Consecutive 5'- and 3'-amide linkages stabilise antisense oligonucleotides and elicit an efficient RNase H response

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List of Oligonucleotides

Table S1 List of oligonucleotides (ONs) used in this study with calculated and found mass spectrometry (MS) data.

ON code	Sequence (5'→3')	Calcd (Da)	Found (Da)
ON1	TTTTTTTTTTTTTT	3588	3589
ON2	TTTTTTTTTTTTTT	3472	3473
ON3	uuuTTTTTTTTuuu	3683	3685
ON4	uuuTTTTTTTTuuu	3645	3646
ON5	uuuTTTTTTTTuuu	3606	3607
ON6	TTTTTCCTGATAGTTTTTT	5140	5141
ON7	uuuuuCCTGATAGuuuuu	5610	5610
ON8	TTTTTCCTGATAGTTTTT	5451	5451
ON9	FL-TTTTTCCTGATAGTTTTT	5677	5678
ON10	FL-uuuuuCCTGATAGuuuuu	6147	6149
ON11	FL-TTTTTCCTGATAGTTTTT	5988	5989
FL-RNA1	FL-aaaaaaaaaaaaa	4425	4426
FL-RNA2	FL-aaaaacuaucaggaaaaa	6338	6339

Ⓝ DNA ⓝ 2'-OMe ⓝ RNA Phosphodiester (PO) Amide (AM) FL Fluorescein

Chromatography Traces of Crude and Purified ONs

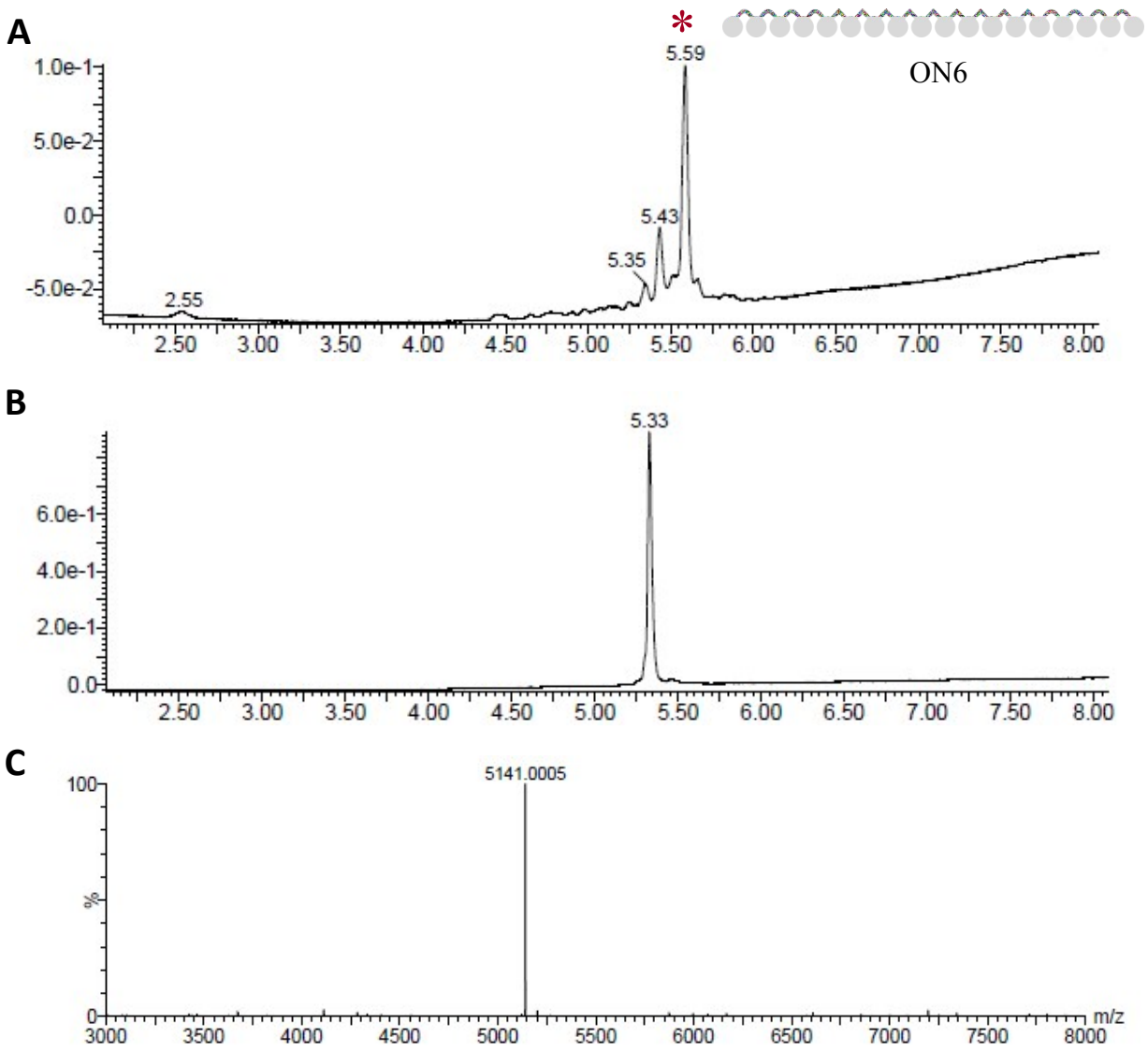


Fig. S1 (A) Reversed-phase ultra performance liquid chromatography (RP-UPLC) trace of crude ON6. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. *Peak corresponding to ON6. (B) RP-UPLC trace of purified ON6. (C) De-convoluted mass spectrum for ON6.

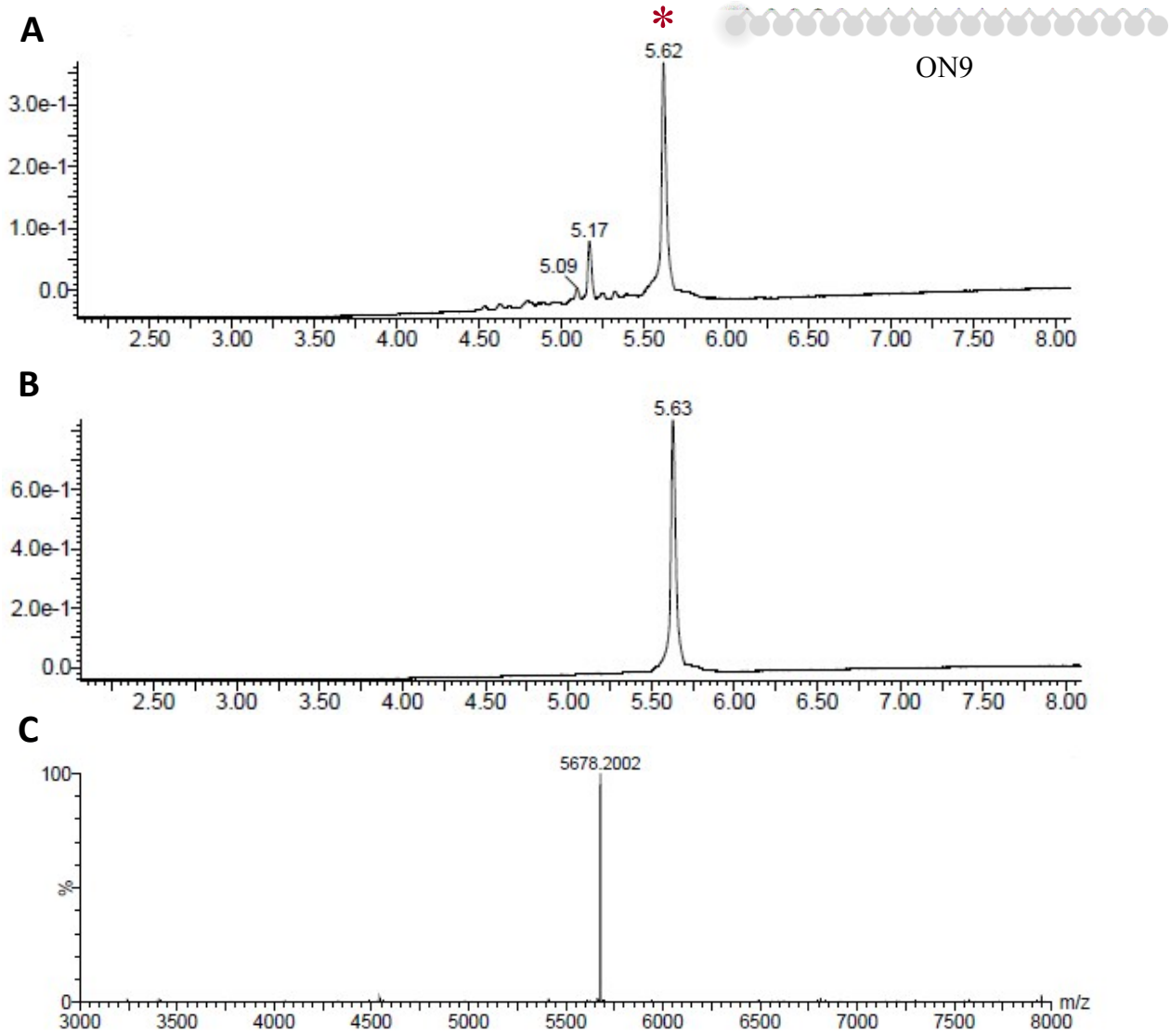


Fig. S2 (A) RP-UPLC trace of crude ON9. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. *Peak corresponding to ON9. (B) RP-UPLC trace of purified ON9. (C) De-convoluted mass spectrum for ON9.

Additional RNase H Digestion Gels

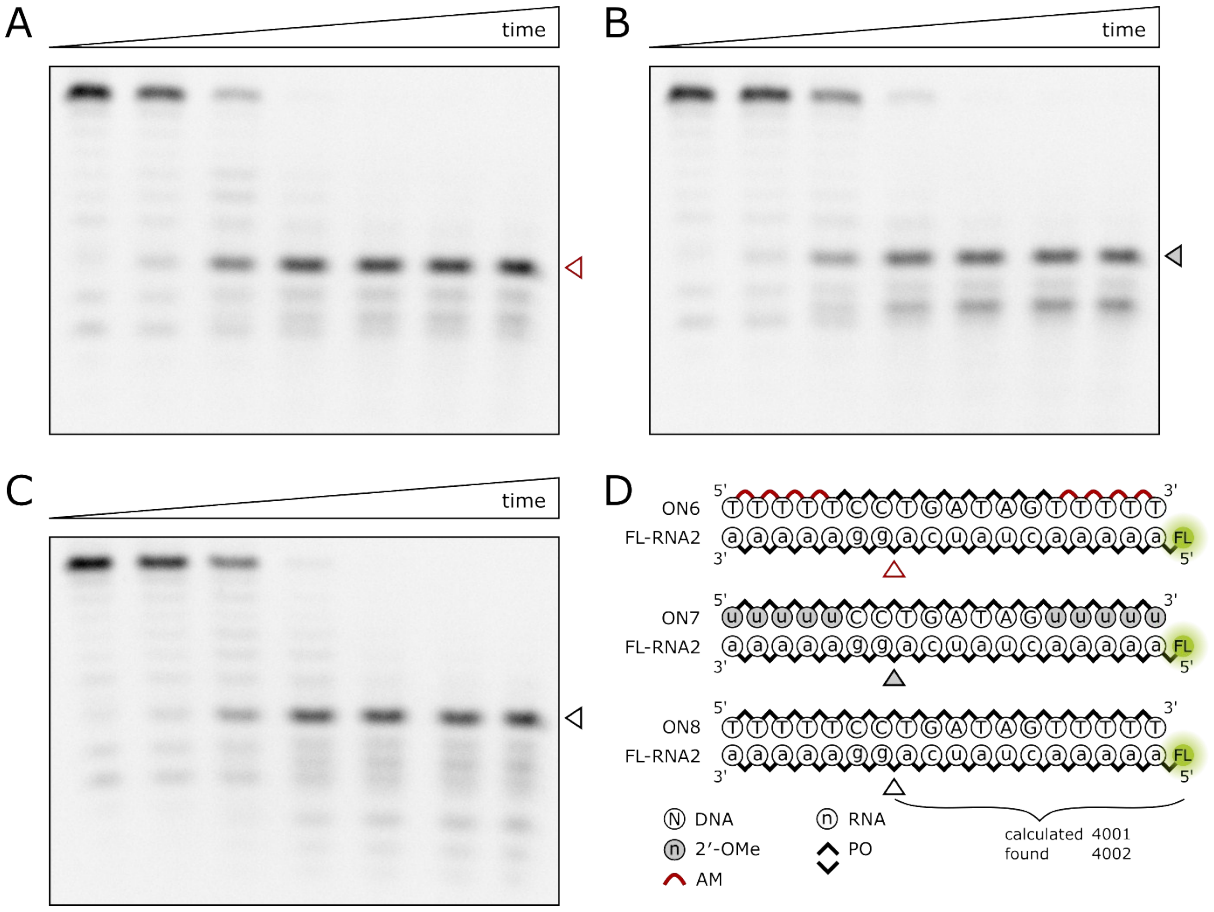


Fig. S3 (A–C) 20% Denaturing polyacrylamide gel electrophoresis (PAGE) results showing degradation of FL-RNA2 by RNase H after incubation with antisense oligonucleotides (ASOs) at 37 °C over time. ASOs are (A) ON6, (B) ON7 and (C) ON8. ASO/FL-RNA2, 1/4 (mole/mole). Time points are 0 min, 1 min, 5 min, 15 min, 30 min, 1 h and 2 h. Triangles indicate the bands that were extracted and analysed by RP-UPLC followed by MS. (D) Representation of ASO:FL-RNA2 heteroduplexes. Triangles show the major cutting sites determined from the gel extraction of the bands indicated in (A), (B) and (C).

