Supporting information

Thio-Modification Effects on mRNA Translation Using a PureCap-Based Capping Method

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Experimental Procedures

General Materials and Instrumentation

Reagents and solvents were obtained from TCI, FUJIFILM Wako, KANTO Chemical, Sigma-Aldrich, Angene, and ChemGenes as reagent-grade, first-grade, or special-grade materials unless otherwise specified, and were used without further purification. Thin-layer chromatography (TLC) was performed on Silica Gel 70 F254 plates (Wako). Silica gel column chromatography was carried out using spherical Silica Gel 60 (40–50 μm, KANTO Chemical), and flash column purification was conducted using a CombiFlash® NextGen 300$^+$ system. High-performance liquid chromatography (HPLC) analysis and purification were performed on a Shimadzu HPLC system. Nuclear magnetic resonance (NMR) spectra were recorded on a JNM-ECZ400 or JNM-ECA600 spectrometer (JEOL). Chemical shifts for ^{1}H and ^{13}C NMR spectra were referenced to residual solvent signals, whereas ^{31}P NMR spectra were reported without external or internal standardization. Mass spectrometric analysis was performed using a Bruker compact mass spectrometer (ESI-MS) or an Agilent 6530 quadrupole-time-of-flight (Q-TOF) system. LC-MS analysis of PureCap analog purity was conducted on an Agilent 1290 Infinity II equipped with a 6530 LC/Q-TOF.

Experimental Details for Organic Synthesis

General synthesis of 22 and 23

A mixture of molecular sieve 3Å, *N*-acetyl-2',3'-acetyl-guanosine (1–2 eq.) and phosphoramidite (1 eq., **20** for synthesis of **22**, **21** for synthesis of **23**) was dissolved in anhydrous acetonitrile (0.1 M solution) and stirred at room temperature under an Ar atmosphere for 10–20 minutes. *1H*-Tetrazole (4.5 eq.) was then added, and the reaction was allowed to continue for another hour. Afterward, *tert*-butyl hydroperoxide (TBHP, 1 M in toluene, 1.5 eq.) was added, and the reaction was allowed to proceed for 1.5–2 hours. The reaction mixture was extracted with ethyl acetate and H_2O, and the organic phase was washed with saturated $NaHCO_3$, followed by brine, dried over Na_2SO_4, and concentrated under reduced pressure to afford the crude product. The purification was conducted on a silica gel column to give **22** (50%) or **23** (41%) as white solids.

(22)

^{1}H-NMR (400 MHz, *ACETONITRILE-D3*) δ 12.09 (d, J = 18.8 Hz, 3H), 9.49–9.59 (1H), 8.23 (d, J = 10.8 Hz, 2H), 7.98–7.93 (m, 5H), 7.78 (d, J = 8.6 Hz, 2H), 7.61–7.57 (m, 2H), 7.53–7.45 (m, 10H), 7.37–7.28 (m, 14H), 7.24–7.22 (m, 2H), 6.89–6.84 (m, 10H), 6.04–6.01 (m, 2H), 5.99–5.95 (m, 3H), 5.93–5.87 (m, 1H), 5.80 (t, J = 6.0 Hz, 1H), 5.61–5.50 (m, 5H), 5.11–5.22 (m, 2H), 4.94–5.06 (m, 2H), 4.51–4.45 (m, 3H), 4.44–4.38 (m, 2H), 4.29 (s, 1H), 4.20 (d, J = 5.9 Hz, 3H), 3.60 (s, 4H), 2.81 (d, J = 6.0 Hz, 2H), 2.72–2.68 (m, 5H), 2.11–2.06 (m, 11H), 1.96 (s, 7H), 1.93–1.90 (m, 22H), 1.82 (s, 25H), 1.17–0.99 (m, 22H), 0.67 (s, 9H), 0.58 (s, 7H), -0.17 (s, 3H), -0.23 (s, 2H), -0.45 (d, J = 5.6 Hz, 5H).
^{13}C-NMR (100 MHz, *ACETONITRILE-D3*) δ 180.5, 180.4, 180.3, 172.4, 170.1, 170.0, 169.9, 169.7, 169.5, 165.4, 158.8, 155.5, 155.4, 151.7, 148.7, 148.5, 148.4, 148.3, 144.9, 143.8, 143.5, 139.4, 139.1, 138.2, 136.1, 136.0, 135.7, 132.7, 130.1, 130.0, 128.7, 128.2, 128.1, 128.0, 127.1, 122.3, 119.9, 119.4, 119.0, 117.4, 116.7, 115.9, 115.7, 113.3, 87.1, 87.0, 86.5, 86.5, 86.0, 84.0, 81.5, 81.4,

81.1, 81.0, 80.8, 76.3, 75.8, 73.1, 71.9, 71.2, 70.7, 70.6, 67.6, 64.6, 63.2, 63.0, 62.6, 61.4, 55.0, 55.0, 48.8, 48.7, 35.7, 35.6, 24.9, 24.8, 24.7, 21.6, 20.0, 19.7, 19.6, 19.4, 19.3, 19.2, 18.5, 18.5, 18.3, 18.3, 18.2, 17.5, 17.4, 1.9, 1.5, 1.3, 1.0, 0.8, 0.6, 0.4, 0.2, -0.0, -0.2, -0.4, -0.6, -5.6, -5.7, -6.1, -6.2, -7.1.
^{31}P-NMR (160 MHz, *ACETONITRILE-D3*) δ -2.5
HRMS (ESI): Calcd. for $C_{65}H_{75}N_{11}O_{16}PSSi^{+}$ 1356.4615 $[M+H]^{+}$; obsd.1356.4611.

(**23**)
^{1}H-NMR (392 MHz, *METHANOL-D3*) δ 8.53–8.44 (m, 4H), 8.07–8.04 (m, 5H), 7.65–7.58 (m, 2H), 7.56–7.49 (m, 4H), 7.49–7.43 (m, 4H), 7.41–7.25 (m, 13H), 7.22–7.17 (m, 2H), 6.87–6.83 (m, 9H), 6.22–6.13 (m, 4H), 5.97–5.94 (m, 1H), 5.88–5.83 (m, 1H), 5.66–5.59 (m, 2H), 5.40–5.34 (m, 1H), 5.33–5.27 (m, 1H), 4.50–4.45 (m, 6H), 4.33–4.26 (m, 4H), 4.10–4.04 (m, 1H), 3.88–3.81 (m, 2H), 3.74–3.73 (m, 12H), 3.67–3.61 (m, 2H), 3.59–3.49 (m, 2H), 2.87–2.81 (m, 4H), 2.70–2.65 (m, 2H), 2.10–2.08 (m, 6H), 2.02–1.96 (m, 7H), 1.19–1.10 (m, 13H).
^{13}C-NMR (99 MHz, *METHANOL-D3*) δ 180.4, 170.2, 170.1, 169.8, 166.8, 159.0, 158.9, 156.0, 151.9, 151.7, 149.9, 149.0, 148.5, 144.8, 144.0, 143.8, 139.1, 138.8, 135.7, 135.7, 135.4, 133.6, 132.6, 130.1, 128.4, 128.1, 128.0, 127.6, 126.7, 124.4, 120.8, 120.7, 117.1, 113.0, 86.8, 86.8, 86.6, 86.3, 83.6, 83.5, 81.1, 81.1, 78.3, 72.7, 72.5, 70.5, 67.3, 63.9, 63.9, 63.3, 61.0, 60.7, 58.2, 58.1, 54.4, 49.4, 48.3, 48.1, 47.9, 47.7, 47.4, 47.2, 47.0, 35.6, 19.5, 19.2, 18.9, 18.8, 18.7, 18.0, 18.0, 13.1.
^{31}P-NMR (159 MHz, *METHANOL-D3*) δ -2.5, -2.5
HRMS (ESI): Calcd. for $C_{60}H_{63}N_{11}O_{16}PS^{+}$ 1256.3907 $[M+H]^{+}$; obsd.1256.3903.

General synthesis of 24 and 25
22 or **23** (1 eq.) was dissolved in dichloromethane (DCM, 0.2 M solution), then the same volume of trichloroacetic acid (TCA) solution in dichloromethane (2 M solution, 10 eq.) was added dropwise under an ice-bath. The reaction mixture was warmed to room temperature and stirred for 15–20 minutes. The reaction was quenched with saturated $NaHCO_3$ solution, then extracted with ethyl acetate. The organic layer was washed with brine, then dried over Na_2SO_4, then concentrated. The crude was purified using a silica gel column, a white solid was obtained as the target compound (85% for **24** from **22**; quant. for **25** from **23**).

(**24**)
^{1}H-NMR (594 MHz, *ACETONITRILE-D3*) δ 8.65 (s, 1H), 8.62 (s, 1H), 8.54 (s, 1H), 8.49 (s, 1H), 8.02–7.96 (m, 6H), 7.85 (s, 1H), 7.82 (s, 1H), 7.60–7.57 (m, 3H), 7.48 (t, *J* = 7.8 Hz, 6H), 6.04 (d, *J* = 6.6 Hz, 2H), 6.0–5.95 (m, 3H), 5.94 (t, *J* = 6.0 Hz, 3H), 5.85–5.77 (m, 10H), 5.57 (q, *J* = 3.6 Hz, 2H), 5.54 (dd, *J* = 5.4, 3.6 Hz, 1H), 5.20–5.17 (m, 1H), 5.06–5.02 (m, 3H), 4.93–4.89 (m, 1H), 4.53–4.44 (m, 6H), 4.43–4.35 (m, 3H), 4.29 (q, *J* = 6.6 Hz, 3H), 4.23–4.19 (m, 4H), 4.03 (q, *J* = 7.2 Hz, 1H), 3.95–3.86 (m, 3H), 3.82–3.78 (m, 5H), 3.73–3.70 (m, 3H), 2.82 (t, *J* = 6.0 Hz, 3H), 2.75–2.70 (m, 7H), 2.09–2.07 (m, 9H), 1.96–1.91 (m, 15H), 1.18–1.12 (m, 20H), 0.70 (s, 13H), 0.65 (s, 12H), -0.09 (s, 4H), -0.16 (s, 3H), -0.29– -0.32 (m, 8H).
^{13}C-NMR (149 MHz, *ACETONITRILE-D3*) δ 180.5, 180.4, 172.8, 170.9, 170.1, 170.0, 170.0, 169.7, 169.7, 165.8, 158.8, 155.6, 152.1, 151.6, 150.4, 150.3, 148.7, 148.5, 148.5, 148.4, 143.7, 143.5, 139.2, 139.1, 138.1, 133.9, 132.7, 130.1, 130.0, 128.7, 128.3, 128.1, 125.0, 124.9, 122.1, 122.0,

121.2, 118.8, 118.3, 117.5, 116.4, 113.3, 112.9, 86.9, 86.7, 85.8, 84.0, 81.3, 81.2, 81.2, 81.1, 81.0, 77.7, 73.2, 72.1, 71.6, 71.3, 70.6, 70.6, 67.6, 67.6, 67.5, 63.3, 63.2, 63.0, 62.9, 62.6, 62.2, 61.4, 60.1, 55.0, 54.4, 51.5, 51.4, 35.6, 25.2, 25.0, 21.7, 20.3, 20.0, 20.0, 19.7, 19.4, 19.3, 19.3, 19.2, 18.6, 18.5, 18.4, 18.2, 17.5, 17.5, 13.6, 2.5, 1.5, 1.2, 0.8, 0.7, 0.5, 0.4, 0.3, 0.1, -0.0, -0.1, -1.3, -1.4, -3.1, -5.7, -5.8, -6.1.

^{31}P-NMR (241 MHz, *ACETONITRILE-D3*) δ -2.5.

HRMS (ESI): Calcd. for $C_{44}H_{57}N_{11}O_{14}PSSi^{+}$ 1054.3309 $[M+H]^{+}$; obsd.1054.3308.

(**25**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.76–8.62 (m, 5H), 8.18–7.98 (m, 10H), 7.64–7.54 (m, 9H), 6.29–6.23 (m, 1H), 6.20–6.14 (m, 5H), 5.95–5.86 (m, 2H), 5.67–5.59 (m, 2H), 5.33 (s, 1H), 5.24 (s, 1H), 4.95–4.90 (m, 2H), 4.53–4.34 (m, 17H), 3.93–3.84 (m, 6H), 3.80–3.75 (m, 3H), 3.48–3.29 (m, 20H), 2.97–2.90 (m, 6H), 2.77–2.67 (m, 4H), 2.19–1.95 (m, 23H), 1.29–1.12 (m, 24H).

^{13}C-NMR (149 MHz, *METHANOL-D3*) δ 180.5, 170.2, 170.1, 169.9, 169.8, 166.8, 164.8, 156.1, 152.2, 151.9, 149.9, 149.0, 148.6, 148.5, 143.8, 143.6, 139.1, 139.0, 133.6, 132.6, 128.4, 128.1, 120.8, 117.2, 86.5, 86.4, 85.2, 81.3, 81.1, 78.6, 78.3, 72.7, 72.5, 70.5, 70.5, 67.4, 67.2, 63.4, 63.3, 62.3, 61.6, 60.6, 60.2, 58.2, 58.0, 52.0, 51.7, 48.1, 48.0, 47.8, 47.7, 47.5, 47.4, 47.3, 35.6, 31.4, 22.4, 19.2, 18.9, 18.9, 18.8, 18.2, 18.1, 18.0, 17.9, 13.1.

^{31}P-NMR (241 MHz, *METHANOL-D3*) δ -0.4, -2.7, -2.8, -3.0.

HRMS (ESI): Calcd. for $C_{39}H_{45}N_{11}O_{14}PS^{+}$ 954.2600 $[M+H]^{+}$; obsd.954.3247.

General synthesis of 28, 29, and 30

A mixture of molecular sieve 4Å, **24** or **25** (1 eq.) and phosphoramidite (**26** or **27**, 1.25 eq.) was dissolved in a mixture of anhydrous acetonitrile and dichloroethane v/v = 1:8 (0.13 M solution) and stirred at room temperature under an Ar atmosphere for 15–60 minutes. *1H*-Tetrazole (4.5 eq.) was then added, and the reaction was allowed to continue for another hour. Afterward, *tert*-butyl hydroperoxide (TBHP, 1 M in toluene, 1.5 eq.) was added, and the reaction was allowed to proceed for 1.5–2 hours. The reaction mixture was extracted with ethyl acetate and H_2O, and the organic phase was washed with saturated $NaHCO_3$, followed by brine, dried over Na_2SO_4, and concentrated under reduced pressure to afford the crude product. The purification was conducted on a silica gel column to give the desired compound as a white solid. **28** was synthesized from **24** and **26** (38%), **29** was synthesized from **24** and **27** (76%), **30** was synthesized from **25** and **27** (25%).

(**28**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.66–8.54 (m, 3H), 8.11–8.07 (m, 3H), 7.90–7.85 (m, 2H), 7.66–7.24 (m, 21H), 7.00–6.80 (m, 4H), 6.18–5.91 (m, 5H), 5.65–5.55 (m, 1H), 5.22–4.95 (m, 3H), 4.72–4.62 (m, 2H), 4.54–4.38 (m, 4H), 4.28–4.01 (m, 5H), 3.89 (s, 1H), 3.70 (s, 3H), 2.82–2.68 (m, 6H), 2.14–2.09 (m, 3H), 2.05–2.01 (m, 3H), 1.20–1.15 (m, 7H), 0.97–0.91 (m, 11H), 0.84–0.75 (m, 12H), 0.26–0.19 (m, 6H), 0.07– -0.07 (m, 6H).

^{13}C-NMR (149 MHz, *METHANOL-D3*) δ 180.4, 170.1, 170.1, 169.8, 166.4, 163.2, 159.0, 157.0, 156.0, 151.9, 150.0, 149.0, 148.5, 146.7, 144.5, 143.7, 143.5, 143.4, 138.9, 138.8, 135.4, 133.7, 132.8, 132.6, 130.3, 128.5, 128.3, 128.1, 127.7, 126.9, 124.3, 120.8, 117.4, 117.3, 117.2, 117.1, 113.0, 97.7,

97.4, 87.4, 86.4, 86.3, 81.0, 79.0, 78.0, 77.6, 73.3, 72.6, 70.4, 70.3, 70.3, 68.2, 67.5, 67.4, 67.0, 63.4, 63.1, 58.3, 54.5, 49.7, 48.7, 48.2, 48.1, 48.0, 47.8, 47.7, 47.5, 47.4, 47.2, 44.3, 35.6, 25.0, 24.8, 19.3, 19.2, 19.0, 18.9, 18.8, 18.1, 18.0, 17.7, 17.4, 17.4, -5.6, -5.7, -5.8, -5.8, -6.0, -6.1.

^{31}P-NMR (241 MHz, *METHANOL-D3*) δ -1.7, -2.2, -2.2, -2.3, -3.0, -3.4.

HRMS (ESI): Calcd. for $C_{90}H_{108}N_{15}O_{23}P_2S_2Si_2^+$ 1948.6192 $[M+H]^+$; obsd.1948.6191.

(**29**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.71–8.52 (m, 9H), 8.13–8.05 (m, 8H), 7.98–7.92 (m, 11H), 7.67–7.58 (m, 8H), 7.56–7.44 (m, 32H), 7.38–7.31 (m, 34H), 7.29–7.20 (m, 6H), 6.93–6.87 (m, 19H), 6.20–6.14 (m, 7H), 6.11–6.05 (m, 2H), 5.96–5.90 (m, 2H), 5.65–5.63 (m, 1H), 5.62–5.60 (m, 1H), 5.19–5.15 (m, 1H), 5.13–5.04 (m, 6H), 4.62–4.57 (m, 1H), 4.55–4.44 (m, 10H), 4.37–4.22 (m, 14H), 4.21–4.15 (m, 6H), 4.10 (q, *J* = 7.2 Hz, 1H), 4.06–4.00 (m, 2H), 3.93–3.85 (m, 2H), 3.79–3.77 (m, 14H), 3.67–3.65 (m, 5H), 3.64–3.60 (m, 7H), 3.59–3.55 (m, 12H), 2.92–2.78 (m, 13H), 2.74–2.67 (m, 2H), 2.12 (dd, *J* = 7.8, 5.4 Hz, 7H), 2.04–2.01 (m, 10H), 1.33–1.16 (m, 24H), 0.90 (t, *J* = 7.2 Hz, 3H), 0.79–0.71 (m, 23H), 0.05– -0.04 (m, 7H), -0.12– -0.19 (m, 7H).

^{13}C-NMR (149 MHz, *METHANOL-D3*) δ 180.4, 180.1, 179.8, 179.6, 179.4, 170.2, 170.1, 169.8, 167.7, 167.6, 167.4, 166.7, 166.6, 163.4, 163.3, 159.1, 159.1, 159.0, 159.0, 158.8, 156.9, 156.8, 156.0, 152.0, 151.9, 150.1, 149.0, 148.5, 146.4, 146.2, 144.6, 144.4, 144.3, 143.8, 143.7, 143.6, 138.9, 138.8, 135.8, 135.6, 135.3, 135.3, 133.6, 133.3, 133.1, 132.8, 132.6, 130.9, 130.3, 130.2, 130.1, 130.1, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 127.9, 127.7, 127.6, 127.0, 127.0, 126.8, 120.8, 117.3, 117.2, 117.1, 117.0, 113.0, 112.9, 98.0, 97.9, 97.3, 87.6, 87.3, 87.3, 86.9, 86.4, 85.7, 85.6, 85.3, 85.2, 81.0, 72.9, 72.8, 72.7, 72.6, 71.7, 70.4, 70.4, 67.6, 67.4, 64.4, 64.0, 63.8, 63.4, 63.3, 63.3, 62.2, 62.0, 61.9, 61.7, 60.7, 60.6, 60.6, 60.5, 60.3, 60.2, 60.1, 58.1, 58.0, 57.9, 57.7, 57.6, 57.5, 57.1, 54.5, 54.5, 54.4, 50.7, 50.4, 50.3, 49.3, 49.2, 49.0, 48.2, 48.1, 48.0, 47.8, 47.7, 47.5, 47.4, 47.2, 35.6, 31.4, 24.7, 23.9, 22.4, 20.5, 20.5, 19.3, 19.2, 18.9, 18.9, 18.8, 18.8, 18.1, 18.1, 18.0, 17.4, 17.4, 13.1, 13.1, -5.9, -5.9, -6.0, -6.0, -6.3, -6.3.

^{31}P-NMR (159 MHz, *METHANOL-D3*) δ -2.2, -2.2, -2.4, -2.6, -2.7, -2.9, -3.0.

HRMS (ESI): Calcd. for $C_{85}H_{96}N_{15}O_{23}P_2S_2Si^+$ 1848.5484 $[M+H]^+$; obsd. 1848.5354.

(**30**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.68–8.64 (m, 3H), 8.57–8.54 (m, 2H), 8.10–8.05 (m, 5H), 7.96–7.92 (m, 5H), 7.68–7.61 (m, 5H), 7.58–7.46 (m, 15H), 7.37–7.31 (m, 15H), 7.28–7.21 (m, 2H), 6.92–6.88 (m, 9H), 6.30–6.26 (m, 1H), 6.25–6.17 (m, 5H), 5.96–5.93 (m, 1H), 5.88–5.85 (m, 1H), 5.66 (s, 1H), 5.63 (dd, *J* = 11.4, 5.4 Hz, 1H), 5.39–5.34 (m, 1H), 5.32 (s, 1H), 5.14–5.12 (m, 2H), 5.00–4.96 (m, 1H), 4.94–4.90 (m, 2H), 4.72–4.69 (m, 1H), 4.63–4.60 (m, 1H), 4.54–4.19 (m, 24H), 4.02–3.97 (m, 2H), 3.90–3.85 (m, 2H), 3.79–3.77 (m, 15H), 3.68–3.57 (m, 12H), 3.53–3.52 (m, 1H), 2.95–2.82 (m, 12H), 2.74–2.69 (m, 4H), 2.13–2.11 (m, 7H), 2.03–2.01 (m, 8H), 1.31–1.16 (m, 22H), 0.10 (s, 4H).

^{13}C-NMR (149 MHz, *METHANOL-D3*) δ 180.4, 170.2, 170.1, 169.8, 163.3, 159.1, 159.0, 156.8, 156.0, 151.9, 150.1, 150.0, 149.0, 148.8, 148.6, 146.2, 144.4, 144.0, 143.4, 138.9, 138.6, 135.3, 135.3, 133.6, 133.2, 132.8, 132.6, 132.3, 131.1, 130.9, 130.6, 130.3, 130.1, 128.5, 128.4, 128.3, 128.1, 127.9, 127.7, 126.9, 122.5, 121.1, 120.8, 120.6, 117.3, 116.9, 116.6, 113.0, 87.4, 86.1, 85.2, 84.2,

84.1, 84.0, 81.2, 81.1, 80.9, 77.2, 72.9, 72.8, 72.6, 70.4, 70.3, 67.8, 67.6, 67.4, 67.2, 63.5, 63.3, 63.2, 62.2, 62.0, 60.2, 60.2, 60.1, 58.3, 58.1, 58.0, 58.0, 57.9, 54.5, 49.1, 48.2, 48.1, 47.9, 47.8, 47.7, 47.5, 47.4, 47.2, 46.8, 35.6, 31.8, 31.7, 31.7, 30.4, 30.3, 30.2, 29.4, 29.2, 29.1, 28.8, 28.8, 28.7, 28.6, 23.9, 23.9, 23.7, 23.6, 23.5, 23.1, 22.8, 22.7, 22.6, 22.5, 22.4, 22.4, 21.9, 21.9, 19.5, 19.2, 19.2, 19.0, 18.9, 18.8, 18.1, 18.0, 13.2, 13.1, 13.0, 0.1.
31P-NMR (241 MHz, *METHANOL-D3*) δ -2.6, -2.7, -2.8, -3.0, -3.1
HRMS (ESI): Calcd. for $C_{80}H_{84}N_{15}O_{23}P_2S_2^+$ 1748.4776 $[M+H]^+$; obsd.1748.5812.

General synthesis of 31, 32, and 33

28, **29**, or **30** (1 eq.) was dissolved in dichloromethane (0.2 M solution), then trichloroacetic acid solution in dichloromethane (2 M solution, 10 eq.) was added dropwise under an ice-bath. The reaction mixture was warmed to room temperature and stirred for 5–15 minutes. The reaction was quenched with saturated $NaHCO_3$ solution, then extracted with ethyl acetate. The organic layer was washed with brine, then dried over Na_2SO_4, then concentrated. The crude was purified using a silica gel column, a white solid was obtained as the target compound. **31** was synthesized from **28** (quant.), **32** was synthesized from **29** (61%), **33** was synthesized from **30** (60%).

(**31**)

^{1}H-NMR (392 MHz, *METHANOL-D3*) δ 8.92 (q, *J* = 6.8 Hz, 2H), 8.71–8.58 (m, 4H), 8.15–8.06 (m, 6H), 7.90–7.83 (m, 4H), 7.74–7.39 (m, 21H), 6.19 (dd, *J* = 6.0, 2.4 Hz, 1H), 6.15–6.12 (m, 1H), 6.09 (d, *J* = 5.6 Hz, 1H), 6.05–6.00 (m, 2H), 5.97 (s, 1H), 5.96–5.85 (m, 2H), 5.66 (dd, *J* = 6.0, 3.6 Hz, 1H), 5.60–5.57 (m, 1H), 5.27–5.19 (m, 1H), 5.18–5.12 (m, 1H), 5.09–4.95 (m, 2H), 4.90 (s, 1H), 4.79–4.68 (m, 3H), 4.58–4.49 (m, 8H), 4.44–4.28 (m, 10H), 4.23–4.19 (m, 5H), 4.08–3.99 (m, 5H), 3.97–3.91 (m, 1H), 3.88–3.82 (m, 2H), 3.01–2.93 (m, 7H), 2.84 (dd, *J* = 13.2, 6.0 Hz, 2H), 2.76–2.68 (m, 2H), 2.16–2.11 (m, 7H), 2.06–2.01 (m, 7H), 1.73–1.64 (m, 6H), 1.49–1.29 (m, 23H), 1.21–1.16 (m, 13H), 1.00–0.90 (m, 39H), 0.82–0.77 (m, 19H), 0.25–0.19 (m, 12H), 0.07–0.01 (m, 6H), -0.06– -0.11 (m, 6H).
^{13}C-NMR (99 MHz, *METHANOL-D3*) δ 180.4, 170.2, 170.1, 169.8, 169.8, 168.0, 167.3, 166.5, 163.1, 156.0, 152.0, 151.9, 150.0, 149.0, 148.6, 148.5, 147.0, 143.6, 143.4, 139.0, 138.9, 133.6, 132.8, 132.6, 132.3, 131.1, 128.5, 128.4, 128.2, 128.1, 127.8, 124.3, 120.8, 117.3, 117.2, 117.0, 86.4, 86.4, 81.1, 81.0, 79.2, 78.4, 72.6, 70.4, 70.3, 67.8, 67.6, 67.5, 63.5, 63.5, 63.0, 50.5, 48.3, 48.1, 47.9, 47.7, 47.4, 47.2, 47.0, 38.8, 35.6, 30.3, 28.8, 25.0, 25.0, 24.8, 23.6, 22.7, 19.3, 19.2, 19.0, 18.9, 18.1, 18.0, 18.0, 17.6, 17.4, 13.1, 10.1, -5.7, -5.7, -5.8, -5.8, -5.9, -6.0, -6.2, -6.2, -6.3.
^{31}P-NMR (159 MHz, *METHANOL-D3*) δ -1.9, -2.1, -2.2, -2.9, -3.2.
HRMS (ESI): Calcd. for $C_{69}H_{90}N_{15}O_{21}P_2S_2Si_2^+$ 1646.4885 $[M+H]^+$; obsd.1646.4770.

(**32**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.83–8.77 (m, 2H), 8.74–8.69 (m, 3H), 8.66–8.63 (m, 1H), 8.16–8.13 (m, 2H), 8.10–8.07 (m, 5H), 7.95–7.91 (m, 4H), 7.66–7.59 (m, 7H), 7.56–7.49 (m, 13H), 6.28–6.25 (m, 2H), 6.22–6.15 (m, 3H), 6.10 (dd, *J* = 10.8, 6.0 Hz, 1H), 5.96–5.93 (m, 2H), 5.68–5.65 (m, 1H), 5.64–5.60 (m, 2H), 5.31–5.24 (1H), 5.24–5.16 (m, 1H), 5.15–5.05 (m, 5H), 4.75–4.72 (m, 2H), 4.60–4.55 (m, 6H), 4.51–4.45 (m, 6H), 4.42–4.30 (m, 13H), 4.12–4.05 (m, 4H), 3.99–3.95 (m, 2H), 3.91–3.86 (m, 2H), 3.82–3.79 (m, 2H), 3.64–3.60 (m, 6H), 3.00–2.95 (m, 8H), 2.89–2.84 (m, 2H),

2.75–2.70 (m, 2H), 2.14 (dd, *J* = 12.0, 3.6 Hz, 8H), 2.05–2.01 (m, 11H), 1.32–1.18 (m, 34H), 0.90 (t, *J* = 6.6 Hz, 10H), 0.79–0.71 (m, 24H), 0.05 (d, *J* = 6.6 Hz, 2H), -0.00 (s, 4H), -0.12– -0.18 (m, 7H).
^{13}C-NMR (149 MHz, *METHANOL-D3*) *δ* 180.3, 170.2, 170.1, 169.8, 169.8, 167.6, 166.7, 163.4, 160.7, 157.1, 156.1, 152.2, 151.9, 150.1, 149.0, 149.0, 148.6, 146.6, 146.6, 143.7, 139.0, 138.8, 133.6, 132.8, 132.6, 128.8, 128.7, 128.7, 128.6, 128.4, 128.1, 128.0, 127.9, 127.7, 127.6, 127.4, 127.4, 127.3, 127.2, 127.1, 124.4, 124.3, 120.7, 117.3, 99.9, 97.8, 86.5, 86.3, 85.5, 80.8, 77.8, 77.8, 77.6, 77.1, 76.8, 72.7, 72.6, 72.5, 70.4, 67.9, 67.7, 67.6, 67.5, 67.3, 63.5, 63.0, 62.7, 61.0, 60.2, 58.1, 57.9, 51.5, 48.2, 48.1, 47.9, 47.8, 47.7, 47.5, 47.4, 47.2, 35.4, 31.3, 24.7, 22.3, 19.4, 18.8, 18.1, 17.3, 17.2, 12.9, -6.0, -6.3.
^{31}P-NMR (241 MHz, *METHANOL-D3*) *δ* 2.5, -2.1, -2.3, -2.4, -2.6, -2.7, -2.8.
HRMS (ESI): Calcd. for $C_{64}H_{78}N_{15}O_{21}P_2S_2Si^+$ 1546.4177 $[M+H]^+$; obsd.1546.4179.

(**33**)
^{1}H-NMR (594 MHz, *DMSO-D6*) *δ* 12.09 (s, 2H), 11.55 (s, 2H), 11.20 (s, 2H), 8.78–8.70 (m, 5H), 8.45 (s, 2H), 8.25–8.23 (m, 2H), 8.02–7.96 (m, 12H), 7.61–7.39 (m, 25H), 6.24–6.17 (m, 4H), 6.12–6.07 (m, 4H), 5.79–5.75 (m, 2H), 5.53–5.47 (m, 2H), 5.41–5.34 (m, 2H), 5.19 (s, 2H), 5.05 (s, 2H), 4.63 (s, 3H), 4.45–4.24 (m, 44H), 4.06–3.97 (m, 2H), 3.92–3.85 (m, 4H), 3.48–3.36 (m, 11H), 3.15–3.12 (m, 2H), 2.95–2.89 (m, 26H), 2.73 (s, 2H), 2.10–2.06 (m, 8H), 1.99–1.95 (m, 10H), 1.24–1.08 (m, 31H), 0.84–0.80 (m, 7H).
^{13}C-NMR (149 MHz, *METHANOL-D3*) *δ* 180.5, 170.1, 167.7, 166.7, 166.7, 166.0, 163.2, 160.0, 156.1, 155.3, 154.1, 152.1, 150.0, 149.0, 148.5, 138.9, 133.6, 132.9, 128.6, 128.2, 127.9, 117.4, 117.2, 117.1, 116.8, 98.1, 86.5, 86.1, 81.3, 80.9, 78.0, 77.8, 77.6, 70.5, 67.4, 63.4, 63.3, 58.5, 58.5, 58.3, 51.4, 35.7, 29.5, 28.8, 23.7, 19.5, 19.1, 18.4, 18.3, 13.5.
^{31}P-NMR (241 MHz, *METHANOL-D3*) *δ* -2.5, -2.7, -2.8.
HRMS (ESI): Calcd. for $C_{59}H_{66}N_{15}O_{21}P_2S_2^+$ 1446.3469 $[M+H]^+$; obsd. 1446.3467.

General synthesis of 34, 35, and 36
A mixture of molecular sieve 4Å, **31**, **32,** or **33** (1 eq.) and bis (2-cyanoethyl)-*N, N*-diisopropylphosphoramidite (5 eq.) was dissolved in a mixture of anhydrous acetonitrile and dichloroethane v/v = 1:8 (0.13 M solution) and stirred at room temperature under an Ar atmosphere for 15–60 minutes. *1H*-Tetrazole (4.5 eq.) was then added, and the reaction continued for another hour. Afterward, tert-butyl hydroperoxide (1.5–2 eq.) was introduced, and the reaction proceeded for 2 hours. The reaction mixture was extracted with ethyl acetate and H_2O, and the organic phase was washed with saturated $NaHCO_3$, followed by brine, dried over Na_2SO_4, and concentrated under reduced pressure to afford the crude product. Then the reaction crude was dissolved in ethyl acetate and then suspended in hexane. The suspension was centrifuged, and the pellet was washed 3 times with hexane. The final pellet was dried to give **34**, **35,** or **36** as a white solid. **34** was synthesized from **31** (70%), **35** was synthesized from **32** (94%), **36** was synthesized from **33** (85%).

(**34**)
^{1}H-NMR (594 MHz, *METHANOL-D3*) *δ* 8.70–8.58 (m, 7H), 8.53 (s, 1H), 8.16–8.05 (m, 12H), 7.89 (d, *J*

= 7.8 Hz, 2H), 7.84 (dd, *J* = 13.6, 7.8 Hz, 4H), 7.78 (d, *J* = 7.8 Hz, 1H), 7.64–7.41 (m, 27H), 6.18 (q, *J* = 6.0 Hz, 2H), 6.14–6.11 (m, 2H), 6.07 (q, *J* = 5.4 Hz, 2H), 6.03 (d, *J* = 4.8 Hz, 1H), 5.98–5.93 (m, 5H), 5.91–5.84 (m, 4H), 5.67–5.61 (m, 2H), 5.59 (t, *J* = 4.2 Hz, 1H), 5.55–5.53 (m, 1H), 5.24 (s, 1H), 5.19–5.12 (m, 1H), 5.12–5.05 (m, 1H), 5.03–4.97 (m, 2H), 4.91 (s, 3H), 4.78–4.74 (m, 5H), 4.69–4.62 (m, 6H), 4.58–4.49 (m, 13H), 4.47–4.31 (m, 37H), 4.31–4.26 (m, 3H), 4.07–4.01 (m, 7H), 2.99–2.81 (m, 34H), 2.71–2.63 (m, 3H), 2.13–2.08 (m, 12H), 2.04–1.99 (m, 38H), 1.33–1.13 (m, 31H), 0.95–0.65 (m, 70H), 0.28–0.18 (m, 23H), 0.07– -0.09 (m, 12H).

^{13}C-NMR (149 MHz, *METHANOL-D3*) *δ* 180.3, 170.2, 170.1, 170.1, 170.1, 169.8, 169.8, 156.8, 156.1, 155.8, 152.0, 151.9, 150.1, 149.1, 149.0, 148.9, 148.6, 148.5, 148.5, 143.5, 138.8, 133.6, 132.8, 132.6, 128.4, 128.4, 128.3, 128.1, 127.8, 127.8, 124.3, 120.7, 117.4, 117.1, 117.0, 116.8, 97.5, 97.2, 86.2, 86.1, 81.2, 81.0, 80.8, 77.4, 77.0, 72.8, 72.6, 72.6, 72.5, 70.5, 70.4, 70.3, 70.2, 67.8, 67.8, 67.6, 67.5, 63.8, 63.7, 63.6, 63.6, 63.5, 63.5, 63.4, 63.0, 53.5, 48.1, 47.9, 47.8, 47.7, 47.5, 47.4, 47.2, 35.6, 25.5, 25.4, 25.3, 25.3, 25.2, 25.0, 24.9, 24.9, 24.8, 24.7, 24.6, 24.6, 24.5, 19.0, 18.9, 18.8, 18.8, 18.7, 18.2, 18.2, 18.1, 18.0, 18.0, 17.7, 17.6, 17.6, 17.5, -0.7, -0.7, -5.6, -5.7, -5.7, -5.8, -5.9, -5.9, -6.0, -6.1, -6.3.

^{31}P-NMR (241 MHz, *METHANOL-D3*) *δ* -1.6, -1.9, -2.2, -2.2, -2.6, -3.0.

HRMS (ESI): Calcd. for $C_{75}H_{97}N_{17}O_{24}P_3S_2Si_2^+$ 1832.5080 $[M+H]^+$; obsd.1832.5081.

(**35**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) *δ* 8.75–8.65 (m, 3H), 8.55–8.52 (m, 1H), 8.17–8.05 (m, 7H), 7.94–7.87 (m, 4H), 7.64–7.59 (m, 5H), 7.54–7.44 (m, 11H), 6.26–6.10 (m, 6H), 6.07–6.02 (m, 1H), 5.90 (d, *J* = 6.1 Hz, 2H), 5.68–5.58 (m, 2H), 5.28–5.01 (m, 5H), 4.77–4.70 (m, 3H), 4.60–4.29 (m, 39H), 4.11–4.01 (m, 7H), 3.67–3.60 (m, 7H), 2.96–2.67 (m, 31H), 2.15–2.08 (m, 9H), 2.04–1.99 (m, 12H), 1.36–1.16 (m, 37H), 0.90–0.86 (t, *J* = 6.8 Hz, 8H), 0.77–0.70 (m, 22H), 0.04– -0.06 (m, 7H), -0.14– -0.23 (m, 6H).

^{13}C-NMR (149 MHz, *METHANOL-D3*) *δ* 180.4, 169.9, 167.7, 166.7, 163.4, 156.8, 156.1, 152.1, 151.9, 150.1, 149.1, 148.8, 148.5, 146.5, 146.3, 143.9, 143.6, 138.9, 138.7, 133.6, 132.8, 132.6, 128.5, 128.2, 127.9, 124.4, 122.7, 120.8, 117.5, 98.1, 97.9, 86.3, 84.6, 84.5, 81.0, 79.5, 76.9, 72.9, 70.4, 67.6, 63.6, 63.4, 62.9, 58.3, 58.2, 48.1, 48.0, 47.8, 47.7, 47.6, 47.4, 47.3, 35.6, 34.0, 31.4, 29.4, 28.7, 24.7, 23.9, 22.9, 22.4, 19.3, 19.0, 18.1, 17.4, 13.1, 0.2, -5.8, -6.3.

^{31}P-NMR (241 MHz, *METHANOL-D3*) *δ* -2.2, -2.5.

HRMS (ESI): Calcd. for $C_{70}H_{85}N_{17}NaO_{24}P_3S_2Si^+$ 1754.6451 $[M+Na]^+$; obsd. 1754.3840.

(**36**)

^{1}H-NMR (594 MHz, *DMSO-D6*) *δ* 12.09 (s, 2H), 11.55 (s, 2H), 11.20 (s, 1H), 8.80–8.70 (m, 3H), 8.45 (s, 1H), 8.25–8.23 (m, 2H), 8.01 (d, *J* = 7.2 Hz, 4H), 7.96 (s, 5H), 7.65–7.57 (m, 5H), 7.53–7.46 (m, 8H), 7.39 (s, 1H), 6.24–6.17 (m, 2H), 6.12–6.07 (m, 4H), 5.79–5.75 (m, 2H), 5.52–5.46 (m, 2H), 5.40–5.34 (m, 2H), 5.19 (s, 2H), 5.05 (s, 2H), 4.63 (s, 2H), 4.45–4.24 (m, 35H), 4.05 (q, *J* = 5.1 Hz, 1H), 4.02–3.97 (m, 1H), 3.92–3.85 (m, 3H), 3.48–3.36 (m, 9H), 3.13 (d, *J* = 3.0 Hz, 2H), 2.95–2.89 (m, 20H), 2.73 (s, 2H), 2.10–2.06 (m, 6H), 1.99–1.95 (m, 8H), 1.24–1.08 (m, 24H), 0.85–0.80 (m, 5H).

^{13}C-NMR (149 MHz, *DMSO-D6*) *δ* 180.8, 170.0, 169.8, 167.9, 167.9, 166.2, 163.6, 155.3, 152.6, 152.2, 151.1, 149.2, 149.0, 139.6, 138.2, 133.8, 133.5, 133.4, 133.0, 129.0, 126.4, 121.0, 120.9,

118.7, 98.0, 84.9, 84.7, 83.8, 83.5, 83.5, 83.2, 83.0, 81.1, 76.8, 72.6, 70.6, 67.9, 67.3, 63.6, 63.4, 60.3, 58.9, 48.9, 40.5, 40.4, 40.2, 40.1, 39.9, 39.8, 39.6, 35.4, 31.5, 22.6, 21.4, 20.9, 20.7, 20.2, 19.6, 19.4, 14.6, 14.5.

^{31}P-NMR (241 MHz, *DMSO-D6*) δ -1.9, -2.2, -2.6.

HRMS (ESI): Calcd. for $C_{65}H_{73}N_{17}O_{24}P_3S_2^+$ 1654.4091 $[M+Na]^+$; obsd. 1654.3721.

General synthesis of 11 and 12

34 (48 μmol) or **35** (8.6 μmol) was dissolved in NH_4OH and MeOH (v/v = 1:1), and the reaction was incubated at 50 °C. Additional NH_4OH and methanol were added to achieve complete deprotection. The mixture was then concentrated to remove the solvent. Then the residue was dissolved in methanol (800 μL for **37**, 400 μL for **38**), and $Et_3N{\cdot}3HF$ (800 μL for **37**, 400 μL for **38**) and Et_3N (500 μL for **37**, 250 μL for **38**) were added. After reacting at 65 °C for 4–7 hours, the reaction was quenched by the addition of 1 M TEAA buffer (1 mL, pH 7.0). The crude was purified by HPLC preparation (column, YMC-Actus Triart C8, 250 x 20.0 mm I.D., S-5 μm, 12 nm; solvent A, 50 mM TEAA (pH 7.0) + 0.5% ACN; solvent B, ACN; B%: 0.01–25 min 5%–80%, 25.01–35.00 min 80%–100%, 35–35.01 min 100%–0%, 35.01–45.01 min 0%; flow rate, 10 mL/min). Fractions containing the target compound were collected and lyophilized, yielding a white solid (determined by NanoDrop, 14% for **11** from **37**, and 12% for **12** from **38**, over 2 steps).

(**11**)

^{1}H-NMR (400 MHz, D_2O) δ 8.34–8.28 (m, 2H), 7.90–7.85 (m, 1H), 7.65–7.60 (m, 1H), 5.64–5.56 (m, 3H), 5.50 (t, *J* = 4.0 Hz, 1H), 4.54–4.48 (m, 2H), 4.46–4.42 (m, 1H), 4.41–4.34 (m, 1H), 4.27 (q, *J* = 4.8 Hz, 1H), 4.17–4.04 (m, 7H), 3.97–3.91 (m, 1H), 3.76–3.63 (m, 2H), 3.14–3.10 (m, 1H), 3.01 (qd, *J* = 7.3, 4.3 Hz, 55H), 1.80 (td, *J* = 14.7, 4.3 Hz, 17H), 1.15–1.02 (m, 85H).

^{13}C-NMR (100 MHz, D_2O) δ 179.2, 163.1, 158.3, 154.8, 154.3, 153.6, 151.4, 150.9, 148.4, 143.8, 140.6, 136.7, 118.3, 116.0, 95.4, 87.9, 82.9, 82.8, 77.1, 76.7, 76.4, 76.1, 73.8, 69.3, 66.1, 64.7, 64.1, 63.9, 62.5, 49.0, 48.6, 48.4, 48.2, 48.0, 47.8, 47.5, 47.3, 46.6, 21.9, 8.2, 8.0.

^{31}P-NMR (160 MHz, D_2O) δ 0.6, -0.8, -1.0.

HRMS (Q-Tof): Calcd. for $C_{29}H_{37}N_{13}O_{19}P_3S_2^-$ 1028.0988 $[M-H]^-$; obsd. 1028.0975.

(**12**)

^{1}H-NMR (594 MHz, D_2O) δ 8.35 (s, 1H), 8.25 (d, *J* = 6.6 Hz, 1H), 7.92 (s, 1H), 7.61 (s, 1H), 5.81 (s, 1H), 5.60 (s, 1H), 5.57 (d, *J* =7.2, 1H), 5.55 (d, *J* =4.2, 1H), 4.82–4.76 (m, 1H), 4.54–4.49 (m, 2H), 4.37 (s, 1H), 4.25 (t, *J* = 4.8 Hz, 1H), 4.15–4.10 (m, 5H), 4.08–4.03 (m, 2H), 3.95 (d, *J* = 12.0 Hz, 1H), 3.85 (s, 1H), 3.79 (s, 1H), 3.64 (t, *J* = 3.0 Hz, 1H), 3.48 (s, 3H), 3.11–2.95 (m, 55H), 1.21–1.00 (m, 84H).

^{13}C-NMR (149 MHz, D_2O) δ 181.5, 165.4, 159.1, 157.7, 155.2, 154.1, 152.5, 150.9, 148.5, 143.2, 140.5, 136.7, 118.6, 116.4, 95.9, 88.0, 85.8, 82.8, 82.8, 77.0, 76.3, 75.2, 73.8, 69.2, 65.0, 64.6, 64.0, 63.3, 62.5, 58.3, 49.8, 49.0, 46.7, 46.5, 23.3, 8.3, 8.1.

^{31}P-NMR (241 MHz, D_2O) δ 4.1, -0.9.

HRMS (Q-Tof): Calcd. for $C_{30}H_{39}N_{13}O_{19}P_3S_2^-$ 1042.1145 $[M-H]^-$; obsd. 1042.1137.

Synthesis of 13

36 (9.3 μmol) was dissolved in 150 μL of NH_4OH and 150 μL of MeOH, and the reaction was incubated at 50 °C. Additional NH_4OH and methanol were added to achieve complete deprotection.

The mixture was then concentrated to remove the solvent. The crude was purified by HPLC preparation (column, YMC-Actus Triart C8, 250 x 20.0 mm I.D., S-5 µm, 12 nm; solvent A, 50 mM TEAA (pH 7.0) + 0.5% ACN; solvent B, ACN; B%, 0.01–25 min 5%–80%, 25.01–35.00 min 80%–100%, 35–35.01 min 100%–0%,35.01–45.01 min 0%; flow rate, 10 mL/min). Fractions containing the target compound were collected and lyophilized, yielding a white solid (18%, determined by NanoDrop).

^{1}H-NMR (591 MHz, D_2O) δ 8.37 (s, 1H), 8.26 (s, 1H), 7.94 (s, 1H), 7.67 (s, 1H), 5.90–5.80 (m, 2H), 5.66–5.50 (m, 2H), 4.40–3.98 (m, 14H), 3.79–3.63 (d, *J* = 59.5 Hz, 4H), 3.59–3.53 (m, 3H), 3.52–3.47 (m, 6H), 2.94–2.41 (m, 6H), 1.82–1.72 (m, 5H), 1.01–0.93 (m, 2H).

