Supplemental Information

Desthiobiotin (DTB)-modified and TAMRA-modified 2''OMeNAD$^+$ are RNA 2'-phosphotransferase (Tpt1) poisons that enable affinity-tagging and fluorescence-tagging of internal RNA 2'-phosphate groups

Renata Kasprzyk, Shreya Ghosh, and Stewart Shuman

Supplemental Methods

Figure S1

HPLC profiles, HR MS and NMR spectra

General methods for chemical synthesis

All solvents and chemical reagents used for compound synthesis were purchased from Sigma-Aldrich, VWR Chemicals, and Acros Organics and were used without any prior treatment. The synthesis of triethylammonium salt of 1-(2′-methoxy-*β*-D-ribofuranosyl)nicotinamide-5′-*O*-monophosphate (2”OMeNMN) has been reported in our previous work (Arnold et al. 2025). Triethylammonium salt of *N*6-(6-aminohexyl)adenosine 5’-monophosphate (*N*6-hex-AMP) was synthesized as described previously (Szczepaniak et al. 2012). Desthiobiotin NHS ester (DTB-NHS), *P*-imidazolides of adenosine 5’-monophosphates modified with desthiobiotin and TAMRA at the *C*2 position (*C*2-DTB-AMP-Im and *C*2-TAMRA-AMP-Im), their precursors, NAD-2-DTB, and NAD-2-TAMRA were synthesized as described previously (Kasprzyk et al. 2024; Lehner et al. 2022).

The progress of nucleotide synthesis was monitored using analytical RP-HPLC. The analysis was performed using a Shimadzu system consisting of LC-20AT pumps, an SPD-M20A absorption detector, a SIL-20A autosampler, and a CBM-20A communication module. The system was equipped with an EC HPLC NUCLEODUR C18 Pyramid column (5 µm, 250 x 4.6 mm, flow rate of 1.0 mL/min), operating with a linear gradient of acetonitrile in 0.1 % formic acid. UV-detection was conducted at 254 nm, with additional detection at 555 nm for TAMRA-labelled nucleotides.

Compounds **1**, **3**, and **4** (Fig. S1) were purified using semipreparative RP-HPLC on a Shimadzu system consisting of LC-20AP pumps, an SPD-M20A absorption detector, an FRC-10A fraction collector, and a CBM-20A communication module. The system was equipped with a VP HPLC NUCLEODUR C18 Pyramid column (5 µm, 250 x 16 mm, flow rate 8.0 mL/min) using different buffers and eluents (see the table below). UV-detection was performed at 254 nm, with additional detection at 555 nm for TAMRA-labelled nucleotides. The specific HPLC methods used in the studies are outlined below:

Method name	Eluent A	Eluent B	Eluent gradient		Column
P1	0.05 M triethylammonium acetate buffer (pH ~ 6)	ACN	5 % 5 – 80 % 80 – 100 % 100 % 100 – 5 % 5 %	0 – 10 min 5 – 60 min 60 – 65 min 65 – 70 min 70 – 75 min 75 – 80 min	VP HPLC NUCLEODUR C18 HTec, 5 µm, 250 x 21 mm
P2	0.1 % formic acid	ACN	0 % 0 – 55 % 55 – 100 % 100 % 100 – 0 % 0 %	0 – 5 min 5 – 40 min 40 – 45 min 45 – 50 min 50 – 55 min 55 – 60 min	VP HPLC NUCLEODUR C18 Pyramid, 5 µm, 250 x 16 mm
P3	0.1 % formic acid	ACN	0 % 0 – 45 % 45 – 100 % 100 % 100 – 0 % 0 %	0 – 5 min 5 – 40 min 40 – 45 min 45 – 50 min 50 – 55 min 55 – 60 min	VP HPLC NUCLEODUR C18 Pyramid, 5 µm, 250 x 16 mm
P4	0.05 M ammonium acetate buffer (pH 5.9)	ACN	0 % 0 – 65 % 65 – 100 %	0 – 5 min 5 – 60 min 60 – 70 min	VP HPLC NUCLEODUR C18 Pyramid, 5 µm,

			100 % 100 – 0 % 0 %	70 – 75 min 75 – 80 min 80 – 85 min	250 x 16 mm
P5	0.05 M ammonium acetate buffer (pH 5.9)	ACN	0 % 0 – 45 % 45 – 100 % 100 % 100 – 0 % 0 %	0 – 5 min 5 – 65 min 65 – 70 min 70 – 75 min 75 – 80 min 80 – 85 min	VP HPLC NUCLEODUR C18 Pyramid, 5 µm, 250 x 16 mm

Yields after purification were calculated based on sample absorption, measured using the ND-1000 NanoDrop spectrophotometer (PEQLAB). The structure and homogeneity of the final product were confirmed using a combination of analytical techniques, including RP-HPLC, high-resolution mass spectrometry (HRMS) in ESI+ mode, and various NMR methods: ^{1}H NMR, ^{1}H-^{1}H COSY, ^{13}C NMR, ^{1}H-^{13}C HSQC NMR, ^{31}P NMR, and ^{1}H-^{31}P HMBC NMR spectroscopy. High resolution mass spectra were recorded on an LTQ Orbitrap Discovery. Routine NMR spectra were recorded at 25°C with nucleotide concentrations ranging from 5 to 10 mM, using a Bruker AVANCE NEO spectrometer operating at 800 MHz (^{1}H, ^{1}H-^{1}H COSY), 201 MHz (^{13}C), and 324 MHz (^{31}P), a Bruker Avance NEO 600 spectrometer operating at 151 MHz (^{13}C), a Jeol ECZ 500R spectrometer operating at 500 MHz (^{1}H, ^{1}H-^{1}H COSY), 126 MHz (^{13}C), and 202 MHz (^{31}P) or a Bruker Avance III 400 spectrometer operating at 400 MHz (^{1}H, ^{1}H-^{1}H COSY). The ^{1}H chemical shifts were referenced to the solvent signal, and the ^{31}P and ^{13}C chemical shifts were adjusted based on correlation spectra with the ^{1}H signals.

References:

Arnold J, Ghosh S, Kasprzyk R, Brakonier M, Hanna M, Marx A, Shuman S. 2024. Chemical synthesis of 2''OMeNAD+ and its deployment as an RNA 2'-phosphotransferase (Tpt1) ‘poison’ that traps the enzyme on its abortive RNA-2'-PO4 -(ADP-2''OMe-ribose) reaction intermediate. Nucleic Acids Res 23: 10533-10542.

Kasprzyk R, Rieth S, Heid P, Stengel F, Marx A. 2024. Cell-permeable nicotinamide adenine dinucleotides for exploration of cellular protein ADP-ribosylation. Angew Chem Int Ed 63: e202411203.

Lehner M, Rieth S, Höllmüller E, Spliesgar D, Mertes B, Stengel F, Marx A. 2022. Profiling of the ADP-ribosylome in living cells. Angew Chem Int Ed 61: e202200977.

Szczepaniak SA, Zuberek J, Darzynkiewicz E, Kufel J, Jemielty J. (\2012. Affinity resins containing enzymatically resistant mRNA cap analogs—a new tool for the analysis of cap-binding proteins. RNA 18: 1421-1432.

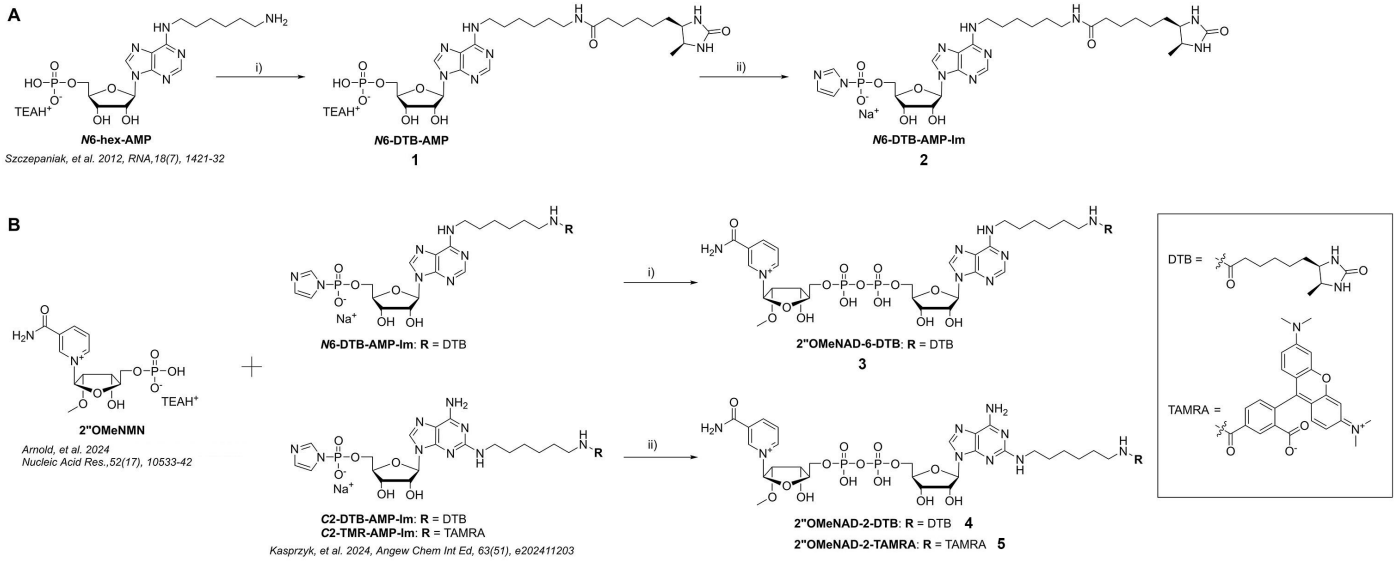
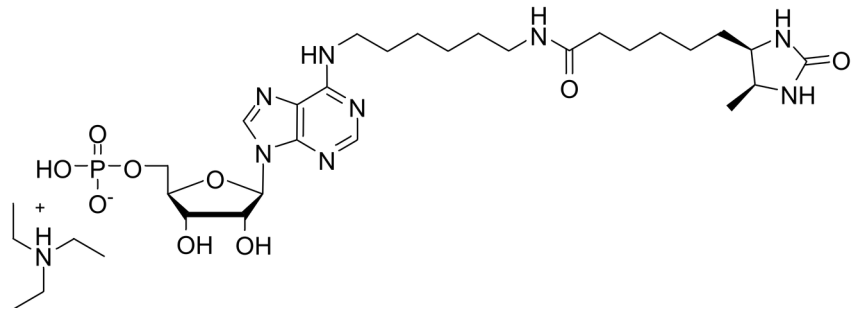


Figure S1. **Synthesis of desthiobiotin (DTB)-modified and 5-carboxytetramethylrhodamine (TAMRA)-modified 2''OMeNAD$^+$ analogs**. (A) (i) N-Hydroxysuccinimide ester of DTB (DTB-NHS), 50 mM borate buffer pH 8.2, r.t., 2 h, 75%. (ii) imidazole, 2,2'-dithiodipyridine, triethylamine, triphenylphosphine, dimethylformamide, r.t., 18 h, then $NaClO_4$, acetone, 4°C, precipitation, 73%. (B) (i) formamide/dimethyl sulfoxide (1:1, v/v), $MgCl_2$, r.t., 24 h, 35%. (ii) formamide/dimethyl sulfoxide (1:1, v/v), $MgCl_2$, r.t., 20 h, 42% for 2''OMeNAD-2-DTB or formamide/dimethyl sulfoxide (3:2, v/v), $MgCl_2$, $MnCl_2$, r.t., 22h, 42% for 2''OMeNAD-2-TAMRA.

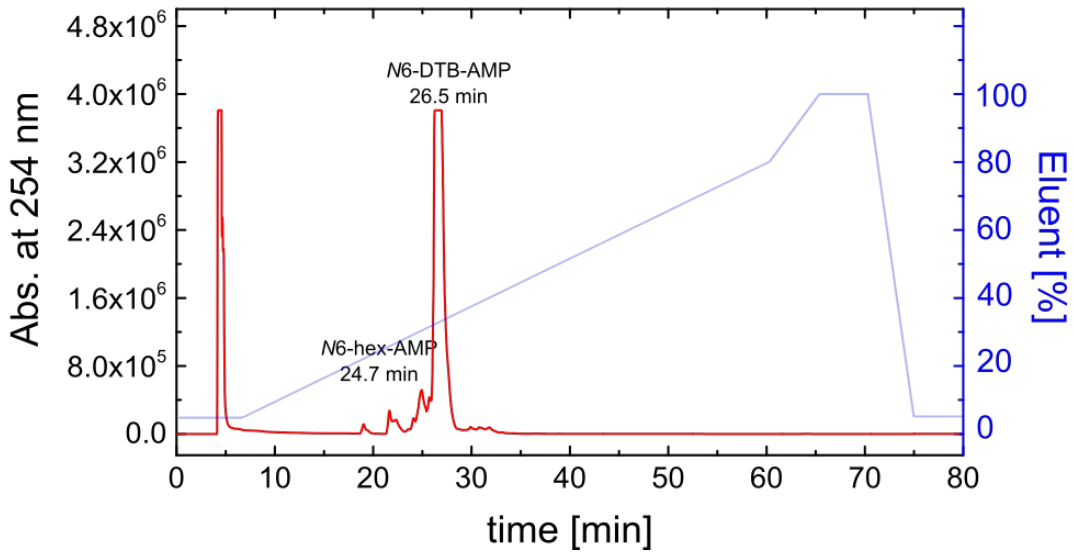
HPLC profiles, HR MS and NMR spectra

*N*6-DTB-AMP (**1**)

