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Supplementary Information for
Thiomorpholino oligonucleotides as a robust next generation antisense platforms for therapeutic alternate splicing

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Supplementary Information Text

General. All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Standard reagents used in automated DNA synthesis as well as 5'-dimethoxytrityl-2'-deoxyribonucleoside 3'-[(Cyanoethyl)-(N,N-diisopropylamino)]-phosphoramidites (**9, 10, 11** or **12**) were purchased from Glen Research. Nucleosides and 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropyl-phosphorodiamidite were purchased from ChemGenes Corporation. Morpholino 3'-phosphorodiamidite synthons were prepared according to a published procedure (1) or purchased from ChemGenes Corporation. Deuterated solvents were purchased from Cambridge Isotopes.

NMR. Experiments were carried out on a Bruker Avance-III 400 (1H = 400.13 MHz). Chemical shifts are given in ppm with positive shifts downfield. All ^{1}H and ^{13}C chemical shifts were referenced relative to internal residual protons from a lock solvent.^{31}P Chemical shifts are referenced to 0.0 ppm in the ^{1}H NMR spectrum according to the standard IUPAC method.

LCMS. Analyses were carried out on an Agilent 6530 series Q-TOF LC/MS spectrometer. A Waters ACQUITY UPLC BEH C18, 1.7 µm, 2.1 X 100 nm column was used with a gradient of 0-100% of

buffer B in 50 min with a flow rate of 0.2 mL/min at 75 ^{o}C (buffer A was a 1:80:9.5:9.5 mixture of 500mM dibutylammonium acetate:water:isopropanol:acetonitrile and buffer B was a 1:10:44.5:44.5 mixture of 500 mM dibutylammonium acetate:water: isopropanol:acetonitrile).

Chemical Synthesis of Morpholino Phosphorodiamidite Synthons 5-8.
The general procedure for building the morpholino ring (Fig. S3) is as follows. The 5′ dimethoxytrityl protected nucleoside was dissolved in methanol followed by addition of 1.2 equivalents of sodium periodate and ammonium biborate tetrahydrate (1.2 equivalents). The mixture was stirred at room temperature for three hours when TLC indicated complete consumption of the starting material. The reaction mixture was filtered through a pad of celite, activated powdered 4 A^{o} molecular sieves (0.4 g/mmol) and then 2.0 equivalents of sodium cyanoborohydride and glacial acetic acid (2.0 equivalents) were added. The reaction mixture was stirred for another 4-5 h when the intermediate diol had been completely reduced (TLC analysis). The reaction mixture was filtered through a pad of celite and evaporated to dryness. The residue was dissolved in chloroform and washed with saturated $NaHCO_3$ and then brine. The organic layer was collected, dried over Na_2SO_4 and chloroform was removed under reduced pressure. Each product was purified by flash chromatography on a silica gel column. In all cases the silica gel slurry was prepared with the starting eluant mixture containing an additional 5% triethylamine. After pouring the slurry, the column was washed with two column volumes of the starting solvent mixture containing no triethylamine. **1**, **2, 3** and **4** were eluted using a gradient of chloroform to 19:1 chloroform-methanol. All yields as described in the following sections represent those obtained over two steps starting from the 5'-Dimethoxytrityl-N-protected nucleosides.

The general procedure for the synthesis of morpholinophosphorodiamidites (**5**, **6**, **7** or **8**) is as follows: 5'-O-DMT-protected morpholino nucleoside (**1, 2, 3** or **4**) was dried overnight in vacuum and dissolved in anhydrous CH_2Cl_2 followed by addition of 1.2 equivalents of 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphorodiamidite under argon. After adding 0.5 equivalent of 4,5-dicyanoimidazole, the reaction mixture was allowed to stir for 5 h under argon at room temperature. TLC analysis indicated complete conversion of starting material. The reaction mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ solution and brine. The organic layer was dried over Na_2SO_4, filtered, and solvent removed by evaporation to dryness. The silica gel slurry was prepared with the starting eluant mixture containing an additional 5% triethylamine. After pouring the slurry, the column was washed with two column volumes of the starting solvent mixture containing no triethylamine. Compounds **5, 6, 7** and **8** (Fig. S4) were purified using a gradient of 3:7 ethylacetate-hexanes to 9:1 ethylacetate-hexane.

A general procedure for the synthesis of morpholinophosphorodiamidites is as follows: 5'-O-DMT-protected morpholino nucleoside was dried overnight *in vacuo*, dissolved in anhydrous CH_2Cl_2, and 1.2 equivalents of 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropyl-phosphoroamidite was added under argon. After further addition of 0.5 equivalent of 4,5-dicyanoimidazole, the reaction mixture was allowed to stir for 5 h under argon at room temperature. At that time, TLC indicated complete conversion of starting material. The reaction mixture was diluted with CH_2Cl_2, washed with 5% $NaHCO_3$ solution and then brine. The organic layer was dried over Na_2SO_4, filtered, and solvent removed by evaporation to dryness. A silica gel slurry was prepared with the starting eluant mixture containing an additional 5% triethylamine. After pouring the slurry, the column was washed with two column volumes of the starting solvent mixture containing no triethylamine. The crude morpholinophosphorodiamidites were purified using a gradient of 3:7 ethylacetate-hexanes to 9:1 ethylacetate-hexane. The characterization data for phosphorodiamidites obtained using this method were as follows:

***5'-dimethoxytrityl-morpholinothymidine-3'-N-cyanoethyl-N,Ndiisopropylphosphoradiamidite (5)*:** Yield: 86%. ^{31}P NMR (CD_2Cl_2) δ: 127.21, 126.08. ^{1}H NMR (CD_2Cl_2, 400 MHz) δ: 9.51 (1H, bs), 7.50-7.47 (2H, m), 7.38-7.25 (8H, m), 6.89-6.86 (4H, m), 5.78-5.75 (0.5H, dd), 5.65-5.62 (0.5H, dd), 4.08-4.02 (1H, m), 3.99-3.86 (3H, m), 3.82 (6H, s), 3.65-3.52 (2H, m), 3.48-3.34 (1H, m), 3.31-3.27 (1H, m), 3.13-3.09 (1H, m), 2.75-2.68 (2H, m), 2.53-2.47 (2H, m), 1.96 (3H, m), 1.25-1.18 (12H, m). ^{13}C NMR (CD_2Cl_2) δ: 164.02, 163.96, 158.66,

150.13, 144.95, 135.92, 135.90, 135.79, 135.68, 135.52, 129.99, 128.07, 127.77, 126.77, 117.89, 117.74, 113.05, 110.48, 110.39, 86.05, 80.54, 80.49, 80.20, 77.36, 77.21, 64.37, 60.09, 59.84, 55.20, 49.05, 48.83, 47.58, 47.06, 46.83, 45.87, 45.78, 43.91, 43.74, 24.36, 24.29, 24.22, 24.20, 20.77, 20.33, 20.66, 12.29. ESI-MS (m/z): 727.4047 $(M+H)^+$.