^{13}C-NMR (149 MHz, D_2O) δ 165.4, 158.6, 157.5, 155.2, 153.7, 152.6, 151.0, 148.6, 143.1, 140.4, 137.0, 118.5, 116.4, 100.1, 100.0, 100.0, 99.9, 95.7, 88.0, 85.9, 85.8, 82.9, 75.0, 74.9, 73.7, 69.4, 64.6, 64.3, 63.4, 60.0, 59.5, 58.3, 49.5, 46.7, 34.5, 34.4, 30.8, 23.3, 18.7, 10.6, 8.3.

^{31}P-NMR (241 MHz, D_2O) δ 2.5, -1.0.

HRMS (Q-Tof): Calcd. for $C_{31}H_{41}N_{13}O_{19}P_3S_2^-$ 1056.1301 $[M-H]^-$; obsd. 1056.1213

General procedure of synthesis of 14 to 19

Syntheses of trinucleotides were performed by ÄKTAoligosynt™ oligonucleotide synthesizer (Cytiva) on Primer Support 5G riboG 300 (Cytiva, 255 µmol scale synthesis). In the coupling steps, 150 mM 2'-*O*-Methyl Adenosine (n-bz) CED phosphoramidite in CH_3CN, 150 mM 5'-DMT-2'-TOM-ribo Adenosine (n-acetyl) OP in CH_3CN, 150 mM 5'-DMT-2'-TOM-ribo Cytidine (n-acetyl) OP in CH_3CN, 2'-*O*-Methyl Cytidine (n-acetyl) CED phosphoramidite in CH_3CN, 200 mM bis(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite in CH_3CN, and 0.25 M 5-(benzylthio)-*1H* tetrazole in CH_3CN (activator) were circulated through the column. A solution of 3% dichloroacetic acid in toluene was used as a detritylation reagent, and 0.05 M I_2 in pyridine/H_2O (9:1, v/v) for the oxidation, 0.1M ((dimethylamino-methylidene)amino)-3*H*-1,2,4-dithiazoline-3-thione in pyridine for thiolation, 10% Ac_2O in THF /pyridine (8:1, v/v) as Cap-A, 10% 1-methylimidazole in THF as Cap-B. After the last cycle of the synthesis, the solid support was treated by a 1:1 mixture of 28% ammonium hydroxide/40% methylamine aq. (1.00 mL/100 mg of dried solid support) at 65 °C for 1–2 hours to remove the protecting groups and cleave the nucleotides from the solid support. Remained ammonium salt was removed by rotary evaporation, then DMSO/triethylamine trihydrofluoride 1:1 v/v was added, then incubated under 65 °C for 6–7 hours, quenched with Na_2CO_3 solution, the crude was purified by C18 column (0.05 M TEAA buffer (pH 7.0) containing 0.5% CH_3CN as solvent A, and ACN as solvent B, in a linear gradient of 0–80% B from 0.00 min to 80.00 min, flow rate 6 mL/min., followed by ion exchange column using water as solvent A and 1.0 M TEAA buffer (pH 7.0) containing 10% CH_3CN as solvent B, in a linear gradient of 0–100% B from 0.00 min to 270.00 min, flow rate 6 mL/min. Fractions containing the desired compound was collected and then lyophilized. A white solid was obtained.

(**14**)

^{1}H-NMR (400 MHz, D_2O) δ 8.35 (d, *J* = 6.4 Hz, 2H), 7.92–7.85 (m, 4H), 7.69 (d, *J* = 7.6 Hz, 2H), 5.83–5.74 (m, 6H), 5.64–5.60 (m, 2H), 4.59–4.50 (m, 7H), 4.48–4.44 (m, 1H), 4.36–4.13 (m, 8H), 4.08–3.91 (m, 17H), 3.17–3.06 (m, 4H), 2.94 (q, *J* = 7.4 Hz, 168H), 2.84–2.70 (m, 11H), 1.13–1.01 (m,

267H), 0.73–0.64 (m, 5H)
^{13}C-NMR (101 MHz, D_2O) δ 179.4, 165.0, 165.0, 158.4, 156.3, 155.1, 153.8, 152.5, 151.5, 151.4, 148.9, 148.8, 141.5, 139.7, 139.6, 139.6, 137.5, 137.5, 137.4, 137.3, 118.3, 116.1, 116.0, 96.2, 88.7, 88.6, 88.5, 87.5, 87.4, 87.2, 87.0, 87.0, 86.9, 83.6, 83.5, 83.4, 82.9, 82.8, 82.6, 82.5, 82.5, 75.2, 75.1, 74.4, 73.8, 73.6, 73.4, 73.2, 70.4, 70.4, 65.4, 65.2, 65.1, 64.2, 64.1, 54.9, 48.6, 48.5, 48.4, 48.2, 47.9, 47.7, 47.5, 47.3, 46.9, 46.8, 46.6, 46.4, 46.3, 46.1, 42.6, 42.1, 39.7, 39.1, 34.8, 28.8, 25.5, 22.8, 22.6, 19.1, 19.1, 19.0, 18.2, 14.7, 12.7, 11.8, 10.6, 10.5, 10.5, 10.2, 8.7, 8.3, 8.1, 8.0, 7.2
^{31}P-NMR (162 MHz, D_2O) δ 56.8, 56.6, 0.5
HRMS (ESI): Calcd. for $C_{29}H_{37}N_{13}O_{19}P_3S_2^-$ 1028.0988 [M-H]$^-$; obsd. 1028.1092.

(**15**)
^{1}H-NMR (400 MHz, D_2O) δ 8.35–8.32 (m, 2H), 7.95–7.75 (m, 6H), 5.90–5.74 (m, 6H), 5.68–5.60 (m, 2H), 4.48–4.28 (m, 7H), 4.22–3.94 (m, 18H), 3.32–3.29 (m, 6H), 3.04–2.90 (m, 90H), 1.09 (t, J = 7.2 Hz, 146H), 0.80–0.71 (m, 4H)
^{13}C-NMR (101 MHz, D_2O) δ 179.1, 164.5, 164.4, 158.3, 158.2, 155.4, 154.8, 153.7, 152.3, 151.4, 151.3, 151.2, 148.7, 148.6, 141.4, 139.6, 137.4, 137.4, 137.3, 137.2, 118.2, 115.9, 115.8, 96.1, 87.4, 87.3, 87.1, 87.0, 86.9, 83.4, 83.4, 82.8, 82.7, 82.6, 82.4, 82.4, 82.3, 82.3, 81.5, 75.0, 75.0, 73.8, 73.7, 72.5, 70.4, 65.4, 65.4, 65.2, 65.2, 65.1, 63.7, 63.7, 57.8, 54.8, 48.4, 46.7, 46.4, 46.0, 42.5, 42.0, 39.7, 39.1, 28.7, 25.5, 22.7, 22.5, 19.2, 19.1, 19.0, 18.2, 14.8, 12.8, 10.7, 10.6, 10.3, 8.8, 8.7, 8.6, 8.2, 8.0, 7.8
^{31}P-NMR (162 MHz, D_2O) δ 56.6, 56.0, 0.4
HRMS (ESI): Calcd. for $C_{30}H_{39}N_{13}O_{19}P_3S_2^-$ 1042.1145 [M-H]$^-$; obsd. 1042.1146.

(**16**)
^{1}H-NMR (400 MHz, D_2O) δ 8.35–8.29 (m, 2H), 7.91–7.80 (m, 4H), 7.75–7.71 (m, 2H), 5.91–5.88 (m, 2H), 5.83–5.77 (m, 2H), 5.73–5.70 (m, 2H), 5.66–5.58 (m, 2H), 4.92–4.85 (m, 3H), 4.55–4.44 (m, 3H), 4.35–4.20 (m, 8H), 4.11–3.86 (m, 18H), 3.27–3.15 (m, 13H), 3.00–2.91 (m, 87H), 1.10–1.01 (m, 131H), 0.85–0.80 (m, 10H).
^{13}C-NMR (101 MHz, D_2O) δ 181.2, 179.6, 175.0, 170.4, 164.5, 164.5, 158.4, 158.4, 158.3, 155.3, 154.8, 154.7, 153.7, 153.6, 153.6, 152.2, 151.4, 151.3, 151.2, 151.0, 148.7, 148.6, 148.5, 141.5, 141.4, 139.5, 139.4, 137.7, 137.6, 137.6, 137.3, 137.3, 133.1, 131.9, 128.6, 126.7, 118.3, 118.2, 118.2, 117.4, 116.3, 116.1, 116.1, 116.0, 96.2, 96.0, 87.9, 87.5, 87.3, 87.2, 87.0, 85.8, 85.5, 85.4, 83.4, 83.3, 83.2, 82.9, 82.8, 82.4, 82.3, 82.2, 82.2, 82.0, 81.6, 81.5, 73.8, 73.6, 73.5, 73.2, 70.5, 70.4, 70.3, 65.4, 65.3, 65.1, 64.8, 63.7, 61.5, 58.2, 58.2, 58.1, 58.1, 57.8, 51.1, 46.8, 46.7, 46.5, 46.3, 44.0, 34.9, 32.9, 31.1, 26.4, 25.8, 25.8, 22.5, 18.7, 18.7, 14.7, 14.4, 8.4, 8.2, 8.0
^{31}P-NMR (162 MHz, D_2O) δ 56.8, 56.4, 56.0, 0.5
HRMS (ESI): Calcd. for $C_{31}H_{41}N_{13}O_{19}P_3S_2^-$ 1056.1301 [M-H]$^-$; obsd. 1056.1329.

(**17**)
^{1}H-NMR (400 MHz, CD_3OD) δ 8.58 (s, 1H), 8.18 (s, 1H), 8.02 (d, J = 7.1 Hz, 2H), 6.12 (t, J = 7.0 Hz, 2H), 5.96 (d, J = 7.5 Hz, 1H), 5.87 (d, J = 6.1 Hz, 1H), 4.91 (d, J = 6.5 Hz, 1H), 4.86–4.77 (m, 3H), 4.69 (s, 1H), 4.45 (s, 2H), 4.36 (s, 2H), 4.24 (s, 2H), 4.18–4.11 (m, 4H), 3.09 (q, J = 7.5 Hz, 24H), 1.21 (t, J =

7.5 Hz, 36H).
^{13}C-NMR (101 MHz, *CD_3OD*) *δ* 167.51, 159.97, 158.77, 157.19, 155.64, 154.10, 153.34, 151.15, 143.00, 140.96, 138.38, 119.98, 117.69, 96.97, 89.76, 89.15, 88.33, 85.41, 84.97, 84.32, 76.90, 76.14, 75.18, 75.08, 72.33, 66.78, 65.64, 59.92, 47.30, 9.17.
^{31}P-NMR (162 MHz, *CD_3OD*) *δ* 1.30, 0.24, 0.09.
HRMS (ESI): Calcd. for $C_{29}H_{37}N_{13}O_{21}P_3^-$, 996.1445 [M-H]$^-$; obsd. 996.1454.

(**18**)
^{1}H-NMR (400 MHz, *CD_3OD*) *δ* 8.64 (s, 1H), 8.18 (s, 1H), 8.08–8.01 (m, 2H), 6.13 (t, *J* = 5.9 Hz, 2H), 5.98 (d, *J* = 7.5 Hz, 1H), 5.88 (d, *J* = 5.8 Hz, 1H), 4.96 (t, *J* = 5.4 Hz, 1H), 4.87–4.76 (m, 3H), 4.45 (d, *J* = 7.5 Hz, 2H), 4.36 (s, 1H), 4.29–4.10 (m, 7H), 4.03–3.96 (m, 1H), 3.26 (s, 3H), 3.10 (q, *J* = 7.3 Hz, 18H), 1.22 (t, *J* = 7.3 Hz, 27H).
^{13}C-NMR (101 MHz, *CD_3OD*) *δ* 167.52, 159.89, 158.47, 157.20, 155.62, 154.14, 153.34, 151.17, 142.78, 140.88, 138.27, 119.88, 117.69, 96.98, 89.09, 88.37, 88.00, 85.48, 85.07, 84.51, 83.45, 77.02, 75.09, 74.03, 72.28, 66.81, 65.36, 59.83, 58.59, 47.31, 9.15.
^{31}P-NMR (162 MHz, *CD_3OD*) *δ* 1.22, 0.25, -0.53.
HRMS (ESI):Calcd. for $C_{30}H_{39}N_{13}O_{21}P_3^-$, 1,010.1602 [M-H]$^-$; obsd. 1,010.1628.

(**19**)
^{1}H-NMR (600 MHz, *D_2O*) *δ* 8.32 (s, 1H), 8.04 (d, *J* = 1.2 Hz, 1H), 7.87 (d, *J* = 7.6 Hz, 2H), 6.12 (d, *J* = 3.7 Hz, 1H), 5.89 (d, *J* = 7.6 Hz, 1H), 5.82 (d, *J* = 3.1 Hz, 1H), 5.76 (d, *J* = 5.4 Hz, 1H), 4.87 (dt, *J* = 7.9, 5.2 Hz, 1H), 4.67 (t, *J* = 5.4 Hz, 1H), 4.60 (q, *J* = 7.6 Hz, 1H), 4.42 (dt, *J* = 6.0, 3.7 Hz, 2H), 4.30 (dt, *J* = 5.9, 3.4 Hz, 3H), 4.21–4.10 (m, 5H), 4.09–4.02 (m, 2H), 3.53 (d, *J* = 0.8 Hz, 3H), 3.45 (s, 3H), 3.31 (q, *J* = 7.2 Hz, 2.5H), 3.15 (qd, *J* = 7.3, 0.8 Hz, 21.5H), 1.23 (td, *J* = 7.3, 0.9 Hz, 36H).
^{13}C-NMR (151 MHz, *D_2O*) *δ* 165.36, 158.50, 156.41, 155.14, 153.60, 152.67, 151.33, 148.30, 140.90, 138.60, 137.62, 118.45, 116.29, 95.96, 87.81, 87.40, 85.69, 83.50, 83.44, 82.15, 81.62, 73.29, 71.78, 71.22, 70.06, 64.87, 63.94, 63.02, 58.89, 58.00, 57.58, 46.68, 8.26.
^{31}P-NMR (243 MHz, *D_2O*) *δ* 0.94, -0.44, -0.71.
HRMS (ESI): Calcd. for $C_{31}H_{41}N_{13}O_{21}P_3^-$, 1,024.16583 [M-H]$^-$; obsd. 1,024.17943.

General procedure of synthesis of PureCap analogs (1 to 9)
The capping reagent (**10**) was synthesized following the reported procedure. To a 0.05 M trinucleotide (**11** to **19**) DMSO solution, capping reagent (**10,** 2–10 eq.) and $ZnCl_2$ (20–30 eq.) were added. The reaction mixture was stirred at 37 °C for 48–66 hours before being quenched by 500 mM EDTA/NaOH solution (pH 8.0, 25–60 eq.), then diluted with water. The crude was purified by HPLC preparation (column, YMC-Actus Triart C8, 250 x 20.0 mm I.D., S-5 µm, 12 nm; solvent A, 50 mM TEAA (pH 6.0) + 0.5% ACN, solvent B, ACN; B%, 0.01–25 min 5%–80%,25.01–35.00 min 80%–100%, 35–35.01 min 100%–0%,35.01–45.01 min 0%; flow rate, 10 mL/min). The fractions containing the target chemical compounds were collected, and the solvent was removed via rotary evaporation and lyophilization to give PureCap analogs in the form of triethylamine salts. The compound was dissolved in 1–2 mL of methanol and then suspended in 190 mM sodium perchlorate in acetone. The suspension was centrifuged at 4000 rpm for 5 minutes, and the

resulting pellet was washed with acetone three more times. The supernatant was discarded, and the pellet was vacuum-dried, resulting in the desired PureCap analogs as sodium salts. The NMR was conducted using either triethylamine salt or sodium salt. The yield was calculated using absorbance at 260 nm by NanoDrop as a sodium salt (25% for **1**, 16% for **2**, 30% for **3**, 7.6% for **4**, 19% for **5**, 17% for **6**, 22% for **7**, 26% for **8**, 17% for **9**). The purity of the final cap analogs was confirmed by LC-MS.

(**1**)

^{1}H-NMR (594 MHz, D_2O) δ 8.36 (s, 1H), 8.04–8.03 (m, 1H), 7.97–7.91 (m, 1H), 7.66–7.57 (m, 2H), 7.49–7.33 (m, 3H), 5.69–5.67 (m, 2H), 5.63–5.57 (m, 4H), 4.91 (s, 1H), 4.84–4.82 (m, 1H), 4.62–4.50 (m, 3H), 4.45–4.38 (m, 3H), 4.35–4.34 (m, 1H), 4.29–4.23 (m, 3H), 4.20–4.09 (m, 12H), 3.98–3.95 (m, 3H), 3.90 (s, 2H), 3.78–3.71 (m, 3H), 3.14–3.10 (m, 1H), 3.01 (q, J = 7.2 Hz, 125H), 2.92–2.87 (m, 1H), 2.83–2.80 (m, 1H), 2.76–2.70 (m, 1H), 1.11 (t, J = 7.2 Hz, 179H), 0.58–0.55 (m, 5H), 0.48 (s, 4H), 0.39 (s, 3H).

^{13}C-NMR (149 MHz, D_2O) δ 181.5, 177.1, 165.4, 155.2, 152.5, 151.0, 149.2, 148.0, 142.9, 142.6, 140.5, 140.4, 136.3, 133.9, 131.9, 129.9, 123.8, 123.4, 118.5, 116.7, 108.7, 93.8, 87.7, 86.9, 82.9, 80.9, 80.2, 79.2, 77.1, 77.0, 76.9, 73.7, 69.4, 65.3, 65.1, 65.1, 65.0, 64.2, 62.2, 59.0, 49.1, 46.6, 36.5, 36.2, 35.9, 35.6, 25.0, 24.8, 24.8, 24.6, 24.6, 23.3, 8.3.

^{31}P-NMR (241 MHz, D_2O) δ -0.7, -0.9, -11.1, -11.2, -22.4.

HRMS (Q-Tof): Calcd. for $C_{52}H_{66}N_{19}O_{32}P_5S_2^{2-}$ 843.6131 $[M-2H]^{2-}$; obsd. 843.6503.

(**2**)

^{1}H-NMR (400 MHz, *METHANOL-D3*) δ 8.58 (s, 2H), 8.30 (d, J = 6.8 Hz, 2H), 8.12 (s, 2H), 7.93 (s, 1H), 7.75–7.72 (m, 1H), 7.67–7.63 (m, 3H), 7.59–7.55 (m, 1H), 7.49–7.45 (m, 1H), 7.37 (t, J = 8.0 Hz, 1H), 6.10 (s, 1H), 6.05–6.03 (m, 1H), 5.96 (d, J = 6.4 Hz, 2H), 5.87 (s, 1H), 5.78 (d, J =6.0 Hz, 2H), 5.20–5.10 (m, 2H), 4.57 (t, J = 5.4 Hz, 5H), 4.44 (s, 2H), 4.40–4.28 (m, 7H), 4.11 (t, J = 6.1 Hz, 7H), 3.87–3.80 (m, 4H), 3.46 (d, J = 22.0 Hz, 6H), 3.15 (q, J = 7.6 Hz, 48H), 1.27 (t, J = 7.6 Hz, 76H), 0.83–0.71 (m, 17H).

^{13}C-NMR (150 MHz, *METHANOL-D3*) δ 162.1, 158.4, 155.7, 154.4, 153.8, 151.6, 149.2, 140.8, 136.9, 133.2, 132.3, 132.1, 130.3, 130.1, 128.7, 128.3, 123.7, 123.4, 120.6, 118.7, 117.8, 116.3, 107.7, 96.0, 88.6, 87.6, 85.4, 84.6, 83.9, 79.6, 77.4, 75.0, 73.7, 70.5, 68.5, 66.1, 64.6, 64.0, 62.8, 62.1, 57.9, 49.9, 49.9, 49.7, 48.3, 48.1, 48.0, 47.8, 47.7, 47.6, 47.4, 46.5, 36.3, 36.1, 35.9, 25.0, 8.1.

^{31}P-NMR (160 MHz, METHANOL-D3) δ -0.1, -1.0, -10.4, -10.5, -20.9.

HRMS (Q-Tof): Calcd. for $C_{53}H_{68}N_{19}O_{32}P_5S_2^{2-}$ 850.6209 $[M-2H]^{2-}$; obsd. 850.6349.

(**3**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.62 (s, 1H), 8.16–8.12 (m, 2H), 7.88 (s, 1H), 7.74–7.60 (m, 7H), 7.59–7.55 (m, 1H), 7.48–7.42 (m, 1H), 7.36 (s, 1H), 6.16–6.28 (1H), 6.10–6.02 (m, 4H), 5.79–5.93 (1H), 5.77 (s, 2H), 5.22–5.17 (m, 2H), 5.06–4.94 (m, 3H), 4.69–4.55 (m, 8H), 4.41–4.09 (m, 33H), 3.92–3.80 (m, 4H), 3.53–3.48 (m, 12H), 3.14 (q, J = 6.4 Hz, 56H), 3.05 (s, 2H), 1.26 (t, J = 6.4 Hz, 98H), 0.77–0.69 (m, 20H).

^{13}C-NMR (149 MHz, *ACETONITRILE-D3*) δ 156.2, 154.8, 152.8, 151.3, 148.6, 148.1, 146.5, 145.2,

140.1, 139.9, 139.8, 139.7, 138.1, 134.4, 134.4, 126.6, 126.3, 121.6, 116.2, 115.9, 114.2, 105.7, 95.5, 94.2, 92.4, 92.1, 86.3, 85.8, 83.3, 82.9, 81.1, 80.5, 78.2, 76.5, 72.4, 72.3, 72.2, 71.7, 67.1, 64.8, 62.3, 61.8, 60.5, 60.3, 57.2, 55.8, 55.7, 47.0, 43.2, 33.9, 33.8, 33.7, 23.0, -0.8, -1.3, -1.5, -1.6, -1.8, -1.9, -2.0, -2.2, -2.3, -2.3.

^{31}P-NMR (241 MHz, *ACETONITRILE-D3*) δ -3.3, -3.6, -12.6, -12.9, -23.7.

HRMS (Q-Tof): Calcd. for $C_{54}H_{70}N_{19}O_{32}P_5S_2^{2-}$ 857.6287 $[M-2H]^{2-}$; obsd. 857.6541.

(**4**)

^{1}H-NMR (594 MHz, *METHANOL-D3*) δ 8.17 (s, 2H), 8.12–8.06 (m, 1H), 8.06–7.98 (m, 1H), 7.73 (d, *J* = 7.8 Hz, 1H), 7.66–7.57 (m, 3H), 7.48–7.46 (m, 1H), 7.40–7.33 (m, 1H), 6.12–6.09 (m, 5H), 5.87–5.82 (m, 2H), 5.14–5.00 (m, 7H), 4.68–4.60 (m, 2H), 4.55–4.48 (m, 4H), 4.42–4.10 (m, 20H), 3.29–3.26 (m, 14H), 3.16 (q, *J* = 7.4 Hz, 363H), 1.35–1.27 (m, 575H), 0.70 (d, *J* = 14.6 Hz, 11H).

^{13}C-NMR (149 MHz, D_2O) δ 181.9, 164.1, 162.7, 156.8, 155.4, 152.7, 149.6, 148.0, 140.7, 139.4, 139.3, 136.4, 133.9, 132.5, 131.8, 129.9, 128.6, 127.6, 123.6, 123.4, 118.6, 117.1, 108.7, 96.3, 95.5, 87.5, 86.8, 79.2, 77.3, 73.9, 73.7, 73.2, 70.3, 70.0, 69.4, 65.2, 65.1, 64.8, 46.5, 36.5, 35.9, 24.8, 23.3, 8.5.

^{31}P-NMR (159 MHz, *METHANOL-D3*) δ 58.1, 57.9, 57.2, 57.1, -11.0, -21.8

HRMS (Q-Tof): Calcd. for $C_{52}H_{66}N_{19}O_{32}P_5S_2^{2-}$ 843.6131 $[M-2H]^{2-}$; obsd. 843.6151.

(**5**)

^{1}H-NMR (400 MHz, *METHANOL-D3*) δ 8.69–8.60 (m, 1H), 8.18–8.06 (m, 4H), 7.76–7.56 (m, 5H), 7.45–7.37 (m, 2H), 6.11 (s, 5H), 5.87–5.82 (m, 2H), 4.87–4.22 (m, 26H), 4.11 (d, *J* = 7.2 Hz, 7H), 3.47–3.31 (m, 3H), 3.16 (q, *J* = 7.2 Hz, 157H), 1.35–1.25 (m, 239H), 0.73 (s, 16H)

^{13}C-NMR (100 MHz, *METHANOL-D3*) δ 176.7, 165.8, 165.7, 158.4, 156.8, 156.2, 156.1, 155.7, 152.6, 152.5, 151.9, 150.6, 150.3, 149.9, 149.8, 149.7, 149.7, 149.6, 149.5, 149.3, 142.0, 140.1, 140.0, 137.2, 137.1, 137.0, 133.7, 133.3, 132.1, 131.9, 130.2, 130.1, 128.6, 128.3, 123.7, 123.5, 118.7, 118.6, 116.3, 116.2, 107.5, 96.4, 96.4, 96.3, 94.2, 94.1, 88.4, 87.7, 87.5, 87.5, 86.9, 86.8, 86.6, 84.2, 84.1, 83.8, 83.4, 83.3, 83.2, 82.1, 79.9, 79.7, 79.6, 74.5, 74.4, 74.3, 74.1, 74.0, 73.9, 71.3, 71.1, 68.2, 68.0, 65.6, 63.7, 63.6, 57.6, 48.4, 48.2, 47.9, 47.7, 47.5, 47.3, 47.1, 46.8, 46.8, 46.7, 46.3, 46.2, 46.0, 45.8, 38.9, 36.2, 35.9, 35.8, 31.1, 25.1, 21.7, 21.4, 21.1, 19.8, 12.8, 10.6, 8.8, 8.5, 8.0, 7.9, 7.7.

^{31}P-NMR (160 MHz, *METHANOL-D3*) δ 58.5, 58.3, 57.9, -11.1, -21.8.

HRMS (Q-Tof): Calcd. for $C_{53}H_{68}N_{19}O_{32}P_5S_2^{2-}$ 850.6209 $[M-2H]^{2-}$; obsd. 850.6247.

(**6**)

^{1}H-NMR (400 MHz, *METHANOL-D3*) δ 8.78–8.862 (m, 1H), 8.18–8.12 (m, 1H), 8.09–8.04 (m, 2H), 7.72 (d, *J* =8.0 Hz, 0H), 7.65–7.59 (m, 2H), 7.57–7.41 (m, 1H), 7.33 (t, *J* = 8.4 Hz, 1H), 6.21–6.09 (m, 4H), 5.85–5.81 (m, 2H), 5.23–5.13 (m, 3H), 5.06–4.98 (m, 4H), 4.70–4.59 (m, 7H), 4.55–4.43 (m, 3H), 4.30–4.13 (m, 18H), 3.48–3.44 (m, 3H), 3.37 (s, 4H), 3.29 (q, *J* = 1.6 Hz, 4H), 3.14 (q, *J* = 7.0 Hz, 79H), 1.33–1.25 (m, 136H), 0.71 (d, *J* = 7.4 Hz, 11H).

^{13}C-NMR (100 MHz, *METHANOL-D3*) δ 179.4, 166.2, 158.5, 157.7, 157.4, 157.3, 155.8, 154.1, 152.7, 151.9, 150.6, 149.9, 149.7, 149.5, 149.4, 141.7, 139.9, 137.7, 137.5, 133.8, 133.2, 132.2, 131.9, 130.2, 130.1, 128.6, 128.4, 128.1, 127.3, 126.9, 123.6, 123.4, 118.5, 116.5, 107.9, 96.5, 88.0, 87.8,

86.8, 84.5, 84.0, 82.9, 82.1, 80.3, 79.7, 74.1, 73.7, 73.6, 71.3, 71.2, 71.1, 68.3, 65.6, 63.7, 57.9, 57.8, 57.6, 48.4, 48.2, 48.0, 47.8, 47.6, 47.4, 47.1, 46.3, 36.3, 35.9, 35.9, 25.0, 24.9, 23.1, 8.1, 7.9.
^{31}P-NMR (159 MHz, *METHANOL-D3*) δ 58.1, 57.7, 57.5, -10.9, -11.0, -11.2, -22.0
HRMS (Q-Tof): Calcd. for $C_{54}H_{70}N_{19}O_{32}P_5S_2^{2-}$ 857.6287 $[M-2H]^{2-}$; obsd. 857.6326.

(**7**)
^{1}H-NMR (400 MHz, D_2O) δ 9.14–8.82 (m, 1H), 8.26 (s, 1H), 7.99 (s, 1H), 7.80 (s, 1.5H), 7.60–7.15 (m, 4.5H), 6.01 (s, 1H), 5.84 (s, 1H), 5.65 (s, 3H), 4.91–4.77 (m, 3H), 4.56–4.45 (m, 1H), 4.36–3.89 (m, 18H), 0.47 (d, *J* = 17.8 Hz, 9H).
^{13}C-NMR (101 MHz, D_2O) δ 162.35, 158.61, 155.46, 155.25, 154.69, 154.54, 153.70, 152.80, 150.37, 149.71, 149.47, 149.05, 148.60, 142.32, 136.36, 133.65, 132.63, 132.28, 130.08, 128.77, 128.13, 123.79, 123.32, 107.86, 107.53, 96.24, 94.83, 93.54, 88.55, 88.14, 87.84, 85.50, 83.36, 82.45, 82.15, 81.63, 80.46, 79.18, 78.83, 74.19, 73.51, 69.98, 68.63, 68.26, 64.90, 64.55, 36.36, 36.09, 35.69, 30.26, 24.81, 24.73.
^{31}P-NMR (162 MHz, D_2O) δ -0.11, -10.88, -22.13.
HRMS (Q-Tof) Calcd. for $C_{52}H_{66}N_{19}O_{34}P_5^{2-}$ 827.6359, $[M-2H]^{2-}$; obsd. 827.6461.

(**8**)
^{1}H-NMR (600 MHz, D_2O) δ 8.11 (d, *J* = 7.8 Hz, 1H), 7.72 (s, 2H), 7.66–7.54 (m, 2H), 7.52–7.37 (m, 3H), 7.34–7.16 (m, 1H), 5.95–5.69 (m, 4H), 5.66–5.59 (m, 1H), 5.52–5.40 (m, 1H), 4.96 (s, 2H), 4.45 (dd, *J* = 9.7, 4.9 Hz, 3H), 4.36–4.29 (m, 3H), 4.21–4.15 (m, 6H), 4.10–3.92 (m, 11H), 3.36 (2s, 3H), 0.55–0.48 (m, 9H).
^{13}C-NMR (151 MHz, D_2O) δ 165.68, 158.42, 156.49, 155.13, 153.58, 152.38, 151.12, 149.68, 149.48, 149.13, 148.79, 147.99, 140.18, 138.36, 137.24, 133.65, 132.68, 132.30, 129.99, 128.77, 128.18, 123.85, 123.48, 118.50, 116.16, 107.64, 96.35, 95.04, 88.47, 87.76, 87.31, 84.91, 83.25, 81.51, 79.62, 79.28, 77.48, 73.79, 73.62, 72.88, 71.46, 69.86, 68.46, 66.62, 64.63, 63.97, 57.56, 48.94, 36.32, 36.22, 35.80, 30.30, 24.92, 24.85.
^{31}P-NMR (243 MHz, D_2O) δ -0.36, -10.11 – -11.10, -21.92.
HRMS (Q-Tof) Calcd. for $C_{53}H_{68}N_{19}O_{34}P_5^{2-}$, 834.6438 $[M-2H]^{2-}$; obsd. 834.6457.

(**9**)
^{1}H-NMR (600 MHz, D_2O) δ 8.36 (s, 1H), 8.08 (s, 1H), 7.90 (s, 2H), 7.63 (d, *J* = 6.5 Hz, 0.5H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.49 (s, 1.5H), 7.37 (s, 0.5H), 7.31 (s, 0.5H), 6.12–6.05 (m, 2H), 6.01 (s, 0.5H), 5.83 (d, J = 4.3 Hz, 0.5H), 5.79–5.75 (m, 1H), 5.72 (s, 1H), 5.03–4.96 (m, 1H), 4.91 (s, 1H), 4.85 (d, *J* = 7.6 Hz, 1H), 4.71–4.68 (m, 2H), 4.57 (s, 1H), 4.47–4.42 (m, 3H), 4.36–4.28 (m, 6H), 4.20–4.04 (m, 11H), 3.48 (d, *J* = 2.6 Hz, 2.5H), 3.45–3.43 (m, 2.5H), 3.34 (d, *J* = 2.9 Hz, 1H), 0.61–0.54 (m, 9H).
^{13}C-NMR (151 MHz, D_2O) δ 163.29, 158.41, 155.35, 154.61, 153.64, 150.47, 149.74, 149.11, 148.78, 148.27, 141.81, 139.70, 132.61, 130.06, 128.22, 123.81, 123.37, 118.47, 116.13, 107.78, 107.53, 96.21, 87.92, 87.69, 86.94, 85.88, 83.42, 82.23, 81.53, 80.42, 79.01, 73.37, 72.17, 70.17, 68.10, 66.07, 65.01, 64.27, 57.98, 57.63, 48.92, 36.30, 36.14, 35.71, 30.28, 24.87, 24.79.
^{31}P-NMR (243 MHz, D_2O) δ -0.48, -10.80, -21.97.
HRMS (Q-Tof) Calcd. for $C_{54}H_{70}N_{19}O_{34}P_5^{2-}$, 841.6516 $[M-2H]^{2-}$; obsd. 841.6473.

DNA Template Preparation

The DNA templates were prepared by polymerase chain reaction (PCR) using 0.3 μM primers, 1 ng/μL pNL1.1 TK vector (Promega), 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 1× KOD-Plus-Neo PCR buffer, and 0.02 units/μL KOD-Plus-Neo polymerase (Toyobo). The cycling program was as follows: 95 °C for 2 min; then 30 cycles of 98 °C for 15 s, 55 °C for 15 s, and 72 °C for 15 s. PCR products were purified using the Wizard PCR Preps DNA Purification System, and the final DNA templates were verified by electrophoresis on a 1% agarose gel and imaged with a ChemiDoc™ MP imaging system.

IVT and mRNA Sample Preparation

The *in vitro* transcription (IVT) reaction was conducted in a solvent system of 10 ng/μL DNA, 40 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-KOH, pH 7.5), 8 mM $Mg(OAc)_2$, 2 mM spermidine, 5 mM dithiothreitol (DTT), 2 mM nucleoside triphosphate (NTPs), 1 mM PureCap analog, 0.002 U/μl Pyrophosphatase (house made), 10% T7 RNA polymerase (house made). The mixture was incubated at 37 °C for 2 hours, then added 2% DNase I (Takara) and incubated for another 15 minutes. The LiCl precipitation was performed. For yield calculation, the IVT reaction was performed at a 10 μL scale. IVT yield and capping efficiency were determined by integrating peak areas obtained from Shimadzu HPLC reverse-phase analysis and quantified using a standard calibration curve. 100–400 μL scale reaction was also performed for mRNA preparation under the same condition. The LiCl-precipitated crude mRNA was subjected to reverse-phase HPLC using a YMC-Triart Bio C4 column. The elution conditions were as follows: solvent A, 100 mM triethylammonium acetate (TEAA) (pH 7.0) + 5% acetonitrile; solvent B, 100 mM TEAA (pH 7.0) + 50% acetonitrile. A linear gradient from 10% to 30% B was applied over 20 minutes at 50 °C. Fractions containing capped mRNA were collected. To remove the hydrophobic tags introduced by the capping reagent, the collected fractions were irradiated under 365 nm UV light. The resulting deprotected mRNA was further purified by reverse-phase HPLC using the same gradient conditions for samples prepared for the *in vitro* experiments. Fractions containing the target mRNA were precipitated with isopropanol (iPrOH) and washed with 80% ethanol (EtOH) to obtain the final full-length, high-purity capped mRNA product. For samples prepared for *in vivo* experiments, LiCl precipitated IVT crude mRNA was first dissolved in buffer of 10 mM Tris-HCl (tris(hydroxymethyl)aminomethane, pH 7.4), 1 mM EDTA, 500 mM NaCl and was then subjected to poly(A) purification using a Thermo Fisher POROS dT25 column for poly(A) purification. The chromatography was carried out using the following solvents, solvent A, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 500 mM NaCl; solvent B, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA. Elution was performed at a flow rate of 1.0 mL/min with the following gradient: 0–10 min 100% A, 10–20 min 50% A, 20–30 min 0% A. Fractions containing poly(A)-tailed mRNA were collected for further purification. Next, the dT-purified fractions were further purified by reverse-phase HPLC, followed by photoirradiation as described above. The resulting mRNA was precipitated with iPrOH. No additional HPLC purification was performed.

mRNA with CleanCap AG (Cap 1) was also synthesized under the following conditions: 40 ng/μL DNA, 40 mM Tris-HCl (pH 8.0), 8 mM $Mg(OAc)_2$, 2 mM spermidine, 10 mM DTT, 5 mM NTPs, 4 mM

cap analogs, 0.002 U/μL Pyrophosphatase (house made), 10% T7 RNA polymerase (house made) at 37 °C for 1 hour, then added 2% DNase I and incubated for another 15 minutes. The LiCl precipitation and purification utilizing Monarch Total RNA Miniprep Kit were performed. The IVT yield was approximately 2.2 mg RNA per mL reaction after Monarch column purification.
The final product was confirmed by 5% denaturing urea polyacrylamide gel electrophoresis (dPAGE) analysis and imaged with a ChemiDoc™ MP imaging system.

dsRNA Amount Quantification

Double-stranded RNA (dsRNA) contamination in IVT products was evaluated by dot blot using an anti-dsRNA monoclonal antibody J2 (Jena Bioscience). Poly(I:C) (InvivoGen, tlrl-picw) at concentrations of 50, 5, 0.5, and 0.05 ng per 3 μL was used as a positive control.
Three microliters of each 500 ng RNA sample or Poly(I:C) solution was spotted onto a Biodyne B Nylon Membrane (0.45 μm) and air-dried for 20 min. The membrane was then blocked with 5% skim milk in TBS-T (Tris-buffered saline containing 0.05% Tween-20) for 1 h at room temperature, followed by incubation with anti-dsRNA antibody (clone J2) diluted 1:1000 in blocking buffer for 1 h. After washing with TBS-T, the membrane was incubated with HRP-conjugated anti-mouse IgG secondary antibody (Sigma, A9044) diluted 1:5000 for 1 h.
Chemiluminescent signals were developed using the SuperSignal™ West Femto substrate (Thermo Fisher Scientific) and visualized with a ChemiDoc™ MP imaging system.

Cell Transfection and Luciferase Assay

HeLa cells (RIKEN Cell Bank) were cultured in Dulbecco's Modified Eagle Medium (DMEM, WAKO) with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C and 5% CO_2. Cells were seeded in 96-well plates (0.5×10^4 cells/well) one day prior to transfection. For transfection, 5 ng mRNA and 0.15 μL Lipofectamine MessengerMAX (Invitrogen) were diluted in Opti-MEM (Thermo Fisher Scientific), incubated for 10 min to form complexes, and then added to the cells. After 6 h, 24 h, 48 h, or 72 h of incubation, cells were lysed using 1× Glo Lysis Buffer (Promega), and luciferase activity was measured using the Nano-Glo Luciferase Assay System (Promega) on a TriStar5 plate reader (Berthold Technologies).

Preparation and Characterization of mRNA-Loaded LNPs

Lipid nanoparticles (LNPs) encapsulating Nluc mRNA were prepared by a rapid mixing method. Briefly, NanoLuc (Nluc) mRNA (25 μg) was dissolved in 600 μL of citric acid buffer (10 mM, pH 4) in a 5 mL microtube. A mixture of 3.125 mM SM-102 (Selleck Chemicals), 0.625 mM DSPC (FUJIFILM), 2.4 mM cholesterol (Sigma-Aldrich) and 0.09375 mM DMG-PEG2000 (Sigma-Aldrich) in ethanol was prepared as the lipid solution. During vortex mixing, 200 μL of lipid solution was rapidly added to the 600 μL mRNA solution and mixed for 5–10 s. Subsequently, 1 mL of PBS was added dropwise under continuous vortex mixing for an additional 5–10 s to neutralize the formulation and facilitate particle formation.
The resulting LNP suspension was purified by ultrafiltration at 1500 × g, 25 °C, 30 min, using Amicon Ultra-4 100 kDa MWCO centrifugal filters (Merck Millipore). The retained LNPs were washed and collected in 300 μL of PBS. The concentration of encapsulated mRNA was determined using the

Quant-it RiboGreen Reagent (Thermo Fisher Scientific). The size of the LNPs was measured by dynamic light scattering method using a Zetasizer Pro (Malvern Panalytical).

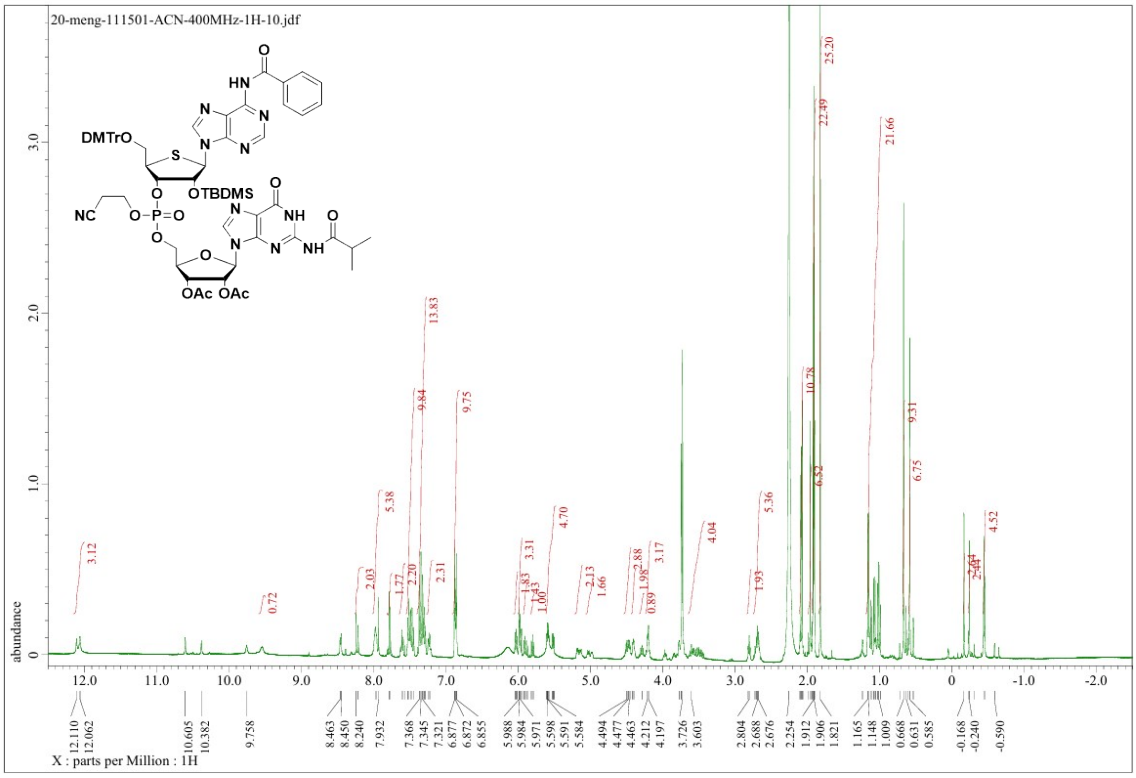
***In vivo* Luminescence Imaging**

Fluorofurimazine (FFz, Chemspace LLC) substrate solution was prepared by dissolving FFz powder in 50 µL DMSO as 86 mg/mL, followed by sequential addition of 400 µL PEG 300 (FUJIFILM), 50 µL polysorbate 80 (FUJIFILM), and 500 µL Milli-Q water, with vortex mixing at each step. The final concentration was 10 mM (4.3 mg/mL).

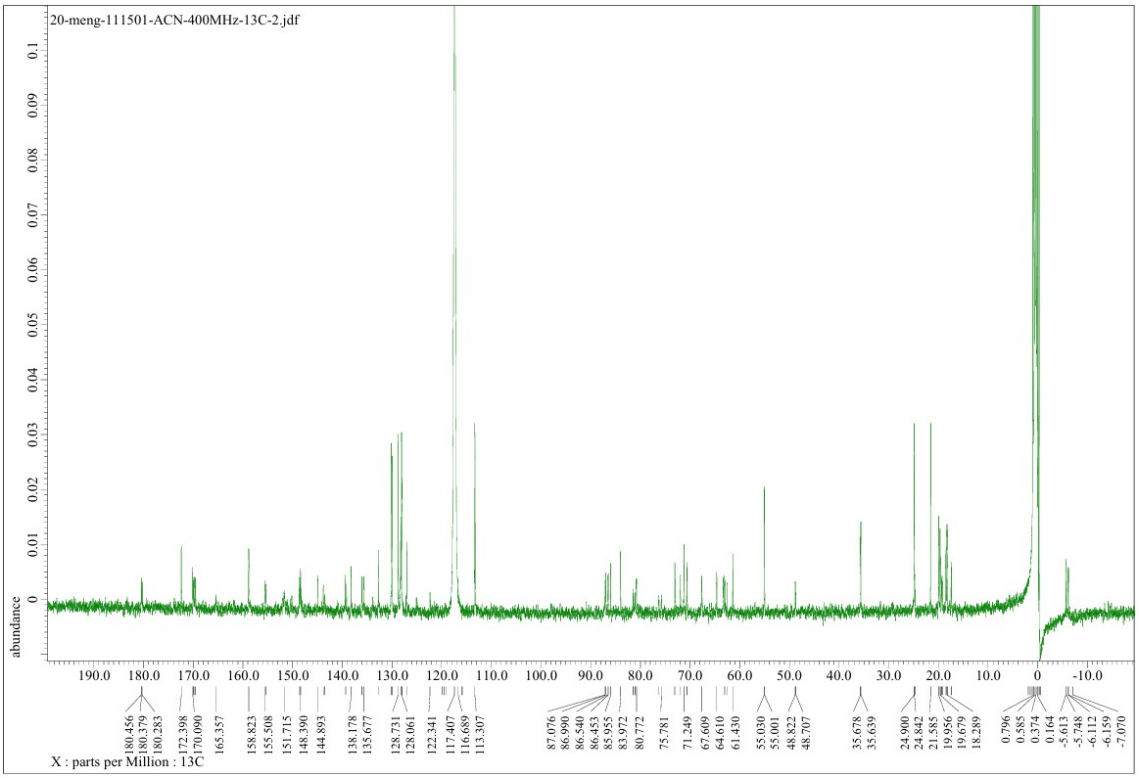
The mRNA LNPs were subcutaneously injected on the back of female ICR mice (4-weeks-old, n=5) at a dose of 2 µg mRNA (in 50 µL PBS) per mouse. Nluc luminescence imaging was performed at 6, 24, and 48 h post-injection using an IVIS Lumina LT system (PerkinElmer). For imaging, mice were anesthetized with isoflurane, and the FFz substrate solution (150 µL, 1.5 µmol) was injected intraperitoneally into each mouse at 5 min before imaging. The luminescent intensity was quantified using Living Image 4.0.

