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Synthesis and Triple Helix-Forming Ability of Oligonucleotides with *N,N*-Dimethylaminoethyl Phosphoramidate Linkages

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Abstract: Two oligonucleotides, partially modified with *N,N*-dimethylaminoethyl phosphoramidate groups, were obtained by an optimized solid-phase synthesis cycle based on *H*-phosphonate chemistry. Their use as third strands in parallel triple helices was shown to produce a decrease in stability with respect to all-phosphodiester oligonucleotide complexes, most probably due to unfavourable steric effects. Phosphoramidate-modified oligonucleotides were shown to be notably stable to exonucleases.

Triple helices of nucleic acids[1] have biological and biomedical interest. However, their therapeutic application is usually hampered by sequence stringency, low thermal stability, dependence on ionic media (pH, $K^{\oplus}$), and severe enzymatic degradation of oligonucleotides in biological media.

Various alternatives have been assayed[2] to increase the affinity of the third strand for dsDNA. On one hand, triple-helix formation has been facilitated by the addition of DNA ligands or polyamines. On the other hand, chemical modification of the third strand either at the sugar or nucleobase moieties or at internucleoside phosphate groups,[3] which often increases the stability to nucleases, provides additional interactions that favour triplex formation or reduces the electrostatic repulsion between negatively charged chains, respectively. Several researchers have recently synthesized oligonucleotides modified with aminoalkyl phosphoramidates, obtaining cationic oligomers,[4] when totally modified, or zwitterionic analogues[5] in the case of every other phosphoramidate-modified internucleoside linkage. Some of these phosphoramidate analogues form more stable triple helices than phosphodiester unmodified oligonucleotides.[4]

Here we report preliminary results on the effect on triple helix formation of modification of internucleoside linkages by cationic *N,N*-dimethylaminoethyl phosphoramidate groups (Fig. 1a). The reduction in charge may improve the stability of triple helices and the modified chain should be more robust to degradation by nucleases.

Two main strategies have been used to synthesize oligonucleotides with phosphoramidate bonds: i) chain elongation using *H*-phosphonate chemistry and final oxidation of all of the *H*-phosphonate diesters with amines in a single step, which produces mixtures of diastereomers,[6] ii) synthesis of stereochemically pure dinucleoside phosphoramidates for use as building blocks in the synthesis of stereoregular oligomers.[7] We have developed a simple strategy, also based on *H*-phosphonate chemistry, which generates phosphoramidate functions at the desired positions *via* oxiamidation after the coupling step (Fig. 1b) to obtain two pyrimidine oligomers, $^{5'}$T-T-T-T-C-T-C-T-T-C-T+T+T+T+T$^{3'}$ (**1**) and $^{5'}$T+T+T+T+C-T-C-T-T-C-T-T-T-T-T$^{3'}$ (**2**),[8] partially modified with dimethylaminoethyl phosphoramidate linkages at either end.

In our hands, preliminary assays using the synthetic procedures previously described[4a,6] for the synthesis of similar products failed. Complex mixtures of products were obtained and different operational protocols were optimized.

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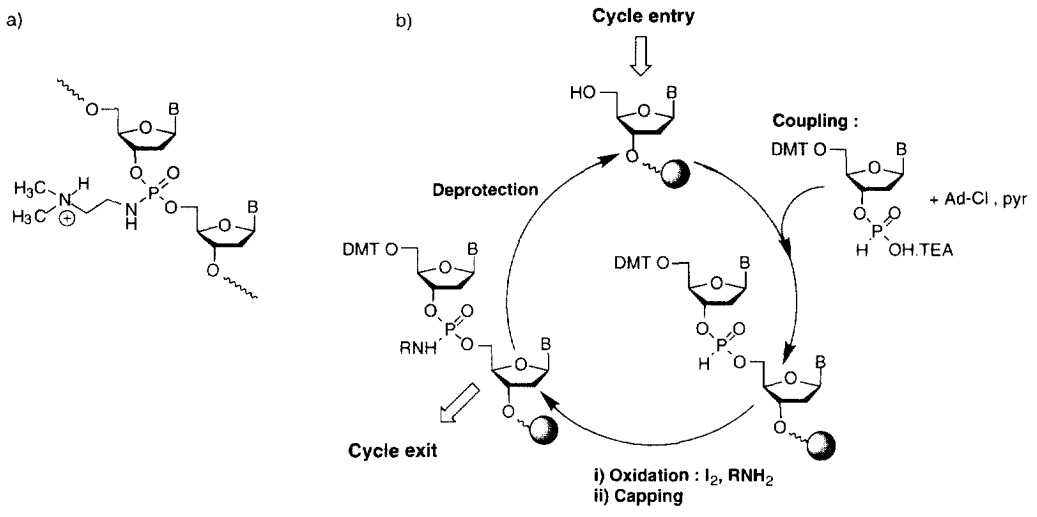

Fig. 1 : a) Cationic internucleoside linkages with *N,N*-dimethyaminoethyl phosphoramidate groups; b) Synthesis cycle for the preparation of phosphoramidate-modified oligonucleotide fragments.

