Supporting Information

A modified guanosine phosphoramidite for click functionalization of RNA on the sugar edge

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RNA oligoribonucleotide synthesis and HPLC purification

Unmodified RNA strands, as well as dye labeled constructs were obtained from IBA (Götingen, Germany). All other oligoribonucleotides were custom-synthesized by Purimex (Grebenstein, Germany) on a 1 µmol scale, starting from preloaded controlled pore glass. The dimethoxytrityl group of the 5'-terminal nucleotide was removed before release from the solid support, RNA oligoribonucleotides were deprotected and cleaved from the solid support, using a mixture of concentrated aqueous ammonia (28-33%) and ethanol (1 mL) for 3h at RT. The supernatant was collected and the residue was washed with a mixture of MilliQ water, ethanol and acetonitrile (1:3:1). Excess ammonia and water were removed from the combined aqueous solutions with the aid of a SpeedVac. TBAF (1M in THF) was used for the deprotection step of the 2' silyl containing protection groups – TBDMS for all commercial phosphoramidites and TOM for the new building block, CTDNPI. After desalting, using NAP-10 pre-packed columns and lyophilization, the crude oligoribonucleotide was dissolved in MilliQ water and "clicked" with the Atto 590 azide dye. The resulting crude mixture was subjected to a NAP-10 column to remove all unreacted dye molecules, after which it was purified by means of semi-prep HPLC (Agilent 1100 series, Waldbronn, Germany), with a gradient from 5 to 85% CH_3CN in 20 min, at a flow rate of 3 mL/min. This single, conventional HPLC step on a C18 reversed-phase column (LiChroCART 250-10, Merck, Darmstadt, Germany) provided the labeled oligoribonucleotides in high yields and purity, as demonstrated by MALDI-TOF mass spectrometry (Figure S4).

UV melting profile

Melting curves of the siRNAs (~ 1.0 µM; 150 µL) in 10 mM NaH_2PO_4 (pH 8); 50 mM NaCl were acquired on a Jasco V-650 spectrophotometer equipped with a Jasco ETC-505T Peltier temperature programmer, using a 10 mm path-length quartz cell. Melting curves were recorded at 260 nm, using a heating rate of 0.4 °C/min, a slit of 2 nm and a response of 0.2 s.

Determination of gene-silencing activity

HEK 293T cells were cultivated under standard conditions in DMEM (Dulbecco's Modified Eagle Medium) (Invitrogen, Darmstadt, Germany), containing 10 % fetal calf serum (Invitrogen) and 1 % penicillin/streptomycin (Invitrogen). One day prior to transfection, 8 x 10^5 cells were seeded in 1 mL medium without antibiotics in 24-well tissue culture treated plates. At the time of transfection, cells had a confluence of 70-80 %. Medium was aspirated off and 500 µL fresh medium was added. 400 ng of pEGFP-N1 (GenBank Accession #U55762) and the respective amount of siRNA were diluted in a final volume of 50 µL OptiMem (Invitrogen). After addition of 1 µL Lipofectamine 2000 (Invitrogen) in a final volume of 50 µL OptiMem, the 100 µL transfection mix was incubated for 20 min at room temperature. The mix was added dropwise onto each well.

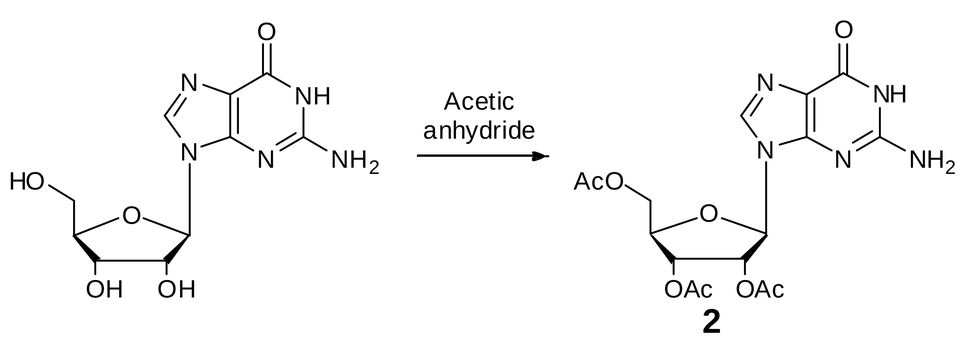
After 24 h incubation, cells were washed once with PBS, trypsinated and dissolved in 600 µL PBS. eGFP expression was analyzed by flow cytometry on an LSR-FortessaSORP, BD, Heidelberg, Germany, with excitation at 488 nm and detection at 530/30nm. The eGFP signal was calculated as the product of mean fluorescence intensity and number of eGFP positive cells compared to the value of a positive control. IC_{50} curves (Fig. 3) resulted of three biological triplicates, each done in technical triplicate. Curves were calculated with GraphPad Prism (asymmetric 5 parameter dose-respond model).

Cell imaging of labeled oligonucleotides

RBE4[1] cells were cultivated under standard conditions in a medium consisting of 45 % v/v DMEM, 45 % v/v HAM's F-10, 10 % v/v serum (*e.g.* fetal calf or bovine serum), 100 µg/mL penicillin/streptomycin mix and 1 ng/mL basic fibroblast growth factor (all Invitrogen). For confocal imaging, the cells were grown on 8-well µ-slides (Ibidi, Munich, Germany). One day before transfection, ~ 20 000 cells per well were seeded in 250 µL growth medium without antibiotics, resulting in 50 % confluency at the day of transfection. An optimized standard protocol for Oligofectamine was used to transfect RBE4 cells with labeled siRNA duplexes.
0.8 µL of a 5 µM siRNA solution were diluted with 14.5 µL of Opti-Mem medium (Invitrogen) and combined with a mixture of 0.85 µL Oligofectamine (Invitrogen) and 3.5 µL Opti-Mem and incubated for 20 min. Before adding the formed lipoplexes dropwise to the cells, the medium was replaced with transfection medium (medium without serum and antibiotics), resulting in a final culture volume of 150 µL. Cells were incubated at 37 °C until fixation. For fixation, cells were washed once with PBS and fixated for 10 min in 4 % formaldehyde diluted in PBS. After two further washing steps with PBS and one with water, cells were mounted by adding three droplets of IBIDI Mounting medium (IBIDI) to the well. Samples were stored at 4 °C until imaging. Fixed cell samples were imaged on an inverted confocal microscope TCS SP5 (Leica, Wetzlar, Germany), equipped with an oil-immersion objective (63x magnification; NA 1.4) and a 561 nm laser. Images were recorded at 512x512 8-bit-pixel resolution with a pinhole of 130 µm and confocal plane depth of 1.0 µm, resulting in a total image size width and height of 246x246 µm. Atto 590 emission was recorded at 561 nm excitation and the emission was recorded between 605 - 635 nm.

