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Hexofuranosyl thymidines inserted into oligodeoxynucleotides via their two exocyclic hydroxy groups. Oligo synthesis and RNase H activity

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Abstract—Hexofuranosyl nucleosides are considered as conformationally restricted acyclic nucleosides using a furanose ring to link the diol backbone to the nucleobase. The phosphoramidite of 1-(2,3-dideoxy-β-D-*erythro*-hexofuranosyl)thymine was synthesized from thymidine with formation of a new stereocentre at C-5′ and the nucleoside was used in oligodeoxynucleotide (ODN) synthesis. Binding of mixed sequence ODNs towards complementary DNA and RNA showed decreased affinity compared to the wild-type oligos. Insertion in the middle of poly αT sequence led to stabilization of ODN/dA_{14} duplexes at low ionic strength, but a decrease was observed in medium and high salt buffers compared to $d(\alpha T)_{14}/dA_{14}$. Both β and α hexofuranosyl thymidines allowed cleavage of complementary mixed-sequence RNA by RNase H to the 3′-site of the modification in ODNs whereas a limited inhibition was detected from the 5′-site.

1. Introduction

In the last decades, synthesis and incorporation of modified nucleosides into oligodeoxynucleotides (ODNs) have become interesting for biochemists.[1] A number of DNA analogues with improved hybridization affinity, exo- and endonuclease stability have been synthesized and their antisense properties evaluated. Since the discovery of PNA (peptide nucleic acid, Fig. 1) constructed from 2-aminoethyl glycine units to which nucleobases are attached by methylenecarbonyl linkers,[2] a number of ODNs possessing acyclic DNA backbone have been obtained.[3] The insertion of acyclic moieties into ODNs leads to loss in entropy upon duplex formation and lower thermal stability is usually observed. The decrease in duplex stability was found to be smaller when acyclonucleotides were inserted near the end than in the middle of duplexes[3b] with acyclo DNA (T) (Fig. 1) showing the largest decrease with ΔT_m of −13.4 °C.[4] However, rigid groups as carboxamide groups in PNA could significantly improve hybridization properties. The conformational restriction by a C=C bond[5] and

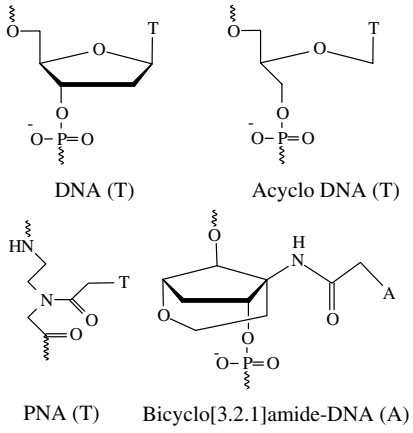

Figure 1.

Keywords: Hexofuranosyl nucleoside; Exocyclic DNA backbone; Duplex stability; Salt effect; RNase H activity.
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ODNs with (N-thymin-1-ylacetyl)-1-arylserinol backbone[6] have binding capacity towards DNA and RNA duplexes, but decreased melting temperatures with ΔT_m of −5.5 and −9.0 °C, respectively, have been observed when the monomers have been inserted in the middle of a sequence. The use of acyclic linkers can provide extra properties, which are not available in native nucleic acids. For example, homobasic adenine containing bicyclo[3.2.1]amide-DNA (Fig. 1) forms strictly antiparallel duplexes with D- and L-complements of similar stability and which are enantiomorphic in structure.[7]

Special attention is paid in nucleic acid research to find chemically modified nucleotides with good resistant towards enzymatic degradation and with ability to activate the cellular enzymes as, for example, RNase H or RNase L, which recognize DNA/mRNA duplexes and rapidly degrade the mRNA at the site of complexation.[1] However, the detailed mechanism of action of the enzymes and the criteria for design of nonnatural nucleotides for being a substrate are not well characterized. ODNs containing acyclonucleotides were studied towards nucleolytic degradation and found to be more stable than the wild-type oligos.[3b,6] PNA/RNA hybrids were not substrates for RNase H[2b] and no evaluation of activity of other acyclonucleotides were done.

Recently we proposed a new conformationally restricted acyclic nucleosides using the furanose ring to link the phosphate backbone to the nucleobase.[8] 1-(2,3-Dideoxy-3-amino-α-D-*arabino*-hexofuranosyl)thymine (**1**, Fig. 2) as the first representative of this type of molecules was inserted into ODNs via its two exocyclic OH-groups. Due to free rotation around C-4′–C-5′ bond, hexofuranosyl nucleosides can exist in two conformations **1** and **1′** corresponding to α and β anomers, respectively, of natural nucleosides (Fig. 2). Therefore, oligos from this type of nucleosides may have the advantage like PNA in forming duplexes simulating both β and α anomers and may hybridize both in a parallel and an antiparallel fashion. This property seems to be a prerequisite for strand invasion.[2b] We found that the insertion of the nucleoside **1** having relatively short ethyleneglycol backbone and thymine situated in the α-position of the furanose ring into ODNs did not diminish the hybridization properties more than other acyclic nucleotide analogues, apart from PNA. From C/T mismatch studies it was concluded that the nucleoside **1** obeyed Watson–Crick base pairing. When the monomer **1** was inserted into an αT sequence and a βT sequence as next-nearest neighbours, the drop in melting temperature per modification was nearly the same for both sequences towards RNA complement. This might indicate that nucleotide **1** behaved both as α and β nucleotide.