N^2-benzoyl-5'-dimethoxytrityl-morpholinocytidine-3'-N-cyanoethyl-N,N-diisopropylphosphoradiamidite(6): Yield: 79%. ^{31}P NMR (CD_2Cl_2) δ: 126.24, 125.70. ^1H NMR (CD_2Cl_2, 400 MHz) δ: 7.99-7.96 (3H, m), 7.67-7.64 (1H, m), 7.56-7.49 (5H, m), 7.39-7.26 (7H, m), 6.89-6.87 (4H, m), 5.83-5.67 (1H, m), 4.11-4.07 (1H, m), 4.09-3.87 (2H, m), 3.83 (6H, s), 3.81-3.77 (1H, m), 3.65-3.48 (3H, m), 3.37-3.25 (2H, m), 3.19-3.15 (1H, m), 2.78-2.75 (1H, m), 2.72-2.68 (1H, m), 2.56-2.53 (1H, m), 2.39-2.33 (1H, m), 1.25-1.18 (12H, m). ^{13}C NMR (CD_2Cl_2) δ: 162.22, 158.65, 144.94, 135.92, 135.78, 133.01, 130.05, 130.00, 128.89, 128.06, 127.80, 127.64, 126.79, 117.91, 117.77, 113.05, 86.04, 82.17, 81.93, 77.36, 64.40, 60.22, 60.11, 59.87, 49.85, 49.62, 48.22, 48.17, 47.05, 46.81, 45.70, 45.63, 43.94, 43.88, 43.82, 43.77, 24.38, 24.30, 24.24, 24.16, 20.34, 20.30, 20.22. ESI-MS (m/z): 833.3801 $(M+H)^+$.

N^2-benzoyl-5'-dimethoxytrityl-morpholinoadenosine-3'-N-cyanoethyl-N,N-diisopropylphosphoradiamidite(7)**:** Yield: 87%. ^{31}P NMR (CD_2Cl_2) δ: 127.90, 125.73. ^1H NMR (CD_2Cl_2, 400 MHz) δ: 9.15 (1H, s), 8.78 (1H, s), 8.25-8.24 (1H, d), 8.03-8.01 (2H, m), 7.67-7.63 (1H, m), 7.58-7.54 (2H, m), 7.51-7.47 (2H, m), 7.38-7.24 (7H, m), 6.04-5.90 (1H, m), 4.18-4.11 (1H, m), 4.09-4.05 (1H, m), 4.00-3.89 (2H, m), 3.86 (1H, bs), 3.83 (6H, s), 3.70-3.54 (3H, m), 3.42-3.31 (2H, m), 3.19-3.15 (1H, m), 2.99-2.89 (1H, m), 2.77-2.74 (1H, m), 2.72-2.62 (2H, m), 1.27-1.21 (12H, m). ^{13}C NMR (CD_2Cl_2) δ: 158.64, 152.41, 152.32, 149.56, 144.92, 140.71, 135.86, 135.74, 134.02, 132.60, 130.00, 128.79, 128.04, 127.78, 126.77, 123.19, 123.11, 117.81, 117.74, 113.04, 86.08, 80.81, 80.60, 77.15, 64.27, 60.14, 59.80, 50.27, 50.04, 48.66, 47.29, 47.06, 45.97, 43.92, 43.72, 24.24, 24.19, 20.29. ESI-MS (m/z): 857.3911 $(M+H)^+$.

N^2-isobutyryl-5'-dimethoxytrityl-morpholinoguanosine-3'-N-cyanoethyl-N,N-diisopropylphosphoradiamidite (8)**:** Yield: 82%. ^{31}P NMR (CD_2Cl_2) δ: 132.21, 118.40. ^1H NMR (CD_2Cl_2, 400 MHz) δ: 12.02 (1H, s), 7.89-7.86 (1H, m), 7.52-7.47 (2H, m), 7.40-7.27 (7H, m), 6.90-6.86 (4H, m), 5.67-5.66 (1H, m), 4.14-4.09 (1H, m), 4.06-4.02 (1H, m), 4.00-3.85 (2H, m), 3.83 (6H, s), 3.74-3.68 (1H, m), 3.52-3.27 (3H, m), 3.21-3.17 (1H, m), 2.88-2.60 (5H, m), 1.27-1.22 (12H, m), 1.19-1.15 (6H, m). ^{13}C NMR (CD_2Cl_2) δ: 179.29, 178.97, 158.67, 147.82, 144.93, 136.17, 135.74, 130.04, 128.04, 127.79, 126.79, 121.16, 121.06, 118.49, 118.07, 113.06, 86.12, 81.61, 81.46, 77.15, 77.03, 76.77, 64.31, 64.24, 60.23, 59.51, 59.27, 50.39, 50.20, 48.20, 47.53, 47.19, 46.67, 46.64, 45.54, 45.48, 43.81, 43.69, 43.53, 43.42, 36.22, 35.97, 24.55, 24.47, 24.43, 24.35, 24.24, 24.17, 24.03, 20.79, 20.71, 20.62, 20.56, 20.48, 18.76, 18.63, 18.58. ESI- ESI-MS (m/z): 839.4019 $(M+H)^+$.

Solid-phase synthesis of TMOs. All TMO syntheses were carried out using ABI-394 synthesizers on a 1.0 μmol scale using a 5′-DMTr-2′-OMe-ribouridine joined to a CPG solid support via a succinate linkage. TMO1(-2), TMO1(-4) and TMO-FI (Table 1) were synthesized as described previously(1). The 5'-fluorescein linkage in case of TMO-FI was introduced using fluorescein phosphoramidite (Glen Research) followed by sulfurization with 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT, Glen Research). The synthesis cycle used for the oligonucleotide synthesis of TMOs (**1-4, TMO1(-2), TMO1(-4)**) is shown below in Figure S5 and Table S1.

The procedure used to synthesize TMO and TMO-DNA chimeras is outlined in following scheme. Prior to synthesis, the 5'-O-DMTr group on the 2'-OMe ribouridine linked to a controlled pore glass support was removed with 3% trichloroacetic acid in dichloromethane. The 5'-unprotected-2'-deoxyribonucleoside (**A**) was then allowed to react (5 minutes) with **5**, **6**, **7** or **8** in anhydrous acetonitrile containing a 0.12 molar solution of 4,5-dicyanoimidazole (DCI) in order to generate a dimer having a phosphoramiditediester internucleotide linkage (**B**).[13]This intermediate was converted to (**D**) by treatment with 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT) for sulfurization and the support was next treated with a solution of acetic anhydride in order to cap any unreacted 5'-hydroxyl groups. For the preparation of thiophosphoroamidate morpholino DNA chimeras (TMO/DNA), the synthesis cycle was similar except that 5-(ethylthio)-1H-tetrazole was the activator with 5'-O-dimethoxytrityl-2'-deoxyribonucleoside-3'-phosphoramidite synthons (**9**, **10**, **11** or **12**) in order to generate (**C**). This

phosphite triester was then converted to (**E**) by oxidation with DDTT. The products of these condensations (**D** or **E**) were then capped with acetic anhydride. Following treatment with 3% trichloroacetic acid in dichloromethane, (**F**) or (**G**) were ready for repetitions of the appropriate cycle in order to generate the TMO or TMO-DNA chimera. As observed previously during the synthesis of various PMO analogues(2), selective activation, *via* dicyanoimidazole, of the diisopropylamino group of the morpholino phosphordiamidite during coupling was a key step for assembling these analogs. 5-(Ethylthio)-1H-tetrazole cannot be used with **5**, **6**, **7**, and **8** as it activates both the morpholino and diisopropylamino components of these synthons.
Following the protocol described above, TMOs 1-4, TMO1(-2), and TMO1(-4) were synthesized as 5'- DMT ON by avoiding the final detritylation step following the last synthesis cycle during solid-phase synthesis.

