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Expanding the Chemical Diversity of TNA with tUTP Derivatives that are Substrates for a TNA Polymerase

Hui Mei and John C. Chaput*

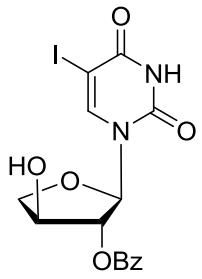
Departments of Pharmaceutical Sciences, Chemistry, Molecular Biology and Biochemistry, University of California, Irvine, CA 92697-3958.

* To whom correspondence should be addressed. E-mail: jchaput@uci.edu

Table of Contents

Experimental Section

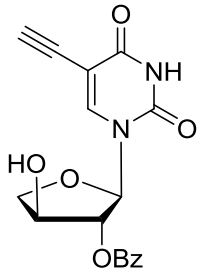
General Methods and Materials. All non-aqueous reactions were performed using oven-dried glassware under an atmosphere of argon or nitrogen. All chemicals and solvents were purchased from Sigma-Aldrich and used without further purification. Reactions were monitored by thin layer chromatography using UV-activated TLC plates with silica gel 60 F254 and aluminium backing (Sigma-Aldrich, St. Louis, MO). Flash column chromatography was performed using SiliCycle 40-60 mesh silica gel (SiliCycle Inc., Quebec City, Canada). Yields are reported as isolated yields of pure compounds. UV quantification data are analyzed on NanoDrop 2000c using Beer's Law. ^{1}H, ^{13}C and ^{31}P NMR spectra were obtained using Bruker DRX400 and Bruker DRX500 NMR spectrometers (Bruker, Billerica, MA). ^{1}H and ^{13}C NMR values are reported in parts per million (ppm) relative to Me_4Si as internal standard or corresponding deuterium solvents as internal standard. ^{31}P NMR values are reported in ppm relative to an external standard of 85% H_3PO_4. Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. HPLC purification was performed with a C18 reverse-phase 250 x 9.4 mm HPLC column (Thermo Scientific, US) using a mobile phase of 0.1 M triethylammonium acetate buffer (pH 7.0)/acetonitrile. α-L-threofuranosyl nucleoside 3'-monophosphates (**6a-c**), 3'-phosphoro(2-methyl) imidazolides (**7a-c**), and 3'-triphosphates (**8a-c**) were analyzed by analytical HPLC with a reverse-phase column (C18 150 × 4.6 mm, 5 μm particle size, Thermo Scientific, US). Thermo Pol buffer was purchased from New England Biolabs (Ipswich, MA). DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA), purified by denaturing polyacrylamide gel electrophoresis, electro-eluted, precipitated by ethanol, re-suspended in water, and quantified by UV absorbance. Recombinant Kod-RI polymerases was expressed and purified from *E. coli* as previously described[1]. TNA triphosphates bearing natural bases were synthesized as previously described[2].



3

5-iodo-1-(2'-*O*-benzoyl-α-L-threofuranosyl)-uracil (3).

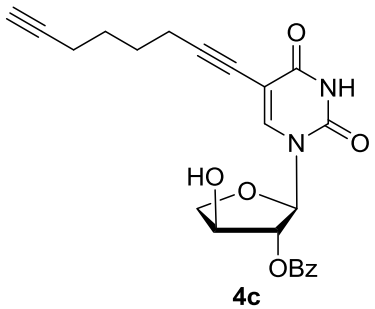
To a suspension of 5-iodo-uracil (**1**) (9.1 g, 38.5 mmol) in 100 mL anhydrous acetonitrile was added *N*,*O*-bis(trimethylsilyl)acetamide (19.6 mL, 80.2 mmol) and the mixture was stirred for 1 h at 60 °C. After cooling to 24 °C, 1-*O*-acetyl-2-*O*-benzoyl-3-*O*-*tert*-butyldiphenylsilyl-L-threofuranose (**2**) (16.2 g, 32.1 mmol) in 50 mL anhydrous acetonitrile was added dropwise to the reaction mixture. TMSOTf (17.4 mL, 96.3 mmol) was then added and the mixture was heated for 2.5 h at 60 °C. After removal of acetonitrile, the syrup was diluted with 200 mL of EtOAc. The solution was washed with aqueous saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was directly used for the next step. The residue was dissolved in THF (100 mL), then cooled to 0-5 ºC. To the cold THF solution was added dropwise tetrabutylammonium fluoride (TBAF, 1 M solution in THF, 32.1 mL, 32.1 mmol) and the mixture was stirred for 2 h at 0 °C. The solvent was evaporated under reduced pressure and the residue was dissolved in 200 mL of EtOAc. The organic layer was washed with H_2O (100 mL x 2) and brine (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give a yellow syrup. The syrup was purified by flash chromatography (silica gel, hexane/EtOAc/CH_2Cl_2, 1:1:1) to afford **3** (10 g, 70%) as a light yellow form. TLC (hexane/EtOAc, 1:1): R_f = 0.23. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.07 (s, 1H), 8.02-8.00 (m, 2H), 7.62-7.59 (m, 1H), 7.48-7.45 (m, 2H), 7.26 (s, 1H), 5.98 (s, 1H), 5.50 (s, 1H), 4.51 (s, 1H), 4.40-4.38 (m, 1H), 4.27-4.25 (m, 1H), 3.75 (s, 1H). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 165.9, 160.3, 150.1, 145.9, 134.1, 130.1, 128.8, 128.5, 91.4, 82.6, 74.1, 67.7. HRMS (ESI-TOF) calcd for $C_{15}H_{13}N_2O_6NaI$ $[M + Na]^+$ 466.9716; observed 466.9709.



4a

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-ethynyl-uracil (4a).

To a solution of **3** (1.16 g, 2.61 mmol), [$Pd(PPh_3)_4$] (0.30 g, 0.26 mmol) and CuI (0.10 g, 0.52 mmol) in anhydrous DMF (15 mL) were added anhydrous Et_3N (0.73 mL, 5.2 mmol), and trimethylsilylacetylene (1.8 mL, 13.0 mmol). The reaction mixture was stirred at rt under a nitrogen atmosphere for 16 h. The solvent was diluted with ethyl acetate (80 mL), and washed with saturated Na_2EDTA aqueous solution (80 mL x 6) and brine (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give a black residue. The residue was dissolved in THF (20 mL), and treated with TBAF (1 M solution in THF, 13.0 mL, 13.0 mmol) at 0 ^oC for 20 min. After removal of THF, the residue was purified by flash chromatography (silica gel, CH_2Cl_2/acetone, 10:1) to give the product **4a** (0.79 g, 88%) as a light yellow foam. TLC (CH_2Cl_2/acetone, 10:1): R_f = 0.14. ^1H NMR (500 MHz, DMSO-*d6*): *δ* 11.75 (s, 1H), 8.07 (s, 1H), 8.01 (d, *J* = 7.5 Hz, 2H), 7.72-7.69 (m, 1H), 7.58-7.55 (m, 2H), 5.96 (s, 1H), 5.33 (s, 1H), 4.36 (s, 1H), 4.25 (d, *J* = 9.5 Hz, 1H), 4.16-4.14 (m, 2H). ^{13}C NMR (125.8 MHz, DMSO-*d6*): *δ* 164.6, 161.7, 149.4, 145.0, 134.0, 129.6, 128.9, 128.8, 97.3, 89.5, 83.7, 81.7, 76.3, 75.7, 72.3. HRMS (ESI-TOF) calcd for $C_{17}H_{14}N_2O_6Na$ $[M + Na]^+$ 365.0750; observed 365.0748.

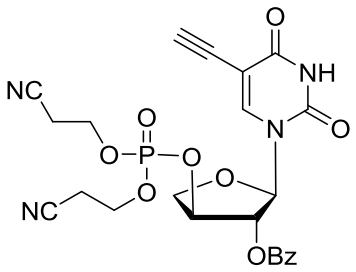


4c

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(octa-1,7-diynyl)-uracil (4c).

To a solution of **3** (1.25 g, 2.82 mmol), [$Pd(PPh_3)_4$] (0.32 g, 0.28 mmol) and CuI (0.11 g, 0.57 mmol) in anhydrous DMF (15 mL) were added anhydrous Et_3N (0.59 mL, 4.2 mmol),

and octa-1,7-diyne (3.75 mL, 28.0 mmol). The reaction mixture was stirred at rt under a nitrogen atmosphere for 16 h. The solvent was diluted with ethyl acetate (80 mL), and washed with saturated Na_2EDTA aqueous solution (80 mL x 6) and brine (100 mL), dried over $MgSO_4$, and concentrated under reduced pressure to give a black residue. The residue was purified by flash chromatography (silica gel, hexane/EtOAc, 1:1) to give the product **4c** (0.66 g, 58%) as a light yellow foam. TLC (hexane/EtOAc, 1:1): R_f = 0.41. ^{1}H NMR (500 MHz, DMSO-*d6*): *δ* 9.56 (s, 1H), 7.99 (d, *J* = 5.0 Hz, 2H), 7.79 (s, 1H), 7.57 (t, *J* = 7.0 Hz, 1H), 7.42 (t, *J* = 8.0 Hz, 2H), 5.96 (s, 1H), 5.54 (s, 1H), 4.46 (s, 1H), 4.38 (d, *J* = 10.0 Hz, 1H), 4.28-4.22 (m, 2H), 2.40-2.38 (t, *J* = 6.5 Hz,2H), 2.24-2.21 (m, 2H), 1.97-1.96 (m, 1H), 1.69-1.64 (m, 4H). ^{13}C NMR (126 MHz, DMSO-*d6*): *δ* 165.7, 162.6, 149.5, 143.0, 133.9, 130.0, 128.7, 100.0, 94.5, 91.2, 84.4, 82.2, 76.3, 73.8, 71.5, 68.7, 27.7, 27.5, 19.2, 18.1. HRMS (ESI-TOF) calcd for $C_{23}H_{22}N_2O_6Na$ $[M + Na]^+$ 445.1375; observed 445.1375.

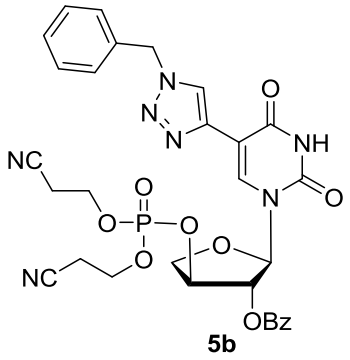


5a

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-ethynyl-uracil 3'-*O*-bis(2-cyanoethyl)-phosphotriester (5a).

To a stirring solution of **4a** (0.43 g, 1.0 mmol) and DMAP (25 mg, 0.20 mmol) in CH_2Cl_2 (10 mL) was added *N*,*N*-Diisopropylethylamine (DIPEA) (261 µL, 1.5 mmol) followed by the addition of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (268 µL, 1.2 mmol). After stirring for 20 min at rt, the solution was diluted with CH_2Cl_2 (40 mL) and extracted with saturated aqueous $NaHCO_3$ (40 mL). The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to dryness for the next step.To a stirring solution of the residue in acetonitrile (15 mL) was added 3-hydroxypropionitrile (137 µL, 2.0 mmol) followed by a solution of 0.45 M tetrazole in acetonitrile (4.45 mL, 2.0 mmol). After being stirred for 2 h at room temperature, H_2O_2 (30% in H_2O) (306 µL, 3.0 mmol) was added to the solution. After being stirred at rt for 20 min, the solution was diluted with CH_2Cl_2 (50

mL) and washed with brine. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by flash chromatography (silica gel, CH_2Cl_2/ MeOH, 20:1) to afford **5a** (0.21 g, 40%) as a white foam. TLC (CH_2Cl_2/MeOH, 10:1): R_f = 0.31. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.59 (s, 1H), 8.02 (d, J = 7.5 Hz, 2H), 7.83 (s, 1H), 7.63-7.60 (t, J = 7.5 Hz, 1H), 7.48-7.45 (t, J = 7.5 Hz, 2H), 6.07 (s, 1H), 5.73 (s, 1H), 5.09-5.07 (m, 1H), 4.65 (d, J = 11.0 Hz, 1H), 4.42-4.34 (m, 5H), 3.25 (s, 1H), 2.85-2.83 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 164.9, 161.7, 149.2, 143.4, 134.3, 130.1, 128.8, 128.2, 116.8, 98.6, 90.4, 82.3, 79.8 (d, $J_{C,P}$ = 6.3 Hz), 78.8 (d, $J_{C,P}$ = 5.0 Hz), 75.1, 74.7 (d, $J_{C,P}$ = 5.0 Hz), 63.4 (t, $J_{C,P}$ = 5.0 Hz), 19.9 (t, $J_{C,P}$ = 5.0 Hz, $J_{C,P}$ = 6.3 Hz). ^{31}P NMR (162 MHz, $CDCl_3$): δ -3.29. HRMS (ESI-TOF) calcd for $C_{23}H_{21}N_4O_9PNa$ $[M + Na]^+$ 551.0944; observed 551.0950.

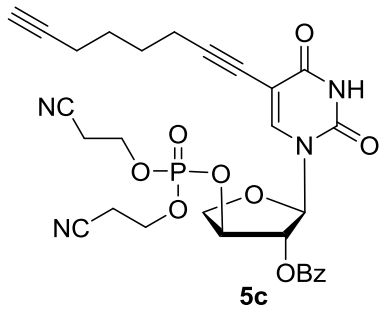


5b

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(1-phenyl-1*H*-1,2,3-triazol-4-yl)-uracil 3'-*O*-bis(2-cyanoethyl)-phosphotriester (5b).

Phosphotriester **5a** (155 mg, 0.29 mmol) and benzyl azide (1.0 mmol) were dissolved in THF/H_2O/t-BuOH (3:1:1, v/v, 6 mL), then sodium ascorbate (0.50 mL, 0.50 mmol) of a freshly prepared 1 M solution in water was added, followed by the addition of copper(II) sulfate pentahydrate 7.5% in water (333 mL, 0.10 mmol). The reaction mixture was stirred for 30 min at room temperature. The solvent was evaporated and the residue was diluted with CH_2Cl_2 (50 mL). The organic layer was washed with saturated Na_2EDTA aqueous solution (50 mL x 3) and brine (50 mL), dried over $MgSO_4$, and concentrated. The residue was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH, 30:1) to give **5b** (127 mg, 66%) as a white foam. TLC (CH_2Cl_2/MeOH, 10:1): R_f = 0.43. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.41 (s, 1H), 8.49 (s, 1H), 8.20 (s, 1H), 8.02 (d, J = 7.0 Hz, 2H), 7.83 (s, 1H), 7.61 (t, J = 7.5 Hz, 1H), 7.47-7.45 (m, 2H), 7.38-7.27 (m, 5H), 6.25 (s, 1H), 5.65 (s, 1H), 5.51-5.50 (m,

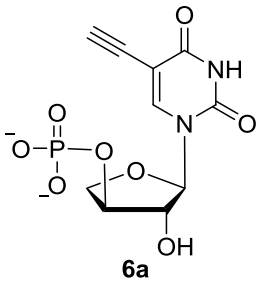
2H), 5.16-5.14 (m, 1H), 4.68 (d, *J* = 11.5 Hz, 1H), 4.44-4.30 (m, 5H), 2.81-2.73 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$): *δ* 165.1, 161.1, 149.4, 139.4, 135.7, 134.7, 134.2, 130.1, 129.3, 128.9, 128.8, 128.3, 128.1, 122.6, 116.7, 116.6, 106.5, 90.1, 80.8 (d, $J_{C,P}$ = 6.3 Hz), 79.3 (d, $J_{C,P}$ = 5.0 Hz), 74.1 (d, $J_{C,P}$ = 3.8 Hz), 63.4 (d, $J_{C,P}$ = 5.0 Hz), 63.3 (d, $J_{C,P}$ = 3.8 Hz), 19.6 (d, $J_{C,P}$ = 8.8 Hz). ^{31}P NMR (162 MHz, $CDCl_3$): *δ* -2.56. HRMS (ESI-TOF) calcd for $C_{30}H_{28}N_7O_9PNa$ $[M + Na]^+$ 684.1584; observed 684.1569.



5c

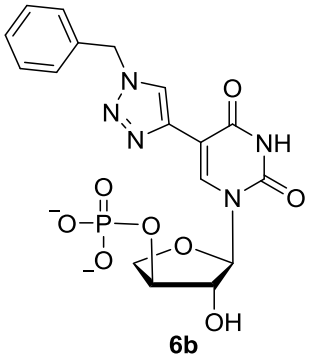
1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(octa-1,7-diynyl)-uracil 3'-*O*-bis(2-cyanoethyl)-phosphotriester (5c).

As described for **5a**, compound **5c** was prepared from nucleoside **4c** (0.38 g, 0.90 mmol), purified by flash chromatography (silica gel, CH_2Cl_2/MeOH, 30:1), and obtained as a white foam (0.24 g, 44%). TLC (CH_2Cl_2/MeOH, 10:1): R_f = 0.42. ^{1}H NMR (500 MHz, $CDCl_3$): *δ* 9.73 (s, 1H), 8.02 (d, *J* = 7.5 Hz, 2H), 7.67 (s, 1H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.46 (t, *J* = 7.5 Hz, 2H), 6.04 (s, 1H), 5.71 (s, 1H), 5.09-5.07 (m, 1H), 4.62 (d, *J* = 11.0 Hz, 1H), 4.39-4.33 (m, 5H), 2.85-2.83 (m, 4H), 2.43 (t, *J* = 7.0 Hz, 2H), 2.25-2.22 (m, 2H), 1.99 (t, *J* = 2.5 Hz, 1H), 1.72-1.64 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$): *δ* 164.9, 162.2, 149.3, 141.3, 134.2, 130.1, 128.7, 128.2, 116.8, 116.7, 100.2, 94.7, 90.4, 84.2, 79.8 (d, $J_{C,P}$ = 6.3 Hz), 78.8 (d, $J_{C,P}$ = 5.0 Hz), 74.5 (d, $J_{C,P}$ = 5.0 Hz), 71.7, 68.8, 63.3 (dd, $J_{C,P}$ = 5.0 Hz, $J_{C,P}$ = 8.8 Hz), 27.6, 19.8 (t, $J_{C,P}$ = 8.8 Hz), 19.2, 18.0. ^{31}P NMR (162 MHz, $CDCl_3$): *δ* -2.45. HRMS (ESI-TOF) calcd for $C_{29}H_{29}N_4O_9PNa$ $[M + Na]^+$ 631.1570; observed 631.1556.



6a

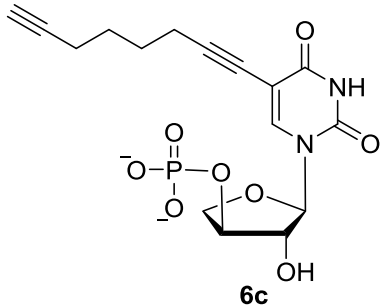
1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-ethynyl-uracil 3'-*O*-monophosphate (6a). Compound **5a** (236 mg, 0.45 mmol) was dried by co-evaporation with dry pyridine (5 mL x 2) and dissolved in 10 ml dry pyridine. To this solution *N*,*O*-Bis(trimethyl silyl)acetamide (4.4 mL, 18.0 mmol) and DBU (404 µL, 2.7 mmol) were added and the resulting solution was stirred at room temperature for 5 h. The reaction was quenched with 30 ml water and diluted with 50 mL diethyl ether. The organic layer was extracted with 2 × 10 mL water and the combined aqueous phases were evaporated to dryness. The residue was co-evaporated with 3 × 5 mL toluene and 2 × 5 mL pyridine, and treated with ammonia in methanol (2.5 M, 10 mL) for 3 h under stirring at room temperature. The solvent was evaporated, the residue was dissolved in water (20 mL), and washed with CH_2Cl_2 (4 x 30 mL). The aqueous layer was lyophilized, and the resulting material was suspended in ammonia-MeOH (2.5 M, 1 mL) and diluted with acetone (20 mL). The precipitate was collected by centrifugation at 4400 rpm at room temperature for 15 min and the resulting pellet was washed twice with 20 mL of acetone and dried under high vacuum. Monophosphate **6a** was obtained as the ammonium salt (white solid) in near quantitative yield (150 mg, 95%). ^{1}H NMR (500 MHz, D_2O): *δ* 8.18 (s, 1H), 5.84 (s, 1H), 4.66-4.64 (m, 1H), 4.55-4.52 (m, 2H), 4.43-4.39 (m, 1H), 3.65 (s, 1H). ^{13}C NMR (126 MHz, D_2O): *δ* 165.7, 151.1, 146.6, 110.5, 97.9, 93.2, 83.5, 79.6, 78.2, 78.1, 76.4, 76.3, 75.6. ^{31}P NMR (162 MHz, D_2O): *δ* 2.83. HRMS (ESI-TOF) calcd for $C_{10}H_{12}N_2O_8P$ $[M + H]^+$ 319.0331; observed 319.0330.



