Supporting Information

Synthesis and Relaxivity of Gadolinium-Based DOTAGA Conjugated 3-Phosphoglycerate

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Section 1: Materials

Solvents were purchased from Thermo-Fisher or Sigma-Aldrich. Methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate and (R)-5-Oxotetrahydrofuran-2-carboxylic acid were purchased from ChemScene. DOWEX 50X8 was purchased from Thermo-Scientific. TBS-Cl, 1*H*-Tetrazole (0.4 M in ACN) subsequently dried with activated 4 Å molecular sieves, trifluoroacetic acid, and p-NsCl were purchased from Oakwood. Imidazole, tBuOH, DCC, DMAP, DIBAL in hexane, LiOH, tert-Butyl tetraisopropylphosphorodiamidite and di-tertbutyl peroxide, were purchased from Sigma-Aldrich. Glacial acetic acid was purchased from Fisher. Diisopropylammonium tetrazolide salt was purchased from 1ClickChemistry. $GdCl_3 \bullet XH_2O$ was purchased from Strem. Phosphyglycerate Kinase was purchased from proteintech. Kinase Buffer was purchased from Cell Signaling Technology. ADP-Glo Kinase Assay was purchased from Promega. ATP was purchased from TCI.

Section 2: Relaxivity

Gd-3PG was studied in x1 PBS and citric acid at varying pH. Relaxation times were measured on a Bruker mq60 NMR analyzer equipped with Minispec v 2.51 Rev.00/NT software (Bruker Biospin, Billerica, MA, USA) operating at 1.41 T (60 MHz) and 37 °C. Measurement of T_1 relaxation times were made using an inversion recovery pulse sequence with the following parameters: 4 scans per point, 10 data points, monoexponential curve fitting, phase cycling, 10 ms first pulse separation, and a recycle delay and final pulse separation ≥ 5 T1. The inverse of the relaxation time ($1/T$, s^{-1}) was plotted against the Gd(III) concentration (mM) determined by ICP-MS for each sample. By applying a linear fit to this data, the slope generated was defined as the relaxivity (r_1) of the agent in units of mM^{-1} s^{-1}.

Relaxivity measurements that were performed at 7 T, dilutions were added to flame-sealed pipets, cut with a glass cutter, and sealed at the top with parafilm. These capillaries were imaged using a Bruker PharmaScan 7 T MR imaging spectrometer (Bruker BioSpin, Billerca, MA, USA). T_1 relaxation times were

measured using a rapid-acquisition rapid-echo (RARE-VTR) T_1-map pulse sequence with static TE (10 ms) and variable TR (100, 200, 400, 500, 750, 1000, 2500, 7500, 10000 ms) values. Imaging parameters were as follows: field of view, 25 x 25 mm2 ; matrix size, 256 x 256; number of axial slices, 5; slice thickness, 1.0 mm; and averages, 4. T_1 analysis was carried out using the image sequence analysis tool in Paravision 6.0 software (Bruker) with mono-exponential curve-fitting of image intensities of selected ROIs for each axial slice. The relaxation rates ($1/T_1$, s^{-1}) was plotted against the Gd(III) concentration (mM) determined by ICP-MS for each sample. By applying a linear fit to this data, the generated was defined as the relaxivity (r_1) of the agent in units of mM^{-1} s^{-1}.

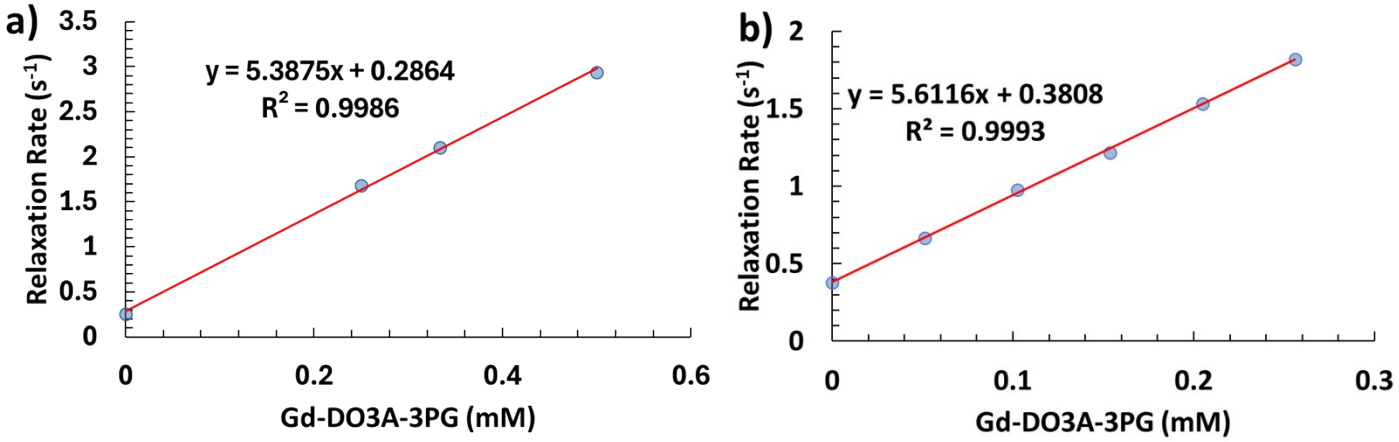


Figure S1. Relaxivity curves of *Gd-3PG* at **a)** 1.4 T at 37 °C and **b)** 7 T at 21 °C in 1xPBS. The uncertainty was determined from the standard deviation of a linear regression. Concentrations determined with ICP-MS.

Data for Figure 1b was extracted using https://plotdigitizer.com/.

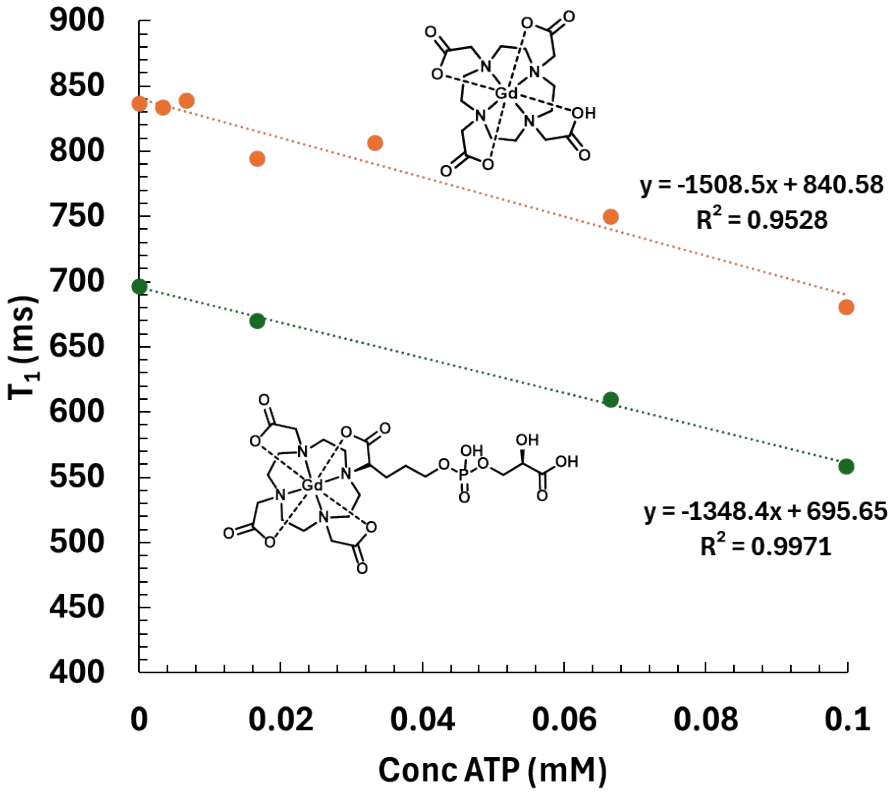


Figure S2. Monitoring GBCAs with an increase in ATP concentrations. This shows a dependence of relaxivity on ATP independent of the 3PG arm as the slops of Gd-DOTA and GD-3PG are similar.

Section 3: Enzyme Kinetics

The kinetics of the 3PG-Arm was determined with PGK protein (10 nM) in kinase buffer (Cell Signaling Technology). The assay was in accordance with the ADP-Glo Kinase Assay manufacturer's protocol. For reactions, ATP (4 mM) needed to be added last to initiate the reaction or the results were inconclusive. All concentrations mentioned are the final once diluted. Staurosporine was used as a positive control.

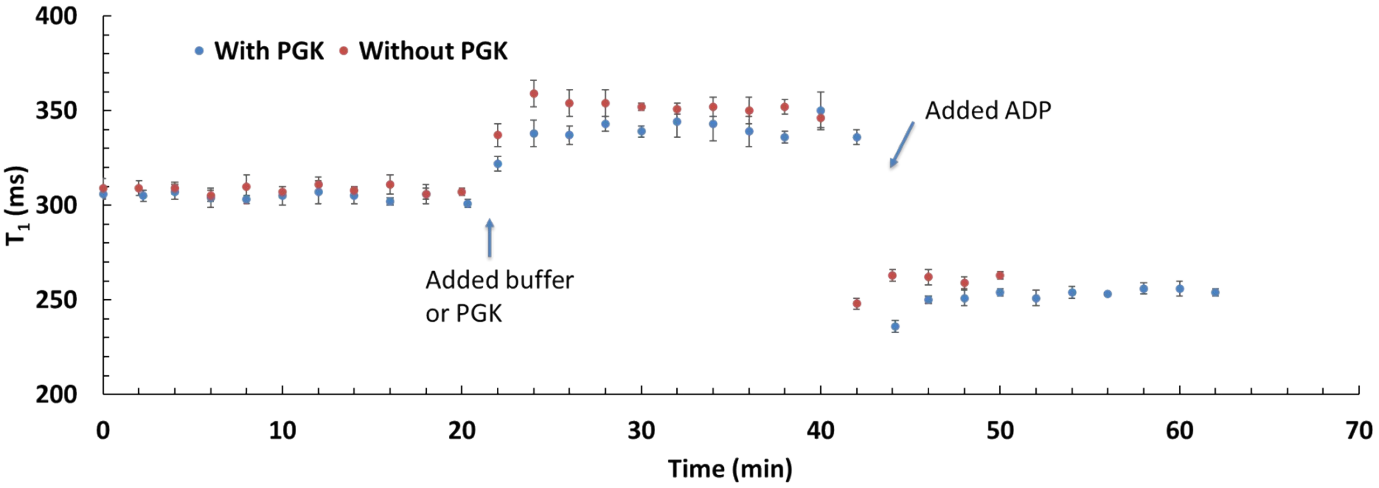
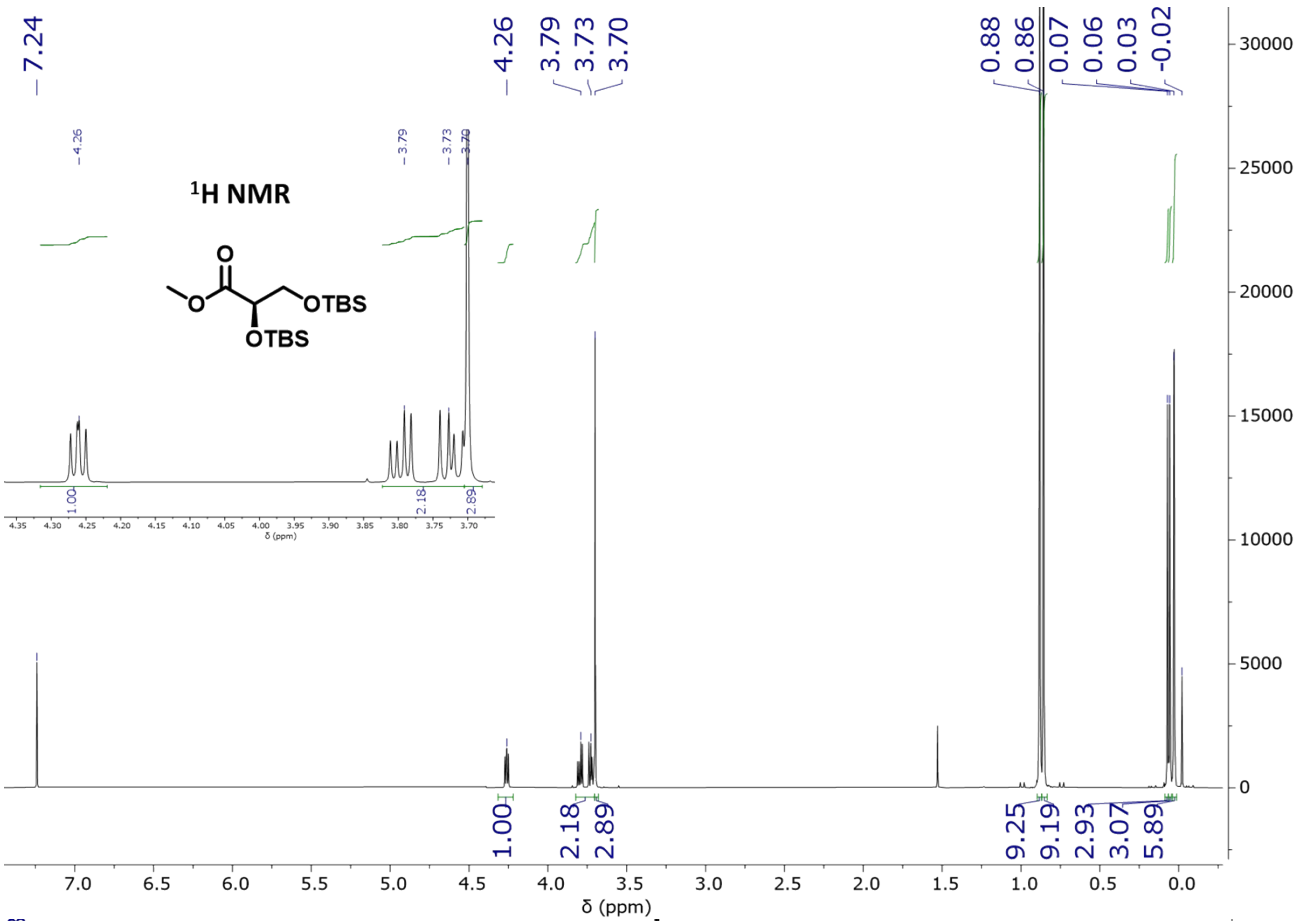
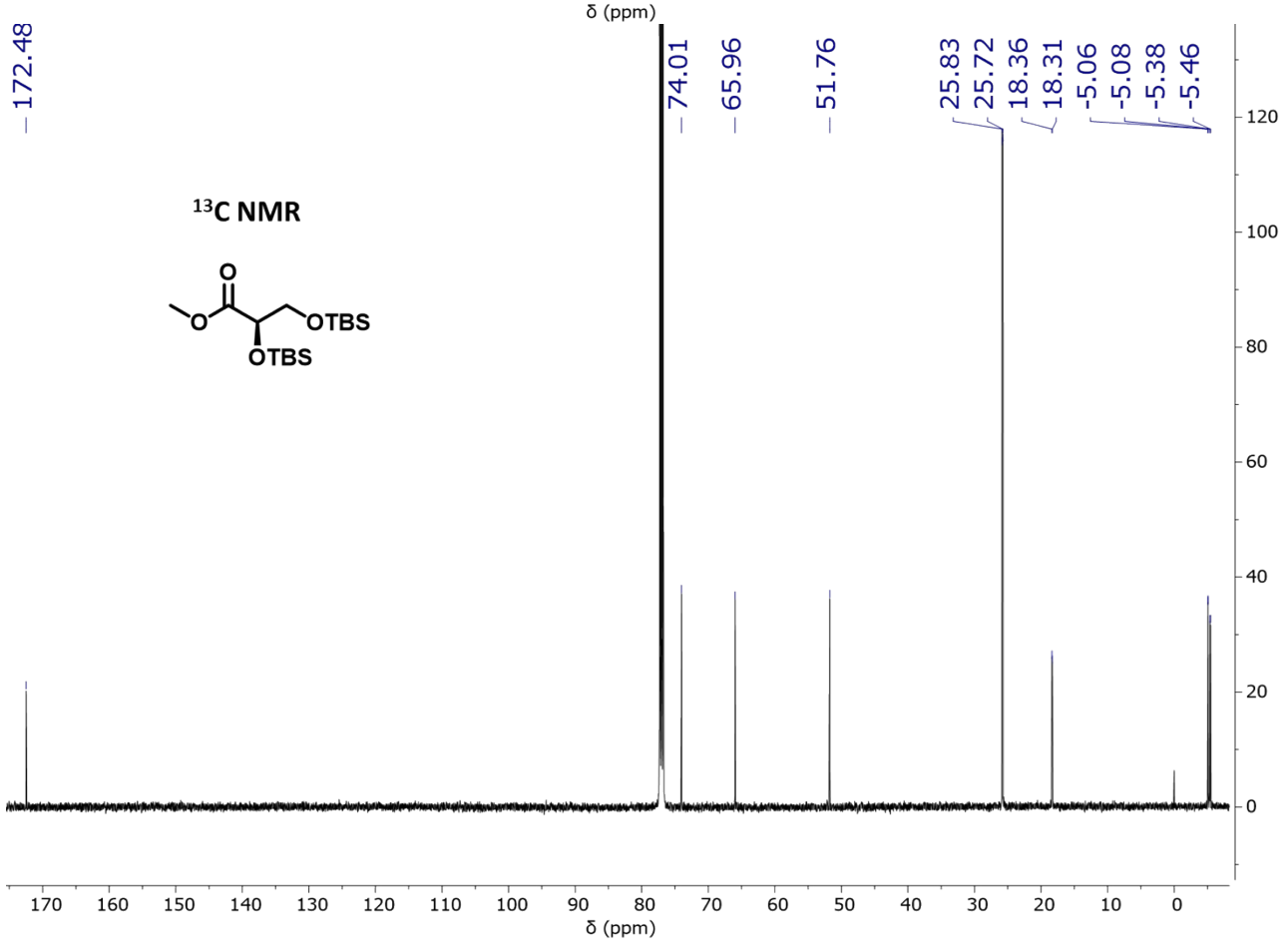
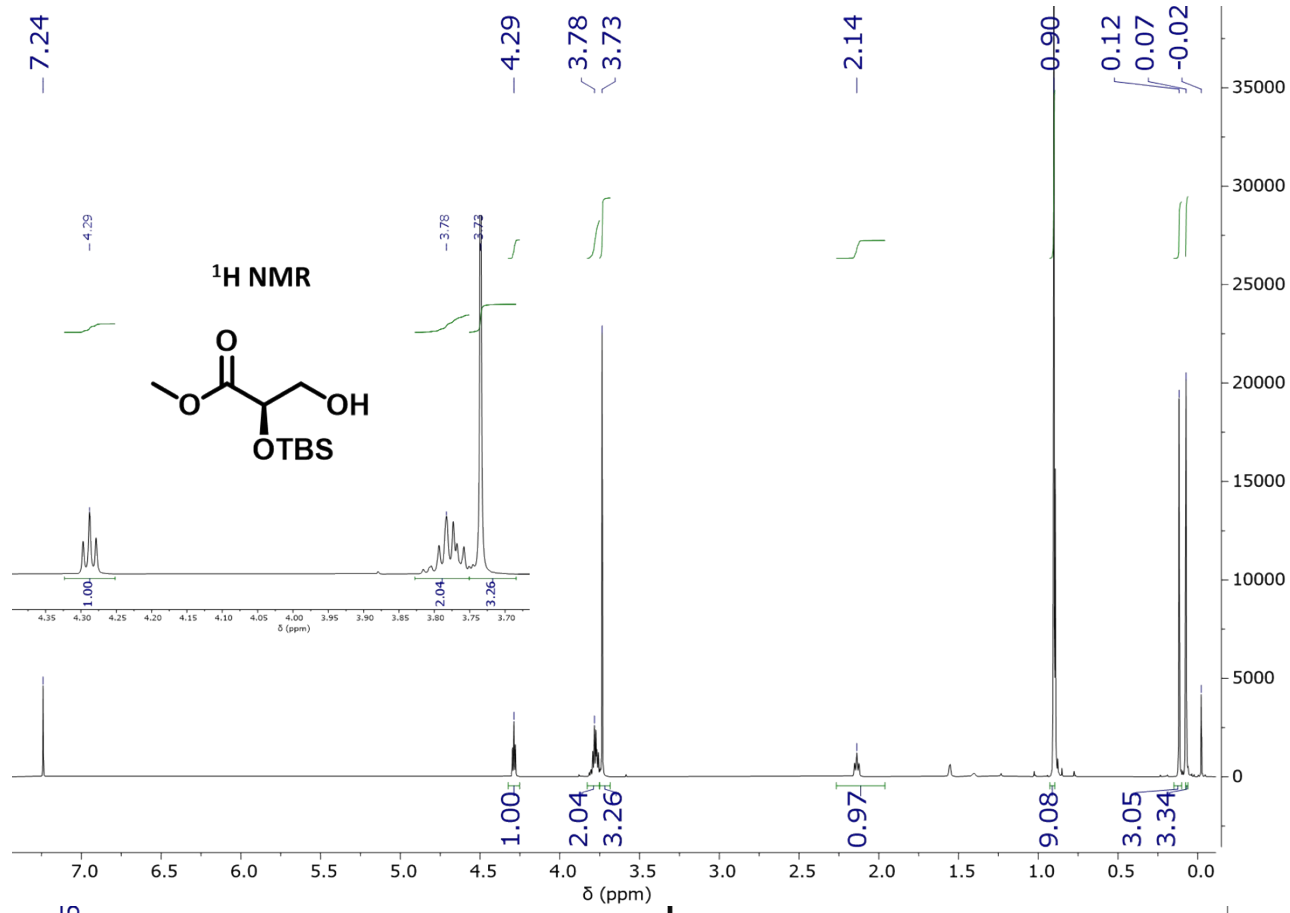


