Synthesis and cellular activity of stereochemically-pure 2’-O-(2-methoxyethyl)-phosphorothioate oligonucleotides

Meiling Li, Helen L. Lightfoot, François Halloy, Anna L. Malinowska, Christian Berk, Alok Behera, Daniel Schümperli and Jonathan Hall

Supplementary Information

Contents
Reagents and Instruments ........................................................................................................................................... 3
The synthesis of 5’-ODMT-2’-OMOE-N-benzoyl adenosine (S3) ........................................................................... 3
2’-OMOE adenosine (S1) ........................................................................................................................................ 3
2’-OMOE-N-benzoyl adenosine (S2) ...................................................................................................................... 4
5’-DMT-2’-OMOE-N-benzoyl adenosine (S3) ........................................................................................................ 5
The synthesis of 5’-ODMT-2’-OMOE-N\textsuperscript{2}-isobutyryl-Guanosine (S9) ........................................... 5
2’-OMOE-2-amino-adenosine (S5) ........................................................................................................................ 5
2’-OMOE-N\textsuperscript{2}-isobutyryl-adenosine (S6) ................................................................................................... 6
2’-OMOE-N\textsuperscript{2}-isobutyryl-guanosine (S8) ................................................................................................... 7
5’-ODMT-2’-OMOE-N\textsuperscript{2}-isobutyryl-guanosine (S9) ................................................................................... 8
The synthesis of chiral phosphoramidites (4a-4h) .................................................................................................. 9
Sp-T OAP monomer (Sp-4a) ................................................................................................................................ 9
Sp-G\textsuperscript{4bu} OAP monomer (Sp-4d) .............................................................................................................. 10
Sp-mC\textsuperscript{Bz} OAP monomer (Sp-4b) ............................................................................................................... 11
Sp-A\textsuperscript{Bz} OAP monomer (Sp-4c) ................................................................................................................ 12
Rp-A\textsuperscript{Bz} OAP monomer (Rp-4g) ................................................................................................................ 13
Rp-T OAP monomer (Rp-4e) ................................................................................................................................ 14
Rp-mC\textsuperscript{Bz} OAP monomer (Rp-4f) ................................................................................................................ 14
Rp-G\textsuperscript{4bu} OAP monomer (Rp-4h) ................................................................................................................ 15
Materials and methods for properties .................................................................................................................. 16
Oligoribonucleotide phosphorothioate synthesis ................................................................................................. 16
Thermal stability studies (T\textsubscript{m}) ............................................................................................................. 17
Cell culture and gapmers (Mp (16), Mp-Rp (17) and Mp-Sp (18)) transfection .................................................. 17
RNA extraction and purification

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Cell culture and SSO (FCH (20), FCH-Rp (21), FCH-Sp (22) and FCH-PO (24)) minigene assay

RNA extraction and purification

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Agarose gel electrophoresis

Data analysis

Plasmid for SSO minigene assay

HPLC-MS profiles of purified FCH-Sp (22), FCH-Rp (21), Mp-Sp (18) and Mp-Rp (17)

Melting temperature (T_m) curves of stereodefined PS-oligonucleotides

Spectra of synthesized nucleosides and phosphoramidites

1H and 13C NMR spectra of 2'-OMOE adenosine (S1)

1H and 13C NMR spectra of 2'-OMOE-2-amino-adenosine (S5)

1H and 13C NMR spectra of 2'-OMOE-N-benzoyl adenosine (S2)

1H and 13C NMR spectra of 5'-ODMT-2'-OMOE-N-benzoyl adenosine (S3)

1H and 13C NMR spectra of 2'-OMOE-N2-isobutyryl-adenosine (S6)

1H and 13C NMR spectra of 2'-OMOE-N2-isobutyryl-guanosine (S8)

1H and 13C NMR spectra of 5'-ODMT-2'-OMOE-N2-isobutyryl-guanosine (S9)

1H, 13C and 31P NMR spectra of Sp-A⁴⁸ (Sp-4c) OAP monomer

1H, 13C and 31P NMR spectra of Rp-A⁴⁸ (Rp-4g) OAP monomer

1H, 13C and 31P NMR spectra of Sp-G⁴⁸ (Sp-4d) OAP monomer

1H, 13C and 31P NMR spectra of Rp-G⁴⁸ (Rp-4h) OAP monomer

1H, 13C and 31P NMR spectra of Sp-T (Sp-4a) OAP monomer

1H, 13C and 31P NMR spectra of Rp-T (Rp-4e) OAP monomer

1H, 13C and 31P NMR spectra of Sp-·mC⁸ (Sp-4b) OAP monomer

1H, 13C and 31P NMR spectra of Rp-·mC⁸ (Rp-4f) OAP monomer

The crude HPLC-MS profiles of stereodefined PS-ORNs

Crude HPLC-MS profiles of DMT-T₅₆CGTAGT (5) and DMT-T₅₆CGTAGT (11)

Crude HPLC-MS profiles of DMT-C₅₆CGTAGT (6) and DMT-C₅₆CGTAGT (12)

Crude HPLC-MS of DMT-A₅₆CGTAGT (7) and DMT-A₅₆CGTAGT (13)

Crude HPLC-MS of DMT-·mC₅₆CGTAGT (6) and DMT-·mC₅₆CGTAGT (12)

Crude HPLC-MS of DMT-G₅₆CGTAGT (8) and DMT-G₅₆CGTAGT (14)

Crude HPLC-MS of DMT-T₅₆AGTAGT (10)

Crude HPLC-MS of DMT-T₅₆TGTAGT (9)
Reagents and Instruments

All NMR spectra were recorded on a Bruker AV400. $^1$H NMR, $^{13}$C NMR and $^{31}$P NMR were measured in deuterated solvents. ESI Mass spectra were recorded on a Bruker’s solariX (ESI/MALDI-FTICR-MS).

All glassware were dried thoroughly prior to use. Triethylamine was distilled from CaH$_2$, and stored over KOH pellets under Argon. N-methyl morpholine was distilled from BaO, and stored over KOH pellets under Argon. Phosphorus trichloride was purchased from Aldrich and used as received. Tetrahydrofuran and toluene were purchased from Aldrich and stored over molecular sieves. The other organic solvents were reagent grade and used as received. Silica gel column chromatography was carried out using Merck Silica gel 40-60 µm (230 – 400 mesh). Analytical TLC was performed on Merck Kieselgel 60 F$_{254}$ aluminium sheet. The activator N-phenyl imidazolium triflate was prepared according to the literature$^1$.

Chiral auxiliaries D-I and L-I were synthesized as previously reported$^2$. Compound 5’-ODMT-2’-OMOE-thymidine and 5’-DMT-2’-OMOE-5-methyl-cytidine were synthesized according to the literature$^3$.

