Rational optimization of siRNA to ensure strand bias in the interaction with the RNA-induced silencing complex

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Supporting Information

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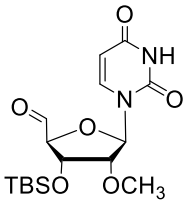
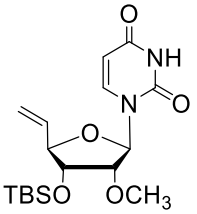
Synthesis of building blocks

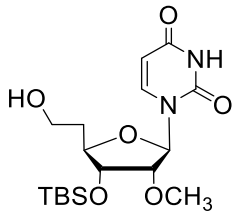
General conditions: TLC was performed on Merck silica gel 60 plates coated with F254. Compounds were visualized under UV light (254 nm) or after spraying with the p-anisaldehyde staining solution followed by heating. Flash column chromatography was performed using a Teledyne ISCO Combi Flash system with pre-packed RediSep Teledyne ISCO silica gel cartridges and Prep-Achiral supercritical fluid chromatography. All moisture-sensitive reactions were carried out under anhydrous conditions using dry glassware, anhydrous solvents, and argon atmosphere. All commercially available reagents and solvents were purchased from Sigma-Aldrich unless otherwise stated and were used as received. ESI-MS spectra were recorded on a Waters QTof Premier instrument using the direct flow injection mode. ^{1}H NMR spectra were recorded at 300, 400, and 500 MHz. ^{13}C NMR spectra were recorded at 75, 101, and 126 MHz. ^{31}P NMR spectra were recorded at 162 and 202 MHz. Chemical shifts are given in ppm referenced to the solvent residual peak (DMSO-d_6 – ^{1}H: δ at 2.50 ppm and ^{13}C δ at 39.5 ppm; $CDCl_3$ – ^{1}H: δ at 7.26 ppm and ^{13}C δ at 77.16 ppm). Coupling constants are given in Hertz. Signal splitting patterns are described as singlet (s), doublet (d), triplet (t), septet (sept), broad signal (brs), or multiplet (m).



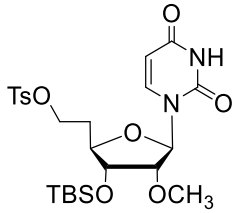
***(2S,5R)-3-[tert-butyl(dimethyl)silyl]oxy-5-(2,4-dioxopyrimidin-1-yl)-4-methoxy-tetrahydrofuran-2-carbaldehyde* (2):** The aldehyde was synthesized following the literature procedure.[1] 2-Iodoxybenzoic acid (2.82 g, 10.07 mmol) was added to **1**[2] (1.25 g, 3.36 mmol) in anhydrous acetonitrile (30 mL) under argon atmosphere. The mixture was refluxed at 81 °C for 0.75 h and then cooled. The reaction mixture was filtered through a celite bed, and the solid residue was washed with ethyl acetate (EtOAc) (50 mL). The combined filtrate was evaporated at 30 °C. The gummy residue thus obtained was further co-evaporated with toluene (30 mL) to afford **2** (1.15 g, 93% yield) as an amorphous white solid that was used in the next step without further purification. The product was stored at −20 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.79 (s, 1H), 9.75 (s, 1H), 7.68 (d, *J* = 8.1 Hz, 1H), 5.88 – 5.72 (m, 2H), 4.55 (d, *J* = 4.5 Hz, 1H), 4.43 (t, *J* = 4.5 Hz, 1H), 3.96 (t, *J* = 4.7 Hz, 1H), 3.47 (s, 3H), 0.93 (s, 9H), 0.14 (d, J = 7.7 Hz, 6H) ppm.



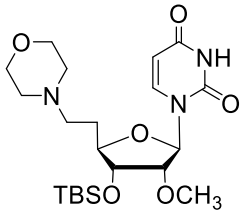
1-[(2R,5R)-4-[tert-butyl(dimethyl)silyl]oxy-3-methoxy-5-vinyl-tetrahydrofuran-2-yl]pyrimidine-2,4-dione (3): Compound **3** was obtained following the literature procedure.[1] To a well-stirred suspension of methyltriphenylphosphonium bromide (7.08 g, 19.43 mmol) in tetrahydrofuran (THF) (30 mL) was added potassium-tert-butoxide (2.23 g, 19.43 mmol). The bright yellow suspension was stirred at 0 °C for 10 min and then for 1 h. The crude aldehyde **2** (2.4 g, 6.48 mmol) was dissolved in THF (20 mL), transferred into a dropping funnel, and slowly added to the solution of ylide at 0 °C. The mixture was vigorously stirred at 0 °C for 10 min and then at 22 °C for 16 h. The mixture was diluted with DCM (30 mL), and the organic layer was washed with saturated NH_4Cl solution (30 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered, and the filtrate was evaporated to dryness. The crude compound was purified by column chromatography (gradient: 0-50% EtOAc in hexanes) to afford **3** (1.93 g, 81% yield) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.26 (s, 1H), 7.38 (d, *J* = 8.1 Hz, 1H), 5.90 (ddd, *J* = 17.1, 10.5, 6.5 Hz, 1H), 5.83 (d, *J* = 2.0 Hz, 1H), 5.77 (dd, *J* = 8.1, 1.9 Hz, 1H), 5.45 (dt, *J* = 17.1, 1.3 Hz, 1H), 5.35 (dt, *J* = 10.5, 1.3 Hz, 1H), 4.42 (tt, *J* = 6.5, 1.3 Hz, 1H), 3.91 (dd, *J* = 7.7, 5.0 Hz, 1H), 3.72 (dd, *J* = 5.0, 2.1 Hz, 1H), 3.56 (s, 3H), 0.90 (s, 9H), 0.09 (d, *J* = 7.2 Hz, 6H) ppm. ^{13}C (101 MHz, $CDCl_3$) δ 163.4, 150.0, 139.8, 134.6, 119.3, 102.6, 89.8, 8.1, 83.6, 74.6, 58.8, 25.8, 18.3, -4.5, -4.5 ppm. HRMS calc. for $C_{17}H_{29}N_2O_5Si$ $[M + H]^+$ 369.1846, found 369.1846.



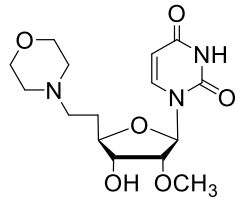
1-[(2R,5R)-4-[tert-butyl(dimethyl)silyl]oxy-5-(2-hydroxyethyl)-3-methoxy-tetrahydrofuran-2-yl]pyrimidine-2,4-dione (4): Conditions used for optimization are shown in Table S1. Hydroboration of **3** was done following the literature procedure.[3] To a solution of **3** (2.0 g, 5.43 mmol) in THF (25 mL) was added 9-borabicyclo[3.3.1]nonane (3.97 g, 32.56 mmol, 4.44 mL) at 0 °C. The mixture was allowed to warm and stirred at 22 °C for 20 h. The reaction mixture was cooled, and methanol (20 mL) was added dropwise. When the gas evolution ceased, water (30 mL) was added followed by sodium perborate tetrahydrate (20.88 g, 130.26 mmol). The resulting mixture was vigorously stirred for 30 h at 0 °C and then filtered. The filtrate was washed with EtOAc (50 mL). The organic layer was further washed with brine (40 mL), dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to dryness. The crude residue thus obtained was purified by column chromatography (gradient: 20-75% EtOAc in hexanes) to afford **4** (1.57 g, 75% yield) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.38 (s, 1H), 7.62 (d, J = 8.0 Hz, 1H), 5.77 (d, J = 4.5 Hz, 1H), 5.66 (d, J = 8.0 Hz, 1H), 4.56 (t, J = 5.0 Hz, 1H), 4.12 (t, J = 5.2 Hz, 1H), 3.88 (dt, J = 7.0, 4.4 Hz, 2H), 3.56 – 3.42 (m, 2H), 3.32 (s, 3H), 1.80 (dtd, J = 14.3, 7.4, 4.6 Hz, 1H), 1.71 (ddt, J = 14.0, 8.0, 5.5 Hz, 1H), 0.88 (s, 9H), 0.09 (d, J = 3.6 Hz, 6H) ppm. ^{13}C NMR (151 MHz, DMSO-d_6) δ 163.1, 150.4, 141.0, 102.2, 87.1, 81.5, 80.7, 73.4, 57.5, 57.4, 35.9, 25.7, 17.8, -4.7, -4.9 ppm. HRMS calc. for $C_{17}H_{30}N_2O_6SiNa$ $[M + Na]^+$ 409.1771, found 409.1767.



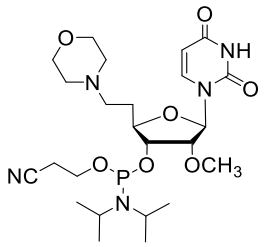
2-[(2R,5R)-3-[tert-butyl(dimethyl)silyl]oxy-5-(2,4-dioxopyrimidin-1-yl)-4-methoxy-tetrahydrofuran-2-yl]ethyl-4-methylbenzenesulfonate (5): To a clear solution of **4** (1.00 g, 2.59 mmol) in dry DCM (30 mL) and pyridine (620.15 mg, 7.76 mmol, 0.63 mL) was added 4-(dimethylamino)pyridine (638.55 mg, 5.17 mmol), and reaction mixture was cooled to 0 °C. To the resulting solution, p-toluenesulfonyl chloride (747.36 mg, 3.88 mmol) was added in single portion, and the reaction mixture was stirred for 12 h at 22 °C. The reaction mixture was diluted with DCM (20 mL) and washed with $NaHCO_3$ solution (30 mL). The DCM layer was dried over anhydrous Na_2SO_4 and filtered, and the filtrate was evaporated to dryness. The crude mass thus obtained was purified by column chromatography (gradient: 0-60% EtOAc in hexanes) to afford **5** (0.92 g, 66% yield) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.61 (s, 1H), 7.81 – 7.76 (m, 2H), 7.37 – 7.32 (m, 2H), 7.23 (d, J = 8.1 Hz, 1H), 5.76 (dd, J = 8.1, 1.8 Hz, 1H), 5.64 (d, J = 2.6 Hz, 1H), 4.23 (ddd, J = 10.1, 7.1, 5.3 Hz, 1H), 4.13 (ddd, J = 10.1, 7.7, 6.4 Hz, 1H), 3.94 (ddd, J = 9.4, 7.3, 3.3 Hz, 1H), 3.86 (dd, J = 7.3, 5.3 Hz, 1H), 3.73 (dd, J = 5.3, 2.6 Hz, 1H), 3.48 (s, 3H), 2.45 (s, 3H), 2.14 (dtd, J = 14.6, 7.4, 3.4 Hz, 1H), 1.90 (dddd, J = 14.5, 9.4, 6.5, 5.4 Hz, 1H), 0.89 (s, 9H), 0.09 (s, 3H), 0.07 (s, 3H) ppm. ^{13}C NMR (151 MHz, $CDCl_3$) δ 162.9, 149.7, 145.1, 140.3, 132.9, 130.0, 128.1, 102.8, 90.4, 83.0, 79.5, 74.4, 67.0, 58.6, 32.5, 25.8, 21.8, 18.2, -4.4, -4.7 ppm. HRMS calc. for $C_{24}H_{37}N_2O_8SSi$ $[M + H]^+$ 541.2040, found 541.2045.



1-[(2R,5R)-4-[tert-butyl(dimethyl)silyl]oxy-3-methoxy-5-(2-morpholinoethyl)tetrahydrofuran-2-yl]pyrimidine-2,4-dione (6): Morpholine (4 mL) was added to **5** (0.5 g, 0.924 mmol), and the clear solution was heated at 70 °C for 8 h. All the volatile matters were evaporated, and the residue was purified by column chromatography (gradient: 0-5% MeOH in DCM) to afford **6** (0.35 g, 83% yield) as a hygroscopic solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.05 (s, 1H), 7.33 (d, *J* = 8.1 Hz, 1H), 5.78 (d, *J* = 2.4 Hz, 1H), 5.76 (d, *J* = 8.1 Hz, 1H), 4.03 (ddd, *J* = 9.0, 7.4, 3.8 Hz, 1H), 3.84 (dd, *J* = 7.4, 5.2 Hz, 1H), 3.75 – 3.63 (m, 5H), 3.52 (s, 3H), 2.67 – 2.34 (m, 6H), 2.05 – 1.88 (m, 1H), 1.79 – 1.64 (m, 1H), 0.91 (s, 9H), 0.10 (d, *J* = 5.0 Hz, 6H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.2, 149.9, 139.9, 102.6, 89.6, 83.6, 81.5, 74.7, 67.0, 58.5, 55.4, 53.8, 30.4, 25.8, 18.3, -4.3, -4.6 ppm. HRMS calc. for $C_{21}H_{38}N_3O_6Si$ $[M + H]^+$ 456.2530, found 456.2529.

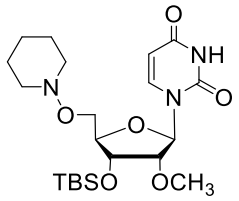


1-[(2R,5R)-4-hydroxy-3-methoxy-5-(2-morpholinoethyl)tetrahydrofuran-2-yl]pyrimidine-2,4-dione (7): To a clear solution of **6** (0.6 g, 1.32 mmol) in THF (15 mL) at 22 °C, tetrabutylammonium fluoride, 1 M in THF (1.71 mmol, 1.71 mL), was added slowly in a single portion and then stirred for 3 h. All the volatile matters were removed under high vacuum, and the residue thus obtained was purified by column chromatography (gradient: 0-10% MeOH in DCM) to afford **7** (0.37 g, 82% yield) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.50 – 11.08 (m, 1H), 7.59 (d, *J* = 8.1 Hz, 1H), 5.77 (d, *J* = 4.3 Hz, 1H), 5.65 (dd, *J* = 8.1, 1.9 Hz, 1H), 5.37 – 5.18 (m, 1H), 3.90 (s, 1H), 3.84 (dd, *J* = 5.3, 4.3 Hz, 1H), 3.79 (dt, *J* = 8.0, 5.4 Hz, 1H), 3.57 (t, *J* = 4.7 Hz, 4H), 3.36 (s, 3H), 2.40 – 2.26 (m, 6H), 1.87 (dtd, *J* = 13.1, 7.8, 5.2 Hz, 1H), 1.71 (dtd, *J* = 13.5, 7.7, 5.5 Hz, 1H) ppm. ^{13}C NMR (126 MHz, DMSO-d_6) δ 163.0, 150.4, 140.8, 102.1, 87.0, 82.0, 81.7, 72.1, 66.1, 57.6, 54.6, 53.2, 29.7 ppm. HRMS calc. for $C_{15}H_{24}N_3O_6$ $[M + H]^+$ 342.1665, found 342.1660.

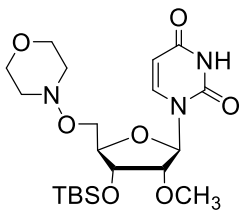


3-[(diisopropylamino)-[(2R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-methoxy-2-(2-morpholinoethyl)tetrahydrofuran-3-yl]oxy-phosphanyl]propanenitrile (8): To a clear solution of **7** (0.34 g, 1.0 mmol) in DCM (20 mL) was added DIPEA (650.13 mg, 4.98 mmol, 0.88 mL) and

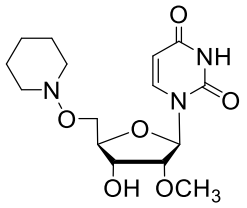
N-methylimidazole (123.90 mg, 1.49 mmol, 0.12 mL) in single portions. To the resulting mixture was added 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (248.15 mg, 996.02 μmol, 0.23 m μL) at 22 °C. After stirring for 1 h, when TLC showed completion of reaction, the mixture was diluted with DCM (20 mL) and quenched by adding $NaHCO_3$ solution (20 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to dryness. The crude material was triturated with 1:1 hexanes in ether. The precipitate thus obtained was purified by column chromatography (gradient: 0-3% MeOH in DCM containing 3% TEA) to afford **8** (0.36 g, 69% yield) as a yellowish-white hygroscopic foam. ^{1}H NMR (400 MHz, CD_3CN) δ 8.84 (s, 1H), 7.40 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.14 – 6.73 (m, 1H), 5.83 (dd, *J* = 4.5, 1.1 Hz, 1H), 5.63 (d, *J* = 8.1 Hz, 1H), 4.29 – 3.97 (m, 2H), 3.91 – 3.75 (m, 2H), 3.69 – 3.55 (m, 6H), 3.43 (d, *J* = 14.2 Hz, 3H), 2.83 – 2.60 (m, 2H), 2.49 – 2.26 (m, 4H), 1.94 (dt, *J* = 4.9, 2.5 Hz, 1H), 1.76 (ddd, *J* = 9.9, 6.3, 2.2 Hz, 1H), 1.29 – 0.97 (m, 13H) ppm. ^{13}C NMR (126 MHz, CD_3CN) δ 164.1, 151.5, 151.5, 141.1, 138.9, 129.5, 121.3, 119.6, 103.2, 103.1, 88.9, 88.5, 83.2, 83.2, 82.8, 82.7, 82.4, 82.3, 82.0, 81.9, 75.3, 75.2, 75.1, 74.9, 74.5, 67.5, 67.5, 59.8, 59.6, 59.2, 59.0, 58.9, 58.8, 58.7, 58.7, 58.0, 55.6, 54.6, 54.6, 47.3, 46.6, 46.4, 46.3, 46.0, 46.0, 45.6, 44.2, 44.1, 44.1, 44.0, 33.6, 30.9, 25.0, 24.9, 24.9, 24.9, 23.6, 23.2, 23.2, 23.1, 23.1, 22.6, 21.1, 21.0, 21.0, 20.4, 20.4 ppm. ^{31}P NMR (202 MHz, CD_3CN) δ 150.84, 150.78 ppm. HRMS calc. for $C_{24}H_{41}N_5O_7P$ $[M + H]^+$ 542.2744, found 542.2744.



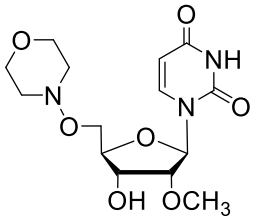
1-[(2R,5R)-4-[tert-butyl(dimethyl)silyl]oxy-3-methoxy-5-(1-piperidyloxymethyl)tetrahydro furan-2-yl]pyrimidine-2,4-dione (10): To a clear solution of **9**[4] (0.4 g, 1.03 mmol) in acetic acid (5 mL) and DCM (10 mL) was added glutaraldehyde (0.1 g, 1.03 mmol). To the resulting mixture, sodium cyanoborohydride (0.74 g, 11.56 mmol) was added in portions at 15 °C. The reaction mixture was further diluted with DCM (70 mL) and stirred for 8 h. Volatile matters were removed under high vacuum, and the residue thus obtained was diluted with DCM (50 mL) and washed with water (3 x 30 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to dryness. The crude compound thus obtained was purified by column chromatography to afford **10** (0.32 g, 68.0% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (s, 1H), 8.07 (d, *J* = 8.2 Hz, 1H), 5.88 (d, *J* = 1.9 Hz, 1H), 5.69 (dd, *J* = 8.1, 2.1 Hz, 1H), 4.36 – 3.96 (m, 3H), 3.97 – 3.72 (m, 1H), 3.61 (dd, *J* = 4.7, 1.9 Hz, 1H), 3.54 (s, 3H), 3.41 – 3.24 (m, 2H), 2.36 (s, 2H), 1.74 (s, 2H), 1.56 (d, *J* = 18.3 Hz, 3H), 1.24 – 1.11 (m, 1H), 0.90 (s, 9H), 0.09 (d, *J* = 3.4 Hz, 6H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.5, 150.2, 140.5, 101.7, 88.1, 84.2, 82.5, 69.4, 68.7, 58.5, 56.9, 25.8, 25.5, 23.5, 18.3, -4.5, -4.7 ppm. HRMS calc. for $C_{21}H_{38}N_3O_6Si$ $[M + H]^+$ 456.2530, found 456.2520.



1-[(2R,5R)-4-[tert-butyl(dimethyl)silyl]oxy-3-methoxy-5-(morpholinooxymethyl)tetrahydrofuran-2-yl]pyrimidine-2,4-dione (11): To a clear solution of **9** (2.4 g, 6.19 mmol) in acetic acid (20 mL) was added 2-(2-oxoethoxy)acetaldehyde[5] (0.63 g, 6.19 mmol) followed by sodium cyanoborohydride (4.13 g, 64.4 mmol) in portions. After stirring at 15 °C for 12 h, the mixture was diluted with DCM (50 mL), and the organic layer was washed with water (2 x 30 mL). The DCM layer was dried over anhydrous Na_2SO_4 and filtered. The filtrate was evaporated to dryness. The crude residue was purified by column chromatography to afford **11** (0.88 g, 31% yield) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.50 (s, 1H), 7.93 (d, *J* = 8.2 Hz, 1H), 5.86 (d, *J* = 2.0 Hz, 1H), 5.70 (dd, *J* = 8.2, 2.0 Hz, 1H), 4.23 – 4.06 (m, 3H), 3.92 (dd, *J* = 11.9, 3.0 Hz, 3H), 3.67 – 3.57 (m, 3H), 3.55 (s, 3H), 3.23 (dd, *J* = 28.2, 10.2 Hz, 2H), 2.65 (q, *J* = 10.1 Hz, 2H), 0.91 (s, 9H), 0.10 (d, *J* = 5.8 Hz, 6H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.4, 150.1, 140.2, 101.8, 88.4, 84.1, 82.1, 69.4, 68.9, 66.4, 58.6, 56.4, 25.8, 18.3, -4.4, -4.7 ppm. HRMS calc. for $C_{20}H_{36}N_3O_7Si$ $[M + H]^+$ 458.2323, found 458.2315.

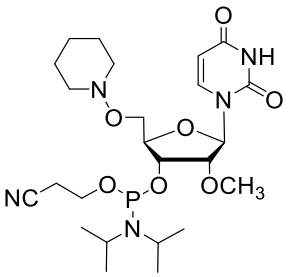


1-[(2R,5R)-4-hydroxy-3-methoxy-5-(1-piperidyloxymethyl)tetrahydrofuran-2-yl]pyrimidine-2,4-dione (12): To a solution of **10** (0.30 g, 0.66 mmol) in THF (5 mL) at 25 °C, tetrabutylammonium fluoride, 1 M in THF (0.99 mmol, 0.99 mL), was added slowly in a single portion and then stirred for 5 h. Volatile matters were removed under high vacuum, and the crude residue thus obtained was purified by column chromatography (gradient: 10-60% EtOAc in hexanes) to afford **12** (0.17 g, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (s, 1H), 7.97 (d, *J* = 8.2 Hz, 1H), 5.94 (d, *J* = 2.2 Hz, 1H), 5.71 (dd, *J* = 8.2, 1.8 Hz, 1H), 4.20 (td, *J* = 7.4, 5.2 Hz, 1H), 4.16 – 4.11 (m, 1H), 4.08 (dt, *J* = 7.1, 2.6 Hz, 1H), 3.95 (dd, *J* = 11.2, 2.7 Hz, 1H), 3.77 (dd, *J* = 5.2, 2.3 Hz, 1H), 3.61 (s, 3H), 3.36 (s, 2H), 2.84 (d, *J* = 7.7 Hz, 1H), 2.39 (t, *J* = 11.4 Hz, 2H), 1.76 (d, *J* = 13.0 Hz, 2H), 1.59 (brs, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.2, 150.2, 140.2, 102.0, 87.6, 84.0, 83.1, 69.5, 69.1, 58.8, 57.0, 56.7, 25.5, 23.5 ppm. HRMS calc. for $C_{15}H_{24}N_3O_6$ $[M + H]^+$ 342.1665, found 342.1656.

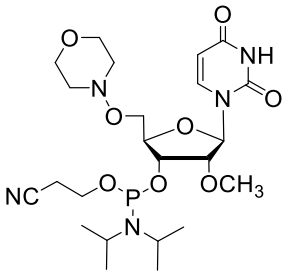


1-[(2R,5R)-4-hydroxy-3-methoxy-5-(morpholinooxymethyl)tetrahydrofuran-2-yl]pyrimidine-2,4-dione (13): To a solution of **11** (0.85 g, 1.86 mmol) in THF (15 mL) at 22 °C, tetrabutylammonium fluoride, 1 M in THF (2.41 mmol, 2.41 mL), was added slowly in single portion and then stirred for 3 h. Volatile matters were removed under high vacuum, and the crude residue thus obtained was purified by column chromatography (gradient: 0-5% MeOH in DCM) to afford **13** (0.52 g, 81% yield) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.26 (s, 1H), 7.85 (d, *J* = 8.2 Hz, 1H), 5.93 (d, *J* = 2.1 Hz, 1H), 5.73 (d, *J* = 8.2 Hz, 1H), 4.23 – 4.14 (m, 2H), 4.08 (dt, *J* = 7.3, 2.9 Hz, 1H), 3.98 (dd, *J* = 11.3, 3.2 Hz, 1H), 3.92 (d, *J* = 11.7 Hz, 2H), 3.81 – 3.71

(m, 1H), 3.62 (s, 5H), 3.25 (t, *J* = 9.1 Hz, 2H), 2.81 (d, *J* = 8.3 Hz, 1H), 2.67 (td, *J* = 10.9, 3.2 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.4, 150.2, 140.0, 102.1, 87.7, 83.7, 82.7, 69.6, 68.9, 66.3, 58.9, 56.5, 56.23ppm. HRMS calc. for $C_{14}H_{22}N_3O_7$ $[M + H]^+$ 344.1458, found 344.1465.



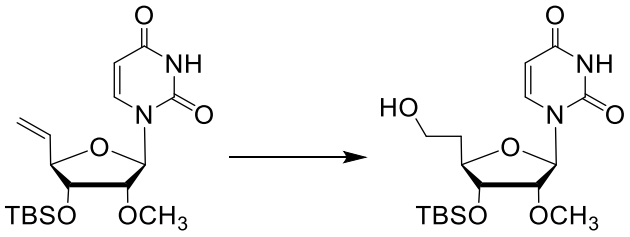
3-[(diisopropylamino)-[(2R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-methoxy-2-(1-piperidyloxy methyl)tetrahydrofuran-3-yl]oxy-phosphanyl]oxypropanenitrile (14): To a clear solution of **12** (0.60 g, 1.76 mmol) in DCM (20 mL), diisopropylethylamine (1.15 g, 8.79 mmol, 1.55 mL) and N-methylimidazole (0.51 g, 6.15 mmol, 0.49 mL) were added at 22 °C. After 5 min, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.88 g, 3.52 mmol, 0.82 mL) was added slowly. After stirring for 0.5 h, the reaction mixture was diluted with DCM (10 mL) and quenched with 10% $NaHCO_3$ solution (20 mL). The organic layer was separated, dried on anhydrous Na_2SO_4, and filtered. The filtrate was evaporated to dryness. The crude compound thus obtained was purified by column chromatography (gradient: 20-80% EtOAc in hexanes) to afford **14** (0.66 g, 70% yield) as a hygroscopic solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 7.96 (dd, *J* = 10.9, 8.1 Hz, 1H), 5.97 (d, *J* = 3.7 Hz, 1H), 5.69 (d, *J* = 8.1 Hz, 1H), 4.49 – 4.16 (m, 2H), 4.09 (td, *J* = 11.2, 2.3 Hz, 1H), 3.98 – 3.77 (m, 4H), 3.72 – 3.58 (m, 2H), 3.51 (d, *J* = 14.2 Hz, 3H), 3.39 – 3.23 (m, 2H), 2.64 (dt, *J* = 11.9, 6.3 Hz, 2H), 2.39 (d, *J* = 10.5 Hz, 2H), 1.73 (s, 2H), 1.58 (s, 3H), 1.30 – 1.06 (m, 15H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.4, 163.4, 150.4, 150.3, 140.4, 140.2, 117.8, 117.6, 102.1, 102.0, 87.8, 87.5, 83.5, 83.4, 83.1, 83.1, 82.4, 82.4, 82.2, 82.2, 70.9, 70.7, 70.1, 70.0, 69.6, 69.5, 58.9, 58.7, 58.7, 58.3, 58.3, 58.2, 58.1, 58.0, 56.9, 53.6, 43.6, 43.5, 43.4, 43.4, 25.4, 24.8, 24.7, 24.7, 24.7, 24.7, 23.5, 23.4, 20.6, 20.5, 20.5, 20.5 ppm. ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.98, 150.54 ppm. HRMS calc. for $C_{24}H_{41}N_5O_7P$ $[M + H]^+$ 542.2744, found 542.2747.



