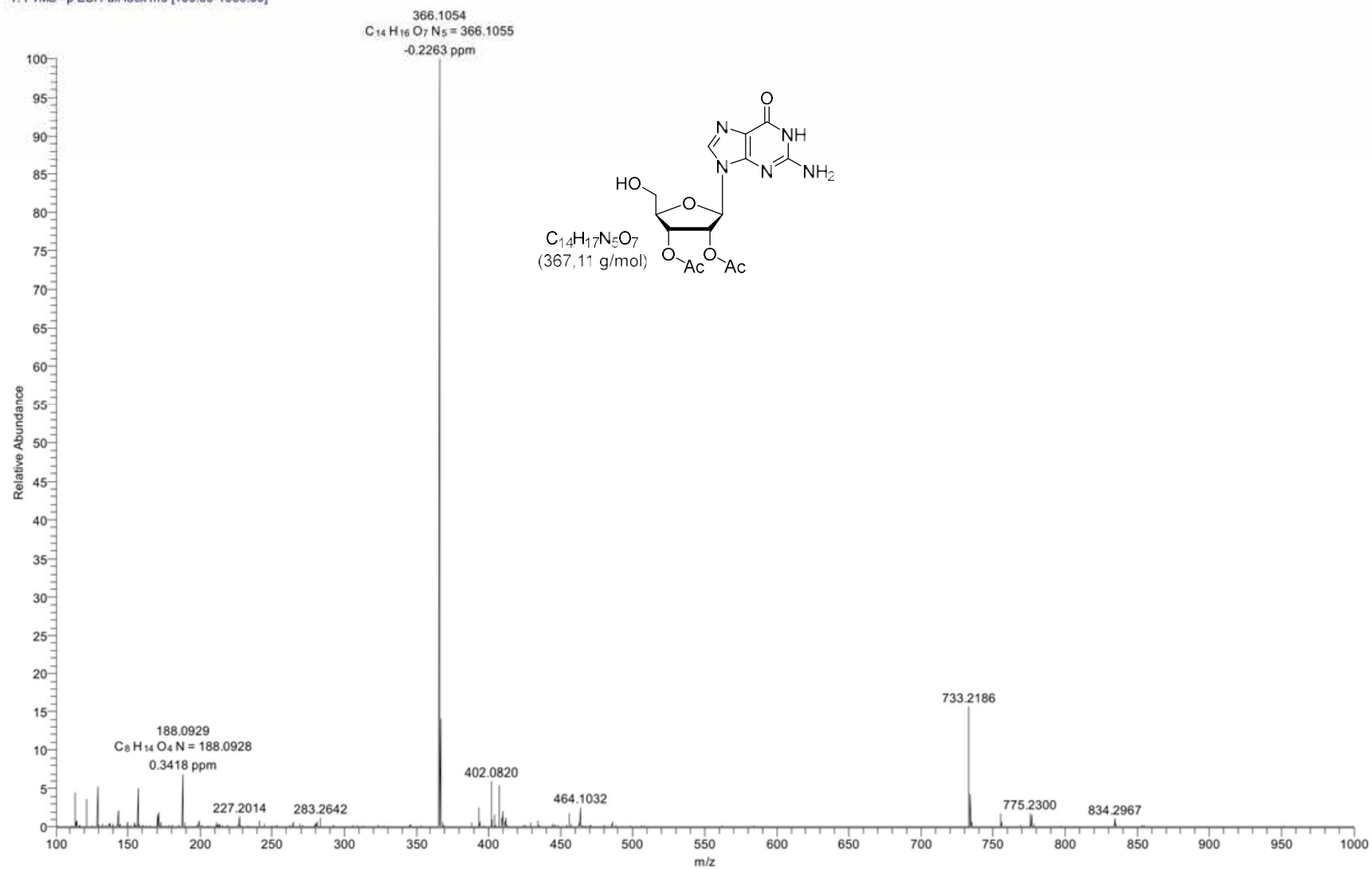
HRMS (ESI) analysis of 2',3'-diacetoxy guanosine (18):

D:\data_2022\vpjeb19shr2

5/3/2022 3:49:22 PM

ar218

vpjeb19shr2 #1 RT: 0.02 AV: 1 NL: 9.15E6
T: FTMS - p ESI Full lock ms [100.00-1000.00]



HRMS (ESI) analysis of 2',3'-diacetoxy 5'-TBS N⁷-methylguanosine iodide (SI-09):

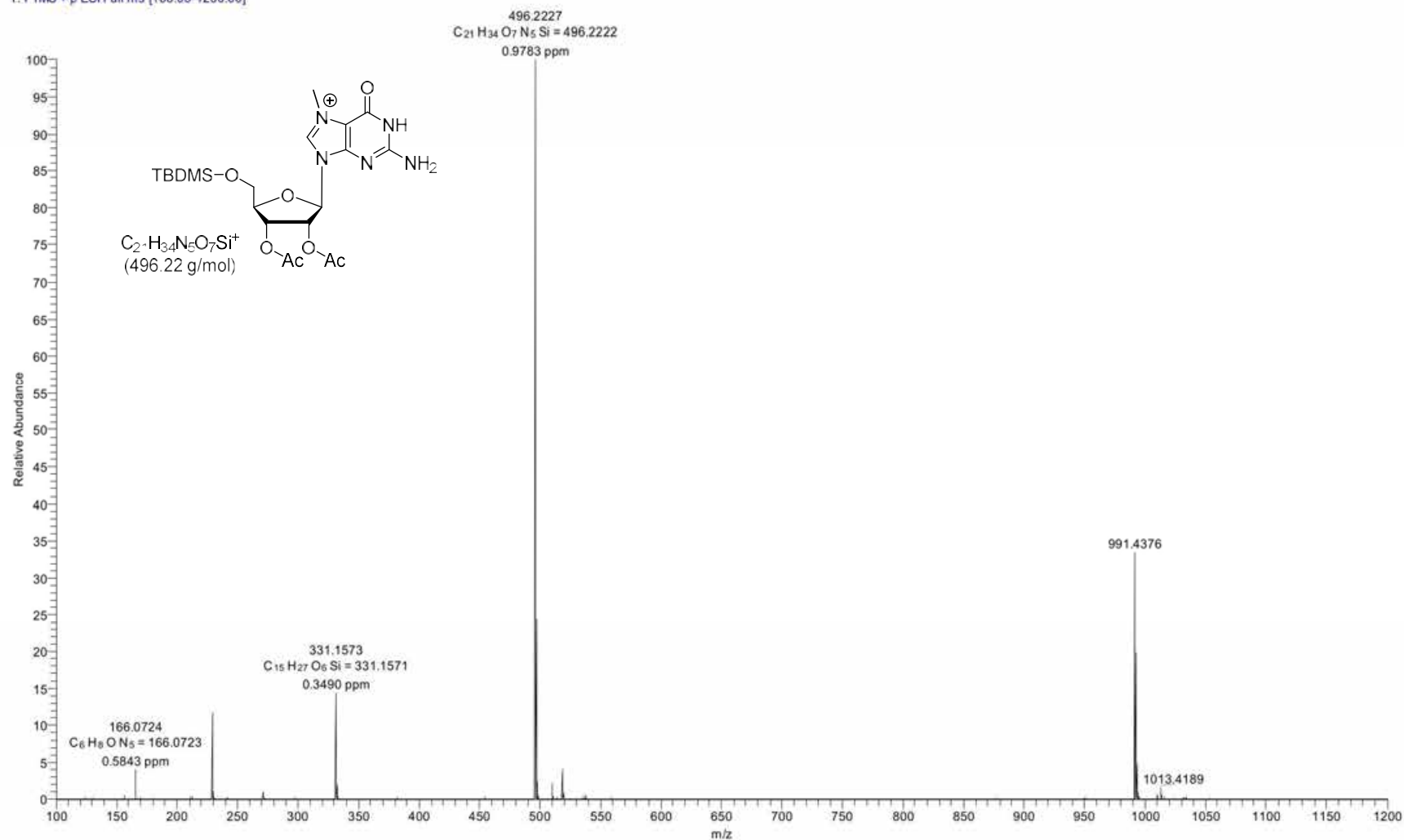
D:\data_2022\vpjeb46shr3

10/31/2022 11:42:30 AM

ar282.1

vpjeb46shr3 #1 RT: 0.02 AV: 1 NL: 8.95E7

T: FTMS + p ESI Full ms [100.00-1200.00]



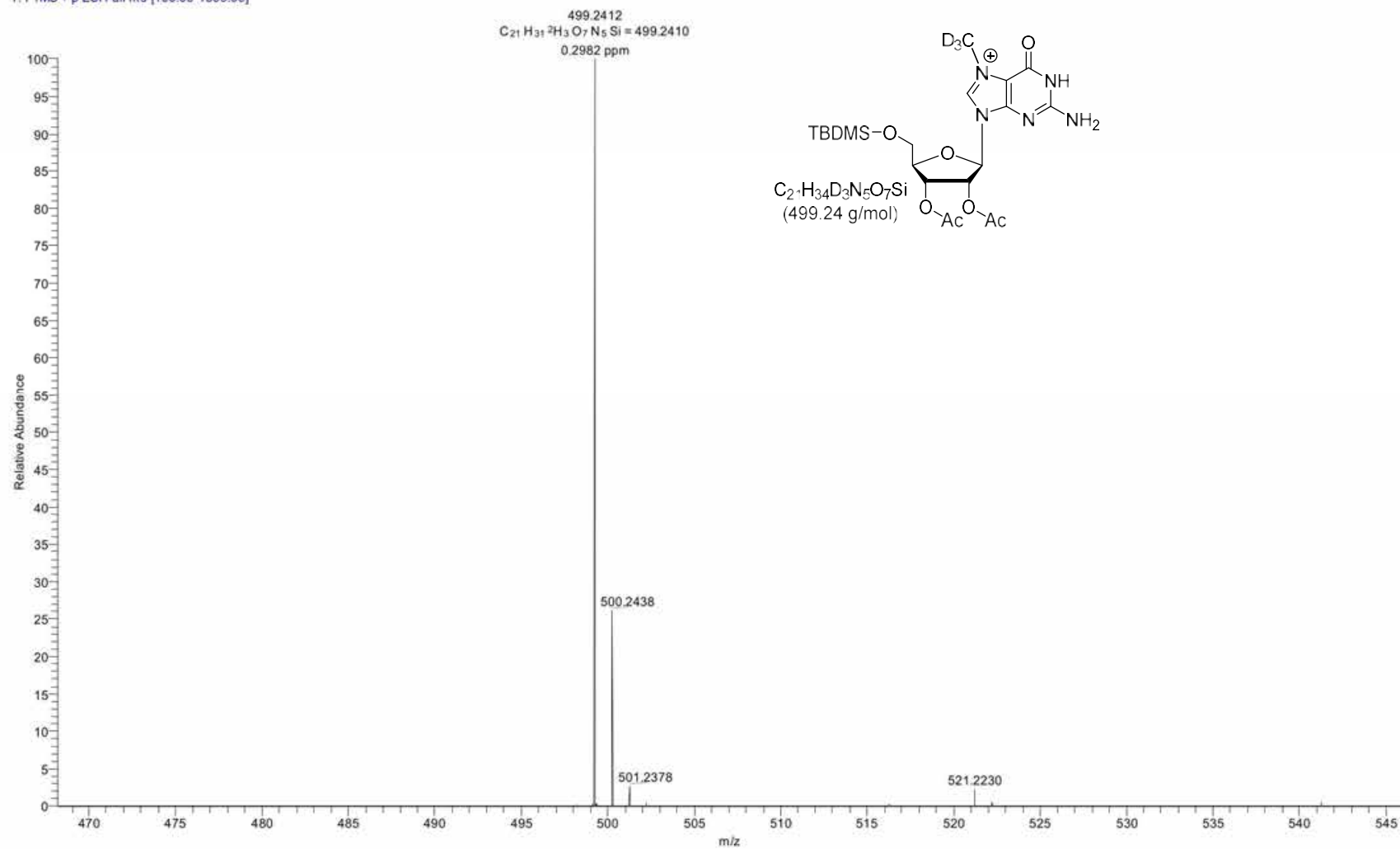
HRMS (ESI) analysis of 2',3'-diacetoxy 5'-TBS N⁷-methyl-d₃-guanosine iodide (SI-10):

D:\data_2023\hcjea06shr1

5/24/2023 1:32:39 PM

thu032.02

hcjea06shr1 #1 RT: 0.02 AV: 1 NL: 8.50E7
T: FTMS + p ESI Full ms [100.00-1300.00]



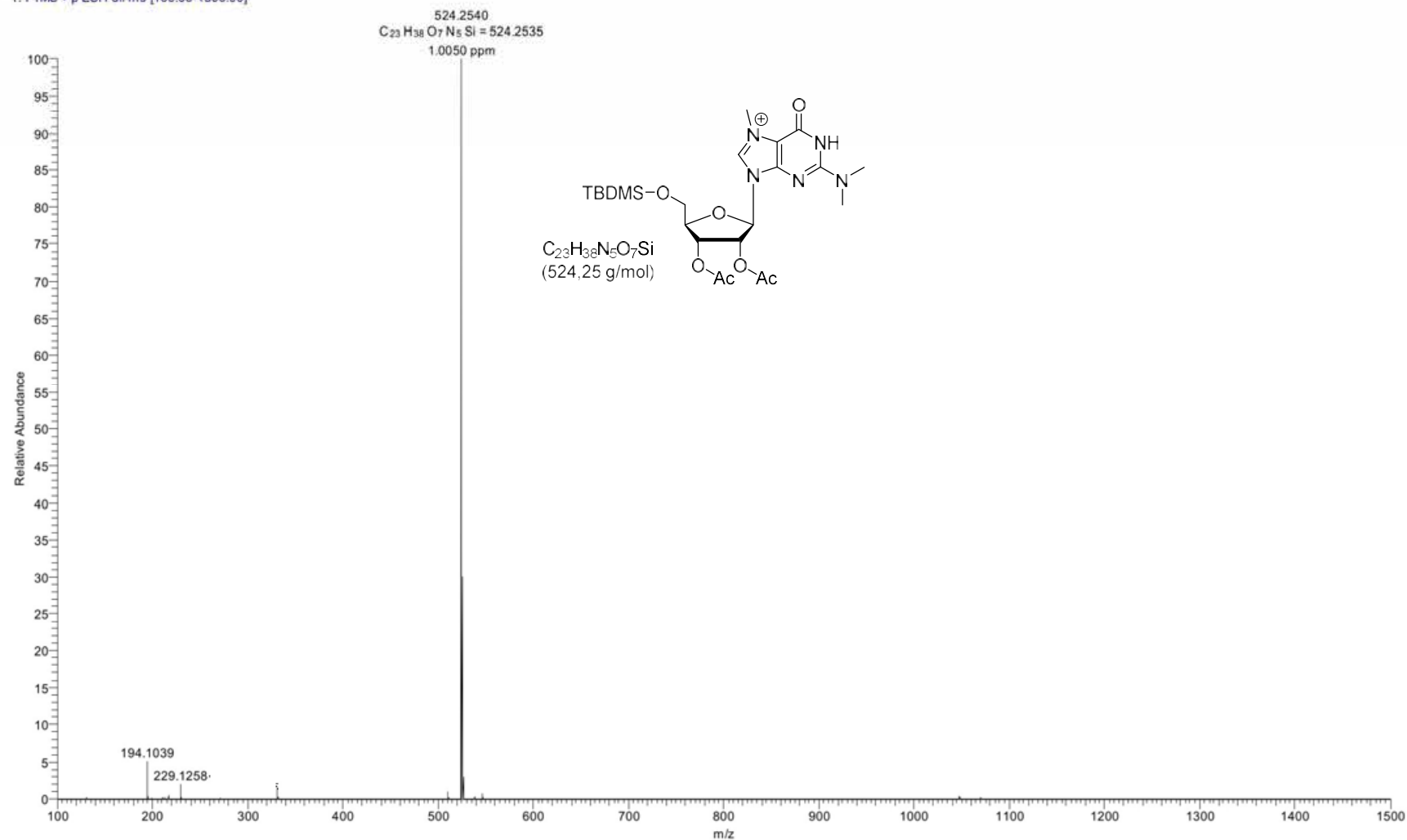
HRMS (ESI) analysis of 2',3'-diacetoxy 5'-TBS N^{2,2,7}-trimethylguanosine iodide (SI-11):

D:\data_2023\pjeb73shr1

4/28/2023 11:36:54 AM

ar335f2

rpjeb73shr1 #1 RT: 0.02 AV: 1 NL: 1.72E8
T: FTMS + p ESI Full ms [100.00-1500.00]



