Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Synthesis of an acp^{3}U phosphoramidite and incorporation of the hypermodified base into RNA

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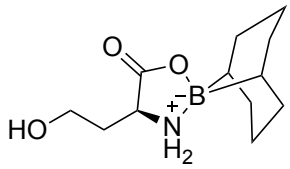
1. General Experimental Methods

Chemicals were purchased from Sigma-Aldrich, TCI, Fluka, ABCR, Carbosynth or Acros organics and used without further purification. Strands containing canonical bases were purchased from Metabion. The solvents were of reagent grade or purified by distillation, unless otherwise specified. Reactions and chromatography fractions were monitored by qualitative thin-layer chromatography (TLC) on silica gel F254 TLC plates from Merck KGaA. Flash column chromatography was performed on Geduran® Si60 (40-63 μm) silica gel from Merck KGaA. NMR spectra were recorded on Bruker AVIIIHD 400 spectrometers (400 MHz). ^{1}H NMR shifts were calibrated to the residual solvent resonances: DMSO-d_6 (2.50 ppm), $CDCl_3$ (7.26 ppm), Acetone-d_6 (2.05 ppm). ^{13}C NMR shifts were calibrated to the residual solvent: DMSO-d_6 (39.52 ppm), $CDCl_3$ (77.16 ppm), Acetone-d_6 (29.84 ppm). All NMR spectra were analysed using the program MestRENOVA 10.0.1 from Mestrelab Research S. L. High resolution mass spectra were measured by the analytical section of the Department of Chemistry of the Ludwigs-Maximilians-Universität München on the spectrometer MAT 90 (ESI) from Thermo Finnigan GmbH. IR spectra were recorded on a PerkinElmer Spectrum BX II FT-IR system. All substances were directly applied as solids or on the ATR unit. Analytical RP-HPLC was performed on an analytical HPLC Waters Alliance (2695 Separation Module, 2996 Photodiode Array Detector) equipped with the column Nucleosil 120-2 C18 from Macherey Nagel using a flow of 0.5 mL/min, a gradient of 0-30% of buffer B in 45 min was applied. Preparative RP-HPLC was performed on a HPLC Waters Breeze (2487 Dual λ Array Detector, 1525 Binary HPLC Pump) equipped with the column VP 250/32 C18 from Macherey Nagel using a flow of 5 mL/min, a gradient of 0-25% of buffer B in 45 min was applied for the purifications. Oligonucleotides were purified using the following buffer system: buffer A: 100 mM NEt_3/HOAc (pH 7.0) in H_2O and buffer B: 100 mM NEt_3/HOAc in 80% (v/v) acetonitrile. The pH values of buffers were adjusted using a MP 220 pH-meter (Metter Toledo). Oligonucleotides were detected at wavelength: 260 nm. Melting profiles were measured on a JASCO V-650 spectrometer. Calculation of concentrations was assisted using the software OligoAnalyzer 3.0 (Integrated DNA Technologies: https://eu.idtdna.com/calc/analyzer). For strands containing artificial bases, the extinction coefficient of their corresponding canonical-only strand was employed without corrections. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker Autoflex II. For MALDI-TOF measurements, the samples were desalted on a 0.025 μm VSWP filter (Millipore) against ddH_2O and co-crystallized in a 3-hydroxypicolinic acid matrix (HPA).

2. Synthesis of the Phosphoramidite Building-Block

Compound 3b

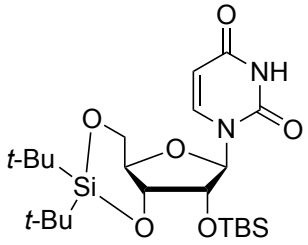


The reaction was conducted according to a published procedure with minor modifications.[1]

L-homoserine (**2**) (0.371 g, 3.12 mmol) was suspended in methanol (25 mL) and heated under reflux until the mixture became clear. Then, a solution of 9-BBN, 9-borabicyclo(3.3.1)nonane (6.7 mL, 3.35 mmol) in tetrahydrofuran (0.5 M) was added dropwise. The reaction mixture was refluxed for 3 hours under inert atmosphere. The reaction mixture was concentrated and the crude product purified by silica gel chromatography eluting with 50% ethyl acetate-hexane to 100% ethyl acetate. The 9-BBN protected L-homoserine (**3b**) was obtained as a white solid (yield 80%); mp 112 – 115 ^{o}C.

^{1}H NMR (400 MHz, DMSO-d_6) δ 0.49 (d, J = 0.5 Hz, 2H), 1.33 – 1.83 (m, 13H), 1.94 – 2.01 (m, 1H), 3.58 – 3.67 (m, 3H), 4.80 (t, J = 4.8 Hz, 1H), 5.86 – 5.91 (m, 1H), 6.41 – 6.46 (m, 1H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 23.9, 24.4, 30.9, 31.3, 33.1, 52.3, 57.9, 174.0; **IR** (ν_{max}) 3425, 3227, 2843, 1721, 1595, 1276; **HRMS** (ESI): calculated for $C_{12}H_{23}BNO_3^+$ $[M + H]^+$: 240.1771; found 240.1764.

Compound 5



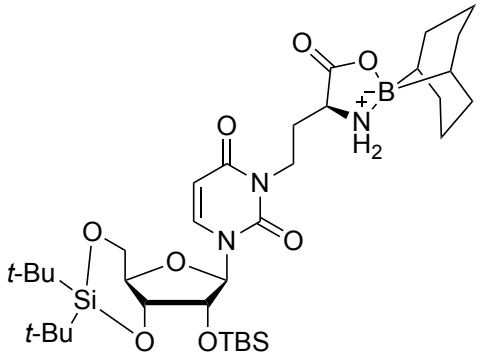
The reaction was conducted according to a published procedure with minor modifications.[2]

Uridine (**4**) (1 g, 4.1 mmol) was dissolved in dry DMF (5 ml) and stirred at 0 ^{o}C. Di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (1.6 ml, 2.2 g, 4.95 mmol, 1.2 eq.) was added dropwise. After 45 min, imidazole (1.4 g, 20.5 mmol, 5.0 eq.) was added and then the reaction was warmed to room temperature over the period of 30 min. Then, di-*tert*-butyldimethylsilyl chloride (0.75 g, 4.95 mmol, 1.2 eq.) was added and the reaction was left to stir overnight. Afterwards the reaction mixture was diluted with ethyl acetate and extracted with saturated $NaHCO_3$ and water. The organic layer was dried over Na_2SO_4 and evaporated. The crude product was then purified by silica gel

chromatography eluting with hexane/ethyl acetate (7/3, v/v) to afford the product **5** as a white solid (yield 86%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.13 (s, 3H), 0.18 (s, 3H), 0.92 (s, 9H), 1.01 (s, 9H), 1.04 (s, 9H), 3.86 (dd, *J* = 9 Hz, *J* = 5 Hz, 1H), 3.97 (d, *J* = 9 Hz, 1H), 4.09 – 4.18 (m, 1H), 4.28 (d, *J* = 5 Hz, 1H), 4.50 (dd, *J* = 9 Hz, *J* = 5 Hz, 1H), 5.65 (s, 1H), 5.74 (d, *J* = 8 Hz, 1H), 7.25 (d, *J* = 8 Hz, 1H), 9.47 (s, 1H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ -4.9, -4.2, 18.4, 20.5, 22.9, 25.9, 27.1, 27.6, 67.7, 74.6, 75.5, 76.2, 94.0, 102.5, 139.5, 149.7, 163.1; **IR** (ν_{max}) 3227, 2920, 2844, 1707, 1597, 1452, 1357, 1259; **HRMS** (ESI): calculated for $C_{23}H_{43}O_6N_2Si_2^+$ $[M + H]^+$: 499.2660; found 499.2657.

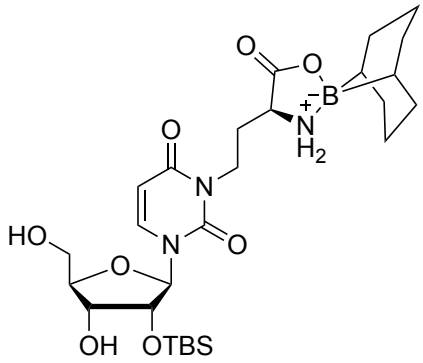
Compound 6



2'-*O*-(*tert*-butyldimethylsilyl)-3'-5'-*O*-(di-*tert*-butylsilylene)-uridine (**5**) (0.38 g, 0.7 mmol) was dissolved in dry tetrahydrofuran (7 mL) under nitrogen. Then 9-BBN protected homoserine (**3b**) (0.22 g, 0.91 mmol, 1.2 eq.) and triphenylphosphine (0.24 g, 0.91 mmol, 1.2 eq.) were added. Afterwards DIAD (0.19 ml, 0.2 g, 0.99 mmol, 1.3 eq.) was added dropwise at 0 °C. The reaction mixture was left to stir for 3 hours at room temperature and then volatiles were removed *in vacuo*. The residue was purified by silica gel chromatography eluting with hexane/ethyl acetate (1/1, v/v) to afford the compound **6** as a white solid (yield 88%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.13 (s, 3H), 0.18 (s, 3H), 0.56 – 0.63 (m, 2H), 0.92 (s, 9H), 1.02 (s, 9H), 1.05 (s, 9H), 1.29 – 1.91 (m, 12H), 1.96 – 2.06 (m, 1H), 2.49 – 2.58 (m, 1H), 3.35 – 3.41 (m, 1H), 3.84 (dd, *J* = 9 Hz, *J* = 5 Hz, 1H), 3.95 – 4.03 (m, 2H), 4.16 – 4.25 (m, 2H), 4.28 (d, *J* = 5 Hz, 1H), 4.39 – 4.4.6 (m, 1H), 4.50 (dd, *J* = 9 Hz, *J* = 5 Hz, 1H), 5.62 (s, 1H), 5.76 – 5.79 (m, 1H), 5.81 (d, *J* = 8 Hz, 1H), 7.33 (d, *J* = 8 Hz, 1H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ -4.9, -4.1, 18.3, 20.5, 22.9, 25.9, 27.1, 27.6, 29.0, 30.9, 31.3, 31.6, 32.2, 53.4, 67.7, 74.8, 75.4, 76.0, 94.8, 101.6, 138.2, 150.7, 163.5, 173.0; **IR** (ν_{max}) 2929, 2858, 1695, 1458, 1266, 1053, 800; **HRMS** (ESI): calculated for $C_{35}H_{63}BN_3O_8Si_2^+$ $[M + H]^+$: 720.4247; found 720.4243.

