SiRNAs with Neutral Phosphate Triester Hydrocarbon Tails Exhibit Carrier-Free Gene-Silencing Activity

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Materials and Methods

General Methods

During the synthesis, no unexpected or unusually high safety hazards were encountered. Unless otherwise indicated all starting reagents used were obtained from commercial sources without additional purification. Anhydrous CH_2Cl_2 and THF were purchased from Sigma-Aldrich and run through a PureSolv 400 solvent purification system to maintain purity. Flash column chromatography was performed with Silicycle Siliaflash 60 (230-400 mesh), using the procedure developed by Still, Kahn and Mitra.[1] NMRs were performed on a Bruker 400 MHz spectrophotometer or 500 MHz. All ^{1}H NMRs were recorded for 16 or 64 transients at 400 or 500 MHz, all ^{13}C NMRs were run for 1024 or 1500 transients at 101 or 125 MHz, all ^{31}P NMRs were recorded for 64 transients at 167 MHz and all ^{19}F NMRs were recorded for 16 or 32 transients at 377 or 470 MHz. Spectra were processed and integrated using ACD labs NMR Processor Academic Edition.

Synthesis of Compound 2 - (2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) octadecyl phosphate

To a toluene solution (2.5 ml) of stearyl alcohol (163 mg, 0.6 mmol) and flame-dried MS4A (549 mg) was added 1,5,7-triazabicyclo [4.4.0] dec-5-ene (TBD) (83 mg, 0.6 mmol). After stirring for 30 min at room temperature, a toluene solution of dinucleotide **1** (549 mg, 0.5 mmol) was added to the mixture at 0 ℃. After stirring for 3 h, the reaction quenched by addition of a toluene solution (0.6 mL) of acetic acid (24 mg, 0.6 mmol) at 0 °C. The mixture was filtered on Celite and washed with ethyl acetate. The filtrate was washed successively with phosphate buffer (pH 7), water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (hexane/ethyl acetate = 1/1 with 1% Et_3N) to give **compound 2** (507 mg, 80%) as a diastereomer mixture (1.3:1.0) (white solid).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.95 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.89 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.85 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.36-7.34 (m, 9H), 6.84-6.82 (m, 4H), 6.36 (t, *J* = 6.4 Hz, 1H), 6.24 (t, *J* = 6.3 Hz, 1H, one diastereomer), 6.23 (t, *J* = 6.23, *J* = 6.4 Hz, the other diastereomer), 6.06-5.98 (m, 2H), 5.903 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.899 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.62 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.62 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.40-5.34 (m, 2H), 5.30-5.25 (m, 2H), 5.15-5.11 (m, 1H), 4.88-4.85 (m, 4H), 4.34-4.15 (m, 4H), 4.07-3.93 (m, 3H), 3.791 (s, 6H, one diastereomer), 3.788 (s, 6H, the other diastereomer), 3.49-3.41 (m, 2H), 2.88-2.82 (m, 1H), 2.54-2.46 (m, 1H), 2.35-2.27 (m, 1H), 2.13-2.06 (m, 1H), 1.67 (quin, *J* = 7.1 Hz, 2H, one diastereomer), 1.61 (quin, *J* = 7.2 Hz, 2H, the other diastereomer), 1.25 (m, 30H), 0.89-0.86 (m, 12H), 0.08 (s, 3H, one diastereomer), 0.075 (s, 3H, one diastereomer), 0.067 (s, 3H, the other diastereomer), 0.06 (s, 3H, the other diastereomer).**^{13}C NMR (126 MHz, $CDCl_3$)** δ 170.96, 170.94, 158.67, 155.53, 155.50, 155.47, 143.96, 142.44, 142.40, 142.30, 142.22, 135.02, 134.97, 134.87, 134.85, 131.94, 131.91, 131.85, 129.99, 128.01, 127.94, 127.12, 118.70, 118.56, 113.22, 87.11, 87.07, 86.66, 86.55, 86.40, 86.30, 84.97 (d, *J* = 7.3 Hz), 84.94 (d, *J* = 7.7 Hz), 84.81 (d, *J* = 6.3 Hz), 84.79 (d, *J* = 6.3 Hz), 77.74 (d, *J* = 4.9 Hz), 70.70, 70.56, 68.65, 68.61, 68.59, 68.54, 67.69, 67.63, 67.62, 66.37 (d, *J* = 5.9 Hz), 66.20 (d, *J* = 5.7 Hz), 62.62, 62.58, 41.60, 41.52, 40.17 (d, *J* = 3.9 Hz), 30.21 (d, *J* = 2.8 Hz), 30.16 (d, *J* = 2.9 Hz), 29.62, 29.57, 29.51, 29.43, 29.27, 29.06, 17.83, 14.04, -4.70, -4.73, -4.96, -4.99. **^{31}P NMR** (202 MHz, $CDCl_3$, External

standard: 85% H_3PO_4) δ -0.78 (one diastereomer), -0.97 (the other diastereomer). **IR (ATR)** 2924, 2853, 1667, 1628, 1540, 1508, 1468, 1397, 1300, 1249, 1176, 1110, 1075, 1005, 829, 780, 701, 673. **MS (ESI)** *m/z* 1290 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{69}H_{99}N_4Na_1O_{14}P_1Si_1$ $(M+Na)^+$1289.65623, found 1289.65850.

Synthesis of Compound 3 - (2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) ((*R*)-2,3-bis(octadecyloxy)propyl) phosphate

To a toluene solution (2.5 ml) of (*S*)-2,3-bis(octadecyloxy)propan-1-ol (358 mg, 0.6 mmol) and flame-dried MS4A (549 mg) was added 1,5,7-triazabicyclo [4.4.0] dec-5-ene (TBD) (83 mg, 0.6 mmol). After stirring for 30 min at room temperature, a toluene solution of dinucleotide **1** (549 mg, 0.5 mmol) was added to the mixture. After stirring for 1.5 h at room temperature, the reaction quenched by addition of a toluene solution (0.6 mL) of acetic acid (24 mg, 0.6 mmol) at 0 °C. The mixture was filtered on Celite and washed with ethyl acetate. The filtrate was washed successively with phosphate buffer (pH 7), water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (hexane/ethyl acetate = 1/1 with 1% Et_3N) to give **compound 3** (562 mg, 71%) as a diastereomer mixture (colorless oil).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.93 (d, *J* = 7.3 Hz, 1H, the other diastereomer), 7.91 (d, *J* = 7.3 Hz, 1H, one diastereomer), 7.87 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.36-7.34 (m, 2H), 7.30-7.22 (m, 7H), 6.84-6.82 (m, 4H), 6.37 (t, *J* = 7.2 Hz, 1H, one diastereomer), 6.35 (t, d, *J* = 7.5 Hz, 1H, the other diastereomer), 6.24 (t, *J* = 5.9 Hz, 1H, one diastereomer), 6.23 (t, *J* = 5.9 Hz, 1H, the other diastereomer), 6.06-5.97 (m, 2H), 5.929 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.926 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.61 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.60 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.40-5.34 (m, 2H), 5.29-5.24 (m, 2H), 5.19-5.14 (m, 1H), 4.88-4.84 (m, 4H), 4.37-4.13 (m, 5H), 4.09-4.04 (m, 1H), 4.00-3.93 (m, 1H), 3.800 (s, 6H, one diastereomer), 3.786 (s, 6H, the other diastereomer), 3.64-3.40 (m, 9H), 2.90-2.83 (m, 1H), 2.54-2.46 (m, 1H), 2.35-2.27 (m, 1H), 2.12-2.05 (m, 1H), 2.12-2.05 (m, 1H), 1.52-1.48 (m, 1H), 1.31-1.25 (m, 60H), 0.89-0.86 (m, 15H), 0.083 (s, 3H, one diastereomer), 0.078 (s, 3H, one diastereomer), 0.066 (s, 3H, the other diastereomer), 0.058 (s, 3H, the other diastereomer). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 170.95, 170.94, 158.67, 155.49, 143.98, 142.49, 142.43, 142.33, 142.25, 135.05, 135.01, 134.87, 134.84, 131.97, 131.95, 131.97, 130.00, 129.96, 128.03, 128.01, 127.95, 127.11, 118.68, 118.50, 113.25, 113.23, 95.74, 95.69, 95.58, 87.12, 87.09, 86.63, 86.54, 86.40, 86.37, 85.01, 84.98, 84.95, 84.91, 84.85, 78.25 (d, *J* = 4.7 Hz), 78.08 (d, *J* = 4.9 Hz), 71.81 (d, *J* = 4.2 Hz), 70.76, 70.65, 70.62, 69.22, 67.84 (d, *J* = 4.9 Hz), 67.69, 67.40, 66.48 (d, *J* = 5.6 Hz), 66.25 (d, *J* = 5.6 Hz), 62.80, 62.69, 55.15, 41.65, 41.57, 40.25 (d, *J* = 4.3 Hz), 40.13, 31.85, 29.94, 29.64, 29.60, 29.59, 29.54, 29.44, 29.29, 26.00, 25.95, 25.63, 25.62, 22.61, 17.84, 17.82, 14.05, -4.69, -4.73, -4.94, -4.96. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -0.81. **IR (ATR)** 2924, 2853, 1665, 1629, 1541, 1508, 1468, 1398, 1301, 1251, 1176, 1113, 1204, 940, 830, 783, 753, 664 cm^{-1}. **MS (ESI)** *m/z* 1616 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{90}H_{141}N_4Na_1O_{16}P_1Si_1$ $(M+Na)^+$1615.97471, found 1615.97705.

Synthesis of Compound 4 - (2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) (3,4,5-tris(octadecyloxy)phenethyl) phosphate

To a toluene solution (2.5 ml) of 2-(3,4,5-tris(octadecyloxy)phenyl)ethan-1-ol (482 mg, 0.52 mmol) and flame-dried MS4A (475 mg) was added 1,5,7-triazabicyclo [4.4.0] dec-5-ene (TBD) (72 mg, 0.52 mmol). After stirring for 30 min at room temperature, a toluene solution of dinucleotide **1** (475 mg, 0.43 mmol) was added to the mixture. After stirring for 1.5 h at room teperature, the reaction

quenched by addition of a toluene solution (0.52 mL) of acetic acid (31 mg, 0.52 mmol) at 0°C. The mixture was filtered on Celite and washed with ethyl acetate. The filtrate was washed successively with phosphate buffer (pH = 7), water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica (hexane / ethyl acetate = 2 / 3 + 1% Et_3N, then 4 / 5) to give **compound 4** (600 mg, 72 %) as a diastereomer mixture (1:1.3) (white solid).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.96 (d, *J* = 7.4 Hz, the other diastereomer), 7.93 (d, *J* = 7.4 Hz, one diastereomer), 7.86 (d, *J* = 7.4 Hz, one diastereomer), 7.81 (d, *J* = 7.4 Hz, the other diastereomer), 7.35-7.33 (m, 2H), 7.28-7.21 (m, 7H), 6.83-6.81 (m, 4H), 6.36-6.33 (m, 3H), 6.22 (t, *J* = 6.0 Hz, 1H), 6.06-5.96 (m, 2H), 5.88 (d, *J* = 7.4 Hz, the other diastereomer), 5.88 (d, *J* = 7.4 Hz, one diastereomer), 5.63 (d, *J* = 7.4 Hz, the other diastereomer), 5.62 (d, *J* = 7.4 Hz, one diastereomer), 5.38 (dt, J_d = 17.2 Hz, J_t = 1.5 Hz, 2H, the other diastereomer), 5.36 (dq, J_d = 17.2 Hz, J_t = 1.6 Hz, 2H, one diastereomer), 5.29-5.24 (m, 2H), 5.13 (br, 1H), 4.88-4.85 (m, 4H), 4.30-4.27 (m, 2H), 4.24-4.08 (m, 4H), 4.03-4.02 (m, 1H, one diastereomer), 3.970-3.966 (m, 1H, the other diastereomer), 3.92-3.88 (m, 6H), 3.775 (s, 3H, the other diastereomer), 3.772 (s, 3H, one diastereomer), 3.766 (s, 3H, the other diastereomer), 3.763 (s, 3H, one diastereomer), 3.48-3.40 (m, 2H), 2.90 (t, *J* = 7.6 Hz), 2.86-2.82 (m, 2H), 2.52-2.45 (m, 1H), 2.33-2.28 (m, 1H), 2.12-2.03 (m, 1H), 1.79-1.69 (m, 6H), 1.45-1.43 (m, 6H), 1.30-1.26 (m, 90H), 0.89-0.86 (m, 18H). 0.076 (s, 3H, the other diastereomer), 0.066 (s, 3H, one diastereomer), 0.059 (s, 3H, the other diastereomer), 0.046 (s, 3H, one diastereomer). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 170.96, 170.93, 170.91, 158.66, 155.50, 155.47, 155.42, 153.19, 143.95, 142.41, 142.37, 142.33, 142.24, 137.09, 134.98, 134.93, 134.81, 131.91, 131.90, 131.82, 131.00, 129.97, 129.93, 127.97, 127.93, 127.10, 118.68, 118.54, 113.21, 107.24, 95.64, 95.61, 87.09, 87.06, 86.71, 86.62, 86.43, 86.33, 84.90 (d, *J* = 7.5 Hz), 84.81, 84.78, 78.01 (d, *J* = 4.3 Hz), 73.30, 70.77, 70.63, 69.07 (d, *J* = 1.4 Hz), 68.65 (d, *J* = 5.8 Hz), 67.65 (d, *J* = 7.1 Hz), 66.44 (d, *J* = 5.4 Hz), 66.29 (d, *J* = 5.6 Hz), 62.62 (d, *J* = 4.0 Hz), 55.11, 41.43 (d, *J* = 7.6 Hz), 40.10, 36.94 (d, *J* = 6.7 Hz), 31.84, 30.28, 29.69, 29.68, 29.64, 29.59, 29.40, 29.28, 26.07, 25.60, 22.60, 17.80, 14.03, -4.72, -4.75, -4.97, -4.99. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.07 (one diastereomer), -1.15 (the other diastereomer). **IR (ATR)** 2916, 1849, 1664, 1629, 1541, 1508, 1467, 1302, 1250, 1114, 1004, 830, 752 cm^{-1}. **MS (ESI)** *m/z* 1946 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{113}H_{179}N_4Na_1O_{17}P_1Si_1$ $(M+Na)^+$ 1946.26698, found 1946.27047.

