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Novel Conformationally Constrained 2′-C-Methylribonucleosides: Synthesis and Incorporation into Oligonucleotides

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Abstract. Synthesis of two novel conformationally constrained bicyclic ribonucleoside phosphoramidites bearing a 2′-C-methyl substituent has been accomplished. These phosphoramidites were used to incorporate the corresponding 2′-C-methyl nucleotides into oligonucleotides and to study their effects on duplex thermal stability. Whereas the C2′-O4′-linked LNA-type derivative induced severe destabilization of duplexes formed with complementary DNA and RNA, the C3′-O4′-linked derivative induced RNA-selective hybridization with increased affinity relative to that of the unmodified DNA-based probe.

Electronic Supplementary Information (ESI) available [Details of crystal structure determinations. Copies of IE-HPLC curves, MAIDI-TOF mass spectra and NMR spectra for new compounds]. See DOI: 10.1039/c000000x/
Introduction

The use of conformationally restricted nucleotide monomers in antisense oligonucleotides is of significant interest due to their constrained sugar puckering, which can mimic a DNA- or RNA-type furanose ring conformation. This may promote several advantages with respect to antisense capability such as increasing thermal stability towards targeted mRNA, efficient cleavage of targeted mRNA by activation of RNase H, and stability against nucleolytic degradation.\(^1,2\)

Prominent examples of conformationally restricted oligonucleotides are locked nucleic acid (LNA)\(^3\) and its stereoisomer α-L-LNA,\(^4\) both having a C2′-O4′ methylene bridge (Fig. 1), which efficiently locks the furanose rings in a 3′-endo (North-type) pseudorotational conformation. When singly incorporated into oligonucleotides, these two monomers increase the thermal stability of duplexes with complementary DNA or RNA very significantly when compared to the thermal stability of the corresponding unmodified oligonucleotides.\(^5\) Oligonucleotides containing LNA in the wings of so-called gapmers have been shown to successfully activate RNase H and thus to mediate cleavage of targeted RNA. In addition, incorporation of several LNA nucleotides lead to a substantial increase in the stability in human serum compared to an unmodified oligonucleotide.\(^6\)

Another group of conformationally restricted nucleotides contains an oxetane ring system where the methylene bridging is linking either C1′-O2′,\(^7,7\)-10 O2′-C3′\(^11,12\) or O3′-C4′.\(^5,13,15\). The C1′-O2′ oxetane derivatives (Fig. 1) adopt a North-East pseudorotational conformation when incorporated into oligonucleotides (phosphodiester 3′-5′-linkage) and affects the thermodynamic stability of duplexes formed with complementary RNA by a decrease of approximately 6 °C per incorporated pyrimidine monomer while incorporation of purine monomers has no effect.\(^9\) In certain configurations this oxetane nucleotide monomer has been shown to be compatible with RNase H activity when hybridized to RNA,\(^2\) to give protection against endonucleases,\(^7\) and to be useful as modification in siRNA constructs.\(^16\) Other members of the oxetane nucleotide family include oxetane nucleic acid (ONA)\(^13\) and α-L-ONA\(^5\) with a O3′-C4′ methylene bridge (Fig. 1). These adopt a 2′-endo (South-type) pseudorotational conformation, and when incorporated into oligonucleotides (phosphodiester 2′-5′-linkage) they show a selectivity for RNA over DNA and a significant decrease in thermal stability against complementary DNA and RNA.\(^17\) It is also
relevant to mention that several 2'-C-methylribonucleosides are selective inhibitors of HCV replication including the FDA approved drug Sofosbuvir as the most prominent compound.\textsuperscript{18}

Herein we describe the synthesis of two novel constrained ribonucleotide phosphoramidites bearing a 2'-C-methyl substituent and either a O2'-C4' (LNA-analogue) or a O3'-C4' (ONA-analogue) methylene bridge. Furthermore, their incorporation into oligonucleotides as monomers X and Y (Fig. 1) and their effects on duplex stability were studied.

Fig. 1 Chemical structures of conformational restricted nucleic acid analogues incorporated as phosphodiester into oligonucleotides. B = nucleobases, U = uracil-1-yl and T = thymin-1-yl. LNA,\textsuperscript{3} α-L-LNA,\textsuperscript{4} C1'-O2'-ONA,\textsuperscript{1,7-10} O2'-C3'-oxetane-ANA,\textsuperscript{11,12} ONA\textsuperscript{5,13-15} and α-L-ONA.\textsuperscript{5}

**Results and discussion**

**Synthesis of phosphoramidites**

Starting from commercially available 2'-C-methyluridine (1), the conversions into the phosphoramidite derivatives 9a and 9b with either an LNA-type (O2'-C4' linked) or an ONA-type (O3'-C4' linked) bicyclic scaffold are shown in Schemes 1 and 2, and Table 1. The desired precursor for the LNA- and ONA-type analogues were synthesized in five steps (Scheme 1). In
the first step the 2'- and 3'-hydroxy groups were protected using a cyclohexane ketal protecting group affording intermediate 2 in 90% yield. A Swern-oxidation followed by an Aldol-Cannizarro reaction gave the 4'-hydroxymethyl derivative 3 in 40% yield over three steps from 2. The two primary hydroxy groups were mesylated giving nucleoside 4 as an intermediate and the cyclohexane ketal protecting group was subsequently removed using 80% aqueous TFA giving the key intermediate 5 suitable for the subsequent ring closure reaction (76% yield over two steps).

Scheme 1 Synthesis of the bicyclic precursors 6a and 6b. U = uracil-1-yl.

The ring closure of the bis-mesylate 5 into a mixture of the corresponding bicyclic nucleosides 6a or 6b was optimized. Our expectation was that the presence of the 2'-C-methyl substituent would exert a conformational effect due to its preference for an axial orientation enabling a higher yield of the LNA-type derivative 6a than observed for the corresponding reaction with a similar precursor having a H-atom in the 2'-beta position. Using NaH and performing the reaction at room temperature (Table 1, entry 1) resulted in isolation of 6a and 6b in yields of 22% and 12%, respectively. Performing the cyclization reaction at a temperature of 120 °C (Table 1, entry 2) reversed the selectivity giving 6a and 6b in yields of 15% and 25%, respectively. Substantial amounts of material from side reactions were, however, isolated under both conditions. Following these observations, attempts to improve the reaction were made. In one setting, the solution containing 5 was cooled to 0 °C followed by addition of NaH in two
equal portions and then allowing the mixture to react at room temperature overnight (Table 1, entry 3), which afforded 6a and 6b in approximately 34% yield of each. A subsequent attempt splitting the amount of NaH into three portions and allowing the reaction mixture to react for one day at 0 °C followed by another one day at room temperature (Table 1, entry 4) did not improve the outcome of the reaction. The structure assigned to the LNA- and ONA-type derivatives 6a and 6b were confirmed by comparison of data reported earlier for similar LNA3,5 and ONA nucleosides.14

Table 1 Ring closure of compound 5 into the LNA-analogue 6a and ONA-analogue 6b

<table>
<thead>
<tr>
<th>Entry</th>
<th>Ratio 5 : NaH</th>
<th>Anhydrous DMF</th>
<th>Temp.</th>
<th>Time</th>
<th>6a&lt;sup&gt;a&lt;/sup&gt;</th>
<th>6b&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 : 2.2</td>
<td>4 mL/mmol</td>
<td>RT</td>
<td>3 days</td>
<td>22%&lt;sup&gt;[d]&lt;/sup&gt;</td>
<td>12%&lt;sup&gt;[d]&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1 : 2.2</td>
<td>22 mL/mmol</td>
<td>120 ºC</td>
<td>2 hours</td>
<td>15%&lt;sup&gt;[d]&lt;/sup&gt;</td>
<td>25%&lt;sup&gt;[d]&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>1 : 2.2&lt;sup&gt;[b]&lt;/sup&gt;</td>
<td>66 mL/mmol</td>
<td>0 ºC (2 hours) → RT (overnight)</td>
<td></td>
<td>34%&lt;sup&gt;[d]&lt;/sup&gt;</td>
<td>34%&lt;sup&gt;[d]&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>1 : 2.2&lt;sup&gt;[c]&lt;/sup&gt;</td>
<td>66 mL/mmol</td>
<td>0 ºC (1 day) → RT (1 day)</td>
<td></td>
<td>37%&lt;sup&gt;[d]&lt;/sup&gt;</td>
<td>24%&lt;sup&gt;[d]&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Yields of isolated products. <sup>b</sup>NaH was added in 2 portions. <sup>c</sup>NaH was added in 3 portions. <sup>d</sup>Yields are only estimated due to the presence of minor impurities as evidenced by NMR analysis.

