Supporting Information

Synthesis and Evaluation of Neutral Phosphate Triester Backbone-Modified siRNAs

Kouta Tsubaki,[1,2] Matthew L. Hammill,[2] Andrew Varley,[2] Mitsuru Kitamura,[1] Tatsuo Okauchi,[1] and Jean-Paul Desaulniers[2*]

[1] *Department of Applied Chemistry, Graduate School of Engineering, Kyushu Institute of Technology, 1-1 Sensui-cho, Tobata, Kitakyushu 804-8550, Japan*

[2] *Faculty of Science, University of Ontario Institute of Technology, 2000 Simcoe Street North, Oshawa, Ontario L1G 0C5 Canada*

Table of Contents

Experimental Procedures for the Synthesis of Novel Compounds and Characterizations:

Nucleic Acid and Biological Procedures

Figures and Tables

^{1}H/^{13}C/^{19}F/^{31}P NMR Spectra of Compounds

Experimental Procedures for the Synthesis of Novel Compounds and Characterizations

General

All reactions were performed under a nitrogen atmosphere with magnetic stirring. Column chromatography on silica gel was performed with Fuji Silysia BW-127ZH. Preparative TLC was performed on Wako gel B-5F/TLC-cards (20´20´0.7 cm). Materials were purchased from commercial suppliers (Aldrich, TCI, Kanto) and used without further purification. Organic solvents were purified and dried by standard procedures. NMR spectra were recorded on a Bruker spectrometer Avance III HD 500 at 500 MHz (^{1}H NMR), 126 MHz (^{13}C NMR) and 202 MHz (^{31}P NMR). In $CDCl_3$, tetramethylsilane (0.00 ppm) was used as an internal standard in ^{1}H NMR, whereas the middle line of the solvent signal (77.0 ppm) was used in ^{13}C NMR. 85% H_3PO_4 (0.0 ppm) was used as external standard in ^{31}P NMR. ESI-MS was recorded on JEOL AccuTOF JMS-T100 LC mass spectrometer. Infrared spectra were obtained on an Agilent Technologies Cary 630 FTIR spectrometer. Unless otherwise indicated all starting reagents used were obtained from commercial sources (Sigma, TCI, Fisher Scientific, or ChemGenes) without additional purification. Oligonucleotides were synthesized according to our group's previously published protocol, using standard solid phase synthesis and materials. Antisense luciferase siRNA purchased from Integrated DNA Technologies (IDT) was used for this study.

((2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl bis(2,2,2-trifluoroethyl) phosphate-Compound (5)

To a solution of tris(2,2,2-trifluoroethyl) phosphate (894 mg, 2.60 mmol), **1** (870 mg, 2.20 mmol) and MS4A 870 mg in toluene (22 mL) was added to DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (334 mg, 2.20 mmol) at 0 °C under N_2 atmosphere. After stirring for 5 h at room temperature, the reaction was quenched by addition of phosphate buffer (pH 7.00) at 0 °C. The mixture was extracted twice with ethyl acetate. The combined extracts were washed with aqueous brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (2:3 hexane/ethyl acetate) to give **5** as a white solid (1.31 g, 93%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57-7.56 (m, 1H), 6.27 (t, J = 6.2 Hz, 1H), 6.05 (ddt, J_d = 17.2, 10.6 Hz, J_t = 5.5 Hz, 1H), 5.39 (ddt, J_d = 17.2, 1.5 Hz, J_t = 1.5 Hz, 1H), 5.27 (ddt, J_d = 10.5, 1.3 Hz, J_t = 1.3 Hz, 1H), 4.90 (ddt, J_d = 5.6, 1.4 Hz, J_t = 1.4 Hz, 2H), 4.45-4.28 (m, 7H), 4.06-4.02 (m, 1H), 2.46 (ddd, J = 13.7, 6.6, 5.1 Hz, 1H), 2.15 (ddd, J = 13.4, 7.1, 6.0 Hz, 1H), 1.98 (d, J = 0.9 Hz, 3H), 0.89 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.1, 155.6, 139.3, 132.1, 122.2 (dq, J_d = 9.2 Hz, J_q = 277.6 Hz), 118.1, 104.9, 86.6, 84.5 (d, J = 7.4 Hz), 70.5, 67.7, 67.6 (d, J = 6.2 Hz), 64.1 (dq, J_d = 4.0 Hz, J_q = 38.6 Hz), 41.3, 25.6, 17.9, 12.0, -4.7, -5.1.^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.1. ^{19}F NMR (470 MHz, $CDCl_3$, Internal standard: Hexafluorobenzene) δ 86.48-86.43 (m). IR (ATR) 2953, 2933, 2858, 1666, 1533, 1470, 1417, 1270, 1168, 1075 cm^{-1}. MS (ESI) *m/z* 663 $(M+Na)^+$. HRMS (ESI) calcd for $C_{23}H_{35}F_6N_2Na_1O_8P_1Si_1$ $(M+Na)^+$ 663.17022, found 663.17099

((2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl bis(2,2,2-trifluoroethyl) phosphate- Compound (6)

To a solution of tris(2,2,2-trifluoroethyl) phosphate (991 mg, 2.88 mmol), **2** (917 mg, 2.40 mmol) and MS4A 917 mg in toluene (24 mL) was added DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) (365 mg, 2.40 mmol) at 0 °C under N_2. After stirring for 5 h at room temperature, the reaction was quenched by addition of phosphate buffer (pH 7.00) at 0 °C. The mixture was extracted twice with ethyl acetate. The combined extracts were washed with aqueous brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (1:2 hexane/ethyl acetate) to give **6** as a white solid (1.38 g, 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, J = 7.3 Hz, 1H), 6.24 (t, J = 6.2 Hz, 1H), 6.03 (ddt, J_d = 17.1, 10.4 Hz, J_t = 5.7 Hz, 1H), 5.93 (d, J = 7.4 Hz, 1H), 5.38 (ddt, J_d = 17.2, 1.5 Hz, J_t = 1.5 Hz, 1H), 5.28 (ddt, J_d = 10.5, 1.2 Hz, J_t = 1.2 Hz, 1H), 4.90 (ddt, J_d = 5.8, 1.2 Hz, J_t = 1.2 Hz, 2H), 4.45-4.29 (m, 7H), 4.06-4.05 (m, 1H), 2.51 (ddd, J = 18.9, 6.9, 5.3 Hz, 1H), 2.15 (ddd, J = 19.4, 7.1, 5.7 Hz, 1H), 0.89 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H).^{13}C NMR (126 MHz, $CDCl_3$) δ 171.0, 155.5, 142.1, 131.9, 122.1 (dq, J_d = 9.3 Hz, J_q = 278.3 Hz), 118.7, 95.8, 86.9, 84.6 (d, J = 7.3 Hz), 70.4, 67.7, 67.4 (d, J = 6.7 Hz), 64.1 (dq, J_d = 4.3 Hz, J_q = 38.5 Hz), 41.4, 25.6, 17.9, -4.8, -5.2.^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.1. ^{19}F NMR (470 MHz, $CDCl_3$, Internal standard: Hexafluorobenzene) δ 86.47 (t, J = 7.9 Hz). IR (ATR) 2951, 2858, 1663, 1628, 1545, 1451, 1399, 1302, 1274, 1163 cm^{-1}. MS (ESI) *m/z* 649 $(M+Na)^+$. HRMS (ESI) calcd for $C_{22}H_{33}F_6N_2Na_1O_8P_1Si_1$ $(M+Na)^+$ 649.15457, found 649.15427.

(2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) (2,2,2-trifluoroethyl) phosphate (1.6:1 diastereomeric mixture)- Compound (7)

To a toluene (10 mL) and THF (5 ml) solution of **3** (1.2 g, 2.1 mmol) was added 2.1 mL of a 1.0 M hexane solution of lithium *tert*-butoxide (2.1 mmol) at 0 °C. After stirring for 1 h at 0 °C, a toluene solution (5 mL) of **5** (1.3 g, 2.0 mmol) was added to the mixture at –45 °C. After being stirred for 5 h at –45 °C and then 1.5 h at 0 °C, the reaction was quenched by addition of phosphate buffer (pH 7.00) at room temperature. The mixture was extracted twice with ethyl acetate. The combined extracts were washed with aqueous brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (1:2 hexane/ethyl acetate) to give **7** as a white solid (2.0 g, 88 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (d, J = 1.0 Hz, 1H, one diastereomer), 7.80 (d, J = 1.0 Hz, 1H, the other diastereomer), 7.58 (d, J = 1.0 Hz, 1H, the other diastereomer), 7.55 (d, J = 1.0 Hz, 1H, one diastereomer), 7.37-7.33 (m, 2H), 7.31-7.22 (m, 7H), 6.84-6.81 (m, 4H), 6.46 (ddd, J = 5.4, 7.9, 13.1 Hz, 1H), 6.24 (dt, J_d = 12.0 Hz, J_t=6.0 Hz, 1H), 6.08-5.99 (m, 2H), 5.41-5.35 (m, 2H), 5.29-5.24 (m, 2H), 5.18-5.10 (m, 1H), 4.93-4.86 (m, 4H), 4.41-4.14 (m, 6H), 4.03-4.00 (m, 1H, the other diastereomer), 3.99-3.95 (m, 1H, one diastereomer), 3.79 (s, 6H), 3.56-3.51 (m, 1H), 3.41-3.33 (m, 1H), 2.85-2.79 (m, 1H), 2.48-2.40 (m, 1H), 2.39-

2.32 (m, 1H), 2.17-2.09 (m, 1H), 1.95 (d, *J* = 1.0 Hz, 3H, one diastereomer), 1.94 (d, *J* = 1.0 Hz, 3H, the other diastereomer), 1.55 (d, *J* = 0.9 Hz, 3H, one diastereomer), 1.53 (d, *J* = 0.9 Hz, 3H, the other diastereomer), 0.88 (s, 9H, the other diastereomer), 0.88 (s, 9H, one diastereomer), 0.08 (s, 3H, the other diastereomer), 0.07 (s, 3H, the other diastereomer), 0.062 (s, 3H, one diastereomer), 0.06 (s, 3H, one diastereomer).^{13}C NMR (126 MHz, $CDCl_3$) δ 170.07, 170.05, 170.01, 158.77, 158.76, 155.64, 155.63, 155.5, 144.00, 143.98, 139.4, 139.3, 139.2, 135.00, 134.98, 132.13, 132.10, 132.0, 130.0, 128.02, 127.97, 127.21, 127.19, 122.3 (q, *J* = 278.3 Hz), 118.2, 118.1, 113.3, 105.2, 105.1, 104.9, 104.8, 87.2, 86.6, 86.4, 86.0, 85.8, 84.7-84.4 (m), 79.52 (d, *J* = 5.1 Hz), 79.48 (d, *J* = 4.4 Hz), 70.7, 70.5, 67.7 (d, *J* = 8.3 Hz), 67.4 (d, *J* = 6.2 Hz), 67.2 (d, *J* = 6.2 Hz), 64.3-63.3 (m), 62.9 (d, *J* = 3.1 Hz), 55.2, 41.3 (d, *J* = 1.9 Hz), 40.0 (d, *J* = 4.2 Hz), 39.9 (d, *J* = 4.1 Hz), 25.6, 17.8, 12.0, 11.60, 11.56, -4.70, -4.73, -5.0.^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.5 (one diastereomer), -1.6 (the other diastereomer). ^{19}F NMR (470 MHz, $CDCl_3$, Internal standard: Hexafluorobenzene) δ 86.68 (dd, *J* = 7.8, 7.8 Hz, one diastereomer), 86.59 (dd, *J* = 8.2, 8.2 Hz, the other diastereomer). IR (ATR) 2930, 2855, 1665, 1607, 1530, 1508, 1465, 1404, 1322, 1173, 1101, 829, 700 cm^{-1}. MS (ESI) *m/z* 1147 $(M+Na)^+$. HRMS (ESI) calcd for $C_{55}H_{68}F_3N_4Na_1O_{14}P_1Si_1$ $(M+Na)^+$ 1147.40887, found 1147.41202.

(2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) (2,2,2-trifluoroethyl) phosphate (2.3:1 diastereomeric mixture)- Compound (8)

To a toluene (10 mL) and THF (5 ml) solution of **4** (1.3 g, 2.3 mmol) was added 2.3 mL of a 1.0 M hexane solution of lithium *tert*-butoxide (2.3 mmol) at 0 °C. After stirring for 1 h at 0 °C, a toluene solution (5 mL) of **6** (1.3 g, 2.2 mmol) was added to the mixture at –45 °C. After being stirred for 3 h at –45 °C and then 2 h at 0 °C, the reaction was quenched by addition of phosphate buffer (pH 7.00) at room temperature. The mixture was extracted twice with ethyl acetate. The combined extracts were washed with aqueous brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (1:2 hexane/ethyl acetate) to give **8** as a white solid (2.0 g, 84 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.95 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.94 (d, *J* = 7.3 Hz, 1H, the other diastereomer), 7.78 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.75 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.35-7.22 (m, 9H), 6.85-6.81 (m, 4H), 6.36-6.32 (m, 1H), 6.21 (dt, J_d = 12.2 Hz, J_t=6.1 Hz, 1H), 6.07-5.97 (m, 2H), 5.900 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.9 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.65 (d, *J* = 7.3 Hz, 1H, one diastereomer), 5.64 (d, *J* = 7.0 Hz, 1H, the other diastereomer), 5.41-5.34 (m, 2H), 5.30-5.25 (m, 2H), 5.16-5.08 (m, 1H), 4.89-4.85 (m, 4H), 4.43-4.17 (m, 6H), 4.06-4.02 (m, 1H, the other diastereomer), 4.02-3.99 (m, 1H, one diastereomer), 3.52-3.39 (m, 2H), 2.87 (ddd, *J* = 2.6, 5.7, 14.3 Hz, 1H), 2.53-2.45 (m, 1H), 2.36-2.28 (m, 1H), 2.17-2.10 (m, 1H), 0.88 (s, 9H, the other diastereomer), 0.88 (s, 9H, one diastereomer), 0.08 (s, 3H, the other diastereomer), 0.07 (s, 3H, the other diastereomer), 0.07 (s, 3H, one diastereomer), 0.06 (s, 3H, one diastereomer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.05, 171.00, 155.54, 155.50, 155.45, 143.9, 142.35, 142.31, 142.28, 142.19, 134.9, 134.8, 131.94, 131.91, 131.85, 130.0, 128.0, 127. 2, 122.3 (q, *J* = 268.2 Hz),118.8, 118.7, 113.3, 95.82, 95.78, 95.74, 87.22, 87.20, 86.9, 86.7, 86.5, 86.3, 84.8-84.5 (m), 79.0 (d, *J* =

5.2 Hz), 78.8 (d, *J* = 4.4 Hz), 70.7, 70.5, 67.7 (d, *J* = 7.1 Hz), 67.4 (d, *J* = 6.2 Hz), 67.1 (d, *J* = 5.2 Hz), 64.3-63.4 (m), 62.6, 62.5, 55.2, 41.41, 41.38, 40.2, 40.0 (d, *J* = 4.3 Hz), 31.5, 25.6, 22.6, 14.1, -4.69, -4.72, -5.0, -5.1. ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.5. ^{19}F NMR (470 MHz, $CDCl_3$, Internal standard: Hexafluorobenzene) δ 86.70 (dd, *J* = 8.1, 8.1 Hz, one diastereomer), 86.65 (dd, *J* = 7.9, 7.9 Hz, the other diastereomer). IR (ATR) 2932, 2856, 1663, 1628, 1540, 1508, 1469, 1399, 1249, 1173, 1022, 829, 701cm^{-1}. MS (ESI) *m/z* 1119 $(M+Na)^+$. HRMS (ESI) calcd for $C_{53}H_{64}F_3N_4Na_1O_{14}P_1Si_1$ $(M+Na)^+$ 1119.37757, found 1119.38020.

(2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) benzyl phosphate (1.1:1 diastereomeric mixture)- Compound (9)

To a toluene (1.5 mL) solution of 2-Phenylethyl Alcohol (60 mg, 0.5 mmol) was added 0.5 mL of a 1.0 M hexane solution of lithium *tert*-butoxide (0.5 mmol) at 0 °C. After stirring for 1 h at 0 °C, a toluene solution (2 mL) of **7** (371 mg, 0.3 mmol) was added to the mixture at –45 °C. After being stirred for 5 h at –45 °C, the reaction was quenched by addition of a toluene solution (1 ml) of acetic acid (30 mg, 0.5 mmol) at -45 ℃, followed by addition of phosphate buffer (pH 7.00). The mixture was extracted twice with ethyl acetate. The combined extracts were washed with aqueous brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (1:4 hexane/ethyl acetate) to give **9** as a diastereomer mixture (315 mg, 83 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, *J* = 1.2 Hz, 1H, one diastereomer), 7.77 (d, *J* = 1.0 Hz, 1H, the other diastereomer), 7.65 (d, *J* = 1.0 Hz, 1H, the other diastereomer), 7.60 (d, *J* = 1.0 Hz, 1H, one diastereomer), 7.37-7.33 (m, 2H), 7.30-7.20 (m, 9H), 7.19-7.11 (m, 3H), 6.83-6.80 (m, 4H), 6.45 (ddd, *J* = 5.6, 8.1, 15.5 Hz, 1H), 6.25 (dt, J_d = 12.7 Hz, J_t=6.3 Hz, 1H), 6.08-5.99 (m, 2H), 5.41-5.38 (m, 2H, one diastereomer), 5.37-5.35 (m, 2H, the other diastereomer), 5.29-5.24 (m, 2H), 5.12-5.08 (m, 1H, one diastereomer), 5.06-5.02 (m, 1H, the other diastereomer), 4.92-4.87 (m, 4H), 4.31-4.03 (m, 6H), 3.99-3.95 (m, 1H, the other diastereomer), 3.93-3.90 (m, 1H, one diastereomer), 3.78 (s, 3H, the other diastereomer), 3.77 (s, 3H, one diastereomer), 3.77 (s, 3H, the other diastereomer), 3.77 (s, 3H, one diastereomer), 3.48 (ddd, *J* = 3.0, 10.8, 14.0 Hz, 1H), 3.32 (ddd, *J* = 2.7, 10.7, 20.7 Hz, 1H), 2.96 (t, 1H, *J* = 6.9 Hz), 2.89 (dt, 1H, J = 1.8, 7.0 Hz), 2.74 (ddd, *J* = 1.9, 5.5, 14.1 Hz, 1H, one diastereomer), 2.69 (ddd, *J* = 2.2, 5.6, 14.1 Hz, 1H, the other diastereomer), 2.43 (1H, dddd, *J* = 5.0, 6.6, 13.8, 20.5 Hz), 2.31-2.21 (m, 1H), 2.08-1.99 (m, 1H), 1.92 (d, *J* = 0.9 Hz, 3H, the other diastereomer), 1.91 (d, *J* = 0.9 Hz, 3H, one diastereomer), 1.51 (d, *J* = 0.9 Hz, 3H, the other diastereomer), 1.50 (d, *J* = 0.8 Hz, 3H, one diastereomer), 0.88 (s, 9H, one diastereomer), 0.87 (s, 9H, the other diastereomer), 0.07 (s, 3H, one diastereomer), 0.06 (s, 3H, one diastereomer), 0.05 (s, 3H, the other diastereomer), 0.03 (s, 3H, the other diastereomer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.1, 170.03, 169.98, 158.8, 155.71, 155.65, 155.63, 144.1, 139.41, 139.36, 139.33, 136.52, 136.46, 135.2, 135.12, 135.11, 135.07, 132.21, 132.19, 132.10, 130.1, 128.9, 128.60, 128.58, 128.1, 128.0, 127.2, 126.9, 118.2, 118.1, 113.3, 105.14, 105.05, 104.8, 104.7, 87.1, 86.4, 86.2, 85.9, 85.8, 84.9-84.7 (m), 78.8 (d, *J* = 5.5 Hz), 78.6

(d, *J* = 5.3 Hz), 70.8, 70.7, 68.59 (d, *J* = 6.0 Hz), 68.57 (d, *J* = 6.6 Hz), 67.75, 67.67, 66.43, 66.40, 63.2, 63.1, 55.2, 41.53, 41.49, 40.0 (d, *J* = 4.4 Hz), 25.69, 25.67, 17.9, 12.1, 11.6, -4.6, -4.7, -4.99, -4.91. ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.2 (one diastereomer), -1.3 (the other diastereomer). IR (ATR) 3059, 2928, 2854, 1655, 1533, 1465, 1402, 1322, 1249, 1176, 999, 828, 699 cm^{-1}. MS (ESI) *m/z* 1169 $(M+Na)^+$. HRMS (ESI) calcd for $C_{61}H_{75}N_4Na_1O_{14}P_1Si_1$ $(M+Na)^+$ 1169.46843, found 1169.46992.