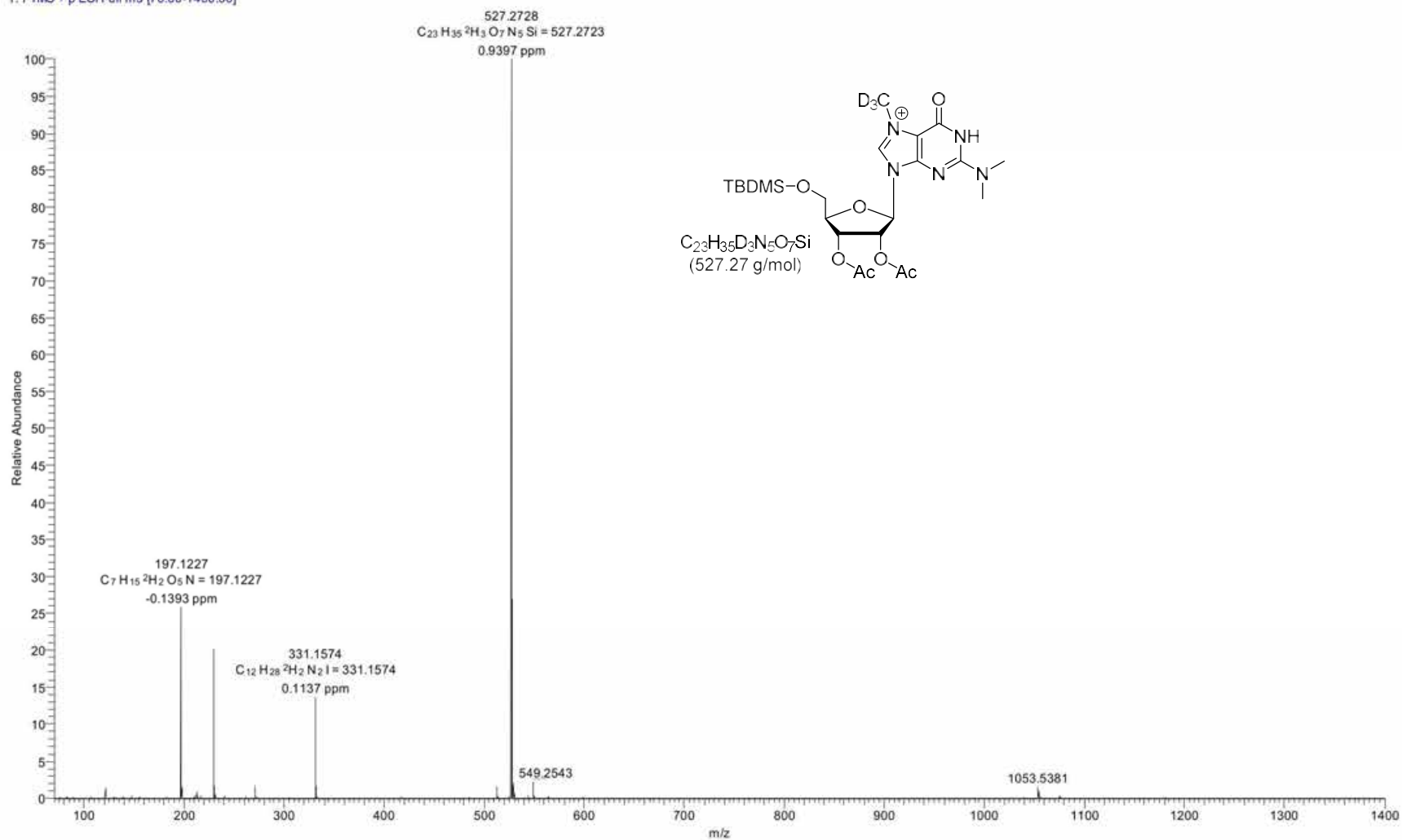
HRMS (ESI) analysis of 2',3'-diacetoxy 5'-TBS N^{2,2,7}-trimethyl-⁷d₃-guanosine iodide (SI-12):

D:\data_2023\hjea17shr1

6/27/2023 2:30:43 PM

thu060.01

hjea17shr1 #1 RT: 0.02 AV: 1 NL: 4.73E7
T: FTMS + p ESI Full ms [70.00-1400.00]



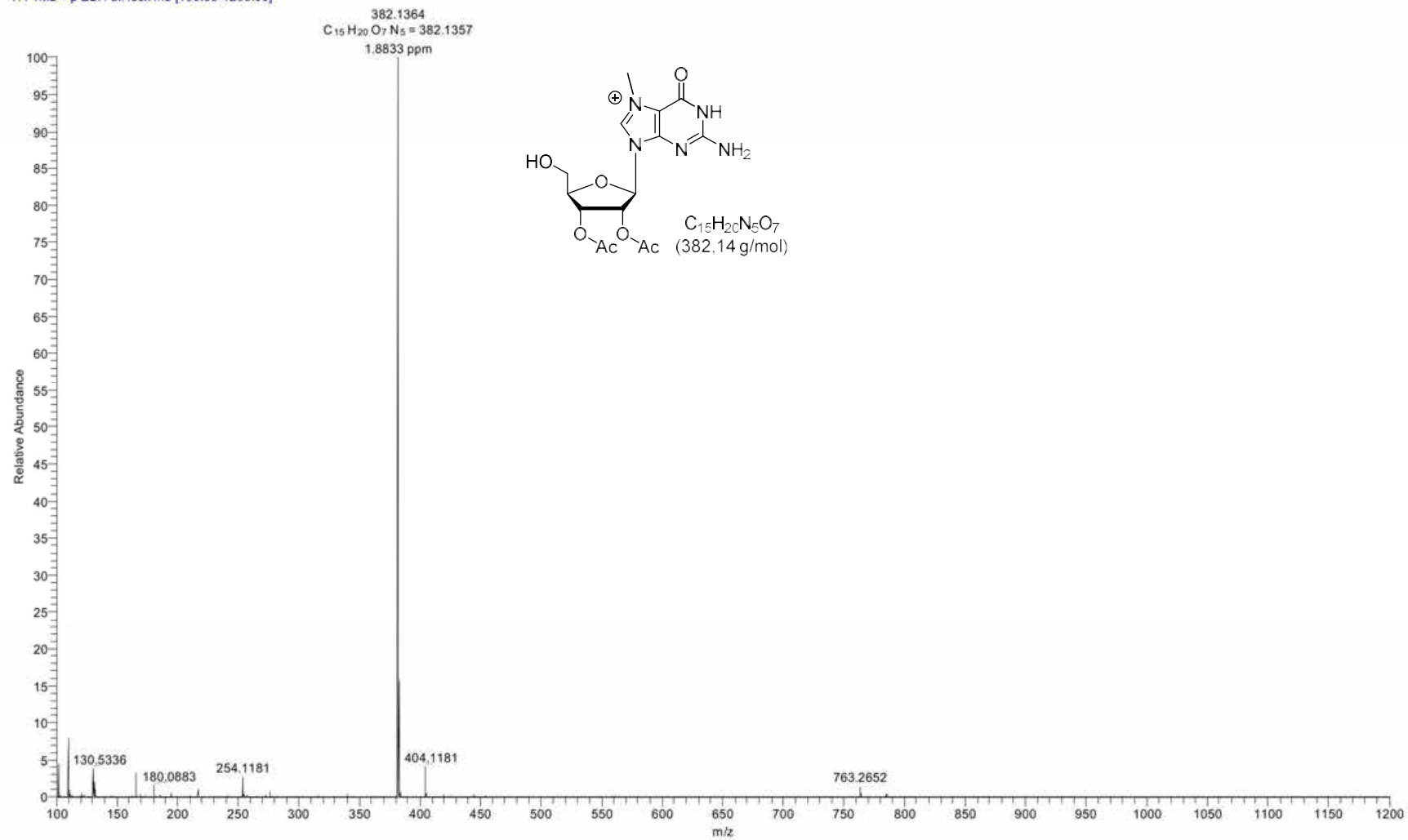
HRMS (ESI) analysis of 2',3'-diacetoxy N⁷-methylguanosine (19):

D:\data_2023\vpjeb54shr2

2/7/2023 12:14:13 PM

ar298

vpjeb54shr2 #1 RT: 0.02 AV: 1 NL: 1.27E7
T: FTMS + p ESI Full lock ms [100.00-1200.00]



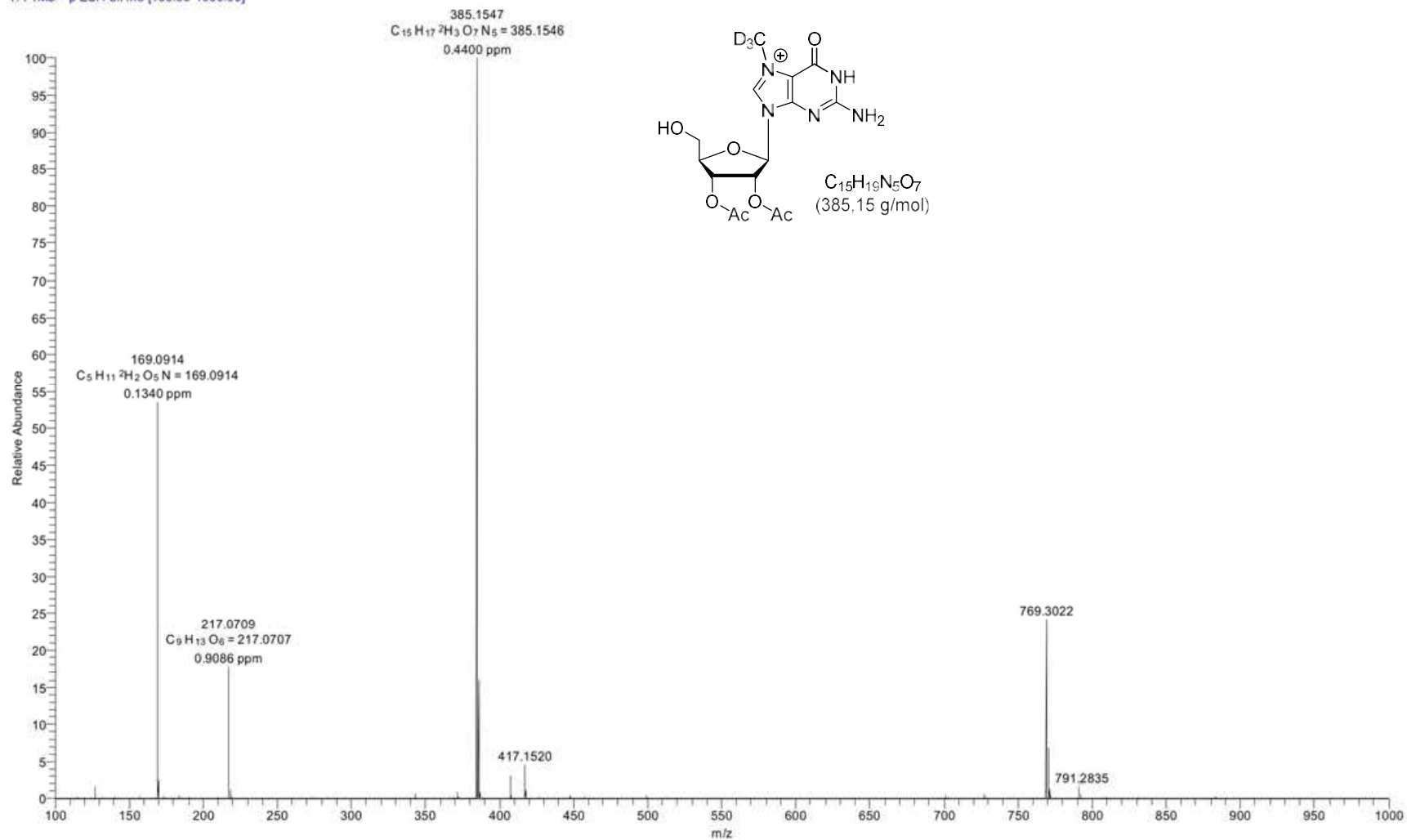
HRMS (ESI) analysis of 2',3'-diacetoxy N⁷-methyl-d₃-guanosine (20):

D:\data_2023\hcjea07shr1

5/24/2023 1:44:21 PM

thu037.01

hcjea07shr1 #1 RT: 0.02 AV: 1 NL: 8.28E7
T: FTMS + p ESI Full ms [100.00-1000.00]



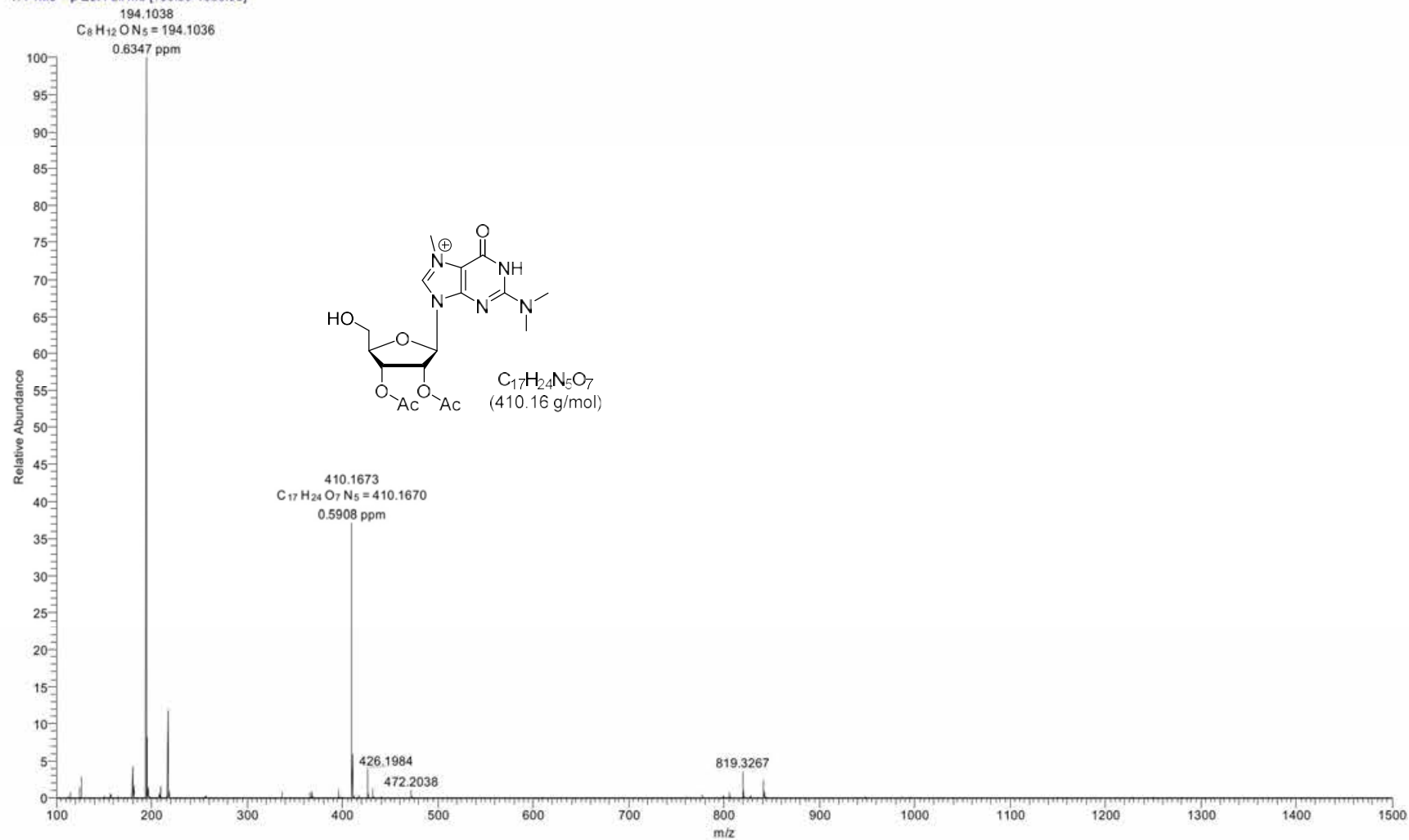
HRMS (ESI) analysis of 2',3'-diacetoxy N^{2,2,7}-trimethylguanosine (21):

D:\data_2023\hjea18shr1

6/29/2023 9:28:51 AM

thu059.01

hjea18shr1 #1 RT: 0.02 AV: 1 NL: 2.15E7
T: FTMS + p ESI Full ms [100.00-1500.00]