SYNTHETIC PROCEDURES

*Synthesis of 2',3',5'-O-triacetylguanosine (**2**)*[2]



2

Guanosine (9.5 g) was weighed into a rb flask, to which was added acetic anhydride (30 mL), pyridine (15 mL) and DMF (40 mL). The mixture was stirred and the temperature was raised to 75 °C and further stirred for 4 h, after which the temperature was lowered to 25 °C and stirring continued overnight. The volume of solvent was reduced with the aid of a rotary evaporator. The off-white crude mixture was purified by recrystalization, using hot isopropanol. The product, which was obtained in a yield of 93 % was used in concomitant steps without further analysis.

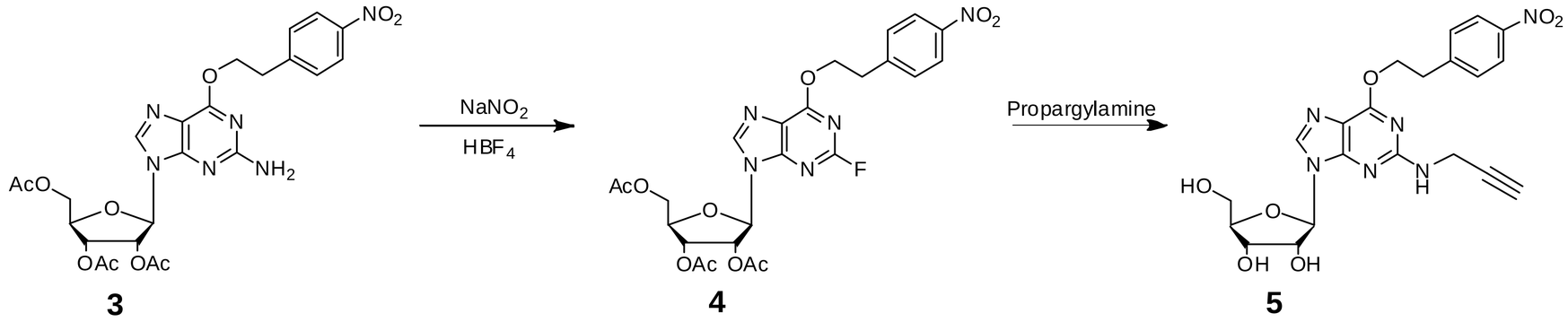
*Synthesis of 2',3',5',-O-triacetyl-O^6-nitrophenylguanosine (**3**)*[2]

2 **3**

2 (3.5 g) was weighed into a rb flask, to which was added 120 mL of dioxane and PPh_3 (1.2 equivalents). The mixture was stirred at 60 °C for 1 h and 2-(*p*-nitrophenyl)ethanol (1.5 equivalents) was added and further stirred for 0.5 h. DIAD (1.5 equivalents) was added and stirred for additionally 5 h. The volume of solvent was reduced with the aid of a rotary evaporator under reduced pressure. The off-white crude mixture was purified *via* flash column chromatography, using a gradient system

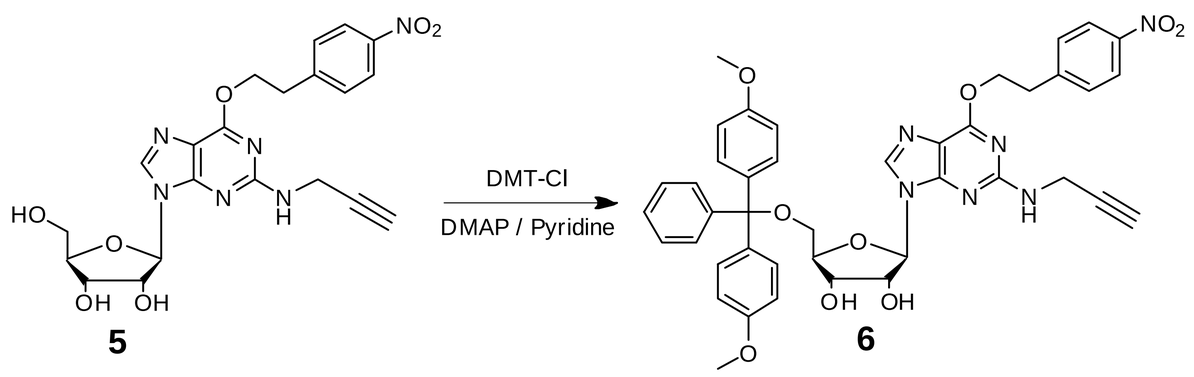
(Ether:DCM, 20:1, 10:1, 1:1, 1:5). The product was obtained in a yield of 58 %. ^{1}H NMR: J values are given in Hz (400 MHz, $CDCl_3$) δ_H 8.07 (1 H, s, 8-H), 7.71 (2 H, d, 14-H & 16-H), 7.40 (2 H, d, 13-H & 17-H), 5.98 (1 H, d, 1'-H), 5.92 (1 H, t, 2'-H), 5.74 (1 H, t, 3'-H), 4.65 (2 H, t, 10-H), 4.40 (3 H, m, 4'-H & 5'-H), 3.20 (2 H, t, 11-H), 2.08 (3 H, s, 5'-OAc-CH_3), 2.03 (3 H, s, OAc-CH_3), 2.02 (3 H, s, OAc-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C = ppm 170.49; 169.61; 160.89; 159,29; 153,42; 146,73; 146,00; 138,02; 132,08; 131.98; 129.91; 128.55; 128.43; 123.64; 115.75; 86.43; 79.78; 72.82; 70.52; 66.25; 63.02; 35.07; 20.69; 20.52; 20.41. FD-MS *m/z* 558.4 (M^+, 100%).

*Synthesis of O^6-nitrophenyl-2-propargylaminylinosine (**5**)*[3]



O^6-npe-2',3',5'-O-triacetylguanosine (**3**) (0.7 g) was weighed into a rb flask, to which was added 20 mL of acetone. To the reaction mixture was added HBF_4 (14 mL of 50 % in H_2O). The reaction mixture was stirred at -40 °C for 1 h, after which $NaNO_2$ (3 equivalents) was added dropwise as a solution in H_2O. The mixture was stirred overnight, after which it was neutralized using NaOH (8 M). It was then extracted three times into DCM, the organic medium was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. To the crude was added propargylamine as a solution in MeOH (8 M) and stirred for three days. The solvent was evaporated and the crude purified by flash cc. with a gradient of DCM:MeOH(1:0, 15:1). The combined fractions, containing the product, was pulled together and concentrated to give a final mass of 320 mg. A final yield of 54 % was recorded for the three steps. ^{1}H NMR: J values are given in Hz (400 MHz, DMSO-d_6) δ_H 8.17 (2 H, d, ^{3}J =8.7, 14-H & 16-H), 8.13 (1 H, s, 8-H), 7.62 (2 H, d, ^{3}J = 8.7, 13-H & 17-H), 7.34 (1 H, t, ^{3}J =1.3, 18-H), 5.79 (1 H, d, ^{3}J =5.4, 1'-H), 5.38 (1 H, d, ^{3}J =4.8, 2'-OH), 5.16 (1 H, d, ^{3}J =4.8, 3'-OH), 4.97 (1 H, t, 5'-OH), 4.73 (2 H, t, ^{3}J =6.9, 10-H), 4.56 (1 H, m, 2'-H), 4.13 (1 H, m, 3'-H), 4.07 (1 H, dd, ^{3}J = 1.2, 19-H), 3.89 (1 H, m, 4'-H), 3.59 (2 H, m, 5'-H), 3.29 (2 H, t, ^{3}J=6.9, 11-H), 3.00 (1 H, s, 21-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 160.06; 158.02; 146.73, 138.86; 130.30; 123.57; 114.34; 87.04; 85.61; 82.65; 73.07; 72.10; 70.35; 65.68; 61.57; 54.91; 34.29; 25.56. FD-MS *m/z* 470.3 (M^+, 100%). Mp 82 – 83 °C.

