

Synthesis of an acp³U phosphoramidite and incorporation of the hypermodified base into RNA

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The article was first published online 17.09.2019 in Chemical Communications, doi: 10.1039/C9CC06314E

Print version: **Chem. Commun.**, 2019, **55**, 12216-12218

acp³U is a hypermodified base that is found in the tRNAs of prokaryotes and eukaryotes and also in ribosomal RNA of mammals. The function is so far unknown but it is speculated that acp³U complexes Mg ions, which may contribute to the stabilization of the RNA structure. As a hypermodified base in which a nucleoside is covalently connected to an amino acid, acp³U is a natural nucleoside between genotype and phenotype and hence also of particular importance for theories about the origin of life. Herein, we report the development of a phosphoramidite building block and of a solid phase protocol that allows to synthesize RNA containing acp³U.

RNA contains a vast majority of modified bases.¹ Until recently it was believed that such bases are predominantly present in transfer-RNAs (tRNAs) and ribosomal-RNAs (rRNA), but with the discovery that also messenger-RNAs (mRNAs) contain modified bases the whole field has regained enormous attention.²⁻⁵ Modified bases can be divided into two groups. Most modified nucleosides (first group) deviate from the canonical bases by just small structural changes such as methylation(s) or exchange of oxygen by sulfur atoms. Other modified bases can be categorized into the group of hypermodified bases, which feature large structural changes compared to the canonical bases.⁶ The biosynthesis of the bases in this group requires complex and often not completely understood biosynthesis machinery.

Related to the question of the origin of life, those hypermodified bases, which are modified with amino acids, are of particular interest.⁷ These nucleosides feature properties of the genotype (information encoding potential) and the phenotype in a sense that they are in principle able to catalyse chemical transformations similar to the amino acids present in proteins. Indeed, hypermodified bases, which are “charged” with an amino acid, such as acp³U, hn/g/t⁶A, tm⁵s²U, k²C and agm²C, were suggested to be relics of an ancient code (Fig. 1).⁸⁻¹⁰ As such they could have been part of an early translational system, within the concept of a prebiotic RNA-peptide world.

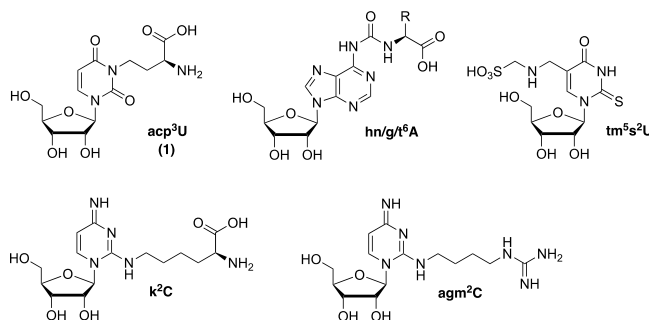
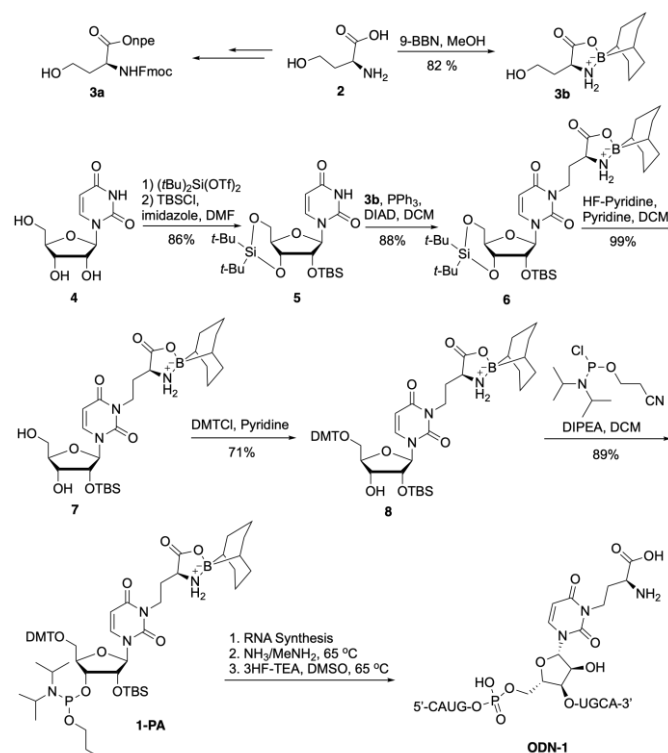


Fig. 1. Depiction of hypermodified bases that are charged with an amino acid.

In order to investigate the properties of the hypermodified, amino acid charged nucleosides, it is essential to have phosphoramidite building blocks and solid phase synthesis procedures for their incorporation into RNA. Here we report the development of a phosphoramidite building block for acp³U (Fig. 1) together with an efficient solid phase RNA synthesis protocol that allows preparing RNA containing this hypermodified base acp³U (**1**).

For the synthesis of the phosphoramidite building block of **1** (**1-PA**), we first prepared the properly protected L-homoserine **2**. The design of appropriate protecting group strategy for L-homoserine **2** was guided by the need to have sufficient stability during RNA solid phase oligonucleotide synthesis. Also, the protecting groups need to be easily removable after the synthesis. Importantly this has to be possible without racemization of the L-amino acid. Based on previous studies regarding the compatibility of protecting groups with solid phase oligonucleotide synthesis, we initially selected the 9-fluoromethoxycarbonyl (Fmoc)^{11,12} and 4-nitrophenylethyl (npe)¹³ for deeper investigation. *N*-Fmoc protection was successfully achieved, while attempts to form the ester

3a (Scheme 1) lead to the formation of a stable lactone.¹⁴ Although in principle, it was possible to obtain compound **3a** via blocking of the γ -OH group, experiments in parallel to protect the amino- and carboxy group simultaneously utilising 9-BBN gave better yields and allowed to avoid lactonization. Stirring of **2** in MeOH under reflux with 9-BBN for 4h provided the protected L-homoserine building block **3b** in 82% yield.¹⁵ In parallel, uridine **4** was first 5'-3'-silylprotected followed by protection of the still free 2'-OH with TBS-Cl using imidazole as the base.¹⁶⁻¹⁸ The fully protected uridine building block **5** was obtained in 86% yield. We next connected the protected L-homoserine unit **3b** with **5** to afford **6** (88% yield) using a Mitsunobu reaction with Ph_3P and DIAD in dichloromethane.¹⁹ Subsequent deprotection of the cyclic 5'-3' protecting group with HF in pyridine furnished compound **7** (99% yield) which was then converted into the 5'-DMT protected acp³U compound **8**.²⁰⁻²³ Reaction of **8** with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite²⁴ gave finally the target compound **1-PA** in excellent 89% yield.



Scheme 1. Synthesis of the acp³U phosphoramidite (**1-PA**) with a 9-BBN protecting group and incorporation of **1-PA** into the oligonucleotide **ODN1**.

We next investigated the incorporation of **1-PA** using standard RNA solid phase oligonucleotide synthesis.²⁵⁻³¹ The main question was how the 9-BBN group would behave during the complex protocol. To the best of our knowledge, the 9-BBN protecting group was never used before in solid phase oligonucleotide chemistry. We used a standard RNA synthesis protocol and were surprised that **1-PA** could be incorporated into strands such as **ODN1** just like the canonical bases. Neither an extension of the coupling time nor double coupling often required to obtain decent yield for the insertion of a modified unit were required. The 9-BBN protecting group did create no problems making it ideally suited for the incorporation of amino acid modified bases. We next cleaved the synthesized strand from the CPG-solid support material under concomitant deprotection of the base protecting groups with an aqueous solution of NH_3 and MeNH_2 followed by deprotection of the 2'-OTBS group with triethylamine trihydrofluoride in DMSO over 1.5 h at 65 °C. This leads to complete 2'-OTBS deprotection. Under these conditions we also noted cleavage of the 9-BBN group. An additional deprotection step with **ODN1** to our delight was not required.

Fig. 2a shows the raw HPL chromatograms obtained directly after cleavage of **ODN1** from the support and of all protecting groups. The inset depicts **ODN1** after one round of HPLC purification using a C18-column and a water (acetic acid, triethylamine buffer)-acetonitrile gradient. The MALDI-TOF spectrum depicted in Fig. 2b shows a clean spectrum with the expected m/z -value for $[\text{M}-\text{H}]^-$ of 2913 (m/z -value expected = 2913 for $[\text{M}-\text{H}]^-$). Both the already clean raw-HPLC chromatogram and the mass spectrum prove the successful incorporation of acp³U (**1**) into RNA.

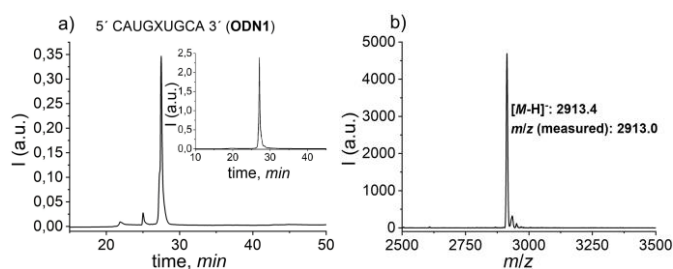


Fig. 2. (a) raw-HPL chromatogram of **ODN1** with the inset showing the HPL chromatogram of purified **ODN1**. (b) MALDI-TOF mass spectrum of **ODN1** after purification.