Cleavage from the support, DMT ON purification, and LCMS characterization of TMOs 1-4, TMO1(-2), and TMO1(-4). All oligonucleotide syntheses were carried out as DMT ON. Following synthesis, CPG resins were transferred to 1.5 mL screw cap vials and treated with 1.0 mL NH_4OH (37 %) 18 h at 55 ^oC.
The purification of each oligonucleotide involved several steps. First, the above reaction mixture was cooled to room temperature, filtered, and 3.0 mL of deionized water was added to the deprotected DMT-ON oligonucleotide suspended in the ammonium hydroxide solution (Step 1).
Each reaction mixture was then passed through a Poly Pak cartridge following the manufacturer's protocols (3) to remove failure oligonucleotides (Step 2). A syringe was connected to the female luer of the cartridge (Poly-Pak II; Glen Research) and the male luer terminated in a waste vessel. The cartridge was flushed with 4mL acetonitrile followed by 4mL 2M triethyl ammonium acetate. The DMT-ON oligonucleotide was deprotected in ammonium hydroxide (1.0 mL) at 55 ^oC for 18 h. The solution was cooled to room temperature and filtered. 3.0 mL of deionized water was added to the deprotected DMT-ON oligonucleotide in the ammonium hydroxide solution. The sample solution was loaded onto the cartridge. The eluted fraction was collected and again pushed through the cartridge. The cartridge was first flushed with 6mL of ammonium hydroxide (1:20) and then 4mL of deionized water. The support-bound oligonucleotide was detritylated by flushing the cartridge with 4mL of 3% TFA. The cartridge was then flushed with 4mL of deionized water. The purified, detritylated oligonucleotide was eluted by washing the cartridge with 20% acetonitrile and the eluted fractions collected. A flow rate of 1-2 drops/sec was maintained throughout the process.
The eluants were concentrated in a speed vac to remove solvent and then further purified *via* RP-HPLC. Following a desalting step (Step 3) using NAP columns, the RP-HPLC purifications of TMO1 and TMO-DNA chimeras 2-4 were carried out. A linear gradient of 0 to 100% B over 40 min at a flow rate of 4.0 mL/min; buffer A: 50 mM TEAB, pH 8.5; buffer B: acetonitrile, 60 ^oC. Column specifications: XBridge Oligonucleotide BEH C18 Prep Column, 130Å, 2.5 µm, 10 mm X 50 mm.
LC-MS Analyses were carried out on an Agilent 6530 series Q-TOF LC/MS spectrometer. A waters ACQUITY UPLC BEH C18, 1.7 µm, 2.1 X 100 nm column was used with a gradient of 0-100% of buffer B in 50 min with a flow rate of 0.2 mL/min at 75 ^oC (Buffer A was 1:380:10:10.4 mixture of triethylamine:water:methanol:hexafluoro-2-propanol and Buffer B was 1:370:20:10.4 mixture of triethylamine:methanol:water:hexafluoro-2-propanol). LCMS data of the TMOs are shown in Fig. S6-13.

Protocol for mild detritylation of phosphoramidate oligonucleotide MO. DMT-ON MO oligonucleotide was synthesized using published procedures and isolated using RP-HPLC (4). The MO oligonucleotide was deprotected following a modified procedure that was based upon previous literature reports (5). The combined pure fractions of MO after RP-HPLC were pooled and evaporated to dryness in a 2.0 mL Eppendorf vial. The dry residue obtained from 1.0 uM synthesis was treated with 500 uL of aqueous sodium acetate (100 mM, pH 7) and warmed at 40 ^oC for 3 hours. After filtration, the reaction mixture was re-purified by RP-HPLC and the pure fractions were evaporated to dryness to obtain the final product (Fig. S11).

Hybridization of TMOs 1-4 with complementary RNA. All oligonucleotides were prepared as 2 µM concentration in buffer solution containing 10 mM NaCl, 0.01 mM EDTA and 10 mM sodium phosphate (pH 7.0). Before loaded onto a quartz cuvettes of 1 mm path-length, the ASOs were

hybridized with the same molar concentration of a synthetic complementary RNA sequence (5'-r(AG GUA AGC CGA GGU UUG GCC)-3', purchased from IDT (Coralville, Iowa, United States) in equal volume by denaturing at 95 ^{o}C followed by slow cooling to room temperature. Melting temperature measurement was then performed using Shimadzu UV-1800 UV spectrophotometer (Rydalmere, NSW, Australia) with a temperature range of 20-95 °C (ramp rate = 1.0 ^{o}C/ min). Melting temperature curves were shown in Fig. S14 and T_m values were calculated by the first derivative (Table S2).

Supplementary Figures

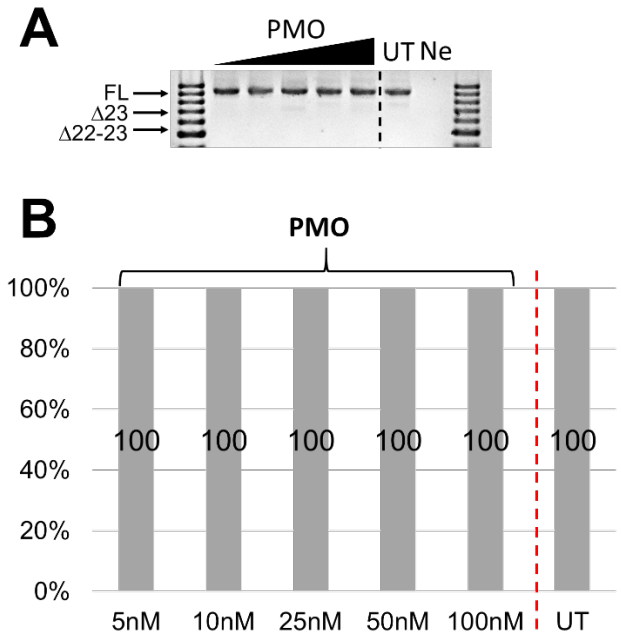

Fig. S1 (A) RT-PCR and (B) densitometry analysis of RNA prepared from *H2K mdx* mouse myotubes transfected with PMO ASO in the presence of lipofectin (24hrs timepoint).

