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Synthesis and Evaluation of Oligodeoxynucleotides Containing Acyclic Nucleosides: Introduction of Three Novel Analogues and A Summary

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Abstract—Novel flexible oligodeoxynucleotides containing (*S*)-1-(2,3-dihydroxypropyl)thymine or 2',3'-*seco*-thymidine nucleoside analogues were synthesized on an automated DNA-synthesizer. Oligodeoxynucleotides with one, two or three acyclic nucleosides incorporated in the middle or in the ends of 17-mers have been evaluated. 3'-End-modified oligomers were significantly stabilized towards 3'-exonucleolytic degradation compared to unmodified analogues and showed acceptable hybridization properties as measured by UV experiments. For oligodeoxynucleotide analogues containing the three novel acyclic monomers in the middle, a more pronounced reduction in duplex stability was observed. All oligodeoxynucleotides containing acyclic nucleoside analogues made so far are evaluated with respect to stability towards 3'-exonucleolytic degradation and hybridization properties.

Introduction

Oligodeoxynucleotide analogues are promising therapeutic agents for treatment of infectious diseases and cancers by acting as inhibitors of gene expression.[1,2] This requires design and synthesis of oligodeoxynucleotides, e.g. resistant towards cellular nucleases and capable of hybridizing with appropriate specificity and affinity to complementary target nucleic acids. Therefore, oligonucleotides have to be chemically modified, and modifications in the phosphate moiety[3–9] or in the carbohydrate part[3,10–21] have been intensively studied, but whereas enhanced resistance towards nucleases often has been achieved, a decrease in the stability of the resulting duplexes has frequently been observed.

The replacement of the sugar moiety by an acyclic structure has been used to increase the enzymatic stability of oligonucleotides, and eight different acyclic nucleosides have been investigated so far as monomeric substitutes. Thus, glyceronucleoside analogues, which lack two bonds (C1'–C2'–C3') compared to the natural 2'-deoxy-D-ribose,[22–25] and three different nucleosides lacking the C4'–O–C1' moiety [(*S*)-9-(3,4-dihydroxybutyl)adenine,[26] 3(*S*),5-dihydroxypentyl- and 4(*R*)-methoxy-3(*S*),5-dihydroxypentyladenine[27]] have been incorporated into oligodeoxynucleotides. Additionally, we recently described the synthesis of oligodeoxynucleotides containing four different nucleosides lacking the C3'–C4' bond.[28] The hybridization properties and the stability towards exonucleases of all these analogues are described in the Discussion section.

As an attempt to further investigate and improve the potential of acyclic oligodeoxynucleotide analogues with respect to nucleic acid recognition and biological applications, this paper describes the synthesis and incorporation of three different acyclic nucleosides into novel oligodeoxynucleotide analogues. The glycerol derivative **2** was chosen because it induces an extremely contracted and small oligonucleotide backbone being less flexible than most other acyclic analogues. Furthermore, the synthesis of the nucleoside **2** is known and straightforward with possibilities to be even further simplified. The 2',3'-*seco*-nucleoside **11** was synthesized as this represents a hitherto non-investigated possibility for cleaving the sugar ring in DNA-substitutes.[27] In this nucleoside the C2'–C3' bond is missing, but as it contains a 2'-hydroxyl-group it is not a true deoxy-analogue. Nevertheless, this nucleoside was chosen because the extra hydroxyl group via hydrogen bonding between the 2'- and 3'-functionalities might stabilize a duplex compared to oligodeoxynucleotide analogues containing acyclic nucleosides lacking C-2'.[23] The nucleoside **11** was incorporated into oligonucleotides both through *O*-3' and *O*-2'. Incorporation of **13** (through *O*-3') resulted in DNA-analogues with an intact backbone comparable with other acyclic analogues lacking one or two of the bonds C4'–O'–C1'–C2'–C3'.[23,26,27] Incorporation of **15** (through *O*-2') resulted in DNA-analogues in which the backbone was one atom shorter than for the earlier synthesized 3',4'-*seco*-DNA[28] which might induce improved base-pairing properties.

Finally, as almost all obvious possibilities for cleaving the ribose ring in DNA substitutes have been carried out, we compare the results obtained from melting and enzymatic stability experiments on all hitherto synthesized oligodeoxynucleotide analogues containing acyclic nucleosides.

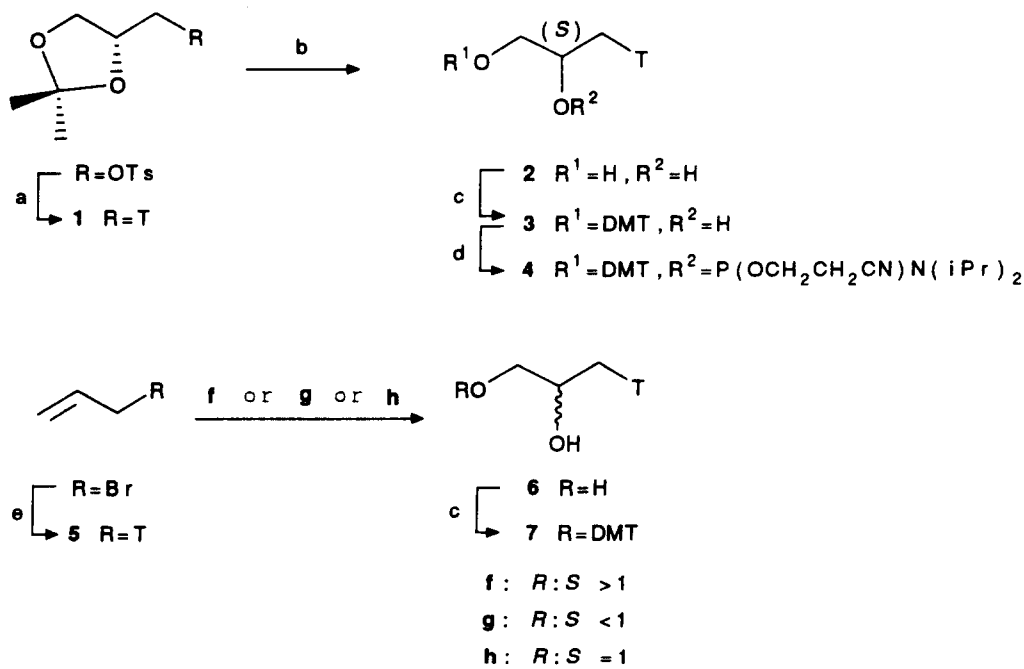
Results and Discussion

Synthesis of the acyclic nucleosides

Synthesis of (*S*)-1-(2,3-dihydroxypropyl)thymine (**2**) has been reported earlier from different starting compounds, e.g. 1-*O*-tosyl-2,3-*O*-isopropylidene-D-glycerol using the sodium salt of thymine.[29] The racemic mixture **6h** (**2** and its enantiomer) has been reported in a number of papers. Thus, reaction of allylbromide with dimethylated thymine[30] or disilylated thymine[31] and subsequent dihydroxylation of allylthymine using osmium tetroxide has been accomplished. Besides, reaction of thymine with 2,3-*O*-isopropylideneglycerol chlorohydrin[32] or with glycidol[33] using potassium carbonate afforded the racemic mixture.

