

Gapmer Antisense Oligonucleotides Containing 2',3'-Dideoxy-2'-fluoro-3'-C-hydroxymethyl- β -d-lyxofuranosyl Nucleotides Display Site-Specific RNase H Cleavage and Induce Gene Silencing

Danielsen, Mathias B.; Lou, Chenguang; Lisowiec-Wachnicka, Jolanta; Pasternak, Anna; Jørgensen, Per T.; Wengel, Jesper

Published in:
Chemistry - A European Journal

DOI (link to publication from Publisher):
[10.1002/chem.201904540](https://doi.org/10.1002/chem.201904540)

Publication date:
2020

Document Version
Accepted author manuscript, peer reviewed version

[Link to publication from Aalborg University](#)

Citation for published version (APA):

Danielsen, M. B., Lou, C., Lisowiec-Wachnicka, J., Pasternak, A., Jørgensen, P. T., & Wengel, J. (2020). Gapmer Antisense Oligonucleotides Containing 2',3'-Dideoxy-2'-fluoro-3'-C-hydroxymethyl- β -d-lyxofuranosyl Nucleotides Display Site-Specific RNase H Cleavage and Induce Gene Silencing. *Chemistry - A European Journal*, 26(6), 1368-1379. <https://doi.org/10.1002/chem.201904540>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -

Take down policy

If you believe that this document breaches copyright please contact us at vbn@aub.aau.dk providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from vbn.aau.dk on: December 11, 2025

Author Manuscript

Title: Gapmer antisense oligonucleotides containing 2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxofuranosyl nucleotides display site-specific RNase H cleavage and induce gene silencing.

Authors: Mathias B. Danielsen; Chenguang Lou; Jolanta Lisowiec-Wachnicka; Anna Pasternak; Per T. Jørgensen; Jesper Wengel, Prof.

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record.

To be cited as: 10.1002/chem.201904540

Link to VoR: <https://doi.org/10.1002/chem.201904540>

Gapmer antisense oligonucleotides containing 2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxofuranosyl nucleotides display site-specific RNase H cleavage and induce gene silencing

Mathias B. Danielsen^[a], Chenguang Lou^[a], Jolanta Lisowiec-Wachnicka^[b], Anna Pasternak^[b], Per T. Jørgensen^[a] and Jesper Wengel^{*[a]}

Abstract: Off-target effects remains a significant challenge in the therapeutic use of gapmer antisense oligonucleotides (AONs). Over the years various modifications has been synthesized and incorporated into AONs, however precise control of RNase H induced cleavage and target sequence selectivity has yet to be realized. Herein, synthesis of the uracil and cytosine derivatives of a novel class of 2'-deoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxo-configured nucleotides has been accomplished and incorporated into AONs. Experiments on exonuclease degradation showed improved nucleolytic stability relative to the unmodified control. Upon the introduction of one or two of the novel 2'-fluoro-3'-C-hydroxymethyl nucleotides as modifications in the gap region of a gapmer AON was associated with efficient RNase H mediated cleavage of the RNA strand of the corresponding AON:RNA duplex. Notably, a tailored single cleavage event could be engineered depending on the positioning of a single modification. The effect of single mismatched base pairs was scanned along the full gap region demonstrating that the modification enables a remarkable specificity of RNase H cleavage. A cell-based model system was used to demonstrate the potential of gapmer AONs containing the novel modification to mediate gene silencing.

Introduction

In the past few decades, extensive research has been invested on antisense oligonucleotides (AONs) resulting in several AONs being approved by the FDA. For ASOs, recruitment of RNase H has proved to be a successful strategy to induce cleavage of the target RNA-strand of an AON:RNA heteroduplex.^[1] Many chemical modifications have been introduced into AONs to address the long-lasting challenges including biostability, targeting specificity and delivery.^[2-5] However, some nucleotide modifications that increase the binding affinity of AONs towards

RNA targets have been reported to impede the recruitment of RNase H.^[1] For instance, incorporation of modified nucleotides with a C3'-endo ribose-ring conformation, such as locked nucleic acid (LNA) or O2'-alkylated-RNA nucleotides, significantly reduce or halt RNase H recruitment.^[1,6,7] This can be circumvented using so-called antisense gapmers having high-affinity nucleotides towards the two terminal regions flanking a central contiguous stretch of DNA nucleotides ("the gap"),^[8,9] a design which most frequently has been explored as oligonucleotides containing phosphorothioate linkages throughout (all-PS).

Concerns have risen with respect to the hepatotoxicity/cytotoxicity of gapmers due to off-target effects, especially for those having too strong binding affinity.^[10-13] To minimize non-target recognition, an approach like to one reported for LNA all-PS gapmers, namely in silico methods for sequence optimization, has shown promise as a strategy to minimize off-target effects.^[12] In addition, exploitation of nucleoside modifications that can be combined with common affinity-enhancing monomers like 2'-O-methyl-RNA (2'-OMe-RNA), 2'-O-methoxyethyl-RNA (2'-O-MOE-RNA), 2'-O-constrained ethyl (cEt) residues and LNA^[14] has been pursued to address the issue. Such nucleosides designed for incorporation into the gap-region of antisense gapmers need to be compatible with RNase H activity. Furthermore, they ideally should improve the sequence specificity and resistance to nucleolytic degradation and mediate high-affinity RNA-targeting.

One study aiming for allele selectivity has shown that fluorinated nucleotides such as 2'-fluoro-hexitol nucleic acid (FHNA), positioned as T6 in an AON-gapmer (3-9-3 design), induce RNase H cleavage of the complementary mutated huntingtin (mtHTT) mRNA. However, changing A to G in the target to give the wild-type HTT mRNA completely blocked RNase H1 activity.^[15] Gapmer ASOs containing 2'-MOE-RNA with an all PS backbone constitute the most widely investigated class of nucleic acids therapeutics in the clinic.^[16] LNA and constrained ethyl (cEt) have been used instead of MOE in gapmer PS-AONs to improve duplex stability.^[17] However, incidences of hepatotoxicity, also for ASOs containing 2'-MOE, LNA and cEt, is an issue that needs to be taking into consideration when designing gapmer PS-AONs.^[11,13,18-20]

Recently it has been reported that modification with 2'-OMe-RNA at gap position 2 reduced protein binding substantially leading to a decrease in hepatotoxicity while only having a small impact on antisense activity of gapmer PS-AONs.^[16] A 3'-deoxy-3'-C-hydroxymethyl nucleotide with *threo*-configuration (Figure 1, A) has previously been introduced as a DNA mimic which had

[a] M. B. Danielsen, Dr. C. Lou, P. T. Jørgensen, Prof. J. Wengel
Biomolecular Nanoscale Engineering Center, Department of
Physics, Chemistry and Pharmacy
University of Southern Denmark, Odense M, Campusvej 55, 5230,
(Denmark)
E-mail: jwe@sdu.dk

[b] J. Lisowiec-Wachnicka, Prof. A. Pasternak
Institute of Bioorganic Chemistry, Polish Academy of Sciences, Z.
Noskowskiego 12/14, 61-704 Poznań, (Poland)

Supporting information and the ORCID identification number(s) for
the author(s) of this article can be found under:

improved resistance towards snake venom phosphodiesterase and encouraging duplex stability.^[21] It was thought that the hydroxymethyl group is adopting an equatorial position and the pentofuranose ring thus adopting a DNA-mimicking C2'-*endo* conformation.

Similarly, for the 2'-deoxy-2'-fluoro- β -D-arabinonucleic acid (3'-F-ANA) nucleotide, the gauche effect induced by the electronegative 2'-fluoro substituent constrains the furanose ring of this nucleotide in a DNA-mimicking O4'-*endo* conformation^[22] (Figure 1, B). Besides the ability of FANA to recruit RNase H, enhanced affinity for RNA has also been reported.^[22] In an attempt of combining the effects of nucleotides A and B (Figure 1), we have designed the novel nucleotide C (Figure 1) having a 2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxofuranosyl constitution. In the present study, we describe the synthesis of the uracil and cytosine monomers and analyze their properties as gap-region modifications with a particular focus on the aspects of the specificity of RNA-recognition and RNase H mediated RNA-cleavage. The long-term perspective is to contribute to design of ASOs with reduced toxicities and off-target effects.

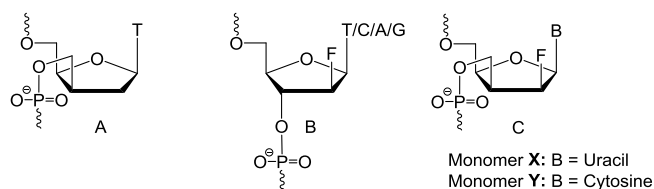


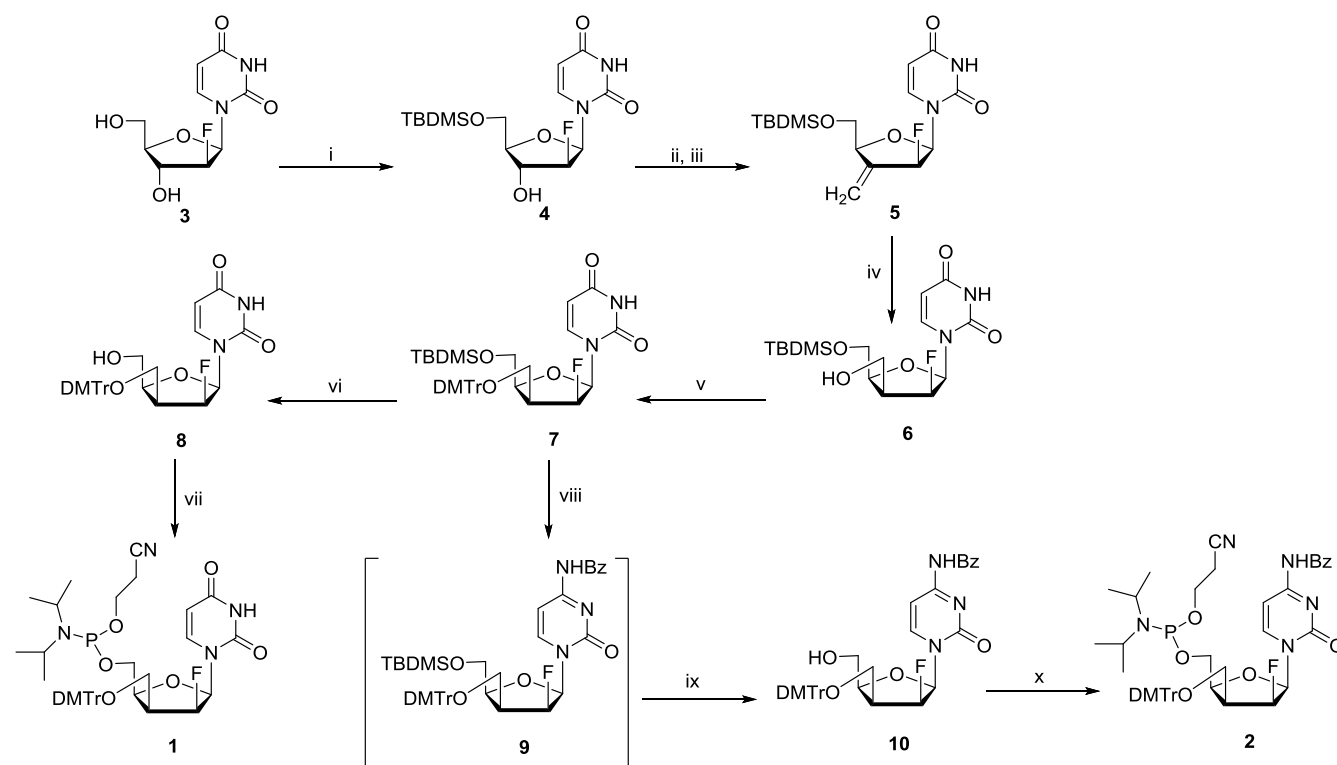
Figure 1. Caption Structure of DNA-mimicking nucleotides A, B (FANA) and C, with the two new hybrid monomers X and Y explored in the present study

