D-Altritol Nucleic Acids (ANA): Hybridisation Properties, Stability, and Initial Structural Analysis

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Abstract: Oligonucleotides composed of a phosphorylated D-altritol backbone with nucleobases introduced in the 2′-position, hybridise strongly and sequence-selectively with RNA in an antiparallel way. The order of hybridisation strength is: dsANA > ANA:RNA > ANA:DNA. Complexes between ANA and RNA or DNA are more stable than between HNA and natural oligonucleotides. The dsANA hybrid is extremely stable. When compared with an identical dsDNA sequence, the ΔT_m per modification is $+10.2\,^{\circ}C$ for a hexamer. CD spectral analysis indicates that ANA complexes are very similar to the A-form dsRNA duplex. ANA are stable in alkaline medium up to pH 12 and, likewise, are not degraded in human serum.

Keywords: altritol nucleic acids · antisense agents · hexitol nucleic acids · oligonucleotides · thermal stability data

Introduction

Control of translation processes is a continuously growing research area, and the use of antisense oligonucleotides reflects one of the possibilities enabling such control. This relies mainly on the degradation of the mRNA target through the assistance of RNase H, which becomes activated by recognition of the mixed DNA:RNA duplex. Oligonucleotides that do not activate RNase H after hybridisation with complementary RNA have to rely on a strong association within the duplex to obtain an antisense effect. If oligomers can be obtained that are able to induce strand displacement in double-stranded RNA structures, targeting of RNA becomes independent of the secondary and tertiary structure of the mRNA, and the number of possible RNA targets will increase considerably. One way to approach this problem is to synthesise carbohydrate-modified oligonucleotides, exemplified by hexitol nucleic acids,[1–3] 2′-*O*-(2-methoxy)ethyl oligonucleotides[4] and bicyclic oligonucleotides,[5] with the compounds of the Wengel group[6] showing the strongest affinity for RNA. The strong hybridisation characteristics between these structures and complementary RNA are generally attributed[7] to the formation of a preorganized conformation, which fits the A-form of dsRNA, good stacking interactions between the bases, which interact in a Watson–Crick type geometry with their complement, and efficient hydration of the double-stranded helix.

Hexitol nucleic acids (HNA) are composed of phosphorylated 2,3-dideoxy-D-arabino-hexitol units with a nucleobase situated in the 2-(*S*)-position. They hybridise sequence-selectively with RNA in an antiparallel way. The observed increase in T_m per modification of an HNA:RNA duplex versus duplexes of natural nucleic acids is sequence and length dependent and varies from $+0.9\,^{\circ}C$ per modification[3] to $+5.8\,^{\circ}C$ per modification.[2] HNA is an efficient steric-blocking agent, as observed during investigations of HNA in cell-free translation experiments (giving IC_{50} values of 50 nM as inhibitors of Ha-ras mRNA translation).[8]

An interesting observation made during hybridisation experiments is that the HNA:RNA duplex is invariably more stable than the HNA:DNA duplex. Molecular-dynamics simulation of HNA:RNA and HNA:DNA hybrids revealed that minor-groove solvation contributes to this difference in duplex stability.[9] In order to increase minor-groove hydration further, in an effort to influence hybridisation in a beneficial way, we synthesised D-altritol nucleic acids (ANA). D-Altritol

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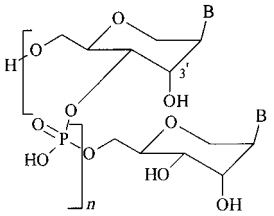


Figure 1. Structure of D-altritol nucleic acids (ANA).

nucleic acids consist of a phosphorylated D-altritol backbone with nucleobases inserted in the 2′-position of the carbohydrate moiety (Figure 1). They differ structurally from HNA[1, 3] by the presence of a supplementary hydroxy group in the 3′-α-position, this means that the 3′-carbon of the altritol moiety adopts the (*S*)-configuration. Inversion of configuration, giving the 3′-(*R*)-form, gives D-mannitol nucleic acids (MNA), which lack hybridisation capabilities with natural nucleic acids.[10]

The introduction of an additional hydroxy group in the 2′-position of natural furanose nucleosides or in the 3′-position of 1,5-anhydrohexitol nucleosides might influence hybridisation owing to one or more of the following effects: a) influence on the conformation of the nucleoside itself, which might become locked in one of the extreme furanose conformations, b) influence on the polarity of the solvent-accessible surface and c) stabilisation of a particular oligonucleotide conformation because of the formation of hydrogen bonds and/or steric effects. Six-membered nucleosides are generally conformationally more pure than five-membered nucleosides,[11, 12] therefore, in these cases, the influence of a) is of less importance and the effects of b) and c) are easier to study. Unfortunately only very weak hybridisation was noticed for MNA[10] with complementary RNA sequences. This is due to conformational restriction of single-stranded MNA in a partially unwound form by formation of intrastrand hydrogen bonds between the 3′-hydroxy and the 6′-*O* of the phosphate of the next nucleotide (point c). This hydrogen bond, however, cannot be formed with 2-deoxy-1,5-anhydro-D-altritol nucleosides as the repeating unit in the backbone structure (ANA). The 3′-hydroxy group of this nucleoside analogue points into the minor groove of the ANA:RNA duplex and might positively influence hybridisation, either by increasing hydration of the groove or by further stabilisation of a preorganized single-stranded structure. The D-altritol nucleoside analogue with a uracil-base moiety has been synthesised before[13] as an unprotected compound and was shown to adopt the C1 conformation as found in the building units of HNA.