3-[(diisopropylamino)-[(2R,5R)-5-(2,4-dioxopyrimidin-1-yl)-4-methoxy-2-(morpholinooxy methyl)tetrahydrofuran-3-yl]oxy-phosphanyl]oxypropanenitrile (15): To a solution of **13** (0.3 g, 0.87 mmol) in dry acetonitrile (10 mL) was added 5-(ethylthio)-*1H*-tetrazole (0.12 g, 0.87 mmol). 2-Cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphordiamidite (1.14 mmol, 0.37 mL) was added slowly to the reaction mixture and stirred at 22 °C for 3 h. The reaction mixture was filtered, volatile matters were removed under high vacuum, and the residue was purified by flash column chromatography using a gradient of EtOAc in hexanes containing 0.2% triethylamine to yield **15** as a white solid. To remove P (V) impurities from the column-purified compound, **15** was

dissolved in methyl *tert*-butylether (25 mL) and washed with 50% DMF in water (2 x 10 mL) and the with brine (3 x 20 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, and filtered. The filtrate was evaporated under high vacuum to obtain **15** (0.31 g, 66% yield) as a white foam. ^{1}H NMR (400 MHz, CD_3CN) δ 8.94 (s, 1H), 7.76 (dd, *J* = 8.9, 8.2 Hz, 1H), 5.88 (dd, *J* = 7.3, 4.6 Hz, 1H), 5.65 (dd, *J* = 8.2, 3.0 Hz, 1H), 4.50 – 4.14 (m, 2H), 4.08 – 3.98 (m, 1H), 3.92 – 3.74 (m, 5H), 3.65 (dtd, *J* = 10.3, 6.8, 4.7 Hz, 2H), 3.56 – 3.36 (m, 6H), 3.21 (d, *J* = 10.2 Hz, 2H), 2.75 – 2.51 (m, 4H), 1.28 – 1.10 (m, 17H) ppm. ^{13}C NMR (126 MHz, CD_3CN) δ 163.9, 163.9, 151.4, 151.4, 141.0, 141.0, 119.6, 119.6, 102.8, 102.7, 88.4, 87.9, 83.5, 83.5, 83.2, 83.2, 83.2, 83.1, 82.7, 82.7, 72.2, 72.0, 71.6, 71.5, 71.0, 70.9, 66.8, 66.8, 59.8, 59.6, 59.2, 59.1, 58.9, 58.9, 58.6, 58.6, 57.2, 57.0, 49.5, 44.2, 44.2, 44.1, 44.1, 27.2, 25.0, 25.0, 24.9, 24.9, 24.9, 24.8, 21.1, 21.0, 21.0 ppm. ^{31}P NMR (202 MHz, CD_3CN) δ 151.48, 151.16 ppm HRMS calc. for $C_{23}H_{38}N_5O_8PNa$ $[M + Na]^+$ 566.2356, found 566.2379.

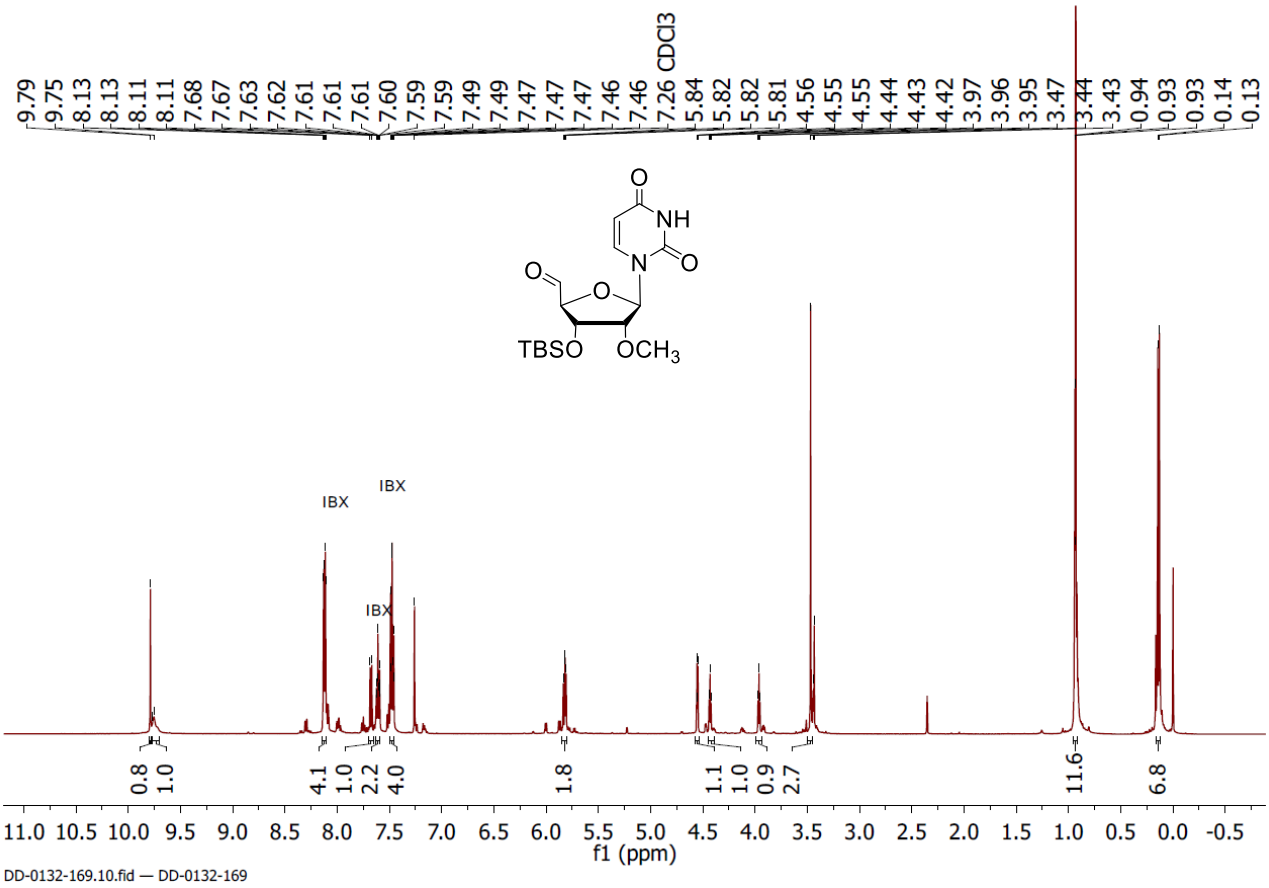
Table S1: Optimization of conditions for compound **4**



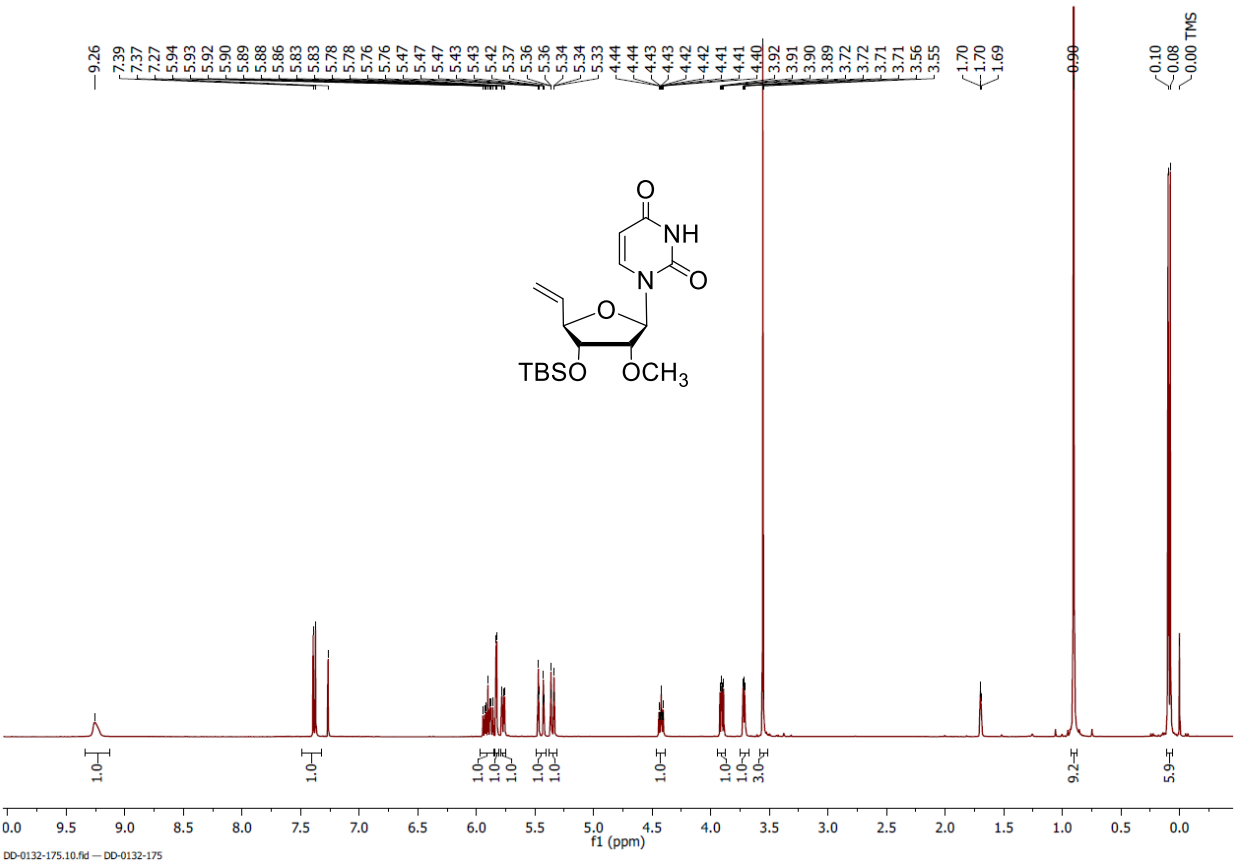
3 **4**

Entry	Conditions[a]	Product(s) (based on LCMS)
1	9-BBN, sodium perborate tetrahydrate, THF, MeOH, H_2O, 0-25 ^oC, 17 hr	**4** (7%), **3** (70%)
2	9-BBN, sodium perborate tetrahydrate, THF, MeOH, H_2O, 0-25 ^oC, 17 hr	**4** (14%), **3** (69%)
3	9-BBN, sodium perborate tetrahydrate, THF, MeOH, H_2O, 30 ^oC, 17 hr	**4** (32%), **3** (21%)
4	BH_3.THF, sodium perborate tetrahydrate, THF, MeOH, H_2O, 0-25 ^oC, 3 hr	85% unidentified byproduct
5	9-BBN, sodium perborate tetrahydrate, THF, MeOH, H_2O, 40 ^oC, 3 hr	**4** (43%)
6	9-BBN,sodium perborate tetrahydrate, THF, MeOH, H_2O, 0 ^oC-rt, 50 hr	**4** (75%)
[a] Entry 5 is the optimized condition used during large scale synthesis of **4**.		

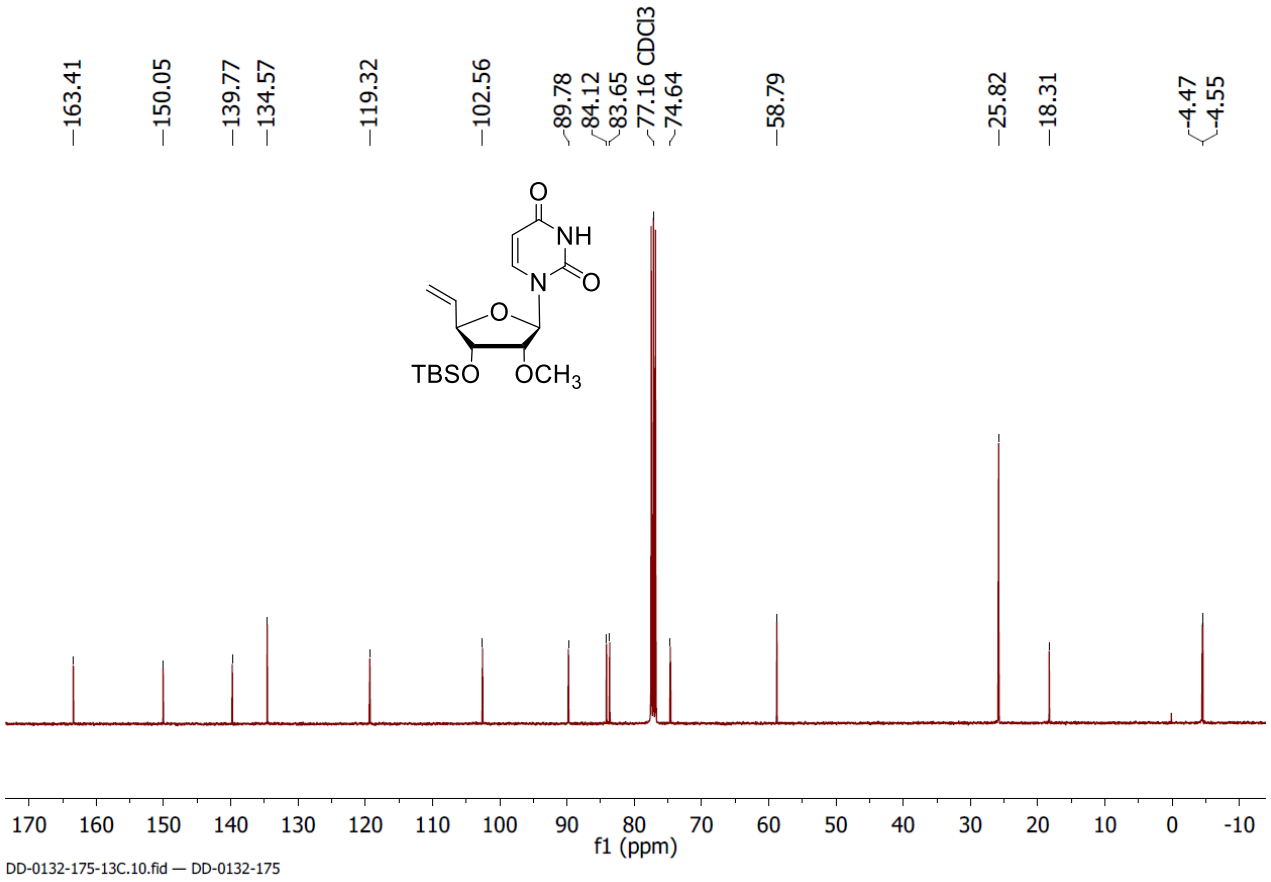
^{1}H, ^{13}C and ^{31}P NMR data



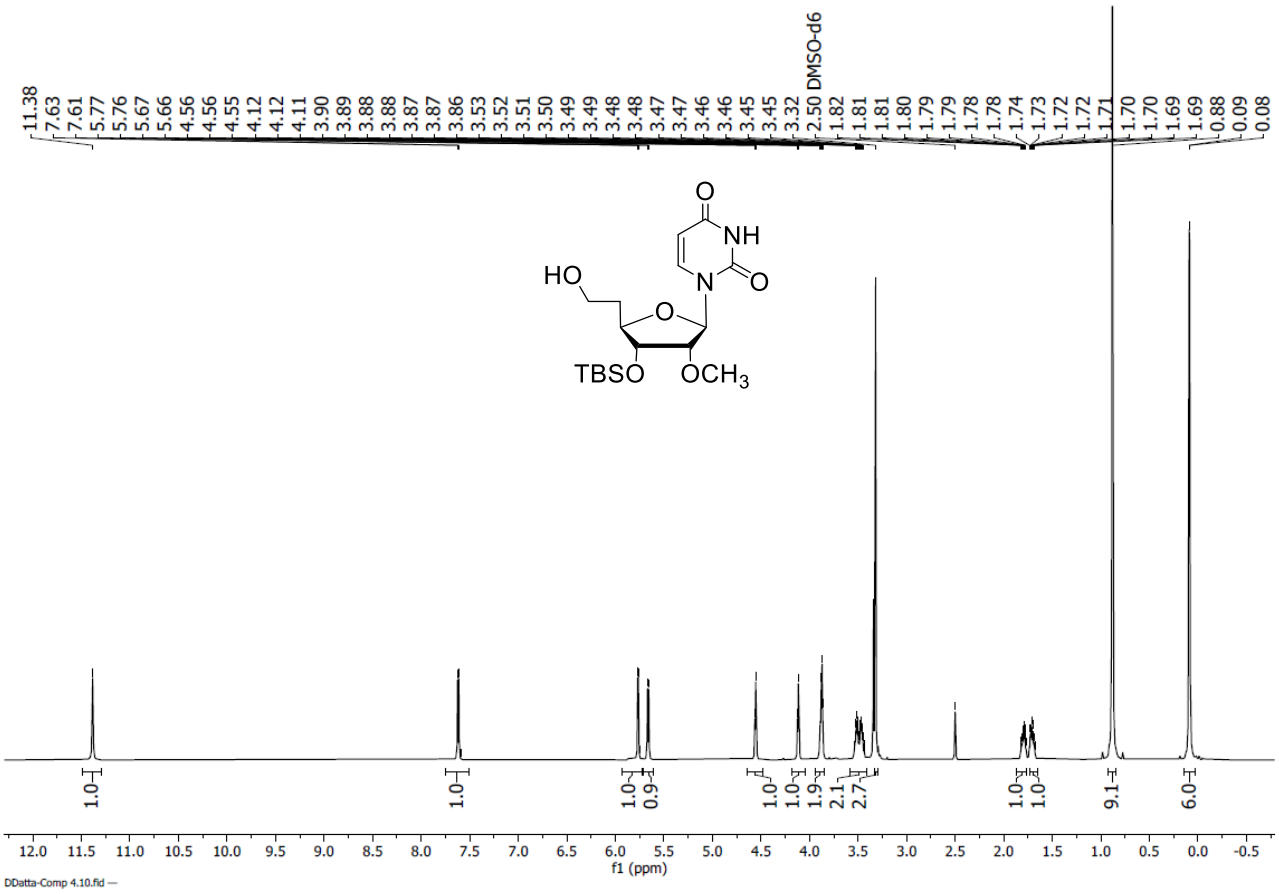
^{1}H NMR (500 MHz, $CDCl_3$) of **2**.



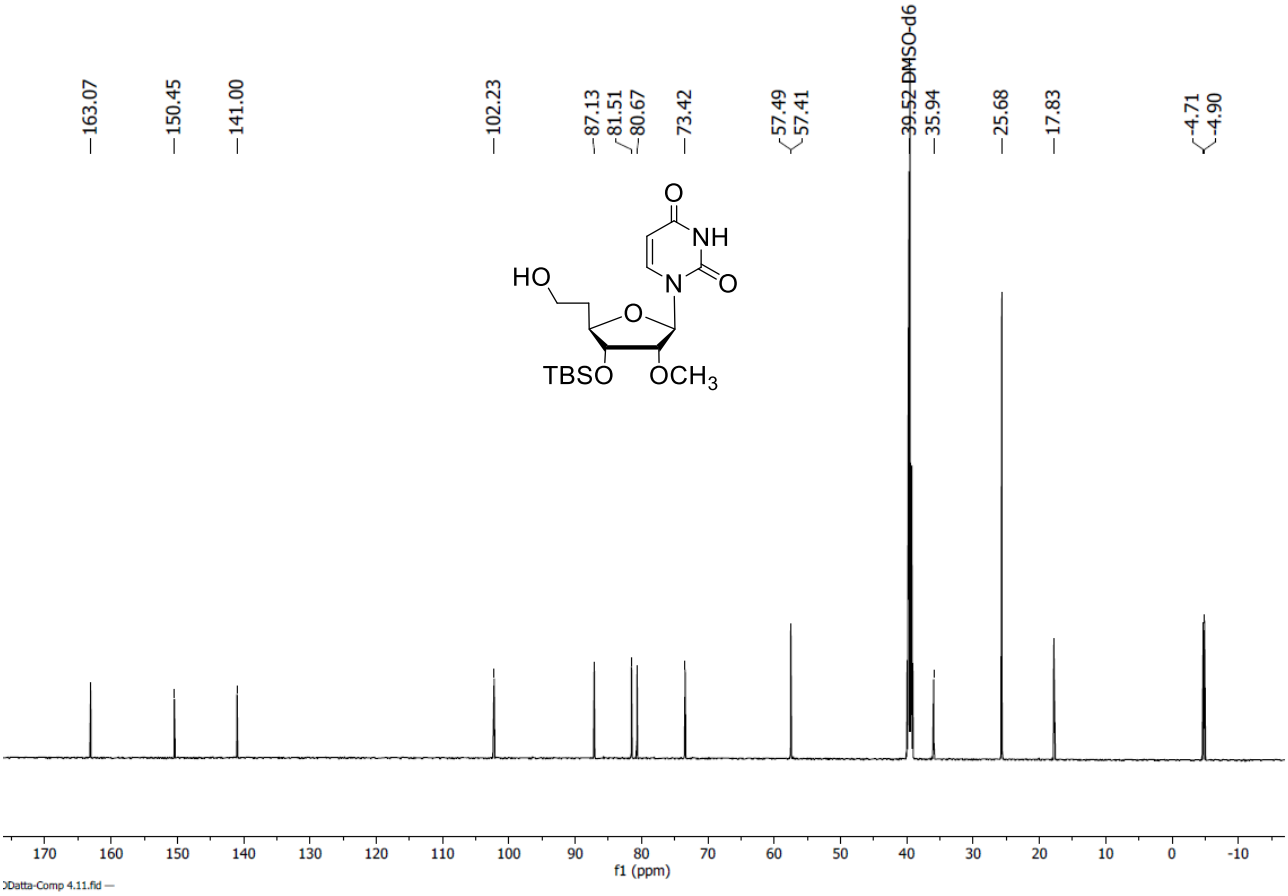
^{1}H NMR (400 MHz, $CDCl_3$) of **3**.



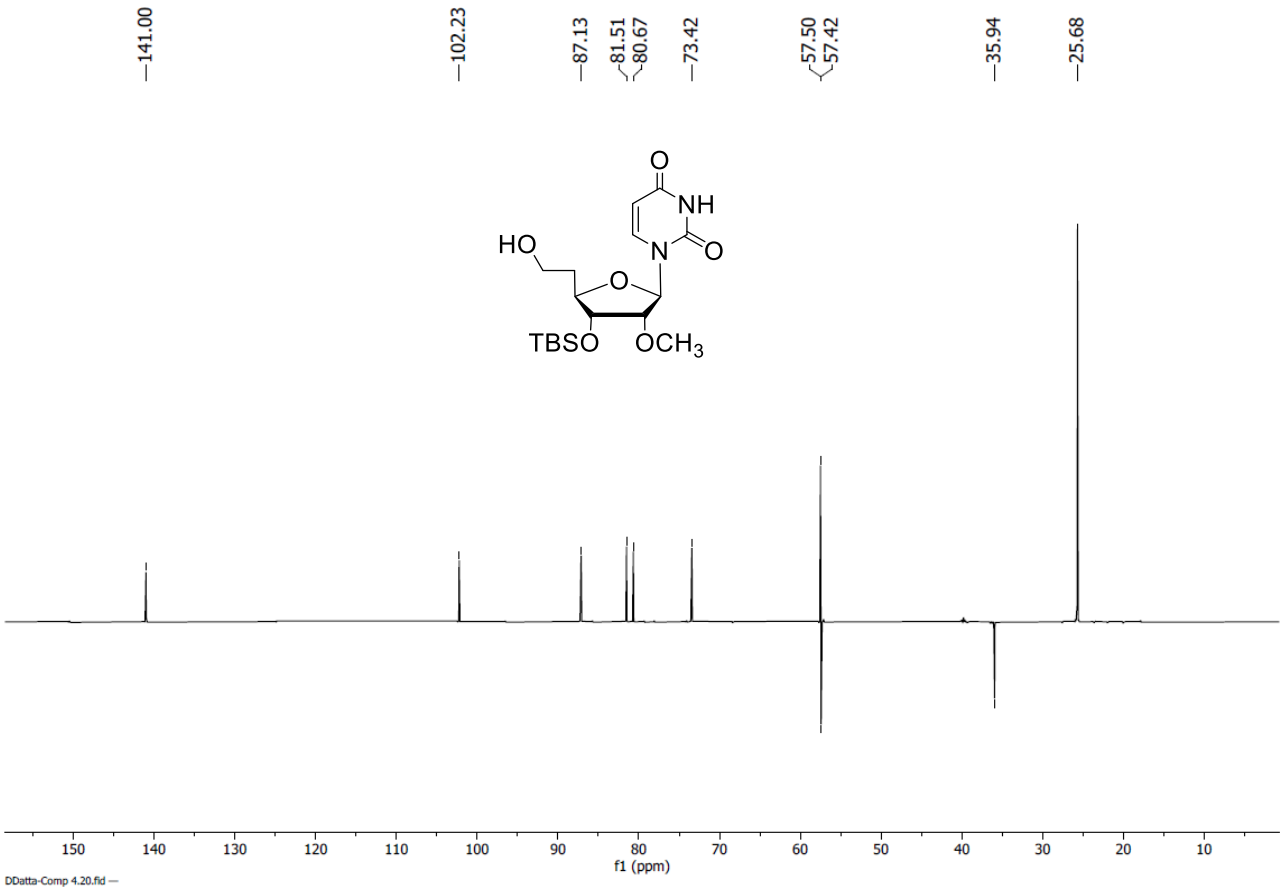
^{13}C (101 MHz, $CDCl_3$) of **3**.