Results and Discussion

Synthesis of compound **1** was accomplished in six steps starting from the commercially available nucleoside 1-(2'-deoxy-2'-fluoro- β -D-arabinofuranosyl)uracil (**3**, Scheme 1). Compound **4** and the corresponding 3'-oxy derivative have been reported earlier,^[25] whereas, to the best of our knowledge, only the β -L-*erythro*- and the β -L-*threo* and not the β -D-*threo* pentofuranose configuration of compound **5** (Scheme 1) has been reported.^[26] The primary hydroxy group of compound **3** was reacted with *tert*-butyldimethylsilyl chloride in anhydrous DMF with imidazole as the basic activator to give the O5'-protected nucleoside **4** in 73% yield. The secondary alcohol of **4** was then oxidized with the Dess-Martin periodinane reagent in *tert*-butanol and dichloromethane (DCM) to furnish the corresponding keto-nucleoside as a suitable intermediate for the following methylation reaction with Nysted reagent and titanium tetrachloride in anhydrous tetrahydrofuran (THF) and DCM. The 3'-methylene nucleoside **5** was isolated with 40% yield (from compound **4**). Hydroboration of compound **5** was done with borane dimethyl sulfide ($\text{BH}_3 \cdot \text{Me}_2\text{S}$) in anhydrous THF at 0 °C for five days whereupon the intermediate was oxidized with sodium-perborate tetrahydrate ($\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$) in THF/MeOH/H₂O (5/2/3; v/v/v), producing the key nucleoside **6** in 24% yield. The newly formed hydroxy group on nucleoside **6**

was then protected using 4,4'-dimethoxytrityl chloride (DMTCl) in anhydrous pyridine resulting in only 27% yield of nucleoside **7**, most likely due to steric effects hindering straight-forward protection of the hydroxymethyl functionality positioned to the same face of the furanose ring as the remaining three substituents. The yield was improved significantly by using 2,6-lutidine as a base instead of pyridine and silver triflate as activator producing nucleoside **7** in 95% yield. The configuration of compound **7** was confirmed by a NOESY experiment by observing a coupling between the H3' and H1' protons (see Supporting Information, NOESY). Desilylation of **7** was achieved using tetrabutylammonium fluoride (TBAF) in anhydrous THF to give nucleoside **8** in 72% yield. Eventually, **8** was phosphitylated using 2-cyanoethyl *N,N,N',N'*-tetraisopropyl-phosphordiamidite diisopropylammonium tetrazolide to furnish the desired phosphoramidite building block **1** (75% yield) to be used on an automated DNA-synthesizer. To obtain the corresponding cytosine building block **2** (Scheme 1), nucleoside **7** was first reacted with 1,2,4-triazole and POCl_3 in anhydrous acetonitrile and trimethylamine and then with 28-30% aq. ammonia in 1,4-dioxane. As a next step, benzoyl protection of the primary amine in anhydrous pyridine gave nucleoside **9** which was used without further purification. As the next step desilylation as described above furnished nucleoside **10** (51% yield from **7**). Nucleoside **10** was converted in 85% yield into the desired phosphoramidite building block **2**, as described for synthesis of compound **1**. Nysted's olefination using DMT as a protecting group on the 5'-position was unsuccessful. Attempts with TBDMS as the protecting group on the 5'-position resulted in two consecutive steps as mentioned above (**4** to **6**) with a low yield. It was then determined to keep the silyl group on for ease of synthesis and to introduce compound **1** and **2** as inverted phosphoramidites. The conformation of the nucleoside seems to point towards the eastern-type. Since the H1' is split in to a dd close to 21.1 Hz and 2.6 Hz corresponding to $J(\text{H1}'\text{F})$ and $J(1',2')$ respectively take from compound **10**. Similar observations have been made for the 2'-F-ANA with J-couplings of $\text{H1}' = (\text{dd}, J_{1',2'} = 4 \text{ Hz}; J_{\text{H1}',\text{F}} = 17 \text{ Hz})$.^[22]

ON synthesis was carried out using solid-phase phosphoramidite chemistry on an automated DNA synthesizer.^[2] Due to the fact that compounds **1** and **2** were obtained as O5'-phosphoramidite derivatives instead of standard O3'-phosphoramidite derivatives, inverted oligonucleotide syntheses (in a 5' to 3' direction) were carried out using inverted commercially available so-called inverted phosphoramidites. Compound **1** and **2** were introduced via a hand-coupling procedure.^[27] The stepwise coupling efficiency for **1** and **2** was above 97% and of unmodified DNA or commercially available inverted 2'-OMe-RNA phosphoramidites above 98%. The synthesized ONs were purified by ion-exchange HPLC and their composition confirmed by MALDI-TOF MS and their purity (>90%) by analytical ion-exchange HPLC analysis after purification.



Scheme 1. Reagents and conditions: (i) TBDMSCl, DMF, imidazole, 0 °C → RT, 4 h, 73%; (ii) Dess-Martin periodinane, t-BuOH, DCM, RT, 2.5 h; (iii) Nysted reagent, THF, TiCl₄, -78 °C → RT, 17.5 h, 40%; (iv) a) BH₃·Me₂S, THF, 0 °C, 125 h; b) NaBO₃·4H₂O, THF/MeOH/H₂O (5/2/3; v/v/v), RT, 21 h, 24%; (v) DMTrCl, silver triflate, 2,6-lutidine, CH₂Cl₂, RT, 48 h, 95%; (vi) tetra-*n*-butylammonium fluoride, anhydrous THF, RT, 19.5 h, 72%; (vii) anhydrous CH₂Cl₂, diisopropylamine tetrazolide, 2-cyanoethyl N,N,N',N'-tetraisopropyl-phosphorodiamidite, RT, 24 h (for compound **1**), 75% and 23 h (for compound **2**), 85%; (viii) a) anhydrous acetonitrile, POCl₃, triethylamine, 1,2,4-triazole, 6 h; b) dioxane, 28–30% aq. ammonia, 6 h; c) anhydrous pyridine, benzoyl chloride, 2 h; (ix) anhydrous THF, tetra-*n*-butylammonium fluoride, RT, 21 h, overall yield 51% (from **7** to **10**).

Thermal denaturation studies

A 9-mer DNA-sequence^[6,28,29] was chosen for the preliminary evaluation of the binding affinity effects of monomer **X** (Figure 1) containing ONs (Table 1). A single incorporation of 1-(2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl-β-D-lyxofuranosyl)uracil monomer **X** in the middle of a 9-mer DNA sequence (**ON2**), showed a preference for binding to its RNA complement ($\Delta T_m = +2$ °C) relative to its DNA complement ($\Delta T_m = -1$ °C). The increase in T_m for monomer **X** was however, not cumulative since three incorporations (**ON3**) induced a ΔT_m of -4 °C for the DNA/RNA duplex and -11 °C for the DNA/DNA duplex. The stabilizing effect against the RNA complement is similar to that observed for 2'-F-ANA.^[22] Compared to 3'-deoxy-3'-C-threo-hydroxymethylthymidine (Figure 1A), monomer **X** seems to have similar stability against DNA^[21] but with improved stability against complement RNA.^[1] Notably, modification **X** shows improved selectivity against a mismatch in the 9-mer relative to the strand carrying a thymidine (Table S2; Figure S4-S5). The thermal stability assessment for the 16-mer (containing **ON4-ON7**) and 18-mer (containing **ON8-ON10**) gapmer sequences, containing monomers **X** and **Y** respectively, revealed a modest effect only, and essentially unchanged T_m values against RNA.

The 16-mer sequence (5'-UCCGtcatcgcctCCUC) was chosen based on a former paper assessing the ability of unlocked nucleic acid (UNA) and 4'-C-hydroxymethyl DNA nucleotides to recruit RNase H in LNA-gapmers.^[8] The 18-mer ON (UGCAAgccttagccCAUUAU) was chosen as a biological active sequence in the cell assay performed in this study. Introducing monomer **X** in the middle of the 16-mer sequence (**ON5**) resulted in a slightly decreased thermal stabilities ($\Delta T_m = -2$ °C against DNA; $\Delta T_m = -0.5$ °C against RNA relative to the reference duplex **ON4**). Changing the two thymidines near the flanks for the modification **X** (**ON6**) led to an insignificant drop in T_m of -0.5 °C against both DNA and RNA duplexes relative to the centrally modified **ON5**. When all three thymidines in the gap region were exchanged with monomer **X** (**ON7**), notably the destabilization towards RNA was remarkably small ($\Delta T_m = -1.5$ °C). A single insertion of 1-(2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl-β-D-lyxofuranosyl)cytosine monomer **Y** in the biological active 5-8-5 gapmer sequence (**ON9**) gave similar hybridization properties as incorporation of monomer **X** (**ON9** compared to **ON5**). Two incorporations of monomer **Y** in the gap caused a destabilizing effect ($\Delta T_m = -2.5$ °C) for the DNA/DNA

duplex and only -1°C ($\Delta T_m = -0.5^{\circ}\text{C}/\text{modification}$) for the DNA/RNA heteroduplex (**ON10**). An all-DNA reference strand was also tested together with a scrambled gapmer sequence which were used in the transfection assay. The all-DNA (**ON11**)

had improved DNA binding with poor RNA binding relative to the reference (**ON8**), while the scrambled sequence (**ON12**) did not show any hybridization.

Table 1. Thermal denaturation temperatures of duplexes^a

Sequence (5'-3')		Complementary DNA ($^{\circ}\text{C}$)	Complementary RNA ($^{\circ}\text{C}$)
gtgatatgc	ON1	31.5	29.5
gtga X atgc	ON2	30.5 (-1)	31.5 (+2)
g X ga XaX gc	ON3	20.5 (-11)	25.5 (-4)
UCCgtcatcgctCCUC	ON4	60.5	73.0
UCCgtca X cgcctCCUC	ON5	58.5 (-2)	72.5 (-0.5)
UCCg X catgcg X CCUC	ON6	58.0 (-2.5)	72.0 (-1)
UCCg X ca X cgc X CCUC	ON7	55.5 (-5)	71.5 (-1.5)
UGCAAgcctagccCAUUAU	ON8	60.5	72.0
UGCAAgcctag Y cCAUUAU	ON9	60.0 (-0.5)	72.0 (0.0)
UGCAAgc Y tag Y cCAUUAU	ON10	57.5 (-2.5)	71.0 (-1.0)
tgcaagcctagcccatat	ON11	64.5 (+4.5)	67.0 (-5.0)
AUCCAcgacctcgAAUUG	ON12	<10.0	<10.0

^a) T_m values ($^{\circ}\text{C}$) of unmodified and modified [**X**, **Y** (Figure 1c)] DNA–DNA, DNA–RNA, gapmer–DNA and gapmer RNA duplexes measured as an average of two independent melting temperature determinations with a deviation $<0.5^{\circ}\text{C}$. Numbers in brackets are ΔT_m measured as the difference in T_m values between modified and unmodified duplexes. The experiments were carried out at pH 7.0 in medium salt buffer (5.8 mM NaH_2PO_4 – Na_2HPO_4 buffer, containing 100 mM NaCl and 0.10 mM EDTA). Complementary sequences (for **ON1–ON3** [DNA = 5'-gcatatcac, RNA = 5'-gcauauacac]; for **ON4–ON7** [DNA = 5'-gaggagcgatgacgga, RNA = 5'-gaggagcgatgacgga]; for **ON8–ON12** [DNA = 5'-atatgggctaggcttga, RNA = 5'-auaugggcuaggcuugca]; g,t,c,a = DNA monomers, U,C,A,G = 2'-O-methyl-RNA monomers, nd = not determined. **ON1**, **ON4** and **ON8** are reference duplexes with respect to T_m values. Oligo sequences. Oligo sequences and curves can be found in supporting information (Table S1, S3 and S4; Figure S3, S10 and S14).