(2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) benzyl phosphate (2.1:1 diastereomeric mixture)- Compound (10)

To a toluene (5 mL) solution of 2-Phenylethyl Alcohol (322 mg, 2.6 mmol) was added 2.6 mL of a 1.0 M hexane solution of lithium *tert*-butoxide (2.6 mmol) at 0 °C. After stirring for 1 h at 0 °C, a toluene solution (3 mL) of **8** (968 mg, 0.9 mmol) was added to the mixture at –45 °C. After being stirred for 4 h at –45°C, the reaction was quenched by addition of a toluene solution (1 ml) of acetic acid (156 mg, 2.6 mmol) at -45 °C, followed by addition of phosphate buffer (pH 7.00). The mixture was extracted twice with ethyl acetate. The combined extracts were washed with aqueous brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (1:4 hexane/ethyl acetate) to give **10** as a diastereomer mixture (824 mg, 83 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.92 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.83 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.79 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.35-7.32 (m, 2H), 7.30-7.21 (m, 9H), 7.20-7.12 (m, 3H), 6.84-6.81 (m, 4H), 6.33 (t, *J* = 6.5 Hz, the other diastereomer), 6.32 (t, *J* = 6.4 Hz, one diastereomer), 6.21 (t, *J* = 6.1 Hz, the other diastereomer), 6.20 (t, *J* = 6.1 Hz, one diastereomer), 6.07-5.97 (m, 2H), 5.85 (d, *J* = 7.4 Hz, 1H), 5.62 (t, *J* = 7.4 Hz, one diastereomer), 5.61 (t, *J* = 7.4 Hz, the other diastereomer), 5.41-5.34 (m, 2H), 5.30-5.25 (m, 2H), 5.10-5.05 (m, 1H, the other diastereomer), 5.03-4.99 (m, 1H, one diastereomer), 4.89-4.84 (m, 4H), 4.29-4.05 (m, 6H), 4.01-3.98 (m, 1H, the other diastereomer), 3.96-3.93 (m, 1H, one diastereomer), 3.78 (s, 3H, one diastereomer), 3.78 (s, 3H, one diastereomer), 3.77 (s, 3H, the other diastereomer), 3.78 (s, 3H, the other diastereomer), 3.46-3.32 (m, 2H), 2.97 (t, *J* = 6.9 Hz, 2H, the other diastereomer), 2.91 (t, *J* = 7.4 Hz, 2H, one diastereomer), 2.79 (ddd, *J* = 2.8, 5.8, 14.2 Hz, 1H, the other diastereomer), 2.73 (ddd, *J* = 2.9, 5.8, 14.2 Hz, 1H, one diastereomer), 2.52-2.43 (m, 1H), 2.29-2.16 (m, 1H), 2.08-1.99 (m, 1H), 0.88 (s, 9H, the other diastereomer), 0.87 (s, 9H, one diastereomer), 0.07 (s, 3H, the other diastereomer), 0.06 (s, 3H, the other diastereomer), 0.05 (s, 3H, one diastereomer), 0.04 (s, 3H, one diastereomer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.01, 170.99, 170.96, 158.7, 155.6, 155.53, 155.50, 144.0,142.5, 142.4, 142.3, 142.2, 136.53, 136.45, 135.1, 135.0, 134.9, 131.99, 131.89, 130.03, 130.01, 128.90, 128.87, 128.62, 128.59, 128.04, 127.98, 127.2, 126.9, 118.8, 118.64, 118.62, 113.3, 95.7, 87.13, 87.10, 86.7, 86.6, 86.4, 86.3, 84.9 (d, *J* = 7.6 Hz), 84.8 (d, *J* = 6.2 Hz), 78.0 (d, *J* = 4.9 Hz), 70.8, 70.7, 68.65 (d, *J* = 5.4 Hz), 68.61 (d, *J* = 6.0 Hz), 67.73, 67.66, 66.5, 66.4, 62.69, 62.65, 55.2, 41.6, 41.5, 40.2 (d, *J* = 3.5 Hz), 36.58 (d, *J* = 6.8 Hz), 36.56, (d, *J* = 7.0 Hz), 25.7, 17.9, -4.6, -4.7, -4.92, -4.94. ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.1 (one diastereomer), -

1.2 (the other diastereomer). IR (ATR) 3059, 2928, 2855, 1664, 1627, 1540, 1508, 1468, 1397, 1248, 1175, 999, 780 cm^{-1}. MS (ESI) *m/z* 1141 $(M+Na)^+$. HRMS (ESI) calcd for $C_{59}H_{71}N_4Na_1O_{14}P_1Si_1$ $(M+Na)^+$ 1141.43713, found 1141.43633.

(2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2R,3S,5R)-5-(4-(allyloxy)-5-methyl-2-oxopyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) benzyl phosphate (1:1 diastereomeric mixture)- Compound (11)

To a THF solution (3 mL) of **9** (355 mg, 0.3 mmol), triethylamine (0.65 mL, 4.7 mmol) was added triethylamine trihydrofluoride (3HF•Et_3N) (0.5 mL, 3.1 mmol) at 0 °C. After being stirred for 10 h at 40 °C, the reaction mixture was quenched by addition of saturated sodium bicarbonate at 0 °C. The mixture was extracted twice with ethyl acetate. The combined extracts were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on silica (9:1 = $CHCl_3$/MeOH) to give **11** as a diastereomer mixture (261 mg, 82 %). ^1H NMR (500 MHz, $CDCl_3$) δ 7.82 (d, *J* = 1.0 Hz, 1H, one diastereomer), 7.80 (d, *J* = 1.0 Hz, 1H, the other diastereomer), 7.64 (d, *J* = 1.0 Hz, 1H, one diastereomer), 7.60 (d, *J* = 1.0 Hz, 1H, the other diastereomer), 7.37-7.33 (m, 2H), 7.30-7.21 (m, 9H), 7.18-7.12 (m, 3H), 6.41-6.35 (m, 1H), 6.31-6.25 (m, 1H), 6.08-5.99 (m, 2H), 5.40-5.38 (m, 2H, one diastereomer), 5.37-5.35 (m, 2H, the other diastereomer), 5.29-5.24 (m, 2H), 5.09-5.06 (m, 1H, one diastereomer), 4.99-4.95 (m, 1H, the other diastereomer), 4.91-4.87 (m, 4H), 4.46-4.17 (m, 6H), 4.11-4.08 (m, 1H), 4.07-3.98 (m, 1H), 3.78 (s, 3H, one diastereomer), 3.78 (s, 3H, one diastereomer), 3.78 (s, 3H, one diastereomer), 3.78 (s, 3H, one diastereomer), 3.77 (s, 3H, the others diastereomer), 3.78 (s, 3H, the other diastereomer), 3.48 (ddd, *J* = 3.2, 10.7, 20.1 Hz, 1H), 3.31 (ddd, *J* = 2.8, 10.7, 16.1 Hz, 1H), 2.96 (t, 1H, *J* = 6.8 Hz, the other diastereomer), 2.93 (t, 1H, *J* = 6.9 Hz, one diastereomer), 2.89 (dd, 1H, *J* = 5.1, 13.6 Hz, one diastereomer), 2.71 (ddd, *J* = 2.1, 5.5, 14.1 Hz, 1H, the other diastereomer), 2.60-2.49 (m, 1H), 2.28-2.02 (m, 2H), 1.93 (d, *J* = 0.9 Hz, 3H, the other diastereomer), 1.90 (d, *J* = 0.9 Hz, 3H, one diastereomer), 1.56 (d, *J* = 0.9 Hz, 3H, one diastereomer), 1.55 (d, *J* = 0.8 Hz, 3H, the other diastereomer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.2, 170.1, 170.0, 169.9, 158.8, 158.7, 155.9, 155.8, 155.7, 155.6, 144.1, 144.0, 139.5, 139.35, 139.28, 139.24, 136.54, 136.50, 135.13, 135.07, 135.04, 132.2, 132.1, 132.0, 131.9, 130.06, 130.04, 130.00, 128.9, 128.6, 128.5, 128.1, 128.02, 128.00, 127.2, 126.9, 126.8, 118.3, 118.2, 118.13, 118.08, 113.3, 105.6, 105.3, 104.9, 104.5, 87.2, 87.1, 86.17, 86.15, 86.0, 85.9, 84.8 (d, *J* = 8.0 Hz), 84.6 (d, *J* = 6.3 Hz), 84.4 (d, *J* = 6.5 Hz), 84.1 (d, *J* = 7.4 Hz), 79.6 (d, *J* = 5.1 Hz), 78.8 (d, *J* = 6.3 Hz), 70.2, 69.3, 68.7 (d, *J* = 5.4 Hz), 68.5 (d, *J* = 5.2 Hz), 67.9, 67.8, 67.7, 67.6, 66.8 (d, *J* = 5.7 Hz), 66.2 (d, *J* = 5.6 Hz), 63.3, 63.0, 55.2, 41.1, 41.0, 40.1, 39.8, 36.6 (d, *J* = 7.2 Hz), 36.5 (d, *J* = 7.3 Hz), 12.15, 12.11, 11.6. ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.0 (one diastereomer), -1.6 (the other diastereomer). IR (ATR) 3335, 2930, 1661, 1530, 1465, 1403, 1321, 1248, 1175, 1114, 997, 826, 780, 699 cm^{-1}. MS (ESI) *m/z* 1055 $(M+Na)^+$. HRMS (ESI) calcd for $C_{55}H_{61}N_4Na_1O_{14}P_1$ $(M+Na)^+$ 1055.38196, found 1055.38383.

(2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2R,3S,5R)-5-(4-(allyloxy)-2-oxopyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) benzyl phosphate (2.2:1 diastereomeric mixture)- Compound (12)

To a THF solution (3 mL) of **10** (797 mg, 0.7 mmol), triethylamine (1.5 mL, 10.5 mmol) was added triethylamine trihydrofluoride (3HF•Et3N) (1.1 mL, 7 mmol) at 0 °C. After being stirred for 12 h at 40 °C, the reaction mixture was quenched by addition of saturated sodium bicarbonate at 0 °C. The mixture was extracted twice with ethyl acetate. The combined extracts were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on silica (9:1 = $CHCl_3$/MeOH) to give **11** as a diastereomer mixture (600 mg, 84 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.90 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.83 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.77 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.35-7.31 (m, 2H), 7.31-7.21 (m, 9H), 7.20-7.15 (m, 3H), 6.85-6.81 (m, 4H), 6.31-6.20 (m, 2H), 6.06-5.96 (m, 2H), 5.86 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.80 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.70 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.66 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.41-5.35 (m, 2H), 5.30-5.25 (m, 2H), 5.09-5.05 (m, 1H, the other diastereomer), 4.98-4.94 (m, 1H, one diastereomer), 4.89-4.84 (m, 4H), 4.43-4.00 (m, 9H), 3.79 (s, 6H), 3.49-3.34 (m, 2H), 2.97 (t, *J* = 6.8 Hz, 2H, one diastereomer), 2.94 (t, *J* = 6.9 Hz, 2H, the other diastereomer), 2.88 (ddd, 1H, *J* = 1.5, 5.2,14.2 Hz, the other diastereomer), 2.76 (ddd, 1H, *J* = 2.8, 5.7, 14.2 Hz, one diastereomer), 2.63-2.53 (m, 1H), 2.22-2.13 (m, 2H, the other diastereomer), 2.05 (dt, J_d = 13.8 Hz, J_t = 6.4 Hz, 2H, one diastereomer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.2, 171.1, 171.00, 170.95, 158.7, 155.8, 155.64, 155.58, 155.54, 144.02, 143.97, 142.4, 142.30, 142.28, 142.16, 136.6, 136.5, 135.0, 134.9, 132.0, 131.9, 131.8, 131.7, 130.1, 130.0, 128.9, 128.62, 128.61, 128.02, 128.00, 127.2, 126.9, 118.9, 118.8, 118.7, 118.6, 113.3, 96.1, 95.84, 95.76, 95.5, 87.14, 87.13, 86.62, 86.56, 86.44, 86.39, 84.8 (d, *J* = 7.3 Hz), 84.7 (d, *J* = 6.3 Hz), 84.5 (d, *J* = 6.1 Hz), 84.3 (d, *J* = 7.3 Hz), 79.0 (d, *J* = 4.5 Hz), 78.1 (d, *J* = 5.6 Hz), 70.3, 69.5, 68.7 (d, *J* = 5.7 Hz), 68.6 (d, *J* = 6.2 Hz), 67.9, 67.8, 67.70, 67.66, 66.7 (d, *J* = 6.4 Hz), 66.3 (d, *J* = 5.2 Hz), 62.9, 62.6, 41.1, 41.0, 40.2, 40.1, 36.6 (d, *J* = 7.2 Hz), 36.5 (d, *J* = 6.9 Hz). ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.1 (the other diastereomer), -1.4 (one diastereomer). IR (ATR) 3334, 2936, 16511, 1627, 1540, 1469, 1397, 1301, 1248, 1175, 1114, 998, 827, 699 cm^{-1}. MS (ESI) *m/z* 1027 $(M+Na)^+$. HRMS (ESI) calcd for $C_{53}H_{57}N_4Na_1O_{14}P_1$ $(M+Na)^+$ 1027.35066, found 1027.35092.

Benzyl ((2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl) (((2R,3S,5R)-3-hydroxy-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl) phosphate (1.1:1 diastereomeric mixture)- Compound (13)

To a heterogeneous mixture of **11** (228 mg, 0.22 mmol) and diethylammonium hydrogencarbonate (356 mg, 2.6 mmol) in CH_2Cl_2 (2 ml) was added $Pd(PPh_3)$ (13mg, 0.01 mmol) and PPh_3 (2 mg, 0.008 mmol). After being stirred for 3 h at room temperature, the reaction mixture was

concentrated and the residue was purified by column chromatography on silica ($CHCl_3$/MeOH=100:1→10:1) to give **13** as a diastereomer mixture (200 mg, 96 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.30-9.40 (br, 2H), 7.54 (dd, *J* = 0.9, 6.4 Hz, 1H), 7.35 (d, *J* = 7.6 Hz, 2H), 7.32-7.20 (m, 10H), 7.19-7.12 (m, 3H), 6.85-6.81 (m, 4H), 6.36 (dt, J_d = 9.2 Hz, J_t = 4.6 Hz, 1H), 6.26 (dt, J_d = 6.9 Hz, J_t = 6.9 Hz, 1H), 5.09 (t, *J* = 5.0 Hz, 1H, one diastereomer), 4.97 (t, *J* = 5.4 Hz, 1H, the other diastereomer), 4.47-4.43 (m, 1H, one diastereomer), 4.40-4.36 (m, 1H, the other diastereomer), 4.30-4.09 (m, 5H), 4.08-4.00 (m, 1H), 3.77 (s, 6H), 3.46 (dt, J_d = 2.7 Hz, J_t = 11.0 Hz, 1H), 3.33 (dd, *J* = 2.3, 10.6 Hz, 1H, the other diastereomer), 3.28 (dd, *J* = 2.2, 10.6 Hz, 1H, one diastereomer), 2.95 (t, *J* = 6.8 Hz, 2H, one diastereomer), 2.91 (t, *J* = 7.0 Hz, 2H, the other diastereomer), 2.66 (dd, *J* = 5.1, 13.7 Hz, 1H, one diastereomer), 2.66 (dd, *J* = 5.1, 13.7 Hz, 1H, one diastereomer), 2.49 (dd, J = 5.5, 13.2 Hz, 1H, the other diastereomer), 2.43-2.27 (m, 2H), 2.17-2.06 (m, 1H), 1.86 (d, *J* = 0.9 Hz, 3H, the other diastereomer), 1.85 (d, *J* = 0.9 Hz, 3H, one diastereomer), 1.42 (d, *J* = 0.8 Hz, 3H, the other diastereomer), 1.40 (d, *J* = 0.8 Hz, 3H, one diastereomer). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.0, 163.9, 163.81, 163.80, 158.8, 151.0, 150.9, 150.5, 144.1, 144.0, 136.6, 136.5, 135.7, 135.5, 135.1, 135.03, 135.00, 134.96, 130.1, 128.9, 128.60, 128.57, 128.1, 128.0, 127.3, 126.9, 113.3, 112.0, 111.8, 111.3, 111.1, 87.3, 87.2, 85.3, 85.2, 84.6-84.3 (m), 79.6 (d, *J* = 4.8 Hz), 79.2 (d, *J* = 5.3 Hz), 71.0, 70.9, 68.70 (d, *J* = 5.9 Hz), 68.68 (d, *J* = 5.3 Hz), 67.3 (d, *J* = 6.3 Hz), 67.0 (d, *J* = 5.4 Hz), 63.4, 63.3, 40.6, 39.9, 39.1, 38.7, 36.6 (d, *J* = 7.2 Hz), 36.5 (d, *J* = 6.2 Hz), 12.4, 11.7. ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.3 (the other diastereomer), -2.0 (one diastereomer). IR (ATR) 3167, 2959, 2831, 1677, 1605, 1507, 1366, 1248, 1175, 999, 825, 699 cm^{-1}. MS (ESI) *m/z* 975 $(M+Na)^+$. HRMS (ESI) calcd for $C_{49}H_{53}N_4Na_1O_{14}P_1$ $(M+Na)^+$ 975.31936, found 975.32148.