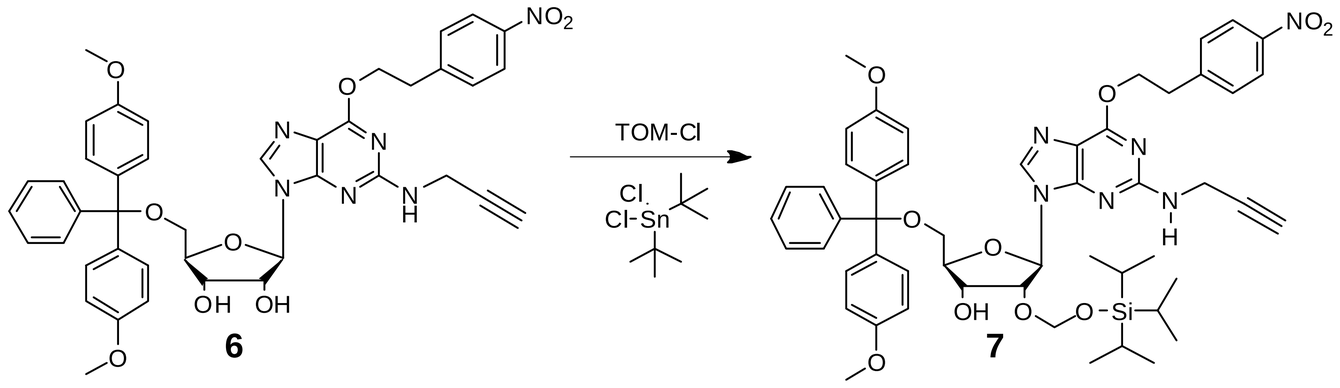
*Synthesis of 5'-O-DMT-O^6-nitrophenyl-2-propargylaminylinosine (**6**)*



To a rbf containing vacuum-dried **5** (110 mg, 0.24 mmol), which was resuspended in pyridine (3 mL) was added DMT-Cl (1.2 equiv., 100 mg, 0.28 mmol) as a solution in pyridine. DMAP (10 mg) was added as a solution in pyridine. The reaction mixture was stirred at rt and monitored hourly. The reaction was finished after 12 hours, quenched with water and evaporated to dryness at 25 °C. fCC, using ether/ethyl acetate 2:3 with 3 % Et_3N , then pure DCM with 3 % Et_3N and then DCM /MeOH 10:1 with 3 % Et_3N gave the product in pure yield (> 95 %). ^{1}H NMR: J values are given in Hz (400 MHz,DMSO-d_6) δ_H 8.17 (2 H, d, ^{3}J=8.7, 4-nitrophenyl 14-H & 16-H), 8.03 (1 H, s, 8-H), 7.62 (2 H, d, ^{3}J=8.7, 4-nitrophenyl 13-H & 17-H), 7.36– 7.43 (1 H, t, 18-H), 7.19–7.32 (9 H, m, trityl-H), 6.81 (4 H, m, trityl-H, 31-H, 33-H, 38-H & 40-H), 5.85 (1 H, d, ^{3}J=5.4, 1'-H), 5.56 (1 H, d, ^{3}J=5.0, 2'-OH), 5.20 (1 H, d, *J*=5.6, 3'-OH), 4.71 (2 H, t, ^{3}J=6.9, 11-H), 4.63 (1 H, m, 2'-H), 4.27 (1 H, m, 3'-H), 4.02

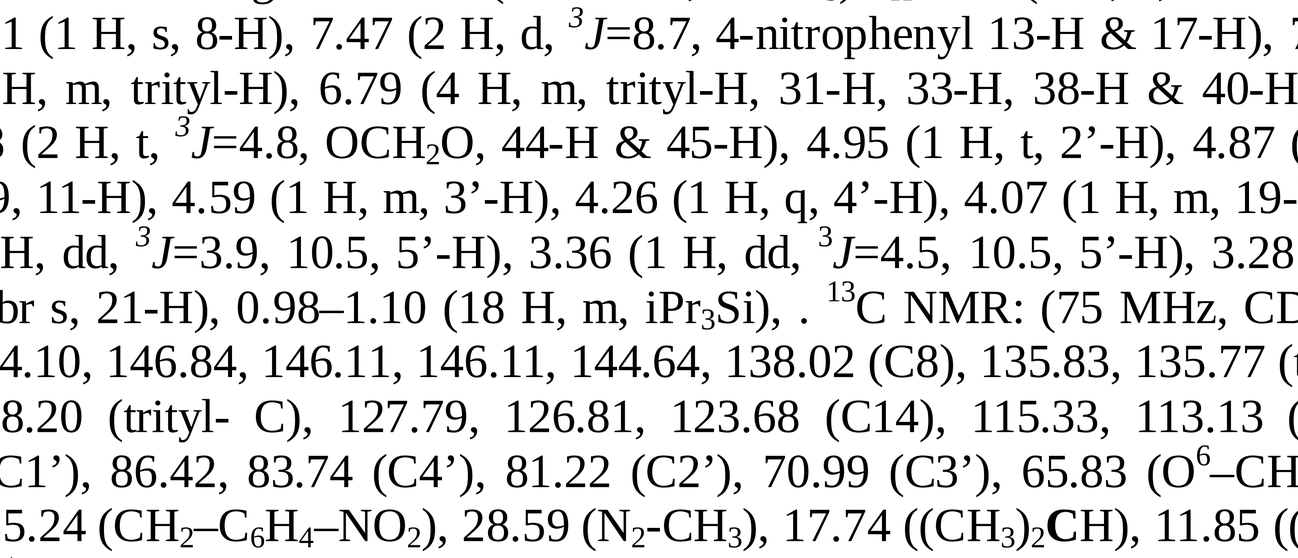
(1 H, q, 4'-H), 3.97 (2 H, br s, 19-H), 3.70, 3.71 (6 H, s, 2xOCH_3, 42-H & 43-H), 3.28 (2 H, t, ^3J=6.9, CH_2–C_6H_4–NO_2), 3.19 (2 H, m, 5'-H), 2.96 (1 H, s, 21-H). ^{13}C NMR: (100 MHz, DMSO-d_6) δ_C 160.46; 158.49; 147.10; 146.73; 145.32; 135.99; 130.88; 128.42; 127.28; 124.06 (4-nitrophenyl C14=C16); 113.84 (trityl-C); 85.91 (OCH_2O); 83.53; 73.49; 72.53; 71.03 (3'C); 66.11 (O^6–CH_2); 64.53 (5'C); 55.68 (2 CH_3O); 34.79 (CH_2–C_6H_4–NO_2); 31.16. FD-MS *m/z*: 773.675 (M^+, 13.9%) 303.15 (100).