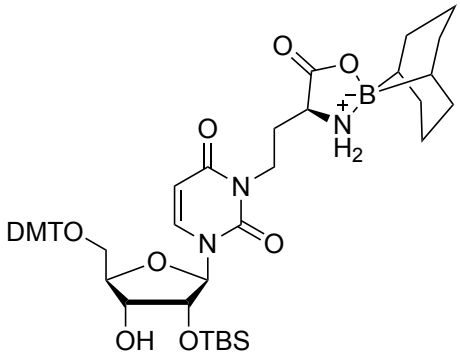
Compound 7



Compound **6** (0.5 g, 0.69 mmol) was dissolved in dry CH_2Cl_2 (7 ml), transferred in a falcon tube and cooled to 0 ^oC. Then pyridine was added (1 ml) followed by addition of HF-Pyridine (0.131 ml). After 1 hour the reaction was quenched with saturated $NaHCO_3$, washed with CH_2Cl_2, dried and evaporated. The residue was purified by silica gel chromatography eluting with CH_2Cl_2/CH_3OH (9/1, v/v) to afford alcohol **7** as a white solid (yield 99%).

^{1}H NMR (400 MHz, Acetone-d_6) δ 0.13 (s, 3H), 0.14 (s, 3H), 0.51 – 0.67 (m, 2H), 0.93 (s, 9H), 1.36 – 1.94 (m, 12H), 1.95 – 2.02 (m, 1H), 2.38 – 2.48 (m, 1H), 3.66 – 3.74 (m, 1H), 3.83 (dd, J = 12 Hz, J = 2.5 Hz, 1H), 3.94 (dd, J = 12 Hz, J = 2.5 Hz, 1H), 4.06 – 4.10 (m, 1H), 4.11 – 4.16 (m, 2H), 4.21 (t, J = 5 Hz, 1H), 4.39 (t, J = 4 Hz, 1H), 5.69 (t, J = 11 Hz, 1H), 5.77 (d, J = 8 Hz, 1H), 5.92 (d, J = 4 Hz, 1H), 6.05 – 6.18 (m, 1H), 8.27 (d, J = 8 Hz, 1H); **^{13}C NMR** (101 MHz, Acetone-d_6) δ -4.7, -4.6, 18.7, 24.9, 25.4, 26.1, 31.9, 32.1, 32.2, 32.6, 38.4, 53.7, 61.2, 70.4, 77.4, 85.4, 91.0, 101.7, 140.2, 152.5, 164.3, 172.9; **IR** (ν_{max}) 3220, 3131, 2924, 2854, 1699, 1662, 1622, 1462, 1260, 1219, 1147, 1098, 966, 864, 837; **HRMS** (ESI): calculated for $C_{27}H_{46}O_8N_3BNaSi^+$ $[M + Na]^+$: 602.3045; found 602.3044.

Compound 8

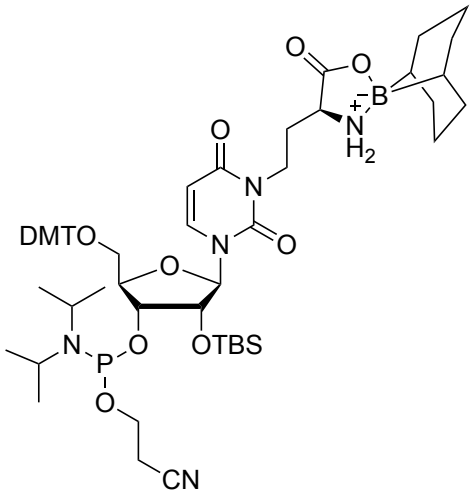


To the solution of compound **7** (0.25 g, 0.34 mmol) in dry pyridine (3 ml) 4,4′-dimethoxytrityl chloride (0.22 g, 0.65 mmol, 1.5 eq.) was added. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. After evaporation of reaction mixture the residue was

dissolved in CH_2Cl_2, washed with saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with CH_2Cl_2/CH_3OH (9/1, v/v) containing 0.1 % of pyridine to afford the product **8** as white foam (yield 71%).

^{1}H NMR (400 MHz, Acetone-d_6) δ 0.16 (s, 3H), 0.20 (s, 3H), 0.51 – 0.63 (m, 2H), 0.95 (s, 9H), 1.42 – 2.01 (m, 13H), 2.38 – 2.47 (m, 1H), 3.55 (dd, *J* = 11 Hz, *J* = 3 Hz, 1H), 3.47 (dd, *J* = 11 Hz, *J* = 3 Hz, 1H), 3.68 – 3.77 (m, 1H), 3.80 (s, 6H), 4.10 – 4.20 (m, 3H), 4.41 – 4.4.6 (m, 2H), 5.39 (d, *J* = 8 Hz, 1H), 5.69 (t, *J* = 11 Hz, 1H), 5.86 (d, *J* = 2 Hz, 1H), 6.05 – 6.18 (m, 1H), 6.91 (d, *J* = 8.9 Hz, 4H), 7.26 – 7.49 (m, 9H), 8.07 (d, *J* = 8 Hz, 1H); **^{13}C NMR** (101 MHz, Acetone-d_6) δ -4.54, -4.5, 18.7, 22.2, 24.9, 25.5, 26.2, 31.9, 32.0, 32.2, 32.6, 38.4, 53.6, 55.5, 62.8, 69.1, 70.4, 77.4, 83.5, 87.5, 91.6, 101.6, 114.0, 126.1, 127.8, 128.7, 128.9, 129.7, 131.0, 136.1, 136.5, 139.8, 145.7, 152.4, 157.2, 159.7, 164.1, 172.9; **IR** (ν_{max}) 2925, 2855, 1705, 1652, 1607, 1508, 1458, 1301, 1249, 1219, 1176, 1110, 833; **HRMS** (ESI): calculated for $C_{48}H_{64}O_{10}N_3BNaSi^+$ $[M + Na]^+$: 904.4352; found 904.4333.

Compound 1-PA



The DMT-protected nucleoside **8** (0.1 g, 0.11 mmol) was co-evaporated with pyridine and dried under high vacuum overnight. It was further dissolved in anhydrous CH_2Cl_2 (3 ml) and cooled to 0 °C. Then DIPEA (0.08 mL, 0.44 mmol, 4 eq.) and 2-cyanoethyl-*N*,*N*,-diisopropyl chlorophosphoramidite **(**0.063 mL, 0.28 mmol, 2.5 eq.) were added under nitrogen atmosphere. The reaction mixture was brought to room temperature and stirred for 3 hours. The reaction mixture was quenched by the addition of saturated $NaHCO_3$ solution and extracted with CH_2Cl_2. The combined organic extract was dried over anhydrous $NaSO_4$, filtered, concentrated *in vacuo*, and the residue was purified by silica gel column chromatography eluting with hexane/ethyl acetate (2/1, v/v, HPLC

grade solvents) containing 0.1 % of pyridine to afford phosphoramidite **1-PA** as a white solid after lyophilization from benzene (yield of the mixture of diastereomers 89%).

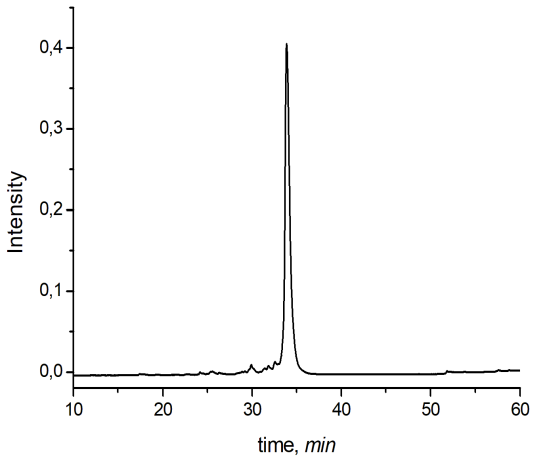
Diastereomers can be separated during column chromatography; ^{1}H and ^{13}C spectra are given of one of the isomers. **^{1}H NMR** (400 MHz, Acetone-d_6) δ 0.22 (s, 3H), 0.24 (s, 3H), 0.60 – 0.62 (br s, 2H), 0.95 (s, 9H), 1.07 (d, J = 6.8 Hz, 6H), 1.18 (d, J = 6.8 Hz, 6H), 1.43 – 1.97 (m, 12H), 1.98 – 2.01 (m, 1H), 2.38 – 2.47 (m, 1H), 2.71 – 2.79 (m, 2H), 3.51 (dd, J = 11, J = 3 Hz, 1H), 3.62 (dd, J = 11 Hz, J = 3 Hz, 1H), 3.60 – 3.73 (m, 2H), 3.80 (s, 6H), 3.82 – 3.86 (m, 1H), 3.94 – 4.16 (m, 3H), 4.29 – 4.32 (m, 1H), 4.45 – 4.51 (m, 1H), 4.57 (t, J = 4 Hz, 1H), 5.26 (d, J = 8 Hz, 1H), 5.60 – 5.66 (m, 1H), 5.85 (d, J = 3 Hz, 1H), 6.04 – 6.10 (m, 1H), 6.89 – 6.92 (m, 4H), 7.26 – 7.36 (m, 7H), 7.45 – 7.49 (m, 2H), 8.05 (d, J = 8 Hz, 1H); **^{13}C NMR** (101 MHz, Acetone-d_6) δ -4.4, -4.2, 18.6, 21.1, 21.2, 24.8, 24.9, 25.0, 25.1, 25.5, 26.3, 32.0, 32.1, 32.2, 32.6, 38.3, 43.7, 43.8, 53.6, 55.5, 59.0, 59.2, 62.4, 72.5, 72.7, 76.5, 82.7, 82.8, 87.7, 91.1, 101.7, 114.0, 119.5, 127.9, 128.8, 129.1, 131.2, 135.9, 136.1, 139.6, 145.4, 152.1, 159.8, 164.4, 172.9; **^{31}P NMR** (162 MHz, Acetone-d_6) δ 150.1, 148.8; **IR** (ν_{max}) 2927, 2853, 1703, 1654, 1608, 1508, 1459, 1363, 1302, 1252, 1178, 834, 809; **HRMS** (ESI): calculated for $C_{57}H_{82}BN_5O_{11}PSi^+$ $[M+H]^+$: 1082.5611; found 1082.5603.