Next the LNA-type and ONA-type derivatives 6a and 6b were converted into the corresponding O3′ and O2′ phosphoramidites, respectively in four synthetic steps (Scheme 2). Converting the 5′-C-mesyloxy groups into the corresponding 5′-hydroxy groups was performed through formation of 5′-O-benzoyl intermediates followed by alkaline ester hydrolysis as reported earlier for an analogous substrate<sup>20</sup> giving derivatives 7a and 7b, respectively. Compounds 7a and 7b were 5′-O-DMTr-protected giving 8a and 8b, respectively. Eventually, phosphitylation was carried out giving the phosphoramidite derivatives 9a and 9b by using 2-cyanoethyl-N,N,N′,N′-tetraisopropylphosphordiamidite as the phosphitylating reagent and N,N-diisopropylamine tetrazolide (DIPAT) as activator. All conversions described above proceeded in satisfactory yields (all estimated to be above 70%), but relative yields were not calculated due to the presence of minor impurities as evidenced by NMR analyses (see ESI).
Scheme 2 Synthesis of the phosphoramidite derivatives 9a and 9b. U = uracil-1-yl, DMTr = 4,4'-dimethoxytrityl, PN2-reagent = 2-cyanoethyl-N,N',N,N'-tetraisopropylphosphordiamidite, DIPAT = N,N-diisopropylamine tetrazolide.

Oligonucleotide synthesis
The 2’-C-Me-LNA and 2’-C-Me-ONA phosphoramidites 9a and 9b, respectively, were successfully used in automated ON synthesis on a commercial nucleic acid synthesizer. The stepwise coupling yields were ~80% for the 2’-C-Me-LNA phosphoramidite 9a employing “hand coupling conditions” and ~99% for standard DNA and LNA phosphoramidites. Following the coupling of the 2’-C-Me-ONA phosphoramidite 9b, an oxidation step using a 0.5 M solution of (1S)-(+)-(10-camphorsulfonyl)oxaziridine (CSO) in anhydrous MeCN was applied followed by capping. The non-aqueous oxidizer CSO was used as we expected that the phosphotriester, formed from coupling using the tertiary phosphoramite 9b, would be liable to an elimination reaction if the standard aqueous iodine oxidation was used. The coupling yield of 9b after CSO-oxidation was approximately 75%. Deprotection and cleavage from the solid support (32% ammonia, 50 °C, 16 h) yielded oligonucleotides containing the monomers X and Y after purification by reversed phase HPLC. The composition and purity (>80% yield) of the oligonucleotides were verified by MALDI-TOF MS and ion-exchange HPLC, respectively (Experimental section).
Thermal denaturation experiments

9-Mer DNA-based sequences were synthesized with incorporations of either the 2’-C-Me-LNA monomer X (ON2 and ON5) or the 2’-C-Me-ONA monomer Y (ON7-ON9). In order to evaluate the effect of the monomers X and Y on duplex stability, ON1-ON9 were hybridized to complementary DNA or RNA and thermal denaturation experiments were conducted in a medium salt buffer (Table 2).

When ON2 with one incorporation of monomer X was hybridized to DNA or RNA a significant drop in thermal denaturation temperatures ($T_m$) of 10.5 °C and 7.5 °C respectively, was observed, in comparison to control ON1. Replacing the 2’-Me-C-LNA-U with LNA-T (ON3) gave the expected large increase in $T_m$ when measured against complementary DNA and RNA. A similar destabilizing effect for an LNA-constitution type monomer has been shown only for α-L-amino-LNA being either N2’-alkylated or N2’-acetylated, which resulted in a decrease in thermal denaturation temperatures of 12 °C and 16 °C per modification, respectively.\(^1\)

Next we investigated if incorporation of LNA-T was able to reverse the destabilizing effect exerted by monomer X. A central modification with monomer X and all other thymidine monomers replaced by LNA-T was synthesized (ON5). When ON5 was hybridized to complementary strands, a decrease in $T_m$ by 2.5 °C was observed towards complementary DNA but an increase of 8.5 °C towards complementary RNA. When the results for ON5 are compared to the results for ON4 and ON6 containing two and three incorporations of LNA-T, respectively, it is evident that monomer X has an overall destabilizing effect.

When ON7 with one incorporation of monomer Y was hybridized to complementary DNA or RNA an increase in $T_m$ of 0.5 °C and 2.5 °C, respectively, were observed. For three incorporations of monomer Y (ON8) a decrease in $T_m$ against complementary DNA of 6.0 °C and an increase against complementary RNA of 4.5 °C was observed. This shows an interesting selectivity for RNA over DNA for the ON containing several incorporations of monomer Y. This is in accordance with the trends observed in previous data reported for ONA,\(^12\)\(^-\)\(^14\) though only in the case of monomer Y reported herein was an affinity increase with complementary RNA observed. Next, the effect on duplex stability upon combining LNA-T and monomer Y (ON9) was investigated which revealed an increase in $T_m$ of 7 °C against complementary DNA and an increase of 14.5 °C against complementary RNA, thus demonstrating compliance between LNA monomers and monomer Y.
Table 2 Thermal denaturation studies of duplexes

<table>
<thead>
<tr>
<th>ON</th>
<th>Sequence</th>
<th>( T_m (\Delta T_m) )°C DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON1</td>
<td>5'-GTG-ATA-TGC-3'</td>
<td>30.5 (ref)</td>
<td>28.5 (ref.)</td>
</tr>
<tr>
<td>ON2</td>
<td>5'-GTG-AXA-TGC-3'</td>
<td>20.0 (-10.5)</td>
<td>21.0 (-7.5)</td>
</tr>
<tr>
<td>ON3</td>
<td>5'-GTG-AT(^4)A-TGC-3'</td>
<td>37.5 (+7.0)</td>
<td>37.0 (+8.5)</td>
</tr>
<tr>
<td>ON4</td>
<td>5'-GT(^4)G-ATA-T(^4)GC-3'</td>
<td>39.0 (+8.5)</td>
<td>43.0 (+14.5)</td>
</tr>
<tr>
<td>ON5</td>
<td>5'-GT(^4)G-AXA-T(^4)GC-3'</td>
<td>28.0 (-2.5)</td>
<td>36.5 (+8.0)</td>
</tr>
<tr>
<td>ON6</td>
<td>5'-GT(^4)G-AT(^4)A-T(^4)GC-3'</td>
<td>44.0 (+13.5)</td>
<td>50.5 (+22.0)</td>
</tr>
<tr>
<td>ON7</td>
<td>5'-GTG-AYA-TGC-3'</td>
<td>31.0 (+0.5)</td>
<td>31.0 (+2.5)</td>
</tr>
<tr>
<td>ON8</td>
<td>5'-GYG-AYA-YGC-3'</td>
<td>24.5 (-6.0)</td>
<td>33.0 (+4.5)</td>
</tr>
<tr>
<td>ON9</td>
<td>5'-GT(^4)G-AYA-T(^4)GC-3'</td>
<td>37.5 (+7.0)</td>
<td>43.0 (+14.5)</td>
</tr>
</tbody>
</table>

\(^a\)Thermal denaturation temperatures (\( T_m \)-values) of synthesized oligonucleotides against complementary sequences DNA:DNA or DNA:RNA duplexes measured as the average of the maxima of the first derivatives of the melting curves (\( A_{260} \) versus temperature) from two independent melting temperature determination with a deviation <0.5°C. Numbers in parentheses are \( \Delta T_m \) values measured in degrees Celsius as the difference in \( T_m \)-values between modified and unmodified duplexes including ON1. The experiment was carried out in medium salt buffer (6.1 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 \( \mu \)M concentrations of the two complementary strands (assuming identical extinction coefficients for all modified and unmodified oligonucleotides); \( X = 2'-C\)-Me-LNA-monomer, \( T^L = LNA-T \) monomer, \( Y = 2'-C\)-Me-ONA uridine phosphodiester monomer, \( A = \) adenin-9-yl monomer, \( C = \) cytosin-1-yl monomer, \( G = \) guanin-9-yl monomer and \( T = \) thymin-1-yl monomer.

