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Incorporation of (*R*)- and (*S*)-3′,4′-*seco*-thymidine into oligodeoxynucleotides: hybridization properties and enzymatic stability

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ABSTRACT

Novel flexible oligodeoxynucleotide analogues containing (*R*)- and (*S*)-3′,4′-*seco*-thymidine were synthesized on an automated DNA-synthesizer using the phosphoramidite approach. Oligodeoxynucleotide analogues (17-mers) having one or three modifications in the middle or one or two modifications in the ends were evaluated with respect to hybridization properties and enzymatic stability. 3′-End-modified oligomers were stable towards 3′-exonuclease degradation and displayed acceptable hybridization properties.

INTRODUCTION

The appearance of oligonucleotide analogues as promising therapeutic agents has stimulated much effort towards the development of novel nuclease resistant oligonucleotides, which are capable of hybridizing with appropriate specificity and affinity to complementary sequences thus acting as effective inhibitors of gene expression.[1,2] To avoid rapid degradation by cellular nucleases, synthetic oligonucleotides need to be modified chemically.[1,3,4] Modification of an oligonucleotide is possible in the base moiety, in the sugar moiety or in the internucleoside linkage. Modifications in the phosphate moiety have been intensively studied, [1–3,5–8] and although enhanced nuclease resistance often is achieved, such modifications (e.g. phosphorothioates, phosphoramidates and methylphosphonates) induce chirality at phosphorus resulting in highly heterogeneous oligomers, which lowers the binding to the target. We concentrate our research on synthesizing analogues with neutral achiral backbones and/or modified sugar moieties. A number of oligonucleotides containing monomers with unnatural sugar configurations or skeletons have been reported e.g. α-DNA,[9] α-RNA,[10] 2′-*O*-methyl-RNA,[11] carbocyclic-DNA,[12] 4′-thio-RNA,[13] L-DNA,[14] 3′-deoxy-*threo*-hydroxymethyl-DNA,[15] xylo-DNA,[16] and hexose-DNA,[17,18] but acyclic oligonucleotides have received little attention. Glyceronucleoside phosphoramidite synthons have been synthesized and incorporated into oligodeoxynucleotides.[19,20] The melting experiments with these analogues were disappointing, as introduction of one or two of these flexible glyceronucleosides in the middle of a sequence caused a significant decrease in duplex stability (ΔT_m/modification > 10°C).[20] All modified glycerooligonucleotides were resistant to snake venom and spleen phosphodiesterases.[19] The results obtained with oligodeoxynucleotides incorporating acyclic nucleosides with 3,4-dihydroxybutyl-[21] and 3,5-dihydroxypentyl[22] side chains were more encouraging as endT-modified oligomers showed acceptable base-pairing capacities and stability towards nucleases.

It is known that 3′-phosphodiesterases play a predominant role in the degradation of oligonucleotides *in vivo*.[23,24] Thus, 3′-end protected oligonucleotide analogues containing the right mixture of normal and modified nucleosides could comply with all requirements for successful *in vivo* inhibitors of gene expression. In this view, we decided to examine the novel acyclic 3′,4′-*seco*-DNA, and this report describes the synthesis of the acyclic thymine phosphoramidite monomers **13**–**16** and their subsequent incorporation into oligodeoxynucleotides. Besides, the base-pairing properties and enzymatic stability of the novel 3′,4′-*seco*-DNAs are evaluated.

RESULTS AND DISCUSSION

Synthesis of the acyclic nucleosides 5–12

Condensation of 1,3,4-tri-*O*-benzoyl-2-deoxy-D-ribose (**1**)[25] and silylated thymine using trimethylsilyltriflate as catalyst[26] gave in 97% yield an anomeric mixture (α:β = 5:2; ^{1}H NMR) of nucleosides **2**. Reaction of **2** with methanolic ammonia afforded an anomeric mixture of deprotected nucleosides which were separated on HPLC giving the pure β-anomer **3** in 20% yield and the pure α-anomer **4** in 45% yield. The bond between C-3′ and C-4′ in **3** and **4** were oxidatively cleaved with periodate, and after reduction the enantiomers (*R*)-3′,4′-*seco*-thymidine (**5**) and (*S*)-3′,4′-*seco*-thymidine (**6**) were obtained in 94% and 93% yield, respectively. The synthesis of the two enantiomers **5** and **6** has been reported earlier[27] and a stereoselective synthesis of the (*R*)-isomer (**5**) from L-arabinose is known.[28] Furthermore, the racemic mixture (**5** + **6**) has been obtained using Michael-type additions.[29,30] Reaction of **5** with 1.0 eqv. of 4,4′-dimeth-