It is clear from the thermal melting studies that both monomers **X** and **Y** have very similar tendencies, that one to three incorporations do not disrupt the duplex structures, that RNA binding is better than DNA binding, and that the binding towards RNA with one to three modifications in the gap region of 2'-O-methyl gapmers is essentially the same as for the gapmers with an all-DNA gap.

Enzymatic stability

Previous work had established that the 2'-fluoro modification such as that on the 2'-F-ANA, increases the stability of an ON against endo- and exonucleases.^[30,31] In order to evaluate the effects of the novel class of 2'-deoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxo-configured nucleotides in this respect,

the stability of 5'-³²P labeled **ON1** and **ON2** was studied upon incubation with snake venom phosphodiesterase (SVPDE; 3'-exonuclease), using a procedure that has been modified prior to this work.^[32] Figure 2 shows that the unmodified **ON1** rapidly degrades and that no

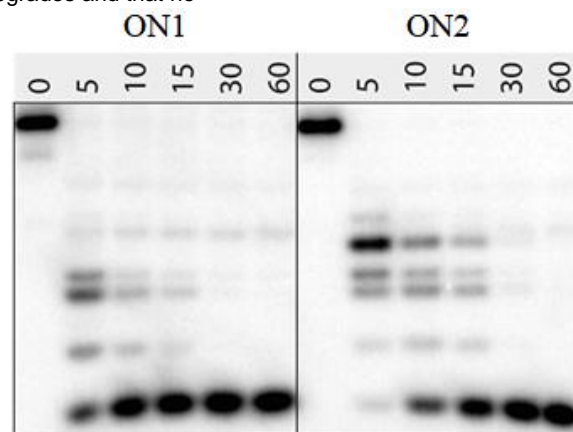


Figure 2. 20% PAGE denaturing gel showing the time-course of SVPDE-mediated degradation of 5'-³²P-GTGA1ATGC (**ON1**, 1 = T), and (**ON2**, 1 = monomer **X**), pH = 8.0 at 21 °C. Samples were incubated for 5, 10, 15, 30 and 60 min. The reference point (0 min) was taken before the enzyme was added. The gel was visualized by autoradiography. trace of the full ON was detectable after 5 min incubation. **ON2** containing monomer **X** showed rapid degradation at the 5-mer, 5'-GTGAX which was detectable up to 15 min after incubation but fully degraded at 30 and 60 min. A reduced of the 3'-exonuclease sequential degradation activity of the oligomer was observed when **ON3** was evaluated relative to **ON2** (supporting information; S8). These results indicate that ONs modified with monomer **X** have increased 3'-exonucleolytic resistance compared to the unmodified ONs which constitute a further impetus to evaluate its potential for antisense applications.

Cleavage by RNase H

The ability of 2'-O-Me gapmers **ON4-ON7** to induce RNase H cleavage of the corresponding duplexes formed with the complementary RNA sequence 5'-r(GAGGAGCGAUGACGGA)-3' (Target-RNA₁₆; which was [³²P]-labelled at its 5'-end) was studied. For determining cleavage points, an RNA ladder was included (using SVPDE; see Supporting Information, Target-RNA₁₆). The reference gapmer **ON4** showed three cleavage points resulting in three shortened RNA strands (5'-GAGGAGCGAUGAC; 5'-GAGGAGCGA; 5'-GAGGAGCG; all RNA fragments shown in this section are tentatively assigned based on gel mobility). Exchanging thymidine with monomer **X** centrally in the gap-region (**ON5**) caused the RNase H to only cleave close to the modification. Interestingly, displacement of the two thymidine residues located next to the two flanking regions by monomer **X** (**ON6**) shifted the cleavage pattern thus only allowing the RNA to be cut in the middle and near the 3'-end. Finally, **ON7** with three monomer **X** insertions resulted in only a single cut in the middle of the RNA-strand, leading to the 5'-r(GAGGAGCGA) to be the only cleaved [5'-³²P]-product present. The overall cleavage efficiencies with **ON4-ON7** were comparable (Figure 3).

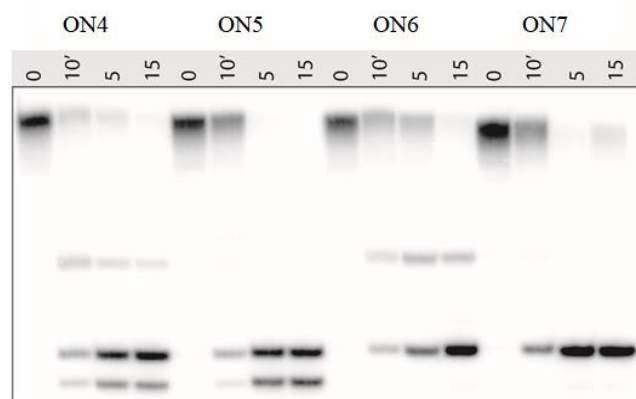


Figure 3. 20% PAGE denaturing gel showing the time-course of RNase H cleavage of 5'-³²P-r(GAGGAGCGAUGACGGA)-3' with complementary

sequence **ON4** (control) and **ON5**, **ON6** and **ON7** with one, two and three insertions of monomer **X**, respectively; pH = 7.8 at 37 °C. Samples were incubated for 10 seconds, 5 minutes and 15 minutes. The reference point (0 min) was taken before the enzyme was added.

Next, the cytosine monomer **Y** was investigated to further understand the diverse cleaving pattern induced in the RNase H experiment. The targeted RNA strand ([³²P]-5'-r(AUAUGGGCUAGGCUUGCA); Target-RNA₁₈) was cleaved at three different positions resulting in three cleavage products (5'-r(AUAUGGGCUAGG); 5'-r(AUAUGGGCUAG); 5'-r(AUAUGGGCU)). The unmodified gapmer **ON8** gave a primary RNA cleavage product (5'-AUAUGGGCU) with secondary cleavage products barely visible. Introducing the monomer **Y** near the 3'-end in the gap-region (**ON9**) induced a somewhat different cleaving pattern as two preferred cleavage products appeared (5'-r(AUAUGGGCUAGG); 5'-r(AUAUGGGCUAG)), with the 12-mer being the major one. Introducing monomer **X** in the gap-region (**ON10**) caused the RNA strand to be selectively cleaved leading to the strand 5'-r(AUAUGGGCUAG) as being the only product (Figure 4).

All things considered, the novel 2'-deoxy-2'-fluoro-3'-C-hydroxymethyl-β-D-lyxo-configured nucleotides allows RNase H to recognize the DNA-gap in the AON gapmer, when incorporated in the DNA region and efficiently cleaves the target RNA. Previously it has been shown that nucleotide conformation and orientation of substituents play an important role for activity. The incorporation of a 3'-C-hydroxymethyl-arabino nucleotide, with a flexible S-type conformation, or a piperazino-functionalized-C3'-O2'-linked-arabino nucleotide, with a restricted E-type conformation enhanced cleavage activity. The orientation of substituents into the major groove, away from the minor groove where RNase H is known to bind in a DNA/RNA duplex, were argued as important for tolerability of the modified nucleotides in the initial binding region of RNase H.^[1] Combining the 2'-F-ANA modification with nucleotides that have reduced recognition for RNase H seems to be a successful strategy. However, previous work has established that combining the 2'-F-ANA modification with the tricyclo-ANA modification maintained the good binding properties and nuclease resistance while allowing for RNase H activation.^[5]

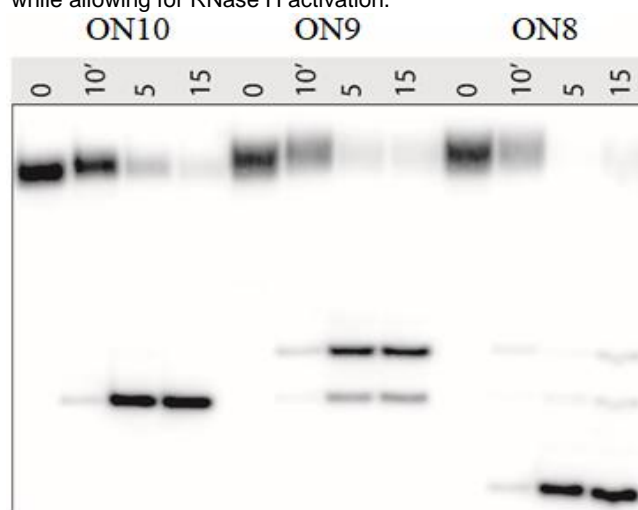


Figure 4. 20% PAGE denaturing gel showing the time-course of RNase H cleavage of 5'-³²P-r(AUAUGGGCUAGGCUUGCA)-3' with complementary sequence **ON8** (control) and **ON9** and **ON10** with one and two insertions of

monomer **Y**, respectively; pH 7.8 at 37 °C. Samples were incubated for 10 seconds, 5 minutes and 15 minutes. The reference point (0 min) was taken before the enzyme was added.

Selectivity of AON-gapmers should be of great interest for therapeutic AONs, since off-target effects caused by low specificity potentially lead to toxicity.^[10,11] This prompted us to investigate the mismatch discrimination of **ON10** with respect to RNase H cleavage to establish whether improved discrimination towards single nucleotide polymorphism (SNP) could be observed for a gap-region modified antisense gapmer relative to the reference gapmer with an all-DNA gap-region. By mutating one nucleobase at a time along the 8-mer DNA-based gap-region on the complementary RNA, resulting in eight singly-mismatched target RNA-sequences (**T1-T8**), the RNase H induced cleavage patterns were evaluated relative to the reference **ON8**. The results are found in Table 2.

Table 2: Cleavage patterns of double modified and reference gapmers against wild-type-RNA and eight mismatched RNA-sequences.

Target RNA	Cleavage pattern of reference gapmer ON8	Amount cleaved
T0	3'-A C G U U C↓G↓G A↓U C G G G U A U A-5'	++
T1	3'-A C G U U <u>G</u> ↓G↓G A↓U C G G G U A U A-5'	++
T2	3'-A C G U U C C↓G↓G A↓U C G G G U A U A-5'	++
T3	3'-A C G U U C↓G↓G A↓U C G G G U A U A-5'	+
T4	3'-A C G U U C↓G↓G A↓U C G G G U A U A-5'	+++
T5	3'-A C G U U C↓G↓G A↓A C G G G U A U A-5'	++
T6	3'-A C G U U C↓G↓G A↓U <u>A</u> G G G U A U A-5'	++
T7	3'-A C G U U C G↓G↓G A↓U C <u>C</u> G G U A U A-5'	+++
T8	3'-A C G U U C↓G↓G A↓U C G <u>C</u> G U A U A-5'	+++
5'-U G C A A g c c t a g c c C A U A U-3'		
Target RNA	Cleavage pattern of modified gapmer ON10	Amount cleaved
T0	3'-A C G U U C G↓G A U C G G G U A U A-5'	+++
T1	3'-A C G U U <u>G</u> ↓G A U C G G G U A U A-5'	++
T2	3'-A C G U U C C↓G A U C G G G U A U A-5'	++
T3	3'-A C G U U C G <u>C</u> A U C G G G U A U A-5'	
T4	3'-A C G U U C G G <u>G</u> U C G G G U A U A-5'	
T5	3'-A C G U U C G↓G A <u>A</u> C G G G U A U A-5'	++
T6	3'-A C G U U C G↓G A U <u>A</u> G G G U A U A-5'	++
T7	3'-A C G U U C G↓G A U C <u>C</u> G G U A U A-5'	++
T8	3'-A C G U U C G↓G A U C G <u>C</u> G U A U A-5'	+++
5'-U G C A A g c <u>Y</u> t a g <u>Y</u> c C A U A U-3'		

Key: + = weak cleavage; ++ = medium cleavage; +++ = complete cleavage of RNA sequence after 15 min incubation. ↓ = weak cleavage point; ↓↓ = strong cleavage point.