Crystal structure of LNA intermediates

To gain insight into why the 2'-C-Me-LNA monomer \( X \) has such a pronounced duplex destabilizing effect, the possibility of obtaining a crystal structure of a relevant nucleoside intermediate was investigated. It was possible to crystallize 6a as well as to synthesize and crystallize 5'-OMs-LNA-T 11\(^23\) (Scheme 3), and the crystals of these two structures were analyzed and compared to study the steric effects of the 2'-Me substituent present in 6a.

![Scheme 3 Synthesis of 5'-OMs-LNA-T 11.](image-url)
The molecular structures of 6a and 11 are shown in Fig. 2A. Four independent (but very similar) molecules of 11 are found in the unit cell, however only one is displayed in Fig. 2. In both compounds the molecules are paired by Watson-Crick type hydrogen bonding involving N2 and O4. From these structures it can be determined that 6a has a phase angle $P = 19.1^\circ$ whereas the phase angles for the four molecules of 11 lie in the range 16.4-18.2$^\circ$ which implies, as expected, that both compounds have a C3'-endo (North-type)-conformation. When the structures are viewed along the plane of the pyrimidine ring (with C1'-N1 horizontal) (Fig. 2B) and in a space-filling style (Fig. 2C), the steric effect of 2'-Me substituent in 6a is evident compared to the LNA control 11. The 2'-Me substituent bulges towards the uracil nucleobase at a -48.35$^\circ$ torsion angle (2'Me-C2'-C1'-N1) and would likely impede the nucleobases ability to form adequate Watson-Crick base paring when incorporated into an oligonucleotide. Additionally for 6a and 11 their glyosidic bond angle $\chi$ were found to be $-157^\circ$ (or +203$^\circ$) and $-167.9^\circ$ to $-173.3^\circ$ (or +186.7$^\circ$ to +192.1$^\circ$), respectively. These differences in $\chi$-values of 10.9$^\circ$ to 16.3$^\circ$ might hinder the nucleobase ability to base-stack. These results would likely explain the observed thermal destabilizing effect when monomer X is incorporated into an oligonucleotide, which is in sharp contrast to the high affinity hybridization generally induced by LNA monomers.
Fig. 2 A) Perspective views of 6a and 11 drawn with 50% probability ellipsoids, only one of the four independent molecules in the structure of 11 is shown, B) views in the plane of the pyrimidine ring with the N1-C1’ bond horizontal, C) space-filling view in the same orientation as in B. Compound 6a has a phase angle $\varphi = 19.1^\circ$ and $\chi = -157^\circ$ (or +203°) 11 has a phase angle $\varphi = 16.4$-18.2° and $\chi = 167.9^\circ$ to -173.3° (or +186.7° - +192.1°).
**Conclusion**

Two novel conformationally constrained bicyclic ribonucleotide phosphoramidites bearing a 2'-C-methyl substituent and with either a C2'-O4'-linked (LNA-type) or C3'-O4'-linked (ONA-type) methylene bridge were synthesized. A key synthetic step was the ring-closure of compound 5 into a mixture of two bicyclic intermediates. The phosphoramidites 9a and 9b were successfully used on an automated DNA synthesizer resulting in incorporation into oligonucleotides of monomers X and Y. Monomer X (LNA-type) showed an overall destabilizing effect, whereas for monomer Y (ONA-type) a selectivity for duplex formation with complementary RNA over DNA was observed, along with increased thermal stability relative to the native reference duplex with the RNA complement. The crystal structure of a key intermediate showed that the steric effect from the 2'-Me substituent likely impeded the ability of the nucleobases bases to form adequate Watson-Crick base paring in the case of the LNA-type crystal.

**Experimental section**

NMR spectra were recorded on a Bruker AVANCE III 400 (1H at 400 MHz, 13C at 101 MHz and 31P at 162 MHz). All samples were dissolved in DMSO-d6. Chemical shifts are reported in ppm relative to solvent peak (1H, internal standard, δH 2.500 ppm; 13C, internal standard, δC 39.520 ppm) or 85% H3PO4 (31P, external standard, δP 0.000 ppm). Assignments of the NMR signals are based on 2D correlation spectroscopy. Standard nucleoside atom-numbering is used for assignment of NMR signals. High-resolution MS spectra were recorded by electrospray ionization in positive mode on a Bruker MicrOTOF-Q II. ONs were synthesized on an Expedite™ nucleic acid synthesis system (PerSeptive Biosystems). The compositions of ONs were confirmed by MALDI-MS on a Microflex MALDI-TOF MS (Bruker Daltronics), purifications were performed by reversed-phase HPLC on a Waters 600 system, and purities were determined by ion-exchange HPLC on a LaChrom L-7000 system (Merck Hitachi).

**2',3'-O-Cyclohexylidene-2'-C-methyluridine (2)**

To a flame-dried 250 mL RB was added 2’-C-methyluridine (10.0 g, 38.73 mmol), p-toluenesulfonic acid monohydrate (744 mg, 3.873 mmol), anhydrous THF (65 mL) and 1,1-
dimethoxycyclohexane (10 mL, 65.84 mmol) under an atmosphere of argon. The resulting reaction mixture was refluxed at 90 °C overnight and then evaporated to dryness under reduced pressure into a yellow oil. This oil was re-dissolved in DCM (200 mL) and washed with sat. aq. NaHCO$_3$ (50 mL) and the aqueous phase back-extracted with DCM (2 × 25 mL). The organic fractions were combined and dried (Na$_2$SO$_4$), filtered and evaporated to dryness under reduced pressure into a beige foam which was purified by silica gel column chromatography (5% MeOH/DCM). The product (11.75 g, 90% yield) was isolated as a white foam. $R_f$ = 0.5 (10% MeOH/DCM v/v). $^1$H NMR (DMSO-$d_6$) δ (ppm) 11.37 (s, 1H, NH), 7.85 (d, $J$ = 8.1 Hz, 1H, H6), 6.01 (s, 1H, H1'), 5.63 (dd, $J$ = 8.1, 2.0 Hz, 1H, H5), 5.25 (t, $J$ = 5.1 Hz, 1H, 5'-OH), 4.46 (d, $J$ = 2.8 Hz, 1H, H3'), 4.14 (q, $J$ = 3.4 Hz, 1H, H4'), 3.83–3.54 (m, 2H, H5'), 1.81–1.64 (m, 2H, cyclohexylidene), 1.67–1.44 (m, 6H, cyclohexylidene), 1.45–1.28 (m, 2H, cyclohexylidene), 1.22 (s, 3H, 2'-CH$_3$). $^{13}$C NMR (DMSO-$d_6$) δ (ppm) 162.9 (C4), 150.2 (C2), 140.7 (C6), 113.4 (cyclohexylidene), 101.0 (C5), 92.2 (C1'), 89.3 (C2'), 85.1 (C3'), 84.2 (C4'), 61.1 (C5'), 37.4, 36.1, 24.3, 23.5, 23.3 (5 × CH$_2$, cyclohexylidene), 19.3 (C2'-CH$_3$). HRMS (ESI) $m/z$ 361.1373 ([M + Na$^+$], C$_{16}$H$_{22}$N$_2$O$_6$·Na$^+$ calc. 361.1370).