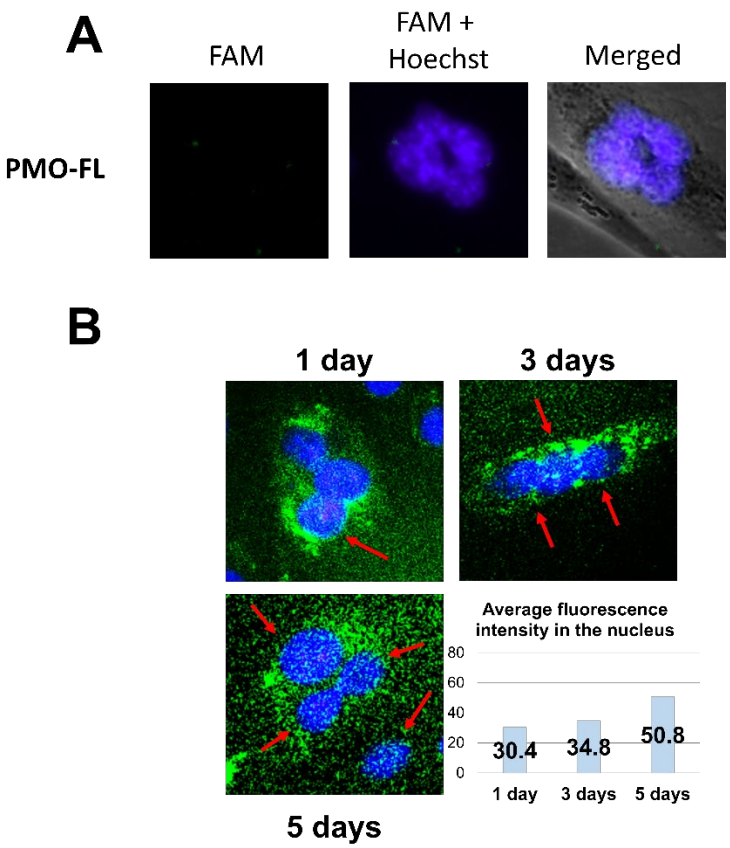

Fig S2. (A) Fluorescence images of PMO-FL at 100nM when transfected with lipofectin (24hrs timepoint); (B) Fluorescence images of PMO-FL at 200nM without a transfection reagent (1, 3, 5-day time points). The graphical illustration shows average fluorescence intensity of the corresponding images inside the nucleus. Red arrows indicate representative cells with internalized ASOs (green dots).

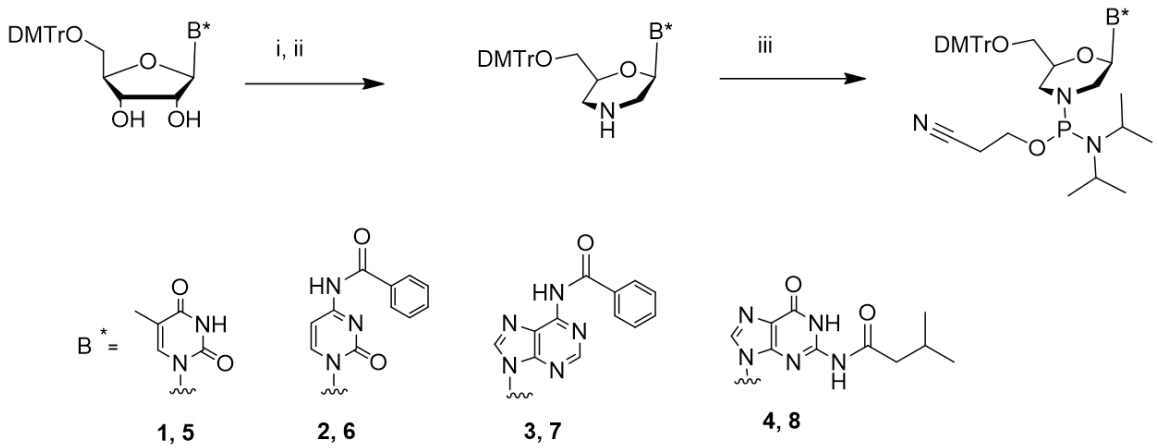

Fig. S3 (i) $NaIO_4$ (1.1 equiv), $(NH_4)_2B_4O_7$ (1.1 equiv), MeOH; (ii) $NaCNBH_3$ (2.0 equiv), AcOH (2.0 equiv), MeOH; (iii) $P(OCH_2CH_2CN)(N\textit{i}Pr_2)_2$ (1.2 equiv), 4,5-dicyanoimidazole (0.5 equiv), and CH_2Cl_2.

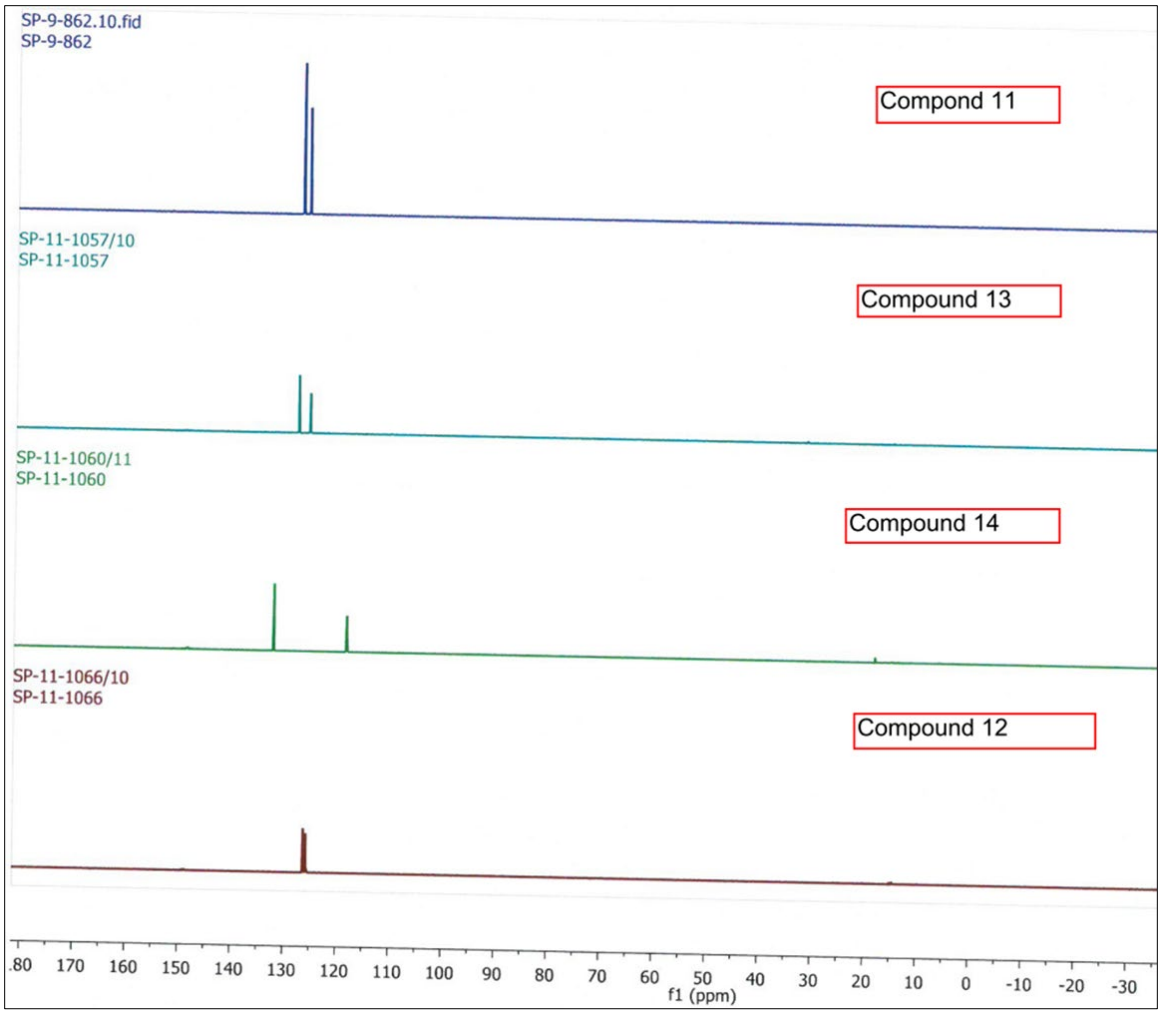

Fig. S4 The 31P NMR spectra of 5 (Compound 11), 6 (Compound 13), 7 (Compound 14) and 8 (Compound 12) are shown.