By reaction of thymine with 1-*O*-tosyl-2,3-*O*-isopropylidene-D-glycerol and caesium carbonate we obtained the desired N-1 alkylated thymine derivative **1** in 37% yield together with the N-3 alkylated product **1a** (6%) and the dialkylated thymine derivative **1b** (25%). Compound **1** was deprotected in acetic acid to give **2** in 95% yield. Reaction of **2** with 4,4'-dimethoxytrityl-chloride (DMTCl) in anhydrous pyridine afforded **3** in 89% yield (Scheme 1).

As other potential strategies for the synthesis of **2**, we investigated alkylation of disilylated thymine with 1-*O*-tosyl-2,3-*O*-isopropylidene-D-glycerol, reaction of thymine with (*R*)-glycidol and asymmetric dihydroxylation of allylthymine using Sharpless reagent. Reaction of disilylated thymine with 1-*O*-tosyl-2,3-*O*-isopropylidene-D-glycerol did not succeed in our hands. The alkylation of thymine with (*R*)-glycidol was possible but the N-1 and N-3 alkylated products were impossible to separate. Reaction of thymine with allylbromide using *in situ* silylation as reported[31] afforded **5** in 90% yield (Scheme 1). Catalytic dihydroxylation of the allyl-moiety using osmium tetroxide and *N*-methylmorpholine *N*-oxide (NMO) afforded the racemic mixture **6h** in 52% yield. Attempts to asymmetrically dihydroxylate allylthymine (**5**) using Sharpless reagents, afforded with AD-mix-α[34] and AD-mix-β[34] in good yields (80 and 90%) products **6f** and **6g**, respectively. The three products **6f–h** were reacted with DMTCl to give enantiomeric mixtures **7f–h**. Measurements of specific rotations of **7f** and **7g** showed that these products were contaminated with the enantiomer and that the enantiomeric excess were around 23%. Sharpless reagents are thus inapplicable for asymmetric dihydroxylation of allylthymine. These disappointing results were verified by the experiment shown in Figure 1. The tritylated products **3** and **7f** were reacted with commercial thymidine 3'-*O*-2-(cyanoethyl)phosphoramidite in the presence of tetrazole as activator, and the dinucleotide phosphites **8** and **9** were obtained in good yields. Because of the chirality of phosphorus, a dinucleotide phosphite derived from an enantiomerically pure glycerol derivative will consist of two different diastereomeric compounds appearing as two different signals in a ^{31}P NMR spectrum. Analogously, a dinucleotide derivative, which is synthesized from an enantiomeric mixture **7**, will contain four diastereomeric compounds causing four different signals in a ^{31}P NMR spectrum. In Figure 1, the two ^{31}P NMR spectra obtained from **8** and **9** are shown and the results obtained with measurements of specific rotations are confirmed. Thus, in the spectrum of **9**, the peaks from the (*S*)-isomer (which are identified in the spectrum of compound **8** synthesized



Scheme 1. (a) Thymine, Cs_2CO_3, NaI, DMF, (b) 80% AcOH, (c) DMTCl, pyridine, (d) $NCCH_2CH_2OP(Cl)N(iPr)_2$, CH_2Cl_2, $EtN(iPr)_2$, (e) thymine, HMDS, CH_3CN, DMSO, (f) AD-mix α, *t*-BuOH, H_2O, (g) AD-mix β *t*-BuOH, H_2O, (h) OsO_4, NMO, *t*-BuOH, H_2O. T = thymin-1-yl.