6b

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(1-phenyl-1*H*-1,2,3-triazol-4-yl)-uracil 3'-*O*-monophosphate (6b).

In a sealed tube, compound **5b** (116 mg, 0.175 mmol) was combined with NH_3-MeOH (2 mL) and NH_4OH (4 mL), and stirred for 16 h at 37 °C. The solution was cooled down to room temperature, diluted with water, and washed with CH_2Cl_2 (3 x 40 mL). The aqueous layer was lyophilized to afford the product as ammonium salt. The precipitate was collected by centrifugation at 4400 rpm at room temperature for 15 min and the resulting pellet was washed twice with 20 mL of acetone and dried under high vacuum. The nucleoside 3'-monophosphate **6b** was obtained as the ammonium salt (white solid) in quantitative yield (85 mg, 100%). ^{1}H NMR (400 MHz, D_2O): *δ* 8.29-8.27 (m, 2H), 7.42-7.32 (m, 5H), 5.85 (s, 1H), 5.57 (s, 2H), 4.70-4.69 (m, 1H), 4.63-4.59 (m, 2H), 4.45-4,44 (m, 1H). ^{13}C NMR (100 MHz, D_2O): *δ* 163.2, 151.0, 138.2, 135.1, 129.4, 129.0, 128.2, 124.0, 104.6, 92.7, 79.2, 79.1, 78.2, 75.7, 69.6, 54.2. ^{31}P NMR (162 MHz, D_2O): *δ* 0.46. HRMS (ESI-TOF) calcd for $C_{17}H_{18}N_5O_8PNa$ $[M + Na]^+$ 474.0791; observed 474.0802.

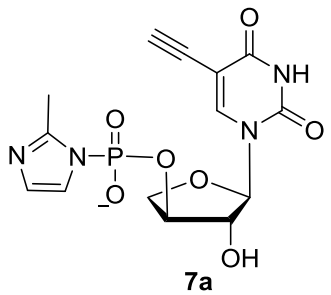


6c

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(octa-1,7-diynyl)-uracil 3'-*O*-monophosphate (6c).

As described for **6b**, compound **6c** was prepared from nucleoside **5c** (184 mg, 0.30 mmol), and obtained as the ammonium salt (white solid, 105 mg, 81%). ^{1}H NMR (500

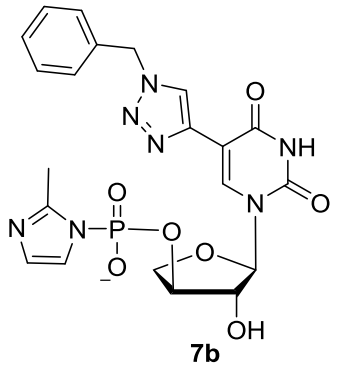
MHz, D_2O): *δ* 7.98 (s, 1H), 5.81 (s, 1H), 4.65-4.64 (m, 1H), 4.55-4.50 (m, 2H), 4.43-4.40 (m, 1H), 2.55-2.47 (m, 2H), 2.41 (s, 1H), 2.32-2.29 (m, 2H), 1.74-1.68 (m, 4H). ^{13}C NMR (126 MHz, D_2O): *δ* 165.3, 150.6, 143.9, 98.9, 95.9, 92.6, 86.1, 79.0, 77.8, 77.7, 75.7, 71.5, 69.4, 27.1, 27.0, 18.4, 17.2. ^{31}P NMR (162 MHz, D_2O): *δ* 1.38. HRMS (ESI-TOF) calcd for $C_{16}H_{19}N_2O_8PNa$ $[M + Na]^+$ 421.0777; observed 421.0762.



7a

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-ethynyl-uracil 3'-*O*-phosphor-(2-methyl)imidazolide (7a). General procedure for the preparation of 7a-c.

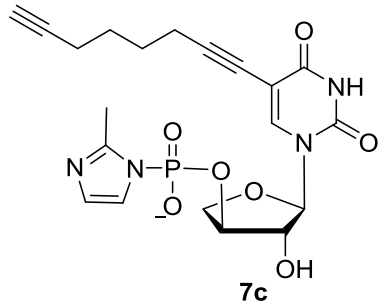
To a stirring solution containing nucleoside 3'-monophosphate **6a** (88 mg, 0.25 mmol) and 2-methylimidazole (200 mg, 2.44 mmol, 9.7 equiv) in anhydrous DMSO (1 mL) and DMF (1 mL), and was added triethylamine (0.2 mL, 1.44 mmol, 5.8 equiv), triphenylphosphine (200 mg, 0.76 mmol, 3.0 equiv) and 2,2'-dipyridyldisulfide (240 mg, 1.1 mmol, 4.4 equiv). The reaction was stirred under a nitrogen atmosphere for 3 h at room temperature with monitoring by analytical HPLC. After consumption of the starting material, the product was precipitated by the dropwise addition of the reaction mixture to a stirring solution containing 60 mL of acetone, 60 mL of diethyl ether, 4 mL of triethylamine and 1 mL of saturated $NaClO_4$ in acetone. The precipitate was collected by centrifugation at 4400 rpm for 15 min at room temperature. The pellet was washed twice with 30 mL of washing solution (acetone/diethyl ether 1:1) and dried under high vacuum to afford **7a** as sodium salt (white solid, 73 mg, 72%). ^1H and ^{13}C spectrum were not taken for nucleoside 3'-phosphoro(2-methyl) imidazolides (**7a-c**), as these compounds were used without further purification. ^{31}P NMR (162 MHz, DMSO-*d6*): *δ* -9.86. HRMS (ESI-TOF) calcd for $C_{14}H_{14}N_4O_7PNa_2$ $[M -H + 2Na]^+$ 427.0396; observed 427.0394.



7b

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(1-phenyl-1*H*-1,2,3-triazol-4-yl)-uracil 3'-*O*-phosphor-(2-methyl)imidazolide (7b).

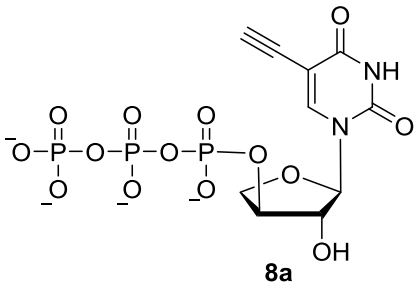
As described for **7a**, compound **7b** was prepared from nucleoside **6b** (78 mg, 0.16 mmol), and obtained as sodium salt (white solid, 85 mg, 98%). ^{31}P NMR (162 MHz, D_2O) *δ* -9.73. HRMS (ESI-TOF) calcd for $C_{21}H_{22}N_7O_7PNa$ $[M + Na]^+$ 538.1216; observed 538.1224.



7c

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(octa-1,7-diynyl)-uracil 3'-*O*-phosphor-(2-methyl)imidazolide (7c).

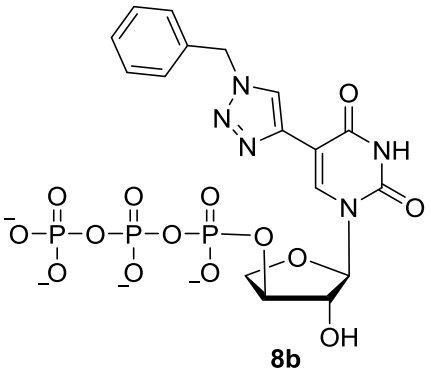
As described for **7a**, compound **7c** was prepared from nucleoside **6c** (40 mg, 0.093 mmol), and obtained as sodium salt (white solid, 40 mg, 89%). ^{31}P NMR (162 MHz, DMSO-*d6*): *δ* -9.77. HRMS (ESI-TOF) calcd for $C_{20}H_{23}N_4O_7PNa$ $[M +Na]^+$ 485.1202; observed 485.1191.



8a

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-ethynyl-uracil 3'-*O*-triphosphate (8a). General procedure for the preparation of 8a-c.

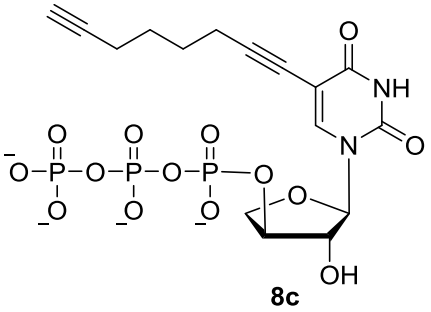
To a anhydrous DMF (2 mL) solution of compound **7a** (30 mg, 78 µmol) was added tributylamine (48 µL, 0.20 mmol, 2.56 equiv) and tributylammonium pyrophosphate (110 mg, 0.20 mmol, 2.56 equiv), and the reaction was stirred for 6 h at 24°C under N_2 with monitoring by analytical HPLC. After the reaction was finished, the reaction mixture was added dropwise to a stirring solution containing 20 mL of acetone and 3 mL of saturated $NaClO_4$ in acetone. The precipitate was collected by centrifugation at 4400 rpm for 15 min at room temperature and dried under vacuum for 1 h. The crude precipitate was dissolved in 2 ml of 0.1 M triethylammonium acetate buffer and purified by a semi-preparative HPLC. Fractions containing triphosphates were collected, concentrated, pH adjusted by triethylamine to 8.0, and lyophilized to afford the product as a triethylammonium salt. The solid product was resuspended in 1 mL of methanol and was added dropwise to a solution containing 30 mL of acetone and 1 mL of saturated $NaClO_4$ in acetone. The solution was centrifuged at 4400 rpm for 15 min at room temperature. The supernatant was discarded, and the pellet was washed with 30 mL acetone and dried under vacuum. The desired triphosphate **8a** was obtained as sodium salt in a white solid form. The yield was measured by the NanoDrop 2000c, assuming an extinction coefficient[2-3] of 3.8 mM^{-1} cm^{-1} and found to be 22% (17 µmol). The 3'-*O*-triphosphates were dissolved and stocked in a 10 mM Tris (pH 8.0) buffer and used for enzymatic TNA synthesis. ^{1}H and ^{13}C spectra were not recorded for nucleoside 3'-*O*-triphosphates (**8a-c**), because of the difficulty in recovery the compounds from the Tris buffer. ^{31}P NMR (162 MHz, D_2O): δ –4.42 (d, J = 21.7 Hz), −10.09 (d, J = 20.7 Hz), −20.33 (t, J = 24.8 Hz). HRMS (ESI-TOF) calcd for $C_{10}H_{10}N_2O_{14}P_3Na_4$ $[M -3H + 4Na]^+$ 566.8936; observed 566.8959.



8b

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(1-phenyl-1*H*-1,2,3-triazol-4-yl)-uracil 3'-*O*-triphosphate (8b).

As described for **8a**, compound **8b** was prepared from nucleoside **7b** (65 mg, 0.12 mmol), and obtained as sodium salt in a white solid form. The yield was measured by the NanoDrop 2000c, assuming an extinction coefficient[4] of 3.8 mM^{-1} cm^{-1} and found to be 28% (33.8 µmol). ^{31}P NMR (162 MHz, D_2O): *δ* –4.64 (d, *J* = 20.7 Hz), −11.19 (d, *J* = 20.6 Hz), -20.63 (t, *J* = 20.3 Hz). HRMS (ESI-TOF) calcd for $C_{17}H_{17}N_5O_{14}P_3Na_4$ $[M -3H + 4Na]^+$ 699.9576; observed 699.9598.



8c

1-(2'-*O*-benzoyl-α-L-threofuranosyl)-5-(octa-1,7-diynyl)-uracil 3'-*O*-triphosphate (8c).

As described for **8a**, compound **8c** was prepared from nucleoside **7c** (30 mg, 62 µmol), and obtained as sodium salt in a white solid form. The yield was measured by the NanoDrop 2000c, assuming an extinction coefficient[5] of 4.0 mM^{-1} cm^{-1} and found to be 32% (19.8 µmol). ^{31}P NMR (162 MHz, D_2O): *δ* –4.46 (d, *J* = 20.3 Hz), −10.99 (d, *J* = 20.4 Hz), −20.38 (t, *J* = 20.3 Hz). HRMS (ESI-TOF) calcd for $C_{16}H_{18}N_2O_{14}P_3Na_4$ $[M -3H + 4Na]^+$ 646.9562; observed 646.9591.

Primer extension assay. Primer-extension reactions were performed in a final volume of 10 µL using 10 pmol of PBS8-IR680 primer (5'-IR680-GGATA CCACC) and 15 pmol of 30-mer DNA template template (5'- TCTCT ATAGT GAGTC GTATA GGTGG TATCC). Each reaction contained primer/template complex, 1x ThermoPol buffer [20 mM Tris-HCl, 10 mM $(NH4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8], 1 µM KOD-RI, 100 µM of each tNTP, and 1 mM $MnCl_2$. Reactions were incubated for 1 h or 2 h at 55°C, quenched with stop buffer (8 M urea, 45 mM EDTA) and analyzed by 15% denaturing urea PAGE.

Large-scale TNA synthesis. Primer-extension reactions were performed in a final volume of 500 µL using 0.5 nmol of PBS8-IR680 primer (5'-IR680-GGATA CCACC) and 0.75 nmol of 50-mer DNA template template (5'-TCTCT ATAGT GAGTC GTATA GGTGG TATCC TTTTT TTTTT TTTTT TTTTT) or (5'-TCTCT ATTGT GTGTC GTCTC GGTGG TATCC TTTTT TTTTT TTTTT TTTTT). Each reaction contained primer/template complex, 1x ThermoPol buffer [20 mM Tris-HCl, 10 mM $(NH4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8], 1 µM KOD-RI, 100 µM of tNTP containing 5-alkynyl-tUTP **8a** or **8c**, and 1 mM $MnCl_2$. Reaction was incubated for 1 h at 55°C, and the aqueous part was removed by SpeedVac concentrator and the residue was purified by 10% denaturing urea PAGE. The incorporation of 5-alkynyl-tUTP **8a** or **8c** into the TNA oligonucleotides was confirmed by mass spectrum (for **8a**, calculated: 10456.8, found: 10460.0; for **8c**, calculated: 10790.1, found: 10786.6).

Click reactions performed on purified TNAs with benzyl azide. To 5 µL of a 4 µM TNA aqueous solution (20 pmol) obtained from TNA synthesis step based on DNA template (5'-TCTCT ATAGT GAGTC GTATA GGTGG TATCC TTTTT TTTTT TTTTT TTTTT), 1 µL of benzyl azide solution (in DCM, 100 mM), 10 µL of a freshly prepared solution containing 0.1 M CuBr and 0.1 M TBTA ligand in a 1.2 ratio in 3:1 DMSO/*t*-BuOH was added. The mixture was thoroughly mixed and shaken at 37 ^oC for 16 h. The reaction was subsequently diluted with 0.3 M NaOAc (10 µL) and the TNA precipitated using 0.5 mL cold EtOH. The washed residue was redissolved in pure water (40 µL) and 10 µL was

used for PAGE analysis without further purification.

Click reactions performed on purified TNAs with IR-800 azide. To 10 µL of a 12 µM TNA aqueous solution (120 pmol) obtained from TNA synthesis step based on DNA template (5'- TCTCT ATTGT GTGTC GTCTC GGTGG TATCC TTTTT TTTTT TTTTT TTTTT), 1 µL of IR-800 azide solution (in DMSO, 100 mM), 10 µL of a freshly prepared solution containing 0.1 M CuBr and 0.1 M TBTA ligand in a 1.2 ratio in 3:1 DMSO/*t*-BuOH was added. The mixture was thoroughly mixed and shaken at 37 ^{o}C for 16 h. The reaction is subsequently diluted with 0.3 M NaOAc (20 µL) and the TNA precipitated using 1 mL cold EtOH. The washed residue was purified by denaturing PAGE and then used for PAGE analysis.

References:

1. Nikoomanzar, A.; Dunn, M. R.; Chaput, J. C., *Curr. Protoc. Nucleic Acid Chem.* **2017,** *69*, 4.75.1-4.75.20.
2. Bala, S.; Liao, J. Y.; Mei, H.; Chaput, J. C., *J. Org. Chem.* **2017,** *82*, 5910-5916.
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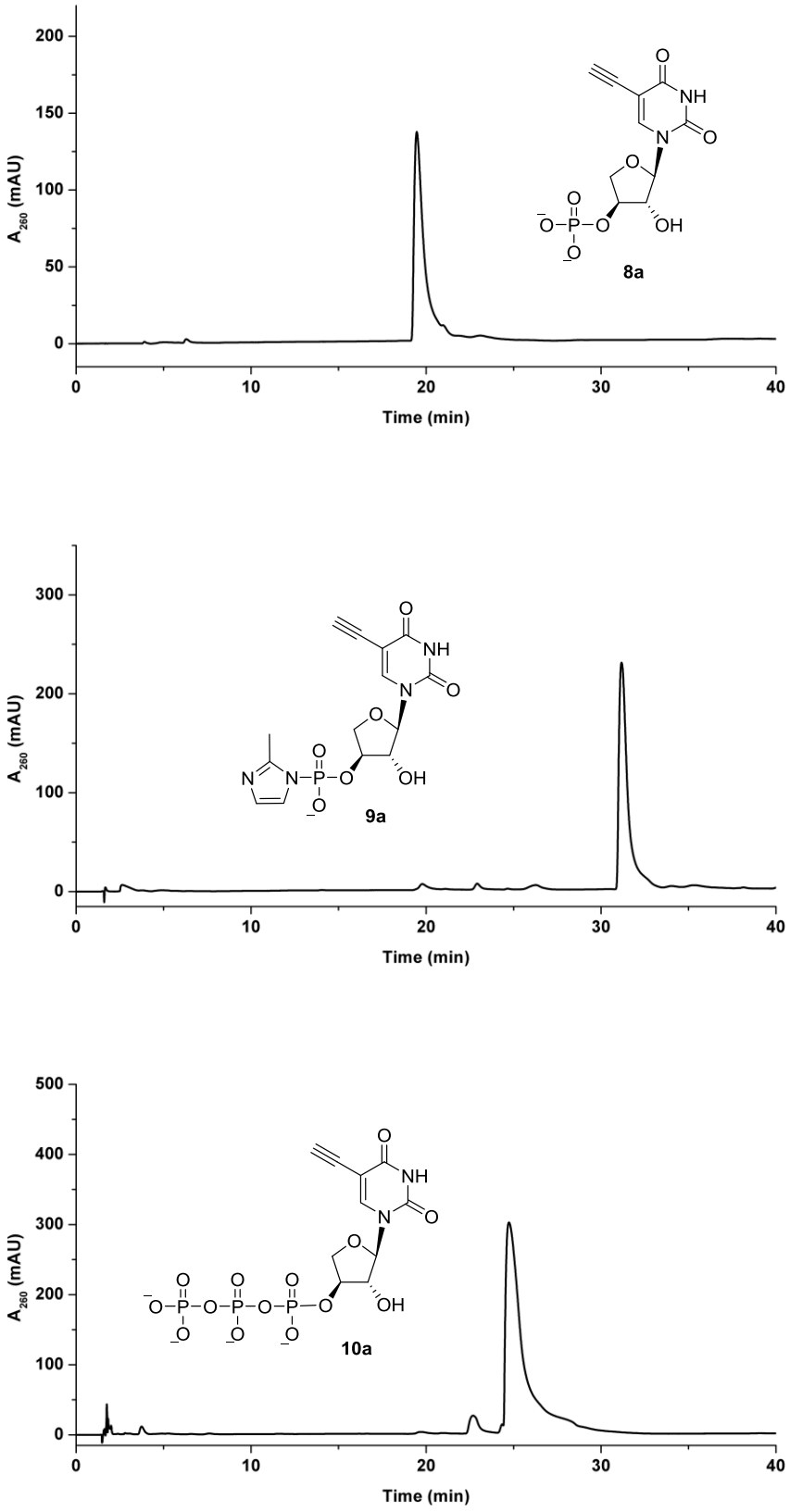
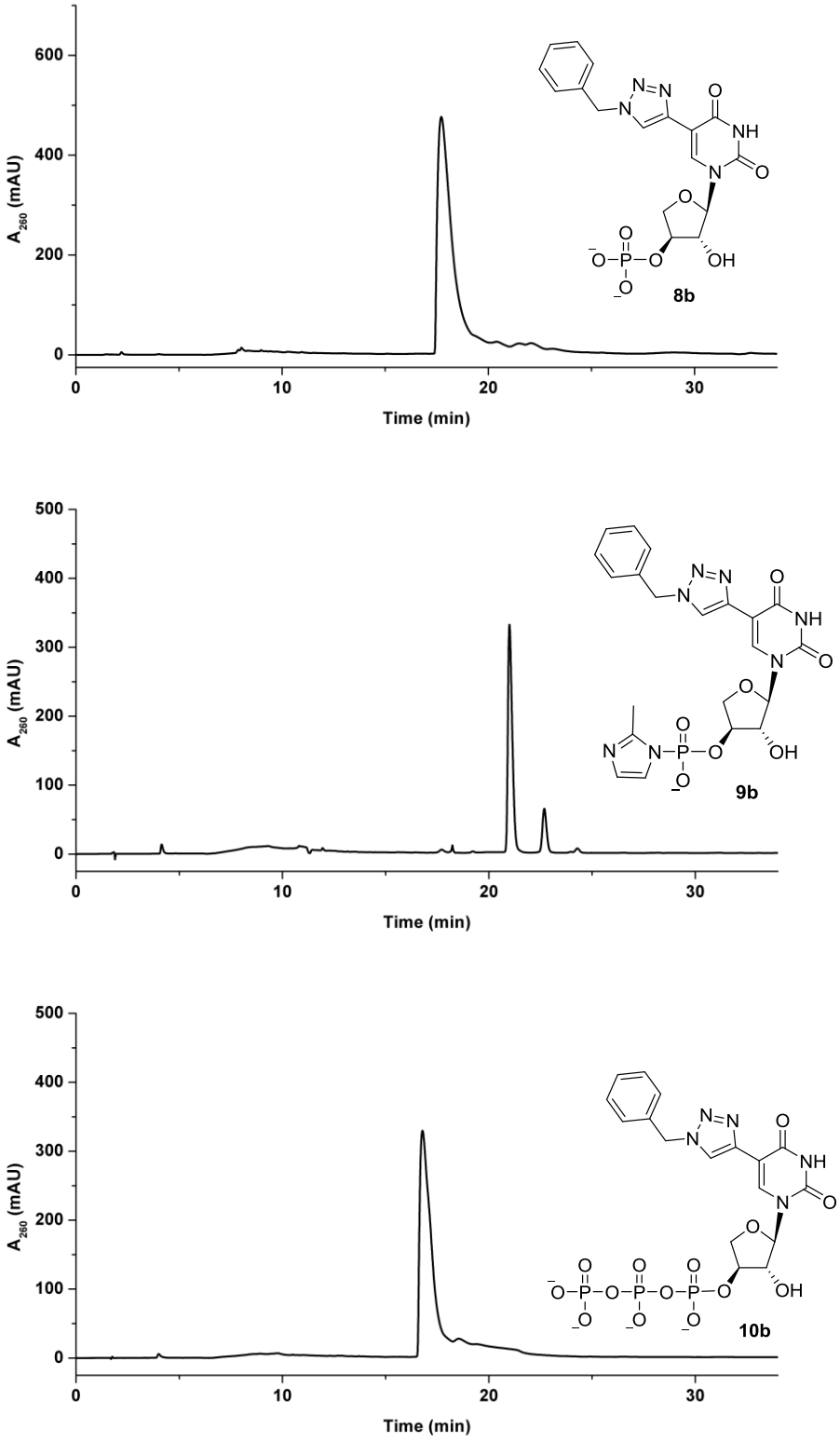
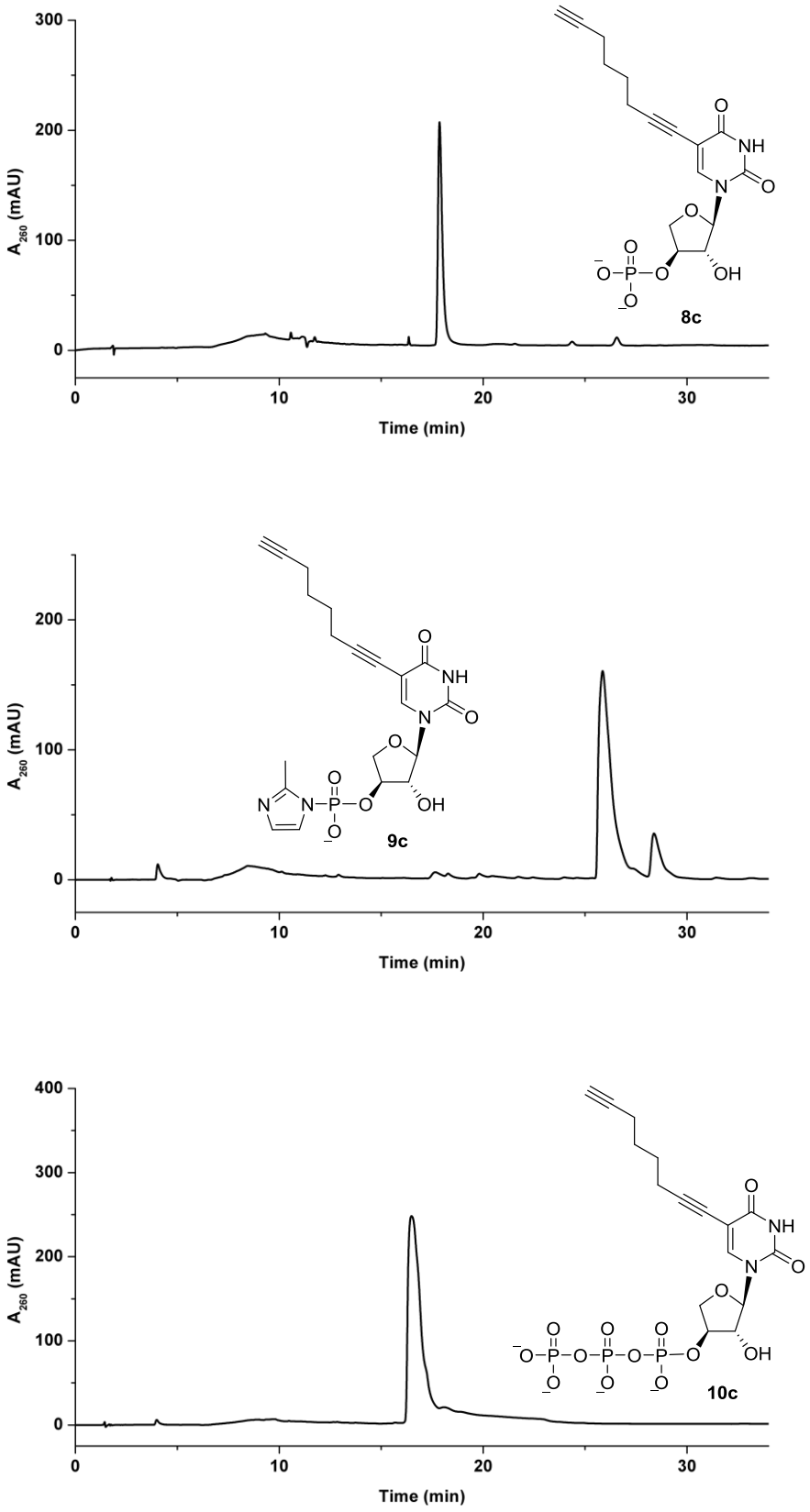
4. Kocalka, P.; Andersen, N. K.; Jensen, F.; Nielsen, P., *ChemBioChem* **2007,** *8*, 2106-2116.
5. Seela, F.; Sirivolu, V. R., *Helv. Chim. Acta* **2007,** *90*, 535-552.



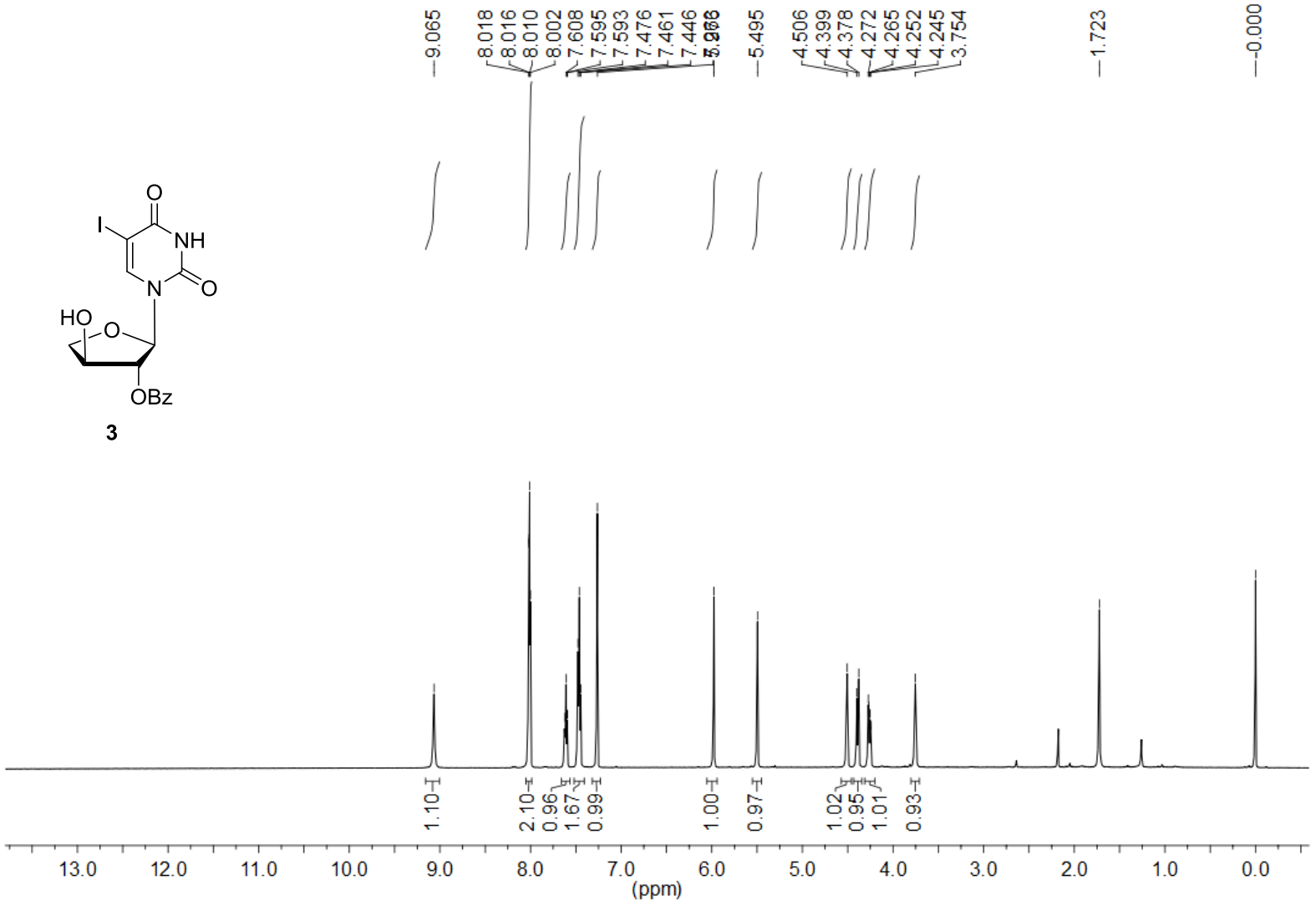
HPLC analysis of the TNA nucleoside 3'-monophosphate **8a**, 3'-phosphor-imidazolide **9a**, 3'-triphosphate **10a**.



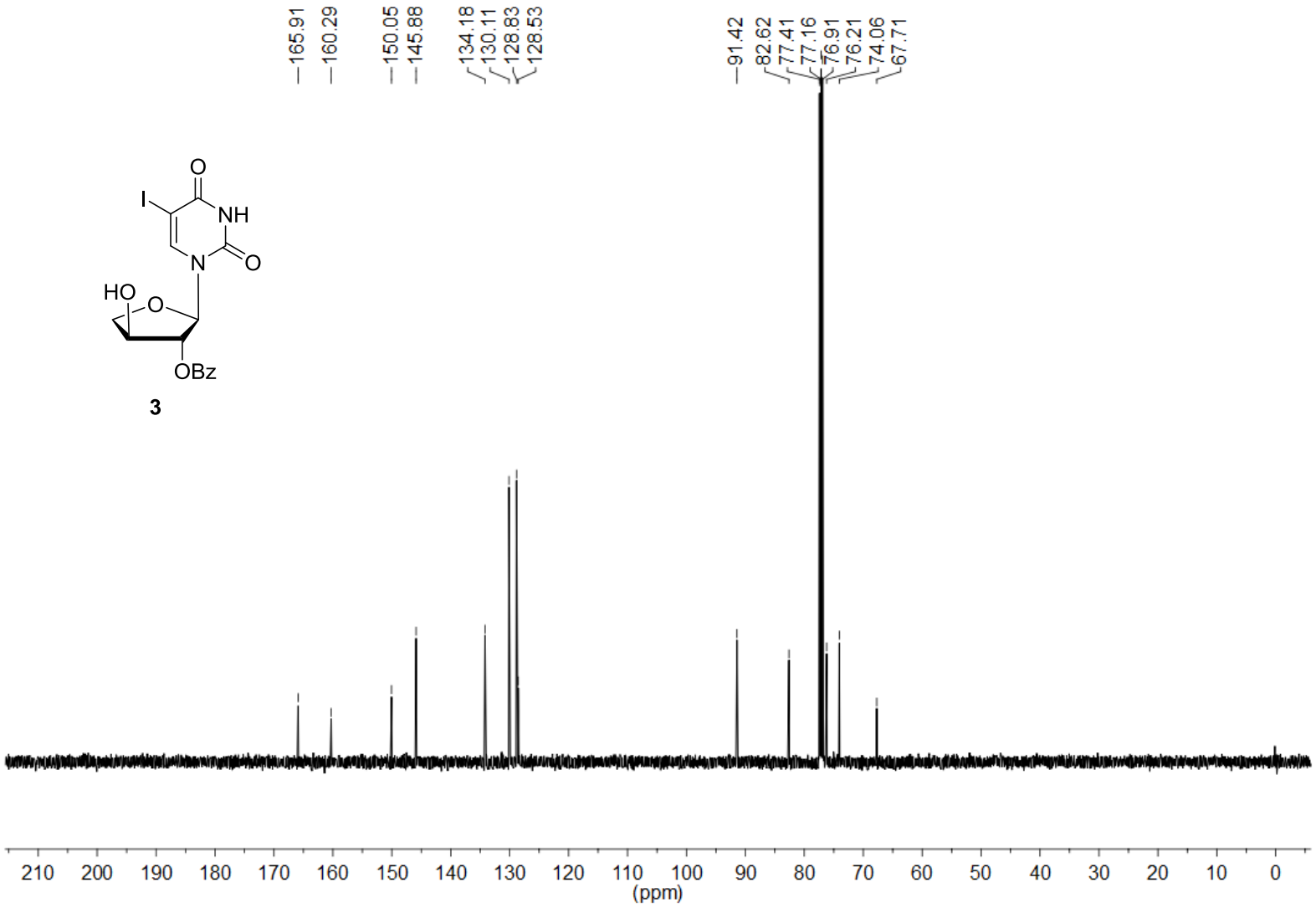
HPLC analysis of the TNA nucleoside 3'-monophosphate **8b**, 3'-phosphor-imidazolide **9b**, 3'-triphosphate **10b**.



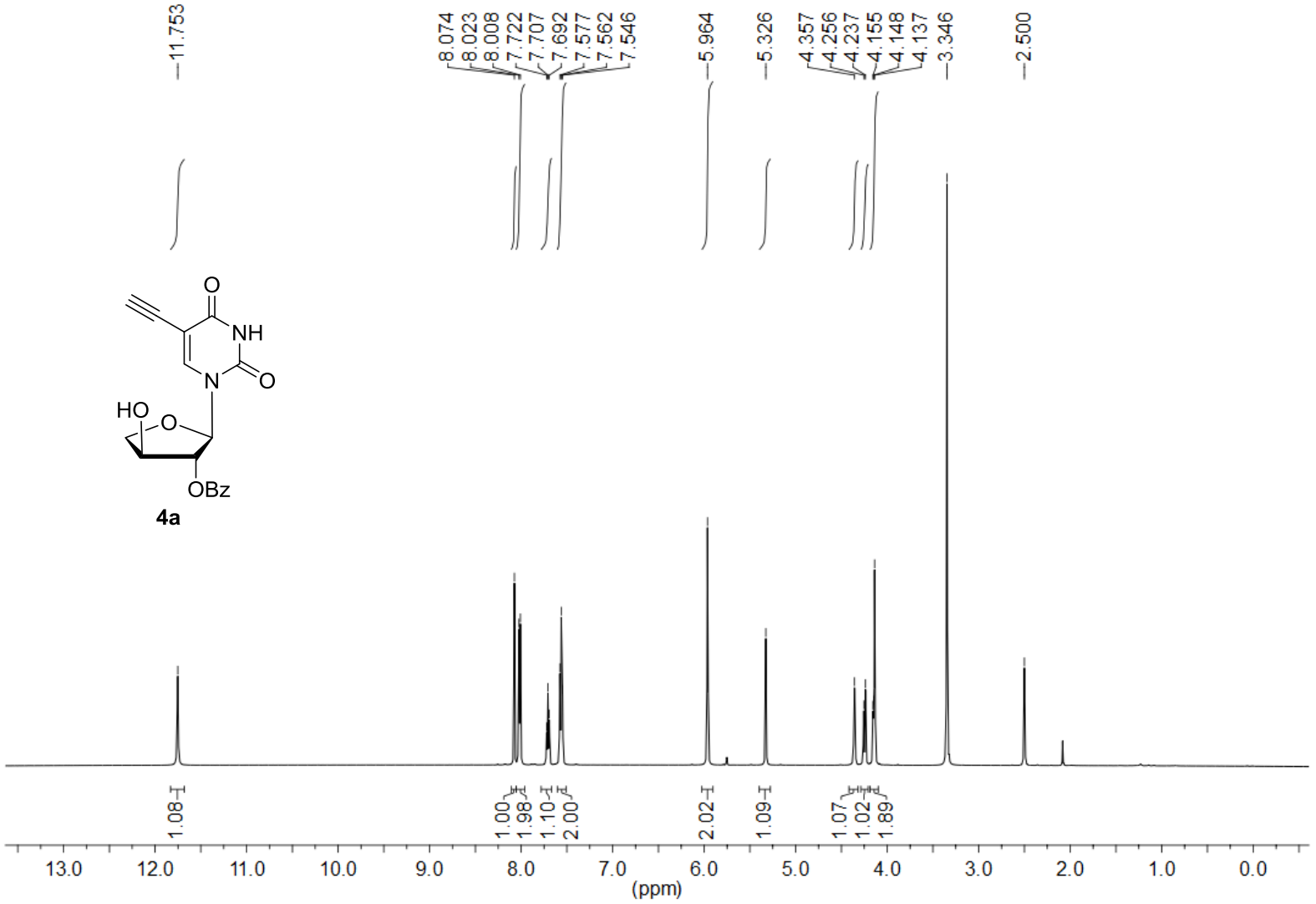
HPLC analysis of the TNA nucleoside 3'-monophosphate **8c**, 3'-phosphor-imidazolide **9c**, 3'-triphosphate **10c**.