Additional Melting Temperature Data

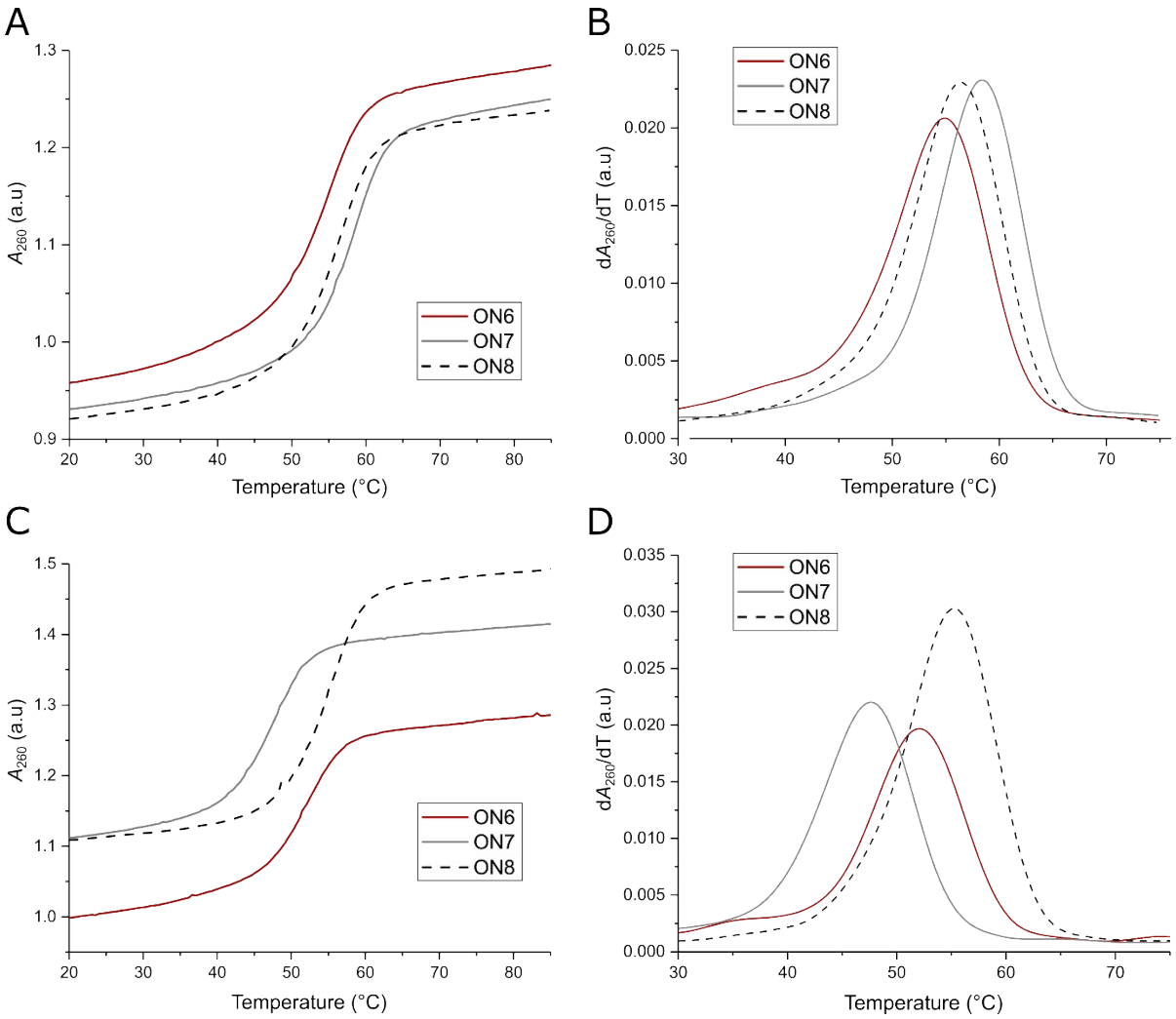


Fig. S4 (A) UV-melting curves of ON6–ON8 against a complementary RNA target containing 3 μM of ASO and 3 μM of the RNA target in 10 mM phosphate buffer, 200 mM NaCl, pH 7.0. Data points were taken as an average of six ramps from 20 to 85 °C. RNA target: 5'-AAAAACUAUCAGGAAAAA-3'. (B) First derivatives of (A). (C) UV-melting curves of ON6–ON8 against a complementary DNA target containing 3 μM of ASO and 3 μM of the DNA target in 10 mM phosphate buffer, 200 mM NaCl, pH 7.0. Data points were taken as an average of six ramps from 20 to 85 °C. DNA target: 5'-AAAAACTATCAGGAAAAA-3'. (D) First derivatives of (C).

Circular Dichroism Spectra

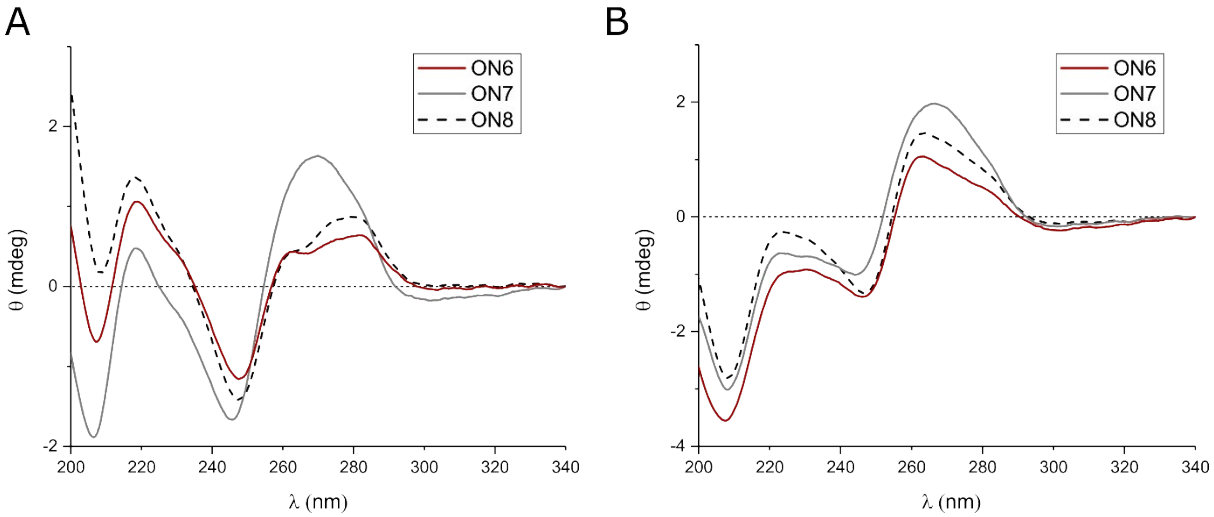


Fig. S5 (A) Circular dichroism (CD) spectra of ASOs ON6–ON8 in duplex with a DNA target containing 2 μM of ASO and 2 μM of the DNA target in 10 mM phosphate buffer, 200 mM NaCl, pH 7.0. Data points were taken as an average of four scans at 25 °C. DNA target: 5'-AAAAACTATCAGGAAAAA-3' (B) CD spectra of ASOs ON6–ON8 in duplex with an RNA target containing 2 μM of ASO and 2 μM of the RNA target in 10 mM phosphate buffer, 200 mM NaCl, pH 7.0. Data points were taken as an average of four scans at 25 °C. RNA target: 5'-AAAAACUAUCAGGAAAAA-3'.

Foetal Bovine Serum Digestion Assay

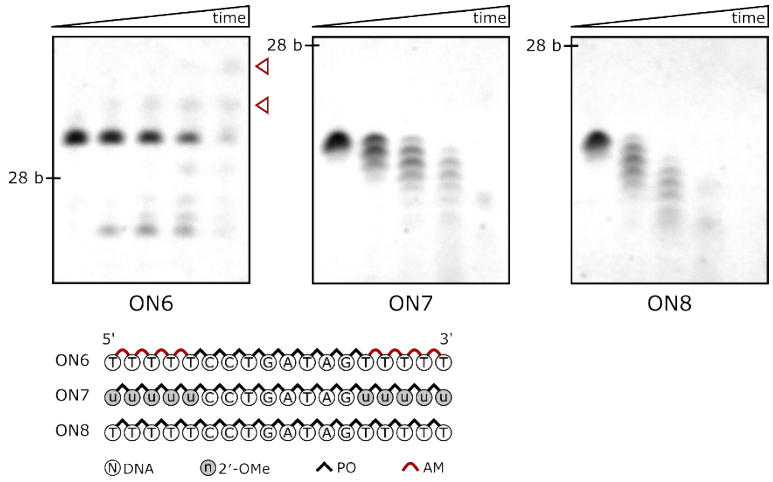


Fig. S6: 20% PAGE results from foetal bovine serum (FBS) assay of ASOs ON6, ON7 and ON8 visualised by UV. Samples were incubated with FBS in phosphate buffered saline (PBS) 1/1 (v/v) at 37 °C and aliquots were quenched over time. Time points are 0 min, 1 h, 3 h, 6 h and 12 h. Approximate sizes of 28 bases (28 b) were determined by migration of xylene cyanol. The apparent higher molecular weight of full-length ON8 (slower migration) was rationalised by the overall higher mass-to-charge ratio which is further increased in degraded fragments as indicated by the triangles.

Additional Microscopy Data

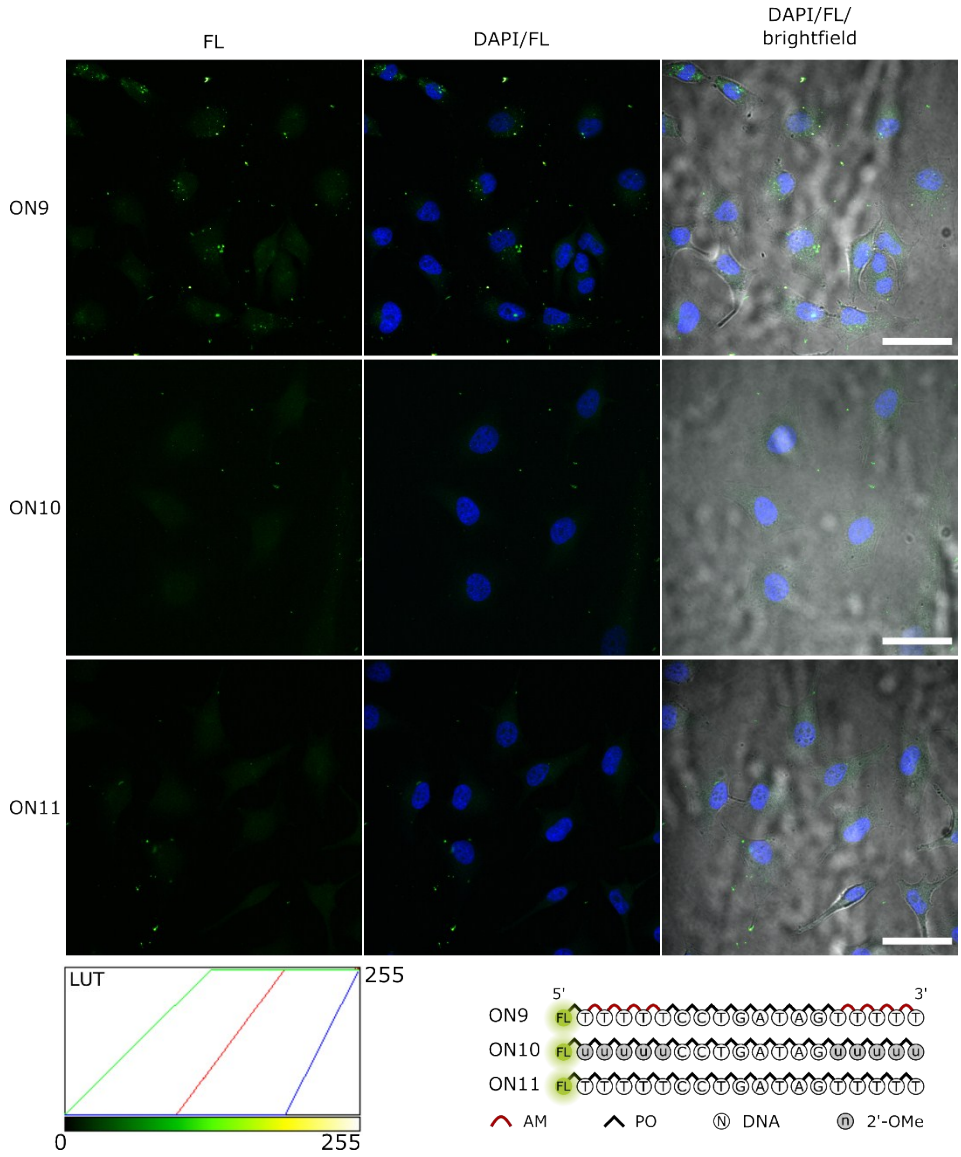


Fig. S7 Zoom-out of HeLa cells presented in Fig. 5. Cells were incubated with 5 µM ON9–ON11 in serum-free Opti-MEM™ for 16 h at 37 °C. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and cells were fixed before confocal laser scanning microscopy (CLSM) analysis. Scale bar: 50 µm. Look-up table (LUT) is displayed for the FL-channel.

