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The Effects of 2'- and 3'-Alkyl Substituents on Oligonucleotide Hybridization and Stability

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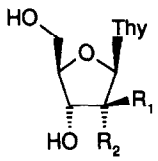
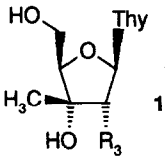
Abstract: *The hybridization properties and nuclease resistance of 2'- and 3'-alkyl, -heteroalkyl, -alkenyl, and -aryl substituted oligodeoxyribonucleotides have been investigated. While such modified oligonucleotides generally exhibit reduced binding affinity for complementary RNA and DNA, a dramatic increase in stability against 3'-exonucleases was observed for certain 2'-substituents.*

The inhibition of protein expression by antisense oligonucleotides has recently emerged as an attractive alternative to classical drug design strategies.[1] The concept is based on the sequence-specific binding of a synthetic (antisense) oligonucleotide to the mRNA of a disease-related protein, which may subsequently result in suppression of RNA translation and thus in a very specific inhibition of protein synthesis.[1] The successful *in vivo* implementation of this strategy, however, depends on the use of chemically modified oligonucleotides or oligonucleotide analogs with significantly improved resistance to nuclease degradation (as compared to natural single-stranded DNA).[1] One possible approach to metabolically more stable oligonucleotides consists in the use of certain 2'-α-O-alkyl substituted nucleotide building blocks,[1,2] some of which have additionally been demonstrated to also increase the affinity of the corresponding oligonucleotides for complementary RNA.[2b,c,3] The latter finding is generally attributed to the predominance of a 3'-endo conformation for the 2'-O-alkyl substituted sugar units, which in turn results in a significant preorganization of the modified single-stranded oligonucleotide for an A-type DNA/RNA heteroduplex.[3b,4,5] This notion is supported by the dramatic increase in DNA/RNA heteroduplex stability that is observed for 2'-α-fluoro substituted DNA,[6] as the fraction of 3'-endo conformation in 2'-α-heterosubstituted deoxyribonucleosides has been shown to linearly increase with substituent electronegativity.[4]

Fig. 1

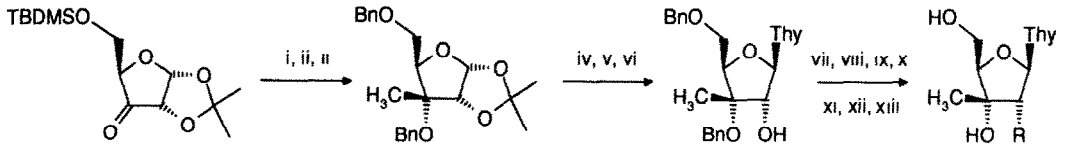


1: $R_1 = H, R_2 = CH_3$
2: $R_1 = CH_3, R_2 = H$
3: $R_1, R_2 = CH_2$
4 - 8: $R_1 = H$; $R_2 = CH_2F$ (**4**), CF_3 (**5**), CH_2OH (**6**), CH_2OCH_3 (**7**), Ph (**8**)



9: $R_3 = H$
10: $R_3 = OCH_3$

While the preference of various 2'-α-heterosubstituted deoxyribonucleosides for a 3'-endo conformation[4] can be satisfactorily explained on the basis of the gauche effect between the electronegative 2'-substituent and $O4'$ of the furanose ring,[7] the sugar conformation of any 2'-substituted nucleotide unit within the context of either a single stranded oligonucleotide or a nucleic acid duplex may be additionally affected by dipole-dipole and dipole-charge interactions (involving the dipole associated with the 2'-substituent), by solvation effects, or by the capacity of the 2'-substituent for intrastrand hydrogen-bond formation.[8] It is, therefore, not *a priori* clear whether enhancement of DNA/RNA heteroduplex stability by 2'-substituted building blocks invariably



11 **12** **13** **9**: R = H **10**: R = OCH_3

Scheme 1

i: MeMgBr, Et_2O, -78°→ r.t., 89%; ii: Bu_4NF, THF, r.t., 10 min; iii: NaH, BnBr, THF, 0°→ r.t., 57% (2 steps); iv: CF_3COOH, 10% H_2O, r.t.; v: Ac_2O, pyridine, r.t., 82% (2 steps); vi: $(TMS)_2$-thymine, $CF_3SO_3SiMe_3$, CH_3CN, r.t., 12h, 90%. For R = H: vii: MeONa/MeOH, r.t., 81%; viii: $C_6F_5OC(S)Cl$, DMAP, CH_2Cl_2, -15°, 91%; ix: Bu_3SnH (2 eq.), AIBN (0.3 eq.), 1M, benzene, refl., 30 min, 47%; x: H_2, 20% $Pd(OH)_2$-C, MeOH, 12h, quant.. For R = OCH_3: xi: $BnOCH_2OCH_2Cl$, DBU, r.t., 12h, 94%; xii: NaH, CH_3I, THF, r.t., 72%; xiii: H_2, Pd-C, MeOH, 78%.

