Synthesis and biological properties of triazole-linked locked nucleic acid

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Supporting Information

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General Information

^{1}H-, ^{13}C- and ^{31}P NMR spectra were recorded on 300/400/500 MHz Bruker Avance spectrometers using TMS as internal standard or normalizing based on the signal from the deuterated solvent. Coupling constants (*J*) are given in Hz. HR-ESI-TOF-MS analyses were carried out on 6530 Accurate-Mass Q-TOF (Agilent Technologies). Analytical TLCs were performed on precoated Merck silica-gel 60F_{254} plates; the spots were detected either under UV light or by charring with 4% alcoholic H_2SO_4. Compounds were purified by silica gel column chromatography or automated flash column chromatography (Biotage) with pre-packed flash chromatography cartridges.

Synthesis and characterization of 2-7

The structure of all the synthesized compounds **2-7** was unambiguously established on the basis of their spectral (^{1}H-, ^{13}C NMR, and HRMS) data analysis. The structure of known compounds **2**,[1] **3a-c,**[2-4] **4b**[3] and **4c**[4] was further confirmed by the comparison of their physical and spectral data with those reported in the literature.

General procedure for the synthesis of dinucleosides 4a-c. Azidonucleoside **5** (ref 1) (0.44 g, 1.49 mmol) and 5′-ethynyl nucleoside **6-8** (ref 2-4) (1.25 mmol) were suspended in a mixture of THF:*t*-BuOH:H_2O (1:1:1) (60 mL). Sodium ascorbate (0.10 g, 0.51 mmol) was then added into the reaction followed by $CuSO_4.5H_2O$ (0.063 g, 0.25 mmol). The reaction mixture was stirred for 12 h. After completion of the reaction, excess solvent was evaporated and traces of moisture were removed by co-evaporation with toluene (2 X 50 mL). The crude thus obtained was purified by silica gel column chromatography using MeOH:$CHCl_3$ (1:4) to afford the triazole-linked dinucleosides **9**, **10** (ref 3) and **11**(ref 4) in 92, 91 and 91 % yields, respectively.

1-(3′-deoxy-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(5′′-deoxy-thymidin-5′′-*yl*)-1,2,3-triazole (4a)

^{1}H NMR (DMSO-d_6, 300 MHz): δ 11.42 (1H, s), 11.29 (1H, s), 8.07 (1H, s), 7.71 (1H, s), 7.41 (1H, s), 6.13 (1H, t, *J* = 6.9 Hz), 5.66 (1H, s), 5.45 (1H, t) 5.32 (1H, d, *J* = 3.3 Hz), 5.07 (1H, s), 4.81 (1H, s), 4.18-4.00 (4H, m), 3.98 (1H, d, *J* = 7.5Hz), 3.59 (1H, d, J = 7.5Hz), 3.02 (2H, m), 2.08 (2H, m), 1.80 (3H, s), 1.77 (3H, s).

^{13}C NMR (DMSO-d_6, 75.5 MHz): δ 163.99, 163.70, 150.44, 150.08, 143.01, 135.99, 134.63, 123.49, 109.73, 108.65, 90.27, 86.15, 84.95, 83.65, 79.28, 72.42, 71.01, 59.40, 56.79, 38.21, 29.22, 12.42, 12.07.

HRMS: *m/z* calculated for [$C_{23}H_{27}N_7O_9H^+$] 546.1943, observed 546.1943.

General procedure for the 5'-DMT protection of triazole-linked dinucleosides 4a-c.

Dimethoxytrityl chloride (675 mg, 2.0 mmol) was added to a solution of dinucleoside **4a-c** (1.5 mmol) in 10 mL of anhydrous pyridine under argon atmosphere. The reaction mixture was then stirred at rt for 6-8 h, quenched with saturated aq. sodium bicarbonate solution (50 mL) and extracted with EtOAc (3 x 50 mL). The organic phase was washed with brine (2 x 100 mL), dried over anhydrous sodium sulphate and concentrated under reduced pressure. The residue thus obtained was purified by flash column chromatography using methanol in chloroform containing 1% TEA to afford the tritylated dinucleosides **5a-c** in 77, 83 and 81 % yields, respectively.

1-(3′-deoxy-5′-*O*-dimethoxytrityl-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(5′′-deoxy-thymidin-5′′-*yl*)-1,2,3-triazole (**5a**)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.47 (1H, s), 11.31 (1H, s), 8.01 (1H, s), 7.64 (1H, s), 7.47-7.43 (3H, m), 7.32-7.29 (7H, m), 6.89 (4H, dd, *J* = 8.8 & 6.0 Hz), 6.14 (1H, t, *J* = 6.69 Hz), 5.69 (1H, s), 5.34 (1H, d, *J* = 4.0 Hz), 5.12 (2H, d, *J* = 5.4 Hz), 4.19-4.18 (1H, m), 3.98-3.85 (2H, m), 3.74 (6H, s), 3.66-3.62 (2H, m), 3.52 (1H, d, *J* = 8.8 Hz), 3.07-3.03 (3H, m), 2.07 (1H, m) , 1.75 (3H, s), 1.56 (3H, s).

^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 164.36, 164.14, 158.66, 150.87, 150.45, 145.07, 143.63, 136.47, 135.62, 135.40, 134.49, 130.29, 130.17, 128.41, 128.07, 127.31, 123.73, 113.76, 113.72, 110.14, 109.24, 89.05, 86.73, 86.56, 85.45, 84.16, 79.66, 79.52, 72.95, 71.67, 60.25, 59.12, 55.51, 45.97, 38.75, 29.73, 12.78, 12.51, 9.03.

HRMS: *m/z* calculated for [$C_{44}H_{45}N_7O_{11}H^+$] 848.3250, observed 848.3269.

1-(3′-deoxy-5′-*O*-dimethoxytrityl-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(5′′-deoxy-2′′-*O*,4′′-*C*-methylene-*xylo*-thymidin-5′′-*yl*)-1,2,3-triazole (**5b**)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.49 (1H, s), 11.30 (1H s), 8.07 (1H, s), 7.66 (1H, s), 7.59 (1H, d, *J* = 1.2 Hz), 7.45 (2H, d, *J* = 7.6 Hz), 7.33-7.29 (7H, m), 6.89 (4H, dd, 9.0 & 7.4 Hz), 5.88 (1H, d, *J* = 3.1 Hz), 5.69 (1H, s), 5.44 (1H, s), 5.14 (2H, d, 7.5), 4.22 (1H, d, *J* = 2.2 Hz), 4.02 (1H,

m), 3.85-3.81 (2H, dd, *J* = 18.8 & 8.6 Hz), 3.74 (6H, d, *J* = 1.1 Hz), 3.63-3.62 (3H, m), 3.55 (1H, d, 9.0 Hz), 3.27 (2H, d, 2.2 Hz), 1.73 (3H, d, 1.0 Hz), 1.57 (3H, s).

^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 164.49, 164.36, 158.67, 150.60, 145.08, 141.75, 137.56, 135.55, 135.44, 134.48, 130.25, 130.18, 128.41, 128.06, 127.33, 124.30, 113.76, 113.71, 109.26, 106.48, 89.02, 88.66, 86.81, 86.56, 79.64, 79.35, 77.95, 73.78, 73.26, 71.69, 60.30, 59.02, 55.50, 23.44, 12.88, 12.81.

HRMS: *m/z* calculated for [$C_{45}H_{45}N_7O_{12}H^+$] 876.3199, observed 876.3207.

1-(3′-deoxy-5′-*O*-dimethoxytrityl-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(5′′-deoxy-2′′-*O*,4′′-*C*-methylenethymidin-5′′-*yl*)-1,2,3-triazole (**5c**)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.49 (1H, s), 11.35 (1H, s), 8.08 (1H, s), 7.66 (1H, s), 7.45 (2H, d, *J* = 7.5), 7.32-7.21 (8H, m), 6.88 (4H, t *J* = 8.5), 5.84 (1H, s), 5.70 (1H, s), 5.38 (1H, s), 5.15 (2H, d, *J* = 4.6), 4.11 (1H, s), 3.92-3.90 (3H, m) 3.74 (6H, s), 3.65-3.62 (3H, m), 3.54-3.51 (1H, m), 3.25 (1H, s), 3.17 (1H, s), 1.62 (3H, s), 1.57 (3H, s).

^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 164.35, 164.21, 158.66, 150.45, 150.33, 145.12, 141.90, 135.52, 135.44, 135.16, 134.46, 130.25, 130.19, 128.41, 128.04, 127.32, 124.82, 113.77, 113.72, 109.26, 108.78, 89.07, 86.92, 86.83, 86.59, 79.65, 79.54, 79.42, 73.09, 71.67, 71.05, 60.26, 55.52, 45.99, 22.90, 12.80, 12.48, 8.99.

HRMS: *m/z* calculated for [$C_{45}H_{45}N_7O_{12}H^+$] 876.3199, observed 876.3211.

General procedure for the synthesis of phosphoramidites 6a-c.

To a stirred solution of trityl-protected dinucleoside **5a-c** (0.5 mmol) in dry DCM (5 ml), *N*,*N*-diisopropylethylamine (0.22 mL, 1.2 mmol) and 2-cyanoethyl *N*,*N*-diisopropylamino chlorophosphoramidite (164 µL, 0.65 mmol) were added under argon atmosphere. The reaction mixture was stirred at rt for 4-6 h. After completion of reaction on analytical TLC examination, the reaction mixture was diluted with EtOAc (50 mL) and washed with saturated KCl solution (2 x 20 mL). The organic phase was dried over anhydrous sodium sulphate and concentrated under reduced pressure. Purification was done by flash column chromatography using methanol in dichloromethane containing 1% TEA to afford the phosphoramidites **6a-c** in 83-89% yields as white foams.

1-(3′-deoxy-5′-*O*-dimethoxytrityl-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(3"-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite-5′′-deoxy-thymidin-5′′-*yl*)-1,2,3-triazole (**6a**)

^{31}P NMR (162 MHz, $CDCl_3$): δ 148.59, 148.32.

MS: *m/z* calculated for [$C_{45}H_{45}N_7O_{12}Na^+$] 1070.4, observed 1069.1.

1-(3′-deoxy-5′-*O*-dimethoxytrityl-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(3"-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite-5′′-deoxy-2′′-*O*,4′′-*C*-methylene-*xylo*-thymidin-5′′-*yl*)-1,2,3-triazole (**6b**)

^{31}P NMR (162 MHz, $CDCl_3$): δ 152.11, 151.03

MS: *m/z* calculated for [$C_{45}H_{45}N_7O_{12}Na^+$] 1098.4, observed 1097.6.

1-(3′-deoxy-5′-*O*-dimethoxytrityl-2′-*O*,4′-*C*-methylenethymidin-3′-*yl*)-4-(3"-*O*-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite-5′′-deoxy-2′′-*O*,4′′-*C*-methylenethymidin-5′′-*yl*)-1,2,3-triazole (**6c**)

^{31}P NMR (202.5 MHz, CD_3CN): δ 149.06, 148.48

MS: *m/z* calculated for [$C_{45}H_{45}N_7O_{12}Na^+$] 1098.4, observed 1097.5.

General procedure for LCAA-CPG derivatization with dinucleosides 5a-c.

A solution of dinucleoside **5a-c** (0.1 mmol), succinic anhydride (25 mg, 0.25 mmol), DMAP (5mg), TEA (.07 mL, 0.5 mmol) in DCM (2 mL) was stirred at rt for 3 h. The reaction was diluted with DCM (50 mL) and washed with brine (50 mL). The organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude residue was coevaporated with dry pyridine (3 x 2 mL). The triethylammonium salt thus obtained was stirred with HATU (76 mg, 0.2 mmol) and DIPEA (0.35 mL, 2.0 mmol) in DMF (7.0 mL) at rt for 10 minutes. This reaction mixture was then transferred to a centrifuge tube containing 1.0 g of LCAA CPG 1000Å. The reaction mixture was gently shaken at rt overnight. The solid support was filtered and successively washed with DCM, MeOH, DCM and diethyl ether (25 mL each).

For capping, the derivatized CPG was suspended with acetic anhydride (3 mL), pyridine (3 mL) and DMAP (50 mg) and was gently shaken at rt for 2 h. The support was filtered, successively washed with DCM, MeOH, DCM, diethyl ether (25 mL each) and then air dried. The loading of CPG $T^{L}{}_{T}T$ **7a**, $T^{L}{}_{T}T^{xylo\text{-}L}$ **7b** and $T^{L}{}_{T}T^{L}$ **7c** was 32, 35 and 30 μ mol/g as determined by absorbance of DMT cation at 495 nm.

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer at 1 µmol scale. Oligonucleotides with trizole-modified 3′-end were synthesized on 1000Å LCAA CPG functionalized with the corresponding dinucleoside blocks (30-35 µmol/g) which was prepared according to the procedure described above. The remaining oligonucleotides were synthesized on Unylinker CPG (ChemGenes, 40 µmol/g). Activation was achieved with 5-benzylthio-1*H*-tetrazole (BTT, 0.3 M in acetonitrile). Oxidation was achieved using 0.02 M iodine in a mixture of THF, water, and pyridine. Sulfurization was accomplished with DDTT (0.1 M, ChemGenes). LNA phosphoramidites were synthesized using standard procedures from 3′-hydroxyl precursors purchased from Rasayan. DNA and 2'-*O*-TBDMS-protected RNA phosphoramidites were purchased from ChemGenes. For coupling, 0.10 mM solutions of DNA and LNA phosphoramidites and 0.15 mM solutions of RNA and triazole-modified phosphoramidites were prepared in acetonitrile. The phosphoramidite coupling efficiency of LNA and triazole-linked dinucleosides was 95-96% except in case of 3'-*xylo*-configured dinucleoside $T^{L}{}_{T}T^{xylo\text{-}L}$ where only 69% coupling was observed. Standard conditions were used for synthesis and deprotection of unmodified DNA and RNA. Coupling time for RNA and modified phosphoramidites was extended to 10 minutes.

Oligonucleotides were deprotected with concentrated NH_4OH (1 mL) at 55 ºC overnight. Then the oligonucleotides were evaporated to dryness in a centrifugal evaporator and resuspended in 1 mL RNase-free water. RNA oligonucleotides were deprotected using NH_4OH/EtOH (3:1, 2 mL) for 48 h at room temperature, then the TBDMS protecting groups were cleaved with a DMSO/TEA.3HF (4:1) solution (500 µL) at 65 ºC for 3 h. The deprotected RNA oligonucleotides were then precipitated by addition of 3M NaOAc (25 µL) and *n*-BuOH (1 mL), and the pellet was washed with 70% EtOH and resuspended in 1 mL RNase-free water.

Oligonucleotides were purified by reverse phase chromatography using an Agilent 1200 HPLC using C-18 columns and triethylammonium acetate (TEAA, pH 6.0) buffers. Pure fractions were desalted using NAP-25 columns (GE Healthcare). Oligonucleotide masses were verified by LC-MS analysis on an Agilent 6530 Accurate-Mass Q-TOF using gradients of water and methanol, both containing 9 mM Et_3N and 100 mM hexafluoroisopropanol.

UV melting experiments

UV melting was monitored on a Cary UV-Visible Spectrophotometer at 2 µM concentration of each oligonucleotide in 9 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.4 mM KCl, 123 mM NaCl, pH 7.4. Spectra were recorded at 260 nm. The samples were initially heated to 40 ºC then cooled to 15 ºC at 2 ºC min^{-1}. Then the samples were heated to 95 ºC and cooled to 15 ºC at 1 ºC min^{-1}. Six successive melting curves were measured and T_m values were calculated from derivative analysis using Cary Win UV Thermal application Software.

CD spectroscopy

Circular dichroism (CD) spectra of duplexes were collected from 200 to 350 nm on a Jasco-810 spectropolarimeter with a thermoelectric temperature control system in a 0.2 cm cuvette at 4 ºC. The duplexes were prepared by annealing oligonucleotides at 2.0 µM concentration in 9 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.43 mM KCl, 123.3 mM NaCl, pH 7.4. Three spectra were obtained and averaged, and baseline-corrected with blank containing the buffer only.

Nuclease Stability Assays

SVPD stability: In 200-µL PCR tubes, 5 nmol of oligonucleotide was dissolved in 20 µL of Snake venom phosphodiesterase (SVPD) buffer (100 mM Tris-HCl, 100 mM NaCl, 14 mM $MgCl_2$, pH 8.9) and warmed to 37 ºC. From this mixture 2.5 µL was removed as a zero point. SVPD (Phosphodiesterase I from Crotalus adamanteus venom, USB Corporation, Cleveland, Ohio; 0.5 Units in 17.5 µL of SVPD buffer) was warmed to 37 ºC, and then added to start the reaction. The reactions were topped with 10 µL of mineral oil to avoid any concentration changes by evaporation. Aliquots (5 µL) were removed at various time points, pipetted onto 5 µL formamide, heated to 95 ºC for 1 minute and then stored at -20 ºC. The samples were analyzed by 24% denaturing PAGE (7 M urea) and visualized using Stains-All dye.

