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Increased Affinity of 2'-*O*-(2-methoxyethyl)-Modified Oligonucleotides to RNA through Conjugation of Spermine at Cytidines

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Dedicated to François Diederich on the occasion of his retirement

Structural modification at the 2'-*O*-position of riboses in oligonucleotide therapeutics is of critical importance for their use as drugs. To date, the methoxyethyl (MOE) substituent is the most important and features in dozens of antisense oligonucleotides that have been tested in clinical trials. Yet, the search for new improved modifications continues in a quest for increased oligonucleotide potency, improved transport *in vivo* and favorable metabolism. Recently, we described how the conjugation of spermine groups to pyrimidines in oligonucleotides vastly increases their affinity for complementary RNAs through accelerated binding kinetics. Here we describe how spermines can be linked to the exocyclic amino groups of cytidines in MOE-oligonucleotides employing a straightforward "convertible nucleoside approach" during solid phase synthesis. Singly- or doubly-modified oligonucleotides show greatly enhanced affinity for complementary RNA, with potential for a new generation of MOE-based oligonucleotide drugs.

Keywords: oligonucleotide conjugate • spermine • MOE oligonucleotide • hybridization • convertible nucleoside approach

Introduction

Single- and double-stranded oligonucleotides are a new class of therapeutics that have recently come of age.[1] They bind to their cellular RNA targets with high affinity and extraordinary selectivity according to the rules of Watson-Crick (W-C) base-pairing, whereupon they elicit a variety of inhibitory mechanisms determined by their structural modifications and the class of RNA target. A large part of their current success in the clinical setting is due to two decades of medicinal chemistry efforts. These have produced a handful of innovative nucleic acid analogs which satisfy the principal requirements for therapeutic use in man: a structure that maintains WC-binding affinity and selectivity, one that is stable to ubiquitous nucleases *in vivo*, one that tolerates the cellular factors responsible for processing the RNA target (e.g. RNaseH enzymes) and one that is accessible on large scale through solid-phase chemistry processes. The mainstay of clinically-validated oligonucleotide modifications is the 2'-O-(2-methoxyethyl) (2'-MOE)-ribose connected by backbone of phosphorothioate (PS) linkages.[2] Dozens of 2'-MOE-oligonucleotides have shown useful activity in patients including the approved drugs Mipomersen[3] and Nusinersen.[4] Other prominent chemistries that have reached clinical testing include the bicyclic ribonucleotides – the so-called locked nucleic acid (LNA)[5-7] and the constrained ethyl derivatives (cEt)[8] – and the structurally-distinct 6-ring morpholino phosphordiamidates (PMO).[9]

The drive to develop new structural modifications for oligonucleotides in industry and in academia continues, though priorities for medicinal chemists have changed in line with evolving challenges in the field. For instance, the development of carrier groups to deliver drugs more efficiently and to a wider range of tissues has become a major focus since the discovery that N-acetylgalactosamine (GalNac) conjugated groups are able to increase 100-1000-fold delivery of siRNAs to hepatocytes.[10-12] Also, the push for stereochemically-pure PS drugs has been boosted by advances in phosphoramidite chemistry.[13] Both of these strategies are being applied to 2'-MOE- and LNA-oligonucleotides, since they may improve the pharmacokinetics properties and thereby show pharmacological activity in cell/tissue types where delivery is as yet limiting.

Conjugated cationic oligospermines have been investigated extensively as carrier groups to improve the cellular delivery of oligonucleotides, and found to an act in similar fashion to polyamine-type delivery vectors. [14, 15] [16] We recently described how conjugation of polyamine chains derived from spermine to the C5 (but not to the 2'-hydroxyl) of uridines greatly increased the binding affinity of 2'-*O*-methyl (OMe)-modified oligoribonucleotides for complementary RNAs.[17] We postulated that the polyamine likely positions itself in the duplex major groove, mimicking natural polyamines stabilizing cellular RNAs. This result was striking; however, the synthesis of these derivatives is rather tedious and the use of 2'-OMe oligonucleotides as antisense agents is outdated. We therefore searched for an improved means to extend these findings to 2'-MOE oligonucleotides.

In a previous work we applied the "convertible nucleoside" approach[18] to introduce methyl- and dimethylamine to the 4-position of cytidine by nucleophilic displacement of triazole.[19] The triazole derivative is very conveniently prepared just before or just after phosphitylation of the protected nucleoside. Furthermore, this chemistry offers the possibility to introduce the nucleophiles during solid-phase synthesis, i.e. before protecting group removal and cleavage from the controlled pore glass (CPG) solid support. Mono-substitution of the substituted amino group on cytidine permits a regular G-C base-pair formation during hybridization if the substituent – in our case, a spermine group – adopts a *trans* conformation with cytidine N^3.

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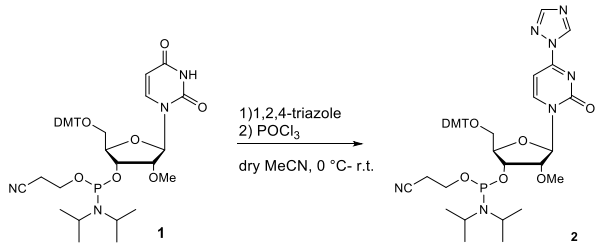
Here, we describe a convenient and straightforward synthesis of polyamine-modified 2'-OMe- and 2'-MOE-oligonucleotides. Analogously to polyamine modification at the uridine 5-position, spermine conjugation to N^4-of cytidine greatly increases hybridization affinities of modified oligonucleotides to complementary RNAs.

