Replacement of oxygen with sulfur on the furanose ring of cyclic dinucleotide enhances the immunostimulatory effect by STING activation.

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Table of Contents

General Information

Physical data were measured as follows; ^{1}H, and ^{31}P NMR spectra were recorded at 400, or 500 MHz and 162 or 202 MHz instruments (Bruker FT-NMR AV400 or AV500), respectively in $CDCl_3$ or DMSO-d_6 as the solvent with tetramethylsilane (for ^{1}H NMR) or phosphoric acid (^{31}P NMR). Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Mass spectra were measured on a SQD2 Waters), BioAccord LC-MS (Waters). TLC was done on Merck Kieselgel F254 precoated plates. Silica gels used for column chromatography were KANTO Chemical silica gel 60 and KANTO Chemical silica gel 60N (neutral). The c-di-AMP was purchased from Invivogen (California, USA).

Experimental Procedures

***N*-(Phenyl)imidazolium triflate (*N*-PhIMT)**[43]

Trifluoromethanesulfonic acid (3.0 mL, 34 mmol) was added to a solution of an *N*-phenylimidazole (4.39 mL, 34 mmol) in dry CH_2Cl_2 (25 mL) over 30 minutes, and the mixture was stirred for 30 min. The reaction mixture was diluted with dry diethyl ether (20 mL). The resultant precipitate was collected by filtration to give *N*-PhIMT. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.60 (1 H, s), 8.28 (1 H, s), 7.89 (1 H, s), 7.80 (2 H, d, J = 8.2 Hz), 7.65 (2 H, t, J = 8.2 Hz), 7.57 (1 H, t, J = 7.3 Hz).

N^6-Benzoyl-2'-*O*-*tert*-butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)adenosine-{3'-(2-cyanoethyl)phosphono-5'}-N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)adenosine (5).

Under argon atmosphere, a mixture of **3** (543 mg, 0.55 mmol) and **4** (243 mg, 0.50 mmol) in dry CH_3CN (10 mL) containing 3Å MS (100 mg) was stirred for 1 hour at room temperature. To the above solution, *N*-PhIMT (161 mg, 0.55 mmol) was added, and the whole was stirred for 1 hour at room temperature. Then, a solution of TBHP/toluene (1.0 M, 1.0 mL) was added to the reaction mixture, and the whole was stirred for 2 hours at the same temperature. The reaction mixture was partitioned between AcOEt and H_2O. The separated organic layer was further washed with sat. $NaHCO_3$, followed by brine, dried (Na_2SO_4) and concentrated *in*

vacuo. The residue was purified by a silica gel column, eluted with hexane/AcOEt (1/2–0/1), then AcOEt/acetone (1/0-3/1), to give **5** (676 mg, 97%) as a white foam. ESI-LRMS *m/z* 1411 (MNa^+); ESI-HRMS calcd for $C_{70}H_{83}N_{11}O_{14}PSi_2$ 1388.5397, found 1388.5393; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.96, 8.96, 8.95, and 8.94 (2 H, 4 brs, exchangeable with D_2O), 8.82, 8.81, 8.68, 8.66, 8.24, 8.21, 8.20, and 8.19 (4 H, 8 s), 8.03–7.97 (4 H, m), 7.64–7.43 (8 H, m), 7.35–7.28 (6 H, m), 7.24–7.19 (1 H, m), 6.84–6.81 (4 H, m), 6.11–6.01 (2 H, m), 5.28–5.24 (1 H, m), 5.02–4.89 (2 H, m), 4.57–4.38 (4 H, m), 4.32–4.10 (3 H, m), 3.77, 3.76 (6 H, 2 s), 3.65–3.54 (1 H, m), 3.40–3.34 (1 H, m), 2.82–2.52 (3 H, m), 0.89, 0.88, 0.72, and 0.71 (18 H, 4 s), 0.06, 0.04, –0.01, –0.02, –0.04, –0.05, –0.24, and –0.27 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –2.18, –2.42.

N^6-Benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)adenosine-{3'-(2-cyanoethyl)phosphono-5'}-N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)adenosine (6).

To a solution of **5** (654 mg, 0.47 mmol) in CH_2Cl_2 (4.8 mL) was added dichloroacetic acid (1.2 mL) dropwisely at 0 °C, and the whole was stirred for 30 minutes at the same temperature. The reaction was quenched by addition of sat. $NaHCO_3$, and the reaction mixture was partitioned between $CHCl_3$ and sat. $NaHCO_3$ The separated organic layer was washed with H_2O, followed by brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by a silica gel column, eluted with MeOH in $CHCl_3$ (0–10%), to give **6** (495 mg, 97%) as a white foam. ESI-LRMS *m/z* 1108 (MNa^+); ESI-HRMS calcd for $C_{49}H_{65}N_{11}O_{12}PSi_2$ 1086.4090, found 1086.4113; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.08, 9.04, 9.03, and 8.97 (2 H, 4 brs, exchangeable with D_2O), 8.84, 8.83, 8.79, 8.78, 8.25, 8.24, 8.20, and 8.15 (4 H, 8 s), 8.01–7.95 (4 H, m), 7.65–7.45 (6 H, m), 6.04–5.84 (3 H, m), 5.23–5.17 (1 H, m), 5.03–4.96 (2 H, m), 4.55–4.47 (3 H, m), 4.35–4.26 (4 H, m), 3.93–3.74 (2 H, m), 2.80–2.76 (3 H, m), 0.90, 0.89, 0.72, and 0.70 (18 H, 4 s), 0.07, 0.06, 0.01, −0.01, −0.15, −0.16, −0.35, and −0.37 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –2.21, –2.31.

(3',5')-Cyclic-bis-{N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-(2-cyanoethyl)}phosphonoadenosine (8).[42]

Under argon atmosphere, a solution of **6** (477 mg, 0.43 mmol) in dry CH_2Cl_2 (10 mL) containing 4Å MS

(100 mg) was stirred for 1 hour at room temperature. To the above solution, 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (149 μL, 0.47 mmol) and *N*-PhIMT (138 mg, 0.47 mmol) were added, and the whole was stirred at room temperature. After 3 hours, additional *N*-PhIMT (253 mg, 0.86 mmol) was added to the reaction mixture. After being stirred for 3 hours, a solution of TBHP/toluene (1.0 M, 1.5 mL) was added to the reaction mixture, and the whole was stirred for 1 hour at the same temperature. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The separated organic layer was further washed with sat. $NaHCO_3$, followed by brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by a silica gel column, eluted with Hexane/AcOEt (1/8–0/1), then AcOEt/acetone (1/0–3/1), to give **8** (336 mg, 3 steps 63%) as a white foam. ESI-LRMS *m/z* 1223 (MNa^+); ESI-HRMS calcd for $C_{52}H_{67}N_{12}O_{14}P_2Si_2$ 1201.3914, found 1201.3938; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.00 and 8.95 (2 H, 2 brs, exchangeable with D_2O), 8.73, 8.81, 8.94, and 8.99 (4 H, 4 s), 8.18–8.00 (6 H, m), 7.64–7.50 (6 H, m), 6.02–5.96 (2 H, m), 5.58–5.38 (2 H, m), 5.16–5.01 (2 H, m), 4.83–4.54 (4 H, m), 4.47–4.16 (6 H, m), 2.95–2.58 (4 H, m), 0.80 and 0.93 (18 H, 2 s), 0.18, 0.11, 0.06, 0.04, 0.01, −0.12, −0.22, and −0.23 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 1.07, −0.05, –4.17, −5.07.

c-di-AMP[42]

A solution of **8** (266 mg, 0.2 mmol) in NH_3/MeOH (20 mL) was allowed to stand for 18 hours, and then the solvent was removed *in vacuo*. After the resulting crude was dissolved in dry pyridine (1 mL), Et_3N (2.5 mL) and triethylamine trihydrofluoride (1.0 mL) were added, and the whole was heated for 2.5 hours at 65 °C. After being cooled to a room temperature, HPLC grade acetone (50 mL) was immediately added in a slow, steady stream to the stirring mixture. After 10 minutes of stirring, the crystals were collected by filtration and washed thoroughly with 5 mL portions of acetone (× 5). The crystals were dried in a desiccator overnight over KOH, giving c-di-AMP (triethylammonium salt, 46 mg, 2 steps 24%) as a brown foam. ESI-LRMS *m/z* 657 (MH^-); ^{1}H NMR (D_2O 400 MHz) δ 8.14 (2 H, H-2 or H-8, s), 7.96 (2 H, H-2 or H-8, s), 6.11 (2 H, H-1', s), 4.61–4.58 (4 H, H-3' and H-2', m), 4.47–4.44 (4 H, H-4' and H-5'a, m), 4.05 (2 H, H-5'b, d, J = 10.0 Hz), 3.18 (12 H, Et_3N, q, J = 7.3 Hz), 1.25 (18 H, Et_3N, t, J=7.3 Hz); ^{31}P NMR (D_2O, 162 MHz) δ –2.31.

N^6-Benzoyl-2'-*O*-*tert*-butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)-4'-thioadenosine-{3'-(2-cyanoethyl)phosphono-5'}-N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-4'-thioadenosine (11)

In the similar manner as described for **5**, **9** (552 mg, 0.55 mmol) and **10** (250 mg, 0.5 mmol) in dry MeCN (15 mL) containing 3 Å MS (150 mg) was treated with *N*-PhIMT (162 mg, 0.55 mmol), and then TBHP/toluene (1.0 M, 1.0 mL) to give **11** (660 mg, 2 steps 93%) as a white foam. ESI-LRMS *m/z* 1443 (MNa^+); ESI-HRMS calcd for $C_{70}H_{83}N_{11}O_{12}PS_2Si_2$ 1420.4940, found 1420.4931; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.09 and 9.04 (2 H, 2 brs, exchangeable with D_2O), 8.77, 8.69, 8.68, 8.54, 8.48, 8.31, 8.29, and 8.09 (4 H, 8 s), 8.07–7.89 (4 H, m), 7.63–7.46 (9 H, m), 7.38–7.28 (6 H, m), 6.88–6.83 (4 H, m), 6.02–5.88 (2 H, m), 5.35–4.92 (3 H, m), 4.72–3.86 (7 H, m), 3.78 and 3.77, (6 H, 2 s), 3.75–3.60 (2 H, m), 2.89–2.62 (3 H, m), 0.90, 0.89, 0.80, and 0.77 (18 H, 4 s), 0.08, 0.08, 0.07, 0.06, −0.04, −0.05, −0.19, and −0.24 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 202 MHz) δ −2.52, −3.10.

N^6-Benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-4'-thioadenosine-{3'-(2-cyanoethyl)phosphono-5'}-N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-4'-thioadenosine (12).

In the similar manner as described for **6**, **11** (209 mg, 0.14 mmol) in dry CH_2Cl_2 (5 mL) was treated with dichloroacetic acid (0.5 mL) to give **12** (135 mg, 82%) as a white foam. ESI-LRMS *m/z* 1140 (MNa^+); ESI-HRMS calcd for $C_{49}H_{65}N_{11}O_{10}PS_2Si_2$ 1118.3633, found 1118.3663; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.02, 8.99, 8.95, 8.95, (2 H, 4 brs, exchangeable with D_2O), 8.83, 8.82, 8.81, 8.80, 8.51, 8.43, and 8.24 (4 H, 7 s), 8.03–8.00 (4 H, m), 7.71–7.49 (6 H, m), 6.01–5.94 (2 H, m), 5.44–5.08 (1 H, m, exchangeable with D_2O), 5.05–5.02 (1 H, m), 4.80–4.74 (1 H, m), 4.63–4.54 (2 H, m), 4.41–4.31 (3 H, m), 4.25–4.17 (1 H, m), 4.14–4.09 (1 H, m), 3.96–3.87 (2 H, m), 3.81–3.75 (1 H, m), 2.88–2.81 (2 H, m), 2.69–2.63 (1 H, m, exchangeable with D_2O), 0.91, 0.90, 0.77, and 0.76, (18 H, 4 s), 0.10, 0.08, 0.08, 0.05, –0.06, –0.09, –0.30, and –0.34 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –2.39, –2.74.

(3',5')-Cyclic-bis-{*N*6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-(2-cyanoethyl)}phosphono-4'-thioadenosine (14).[33]

Under argon atmosphere, a solution of **12** (498 mg, 0.44 mmol) in dry CH_2Cl_2 (15 mL) containing 4Å MS (150 mg) was stirred for 1 hour at room temperature. To the above solution, 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (154 μL, 0.48 mmol) and *N*-PhIMT (141 mg, 0.48 mmol) were added to the above solution, and the whole was stirred at room temperature. After 2.5 hours, additional *N*-PhIMT (259 mg, 0.88 mmol) was added to the reaction mixture. After being stirred for 3 hours, a solution of TBHP/toluene (1.0 M, 1.7 mL) was added to the reaction mixture, and the whole was stirred for 1 hour at the same temperature. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The separated organic layer was washed with saturated aqueous $NaHCO_3$, followed by brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by a silica gel column, eluted with AcOEt/acetone (1/0–5/1) to give **14** (366 mg, 3 steps 66%) as a white foam. ESI-LRMS *m/z* 1255 (MNa^+); ESI-HRMS calcd for $C_{52}H_{67}N_{12}O_{12}P_2S_2Si_2$ 1233.3457, found 1233.3497; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.46, 9.07, 9.06, and 9.03 (2 H, 4 s, exchangeable with D_2O), 8.86, 8.85, 8.69, and 8.37 (2 H, 4 s), 8.09–7.93 (5 H, m), 7.75–7.44 (7 H, m), 6.15–6.06 (2 H, m), 5.57–5.37 (2 H, m) 5.21–5.09 (1 H, m), 4.86–4.75 (1 H, m), 4.64–4.49 (1 H, m), 4.45–4.40 (2 H, m), 4.29–4.08 (4 H, m), 3.87 (1 H, dd, *J* = 3.2, 11.2 Hz), 2.90–2.73 (4 H, m), 0.92,0.72, 0.71, and 0.70 (18 H, 4 s), 0.15, 0.14, 0.09, 0.06, –0.02, –0.40, –0.44, and –0.47 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 0.67, –0.09, –4.60, –5.64.

c-di-4'-thioAMP (1).[33]

A solution of **14** (10 mg, 0.01 mmol) in NH_3/MeOH (20 mL) was allowed to stand for 19 hours, and then the solvent was removed *in vacuo*. After the resulting crude was dissolved in MeOH (0.5 mL), Et_3N (0.25 mL) and triethylamine trihydrofluoride (0.4 mL) were added, and the whole was heated for 8 hours at 65 °C. After being cooled to a room temperature, the resulting mixture containing **1** was diluted in 1.0 M triethylammonium acetate (TEAA) buffer (1 mL, pH 7.0) and purified on a C18 cartridge column (YMC Dispo SPE C18), eluted with 20% MeCN to give **1** (triethylammonium salt, 5 mg, 2 steps 66%) as a white foam. ESI-LRMS *m/z* 689 (MH^-); ESI-HRMS calcd for $C_{20}H_{23}N_{10}O_{10}P_2S_2$ 689.0528, found 689.0550; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.51 (2 H, s, H-2 or H-8), 8.43 (2 H, d, *J*=5.4 Hz, exchangeable with D_2O, -OH), 8.15 (2 H, s, H-2 or H-8), 7.26 (4 H, br s, exchangeable with D_2O, NH_2), 5.88 (2 H, d, *J* = 9.2 Hz, H-1'), 5.02–4.99 (2 H, m, H-2'), 4.85 (2 H,

dd, J = 2.2, 7.2 Hz, H-3'), 4.13–4.01 (4 H, m, H-4' and H-5'a), 3.49 (2 H, dd, J = 7.2, 12.0 Hz, H-5'b), 3.07 (12 H, Et_3N, q, J = 7.3 Hz), 1.17 (18 H, Et_3N, t, J=7.3 Hz); ^{31}P NMR (DMSO-d_6, 202 MHz) δ –0.59.

N^6-Benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)adenosine-{3'-(2-cyanoethyl)phosphono-5'}-N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-4'-thioadenosine (15).

In the similar manner as described for **5**, **3** (850 mg, 0.86 mmol) and **10** (391 mg, 0.78 mmol) in dry MeCN (20 mL) containing 3 Å MS (200 mg) was treated with *N*-PhIMT (253 mg, 0.86 mmol), and then TBHP/toluene (1.0 M, 5 mL) to give **15** (746 mg, 2 steps 68% from **10**) as a white foam. ESI-LRMS *m/z* 1427 (MNa^+); ESI-HRMS calcd for $C_{70}H_{83}N_{11}O_{13}PSSi_2$ 1404.5163, found 1405.5216; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.03, 9.02 (2 H, 2 brs, exchangeable with D_2O), 8.82, 8.81, 8.69, 8.67, 8.43, 8.40, 8.23, and 8.23 (4 H, 8 s), 8.02–8.00 (4 H, m), 7.63–7.44 (9 H, m), 7.35–7.20 (6 H, m), 6.84–6.80 (4 H, m), 6.14–6.12 (1 H, m), 5.98–5.95 (1 H, m), 5.31–5.23 (1 H, m), 5.05–4.99 (1 H, m), 4.49–4.71 (1 H, m), 4.65–4.59 (1 H, m), 4.54–4.46 (2 H, m), 4.35–4.11 (3 H, m), 3.77 and 3.76 (6 H, 2 s), 3.71–3.62 (2 H, m), 3.42 (1 H, dd, J = 3.2, 10.8 Hz), 2.79–2.58 (3 H, m), 0.89, 0.88, 0.75, and 0.74 (18 H, 4 s), 0.08, 0.07, 0.02, 0.02, –0.01, –0.01, –0.20, and –0.22 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –2.45, –2.62.

N^6-Benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)adenosine-{3'-(2-cyanoethyl)phosphono-5'}-N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-4'-thioadenosine (16).

In the similar manner as described for **6**, **15** (825 mg, 0.58 mmol) in dry CH_2Cl_2 (15 mL) was treated with dichloroacetic acid (1.0 mL) to give **16** (532 mg, 82%) as a yellow foam. ESI-LRMS *m/z* 1124 (MNa^+); ESI-HRMS calcd for $C_{49}H_{65}N_{11}O_{11}PSSi_2$ 1102.3862, found 1102.3876; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.12, 9.09, 9.03, 9.00 (2 H, 4 brs, exchangeable with D_2O), 8.83, 8.82, 8.80, 8.80, 8.46, 8.43, 8.17, and 8.16 (4 H, 8 s), 8.03–8.01 (4 H, m), 7.65–7.49 (6 H, m), 6.10–5.97 (3 H, m), 5.30–5.25 (1 H, m), 5.10–5.05 (1 H, m), 4.82–4.77 (1 H, m), 4.65–4.56 (3 H, m), 4.43–4.32 (3 H, m), 4.01–3.96 (1 H, m), 3.88–3.77 (2 H, m), 2.88–2.83 (2 H, m), 2.74–2.67 (1 H, m, exchangeable with D_2O), 0.91, 0.90, 0.74, and 0.72 (18 H, 4 s), 0.09, 0.05, –0.11, –0.12, –0.13, –0.31, –0.34, and –0.41 (12 H, 8 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ –2.32, –2.49.

(3',5')-Cyclic-[N^6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-{(2-cyanoethyl)phosphono}adenosine]-[*N*6-benzoyl-2'-*O*-(*tert*-butyldimethylsilyl)-3'-*O*-(2-cyanoethyl)}phosphono-4'-thioadenosine] (18).