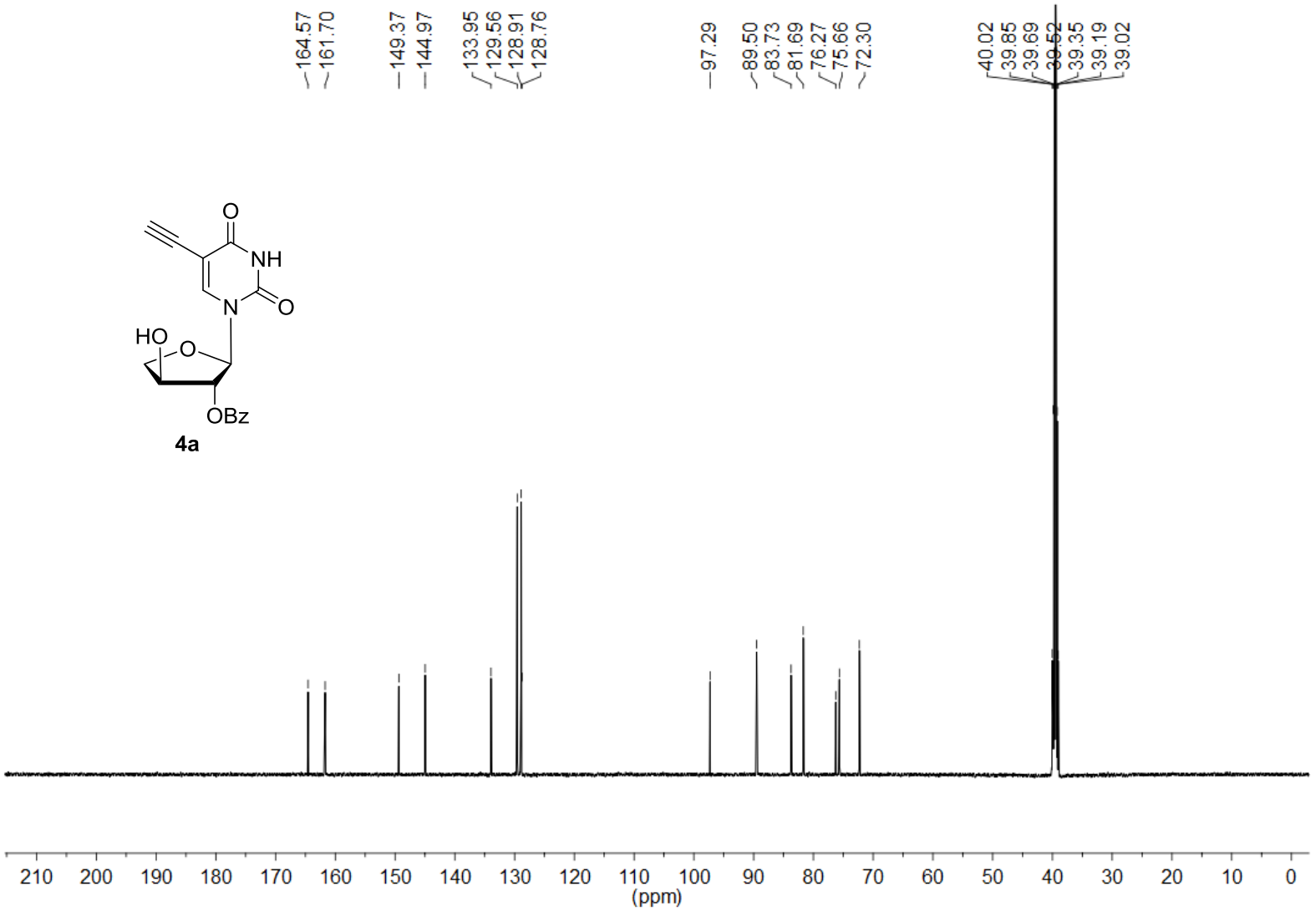
^{1}H NMR spectrum of compound **3** (500 MHz, $CDCl_3$)



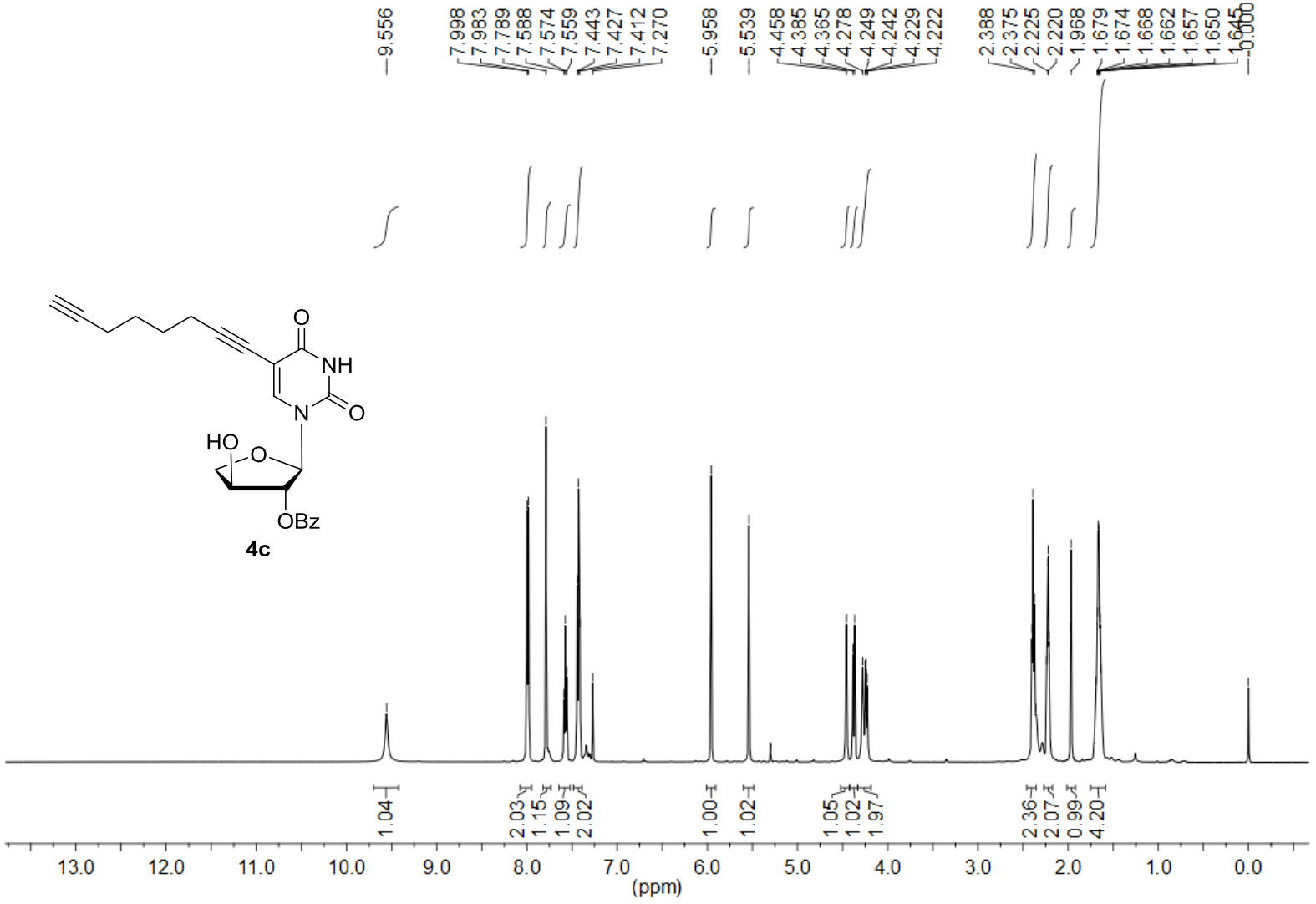
^{13}C NMR spectrum of compound **3** (126 MHz, $CDCl_3$)



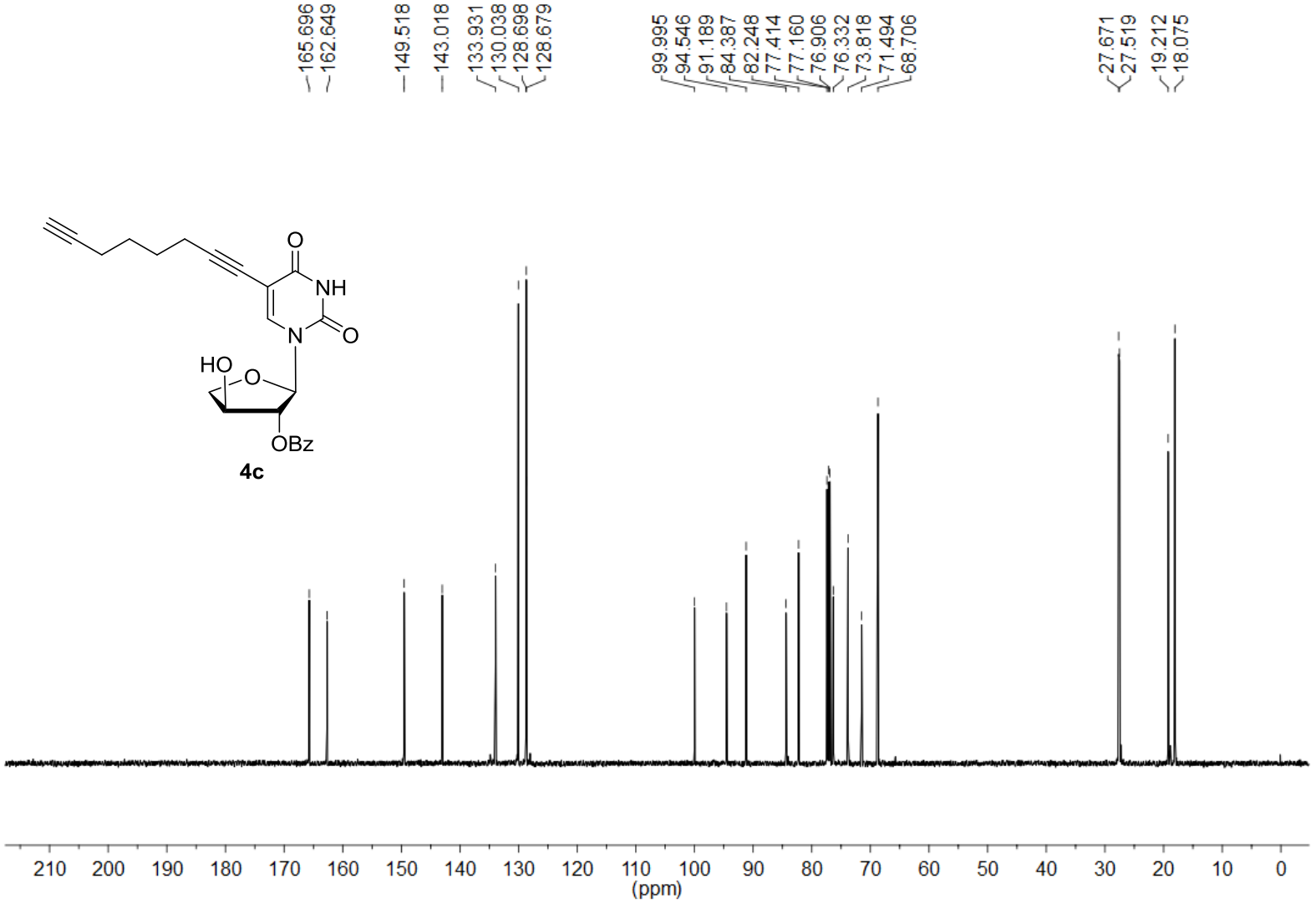
^{1}H NMR spectrum of compound **4a** (500 MHz, DMSO-d6)



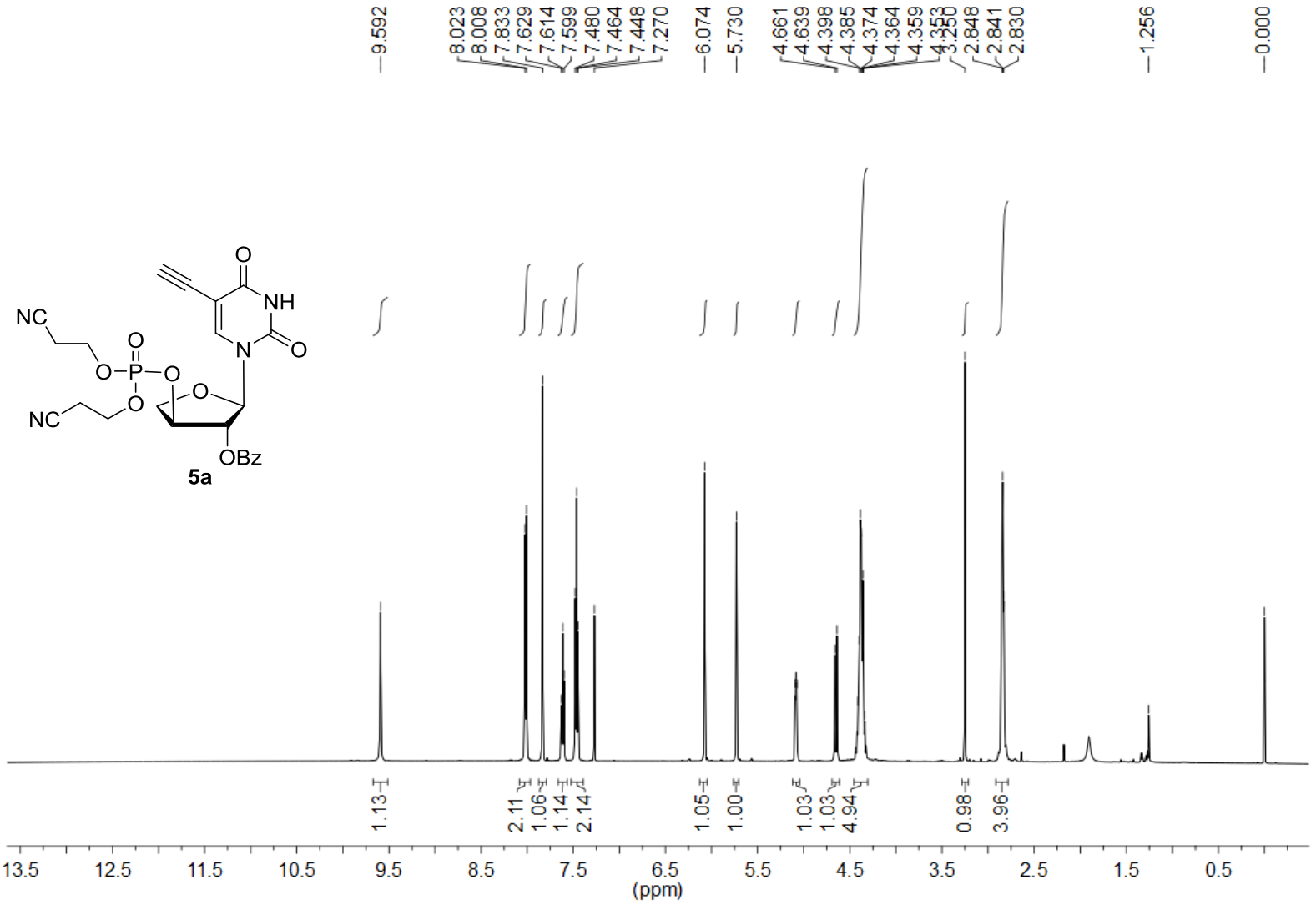
^{13}C NMR spectrum of compound **4a** (126 MHz, DMSO-d6)



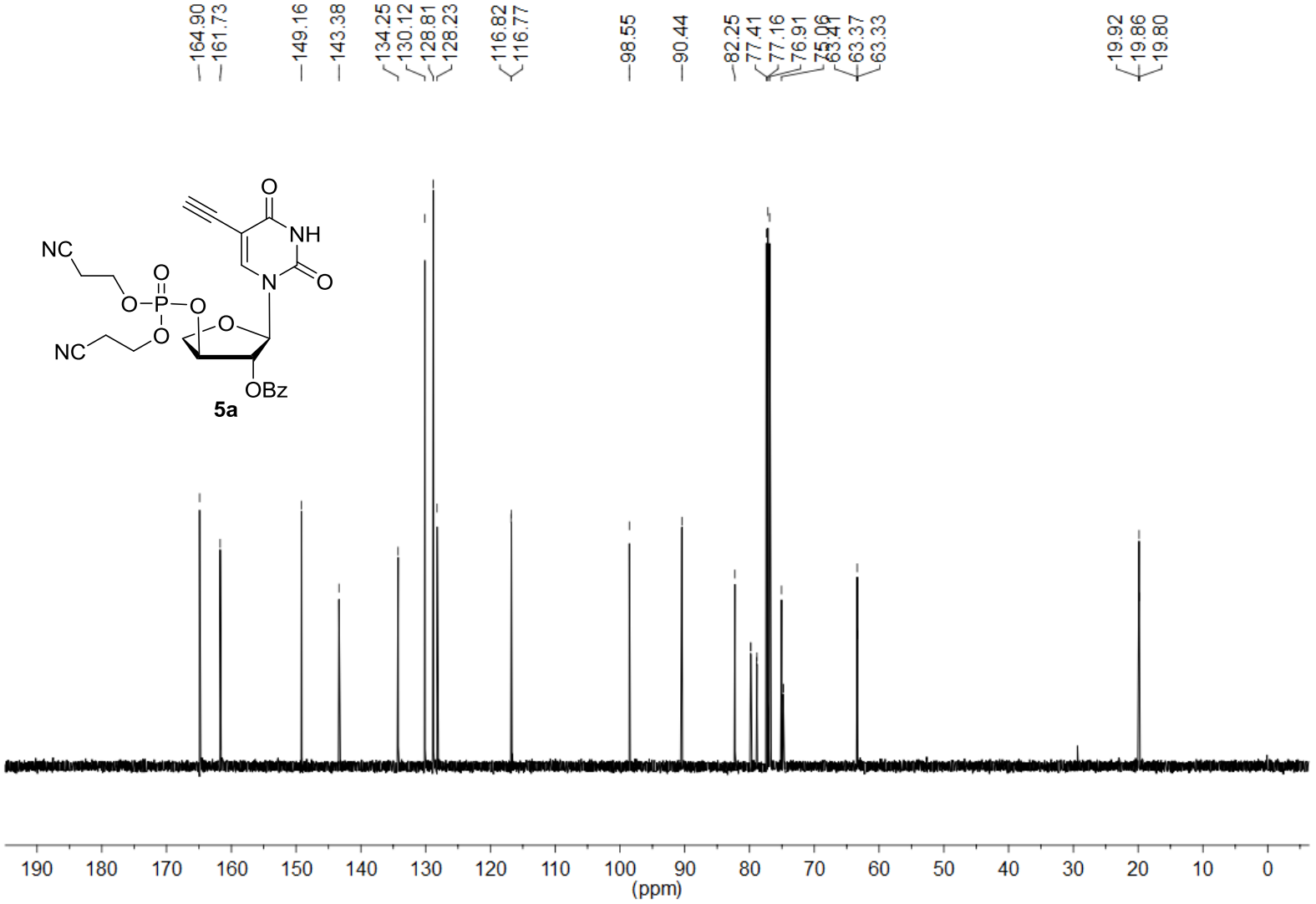
^{1}H NMR spectrum of compound **4c** (500 MHz, $CDCl_3$)



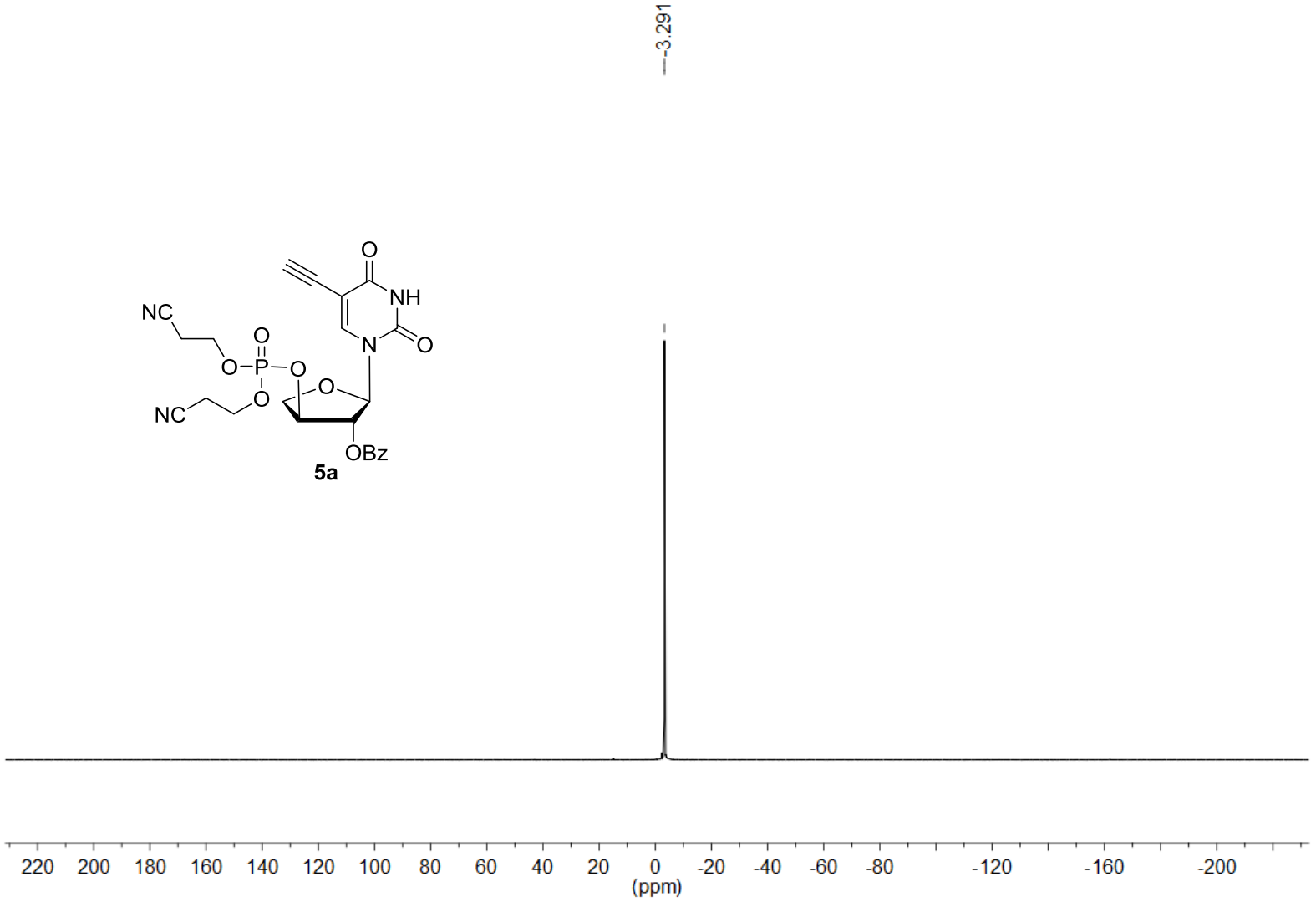
^{13}C NMR spectrum of compound **4c** (126 MHz, $CDCl_3$)