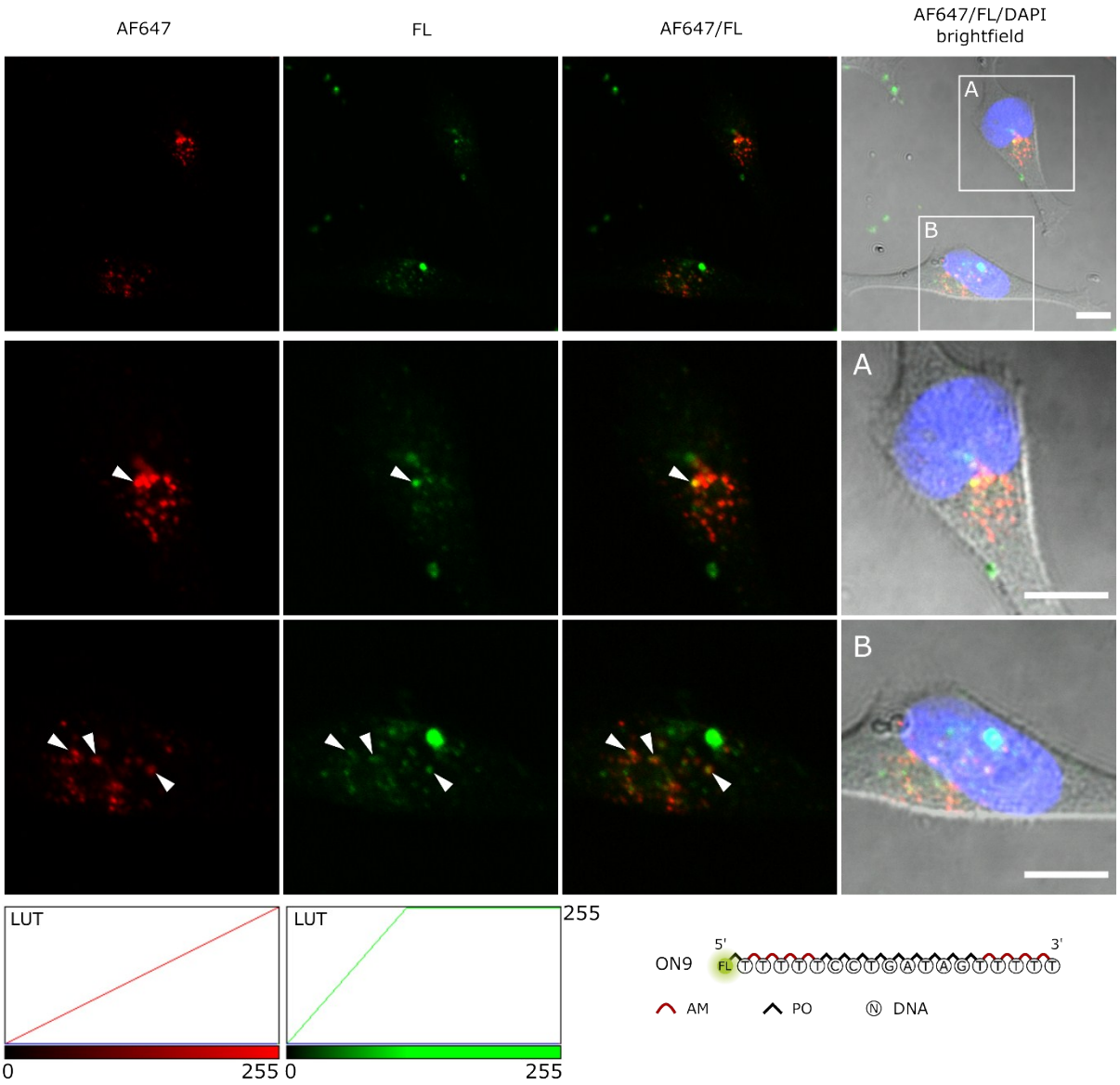


Fig. S8 HeLa cells were incubated with 5 µM ON9 in serum-free Opti-MEM™ for 16 h at 37 °C. Endosomes resulting from receptor-mediated endocytosis were stained by incubation with epidermal growth factor (EGF) complexed to Alexa Fluor 647 (AF647) for 30 min and nuclei were stained with DAPI. Cells were fixed before CLSM analysis. Arrows indicate co-localisation. Scale bar: 10 µm. LUTs are displayed for the AF647- and the FL-channel at the bottom of the corresponding panels.

Experimental

Cell Growth Conditions

HeLa cells (regularly tested for mycoplasma contamination) were cultured in Dulbecco's modified eagle media (DMEM) [(high glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered, no phenol red, with glutamine (*Thermo Fisher Scientific*, cat. No. 21063029)], supplemented with 10% (v/v) FBS (*Thermo Fisher Scientific*, cat. No. 10270098) in a humidified incubator at 37 °C with 5% CO_2.

Cell Preparation for Microscopy

8-Well chambered cover glass slides (Nunc™ Lab-Tek™ from *VWR Int.*, cat. No. 734-2062) were coated with a 0.01% poly-L-lysine solution (*Sigma Aldrich*, cat. No. P8920) for 1 h at 37 °C. The poly-L-lysine solution was removed, and wells were washed two times with PBS. Then, HeLa cells (passage between 11–35) were seeded (5000 cells/well) and allowed to attach in DMEM supplemented with 10% (v/v) FBS for 24 h at 37°C. The medium was removed, and the wells were washed two times with PBS. Fluorescently labelled ASOs (5 μM in serum-free, colourless Opti-MEM™) were added and the cells were incubated at 37 °C for 16 h. Optionally EGF-AF647 (*Thermo Fischer Scientific*, cat. E35351) was added at a final concentration of 2 $\mu g \cdot mL^{-1}$ 30 min before removal of the medium. The medium was removed, the wells were washed with PBS and the cells fixed with 4% paraformaldehyde in PBS for 20 min at rt. After removal of the fixation buffer, the wells were washed with PBS, disassembled and a coverslip was mounted using ProLong™ Diamond Antifade Mountant with DAPI (*Thermo Fisher Scientific*, cat. No. P36966).

Confocal Microscopy

Cell imaging was performed on an Olympus FluoView FV1200 confocal microscope with a 40× UPlan FLN 1.30 oil immersion inverted objective. The samples were excited using internal lasers and emission detected using the inbuilt filter systems. Different channels were imaged using sequential excitation and detection of emission. Images were then processed using ImageJ software. DAPI, FL and AF647 were excited at 405, 488 and 635 nm respectively and emission detected using the corresponding inbuilt filter set ups (DAPI, AF488 and AF647 respectively).

RNase H Assay

Escherichia coli RNase H was purchased from *New England Biolabs*. ASO (1.4 μM for ON1–ON5; 0.33 μM for ON6–ON8), FL-RNA1 or FL-RNA2 (1.3 μM) and *E. coli* RNase H (20 U/mL) were incubated in 1 × RNase H reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), pH 8.3) at 37 °C. At each time point, 15 μL aliquots were taken and quenched with ethylenediaminetetraacetic acid (EDTA) (5 μl, 160 mM) at a concentration of 40 mM, denatured with formamide 1/1 (v/v) and kept at –20 °C in the dark. The aliquots were submitted to a 20% PAGE (400 V) and analysed using a G:Box (*Syngene*) with an excitation wavelength of $\lambda = 460$ nm and an emission filter of $\lambda = 516–600$ nm for detection of the FL-label. Quantification of full-length FL-RNA2 from gel images was performed using ImageJ. Here, rectangular sections of the gel lanes were selected from 8-bit inverted grayscale images and plotted using the inbuilt ImageJ analysis tool. Peaks corresponding to full-length FL-RNA2 were enclosed using the straight-line tool and quantified using the wand (tracing) tool. All obtained values were normalised to t = 0 for the individual data set and plotted in Origin 2017.

Gel Extraction

After 20% PAGE as described above, the gel was visualised under UV and bands of interest were excised. The polyacrylamide bands were then crushed and incubated in a ThermoMixer (*Eppendorf*) at 37 °C, 950 rpm in RNA extraction buffer (50 mM Tris-HCl, 25 mM NaCl, pH 7.5, 7 mL) for 16 h. The polyacrylamide was filtered off and the filtrate concentrated under reduced pressure and desalted using Illustra NAP-10 and NAP-25 columns (*GE Healtcare*) consecutively. Extracted ONs were analysed by MS as described in the corresponding section.

UV Melting Studies

UV DNA melting curves were recorded on a Cary 4000 Scan UV-Visible Spectrophotometer using 3 μM of each ON in a 10 mM phosphate buffer containing 200 mM NaCl at pH 7.0. Samples were annealed by heating to 85 °C (10 °C/min) and then slowly cooling to 20 °C (1 °C/min). As these six successive cycles (heating and cooling) were performed at a gradient of 0.5 °C/min, the change in UV absorbance at 260 nm was recorded. The T_m was calculated from the average of the 1st derivatives of the melting curves using in built software.

Foetal Bovine Serum Assay

5 nmol of ON was dissolved in 50 μL of Dulbecco's PBS and 50 μL of FBS (Gibco, standard sterile filtered). This was then vortexed and 20 μL were removed (t = 0 min; control), mixed with formamide 1/1 (v/v) and kept at –20 °C. The remaining solution was incubated at 37 °C and 20 μL aliquots were removed at specified time points, mixed with formamide 1/1 (v/v) and kept at –20 °C. Samples were analysed by 20% PAGE (400 V) and viewed under short wave UV-light using a G:Box (*Syngene*).

Circular Dichroism Studies

CD was recorded for duplexes formed from 2 μM ASO and 2 μM of a complementary DNA or RNA target in 10 mM phosphate buffer with 200 mM NaCl (pH 7.0). CD was performed on a Chirascan Plus spectrometer using a quartz cuvette with a pathlength of 1 mm. Scans were taken at 25 °C from 200–340 nm with 0.5 a step and 1.0 s time point intervals. The average of four scans was taken and smoothed to 20 points using a third order polynomial (Savitzky-Golay, Origin 2017). The spectra were then baseline corrected to the θ-value at 340 nm.

Synthesis and Purification of Oligonucleotides

Synthesis of DNA Oligonucleotides

Standard DNA phosphoramidites, solid supports and reagents were purchased from *Link Technologies* and *Applied Biosystems*. 5'-Fluorescein-CE phosphoramidite (6-FAM) was purchased from *Link Technologies* (cat. no 2134) and 5'-amino-dT-CE phosphoramidite **8** was purchased from *Glen Research* (cat. no 10-1932-02). Automated solid phase synthesis of ONs (trityl off) was performed on an Applied Biosystems 394 synthesiser. Synthesis was performed on a 1.0 μmol scale involving cycles of acid-catalysed detritylation, coupling, capping, and iodine oxidation. Standard DNA phosphoramidites were coupled for 60 s and chemically modified phosphoramidites for 10 min. Coupling efficiencies and overall synthesis yields were determined by the inbuilt automated trityl cation conductivity monitoring facility. The ONs were then cleaved from the solid support and protecting groups from the nucleobase and backbone were removed by exposure to concd aq NH_3 for 60 min at room temperature followed by heating in a sealed tube at 55 °C for 5 h.

Synthesis of RNA Oligonucleotides

2′-*tert*-Butyldimethylsilyl (2′-TBS) protected RNA phosphoramidite monomers with *tert*-butylphenoxyacetyl protection of the A, G and C nucleobases were used to assemble RNA ONs. Benzylthiotetrazole (BTT) was used as the coupling agent, *tert*-butylphenoxyacetic anhydride as the capping agent and 0.1 M iodine as the oxidizing agent (*Sigma-Aldrich*). Coupling time of 10 min was used and coupling efficiencies of >97% were obtained. Cleavage of ONs from the solid support and protecting groups from the nucleobase and backbone were removed by exposure to concd aq NH_3/EtOH [3/1 (v/v), 4 mL] for 2 h at room temperature followed by heating in a sealed tube for 2 h at 55°C.

Removal of 2′-TBS Protection of RNA Oligonucleotides

After cleavage from the solid support and removal of the protecting groups from the nucleobases and phosphodiesters in concd aq NH_3/EtOH [3/1 (v/v), 4 mL] as described above, ONs were concentrated to a small volume under reduced pressure, transferred to 15 mL plastic tubes and freeze dried (lyophilised). The residue was dissolved in dimethylsulfoxide (DMSO) (300 μL) and $Et_3N{\cdot}3HF$ (300 μL) was added after which the reaction mixtures were kept at 65 °C for 2.5 h. NaOAc (3 M, 50 μL) and *n*-BuOH (3 mL) were added with vortexing and the samples were kept at −80 °C for 30 min then centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was decanted and the precipitate was washed twice with EtOH (0.75 mL) then dried *in vacuo*.

Synthesis of Phosphodiester–Amide Backbone Chimeras

A schematic overview of the method is given in Fig. S9. Chimeras with a terminal amide bond at the 3'-end of the oligomer were synthesised on functionalised resin **S2** (42 mg, loading 23.6 μmol·g^{-1}, 1.0 μmol). Detritylation was performed on an Applied Biosystems 394 synthesiser as for standard DNA synthesis for 80 s. For amide couplings, the synthesis column was removed from the synthesiser and equipped with a 1 mL plastic syringe on each side. In a separate vial, 4-methylmorpholine (3.5 μL, 32 μmol, 32 equiv), (benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate (PyBOP) (5.2 mg, 10 μmol, 10 equiv) and monomer **7**[1] (6 mg, 10 μmol, 10 equiv) were mixed in anhydrous (anhyd) *N*,*N*-dimethylformamide (DMF) (400 μL) and incubated at rt for 5 min. The solution was added to the column containing the growing ON with the 5'-NH_2 via one of the syringes and the solution was pushed back and forth three times through the column using the two attached syringes. The reaction was left at rt for 2 h agitating the reaction mixture using the syringes approximately every 20 min. The solvents were then removed and the resin was washed with anhyd MeCN (3 × 1 mL) using the two syringes as described before. The column was transferred back to the synthesiser for detritylation and then washed with MeCN. This allows for repetitive amide couplings as described above. To switch from amide couplings to standard ON synthesis, the 5'-deprotected oligomer on the solid support was incubated with monomer **3** as described above for amide couplings and transferred back to the synthesiser for detritylation. Standard ON synthesis was performed as described in the section for synthesis of DNA ONs. To switch from standard ON synthesis to amide couplings, commercially available phosphoramidite **8** was coupled with an extended coupling time of 10 min to introduce a 5'-NH_2 functionality (without the capping step) and amide couplings were performed as outlined above. The final amide coupling was performed using monomer **3** to introduce a 5'-OH after standard detritylation. After synthesis, the resin was divided (2 × 25 mg) and one half was cleaved and deprotected as described above for synthesis of DNA ONs to give ON6. The other half was transferred back to the synthesiser a coupled with 6-FAM for an extended coupling

time of 10 min introducing the 5'-terminal FL in ON9. Cleavage from the resin and deprotection was carried out as for standard ONs to obtain ON9. Yields were determined after reversed phase high performance liquid chromatography (RP-HPLC) as described in the corresponding section.