Abstract in Dutch: *Oligonucleotiden opgebouwd uit een gefosforyleerde D-altritol ruggegraat met nucleobasen in de 2′-positie, hybridizeren sterk en sequentieselectief met RNA in antiparallelle richting. De volgorde van hybridizatiesterkte is: dsANA > ANA:RNA > ANA:DNA. Complexen gevormd tussen ANA en RNA of DNA zijn stabieler dan tussen HNA en natuurlijke oligonucleotiden. Het dsANA hybride is uitzonderlijk stabiel; vergeleken met eenzelfde dsDNA sequentie bedraagt de ΔT_m per modificatie +10.2 °C voor een zesmeer. Analyse door middel van CD spectrometrie geeft een aanwijzing dat ANA-complexen sterk gelijken op de A-vorm van een dsRNA duplex. ANA zijn stabiel in alkalisch milieu tot pH 12 en worden niet afgebroken in menselijk serum.*

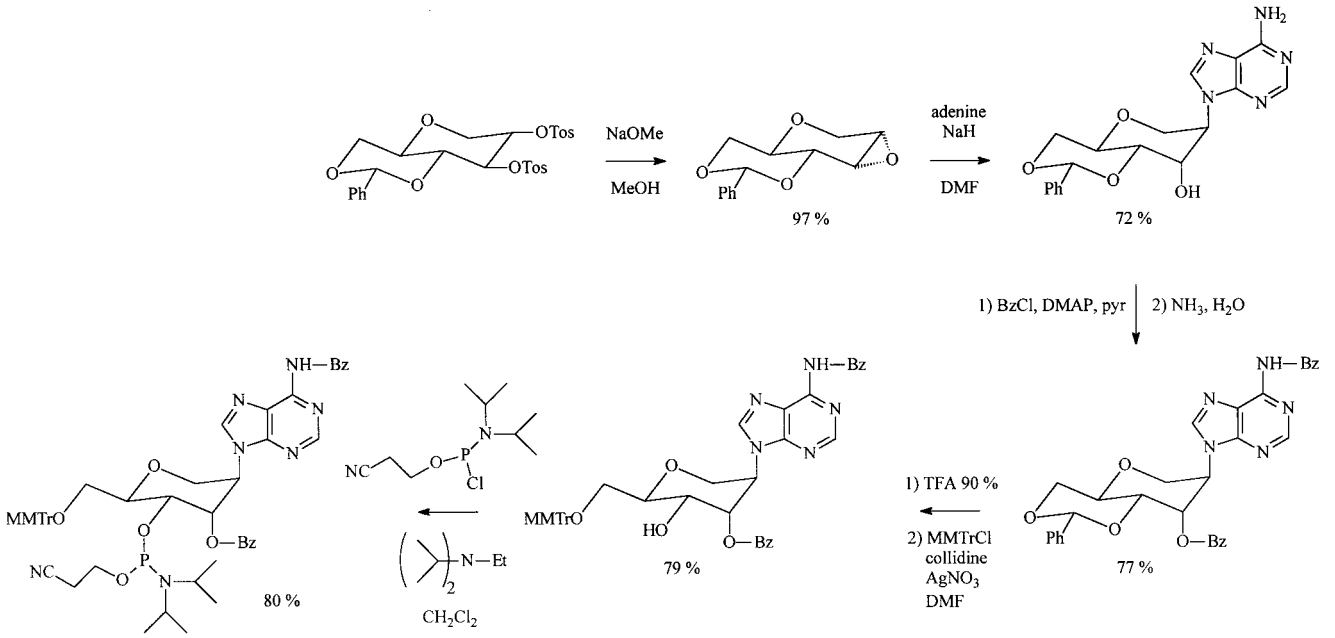
Results and Discussion

Synthesis and analysis of D-altritol nucleic acids: The protected D-altritol nucleosides were synthesised by the cleavage of the 2,3-epoxide of 4,6-benzylidine-1,5-anhydro-D-allitol with the sodium salt of nucleobases, followed by a general protecting–deprotecting strategy resulting in 3-*O*-benzoyl-2-deoxy-4-*O*-[*N*,*N*-diisopropyl(2-cyanoethyl)]phosphoramidite-6-*O*-monomethoxytrityl-D-altritol building blocks (Scheme 1).[14]

The dimethylformamidine group was selected for protection of the 2-NH_2 function of the guanine base instead of the more (basic) stable isobutyryl group, which proved difficult to introduce selectively. The obtained phosphoramidites were used to synthesise oligonucleotides on a 1 μmol scale following standard protocol (concentration phosphoramidites 0.12 M), except for a prolonged coupling time of 10 min to ensure adequate coupling yields (comparable with standard RNA synthesis) and a prolonged acid treatment (2 min) to fully deprotect the monomethoxytrityl group. The solid support was functionalised with a 1,3-propanediol moiety to avoid the formation of the corresponding succinylated D-altritol nucleosides.[1–3] Deprotection was initially carried out in a two-step procedure. In order to avoid a migration reaction, the cyanoethyl protecting group was first removed with piperidine/dioxane (1:4) followed by ammonolysis of the benzoyl groups with concentrated ammonia at 55 °C. However, it was later demonstrated that this procedure is not needed. Therefore, deprotection was carried out with only concentrated ammonia for the second series of ANA oligonucleotides. Purification by ion exchange chromatography followed published procedures.[15] Deconvoluted electrospray ionisation mass spectrometric analysis showed the obtained oligonucleotides to be of correct molecular weight.

Thermal stability studies of D-altritol nucleic acids: For comparison of data with the previously obtained HNA sequences, we synthesised the hexamer 6′-AGGAGA-4′ and the dodecamer 6′-AGGGAGAGGAGA-4′. Table 1 summarises the results of the T_m determinations, which were generally measured in 0.1 M NaCl, 20 mM KH_2PO_4, pH 7.5 and 0.1 mM EDTA. In order to allow comparison with the DNA complexes of the hexamer, T_ms of the latter were determined in NaCl (1 M), as otherwise no dsDNA duplex could be obtained. For the HNA:DNA oligopurine hexamer an increase in T_m (at 1 M NaCl) of 21 °C was obtained relative to that of dsDNA. This ΔT_m further increases to 29 °C for the ANA:DNA duplex, resulting in a ΔT_m per modification of +3.5 °C and +4.8 °C, respectively.