The synthesis of 5’-ODMT-2’-OMOE-N-benzoyl adenosine (S3)

2’-OMOE adenosine (S1)

![S1]

Adenosine (5.34 g, 20 mmol) was dissolved in DMF (130 ml) at 100 °C. Then it was cooled down to 0 °C and NaH (60% in mineral oil, 1.28g, 32 mmol) was added portionwise. The resulting mixture
was stirred at room temperature for 1 h. Tosylated alcohol (5.0 g, 22 mmol) was added over 10 mins. The reaction mixture was stirred for 16 h at 50 °C. DMF was evaporated to dryness under reduced pressure. The residue was dissolved in methanol (100 ml) followed by silica gel (20 g). Methanol was removed until very thin power was formed. The mixture was subjected to purification by chromatography (silica gel, CH2Cl2:MeOH, 10:1) to give the mixture of 2'- and 3'-isomers. The mixture was precipitated from CH2Cl2 to give pure 2'-isomer S1 (2.6 g, 40%). 1H NMR (400 MHz, DMSO-d6) δ 8.38 (s, 1 H), 8.14 (s, 1 H), 7.35 (s, 2 H), 5.99 (d, J = 6.08 Hz, 1 H), 5.42 (dd, J = 6.97, 4.69 Hz, 1 H), 5.13 (d, J = 4.82 Hz, 1 H), 4.55 (dd, J = 6.21, 4.94 Hz, 1 H), 4.35 - 4.28 (m, 1 H), 3.98 (q, J = 3.30 Hz, 1 H), 3.72 - 3.63 (m, 2 H), 3.60 - 3.48 (m, 2 H), 3.37 (t, J = 4.69 Hz, 2 H), 3.12 (s, 3 H). 13C NMR (100 MHz, DMSO-d6) δ 156.4, 153.0, 149.4, 140.6, 119.6, 119.5, 86.7, 86.5, 81.6, 71.5, 69.4, 61.8, 58.4.

2'-OMOE-N-benzoyl adenosine (S2)

Compound S1 (4.87 g, 15 mmol) was coevaporated with anhydrous pyridine (2 x 15 ml) and dissolved in anhydrous pyridine (70 ml) followed by the addition of chlorotrimethylsilane (61.7 mmol, 7.8 ml). After 30 mins, benzoyl chloride (2.3 ml, 19.6 mmol) was added and stirring was continued for 3 h. The reaction mixture was then cooled down to 0 °C and quenched with water (30 ml). Subsequently the reaction mixture was treated with an aqueous concentrated ammonium hydroxide solution (50 ml) and stirred for 30 min at room temperature. The mixture was extracted with CH2Cl2 (3 x 100 ml). The organic layer was dried over Na2SO4 and concentrated under reduced pressure. Flash chromatography (CH2Cl2:MeOH, 95:5) give the desired compound S2 (4.76, 74%) as a colorless foam. 1H NMR (400 MHz, DMSO-d6) δ 11.21 (s, 1 H), 8.75 (d, J = 10.65 Hz, 2 H), 8.04 (d, J = 7.10 Hz, 2 H), 7.68 - 7.61 (m, 1 H), 7.59 - 7.52 (m, 2 H), 6.15 (d, J = 5.83 Hz, 1 H), 5.20 (d, J = 5.32 Hz, 1 H), 5.15 (t, J = 5.58 Hz, 1 H), 4.61 (t, J = 5.32 Hz, 1 H), 4.39 - 4.32 (m, 1 H), 4.00 (q, J = 3.80 Hz, 1 H), 3.77 - 3.65 (m, 2 H), 3.64 - 3.54 (m, 2 H), 3.42 - 3.39 (m, 2 H), 3.14 (s, 3
$^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 166.1, 152.6, 152.2, 151.0, 143.5, 133.8, 133.0, 128.9 (4C), 126.3, 86.6, 86.3, 81.7, 71.6, 69.5, 69.3, 61.6, 58.5.

5'-DMT-2'-OMOE-N-benzoyl adenosine (S3)$^5$

![Chemical structure of S3]

Compound S2 (3.7 g, 8.6 mmol) was coevaporated with anhydrous pyridine (2 x 15 mL) and then dissolved in anhydrous pyridine (40 mL). DMTrCl (3.79 g, 9.50 mmol) was added portionwise at 0 °C. The reaction was stirred for 3 h at 0 °C. Once completed, the reaction mixture was stirred for 30 min after the addition of methanol (5 mL). The solvent was evaporated under reduced pressure. The residue was portioned between ethyl acetate (40 mL) and sat. aq. NaHCO$_3$ (70 mL). The organic phase was separated. The aqueous layer was extracted with ethyl acetate (2 x 40 mL). The combined organic extracts were dried over Na$_2$SO$_4$, filtered and concentrated. Purification on chromatography (silica gel, DCM:MeOH, 97:3) afford compound S3 as a white foam (5.1 g, 81%).

$^1$H NMR (400 MHz, CD$_3$CN) $\delta$ 9.54 (br s, 1 H), 8.58 (s, 1 H), 8.27 (s, 1 H), 7.98 (d, $J = 7.4$ Hz, 2 H), 7.61 - 7.58 (m, 1 H), 7.51 - 7.49 (m, 2 H), 7.42 - 7.39 (m, 2 H), 7.29 - 7.18 (m, 7 H), 6.82 - 6.79 (m, 4 H), 6.12 (d, $J = 4.6$ Hz, 1 H), 4.71 (t, $J = 4.7$ Hz, 1 H), 4.53 (br s 1 H), 4.16 - 4.13 (m, 1H), 3.82 - 3.74 (m, 3 H), 3.72 (s, 6 H), 3.48 - 3.45 (m, 2 H), 3.34 (d, $J = 4.1$ Hz, 2 H), 3.23 (s, 3H). $^{13}$C NMR (100 MHz, CD$_3$CN) $\delta$ 159.7, 153.0, 146.0, 143.5, 136.9, 136.8, 133.6, 131.1, 129.7, 129.4, 129.2, 129.1, 128.9, 128.4, 127.9, 114.1, 88.1, 87.2, 85.1, 82.7, 72.5, 71.1, 70.8, 64.4, 59.1, 55.9.

The synthesis of 5'-ODMT-2'-OMOE-N$^2$-isobutyryl-Guanosine (S9)

2'-OMOE-2-amino-adenosine (S5)

![Chemical structure of S5]
2-Amino adenosine **S4** (22.6 g, 80 mmol) was dissolved in DMF (350 ml) at 100 °C. Then it was cooled down to 0 °C and NaH (60% in mineral oil, 5.12 g, 128 mmol) was added portionwise. The resulting mixture was stirred at room temperature for 1 h. Tosylated alcohol (20.2 g, 88 mmol) was added over 20 min. The reaction mixture was stirred for 16 h at 50 °C. DMF was evaporated to dryness under reduced pressure. The residue was dissolved in MeOH (100 ml) followed by silica gel (60 g). Methanol was removed until very thin power was formed. The mixture was subjected to purification by chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 10:1) to give the mixture of 2'- and 3'- isomers. The mixture was precipitated from CH$_2$Cl$_2$ to give pure 2'- isomer **S5** (9.0 g, 33%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.97 (br s, 1 H), 6.79 (br s, 2 H), 5.83 (d, $J$ = 6.84 Hz, 1 H), 5.78 - 5.72 (m, 2 H), 5.44 (dd, $J$ = 6.59, 4.82 Hz, 1 H), 5.05 (d, $J$ = 4.82 Hz, 1 H), 4.45 (dd, $J$ = 6.59, 5.07 Hz, 1 H), 4.27 (td, $J$ = 4.69, 2.79 Hz, 1 H), 3.93 (q, $J$ = 3.46 Hz, 1 H), 3.72 - 3.60 (m, 2 H), 3.58 - 3.48 (m, 2 H), 3.44 - 3.36 (m, 2 H), 3.16 (s, 3 H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 160.5, 156.7, 151.9, 136.6, 113.9, 86.5, 85.5, 81.4, 71.5, 69.6, 69.3, 62.1, 58.5.