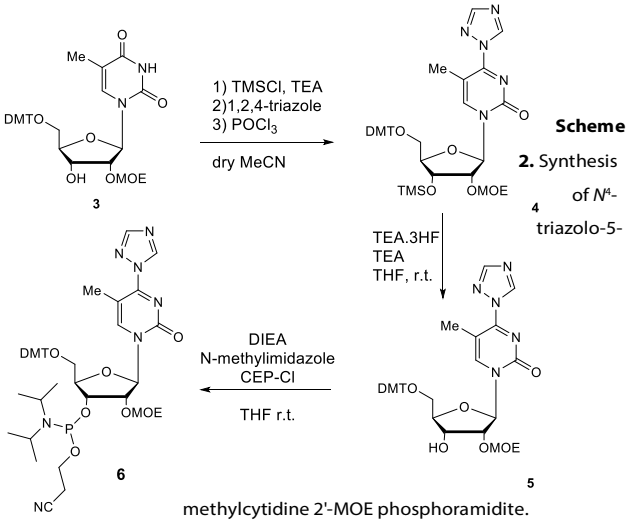
Results and Discussion

The N^4-triazole-modified 2′-OMe cytidine phosphoramidite **2** was obtained in a single step from commercially-available 5′-dimethoxytrityl (DMT)-2′-OMe cytidine phosphoramidite **1**, as described previously.[20]



Scheme 1. Single-step preparation of N^4-triazolo-2'-OMe cytidine.

2'-MOE-modified oligonucleotides are typically used with thymine and 5-methyl cytosine pyrimidines because the 5-methyl group increases hybridization affinity of the oligonucleotide to target mRNAs. Efforts to prepare the 2'-MOE thymidine-convertible nucleoside using a similar protocol to that employed for **2** failed, possibly due to instability of the 2'-MOE phosphoramidite under conditions of the reaction. This was remedied by beginning with commercially-available 5′-DMT-2'-MOE thymidine (**3**; Scheme 2).



Scheme 2. Synthesis of N^4-triazolo-5-methylcytidine 2'-MOE phosphoramidite.

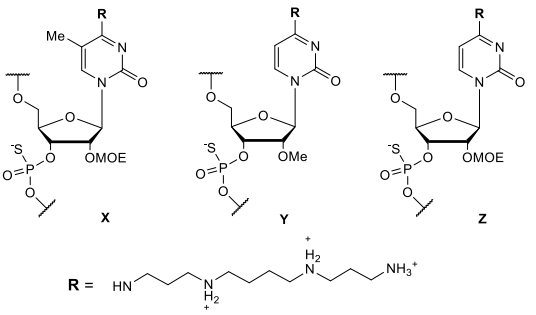
Thus, O^4 of **3** was exchanged for 1,2,4-triazole providing **5** *via* temporary silyl-protection of the 3'-OH. Following deprotection, the nucleoside was directly phosphitylated using 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to provide **6**. The triazole-bearing phosphoramidites were then employed for the solid-phase synthesis of PS oligonucleotides on controlled pore glass (CPG). Due to poor solubility of the modified phosphoramidite **6** in acetonitrile, the solvent was switched to dry isobutyronitrile at a concentration of 0.25 M for solid phase synthesis. After all bases were coupled, spermine was added to the CPG for controlled displacement of the triazole group. The reaction was assumed to occur at terminal position of the spermine, as reported previously[21]. Deprotection, cleavage and purification steps were then performed under standard conditions to provide a library of purified spermine oligonucleotide conjugates (Table 1). The 2'-MOE oligonucleotides were obtained cleanly as shown by LCMS (entries 6-8, Table 1; supplementary material); however, the three 2'-OMe oligonucleotides were accompanied by small amounts of inseparable N-acetylated byproducts, detected as shoulder peaks in the LCMS chromatagrams (entries 2-4, Table 1).

Melting temperature (T_M) experiments were performed to study the influence of the spermine on hybridization with complementary RNAs and to compare the performance of 2'-OMe and 2'-MOE analogs with their unmodified parent sequences (Table 1). Previously, we showed that spermine conjugation through its terminal amino group to C^5 of uridine in 2'-OMe oligonucleotides increases their T_M's with complementary RNAs, by up to 12 ^{0}C per modification. Moving the spermine fragment to the 4-position on the nucleobase also boosted hybridization to RNA compared to the parent oligonucleotide (entry 1, Table 1) for three sequences (Entries 2-4, Table 1) (T_M's +4-6 ^{0}C per modification).

Table 1. Composition and T_M's of modified sequences bearing polyamine fragments.