An increase in T_m with RNA as the complement was also observed, resulting in extremely stable ANA:RNA duplexes, at 1 M NaCl approaching 60 °C. This observation was confirmed by the use of the dodecamer. The ΔT_m per modification for HNA:DNA and ANA:DNA relative to dsDNA is +1.3 °C and +2.0 °C, respectively. With RNA as a complement, the ΔT_m per modification increases to +3.0 °C and +3.3 °C. The difference in stability of ANA:RNA complexes relative to ANA:DNA hybrids confirms the previously observed RNA selectivity for hexitol nucleic acids.[2, 3] When



Scheme 1. General scheme for the synthesis of the modified nucleosides, exemplified by the synthesis of the building block with the adenine base moiety.[14]

Table 1. Melting temperatures [°C, at 260 nm] of fully modified hexamers and dodecamers (1,5-anhydrohexitol oligomers prefix h, altrohexitol oligomers prefix a) determined in 0.1M NaCl, 20mM KH_2PO_4 pH 7.5, 0.1mM EDTA with a concentration of 4μM of each oligonucleotide. Duplexes were formed with the complementary sequences 5′-TCTCCT, 5′-TCTCCTCTCCCT (antiparallel) and 5′-TCCCTCTCCTCT (parallel) as the respective DNA complements; and 5′-TT-r(UCUCCU)-TT, 5′-TT-r(UCUCCUCUCCCU)-TT (antiparallel) and TT-r(UCCCUCUCCUCU)-TT (parallel) as the respective RNA complements.

Sequence	T_m DNA complement	T_m RNA complement
6′-h(AGGAGA)	31.2[a]	44.8
6′-a(AGGAGA)	39.2[a]	48.9 (59.7[a])
5′-AGGAGA	10.0[a]	ND[b]
6′-h(AGGGAGAGGAGA)	64.8	84.0
6′-a(AGGGAGAGGAGA)	73.5	88.1
5′-AGGGAGAGGAGA	49.0	47.6
6′-h(AGGGAGAGGAGA)	52.4 (parallel)	69.7 (parallel)
6′-a(AGGGAGAGGAGA)	60.1 (parallel)	72.7 (parallel)
5′-AGGGAGAGGAGA	31.4 (parallel)	26.8 (parallel)

[a] Determined at 1M NaCl as otherwise no dsDNA could be detected. [b] ND: not determined owing to the low melting temperature.

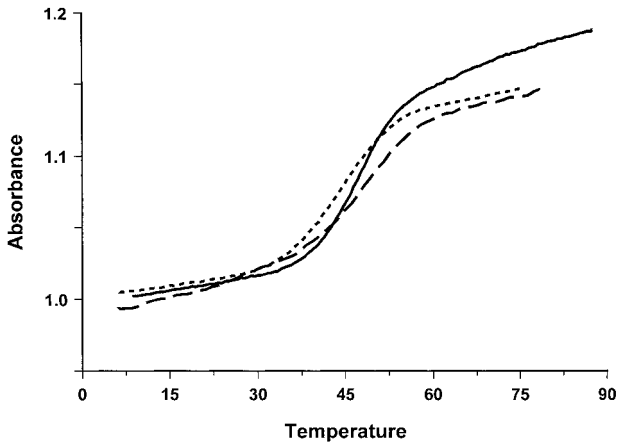


Figure 2. Melting profiles for the HNA and ANA hexamer 6′-AGGAGA-4′ in 0.1M NaCl buffer at 4μM with their RNA complement [HNA:RNA (----); ANA:RNA (– – –)], in comparison with the melting profile for a DNA dodecamer 5′-AGGGAGAGGAGA-3′ with its RNA complement (——); all the profiles are normalized.

compared with HNA, the ANAs invariably demonstrate increased duplex stability. Figure 2 shows the melting profiles of the hexamer sequences of HNA and ANA with their respective RNA complements.

Figure 2 and Table 1 clearly show the hybridising potential of HNA and ANA, where the stability of a hexamer duplex approaches the affinity of a DNA dodecamer for their respective RNA complementary sequences. In summary, duplex stability decreases in the order ANA:RNA > HNA:RNA > ANA:DNA > HNA:DNA > dsDNA > DNA:RNA. Table 1 also provides information about the orientation of both strands in the duplex. In all cases studied, the T_ms of the parallel-oriented strands are considerably lower than the T_ms of the antiparallel strands. The T_m found for the parallel sequences (Table 1) most probably reflects antiparallel pairing of the 8 bases at the 3′(4′)-terminal end of the oligopurine sequence with the 8 bases at the 3′-end of the oligopyrimidine complement. ANA thus represents an antiparallel Watson–Crick pairing system as observed for HNA.

Table 2 summarises investigations on the fidelity of the Watson–Crick pairing system between ANA (purine base) and DNA (pyrimidine base) in comparison with the results obtained with HNA. A G–G, G–A or G–T mismatch results in a decrease of the T_m of −11°C to −14°C for the dsDNA duplex and a decrease of −10°C to −17°C for the HNA:DNA duplex. In accordance with the latter observation, a single mismatch in the ANA:DNA duplex leads to a ΔT_m of −10°C to −17°C. Similar results are obtained upon introduction of A–G, A–A or A–C mismatches. The

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Table 2. Influence of mismatches versus G* and A* on the melting temperature [°C, at 260 nm] of HNA:DNA and ANA:DNA duplexes determined in 0.1M NaCl, 20mM KH_2PO_4 pH 7.5, 0.1mM EDTA with a concentration of 4μM of each oligonucleotide.