XRN1 stability: We used Terminator™ phosphate-dependent 5'-to-3' processive exonuclease (Epicentre) for these assays. Although the exact composition of this enzyme is proprietary, it is closely related to *S. cerevisiae* XRN1,[5, 6] and has been used as an XRN1 analogue before.[7, 8] In 200-µL PCR tubes, 30 pmol of oligonucleotide was dissolved in 8 µl of RNase-free water and 1 µl of 10X reaction buffer A (Epicentre). To this solution, 1 µl of Terminator™ phosphate-dependent 5'-to-3' exonuclease (Epicentre) was added and the reaction was incubated at 37 ºC for 12h. The reaction samples were then heated to 95 ºC for 1 minute and then stored at -20 ºC. The samples were analyzed by 24% denaturing PAGE (7 M urea) and visualized using Stains-All dye.

Mammalian cell culture

MRC-5 human lung fibroblasts were purchased from the American Type Culture Collection (ATCC) and cultured in MEM media supplemented with 10% FBS and 1% NEAA (Sigma-Aldrich Co.) HeLa cells (ATCC) were cultured in DMEM with 10% fetal bovine serum (Gibco) and 100 U/mL penicillin/streptomycin (Invitrogen). Cells were maintained in 5% CO_2 cell culture incubator.

ASO transfection and analysis

MRC-5 cells were seeded in 12 well plates at 80K cells/well (lipid transfection) or 5k cells/well (gymnotic delivery) at least 16 h prior to transfection. ASOs targeting human *ADAM33* were transfected using Lipofectamine RNAiMax reagent (Invitrogen) to a final concentration of 50 nM in OptiMEM for single dose or serially diluting to lower concentrations for dose responses. Gymnotic delivery was achieved by continuously treating the cells with 3 μM ASOs in full media or serially diluting to lower concentrations for dose responses. Cells were harvested 3 days after lipid transfection or, in the case of gymnotic delivery, after 7 days of exposure to oligonucleotide. Total RNA was extracted from the cells using TRI reagent (Sigma-Aldrich) following the manufacturer's protocol. In brief, cell supernatant was removed and washed once with 1X PBS. Cells were lysed directly on the culture plate by adding 250 μL of TRI reagent per well, incubating at rt for 5 min and pipetting several times to form a homogenous lysate. The lysate was then transferred to 1.7-mL microcentrifuge tubes, and 200 μL of chloroform was then added to each tube and shaken vigorously for at least 15 seconds. The tubes were incubated for 10 min at room temperature followed by 15 min centrifugation at 10800 RPM for phase separation. The clear aqueous phase was then transferred into new tubes containing 500 μL of 2-propanol. The mixture was then incubated at -20 ºC for 20 min followed by centrifugation at 10800 RPM for 10 min. The resulting pellet was washed with 1 mL ice-cold 75 % ethanol and briefly air dried. The RNA was resuspended in 20 μL of nuclease-free water and heated at 55 ºC for 5 min. RNA was quantified using Nanodrop One (Thermo scientific). The total RNA yield from this quantity of cells was typically 8–10 ug with A260/A280 in the range of 1.9 – 2.10. 1 μg of RNA was then treated with 2U of DNase I (Worthington Biochemical) at 37 ºC for 10 min followed by heat inactivating the enzyme at 75 ºC for 5 min. The DNase-treated RNA was then reverse transcribed to cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's protocol (incubation with random primers and RT enzyme for 10 min at 25 ºC followed by 2 h at 37 ºC and then heating the reaction mixture to 85 ºC for 5 min). qRT-PCR was performed in

technical duplicate, with 20 ng of cDNA in a 20 μL reaction, using a final concentration of 1x iTaq Universal Probes SuperMix (cat. #172-5132, Bio-Rad). We used PrimeTime qPCR primer-probe sets (IDT) at 500 nM forward and reverse primers and 250 nM probe and carried out the PCR on the CFX96 real-time system (Bio-Rad). Cycling conditions were as follows: hot start of 95 ºC for 10 min, then cycling at 95 ºC for 15 sec, 60 ºC for 1 min, 40 cycles. C_t values were determined using the threshold method, where the threshold was automatically determined by the instrument software. GAPDH was chosen as a reference gene; the C_t values for GAPDH remained constant irrespective of oligonucleotide treatment. The C_t values were within the linear range of the standard curve, and as such, all were well within the limit of detection (the highest C_t values were still under 34). A 'no-RT' control confirmed that the samples were free of confounding genomic DNA. The target mRNA expression was quantified using the $\Delta\Delta C_t$ method and normalized to GAPDH mRNA expression levels. Results were analysed in Microsoft Excel. No replicates were considered outliers or removed. Specificity was optimized by using primer/probe assays rather than SYBR Green methods.

Primer set details including standard curve information:

ADAM33: IDT PrimeTime assay. A standard curve was run from 100 ng/μL to 40 pg/μL total cellular RNA, (~31 to 38 C_t,) and was linear throughout this range. Slope = -3.224; y-intercept = 37.9; R^2 = 0.9563, efficiency = 104%. Primer and probe sequences:

forward primer, 5′-GGCCTCTGCAAACAAACATAATT-3′;

reverse primer, 5′-GGGCTCAGGAACCACCTAGG-3′;

probe, 5′- /56FAM/ CTTCCTGTT /ZEN/ TCTTCCCACCCTGTCTTCT CT /3IABkFQ/ -3′;

GAPDH: IDT PrimeTime assay. A standard curve was run from 100 ng/μL to 40 pg/μL total cellular RNA, (~18 to 26 C_t,) and was linear throughout this range. Slope = -3.3695; y-intercept = 24.992; R^2 = 0.9993, efficiency = 101%. Primer and probe sequences:

forward primer, 5′-TGGTCCAGGGGTCTTACT-3′;

reverse primer, 5′-CCTCAACGACCACTTTGT-3′;

probe, 5′- /56FAM/ CTCATTTCC /ZEN/ TGGTATGACAACGAATTTGGC /3IABkFQ/ -3′.

RNA integrity. Multiple additional probe sets targeting additional regions of the mRNA gave similar qPCR results; the above probe sets were chosen for optimal efficiency. A random selection of RNA samples were analysed on an Agilent BioAnalyzer capillary electrophoresis instrument and showed RIN numbers between 6.6 and 9.5.

siRNA transfection and analysis

Duplexes of hsiRNA were formed by combining equimolar amounts of sense and antisense strands suspended in water. The solution was heated to 90 ºC for 1 minute and allowed to cool to rt. Duplex formation was validated by PAGE on a 20% TBE gel (*Invitrogen*) and stained with SybrGold for visualization.

Cells were plated in DMEM with 6% FBS at 10,000 cells per well in 96-well tissue culture treated plates. siRNA was diluted to four times the final concentration in OptiMEM, and Lipofectamine® RNAiMAX Transfection Reagent (*Invitrogen*) was diluted to four times the final concentration (final = 0.3 µl/25 µl/well). RNAiMAX and siRNA solutions were mixed 1:1, and 50 µl of the transfection mixture was added to 50 µl of cells resulting in 3% FBS final. Cells were incubated for 72 hours at 37°C and 5% CO_2.

mRNA quantification was done using the QuantiGene 2.0 branched DNA (bDNA) Assay (Affymetrix). Cells were lysed in 250 µL diluted lysis mixture composed of 1 part lysis mixture, 2 parts H_2O, and 0.167 µg / µL proteinase K (Affymetrix) for 30 minutes at 55 ºC. Cell lysates were mixed thoroughly, and 40 µL (~8000 cells) of each lysate was added per well to a capture plate with 40 µL diluted lysis mixture without proteinase K. Probe sets were diluted as specified in the Affymetrix protocol. For HeLa cells, 20 µL human HTT or HPRT probe set (Affymetrix) was added to appropriate wells for a final volume of 100 µL. Signal was amplified according to the Affymetrix protocol. Luminescence was detected on a Tecan M1000 (Tecan).

Huntingtin mRNA levels were normalized to the housekeeping gene HPRT. Data in Fig 4 are presented as percent of untreated control (n=3 wells, mean ± SD). Graph is representative. IC50 and silencing values were averaged across two independent experiments.

Statistical analysis

Data was analyzed using GraphPad Prism 7 software (GraphPad Software, Inc., San Diego, CA). Concentration-dependent IC_{50} curves were fitted using a log(inhibitor) vs. response - variable slope (four parameters).

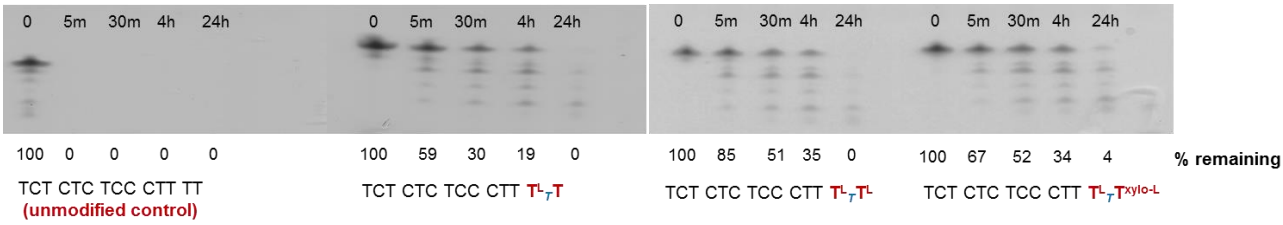


Fig S1. 24% Denaturing PAGE showing stability of triazole-modified oligonucleotides to the 3'-exonuclease SVPD. Oligonucleotides were incubated at 37 °C with 0.5 units SVPD; aliquots were removed at the times indicated in minutes (m) and hours (h).

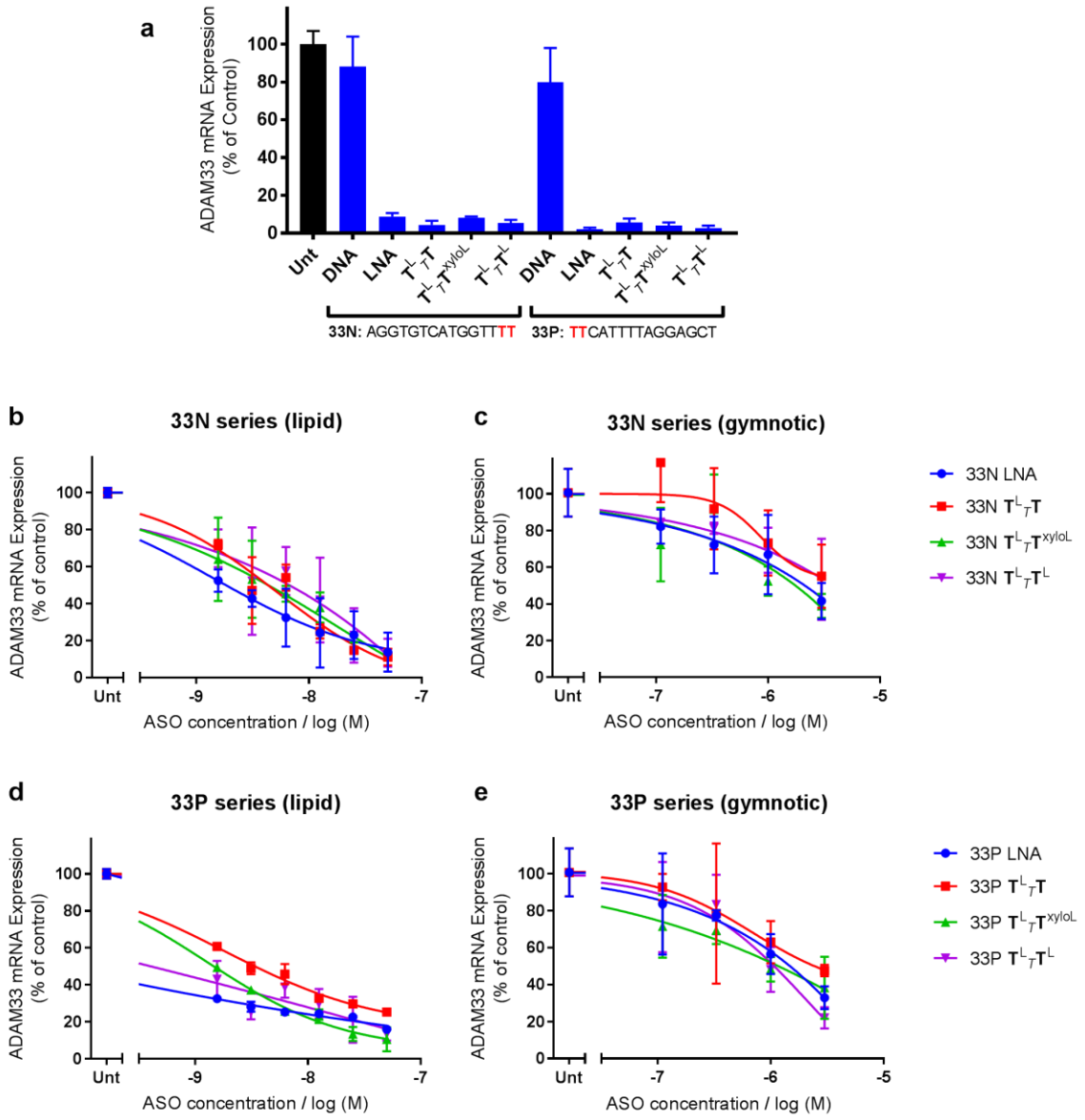


Fig S2. Gapmers containing triazole-linked LNA dimers at the 3'- or 5'-end are of comparable efficacy and potency to the parent LNA gapmers. (a) qRT-PCR results of human ADAM33 mRNA levels when MRC-5 lung fibroblasts are treated with oligonucleotides at 50 nM. **TT** represents the site of modification in the context of what is otherwise a 3-9-3 LNA gapmer (see SI Table 3 for full details of modification patterns). The similar potency of all the gapmer sequences is confirmed by dose responses after (b, d) lipid transfection or (c, e) gymnotic delivery. All data are normalised to GAPDH and represent results from two biological replicates.

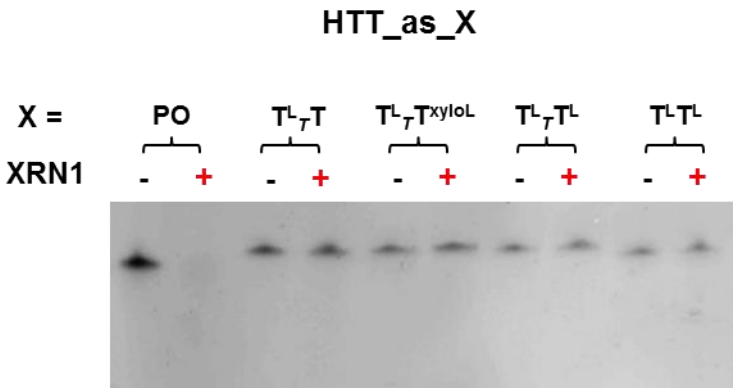


Fig S3. Triazole-modified siRNAs are resistant to the 5'-exonuclease XRN1. Antisense strands (30 pmol) were incubated with (+) and without (-) XRN1 5'-exonuclease at 37 ºC for 12h, then resolved by 24% denaturing PAGE.