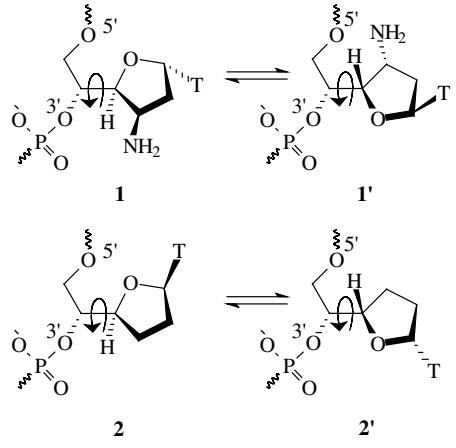


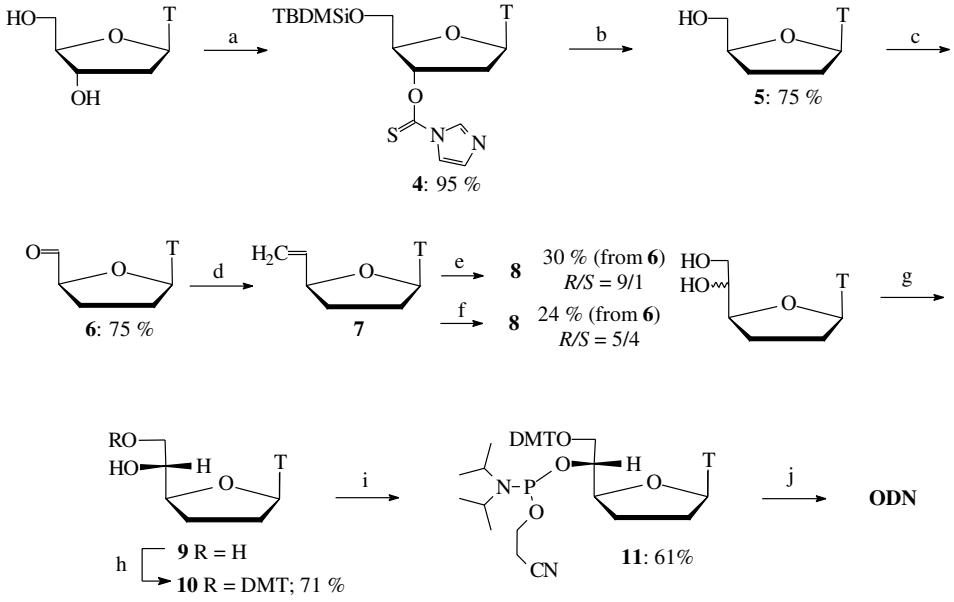
Figure 2.

Following a similar approach, hexofuranosyl nucleosides having thymine in a β-position of the furanose ring could be studied and compared with the above mentioned investigation. We report here a linear synthesis of 1-(2,3-dideoxy-β-D-*erythro*-hexofuranosyl)thymine (**2**), its incorporation into ODNs, the base-pairing properties in complexation with DNA and RNA, and RNase H activity of ODNs possessing either **1** or **2**.

2. Chemistry

Synthesis and biological evaluation of hexofuranosyl nucleosides as potential anticancer, antiviral agents have been published before.[9] In our case we decided to incorporate hexofuranosyl thymidine having no substituents at C-2′ and C-3′ positions of the furanose ring into ODNs. Lau et al.[9a] and Hrebabecky and Holy[9b] have independently published a nonlinear synthesis of **2** starting from carbohydrates with known stereochemistry at C-5 position. In both cases long, more than eight-steps syntheses have been applied. Instead we used a linear approach to synthesize 1-(2,3-dideoxy-β-D-*erythro*-hexofuranosyl)thymine (**2**) from thymidine via the formation of the new stereocentre at C-5′ position using Sharpless asymmetric dihydroxylation (Scheme 1).

Sekine and Nakanishi[10] have obtained 3′-deoxythymidine (**5**) via the synthesis of 3′,5′-bis-*O*-(phenoxythiocarbonyl)thymidine followed by the predominately deoxygenation reaction at the 3′-position and alkaline deprotection. Instead of expensive and unstable phenyl chlorothioformate we applied the same approach using 1,1′-thiocarbonyldiimidazole. However, after the Barton reaction under the same conditions an inseparable mixture was obtained, which means that 5′-*O*-(1-imidazolylthiocarbonyl) group did not serve here as 'protective group' and was involved in side reactions. We synthesized 3′-deoxythymidine (**5**) in two one-pot reactions starting with silyl protection of the primary OH-group of thymidine in the presence of imidazole in DMF at 0–5 °C for 3 h followed by adding of 1,1′-thiocarbonyldiimidazole and heating at 80 °C for 2.5 h affording 5′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(1-imidazolylthiocarbonyl)thymidine (**4**)[11] in 95% yield after precipitation from water. Reduction of **4** with $(n\text{-Bu})_3\text{SnH}$ in the presence of AIBN in refluxing dioxane for 7 h and final deprotection by trifluoroacetic acid gave 3′-deoxythymidine (**5**) in 75% yield after silica gel purification.



Scheme 1. Reagents and conditions: (a) TBDMSiCl, imidazole, DMF, 0 °C, 2 h then 1,1′-thiocarbonyldiimidazole, DMF, 80 °C, 3 h; (b) $(n\text{-Bu})_3SnH$, AIBN, dioxane, reflux, 6 h then 50% aq CF_3COOH in dioxane, rt, 4.5 h; (c) *o*-iodoxybenzoic acid, MeCN, reflux, 1 h; (d) $MePPh_3Br$, LiHMDS, THF, rt, 4 h; (e) AD-mix-α, *t*-BuOH/H_2O, rt, overnight; (f) AD-mix-β, *t*-BuOH/H_2O, rt, overnight; (g) crystallization from acetone; (h) DMTCl, Py, rt, overnight; (i) $NC(CH_2)_2OP(NPr^i_2)_2$, *N*,*N*-diisopropylammonium tetrazolide, CH_2Cl_2; (j) DNA-synthesizer.