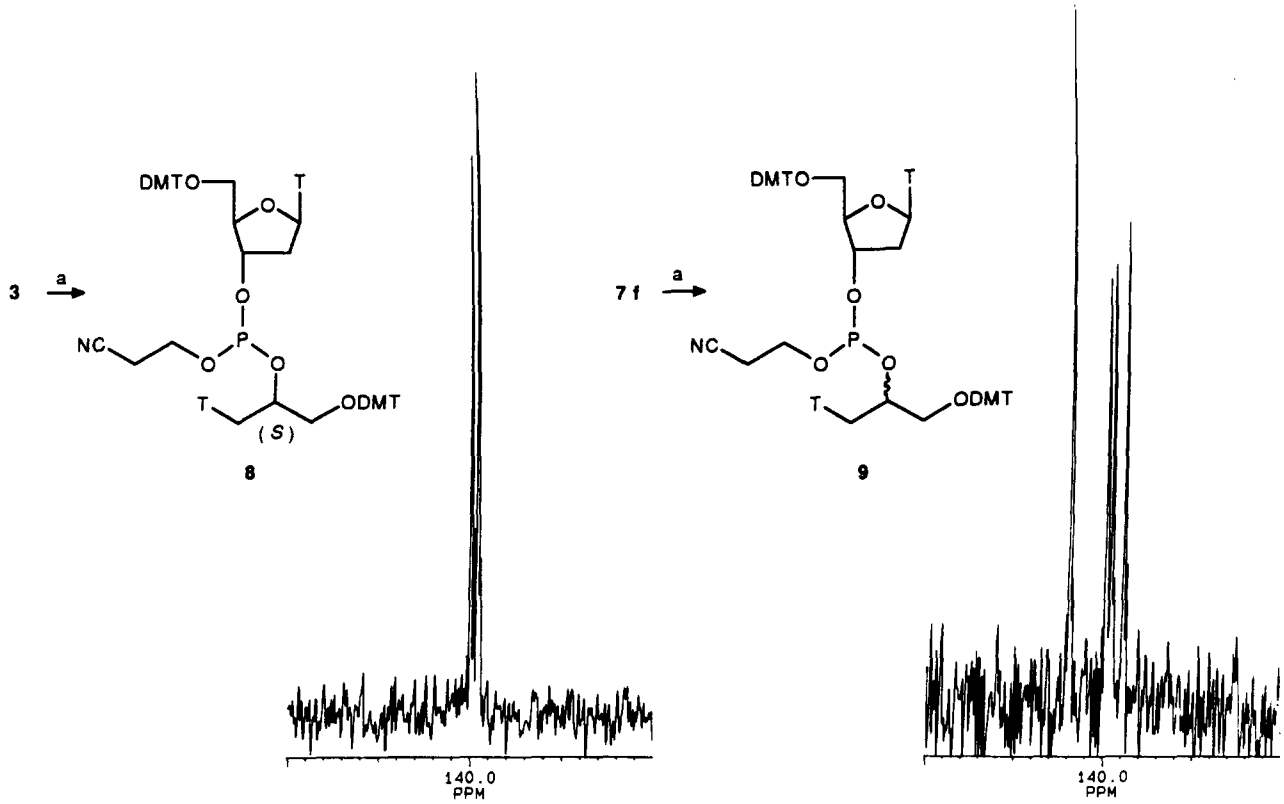


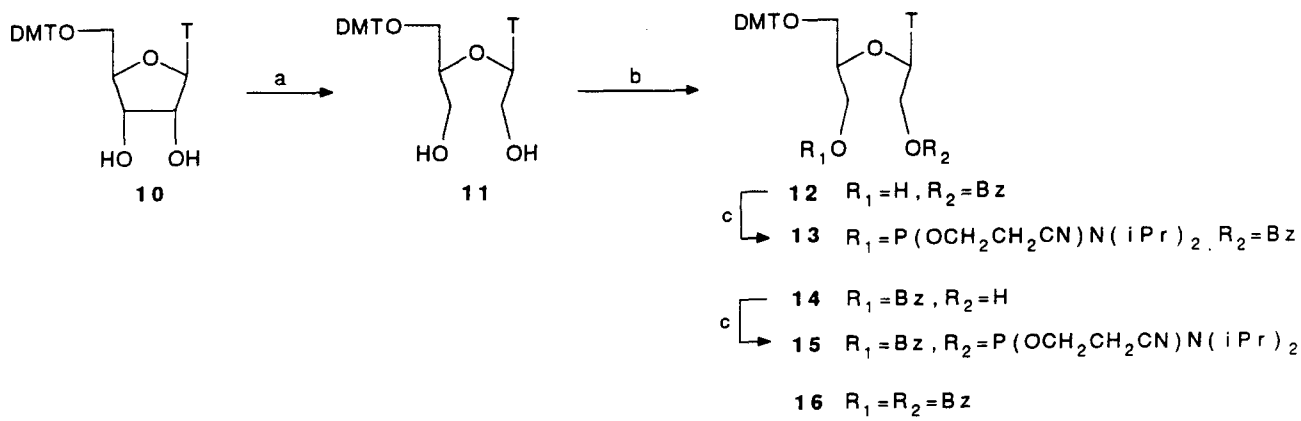
Figure 1. Synthesis and ^{31}P NMR spectra of dinucleotide phosphites.

from the enantiomeric pure glycerol analogoue **2**) are only of slightly less intensity than the peaks from the expected (*R*)-isomer.

For the incorporation of the acyclic nucleoside **11** into oligodeoxynucleotides, 2'-hydroxythymidine was reacted with DMTCl in dry pyridine to give 5'-*O*-(4,4'-dimethoxytrityl)-2'-hydroxythymidine (**10**).[35] Using an earlier published method, the ribose ring was opened by periodate oxidation followed by reduction with sodium borohydride to give 5'-*O*-(4,4'-dimethoxytrityl)-2',3'-*seco*-2'-hydroxythymidine (**11**)[36] in 88% yield. Benzoylation was carried out at −55 °C in dry pyridine with benzoylchloride[36] to give the 2'-*O*- and 3'-*O*-benzoylated derivatives **12** and **14** in 38% yield and 17% yield, respectively, together with the dibenzoylated derivative **16** in 12% yield (Scheme 2).

*Building blocks **4**, **13** and **15** and their incorporation into oligodeoxynucleotides*

The three DMT-protected nucleosides **3**, **12** and **14** were reacted with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite in the presence of *N*,*N*-diisopropylethylamine[37,38] affording **4**, **13** and **15** in good yields (85–93%) after precipitation in cold petroleum ether. The oligodeoxynucleotides **17–28** (Table 1) were synthesized by standard phosphoramidite methodology on an automated DNA-synthesizer using the appropriate building blocks (**4**, **13**, **15** and commercial 2'-deoxynucleoside 3'-*O*-2-(cyanoethyl) phosphoramidites). The coupling efficiencies of each of the acyclic phosphor-



Scheme 2. (a) $NaIO_4$, dioxane, H_2O; $NaBH_4$, (b) BzCl, pyridine, (c) $NCCH_2CH_2OP(Cl)N(iPr)_2$, CH_2Cl_2, $EtN(iPr)_2$. T = thymin-1-yl.

Table 1. Sequences and melting experiments of synthesized oligodeoxynucleotides

Sequence	No.	T_m / °C	ΔT_m / °C	H_m
5'-CACCAACTTCTTCCACA-3'	**17**	60.5	-	1.14
5'-CACCAACTTCTXCCACA-3'	**18**	52.0	8.5	1.12
5'-CACCAACXXCTXCCACA-3'	**19**	37.5	7.7	1.09
5'-CACCAACYTCTTCCACA-3'	**20**	50.0	10.5	1.22
5'-CACCAACYTCTYCCACA-3'	**21**	42.0	9.3	1.12
5'-CACCAACZTCTZCCACA-3'	**22**	47.5	6.5	1.18
5'-TTAACTTTCTTCACATTC-3'	**23**	51.5	-	1.16
5'-XTAACTTCTTCACATXC-3'	**24**	46.5	2.5	1.13
5'-TTAACTTCTTCACAXXC-3'	**25**	45.5	3.0	1.14
5'-TTAACTTCTTCACATYC-3'	**26**	49.0	2.5	1.20
5'-TTAACTTCTTCACAYYC-3'	**27**	46.0	2.8	1.18
5'-TTAACTTCTTCACAZZC-3'	**28**	41.5	5.0	1.19

A = 2'-deoxyadenosine, C = 2'-deoxycytidine, T = thymidine, X, Y and Z = acyclic monomers derived from **4**, **13** and **15**, respectively. T_m = melting temperature, ΔT_m = decrease in T_m per modification, H_m = thermal hyperchromicity.

amidites were approximately 90% as monitored by the release of the dimethoxytrityl cation after each coupling step compared to approximately 99% for unmodified monomers. The oligonucleotides were removed from the support and all protecting groups except 5'-*O*-DMT removed with concentrated ammonia. Purification of oligomers was performed using disposable reverse-phase chromatography cartridges.

The composition of the oligonucleotides **19**, **26** and **28** was verified by matrix assisted laser desorption mass spectrometry. Only negligible deviations between the calculated masses of **19** (4909.2 Da), **26** (5089.4 Da) and **28** (5107.4 Da) and the measured masses of **19** (4907.4 Da), **26** (5090.2 Da) and **28** (5107.3 Da) were observed. Because of the homogeneous results from the syntheses of all the modified oligodeoxynucleotides we consider their composition verified. Thus, during incorporation of monomer **4** containing a vicinal diol functionality in the backbone, we did not observe any problems comparable to those experienced earlier during incorporation of 1-(3,4-dihydroxybutyl) adenine.[26,27]

Melting experiments

The hybridization properties of the synthesized oligodeoxynucleotides were examined by mixing each oligomer with its complementary DNA-strand and determining the melting points of the DNA–DNA hybrids by UV measurements. In Table 1 the melting temperatures (T_m) and the differences between modified and unmodified oligomers as the decrease in melting temperature per modification (ΔT_m) are shown together with thermal hyperchromicities (H_m).