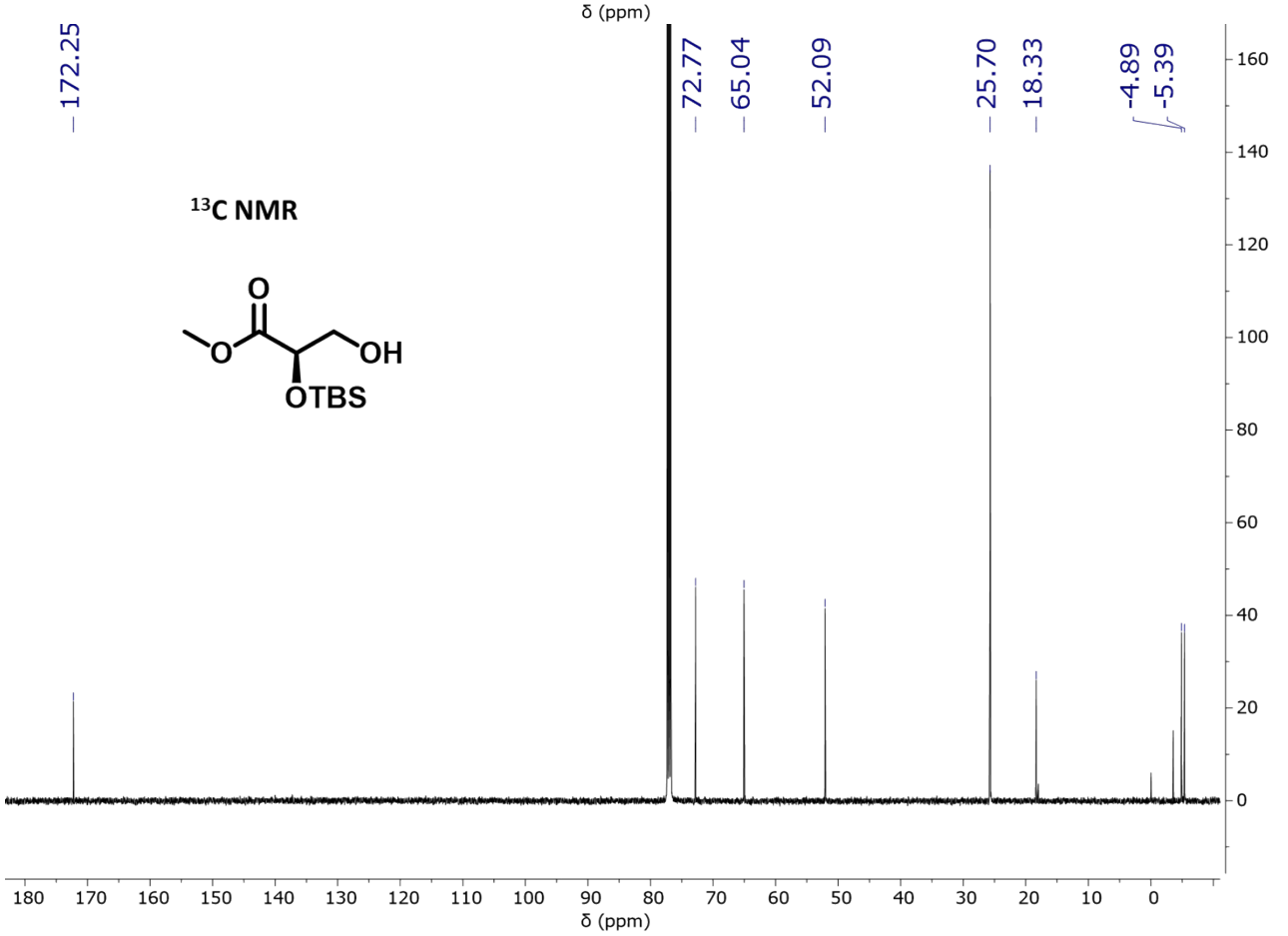
Figure S3. Monitoring T_1 of the phosphorylation of Gd-3PG. A baseline of Gd-3PG (0.325 mM) in the enzyme buffer was obtained over 20 mins. The ATP concentration was increased to drive the reaction to phosphorylation (0.1 M ATP). After 20 mins, either buffer or buffer with PGK was added to give a final concentration of 8 µg/mL PGK and T_1 monitored for an additional 40 mins. ADP was then added to a final concentration of 1.8 M and the relaxivity was further monitored. These results with the control show no significant change in relaxivity from control to sample.