		HNA:DNA 6′-(AGG GAG AGG AGA)* 3′- TCC CT**C** **T**CC TCT-5′[a]		ANA:DNA 6′-(AGG GAG AGG AGA)* 3′- TCC CT**C** **T**CC TCT-5′[a]		DNA:DNA 5′-AGG GAG AGG AGA-3′ 3′-TCC CT**C** **T**CC TCT-5′[a]	
x	y	T_m [°C]	ΔT_m [°C]	T_m [°C]	ΔT_m [°C]	T_m [°C]	ΔT_m [°C]
Control		64.8		73.5		49.0	
1 × G		49.8	−15.0	57.0	−16.5	37.0	−12.0
1 × A		47.9	−16.9	56.2	−17.3	37.8	−11.2
1 × T		55.0	−9.8	62.9	−10.6	35.4	−13.6
	1 × G	44.9	−19.9	53.0	−20.5	35.9	−13.1
	1 × A	54.3	−10.5	61.6	−11.9	38.0	−11.0
	1 × C	56.2	−8.6	62.9	−10.6	34.3	−14.7

[a] **C** indicates position x and **T** indicates position y.

decrease in T_m for an A–A mismatch is similar for the dsDNA, HNA:DNA and ANA:DNA duplexes (−11°C). The A–G mismatch gives a more profound decrease in T_m when considering ANA:DNA and HNA:DNA duplexes rather than dsDNA. The opposite is observed for A–C mismatches. In summary, the experiment with the polypurine sequence demonstrates the sequence selectivity of the Watson–Crick ANA base-pairing system.

In a second set of experiments, we evaluated the thermal stability of ANA-containing complexes that contain all four natural nucleobases. The previously observed order of stability [ANA:RNA > HNA:RNA > dsRNA] was confirmed, though the ΔT_m per modification with dsRNA as a reference is lower (ΔT_m per modification = +1.5°C for ANA and ΔT_m per modification = +0.85°C for HNA) than with dsDNA or DNA:RNA as a reference (as used in Table 1). For the evaluation of these compounds as potential RNA-strand displacement agents, a comparison with the thermal stability of dsRNA is important. One also has to take into account that in the ANA sequence, uracil is the replacement nucleobase for thymine; this results in a slight drop in affinity. The actual influence of the extra 3′-hydroxy group might be slightly higher than reflected by the ΔT_m here. The synthesis of the mixed sequence also allows us to evaluate the destabilisation of duplexes for all mismatches. As can be observed in Table 3, the ΔT_m per mismatch ranges from −11°C (G–U) to −30°C (G–A) for the RNA:ANA duplex and the given sequence. This difference is similar to the destabilisation observed when introducing mismatches in dsRNA and HNA:RNA [except for a G–U mismatch in dsRNA with a ΔT_m of −4°C (Table 3, entry 5), but the U–G mismatch in position 3 (Table 3, entry 9) yields a ΔT_m of −12°C]. Even in a completely mixed ANA sequence, the selectivity of the Watson–Crick base pairing could be observed.

This sequence, however, does not allow a comparison between the stability of ANA:RNA and ANA:DNA duplexes at the level of mixed A,U,G,C sequences, because the DNA sequence 5′-GCGTAGCG-3′ appears as an unusual hairpin structure.[16] Therefore, we repeated the T_m measurement for another mixed sequence (Table 4). The expected order of stability [ANA:RNA > HNA:RNA > ANA:DNA > HNA:DNA] was confirmed. The difference in stability between ANA:RNA and ANA:DNA is very large (−2.55°C per base). Therefore, in completely mixed systems ANA is like HNA, an RNA-selective oligomer.

Table 3. Influence of mismatches on the melting temperatures [°C, at 260 nm] of a mixed HNA 8-mer and its DNA and RNA analogues with their RNA complement containing one mismatch (bold and underlined), determined in 0.1M NaCl, 20mM KH_2PO_4 pH 7.5, 0.1mM EDTA with a concentration of 4 μM of each oligonucleotide.

	6′-GCGUAGCG-4′ ANA		6′-GCGTAGCG-4′ HNA		5′-GCGUAGCG-3′ RNA	
RNA	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
1 3′-CGCAUCGC-5′	59.6		54.4		47.6	
2 3′-CGCA**A**CGC-5′	40.2	−19.4	32.7	−21.7	27.3	−20.3
3 3′-CGCA**G**CGC-5′	41.0	−18.6	37.0	−17.4	32.9	−14.7
4 3′-CGCA**C**CGC-5′	47.3	−12.3	40.8	−13.6	30.5	−17.1
5 3′-CGC**G**UCGC-5′	48.8	−10.8	41.5	−12.9	43.7	−3.9
6 3′-CGC**C**UCGC-5′	42.2	−17.4	31.0	−23.4	30.1	−17.5
7 3′-CGC**U**UCGC-5′	43.4	−16.2	33.8	−20.6	30.1	−17.5
8 3′-CG**A**AUCGC-5′	29.5	−30.1	16.2	−38.2	19.7	−27.9
9 3′-CG**U**AUCGC-5′	45.2	−14.4	40.8	−13.6	35.0	−12.6
10 3′-CG**G**AUCGC-5′	38.7	−20.9	30.6	−23.8	28.1	−19.5
11 3′-C**C**CAUCGC-5′	41.9	−17.7	36.2	−18.2	36.5	−11.1
12 3′-C**A**CAUCGC-5′	44.8	−14.8	41.6	−12.8	38.5	−9.1
13 3′-C**U**CAUCGC-5′	42.2	−17.4	37.4	−17	35.8	−11.8

The complex formed between the polypurine ANA [6′-a(AG_2AGA)] and its polypyrimidine RNA complement is considerably more stable than the reverse complex formed between the polypyrimidine ANA sequence [6′-a($UCUC_2U$] and its polypurine RNA complement (Tables 1 and 4). The T_m in 1.0M NaCl buffer of 6′-a(AG_2AGA)–3′-r(UC_2UCU) is

Table 4. Melting temperatures [°C, at 260 nm] of fully modified hexamers and decamers determined in 0.1M NaCl, 20mM KH_2PO_4 pH 7.5, 0.1mM EDTA with a concentration of 4μM of each oligonucleotide. Duplexes were formed with the complementary sequences 6′-a(AGGAGA), 5′-TT-r(AGGACA)-TT, 5′Tr(GGCAUUACGG)-T and 5′-GGCATTACGG.