Camarasa et al.[12] and O-Yang et al.[13] have reported the conversion of 3′-deoxythymidine to the corresponding aldehyde **6** using CrO_3/pyridine/Ac_2O and applying Moffat oxidation (DCC/TFA/pyridine/DMSO), respectively. Despite of the instability of the aldehyde **6**, silica gel chromatography had to be used in order to avoid contaminations of toxic chromium salts in the first case and methylthioalkyl ethers formed as a by-product in the second case. Recently, some alternative protocols for the oxidation of primary alcohols to aldehydes without any over oxidation to carboxyl compounds and without silica gel purification have been presented.[14] Dess–Martin periodane[14a] could be a good choice in nucleoside chemistry; however hygroscopic sensitivity and price limit its applicability. More and Finney[15] have published effective oxidation of alcohols in hot or refluxing solvents in the presence of suspended *o*-iodoxybenzoic acid (IBX), which is filtered afterwards. The alcohol **5** was converted to the aldehyde **6** using IBX in refluxing acetonitrile for 1 h in 90% yield.

The Wittig reaction of **6** with CH_3PPh_3Br using lithium bis(trimethylsilyl)amide in dry THF led to the alkene **7** that unfortunately was difficult to separate from $Ph_3P{=}O$ using either silica gel column or crystallization. Dihydroxylation of **7** with two commercially available Sharpless asymmetric reagents AD-mix-α and AD-mix-β in a 1:1 mixture of water and *tert*-butyl alcohol at rt gave the epimeric diol product mixtures **8** after silica gel purification in 30% and 24% yields, respectively, calculated from the aldehyde **6**. From the ^1H NMR spectrum (H-6 signal), the ratio of the two epimers was estimated as 9:1 (*R*:*S*) for the reaction with AD-mix-α and 5:4 (*R*:*S*) for AD-mix-β. The structural assignment of *S* and *R* epimers at the new C-5′ stereocentre was done by comparison of the NMR data for 1-(2,3-dideoxy-β-D-*erythro*-hexofuranosyl)thymine (**9**) published by Lau et al.[9a] The predominately formation of one epimer using one of the asymmetric reagents is due to the sterically hindered double bond. This is also in agreement with previous studies of Fensholdt and Wengel[16] on the dihydroxylation of the 5′-*C*-methylene-3′-*O*-silyl protected thymidine. For the synthesis of the fully modified ODN the pure epimer is needed in order to get sharp melting curves. Recrystallization from acetone of the obtained product using AD-mix-α led to the substance **9**, which was >98% diastereomeric pure. This compound was treated with 4,4′-dimethoxytrityl chloride (DMTCl) in dry pyridine. However, the presence of pyridine hydrochloride formed during the reaction led to the partial cleavage of DMT-group upon the concentration of products. Instead, the reaction mixture was evaporated to the half volume under reduced pressure followed by dilution with ethyl acetate and extraction by saturated aqueous solution of $NaHCO_3$, which gave DMT-protected thymidine **10** in 71% yield after silica gel purification.

Compound **10** was treated with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite in the presence of *N*,*N*-diisopropylammonium tetrazolide in anhydrous CH_2Cl_2 at rt overnight affording the required phosphoramidite **11** in 61% yield after silica gel column chromatography.

The phosphoramidite **11** was incorporated into different oligonucleotide sequences on an automated solid phase DNA synthesizer using an increased coupling time (5 min). The coupling efficiencies using 4,5-dicyanoimidazole as an activator were approximately 90–95% compared to approximately 99% for commercial phosphoramidites (2 min coupling).

3. Hybridization studies

The hybridization properties of the modified ODNs towards complementary DNA and RNA single strands were evaluated by thermal denaturation studies. The melting temperature (T_m) as a first derivative of the melting curve and the differences between modified and unmodified oligomers as the change in melting temperature per modification (ΔT_m) are listed in Tables 1–3.

The insertion of **2** in the central region of mixed sequences decreased the T_m significantly (Table 1, ΔT_m −7.0 to −10.0 °C). The smallest decrease and a slight discrimination in destabilization of RNA (ΔT_m −2.0 °C) over DNA (ΔT_m −6.0 °C) were obtained for ODNs possessing nucleotide **2** near the end of the sequence. Both these effects were probably observed because the neighbouring nucleotides near the modification compensated for the shortness of the ethyleneglycol linker. Compensation was probably not possible when the monomer was situated in the middle of the oligomer. Also shown in Table 1 are T_m values for **ODN 1** and **ODN 2** towards a single C/T mis-matched complement. These data demonstrated that β-hexofuranosyl thymidine **2** discriminated towards mis-match less efficiently than α analogue **1** (ΔT_m −5.0 °C).[8]

The shortness of the diol linker in the middle of the strand may possibly be compensated by the complementary ODN possessing the same modification. We studied the thermal stability of DNA duplexes containing the monomer **2** in the neighbouring AT-pairs in the middle of strands (Table 2). The decrease in T_m per modification was much lower when hexofuranosyl nucleotide was situated in both strands (T_m −4.8 and −3.7 °C for one and two modifications in each strand, respectively). The fully modified 14-mer sequence of **2** gave no melting transitions towards DNA and RNA complements. These results in conjunction with previous research of the nucleotide **1** clearly indicate that the diol linker is too short to mimic natural nucleic acids backbone.

