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Dramatic Effect of the Anomeric Configuration on the Thermal Stability of Duplex Formed Between Novel Dodecathymidine Phosphoramidate ($P\text{-}NH_2$) and Complementary DNA and RNA Strands

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Abstract. The inversion of the anomeric configuration of the sugar moieties of a dodecathymidine combined with the replacement of the phosphodiester backbone by non-ionic phosphoramidate ($P\text{-}NH_2$) produce a novel oligonucleotide analogue exhibiting unexpected high affinity for DNA and RNA targets. In comparison the phosphoramidate oligomer with the natural sugar anomer binds much less efficiently.

Due to their ability to hybridize in a sequence-specific way mRNA or DNA targets, antisense oligonucleotides are potential therapeutics for the treaments of various diseases[1-3]. However, the efficacy of unmodified oligodeoxynucleotides (ODNs) in serum and cultured cells is restricted because of their rapid degradation by 3'-exonucleases[4]. Nuclease-resistant ODN analogues may be obtained with the replacement of natural phosphodiester linkages by isoelectric phosphorothioate, non-ionic methylphosphonate or phosphoramidate linkages or by dephosphono linkers[5]. We recently reported on the synthesis, nuclease-resistance and base-pairing properties of novel ODN phosphoramidates[6]. In these analogues, an amino (NH_2) group was substituted for one of the two non-bridged oxygen atom of each internucleoside linkage. These non-ionic ODN analogues are strongly resistant to hydrolysis by various nucleases and hybridize complementary single-stranded DNA or RNA according to the Watson and Crick model, although with lower affinity.

Modifications introduced in the sugar moieties of ODNs may also generate nuclease-resistant analogues. For instance, α-ODNs which are constituted of unnatural α-anomeric nucleotide units[7], while they retain the base-pairing capacity of natural ODNs, have been shown to be more nuclease-resistant both *in vitro*[8] and *ex vivo*[9] systems, though recent studies indicate that sequence specificity exists[10]. α-ODNs are able to bind tightly to complementary DNA and RNA single strands. In these duplexes, the two strands were found to have parallel orientation[11,12].

More recently, we found that replacement, in a non stereospecific way, of phosphodiesters by phosphorothioate linkages in the ODN strand of ODN•RNA duplexes was less destabilizing when the ODN strand was in α-anomeric configuration ($-0.2°C \leq \Delta Tm/modification$) than when it was in a natural β one ($-0.9 \leq \Delta Tm/modification \leq -0.3°C$)[13,14]. As a result, α-ODN phosphorothioates consistently formed more stable

duplexes with complementary RNA strands than did the corresponding β-ODN phosphorothioates. Whether this discrepancy could be extended to other phosphate modifications, i.e. phosphoramidate ($P\text{-}NH_2$), is the matter of the present work.

The automated solid-phase synthesis of α-anomeric dodecathymidine undecaphosphoramidate (α-$d(Tpn)_{11}T$) was performed using H-phosphonate chemistry[15,16] as already described for the synthesis of β-anomeric analogue, β-$d(Tpn)_{11}T$[6]. After completion of the elongation steps, oxidative amidation of the hydrogenphosphonate diester linkages was carried out by treatment of the support bound oligomer with a saturated solution of ammonia in carbon tetrachloride-dioxan (4:1, v:v, 0°C, 30min)[17]. To avoid breakage of the base-labile phosphoramidate linkages, regular succinyl linker between control pore glass-long chain alkylamine solid support and 3'-nucleoside unit was replaced by a more base-labile oxalyl anchor[18]. This modification allowed the use of milder releasing conditions (saturated methanolic ammonia, room temperature, 5 min). The ^{31}P-NMR spectrum of the crude mixture consisted exclusively of a single broad peak centred at δ 12.34 ppm consistent with phosphoramidate diesters chemical shifts[19] and provided evidence that the phosphoramidate linkages survived the cleavage conditions of the oxalyl linker. In order to ascertain the integrity of the sugar-phosphate backbone, a 90% aqueous formic acid solution of HPLC-purified α-$d(Tpn)_{11}T$ was heated at 90°C for 30 min. Reverse-phase HPLC analysis revealed conversion to a product showing identical retention times as an authentic sample of α-anomeric dodecathymidine undecaphosphate (α-$d(Tp)_{11}T$).

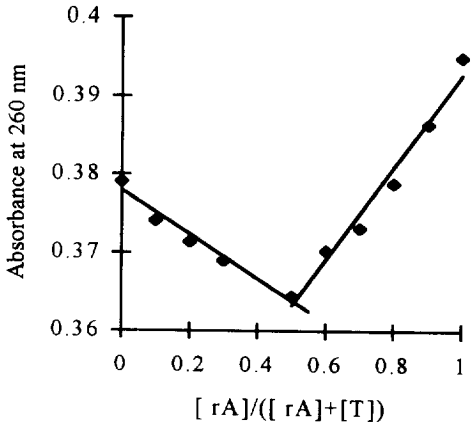

Figure 1. Mixing curve titration of the complex α-$d(Tpn)_{11}T$•poly rA at 5°C. Measurements were carried out at 48 μM (A+T) nucleotide concentration in 0.1 M NaCl, 10 mM sodium cacodylate (pH 7).