NMR Spectra of Synthesized Compounds

^{1}H-NMR chart of **22**



^{13}C-NMR chart of **22**



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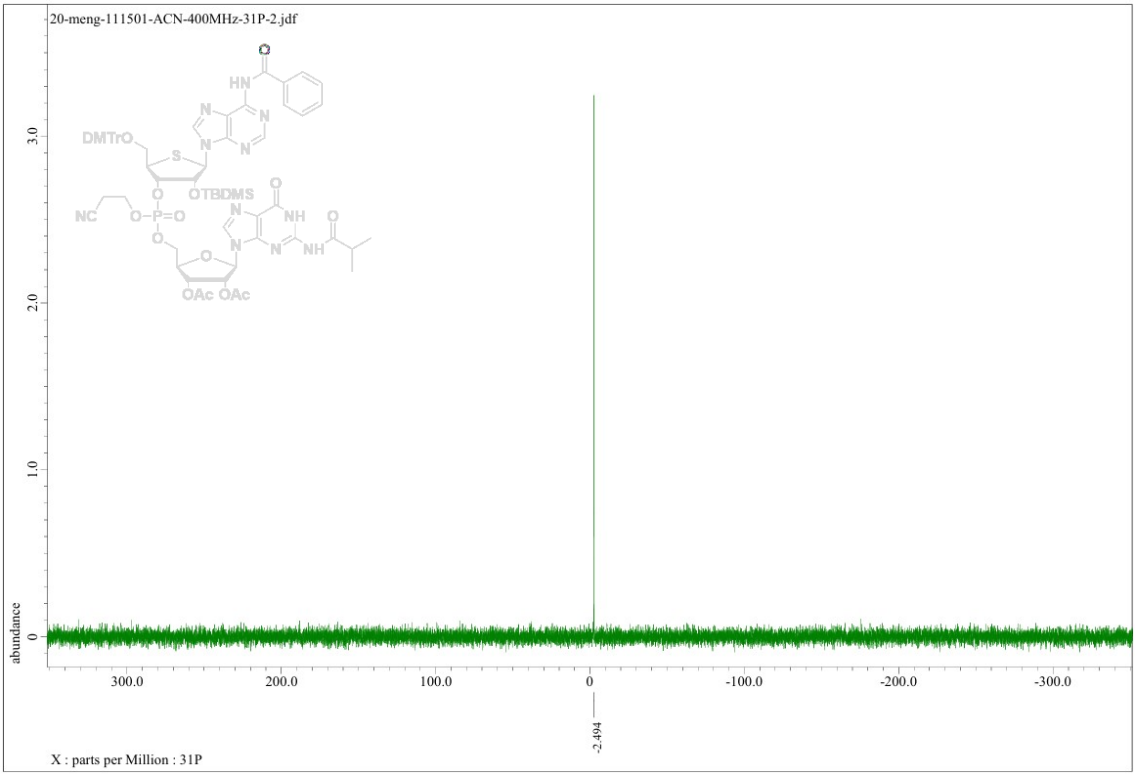

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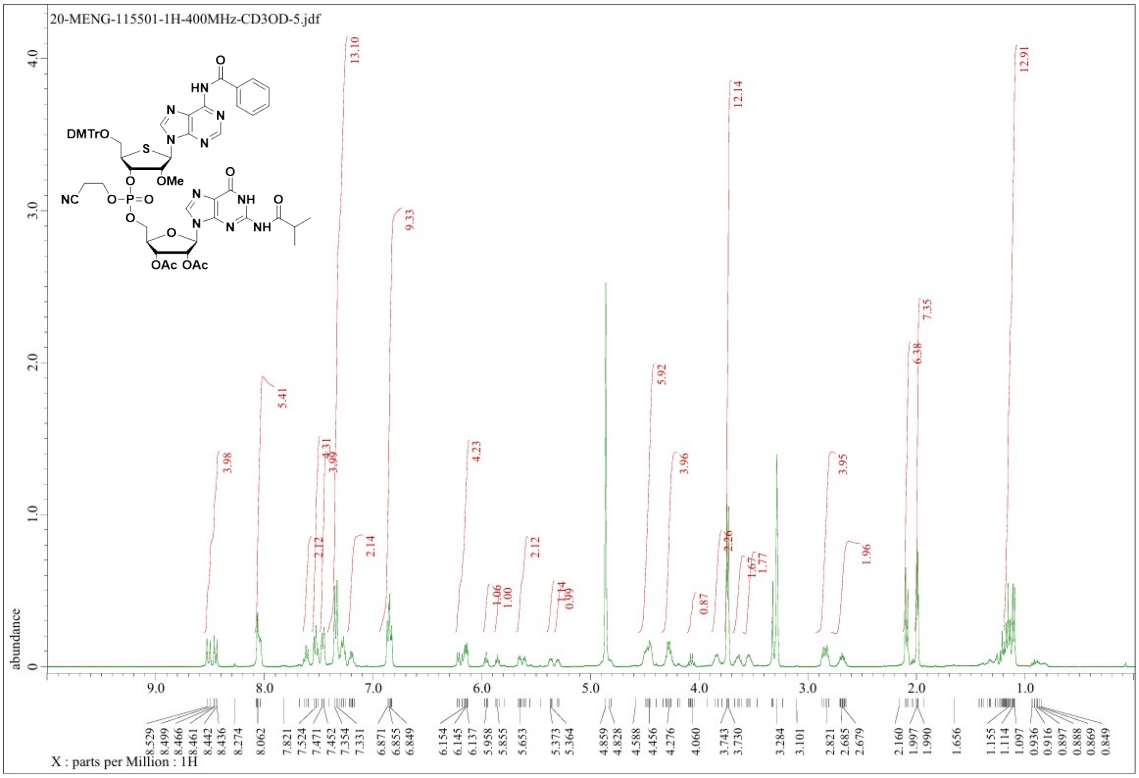

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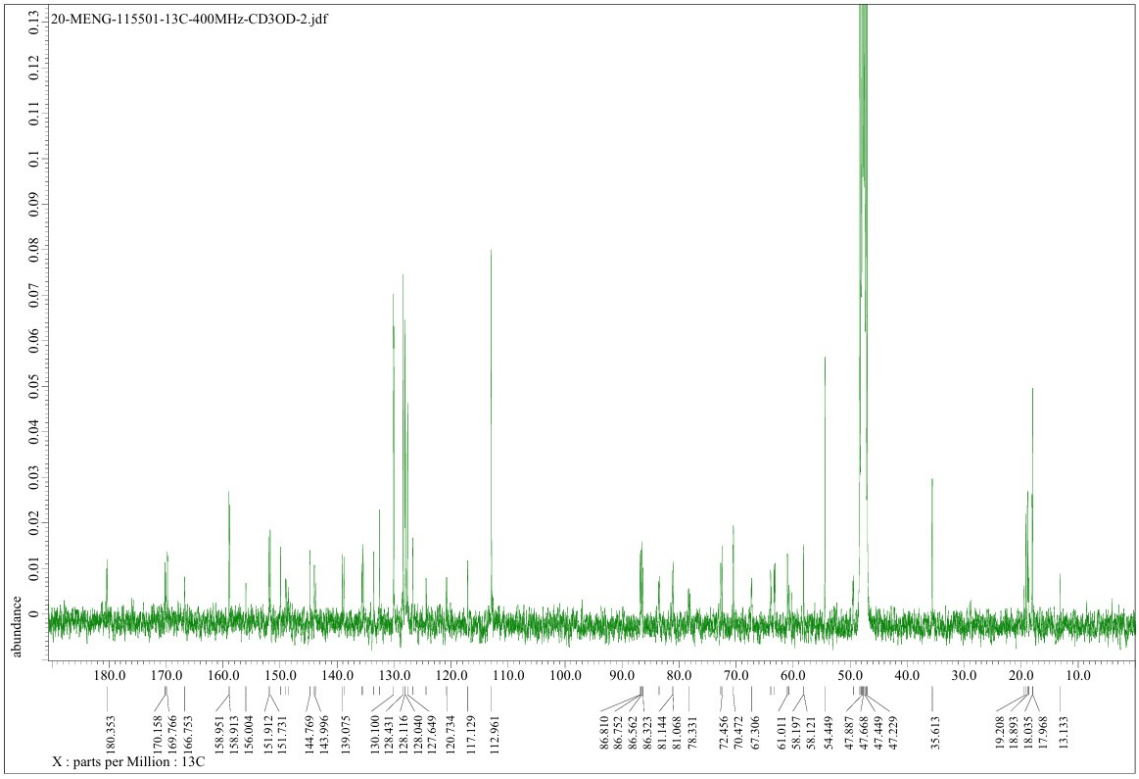

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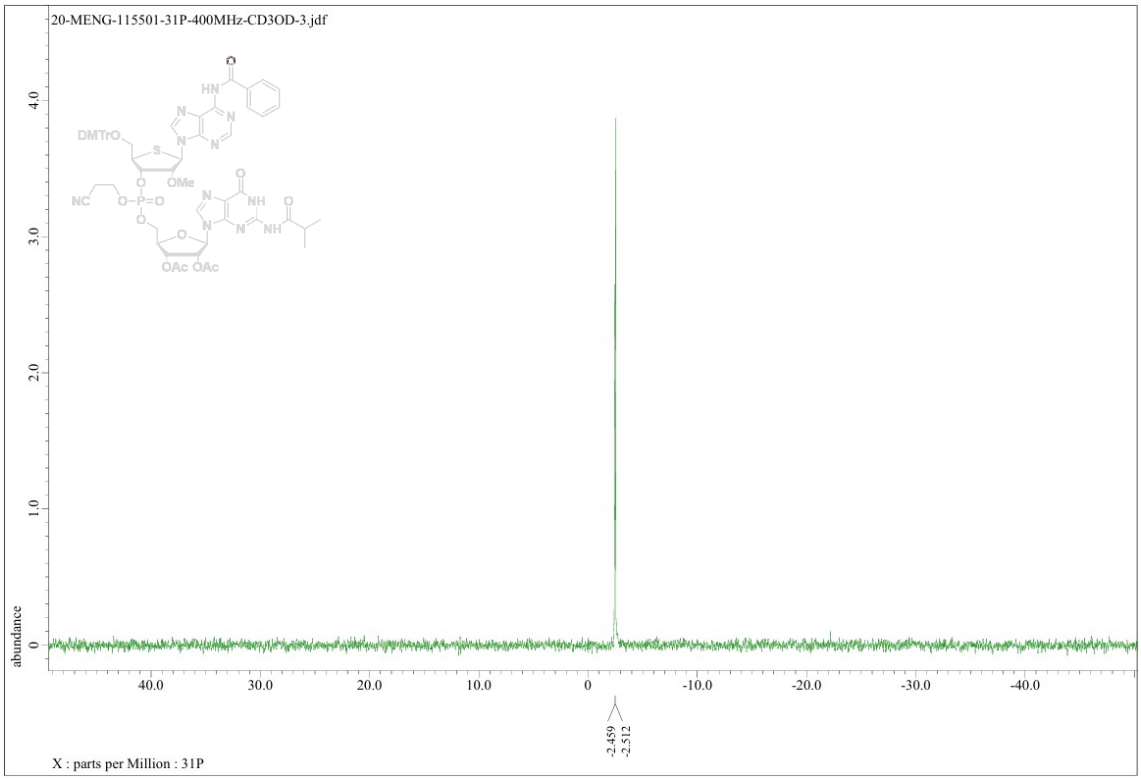
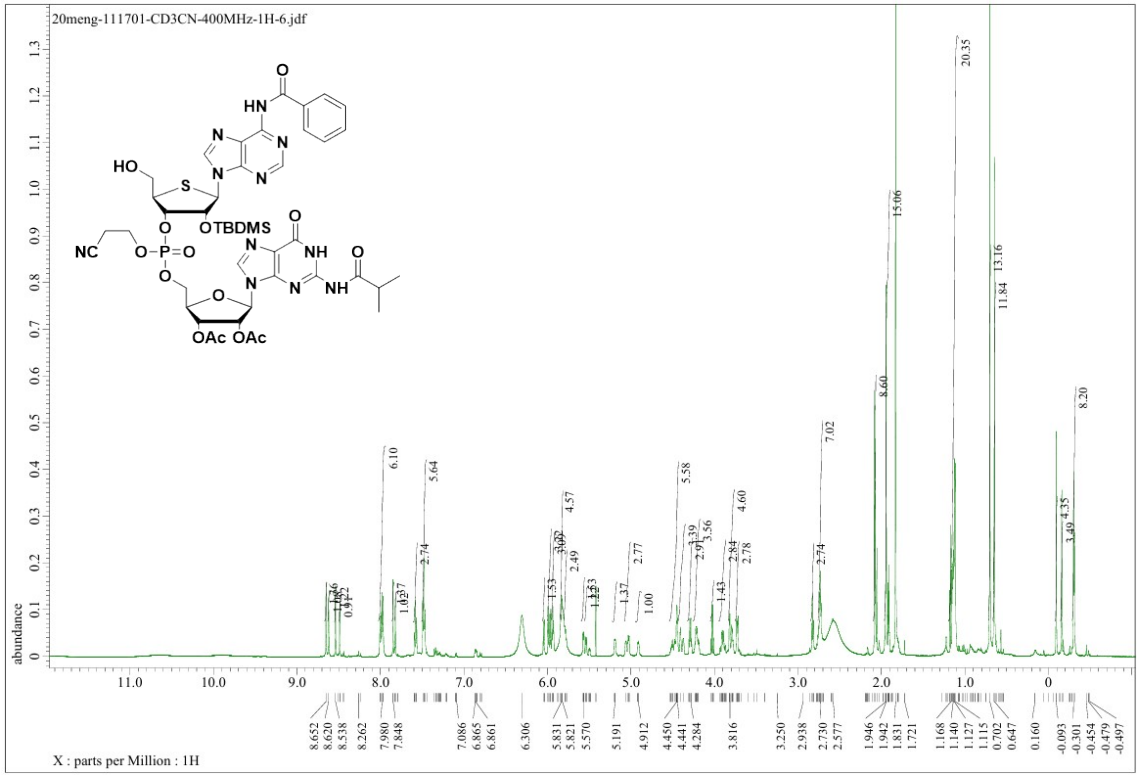


^{1}H-NMR chart of **24**



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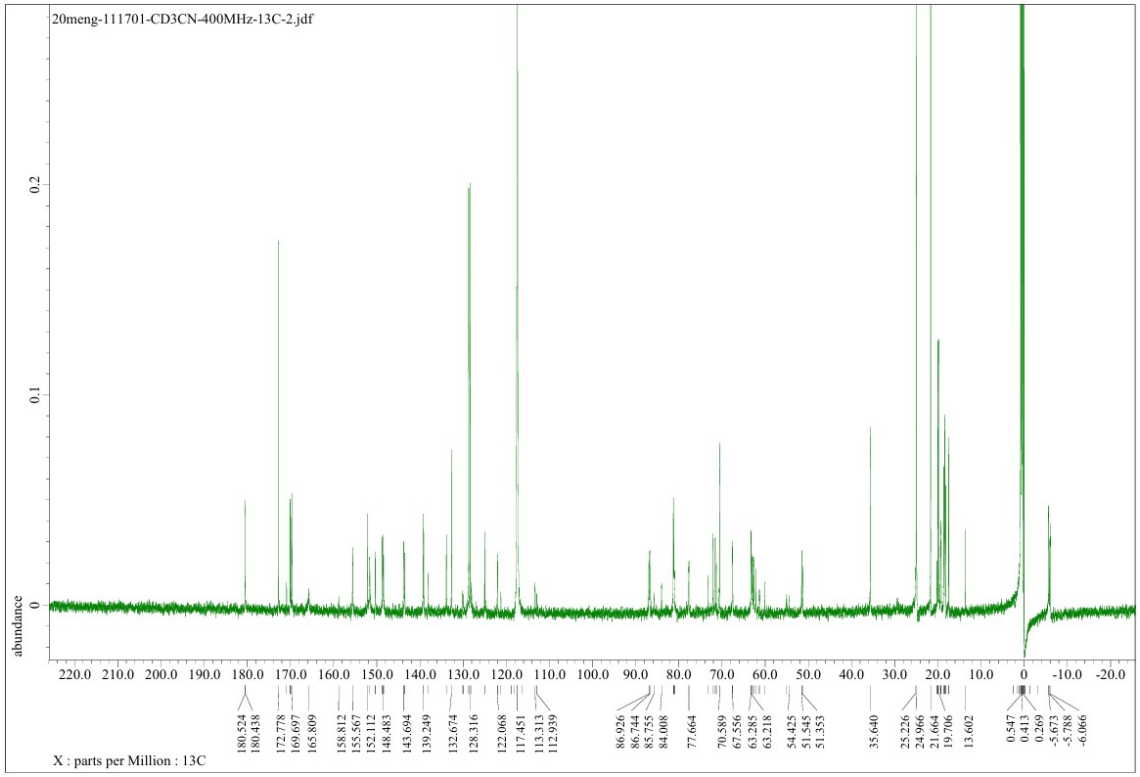

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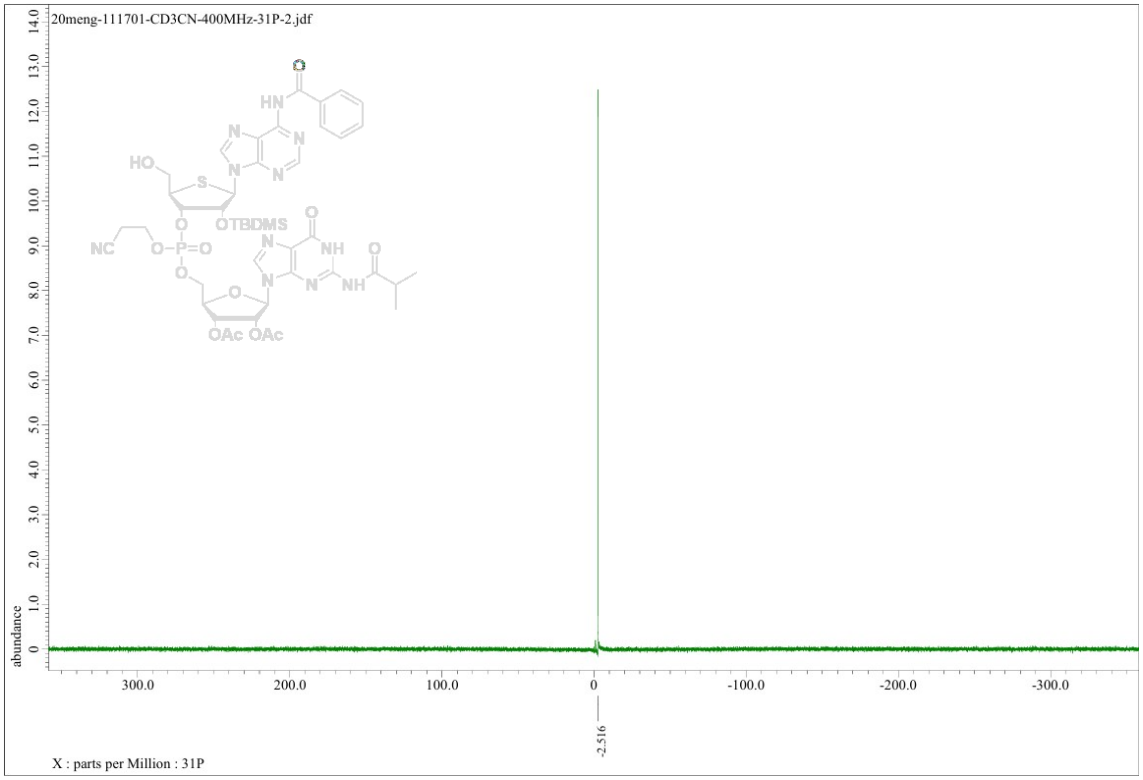
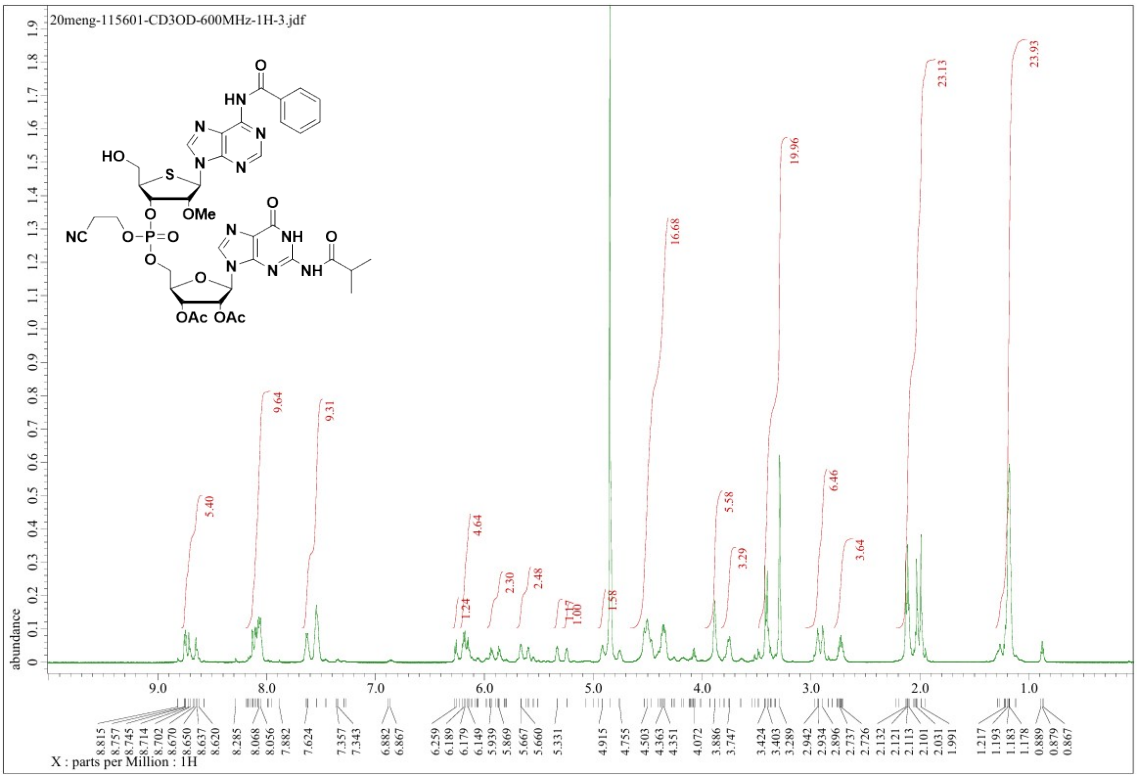


^{1}H-NMR chart of **25**



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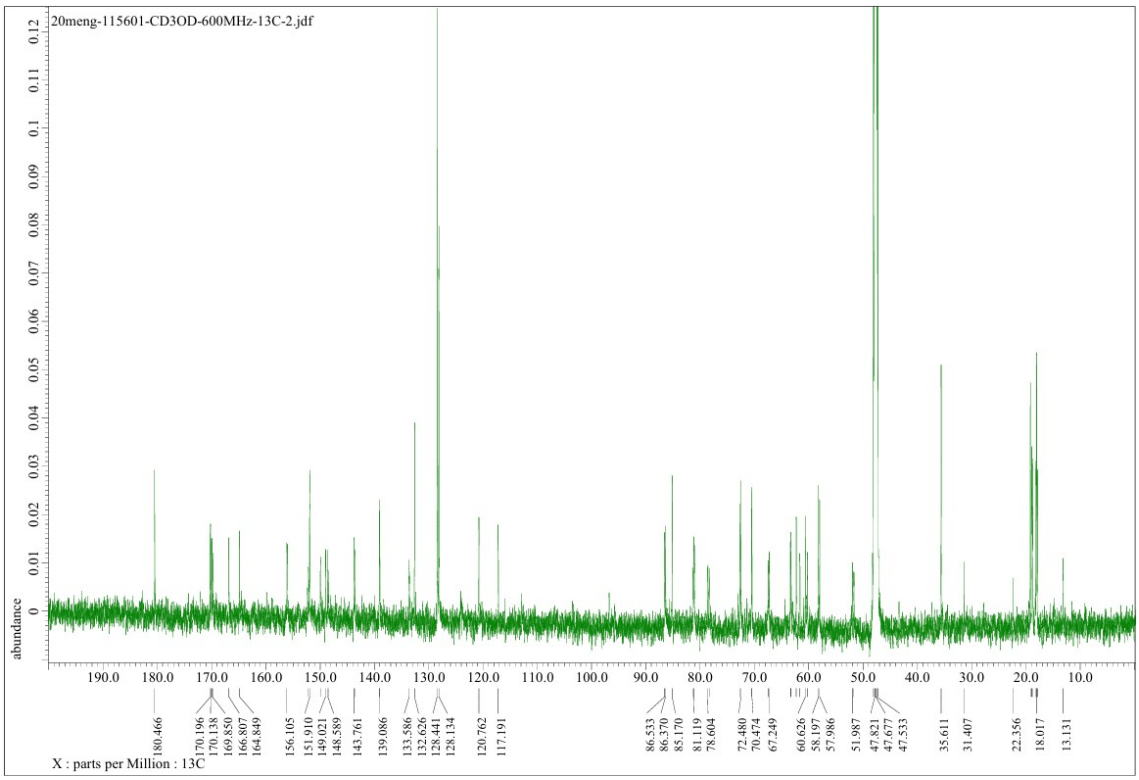

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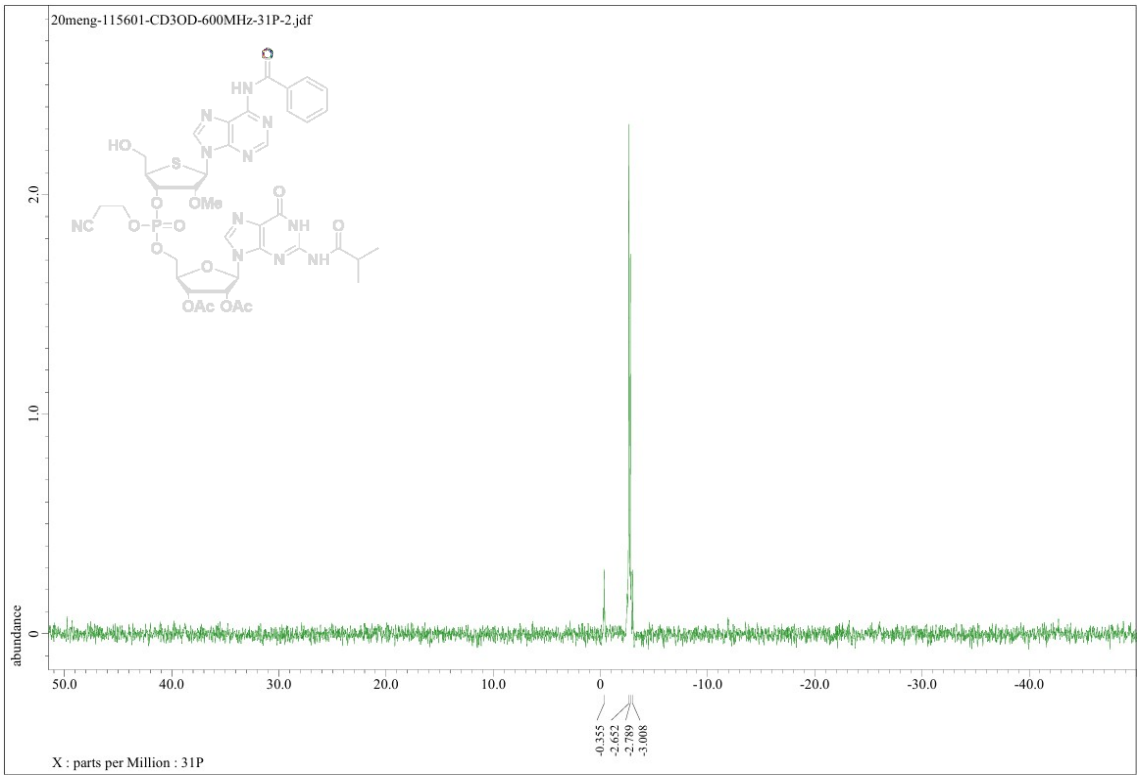
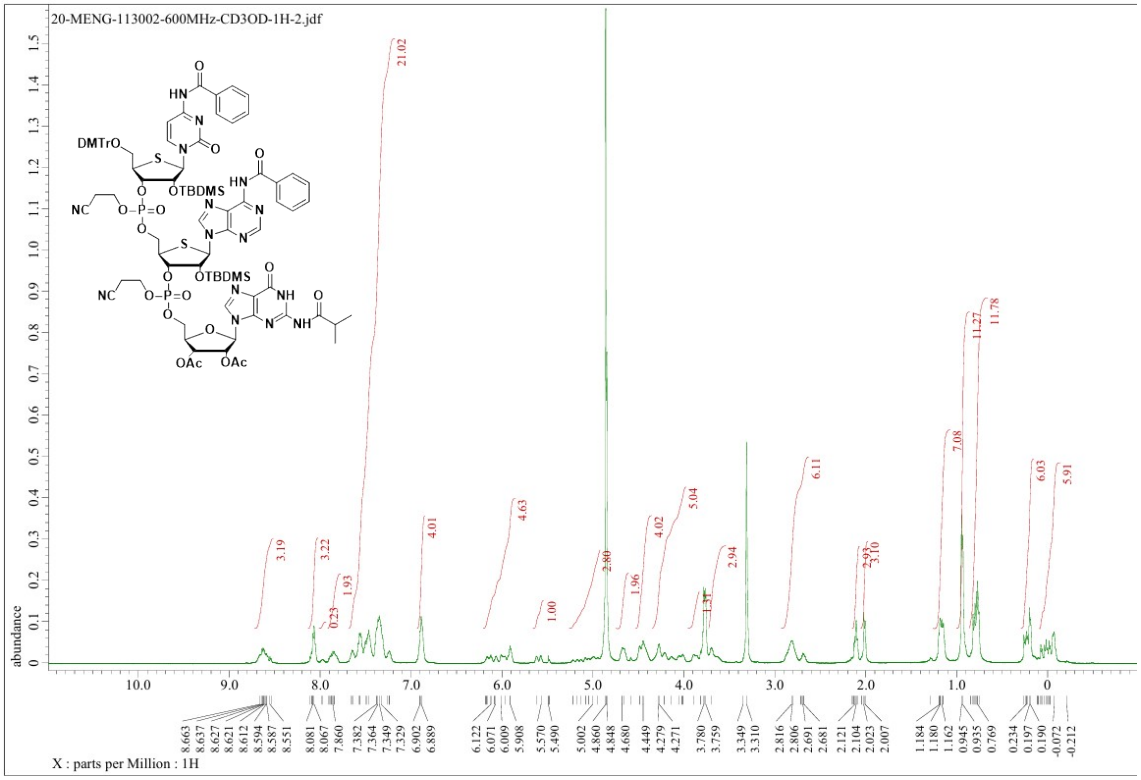
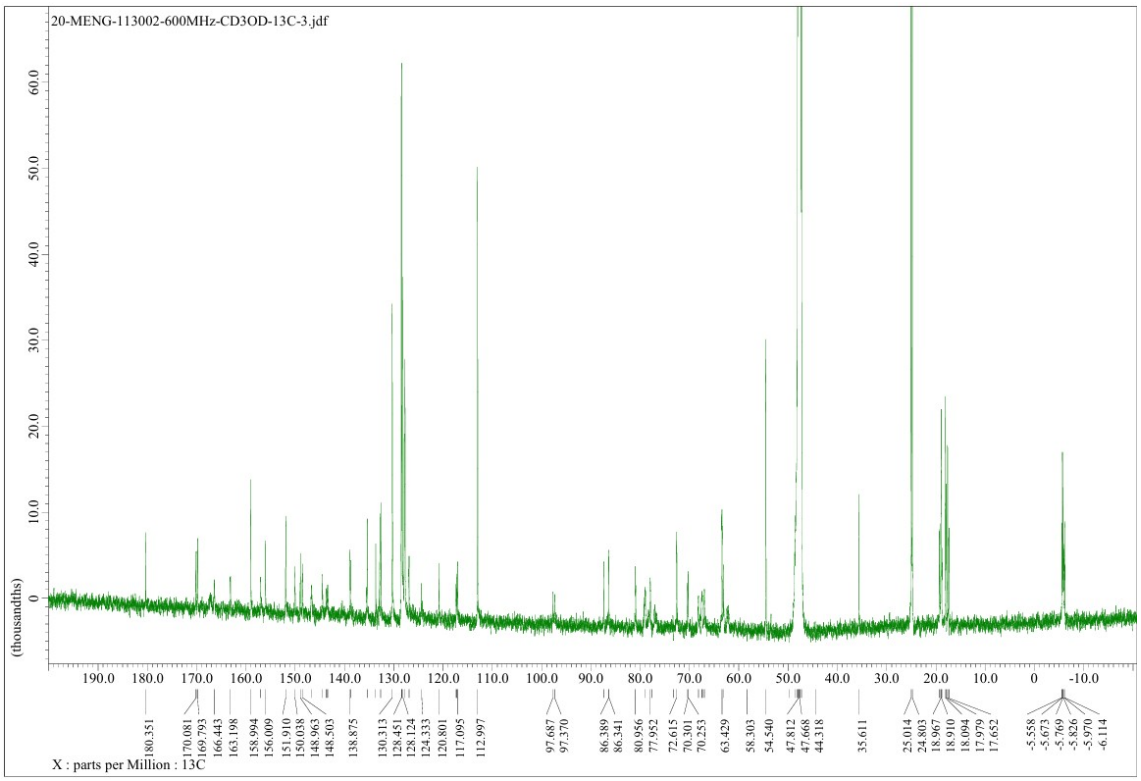


^{1}H-NMR chart of **28**



^{13}C-NMR chart of **28**



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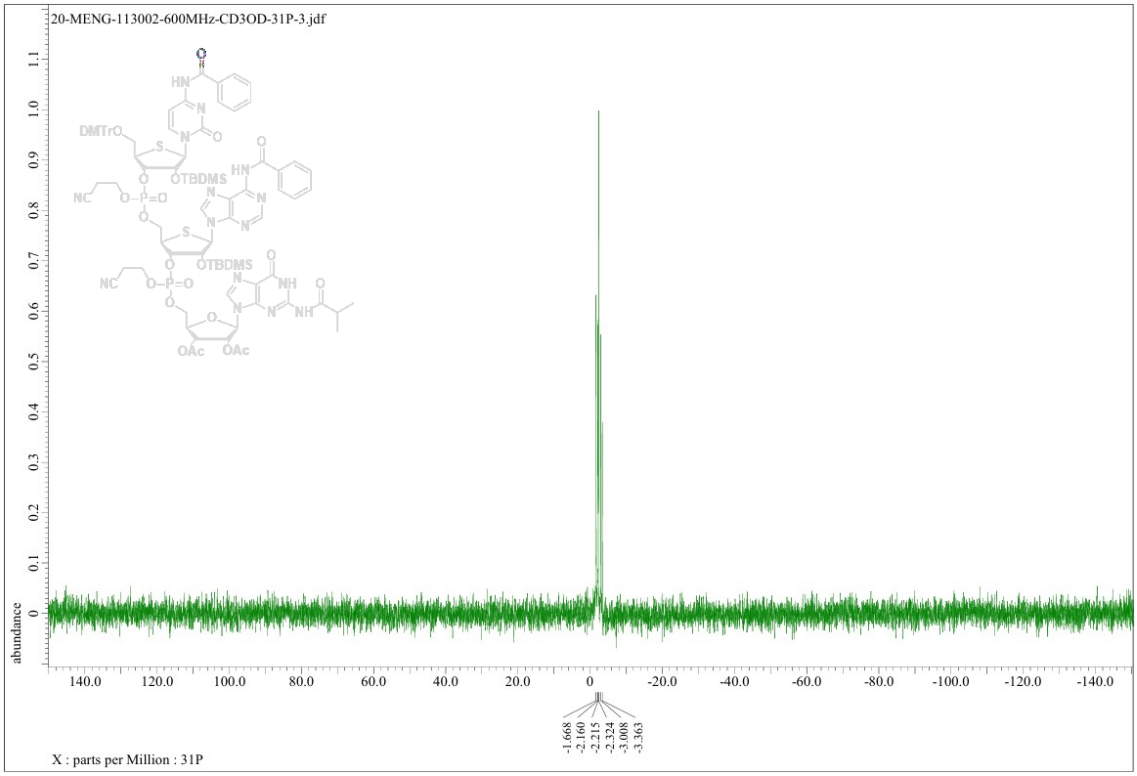

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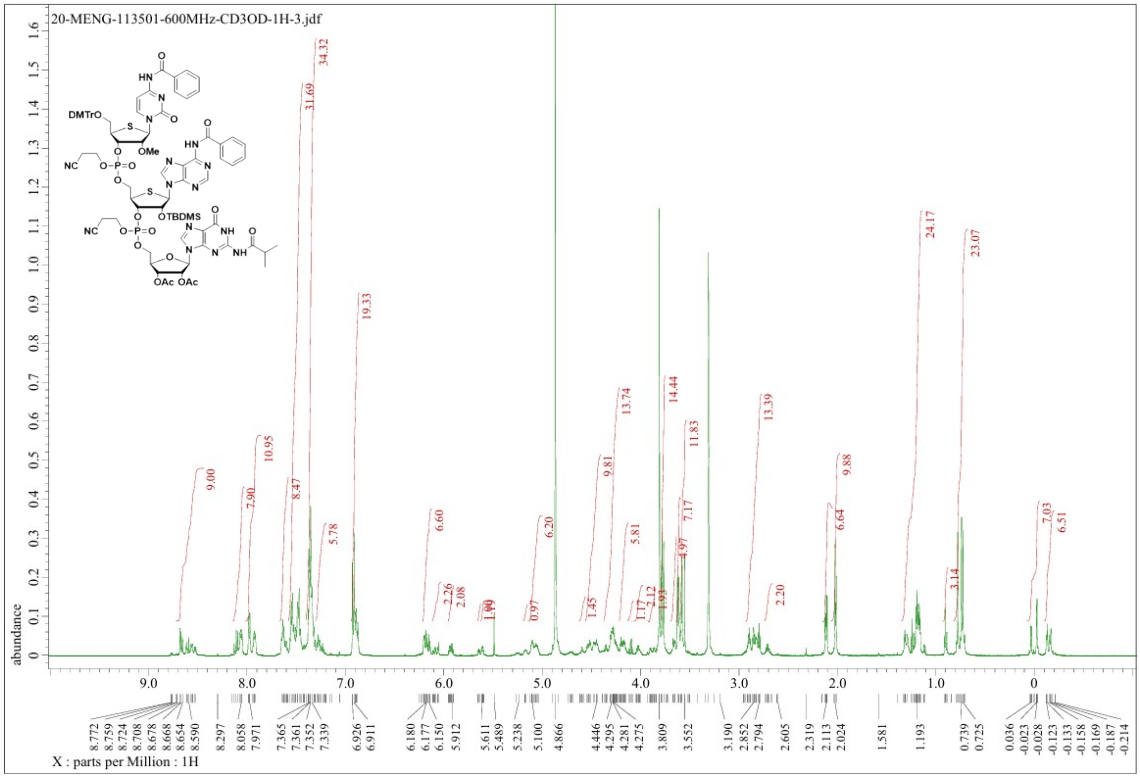
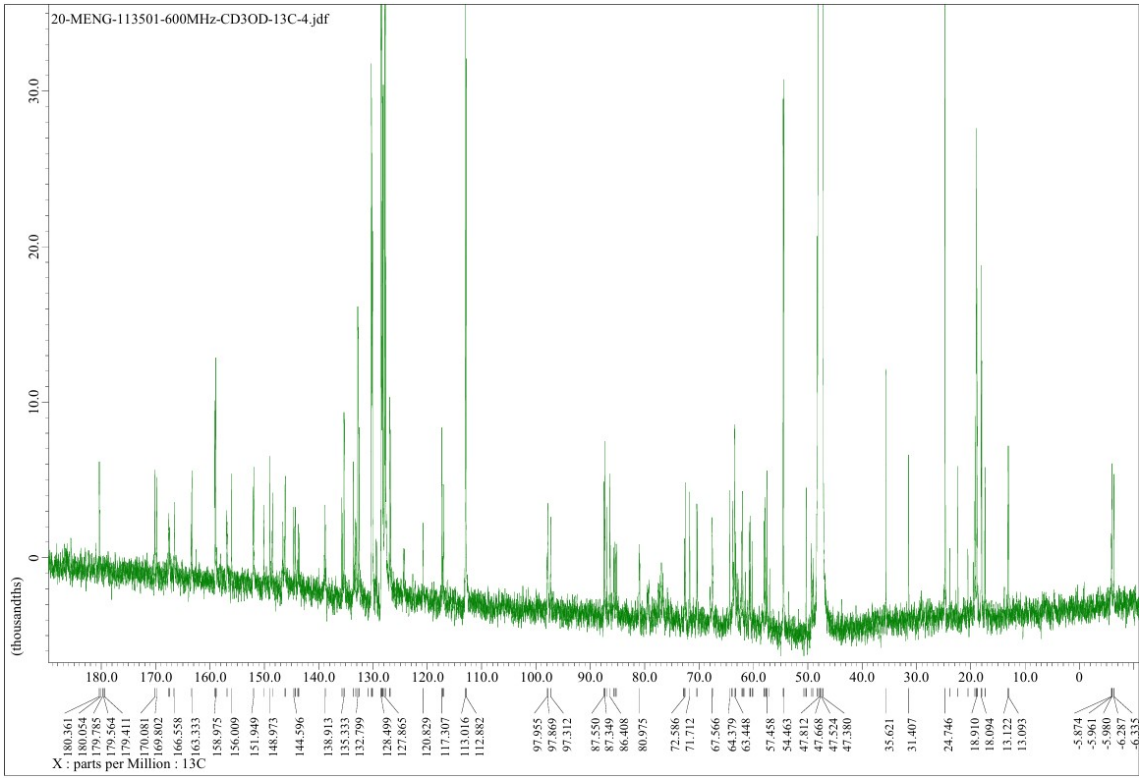


^{13}C-NMR chart of **29**



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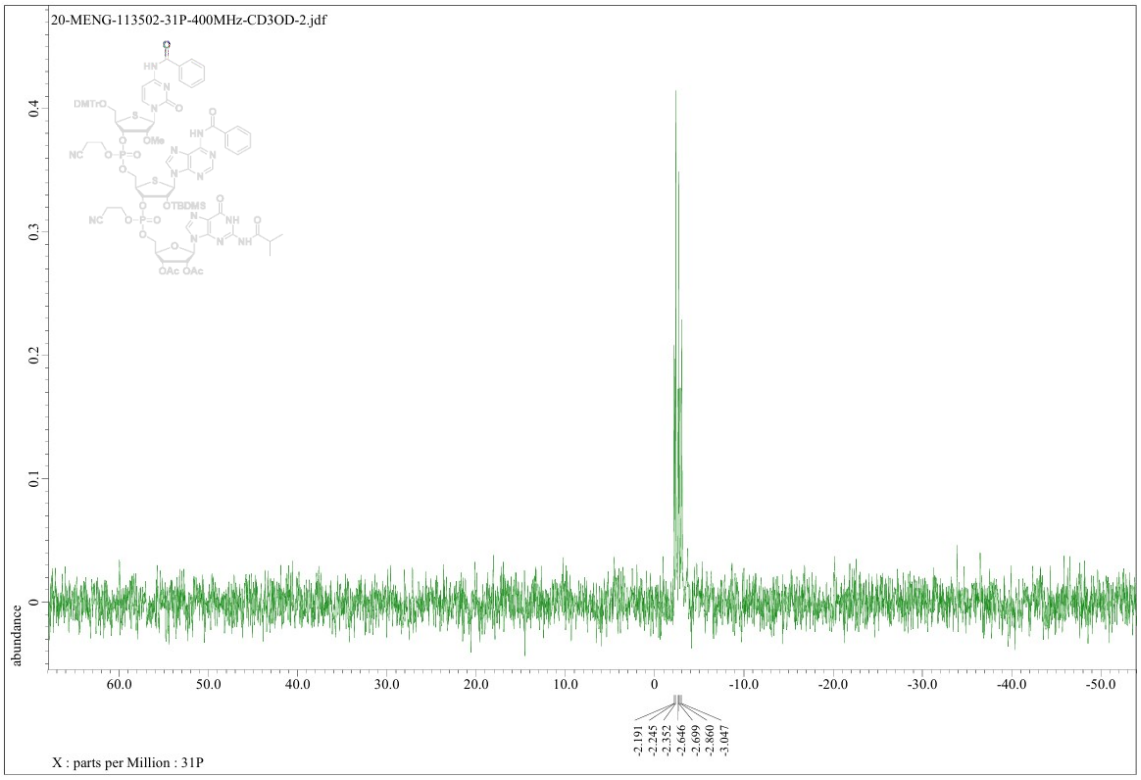
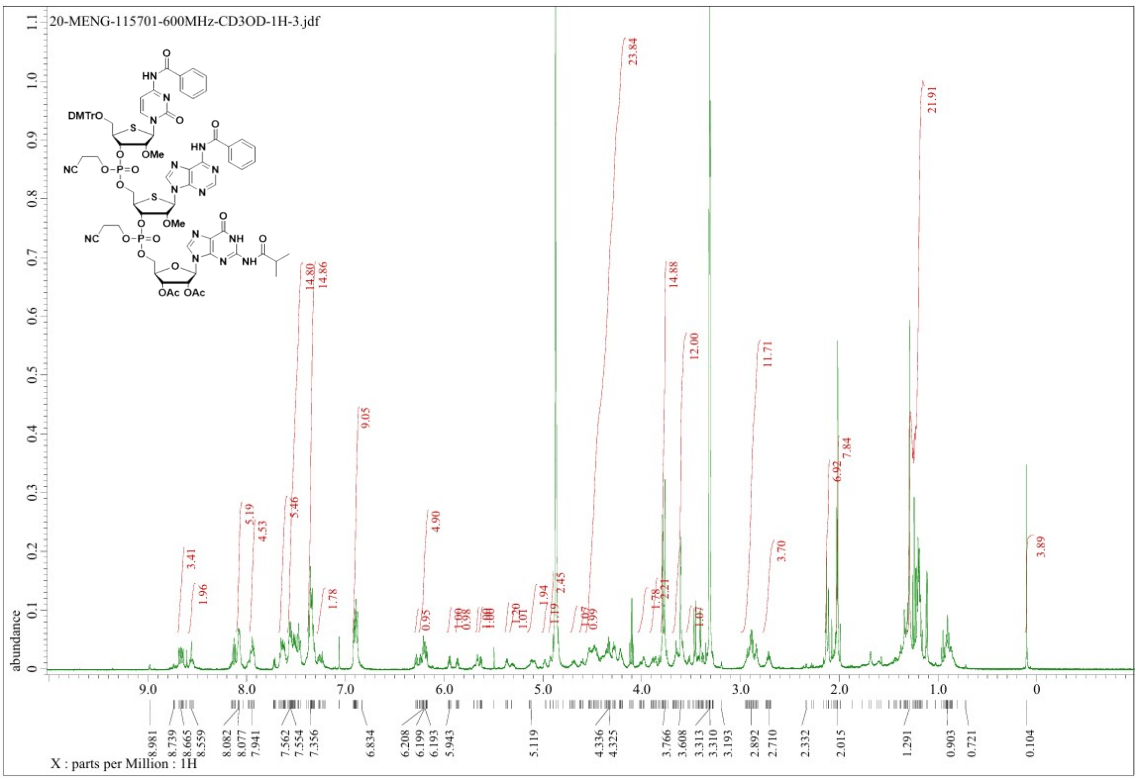