3. Synthesis and Purification of Oligonucleotides

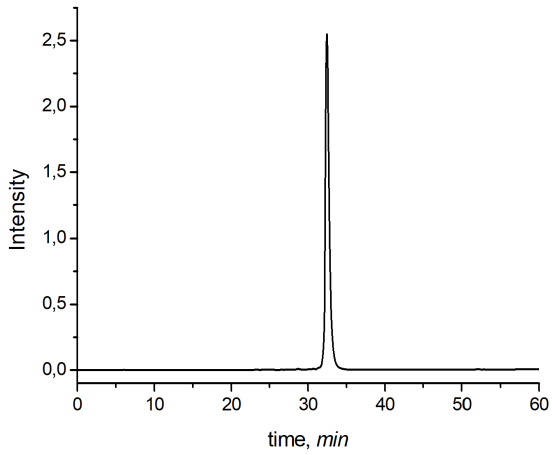
Sequence: 5' CAUGacp^{3}UUGCA 3' (ODN1)
Sequence: 5' GACUGACacp^{3}UCGUAGCacp^{3}UAACUCAU 3' (ODN4)

All of the oligonucleotides used in this study were synthesized on a 1 μmol scale using a DNA automated synthesizer (Applied Biosystems 394 DNA/RNA Synthesizer) with standard phosphoramidite chemistry. The phosphoramidites of canonical ribonucleosides were purchased from Glen Research and Sigma-Aldrich. Oligonucleotide containing acp^{3}U nucleoside was synthesized in DMT-OFF mode using phosphoramidites (Bz-A, Dmf-G, Ac-C, U) with BTT in CH_3CN as an activator, DCA in CH_2Cl_2 as a deblocking solution and Ac_2O in pyridine/THF as a capping reagent. The cleavage and deprotection of the CPG bound oligonucleotides were performed with aqueous $NH_4OH/MeNH_2$ (1/1, v/v, 1 mL) at room temperature for 1 h. The resin was removed by filtration and the solution was evaporated at under reduced pressure. The residue was subsequently heated with a solution of triethylamine trihydrofluoride (125 μL) in DMSO (50 μL) at 65 °C for 1.5 h. Upon cooling on ice bath, NaOAc (3.0 M, 25 μL) and n-BuOH (1 mL) were added. The resulting suspension was vortexed and cooled in a freezer (-80 °C) for 1 h. After the centrifugation, supernatant was removed and the remaining oligonucleotide pellet was dried under vacuum. The oligonucleotides were further purified by reverse-phase HPLC using a Waters Breeze (2487 Dual λ Array Detector, 1525 Binary HPLC Pump) equipped with the column VP 250/32 C18 from Macherey Nagel. Oligonucleotides were purified using the following buffer system: buffer A: 100 mM NEt_3/HOAc, pH 7.0 in H_2O and buffer B: 100 mM NEt_3/HOAc in 80 % (v/v) acetonitrile. A flow rate of 5 mL/min with a gradient of 0-25 % of buffer B in 30 min was applied for the purifications. Analytical RP-HPLC was performed on an analytical HPLC Waters Alliance (2695 Separation Module, 2996 Photodiode Array Detector) equipped with the column Nucleosil 120-2 C18 from Macherey Nagel using a flow of 0.5 mL/min, a gradient of 0-30% of buffer B in 45 min was applied. Calculation of concentrations was assisted using the software OligoAnalyzer 3.0 (Integrated DNA Technologies: https://eu.idtdna.com/calc/analyzer). For strands containing non-canonical base, the extinction coefficient of their corresponding canonical-only strand was employed without corrections. The structural integrity of the synthesized oligonucleotides was analyzed by MALDI-TOF mass measurement.

a)



b)



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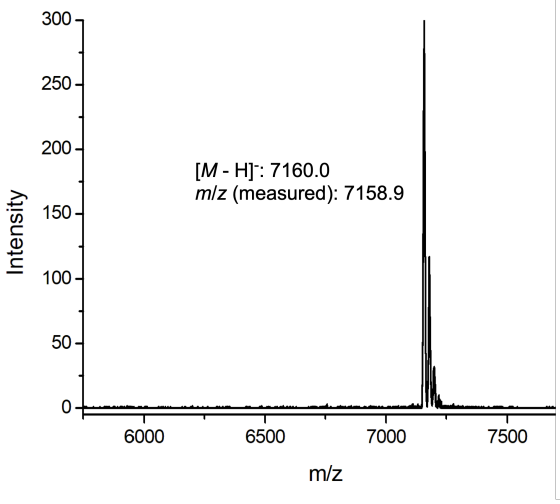

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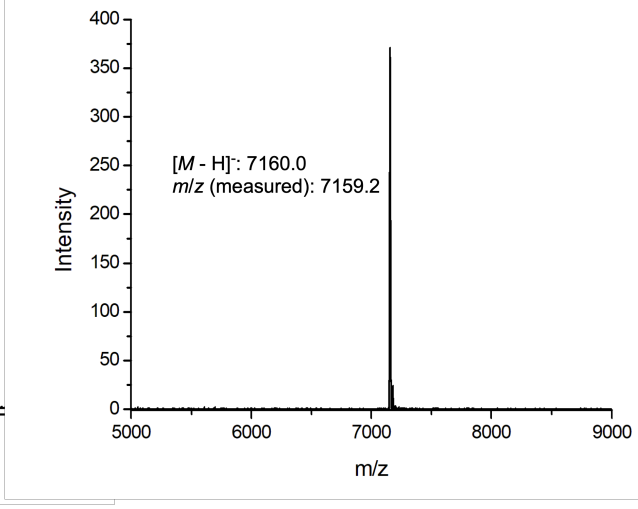


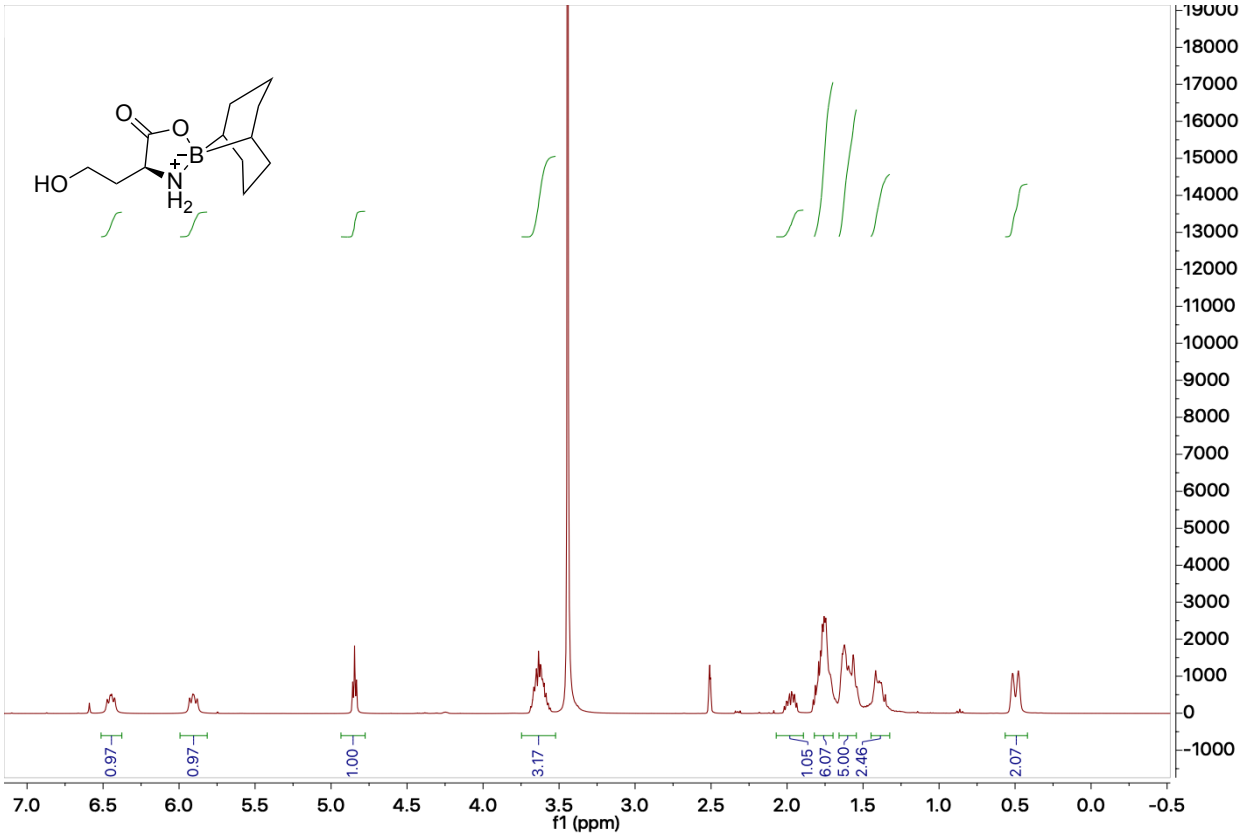
Figure S1. (a) raw-HPL chromatogram of **ODN4**; (b) HPL chromatogram of purified **ODN4**; (c) MALDI-TOF mass spectrum of raw **ODN4**; (d) MALDI-TOF mass spectrum of purified **ODN4**.