With the reference gapmer **ON8**, a mutation of C and G in **T1** and **T2**, resulted in a similar cleavage pattern and cleavage rate

as with the fully matched RNA target. Moving to RNA-**T3**, a change of cleavage rate and pattern was observed when G was exchanged with a C, reflecting that RNase H is hampered in cutting right at the mutation site. The **T4** strand gives an almost similar result but with a much higher amount of RNA cleavage. When the mismatch is placed after the major cleavage site, a complete change of cleavage pattern is observed. The minor cleavage point observed for **T3** and **T4** is now the major one (5'-r(AUAUGGGCUAGG)) for RNA **T5** and **T6**. The **T6** RNA strand has an adenosine mismatch which was chosen since the guanosine mismatch caused four consecutive guanosine residues to be placed right after each other, resulting in the likely formation of a quadruplex structure (see Supporting Information, S7B). It is noticeable that the **T6**-RNA strand only gave a single cleavage product. Finally, when the reference AON (**ON8**) is hybridized to **T7** and **T8**, the major cleavage point again is 5'-r(AUAUGGGCU).

Switching to the modified AON-gapmer (**ON10**) a different picture appears. Both **T1** and **T2** display the same cleavage pattern as the fully matched target **T0**, however with reduced cleavage efficiency. Interestingly, mutating G to C complementary to the modified **Y** (cytosine) nucleotide completely abolishes RNase H activity. Changing the neighbouring nucleobase from A to U has the same effect. This shows that the modified nucleotide has the ability to block RNase H recruitment if a single nucleotide is mutated directly opposite or adjacent to **Y** (**T3** and **T4**). Moving the mutation further away towards the 5'-end of the RNA (**T5-T7**) restores RNase H activity, still with less cleaved product compared to **T0**. Only the **T8** target induces same overall cleavage efficiently as the fully matched RNA target strand (**T0**). The RNase H mismatch experiments show that precise control has been realized down to site-specific cleavage of a target RNA, despite different mutations being introduced in the target RNA. This is combined with complete termination of enzymatic activity when a single mismatch (**T3** and **T4**) caused the AON-gapmer/RNA heteroduplex to be unrecognizable by the RNase H enzyme. It is further noticeable that all other single nucleoside mismatched target strands besides **T8** gave reduced cleavage rates, all relative to the reference gapmer **ON8**. Overall, when **ON10** was hybridized to the mismatched RNA strands (**T1-T8**) a single cleavage product was observed, but with reduce RNase H activity relative to the matched RNA strand. Still **T4** and **T3** showed complete RNase H termination. Cleavage products generated by a gapmer AON are processed in the body by the RNA surveillance mechanism (33). This mechanism is used to ensure elimination of unwanted RNA stands, from the transcription process, which prevents the build-up of defective proteins^[34,35] Whether site-specific cleavage is of importance for toxicity remains to be tested. However, it has been speculated that toxicity for some high-affinity LNA-single stranded ONs might be related to the generated RNA products, but it has not been confirmed.^[20]

ASO transfection assay

To determine whether the gapmer oligonucleotides with monomer **Y** had an antisense effect a study was carried out using a HeLa cell line. The experiment was carried out with the introduction of a plasmid and one of the tested 18-mer gapmers

(5-8-5 design; with DNA nucleotides in the gap-region flanked by 2'-O-methyl-RNA nucleotides at each end) to the cells. The plasmid encoded the ZsGreen1 fluorescent protein that had a cloned fragment which was complementary to the tested gapmers.

A gapmer at a concentration of 200 nM and 1 µg of plasmid was introduced into a determined amount of HeLa cells. Measurements of ZsGreen1 mRNA levels were done using the quantitative polymerase chain reaction (qPCR) technique and on this basis the activity of each individual gapmer as an expression silencing capability was determined. Three gapmers containing modified nucleotides were tested: (i) **ON10** that contained modified nucleotide at position 8 and 12, (ii) gapmer **ON13**, (see Supporting Information) with one modified nucleotide at position 8, and (iii) gapmer **ON9** with one modified nucleotide at position 12. In addition, three controls were used in the studies: the unmodified reference gapmer **ON8**, isosequential DNA oligomer (**ON11**), and **ON12** with the gapmer structure and scrambled sequence.

Quantitative results obtained by the qPCR method were normalized to the control which has been set to the expression value of 1. Values below 1 indicate silencing of the gene, whereas values above 1 mean that the expression of mRNA of ZsGreen1 protein is higher relative to the control. Comparison of the modified gapmers with **ON12** (scrambled gapmer sequence) as a control revealed that the gapmer having modified nucleotide **Y** at position 12 (**ON9**) has no silencing activity with a normalized ZsGreen mRNA expression level similar to the control (Figure 5A). **ON13** with nucleotide **Y** at position 8 caused the silencing of the gene expression by 19% relative to the control, however without any statistical significance. The most favourable result is observed for the gapmer carrying two modifications, one at position 8 and one at position 12 (**ON10**). Treatment of cells with this oligonucleotide resulted in a silencing of the gene expression by 66% relative to the control (**ON12**).

Given that the gapmer containing two modified nucleotides **Y** had significantly improved silencing capabilities over the scrambled sequence a study comparing the modified gapmers relative to the isosequential DNA (**ON11**) as a control was carried out (Figure 5B). As in the previous case, gapmers with one modification (**ON13** and **ON9**), silenced gene expression by the same value or even slightly less efficiently than the control (**ON11**). Notably, the gapmer containing two modified nucleotides **Y** resulted in an improved silencing activity mounting to 44% relative to the DNA-control. As improved gene silencing for the double modified gapmer were observed, relative to both the scrambled and the isosequential DNA control, a study with a gapmer containing the same sequence as control (**ON8**) was carried out together with the modified gapmers (Figure 5C). The results indicate that the presence of modified nucleotide at position 8 (**ON13**) in the gapmer does not change silencing potential significantly relative to the control, whereas the gapmer with the modification at position 12 (**ON9**) is less efficient in gene silencing. Interestingly, the gapmer with two modified nucleotides (**ON10**) is characterized by gene silencing enhanced by 27% compared to the control gapmer, demonstrating that the presence of two monomers **Y** is favourable for improvement of antisense potential of gapmers.

As toxicity could be the leading factor for the observed gene silencing of the tested sequences, an MTT assay was performed.

The cells were incubated with different concentrations of gapmers to assess cell viability. Based on the dose-response curves, the IC₅₀ was evaluated (Table 3).

Table 3. Half maximal inhibitory concentration IC₅₀ [nM] of oligonucleotides in HeLa cell line after 24 h of incubation.

IC ₅₀	ON8	ON9	ON10	ON11	ON12	ON13
[nM]	478.3	571.3	623.8	874.7	1416.5	394.8

In all cases, the IC₅₀ parameter was found to be much higher than the concentration used in the silencing activity experiments. The cellular studies show that monomer **Y** can be used in the design of molecular tools based on nucleic acids. Gapmers with a single **Y** modification as well as with two **Y** modifications in experimental conditions are not toxic to the HeLa cell line. Gapmers containing one modified residue **Y**, depending on positioning, might work at a similar level to the DNA antisense oligonucleotide with the same sequence but their silencing potential is comparable or decreased relative to the gapmer without **Y** modification. Interestingly, a molecule with two **Y** modifications is more efficient gene silencer by 44% compared to all-DNA ASO and by 27% in reference to isosequential gapmer containing no monomer **Y**.

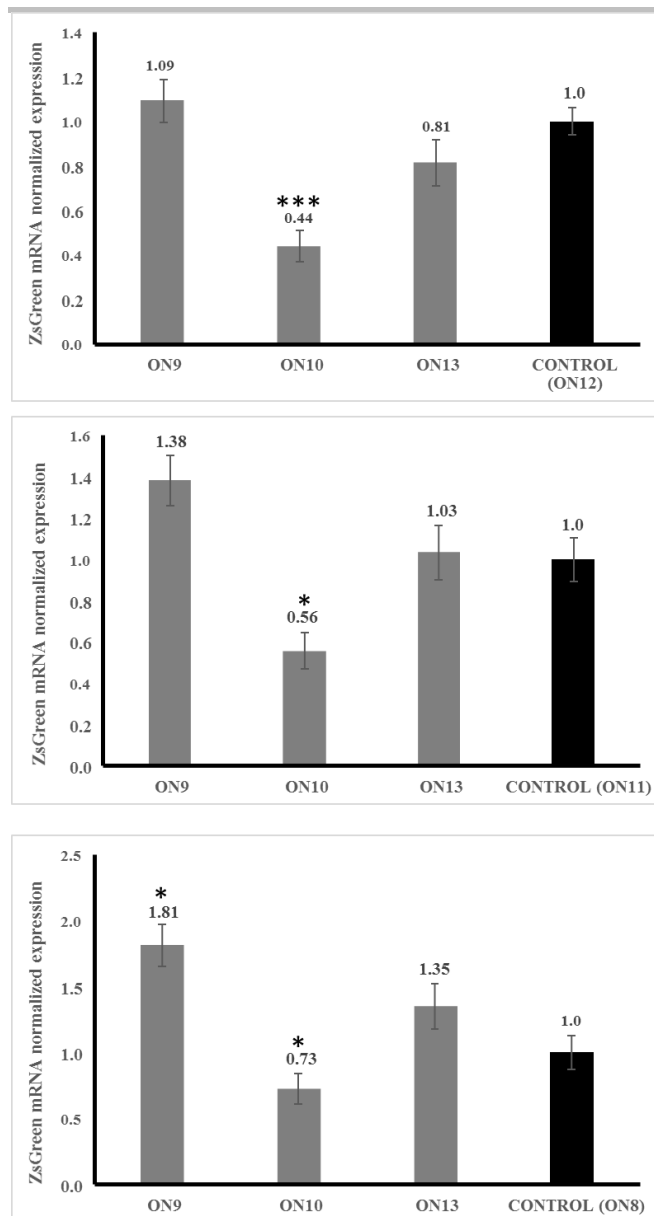


Figure 5. The relative level of ZsGreen1 mRNA normalized expression induced by antisense oligonucleotide: **ON9**, **ON10**, and **ON13**. A) ZsGreen1 mRNA normalized expression in reference to gapmer with scrambled sequence (**ON12**), B) ZsGreen1 mRNA relative normalized expression in reference to all-DNA antisense oligonucleotide with the same sequence (**ON11**), C) ZsGreen1 mRNA normalized expression in reference to gapmer without modified nucleotide **Y** (**ON8**). Each bar represents the mean value \pm standard error of the mean (SEM) of $n = 3-5$. *statistical significance at a level of $P < 0.05$; ***statistical significance at a level of $P < 0.01$.

Conclusion

Synthesis of the uracil and cytosine derivatives of the novel class of 2'-deoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxo-configured nucleotides has been accomplished. Both monomers had negligible effects on the binding affinity towards complementary RNA when substituting the corresponding DNA nucleotides. However, improved exonuclease stability was achieved, and RNase H experiments showed that 2'-deoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxo-configured nucleotides when incorporated in the gap-region of a gapmer antisense ON preserve the ability of RNase H recruitment and has potential for

engineering of precise cleavage control of the target RNA-strand. Thus, mismatch studies showed that when the target RNA was mutated through the gap-region, a single cleavage product was observed, with the complete termination of the enzymatic activity when a mismatched nucleobase was placed complementary or adjacent to the modification site. This observation is in sharp contrast to results with the reference gapmer, which allowed multiple cleavage products no matter where the mutation was positioned in the complementary RNA, and generally higher overall cleavage efficiencies than for the modified **ON10**. Finally, a significantly improved effect was observed in transfection mediated silencing assay of 44% comparing the double modified gapmer to isosequential DNA and 27% when the double modified gapmer was compared to the unmodified reference gapmer.