	T_m RNA complement	T_m ANA complement	T_m DNA complement
6′-a(UCUCCU)	38.4 (47.6[a])	61.8 (71.2[a])	–[c]
6′-h(UCUCCU)	30.5 (40[a])	50.8[b] (61.2[a,b])	–[c]
6′-a(CCGUAAUGCC)	64.0	ND[d]	38.5
6′-h(CCGTAATGCC)	59.4	ND[d]	31.6
5′-CCGTAATGCC	44.5	ND[d]	46.5

[a] Determined at 1M NaCl. [b] HNA complement h(AGGAGA). [c] No T_m detected. [d] ND: not determined.

59.7 °C, while the T_m of 4′-a(UC_2UCU) – 5′-r(AG_2AGA) is 47.6 °C. The same difference is observed in 0.1 M NaCl (48.9 °C and 38.4 °C, respectively). This may be a result of a combination of a) higher conformational stress of D-altritol pyrimidine nucleosides versus D-altritol purine nucleosides when incorporated in oligonucleotides and b) differences in stacking energy within both complexes.

A last observation from T_m measurements is that self-complementary ANAs are extremely stable and exceed the stability of all other complexes. While the T_m for the dsDNA sequence 5′-d(AG_2AGA) – 3′-d(TC_2TCT) is only 10 °C with 1 M NaCl, the T_m for the same dsANA sequence (with uracil bases) is 61.8 °C with 0.1 M NaCl and 71.2 °C with 1 M NaCl, this means a ΔT_m per base pair of +10.2 °C. For this same sequence the difference in stability between dsANA and ANA:RNA is 11.5 °C for 1 M NaCl (+1.9 °C per modification) with the purine sequence as ANA, and 23.6 °C (4 °C per modification) if the pyrimidine strand makes up the ANA part.

Thermodynamic calculations were performed for the mixed sequence octamers (Table 5) with the methods developed by Gralla and Crothers[17] (see the Experimental Section). However, it seems preliminary to make definitive conclusions with respect to the effects on enthalpy and entropy changes caused by the different backbone changes. First of all we have to bear in mind that the HNA sequence contains a thymine base as a replacement for uracil in the ANA and RNA sequence. Recent results confirm that for HNA, replacement of a thymine base with a uracil base results in an increase in T_m of approximately 1.5 °C.[18]

Notwithstanding the presence of uracil in the ANA sequence, a clearly higher gain in enthalpy is noticed for duplex formation of the latter with RNA relative to the HNA:RNA duplex. This is partially compensated by a slightly higher loss in entropy for the ANA:RNA duplex formation. The gain in enthalpy for the mixed sequence octamers was comparable for both duplexes HNA:RNA and RNA:RNA. Owing to the slightly higher loss in entropy, RNA:RNA duplex formation is still accompanied with a lower gain in free energy.

Table 5. Thermodynamic data calculated for the duplexes formed between the mixed HNA 8 mer 6′-GCGTAGCG-4′and its DNA and RNA analogues with their RNA complement 3′CGCAUCGC-5′. See Experimental Section for conditions.

	hybridization of the RNA complement with		
	ANA	HNA	RNA
T_m [°C]	59.6	54.4	47.6
$-\Delta H$ [kcal mol^{-1}]	67.7	58.5	58.9
$-\Delta S$ [cal K^{-1} mol^{-1}]	178	153	158
$-\Delta G$25 °C [kcal mol^{-1}]	14.6	12.8	11.8

Overall, however, one might conclude that the extra hydroxy groups of the ANA sequence generate an enthalpic advantage over HNA for duplex formation. This preliminary observation is more in favour of the increased hydration hypothesis than the increased preorganization hypothesis, when trying to explain the higher stability of ANA:RNA over HNA:RNA duplexes.

CD spectral analysis: CD spectroscopy is a useful technique to identify the general shape of nucleic acid complexes. The difference between an A-type duplex (i.e., dsRNA) and a B-type duplex (i.e., dsDNA) can be easily distinguished by means of this technique. We determined the CD spectrum of the single-stranded ANA sequence 6′-a($C_2GUA_2UGC_2$), of the complex formed with its RNA complement and of the corresponding dsRNA in 0.1 M NaCl, 0.02 M KH_2PO_4 at pH 7.5 (Figure 3). The CD spectrum of the ssANA (A) shows a band at 270 nm. The signals below 250 nm are very weak. For the ANA:RNA complex (B), the positive Cotton effect is situated around 258 nm and the negative band at 212 nm. This last spectrum corresponds well with the CD spectra for dsRNA (C) and HNA:RNA.[3] The hydroxy group in the 3′-position does not influence the general shape of ANA complexes, which resemble the structure of A-like dsRNA duplexes.

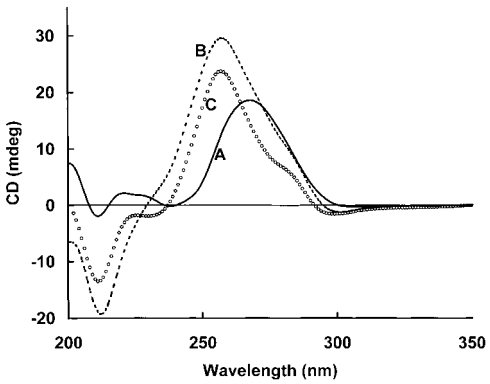

Figure 3. CD spectrum of: A) the single-stranded ANA sequence 6′-a($C_2GUA_2UGC_2$), B) the complex with its RNA complement and C) the corresponding dsRNA sequence in 0.1 M NaCl, 0.02 M KH_2PO_4 at pH 7.5.