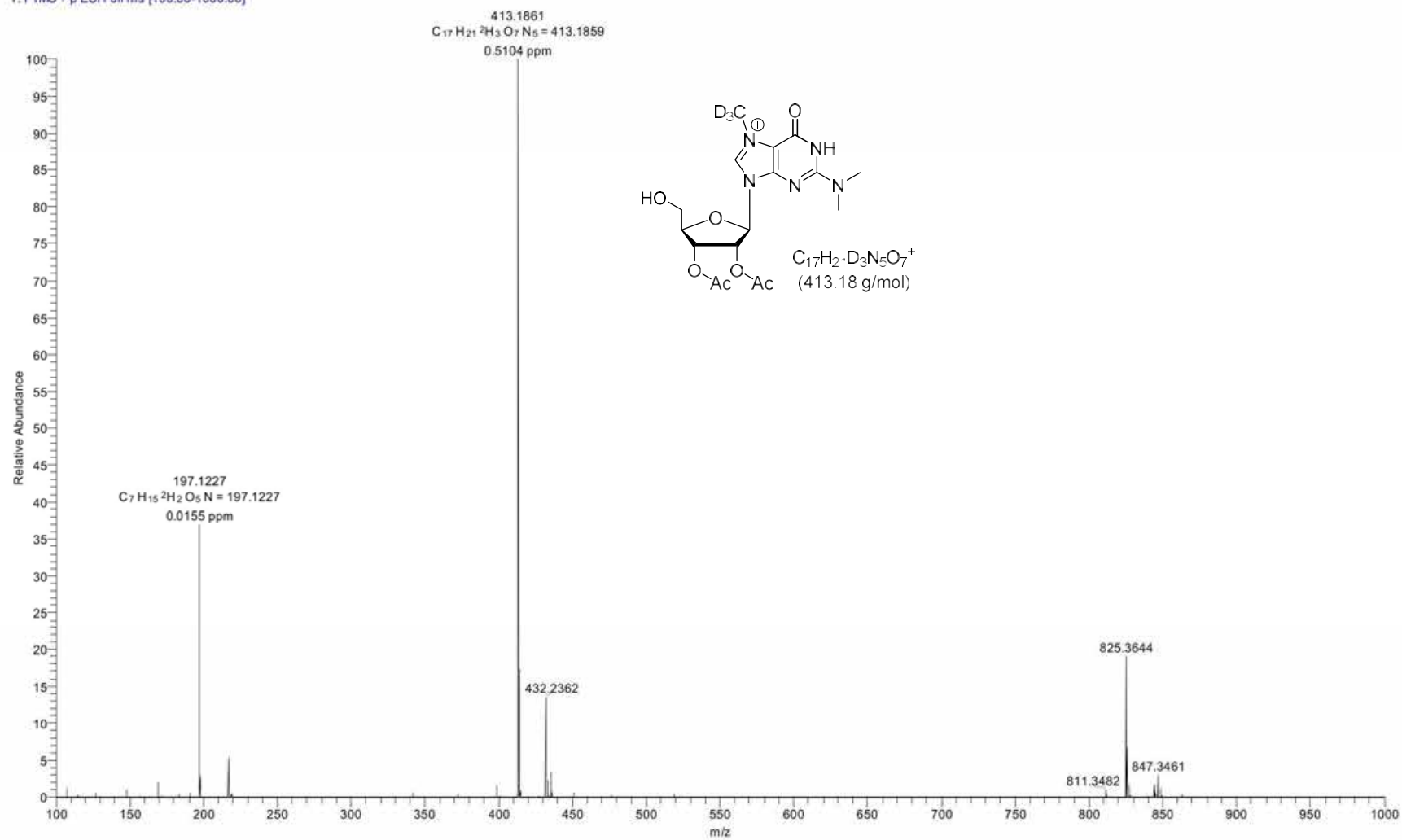
HRMS (ESI) analysis of 2',3'-diacetoxy N^{2,2,7}-trimethyl-⁷d₃-guanosine (22):

D:\data_2023\vpjeb94shr1

9/1/2023 1:02:31 PM

ar367

vpjeb94shr1 #1 RT: 0.02 AV: 1 NL: 9.02E7
T: FTMS + p ESI Full ms [100.00-1000.00]



HRMS (ESI) analysis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-guanosine (23):

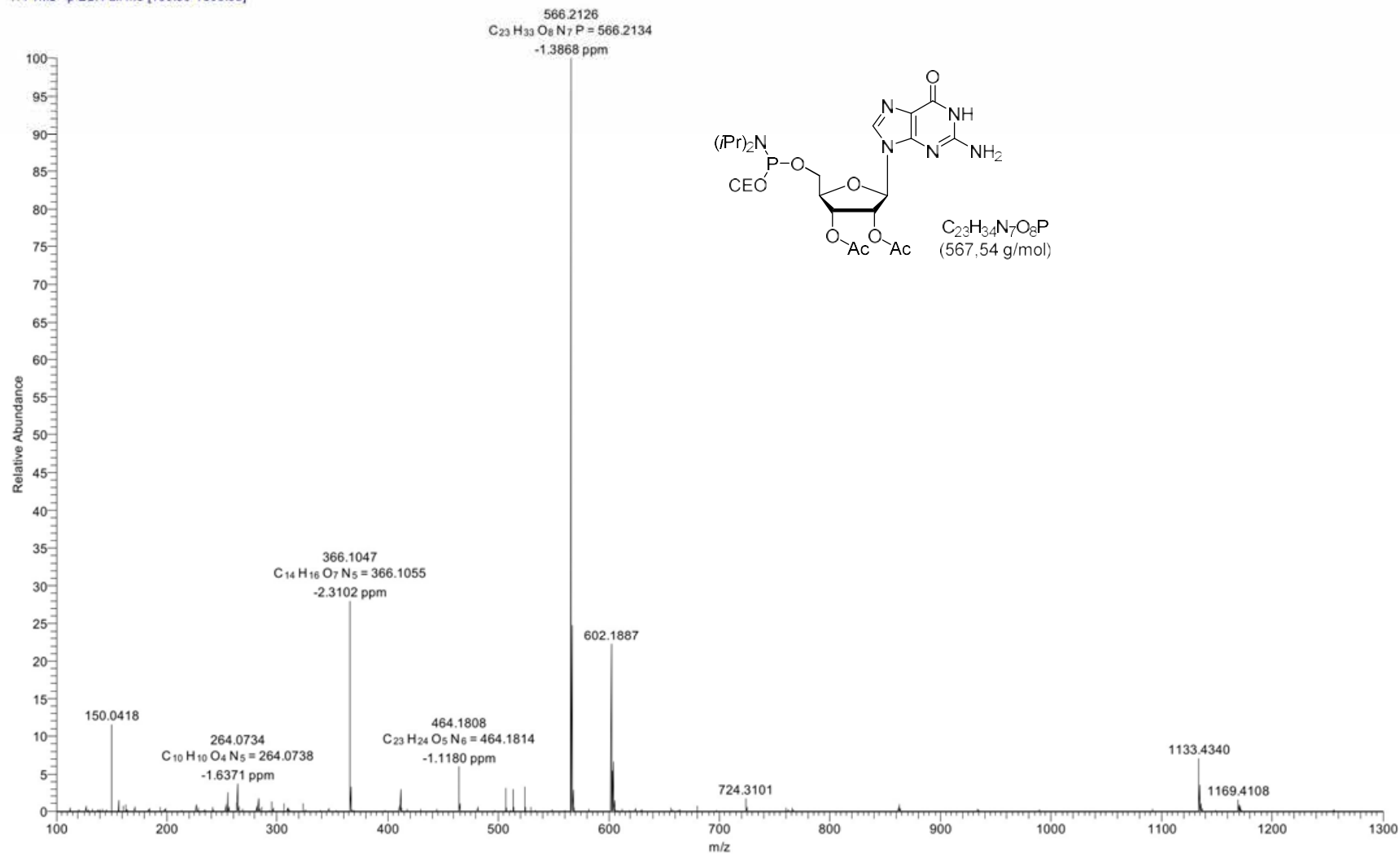
D:\data_2022\vpjeb40shr3

7/18/2022 12:16:42 PM

ar250

rpjeb40shr3 #1 RT: 0.02 AV: 1 NL: 1.68E7

T: FTMS - p ESI Full ms [100.00-1300.00]



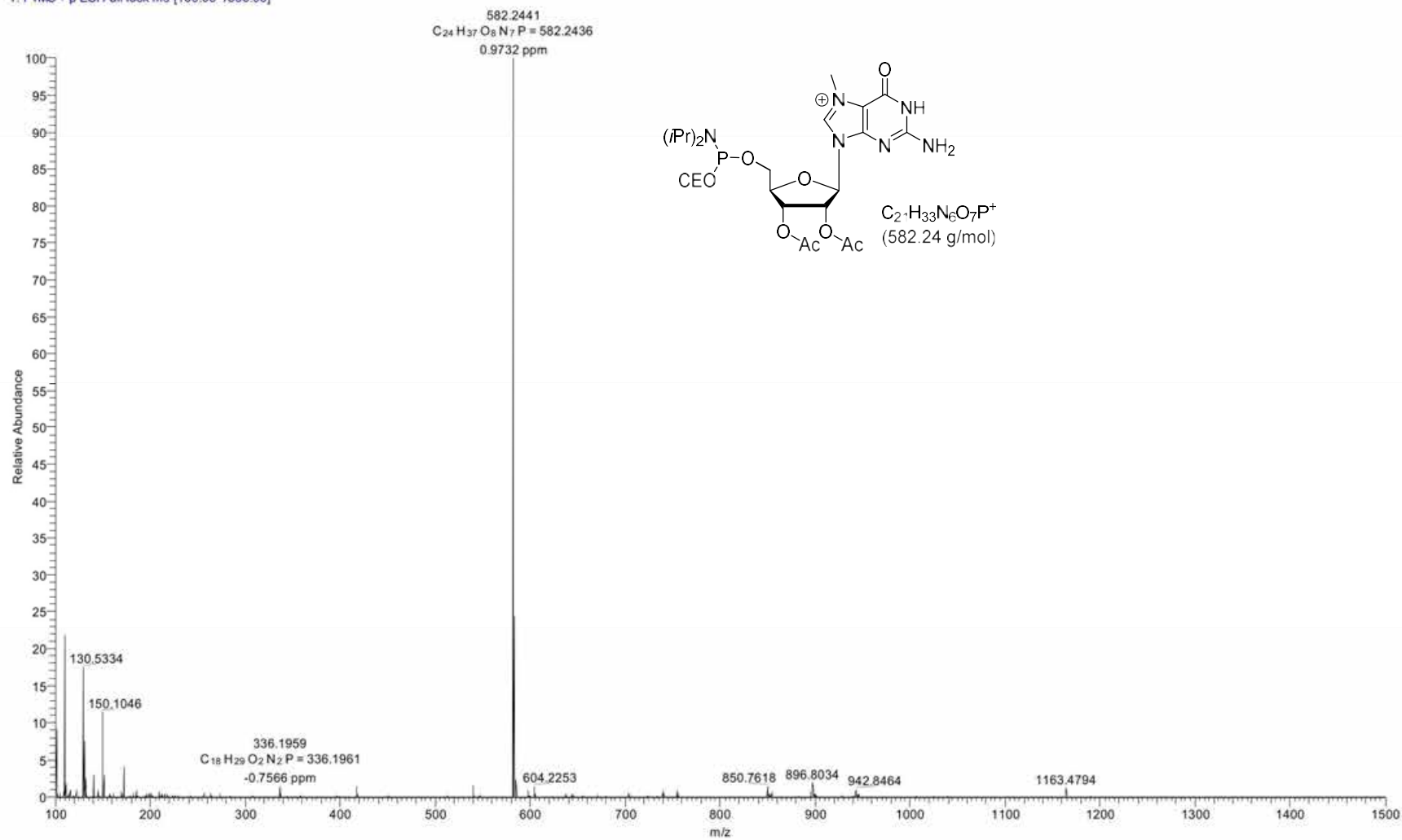
HRMS (ESI) analysis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N⁷-methylguanosine (24):

D:\data_2023\hjea01shr4

2/16/2023 3:59:34 PM

thu030.02

hjea01shr4 #1 RT: 0.02 AV: 1 NL: 1.04E7
T: FTMS + p ESI Full lock ms [100.00-1500.00]



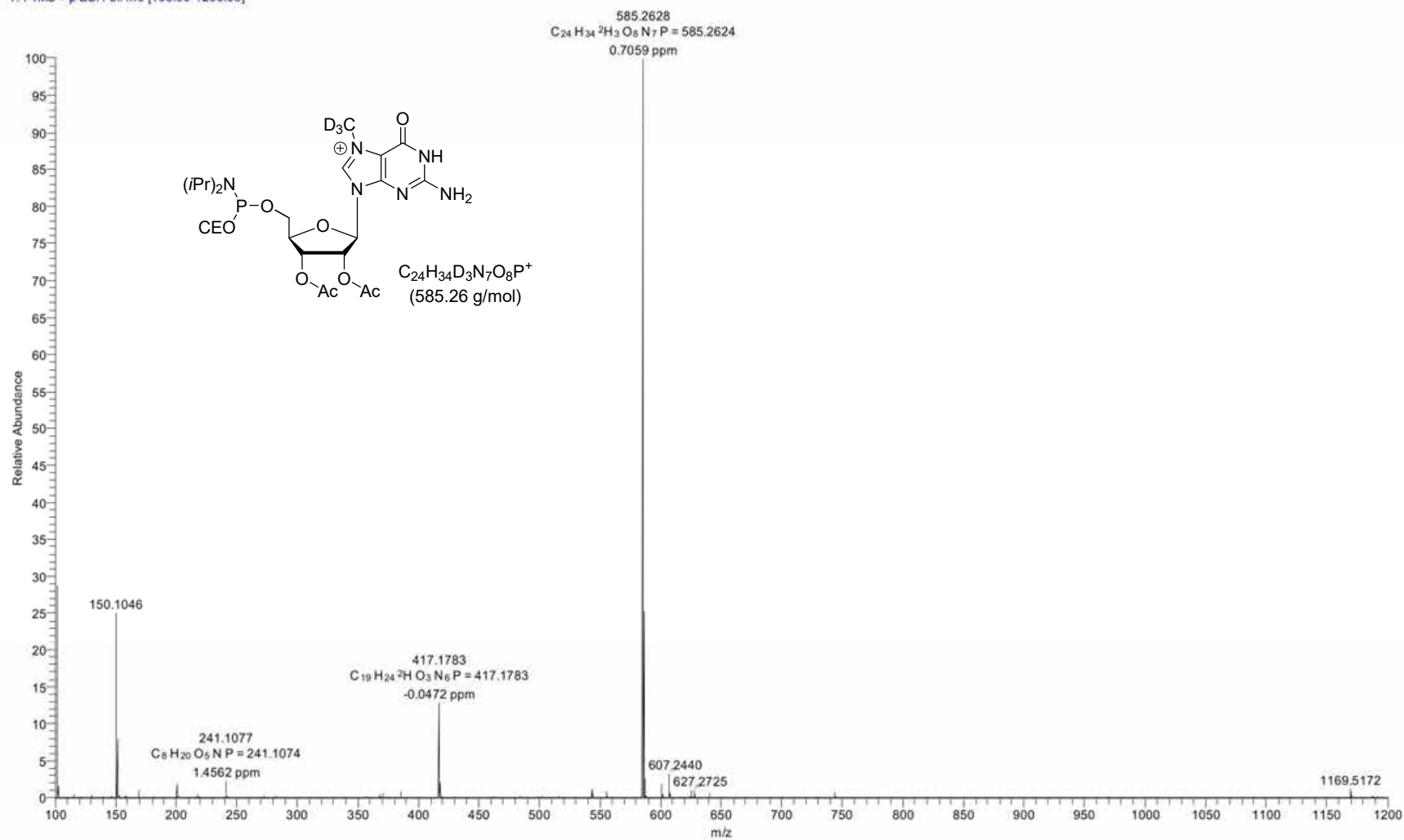
HRMS (ESI) analysis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N⁷-methyl-d₃-guanosine (25):

D:\data_2023\hjea08shr1

5/24/2023 1:52:03 PM

thu040.01

hjea08shr1 #1 RT: 0.02 AV: 1 NL: 3.03E7
T: FTMS + p ESI Full ms [100.00-1200.00]