^{1}H-NMR chart of **30**



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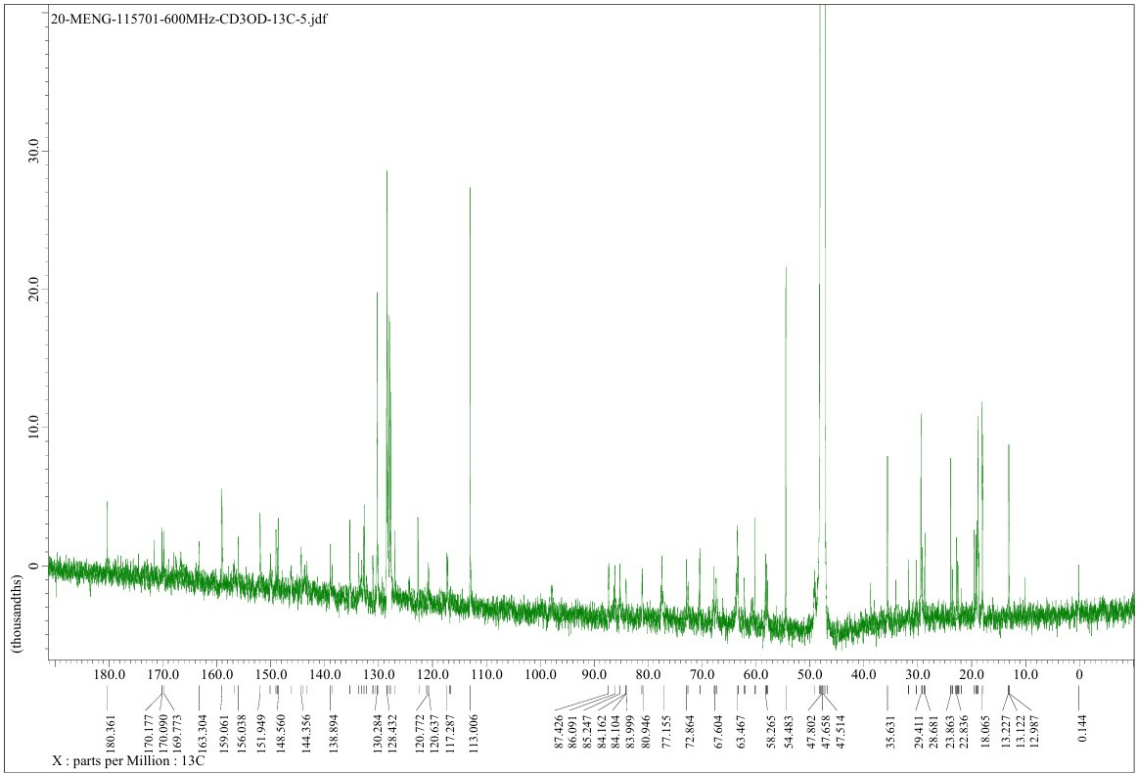

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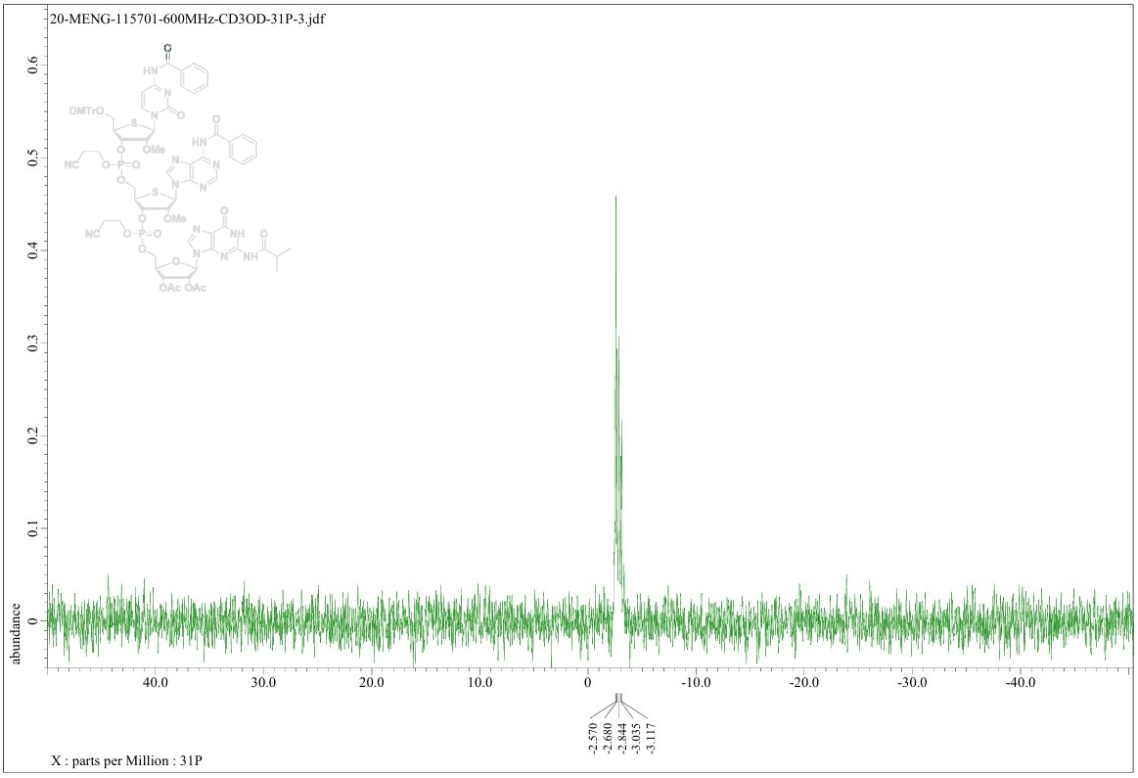

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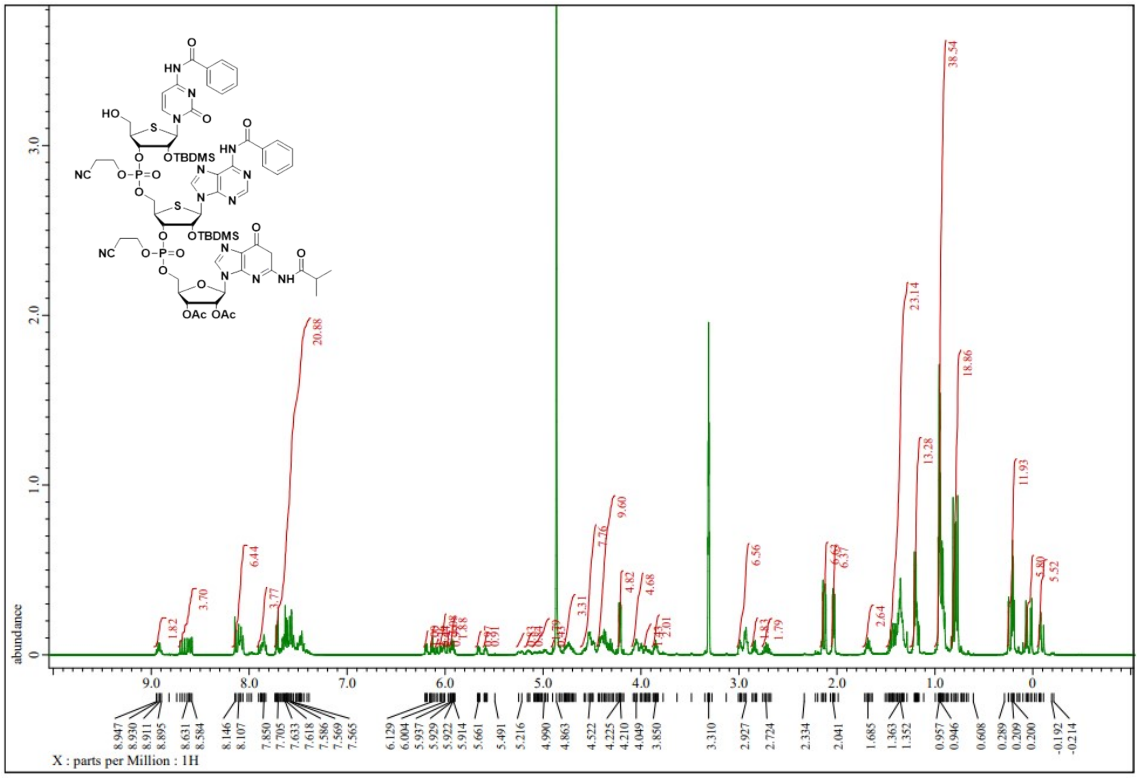

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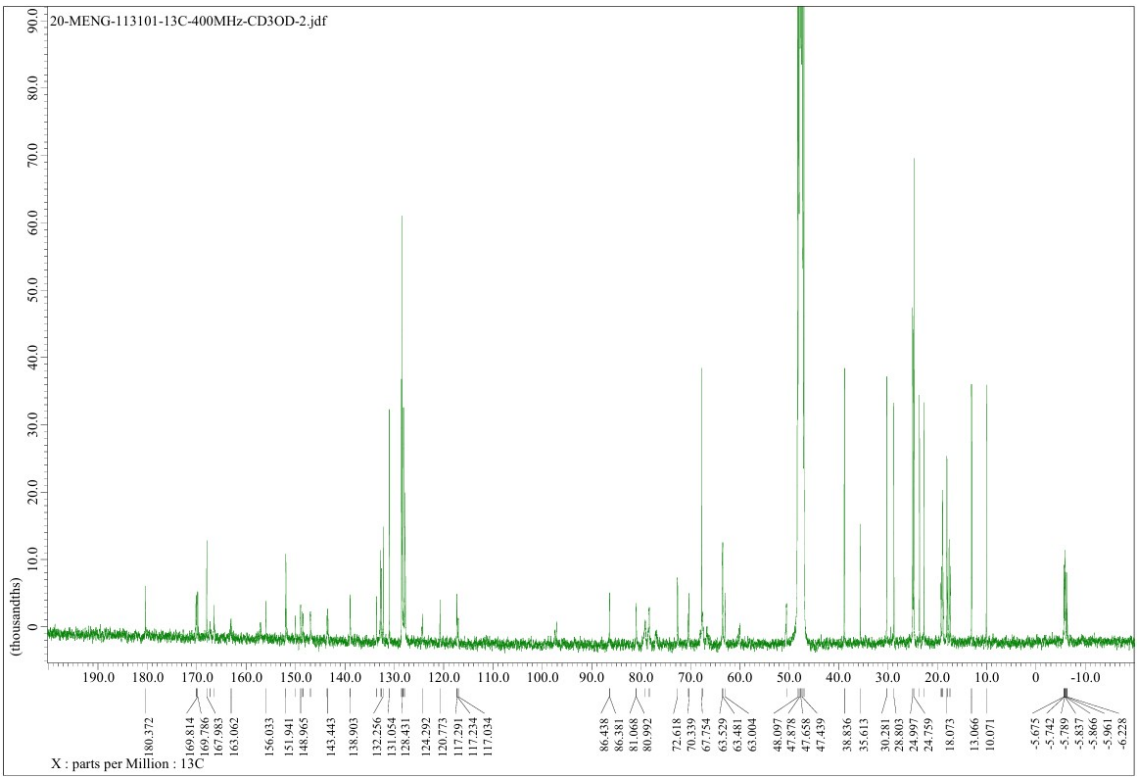

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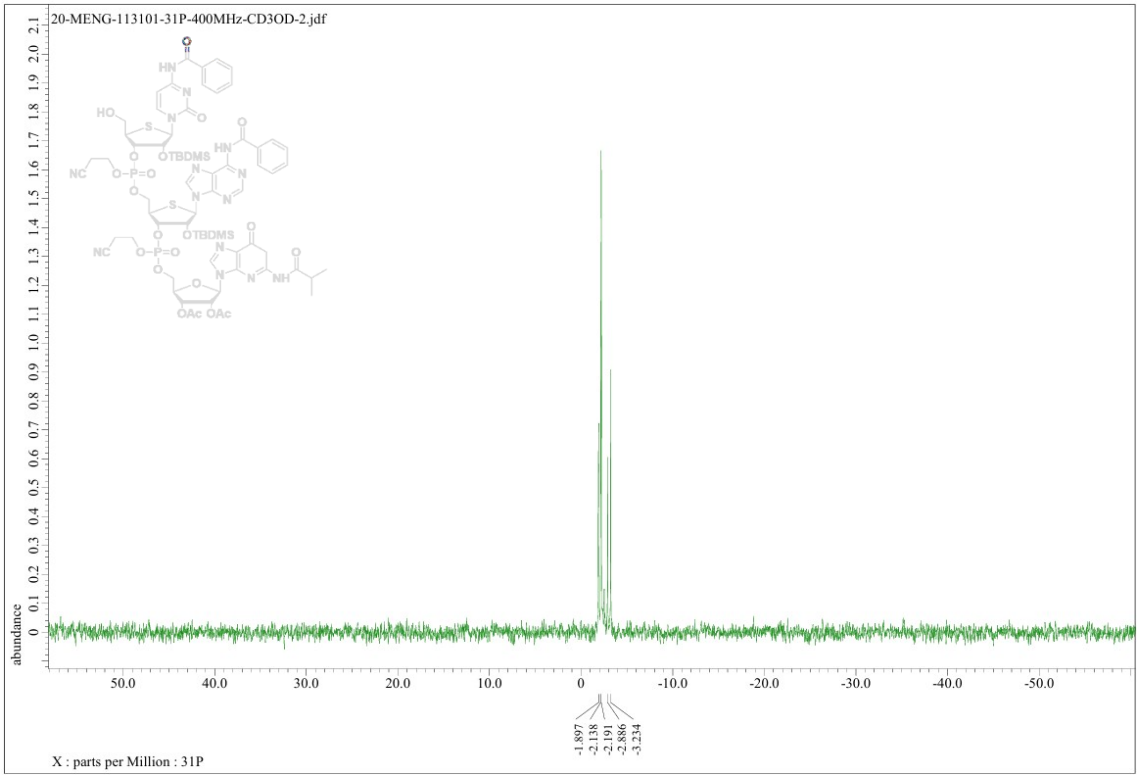

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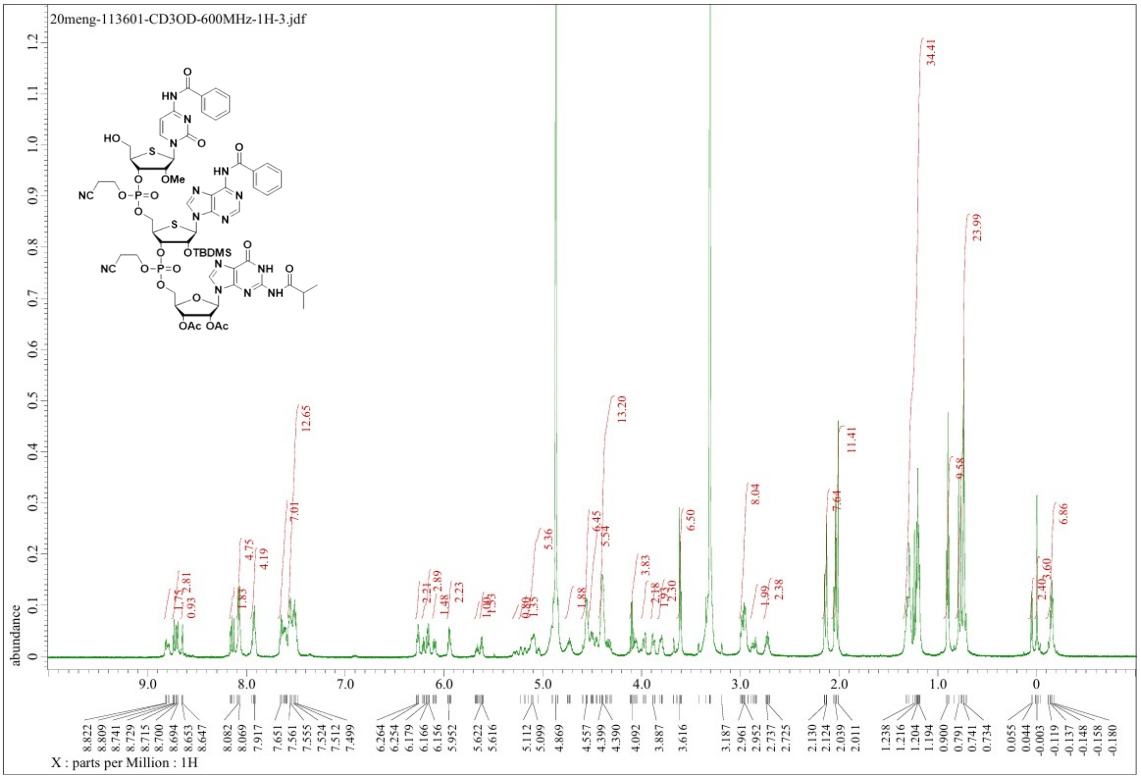
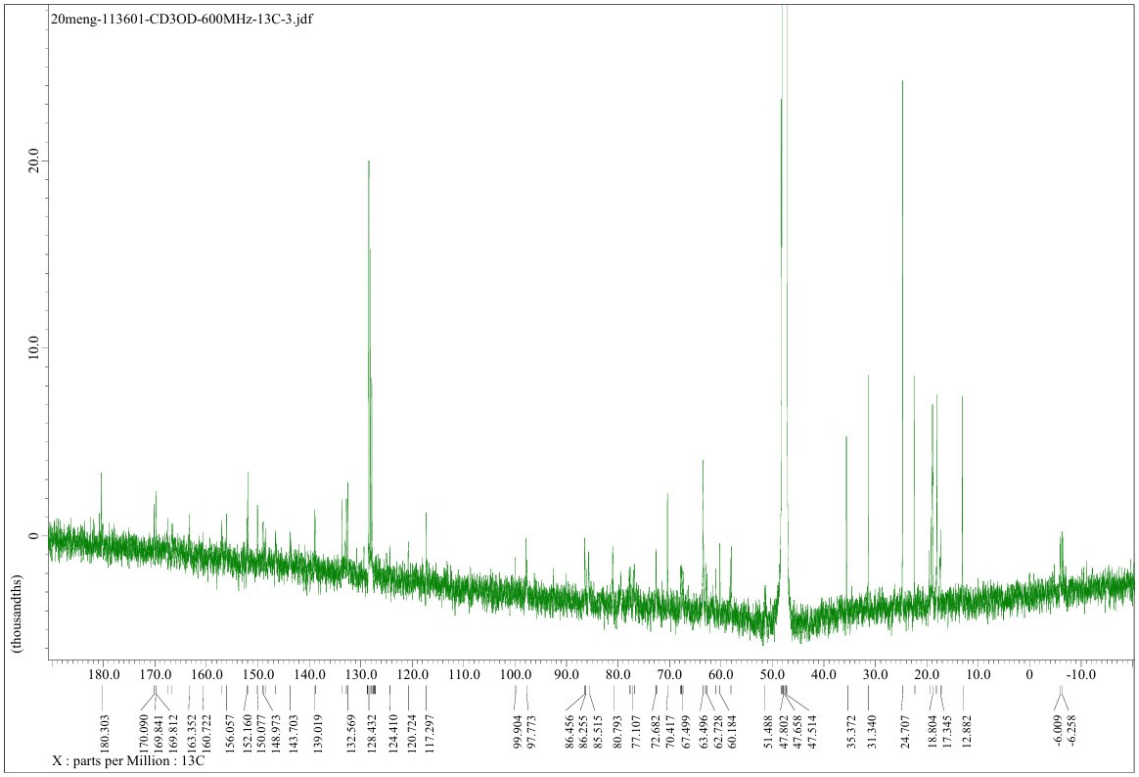
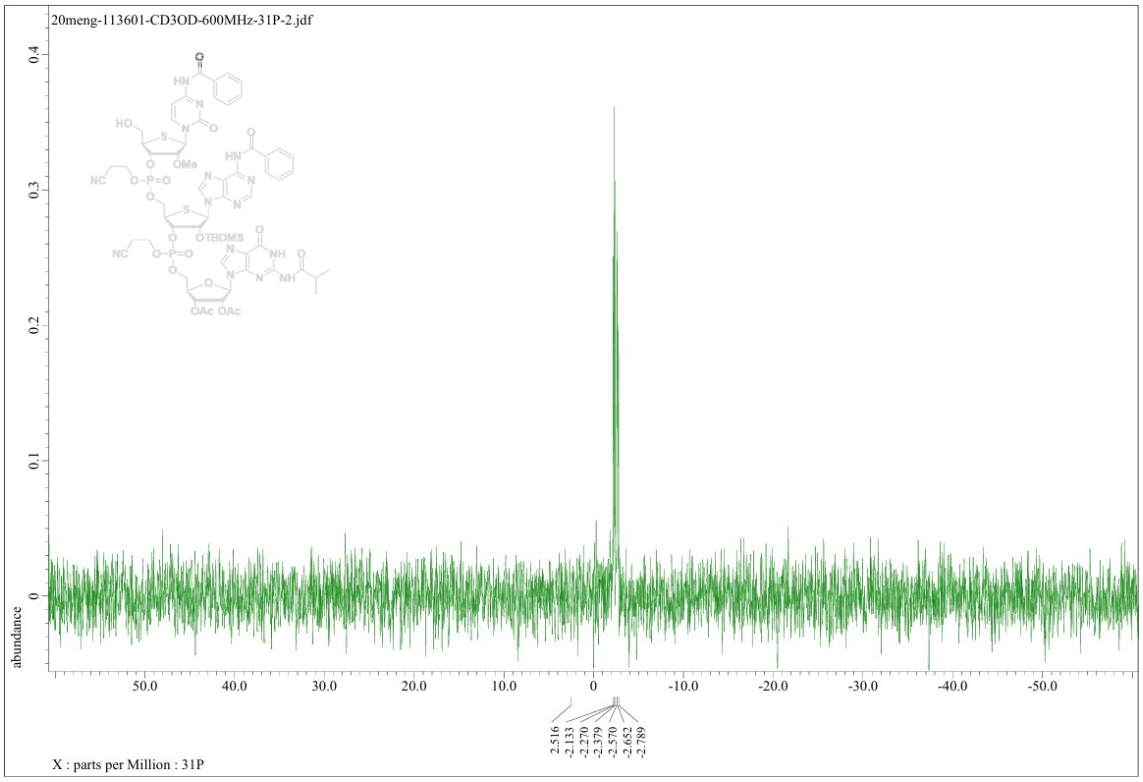