Entry	Sequence[a]	Chemistry (ribose)	T_M[b] (°C)	ΔT_M (°C)
1	CUUAGCAACCUG	2'-OMe, PS	55.5	-
2	**Y**UUAGCAACCUG	2'-OMe, PS	58.5	+3.5
3	CUUAG**Y**AACCUG	2'-OMe, PS	61.0	+5.5
4	**Y**UUAG**Y**AACCUG	2'-OMe, PS	63.8	+8.3
5	CTTAGCAACCTG	2'-MOE, PS	58.3	-
6	**X**TTAGCAACCTG	2'-MOE, PS	62.4	+4.1
7	CTTAG**X**AACCTG	2'-MOE, PS	52.9	-5.4
8	**X**TTAG**X**AACCTG	2'-MOE, PS	49.8	-8.5
9	**Y**TTAGCAACCTG	2'-MOE, PS	62.2	+3.9
10	CTTAG**Y**AACCTG	2'-MOE, PS	64.4	+6.1
11	**Z**TTAGCAACCTG	2'-MOE, PS	64.7	+6.4
12	CTTAG**Z**AACCTG	2'-MOE, PS	65.4	+7.1
13	**Z**TTAG**Z**AACCTG	2'-MOE, PS	74.9	+16.6

[a]



[b] Complementary phosphodiester RNA strand (CAGGUUGCUAAG)

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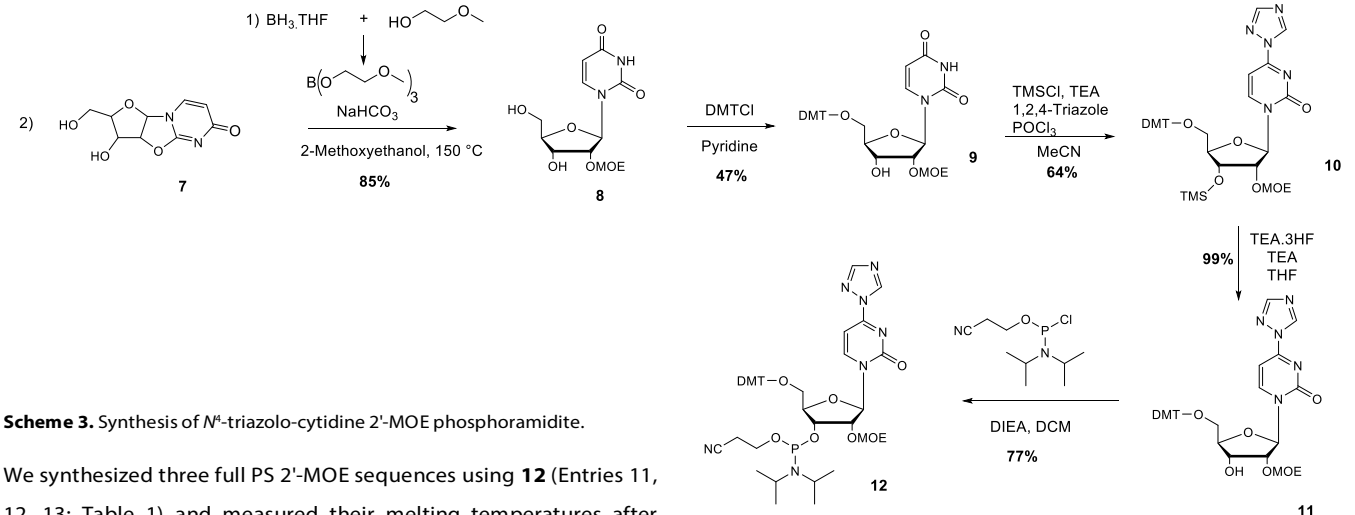
Next, we performed the melting experiments under the same conditions for the isosequential 2'-MOE oligonucleotides. The terminally-modified sequence showed a T_M increase of 4.1 ^{0}C over the parent sequence (Entries 6 and 5, respectively, Table 1). However, modification at an internal position dramatically lowered the hybridization affinity (T_M's of -5.4 and -8.5 ^{0}C, Entries 7 and 8, respectively; Table 1). It seemed most likely that this undesired effect derived from interference of the spermine fragment with the neighboring ring methyl group during hybridization. This finding was consistent with the work of Prakash *et al*., who reported decreased binding for an N^4-spermine group on thymidine in DNA conjugates.[22] To clarify this, we synthesized two 2'-MOE sequences containing spermine-substituted 2'-OMe uridines (Entries 9 and 10, Table 1). Indeed, these showed increased binding affinity to the target RNA with T_M's of +3.9 and +6.1 ^{0}C, respectively.

Hence, we prepared the 2'-MOE analog beginning from uracil, rather than from thymine. We synthesized the required DMT-protected 2'-MOE uridine phosphoramidite in five steps from the anhydro-derivative **7** using literature protocols. Thus, opening of the bicyclic anhydro ring (to **8**) and dimethoxytritylation at the 5'-hydroxyl group produced the nucleoside precursor **9**. The triazole group was subsequently introduced after temporary silylation of the 3'-hydroxyl group (Scheme 3). Phosphitylation then yielded the phosphoramidite **12** ready for oligonucleotide synthesis.



Scheme 3. Synthesis of N^4-triazolo-cytidine 2'-MOE phosphoramidite.

We synthesized three full PS 2'-MOE sequences using **12** (Entries 11, 12, 13; Table 1) and measured their melting temperatures after hybridization to complementary RNAs. We observed increase in T_M of 6.4 ^{o}C and 7.1 ^{o}C for the terminally-modified and internally-modified sequences (Entries 11 and 12), respectively. With a double incorporation of the building block (entry 13), the T_M was increased by 16.6 ^{o}C.