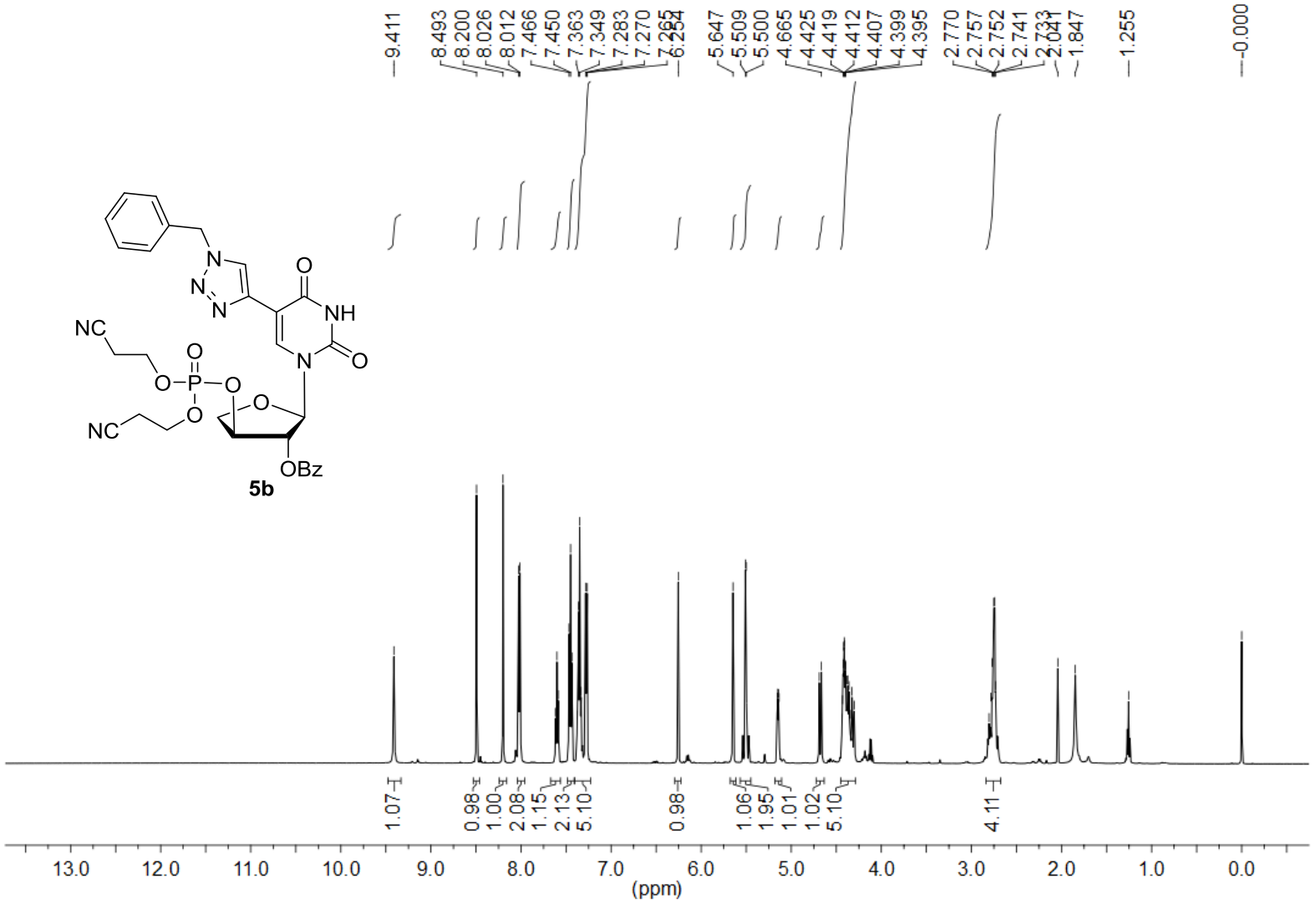
^{1}H NMR spectrum of compound **5a** (500 MHz, $CDCl_3$)



^{13}C NMR spectrum of compound **5a** (126 MHz, $CDCl_3$)



^{31}P NMR spectrum of compound **5a** (162 MHz, $CDCl_3$)



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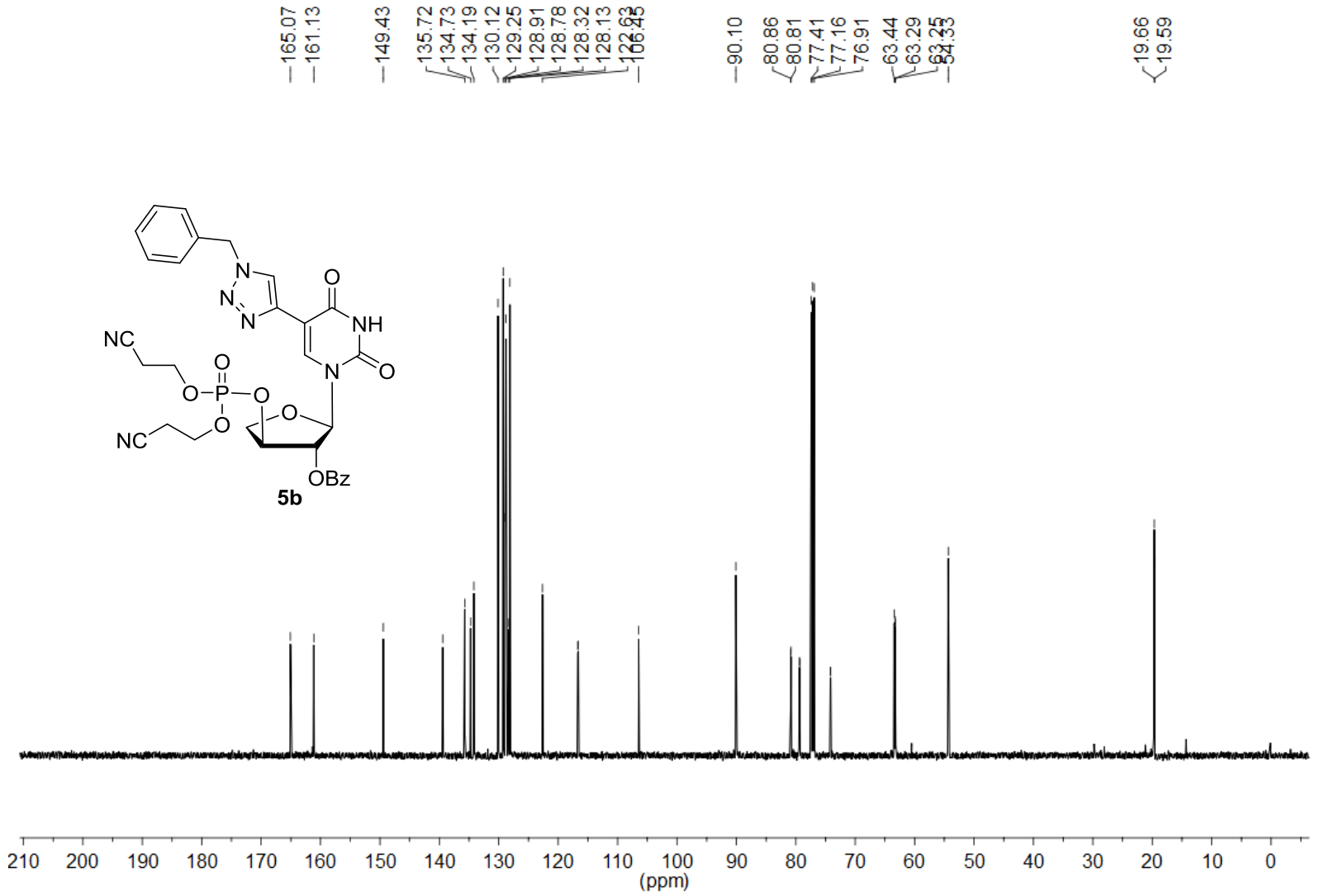
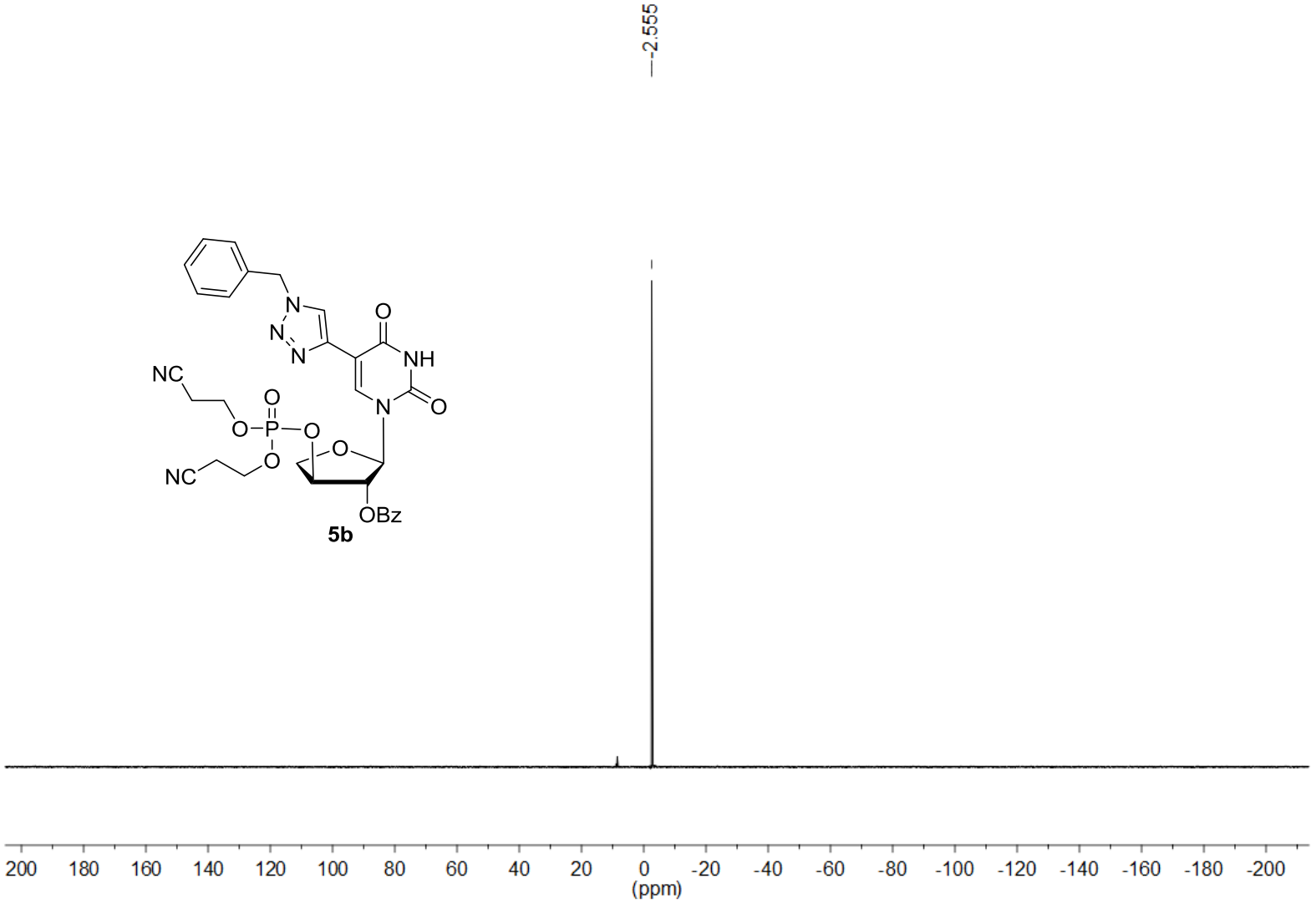
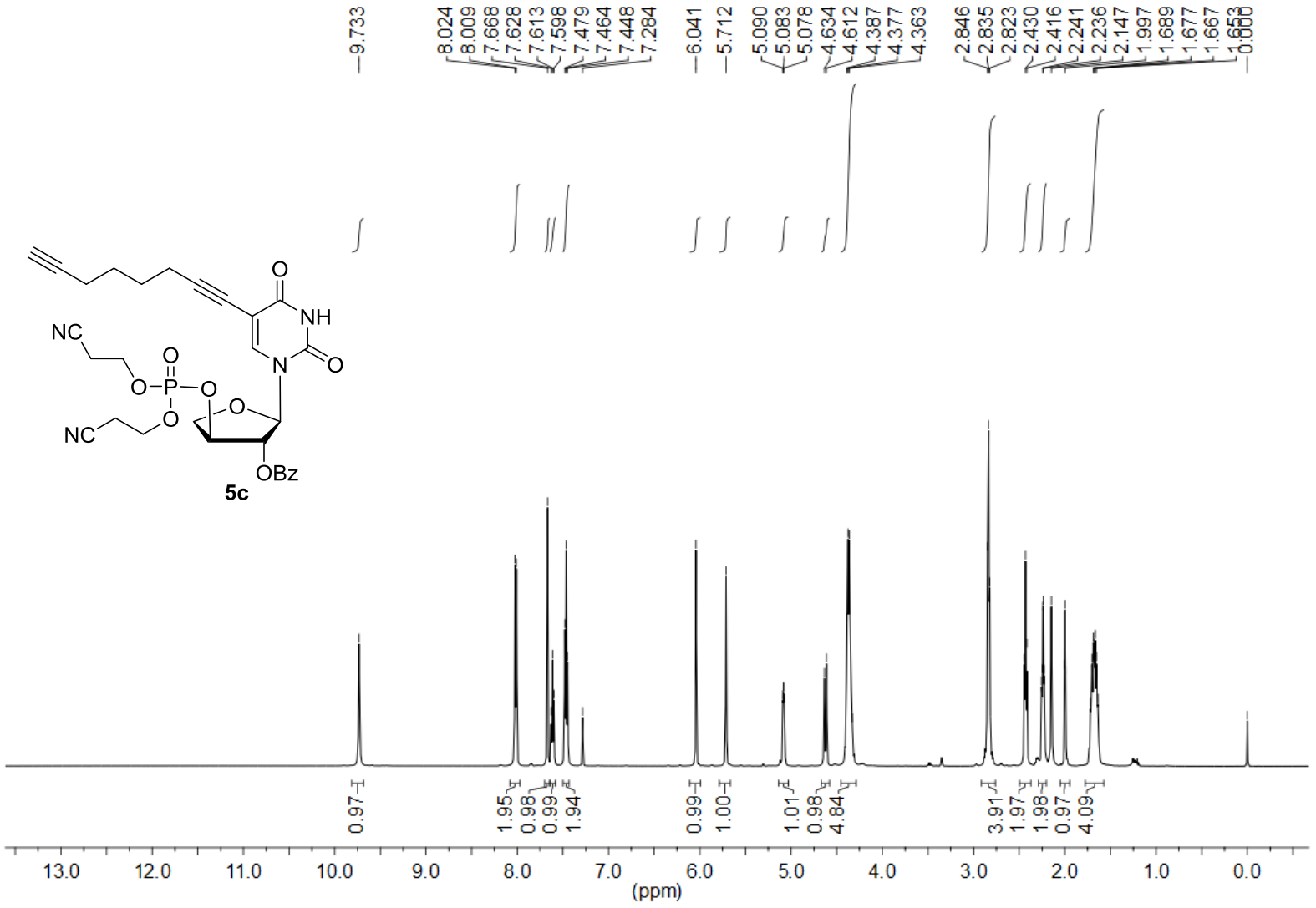


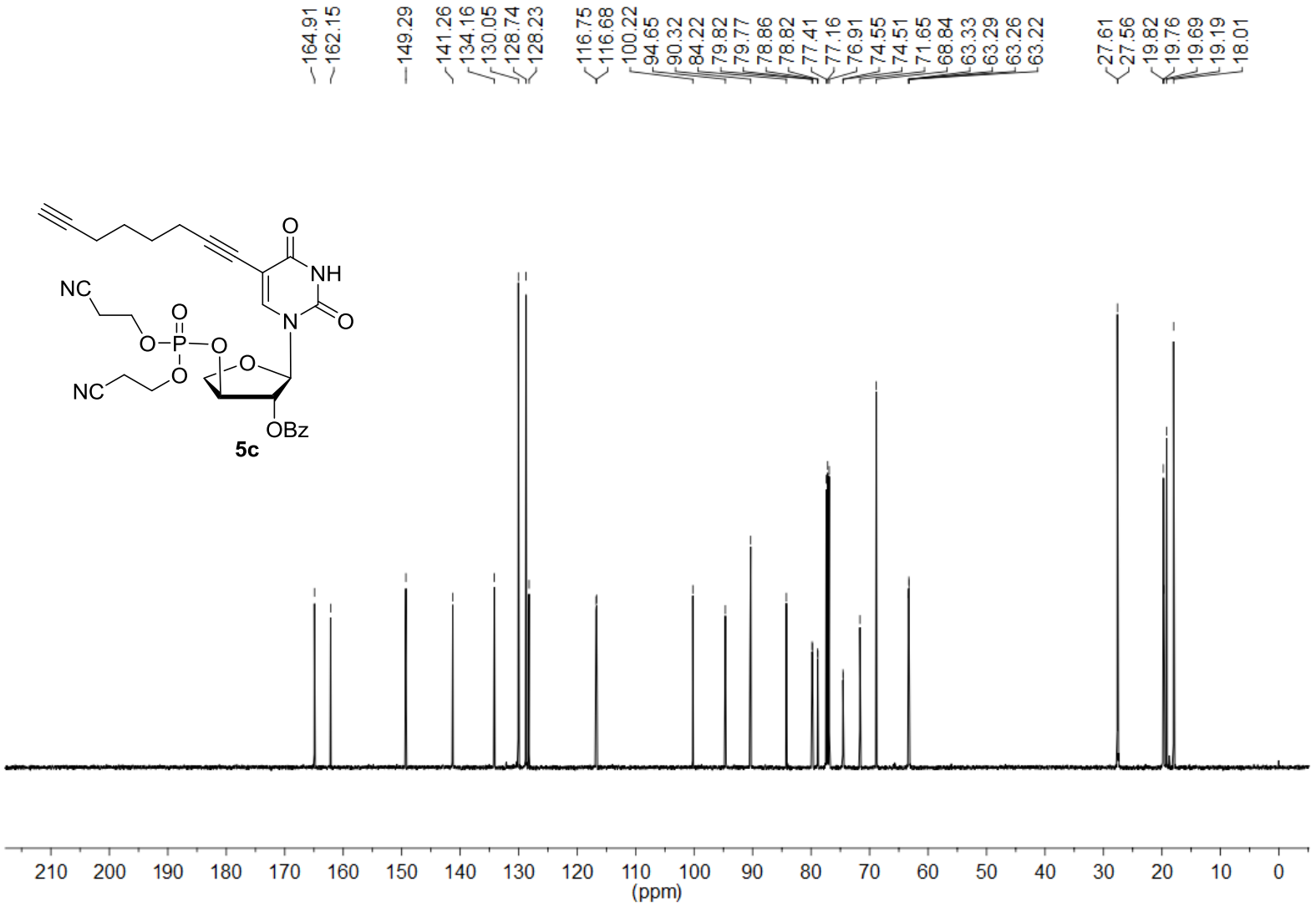
^{13}C NMR spectrum of compound **5b** (126 MHz, $CDCl_3$)