**2'-OMOE-N2-isobutyryl-adenosine (S6)**

![Chemical structure](image)

Compound **S6** was prepared using a protocol from ref$^6$. **S5** (10.48 g, 30.8 mmol) was dried by repeated co-evaporation with anhydrous pyridine (3 X 100 ml) and dissolved in anhydrous pyridine (350 ml). Trimethylsilyl chloride (19.5 ml, 154 mmol) was added and the mixture was stirred at room temperature for 30 min. The solution was cooled to - 20 °C and isobutyryl chloride (3.55 ml, 34.0 mmol) was added dropwise over 30 min. The reaction mixture was stirred at - 20 °C for 2.5 h followed by 1 hour at room temperature. After completion of the reaction (by TLC), the reaction mixture was cooled (ice-bath), ethanol (100 ml) was added, and after 20 min, 28% aqueous ammonia (200 ml) was added. Stirring was continued for 30 min at room temperature. The solvents were evaporated. The resulting oily residue was coevaporated with toluene and
subjected to flash chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 95:5) to give mono-protected S6 (6.82 g) and double-protected S7 (3.70 g). The isolated product S7 was dissolved in methanol (25 ml) followed by the addition of trimethylamine (1.15 ml, 8.25 mmol). The resulting reaction mixture was stirred for 24 h at room temperature. The reaction was condensed and the residue was purified on flash chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 94:6) to give the product S6 (3.16 g) with an overall yield of 79% over two steps. $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 9.82 (s, 1 H), 8.27 (s, 1 H), 7.22 (br s, 2 H), 5.93 (d, $J = 6.34$ Hz, 1 H), 5.09 (d, $J = 5.07$ Hz, 1 H), 5.04 (t, $J = 5.70$ Hz, 1 H), 4.53 (dd, $J = 6.21$, 4.94 Hz, 1 H), 4.30 (td, $J = 4.75$, 3.17 Hz, 1 H), 3.93 (d, $J = 3.29$ Hz, 1 H), 3.73 - 3.49 (m, 4 H), 3.43 - 3.37 (m, 2 H), 3.15 (s, 3 H), 2.84 (br s, 1 H), 1.06 (d, $J = 6.59$ Hz, 6 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 175.5, 156.6, 153.3, 150.8, 139.2, 116.7, 86.4, 85.4, 81.5, 71.5, 69.4, 61.9, 58.4, 55.4, 34.6, 19.8 (2C).

$2'$-OMOE-N$^2$-isobutyryl-guanosine (S8)

![Image](image-url)

Compound S8 can be prepared via enzymatic transformation. However, we synthesized it in a protocol similar to that in ref 8. To a stirred solution of compound S6 (7.48 g, 18.2 mmol) in a mixture of acetic acid (7.3 ml) and water (15 ml) was added NaNO$_2$ (15.1 g, 218.4 mmol). The reaction mixture was stirred for 20 h at room temperature. Once it was completed, the solvents were evaporated under reduced pressure. Then acetone (30 ml) was added. The precipitation was filtered off. The filtrate was concentrated and the residue was subjected to chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 12:1) to give an off white powder S8 (7.0 g, 94%). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 12.08 (br s, 1 H), 11.67 (br s, 1 H), 8.28 (s, 1 H), 5.90 (d, $J = 6.59$ Hz, 1 H), 5.14 - 5.04 (m, 2 H), 4.41 (dd, $J = 6.59$, 4.82 Hz, 1 H), 4.29 (d, $J = 3.04$ Hz, 1 H), 3.93 (d, $J = 3.04$ Hz, 1 H), 3.73 - 3.65 (m, 1 H), 3.64 - 3.50 (m, 3 H), 3.42 - 3.37 (m, 2 H), 3.16 (s, 3 H), 2.82 - 2.69 (m, 1 H), 1.12 (d, $J = 6.59$ Hz, 6 H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 180.34, 155.02, 149.11, 148.48, 137.72, 120.25, 86.26, 84.68, 81.74, 71.31, 69.12 (2C), 61.43, 58.20, 34.97, 19.06 (2C).
Compound **S8** (2.05 g, 5.0 mmol) was coevaporated with anhydrous pyridine (2 x 10 mL) and then was dissolved in anhydrous pyridine (30 mL). DMTrCl (2.20 g, 5.5 mmol) was added portionwise at 0 °C. The reaction was stirred for 3 h at 0 °C and 30 mins at room temperature. Once completed, the reaction mixture was stirred for 30 mins after the addition of methanol (3 mL). The solvent was evaporated under reduced pressure. The residue was portioned between ethyl acetate (30 mL) and sat. aq. NaHCO₃ (60 mL). The organic phase was separated. The aqueous layer was extracted with ethyl acetate (2 x 30 mL). The combined organic extracts were dried over Na₂SO₄, filtered and concentrated. Purification on chromatography (silica gel, DCM:MeOH, 96:4) afford the white foam **S9** (2.3 g, 65%). **¹H NMR (400 MHz, CD₃CN) δ 11.96 (s, 1 H), 9.23 (s, 1 H), 7.83 (s, 1 H), 7.43 - 7.38 (m, 2 H), 7.31 - 7.17 (m, 7 H), 6.83 - 6.77 (m, 4 H), 5.90 (d, J = 4.56 Hz, 1 H), 4.59 - 4.54 (m, 1 H), 4.53 - 4.46 (m, 1 H), 4.10 - 4.06 (m, 1 H), 3.84 - 3.77 (m, 1 H), 3.76 - 3.68 (m, 7 H), 3.54 - 3.47 (m, 3 H), 3.38 (dd, J = 10.52, 5.70 Hz, 1 H), 3.29 - 3.22 (m, 4 H), 2.64 - 2.52 (m, 1 H), 1.15 (dd, J = 8.74 Hz, 3 H), 1.13 (dd, J = 8.74 Hz, 3 H). **¹³C NMR (100 MHz, CD₃CN) δ 180.4, 159.3, 155.8, 149.1, 148.5, 145.6, 138.5, 136.4, 136.3, 130.6, 130.5, 128.6 (2C), 128.3 (2C), 127.4 (2C), 122.0 (2C), 117.9, 113.5 (4C), 87.4, 86.7, 84.7, 82.4, 72.1, 70.7, 70.5, 64.5, 58.7, 55.5 (2C), 36.2, 18.8, 18.7.
The synthesis of chiral phosphoramidites (4a-4h)

\[
\begin{align*}
\text{HO} & \quad \text{1) N-methyl morpholine (2 equiv)} \\
\text{Ph} & \quad \text{2) PCl}_3 (0.95 \text{ equiv}), \text{toluene, -78 °C}
\end{align*}
\]

(R)-phenyl((S)-pyrrolidin-2-yl)methanol

\[
\begin{align*}
\text{PH} & \quad \text{B}^{PR0} \\
\text{T} & \quad \text{NC}_{Bz} \\
\text{A}^{Bz} & \quad \text{G}^{Bu}
\end{align*}
\]

Sp-T OAP monomer (Sp-4a)

\[
\begin{align*}
\text{DMTrO} & \quad \text{8} \\
\text{Ph} & \quad \text{8}
\end{align*}
\]