In order to show that our protocol also allows the preparation of longer RNA strands containing even two acp^3U modifications, we finally successfully prepared the 22-mer RNA strand **ODN4** (seq. 5' GACUGAC acp^3U CGUAGC acp^3U AACUCAU 3'). The synthesis went as expected very well. The purity and structural integrity was assessed by HPLC and MALDI-TOF (ESI⁺). We continued our studies with **ODN1**. For acp^3U we expected a strong destabilizing effect on duplex formation due to the substitution of the N3 required for standard Watson-Crick base pairing. In order to investigate to which extent the L-homoserine at N3 causes duplex destabilization, we measured melting temperatures for **ODN1:ODN2** relative to the standard duplex **ODN3:ODN2**. The obtained melting curves are depicted in Fig. 3. Clearly evident is that acp^3U reduces the melting temperature dramatically by an unusual 24°C proving the extremely strong duplex destabilizing nature of this amino acid charged hypermodified base.

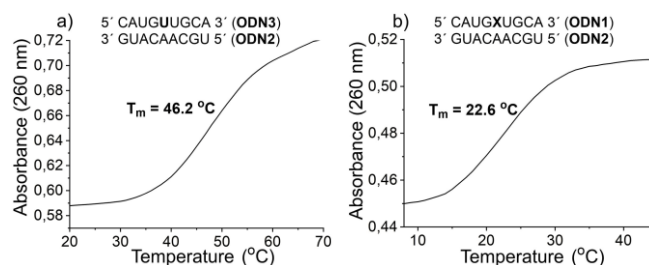


Fig. 3. (a) Depiction of the melting curve obtained with the duplex **ODN3:ODN2** as the reference. (b) Depiction of the melting curve obtained with the duplex **ODN1:ODN2**.

In summary, we report here the development of a phosphoramidite building block for the hypermodified base acp^3U . We show that acp^3U base can be almost perfectly incorporated into small and even long oligonucleotides using the building block **1-PA** with the 9-BBN protecting group for the L-homoserine amino acid. We also show and quantify that the destabilizing effect of acp^3U is unusually high.

This study was supported by the Deutsche Forschungsgemeinschaft (DFG) for financial support via the programs SFB1032 (TP-A5), SFB1309 (TP-A4), SPP-1784 and CA275/11-1. This project has received funding from the European Research Council (ERC-AG) under the European Union's Horizon 2020 research and innovation program (Project ID: 741912).

Notes and references

- 1 M. A. Machnicka, K. Milanowska, O. O. Oglou, E. Purta, M. Kurkowska, A. Olchowik, W. Januszewski, S. Kalinowski, S. Dunin-Howkawicz, K. M. Rother, M. Helm, J. M. Bujnicki, H. Grosjean, *Nucleic Acids Res.*, 2013, **41**, D262–D267.
- 2 W. V. Gilbert, T. A. Bell and C. Schaening, *Science*, 2016, **352**, 14018–1412.
- 3 B. S. Zhao, I. A. Roundtree and C. He, *Nat. Rev. Mol. Cell Biol.*, 2017, **18**, 31–42.
- 4 S. R. Jaffrey, *Nat. Struct. Mol. Biol.*, 2014, **21**, 945–946.
- 5 E. M. Harcourt, A. M. Kietrys and E. T. Kool, *Nature*, 2017, **541**, 339–346.
- 6 M. T. Watts and I. Tinoco, *Biochemistry*, 1978, **17**, 2455–2463.
- 7 C. Schneider, S. Becker, H. Okamura, A. Crisp, T. Amatov, M. Stadlmeier and T. Carell, *Angew. Chem., Int. Ed.*, 2018, **57**, 5943–5946.
- 8 M. Di Giulio, *J. Theor. Biol.*, 1998, **191**, 191–196.
- 9 H. Grosjean, V. de Crecy-Lagard and G. R. Björk, *Trends Biochem. Sci.*, 2004, **29**, 519–522.
- 10 E. Szathmary, *Trends Genet.*, 1999, **15**, 223–229.
- 11 P. Gaytan, J. Yanez, F. Sanchez, H. Mackie and X. Soberon, *Chem Biol.*, 1998, **5**, 519–527.
- 12 H. Schirmeister-Tichy, G. G. Alvarado and W. Pfeleiderer, *Nucleos. Nucleot.*, 1999, **18**, 1219–1220.
- 13 F. Himmelsbach, B. S. Schulz, T. Trichtinger, R. Charubala and W. Pfeleiderer, *Tetrahedron*, 1984, **40**, 59–72.
- 14 A. J. Ozinskas and G. A. Rosenthal, *J. Org. Chem.*, 1986, **51**, 5047–5050.

- 15 W. H. Dent III, W. R. Erickson, S. C. Fields, M. H. Parker and E. G. Tromiczak, *Org. Lett.*, 2002, **4**, 1249–1251.
- 16 C. Chaix, A. M. Duplaa, D. Molko and R. Teoule, *Nucleic Acids Res.*, 1989, **17**, 7381–7393.
- 17 J. Stawinski, R. Strömberg, M. Thelin and E. Westman, *Nucleic Acids Res.*, 1988, **16**, 9285–9298.
- 18 S. M. Ching, W. J. Tan, K. L. Chua and Y. Lam, *Bioorg. Med. Chem.*, 2010, **18**, 6657–6665.
- 19 M. de Champdore, L. De Napoli, G. Di Fabio, A. Messere, D. Montesarchio and G. Piccialli, *J. Chem. Soc. Chem. Commun.*, 2001, 2598–2599.
- 20 B. M. Trost and C. G. Caldwell, *Tetrahedron Lett.*, 1981, **22**, 4999–5002.
- 21 M. Sekine, S. Limura and K. Furusawa, *J. Org. Chem.*, 1993, **58**, 3204–3208.
- 22 H. Schaller, G. Weimann, B. Lerch and H. G. Khorana, *J. Am. Chem. Soc.*, 1963, **85**, 3821–3827.
- 23 K. L. Agarwal, A. Yamazaki, P. J. Cashion and H. G. Khorana, *Angew. Chem., Int. Ed. Engl.*, 1972, **11**, 451–459.
- 24 N. Usman, K. K. Ogilvie, M. Y. Jiang and R. J. Cedergren, *J. Am. Chem. Soc.*, 1987, **109**, 7845–7854.
- 25 H. Seliger, D. Zeh, G. Azuru and J. B. Chattopadhyaya, *Chem. Scr.*, 1983, **22**, 95–102.
- 26 M. H. Caruthers, D. Dellinger, K. Prosser, A. D. Brone, J. W. Dubendorff, R. Kierzek and M. Rosendahl, *Chem. Scr.*, 1986, **26**, 25–30.
- 27 R. Kierzek, M. H. Caruthers, C. E. Longfellow, D. Swinton, D. H. Turner and S. M. Freier, *Biochemistry*, 1986, **25**, 7840–7846.
- 28 I. Hirao, M. Ishikawa and K. Miura, *Nucleic Acids Symp. Ser.*, 1985, **16**, 173–176.
- 29 H. Tanimura, T. Fukuzawa, M. Sekine, T. Hata, J. W. Efcavitch and G. Zon, *Tetrahedron Lett.*, 1988, **29**, 577–578.
- 30 H. Tanimura, M. Sekine and T. Hata, *Nucleos. Nucleot.*, 1986, **5**, 363–383.
- 31 H. Tanimura and T. Imada, *Chem. Lett.*, 1990, 1715–1718.

Supporting information

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

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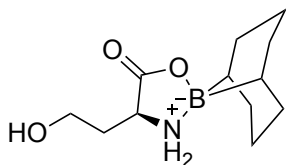
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1. General Experimental Methods

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). ^1H 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 (Mettler 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 $_2\text{O}$ and co-crystallized in a 3-hydroxypicolinic acid matrix (HPA).

2. Synthesis of the Phosphoramidite Building-Block

Compound 3b

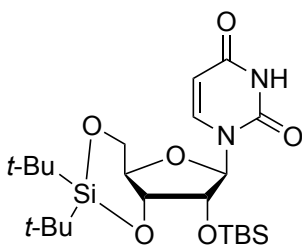


The reaction was conducted according to a published procedure with minor modifications.¹

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 °C.

¹H 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); ¹³C 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 C₁₂H₂₃BNO₃⁺ [M + H]⁺: 240.1771; found 240.1764.