Under argon atmosphere, a solution of **16** (520 mg, 0.47 mmol) in dry CH_2Cl_2 (15 mL) containing 4Å MS (150 mg) was stirred for 1 hour at room temperature. To the above solution, 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (164 μL, 0.52 mmol) and *N*-PhIMT (153 mg, 0.52 mmol) were added, and the whole was stirred at room temperature. After 2 hours, additional *N*-PhIMT (280 mg, 0.94 mmol) was added to the reaction mixture. After being stirred for 3 hours, a solution of TBHP/toluene (1.0 M, 1.7 mL) was added to the reaction mixture, and the whole was stirred for 1 hour at the same temperature. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The separated organic layer was washed with saturated aqueous $NaHCO_3$, followed by brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by a silica gel column, eluted with AcOEt/acetone (1/0–5/1), to give **18** (385 mg, 3 steps 67%) as a yellowfoam. ESI-LRMS *m/z* 1239 (MNa^+); ESI-HRMS calcd for $C_{52}H_{67}N_{12}O_{13}P_2SSi_2$ 1217.3680, found 1217.3709; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.33, 9.16, 9.09, 9.08, 9.08, 9.06, 9.05, and 9.04 (2 H, 8 brs, exchangeable with D_2O), 8.85, 8.84, 8.84, 8.82, 8.74, 8.62, 8.62, and 8.39 (2 H, 8 s), 8.16–7.98 (5 H, m), 7.74–7.45 (7 H, m), 6.15–6.06 (2 H, m), 6.00–5.35 (2 H, m), 5.17–-4.78 (2 H, m), 4.72–4.53 (1 H, m), 4.48–4.34 (3 H, m), 4.24–4.02 (6 H, m), 2.91–2.67 (4 H, m), 0.95, 0.94, 0.79, 0.78, 0.77, 0.74, 0.73, and 0.72 (18 H, 8 s), 0.20, 0.18, 0.16, 0.15, 0.13, 0.06, 0.03, 0.02, 0.01, –0.15, –0.18, –0.21, –0.25, –0.30, –0.36, and –0.48 (12 H, 16 s); ^{31}P NMR ($CDCl_3$, 162 MHz) δ 0.49, 0.29, 0.23, –0.06, –4.59, –4.71, –5.12, –5.80.

Cyclic-adenosine-5'-monophosphate-4'-thioadenosine-5'-monophosphate (2)

A solution of **18** (382 mg, 0.31 mmol) in NH_3/MeOH (20 mL) was allowed to stand still for 17 hours, and then the solvent was removed *in vacuo*. After the resulting crude was dissolved in MeOH (4.0 mL), Et_3N (2.2 mL) and triethylamine trihydrofluoride (1.4 mL) were added, and the whole was heated for 5 hours at 65 °C. After being cooled to a room temperature, the resulting mixture containing **2** was diluted in 1.0 M TEAA buffer (1 mL, pH 7.0) and purified on a C18 cartridge column (YMC Dispo SPE C18), eluted with 20% MeCN to give

2 (70 mg, 2 steps 25%) as a white foam. ESI-LRMS *m/z* 673 (MH^-); ESI-HRMS calcd for $C_{20}H_{23}N_{10}O_{11}P_2S$ 673.0749, found 673.0771; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.51 and 8.44 (2 H, 2 s), 8.26 (2 H, brs, exchangeable with D_2O), 8.14 (2 H, d, *J* = 5.5 Hz), 7.30 and 7.29 (4 H, 2 brs, exchangeable with D_2O), 5.90–5.85 (2 H, m), 4.99–4.95 (2 H, m), 4.79 (1 H, dd, *J* = 2.5, 7.5 Hz), 4.72–4.69 (1 H, m), 4.19–3.89 (4 H, m), 3.55–3.50 (1 H, m), 3.09 (1 H, dd, *J* = 7.5, 13.5 Hz); ^{31}P NMR ((DMSO-d_6, 162 MHz) δ 0.02, –0.40.

Optimization of the nucleotide dimer synthesis by a phosphoramidite coupling.

Under argon atmosphere, a mixture of **3** (543 mg, 0.55 mmol) and **4** (243 mg, 0.50 mmol) in dry CH_3CN (10 mL) containing 3Å MS (100 mg) was stirred for 1 hour at room temperature. To the above solution, promoter (0.55 mmol) was added, and the whole was stirred at room temperature. Then, a solution of TBHP/toluene (1.0 M, 2.5 mL) was added to the reaction mixture, and the whole was stirred for 2 hours at the same temperature. The reaction mixture was partitioned between AcOEt and H_2O. The separated organic layer was further washed with saturated aqueous $NaHCO_3$, followed by brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by a silica gel column, eluted with Hexane/AcOEt (1/2–0/1), then AcOEt/acetone (1/0-3/1), to give **5** as a white foam.

Figure S1 The evaluation of the IRF-3 induction by STING activation. The degree of IRF-3 induction by CDNs were measured in a) 293T-Dual™ Null (5 μM CDNs) and 293T-Dual R232 hSTING cells (5 μM CDNs), and b) 293T-Dual A230 hSTING cells (1 μM CDNs). IRF-3 induction was measured by alkaline phosphatase reporter assay. Luminescence data are the mean relative light units ± SEM of at least three experiments (n = 3). ****P < 0.0001 by Student's t test.

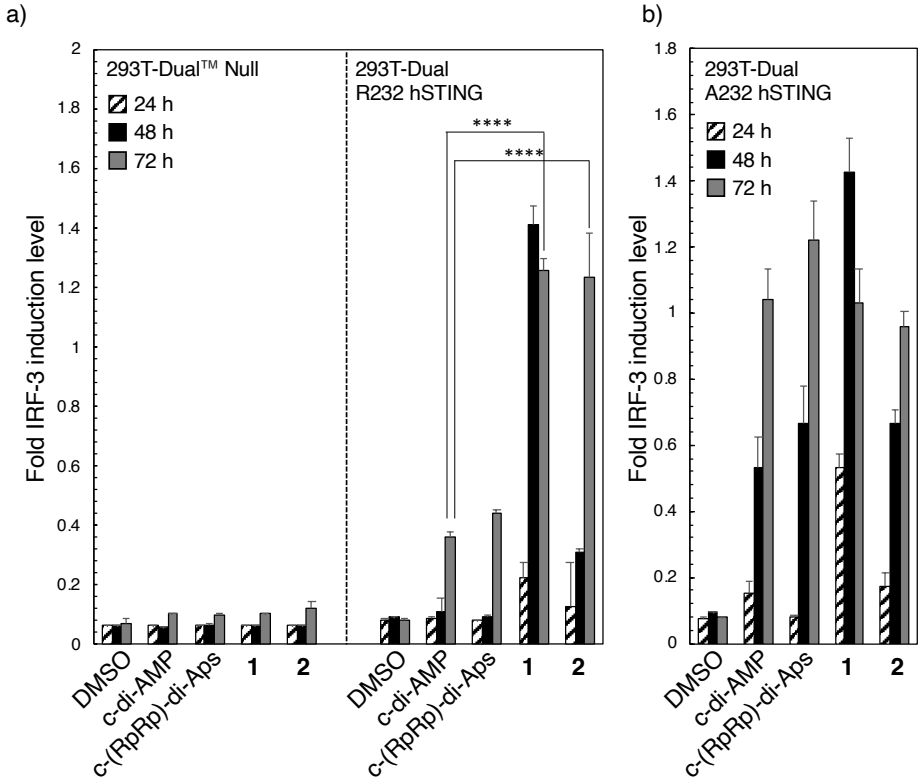
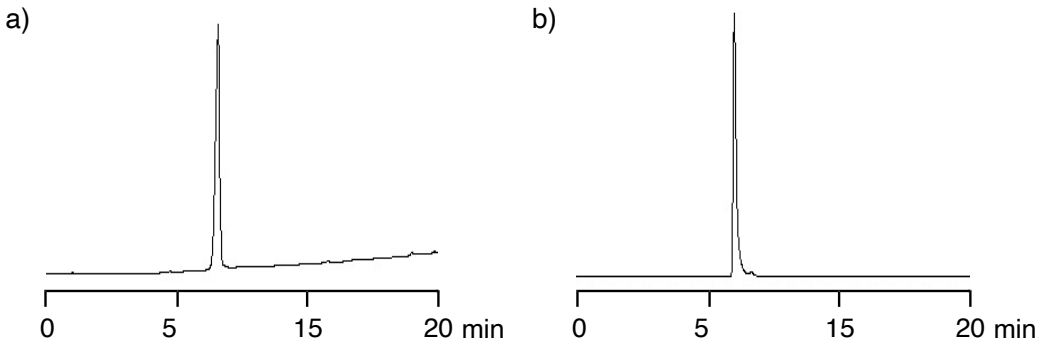
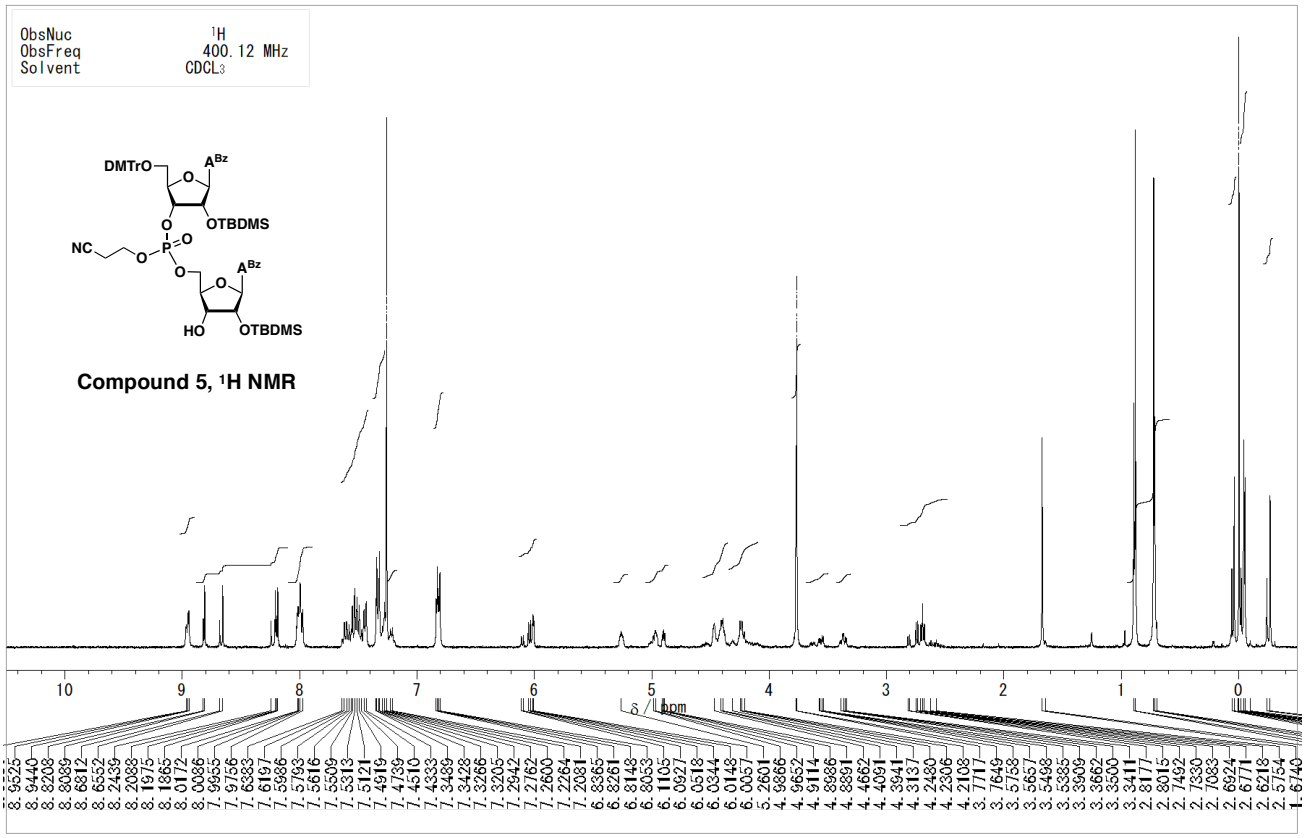
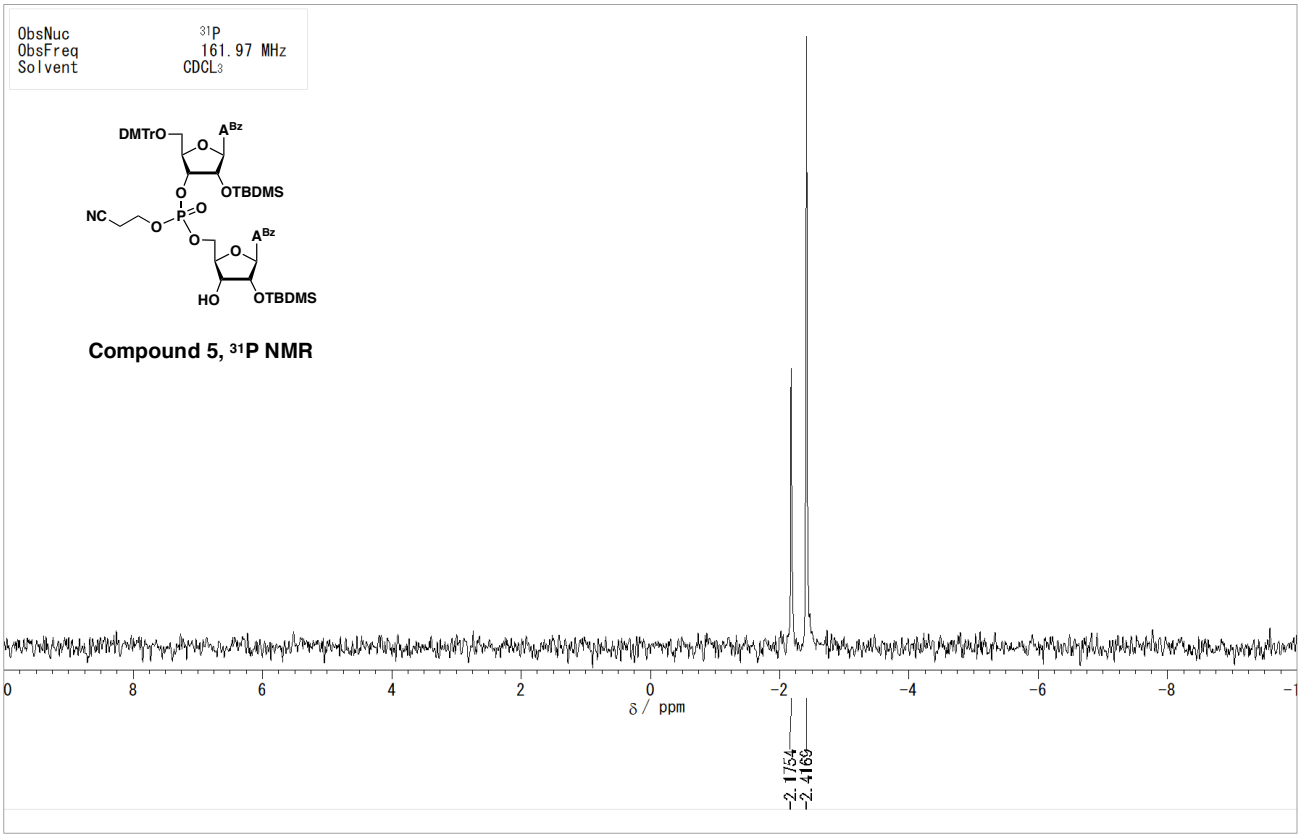


Figure S2 HPLC analyses of 4'-thiomodified c-di-AMP analogues a) **1** and b) **2**. Samples were analyzed by using HPLC with X-Bridge column (4.6 × 250 mm, Waters Corporation, Massachusetts, USA), eluted with linear gradient from 0% to 25% CH_3CN in 0.1 N TEAA buffer solution (pH 7.0).