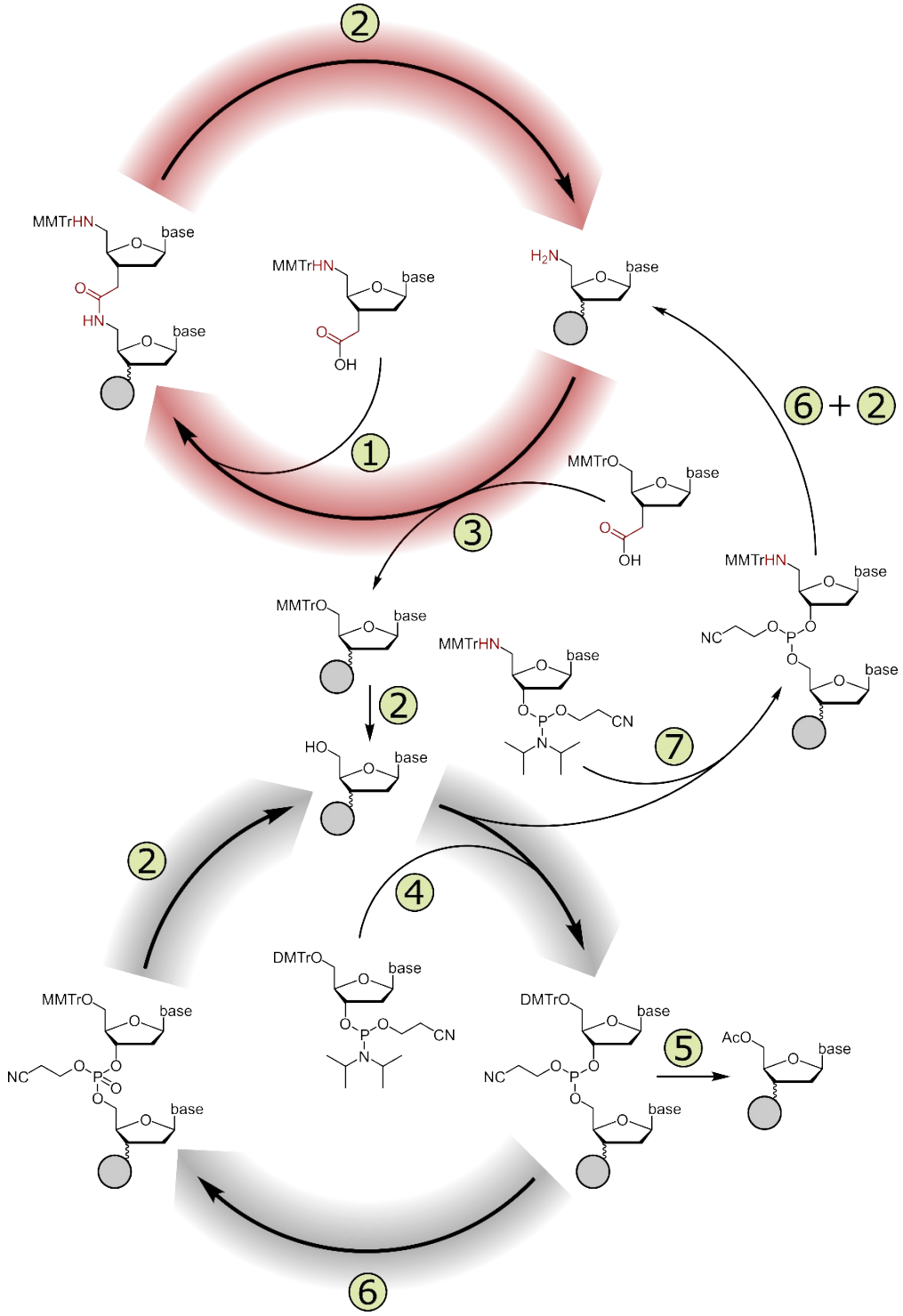


Fig. S9 Chimera synthesis can switch between amide couplings (red cycle) and standard ON synthesis (grey cycle). (1) amide coupling, (2) detritylation, (3) amide coupling to introduce a 5'-OH functionality to switch from amide couplings to standard ON synthesis, (4) coupling of phosphoramidites, (5) capping, (6) oxidation, (7) coupling of phosphoramidite to introduce a 5'-NH_2 functionality to switch from standard ON synthesis to amide couplings. MMTr = 4-methoxytriphenylmethyl, DMTr = 4,4-dimethoxytriphenylmethyl. Modified procedure from Altmann *et al.*,[1] Silverman *et al.*[2] and Rozners *et al.*[3]

Purification of Oligonucleotides (DNA or RNA)

The fully deprotected ONs were purified by RP-HPLC on a Gilson system using a Luna 10 μm C8(2) 100 Å pore Phenomenex column (250 × 10 mm) with a gradient of MeCN in aq triethylammonium bicarbonate (TEAB) (buffer A, 0.1 M TEAB, pH 7.5; buffer B, 50% buffer A in MeCN). Buffer B was increased (ON2, ON4 and ON5, 20–50%; ON6, 30–50%; ON9, 25–50%) over 20 min at a flow rate of 4 mL·min^{-1} and elution was monitored by UV absorption between 260–295 nm.

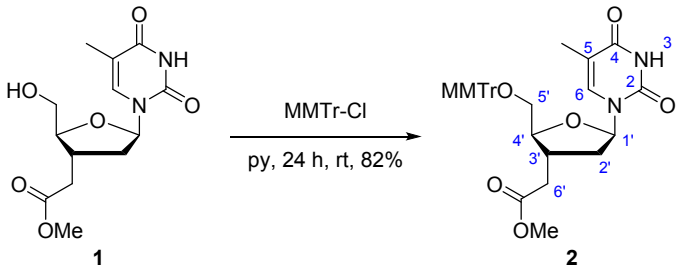
Mass Spectrometry

ON MS was recorded on a UPLC-MS Waters XEVO G2-QTOF (ESI$^-$) using an ACQUITY UPLC ON BEH C18 column, 130 Å (1.7 μm, 2.1 mm × 50 mm). Data was then de-convoluted using MassLynx v4.1. A gradient of CH_3OH in Et_3N and hexafluoroisopropanol (HFIP) was used (buffer A, 8.60 mM Et_3N, 200 mM HFIP in 5% CH_3OH/H_2O (v/v); buffer B, 20% buffer A in CH_3OH). Buffer B was increased from 0–70% over 8 min, at a flow rate of 0.2 mL·min^{-1}.

General Synthetic Methods

All reagents were purchased from *Sigma-Aldrich*, *Acros Organics*, *Fluorochem*, *Carbosynth* and *Fisher Scientific* and used without further purification. Anhyd solvents [pyridine (py), DMF, Et_3N, CH_2Cl_2, tetrahyudrofuran (THF), MeCN] were obtained using an MBraun SPS Bench Top solvent purification system (SPS). All air/moisture sensitive reactions were carried out under inert atmosphere (argon) in oven-dried glassware. Solvents used for phosphitylation reactions and purification were also degassed by bubbling the solvents with argon for 20 min. Reactions were monitored by thin layer chromatography (TLC) using Merck Kieselgel 60 F24 silica gel plates (0.22 mm thickness, aluminium backed). The compounds were visualised by UV irradiation at 254/265 nm and by staining in *p*-anisaldehyde solution followed by gentle heating. Column chromatography was carried out under pressure (Biotage® SP4) using Biotage® SNAP KP-Sil columns. ^{1}H NMR (400 MHz), ^{13}C NMR (101 MHz or 126 MHz) and ^{31}P NMR (162 MHz) spectra were measured on a Bruker AVIIIHD 400 or a Bruker AVII 500 spectrometer. ^{1}H and ^{13}C NMR spectra were referenced to the appropriate deuterated solvent signal and chemical shifts are given in ppm and all coupling constants (*J*) are quoted in Hertz (Hz). Assignment of the compounds was aided by COSY (^{1}H - ^{1}H) and HSQC (^{1}H - ^{13}C) experiments when feasible. IR spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer and selected characteristic are peaks reported in cm^{-1}. High-resolution mass spectra (HRMS) were recorded in HPLC grade CH_3OH or MeCN using electrospray ionisation (ESI$^+$) on a Bruker APEX III FT-ICR mass spectrometer.

3'-Carbmethoxymethyl-3'-deoxy-5'-(monomethoxytrityl)thymidine (**2**)

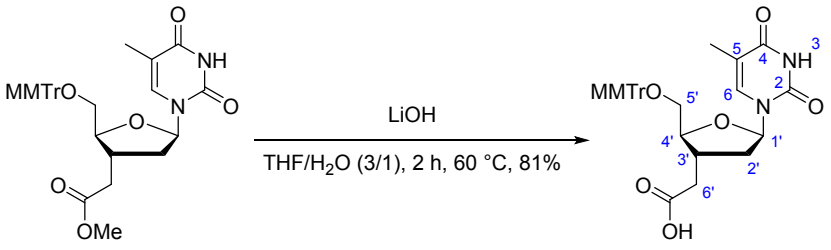


1 **2**

$C_{33}H_{34}N_2O_7$
570.64

MMTr-Cl (2.45 g, 7.94 mmol) was added to a solution of compound **1**[1] (1.50 g, 5.03 mmol) in anhyd py (50 mL) and the reaction was stirred at rt for 24 h. The solvent was removed *in vacuo* and the residue taken up in EtOAc (100 mL) and washed with sat. aq $NaHCO_3$ (3 × 100 mL). The separated organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was purified by column chromatography (petroleum ether (40–60)/EtOAc, 0–100%) to give compound **2** as a white solid (2.35 g, 4.12 mmol, 82%). R_f 0.65 (EtOAc); IR (cm^{-1}): 2950, 1734, 1684 (C=O), 1509, 1446, 1251, 1178; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H, NH), 7.57 (s, 1H, H6), 7.39 (d, *J* = 8.1 Hz, 4H, H-Ar), 7.33 – 7.15 (m, 9H, 8 × H-Ar *and* solvent), 6.80 (d, *J* = 9.0 Hz, 2H, H-Ar), 6.10 (dd, *J* = 6.9, 3.8 Hz, 1H, H1'), 3.84 – 3.71 (m, 4H, OCH_3 *and* H4'), 3.58 (s, 3H, CO_2CH_3), 3.48 (dd, *J* = 10.9, 2.7 Hz, 1H, H5'), 3.24 (dd, *J* = 10.9, 3.7 Hz, 1H, H5''), 2.83 – 2.70 (m, 1H, H3'), 2.46 – 2.29 (m, 2H, H2' *or* H6'), 2.28 – 2.05 (m, 2H, H2' *or* H6'), 1.47 (s, 3H, CH_3-*thymidine*); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.9 ($\underline{C}O_2CH_3$), 163.9 (C4), 158.9 (C-Ar), 150.4 (C2), 144.0 (C-Ar), 135.7 (C6), 135.1 (C-Ar), 130.5 (CH-Ar), 128.5 (CH-Ar), 128.1 (CH-Ar), 127.4 (CH-Ar), 113.4 (CH-Ar), 110.8 (C5), 87.1 (C_{quat}-*MMTr*), 85.0 (C1'), 84.5 (C4'), 63.1 (C5'), 55.4 (OCH_3-*MMTr*), 52.0 ($CO_2\underline{C}H_3$), 39.2 (C2' *or* C6'), 36.4 (C2' *or* C6'), 34.8 (C3'), 12.2 (CH_3-*thymidine*); HRMS–ESI (*m/z*): $[M + Na]^+$ calcd for $C_{33}H_{34}N_2O_7Na$, 593.2258; found, 593.2255.

3'-Carboxymethyl-3'-deoxy-5'-(monomethoxytrityl)thymidine (**3**)

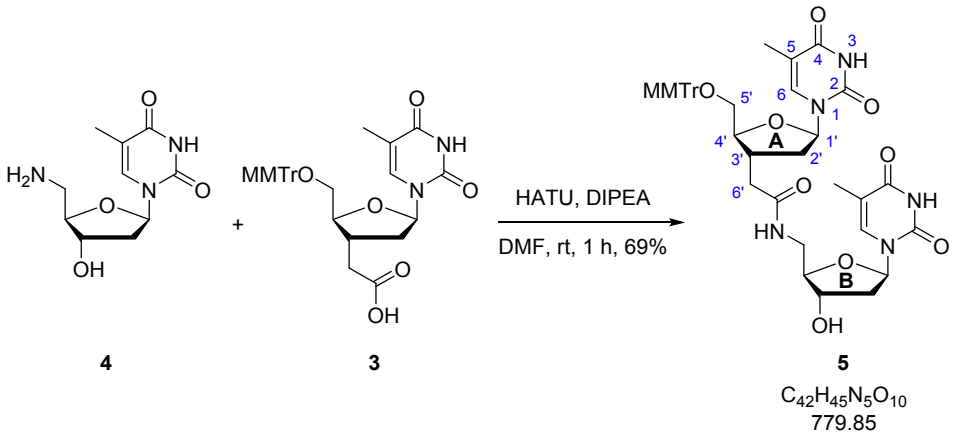


2 **3**

$C_{32}H_{32}N_2O_7$
556.62

LiOH (2.00 g, 83.3 mmol) was added to a solution of ester **2** (1.84 g, 3.22 mmol) in THF/H_2O [3/1 (v/v), 11 mL] and the reaction was stirred at 60 °C for 2 h. The reaction mixture was diluted with H_2O (50 mL) and carefully acidified with 1 M HCl to pH 4.5. The resulting white precipitate was dissolved by addition of EtOAc (100 mL) and the layers were separated. The organic layer was washed with H_2O (3 × 100 mL) and the separated organic layer was dried over Na_2SO_4, filtered and evaporated *in vacuo* to give acid **3** as a white solid (1.45 g, 2.60 mmol, 81%) which was used without further purification. R_f 0.28 (EtOAc); IR (cm^{-1}): 2981 (O-H), 2361, 2340, 1687 (C=O), 1509, 1447, 1251, 1179, 1117, 1033; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.68 (s, 1H, NH), 7.64 (d, *J* = 1.3 Hz, 1H, H6), 7.43 – 7.34 (m, 4H, H-Ar), 7.31 – 7.12 (m, 9H, 8 × H-Ar *and* solvent), 6.86 – 6.72 (m, 2H, H-Ar), 6.06 (dd, *J* = 7.0, 3.2 Hz, 1H, H1'), 3.83 – 3.76 (m, 1H, H4'), 3.74 (s, 3H, OCH_3), 3.50 (dd, *J* = 11.0, 2.6 Hz, 1H, H5'), 3.25 (dd, *J* = 11.0, 3.6 Hz, 1H, H5''), 2.82 – 2.67 (m, 1H, H3'), 2.52 – 2.30 (m, 2H, H2' *and* H6'), 2.26 – 2.08 (m, 2H, H2'' *and* H6''), 1.48 (d, *J* = 1.3 Hz, 3H, CH_3-*thymidine*); ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.9 (CO_2H), 164.9 (C4), 158.9 (C-Ar), 150.6 (C2), 144.0 (C-Ar), 136.2 (C6), 135.0 (C-Ar), 130.5 (CH-Ar), 128.5 (CH-Ar), 128.2 (CH-Ar), 127.4 (CH-Ar), 113.4 (CH-Ar), 110.6 (C5), 87.1 (C_{quat}-*MMTr*), 85.4 (C1'), 84.6 (C4'), 62.8 (C5'), 55.4 (OCH_3-*MMT*), 39.4 (C2'), 36.1 (C6'), 34.4 (C3'), 12.2 (CH_3-*thymidine*); HRMS–ESI (*m/z*): $[M + Na]^+$ calcd for $C_{32}H_{32}N_2O_7Na$, 579.2102; found, 579.2101.