Section 4: NMR






7.24
4.29
3.78
3.73
2.14
0.90
0.12
0.07
-0.02
1H NMR
O
O
OH
OTBS
1.00
2.04
3.26
0.97
9.08
3.05
3.34
δ (ppm)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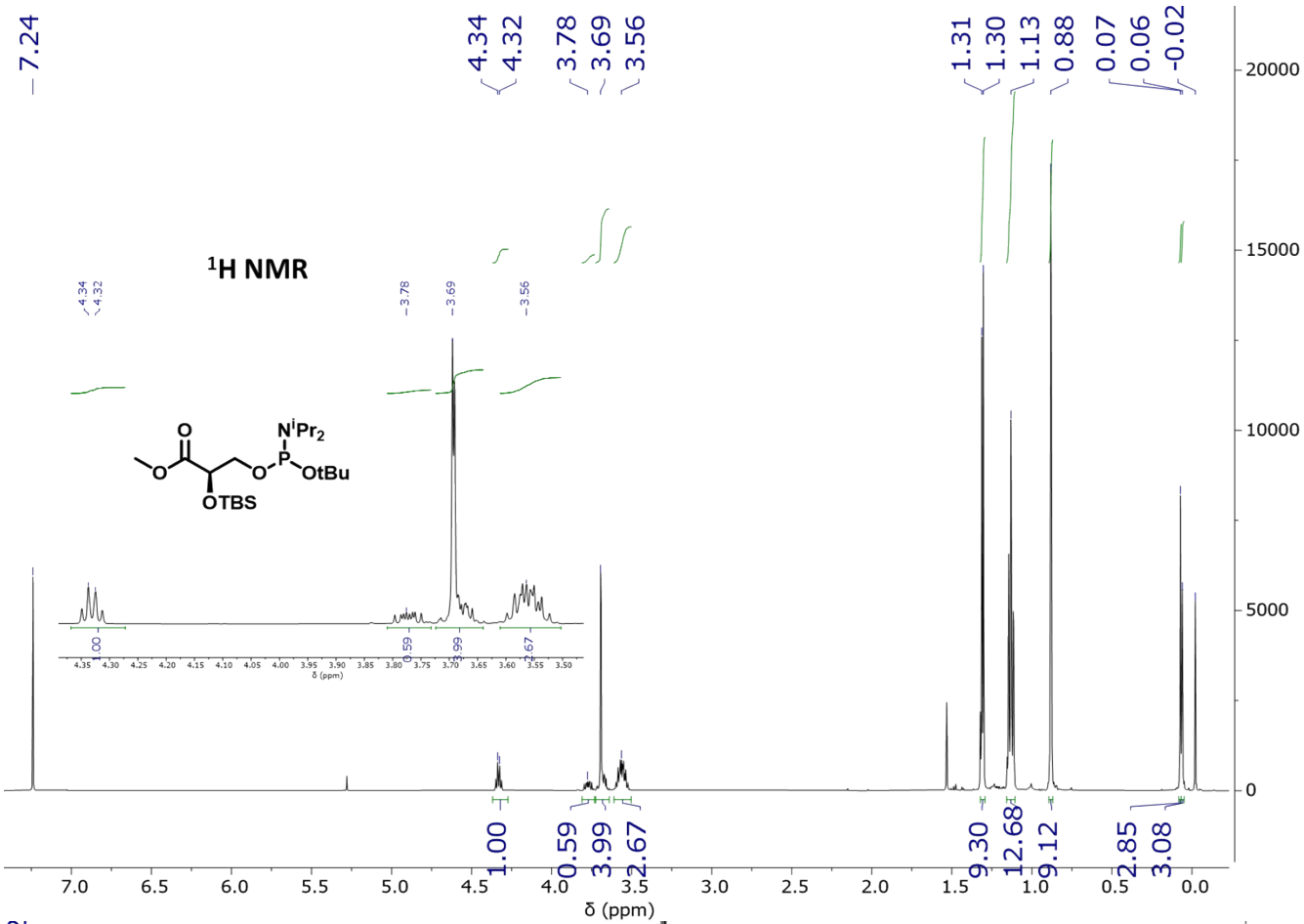
13C NMR
O
O
OH
OTBS
δ (ppm)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4.32
3.78
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3.56
1.31
1.30
1.13
0.88
0.07
0.06
-0.02
1H NMR
N^iPr2
OtBu
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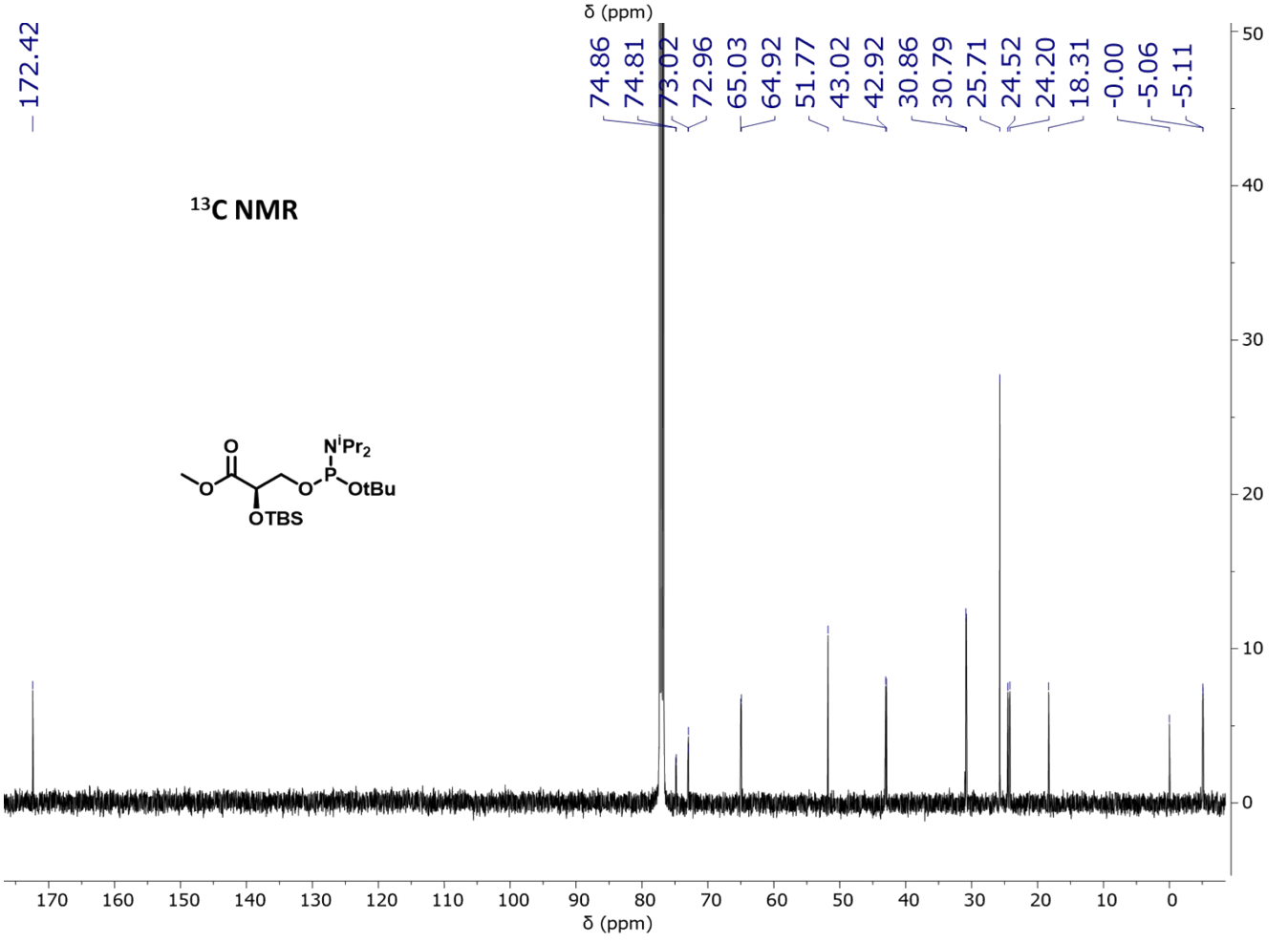
12.68
9.12
2.85
3.08
δ (ppm)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72.96
65.03
64.92
51.77
43.02
42.92
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30.79
25.71
24.52
24.20
18.31
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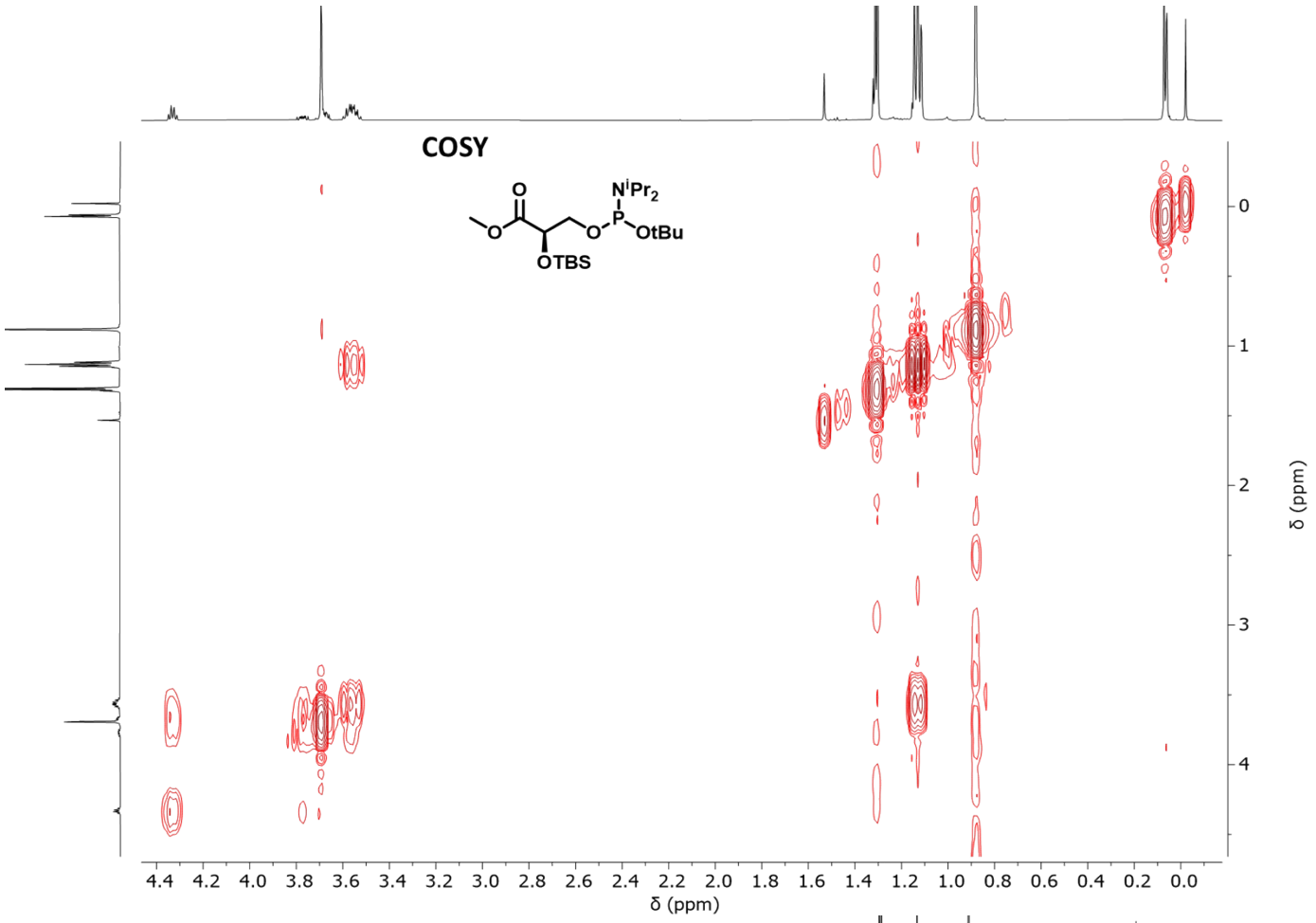
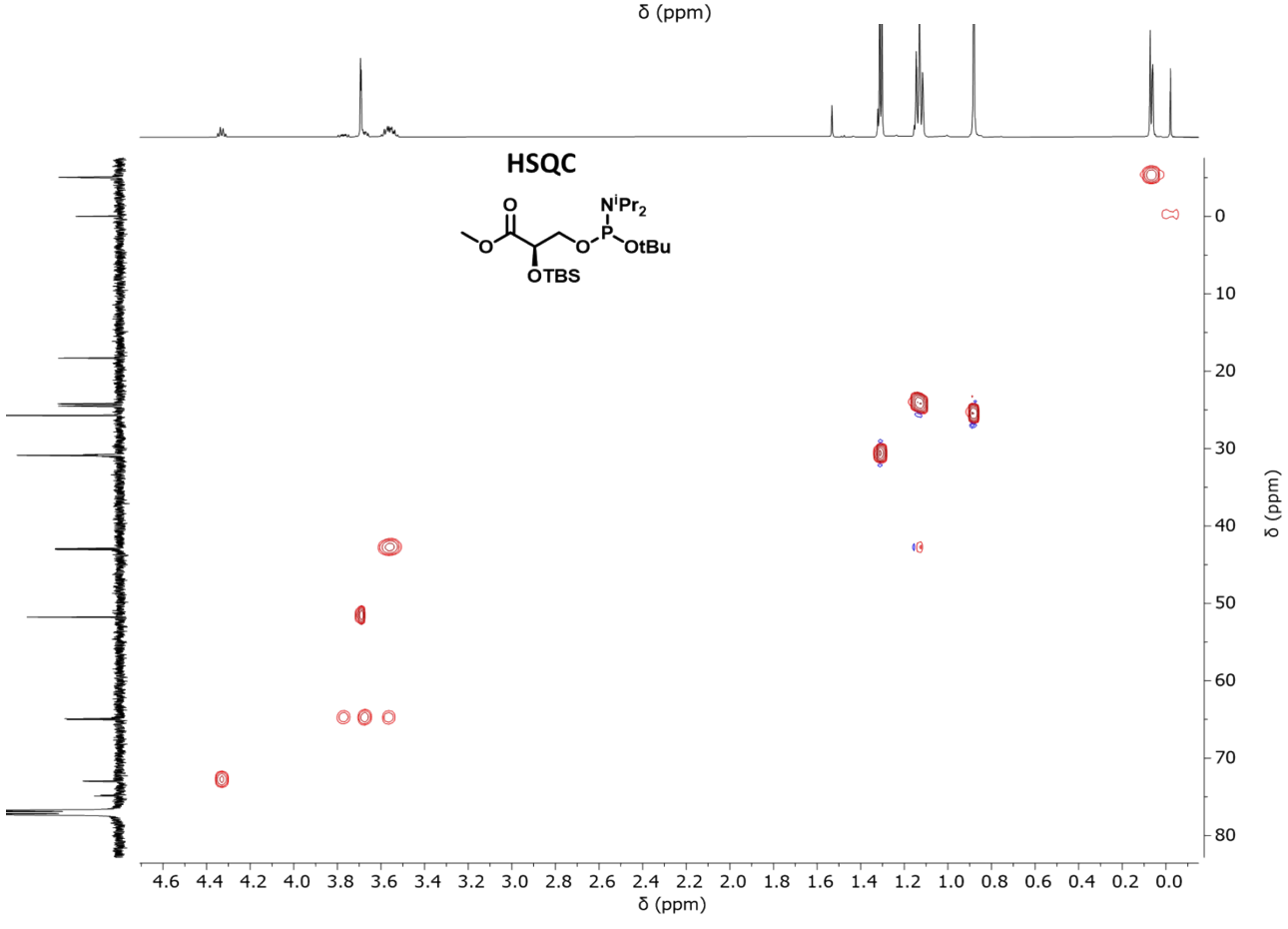
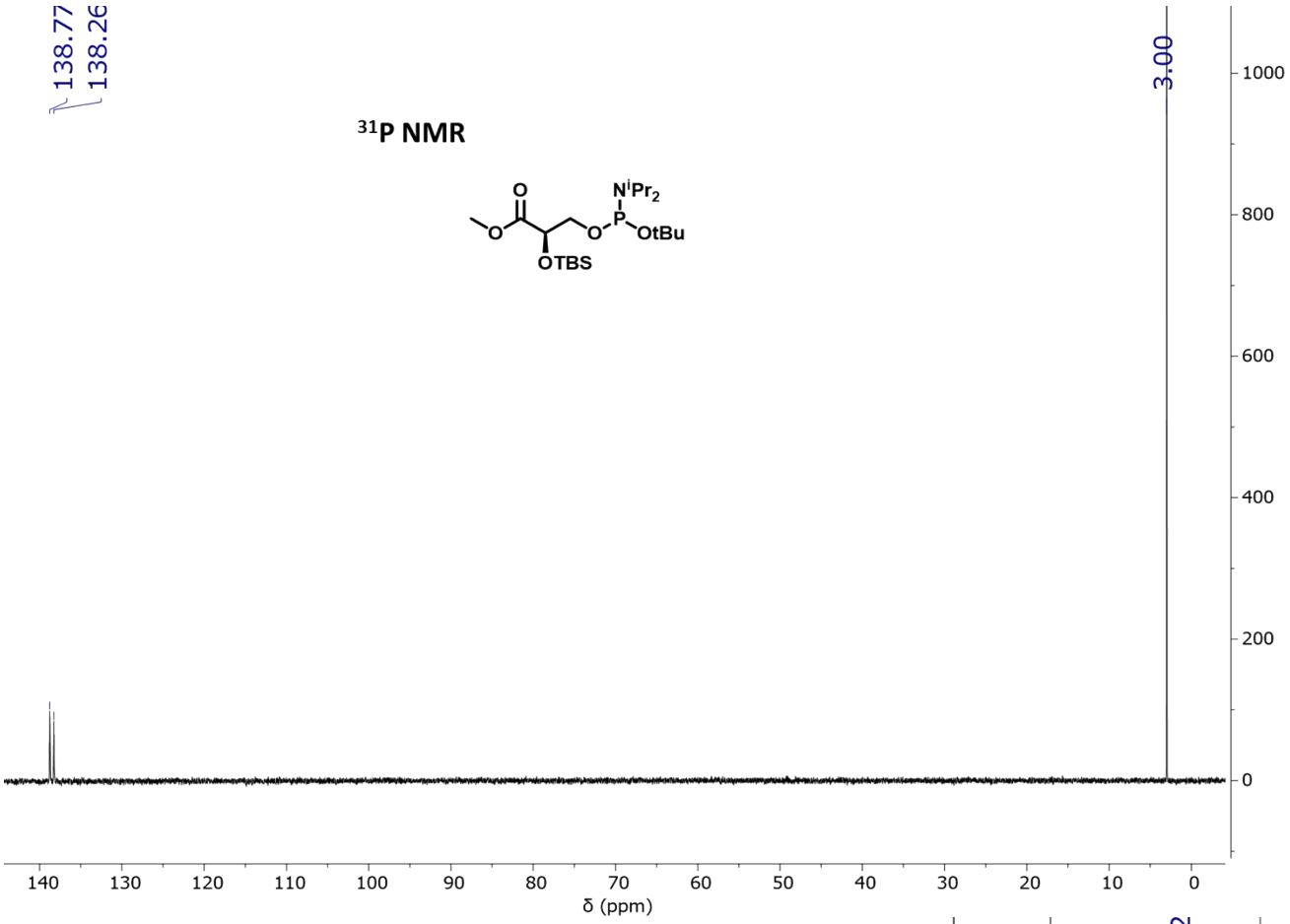
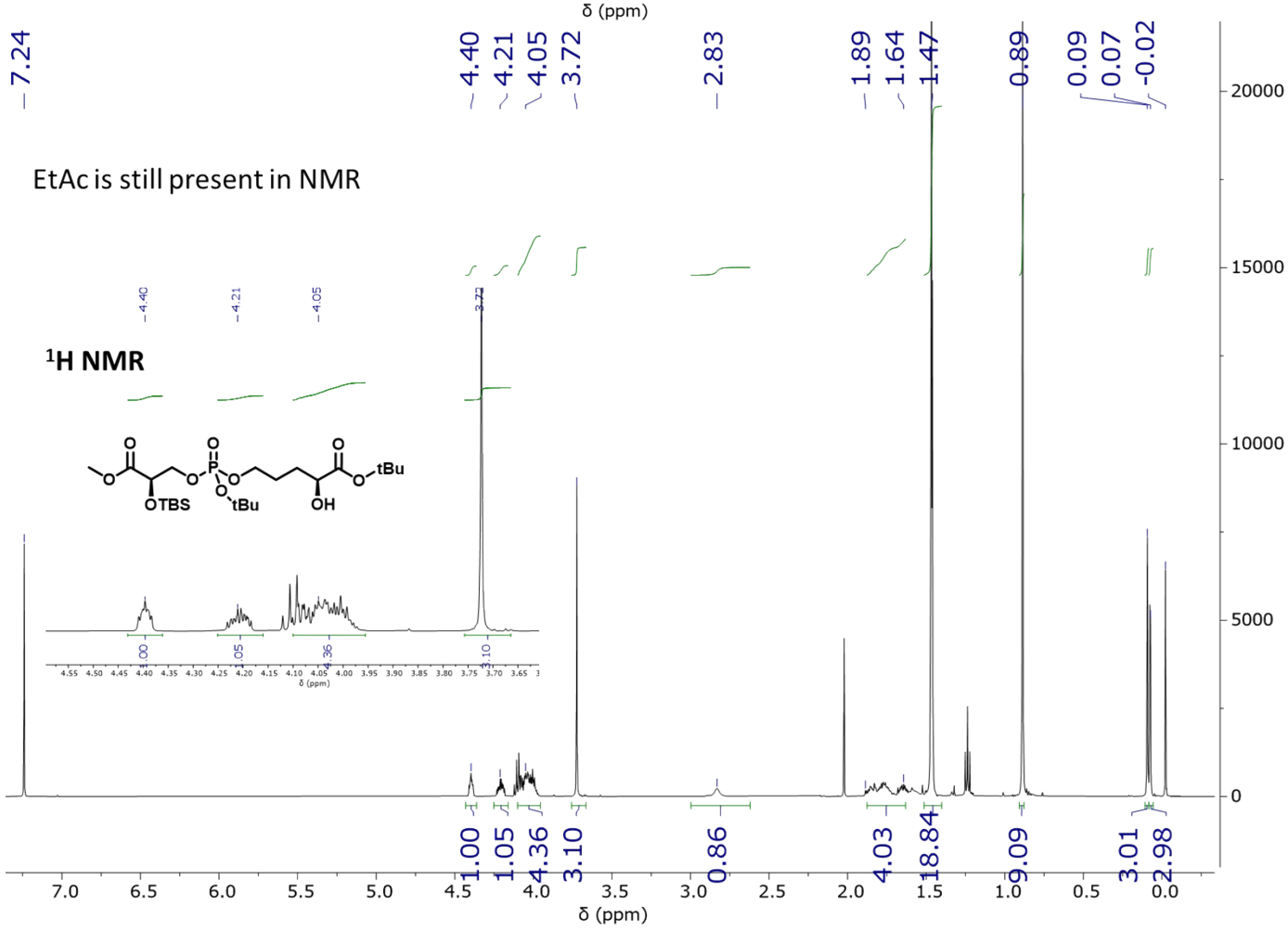
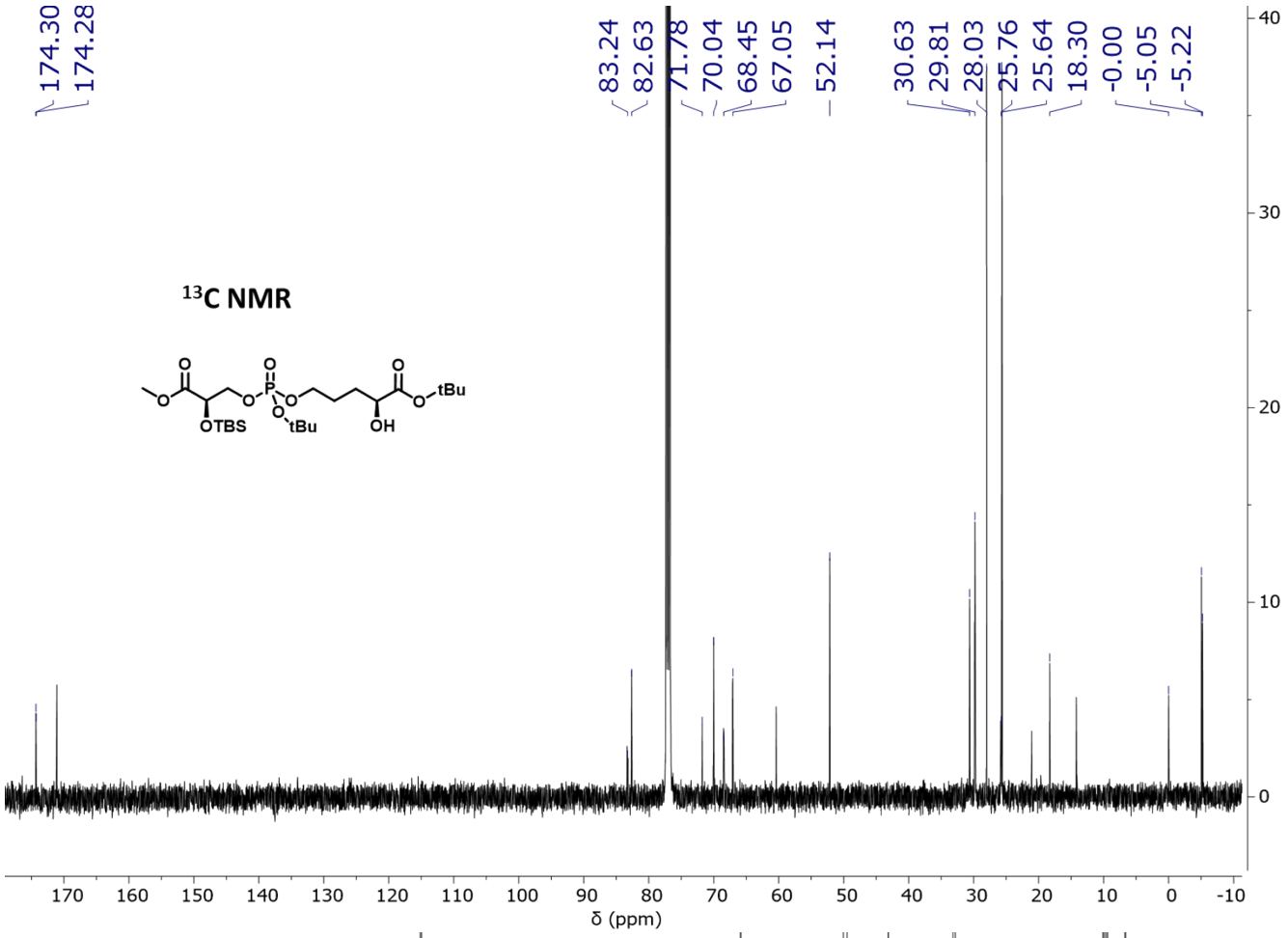
-5.11
13C NMR
N^iPr2
OtBu
OTBS
δ (ppm)


COSY
O
N^{i}Pr$_2$
O
O
P
OtBu
OTBS
0
1
2
3
4
δ (ppm)
4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
δ (ppm)


HSQC
O
N^{i}Pr$_2$
O
O
P
OtBu
OTBS
0
10
20
30
40
50
60
70
80
δ (ppm)
4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2.0 1.8 1.6 1.4 1.2 1.0 0.8 0.6 0.4 0.2 0.0
δ (ppm)


138.77
138.26
3.00
31P NMR
N^iPr2
O
O
O
P
OtBu
OTBS
1000
800
600
400
200
0
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
δ (ppm)


