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Acyclic Oligonucleotides: Possibilities and Limitations

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Abstract. Oligonucleotides containing acyclic nucleosides with a 3(*S*),5-dihydroxypentyl (**1a-e**) or 4(*R*)-methoxy-3(*S*),5-dihydroxypentyl (**2a**) side chain were prepared and their hybridization properties as well as their stability towards degradation with snake venom posphodiesterase were studied. Attachment of an acyclic nucleoside at the 3'-end of an oligonucleotide makes it extremely resistant against enzymatic breakdown. Whereas oligonucleotides consisting completely of acyclic 2'-deoxyadenosine analogues (**1a** or **2a**) can still hybridize with an unmodified oligothymidylate, completely modified oligothymidylates or hetero-oligomers do not hybridize with their unmodified complementary oligonucleotide. This can be explained by the favourable enthalpy change on hybridization for the oligomers with adenine bases because of their higher degree of stacking and the ability to form T-A*.T triplets. In base-pairing with the natural DNA-nucleosides (dA,dC,dG,T), the acyclic nucleoside analogues (**1a-e**) discriminate less compared to the natural 2'-deoxynucleosides. 9-(3(*S*),5-Dihydroxypentyl)hypoxanthine shows the least spreading in melting temperature on hybridization with the four natural 2'-deoxynucleosides. Because of their conformational flexibility, acyclic nucleosides can be considered as universal nucleoside for the design of probes with ambiguous positions.

INTRODUCTION

Oligonucleotides are small pieces of DNA or RNA with a wide applicability in molecular biology and bioorganic chemistry[1]. They can be used to study the mode of action of certain drugs[2] and as diagnostic agents[3]. A potential application is their use as therapeutic agents to treat all kinds of infectious diseases and cancers[4].

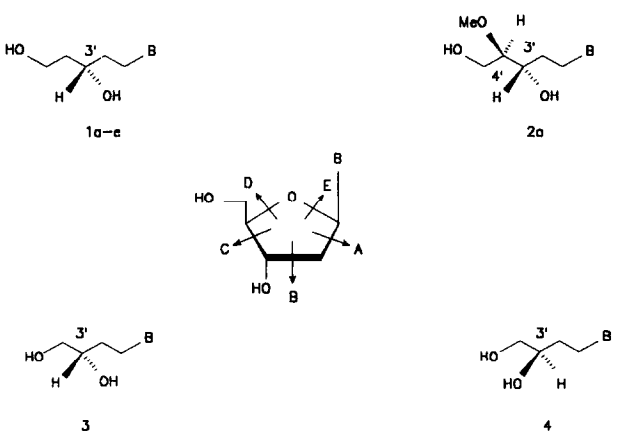
For most of these applications the hybridization properties of the oligonucleotide *via* Watson-Crick or Hoogsteen base-pairing, are of fundamental importance. When one thinks of oligonucleotides as chemotherapeutic agents, it is also necessary that the molecules are enzymatically stable, water soluble and that they can be taken up by cells. Several oligonucleotides, modified in either the carbohydrate part or the phosphate backbone have been described[4]. Almost all of these modifications increase the stability of the oligonucleotide against degradation by endo- and exonucleases to some extent. However, some of these modifications strongly decrease the stability of the duplex, formed between the modified oligo and natural DNA or RNA, whereas other modifications decrease the water solubility, so that their therapeutic potential is strongly reduced.

One of the approaches which can be used to increase the enzymatic stability of the oligonucleotide is the replacement of the 2-deoxy-D-ribose moiety of the nucleoside by an acyclic chain. Acyclic dinucleotide analogues have been reported to be resistant to enzymatic degradation by snake venom and bovine spleen phosphodiesterase[5]. However, the loss in conformational freedom when a duplex is formed between

two unmodified, rigid oligomers is less than the loss in entropy on duplex formation between an oligonucleotide containing flexible nucleoside analogues and a natural oligonucleotide. So, theoretically, a rigid oligonucleotide has a free energy advantage over a flexible oligomer assuming that the same intermolecular forces determine duplex formation. Whether this expected unfavourable entropy factor can be counterbalanced by the enthalpy factor on duplex formation with acyclic oligonucleotides has not been studied yet. From results of others[6], it seemed that acyclic oligonucleotide analogues are not able to form stable duplexes with natural oligonucleotides. It was however not clear whether this feature is typical for the described *glycero*-oligonucleotides or may be common to all acyclic oligonucleotides.

In contrast to the findings with acyclic oligonucleotides having a phosphate containing backbone, analogues with a peptide like, acyclic backbone, peptide nucleic acids (PNA), have been reported to form stable duplexes with DNA and RNA[7]. As mentioned before, restriction of the degrees of freedom, as in the rigid 2-deoxy-D-ribose sugar, leads to a less unfavourable change in entropy on duplex formation than with the synthesized acyclic oligonucleotides. A reason for the good hybridizing properties of the PNA's is the constrained flexibility of the polyamide backbone. Here, we present our studies on the incorporation of acyclic nucleosides in oligonucleotides and their potential applications.