Compound 5



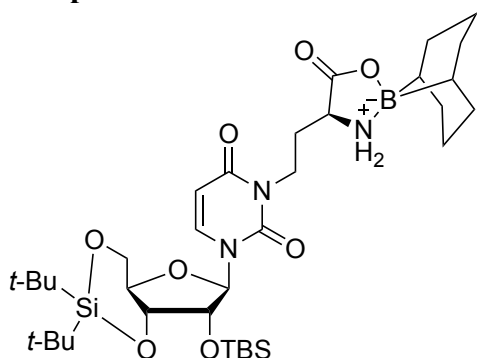
The reaction was conducted according to a published procedure with minor modifications.²

Uridine (**4**) (1 g, 4.1 mmol) was dissolved in dry DMF (5 ml) and stirred at 0 °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 NaHCO₃ and water. The organic layer was dried over Na₂SO₄ 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%).

¹H NMR (400 MHz, CDCl₃) 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); **¹³C NMR** (101 MHz, CDCl₃) 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 C₂₃H₄₃O₆N₂Si₂⁺ [M + H]⁺: 499.2660; found 499.2657.

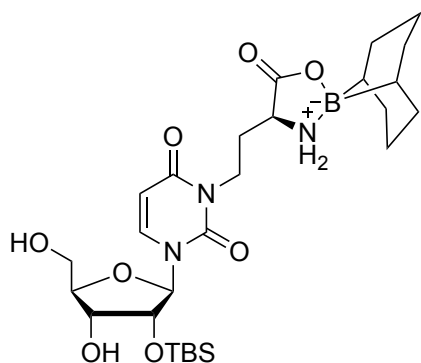
Compound 6



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%).

¹H NMR (400 MHz, CDCl₃) 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 – 4.46 (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); **¹³C NMR** (101 MHz, CDCl₃) 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 C₃₅H₆₃BN₃O₈Si₂⁺ [M + H]⁺: 720.4247; found 720.4243.

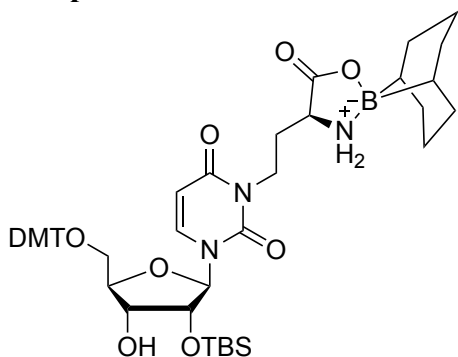
Compound 7



Compound **6** (0.5 g, 0.69 mmol) was dissolved in dry CH₂Cl₂ (7 ml), transferred in a falcon tube and cooled to 0 °C. Then pyridine was added (1 ml) followed by addition of HF-Pyridine (0.131 ml). After 1 hour the reaction was quenched with saturated NaHCO₃, washed with CH₂Cl₂, dried and evaporated. The residue was purified by silica gel chromatography eluting with CH₂Cl₂/CH₃OH (9/1, v/v) to afford alcohol **7** as a white solid (yield 99%).

¹H 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); **¹³C 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 C₂₇H₄₆O₈N₃BNaSi⁺ [*M* + Na]⁺: 602.3045; found 602.3044.

Compound 8

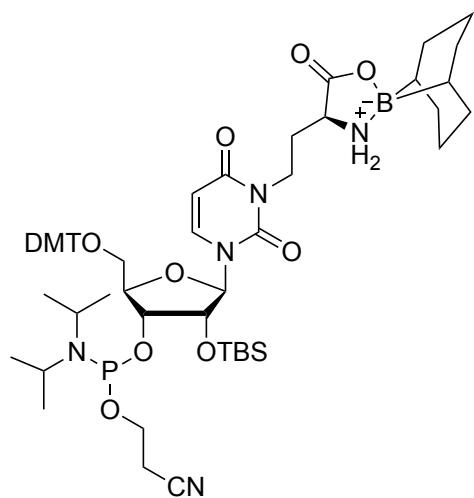


To the solution of compound **7** (0.25 g, 0.34 mmol) in dry pyridine (3 ml) 4,4'-dimethoxytrityl chloride (0.22 g, 0.65 mmol, 1.5 eq.) was added. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. After evaporation of reaction mixture the residue was

dissolved in CH₂Cl₂, washed with saturated NaHCO₃ and brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by silica gel chromatography eluting with CH₂Cl₂/CH₃OH (9/1, v/v) containing 0.1 % of pyridine to afford the product **8** as white foam (yield 71%).

¹H 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); **¹³C 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 C₄₈H₆₄O₁₀N₃BNaSi⁺ [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 CH₂Cl₂ (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 NaHCO₃ solution and extracted with CH₂Cl₂. The combined organic extract was dried over anhydrous NaSO₄, 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; ^1H and ^{13}C spectra are given of one of the isomers. **^1H 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 $\text{C}_{57}\text{H}_{82}\text{BN}_5\text{O}_{11}\text{PSi}^+$ $[\text{M}+\text{H}]^+$: 1082.5611; found 1082.5603.

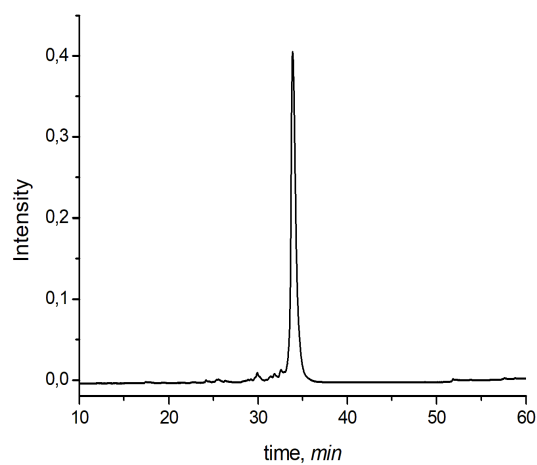
3. Synthesis and Purification of Oligonucleotides

Sequence: 5' CAUGacp³UUGCA 3' (ODN1)

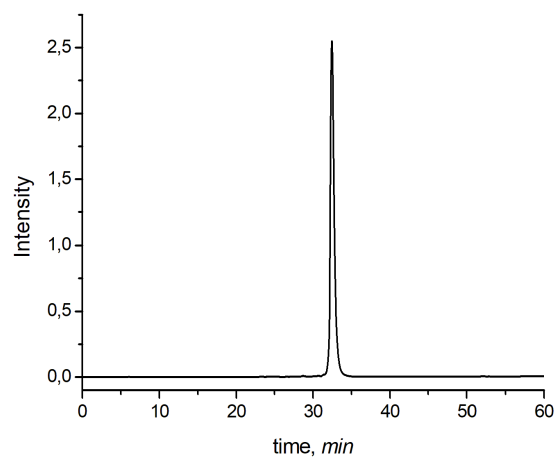
Sequence: 5' GACUGACacp³UCGUAGCacp³UAACUCAU 3' (ODN4)

All of the oligonucleotides used in this study were synthesized on a 1 μ mol 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 acp³U nucleoside was synthesized in DMT-OFF mode using phosphoramidites (Bz-A, Dmf-G, Ac-C, U) with BTT in CH₃CN as an activator, DCA in CH₂Cl₂ as a deblocking solution and Ac₂O in pyridine/THF as a capping reagent. The cleavage and deprotection of the CPG bound oligonucleotides were performed with aqueous NH₄OH/MeNH₂ (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 μ L) in DMSO (50 μ L) at 65 °C for 1.5 h. Upon cooling on ice bath, NaOAc (3.0 M, 25 μ L) 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 NEt₃/HOAc, pH 7.0 in H₂O and buffer B: 100 mM NEt₃/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.

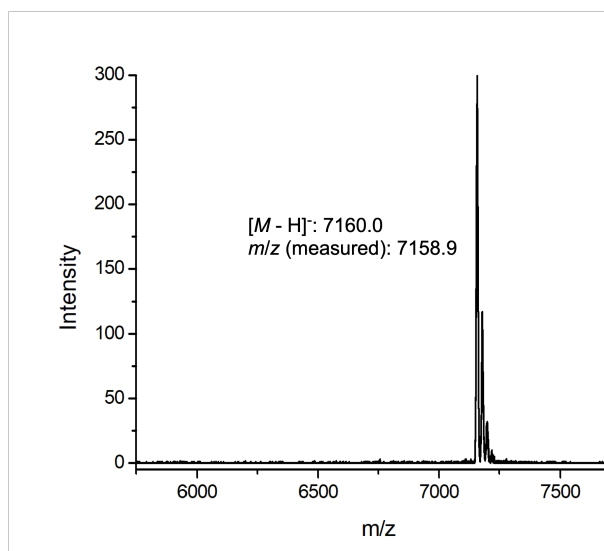
a)



b)



c)



d)

