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Replacement of the Phosphodiester Linkage in Oligonucleotides: Comparison of two Structural Amide Isomers

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Abstract: The two structural amide isomers **2** and **3** as backbone replacement in oligonucleotides lead to very similar results for the binding affinities (T_m) of the duplexes formed with their complementary RNA strands.

Synthetic modifications of oligonucleotides are required in order to allow their therapeutical use in the antisense approach.[1] The binding affinity and specificity for the RNA target have at least to be maintained compared to the natural phosphodiester backbone **1**. However, the stability of these oligonucleotides towards nucleases as well as their cellular uptake should be substantially increased.

We recently proposed to replace the phosphodiester linkage **1** by an amide moiety as in **2** (**Figure 1**).[2] We describe here the synthesis of a structural isomer of **2**, namely the amide backbone replacement **3** and its incorporation into oligonucleotides. If the geometrical factors are predominant for the formation and the thermal stability of the duplex formed between a modified oligonucleotide and its RNA target, then the two amide modifications **2** and **3** should display very similar binding data (T_m). This, indeed, was observed for the amide **2** and **3** which can be regarded as structural analogues of the same fictive *trans* double bond.

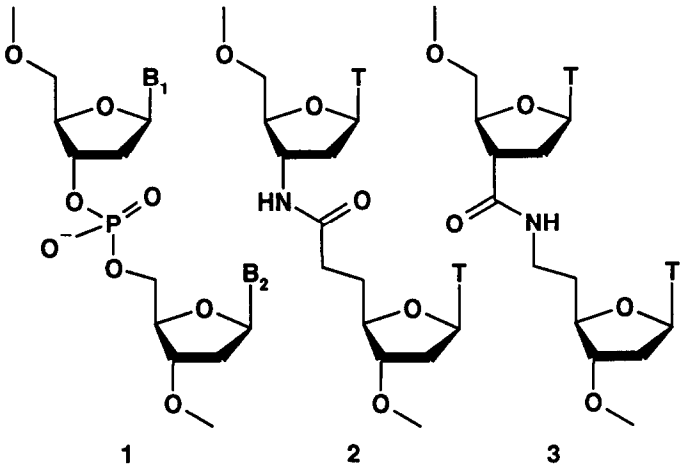
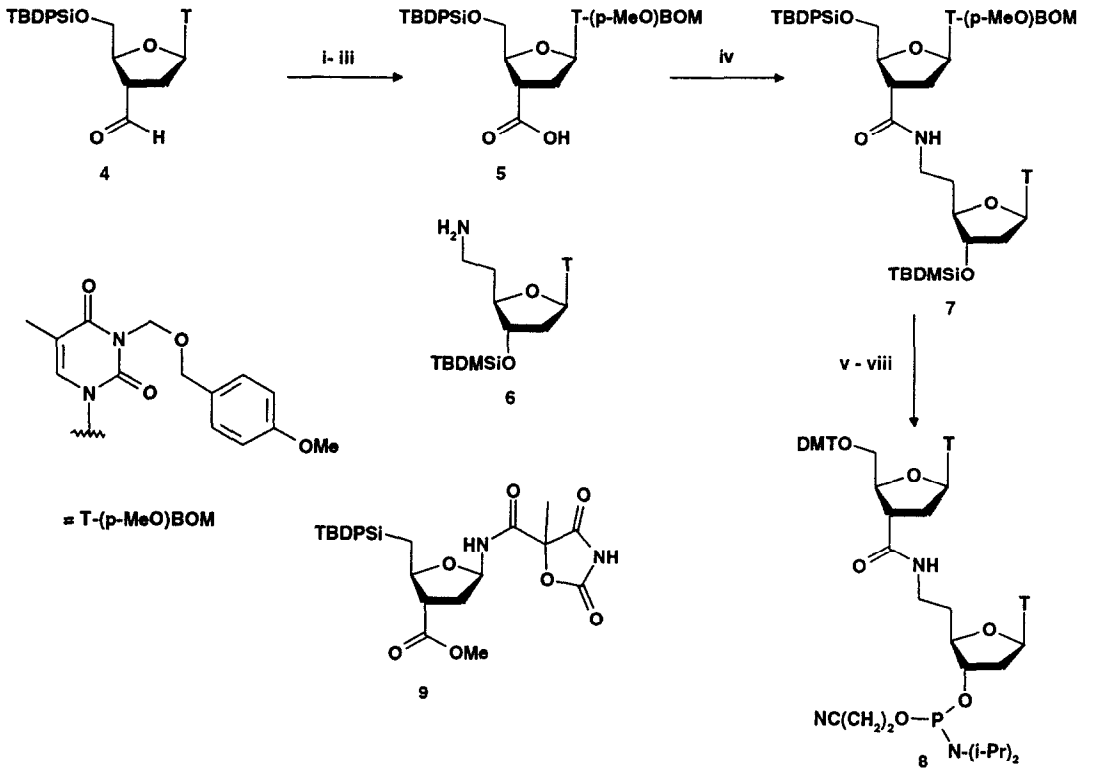