Synthesis of Compound 5 - (2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) octadecyl phosphate

To a THF solution (8 mL) of **2** (987 mg, 0.78 mmol), triethylamine (1.7 mL, 11.7 mmol) and triethylamine trihydrofluoride (3HF•Et3N) (1.3 mL, 7.8 mmol) were added. After being stirred for 14 h at 40 ℃, the reaction mixture was quenched by the addition of saturated sodium bicarbonate at 0 ℃. The mixture was extracted with $CHCl_3$ thrice. The combined extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica ($CHCl_3$ / MeOH = 100 / 1 with 1% Et_3N, then $CHCl_3$ / MeOH = 15 / 1) to give **compound 5** (600 mg, 84%) as a diastereomer mixture (1:1) (colorless oil).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.93 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.92 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.86 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.40-7.22 (m, 9H), 6.84-6.83 (m, 4H), 6.32 (t, *J* = 6.4 Hz, 1H), 6.29-6.24 (m, 1H), 6.05-5.97 (m, 2H), 5.92 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.90 (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), 5.40-5.38 (m, 2H, one diastereomer), 5.36-5.35 (m, 2H, the other diastereomer), 5.29-5.25 (m, 2H), 5.14-5.12 (m, 1H, one diastereomer), 5.10-5.06 (m, 1H, the other diastereomer), 4.87-4.85 (m, 4H), 4.47-3.98 (m, 8H), 3.792 (s, 6H, one diastereomer), 3.789 (s,

6H, the other diastereomer), 3.50-3.41 (m, 2H), 2.92 (ddd, *J* = 14.2, 5.3, 1.9 Hz, 1H, one diastereomer), 2.87 (ddd, *J* = 14.2, 5.8, 3.0 Hz, 1H, the other diastereomer), 2.66-2.59 (m, 1H), 2.33-2.17 (m, 1H), 2.12-2.07 (m, 1H), 1.69-1.61 (m, 2H), 1.31-1.24 (m, 30H), 0.88 (t, *J* = 6.9 Hz). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.04, 170.99, 170.95, 158.68, 155.75, 155.63, 155.58, 143.99, 143.95, 142.40, 142.29, 142.26, 134. 98, 134.91, 134.89, 131.92, 131.86, 131.80, 131.70, 130.12, 129.97, 128.00, 127.97, 127.15, 118.85, 118.77, 118.63, 118.62, 113.27, 96.09, 95.81, 95.78, 95.54, 87.10, 86.63, 86.46, 86.42, 84.86 (d, *J* = 7.8 Hz), 84.72 (d, *J* = 6.6 Hz), 84.67 (d, *J* = 7.0 Hz), 84.44 (d, *J* = 7.3 Hz), 78.66 (d, *J* = 4.9 Hz), 77.90 (d, *J* = 5.3 Hz), 70.25, 69.52, 68.78 (d, *J* = 6.1 Hz), 66.36 (d, *J* = 5.3 Hz), 62.86, 62.59, 55.20, 55.19, 41.16, 41.11, 40.16, 40.04, 31.85, 30.21, 30.16, 29.64, 29.59, 29.53, 29.45, 29.29, 29.07, 25.32, 22.62, 14.06. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -0.73 (one diastereomer), -1.16 (the other diastereomer). **IR (ATR)** 3374, 2922, 2852, 1655, 1627, 1541, 1508, 1468, 1397, 1300, 1248, 1175, 1115, 1000, 783 cm^{-1}. **MS (ESI)** *m/z* 1175 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{63}H_{85}N_4Na_1O_{14}P_1$ $(M+Na)^+$ 1175.56976, found 1175.57163.

Synthesis of Compound 6 - (2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) ((*R*)-2,3-bis(octadecyloxy)propyl) phosphate

To a THF solution (7.5 mL) of **3** (1.2 g, 0.75 mmol), triethylamine (1.6 mL, 11 mmol) and triethylamine trihydrofluoride (3HF•Et3N) (1.2 mL, 7.5 mmol) were added. After being stirred for 14 h at 40 ℃, the reaction mixture was quenched by the addition of saturated sodium bicarbonate at 0 ℃. The mixture was extracted with $CHCl_3$ thrice. The combined extracts were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on silica ($CHCl_3$ / MeOH = 100 / 1 + 1% Et_3N, then 50 / 1) to give **6** (920 mg, 83%) as a diastereomer mixture (1.1:1) (colorless oil).

^{1}H NMR (500 MHz, $CDCl_3$) δ, 7.97 (d, *J* = 7.7 Hz, 1H, one diastereomer), 7.95 (d, *J* = 8.0 Hz, 1H, the other diastereomer), 7.94 (d, *J* = 8.1 Hz, 1H, one diastereomer), 7.87 (d, *J* = 7.7 Hz, 1H, the other diastereomer), 7.36-7.34 (m, 2H), 7.31-7.21 (m, 7H), 6.85-6.82 (m, 4H), 6.34 (t, *J* = 6.5 Hz, 1H, one diastereomer), 6.30-6.23 (m, 1H), 6.05-5.97 (m, 2H), 5.95 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.93 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.68 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 5.64 (d, *J* = 7.4 Hz, 1H, one diastereomer), 5.39 (dt, J_d = 1.4 Hz, J_t = 5.9 Hz, one diastereomer), 5.35 (dt, J_d = 1.4 Hz, J_t = 5.9 Hz, the other diastereomer), 5.29-5.24 (m, 2H), 5.16-5.15 (m, 1H), 4.87-4.84 (m, 4H), 4.48-3.99 (m, 8H), 3.789 (s, 6H, one diastereomer), 3.782 (s, 6H, the other diastereomer), 3.64-3.42 (m, 9H), 2.92 (ddd, *J* = 14.0, 5.8, 2.4 Hz, 1H, one diastereomer), 2.90 (ddd, *J* = 14.4, 5.6, 2.4 Hz, 1H, the other diastereomer), 2.66-2.59 (m, 1H), 2.34-2.24 (m, 1H), 2.22-2.08 (m, 1H), 1.53-1.50 (m, 4H), 1.26 (m, 60H), 0.88 (t, 6.9 Hz, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.02, 170.92, 158.61, 155.63, 155.55, 155.52, 143.93, 143.89, 142.39, 142.32, 142.29, 142.20, 134.93, 134.81, 131.90, 131.84, 131.75, 131.67, 129.94, 129.91, 127.94, 127.91, 127.05, 118.72, 118.64, 118.49, 118.42, 113.20, 95.90, 95.69, 95.57, 87.05, 86.47, 86.43, 86.34, 84.80 (d, *J* = 9.1 Hz), 84.73 (d, *J* = 7.1 Hz), 84.51 (d, *J* = 6.7 Hz), 84.29 (d, *J* = 7.2 Hz), 78.73 (d, *J* = 5.0 Hz), 71.77 (d, *J* = 4.8 Hz), 70.63, 70.55, 69.80, 69.10 (d, *J* = 7.6 Hz), 67.73-67.44 (m), 66.52, 66.30, 62.83, 62.63, 55.09, 41.06 (d, *J* = 4.0 Hz), 40.14, 39.98, 31.79, 29.82, 29.80, 29.58, 29.46, 29.37, 29.34, 29.23, 25.93, 25.86, 22.55, 13.99. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -0.90 (one diastereomer), -0.96 (the other diastereomer). **IR (ATR)** 3380, 2923, 2853, 1654, 1633, 1542, 1508, 1469, 1303, 1251, 1176, 1116, 1014 cm^{-1}. **MS (ESI)** *m/z* 1501 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{84}H_{127}N_4Na_1O_{16}P_1$ $(M+Na)^+$ 1501.88824, found 1501.88704.

Synthesis of Compound 7 - (2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(4-(allyloxy)-2-oxopyrimidin-1(2*H*)-yl)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl) (3,4,5-tris(octadecyloxy)phenethyl) phosphate

To a THF solution (9.5 mL) of **4** (1.8 g, 0.94 mmol), triethylamine (2.0 mL, 14 mmol) and triethylamine trihydrofluoride (3HF•Et3N) (1.6 mL, 9.4 mmol) were added. After being stirred for 13 h at 40 ℃, the reaction mixture was quenched by addition of saturated sodium bicarbonate at 0 ℃. The mixture was extracted with $CHCl_3$ thrice. The combined extracts were dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on silica ($CHCl_3$ + 1% Et_3N, then CH_3Cl / MeOH = 97 / 3) to give **compound 7** (1.4 g, 81 %) as a diastereomer mixture (1 : 1.1).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.96 (d, *J* = 7.4 Hz, 1H, one diastereomer), 7.92 (d, *J* = 7.4 Hz, the other diastereomer), 7.86 (d, *J* = 7.4 Hz, the other diastereomer), 7.78 (d, *J* = 7.4 Hz, 1H, the other diastereomer), 7.35-7.34 (m, 2H), 7.29-7.21 (m, 7H), 6.82 (d, *J* = 8.8 Hz, 4H), 6.37 (d, *J* = 11.7 Hz, 2H), 6.32-6.22 (m, 2H), 6.06-5.96 (m, 2H), 5.88 (d, *J* = 7.7 Hz, one diastereomer), 5.86 (d, *J* = 8.1 Hz, the other diastereomer), 5.68 (d, *J* = 7.4 Hz, the other diastereomer), 5.66 (d, *J* = 7.4 Hz, one diastereomer), 5.40-5.34 (m, 2H), 5.27 (t, *J* = 11.5 Hz, 2H), 5.12-5.08 (m, 1H), 4.87-4.83 (m, 4H), 4.38-4.02 (m, 8H), 3.93-3.90 (m, 6H), 3.77 (s, 6H), 3.48-3.40 (m, 2H), 2.91-2.83 (m, 3H), 2.63-2.55 (m, 1H), 2.32-2.22 (m, 1H), 2.17-2.13 (m, 1H), 2.07-2.02 (m, 1H), 1.77-1.69 (m, 6H), 1.45-1.44 (m, 6H), 1.26 (m, 90 H), 0.88 (t, *J* = 6.9 Hz). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.00, 170.91, 170.89, 158.64, 155.67, 155.58, 155.53, 155.51, 143.96, 143.92, 142.39, 142.34, 142.29, 142.25, 136.99, 136.96, 134.92, 134.91, 134.84, 134.81, 131.88, 131.84, 131.75, 131.68, 131.39, 131.33, 129.96, 129.93, 127.93, 127.09, 118.78, 118.71, 118.55, 113.23, 107.36, 107.32, 95.98, 95.76, 95.70, 95.54, 87.06, 86.67,86.60, 86.43, 84.80 (d, *J* = 7.6 Hz), 84.70 (d, *J* = 6.5 Hz), 84.56 (d, *J* = 6.7 Hz), 84.44 (d, *J* = 7.5 Hz), 78.60 (d, *J* = 4.9 Hz), 78.01 (d, *J* = 5.1 Hz), 73.37, 73.36, 70.28, 69.71, 69.13 (d, *J* = 4.3 Hz), 68.72 (d, *J* = 6.5 Hz), 68.67 (d, *J* = 6.7 Hz), 67.78, 68.70, 67.62, 67.61, 66.80, 66.56 (d, *J* = 5.3 Hz), 62.77, 62.56, 55.12, 45.96, 41.02 (d, *J* = 2.8 Hz), 40.04, 39.97, 36.85, 31.83, 30.25, 29.67, 29.63, 29.58, 29.38, 29.27, 26.05, 22.59, 14.02. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.11 (one diastereomer), -1.35 (the other diastereomer). **MS (ESI)** *m/z* 1832 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{107}H_{165}N_4Na_1O_{17}P_1$ $(M+Na)^+$ 1832.18050, found 1832.18163.

Synthesis of Compound 8 - (2*R*,3*S*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) octadecyl phosphate

To a heterogeneous mixture of **5** (800 mg, 0.69 mmol) and diethylammonium hydrogencarbonate (1118 mg, 8.3 mmol) in CH_2Cl_2 (7 ml) was added $Pd(PPh_3)_4$ (35 mg, 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, then 9 / 1) to give **compound 8** (674 mg, 91%) as a diastereomer mixture (1.0:1.1) (white solid).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.30-9.90 (br, 2H), 7.68 (d, *J* = 8.2 Hz, 1H, one diastereomer), 7.66 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 7.55 (d, *J* = 8.2 Hz, 1H, one diastereomer), 7.49 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 7.35-7.21 (m, 9H), 6.85-6.83 (m, 4H),6.32-6.27 (m, 1H), 6.23 (t, *J* = 6.4 Hz, 1H), 5.73 (d, *J* = 3.9 Hz, 1H, one diastereomer), 5.71 (d, *J* = 3.9 Hz, 1H, the other diastereomer), 5.40 (t, *J* = 7.9 Hz, 1H), 5.16 (t, *J* = 5.3 Hz, 1H, one diastereomer), 5.09 (t, *J* = 5.6 Hz, 1H, the other diastereomer), 4.50-4.46 (m, 1H, one diastereomer), 4.45-4.42 (m, 1H, the other diastereomer), 4.34-4.00 (m, 7H), 3.78 (s, 3H), 3.78 (s, 3H), 3.46-3.42 (m, 2H), 2.74-2.65 (m, 1H), 2.48-2.30 (m, 2H), 2.20-2.10 (m, 1H), 1.68-1.59 (m, 2H), 1.31-1.23 (m, 30H), 0.87 (t, *J* = 6.9 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 163.62, 163.49, 163.41, 163.34, 158.71, 150.89,

150.72, 150.49, 143.99, 143.93, 140.00, 139.84, 139.75, 139.51, 134.96, 134.95, 134.84, 134.81, 130.03, 128.01, 127.21, 113.30, 102.84, 102.74, 102.67, 102.53, 87.26, 87.25, 85.67, 85.53, 84.93, 84.81, 84.93-84.52 (m), 78.69 (d, *J* = 4.8 Hz), 78.49 (d, *J* = 6.6 Hz), 70.88, 70.74, 68.85 (d, *J* = 6.0 Hz), 68.72 (d, *J* = 6.1 Hz), 62.93, 40.20, 40.08, 39.37, 39.09, 31.86, 30.24, 30.19,30.15, 29.66, 29.60, 29.56, 29.48, 29.47, 29.30, 29.11, 29.08, 25.24, 25.31, 22.63, 14.07. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -0.9 (one diastereomer), -1.7 (the other diastereomer). **IR(ATR)** 3413, 3177, 3056, 2922, 2852, 1685, 1508, 1458, 1377, 1247, 1175, 1002, 825, 701; MS (ESI) *m/z* 1095 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{57}H_{77}N_4Na_1O_{14}P_1$ $(M+Na)^+$ 1095.50716, found 1095.50666.

Synthesis of Compound 9 - (2*R*,3*S*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-yl ((*R*)-2,3-bis(octadecyloxy)propyl) (((2*R*,3*S*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3-hydroxytetrahydrofuran-2-yl)methyl) phosphate

To a heterogeneous mixture of **6** (900 mg, 0.61 mmol) and diethylammonium hydrogencarbonate (986 mg, 7.3 mmol) in CH_2Cl_2 (6 ml) was added $Pd(PPh_3)_4$ (35 mg, 0.03 mmol) and PPh_3 (7 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, then 9 / 1) to give **compound 9** (800 mg, 93 %) as a diastereomer mixture (1.0:1.1) (light yellow solid).

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.13-9.74 (br, 2H), 7.66 (d, *J* = 8.2 Hz, 1H, one diastereomer), 7.66 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 7.54 (d, *J* = 8.2 Hz, 1H, one diastereomer), 7.47 (d, *J* = 8.2 Hz, 1H, the other diastereomer), 7.34-7.22 (m, 9H), 6.85-6.82 (m, 4H), 6.34 (dd, *J* = 6.0, 7.7 Hz, 1H, one diastereomer), 6.29 (dd, *J* = 5.5, 8.2 Hz, the other diastereomer, 1H), 5.75 (d, *J* = 8.1 Hz, 1H, one diastereomer), 5.72 (d, *J* = 8.1 Hz, 1H, the other diastereomer), 5.39 (d, *J* = 8.1 Hz, 1H, one diastereomer), 5.36 (d, *J* = 8.1 Hz, 1H, the other diastereomer), 5.17-5.16 (m, 1H), 4.51-3.99 (m, 8H), 3.79 (s, 3H), 3.78 (s, 3H), 3.65-3.41 (m, 9H), 2.76-2.67 (m, 1H), 2.48-2.30 (m, 2H), 2.17 (ddd, *J* = 6.6, 13.5, 20.5 Hz, 1H), 1.54-1.50 (m, 4H), 1.29-1.25 (m, 60H), 0.88 (t, *J* = 6.9 Hz, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 163.52, 163.40, 163.35, 163.26, 158.70, 150.86, 150.69, 150.41, 150.38, 143.96, 143.90, 139.89, 139.76139.51. 134.94, 134.91, 134.77, 130.03, 128.04, 128.00, 127.19, 113.30, 102.79,102.73, 102.62, 102.57, 87.27, 85.53, 85.48, 84.82, 84.63-84.48 (m), 79.03 (d, *J* = 4.3 Hz), 78.90 (d, *J* = 5.5), 71.88, 71.83, 70.68, 70.60, 70.52, 70.49, 69.21, 69.14, 67.81 (d, *J* = 6.9 Hz), 67.67 (d, *J* = 5.7), 66.92, 66.89, 63.06, 40.16, 40.05, 39.39, 39.04, 31.85, 29.88, 29.87, 29.65, 29.60, 29.52, 29.44, 29.42, 29.29, 25.99, 25.92, 22.6214.05. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.0 (one diastereomer), -1.5 (the other diastereomer). **IR(ATR)** 3388, 3178, 3055, 2921, 2851, 1685, 1610, 1508, 1458, 1378, 1249, 1176, 1114, 1012, 825, 760, 701. **MS(ESI)** *m/z* 1421 $(M+Na)^+$. **HRMS (ESI)** calcd for $C_{78}H_{119}N_4Na_1O_{16}P_1$ $(M+Na)^+$ 1421.82564, found 1421.82724.