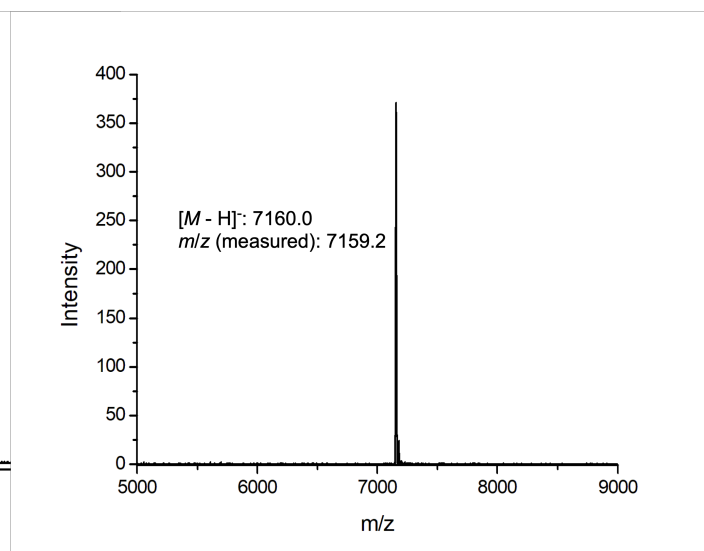


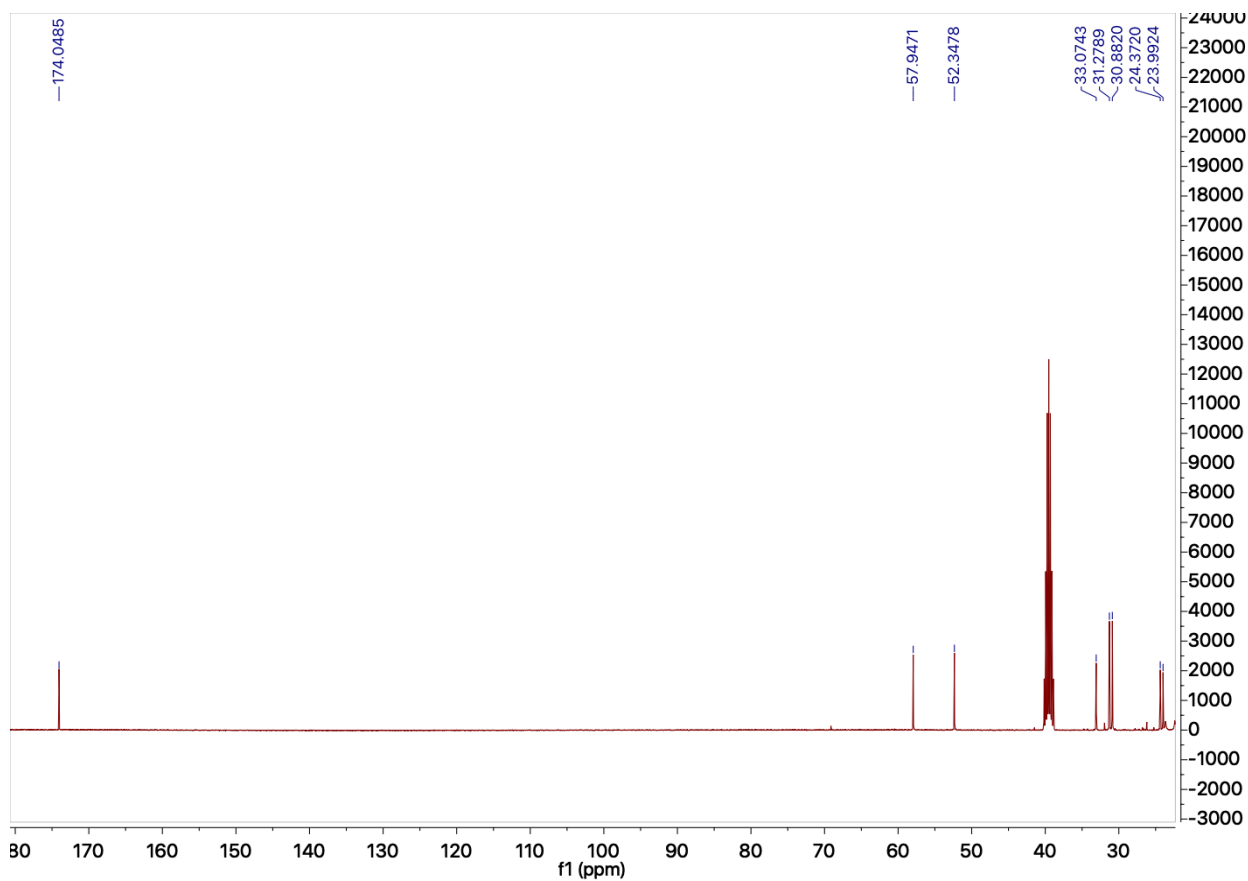
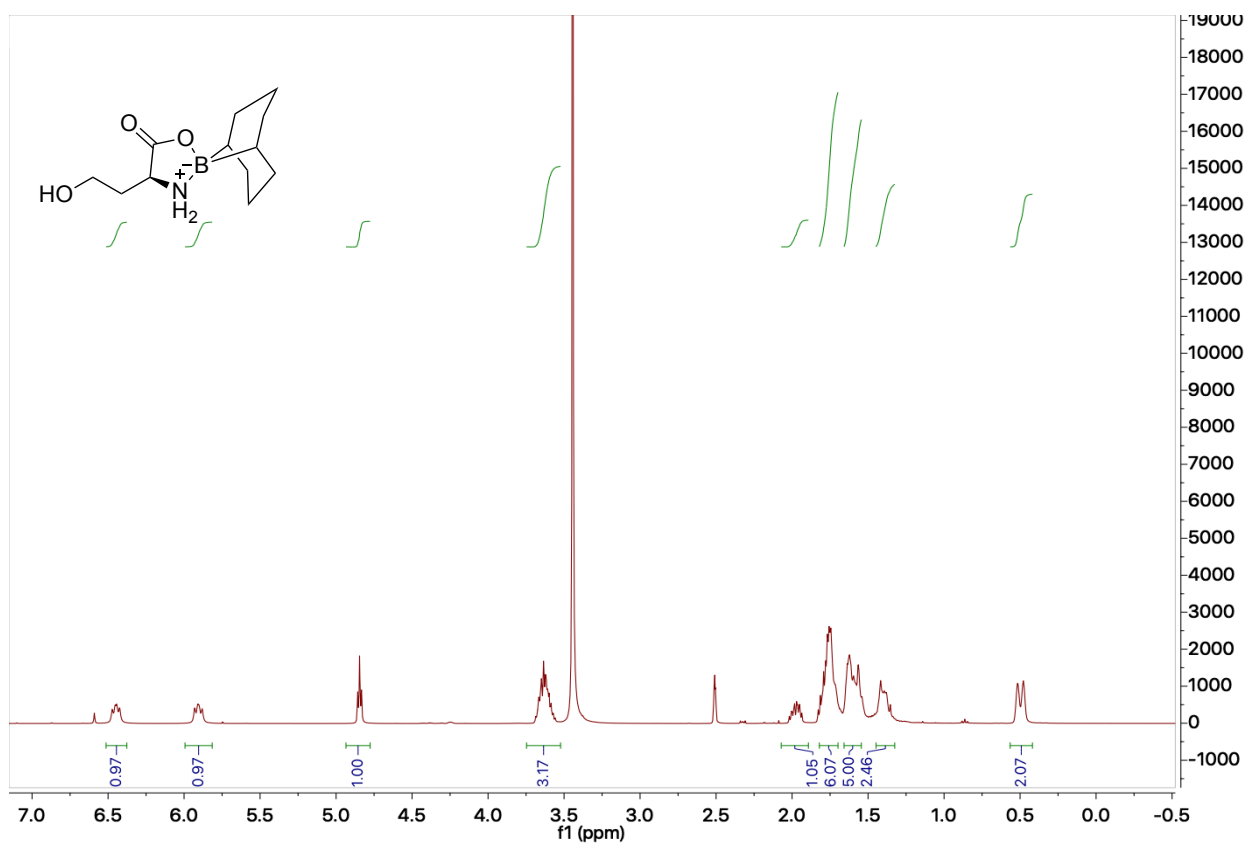
Figure S1. (a) raw-HPL chromatogram of **ODN4**; (b) HPL chromatogram of purified **ODN4**; (c) MALDI-TOF mass spectrum of raw **ODN4**; (d) MALDI-TOF mass spectrum of purified **ODN4**.