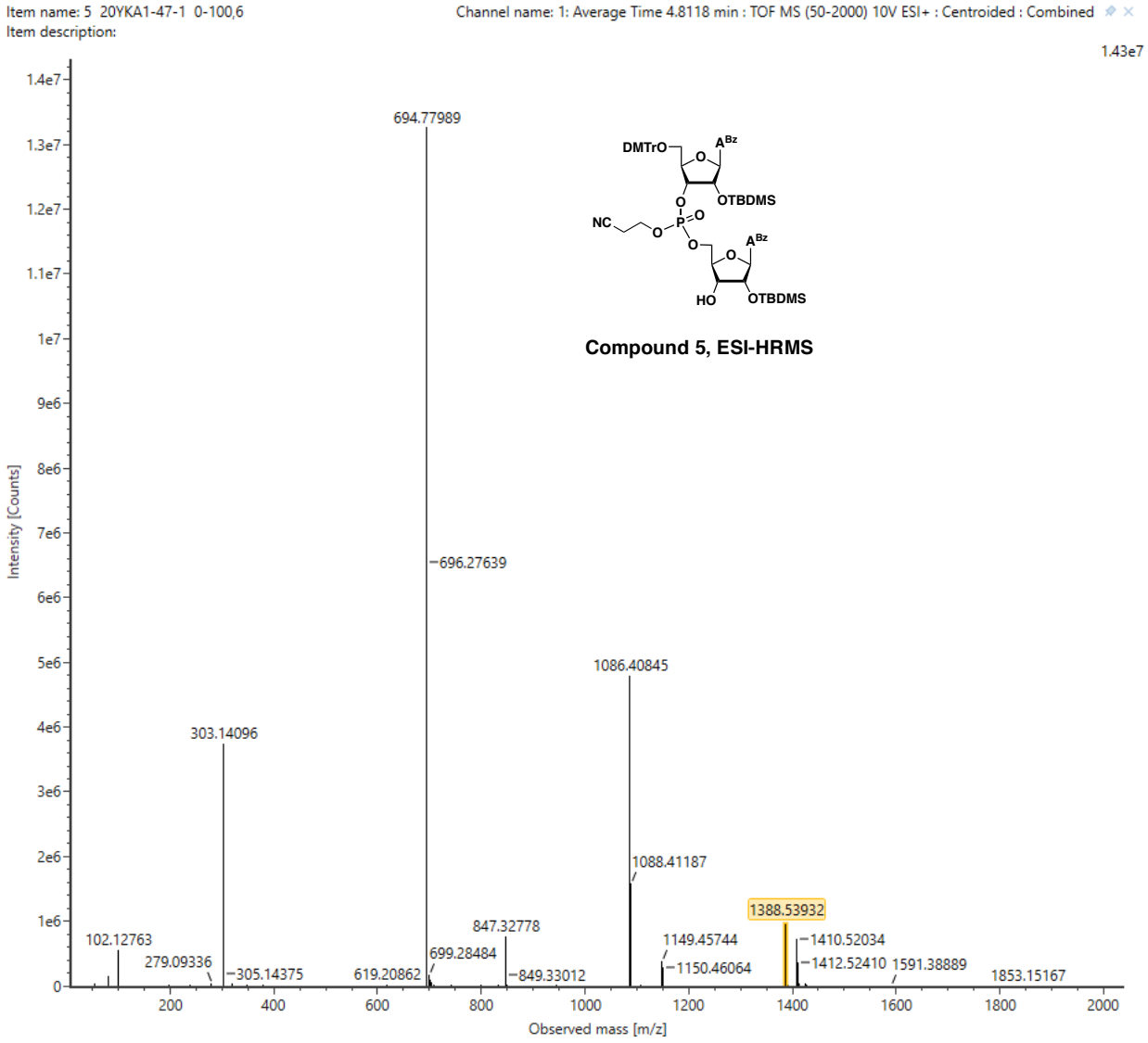




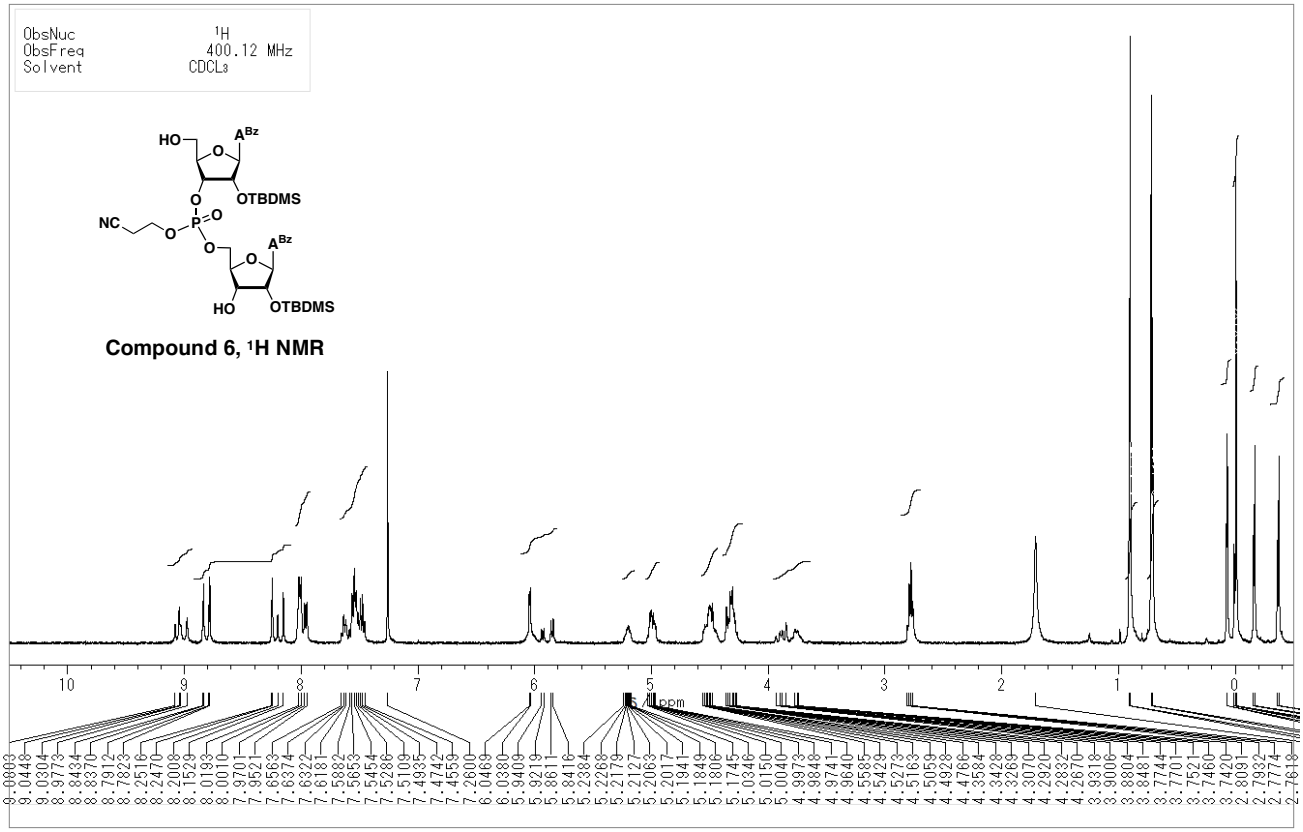
Compound 5, ^{1}H NMR



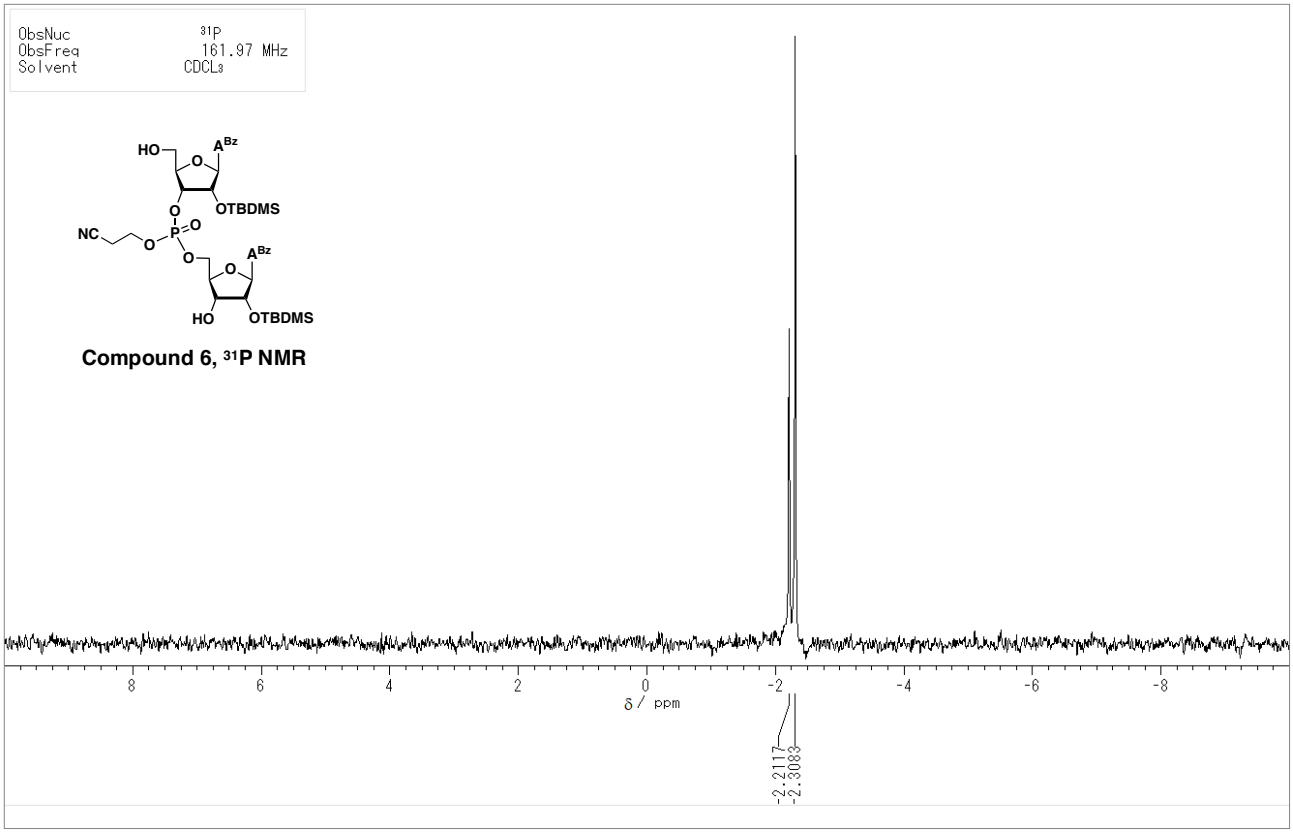
Compound 5, ^{31}P NMR



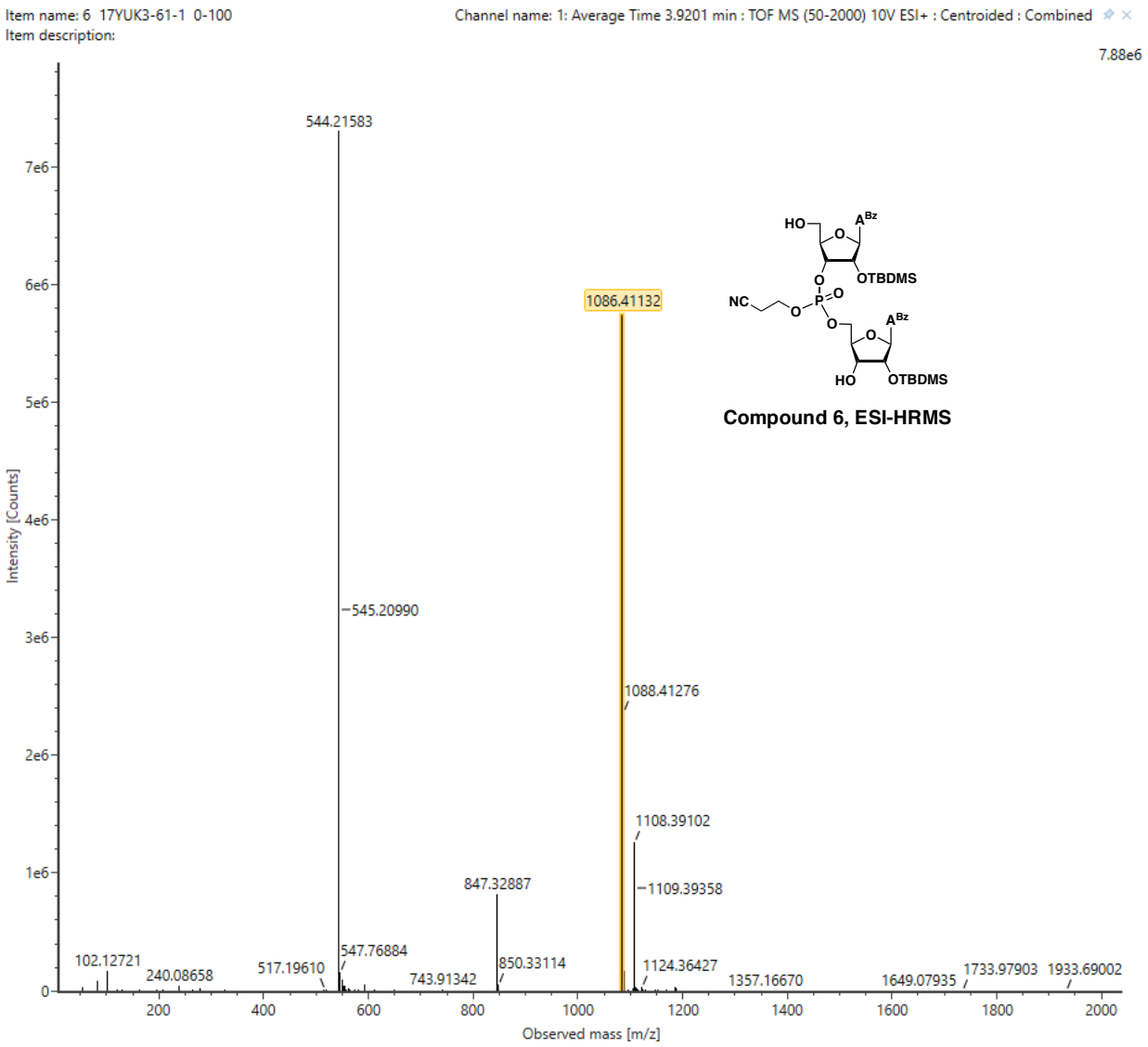
Compound 5, ESI-HRMS



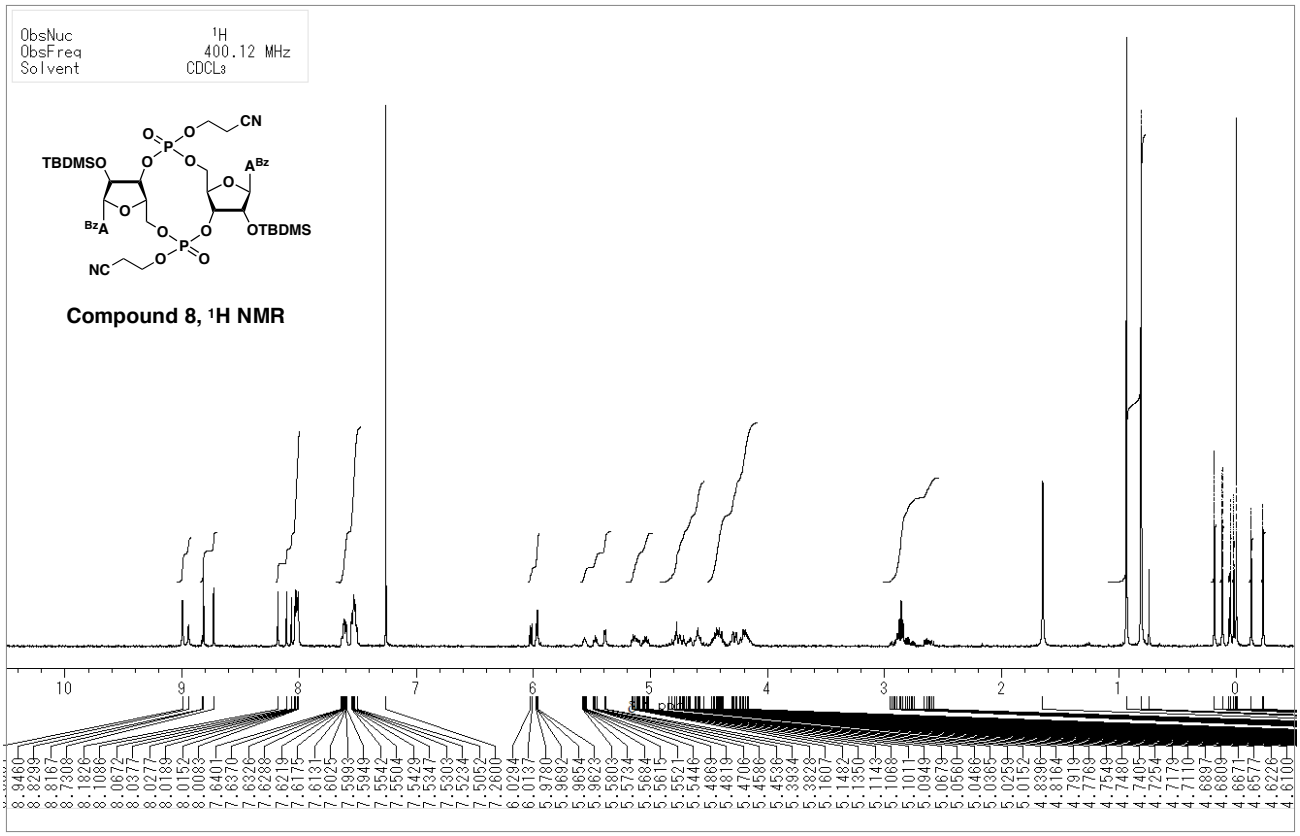
Compound 6, ^{1}H NMR



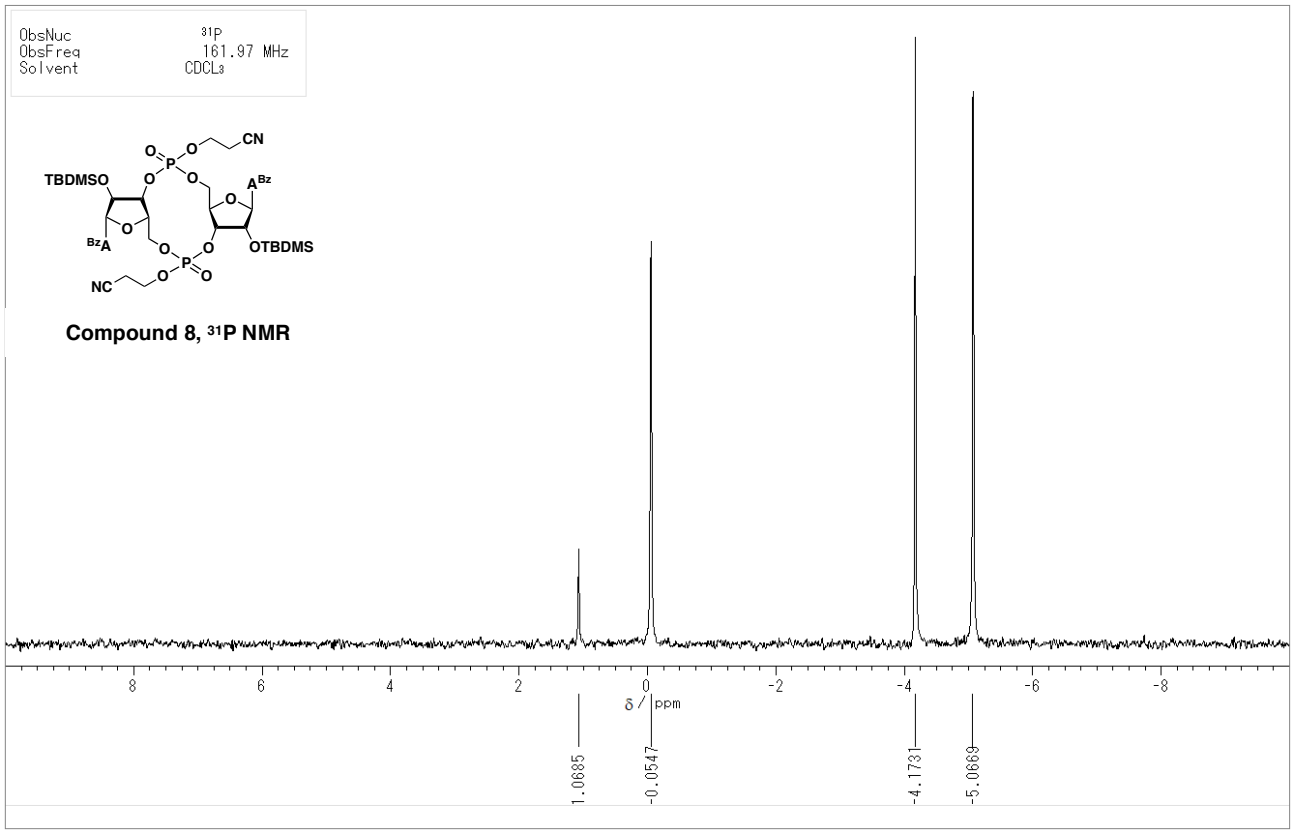
Compound 6, ^{31}P NMR



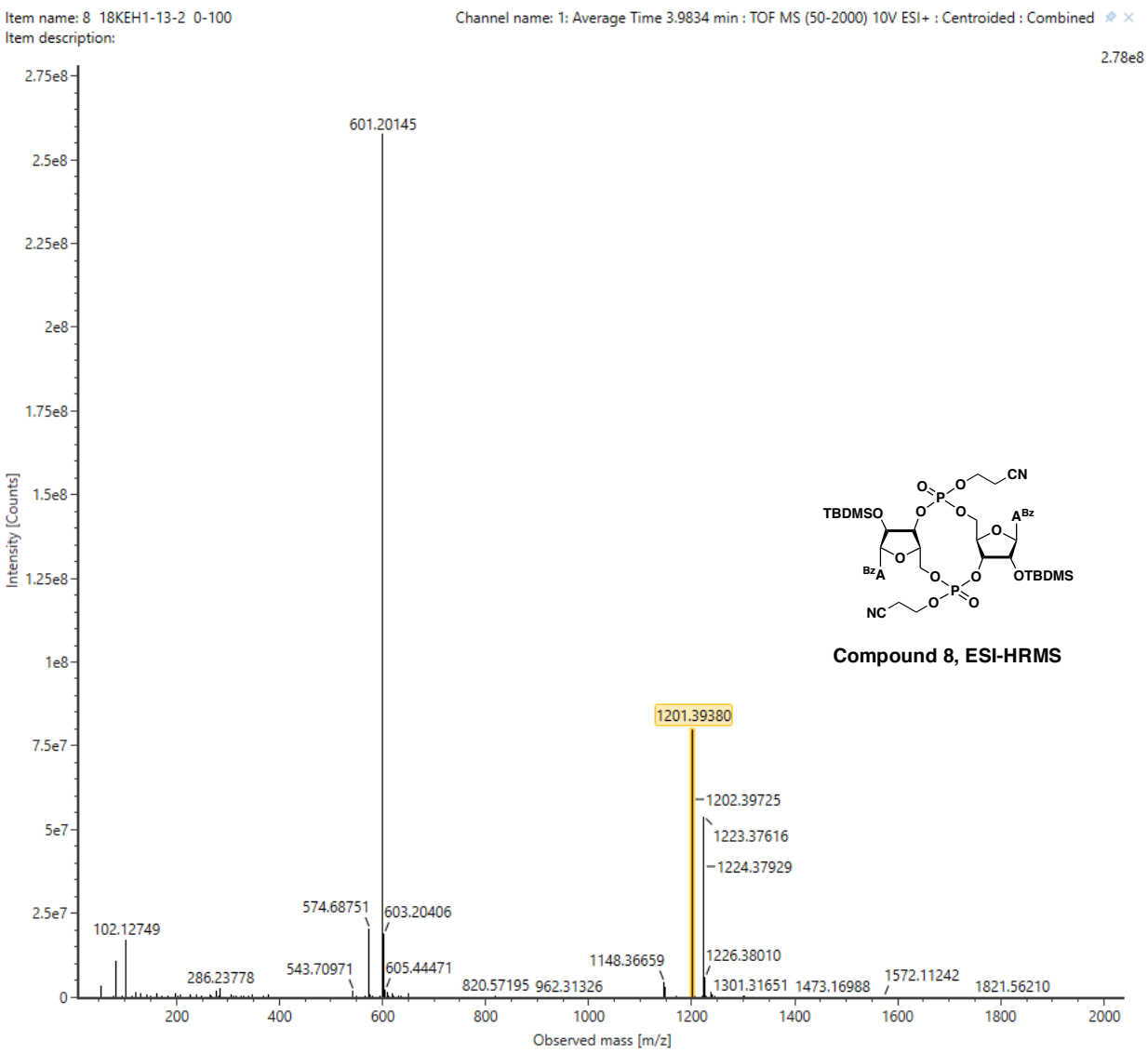
Compound 6, ESI-HRMS