To evaluate the possible rotation around C-4′–C-5′ bond we synthesized ODNs possessing incorporation of **2** either in $d(\beta T)_{14}$ or in $d(\alpha T)_{14}$ (Table 3). In a medium salt buffer decreasing in T_m was observed in all cases. The same tendency of destabilization for incorporation of **2** into the end and into the middle of the strands as we saw in case of mixed-sequences (Table 1) was detected, except for complexes having **2** in α sequences towards complementary DNA. Here the decreasing of T_m was smaller than the ones observed either towards RNA or in case of incorporations into β strand. Lower hyperchromicity for duplexes with α sequences than for β sequences was also observed. Interestingly the two αT sequences possessing modification **2** in the middle of the sequence (**ODN 18**, **ODN 19**) have values of ΔT_ms that changed significantly from decreasing T_ms in high ionic strength buffer to unchanged or increasing T_ms in low ionic strength buffer when compared with $d(\alpha T)_{14}$. Usually it is believed that at low salt concentration the

Table 1. Melting experiments, calculated and experimental masses of ODNs from MALDI-TOF MS of ODNs possessing **2** in mixed sequences[a]

Entry	No	Sequence	T_m (°C) (ODN/DNA)	ΔT_m[b] (°C)	T_m (°C) (ODN/RNA)	ΔT_m[b] (°C)	$\Delta T_{m(RNA)} - \Delta T_{m(DNA)}$	m/z, calcd (Da)	m/z, found (Da)
1	**ODN 1**	5′-GCTTCTCTAGAATG	40.6[c]		41.8[d]				
2	**ODN 2**	5′-GCTTCTC**2**AGAATG	33.5	−7.1	31.8	−10.0	−2.9	4266	4266
3	**ODN 3**	5′-GCT**2**CTCTAGAATG	30.2	−10.4	32.9	−8.9	1.5	4266	4265
4	**ODN 4**	5′-GC**22**CTCTAGAATG	25.6	−7.5	30.9	−5.0	2.5	4280	4280
5	**ODN 5**	5′-GCTTCTCTAGAA**2**G	34.6	−6.0	39.2	−2.0	4.0	4266	4269
		C/T mis-matches	T_m (°C) (ODN/3′-CGAAGAG***C***TCTTAC)						
6	**ODN 1**	5′-GCTTCTCTAGAATG	30.2			−10.4	Entry 1 as reference		
7	**ODN 2**	5′-GCTTCTC**2**AGAATG	30.0			−3.5	Entry 2 as reference		

[a] T_m, °C was determined at 260 nm against increasing temperature (1.0 °C per 1 min) on equimolar mixtures (1.5 μM in each strand) of modified oligomer and its complementary DNA/RNA in medium salt buffer (10 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.0).
[b] ΔT_m, °C = change in T_m per modification.
[c] Target: 3′-CGAAGAGATCTTAC.
[d] Target: 3′-CGAAGAGAUCUUAC.

Table 2. Melting experiments (T_m, °C), calculated and experimental masses from MALDI-TOF MS of ODNs possessing **2** in both strands[a]

5′-GCTTC$\mathbf{X_1}$A$\mathbf{X_2}$AGAATG
3′-CGAAGA$\mathbf{Y_1}$A$\mathbf{Y_2}$CTTAC

	ODN 6 $\mathbf{X_1 = X_2 = T}$	**ODN 7** $\mathbf{X_1 = T; X_2 = 2}$	**ODN 8** $\mathbf{X_1 = X_2 = 2}$	m/z, calcd (found, Da)
ODN 9 $\mathbf{Y_1 = Y_2 = T}$	41.0	32.5 (−8.5)[b]	19.1 (−11.0)[b]	
ODN 10 $\mathbf{Y_1 = T; Y_2 = 2}$	30.5 (−10.5)[b]	31.4 (−4.8)[b]	22.7 (−6.1)[b]	4252 (4257)
ODN 11 $\mathbf{Y_1 = Y_2 = 2}$	20.8 (−10.1)[b]	25.8 (−5.1)[b]	26.3 (−3.7)[b]	4273 (4275)
m/z, calcd (found, Da)		4290 (4289)	4304 (4303)	

[a] T_m was determined under the same conditions as in Table 1.
[b] ΔT_m, °C = change in T_m per modification in brackets.

Table 3. Melting experiments at different ionic strength,[a] calculated and experimental masses from MALDI-TOF MS of ODNs possessing modification **2** in α and β polyT

No.	Sequence	ODN/dA_{14}						ODN/rA_{14}		m/z, calcd (Da)	m/z, found (Da)
		High salt		Medium salt		Low salt		Medium salt			
		T_m (°C)	ΔT_m[b] (°C)	T_m (°C)	ΔT_m^b (°C)	T_m (°C)	ΔT_m^b (°C)	T_m (°C)	ΔT_m^b (°C)		
ODN 12	dT_{14}	44.5		32.5		24.0		29.0			
ODN 13	5′-T_{12}**2**T	42.5	−2.0	29.5	−3.0	22.0	−2.0	27.0	−2.0	4209	4210
ODN 14	5′-T_7**2**T_6	35.0	−9.5	21.0	−11.5	13.0	−11.0	22.5	−6.5	4209	4205
ODN 15	5′-T_4**2**T**2**T_7	23.5	−10.5	<5.0	—	<5.0	—	11.5	−8.4	4223	4221
ODN 16	αdT_{14}	46.5		30.5		20.0		42.5			
ODN 17	α5′-(T_{12}**2**T)	44.0	−2.5	26.5	−3.5	17.0	−3.0	40.5	−2.5	4209	4206
ODN 18	α5′-(T_7**2**T_6)	39.5	−7.0	28.0	−2.0	24.0	+4.0	35.0	−7.5	4209	4210
ODN 19	α5′-(T_4**2**T**2**T_7)	29.5	−8.5	20.0	−5.25	20.0	0.0	23.6	−9.5	4223	4222