5'-*O*-(Monomethoxytrityl)-DNA-thymidine-amide-DNA-thymidine dimer (**5**)



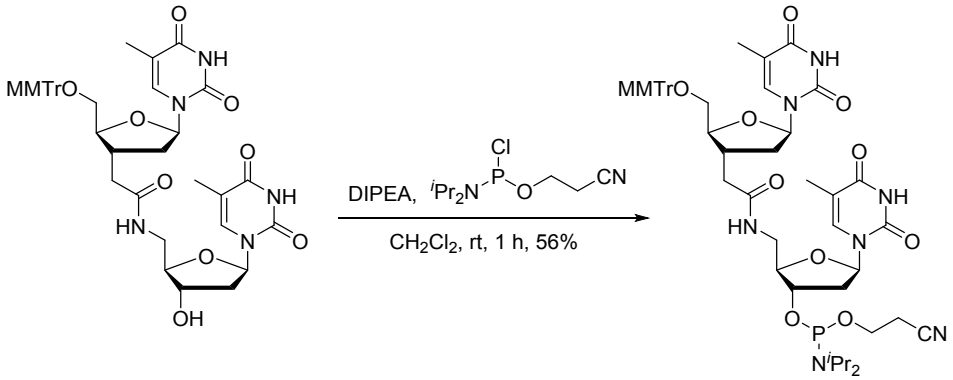
4 **3** **5**

$C_{42}H_{45}N_5O_{10}$

779.85

Carboxylic acid **3** (389 mg, 0.72 mmol) was dissolved in anhyd DMF (5 mL) and stirred at rt for 5 min in the presence of activated 4 Å molecular sieves (200 mg). HATU (271 mg, 0.72 mmol) and anhyd DIPEA (250 µL, 1.44 mmol, pre-dried over activated 4 Å molecular sieves) were added and the solution was stirred at rt for 20 min. A suspension of amine **4**[4] (189 mg, 0.78 mmol, pre-incubated for 10 min with 100 mg of activated 4 Å molecular sieves in 4 mL anhyd DMF) was added and the reaction mixture was stirred at rt for 1 h. The resulting yellow suspension was evaporated *in vacuo* and re-suspended in CH_2Cl_2/py [99/1 (v/v), 75 mL] and filtered through Celite. The filtrate was washed with sat. aq $NaHCO_3$ (50 mL) and the aq layer was re-extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was submitted to column chromatography (CH_2Cl_2/CH_3OH, 0–10% + 0.5% Et_3N). The resulting white solid was re-dissolved in EtOAc (70 mL) and washed with sat. aq $NaHCO_3$ (50 mL) and brine (50 mL) to remove remaining Et_3N. The organic phase was dried over Na_2SO_4, filtered and evaporated to give dimer **5** (387 mg, 0.50 mmol, 69%) as a white solid. R_f 0.25 (CH_2Cl_2/CH_3OH, 9/1); IR (cm^{-1}): 3692, 3026, 2923 (O-H), 2840, 1684 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.28 (bs, 2H, H3A and H3B), 8.09 (t, *J* = 5.8 Hz, 1H, NH-*amide*), 7.49 (d, *J* = 1.3 Hz, 1H, H6A *or* H6B), 7.47 (d, *J* = 1.3 Hz, 1H, H6A *or* H6B), 7.43 – 7.35 (m, 4H, H-Ar), 7.35 – 7.28 (m, 4H, H-Ar), 7.28 – 7.20 (m, 4H, H-Ar), 6.92 – 6.85 (m, 2H, H-Ar), 6.11 (dd, *J* = 7.7, 6.3 Hz, 1H, H1'B), 6.02 (dd, *J* = 7.0, 4.2 Hz, 1H, H1'A), 5.29 (d, *J* = 4.2 Hz, 1H, OH), 4.15 – 4.06 (m, 1H, H3'B), 3.82 (dt, *J* = 7.7, 3.6 Hz, 1H, H4'A), 3.77 – 3.67 (m, 4H, OCH_3 *and* H4'B), 3.29 – 3.14 (m, 4H, H5'A, H5"A, H5'B *and* H5"B), 2.77 – 2.62 (m, 1H, H3'A), 2.26 – 2.13 (m, 3H, H6', H6" *and* H2'A), 2.13 – 1.99 (m, 3H, H2"A, H2'B *and* H2"B), 1.77 (d, *J* = 1.3 Hz, 3H, CH_3), 1.45 (d, *J* = 1.3 Hz, 3H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ = 170.6 (C(O)-*amide*), 163.7 (C4A *or* C4B), 163.7 (C4A *or* C4B), 158.3 ($\underline{C}OCH_3$), 150.4 (C2A *or* C2B), 150.4 (C2A *or* C2B), 144.2 (C-Ar), 144.1 (C-Ar), 136.1 (C6A *or* C6B), 135.6 (C6A *or* C6B), 134.8 (C-Ar), 130.1 (CH-Ar), 128.0 (CH-Ar), 127.9 (CH-Ar), 127.0 (CH-Ar), 113.3 (CH-Ar *o* to OCH_3), 109.7 (C5A *or* C5B), 109.2 (C5A *or* C5B), 86.0 (C_{quat}-*MMTr*), 84.9 (C4'B), 83.9 (C1'A *and* C1'B), 83.4 (C4'A), 71.3 (C3'B), 63.7 (C5'A), 55.1 (OCH_3), 41.0 (C5'B), 38.5 (C2'B), 37.7 (C6'), 37.4 (C2'A), 35.2 (C3'A), 12.0 (CH_3), 11.9 (CH_3); *m/z* (ESI$^+$): 273.145 ([MMTr]$^+$, 100%), 802.326 ([M + Na]$^+$, 83.3%), 1581.633 ([2M + Na]$^+$, 27.1%); HRMS–ESI (*m/z*): [M + Na]$^+$ calcd for $C_{42}H_{45}N_5O_{10}Na$, 802.3059; found, 802.3062.

5'-*O*-(Monomethoxytrityl)-DNA-thymidine-amide-DNA-thymidine phosphoramidite dimer (**6**)

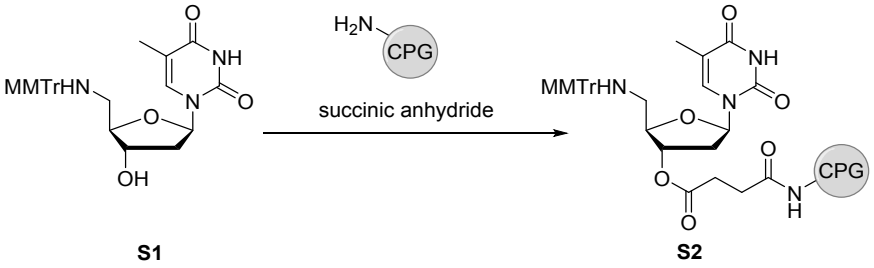


5 **6**

$C_{51}H_{62}N_7O_{11}P$
980.07

Degassed DIPEA (200 µL, 1.15 mmol) and chloro(diisopropylamino)-β-cyanoethoxyphosphine (123 µL, 0.55 mmol) were added to a solution of dimer **5** (359 mg, 0.46 mmol) in degassed, anhyd CH_2Cl_2 (4 mL) and the reaction mixture was stirred at rt for 1 h. The solution was diluted with degassed CH_2Cl_2 (3 mL) and then washed with sat. aq KCl (10 mL). The aq layer was extracted with degassed CH_2Cl_2 (2 × 5 mL) and the combined organic layers were dried over Na_2SO_4, filtered and the solvents evaporated under reduced pressure. The remaining residue was dissolved in a minimum amount of degassed CH_2Cl_2 (2.5 mL) and the product was then precipitated by addition of petroleum ether (40–60) (70 mL). The supernatant was decanted and the solid dried *in vacuo*. The precipitation was repeated as described above to give a white solid which was submitted to column chromatography (CH_2Cl_2/MeCN, 50% with a solvent switch to EtOAc/MeCN, 30%) gave phosphoramidite **6** (254 mg, 0.26 mmol, 56%) as a white solid. R_f 0.39 (CH_2Cl_2/CH_3OH, 19/1); ^{31}P NMR (162 MHz, $CDCl_3$) δ 149.4, 149.0; *m/z* (ESI$^+$): 273.133 ([MMTr]$^+$, 100%), 980.427 ([M + H]$^+$, 15.7%), 1002.457 ([M + Na]$^+$, 42.5%); HRMS–ESI (*m/z*): [M + H]$^+$ calcd for $C_{51}H_{63}N_7O_{11}P$, 980.4318; found, 980.4319.

Functionalisation of CPG resin **S2**



Long chain alkylamine controlled pore glass (LCAA CPG) 1000/110 (*LGC Genomics Ltd.*) resin (1.50 g, loading 81.1 μmol·g^{-1}) was rotated at rt for 1 h in a glass vessel containing a solution of 3% (w/v) trichloroacetic acid in anhyd CH_2Cl_2 (15 mL). The resin was filtered and consecutively washed with Et_3N/DIPEA [9/1 (v/v), 10 mL], anhyd CH_2Cl_2 (10 mL) and Et_2O (10 mL) and dried *in vacuo*. The resin was soaked in freshly distilled anhyd py (4 mL), a solution of succinic anhydride (750 mg, 7.50 mmol) and 4-dimethylaminopyridine (DMAP) (75 mg, 0.62 mmol) in freshly distilled py (15 mL) was added, and the reaction vessel was rotated at rt for 20 h. The solvent was removed by filtration and the resin was consecutively washed with anhyd py (10 mL), anhyd CH_2Cl_2 (10 mL) and Et_2O (10 mL) and dried *in vacuo*. The resin was soaked with anhyd py (4 mL) and a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (386 mg, 2.01 mmol), Et_3N (78 μL, 0.56 mmol) and DMAP (15 mg, 0.12 mmol) in anhyd py (5 mL) was added followed by addition of nucleoside **S1**[5] (70 mg, 0.14 mmol) in anhyd py (2 mL). The reaction mixture was gently agitated at rt for 24 h, pentachlorophenol (45 mg, 0.17 mmol) was then added and the mixture was rotated at rt for another 1 h before filtration and successive washing with anhyd py (3 × 5 mL), anhyd CH_2Cl_2 (3 × 5 mL) and Et_2O (3 × 5 mL). Piperidine/anhyd DMF [1/9 (v/v), 5 mL] was added to the dried resin and the vessel was rotated at rt for 10 min. Afterwards, the resin was filtered and consecutively washed with anhyd CH_2Cl_2 (3 × 5 mL) and Et_2O (3 × 5 mL). ON synthesis grade capping reagent (*Applied Biosystems*) Ac_2O/py/THF/*N*-methyl imidazole in THF [1/1 (v/v), 10 mL] was added and the vessel was rotated for 1 h. The resin was filtered and consecutively washed with anhyd CH_2Cl_2 (3 × 5 mL) and Et_2O (3 × 5 mL) and dried *in vacuo* to give the functionalised resin **S2** as a white solid (1.46 g, loading 23.6 μmol·g^{-1}, 29%). Loading efficiency was determined by measuring A_{478} after MMTr-deprotection in a solution of 3% (w/v) trichloroacetic acid in anhyd CH_2Cl_2 applying an extinction coefficient of ε = 56 mL·cm^{-1}·μmol^{-1} for the MMTr-cation.[5–7]

Characterisation Data

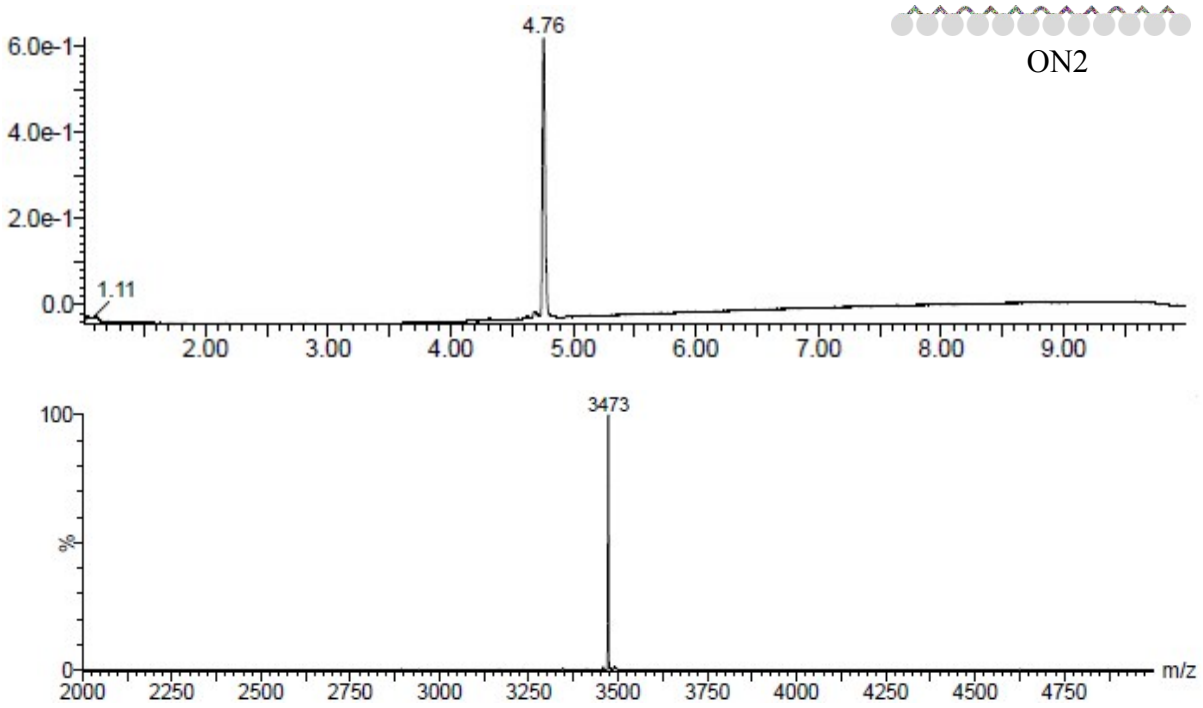


Fig. S10 (top) RP-UPLC trace of ON2. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. (bottom) De-convoluted mass spectrum for ON2.