Experimental Section

General

All reagents used were purchased from Sigma-Aldrich or Fluka and were used without further purification. DNA phosphoramidite monomers, solid supports and additional reagents, however, were purchased from Sigma-Aldrich or Glen Research. Dichloromethane (CH_2Cl_2), tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF) and pyridine were dried over activated molecular sieves (3 Å, 8-12 mesh) and their water content determined on a Karl Fischer titrator (<15 ppm). All reactions were carried out under nitrogen or argon atmosphere using glassware that had been dried at 120°C overnight. Column chromatography was carried out under pressure using Merck Millipore silica gel 60 (0.040-0.063 mm). Thin layer chromatography (TLC) was performed using Merck Kieselgel 60 F254 (0.22 mm thickness, aluminium backed). Compounds were visualized at 254 nm or stained with 5% sulfuric acid in EtOH. ^1H -NMR spectra were measured at 400 MHz on a Bruker AVANCE III 400 spectrometer. ^{13}C -NMR spectra were measured at 101 MHz on the same spectrometer. Chemical shifts are given in ppm, and J values are given in Hz. All assignments for ^1H -NMR and ^{13}C -NMR have been confirmed using 2D correlation experiments (H-H COSY, HMQC and HMBC). ^{31}P -NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer at 162 MHz. DMSO- d_6 were used as solvents for NMR experiments. High resolution mass spectra were recorded in acetonitrile using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer. HPLC grade acetonitrile or methanol were used as the solvent.

1-[5'-O-(*tert*-Butyldimethylsilyl)-2'-deoxy-2'-fluoro- β -D-arabinofuranosyl]uracil (**4**)

To a solution of starting nucleoside **3** (2.00 g, 8.12 mmol) in anhydrous DMF (20 ml) was added *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (1.46 g, 9.74 mmol) and imidazole (1.66 g, 24.30 mmol) at 0°C . The reaction mixture was stirred for 4 h during which the reaction was allowed to reach room temperature. The reaction was quenched by addition of H_2O (40 ml), and the aqueous phase was extracted with Et_2O (3×50 ml). The combined organic phase was then washed with H_2O (3×30 ml), dried (Na_2SO_4), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (0-5% MeOH in CH_2Cl_2) to furnish nucleoside **4** as a white solid material (2.12 g, 73%). $R_f = 0.35$ (5% MeOH in CH_2Cl_2). ^1H NMR (400 MHz, DMSO- d_6): δ 11.47 (s, 1H, NH), 7.62 (dd, $J = 8.1, 1.3$ Hz, 1H, H6), 6.14 (dd, $J = 14.3, 4.6$ Hz, 1H, H1'), 5.95 (d, $J = 5.0$ Hz, 1H, 3'-OH), 5.60 (d, $J = 8.1$ Hz, 1H, H5), 5.08 (m, 1H, H2'), 4.23 (s, 1H, 3'H), 3.82 (m, 3H, H4', H5'), 0.88 (s, 9H, TBDMS), 0.08 (s, 6H, TBDMS). ^{13}C NMR (101 MHz, DMSO- d_6): δ 162.82 (C4), 150.05 (C2), 140.59 (C6), 101.09 (C5), 95.49 (d, $J = 191.6$ Hz, C2'), 82.60 (C4'), (82.28 (d, $J = 16.6$ Hz, C1'), 72.53 (d, $J = 24.0$ Hz, C3'), 61.55 (C5'), 25.68 (TBDMS), 17.95 (TBDMS), -5.52

(TBDMS). HRMS (ESI) m/z calc. for $C_{15}H_{25}FN_2NaO_5Si$ [M+Na]⁺ 383.1409, found 383.1404.

1-[5'-O-(*tert*-Butyldimethylsilyl)-2',3'-dideoxy-2'-fluoro-3'-methylene-β-D-threo-pentofuranosyl]uracil (5)

To a suspension of Dess–Martin periodinane (1.41 g, 3.33 mmol) in anhydrous CH_2Cl_2 (32 ml) was added *tert*-butyl alcohol (0.267 g, 3.61 mmol). The resulting mixture was stirred at room temperature under an argon atmosphere for 10 min whereupon compound 4 (1.00 g, 2.78 mmol) was added in a solution of anhydrous CH_2Cl_2 (25 ml) via a syringe. The reaction mixture was stirred for 2.5 h at room temperature before dilution with EtOAc (79 ml) and subsequent sequential addition of an aqueous solution of $Na_2S_2O_3$ (1.0 M 45 ml), brine (30 ml) and a saturated aqueous solution of $NaHCO_3$ (30 ml). The resulting biphasic mixture was stirred for 1 h followed by separation of the organic and aqueous phases. The aqueous phase was then extracted with EtOAc (60 ml) and the combined organic phase was dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure to give a white solid material which was vacuum-dried for 2 h. R_f = 0.59 (5% MeOH in CH_2Cl_2); HRMS (ESI) m/z calc. for $C_{15}H_{23}FN_2NaO_5Si$ [M+Na]⁺ 381.1252, found 381.2945. This material was dissolved in anhydrous CH_2Cl_2 (12 ml) and dropwise added to a suspension of the Nysted reagent (20% in THF, w/w, 5.38 ml, 3.50 mmol) in anhydrous THF (4.60 ml) at -78 °C. $TiCl_4$ (1 M, 2.80 ml) was then added dropwise at -78 °C and the resulting mixture was stirred for 2 h at -78 °C and subsequently allowed to slowly reach room temperature overnight. The white vapor formed inside the reaction flask during the addition of $TiCl_4$ was immediately removed by suction with a syringe while allowing argon inflow from a balloon filled with argon. Subsequently the reaction mixture was poured into a saturated aqueous solution of $NaHCO_3$ (150 ml), and the resulting suspension was stirred vigorously for 30 min before it was filtered through a short pad of celite on a glass filter. The phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (2 × 30 ml). The combined organic phase was washed with brine (100 ml), dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (50% EtOAc in petroleum ether) to yield nucleoside 5 as a white solid material (0.390 g, 40%). R_f = 0.50 (5% MeOH in CH_2Cl_2). ¹H NMR (400 MHz, DMSO- d_6): δ 11.49 (s, 1H, NH), 7.66 (dd, J = 8.1, 2.2 Hz, 1H, H6), 6.11 (dd, J = 17.0, 3.9 Hz, 1H, H1'), 5.73 (dd, J = 6.0, 2.0 Hz, 1H, 3'-CH₂(b)), 5.60 (dd, J = 8.1, 2.2 Hz, 1H, H5), 5.57–5.54 (m, 1H, 3'-CH₂(a)), 5.54 (m, 1H, H2'), 4.63–4.51 (m, 1H, H4'), 3.90–3.81 (m, 2H, H5'), 0.87 (s, 9H, TBDMS), 0.06 (d, J = 3.8 Hz, 6H, TBDMS). ¹³C NMR (101 MHz, DMSO- d_6): δ 162.84 (C4), 150.11 (C2), 142.73 (d, J = 11.7 Hz, C3'), 141.16 (C6), 117.13 (d, J = 8.4 Hz, 3'-CH₂), 100.83 (C5), 89.66 (d, J = 187.6 Hz, C2'), 83.26 (d, J = 15.8 Hz, C1'), 79.38 (C4'), 64.58 (C5'), 25.66 (TBDMS), 17.93 (TBDMS), -5.51 (d, J = 2.1 Hz, TBDMS). HRMS (ESI) m/z calc. for $C_{16}H_{25}FN_2NaO_4Si$ [M+Na]⁺ 379.1460, found 379.1441.

1-[5'-O-(*tert*-Butyldimethylsilyl)-2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl-β-D-lyxosefuranosyl]uracil (6)

Compound 5 (550.0 mg, 1.543 mmol) was dissolved in anhydrous THF (18.3 ml, 9.1 ppm) and the resulting mixture was cooled to 0 °C under an argon atmosphere. $(CH_3)_2S \cdot BH_3$ in THF (0.405 ml, 4.63 mmol) was then added dropwise at 0 °C via syringe before the reaction mixture was stirred for 125 h (~5 days). The reaction mixture was then concentrated under an argon atmosphere before sodium perborate tetrahydrate (4.270 mg, 27.77 mmol) and a solution of THF/methanol/H₂O (5/2/3; 21.5 ml/8.6 ml/12.2 ml) was added to the caramel like substance. The reaction mixture was stirred vigorously for 21 h before it was filtered through a glass funnel, diluted with EtOAc (100 ml) and washed with a saturated aqueous solution of $NaHCO_3$ (50 ml) followed by H₂O (50 ml). The organic layer was separated, dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (silica neutralized by triethylamine, 0–2% MeOH in CH_2Cl_2) to furnish compound 6 as a white solid (138.5 mg,

24%). R_f = 0.29 (5% MeOH in CH_2Cl_2). ¹H NMR (400 MHz, DMSO- d_6): δ: 11.45 (s, 1H, NH), 7.66 (dd, J = 8.1, 1.6 Hz, 1H, H6), 6.03 (dd, J = 20.9, 2.7 Hz, 1H, H1'), 5.58 (dd, J = 8.1, 2.1 Hz, 1H, H5), 5.25–5.00 (m, 1H, H2'), 4.82 (t, J = 4.9 Hz, 1H, 3'-OH), 4.37–4.23 (m, 1H, H4'), 3.78–3.54 (m, 4H, H5' and 3'-CH₂), 2.92 (m, 1H, H3'), 0.88 (s, 9H, TBDMS), 0.06 (s, 6H, TBDMS). ¹³C NMR (101 MHz, DMSO- d_6): δ: 162.99 (s, C4), 150.08 (s, C2), 140.73 (s, C6), 100.45 (s, C5), 91.14 (d, J = 190.1 Hz, C2'), 85.63 (d, J = 15.3 Hz, C1'), 79.91 (s, C4'), 62.65 (s, C5'), 54.70 (d, J = 8.1 Hz, 3'-CH₂), 46.41 (d, J = 18.1 Hz, C3'), 25.61 (s, TBDMS), 17.78 (s, TBDMS), -5.60 (d, J = 4.4 Hz, TBDMS). HRMS (ESI) m/z calc. for $C_{15}H_{27}FN_2NaO_5Si$ [M+Na]⁺ 397.1565, found 397.1577; m/z calc. for $C_{15}H_{28}FN_2O_5Si$ [M+H]⁺ 375.1746, found 375.1769.

1-[5'-O-(*tert*-Butyldimethylsilyl)-2',3'-dideoxy-3'-C-(4,4'-dimethoxytrityloxymethyl)-2'-fluoro-β-D-lyxosefuranosyl]uracil (7).

Method A: Compound 6 (138.0 mg, 0.224 mmol) was co-evaporated with anhydrous CH_2Cl_2 (10 ml) and vacuum-dried overnight. To a round bottomed flask containing compound 6, was added 4,4'-dimethoxytriphenylmethyl chloride (200.0 mg, 0.589 mmol), anhydrous pyridine (20 ml) and *N,N*-dimethylpyridin-4-amine (32.0 mg, 0.262 mmol) under an argon atmosphere. The reaction was stirred for 24 h at room temperature before the solution was concentrated under reduced pressure. The caramel like residue was dissolved in CH_2Cl_2 (50 ml) and the organic phase was washed with saturated aqueous solutions of $NaHCO_3$ (2 × 30 ml), brine (2 × 30 ml) and H₂O (40 ml). The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (silica neutralized by triethylamine, 0–2.5% MeOH in CH_2Cl_2) to furnish compound 7 as a white solid (67.7 mg, 27%). R_f = 0.57 (5% MeOH in CH_2Cl_2). HRMS (ESI) m/z calc. for $C_{37}H_{45}FN_2NaO_7Si$ [M+Na]⁺ 699.2872, found 699.2840.