7.24
4.40
4.21
4.05
3.72
2.83
1.89
1.64
1.47
0.89
0.09
0.07
-0.02
EtAc is still present in NMR
1H NMR
OTBS
tBu
OH
1.00
1.05
4.36
3.10
0.86
4.03
18.84
9.09
3.01
2.98
20000
15000
10000
5000
0
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
0.5
0.0
δ (ppm)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71.78
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68.45
67.05
52.14
30.63
29.81
28.03
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18.30
-0.00
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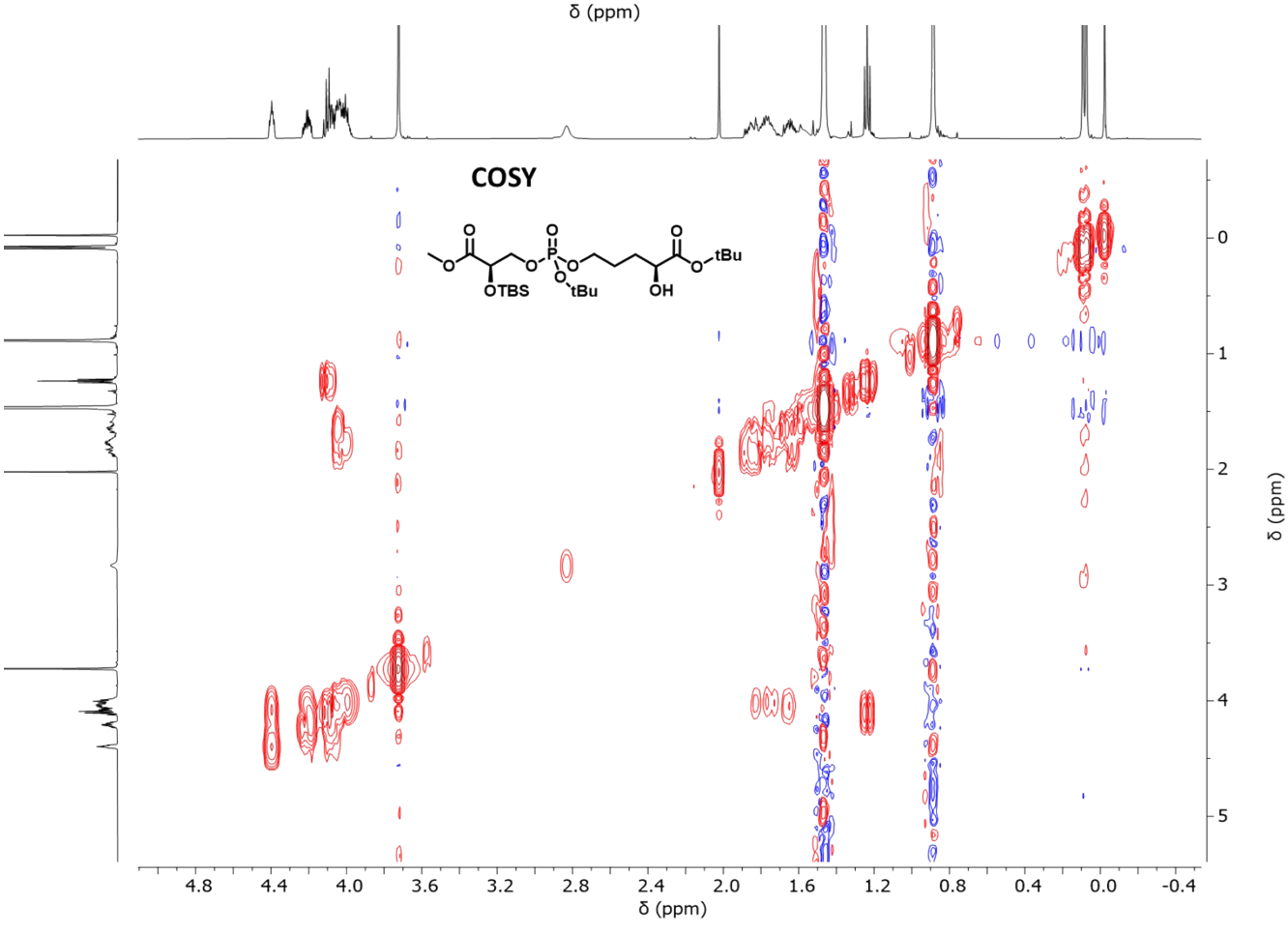
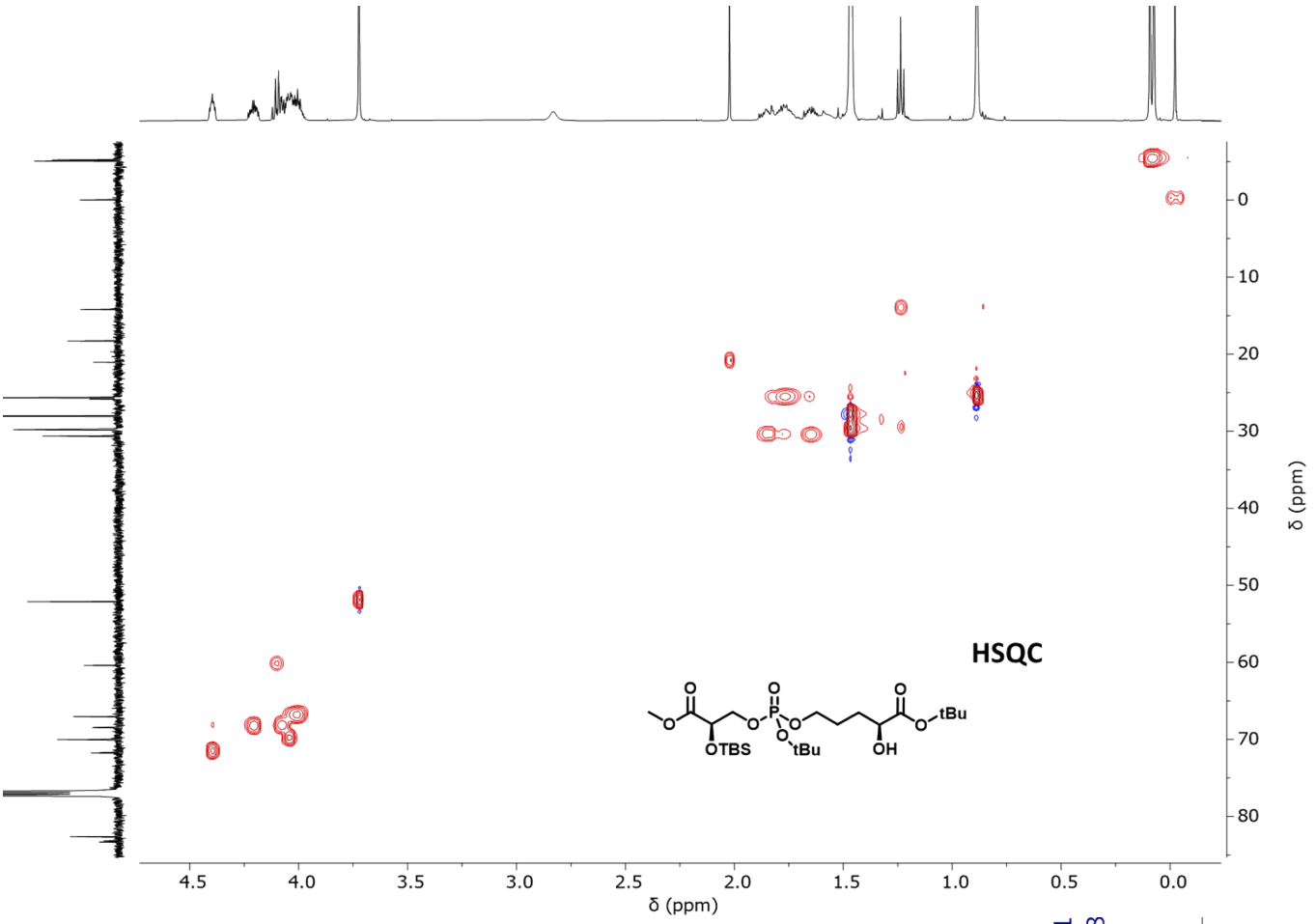
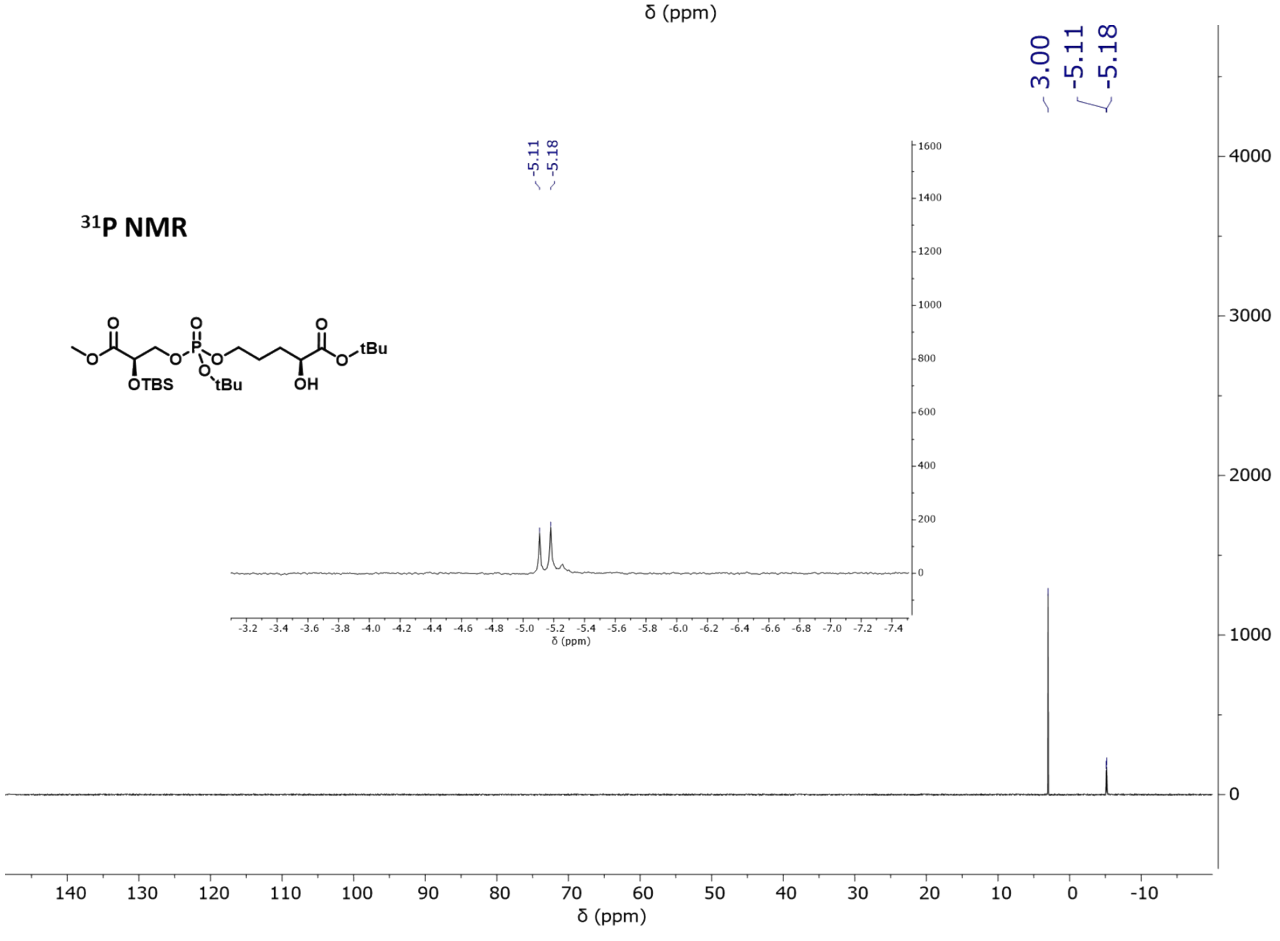
-5.22
^{13}C NMR
OTBS
tBu
OH
δ (ppm)


COSY
OTBS
tBu
OH
δ (ppm)
δ (ppm)


HSQC
δ (ppm)
δ (ppm)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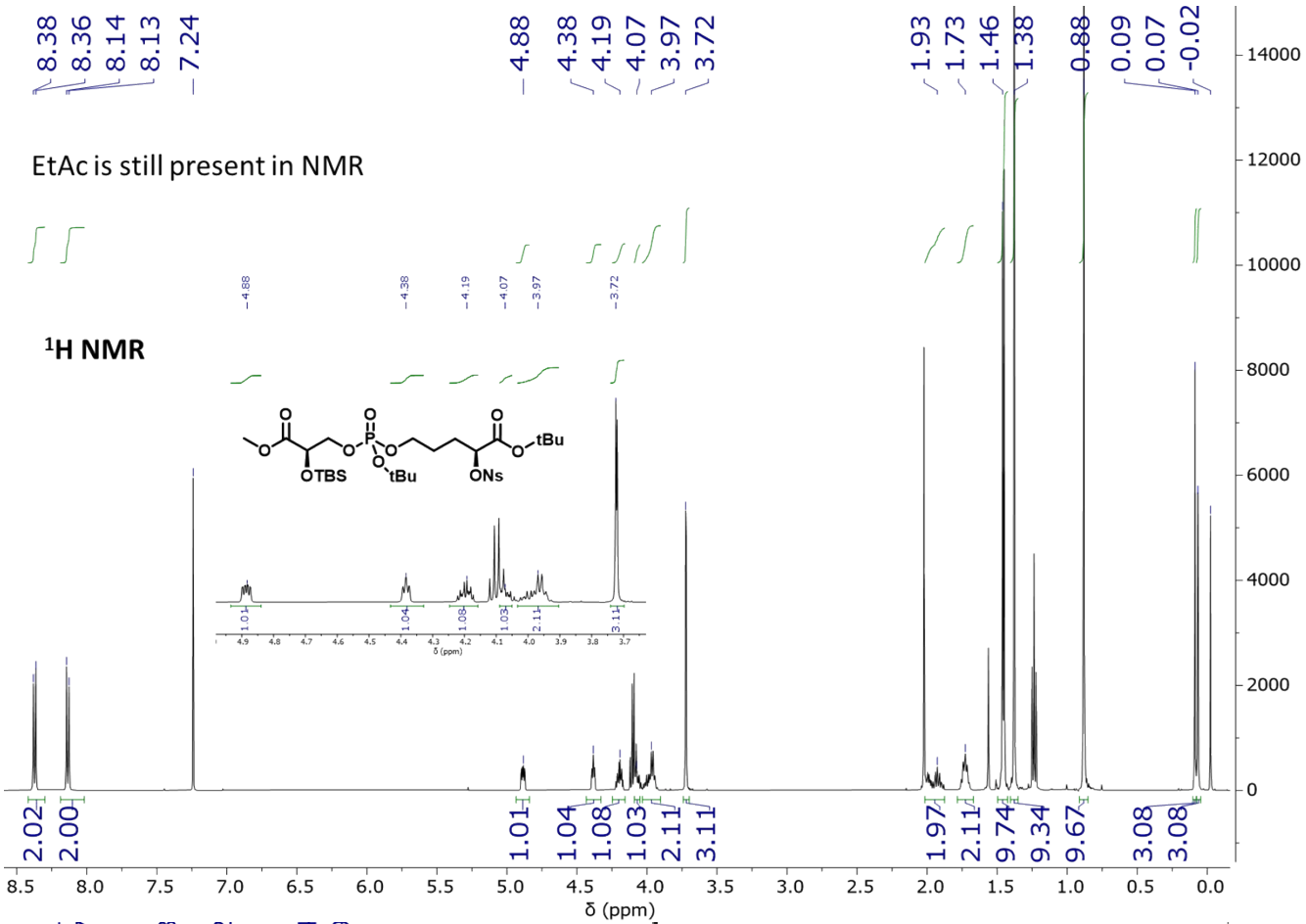
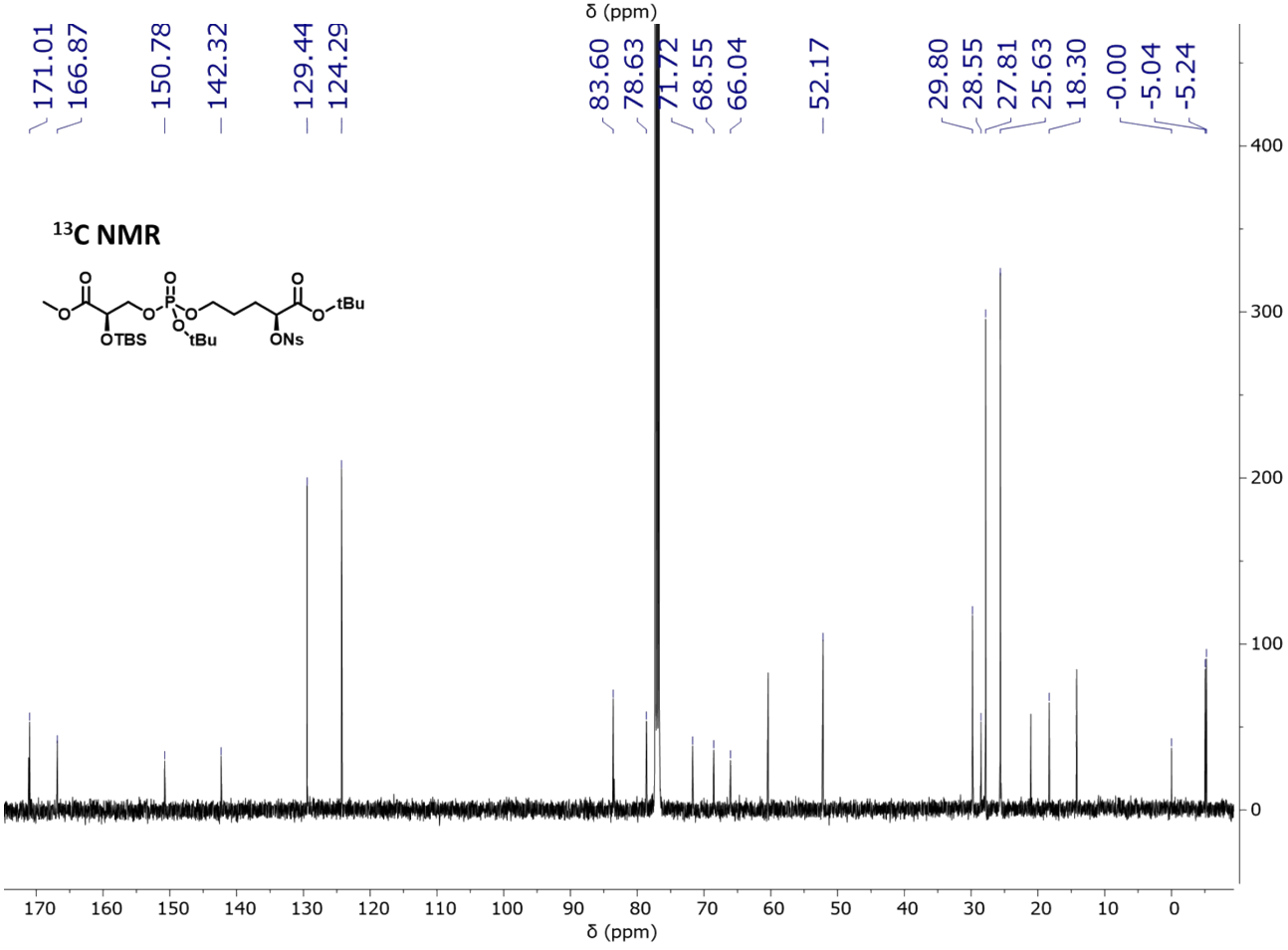
-5.18
δ (ppm)