Synthesis of compound 10 - (2*R*,3*S*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-yl (((2*R*,3*S*,5*R*)-3-((*tert*-butyldimethylsilyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-2-yl)methyl) (3,4,5-tris(octadecyloxy)phenethyl) phosphate

To a heterogeneous mixture of **7** (1.35 g, 0.75 mmol) and diethylammonium hydrogencarbonate (1215 mg, 9.0 mmol) in CH_2Cl_2 (7.5 ml) was added $Pd(PPh_3)$ (43 mg, 0.04 mmol) and PPh_3 (6 mg, 0.02 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 ($CHCl_3$, then $CHCl_3$ / MeOH = 9 / 1) to give **compound 10** (1.17 g, 89%) as a diastereomer mixture (1.0:1.2) (light yellow solid)

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.11-9.52 (br, 2H), 7.66 (d, *J* = 7.8 Hz, 1H, the other diastereomer), 7.65 (d, *J* = 7.8 Hz, 1H, one diastereomer), 7.54 (d, *J* = 8.2 Hz, 1H, one diastereomer), 7.48 (d, *J*

= 8.1 Hz, 1H, one diastereomer), 7.38 (d, *J* = 8.1 Hz, 1H, the other diastereomer), 7.32-7.22 (m, 9H), 6.83 (d, *J* = 8.4 Hz 4H), 6.39 (s, 1H), 6.36 (s, 1H), 6.28 (dd, *J* = 13.9, 7.7 Hz, 1H), 6.18 (dt, J_d = 13.3 Hz, J_t = 6.6 Hz, 1H), 5.72 (d, *J* = 8.1 Hz, 1H, one diastereomer), 5.69 (d, *J* = 8.1 Hz, 1H, the other diastereomer), 5.39 (t, *J* = 8.3 Hz, 1H), 5.13 (br, 1H, one diastereomer), 5.08 (br, 1H, the other diastereomer), 4.37-3.97 (m, 8H), 3.92-3.87 (m, 6H), 3.77 (6H), 3.44-3.42 (m, 2H), 2.89-2.84 (m, 2H), 2.72-2.61 (m, 2H), 2.42-2.31 (m, 2H), 2.17-2.07 (m, 1H), 1.77-1.71 (m, 6H), 1.25 (m, 90H), 0.88 (t, *J* = 6.7 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 163.44, 163.28, 163.25, 163.15, 158.73, 153.20, 150.82, 150.64, 150.33, 150.24, 140.07, 139.93, 139.75, 139.50, 136.97, 134.91, 134.80, 134.77, 131.52, 131.41, 130.04, 128.03, 127.23, 113.32, 107.47, 107.38, 102.84, 102.76, 102.58, 87.27, 85.84, 85.70, 84.96, 84.96-84.55 (m), 78.74 (d, *J* = 5.0 Hz), 78.57 (d, *J* = 6.4 hz), 73.52, 73.48, 70.83, 70.74, 69.27, 69.19, 68.80, 67.02, 62.93, 39.97, 39.30,39.06, 36.89, 31.89, 30.30, 29.74, 29.71, 29.66, 29.64, 29.44, 29.34, 26.12, 22.66, 14.09. **^{31}P NMR** (202 MHz, $CDCl_3$, External standard: 85% H_3PO_4) δ -1.2 (one diastereomer), -1.8 (the other diastereomer). **IR(ATR)** 3380, 3198, 3059, 2918, 2850, 1685, 1605, 1586, 1508, 1465, 1379, 1248, 1176, 1115, 1009, 825, 721; MS(ESI) *m/z* 1752 $(M+Na)^+$. **HRMS(ESI)** calcd for $C_{101}H_{157}N_4Na_1O_{17}P_1$ $(M+Na)^+$ 1752.11790, found 1752.12147.

Synthesis of Compound 11- *(2R,3S,5R)*-2-((((((*2R,3S,5R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)oxy)(octadecyloxy)phosphoryl)oxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite

To a solution of 4.00 mL of anhydrous DCM 0.287 g of compound **8** (0.27 mmol, 1.00 equiv.) was added to a flame dried flask. To that solution 0.378 mL of anhydrous triethylamine (2.41 mmol, 10.0 equiv.) was added along with 0.200 mL of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite with (0.9 mmol, 3.00 equiv.) and stirred at room temperature until TLC showed starting materials were consumed (2 h). After rotary evaporation, the compound was then purified on silica gel using a 50%:48%:2% hexanes/ethyl acetate/triethylamine mobile phase. This afforded compound **11** as a white powder (0.31 g, 88%). **^{31}P NMR** (162 MHz, $CDCl_3$) d ppm 149.5, 149.4 ,16.3, 16.2, 12.3, 12.2, -1.7 (one diastereomer) (s), -1.8 (the other diastereomer).

Synthesis of Compound 12- (*2R,3S,5R*)-2-((((((*2R,3S,5R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)oxy)(2,3-bis(octadecyloxy)propoxy)phosphoryl)oxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite

To a solution of 4.00 mL of anhydrous DCM 0.277 g of compound **9** (0.178 mmol, 1.00 equiv.) was added to a flame dried flask. To that solution 0.248 mL of anhydrous triethylamine (1.78 mmol, 10.0 equiv.) was added along with 0.126 mL of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite with (0.534 mmol, 3.00 equiv.) and stirred at room temperature until TLC showed starting materials were consumed (2 h). After rotary evaporation, the compound was then purified on silica gel using a 50%:48%:2% acetone/hexanes/triethylamine mobile phase. This afforded compound **12** as a white powder (0.31 g, 95%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ ppm 7.72- 7.19 (m, 10 H), 6.8 (d, 4 H), 6.2 (s, 1 H), 5.7 (s, 1 H), 5.3 (t, 1 H), 3.7 (s, 6 H), 3.5 (m, 13 H), 2.7 (m, 1 H), 2.6 (m, 5 H), 1.14-1.26 (m, 85 H), 0.8 (m, 5 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ ppm 149.4, 148.9, 139.2, 138.9, 16.2, 12.3, -1.2 (one diastereomer), -1.8 (the other diastereomer).

Synthesis of Compound 13- (*2R,3S,5R*)-2-((((((*2R,3S,5R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl)oxy)(3,4,5-tris(octadecyloxy)phenethoxy)phosphoryl)oxy)methyl)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite

To a solution of 5.00 mL of anhydrous DCM 0.360 g of compound **10** (0.21 mmol, 1.00 equiv.) was added to a flame dried flask. To that solution 0.210 mL of anhydrous triethylamine (2.1 mmol, 10.0 equiv.) was added along with 0.200 mL of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite with (0.63 mmol, 3.00 equiv.) and stirred at room temperature until TLC showed starting materials were consumed (2 h). After rotary evaporation, the compound was then purified on silica gel using a 50%:48%:2% acetone/hexanes/triethylamine mobile phase. This afforded compound **13** as a white powder (0.38 g, 95%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ ppm 7.7 (s, 2 H), 7.3 (s, 7 H), 7.2 (d, 6 H), 6.8 (m, 4 H), 6.1 (s, 2 H), 5.7 (d, 1 H), 5.3 (d, 1 H), 4.2 (m, 14 H), 3.9 (s, 3 H), 3.9 (s, 3 H), 3.8 (d, 3 H), 3.8 (d, 3 H), 3.5 (dt, 10 H), 2.8 (td, 9 H), 2.6 (q, 16 H), 1.3 (m, 107 H), 1.0 (t, 22 H), 0.9 (m, 10 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ ppm 149.4, 148.9, 16.23, 12.4, -1.8 (one diastereomer) -2.0 (the other diastereomer).

Oligonucleotide Synthesis

Oligonucleotides were prepared on an Applied Biosystems 394 DNA/RNA synthesizer using 1.0 µM controlled-pore glass (CPG) support columns and a 1.0 µM cycle with a 999-second coupling time. Immediately before synthesis, phosphoramidites were resuspended to a final concentration of 0.1 M. Cleavage of the oligonucleotides from the solid support was achieved by flushing each CPG column with 1 mL EMAM solution (1:1 methylamine 33 wt% in ethanol/methylamine 40 wt% in water) for 1 hour at room temperature followed by overnight incubation EMAM to deprotect the bases. Oligonucleotides were concentrated in a MiVac Quattro Concentrator and subsequently desilylated by incubation in DMSO (100 µL) and 3HF-Et_3N (125 µL) for 3 hours at 65 °C. Crude oligonucleotides were precipitated in ethanol and desalted using Millipore Amicon Ultra 3000 MW cellulose centrifugal filters. Strands were then purified using reverse-phase HPLC eluting from 5% to 95% ACN in 0.1 M TEAA buffer (pH 7.0).

Biophysical Characterizations

Each modified strand was annealed to its complementary antisense strand by combining equimolar amounts of each strand in 300 µL pH 7 sodium phosphate buffer (90.0 mM NaCl, 10.0 mM Na_2HPO_4, 1.00 mM EDTA). Each sample was heated for 2 minutes at 90 °C and then allowed to equilibrate to room temperature. To biophysically characterize each duplex, we performed CD and thermal denaturation studies using a Jasco J-815 Circular Dichroism (CD) Spectropolarimeter equipped with a temperature controller. CD spectra were recorded at 25 °C, scanning from 200 to 350 nm with a screening rate of 50 nm/min and a 0.20 nm data pitch. We then obtained melting temperature curves for each duplex by measuring the change in absorbance at 260 nm against a temperature gradient (15 to 90 °C) at a rate of 1 °C per minute. Data were analysed using Meltwin v3.5 software to determine the melting temperature (Tm) of each duplex.

Cell culture

HeLa cells were maintained in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Sigma) at 37 °C in a humidified atmosphere with 5% CO_2.

Transfection with Lipofectamine

The day before transfection, HeLa cells were seeded into 24-well plates at a density of 5.0×10^4 cells per well. To each well was added 400 µL DMEM (10% FBS, no antibiotics). Plates were then incubated for an additional 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. Each

transfection sample required a mixture of 1 µL of Lipofectamine 2000™ (Invitrogen) and 49 µL of Gibco′s 1X Opti-Mem Reduced Serum Medium which was incubated at room temperature for 5 minutes. Each siRNA was then diluted in 1X Gibco′s Opti-MEM Reduced Serum Medium (Invitrogen) on ice and mixed with 200 ng pGL3 and 50 ng pRLSV40 plasmids to a final volume of 50 µL. The diluted siRNA/plasmid mixture was combined with the diluted Lipofectamine 2000™ mixture and incubated at room temperature for 35 minutes. The complexes were then transferred to each well and the plates were incubated for an additional 24 hours at 37 °C in a humidified atmosphere with 5% CO_2.

Carrier-free Transfection

The day before transfection, HeLa cells were seeded into 96-well plates at a density of 1.0×10^4 cells per well. To each well was added 50 µL DMEM (10% FBS, no antibiotics). Plates were the incubated for an additional 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. Plasmids coding for firefly luciferase (pGL3, 200 ng) and *Renilla* luciferase (pRLSV40, 50 ng) were co-transfected using 1 µL of Lipofectamine 2000™ as described above. Plates were incubated for 4 hours at 37 °C in a humidified atmosphere with 5% CO_2 after which the medium was removed from each well. Cells were washed twice with 1X phosphate-buffered saline (PBS) after which fresh DMEM (10% FBS, no antibiotics) was added to each well. Each siRNA sample was diluted in 50 µL 1X Gibco′s Opti-MEM Reduced Serum Medium (Invitrogen) on ice and the samples were immediately transferred to the respective wells of the 96-well plate. Plates were incubated for an additional 16 h prior to cell lysis.

Cell Lysis and Dual-Luciferase® Reporter Assay

Cells were lysed with 1X passive lysis buffer for 30 min at room temperature. Cell lysates were transferred to microcentrifuge tubes and were immediately used to assess the gene-silencing activity of siRNAs using a Dual-Luciferase® Reporter Assay (Promega). Luciferase Assay Reagent II (LAR II) and Stop & Glo® Reagent were prepared following the manufacturer′s protocol. Cell lysates (10 µL) were transferred to Costar 96-well plates in triplicate. LAR II reagent (50 µL) was then added to each well and the first luminescence measurement was taken on a Synergy HT (Bio-Tek) plate luminometer. Stop & Glo® Reagent (50 µL) was then added to each well and the second luminescence measurement was taken. Results are expressed as the ratio of firefly/*Renilla* luminescence taken as a percentage of an untreated control. Each value is the average of at least two biological replicates and error bars indicate standard deviation.

Procedure for *in vitro* strand selection assay

In a 24-well culture dish, 500 µL of 1 × 105 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 1.0 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 OptiMEM 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 to 2.5 to 6.25 ng/μL for qPCR amplification within a linear range. 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 antisense (pGL3 expressed) and sense (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.

Procedure for Nuclease Stability Assay

Wild-type and modified siRNAs (1, 4 and 7) 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 (13.5 % 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).

Figures and Tables

Table S-1. Oligonucleotide sequences and mass spectrometry data

#	*Sequence*	*Mass (predicted)*	*Mass (found)*
wt	5′ CUUACGCUGAGUACUUCGAdTdT 3′	6683.9	6684.0
1	5′ CUUACGCUGAGUACUUCGA**dU$_1$dU** 3′	6828.1	6829.0
2	5′ CUUACGCUGAGUAC**dU$_1$dU**CGAdTdT 3′	6823.1	6824.7
3	5′ C**dU$_1$dU**ACGCUGAGUACUUCGAdTdT 3′	6824.2	6824.6
4	5′ CUUACGCUGAGUACUUCGA**dU$_2$dU** 3′	7154.5	7154.0
5	5′ CUUACGCUGAGUAC**dU$_2$dU**CGAdTdT 3′	7153.1	7153.4
6	5′ C**dU$_2$dU**ACGCUGAGUACUUCGAdTdT 3′	7154.1	7154.9
7	5′ CUUACGCUGAGUACUUCGA**dU$_3$dU** 3′	7484.8	7483.4
8	5′ C**dU$_x$dU**ACGCUGAGUAC**dUxdU**CGAdTdT 3′	6831.3	6830.9
9	5′ CUUACGCUGAGUACUUCGA**dU$_x$dU** 3′	7067.3	7066.9

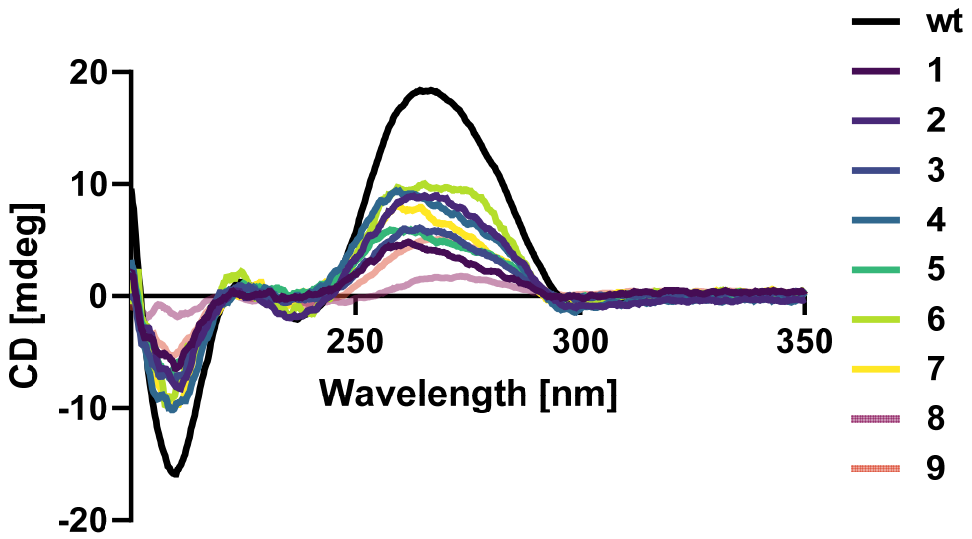


Figure S-1. Circular dichroism spectra of wild-type (wt) and modified siRNAs.