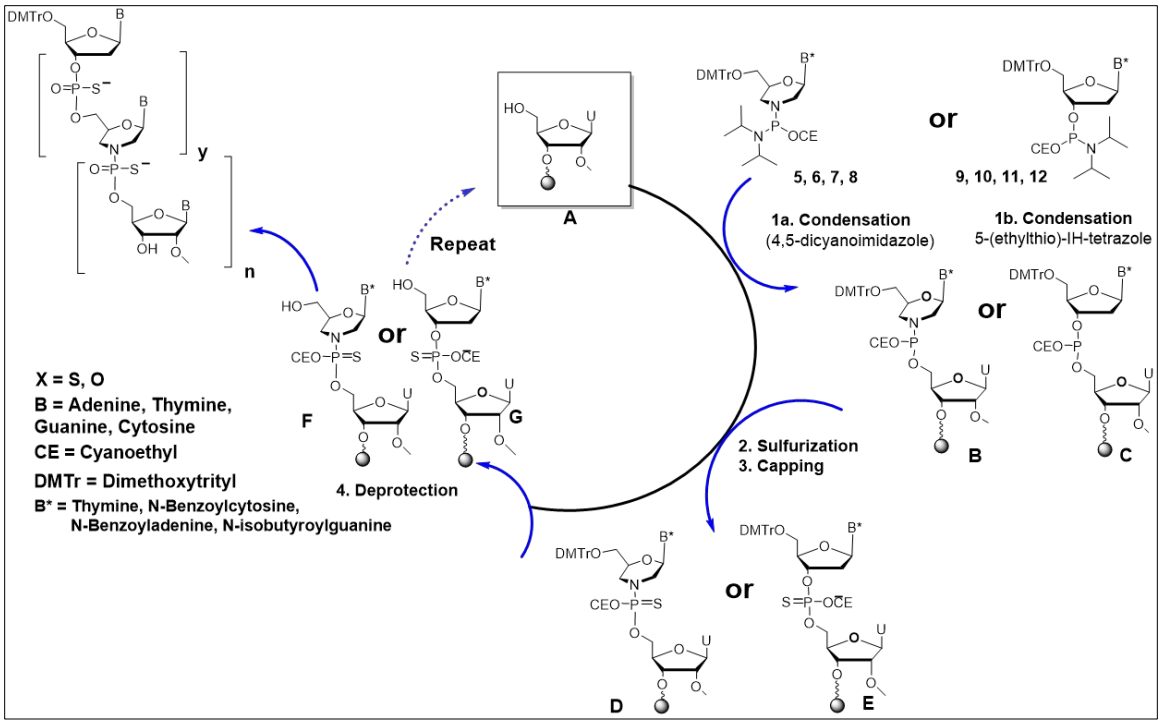

Fig. S5 The solid phase synthesis cycle for preparing TMOs 1-4, TMO1(-2) and TMO1(-4).

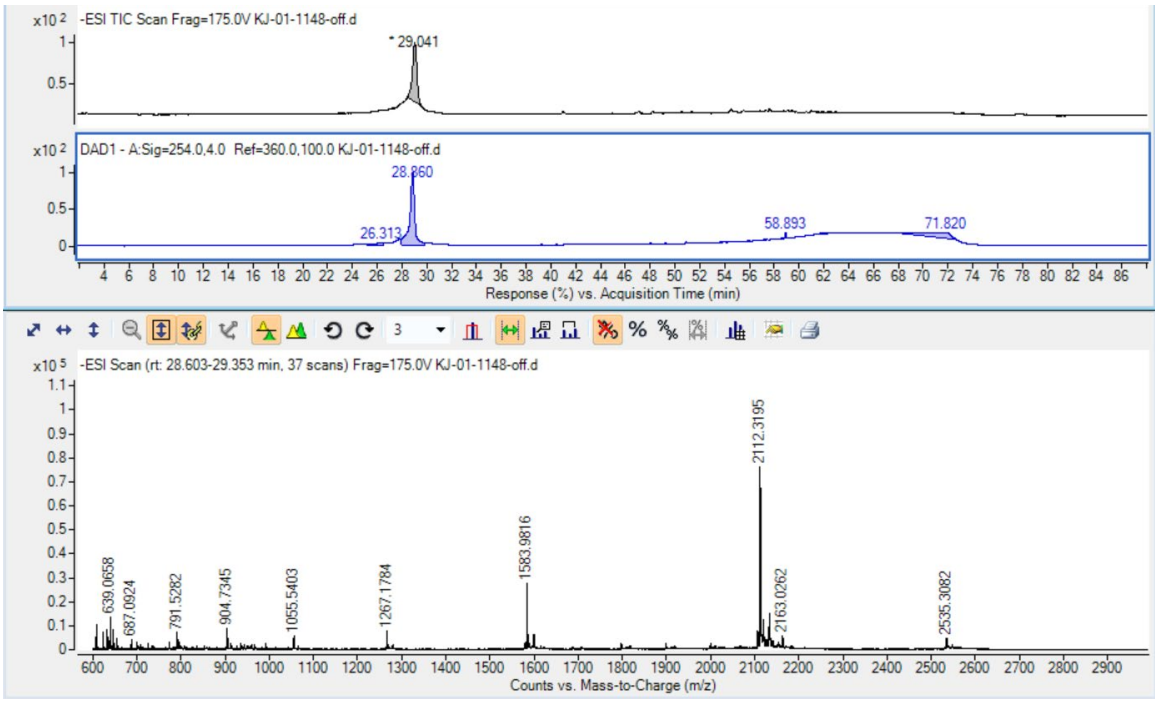

Fig. S6 LCMS Characterization of a sample of TMO-1 used in this study.