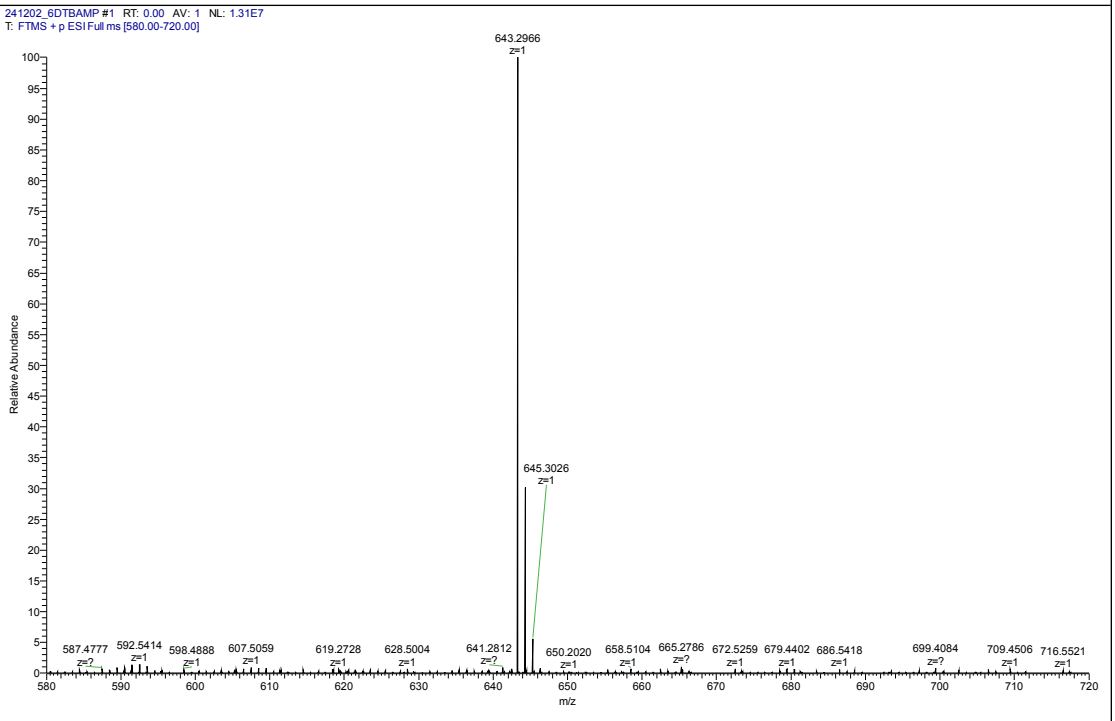
Formula



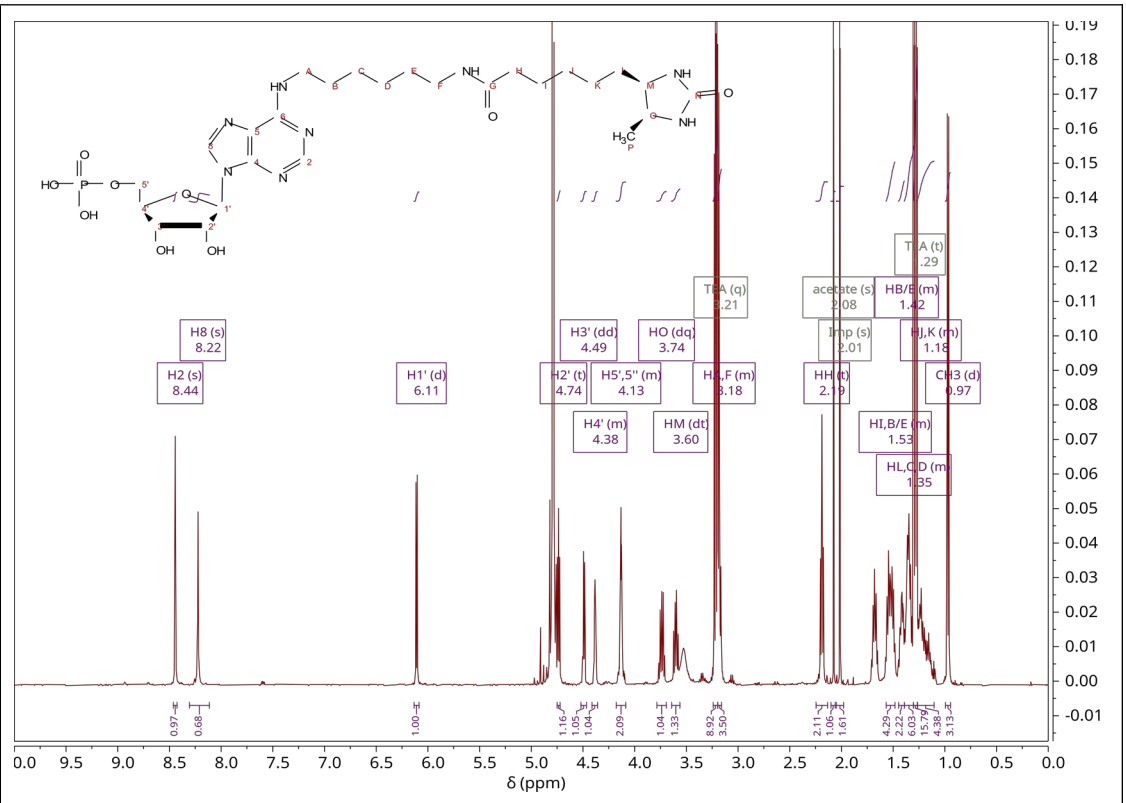
HPLC purification profile (Method P1)



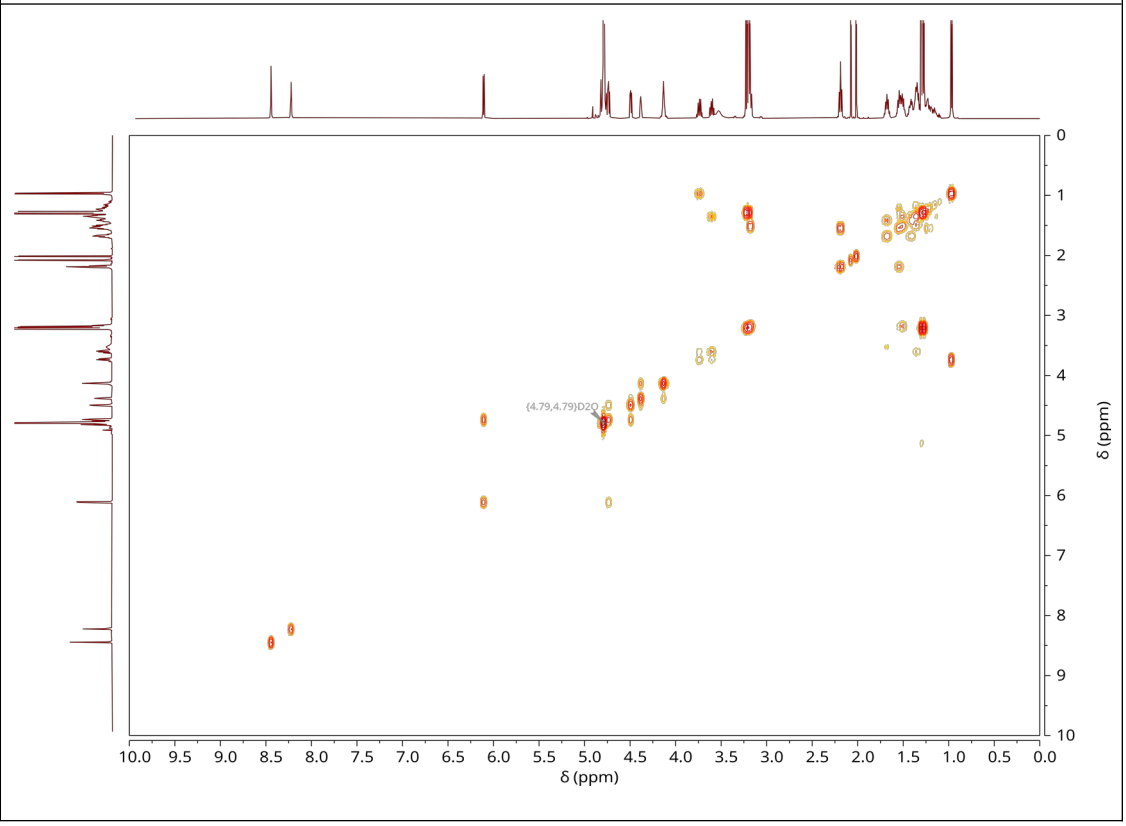
HRMS (+)ES



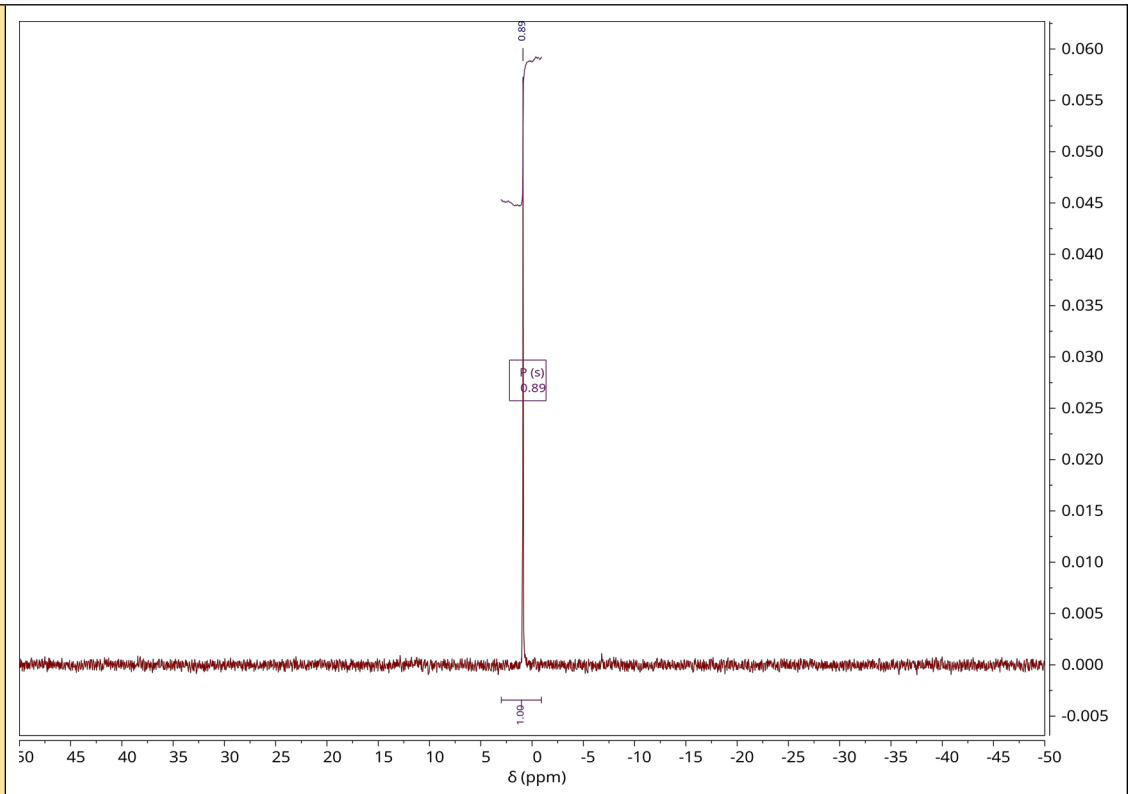
^{1}H NMR (500 MHz, D_2O, 25°C)



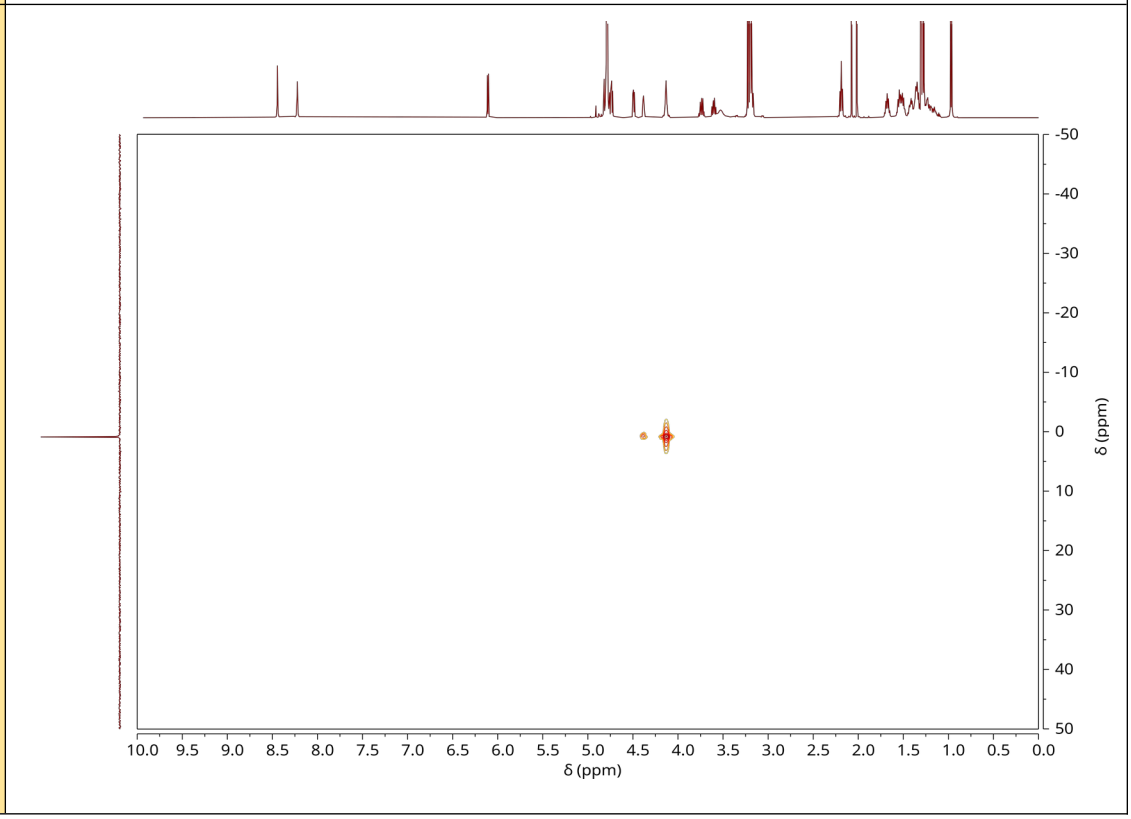
^{1}H-^{1}H COSY (D_2O, 25°C)



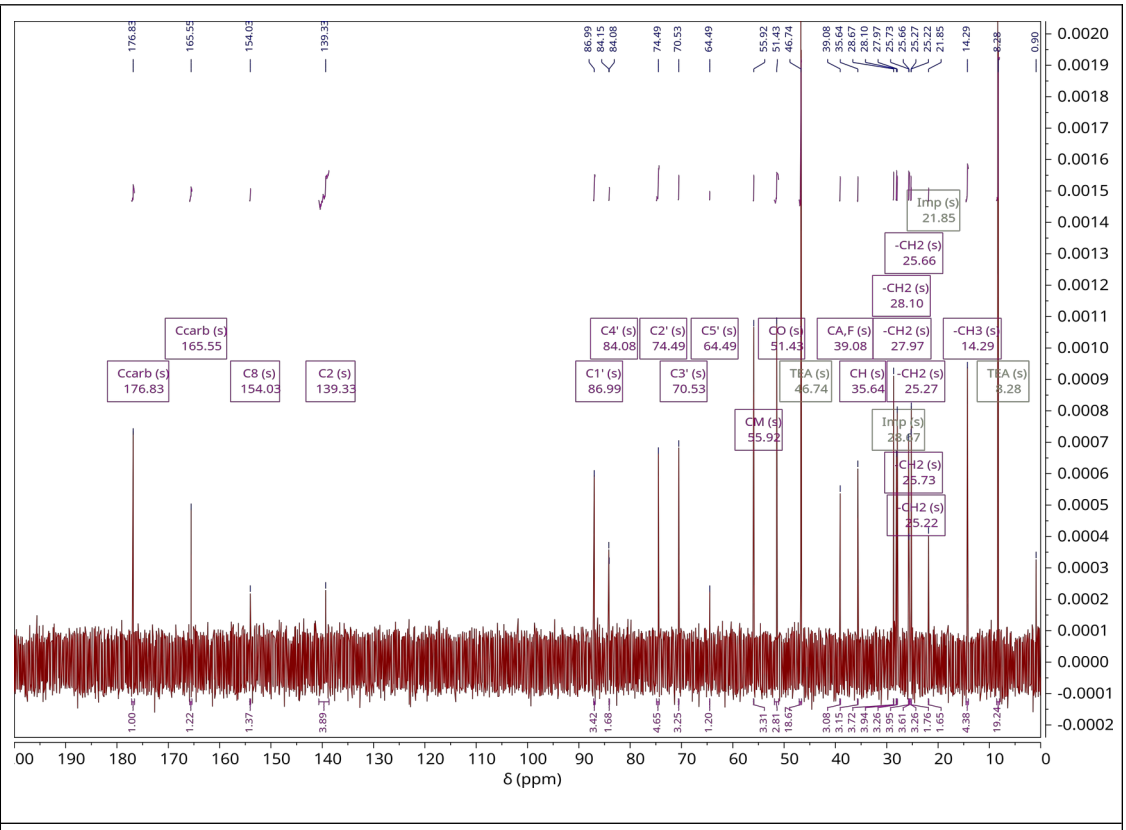
^{31}PCPD NMR (202 MHz, D_2O, 25°C)