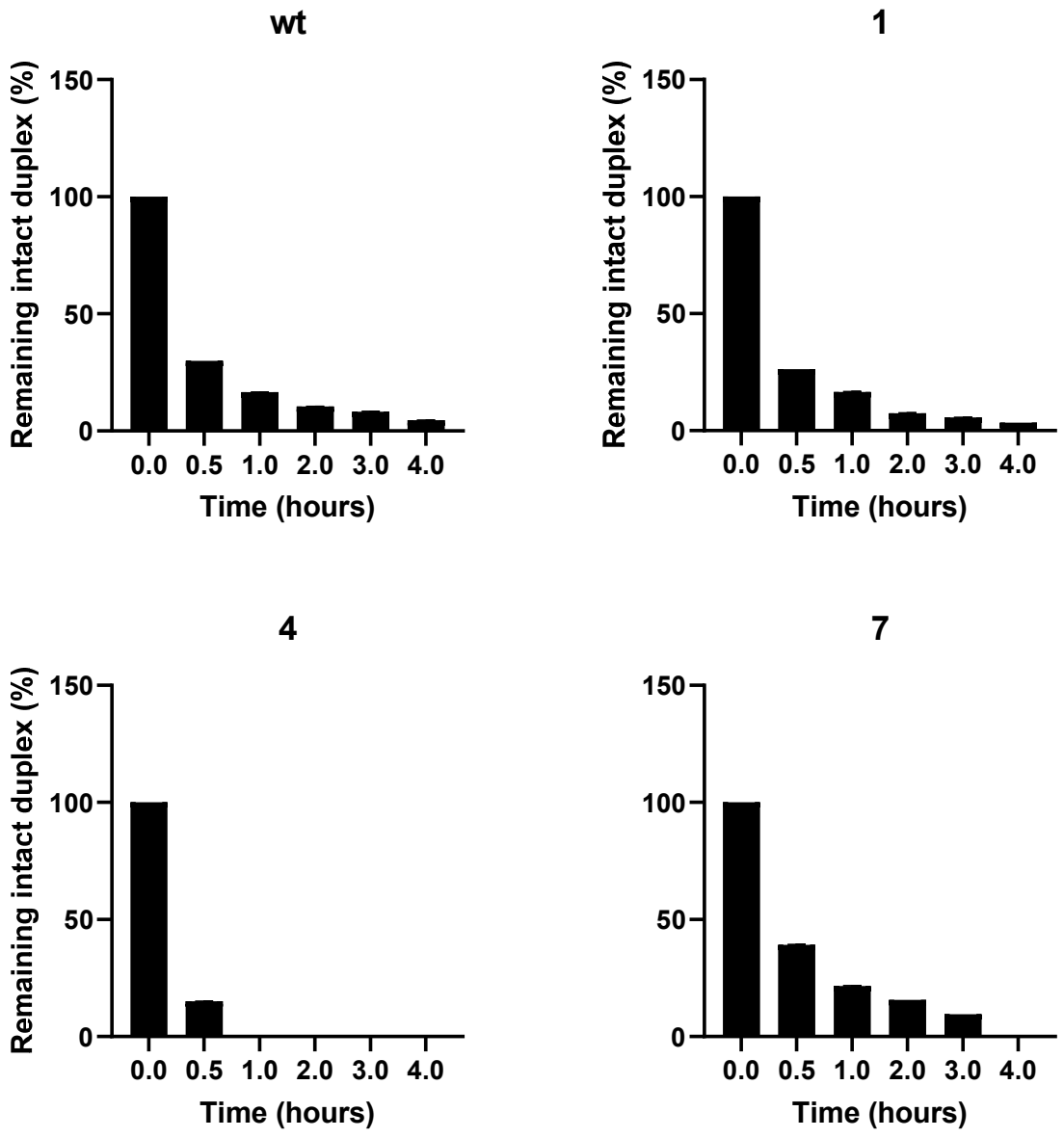
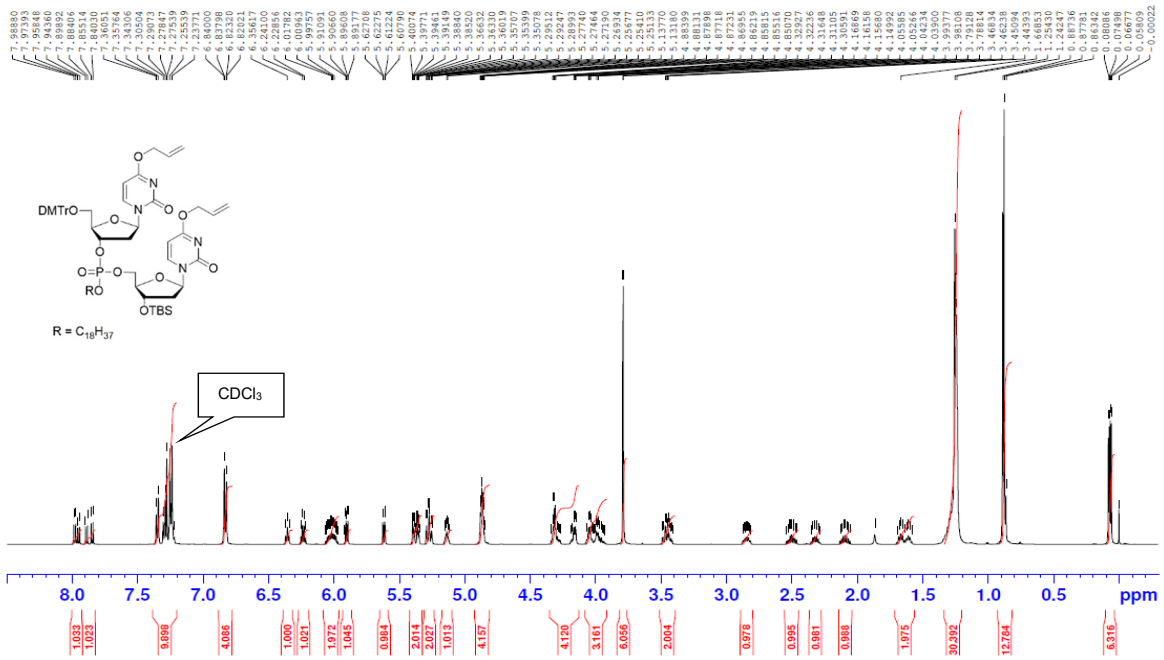


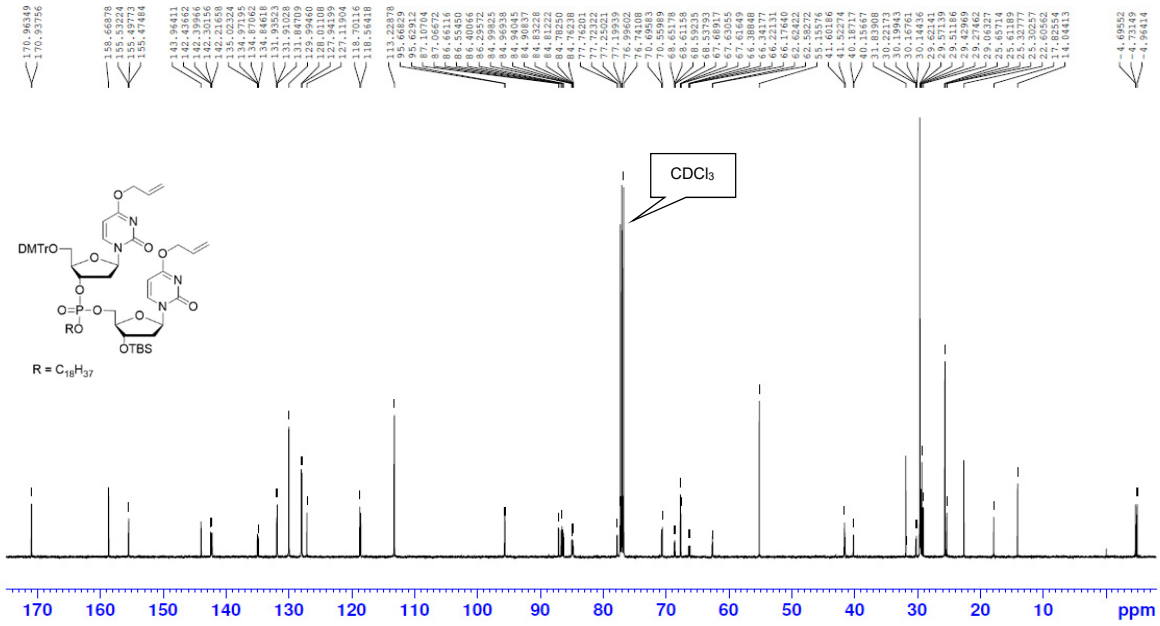
Figure S-2. Nuclease stability assay. Wild-type and siRNAs 1, 4, and 7 were incubated with 13.5% fetal bovine serum at 37 °C from 0 to 4 hours and samples were then run on a 20% non-denaturing polyacrylamide gel.