[a] T_m, °C was determined at 260 nm against increasing temperature (1.0 °C per 1 min) on equimolar mixtures (1.5 μM in each strand) of modified oligomer and its complementary DNA/RNA in high salt buffer (10 mM NaH_2PO_4, 700 mM NaCl, 0.1 mM EDTA, pH 7.0), medium salt buffer (10 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.0) and low salt buffer (10 mM NaH_2PO_4, 30 mM NaCl, 0.1 mM EDTA, pH 7.0).
[b] ΔT_m, °C = change in T_m per modification.

electrostatic repulsion between phosphodiester backbones increases thus leading to destabilization of DNA complexes. For the native $d(\alpha T)_{14}/dA_{14}$ in low salt concentration the destabilization of 10.5 °C was observed in comparison with the medium buffer. However, for the complex of **ODN 19** with two modifications, T_m values in medium and low salt buffers were the same. To the best of our knowledge this is the first example of a oligonucleotide possessing a phosphodiester linker without positively charged groups showing such a dependence of ionic strength. According to the polyelectrolyte theory based on counterion condensation, the linearity of charges has an influence on the duplex stability.[17] Compound **2** is likely to change this linearity.

From the hybridization studies it can be concluded that hexofuranosyl thymidine **2** behaves more likely as αT whereas the α-analogue **1** has slightly better affinity towards β-nucleotides.[8] The single insertion of **2** in the middle of αT sequence led to slight destabilization of ODN/DNA duplex at medium salt concentration and gave more stable duplex than the reference at low salt concentration. However, the short diol linker could not mimic the native backbone for the fully modified oligomer of **2**.

4. RNase H activity

The mixed ODNs possessing hexofuranosyl thymidines **1** or **2** in the complex with complementary RNA were studied as substrates for RNase H. Our interest in restricted acyclonucleotides in this area was stimulated by the fact that only PNA has been studied and found not to be a substrate for RNase H. However, in contrary to polypeptide chain in PNA, modified nucleotides **1** or **2** were incorporated in ordinary DNA sequences making them more likely to support RNase H degradation. To characterize the effect of the modifications the cleavage of complementary RNA versus the position of modified nucleotide were investigated. We studied the degradation of [^{32}P]-labelled RNA that was complementary to **ODN 1** (Table 4). As seen in Figure 3 not all phosphodiester bonds are cleaved by the enzyme and the cleavage pattern varies from sequence to sequence. Basic hydrolysis of the RNA (Fig. 3) were used to identify the cleaved bonds. Initial cut occurred at the third nucleotide from the 3′-end of RNA and the last cut was at the sixth nucleotide from the 5′-end. Due to low hybridization affinity of modified ODNs the samples were incubated with RNase H at 30 °C. The cleaved RNA was visualized by autoradiography after separation on PAGE. Results of 5 and 30 min cleavage and its interpretation are presented in Figure 3 and Table 4.

Hexofuranosyl nucleotides **1** and **2** allowed RNase H to cleave the RNA complement to the 3′-side of the modifications. Some inhibition of cleavage was observed from the 5′-side where **1** affected cleavage four nucleotides away (**ODN 20** and **21**) whereas **2** affected cleavage after the next neighbouring nucleotide (**ODN 2**). Two nucleotides **1** inserted in the middle of the sequence (**ODN 22**), inhibited cleavage, except for one cut in the complementary RNA to the 3′-side of the modification. However, this complex also showed a T_m (25.3 °C) lower

Table 4. RNA cleavage by RNase H versus position of **1**[8] and **2** in ODNs[a]

No.	3′-C G A A G A G A U C U U A C-[^{32}P]
ODN 1	5′-G C T/T/C |T‖C T|A G A A T G
ODN 20	5′-G C T T C **1**‖C/ T|A G A A T G
ODN 21	5′-G C/T|T C T C **1**‖A G A A T G
ODN 2	5′-G C|T‖T/C/T C **2**|A G A A T G
ODN 22	5′-G C T T C **1** C **1** |A G A A T G
ODN 23	5′-G C T **1** C‖T ‖C T‖A G A A T G
ODN 3	5′-G C T **2** C‖T ‖C T‖A G A A T G
ODN 24	5′-G C **1** **1** C| T ‖C T |A G A A T G
ODN 4	5′-G C **2** **2** C| T ‖C T |A G A A T G
ODN 25	5′-G C T|T/C|T ‖C/T/A G A A **1** G
ODN 5	5′-G C T|T/C|T ‖C/T/A G A A **2** G

[a] / - weak, | - medium, || - strong cleavage of RNA.

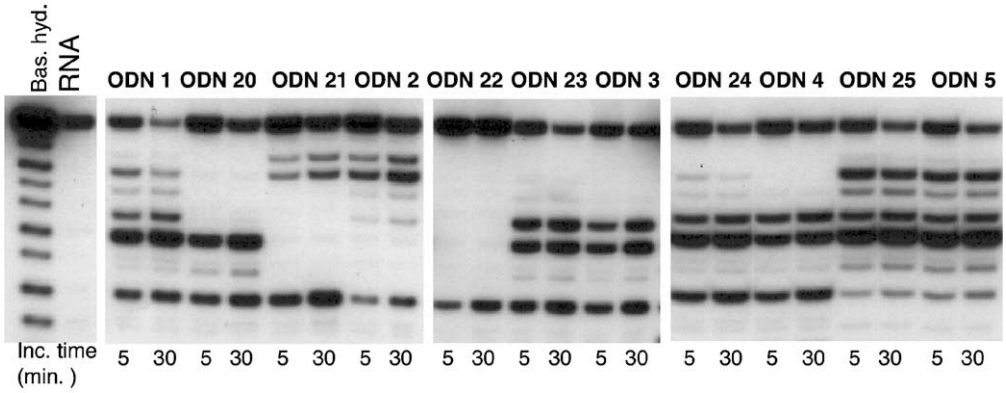


Figure 3. Gel-assay of RNA cleavage by RNase H. ODN/RNA samples were incubated for 5 and 30 min.

than the assay temperature. The presence of modifications close to the 3′-end of ODN only had a minor effect on the cleavage pattern (**ODN 5** and **25**).