2',3'-O-Cyclohexylidene-4'-C-hydroxymethyl-2'-C-methyluridine (3)

A flame-dried 3-necked 500 mL RB was evacuated and filled with argon gas several times whereupon anhydrous DCM (200 mL) was added and cooled to -78 °C under an atmosphere of argon. Oxalyl chloride (5.46 mL, 63.4 mmol) was added, and the resulting mixture was stirred for 30 min. Anhydrous DMSO (9.1 mL, 126.7 mmol) was slowly added dropwise to ensure that the temperature did not rise above -70 °C, and afterwards was stirring continued for 20 min at -78 °C. To a flame-dried flask was added compound 2 (8.58 g, 25.35 mmol), and the flask was slowly evacuated and filled with argon. Anhydrous DCM (31 mL) was added, and the resulting mixture was cooled to near -78 °C and slowly added dropwise to the above mixture. Stirring was continued for 40 min whereupon DIPEA (22 mL, 126.7 mmol) was added, and after continued stirring for 5 min the mixture was allowed to warm to r.t. giving a light orange solution. H$_2$O (75 mL) was added and the organic phase was separated and washed with H$_2$O (3 × 10 mL) and evaporated to dryness under reduced pressure to give a beige/orange foam as a crude intermediate which was used in the next step without further purification. To this foam was added dioxane (58 mL), 37% aq. CH$_3$O (5 mL, 63.4 mmol, 2.5 eq.) and 2M NaOH (8.9 mL,
17.74 mmol). The resulting mixture was stirred at r.t. for 4 h and then cooled to 0 °C. NaBH₄ (1.15 g, 30.42 mmol) was added and the resulting mixture was under stirring allowed to warm to r.t. whereupon stirring was continued for 1 h. A 4:1 mixture of anhydrous pyridine:AcOH (9.6 mL:2.4 mL) was added and stirring was continued at r.t. for 5 min. The mixture was evaporated to dryness under reduced pressure and the residue was purified by silica gel column chromatography (0→10% MeOH/DCM v/v) to furnish compound 3 (3.73 g, 40% yield) as a white foam. $R_f = 0.42$ (10% MeOH/DCM v/v). $^1$H NMR (DMSO-d$_6$) $\delta$ (ppm) 11.34 (s, 1H, NH), 7.85 (d, $J = 8.2$ Hz, 1H, H6), 6.02 (s, 1H, H1’), 5.62 (d, $J = 8.2$ Hz, 1H, H5), 5.21 (t, $J = 5.3$ Hz, 1H, 5’-OH), 4.62 (t, $J = 5.8$ Hz, 1H, 5’’-OH), 4.48 (s, 1H, H3’), 3.74–3.48 (m, 4H, H5’’, H5’’’), 1.76–1.63 (m, 2H, cyclohexylidene), 1.64–1.41 (m, 6H, cyclohexylidene), 1.38–1.28 (m, 2H, cyclohexylidene), 1.28 (s, 3H, 2’GCH$_3$). $^{13}$C NMR (DMSO-d$_6$) $\delta$ (ppm) 163 (C4), 150.2 (C2), 140.7 (C6), 113.5 (cyclohexylidene), 100.7 (C5), 91.2 (C1’), 89.6 (C2’), 86.6 (C3’), 86 (C4’), 62.6 (C5’’), 60.3 (C5’), 36.9, 35.4, 24.4, 23.5, 23.1 (5 × CH$_2$, cyclohexylidene), 19.4 (2’-CH$_3$). HRMS (ESI) $m/z$ 391.1476 ([M + Na$^+$], C$_{17}$H$_{24}$N$_2$O$_7$·Na$^+$ calc. 391.1486).

2’,3’-O-Cyclohexylidene-5’-O-mesyl-4’-C-mesyoxyethyl-2’-C-methyluridine (4)

To a flame dried 100 mL RB was added compound 3 (2.658 g, 7.216 mmol) and co-evaporation under reduced pressure with anhydrous pyridine (3 × 5 mL). The resulting foam was re-dissolved in anhydrous pyridine (22 mL) and cooled to 0 °C under an atmosphere of argon. Methanesulfonyl chloride (1.70 mL, 21.64 mmol) was added dropwise resulting in a cloudy yellowish mixture which under stirring was allowed to warm to r.t.. After continued stirring for 4 h at r.t. the resulting intense red reaction mixture was evaporated to dryness under reduced pressure. The residue was co-evaporated under reduced pressure with toluene (2 × 5 mL) resulting in a red-brown foam which was subsequently subjected to silica gel column chromatography (0→10% MeOH/DCM v/v) to give a product assigned as compound 4 (3.41 g, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) as a white foam. $R_f = 0.64$ (10% MeOH/DCM v/v). $^1$H NMR (DMSO-d$_6$) $\delta$ (ppm) 11.45 (s, 1H, NH), 7.58 (d, $J = 8.2$ Hz, 1H, H6’), 6.18 (s, 1H, H1’), 5.65 (dd, $J = 8.2$, 2.1 Hz, 1H, H5’), 4.61 (s, 1H, H3’), 4.53–4.32 (m, 4H, H5’, H5’’’), 3.35 (s, 3H, 5’-OMs), 3.26 (s, 3H, 5’’-OMs), 1.86–1.36 (m, 10H, cyclohexylidene), 1.33 (s, 3H, 2’-CH$_3$). $^{13}$C NMR (DMSO-d$_6$) $\delta$
(ppm) 162.7 (C4), 150.0 (C2), 139.8 (C6), 115.1 (cyclohexylidene), 101.4 (C5), 90.6 (C1'), 88.9 (C2'), 85.2 (C3'), 81.4 (C4'), 68.6 (C5''), 67.2 (C5'), 36.84 (5'-OMs), 36.81 (5''-OMs), 36.6, 35.3, 24.2, 23.5, 23.1 (5 × CH2 cyclohexylidene), 19 (2'-GCH3). HRMS (ESI) m/z 547.1027 ([M + Na]+, C19H28N2O11S2·Na+ calc. 547.1020).

5'-O-Mesyl-4'-C-mesyloxyethyl-2'-C-methyluridine (5)

Compound 4 (3.23 g) was suspended in cold 80% aqueous TFA (25 mL) resulting in a red/orange solution. After stirring for 4 days at r.t. TLC showed full consumption of starting material, and the mixture was evaporated to dryness under reduced pressure to give an orange-brownish oil. Upon addition of DCM, a precipitate was formed, filtered and collected. The filtrate was evaporated to dryness under reduced pressure and another precipitation was performed. The combined product 5 (2.19 g, 76% yield calculated for two steps from compound 3) was isolated as an off-white powder. Rf = 0.41 (10% MeOH/DCM v/v). 1H NMR (DMSO-d6) δ (ppm) 11.46 (s, 1H, NH), 7.57 (d, J = 8.1 Hz, 1H, H6), 5.94 (bs, 2H, 2'-OH, 3'-OH), 5.75 (s, 1H, H1'), 5.61 (dd, J = 8.1, 2.2 Hz, 1H, H5), 4.81 (d, J = 11.5 Hz, 1H, H5'), 4.44 (q, J = 10.6 Hz, 2H, H5''), 4.33 (d, J = 11.5 Hz, 1H, H5'), 4.05 (s, 1H, H3'), 3.28 (s, 3H, 5'-OMs), 3.21 (s, 3H, 5''-OMs), 1.06 (s, 3H, 2'-CH3). 13C NMR (DMSO-d6) δ (ppm) 162.7 (C4), 150.4 (C2), 101.9 (C5), 82.2 (C1'), 78.2 (C4'), 75.4 (C3'), 68.5 (C5'), 68.2 (C5''), 54.8 (C2'), 36.9 (5'-OMs), 36.8 (5''-OMs), 21.1 (2'-Me). HRMS (ESI) m/z 467.0411 ([M + Na]+, C13H20N2O11S2·Na+ calc. 467.0401).

