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Binding Properties of Oligonucleotides Containing a Modified 2′-Deoxyuridine with a Thymine Ended Linker to Pair with 2′-Deoxyadenosine

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Abstract—Oligonucleotides containing in the place of thymidine the nucleoside **2**, a 2′-deoxyuridine harbouring at C-5 a thymine ended linker, were found to undergo base pairing with the opposite 2′-deoxyadenosine. However, the corresponding duplexes are significantly destabilised as compared to the fully natural ones.

The stability of DNA duplexes is controlled by an intricate interplay of stabilising (hydrogen bonding to form base pairs, base π-stacking) and destabilising (electrostatic repulsion, steric effects) forces, not to mention water molecule and metal ion interactions.[1]

With the ongoing developments of DNA technologies[2] and oligonucleotide antisense applications,[3] the need has appeared to devise modified oligomers able to form, with a complementary DNA or RNA strand, a duplex with enhanced stability compared to a fully natural one.[4] To date, many elegant proposals have been worked out in order to modify the thermodynamics of formation of nucleic acid complexes by influencing either the enthalpic or the entropic contributing factors, or both. In this regard, the most remarkable achievements in this field are probably the developments of peptide nucleic acids[5] (PNA) and that of oligonucleotides containing constrained nucleosides.[6]

However, along this research line, other strategies have been explored such as the one illustrated by the design of G-clamp-containing oligonucleotides.[7] A G-clamp base corresponds to a cytosine analogue which can form an additional hydrogen bond to guanine, contributing to enhance considerably the helical thermal stability. With this in mind, we proposed to consider another approach, wondering if a pyrimidine nucleoside construct featuring a linker bearing a terminal pyrimidine could exercise a stabilising effect by forming with an opposite adenosine, across the major groove, the unusual bonding outlined in Figure 1.[8] We based our assumption on the well known capacity of nucleic bases to form Hoogsteen or reverse Hoogsteen hydrogen bonds under a variety of conditions in the DNA as well as in the RNA series.[9] Furthermore, we established, in a previous work, that an oligonucleotide containing internally the nucleoside **1** bearing the 4-thiothymine photolabel was able to cross-link residues located in the complementary strand under irradiation.[10] This suggested that, as least transiently, the photoactive base could thread into the major groove of the duplex to interact with the base acceptors of the opposite strand, and this with a better efficiency for DNA than for RNA. Moreover, this experiment suggested that the length of the arm of compound **1** should be well designed for our purpose.

Herein, we describe our experiments aimed at the validation of the proposed concept. Nucleoside **2** was prepared classically starting from intermediate **3**[11] which in the presence of ethylenediamine gave compound **4**.[12] This derivative was conjugated with *N*-1-thyminyl-2-acetic acid to provide intermediate **5**[13] which was phosphitylated to furnish phosphoramidite **6** (Scheme 1).[14] With this material in hand we could synthesise a series

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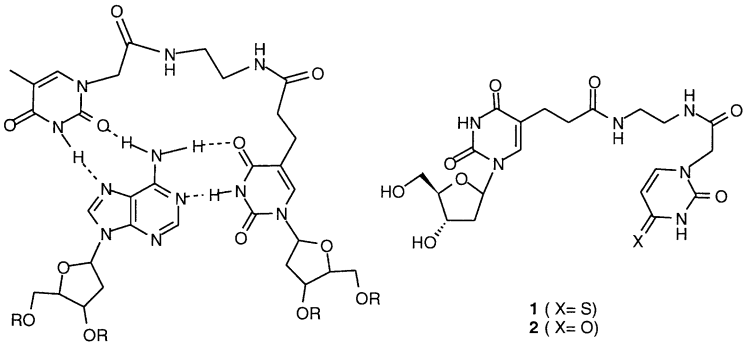
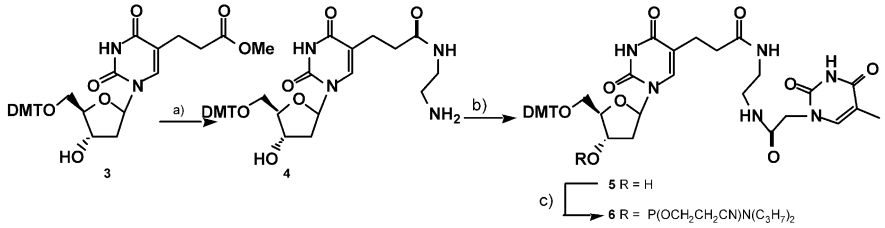


Figure 1.