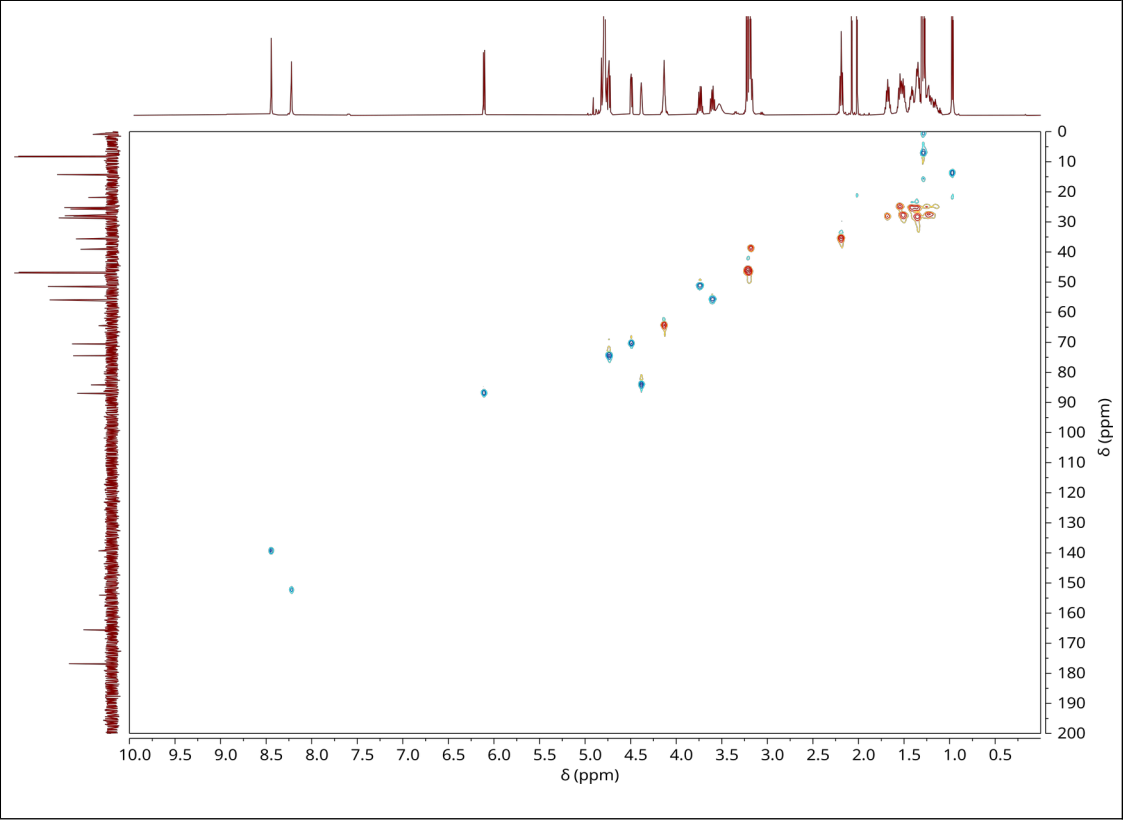
^{1}H-^{31}P HMBC (D_2O, 25°C)



^{13}C NMR 125 MHz, D_2O, 25°C)

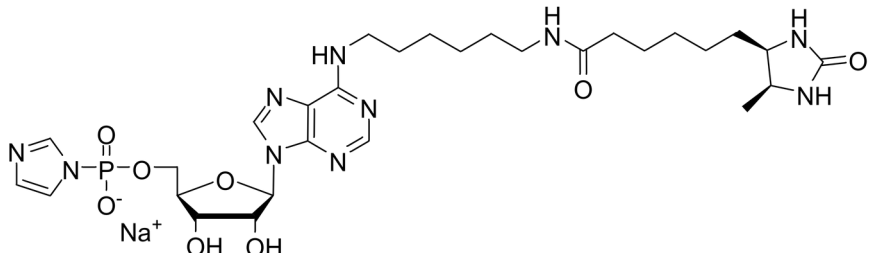


^{1}H-^{13}C HSQC (D_2O, 25°C)

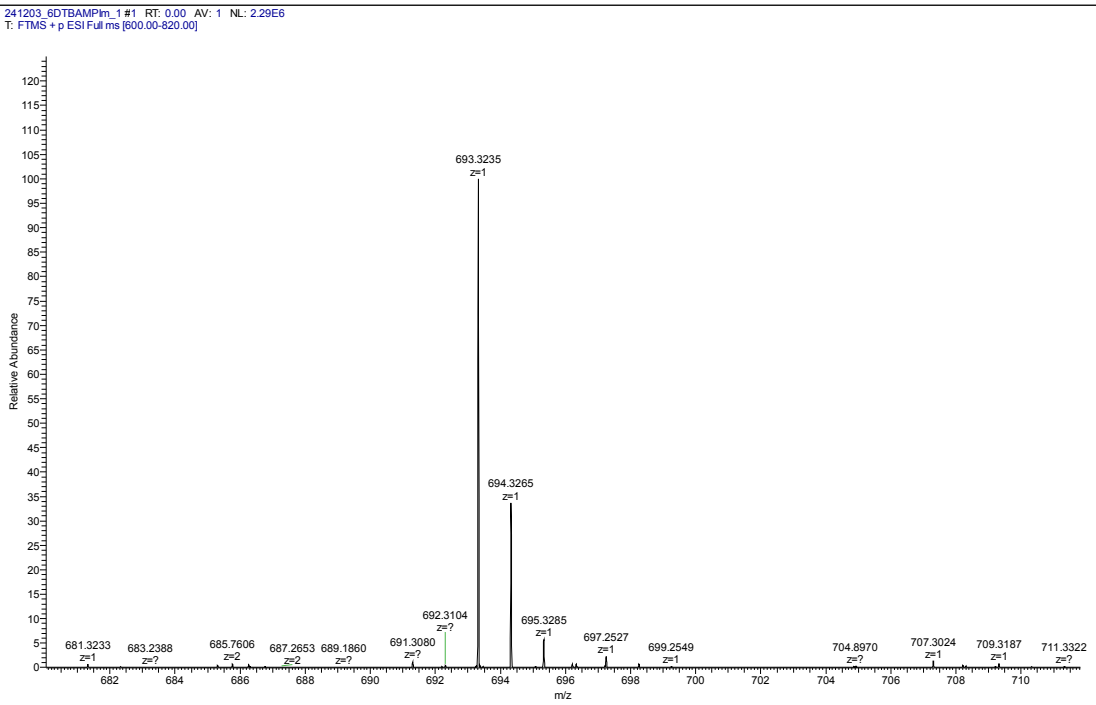


*N*6-DTB-AMP-Im (**2**)

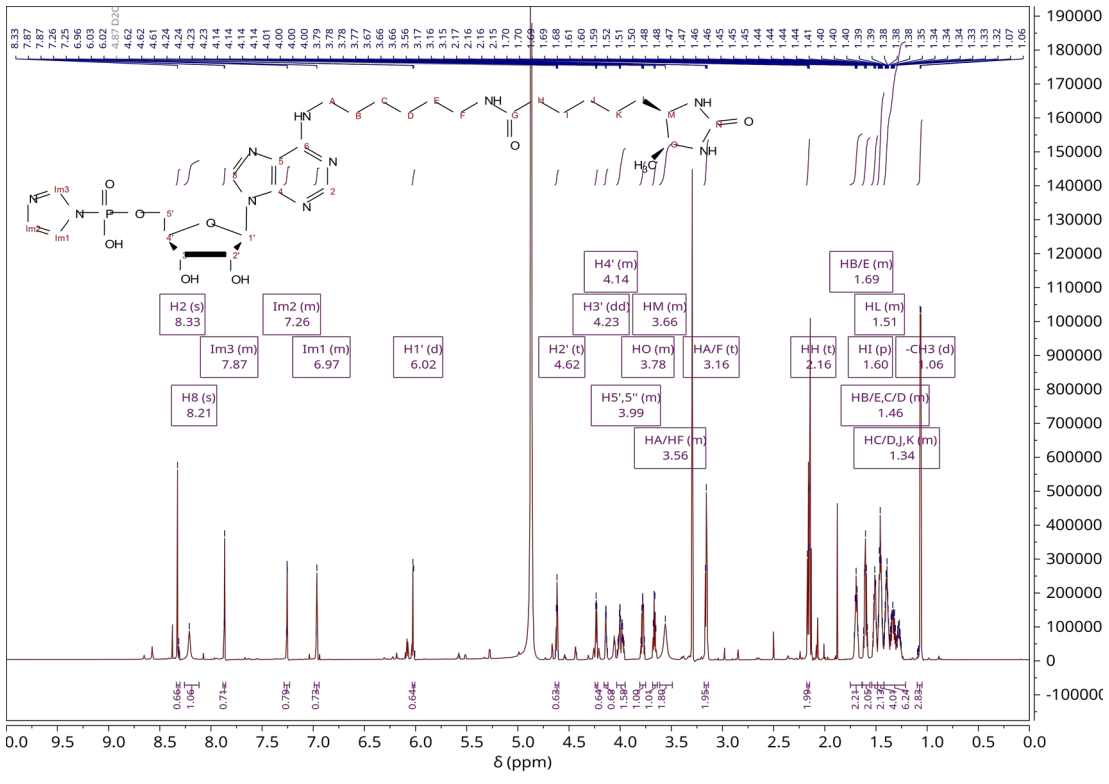
Formula



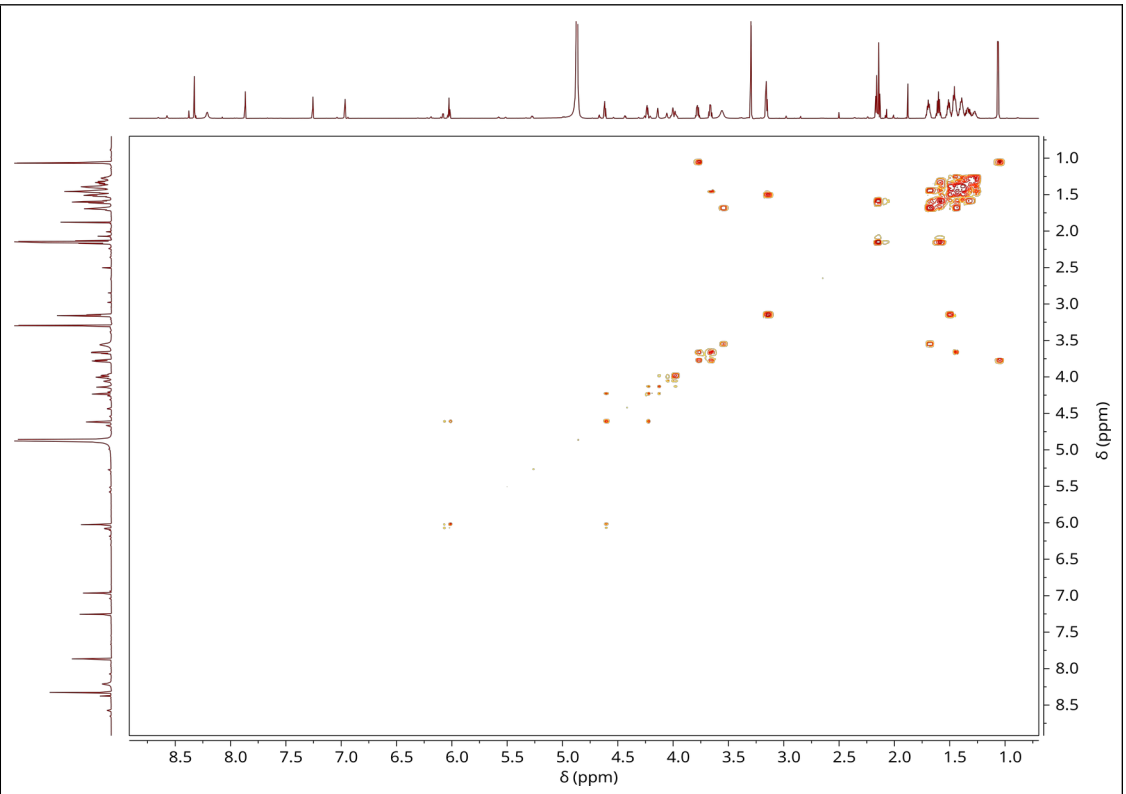
HRMS (+)ES



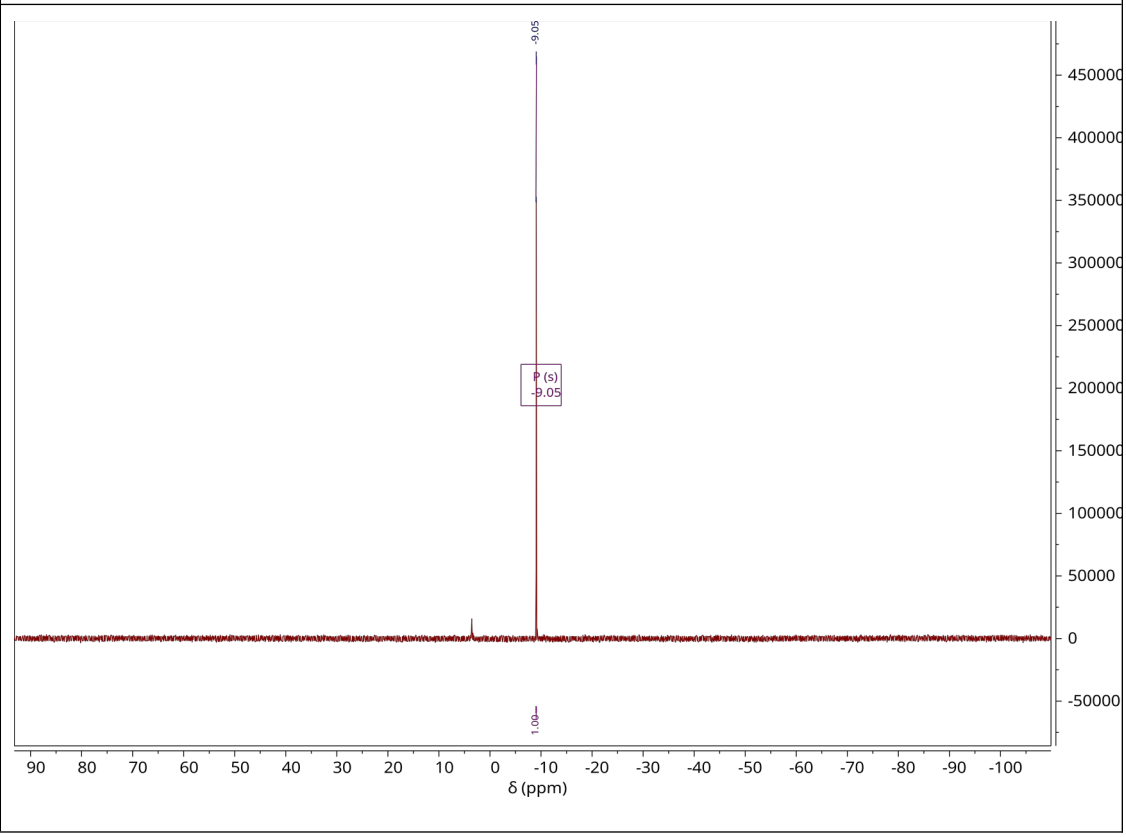
^{1}H NMR (800 MHz, MeOD, 25°C)



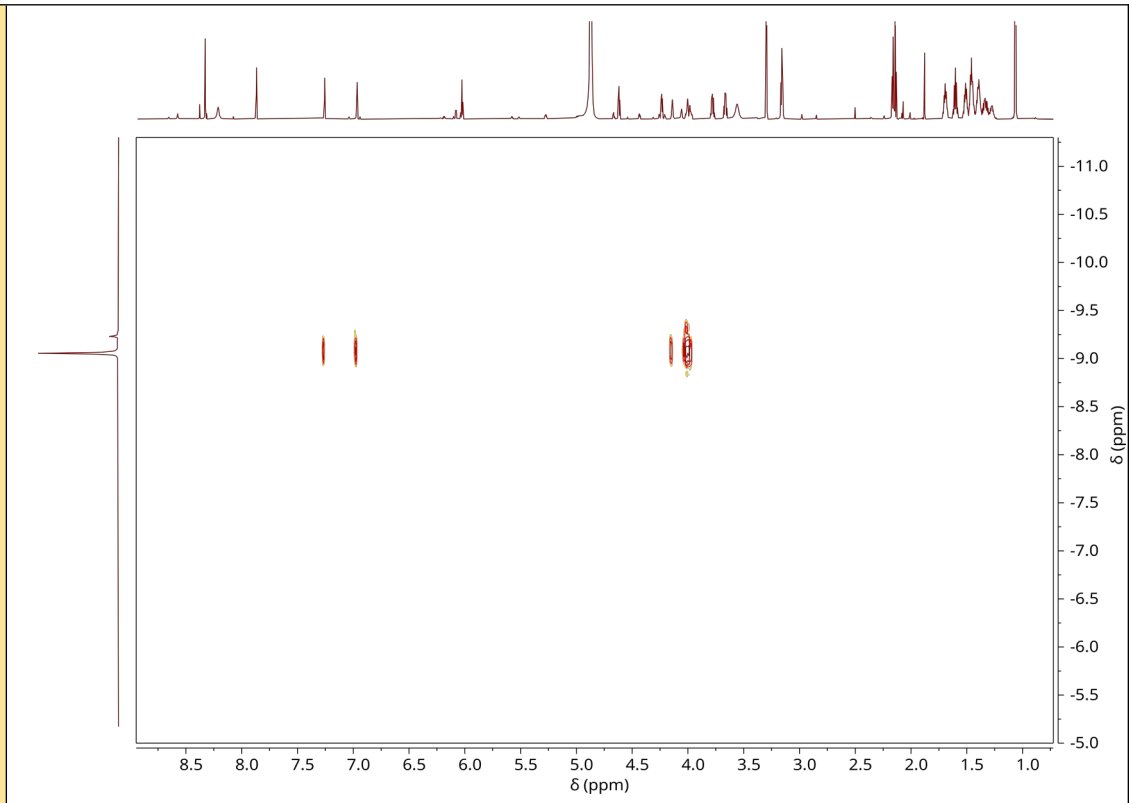
^{1}H-^{1}H COSY (MeOD, 25°C)