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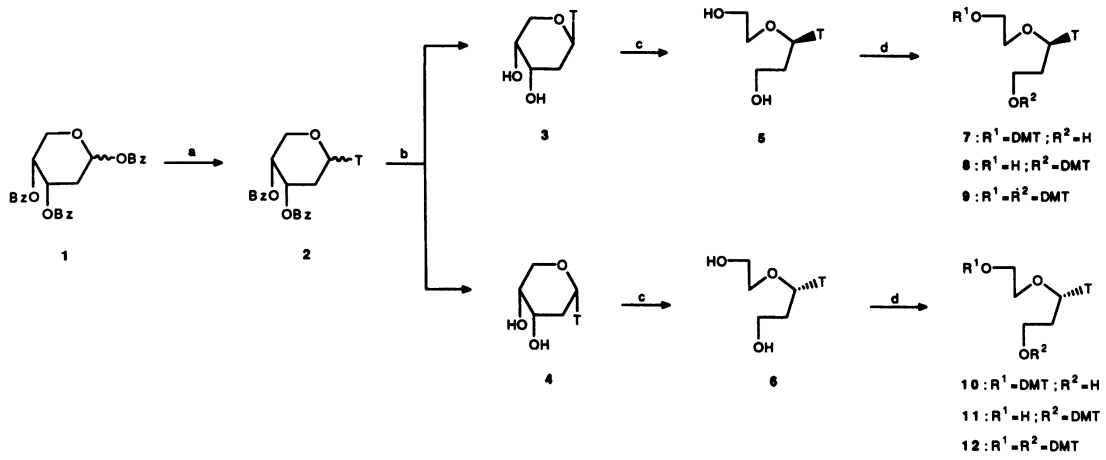
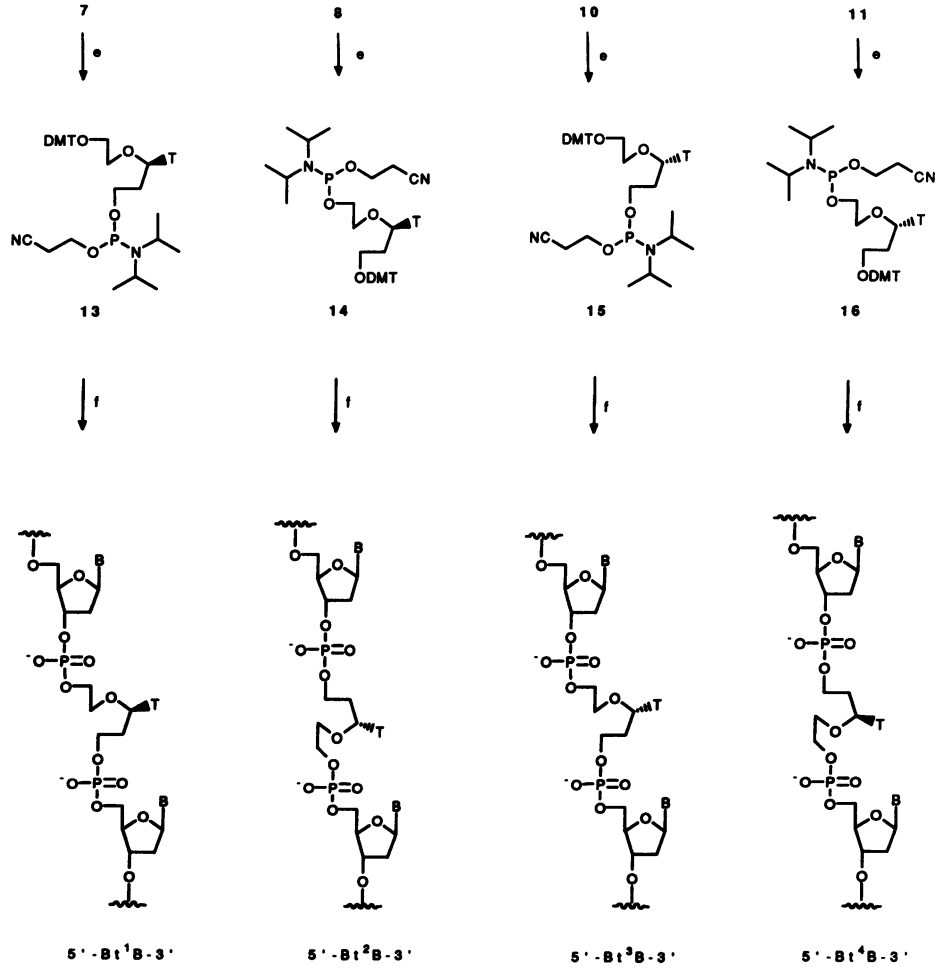


Scheme 1. a) 1) HMDS, $(NH_4)_2SO_4$, thymine, 2) TMS-triflate, CH_3CN, b) NH_3, MeOH, c) 1) $NaIO_4$, H_2O, dioxane, 2) $NaBH_4$, d) DMTCl, pyridine. T = thymin-1-yl.



Scheme 2. e) $NCCH_2CH_2OP(Cl)N(iPr)_2$, CH_2Cl_2, $EtN(iPr)_2$, f) DNA-synthesizer.

oxytritylchloride (DMTCl) in anhydrous pyridine gave **7** (10.2% yield), **8** (22.7% yield) and **9** (11.8% yield), together with recovered **5** (40% yield). Reaction of **6** with 1.0 eqv. of DMT-Cl gave **10**–**12** in equivalent yields. (Scheme 1). The products were separated by aluminum oxide column chromatography and their structures confirmed by a ^1H NOE experiment on **11**: saturation of the aromatic signals from the DMT-group gave enhancements of the signals from the 2'- (1%) and 3'-protons

Table 1. Sequences and melting experiments of synthesized oligodeoxynucleotides

Sequence	No.	T_m/°C	ΔT_m/ °C	h_m/%
5′-TTAACTTCTTCACATTC-3′	**17**	52.0	–	15.6
5′-t[1]TAACTTCTTCACATt[1]C-3′	**18**	46.5	2.8	16.0
5′-t[2]TAACTTCTTCACATt[2]C-3′	**19**	47.5	2.3	14.0
5′-t[3]TAACTTCTTCACATt[3]C-3′	**20**	47.0	2.5	14.9
5′-t[4]TAACTTCTTCACATt[4]C-3′	**21**	47.0	2.5	17.1
5′-TTAACTTCTTCACAt[2]t[2]C-3′	**22**	44.0	4.0	7.3
5′-TTAACTTCTTCACAt[3]t[3]C-3′	**23**	44.0	4.0	8.2
5′-CACCAACTTCTTCCACA-3′	**24**	60.0	–	13.9
5′-CACCAACTTCTt[2]CCACA-3′	**25**	50.0	10.0	10.3
5′-CACCAACTTCTt[3]CCACA-3′	**26**	49.0	11.0	10.3
5′-CACCAACTTCTt[4]CCACA-3′	**27**	48.0	11.5	10.3
5′-CACCAACt[2]t[2]CTt[2]CCACA-3′	**28**	33.0	9.0	7.4
5′-CACCAACt[3]t[3]CTt[3]CCACA-3′	**29**	30.0	10.0	6.4
5′-CACCAACt[4]t[4]CTt[4]CCACA-3′	**30**	30.0	10.0	6.3
5′-TTTTTTTTTTTTT-3′	**31**	35.0	–	18.2
5′-TTTTTTTt[3]t[3]t[3]t[3]t[3]T-3′	**32**	<10	>5	–