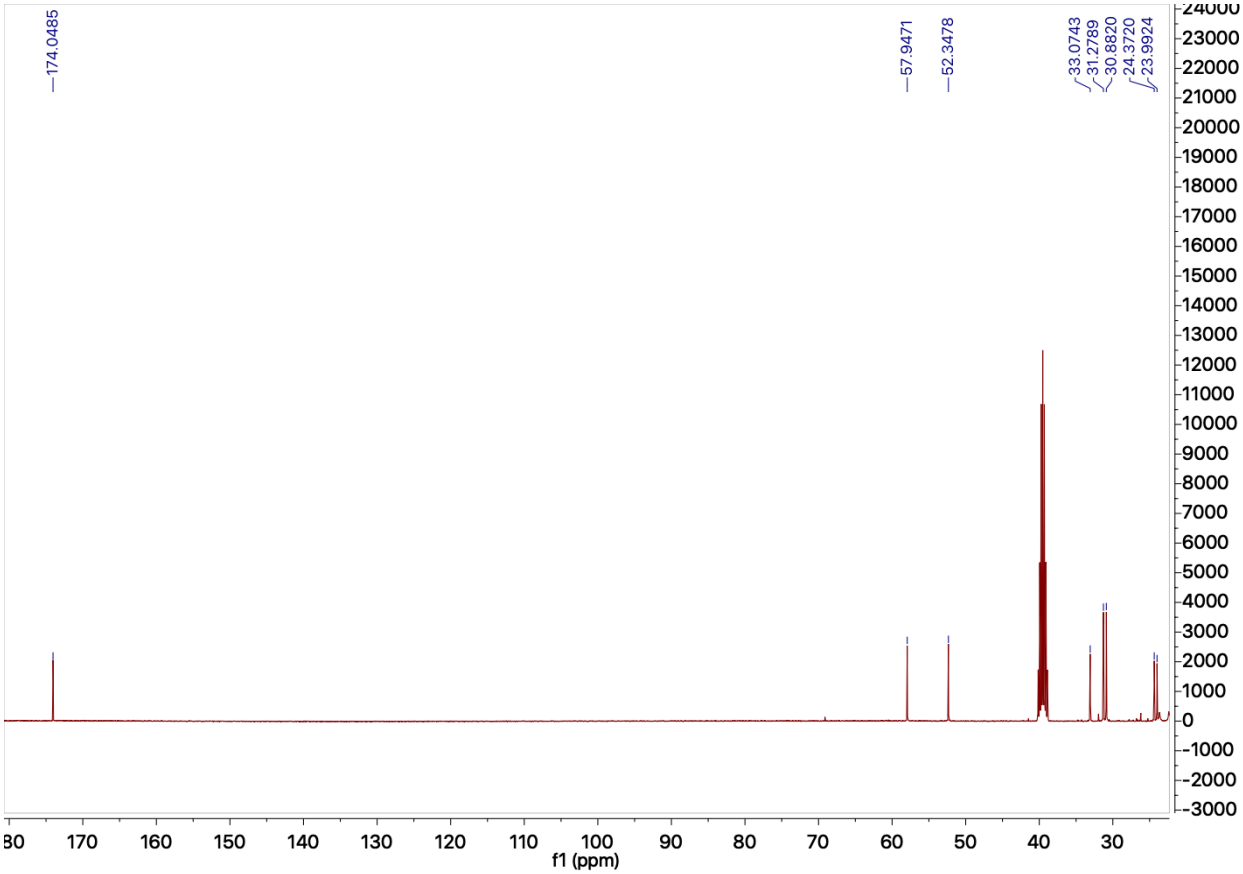
4. UV Melting Curve Measurements

The UV melting curves were measured on JASCO V-650 spectrometer using 10 mm QS cuvettes, purchased from Hellma Analytics. A solution (80 μL) of equimolar amounts of oligonucleotides (4 μM each) in the buffer solution containing 10 mM sodium phosphate buffer (pH 7.0) and 150 mM NaCl was heated at 50 °C for 5 min and gradually cooled to 4 °C prior to the measurement. Melting profiles were recorded at temperatures between 5 and 75 °C with a ramping and scanning rate of 1 °C/min at 260 nm. All samples were measured at least three times. T_m values from each measurement were calculated using the "fitting curve" method and presented as an average of three independent measurements.