4. UV Melting Curve Measurements

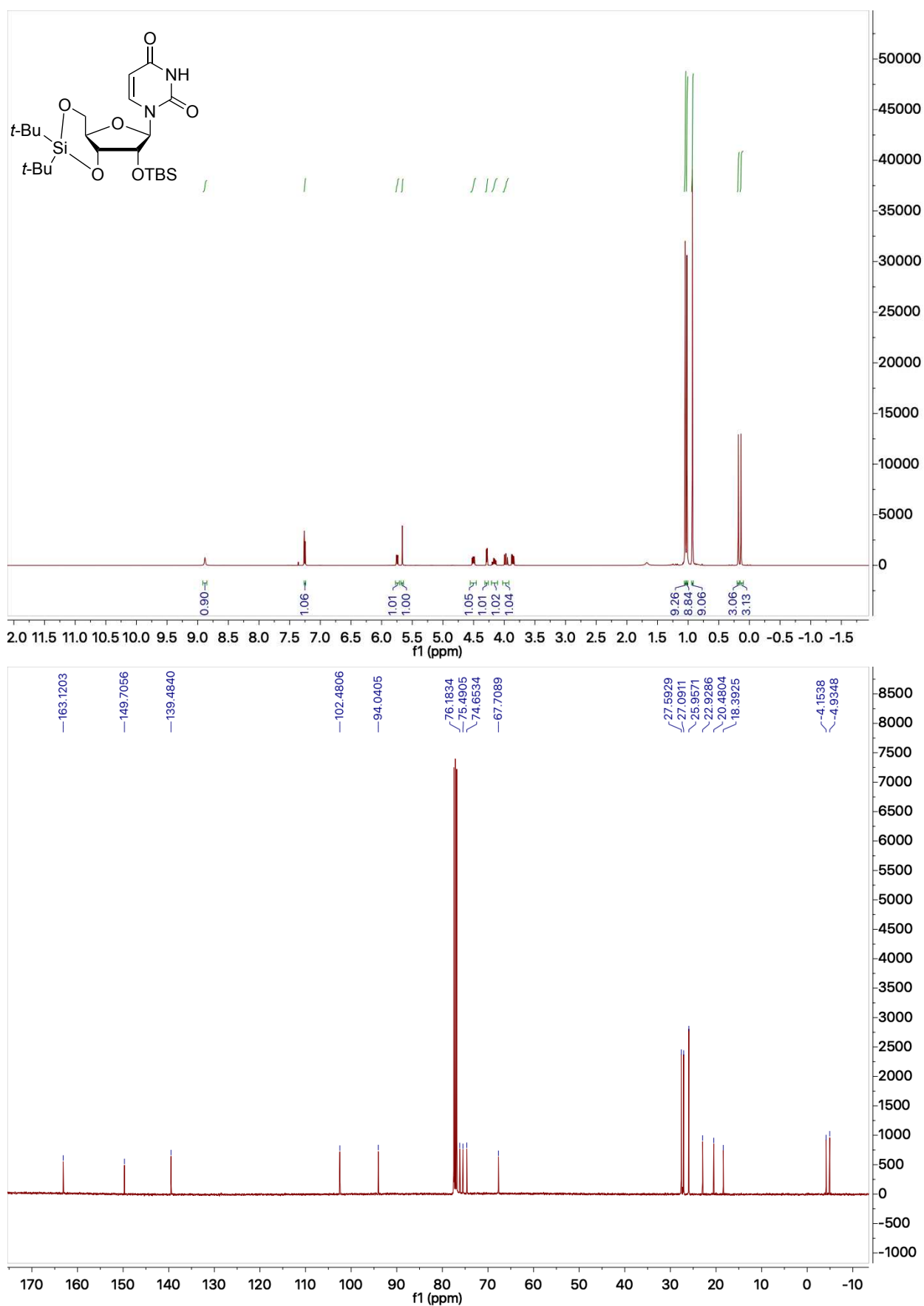
The UV melting curves were measured on JASCO V-650 spectrometer using 10 mm QS cuvettes, purchased from Hellma Analytics. A solution (80 μ L) of equimolar amounts of oligonucleotides (4 μ M each) in the buffer solution containing 10 mM sodium phosphate buffer (pH 7.0) and 150 mM NaCl was heated at 50 °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

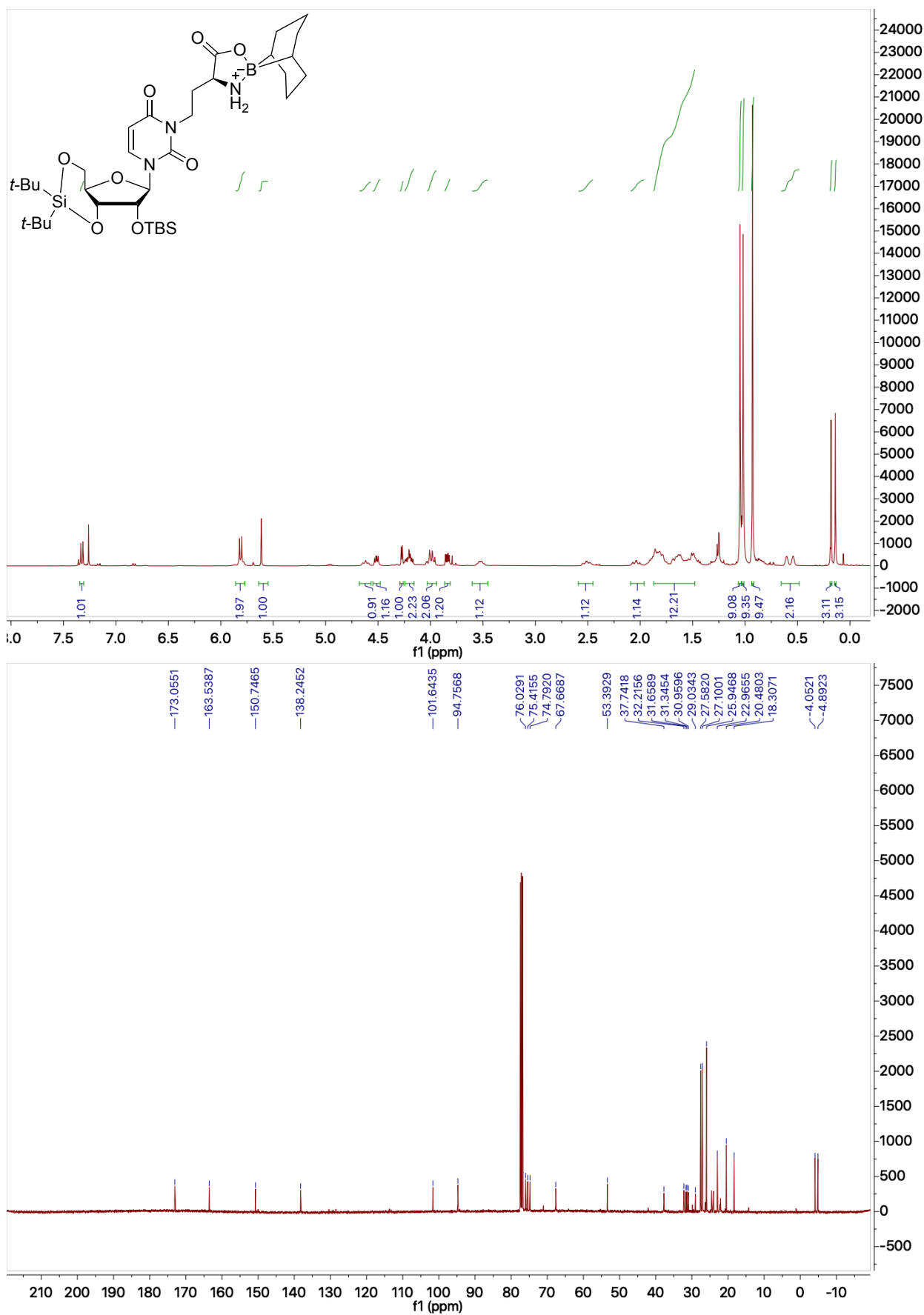
^1H NMR and ^{13}C NMR of compound **3b**