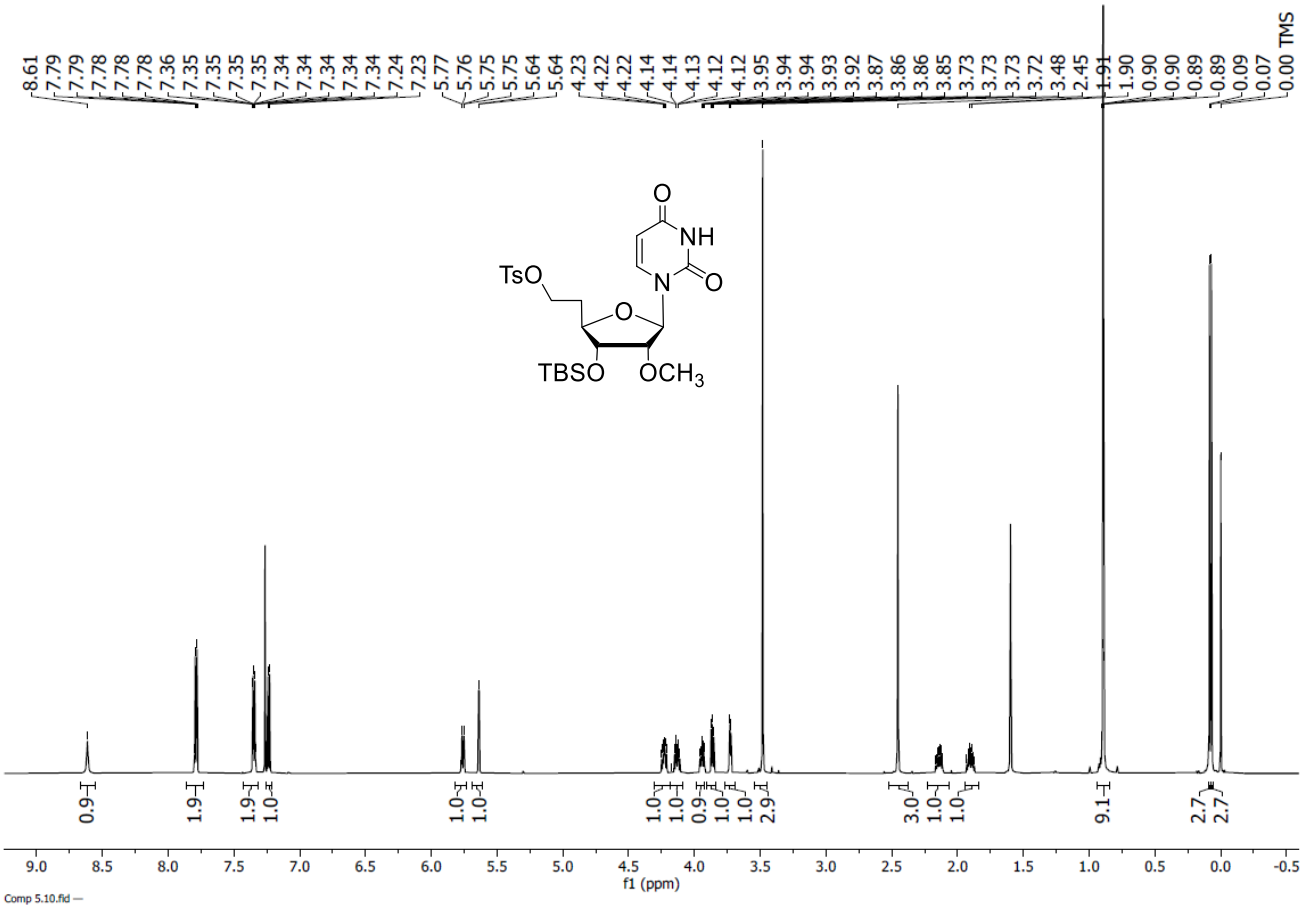
^{1}H NMR (600 MHz, DMSO-d_6) of **4**.



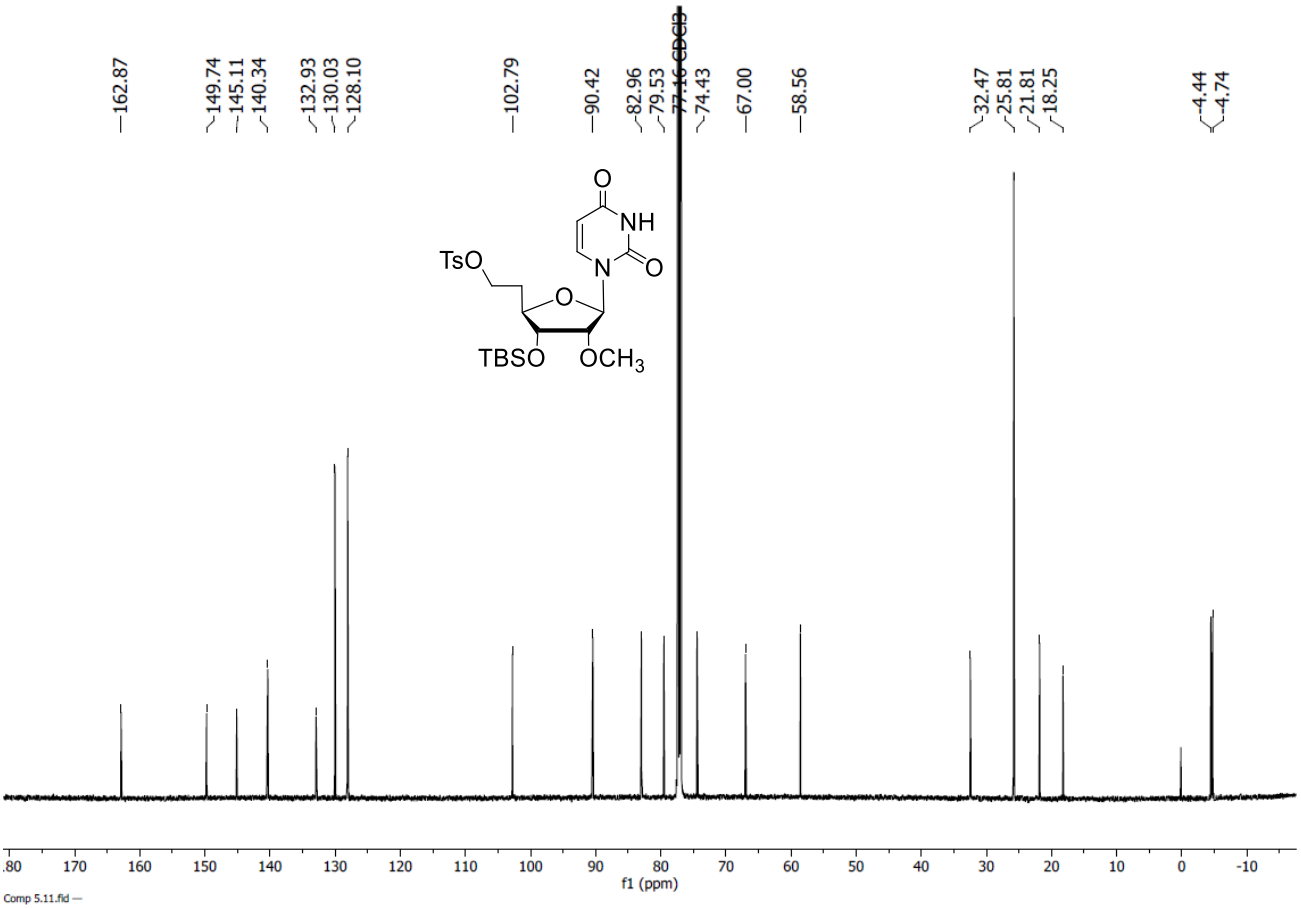
^{13}C NMR (151 MHz, DMSO-d_6) of **4**.



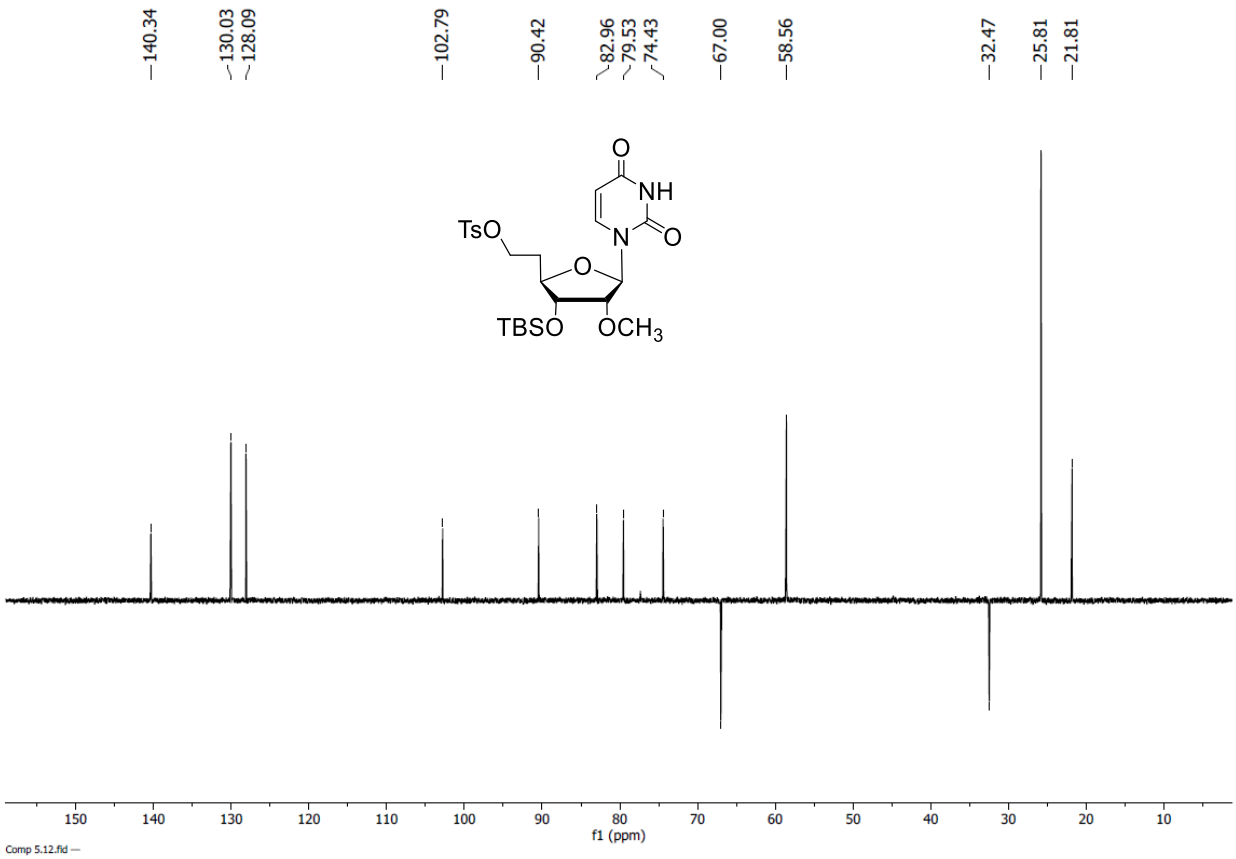
^{13}C NMR (151 MHz, DMSO-d_6) of **4**.



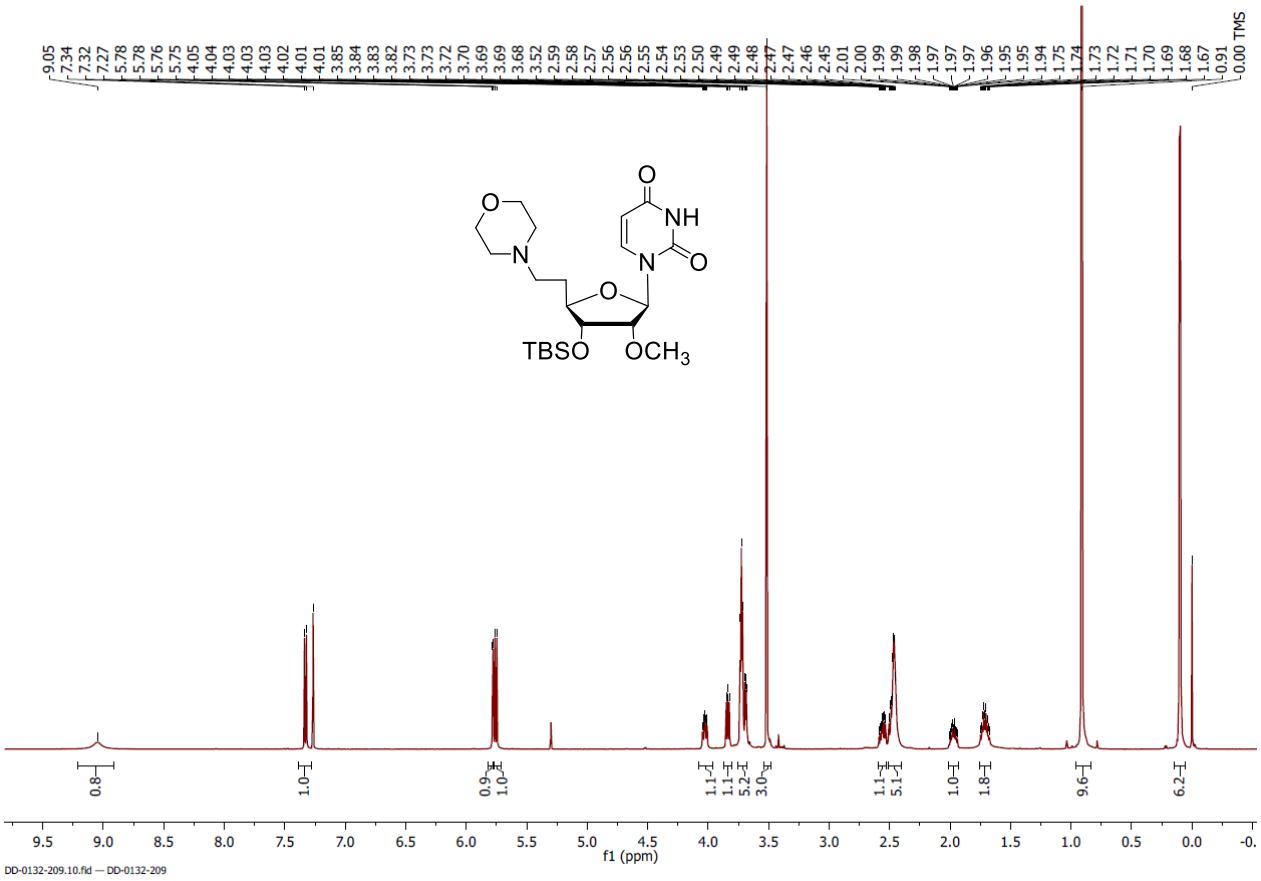
^{1}H NMR (500 MHz, $CDCl_3$) of **5**.



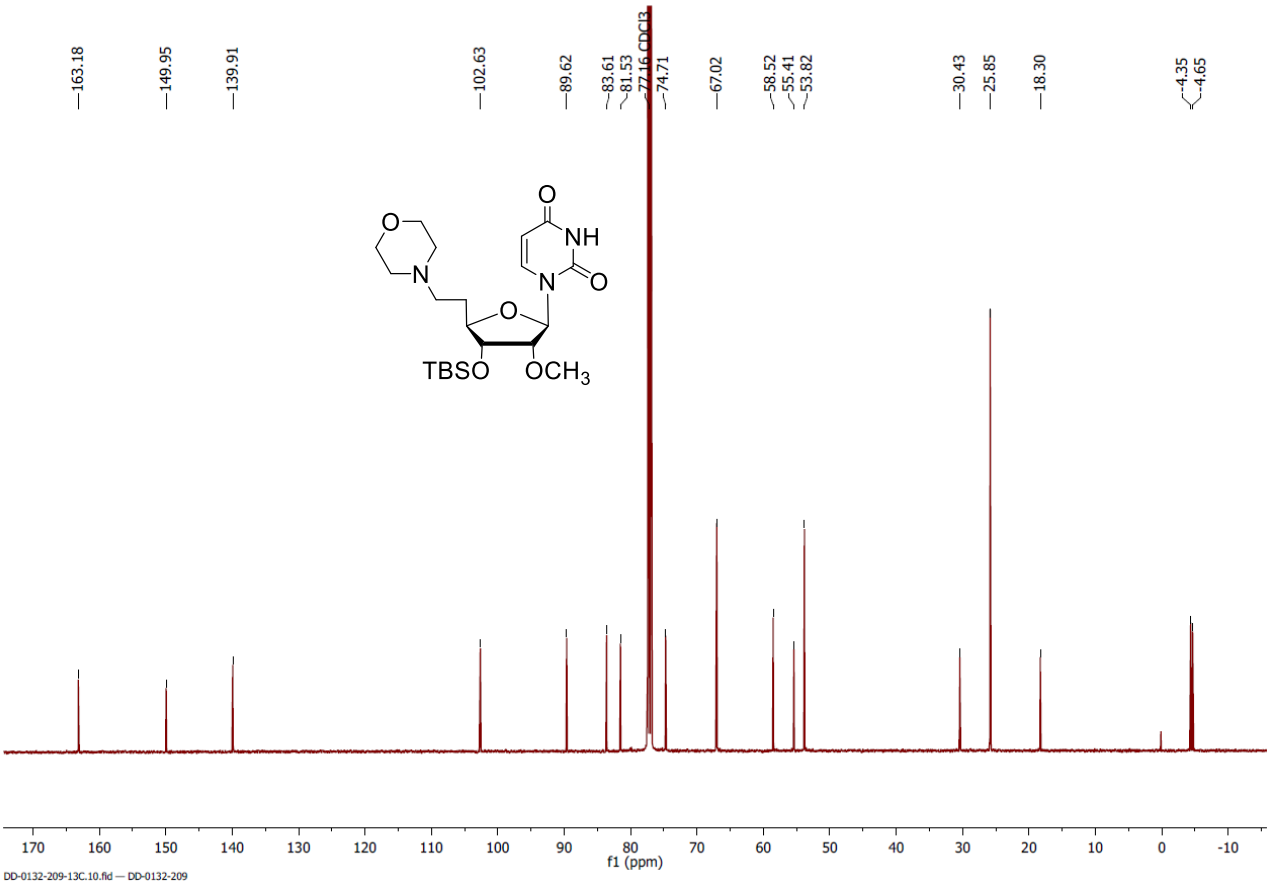
^{13}C NMR (126 MHz, $CDCl_3$) of **5**.



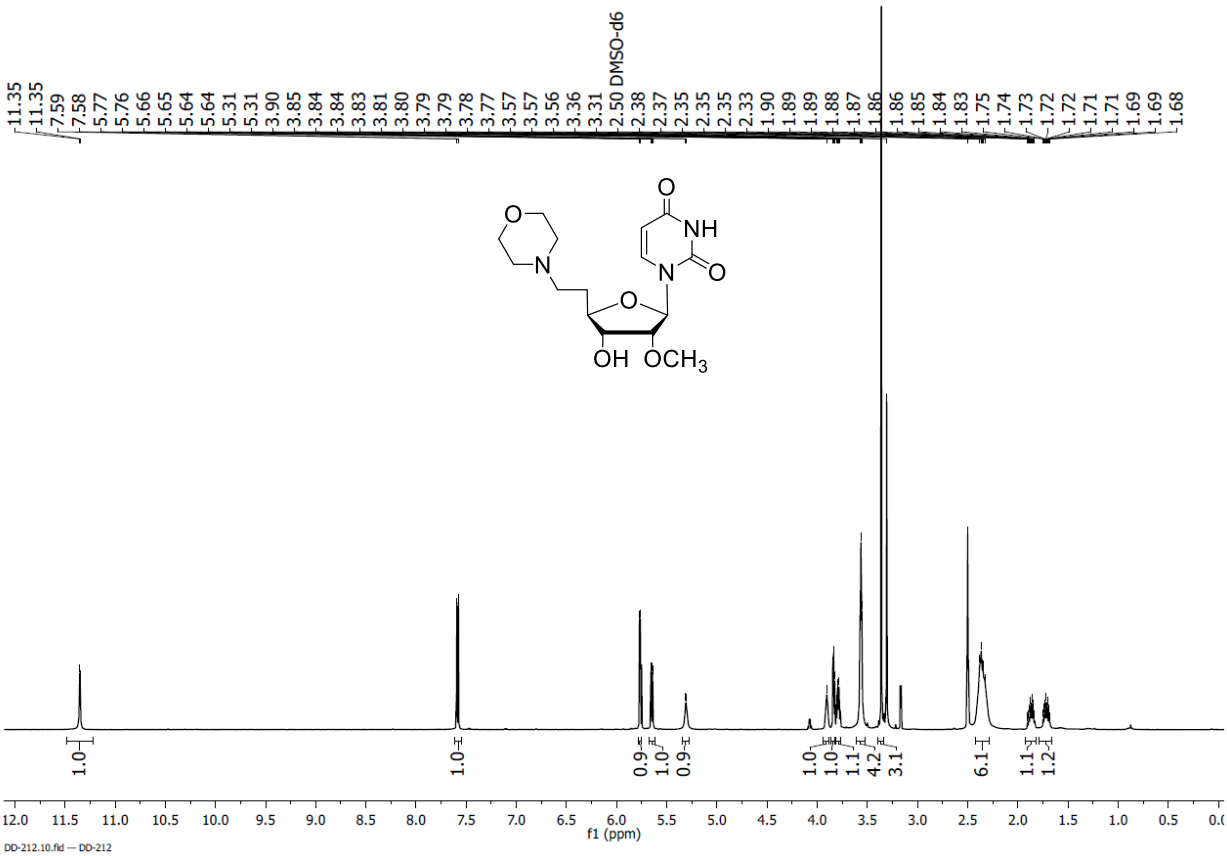
^{13}C NMR (126 MHz, $CDCl_3$) of **5**.



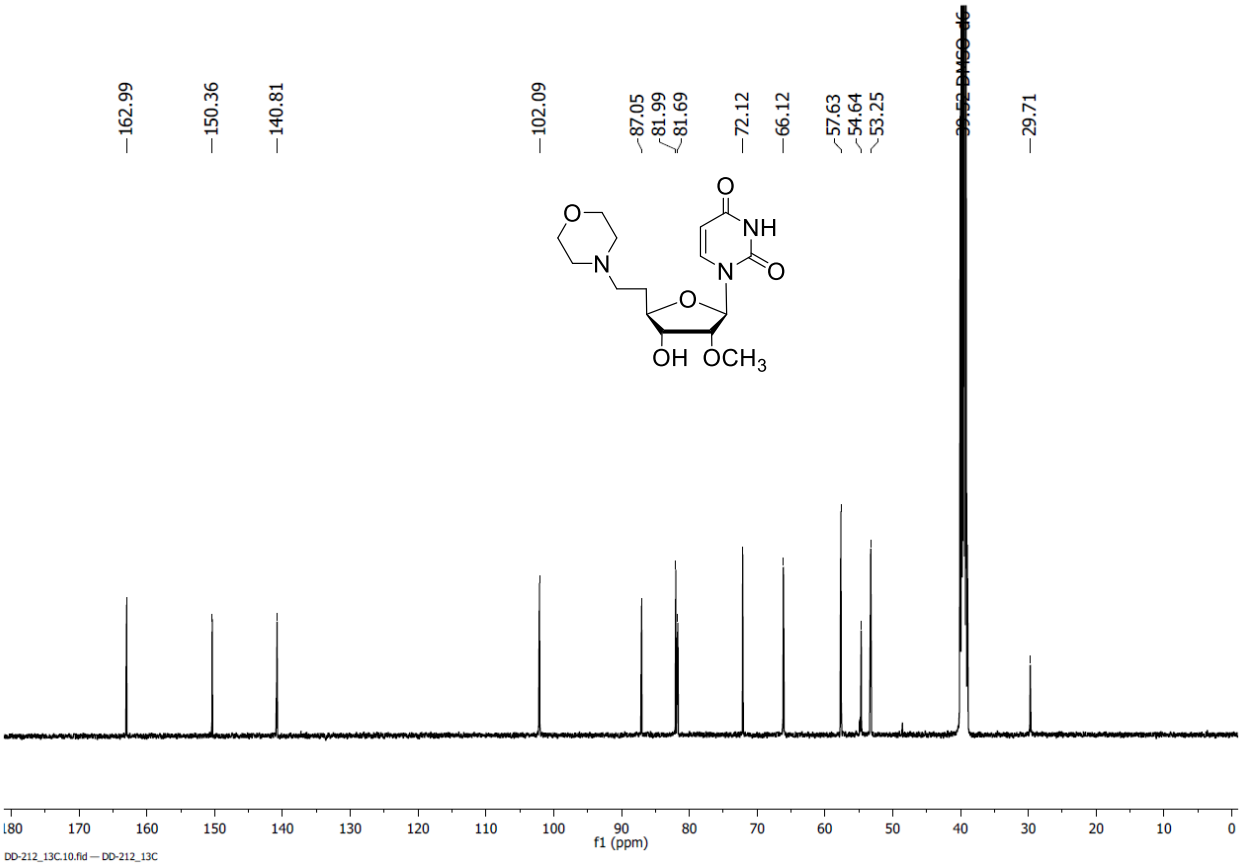
^{1}H NMR (500 MHz, $CDCl_3$) of **6**.



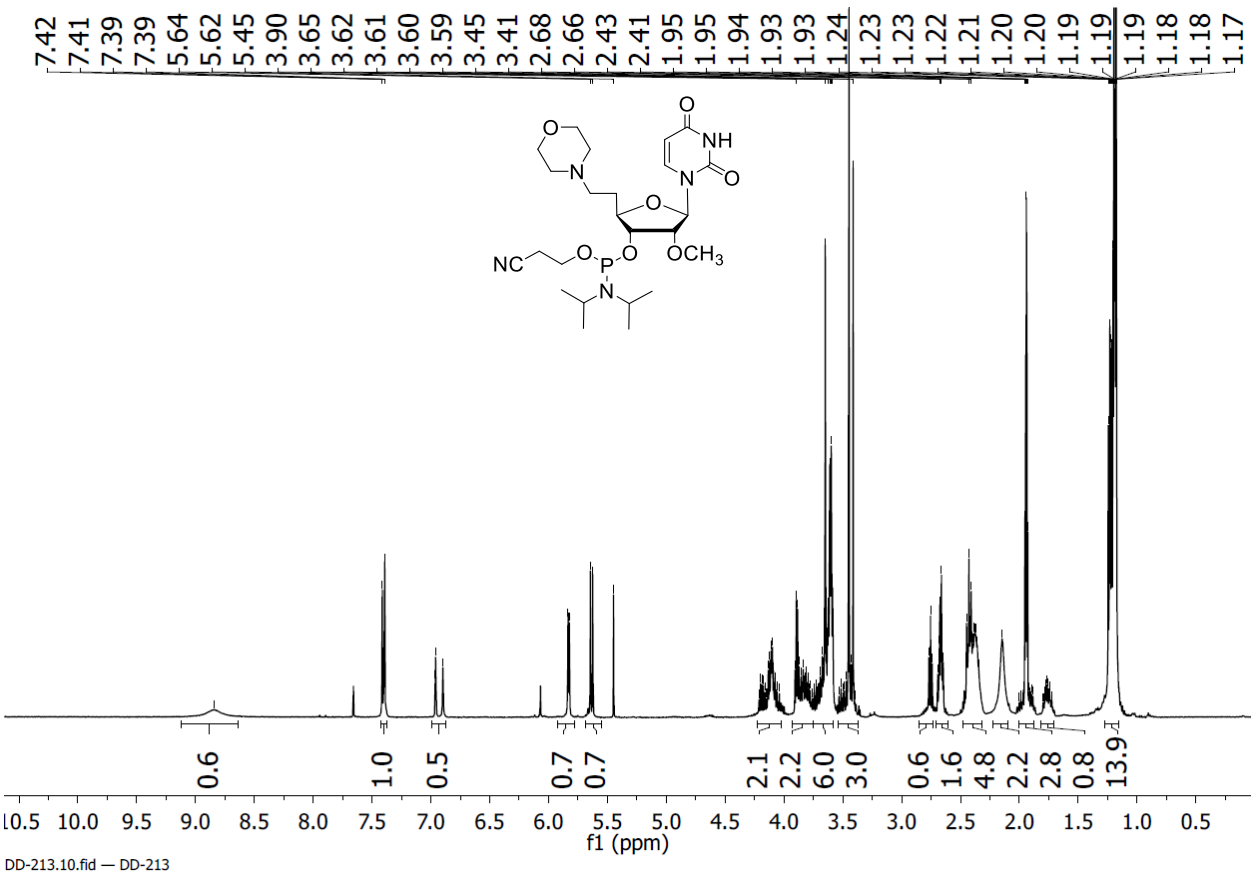
^{13}C NMR (126 MHz, $CDCl_3$) of **6**.