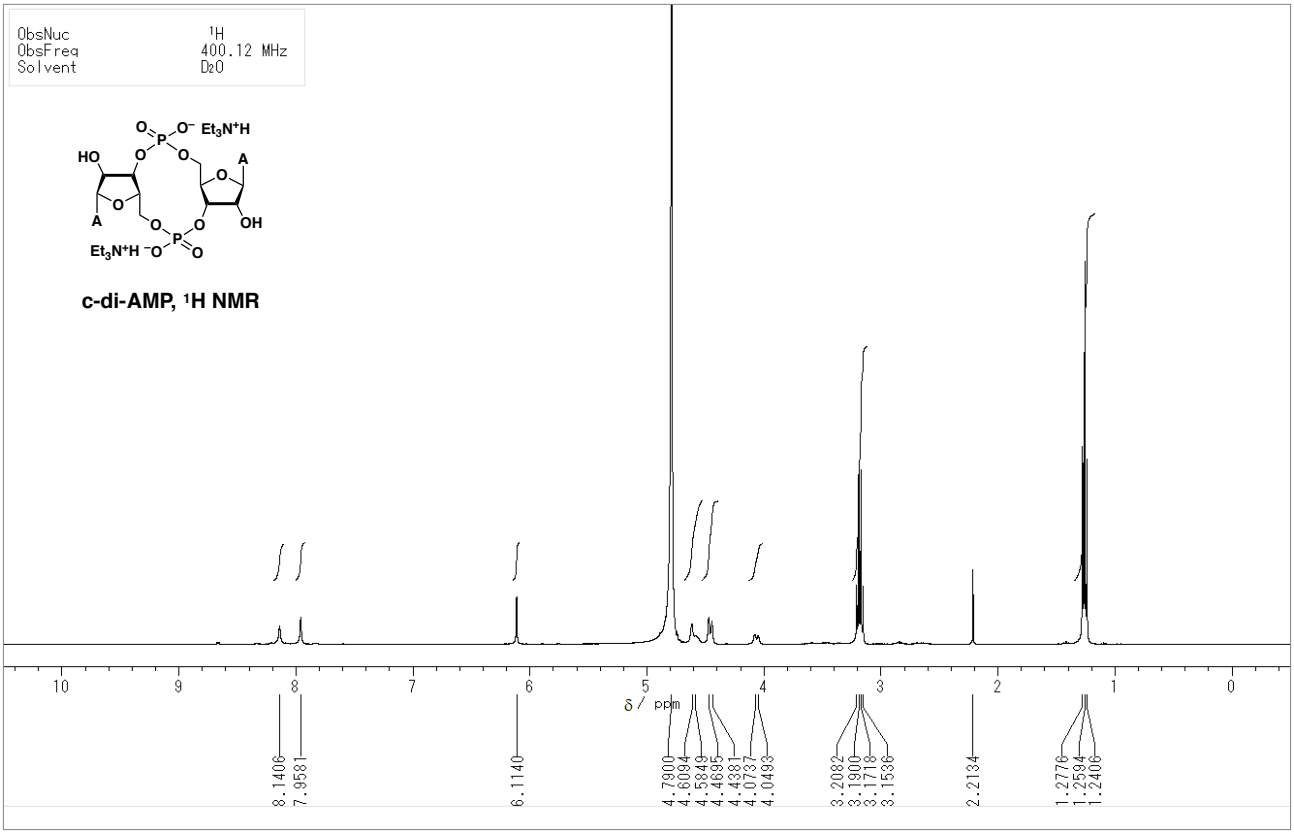
Compound 8, ^{1}H NMR



Compound 8, ^{31}P NMR



Compound 8, ESI-HRMS


ObsNuc 1H
ObsFreq 400.12 MHz
Solvent D2O
c-di-AMP, 1H NMR
δ / ppm
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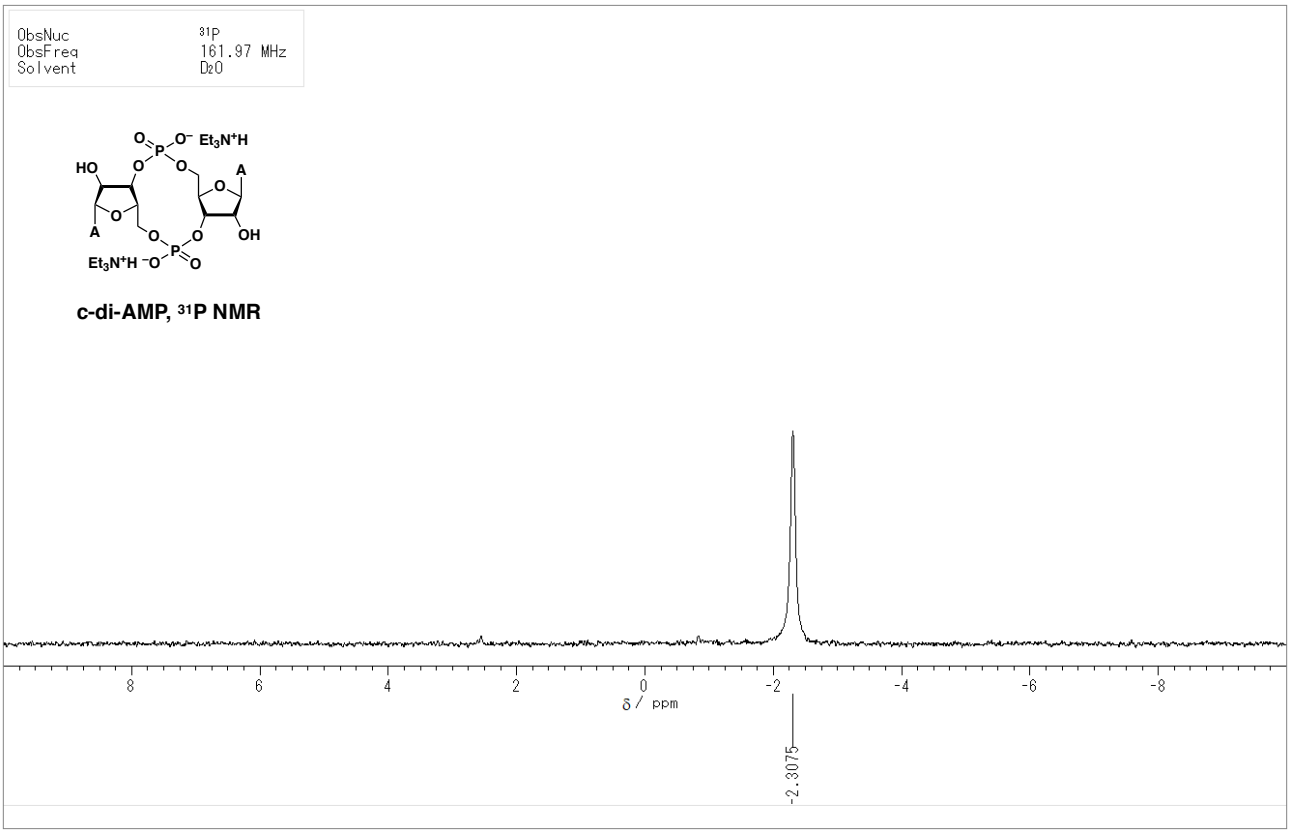
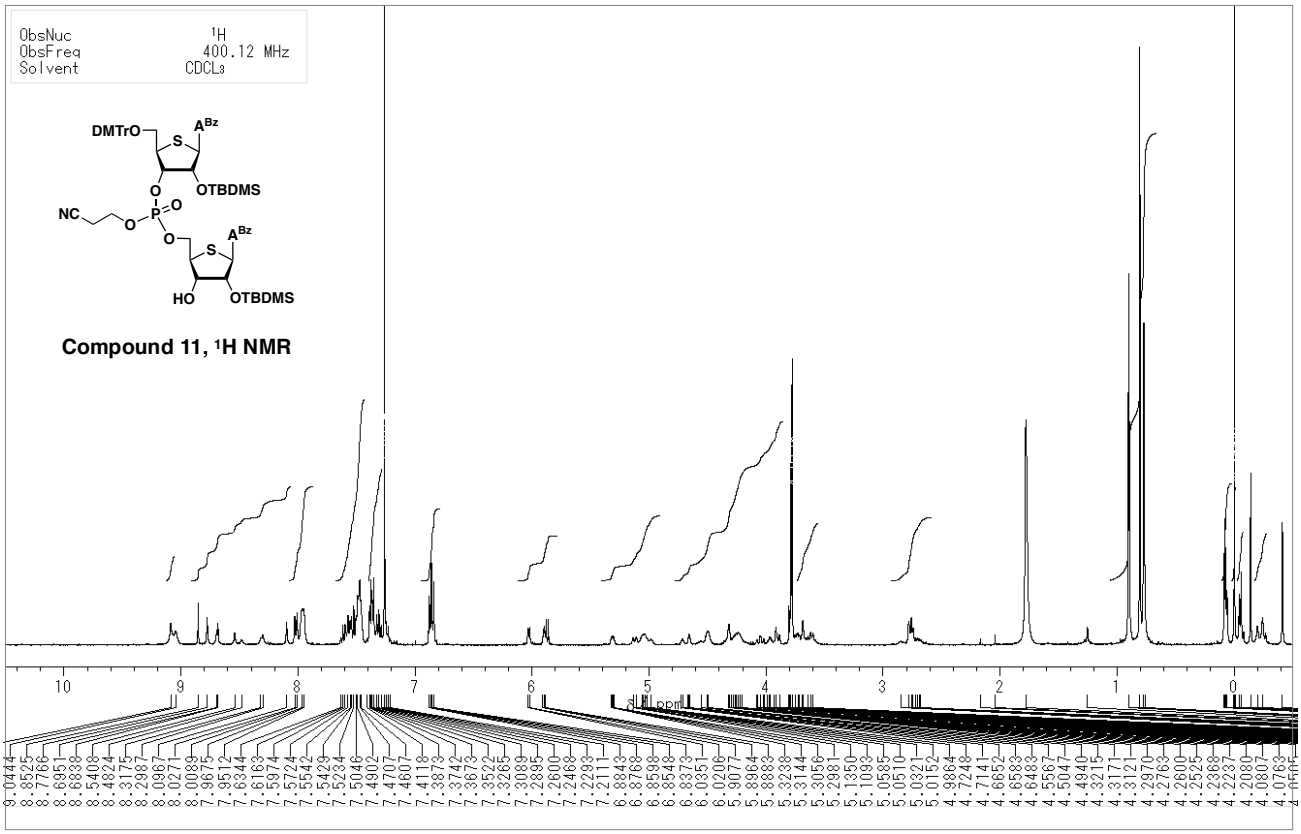
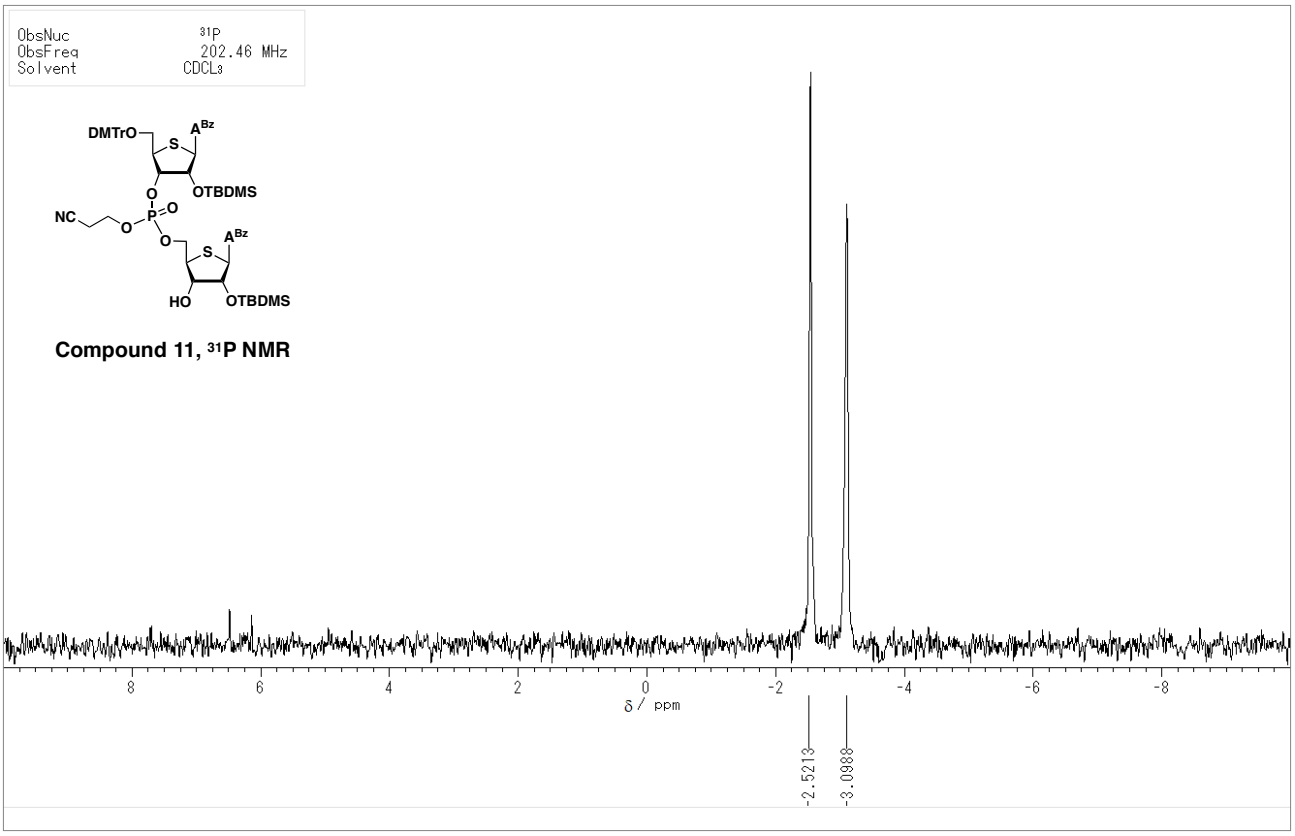
7.9581
6.1140
4.7900
4.6034
4.5849
4.4695
4.4381
4.0737
4.0493
3.2082
3.1900
3.1718
3.1538
2.2134
1.2776
1.2594
1.2406