Conclusions

The 2'-MOE alkylation of ribose is inarguably the most important structural modification to single-stranded oligonucleotide drugs.[2] It led to the clinical success of Nusinersen[4] and Mipomersen,[3] as well as several other 2'-MOE-based drugs that are undergoing late-stage clinical testing.[23]

Increased binding affinity from chemical modification of therapeutic oligoribonucleotides has been a major goal of the RNA field for decades, since it produces greater inhibition of target mRNAs at lower concentrations and with less frequent dosing *in vivo*. In this work, we introduced a single modification to the cytidines of 2'-MOE oligonucleotides, and increased its binding affinity for complementary RNAs. Two spermine additions on a 12-mer 2'-MOE PS oligonucleotide increased its T_M to complementary RNA by more than 16 ^{o}C. The precedent for this work was our earlier observation that positioning a polyamine in the major groove increases dramatically the rate of association of the antisense sequence for its target.[17] In that case the polyamine was conjugated through several synthetic steps to the 5-position of uracils. The chemistry in this work is especially attractive since the polyamine fragment is relatively easily introduced to the clinically-proven 2'-MOE modification by shifting the substituent around the pyrimidine by one position to N^4. Our investigation confirmed that attachment of affinity-enhancing groups to N^4 is permitted if the exocyclic amino group can adopt a *trans* conformation with N^3 during base-pairing with guanidine, and there is no C^5-methyl group.

Most chemical modifications to oligonucleotides are made on the ribose and protrude into the minor groove.[24] This usually attenuates the ability of the oligonucleotide to induce RNase H effector enzymes, but it offers an improved protection against ubiquitous nucleases *in vivo*; PS 2'-MOE oligonucleotides are highly resistant to nucleases.

On the other hand, modifications of nucleobases that protrude into the major groove of an 2'-MOE-mRNA duplex are useful because of the possibility to raise the hybridization affinity without adversely affecting RNase H activity. Previous efforts to achieve this for 2'-MOE oligonucleotides include alkyne and heterocycle modifications fusions to the pyrimidines.[25] Although they raised binding affinity *in vitro*, they did not improve cellular activity unless delivered with cationic lipids. Incorporation of this chemistry into pharmacologically-interesting 2'-MOE oligonucleotides with the aim to improve cellular activity is ongoing.

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Experimental Section

General information.

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 was 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.

Synthesis of compounds.

(2*R*,3*R*,4*R*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-methoxy-5-(2-oxo-4-(1H-1,2,4-triazol-1-yl)pyrimidin-1(2H)-yl) tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (2). U-OMe (0.64 mmol, 500 mg) was co-evaporated three times in dry acetonitrile (5 ml). In the meantime, 1,2,4-triazole (12.86 mmol, 1.39 g) was dissolved in dry acetonitrile (10 ml), to which dry triethylamine (13.2 mmol, 2 ml) was added subsequently. U-OMe was redissolved in dry acetonitrile (5 ml) and was added to the 1,2,4-triazole solution *via* syringe. The reaction was then stirred for 10 min at room temperature. An ice bath was used to cool the reaction to 0 °C. Then phosphorus oxychloride (1.3 mmol, 0.1 ml) was added dropwise. The reaction was stirred for 4 h at 0 °C. The reaction mixture was then directly partitioned between ethylacetate and aqueous sodium bicarbonate and washed twice with brine. The organic layer was dried over sodium sulphate. Solvents were then removed under reduced pressure. The crude product was further purified by column chromatography (EtOAc/Hexane 80:20, 2% TEA). Compound was obtained as a white foam: 296 mg, 58% overall yield. ^{1}H NMR ($CDCl_3$): δ (ppm) 9.17 (s, 1H), 8.83 (dd, *J* = 8Hz, 1H), 8.01 (s, 1H), 7.31–7.20 (m, 9H), 6.83–6.76 (m, 4H), 6.38 (d, *J* = 8Hz, 1H), 5.97 (s, 1H), 4.54 (td, *J* = 8Hz, 4Hz, 1H), 4.25–4.22 (m, 1H) 3.94 (d, *J* = 4Hz, 1H), 3.86–3.77 (m, 2H), 3.75 (s, 6H), 3.65 (s, 3H) 3.64–3.40 (m, 4H), 2.54 (t, *J* = 8Hz, 1H), 2.34 (t, *J* = 8Hz, 1H), 1.43 (d, *J* = 8Hz, 1H), 1.10 (dd, *J* = 12Hz, 8Hz, 9H), 0.96 (d, *J* = 8Hz, 2H); ^{31}P NMR ($CDCl_3$): δ (ppm) 150.51; ^{13}C NMR ($CDCl_3$): δ (ppm) 159.27, 158.82 (2C), 153.82, 147.42, 1433.99, 143.2, 135.4, 137.07, 130.42, 128.50, 128.05 (2C), 127.32, 117.50, 11.32 (4C), 94.75, 89.87, 87.25, 83.65, 82.66, 81.92, 69.06, 68.55, 60.42, 60.11, 59.80, 58.89, 58.61, 58.17, 57.95, 55.28, 43.24, 24.77, 24.71, 24.59, 24.50, 20.40, 14.22. ESI-HRMS: m/z calcd for $C_{42}H_{50}N_7O_8P$ [(M+H)$^+$] 812.3531, found 812.3520.