Benzyl ((2R,3S,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl) (((2R,3S,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) phosphate (2.2:1 diastereomeric mixture)- Compound (14)

To a heterogeneous mixture of **12** (570 mg, 0.57 mmol) and diethylammonium hydrogencarbonate (923 mg, 6.8 mmol) in CH_2Cl_2 (6 ml) was added $Pd(PPh_3)$ (33mg, 0.03 mmol) and PPh_3 (5 mg, 0.02 mmol). After being stirred for 2 h at room temperature, the reaction mixture was concentrated and the residue was purified by column chromatography on silica ($CHCl_3$/MeOH=100:1→10:1) to give **14** as a diastereomer mixture (500 mg, 95 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.28-9.82 (br, 2H), 7.64 (d, *J* = 8.1 Hz, 1H, one diastereomer), 7.63 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 7.48 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 7.42 (d, *J* = 8.2 Hz, 1H, one diastereomer), 7.35-7.20 (m, 10H), 7.19-7.12 (m, 3H), 6.85-6.81 (m, 4H), 6.26 (dt, J_d = 7.9 Hz, J_t = 5.4 Hz, 1H), 6.20 (t, *J* = 6.5 Hz, 1H), 5.68 (d, *J* = 8.1 Hz, 1H, one diastereomer), 5.67 (d, *J* = 8.1 Hz, 1H, the other diastereomer), 5.40 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 5.38 (d, *J* = 8.4 Hz, 1H, one diastereomer), 5.10-5.06 (m, 1H, the other diastereomer), 4.97-4.93 (m, 1H, one diastereomer), 4.44-4.40 (m, 1H, the other diastereomer), 4.39-4.35 (m, 1H, one diastereomer), 4.30-4.01 (m, 7H), 3.76 (s, 3H), 3.76 (s, 3H), 3.43-3.31 (m, 2H), 2.95 (t, *J* = 6.7 Hz, 2H, one diastereomer), 2.91 (t, *J* = 6.9 Hz, 2H, the other diastereomer), 2.69-2.64 (m, 1H, the other diastereomer), 2.53-2.47 (m, 1H, one diastereomer), 2.44-2.04 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.6, 163.5, 163.38, 163.35, 158.7, 150.9, 150.7, 150.5, 144.0, 143.9, 140.0, 139.85,

139.76, 139.5, 136.6, 136.5, 134.9, 134.8, 130.1, 130.0, 128.93, 128.91, 128.6, 128.0, 127.2, 126.9, 113.3, 102.8, 102.75, 102.66, 102.5, 87.3, 85.7, 85.5, 84.81, 84.76, 84.6-84.5 (m), 78.8 (d, *J* = 4.3 Hz), 70.83, 70.76, 68.75 (d, *J* = 5.8 Hz), 67.0 (d, *J* = 4.9 Hz), 63.0, 55.2, 40.1, 40.0, 39.3, 36.53 (d, *J* = 6.6 Hz), 36.48 (d, *J* = 5.5 Hz). ^{31}P NMR (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.3 (the other diastereomer), -1.9 (one diastereomer). IR (ATR) 3053, 1675, 1507, 1458, 1379, 1246, 1175, 999, 825, 749, 699 cm^{-1}. MS (ESI) *m/z* 947 $(M+Na)^+$. HRMS (ESI) calcd for $C_{47}H_{49}N_4Na_1O_{14}P_1$ $(M+Na)^+$ 947.28806, found 947.28974.

Experimental Nucleic Acid and Biological Procedures

Procedure for Oligonucleotide Synthesis and Purification

All standard β-cyanoethyl PAC ultra mild 2'-*O*-TBDMS protected phosphoramidites, reagents and solid supports were purchased from Chemgenes Corporation and Glen Research. Wild-type luciferase strands including the sense and 5'-phosphorylated antisense strand were synthesized. All commercial phosphoramidites were dissolved in anhydrous acetonitrile to a concentration of 0.10 M. The chemically synthesized (phosphonate derivative) phosphoramidites were dissolved in acetonitrile (anhydrous) to a concentration of 0.10 M. The reagents that were used for the phosphoramidite coupling cycle were: PAC ultra mild acetic anhydride/pyridine/THF (Cap A), 16% *N*-methylimidazole in THF (Cap B), 0.25 M 5-ethylthio tetrazole in ACN (activator), 0.02 M iodine/pyridine/H2O/THF (oxidation solution), and 3% trichloroacetic acid/dichloromethane. All sequences were synthesized on 0.20 μM or 1.00 μM dT solid supports except for sequences that were 3'-modified, which were synthesized on 1.00 μM Universal III solid supports. The entire synthesis ran on an Applied Biosystems 394 DNA/RNA synthesizer using 0.20 μM or 1.00 μM cycles kept under argon at 55 psi. Standard and synthetic phosphoramidites ran with coupling times of 999 seconds.

Antisense sequences were chemically phosphorylated at the 5'-end by using 2-[2-(4,4'-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite. At the end of every cycle, the columns were removed from the synthesizer, dried with a stream of argon gas, sealed and stored at 4 °C. Cleavage of oligonucleotides from their solid supports was performed through on-column exposure to 1.50 mL of 30% $NH_4OH_{(aq)}$ for 2 hours at room temperature with the solution in full contact with the controlled pore glass. The oligonucleotides were then concentrated on a Speedvac evaporator overnight, resuspended in a solution of DMSO:3HF/TEA (100 μL:125 μL) and incubated at 65 °C for 3 hours in order to remove the 2'-*O*-TDBMS protecting groups. Crude oligonucleotides were precipitated in EtOH and desalted through Millipore Amicon Ultra 3000 MW cellulose. Oligonucleotides were characterized on a 20% acrylamide gel and further purified on a reverse phase HPLC C18 column, after which they were used for annealing and transfection. Equimolar amounts of complementary RNAs were annealed at 95 °C for 2 min in a binding buffer (75.0 mM KCl, 50.0 mM Tris-HCl, 3.00 mM $MgCl_2$, pH 8.30) and this solution was cooled slowly to room temperature to generate siRNAs used for biological assays. A sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na_2HPO_4, 1.00 mM EDTA, pH 7.00) was used to anneal strands for biophysical measurements.

Procedure for HPLC Purification

HPLC chromatograms were obtained on a Waters 1525 binary HPLC pump with a Waters 2489 UV/Vis detector using the Empower 3 software. A C18 4.6 mm x 150 mm reverse phase column was used. Conditions were 5% acetonitrile in 95% 0.1 M TEAA (Triethylamine-Acetic Acid) buffer up to 100% acetonitrile over 40 min. The main peak was collected, dried down and used for annealing and transfections without further purification.

Procedure for ESI Q-TOF Measurements

All single-stranded RNAs were gradient eluted through a Zorbax Extend C18 HPLC column with a MeOH/H_2O (5:95) solution containing 200 mM hexafluoroisopropyl alcohol and 8.1 mM triethylamine, and finally with 70% MeOH. The eluted RNAs were subjected to ESI-MS (ES^-), producing raw spectra of multiply-charged anions and through resolved isotope deconvolution, the molecular weights of the resultant neutral oligonucleotides were confirmed for all the RNAs.

Procedure for Performing CD Experiments

Circular Dichroism (CD) spectroscopy was performed on a Jasco J-815 CD equipped with temperature controller. Equimolar amounts of each siRNA (10 μM) were annealed to their compliment in 500 μL of a sodium phosphate buffer by incubating at 95 ^{0}C for two minutes and allowing to cool to room temperature. CD measurement of each duplex were recorded in triplicate from 200-500 nm at 25 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. The average of the three replicates was calculated using Jasco's Spectra Manager version 2 software and adjusted against the baseline measurement of the sodium phosphate buffer.

Procedure for Nuclease Stability Assays

SiRNAs 1-5 and wt were tested for nuclease stability at a concentration of 12 μM. The time points tested for the stability were 0, 0.5, 1, 2, 3 and 4 hours for each siRNA. In micro-centrifuge tubes 1 μL of 12 μM siRNA stock solution was added to 9 μL distilled water (10 μL total volume, 0 h time point) or 7.65 μL distilled water along with 1.35 μL fetal bovine serum (FBS) (10 μL total volume, all other time points), mixed and then incubated at 37°C for each time point. At each hour, the sample was prepared and placed in the incubator in a sequential order, starting with the 4-hour sample first. After the incubation, samples were run on a 20% non-denaturing polyacrylamide gel. Samples were mixed with 10 μL of non-denaturing loading dye and loaded onto the gel. The gel was run using a stacking method, in which the gel was first run at 30V for approximately 2 hours until the siRNA was evenly loaded. The gel was then run for an additional 20 hours at 70V. The gel was stained using 3X GelRed nucleic acid dye for 30-45 minutes and was visualized via Flurochem SP (Fisher Scientific).

Procedure for Maintaining Cell Cultures of HeLa Cells

For biological analysis of these siRNAs in a live environment, human epithelial cervix carcinoma cells were used (HeLa cells). They were kept in 250 mL vented culture flasks using 25.0 mL of DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma) in an incubator set for 37 °C @ 5% CO_2 humidified atmosphere.

Once cell lines became confluent (80-90%) they were passaged by washing 3 times with 10 mL of phosphate buffered saline (NaCl 137 mM, KCl 2.70 mM, PO_4^{3-} 10.0 mM, pH 7.40) and incubated with 3.00 mL of 0.25% trypsin (SAFC bioscience) for 4 min @ 37 °C to detach the cells. The cells were transferred to a 50.0 mL centrifuge tube after the addition of 10.0 mL of DMEM solution and pelleted at 300 g for 5 minutes. The supernatant was discarded and the pellet resuspended in 5.0 mL DMEM with 10% FBS.

A standard haemocytometer was used to obtain cell counts, after which the cells were diluted to a final concentration of 1.00×10^6 cells/mL for subsequent assays. To continue the cell line 1.00 mL of freshly passaged cells was added to 24.0 mL of DMEM/10% FBS and 1% penicillin-streptomycin at 37 °C in a new culture flask while the rest were used for assays.

Procedure for siRNA Transfections

One hundred μL of cells (total 1.00×10^5) were transfected into 12 well plates (Falcon®) with 1 mL of DMEM (10% FBS, 1% penicillin-streptomycin) and incubated at 37 °C with 5% CO_2. After 24 hours the cells were transfected with various concentrations of siRNAs, along with both pGL3 (Promega) and pRLSV40 luciferase plasmids using Lipofectamine 2000 (Invitrogen) in Gibco's 1X Opti-Mem reduced serum media (Invitrogen) according to the manufacturer's instructions. 1.00 μL of siRNA was added along with 2.00 μL (pGL3 200 ng) and 0.50 μL pRLSV40 (50.0 ng) to 100 μL of 1X Opti-Mem in a microcentrifuge tube and kept on ice for 5 min. In a different microcentrifuge tube, 1.00 μL of Lipofectamine 2000 (Invitrogen) was mixed with 100 μL of Gibco's 1X Opti-Mem reduced serum media (Invitrogen) and incubated at room temperature for 5 min. After 5 minutes the tubes were mixed and incubated at room temperature for 20 min and then the entire contents transferred to the wells of the 12 well plate.

Procedure for *in vitro* Dual-Reporter Luciferase Assay

One hundred μL of cells (total of 1.00×10^5 cells) were added to 12 well plates (Falcon®) with 1 mL of growth media (DMEM 10% FBS, 1% penicillin-streptomycin) and incubated at 37 °C with 5% CO_2. After 24 hours the cells were transfected with 10.0, 50.0, 100, 200, 400 and 800 pM concentrations of siRNAs, along with both pGL3 (Promega) and pRLSV40 luciferase plasmids using Lipofectamine 2000 (Invitrogen) in Gibco's 1X Opti-Mem reduced serum media (Invitrogen) according to the manufacturer's instructions. After a set amount of time (8, 12 or 22h) the cells were incubated at room temperature in 1X passive lysis buffer (Promega) for 20 minutes. The lysates were collected and loaded onto a 96 well, opaque plate (Costar). With a Dual-Luciferase reporter Assay kit (Promega), Lar II and Stop & Glo® luciferase substrates were sequentially added to the lysates and enzyme activity was measured through luminescence of both firefly/*Renilla* luciferase on a Synergy HT (Bio-Tek) plate luminometer. The ratio of

firefly/*Renilla* luminescence is expressed as a percentage of reduction in firefly protein expression to siRNA efficacy when compared to untreated controls. Each value is the average of at least 3 different experiments with standard deviation indicated.

Procedure for *in vitro* strand selection assay

In a 24-well culture dish, 500 μL of 1×10^5 cells/mL (50,000 cells per well) were dispensed and grown for 24 hours. For siRNA treatments, 50 μL of Opti-MEM was mixed with 2.0 μL of Lipofectamine 2000 (both from ThermoFisher) and incubated at room temp for at least 5 minutes. This mixture was added to a separately prepared mixture of 50 μL Opti-MEM containing 100 ng pGL3 (expresses the gene target for the guide strand of siRNA), 100 ng pGL3-Reverse (expresses the gene target the passenger strand of siRNA), 25 ng pRLSV40 (expresses the internal reference control), and an appropriate mass of chemically modified siRNA for the intended treatment concentration. The combined mixture was incubated at room temperature for 20 minutes then added to the 24-hour cultures to yield a final volume of 600 μL and incubated for an additional 24 hours. At 48 hours, RNA was extracted from cells using an RNA Purification Plus Kit including the supplementary on-column DNase I treatment according to manufacturer's instructions (both from Norgen Biotek). RNA was spectrophotometrically analysed on a Bio-Drop DUO (BioDrop) to confirm A260/A280 values of ~2.0. First-strand cDNA was synthesized using 100 to 250 ng RNA in 10.0 μL reactions using the iScript Reverse Transcription Supermix according to manufacturer's instructions (Bio-Rad) and diluted four times for qPCR amplification within a linear range. Approximately 10% of all RNA samples were randomly tested for DNA contamination via cDNA synthesis lacking reverse transcriptase. qPCR was performed (98°C for 30 s; 40 cycles of 96 °C for 5 s, 57°C for 25 s) in duplicate for each biological triplicate using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with a primer concentration of 500 nM on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Forward primers 5'-TCGAGGTGGACATCACTTACGC-3' and 5'-GAGGTGGACATCATCGAAGTACTC-3' were used to measure guide (pGL3 expressed) and passenger (pGL3-Reverse expressed) strand knockdown, respectively. The reverse primer 5'-CTCCGATAAATAACGCGCCC-3' was used for both targets. The forward and reverse primers 5'-GGTAACGCGGCCTCTTCTTA-3' and 5'-ATGGTAACGCGGCCTCTTC-3', respectively, were used to measure the internal reference gene (pRLSV40 expressed). Relative gene expression was calculated with the CFX Manager software (Bio-Rad) using the ΔΔCq method using the reference control gene expressed from pRLSV40.

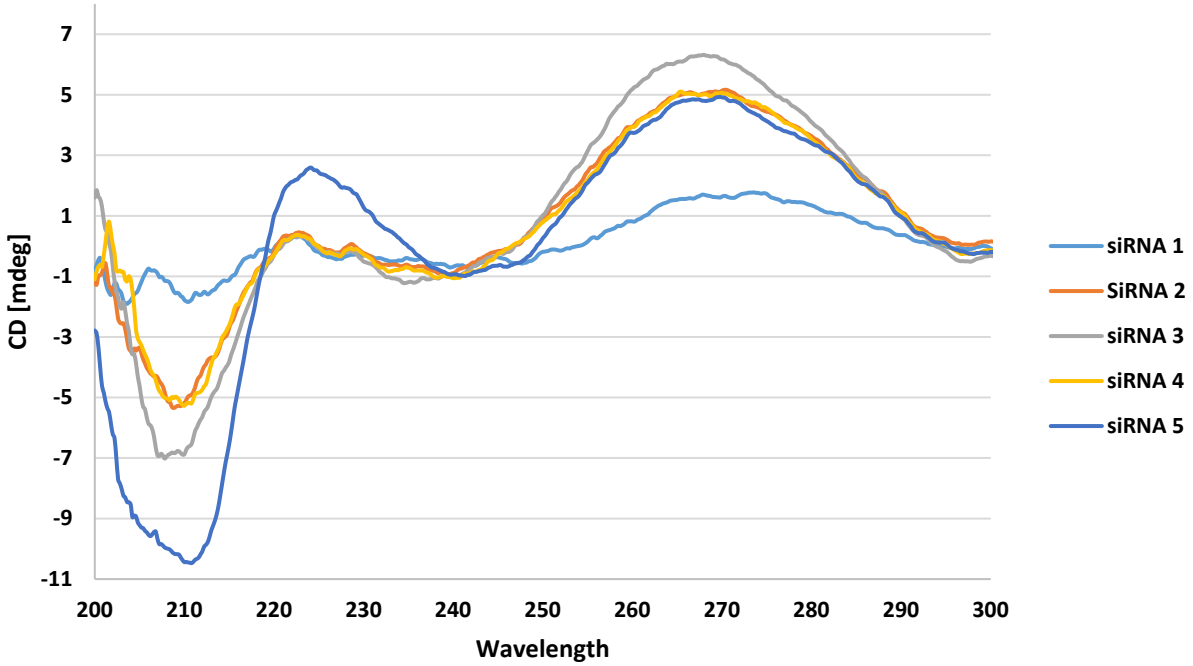


Figure S-1. CD spectra of phosphate modified dimers targeting firefly luciferase mRNAs. Wildtype and modified anti-*firefly* luciferase siRNAs (10 μM/duplex) were suspended in 500 μL of a sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na_2HPO_4, 1.00 mM EDTA, pH 7.00) and scanned from 200-300 nm at 25 °C with a screening rate of 20.0 nm/min and a 0.20 nm data pitch. All scans were performed in triplicate and averaged using Jasco's Spectra Manager version 2.

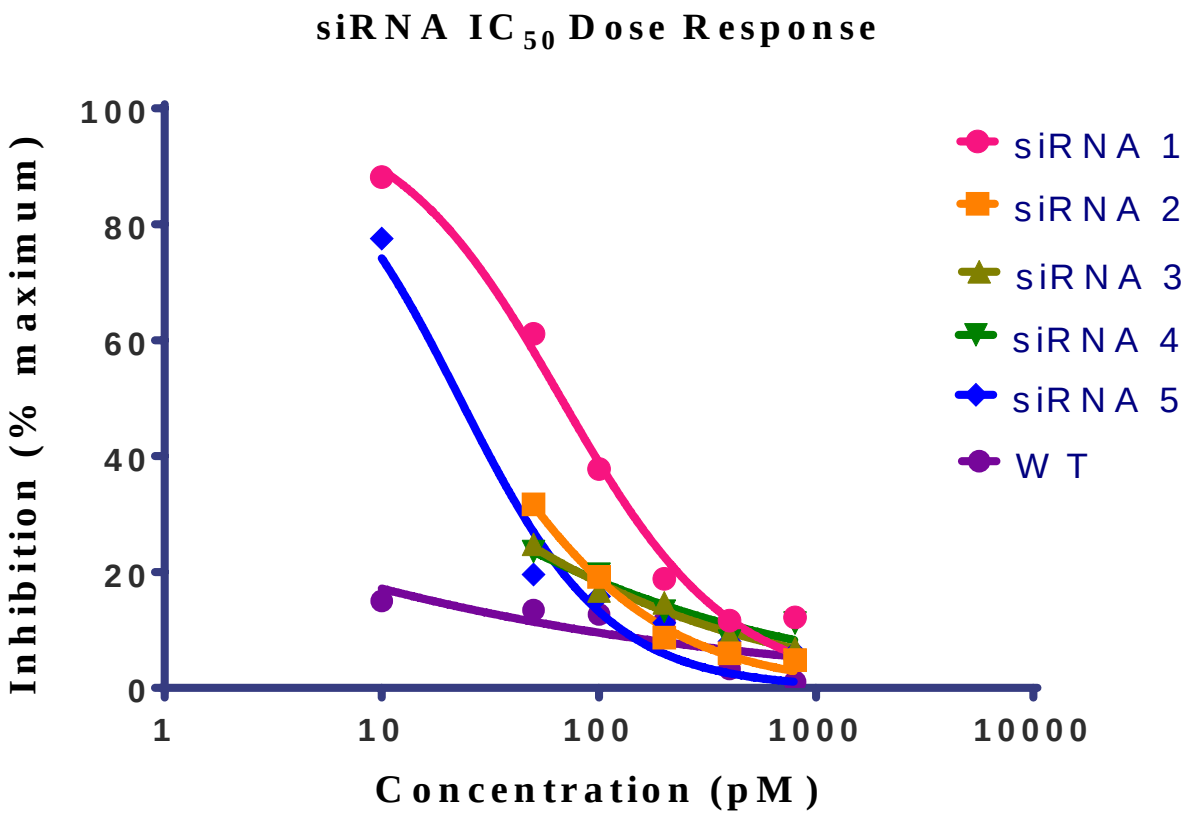


Figure S-2. Dose-responsive curves of siRNAs 1-5 with phosphate modifications using the Dual Reporter Luciferase Assay at 24 h timepoint. The siRNAs were tested at six concentrations from 10.0-800 pM with *firefly* luciferase expression normalized to *Renilla* luciferase. All IC_{50} values were calculated with Prism using the variable slope model. The IC_{50} values were as follows: siRNA 1 (66.9 pM), siRNA 2 (22.7 pM), siRNA 3 (5.94 pM), siRNA 4 (3.51 pM), siRNA 5 (22.8 pM) and wt (5 pM).