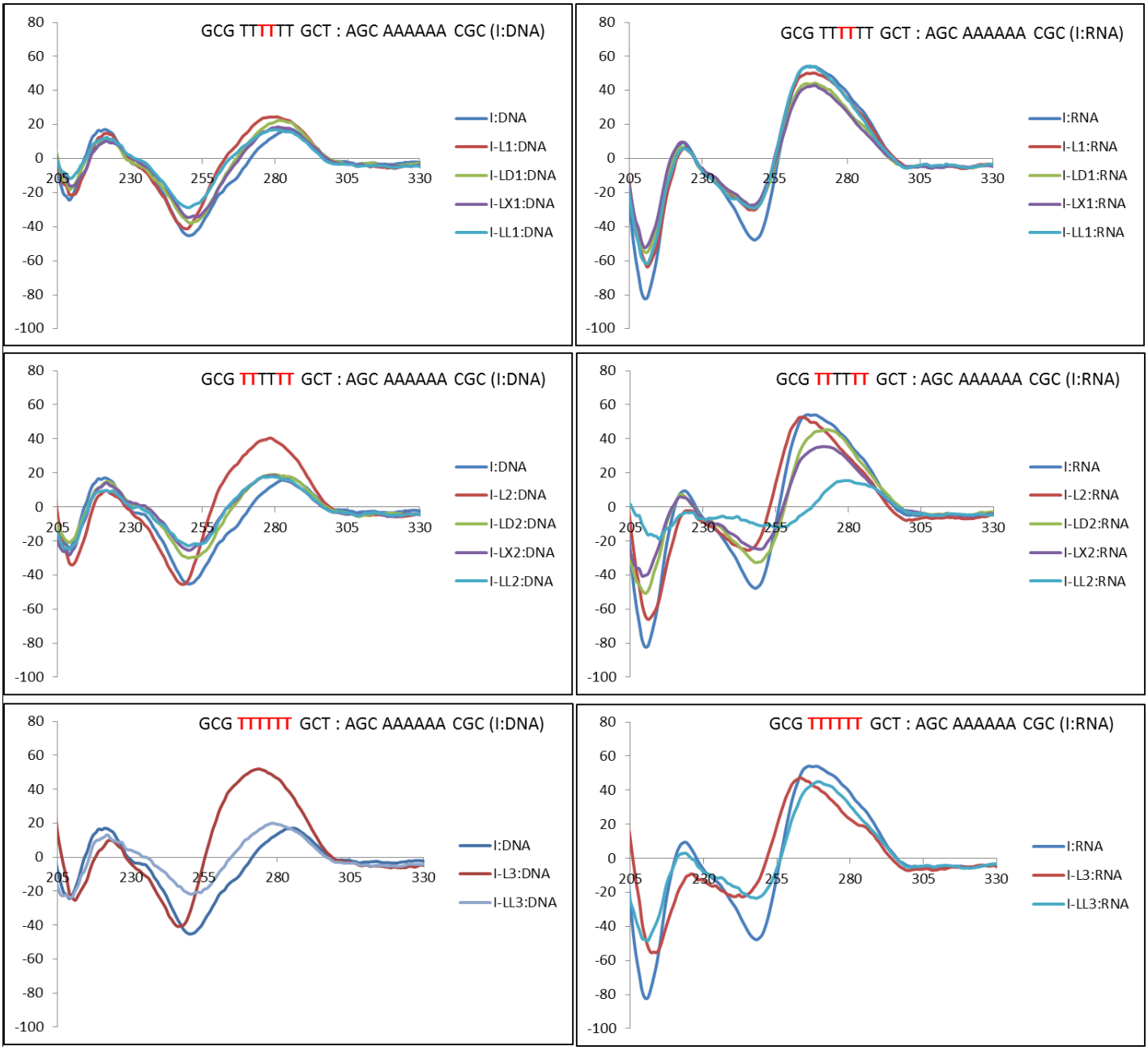


Fig S4. The CD spectra of DNA, LNA and triazole-linked DNA with complementary DNA (left) and RNA (right) at 4°C. The bold letters indicate the site of modification; duplex concentration was 4.0 µM in 9mM Na_2HPO_4, 1.8mM KH_2PO_4, 2.43mM KCl, 123.3 mM NaCl, pH = 7.4. Sequences and modification patterns are spelled out in full in Table 1 (main text).

Discussion of CD data (data shown above in Fig. S4):

With the DNA complementary strand (left column) the unmodified duplex adopts a B-form duplex as expected. With increasing numbers of locked nucleotides, the structure becomes more A-like (eg. see bottom left panel, I-L3:DNA trace). In contrast, the triazole analogues maintain a more B-type helix. With the RNA complementary strand, the helix is closer to A-form but the triazole analogues still appear to prefer more B-form character: for the L2 and L3 series (middle and lower right panels), note that the triazole-containing sequences feature a global maximum that is red-shifted and of lower intensity with respect to the unmodified or the LNA-containing strands, which is consistent with a more B-like helical structure.