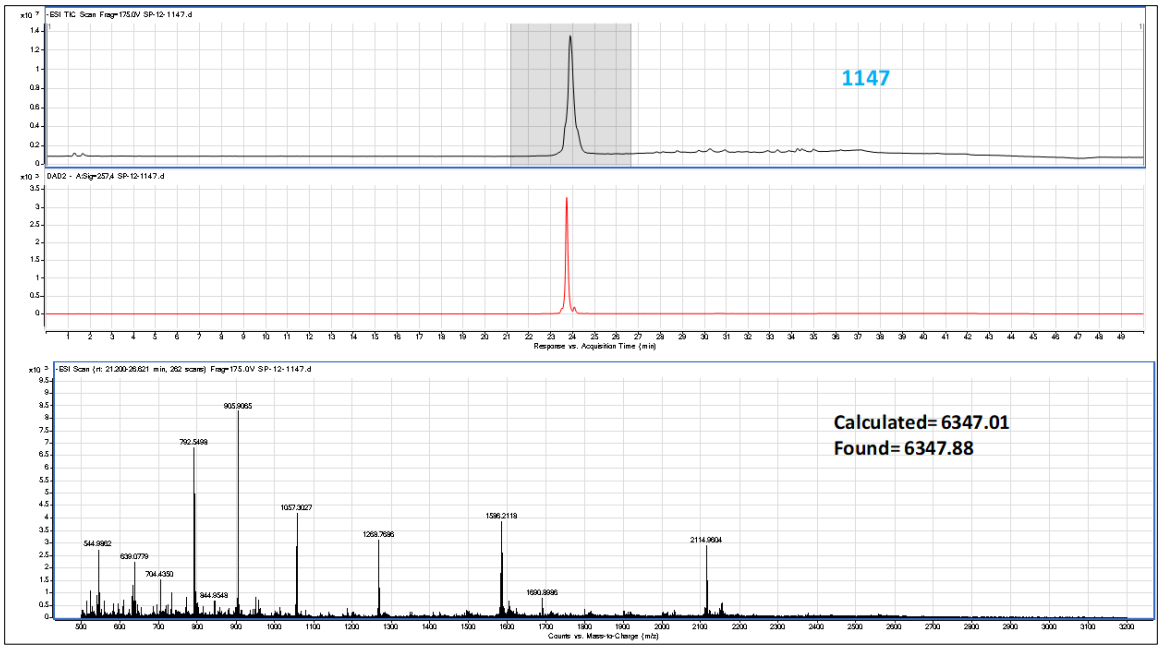

Fig. S7 LCMS Characterization of a sample of TMO-2 used in this study.

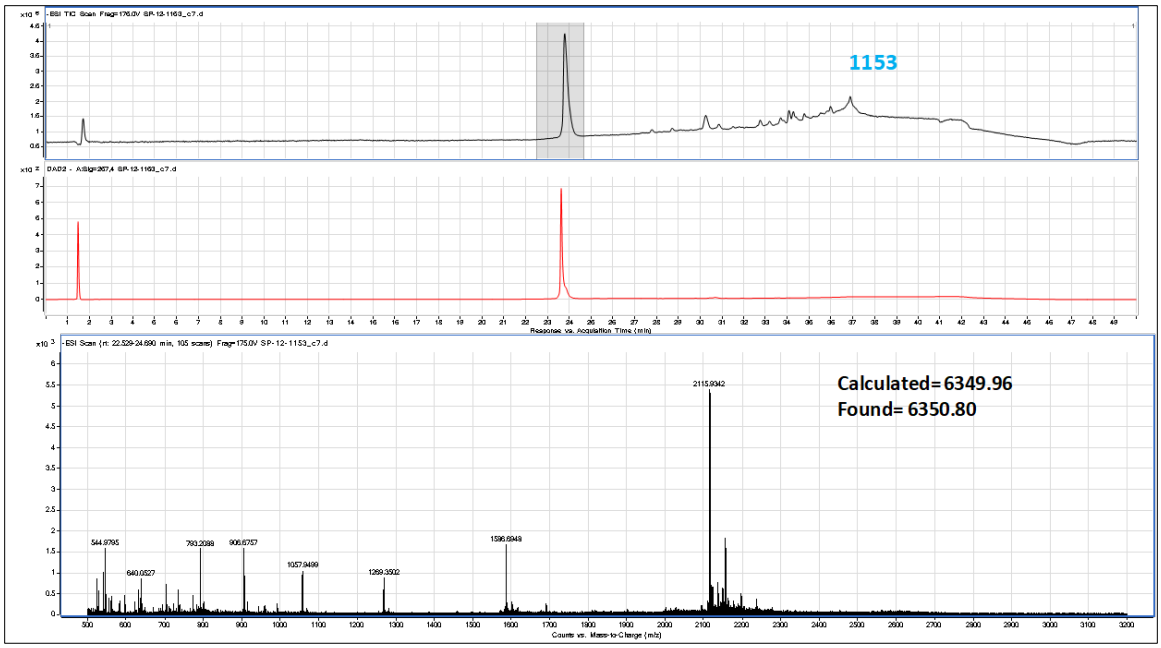

Fig. S8 LCMS Characterization of a sample of TMO-3 used in this study.

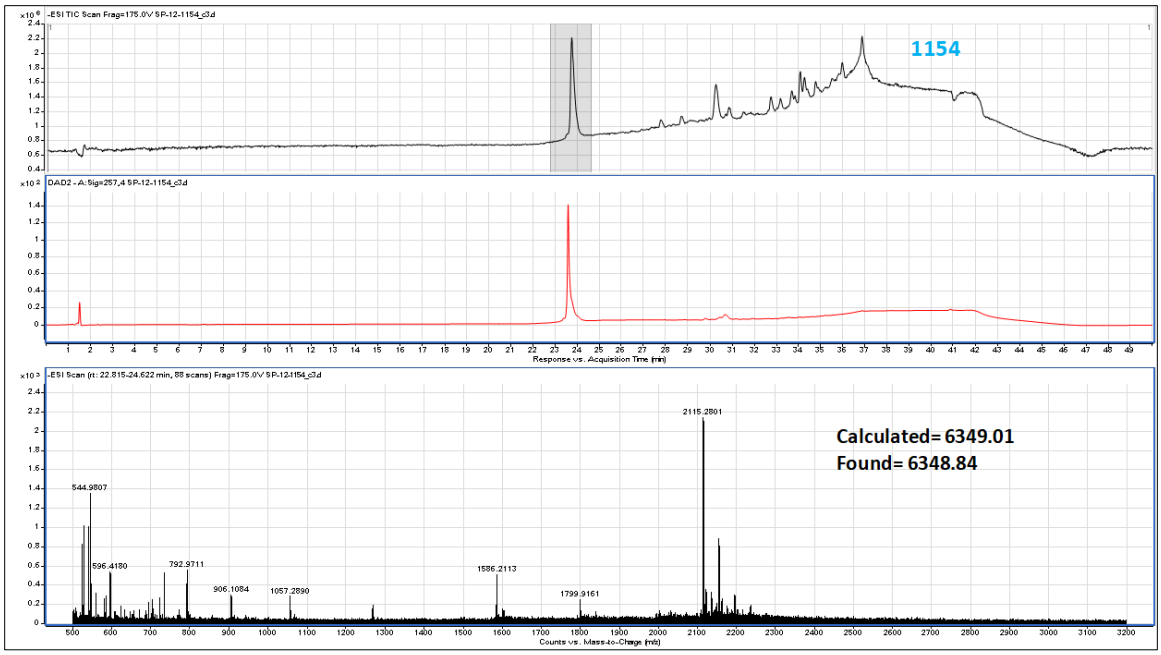

Fig. S9 LCMS Characterization of a sample of TMO-4 used in this study.