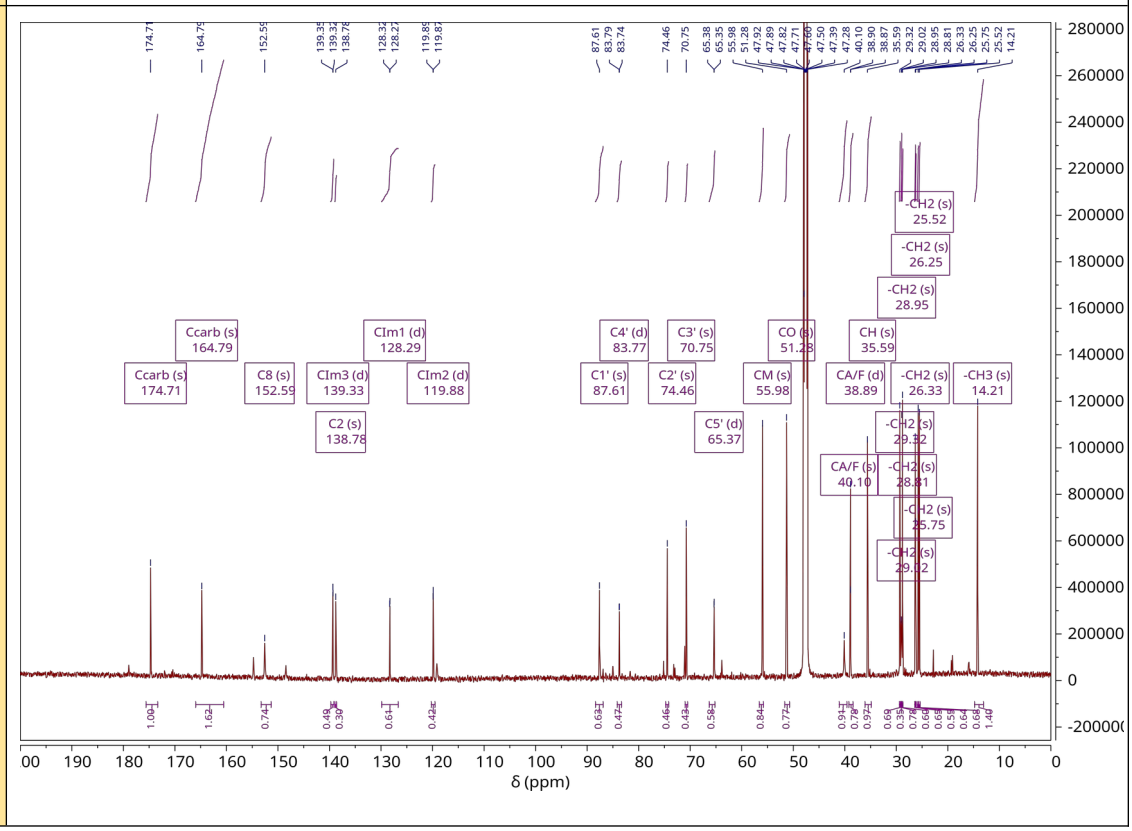
^{31}PCPD NMR (324 MHz, MeOD, 25°C)

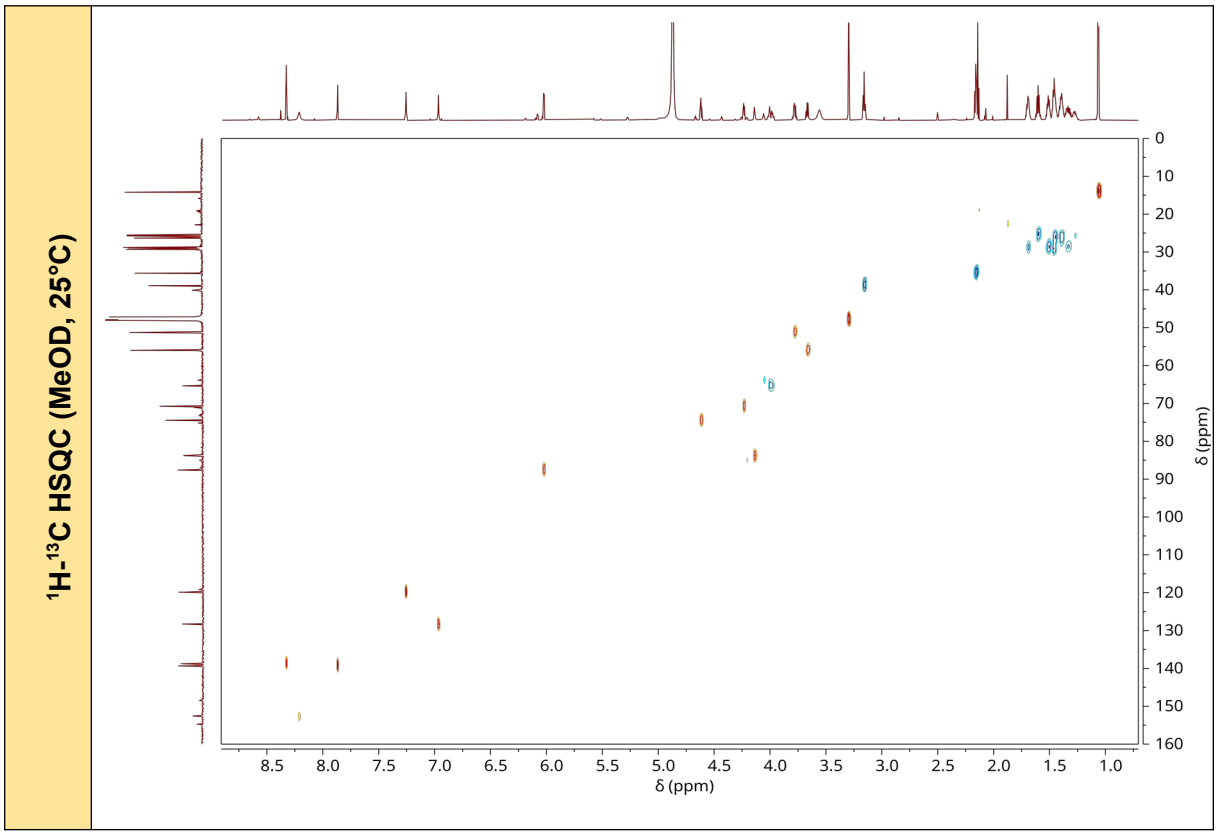


^{1}H-^{31}P HMBC (MeOD, 25°C)



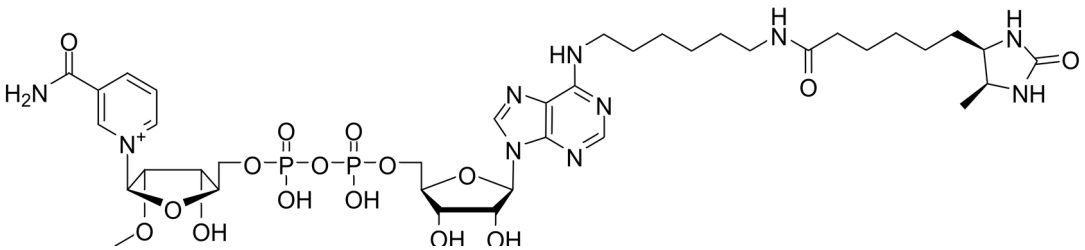
^{13}C NMR 201 MHz, MeOD, 25°C)



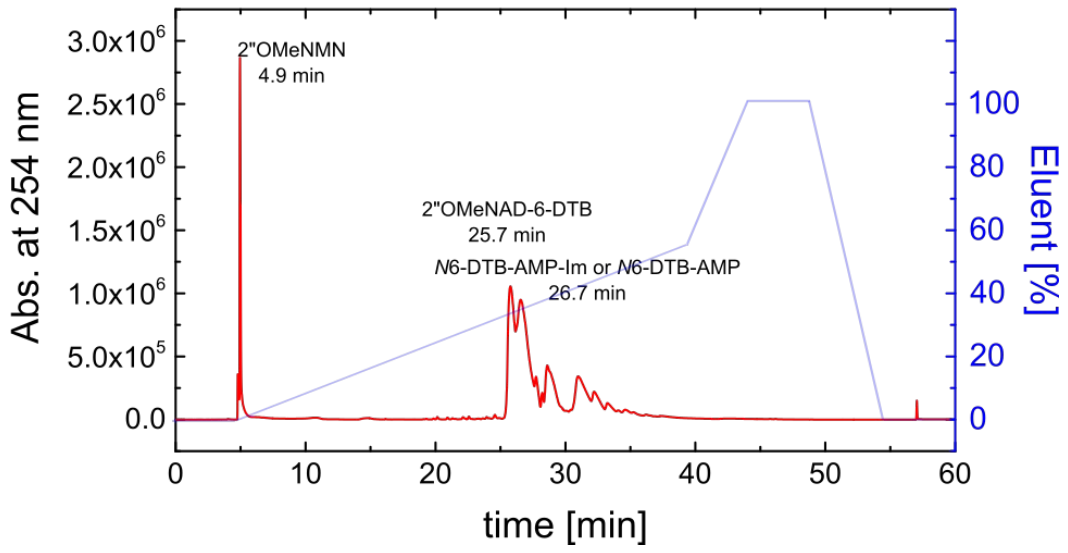

^{1}H-^{13}C HSQC (MeOD, 25°C)
δ (ppm)
δ (ppm)

2”OMeNAD-6-DTB (**3**)

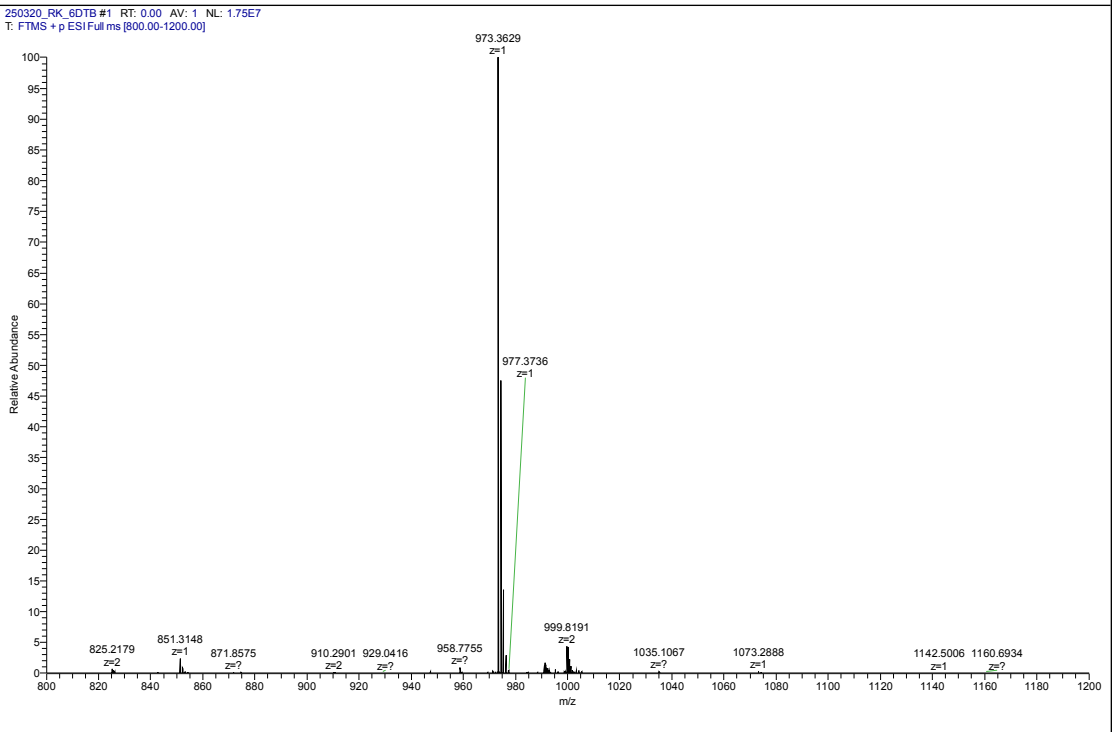
Formula



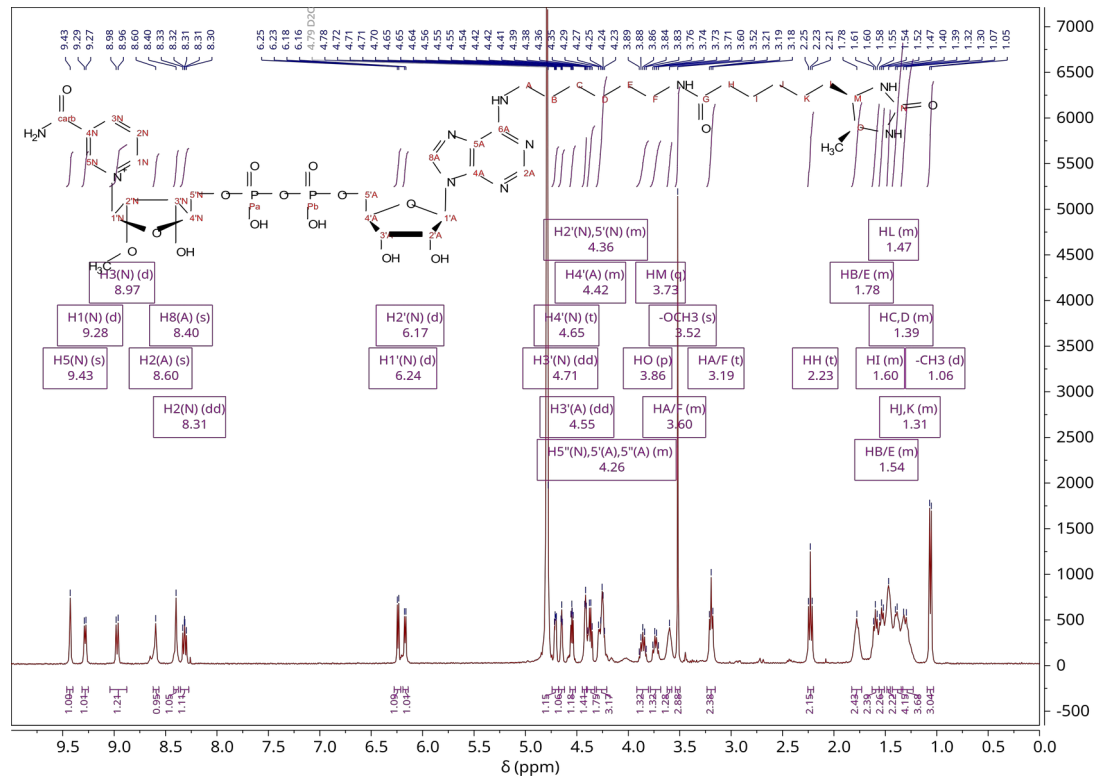
HPLC purification profile (Method P2)