5'-O-Mesyl-2'-O,4'-C-methylene-2'-C-methyluridine (6a) and 5'-O-mesyl-3'-O,4'-C-methylene-2'-C-methyluridine (6b)

5'-O-Mesyl-4'-C-mesyloxyethyl-2'-C-methyluridine (5, 1.42 g, 3.19 mmol) was added to a flame-dried 500 mL RB and stored under high vacuum overnight. The RB was evacuated and filled with argon gas several times, and anhydrous DMF (210 mL) was added resulting in a bright yellow solution which under stirring was cooled to 0 °C. NaH in a 60% oil suspension (280 mg, 7.02 mmol) was added in two portions with 1 h of stirring between the two additions. The resulting mixture was stirred at 0 °C overnight and thereafter allowed to warm to r.t. and stirred again overnight. The reaction mixture was evaporated to dryness under reduced pressure
and co-evaporated under reduced pressure with toluene (2 × 10 mL) resulting in a solid that afterwards was mixed with celite and subsequently purified by silica gel column chromatography (0%→10% MeOH/EtOAc v/v). The LNA product (6a) (378 mg; relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) was isolated as a fine white powder and the ONA product (6b) (378 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) was isolated as a white foam. Compounds 6a and 6b were used without further purification. Compound 6a; Rf = 0.36 (5% MeOH/EtOAc v/v). 1H NMR (DMSO-d6) δ (ppm) 11.41 (s, 1H, NH), 7.73 (d, J = 8.2 Hz, 1H, H6), 6.01 (d, J = 5.0 Hz, 1H, 3'-OH), 5.73 (s, 1H, H1'), 5.62 (d, J = 8.1 Hz, 1H, H5), 4.85 (d, J = 11.9 Hz, 1H, H5'), 4.54 (d, J = 11.9 Hz, 1H, H5'), 3.96 (d, J = 8.0 Hz, 1H, H5''), 3.79 (d, J = 8.0 Hz, 1H, H5''), 3.75 (d, J = 4.9 Hz, 1H, H3'), 3.25 (s, 3H, 5'-GOMs), 1.13 (s, 3H, 2'-GCH3). 13C NMR (DMSO-d6) δ (ppm) 162.8 (C4), 150.4 (C2), 139.3 (C6), 101.4 (C5), 88.0 (C1'), 86.0 (C4'), 85.8 (C2'), 71.1 (C5''), 71.0 (C3'), 66.4 (C5'), 36.8 (5'-OMs), 11.1 (2'-CH3). HRMS (ESI) m/z 371.0528 ([M + Na]+, C12H16N2O8S·Na+ calc. 371.0520). Compound 6b; Rf = 0.18 (5% MeOH/EtOAc v/v). 1H NMR (DMSO-d6) δ (ppm) 11.45 (s, 1H, NH), 7.60 (d, J = 8.2 Hz, 1H, H6), 5.66 (d, J = 8.2 Hz, 1H, H5), 5.37 (s, 1H, 2'-OH), 4.78 (d, J = 8.1 Hz, 1H, H5''), 4.66 (s, 1H, H3'), 4.63 (d, J = 4.4 Hz, 2H, H5'), 4.48 (d, J = 8.2 Hz, 1H, H5'''), 3.27 (s, 3H, 5'-OMs), 0.98 (s, 3H, 2'-CH3). 13C NMR (DMSO-d6) δ (ppm) 162.8 (C4), 150.5 (C2), 141.0 (C6), 101.1 (C5), 88.9 (C1'), 88.2 (C3'), 80.8 (C4'), 76.4 (C5'''), 76.3 (C2'), 68.5 (C5'), 36.8 (5'-OMs), 18.5 (2'-CH3). HRMS (ESI) m/z 371.0518 ([M + Na]+, C12H16N2O8S·Na+ calc. 371.0520).

2'-C-Methyl-2'-O,4'-C-methyleneuridine (7a)

5'-O-Mesyl-2'-O,4'-C-methylene-2'-C-methyluridine (6a, 270 mg prepared and isolated as stated above) was added to a flame-dried 100 mL RB and stored under high vacuum overnight. The RB was evacuated and filled with argon gas several times, and anhydrous DMF (17 mL) and NaOBz (223 mg, 1.55 mmol) were added. The resulting mixture was heated under stirring at 100 °C overnight resulting in a pale yellow clear solution which subsequently was cooled to r.t., evaporated to dryness under reduced pressure and then co-evaporated under reduced pressure with toluene (2 × 5 mL) resulting in a pale yellow amorphous solid. This solid was dissolved in EtOAc (70 mL), washed with H2O (3 × 15 mL) and evaporated to dryness under reduced
pressure to give a beige solid which was dissolved in a mixture of THF and H₂O (1:1; 5.5 mL). 2 M NaOH (1.35 mL, 2.71 mmol) was added and the resulting mixture was stirred at r.t. for 3 h. Concentrated AcOH (0.33 mL) was added, and the resulting mixture was evaporated to dryness under reduced pressure and co-evaporated under reduced pressure with toluene (2 × 5 mL) resulting in a beige solid that was mixed with celite and then purified by silica gel column chromatography (2% → 10% MeOH/DCM v/v). The resulting product (193 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) was isolated as a white foam. \( R_f = 0.25 \) (10% MeOH/DCM v/v). \(^1\)H NMR (DMSO-d<sub>6</sub>) \( \delta \) (ppm) 11.37 (s, 1H, NH), 7.74 (d, \( J = 8.2 \) Hz, 1H, H6), 5.69 (bs, 1H, 3'-OH), 5.66–5.63 (m, 2H, H1', H5), 5.14 (bs, 1H, 5'-OH), 3.83 (d, \( J = 7.8 \) Hz, 1H, H5''), 3.81–3.69 (m, 2H, H5''), 3.68 (d, \( J = 3.9 \) Hz, 1H, H3'), 3.63 (d, \( J = 7.7 \) Hz, 1H, H5''), 1.11 (s, 3H, 2'-CH₃). \(^{13}\)C NMR (DMSO-d<sub>6</sub>) \( \delta \) (ppm) 162.9 (C4), 150.5 (C2), 139.4 (C6), 101.2 (C5), 89.3 (C4'), 87.8 (C1'), 85.5 (C2'), 71.4 (C5''), 70.0 (C3'), 56.4 (C5'), 11.4 (2'-CH₃). HRMS (ESI) \( m/z \) 293.0748 ([M + Na<sup>+</sup>], C<sub>11</sub>H<sub>14</sub>N<sub>2</sub>O<sub>6</sub>·Na<sup>+</sup> calc. 293.0744).