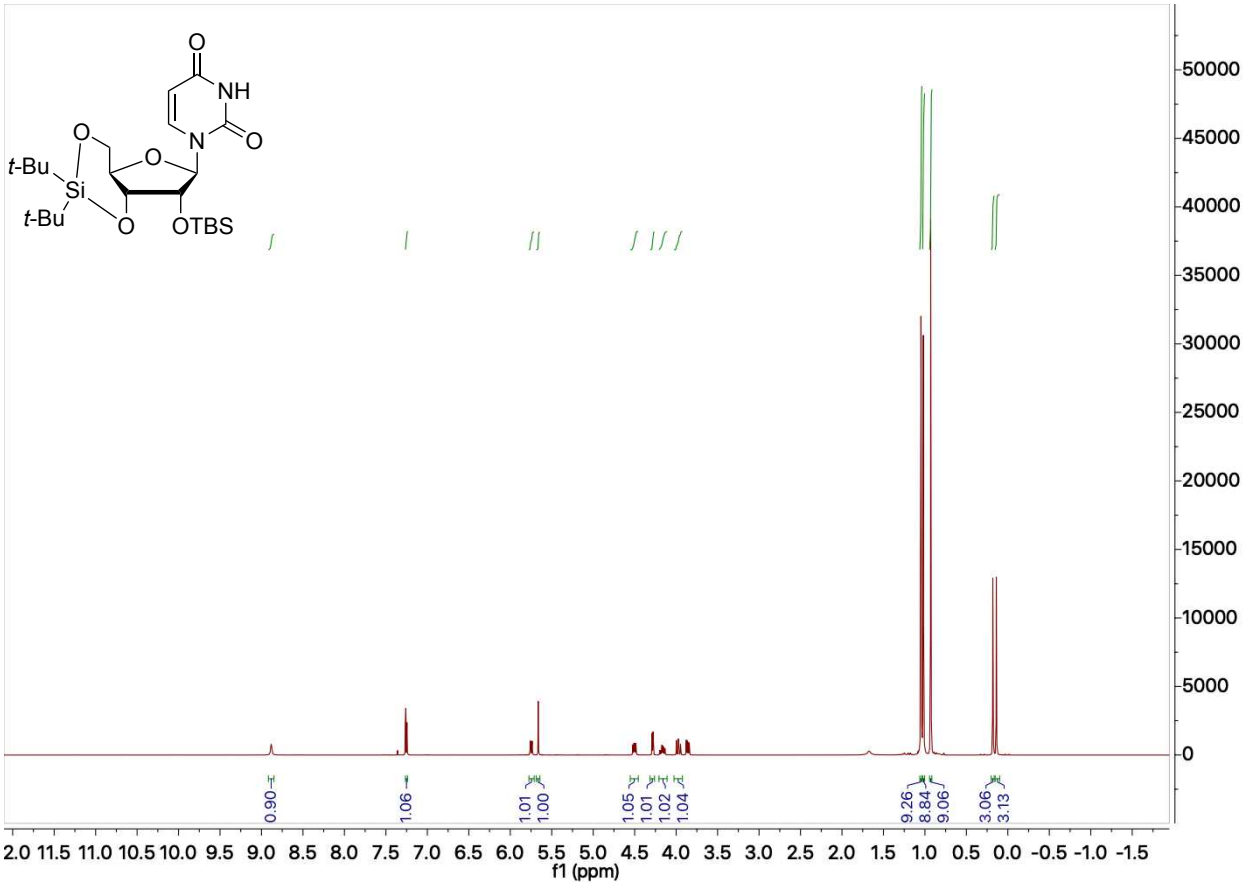
5. NMR Spectra of Synthesized Compounds

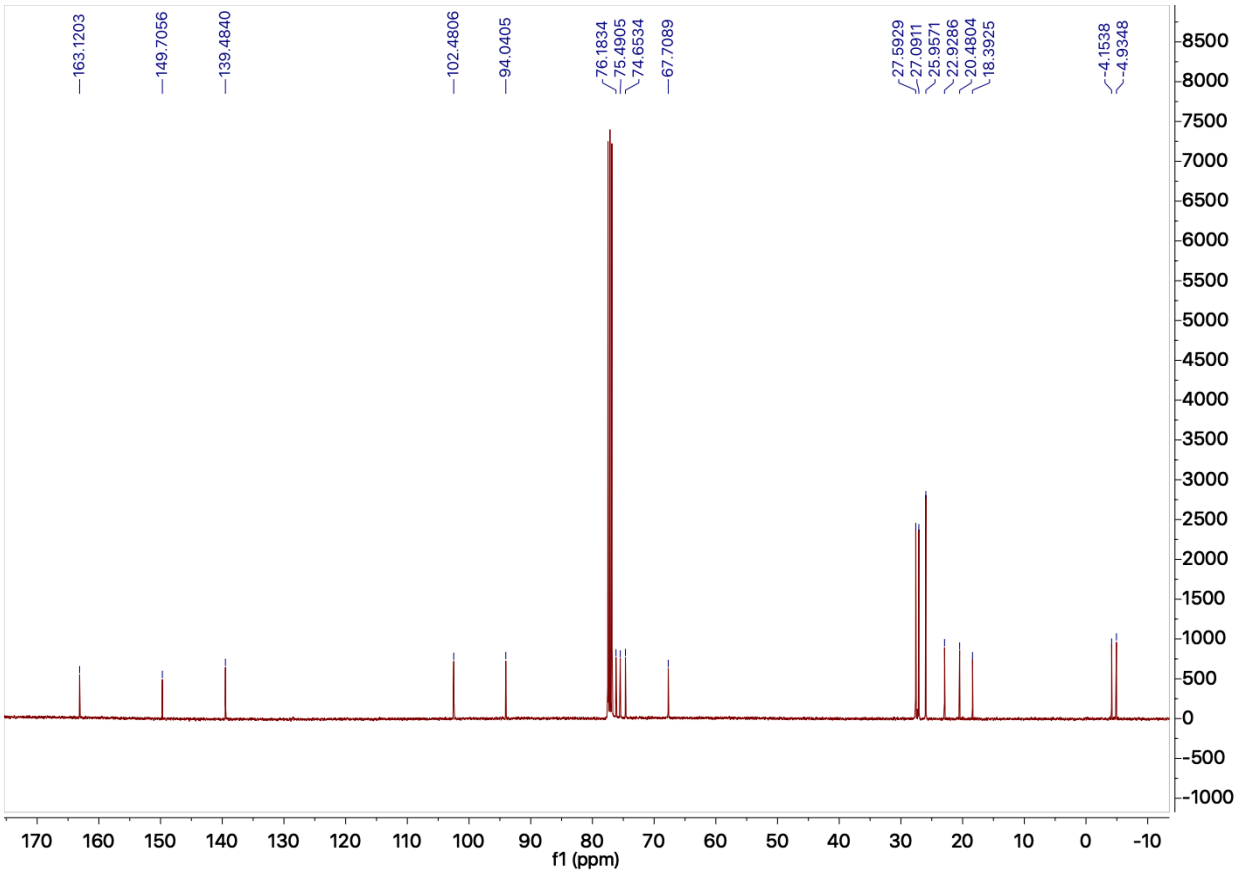
^{1}H NMR and ^{13}C NMR of compound **3b**



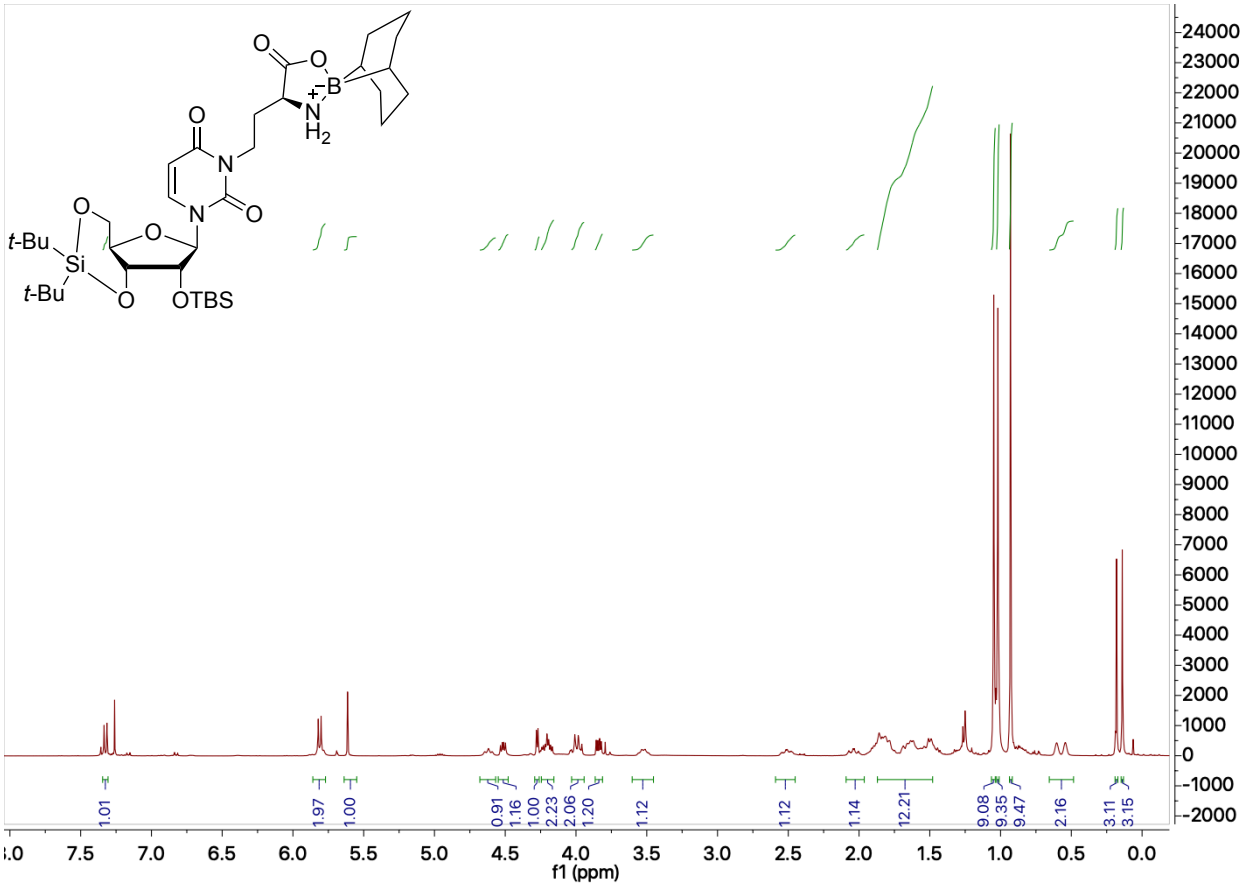


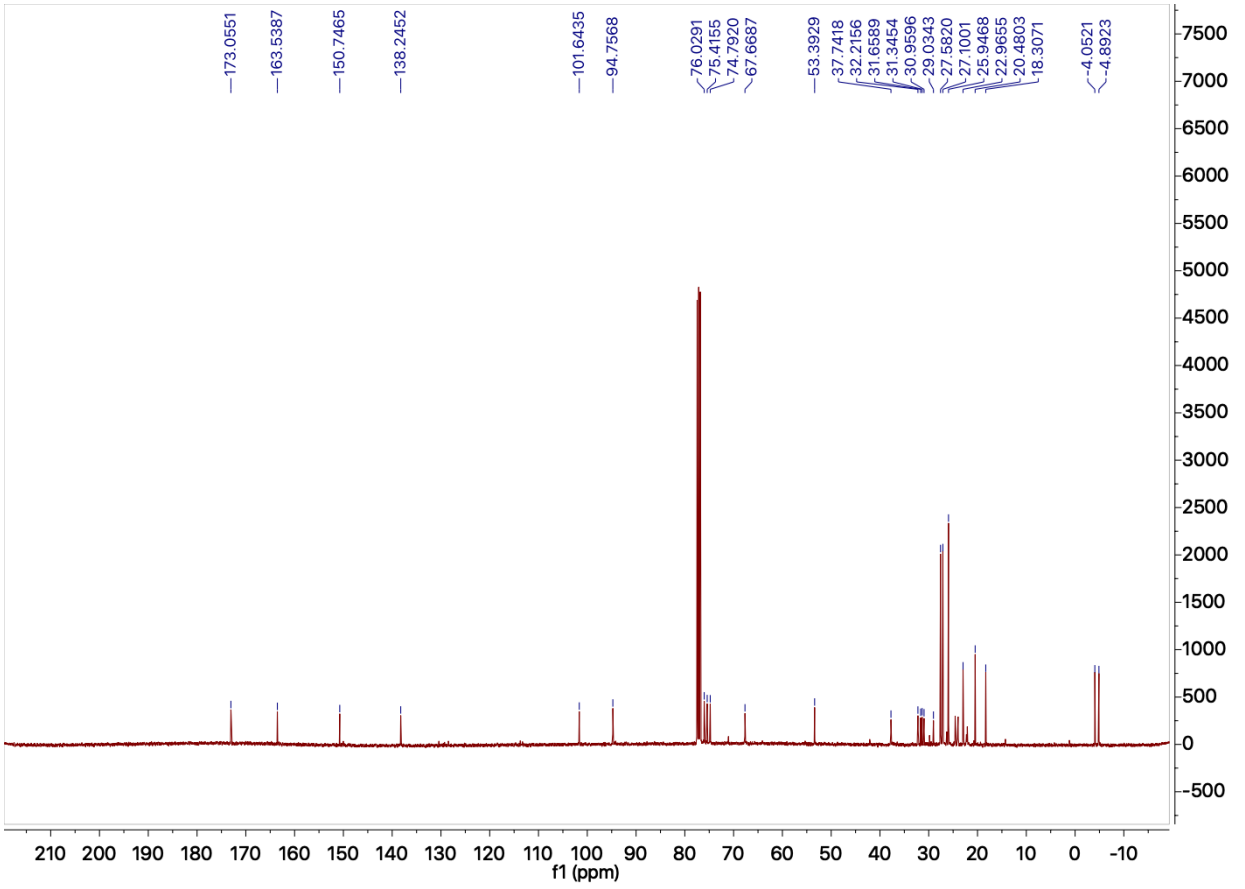
^{1}H NMR and ^{13}C NMR of compound **5**