1-((2*R*,3*R*,4*R*,5*R*)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-3-(2-methoxyethoxy)-4-((trimethylsilyl)oxy)tetrahydrofuran-2-yl)-5-methyl-4-(1H-1,2,4-triazol-1-yl)pyrimidin-2(1H)-one (4). T_{MOE} (1.6 mmol, 1g) was co-evaporated three times in dry acetonitrile (10 ml). T_{MOE} was redissolved in dry acetonitrile (25 ml) under argon, to which first dry triethylamine (35.5 mmol, 5 ml) and then trimethylsilyl chloride (8.1 mmol, 1 ml) were added dropwise. The reaction mixture was stirred for 30 min at room temperature. Then 1,2,4-triazole (38.7 mmol, 2.677 g) was added with further stirring. After 10 min the reaction mixture was cooled to 0 °C and phosphorus oxychloride (4.8 mmol, 0.5 ml) was added drop wise. After 15 min the ice bath was removed and the reaction was stirred for 4 h. The solvent was removed under vacuum and the obtained crude material was partitioned between aqueous sodium bicarbonate and ethylacetate (30 ml) and washed with brine (30 ml). The organic layer was dried over sodium sulphate. Solvents were then removed under reduced pressure. The crude product was purified by column chromatography (EtOAc/Hexane 80:20). Compound was obtained as a white foam: 1.03 g, 87% overall yield. ^{1}H NMR ($MeOD_4$): δ (ppm) 9.25 (s, 1H), 8.54 (s, 1H), 8.13 (s, 1H), 7.41–7.37 (m, 2H), 7.29–7.19 (m, 7H), 6.82–6.79 (m, 4H), 5.91 (d, J = 16Hz, 1H), 4.49–4.45 (m, 1H), 4.17–4.13 (m, 1H), 4.12–4.03 (m, 2H), 3.88–3.79 (m, 1H), 3.70 (s, 6H), 3.64 (dd, J = 11Hz, 2Hz, 1H), 3.60–3.44 (m, 3H), 3.29 (s, 3H), 1.69 (s, 3H), 0.01 (s, 9H); ^{13}C NMR ($MeOD_4$): δ (ppm) 159.11, 158.53, 154.7, 152.7, 147.2, 145.0, 144.3, 135.2, 130.1 (4C), 128.2 (2C), 127.6 (2C), 126.8, 112.9 (4C), 106.6, 90.6, 86.7, 83.0, 82.4, 71.6, 69.9, 68.8, 60.7, 57.8, 57.4 (4C), 15.2, -1.27 (3C). ESI-HRMS: m/z calcd for $C_{39}H_{47}N_5O_8Si$ [(M+Na)$^+$] 764.3086, found 764.3078.

1-((2*R*,3*R*,4*R*,5*R*)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxy-3-(2-methoxyethoxy)tetrahydrofuran-2-yl)-5-methyl-4-(1H-1,2,4-triazol-1-yl)pyrimidin-2(1H)-one (5). T_{MOE}-C_4-1,2,4-triazole **4** (0.9 mmol, 657 mg) was dissolved in dry tetrahydrofurane (10 ml). Then dry triethylamine (0.45 mmol, 4.5 ml) was added followed by slowly adding triethylamine•3 hydrofluoric acid (9.0 mmol, 1.3 ml). The reaction was stirred at room temperature for 2 h. The reaction mixture was directly partitioned between ethylacetate and aqueous sodium bicarbonate and washed twice with brine. The organic layer was dried over sodium sulphate. Solvents were then removed under reduced pressure to yield the final product: 536 mg, 89% overall yield. ^{1}H NMR ($MeOD_4$): δ (ppm) 9.21 (s, 1H), 8.40 (s, 1H), 8.09 (s, 1H), 7.37–7.34 (m, 2H), 7.26–7.13 (m, 7H), 6.78-6.74 (m, 4H), 5.85 (s, 1H), 4.43 (dd, J = 8.8Hz, 4.8Hz, 1H), 4.11–4.05 (m, 3H), 3.85–3.80 (m, 1H), 3.66 (s, 6H), 3.56–3.54 (m, 2H), 3.44 (dq, J = 10.4Hz, 2.4Hz, 2H), 3.28 (s, 3H), 1.68 (s, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 158.9, 158.4, 154.7, 152.7, 147.3, 145.0, 144.5, 135.5, 135.2, 130.0 (2C), 128.1 (2C), 127.6 (2C), 126.7 (2C), 112.9 (4C), 106.5, 90.3, 86.7, 82.9, 82.6, 71.6, 69.7, 68.1, 61.1, 57.8, 54.3 (4C), 15.2. ESI-HRMS: m/z calcd for $C_{36}H_{39}N_5O_8$ [(M+Na)$^+$] 692.2691, found 692.2677.