Our main concern was the oxiamidation step. MALDI-TOF MS analysis of oligonucleotide crudes obtained after a final one-step oxiamidation of *H*-phosphonate linkages[6] revealed products lacking consecutive nucleotide units, and incomplete oxiamidation was also detected. Other procedures for obtaining phosphoramidates such as the oxiamidation of *H*-phosphonate intermediates with CCl_4/amine[6] or the treatment of phosphite triester derivatives with I_2/amine[9] were less effective or produced loss of functionality on the resin. We found that stepwise oxidation of *H*-phosphonate linkages was necessary to ensure complete transformation into phosphoramidate groups. This reaction was successfully performed with a solution containing 0.5 M I_2 and 1 M *N,N*-dimethylaminoethanediamine in pyridine/acetonitrile (1:1) for 15 min.

Other synthesis procedures were optimized. A sarcosyl residue was introduced between the resin and the nucleoside to ensure the stability of the oligomer-resin linkage during the oxiamidation step carried out at every cycle. The amines used for this purpose were shown to produce transamination[10] at benzoyl- and isobutiryl-protected cytidine. The *t*-butylphenoxyacetyl derivative remained stable, so this protecting group was used instead. Finally, the standard deprotection treatment with ammonia was found to partially cleave phosphoramidate internucleoside linkages. Best results were achieved by reaction with 0.05 M LiOH in MeOH/water (9:1) at room temperature for the final overall deprotection.

To prepare oligonucleotide **1**, the phosphoramidate fragment was first assembled using the synthesis procedure depicted in Fig. 1b.[11] The phosphate fragment was subsequently elongated using standard phosphoramidite chemistry. Oligonucleotide **2** was prepared similarly, by changing the order in which the two synthesis protocols were used. Once deprotected, **1** and **2** were purified by preparative PAGE and characterized by MALDI-TOF mass spectrometry.[12] Both **1** and **2** showed, as expected, decreased mobility with respect to the corresponding all-phosphodiester sequence **3** in gel electrophoresis analysis (Fig. 2). Oligonucleotide **1**, with the phosphoramidate fragment at the 3' end, was much more stable to a 3'-exonuclease (snake venom phosphodiesterase) than **3**.[13] In the same conditions, **3** was completely degraded in 5 min, whereas 50 % of **1** was still present after 20 min and 25% after 100 min.

Fig. 2: PAGE analysis of purified strands

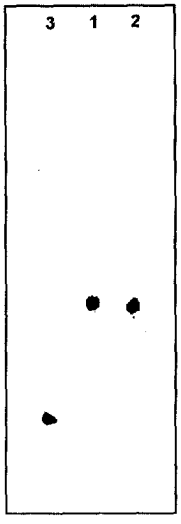

Table 1: T_m values from UV melting experiments

Entry	Complexes	pH	T_{m1}	T_{m2}
1	**4**	6.5	----	69
2	**3** + **4**	6.5	26	69
3	**1** + **4**	6.5	21	69
4	**2** + **4**	6.5	23	69
5	**3** + **4**	7.0	20	69
6	**1** + **4**	7.0	17	69
7	**2** + **4**	7.0	19	69

1 $^{5'}$T-T-T-T-C-T-C-T-T-C-T-C-T+T+T+T+T$^{3'}$

2 $^{5'}$T+T+T+T+C-T-C-T-T-C-T-C-T-T-T-T-T$^{3'}$

3 $^{5'}$T-T-T-T-C-T-C-T-T-C-T-C-T-T-T-T-T$^{3'}$

4 $^{5'}$A-A-A-A-G-A-G-A-A-G-A-G-A-A-A-A-A-(eg)$_6$

$^{3'}$T-T-T-T-C-T-C-T-T-C-T-C-T-T-T-T-T

To study the effect of dimethylaminoethyl phosphoramidate groups on triple helix formation, oligonucleotides **1** or **2** were hybridized with the self-complementary duplex **4**, $^{5'}$AAAAGAGAAGAAAAA-$(eg)_6$-TTTTTCTTCTCTTTT$^{3'}$.[14] The stability of the complexes formed was measured from UV melting experiments[15] in a medium reproducing the cellular concentrations of ions[16] at pH 6.5 and 7.0. In all cases biphasic curves were obtained, so melting temperatures could be determined for both triplex (T_{m1}) and duplex (T_{m2}) transitions. Table 1 summarizes the results obtained for the triple helices formed between duplex **4** and the modified oligonucleotides (**1** + **4** and **2** + **4**), and for the all-phosphate complex (**3** + **4**).