A typical procedure for the synthesis of phosphoramidites 4a-4h

(S)-phenyl((R)-pyrrolidin-2-yl)methanol (533 mg, 3 mmol) was co-evaporated with anhydrous toluene three times and was dissolved in anhydrous toluene (3 mL) in a 25 ml round bottom flask with stirring under Argon. To this solution was added N-methyl morpholine (0.66 ml, 6 mmol). A second round bottom flask was charged with anhydrous toluene (5 ml) and phosphorus trichloride (0.25 ml, 2.85 mmol) at –70 °C with stirring under Argon. The amino alcohol solution was transferred to the solution of PCl3 over a period of 5 mins at –70 °C. The reaction mixture was allowed to warm to room temperature and was stirred for 1 hour.
In a third round bottom flask (50 ml), 5′-ODMTr-2′-OMOE-thymidine (930 mg, 1.5 mmol) was co-evaporated three times with anhydrous toluene and was dissolved in THF (7.5 ml). Et₃N (2.1 ml, 15 mmol) was then added. The solution of 2-chloro-oxazaphospholidine intermediate D-I was added via syringe over a period of 20 mins at – 70 °C. The reaction was allowed to warm up to room temperature and was stirred for 3 h. The reaction mixture was cooled to – 20 °C and was quenched with saturated aqueous sodium bicarbonate solution (30 ml). The mixture was diluted with ethyl acetate (30 ml). The organic layer was separated and the aqueous layer was extracted twice with ethyl acetate (2 X 30 ml). The combined organic phases were washed with brine, dried over Na₂SO₄, filtered and concentrated. The crude material was passed through a very short of silica gel, which was flushed with elute (EA:hexane:Et₃N, 80:20:2). The fractions containing the product were collected and concentrated to give the amidite (Sp)-4a as a white powder (1.10 g, 89%). ¹H NMR (400 MHz, CD₃CN) δ 9.06 (br s, 1 H), 7.54 (s, 1 H), 7.48 - 7.44 (m, 2 H), 7.36 - 7.21 (m, 12 H), 6.86 (dd, J = 8.74, 0.89 Hz, 4 H), 5.86 (d, J = 4.31 Hz, 1 H), 5.66 (d, J = 6.59 Hz, 1 H), 4.80 (d, J = 9.38 Hz, 1 H), 4.22 - 4.17 (m, 1 H), 4.15 - 4.09 (m, 1 H), 3.90 - 3.82 (m, 1 H), 3.80 - 3.75 (m, 8 H), 3.69 - 3.65 (m, 1 H), 3.58 - 3.49 (m, 1 H), 3.47 - 3.43 (m, 2 H), 3.42 (d, J = 2.28 Hz, 1 H), 3.28 (dd, J = 11.15, 3.04 Hz, 2 H), 3.24 (s, 3 H), 3.11 - 3.01 (m, 2 H), 1.67 - 1.49 (m, 2 H), 1.21 - 1.13 (m, 1H), 0.90 - 0.85 (m, 1 H). ¹³C NMR (100 MHz, CD₃CN) δ 164.7, 159.9, 151.6, 145.8, 139.8 (d, 3Jpc = 4.02 Hz), 136.6, 136.5, 131.2, 129.2, 129.1, 129.0, 128.5, 128.1, 126.6, 114.2, 111.4, 88.5, 87.7, 84.1 (d, 3Jpc = 9.1 Hz), 83.8 (d, 3Jpc = 3.02 Hz), 82.2 (d, 3Jpc = 2.01 Hz), 72.8, 70.9 (d, 3Jpc = 5.03 Hz), 70.8, 68.1 (d, 3Jpc = 3.02 Hz), 63.3, 59.2, 56.0, 47.7 (d, 3Jpc = 3.42 Hz), 28.8, 26.7 (d, 3Jpc = 3.02 Hz), 12.4. ³¹P NMR (161 MHz, CD₃CN) δ 151.63. ESI-HRMS: m/z calcd for C₄₅H₅₀N₃O₁₀P⁺ [(M + H)⁺] 823.3234, found 823.3235.

**Sp-G¹Bu OAP monomer (Sp-4d)**
Crude (Sp)-4d was synthesized from 5’-ODMTr-2’-OMOE-guanosine S9 (715 mg, 1 mmol) and (S)-phenyl([R]-pyrrolidin-2-yl)methanol (355 mg, 2 mmol) following the procedure described above and purified by silica gel column chromatography (EA:Acetone:Et3N, 80:20:2) to give (Sp)-4d as a white powder (500 mg, 49%). 1H NMR (400 MHz, CD3CN) δ 7.90 (s, 1 H), 7.49 - 7.42 (m, 2 H), 7.36 - 7.19 (m, 12 H), 6.83 (d, J = 8.87 Hz, 4 H), 5.90 (d, J = 5.58 Hz, 1 H), 5.62 (d, J = 6.59 Hz, 1 H), 4.87 (dt, J = 9.70, 4.66 Hz, 1 H), 4.68 - 4.61 (m, 1 H), 4.26 - 4.18 (m, 1 H), 3.85 (dt, J = 13.31, 6.53 Hz, 1 H), 3.75 (s, 6 H), 3.73 - 3.69 (m, 2 H), 3.60 - 3.47 (m, 1 H), 3.45 - 3.39 (m, 3 H), 3.31 (dd, J = 10.90, 4.31 Hz, 1 H), 3.19 (s, 3 H), 3.09 - 3.01 (m, 1 H), 2.53 - 2.44 (m, 1 H), 1.65 - 1.49 (m, 2 H), 1.19 - 1.13 (m, 1 H), 1.13 (d, J = 6.84 Hz, 3 H), 1.09 (d, J = 6.84 Hz, 3 H), 0.93 - 0.83 (m, 1 H). 13C NMR (100 MHz, CD3CN) δ 180.4, 159.3, 156.0, 149.3, 148.7, 145.5, 139.2 (d, J = 4.02 Hz), 137.8, 136.3, 136.1, 130.7, 130.6, 128.7, 128.6, 128.5, 128.0, 127.5, 126.1, 113.7, 86.9, 86.6, 84.2 (d, J = 3.01 Hz), 83.7 (d, J = 11.1 Hz), 81.3 (d, J = 2.01 Hz), 72.1, 70.7 (d, J = 4.02 Hz), 70.3, 67.6 (d, J = 3.01 Hz), 63.6, 58.6, 55.5, 47.2 (d, J = 35.2 Hz), 36.2, 28.3, 26.2 (d, J = 3.01 Hz), 18.8, 18.7. 31P NMR (161 MHz, CD3CN) δ 151.26. ESI-HRMS: m/z calcd for C49H55N6O10P+ [(M + H)+] 918.3717, found 918.3715.

Sp-3C3 OAP monomer (Sp-4b)