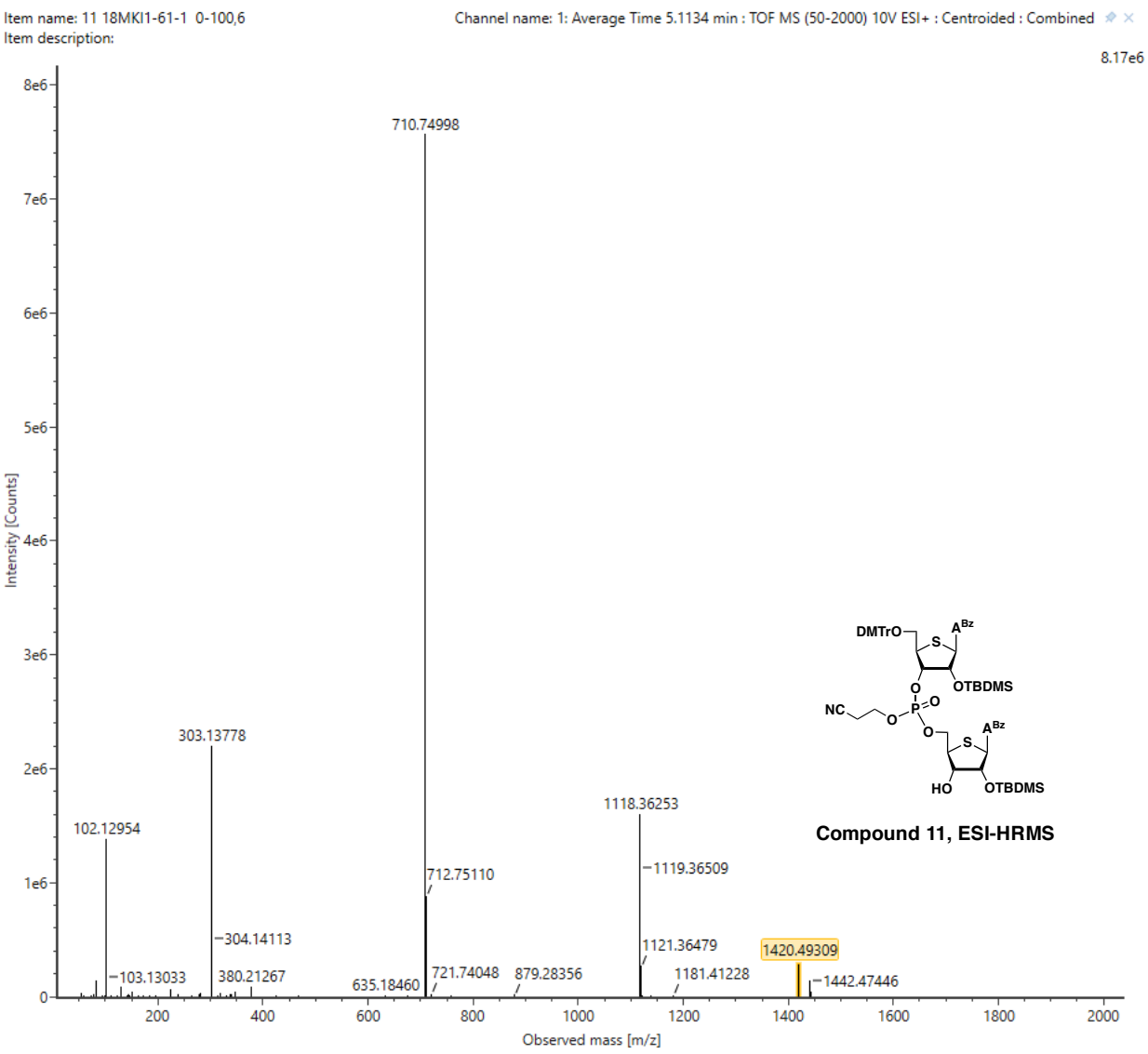

ObsNuc 31P
ObsFreq 161.97 MHz
Solvent D2O
c-di-AMP, 31P NMR
δ / ppm
-2.3075



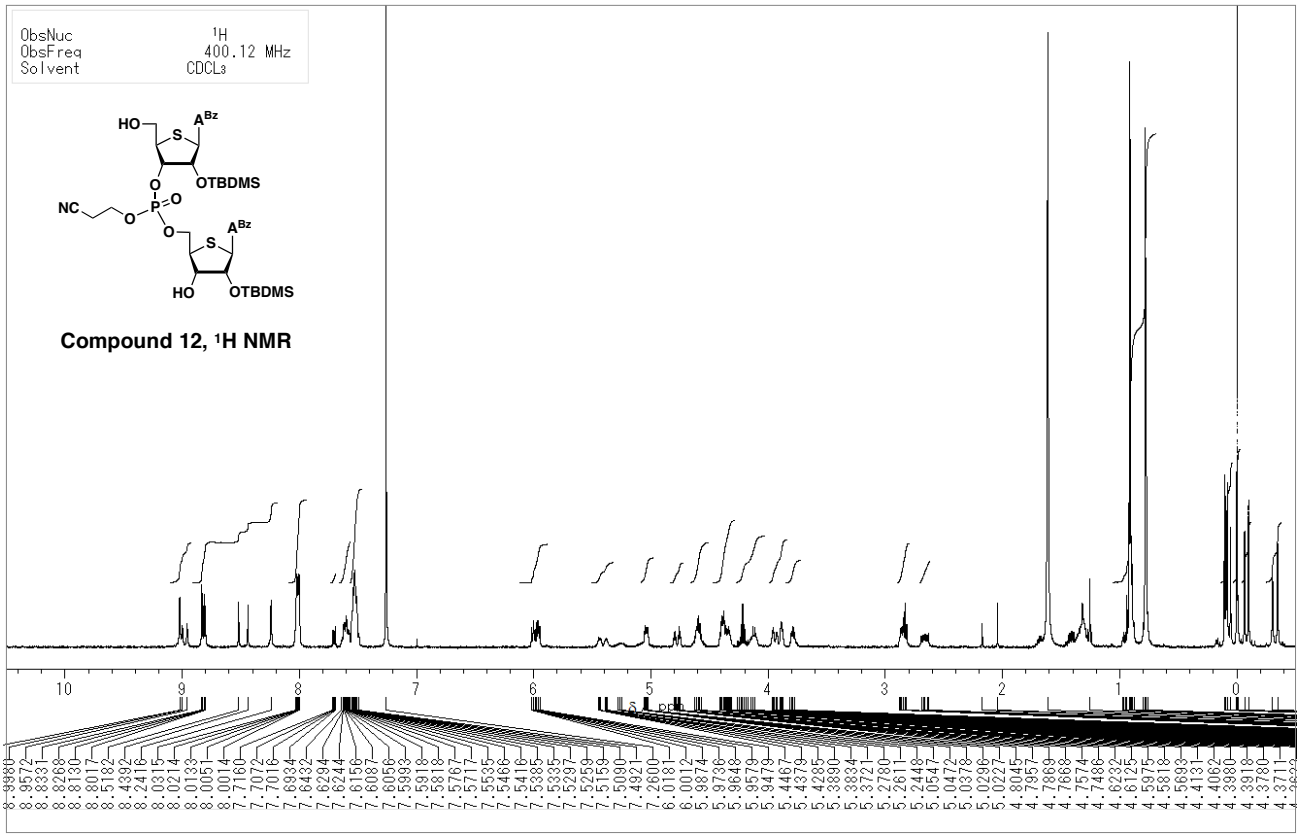
Compound 11, ^{1}H NMR



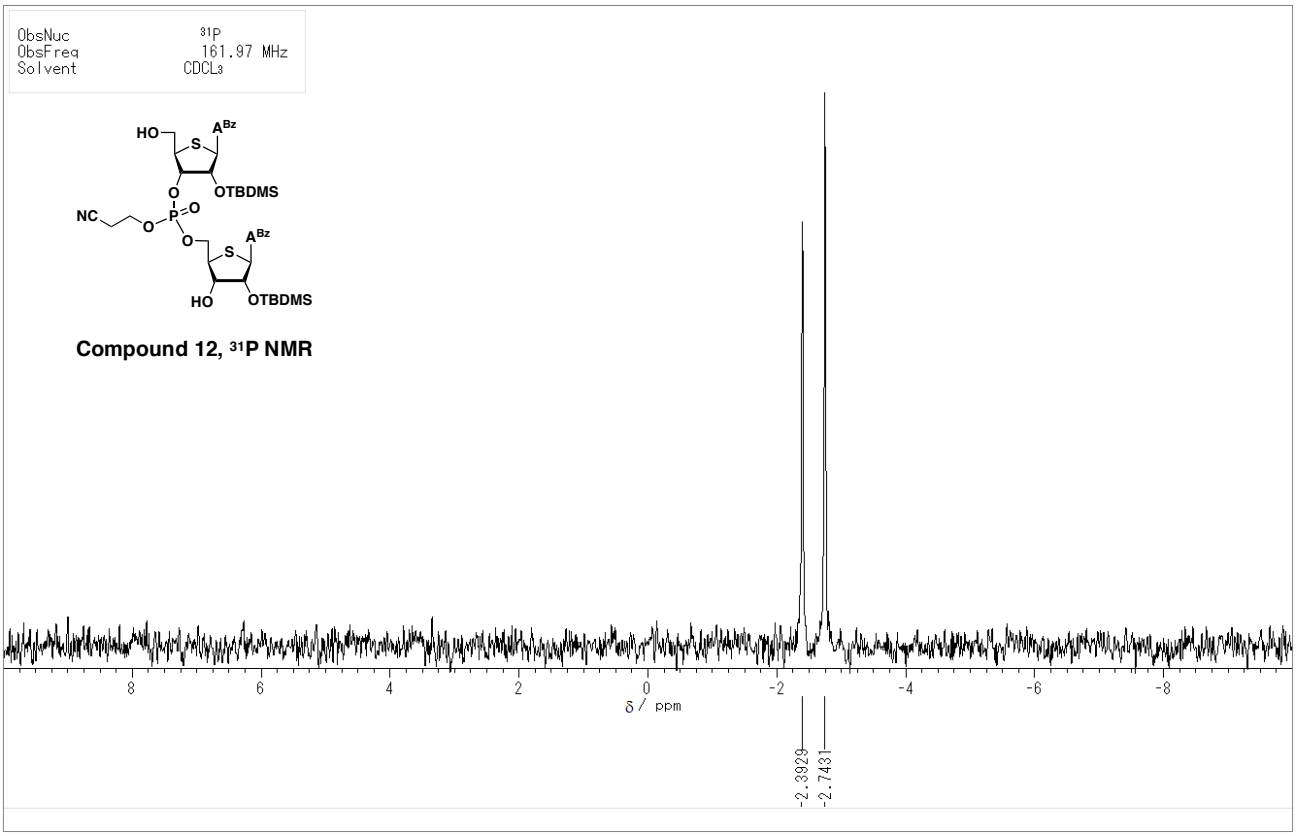
Compound 11, ^{31}P NMR



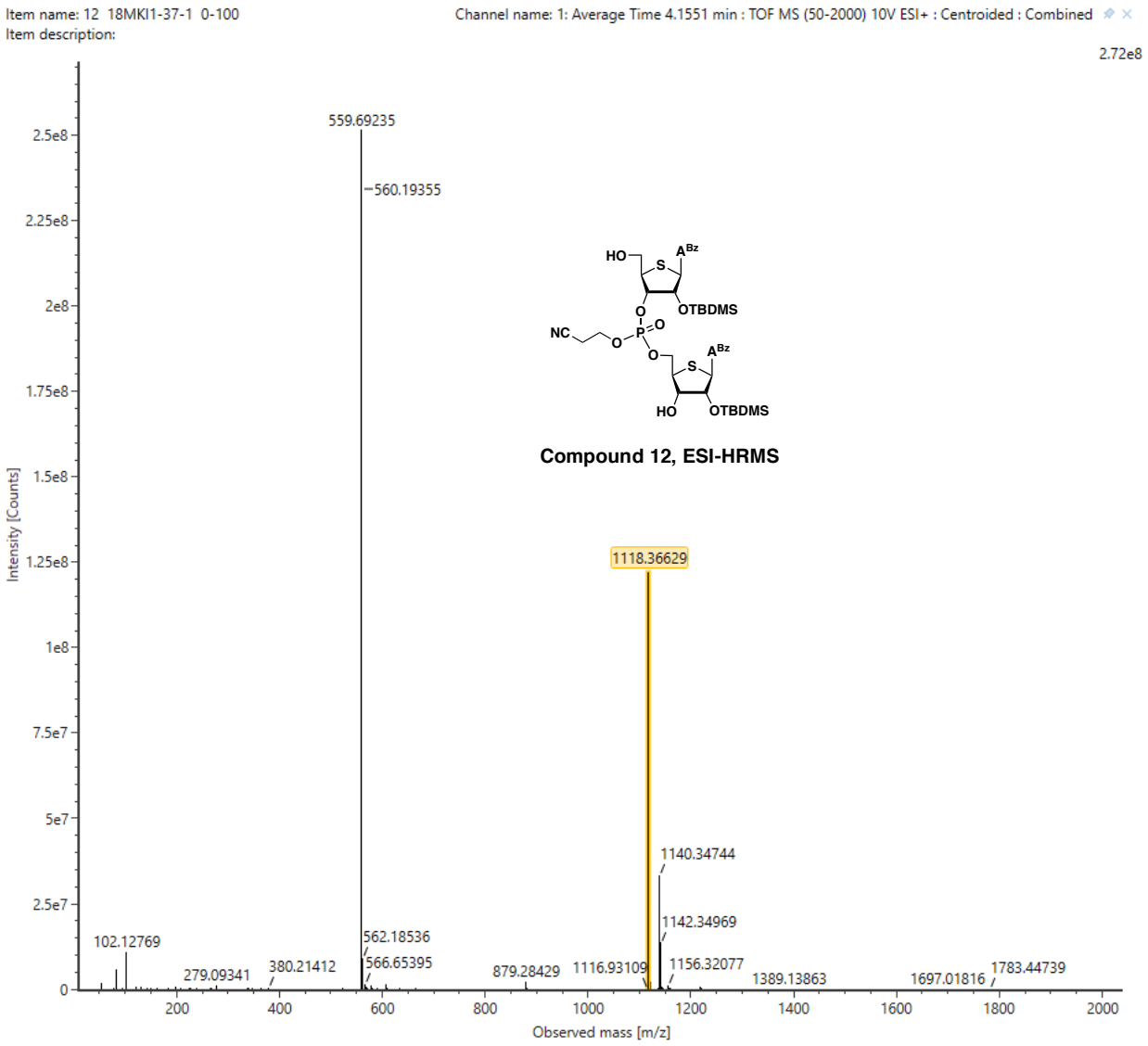
Compound 11, ESI-HRMS



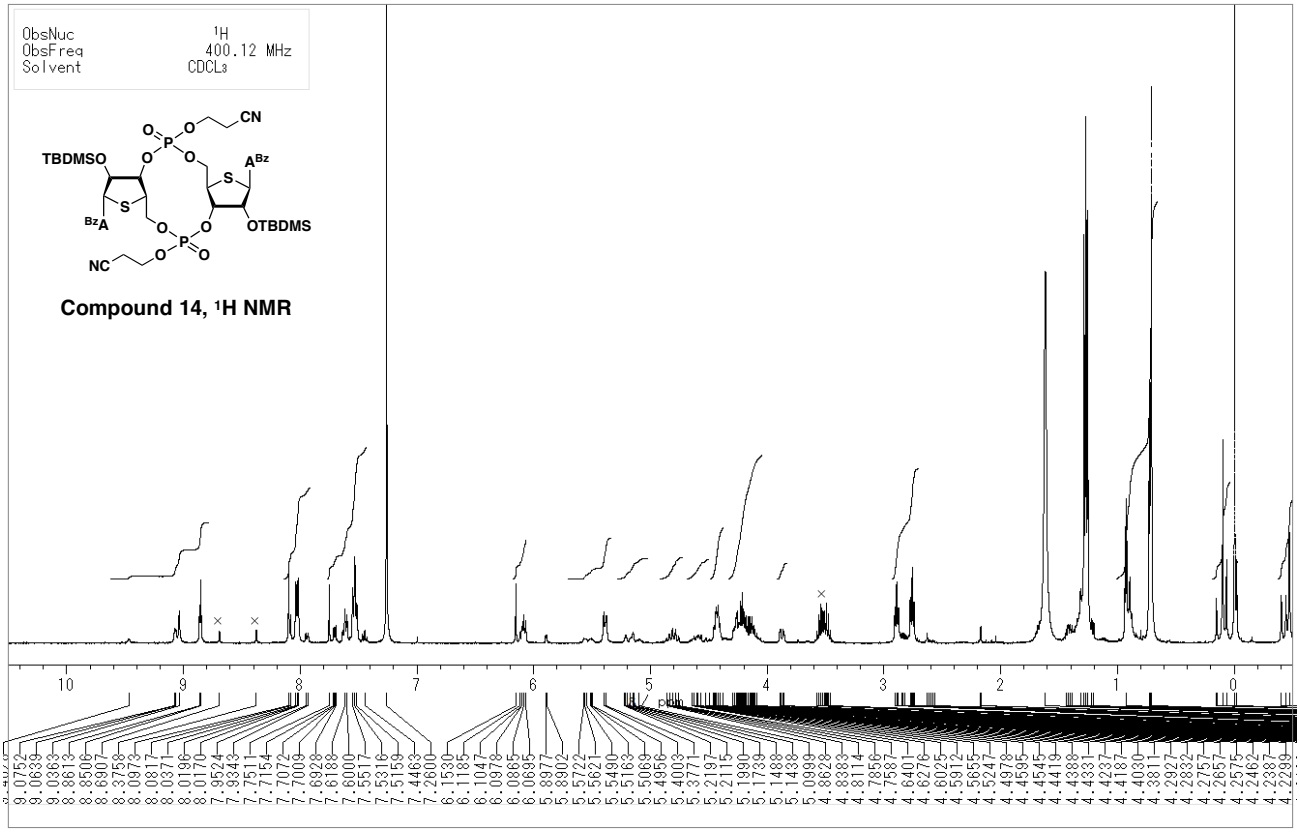
Compound 12, ^{1}H NMR



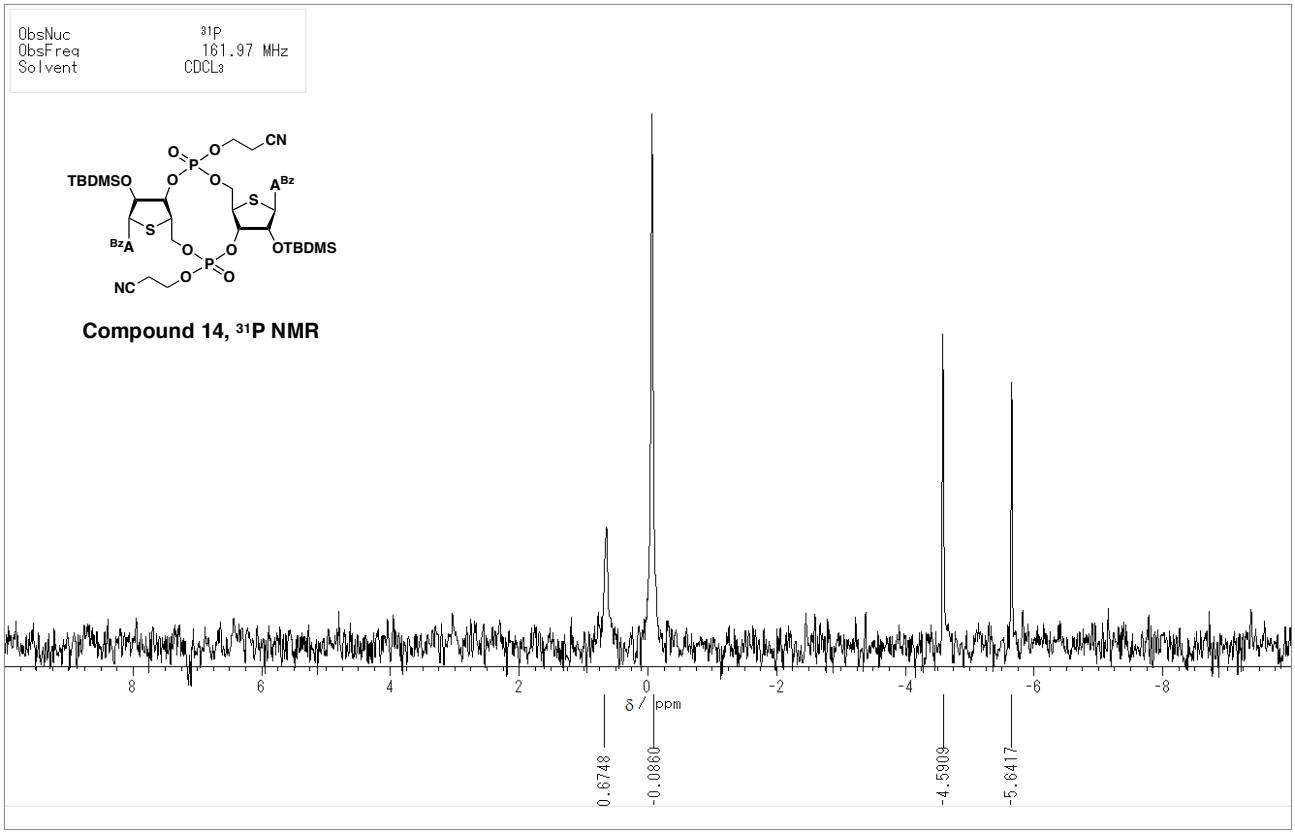
Compound 12, ^{31}P NMR



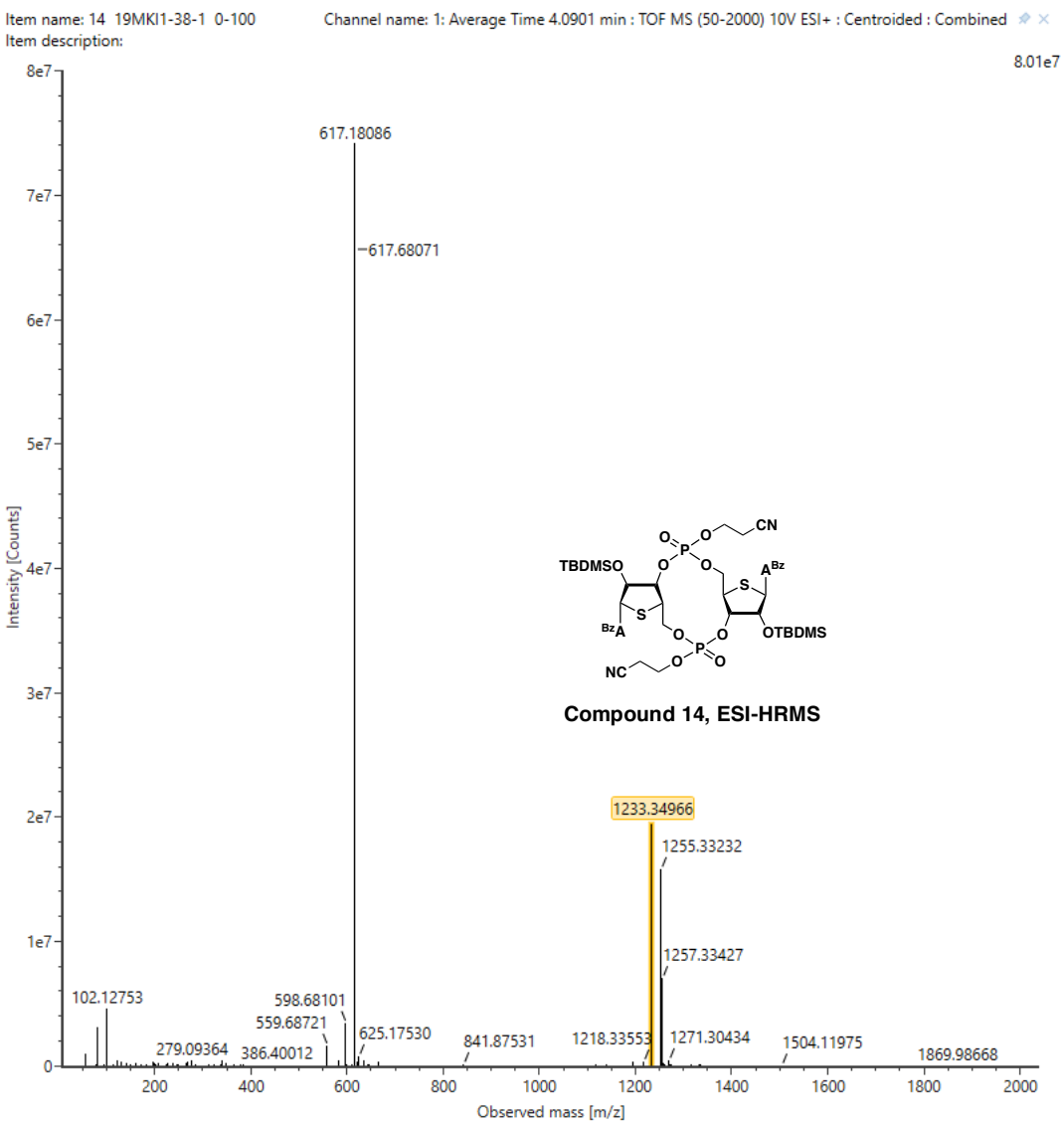
Compound 12, ESI-HRMS



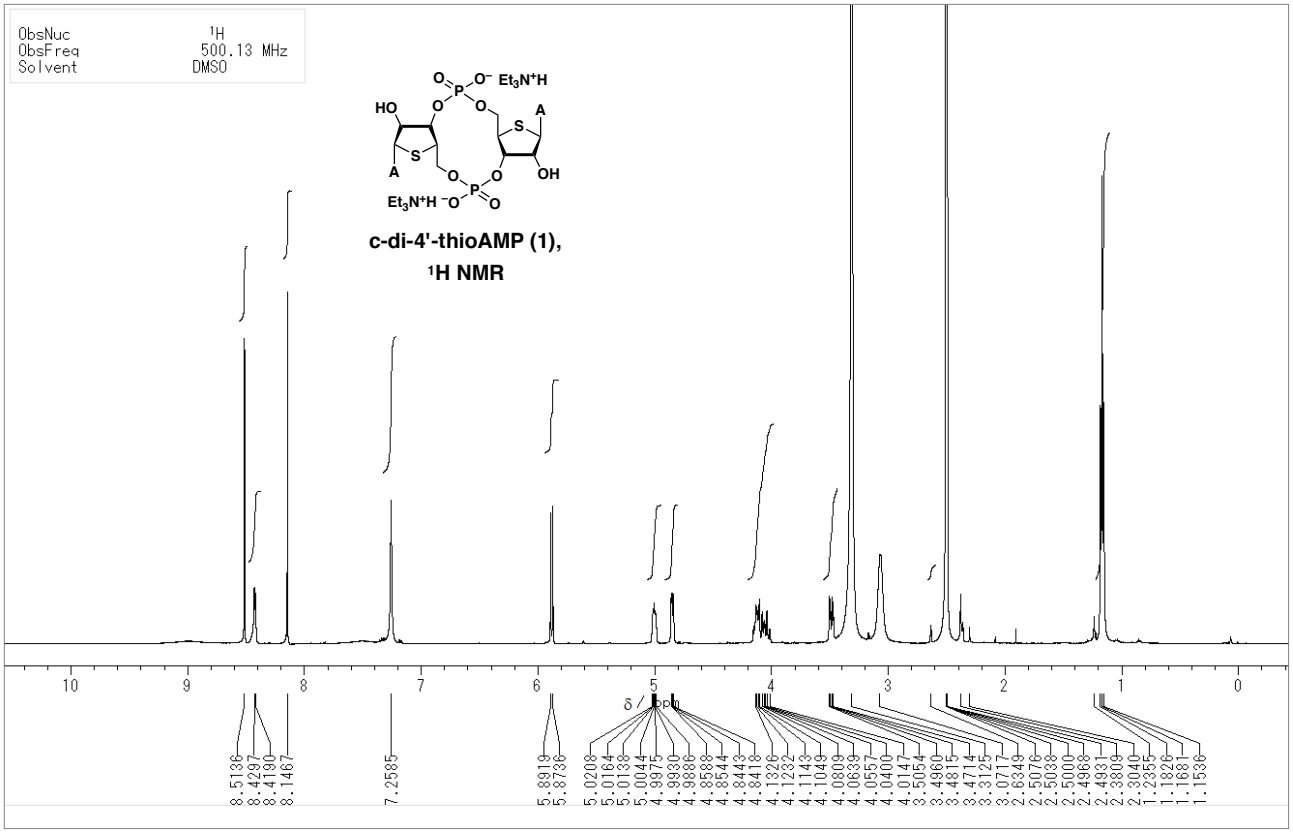
Compound 14, ^{1}H NMR



Compound 14, ^{31}P NMR



Compound 14, ESI-HRMS


ObsNuc 1H