Synthesis of 2'-O-TOM-5'-O-DMT-O^6-nitrophenyl-2-propargylaminylinosine (7)



To a rbf containing vacuum-dried 5'-O-DMT-O^6-nitrophenyl-2-propargylaminylinosine (**6**) (110 mg, 0.14 mmol), which was redissolved in DCE (6 mL) was added di-*tert*butyltin dichloride (1.1 equiv., 66 mg) and warmed to 65°C. Subsequently, TOM-Cl (1.2 equiv., 45 mg, 0.18 mmol), as a solution in DCE (0.5 mL) and triethylamine (0.4 mL) were added counter-respectively and the reaction mixture was stirred and allowed to cool to rt. The progress of the reaction was monitored by TLC. The reaction was finished after 5 h and was quenched with MeOH. The product was purified by fCC using the following gradient solvent system: DCM with 3% Et_3N - 70:1 DCM/MeOH with 1% Et_3N. The TLC plates were developed using DCM/MeOH/Et_3N 48:2:1. The regioselectivity of 2'-O-alkylated over 3'-O-alkylated product was approximately 7:2. YIELD: 54 %. TLC (silica gel, *n*Hex:EtOAc=1:1): R_f=0.41. ^{1}H NMR: J values are given in Hz (400 MHz, $CDCl_3$) δ_H 8.16 (2 H, d, ^3J=8.7, 4-nitrophenyl, 14-H & 16-H), 7.71 (1 H, s, 8-H), 7.47 (2 H, d, ^3J=8.7, 4-nitrophenyl 13-H & 17-H), 7.43 (1 H, t, 18-H), 7.18– 7.33 (9 H, m, trityl-H), 6.79 (4 H, m, trityl-H, 31-H, 33-H, 38-H & 40-H), 6.03 (1 H, d, ^3J=5.4, 1'-H), 4.98 (2 H, t, ^3J=4.8, OCH_2O, 44-H & 45-H), 4.95 (1 H, t, 2'-H), 4.87 (1 H, br s, NH), 4.75 (2 H, t, ^3J=6.9, 11-H), 4.59 (1 H, m, 3'-H), 4.26 (1 H, q, 4'-H), 4.07 (1 H, m, 19-H), 3.78 (6H, s, 2xOCH_3), 3.46 (1 H, dd, ^3J=3.9, 10.5, 5'-H), 3.36 (1 H, dd, ^3J=4.5, 10.5, 5'-H), 3.28 (2 H, t, ^3J=6.9, 10-H), 3.06 (1 H, br s, 21-H), 0.98–1.10 (18 H, m, iPr_3Si), . ^{13}C NMR: (75 MHz, $CDCl_3$) δ_C 160.51, 159.64, 158.52, 154.10, 146.84, 146.11, 146.11, 144.64, 138.02 (C8), 135.83, 135.77 (trityl C), 130.08 (C12), 129.88, 128.20 (trityl- C), 127.79, 126.81, 123.68 (C14), 115.33, 113.13 (trityl-C), 90.79 (OCH_2O), 86.70 (C1'), 86.42, 83.74 (C4'), 81.22 (C2'), 70.99 (C3'), 65.83 (O^6–CH_2), 63.55 (C5'), 55.16 (2xCH_3O), 35.24 (CH_2–C_6H_4–NO_2), 28.59 (N_2-CH_3), 17.74 ((CH_3)$_2$**C**H), 11.85 ((**C**H_3)$_2$CH). FD-ESI *m/z* 959.44 (M^+, 10%) 303.15 (100).

*Synthesis of 3'-O-CEP-2'-O-TOM-5'-O-DMT-O^6-nitrophenyl-2-propargylaminylinosine (**CTDNPI**)*



To a rbf containing vacuum-dried *2'-O-TOM-5'-O-DMT-O^6-nitrophenyl-2-propargylaminylinosine (7)* (120 mg, 0.56 mmol), dissolved in DCM (4 mL) was added dimethylethylamine (0.3 mL) and CEP-Cl (1.5 equiv., 100 mg, 0.18 mmol) as a solution in DCM (1 mL) at rt. The reaction mixture was stirred

and was monitored by TLC. The reaction was complete within 2 h. Column chromatography, using silica gel (cHexane:EtOAc) 2:1, 1:1 with 2 % Et_3N gave the product as diastereomers in the ratio 3:2. ^{1}H NMR: J values given in Hz (72%, 3:2 mixtures of diastereoisomers). (400 MHz, $CDCl_3$) δ_H 8.15 (4 H, d, ^3J=7.5, 4-nitrophenyl 14-H & 16-H), 7.69 (2 H, s, 8-H), 7.48 (4 H, d, ^3J=7.5, 4-nitrophenyl 13-H & 17-H), 7.40–7.44 (4 H, m, 13-H, 14-H & 18-H), 7.18– 7.35 (16 H, m, trityl Hs), 6.75–6.83 (8 H, m, trityl Hs), 6.00, 6.05 (2 H, d, ^3J=6.0, 1'-H), 5.12 (2 H, t, 2'-H), 4.89–4.96 (4 H, m, OCH_2O), 4.79 (2 H, m, 3'-H), 4.73 (4 H, t, 11-H), 4.64 (2 H, br q, NH), 4.31, 4.36 (2 H, q, 4'-H), 4.02-4.12 (4H, m, 19-H), 3.82–3.96 (2 H, m, $POCH_2$), 3.77, 3.78 (12 H, s, OCH_3), 3.45–3.66 (1 H, 5'-H), (2 H, $POCH_2$) (4 H, $((CH_3)_2\mathbf{CH})_2N$), 3.35–3.40 (1 H, m, 5'-H), 3.27 (4 H, t, ^3J=6.9, 10-H), 2.85 (4 H, d, ^3J=2.1, N^2-CH_2-), 2.34, 2.65 (4 H, m, CH_2CN), 0.89–1.24 (42 H, m, iPr_3Si) ppm. ^{31}P NMR (200 MHz, $CDCl_3$) δ_P = 151.17; 151.60 ppm. FD-ESI *m/z* 1159.57 (M^+, 14%), 1160.58(10), 1181.56 (8), 1198.55(5), 303.15 (100).