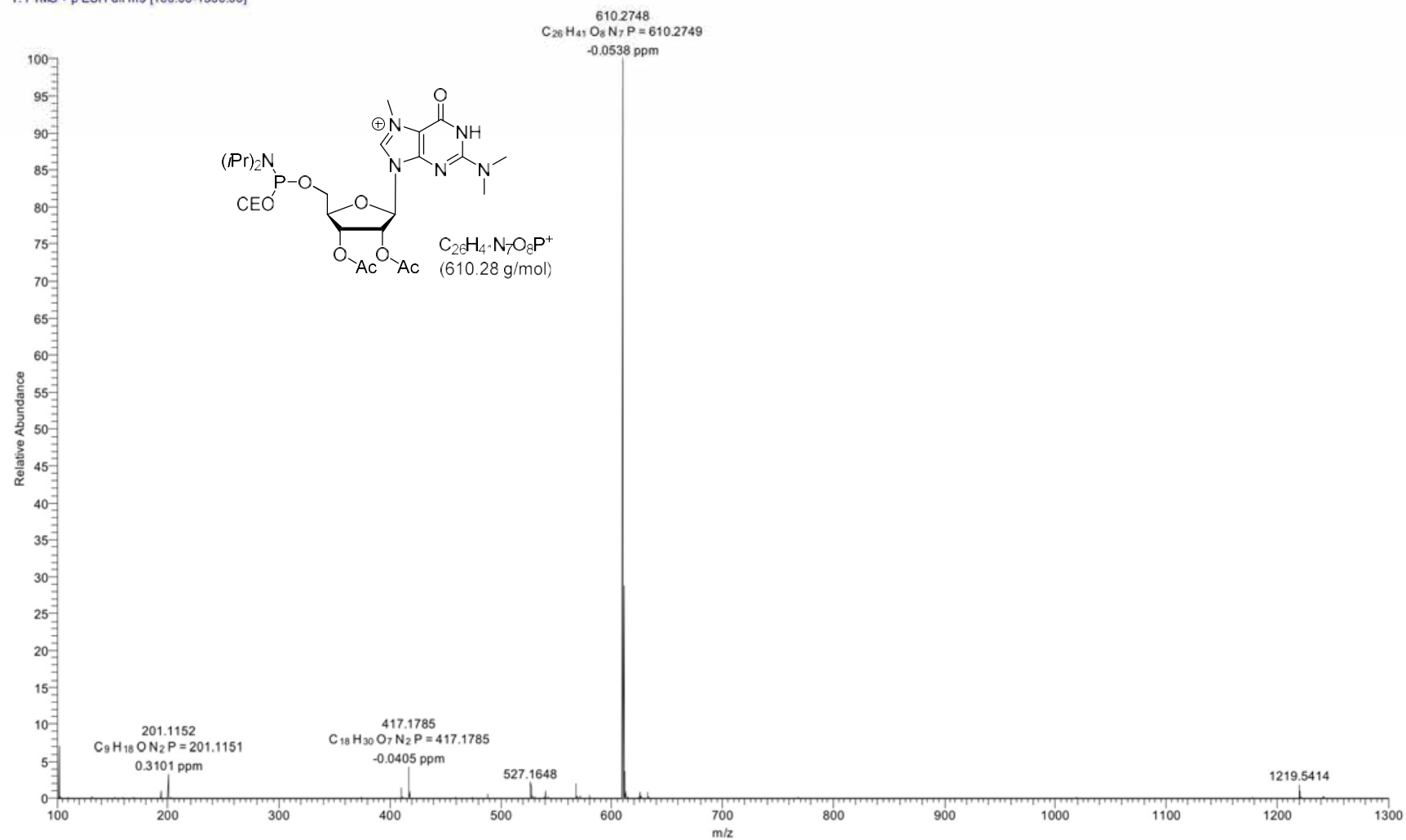
HRMS (ESI) analysis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N^{2,2,7}-trimethylguanosine (26):

D:\data_2023\vpjeb93shr1

8/31/2023 4:21:36 PM

ar366

vpjeb93shr1 #1 RT: 0.02 AV: 1 NL: 1.64E8
T: FTMS + p ESI Full ms [100.00-1300.00]



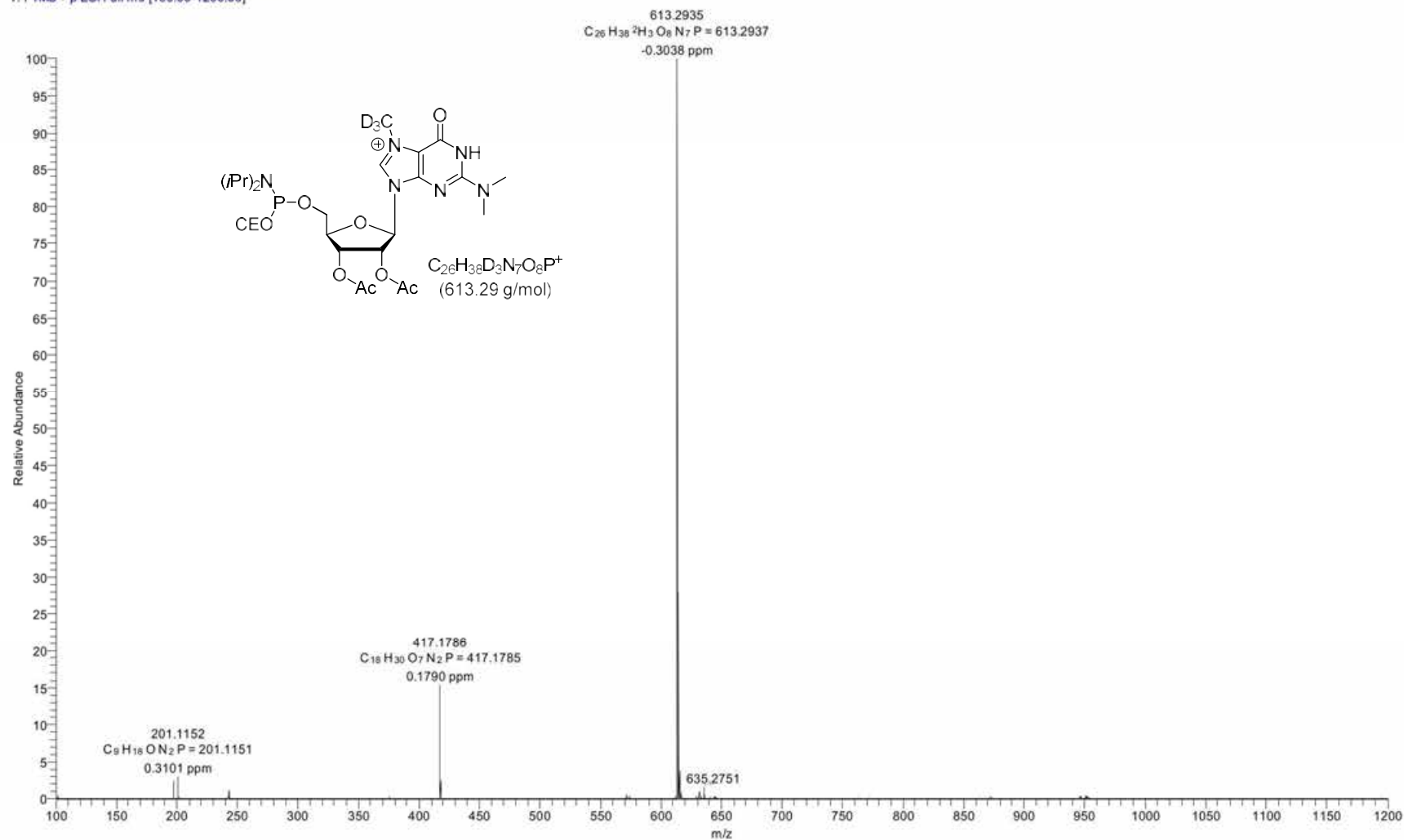
HRMS (ESI) analysis of 2',3'-diacetoxy-5'-((2-cyanoethoxy)(diisopropylamino)phosphaneyl)-N^{2,2,7}-trimethyl-⁷d₃-guanosine (27):

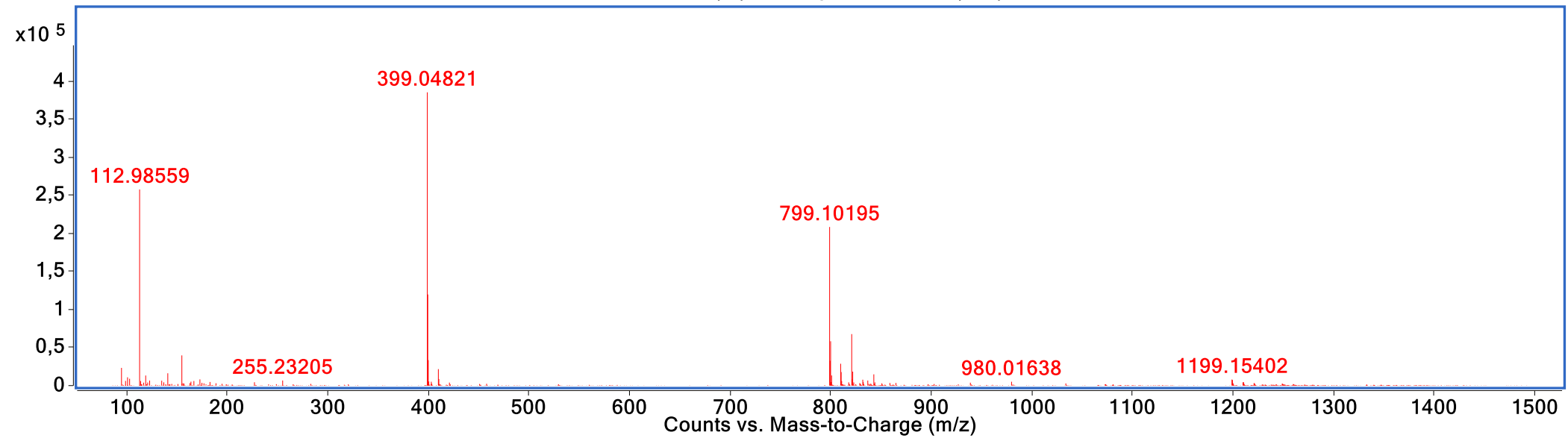
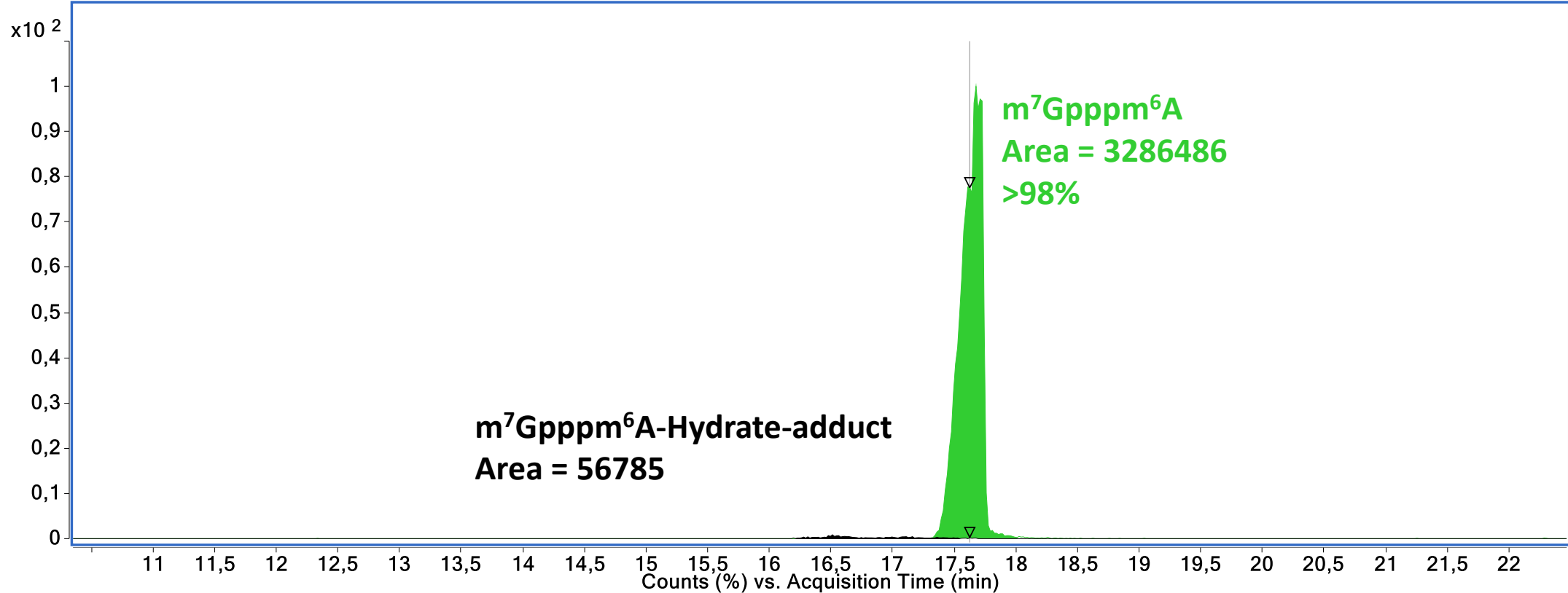
D:\data_2023\vpjec02shr2

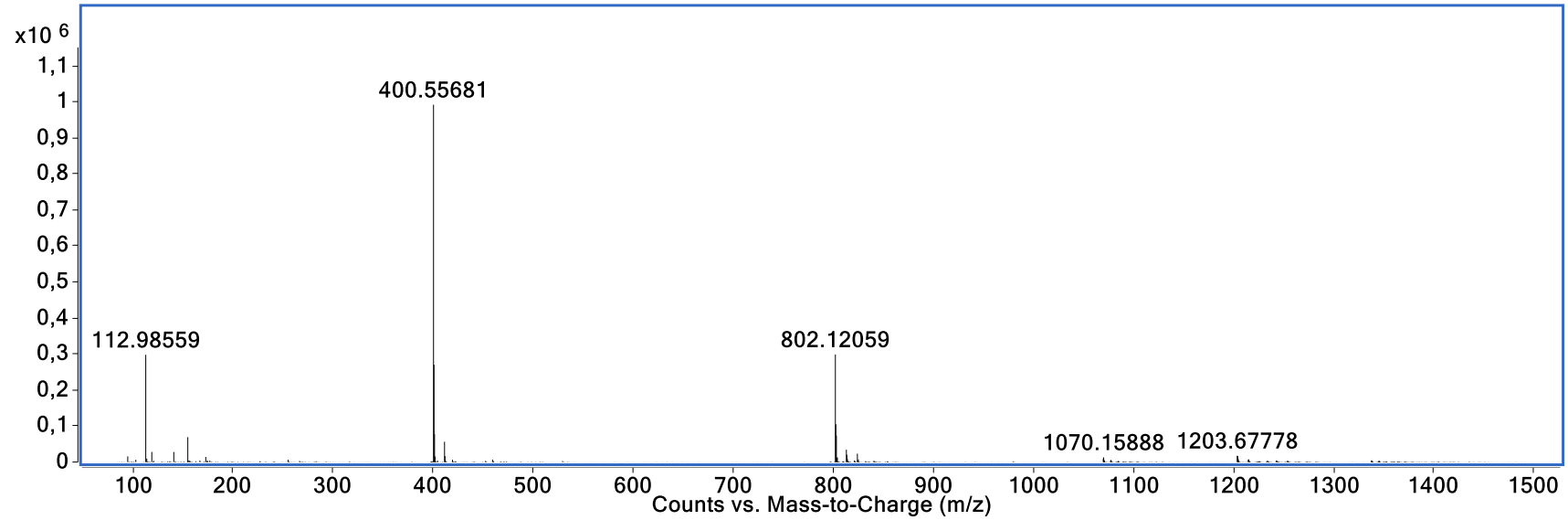
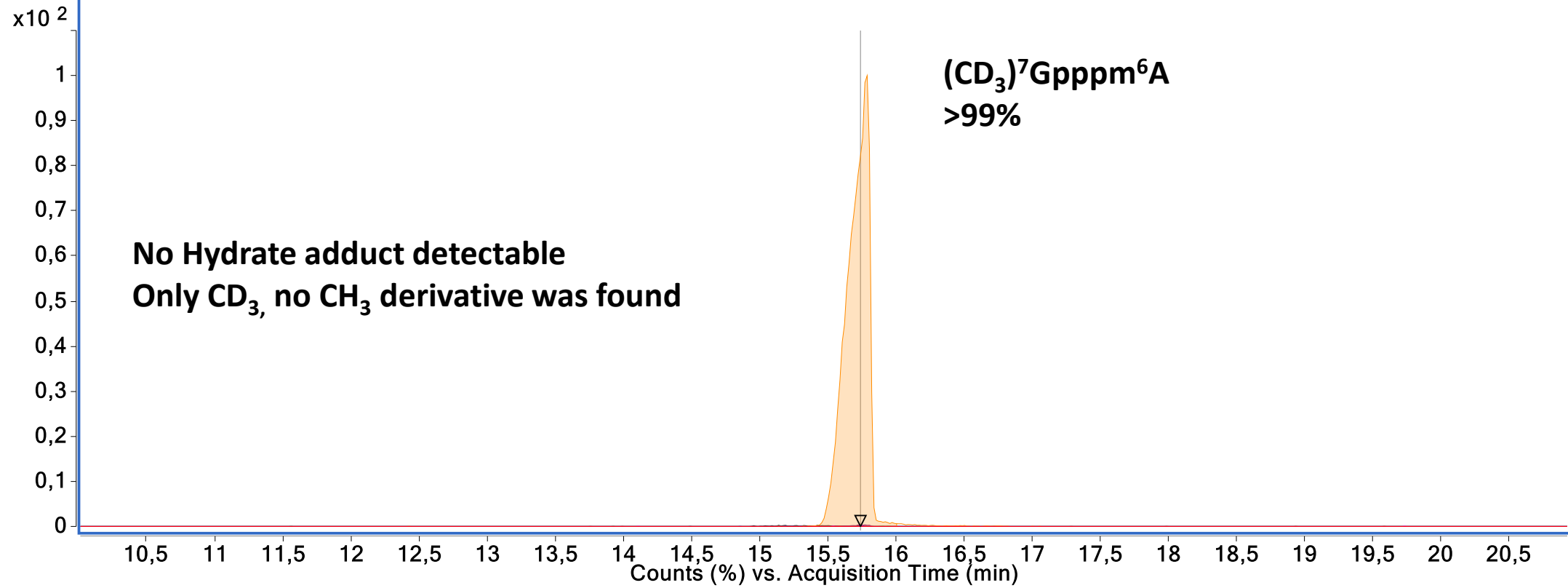
9/8/2023 2:24:02 PM