Crude (Sp)-4b was synthesized from 5’-ODMTr-2’-OMOE-5-methyl-cytidine (1.44 g, 2 mmol) and (S)-phenyl([R]-pyrrolidin-2-yl)methanol (709 mg, 4 mmol) following the procedure described above and purified by silica gel column chromatography (EA:hexane:Et3N, 60:40:2) to give (Sp)-4b as a white powder (1.70 g, 91%). 1H NMR (400 MHz, CD3CN) δ 13.25 (br s, 1 H), 8.23 - 8.33 (m, 2 H), 7.87 (d, J = 1.01 Hz, 1 H), 7.62 - 7.53 (m, 1 H), 7.52 - 7.44 (m, 4 H), 7.38 - 7.25 (m, 12 H), 6.91 - 6.85 (m, 4 H), 5.89 (d, J = 3.30 Hz, 1 H), 5.73 (d, J = 6.59 Hz, 1 H), 4.93 - 4.81 (m, 1 H), 4.24 (dd, J
Crude (Sp)-4c was synthesized from 5'-ODMTr-2'-OMOE-N-benzoyl-adenosine S3 (732 mg, 1 mmol) and (S)-phenyl((R)-pyrrolidin-2-yl)methanol (355 mg, 2 mmol) following the procedure described above and purified by silica gel column chromatography (EA:Et3N, 100:2) to give (Sp)-4c as a white powder (700 mg, 78%). 1H NMR (400 MHz, CD3CN) δ 9.32 (br s, 1 H), 8.68 (s, 1 H), 8.35 (s, 1 H), 8.03 (d, J = 7.60 Hz, 2 H), 7.66 (d, J = 7.60 Hz, 1 H), 7.62 - 7.53 (m, 2 H), 7.44 - 7.18 (m, 14 H), 6.86 - 6.73 (m, 4 H), 6.14 (d, J = 4.31 Hz, 1 H), 5.82 (d, J = 6.59 Hz, 1 H), 5.25 - 5.14 (m, 1 H), 4.94 (t, J = 4.56 Hz, 1 H), 4.26 (d, J = 5.32 Hz, 1 H), 4.04 - 3.94 (m, 1 H), 3.83 - 3.69 (m, 7 H), 3.65 - 3.44 (m, 4 H), 3.30 (dd, J = 10.90, 4.06 Hz, 1 H), 3.21 (s, 3 H), 3.12 (dd, J = 8.11, 5.58 Hz, 1 H), 1.72 - 1.51 (m, 2 H), 1.26 - 1.16 (m, 2 H), 0.91 (dd, J = 12.42, 8.36 Hz, 1 H). 13C NMR (100 MHz, CD3CN) δ 159.7, 152.9, 151.1, 146.0, 144.0, 139.8 (d, Jpc = 4.02 Hz), 136.9, 136.8, 133.6, 131.1, 131.0, 129.7, 129.2, 129.0, 128.8, 128.4, 127.9, 126.6, 126.0, 114.1, 88.8, 87.3, 84.4 (d, Jpc = 10.1 Hz), 84.0 (d, Jpc = 4.02 Hz), 81.5 (d, Jpc = 2.01 Hz), 72.6, 71.3 (d, Jpc = 2.01 Hz), 71.1, 68.1 (d, Jpc = 4.02 Hz).
3.02 Hz), 63.4, 59.0, 55.9, 47.7 (d, \( J_{pc} = 34.2 \) Hz), 28.9, 26.7 (d, \( J_{pc} = 3.02 \) Hz). \(^{31}\)P NMR (161 MHz, CD\(_3\)CN) δ 150.21. ESI-HRMS: \( m/z \) calcd for C\(_{52}H\(_{53}\)N\(_6\)O\(_9\)P\(^+\) [(M + H\(^+\)] 936.3612, found 936.3615.

\( \text{Rp-A}^{\text{Rt}} \) OAP monomer (\( \text{Rp}-4g \))

Crude (\( \text{Rp}-4g \)) was synthesized from \( 5'\)-ODMTr-2'\(-\text{OMOE- N-benzoyl-adenosine 53 (732 mg, 1 mmol) and (R)-phenyl((S)-pyrrolidin-2-yl)methanol (355 mg, 2 mmol) following the procedure described above and purified by silica gel column chromatography (EA:Et\(_3\)N, 100:2) to give (\( \text{Rp}-4g \) as a white powder (500 mg, 52%). \(^1\)H NMR (400 MHz, CD\(_3\)CN) δ 9.26 (br s, 1 H), 8.63 (s, 1 H), 8.29 (s, 1 H), 8.00 (d, \( J = 7.35 \) Hz, 2 H), 7.64 (d, \( J = 7.35 \) Hz, 1 H), 7.60 - 7.52 (m, 2 H), 7.44 - 7.34 (m, 4 H), 7.33 - 7.17 (m, 10 H), 6.78 (dd, \( J = 8.87 \) Hz, 2.28 Hz, 4 H), 6.09 (d, \( J = 4.56 \) Hz, 1 H), 5.88 (d, \( J = 6.34 \) Hz, 1 H), 5.14 - 5.08 (m, 1 H), 4.88 (t, \( J = 4.82 \) Hz, 1 H), 4.25 (d, \( J = 4.31 \) Hz, 1 H), 4.00 - 3.90 (m, 1 H), 3.85 - 3.77 (m, 1 H), 3.76 - 3.64 (m, 7 H), 3.58 - 3.48 (m, 1 H), 3.46 - 3.44 (m, 2 H), 3.41 (d, \( J = 3.80 \) Hz, 1 H), 3.31 (dd, \( J = 10.65 \), 4.82 Hz, 1 H), 3.19 (s, 3 H), 3.02 - 2.92 (m, 1 H), 1.68 - 1.49 (m, 2 H), 1.22 - 1.12 (m, 1 H), 0.94 - 0.85 (m, 1 H). \(^{13}\)C NMR (100 MHz, CD\(_3\)CN) δ 159.7, 152.9, 152.7, 151.1, 146.1, 144.0, 139.8 (d, \( J_{pc} = 4.02 \) Hz), 136.9, 136.8, 135.0, 133.6, 131.1, 131.0, 129.7, 129.2, 129.1, 129.0, 128.8, 128.5, 127.8, 126.7, 114.0, 88.6, 87.2, 84.5 (d, \( J_{pc} = 2.01 \) Hz), 84.2 (d, \( J_{pc} = 11.1 \) Hz), 81.5 (d, \( J_{pc} = 2.01 \) Hz), 72.7, 71.6 (d, \( J_{pc} = 5.03 \) Hz), 71.1, 68.1 (d, \( J_{pc} = 4.02 \) Hz), 63.8, 59.0, 55.9, 47.5 (d, \( J_{pc} = 35.2 \) Hz), 28.9, 26.7 (d, \( J_{pc} = 3.01 \) Hz). \(^{31}\)P NMR (161 MHz, CD\(_3\)CN) δ 151.88. ESI-HRMS: \( m/z \) calcd for C\(_{52}H\(_{53}\)N\(_6\)O\(_9\)P\(^+\) [(M + H\(^+\)] 936.3612, found 936.3614.
Crude (Rp)-4e was synthesized from 5'-ODMTr-2'-OMOE-thymidine (930 mg, 1.5 mmol) and (R)-phenyl((S)-pyrrolidin-2-yl)methanol (535 mg, 3 mmol) following the procedure described above and purified by silica gel column chromatography (EA:hexane:Et₃N, 80:20:2) to give (Rp)-4e as a white powder (810 mg, 68%). ¹H NMR (400 MHz, CD₃CN) δ 8.99 (br s, 1 H), 7.52 - 7.41 (m, 2 H), 7.40 - 7.20 (m, 13 H), 6.85 (dd, J = 8.87, 1.77 Hz, 4 H), 5.89 (d, J = 3.60 Hz, 1 H), 5.77 (d, J = 6.70 Hz, 1 H), 4.71 - 4.76 (m, 1 H), 4.24 - 4.21 (m, 1 H), 4.14 - 4.12 (m, 1 H), 3.83 - 3.74 (m, 9 H), 3.56 - 3.45 (m, 3 H), 3.41 - 3.35 (m, 1 H), 3.31 - 3.27 (m, 4 H), 3.04 - 2.96 (m, 1 H), 1.61 - 1.50 (m, 2 H), 1.42 (d, J = 1.01 Hz, 3 H), 1.18 - 1.09 (m, 1 H), 0.91 - 0.83 (m, 1 H). ¹³C NMR (100 MHz, CD₃CN) δ 164.2, 159.4, 159.3, 151.2, 145.3, 139.3 (d, ³Jpc = 4.02 Hz), 136.0, 136.1, 130.7, 130.6, 128.7, 128.6, 128.5, 128.0, 127.6, 126.2, 113.7, 111.1, 87.5, 87.2, 83.8 (d, ³Jpc = 3.01 Hz), 83.7 (d, ²Jpc = 10.1 Hz), 81.4 (d, ³Jpc = 3.01 Hz), 72.4, 71.0 (d, ²Jpc = 7.04 Hz), 70.4, 67.5 (d, ²Jpc = 3.01 Hz), 63.1, 58.7, 55.5, 47.0 (d, ²Jpc = 35.2 Hz), 28.4, 26.2 (d, ³Jpc = 3.01 Hz), 11.8. ³¹P NMR (161 MHz, CD₃CN) δ 152.95. ESI-HRMS: m/z calcd for C₄₅H₅₀N₃O₁₀P⁺ ([M + H]⁺) 823.3234, found 8223.3235.