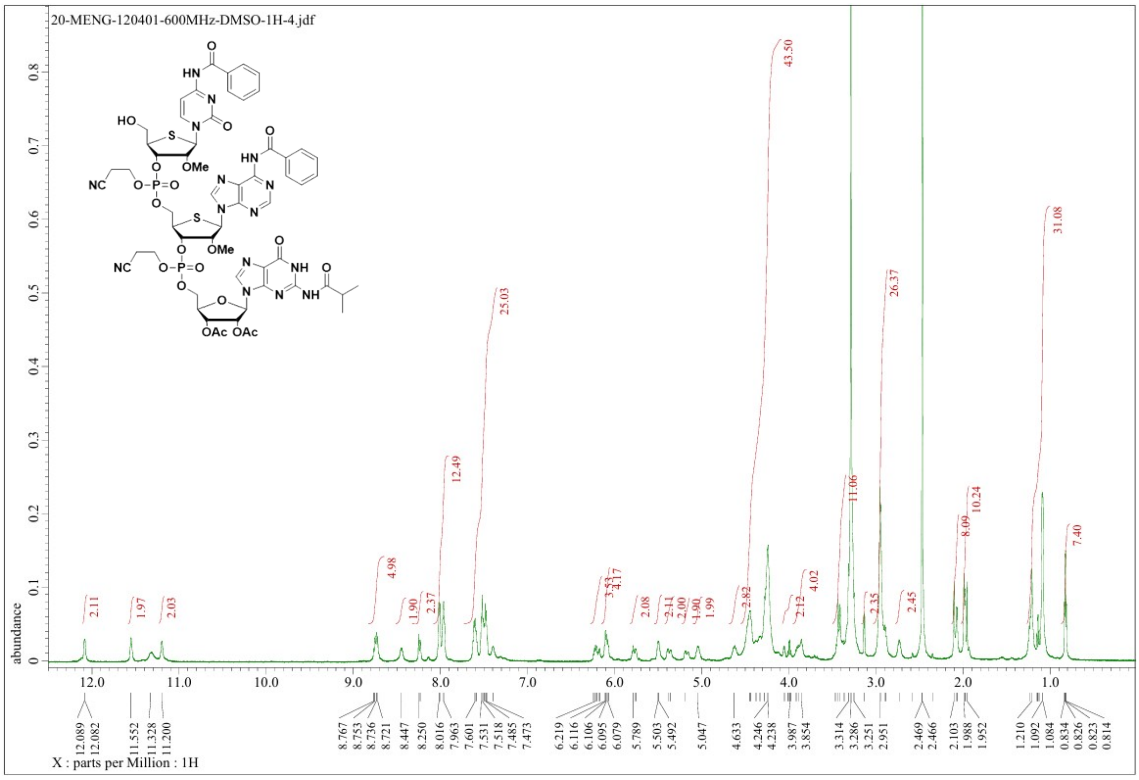
^{13}C-NMR chart of **32**



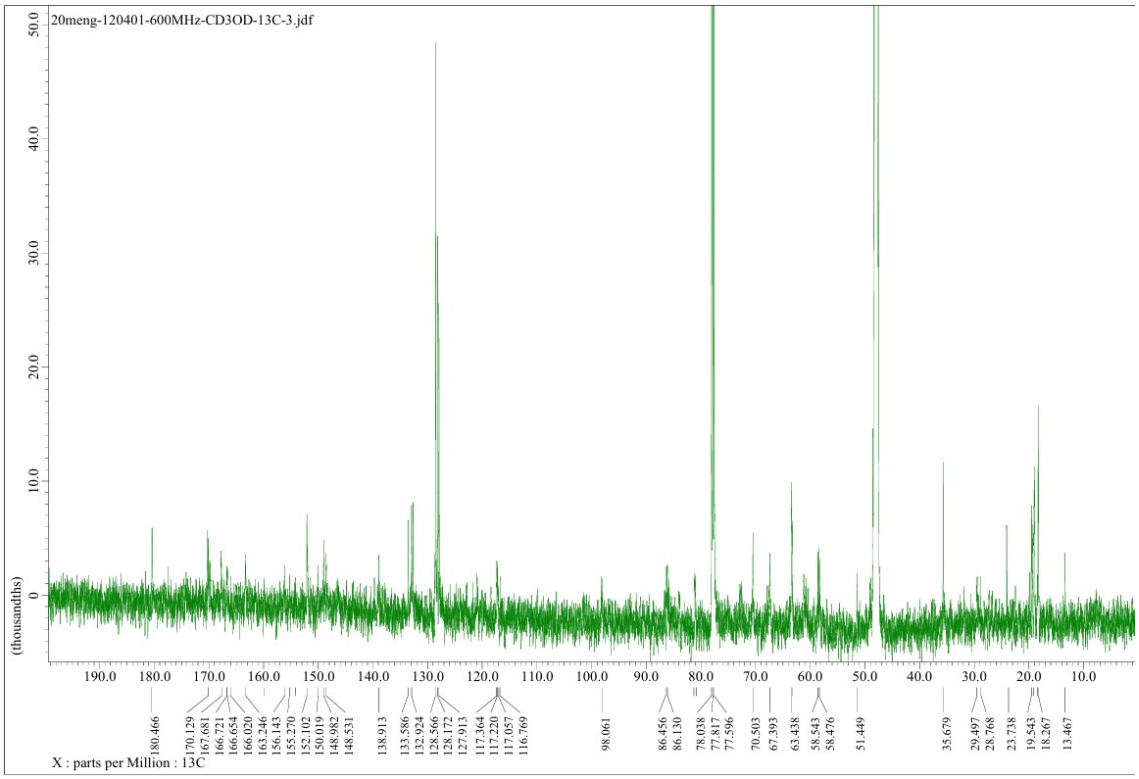
^{31}P-NMR chart of **32**



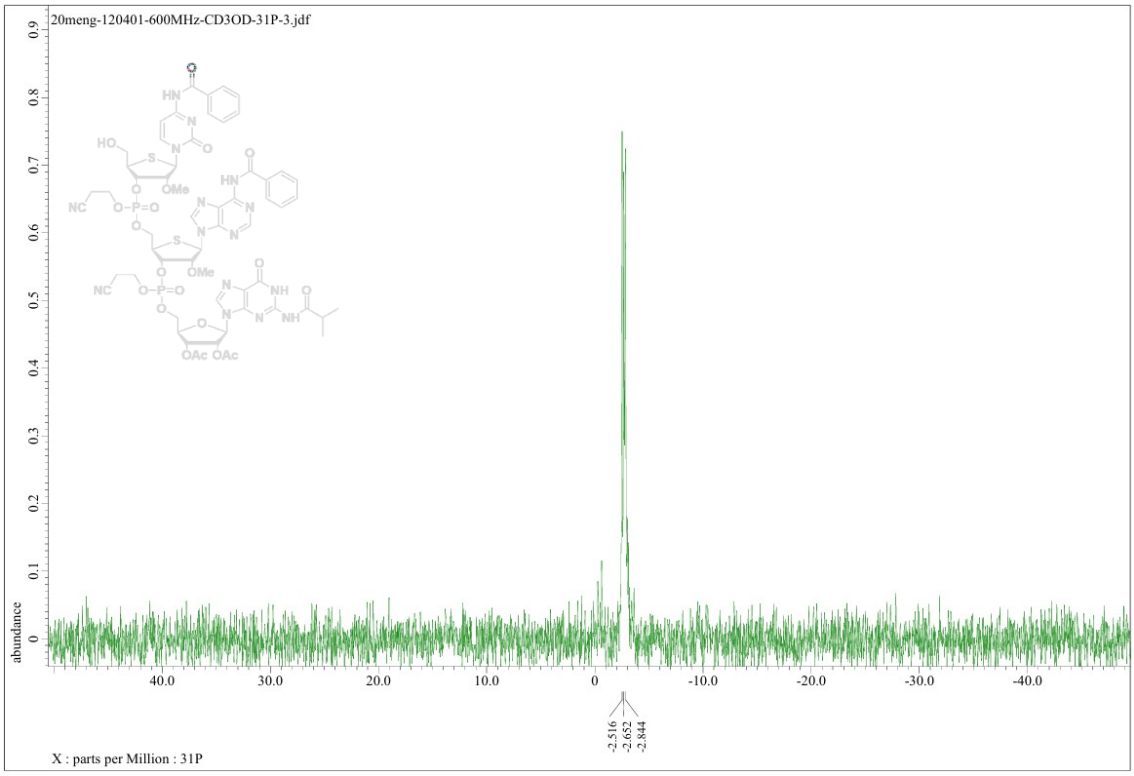
^{1}H-NMR chart of **33**



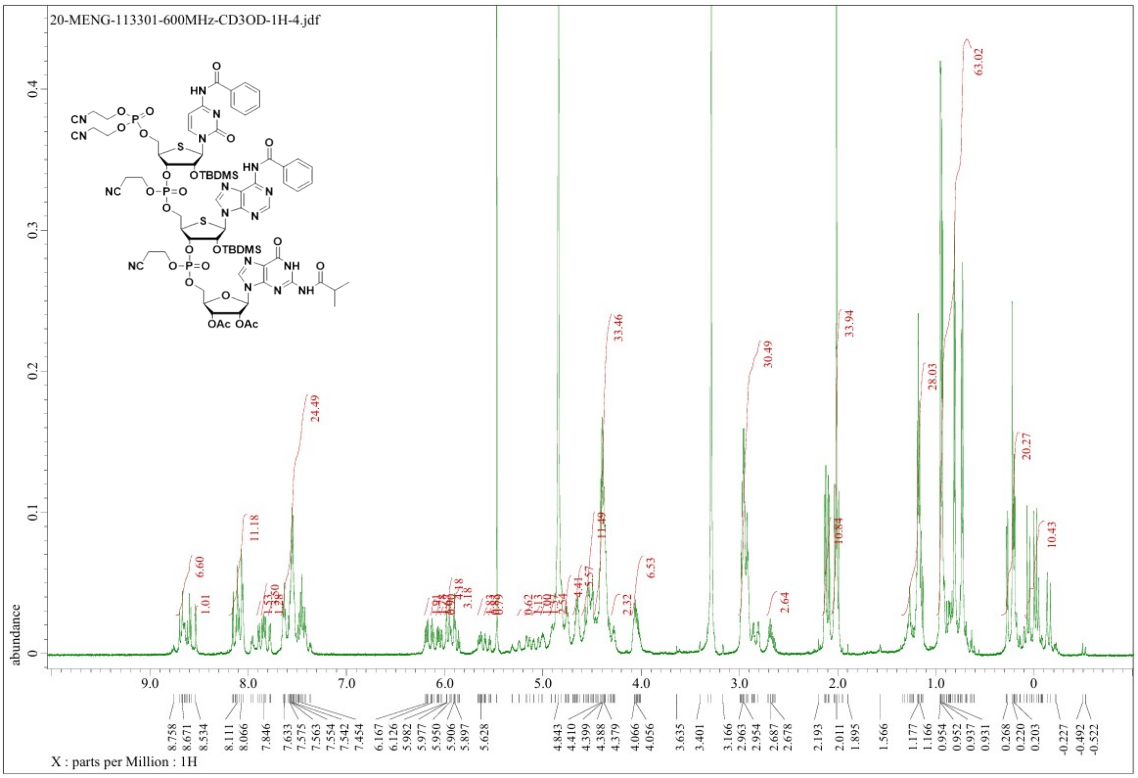
^{13}C-NMR chart of **33**



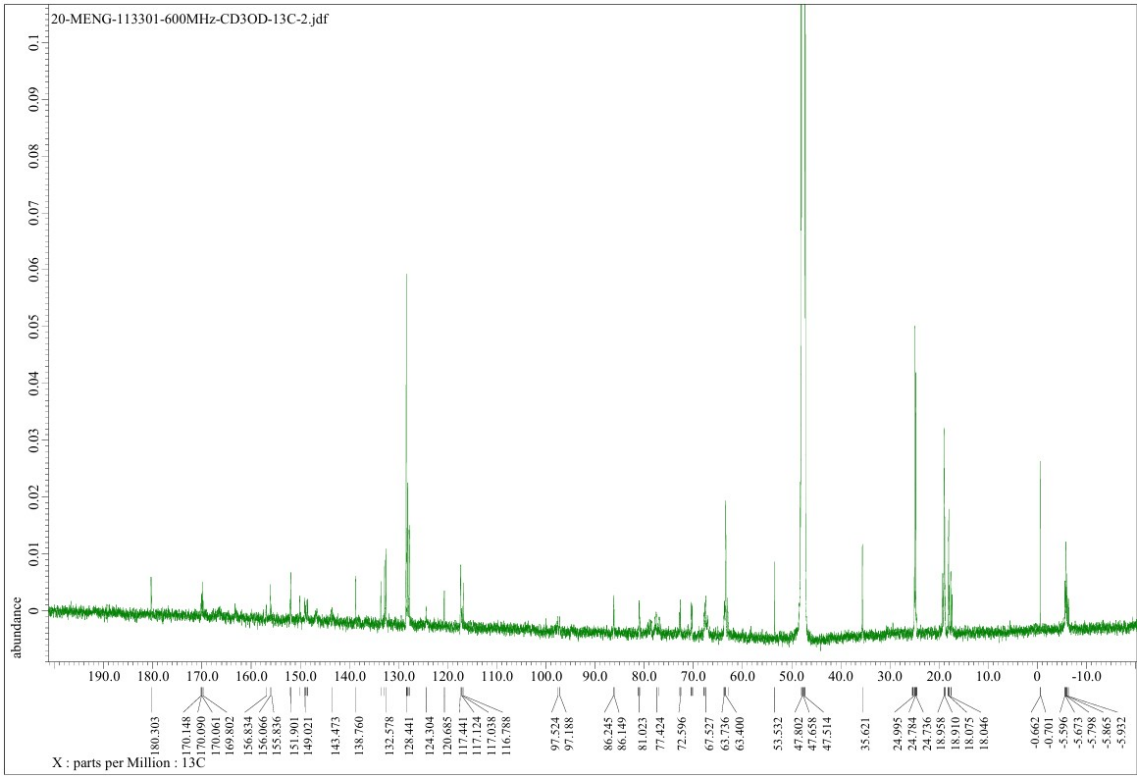
^{31}P-NMR chart of **33**



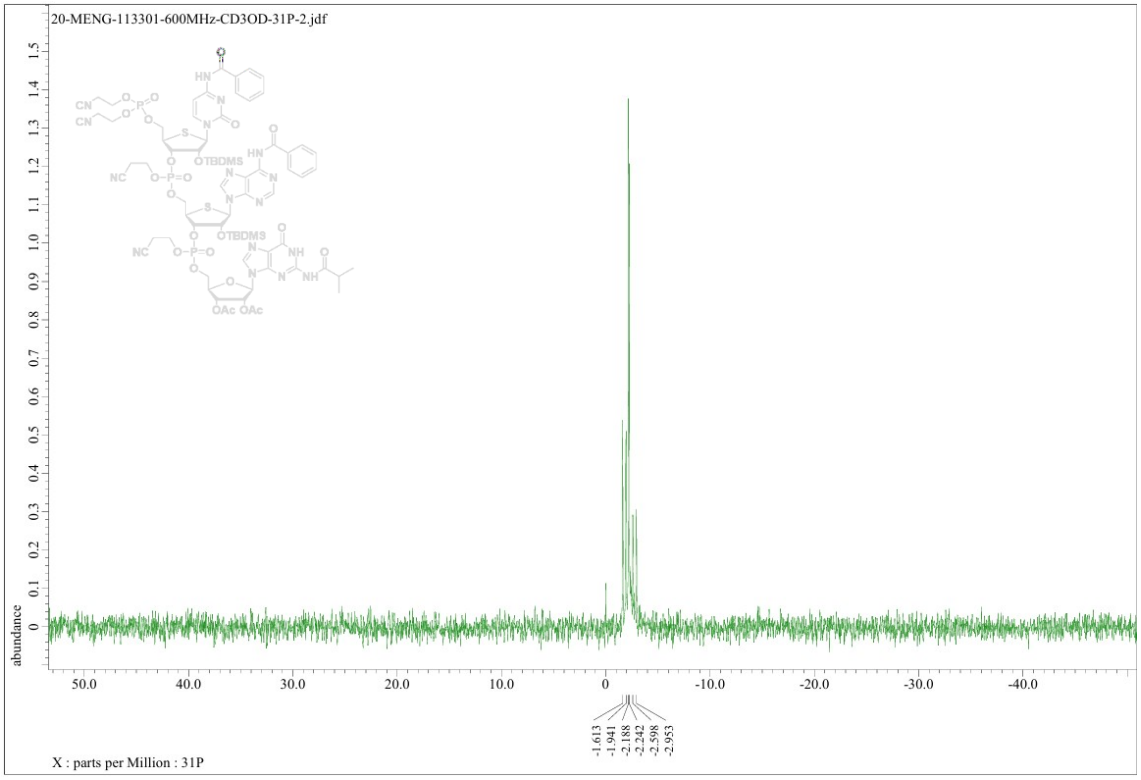
^{1}H-NMR chart of **34**



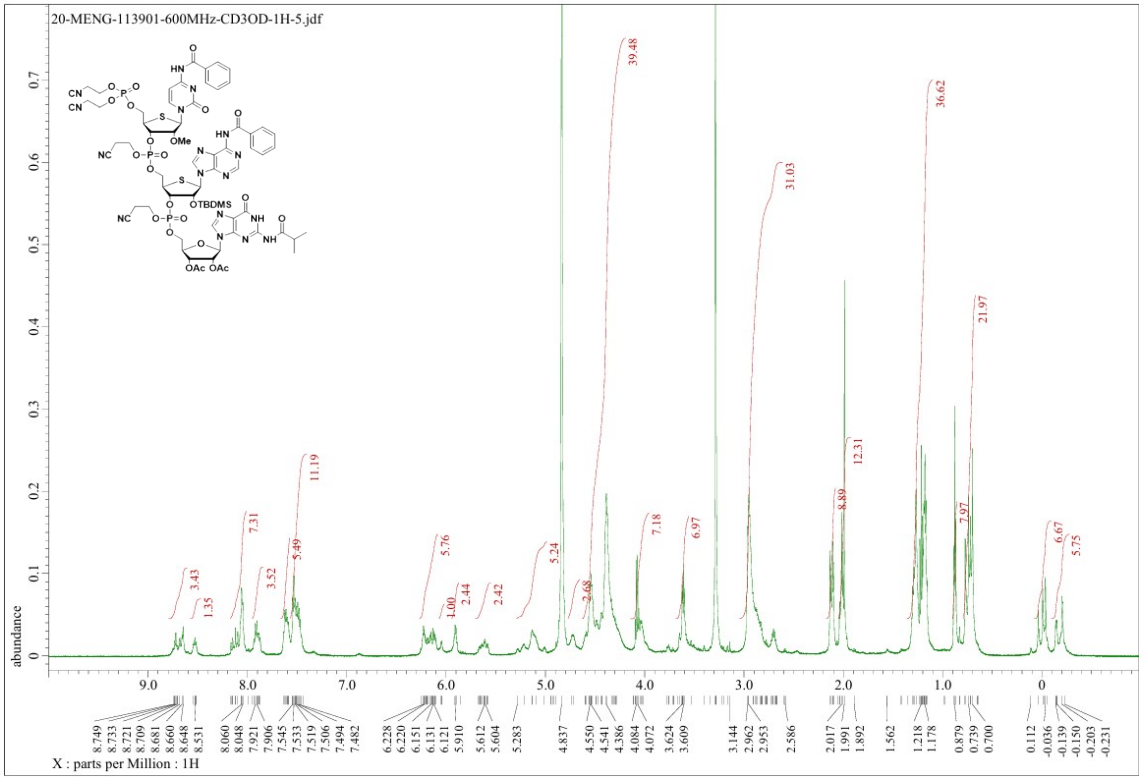
^{13}C-NMR chart of **34**



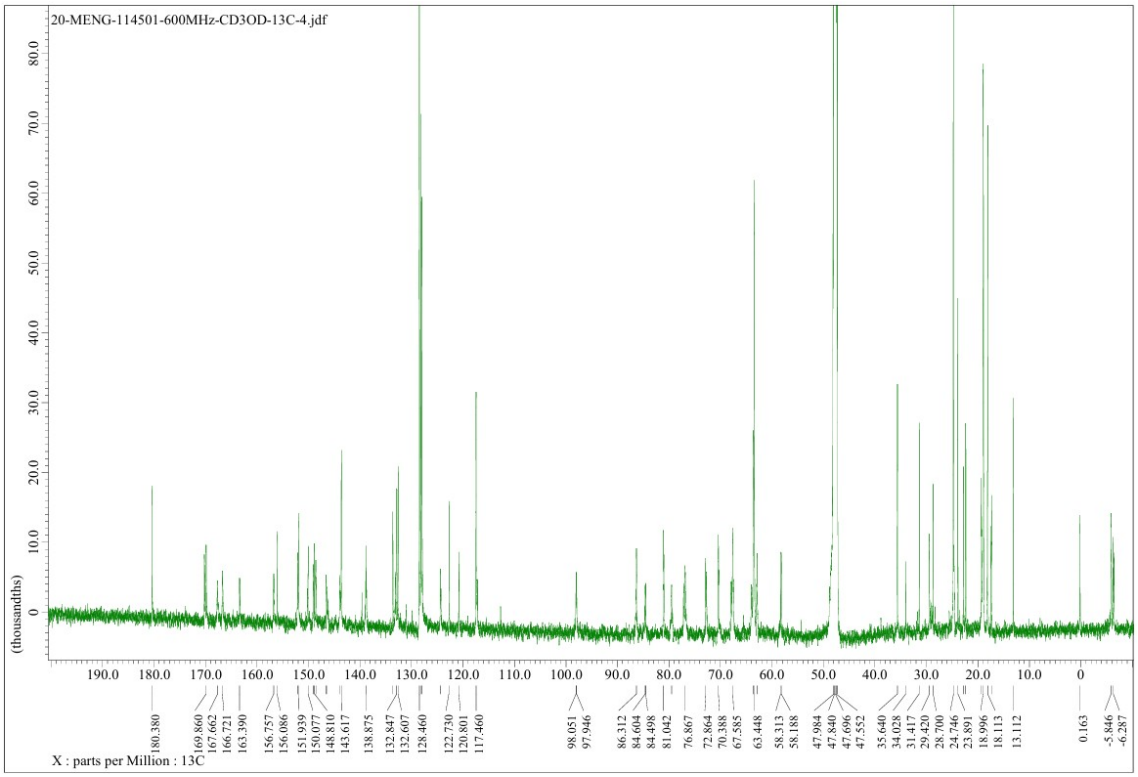
^{31}P-NMR chart of **34**



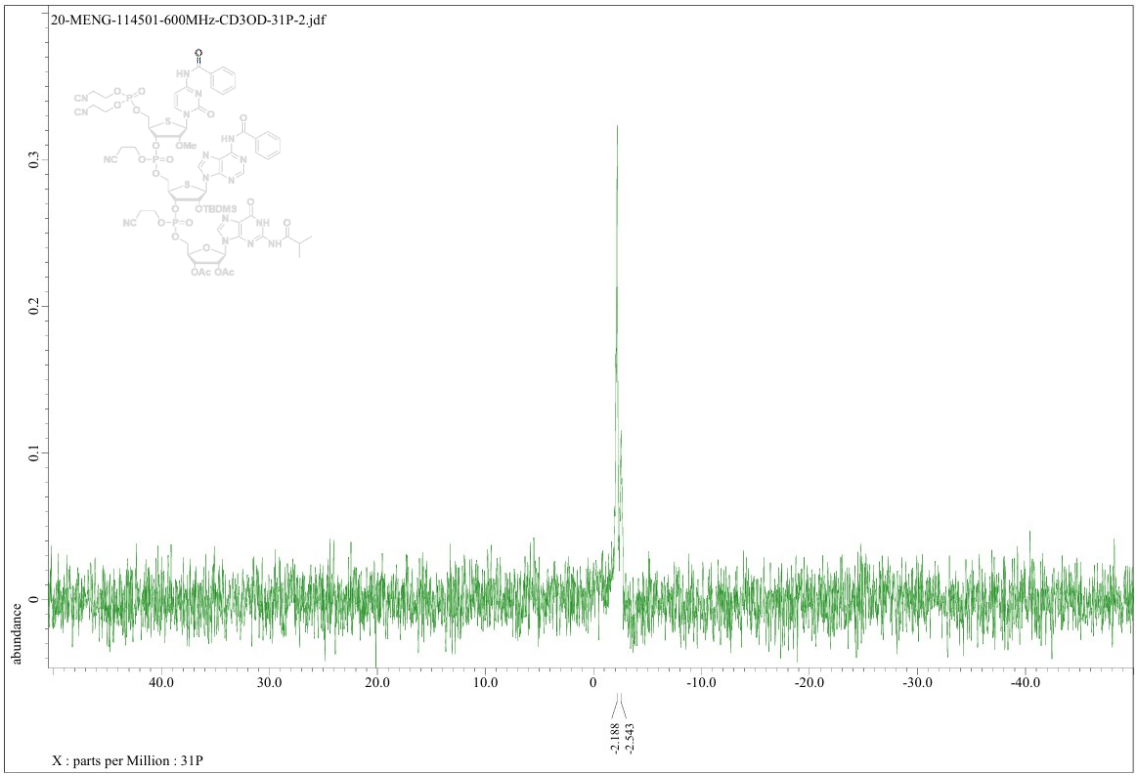
^{1}H-NMR chart of **35**



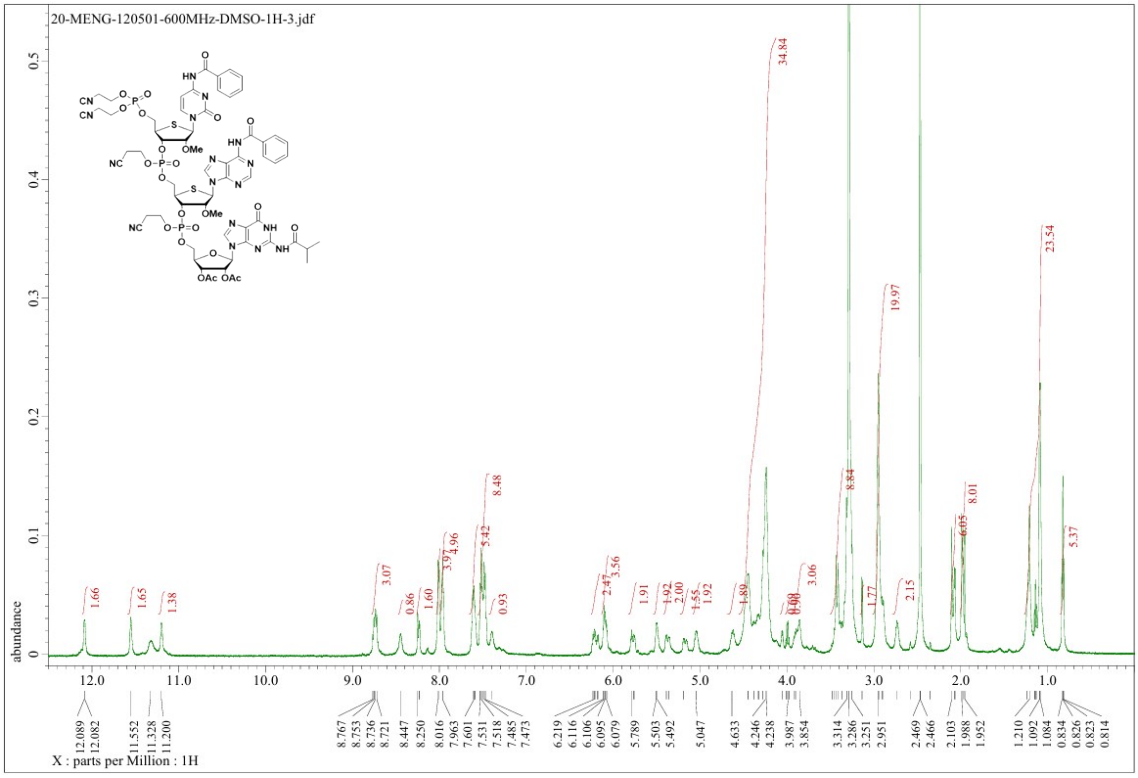
^{13}C-NMR chart of **35**



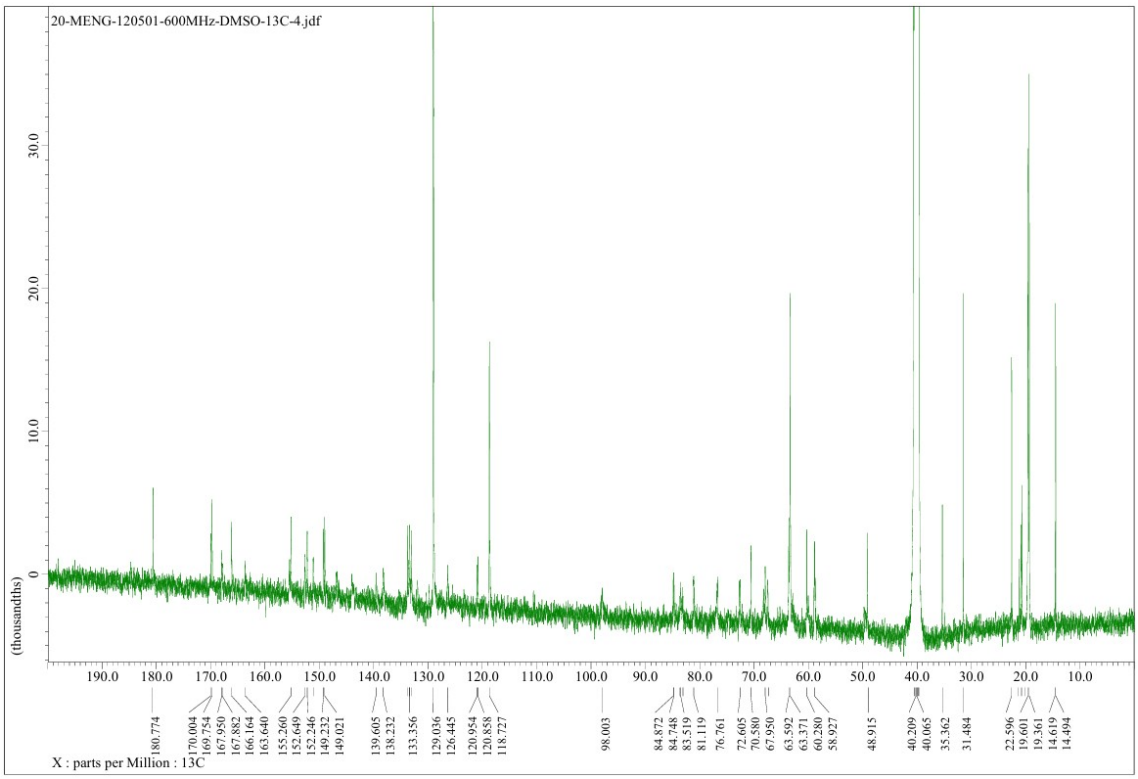
^{31}P-NMR chart of **35**



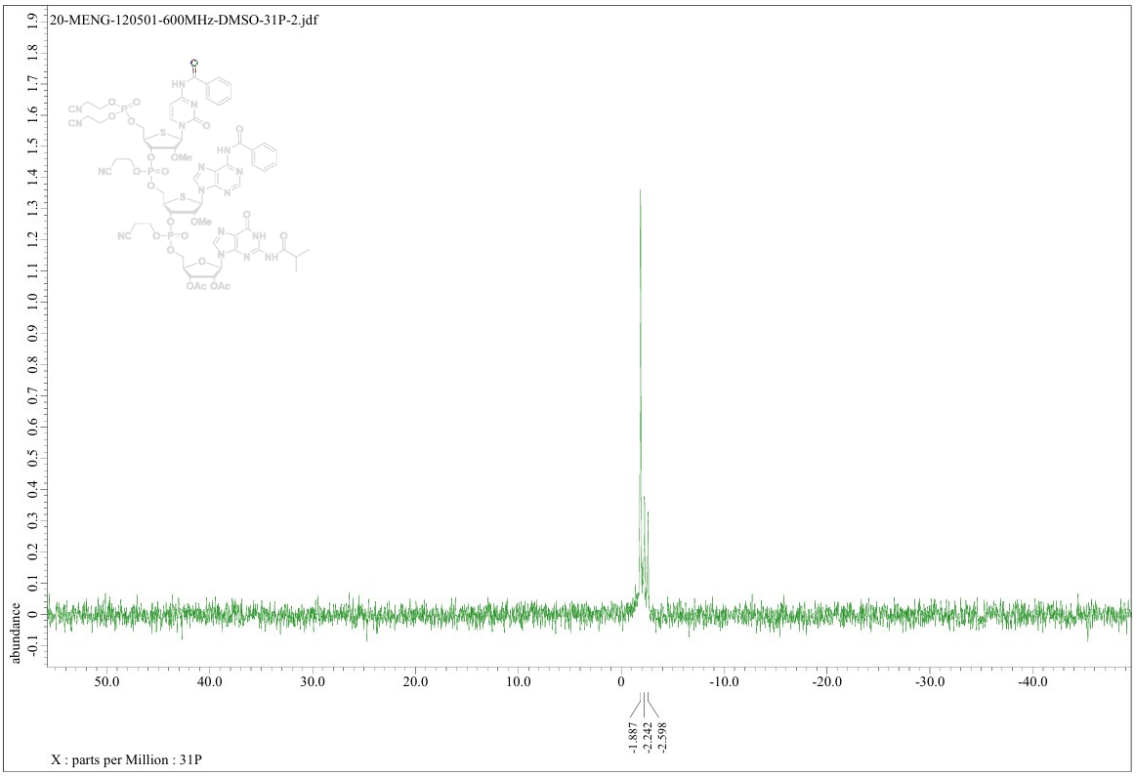
^{1}H-NMR chart of **36**



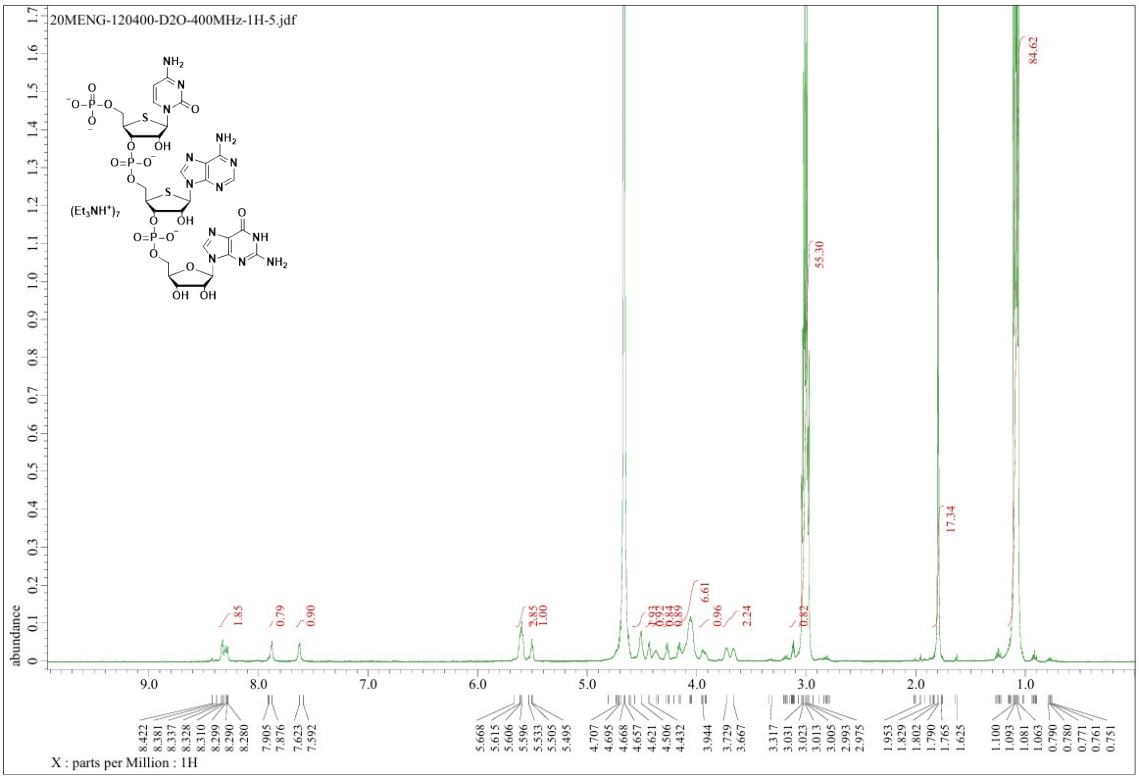
^{13}C-NMR chart of **36**



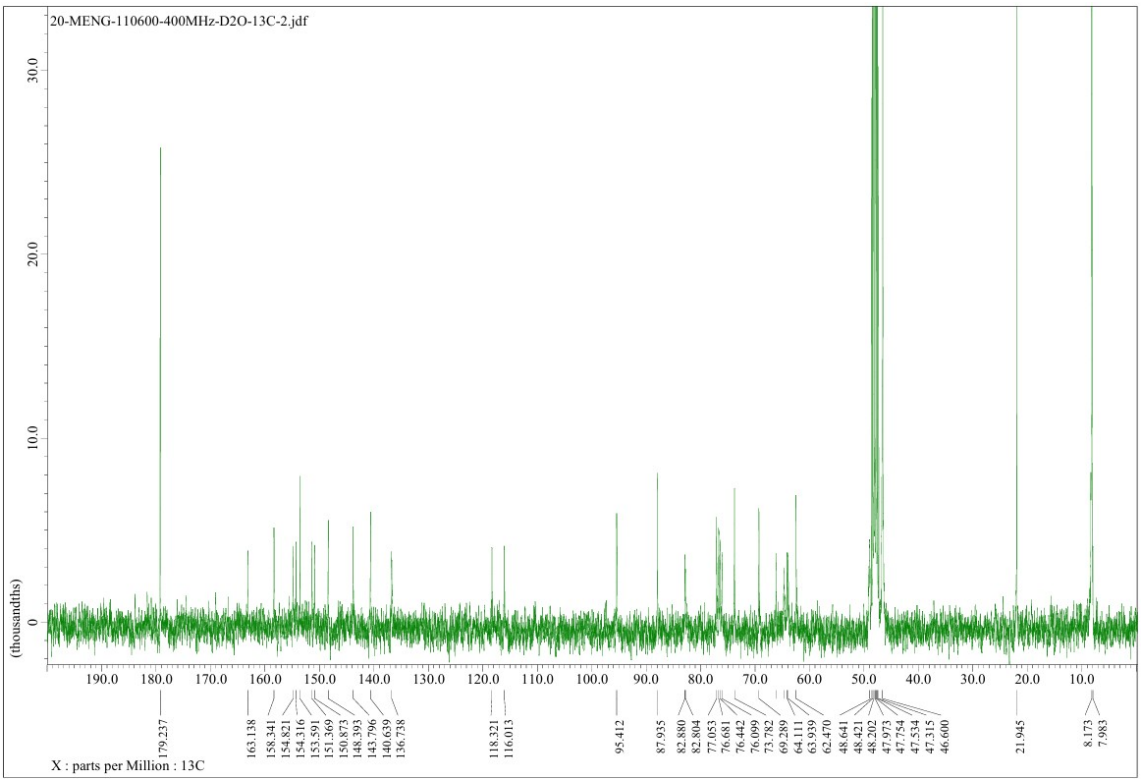
^{31}P-NMR chart of **36**



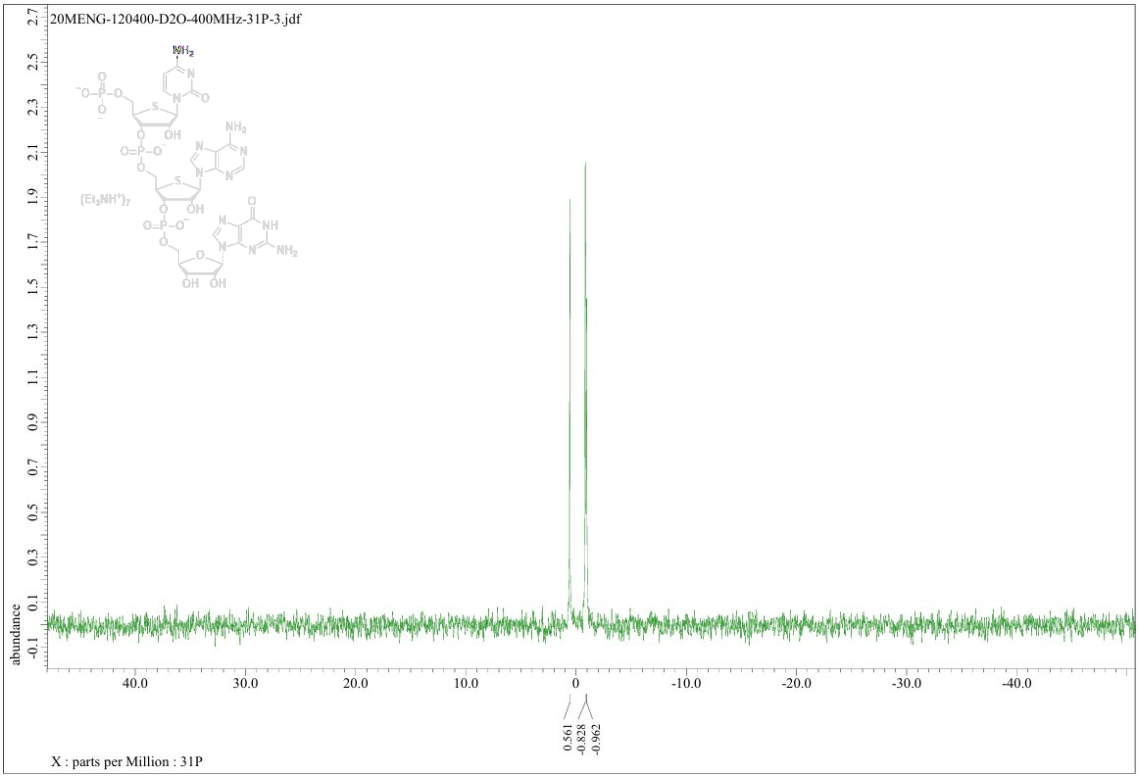
^{1}H-NMR chart of **11**



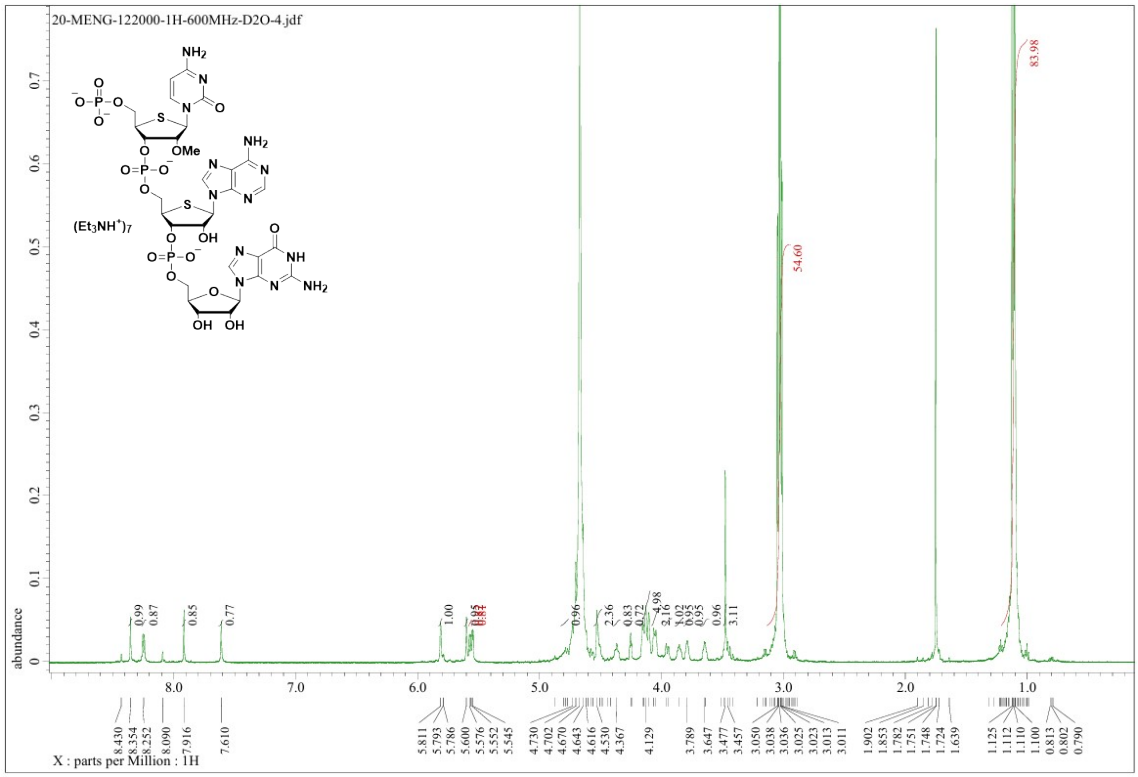
^{13}C-NMR chart of **11**



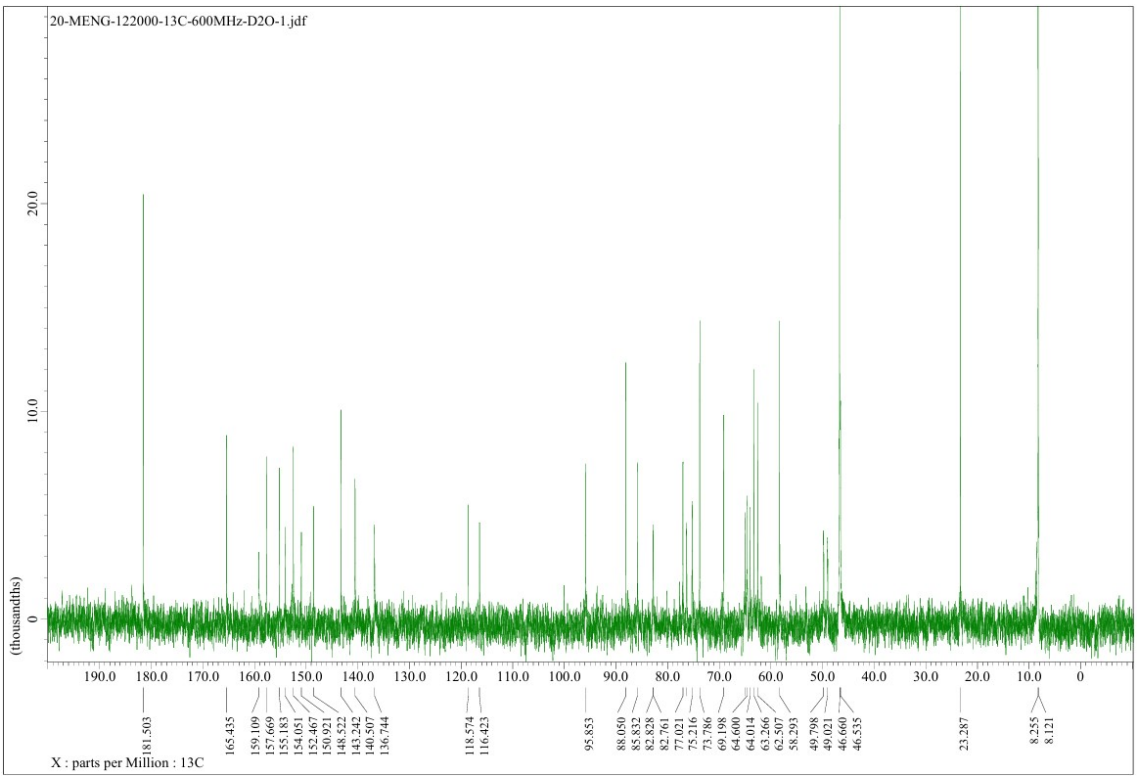
^{31}P-NMR chart of **11**



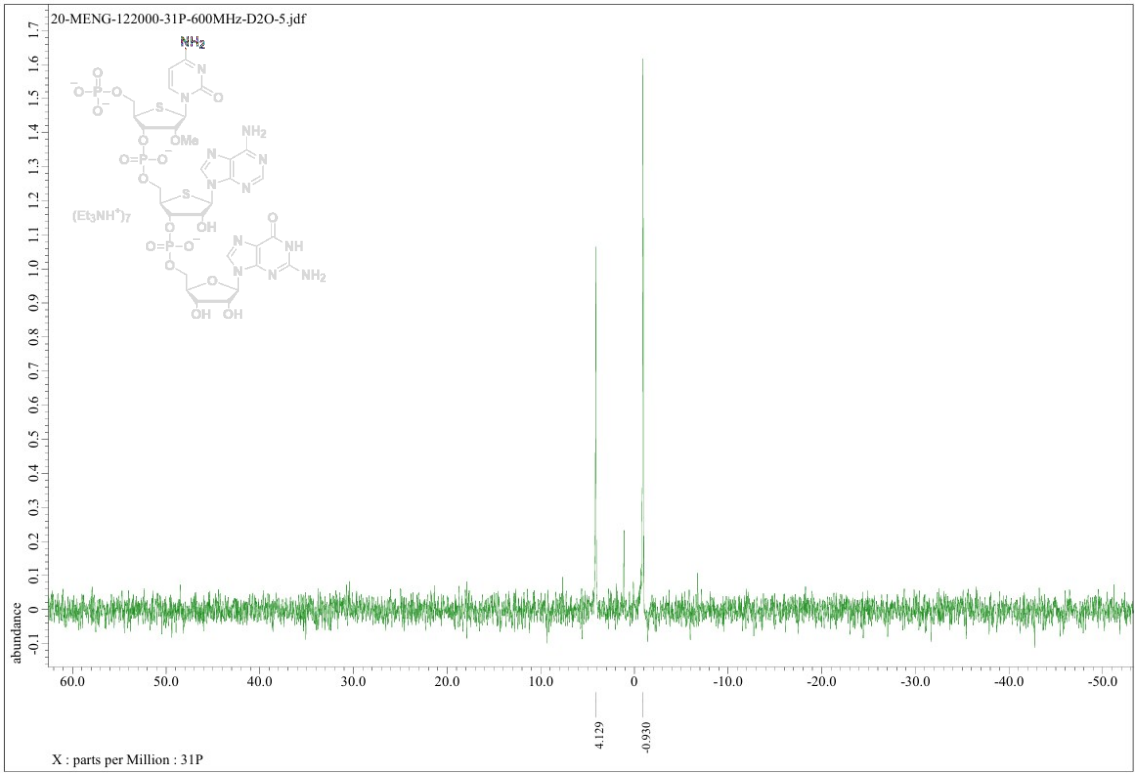
^{1}H-NMR chart of **12**



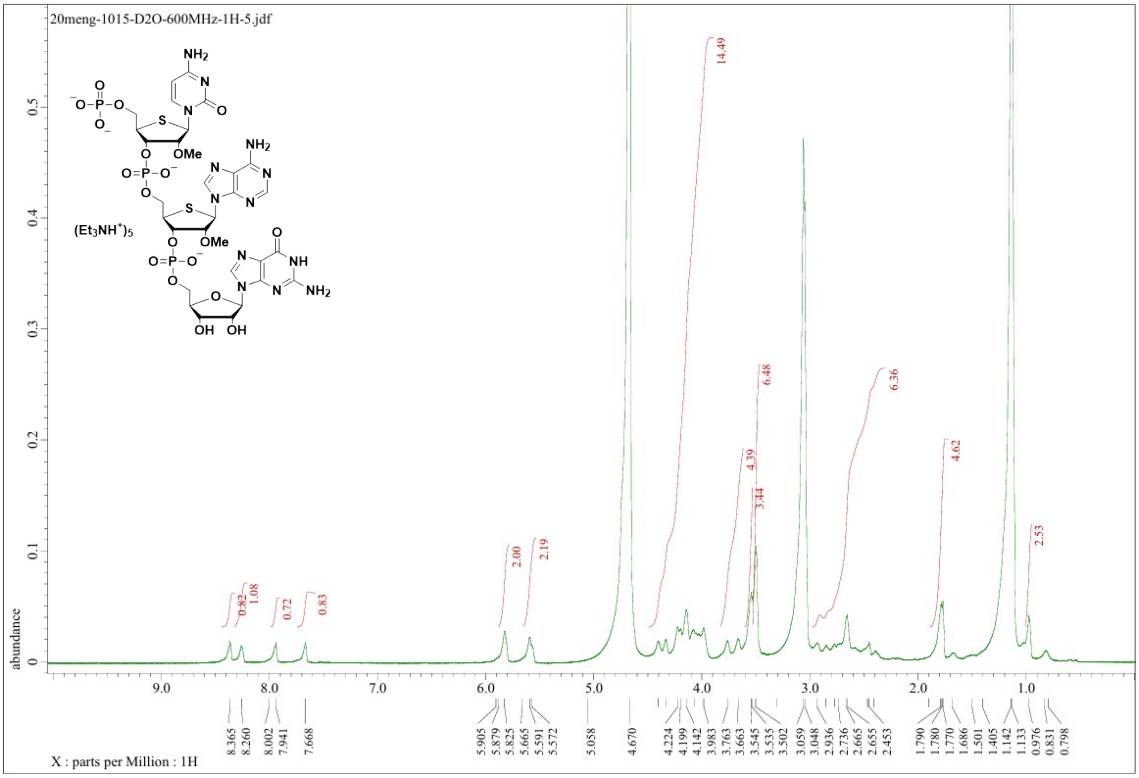
^{13}C-NMR chart of **12**



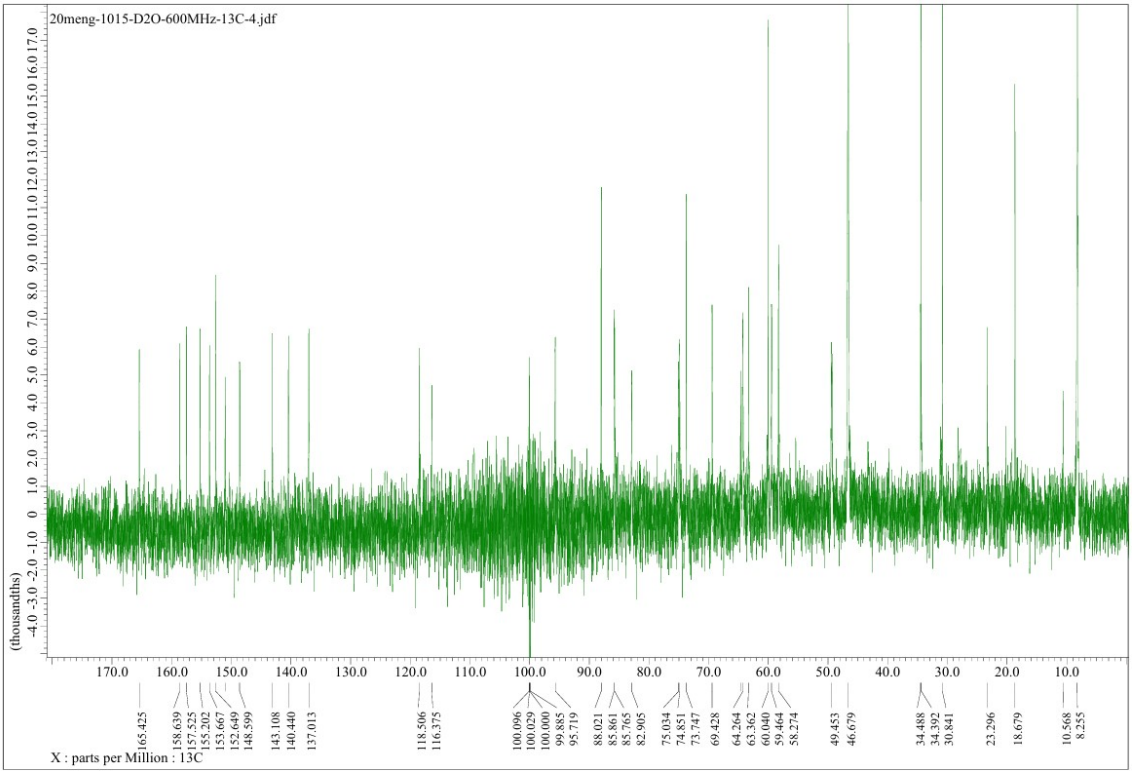
^{31}P-NMR chart of **12**



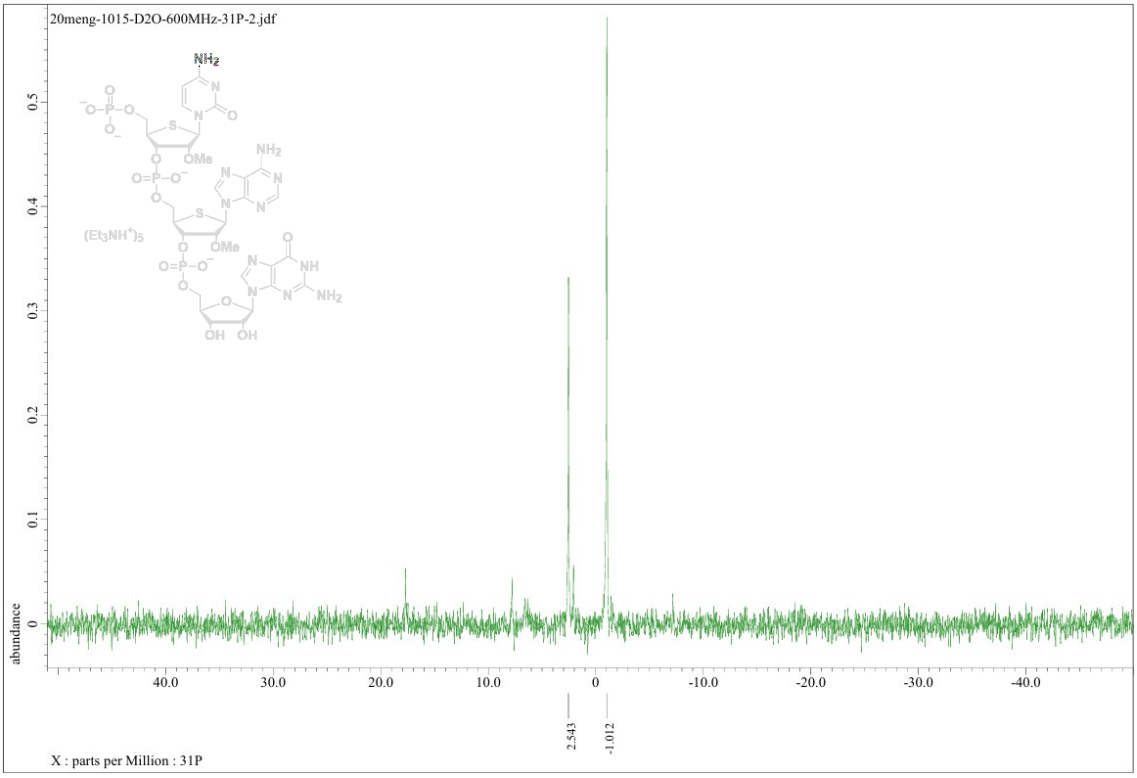