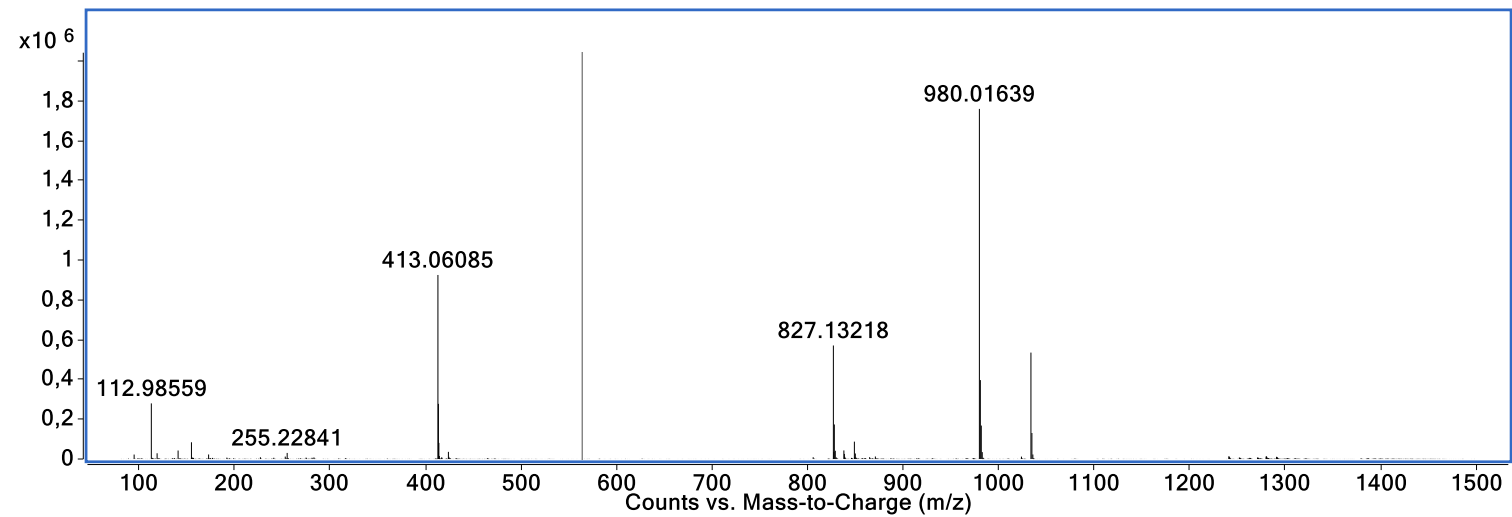
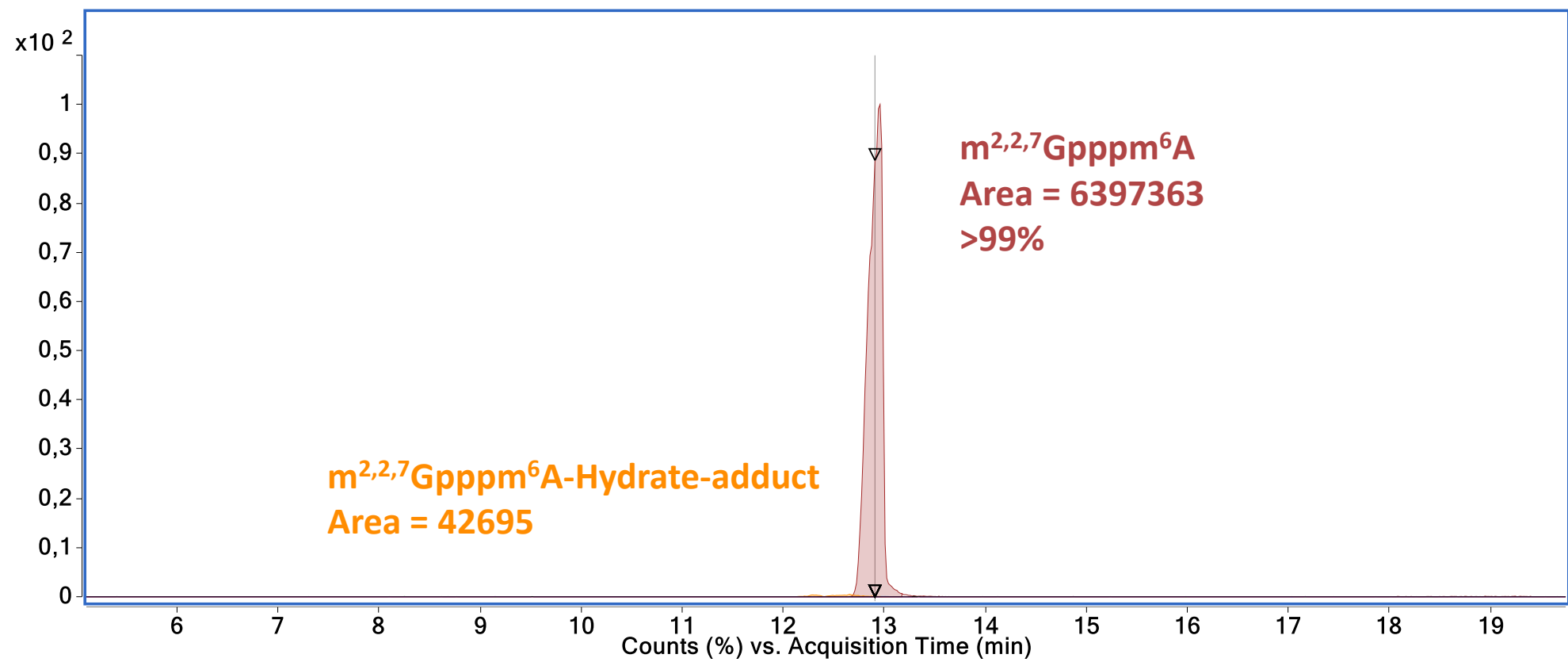
ar373final

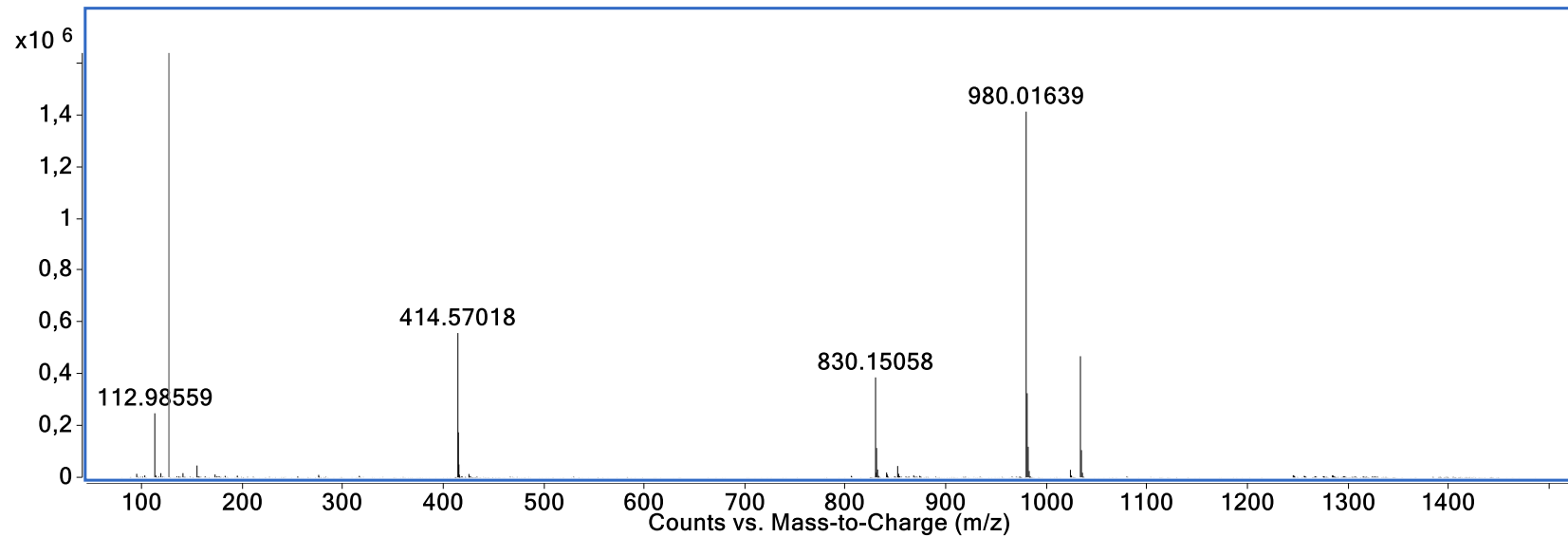
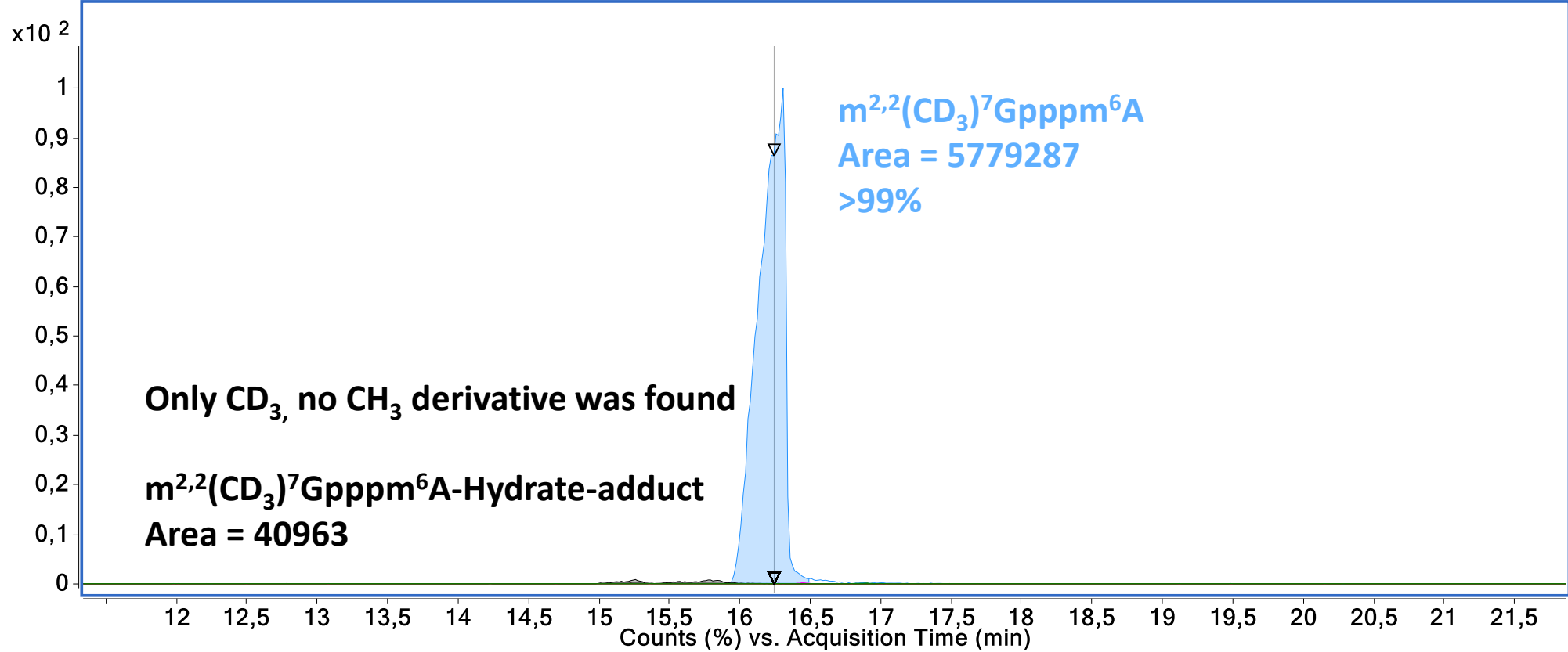
vpjec02shr2 #1 RT: 0.02 AV: 1 NL: 9.41E7
T: FTMS + p ESI Full ms [100.00-1200.00]

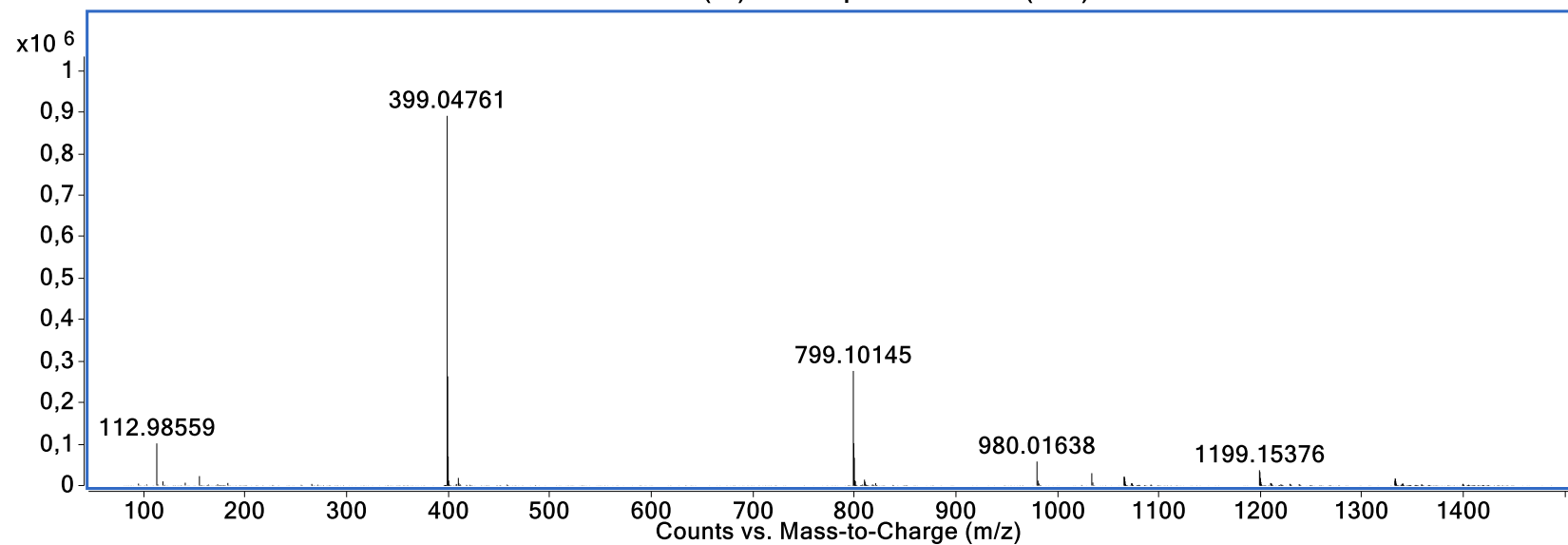
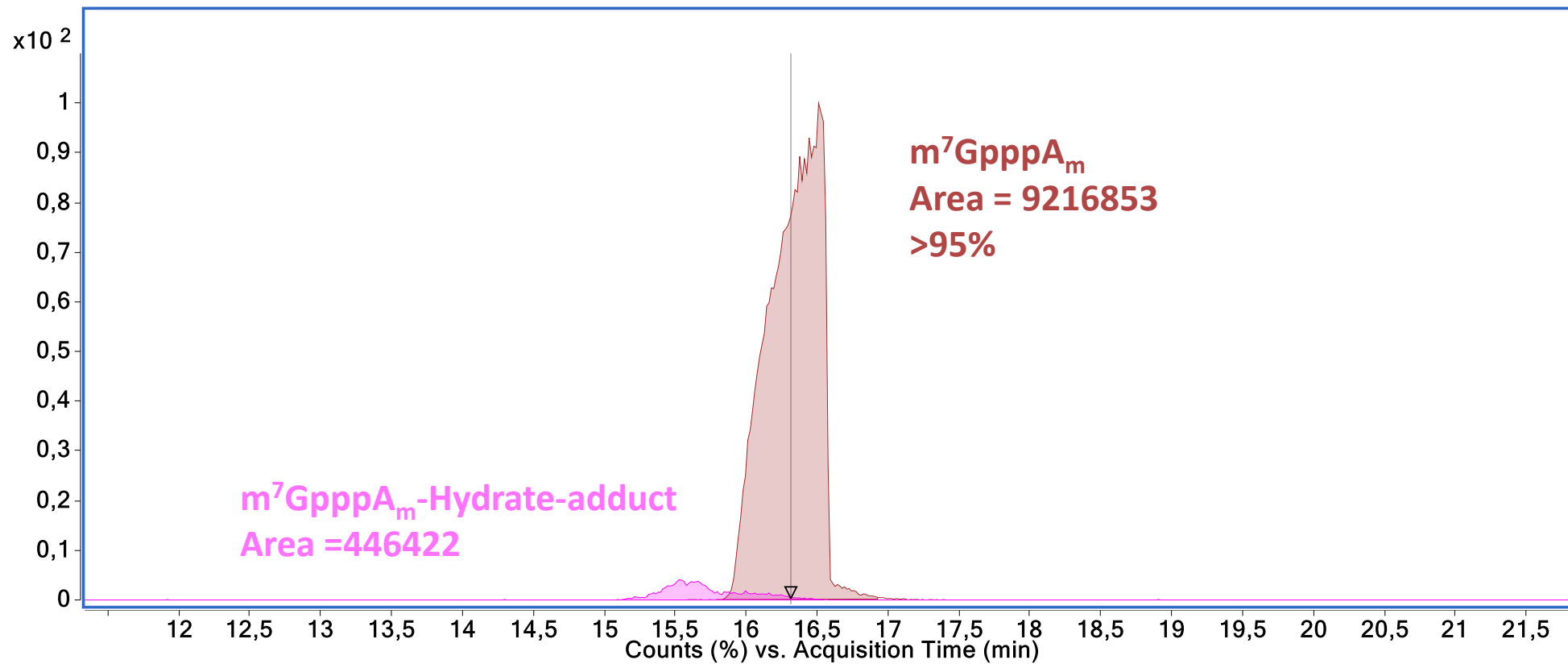


