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Probing the effect of a 3′-*S*-phosphorothiolate link on the conformation of a DNA:RNA hybrid; implications for antisense drug design†

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The effects of a single 3′-*S*-phosphorothiolate link in the DNA strand of a DNA:RNA dodecamer duplex is described; the sulfur induces a conformational shift in the (attached) sugar pucker, as shown by ^{1}H NMR studies, and increases the thermal stability of the duplex compared to the non-modified system.

In designing a DNA-based drug for use in an antisense therapeutic strategy[1] a number of factors must be considered. These include specificity/selectivity of mRNA recognition, the biochemical stability and delivery of the antisense oligodeoxynucleotide-based agent (asODN) and the lifetime of the asODN:RNA complex; the target DNA:RNA system, which when of mixed sequence, is less thermodynamically stable than its DNA:DNA or RNA:RNA counterparts.[2] Furthermore, the antisense efficiency is enhanced if the asODN:RNA hybrid is amenable to RNase H-mediated degradation.[3] A number of chemical variants have been produced and have been shown to have advantages over the natural ODNs in one or more of the problem areas.[4] A modification which we have previously used in studies of enzymes that are involved in cleaving and processing DNA and RNA[5–7] is the 3′-*S*-phosphorothiolate link. This has potential for use in asODNs due to its resistance to nuclease activity[5,6a,c] and decreased hydrophilicity. In studies of dinucleotides containing this modification it has been found that the sugar pucker of the ring to which the sulfur is attached is predominantly north ($C_{3'}$-*endo*/$C_{2'}$-*exo*), which is also the conformation favoured by sugars of ribonucleotides.[8] Moreover this shift in the north–south conformational equilibrium was also found to be transmitted to the subsequent (n + 1) sugar. If both of these features were to be retained in a longer sequence, and in a duplex, we would expect such sequences to have enhanced RNA binding affinity and therefore considerable potential as antisense agents. To investigate this a single 3′-*S*-phosphorothiolate link was incorporated in a DNA dodecamer (**1**, 5′d($C_1C_2T_3$ $A_4A_5A_6$ $T_7T(S)_8T_9$ $G_{10}C_{11}C_{12}$), where 'S' indicates a 3′-*S*-phosphorothiolate link between thymines 8 and 9)† [9] and its complex with its complementary RNA dodecamer (3′ r($G_{24}G_{23}A_{22}$ $U_{21}U_{20}U_{19}A_{18}A_{17}A_{16}$ $C_{15}G_{14}G_{13}$)) was analysed using 2D ^{1}H NMR. The non-modified DNA : RNA hybrid (**2**, 5′d(CCT AAA TTT GCC)/3′r(GGA UUU AAA CGG)) was similarly analysed for comparison. Significant, but localised, conformational changes were observed together with the anticipated enhancement of thermodynamic stability.

Proton NMR chemical shift assignment, for each duplex, was achieved using established procedures.†[10] In comparing proton chemical shifts for the non-modified and modified duplex it was clear that the effects of the sulfur were localised (see Table 1). Further support for this conclusion came from an analysis of cross-peak intensities in the NOESY spectra for (**1**) and (**2**). NOE cross-peak patterns and intensities only varied between the two molecules at or around the substitution site.‡ To establish the nature of this difference a number of key NOE intensities[11,12] were examined more closely; more specifically those connecting protons H2′ to H6/H8 (Fig. 1), H1′ to intra-nucleoside H2′/H2″, and adenine H2 to H1′, in residues adjacent to, or opposite, the site of modification. For nucleotides in which the sugar adopts a north conformation, the distance between an H6/H8 and the H2′ proton (intra-residue) is longer than that to the H2' proton of the preceding residue. In a north-puckered sugar the difference between the H1′–H2′ and H1′–H2″ distances is less than observed for a south-puckered sugar,

Table 1 Proton chemical shift changes on introducing a 3′-*S*-phosphorothiolate link between residue T_8 and T_9

Residue	Chemical shift changes[a]
A6	**H3′** − 0.06
T7	**H1′** − 0.06, **H2′** + 0.14, **H3′** − 0.07
T8	**H1′** − 0.19, **H2′** + 0.23, **H3′** − 1.29, **H4′** − 0.14, **H6** + 0.08
T9	**H2′** + 0.08, **H4′** + 0.09, **H6** − 0.33
G10	**H8** + 0.05
G13	**H1′** + 0.07
A17	**H1′** − 0.06, **H2** − 0.11, **H8** − 0.07
A18	**H2** − 0.08, **H8** − 0.05

[a] ppm; only shifts of magnitude greater than 0.05 ppm noted.