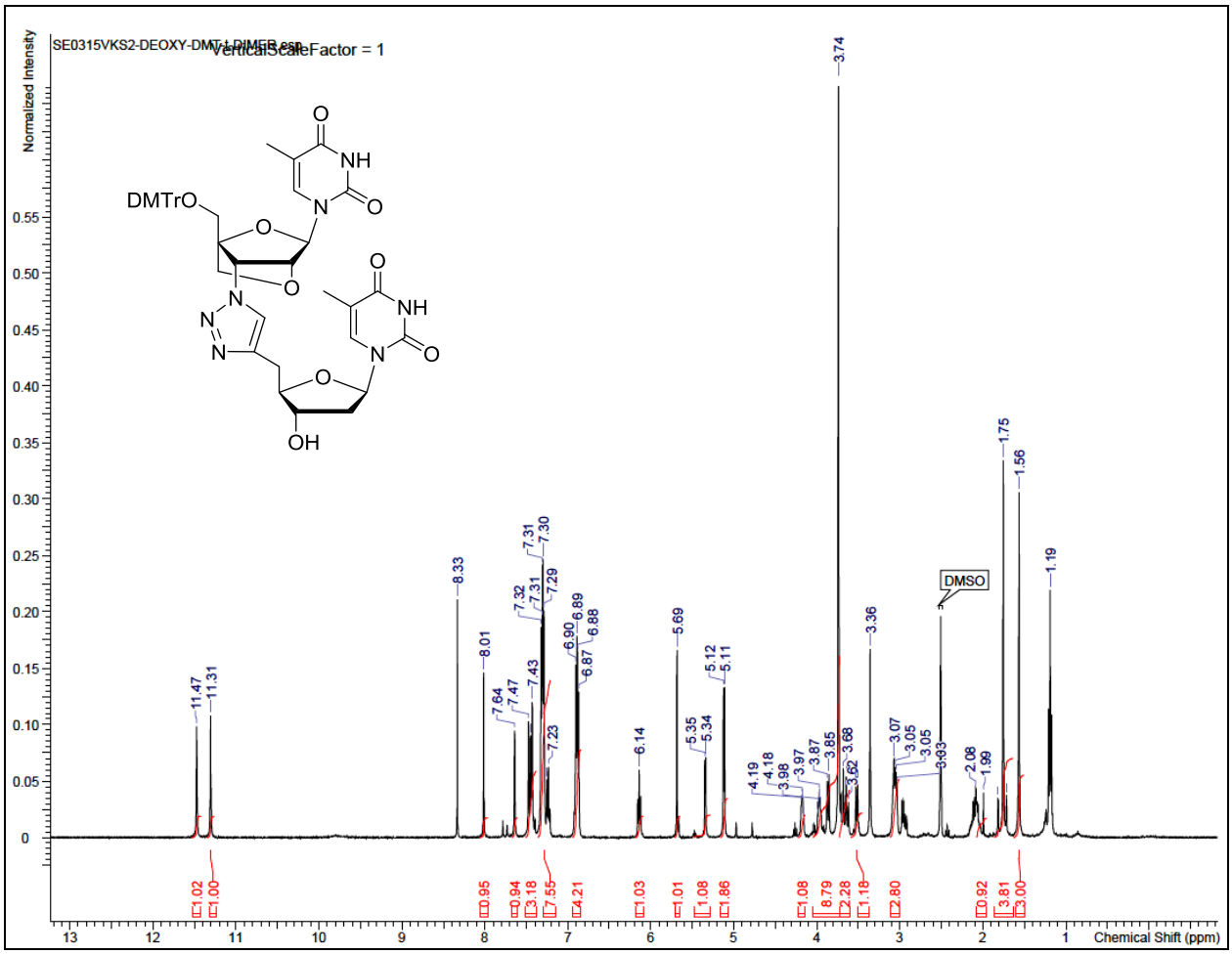


Fig S5. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound **5a**

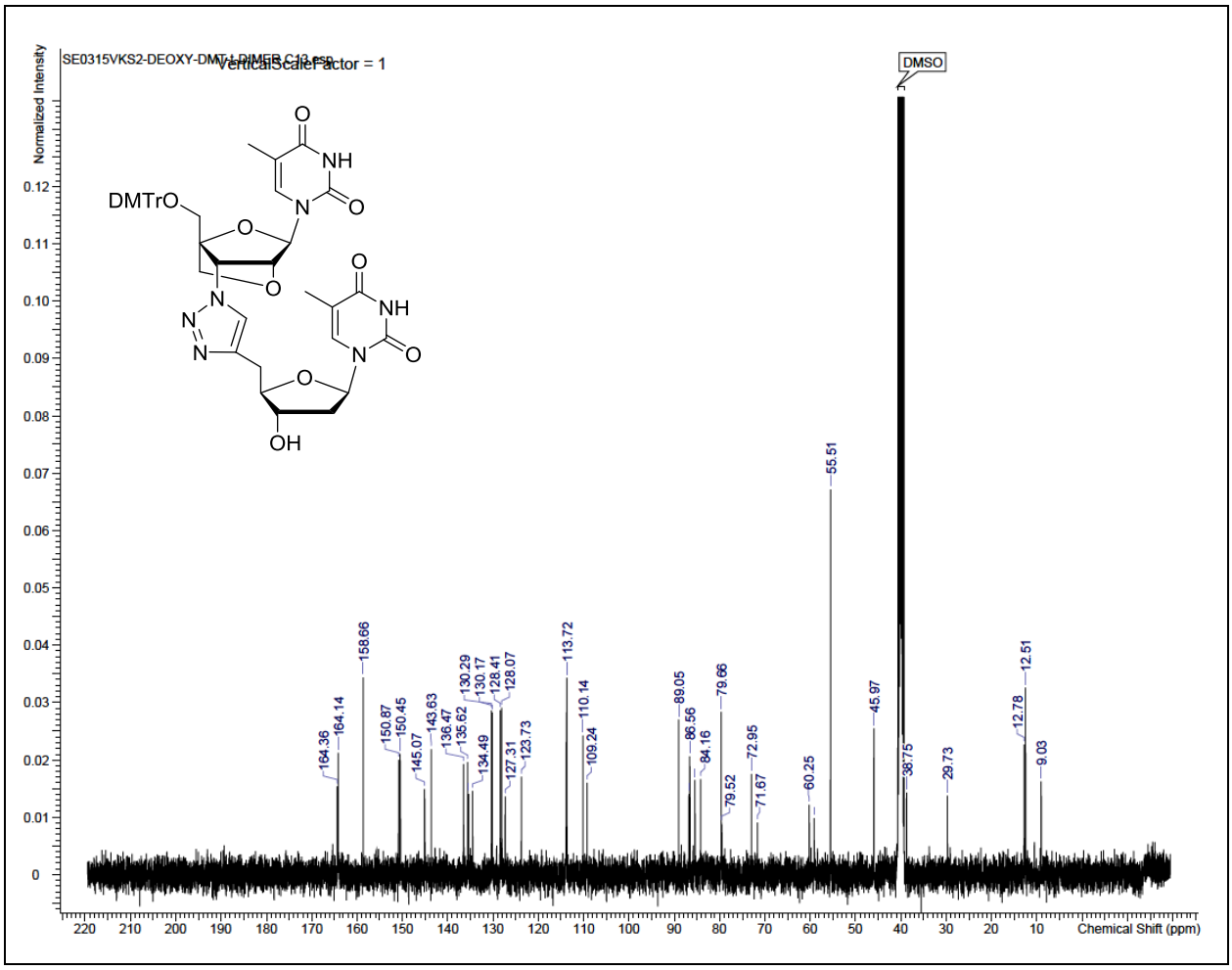


Fig S6. ^{13}C NMR spectrum (100.6 MHz, DMSO-d_6) of compound **5a**

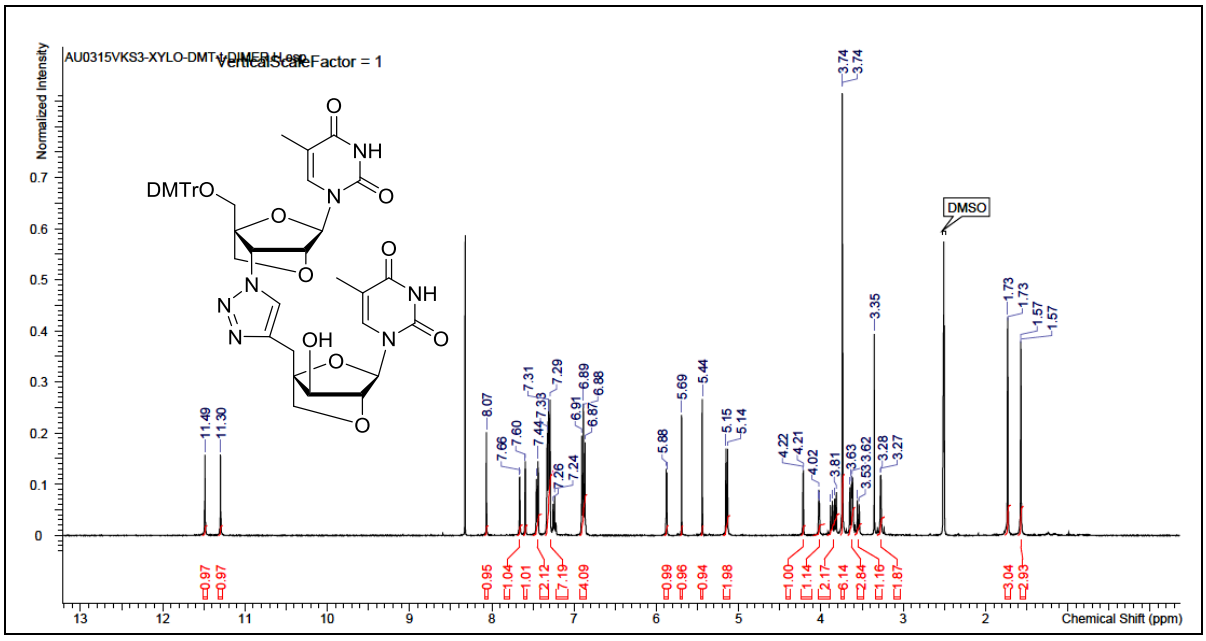


Fig S7. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound **5b**

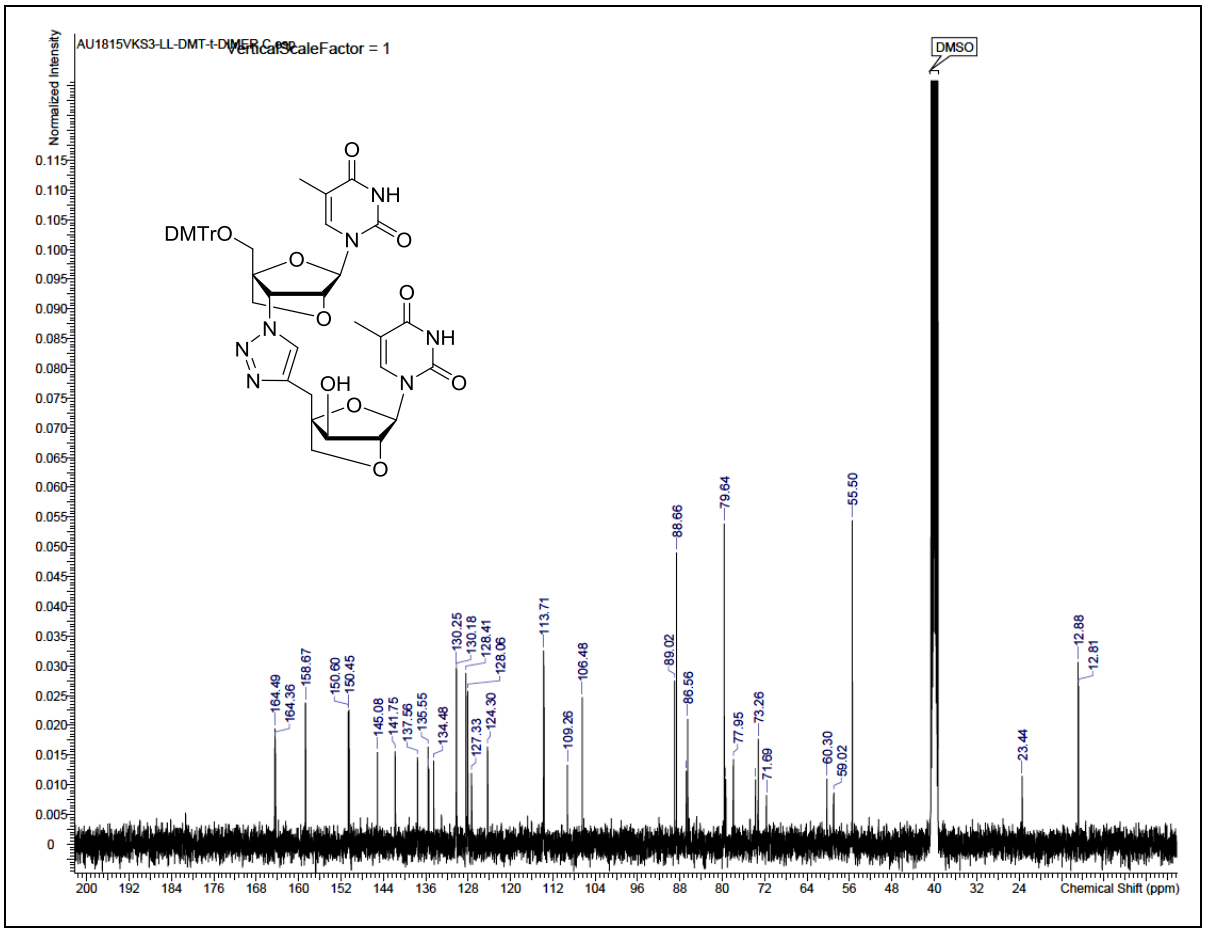


Fig S8. ^{13}C NMR spectrum (100.6 MHz, DMSO-d_6) of compound **5b**

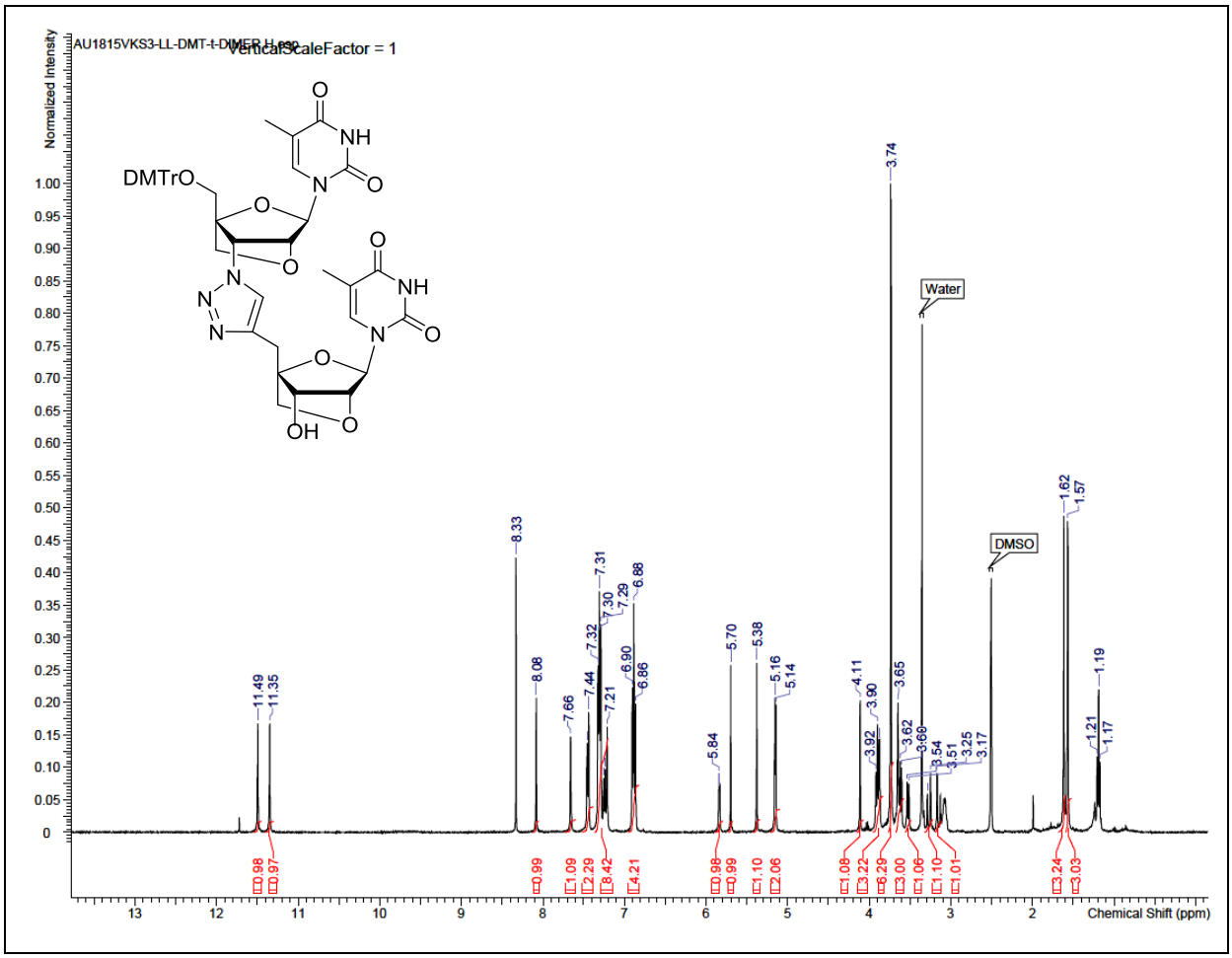


Fig S9.[1]H NMR spectrum (400 MHz, DMSO-d_6) of compound **5c**

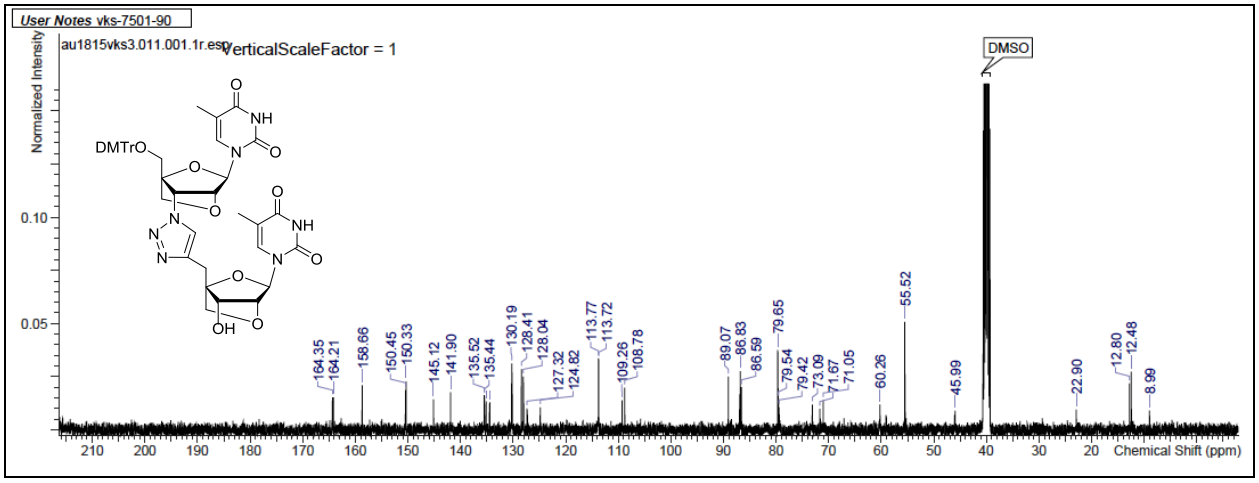


Fig S10.[13]C NMR spectrum (100.6 MHz, DMSO-d_6) of compound **5c**

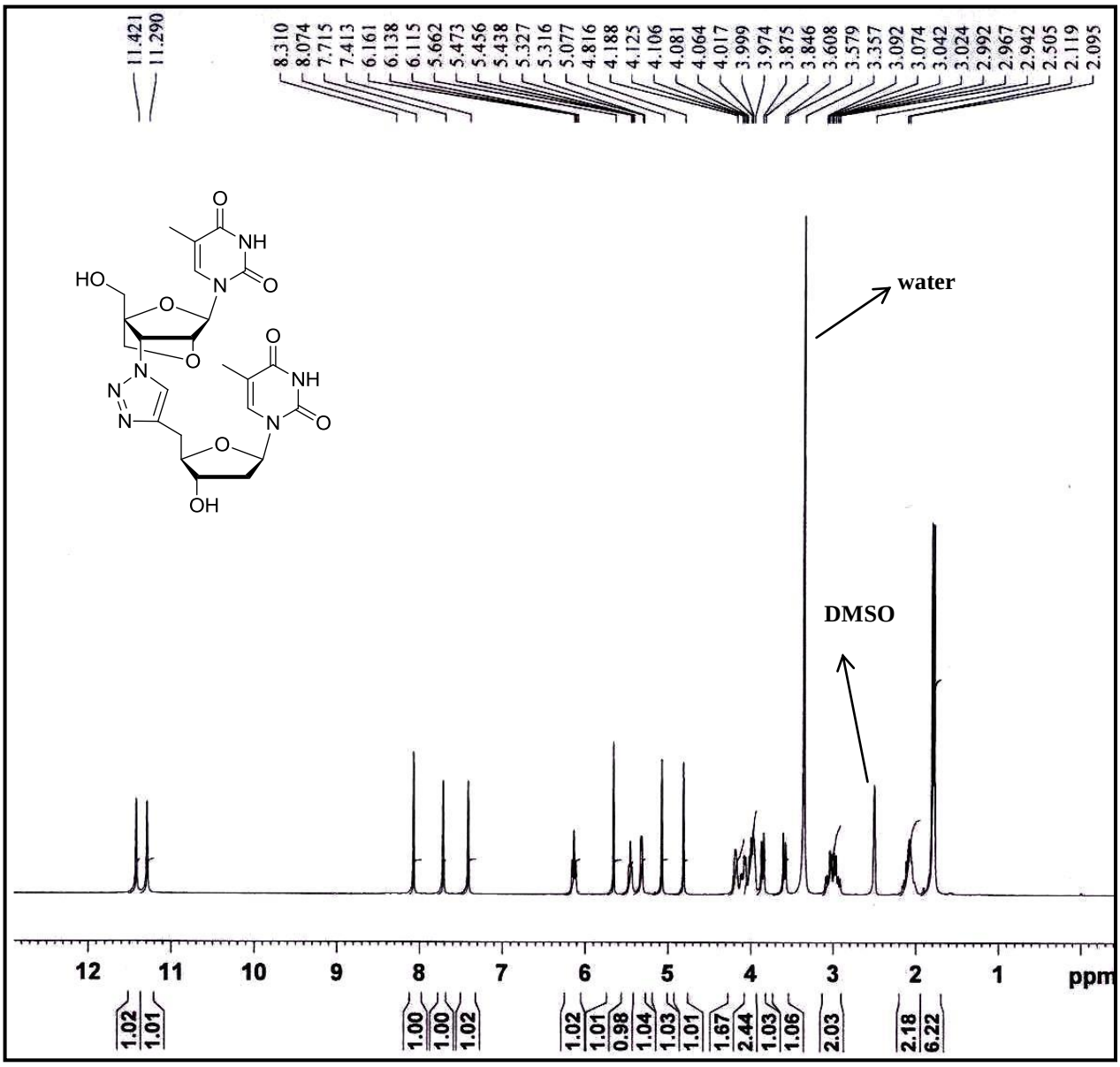


Fig S11. ^{1}H NMR spectrum (300 MHz, DMSO-d_6) of compound **4a**

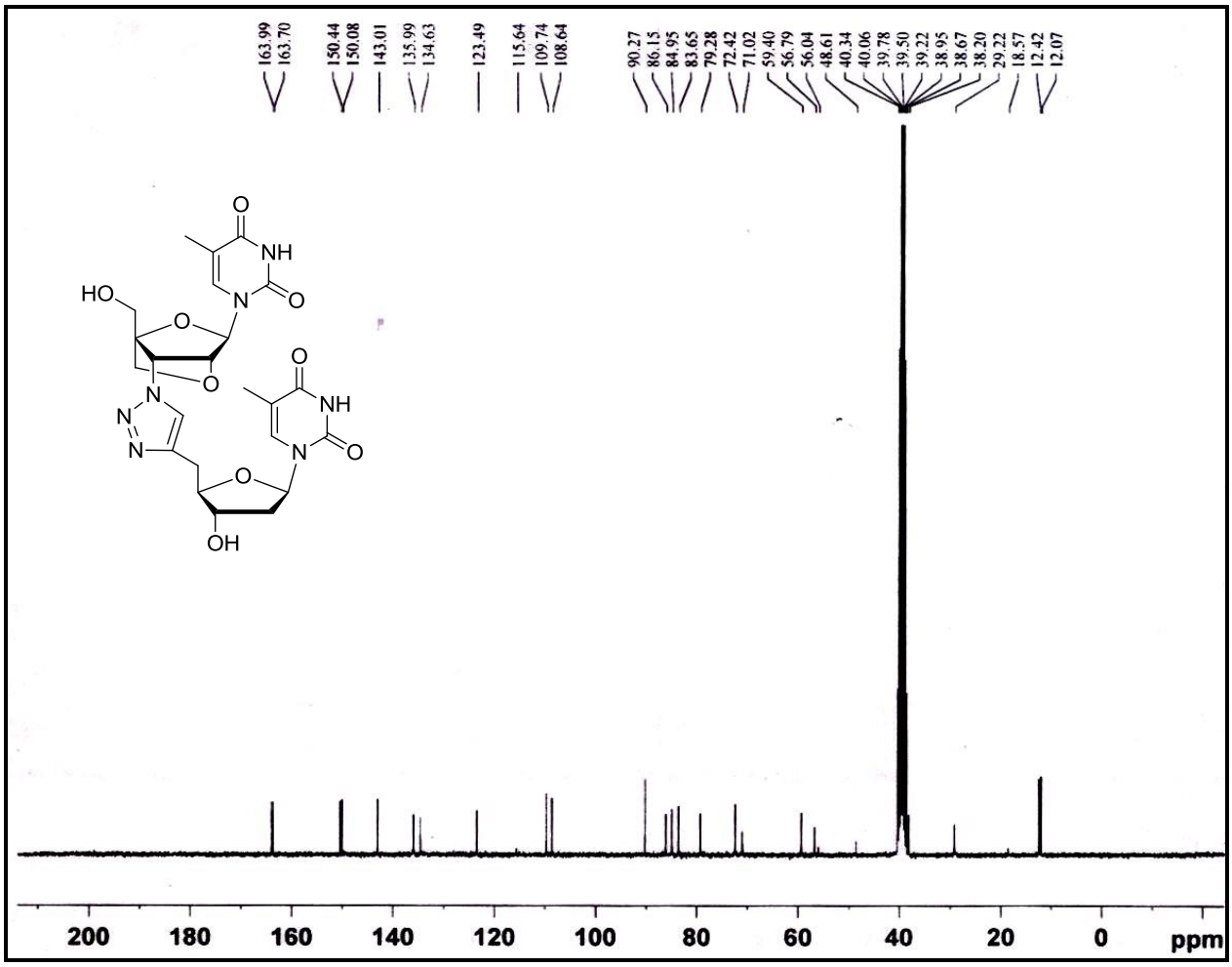


Fig S12. ^{13}C NMR spectrum (75.5 MHz, DMSO-d_6) of compound **4a**

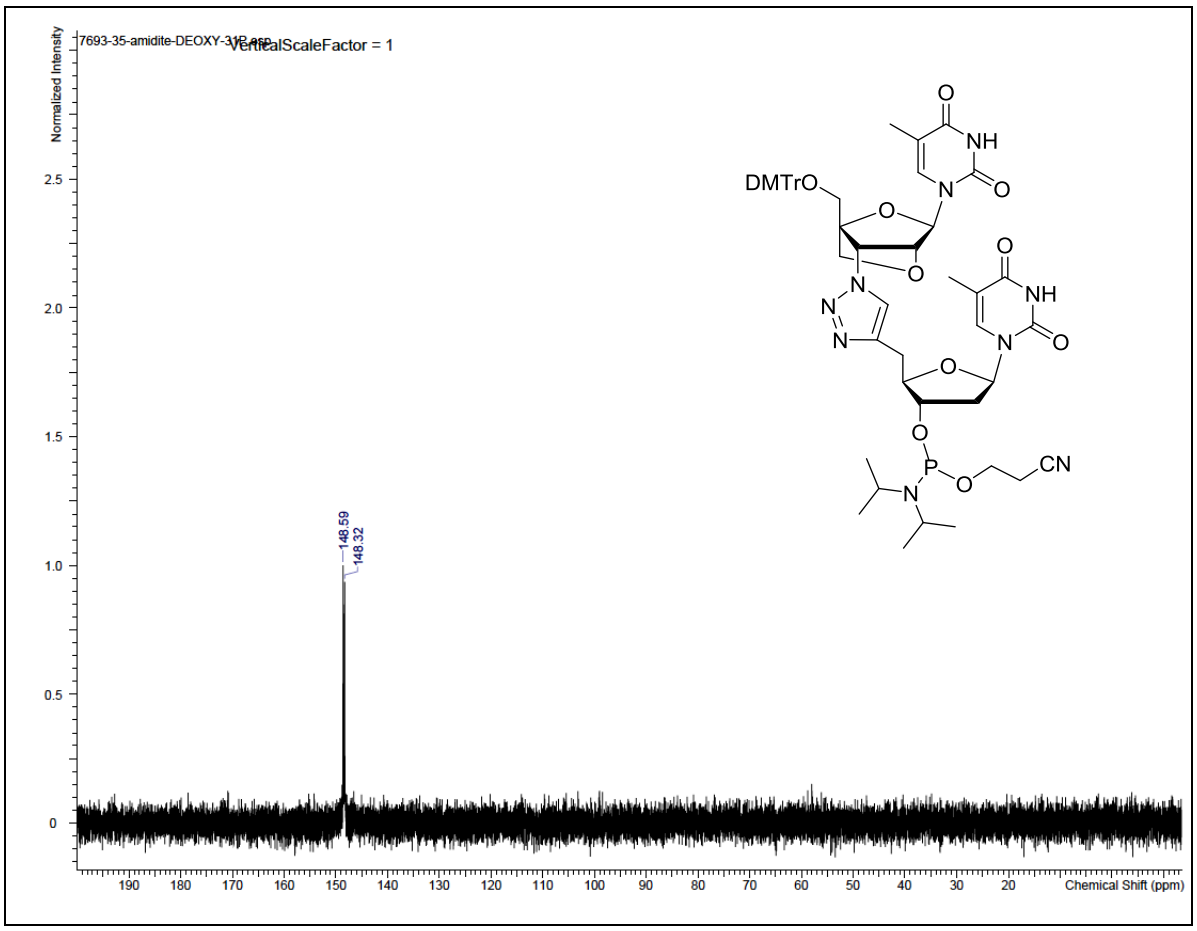


Fig S13. ^{31}P NMR spectrum (162 MHz, $CDCl_3$) of compound **6a**

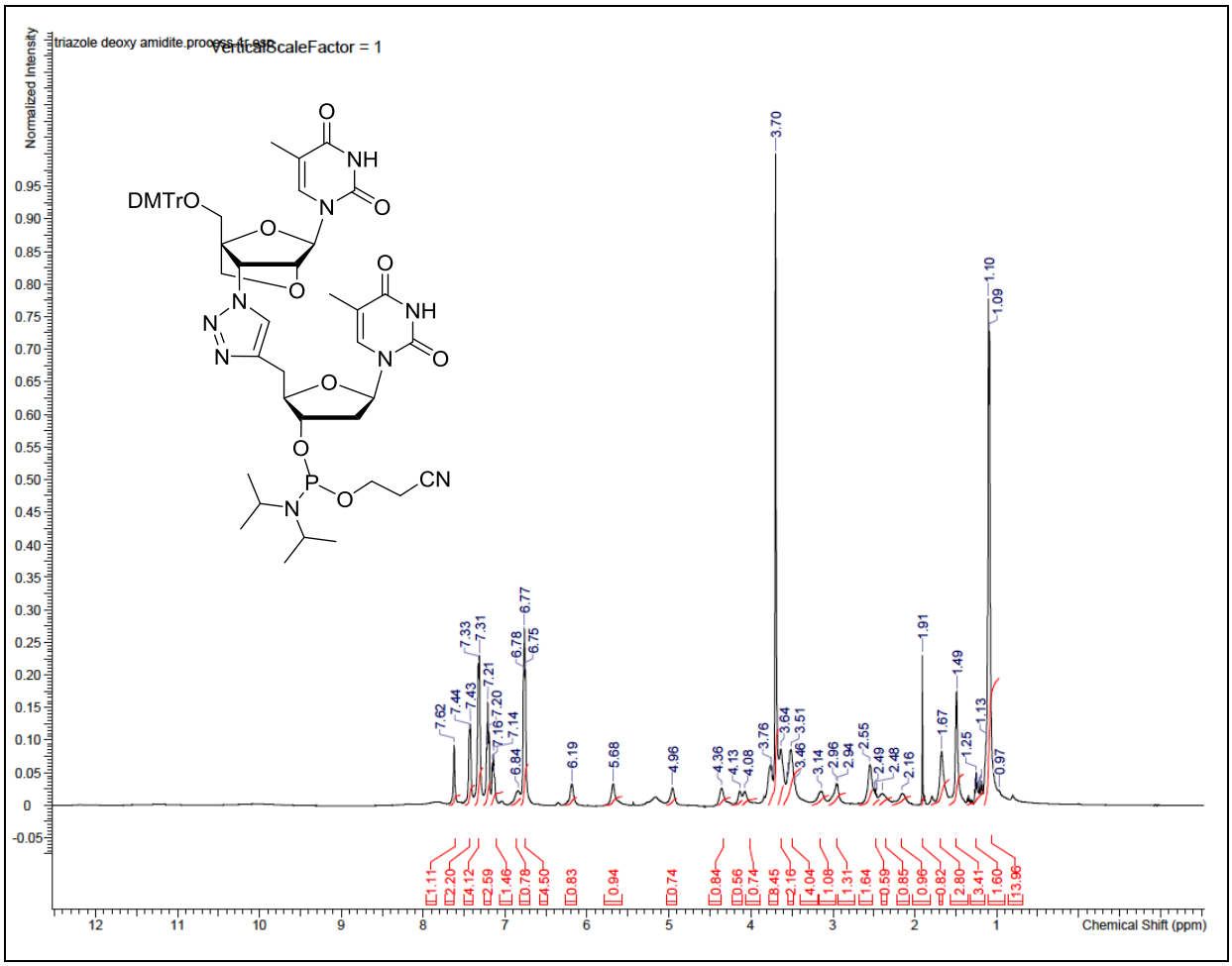


Fig S14. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **6a**

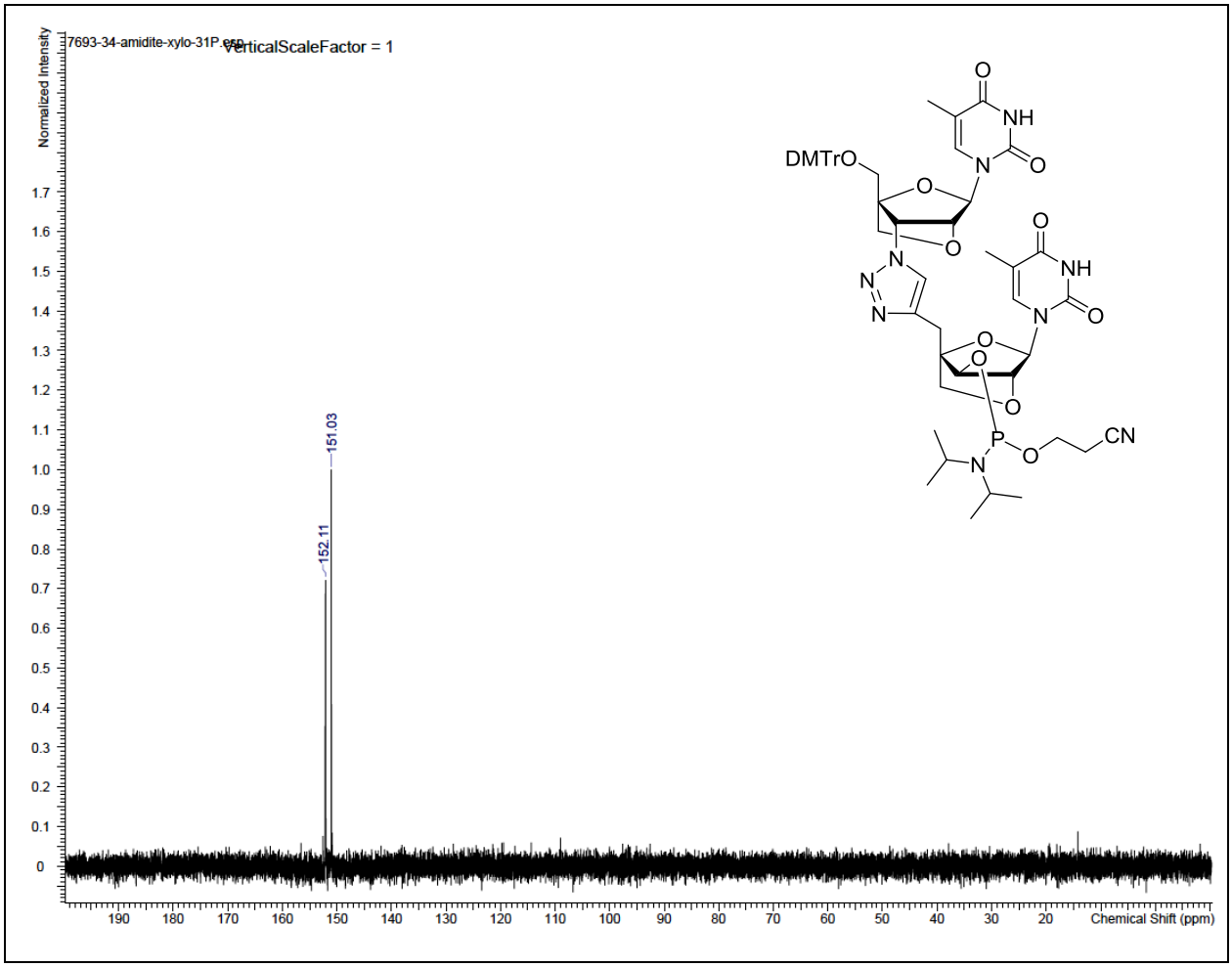


Fig S15. ^{31}P NMR spectrum (162 MHz, $CDCl_3$) of compound **6b**

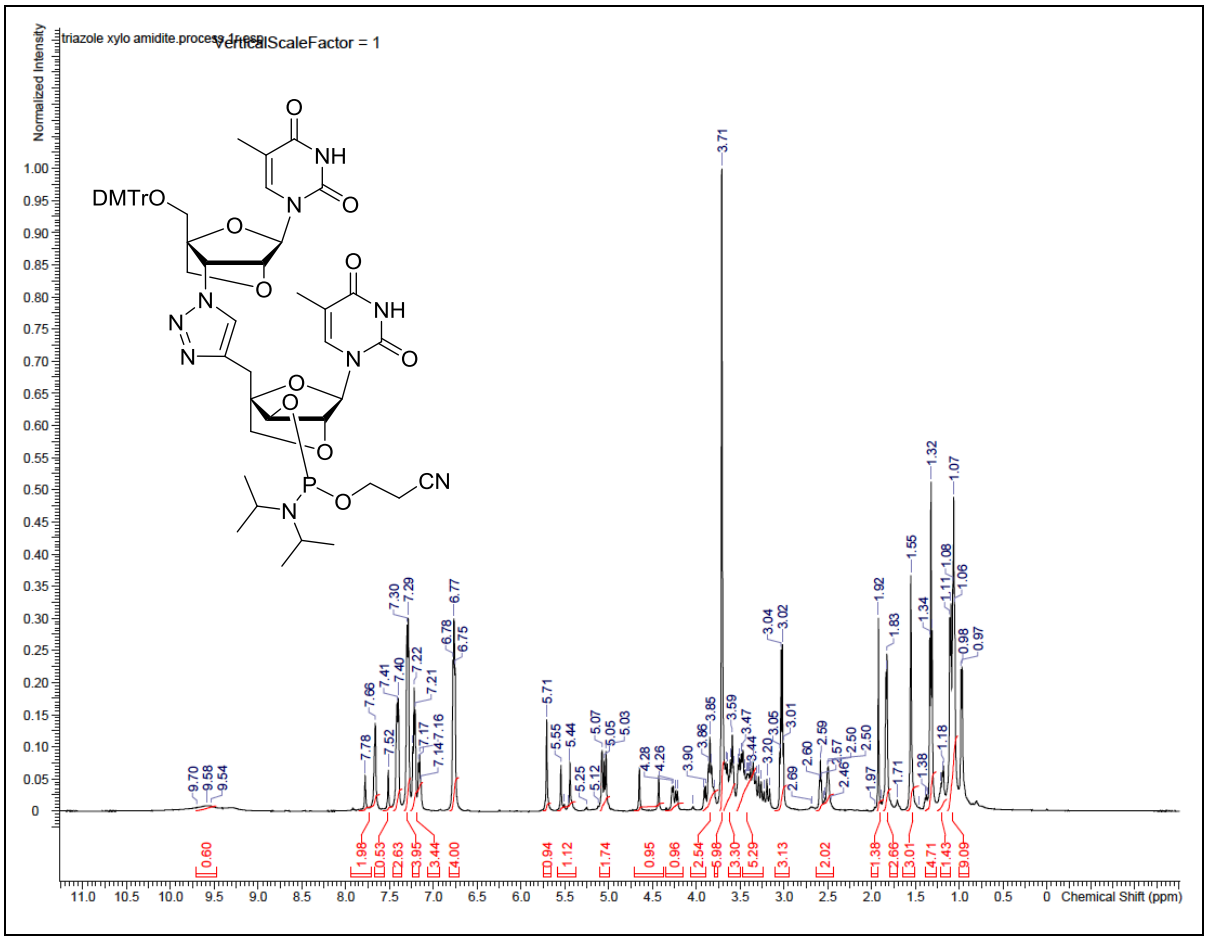


Fig S16. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **6b**

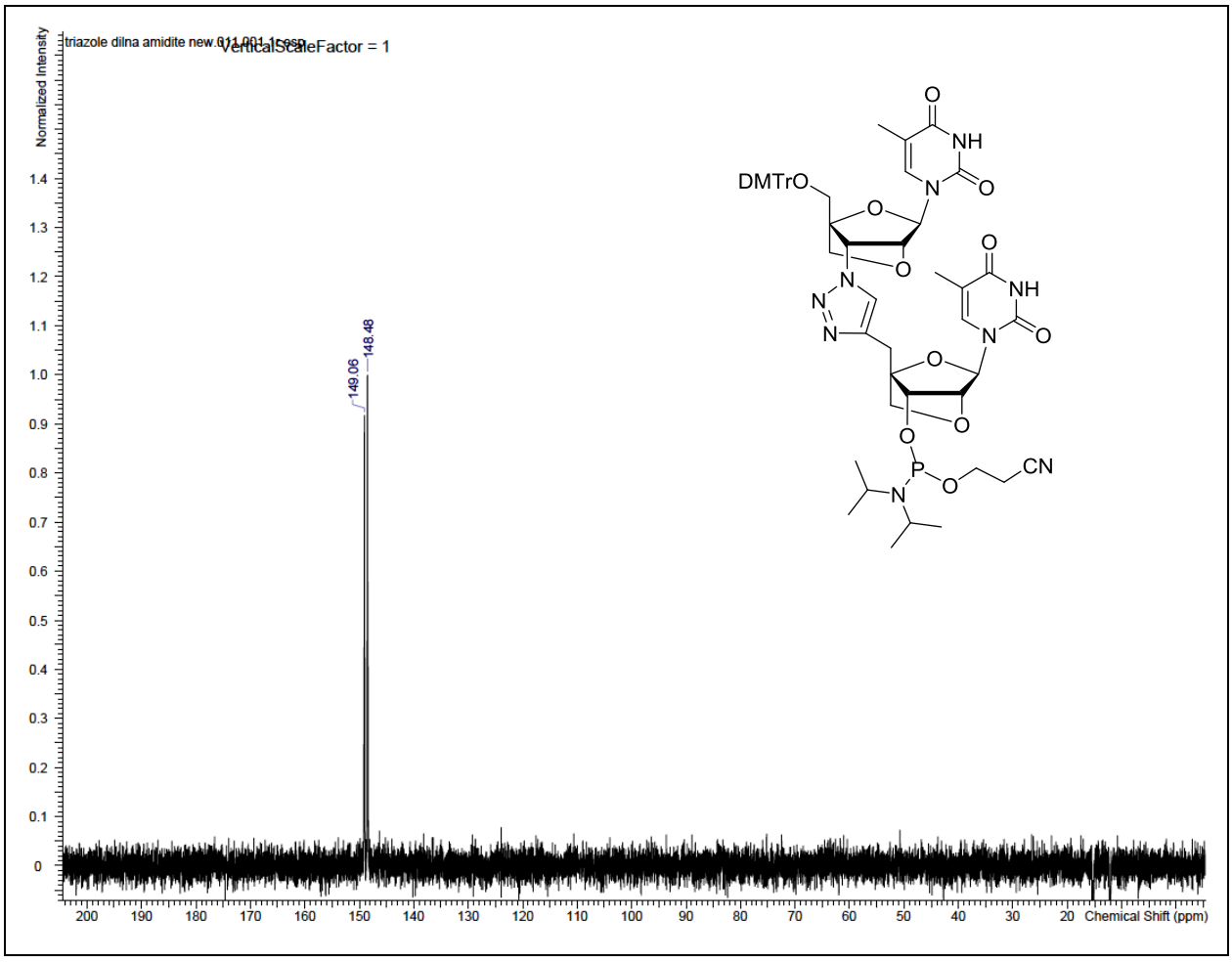


Fig S17. ^{31}P NMR spectrum (202.5 MHz, CD_3CN) of compound **6c**

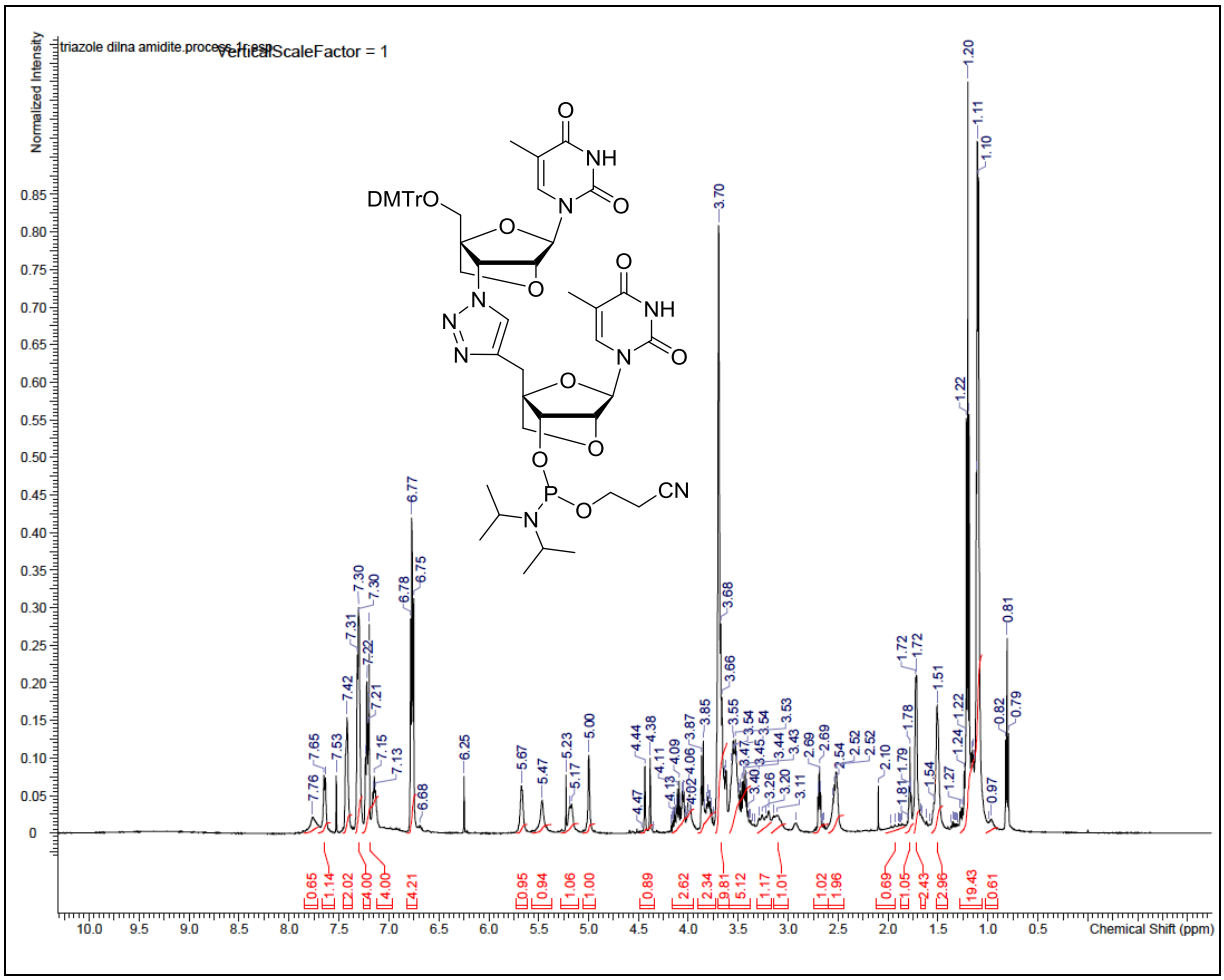


Fig S18. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **6c**

Table S1. Sequences, melting temperatures (T_m) and mass spectrometric analysis of oligonucleotides containing triazole-linked LNA dimers at internal positions.

name	DNA sequence (5'-3')[a]	MW (observed/calcd; (M-H)$^-$)	Target DNA[b]		Target RNA[b]	
			*T*m (ºC)	Δ*T*m[c] (ºC)	*T*m (ºC)	Δ*T*m (ºC)
I	GCG TTTTTT GCT	3632.63/3632.40	53.1	-	49.3	-
I-L1	GCG TTT^{L}T^{L}TT GCT	3688.62/3688.39	56.4	+3.3	59.0	+9.7
I-LD1	GCG TT(**T^{L}$_{T}$T**)TT GCT	3631.68/3631.45	30.0	-23.1	28.6	-20.7
I-LX1	GCG TT(**T^{L}$_{T}$T^{xylo-L}**)TT GCT	3659.68/3659.46	26.2	-26.9	27.8	-21.5
I-LL1	GCG TT(**T^{L}$_{T}$T^{L}**)TT GCT	3659.68/3659.46	26.8	-26.3	35.9	-13.4
I-L2	GCG T^{L}T^{L}TTT^{L}T^{L}GCT	3744.61/3744.41	62.0	+8.9	72.2	+22.9
I-LD2	GCG (**T^{L}$_{T}$T**)TT(**T^{L}$_{T}$T**) GCT	3630.73/3630.53	<20	-	<20	-
I-LX2	GCG (**T^{L}$_{T}$T^{xylo-L}**)TT(**T^{L}$_{T}$T^{xylo-L}**) GCT	3686.72/3686.55	<20	-	<20	-
I-LL2	GCG (**T^{L}$_{T}$T^{L}**)TT(**T^{L}$_{T}$T^{L}**) GCT	3686.72/3686.55	<20	-	<20	-
I-L3	GCG T^{L}T^{L}T^{L}T^{L}T^{L}T^{L} GCT	3800.59/3800.43	71.1	+18.0	82.9	+33.6
I-LL3	GCG (**T^{L}$_{T}$T^{L}**)(**T^{L}$_{T}$T^{L}**)(**T^{L}$_{T}$T^{L}**) GCT	3713.76/3713.64	<20	-	<20	-