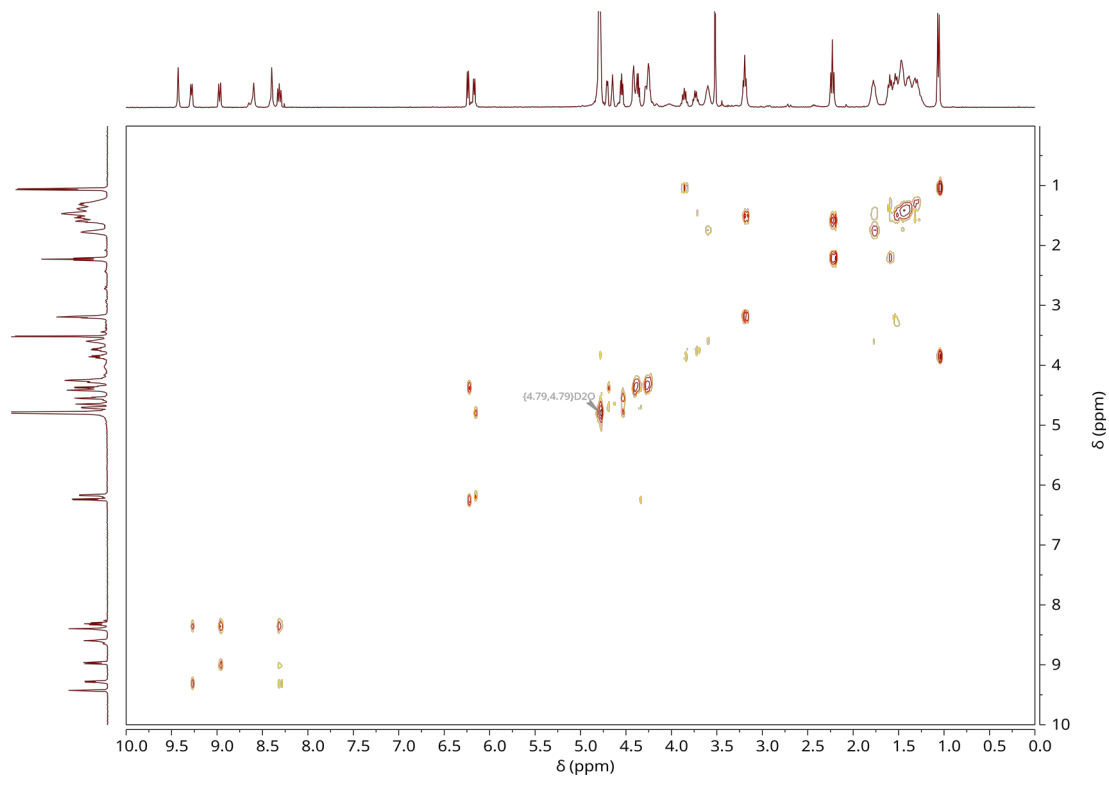
HRMS (+)ES



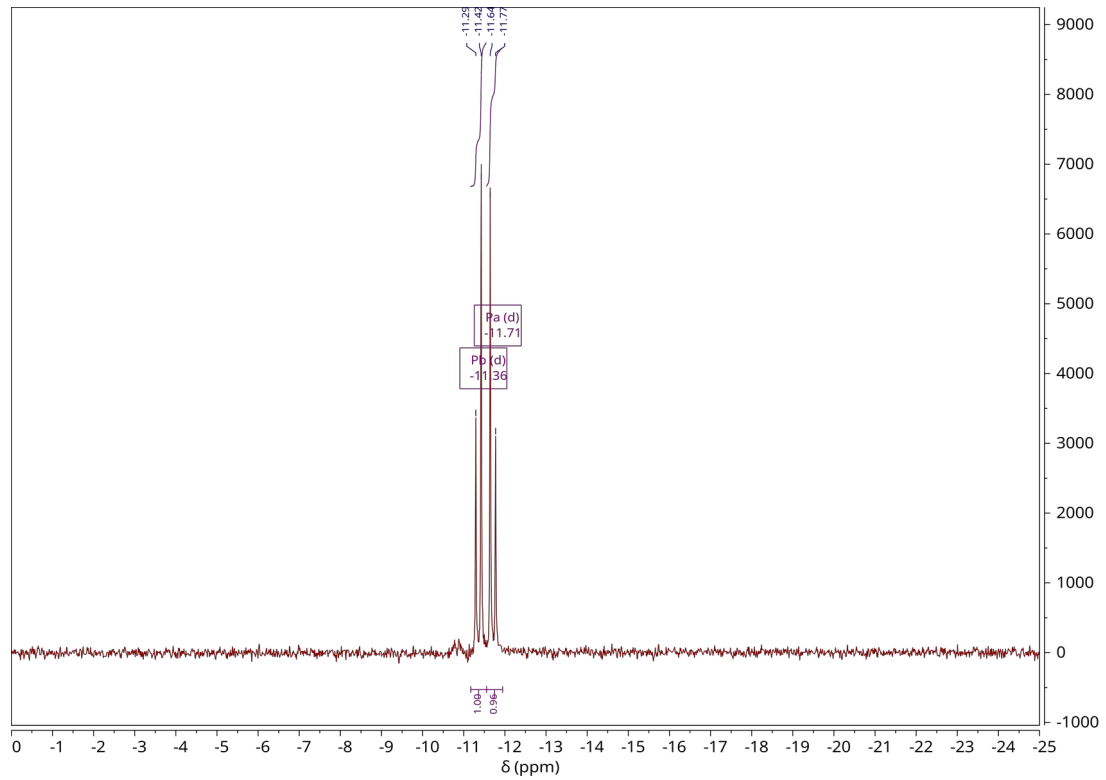
^{1}H NMR (400 MHz, D_2O, 25°C)



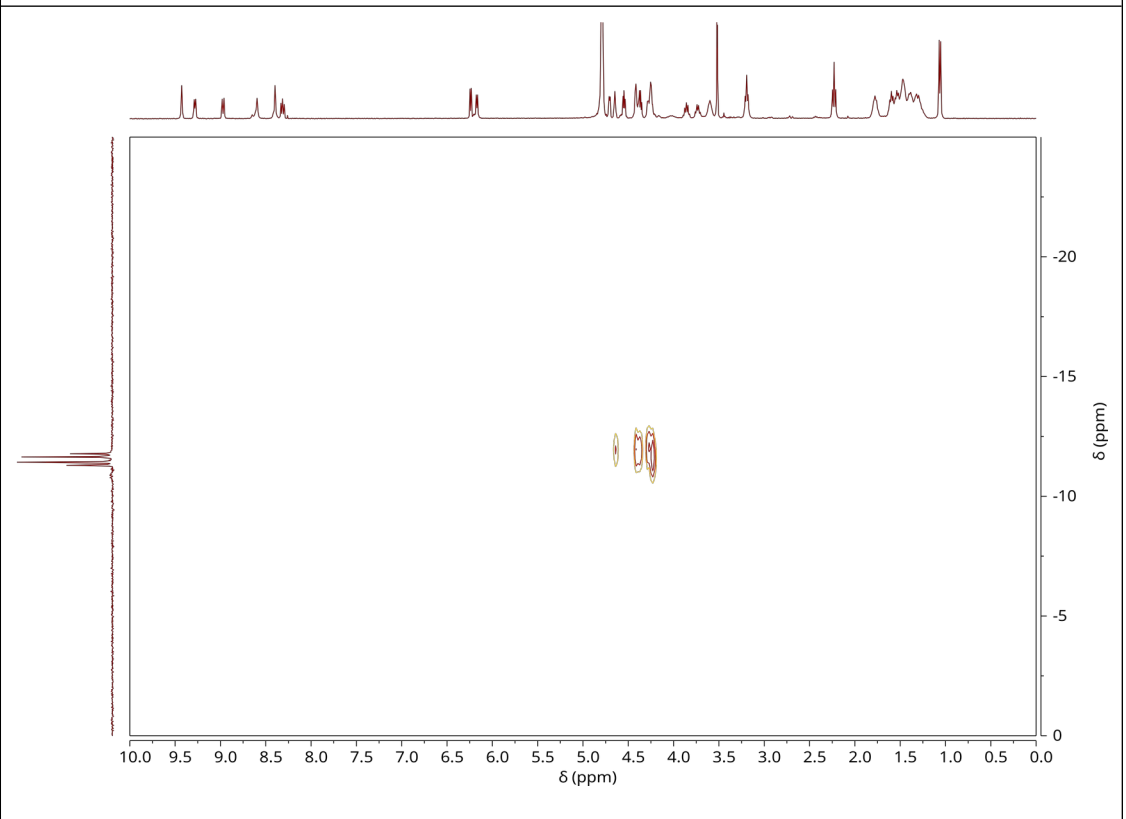
^{1}H-^{1}H COSY (D_2O, 25°C)



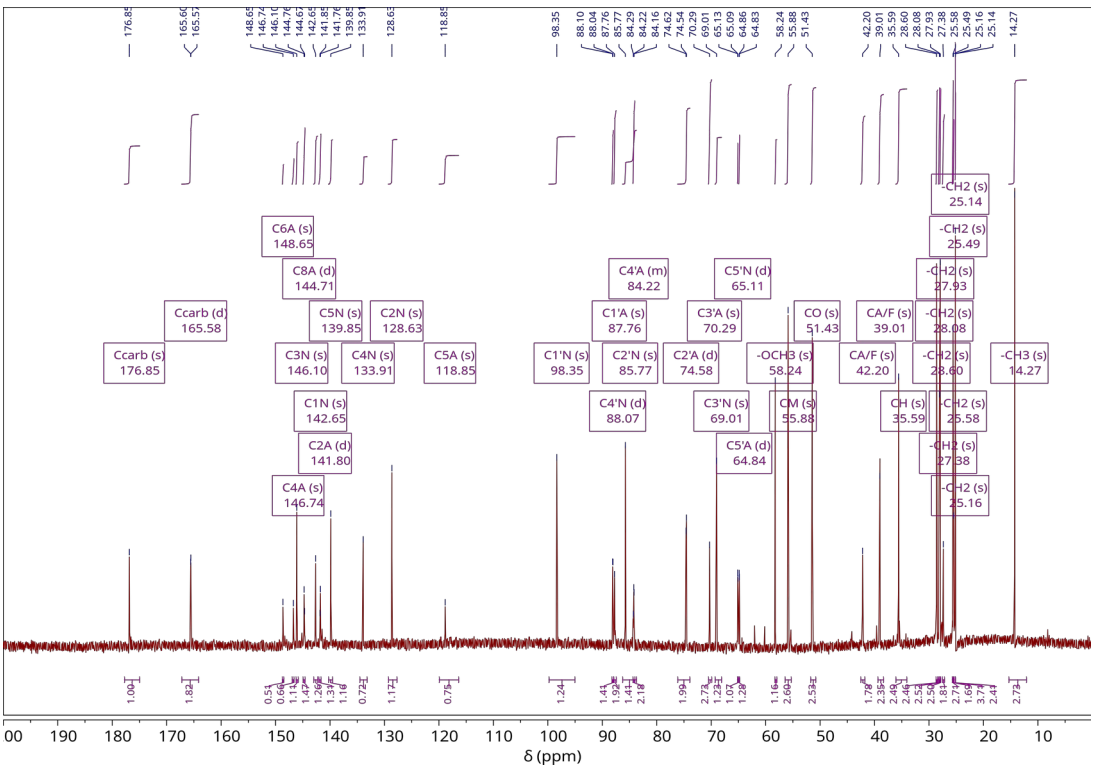
^{31}PCPD NMR (162 MHz, D_2O, 25°C)



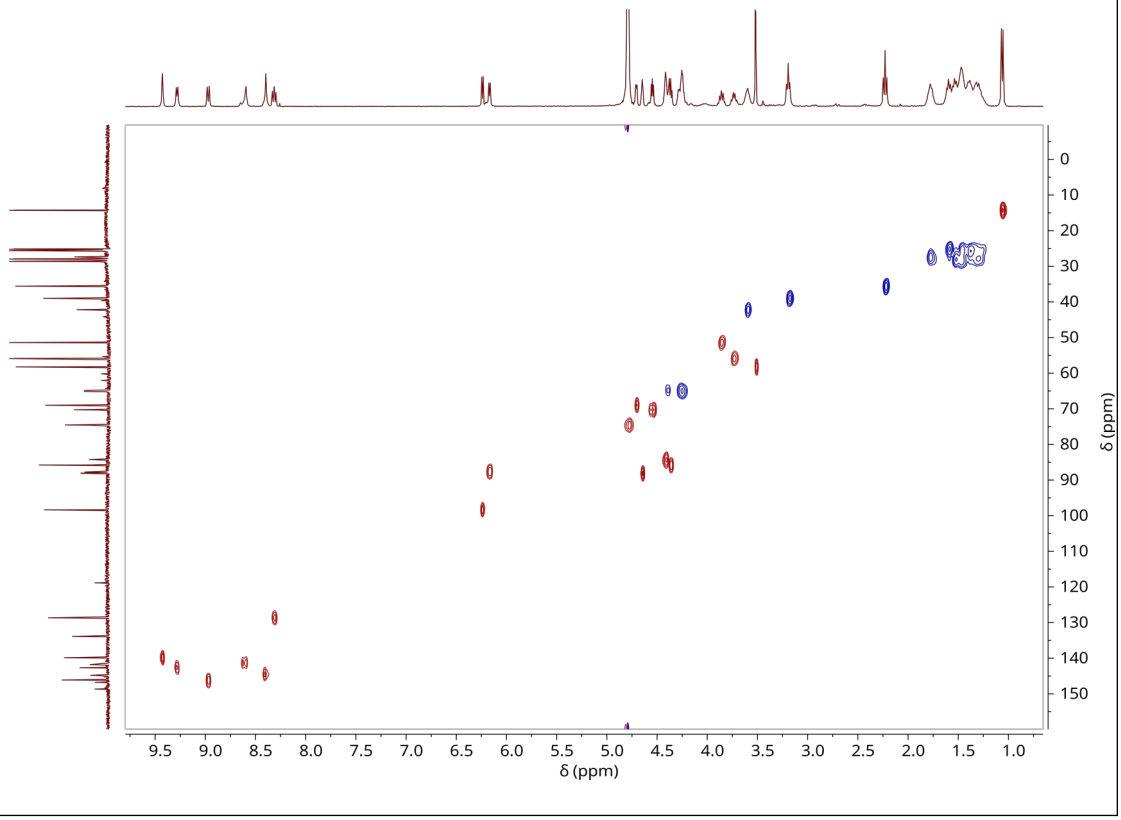
^{1}H-^{31}P HMBC (D_2O, 25°C)