depends on the presence of a C-2' - *heteroatom* bond, although it has been recently demonstrated that certain 2'-α-alkyl (ethyl, allyl) or alkenyl ((E)-styryl) substituents do indeed significantly decrease hybridization affinity of the corresponding oligonucleotides for complementary RNA.[9] In order to assess the generality of these effects, we have now extended this study to a variety of other 2'- and 3'-alkyl, -alkenyl, and especially -heteroalkyl substituted building blocks (Fig.1), whose effects on oligonucleotide hybridization and stability are the subject of this report.

The synthesis of compounds **1** and **4** - **8** is described elsewhere.[10] Compound **3** was prepared according to literature procedures;[11] **2** was obtained by catalytic hydrogenation of 3',5'-tetraisopropyldisiloxan-diyl (TIPSi-) protected **3**[11] and subsequent removal of the protecting group with Bu_4NF.[12] The synthesis of compounds **9** and **10** is summarized in *Scheme 1*.[13] It relies on the diastereoselective methylation of protected furanoside **11** with CH_3MgBr as the first key step. The resulting tertiary alcohol was then converted to the partially protected ribo-thymidine derivative **13** (*via* **12**), which was elaborated into **9** and **10**, respectively, via deoxygenation or methylation of the 2'-OH group. It should be noted that the radical deoxygenation of **13** could only be achieved *via* the corresponding *pentafluorophenyl*-thionocarbonate, while the *phenyl*-thionocarbonate proved to be completely unreactive under a variety of conditions. The moderate yield of 47% in the radical reduction step is due to competing ionic reduction of the thionocarbonate, which is then followed by radical reduction of the resulting thionoformate.[14]

Modified building blocks **1** - **10** were incorporated into five different oligonucleotide sequences with varying number and location of modifications,[15] and the data for the hybridization of these modified oligonucleotides with complementary RNA are summarized in *Tables 1* and *2*. As shown in *Table 1*, all 2'-α-substituents investigated so far lead to a significant decrease in DNA/RNA heteroduplex stability (lower melting temperatures (T_m's) as compared to the unmodified wild-type duplex), albeit to a very different extent: With the exception of sequence **IV** the destabilizing effect is smallest for a 2'-α-CH_3 group (**1**) (average ΔT_m = - 1.7 °C/modification), while any further increase in size and hydrophobicity of the 2'-α-substituent is inevitably associated with more unfavourable hybridization properties. These findings are particularly noticeable for those cases where one (CH_2F, CH_2OH, CH_2OCH_3) or all (CF_3) of the hydrogen atoms of the 2'-α-methyl group were replaced by more electronegative heteroatoms. In analogy to 2'-α-hetero substituents[3,6] the electron-withdrawing properties of these heteromethyl groups[17] together with favourable dipole effects and/or "RNA-like" hydrogen-bonding (CH_2OH-group)[8] were originally conceived to lead to *higher* binding affinity than observed for a simple methyl substituent (*vide supra*).

A possible rationale for the diminished binding affinity of 2'-α-alkyl substituted oligonucleotides for complementary RNA is offered by the results of the conformational analysis of compounds **1** and **4**

Table 1: Relative hybridization affinities of modified oligonucleotides incorporating 2'-substituted thymidines for complementary RNA (ΔTm-values/modification[a]).

Sequence[b]		I (52.3°[c])	II (63.9°[c])	III (61.8°[c])	IV (61.7°[c])	V (48.6°[c])
R_1[d]	R_2[d]					
H	CH_3	- 2.2	- 1.0	- 1.3	- 2.3	non-coop.[g]
CH_3	H	- 3.3	- 3.9	- 2.3	- 3.1	non-coop.
= CH_2		- 3.5	- 5.2	- 1.9	- 3.3	non-coop.
H	CH_2F	- 3.1	n.d.[h]	n.d.	n.d.	n.d.
H	CF_3	- 4.7	n.d.	n.d.	n.d.	n.d.
H	CH_2OH	- 2.6	- 2.9	n.d.	- 3.1	non-coop.
H	CH_2OCH_3	- 3.4	- 4.3	- 2.0	- 1.2	non-coop.
H	CH_2CH_3[e,f]	- 4.4	- 5.8	- 3.1	- 3.5	non-coop.
H	$CH_2CH{=}CH_2$[e]	- 4.7	- 5.0	- 2.4	- 3.7	non-coop.
H	(E)-CH=CH-Ph[e,f]	n.d.	- 7.3	- 3.9	- 3.4	non-coop.
H	Ph	n.d.	- 6.3	- 3.6	- 3.7	non-coop.