General Procedure for Click Chemistry

NaH_2PO_4 (pH 8) was used as buffer for all click reactions. The sodium ascorbate (1 eq.), $CuSO_4.5H_2O$ (0.1 eq.) and tris[4-(3-hydroxy-propyl)-(1,2,3)triazol-1-ylmethyl)amine (TPTA) (0.5 equiv.) were added to the modified oligonucleotide and Atto 590 azide dye (5 eq. in DMSO) and the reaction mixture was stirred at 25 °C for 24 h. After desalting on a NAP-10 column, the crude residue was purified by reverse-phase semi-preparative HPLC. A ramp from 5 % to 85 % acetonitrile was used over 20 min (Buffer B = 0,1 M triethylammonium acetate, pH 7).

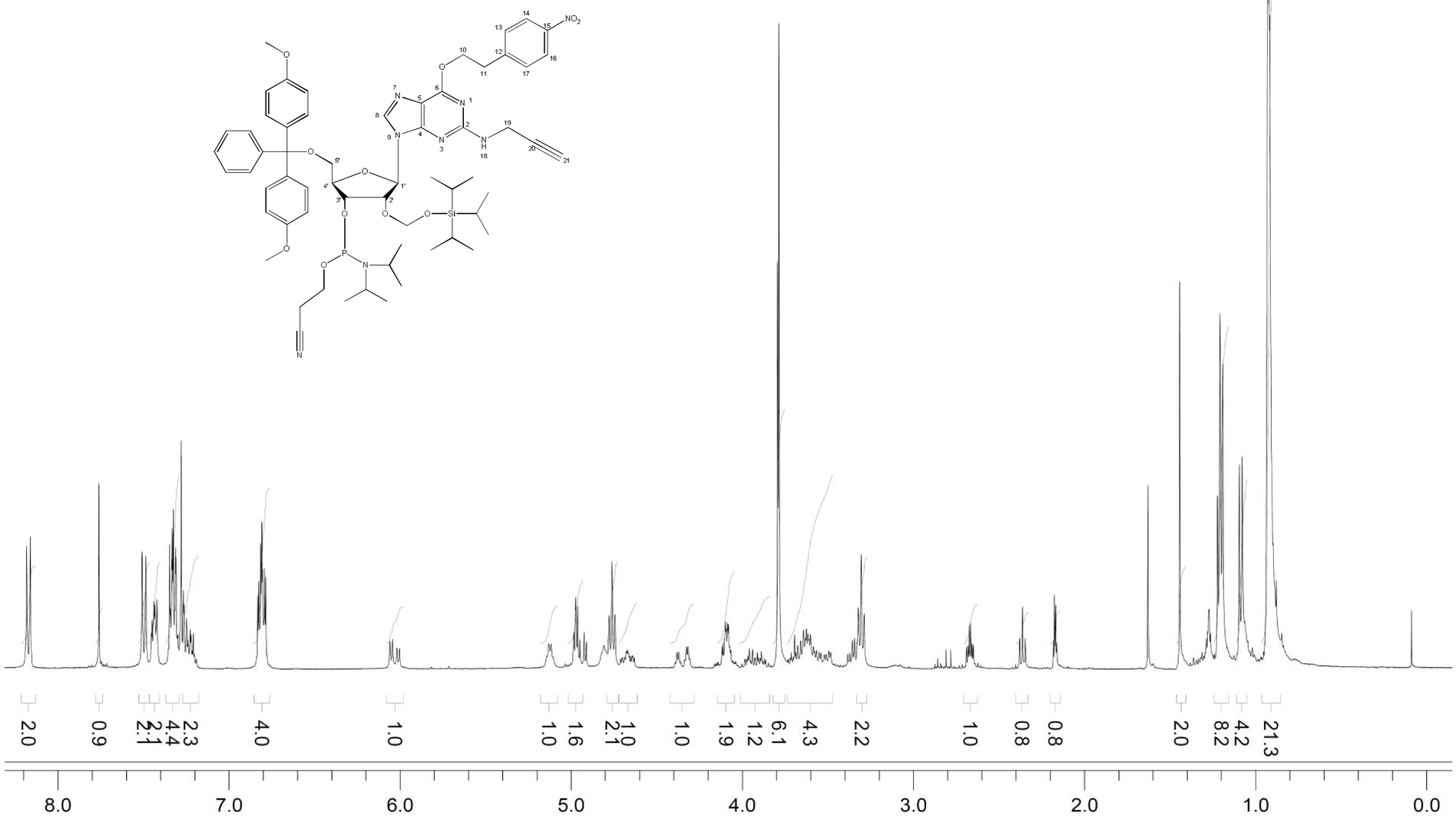


Fig. S1. ^{1}H NMR of the final phosphoramidite, **CTDNPI**.