A = 2′-deoxyadenosine, C = 2′-deoxycytidine, T = thymidine, t^1, t^2, t^3 and t^4 = acyclic monomers, T_m = melting temperature, ΔT_m = decrease in T_m per modification, h_m = melting hypochromicity.

(2%) and no effect on the signals from 4′- and 5′-protons (2D-COSY ^{1}H-^{1}H NMR was used for the interpretation of the ^{1}H NMR spectrum). Moreover, enhancements of the aromatic signals (3%) was only obtained by saturation of the 2′- and 3′-protons. This identification was verified by EI mass spectrometry: in the mass spectrum of **7** a peak at m/z = 363 derived from $DMTOCH_2CH_2O^+$ was identified. This peak was absent in the mass spectrum of **8**.

Synthesis of building blocks 13–16 and oligodeoxynucleotides 17–32

The four monotritylated nucleosides **7, 8, 10** and **11** were reacted with 2-cyanoethyl-*N,N*-diiso-propylphosphoramidochloridite in the presence of *N,N*-diisopropylethylamine affording **13–16** in good yields (59–94%) after precipitation in cold petroleum ether (scheme 2). These acyclic phosphoramidites partially decomposed during silica gel column chromatography which therefore was omitted.

The oligodeoxynucleotides **17–32** (table 1) were synthesized by standard phosphoramidite methodology on an automated DNA-synthesizer using the appropriate buildingblocks (**13–16** and commercial 2′-deoxynucleoside β-cyanoethylphosphoramidites). The coupling efficiencies for the acyclic phosphoramidites were app. 90% compared to app. 99% for the commercial phosphoramidites as monitored by the release of the dimethoxytrityl cation after each coupling step. The oligonucleotides were removed from the support and deblocked with concentrated ammonia at 55°C. Purification of the oligomers was performed by filtration through a NAP-10^R column. The composition of the oligonucleotide **32** was verified by matrix assisted laser desorption mass spectrometry. This method has become a powerful tool for the mass analysis of natural and modified oligonucleotides,[15,31,32] and as oligomer **32** includes 5 modifications, the significant enhancement of the mass (10 Da) compared to unmodified 13-mer **31** allows a safe conclusion to be drawn from such mass analysis. As only a negligible deviation between the calculated mass (3903,7 Da) and the measured mass (3904,1 Da) was observed, we conclude that the modified monomeric buildingblock **15** is incorporated 5 times in oligomer **32** as contemplated. Because of the homogeneous results from the syntheses of all the modified oligodeoxynucleotides, we consider their composition verified.

Melting experiments

The hybridization properties of the 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 hypochromicities (h_m). The results depicted in table 1 indicate that introduction of 3′,4′-*seco*-nucleoside(s) in the middle of a sequence (**25–30**) results in a decrease in T_m by app. 10°C / modification. Substitution in both ends with one nucleoside (**18–21**) leads to a depression of T_m by app. 2,5°C / modification, while two 3′-end-modifications decreases the T_m by 4°C / modification. No cooperative melting was observed below 10°C for the 5 times modified 13-mer **32**. The hybridization data described here for oligodeoxynucleotide analogues containing 3′,4′-*seco*-nucleosides are comparable with data obtained for other flexible oligodeoxynucleotides.[20,22] The observed decreases in melting temperature compared to unmodified oligodeoxynucleotides are due to the more flexible structures of 3′,4′-*seco*-oligodeoxynucleotides. This flexibility reduces the ability of the oligos to form duplexes, because the concomitant loss of entropy is much larger for flexible structures which have a great deal of entropy to lose. The degree of destabilization seems to be independent of the structure and configuration of the 3′,4′-*seco*-thymidine isomer incorporated, as there are no significant differences in melting temperatures between **25**, **26** and **27**, between **28**, **29** and **30** or between **18**, **19**, **20** and **21**. Thus, whether the nucleoside with (*R*)- (t^1 and t^2) or (*S*)- configuration (t^3 and t^4) or whether the '5′-*O*-DMT' protected (t^1 and t^3) or the '3′-*O*-DMT' protected nucleoside (t^2 and t^4) is incorporated in the oligodeoxynucleotide (scheme 2) has no apparent influence on the hybridization properties. This indicates that a mixture of phosphoramidite building blocks **13–16** can be used in syntheses of 3′,4′-*seco*-thymidine (t_1–t_4) containing oligodexoynucleotide analogues without compromising binding affinity compared to similar analogues synthesized using single isomer 3′,4′-*seco*-phosphoramidites. Hereby the accessibility of '3′,4′-*seco*-DNA' is improved as separations of diastereomeric nucleosides (e.g. **3** and **4**) and different dimethoxytritylated products can be

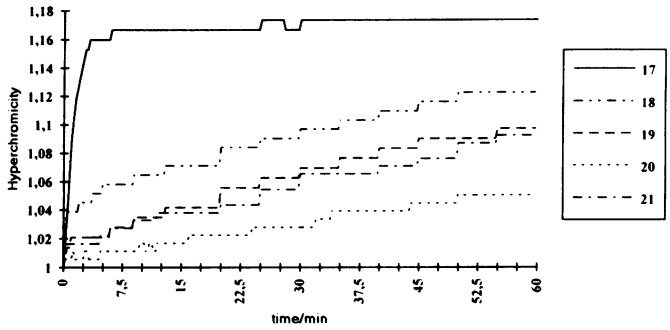


Figure 1. Time course of snake venom phosphodiesterase digestion of modified oligodeoxynucleotides with one modification in each end.