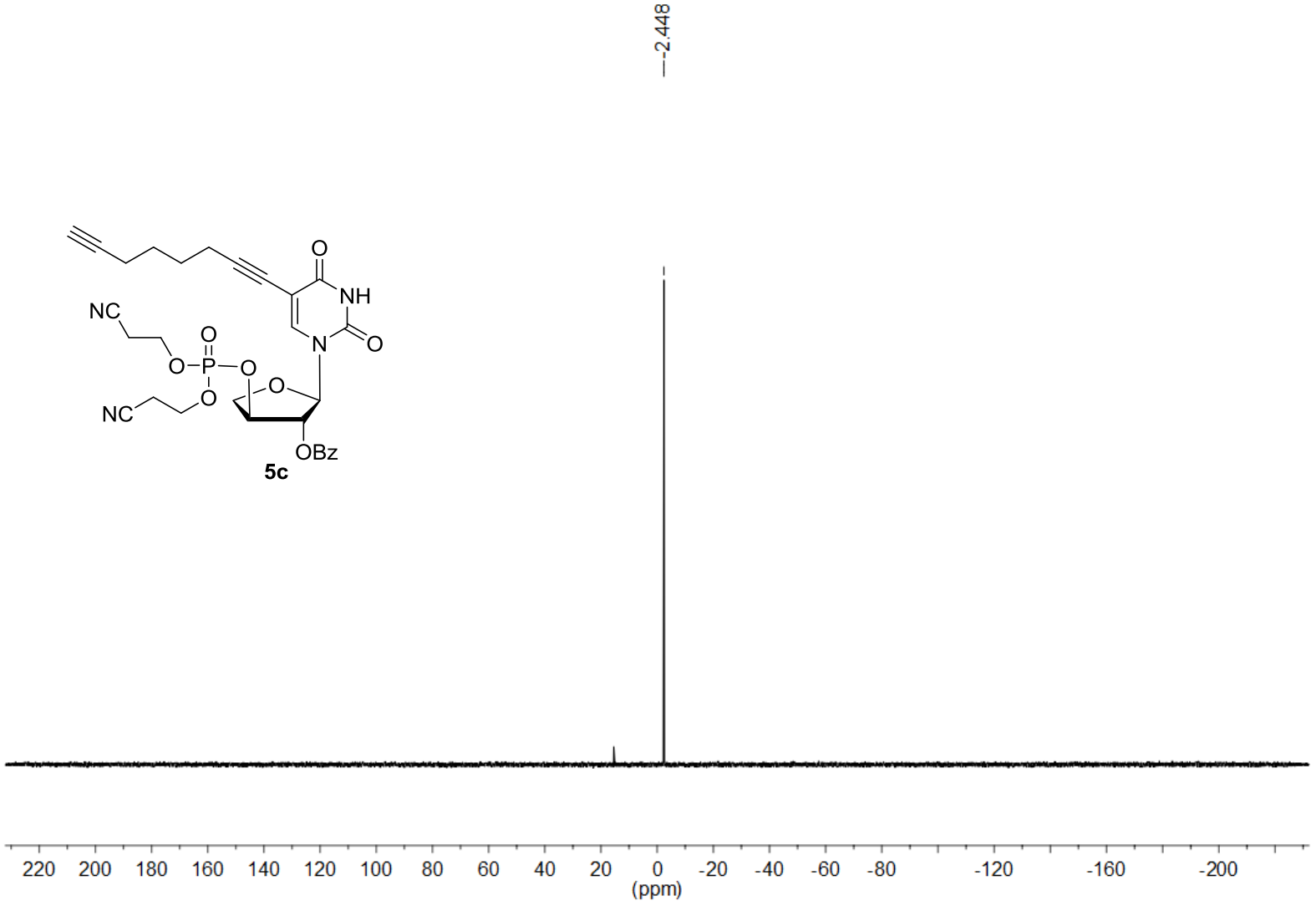
^{31}P NMR spectrum of compound **5b** (162 MHz, $CDCl_3$)



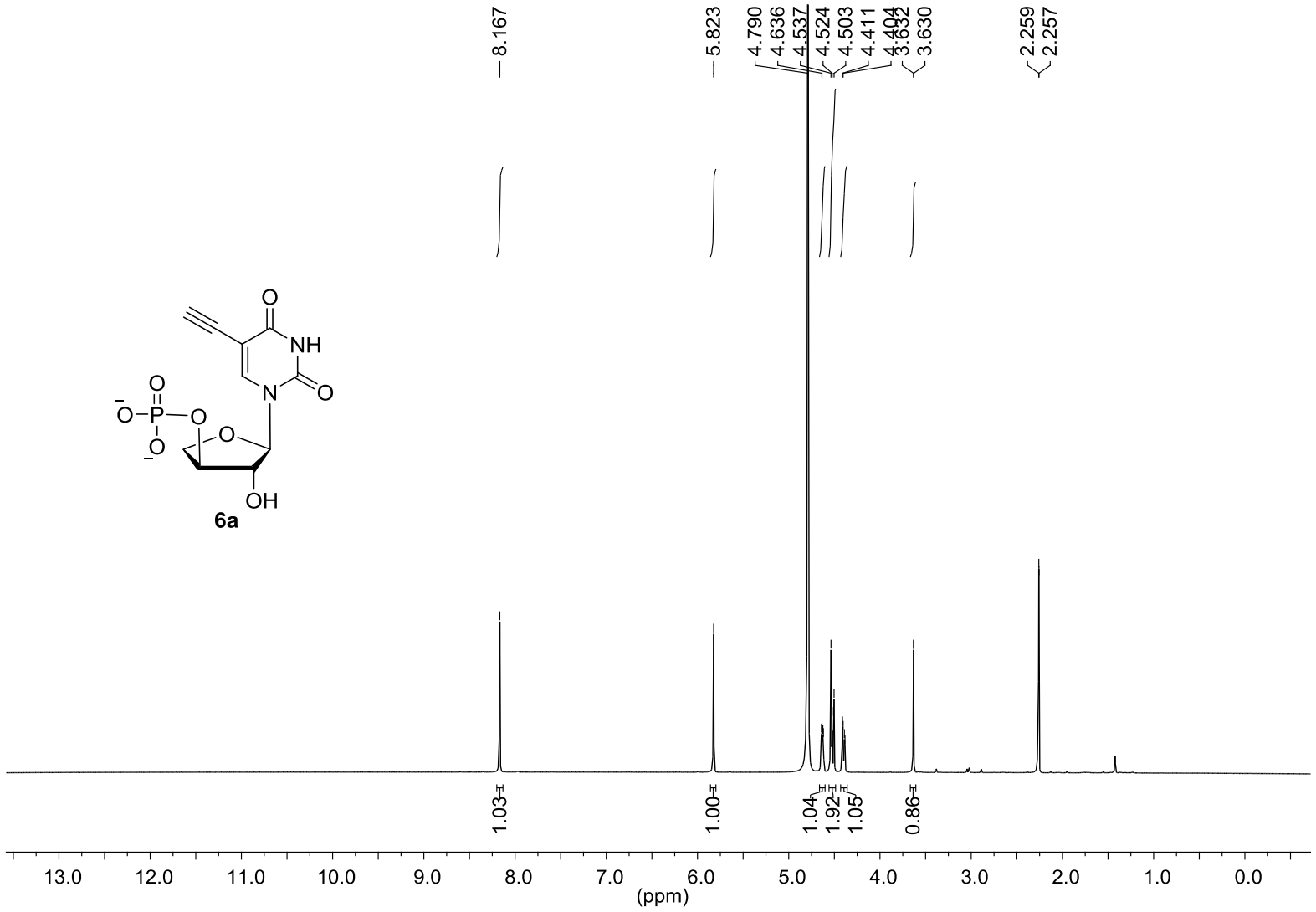
^{1}H NMR spectrum of compound **5c** (500 MHz, $CDCl_3$)



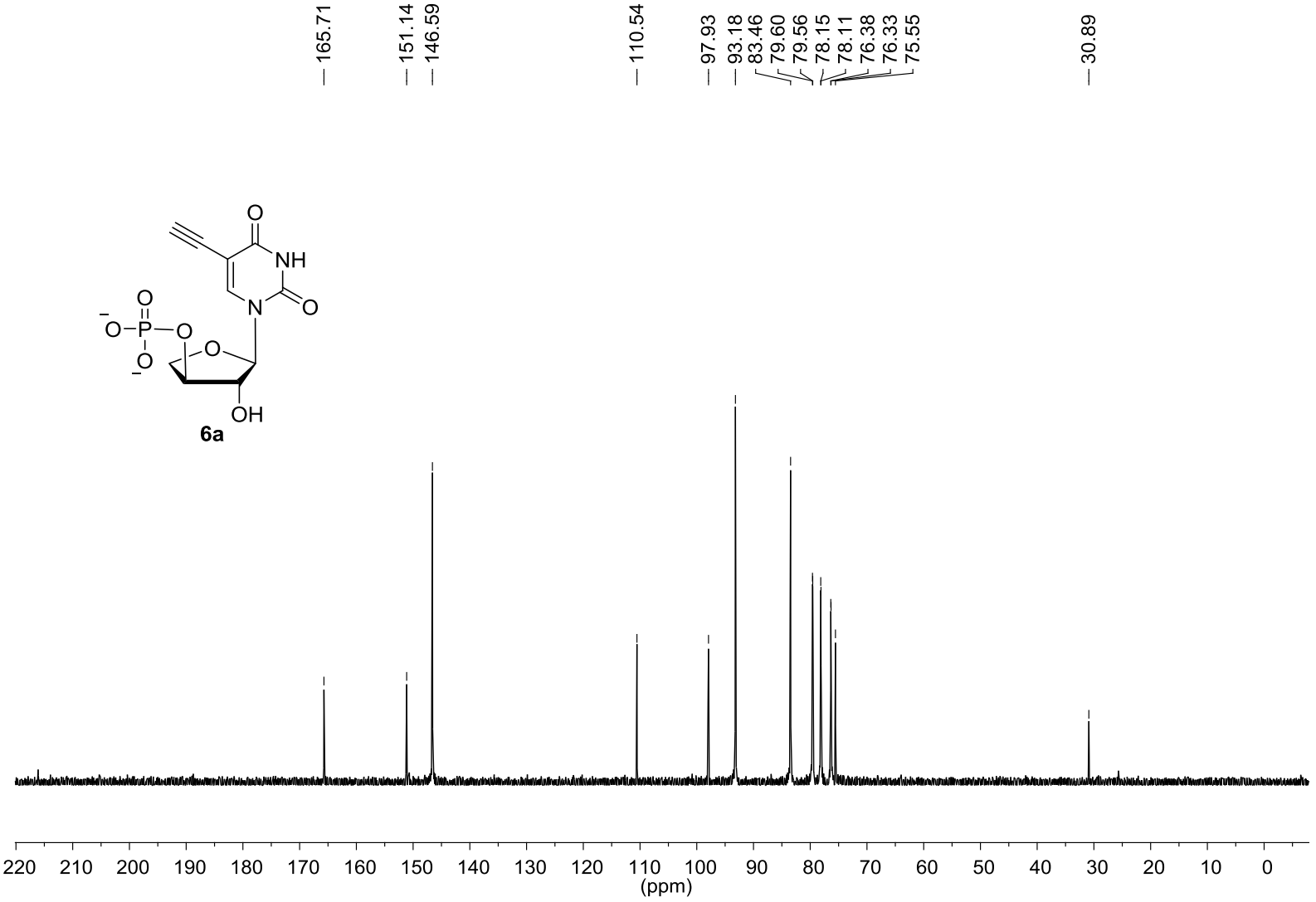
^{13}C NMR spectrum of compound **5c** (126 MHz, $CDCl_3$)



^{31}P NMR spectrum of compound **5c** (162 MHz, $CDCl_3$)



^{1}H NMR spectrum of compound **6a** (500 MHz, D_2O)



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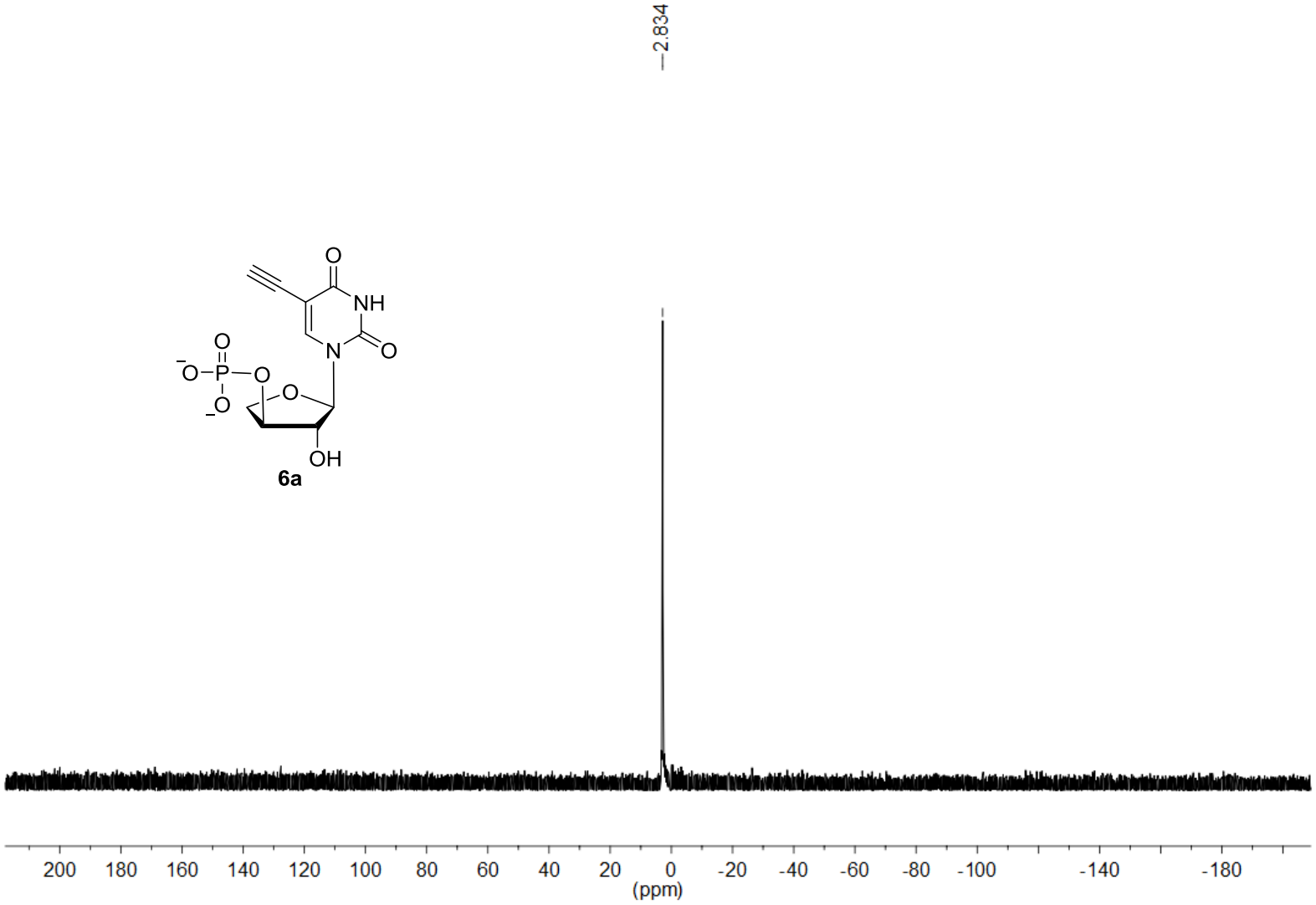