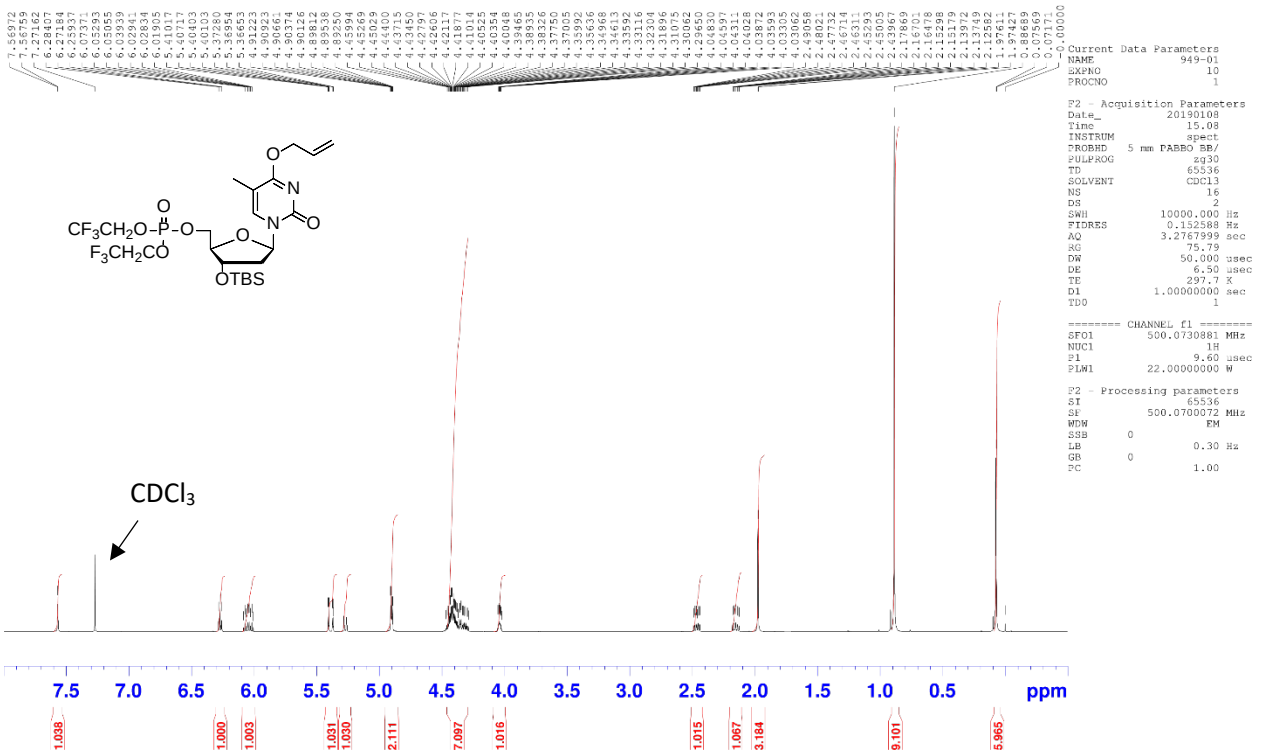
Table S-1 Sequences of anti-luciferase siRNAs, predicted and recorded mass of siRNAs containing the neutral triester phosphate modifications.[a]

siRNA	*siRNA Duplex*	*Predicted Mass*	*Actual Mass*[b]
wt	5'- CUUACGCUGAGUACUUCGAdTdT -3' 3'- dTdTGAAUGCGACUCAUGAAGCU - 5'	N/A	N/A
1	5' - C**dUxdU**ACGCUGAGUAC**dUxdU**CGAdTdT - 3' 3'- dTdTGAAUGCGACUCAUGAAGCU - 5'	6831.3	6830.9
2	5' - CUUACGCUGAGUACUUCGA**dUxdU** - 3' 3'- dTdTGAAUGCGACUCAUGAAGCU - 5'	6763.1	6762.9
3	5' - CUUACGCUGAGUACUUCGA**dUxdU** dT - 3' 3'- dTdTGAAUGCGACUCAUGAAGCU - 5'	7067.3	7066.9
4	5' - CUUACGCUGAGUACUUCGA**dTxdT**dT - 3' 3'- dTdTGAAUGCGACUCAUGAAGCU - 5'	7091.9	7089.9
5	5' - CUUACGCUGAGUACUUCGA**dTxdT** - 3' 3'- dTdTGAAUGCGACUCAUGAAGCU - 5'	6787.9	6786.9

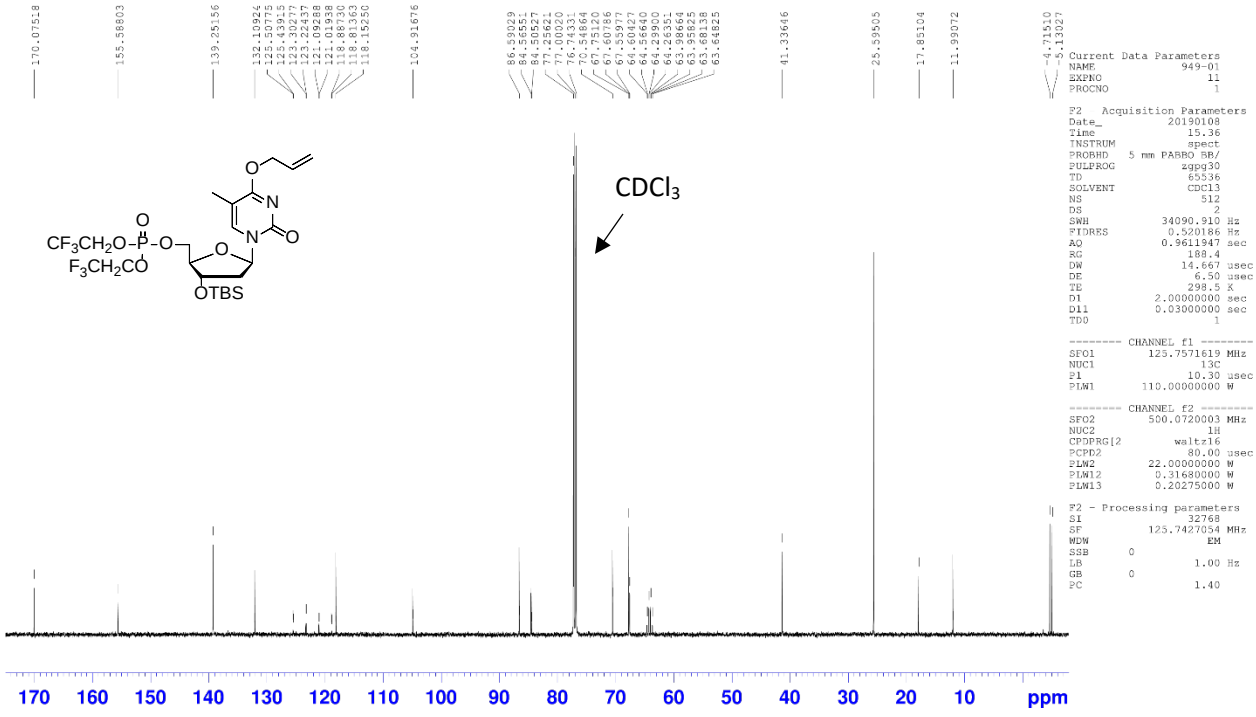
[a] **dUxdU** corresponds to the deoxyuridine-based modification; **dTxdT** corresponds to the deoxythymidine-based modification. The top strand is the sense strand; the bottom strand is the antisense strand. The antisense strand is 5'-phosphorylated. [b] Deconvolution results for siRNAs. ESI-HRMS (ES^+) m/z calculated for siRNAs **1-5** $[M+H]^+$