Incorporation of acyclic nucleoside(s) **4**, **13** or **15** in the middle of a sequence (**18–22**) results in ΔT_m of 6.5–10.5 °C compared to unmodified oligodeoxynucleotide **17**. Incorporation of one or two acyclic nucleosides in one or both ends (**24–28**) results in decreases in ΔT_m of 2.5–5.0 °C/modification compared to unmodified **23**. Incorporation of 2',3'-*seco*-thymidine monomer **13** in the middle of a sequence caused a decrease in T_m of around 10 °C/modification. This result is better than that obtained by Schneider *et al.*[23] indicating that the additional 1'-hydroxymethyl group may cause some stabilization of the DNA–DNA duplex. Nevertheless, the results are similar to those obtained with 3',4'-*seco*-DNA.[28] Incorporation of **4** caused decreases of around 8 °C/modification which are comparable to results for other modifications lacking the oxygen atom of the sugar ring.[27] Finally, the incorporation of **15** twice in the middle caused a relatively small decrease in T_m of 6.5 °C/modification. This is better than the results obtained for 3',4'-*seco*-DNA indicating either that the backbone is too long in 3',4'-*seco*-DNA and/or that the extra hydroxyl group present in **22** causes a stabilization of the DNA–DNA duplex. For all three novel analogues, the effect on duplex stability is significantly less negative in end-modified analogues **24–28**.

Enzymatic stability of the oligomers

As resistance towards destructive nucleases is an important requirement for antisense oligonucleotides, we studied the stability of the oligodeoxynucleotide analogues towards SVPDE (3'-exonuclease) by measuring the hyperchromicity.[16,28] Table 2 shows the results of the enzymatic hyperchromicity experiments. Incorporation of one or two modified acyclic nucleosides in one or both ends of the oligomer (**24–28**) results in a large increase in half-life and a decrease in the enzymatic hyperchromicity (calculated after 60 min digestion). One, two or three modifications in the middle of the sequences apparently have no effect on the stability of the full-length oligodeoxynucleotide analogues. However, the hyperchromicities calculated for **18–22** are smaller than the one calculated for the unmodified oligomer **17**, thus indicating rapid 3'-exonucleolytic degradation of the first five or nine monomers until increased enzymatic stability is induced by an acyclic monomer. There seems to be no significant differences between the three different acyclic modifications concerning the effect on the enzymatic stability of the oligodeoxynucleotides. In our earlier work on 3',4'-*seco*-nucleotides,[28] comparison of the results from hyperchromicity experiments and those obtained by following the digestion (SVPDE) of the 5'- end ^{32}P-labelled oligodeoxynucleotides using denaturating gel electrophoresis showed that the results obtained by UV-

Table 2. Enzymatic digestion of oligonucleotides

No.	$t_{1/2}$ / min	H_e	No.	$t_{1/2}$ / min	H_e
17	< 1	1.17	**23**	< 1	1.18
18	< 1	1.10	**24**	18	1.09
19	< 1	1.08	**25**	22	1.10
20	< 1	1.07	**26**	20	1.05
21	< 1	1.05	**27**	25	1.05
22	< 1	1.08	**28**	33	1.04

$t_{1/2}$ = half-life, H_e = enzymatic hyperchromicity after 60 min digestion.

experiments are reliable and can be used as a good indication of the effect on enzymatic stability caused by incorporation of unnatural monomers.

Acyclic nucleosides incorporated into oligodeoxynucleotides. A summary

A number of acyclic oligonucleotide substitutes have been introduced including polymers with a peptide backbone (PNA) instead of the natural phosphate containing backbone. PNA, which has been reported to form stable duplexes with DNA and RNA,[39–41] is excluded in this summary that concentrates on monomeric acyclic nucleoside analogues incorporated into oligonucleotides containing natural phosphate internucleoside linkages.

In Figure 2, the structures of the acyclic monomers which have been incorporated into oligodeoxynucleotides are shown. The nine acyclic analogues have been evaluated with respect to stability towards 3'-exonucleolytic degradation (SVPDE) and hybridization properties. The results are summarized in Table 3.

Obviously, it is difficult to draw definitive conclusions on the basis of the rather limited amount of data available. For instance, the dependence of melting temperature on base sequence and salt concentration is well established and e.g. oligomers containing homo-adenine sequences are more prone to form stable base-stacked structures compared to oligomers containing homo-thymine sequences. Therefore, good hybridization results are more probably obtained when the modified monomers are incorporated as adenosine analogues into poly-A-strands as indicated by the promising results obtained with the adenine monomer **G**.[27] Generally, the best results concerning hybridization properties of flexible DNA-analogues have been obtained after incorporation of acyclic nucleosides lacking one or both of the bonds connecting the ring-oxygen of the ribose to C-4' or/and C-1' (**F**, **G** and **I**). A backbone resembling the natural C3'–C4'–C5' have to be present as indicated by the disappointing results obtained with 3',4'-*seco*-nucleosides cleaved in the ribose-ring between C-3' and C-4' (**C** and **D**). Nucleoside **E**, however, shows acceptable hybridization which may be due to the OH-group in the 3'-position for which reason a similar acyclic nucleoside without OH-3' or C-3' should be incorporated into oligomers for comparison. The nucleoside lacking C-3' has hitherto only been tested in dimers for stability towards SVPDE.[42] The influence of the extra primary hydroxy group may also be the reason for the good hybridization of oligodeoxynucleotides containing nucleoside **B** compared to nucleoside **A**. Nucleoside **F** contains an extra carbon compared to unmodified

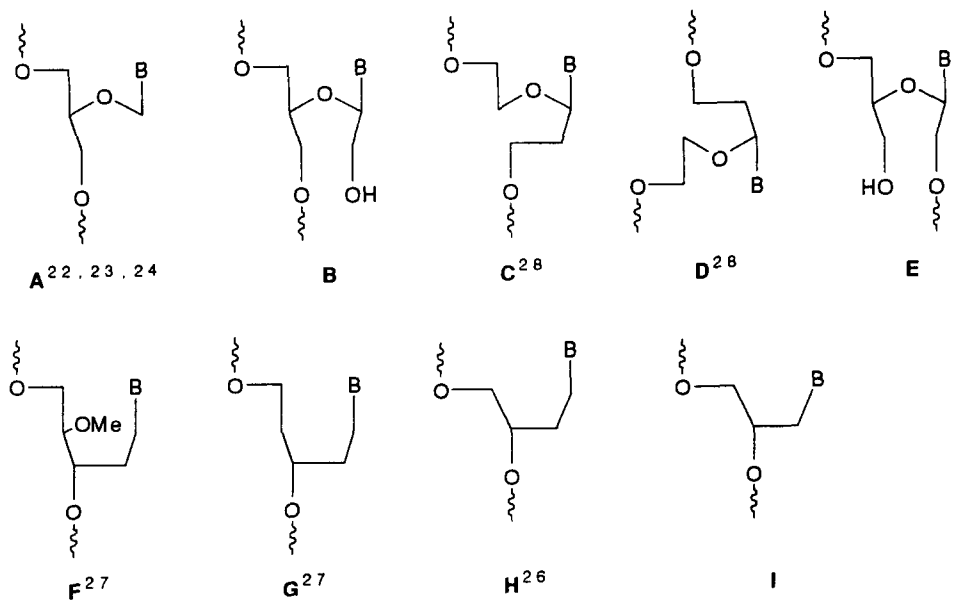


Figure 2. Structures of the different acyclic nucleosides so far incorporated into oligodeoxynucleotides.