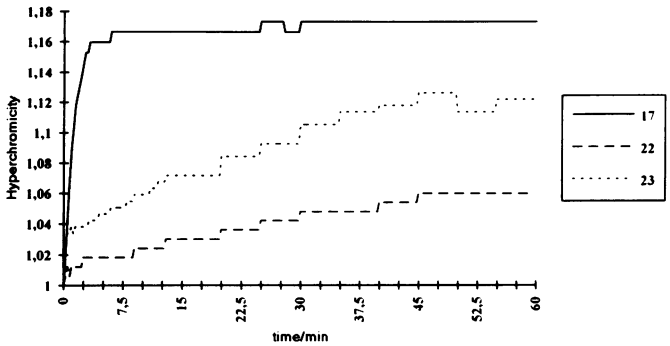


Figure 2. Time course of snake venom phosphodiesterase digestion of modified oligodeoxynucleotides with two modifications in the 3′-end.

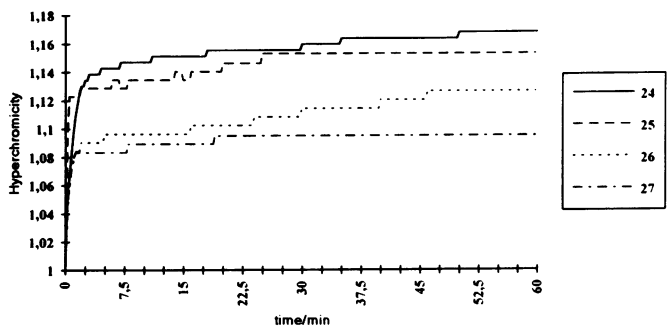


Figure 3. Time course of snake venom phosphodiesterase digestion of modified oligodeoxynucleotides with one modification in the middle.

omitted. The melting hypochromicities for the oligomers are similar for the different modified oligodeoxynucleotides with the same sequences, indicating that the secondary structure of the resulting DNA−DNA duplexes are alike.

Enzymatic stability of the oligomers

We studied the stability of the oligodeoxynucleotide analogues towards SVPDE (3′-exonuclease) by measuring the hyperchromicity and by evaluating the degradation of 5′-end [^{32}P]-labelled oligos. The hyperchromicity (the increase in absorbance at 260 nm during digestion) arises as a consequence

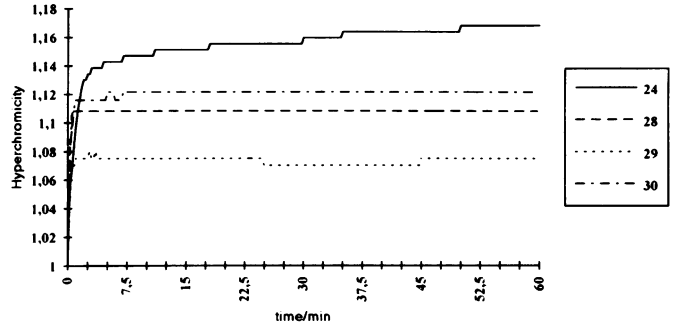


Figure 4. Time course of snake venom phosphodiesterase digestion of modified oligodeoxynucleotides with three modifications in the middle.

of the base stacking of oligonucleotides and consequent $\pi-\pi$-orbital overlap,[33] which is destroyed as the oligonucleotide is converted to its nucleoside constituents during the enzymatic digestion. Figure 1 shows the time dependent hydrolysis of **18**, **19**, **20** and **21** compared to the unmodified oligomer **17**. It is evident that incorporation of one modified acyclic nucleoside in both ends of the oligomers results in a large increase in the half-life ($t_{1/2}$) and a decrease in the enzymatic hypochromicity (calculated after 60 min digestion). Figure 2 shows the hydrolysis of **22** and **23** compared to **17**. The increase in half-life and decrease in hypochromicity are similar with two modifications in the 3′-end as with one in each end. Figure 3 and 4 illustrate the hydrolysis of oligomers **25**−**30** compared to the unmodified oligomer **24**. One or three modifications in the middle of the sequences apparently have no effect on the stability of the full-length oligodeoxynucleotide analogues. However, the hypochromicities calculated for **25**−**30** are smaller than the one calculated for unmodified oligomer **24** thus indicating rapid 3′-exonucleolytic degradation of the first five monomers until increased enzymatic stability is induced by an acyclic 3′,4′-*seco*-monomer. The results of the enzymatic hyperchromicity experiments are summarized in table 2. There seems to be no significant differences between the four different acyclic modifications concerning the effect on the enzymatic stability of the oligodeoxynucleotides.