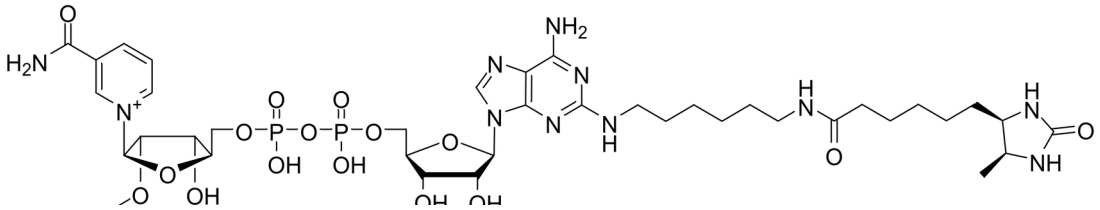
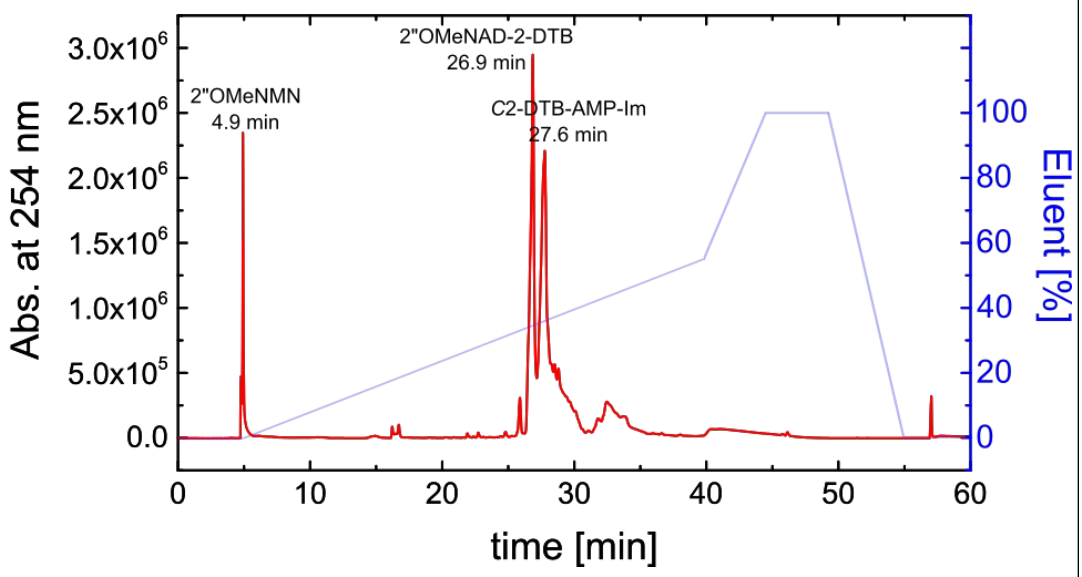
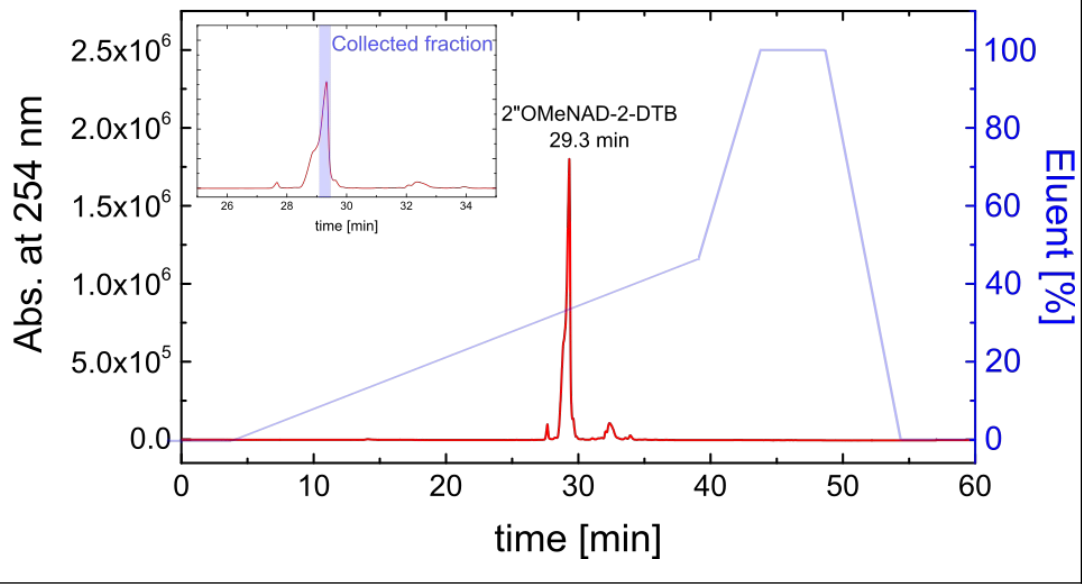


^{13}C NMR 151 MHz, D_2O, 25°C)

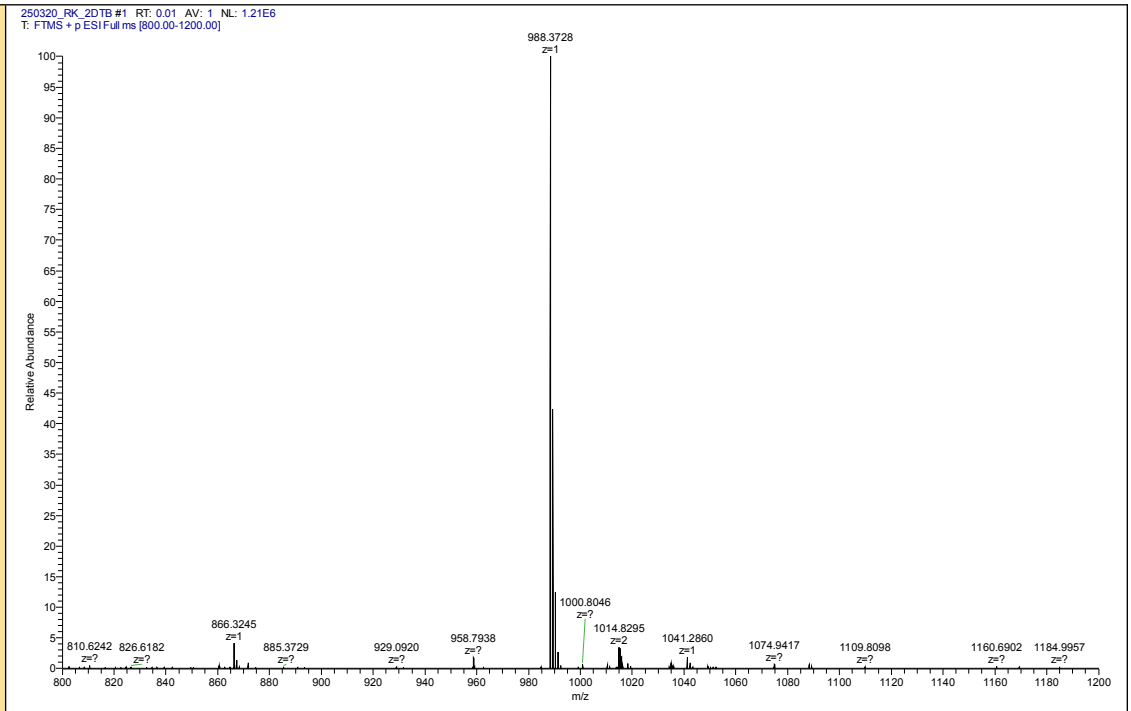


^{1}H-^{13}C HSQC (D_2O, 25°C)

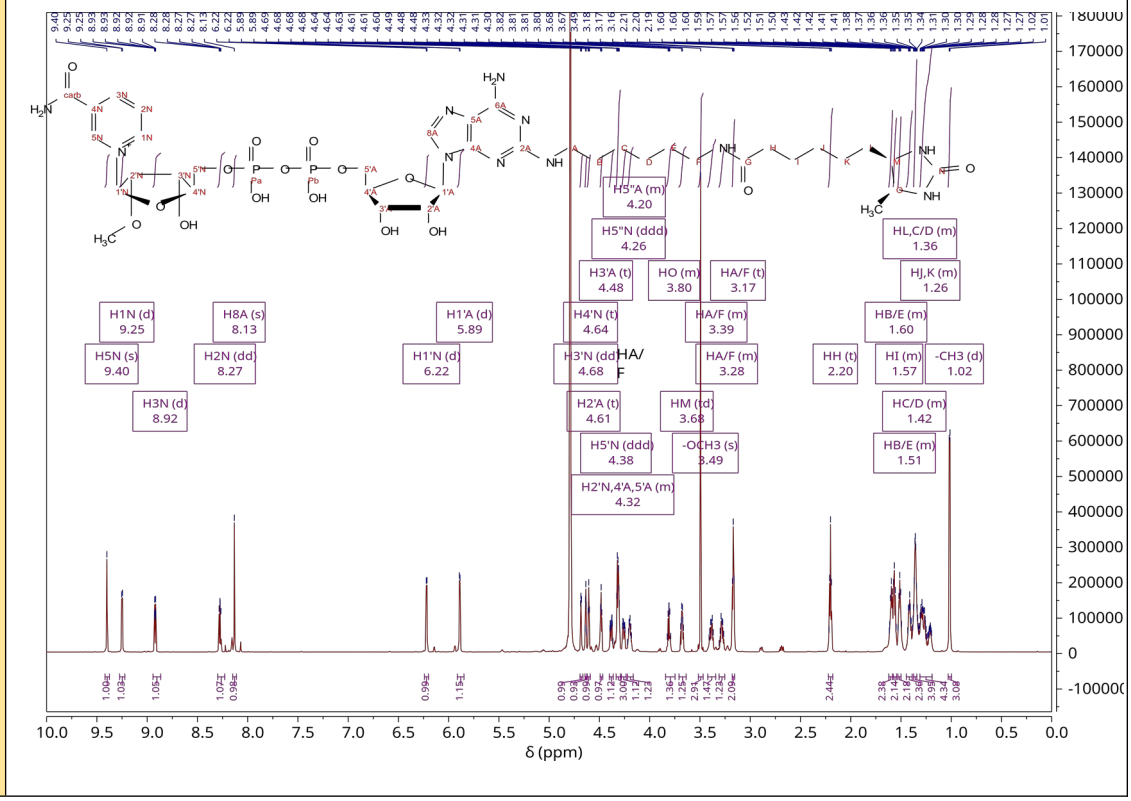


2”OMeNAD-2-DTB (**4**)	
Formula	
HPLC purification profile (Method P2)	
HPLC repurification profile (Method P3)	

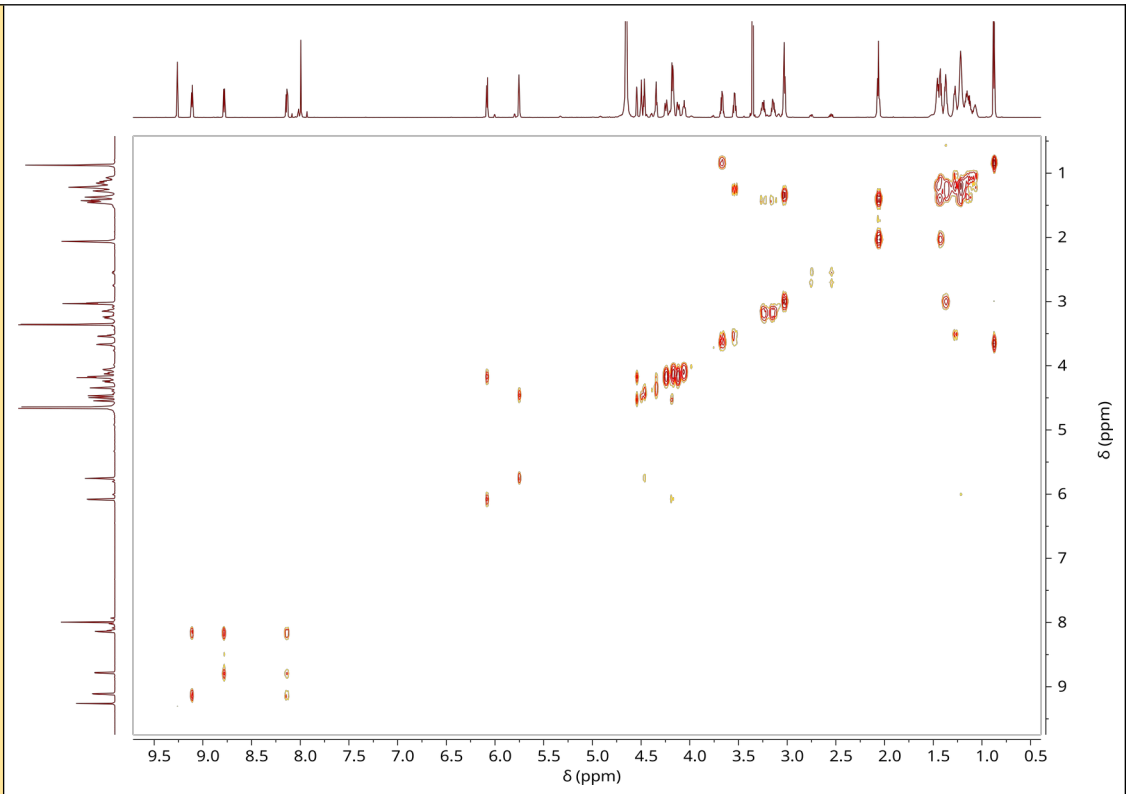
HRMS (+)ES



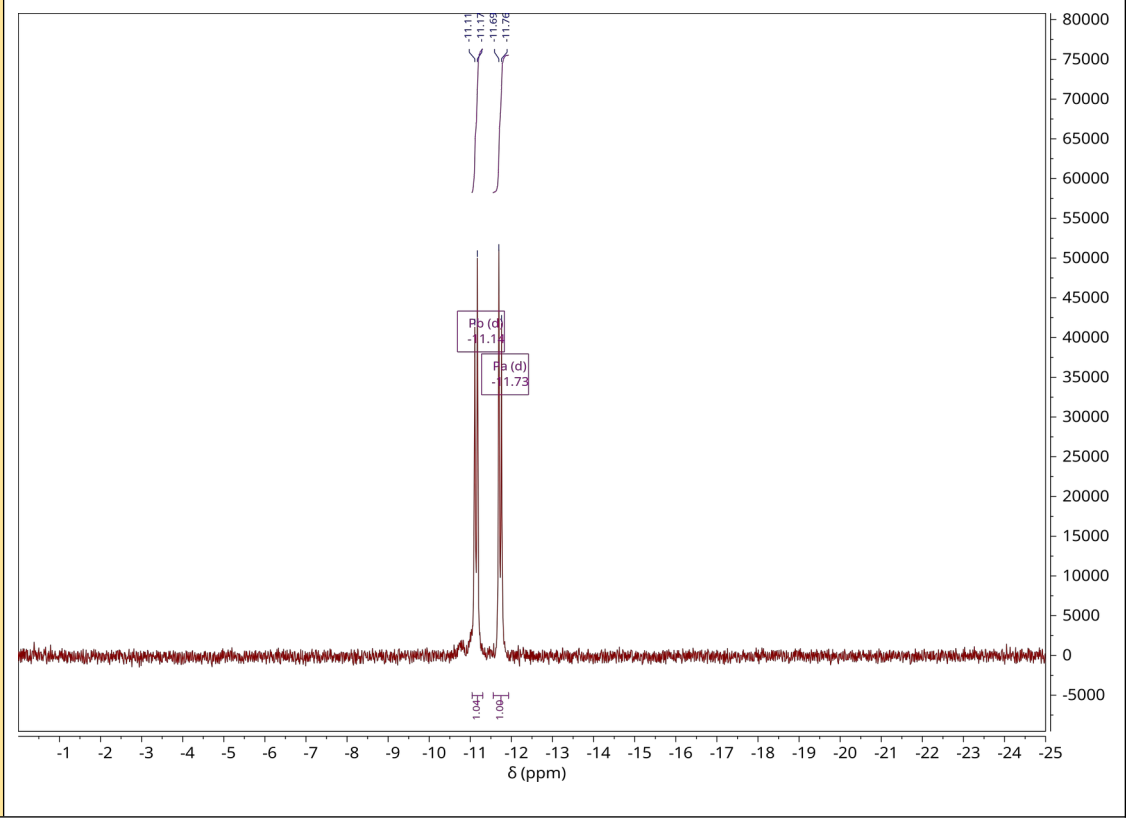
^{1}H NMR (800 MHz, D_2O, 25°C)