Table 3. Evaluation of oligodeoxynucleotides containing acyclic monomers

Acyclic nucleoside incorporated	Bases incorporated	Average ΔT_m/°C *	Enzymatic stability $t_{1/2}$/ min. **
A[22,23,24,25]	T	13.4	stable***
B	T	9.9	20–25
C[28]	T	10.5	>10–20
D[28]	T	10.1	>10
E	T	6.5	33
F[27]	A	6.4	>200
G[27]	A, T, G, C, I	4.3 +, 6.3 ++	>200 +
H[26,27]	A	-	stable***
I	T	8.1	18–22

* Sequences with substitutions in the middle.
** Sequences with substitutions in the ends.
*** Dimer.
+Base = A.
++ Base = T.

nucleosides which may explain the lower melting points obtained with this nucleoside compared to nucleoside **G**. Nucleoside **I** lacks a carbon in the branch to the base compared to **G** which may be a reason for the lower melting points achieved with this nucleoside when compared to **G**.

It is clear that none of the oligodeoxynucleotide analogues containing acyclic monomers show hybridization properties comparable with those obtained for natural DNA or for analogues incorporating one of the more promising cyclic carbohydrate modifications.[19,21,43] This is a result of the more flexible nature of the acyclic monomers causing an unfavourable large loss in entropy upon duplex-formation. As shown in Table 3, all oligodeoxynucleotide analogues synthesized so far containing acyclic monomers in the 3'-end are significantly stabilized towards 3'-exonucleolytic degradation compared to unmodified analogues.

In conclusion, three novel acyclic monomers have been incorporated into oligodeoxynucleotides. Consistent with results obtained for other DNA-substitutes containing flexible acyclic monomers, a considerable decrease in melting temperature was observed with analogues modified in the middle of a sequence. A significantly less pronounced decrease in hybridization properties was experienced using 3'-end modified analogues. Effective protection against degradation by a 3'-exonuclease was observed for oligodeoxynucleotide analogues containing one or two 3'-end modifications. These results suggest that 3'-end capping of oligonucleotides with acyclic nucleoside monomers may be a useful strategy for design of antisense derivatives with adequate base-pairing capacity and excellent stability towards 3'-exonucleolytic degradation. However, development of all- or mid-modified acyclic oligonucleotide analogues into biologically active agents will require design and synthesis of less flexible monomers thereby potentially eliminating the unfavourable entropy contribution upon duplex-formation generally reflected in lowered melting points for acyclic analogues. Alternatively, incorporation of acyclic monomers introducing additional binding interactions may be a possible way of improving the hybridization properties. The data described here indicate that both these strategies depend on future syntheses of acyclic nucleoside monomers and oligonucleotides structurally different from the ring-cleaved analogues of the natural 2'-deoxynucleosides of which the vast majority hereby has been synthesized and evaluated.

Experimental

NMR spectra were recorded at 250 MHz for ^{1}H NMR and 63 MHz for ^{13}C NMR on a Bruker AC 250 spectrometer and at 202.33 MHz for ^{31}P NMR on a Varian Unity 500 spectrometer; δ values are in ppm relative to tetramethylsilane as internal standard (^{1}H and ^{13}C NMR) and relative to 85% H_3PO_4 as external standard (^{31}P NMR). EI mass spectra were recorded on a Varian Mat 311A spectrometer and FAB mass spectra were recorded on a Kratos MS50TC spectrometer. Optical rotations were obtained on a Perkin-Elmer 141 polarimeter. The silica gel (0 040–0 063 mm) used for column chromatography was purchased from Merck. Oligodeoxynucleotides were synthesized on a Pharmacia Gene Assemble Special® DNA-Synthesizer. Purification of 5'-*O*-DMT-ON oligodeoxynucleotides was accomplished using disposable Oligopurification Cartridges (COP, Cruachem). Snake venom phosphodiesterase (*Crotalus adamanteus*) was obtained from Pharmacia. Matrix assisted laser desorption mass spectra were obtained on a prototype laser desorption mass spectrometer from Applied Biosystem Sweden AB, Uppsala, Sweden.

(S)-2,2-Dimethyl-4-(thymin-1-ylmethyl)dioxolane (1)[29]

To a stirred solution of (*R*)-2,2-dimethyl-4-(tosyloxymethyl)dioxolane (500 mg, 1.75 mmol) and thymine (247 mg, 1.96 mmol) in anhydrous DMF (27 mL) was added Cs_2CO_3 (1.38 g, 4.24 mmol) and NaI (318 mg, 2.12 mmol). The mixture was stirred at 90 °C for 18 h and evaporated. The residue was dissolved in EtOAc (30 mL), washed with a saturated aqueous solution of $NaHCO_3$ (2 × 20 mL) and water (2 × 20 mL), dried (Na_2SO_4) and

evaporated. The crude product was purified by silica gel column chromatography (0–2% MeOH in CH_2Cl_2) to give **1** as a white solid material in 37% yield (154 mg, 0.62 mmol) (Lit.[29] 43% yield). Besides, (*S*)-2,2-dimethyl-4-(thymin-3-ylmethyl)dioxolane (**1a**) was isolated in 6% yield (25 mg, 0.10 mmol) (Lit.[29] 35% yield) and (*S,S*)-1,3-di-(2,2-dimethyldioxolan-4-ylmethyl)thymine (**1b**) in 25% yield (113 mg, 0.32 mmol). **1**: ^{13}C NMR ($CDCl_3$): δ 12.21 (CH_3(T)), 25.05, 26.60 (CH_3a,b), 49.91 (C-1'), 66.33 (C-3'), 74.09 (C-2'), 109.90 ($CH_3\underline{C}CH_3$), 110.22 (C-5), 141.58 (C-6), 151.29 (C-2), 164.22 (C-4). ^{1}H NMR ($CDCl_3$): δ 1.35 (*s*, 3H, CH_3b), 1.42 (*s*, 3H, CH_3a), 1.92 (*s*, 3H, CH_3(T)), 3.69 (*dd*, 1H, *J* = 6.7 Hz, *J* = 14.2 Hz, H-1'a), 3.70 (*dd*, 1H, *J* = 6.2 Hz, *J* = 8.8 Hz, H-3'a), 4.01 (*dd*, 1H, *J* = 2.9 Hz, *J* = 14.2 Hz, H-1'b), 4.10 (*dd*, 1H, *J* = 6.6 Hz, *J* = 8.8 Hz, H-3'b), 4.39 (*m*, 1H, H-2'), 7.14 (*s*, 1H, H-6), 9.24 (*s*, 1H, NH). R_f 0.42 (MeOH:CH_2Cl_2, 1:19, v:v). **1a**: ^{13}C NMR ($CDCl_3$): δ 12.84 (CH_3(T)), 25.48, 26.64 (CH_3a,b), 43.34 (C-1'), 67.72 (C-3'), 73.17 (C-2'), 109.48 ($CH_3\underline{C}CH_3$), 110.03 (C-5), 134.49 (C-6), 153.12 (C-2), 163.96 (C-4). ^{1}H NMR ($CDCl_3$) δ: 1.33 (*s*, 3H, CH_3b), 1.46 (*s*, 3H, CH_3a), 1.93 (*s*, 3H, CH_3(T)), 3.85 (*dd*, 1H, *J* = 5.5 Hz, *J* = 8.6 Hz, H-3'a), 3.95 (*dd*, 1H, *J* = 5.6 Hz, *J* = 13.0 Hz, H-1'a), 4.05 (*dd*, 1H, *J* = 6.1 Hz, *J* = 8.6 Hz, H-3'b), 4.30 (*dd*, 1H, *J* = 6.4 Hz, *J* = 13.0 Hz, H-1'b), 4.43 (*m*, 1H, H-2'), 7.03 (*s*, 1H, H-6), 9.9 (*s*, 1H, NH). R_f 0.32 (MeOH:CH_2Cl_2, 1:19, v:v). **1b**: ^{13}C NMR ($CDCl_3$): δ 12.88 (CH_3(T)), 25.04, 25.54, 26.55, 26.63 (2 × CH_3a,b), 43.92 (C-1'$_{N-3}$), 50.93 (C-1'$_{N-1}$), 66.32 (C-3'$_{N-1}$), 67.80 (C-3'$_{N-3}$), 73.23 (C-2'$_{N-3}$), 74.04 (C-2'$_{N-1}$), 109.23, 109.36 (2 × $CH_3\underline{C}CH_3$), 109.75 (C-5), 139.71 (C-6), 151.83 (C-2), 163.62 (C-4). ^{1}H NMR ($CDCl_3$): δ 1.32 (*s*, 3H, CH_3b$_{N-3}$), 1.34 (*s*, 3H, CH_3b$_{N-1}$), 1.41 (*s*, 3H, CH_3a$_{N-1}$), 1.45 (*s*, 3H, CH_3a$_{N-3}$), 1.93 (*s*, 3H, CH_3(T)), 3.65–3.76 (*m*, 2H, H-1'a$_{N-1}$, H-3'a$_{N-1}$), 3.82 (*dd*, 1H, *J* = 5.6 Hz, *J* = 8.5 Hz, H-3'a$_{N-3}$), 3.92–4.12 (*m*, 5H, H-1'a$_{N-3}$, H-3'b$_{N-3}$, H-3'b$_{N-1}$, H-1'b$_{N-1}$, H-1'b$_{N-3}$), 4.29–4.43 (*m*, 2H, H-2'$_{N-1}$, H-2'$_{N-3}$), 7.12 (*d*, 1H, *J* = 0.8 Hz, H-6). R_f 0.72 (MeOH:CH_2Cl_2, 1:19, v:v).