Stability of D-altritol nucleic acids in alkaline buffer and in serum: The structural difference between a D-altritol nucleoside and the previously described D-anhydrohexitol nucleosides is the presence of a hydroxy group in the 3′-position. This is similar to the difference between a ribonucleoside and a deoxyribonucleoside. Because of this difference, RNA is more labile in basic medium than DNA with the 2′-hydroxy group assisting the phosphodiester cleavage reaction. As ribonucleases are more ubiquitous than deoxyribonucleases, RNA is degraded much faster in a biological environment than DNA. Also the presence of a six-membered carbohydrate ring in ANA with an equatorially oriented phosphate group at C-4′ and an axial hydroxy group at C-3′, led us to expect ANA to be somewhat more base stable than RNA. However, this had to be experimentally verified, together with the enzymatic stability of ANA. An increase in both chemical and enzymatic stability, when compared with RNA, would provide ANA with more interesting antisense properties.

The ANA sequences evaluated all have a propanediol at the 3′-end (see legend of Figure 4) to aid synthesis. To

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circumvent the loading of the solid support with four different nucleoside analogues, we prefer to use a more universal propanediol-functionalized solid support.[1, 3] This propanediol may increase the stability of the oligonucleotide against exonuclease degradation even though it does not interfere with the hybridisation strength. As the propanediol is present in all the oligonucleotides subjected to biological evaluation, and as we would like to evaluate the potential of our new oligomers to function as antisense oligonucleotide in vitro and in vivo, the investigation of the stability of propanediol-modified ANA seems to us to be at least as relevant as the investigation of naked ANA. The RNA, used as reference oligomer, was protected at both ends with two thymidine units, increasing somewhat its enzymatic stability. For the study of the stability in basic medium one polypurine and one polypyrimidine oligomer was selected (Figure 4). A mixed

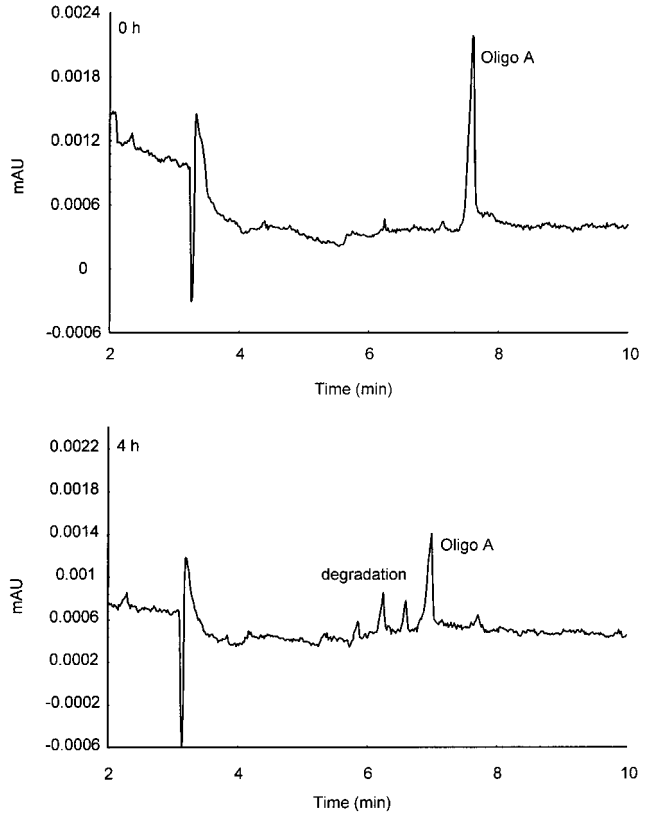

Figure 4. Oligonucleotide sequences which were subjected to enzymatic and base catalysed degradation reactions: A) 6′-a(AGGACA)-propanediol, B) TT-r(AGGAGA)-TT, C) 6′-a(UCUCCU)-propanediol, D) TT-r(UCUCCU)-TT, E) 6′-a(CCGUAAUGCC)-propanediol, F) T-r(GGCAUUACGG)-T. Electropherogram of oligo A analysed after 0 and 4 hours of degradation at pH 13. See Experimental Section for SFC conditions.

sequence was used to carry out the enzymatic degradation study. Oligomers A and C (see legend of Figure 4) were first evaluated at pH 8, 10 and 12. Both compounds were completely stable for at least 24 hours. No traces of shorter length degradation products could be detected. When the RNA analogue was incubated at pH 12, 10% degradation was noticed after 20 hours. Oligomer (oligo) A and its RNA analogue (oligo B) were then subjected to degradation at pH 13. At this pH, oligo A was degraded with a $t_{1/2}$ of 3.5 hours. Figure 4 shows such an electropherogram after 0 and 4 hours at pH 13. The RNA analogue, however, was degraded at least twice as fast as ANA. The observed $t_{1/2}$ at pH 13 is 1.6 hours. The sensitivity of the technique allows the exclusion of extensive 4′-*O* to 3′-*O* migration of the phosphodiester group.

When incubated in serum, oligo E showed no degradation when analysed after a 24 hours incubation. On the other hand, its RNA analogue (oligo F) showed extensive degradation after 1 hour of incubation. These preliminary degradation experiments suggest that a) the propanediol-labelled ANA is sufficiently stable to survive conditions of biological media and b) the increased alkaline stability allows us to use basic conditions, similar to those used for RNA synthesis, during the deprotection of oligomers after solid-phase synthesis.