Method B: Compound 6 (84.0 mg, 0.224 mmol) was co-evaporated with 1,2-dichloroethane (10 ml) and vacuum-dried overnight. To the round bottomed flask, containing compound 6, was added anhydrous CH_2Cl_2 (2.5 ml) and anhydrous 2,6-lutidine (2.5 ml) before 4,4'-dimethoxytriphenylmethyl chloride (133.8 mg, 0.395 mmol), and silver triflate (115 mg, 0.449 mmol; vacuum-dried at 100 °C overnight) were added. The solution turned deep red with a white precipitate forming immediately. The reaction mixture was magnetically stirred for 48 h under an argon atmosphere before the reaction was quenched with MeOH (0.5 ml). The reaction mixture was diluted with CH_2Cl_2 (20 ml) and filtered through a short pad of celite on a glass filter, and CH_2Cl_2 (15 ml) was washed through the glass filter afterwards. The organic phase was washed successively with a saturated aqueous solution of $NaHCO_3$ (2 × 20 ml) and brine (2 × 20 ml) before the solution was dried ($MgSO_4$), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (silica neutralized by triethylamine, 33% EtOAc in petroleum ether) to furnish compound 7 as a white foam (141.6 mg, 95%). R_f = 0.57 (5% MeOH in CH_2Cl_2). ¹H NMR (400 MHz, DMSO- d_6): δ: 11.47 (d, J = 1.9 Hz, 1H, NH), 7.59 (dd, J = 8.2, 1.9 Hz, 1H, H6), 7.41–7.36 (m, 2H, DMT), 7.30–7.19 (m, 8H, DMT), 6.90–6.83 (m, 4H, DMT), 6.10 (dd, J = 21.1, 2.6 Hz, 1H, H1'), 5.56 (dd, J = 8.1, 2.1 Hz, 1H, H5), 5.40–5.24 (m, 1H, H2'), 4.30–4.25 (m, 1H, H4'), 3.73 (d, J = 1.9 Hz, 6H, 2 × OMe), 3.54 (dd, J = 10.9, 5.6 Hz, 1H, H5'), 3.45–3.40 (m, 1H, H5'), 3.37 (d, J = 8.9 Hz, 1H, 3'-CH₂), 3.29–3.15 (m, 1H, H3'), 3.14–3.06 (m, 1H, 3'-CH₂), 0.72 (s, 9H, TBDMS), -0.11 (d, J = 7.8 Hz, 1H, TBDMS). ¹³C NMR (101 MHz, DMSO- d_6): δ: 162.94 (s, C4), 158.02 (s, DMT), 150.09 (s, C2), 144.75 (s, DMT), 140.72 (s, C6), [129.50 (s), 128.85 (s), 127.74 (s), 127.53 (d, J = 7.9 Hz), 127.33 (s), 126.61 (s), DMT], 112.69 (s, DMT), 100.54 (s, C5), 91.22 (d, J = 193.4 Hz, C2'), 85.42 (d, J = 13.1 Hz, C1'), 79.57 (d, J = 56.6 Hz, C4'), 62.49 (s, C5'), 56.77 (s, 3'-CH₂), 54.94 (s, DMT), 43.89 (s, C3'), 25.49 (s, TBDMS), 17.57 (s, TBDMS), -5.83 (s, TBDMS). HRMS (ESI) m/z calc. for $C_{37}H_{45}FN_2NaO_7Si$ [M+Na]⁺ 699.2872, found 699.2848.

1-[2',3'-Dideoxy-3'-C-(4,4'-dimethoxytrityloxymethyl)-2'-fluoro-β-D-lyxofuranosyl]uracil (8)

Compound 7 (180 mg, 0.266 mmol) was co-evaporated with anhydrous 1,2-dichloroethane (10 ml) and vacuum-dried for 2 h. The protected nucleoside was then dissolved in anhydrous THF (5 ml) and a solution of tetrabutylammonium fluoride in THF (1 M, 0.350 ml, 0.350 mmol) was added via syringe under an argon atmosphere. The reaction mixture was then stirred for 19.5 h at room temperature before it was concentrated to dryness under reduced pressure. The caramel like residue was dissolved in CH_2Cl_2 (50 ml) and washed with H_2O (3 × 30 ml). The organic phase was dried (MgSO_4), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (silica neutralized in triethylamine, 0–5 %, MeOH in CH_2Cl_2) to give compound 8 as a white foam (108 mg, 72%) after co-evaporation with CH_2Cl_2 in petroleum ether. $R_f = 0.37$ (5% MeOH in CH_2Cl_2). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 11.46 (s, 1H, NH), 7.66 (dd, $J = 8.2, 1.6$ Hz, 1H, H6), 7.41–7.36 (m, 2H, DMT), 7.34–7.21 (m, 7H, DMT), 6.94–6.84 (m, 4H, DMT), 6.07 (dd, $J = 21.2, 2.5$ Hz, 1H, H1'), 5.61 (dd, $J = 8.1$; 2.1 Hz, 1H, H5), 5.32 (m, $J = 54.7$ Hz, 1H, H2'), 4.80 (t, $J = 5.4$ Hz, 1H, 5'-OH), 4.28 (m, 1H, H4'), 3.74 (s, 6H, 2 × OMe), 3.34–3.08 (m, 4H, H5', 3'-CH₂, H3'), 3.06–2.99 (m, 1H, 3'-CH₂). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ : 163.08 (s, C4), 158.08 (s, DMT), 150.16 (s, C2), 144.75 (s, DMT), 140.88 (s, C6), 135.52 (s, DMT), 129.57 (s), 127.80 (s), 127.56 (s), 126.67 (s), 113.16 (s, DMT), 100.60 (s, C5), 91.41 (d, $J = 189.0$ Hz, C2'), 85.70 (d, $J = 28.0$ Hz, C1'), 80.43 (s, C4'), 61.12 (s, C5'), 56.71 (d, $J = 5.2$ Hz, 3'-CH₂), 54.99 (s, DMT), 43.93 (d, $J = 18.9$ Hz, C3'). HRMS (ESI) m/z calc. for $\text{C}_{31}\text{H}_{31}\text{FN}_2\text{NaO}_7$ [$\text{M}+\text{Na}$]⁺ 585.2008, found 585.1980.

1-{5'-O-[2-Cyanoethyl(diisopropylamino)phosphino]-2',3'-dideoxy-3'-C-(4,4'-dimethoxytrityloxymethyl)-2'-fluoro-β-D-lyxosefuranosyl]uracil (1)

Compound 8 (90.0 mg, 0.160 mmol) was stored in a round bottomed flask under vacuum for 2 days before diisopropylamine tetrazolide (66.0 mg, 0.385 mmol) and anhydrous CH_2Cl_2 (7.0 ml) was added. Subsequently, 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (0.0810 ml, 0.253 mmol) at room temperature was added under an argon atmosphere. After 24 h the reaction mixture was evaporated to dryness, resulting in a white solid. The residue was purified by flash column chromatography on silica gel (the silica gel was neutralized with 5% Et_3N in CH_2Cl_2 before use and eluents were purged with argon; 50% EtOAc in CH_2Cl_2) followed by precipitation in petroleum ether/ EtOAc (purged with argon) (60 ml/1.5 ml) to furnish compound 1 as a white foam (91.6 mg, 75%). $R_f = 0.12$ (50/50; v/v $\text{EtOAc}/\text{CH}_2\text{Cl}_2$). $R_f = 0.65$ (5% MeOH in CH_2Cl_2). ^{31}P NMR (162 MHz, CDCl_3) δ : 149.17, 148.65 ($J = 85.3$ Hz). HRMS (ESI) m/z calc. for $\text{C}_{40}\text{H}_{48}\text{FN}_4\text{NaO}_8\text{P}$ [$\text{M}+\text{Na}$]⁺ 785.3091, found 785.3086; m/z calc. for $\text{C}_{41}\text{H}_{48}\text{FN}_4\text{O}_8\text{P}$ [$\text{M}+\text{H}$]⁺ 763.3267, found 763.3237.

1-[2',3'-Dideoxy-3'-C-(4,4'-dimethoxytrityloxymethyl)-2'-fluoro-β-D-lyxosefuranosyl]-4-*N*-benzoylcytosine (10)

Compound 7 (180.0 mg; 0.266 mmol) was dissolved in anhydrous acetonitrile (5 ml) before 1,2,4-triazole (187 mg; 2.71 mmol) and POCl_3 (0.0600 ml; 0.588 mmol) were dissolved in anhydrous acetonitrile (5 ml) and added via syringe. The reaction mixture was cooled to 0 °C before anhydrous triethylamine (0.4 ml) was added, whereupon the reaction mixture was kept at room temperature for 6 h (the formed product turns blue under tUV-light at 254 nm). Triethylamine (0.5 ml) and H_2O (0.1 ml) were added and the reaction mixture was stirred for 10 min before the solvents was removed under reduced pressure. The residue was re-dissolved in EtOAc (50 ml) and washed successively with a saturated aqueous solution of NaHCO_3 (2 × 20 ml), and H_2O (20 ml). The combined aqueous phases were extracted with CH_2Cl_2 (3 × 20 ml) and the

combined organic phase was evaporated to dryness under reduced pressure and re-dissolved in 1,4-dioxane (5 ml) and 30% aqueous ammonia (0.8 ml). After stirring for 6 h the reaction mixture was evaporated to dryness under reduced pressure and vacuum-dried overnight. The residue was then co-evaporated with anhydrous 1,2-dichloroethane (5 ml) before it was re-dissolved in anhydrous pyridine (10 ml). Benzoyl chloride (0.190 ml, 1.60 mmol) was added and stirring was continued at room temperature for 2 h before the reaction was quenched by addition of H_2O (1 ml). The resulting mixture was evaporated to dryness under reduced pressure. The resulting residue was re-dissolved in EtOAc (50 ml) and washed with H_2O (3 × 40 ml). The aqueous phase was extracted with CH_2Cl_2 (20 ml) and the combined organic phase was evaporated to dryness and vacuum-dried overnight. The resulting residue as purified by flash column chromatography on silica gel (the silica gel was neutralized with 5% Et_3N in CH_2Cl_2 ; 0–0.5% MeOH in CH_2Cl_2) followed by precipitation in petroleum ether to yield a white foam (131.0 mg) which tentatively was assigned as compound 9 and used without further purification in the next step. $R_f = 0.64$ (5% MeOH in CH_2Cl_2). HRMS (ESI) m/z calc. for $\text{C}_{44}\text{H}_{50}\text{FN}_3\text{NaO}_7\text{Si}$ [$\text{M}+\text{Na}$]⁺ 802.3294, found 802.3116; m/z calc. for $\text{C}_{44}\text{H}_{51}\text{FN}_3\text{O}_7\text{Si}$ [$\text{M}+\text{H}$]⁺ 780.3475, found 780.3308.