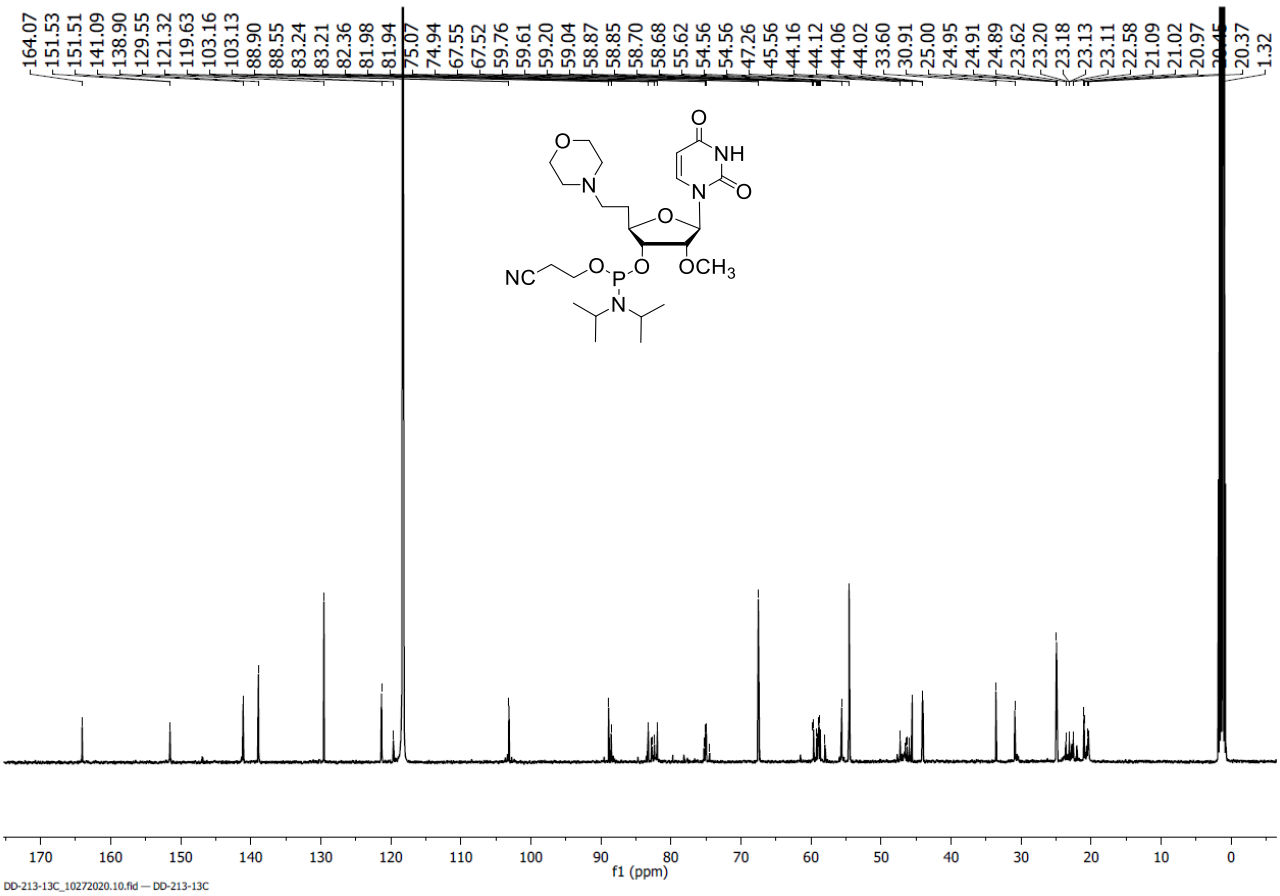
^{1}H NMR (500 MHz, DMSO-d_6) of **7**.



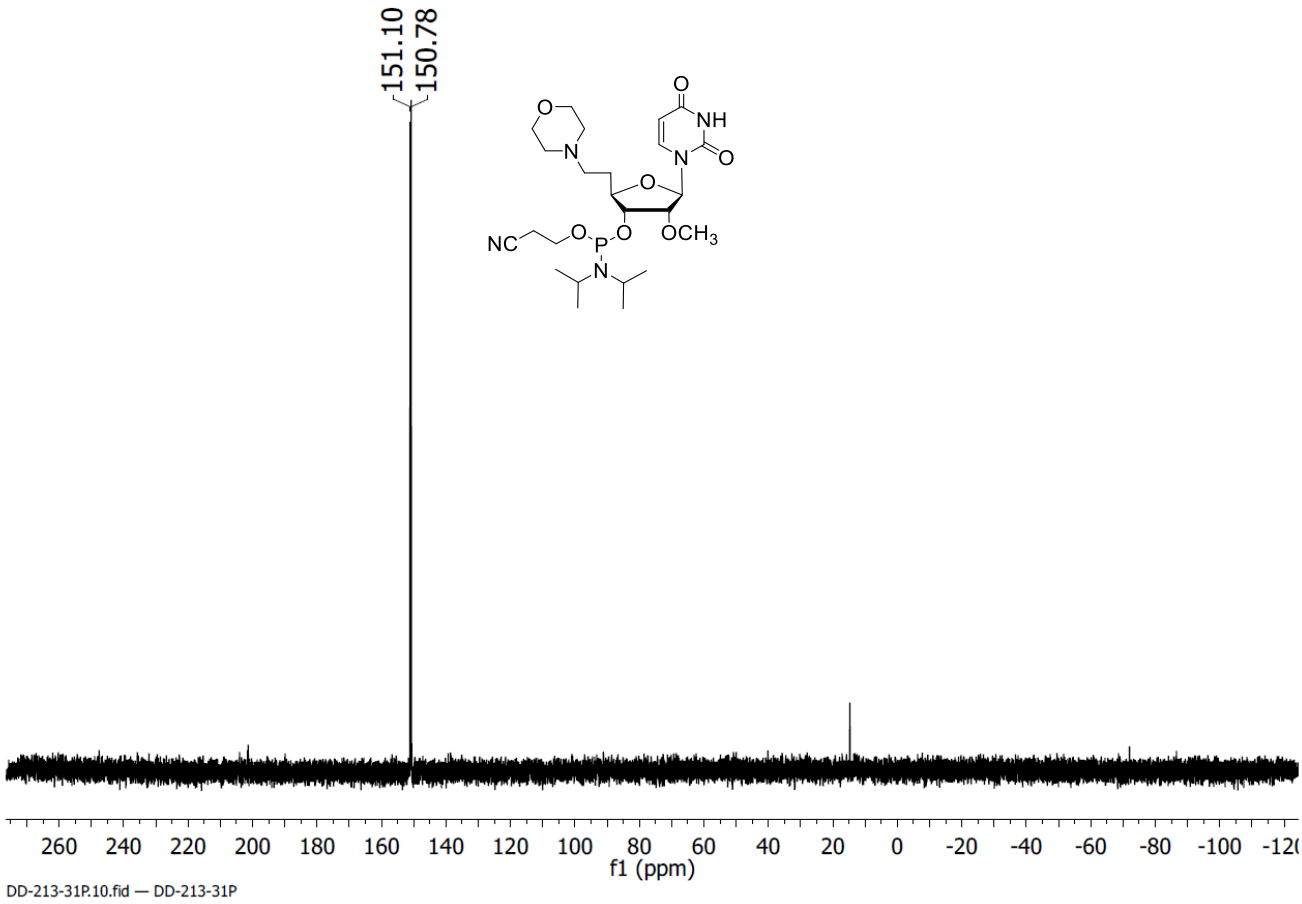
^{13}C NMR (126 MHz, DMSO-d_6) of **7**.



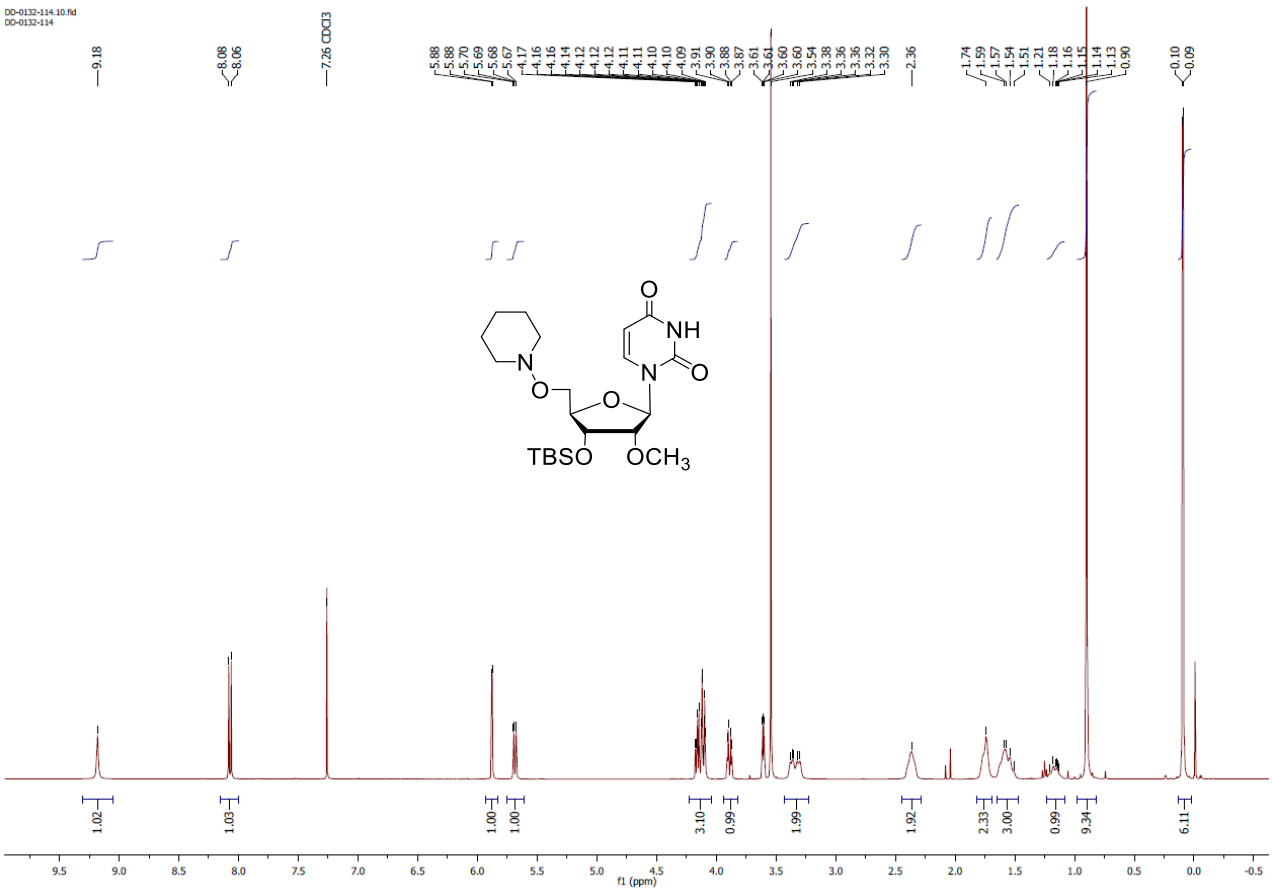
^{1}H NMR (400 MHz, CD_3CN) of **8**.



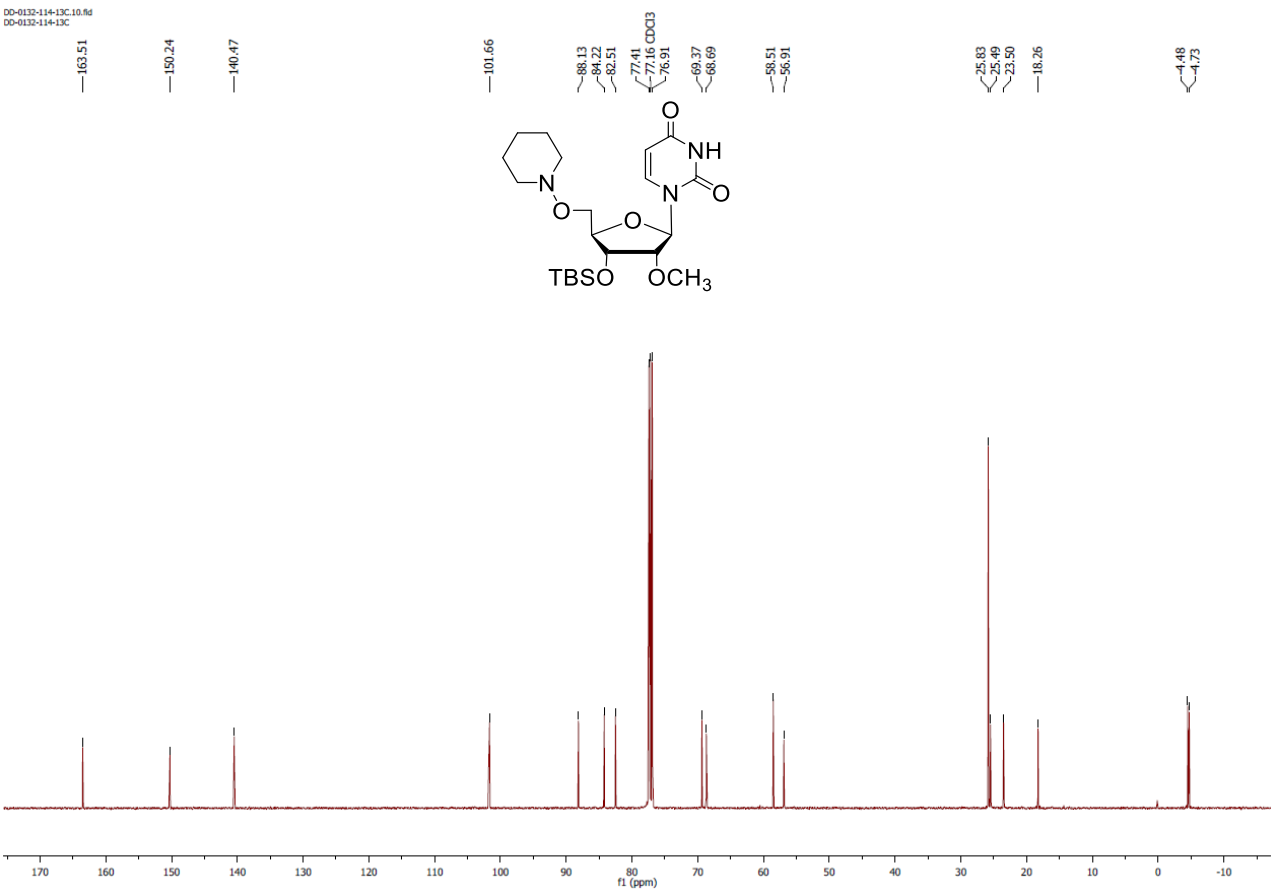
^{13}C NMR (126 MHz, CD_3CN) of **8**.



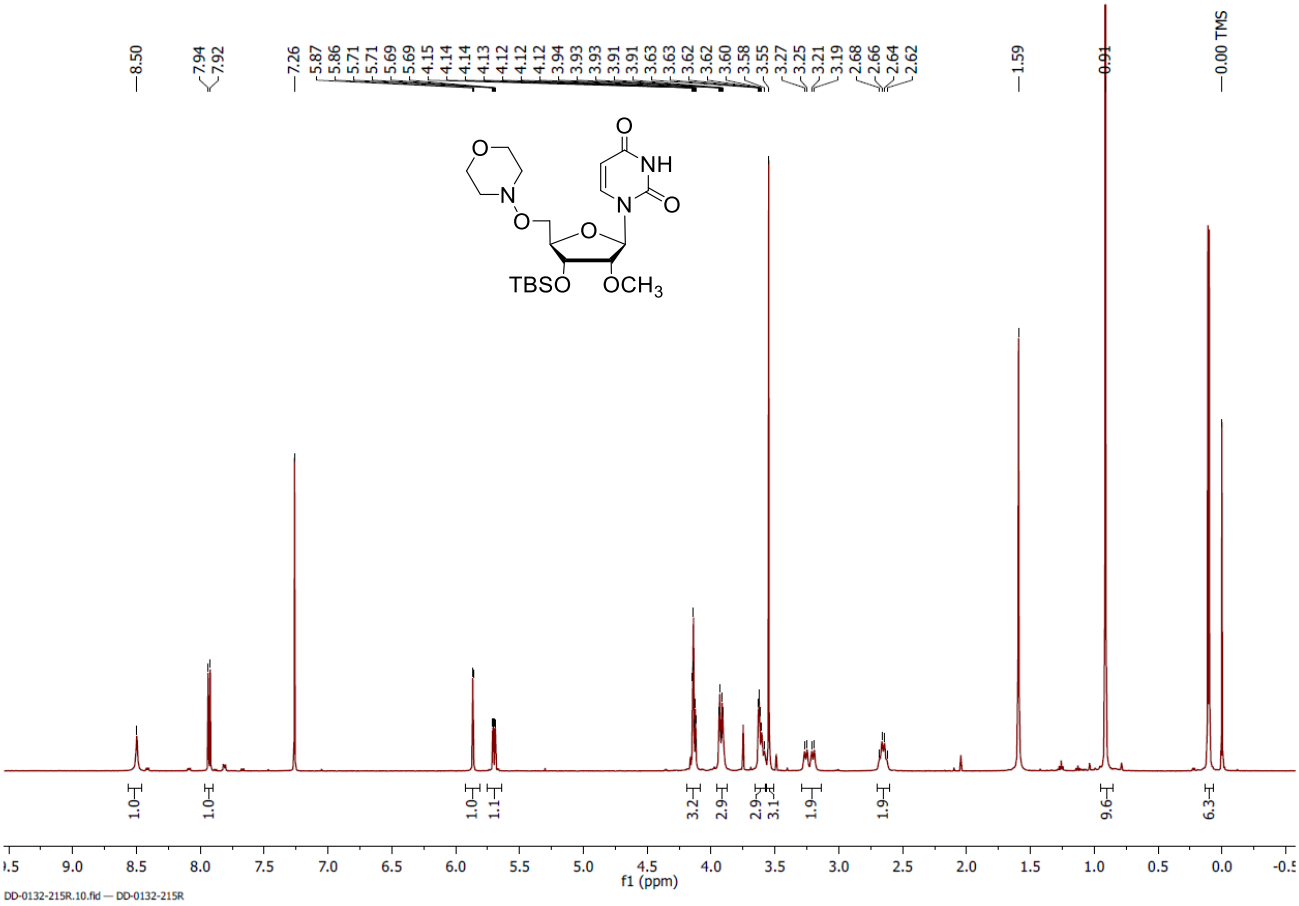
^{31}P NMR (202 MHz, CD_3CN) of **8**.



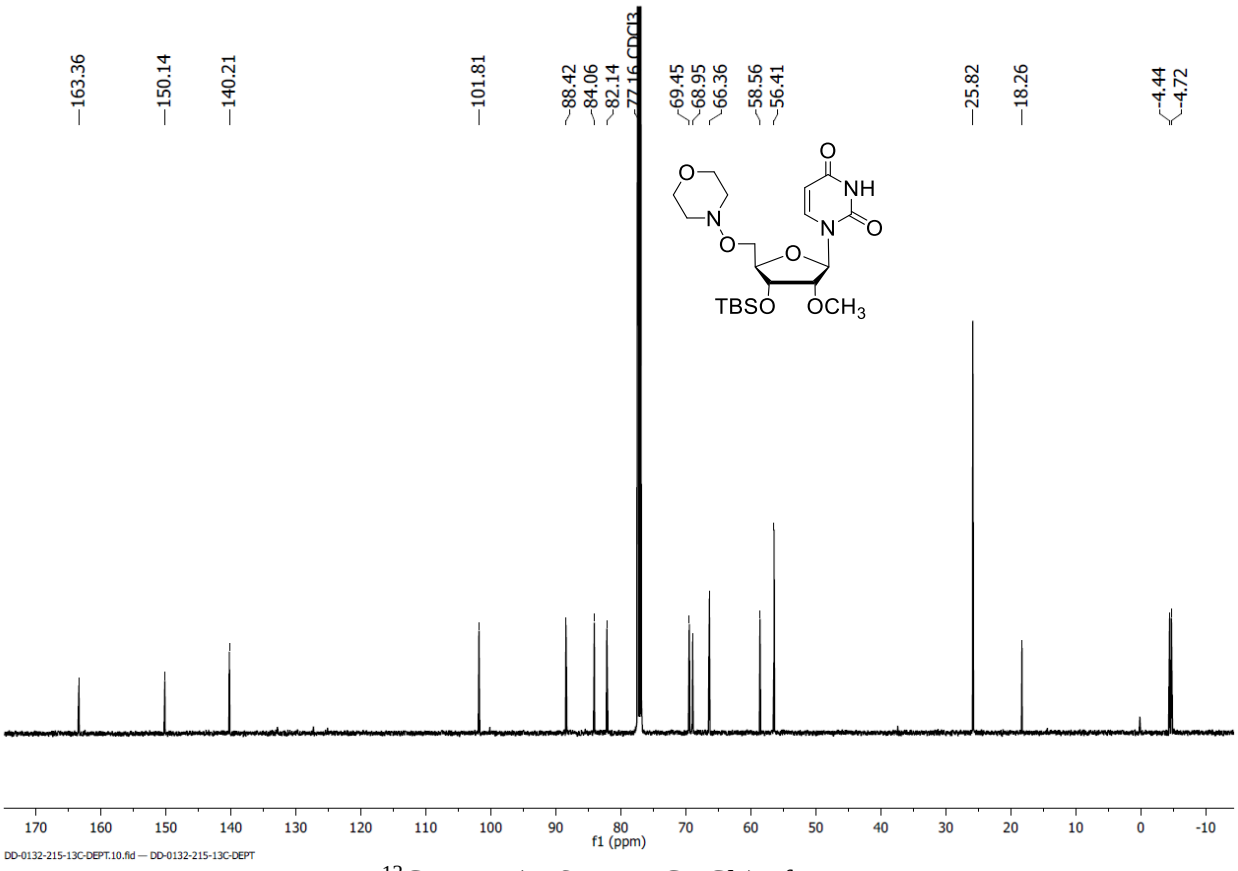
^{1}H NMR (400 MHz, $CDCl_3$) of **10**.



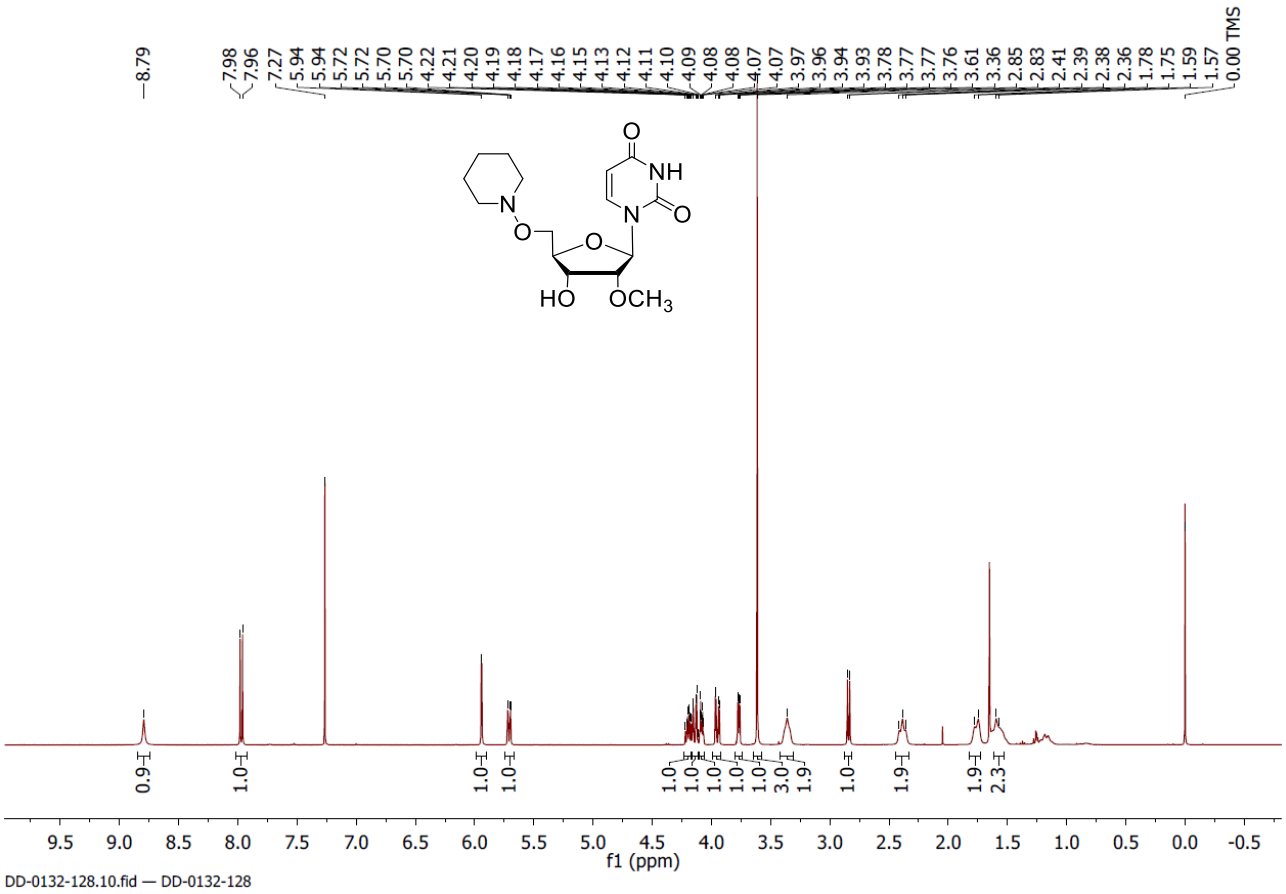
^{13}C NMR (126 MHz, $CDCl_3$) of **10**.



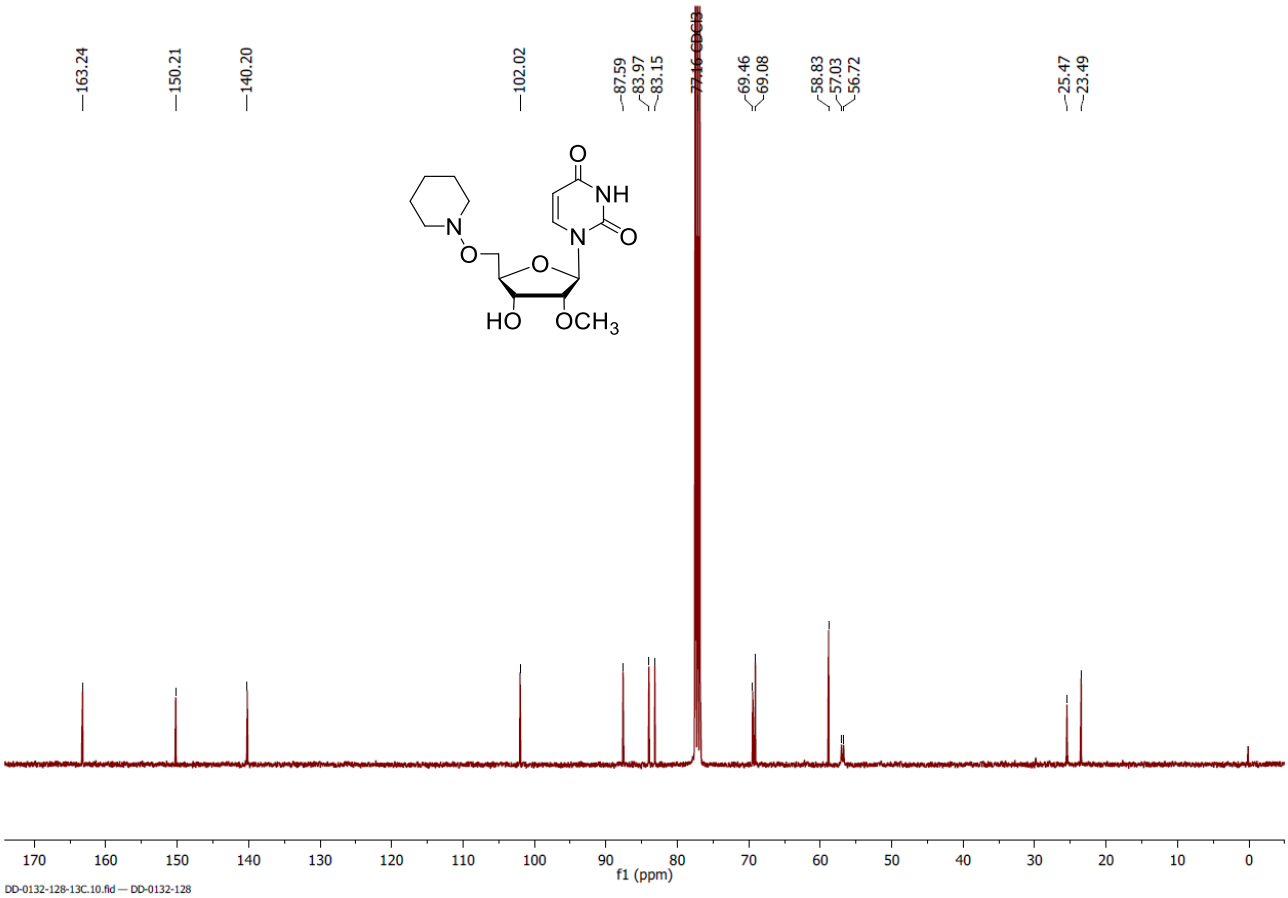
^{1}H NMR (500 MHz, $CDCl_3$) of **11**.