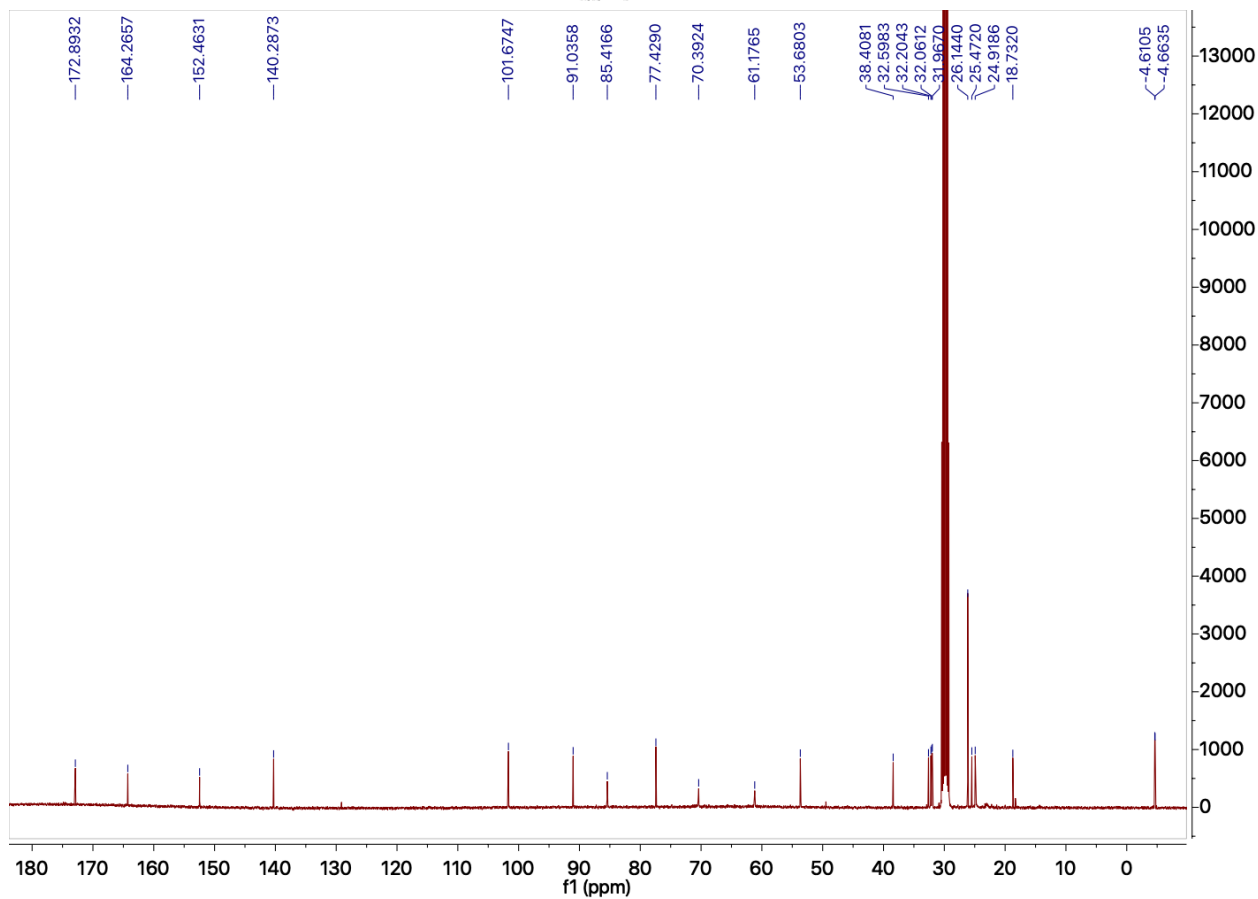
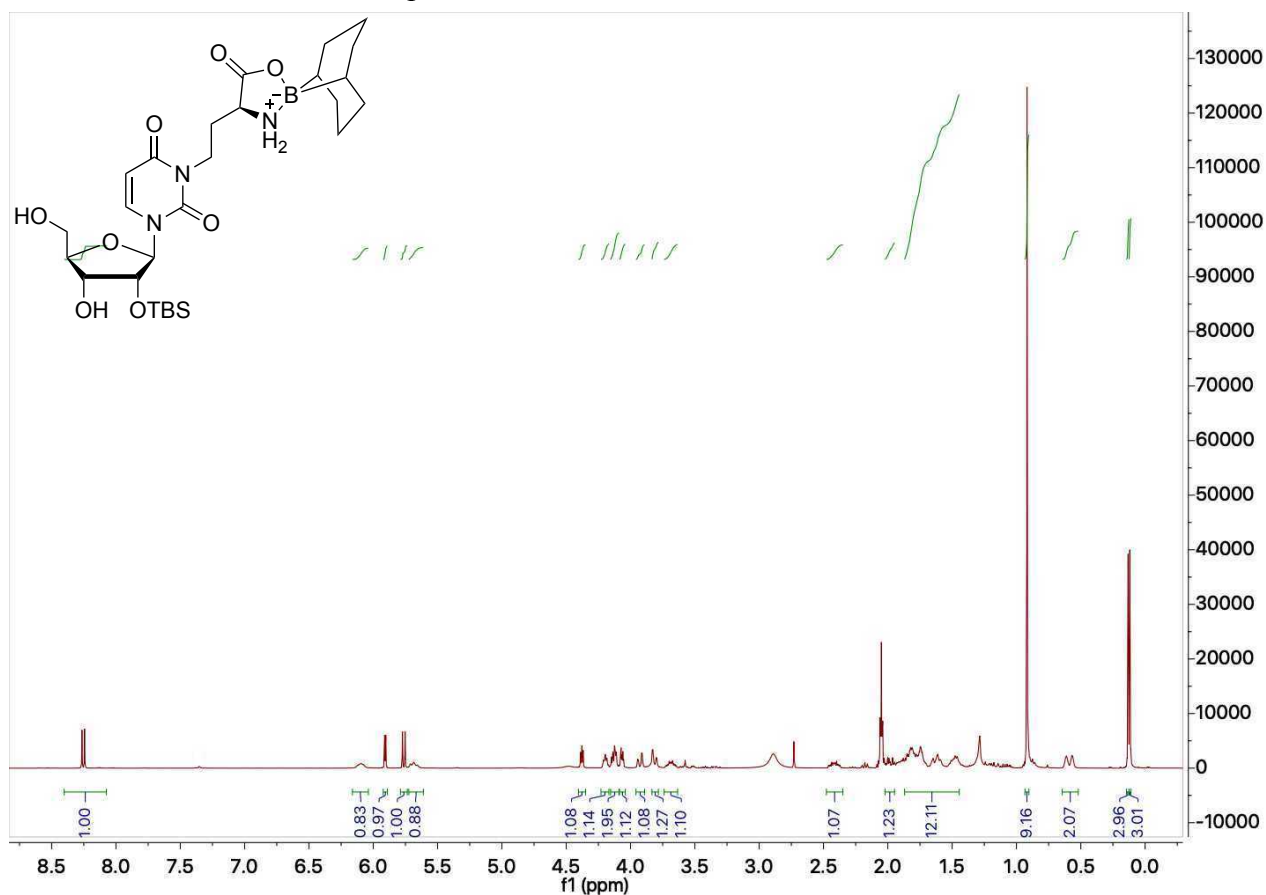
^1H NMR and ^{13}C NMR of compound **5**