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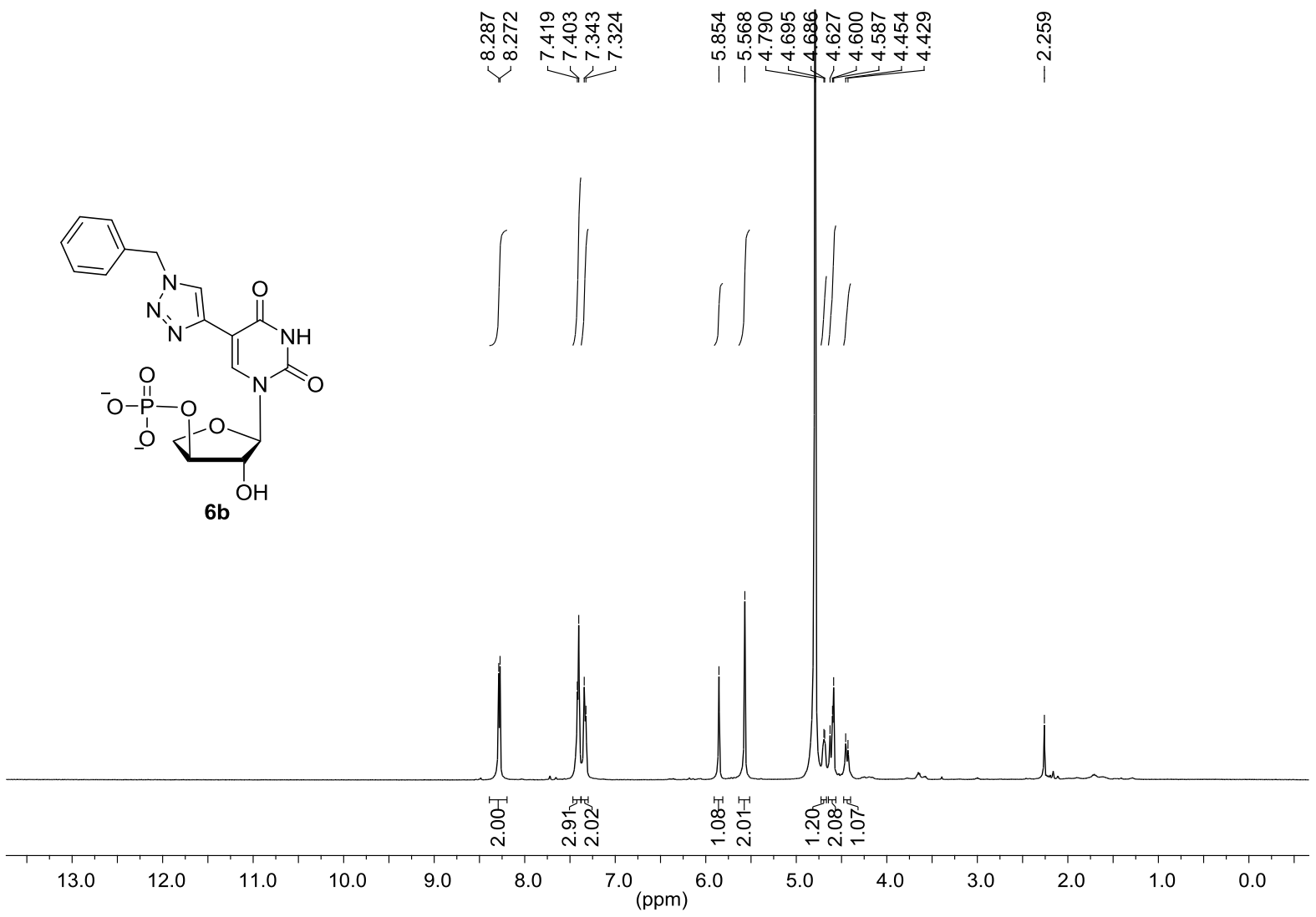
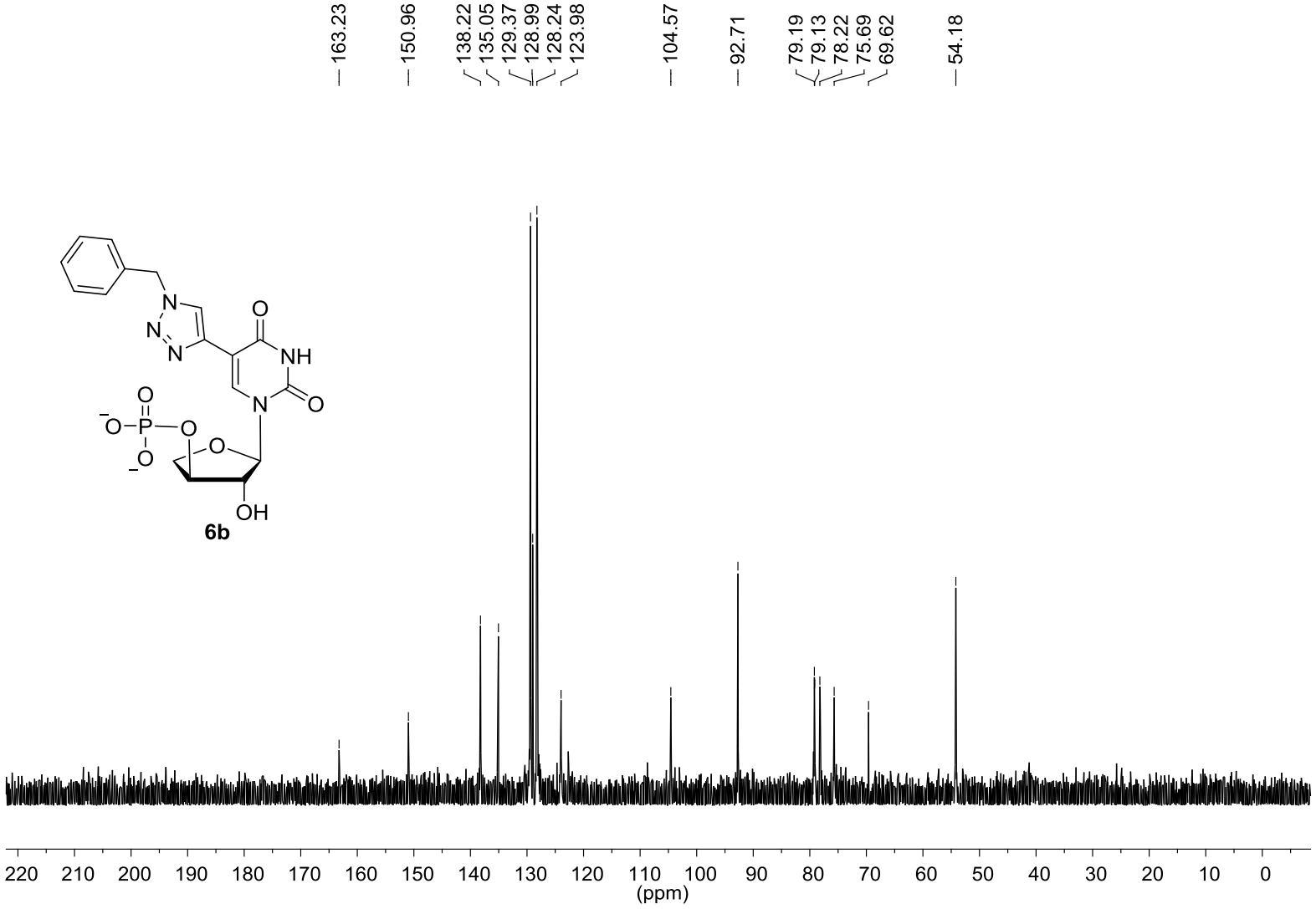
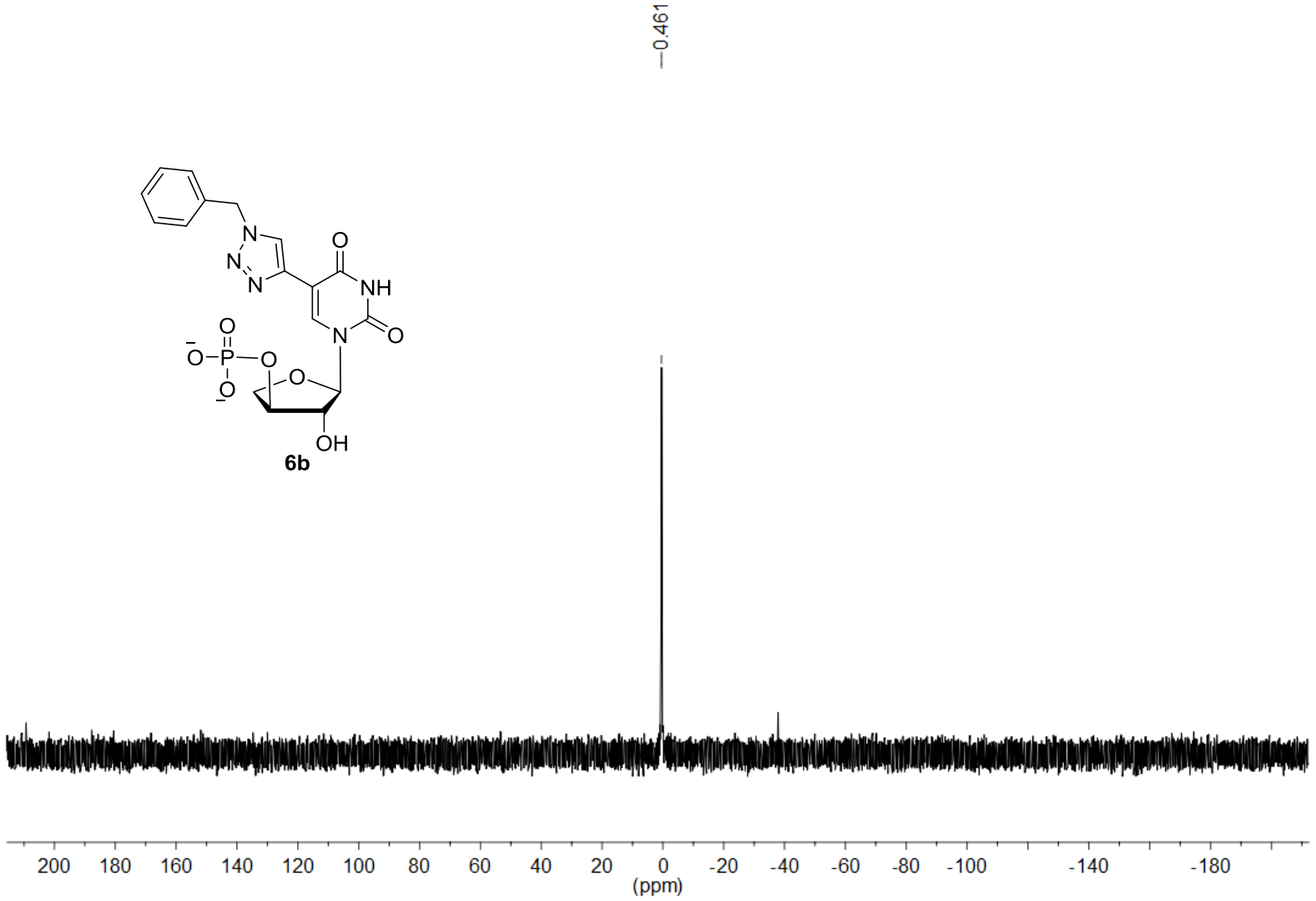


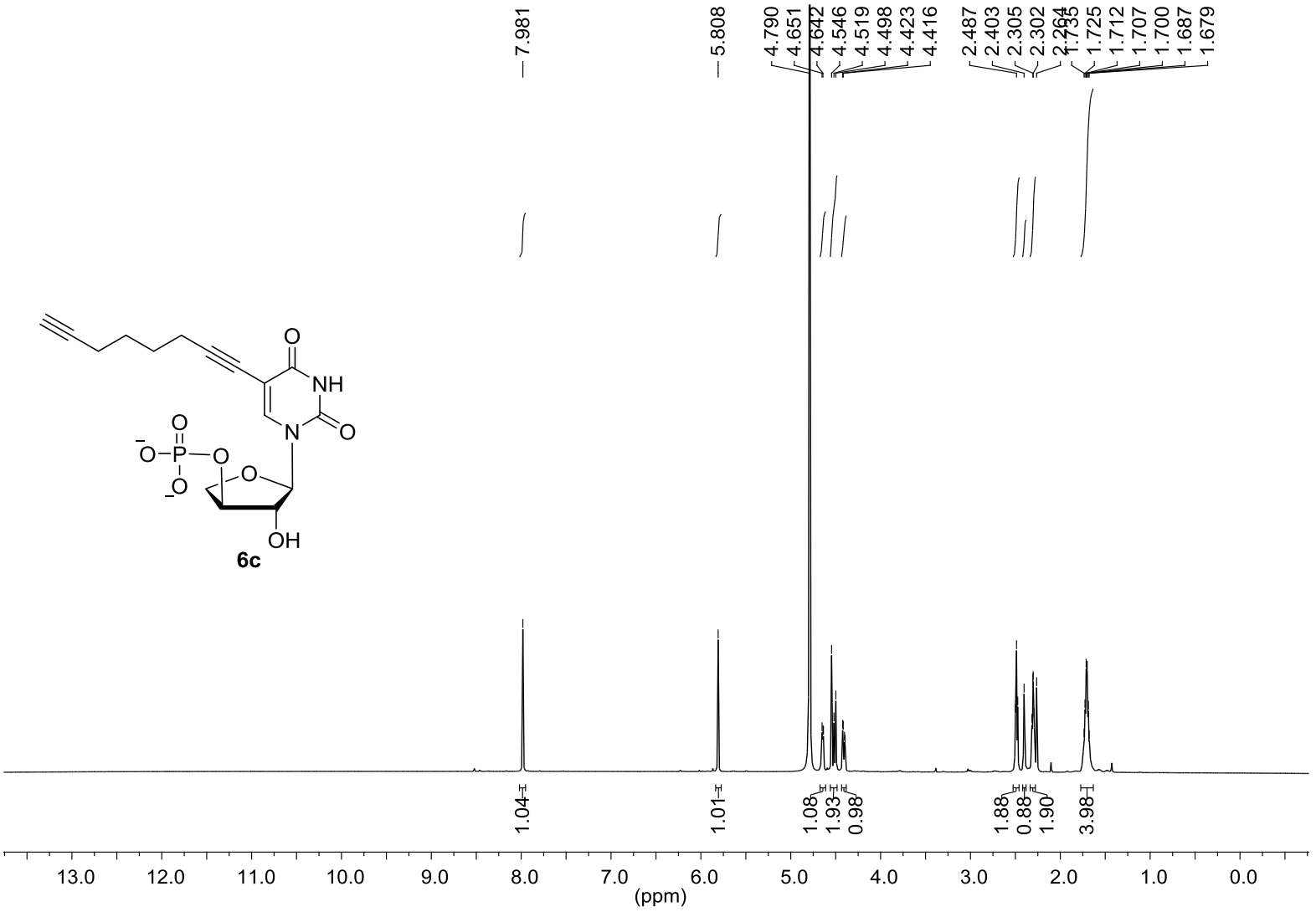
^{1}H NMR spectrum of compound **6b** (400 MHz, D_2O)



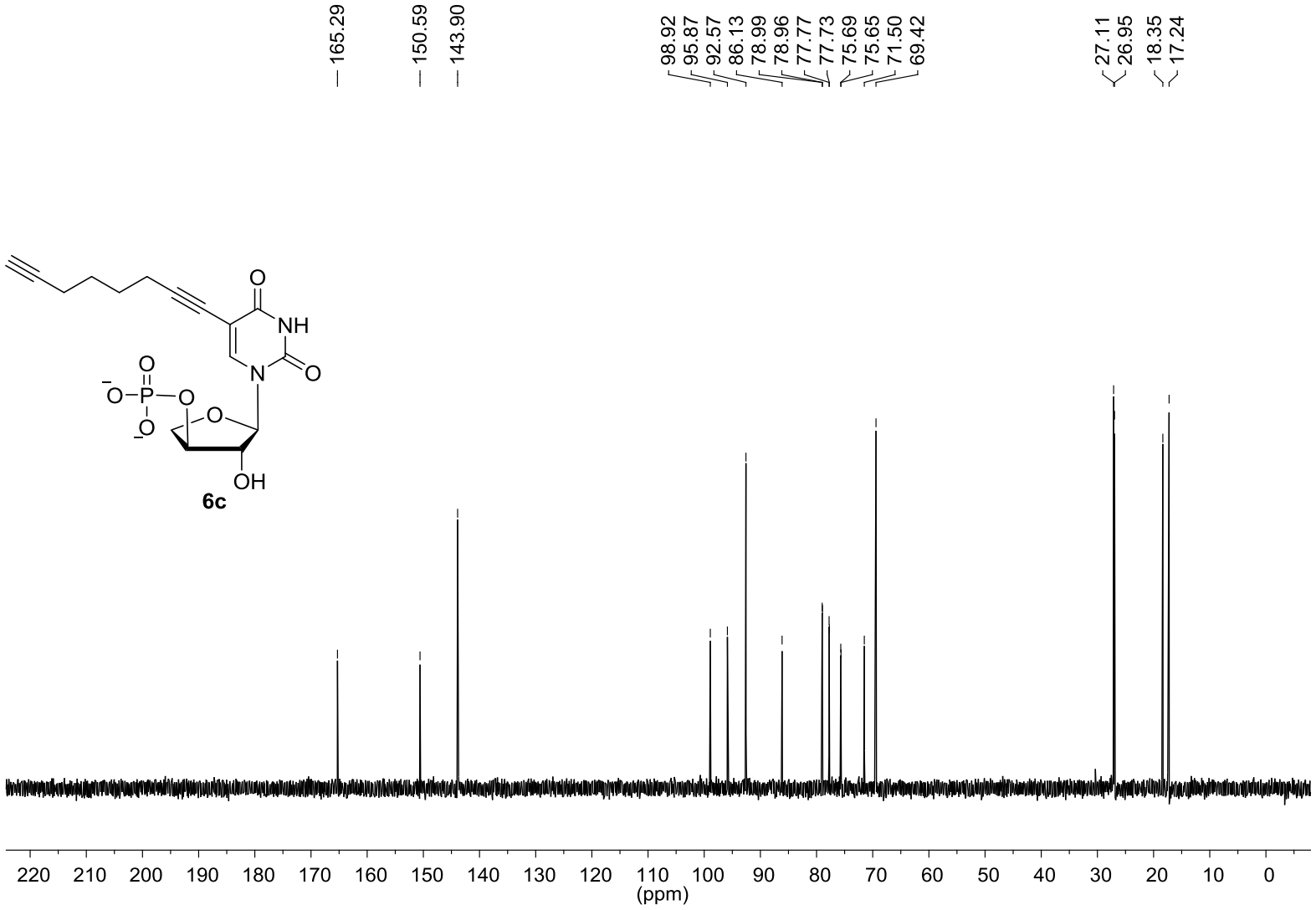
^{13}C NMR spectrum of compound **6b** (100 MHz, D_2O)



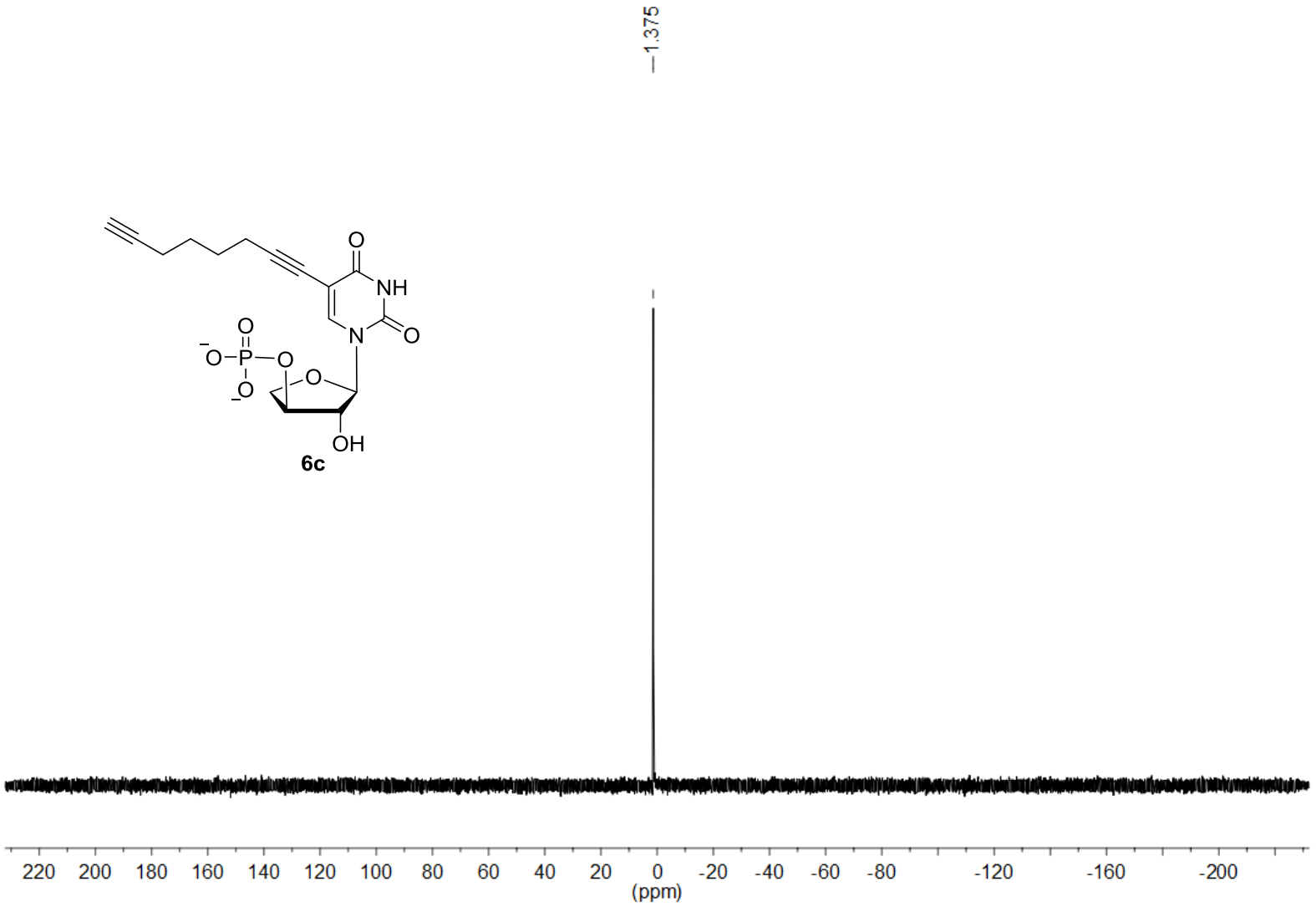
^{31}P NMR spectrum of compound **6b** (162 MHz, D_2O)



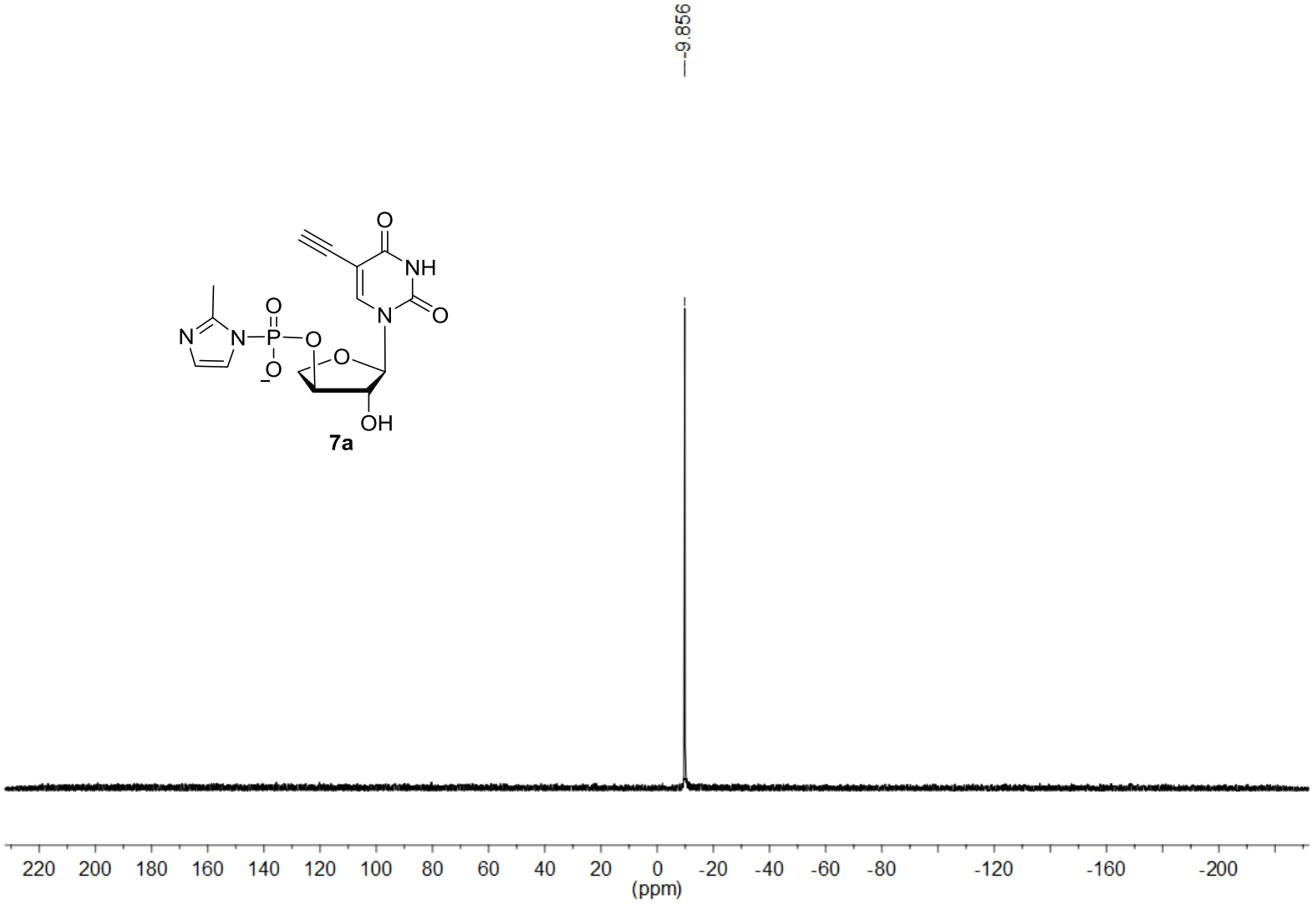
^{1}H NMR spectrum of compound **6c** (500 MHz, D_2O)