Two different types of acyclic nucleosides were incorporated in oligonucleotides. Nucleoside analogues with the 3(*S*),5-dihydroxypentyl side chain (**1a-e**) (Figure 1) lack the C_4'-O-C_1' moiety of the natural sugar, 2'-deoxy-D-ribose. This oxygen may play a role in the enzymatic recognition by exo- and endonucleases. The oxygen atom is present under the form of a methoxy group in the second type of acyclic nucleosides with a 4(*R*)-methoxy-3(*S*),5-dihydroxypentyl (**2a**) side chain. Both analogues lack the glycosidic linkage. Therefore these monomers are stable against phosphorylases. In both nucleosides, the number of bonds between the nucleobase and the primary and secondary hydroxyl group as well as the configuration of the optically active centers is the same as in 2'-deoxy-D-ribose.

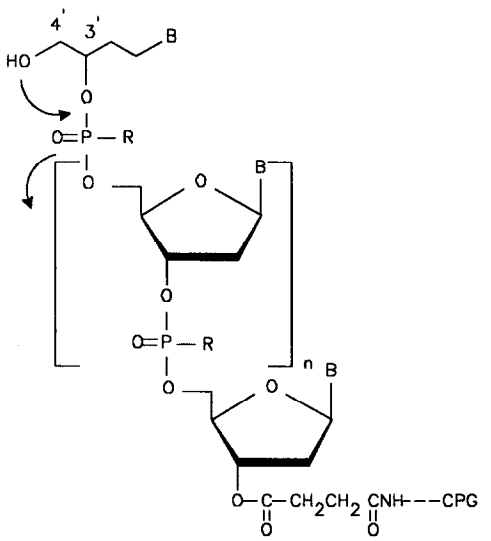


a: B = adenin-9-yl; b: B = thymin-1-yl; c: B = cytosin-1-yl
d: B = guanin-9-yl; e: B = hypoxanthin-9-yl

Figure 1. Different acyclic nucleosides.

The choice of **1** (cleavage of C_4'-O_4' and O_4'-C_1' bond (**D,E**)) and **2** (cleavage of O_4'-C_1' bond (**E**)) as acyclic monomeric nucleoside analogues, is further justified by the fact that cutting the C_2'-C_3' bond (**B**) or the C_3'-C_4' bond (**C**) gives acyclic nucleosides with two primary hydroxyl groups. Protecting one of these groups (dimethoxytritylation) creates a chiral center (**B**) or gives a mixture of two products (**C**) and difficult separation problems can be expected. Cutting the C_1'-C_2' bond (**A**) could be an alternative. However, the synthesis of this monomer is more difficult. The proposed structure has a 1,3-diol functionality and not a 1,2-diol functionality. Besides the close resemblance with the structure of natural nucleosides, the selection of the acyclic nucleosides **1** and **2** for constructing oligonucleotides and not **3** and **4**, was prompted by our previous experience with the latter compounds. In a previous paper[8], we described the synthesis of oligonucleotides containing 3(*S*),4-dihydroxybutyl analogues (**3**) at several internal positions using *H*-phosphonate chemistry. However, degradation of the isolated oligomers using snake venom phosphodiesterase and alkaline phosphatase followed by HPLC analysis, indicated that only the acyclic nucleoside attached at the 3'-end remained incorporated in the different oligonucleotides. Acyclic nucleoside analogues added during chain elongation were generally eliminated. Similar problems were encountered during the incorporation of 3(*R*),4-dihydroxybutyl analogues (**4**) using phosphoramidite chemistry.

These results can be explained by the presence of a vicinal diol functionality in both analogues (**3** and **4**). During the synthetic cycle on a DNA synthesizer, the acyclic nucleoside is removed instantly during the detritylation step (Figure 2). By choosing nucleoside analogues like **1** and **2**, with a 1,3-diol functionality, side reactions during incorporation of the nucleosides into oligonucleotides could be minimized.



R=H or R=OCH_2CH_2CN
CPG = controlled pore glass (solid support)

Figure 2. Elimination of acyclic nucleosides with a 1,2-diol functionality, during DNA synthesis.

SYNTHESIS

The synthesis of the acyclic nucleoside analogues **1a-d** and **2a-d** has been described elsewhere[9]. The 2'-deoxyinosine analogue **1e** was obtained by enzymatic deamination of **1a**[5a]. The adenine and cytosine nucleobase were protected at their amino group with a benzoyl moiety and the guanine base was protected with an isobutyryl group following standard procedures[10]. All nucleosides were protected at their 5'-O-position with a dimethoxytrityl group and phosphitylated to give the phosphoramidite building blocks which were incorporated in oligonucleotides using standard DNA-synthesis on a solid support[11]. Supports with the acyclic 2'-deoxyadenosine analogues **1a** and **2a** were prepared following the procedure described by Pon et al.[12] .