As expected, triple helix stability was lower at pH 7, since the formation of C^+(G.C) triplets is less favoured.[17] The complexes formed with modified oligonucleotides as the third strand (**1** + **4** and **2** + **4**) were less stable than the all-phosphate complex (**3** + **4**) at the two pH, with lower hyperchromicity and T_{m1} values for triplex-duplex transitions (cf entry 2 with entries 3 and 4, and 5 with 6 and 7). These results may be ascribed to the size of the dimethylaminoethyl moieties in phosphoramidate linkages, so that the possible stabilization caused by reduction of negative charge is counterbalanced by steric hindrance. Nevertheless, at high pH, at which triple helices are less stable, there seems to be a slight favourable contribution of positive charge from amino groups, in other words, the destabilization produced by dimethylaminoethyl groups is smaller. The decrease in T_{m1} caused by replacing **3** by **1** is 5 °C at pH 6.5 and 3 °C at pH 7, and the comparison between triplex systems **2** + **4** and **3** + **4** shows that using **2** instead of **3** as the third strand causes a decrease in T_{m1} of 3 °C at pH 6.5 and of only 1 °C at pH 7.

Finally, the T_{m1} values of the complexes in which **2** is the third strand (entries 4 and 7) are 2 °C higher than those formed by **1** (entries 3 and 6), showing that the triple helices formed by **2** are more stable. This difference may reflect additional unfavourable interactions between the 3' phosphoramidate fragment of **1** and the hexaethyleneglycol linker, which are in close proximity when the parallel triple helix is formed.

In summary, the synthetic protocol for the stepwise solid-phase synthesis of oligonucleotides partially modifed with dimethylaminoethyl phosphoramidate groups has been optimized. Only by combining protection of the cytosine exocyclic amine with the *t*-butylphenoxyacetyl group, I_2/amine-mediated oxiamidation of *H*-phosphonate diesters at every synthesis cycle, and reaction with LiOH instead of ammonia for the final deprotection were we able to obtain reasonably homogeneous phosphoramidate-modified oligonucleotides. These analogues, as expected, are more resistant to exonuclease degradation than unmodified oligomers. The

sterically demanding dimethylaminoethyl groups do not appear to facilitate triple helix formation, but the favourable effect of these positively charged groups can be appreciated at higher pH.

Work is in progress to extend this method of synthesis to the preparation of other oligonucleotide analogues in order to assess their effect on the stability of triple helices.

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8. In modified strands, "+" is used to indicate cationic phosphoramidate internucleoside linkages and "-" anionic phosphate diesters.
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11. Oligonucleotide syntheses were performed on CPG in an ABi 380B synthesizer, at the 2-3 μmol scale. The synthesis cycle for the phosphoramidate fragment, which was adapted from standard *H*-phosphonate chemistry except for the oxiamidation reaction, was as follows : 1) deprotection: TCA 3% DCM; 2) coupling (5 x) : 0.035 M nucleoside *H*-phosphonate + 0.17 M adamantoyl chloride in pyridine/ACN (1:3), 1 min ; 3) oxidation : 0.5 M iodine + 1 M *N,N*-dimethylethanediamine in pyridine/ACN (1:1), 15 min; 4) capping : Ac_2O + *N*-methylimidazole in lutidine/THF, 2 min.
12. **1** and **2** were obtained in 3 % and 8 % synthesis and purification yield, respectively, and characterized by MALDI-TOF mass spectrometry (negative mode): **1** (m/z): 2379.4, 4739.9; **2** (m/z): 2362.4, 4742.0. Theoretical average mass for **1** and **2** (neutral form): 4736.4 Da.
13. The oligonucleotides (**1** or **3**; approx. 0.6 OD_{260}) and 2'-deoxyadenosine (approx. 0.6 OD_{260}; internal standard) were dissolved in 250 μL of a buffer containing 40 mM phosphates, 140 mM NaCl and 1 mM $MgCl_2$. 1 μg of snake venom phosphodiesterase (E.C. 3.1.3.1, Boehringer Mannheim) was added to 250 μL of the buffer solution, and the reaction mixture was incubated at 37 °C. At regular intervals, aliquots were removed, frozen and lyophilized. These aliquots were subsequently analyzed by reversed phase HPLC to determine the ratio of undegraded oligonucleotide.
14. **3** and **4** were obtained using standard phosphoramidite chemistry (DMT-on) and purified by reversed phase HPLC and gel filtration (Sephadex G-10). In the case of **4**, the hexaethyleneglycol $(eg)_6$ linker was introduced as the corresponding phosphoramidite derivative, which was prepared as described in Rumney, S.; Kool, E. T. *J. Am. Chem. Soc.*, **1995**, *117*, 5635-5646.
15. UV melting experiments were performed in a Varian Cary 5E spectrophotometer. Curves were recorded by measuring the change in absorbance at 260 nm upon heating the samples from 5 °C to 9 °C at 0.5 °C/min rate. T_m (melting temperature) refers to the midpoint of the region of maximun slope for each transition in the the plot of absorbance versus temperature.
16. Solutions contained 40 mM phosphate buffer, 140 mM NaCl and 1 mM $MgCl_2$, and oligonucleotides were present at approximately 1 μM concentration.
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