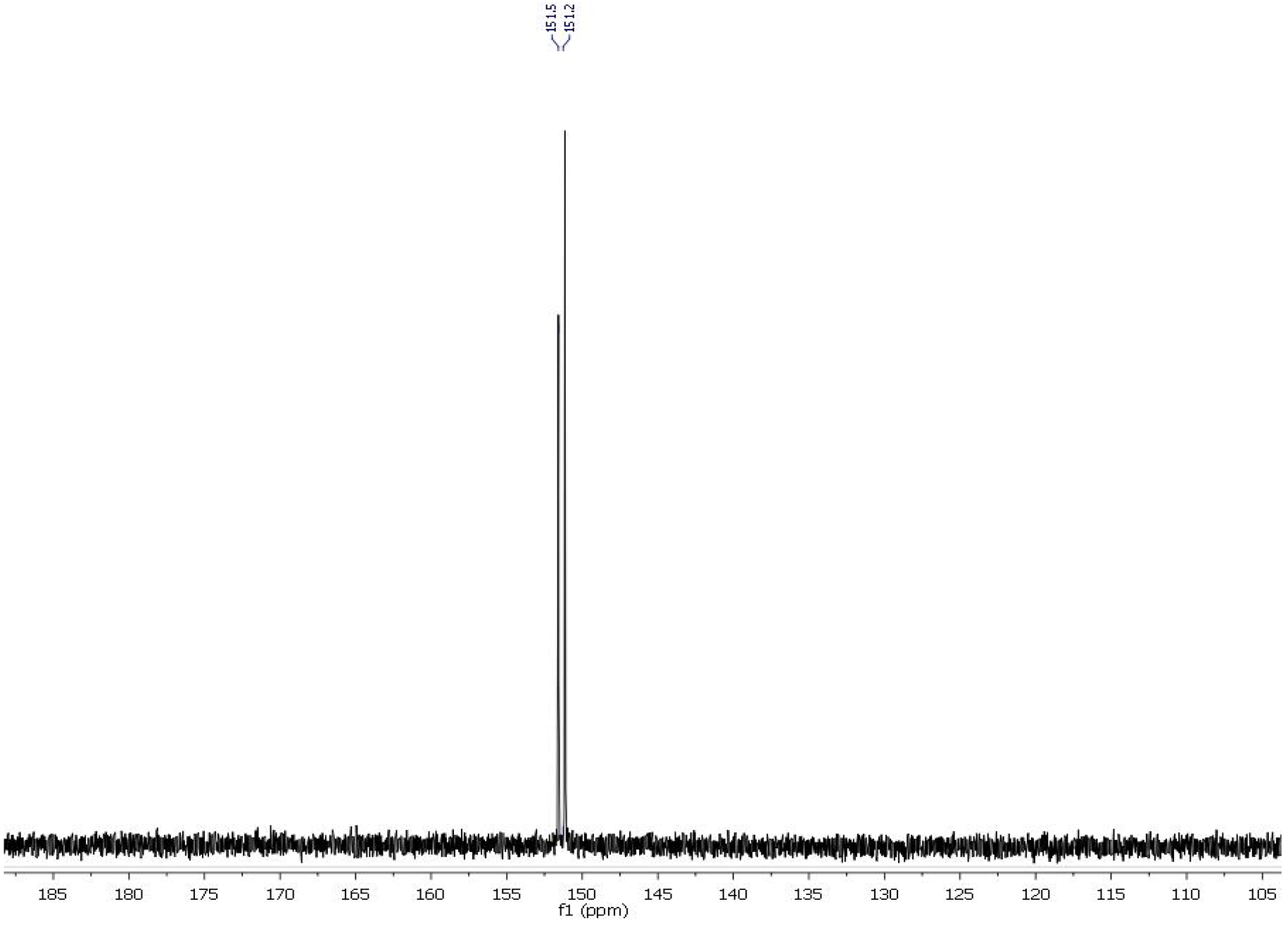


Fig. S2. ^{31}P NMR of the final phosphoramidite, **CTDNPI**.

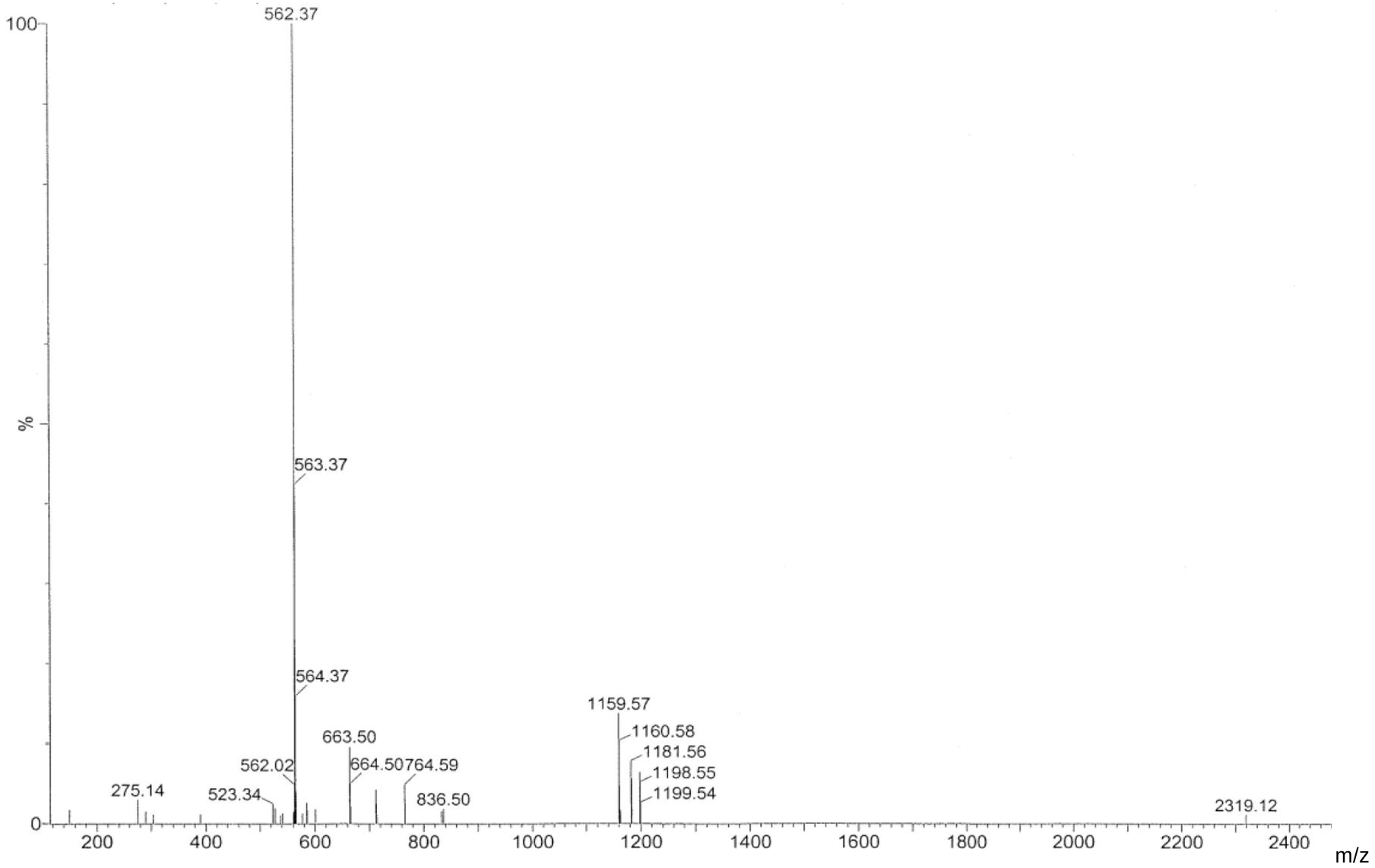


Fig. S3. ESI-MS of the final phosphoramidite, **CTDNPI**. Calculated mass: 1159,52 g/mol.