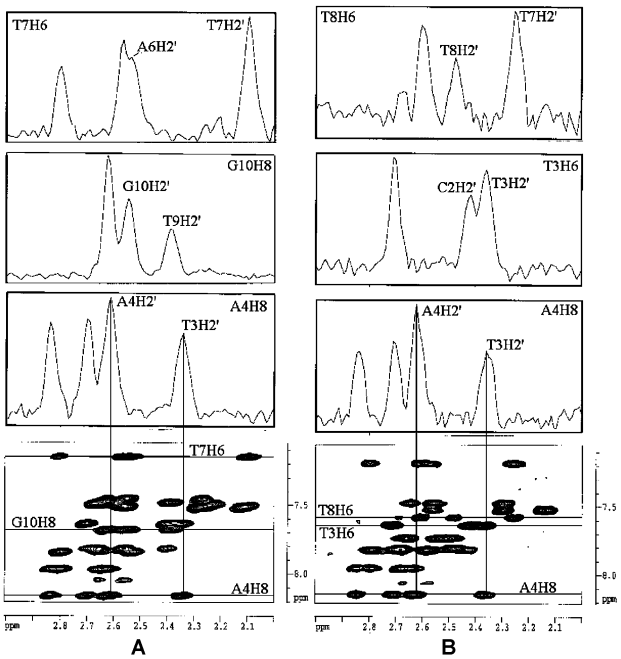

Fig. 1 Sections through the 500 MHz NOESY spectrum recorded for (**A**) the non-modified and (**B**) the modified duplex, showing the relative size of the *n*H6/H8–*n*H2′ and *n*H6/H8–(*n* −1)H2′ cross-peaks. The T8H6 to T7H2′ NOE (in B) is indicative of north-puckering.

† Electronic supplementary information (ESI) available: diagram showing sulfur link, UV melting curves, sample PE-COSY spectra, further NOE intensity data. See: http://www.rsc.org/suppdata/cc/b2/b203582k/

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with the H1'–H2″ distance always shorter. An adenine with a south-type sugar pucker has an H2 proton which is closer in space to its cross-strand H1′ than to the H1′ of its n + 1 (intrastrand) sugar unit.[11] All of the observations made in these regions indicated that the T_8 sugar ring adopts a north conformation in (**1**).

Further conformational information was obtained from an analysis of a number of proton–proton scalar coupling constants. A complete pseudorotational analysis of the sugar puckers,[13] as performed for the dinucleotides,[8] was not possible due to cross-peak overlap. However, it has been previously shown that sugar conformations may be elucidated from combinations of H1′ to H2′ and H1′ to H2″ couplings,[14] *i.e.* using cross-peaks in less crowded regions. These couplings were thus measured for the DNA strand from PE-COSY[15] spectra recorded for both (**1**) and (**2**) (see Table 2). These data indicate that the sugar of T_8 adopts a predominantly south pucker in (**2**) and predominantly north in (**1**). This is consistent with the NOE data. In addition the sugar of T_9 was found to undergo a conformational shift in the same direction but to a lesser extent. The sugar pucker determinations based on the H1′ to H2′ and H1′ to H2″ couplings are very similar to those for d(TpT) and d(TSpT).[8]

This is an important piece of information and goes some way towards supporting the use of minimal sequences to assess the usefulness or otherwise of chemical modifications. These observations are similar to, though less dramatic than, those made for a nonamer containing a single locked nucleic acid.[16] In the LNA system the n + 1 sugar conformation was shifted to > 90% north.

That the 3′-*S*-phosphorothiolate link can make the DNA conformation more like that of the RNA has been demonstrated. It remained to establish that this change would bring with it enhanced stability of the complex. To investigate this we performed UV thermal melting studies for (**1**) and (**2**) under identical conditions.[17]

A reproducible 2.5 °C increase in the melting temperature was observed for (**1**) compared to (**2**). Increases in T_m of 4–9 °C per modification have been observed for LNA:RNA systems.[18] The conformational changes induced by the phosphorothiolate linkage are more subtle than those observed in LNA and this is evidently reflected in the comparison of the T_m values.

In extending the comparison it should be noted that the conformational shift induced by the 3′-*S*-phosphorothiolate link is much more localised than that observed for LNA. In addition to the effect noted at residue n + 1, the n − 1 residue is shifted 60% further north in the LNA system compared with a shift of less than 10% in (**1**). This has considerable implications for the design of asODNs;[19] for example only partial phosphorothiolate substitution towards the ends of an asODN should result in a asODN:RNA hybrid which benefits from the thermodynamic stability of RNA-like 'wings' yet retains susceptibility to RNase H in its centre. The effect of multiple phosphorothiolate substitution is currently under investigation.

Table 2 Coupling constant measurements and approximate mole fraction of south conformer

Residue	J (H1′–H2′)[a]	J(H1′–H2″)[a]	Approximate mole fraction '*S*' conformer
C1[b]	5.4/[d]	5.3/[d]	0.2/[d]
C2[b]	5.0/5.4	6.5/7.0	0.3/0.5
T3[c]	7.2/6.7	6.2/7.5	0.6/0.7
A4[b]	8.7/6.6	6.7/6.6	0.9/0.6
A5[b]	8.3/[d]	6.8/[d]	0.9/[d]
A6[c]	6.7/6.5	6.3/5.7	0.6/0.4
T7[b]	7.2/6.6	6.3/5.9	0.6/0.5
T8[c]	8.0/0	7.1/6.0[e]	0.9/0.0
T9[b]	7.5/5.3	6.4/5.9	0.7/0.3
G10[b]	7.5/6.7	5.9/5.8	0.6/0.5
C11[c]	6.7/6.3	6.7/5.6	0.6/0.4
C12[c]	6.3/6.0	6.9/6.5	0.6/0.5

[a] Hz, non-modified/modified. [b] Couplings accurate ±0.6 Hz. [c] Couplings accurate ±0.3 Hz. [d] PE-COSY cross-peak too overlapped to extract information. [e] Coupling accurate to only ±1 Hz, due to some overlap.

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Notes and references

‡ In both (**1**) and (**2**) the RNA strand is essentially A-type and the DNA strand essentially B-type right handed helices. Full details will be published elsewhere.

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