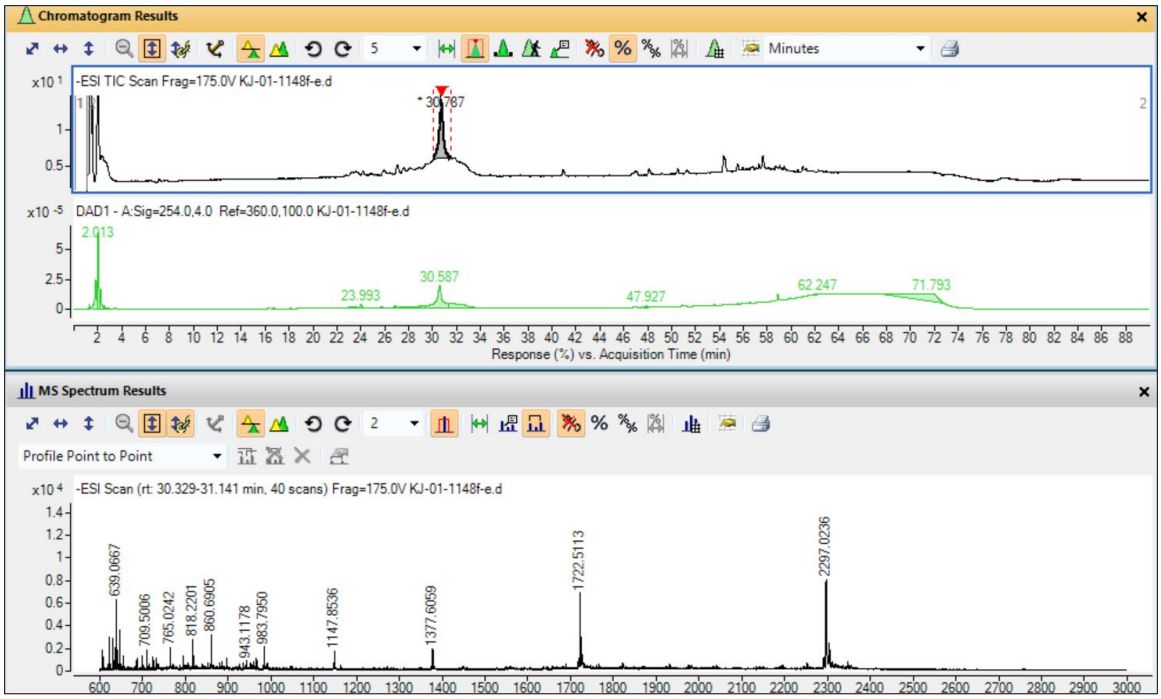

Fig. S10 LCMS Characterization of a sample of TMO1-FL used in this study.

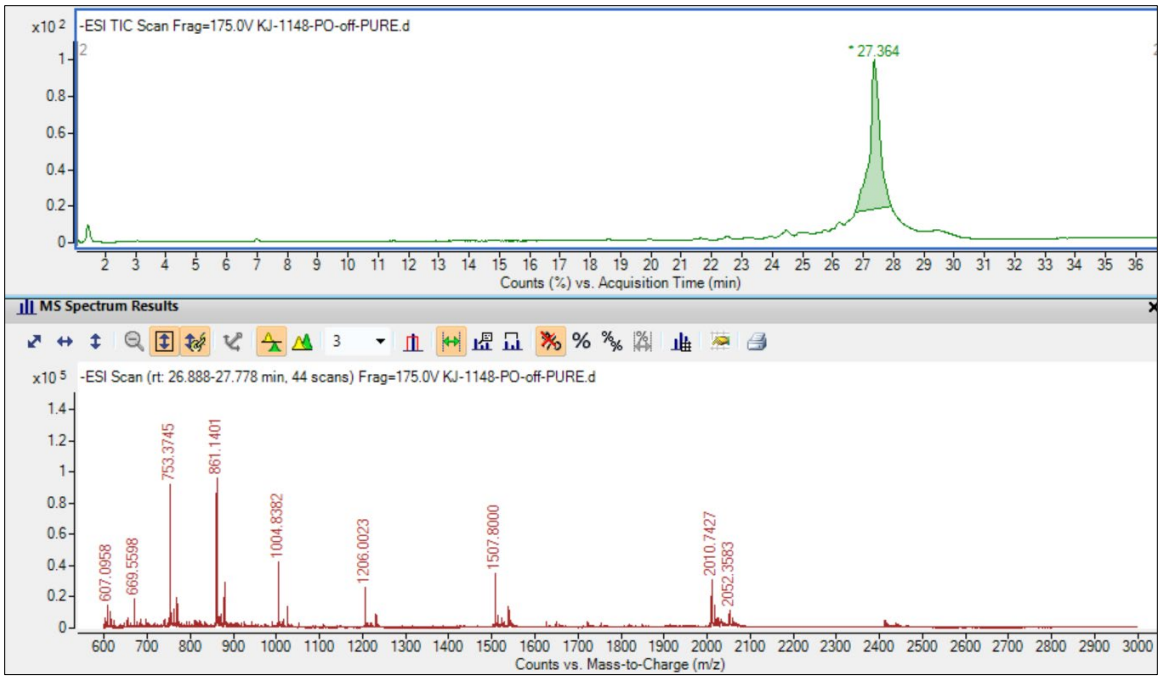

Fig. S11 LCMS Characterization of a sample of MO used in this study.

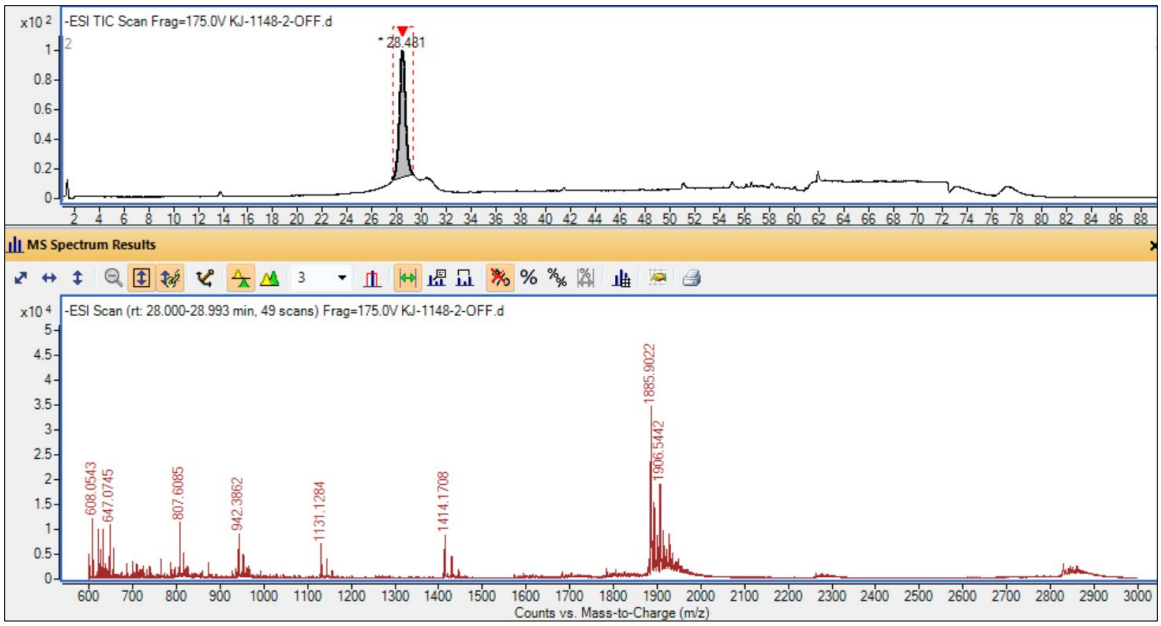

Fig. S12 LCMS Characterization of a sample of TMO1(-2) used in this study.

