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Comparison of Rigid and Flexible Backbones in Antisense Oligonucleotides

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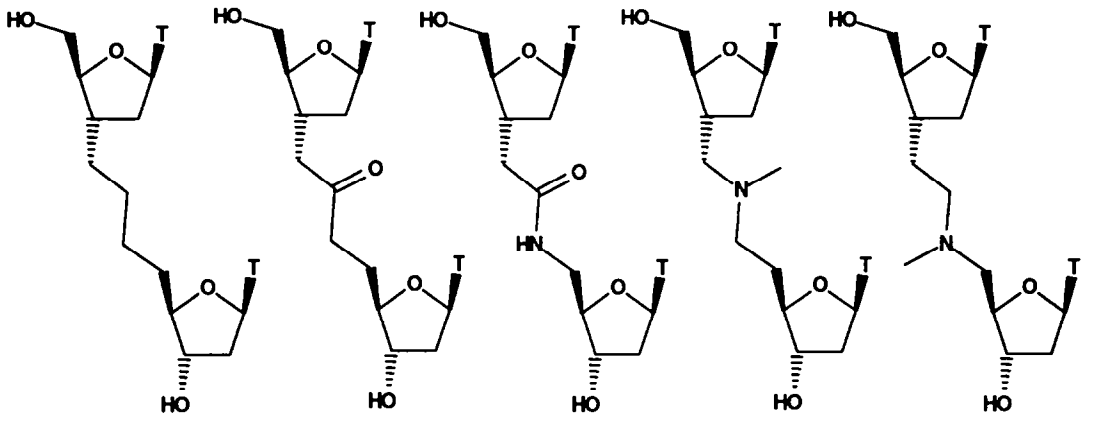
Abstract: Stepwise restriction of free rotation, as well as introduction of positive charges in the internucleosidic linkage are factors which can positively influence the thermal melting behaviour of the corresponding duplexes formed between modified DNA and their RNA complement. By correct positioning of the rigidity within backbone, it is possible to achieve even higher binding affinity (T_m's) than with natural oligodeoxyribonucleotides (ODNs).

Modified oligodeoxyribonucleotides (ODNs) have become an area of increased activity in recent years.[1] The capacity of base pairing of ODNs combined with the sequence specific recognition of nucleic acids has led to their development as diagnostic reagents and tools in molecular biology. The application of ODNs in medicinal chemistry, however, is limited because of their inadequate stability against nucleases as well as their poor ability to penetrate the cell membrane. For these reasons, backbone modified ODNs have become a major field of investigations for chemists.[2]



1 **2** **3** **4** **5**

In continuation of our work on backbone modified oligonucleotides,[3] we now wish to report the results on the new modifications **1, 2, 4** and **5** and compare them to **3**.[4] The linkages in these dimers are achiral (in contrast to phosphorothioates and methylphosphonates) and neutral (in contrast to phosphorothioates), therefore avoiding mixtures of diastereomers and, in comparison to phosphorothioates, favoring penetration through the negatively charged cell membranes. They differ considerably in their conformational flexibility due to restriction of free

rotation within the backbones (compare **3** with **2** and **5**). These modifications were incorporated in different sequences as dimers (**TmT**) by standard phosphoramidite chemistry[5] using a solid support. The melting temperatures of the duplexes formed with their RNA[6] as well as their DNA complements were determined.[7] The differences of T_m's per modification (ΔT_m's) compared to the unmodified oligonucleotides are listed in the **Table**.

Table: Differences of melting temperatures with RNA or DNA complement compared to wild type (w.t.)

Sequences (5'→3')[a]	T_m(°C)	ΔT_m/modification (°C)				
	w.t	**1**	**2**	**3**[3b]	**4**	**5**
A CTCGTACC**TmT**TCCGGTCC	63.3	-4.3	n.m.[b]	+0.9	-2.5	-3.8
B CTCGTAC**TmTTmT**CCGGTCC	61.6	-3.6	n.m.[b]	+0.5	-2.1	-3.3
C GCG**TmTTmTTmTTmTTmT**GCG	50.2	-5.9	-2.8	-0.3	-2.6	-4.4
D TTT**TmT**CTCTCTCTCT	51.6	-3.2	-2.1	+0.4	n.m.[b]	n.m.[b]
E GCG**TmTTmTTmTTmTTmT**GCG	53.8	n.c.[c]	-3.4	-1.5	-3.8	-4.4

a) **A** - **D** measured against RNA complement, **E** measured against DNA complement

b) not measured c) non cooperative (no duplex formation > 20°C)

The most accentuated decrease of thermal stability compared to the wild type was observed for the alkane linkage **1**, which even does not lead to a duplex formation with its DNA complement (see entries **E**). Replacing one of the carbon atoms by a tertiary nitrogen atom as in **4** and **5** leads in dependence of the position of the heteroatom to a minor or a significant increase of thermal stability compared to **1**. On the other hand, changing the hybridization of one carbon atom from sp^3 to sp^2, and therefore restricting the flexibility of the linkage, as in the keto-derivative **2**, also leads to an increased thermal stability of the duplexes compared to the very flexible **1**. Further restriction of free rotation, achieved with the amide linkage **3**, finally leads to duplexes, which exhibit even a higher thermal stability than the natural phosphodiester linkage.