5'-O-(4,4'-Dimethoxytrityl)-2'-C-methyl-2'-O,4'-C-methyleneuridine (8a)

2'-C-Methyl-2'-O,4'-C-methyleneuridine (7a, 140 mg, prepared and isolated as stated above) was added to a flame-dried 25 mL RB and stored under high vacuum overnight. The RB was evacuated and filled with argon gas several times whereupon anhydrous pyridine (8 mL), DMTrCl (211 mg, 0.624 mmol) and DMAP (6.1 mg, 0.05 mmol) were added resulting in a yellow-orange solution which was stirred for 24 h at r.t. followed by evaporated to dryness under reduced pressure. The resulting yellow oil was dissolved in DCM (50 mL), washed with H₂O (3 × 15 mL), dried (MgSO₄), filtered and evaporated to dryness under reduced pressure. The resulting yellow solid was subjected to flash chromatography on NEt₃-neutralized silica gel (1% → 10% MeOH/DCM v/v) to furnish the product (238.2 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) as a white foam. \( R_f = 0.52 \) (10% MeOH/DCM v/v). \(^1\)H NMR (DMSO-d<sub>6</sub>) \( \delta \) (ppm) 11.42 (s, 1H, NH), 7.81 (d, \( J = 8.2 \) Hz, 1H, H6), 7.43–7.31 (m, 4H, DMTr), 7.31–7.22 (m, 5H, DMTr), 6.95–6.89 (m, 4H, DMTr), 5.77 (d, \( J = 5.5 \) Hz, 1H, 3'-OH), 5.71 (s, 1H, H1'), 5.45 (d, \( J = 8.1 \) Hz, 1H, H5), 3.87 (d, \( J = 5.5 \) Hz, 1H, H3'), 3.79 (d, \( J = 7.9 \) Hz, 1H, H5''), 3.77–3.69 (m, 7H, DMTr-OMe, H5''), 3.52 (d, \( J = 6.0 \) Hz, 1H, H5'), 3.28 (d, \( J = 10.9 \) Hz, 1H, H5'), 1.13 (s, 3H, 2'-CH₃). \(^{13}\)C NMR (DMSO-d<sub>6</sub>) \( \delta \)
(ppm) 162.8 (C4), 158.2 (DMTr), 150.4 (C2), 144.6 (DMTr), 139.2 (C6), 135.3, 135, 129.7, 129.7, 127.9, 127.6, 126.8, 113.2 (DMTr), 101.1 (C5), 88 (C1’), 87.7 (DMTr), 85.7 (C4’), 85.4 (C2’), 71.7 (C5’), 70.6 (C3’), 59.2 (C2’), 55.0 (DMTr-OMe), 11.3 (2’GCH3). HRMS (ESI) m/z 595.2049 ([M + Na]+, C32H32N2O8·Na+ calc. 595.2051).

3’-O-[2-Cyanoethoxy(diisopropylamino)phosphino]-5’-O-(4,4’-dimethoxytrityl)-2′-O,4′-C-methyleneuridine (9a)

5’-O-(4,4’-Dimethoxytrityl)-2’-C-methyl-2′-O,4′-C-methyleneuridine (8a, 122.3 mg, prepared and isolated as stated above) was added to a flame-dried 25 mL RB and stored under high vacuum for several days. The RB was evacuated and filled with argon gas several times before diisopropylamine tetrazolide (73.0 mg, 0.473 mmol), anhydrous DCM (6 mL) and 2-cyanoethyl-N,N,N’,N’-tetraisopropylphosphorodiamidite (0.1 mL, 0.32 mmol) were added resulting in a clear solution. The reaction mixture was stirred at r.t. overnight whereupon two drops of 99.9% EtOH was added. The resulting mixture was evaporated to dryness under reduced pressure to give a white solid which was subjected to flash chromatography on NEt3-neutralized silica gel using deoxygenated eluents (0% → 2.5% MeOH/DCM v/v). The product (143.1 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) was isolated as an off-white foam. Rf = 0.4 (3% MeOH/DCM v/v). 31P NMR (CD3CN) δ (ppm) 149.48, 148.69. HRMS (ESI) m/z 795.3093 ([M + Na]+, C32H32N2O8·Na+ calc. 795.3129).

2’-C-Methyl-3’-O,4′-C-methyleneuridine (7b)

5’-O-Mesyl-3’-O,4′-C-methylene-2′-C-methyluridine (6b, 146 mg, prepared and isolated as stated above) was added to a flame-dried 100 mL RB and stored under high vacuum overnight. The RB was filled and evacuated several times with argon gas whereupon anhydrous DMF (13 mL) and NaOBz (121 mg, 0.84 mmol) were added. The resulting mixture was heated at 100 °C overnight under stirring resulting in an orange-yellow solution with white precipitation. The mixture was cooled to r.t., evaporated under reduced pressure and co-evaporated under reduced pressure with toluene (2 × 5 mL) resulting in pale yellow solid. This solid was dissolved in EtOAc (70 mL), washed with H2O (3 × 15 mL) and evaporated to dryness under reduced pressure to give an orange oil which was dissolved in a mixture of THF and H2O (1:1; 4 mL). 2 M NaOH (0.73 mL, 1.47 mmol) was added and stirring was continued at r.t. for 3 h.
Concentrated AcOH (0.2 mL) was added and the resulting mixture was evaporated to dryness under reduced pressure and co-evaporated under reduced pressure with toluene (2 × 5 mL) to give a beige solid which was mixed with celite and then subjected to silica gel column chromatography (2%→10% MeOH/DCM v/v) to give the product (92.3 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) as a white foam. Rf = 0.14 (10% MeOH/DCM v/v). 1H NMR (DMSO-d6) δ (ppm) 11.42 (s, 1H, NH), 7.70 (d, J = 8.2 Hz, 1H, H6), 6.41 (s, 1H, H1'), 5.63 (d, J = 8.1 Hz, 2H, H5), 5.30–5.00 (bs, 2H, 2'-OH, 5'-OH), 4.68 (d, J = 7.7 Hz, 1H, H5''), 4.57 (s, 1H, H3'), 4.37 (d, J = 7.7 Hz, 1H, H5''), 3.74–3.59 (m, 2H, H5'), 0.95 (s, 3H, 2'-CH3). 13C NMR (DMSO-d6) δ (ppm) 162.9 (C4), 150.5 (C6), 141.4 (C2), 100.7 (C5), 89.6 (C3'), 88.2 (C1'), 84.1 (C4'), 76.8 (C2'), 76.5 (C5'), 60.6 (C5'), 18.6 (2'-CH3). HRMS (ESI) m/z 293.0747 ([M + Na]+, C11H14N2O6·Na+ calc. 293.0744).

5'-O-(4,4'-Dimethoxytrityl)-2'-C-methyl-3'-O,4'-C-methyleneuridine (8b)

2'-C-Methyl-3'-O,4'-C-methyleneuridine (7b, 93 mg, prepared and isolated as stated above) was added to a flame-dried 25 mL RB and stored under high vacuum overnight. The RB was evacuated and filled with argon several times whereupon anhydrous pyridine (8 mL), DMTrCl (139 mg, 0.41 mmol) and DMAP (5.1 mg, 0.034 mmol) were added. The resulting mixture was stirred at r.t. for 24 h and then evaporated to dryness under reduced pressure. The residue was subjected to flash chromatography on NEt3-neutralized silica gel (0%→10% MeOH/DCM v/v) to furnish the product (152.4 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) as a white foam. Rf = 0.52 (10% MeOH/DCM v/v). 1H NMR (DMSO-d6) δ (ppm) 11.40 (s, 1H, NH), 7.49 (d, J = 8.1 Hz, 1H, H6'), 7.42–7.30 (m, 4H, DMTr), 7.30–7.21 (m, 5H, DMTr), 6.92 (d, J = 8.4 Hz, 4H, DMTr), 6.41 (s, 1H, H1'), 5.61 (d, J = 8.1 Hz, 1H, H5), 5.27 (s, 1H, 2'-OH), 4.68 (d, J = 7.9 Hz, 1H, H5''), 4.50 (s, 1H, H3'), 4.42 (d, J = 7.8 Hz, 1H, H5''), 3.75 (s, 6H, 2 × OMe), 3.39 (s, 2H, H5'), 0.91 (s, 3H, 2'-CH3). 13C NMR (DMSO-d6) δ (ppm) 162.7 (C4), 158.1 (DMTr), 150.4 (C2), 144.4 (DMTr), 140.7 (C4), 135.1, 129.7, 127.9, 127.6, 126.8, 113.2 (DMTr), 100.9 (C5), 89.4 (C3'), 88.10 (C1'), 88.08 (DMTr), 85.9 (C4'), 82.6 (C2'), 76.6 (C5'), 62.5 (C5') 55.0 (DMTr-OMe), 18.9 (2'-CH3). HRMS (ESI) m/z 595.2028 ([M + Na]+, C32H32N2O8·Na+ calc. 595.2051).
3′-O-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-O-(4,4′-dimethoxytrityl)-3′-O,4′-C-methyleneuridine (9b)