(Rp)-mCBz OAP monomer (Rp-4f)
Crude (Rp)-4f was synthesized from 5’-ODMTr-2’-OMOE-5-methyl-cytidine (1.44 g, 2 mmol) and (R)-phenyl((S)-pyrrolidin-2-yl)methanol (709 mg, 4 mmol) following the procedure described above and purified by silica gel column chromatography (EA:hexane:Et3N, 60:40:2) to give (Rp)-4f as a white powder (1.30 g, 70%). 1H NMR (400 MHz, CD3CN) δ 13.09 (br s, 1 H), 8.28 - 8.26 (m, 2 H), 7.79 (d, J = 1.01 Hz, 1 H), 7.59 - 7.55 (m, 1 H), 7.50 - 7.45 (m, 4 H), 7.41 - 7.23 (m, 12 H), 6.89 - 6.84 (m, 4 H), 5.93 (d, J = 4.31 Hz, 1 H), 5.78 (d, J = 6.34 Hz, 1 H), 4.81 - 4.76 (m, 1 H), 4.27 (t, J = 4.82 Hz, 1 H), 4.20 - 4.18 (m, 1 H), 3.88 - 3.81 (m, 3 H), 3.77 - 3.74 (m, 7 H), 3.57 - 3.47 (m, 2 H), 3.45 - 3.42 (m, 1 H), 3.37 - 3.34 (m, 1 H), 3.28 (s, 3 H), 3.06 - 2.96 (m, 1 H), 1.62 (d, J = 1.01 Hz, 3 H), 1.60 - 1.48 (m, 2 H), 1.20 - 1.12 (m, 1 H), 0.93 - 0.84 (m, 1 H). 13C NMR (100 MHz, CD3CN) δ 161.2, 159.7, 148.8, 145.6, 139.6, 138.6, 138.0, 136.3, 133.3, 131.1, 130.0, 130.0, 129.1, 128.9, 128.8, 128.3, 127.9, 126.5, 114.0, 112.2, 88.9, 87.5, 84.0, 83.9, 82.2, 72.7, 70.9 (d, 2Jpc = 7.04 Hz), 70.8, 67.8, 62.9, 59.0, 55.8, 47.4 (d, 2Jpc = 35.2 Hz), 28.7, 26.5, 13.0. 31P NMR (161 MHz, CD3CN) δ 153.03. ESI-HRMS: m/z calcd for C52H55N4O10P+ [(M + H)+] 926.3656, found 926.3655.

Rp-G^Bu OAP monomer (Rp-4h)

Crude (Rp)-4h was synthesized from 5’-ODMTr-2’-OMOE-guanosine S9 (715 mg, 1 mmol) and (R)-phenyl((S)-pyrrolidin-2-yl)methanol (355 mg, 2 mmol) following the procedure described above after 16 h stirring at room temperature and purified by silica gel column chromatography (3-Aminopropyl functionalized silical gel, EA:Acetone:Et3N, 80:20:2) to give (Rp)-4h as a white powder (600 mg, 40%). 1H NMR (400 MHz, CD3CN) δ 7.86 (s, 1 H), 7.45 - 7.15 (m, 14 H), 6.81 (dd, J = 9.00, 2.41 Hz, 4 H), 5.89 (d, J = 6.08 Hz, 1 H), 5.76 (d, J = 6.34 Hz, 1 H), 4.78 (dd, J = 9.76, 3.93 Hz, 1 H), 4.71 - 4.68 (m, 1 H), 4.22 (d, J = 3.80 Hz, 1 H), 3.80 - 3.65 (m, 10 H), 3.49-3.44 (m, 3H), 3.35 - 3.33 (m, 1 H), 3.26 - 3.23 (m, 1 H), 3.21 (s, 3 H), 3.15 - 3.08 (m, 1 H), 2.55 - 2.49 (m, 1 H),
1.60 - 1.50 (m, 2 H), 1.18 - 1.05 (m, 7 H), 0.90 - 0.84 (m, 1H). $^{13}$C NMR (100 MHz, CD$_3$CN) $\delta$ 180.9, 159.8, 156.4, 149.9, 149.3, 146.0, 142.1, 139.7 (d, $^3$J$_{PC}$ = 3.02 Hz), 138.4, 136.8, 136.7, 131.2, 131.1, 129.2, 129.1, 128.9, 128.5, 128.0, 127.4, 127.1, 126.7, 122.3, 114.1, 87.4, 86.9, 85.2, 84.1, 81.7, 72.7, 68.0, 64.2, 59.1, 56.0, 53.7, 51.5, 47.5 (d, $^2$J$_{PC}$ = 35.2 Hz), 36.7, 28.8, 27.3, 21.4, 19.2. $^{31}$P NMR (161 MHz, CD$_3$CN) $\delta$ 153.47. ESI-HRMS: m/z calcd for C$_{48}$H$_{55}$N$_6$O$_{10}$P$^+$ [(M + H)$^+$] 918.3717, found 918.3718.

Materials and methods for properties

Oligoribonucleotide phosphorothioate synthesis

Oligoribonucleotides were prepared on MerMade 192 DNA/RNA synthesizer using CPG 500 Å unynlinker support (44.9 µmol/g). Fully protected stereopure nucleoside phosphoramidites were incorporated using standard solid-phase oligonucleotide synthesis conditions: i.e. 3% dichloroacetic acid in dichloromethane (DCM) for deblocking, 1.4 M N-phenyl imidazolium triflate in anhydrous acetonitrile as activator, capping reagent A (THF/lutidine/acetic anhydride, 8:1:1) and capping reagent B (16% N-imidazole/THF) for capping, a 0.05 M solution of 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithia-zole-5-thione (DDTT; Sulfurizing Reagent II; Glen Research, Virginia) in dry pyridine/ACN (60:40) for sulfurization, a 0.5 M solution of (1S)-(+)-(10-camphorsulfonyl)oxaziridine in anhydrous acetonitrile (0.5 M CSO) was evaluated as an oxidizing solution. OAP amidites were prepared at 0.11 M in anhydrous acetonitrile and were coupled utilizing three application of OAP amidites, with a 4 min contact time for each pass. After the completion of the synthesis, the solid support was suspended in aqueous ammonia (25%, 300 µl) and heated at 55 °C for 24 hours. The reaction was cooled down to room temperature, and the solid support was filtered and washed with 400 µl of EtOH and H$_2$O 1:1 (v/v). The filtrate was concentrated to dryness and the residue was dissolved in 200 µl water for the analysis on LCMS and the purification on RP-HPLC. The oligonucleotides were purified on an Agilent 1200 series preparative HPLC fitted with a WatersXBridge OST C-18 column, 10 x 50 mm, 2.5 µm, at 65°C). Running buffer: buffer A (0.1 M triethylammonium acetate), buffer B (methanol); gradient for the DMT-on purification: 5–80% buffer B over 6 min; gradient for the DMT-off purification: 5 –35% buffer B over 5 min. Fractions containing the product were collected and dried in a miVac duo SpeedVac from Genevac. The oligonucleotides were analysed by LC-MS (Agilent 1200/6130
system) on a Waters Acquity OST C-18 column, 2.1 x 50 mm, 1.7μm, 65°C. Buffer A: 0.4 M HFIP, 15 mM triethylamine; buffer B: MeOH. Gradient: 10 - 50% B in 20 min; flow rate: 0.3 ml min⁻¹.