(2*R*,3*R*,4*R*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(2-methoxyethoxy)-5-(5-methyl-2-oxo-4-(1H-1,2,4-triazol-1-yl)pyrimidin-1(2H)-yl) tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (6). Compound **5** (0.3 mmol, 200 mg) was co-evaporated three times in dry acetonitrile (5 ml) and dissolved in dry dichlormethane (5 ml); the solvent was subsequently degased for 2 min. Then, first dry diisopropylethylamine (0.45 mmol, 0.08 ml) was added

followed by the dropwise addition of 2-cyanoethyl-N,N-diisopropylaminophosphite (0.45 mmol, 0.1 ml). The reaction was stirred for 2 h at room temperature. The reaction mixture was then directly partitioned between ethylacetate and aqueous sodium bicarbonate and washed twice with brine. The organic layer was dried in sodium sulphate. Solvents were then removed under reduced pressure. The crude product was purified by column chromatography (EtOAc/Hexane 80:20, 1% TEA). Compound was obtained as a white foam: 112 mg, 43% overall yield ^{1}H NMR ($MeOD_4$): δ (ppm) 9.22 (s, 1H); 8.54 (d, J = 4Hz, 1H); 8.11 (s, 1H), 7.44–7.39 (m, 2H); 7.32–7.18 (m, 7H); 6.83 (m, 4H); 5.90 (d, J = 4 Hz, 1H); 4.72–4.60 (m, 1H), 4.33–4.20 (m, 2H); 4.17–4.03 (m, 1H), 3.31 (s, 3H); 2.65 (t, J = 5.6 Hz, 1H); 2.53–2.46 (m, 1H); 1.57 (s, 3H); 1.12–1.05 (m, 9H); 0.96 (d, J = 6.8Hz, 3H); ^{31}P NMR ($CDCl_3$): δ (ppm) 150.50, 149.71; ^{13}C NMR ($MeOD_4$): δ (ppm) 159.04; 158.40; 154.82; 152.59; 147.20; 144.96; 144.32; 135.23; 130.27; 128.36; 127.66 (2C); 126.92; 118.46; 112.89 (2C); 106.86; 91.14; 86.78; 82.44; 82.06; 80.89; 71.74; 70.17; 69.82; 69.52; 68.95; 60.24; 58.00; 57.92; 57.83; 57.69; 54.34; 43.10; 42.96; 23.82; 23.75; 23.62; 19.74; 19.61; 15.23; 14.96. ESI-HRMS: m/z calcd for $C_{42}H_{56}N_7O_9P$ [(M+Na)$^+$] 892.3769, found 892.3755.

1-((2*R*,3*R*,4*R*,5*R*)-4-hydroxy-5-(hydroxymethyl)-3-(2-methoxyethoxy) tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (8). BH_3 THF (5.5 mmol, 5.5 ml) was added to an ice-cooled flask. Then 2'-methoxyethanol (24 mmol, 1.9 ml) was added dropwise. After a few minutes the flask was allowed to warm up to room temperature, and it was stirred for 30 min. Tetrahydrofuran was then evaporated under reduced pressure. 2'methoxyethanol (7 ml) was added as a solvent followed by starting compound **7** (4.4 mmol, 1.0 g) and sodium bicarbonate (26 mg). The reaction was stirred at 150 °C under reflux for 48 h. The reaction mixture was directly prepared for column chromatography for purification (DCM/MeOH 95:5). Compound was obtained as a dark yellow foam: 1.1 g, 85% overall yield. ^{1}H NMR ($MeOD_4$): δ (ppm) 8.07 (d, J = 8Hz, 1H); 5.95 (d, J = 4Hz, 1H); 5.69 (d, J = 8Hz, 1H); 4.23 (t, J = 5.6 Hz, 1H); 4.05 (dd, J = 5.2 Hz, 4 Hz, 1H); 3.99 (dt, J = 5.6Hz, 2.8Hz, 1H); 3.89–3.84 (m, 2H); 3.81–3.72 (m, 2H); 3.59 (ddd, J = 5.2Hz, 3.6Hz, 1.6Hz, 2H); 3.37 (s, 3H); ^{13}C NMR ($MeOD_4$): δ (ppm) 166.18; 152.18; 142.55; 102.49; 89.12; 86.06; 83.78; 73.01; 70.87; 70.04; 61.75; 59.15. ESI-HRMS: m/z calcd for $C_{12}H_{18}N_2O_7$ [(M+Na)$^+$] 325.1006, found 325.1011.

1-((2*R*,3*R*,4*R*,5*R*)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl) -4-hydroxy-3-(2-methoxyethoxy)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione (9). Compound **8** (3.71 mmol, 1.12 g) was co-evaporated three times in dry pyridine (10 ml). The flask was cooled to 0 °C in an ice bath and redissolved in dry pyridine (10 ml). Dimethyoxytriphenylmethylchloride (2.41mmol, 0.71 g) was added to the reaction. After a few minutes the ice bath was removed and the reaction was stirred overnight at room temperature. Another portion of dimethyoxytriphenylmethylchloride (2.41 mmol, 0.71 g) was added and the reaction was further stirred at room temperature for 4 hours. Methanol (1 ml) was finally added and the reaction was again stirred at room temperature for 30 min. Solvents were removed under reduced pressure and the mixture was then partitioned between ethylacetate and aqueous sodium bicarbonate and washed twice with brine. The organic layer was dried in sodium sulphate. Solvents were then removed under reduced pressure. The crude product was purified by column chromatography (DCM/MeOH 95:5). Compound was obtained as a yellow foam: 1.6 g, 77% overall yield. ^{1}H NMR ($MeOD_4$): δ (ppm) 8.03 (d, J = 8Hz, 1H); 7.49–7.41 (m, 2H); 7.31–7.23 (m, 5H); 7.12–7.10 (m, 2H); 6.87–6.80 (m, 4H); 5.91 (d, J = 2.4Hz, 1H); 5.21 (d, J = 8Hz, 1H); 4.44 (dd, J = 7.2Hz, 5.2Hz, 1H); 4.07–4.05 (m, 2H); 3.98–3.93 (m, 1H); 3.84–3.81 (m, 1H); 3.77 (s, 6H); 3.61–3.58 (m, 2H); 3.48–3.47 (m, 2H), 3.36 (s, 3H); ^{13}C NMR ($MeOD_4$): δ (ppm) 166.15; 160.29; 151.96; 150.04; 145.98; 142.28; 141.22; 136.88; 136.57; 131.44; 130.45; 129.46; 128.95; 128.08; 114.25; 113.86; 102.28; 89.31; 88.22; 84.32; 84.04; 73.09; 71.00; 70.07; 59.19; 55.74; 55.66. ESI-HRMS: m/z calcd for $C_{33}H_{36}N_2O_9$ [(M+Na)$^+$] 627.2313, found 627.2310.