Compound 9 (131.0 mg; 0.168 mmol) was vacuum-dried for 48 h before it was dissolved in anhydrous THF (15 ml). To the solution was added tetrabutylammonium fluoride in THF (1 M, 0.2 ml). After stirring for 21 h, the reaction mixture was evaporated to dryness under reduced pressure and the resulting residue re-dissolved in CH_2Cl_2 (60 ml). The organic phase was washed successively with brine (40 ml) and H_2O (2 × 40 ml), and the combined aqueous phase was extracted with CH_2Cl_2 (20 mL). The combined organic phase was dried (MgSO_4), filtered and evaporated to dryness under reduced pressure. The residue was purified by flash column chromatography on silica gel (the silica gel was neutralized with 4% Et_3N in CH_2Cl_2 ; 0–0.9% MeOH in CH_2Cl_2) and precipitated in petroleum ether to furnish compound 10 as a white foam (90.0 mg, 51%). $R_f = 0.44$ (5% MeOH in CH_2Cl_2). ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 11.30 (s, 1H, NH), 8.24 (d, $J = 7.5$ Hz, 1H, H6), 8.09–7.94 (m, 2H, H-Bz), 7.68–7.59 (m, 1H, H-Bz), 7.52 (t, $J = 7.6$ Hz, 2H, H-Bz), 7.43–7.20 (m, 10H, Ar-H of DMT and H5), 6.90 (d, $J = 8.5$ Hz, 4H, Ar-H of DMT), 6.11 (dd, $J = 20.1, 2.6$ Hz, 1H, H1'), 5.53 (m, 1H, H2'), 4.99–4.77 (m, 1H, 5'-OH), 4.36 (m, $J = 33.1, 16.5$ Hz, 1H, H4'), 3.74 (d, $J = 1.4$ Hz, 6H, 2 × OMe), 3.32–3.01 (m, 5H, H3', H5' and 3'-CH₂). ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ : 167.29 (s, CO(Bz)), 163.39 (s, C4), 158.08 (s, Ar-C of DMT), 154.20 (s, C2), 145.39 (s, C6), 144.74 (s, Ar-C of Bz), [135.51 (s), 135.34 (s), 133.09 (s), 132.68 (s), 129.57 (d, $J = 3.0$ Hz), 128.40 (d, $J = 3.5$ Hz), 127.81 (s), 127.56 (s), 126.68 (s), 113.17 (s) Ar-C of DMT and Bz], 95.58 (s, C5), 90.93 (d, $J = 189.6$ Hz, C2'), 87.95 (s, C1'), 85.58 (s, *tert* C of DMT), 81.18 (s, C4'), 61.27 (s, C5'), 56.67 (s, 3'-CH₂), 54.99 (s, 2 × OMe), 44.23 (d, $J = 17.4$ Hz, C3'). HRMS (ESI) m/z calc. for $\text{C}_{38}\text{H}_{36}\text{FN}_3\text{NaO}_7$ [$\text{M}+\text{Na}$]⁺ 688.2429, found 688.2397; m/z calc. for $\text{C}_{38}\text{H}_{37}\text{FN}_3\text{O}_7$ [$\text{M}+\text{H}$]⁺ 666.2610, found 666.2579.

1-{5'-O-[2-Cyanoethyl(diisopropylamino)phosphino]-2',3'-dideoxy-3'-C-(4,4'-dimethoxytrityloxymethyl)-2'-fluoro-β-D-lyxosefuranosyl]-4-*N*-benzoylcytosine (2)

Compound 10 (20.0 mg, 0.030 mmol) was co-evaporated in anhydrous CH_2Cl_2 (5 ml) and vacuum-dried overnight before it was re-dissolved in anhydrous 1,2-dichloroethane (5 ml) and the resulting mixture evaporated to dryness. The residue was dissolved in anhydrous CH_2Cl_2 (8 ml) before *N,N*-diisopropylamine tetrazolide (15.0 mg, 0.088 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite (0.02 ml, 0.047 mmol) were added. The resulting mixture was stirred at room temperature for 23 h before it was evaporated to dryness under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel (the silica gel was neutralized with 5% Et_3N in CH_2Cl_2 before use and eluents were purged with argon; 66% EtOAc in

petroleum ether) followed by precipitation in petroleum ether (purged with argon) to give compound 2 as a white solid (23.0 mg, 85%). $R_f = 0.69$ (5% MeOH in CH_2Cl_2). ^{31}P NMR (162 MHz, CD_3CN) δ 148.14 (s), 147.86 ($J = 88.8$ Hz). HRMS (ESI) m/z calc. for $\text{C}_{47}\text{H}_{53}\text{FN}_5\text{NaO}_8\text{P}$ $[\text{M}+\text{Na}]^+$ 888.3508, found 888.3520; m/z calc. for $\text{C}_{47}\text{H}_{54}\text{FN}_5\text{O}_8\text{P}$ $[\text{M}+\text{H}]^+$ 866.3689, found 866.3687.

Oligonucleotide synthesis

Oligonucleotide (ON) synthesis was carried out on a PerSeptive Biosystems expedite 8909 automated DNA/RNA synthesizer in 0.2 or 1.0 μmol scale using the phosphoramidite approach and following manufacturer's standard protocols. The coupling time for DNA monomers was 156 s and stepwise coupling efficiencies were determined as >97.5% by the absorbance of the dimethoxytrityl cation at 495 nm after each coupling step. For synthesis of ONs containing monomers X and Y, polystyrene Glenn Unysupport (Nr. 26-5040-10) and inverted DNA phosphoramidites were used. Phosphoramidites 1 and 2 were incorporated by a manual hand-coupling procedure^[23] using Activator 42[®] (0.25 M, 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole) as activator and extended coupling time (16 min), resulting in stepwise coupling yields of >97% for phosphoramidites 1 and 2. Cleavage from solid support and removal of nucleobase protecting groups were performed using 28% aqueous ammonia (12 h, RT). After evaporation of all solvents, detritylation was performed using an 80% aqueous solution of acetic acid (20 min, RT) which was followed first by desalting using an aqueous solution of sodium acetate (3 M, 15 μl) and sodium perchlorate (5 M, 15 μl) and then by addition of ice-cold (store at -20°C) acetone. The resulting suspension was stored at -20°C for 1 h. After centrifugation (13200 rpm, 5 min, 4°C), the supernatant was removed, and the pellet further washed with cold acetone (2×1 ml), dried at 55°C for 30 min, and dissolved in Milli Q water (1.0 ml). The residue was then purified by anion-exchange HPLC (IE-HPLC) using the DIONEX Ultimate 3000 system equipped with a DNAPac PA100 semi-preparative column (13 μm , 250 mm \times 9 mm) heated to 60°C . Elution was performed with an isocratic hold of buffer B (10%), starting from 2 min hold on 2% Buffer A in Milli-Q water (solvent A), followed by a linear gradient to 25% buffer A in 20 min at a flow rate of 2.0 ml/min (buffer A: 1.0 M sodium perchlorate; buffer B: 0.25 M Tris-Cl, pH 8.0, solvent A: Milli-Q water). After IE-HPLC purification, ONs were desalted using with an aqueous solution of sodium acetate (3 M, 15 μl) and sodium perchlorate (5 M, 15 μl) followed by addition of ice-cold (stored at -20°C) ethanol (1 ml). The resulting suspension was stored at -20°C for 1 h. After centrifugation (13200 rpm, 5 min, 4°C), the supernatant was removed, and the pellet further washed with cold ethanol (2×1 ml), dried at 55°C for 30 min, and dissolved in Milli Q water (1.0 ml). Mass spectra of ONs were recorded on a Bruker Daltonics Microflex LT MAIDITOF MS instrument in ES+ mode (representative mass spectra are shown in Figure S1, S2 S6-S9 and S11-S15). Analytical IE-HPLC diagrams were recorded on a Merck-Hitachi Lachrom system equipped with a DNAPac PA100 analytical column (13 μm , 250 mm \times 4 mm) heated to 60°C . Elution was performed with an isocratic hold of buffer B (10%), starting from 2 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) (representative IE-HPLC diagrams are shown in Figure S1, S2 S6-S9 and S11-S15). Concentrations of purified ONs were determined by UV measurements at 260 nm, assuming identical molar absorptivities for a DNA thymine monomer and monomers X and Y.

Ultraviolet thermal denaturation studies

The representative UV absorption curves of ONs are shown in supporting information. All thermal denaturation temperature curves were recorded at 260 nm 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 concentration of 2.5 μM for each strand and a volume of 1.0 ml in a buffer (5.8 mM sodium phosphate, 100 mM NaCl

and 0.10 mM EDTA). Samples were prepared as follows: The two strands were mixed in a 1:1 ratio (2.5 μM of each) in 2 ml Eppendorf tubes before addition of medium salt buffer (500 μl , 2 times, 11.7 mM sodium phosphate, 200 mM NaCl, 0.20 mM EDTA, pH 7.0, 500 μl). Milli-Q water was then added to give a total volume of 1 ml. The mixtures were heated to 90°C in a water-bath followed by slow cooling to room temperature before they were transferred into the cuvettes. The UV absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 8°C to 65°C at a rate of $1.0^\circ\text{C}/\text{min}$ programmed by a Peltier temperature controller. Three separate melting curves were measured and the duplex thermal denaturation temperatures (T_m values) calculated using UV-WinLab software as an average from the three thermal denaturation curves having deviation no more than 0.5°C .

RNase H experiments

RNA cleavage for the gapmer/RNA heteroduplex against RNase H from *E. coli* MRE-600 cells (Thermo Fisher Scientific) was studied in a solution containing $5'$ - ^{32}P -labeled RNA, non-labelled (3 μM) RNA and the relevant gapmer (12 μM) in 10 mM Tris-HCl (pH 7.5, 50 mM KCl). The solution was incubated at 72°C at 5 min followed by slow cooling to 37°C . A solution containing the enzyme together with the reaction buffer were added resulting in a final concentration of 20 mM Tris-HCl (pH 7.8, 40 mM KCl), 8 mM MgCl_2 , 1 mM DTT together with RNase H 0.2U/ μl (4U) at a total volume of 20 μl . The experiment was run at 37°C . Initial aliquots (3 μl , 0 min) were drawn immediately prior to adding the enzyme. At time points 10 seconds, 5 min and 15 min, aliquots (3 μl) were taken and added to tubes containing 2 μl ice-cold loading buffer (95% formamide, 20 mM EDTA, xylene cyanol, and bromophenol blue). All samples were heated to 95°C for 5 min and then resolved on 20% denaturing polyacrylamide electrophoresis gels with 7 M urea and then visualized. The RNA-ladder was made with the same procedure and concentrations described in the nuclease resistance assays. Initial aliquots (3 μl , 0 min) were drawn immediately prior to adding the enzyme. At time points 15 min, 30 min, 1 h and 2 h, aliquots (3 μl) were taken and added to tubes containing 2 μl ice-cold loading buffer (95% formamide, 20 mM EDTA, xylene cyanol, and bromophenol blue). A control was included with the gapmer/RNA heteroduplexes D9 and D18 at 0 min under the same conditions as the 0 min time-points in the RNase H experiment to optimize comparison between the gels.

HeLa culture and transfection

HeLa cells were cultivated as described earlier.^[24] The passage of 100,000 cells in each of the 24 wells was performed. After 24 h the transfection was performed using Lipofectamine 2000 in accordance with the manufacturer's recommendations. To evaluate silence efficiency of gapmers, HeLa cells were cotransfected with 1 μg of ZsGreen1 plasmid containing a fragment complementary to gapmers and 200 nM concentration of ASO molecules (ON9, ON10, ON13). The transfected cells were grown for 24 h at 37°C in a 5% CO_2 atmosphere without antibiotics in medium. The control included (i) gapmer with scrambled DNA sequence (ON12), (ii) isosequential, all-DNA ASO (ON11), and (iii) isosequential gapmer without modified nucleotide (ON8). The RNA from the cultured cells was isolated using acid guanidinium thiocyanate-phenol-chloroform extraction, and the RNA was treated with DNase I. The quality of the isolated RNA was verified by agarose gel electrophoresis.