THE ACYCLIC NUCLEOSIDES PROTECT OLIGONUCLEOTIDES AGAINST ENZYMATIC DEGRADATION

As 3'-exonuclease activity is reported to be the main cause of degradation of oligonucleotides in serum[13], we studied the stability of oligonucleotides containing an acyclic nucleoside at different positions towards snake venom phosphodiesterase (SV PDE). The increase in absorption at 260 nm (hyperchromicity) after addition of SV PDE was followed. From Table 1 it can be deduced that oligonucleotides with one acyclic analogue (**1a** or **2a**) at the 3'-end are stable to enzymatic degradation for more than 2 h whereas an unmodified oligo is completely broken down after 3.1 min. An oligomer with the acyclic nucleoside in the middle shows an hyperchromicity of about 50% of that observed for the unmodified oligonucleotide. This observation most probably results from the degradation of the 13-mer to a 7-mer which is stabilized by the presence of the acyclic nucleoside at its 3'-end.

Table 1. Enzymatic Stability of Modified Oligoadenylates towards Snake Venom Phosphodiesterase.

Oligo	Half-life (min)	%H[a]	Oligo	Half-life (min)	%H[a]
A_{13}	3.1	39	A_{13}	3.1	39
$A_6A^*A_6$	2.2	19	$A_6A^*A_6$	2.8	14
$A^*A_{11}A^*$	>120	0	$A^*A_{11}A^*$	>120	0
$A^*_2A_9A^*_2$	>120	0	$A^*_2A_9A^*_2$	>120	0
$A^*A_5A^*A_5A^*$	>120	0	$A^*A_5A^*A_5A^*$	>120	0
A^*_{13}	>120	0	A^*_{12}	>120	0
A*: 3(*S*),5-dihydroxypentyladenine (**1a**)			A*: 4(*R*)-methoxy-3(*S*),5-dihydroxypentyladenine (**2a**)		

[a] %H: hyperchromicity = $\frac{(A_{260} \text{ after degradation} - A_{260} \text{ before addition of SV PDE})}{(A_{260} \text{ before addition of SV PDE})}$

The oligo's were also treated with a large excess of SV PDE and alkaline phosphatase. The resulting mixture was analyzed by HPLC to identify the degradation products. For the oligomers containing **2a**, enzymatic digestion not only gives the expected peaks of the acyclic nucleoside analogue and 2'-deoxyadenosine but also, based on the retention time, peaks representing dimers of the type Ap**A*** and **A***p**A*** and trimer Ap**A***p**A*** (**A***=acyclic nucleoside analogue), in which the acyclic nucleoside is linked *via* a phosphate moiety to the 3'-hydroxyl group of 2'-deoxyadenosine. This is in agreement with previous reported results describing that snake venom phosphodiesterase does not recognize a 1,3-butanediol[14] or 1,3-propanediol[15] moiety and can jump over the Ap**A*** segment. The completely modified 12-mer did not give any degradation product. Oligonucleotides containing **1a** show somewhat different results than the oligonucleotides containing **2a**. The oligonucleotides $A_6\mathbf{A}^*A_6$, $\mathbf{A}^*A_{11}\mathbf{A}^*$ and $\mathbf{A}^*A_5\mathbf{A}^*A_5\mathbf{A}^*$ were broken down completely to 2'-deoxyadenosine and the acyclic analogue (**1a**). Degradation of the oligonucleotide with two acyclic analogues at both ends ($\mathbf{A}^*\mathbf{A}^*A_9\mathbf{A}^*\mathbf{A}^*$) gave 2'-deoxyadenosine together with dimeric (**A***p**A***) and trimeric (Ap**A***p**A***) substances. The completely modified $\mathbf{A}^*_{13}$-mer gave no degradation products.

TRIPLE HELICES ARE FORMED BETWEEN HOMO-OLIGONUCLEOTIDES COMPOSED OF ACYCLIC ADENINE NUCLEOSIDES AND NATURAL OLIGOTHYMIDYLATES

The melting temperature of mixtures of different homo-oligonucleotides containing the acyclic nucleoside analogues and their unmodified complementary sequences were determined. The results are given in Table 2. It can be seen that substitution of one nucleoside in the middle of an oligo gives a greater destabilization than replacement of two nucleosides, one at each end. The drop in Tm for replacement of 4 nucleosides, two at both ends, is about the same as for replacement of one nucleoside in the middle. These results hold for the depicted A-T base-pair formation. However, completely modified oligo-**A*** can still form a stable association with an unmodified oligo-T. This is in contrast with the results reported by Schneider[6a] and J.L. Imbach[6b] for the *glycero*-oligothymidylates. The 4(*R*)-methoxy-3(*S*),5-dihydroxypentyl analogue destabilizes the duplex somewhat more than the 3(*S*),5-dihydroxypentyl analogue. This tendency can also be seen when comparing the free energy change on melting (ΔG^{25^oC}), for the different oligonucleotides.