1-((2*R*,3*R*,4*R*,5*R*)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl) -3-(2-methoxyethoxy)-4-((trimethylsilyl)oxy)tetrahydrofuran-2-yl)-4-(1H-1,2,4-triazol-1-yl)pyrimidin-2(1H)-one (10). Compound **8** (1.6 mmol, 1.0 g) was co-evaporated three times in dry acetonitrile (15 ml). U_{MOE} was redissolved in dry acetonitrile (40 ml), to which first dry triethylamine (35.5 mmol, 5 ml) and then trimethylsilyl chloride (8.1 mmol, 1 ml) was added dropwise. The reaction mixture was stirred for 30 min at room temperature. Then 1,2,4-triazole (38.7 mmol, 2.677 g) was added. After 10 min the reaction mixture was cooled down to 0 °C where phosphorus oxychloride (4.8 mmol, 0.5 ml) was added dropwise. After 15 min the ice bath was removed and the reaction was stirred for 4h at room temperature. The solvent was removed under vacuum and the obtained crude was partitioned between aqueous sodium bicarbonate and ethylacetate (30 ml) and washed twice with brine (30 ml). The organic layer was dried in sodium sulphate. Solvents were then removed under reduced pressure. The crude product was purified by column chromatography (EtOAc/Hexane 80:20). Compound was obtained as a white foam: 784 mg, 75% overall yield. ^{1}H NMR ($MeOD_4$): δ (ppm) 9.37 (s, 1H), 9.03 (d, J = 7.2Hz, 1H); 8.23 (s, 1H); 7.43–7.40 (m, 2H); 7.36–7.28 (m, 7H); 6.91–6.89 (m, 4H); 6.55 (d, J = 7.2Hz, 1H); 6.00 (s, 1H); 4.53 (dd, J = 9.2Hz, 4.8 Hz, 1H); 4.23 (dt, J = 9.1Hz, 2.2Hz, 1H); 4.13–4.12 (m, 1H); 4.08–4.06 (m, 2H); 3.90 (ddd, J = 11.1Hz, 5.0Hz, 3.8Hz, 1H); 3.81 (s, 6H); 3.75 (dd, J = 11.1Hz, 2.3Hz, 1H); 3.63 (ddd, J = 5.0Hz, 3.8Hz, 1.0Hz, 1H); 3.49 (dd, J = 11.1Hz, 2.3Hz, 1H); 3.38 (s, 3H); 0.06 (s, 9H); ^{13}C NMR ($CDCl_3$): δ (ppm) 159.38, 158.89 (2C), 154.56, 153.95, 147.56, 143.79, 143.32, 135.33, 135.01, 130.33 (2C), 130.26 (2C), 128.48 (2C), 128.09, (2C), 127.42, 113.37 (4C), 94.93, 90.17, 87.17, 82.63, 82.40, 71.81, 70.00, 68.29, 59.88, 58.97, 55.32, 0.00. ESI-HRMS: m/z calcd for $C_{38}H_{45}N_5O_8Si$ [(M+Na)$^+$] 750.2930, found 750.2929.

1-((2*R*,3*R*,4*R*,5*R*)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl) -4-hydroxy-3-(2-methoxyethoxy)tetrahydrofuran-2-yl)-4-(1H-1,2,4-triazol-1-yl)pyrimidin-2(1H)-one (11). Compound **10** (0.9 mmol, 650 mg) was dissolved in dry tetrahydrofuran (10 ml). Then dry triethylamine (0.45 mmol, 4.5 ml) was added followed by slow addition of triethylamine•3 hydrofluoric acid (9.0 mmol, 1.3 ml). The reaction was stirred at room

temperature for 2 h. The reaction mixture was directly partitioned between ethylacetate and aqueous sodium bicarbonate and washed with brine. The organic layer was dried in sodium sulphate. Solvents were then removed under reduced pressure to yield the final product: 609 mg, 93 % overall yield. ^{1}H NMR ($MeOD_4$): δ (ppm) 9.34 (s, 1H); 8.93 (d, J = 7.2Hz, 1H); 8.22 (s, 1H); 7.45–7.43 (m, 2H); 7.33–7.23 (m, 7H), 6.89–6.86 (m, 4H); 6.58 (d, J = 7.2Hz, 1H); 5.94 (s, 1H); 4.54 (dd, J = 9.4Hz, 4.8Hz, 1H); 4.19–4.14 (m, 2H); 4.09 (d, J = 4.8Hz, 1H); 3.92 (ddd, J = 11.3Hz, 5.7Hz, 3.9Hz, 1H); 3.78 (s, 6H); 3.74–3.70 (m, 1H); 3.67–3.60 (m, 2H); 3.57–3.53 (m, 1H); 3.39 (s, 3H); ^{13}C NMR ($MeOD_4$): δ (ppm) 160.75, 160.35, 156.51, 154.75, 149.27, 145.65, 144.60, 137.05, 136.51, 131.53 (2C), 131.37 (2C), 129.57 (2C), 129.05 (2C), 128.20, 114.34 (4C), 96.06, 91.45, 88.39, 84.07, 83.98, 72.92, 71.03, 69.09, 61.98, 59.16, 55.77 (2C). (1C is missing).