[a] **T^{L}$_{T}$T** = triazole-linked locked dinucleoside block; $_{T}$ = *Triazole-linkage*; ^{L} is LNA. [b] target DNA (3655.71/3655.47) or RNA (3847.65/3847.46) sequence: AGC AAAAAA CGC (5'-3'). [c] ΔT_m: difference between T_m of modified and unmodified duplexes; duplex concentration 2.0 µM in 9 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.43 mM KCl, 123.3 mM NaCl, pH = 7.4.

Table S2. Sequences and mass spectrometric analysis of oligonucleotides containing triazole-linked LNA dimers at terminal positions.

name	DNA sequence (5'-3')	MW (observed/calcd; $(M-H)^-$)	Target DNA[b]	Target RNA[b]
			*T*m	*T*m
D1	TCT CTC TCC CTT TT	4105.70/ 4105.69	50.5 (± 0.1)	63.7 (± 0.1)
LD1	TCT CTC TCC CTT $T^{L}T^{L}$	4161.62/ 4162.70	52.7 (± 0.4)	66.1 (± 0.0)
D1-LD	TCT CTC TCC CTT $\mathbf{T^{L}_{T}T}$	4104.75/4104.77	49.5 (± 0.1)	63.7 (± 0.0)
D1-LX	TCT CTC TCC CTT $\mathbf{T^{L}_{T}T^{xylo\text{-}L}}$	4132.72/4132.78	49.2 (± 0.1)	64.1 (± 0.1)
D1-LL	TCT CTC TCC CTT $\mathbf{T^{L}_{T}T^{L}}$	4132.73/4132.78	49.1 (± 0.1)	64.3 (± 0.1)
R1	UCU CUC UCC CUU UU	4217.51/ 4217.48	41.9 (± 0.2)	69.6 (± 0.2)
LR1	UCU CUC UCC CUU $T^{L}T^{L}$	4269.52/ 4270.60	43.7 (± 0.4)	73.2 (± 0.4)
R1-LD	UCU CUC UCC CUU $\mathbf{T^{L}_{T}T}$	4212.60/4212.58	42.4 (± 0.6)	71.2 (± 0.2)
R1-LX	UCU CUC UCC CUU $\mathbf{T^{L}_{T}T^{xylo\text{-}L}}$	4240.60/4240.59	42.7 (± 0.3)	70.6 (± 0.1)
R1-LL	UCU CUC UCC CUU $\mathbf{T^{L}_{T}T^{L}}$	4240.59/4240.59	42.9 (± 0.5)	71.3 (± 0.0)

$\mathbf{T^{L}_{T}T}$ = triazole-linked locked dinucleoside block; $_{T}$ = *Triazole-linkage*; ^{L} is LNA.
target DNA (4418.84/4417.96) or RNA (4642.77/4641.95) sequence: AA AAG GGA GAG AGA (5'-3'). Results are given as the mean ± standard deviation of four replicates.

Table S3. Sequences, melting temperatures (T_m) and mass spectrometric analysis of terminal triazole-modified gapmer oligonucleotides complementary to *ADAM33* mRNA.

Oligonucleotide[a]	Sequence (5′-3′)[b]	Mass (obs / calcd; M-H)$^-$	T_m (°C) (full match)[c]	T_m (°C) (mismatch)[d]
33N DNA	AGG TGT CAT GGT TTT	4628.83/4628.01	59.3 (± 0.1)	57.1 (± 0.5)
33N LNA	$A^LG^LG^LTGTCATGGTT^LT^LT^L$	5021.44/5021.00	68.9 (± 0.4)	66.4 (± 0.0)
33N T^L_TT	$A^LG^LG^LTGTCATGGTT^L\mathbf{T^L_TT}$	4948.53/4947.99	65.4 (± 0.1)	64.0 (± 0.3)
33N $T^L_TT^{xylo\text{-}L}$	$A^LG^LG^LTGTCATGGTT^L\mathbf{T^L_TT^{xylo\text{-}L}}$	4976.52/4976.00	65.5 (± 0.2)	63.9 (± 0.4)
33N $T^L_TT^L$	$A^LG^LG^LTGTCATGGTT^L\mathbf{T^L_TT^L}$	4976.52/4976.00	66.2 (± 0.5)	64.4 (± 0.0)
33P DNA	TTC ATT TTA GGA GCT	4572.82/4571.99	49.6 (± 0.5)	47.9 (± 0.4)
33P LNA	$T^LT^LC^LATTTTAGGAG^LC^LT^L$	4993.41/4993.03	64.1 (± 0.4)	58.5 (± 0.1)