Scheme 1. Reagents and conditions: (a) ethylene diamine, rt; (b) *N*-1-thymidinyl-2-acetic acid, EDC, HOBt, TEA, DMF, rt; (c) 2-cyanoethyl diisopropylchlorophosphoramidite, *N,N*-disopropylethylamine, CH_2Cl_2, rt.

of five 10-mer oligonucleotides **O-1**, **O-2**, **O-3**, **O-4** and **O-7** incorporating 1, 2, 3, 4 and 7 units of nucleoside **2**, respectively (Table 1).[15] Their respective binding affinity for their natural target **12AG** was determined and compared with that of the corresponding unmodified 10-mer **10-TC** using UV melting experiments (Table 2).

Whatever the number and the position of nucleoside **2** within each oligonucleotide sequence, all the measured melting temperatures (T_m) are inferior to the one observed with the reference duplex **12AG**/**10-TC**. The most salient feature which is worth notice is that there is no linear variation between the T_m decrease and the number of nucleoside **2** being incorporated in the modified oligonucleotides **O-1** to **O-7.** Thus, the sequences **O-1** and **O-3** with one and three modifications, respectively, have the same affinity for their common target **12AG**. Comparing oligonucleotides **O-2** and **O-3** we find that the less modified sequence **O-2** gives rise to a less stable duplex than **O-3**. The two sequences differ essentially by the mode of substitution which is discontinued in one case and continued in the other. The T_ms observed with **O-4** and **O-7**, having one or two blocks of continuous modifications, are almost identical underlining the non-cumulative substitution destabilising effect.

As a consequence of these intriguing observations, we searched to get insight into the nature of the eventual interactions between the thymine moiety of **2** and its corresponding opposed adenine residue. To this end we proposed to investigate the behaviour of oligonucleotides **O-1** to **O-7** in the presence of a modified target in which four adenines have been replaced by *N*-7-deazaadenines (labelled **a**). *N*-7-Deazaadenine can partially prevent Hoogsteen or reverse Hoogsteen hydrogen bonding and thus be used as an internal probe. When the corresponding 12-mer oligonucleotide designed **12AaG** was annealed with **10-TC** the duplex was found less stable than the reference duplex with a T_m of 25 °C corresponding to a 4 °C decrease (1 °C per substitution). Accordingly, in the measurements of the stability of the

Table 1. Oligonucleotide sequences: **a** stands for *N*-7-deaza-2′-deoxyadenosine

3′-TGAAAAGAAAAT-5′	**12AG**
5′- CTTTTCTTTT-3′	**10TC**
3′-TGaAaAGaAaAT-5′	**12aAG**
5′- CTT**2**TCTTTT-3′	**O-1**
5′- CTT**2**TCTT**2**T-3′	**O-2**
5′- CTTTTC**222**T-3′	**O-3**
5′- C**2222**CTTTT-3′	**O-4**
5′- C**2222**C**222**T-3′	**O-7**

Table 2. UV melting temperatures of duplexes between 12-mer and 10-mer sequences listed in Table 1

Sequence	12AG	12aAG	ΔT_m
10TC	29	25	−4
O-1	24	20	−4
O-2	21	15	−6
O-3	24	11	−13
O-4	18	<5	>−13
O-7	17	<5	>−13

DNA thermal denaturation and renaturation experiments were carried out in a 10 mM cacodylate buffer (pH 6.6) containing 100 mM NaCl, 10 mM MgCl2 and 0.5 mM spermine. Estimated accuracy of the melting temperatures: ±1°C.