$^{1}H/^{13}C/^{19}F/^{31}P$ NMR Spectra of Compounds

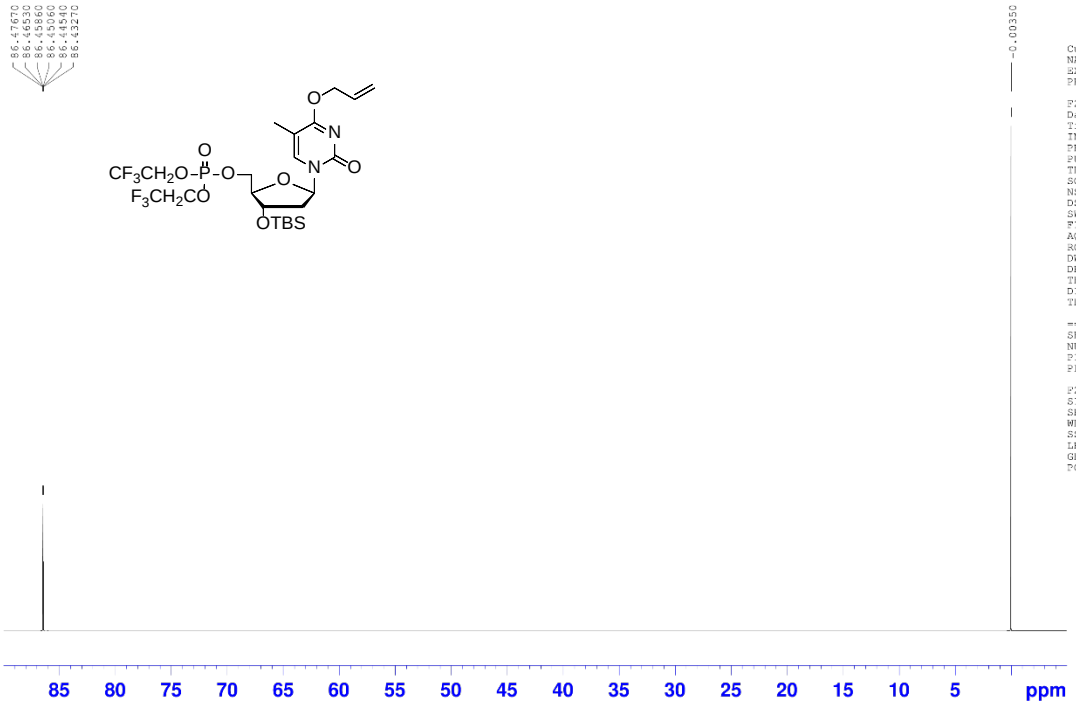
^{1}H NMR Spectra of Compound **5**



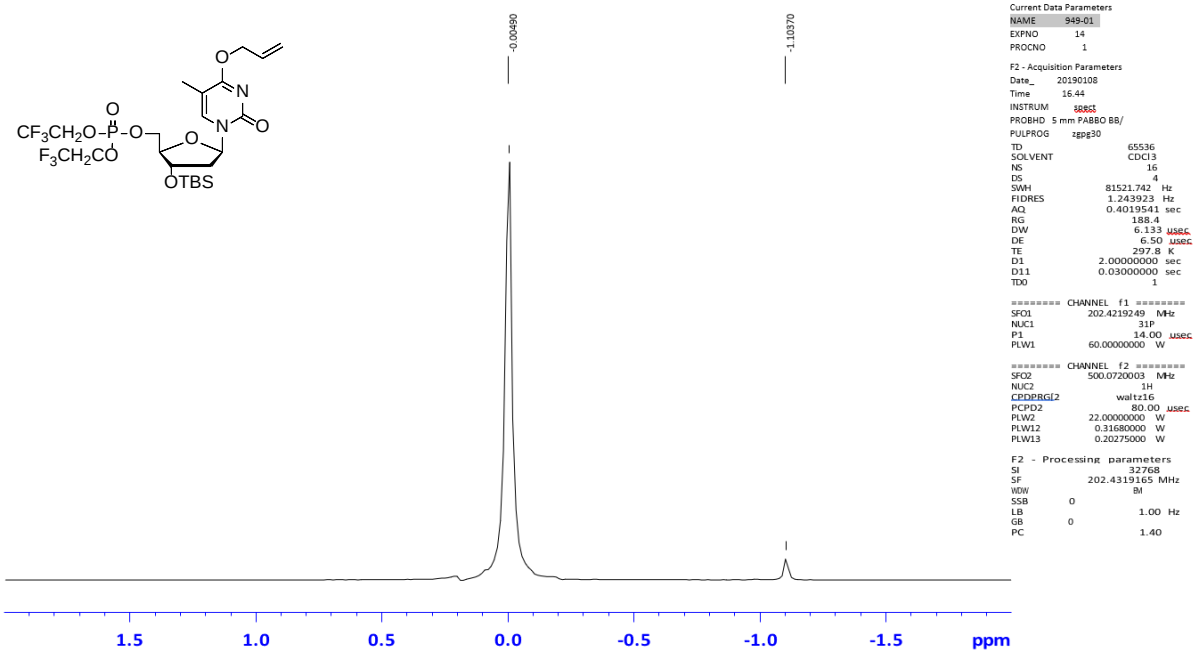
^{13}C NMR Spectra of Compound **5**



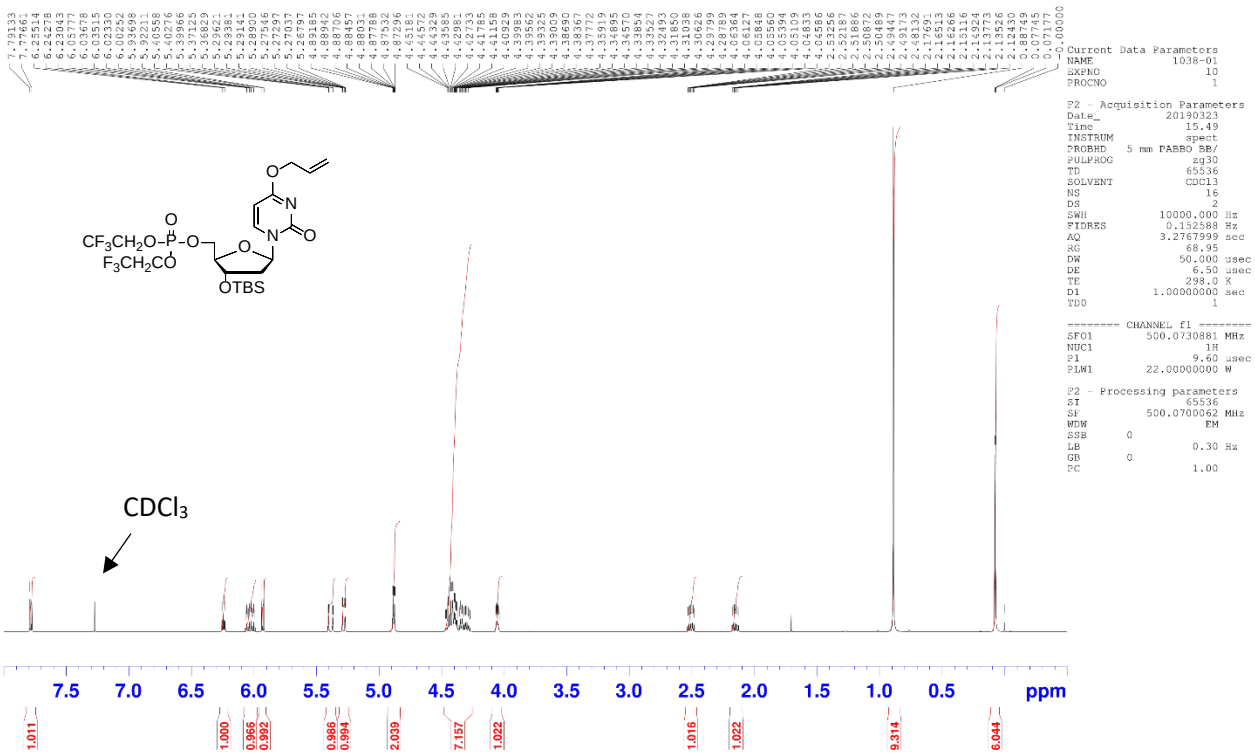
^{19}F NMR Spectra of Compound **5**



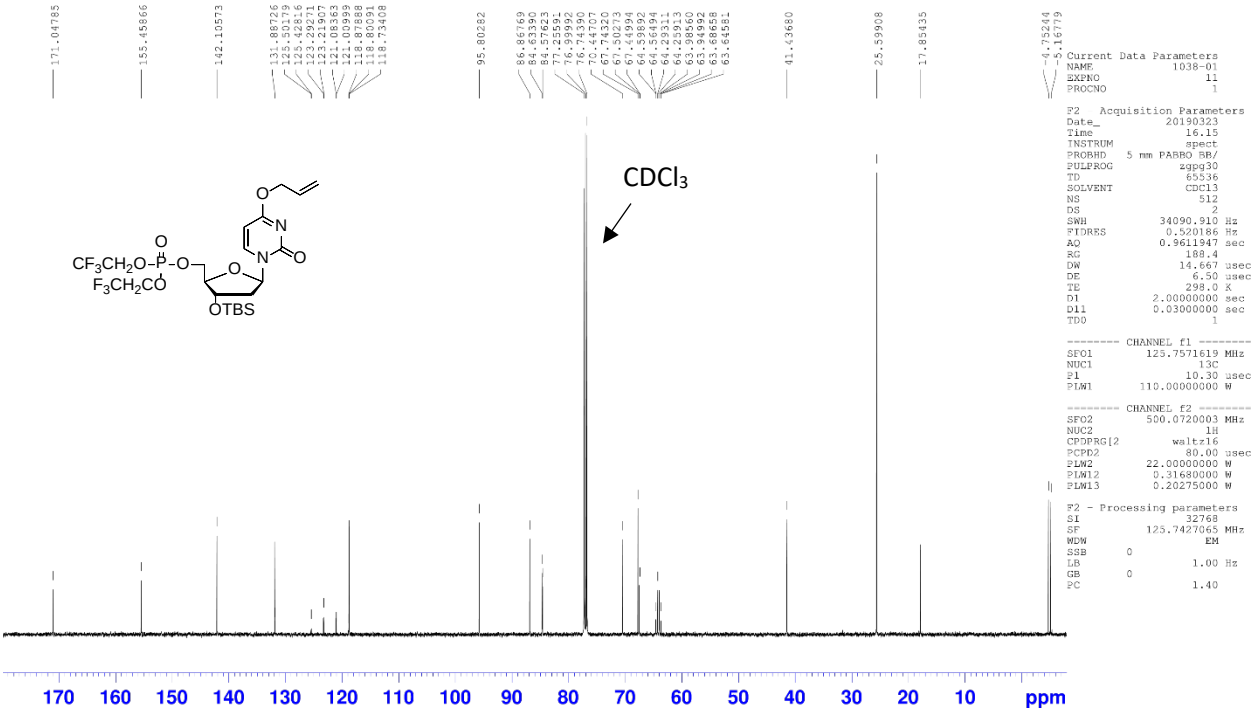
^{31}P NMR Spectra of Compound **5**



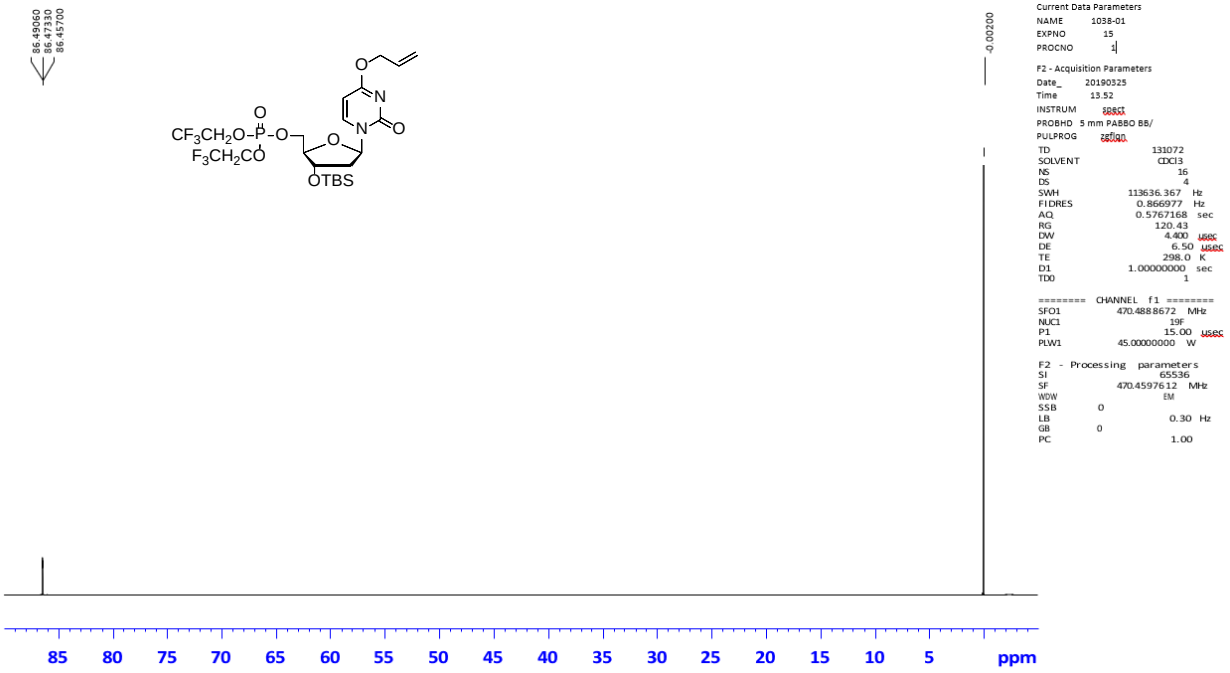
^{1}H NMR Spectra of Compound **6**



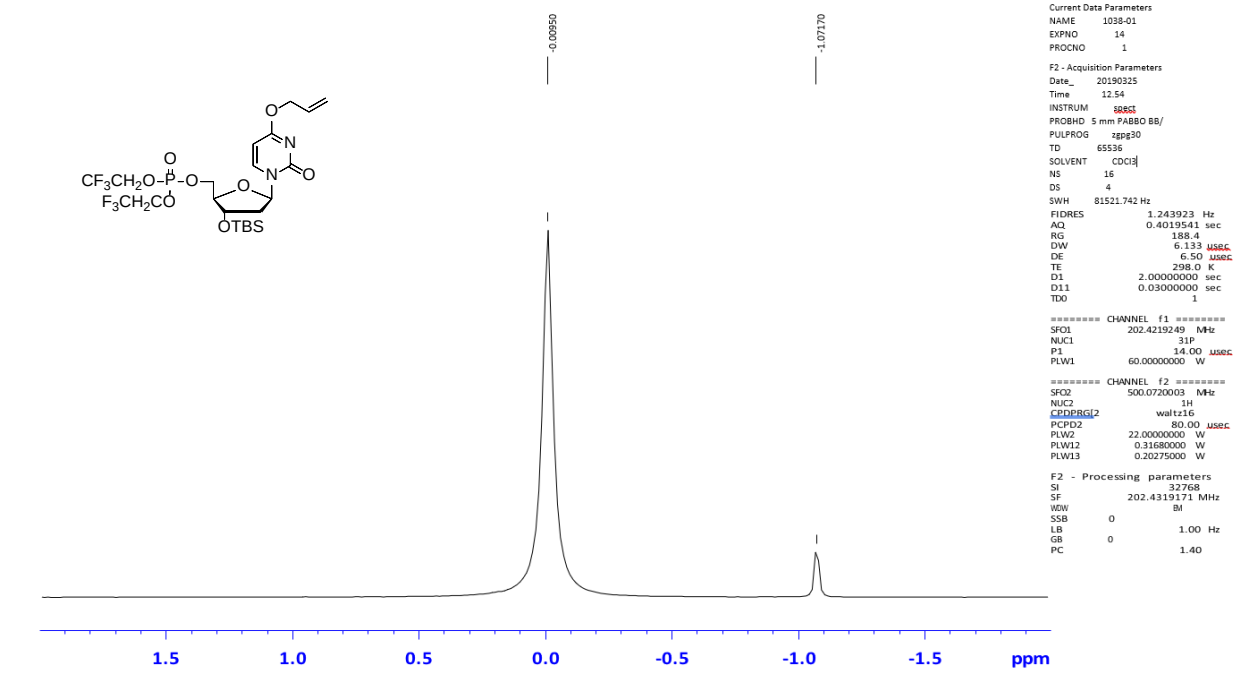
^{13}C NMR Spectra of Compound **6**



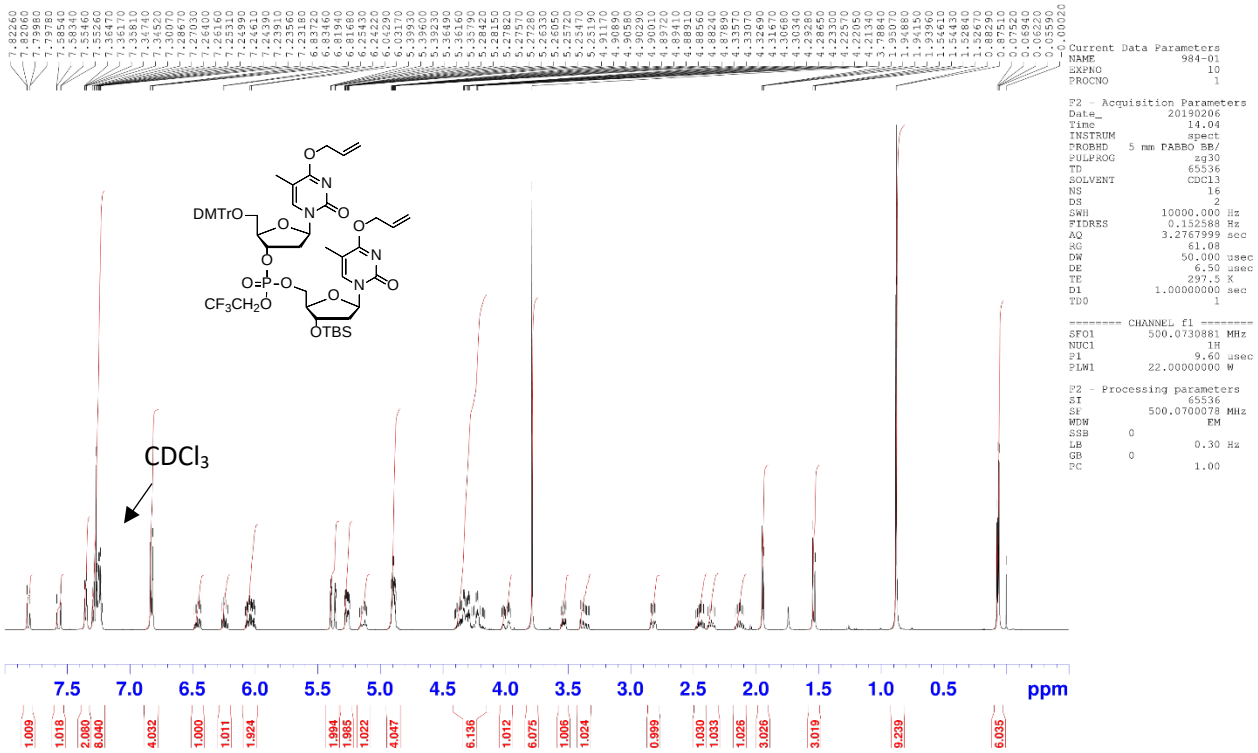
^{19}F NMR Spectra of Compound **6**



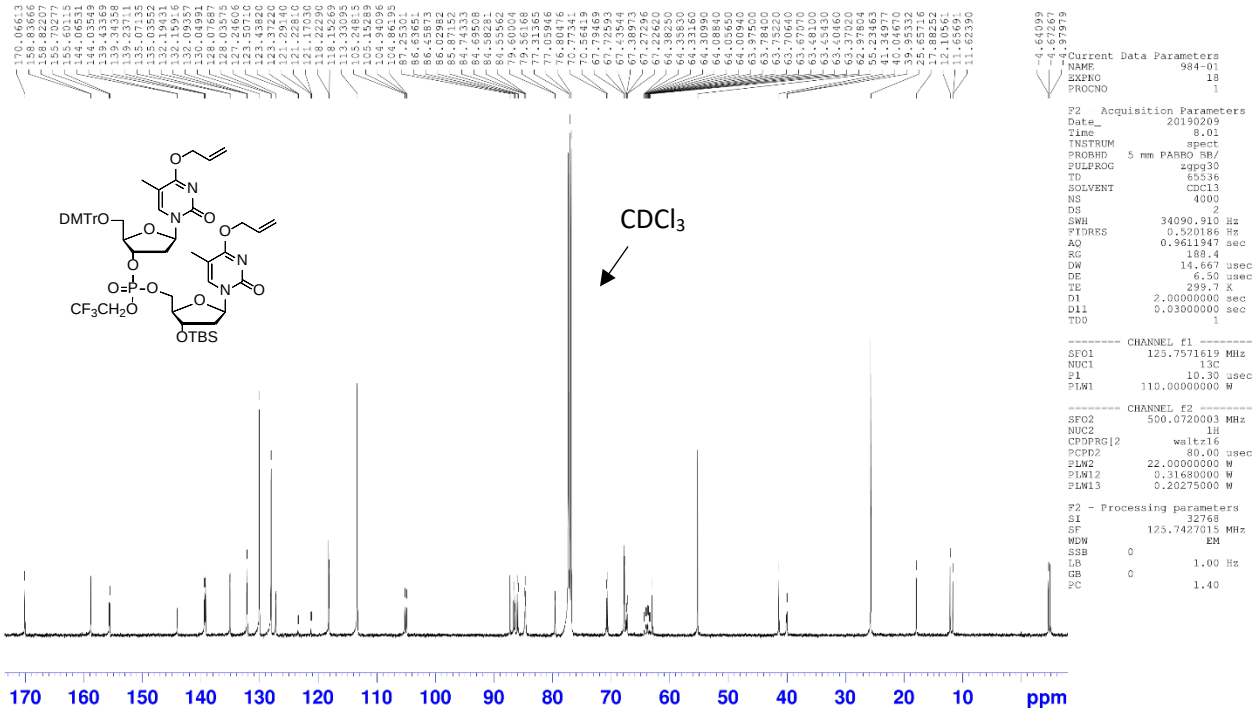
^{31}P NMR Spectra of Compound **6**



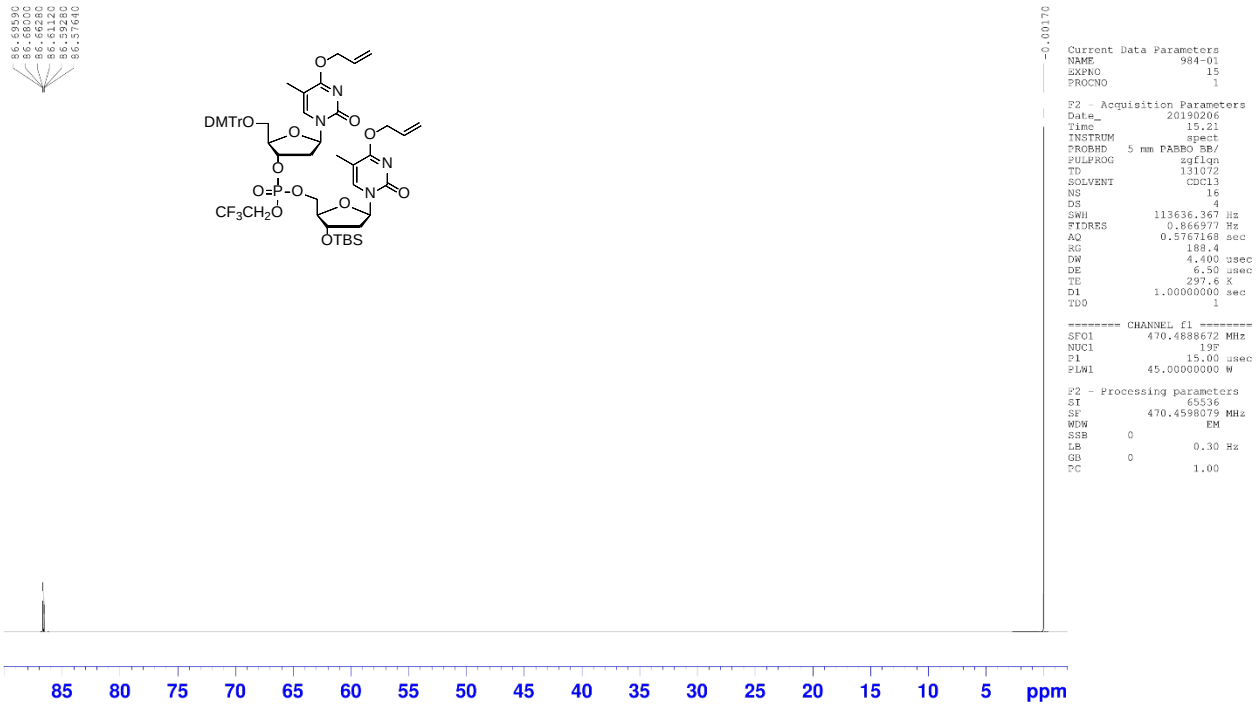
^{1}H NMR Spectra of Compound **7**



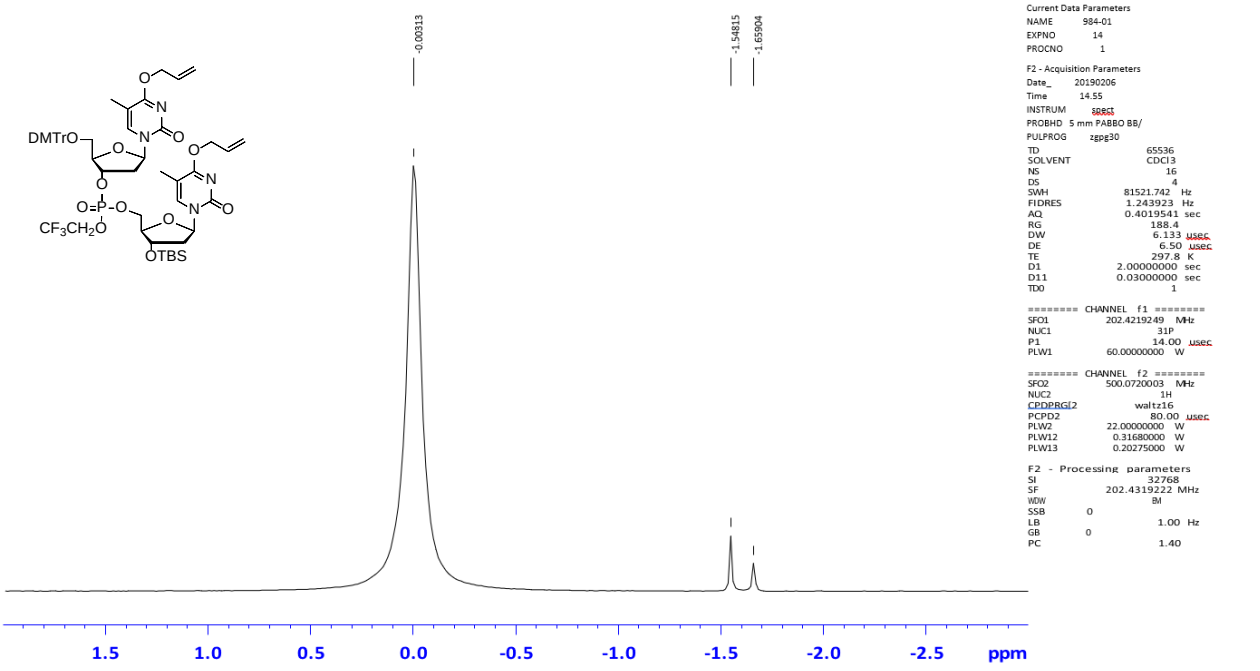
^{13}C NMR Spectra of Compound **7**



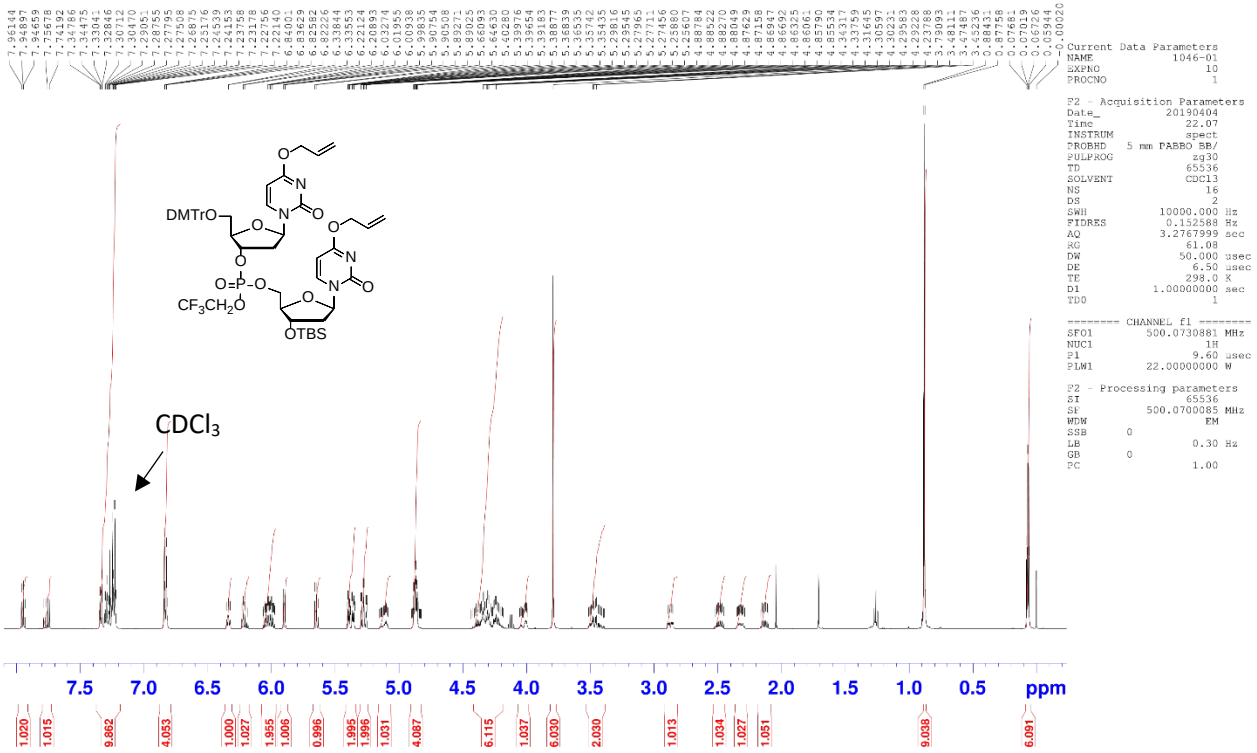
^{19}F NMR Spectra of Compound **7**



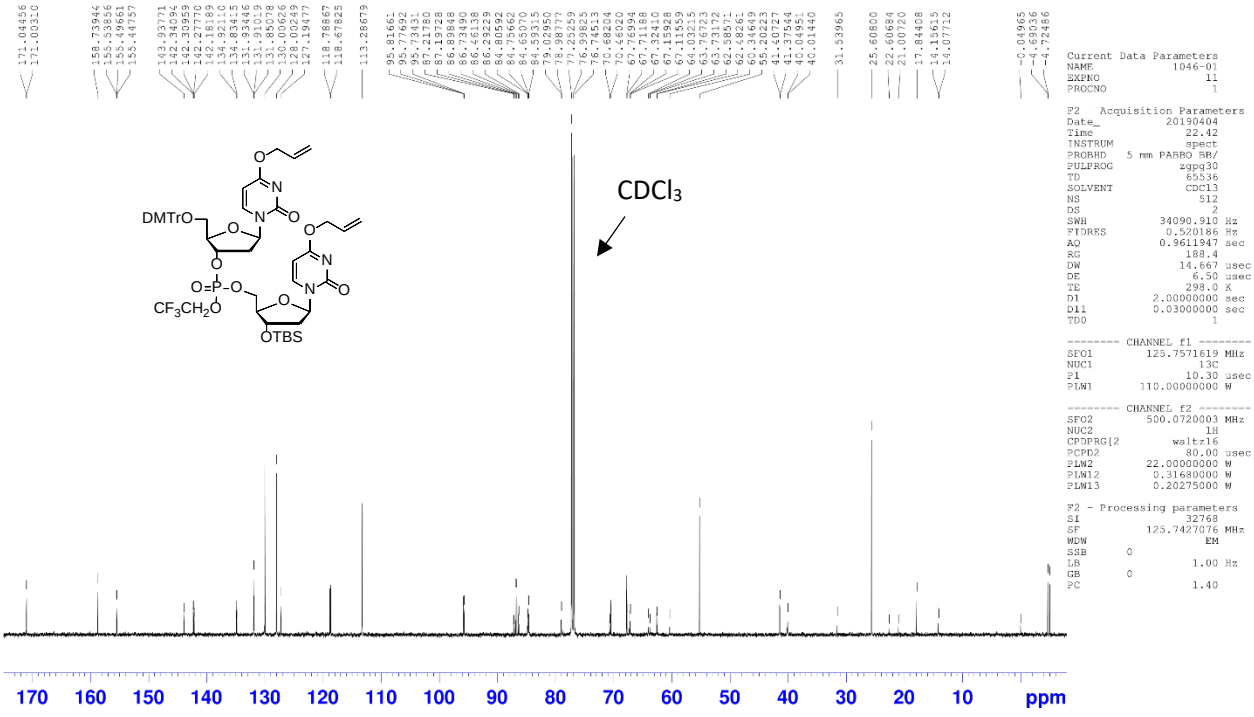
^{31}P NMR Spectra of Compound **7**



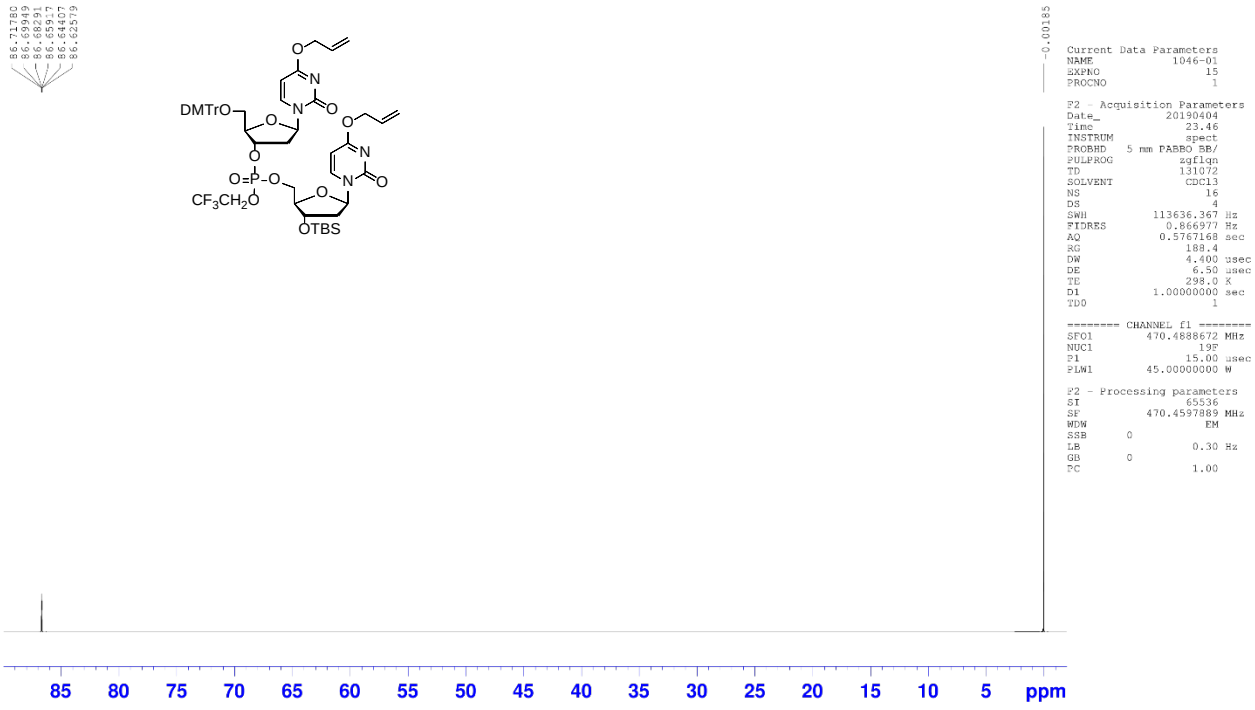
^{1}H NMR Spectra of Compound **8**



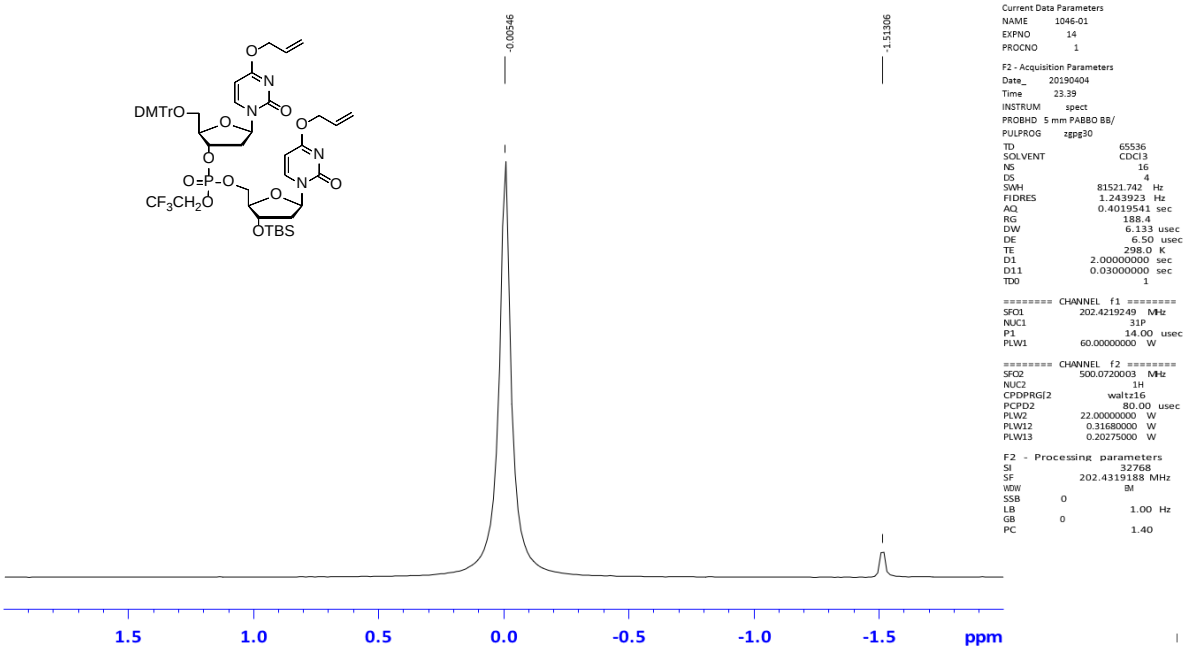
^{13}C NMR Spectra of Compound **8**



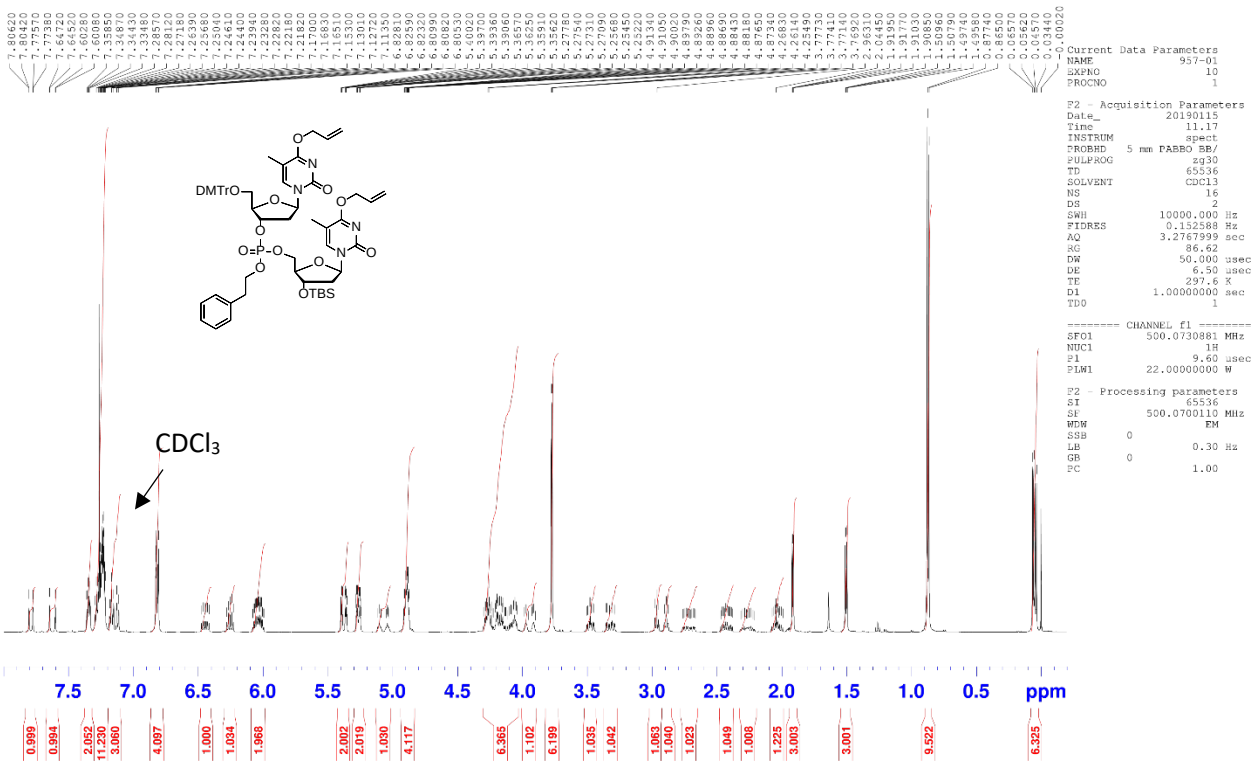
^{19}F NMR Spectra of Compound **8**



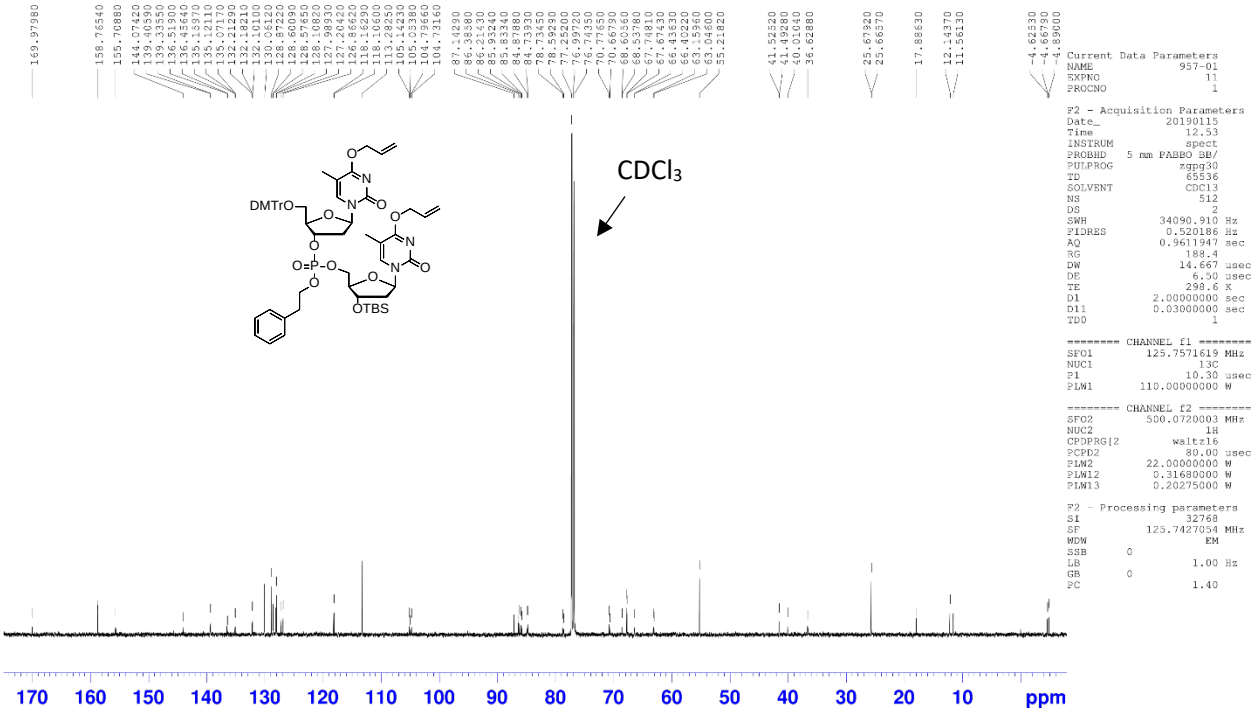