Figure 1

Dimer **3** was obtained starting from the aldehyde **4**[3] which was oxidized to the corresponding methyl ester with pyridinium dichromate (PDC) in the presence of methanol (**Scheme**). The desired product could be isolated by flash chromatography on silica gel in 59% yield. The side product **9** resulting from the oxidation of the thymidine base by PDC was formed in 12% yield.[4] These products were more readily separated after the introduction of the para-methoxybenzyloxy-methyl protective group on N-3 of thymine in the presence of DBU in acetonitrile. The methyl ester was saponified under mild conditions to the corresponding protected acid **5**. No epimerization at 3' was detected by ^{1}H-NMR spectroscopy during these three steps (i-iii, **Scheme**).

Scheme



i) PDC (6 eq.), MeOH (6 eq.), RT, 17 h, 59%; ii) p-MeOPhCH$_2$OCH$_2$Cl (2.5 eq.), DBU (1.2 eq.), CH$_2$Cl$_2$/CH$_3$CN (1:1 v/v), RT, 20 h, 66%; iii) 0.1M aq. NaOH (3 eq.), THF/H$_2$O (2:1 v/v), RT, 16 h; iv) 1-chloro-N,N,2-trimethylpropenylamine (1.3 eq.), **5** (1 eq.), CH$_2$Cl$_2$, RT, **6** (1.1 eq.), EtN$_3$ (1.1 eq.), RT, 20 h, 47%; v) DDQ (2. eq.), CH$_2$Cl$_2$/H$_2$O (18:1 v/v), RT, 4 h, 93%; vi) n-Bu$_4$NF (4 eq.), AcOH (4 eq.), THF, RT, 27 h, 87%; vii) 4,4'-dimethoxytritylchloride (DMTCl), pyridine, RT, 3 d, 48%; viii) (i-Pr$_2$N)$_2$POCH$_2$CH$_2$CN, i-Pr$_2$NH$_2$-tetrazole (1.5 eq.), CH$_2$Cl$_2$, RT, 60 h, 40% (TBDMSi = t-butyldimethylsilyl; TBDPSi = t-butyldiphenylsilyl).

The coupling of amine **6** with various activated esters derived from acid **5** was found to be rather difficult.[5] For example, the amide dimer **7** could be isolated only in 20% yield using O-(1H-benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium tetrafluoroborate (TBTU) and N-hydroxy-benzotriazole as activating agent.[6] The rather unreactive carboxylic acid **5** could, however, be transformed into the corresponding acid chloride by treatment with the *Ghosez* reagent (1-chloro-N,N, 2-trimethyl-propenylamine).[7] Dimer **7** was obtained by reaction with the amine **6** in the presence of Et_3N in 47% yield. The para-methoxybenzyloxy-methyl protective group was cleaved with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) in almost quantitative yield.[8] The choice of this protective group was crucial. The corresponding benzyloxy-methyl group was initially used but it could not be removed efficiently by hydrogenation in the presence of palladium on charcoal in methanol. The cleavage of the silyl protective groups was accomplished with n-Bu_4NF in the presence of acetic acid. The introduction of the 4,4'-dimethoxytrityl group (DMT) at the 5'-end and the phosphoramidite at the 3'-end was performed under standard conditions.[9]

The solid phase syntheses of the oligonucleotides (**Table 1**) and their purification were performed as usual.[10] The melting temperatures of the duplexes formed between the oligonucleotides containing the amide modification **3** and the complementary RNA strands are presented in **Table 1**.[11] The replacement of a single phosphodiester linkage by amide **3** leads to a decrease of the melting temperature of the duplexes between -3.3° C and -3.8°C. This drop of the thermal stability of the duplex for **3** compares well with the one observed for amide **2** (average ΔT_m/modification= -2.9°C).[2c] Both structural isomers adopt similar geometries as deduced from our molecular modelling studies (see below). Therefore, their influence on the melting temperature of the duplexes formed with the complementary RNA strands are of the same order of magnitude.

Table 1: Hybridization data[11]

		T_m(°C)	ΔT_m(°C)/modification	
Entry	Oligomer sequence (5'→3')	Wildtype	**3**[a]	**2**[b]
A	C T C G T A C C **TaT** T C C G G T C C	+63.3	-3.8	-2.2
B	C T C G T A C **TaT TaT** C C G G T C C	+61.8	-3.3	-2.8
C	T T T **TaT** C T C T C T C T C T	+51.8	-3.4	-2.7

a) **a**= 3'-CO-NH-CH_2-CH_2-4', b) **a**= 3'-NH-CO-CH_2-CH_2-4' (see ref. 2c)

The modified oligonucleotides d(CGACTATGCA**T**a**TT**a**T**C), where **a** represents an amide **3** linkage, exhibited an enhanced resistance towards hydrolysis by a factor 3 in 10% fetal calf serum (predominantly 3'-exonuclease) at 37°C as compared to the unmodified oligomer.[12]