33P T^L_TT	$\mathbf{T^L_TT}C^LATTTTAGGAG^LC^LT^L$	4920.61/4920.02	56.4 (± 0.1)	51.1 (± 0.3)
33P $T^L_TT^{xylo\text{-}L}$	$\mathbf{T^L_TT^{xyloL}}C^LATTTTAGGAG^LC^LT^L$	4948.57/4948.03	57.2 (± 0.4)	51.5 (± 0.2)
33P $T^L_TT^L$	$\mathbf{T^L_TT^L}C^LATTTTAGGAG^LC^LT^L$	4948.56/4948.03	61.0 (± 0.4)	53.5 (± 0.1)

[a] uppercase is DNA; triazole-linked locked dinucleoside blocks are shown in bold; *T* = Triazole linkage. L is LNA. [b] The gapmer ASOs are fully phospohorothioate except the inserted triazole. [c]target RNA sequences (5'-3') were AAA ACC AUG ACA CCU (4725.73/4724.95) and AGC UCC UAA AAU GAA (4766.73/4765.96). [d]mismatched target RNA sequences (5'-3') were AGA ACC AUG ACA CCU (4741.73/4741.95) and AGC UCC UAA AAU GGA (4782.71/4783.06). Results are given as the mean ± standard deviation of four replicates.

Discussion of mismatched T_m data

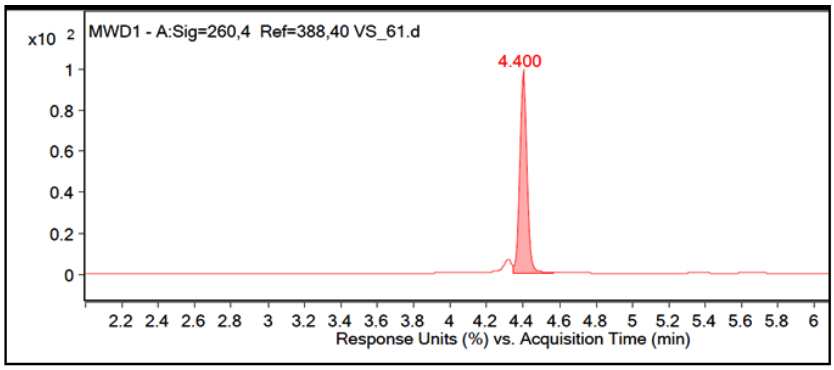
All oligonucleotides, including the xylo-configured triazole-linked dinucleotides, show a significant drop in T_m when paired with a mismatched target (right column) relative to a fully-matched target (second-to-right column). This indicates that base pairing is maintained by all triazole-linked dinucleotides.

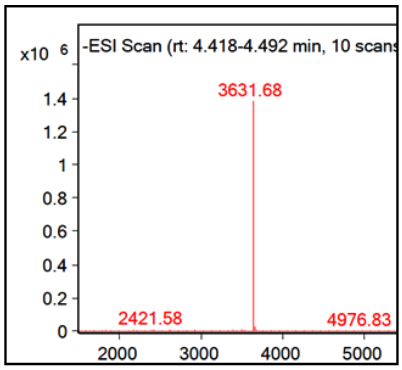
Table S4. Sequences and mass spectrometric analysis of triazole-modified siRNA sequences complementary to huntingtin mRNA (accession number NM_002111.6, starting at position 10150).

Entry	siRNA strand sense (s) or antisense (as)	Sequence (5′-3′)[a]	Mass (obs / calcd; M-H)$^-$
1.	**HTT_as_PO**	P(mU)(fU)(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)(fU)(mG)(fA)(mU)(fA)(mU)(fA)	6475.80/6474.83
2.	**HTT_as_mf**	P(mU)#(fU)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)	6619.70/6619.43
3.	**HTT_ as_T^{L}$_{T}$T**	P(**T^{L}$_{T}$T**)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)	6582.77/6582.48
4.	**HTT_as_T^{L}$_{T}$T^{xyloL}**	P(**T^{L}$_{T}$T^{xyloL}**)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)	6610.77/6610.49
5.	**HTT_as_T^{L}$_{T}$T^{L}**	P(**T^{L}$_{T}$T^{L}**)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)	6610.76/6610.49
6.	**HTT_as_T^{L}T^{L}**	P(**T^{L}T^{L}**)(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)	6623.76/6623.36
7.	**HTT_s_fm**	(fC)#(mA)#(fG)(mU)(fA)(mA)(fA)(mG)(fA)(mG)(fA)(mU)(fU)#(mA)#(fA)-teg-cholesterol	5764.20/5764.34
8.	**HTT_s_fm_Cy3**	Cy3-(fC)#(mA)#(fG)(mU)(fA)(mA)(fA)(mG)(fA)(mG)(fA)(mU)(fU)#(mA)#(fA)-teg-cholesterol	6394.58/6394.69

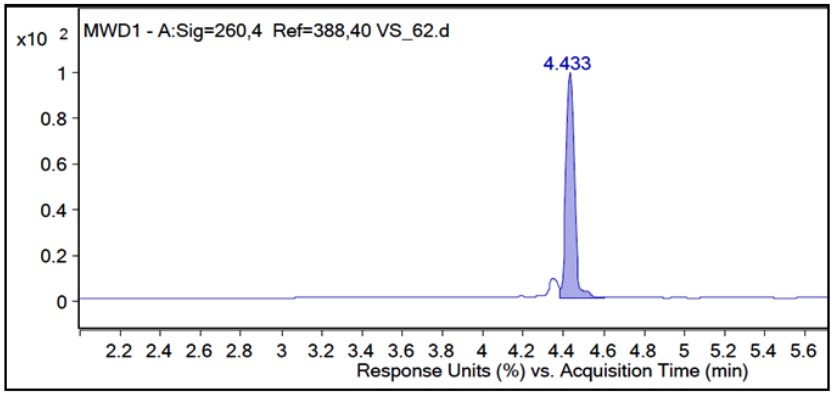
[a] m and f are 2'-*O*-Me and 2'-F modification, respectively; **T^{L}$_{T}$T** = triazole-linked locked dinucleoside block; $_{T}$ = *Triazole-linkage*; ^{L} is LNA; # is PS linkage; teg is tetraethylene glycol; P is 5'-phosphate.