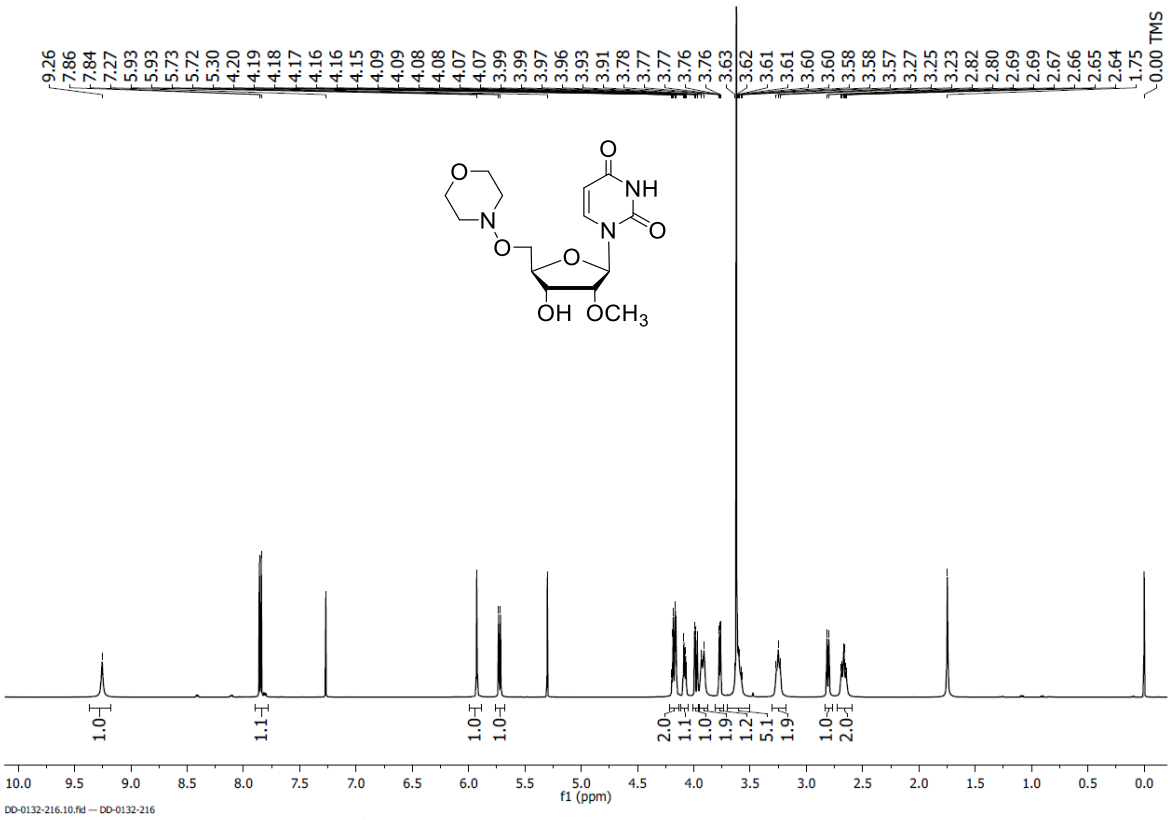
^{13}C NMR (126 MHz, $CDCl_3$) of **11**.



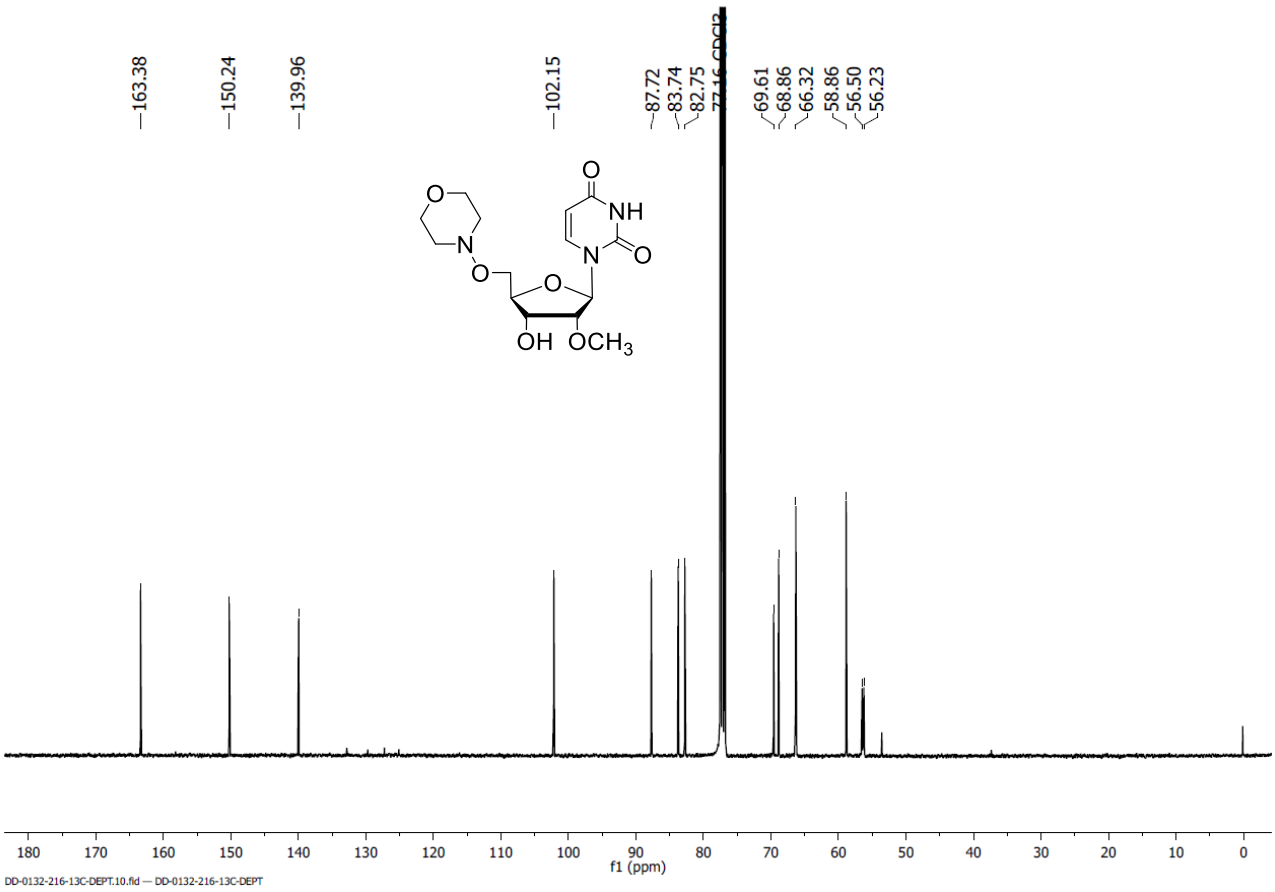
^{1}H NMR (400 MHz, $CDCl_3$) of **12**.



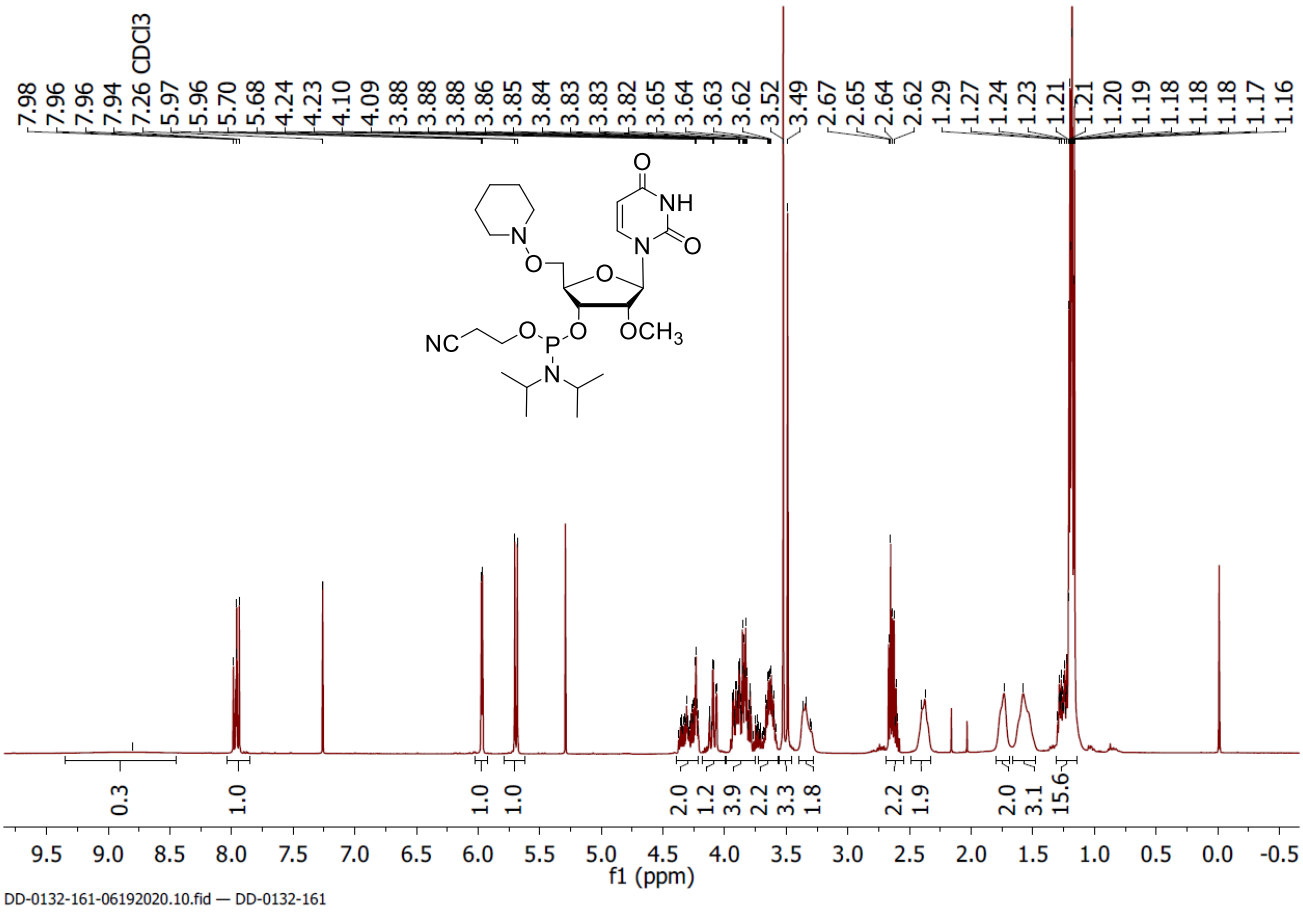
^{13}C NMR (101 MHz, $CDCl_3$) of **12**.



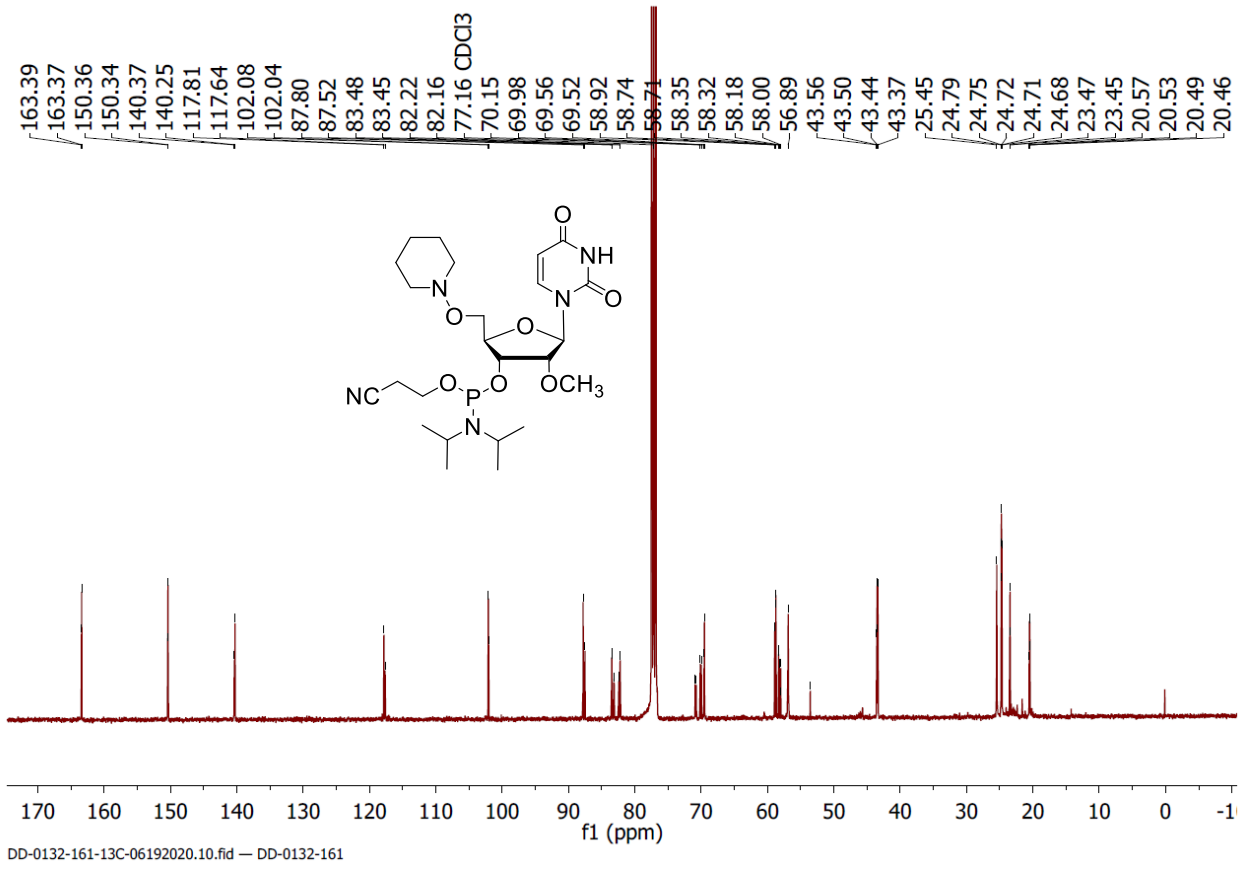
^{1}H NMR (500 MHz, $CDCl_3$) of **13**.



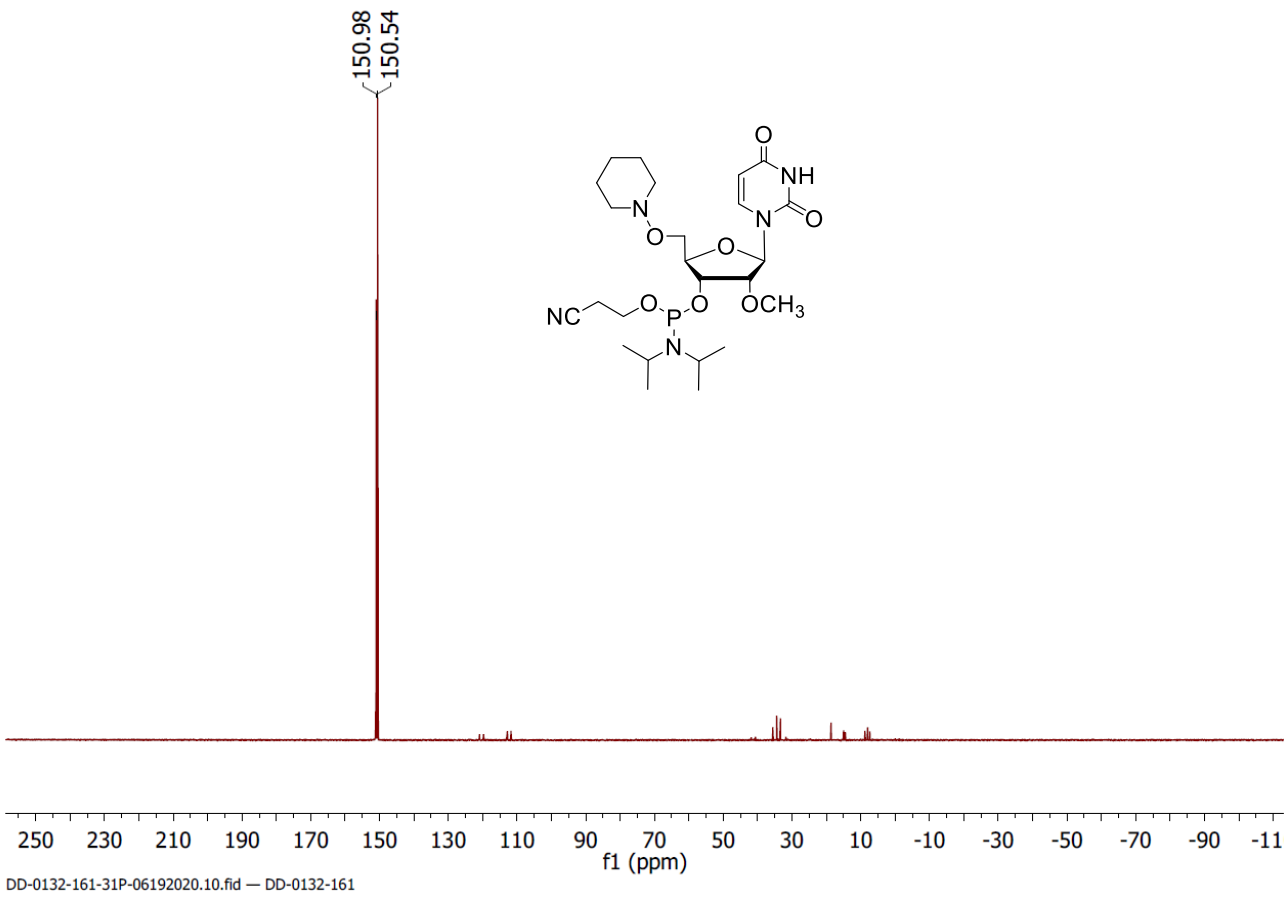
^{13}C NMR (101 MHz, $CDCl_3$) of **13**.



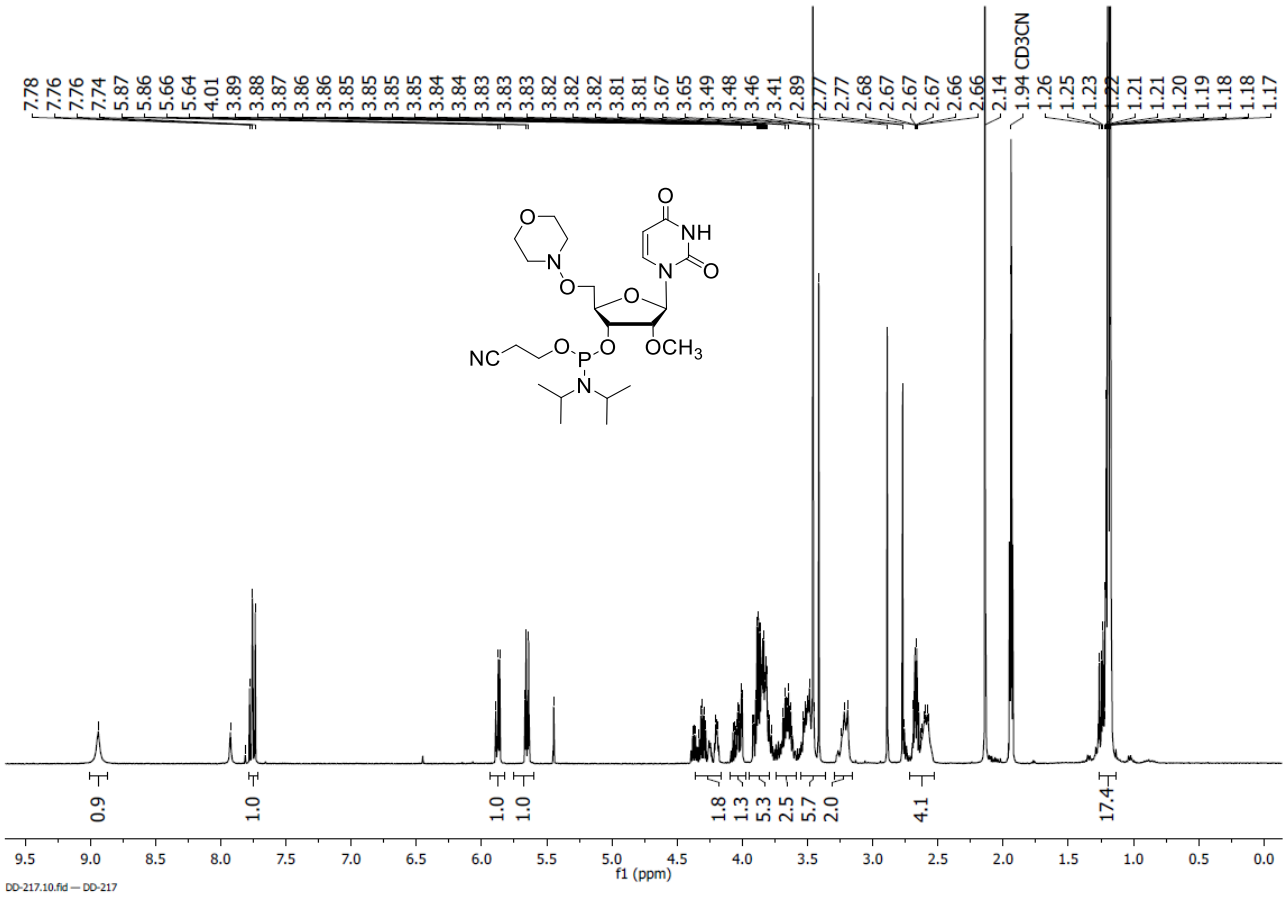
^{1}H NMR (400 MHz, $CDCl_3$) of **14**.



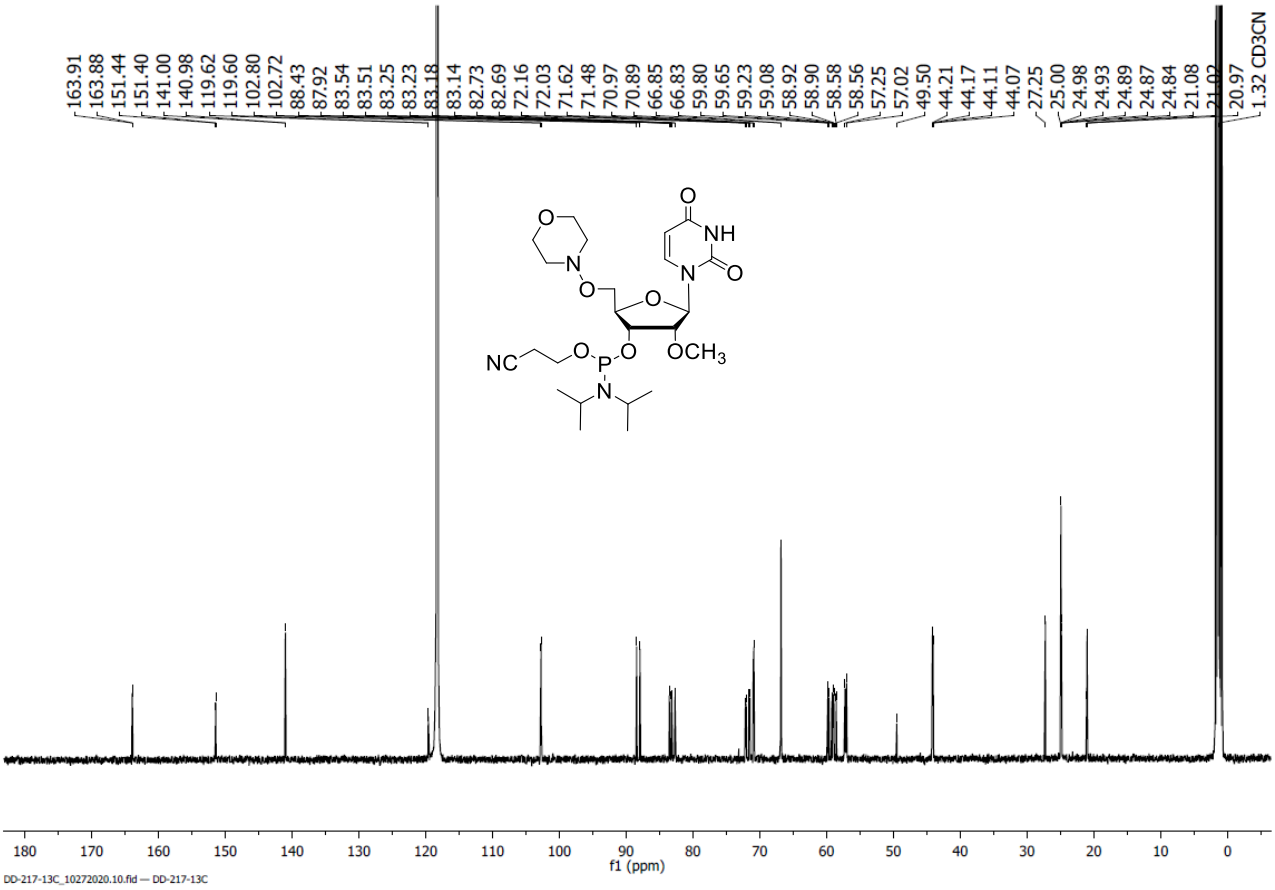
^{13}C NMR (101 MHz, $CDCl_3$) of **14**.