Because of the difference between our results and those of Schneider and Imbach, it seemed valuable to study the influence of the nucleobase on duplex stability as it is known that thymine has less stacking properties compared to adenine, which may have an influence on the hybridizing properties.

Incorporation of one acyclic thymine nucleoside (**1b**) in the middle of an oligonucleotide gives a duplex that is somewhat less stable than the duplex formed between $A_6\mathbf{A}^*A_6$ (**A***=**1a**) and natural oligothymidylate. A completely modified oligothymidylate ($\mathbf{T}^*_{12}T$) does not hybridize with an unmodified A_{13}-mer which is in agreement with the above mentioned results of Schneider and Imbach. These results demonstrate the influence of the nucleobase on the ability to associate with a complementary unmodified oligonucleotide. Completely modified oligonucleotides containing a mixture of **A***, **C*** and **G*** nucleoside analogues, also do not associate with their complementary natural oligonucleotide (Table 3). This table also demonstrates the stability of a G-A mismatch as well as the effect of a penultimate incorporated acyclic nucleoside.

Table 2. Melting Temperatures and Free Energy Change of Homo-oligomers Containing the Acyclic Nucleoside Analogues with their Complementary Sequences.

Oligomer (5'→3')	$\Delta G^{25\ °C}$(kJ/mol)	Tm (°C)
A*:4(*R*)-methoxy-3(*S*),5-dihydroxypentyladenine (**2a**).		
A_{13}	41	33.3
A_{11}+	36	28.7
$A_6A^*A_6$	34	26.7
$A^*A_{11}A^*$	38	30.9
$A^*A_5A^*A_5A^*$	31	24.8
$A_2^*A_9A^*_2$	33	26.8
A^*_{12}‡	34	26.5
A*: 3(*S*),5-dihydroxypentyladenine (**1a**).		
T*: 3(*S*),5-dihydroxypentylthymine (**1b**).		
$A_6A^*A_6$	36	28.6
$A^*A_{11}A^*$	39	31.6
$A^*A_5A^*A_5A^*$	34	27.3
$A^*_2A_9A^*_2$	35	28.5
A^*_{13}	35	27.2
$T_6T^*T_6$	33	25.2
T^*T_{12}	43	33.0
$T^*_2T_{11}$	40	31.4
$T^*_{12}T$	-	no transition

+ A_{11}/T_{13} mixture; ‡ A^*_{12}/T_{13} mixture

Table 3. Melting Temperatures of Hetero-oligomer Duplexes.

Oligonucleotides	Tm (°C)
5'-GGCGCCG*G*CGGTG-3' 3'-CCGCGGC*A*GCCAC-5'	67.4
5'-GGCGCCG*T*CGGTG-3' 3'-CCGCGGC*A*GCCAC-5'	70.0
5'-GGCGCCG*G*CGGTG-3' 3'-C*C*'GCGGC*A*GCCAC-5'	62.5
5'-GGCGCCG*T*CGGTG-3' 3'-C*C*'GCGGC*A*GCCAC-5'	65.7
5'-GGCGCCG*G*CGGTG-3' 3'-C*C*'*G*'*C*'*G*'*G*'*C*'*A*'*G*'*C*'*C*'*A*'*C*'-5'	<6.5
5'-GGCGCCG*T*CGGTG-3' 3'-C*C*'*G*'*C*'*G*'*G*'*C*'*A*'*G*'*C*'*C*'*A*'*C*'-5'	<6.5

X: unmodified nucleoside

X': nucleoside with 3(*S*),5-dihydroxypentyl side chain (III.1)

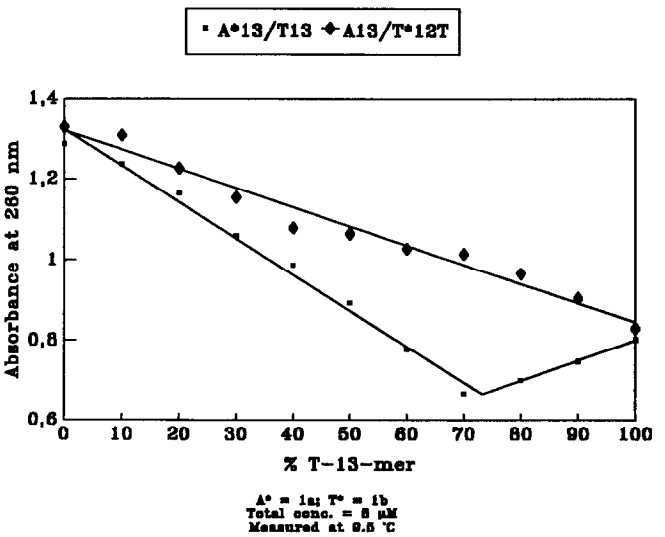
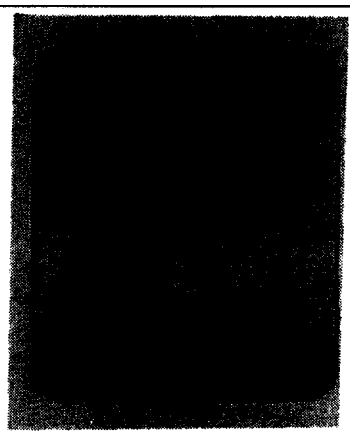