**Thermal stability studies (Tₘ)**

Melting temperatures of 2′-OMOE RNA (PS) duplexes were measured on a CARY 300 (Agilent) equipped with a thermocontroller. 2′-OMOE RNA (PS) and the counter strands (RNA, PO) were combined to yield an equimolar concentration of 2 µM in phosphate-buffered saline (5 mM Na₂HPO₄, 5 mM NaH₂PO₄, 100 mM NaCl, 0.1 mM EDTA). Absorptions at 260 nm were measured in 80 µl quartz cuvettes. The temperature gradient was set to 0.5 K/min⁻¹ for the range of 20 °C – 95 °C. Absorbance readings were taken every 30 sec. Each series was performed three times. Hold time was set to 5 min at 95 °C and 20 °C, respectively, to ensure thermal equilibrium. The melting profiles were fitted to a sigmoidal and Tₘ was determined by the derivative thereof.

**Cell culture and gapmers (Mp (16), Mp-Rp (17) and Mp-Sp (18)) transfection**

Huh7 cells (ATCC) were cultured as monolayers in DMEM GlutaMAX™-I (31966-021, ThermoFisher Scientific) supplemented with 10% of FBS (fetal bovine serum, 10270106, ThermoFisher Scientific). For gapmer transfections, huh7 cells were seeded in 24-well plates (30,000 cells/well). Gapmers (Mp (16), Mp-Rp (17), Mp-Sp (18) and Neg Con (19)) as well as mock transfections were performed with lipofectamine 2000 (11668019, ThermoFisher Scientific), 24 hours post-seeding (0.75 uL Lipofectamine/100ng gapmer). After 24 hours, supernatants were removed and RNA was isolated from the cells.

**RNA extraction and purification**

Total RNA was extracted and purified using TRIzol (15596018, ThermoFisher Scientific).

**Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)**

10 ng of RNA and 500 ng random primer (C1181, Promega) were used for cDNA synthesis with the TaqMan® MicroRNA Reverse Transcription Kit (4366596, ThermoFisher Scientific), according to the manufacturer’s protocol. qPCR reactions were performed using KAPA SYBR® FAST qPCR Master Mix (KK4618, Kapa Biosystems) on a LightCycler 480 Detection System (Roche). Each reaction was carried out in three technical replicates. The mean measured levels of ApoB were
normalized to the housekeeping gene β-Actin. Data from three independent replicates was obtained. Primer pairs utilized are as follows: apoB F: TGC TAA AGG CAC ATA TGG CCT, R: CTC AGG TTG GAC TCT CCA TTG AG; β-Actin F: CCAACCGCGAAGATGA, R: CCAGAGGGCTACAGGGATAG. 2^{ΔΔCt} method was used to calculate the relative transcript abundance.

Cell culture and SSO (FCH (20), FCH-Rp (21), FCH-Sp (22) and FCH-PO (24)) minigene assay

COS-7 cells (ATCC) were cultured as monolayers in DMEM GlutaMAXTM-I (31966-021, ThermoFisher Scientific) supplemented with 10% of FBS (fetal bovine serum, 10270106, ThermoFisher Scientific). For transfections, COS-7 cells were seeded in 24-well plates (135,000 cells/well). FECH-C minigene construct was transfected 24 hours post-seeding at 750 ng/well with XtremeGENE HP reagent (06366236001, Roche Life Sciences, 2.25 µl/1 µg plasmid DNA). SSO (FCH (20), FCH-Rp (21), FCH-Sp (22), FCH-Con (23), FCH-PO (24)) transfection was performed 3 hours later with Lipofectamine 2000 (11668019, ThermoFisher Scientific; 1.25 µL Lipofectamine/50 nM SSO). Depending on the time course desired, supernatants were removed after 24 hours or 48 hours and RNA was isolated from the cells. Emetine (as HCl salt, E2375, Sigma-Aldrich) was used at a final concentration of 3 µM in cell culture well and was added 24 hours prior to cell harvest, i.e. immediately after SSO transfection for 24 hr time points or 1 day after SSO transfection for 48 hr time points.

RNA extraction and purification

Total RNA was extracted and purified using RNeasy Mini Kit (74106, Qiagen). DNase I treatment was doubled to ensure complete plasmid DNA removal.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

1 µg of RNA and 500 pg random primer (C1181, Promega) were used for cDNA synthesis with the TaqMan® MicroRNA Reverse Transcription Kit (4366596, ThermoFisher Scientific), according to the manufacturer's protocol. For PCR reaction, cDNA solutions were diluted 1/3 prior to mixing: 1 µl cDNA solution, 5 µl DreamTaq 10X Buffer, 1 µl dNTPs (18427, ThermoFischer Scientific), 1 µl of FW primer (7.5 µM, 5'-GCTCTCTACCTGGTGTGG-3', Microsynth), 1 µl of RV primer primer (7.5 µM, 5'-GACAATTCATCCACGAGCTTC-3', Microsynth), 0.25 µl DreamTaq Polymerase and 40.75
μl RNase-free water. PCR program was: 1 min, 95°C; 30 cycles (30 s, 95 °C; 30 s; 60 °C; 30 s, 68 °C); 5 min, 68°C.

**Agarose gel electrophoresis**

PCR samples loaded with 5X GelPilot Loading Dye (239901, Qiagen) were allowed to run for 1 hours under 85 V in a 2 % agarose gel in TAE Buffer and 1/10,000 GelRed Nucleic Acid Stain (41003, Chemie Brunschwig).

**Data analysis**

Gels were revealed in a UV chamber (Universal Hood II, BioRad) and ratios of aberrantly spliced product on (normal + aberrant) were calculated using ImageJ software and background subtraction.