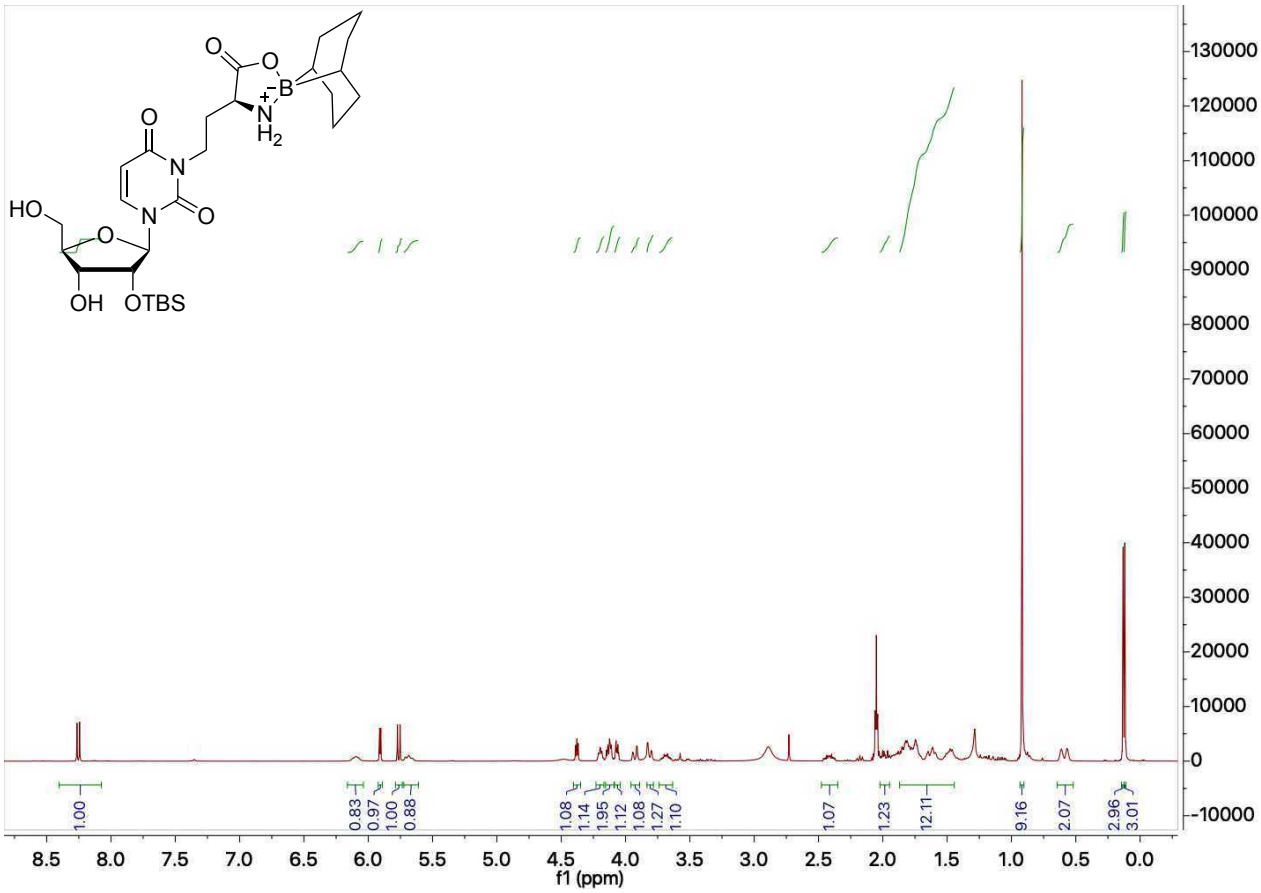


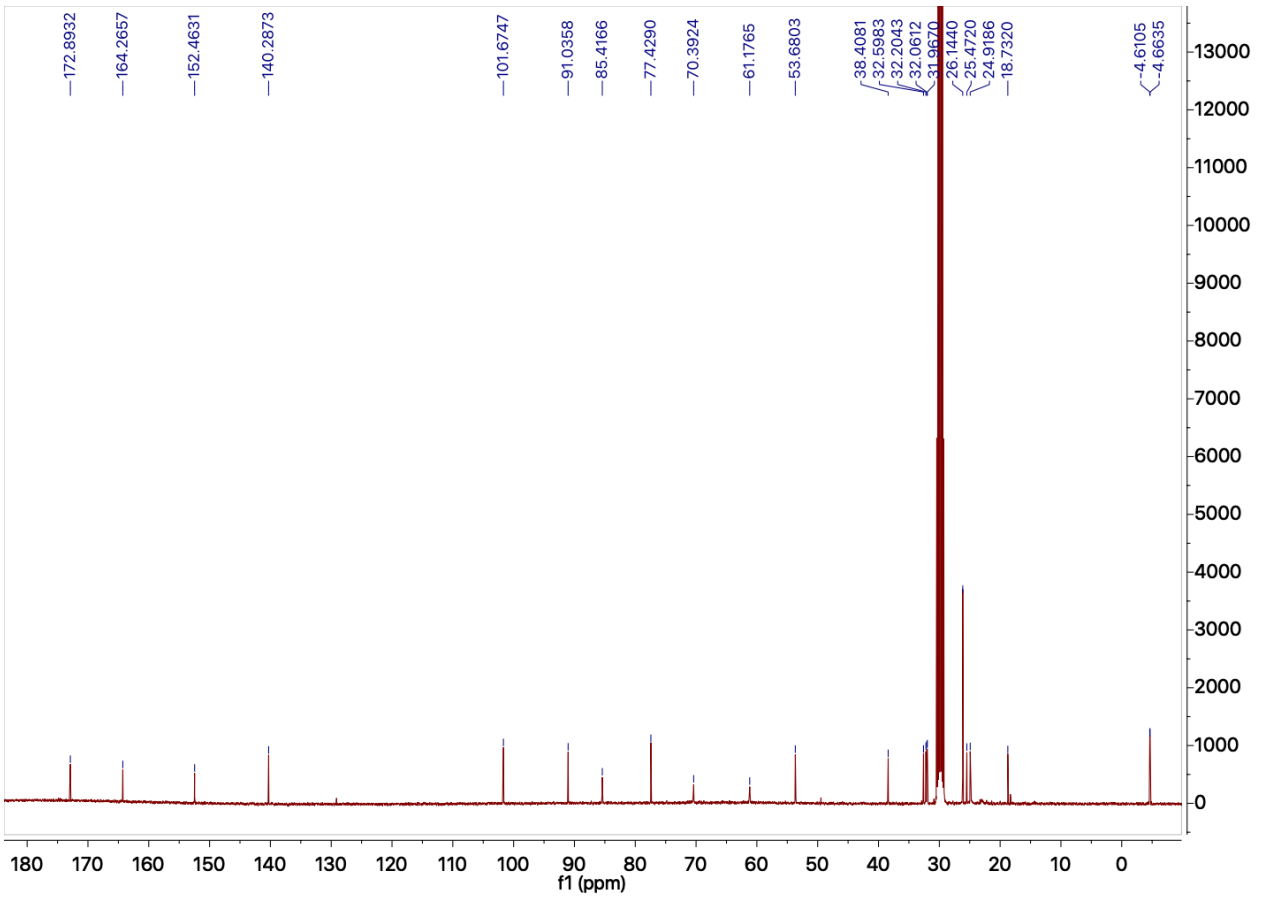
^{1}H NMR and ^{13}C NMR of compound **6**