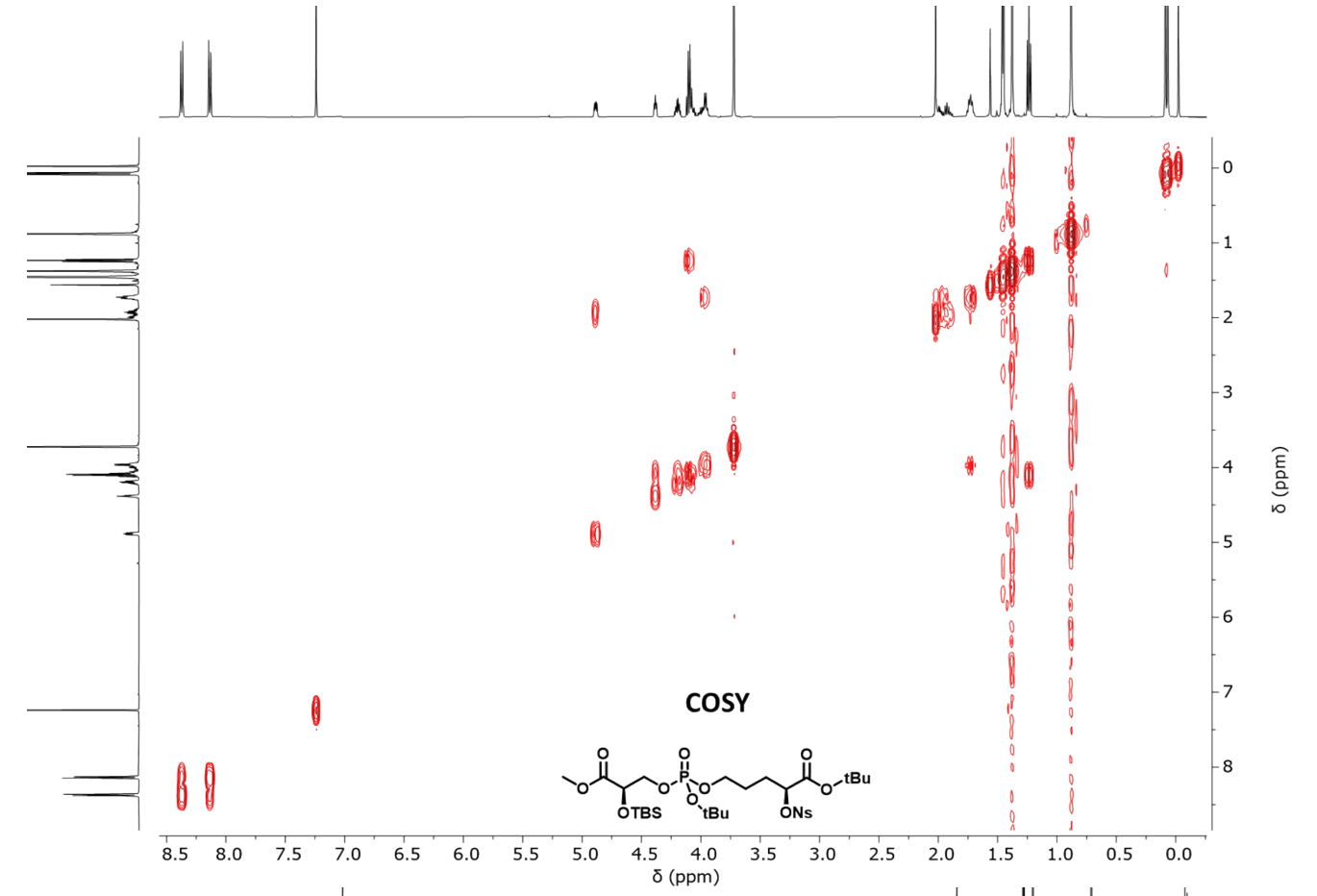
EtAc is still present in NMR

^{1}H NMR



^{13}C NMR

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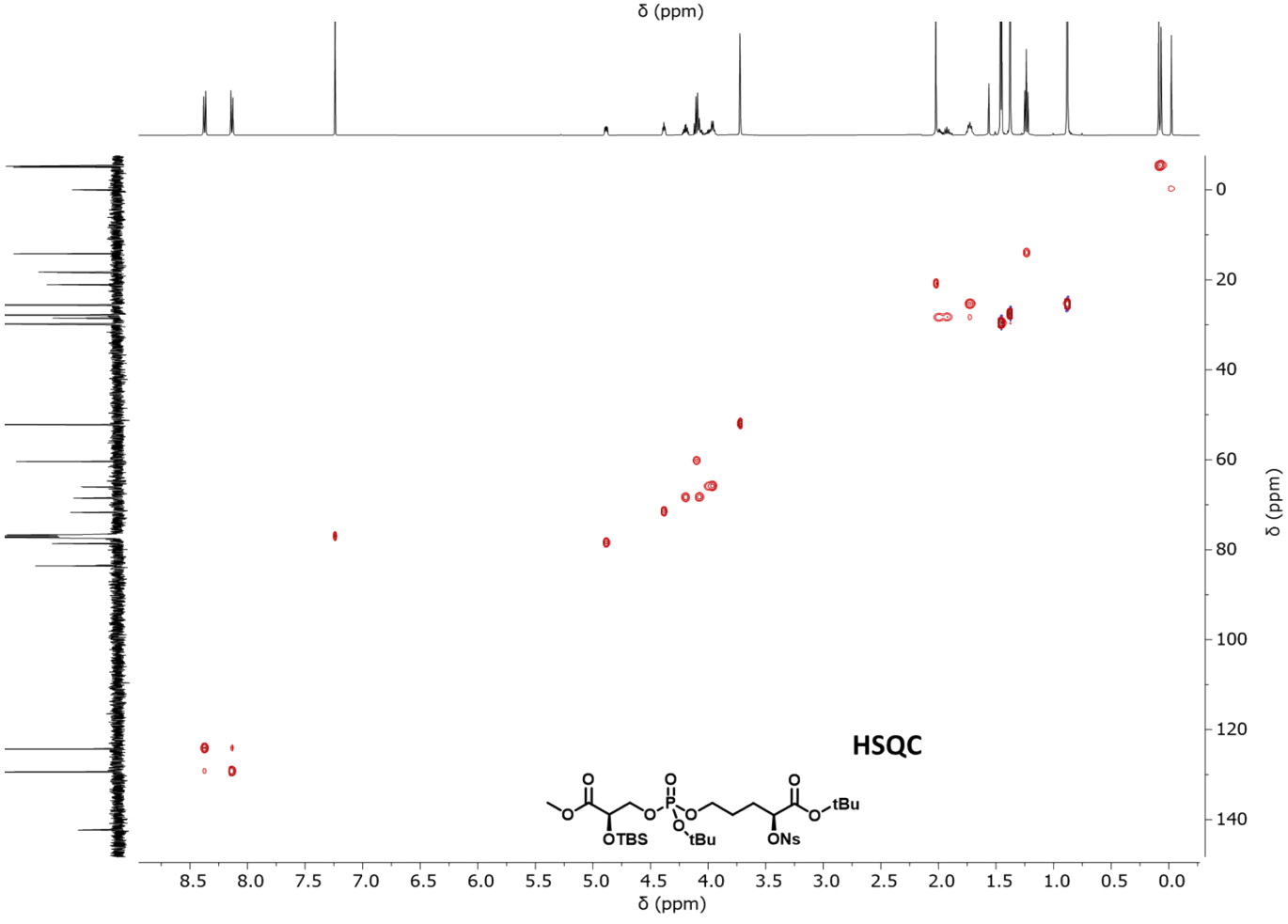
OTBS
tBu
ONs
tBu
δ (ppm)
δ (ppm)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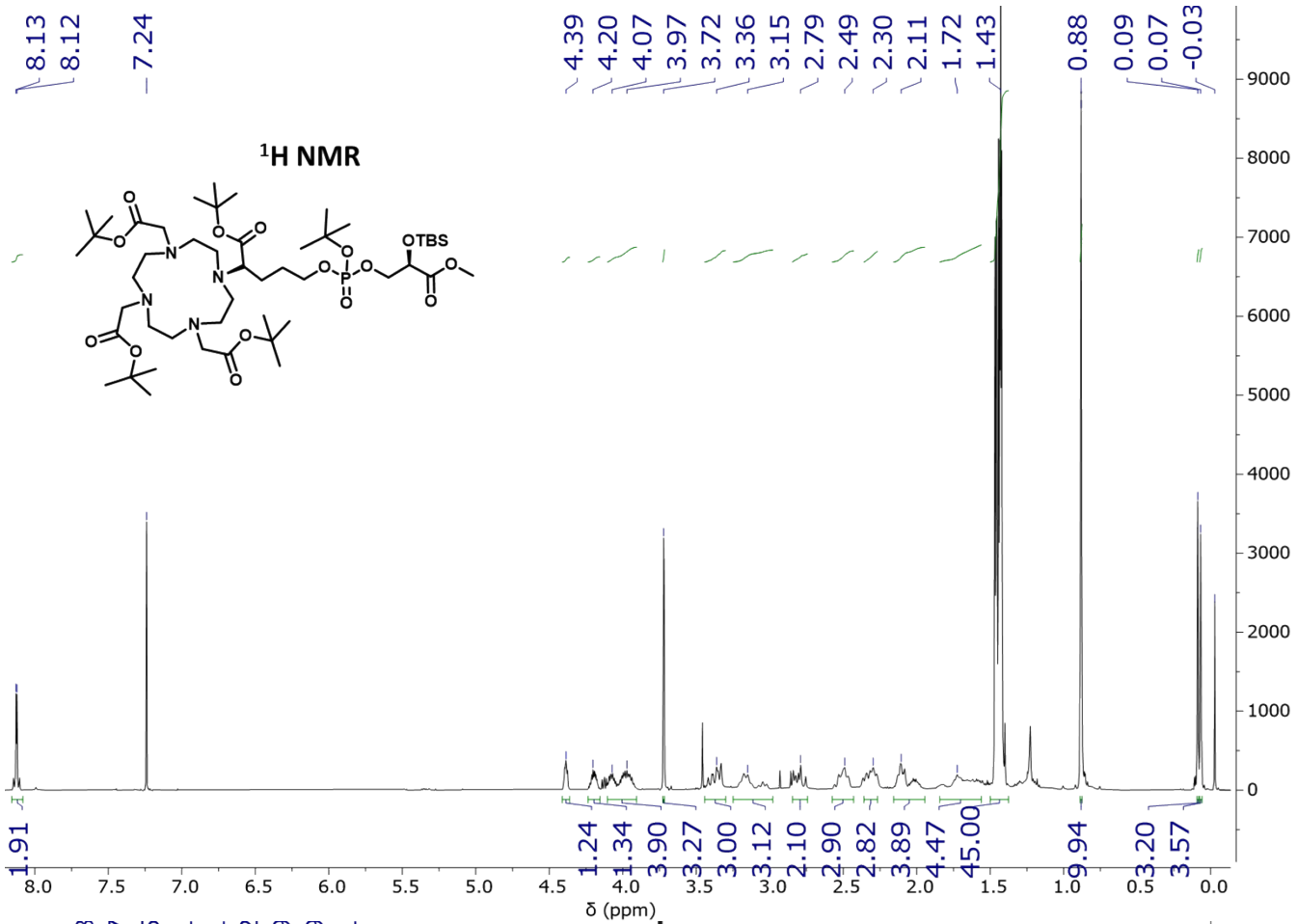
OTBS
tBu
ONs
tBu
δ (ppm)
δ (ppm)



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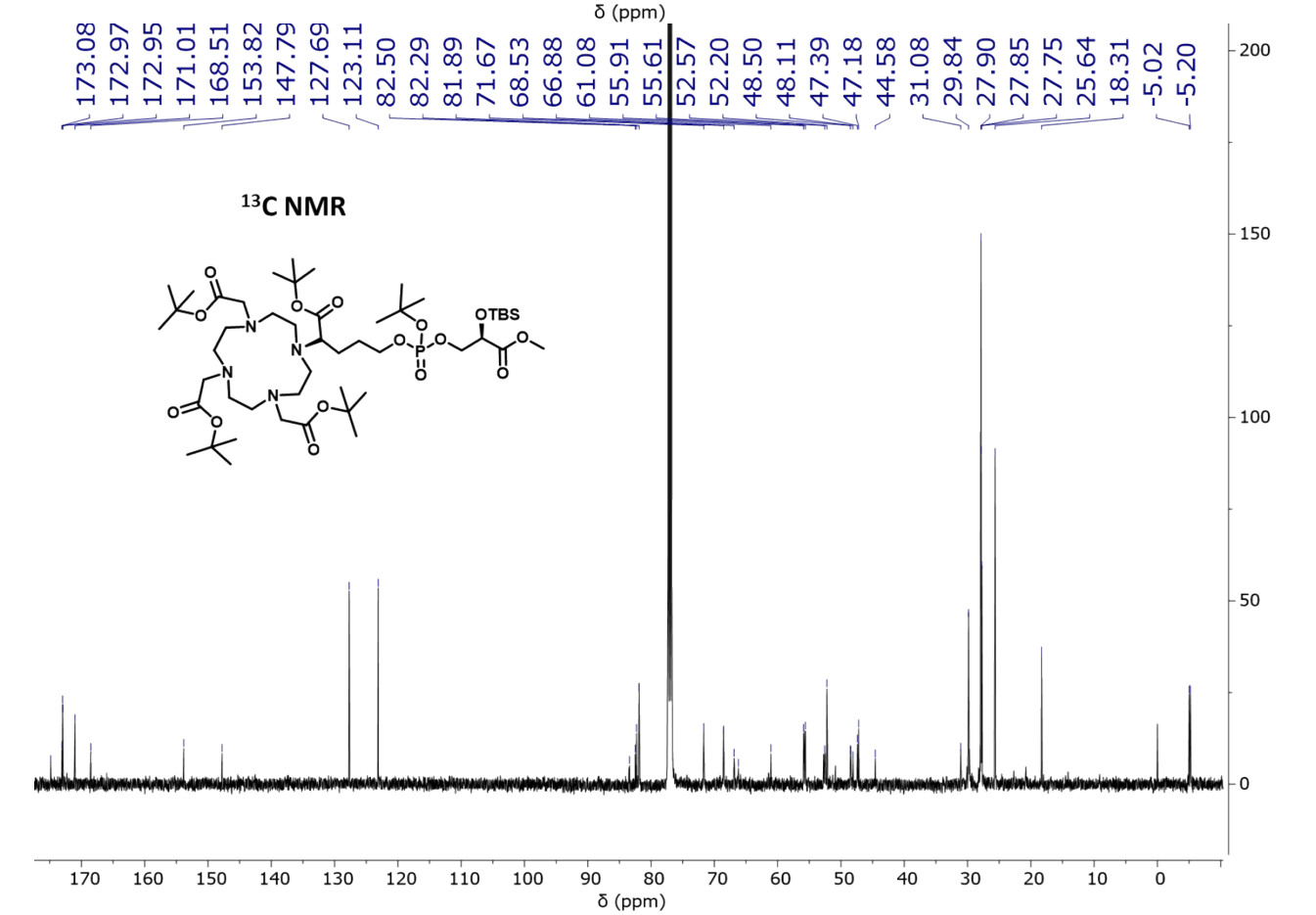