(S)-1-(2,3-Dihydroxypropyl)thymine (2)[29]

Compound **1** (915 mg, 3.81 mmol) was reacted as described[29] to give **2** as white crystals in 98% yield (750 mg, 3.74 mmol). ^{13}C NMR (DMSO-d_6): δ 11.79 (CH_3), 50.71 (C-1'), 63.53 (C-3'), 69.03 (C-2'), 107.28 (C-5), 142.68 (C-6), 150.97 (C-2), 164.26 (C-4). ^{1}H NMR (DMSO-d_6): δ 1.75 (s, 3H, CH_3), 3.27–3.41 (*m*, 3H, 2 × H-3', H-1'a) 3.69 (*m*, 1H, H-2'), 3.88 (*dd*, 1H, *J* = 3.5 Hz, *J* = 13.5 Hz, H-1'b), 4.64 (*t*, 1H, *J* = 5.6 Hz, 3'-OH), 4.93 (*d*, 1H, *J* = 5.5 Hz, 2'-OH), 7.39 (*d*, 1H, *J* = 1.0 Hz, H-6), 11.14 (*br s*, 1H, NH). R_f 0.37 (MeOH:CH_2Cl_2, 3:17, v:v).

(S)-1-(3-(4,4'-Dimethoxytrityloxy)-2-hydroxypropyl) thymine (3)

To a stirred solution of **2** (770 mg, 3.85 mmol) in anhydrous pyridine (5 mL) was added 4,4'-dimethoxytritylchloride (1.43 g, 4.42 mmol). The solution was stirred for 24 h at room temperature, MeOH (0.5 mL) was added and the solvent evaporated under reduced pressure. The residue was diluted with $CHCl_3$ (10 mL), washed with water (3 × 10 mL), dried (Na_2SO_4) and evaporated. The residual gum was crystallized from benzene and cyclohexane (2:1) to give analytically pure **3** as white crystals in 89% yield (1.71 g, 3.40 mmol). ^{13}C NMR ($CDCl_3$): δ 12.15 (CH_3), 51.62 (C-1'), 55.18 (OCH_3), 64.49 (C-3'), 69.36 (C-2'), 86.48 ($Ph_3\underline{C}$), 110.01 (C-5), 113.24, 126.95, 127.90, 127.98, 129.92, 135.59, 144.50, 158.63 (arom.), 141.88 (C-6), 151.71 (C-2), 164.17 (C-4). ^{1}H NMR ($CDCl_3$): δ 1.82 (*d*, 3H, *J* = 0.7 Hz, CH_3), 3.18 (*d*, 2H, *J* = 5.2 Hz, H-1'), 3.64 (*m*, 1H, H-3'a), 3.77 (*s*, 6H, OCH_3), 3.95–4.02 (*m*, 2H, H-2', H-3'b), 4.07 (*br s*, 1H, OH), 6.80–7.42 (*m*, 13H, arom.), 7.04 (*s*, 1H, H-6), 9.15 (*br s*, 1H, NH). R_f 0.68 (MeOH:CH_2Cl_2, 1:9, v:v). MS (EI) *m/z* = 502 ([M]$^+$, 10%). Anal. calcd. for $C_{29}H_{30}N_2O_6$: C, 69.31; H, 6.02; N, 5.13: found: C, 69.66; H, 5.98; N, 5.05. $[\alpha]_D^{25}$ –13.85°.

(S)-1-(3-(4,4'-Dimethoxytrityloxy)-2-((2-cyanoethoxy-(diisopropylamino)phosphin)oxy)propyl)thymine (4)

Nucleoside **3** (316 mg, 0.629 mmol) was dried by co-evaporation with anhydrous CH_3CN (3 × 10 mL) and dissolved under N_2 in anhydrous CH_2Cl_2 (1.61 mL). *N,N*-Diisopropylethylamine (0.52 mL) was added followed by dropwise addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.210 mL, 0.930 mmol). After 45 min analytical TLC showed no more starting material and the reaction was quenched with 3 drops of MeOH and diluted with EtOAc (6 mL). The solution was washed with saturated aqueous solutions of $NaHCO_3$ (3 × 5 mL) and NaCl (3 × 5 mL), dried (Na_2SO_4) and evaporated under reduced pressure. The residual gum was redissolved in anhydrous toluene (1.0 mL) and precipitated in cold (–20 °C) petroleum ether (120 mL). The product was collected by filtration and dried under vacuum to give **4** as a white powder in 92% yield (407 mg, 0.579 mmol). ^{1}H NMR ($CDCl_3$): δ 1.11, 1.14, 1.16, 1.18 (*s,s,s,s*, 4 × CH_3(*i* Pr)), 1.83 (*s*, CH_3), 1.25–1.29 (*m*, 2 × CH_2CN), 2.40 (*t*, *J* = 6.5 Hz, CH_2OP), 2.58 (*t*, *J* = 6.5 Hz, CH_2OP), 3.16–3.83 (*m*, 4 × H-1', 2 × H-2', 2 × CH(*i*Pr)), 3.78, 3.79 (*s,s*, 4 × OCH_3), 4.00–4.23 (*m*, 4 × H-3'), 6.80–7.46 (*m*, arom., 2 × H-6), 8.65 (*br s*, NH). ^{31}P NMR ($CDCl_3$): δ 149.6, 149.7. R_f 0.72 (CH_2Cl_2:EtOAc:Et_3N, 45:45:10, v:v:v). MS(FAB) *m/z* = 703 ([MH]$^+$, 2.5%). Anal. calcd. for $C_{38}H_{47}N_4O_7P \cdot H_2O$: C, 63.32; H, 6.85; N, 7.77: found: C, 63.08; H, 6.76; N, 8.18.