To support the encouraging results from the hyperchromicity experiments, the digestion (SVPDE) of 5′-end [^{32}P]-labelled oligodeoxynucleotide analogues **17, 23, 24** and **26** were followed using denaturing gel electrophoresis. Figure 5 shows the degradation of unmodified oligodeoxynucleotide **17** and the corresponding analogue **23** with two acyclic monomers in the 3′-end. While unmodified **17** is almost completely hydrolyzed after 5 min digestion (lane 10), the 16-mer resulting from rapid removal of one unmodified monomer at the 3′ end of **23** is extraordinary stable (e.g. lane 6: 60 min digestion and almost no degradation of the 16-mer is observed). Thus, incorporation of 3′,4′-*seco*-monomers in the 3′ end makes the oligo (16 mer) very resistant towards 3′-exonucleolytic degradation. Correspondingly, incorporation of one 3′,4′-*seco*-monomer in the middle **26** (as monomer number 12 out of 17) significantly stabilizes the 12-mer resulting from rapid removal of the five unmodified monomers from the 3′ end. These results are shown on figure 6, lane 1−7. As expected, the corresponding unmodified control **24** was rapidly degraded (figure 6, lane 8−14). Comparing the results from hyperchromicity experiments and from digestion of

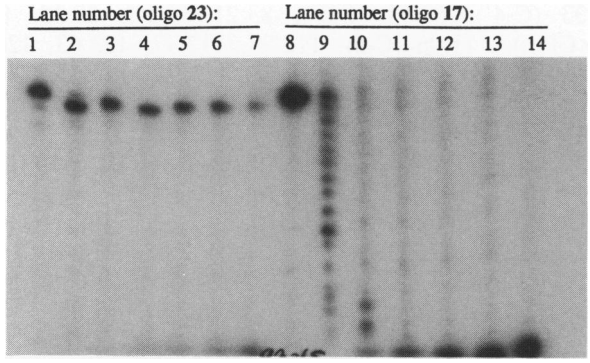


Figure 5. Snake venom phosphodiesterase digestion of [^{32}P]-labelled oligodeoxynucleotides **23** and **17**. Lane 1 and 8: no enzyme added, lane 2 and 9: 1 min digestion, lane 3 and 10: 5 min digestion, lane 4 and 11: 15 min digestion, lane 5 and 12: 30 min digestion, lane 6 and 13: 60 min digestion; lane 7 and 14: 300 min digestion.

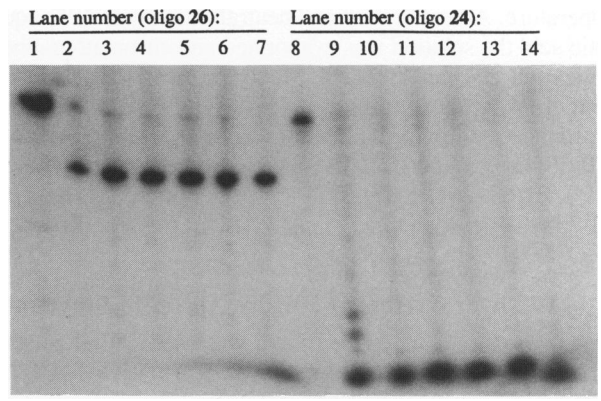


Figure 6. Snake venom phosphodiesterase digestion of [^{32}P]-labelled oligodeoxynucleotides **26** and **24**. Time of digestion for the different lanes as in figure 5.

[^{32}P]-labelled material we conclude that incorporation of 3′,4′-*seco*-nucleosides considerably improves the enzymatic stability of the oligos, and that the results obtained from UV-measurements of the time course of digestion of the oligodeoxynucleotides (table 2) are reliable and can be used as a good indication of the enzymatic stability.

Table 2. Enzymatic digestion of oligonucleotides

No.	$t_{1/2}$/min	h_e/%	No.	$t_{1/2}$/min	h_e/%
17	<1	15	**24**	<1	14
18	>10	11	**25**	<1	13
19	>20	9	**26**	<1	12
20	>10	3	**27**	<1	9
21	>20	8	**28**	<1	10
22	>10	6	**29**	<1	7
23	>10	11	**30**	<1	11

$t_{1/2}$ = half-life, h_e = enzymatic hypochromicity.

CONCLUSION

Oligodeoxynucleotide analogues containing isomers of 3′,4′-*seco*-thymidine (t^1, t^2, t^3 or t^4) have been synthesized. Oligomers with one or two modifications in the 3′-end were effectively protected against 3′-exonuclease degradation and showed only moderately lowered hybridization properties. The results on the synthesis and characteristics of the four stereoisomers of the novel 3′,4′-*seco*-DNA reported here reveals new possibilities for prolonging the intracellular lifetime of synthetic antisense compounds consisting of a mixture of 3′,4′-*seco*- and unmodified deoxynucleosides.

EXPERIMENTAL

NMR-spectra were recorded at 250 MHz for ^{1}H NMR and 62.9 MHz for ^{13}C NMR on a Bruker AC 250 spectrometer and at 202.3 MHz for ^{31}P NMR on a Varian Unity 500 spectrometer; δ-values are in ppm relative to tetramethylsilane as internal standard (^{1}H NMR 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. The silica gel (0.040−0.063 mm) and aluminum oxide (0.063−0.200 mm, activity 1, neutral) used for column chromatography was purchased from Merck. Snake venom phosphodiesterase (*Crotalus adamanteus*) was obtained from Pharmacia. Matrix assisted laser desorption mass spectrum was obtained on a prototype laser desorption mass spectrometer from Applied Biosystem Sweden AB, Uppsala, Sweden.[32]

1-(3,4-Di-*O*-benzoyl-2-deoxy-D-ribopyranosyl)thymine (2)

A mixture of thymine (20 g, 160 mmol) and ammoniumsulfate (50 mg) in hexamethyl-disilazane (800 ml) was stirred at 135°C for 10 h. The solvent was evaporated under reduced pressure at 40°C to give an oily residue. A solution of **1** (40.8 g, 90 mmol) in anhydrous CH_3CN (960 ml) was added and the solution was stirred under a N_2-atmosphere and cooled to −30°C. Trimethylsilyltriflate (19.6 ml, 100 mmol) was added dropwise, and the temperature was increased to room temperature over night. The solution was refluxed at 55°C for 2 h and CH_2Cl_2 (600 ml) was added. The mixture was washed with an ice-cold aqueous solution of $NaHCO_3$ (400 ml) and dried (Na_2SO_4). Yield 30.68 g (77.8 mmol, 97%) as an anomeric mixture (α:β = 5:2).