**Plasmid for SSO minigene assay**

ggtgtgattta cactcaggga agcaatgatt attatccatt tgtactataa atatggattg
ttgctgccct ctctttcttt ttctccttttt tctctctctt cttctctcctt tttttttttc
aaaaatagatt tctattatga aaaattagta catgcaggg ataaaaacta caattagtat
aaaaagaattt acagtaaaag gcaggtgacct ctctcccctt gcacccctcct gtttttctttt
gagagaaagag gctgttcatct ccaagatct tagtgtcata ttacatagc tctctctctt
ttttttttac acagtataaa cagaggtctc cctctctctt ctctctctct ttttttttttt
acattggtta tctctcttta cgtcatctttt cccctctctt ccaccccacac gataggccct
ggtgttctgat gttccctcttct tgttactccaa gttttctctt ctgtcatttt tccacacttga
gtgtagacat gcggtgtttt gttttttgttc cttgtgtagt atggtgggtt
tccagttcctt cctattcctc tctatccttc ctttttttctg gctggctttag
ctatcatac cctctctctt cttgatgact ccagttgccg tttgctgagta gtgaacttctt
tgtgtgtgat cccccctcct ttttcttttt cttttttttt tttttttttttt
tactatccct tataagtgtaa acaagcttact tctttttttt ctttttccatg cttctctctt
ttttcttttt ccttcttttt cttctttttttt ttttttttttt
agaagttttat gtttttttttt ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Legend: (A) Sequence of FECH insert containing intron 3, exon 4 and intron 4 sequences that was cloned by gateway technology into pSpliceExpress (Kishore et al., 2008) as shown in B. Exon 4 is highlighted in yellow. The extra 63 nucleotides present in aberrant transcripts are highlighted in green. The IVS3-48C polymorphism that stimulates the use of the aberrant splice acceptor site is indicated by a larger, red and bold uppercase letter. Splice acceptor and donor sites (AG and GT dinucleotides) are underlined.


HPLC-MS profiles of purified FCH-Sp (22), FCH-Rp (21), Mp-Sp (18) and Mp-Rp (17)

Stereodefined PS ORNs FCH-Sp (22), FCH-Rp (21), Mp-Sp (18) and Mp-Rp (17) were synthesized according to the general procedure for the oligoribonucleotide phosphorothioate synthesis described above.

These stereodefined PS ORNs were obtained with extremely high stereoselectivities albeit in low yields, partly due to the unstability of OAP monomers in anhydrous acetonitrile. After a few hours on the automatic synthesizer, precipitation occurs in the solution of OAP monomers in anhydrous acetonitrile. We assume the precipitation is likely H-phosphonate, which does not dissolve in acetonitrile and does not react with 5’-OH nucleosides under our reaction conditions. The precipitation can dissolve in dichloromethane, however the precipitation still occurs even with 10% anhydrous dichloromethane in anhydrous acetonitrile.
Figure S1 HPLC-MS profiles of purified FCH-Sp (22), FCH-Rp (21), Mp-Sp (18) and Mp-Rp (17). HPLC-MS was performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 20 min at a rate of 0.3 ml min⁻¹.
Melting temperature ($T_m$) curves of stereodefined PS-oligonucleotides

Figure S2 Melting temperature ($T_m$) curves of 12 mers FCH (20), FCH-Rp (21), FCH-Sp (22) and FCH-PO 24 (top), gapmers Mp (16), Mp-Rp (17) and Mp-Sp (18) (bottom).
Spectra of synthesized nucleosides and phosphoramidites

$^1$H and $^{13}$C NMR spectra of 2'-OMOE adenosine (S1)
$^1$H and $^{13}$C NMR spectra of 2'-OMOE-2-amino-adenosine (S5)
$^1$H and $^{13}$C NMR spectra of 2'-OMOE-N-benzoyl adenosine (S2)
$^1$H and $^{13}$C NMR spectra of 5'-ODMT-2'-OMOE-N-benzoyl adenosine (S3)
$^1$H and $^{13}$C NMR spectra of 2'-OMOE-N$^2$-isobutyryl-adenosine (S6)
$^1$H and $^{13}$C NMR spectra of 2'-OMOE-N$^2$-isobutyryl-guanosine (S8)
$^1$H and $^{13}$C NMR spectra of 5'-ODMT-2'-OMOE-N$^2$-isobutyryl-guanosine (S9)
$^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra of Sp-$A^{B(z)}$ (Sp-4c) OAP monomer
$^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra of Rp-A$^{Bz}$ (Rp-4g) OAP monomer
$^{1}H$, $^{13}C$ and $^{31}P$ NMR spectra of Sp-GiBu (Sp-4d) OAP monomer
$1^H$, $13^C$ and $31^P$ NMR spectra of Rp-G$^{\text{Bu}}$(Rp-4h) OAP monomer
$^1$H, $^{13}$C and $^{31}$P NMR spectra of Sp-T (Sp-4a) OAP monomer
$^1$H, $^{13}$C and $^{31}$P NMR spectra of Rp-T (Rp-4e) OAP monomer
$^1\text{H}, ^{13}\text{C}$ and $^{31}\text{P}$ NMR spectra of Sp-$^m\text{C}^\text{Bz}$ (Sp-4b) OAP monomer
$^1$H, $^{13}$C and $^{31}$P NMR spectra of Rp-$^m$C$^{Bz}$ (Rp-4f) OAP monomer
The crude HPLC-MS profiles of stereodefined PS-ORNs

Crude HPLC-MS profiles of DMT-\(T_Rp\)CGTACGT (5) and DMT-\(T_Sp\)CGTACGT (11)

Crude HPLC-MS of DMT-\(T_Rp\)CGTACGT (5) and DMT-\(T_Sp\)CGTACGT (11) were performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 32 min at a rate of 0.3 ml min\(^{-1}\). The peaks at ~ 9.2 min are unreacted 7 mers with PO linkage.
Crude HPLC-MS profiles of DMT-C<sub>Rp</sub>C GTACGT (6) and DMT-C<sub>Sp</sub>C GTACGT (12)

Crude HPLC-MS of DMT-C<sub>Rp</sub>C GTACGT (6) and DMT-C<sub>Sp</sub>C GTACGT (12) were performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 20 min at a rate of 0.3 ml min<sup>-1</sup>. The peaks at ~ 7.4 min are unreacted 7 mers with PO linkage.
Crude HPLC-MS of DMT-A_{RP}CGTACGT (7) and DMT-A_{Sp}CGTACGT (13) were performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 20 min at a rate of 0.3 ml min⁻¹. The peaks at ~ 7.5 min are unreacted 7 mers with PO linkage.
Crude HPLC-MS of DMT-[^m]C<sub>Rp</sub>C GTACGT (6) and DMT-[^m]C<sub>Sp</sub>C GTACGT (12) were performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 20 min at a rate of 0.3 ml min<sup>−1</sup>. The peaks at ~ 7.5 min are unreacted 7 mers with PO linkage.
Crude HPLC-MS of DMT-G_{Rp}CGTACGT (8) and DMT-G_{Sp}CGTACGT (14) were performed with a linear gradient of 10-50\% MeOH in 0.4 M HFIP buffer at 65 °C for 20 min at a rate of 0.3 ml min\(^{-1}\). The peaks at ~ 7.5 min are unreacted 7mers with PO linkage.
Crude HPLC-MS of DMT-TRpAGTACGT (10) was performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 32 min at a rate of 0.3 ml min⁻¹. The peak at ~9.0 min is unreacted 7 mers with PO linkage.

Crude HPLC-MS of DMT-TRpTGTACGT (9)
Crude HPLC-MS of DMT-T<sub>Rp</sub>TGTACGT (9) was performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 32 min at a rate of 0.3 ml min<sup>−1</sup>. The peak at ~ 8.8 min is unreacted 7 mers with PO linkage.

**Crude HPLC-MS of DMT-A<sub>Sp</sub>GGTACGT (15)**

Crude HPLC-MS of DMT-A<sub>Sp</sub>GGTACGT (15) was performed with a linear gradient of 10-50% MeOH in 0.4 M HFIP buffer at 65 °C for 20 min at a rate of 0.3 ml min<sup>−1</sup>. The peak at ~ 7.1 min is unreacted 7 mers with PO linkage.

**References**