1-Allylthymine (5)[31]

A solution of thymine (2.0 g, 15.9 mmol) in HMDS (10 mL) and anhydrous DMF (0.8 mL) was stirred at 140 °C for 12 h and cooled to room temperature. A solution of allylbromide (3.2 ml, 38 mmol) in CH_3CN (28 mL) was added under a N_2-atmosphere, and the mixture was stirred at room temperature for 14 days and evaporated. The residue was dissolved in ethanol (32 mL) and neutralized with 1M methanolic KOH and evaporated. The residue was dissolved in $CHCl_3$ (40 mL), washed with water (3 × 40 mL), dried (Na_2SO_4) and evaporated. The product was purified by silica gel column chromatography (2–5% MeOH in CH_2Cl_2) to give **2** as a white solid in 85% yield (2.20 g, 13.2 mmol). ^{13}C NMR ($CDCl_3$): δ 12.15 (CH_3),

49.65 (C-1'), 110.84 (C-5), 118.96 (C-3'), 131.74 (C-2'), 139.63 (C-6), 151.00 (C-2), 164.44 (C-6). ^{1}H NMR ($CDCl_3$): δ 1.92 (*s*, 3H, CH_3), 4.34 (*d*, 2H, *J* = 5.9 Hz, H-1'), 5.22–5.31 (*m*, 2H, 2 × H-3'), 5.87 (*m*, 1H, H-2'), 6.99 (*s*, 1H, H-6), 9.90 (*br s*, 1H, NH). R_f 0.64 (MeOH:CH_2Cl_2, 1:19, v:v).

(R,S)-*1-(2,3-Dihydroxypropyl)thymine (6)*

f: a solution of 3.21 g AD-mix-α[34] in water (12 mL) was stirred at room temperature. When two separated phases were observed, the solution was cooled to 0 °C and **5** (400 mg, 2.41 mmol) was added. The solution was stirred for 1 h at 0 °C and subsequently for 14 days at 5 °C. Sodium sulfite (3.6 g) was added and the temperature was increased to room temperature. After 1 h the solution was evaporated. The residue was purified by silica gel column chromatography (3–10% MeOH in CH_2Cl_2) to give **6f** as a white solid in 80% yield (385 mg, 1.92 mmol). R_f, ^{1}H and ^{13}C NMR as described for **2**.

g: a solution of 1.60 g AD-mix-β[34] was stirred at room temperature. When two separated phases were observed, the solution was cooled to 0 °C and 200 mg of **5** (1.20 mmol) was added. Following the same procedures as described for **6f** afforded **6g** in 90% yield (220 mg, 1.10 mmol). R_f, ^{1}H and ^{13}C NMR as described for **2**.

h: a solution of **5** (200 mg, 1.20 mmol) in water (2.5 mL) and *tert*-butanol (2.5 mL) was stirred, and *N*-methylmorpholine *N*-oxide (176 mg, 1.50 mmol) and OsO_4 (3 mg, 0.007 mmol) were added. After stirring at room temperature for 24 h, sodium sulfite (378 mg, 1.50 mmol) was added and the solvents evaporated. The product was purified by silica gel column chromatography (3–10% MeOH in CH_2Cl_2) to give **6h** as a white solid in 52% yield (125 mg, 0.625 mmol). R_f, ^{1}H and ^{13}C NMR as described for **2**.

(R,S)-*1-(3-(4,4'-Dimethoxytrityloxy)-2-hydroxypropyl)-thymine (7)*

6f (120 mg, 0.60 mmol) or **6g** (250 mg, 1.25 mmol) or **6h** (85 mg, 0.43 mmol) were reacted as described for **3** to give **7f**, **7g** or **7h**, respectively. Yields: **7f** 60% (182 mg, 0.36 mmol), **7g** 85% (530 mg, 1.05 mmol), **7h** 72% (153 mg, 0.31 mmol). R_f, MS, ^{13}C and ^{1}H NMR as described for **3**. **7f**: $[\alpha]_D^{25}$ +2.35°. **7g**: $[\alpha]_D^{25}$ –2.59°. **7h**: $[\alpha]_D^{25}$ +0.02°.

(S)-*3'-O-(2-Cyanoethoxy-(3-(4,4'-dimethoxytrityloxy)-1-(thymin-1-yl)-2-propoxy)phosphino)-5'-O-(4,4'-dimethoxytrityl)thymidine (8)*

To 0.39 mL of a 0.1 M solution of thymidine 3'-*O*-2-(cyanoethyl)diisopropylphosphoramidite in CH_3CN (0.039 mmol) was added 0.35 mL of a 0.45 M tetrazole solution in CH_3CN (0.161 mmol) and the mixture was stirred under N_2 for 2 min. Compound **3** (20 mg, 0.0398 mmol) dissolved in CH_3CN (0.56 mL) was added dropwise over 10 min. The solution was stirred for 45 min, CH_2Cl_2 (3 mL) was added, and after washing with saturated aqueous solutions of $NaHCO_3$ (3 × 2 mL) and NaCl (3 × 2 mL) and evaporation of the organic phase, **8** was isolated as a white solid in 88% yield (40 mg, 0.035 mmol). R_f 0.24 (CH_2Cl_2:EtOAc:Et_3N, 45:45:10, v:v:v). ^{31}P NMR: δ 139.8; 140.0.

(R,S)-*3'-O-(2-Cyanoethoxy-(3-(4,4'-dimethoxytrityloxy)-1-(thymin-1-yl)-2-propoxy)phosphino)-5'-O-(4,4'-dimethoxytrityl)thymidine (9)*

7f was reacted as described above for **3** to give **9** as a white solid in 79% yield (35 mg, 0.031 mmol). R_f 0.24 (CH_2Cl_2:EtOAc:Et_3N, 45:45:10, v:v:v). ^{31}P NMR: δ 139.5; 139.8; 140.0; 141.0.