^{31}P NMR Spectra of Compound **8**



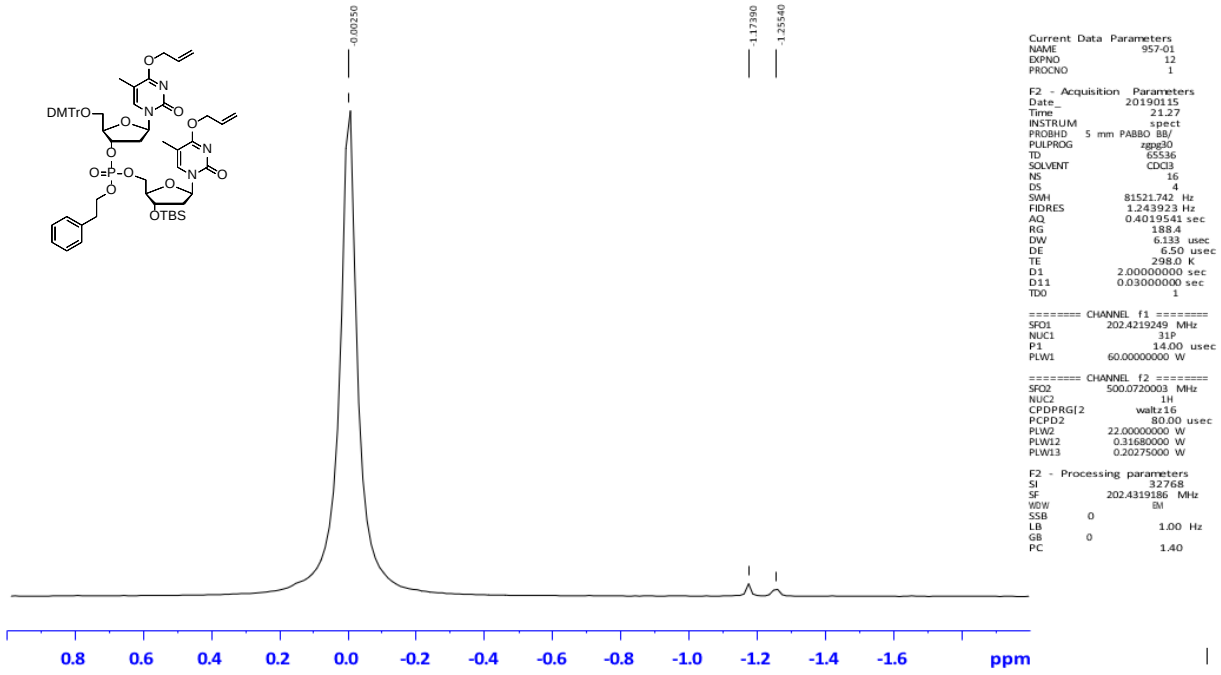
^{1}H NMR Spectra of Compound **9**



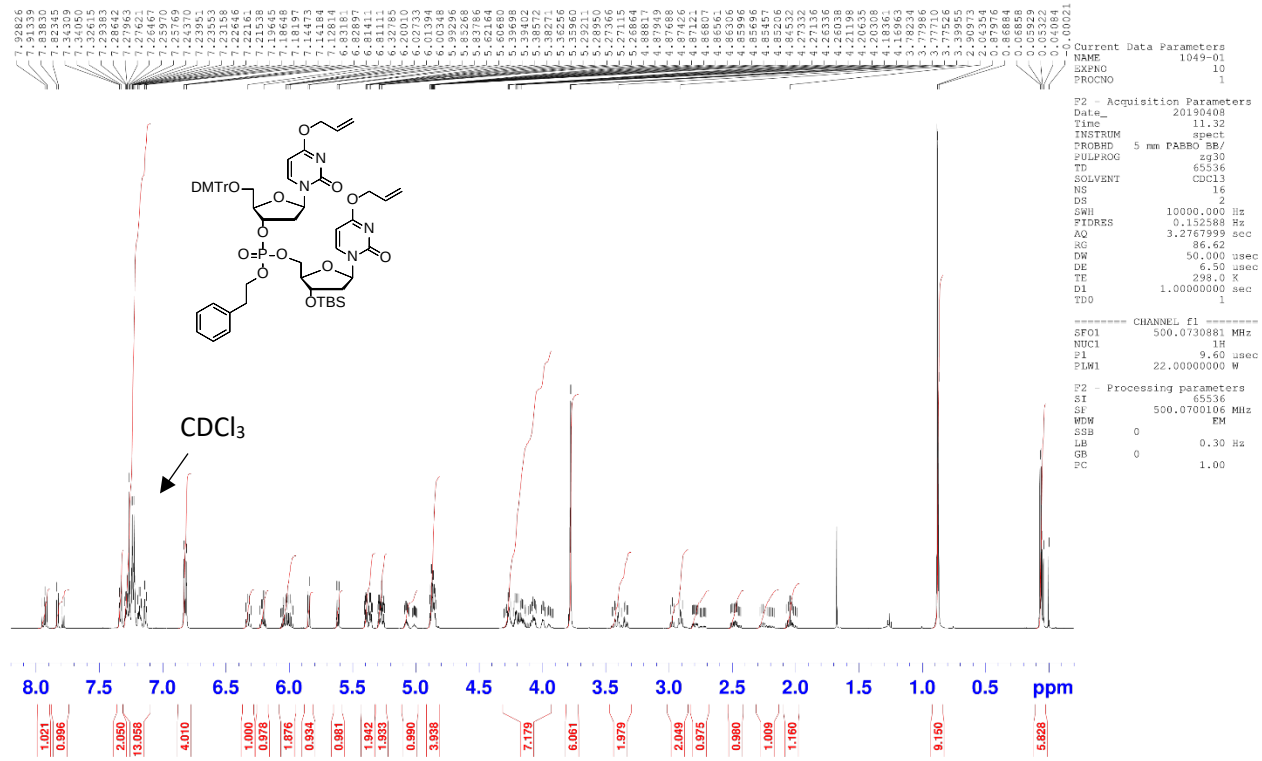
^{13}C NMR Spectra of Compound **9**



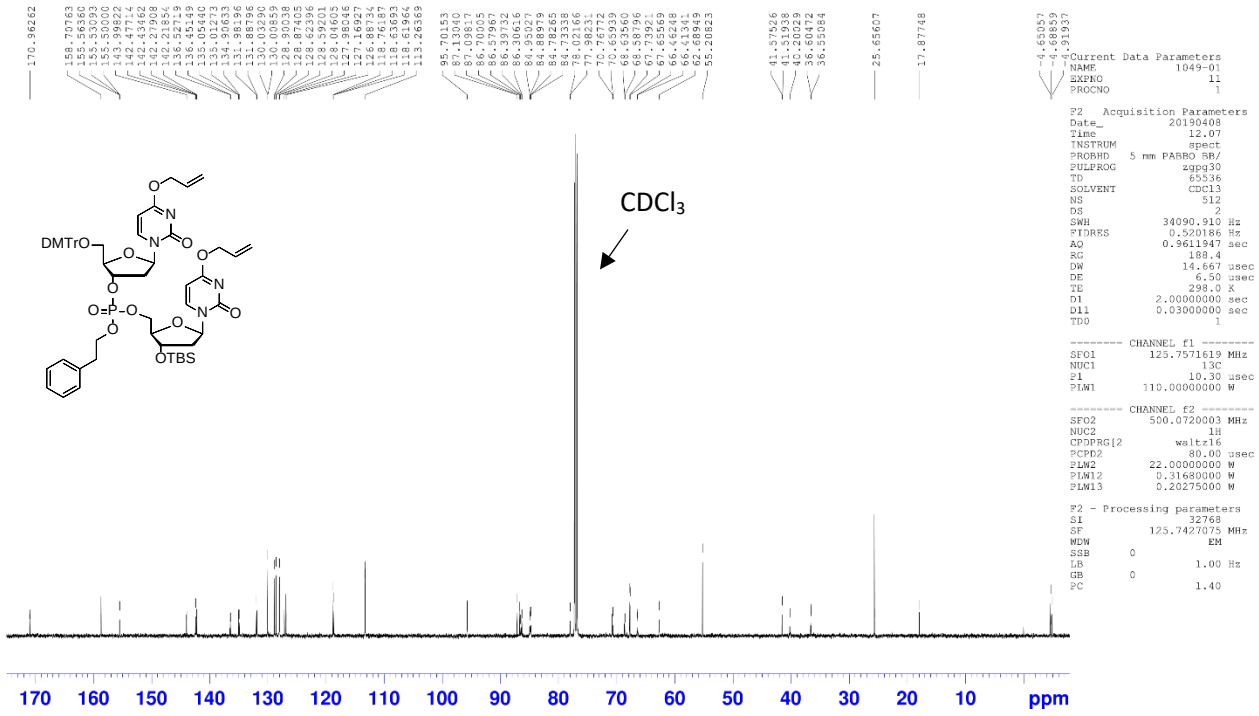
^{31}P NMR Spectra of Compound **9**



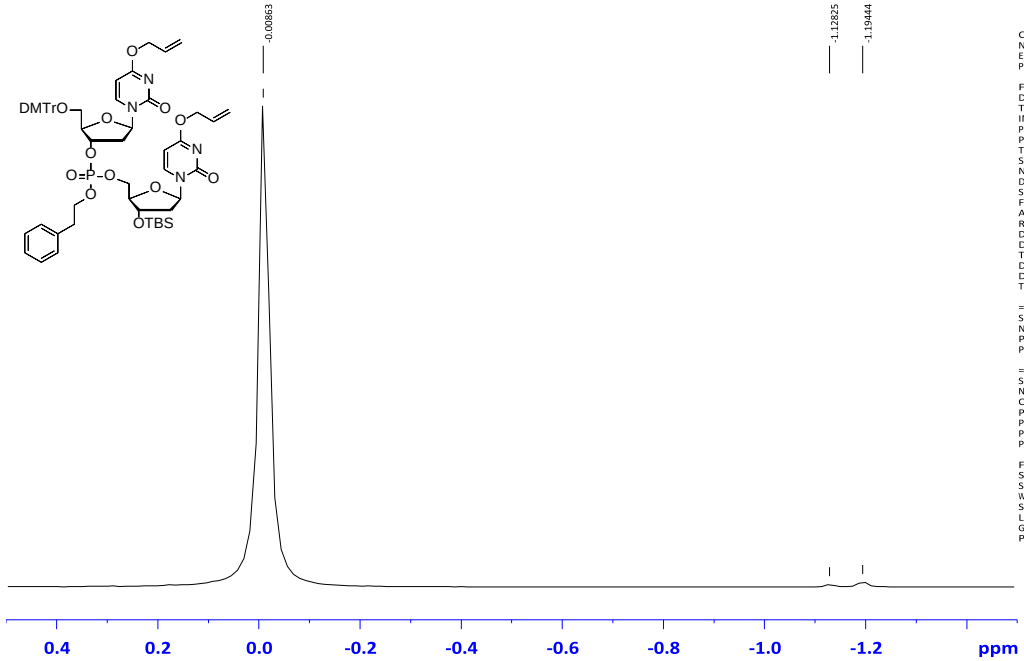
^{1}H NMR Spectra of Compound **10**



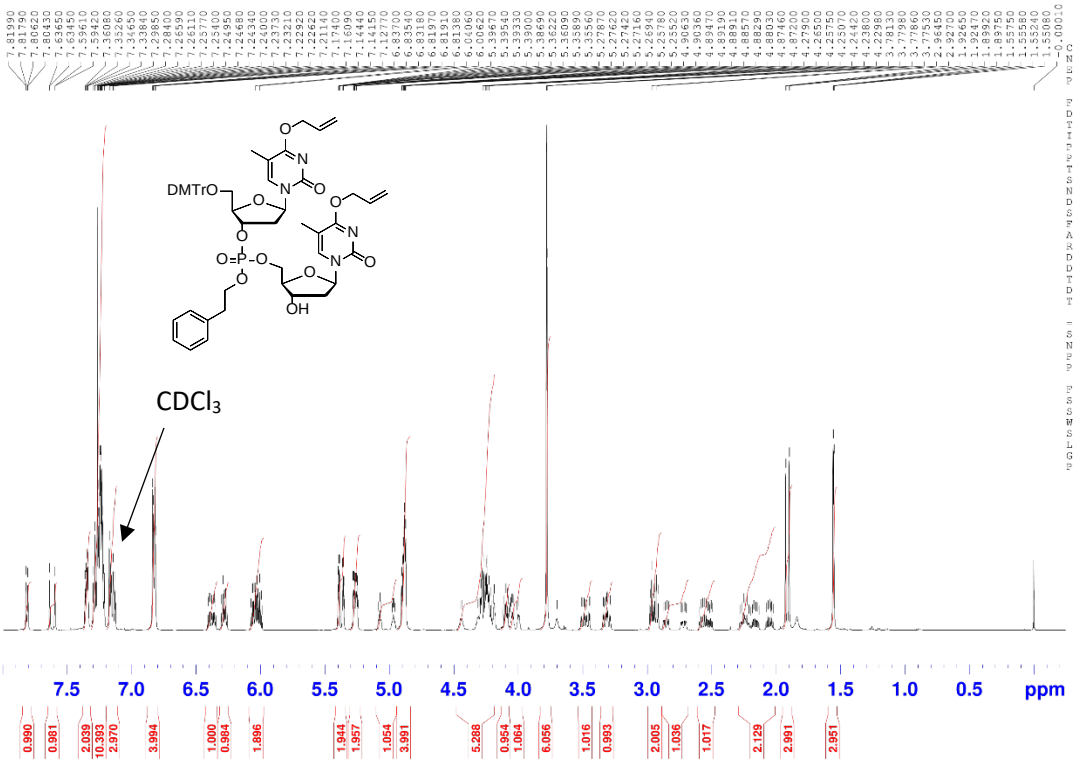
^{13}C NMR Spectra of Compound **10**



^{31}P NMR Spectra of Compound **10**



^{1}H NMR Spectra of Compound **11**



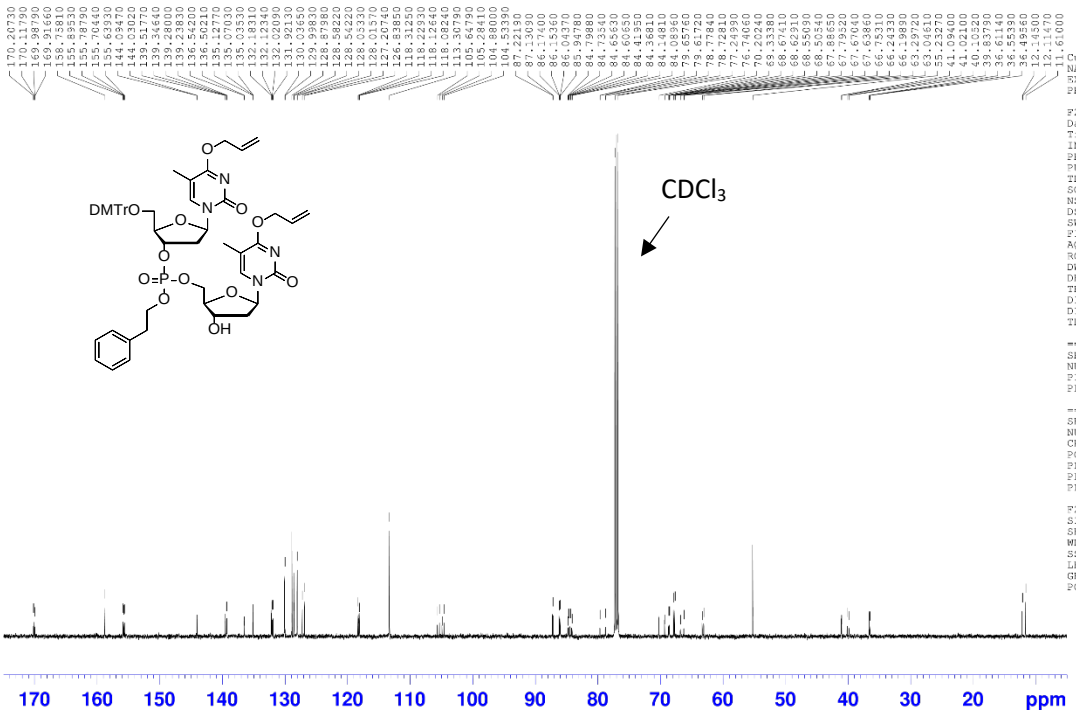
Current Data Parameters
NAME 987-01
EXPNO 10
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190208
Time 13.48
INSTRUM spect
PROBHD 5 mm PABBO BB/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 10000.000 Hz
FIDRES 0.152588 Hz
AQ 3.2767999 sec
RG 68.95
DW 50.000 usec
DE 6.50 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 500.0730881 MHz
NUC1 1H
P1 9.60 usec
PLW1 22.00000000 W

F2 - Processing parameters
SI 65536
SF 500.0700100 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

^{13}C NMR Spectra of Compound **11**



Current Data Parameters
NAME 987-01
EXPNO 11
PROCNO 1

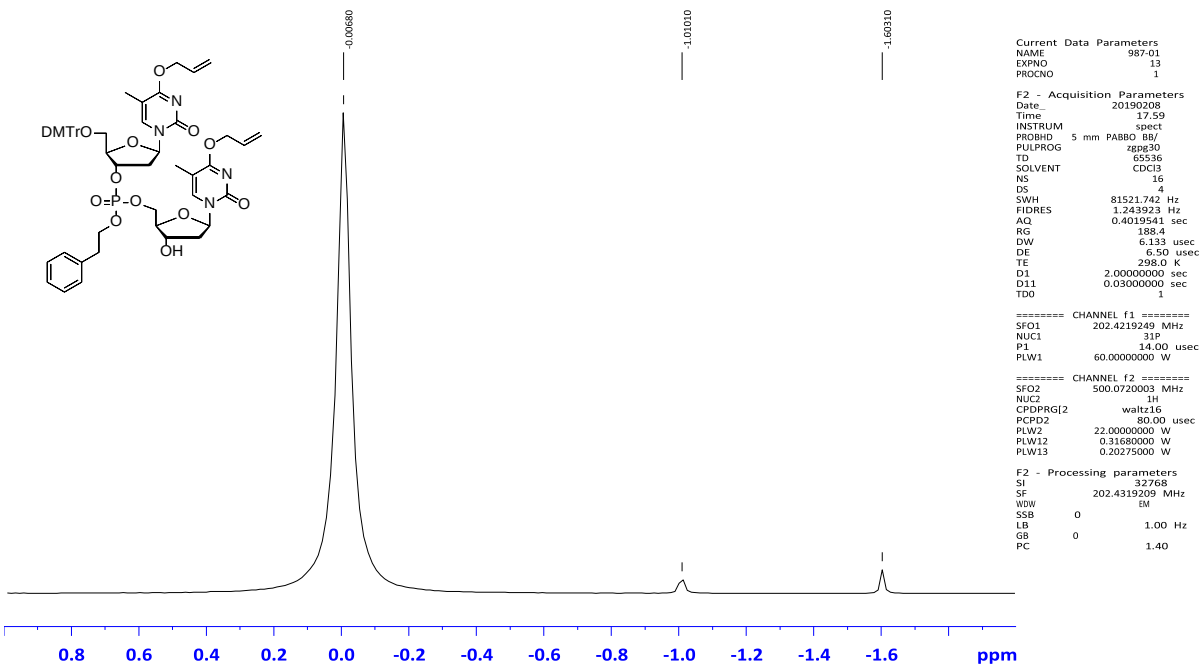
F2 - Acquisition Parameters
Date_ 20190208
Time 14.14
INSTRUM spect
PROBHD 5 mm PABBO BB/