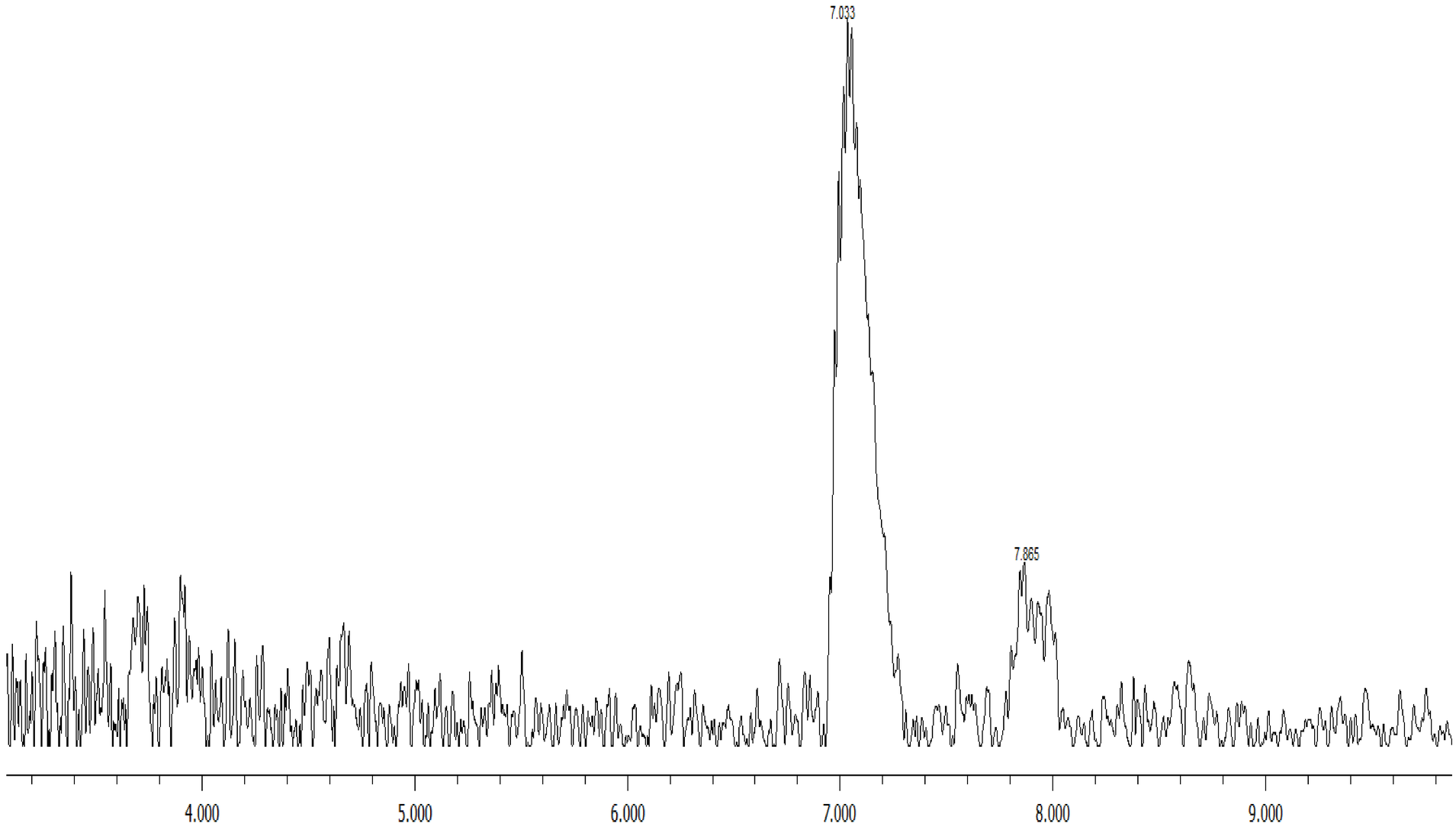


Fig. S4. MALDI-TOF spectrum of the synthesized oligonucleotide, ‘clicked’ with the Atto 590 azide dye (calculated mass: 7862,8 g/mol). The unmodified antisense strand was used as internal standard (calculated mass: 7033 g/mol).

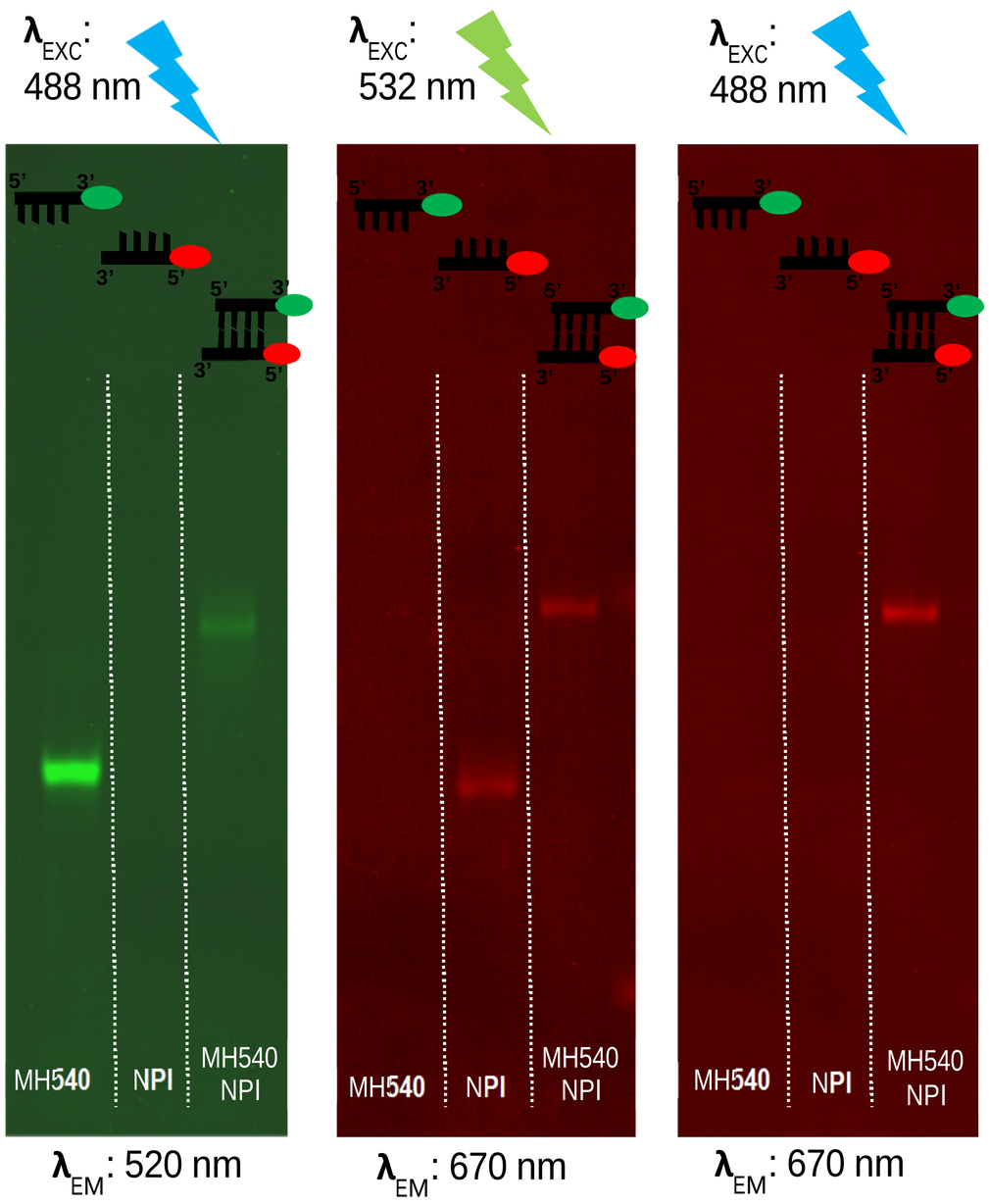


Fig. S5. Polyacrylamide gel electrophoresis of the single stranded siRNAs, MH540 (sense) and NPI (antisense), respectively and the hybridized construct (MH540/NPI). The gels were excited at 488 nm and emission was recorded at 520 nm and 670 nm, respectively (left and right). The middle gel shows excitation at 532 nm and emission at 670 nm.