Conclusions

The introduction of a C-3′-hydroxy group in the hexitol moiety of HNA, giving a 3′-chiral centre in the (*R*)-configuration, leads to oligonucleotides (MNA) that form unstable duplexes with RNA owing to unfavourable preorganization.[10] Inversion of stereochemistry at the 3′-position (3′-(*S*)-configuration) to afford D-altritol nucleic acids results in an oligonucleotide with superior hybridisation properties. Similar studies have been carried out in the past in investigations on the self-pairing of allo-pyranosyl nucleic acids, altro-pyranosyl nucleic acids and gluco-pyranosyl nucleic acids.[19, 20] The allo-pyranosyl oligonucleotides do not display Watson–Crick purine–pyrimidine pairing, but they show weak reverse-Hoogsteen purine–purine pairing. The reason for the weak pairing is the steric hindrance between the equatorially oriented C-2′-hydroxy group and the neighbouring base pair in the 4′-direction.[19, 20] Likewise, self-complementary altro-pyranosyl oligonucleotides demonstrate extremely weak Watson–Crick pairing, this time because of restricted conformational flexibility of the base moiety owing to the presence of the axial C-2′-hydroxy group. Base pairing in the gluco-pyranosyl system is prohibited by unfavourable steric effects because of the equatorial positioning of both the C-2′-hydroxy group and the C-3′ hydroxy group.[19, 20] These systems (hexitol and pyranose), however, cannot be compared as the geometry of the complexes are fundamentally different. Pyranose nucleic acids form quasi-linear structures,[19, 20] while hexitol nucleic acids adopt A-form helical conformations.[7, 9, 21] The former oligomers do not hybridise with natural nucleic acids, while the latter oligonucleotides have the potential to do so. The influence of supplementary hydroxy groups introduced on the six-membered carbohydrate moiety is, therefore, different in both systems. Steric effects and hydrogen bonding might play a role in explaining the weak hybridisation properties of MNA.[10] This is not so for ANA, in which there is no steric hindrance to prevent self-pairing or hybridisation with natural nucleic acids. In this study we demonstrate the higher thermal stability for ANA:RNA relative to HNA:RNA duplexes. Complexes

formed between ANA and natural nucleic acids are, likewise, more stable than complexes between two natural nucleic acid strands. Moreover, the ANA complexes retain their sequence selectivity in both polypurine sequences and completely mixed sequences. Self-complementary ANAs are extremely stable associations. The structure of ANA complexes resembles the A-form helix. Single-stranded ANAs are stable in alkaline conditions up to pH 12. The increased duplex stability of ANA complexes may be due to a more efficient involvement of the 3′-hydroxy group in duplex hydration than in hydration of the isolated single-stranded ANA and/or the formation of a preorganized helical single-stranded ANA, which fits the shape of a dsRNA hybrid better than HNA itself. The present investigations allow us to suggest that the balance inclines in the direction of the former explanation. The CD spectra of single-stranded HNA and ANA of the same sequence are identical, suggesting similar preorganization. Preliminary, thermodynamic calculations indicate an important advantageous enthalpic factor on stabilisation of an ANA:RNA duplex (relative to HNA:RNA). Because of the presence of a methylene function in the 3′-position of the hexitol ring, both flanks of the minor groove of HNA:RNA duplexes have a different polarity. One part (RNA) is considerably more polar than the other part (HNA). The hydroxy group in the 3′-α-position of ANA is expected to be situated in the minor groove pointing in the direction of the solvent. Consequently, the polarity of the hexitol flank of the minor groove increases. Therefore, the solvent-accessible surface of ANA:RNA should be more hydrophilic than the solvent-accessible surface of HNA:RNA. Additionally the 3′-hydroxy group may further restrict the conformational freedom of the backbone (torsion angle ζ), and this effect may contribute to a higher preorganisation of ANA The hybridisation properties together with the strong serum stability make this new oligomer a potential new antisense candidate.

Experimental Section

Oligonucleotide synthesis: The synthesis of the protected monomers will be described elsewhere.[14] Analytical details for the phosphoramidite building blocks are as follows. 2-(N^6-Benzoyl-adenin-9-yl)-3-*O*-benzoyl-2-deoxy-4-*O*-[*N*,*N*-diisopropyl(2-cyanoethyl)]phosphoramidite-6-*O*-monomethoxytrityl-D-altritol: R_f (hexane/acetone/TEA 59:40:1) 0.28; LSI-MS (Thgly-NaOAc) *m*/*z* (%): 984 (50) $[M+Na]^+$, 273 (100) [MMTr]; ^{31}P NMR: $\delta =$ 151.64, 149.54. 2-(N^2-Dimethylformamidino-guanin-9-yl)-3-*O*-benzoyl-2-deoxy-4-*O*-[*N*,*N*-diisopropyl(2-cyanoethyl)]phosphoramidite-6-*O*-monomethoxytrityl-D-altritol: R_f (hexane/acetone/TEA 20:79:1) 0.38; LSI-MS (NPOE) *m*/*z* (%): 929 (2) $[M+H]^+$; ^{31}P NMR: $\delta = 151.49$, 149.97. 2-(N^6-Benzoyl-cytosin-1-yl)-3-*O*-benzoyl-2-deoxy-4-*O*-[*N*,*N*-diisopropyl(2-cyanoethyl)]phosphoramidite-6-*O*-monomethoxytrityl-D-altritol: R_f (hexane/acetone/TEA 59:40:1) 0.33; LSI-MS (Thgly-NaOAc) *m*/*z* (%): 960 (30) $[M+Na]^+$, 273 (100) [MMTr]; ^{31}P NMR: $\delta = 151.54$, 150.07. 2-(Uracil-1-yl)-3-*O*-benzoyl-2-deoxy-4-*O*-[*N*,*N*-diisopropyl(2-cyanoethyl)]phosphoramidite-6-*O*-monomethoxytrityl-D-altritol: R_f (hexane/acetone/TEA 59:40:1) 0.24; LSI-MS (Thgly-NaOAc) *m*/*z* (%): 857 (12) $[M+Na]^+$, 273 (100) [MMTr]; ^{31}P NMR: $\delta = 151.16$, 150.21.