(2*R*,3*R*,4*R*,5*R*)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(2-methoxyethoxy)-5-(2-oxo-4-(1H-1,2,4-triazol-1-yl)pyrimidin-1(2H)-yl) tetrahydrofuran-3-yl (2-cyanoethyl) diisopropylphosphoramidite (12). Compound **11** (1.79 mmol, 1.17 g) was co-evaporated three times in dry acetonitrile (15ml). Then it was dissolved in dry dichlormethane (13 ml), and the solvent was degased for two minutes. Then dry diisopropylethylamine (2.68 mmol, 467 µl) was added followed by the dropwise addition of 2-cyanoethyl N,N-diisopropyl-chlorophosphoramidite (2.15 mmol, 478 µl). The reaction was stirred for 2 h at room temperature. The reaction mixture was then directly partitioned between ethylacetate (25 mL) and aqueous sodium bicarbonate (25 mL) and washed with brine. The organic layer was dried over sodium sulphate and evaporated under vacuum. The crude product was purified by column chromatography (EtOAc/Hexane 80:20, 1% TEA) Compound was obtained as a white foam: 1.3 g, 85% overall yield. ^{1}H NMR ($CDCl_3$): δ (ppm) 9.23 (s, 1H); 8.89 (d, J = 8Hz, 1H); 8.07 (s, 1H); 7.46–7.27 (m, 9H); 6.87–6.74 (m, 4H); 6.46 (d, J = 8Hz, 1H); 6.02 (s. br., 1H); 4.57 (td, J = 12Hz, 8Hz, 1H); 4.39–4.33 (m, 1H); 4.24–4.13 (m, 2H); 3.97–3.89 (m, 1H); 3.81 (s, 6H); 3.78–3.69 (m, 2H); 3.66–3.51 (m, 6H); 3.38 (s, 3H); 2.51 (t, J = 4Hz, 1H); 1.28 (t, J = 8Hz, 3H); 1.19–1.14 (m, 6H); 1.02 (d, J = 8Hz, 3H); ^{31}P NMR ($MeOD_4$): δ (ppm) 150.53, 148.32; ^{13}C NMR ($CDCl_3$): δ (ppm) 160.68, 160.40, 156.67, 149.16, 145.55, 145.43, 144.50, 136.83, 136.57, 136.35, 131.71, 131.63, 129.78, 129.07, 128.30, 119.84, 114.32, 96.34, 92.31, 88.50, 83.77, 83.27, 82.24, 73.03, 71.45, 71.18, 70.66, 70.02, 60.95, 59.37, 59.22, 59.16, 55.74, 44.51, 44.38, 25.14, 24.95, 23.19, 21.17, 20.97. ESI-HRMS: m/z calcd for $C_{44}H_{54}N_7O_9P$ [$(M+H)^+$] 856.3793, found 856.3782.

Oligonucleotide phosphorothioate synthesis.

Oligonucleotides were prepared on MM 12 DNA/RNA synthesizer using CPG 500 Å Unylinker support (44.9 µmol/g). Fully protected phosphoramidites were incorporated using standard solid-phase oligonucleotide synthesis conditions: i.e. 3% Dichloroacetic acid in dichloromethane (DCM) For deblocking, 0.24 M BTT 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-dithiazole-5-thione (DDTT; Sulfurizing Reagent II; Glen Research, Virginia) in dry pyridine/ACN (60:40) for sulfurization. Unmodified 2′-OMe and 2′-MOE phosphoramidites were prepared at 0.1 M in anhydrous acetonitrile and triazole-modofied 2′-OMe and 2′-MOE phosphoramidites were prepared at 0.25 M in anhydrous isobutyronitrile. The CPG support was suspended in water and 8 mg per modification of neat spermine was directly added. The suspension was shaken gently overnight. A complete deprotection was carried out by treatment of 30% aqueous ammonium hydroxide (200 µl) for 5 h at 55 °C. Further work-up was identical to that of unmodified sequences. The reaction was cooled to room temperature and the solid support was filtered and washed with 2x200 µl of EtOH and H_2O 1:1 (v/v). The solvents were removed under reduced pressure and the residue was dissolved in 200 µl water. The crude material was purified by RP-HPLC. 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. DMT-deprotection was performed in 40% of acetic acid (200 µl) for 45 min at room temperature. A final RP-HPLC purification (gradient for the DMT-off purification: 5 –35% buffer B over 5 min) yielded the final products.

Supplementary Material

Supporting information for this article (solid-synthesis LCMS profiles, UV melting curves, and NMR spectra of compounds) is available at http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

ED, AL, FH contributed to study design, synthesis and testing of oligonucleotides, and writing the manuscript. JH contributed to study design and wrote the paper.

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