The observed cleavage pattern is in agreement with previous published results that RNase H starts cleaving complementary RNA ca. 3 nucleotides from the 5′-end of the DNA and tracks the DNA/RNA hybrid until its end where it cleaves the RNA 5-6 nucleotides from the 3′-end of the DNA.[18] The gel pattern indicated that although modifications inhibited one or more cuts, RNase H could slide through the modified region or bind again on the other side of the modification. That means it is possible to design modified ODNs, which allow cleavage by RNase H only at the desired positions of RNA. However good thermal stability with RNA and DNA-like modifications seems to be imperative.

5. Conclusion

We prepared 1-(2,3-dideoxy-β-D-*erythro*-hexofuranosyl)thymine (**2**) starting from thymidine through the stereoselective formation of the new stereocentre at C-5′ position using AD-mix-α. The corresponding phosphoramidite **11** was used in the synthesis of various ODNs. Thermal stability of ODNs with complementary DNA and RNA along with RNase H activity towards mixed-sequence ODN/RNA complexes were studied and compared with 1-(2,3-dideoxy-3-amino-α-D-*arabino*-hexofuranosyl)thymine (**1**). The insertion of **2** in the middle of poly d(αT) sequence gave a stable complex with dA_{14} at low ionic strength whereas a decrease in stability was observed in medium and high salt buffers. RNase H was able to cleave complementary RNA at the 3′-site of **1** and **2** in ODNs whereas some inhibition was observed to the 5′-site.

6. Experimental

NMR spectra were recorded on a Varian Gemini 2000 spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C Internal standards used in ^{1}H NMR spectra were TMS (δ: 0.00) for $CDCl_3$, CD_3OD; in ^{13}C NMR were $CDCl_3$ (δ: 77.0), CD_3OD (δ: 49.0). Accurate ion mass determinations were performed using the 4.7 T Ultima Fourier transform (FT) mass spectrometer (Ion Spec, Irvine, CA). The $[M+H]^+$ and $[M+Na]^+$ ions were peak matched using ions derived from the 2,5-dihydroxybenzoic acid matrix. Thin layer chromatography (TLC) analyses were carried out with use of TLC plates 60 F_{254} purchased from Merck and were visualized in an UV light (254 nm). The silica gel (0.040–0.063 mm) used for column chromatography was purchased from Merck. Solvents used for column chromatography were distilled prior to use, while reagents were used as purchased.

6.1. 3′-Deoxythymidine (5)

To a solution of thymidine (4.84 g, 20 mmol) and *tert*-butyldimethylsilyl chloride (3.77 g, 25 mmol) in DMF (75 mL) at 0 °C was added imidazole (1.7 g, 25 mmol). After 3 h 1,1′-thiocarbonyldiimidazole (4.95 g, 25 mmol) was added and the reaction mixture was heated at 80 °C for 2.5 h. The mixture was allowed to cool to room temperature and poured into chilled H_2O (750 mL). The precipitate was filtered off, washed with H_2O (100 mL × 2), dried in vacuo overnight affording 5′-*O*-(*tert*-butyldimethylsilyl)-3′-*O*-(1-imidazolylthiocarbonyl)thymidine (**4**)[11] as a yellowish solid in 95% yield (8.80 g).

The mixture of **4** (7.90 g, 16.9 mmol), $(n\text{-Bu})_3SnH$ (5.9 mL, 22 mmol) and AIBN (280 mg, 1.7 mmol) in 1,4-dioxane (100 mL) was refluxed under nitrogen for 7 h. The reaction mixture was then cooled to room temperature and 50% aq CF_3COOH (20 mL) was added. The stirring was continued for 4 h, and then solvents were removed in vacuo to the 1/3 of initial volume. CH_3CN (100 mL) was added and the mixture was extracted with cyclohexane (60 mL × 4). The lower layer containing UV-active products was neutralized by 25% NH_4OH (6 mL) and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (0–10% $MeOH/CH_2Cl_2$) to give **5** (75%, 2.86 g) as a solid. ^{1}H NMR is in agreement with the previously published data.[10]

6.2. 3′,5′-Dideoxy-5′-methylenethymidine (7)

A suspension of *o*-iodoxybenzoic acid[19] (4.2 g, 15 mmol), 3′-deoxythymidine (**5**, 2.45 g, 10.1 mmol) in dry CH_3CN (90 mL) was refluxed for 1 h. The reaction mixture was then cooled to room temperature, filtered. The precipitate was washed with CH_3CN (20 mL × 3). The combined CH_3CN phases were concentrated in vacuo to give 3′,5′-dideoxy-5′-oxothymidine (**6**)[12,13] (90%, 2.0 g) as a white foam that was used in the next step without purification.