ObsFreq 500.13 MHz
Solvent DMSO
c-di-4'-thioAMP (1),
1H NMR
δ / ppm
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8.4297
8.4190
8.1467
7.2585
5.8919
5.8736
5.0208
5.0164
5.0138
5.0044
4.9975
4.9930
4.9888
4.8588
4.8544
4.8443
4.8418
4.1326
4.1233
4.1143
4.1049
4.0809
4.0639
4.0557
4.0400
4.0147
3.5054
3.4961
3.4815
3.4714
3.3125
3.0717
2.6349
2.5076
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1.2355
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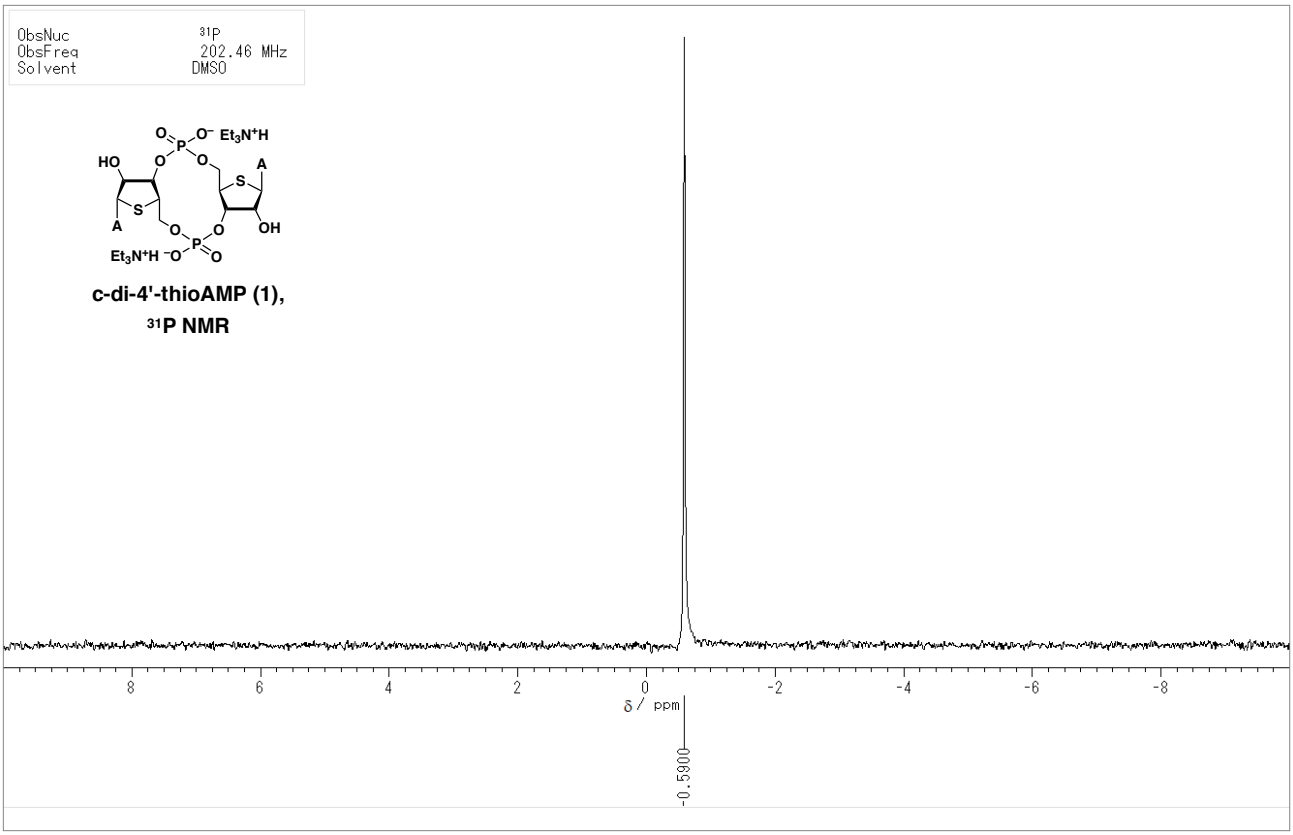
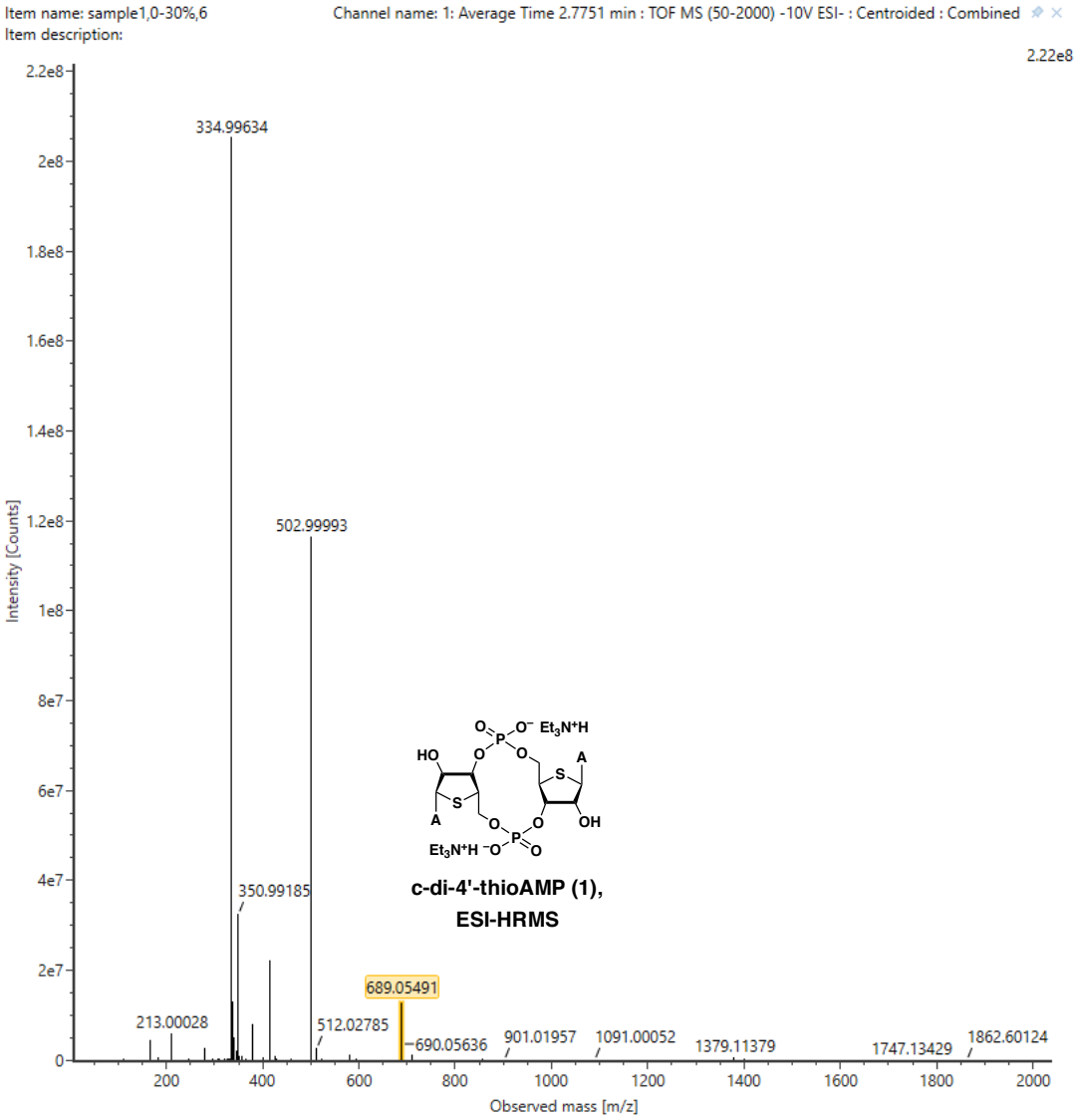
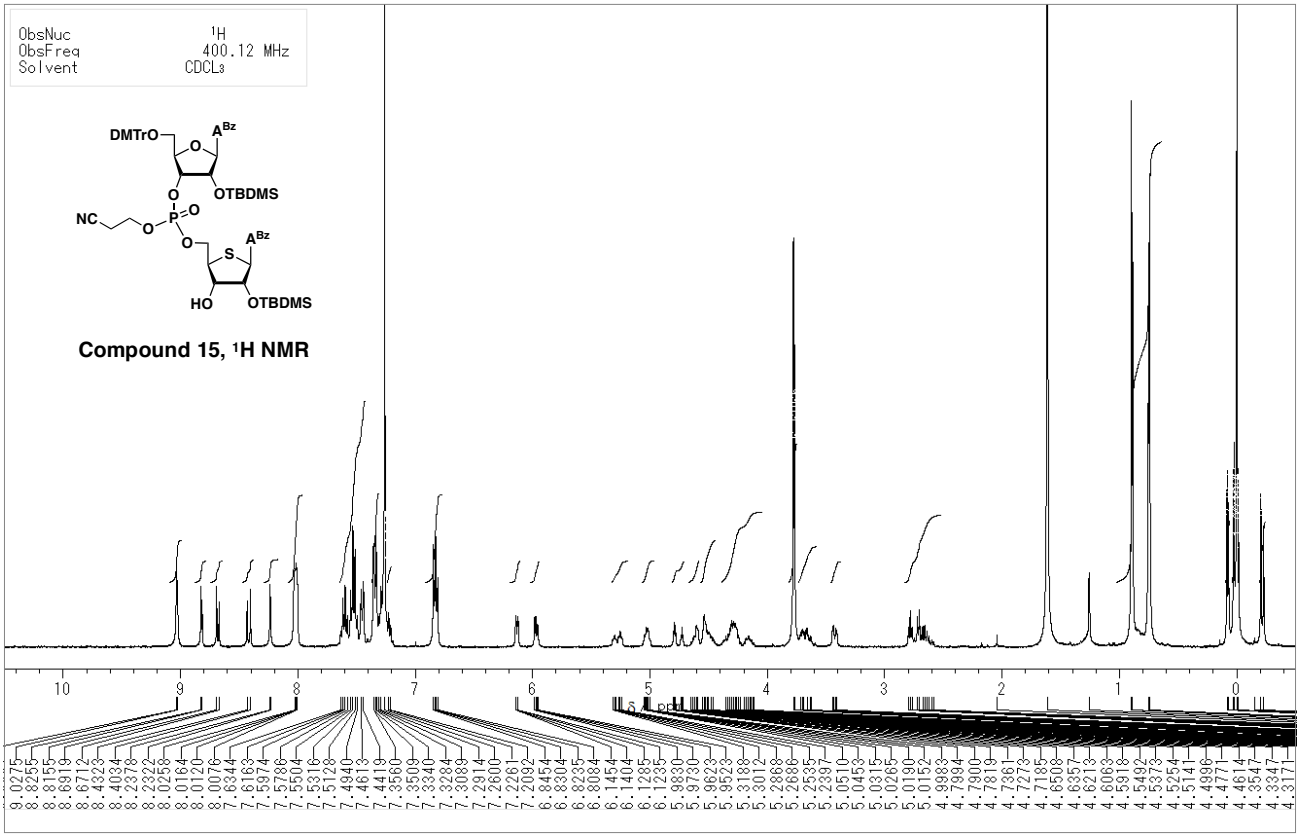
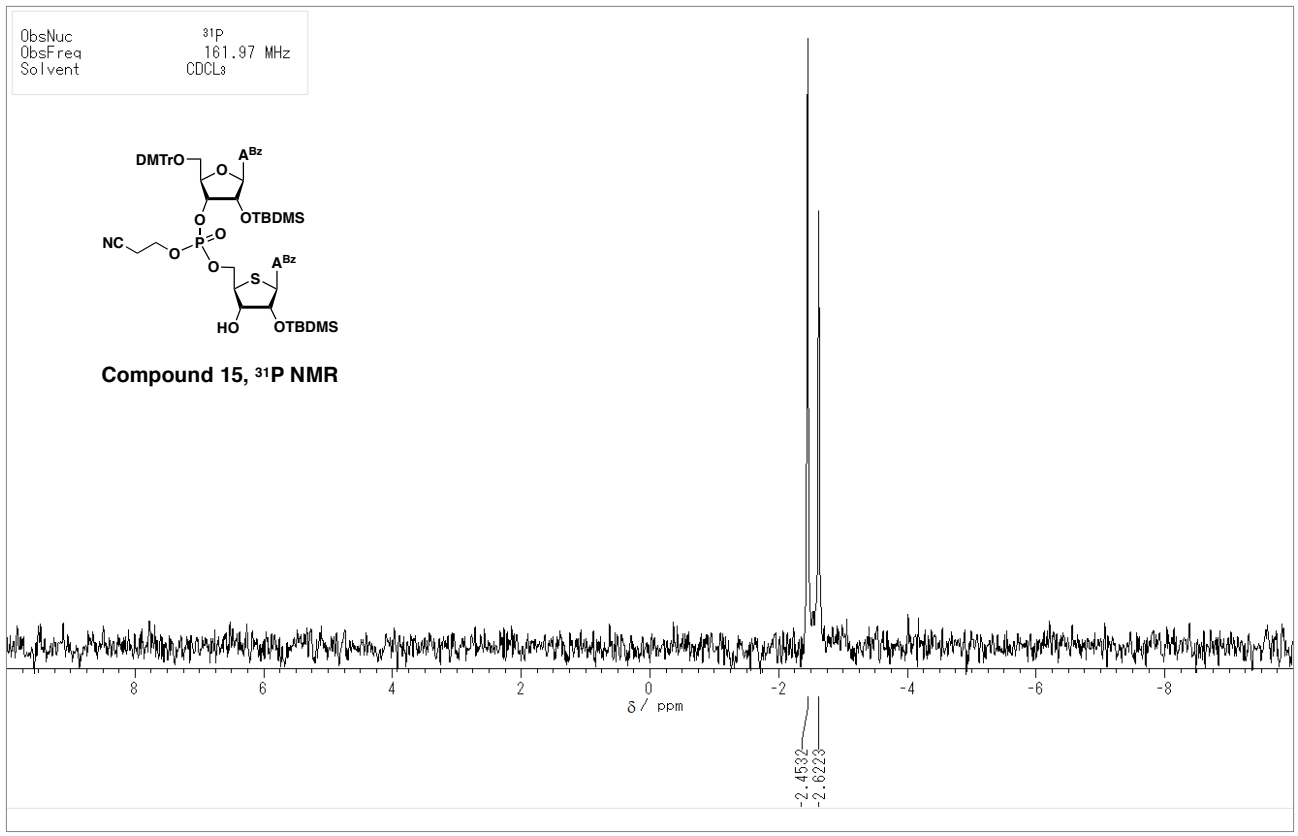
1.1681
1.1538