^{1}H and ^{13}C NMR were in accordance with published data.[27,34]

β- and α-1-(2-deoxy-D-ribopyranosyl)thymine (3 and 4)

A solution of **2** (21.5 g, 54.3 mmol) in a saturated solution of NH_3 in MeOH (700 ml) was stirred at room temperature for 12 h, evaporated under reduced pressure and chromatographed on a silica gel column (0−20% MeOH, rest CH_2Cl_2). The resulting mixture of β- and α-nucleosides (**3** and **4**) was separated on reverse phase HPLC (EtOH/H_2O, 1:99, v/v). The first nucleoside eluted was **4**. Yield 5.86 g (24.2 mmol, 45%). ^{1}H and ^{13}C NMR were in accordance with published data.[27,35] The second nucleoside eluted was **3**. Yield 2.64 g (10.9 mmol, 20%). ^{1}H and ^{13}C NMR were in accordance with published data.[27,35]

(*R*)-1-(1-(2-hydroxyethoxy)-3-hydroxyprop-1-yl)thymine (5)

A solution of **3** (1.10 g, 4.5 mmol) in a 1:1-mixture of H_2O:dioxane (55 ml) was stirred at room temperature. A solution of $NaIO_4$ (0.94 g, 4.5 mmol) in H_2O (8 ml) was added and the mixture was stirred for 90 min. EtOH was added to precipitation and the mixture was filtered. $NaBH_4$ (154 mg, 4.1 mmol) was added and the solution was stirred for 30 min at room

temperature. After filtration and neutralization with 10% aqueous acetic acid the solution was evaporated and chromatographed on a silica gel column (5–12% MeOH, rest CH_2Cl_2) to give **5**. Yield 1.04 g (4.26 mmol, 94%). ^1H NMR data were in accordance with published data.[27,28,34] ^{13}C NMR (DMSO-d_6) δ 12.03 (CH_3), 37.16 (C-2′), 56.47 (C-3′), 59.64 (C-4′), 69.85 (C-5′), 81.88 (C-1′), 109.67 (C-5), 135.74 (C-6), 151.00 (C-2), 163.76 (C-4). MS m/z = 244 (M^+, 6.9%). Rf = 0.51 ($MeOH/CH_2Cl_2$, 15:85, v/v).

(*S*)-1-(1-(2-hydroxyethoxy)-3-hydroxyprop-1-yl)thymine (6)

Same procedure as described for **5** was used with **4** as starting material. Yield 1.04 g (4.22 mmol, 93%). ^1H NMR,^{13}C NMR and Rf were as described above for **5**.

Reaction of 5 with 4,4′-dimethoxytritylchloride

To a stirred solution of **5** (1.03 g, 4.22 mmol) in anhydrous pyridine (4.0 ml) a solution of 4,4′-dimethoxytritylchloride (1.43 g, 4.22 mmol) in anhydrous pyridine (2.0 ml) was added during a period of 20 min followed by stirring for 3 h. The residue was diluted with $CHCl_3$ (10 ml), washed with water (3×5 ml), dried ($NaSO_4$) and evaporated under reduced pressure. The residual oil was chromatographed on an aluminum oxide column (1–30% CH_3OH, rest CH_2Cl_2) to give three analytically pure products together with starting material **5** (410 mg, 1.69 mmol):

(*R*)-1-(1-(2-(4,4′-dimethoxytrityloxy)ethoxy)-3-hydroxyprop-1-yl)thymine (7)

Yield 235 mg (0.43 mmol, 10.2%). $[\alpha]_D^{25}$ +15.0°. ^{13}C NMR ($CDCl_3$): δ 12.41 (CH_3), 37.71 (C-2′), 55.00 (2×OCH_3), 58.00 (C-3′), 62.32 (C-4′), 68.72 (C-5′), 83.99 (C-1′), 86.10 (CPh_3), 111.71 (C-5), 112.97, 127.63, 129.79, 135.76, 135.76, 135.84, 144.61, 158.35 (DMT), 134.55 (C-6), 151.41 (C-2), 163.95 (C-4). ^1H NMR ($CDCl_3$): δ 1.89 (s, 3H, CH_3), 1.93–2.14 (m, 2H, 2×H-2′), 3.16–3.36 (m, 2H, 2×H-4′), 3.53–3.61 (m, 2H, 2×H-5′), 3.67–3.75 (m, 2H, 2×H-3′), 3.75 (s, 6H, 2×OCH_3), 5.92 (t, 1H, J=6.4 Hz, H-1′), 6.81–7.43 (m, 14H, DMT, H-6), 9.91 (s, 1H, NH). Rf = 0.30 (CH_3OH/CH_2Cl_2, 5:95, v/v). MS m/z = 546 (M^+, 2.7%). Anal. Calcd. for $C_{31}H_{34}N_2O_{72}\cdot 0.5H_2O$: C, 67.01; H, 6.35; N, 5.04. Found: C, 67.08; H, 6.18; N, 4.93.