Various elements influence the ability of modified oligonucleotides to form duplexes with their complements; apart from geometrical factors, entropical and electrostatic factors as well as hydration or hydrophobicity are of great importance.[2c] Although all modifications formally carry no charge, it is reasonable to assume that the amines **4** and **5** are partially protonated under physiological conditions (as they are under the conditions used for T_m measurements, pH 7.0)[8] and therefore electrostatic attraction with phosphate groups in the vicinity of the positive charge as well as hydration might play a role.[2c] Hydrophobicity, on the other hand, which could lead to the exclusion of water in the duplex shouldn't be left out of consideration, especially in the case of **1** .

Very often, the thermal stability of RNA/DNA duplexes is higher than the ones for the corresponding DNA/DNA duplexes, suggesting that A-type helices are preferred for these modifications. The alkane linkage in modification **1** was considered to be the most flexible and hence the least preferred for the duplex formation; no beneficial effect, such as electrostatic attraction, rigidity or gauche effect, might support the preferred conformation of the backbone in the duplex.[9] Nevertheless, the drop of thermal stability is not as drastic as expected and even the alternatingly modified oligonucleotide **C** underwent duplex formation. The ketone derivative **2**, in all sequences measured, showed improved thermal stability compared to **1**. Restriction of free rotation and elongation of the backbone, due to change of orbital hybridization of one carbon atom in the backbone (120^{o} angle for sp^2 hybridized carbon), seem to be responsible for this improvement compared to **1**.

Interestingly, both amines **4** and **5** showed improved but significantly different binding affinity compared to the alkane backbone, depending on the position of the nitrogen atom. It is reasonable to assume that the three backbones (**1**, **4** and **5**) do not differ in their flexibility, which therefore does not account for the observed difference. Therefore, electrostatic attraction between the protonated nitrogen atom and the phosphate of the complementary RNA strand is presumably responsible for the increase in thermal stability.[10] Although the pK_a's of **4** and **5** differ, **4** being more basic, both derivatives are supposed to be protonated under the conditions applied for the melting experiments. This illustrates the importance of correctly positioning the positive charge in the backbone. In addition, interaction of the amine with the vicinal sugar and/or the base moiety might be less pronounced in derivative **4** compared to **5** and hence the preferred conformation in the duplex can be adopted without severe distortion of the modified single strand.

The amide **3**, described earlier,[3a,b] formally represents a combination of ketone **2** and amine **5**. This modification, however, is even more restricted with respect to free rotation than ketone **2**. Because all four atoms of the backbone are preferentially fixed in one plane, *trans*-amide **3** is also much less flexible than the natural phosphate linkage. The direction of the dipole in **2** and **3**, as well as its magnitude, most probably differ substantially, which might also have positive consequences on the hydration of the backbone. It this context it is worth mentioning that alkyl substituents on the amide nitrogen atom have no influence on the melting temperatures of the duplexes.[3b]

In conclusion, we have demonstrated that rigidity and introduction of a positive charge into the backbone of dinucleotides strongly influence the thermal melting behaviour of oligodeoxyribonucleotides built from these dimers. Special attention has to be paid where these modifications are placed in the internucleosidic bridge. Emphasis has also to be put to the fact that equal or even higher thermal stability of duplexes with oligonucleotides, which incorporate modifications with higher rigidity than the natural phosphodiester bridge, can be achieved. By correctly designing the modifications, it is possible to evaluate the importance of individual effects on the behaviour of these dimers in the oligonucleotides.

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4. For the synthesis of **1** and **2** see: Lebreton, J., De Mesmaeker, A., Waldner, A., submitted for publication. For the synthesis of **3** see: ref. 3a and 3b. For the syntheses of **4** and **5**: Sanghvi, Y.S. et al., to be published.
5. Each oligonucleotide was prepared on an ABI 390 DNA synthesizer using standard phosphoramidite chemistry according to M. J. Gait, Oligonucleotides synthesis: A Pratical approach, IRL Press, Oxford 1984, but with prolonged times (10 min.) for the coupling step. DMT oligonucleotides were purified by reverse phase HPLC. The purity of the oligodeoxynucleotides were checked by capillary gel electrophoresis and their molecular weight was determined by mass spectrometry (MALDI-TOF: Pieles, U., Zürcher, W., Schär, M., Moser, H. *Nucl. Acids Res.*, **1993**, *21*, 3191).
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7. The thermal denaturation of DNA/RNA hybrides was performed at 260 nm using a UV-spectrophotometer. Absorbance *vs.* temperature profiles were measured at 4 µM of each strand in 10 mM phosphate pH 7.0 (Na salts), 100 mM total $[Na^+]$ (supplemented as NaCl), 0.1 mM EDTA. Tm's were obtained from fits of absorbance *vs.* temperature curves to a two state model with linear slope baselines (Freier, S. M., Albergo, D. D.,Turner, D. H. *Biopolymers*, **1982**, *22*, 1107). All values are averages of at least three experiments.
8. For comparison: pK_a's of n-propylamine and 2-methoxy-ethylamine are 10.6 and 9.45, respectively. Taken from John A. Dean , *Lange's Handbook of Chemistry, 14th*, McGraw-Hill, Inc.: New York, 1992. For comparison: the pK_a of 5'-amino-5'-deoxythymidine was determined to be 8.38 (private communication by Dr. H. Moser, Ciba-Geigy).
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