NMR spectra

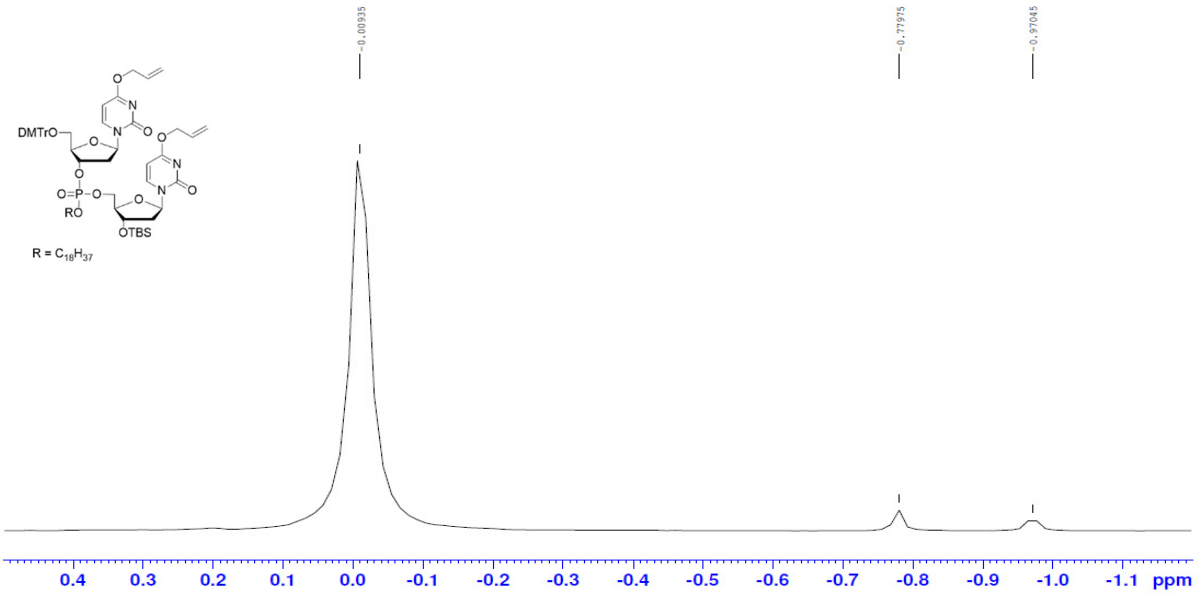
^{1}H NMR of Compound 2



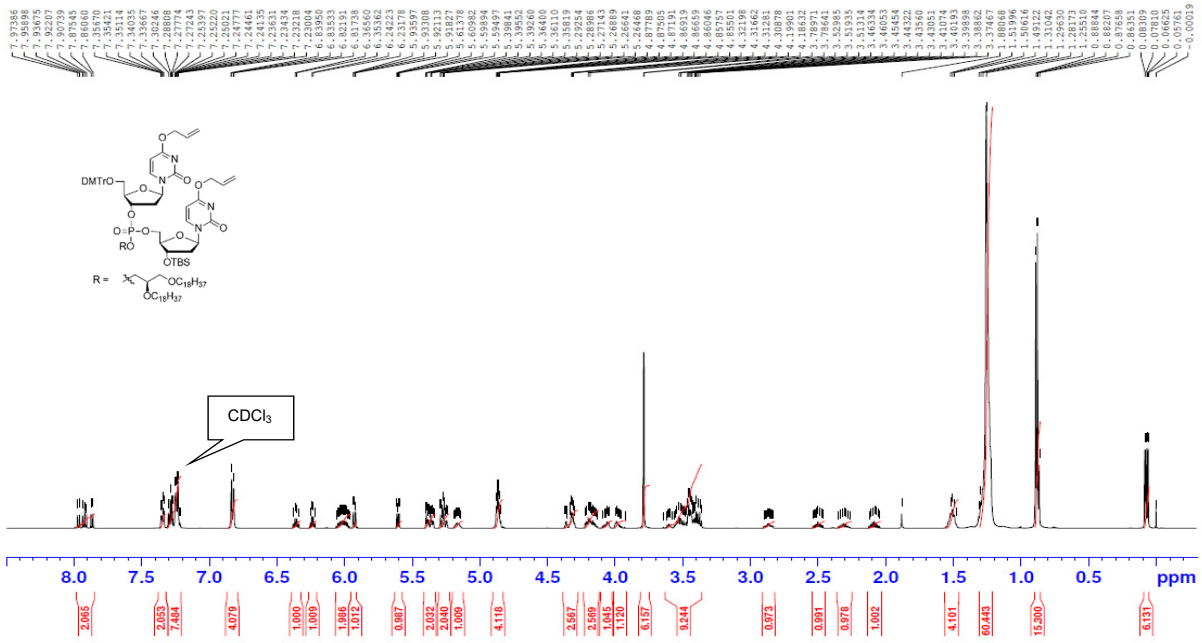
^{13}C NMR of Compound 2



^{31}P NMR of Compound 2



^{1}H NMR of Compound 3



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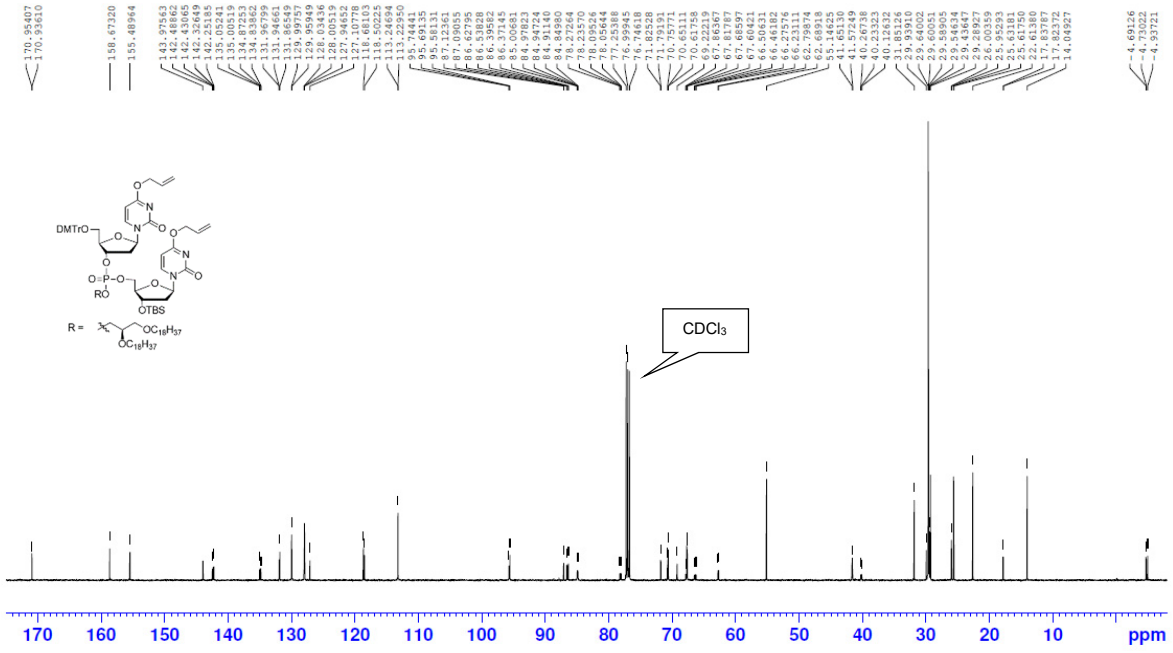
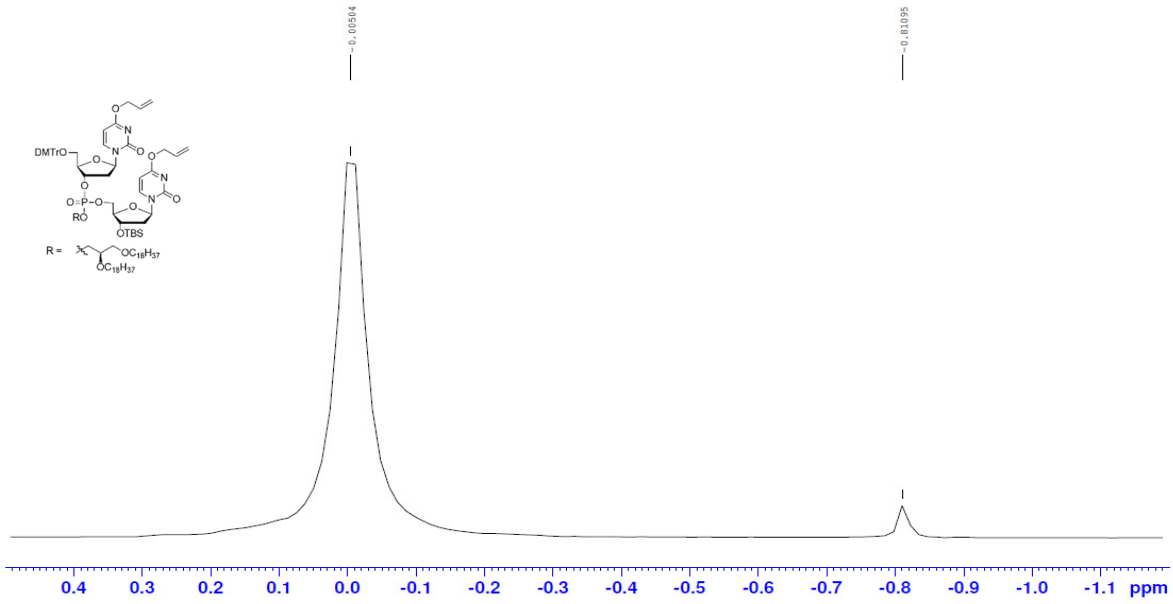


^{31}P NMR of Compound 3



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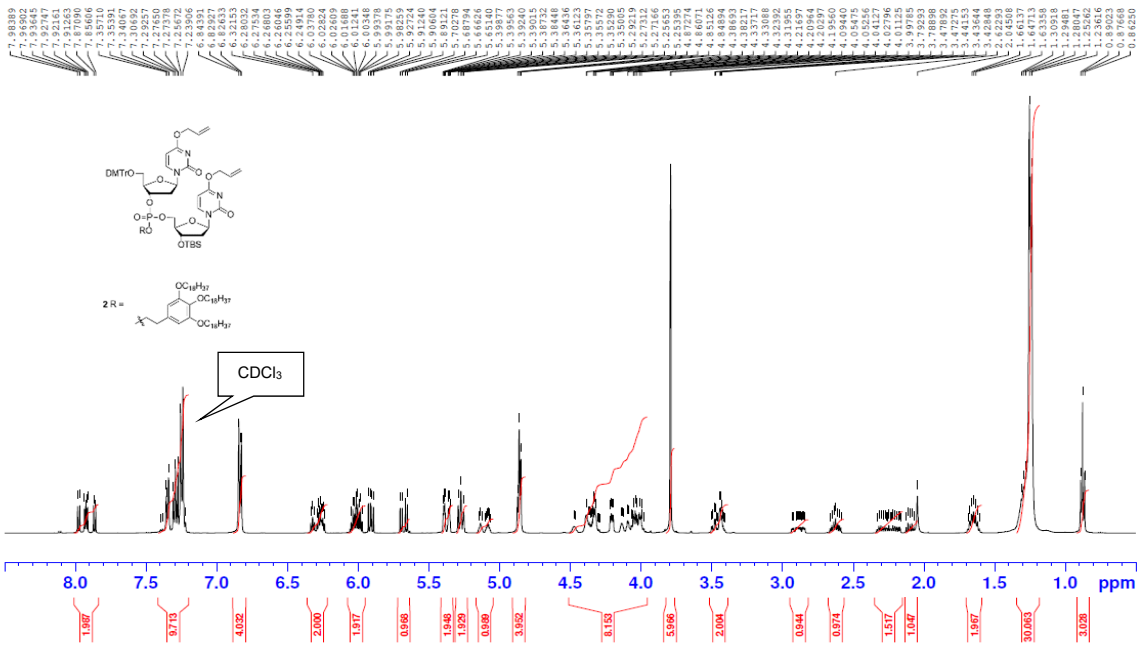
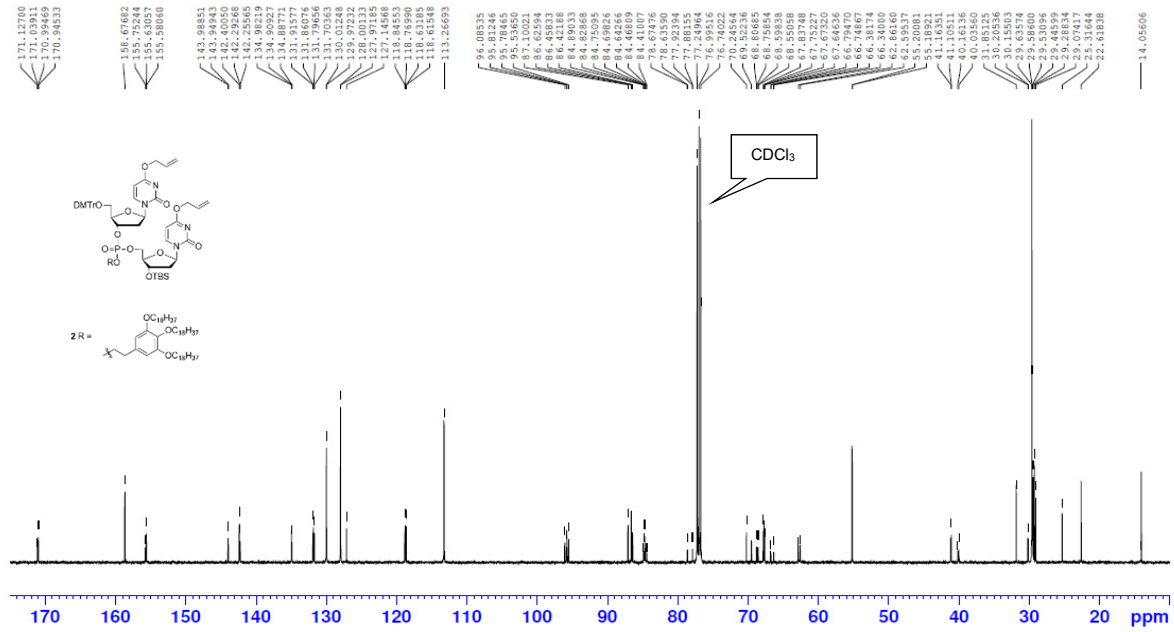
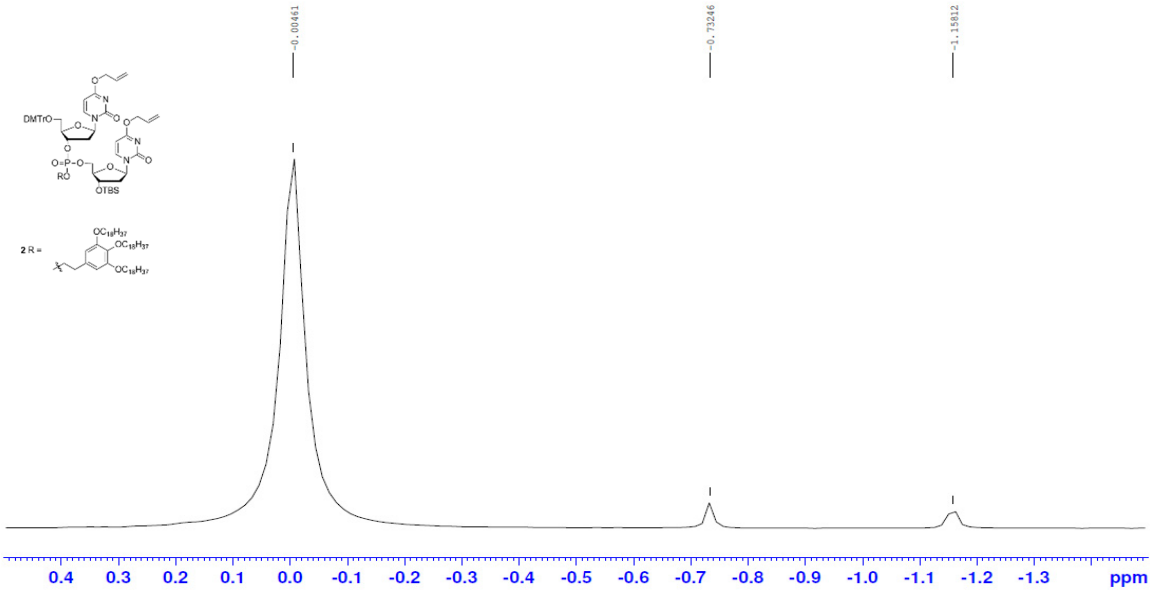


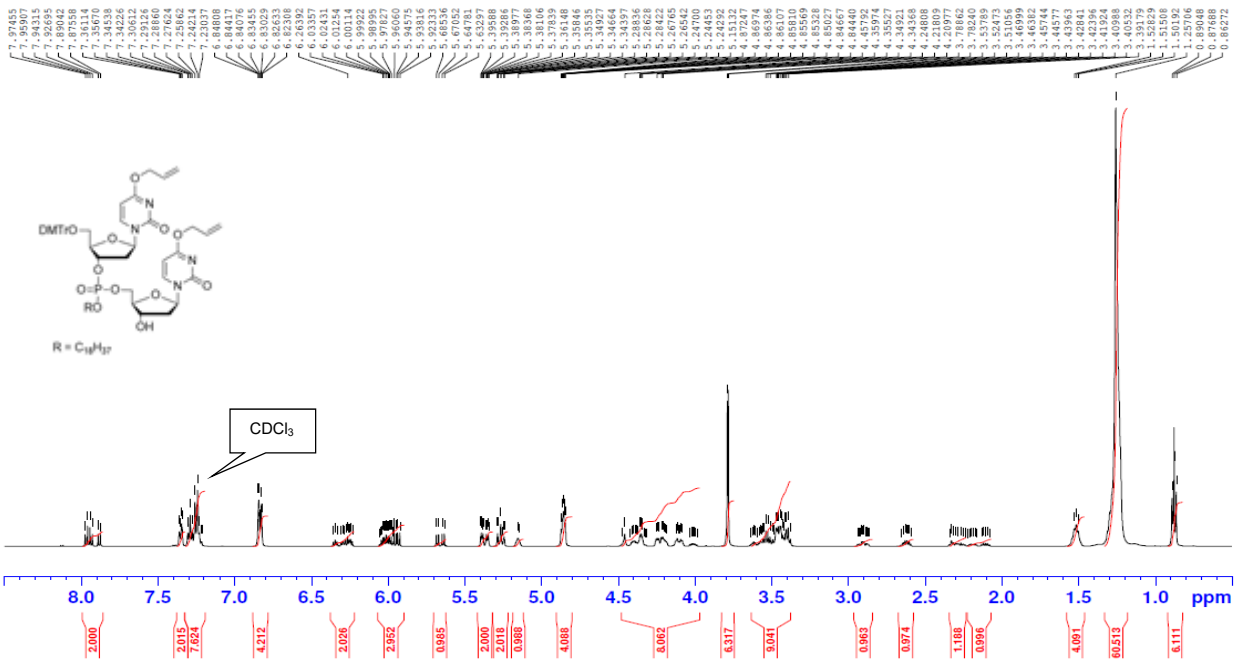
^{13}C NMR of Compound 4



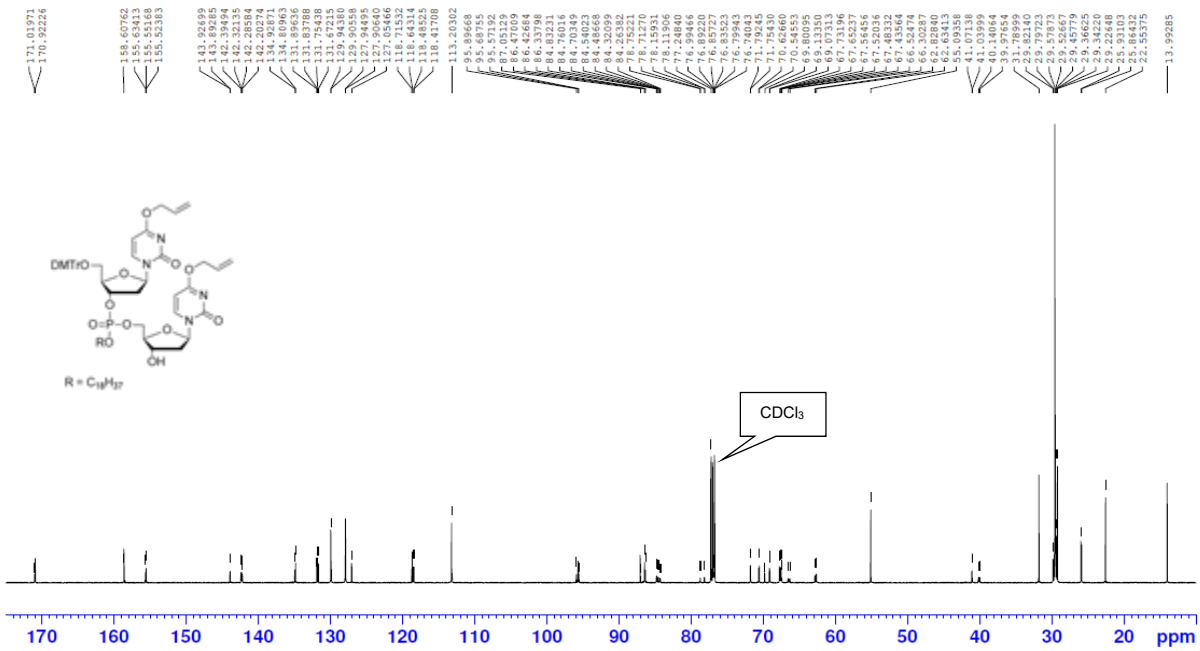
^{31}P NMR of Compound 4



^{1}H NMR of Compound 5



^{13}C NMR of Compound 5



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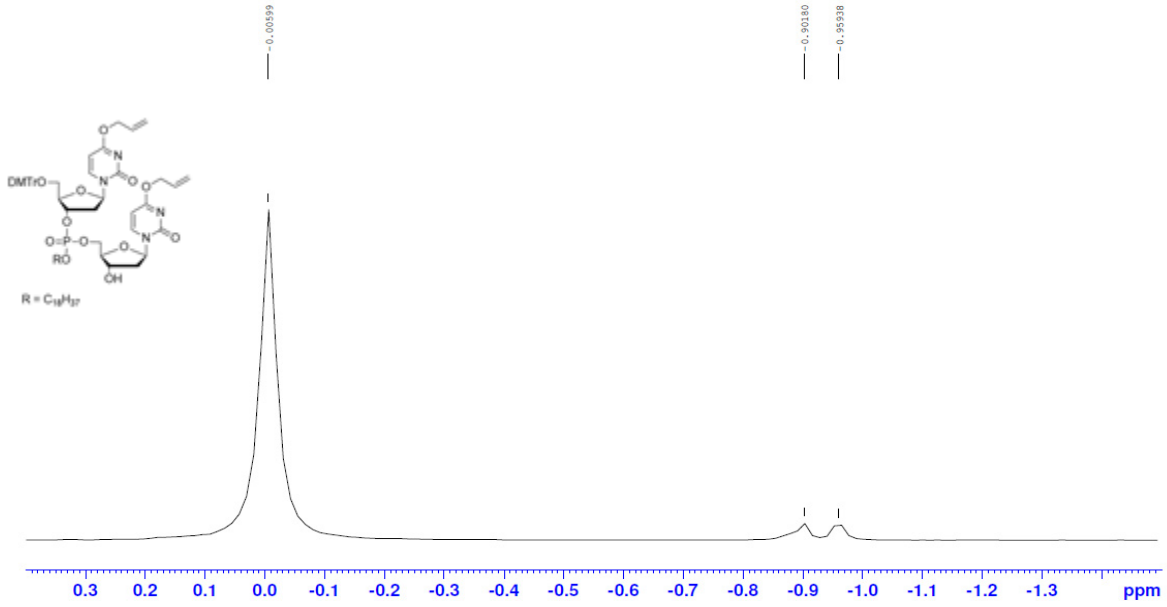