5′-O-(4,4′-Dimethoxytrityl)-2′-C-methyl-3′-O,4′-C-methyleneuridine (8b, 150 mg, prepared and isolated as stated above) was added to a flame-dried 25 mL RB and stored under high vacuum overnight. The RB was evacuated and filled with argon gas several times before diisopropylamine tetrazolide (94 mg, 0.55 mmol), anhydrous DCM (8 mL) and 2-cyanoethyl-N,N,N′,N′-tetraisopropylphosphordiamidite (0.17 mL, 0.52 mmol) were added. The resulting mixture was stirred at r.t. overnight followed by addition of two drops of 99.9% EtOH. The resulting mixture was evaporated to dryness under reduced pressure to give a white solid which was subjected to flash chromatography on NEt₃-neutralized silica gel using de-oxygenated eluents (0%→2.5% MeOH/DCM v/v) to furnish the product (134.4 mg, relative yield not calculated due to the presence of minor impurities as evidenced by NMR analysis) as a white foam. \( R_f = 0.38 \) (3% MeOH/DCM v/v). \(^{31}P\) NMR (CD₂Cl₂) \( \delta \) (ppm) 144.60, 144.37. HRMS (ESI) \( m/z \) 795.3104 ([M + Na\(^+\)], C\(_{32}\)H\(_{32}\)N\(_2\)O\(_8\)·Na\(^+\) calc. 795.3129).

5′-O-Mesyl-2′-O,4′-C-methylene-5-methyluridine (11)

2′-O,4′-C-Methylene-5-methyluridine (10, 100 mg, 0.37 mmol) was added to a flame-dried 10 mL RB and stored under high vacuum overnight. The RB was evacuated and filled with argon gas several times, whereupon anhydrous pyridine (2 mL) was added and the resulting solution was cooled to 0 °C under an atmosphere of argon. Methanesulfonyl chloride (0.03 mL, 0.39 mmol) was added dropwise resulting in a clear mixture which under stirring was allowed to warm to r.t. After continued stirring for 2 h at r.t. the resulting reaction mixture was evaporated to dryness under reduced pressure. The residue was co-evaporated under reduced pressure with toluene (2 × 1 mL) resulting in a white solid which was purified by silica gel column chromatography (0→10% MeOH/DCM v/v) to give compound 11 as a white solid (115 mg, relative yield not calculated due to the presence of pyridine as an impurity as evidenced by NMR analysis). \( R_f = 0.36 \) (10% MeOH/DCM v/v). \(^1H\) NMR (DMSO-d\(_6\)) \( \delta \) (ppm) 11.38 (s, 1H, NH), 7.51 (s, 1H, H6), 5.98 (bs, 1H, 3′-OH), 5.48 (s, 1H, H1′), 4.79 (d, J = 11.9 Hz, 1H, H5′), 4.59 (d, \( J = 11.9 \) Hz, 1H, H5″), 4.21 (s, 1H, H2″), 3.98 (s, 1H, H3′), 3.94 (d, J = 8.0 Hz, 1H, H5′′), 3.77 (d, J = 8.0 Hz, 1H, H5′′′), 3.28 (s, 3H, 5′-OMs), 1.79 (s, 3H, 5-CH₃). \(^13C\) NMR (DMSO-d\(_6\)) \( \delta \)
Oligonucleotide synthesis, purification and analysis

Oligonucleotide synthesis was carried out on a PerSeptive Biosystems expedite 8909 automated DNA/RNA synthesizer in 1.0 µmol scale on polystyrene support material using the phosphoramidite approach and following manufacturer’s standard protocols. The coupling time for standard nucleotide phosphoramidites was 144s; stepwise coupling efficiencies were determined by the absorbance of the liberated trityl cation at 495 nm on a UV-VIS spectrophotometer and were in all cases >98.0%. LNA-T, 2’-C-Me-LNA-U (9a) and 2’-C-Me-ONa-U (9b) phosphoramidite monomers (0.1 M in anhydrous acetonitrile) were incorporated via hand-coupling\textsuperscript{17} using 5-[3,5-bis(trifluoromethyl)phenyl]-H-tetrazole (0.25 M, in anhydrous acetonitrile) as activator and extended coupling time (16 min), resulting in stepwise coupling yields of >95% for LNA-T and >80% for 2’-C-Me-LNA-U and 2’-C-Me-ONa-U. Following the coupling of the 2’-Me-LNA-U phosphoramidite, capping was performed following by oxidation using aqueous iodine. Following the coupling of the 2’-Me-CONa-U phosphoramidite, oxidation was performed using (1S)-(+)-(10-camphorsulfanyl)oxaziridine (CSO, 0.5 M in acetonitrile, 6 min) followed by capping. Cleavage from solid support and removal of nucleobase protecting groups were performed using 28% aqueous ammonia overnight at 55 °C. See Fig. S1 for details. The resulting oligonucleotides were purified by DMT-ON RP-HPLC using a Waters System 600 equipped with a Waters XBridge BEH C18-column (5 µm, 100 mm × 19 mm). Elution was performed starting with an isocratic hold of A-buffer for 5 min followed by a linear gradient to 70% B-buffer over 16.5 min at a flow rate of 5.0 mL/min (A-buffer: 0.05 M triethylammonium acetate in Milli-Q water, pH 7.4; B-buffer: 25% A-buffer, 75% acetonitrile). After removal of all solvents under a flow of nitrogen, oligonucleotides were detritylated using an 80% aqueous solution of acetic acid for 20 min whereupon desalting was performed by addition of an aqueous solution of sodium acetate (3 M, 15 µL) and sodium perchlorate (5 M, 15 µL) followed by addition of cold acetone (1 mL). The resulting suspension was stored at -20 °C. 

(ppm) 163.7 (C4), 149.9 (C2), 134.4 (C6), 108.7 (C5), 86.5 (C1‘), 85.5 (C4‘), 79.0 (C2‘), 70.7 (C5’'), 69.5 (C3‘), 65.8 (C5‘), 36.9 (5’-OMs), 12.1 (5-CH3). HRMS (ESI) m/z 349.0718 ([M + H\textsuperscript{+}], C\textsubscript{12}H\textsubscript{17}N\textsubscript{2}O\textsubscript{8}S calc. 349.0700).
overnight. After centrifugation (13200 rpm, 5 min, 4 °C), the supernatant was removed and the pellet further washed with cold acetone (3 × 1 mL), dried for 10 min, and dissolved in Milli-Q water (1.0 mL); aliquots were taken, evaporated and dissolved in a medium salt buffer (6.1 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0). Mass spectra of oligonucleotides were recorded on a Bruker Daltonics Microflex LT MAIDI-TOF MS instrument in the ES+ mode. Analytical IEGHPLC data were recorded on a Merck-Hitachi Lachrom system equipped with a DNA Pac PA100 analytical column (13 µm, 250 mm × 4 mm) heated to 60 °C. Elution was performed with an isocratic hold of buffer B (10 %), starting from two min hold on 2% Buffer A in Milli-Q water (solvent A), followed by a linear gradient to 30% buffer A in 23 min at a flow rate of 1.1 mL/min (buffer A: 1.0 M sodium perchlorate; buffer B: 0.25 M Tris-Cl, pH 8.0; solvent A: Milli-Q water). Concentrations of purified oligonucleotides were determined by UV absorption at 260 nm.