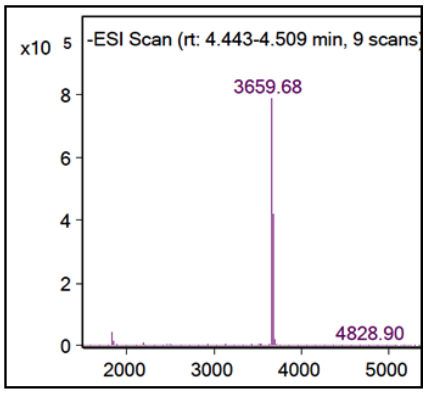
HPLC and LCMS traces of oligonucleotides



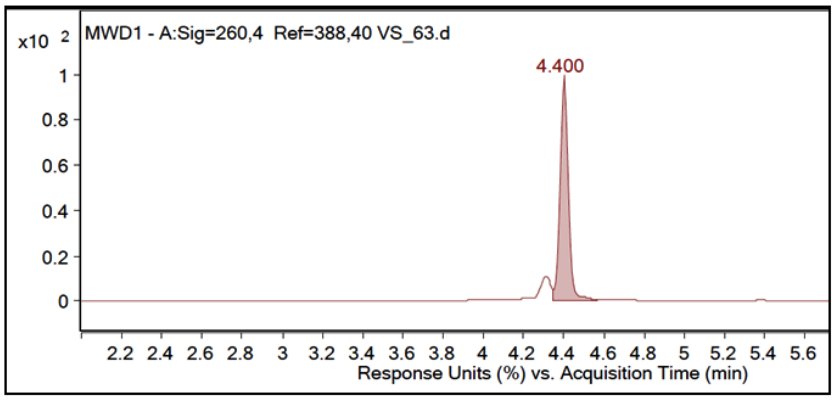


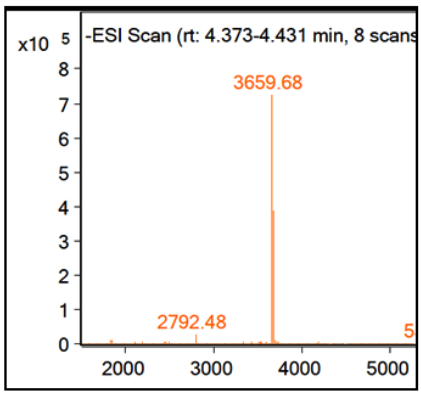
I-LD1: GCG TT($\mathbf{T^{L}_{T}T}$)TT GCT



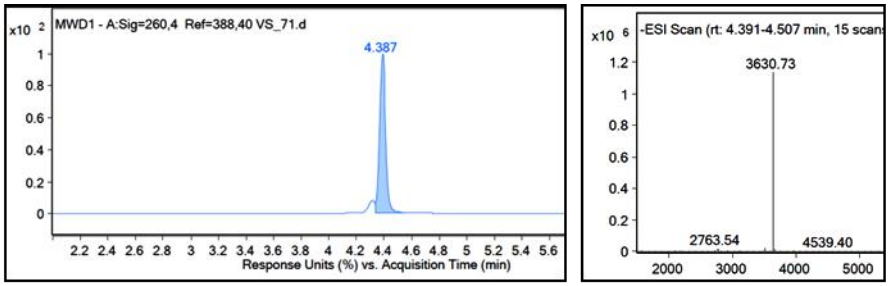


I-LX1: GCG TT($\mathbf{T^{L}_{T}T^{xylo\text{-}L}}$)TT GCT

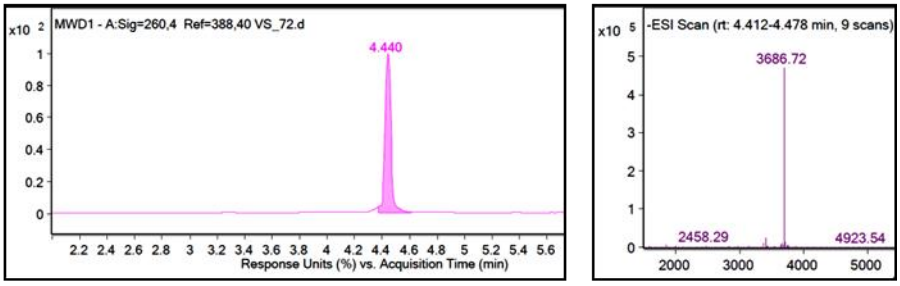




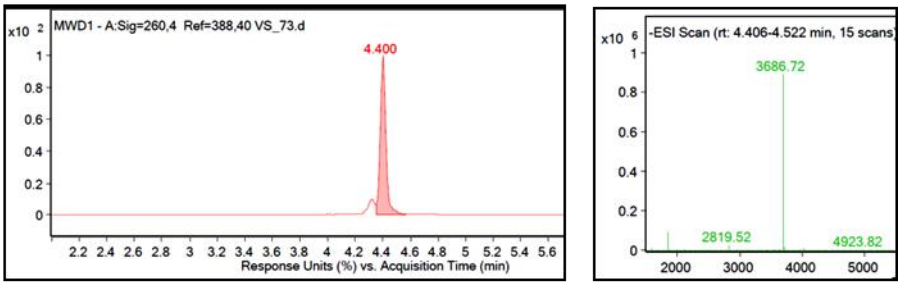
I-LL1: GCG TT($\mathbf{T^{L}_{T}T^{L}}$)TT GCT



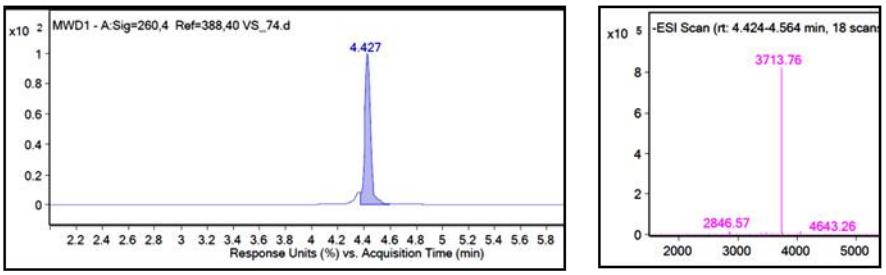
I-LD2: GCG ($T^{L}_{T}T$)TT($T^{L}_{T}T$) GCT



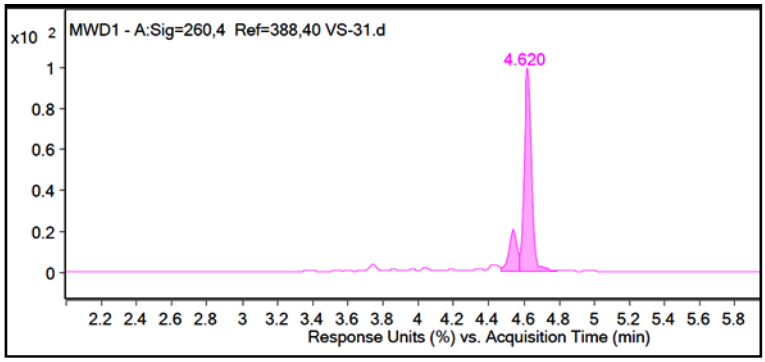
I-LX2: GCG ($T^{L}_{T}T^{xylo\text{-}L}$)TT($T^{L}_{T}T^{xylo\text{-}L}$) GCT

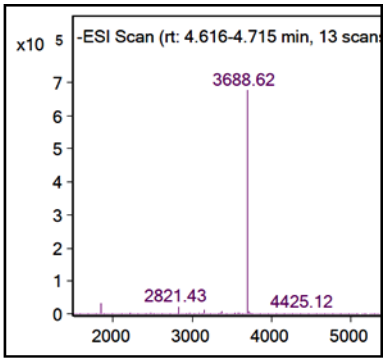


I-LL2: GCG ($T^{L}_{T}T^{L}$)TT($T^{L}_{T}T^{L}$) GCT

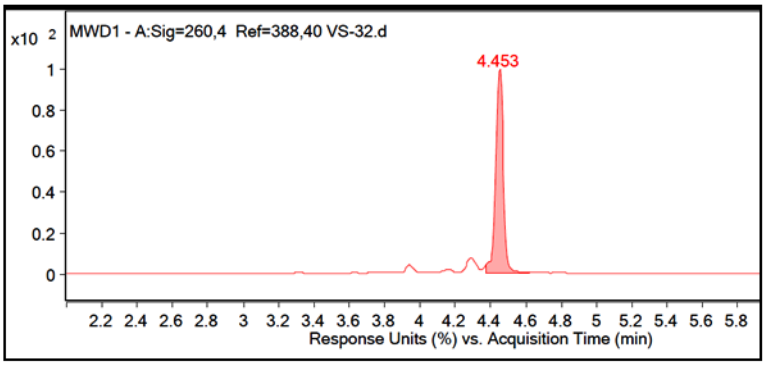


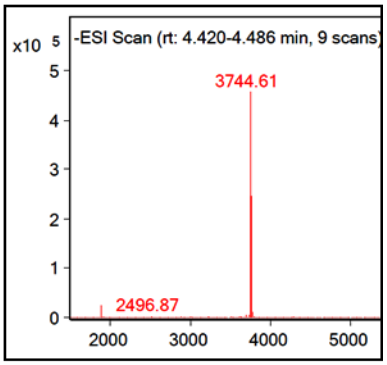
I-LL3: GCG ($T^{L}_{T}T^{L}$) ($T^{L}_{T}T^{L}$)($T^{L}_{T}T^{L}$) GCT



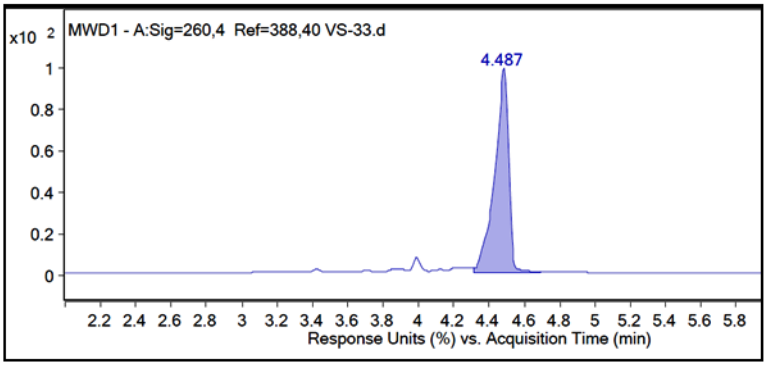


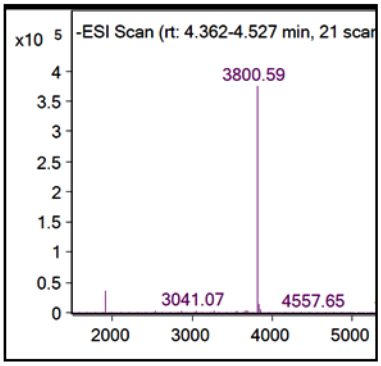
I-L1: GCG TT $\mathbf{T^LT^L}$TTGCT



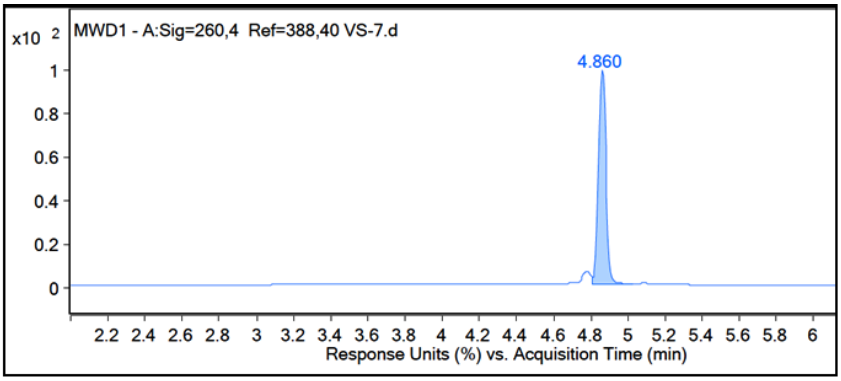


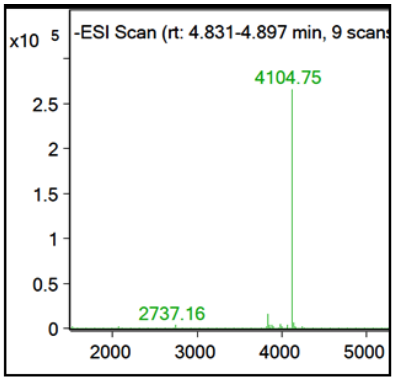
I-L2: GCG $\mathbf{T^LT^L}$TT$\mathbf{T^LT^L}$GCT



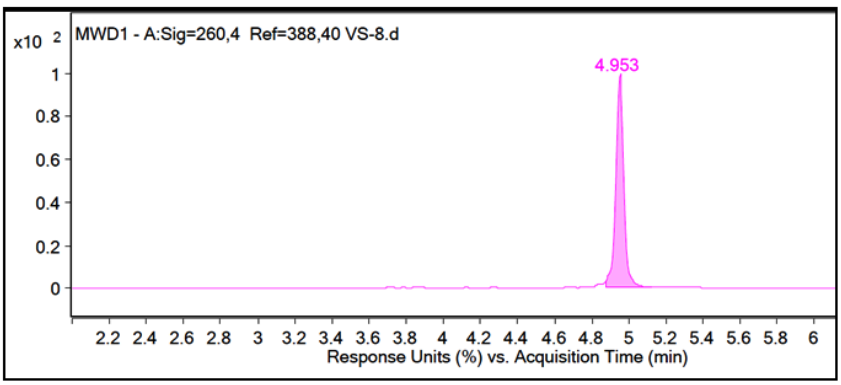


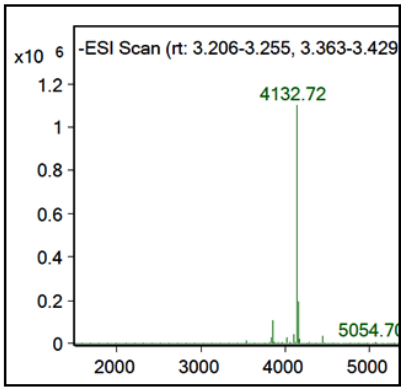
I-L3: GCG $\mathbf{T^LT^LT^LT^LT^LT^L}$ GCT



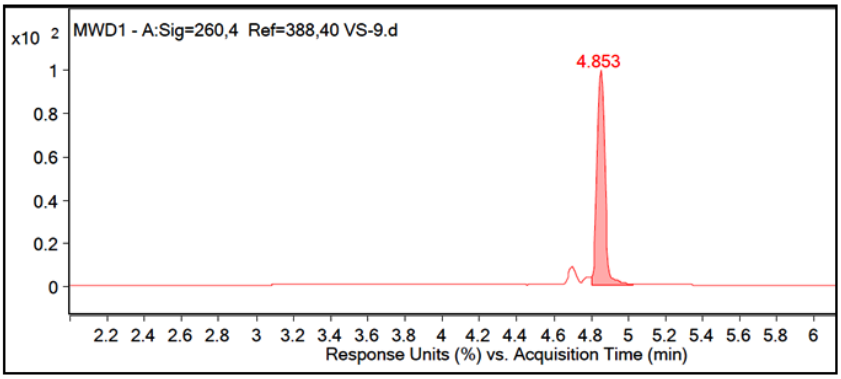


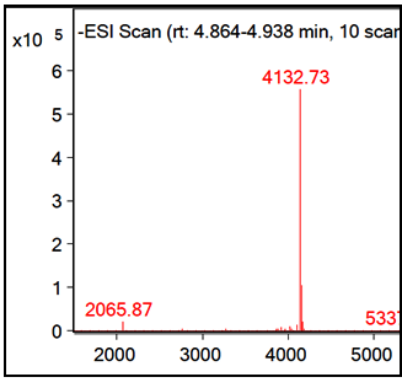
D1-LD: TCT CTC TCC CTT $T^{L}_{T}T$



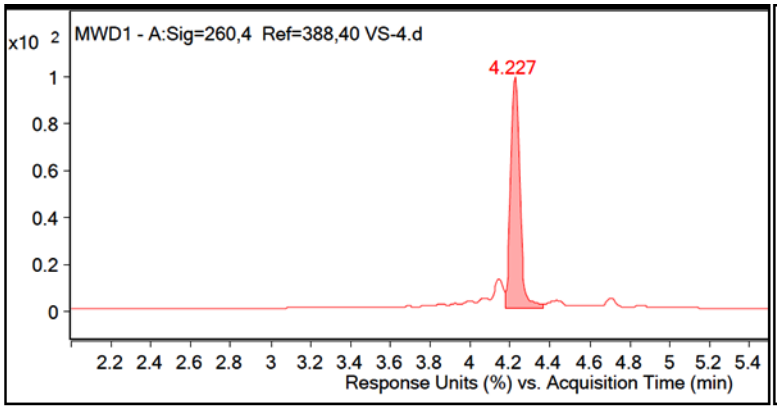


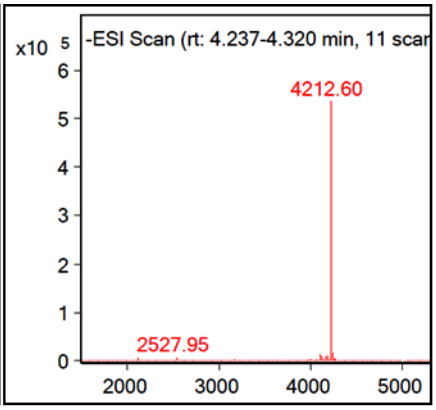
D1-LX: TCT CTC TCC CTT $T^{L}_{T}T^{xylo\text{-}L}$