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 512
DS 2
SWH 34090.910 Hz
FIDRES 0.520186 Hz
AQ 0.96111947 sec
RG 188.4
DW 14.667 usec
DE 6.50 usec
TE 298.7 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 125.7571619 MHz
NUC1 13C
P1 10.30 usec
PLW1 110.00000000 W

======== CHANNEL f2 ========
SFO2 500.0720003 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 80.00 usec
PLW2 22.00000000 W
PLW12 0.31680000 W
PLW13 0.20275000 W

F2 - Processing parameters
SI 32768
SF 125.7427076 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

^{31}P NMR Spectra of Compound **11**



Current Data Parameters
NAME 987-01
EXPNO 13
PROCNO 1

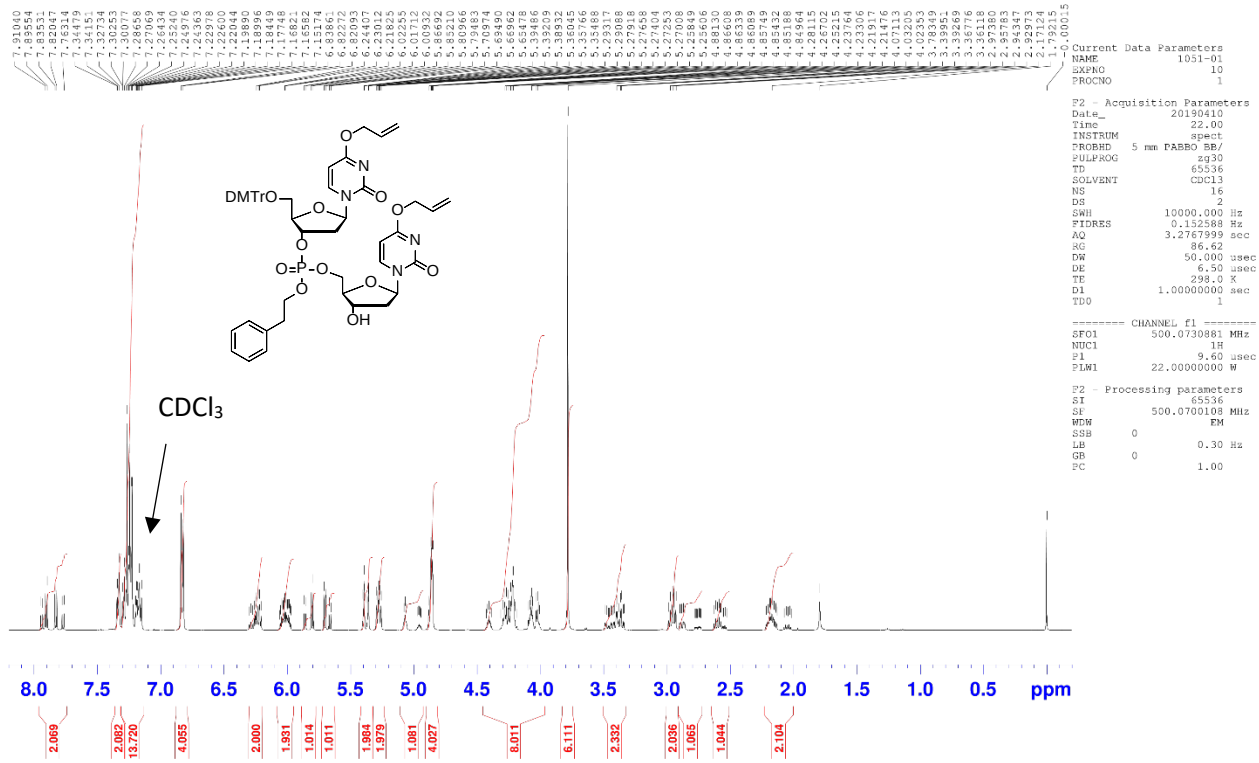
F2 - Acquisition Parameters
Date_ 20190208
Time 17.59
INSTRUM spect
PROBHD 5 mm PABBO BB/
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 16
DS 4
SWH 81521.742 Hz
FIDRES 1.243923 Hz
AQ 0.4019541 sec
RG 188.4
DW 6.133 usec
DE 6.50 usec
TE 298.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 202.4219249 MHz
NUC1 31P
P1 14.00 usec
PLW1 60.00000000 W

======== CHANNEL f2 ========
SFO2 500.0720003 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 80.00 usec
PLW2 22.00000000 W
PLW12 0.31680000 W
PLW13 0.20275000 W

F2 - Processing parameters
SI 32768
SF 202.4319209 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