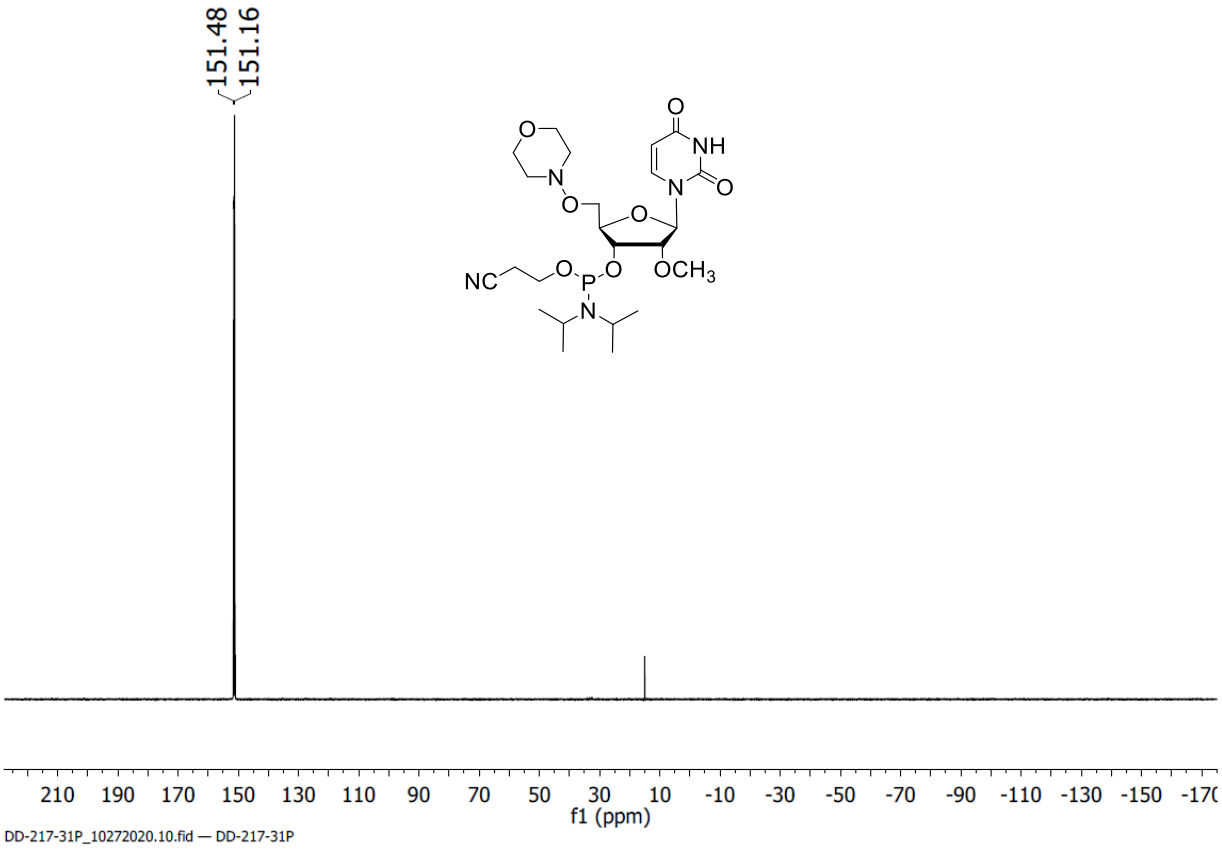
^{31}P NMR (162 MHz, $CDCl_3$) of **14**.



^{1}H NMR (400 MHz, CD_3CN) of **15**.



^{13}C NMR (126 MHz, CD_3CN) of **15**.



^{31}P NMR (202 MHz, CD_3CN) of **15**.

Synthesis of oligonucleotides

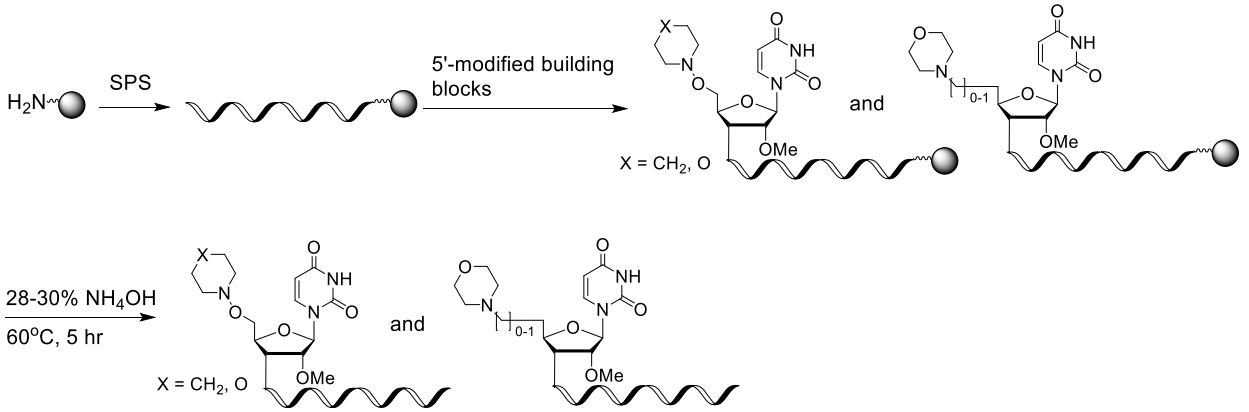


Figure S1: Incorporation of modified amidites at the 5′-end of oligonucleotides. SPS indicates solid-phase synthesis.

Oligonucleotide synthesis and purification: Oligonucleotides were synthesized on K&A H-8-SE at 40-µmol scale using universal supports. A solution of 0.25 M 5-(*S*-ethylthio)-1*H*-tetrazole in acetonitrile (CH_3CN) was used as the activator. The solutions of commercially available phosphoramidites and synthesized phosphoramidities were used at 0.15 M in anhydrous CH_3CN or CH_2Cl_2.The oxidizing reagent was 0.02 M I_2 in THF/pyridine/H_2O. *N*,*N*-Dimethyl-N′-(3-thioxo-3H-1,2,4-dithiazol-5-yl)methanimidamide (DDTT) in 0.1 M in pyridine was used as the sulfurizing reagent. The detritylation reagent was 3% dichloroacetic acid in CH_2Cl_2. Waiting times for coupling, capping, oxidation, and sulfurization step were 450 s, 25 s, 80 s, and 300 s, respectively. After completion of the automated synthesis, the oligonucleotide was manually released from support and deprotected using 28-30% ammonium hydroxide solution at 60 °C for 5h.

After filtration through a 0.45-µm nylon filter, oligonucleotides were purified by ion exchange and/or reverse phase column chromatography. For ion exchange, a preparative HPLC column custom packed with TSKGel SuperQ-5PW(20) (Sigma) was used. Appropriate gradients of mobile phase (buffer A: 20 mM sodium phosphate, 15% CH_3CN, pH 8.5; buffer B: 1 M NaBr, 20 mM sodium phosphate, 15% CH_3CN, pH 8.5) were employed. Oligonucleotides were desalted using size-exclusion chromatography using a column custom packed with Sephadex G25 (GE Healthcare) and water as an eluent. Oligonucleotides were then quantified by measuring the absorbance at 260 nm. Extinction coefficients were calculated using the following extinction coefficients for each residue: A, 13.86; T/U, 7.92; C, 6.57; and G, 10.53 $M^{-1}cm^{-1}$. The identities of modified oligonucleotides were verified by mass spectrometry, respectively. Sequences and mass spectroscopy data are shown in Table S2. Purities were evaluated by analytical reverse-phase HPLC. For reverse-phase HPLC, a C-18 column was used with a gradient of 2-29% buffer B (buffer A: 95 mM hexafluoroisopropanol, 16.3 mM TEA, 0.05 mM EDTA; buffer B: MeOH) over 39 min.

Table S2: Sequences and mass spectroscopy characterization of oligonucleotides.

Entry	Sense and antisense strand (5′-3′)[a]	Target	S / AS	Mass (M-H)$^-$	
				Calcd.	Obsd.
ON1	Mo1●g●*Ug*A*c*A*a*A*U*A*u*G*g*G*c*A*u*C*a*A**L**	*ApoB*	S	8795.45	8796.14
ON2	Mo2●g●*Ug*A*c*A*a*A*U*A*u*G*g*G*c*A*u*C*a*A**L**	*ApoB*	S	8809.48	8809.84
ON3	Pip●g●*Ug*A*c*A*a*A*U*A*u*G*g*G*c*A*u*C*a*A**L**	*ApoB*	S	8809.48	8809.76
ON4	Mo3●g●*Ug*A*c*A*a*A*U*A*u*G*g*G*c*A*u*C*a*A**L**	*ApoB*	S	8811.45	8811.86
ON5	u●g●*Ug*A*c*A*a*A*U*A*u*G*g*G*c*A*u*C*a*A**L**	*ApoB*	S	8726.35	8726.92
ON6	u●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7530.89	7531.36
ON7	Mo1●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7599.99	7600.36
ON8	Mo2●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7614.02	7613.96
ON9	Pip●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7614.02	7614.23
ON10	Mo3●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7615.99	7616.39
ON11[a]	a●a●cagu*G*u*UCU*ugcucuauaa**L**	*TTR*	S	8685.45	8685.35
ON12[a]	VPu●*U*●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7731.12	7730.80
ON13	Mo1●*U*●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7724.22	7724.68
ON14	Mo2●*U*●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7738.25	7736.78
ON15	Pip●*U*●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7738.25	7738.46
ON16	Mo3●*U*●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7740.22	7739.79
ON17	Mo2●u●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7750.28	7749.97
ON18	u●u●aua*G*agcaaga*A*c*A*cuguu●u●u	*TTR*	AS	7667.15	7667.21
ON19	Mo2●u●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7626.05	7626.84
ON20	u●u●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	*ApoB*	AS	7542.92	7542.83
ON21	Mo2●*G*●*Ug*A*c*A*a*A*U*A*u*G*g*G*c*A*u*C*a*A**L**	*ApoB*	S	8795.45	8798.03

[a] **ON11** and **ON12** were synthesized previously.[6]

[b] Chemical modifications are indicated as follows: ●, PS linkage; lower case, 2′-OMe; italicized upper case, 2′-F; **L**, trivalent-GalNAc.

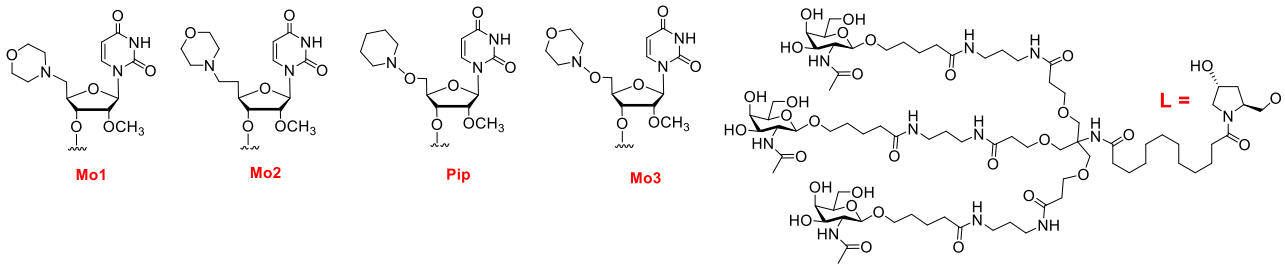
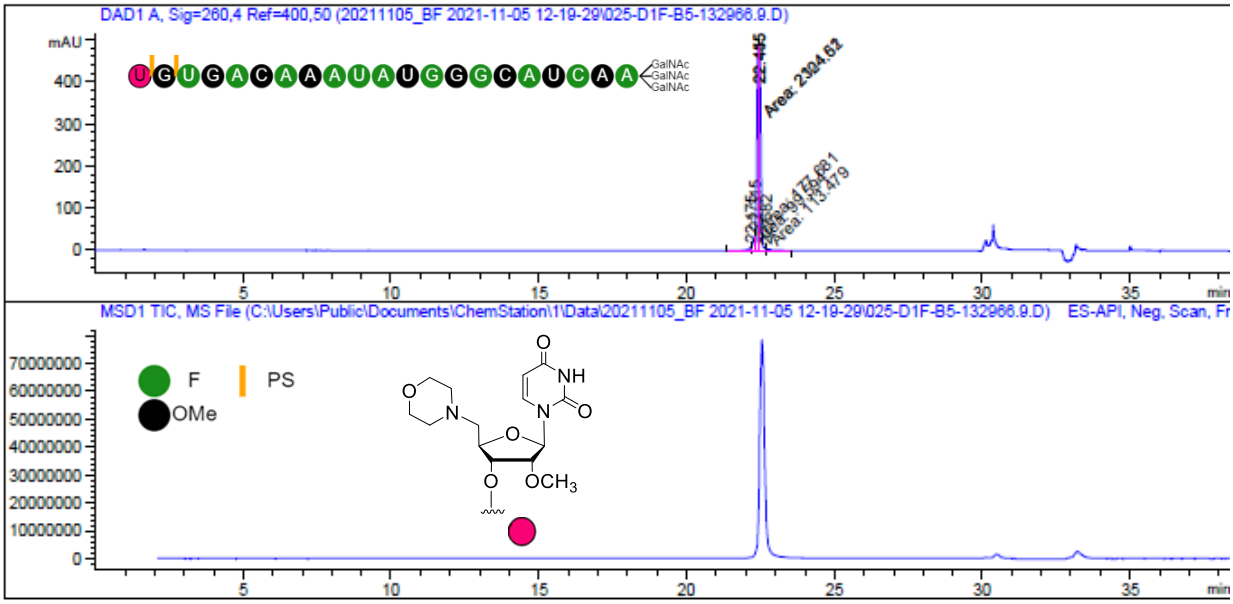


Figure S2: Chemical structures of 5′-end modifications and trivalent-GalNAc.

HPLC chromatograms of oligonucleotides

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   3  22.445 MF    0.0785 2304.50757  489.09137  47.8125
   4  22.455 FM    0.0720 2124.62207  491.51886  44.0804
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Totals :                  4819.88451 1033.99607
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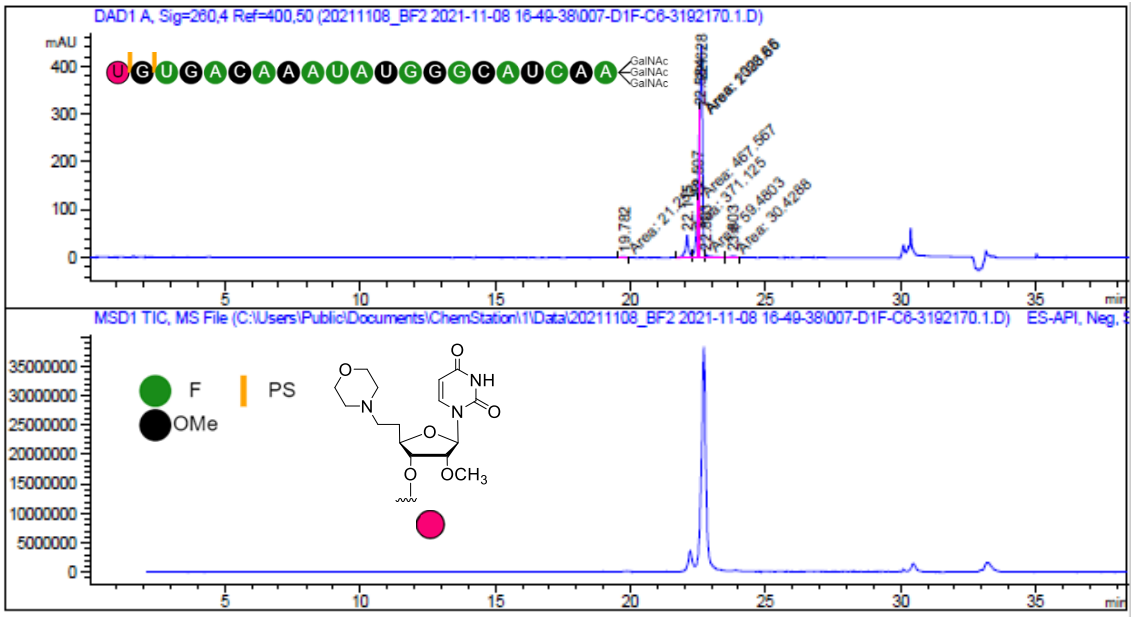
Reverse-phase HPLC profile for **ON1**.

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Signal 1: DAD1 A, Sig=260,4 Ref=400,50

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   5  22.628 FM    0.0898 2398.86304  445.06271  54.8641
   6  22.803 MF    0.1348   59.48031    5.23742   1.3604
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Totals :                  4372.37175  928.01819
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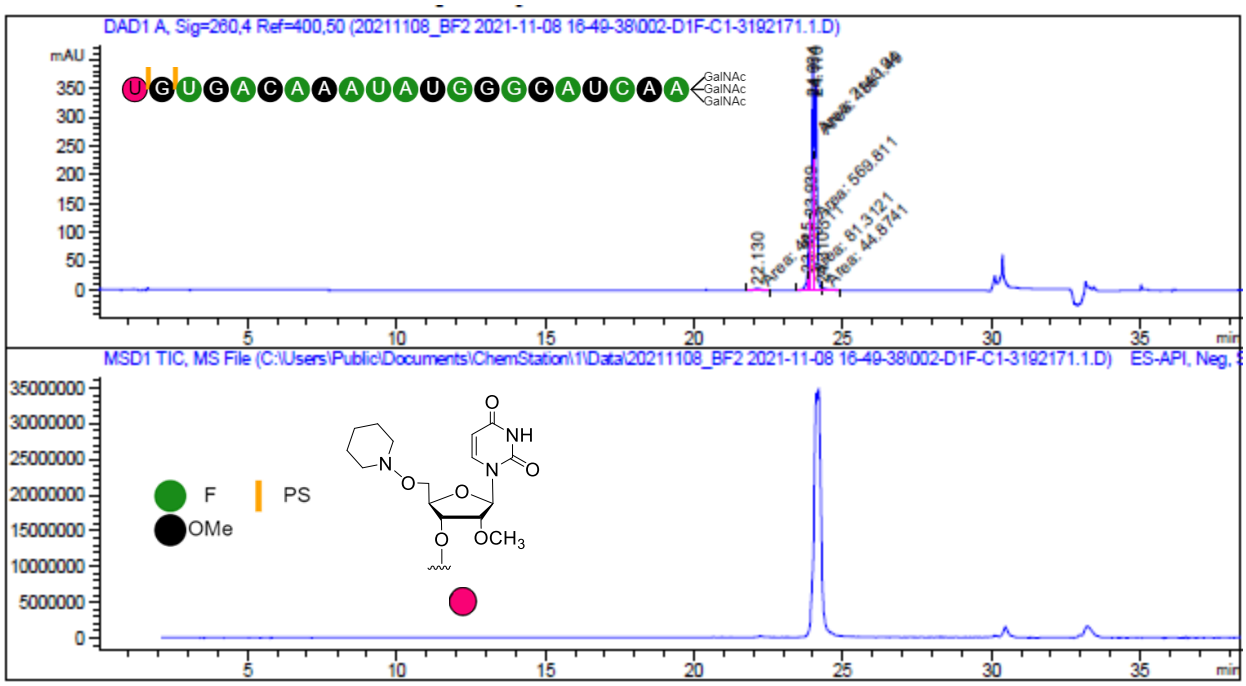
Reverse-phase HPLC profile for **ON2**.

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   1  22.130 MM    0.2470   48.55107    3.27635   1.0185
   2  23.815 MF    0.0618   81.31206   21.91688   1.7057
   3  23.939 FM    0.0798  569.81122  118.93607  11.9533
   4  24.004 MF    0.0885 2140.94141  403.30154  44.9119
   5  24.110 MF    0.0882 1881.48511  355.36322  39.4692
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Totals :                  4766.97496  906.40809
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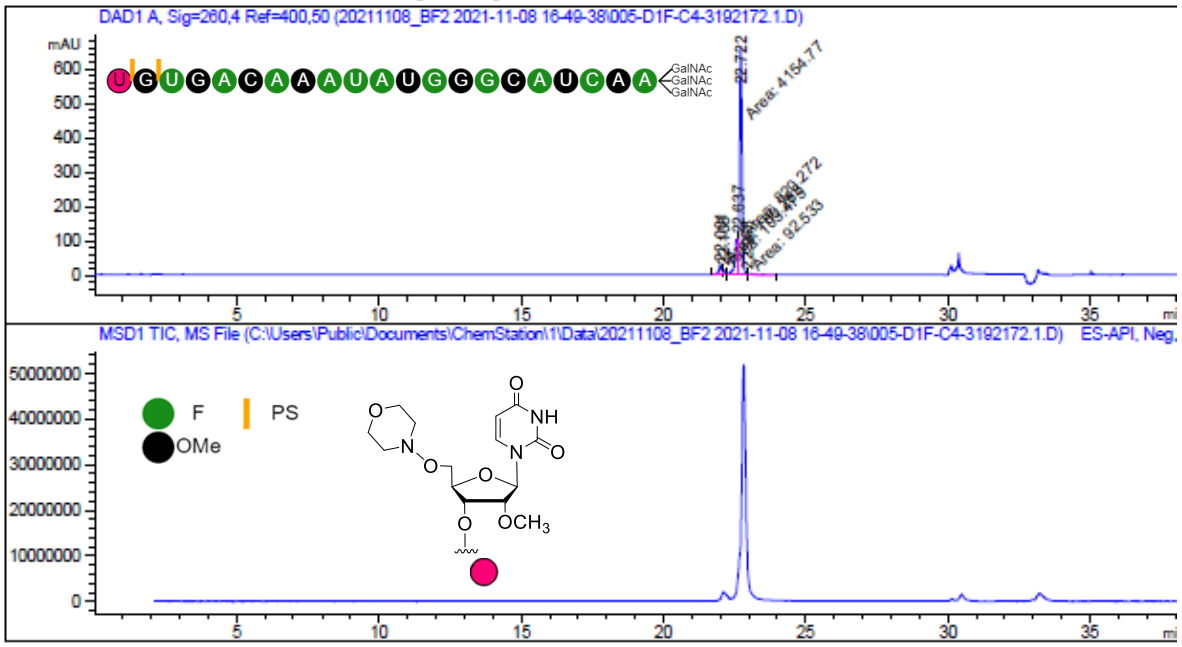
Reverse-phase HPLC profile for **ON3**.

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    2  22.108 MF    0.1140  103.47461   15.13394   1.9336
    3  22.637 MF    0.1291  820.27240  105.89941  15.3284
    4  22.722 FM    0.1047 4154.76611  661.14532  77.6402

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 Totals :                  5351.30862  813.09639
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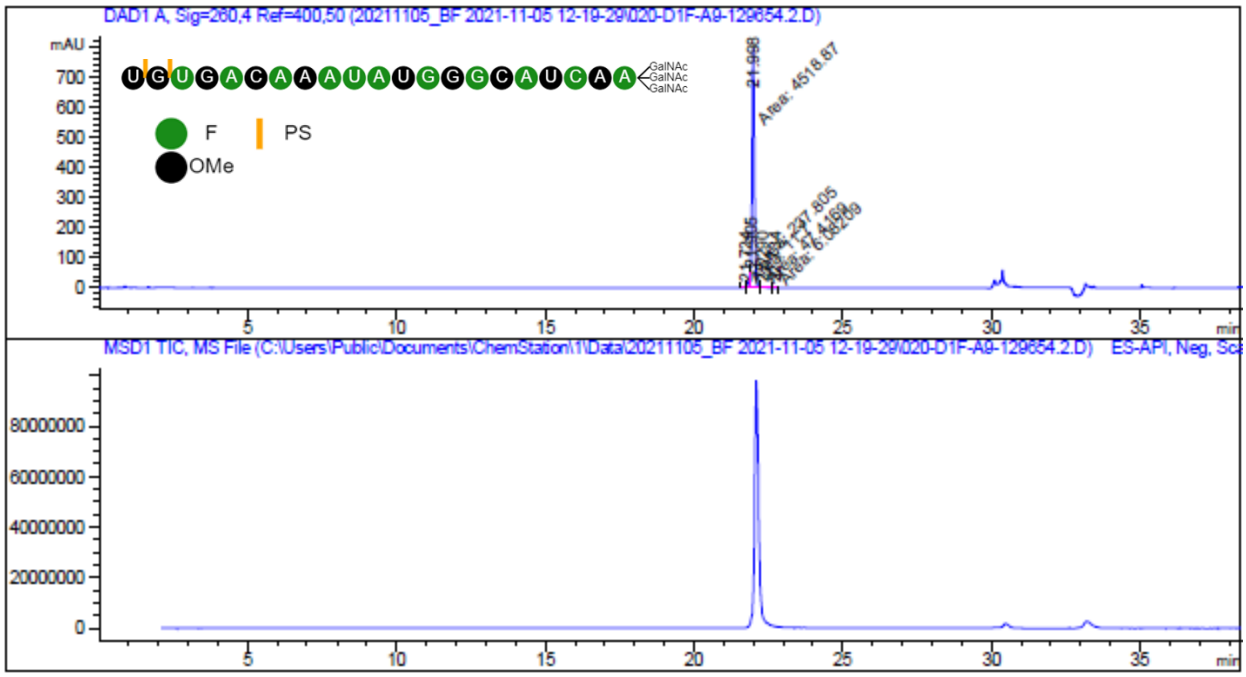
Reverse-phase HPLC profile for **ON4**.

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Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

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   3  21.998 FM    0.0944 4518.87061  798.01074  93.7161
   4  22.290 MF    0.2165   47.41690    3.64943   0.9834
   5  22.734 FM    0.0936    6.08209    1.08352   0.1261

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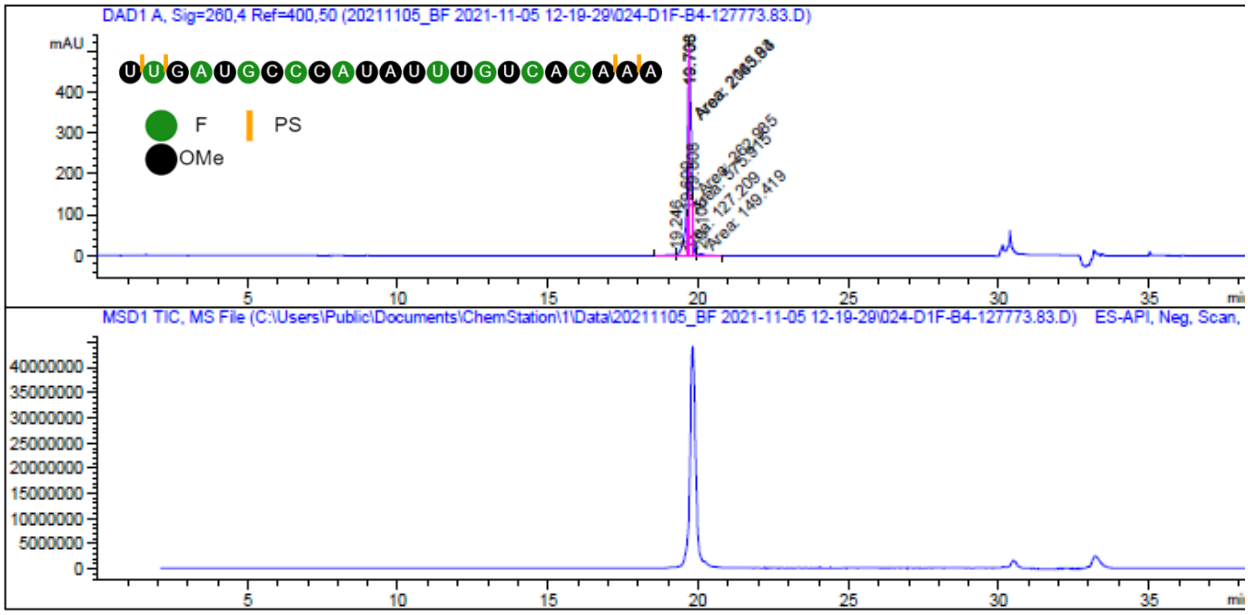
Reverse-phase HPLC profile for **ON5**.