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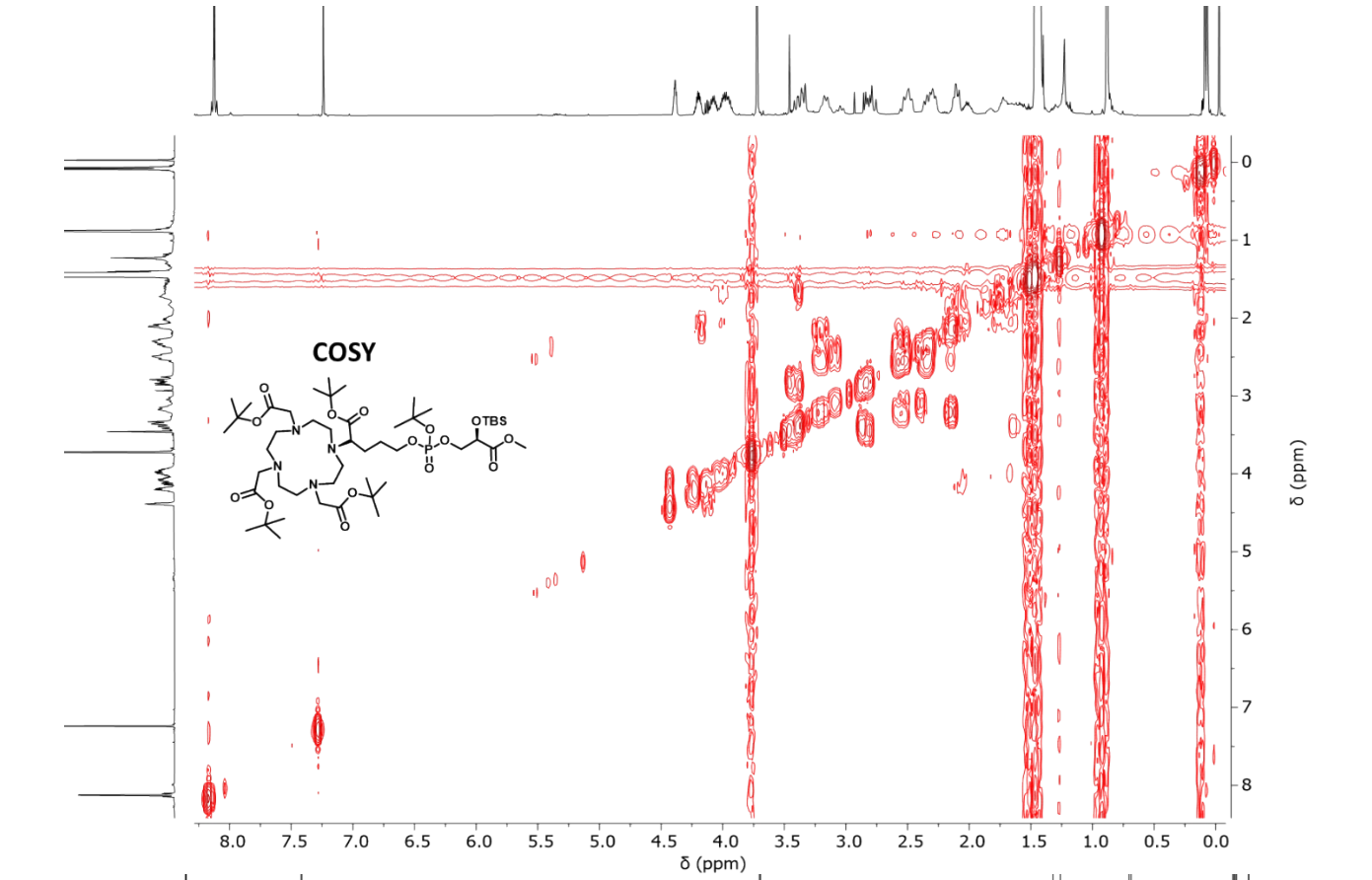
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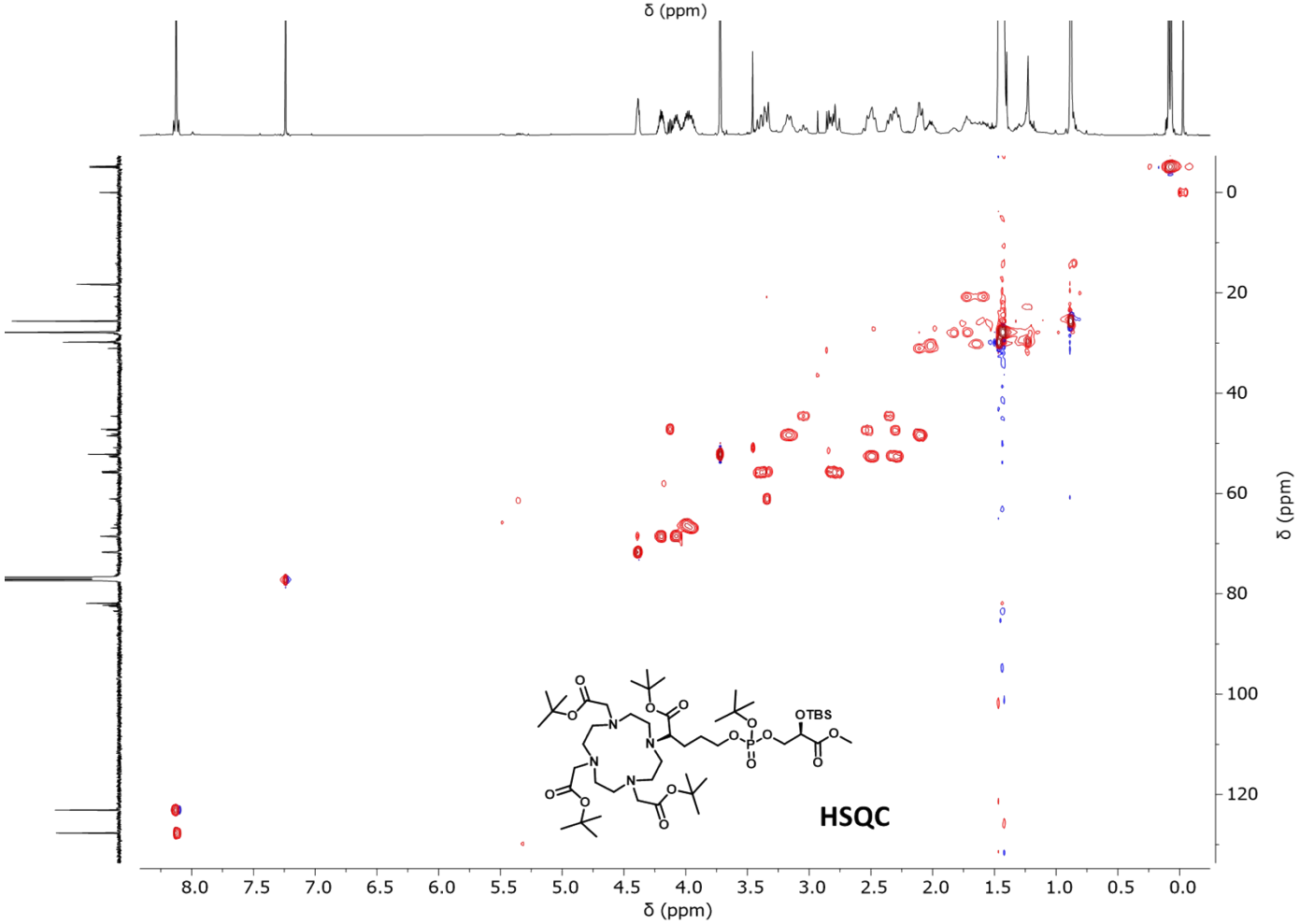
δ (ppm)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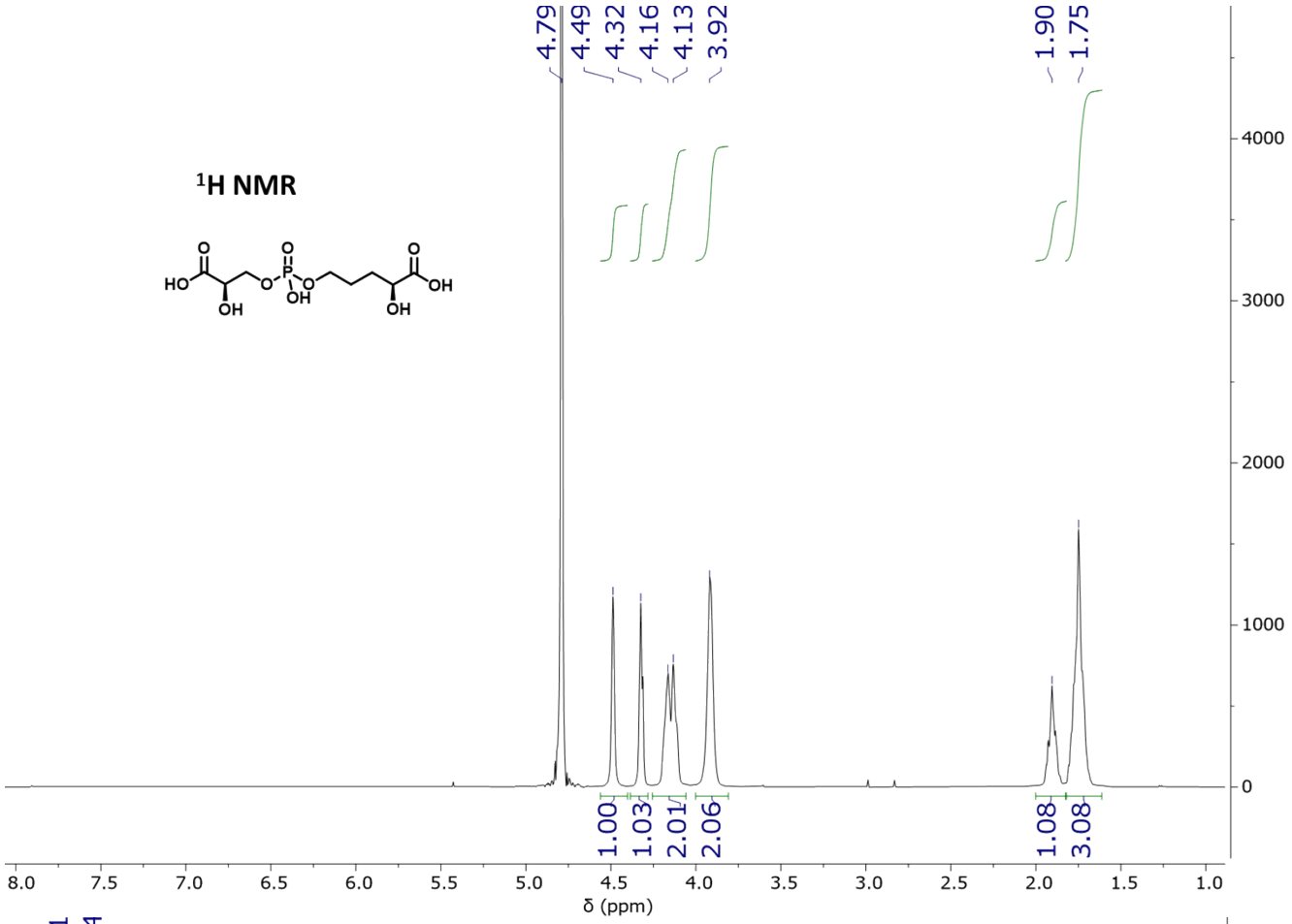
δ (ppm)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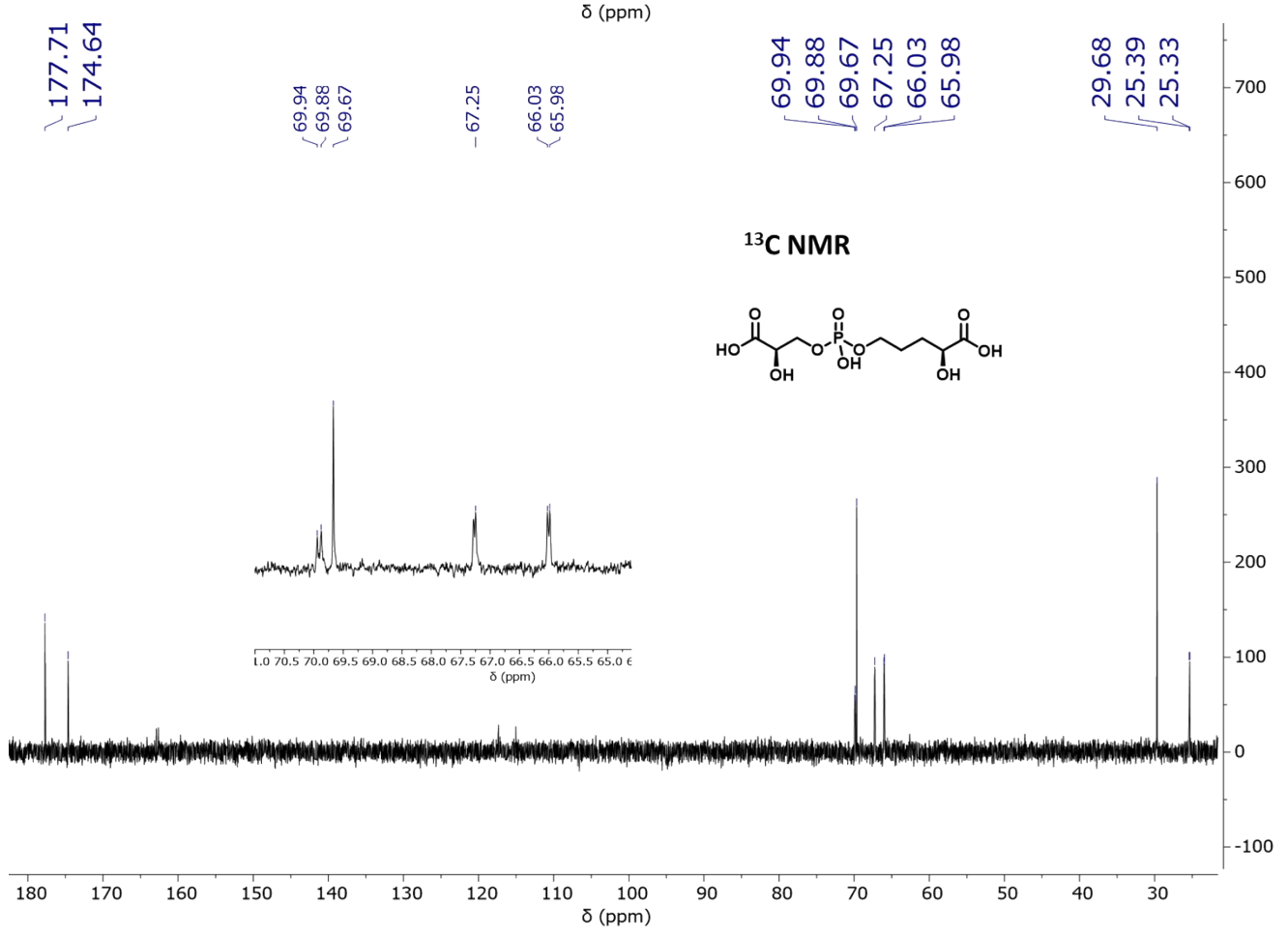
1.08
3.08
δ (ppm)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66.03
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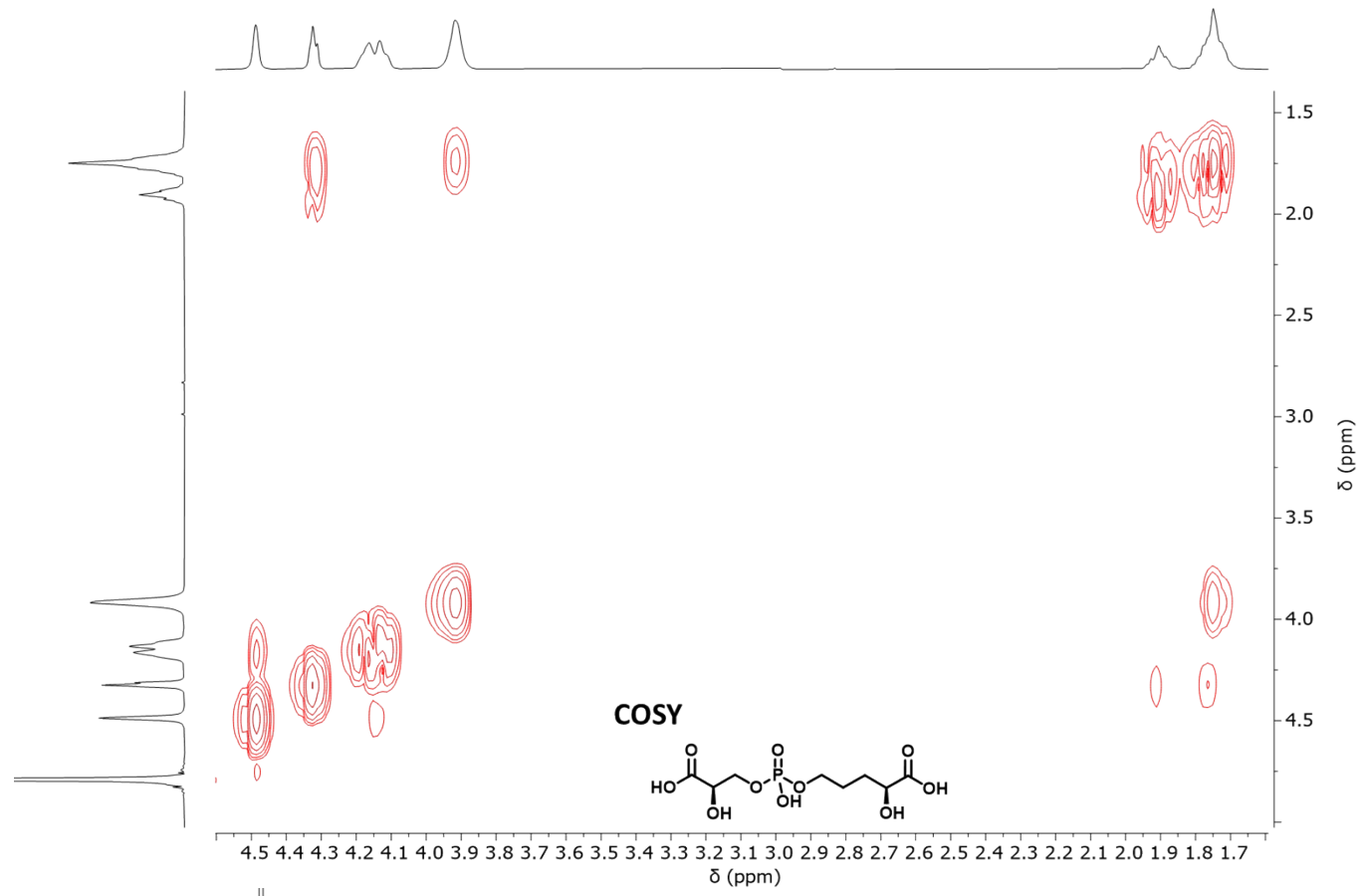
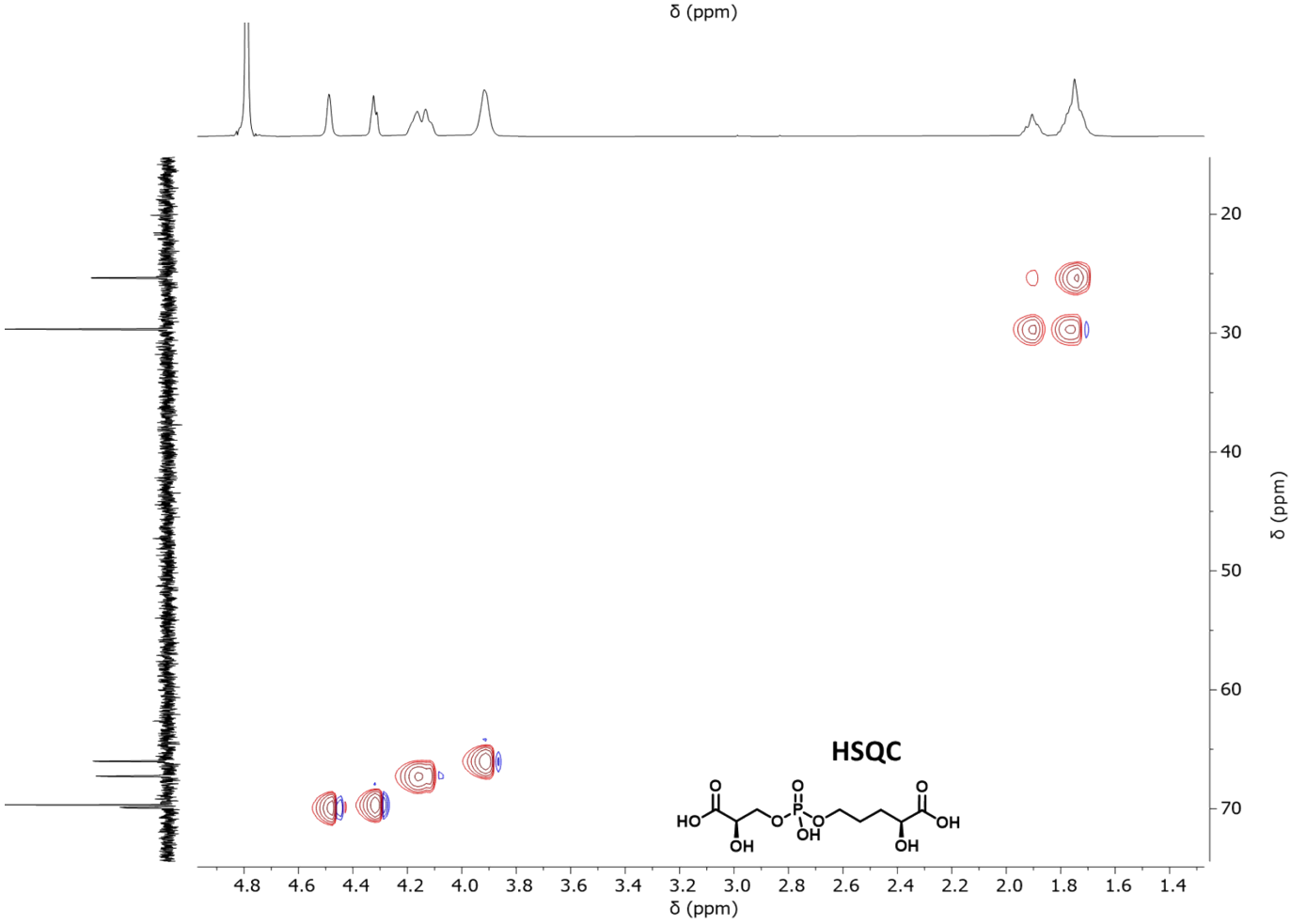
29.68
25.39
25.33
δ (ppm)