Figure 3. Mixing curves.

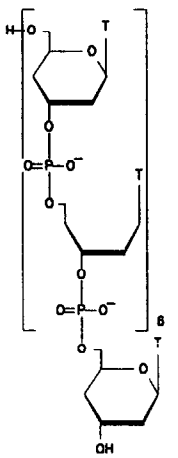
To check the stoichiometry of the T_{13}/A^*_{13} association (A^*=**1a**), a mixing curve was made (Figure 3). The minimum at 72% of T_{13} points to a triple helix like structure (T_{13}:A^*_{13}:T_{13}). These results were confirmed by melting point determinations at 284 nm[16]. Whereas for 1:1 and 1:2 mixtures of unmodified $(dA)_{13}/T_{13}$ no transition could be seen, 1:2 as well as 1:1 mixtures of A^*_{13}/T_{13} showed a transition at 26.3 °C. It therefore can be concluded that completely modified oligoadenylate (A^*_{13}) forms a triple helix with unmodified oligothymidylate in the presence of 100 m*M* NaCl. The absence of triple helix formation for normal $(dA)_{13}/T_{13}$ mixtures (mixing curve not shown) under these conditions is in agreement with literature data[16]. For $T^*_{12}T$ in the presence of $(dA)_{13}$, no minimum in the mixing curve was observed, which is in agreement with the results of the melting experiments (no association detected). The melting experiment (at 284 nm) for a $T^*_{12}T/(dA)_{13}/T_{13}$ (1:1:1) mixture showed no transition indicating that the completely modified $T^*_{12}T$ oligonucleotide was also not able to associate with a $(dA)_{13}$:T_{13} duplex.

The formation of a 2:1 complex of T_{13}:A^*_{13} is also supported by electrophoretic mobility data[17]. Picture 1 shows a non-denaturating PAGE analysis of $(dA)_{13}$, A^*_{13}, $(dA)_{13}/T_{13}$ (1:1 and 1:2 mixtures) and A^*_{13}/T_{13} (1:1 and 1:2 mixtures). From lane 1 and 2 it can be deduced that the single-stranded $(dA)_{13}$ and A^*_{13} have the same electrophoretic mobility. On addition of T_{13} to $(dA)_{13}$ (lane 3 and 4) a new band appears with a lower mobility. This band corresponds to the $(dA)_{13}$:T_{13} duplex. Mixing of A^*_{13} and T_{13} (lanes 5 and 6) gives a band of lower mobility than the band of the $(dA)_{13}$:T_{13} duplex. This band corresponds to a complex of higher molecular weight, a T_{13}:A^*_{13}:T_{13} triple helix. The presence of the band corresponding to A^*_{13} in lane 5 and 6 might result from the slow kinetics of triple helix formation or the equilibrium between single-stranded DNA and the triple helix. It should be mentioned that the T_{13}-mer can not be visualized by silver staining[18].



Picture 1. Electrophoresis of different complexes.

We also synthesized a 13-mer oligonucleotide containing alternating 9-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine[19] and 9-(3(*S*),5-dihydroxypentyl)thymine (Figure 4.). This oligo also does not associate with the complementary natural oligonucleotide. From this result it can be concluded that the 2,4-dideoxy-β-D-*erythro*-hexopyranosyl analogue does not sufficiently reduce the flexibility of the oligonucleotide to give a less unfavourable entropy change on formation of a complex with its unmodified complementary oligo.



T= thymin-1-yl

Figure 4. Mixed Oligonucleotide.

ACYCLIC NUCLEOSIDE ANALOGUES AS UNIVERSAL NUCLEOSIDES

Because of the conformational mobility of the acyclic nucleosides, one could expect that the base-pairing properties of such analogues could be different from the traditional Watson-Crick base-pairing. Eschenmoser demonstrated, based on his results with hexopyranosyl analogues[20], that the selectivity rules for base recognition according to Watson and Crick (A-T,G-C), are not only a consequence of the hydrogen bonding properties of the nucleobases, but also of the furanose structure and conformation of the natural nucleosides. We obtained similar results with our previously described hexopyranosyl analogues[19]. Therefore we also investigated the base-pairing properties of the acyclic nucleosides **1a-e** with all natural nucleosides. Because of the flexibility, some acyclic nucleosides might discriminate less between the four natural bases. This could be useful in the synthesis of probes with degenerate positions (use of a universal nucleoside[4b]). As 2'-deoxyinosine is commonly used as a universal nucleoside, we also studied the hybridizing properties of the 2'-deoxyinosine analogue **1e**.