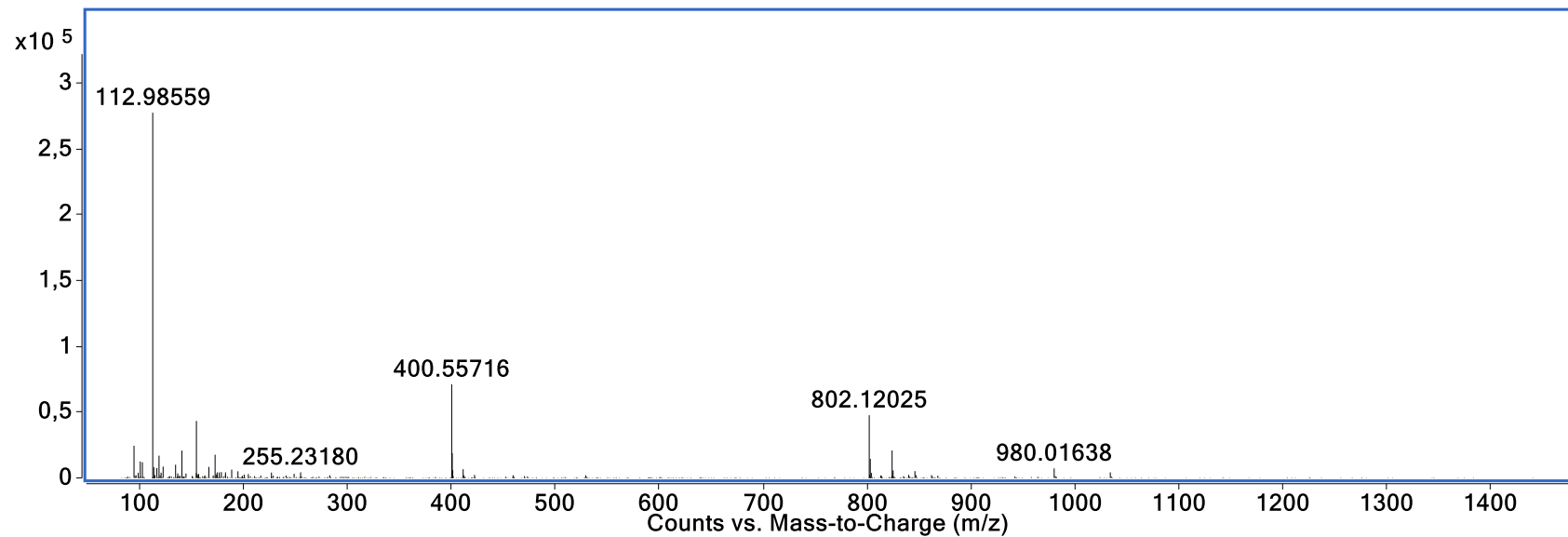
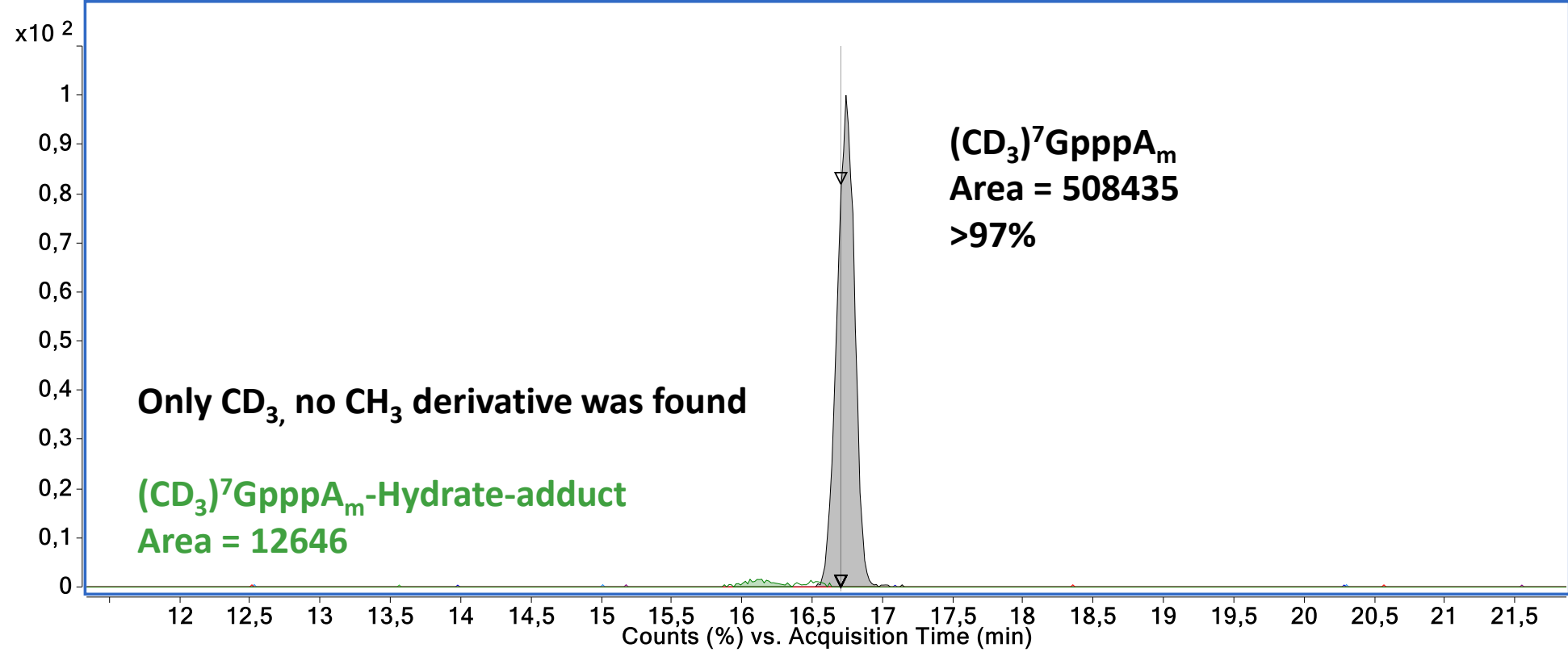


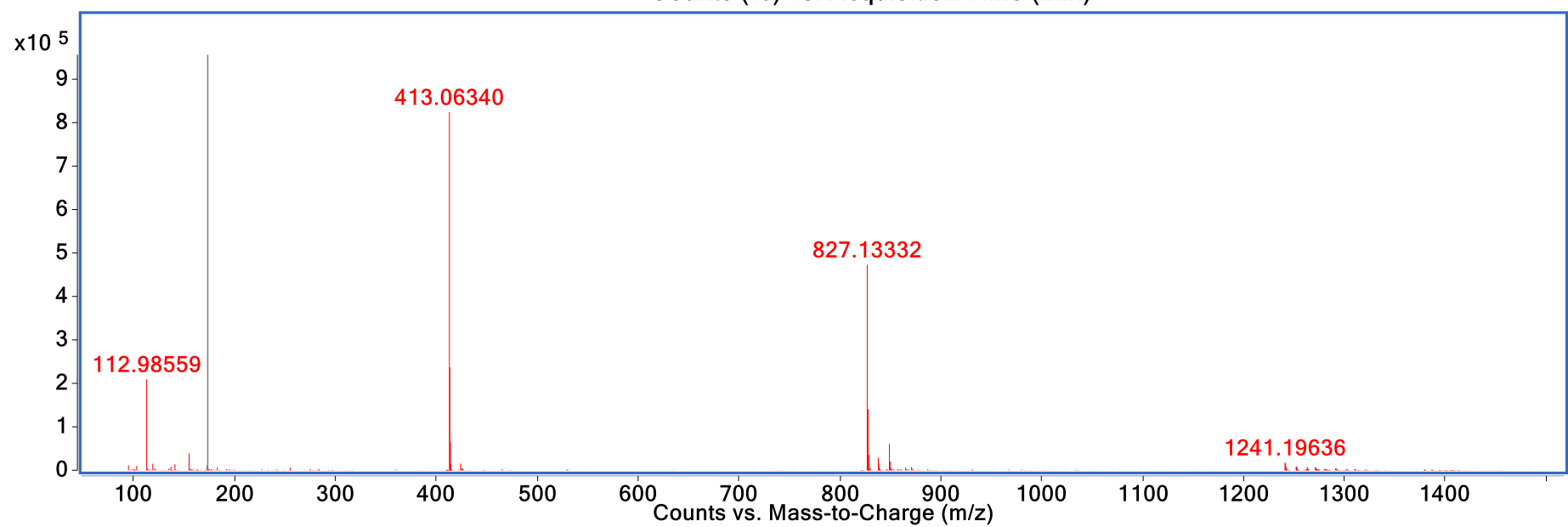
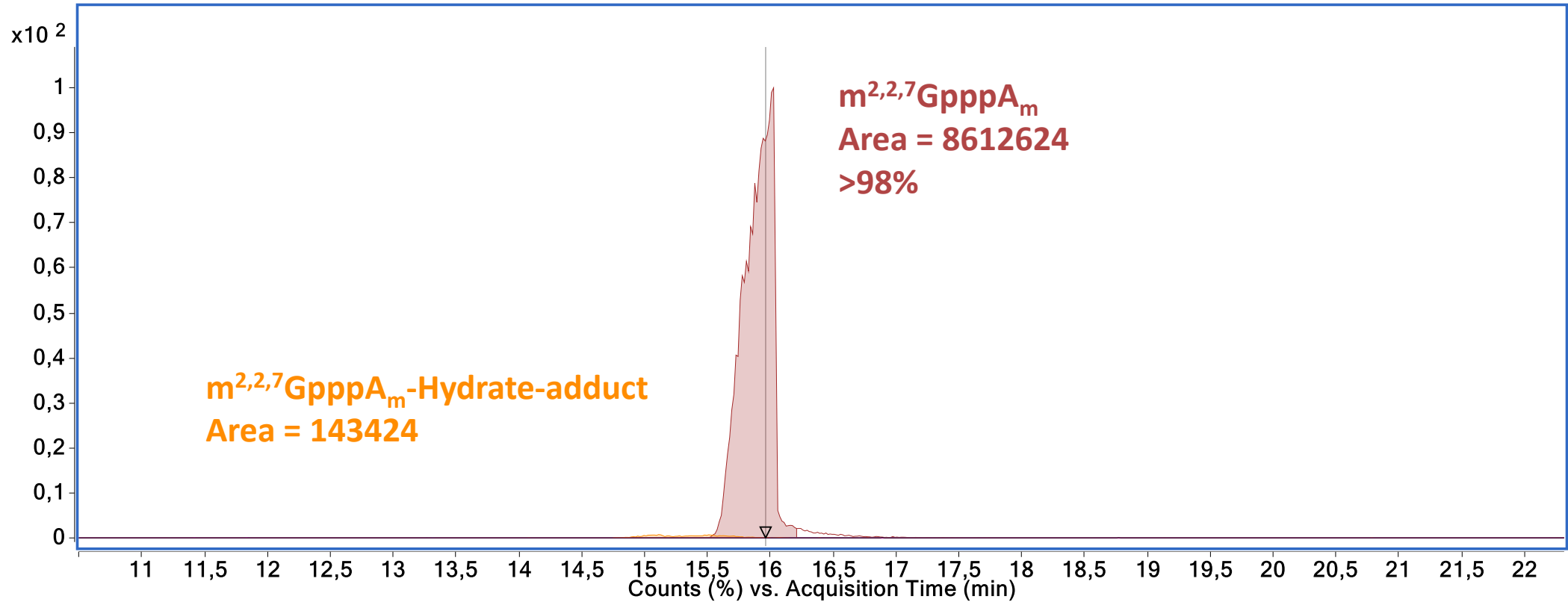


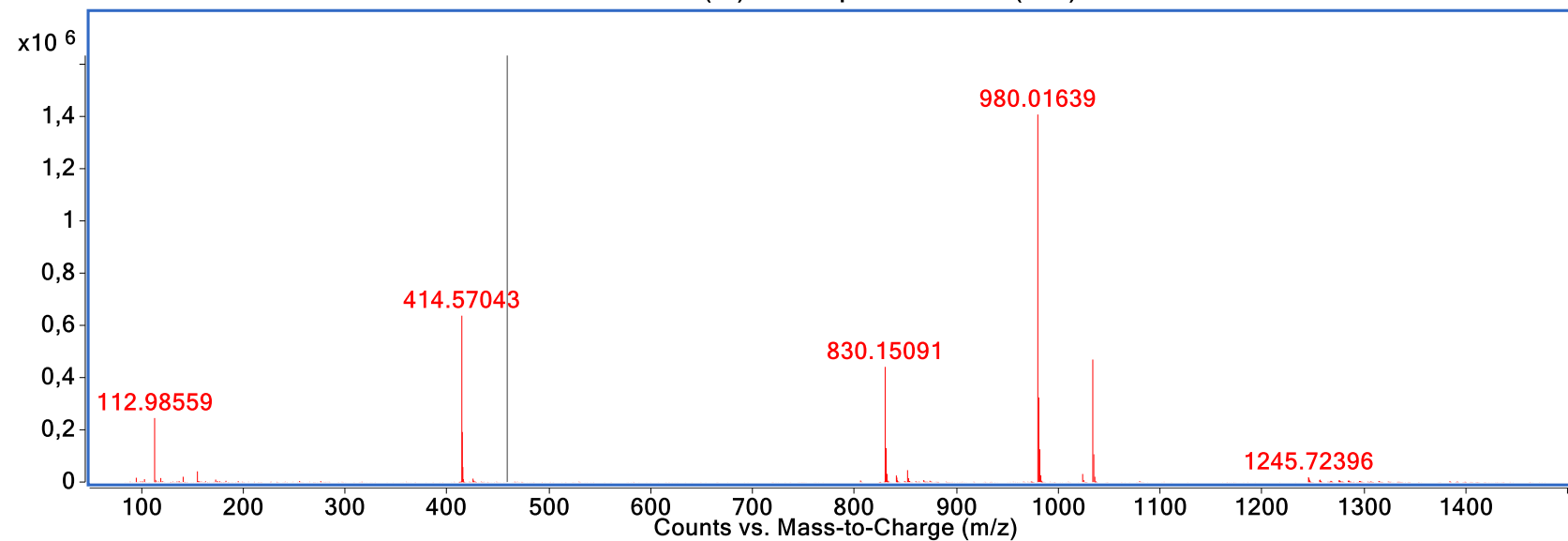
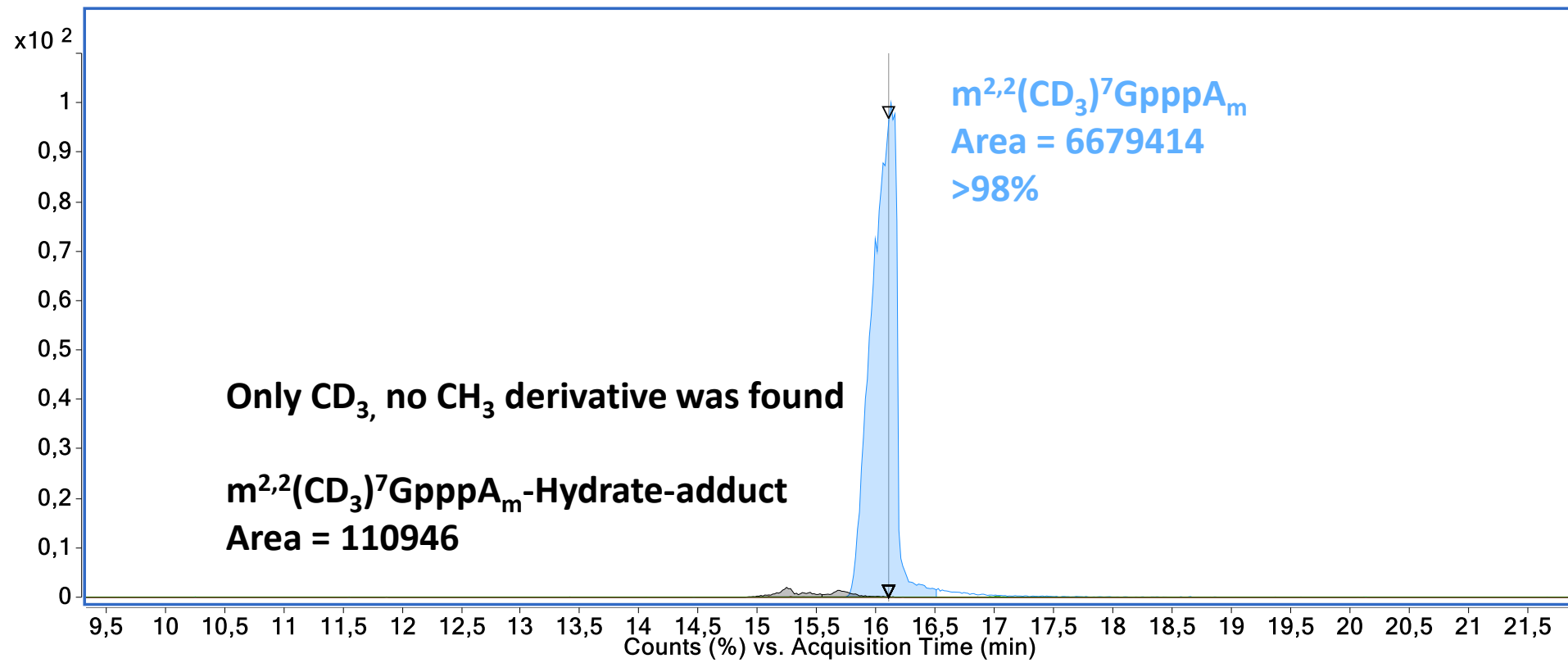