Interaction between α-$d(Tpn)_{11}T$ and poly dA or poly rA was followed by UV absorption spectroscopy and compared to those of β-$d(Tp)_{11}T$, α-$d(Tp)_{11}T$ and β-$d(Tpn)_{11}T$. Adding increasing concentrations of poly rA to a solution of α-$d(Tpn)_{11}T$ at 5°C led to a sharp break when absorbance at 260 nm was plotted versus rA/(rA+T) concentration ratio (Fig. 1). The break occured at a 1/1 ratio, indicating the formation of a double helix. Furthermore, on increasing the temperature of a 1/1 (A to T) mixture formed at 1°C between poly dA and α-$d(Tpn)_{11}T$, a hyperchromic effect was observed at 260 nm, whereas no similar effect was detected at 284 nm again suggesting the formation of a double helix[20]. This hyperchromic effect at 260 nm was found as large as the one observed with α-$d(Tp)_{11}T$, however it occured at higher temperature. Melting temperature (Tm) of duplex α-$d(Tpn)_{11}T$•poly dA was found to be 57.1°C; i.e. 25.6°C higher than that observed between unmodified β-$d(Tp)_{11}T$ and poly dA for the same conditions (Table 1). When considering the influence of the

anomeric configuration on the stability of d(Tpn)$_{11}$T•poly dA, the difference in Tm values is more striking. Inversion from the β- to α-anomeric configuration increased the Tm by 36.6°C, in contrast to what was observed for the corresponding anionic d(Tp)$_{11}$T where a decrease of 7.9°C was found with the same inversion. A similar situation was observed when duplex formation with poly rA was considered. Whereas β-d(Tp)$_{11}$T was not able to hybridize above 0°C[21], its α-anomeric counterpart did form a duplex with a Tm value of 43°C identical to that obtained with α-d(Tp)$_{11}$T and 13.5°C higher than that observed with β-d(Tp)$_{11}$T.

Table 1. Melting temperatures of duplexes formed between dodecathymidine analogues and poly dA or poly rA

ODNs	Versus poly dA		Versus poly rA	
	Tm (°C)	Δ Tm (°C)	Tm (°C)	Δ Tm (°C)
β-d(Tpn)$_{11}$T	20.5	-11.0	< 0	< -29.5
α-d(Tpn)$_{11}$T	57.1	+25.6	43.0	+13.5
β-d(Tp)$_{11}$T	31.5	/	29.5	/
α-d(Tp)$_{11}$T	23.6	-7.9	43.0	+13.5

Measurements were carried out at 60 μM nucleotide concentration for each strand, in 0.1 M NaCl, 10 mM sodium cacodylate (pH 7).

Is the high affinity of α-d(Tpn)$_{11}$T for complementary strands detrimental to its base-pairing specificity ? This question was addressed by measuring the influence of a central single mismatch on the stability of the duplex formed with complementary d(CpCp(Ap)$_{12}$CpC) abbreviated as d($C_2A_{12}C_2$). Data are presented in Table 2. Presence of one CT or GT mismatch in duplex formed with α-d(Tpn)$_{11}$T depressed the melting temperature by 16 and 13°C respectively. These values compare well with those obtained (17 and 15°C respectively) when the same mismatches were introduced in the corresponding duplex formed with unmodified β-d(Tp)$_{11}$T.

Table 2. Influence of a single mismatch on the Tm of duplexes with α-d(Tpn)$_{11}$T

ODNs	Tm (°C)		
	d($C_2A_{12}C_2$)	d($C_2A_6CA_5C_2$)	d($C_2A_6GA_5C_2$)
β-d(Tp)$_{11}$T	27	10	12
α-d(Tpn)$_{11}$T	50	34	37

Measurements were carried out at 5 μM concentration for each strand, in 0.1 M NaCl, 10 mM sodium cacodylate (pH 7).

Although the present results do not allow us to state the orientation of the two strands in duplexes formed between α-oligothymidine phosphoramidate and either complementary DNA or RNA strand, they provide evidence that the combination of two different structural modifications, i.e. the inversion of the anomeric configuration in the sugar moieties and substitution of anionic phosphate diester linkages for non-ionic phosphoramidate (P-NH_2) ones produced novel ODN analogues exhibiting high affinity for both DNA and RNA targets without loss of specificity. Extension of this work to α-oligonucleoside phosphoramidates involving the four common nucleobases is under way and will be reported in due course.

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21. At a fourfold higher concentration of each strand, an hybidization phenomenom was observed (Tm 18°C)[6].

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