Molecular mechanics (MM) and molecular dynamics (MD) studies were carried out using the AMBER force field[13a] as incorporated in the *BIOSYM* software.[13b] No counterions or explicit water was incorporated at this

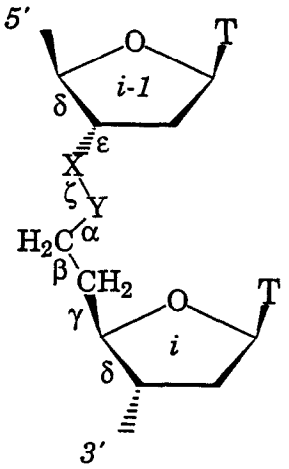


Figure 2
Definition of backbone torsion angles (named and defined as in the natural nucleic acid strand). X and Y are NH and C=O, depending on the amide modification.

stage. The permittivity was modulated by a distance dependent dielectric function $\varepsilon = 4 \cdot r_{ij}$. In order to assess the various possibilities for the modified backbone conformations, a conformational analysis was carried out on a modified octamer d(CTT**TaT**TTC)•r(GA_6G) starting from a standard A-type[13c] duplex. Various local energy minima were collected by enforcing the backbone torsion angles α and β (*cf.* **Figure 2**) by steps of 30° (over 360°), followed each time by a complete relaxation. In **Figure 3**[13d] are shown the lowest-energy geometries (found by this procedure) for the -**TaT**- dimer cut out of the respective fully minimized octamer duplex (**a** being **2** or **3**, respectively). The backbone torsion angles pertaining to these structures are listed in **Table 2**. The only major differences are observed for α and ε and might be attributed to the repulsion between the amide oxygen and the ring oxygen of the following sugar in **2**. MD simulations (100 picoseconds trajectories) were carried out on 14*mer* duplex structures with alternating amide linkages d(CT[**TaT**]$_5$TC)•r($GA_{12}G$), starting with the lowest energy conformation as reported in **Table 2**. During the MD simulation, conformational changes in the backbone were observed sometimes. The riboses stayed conservatively in the C3'-endo domain. The deoxyriboses underwent changes in the puckering state, adopting an average mode in the range of C4'-exo to O4'-endo. However, initially enforcing the C2'-endo puckering mode in both deoxyriboses above and below the amide modification and repeating some of the conformational searches above, no lower-energy structures were found.

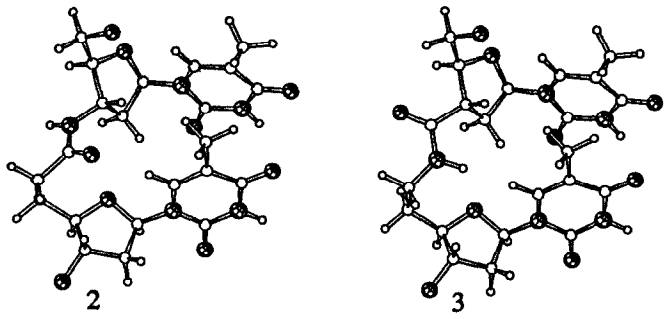


Figure 3
Lowest-energy geometry for the -**TaT**- dimer unit cut out of the fully relaxed octamer duplex d(CTT**TaT**TTC)•r(GAAAAAAG) for **2** and **3**, respectively (see text for details about deoxyribose puckering modes).

As already reported for **2**,[2c] the *cis* amide group in **3**, although higher in energy than the *trans* conformer (+ 2.2 kcal/mol in the above mentioned octamer duplex), shows a higher stability in molecular dynamics simulations, as judged from local distortions. A detailed account on the MD simulations will be given elsewhere.[13e]

Table 2: Backbone torsion angles around the amide modifications in the DNA strand (see **Figure 2** for torsion angle definition).[a)]

amide	residue[b)]	α	β	γ	δ[c)]	ε	ζ
2	*i-1*	*-77*	*173*	*64*	*82*	**-149**	**-162**
	i	**58**	**60**	**59**	*75*	*-173*	*-70*
3	*i-1*	*-75*	*174*	*63*	*79*	**-183**	**-167**
	i	**94**	**63**	**61**	*72*	*-171*	*-68*

a) values in *italic* concern the unmodified portions of the structures, values in **bold** refer to the modified portions, ζ corresponding to the *trans* amide torsion angle; b) see **Figure 2**; c) the angle δ is correlated with the puckering of the deoxyriboses (see text for details).

In conclusion, our present melting temperature (Tm) results indicate that the conformational rigidity introduced by the amide modifications **2** and **3** near the upper sugar moiety disfavors the formation of the duplex with an RNA strand. Consequently, the amide modification should rather be introduced in the middle of the backbone. These derivatives display, indeed, a higher binding affinity for RNA target.[2b]

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