5'-O-(4,4'-Dimethoxytrityl)-2',3'-seco-2'-hydroxythymidine (11)[36]

5'-*O*-(4,4'-Dimethoxytrityl)-2'-hydroxythymidine (**10**)[35] (5.20 g, 9.29 mmol) was reacted as described[36] to give 2',3'-*seco*-nucleoside **11** as a white solid in 88% yield (4.65 g, 8.27 mmol). ^{1}H NMR data were in accordance with published data.[36] ^{13}C NMR (DMSO-d_6) :δ 12.11 (CH_3), 54.94 (OCH_3), 60.59, 61.10 (C-2', C-3'), 63.70 (C-5'), 79.38 (C-1'), 83.57 (C-4'), 85.24 ($Ph_3\underline{C}$), 109.24 (C-5), 113.07, 126.49, 127.47, 127.72, 129.44, 129.51, 135.51, 135.63, 144.90, 157.91 (arom.), 136.58 (C-6), 151.49 (C-2), 163.92 (C-4). R_f 0.40 (MeOH:CH_2Cl_2, 1:9, v:v).

2'-O-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-seco-2'-hydroxythymidine (12)[36], *3'-O-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-seco-2'-hydroxythymidine (14)*[36] *and 2',3'-di-O-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2',3'-seco-2'-hydroxythymidine (16)*[36]

Nucleoside **11** (2.41 g, 4.28 mmol) was reacted as described[36] affording **12**, **14** and **16** as white solid materials. **12**: yield 38% (1.08 g, 1.63 mmol). ^{1}H NMR data were in accordance with published data.[36] ^{13}C NMR ($CDCl_3$): δ 12.26 (CH_3), 55.08 (OCH_3), 62.45, 63.43, 63.99 (C-2', C-3', C-5'), 80.35, 81.59 (C-1', C-4'), 86.43 ($Ph_3\underline{C}$), 111.35 (C-5), 113.05, 126.80, 127.72, 127.84, 128.47, 128.98, 129.57, 129.77, 133.39, 135.07, 144.34, 158.45 (arom.), 135.47 (C-6), 150.73 (C-2), 163.47 (C-4), 165.77 (C=O). R_f 0.67 (MeOH:CH_2Cl_2, 1:9, v:v). **14:** yield 17% (0.48 g, 0.73 mmol). ^{1}H NMR data were in accordance with published data.[36] ^{13}C NMR ($CDCl_3$): δ 12.26 (CH_3), 55.08 (OCH_3), 62.45, 63.43, 63.99 (C-2', C-3', C-5'), 80.35, 81.59, (C-1', C-4'), 86.43 ($Ph_3\underline{C}$), 111.35 (C-5), 113.05, 126.80, 127.72, 127.84, 128.47, 128.98, 129.57, 129.79, 133.39, 135.07, 144.34, 158.47 (arom.), 135.47 (C-6), 150.73 (C-2), 163.47 (C-4), 165.77 (C=O). R_f 0.58 (MeOH:CH_2Cl_2, 1:9, v:v). **16:** yield 12% (0.39 g, 0.51 mmol). ^{1}H NMR data were in accordance with published data.[36] ^{13}C NMR (DMSO): δ 11.88 (CH_3), 54.85 (OCH_3), 62.61, 63.00, 63.42, (C-2', C-3', C-5'), 75.81 (C-1'), 80.42, (C-4'), 85.59 ($Ph_3\underline{C}$), 110.01 (C-5), 112.99, 126.53, 127.38, 127.64, 128.49, 128.59, 128.96, 129.02, 129.38, 133.44, 135.14, 135.27, 135.59, 144.52, 149.14,

157.97 (arom.), 136.41 (C-6), 150.96 (C-2), 163.59 (C-4), 164.87, 165.00 (2 × C=O). R_f = 0.77 (MeOH:CH_2Cl_2, 1:9, v:v).

2'-O-Benzoyl-3'-O-(2-cyanoethoxy(diisopropylamino)-phosphino)-5'-O-(4,4'-dimethoxytrityl)-2',3'-seco-2'-hydroxythymidine (13)

Compound **12** (250 mg, 0.38 mmol) was co-evaporated with dry MeCN (3 × 5 mL) and dissolved in dry CH_2Cl_2 (1.1 mL) and *N,N*-diisopropylethylamine (0.35 mL) was added. 2-Cyanoethyl-*N,N*-diisopropylphosphoramidochloridite (0.15 mL, 0.67 mmol) was added dropwise and the reaction mixture was stirred at 22 °C. After 50 min analytical TLC showed no more starting material, and the mixture was poured into EtOAc (8 mL) and washed with saturated aqueous solutions of $NaHCO_3$ (3 x 4 mL) and NaCl (3 × 4 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was dissoved in toluene (1.1 mL) and precipitated in hexane (80 mL) at –5 °C. The precipitate was collected by filtration to afford **13** as a white solid in 93% yield (301 mg, 0.35 mmol). R_f 0.64 (MeOH:CH_2Cl_2, 1:19, v:v). ^{31}P NMR ($CDCl_3$): δ 148.7, 148.9.

3'-O-Benzoyl-2'-O-(2-cyanoethoxy(diisopropylamino)-phosphino)-5'-O-(4,4'-dimethoxytrityl)-2',3'-seco-2'-hydroxythymidine (15)

The same procedure as described for **12** was used with **14** (250 mg, 0.38 mmol) as starting material to afford **15** in 85% yield (258 mg, 0.30 mmol). R_f 0.59 (MeOH:CH_2Cl_2, 1:19, v:v). ^{31}P NMR ($CDCl_3$) :δ 149.4 (*br* s).

Synthesis of oligodeoxynucleotides

The syntheses of oligonucleotides **17–28** were performed in 0.2 μmol-scale (5 μmol amidite per cycle, Pharmacia primer support™) using commercial 2-cyanoethylphosphoramidites as well as compounds **4, 13** and **15**. The syntheses followed the regular protocol for the DNA-synthesizer for 2-cyanoethylphosphoramidites. The coupling efficiency of **4, 13** and **15** was slightly lower (approximately 90%) than those of the unmodified amidites (approximately 99%). The 5'-*O*-DMT-ON oligodeoxynucleotides were removed from the solid support by treatment with concentrated ammonia at room temperature for 48 h which also removed the nucleobase and phosphate protecting groups. Subsequent purification using disposable reverse-phase chromatography cartridges (including 5'-*O* detritylation) afforded the pure oligomers **17–28**.

Melting experiments

The melting experiments were carried out in medium salt buffer, 1 mM EDTA, 10 mM Na_3PO_4, 140 mM NaCl, pH 7.2 as previously described.[44] The increase in absorbance at 260 nm as a function of time was recorded while the temperature was raised linearily from 10 to 80 °C at a rate of 1 °C min^{-1}.

Enzymatic stability of the oligodeoxynucleotides

A solution of the oligodeoxynucleotide (0.2 OD) in 2 mL of the following buffer (0.1 M Tris–HCl; pH 8.6; 0.1 M NaCl; 14 mM $MgCl_2$) was digested with 1.2 U SVPDE (snake venom phosphordieterase; 34 μl of a solution of the enzyme in the following buffer; 5 mM Tris–HCl pH 7.5; 50% glycerol (v/v)) at 25 °C. During digestion the increase in absorbance at 260 nm was followed. The absorption vs time curve of the digestion was plotted from which the hyperchromicity and half-life of the oligomer were evaluated. The final hyperchromicity (*H*) is defined as the final absorbance at 260 nm divided by the initial absorbance.

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