$MePPh_3Br$ (7.6 g, 21.5 mmol) was suspended in dry THF (125 mL) and cooled to 0 °C. Lithium bis(trimethylsilyl)amide (21.5 mL of an 1 M solution in THF, 21.5 mmol) was added dropwise under nitrogen and the red suspension was stirred for 30 min before the crude 5′-aldehyde (**6**, 2.0 g, 9.1 mmol) dissolved in THF (50 mL) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 1 h and then cooled by ice-water bath. H_2O (100 mL) was added slowly. EtOAc (100 mL) was added and layers were separated. The water phase was extracted with $CHCl_3$ (75 mL × 4) until no UV-active compounds were detected by TLC in the water layer. Combined organic layers were dried ($MgSO_4$) and filtered. Silica gel (5.0 g) was added and solvents were removed in vacuo. The residue was purified using dry column vacuum chromatography[20] and EtOAc (10–50%, v/v) in cyclohexane as an eluent to afford compound **7** (2.5 g). Although contaminated with $Ph_3P{=}O$, this compound was used for the next step. ^{1}H NMR ($CDCl_3$) δ 1.70 (m, 1H, 3′-H), 1.90 (s, 3H, Me), 2.05–2.15 (m, 3H, 3′-H, 2′-H), 2.20 (m, 1H, 2′-H), 4.52 (m, 1H, 4′-H), 5.28 (d, 1H, $J = 10.4$ Hz, 6′-H), 5.38 (d, 1H, $J = 17.0$ Hz, 6′-H), 6.0 (ddd, 1H, $J = 17.0$, 10.4, 4.2 Hz, 5′-H), 6.14 (dd, 1H, $J = 3.4$, 6.6 Hz, 1′-H), 7.35 (s, 1H, 6-H), 9.70 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 12.6 (Me), 29.6 (C3′), 32.6 (C2′), 81.7 (C4′), 86.2 (C1′), 110.2 (C5), 117.5 (C6′), 135.3 (C6), 136.4 (C5′), 150.4 (C2), 164.1 (C4); HR-ESI-MS m/z 467.190 calcd for $C_{22}H_{28}N_4O_6Na$ $[M_2+Na]^+$, found m/z 467.189.

6.3. 1-(2,3-Dideoxy-β-D-*erythro*-hexofuranosyl)thymine (9)

AD-mix-α (12.5 g) dissolved in H_2O (75 mL) and *t*-BuOH (75 mL) were stirred at room temperature until two clear phases were observed (20 min). Compound **7** (2 g) dissolved in *t*-BuOH (20 mL) was added to the mixture and vigorous stirring was continued for 24 h at room temperature. Na_2SO_3 (12.5 g) was added and the mixture was stirred for 3 h. Silica gel (2 g) was added and solvents were removed in vacuo. The residue was chromatographed using dry column vacuum chromatography[20] with MeOH (2–7%, v/v) in $CHCl_3$ to afford epimeric mixture **8** (30% from **6**, 820 mg) as a colourless oil (1:9 ratio of epimers (*S*:*R*), as estimated from the integral of the individual H-6 signals in the ^{1}H NMR spectrum). Crystallization of **8** from acetone gave compound **9** (20% from **6**, 540 mg) as white crystals. R_f, ^{1}H NMR and ^{13}C NMR are in agreement with the previously published data.[9]

Asymmetric dihydroxylation of alkene **7** using AD-mix-β under the same conditions gave the epimeric mixture **8** as a colourless oil (4:5 ratio of epimers (*S*:*R*)). Yield 24% from **6**.

6.4. 1-[2,3-Dideoxy-6-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-hexofuranosyl]thymine (10)

Compound **9** (200 mg, 0.78 mmol) was dissolved in anhyd pyridine (10 mL) and 4,4′-dimethoxytrityl chloride (290 mg, 0.86 mmol) was added under nitrogen. After 3 h MeOH (1 mL) was added and solvents were removed in vacuo to half the volume. EtOAc (30 mL) was added and extracted with satd aq $NaHCO_3$ (10 mL × 3). The water phase was extracted with EtOAc (10 mL × 2). The combined organic layers were dried ($MgSO_4$), filtered and evaporated under diminished pressure. The residue was co-evaporated twice with toluene/EtOH (5 mL, 1:1, v/v). The residue was adsorbed on a silica gel (0.5 g) from $CHCl_3$ (10 mL) and purified using dry column vacuum chromatography[20] with MeOH (0–2%, v/v) in $CHCl_3$ to afford compound **10** (71%, 310 mg) as a white foam. ^{1}H NMR ($CDCl_3$) δ 1.80 (m, 1H, 3′-H), 1.84 (s, 3H, Me), 2.00 (m, 2H, 3′-H, 2′-H), 2.38 (m, 1H, 2′-H), 2.99 (s, 1H, OH), 3.20 (m, 2H, 6′-H), 3.78 (s, 6H, 2 × OMe), 4.18 (m, 1H, 5′-H), 4.21 (m, 1H, 4′-H), 6.06 (dd, 1H, $J = 3.2$, 7.0 Hz, 1′-H), 6.80–6.86, 7.25–7.45 (m, 13H, Ar), 7.69 (d, 1H, $J = 1$ Hz, 6-H), 9.30 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 12.5 (Me), 23.4 (C3′), 32.3 (C2′), 55.1 (OMe), 64.6 (C6′), 71.0 (C5′), 81.6 (C4′), 85.4 (C1′), 86.4 (*C*Ar_3), 110.2 (C5), 113.1, 126.9, 127.8, 128.0, 129.9, 136.4, 144.4, 158.5 (DMT), 135.6 (C6), 150.5 (C2), 164.0 (C4); HR-ESI-MS m/z 581.334 calcd for $C_{32}H_{34}N_2O_7Na$ $[M+Na]^+$, found m/z 581.333.