Table 4. Tm (°C) of the duplexes 5'-CACCGXCGGCGCC-3'
3'-GTGGCYGCCGCGG-5'

Y / X	A	T	G	C	ΔTm(°C)
T	70.3	59.0	65.0	56.5	13.8
T*	64.8	59.6	60.7	55.6	9.2
A	61.5	70.0	67.0	58.3	11.7
A*	60.8	66.0	66.7	58.0	8.7
C	60.1	58.8	73.5	54.9	18.6
C*	61.9	58.8	68.1	55.8	12.3
G	67.0	64.9	66.7	72.8	7.9
G*	63.2	63.4	68.5	67.6	5.3
I	70.2	64.6	65.1	68.5	5.6
I*	65.1	62.6	67.4	65.6	4.8

ΔTm: spreading in Tm for base-pairing of a particular nucleoside with the four natural nucleosides.
X* = nucleoside with 3(*S*),5-dihydroxypentyl side chain

In Table 4 and Figure 5 the stability of duplexes of 13-mer oligonucleotides (5'-CACCG**X**CGGCGCC-3') with X=**1a-e** and the complementary oligomers with the four natural nucleosides (3'-GTGGC**Y**-GCCGCGG-5'; Y=dA, dC, dG and T) are given. Their melting temperatures are compared with these of the unmodified duplexes. With the exceptions of the **T***T, **C***A, **C***C and **G***G pairs, the acyclic analogues form less stable base-pairs compared to the natural nucleosides. This drop in stability on replacement of the natural nucleoside by its acyclic analogue is most pronounced for the Watson-Crick base-pairs (4.5-5.0 °C). The decrease in Tm for the other base-pairs on substitution with the acyclic nucleoside is about 0.5-0.8 °C. As a consequence, the acyclic analogues discriminate less between the four natural nucleosides. This can be expressed by the spreading in Tm (ΔTm). The least discrimination is observed for the acyclic 2'-deoxyinosine analogue (ΔTm= 4.8 °C) (Figure 5). Of particular interest is the higher stability of the **I***-dG base pair than of its natural analogue dI-dG.

As could be expected, the stability of an **I***Y base-pair also depends on its neighbouring bases. This is illustrated in Table 5. In this particular sequence the **I***C pair is the most stable one whereas in the sequence depicted in Table 4, the G**I*** pair is. However, for the duplex of Table 5, the acyclic 2'-deoxyinosine analogue again discriminates less than 2'-deoxyinosine. These results suggest the potential use of the acyclic inosine analogue (**1e**) as universal nucleoside. These studies are now in progress.

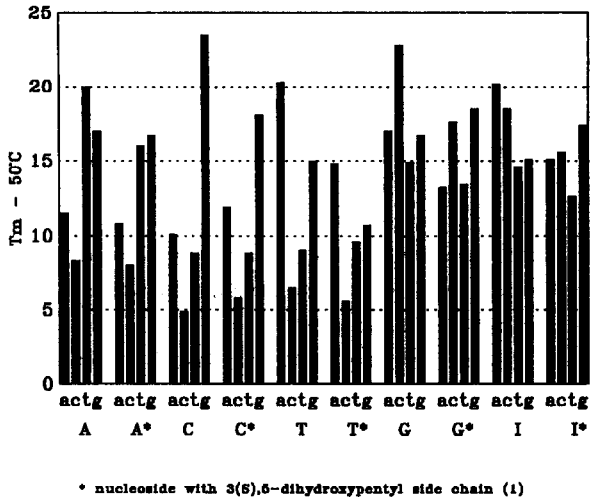


Figure 5. Discrimination properties of the different nucleosides.