COSY
δ (ppm)
δ (ppm)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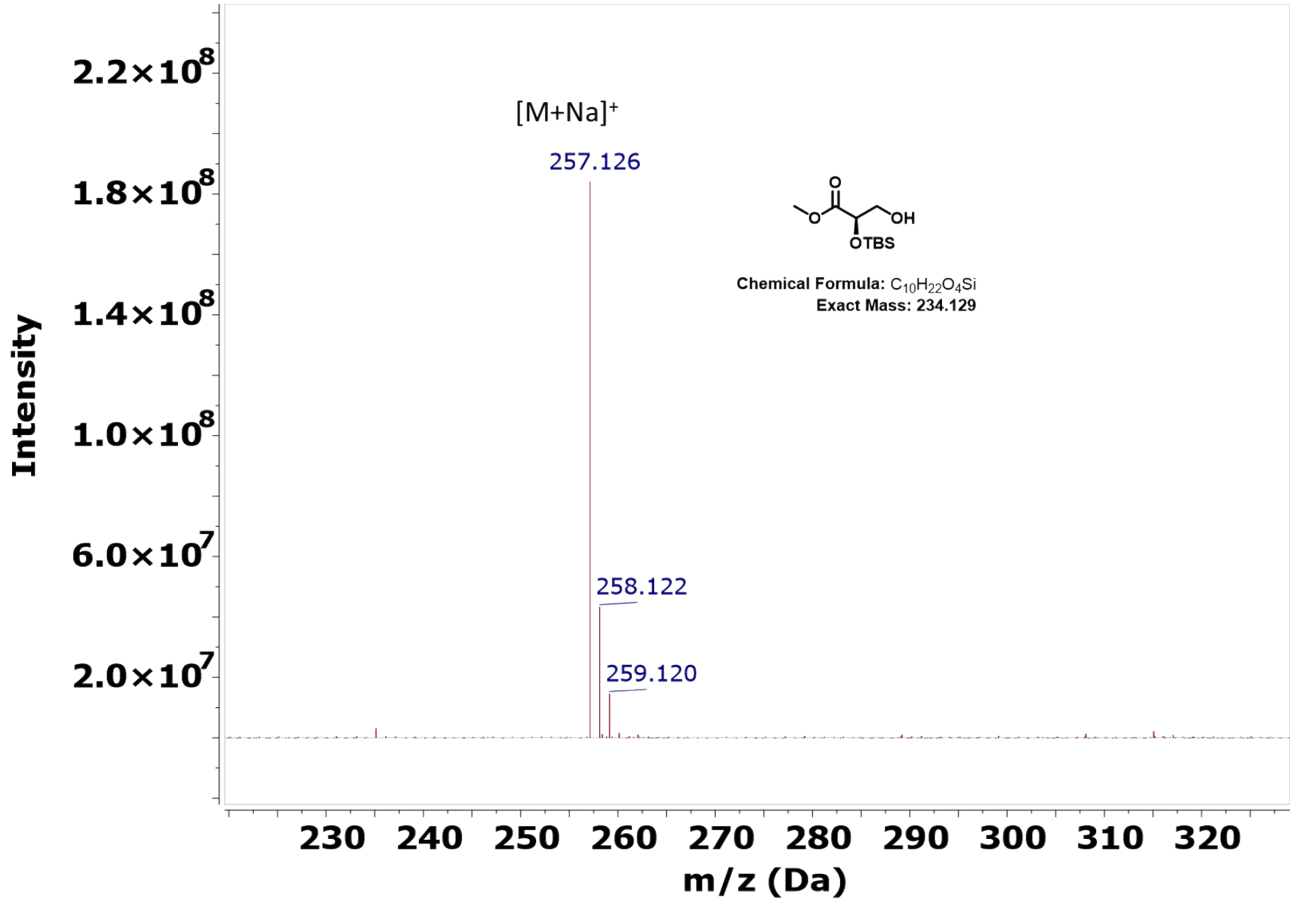
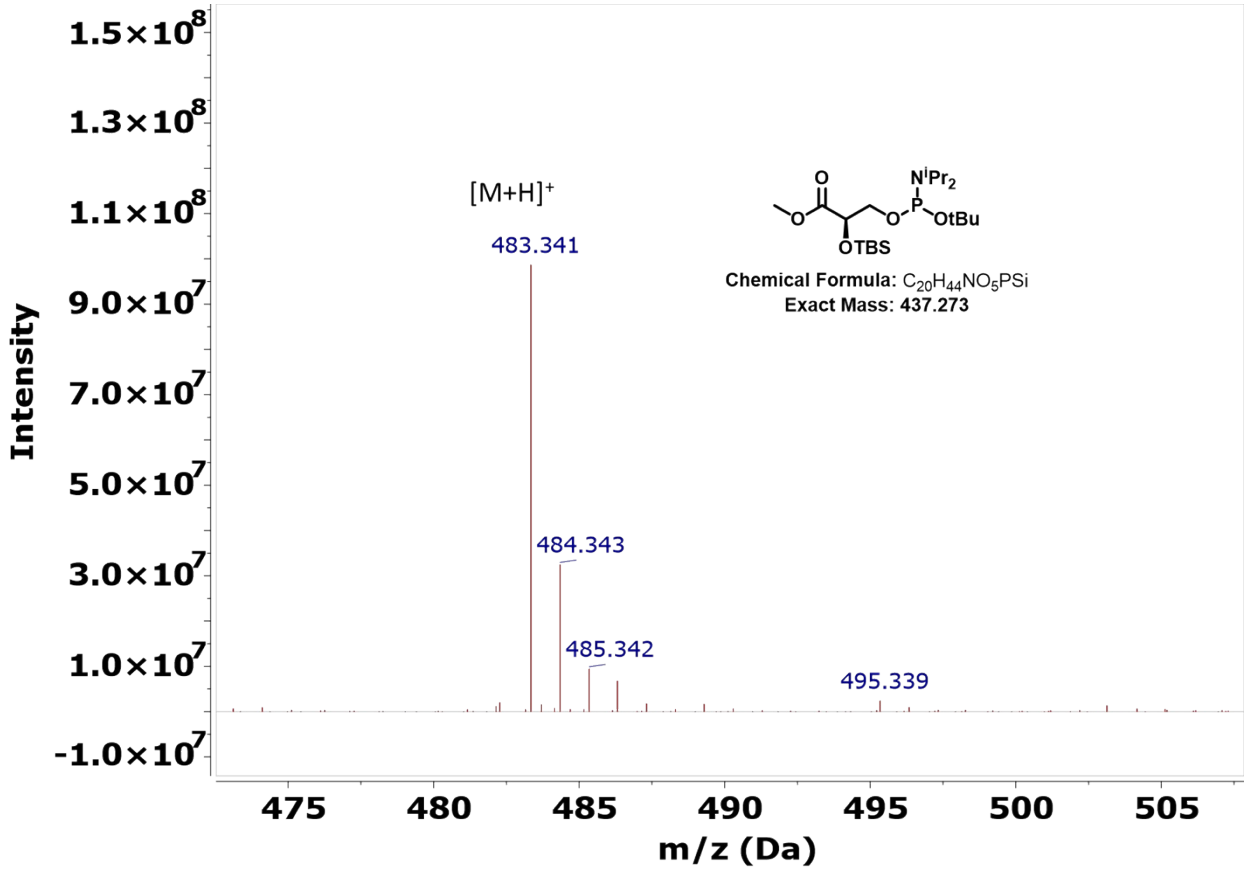
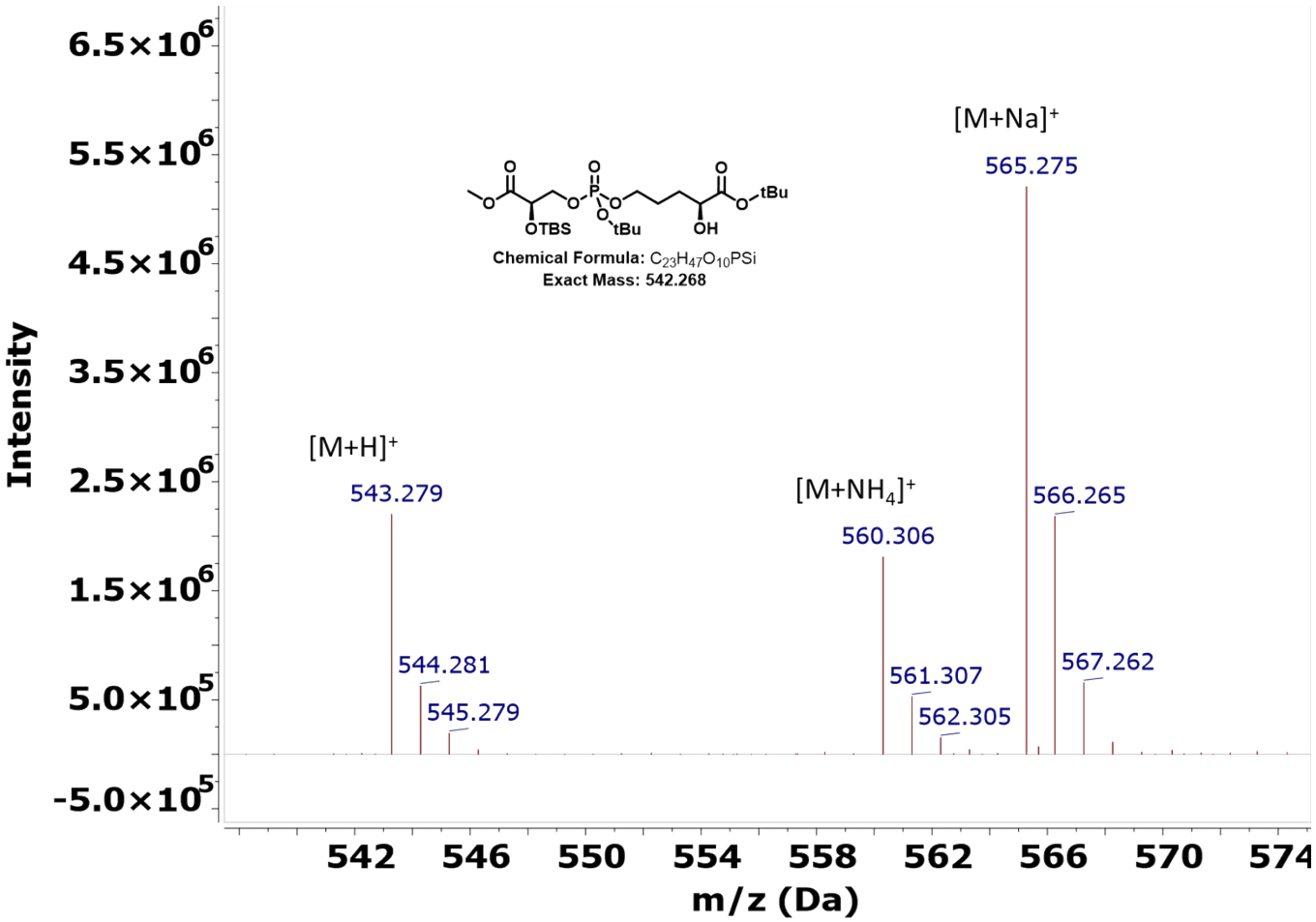
δ (ppm)
δ (ppm)

Section 5: High-Resolution Mass-Spectrometry



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5.5×10^6
4.5×10^6
3.5×10^6
2.5×10^6
1.5×10^6
5.0×10^5
-5.0×10^5
[M+Na]+
565.275
[M+H]+
543.279
[M+NH4]+
560.306
566.265
544.281
561.307
567.262
545.279
562.305
OTBS
tBu
OH
tBu
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Exact Mass: 542.268
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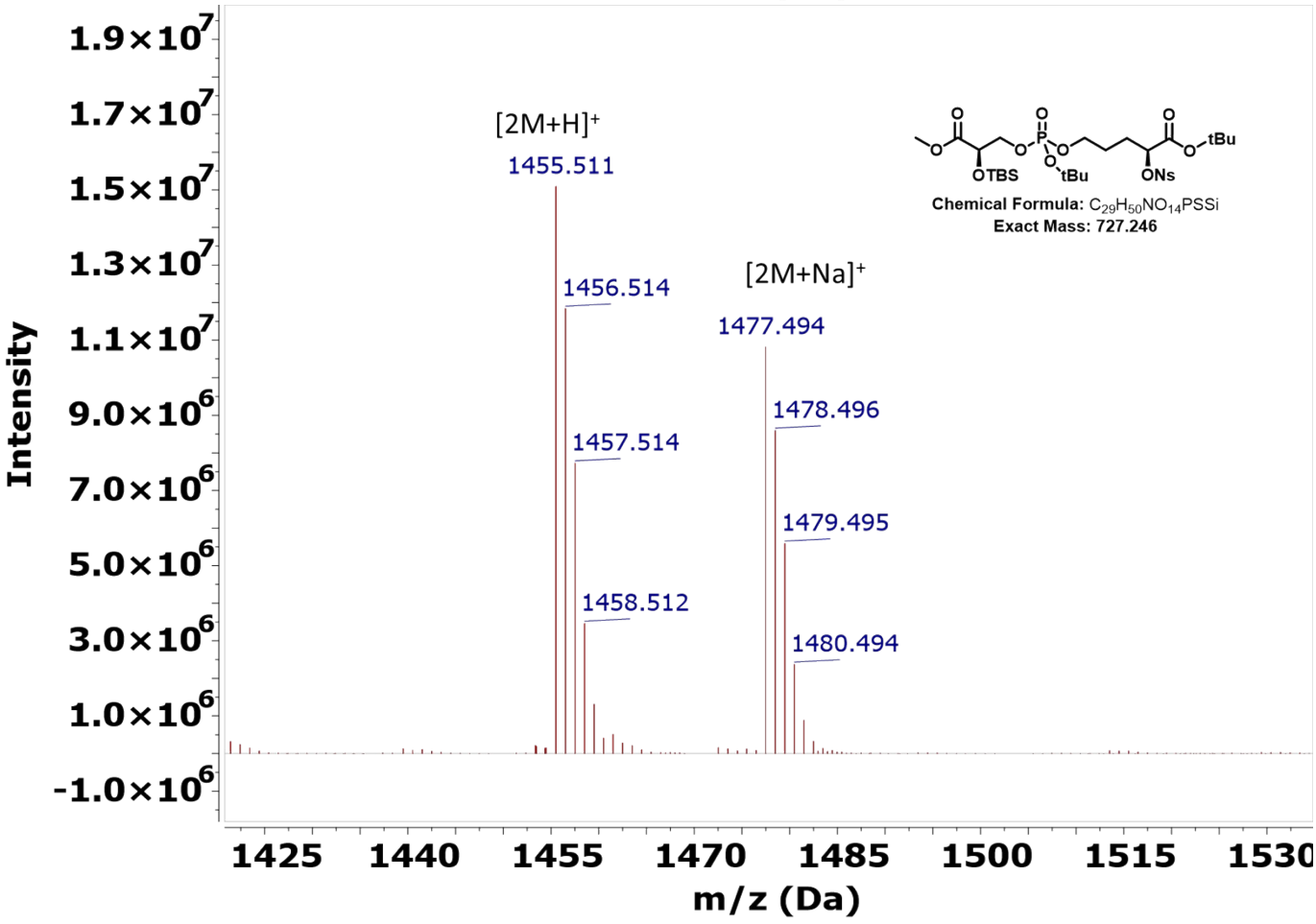
546
550
554
558
562
566
570
574
m/z (Da)