(*R*)-1-(1-(2-hydroxyethoxy)-3-(4,4′-dimethoxytrityloxy)prop-1-yl)thymine (8)

Yield 523 mg (0.96 mmol, 22.7%). $[\alpha]_D^{25}$ +23.3°. ^{13}C NMR ($CDCl_3$): δ 12.44 (CH_3), 35.09 (C-2′), 55.09 (2×OCH_3), 58.68 (C-3′), 61.20 (C-4′), 70.19 (C-5′), 83.01 (C-1′), 86.23 (CPh_3), 111.37 (C-5), 113.02, 127.70, 127.94, 129.80, 135.83, 135,88, 144.55, 158.38, (DMT), 134.85 (C-6), 150.85 (C-2), 163.63 (C-4). ^1H NMR ($CDCl_3$): δ 1.89 (s, 3H, CH_3), 1.94–2.05 (m, 2H, 2×H-2′), 3.20 (t, 2H, J=5.7 Hz, 2×H-3′), 3.51–3.54 (m, 2H, 2×H-5′), 3.62–3.65 (m, 2H, 2×H-4′), 3.77 (s, 6H, 2×OCH_3), 6.01 (br s, 1H, H-1′), 6.82–7.40 (m, 13H, DMT), 7.12 (s, 1H, H-6), 9.04 (s, 1H, NH). Rf = 0.40 (CH_3OH/CH_2Cl_2, 5:95, v/v). MS m/z = 546 (M^+, 5.9%). Anal. Calcd. for $C_{31}H_{34}N_2O_7\cdot 0.25H_2O$: C, 67.56; H, 6.31; N, 5.08. Found: C, 67.82; H, 6.36; N, 4.83.

(*R*)-1-(1-(2-(4,4′-dimethoxytrityloxy)ethoxy)-3-(4,4′-dimethoxytrityloxy)-prop-1-yl)thymine (9)

Yield 400 mg (0.66 mmol, 11.8%). ^{13}C NMR ($CDCl_3$): δ 12.45 (CH_3), 35.42 (C-2′), 55.06, 55.07 (4×OCH_3), 58.59 (C-3′), 62.33 (C-4′), 68.55 (C-5′), 83.21 (C-1′), 86.10, 86.20 (2×CPh_3), 111.06 (C-5), 112.97, 112.97, 123.63, 126.59, 127.96, 129.80, 129.82, 135.92, 135.92, 144.71, 144.75, 158.38, 158.37 (2×DMT), 134.88 (C-6), 149.59 (C-2), 163.50 (C-4). ^1H NMR ($CDCl_3$): δ 1.86 (s, 3H, CH_3), 1.99–2.06 (m, 2H, 2×H-2′), 3.21 (t, 2H, J=5.9 Hz, 2×H-3′), 3.50 (t, 2H, J=4.6 Hz, 2×H-5′), 3.72–3.83 (m, 2H, 2×H-4′), 3.72 (s, 6H, 2×OCH_3), 3.76 (s, 6H, 2×OCH_3), 5.92–5.95 (m, 1H, H-1′), 6.73–7.40 (m, 27H, DMT, H-6). Rf = 0.85 (CH_3OH/CH_2Cl_2, 5:95, v/v). Anal. Calcd. for $C_{52}H_{52}N_2O_9\cdot 2.5H_2O$: C, 69.86; H, 6.43; N, 3.13. Found: C, 69.85; H, 6.33; N, 3.06.

Reaction of 6 with 4,4′-dimethoxytritylchloride

Same procedure as described for **5** was used to give three analytical pure products together with starting material **6** (405 mg, 1.65 mmol):

(*S*)-1-(1-(2-(4,4′-dimethoxytrityloxy)ethoxy)-3-hydroxyprop-1-yl)thymine (10)

Yield 235 mg (0.43 mmol, 11.1%). $[\alpha]_D^{25}$ −10.8°. ^{13}C NMR, ^1H NMR, Rf as described for **7**. Anal. Calcd for $C_{31}H_{34}N_2O_7\cdot 0.5H_2O$: C, 67.01; H, 6.35; N, 5.04. Found: C, 66.83; H, 6.18; N, 4.61.

(*S*)-1-(1-(2-hydroxyethoxy)-3-(4,4′-dimethoxytrityloxy)prop-1-yl)thymine (11)

Yield 609 mg (1.12 mmol, 26.5%). $[\alpha]_D^{25}$ −28.5°. ^{13}C NMR, ^1H NMR, Rf as described for **8**. Anal. Calcd. for $C_{31}H_{34}N_2O_7\cdot 0.5H_2O$: C, 67.01; H, 6.35; N, 5.04. Found: C, 67.26; H, 6.21; N, 4.90.

(*S*)-1-(1-(2-(4,4′-dimethoxytrityloxy)ethoxy)-3-(4,4′-dimethoxytrityloxy)-prop-1-yl)thymine (12)

Yield 231 mg (0.29 mmol, 6.8%). ^{13}C NMR, ^1H NMR, Rf as described for **9**. Anal. Calcd. for $C_{52}H_{52}N_2O_9\cdot 0.25H_2O$: C, 73.18; H, 6.20; N, 3.28. Found: C, 73.11; H, 6.19; N, 3.62.

(*R*)-1-(1-(2-(4,4′-dimethoxytrityloxy)ethoxy)-3-*O*-(2-cyanoethoxy(diisopropylamino)-phosphino)-3-hydroxyprop-1-yl)thymine (13)

7 (100 mg, 0.183 mmol) was dried by coevaporation with anhydrous CH_3CN and dissolved under N_2 in anhydrous CH_2Cl_2 (0.51 ml). *N,N*-diisopropylethylamin (0.16 ml) was added followed by dropwise addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.072 ml, 0.32 mmol). After 60 min when analytical TLC showed no more starting material, the reaction was quenched with MeOH (0.04 ml) and diluted with EtOAc (5 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 (200 ml). The product was collected by filtration and dried under vacuum to give **13** as a white powder. Yield 101 mg (0.14 mmol, 77%). ^1H NMR for the dominating diastereoisomer ($CDCl_3$) δ 1.14–1.36 (m, 14H, 2×$(CH_3)_2C$, CH_2CN), 1.90 (s, 3H, CH_3), 2.03–2.09 (m, 2H, 2×H-2′), 2.57–2.60 (m, 4H, CH_2OP, 2×$CH\underline{C}H_3$), 3.15–3.35 (m, 2H, 2×H-4′), 3.53–3.86 (m, 4H, 2×H-5′, 2×H-3′), 3.78 (s, 6H, 2×OCH_3), 5.82–5.95 (m, 1H, H-1′), 6.78–7.44 (m, 13H, DMT), 7.19 (s, 1H, H-6). ^{31}P NMR ($CDCl_3$) δ 147.9, 148.4. Rf = 0.60 ($EtOAc/CH_2Cl_2/Et_3N$, 45:45:10, v/v/v).