^{1}H-NMR chart of **13**



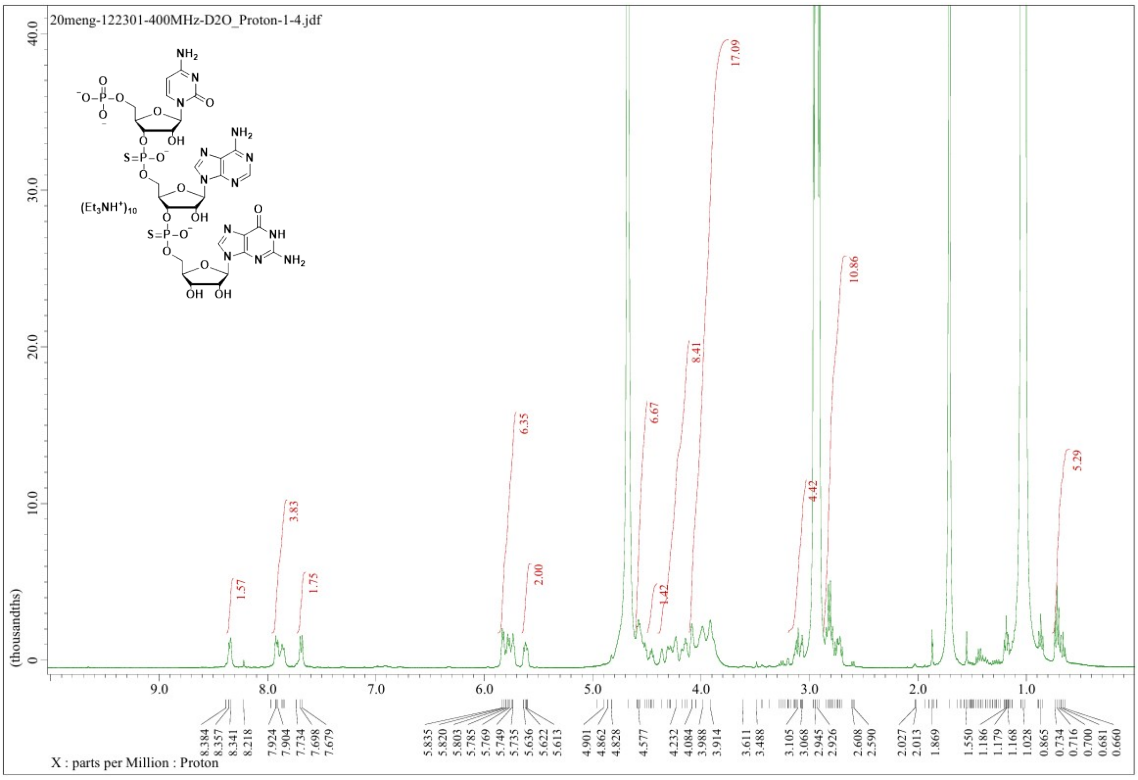
^{13}C-NMR chart of **13**



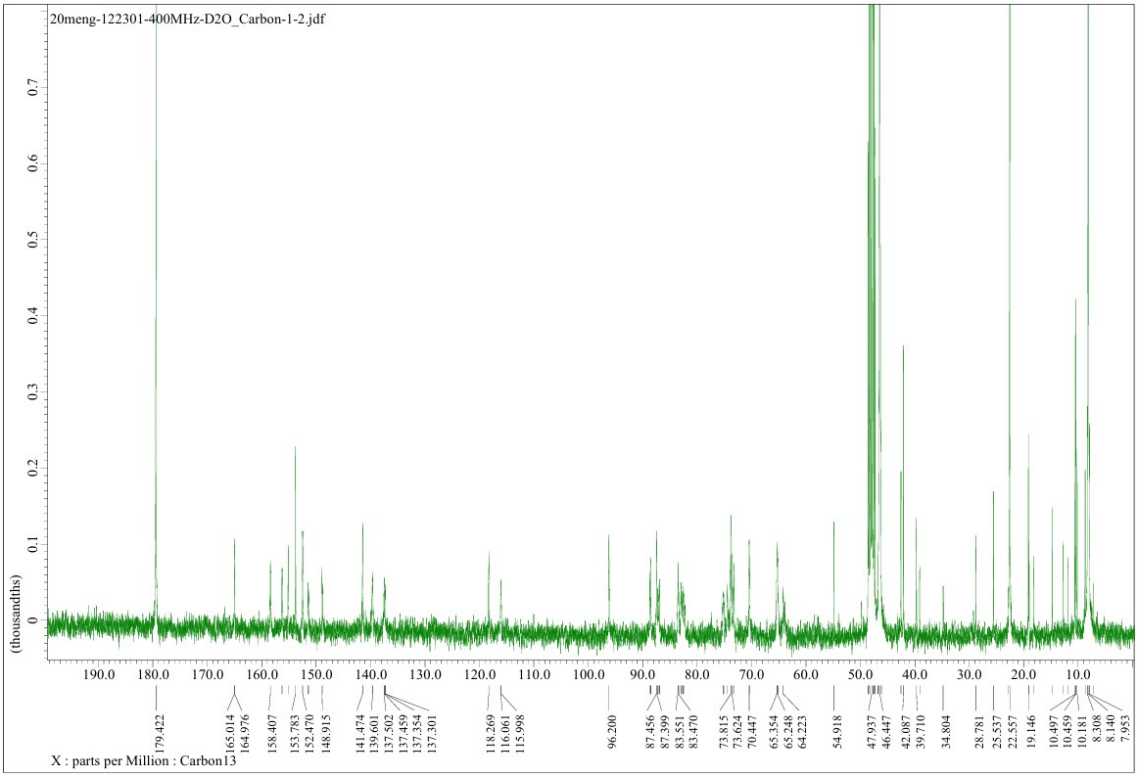
^{31}P-NMR chart of **13**



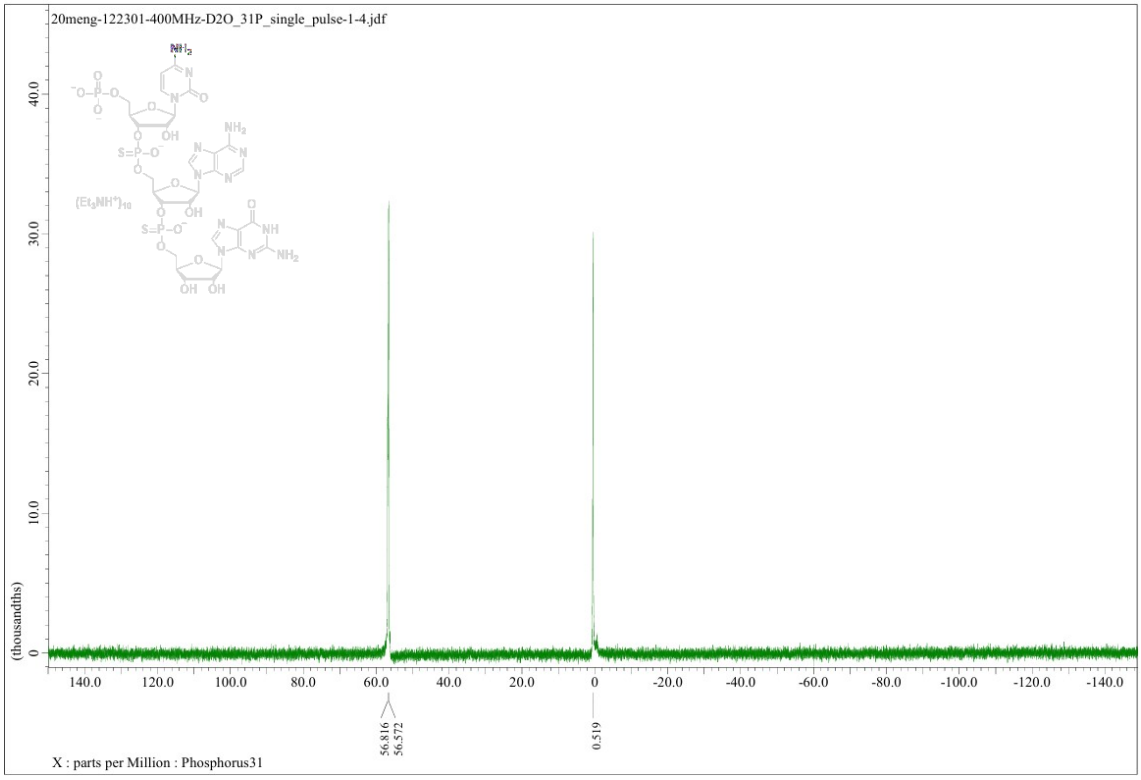
^{1}H-NMR chart of **14**



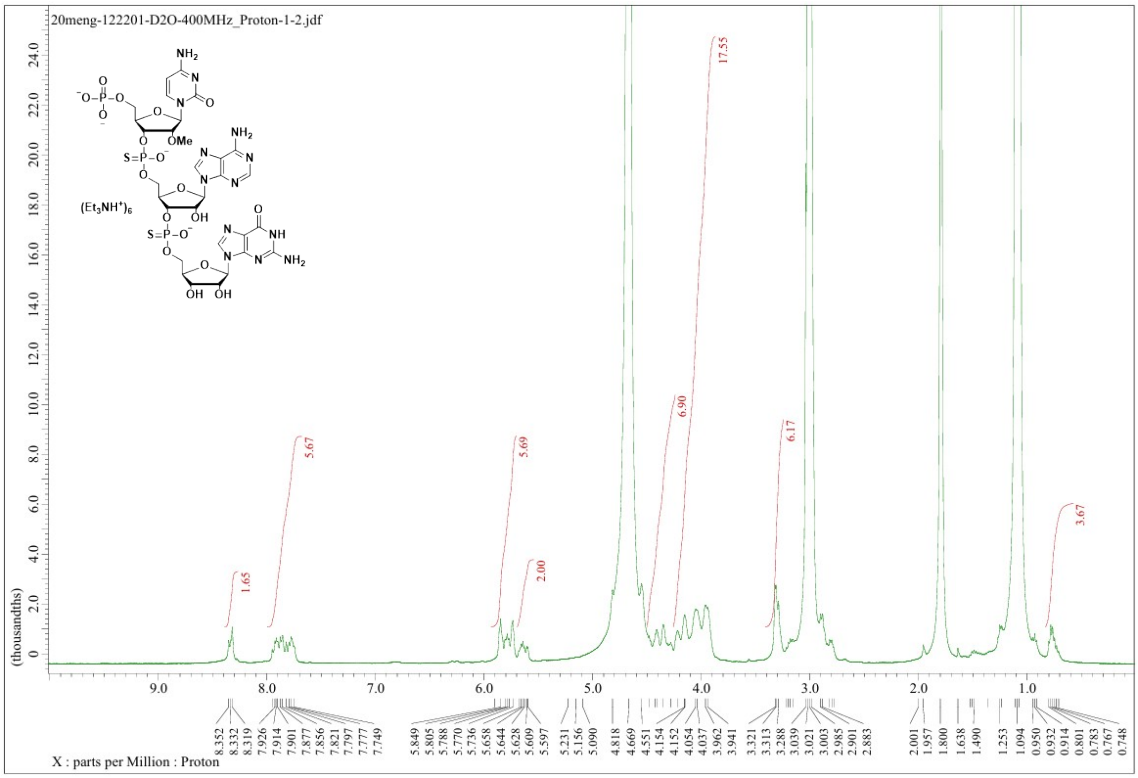
^{13}C-NMR chart of **14**



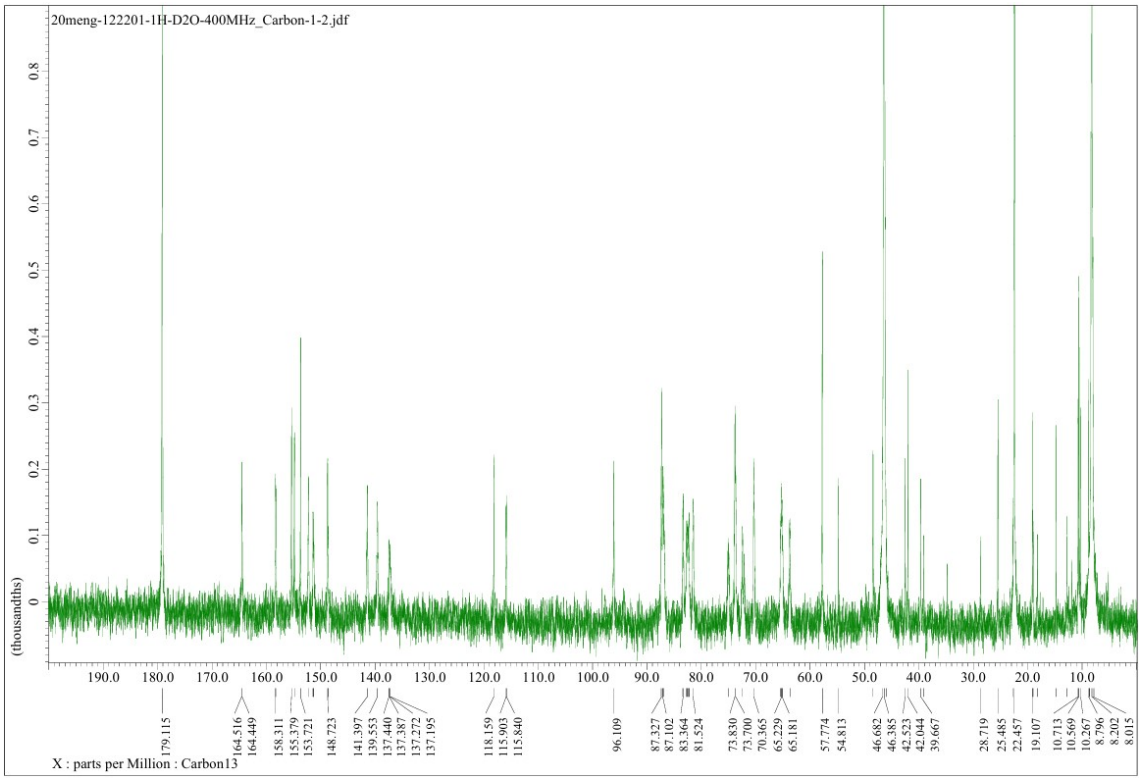
^{31}P-NMR chart of **14**



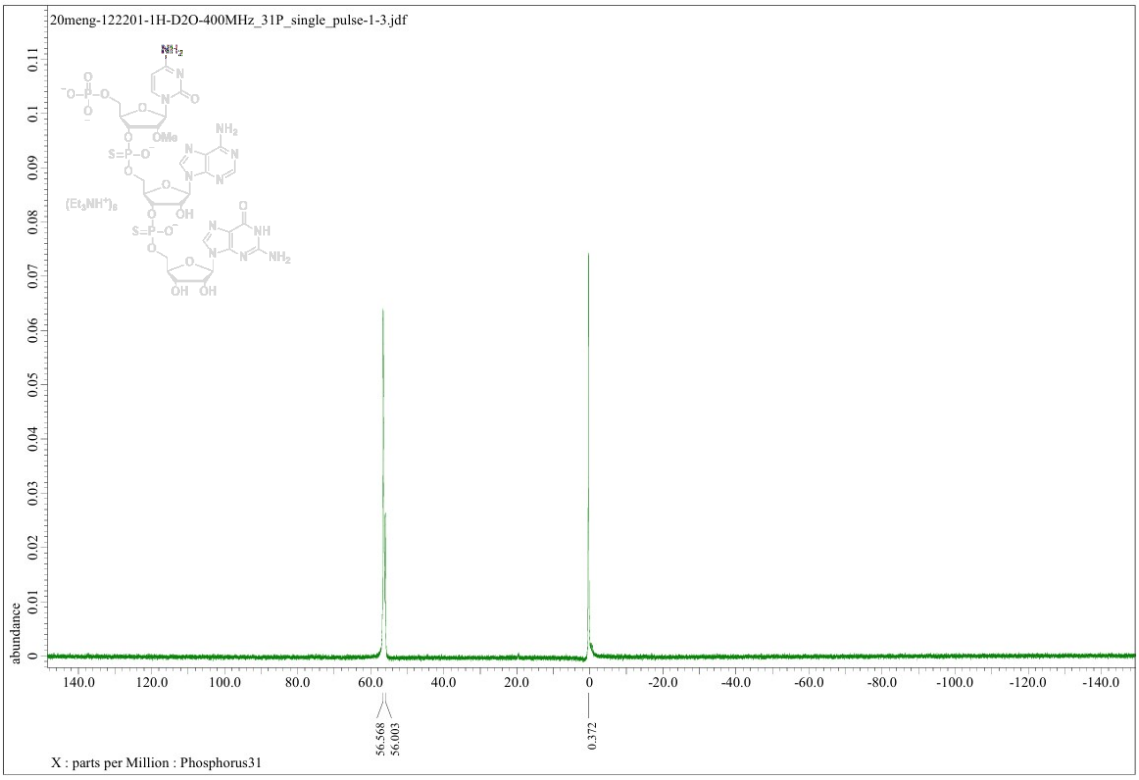
^{1}H-NMR chart of **15**



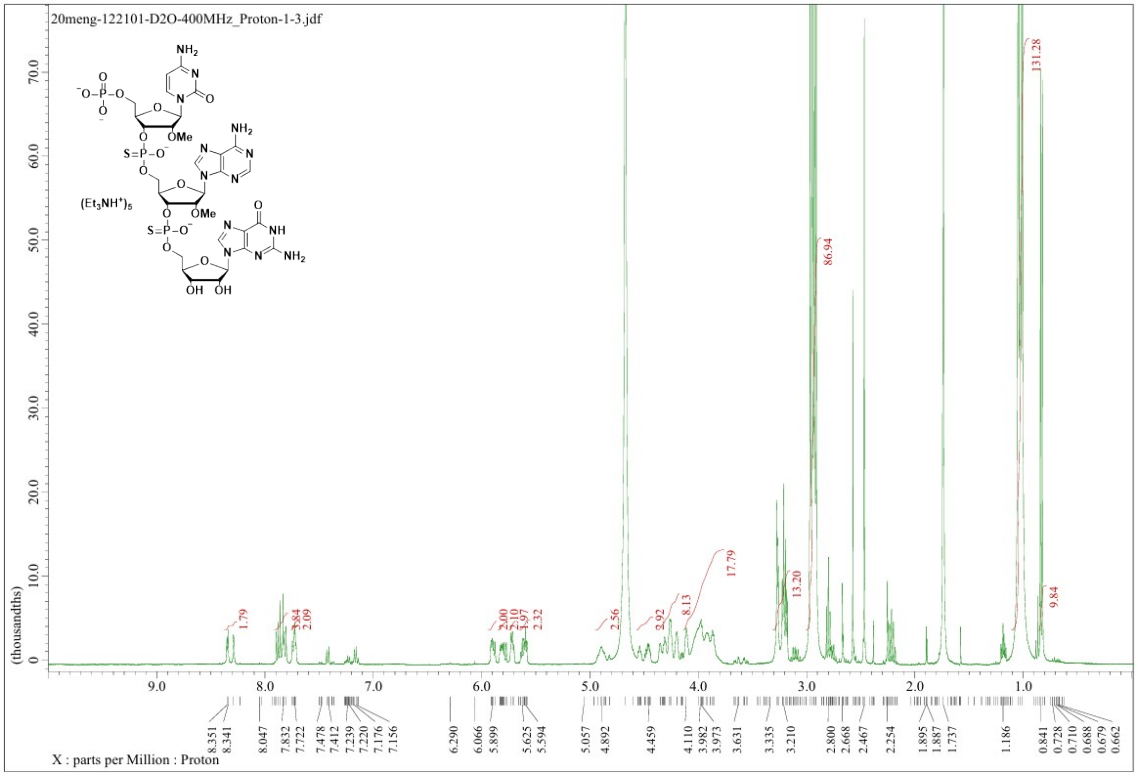
^{13}C-NMR chart of **15**



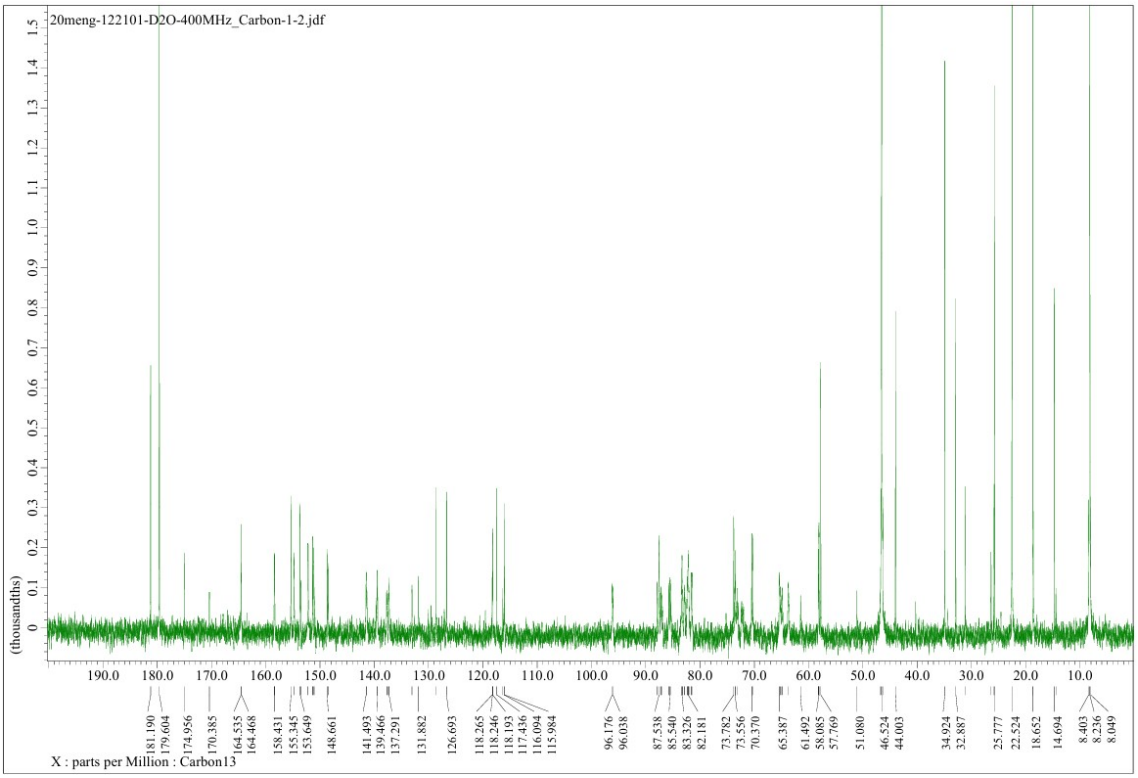
^{31}P-NMR chart of **15**



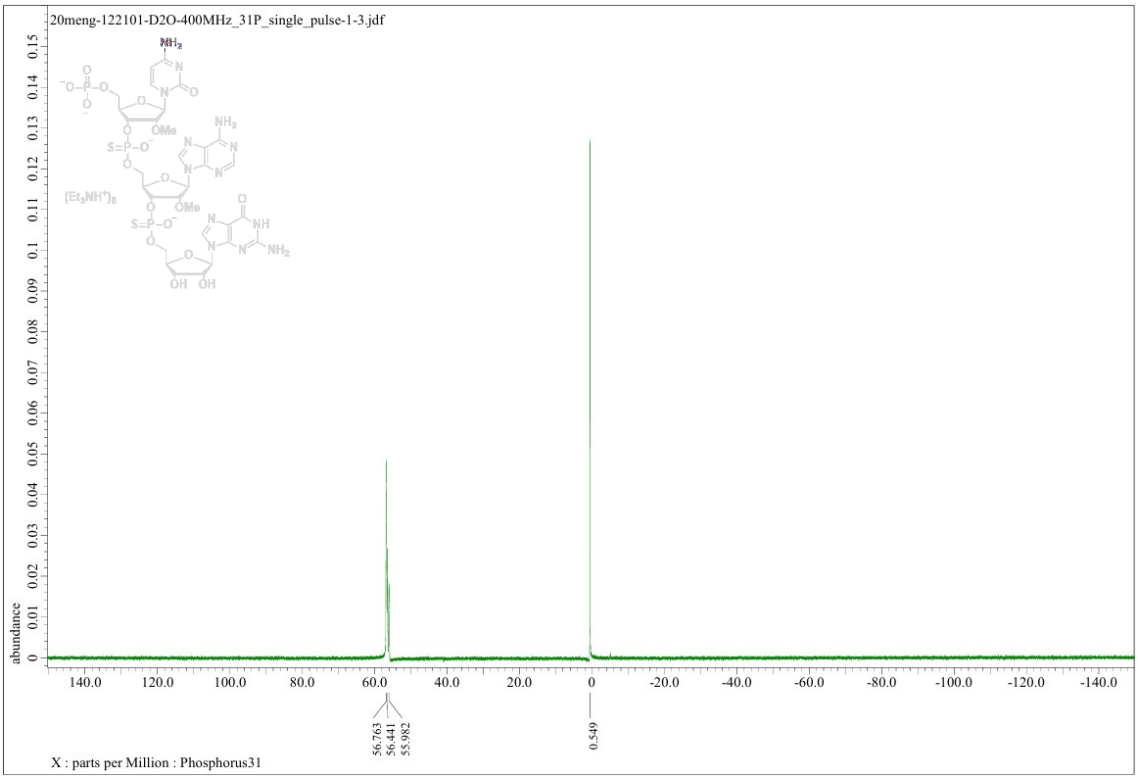
^{1}H-NMR chart of **16**



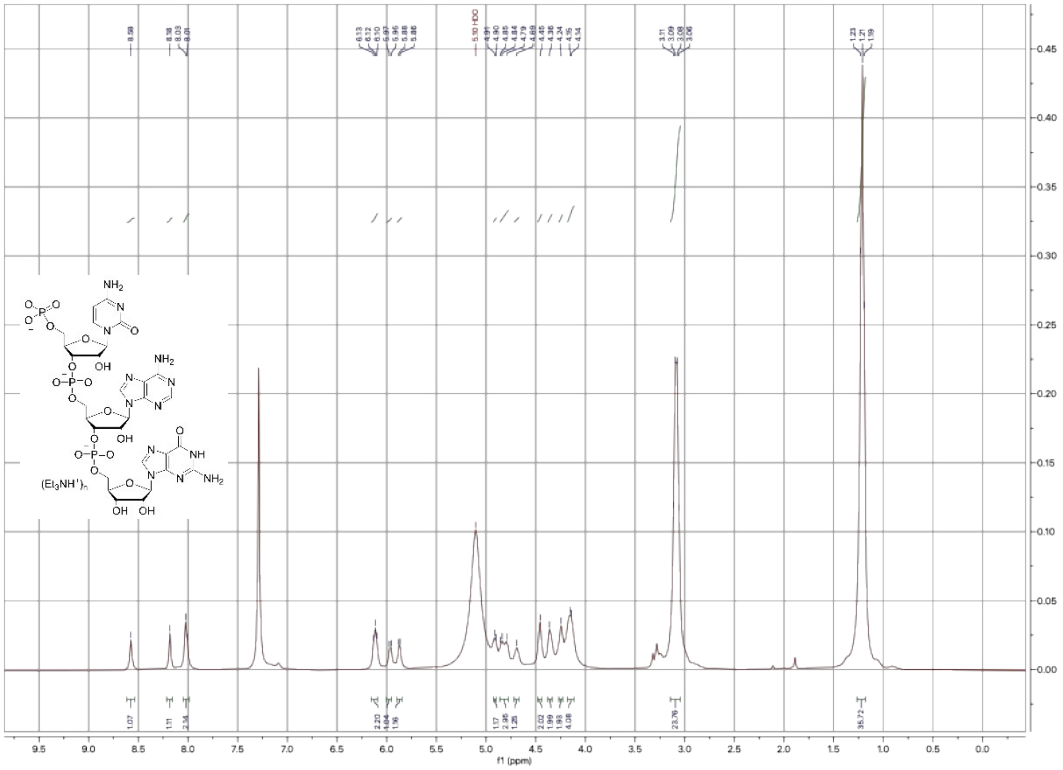
^{13}C-NMR chart of **16**



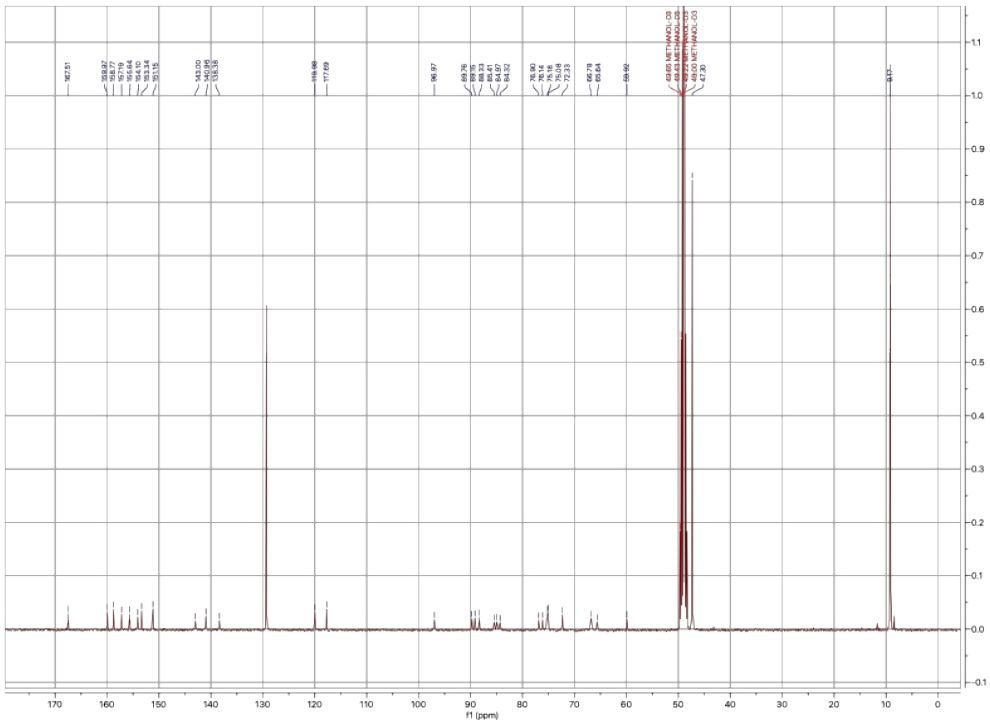
^{31}P-NMR chart of **16**



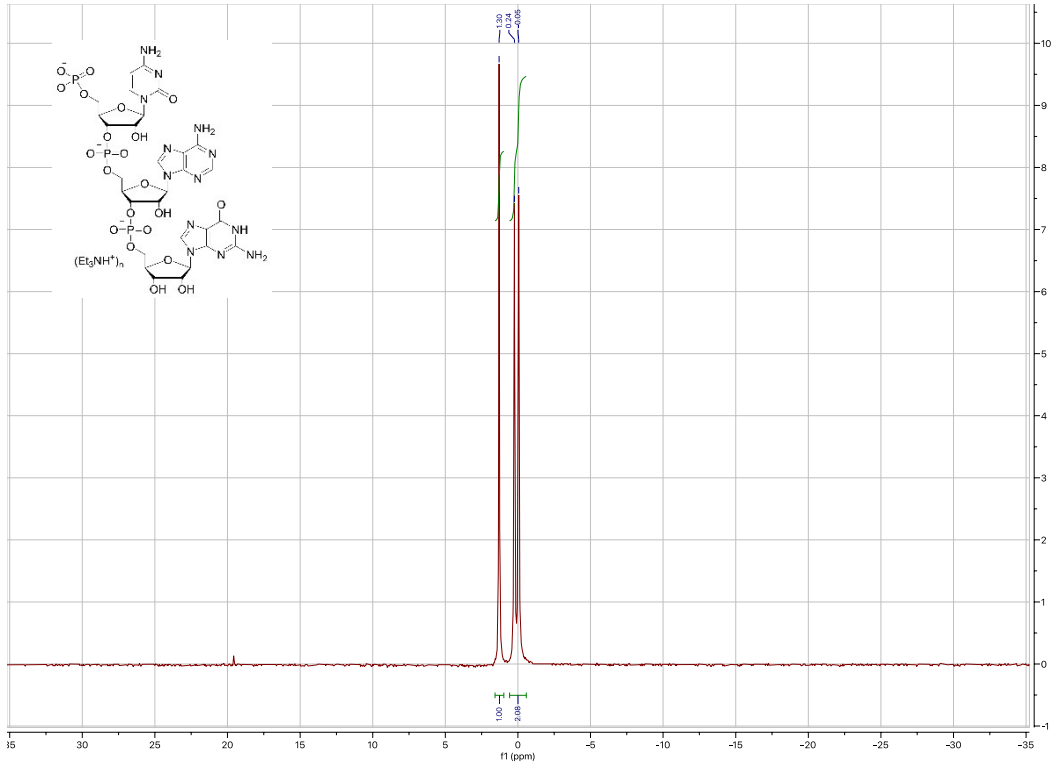
^{1}H-NMR chart of **17**



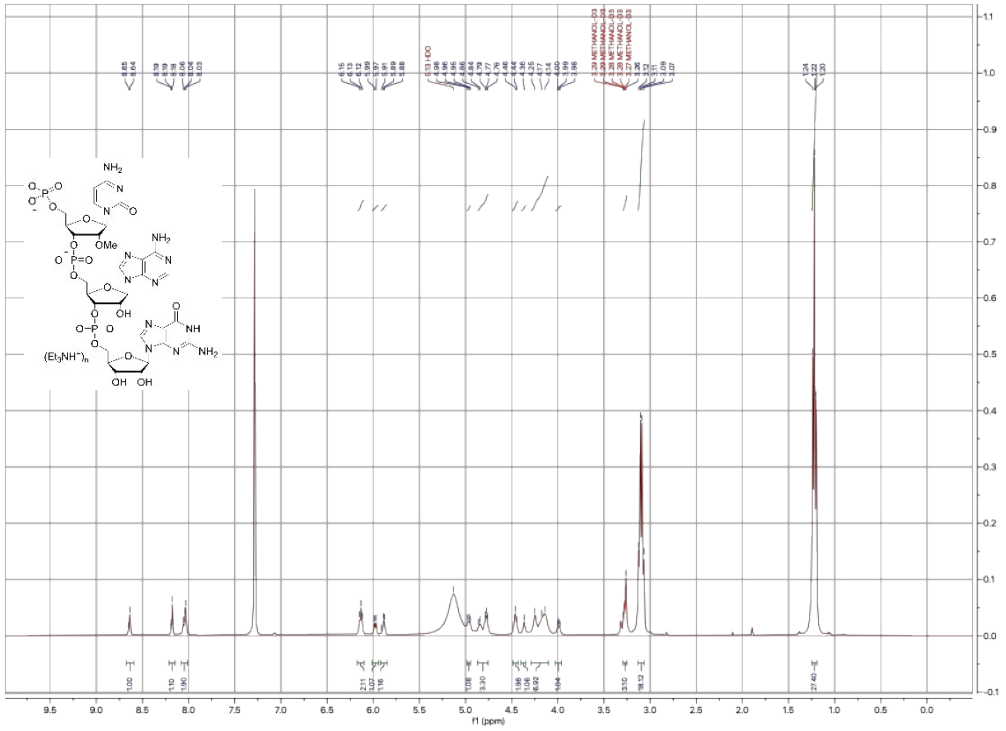
^{13}C-NMR chart of **17**



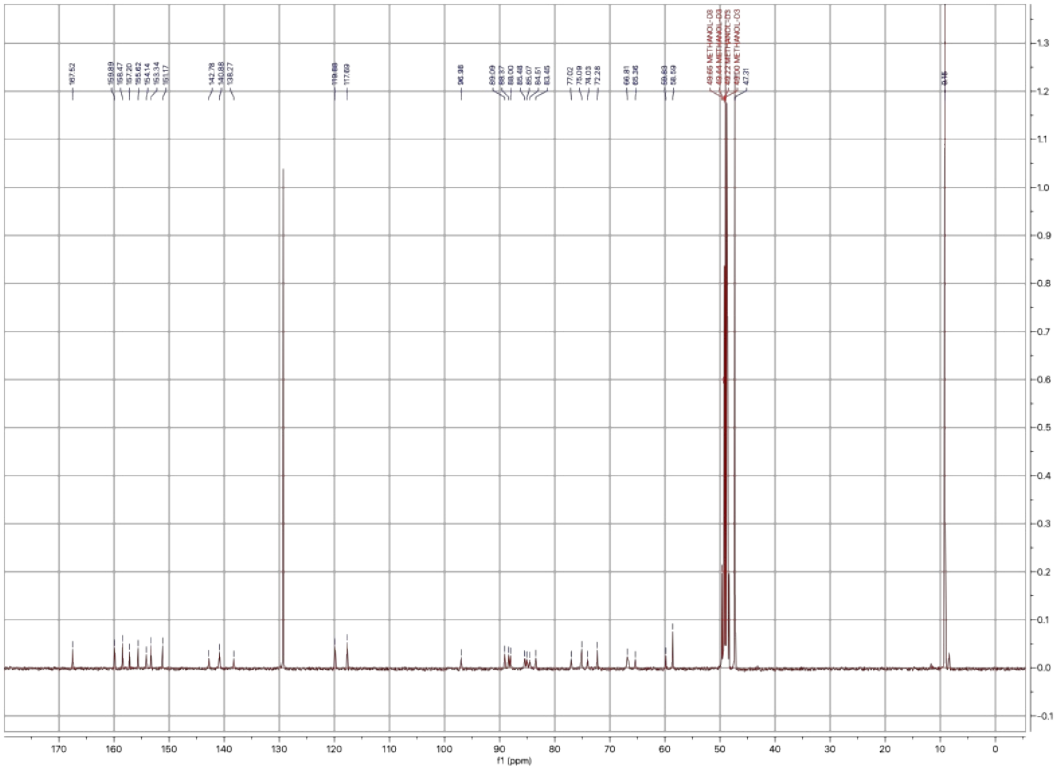
^{31}P-NMR chart of **17**



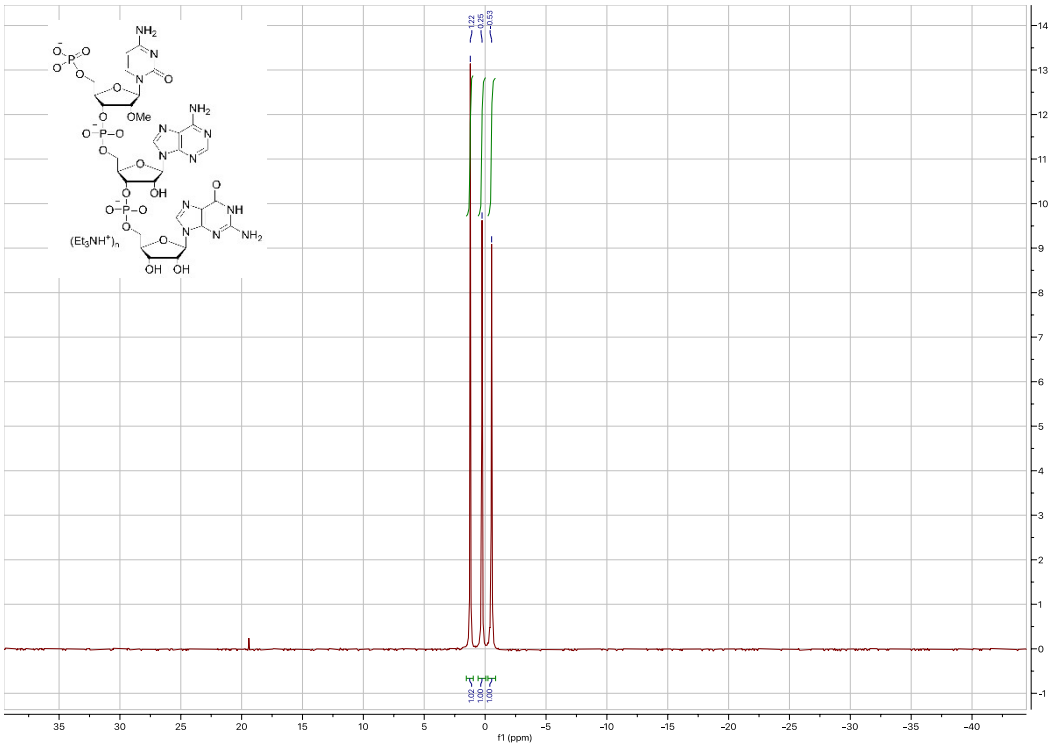
^{1}H-NMR chart of **18**



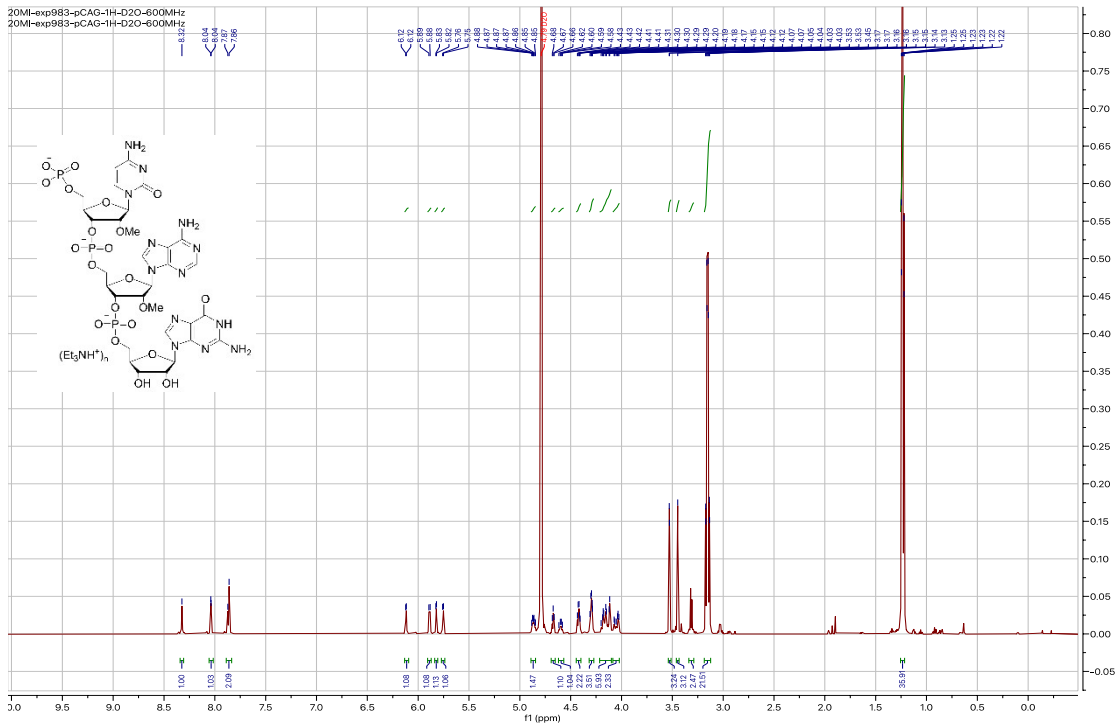
^{13}C-NMR chart of **18**



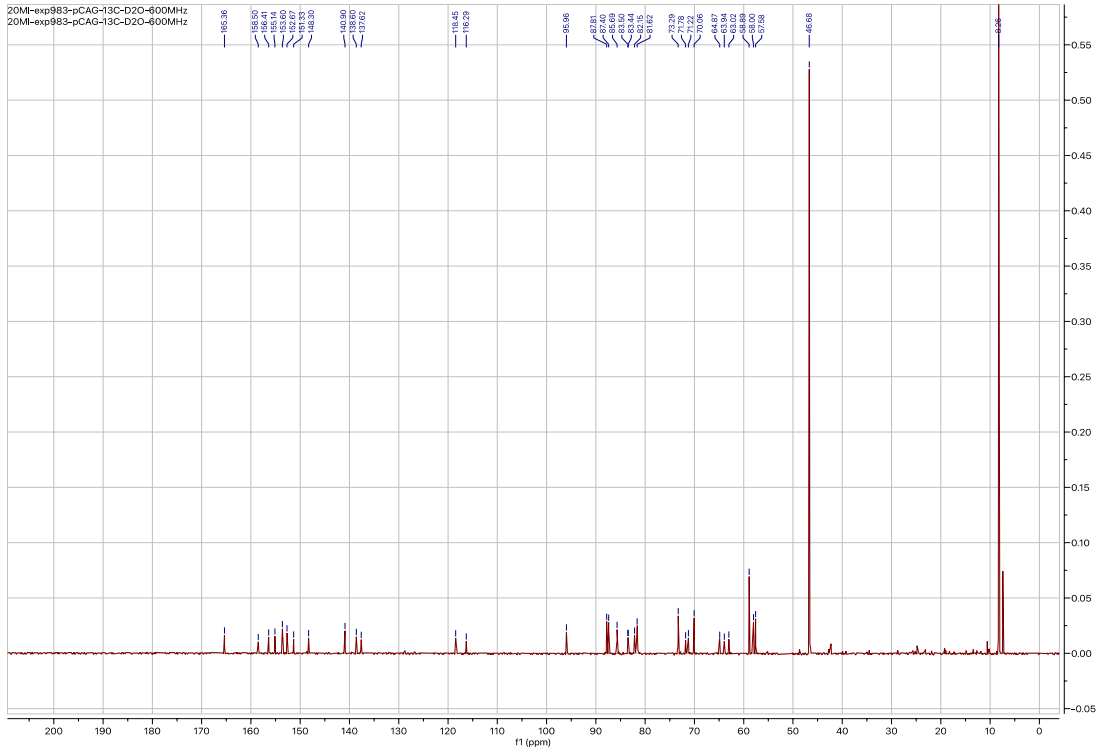
^{31}P-NMR chart of **18**



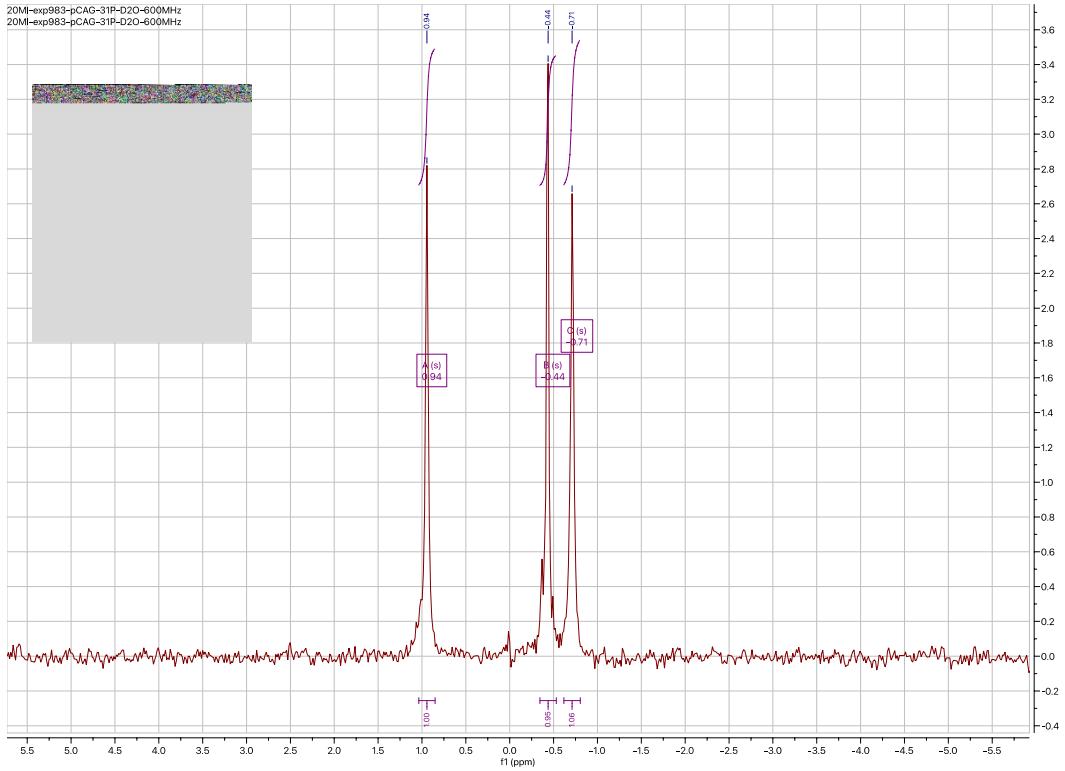
^{1}H-NMR chart of **19**



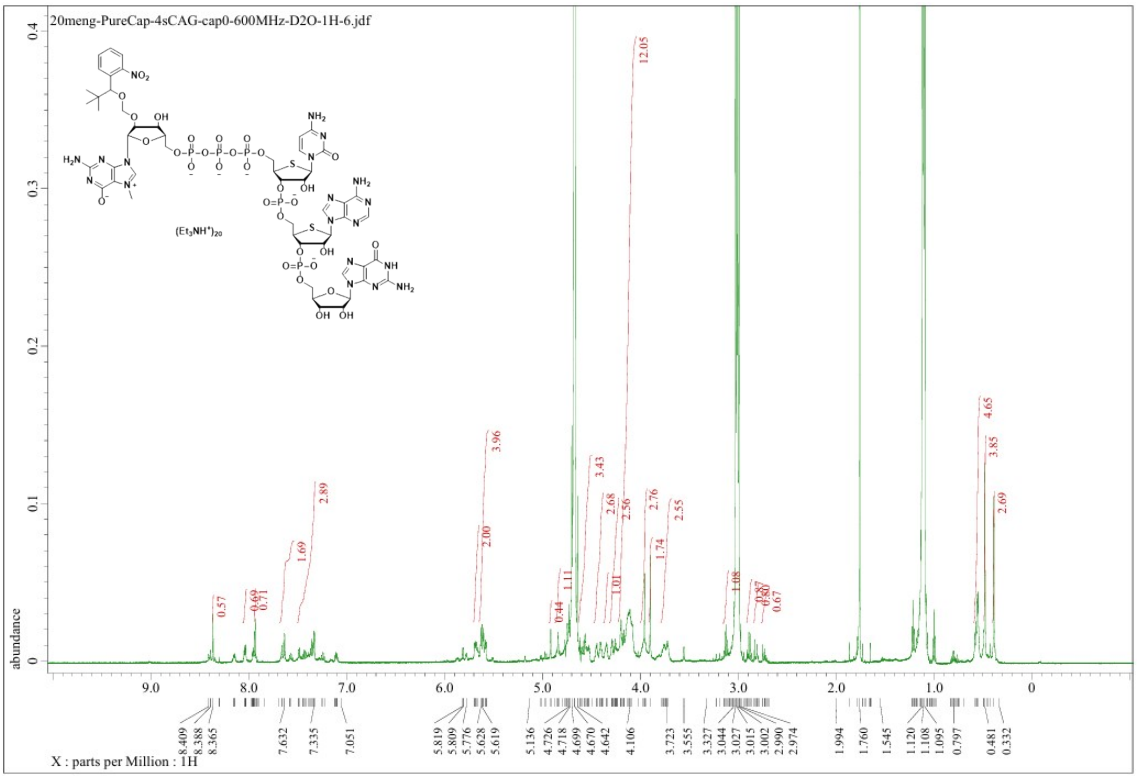
^{13}C-NMR chart of **19**



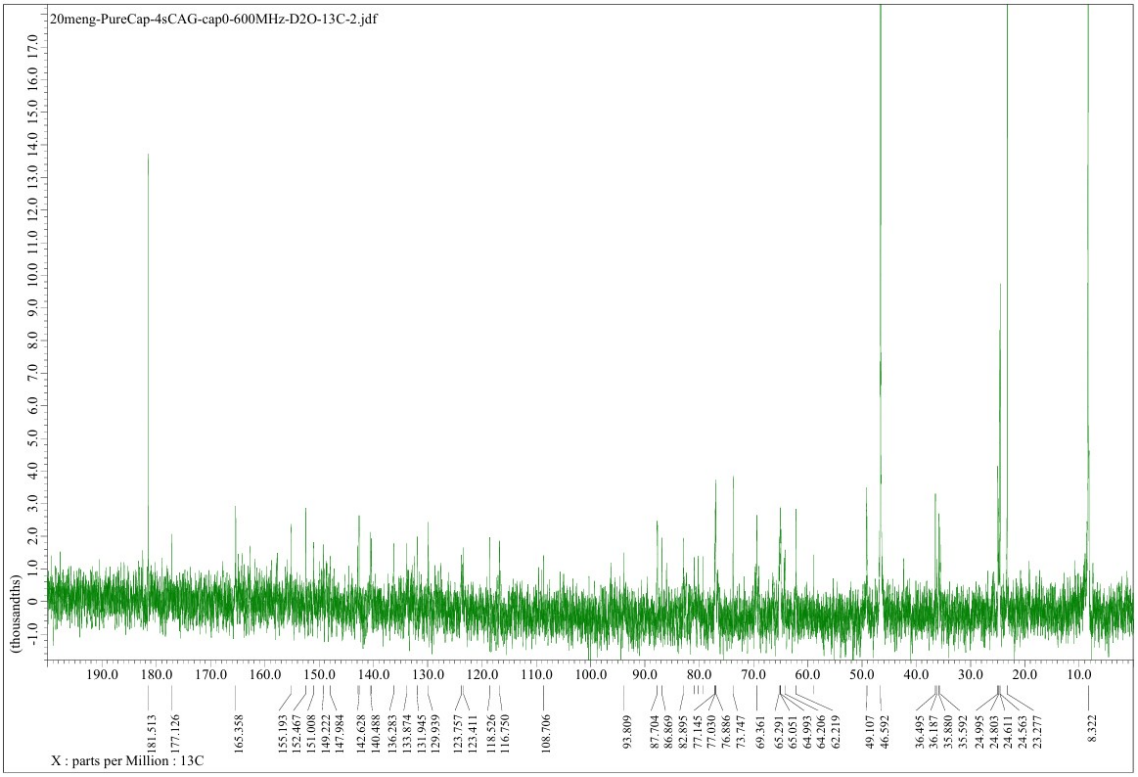