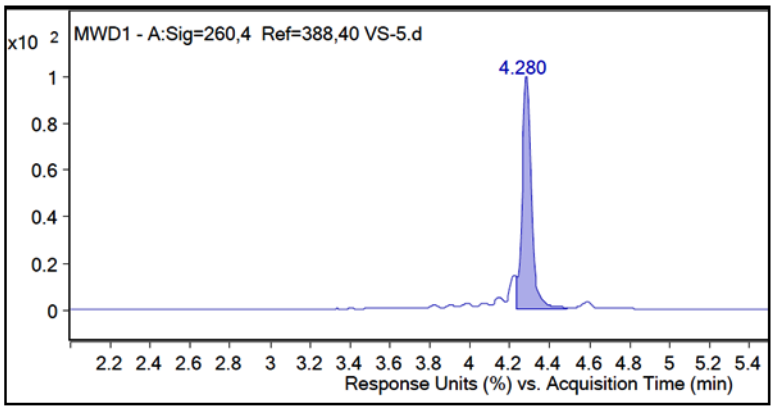


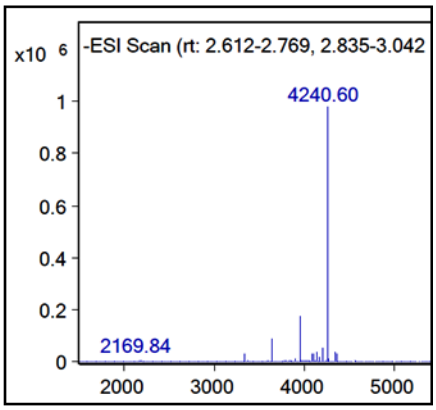
D1-LL: TCT CTC TCC CTT $T^{L}_{T}T^{L}$



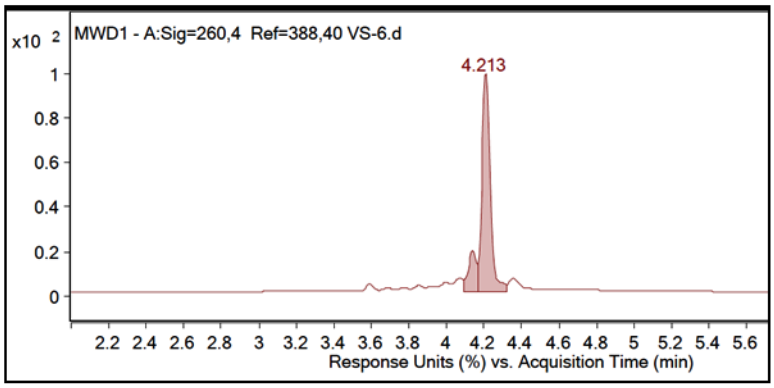


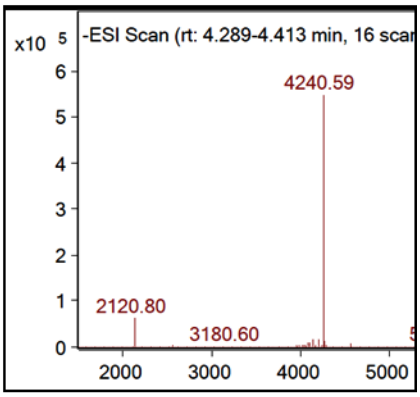
R1-LD: UCU CUC UCC CUU $T^{L}_{T}T$



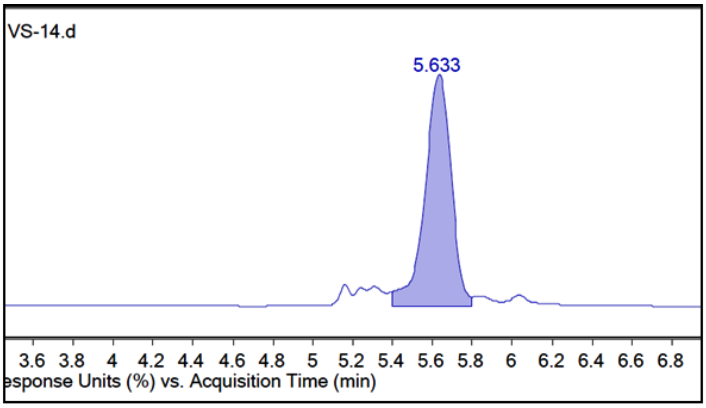


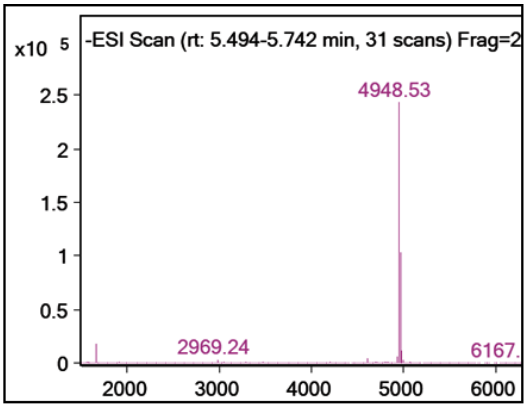
R1-LX: UCU CUC UCC CUU $T^{L}_{T}T^{xylo\text{-}L}$



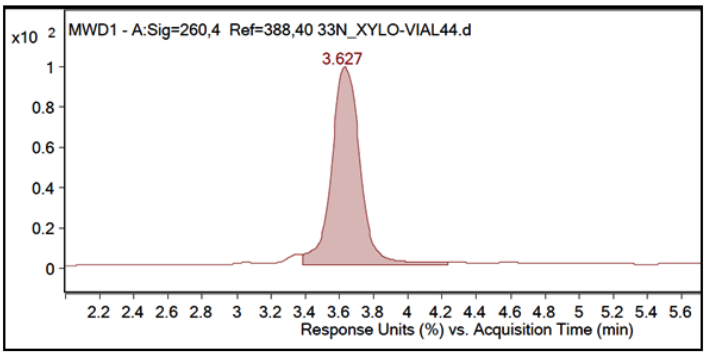


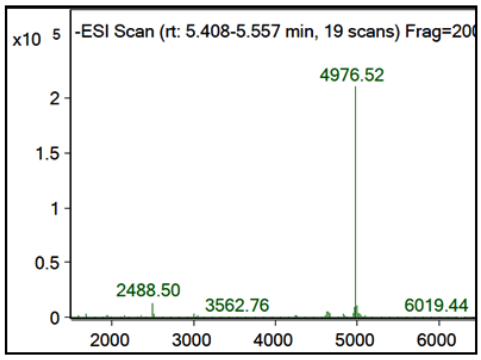
R1-LL: UCU CUC UCC CUU $T^{L}_{T}T^{L}$



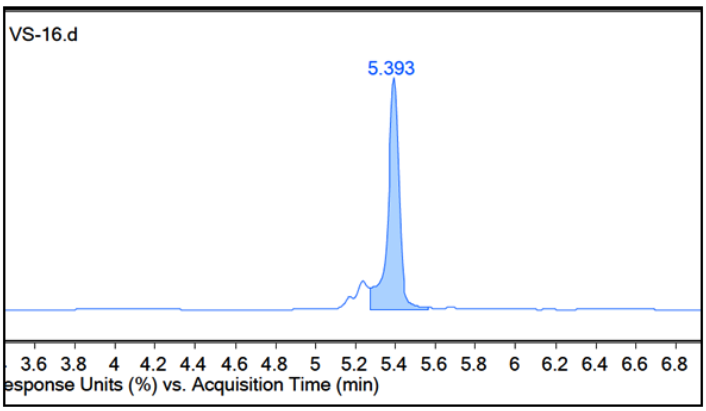


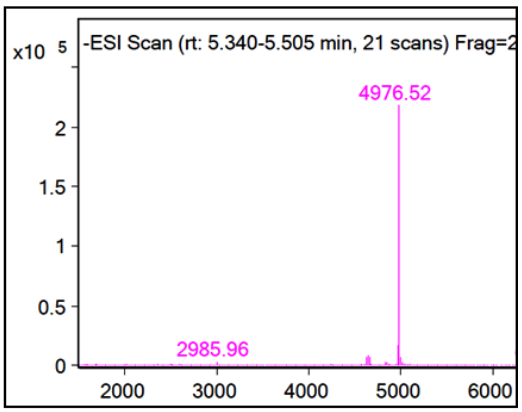
33N $T^{L}_{T}T$: $A^{L}G^{L}G^{L}TGTCATGGTT^{L}T^{L}_{T}T$



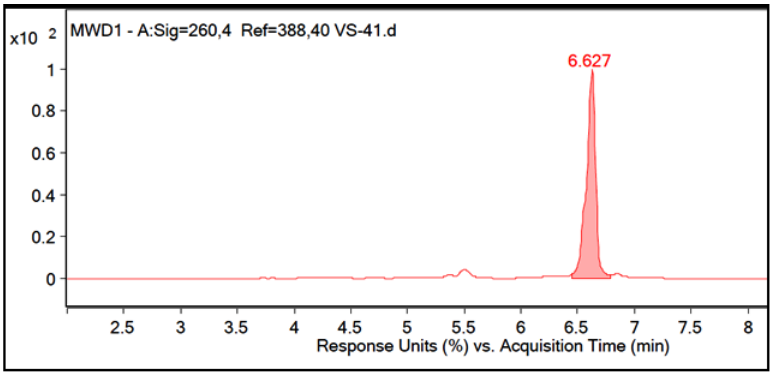


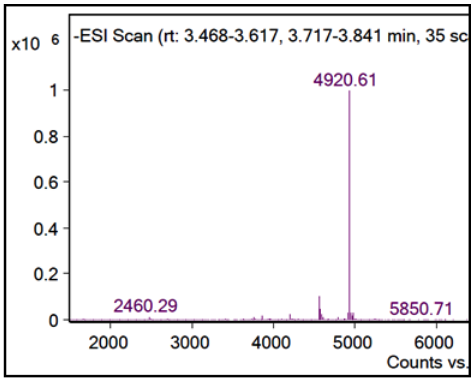
33N $T^{L}_{T}T^{xylo\text{-}L}$: $A^{L}G^{L}G^{L}TGTCATGGTT^{L}T^{L}_{T}T^{xylo\text{-}L}$



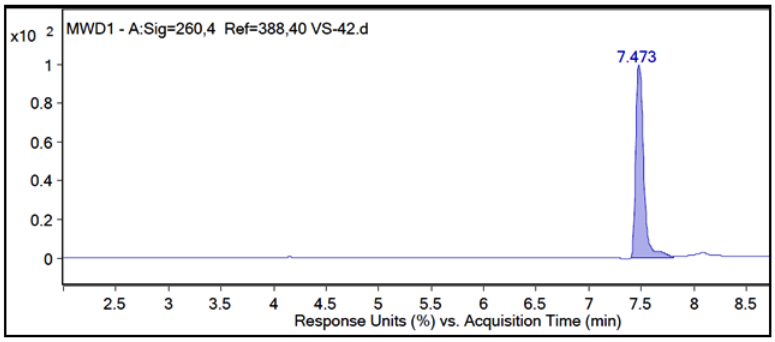


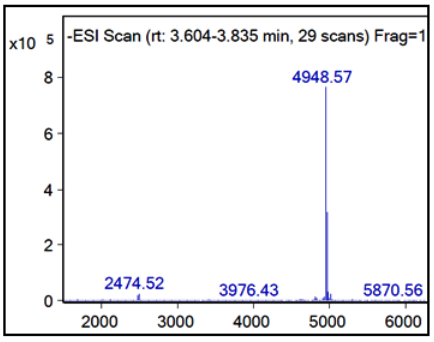
33N $T^{L}_{T}T^{L}$: $A^{L}G^{L}G^{L}TGTCATGGTT^{L}T^{L}_{T}T^{L}$



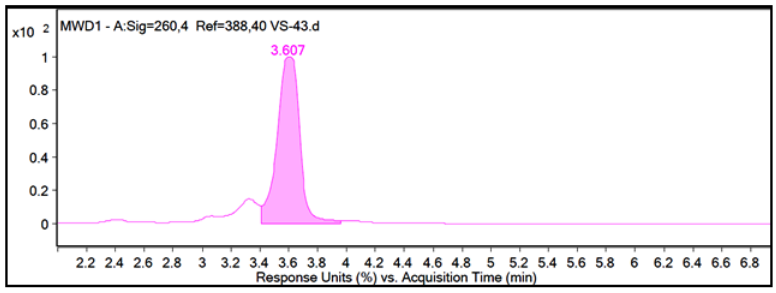


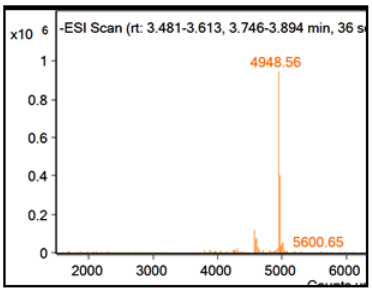
33P $T^{L}_{T}T$: $T^{L}_{T}TC^{L}ATTTTAGGAG^{L}C^{L}T^{L}$



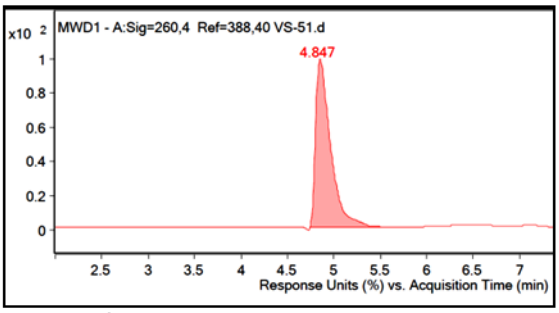


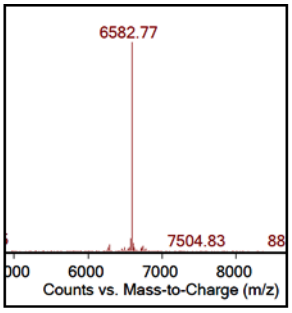
33P $T^{L}_{T}T^{xylo\text{-}L}$: $T^{L}_{T}T^{xyloL}C^{L}ATTTTAGGAG^{L}C^{L}T^{L}$



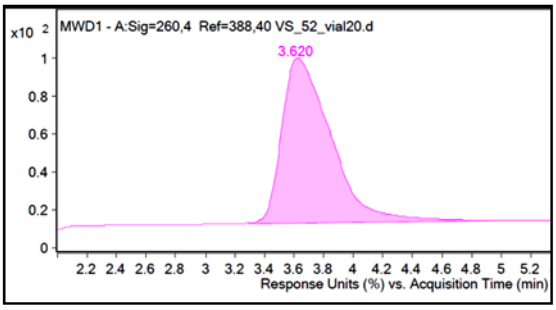


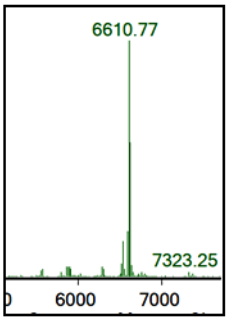
33P $T^{L}_{T}T^{L}$: $T^{L}_{T}T^{L}C^{L}ATTTTAGGAG^{L}C^{L}T^{L}$



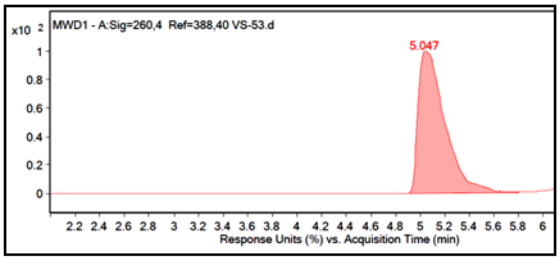


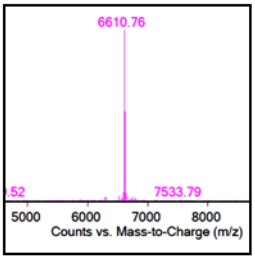
HTT_ as_$T^{L}_{T}T$: P($T^{L}_{T}T$)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)





HTT_as_$T^{L}_{T}T^{xyloL}$: P($T^{L}_{T}T^{xyloL}$)#(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)





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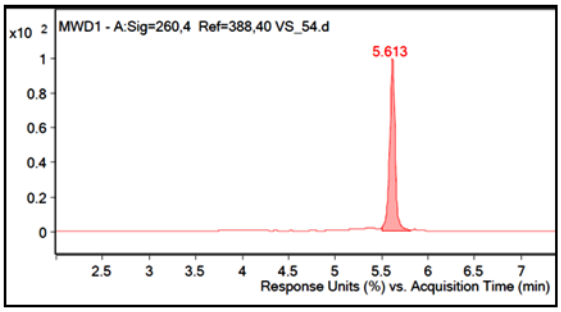

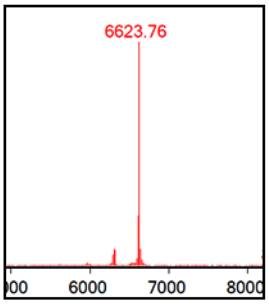

HTT_as_$T^{L}T^{L}$: P($T^{L}T^{L}$)(mA)(fA)(mU)(fC)(mU)(fC)(mU)(fU)(mU)(fA)(mC)#(fU)#(mG)#(fA)#(mU)#(fA)#(mU)#(fA)

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