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Signal 1: DAD1 A, Sig=260,4 Ref=400,50
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    2  19.600 MF    0.0974  575.91504   98.49812  10.8147
    3  19.703 MF    0.0699 2165.92944  516.63629  40.6725
    4  19.708 MF    0.0662 2043.83972  514.25098  38.3798
    5  19.808 FM    0.0309  262.98508  141.66614   4.9384
    6  20.105 FM    0.3964  149.41920    6.28218   2.8058

 Totals :                  5325.29755 1281.94460
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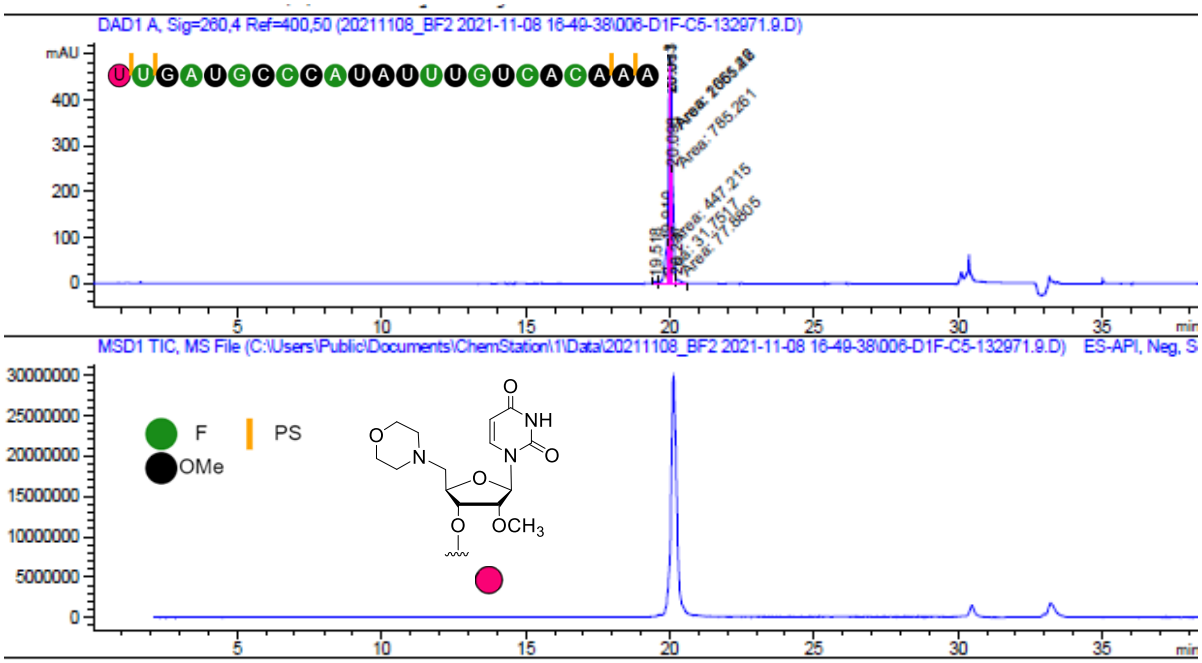
Reverse-phase HPLC profile for **ON6**.

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Signal 1: DAD1 A, Sig=260,4 Ref=400,50

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   1  19.518 MF    0.1089   31.75174    4.86065   0.6277
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   3  20.013 MF    0.0578 1651.22144  476.20313  32.6406
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----|-------|----|-------|----------|----------|--------|
   5  20.098 FM    0.0535  785.26062  244.57695  15.5227
   6  20.237 FM    0.1561   77.88048    8.31264   1.5395

Totals :                  5058.79021 1312.54512
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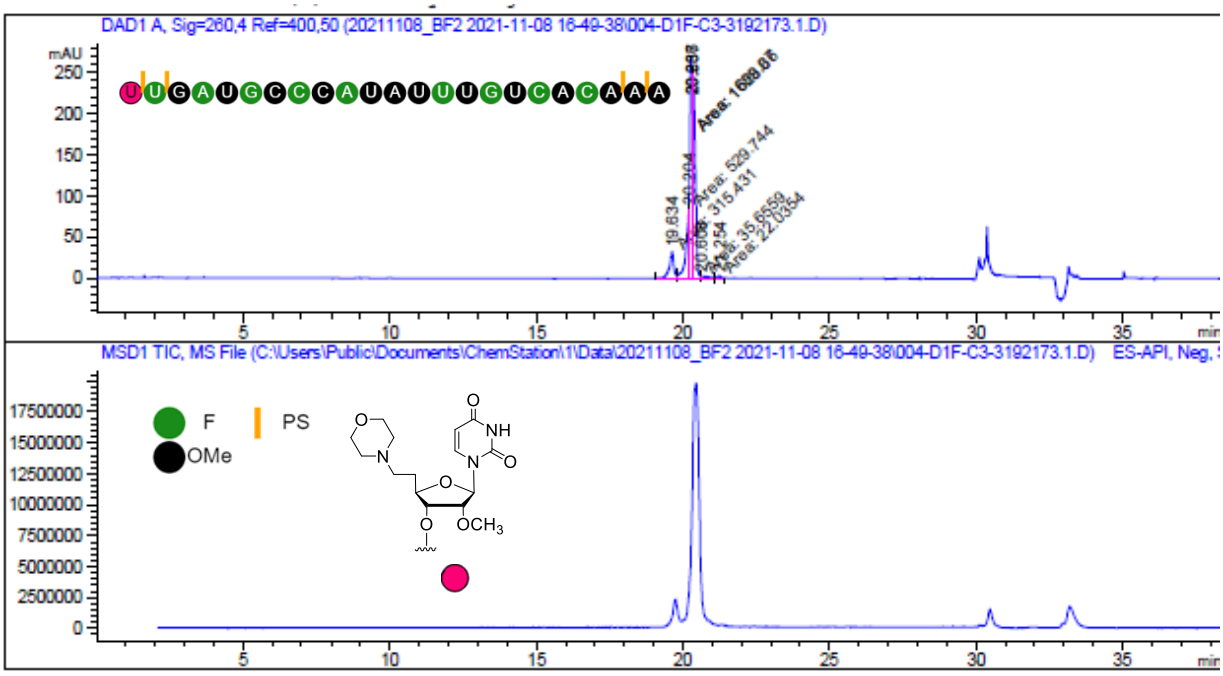
Reverse-phase HPLC profile for **ON7**.

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Dilution              :      1.0000
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Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
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   1  19.634 MF    0.1642  315.43073   32.02452   7.4736
   2  20.204 MF    0.1050  529.74414   84.04922  12.5514
   3  20.286 MF    0.1046 1689.07300  269.13748  40.0197
   4  20.337 FM    0.1089 1628.66138  249.15331  38.5884

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Totals :                  4220.60059  639.56956
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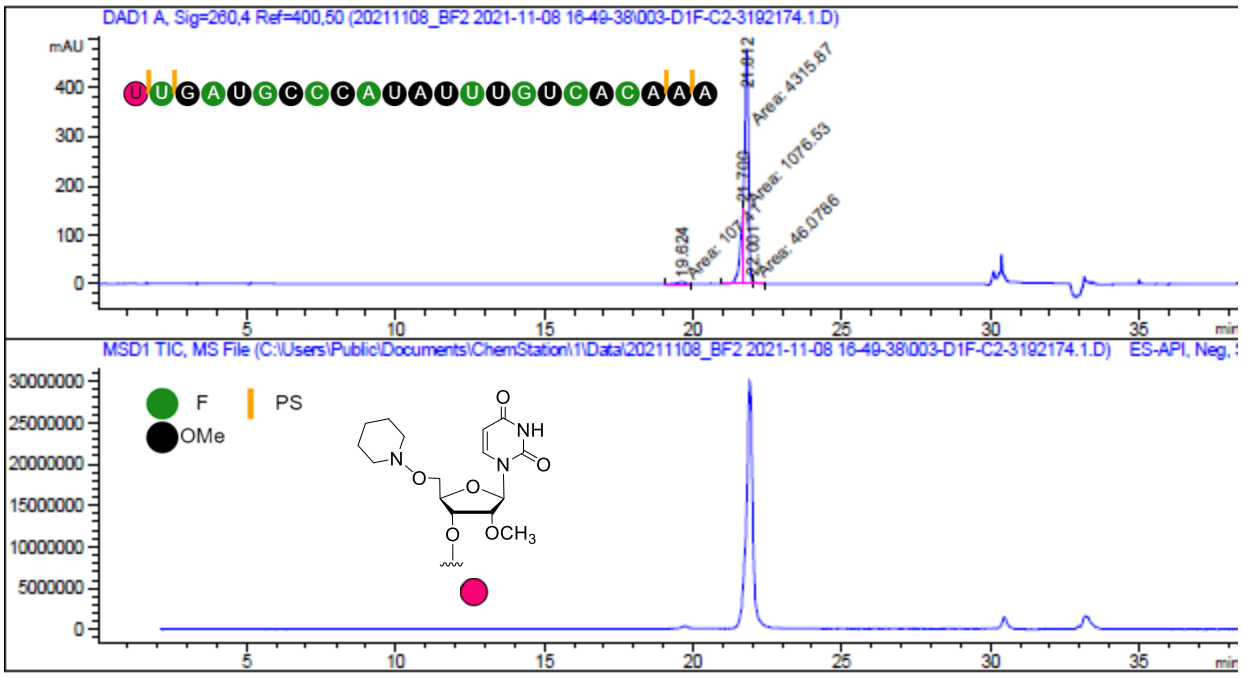
Reverse-phase HPLC profile for **ON8**.

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Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  19.624 MM    0.3687  107.40978    4.85516   1.9367
   2  21.700 MF    0.1146 1076.52722  156.55206  19.4113
   3  21.812 FM    0.1502 4315.87061  478.88672  77.8211
   4  22.001 FM    0.1190   46.07861    6.45469   0.8309

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le Name: 3192174.1

Peak RetTime Type  Width     Area      Height     Area
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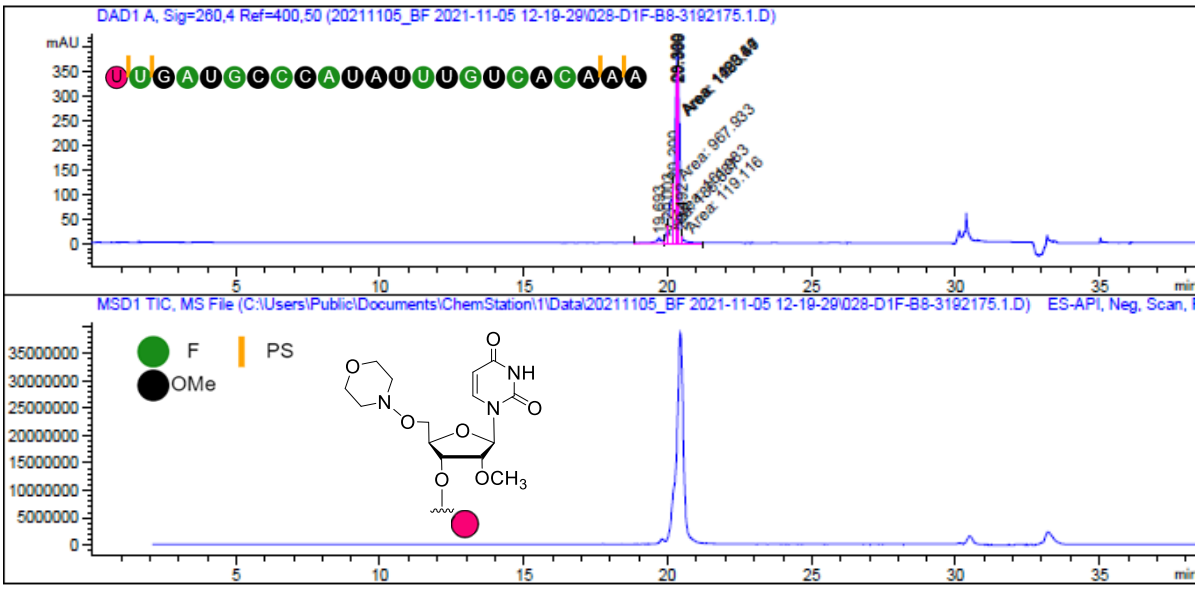
Reverse-phase HPLC profile for **ON9**.

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                        Area Percent Report
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Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50
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 File C:\Users\P...ation\1\Data\20211105_BF 2021-11-05 12-19-29\028-D1F-B8-3192175.1.D
le Name: 3192175.1

Peak RetTime Type  Width     Area      Height     Area
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----|-------|----|-------|----------|----------|--------|
   1  19.693 MF    0.2818  186.88727   11.05233   3.3210
   2  20.003 MF    0.0704  161.98346   38.36491   2.8785
   3  20.200 MF    0.1316  967.93292  122.58730  17.2003
   4  20.300 MF    0.0689 1429.43945  345.58673  25.4013
   5  20.339 MF    0.0616 1498.59497  405.25439  26.6302
   6  20.366 FM    0.0583 1263.47070  360.99738  22.4520
   7  20.492 FM    0.1205  119.11646   16.47668   2.1167

Totals :                 5627.42523 1300.31972
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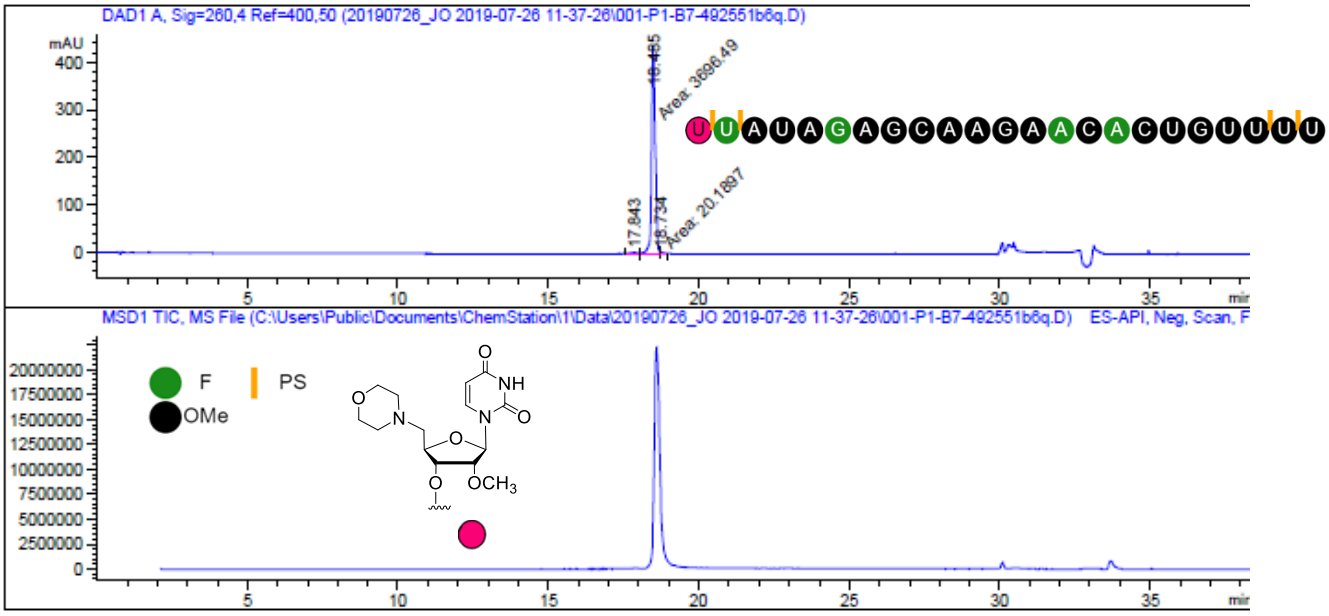
Reverse-phase HPLC profile for **ON10**.

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Sorted By             :      Signal
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Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50
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Peak RetTime Type  Width     Area      Height     Area
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----|-------|----|-------|----------|----------|--------|
   1  17.843 BB    0.1357   23.22223    2.05684   0.6209
   2  18.485 MF    0.1404 3696.49292  438.83374  98.8392
   3  18.734 MF    0.0859   20.18972    3.91767   0.5398

Totals :                  3739.90487  444.80824
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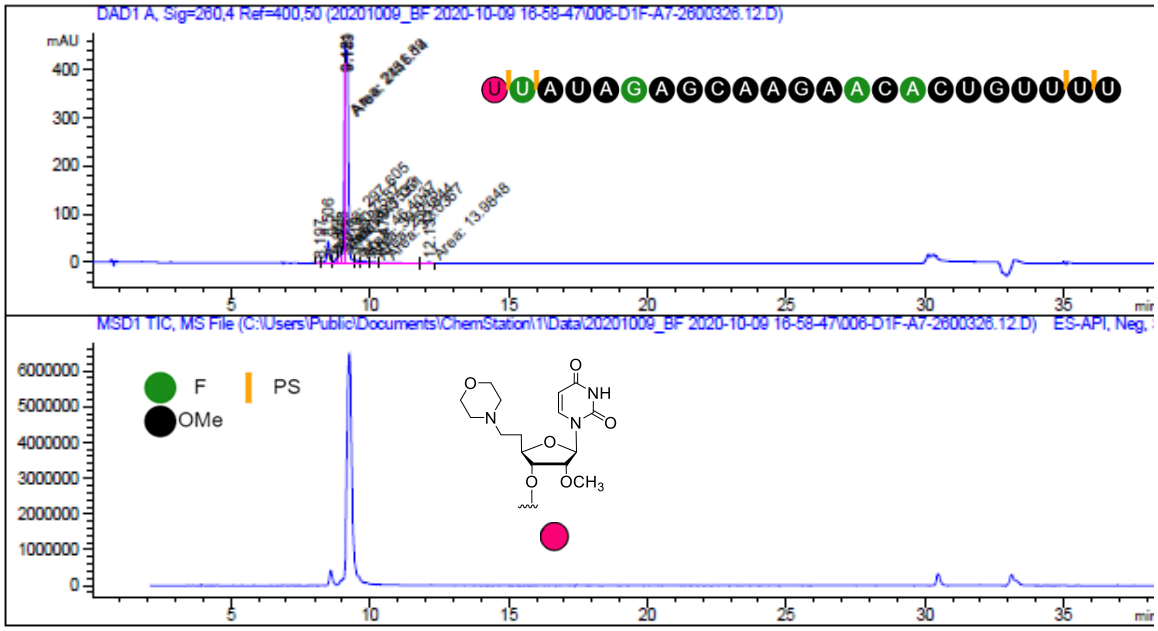
Reverse-phase HPLC profile for **ON13**.

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                          Area Percent Report
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Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   8.197 MF    0.1410   10.75872    1.27193   0.2039
   2   8.506 MF    0.1094  297.60480   45.32123   5.6400
   3   8.855 MF    0.0931   65.55331   11.73782   1.2423
   4   9.003 MF    0.1289  147.05121   19.00803   2.7868
   5   9.121 MF    0.0789 2151.89380  454.54013  40.7812

) Infinity 2 LCMS 2 10/13/2020 2:53:33 PM SYSTEM                    Page   1 of 2

a File C:\Users\P...tion\1\Data\20201009_BF 2020-10-09 16-58-47\006-D1F-A7-2600326.12.D
ole Name: 2600326.12

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   6   9.189 FM    0.0924 2446.13794  441.41931  46.3575
   7   9.516 MF    0.1465   46.40366    5.27857   0.8794
   8   9.789 MF    0.1688   39.87500    3.93666   0.7557
   9  10.123 MF    0.3016   25.38440    1.40286   0.4811
  10  10.478 MF    0.6417   32.03666 8.32079e-1   0.6071
  11  12.137 FM    0.1551   13.98477    1.50238   0.2650
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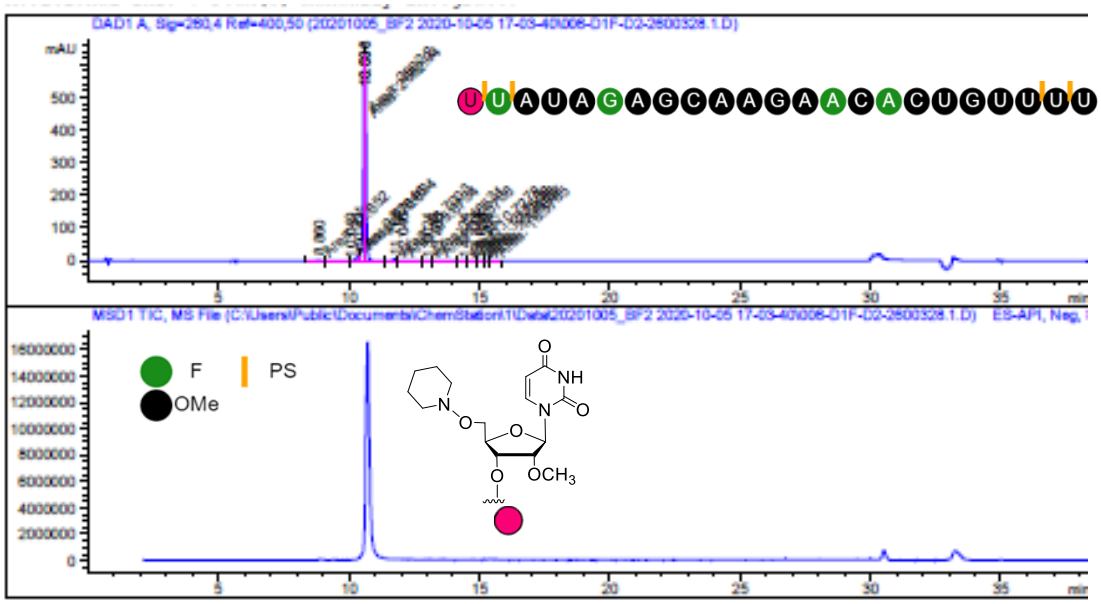
Reverse-phase HPLC profile for **ON14**.



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 A, Sig=260,4 Ref=400,50

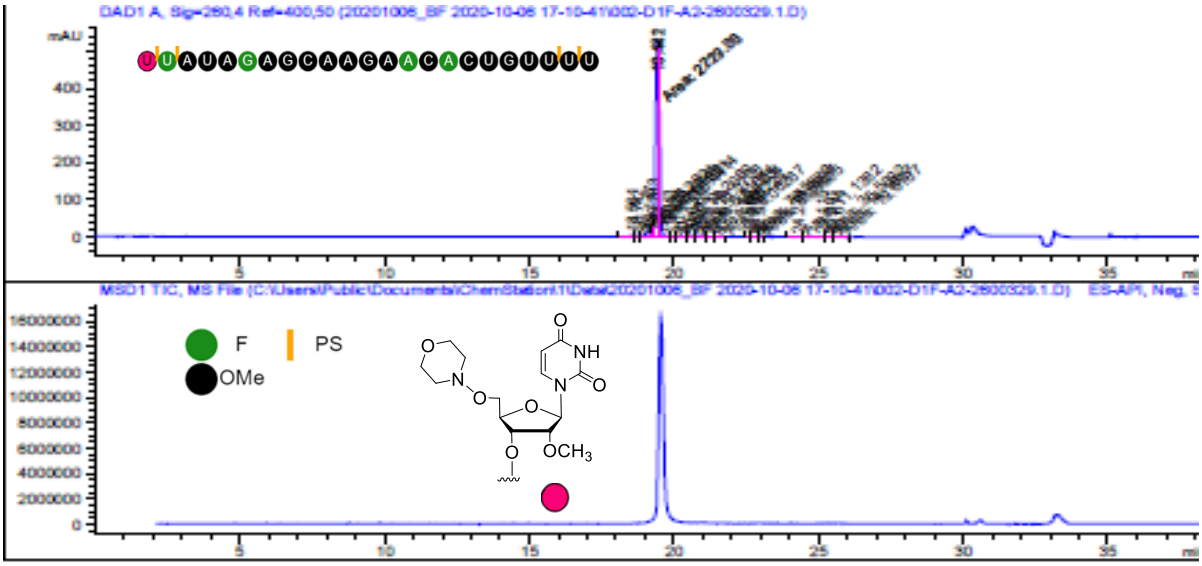
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.860	MF	0.2515	31.65199	2.09716	0.5693
2	10.049	MF	0.2696	9.02646	5.58036e-1	0.1624
3	10.431	MF	0.1220	176.69440	24.14326	3.1781
4	10.598	MF	0.0652	2502.20288	639.62335	45.0052

) Infinity2 LCMS 1 10/6/2020 11:54:45 AM SYSTEM Page 1 of 2

File C:\Users\P...tion\1\Data\20201005_BF2 2020-10-05 17-03-40\006-D1F-D2-2600328.1.D
ple Name: 2600328.1

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
5	10.601	FM	0.0699	2682.03931	639.20264	48.2397
6	11.765	MF	0.1600	46.70032	4.86473	0.8400
7	12.046	FM	0.5437	15.67338	4.80433e-1	0.2819
8	13.024	FM	0.1316	7.48531	9.48274e-1	0.1346
9	13.403	FM	0.1559	15.57684	1.66548	0.2802
10	14.424	MF	0.2091	10.72783	8.55247e-1	0.1930
11	14.833	MF	0.2724	15.75657	9.64212e-1	0.2834
12	15.058	MF	0.1675	15.91831	1.58380	0.2863
13	15.268	MF	0.1409	11.78071	1.39362	0.2119
14	15.541	FM	0.2230	18.57848	1.38881	0.3342

Reverse-phase HPLC profile for **ON15**.



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

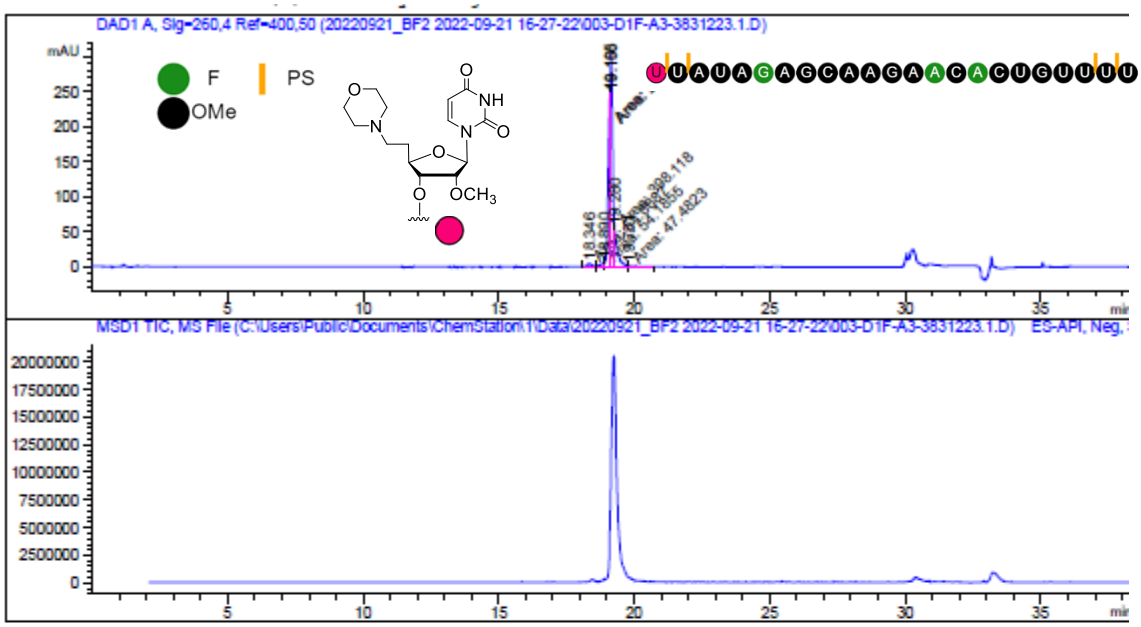
Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	18.561	MF	0.1592	36.28238	3.79760	0.6588
2	18.742	MF	0.1651	28.57434	2.88428	0.5188
3	19.147	MF	0.1573	126.44148	13.39763	2.2959
4	19.283	MF	0.0974	174.01414	29.77535	3.1597
5	19.421	FM	0.0849	2719.76221	533.95935	49.3846
6	19.442	FM	0.0729	2227.03174	509.33322	40.4377
7	19.843	MF	0.1583	11.20692	1.18016	0.2035
8	20.173	MF	0.2157	15.57080	1.20333	0.2827

File C:\Users\P...ation\1\Data\20201006_BF 2020-10-06 17-10-41\002-D1F-A2-2600329.1.D
le Name: 2600329.1

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
9	20.633	MF	0.2348	8.11664	5.76195e-1	0.1474
10	20.861	FM	0.1636	40.42041	4.11726	0.7339
11	21.237	MF	0.1889	6.92250	6.10841e-1	0.1257
12	21.628	MF	0.2338	8.06267	5.74648e-1	0.1464
13	22.562	MF	0.1428	7.45579	8.70007e-1	0.1354
14	22.759	MF	0.1625	10.99369	1.12745	0.1996
15	22.938	FM	0.1608	5.99905	6.21815e-1	0.1089
16	24.279	MF	0.2766	11.13817	6.71229e-1	0.2022

Reverse-phase HPLC profile for **ON16**.