Table 5. Tm (°C) of the duplexes 5'-GGA$_4$XA$_4$GG
3'-CCT$_4$YT$_4$CC

X \ Y	A	T	G	C	ΔTm (°C)
I	37.1	32.1	32.4	41.1	9.0
I*	32.0	29.4	34.1	35.0	5.6

ΔTm: spreading in Tm for base-pairing of a particular nucleoside with the four natural nucleosides.
X* = nucleoside with 3(*S*),5-dihydroxypentyl side chain

DISCUSSION

An important requirement to be met by oligonucleotides as potential therapeutic agents in the antisense or antigene strategy, is their stability against degradation by nucleases present within the cell and in serum. The main nuclease activity present in foetal calf serum is 3'-exonuclease[13]. This has been confirmed by experiments with oligonucleotides that are modified at their 3'-end. These oligonucleotides have an improved half-life in serum compared to unmodified or 5'-end protected analogues. Increased enzymatic stability has been reached by replacement of the diester linkages at the 3'-end (e.g. methylphosphonate,

phosphoramidate[13] or phosphorothioate[13] linkages, 3',3'- and 5',5'-internucleotide linkages[21]), by conjugation with cholesterol, phenazinium[22] and other groups or by substitution of the base moiety by certain modified pyrimidines[23]. Oligomers containing acyclic nucleosides with a 3(*S*),5-dihydroxypentyl or 4(*R*)-methoxy-3(*S*),5-dihydroxypentyl side chain moiety at their 3'-end, show extremely high stability towards snake venom phosphodiesterase. This stability is in agreement with previous reported results with other acyclic oligonucleotides[24]. The results have been confirmed by preliminary experiments in cell culture containing 7% heat inactivated calf serum. Only 2% of the oligo protected at its 3'-end was degraded after 19 h whereas the unmodified oligo was completely broken down under these conditions. From the described degradation experiments it could be concluded that snake venom phosphodiesterase is not able to cleave the phosphodiester moiety between a natural nucleoside and a 4(*R*)-methoxy-3(*S*),5-dihydroxypentylnucleoside, and that the enzyme skips over this dimer moiety.

In contrast with results reported previously on the inability of acyclic oligonucleotides to form stable duplexes with unmodified DNA[6], our completely modified A^{*}_{13}-mers can anneal with unmodified oligothymidylate. The favourable enthalpy change resulting from the association of the oligonucleotides might counterbalance the loss in entropy upon going from a flexible chain to a more ordered structure. It seems therefore reasonable that a T:A*:T triplex can be formed more easily than a T*:dA:T* triplex. A second reason might be the higher degree of stacking of adenine bases than of thymine bases.

Replacement of one nucleoside of a natural Watson-Crick base pair by its acyclic analogue with a 3(*S*),5-dihydroxypentyl side chain, results in a destabilization of the duplex (about 5 °C). This drop in stability is less pronounced on going from a mismatch of two natural nucleosides to a mismatch of one acyclic and one natural nucleoside (0.5-1.5 °C). As a result, the spreading in melting temperature for a particular nucleoside with the four natural nucleosides of DNA, is lower for the acyclic analogues. Substitution of the sugar moiety of 2'-deoxyinosine, commonly used as universal nucleoside in probes with degenerate positions, with the acyclic side chain also results in less discrimination between the four natural nucleosides. This might be explained by the higher flexibility of the incorporated acyclic nucleoside which allows better adjustement of the donor and acceptor position of hypoxanthine to the opposite base. Based on these results, the use of this compound (**1e**) as ambiguous nucleoside in hybridization probes deserves further research.

CONCLUSION

3'-End substituted oligonucleotides with a 3(*S*),5-dihydroxypentyl nucleoside or a 4(*R*)-methoxy-3(*S*),5-dihydroxypentyl nucleoside show high stability against degradation by snake venom phosphodiesterase. As the decrease in hybridizing properties of such constructs is minimal, these end modified oligonucleotides are valuable candidates to be tested in the antisense approach to stop the expression of unwanted genetic information. The hybridization properties of completely modified acyclic oligonucleotides seem to be dependent on the base moiety. Their use as antisense or antigene constructs therefore seems to be limited. The flexibility of the acyclic nucleoside structure could be advantageous when looking for a universal nucleoside. 9-(3(*S*),5-Dihydroxypentyl)hypoxanthine can be considered as a candidate to be tested in probes with degenerate positions.

EXPERIMENTAL SECTION

Methods and reagents are as described previously[9,19].

Dimethoxytritylation of the nucleoside analogues

A solution of 1.5 mmol of the nucleoside and 1.2 equivalents of dimethoxytrityl chloride in 25 mL of pyridine dry was stirred at room temperature for 2 h. After addition of 25 mL of EtOAc, the mixture was washed with $NaHCO_3$ solution (7%) and water, dried, evaporated and coevaporated 3 times with toluene. The residue was purified by column chromatography (CH_2Cl_2-MeOH-Et_3N 95:5:1).

Preparation of the 3'-O-succinylated nucleoside analogues

A mixture of 0.2 mmol of the 5'-*O*-dimethoxytritylated-N^6-benzoylated acyclic adenine nucleoside, 12 mg (0.1 mmol) of DMAP and 60 mg (0.6 mmol) of succinic acid anhydride in 2 mL of anhydrous pyridine was stirred at room temperature for 72 h. The solution was evaporated and coevaporated with toluene. The residue was dissolved in CH_2Cl_2, the organic layer washed with saturated NaCl solution and water, dried and evaporated to give the 3'-*O*-succinylated products.