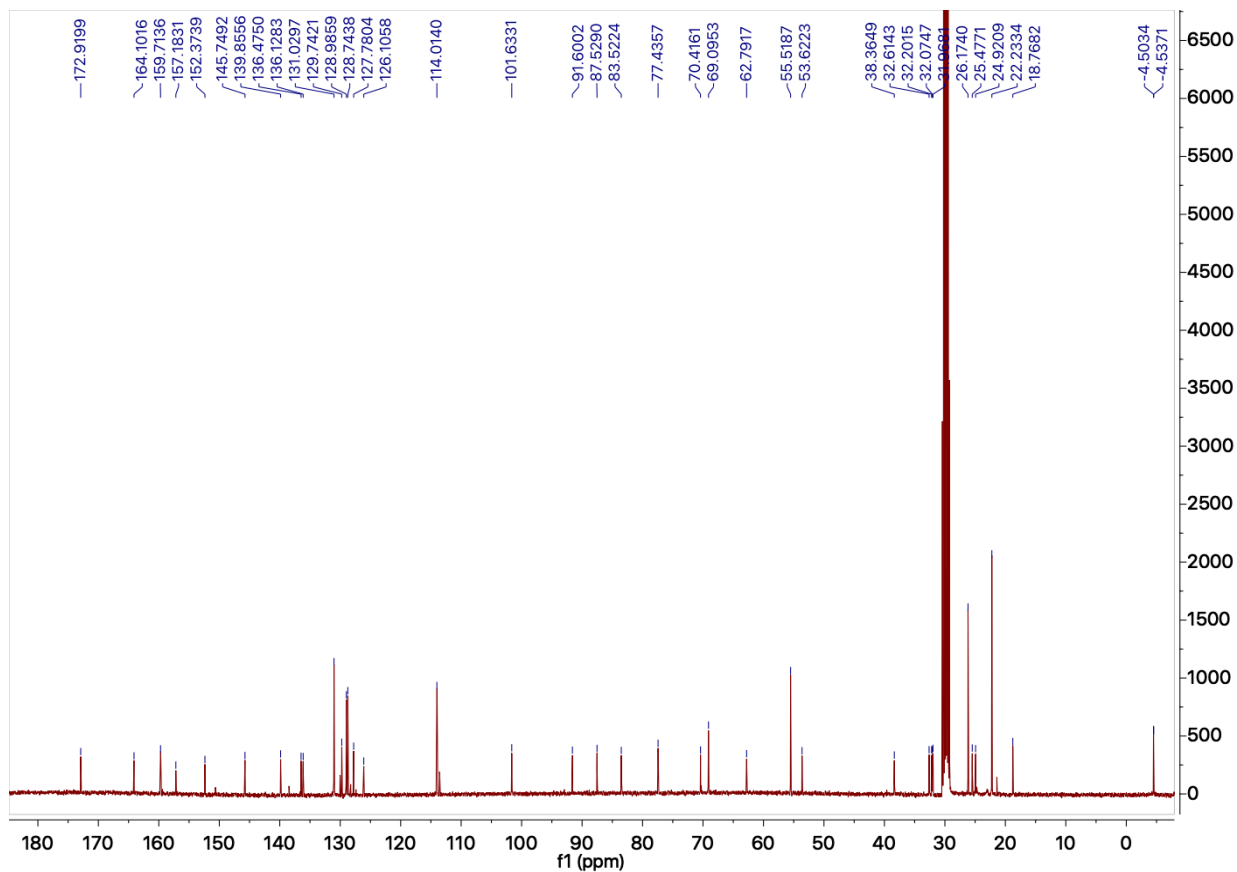
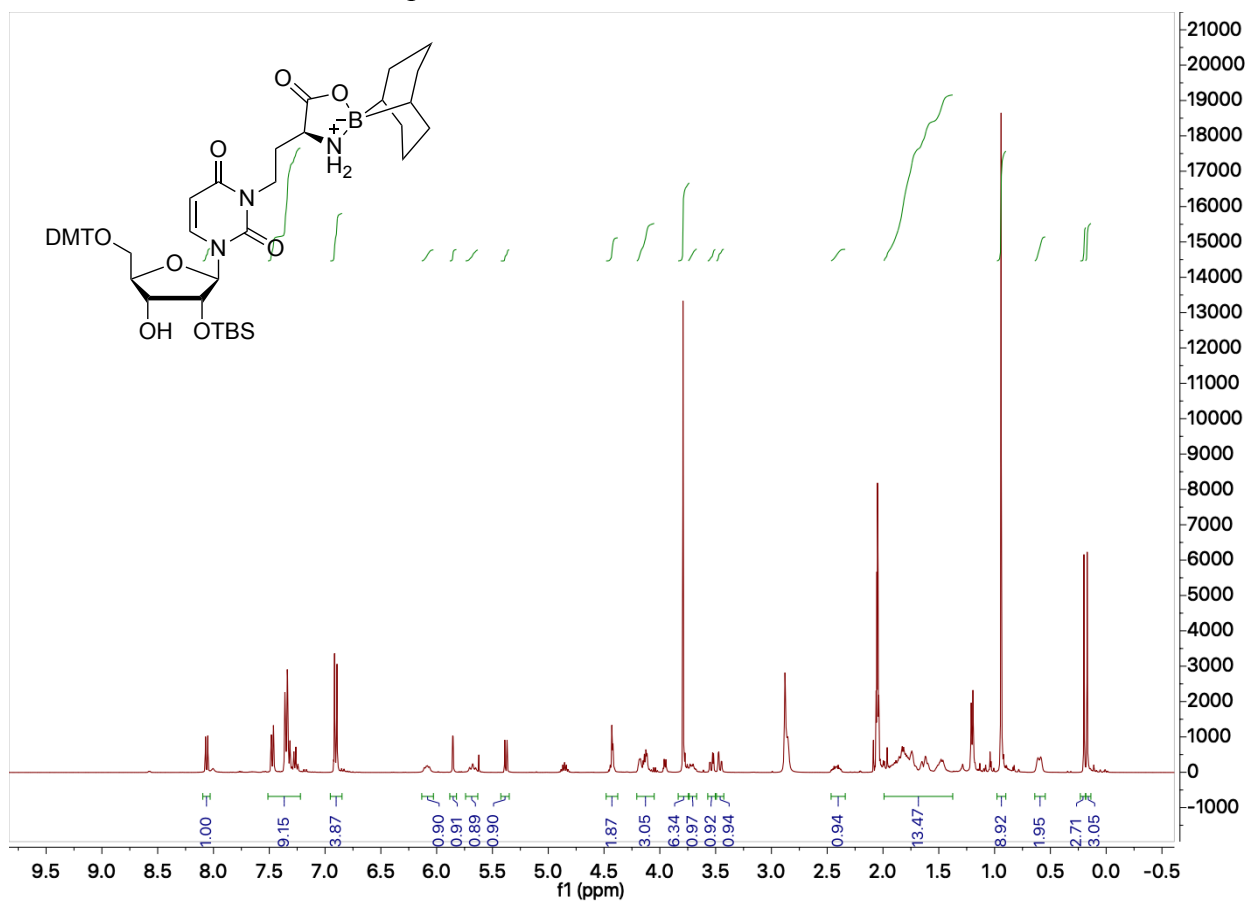
^1H NMR and ^{13}C NMR of compound 6



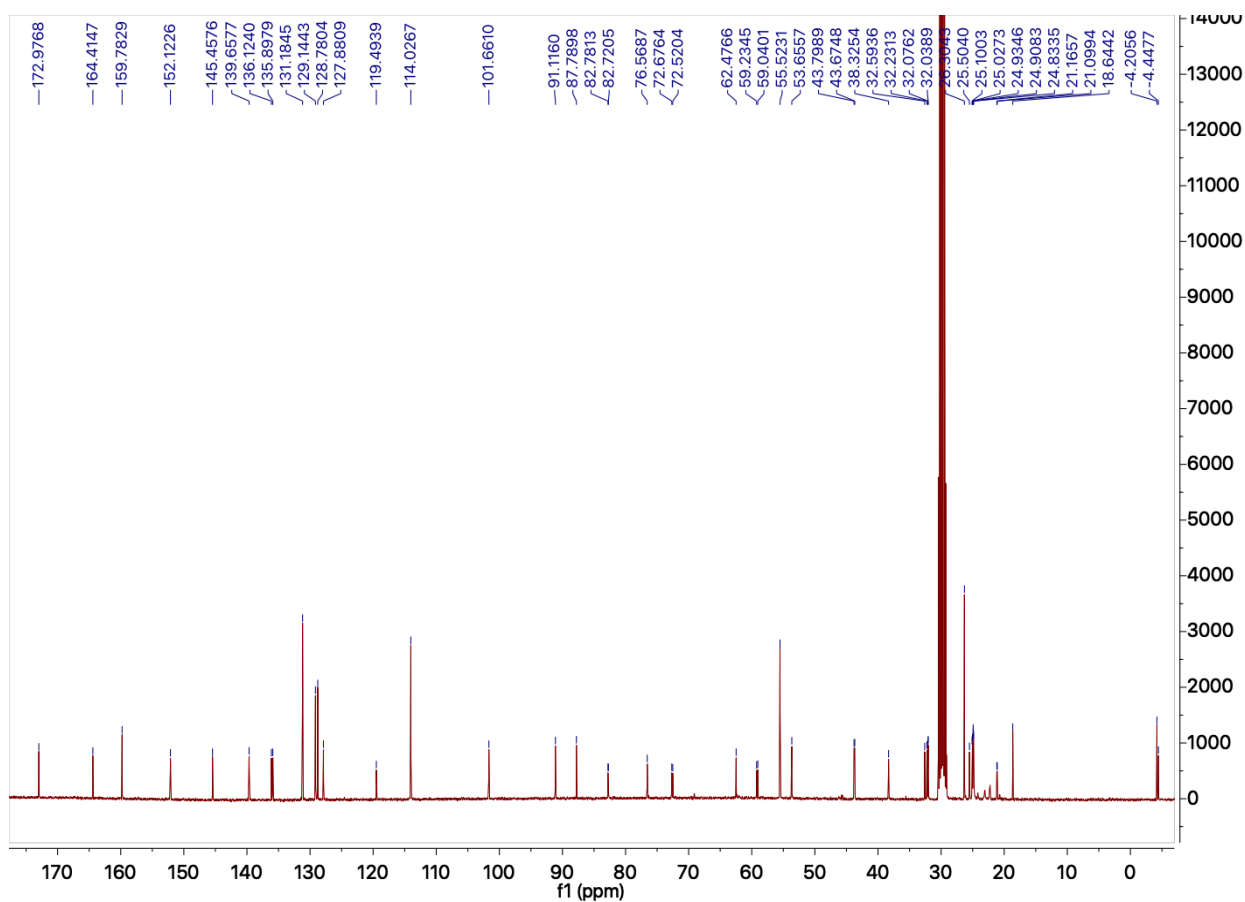
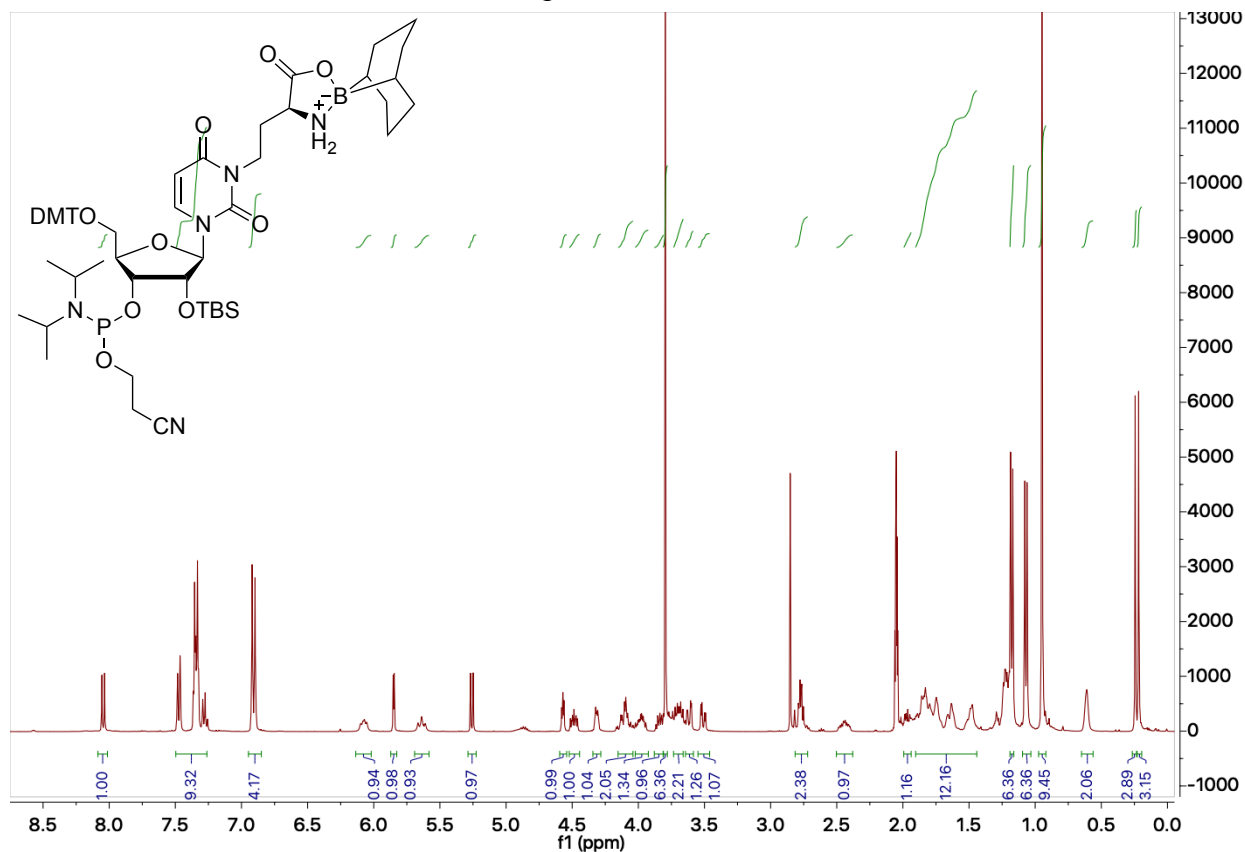
^1H NMR and ^{13}C NMR of compound **7**