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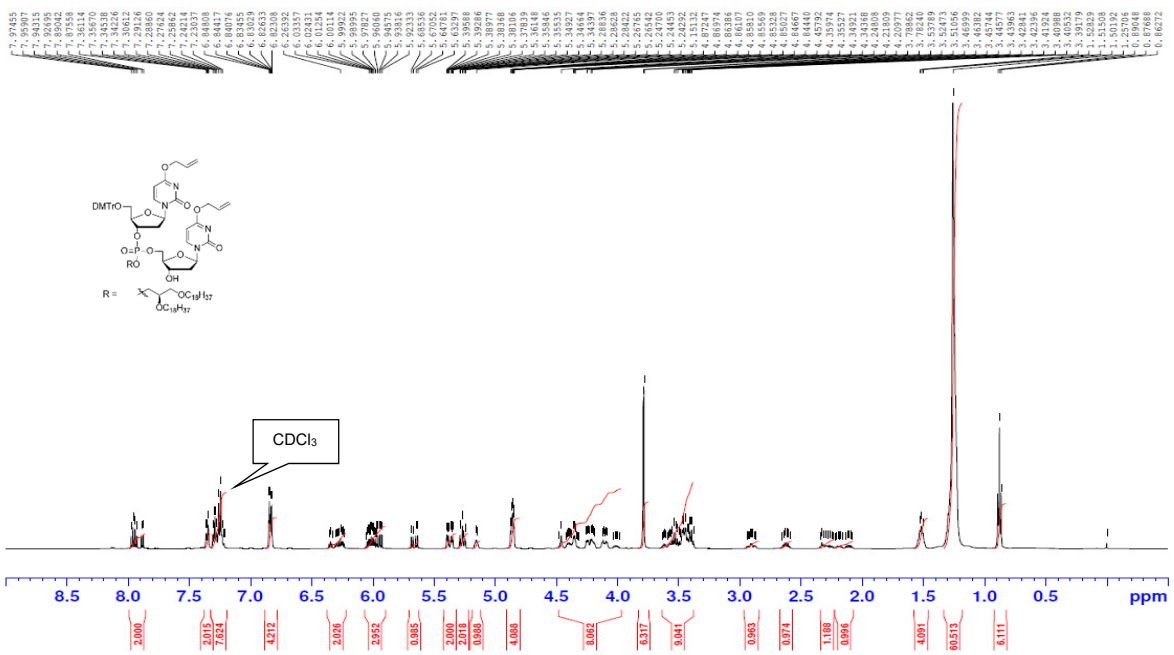
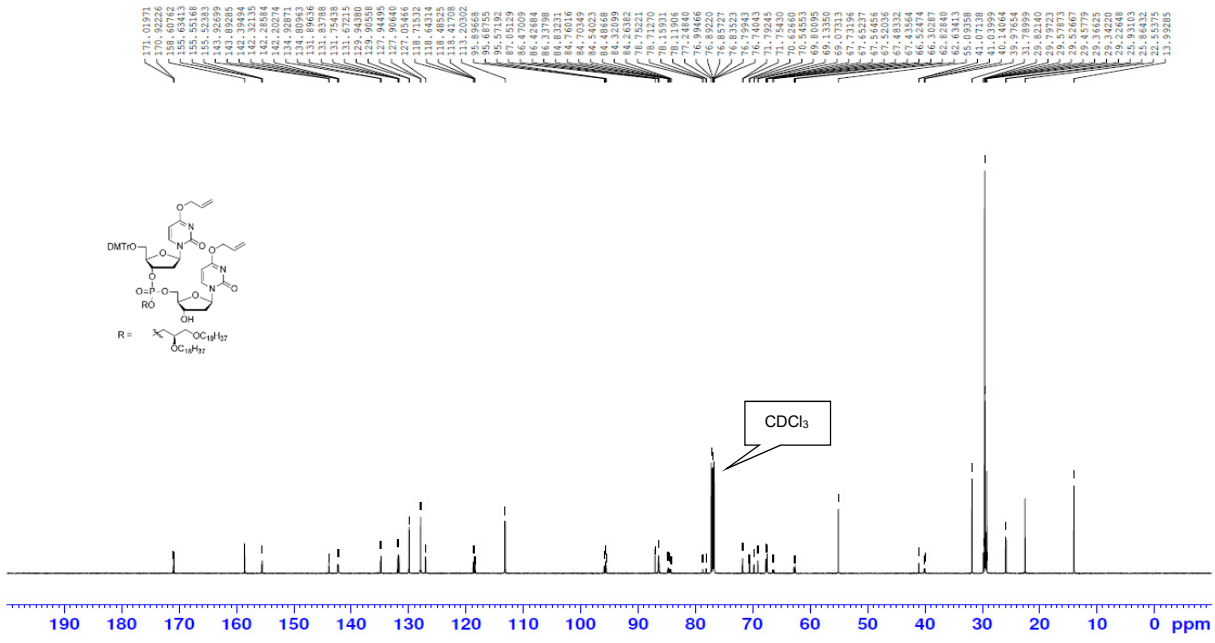
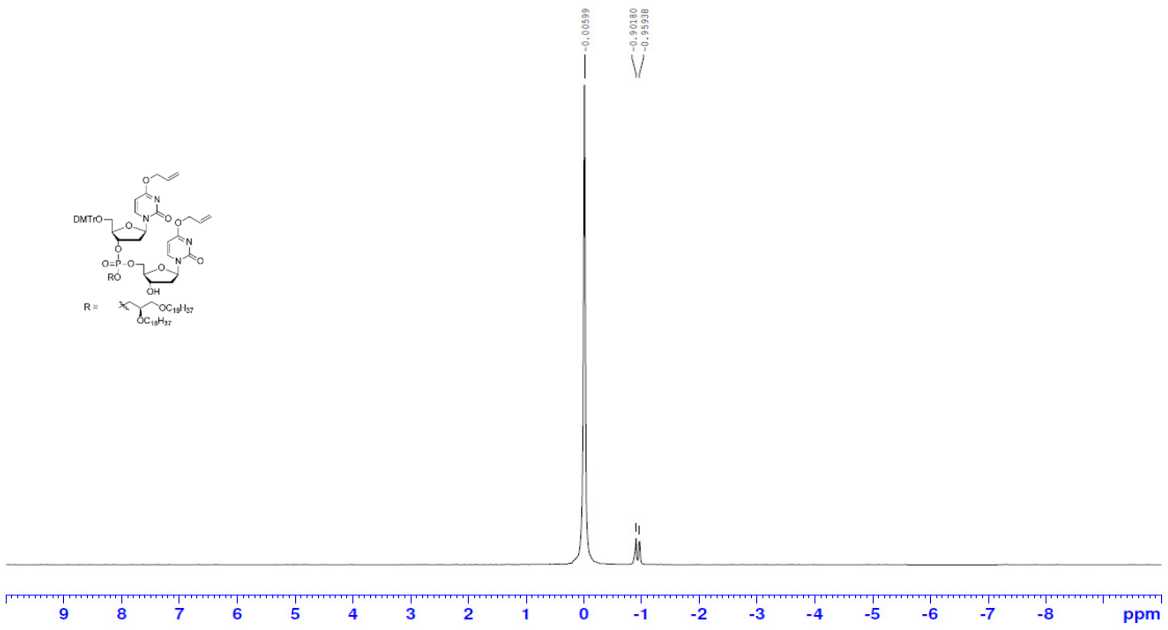


^{13}C NMR of Compound 6



^{31}P NMR of Compound 6



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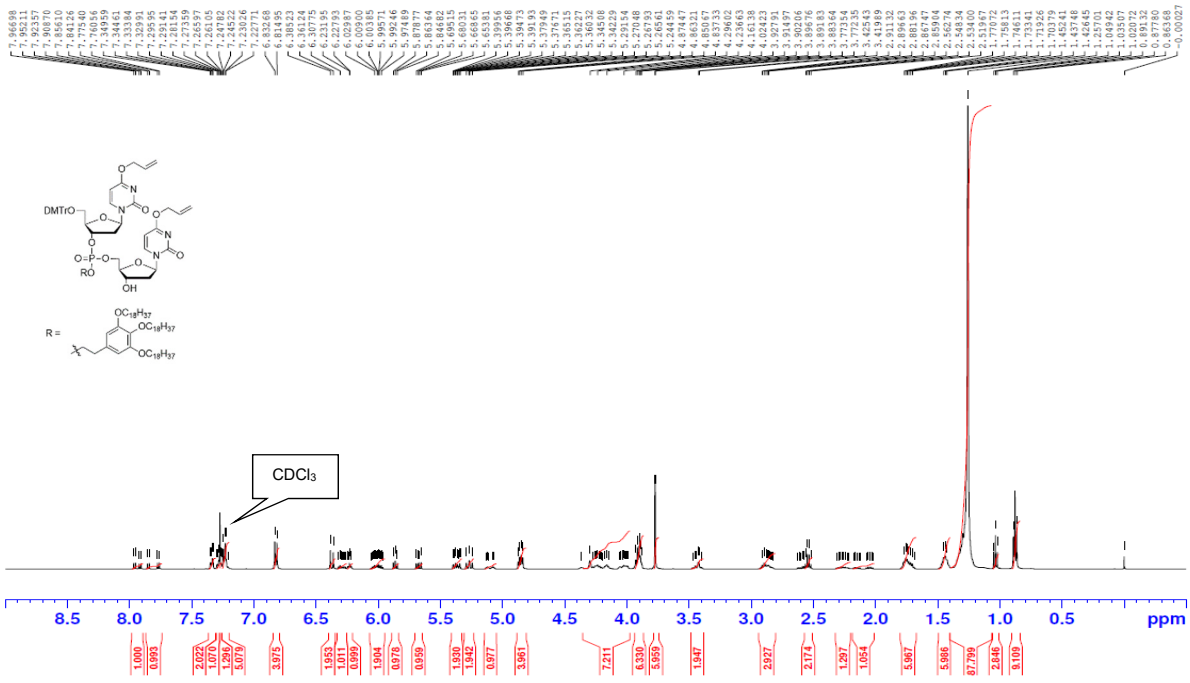

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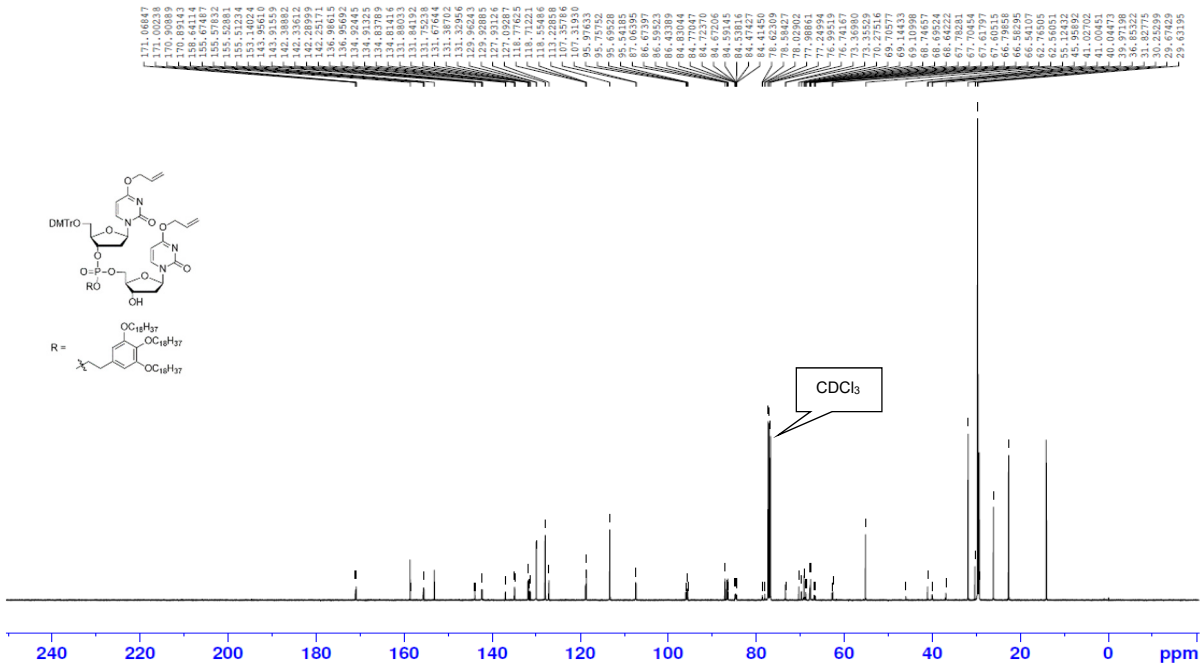