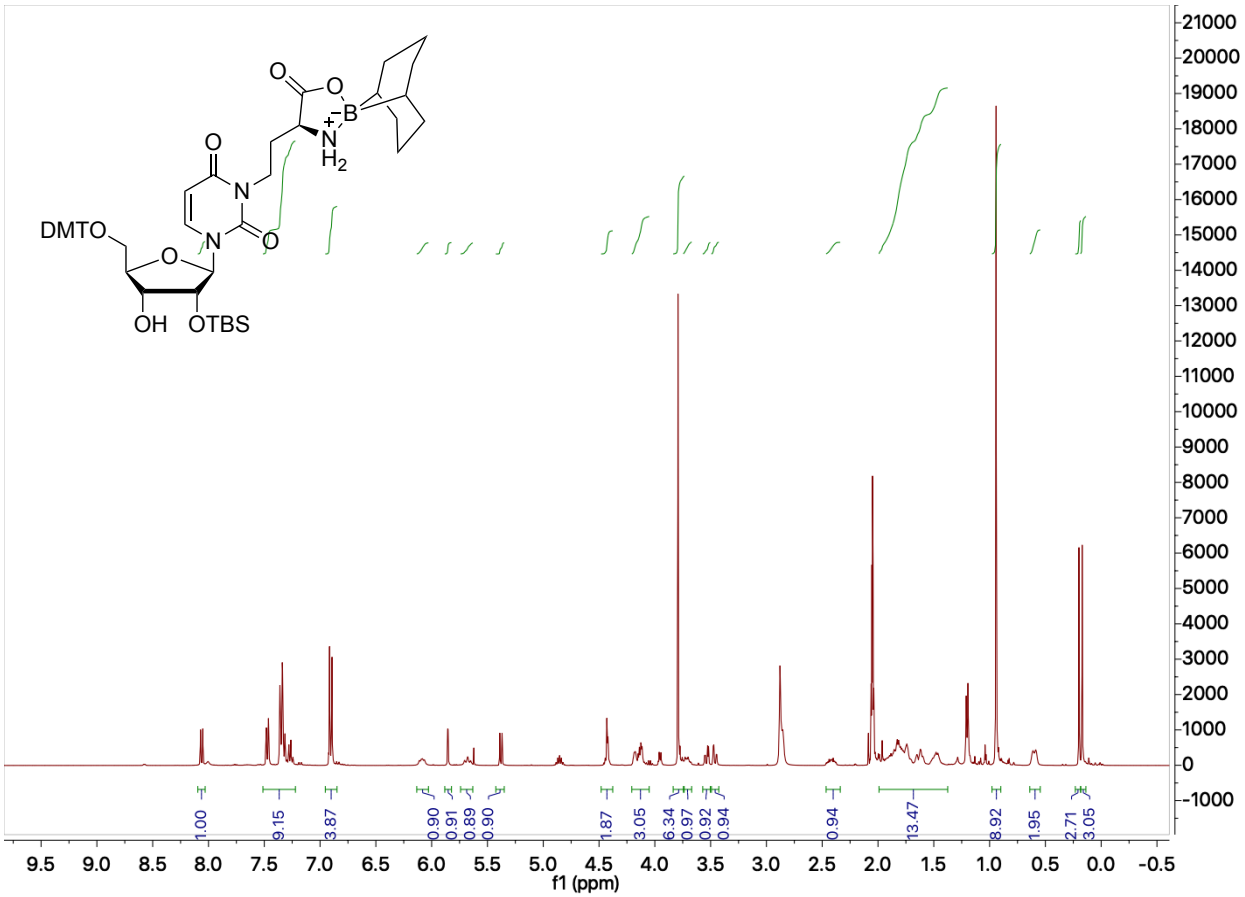


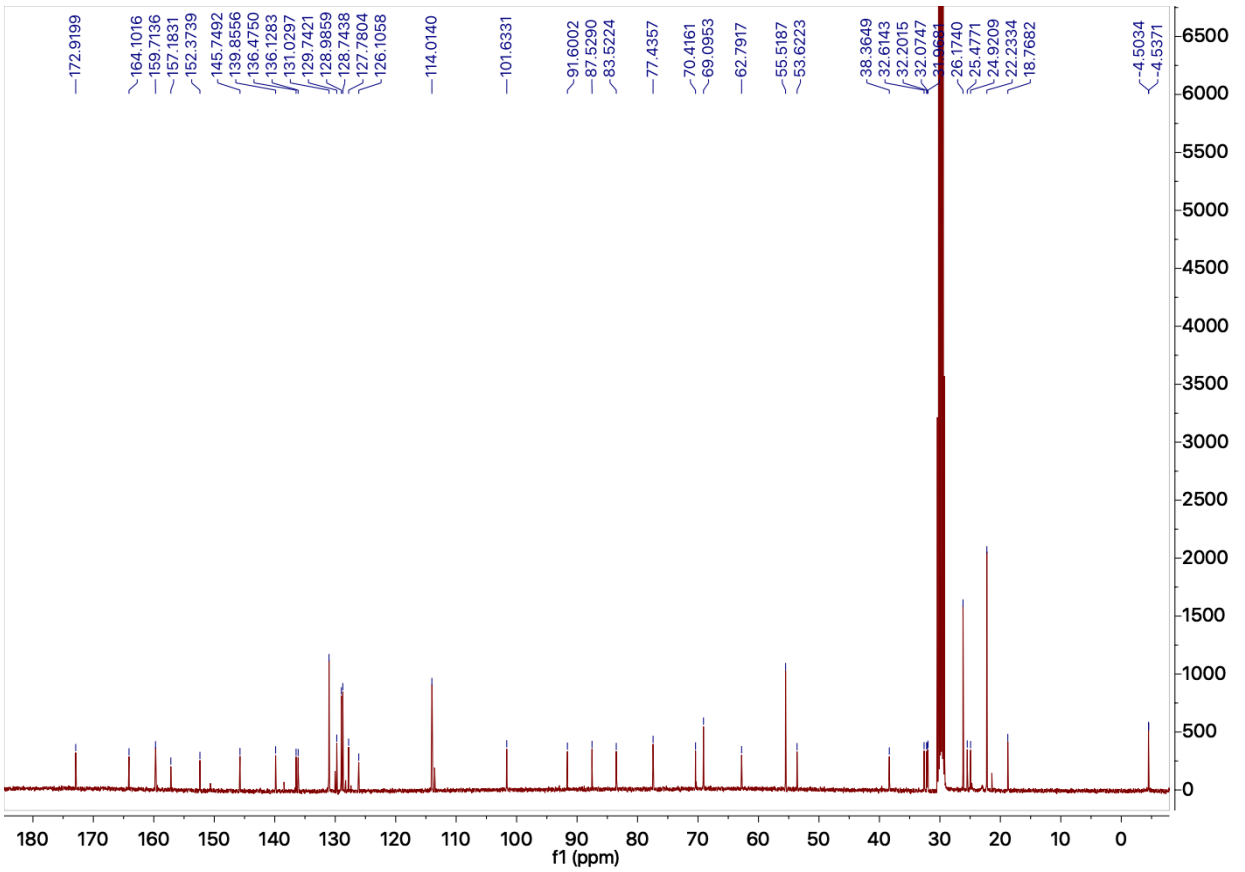
^{1}H NMR and ^{13}C NMR of compound **7**