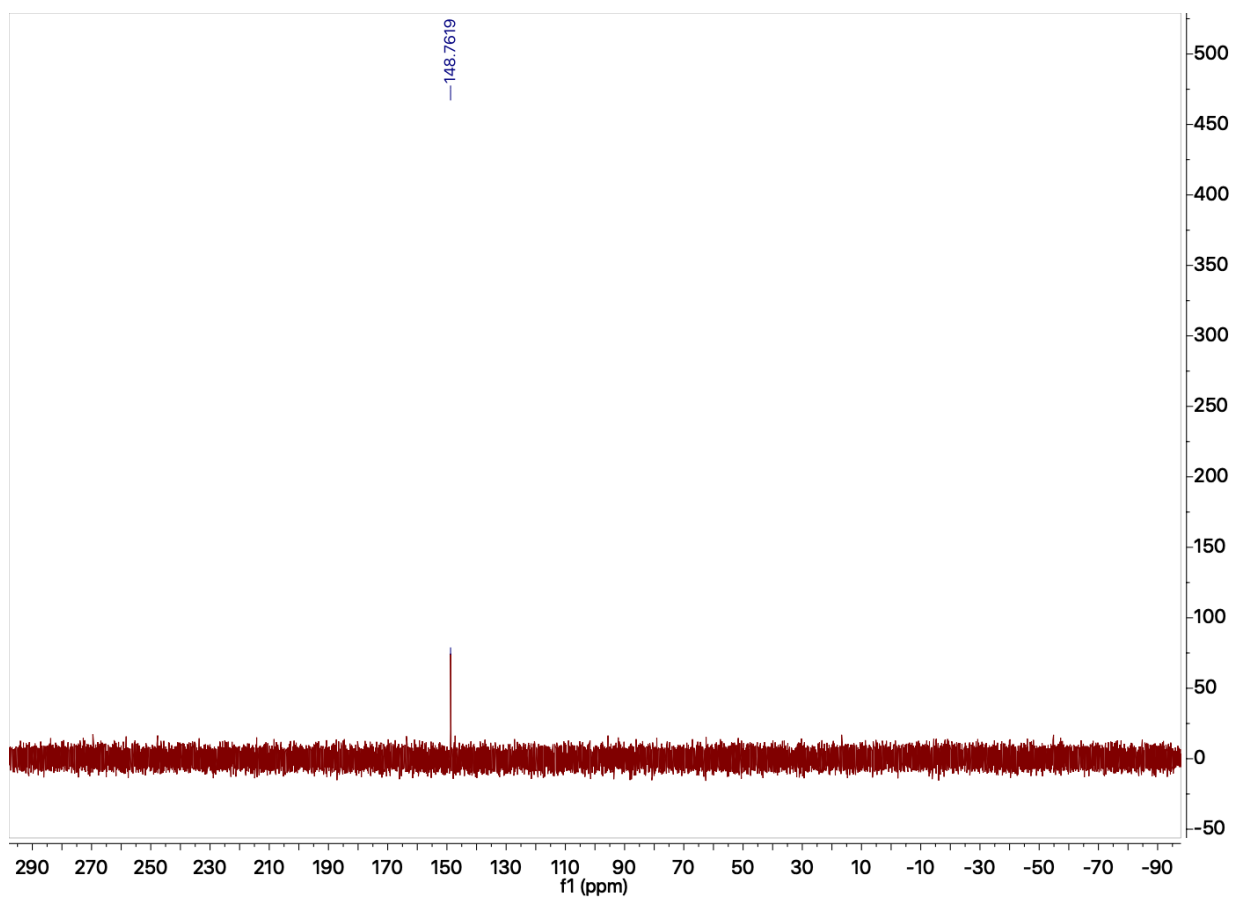
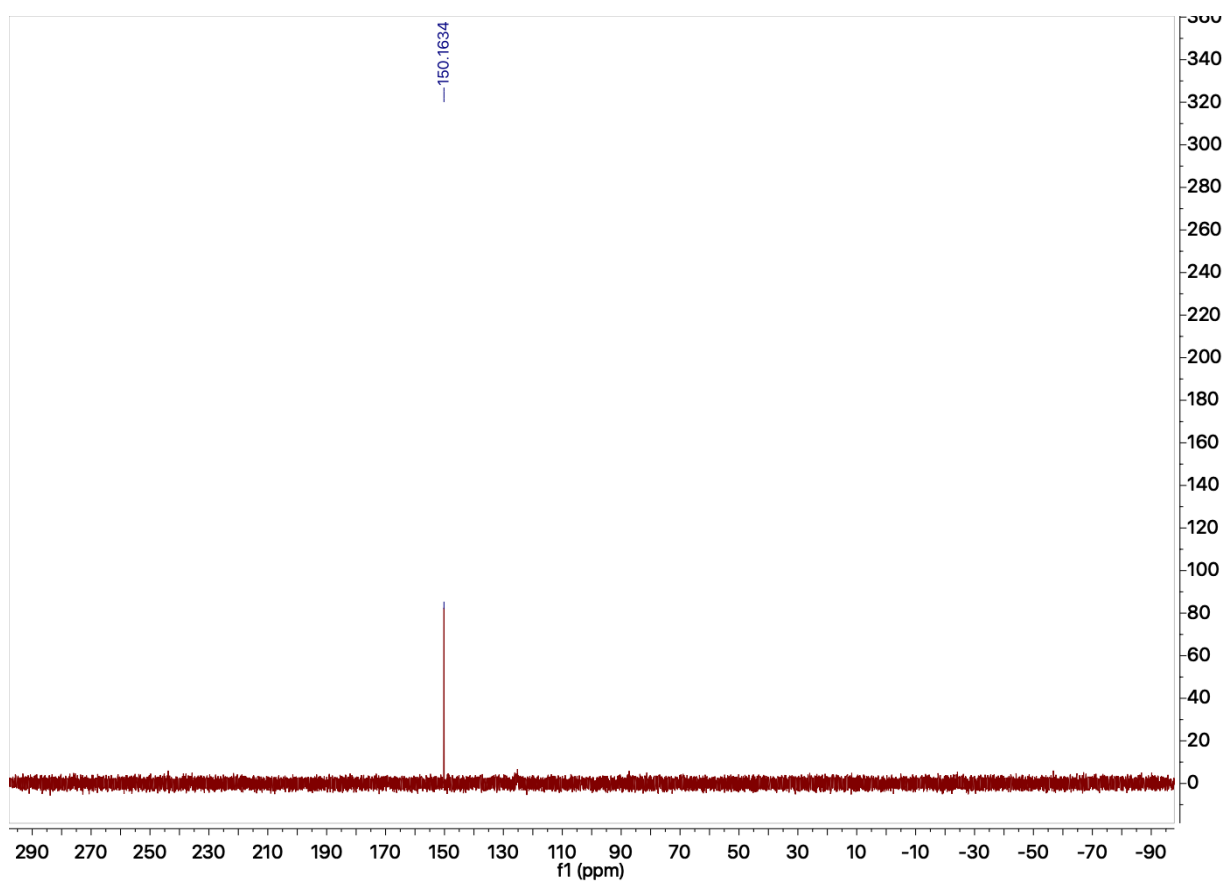


^1H NMR and ^{13}C NMR of compound **8**



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





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.