^{31}P-NMR chart of **19**



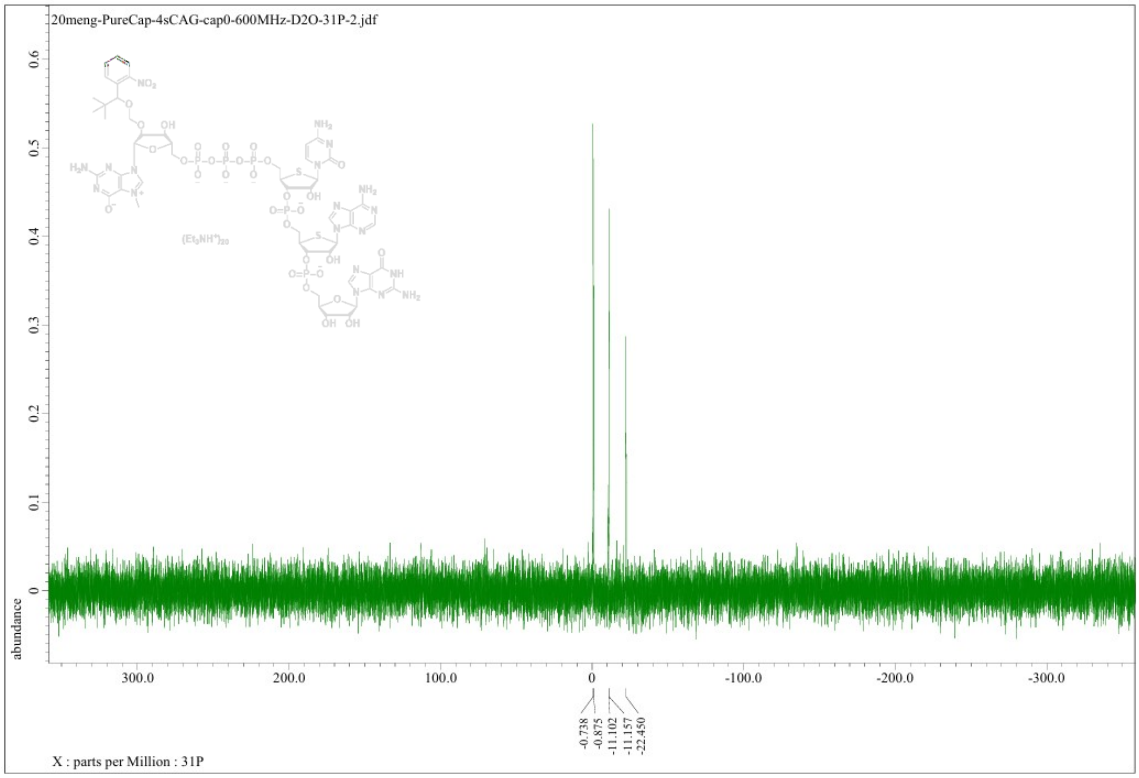
^{1}H-NMR chart of **1**



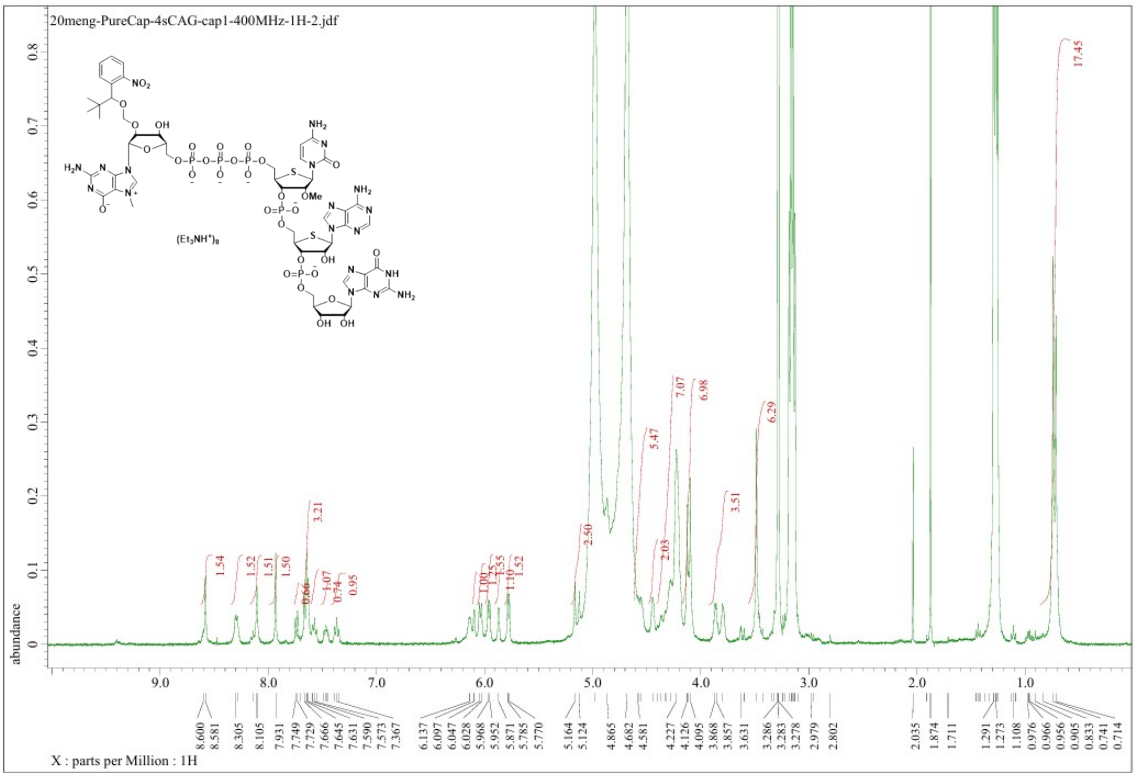
^{13}C-NMR chart of **1**



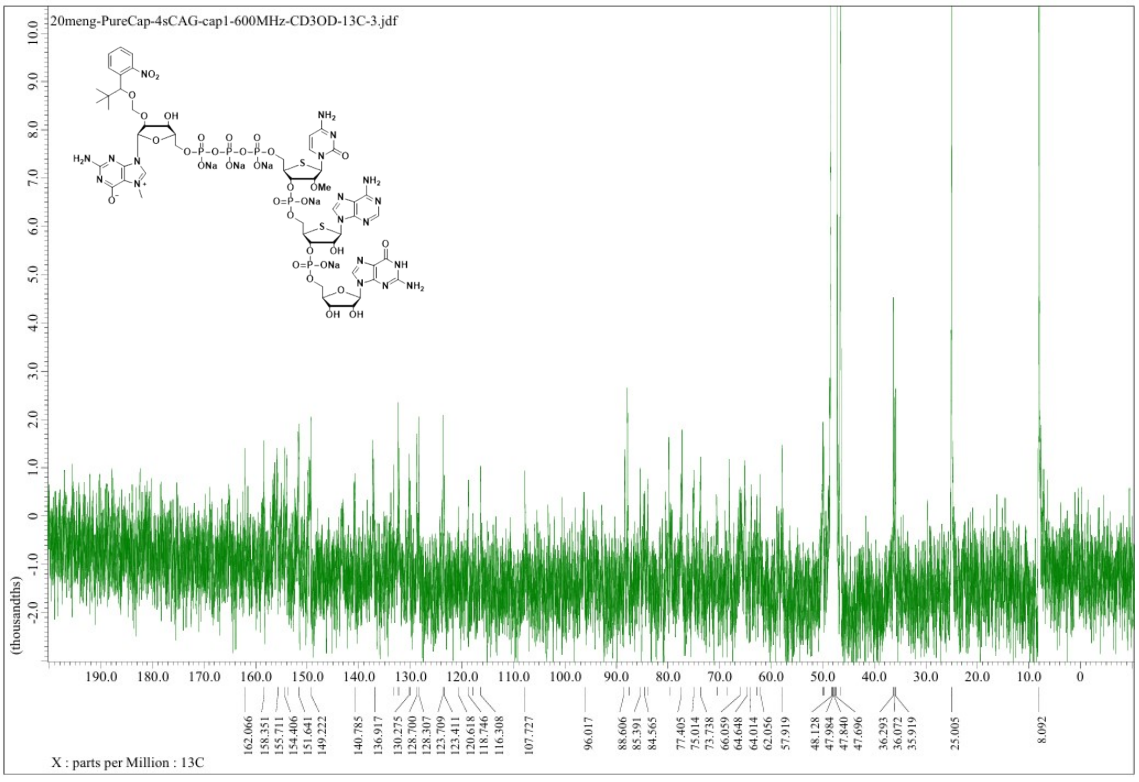
^{31}P-NMR chart of **1**



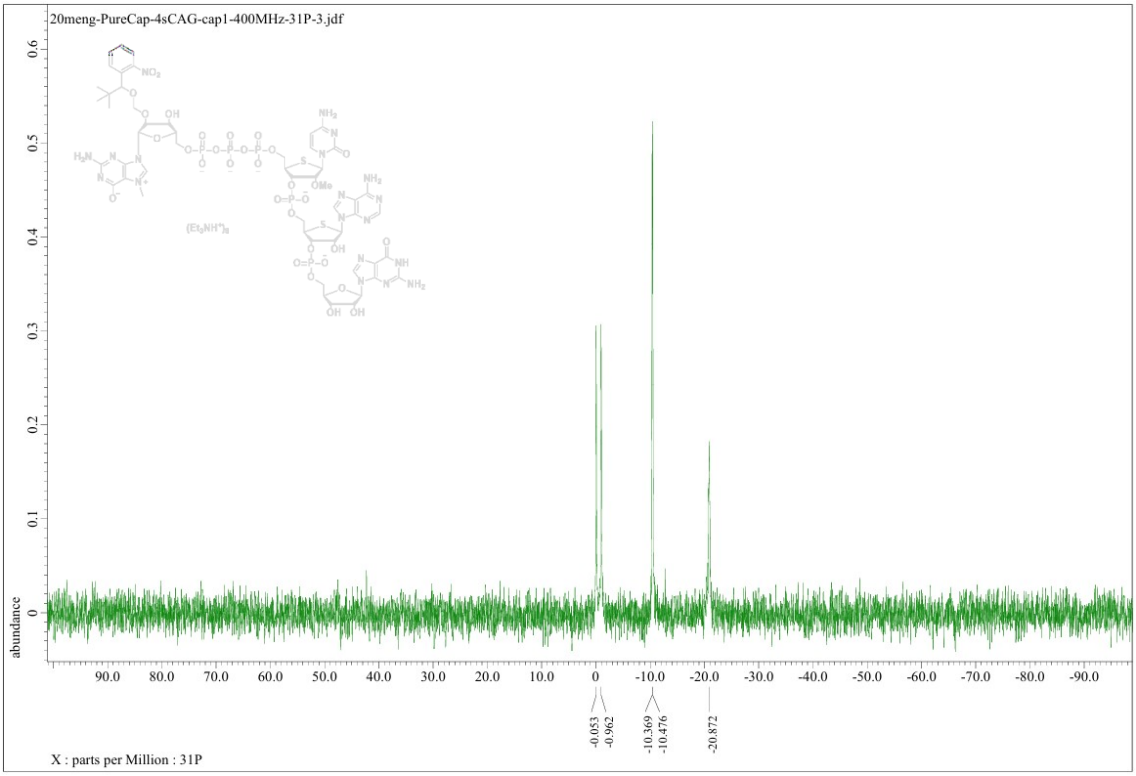
^{1}H-NMR chart of **2**



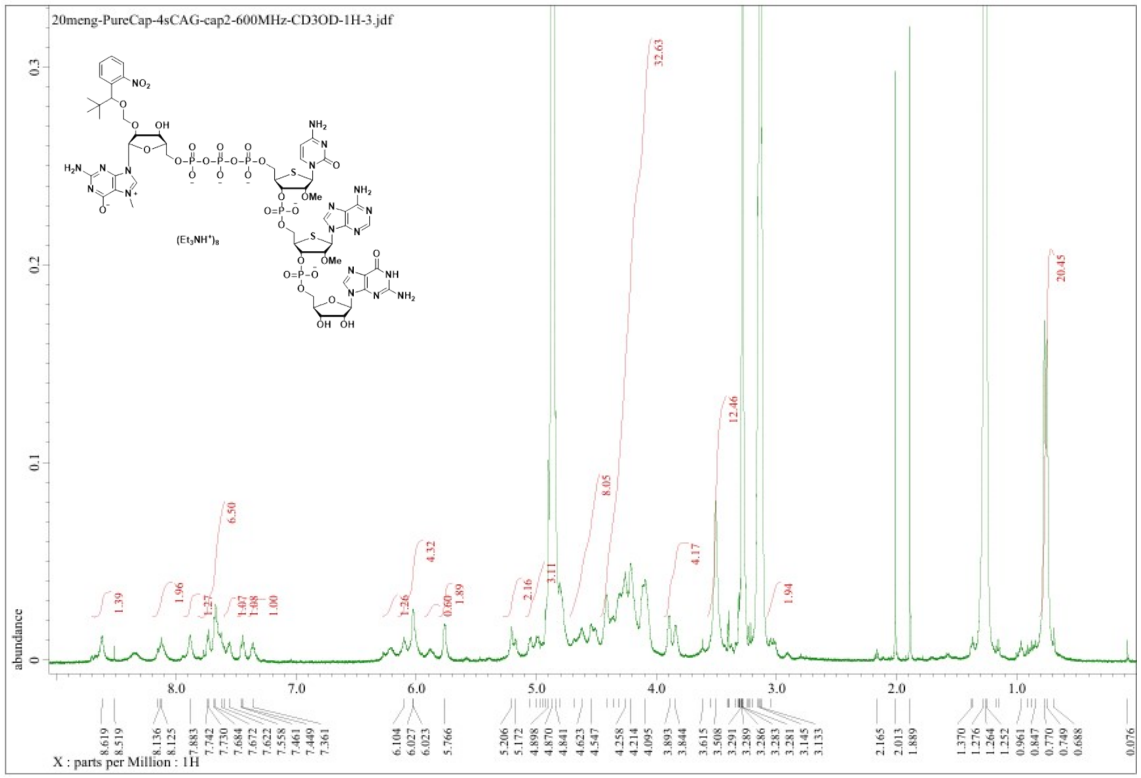
^{13}C-NMR chart of **2**



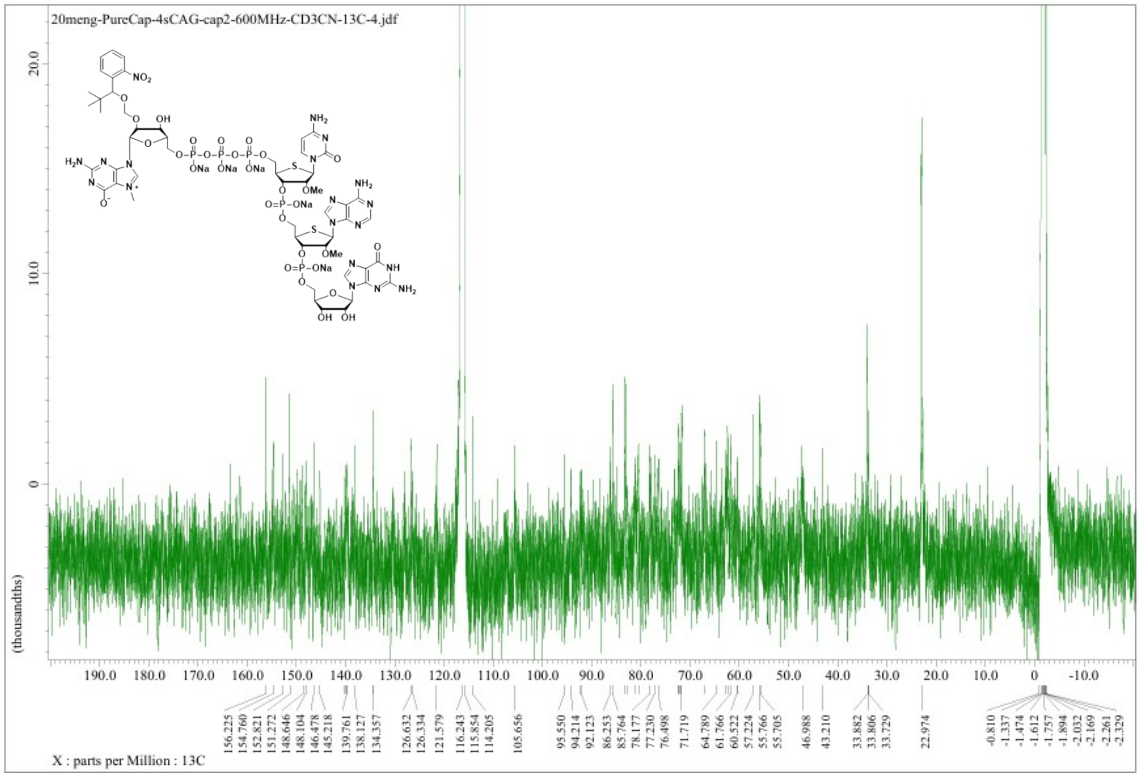
^{31}P-NMR chart of **2**



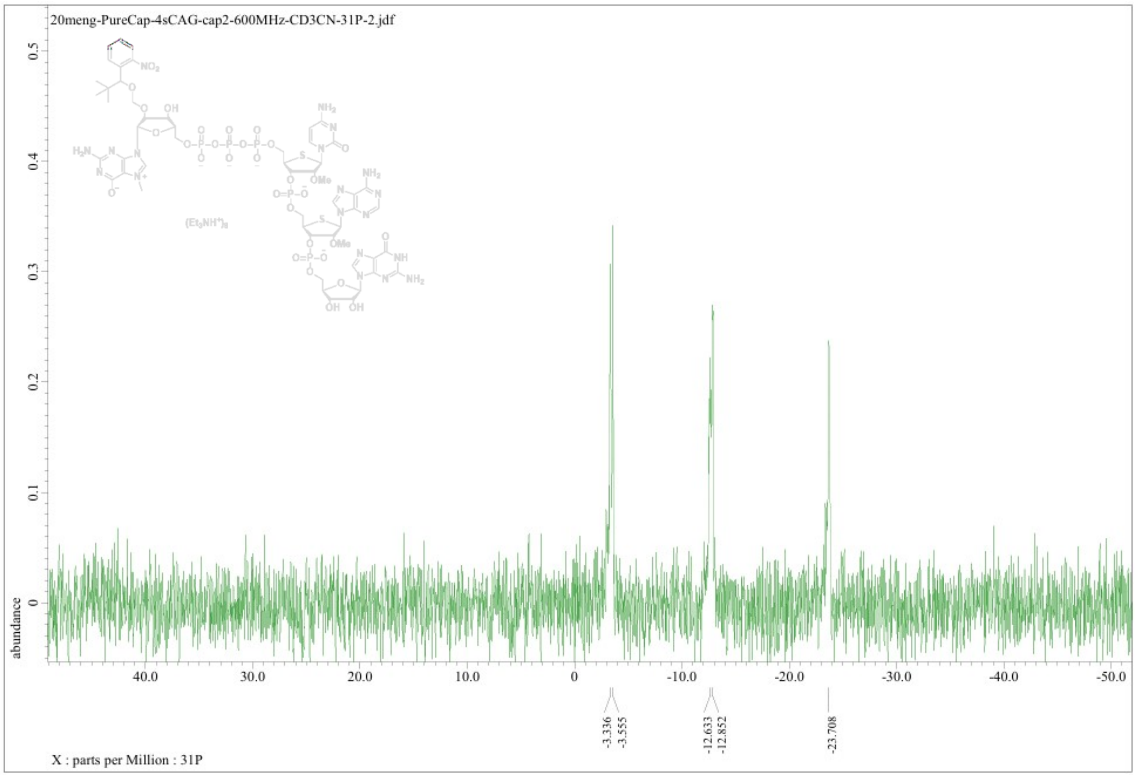
^{1}H-NMR chart of **3**



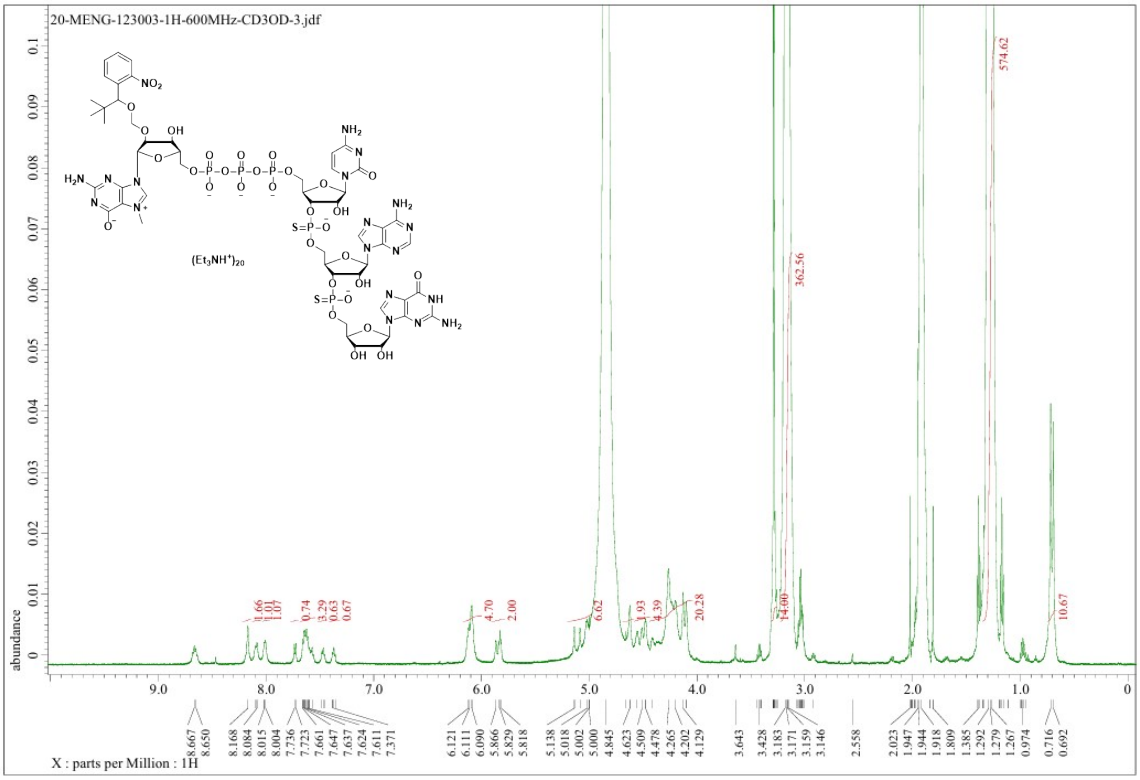
^{13}C-NMR chart of **3**



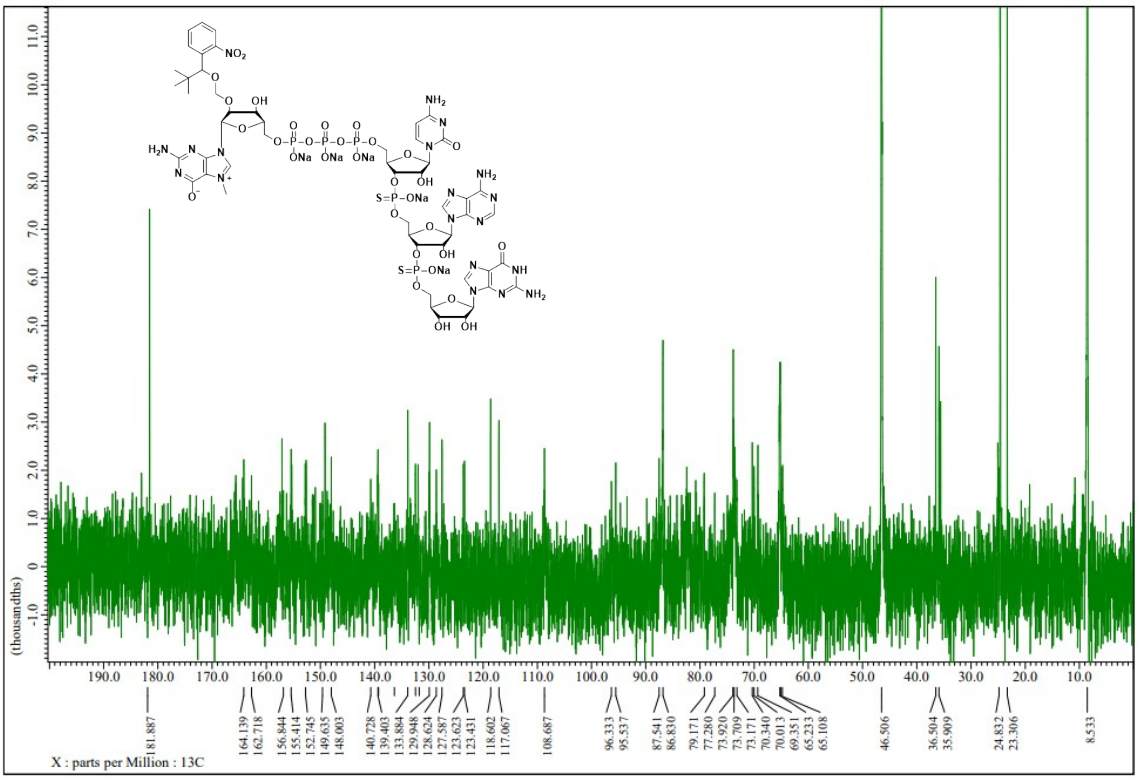
^{31}P-NMR chart of **3**



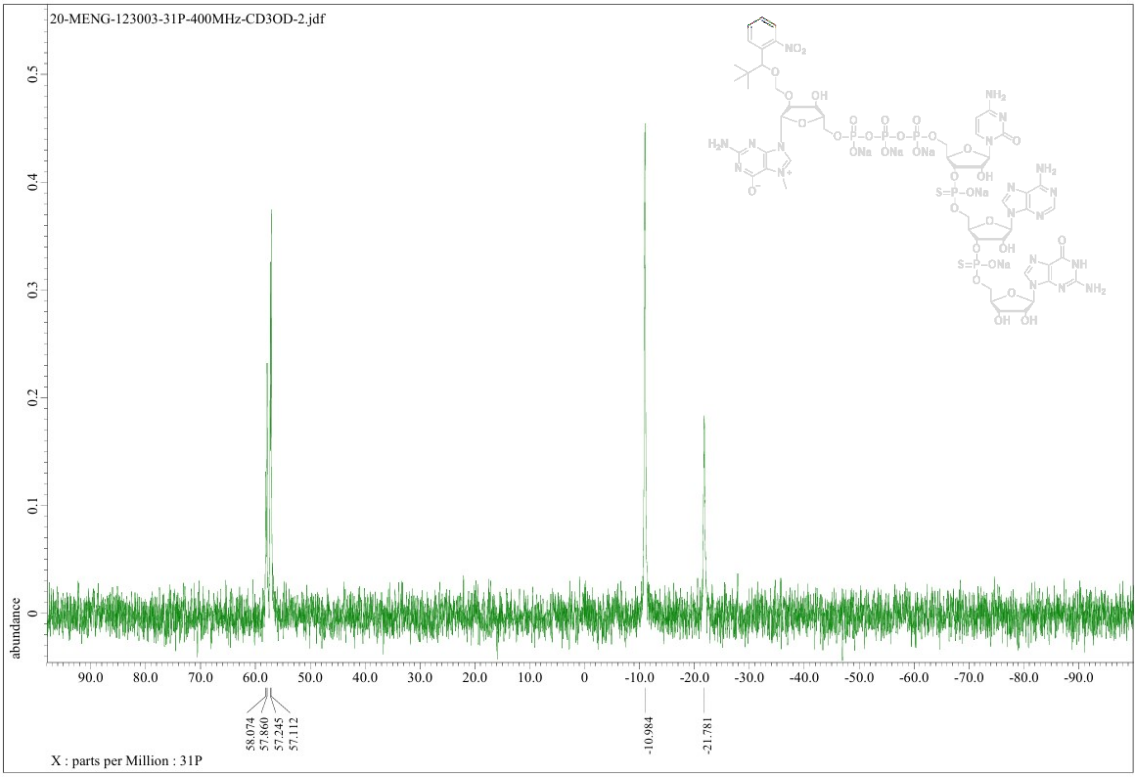
^{1}H-NMR chart of **4**



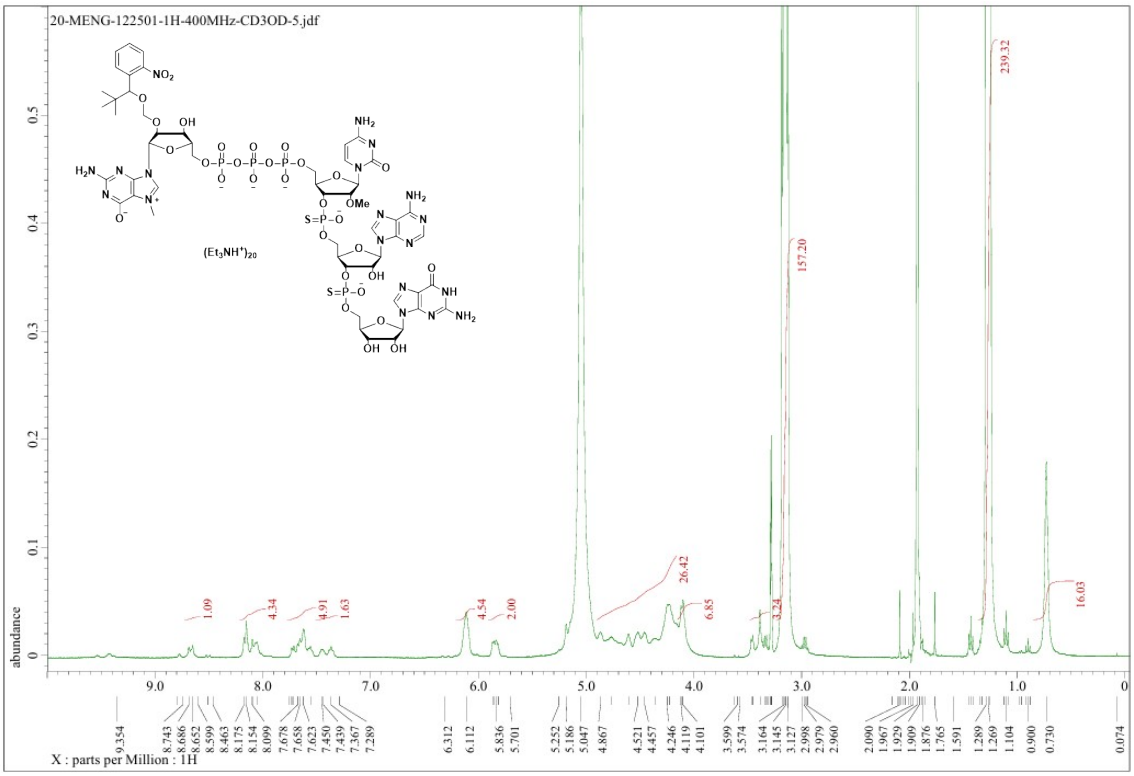
^{13}C-NMR chart of **4**



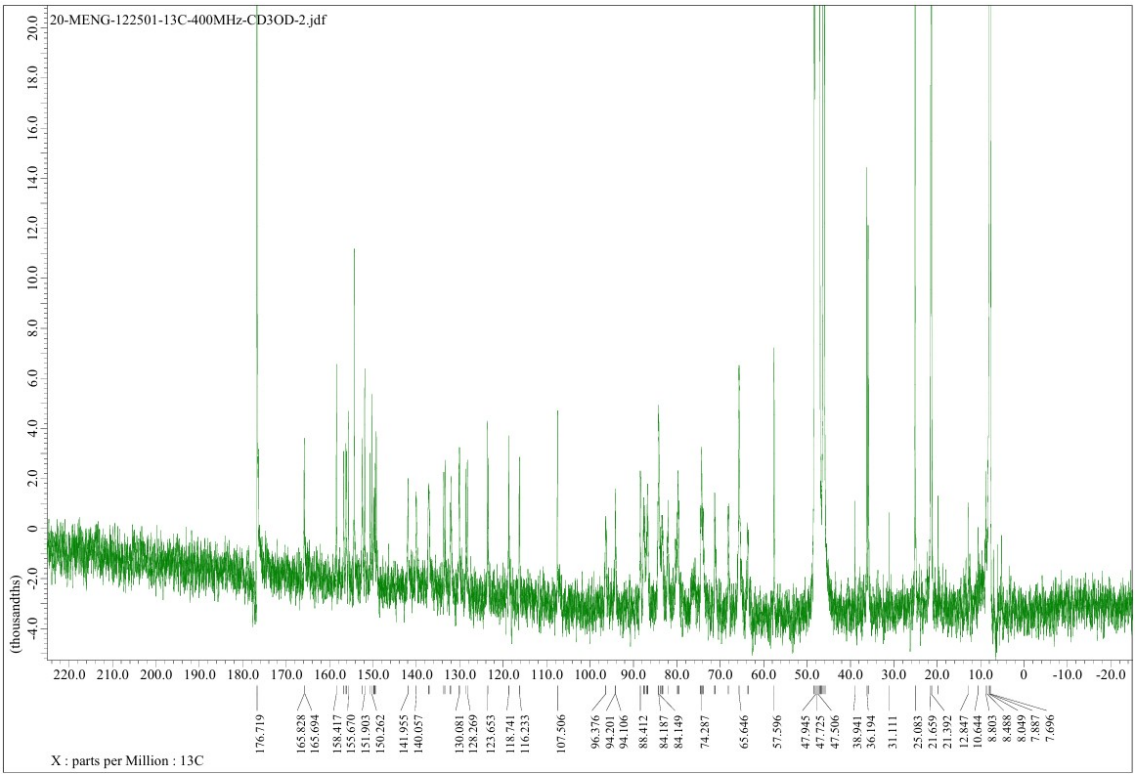
^{31}P-NMR chart of **4**



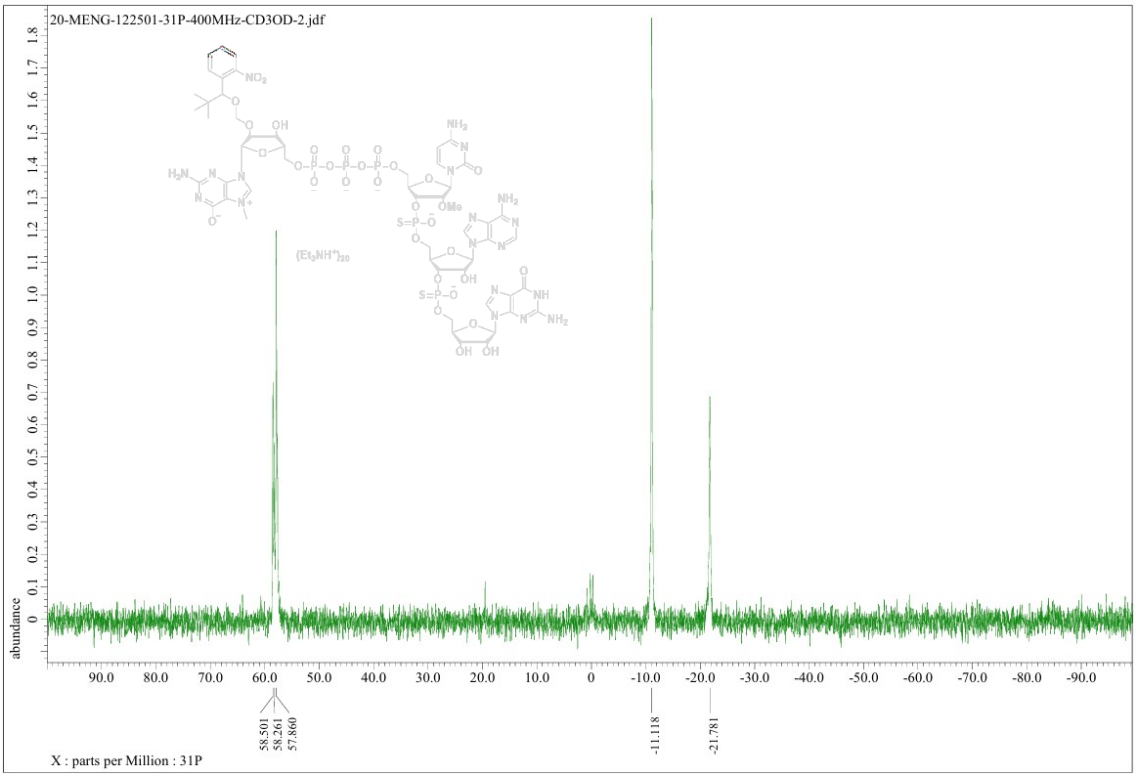
^{1}H-NMR chart of **5**



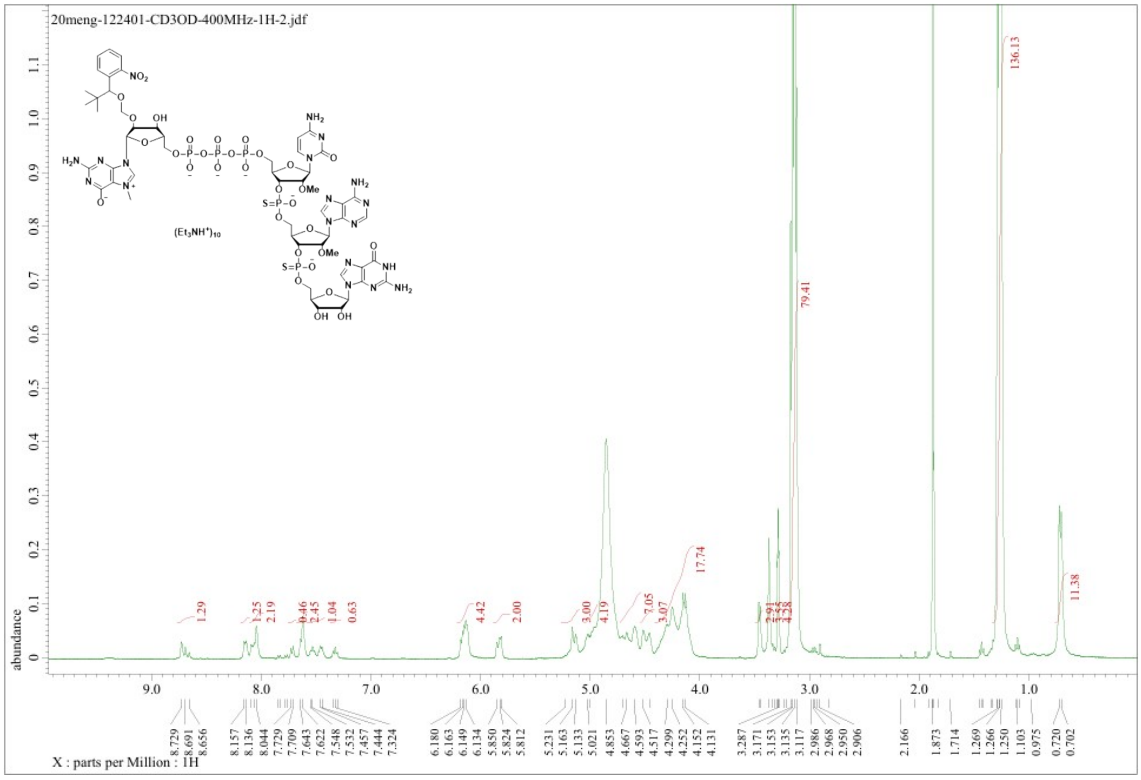
^{13}C-NMR chart of **5**



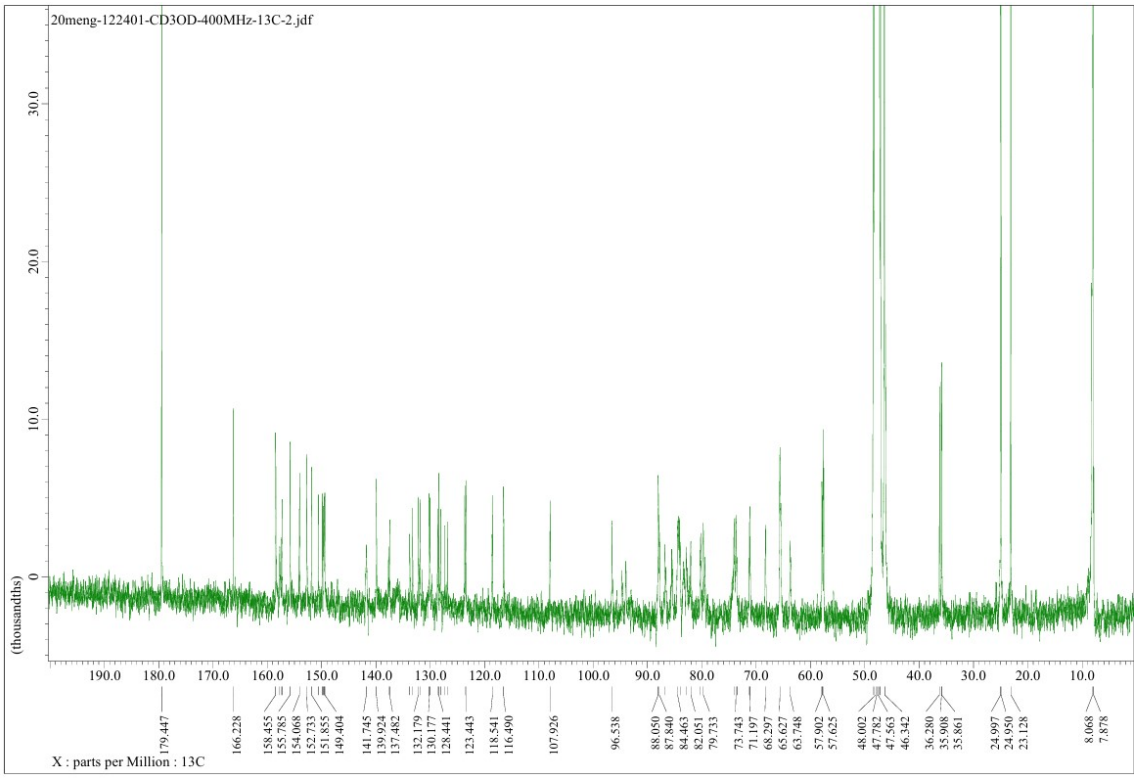
^{31}P-NMR chart of **5**



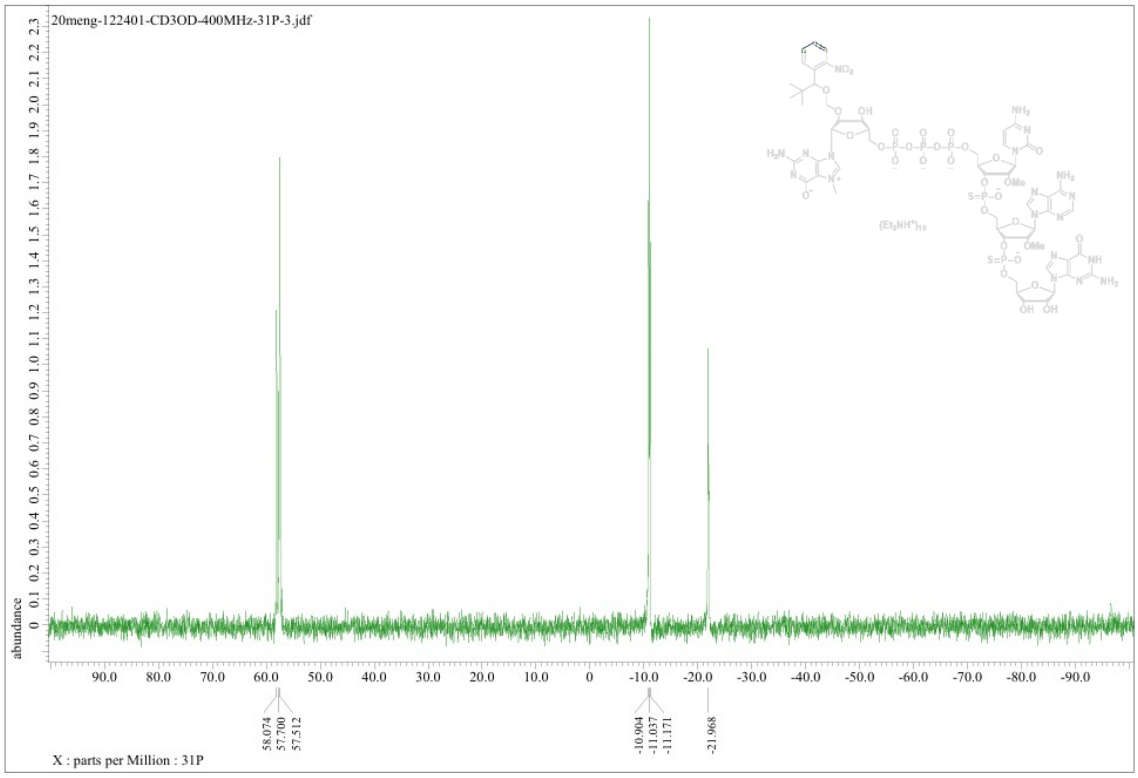
^{1}H-NMR chart of **6**



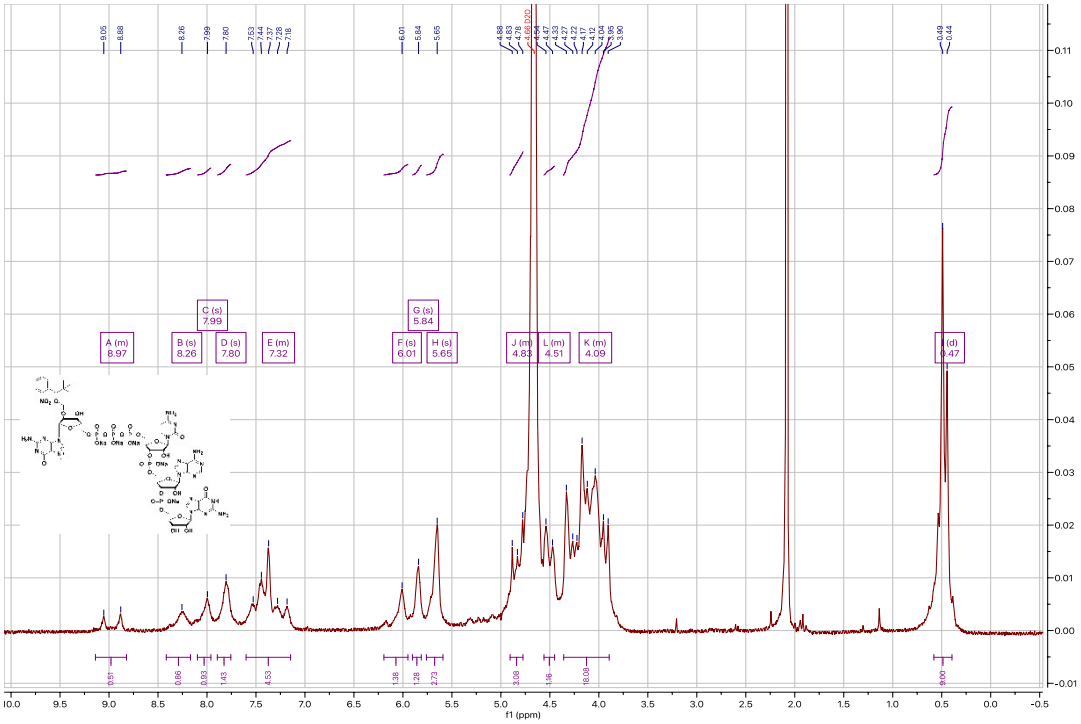
^{13}C-NMR chart of **6**



^{31}P-NMR chart of **6**



^{1}H-NMR chart of **7**



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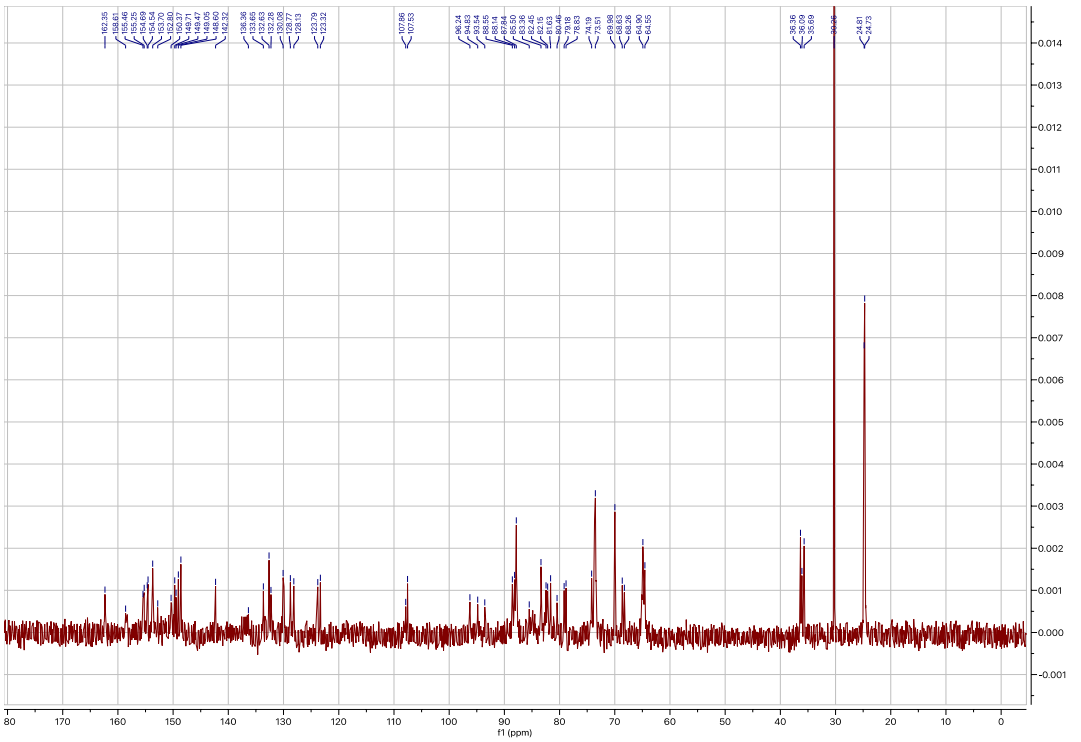
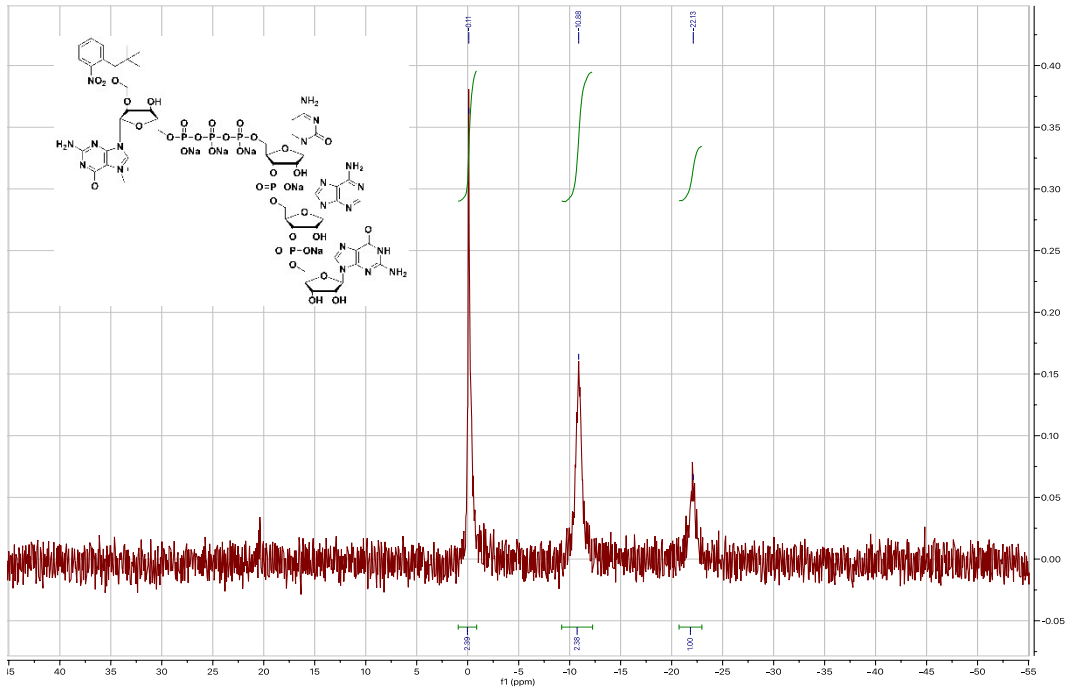
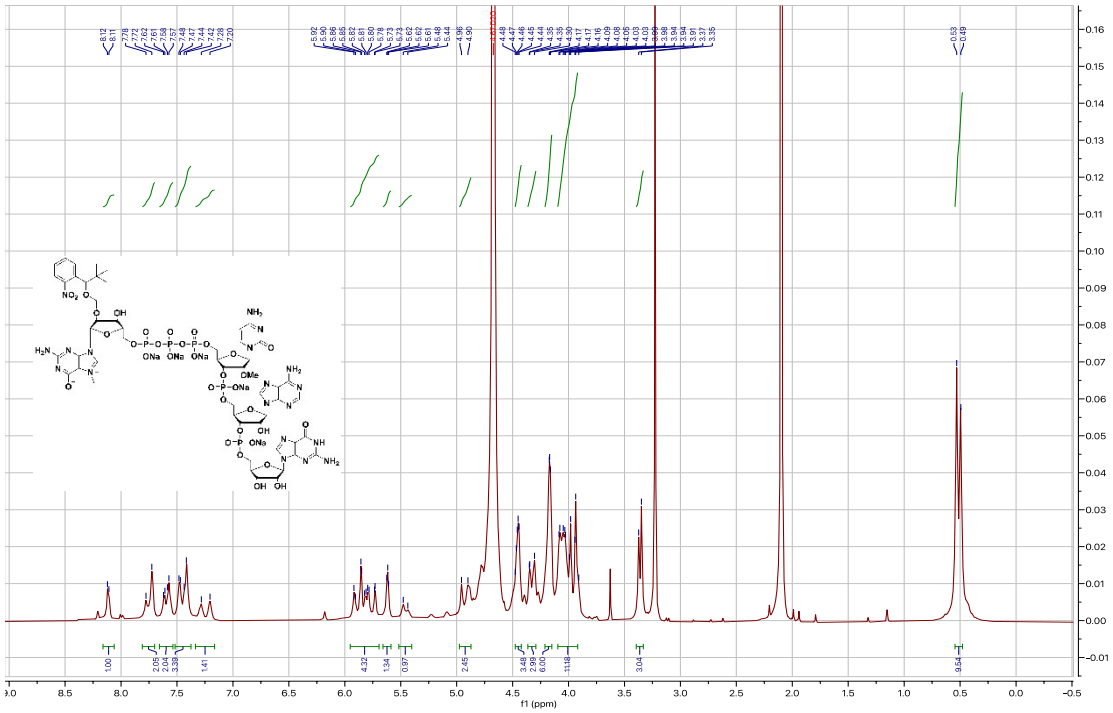