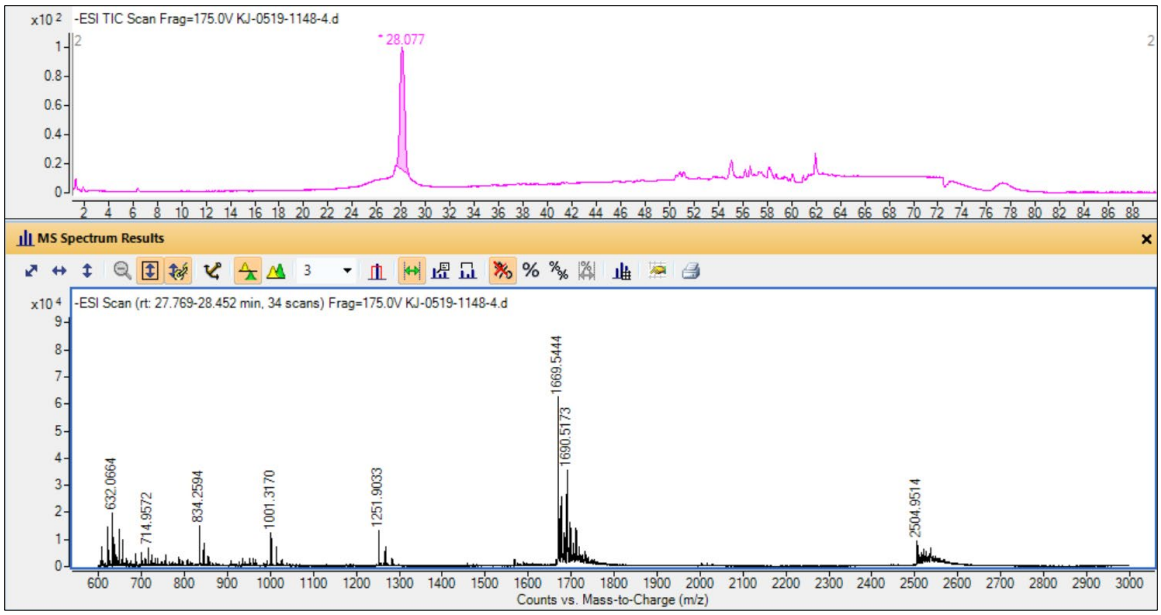

Fig. S13 LCMS Characterization of a sample of TMO1(-4) used in this study.

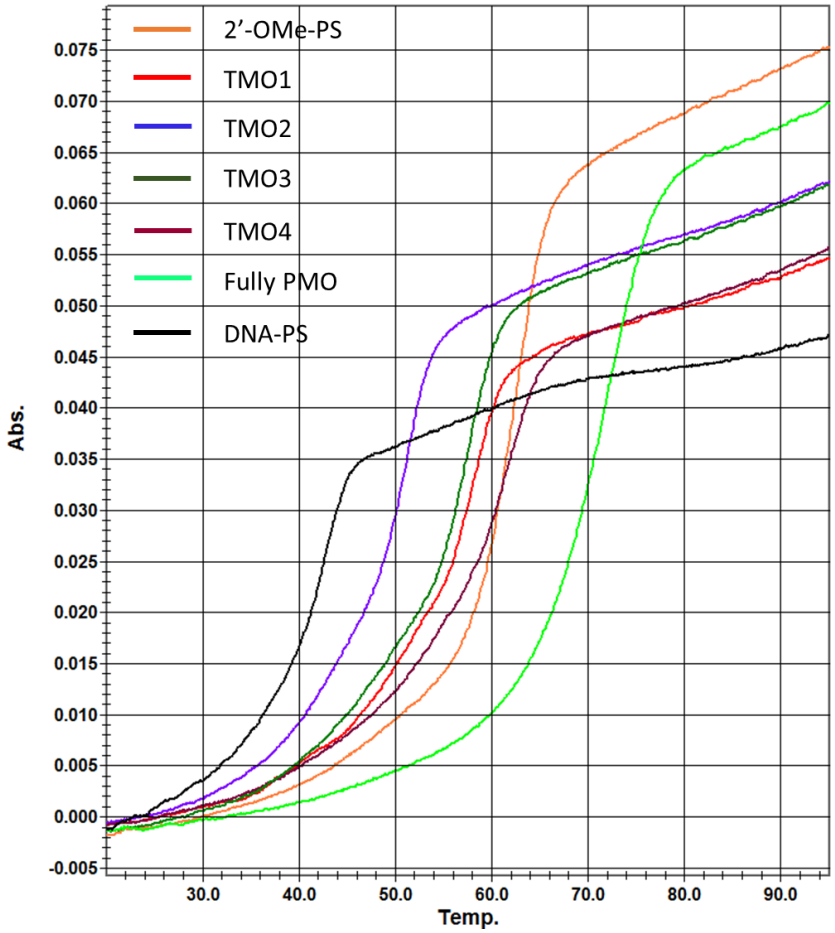

Fig. S14 UV melting profiles of TMOs 1-4, 2’-OMe-PS, PMO and DNA-PS oligonucleotides when duplexed with complementary RNA.

Table S1. Stepwise protocol for the synthesis cycle employed during the synthesis of TMOs 1-4, TMO1(-2) and TMO1(-4).

Reations	Wash/Reagents/Solvents	Time (s)
Detritylation	3% trichloroacetic acid in dichloromethane	Flow 90 s
Condensation	0.1 M phosphordiamidite **5**, **6**, **7** or **8** (in CH_3CN) + Activator* (0.12 M dicyanoimidazole in CH_3CN) Or 0.1 M phosphoramidite **9**, **10**, **11** or **12** in CH_3CN + Activator* (0.25 M etylthio tetrazole in CH_3CN)	Wait 300 s Wait 180 s
Sulfurization	3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione in pyridine, acetonitrile; 3:2	Flow 15 s, wait 90 s
Wash	Anhydrous CH_3CN	Flow 10s
Capping	Cap A* (THF/Pyridine/Ac_2O) + Cap B * (16% 1-methylimidazole in THF)	Flow 10 s, wait 5 s
Wash	Anhydrous CH_3CN	Flow 10 s
Wash	Anhydrous CH_2Cl_2	Flow 25 s

Table S2. Tm data for TMOs 1-4, 2'-OMePS, PMO and DNA.

Duplex	Melting Temperature (°C)
TMO1	58.7
TMO2	51.2
TMO3	57.1
TMO4	61.3
2'-OMe-PS	62.9
PMO	71.6
DNA-PS	42.6

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