MTT assay

The half maximal inhibitory concentration (IC_{50}) of gapmers was determined by MTT assay. The HeLa cells were passaged in 96-well plates in the amount of 5,000 cells per well. The cells were incubated for 24 h at 37°C in a 5% CO_2 atmosphere in the RPMI-1640 medium supplemented with 10% FBS, vitamins and antibiotics. After incubation the cells were transfected with different concentrations of gapmers (1, 5,

20, 50, 100, 200, 500, 1000 nM) using Lipofectamine 2000 and incubated for 24 h. Next the MTT assay was performed. The medium was discarded from the cells, then 150 µl of MTT solution (0.5 mg/ml) in medium without phenol red was added. The MTT reagent was incubated with the cells for 2 h, whereupon the MTT solution was discarded and water-insoluble formazan crystals were dissolved in 40 mM HCl in isopropanol. The absorbance was measured at 550 nm and 650 nm as a reference on a XMark Microplate Spectrophotometer. The IC₅₀ value was estimated using dose-response curves.

RT-qPCR analysis

An earlier prepared 500 ng RNA template was used for cDNA synthesis, using the iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed on a CFX96 real-time PCR system (Bio-Rad) using iTaq SYBR Green Supermix (Bio-rad) and 96-well clear plates. The level of ZsGreen1 mRNA was quantified using target gene primers: J9 5'-GTACCACGAGTCCAAGTTCTAC, J10 5'-CCAGTTGTCTGGTCATCTTCTT, and normalized to human β-actin levels; Reference gene primers: J5 5'-GCCAGCAGCCTCTGATCTG, J6 5'-CTGTTCTTGCCAGCCTCTAG. The Ct values of human β-actin was in the range of 19-21. The qPCR cycles were as follows: 95 °C, 5 min for pre-denaturation step; (95 °C, 10 sec and 66 °C, 20 sec) for 40 cycles.

qPCR statistical analysis

PCR efficiency of each reaction ranged from 94.5% to 96%. The results from replicates for particular samples were gathered to determine the mean normalized expression and its standard error of the mean (Bio-Rad CFX Manager 3.0). The normalized relative expression of ZsGreen1 from replicates for gapmers and controls was compared at a significance level of 0.05 or 0.01 using t-test calculator (GraphPad). Statistically significant differences in mean expression between tested gapmers and controls ($P < 0.05$ or $P < 0.01$) were observed.

ORCID

Jesper Wengel: 0000-0001-9835-1009

Mathias B. Danielsen: 0000-0001-9862-3967

Chenguang Lou: 0000-0002-9521-8285

Per Trolle Jørgensen: 0000-0003-3932-5921

Jolanta Lisowiec-Wachnicka: 0000-0002-3910-9682

Anna Pasternak: 0000-0002-9666-4148

Acknowledgements

The VILLUM FONDEN is thanked for funding the Biomolecular Nanoscale Engineering Center (BioNEC), a VILLUM center of excellence, grant number VKR18333. Joan Hansen and Tina Grubbe Hansen are thanked for technical assistance on oligonucleotide synthesis and purification. This work was supported by the VILLUM FONDEN, Grant Number: VKR18333, from the European Union's Horizon 2020 research and innovation program under grant agreement No 810685 and National Science Center grants UMO-2017/25/ B/ NZ7/00127 to A.P. and UMO-2016/21/D/NZ5/01906 to J.L.W.

Author contributions

J.W. designed the project. J.W., P.T.J. and C.L. supervised the project and edited the manuscript. M.B.D. drafted the manuscript. M.B.D. conducted most of the experimental work. C.L. and M.B.D. designed the enzyme studies. J.L.W. did the transfection studies. A.P. supervised the transfection studies.

Conflicts of interest

The authors declare no competing financial interest.

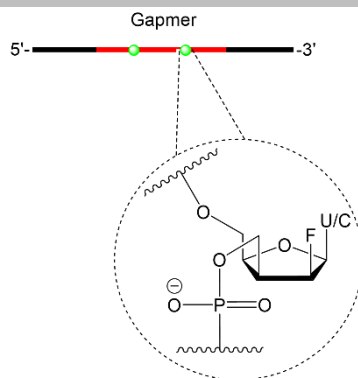
Keywords: Gapmer • Nucleosides • Nucleotides • RNase H • RNA cleavage

- [1] B. Vester, A. M. Boel, S. Lobedanz, B. R. Babu, M. Raunkjær, D. Lindegaard, Raunak, P. J. Hrdlicka, T. Højland, P. K. Sharma, S. Kumar, P. Nielsen, J. Wengel, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2296-2300.
- [2] C. F. Bennett, E. E. Swayze, *Annu. Rev. Pharmacol. Toxicol.* **2010**, *50*, 259-293.
- [3] P. Järver, L. O'Donovan, M. J. Gait, *Nucleic Acid Ther.* **2014**, *24*, 37-47.
- [4] K. E. Lundin, O. Gissberg, E. C. I. Smith, *Human Gene Therapy* **2015**, *26*, 475-485.
- [5] A. Istrate, A. Katolik, A. Istrate, C. J. Leumann, *Chem. Eur. J.* **2017**, *23*, 10310-10318.
- [6] M. D. Sørensen, L. Kværnø, T. Bryld, A. E. Håkansson, B. Verbeure, G. Gaubert, P. Herdewijn, J. Wengel, *J. Am. Chem. Soc.* **2002**, *124*, 2164-2176.
- [7] W. F. Lima, J. G. Nichols, H. Wu, T. P. Prakash, M. T. Migawa, T. K. Wyrzykiewicz, B. Bhat, S. T. Crooke, *J. Biol. Chem.* **2004**, *279*, 36317-36326.
- [8] K. Fluiter, O. R. F. Mook, J. Vreijling, N. Langkjær, T. Højland, J. Wengel, F. Baas, *Mol. Biosyst.* **2009**, *5*, 838-843.
- [9] A. Grünweller, E. Wyszko, B. Bieber, R. Jahnel, V. A. Erdmann, J. Kurreck, *Nucleic Acids Res.* **2003**, *31*, 3185-3193.
- [10] H. Hudlebusch, H. F. Hansen, M. L. Jensen, M. R. Møller, M. Lindow, T. S. Bisgaard, T. Koch, P. H. Hagedorn, M. Pontoppidan, A. Dieckmann, M. Berrera, M. Ebeling, *Nucleic Acids Res.* **2018**, *46*, 5366-5380.
- [11] T. Kasuya, S.-i. Hori, A. Watanabe, M. Nakajima, Y. Gahara, M. Rokushima, T. Yanagimoto, A. Kugimiya, *Sci. Rep.* **2016**, *6*, 30377.
- [12] N. J. Gooderham, P. J. Kamola, J. D. Parry, A. Panjwani, E. L. Koppe, G. Turner, Jeremy D. A. Kitson, K. L. Clark, K. Moores, K. Maratou, L. C. Warnock, M. R. Edbrooke, P. A. Wilson, S. Eriksson, S. A. Hughes, T. W. Gant, G. A. Douillard Guilloux, W. E. Wixted, *Nucleic Acids Res.* **2015**, *43*, 8638-8650.
- [13] S. A. Burel, C. E. Hart, P. Cauntay, J. Hsiao, T. Machemer, M. Katz, A. Watt, H.-H. Bui, H. Younis, M. Sabripour, S. M. Freier, G. Hung, A. Dan, T. P. Prakash, P. P. Seth, E. E. Swayze, C. F. Bennett, S. T. Crooke, S. P. Henry, *Nucleic Acids Res.* **2016**, *44*, 2093-2109.
- [14] P. P. Seth, A. Siwkowski, C. R. Allerson, G. Vasquez, S. Lee, T. P. Prakash, E. V. Wancewicz, D. Witchell, E. E. Swayze, *J. Med. Chem.* **2009**, *52*, 10-13.
- [15] M. E. Østergaard, J. Nichols, T. A. Dwight, W. Lima, M. E. Jung, E. E. Swayze, P. P. Seth, *Mol. Ther. Nucleic Acids* **2017**, *7*, 20-30.
- [16] W. Shen, C. L. De Hoyos, M. T. Migawa, T. A. Vickers, H. Sun, A. Low, T. A. Bell, M. Rahdar, S. Mukhopadhyay, C. E. Hart, M. Bell, S. Riney, S. F. Murray, S. Greenlee, R. M. Crooke, X.-h. Liang, P. P. Seth, S. T. Crooke, *Nat. Biotechnol.* **2019**, *37*, 640-650.
- [17] A. Khvorova, J. K. Watts, *Nat. Biotechnol.* **2017**, *35*, 238.
- [18] A. D. Burdick, S. Sciabola, S. R. Mantena, B. D. Hollingshead, R. Stanton, J. A. Warneke, M. Zeng, E. Martsen, A. Medvedev, S. S. Makarov, L. A. Reed, J. W. Davis, II, L. O. Whiteley, *Nucleic Acids Res.* **2014**, *42*, 4882-4891.

- [19] E. E. Swayze, A. M. Siwkowski, E. V. Wancewicz, M. T. Migawa, T. K. Wyrzykiewicz, G. Hung, B. P. Monia, Bennett, C. Frank, *Nucleic Acids Res.* **2006**, *35*, 687-700.
- [20] A. Dieckmann, P. H. Hagedorn, Y. Burki, C. Brüggmann, M. Berrera, M. Ebeling, T. Singer, F. Schuler, *Mol. Ther. Nucleic Acids* **2018**, *10*, 45-54.
- [21] M. L. Svendsen, J. Wengel, O. Dahl, F. Kirpekar, P. Roepstorff, *Tetrahedron* **1993**, *49*, 11341-11352.
- [22] C. J. Wilds, M. J. Damha, *Nucleic Acids Res.* **2000**, *28*, 3625-3635.
- [23] L. Kværnø, R. Kumar, B. M. Dahl, C. E. Olsen, J. Wengel, *J. Org. Chem.* **2000**, *65*, 5167-5176.
- [24] J. Lisowiec-Wąchnicka, N. Bartyś, A. Pasternak, *Sci. Rep.* **2019**, *9*, 2477.
- [25] O. Moukha-Chafiq, K. N. Tiwari, J. A. Secrist, *Nucleos. Nucleot. Nucl.* **2005**, *24*, 713-715.
- [26] X. X. Zhou, S. Torssell, O. Wallner, P. Sun, **2012**.
- [27] V. K. Rajwanshi, A. E. Håkansson, B. M. Dahl, J. Wengel, *Chem. Commun.* **1999**, 1395-1396.
- [28] N. E. Poopelko, M. Juhl, B. Vester, M. D. Sørensen, J. Wengel, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2285-2290.
- [29] S. K. Singh, R. Kumar, J. Wengel, *J. Org. Chem.* **1998**, *63*, 10035-10039.
- [30] A. Kalota, L. Karabon, C. R. Swider, E. Viazovkina, M. Elzagheid, M. J. Damha, A. M. Gewirtz, *Nucleic Acids Res.* **2006**, *34*, 451-461.
- [31] J. K. Watts, A. Katolik, J. Viladoms, M. J. Damha, *Org. Biomol. Chem.* **2009**, *7*, 1904-1910.
- [32] C. Lou, S. V. Samuelsen, N. J. Christensen, B. Vester, J. Wengel, *Bioconjugate Chem.* **2017**, *28*, 1214-1220.
- [33] C. L. De Hoyos, W. F. Lima, X.-H. Liang, S. T. Crooke, *Nucleic Acids Res.* **2016**, *44*, 3351-3363.
- [34] J. Lykke-Andersen, E. J. Bennett, *J. Cell Biol.* **2014**, *204*, 467-476.
- [35] J. Houseley, J. LaCava, D. Tollervey, *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 529-539.

FULL PAPER

A novel class of 2'-deoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxo-configured nucleotides has been synthesized and incorporated into antisense oligonucleotides for the first time.



Mathias B. Danielsen, Chenguang Lou, Jolanta Lisowiec-Wachnicka, Anna Pasternak, Per T. Jørgensen and Jesper Wengel*

Page No. – Page No.

Gapmer antisense oligonucleotides containing 2',3'-dideoxy-2'-fluoro-3'-C-hydroxymethyl- β -D-lyxofuranosyl nucleotides display site-specific RNase H cleavage and induce gene silencing