ObsNuc 31P
ObsFreq 202.46 MHz
Solvent DMSO
c-di-4'-thioAMP (1),
31P NMR
δ / ppm
-0.5900

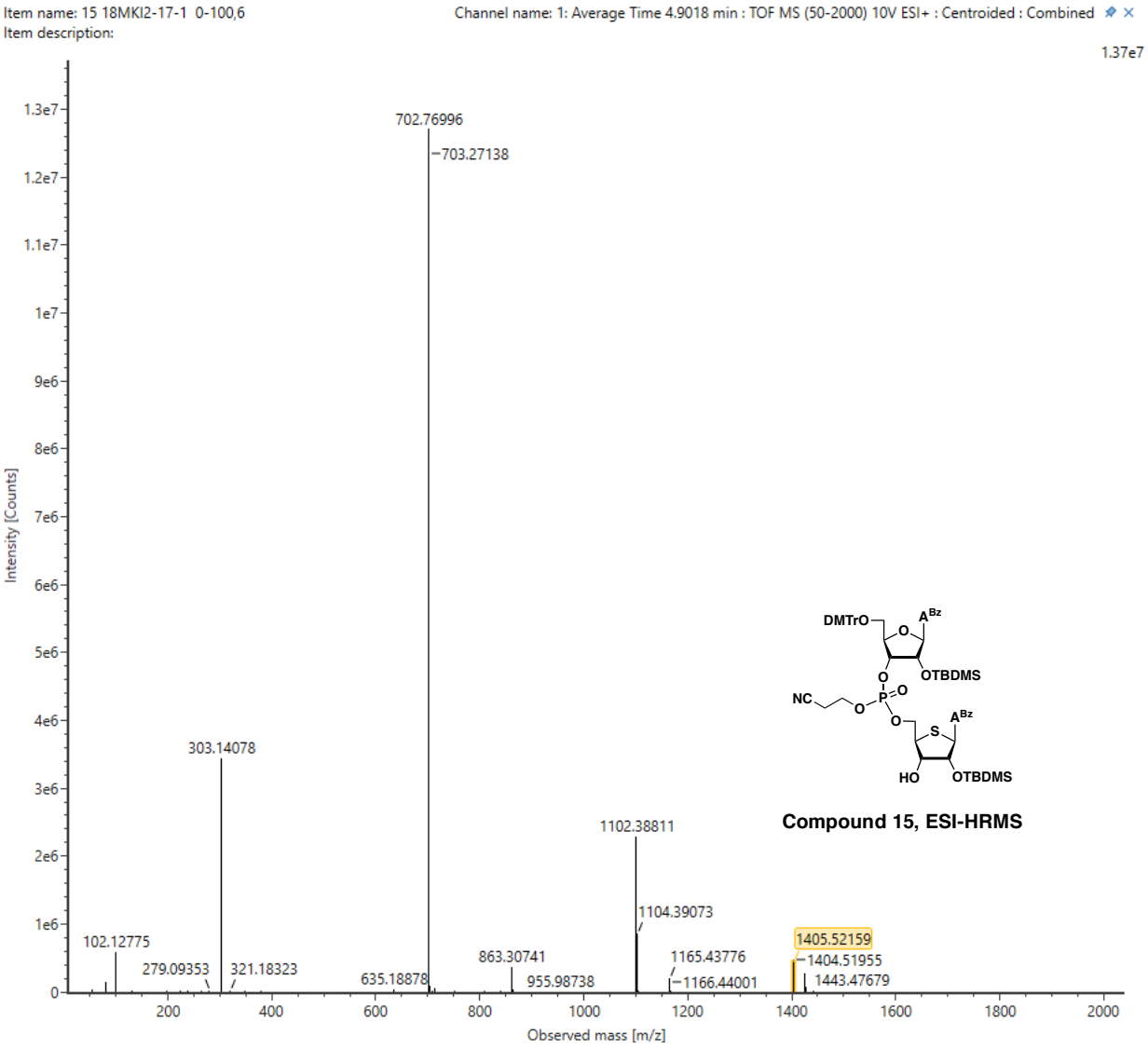

Item name: sample1,0-30%,6
Item description:
Channel name: 1: Average Time 2.7751 min : TOF MS (50-2000) -10V ESI- : Centroided : Combined
2.22e8
Intensity [Counts]
Observed mass [m/z]
334.99634
502.99993
350.99185
689.05491
213.00028
512.02785
690.05636
901.01957
1091.00052
1379.11379
1747.13429
1862.60124
c-di-4'-thioAMP (1),
ESI-HRMS