6.5. 1-{2,3-Dideoxy-6-*O*-(4,4′-dimethoxytrityl)-5-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-β-D-*erythro*-hexofuranosyl}thymine (11)

Compound **10** (150 mg, 0.27 mmol) was dissolved under nitrogen in anhyd CH_2Cl_2 (10 mL). *N*,*N*-Diisopropylammonium tetrazolide (97 mg, 0.56 mmol) was added followed by dropwise addition of 2-cyanoethyl tetraisopropylphosphordiamidite (190 mg, 0.59 mmol). After 24 h analytical TLC showed no more starting material and the reaction was quenched with H_2O (1 mL) followed by addition of CH_2Cl_2 (10 mL). The mixture was washed with satd aq $NaHCO_3$ (10 mL × 2). The org. phase was dried (Na_2SO_4), filtered, silica gel (0.3 g) was added and solvents were removed under reduced pressure. The residue was purified using silica gel dry column vacuum chromatography[20] with cyclohexane/EtOAc (25–50%). Combined UV-active fractions were evaporated in vacuo affording **11** (125 mg, 61%) as a foam that was co-evaporated with dried CH_3CN (30 mL × 3) before using in ODN synthesis. ^{1}H NMR ($CDCl_3$) δ 1.20 (m, 12H, 4 × Me [Pr^i]), 1.80–1.90 (m, 5H, Me, 3′-H), 2.05 (m, 1H, 2′-H), 2.37 (m, 1H, 2′-H), 2.43 (t, 2H, CH_2CN), 2.58 (t, 2H, CH_2CH_2CN), 3.10–3.40 (m, 2H, 6′-H), 3.42–3.60 (m, 2H, 2 × CH [Pr^i]), 3.80 (s, 6H, 2 × OMe), 4.18–4.26 (m, 2H, 5′-H, 4′-H), 6.06 (2 × d, 1H,

$J = 5.0$ Hz, 1′-H), 6.80–6.85, 7.20–7.45 (m, 13H, Ar), 7.52 (s, 1H, 6-H), 9.90 (br s, 1H, NH); ^{31}P NMR ($CDCl_3$) δ 149.7, 150.4 in ratio 5:4, respectively; HR-ESI-MS m/z 759.364 calcd for $C_{41}H_{41}N_4O_8P$ $[M+H]^+$, found m/z 759.363.

6.6. Synthesis and purification of modified oligodeoxynucleotides

ODNs were synthesized on an Expedite™ Nucleic Acid Synthesis System Model 8909 from Applied Biosystems using an increased coupling time (5 min) for the phosphoramidite **11** and 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-α-thymidine. The 5′-DMT-on derivatives were cleaved off from the solid support (rt, 3 h) and deprotected (60 °C, overnight) using 32% aqueous ammonia for standard support or the solution of 2% LiCl in 32% aqueous ammonia (w/w) when using universal CPG support (BioGenex) for α nucleotides. Purification of 5′-*O*-DMT-on ODNs was accomplished using a Waters Delta Prep 4000 Preparative Chromatography System on a Waters Xterra™ MS C_{18} column. Buffer A [950 mL of 0.1 M NH_4HCO_3 and 50 mL of CH_3CN, (pH = 9.0)] and buffer B [250 mL of 0.1 NH_4HCO_3 and 750 mL of CH_3CN, (pH = 9.0)]. Gradients: 5 min 100% A, linear gradient to 70% B in 30 min, 2 min with 70% B, linear gradient to 100% B in 8 min and then 100% A in 15 min (product peak at ~33–37 min). The ODNs were DMT deprotected in 100 μL 80% aq acetic acid, diluted with 1 M aq NaOAc (150 μL) and precipitated from ethanol (550 μL). However for **ODN 13**–**ODN 15**, **ODN 17**–**ODN 19** (Table 1) a second HPLC purification was needed due to failure of the precipitation procedure. In this case volatile salts were removed by several co-evaporations with water. The modified ODNs were confirmed by MALDI-TOF analysis on a Voyager Elite Biospectrometry Research Station from PerSeptive Biosystems. Melting temperature measurements were performed on a Perkin–Elmer UV–vis spectrometer fitted with a PTP-6 temperature programmer. Melting temperature (T_m, °C) was determined by measuring absorbance at 260 nm against increasing temperature (1.0 °C per 1 min) on equimolar mixtures (1.5 μM in each strand) of modified oligomer and its complementary DNA/RNA in phosphate buffers with different NaCl concentration. Before each experiment, all samples were heated at 60 °C for 5 min and then cooled to room temperature. The melting temperature was determined as the maximum of the first derivative plots of the melting curve. All melting temperatures are within the uncertainty ±1.0 °C as determined by repetitive experiments.

6.7. RNase H assay

The 5′-CATTCTAGAGAAGC-3′ RNA was [^{32}P]-labelled at the 5-end with T4 kinase following standard procedures. Radioactive RNA was mixed with unlabelled RNA. 0.2 μM RNA (1 pmol/final sample) was incubated in the presence of a 4-fold excess of various complementary ODNs in hybridization buffer (20 mM Tris–HCl, pH 7.5, 100 mM KCl). The reactions were incubated at 65 °C for 2 min followed by slow cooling to 30 °C. An equal volume of digest buffer (20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, 2 mM DTT) with 0.2 U of *E. coli* RNase H (Amersham) per final sample were added and incubation continued at 30 °C. Aliquotes of 10 μL samples were withdrawn and mixed with 5 μL formamide loading dye with 10 mM EDTA on ice at the time points 5 and 30 min after RNase H addition. A basic hydrolysis of labelled RNA were performed by heating to 90 °C for 15 min in 100 mM Na_2CO_3 (pH 9.0, 2 mM EDTA) followed by cooling on ice and addition of formamide dye. All reactions were analyzed by PAGE (20% polyacrylamide containing 8.3 M urea). The radioactive RNA bands were visualized by autoradiography of the dried gels.

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