Preparation of the solid supports

A mixture of 80 μmol of the 5'-*O*-dimethoxytritylated-3'-*O*-succinylated-N^6-benzoylated acyclic adenine nucleoside, 400 mg of pre-activated LCAA-CPG, 5 mg DMAP (40 μmol), 32 μL of Et_3N and 153 mg (800 μmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.HCl in 4 mL of anhydrous pyridine was first sonicated for 5 min and then shaken at room temperature for 15 h. After shaking, the CPG was filtered off and washed succesively with pyridine, methanol and chloroform and then dried under vacuum. The unreacted sites were capped using 1.5 mL of 1-methylimidazole in THF (Applied Biosystems), and 1.5 mL of acetic anhydride-lutidine-THF 1:1:8 (Applied Biosystems). After shaking for 2 h, the CPG was filtered off, washed with chloroform and dried under vacuum. Colorimetric dimethoxytrityl analysis indicated a loading of 33.1 and 33.9 μmol/g for **1a** and **2a** respectively.

Preparation of the amidite building blocks

A mixture of the 5'-O-protected nucleoside (0.5 mmol), 3 equivalents of dry *N,N*-diisopropylethylamine and 1.5 equivalent of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite in 2.5 mL of dry CH_2Cl_2 was stirred at room temperature for 2 h. After addition of 0.5 mL of EtOH and further stirring for 25 min, the mixture was washed with 5% $NaHCO_3$ solution (15 mL) and saturated NaCl solution, dried and evaporated. Column chromatography with n-hexane/acetone/Et_3N as eluent afforded the amidite as a foam which was dissolved in a minimal volume of dry CH_2Cl_2 and added dropwise to 100 mL of cold (-50 °C) n-hexane. The precipitate was isolated, washed with n-hexane, dried and used as such for DNA synthesis.

Eluting solvent (n-hexane/acetone/Et_3N), yield after precipitation and ^{31}P-NMR for the different amidites: **1a:** (10/90/2), 79% yield, 147.7 and 147.5 ppm; **1b:** (30/70/2), 67% yield, 147.7 and 146.9 ppm; **1c:** (10/90/2), 84% yield, 147.7 and 147.0 ppm; **1d:** (35/65/2), 60% yield, 147.5 and 146.9 ppm; **1e:** (20/80/2), 55% yield; **2a:** (30/70/ 2), 66% yield, 149.3 and 148.2 ppm.

Synthesis of the oligodeoxynucleotides

Oligonucleotide synthesis was performed as described in reference 19.

Study of the enzymatic stability

To a solution of 1.2 OD of the oligo in 2.5 mL of the following buffer [100 m*M* Tris.HCl, pH=8.6, 100 m*M* NaCl, 14 m*M* $MgCl_2$] was added 0.5 U of snake venom phosphodiesterase (Crotalus Atrox Venom, Pharmacia) (solution in following buffer: 5 m*M* Tris.HCl, pH=7.5, 50% glycerol), at 37 °C. The increase in absorption at 260 nm was followed. The curve could be fitted to an exponential curve from which the half-life could be gathered.

Analysis of oligonucleotide products

A mixture of 0.2 OD of the oligo and 1.2 U of snake venom phosphodiesterase in the following buffer [50 m*M* Tris.HCl, pH=8.6, 50 m*M* NaCl, 7 m*M* $MgCl_2$] was kept at 37 °C for 18 h. After addition of 1.2 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) and further incubation for 24 h, the mixtures were analyzed by RP-HPLC.

HPLC Analysis. The HPLC system consisted of a Merck-Hitachi L-6200A Intelligent Pump, a Zorbax C8 reversed phase column, a Uvicord SII 2138 UV detector (Pharmacia-LKB) and a HP 3390A Integrator. Isocratic elution with 5% MeOH in 0.1 *M* triethylammonium acetate solution (pH=6.8) at a flow rate of 1.0 mL/min resulted in elution times of 12.9, 15.8 and 14.6 min for 2'-deoxyadenosine, **2a** and **1a** respectively. Detection was done by monitoring the absorbance at 254 nm. There was no baseline separation between dA and the acyclic analogues.

Melting temperatures

Melting experiments were done as described in reference 19.

Electrophoretic experiments

Polyacrylamide gel electrophoresis (PAGE) was performed using the Phast System™ (Pharmacia). 20% Homogenous Phast Gel media (0.112 *M* acetate, 0.112 *M* Tris.HCl, pH=6.4) (Pharmacia) and Native Phast Gel buffer strips (0.88 *M* L-Alanine, 0.25 *M* Tris.HCl, pH=8.8) (Pharmacia) were used. Gels were prerun at 10 °C for 100 Vh, samples were applicated and separation was done for 78 Vh at 10 °C. Oligonucleotides were detected using silver staining as described in the manual.

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