duplexes involving oligonucleotides **O-1** to **O-7**, this decreased stabilty due to the presence of *N*-7-deazaadenine should be taken into account since it should be observed whatever the nature of the base pair. Thus, with sequences **O-1** and **O-2** the decreased ΔT_m found to be 4 °C and 6 °C, respectively, is comparable to what is observed with the unmodified target **12AG**. Conversely, for the duplexes formed between **12AaG** and oligonucleotides **O-3**, **O-4** and **O-7** the variation of $\Delta T_m \geq 13$ °C is higher than expected for a simple intrinsic lost of stability due to *N*-7-deazaadenine replacement. Considering all these results, we conclude that the presence of a bulky substituent (linker + thymine) at the C-5 position of 2′-deoxyuridine **2** exerts a destabilising effect on duplex formation. However, when nucleosides **2** are introduced blockwise the destabilisation is not cumulative as one could have expected. A reasonable explanation can be suggested in the case of oligonucleotides **O-3**, **O-4** and **O-7** with the contiguous thymine moieties making favourable contacts, presumably by means of π-stacking. Under these conditions, the concerned residues might be pushed inside the major groove of the double helix to escape hydrophobic interactions and take advantage of hydrogen bonding with the N-7 position of the complementary adenine.

At best, this interpretation is tentative and more experimentation will be required to characterise the nature of the interactions that we have discovered with nucleoside **2** and, in particular, to delineate the role of the spacer arm. However, at this stage, it becomes clear that we are dealing with two types of antagonising forces suggesting that the entropic contribution of the linker outbalances the enthalpic gain due to bonding and stacking interactions which finally induces a considerable destabilisation of the duplexes. Further endeavour in this domain will be directed at the construction of a less penalising linker.

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13. Compound **5**: To a mixture of nucleoside **4** (340 mg, 0.5 mmol) and *N*-1-thyminyl-2-acetic acid (116 mg, 0.6 mmol) in DMF (15 mL) were added succesively *N*-hydroxybenzotriazole (110 mg, 0.7 mmol) and EDC (140 mg, 0.7 mmol). After 3 h, the solvent was evaporated and the residue purified by flash silica gel chromatography, elution with CH_2Cl_2/MeOH (2–10% MeOH) containing 1% Et_3N gave nucleoside **5** as a white foam (374 mg, 87%). FAB-MS *m*/*z* 817 $(M + Li)^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 7.62 (s, 1H, H-6), 7.44–7.21 (m, 10H, H-arom and H-6 T), 6.86 (d, $J = 8.7$ Hz, 4H, H-arom), 6.29 (t, $J = 6.6$ Hz, 1H, H-1′), 4.46 (m, 1H, H-3′), 4.01 (m, 1H, H-4′), 3.77 (s, 6H, 2×OMe), 3.39–3.23 (m, 6H, 2×H-5′ and 2×N-CH_2), 2.44–2.09 (m, 4H, 2×CH_2), 1.94 (s, 3H, Me).
14. Compound **6**: To a solution of nucleoside **5** (85 mg, 0.1 mmol) in CH_2Cl_2 (5 mL) was added *N,N*-diisopropylethylamine (0.4 mL) and cyanoethyl diisopropylchlorophosphoramidite (0.2 mL). The solution was stirred at room temperature for 0.5 h, treated with an aqueous solution of sodium bicarbonate (2x2mL), dried over magnesium sulfate and evaporated under reduced pressure. The oily residue was purified by column chromatography over silica gel with heptane/EtOAc (1/1 to 2/8) containing 1% Et_3N to give phosphoramidite **6** (93 mg, 88%). ^{31}P NMR (243 MHz, $CDCl_3$): δ 137.6–137.3.
15. Standard oligonucleotides were purchased from Eurogentec and modified oligonucleotides were synthesised on an Applied Biosystem 392 DNA/RNA synthetiser. The 5′-dimethoxytrityl (DMT) protected oligomers were purified by reversed-phase HPLC (Waters PrepPak Cartridge Delta-Pak C18, 15 mm, 300 A, 25×100 mm) using a 30 min linear gradient of solvent A [0.1 M triethylammonium acetate buffer (pH 7) containing 7% of CH_3CN] and solvent B (CH_3CN) (100:0 to 60:40). The DMT protecting group was finally cleaved by treatment with 80% aq AcOH for 1 h at room temperature. The oligomers were precipitated using an acetate buffer (pH 6.2)/EtOH solution and isolated after centrifugation at 0 °C for 15 min.