[a]Difference in melting temperature (T_m) between the modified DNA/RNA duplex and the unmodified wild-type (WT) duplex per modification ($\Delta T_m = T_m - T_m(WT)$). T_m's were determined in 10 mM phosphate buffer, pH 7, 100 mM Na^+; error limits are ± 0.5°. For details see ref. 3b; [b]Sequences are: **I**: 5'-TTTT**t**CTCTCTCTCT-3'; **II**: CTCGTACC**t**TTCCGGTCC-3'; **III**: 5'-**t**CCAGG**t**G**t**CCGCA**t**C-3'; **IV**: 5'-CTCGTAC**ttt**CCGGTCC-3'; **V**: 5'-GCG**ttttttttt**GCG-3'; **t** = modified thymidine; [c]T_m of the corresponding wild-type duplex in °C; [d]See Fig. 1; [e]ref.9; [f]2'-deoxyuridine derivative; [g]no cooperative melting observed (T_m < 20°); [h]n.d. = not determined.

by ^{1}H-NMR in aqueous solution. Based on the coupling constants $J_{1',2'}$ = 8.9 Hz, $J_{2',3'}$ = 5.8 Hz, and $J_{3',4'}$ = 2.0 Hz the conformation of **1** has been determined to be largely *2'-endo* (≈86%);[19] this contrasts sharply with what is observed for 2'-α-methoxy 2'-deoxyuridine (≈60% *3'-endo*)[4] and even siginificantly exceeds the fraction of 2'-endo conformation found for 2'-deoxyuridine (≈65% 2'-endo).[4] The adverse effect of a 2'-α-methyl substituent on DNA/RNA heteroduplex stability may thus be due, at least in part, to the necessity for the sugar moieties of the modified DNA strand to adopt an intrinsically unfavourable 3'-endo conformation, which is required for A-type duplex formation.[5]

While the preference of **1** for a 2'-endo sugar pucker (with the 2'-α-methyl group in an equatorial orientation) may be predicted on the basis of purely steric arguments, surprisingly, also **4** predominantly adopts a 2'-endo conformation ($J_{1',2'}$ = 8.7 Hz, $J_{2',3'}$ = 6.0 Hz, $J_{3',4'}$ = 2.5 Hz).[19] Thus, even the replacement of the 2'-α-methyl group in **1** by the electron-withdrawing CF_3 group[17] does not alter the 2'-endo preference of the sugar moiety, indicating that the 2'-endo - 3'-endo equilibrium in both cases is largely dominated by steric factors.[20] By analogy, all of the 2'-α-alkyl substituted nucleosides referred to in *Table 1* can be assumed to have a more or less pronounced preference

for a 2'-endo conformation. The corresponding oligonucleotides would, therefore, all suffer from a conformation of the modified sugar units unfavourable for A-type duplex formation, with the specific destabilizing properties of each individual substituent being ultimately determined by additional steric and solvation effects.

Different arguments have to be invoked to rationalize the effects of a 2'-β-CH_3 substituent (**2**). Although no detailed NMR analysis was performed on **2**, the coupling constant $J_{1',2'}$ of 7.2 Hz in this case is strongly suggestive of a 3'-endo sugar conformation (again with the 2'-CH_3 group in an equatorial orientation). However, as indicated by model building, any favourable preorganization of the sugar moieties in a modified *single-stranded* oligonucleotide may be more than offset by unfavourable steric interactions between the 2'-β-CH_3 group and the bases of 3'-adjacent nucleotide units (H-6 of pyrimidines and H-8 of purines) within an actual A-type *duplex* structure.

All of the 2'-substituents listed in *Table 1* increase the stability of oligodeoxynucleotides towards degradation by 3'-exonucleases in 10% heat inactivated fetal calf serum.[21] The most dramatic effects on nuclease resistance were displayed by building blocks **1**, **2**, **7**, and **8**, where half-lives of >48 h were observed for the corresponding 3'-modified oligonucleotides[21] as compared to half-lives of 1 - 2 h for the unmodified wild-type sequence. Somewhat surprisingly, only a 3-fold increase in 3'-exonuclease stability was conferred by a 2'-α-CH_2OH group; this observation may be related to the RNA-like structure of this modification and a resulting susceptibility of the corresponding oligonucleotides to attack by *RNA* degrading enzymes.