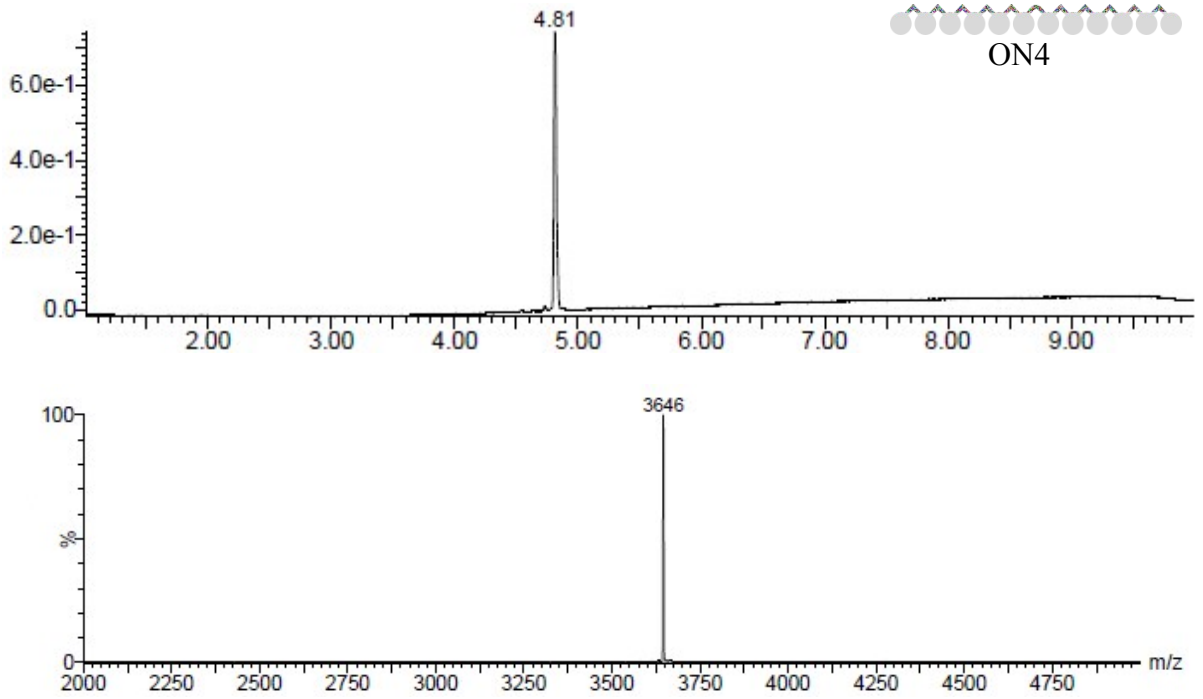


Fig. S11 (top) RP-UPLC trace of ON4. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. (bottom) De-convoluted mass spectrum for ON4.

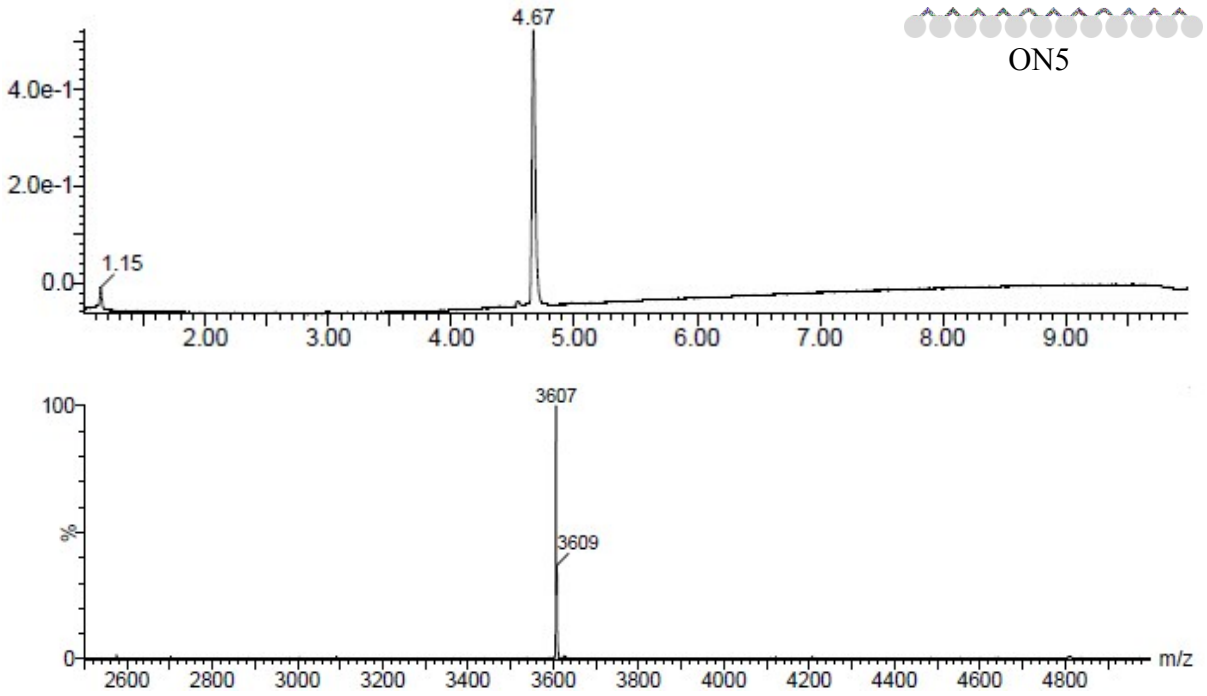


Fig. S12 (top) RP-UPLC trace of ON5. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. (bottom) De-convoluted mass spectrum for ON5.

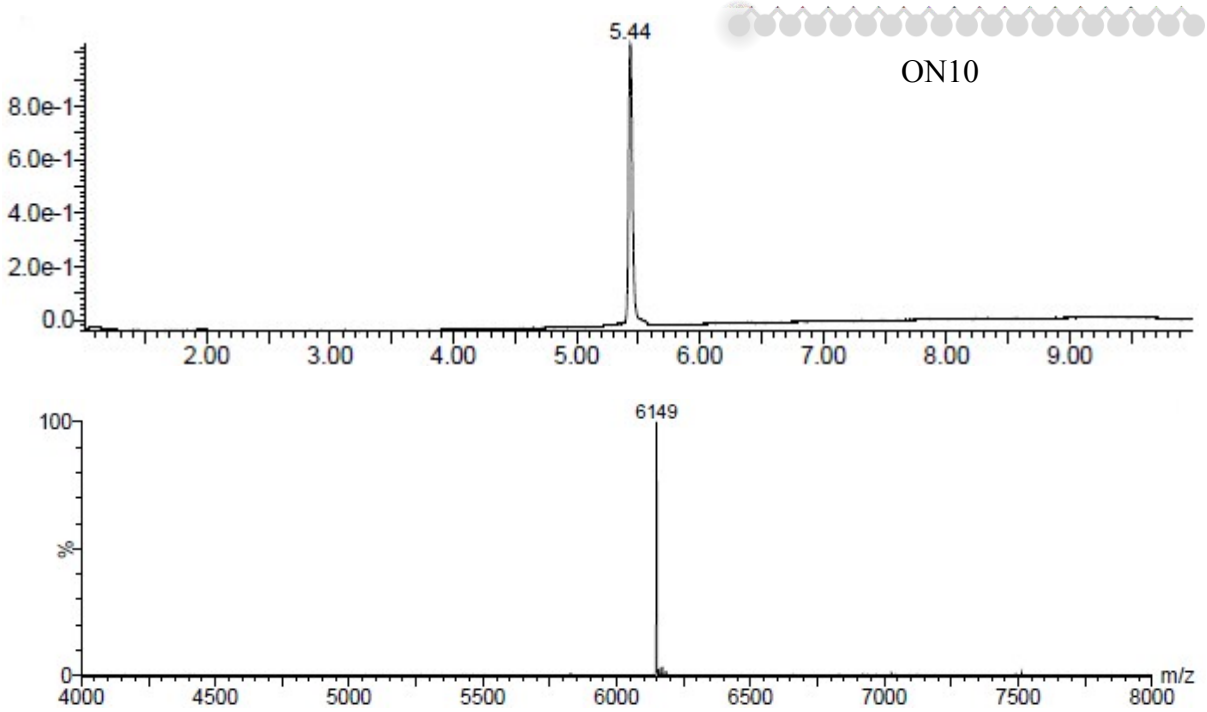


Fig. S13 (top) RP-UPLC trace of ON10. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. (bottom) De-convoluted mass spectrum for ON10.

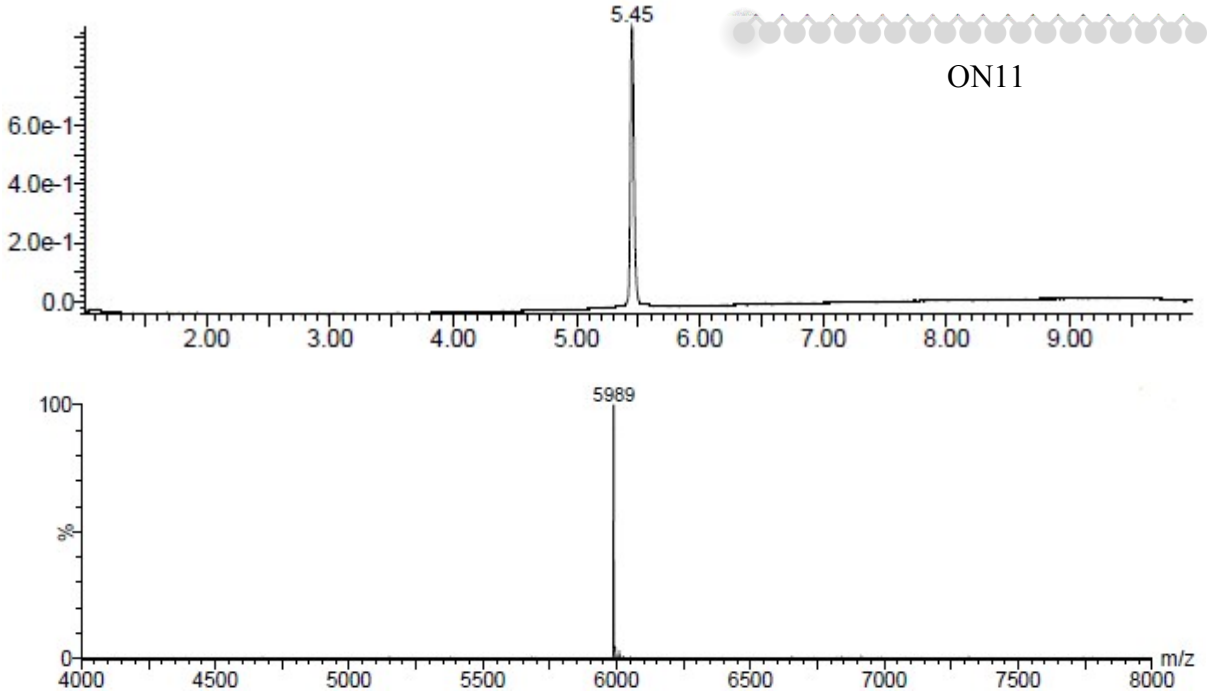


Fig. S14 (top) RP-UPLC trace of ON11. Conditions: BEH C18 column, 130 Å (1.7 µm, 2.1 mm × 50 mm), 0.2 mL·min^{-1}; Method: 0–70% B; 8 min. (bottom) De-convoluted mass spectrum for ON11.