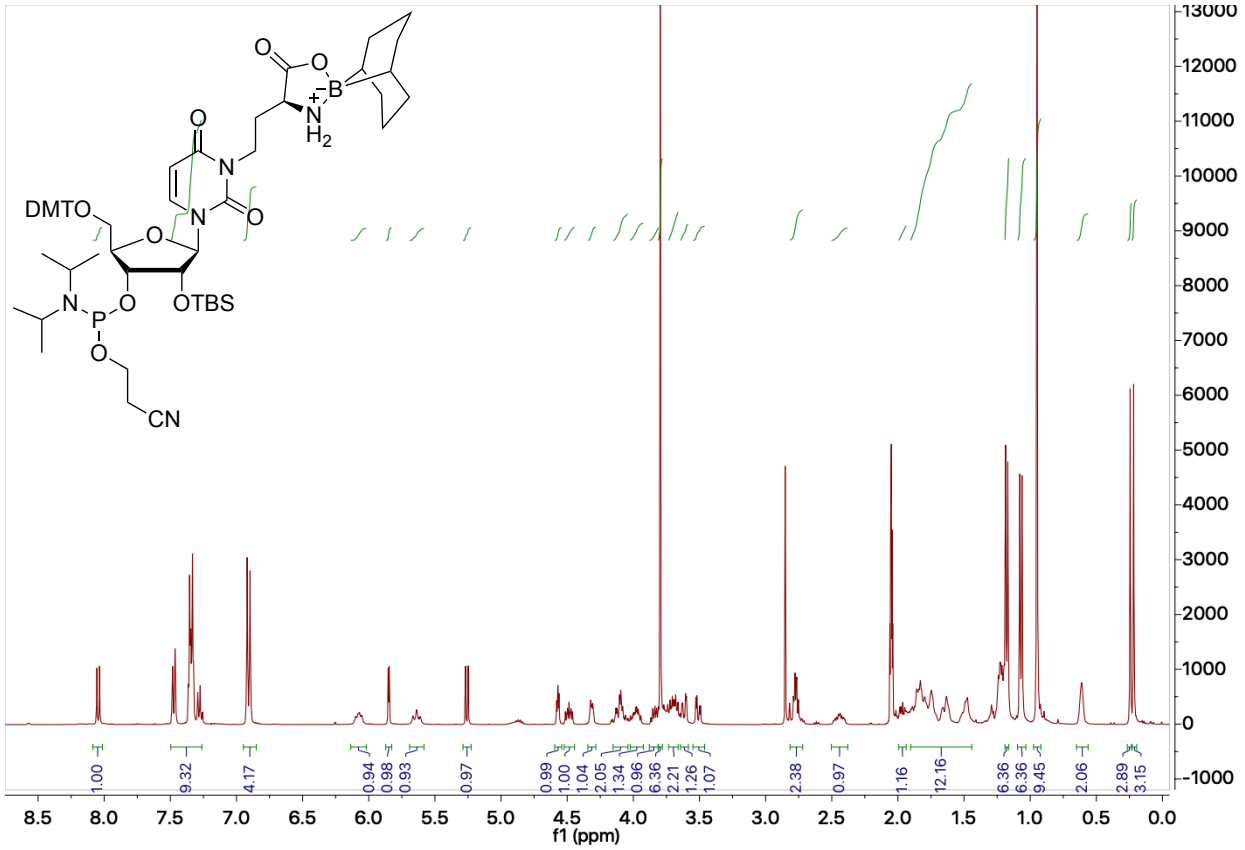


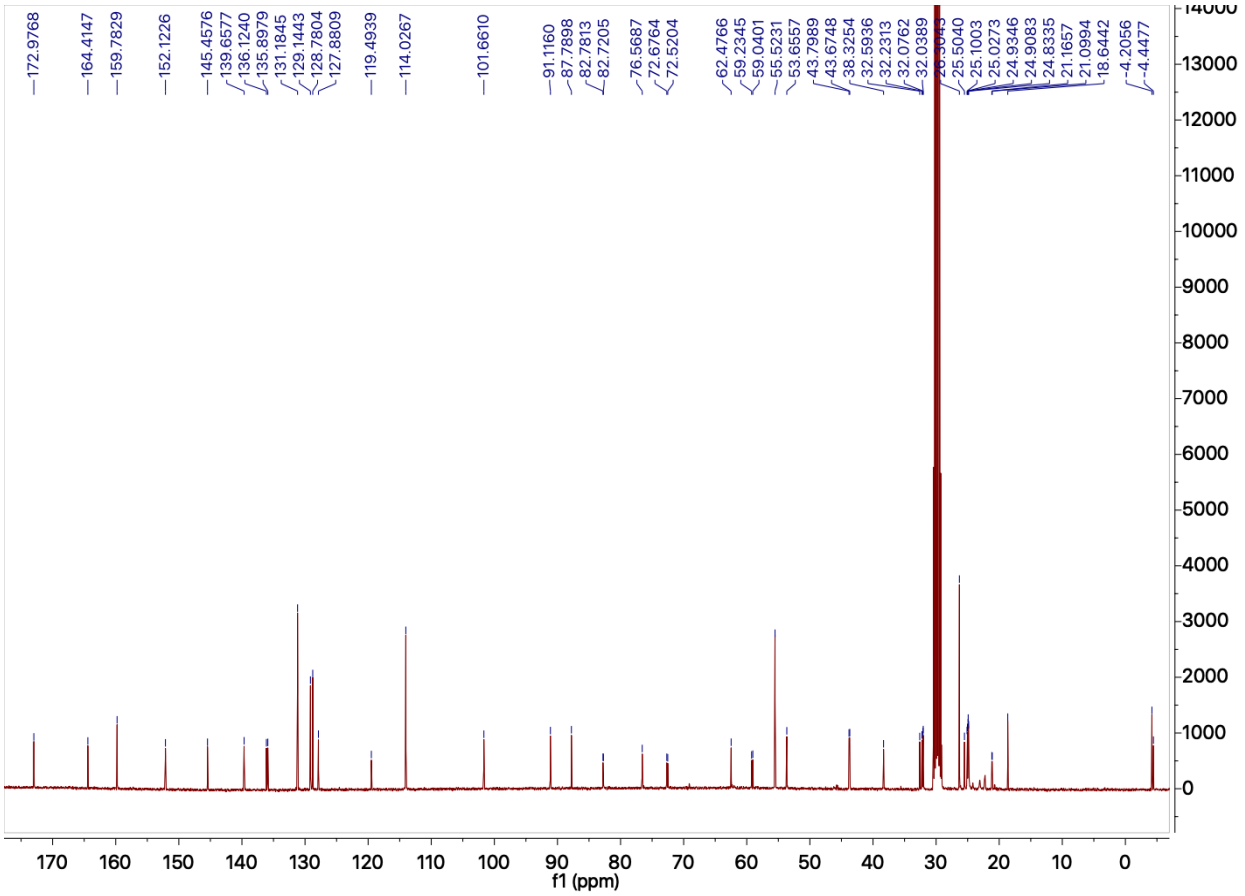
^{1}H NMR and ^{13}C NMR of compound **8**

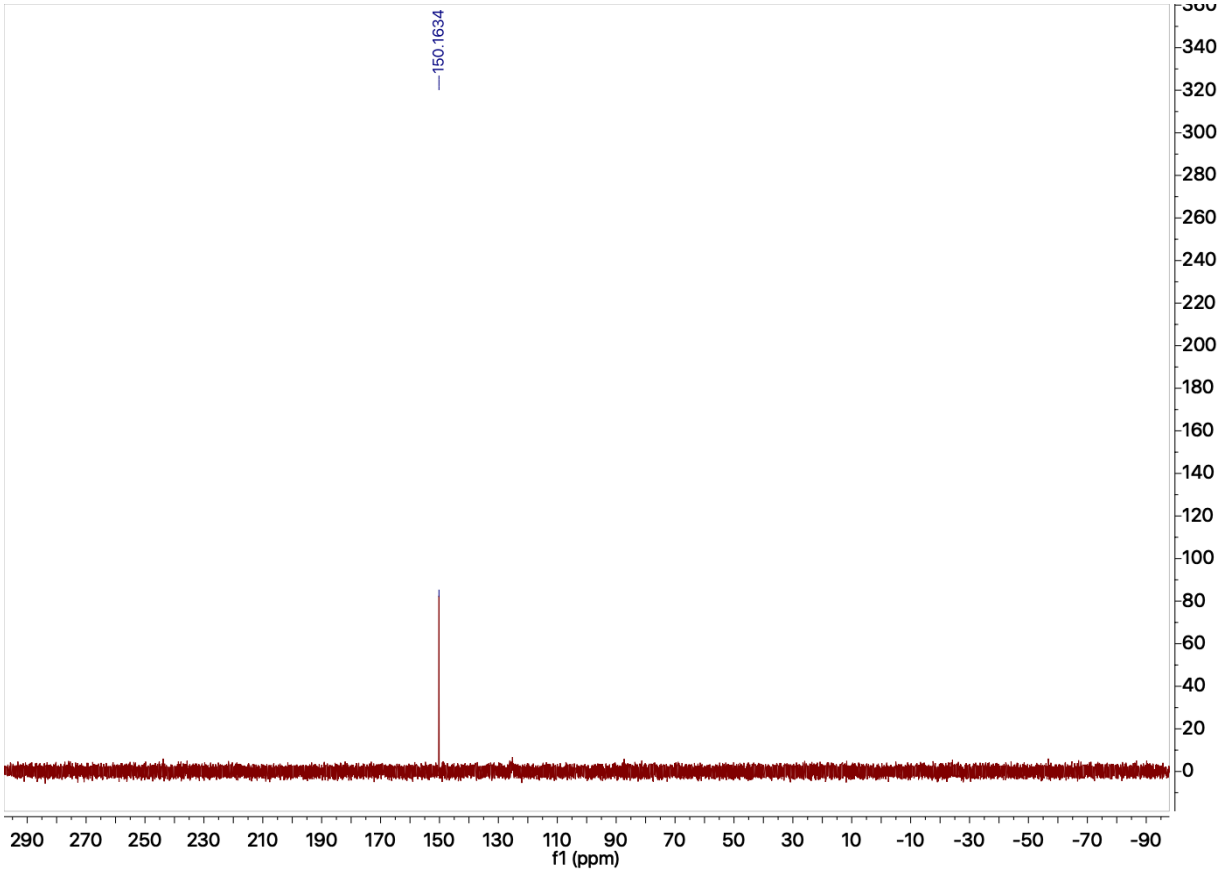




^{1}H NMR, ^{13}C NMR and ^{31}P NMR of compound **1-PA**



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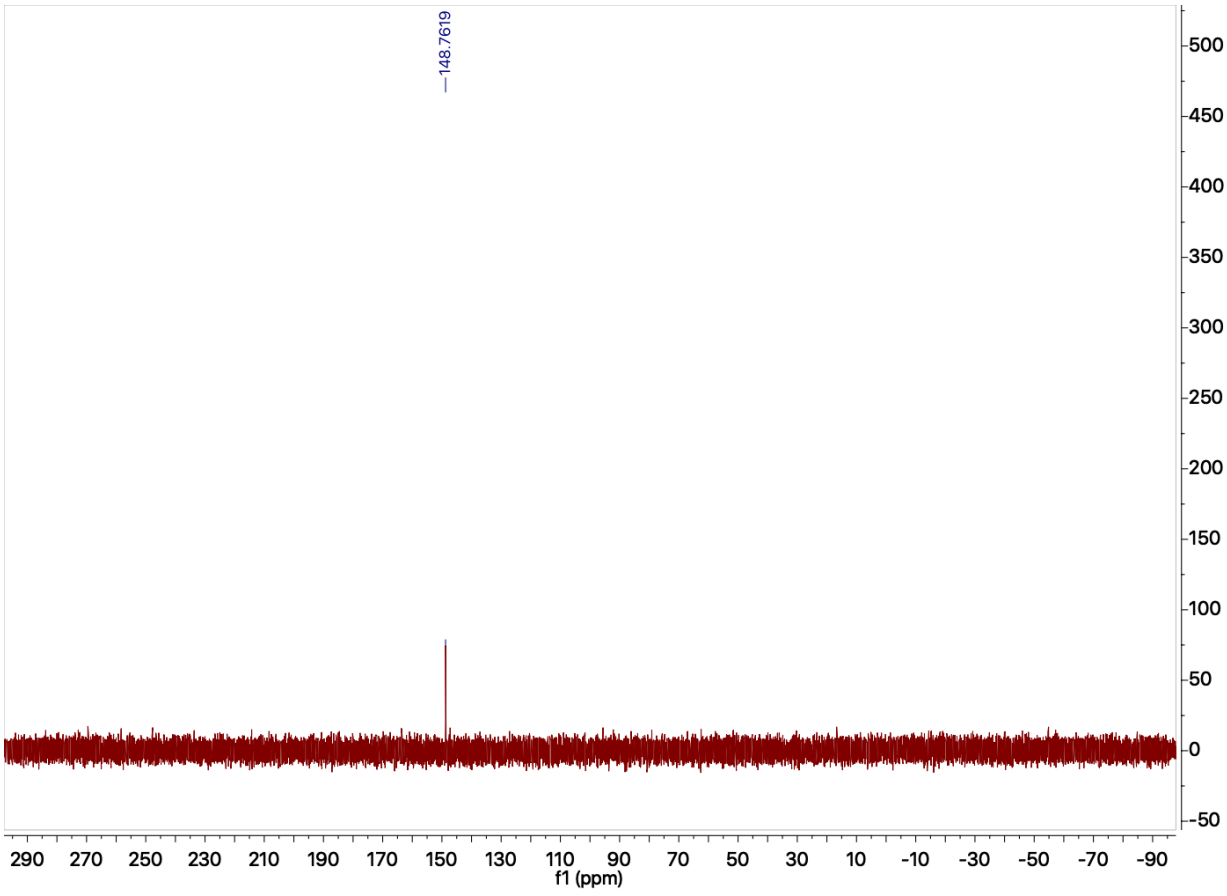
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
290 270 250 230 210 190 170 150 130 110 90 70 50 30 10 -10 -30 -50 -70 -90
f1 (ppm)


148.7619
500
450
400
350
300
250
200
150
100
50
0
-50
290 270 250 230 210 190 170 150 130 110 90 70 50 30 10 -10 -30 -50 -70 -90
f1 (ppm)

6. References

1. H. Taskent-Sezgin, J. Chung, P. S. Banerjee, S. Nagarajan, R. B. Dyer, I. Carrico, D. P. Raleigh, *Angew. Chem. Int. Ed*., 2010, **49**, 7473–7475.
2. C. Riml, T. Amort, D. Rieder, C. Gasser, A. Lusser and R. Micura, *Angew. Chem., Int. Ed*., 2017, **56**, 13479–13483.