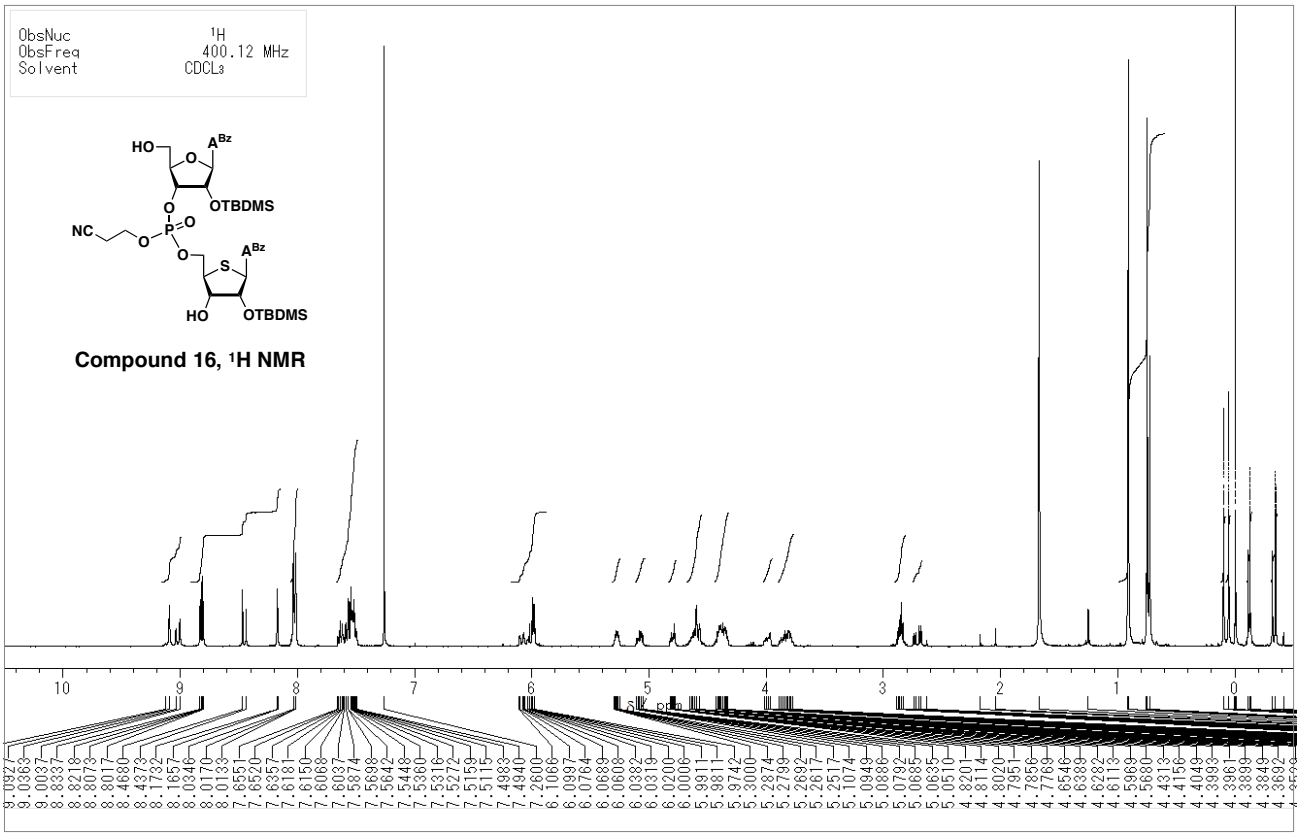
Compound 15, ^{1}H NMR



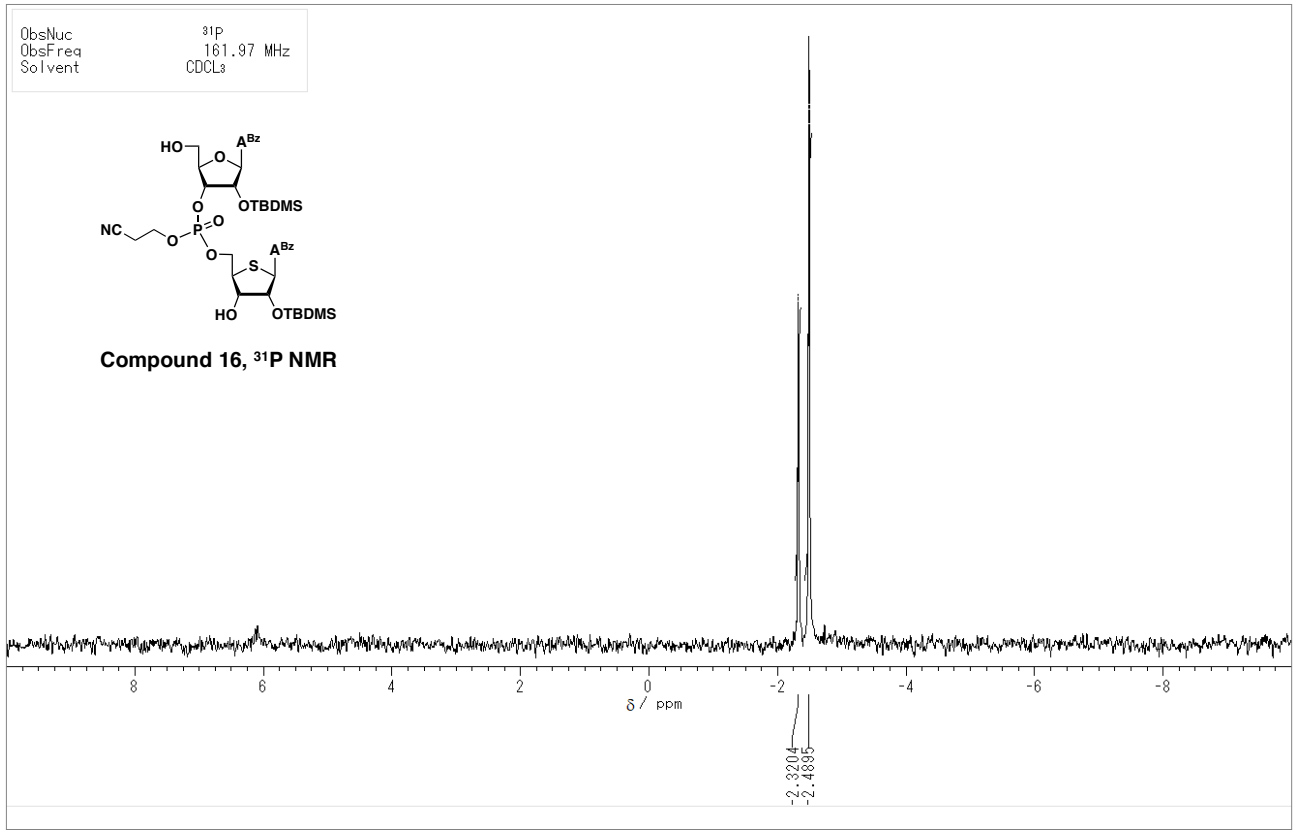
Compound 15, ^{31}P NMR



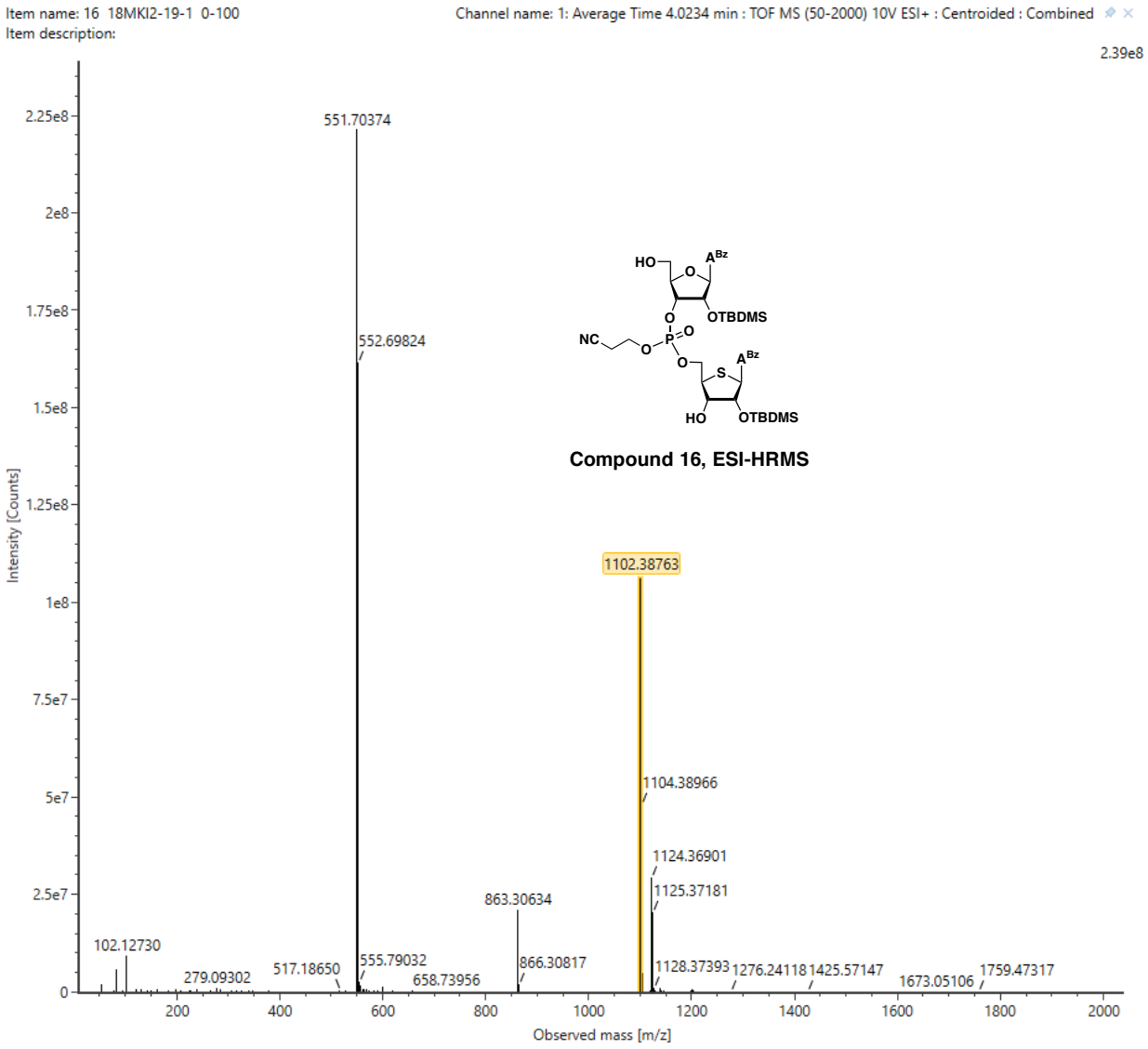
Compound 15, ESI-HRMS



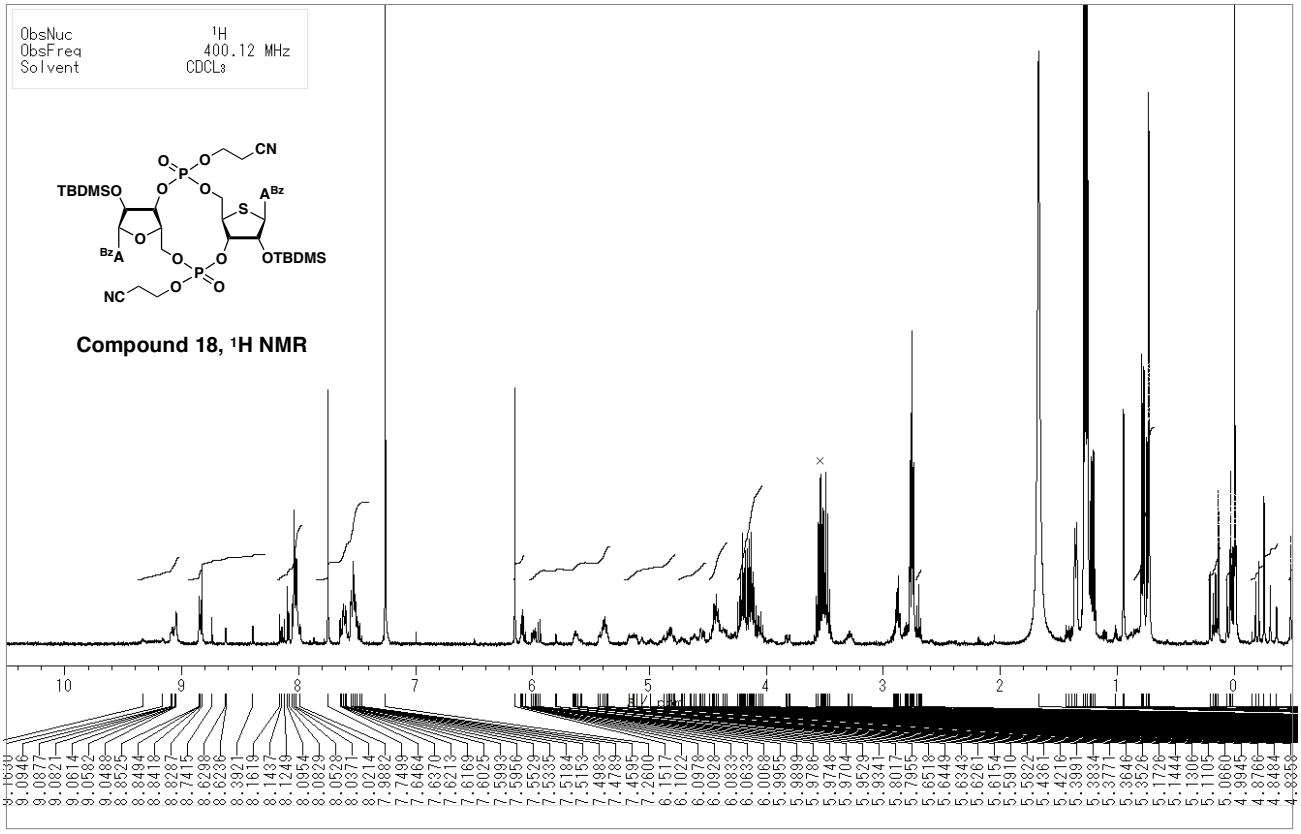
Compound 16, ^{1}H NMR



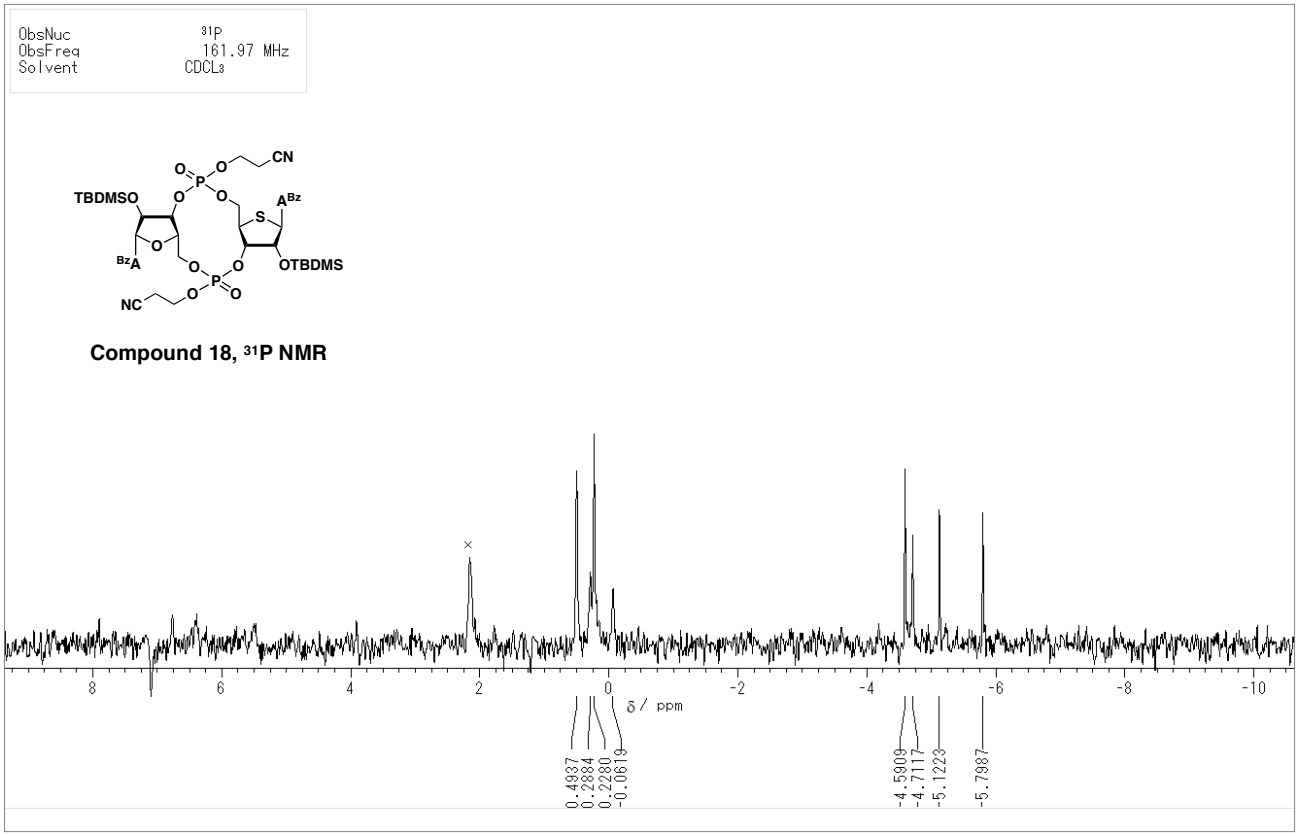
Compound 16, ^{31}P NMR



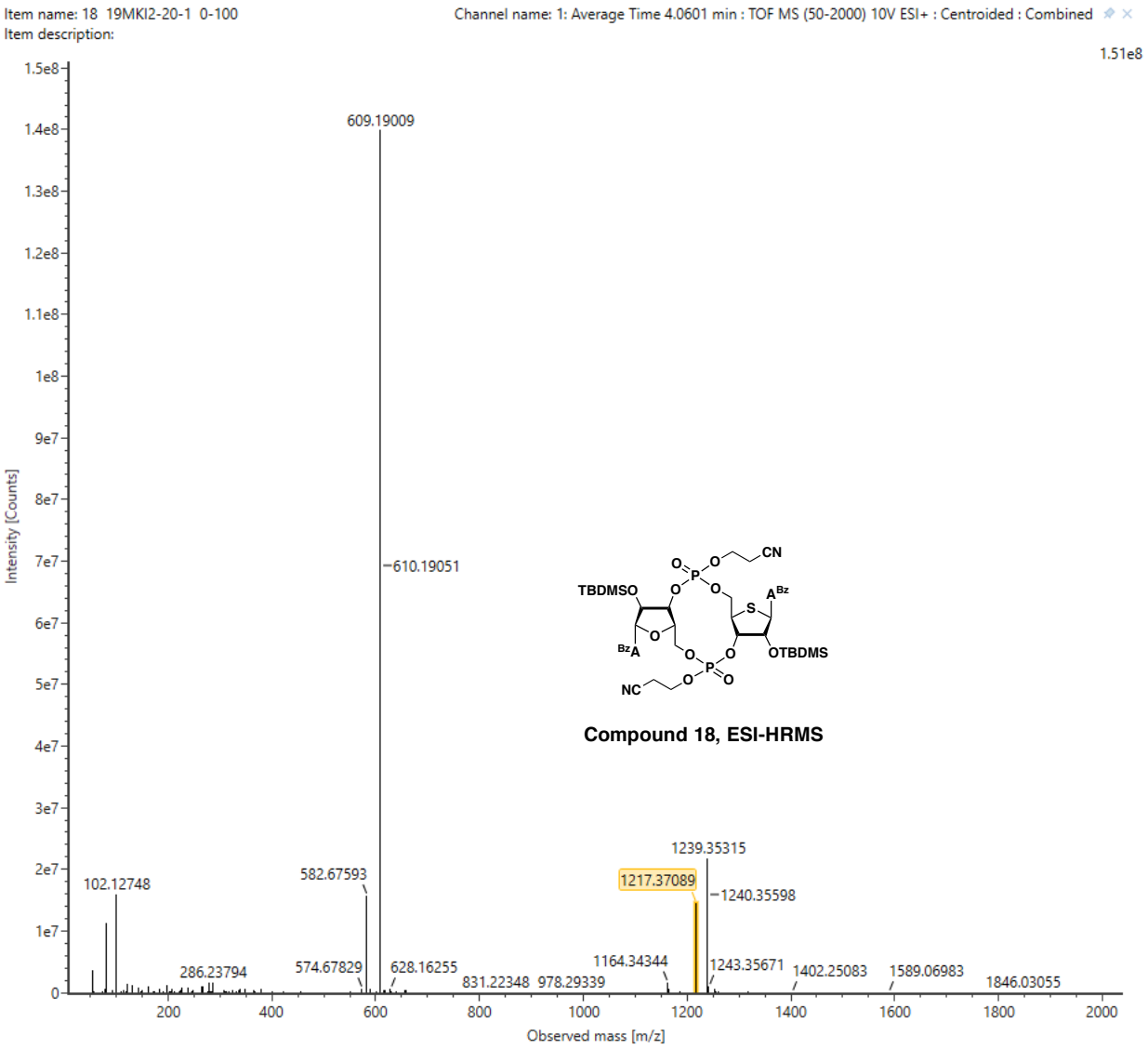
Compound 16, ESI-HRMS



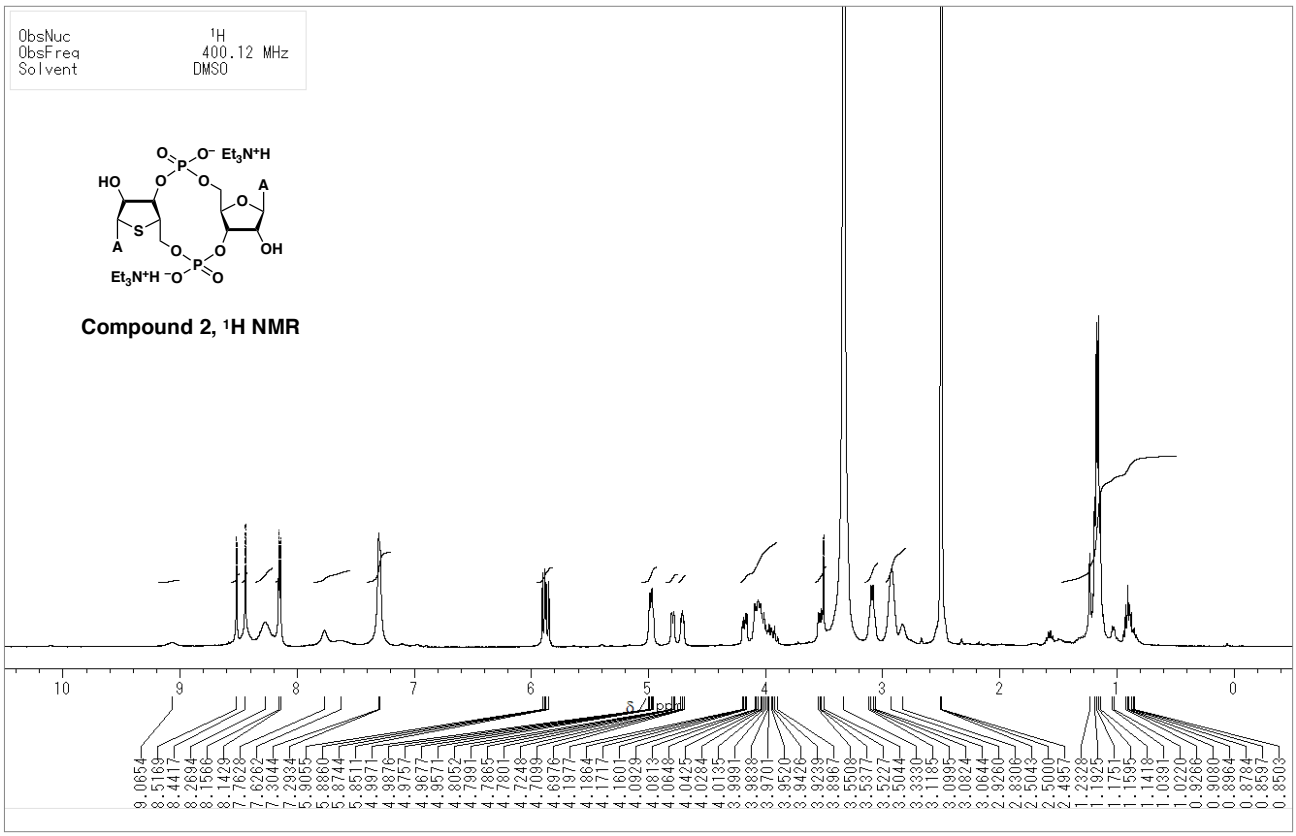
Compound 18, ^{1}H NMR



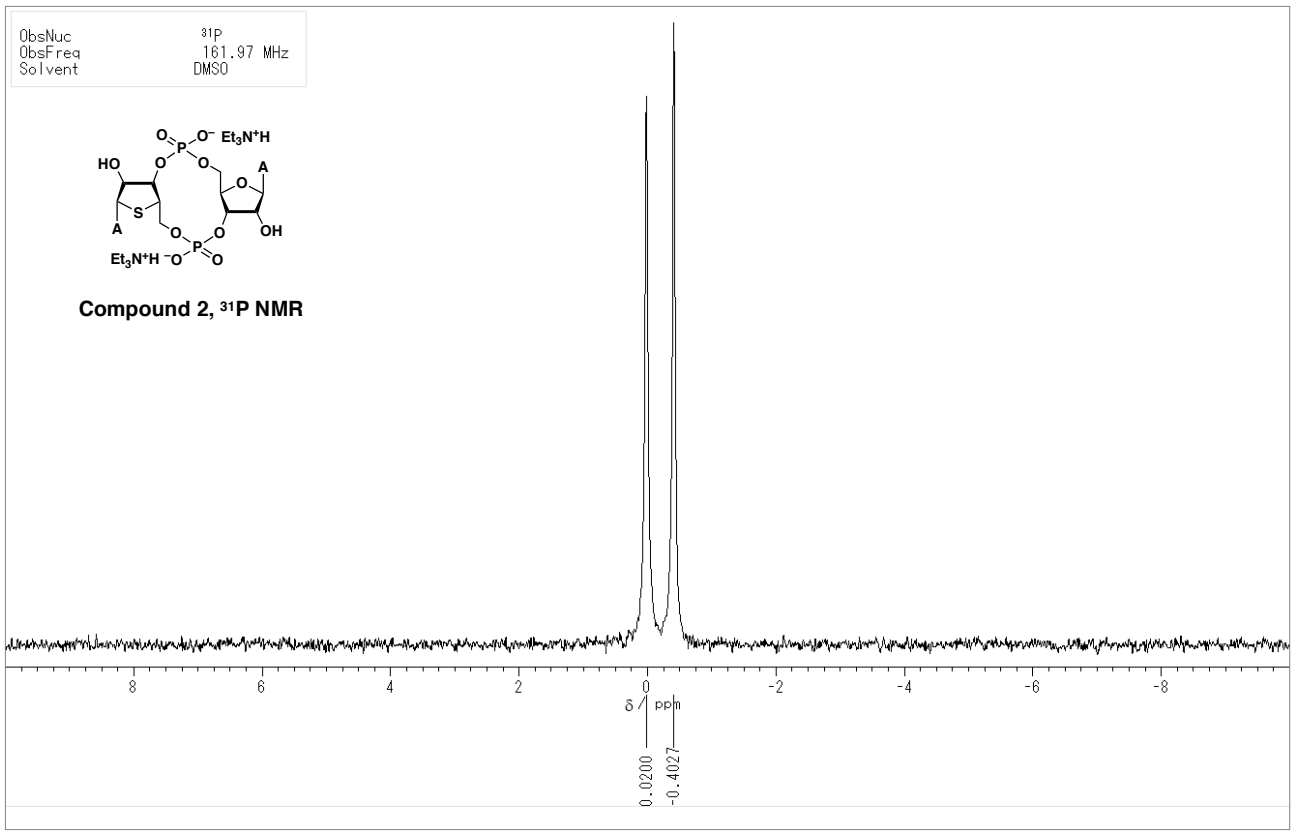
Compound 18, ^{31}P NMR



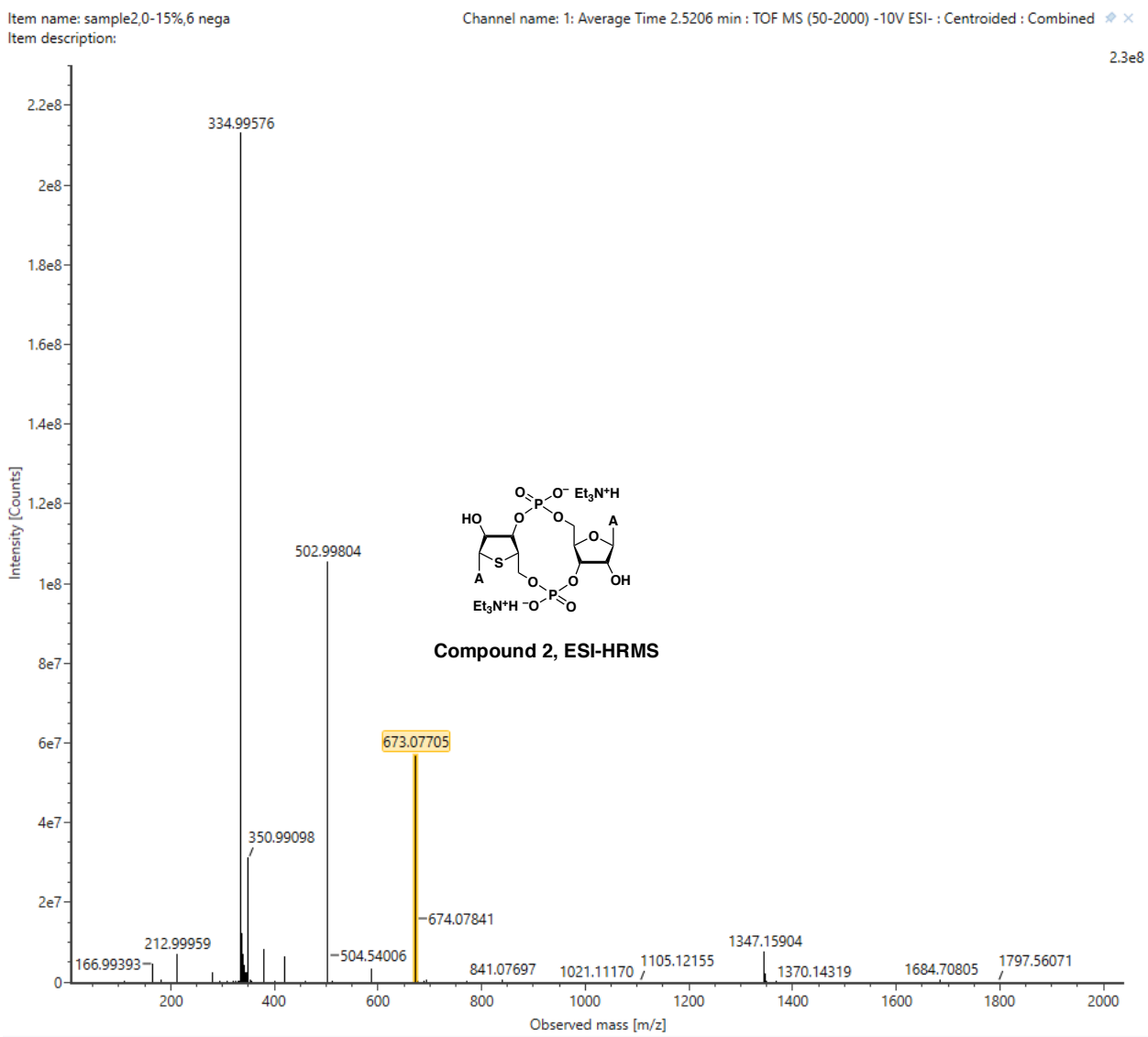
Compound 18, ESI-HRMS



Compound 2, ^{1}H NMR



Compound 2, ^{31}P NMR



Compound 2, ESI-HRMS