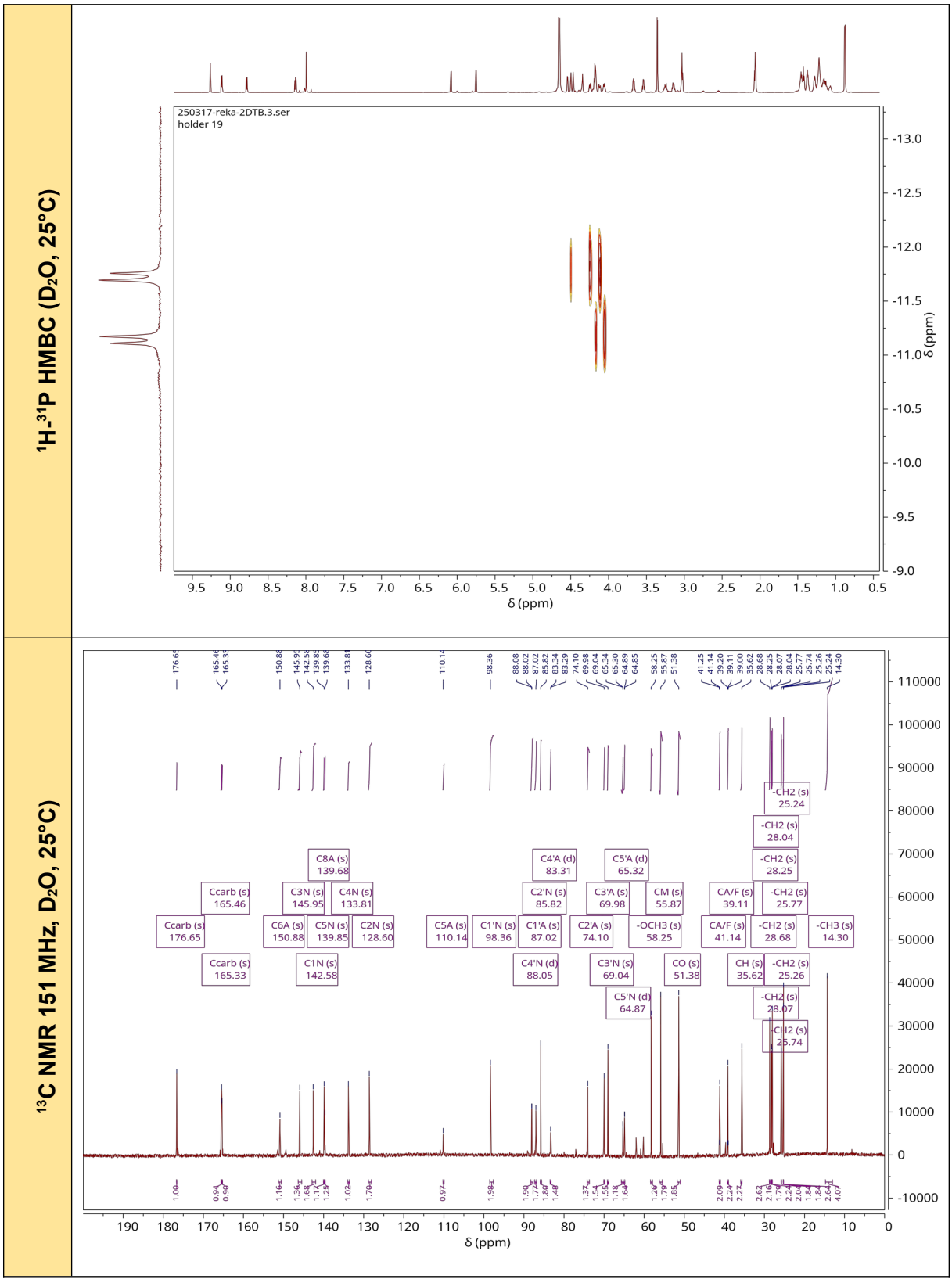


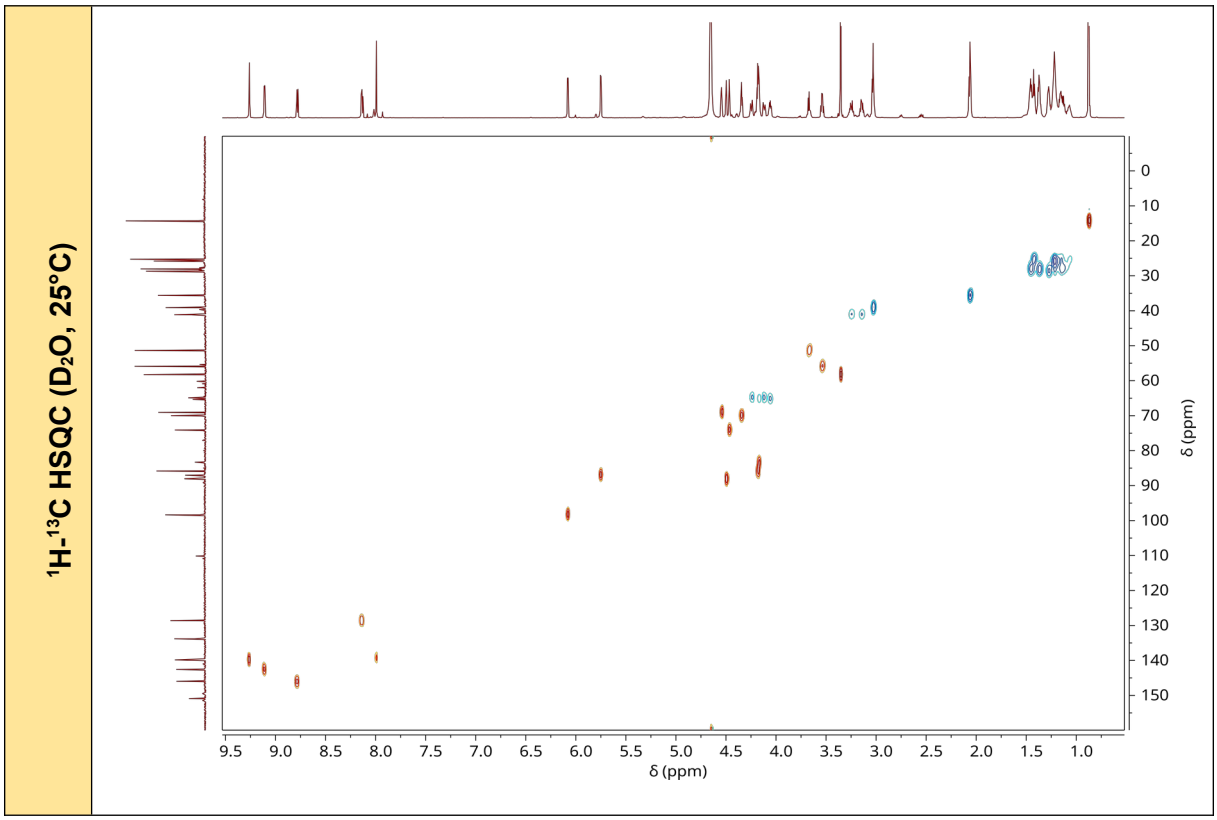
^{1}H-^{1}H COSY (D_2O, 25°C)

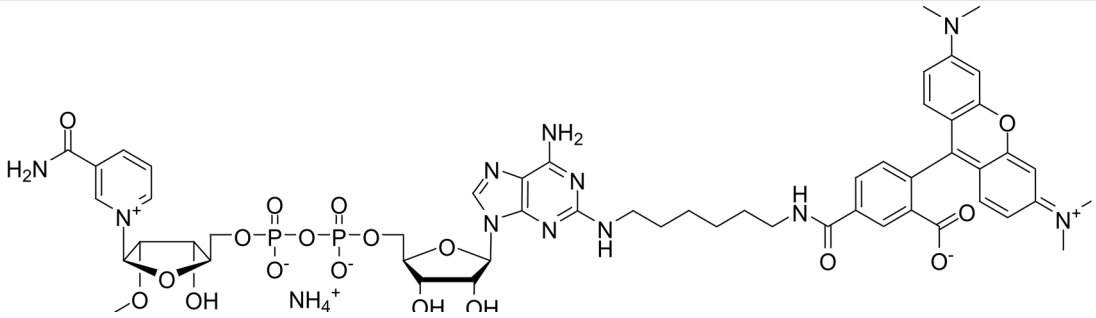
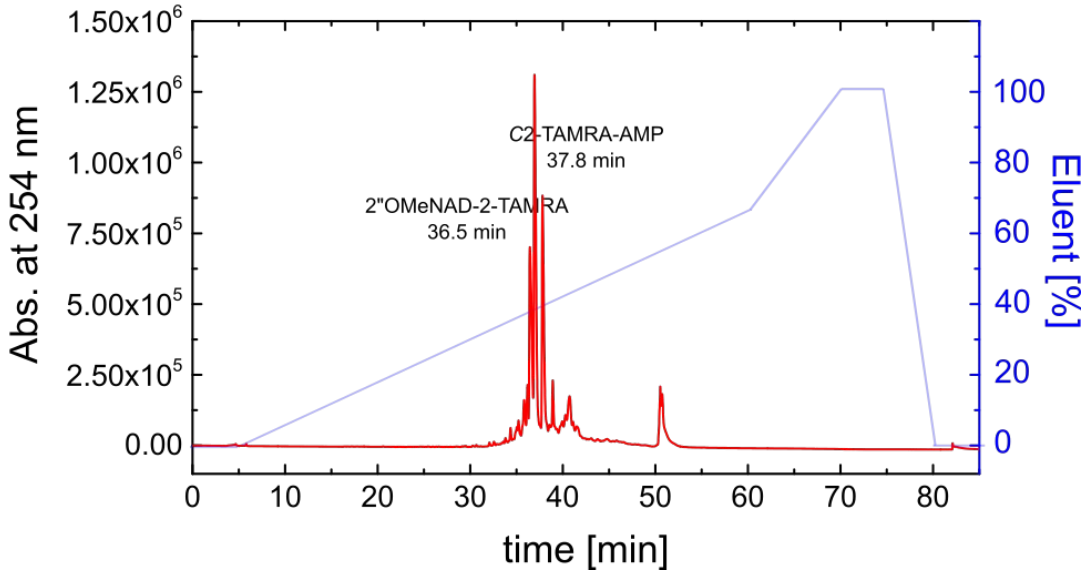
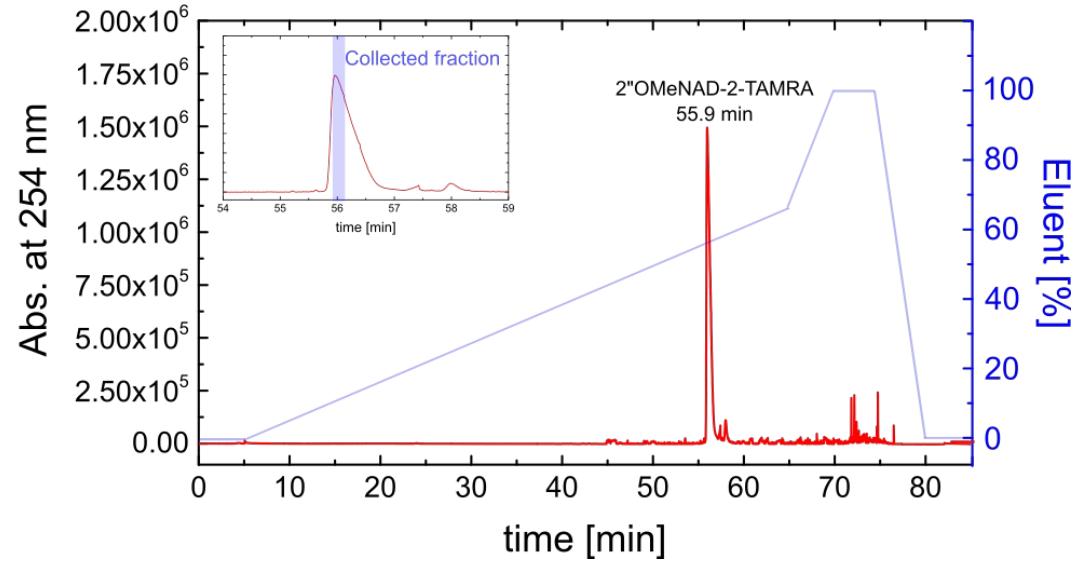


^{31}PCPD NMR (324 MHz, D_2O, 25°C)

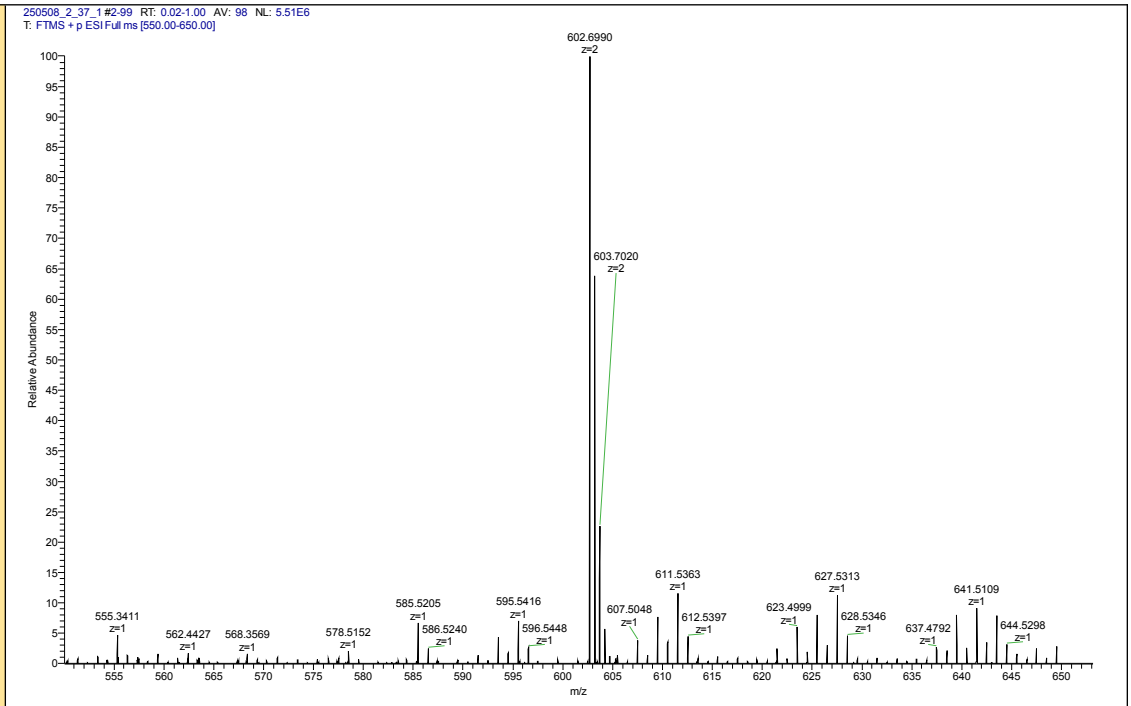



^{1}H-^{31}P HMBC (D$_2$O, 25°C)
250317-reka-2DTB.3.ser
holder 19
δ (ppm)
^{13}C NMR 151 MHz, D$_2$O, 25°C)
δ (ppm)

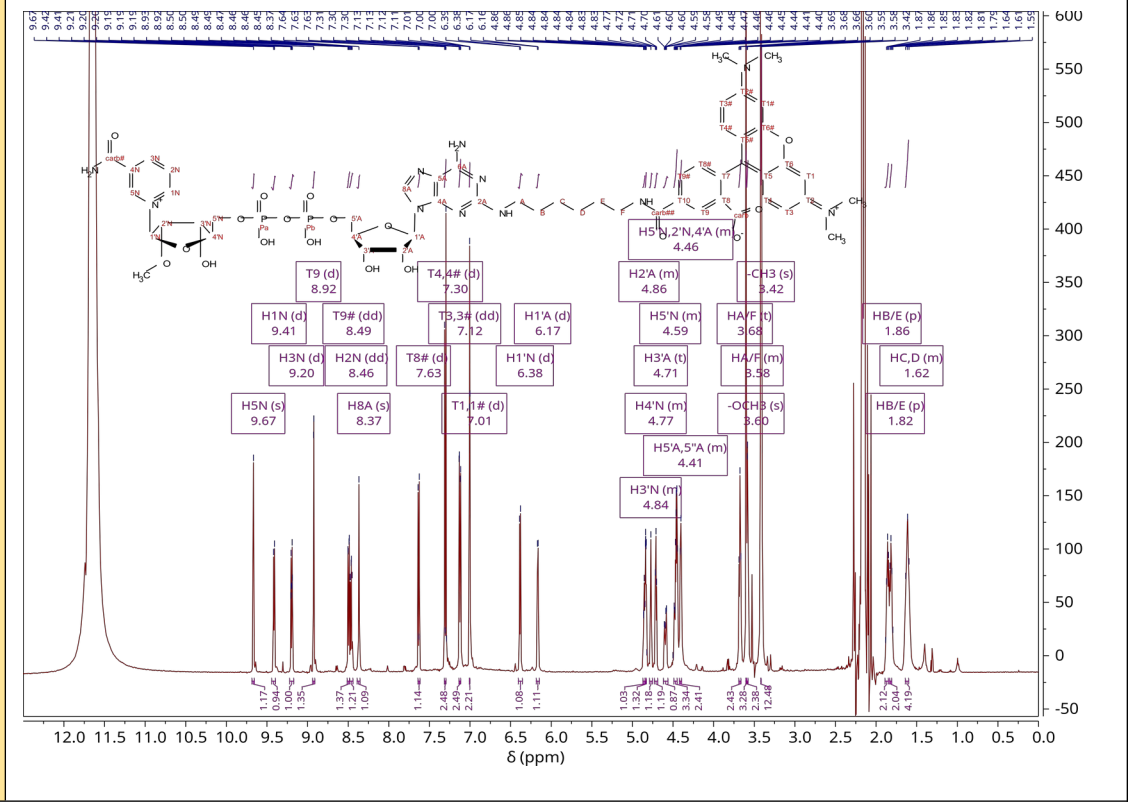

^{1}H-^{13}C HSQC (D_2O, 25°C)
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
δ (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
δ (ppm)