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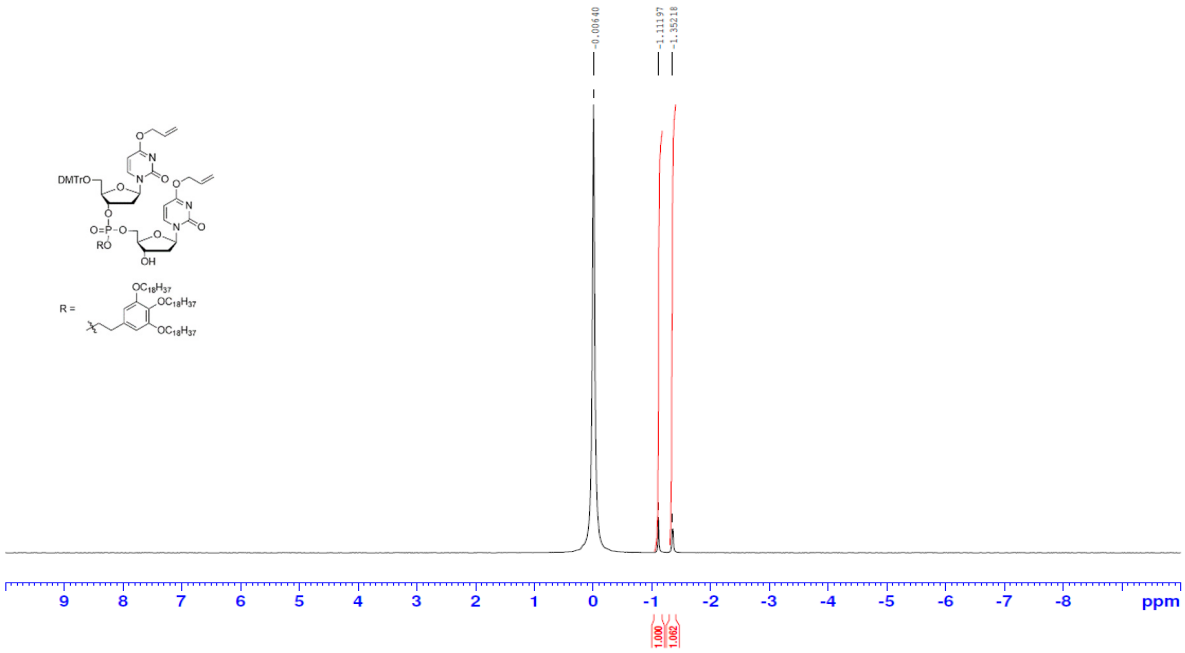
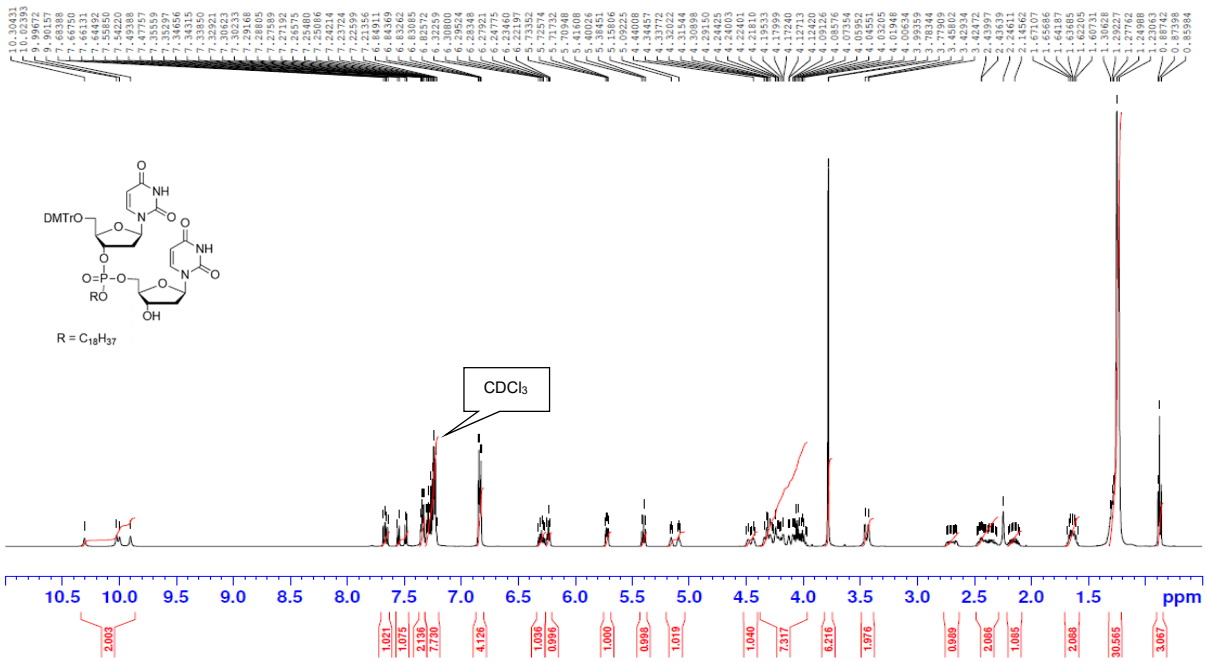


^{1}H NMR of 8



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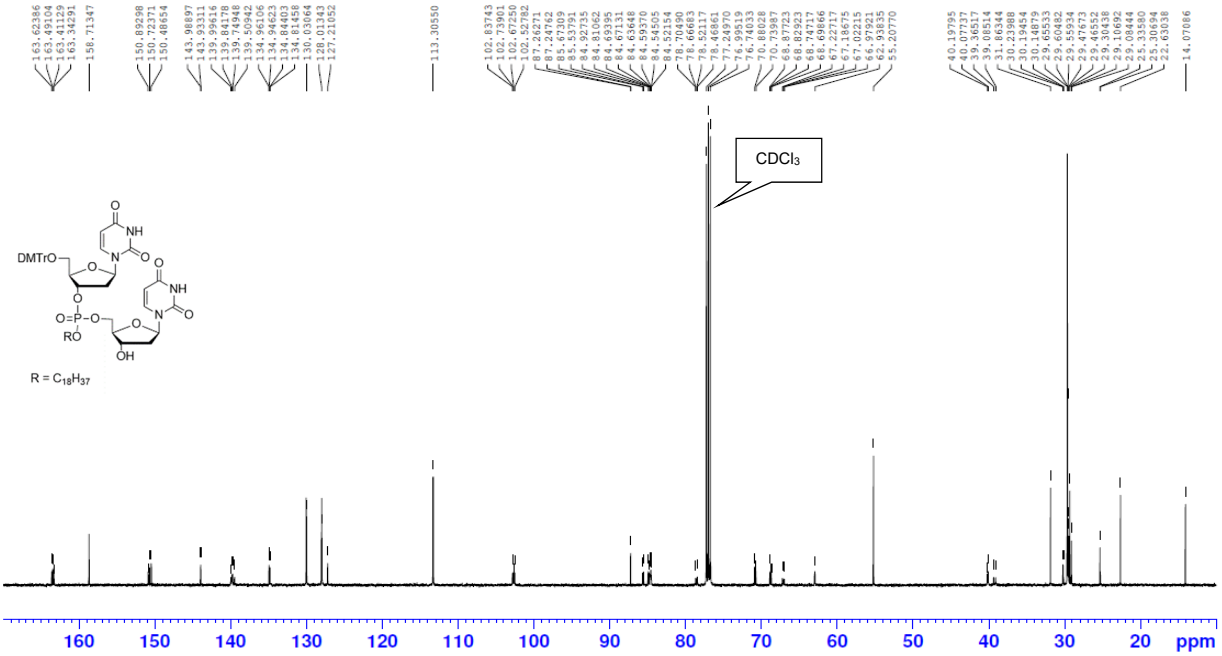

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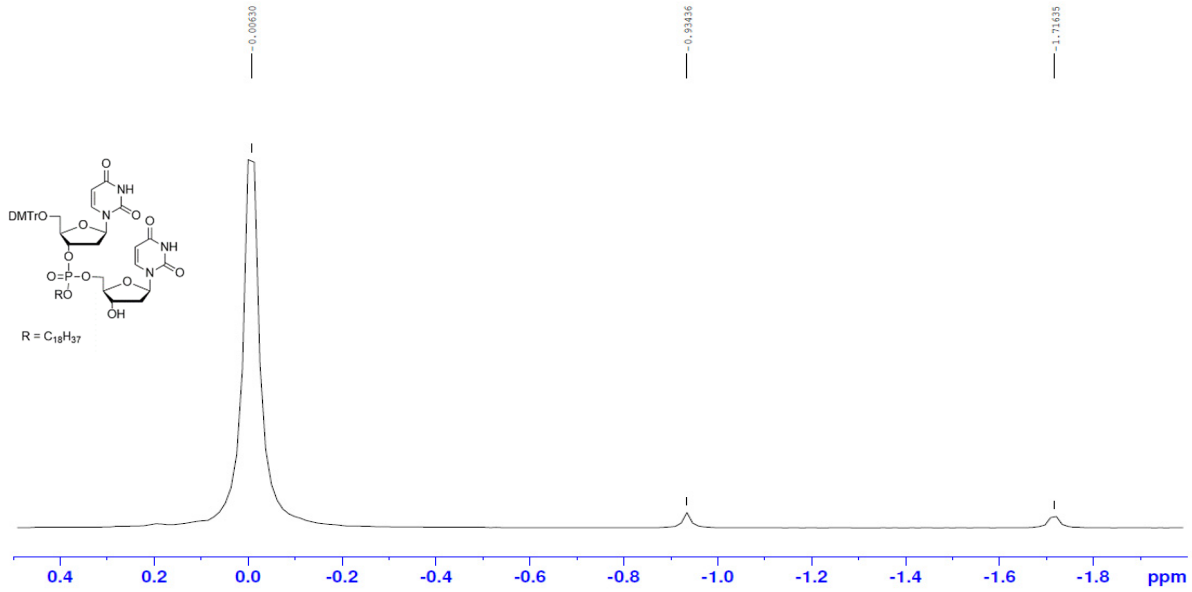
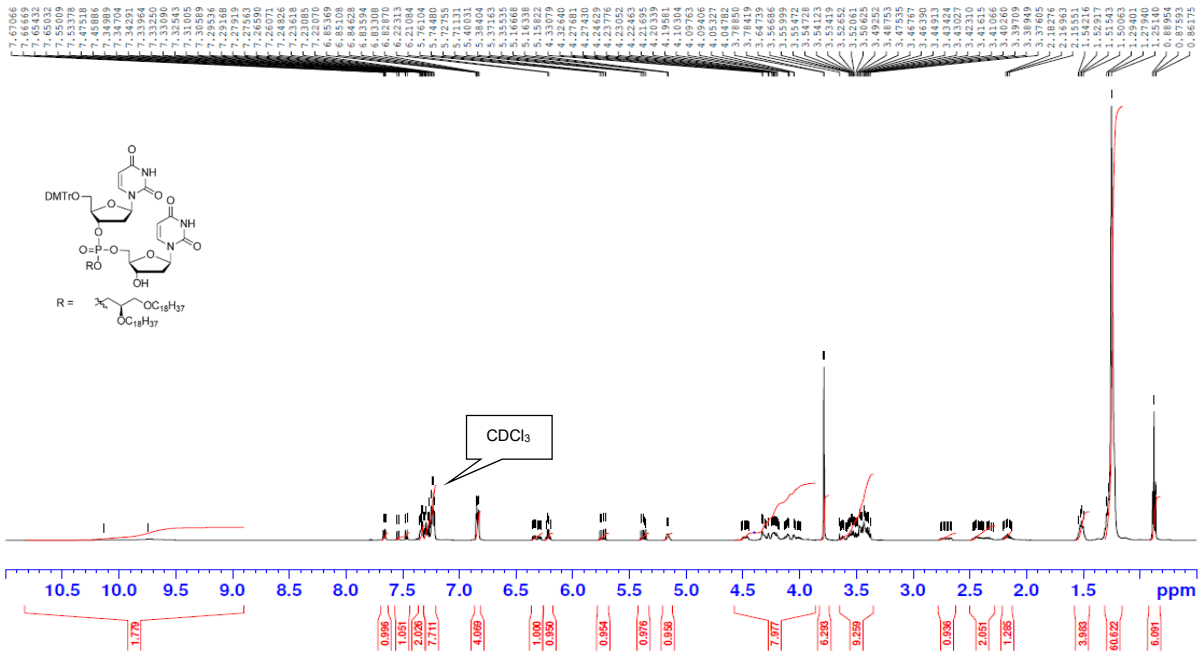


^{1}H NMR of 9



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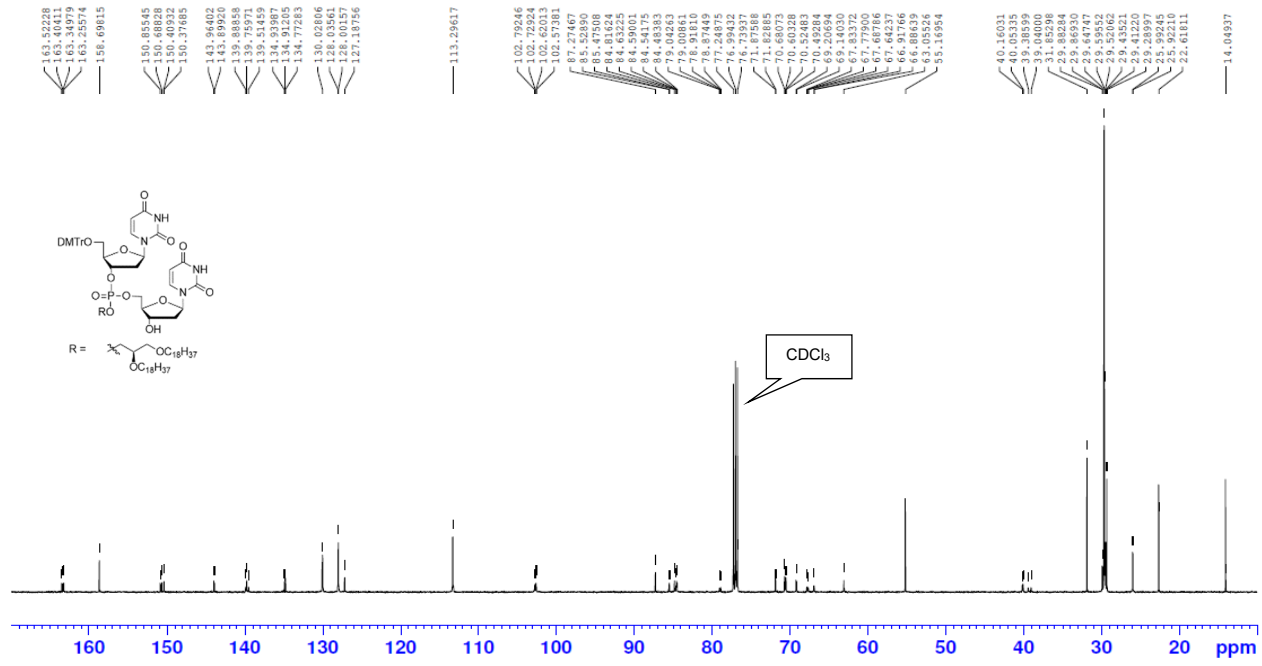

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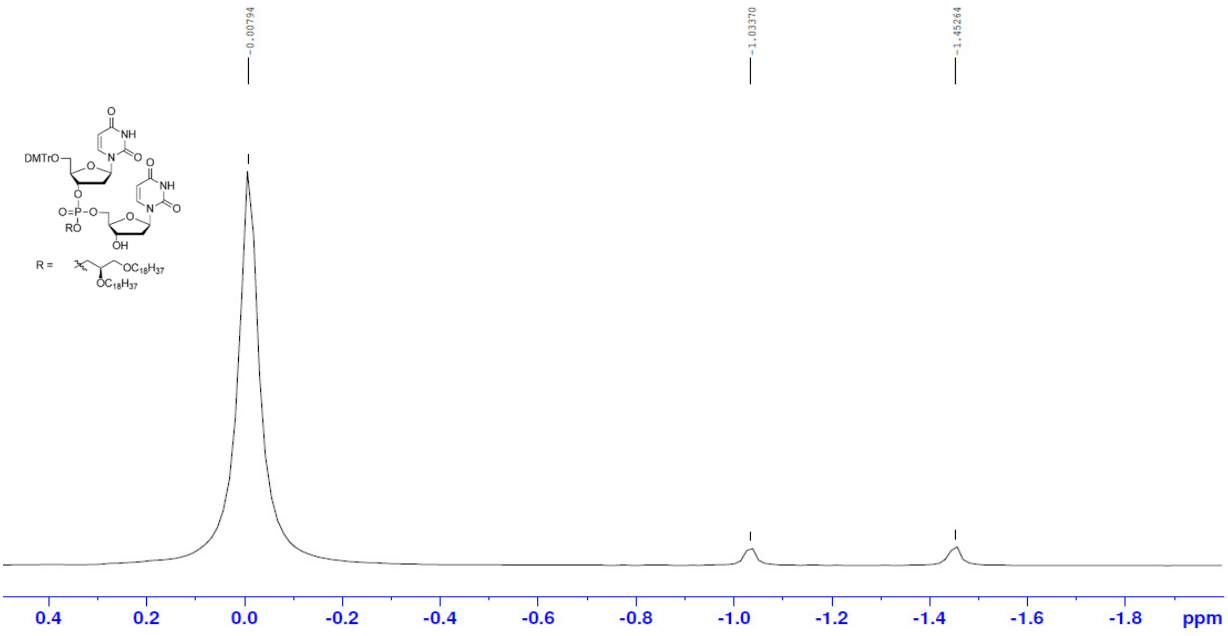