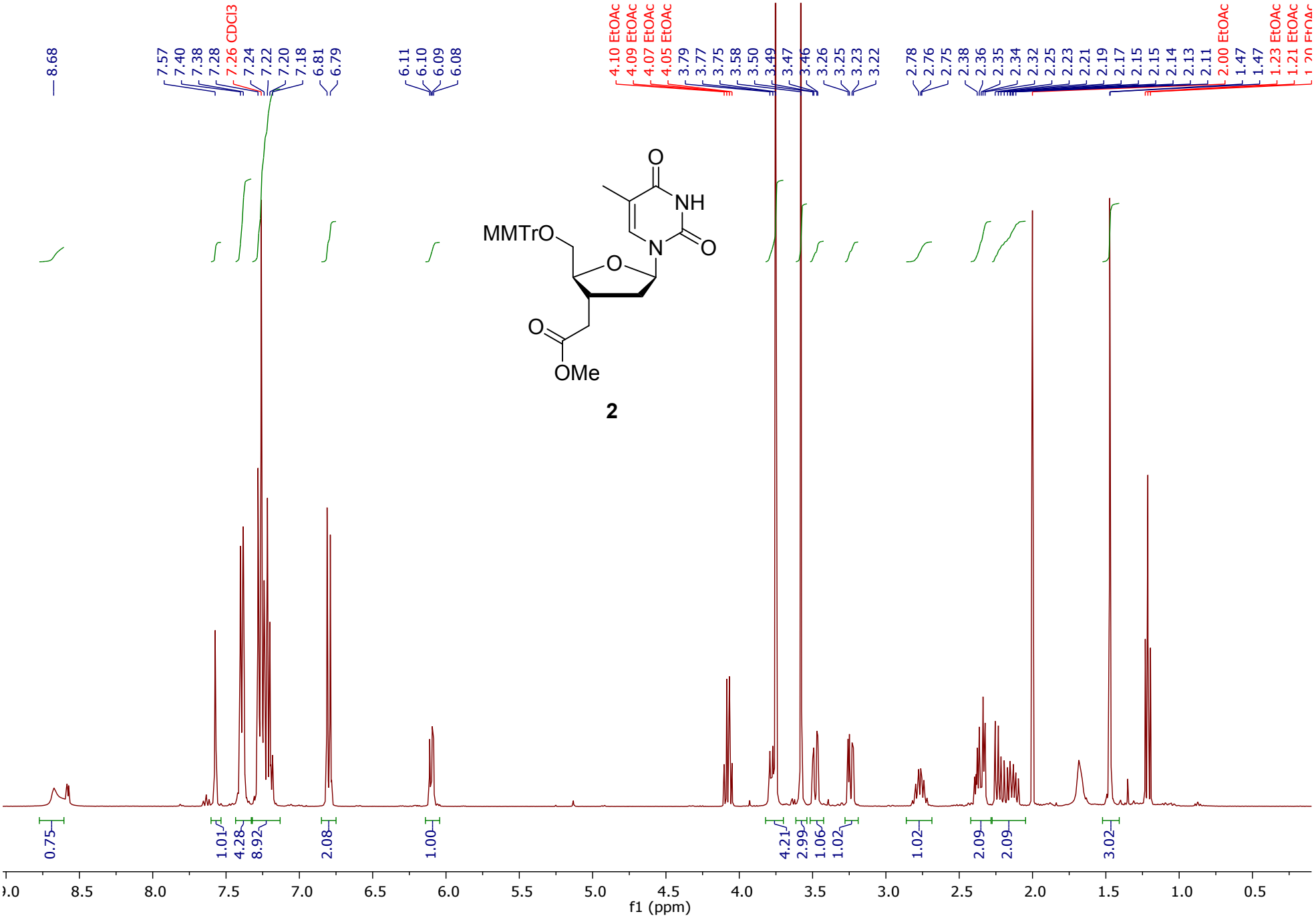


Fig. S15 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of **2**.

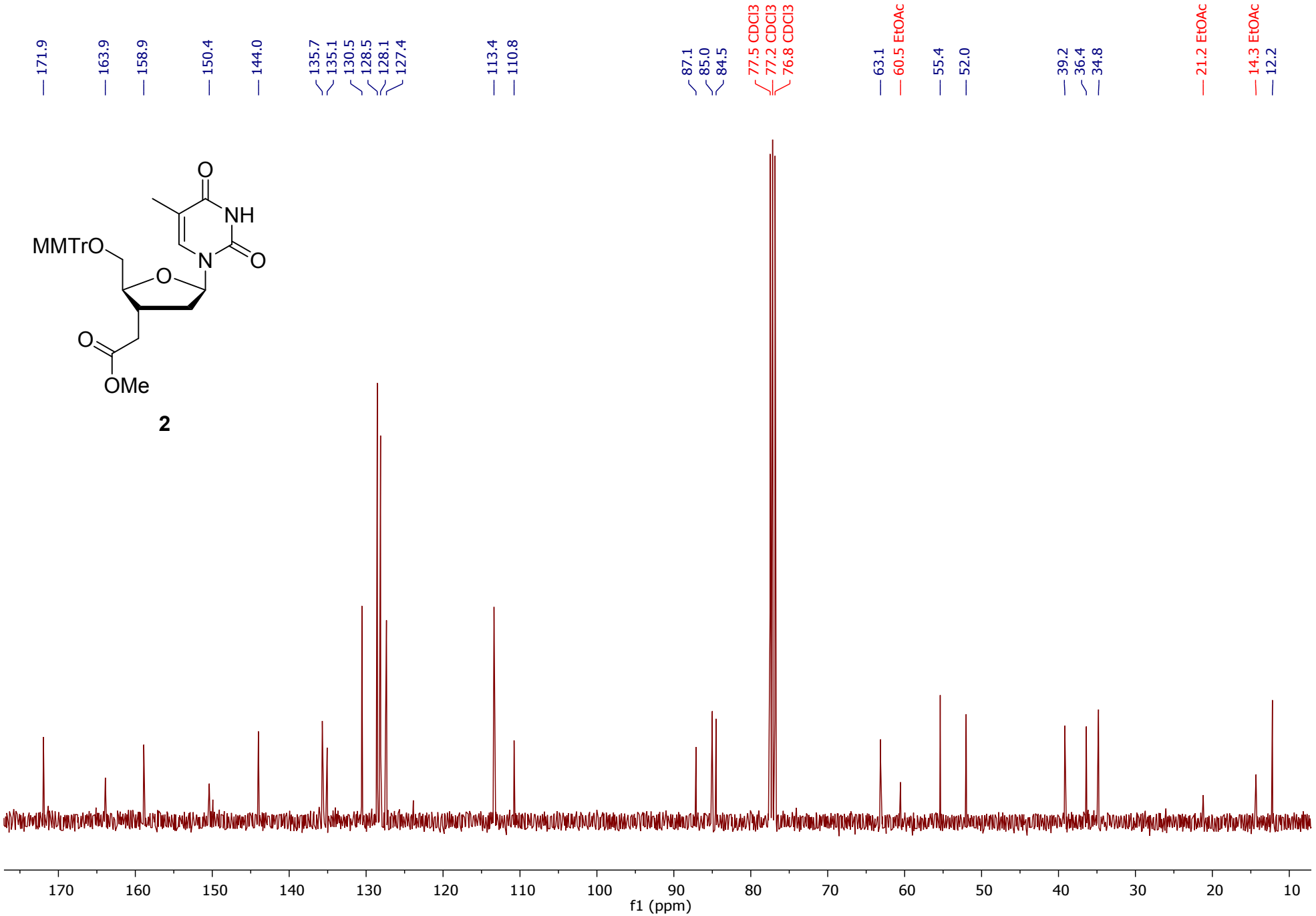


Fig. S16 ^{13}C NMR spectrum (101 MHz, $CDCl_3$) of **2**.

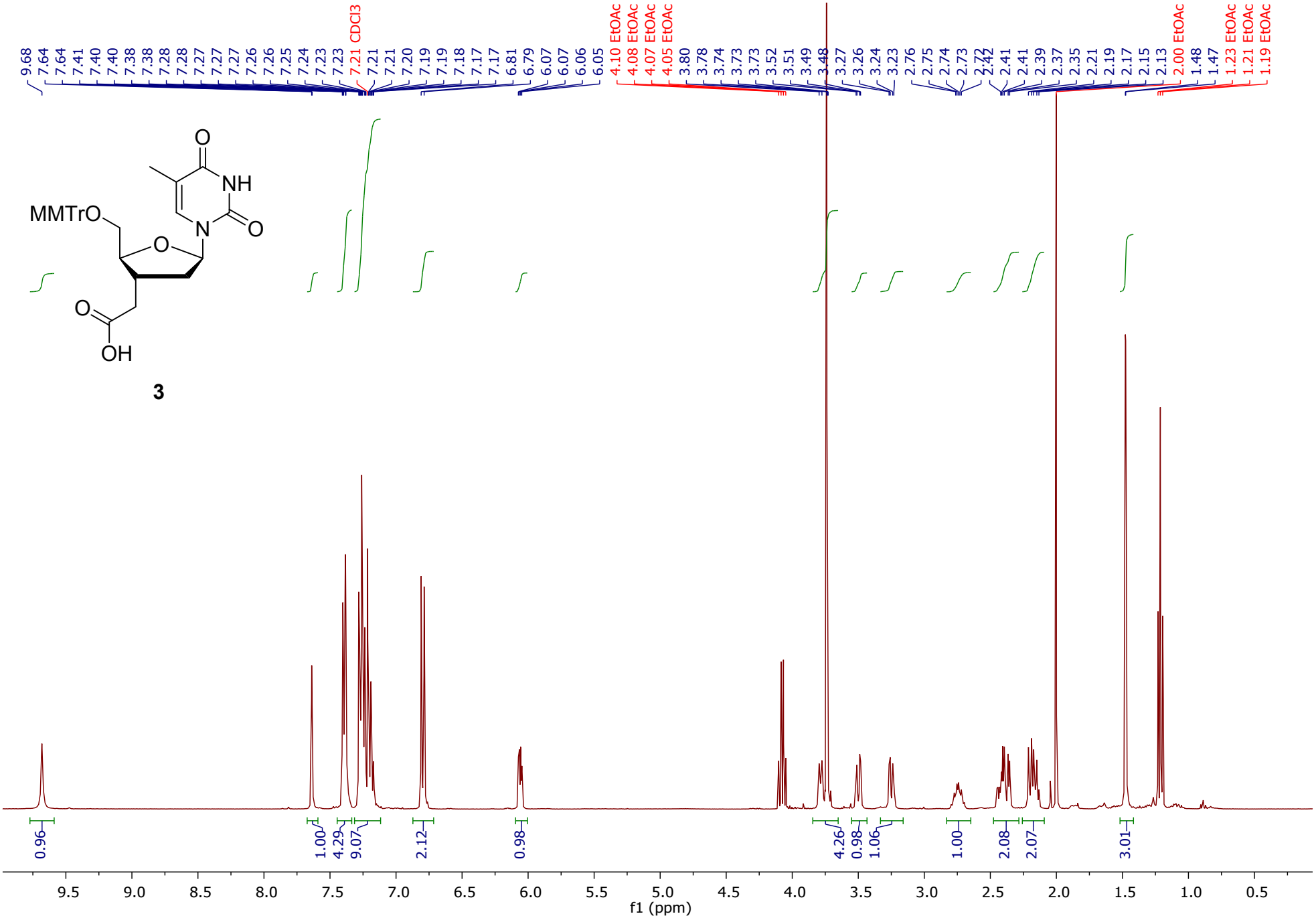


Fig. S17 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of **3**.

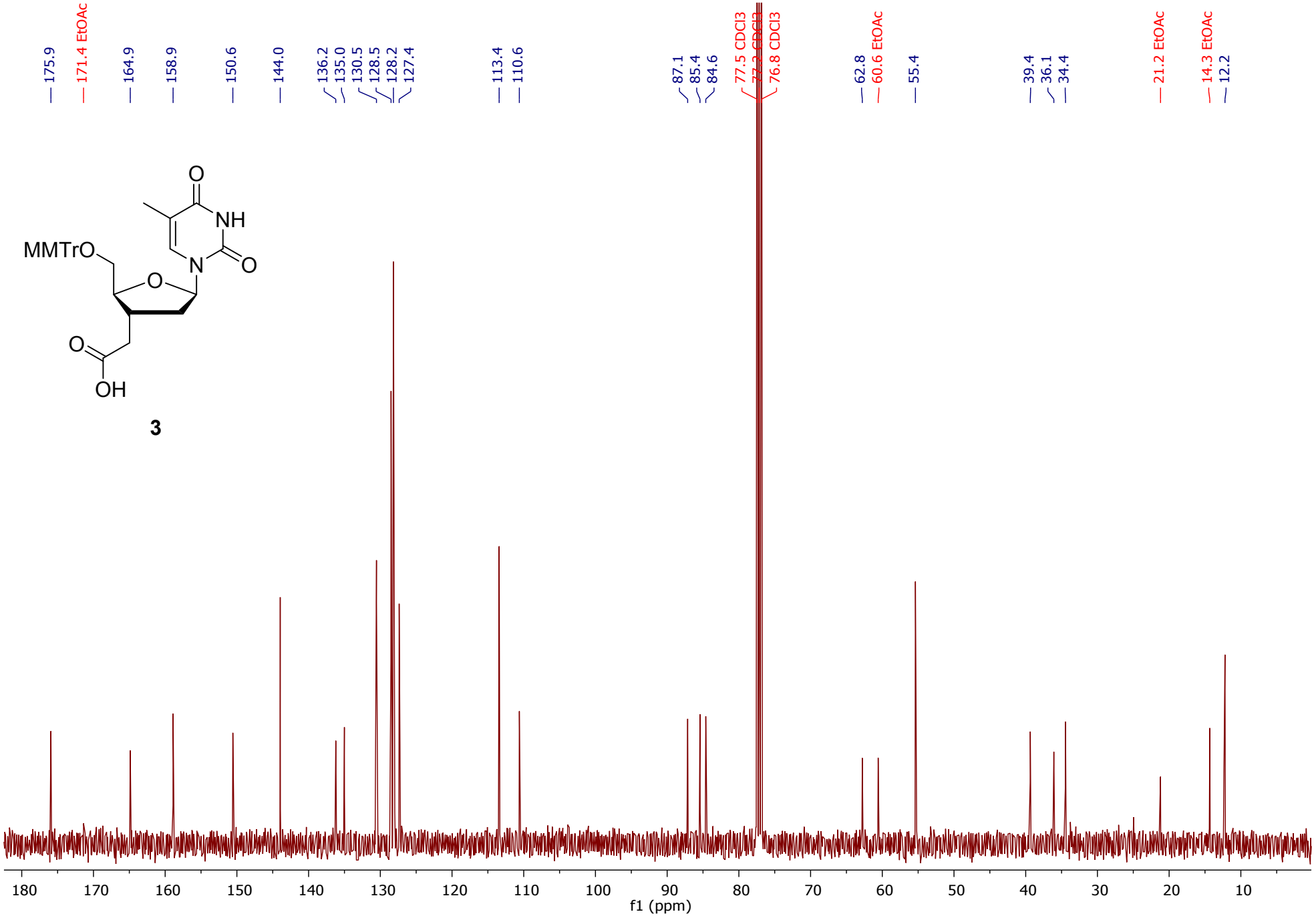


Fig. S18 ^{13}C NMR spectrum (101 MHz, $CDCl_3$) of **3**.