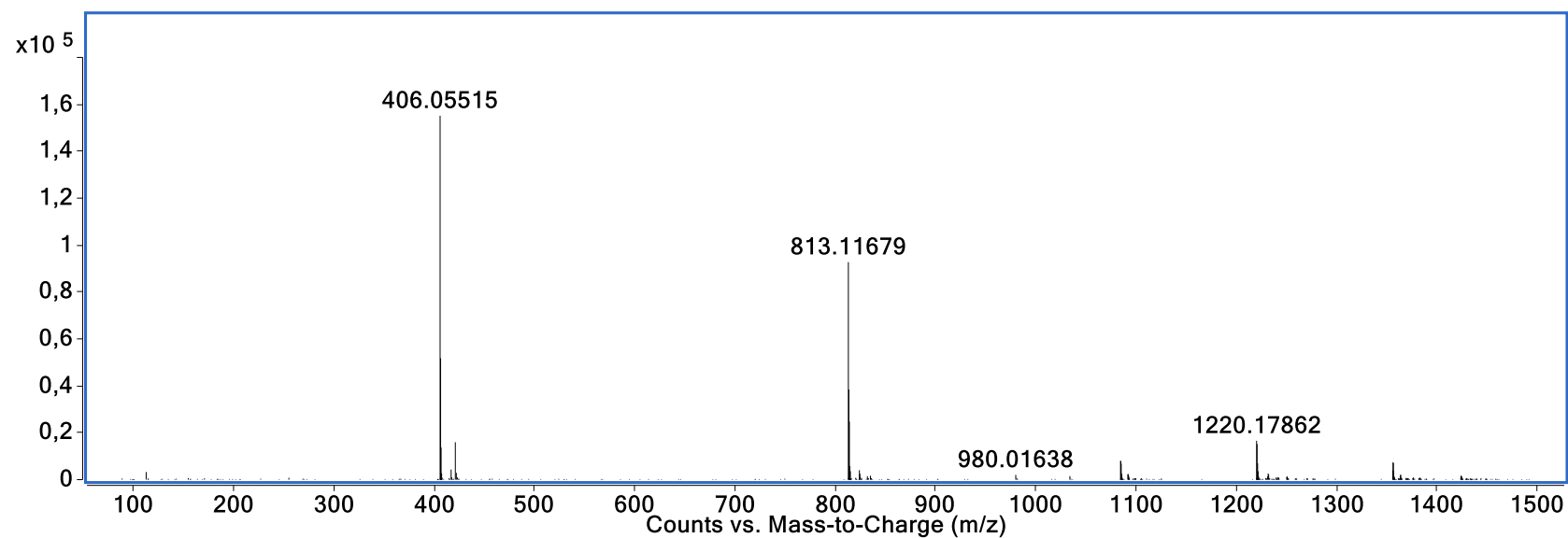
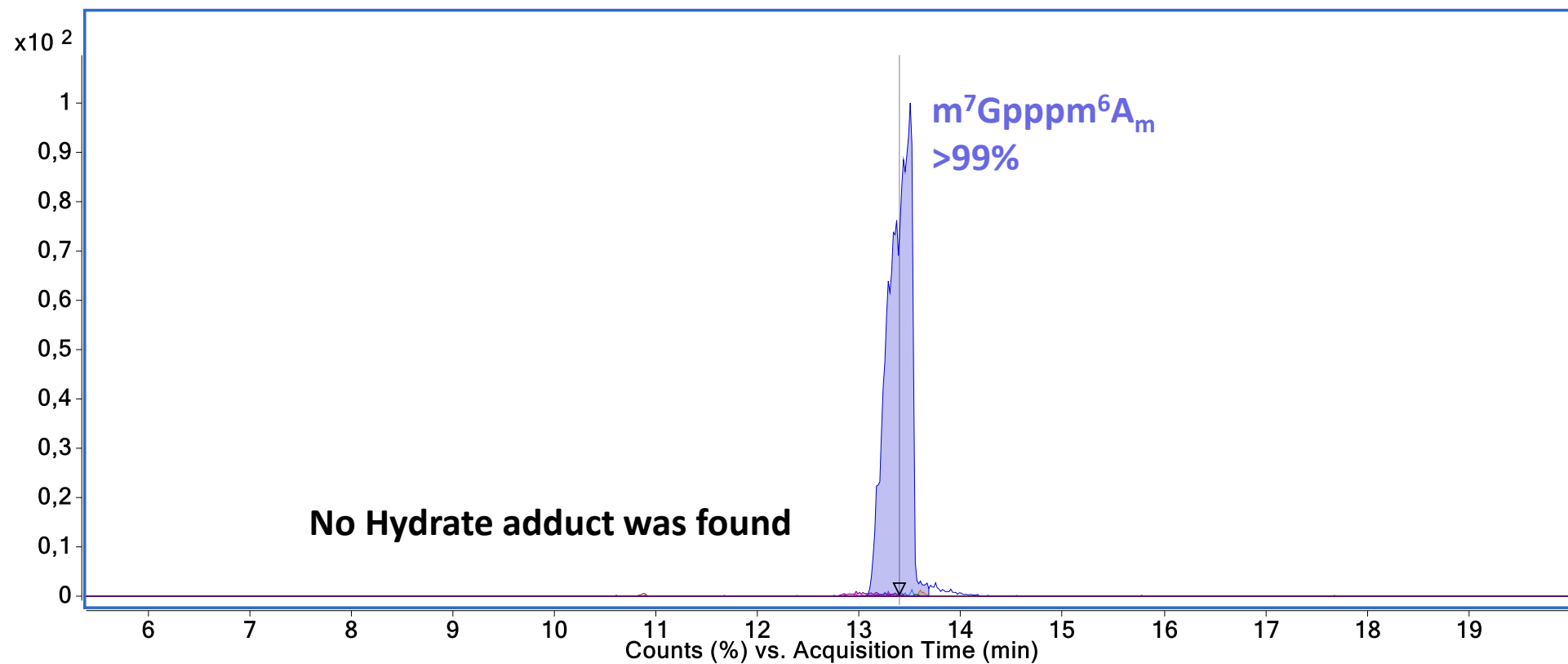


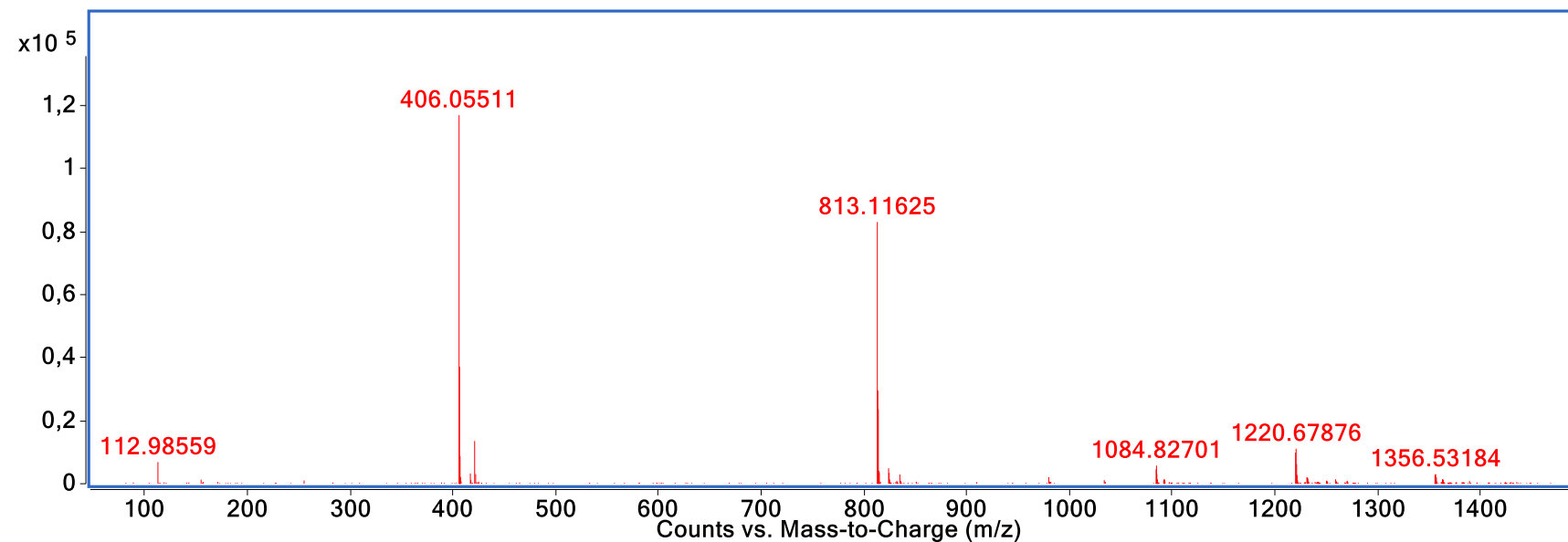
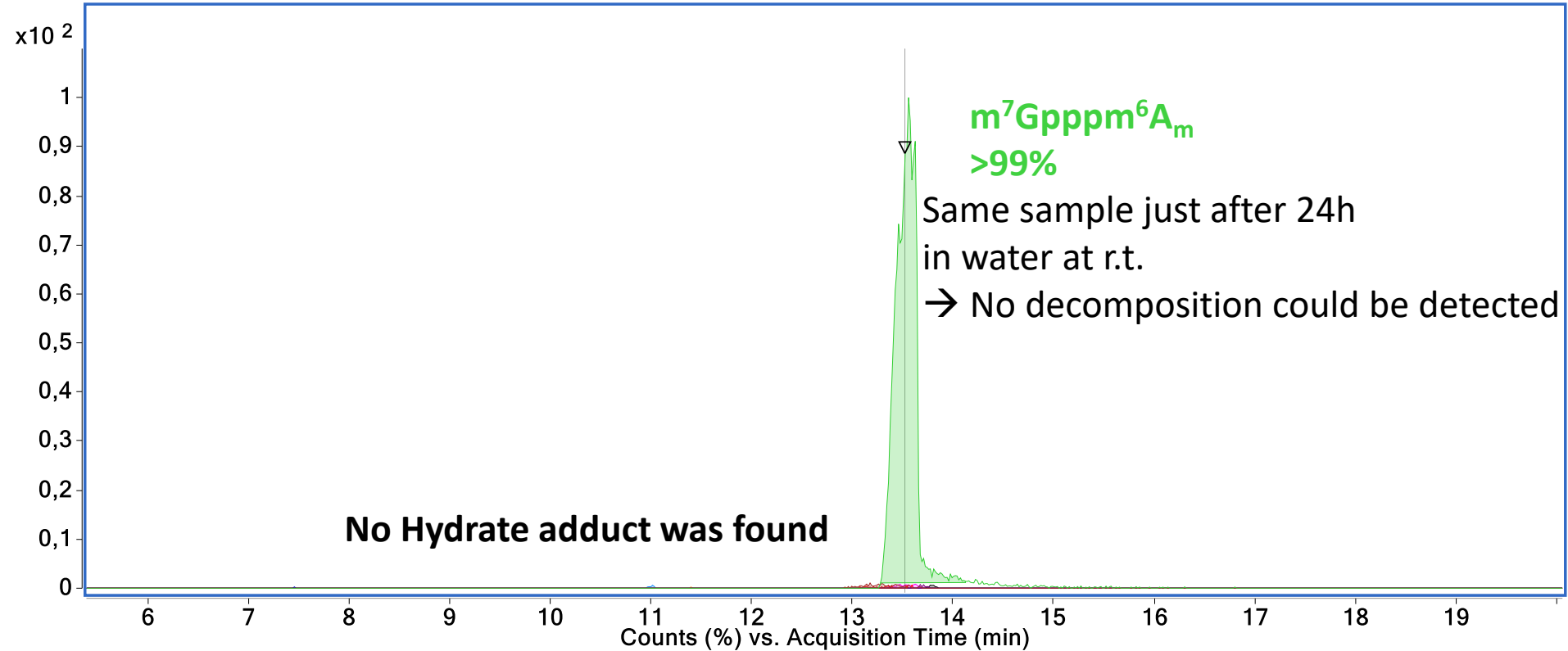


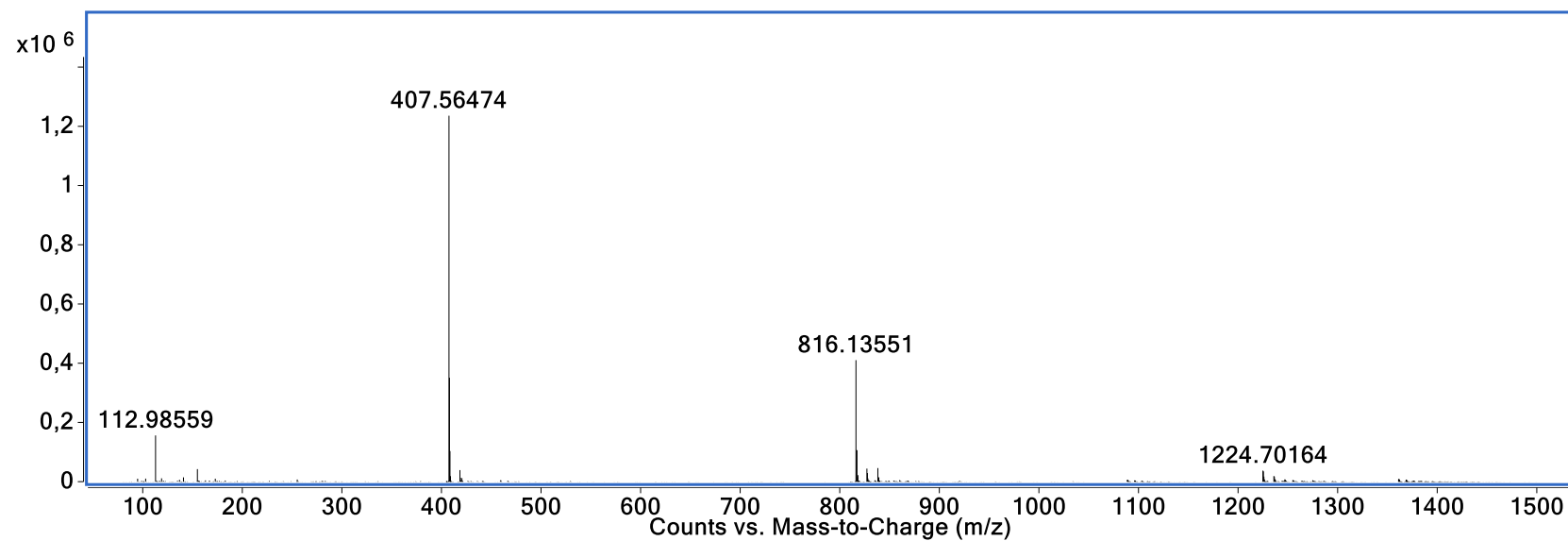
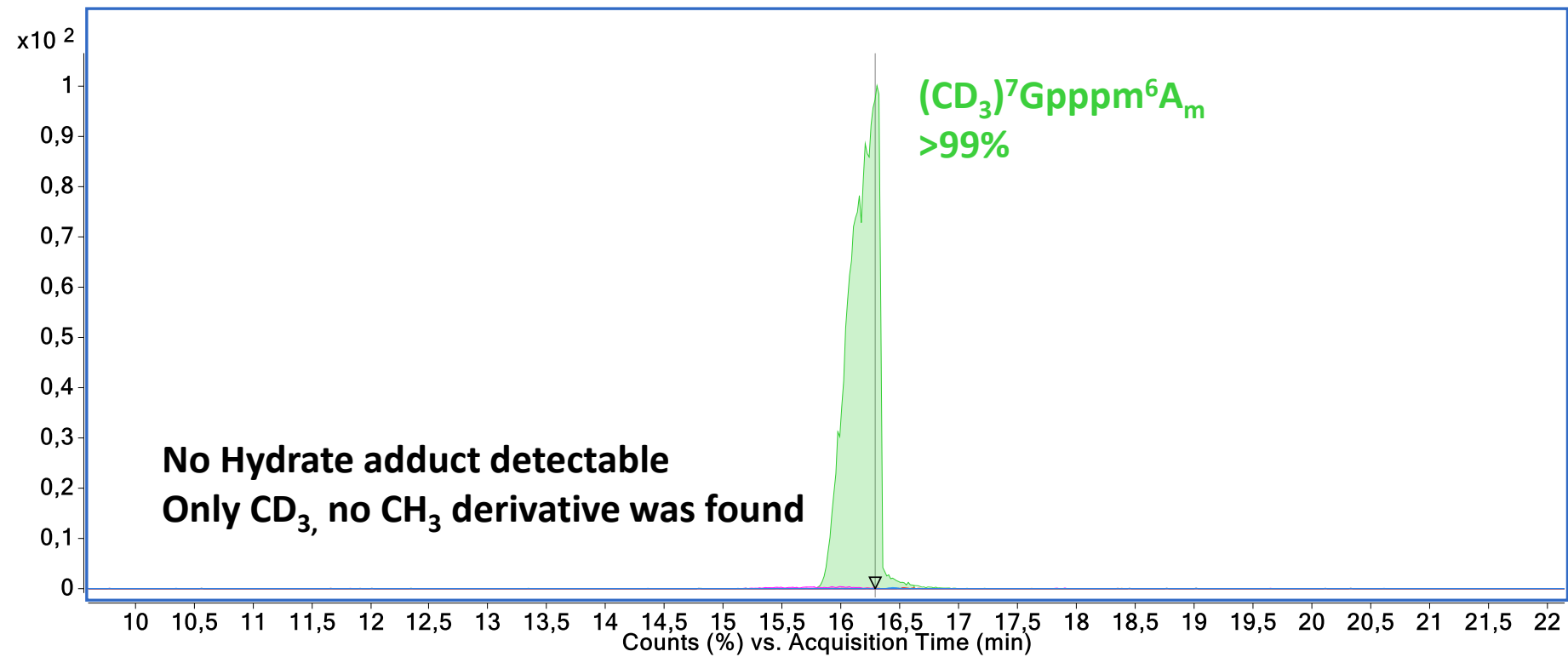