2”OMeNAD-2-TAMRA (**5**)	
Formula	
HPLC purification profile (Method P4)	
	

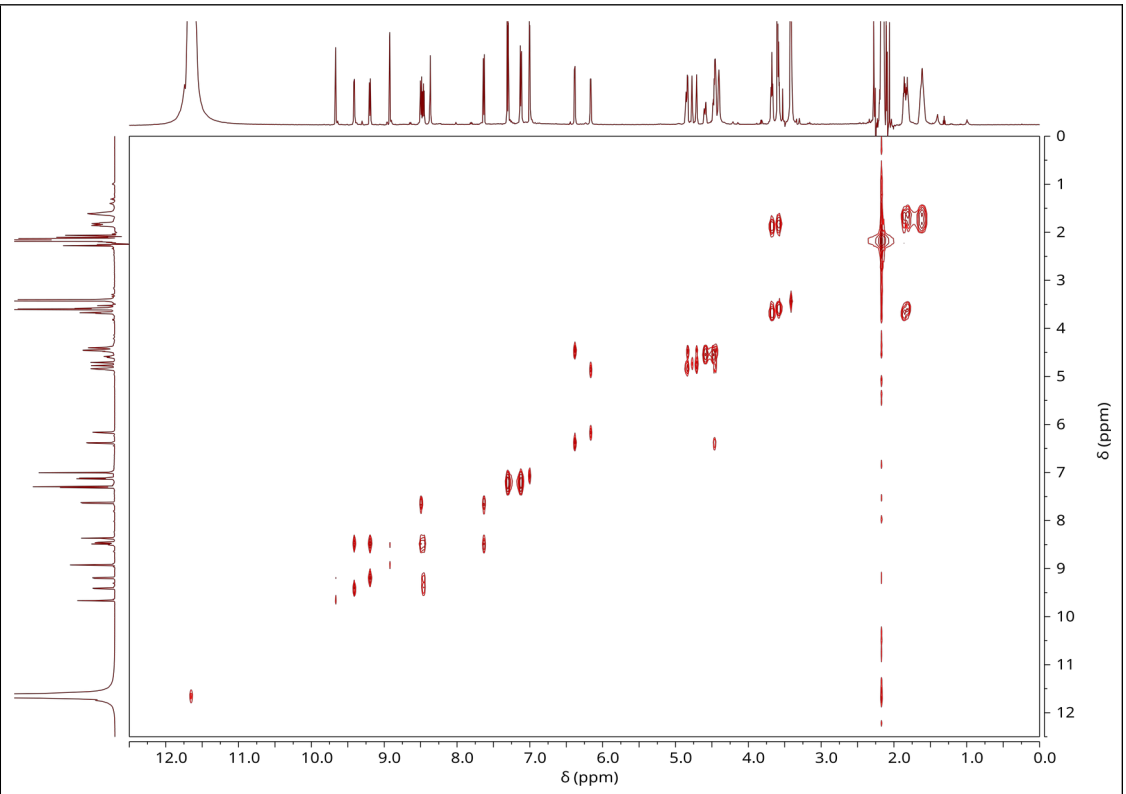
HRMS (+)ES



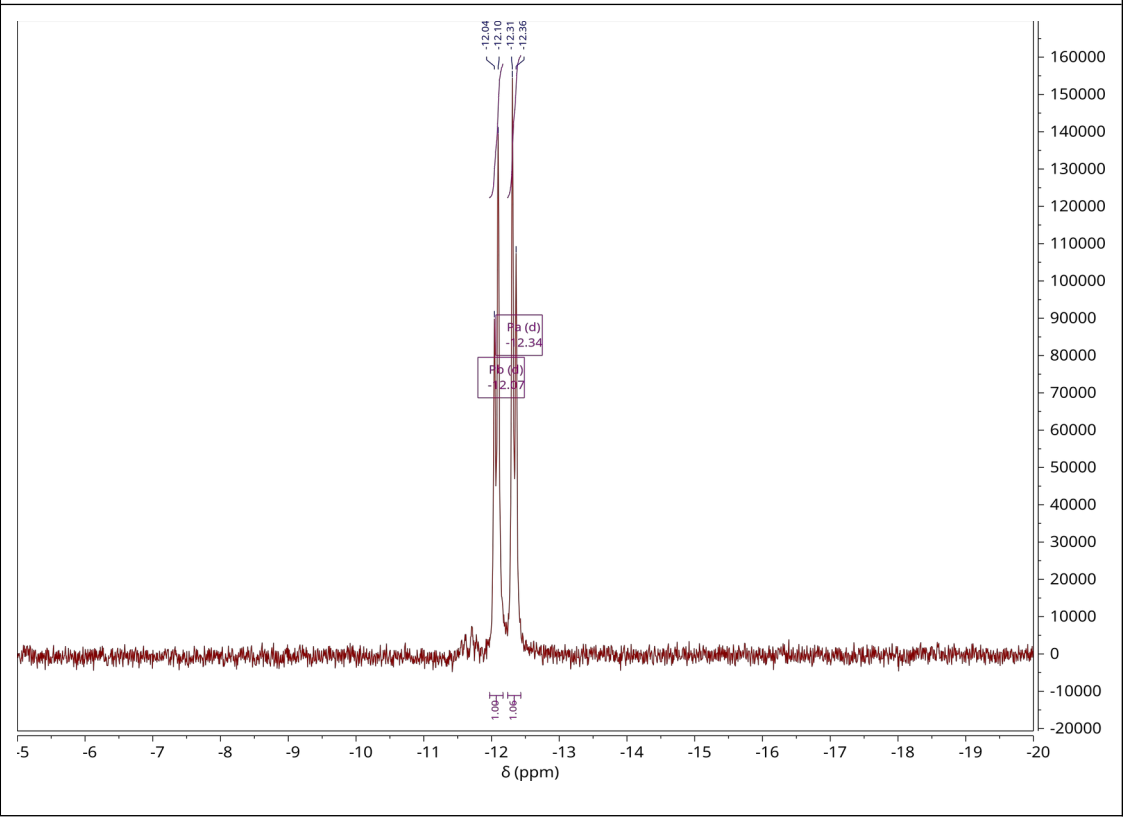
^{1}H NMR (600 MHz, CD_3COOD, 25°C)



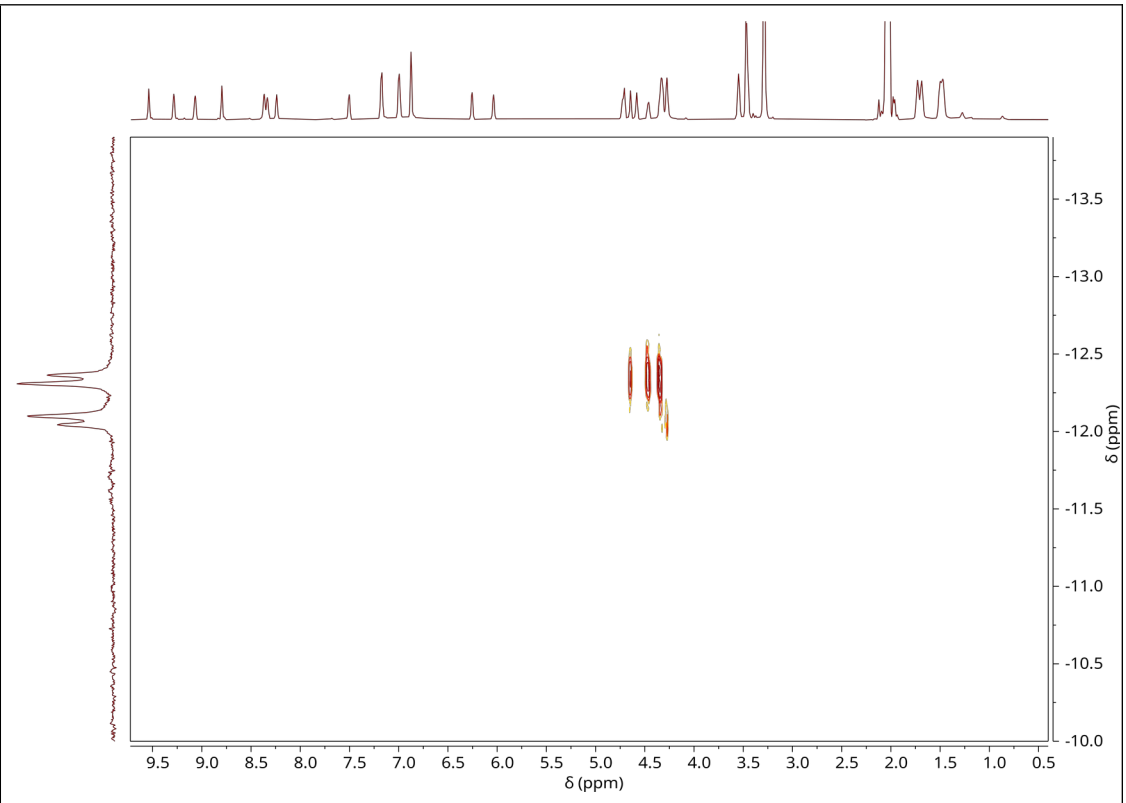
^{1}H-^{1}H COSY (CD_3COOD, 25°C)



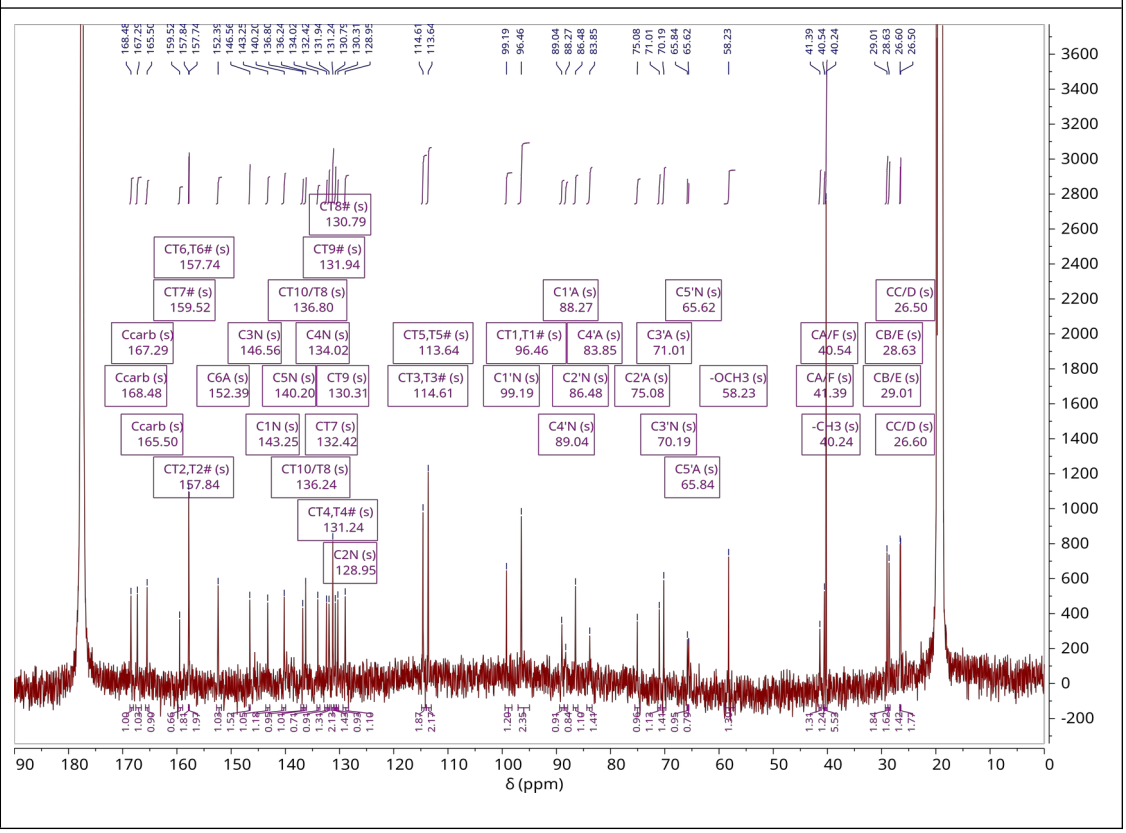
^{31}PCPD NMR (324 MHz, CD_3COOD, 25°C)

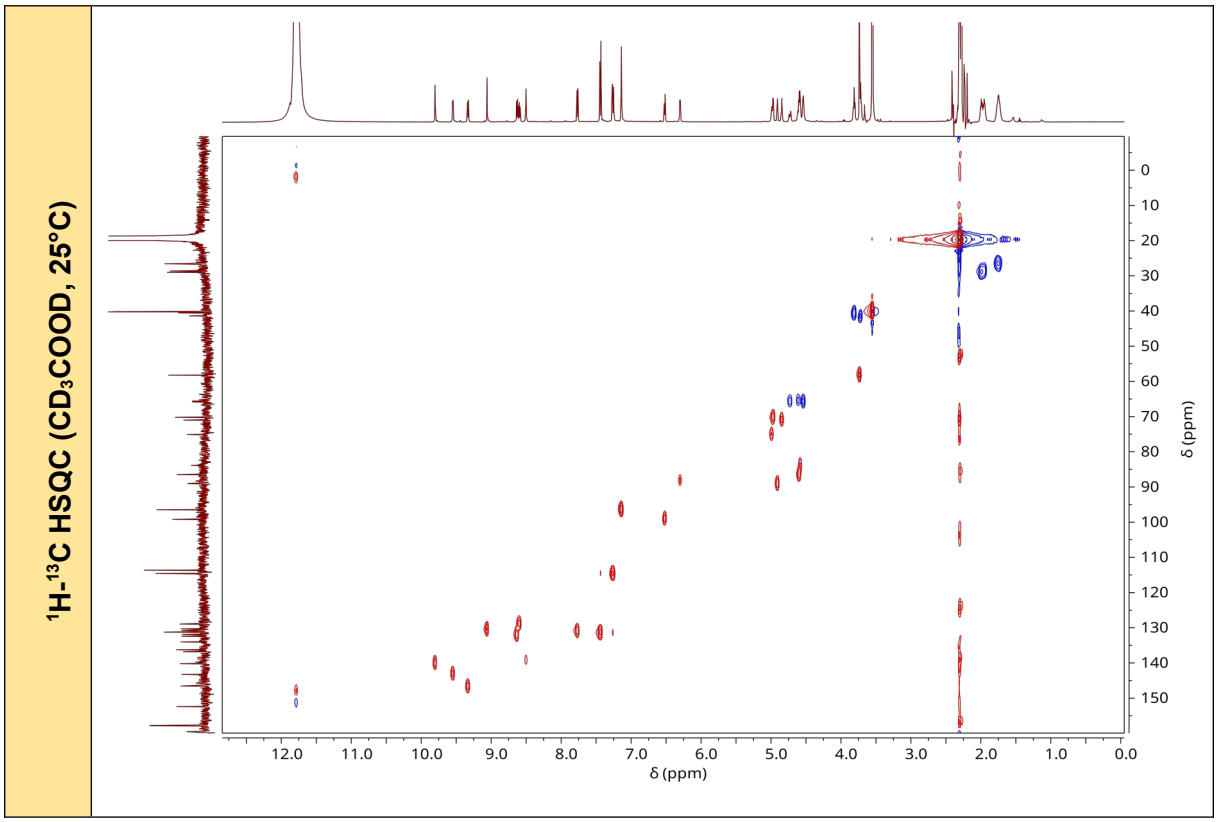


^{1}H-^{31}P HMBC (CD_3COOD, 25°C)



^{13}C NMR 151 MHz, CD_3COOD, 25°C)




^{1}H-^{13}C HSQC (CD_3COOD, 25°C)
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
δ (ppm)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
δ (ppm)