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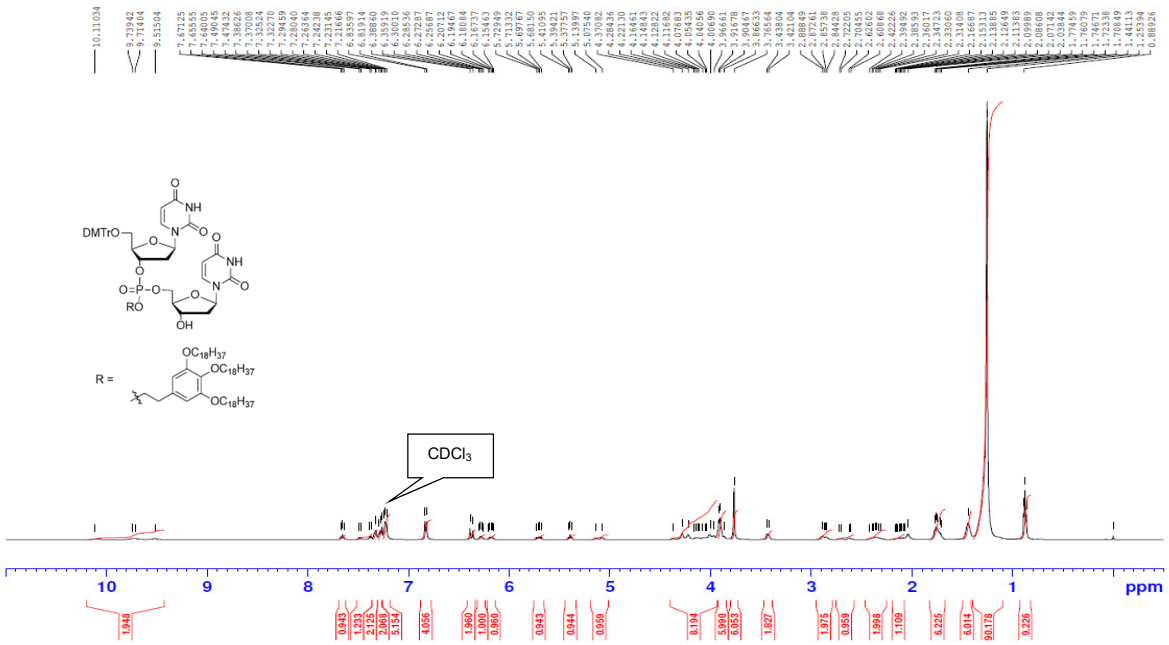

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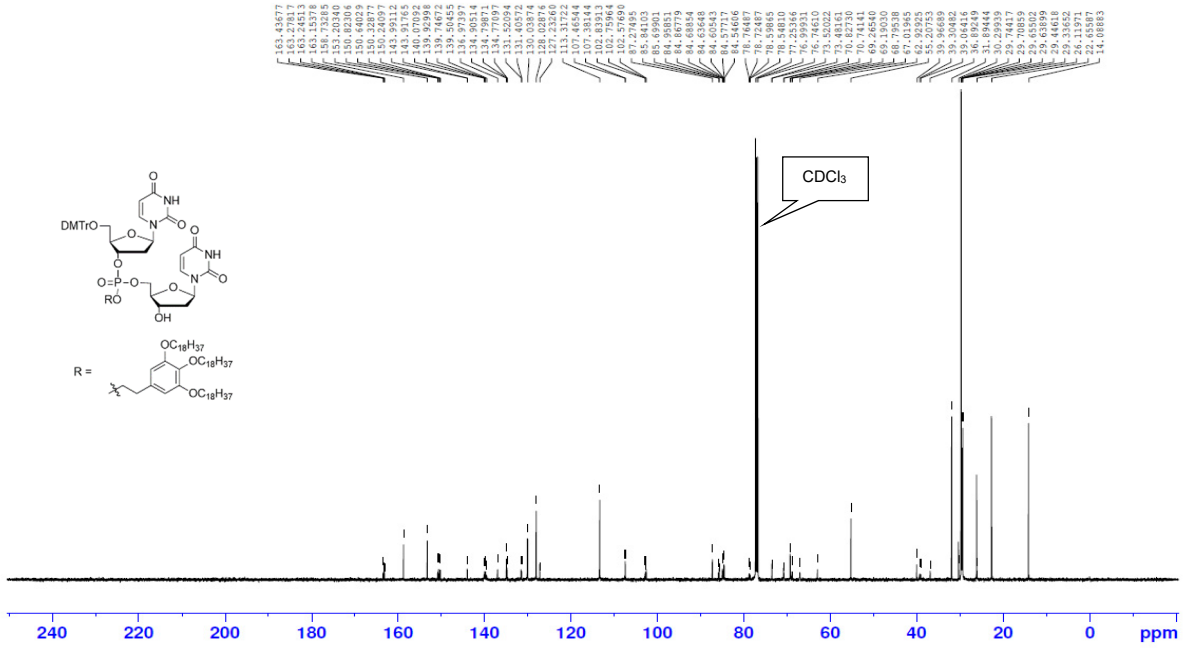

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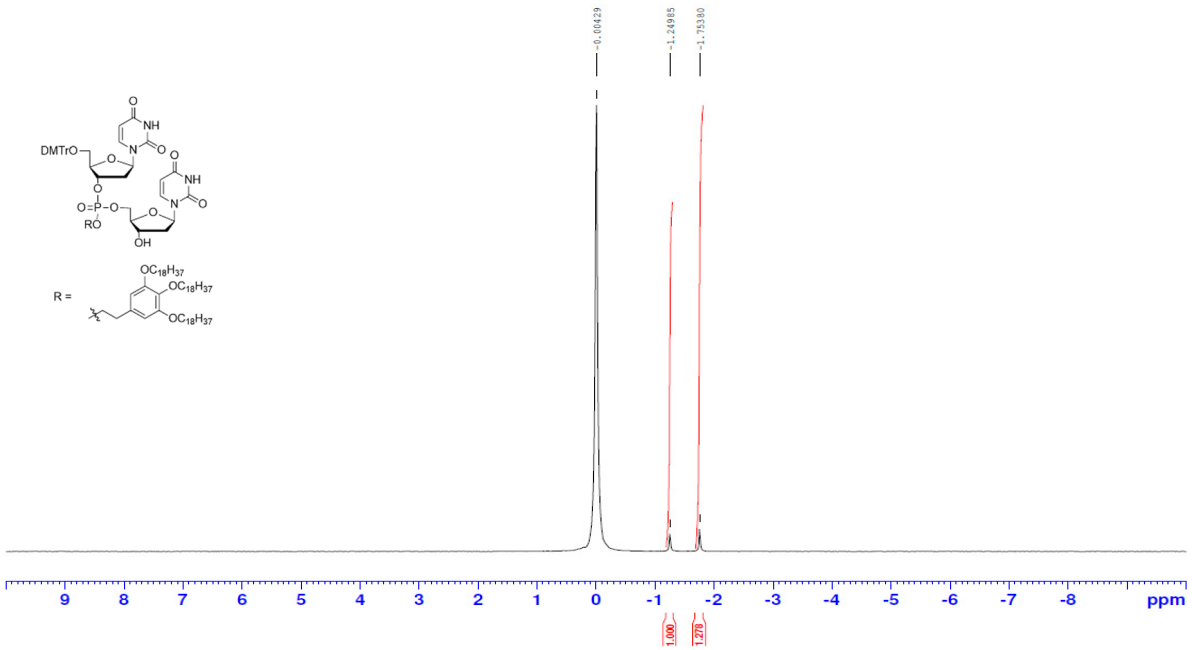
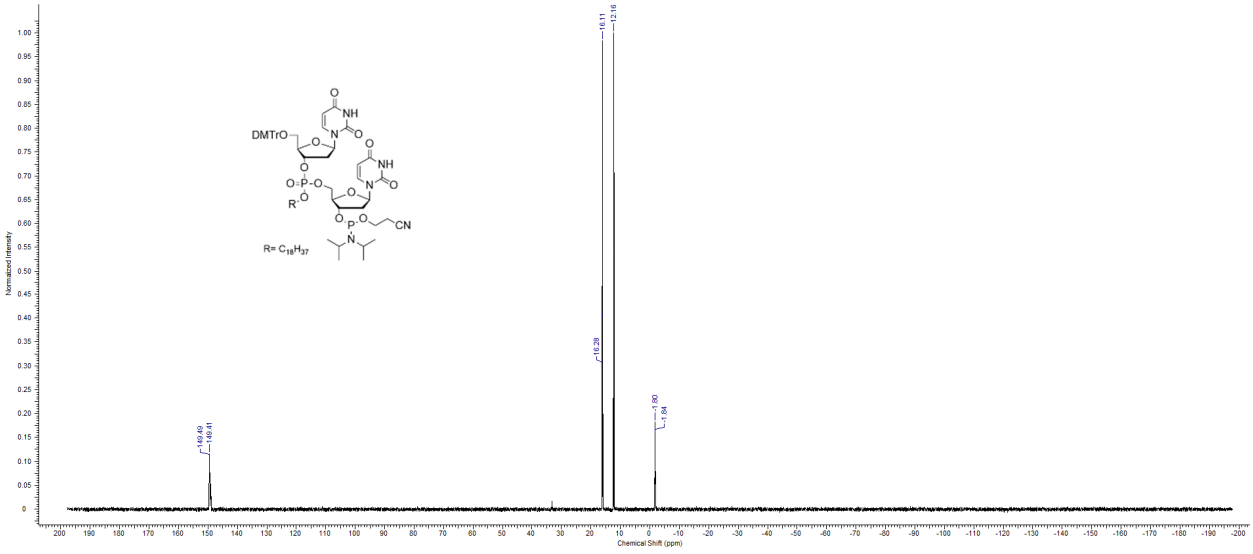
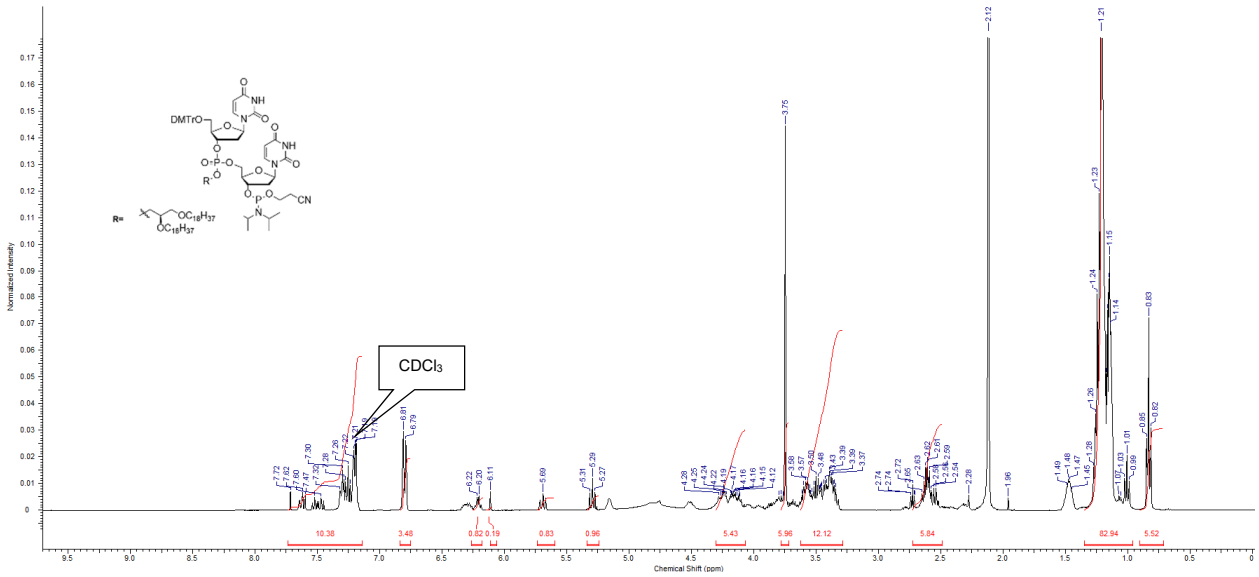


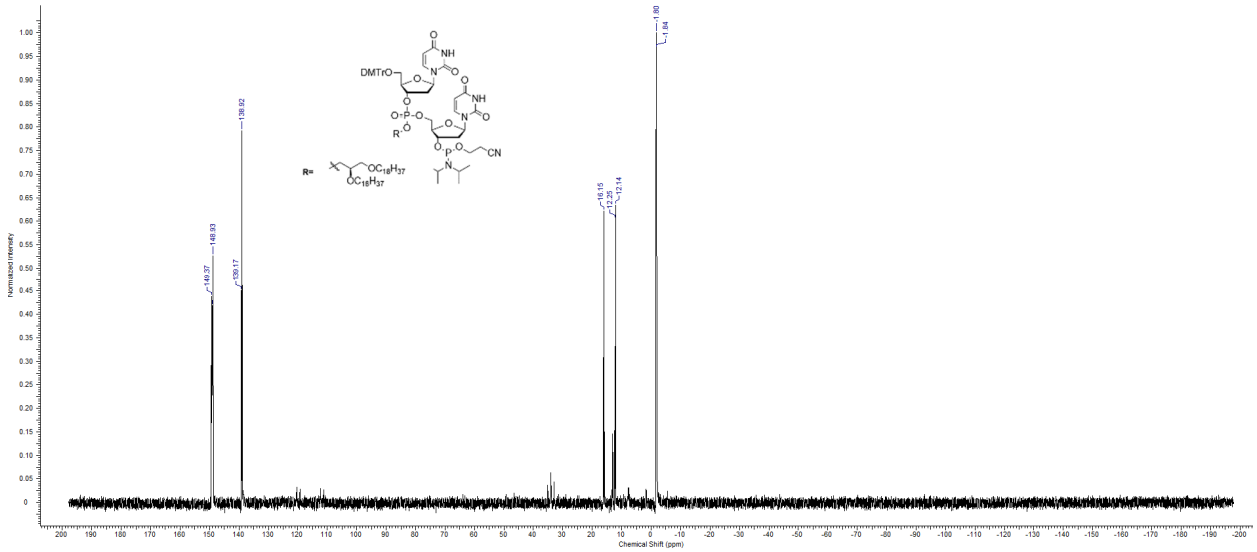
^{31}P NMR of Compound 11



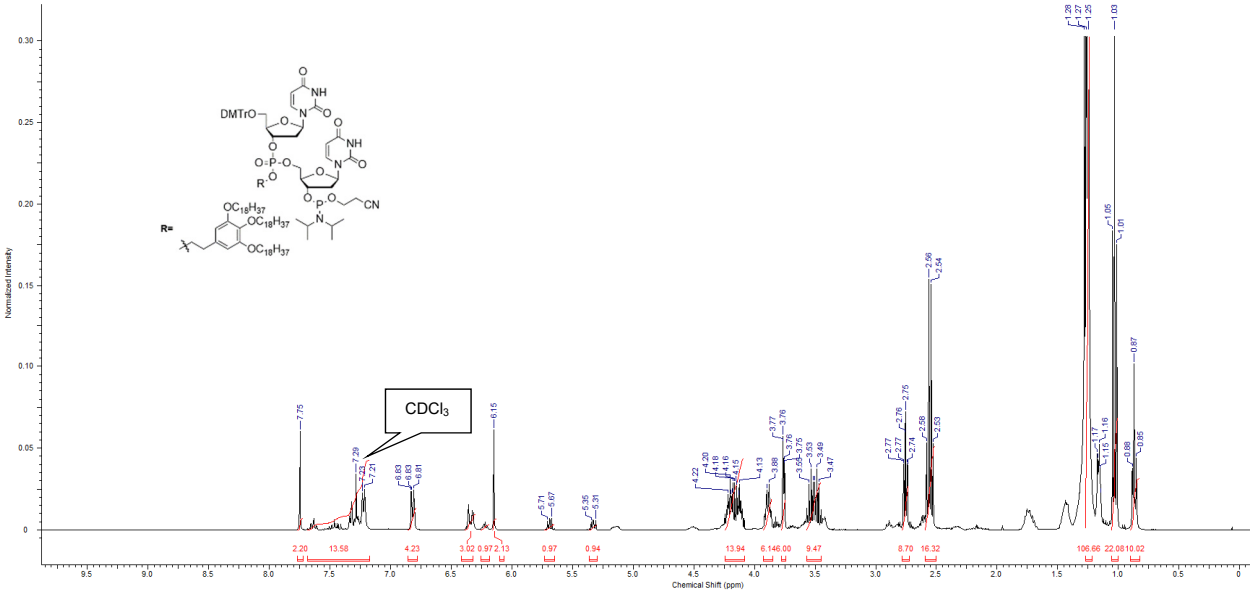
^{1}H NMR of Compound 12



^{31}P NMR of Compound 12



^{1}H NMR of Compound 13



^{31}P NMR of Compound 13