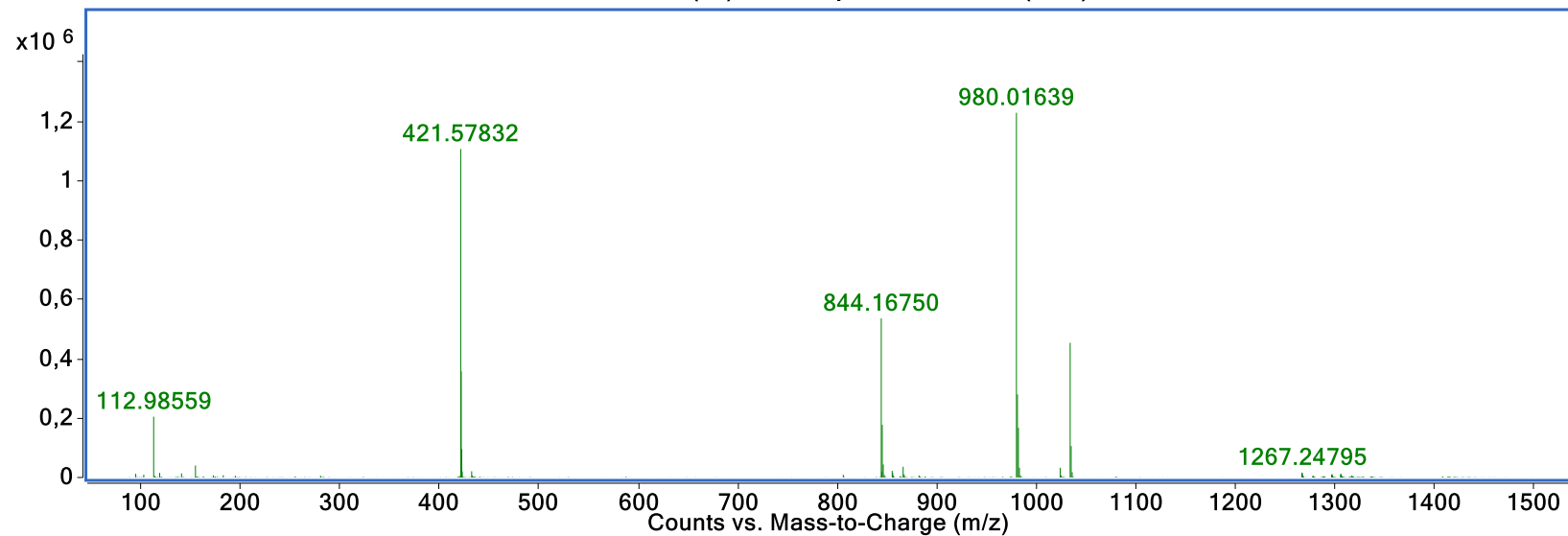
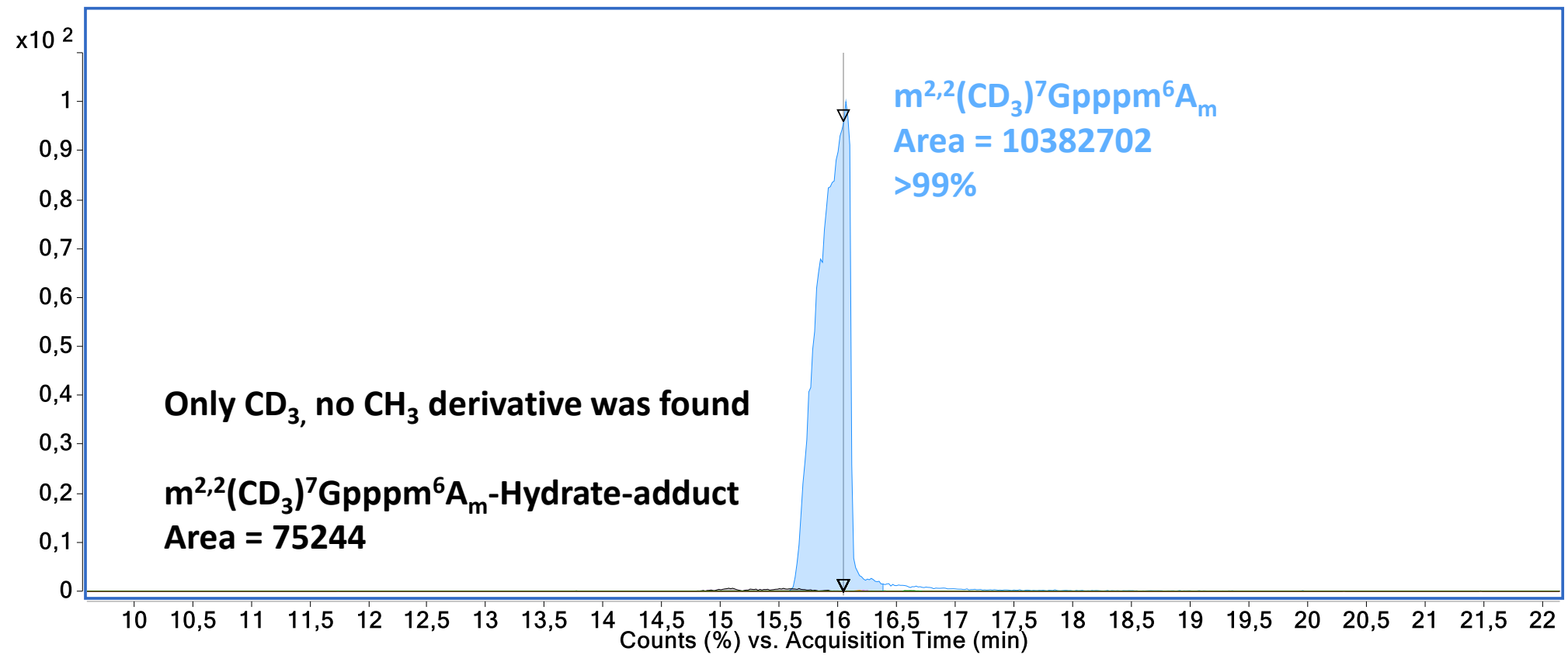




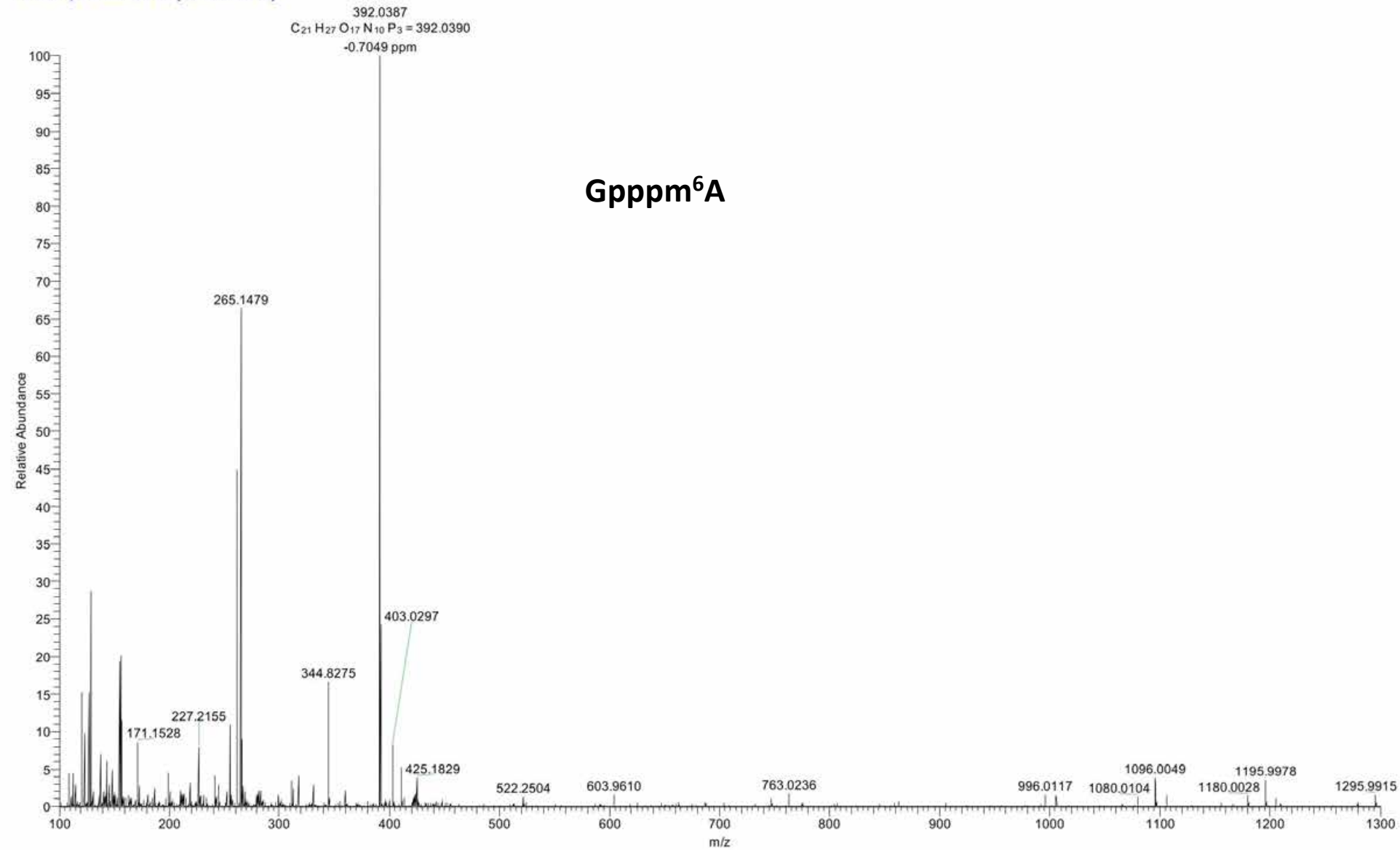




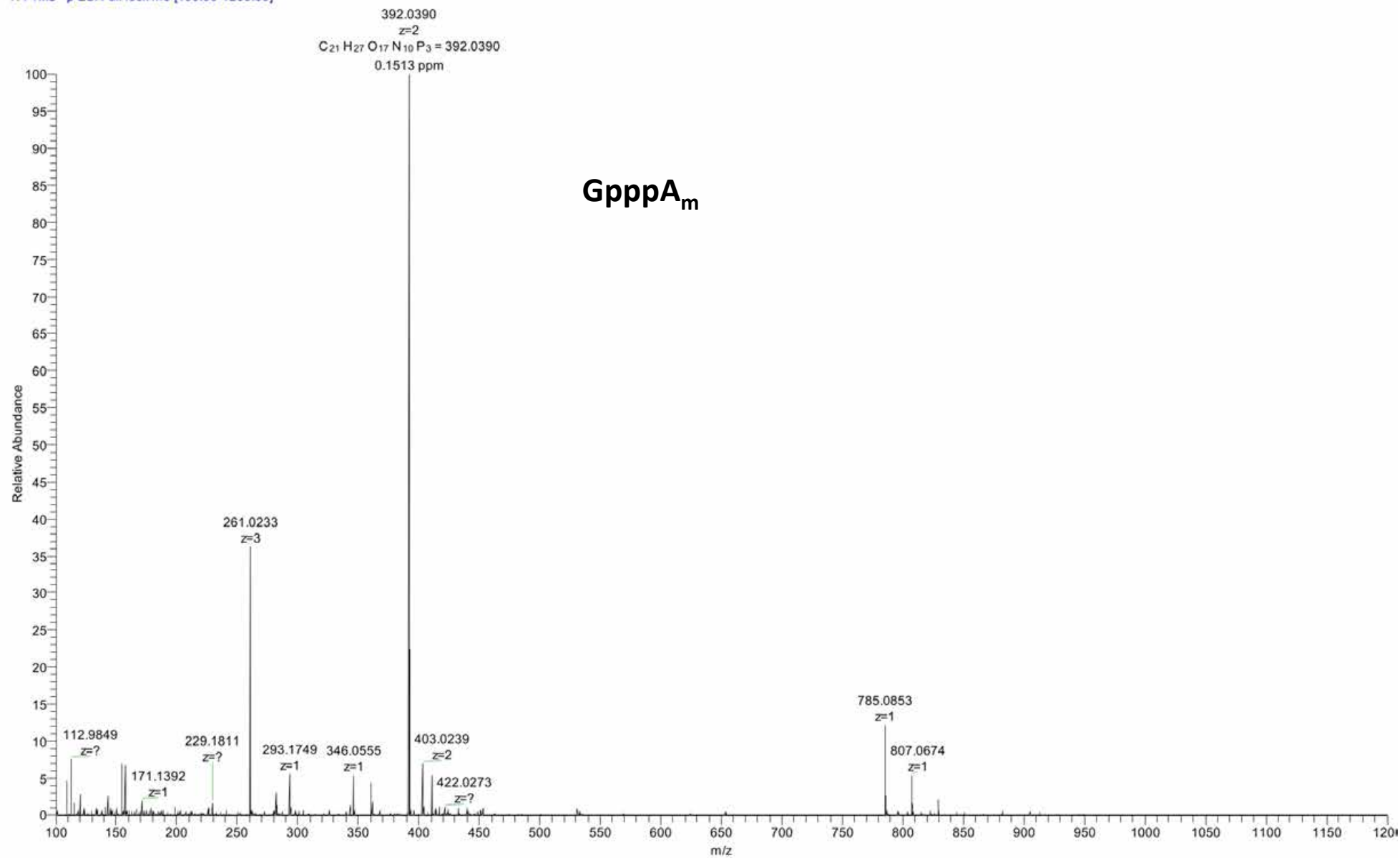




vpjeb31shr5 #1 RT: 0.02 AV: 1 NL: 2.18E6
T: FTMS - p ESI Full lock ms [100.00-1300.00]



rpjeb28shr1 #1 RT: 0.02 AV: 1 NL: 1.31E7
T: FTMS - p ESI Full lock ms [100.00-1200.00]



rpjb35shr1 #1 RT: 0.02 AV: 1 NL: 8.68E5
T: FTMS - p ESI Full ms [100.00-1500.00]