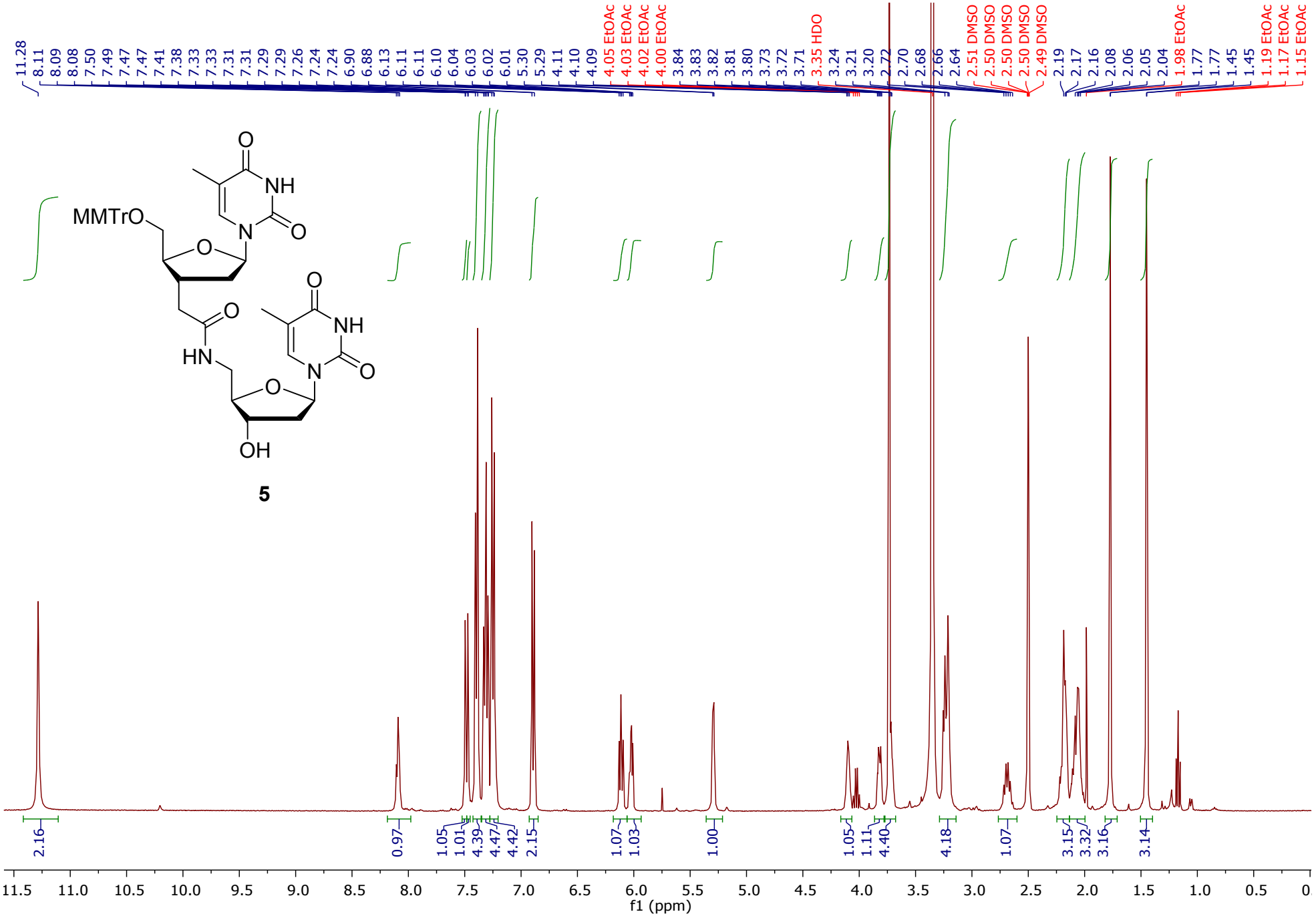


Fig. S19 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of **5**.

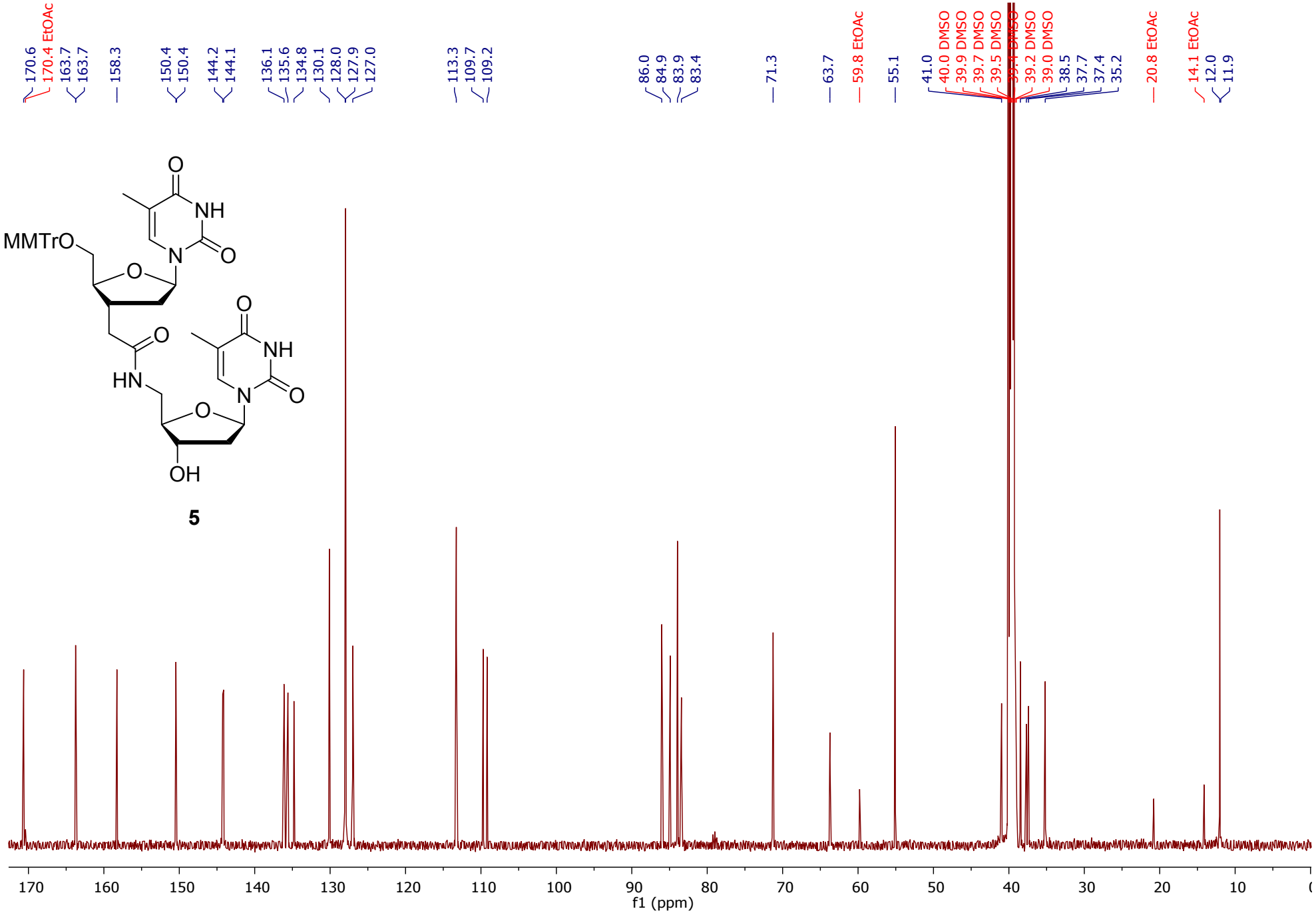


Fig. S20 ^{13}C NMR spectrum (126 MHz, DMSO-d_6) of **5**.

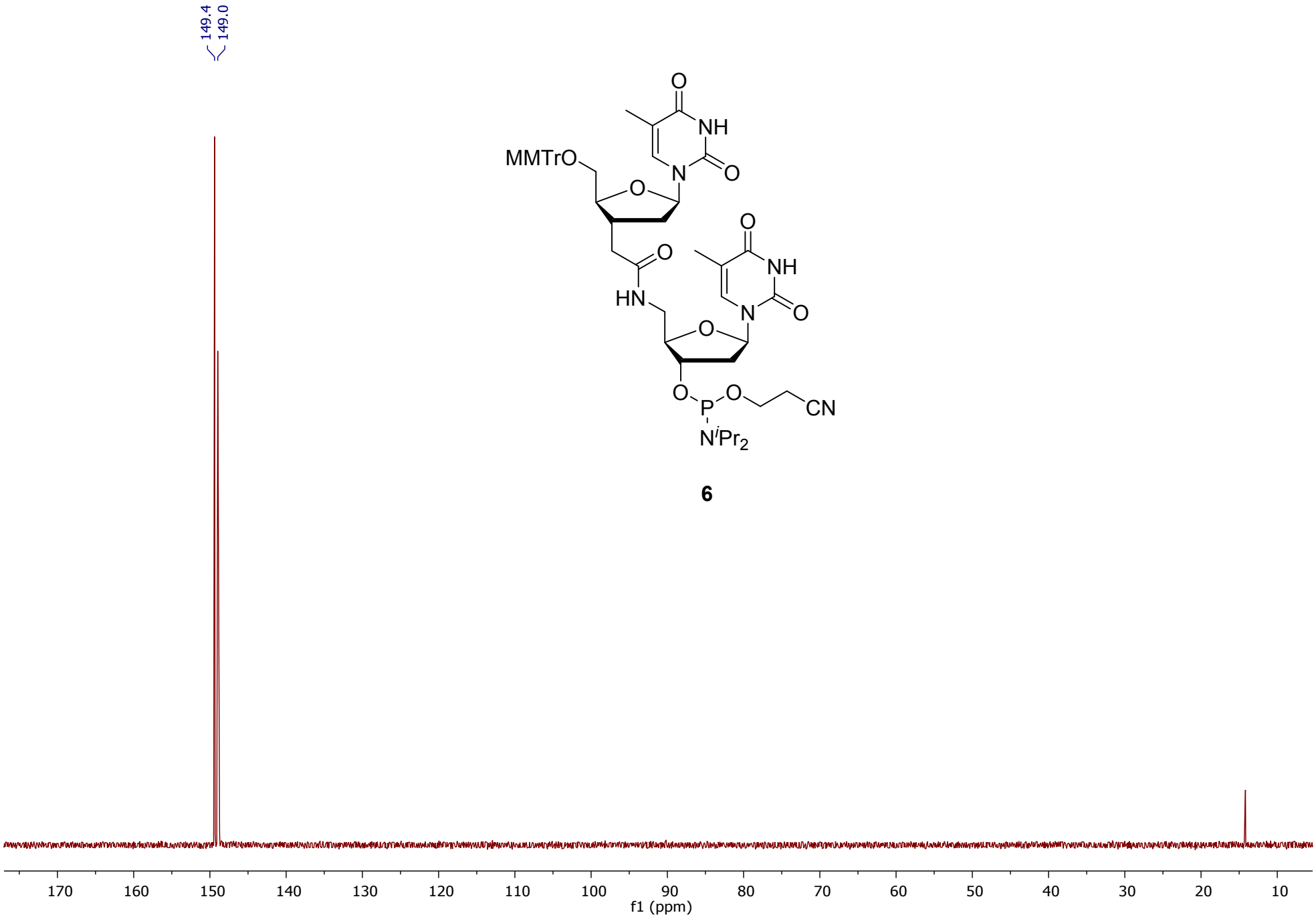


Fig. S21 ^{31}P NMR spectrum (162 MHz, $CDCl_3$) of **6**.

References

1 P. von Matt, A. De Mesmaeker, U. Pieles, W. Zürcher and K. H. Altmann, *Tetrahedron Lett.*, 1999, **40**, 2899–2902.

2 B. M. Brandsen, A. R. Hesser, M. A. Castner, M. Chandra and S. K. Silverman, *J. Am. Chem. Soc.*, 2013, **135**, 16014–16017.

3 P. Tanui, S. D. Kennedy, B. D. Lunstad, A. Haas, D. Leake and E. Rozners, *Org. Biomol. Chem.*, 2014, **12**, 1207–1210.

4 I. Van Daele, H. Munier-Lehmann, M. Froeyen, J. Balzarini and S. Van Calenbergh, *J. Med. Chem.*, 2007, **50**, 5281–5292.

5 P. M. Reddy and T. C. Bruice, *J. Am. Chem. Soc.*, 2004, **126**, 3736–3747.

6 M. J. Damha, P. A. Giannaris and S. V. Zabarylo, *Nucleic Acids Res.*, 1990, **18**, 3813–3821.

7 M. Fonvielle, I. Li De La Sierra-Gallay, A. H. El-Sagheer, M. Lecerf, D. Patin, D. Mellal, C. Mayer, D. Blanot, N. Gale, T. Brown, H. Van Tilbeurgh, M. Ethève-Quelquejeu and M. Arthur, *Angew. Chem., Int. Ed.*, 2013, **52**, 7278–7281.