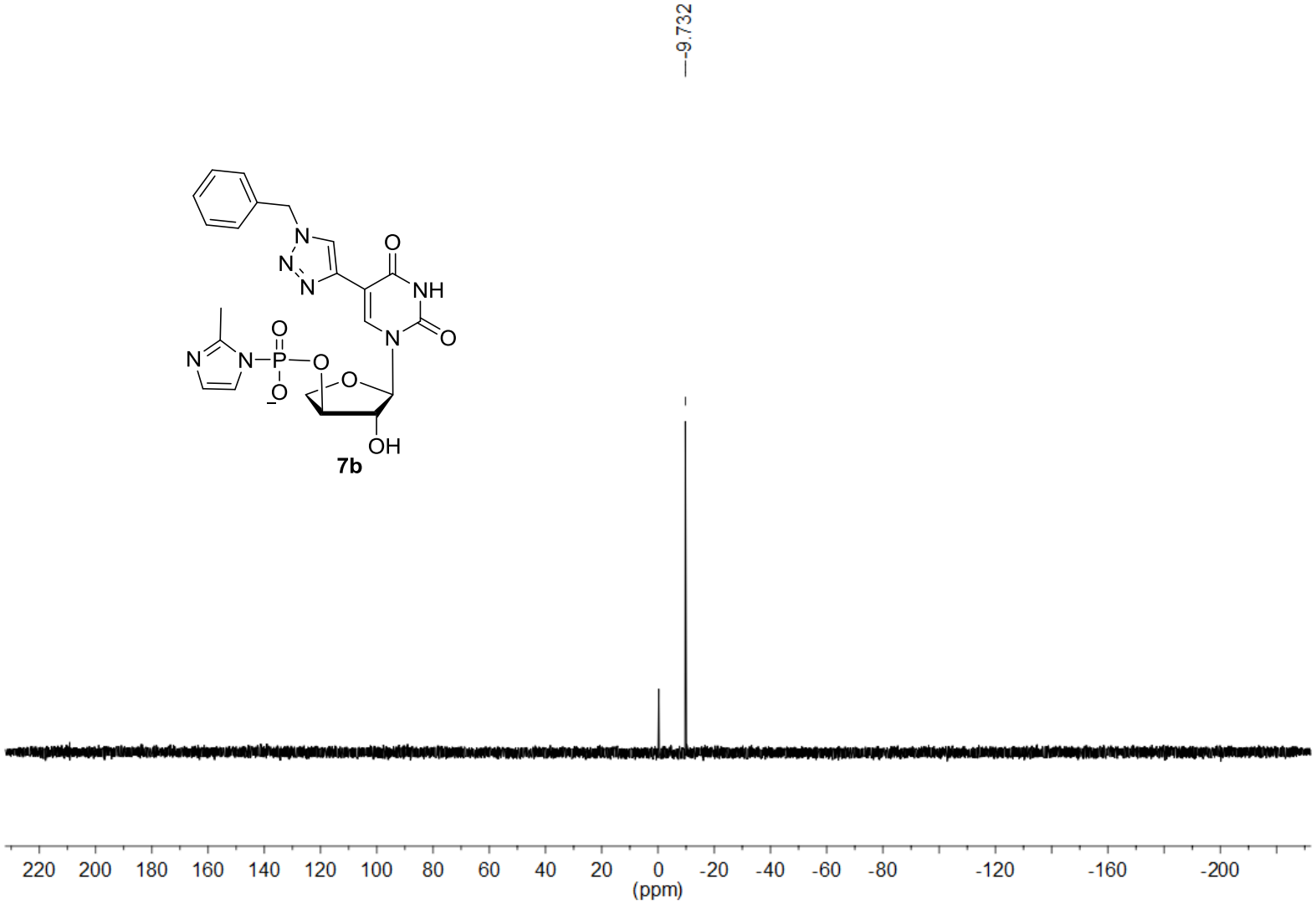
^{13}C NMR spectrum of compound **6c** (126 MHz, D_2O)



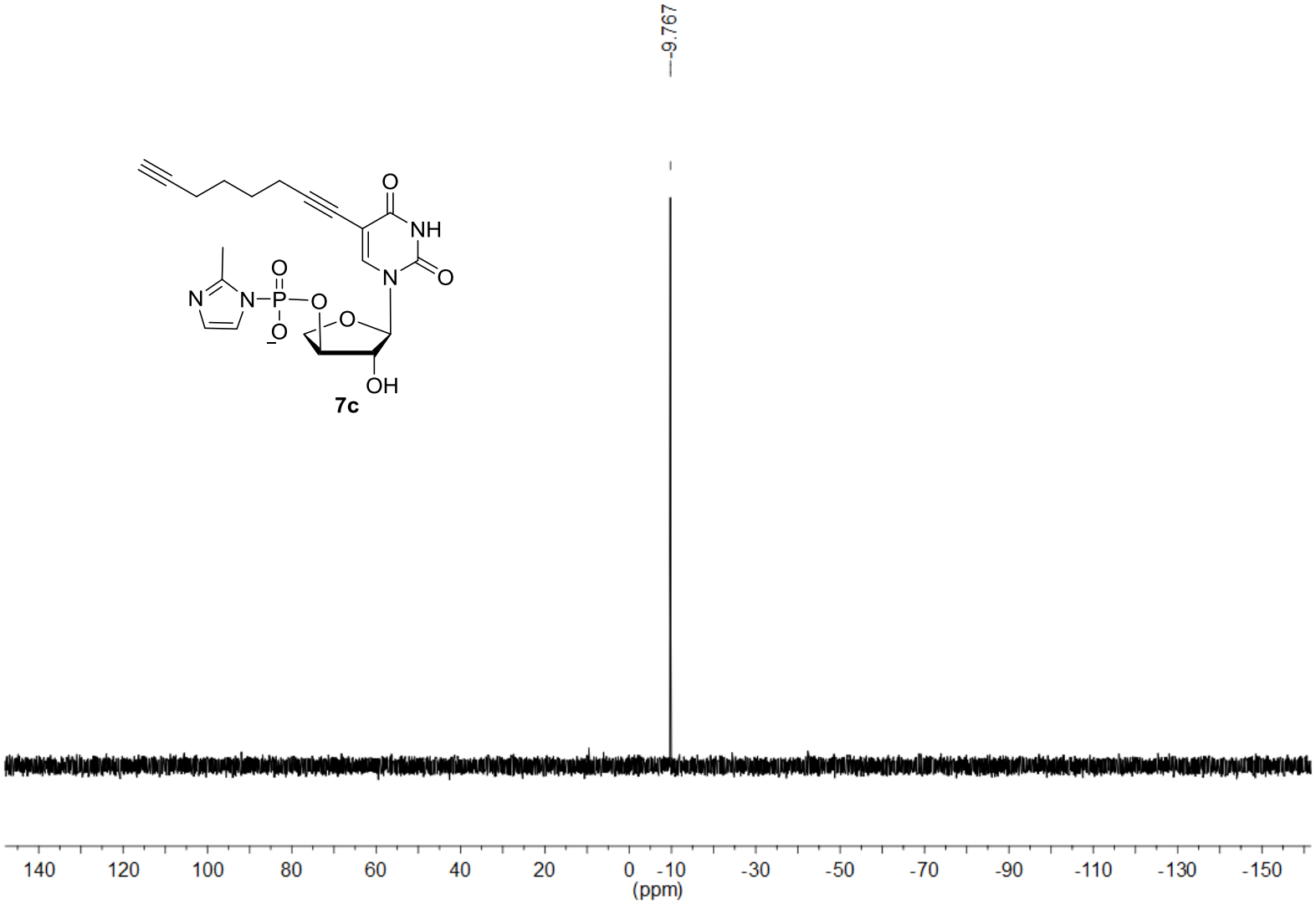
^{31}P NMR spectrum of compound **6c** (162 MHz, D_2O)



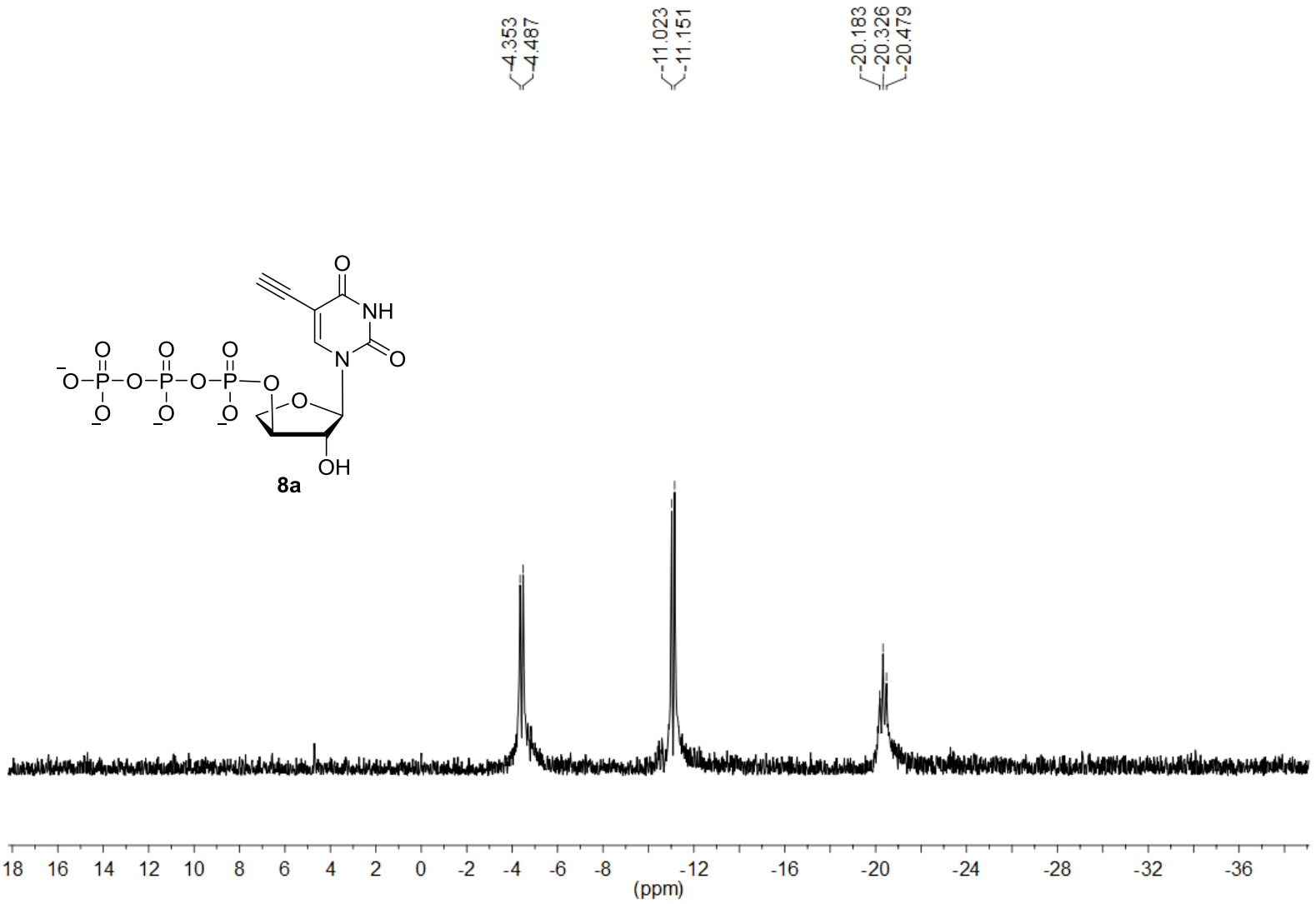
^{31}P NMR spectrum of compound **7a** (162 MHz, D_2O)



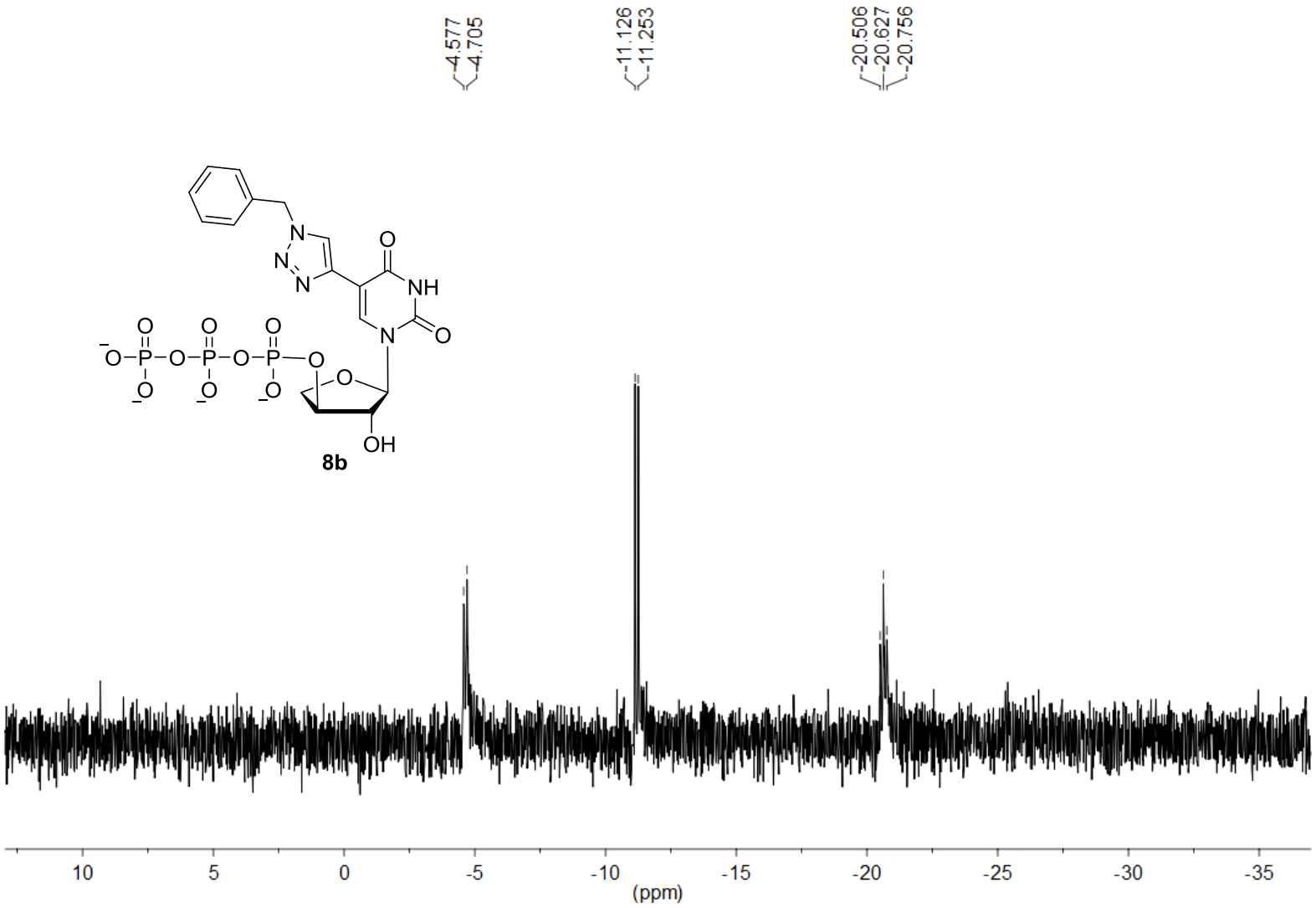
^{31}P NMR spectrum of compound **7b** (162 MHz, D_2O)



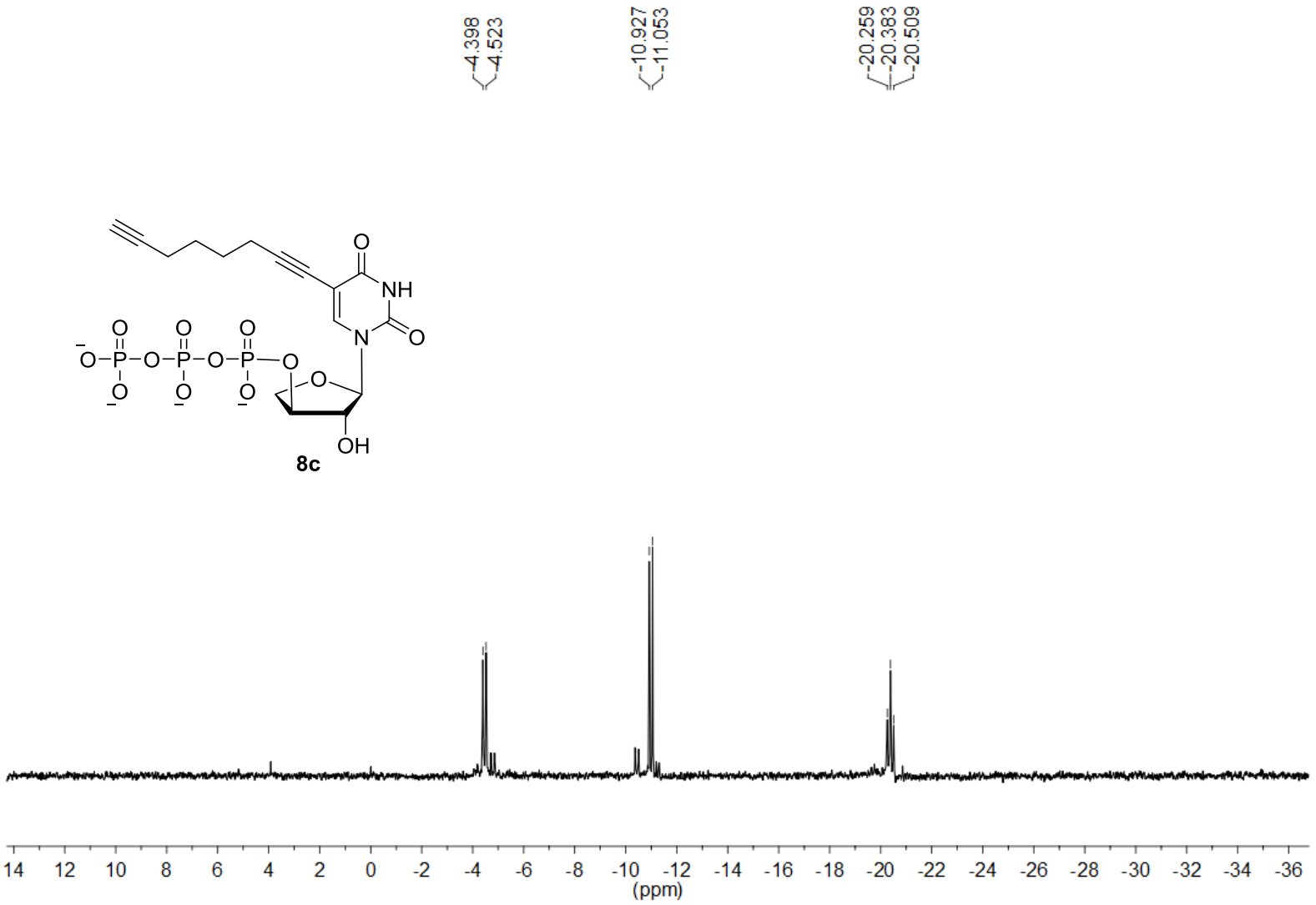
^{31}P NMR spectrum of compound **7c** (162 MHz, D_2O)



^{31}P NMR spectrum of compound **8a** (162 MHz, D_2O)



^{31}P NMR spectrum of compound **8b** (162 MHz, D_2O)



^{31}P NMR spectrum of compound **8c** (162 MHz, D_2O)