<table>
<thead>
<tr>
<th>ON#</th>
<th>Sequence</th>
<th>Calc. m/z</th>
<th>Found m/z</th>
<th>HPLC purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON1</td>
<td>5'-GTG-ATA-TGC-3'</td>
<td>2753.8</td>
<td>2753.8</td>
<td>96.5%</td>
</tr>
<tr>
<td>ON2</td>
<td>5'-GTG-AXA-TGC-3'</td>
<td>2781.8</td>
<td>2781.9</td>
<td>99%</td>
</tr>
<tr>
<td>ON3</td>
<td>5'-GTG-AT4A-TGC-3'</td>
<td>2781.9</td>
<td>2781.9</td>
<td>93.1%</td>
</tr>
<tr>
<td>ON4</td>
<td>5'-GT2G-ATA-T2G-C3'</td>
<td>2809.9</td>
<td>2809.9</td>
<td>80%</td>
</tr>
<tr>
<td>ON5</td>
<td>5'-GT2G-AXA-T2G-C3'</td>
<td>2837.8</td>
<td>2837.9</td>
<td>99%</td>
</tr>
<tr>
<td>ON6</td>
<td>5'-GT2G-AT4A-T4G-C3'</td>
<td>2837.8</td>
<td>2837.9</td>
<td>97.2%</td>
</tr>
<tr>
<td>ON7</td>
<td>5'-GTG-AYA-TGC-3'</td>
<td>2781.9</td>
<td>2781.9</td>
<td>84.6%</td>
</tr>
<tr>
<td>ON8</td>
<td>5'-GYG-AYA-YGC-3'</td>
<td>2837.9</td>
<td>2837.9</td>
<td>93.1%</td>
</tr>
<tr>
<td>ON9</td>
<td>5'-GT2G-AYA-T2G-C3'</td>
<td>2837.9</td>
<td>2837.9</td>
<td>98.1%</td>
</tr>
<tr>
<td>ON10</td>
<td>Comp. DNA</td>
<td>2682.6</td>
<td>2682.8</td>
<td>91.8%</td>
</tr>
<tr>
<td>ON11</td>
<td>Comp. RNA</td>
<td>2798.8</td>
<td>2798.8</td>
<td>93.1%</td>
</tr>
</tbody>
</table>

UV duplex melting studies
To determine duplex melting temperatures \( (T_m) \), UV melting studies were carried out on a Perkin Elmer Lambda 35 UV/Vis Spectrometer using Hellma SUPRASIL synthetic quartz 10 mm path length cuvettes, monitoring at 260 nm with a complementary DNA/RNA strand concentration of each strand of 1.5 µM and a volume of 1.0 mL. Samples were prepared as follows: All oligonucleotide sequences were dissolved in medium salt buffer (100 mM sodium chloride, 6.1 mM sodium phosphates, 0.1 mM EDTA and at pH 7.0); the modified sequences and complementary strands were mixed in a 1:1 ratio and medium salt buffer was added to give a total volume of 1.0 mL. The samples were transferred to cuvettes, denatured by heating to 80 °C and cooling to 5 °C followed by recording UV absorbance as a function of increasing temperature from 5 °C at a rate of 1.0 °C pr. minute programmed by a Peltier temperature controller. Two separate melting curves were measured and \( T_m \)-values were calculated using UV-WinLab Software, taking an average of the two melting curves with a deviation no more than 0.5 °C.

Crystallisation procedure for single crystal diffraction study

Crystals were grown in the following manner; 10 mg of product was placed in a glass vial and dissolved in acetone (3 mL). This vial was placed in beaker containing 20 mL petroleum ether. A lid was added to the beaker which was placed in a 5 °C refrigerator for slow diffusion of solvents and consequent crystallization. The crystals obtained had a white flaky appearance.

Crystal data on (6a) and (11)

Data for both crystals were collected at 100(1)K on a Synergy, Dualflex, AtlasS2 diffractometer using CuKα radiation \((\lambda = 1.54184 \text{ Å})\) and the CrysAlis PRO 1.171.39.12b suite. Using SHELXLE the structure was solved by dual space methods (SHELXT) and refined on \( F^2 \) using all the reflections (SHELXL-2016). All the non-hydrogen atoms were refined using anisotropic atomic displacement parameters. Hydrogen atoms bonded to carbon were inserted at calculated positions using a riding model; those bonded to oxygen or nitrogen were located from difference maps and their coordinates refined. For compound 11, the methylsulfonate group of one molecule (C) was disordered, this was modelled as 56:44% occupancy of two conformations related by rotation about the C6C – S1C bond. Part of the pyrimidine group of molecule B was
also disordered; since this section is close to the disordered methylsulfonate it was modelled with the same 56:44% occupancy of two sites. In both cases, the absolute configuration was unambiguously established from the crystallographic data. Parameters for data collection and refinement are summarised in Table 4.

Table 4. Crystal Data

<table>
<thead>
<tr>
<th></th>
<th>6a</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula</td>
<td>C_{12}H_{16}N_{2}O_{8}S</td>
<td>C_{12}H_{16}N_{2}O_{8}S</td>
</tr>
<tr>
<td>M_r</td>
<td>348.33</td>
<td>348.33</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Tetragonal</td>
<td>Triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P4_2_2</td>
<td>P1</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.54184</td>
<td>1.54184</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100(1)</td>
<td>100(1)</td>
</tr>
<tr>
<td>Unit cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>8.7910 (1)</td>
<td>9.0609 (3)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>8.7910 (1)</td>
<td>10.3679 (2)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>38.3254 (8)</td>
<td>16.4856 (4)</td>
</tr>
<tr>
<td>α (°)</td>
<td>90</td>
<td>82.494 (2)</td>
</tr>
<tr>
<td>β (°)</td>
<td>90</td>
<td>85.478 (2)</td>
</tr>
<tr>
<td>γ (°)</td>
<td>90</td>
<td>74.878 (2)</td>
</tr>
<tr>
<td>V (Å³)</td>
<td>2961.85 (9)</td>
<td>1480.64 (7)</td>
</tr>
<tr>
<td>Z</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>μ (mm⁻¹)</td>
<td>2.39</td>
<td>2.39</td>
</tr>
<tr>
<td>Measured refl.</td>
<td>11649</td>
<td>32404</td>
</tr>
<tr>
<td>Independent refl. R_int</td>
<td>3045, 0.036</td>
<td>10353, 0.051</td>
</tr>
<tr>
<td>Observed refl [I &gt; 2σ(I)]</td>
<td>2807</td>
<td>9332</td>
</tr>
<tr>
<td>R1 [F² &gt; 2σ(F²)]</td>
<td>0.047</td>
<td>0.054</td>
</tr>
<tr>
<td>wR(F²), S</td>
<td>0.115, 1.04</td>
<td>0.146, 1.05</td>
</tr>
<tr>
<td>Refl/restraints/parameters</td>
<td>3045/0/210</td>
<td>10353/82/916</td>
</tr>
<tr>
<td>Δl_{max}, Δl_{min} (e Å⁻³)</td>
<td>0.38, -0.40</td>
<td>0.55, -0.43</td>
</tr>
<tr>
<td>Absolute structure parameter</td>
<td>0.009(12)</td>
<td>-0.005(18)</td>
</tr>
</tbody>
</table>
CCDC 1579074 and 1579075 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Conflicts of interest
There are no conflicts of interest to declare.

Acknowledgements
We thank the WILLUM Foundation for funding the Biomolecular Nanoscale Engineering Center (BioNEC), grant number VKR022710. We are grateful to the Carlsberg Foundation grant CF15-0675 for funding the X-ray diffractometer, and to the University of Southern Denmark/Danish Council for Independent Research|Natural Sciences (grant 4181-00329) for a visiting professorship (to VMcK).

Notes and references


We have demonstrated the synthesis of ONs containing 2’-Me-locked nucleotide analogous (X) and (Y), and examined their biophysical properties.