Oligonucleotide synthesis was carried out on an automated DNA synthesiser with the phosphoramidite approach, model ABI 381 A (Applied Biosystems). Condensations were run at 0.12 M of the respective modified building block for 10 min to ensure adequate coupling yields. The obtained sequences were deprotected and cleaved from the solid support by treatment with concentrated ammonia at 55 °C for 16 hours. After a first purification on a NAP-10^R column (Sephadex G-25-DNA grade), a Mono-QHR 10/10 anion exchange column/Pharmacia) was used with the following gradient system: A) NaOH, pH 12.0 (10 mM), NaCl (0.1 M); B) NaOH, pH 12.0 (10 mM), NaCl (0.9 M). The low-pressure liquid chromatography system consisted of a Merck-Hitachi L6200A Intelligent pump, a Mono-Q HR 10/10 column, a Uvicord SII 2138 UV detector (Pharmacia-LKB) and a recorder. Product-containing fractions were immediately neutralised by addition of aqueous ammonium acetate. Following concentration, the eluent was desalted on a NAP 10 column and lyophilised.

UV-melting experiments and thermodynamic data: UV-melting experiments were recorded with a Uvikon 940 spectrophotometer. Samples were dissolved in a buffer solution containing NaCl (0.1 or 1 M), potassium phosphate (0.02 M, pH 7.5) and EDTA (0.1 mM). The oligomer concentration was determined by measuring the absorbance at 80 °C and assuming extinction coefficients in the denatured state as used for natural DNA. The concentration in all experiments was 4 μM of each strand. Cuvettes were kept at a constant temperature with water circulating through the cuvette holder and with a thermistor immersed directly in the cuvette. For the melting experiments, temperature control and data acquisitions were carried out automatically with an IBM/PC AT-compatible computer. The samples were heated and cooled at a rate of 0.2 °C min^{-1} with data sampling every 30 seconds. T_m values were determined from the maximum of the first-derivative curve. The thermodynamic data ΔH and ΔS (Table 5) were calculated from the melting curves by means of an all or none two-state model for helix-coil transitions developed by Gralla and Crothers.[17] The derivative at each point on the curve was determined by fitting a regression line to the point in a dynamically specified window containing 40 points (4 °C). The transition enthalpy (cal mol^{-1}) can be calculated from the Equation (1). The free energy at $T_{1/2}$ associated with the melting transition and concomitantly the entropy are further calculated as described by Loakes and Brown.[22]

$$\Delta H = -4.37(1/T_{1/2} - 1/T_{3/4}) \quad (1)$$

CD spectra: CD spectra were measured at 10 °C with a Jasco 600 spectropolarimeter in thermostatically controlled 1 cm cuvettes corrected with a Lauda RCS 6 bath. The oligomers were dissolved and analysed in buffer containing NaCl (0.1 M), potassium phosphate (0.2 M, pH 7.5) and EDTA (0.1 mM) and at a concentration of 4 μM of each strand.

Mass spectrometric analysis of oligonucleotides: The appropriate oligonucleotide (20 nmol) was taken up in triethylammonium bicarbonate (TEAB, 0.2 M, pH 8, 0.5 mL) and loaded on a C_{18} cartridge (Waters) preequilibrated with aqueous TEAB. After washing the cartridge with TEAB solution (2 mL), the oligomer was eluted with MeOH/TEAB (0.2 M, 1:1) and the eluate was lyophilised. Immediately before use, the samples were dissolved in a mixture of acetonitrile/ammonium acetate (0.01 M, 1:1, 300 μL; final concentration of the oligonucleotide about 16 pmol μL^{-1}). Electrospray ionisation mass spectra were recorded in negative mode on a VG Platform II mass spectrometer (Micromass, Manchester, UK) equipped with a Mass Lynx data system. The sample spray flow was set to 10 μL min^{-1}. Five spectra were acquired and summed in MCA mode in the *m*/*z* range 500 to 1500. The molecular weights were determined by application of the maximum entropy algorithm.

Degradation studies of oligonucleotides: All oligomers were dissolved in Milli-Q water except for sample 393 028, which was dissolved in NaCl (0.1 M) and KH_2PO_4 (0.02 M). Stock solutions had concentrations between 33 and 45 OD mL^{-1}. Phosphate buffers of pH 8, 10 and 12 were prepared by mixing appropriate volumes of K_2HPO_4 (0.05 M) and K_3PO_4. For pH 13 buffer, K_3PO_4 (0.05 M) was adjusted to pH 13.0 with KOH solution (1 M). Oligomers A-D (Figure 4) were evaluated for stability in alkaline solutions. A 10 μl sample solution was mixed with 60 μl of phosphate buffer (50 mM, pH 8, 10, 12 and 13), kept at room temperature (25 °C) and analysed by micellar electrokinetic capillary chromatography (MECC). Oligomers E and F (Figure 4) were evaluated for their serum stability in 90 % of human serum. Oligomer $(dA)_{21}$ was used as internal standard. After incubation at 37 °C, serum samples were extracted by anion-exchange centrifugal membranes, desalted by drop analysis and analysed by MECC.[23] Conditions for micellar electrokinetic capillary chromatography are as follows; Electrolyte: TAPS (*N*-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid, 20 mM), sodium dodecyl sulfate (70 mM), urea (7 M)

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[pH 7.7 with TRIS (tris(hydroxymethyl)aminomethane)]; Capillary: fused silica (50 μm ID × 44 cm total length L^{-1} and 36 cm length to detector L^{-1}); Temperature: 25 °C; Voltage: 23 kV; Injection: hydrodynamic (2 s); Detection: UV (260 nm).

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