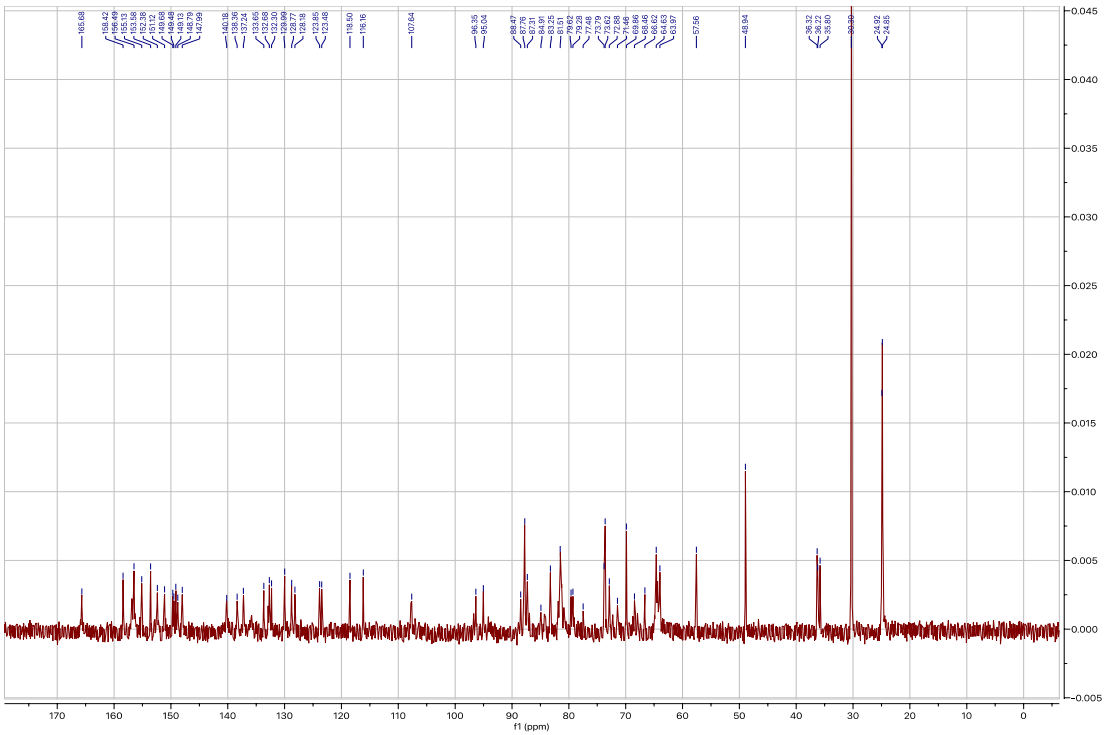
^{31}P-NMR chart of **7**



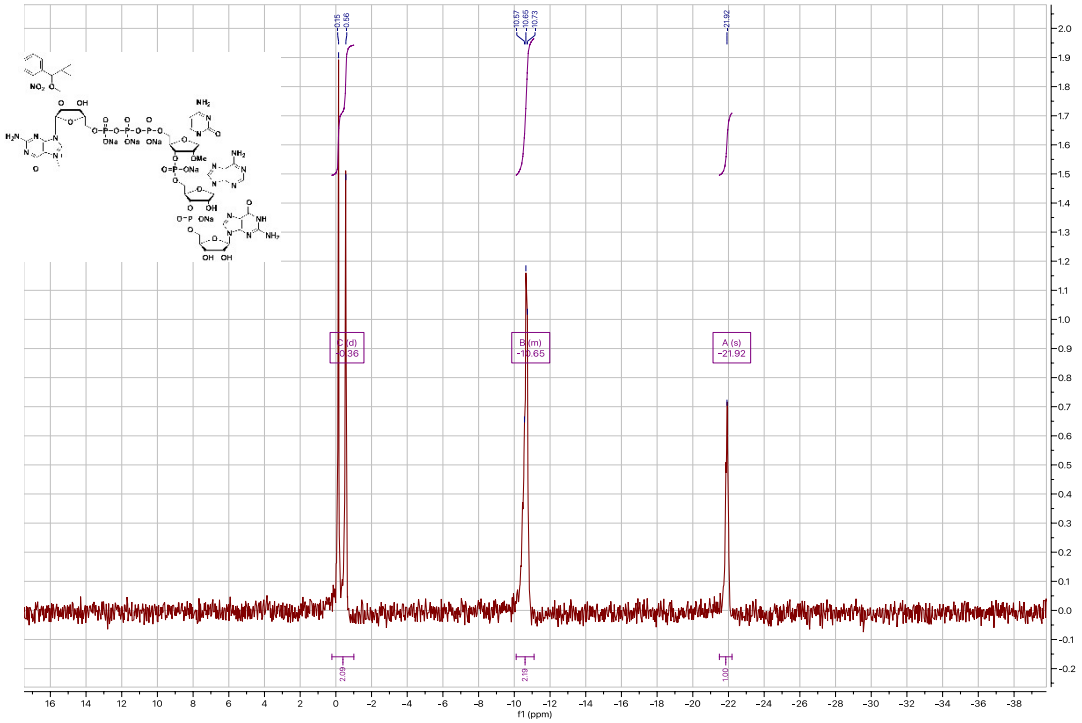
^{1}H-NMR chart of **8**



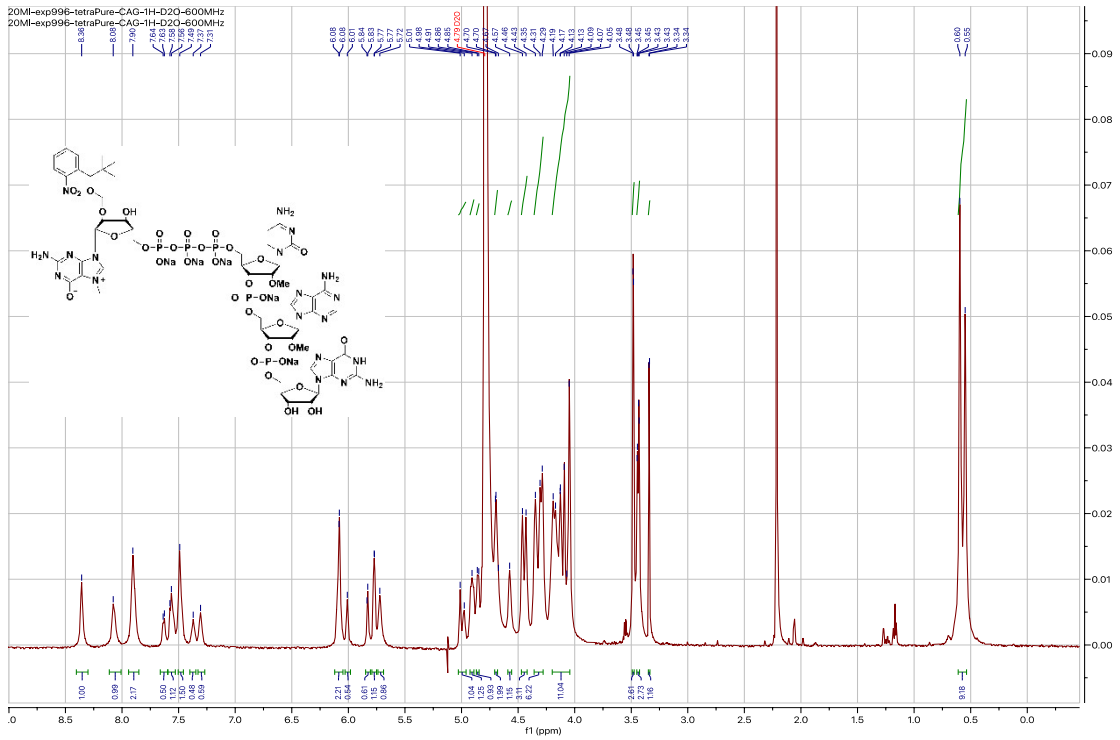
^{13}C-NMR chart of **8**



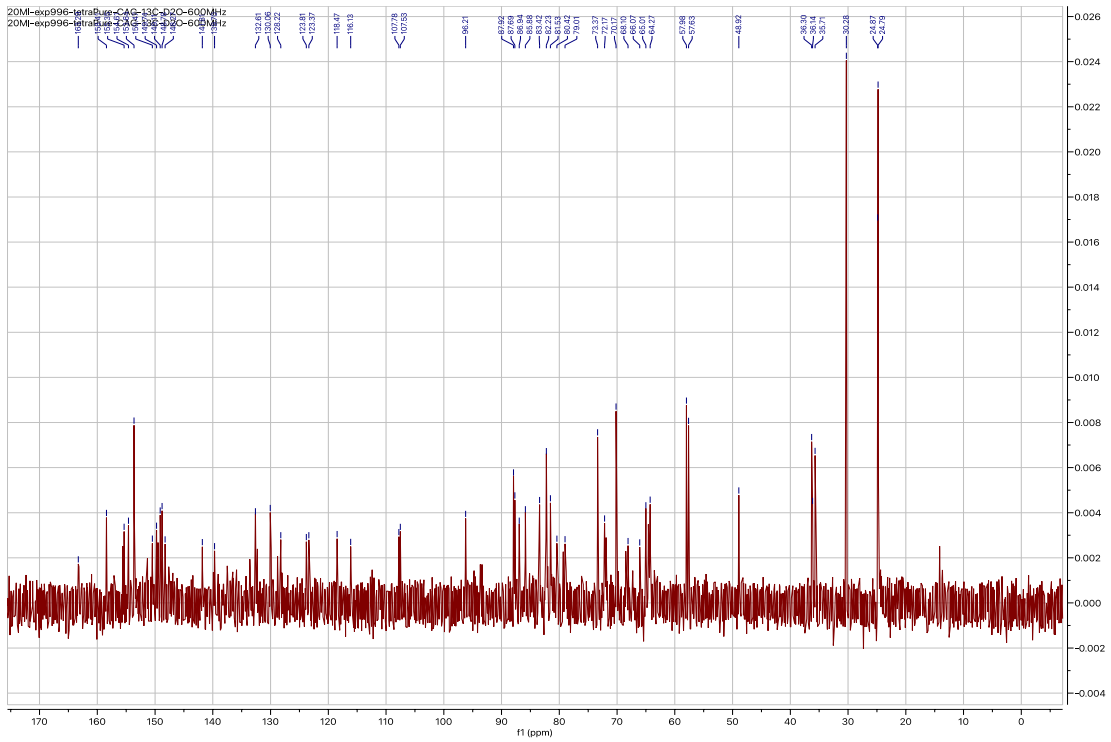
^{31}P-NMR chart of **8**



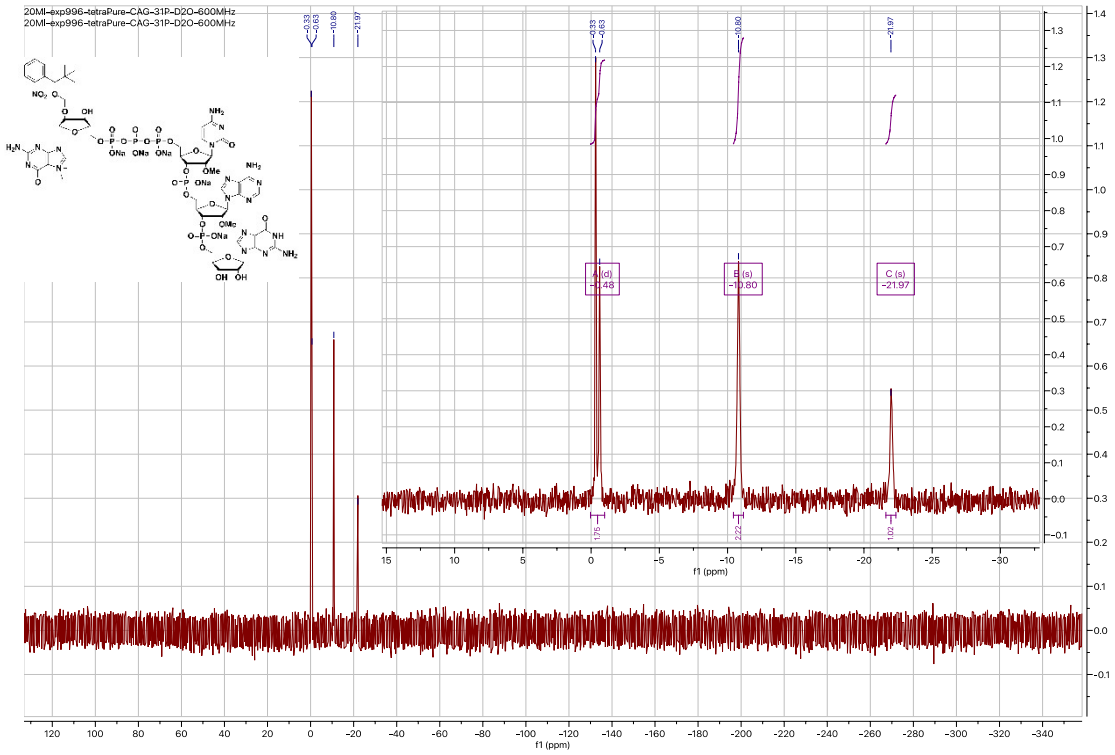
^{1}H-NMR chart of **9**



^{13}C-NMR chart of **9**



^{31}P-NMR chart of **9**



LC-MS Analysis of Cap Analogs

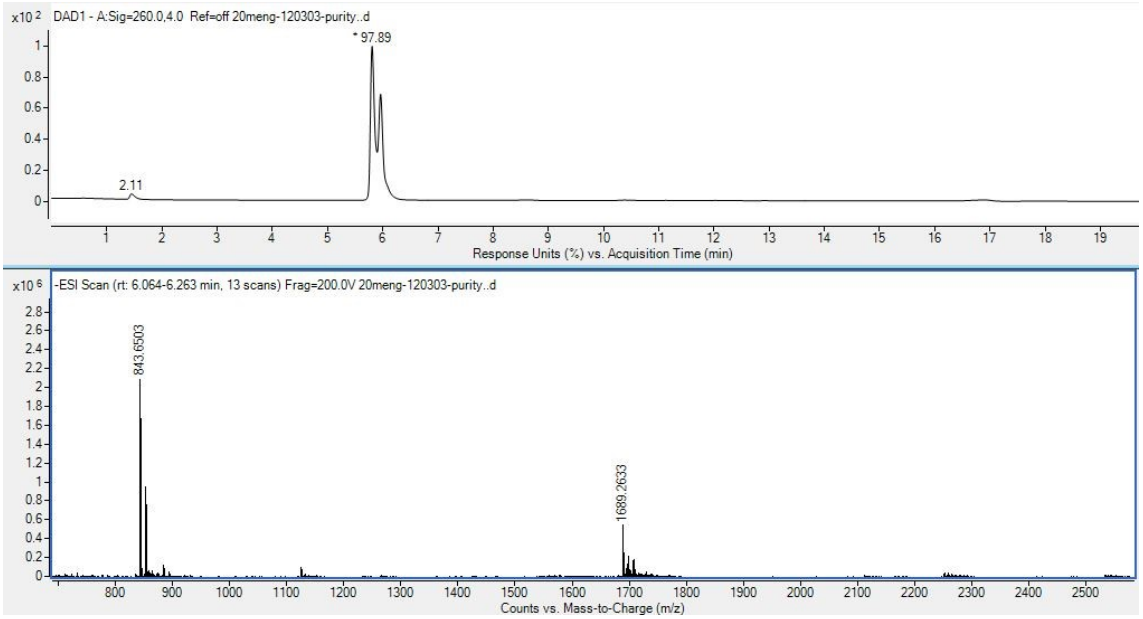


Fig. S1. LC-MS analysis of 1. Purity 97.89% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{52}H_{66}N_{19}O_{32}P_5S_2^{2-}$ 843.6131 $[M-2H]^{2-}$; obsd. 843.6503.

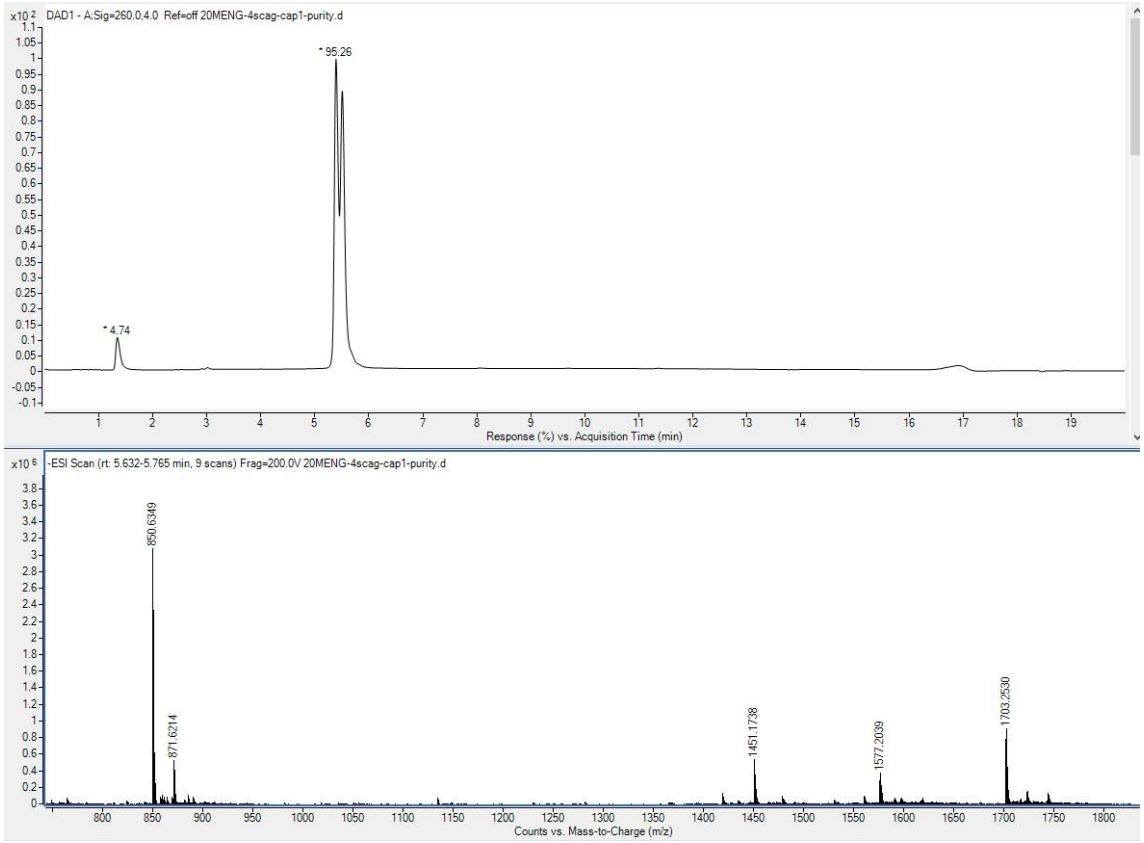


Fig. S2. LC-MS analysis of 2. Purity 95.20% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{53}H_{68}N_{19}O_{32}P_5S_2^{2-}$ 850.6209 $[M-2H]^{2-}$; obsd. 850.6349.

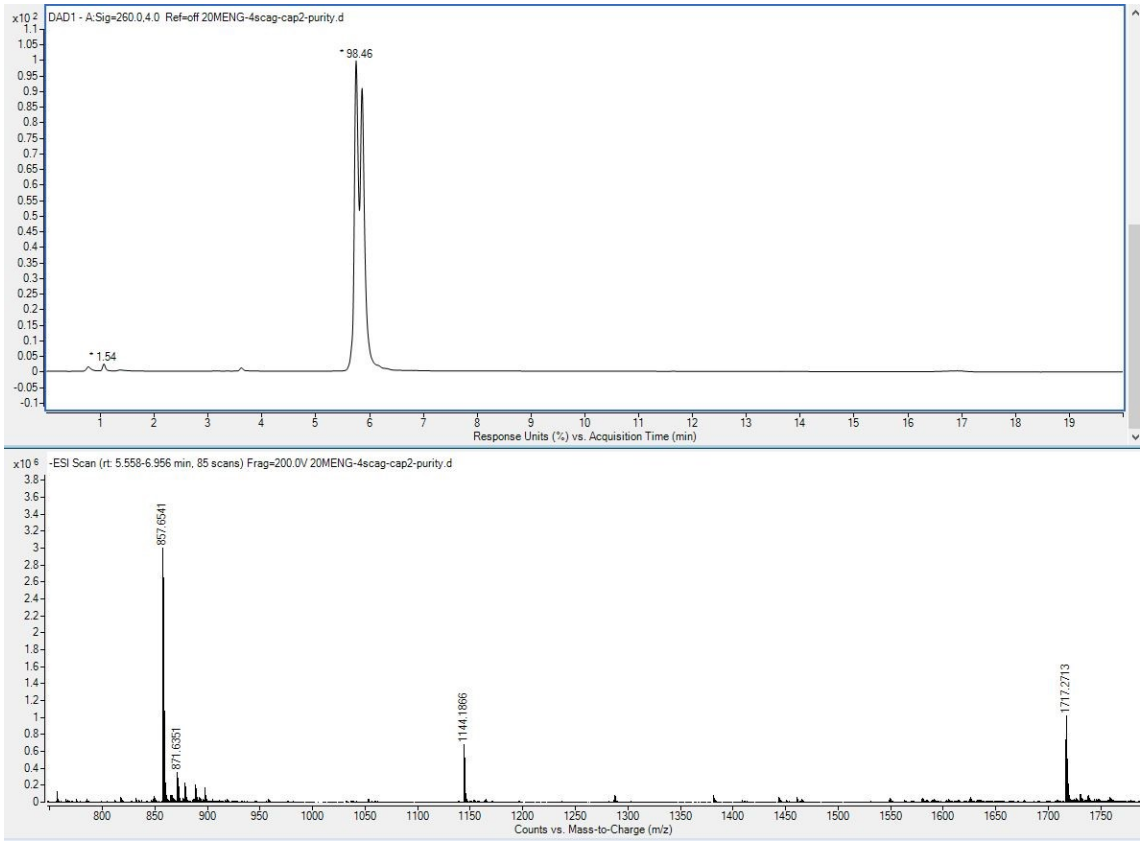


Fig. S3. LC-MS analysis of 3. Purity 98.46% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{54}H_{70}N_{19}O_{32}P_5S_2^{2-}$ 857.6287 $[M\text{-}2H]^{2-}$; obsd. 857.6541.

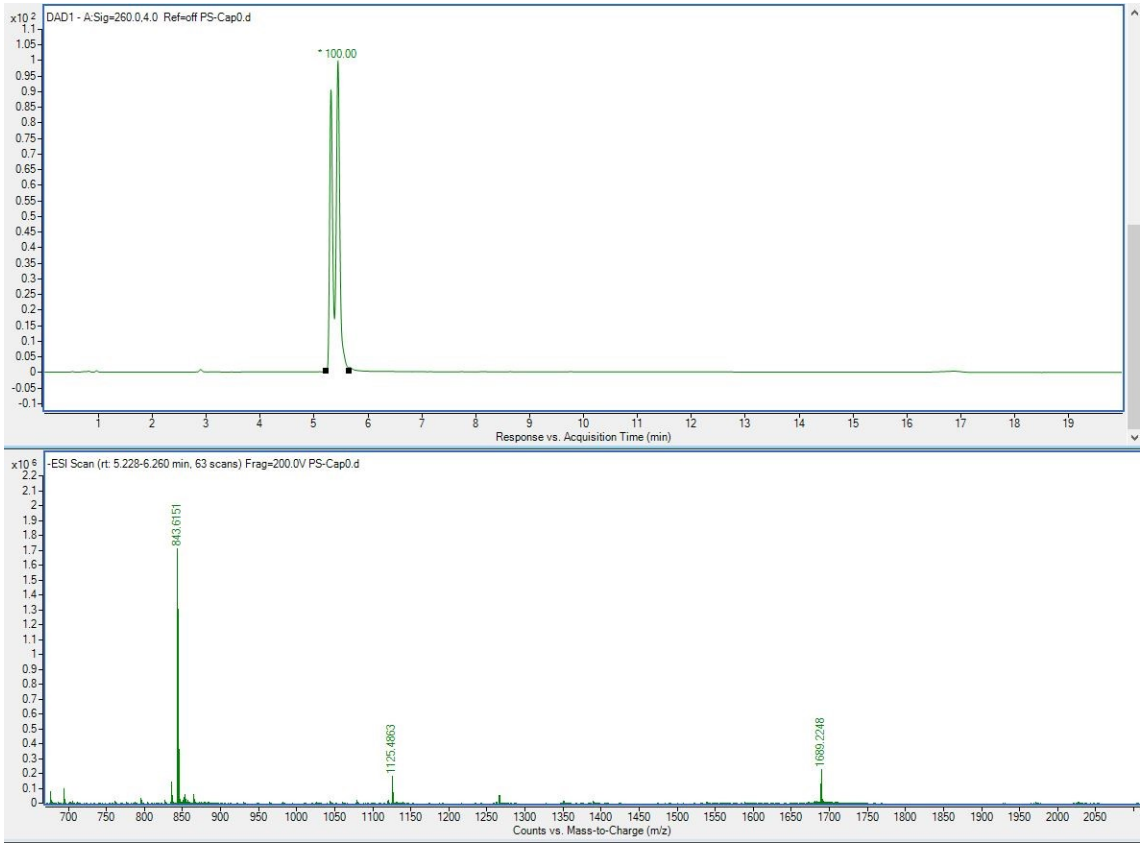


Fig. S4. LC-MS analysis of 4. Purity 100% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A: 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{52}H_{66}N_{19}O_{32}P_5S_2^{2-}$ 843.6131 $[M\text{-}2H]^{2-}$; obsd. 843.6151.

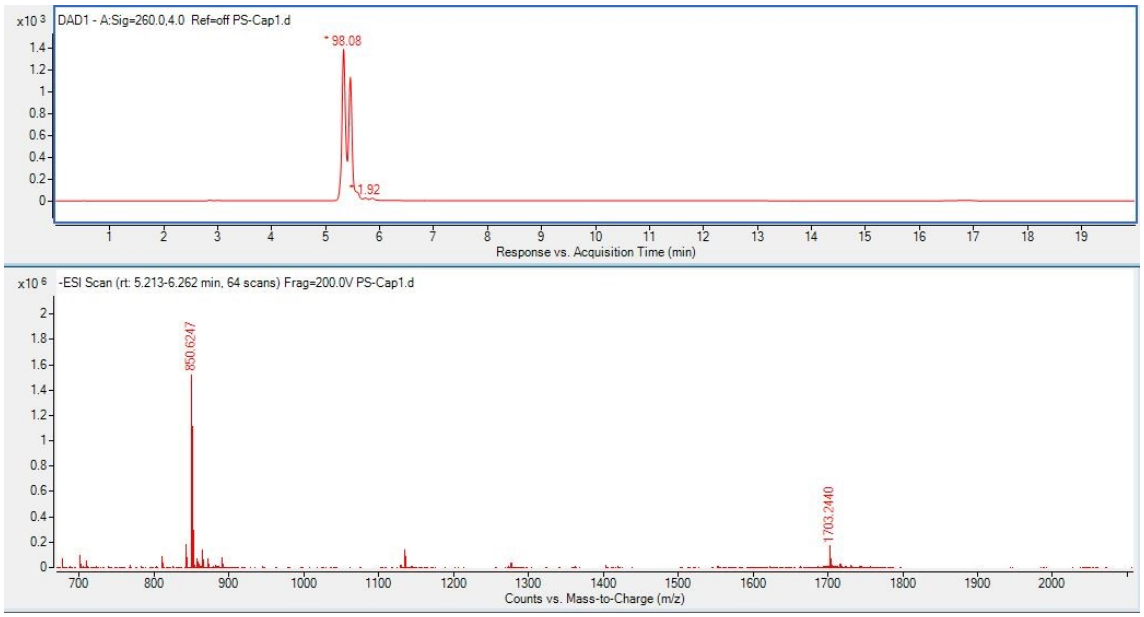


Fig. S5. LC-MS analysis of 5. Purity 98.08% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{53}H_{68}N_{19}O_{32}P_5S_2^{2-}$ 850.6209 $[M-2H]^{2-}$; obsd. 850.6247.

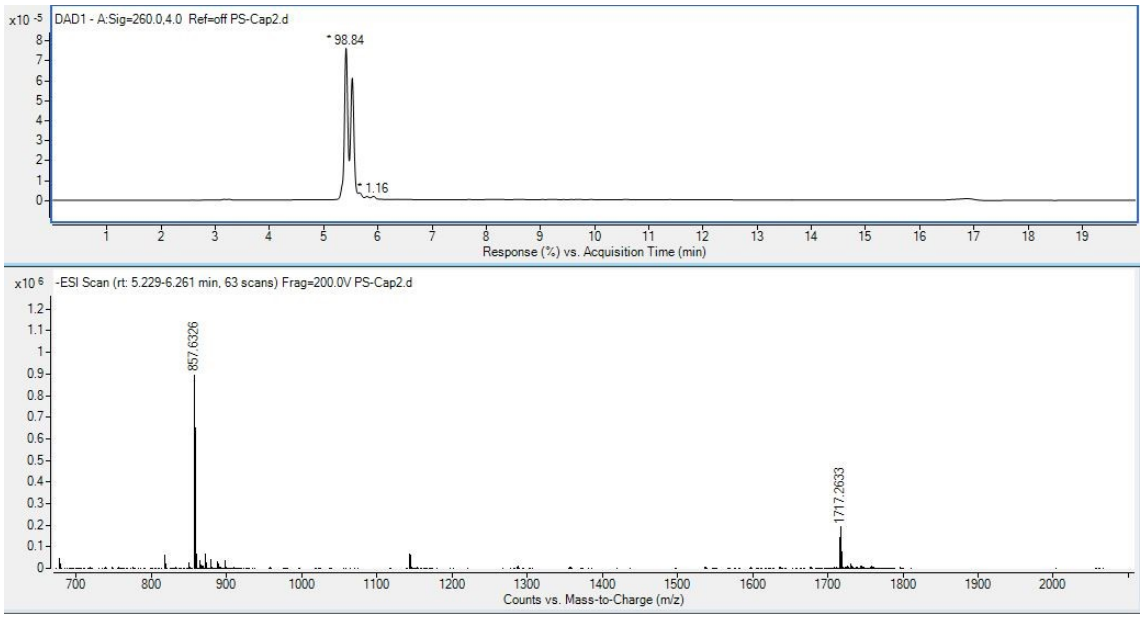


Fig. S6. LC-MS analysis of 6. Purity 98.84% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{54}H_{70}N_{19}O_{32}P_5S_2^{2-}$ 857.6287 $[M\text{-}2H]^{2-}$; obsd. 857.6326.

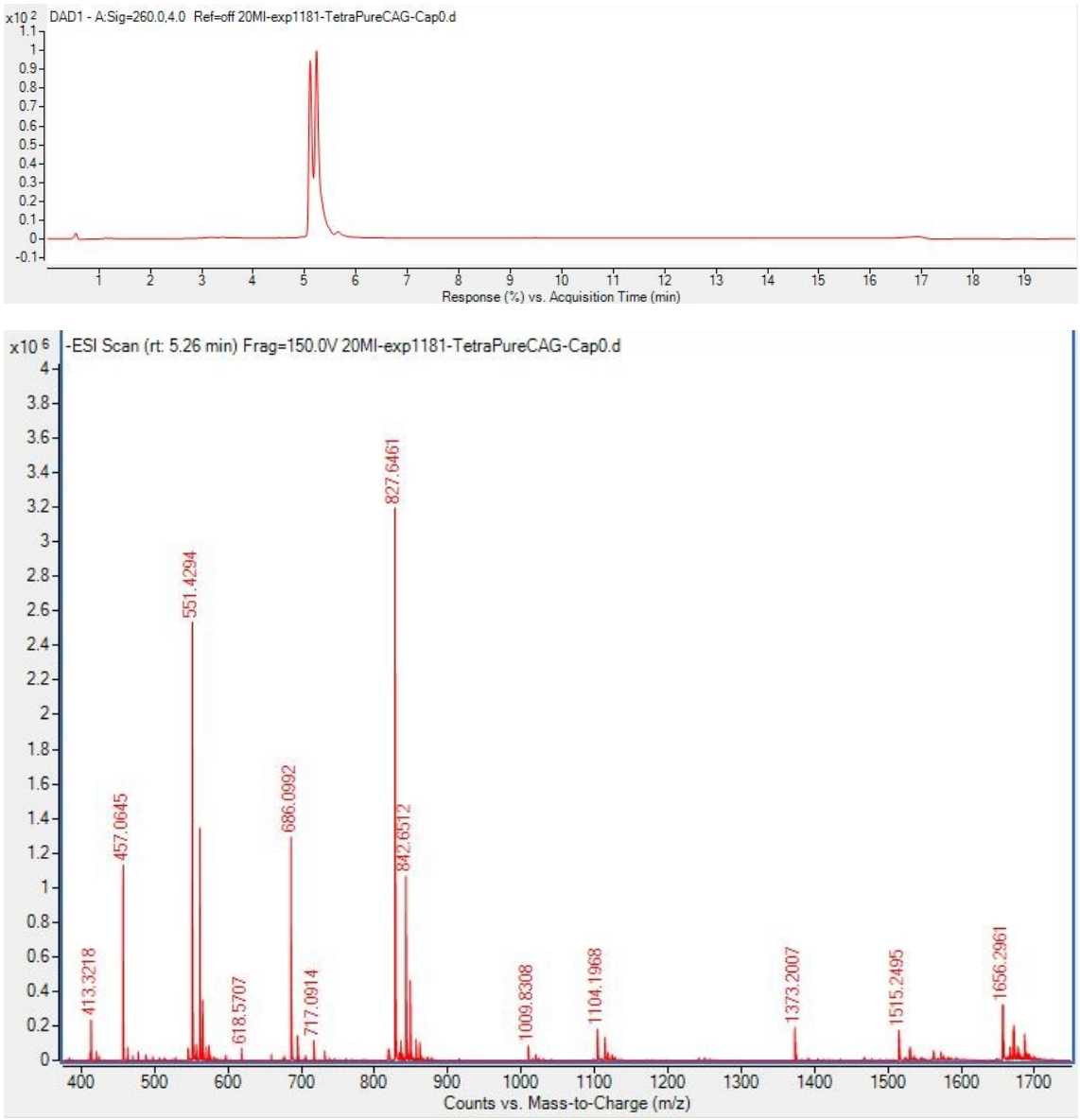


Fig. S7. LC-MS analysis of 7. Purity 98.91% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{52}H_{66}N_{19}O_{34}P_5^{2-}$ 827.6359, $[M\text{-}2H]^{2-}$; obsd. 827.6461.

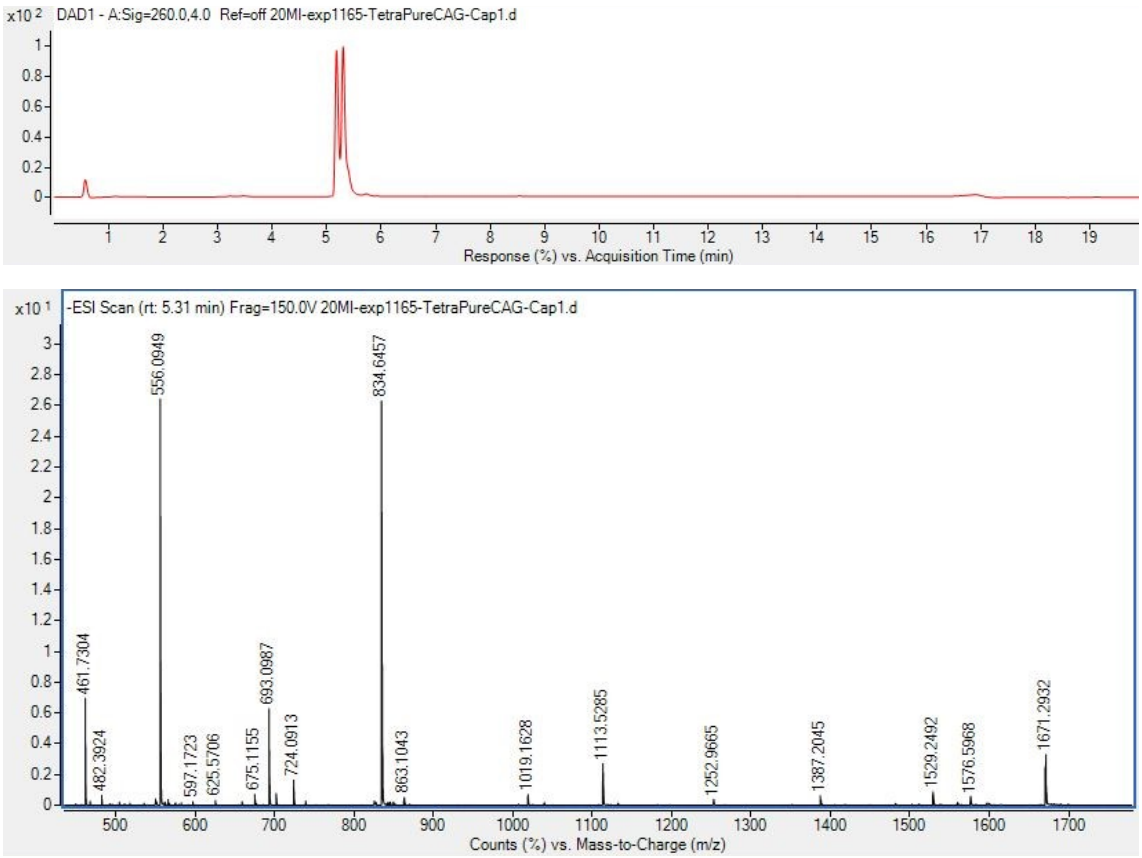


Fig. S8. LC-MS analysis of 8. Purity 97.86% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{53}H_{68}N_{19}O_{34}P_5^{2-}$, 834.6438, $[M-2H]^{2-}$; obsd. 834.6457.

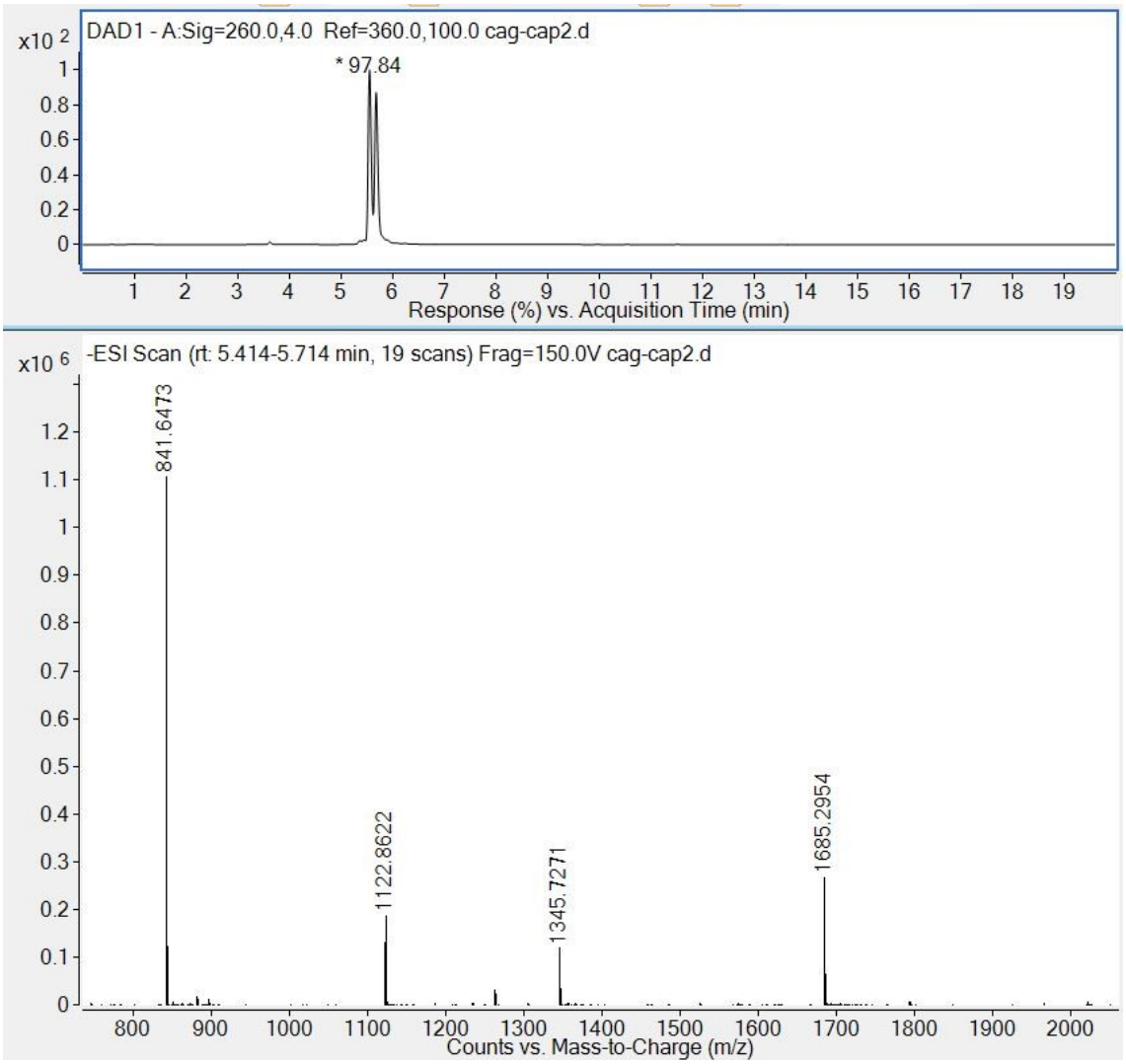


Fig. S9. LC-MS analysis of 9. Purity 97.87% (% of sum peak area). Conditions, ACQUITY UPLC BEH C18 column; solvent A, 8.6 mM TEA/100 mM HFIP; solvent B, MeOH; linear gradient of solvent B, 0 to 90% B (12 min); detected wavelength, 260 nm; flow rate, 0.3 mL/min; column temperature, 60 °C. Calcd. for $C_{54}H_{70}N_{19}O_{34}P_5^{2-}$, 841.6516 $[M-2H]^{2-}$; obsd. 841.6473.

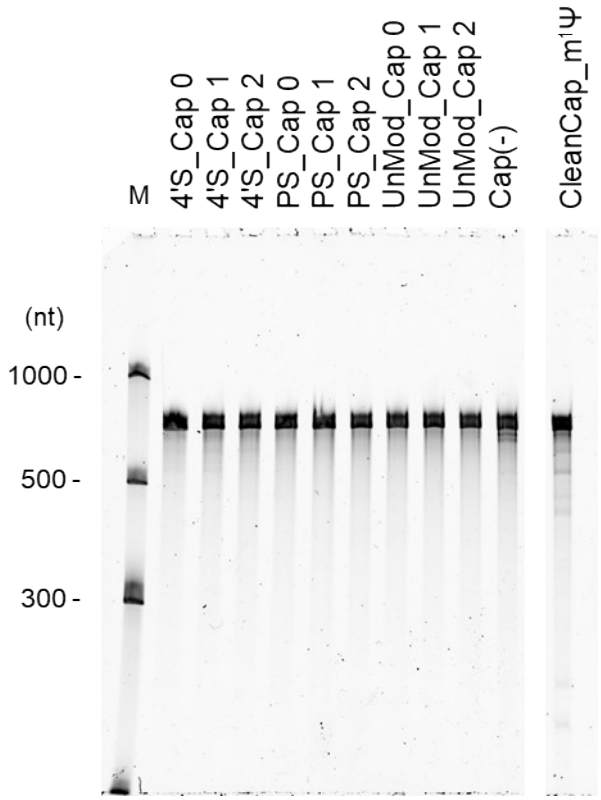
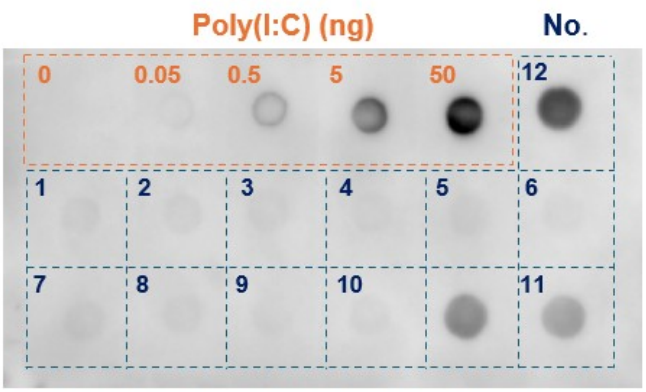


Fig. S10. 5% dPAGE analysis of mRNA samples (50 ng each). Stained by SYBR Green II.

A

Spot No.	Description		Signal Intensity	Calcd dsRNA amount per spot (ng)	dsRNA content (%)
1	mRNA (500 ng each)	4'S_Cap 0	422719	0.12	0.023
2		4'S_Cap 1	443517	0.12	0.024
3		4'S_Cap 2	395147	0.11	0.021
4		PS_Cap 0	308959	0.081	0.016
5		PS_Cap 1	376594	0.10	0.020
6		PS_Cap 2	333434	0.088	0.018
7		UnMod_Cap 0	490827	0.14	0.027
8		UnMod_Cap 1	401979	0.11	0.022
9		UnMod_Cap 2	278181	0.072	0.014
10		Cap(-)	339860	0.090	0.018
11		CleanCap_m$^{1}\Psi$	3142501	1.8	0.36
12		Crude of Cap(-)	10081835	26	5.2
13	poly I:C control	0 ng	10505		
14		0.050 ng	239829		
15		0.50 ng	1692376		
16		5.0 ng	5521083		
17		50 ng	12704197		

B



C

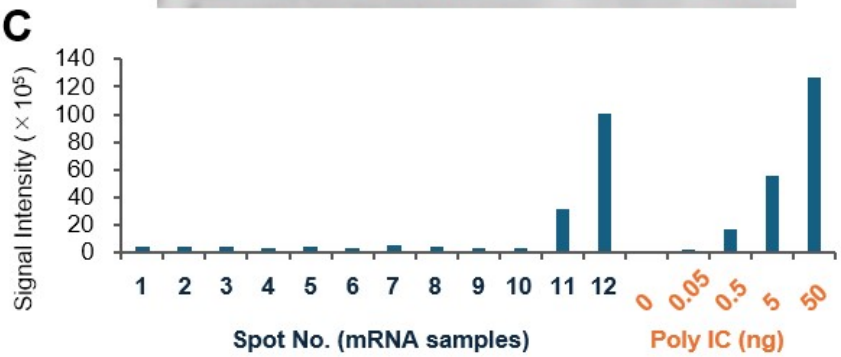


Fig. S11. dsRNA analysis. (A) Sample information and the quantification result summary. (B) Dot-blot image. (C) Signal quantification results of the blot shown in (B).

Table S1. Sequence information for the IVT template: PCR primers used to amplify a region of the pNL1.1TK vector and the corresponding PCR product.

Type	Sequence
F primer	CCCGGATCCTAATACGACTCACTATA**A**GGGAAATATTAAGGTGACGCGT
R primer	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTAGAATTACGCCAGAATGCG
PCR Product	CCCGGATCCTAATACGACTCACTATA**A**GGGAAATATTAAGGTGACGCGTGTGGCCTCGA ACACCGAGCGACCCTGCAGCGACCCGCTTAAAAGCTTGGCAATCCGGTACTGTTGGTAA AGCCACCATGGTCTTCACACTCGAAGATTTCGTTGGGGACTGGCGACAGACAGCCGGCT ACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGTTTCAGAATCTCGGG GTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATGGGCTGAAGATCGA CATCCATGTCATCATCCCGTATGAAGGTCTGAGCGGCGACCAAATGGGCCAGATCGAAA AAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGTGATCCTGCACTATG GCACACTGGTAATCGACGGGGTTACGCCGAACATGATCGACTATTTCGGACGGCCGTAT GAAGGCATCGCCGTGTTCGACGGCAAAAAGATCACTGTAACAGGGACCCTGTGGAACG GCAACAAAATTATCGACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGAGTA ACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGTAATTCTAGAA AAAAAAAAAAAAAAAAAAAAAAAAAAA

Table S2. LNP characterization. PDI represents the polydispersity index, EE represents the efficiency of encapsulation.

Sample	Size (nm)	PDI	EE (%)	Recovery (%)	Volume (µL)	Conc. (µg/mL)
4'S_Cap 2	99	0.12	91	78	390	50
PS_Cap 2	94	0.10	98	90	420	54
UnMod_Cap 2	92	0.13	93	91	400	57
CleanCap_$m^1\Psi$	93	0.10	98	94	410	58