The effects of 3'-β-methyl substituted building blocks **9** and **10** on the binding affinity of oligodeoxyribonucleotides for complementary RNA are summarized in *Table 2*. As for the 2'-alkyl derivatives, the presence of a 3'-β-methyl group leads to reduced duplex stability, with only a very minor destabilization being observed for compound **9** in sequence **III**. Interestingly, the presence of an additional 2'-α-OCH_3 group in **10**, rather than compensating for the destablizing effect of the 3'-β-methyl substituent, even further decreases duplex stability, which is absolutely contrary to the usual affinity-*enhancing* properties of this type of modification (*vide supra*). While the adverse effect of **9** on hybridization affinity may again be rationalized on the basis of a strong preference of the 3'-β-substituted sugar moiety for a 2'-endo conformation (equatorial orientation of the CH_3 group),[22] the reasons for the behaviour of **10** are less clear. It may be speculated, however, that the presence of a 3'-β-methyl group is incompatible with standard A-type duplex formation for steric reasons (unfavourable interactions between an axial 3'-β-methyl group and the base in an anti-orientation);[22b] within the actual duplex structure the modified sugar moieties may thus be forced into a conformation distinctly different from the usual 3'-endo pucker, which might be energetically more unfavourable in the presence of the additional 2'-α-OCH_3 substituent.

Regarding the effects of 2'- and 3'-alkyl substituted building blocks on hybridization affinity for complementary *DNA*, a significantly more pronounced decrease in duplex stability was observed for DNA/DNA than for DNA/RNA hybrids (data not shown).[23] In view of the anticipated *favourable* sugar conformation of 2'-α-alkyl substituted nucleotide units for B-type (DNA/DNA) double helix formation (2'-endo conformation)[5], this finding may reflect major distortions in the normal structure of the DNA/DNA double helix induced by unfavourable steric interactions involving the 2'-α-alkyl substituent. In addition, we would like to point out that based on previous literature reports[24] as well as our own experience with a variety of other types of sugar and also backbone modifications the lower *relative* affinity of modified oligonucleotides for DNA than for RNA (i.e. more negative ΔTm-values for hybridization to DNA than to RNA) appears to be a *rather frequent* phenomenon. These observations, together with the fact that hybridization of oligonucleotide therapeutics to single-stranded DNA is not a critical issue for antisense applications, may put into perspective some recent reports that have particularly emphasized preferential relative binding of modified oligonucleotides to complementary RNA rather than DNA.[25]

Table 2: Relative hybridization affinities of modified oligonucleotides incorporating 3'-substituted thymidines for complementary RNA (ΔTm-values/modification[a]).

Sequence[b]	I (52.3°C)	II (63.9°C)	III (61.8°C)
R3[d]			
H	- 1.5	- 1.3	- 0.1
OCH_3	- 1.3	- 4.8	- 1.4

[a]Difference in melting temperature (T_m) between the modified DNA/RNA duplex and the unmodified wild-type (WT) duplex per modification ($\Delta T_m = T_m - T_m(WT)$). T_m's were determined in 10 mM phosphate buffer, pH 7, 100 mM Na^+; error limits are ± 0.5°. For details see ref. 3b; [b]Sequences are: I: 5'-TTTTtCTCTCTCTCT-3'; II: CTCGTACCtTTCCGGTCC-3'; III: 5'-tCCAGGtGtCCGCAtC-3'; [c]T_m of the corresponding wild-type duplex in °C; [d]See Fig. 1.

In conclusion, we have demonstrated that the modification of oligodeoxyribonucleotides by a large variety of 2'- and 3'-alkyl substituents reduces binding affinity for complementary RNA and also DNA. Given the structural diversity of the pendant groups investigated in this study, this appears to be a rather general finding, thus emphasizing the importance of a C-2' - *heteroatom* bond for DNA/RNA heteroduplex stabilization by 2'-modified oligonucleotides. In view of the RNA binding data compiled in *Tables 1* and *2*, fully modified oligonucleotides based on any of the modifications discussed in this report can be expected to hybridize to complementry RNA only with very low affinity (cf. sequence V, *Table 1*), which renders them inadequate for *in vivo* antisense applications. However, in special instances some of these building blocks may be suitable tools for the stabilization of oligonucleotides against degradation by 3'-exonucleases.

Acknowledgements

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