Intensity
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1.7×10^7
1.5×10^7
1.3×10^7
1.1×10^7
9.0×10^6
7.0×10^6
5.0×10^6
3.0×10^6
1.0×10^6
-1.0×10^6
[2M+H]+
1455.511
[2M+Na]+
1456.514
1477.494
1478.496
1457.514
1479.495
1458.512
1480.494
OTBS
tBu
ONs
tBu
Chemical Formula: C29H50NO14PSSi
Exact Mass: 727.246
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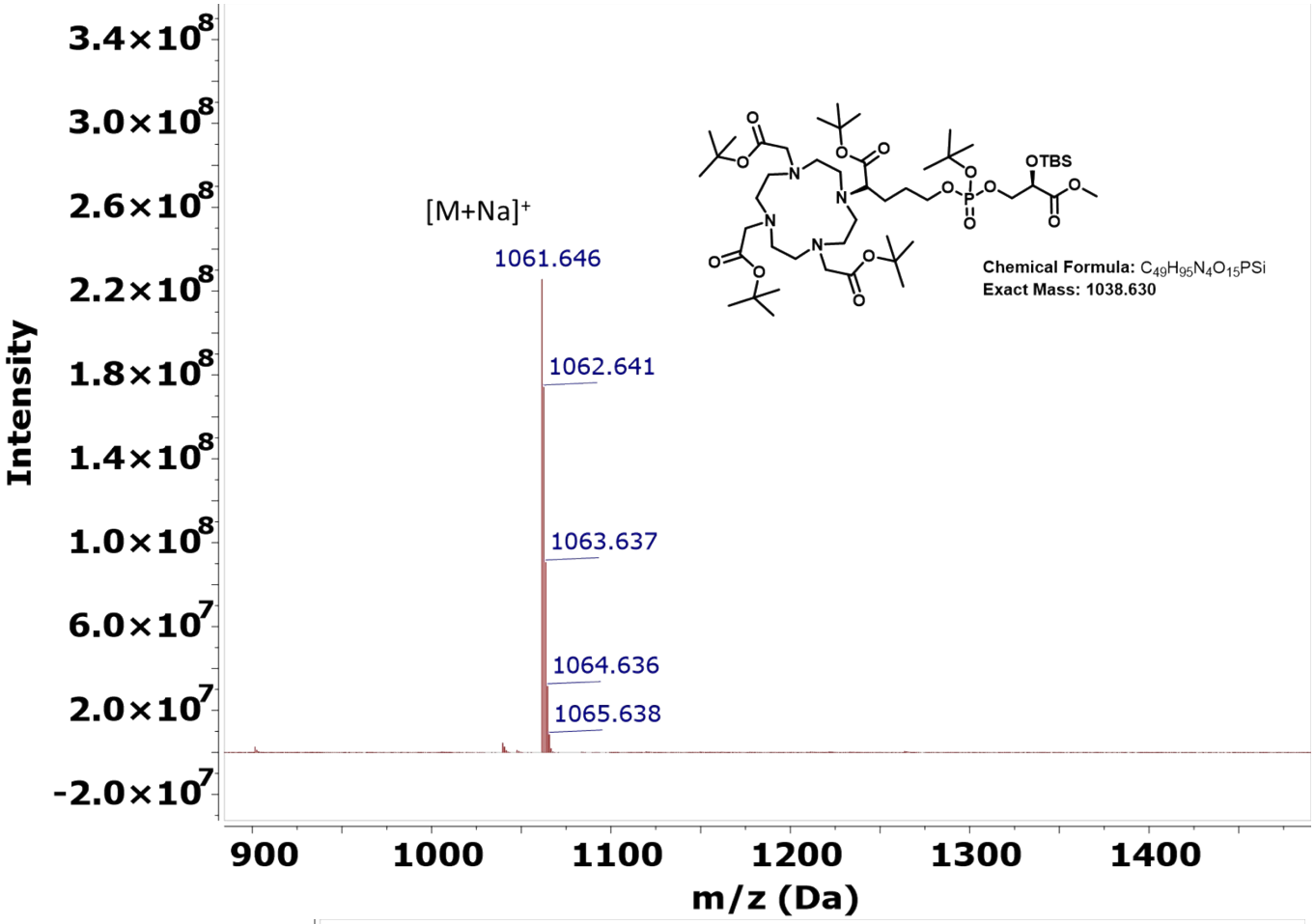
1455
1470
1485
1500
1515
1530
m/z (Da)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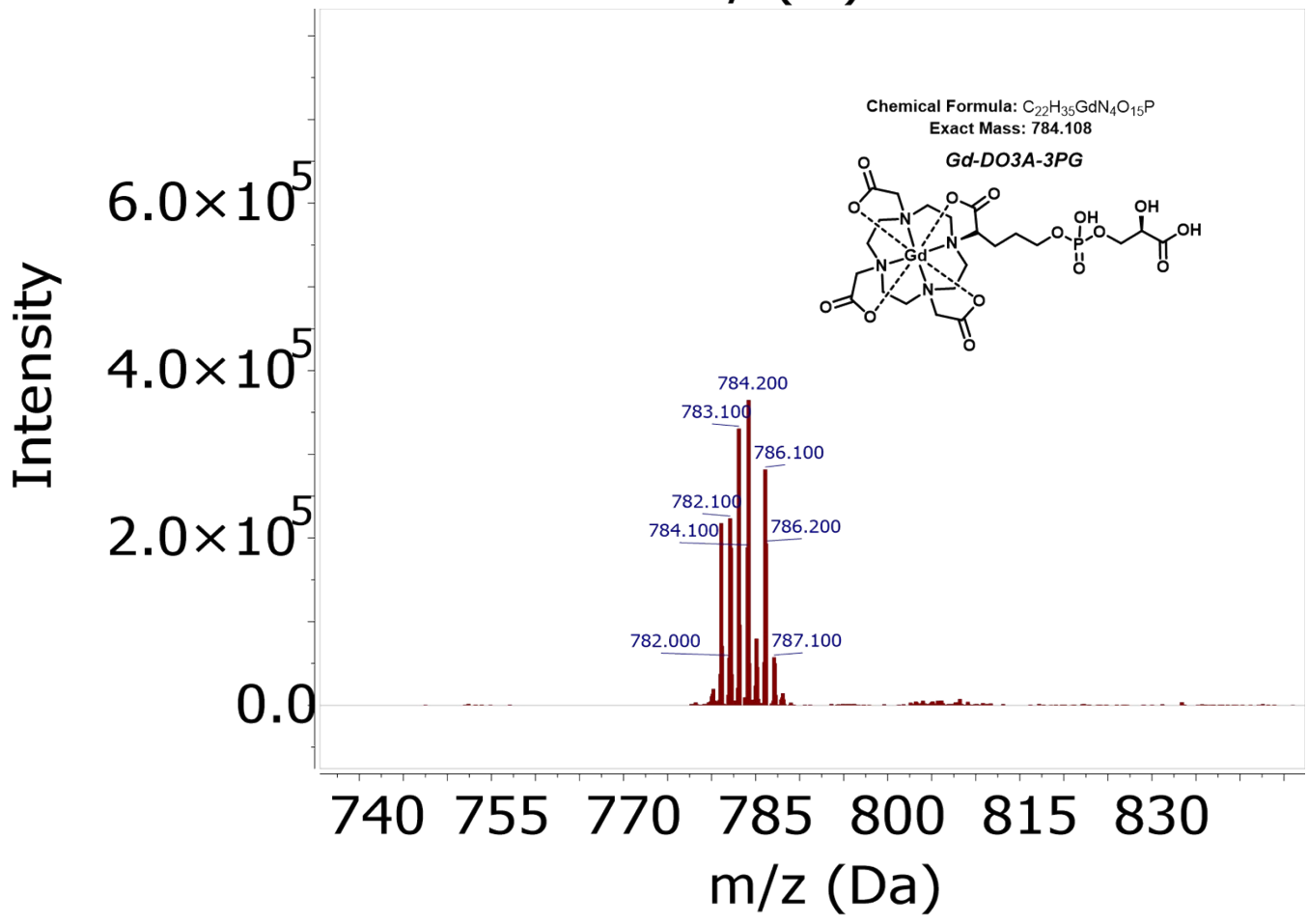
1063.637
1064.636
1065.638
Chemical Formula: C49H95N4O15PSi
Exact Mass: 1038.630
OTBS
Intensity
3.4×10^8
3.0×10^8
2.6×10^8
2.2×10^8
1.8×10^8
1.4×10^8
1.0×10^8
6.0×10^7
2.0×10^7
-2.0×10^7
900
1000
1100
1200
1300
1400
m/z (Da)


Chemical Formula: C22H35GdN4O15P
Exact Mass: 784.108
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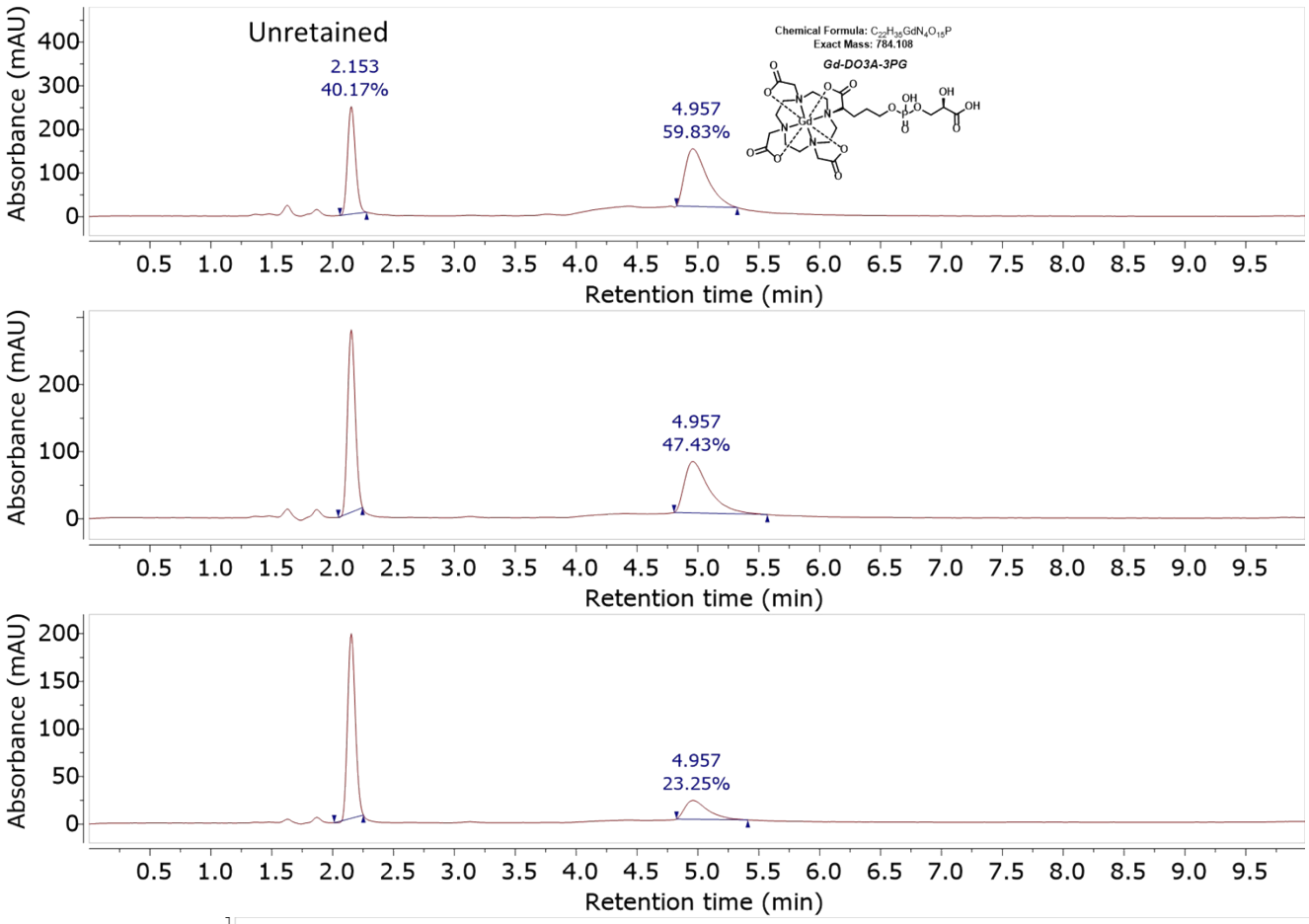
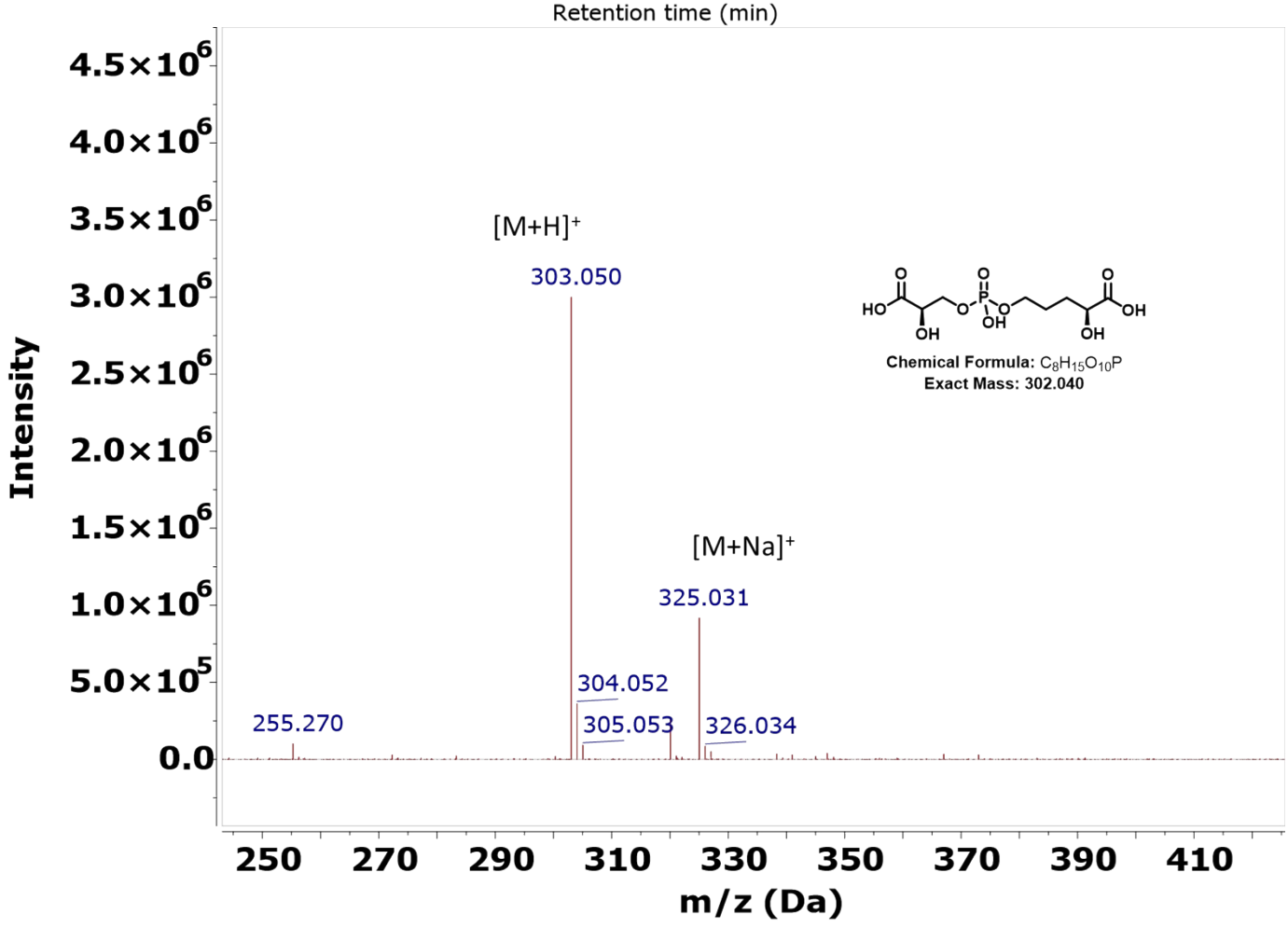
783.100
786.100
782.100
784.100
786.200
782.000
787.100
Intensity
6.0×10^5
4.0×10^5
2.0×10^5
0.0
740
755
770
785
800
815
830
m/z (Da)


Unretained
2.153
40.17%
4.957
59.83%
Chemical Formula: C22H36GdN4O15P
Exact Mass: 784.108
Gd-DO3A-3PG
Absorbance (mAU)
Retention time (min)
4.957
47.43%
4.957
23.25%


Intensity
[M+H]+
303.050
[M+Na]+
325.031
304.052
305.053
326.034
255.270
m/z (Da)
Chemical Formula: C8H15O10P
Exact Mass: 302.040