^{1}H NMR Spectra of Compound **12**



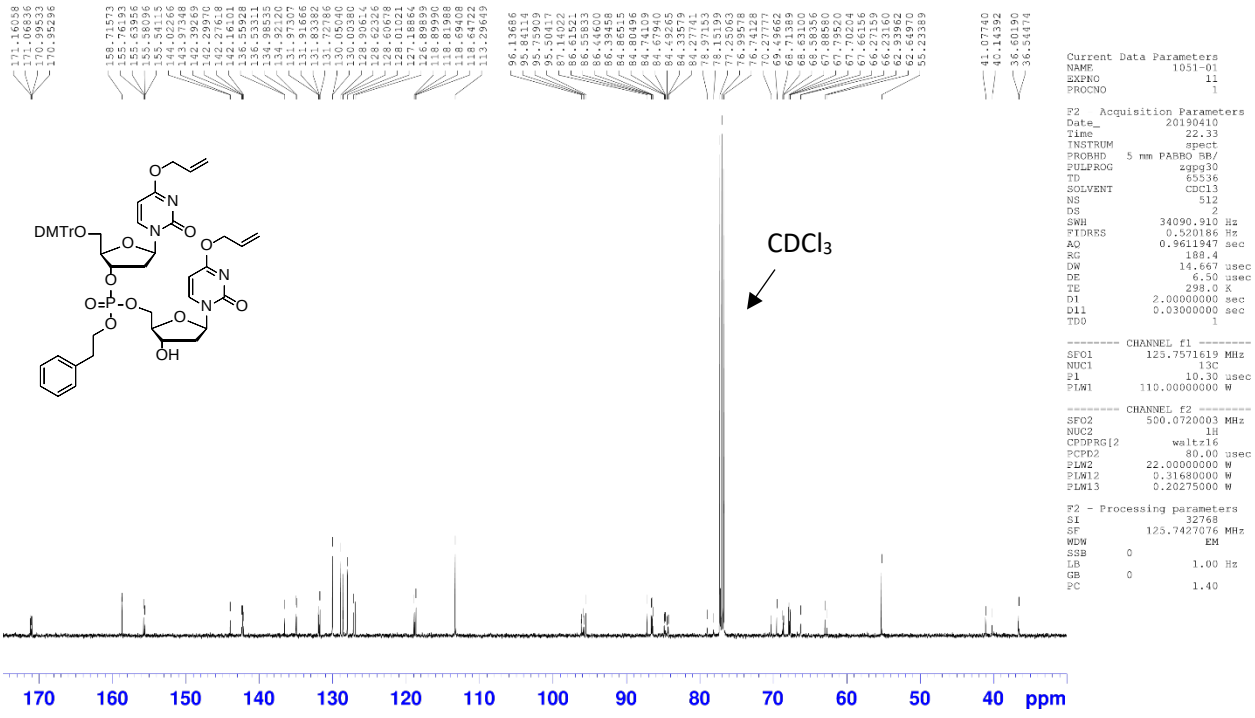
Current Data Parameters
NAME 1051-01
EXPNO 10
PROCNO 1

F2 - Acquisition Parameters
Date_ 20190410
Time 22.00
INSTRUM spect
PROBHD 5 mm PABBO BB/
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 16
DS 2
SWH 10000.000 Hz
FIDRES 0.152588 Hz
AQ 3.2767999 sec
RG 86.62
DW 50.000 usec
DE 6.50 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1

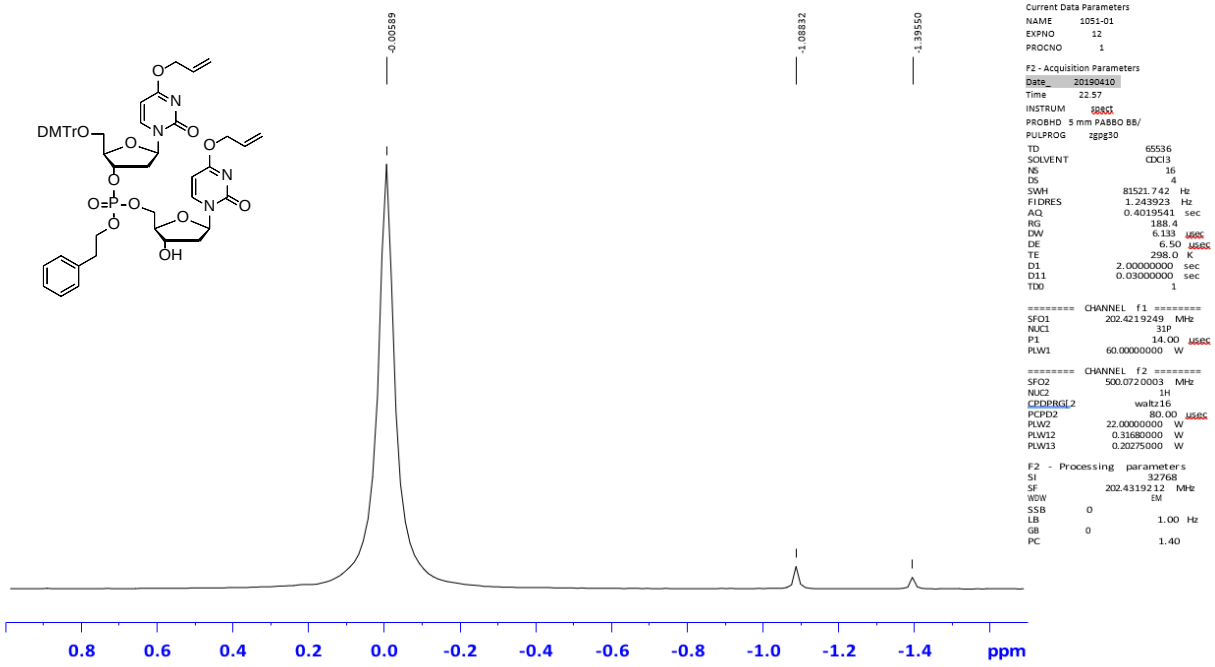
======== CHANNEL f1 ========
SFO1 500.0730881 MHz
NUC1 1H
P1 9.60 usec
PLW1 22.00000000 W

F2 - Processing parameters
SI 65536
SF 500.0700108 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

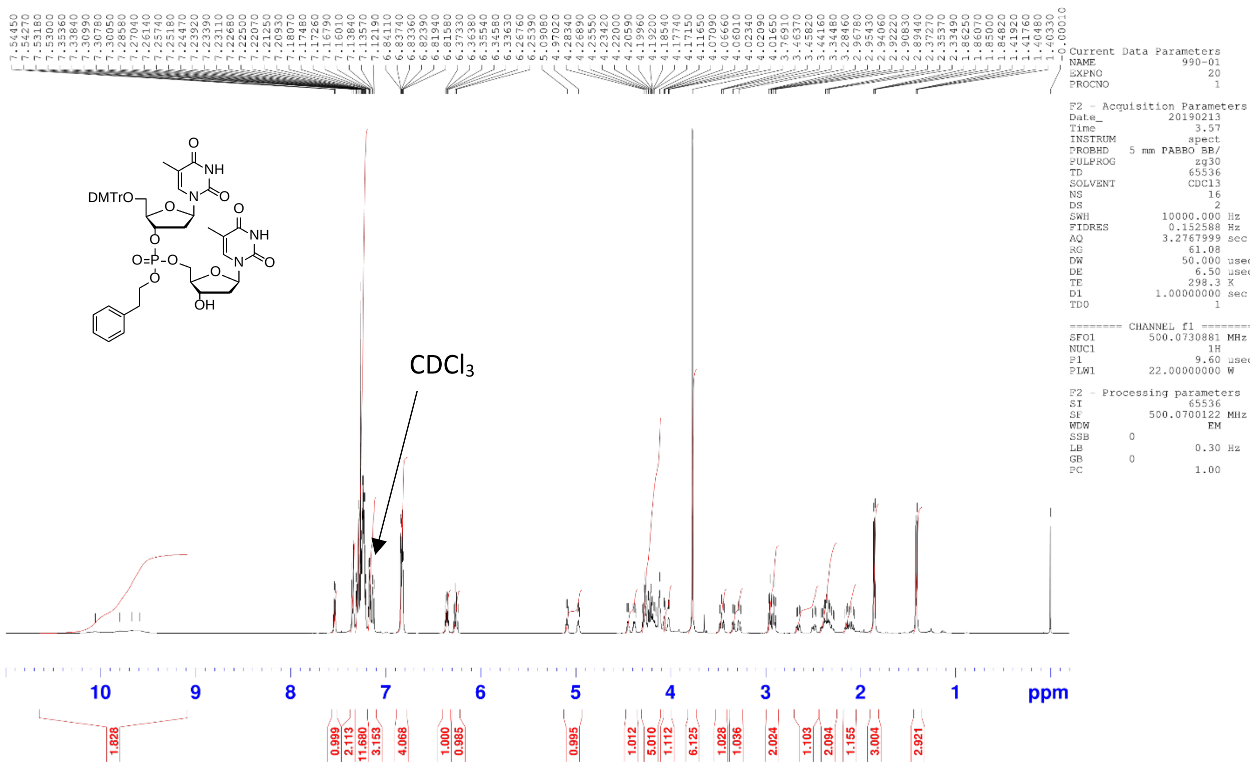
^{13}C NMR Spectra of Compound **12**



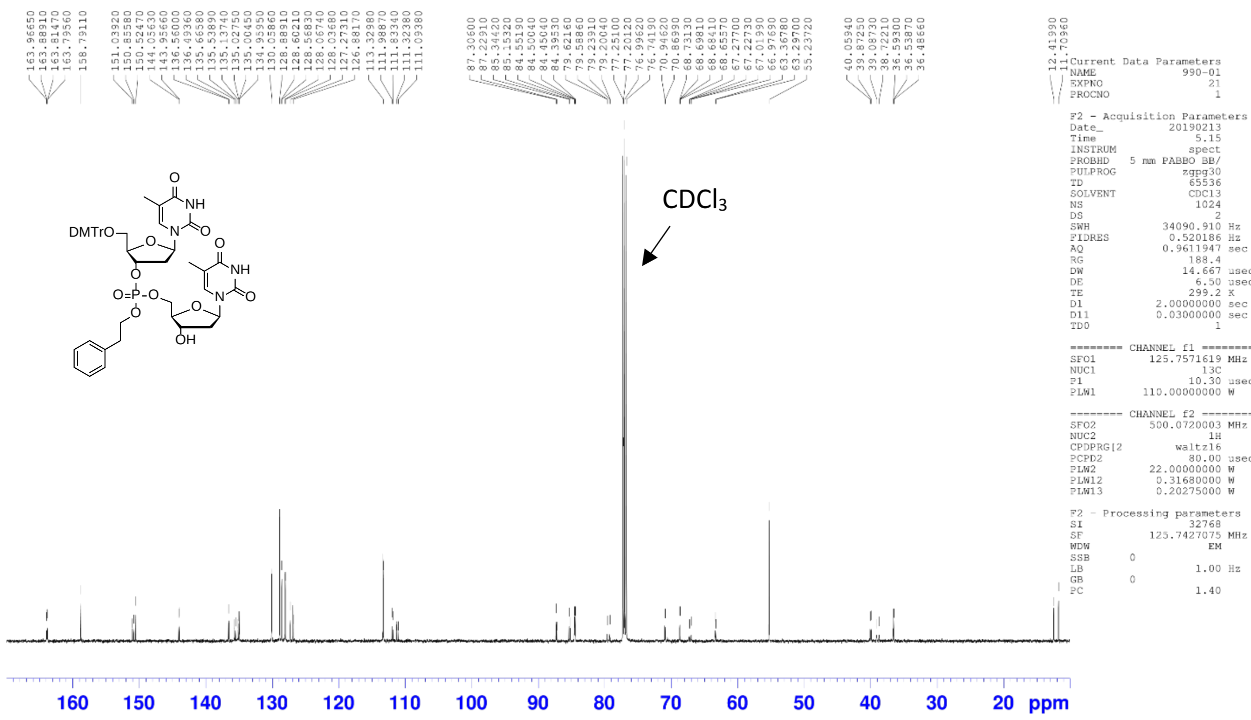
^{31}P NMR Spectra of Compound **12**



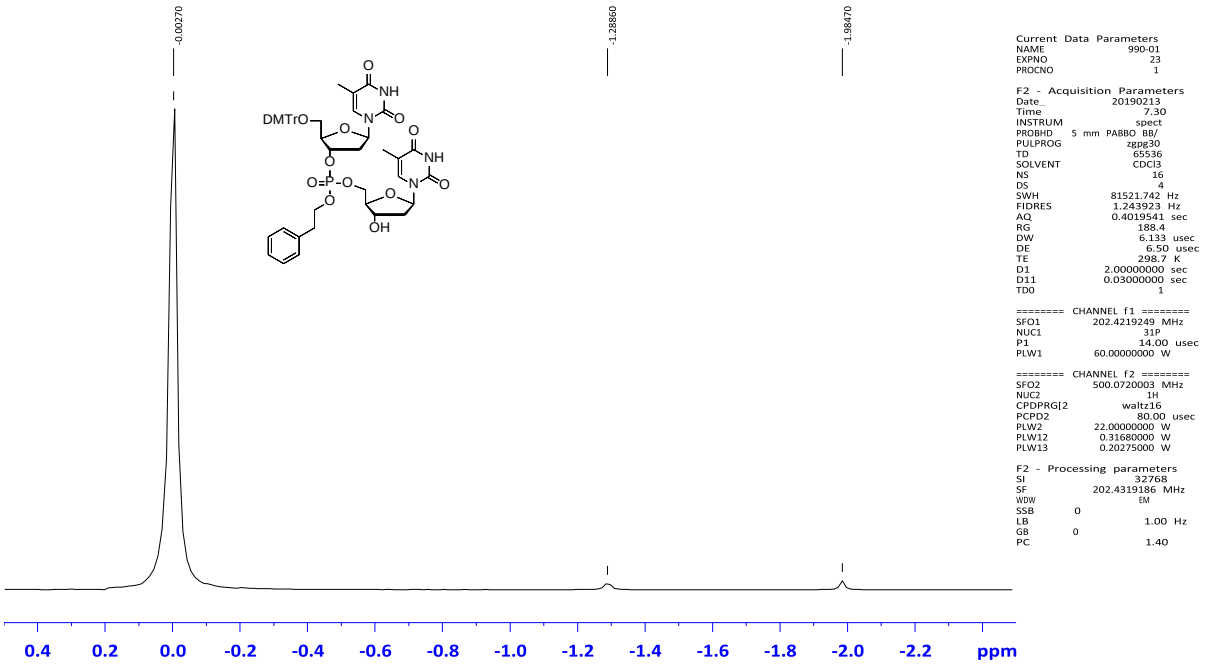
^{1}H NMR Spectra of Compound **13**



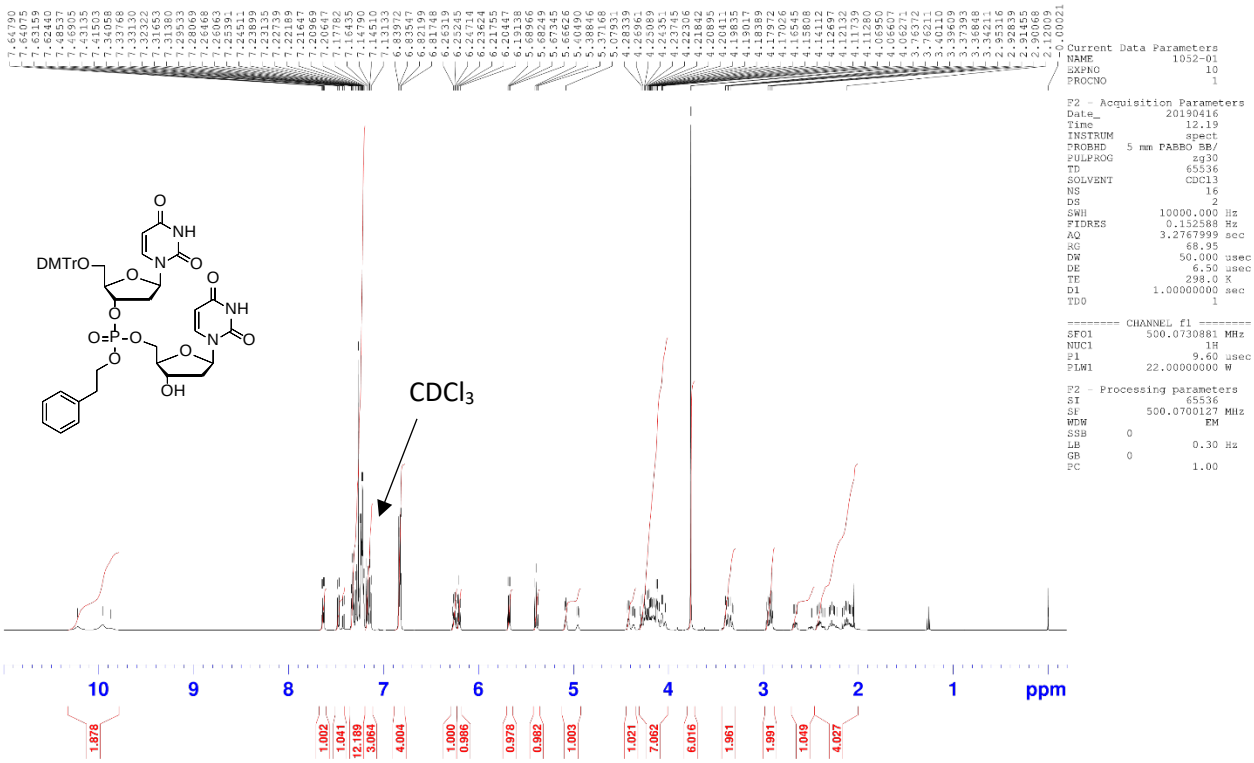
^{13}C NMR Spectra of Compound **13**



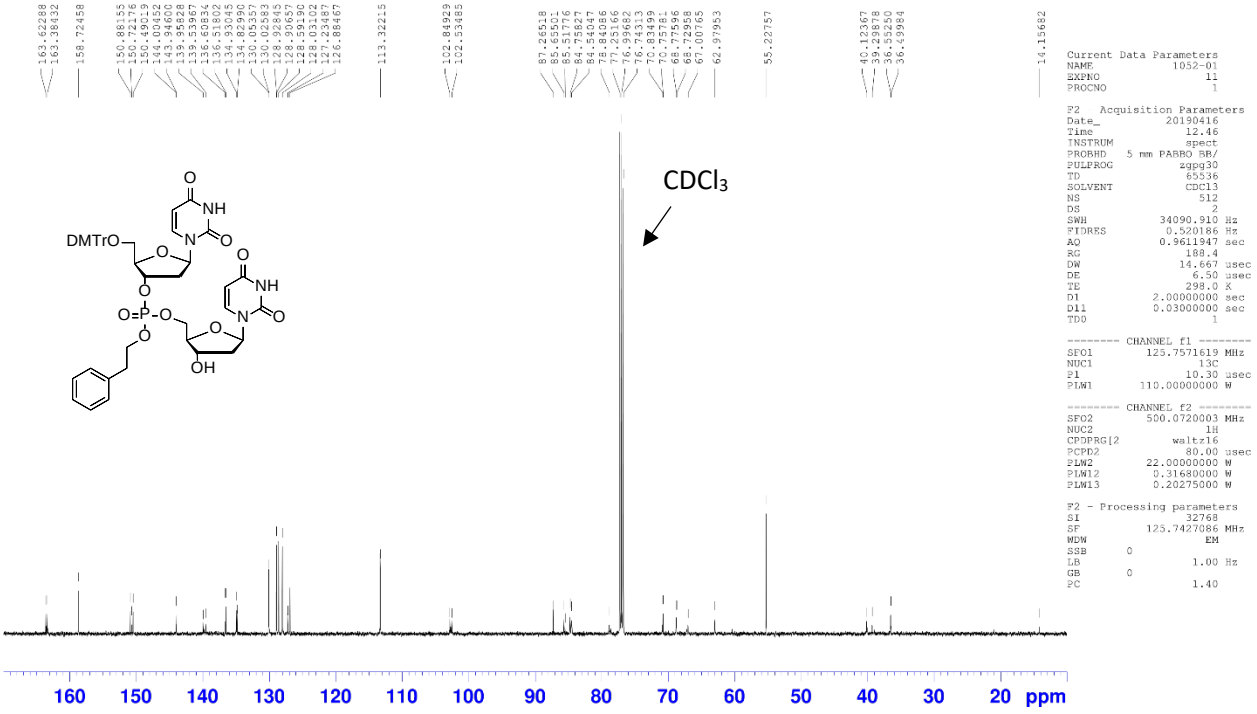
^{31}P NMR Spectra of Compound **13**



^{1}H NMR Spectra of Compound **14**



^{13}C NMR Spectra of Compound **14**



^{31}P NMR Spectra of Compound **14**