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Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  18.346 MF    0.2036   54.86871    4.49061   1.4317
   2  18.890 MF    0.1727   54.18547    5.23053   1.4138
   3  19.105 MF    0.0739 1114.96838  251.36119  29.0922
   4  19.156 MF    0.1181 2162.90601  305.14639  56.4355

290 Infinity2 LCMS 1 9/22/2022 2:15:17 PM SYSTEM                    Page   1 of 2

ata File C:\Users\P...tion\1\Data\20220921_BF2 2022-09-21 16-27-22\003-D1F-A3-3831223.1.D
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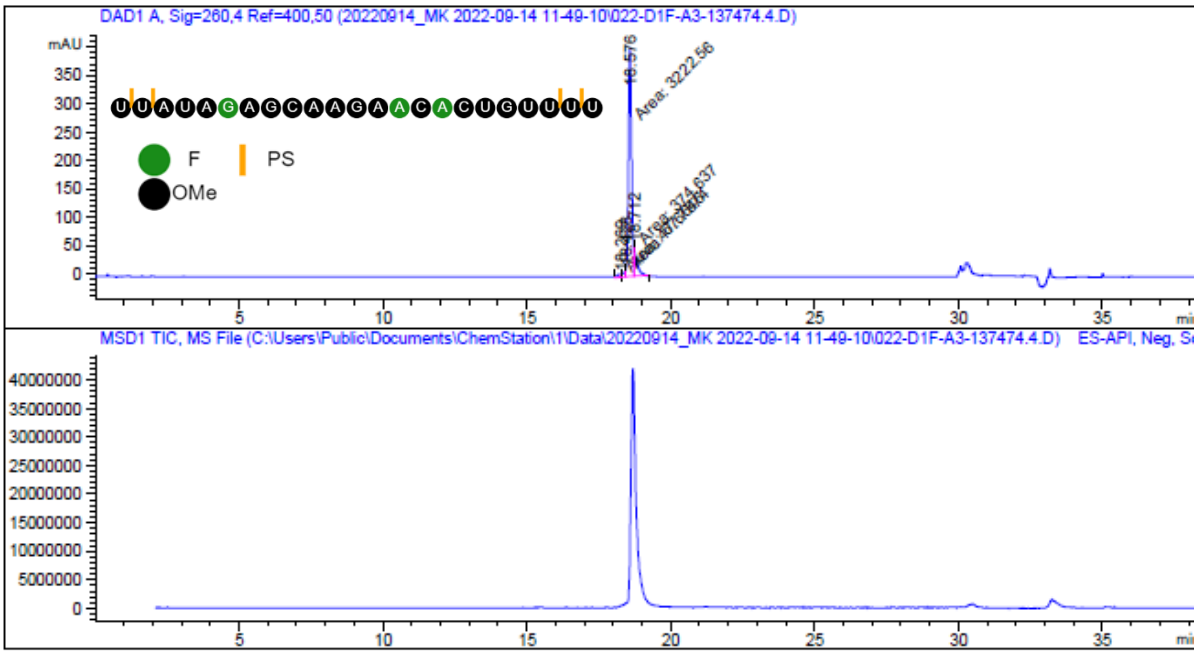
Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   5  19.280 FM    0.1212  398.11752   54.73122  10.3679
   6  19.781 FM    0.4539   47.48226    1.74365   1.2369

Totals :                  3832.52835  622.70360
```

Reverse-phase HPLC profile for **ON17**.

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Sorted By             :      Signal
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Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  18.269 MF    0.1659   47.66455    4.78804   1.2839
   2  18.428 MF    0.1056   67.75739   10.69532   1.8251
   3  18.576 MF    0.1328 3222.56177  404.33585  86.8002
   4  18.712 FM    0.1187  374.63724   52.60560  10.0909
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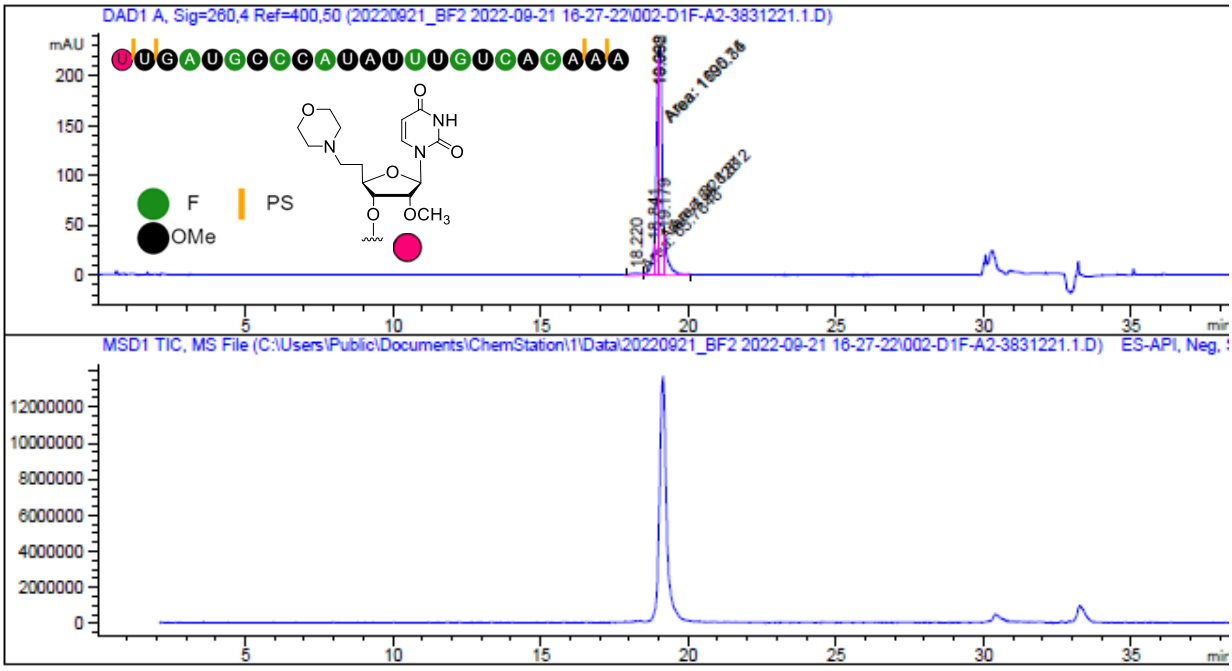
Reverse-phase HPLC profile for **ON18**.

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                        Area Percent Report
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Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  18.220 MF    0.4364   65.76465    2.51170   1.8981
   2  18.841 MF    0.0988  184.12778   31.06525   5.3142
   3  18.998 MF    0.0914 1195.73523  218.11739  34.5110
   4  19.032 FM    0.1228 1690.35413  229.37971  48.7866
   5  19.179 FM    0.1320  328.81210   41.50126   9.4901

Totals :                  3464.79388  522.57530
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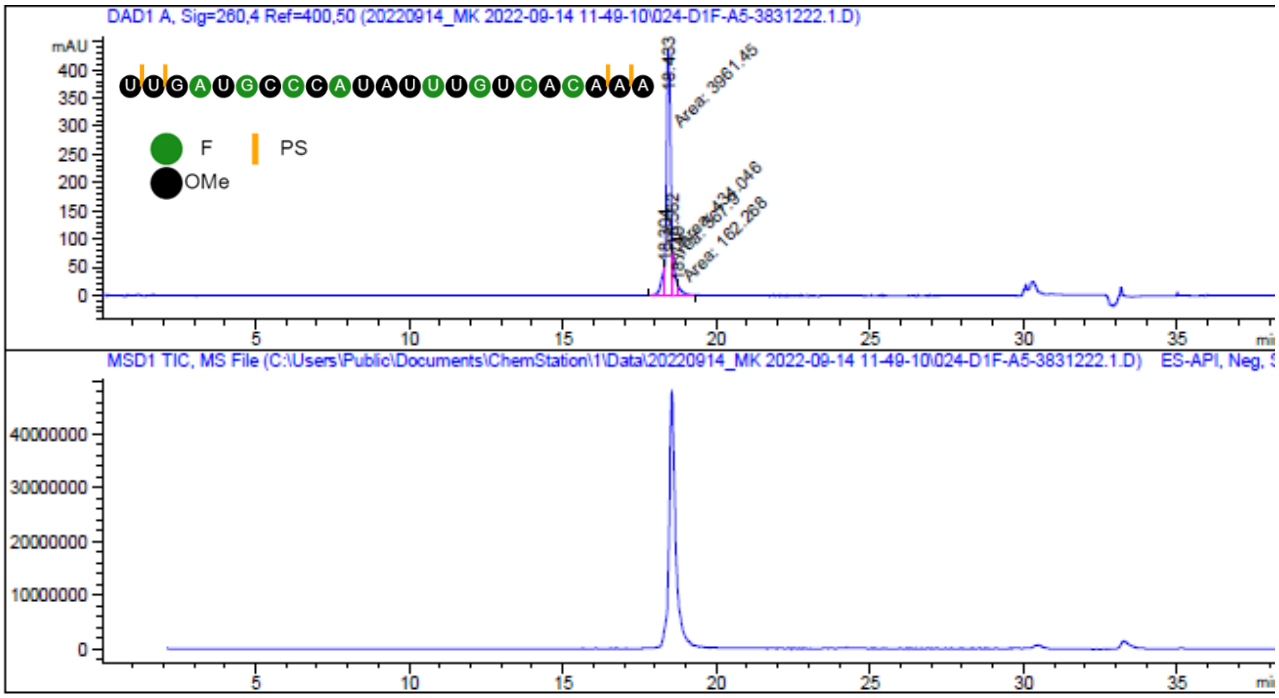
Reverse-phase HPLC profile for **ON19**.

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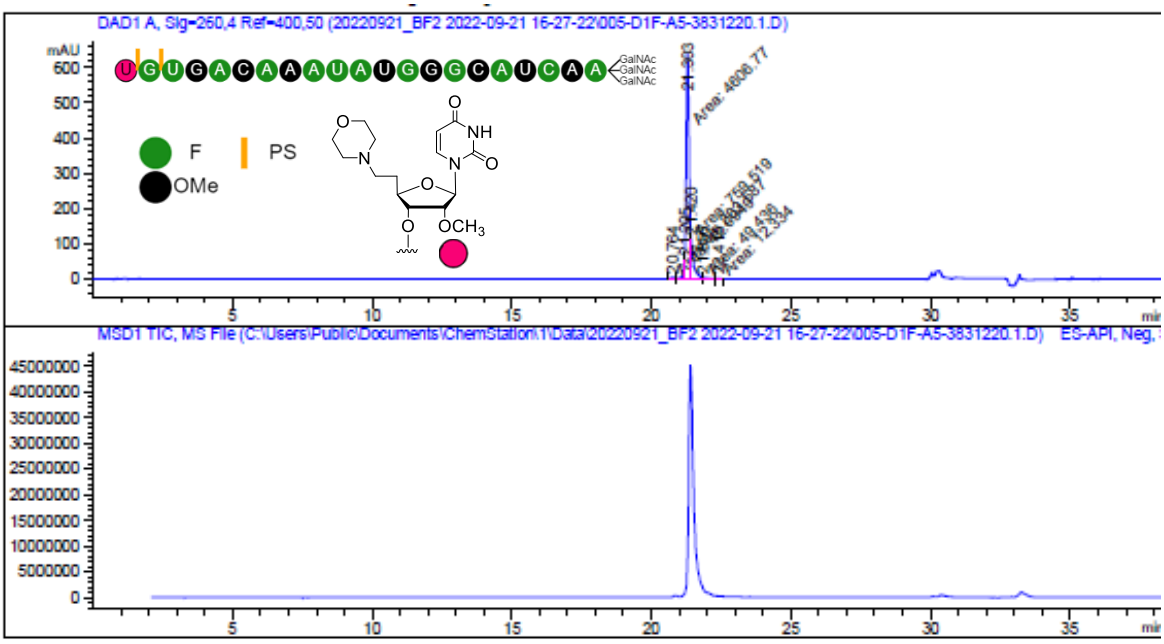
Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  18.304 MF    0.1203  367.90045   50.97963   7.4691
   2  18.433 MF    0.1514 3961.44849  436.09494  80.4247
   3  18.562 MF    0.0744  434.04633   80.59775   8.8119
   4  18.740 FM    0.1454  162.26782   18.60048   3.2943

290 Infinity2 LCMS 1 9/15/2022 1:15:12 PM SYSTEM                Page   1 of 2
```

Reverse-phase HPLC profile for **ON20**.

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Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=260,4 Ref=400,50

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  20.764 MF    0.1403   42.09488    4.99930   0.7419
   2  21.205 MF    0.0595  203.58710   57.04085   3.5882
   3  21.303 MF    0.1198 4606.76953  640.96368  81.1946
   4  21.420 FM    0.1095  759.51910  115.64349  13.3866

1290 Infinity2 LCMS 1 9/22/2022 2:31:46 PM SYSTEM                Page   1 of 2

Data File C:\Users\P...tion\1\Data\20220921_BF2 2022-09-21 16-27-22\005-D1F-A5-3831220.1.D
Sample Name: 3831220.1

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   5  21.856 FM    0.1994   49.43597    4.13282   0.8713
   6  22.415 FM    0.1486   12.33401    1.38323   0.2174
```

Reverse-phase HPLC profile for **ON21**.

Treatment of mice and analysis of *ApoB*

All studies were conducted using protocols consistent with local, state, and federal regulations, as applicable, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Alnylam Pharmaceuticals. Female C57BL/6 mice (Charles River Laboratories) of 6 -8 weeks old used. Mice were treated with test article via subcutaneous injection. There were three mice per group. siRNA was given at a dose of 3 mg/kg on day 0. Plasma samples were collected in EDTA collection tube at days 0 (pre-dose), 7, 14, and 21. Apo-B protein levels were determined using the Mouse ApoB SimpleStep ELISA® Kit (Abcam; catalog number ab230932) in accordance with the manufacturer's protocol, and data were normalized to pre-bleed ApoB levels in each individual mouse.

On- and off-target activity determination in luciferase reporter assay[7]

COS-7 cells were cultured at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells, in 96-well plates (15,000 cells/well), were co-transfected with 10 ng luciferase reporter plasmid and 0.64 pM to 50 nM siRNA in 5-fold dilutions using 2 µL Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were harvested at 48 hours after transfection for the dual luciferase assay (Promega) according to manufacturer's instructions. The on-target reporter plasmid contained a single site perfectly complementary to the antisense strand in the 3' UTR of *Renilla* luciferase. The off-target reporter plasmid contained four tandem seed-complementary sites separated by a 19-nucleotide spacer (TAATATTACATAAATAAAA) in the 3' UTR of *Renilla* luciferase. Both plasmids co-expressed firefly luciferase as a transfection control.

Table S3. siRNA activities in luciferase reporter assay

Duplex ID	Sense strand (upper) and antisense strand (lower)[a] (5′-3′)	IC_{50} (nM)	IC_{50} fold change relative to parent
Parent	a●a●caguGu*UCU*ugcucuauaa**L** u●*U*●auaGagcaagaAcAcuguu●u●u	0.0075	1.0
IX	a●a●caguGu*UCU*ugcucuauaa**L** Mo1●*U*●auaGagcaagaAcAcuguu●u●u	0.0961	13
X	a●a●caguGu*UCU*ugcucuauaa**L** Mo2●*U*●auaGagcaagaAcAcuguu●u●u	0.2216	30
XI	a●a●caguGu*UCU*ugcucuauaa**L** Pip●*U*●auaGagcaagaAcAcuguu●u●u	0.0102	1.4
XII	a●a●caguGu*UCU*ugcucuauaa**L** Mo3●*U*●auaGagcaagaAcAcuguu●u●u	0.0107	1.4
XIII	a●a●caguGu*UCU*ugcucuauaa**L** Mo2●u●auaGagcaagaAcAcuguu●u●u	NA	NA

[a] Chemical modifications are indicated as follows: ●, PS linkage; lower case, 2′-OMe; upper case, 2′-F; **L**, trivalent-GalNAc.

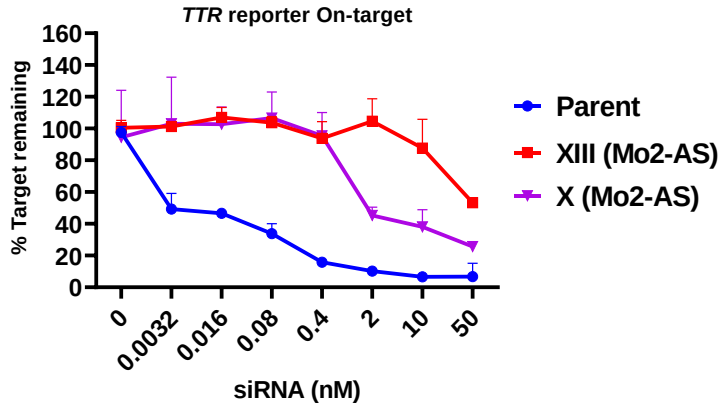


Figure S3: Gene silencing activity is inhibited by **Mo2** modification of the antisense strand. Percent luciferase expression in reporter assay as a function of siRNA concentration.

***In vitro ApoB* assay**

To evaluate modified siRNA silencing activity *in vitro*, oligonucleotides were transfected into primary mouse hepatocytes. Cells were seeded in collagen-coated 96-well plates (50,000 cells/well) and transfected with 0.64 pM to 10 nM siRNA in 5-fold dilutions using 0.3 µl/well Lipofectamine RNAiMax reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were harvested at 48 h after transfection, and RNA was extracted, and ApoB mRNA was quantified by RT-PCR.

Table S4: *In vitro* silencing activity of siRNAs targeting *ApoB*.

Duplex ID	Sense strand (upper) and antisense strand (lower)[a] (5′-3′)	IC_{50} (nM)	max repression (%)
Parent	u●g●*U*g*A*c*A*a*AU*A*uG*g*G*c*A*u*C*a*A**L** u●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	0.244	84.0
III	Mo2●g●*U*g*A*c*A*a*AU*A*uG*g*G*c*A*u*C*a*A**L** u●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	0.135	85.0
VI	u●g●*U*g*A*c*A*a*AU*A*uG*g*G*c*A*u*C*a*A**L** Mo2●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	-	0.2
XIV	Mo2●*G*●*U*g*A*c*A*a*AU*A*uG*g*G*c*A*u*C*a*A**L** u●*U*●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	0.023	88.0
XV	u●g●*U*g*A*c*A*a*AU*A*uG*g*G*c*A*u*C*a*A**L** Mo2●u●g*A*u*G*c*C*c*A*uau*U*u*G*u*C*a*C*a●a●a	-	3.7

[a] Chemical modifications are indicated as follows: ●, PS linkage; lower case, 2′-OMe; upper case, 2′-F; **L**, trivalent-GalNAc.

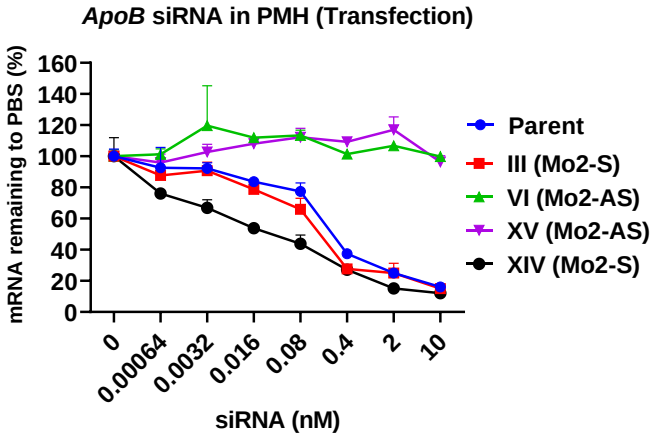
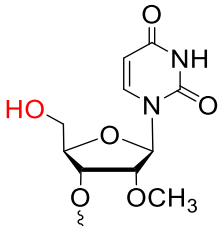
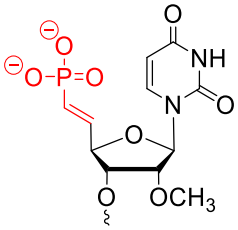
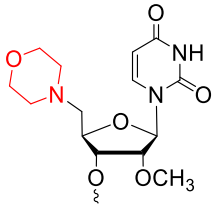
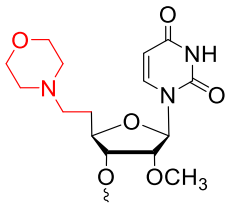


Figure S4: 5′-Mo2 coupled with 2′-fluoro at position 2 of the antisense strand inhibits activity at the highest concentration tested. Percent *ApoB* mRA remaining in cells transfected with indicated siRNA.

Table S5. Details regarding Oligonucleotides shown in Figure 5.

TTR AS strand (5'-3')[a]	5'-end modification	Remarks
ON11 u•*U*•auaGagcaagaAcAcuguu•u•u	-OH 	Poor loading. Inefficient phosphorylation due to chemical modification or de-phosphorylation by phosphatases
ON12 VPu•*U*•auaGagcaagaAcAcuguu•u•u	VP (Vinyl phosphonate) 	Efficient MID domain loading
ON13 Mo1•*U*•auaGagcaagaAcAcuguu•u•u	Mo1 	Inhibitor of loading
ON14 Mo2•*U*•auaGagcaagaAcAcuguu•u•u	Mo2 	Most efficient inhibitor of loading

[a]Chemical modifications are indicated as follows: •, PS linkage; lower case, 2′-OMe; and upper case, 2′-F; respectively.

***In vitro* Ago2 binding assay**

Anti-FLAG M2 antibody (2.5 µg) was incubated with 20 µl of Dynabeads® Protein G (Life Technologies) in PBS supplemented with 0.02% Tween-20. After washing in fresh buffer, 4 µg of N-terminal FLAG-tagged recombinant human Ago2 (Active Motif, catalog number 31486) was incubated for 10 min at room temperature with gentle rotation, and unbound protein was removed by washing in PBS. Beads were resuspended in Ago2 binding and wash buffer (150 mM NaCl, 20 mM Tris pH 8.0, 2 mM $MgCl_2$, 0.5 mM TCEP). Antisense strands of siRNAs were added to Ago2 protein immobilized on Dynabeads to a final concentration of 0.05 µg in 40 µl final volume and allowed to incubate for 1 h at 37° C. After washing beads three times in 1 ml PBS with 0.25% Triton X-100, RNA was quantified using stem-loop RT-qPCR as previously described.[8]

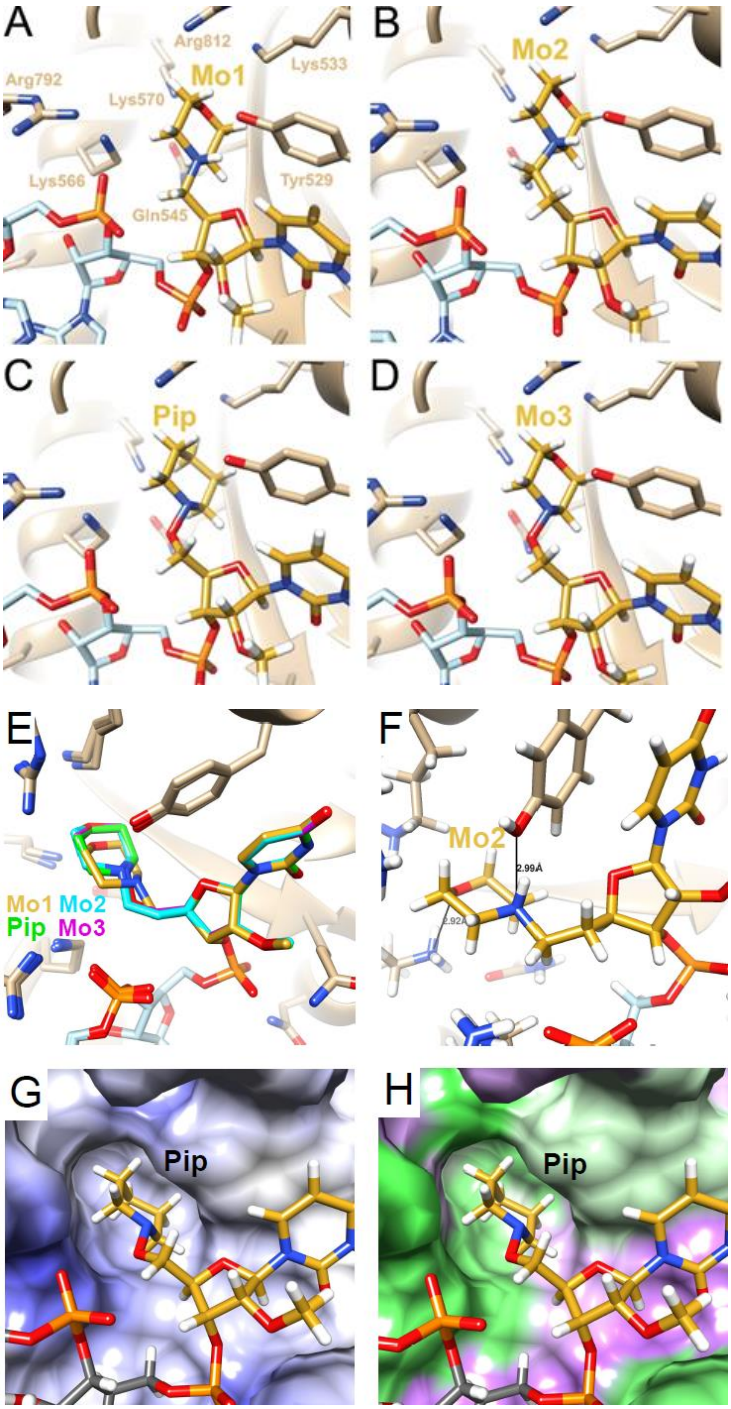


Figure S5. Morpholino analogues disrupt interaction of the 5′-phosphate with the MID domain of Ago2. Models of Ago2 bound to strands with (A) **Mo1**, (B) **Mo2**, (C) **Pip**, and (D) **Mo3**. (E) Overlay of the complexes shown in panels A-D. (F) Potential hydrogen-bond formation with **Mo2**. (G) Ago2 surface colored according to Coulombic potential (blue positive, white neutral). (H) Ago2 surface colored according to hydrophobicity (green lowest and pink highest).

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