(*R*)-1-(1-(2-*O*-(2-cyanoethoxy(diisopropylamino)phosphino)-2-hydroxyethoxy)-3-(4,4'-dimethoxytrityloxy)prop-1-yl)thymine (14)

Same procedure as described for **13** was used with **8** as starting material. Yield 93.1 mg (0.12 mmol, 71%). ^{1}H NMR for the diastereoisomer obtained ($CDCl_3$) δ 1.04 (t, 2H, J=7.2 Hz, CH_2CN), 1.12−1.27 (m, 12H, 2×$(CH_3)_2C$), 1.90 (s, 3H, CH_3), 1.94−2.05 (m, 2H, 2×H-2'), 2.56 (t, 2H, J=7.1 Hz, CH_2OP), 2.58−2.62 (m, 2H, 2×C<u>H</u>CH_3), 3.18 (t, 2H, J=5.9 Hz, 2×H-3'), 3.52−3.81 (m, 4H, 2×H-5', 2×H-4'), 3.77 (s, 6H, 2×OCH_3), 5.96 (t, 1H, J=6.0 Hz, H-1'), 6.78−7.40 (m, 13H, DMT), 7.11 (s, 1H, H-6). ^{31}P NMR ($CDCl_3$) δ 149.2. Rf = 0.60 (EtOAc/CH_2Cl_2/Et_3N, 45:45:10, v/v/v).

(*S*)-1-(1-(2-(4,4'-dimethoxytrityloxy)ethoxy)-3-*O*-(2-cyanoethoxy(diisopropylamino)-phosphino)-3-hydroxyprop-1-yl)thymine (15)

Same procedure as described for **13** was used with **10** as starting material. Yield 77.4 mg (0.10 mmol, 59%). ^{1}H NMR, ^{31}P NMR and Rf as described for **13**.

(*S*)-1-(1-(2-*O*-(2-cyanoethoxy(diisopropylamino(phosphino)-2-hydroxyethoxy)-3-(4,4'-dimethoxytrityloxy)prop-1-yl)thymine (16)

Same procedure as described for **13** was used with **11** as starting material. Yield 123 mg (0.16 mmol, 94%). ^{31}P NMR for the diastereoisomer obtained ($CDCl_3$) δ 148.7. Rf as described for **14**.

Synthesis of oligodeoxynucleotides

The syntheses of oligonucleotides **17−32** were performed on a Pharmacia Gene Assembler Special^R DNA-synthesizer in 0.2 μmol-scale (5 μmol amidite per cycle, Pharmacia primer support^{TM}) using commercial 2-cyanoethylphosphoramidites as well as compound **13−16**. The synthesis followed the regular protocol of the DNA-synthesizer for 2-cyanoethylphosphoramidites. The coupling efficiency of **13−16** was slightly lower (app. 90%) than those of the unmodified amidites (app. 99%). The oligonucleotides were removed from the solid support by treatment with concentrated ammonia at 55°C for 10 h which also removed the protecting groups. The ammonia solutions were desalted on a NAP-10^R-column (Pharmacia) eluting with water.

Melting experiments

The melting experiments were carried out in medium salt buffer, 1 mM EDTA, 10 mM Na_2HPO_4, 140 mM NaCl, pH 7.2 as previously described.[36] The increase in absorbance at 260 nm as a function of time was recorded while the temperature was raised linearly from 10−80°C with a rate 1°C per minute. Melting hypochromicity values were calculated from the initial and final absorbance as described below.

Enzymatic stability of the oligodeoxynucleotides

A solution of the oligodeoxynucleotide (app. 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 phosphodiesterase; 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 versus time curve of the digestion was plotted from which the hyperchromicity and half-life of the oligomer was evaluated. The final hyperchromicity is defined as the final absorbance at 260 nm divided by the initial absorbance. The extinction coefficient of the oligonucleotide is the sum of the extinction coefficients of the constituent monomeric deoxynucleosides divided by the final hyperchromicity. Then the hypochromicity value (h) can be calculated using the equation:[16]

$$H = \frac{\epsilon_{monomer} - \epsilon_{oligo}}{\epsilon_{monomer}} \times 100\%$$

Extinction coefficients for the constituent monomers: ϵ_{260} : A_d, 15400; C_d, 7300; G_d, 11700; T, t, 8800.[16,33]

Digestion of 5'-end [^{32}P]-labelled oligodeoxynucleotides

Oligodeoxynucleotides **17**, **23**, **24** and **26** were [^{32}P]-labelled at the 5'-end using γ-[^{32}P]-ATP and polynucleotide kinase as described.[37] 50 pmol [^{32}P]-labelled oligodeoxynucleotide was digested with 0.035 U SVPDE in 80 μl of 0.1 M Tris.HCl (pH 8.9), 0.1 M NaCl, 14 mM $MgCl_2$ at 25°C. Aliguots were transferred to an equal volume of 20 mM EDTA on ice prior to enzyme addition and at 1, 5, 15, 30, 60 and 300 minutes after enzyme addition. The samples were dried and redissolved in 10 μl de-ionized formamide. The samples were electrophorezed through a 20% polyacrylamide gel containing 8 M urea. The gel was soaked in 30% ethanol, 20% glycerol, dried and exposed to autoradiography.

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