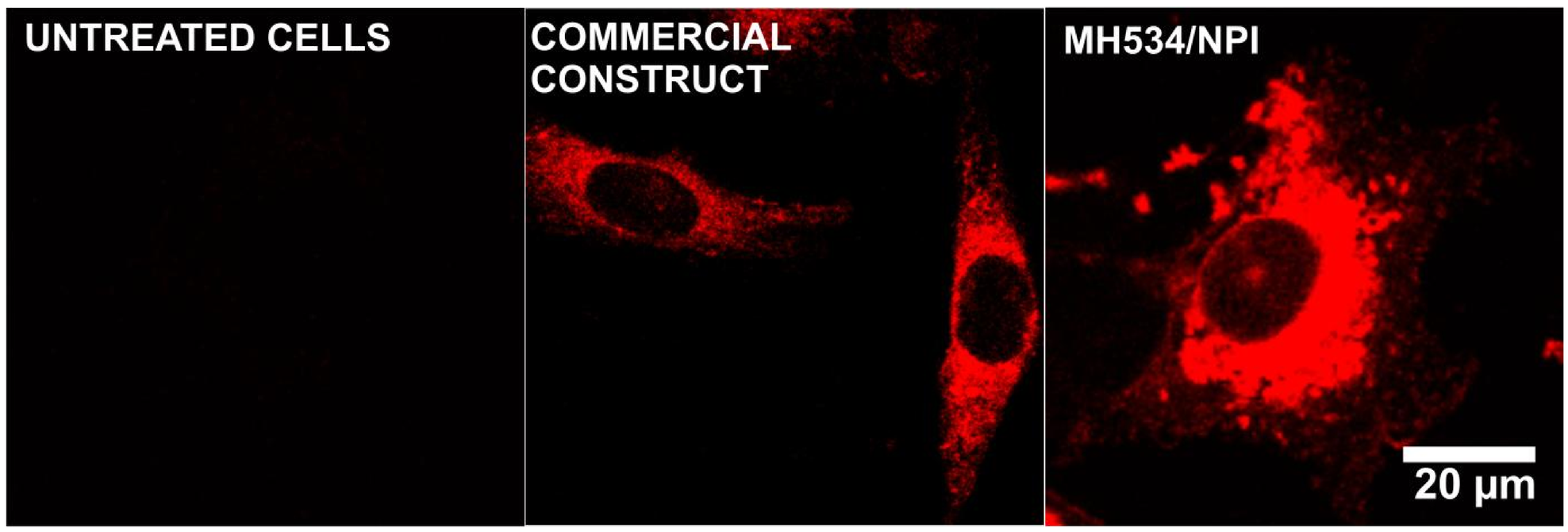


Fig. S6. Cell imaging after excitation at 561 nm of RBE4 cells, transfected with single labeled commercial (middle) and our NPI construct (right), respectively.

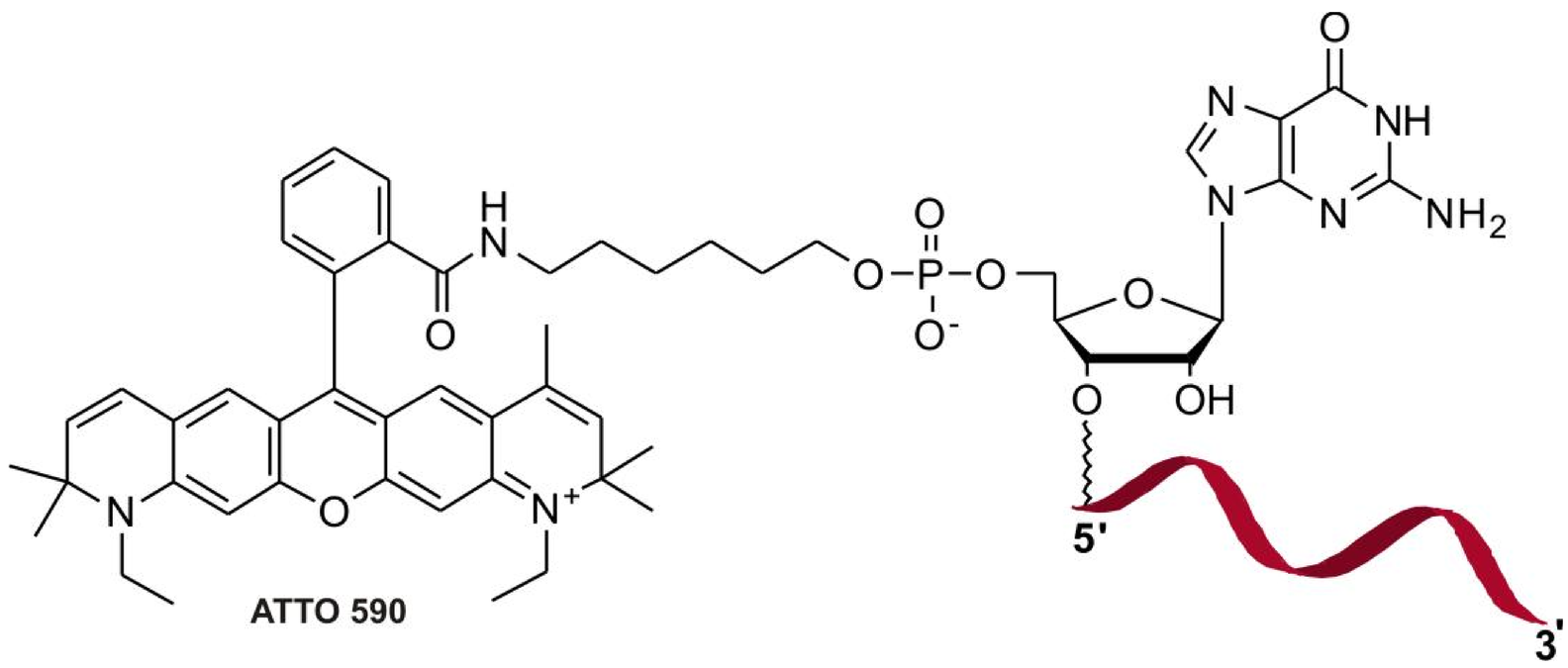


Fig. S7. Commercial antisense strand, containing the Atto 590 dye as label on the 5'-phosphate.

References

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