Duplex stability of DNA·DNA and DNA·RNA duplexes containing 3′-*S*-phosphorothiolate linkages

Joanne Bentley,[a] John A. Brazier,[a] Julie Fisher*[b] and Richard Cosstick*[a]

Received 29th August 2007, Accepted 20th September 2007
First published as an Advance Article on the web 2nd October 2007
DOI: 10.1039/b713292a

3′-*S*-Phosphorothiolate (3′-SP) linkages have been incorporated into the DNA strand of both a DNA·RNA duplex and a DNA·DNA duplex. Thermal melting (T_m) studies established that this modification significantly stabilises the DNA·RNA duplex with an average increase in T_m of about 1.4 °C per modification. For two or three modifications, the increase in T_m was larger for an alternating, as compared to the contiguous, arrangement. For more than three modifications their arrangement had no effect on T_m. In contrast to the DNA·RNA duplex, the 3′-*S*-phosphorothiolate linkage destabilised the DNA·DNA duplex, irrespective of the arrangement of the 3′-SP linkages. The effect of ionic strength on duplex stability was similar for both the phosphorothiolate-substituted and the unmodified RNA·DNA duplexes. The results are discussed in terms of the influence that the sulfur atom has on the conformation of the furanose ring and comparisons are also drawn between the current study and those previously conducted with other modifications that have a similar conformational effect.

Introduction

Knowledge that oligonucleotides can act as potential therapeutic agents, through antisense, antigene and RNA interference mechanisms, has made the chemical modification of nucleic acids a subject of exceptional interest. Chemists have shown considerable ingenuity in developing nucleic acid mimics, which exhibit structural features that deviate considerably from the natural sugar-phosphate backbone, but retain sequence-specific binding to DNA/RNA. Pre-eminent in this class are the amide-based backbone structures such as PNA[1] and its analogues,[2] which show exceptionally high affinity for complementary sequences. However, nucleic acid analogues which are based on backbones that are more closely related to the natural phosphodiester bonds still continue to attract attention. These analogues are typified by linkages in which one oxygen atom of the phosphodiester bond is replaced by another heteroatom. Within this category both the phosphorothioate[3] and the 3′-*N*-phosphoramidate[4] (3′-NP) analogues have been extensively studied as therapeutic agents[5] and the phosphorothioate linkage has also proved valuable in probing the detailed mechanism[6] of phosphodiester bond cleavage in nucleic acids.

Closely related to the phosphorothioate modification, is the 3′-*S*-phosphorothiolate (3′-SP) linkage (Fig. 1), in which the 3′-bridging oxygen is replaced by sulfur and can formerly be considered as the result of incorporating a 3′-thionucleoside into nucleic acids. This modification initially attracted attention for its application in probing enzymatically catalysed cleavage mechanisms in both DNA and RNA.[7] More recently the structural consequences of incorporating a single 3′-SP linkage into the DNA strand of a DNA·RNA hybrid [d(CCTAAATTTGCC)·r(GGCAAAUUUAGG)] has been studied by high resolution NMR spectroscopy and has demonstrated that the conformation of the sugar to which the sulfur is attached shifts to the north pucker (C3′-*endo*/C2′-*exo*).[8] In addition, it was noted that the conformation of the furanose ring 3′ to the site of modification is also shifted to the north. Finally, UV thermal melting studies established that, in comparison to the unmodified system, the incorporation of one or two 3′-*S*-phosphorothiolate linkages into this DNA·RNA hybrid increased the thermal stability of the DNA·RNA duplex by 1–2 °C per modification depending on position.[9] We now report on a systematic investigation of thermal duplex stability in both DNA·DNA and DNA·RNA duplexes that contain multiple 3′-SP linkages and describe the effect of ionic strength on melting temperature.[10] The current study also examines the effect that the pattern of substitution (contiguous *versus* alternating) has on duplex stability and reveals some interesting differences in thermal melting behaviour between duplexes containing the 3′-*S*-phosphorothiolate linkage and the related 3′-*N*-phosphoramidate modification.

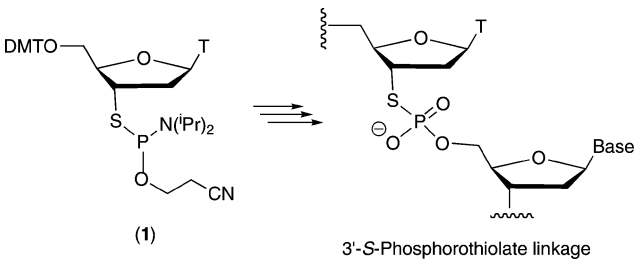

Fig. 1 The 3′-*S*-phosphorothiolate linkage prepared from a thymidine-3′-*S*-phosphorothioamidite monomer (**1**). T = thymin-1-yl, DMT = dimethoxytrityl.

[a]*Department of Chemistry, University of Liverpool, Liverpool, UK L69 7ZD. E-mail: rcosstic@liv.ac.uk; Fax: +44 (0)151 794 3588; Tel: +44 (0)151 794 3514*
[b]*School of Chemistry, University of Leeds, Leeds, UK LS2 9JT. E-mail: j.fisher@chem.leeds.ac.uk; Fax: +44 (0)113 343 6565; Tel: +44 (0)113 343 6577*

Results and discussion

Sequence choice and synthesis

Our previous thermal melting (T_m) studies were limited to the d(CCTAAATTTGCC)·r(GGCAAAUUUAGG) duplex in which only one or two modifications were introduced into the DNA strand.[9] This sequence has limited potential for the incorporation of 3′-*S*-phosphorothiolate linkages, which for synthetic reasons are most easily incorporated in association with pyrimidine nucleotides. In addition, it also contains an ApT step, which is known to induce a bend in the all DNA duplex and thus there was concern that the effects observed with the 3′-*S*-phosphorothiolate linkages could be specific for this sequence.[11] For the present studies, 3′-SP linkages were incorporated into the thymidine tract within the sequence d(GCGTTTTTTTTTTGCG). This strand is known to form duplexes with complementary DNA and RNA and both show well behaved, single-phase melting curves.[12]

Oligodeoxynucleotides containing 3′-SP linkages were prepared using a thymidine-3′-*S*-phosphorothioamidite monomer (**1**, Fig. 1) which was activated with 5-ethylthiotetrazole.[13] Using this procedure the coupling yield for the introduction of each phosphorothiolate linkage was about 92%, which was sufficient to prepare oligomers containing up to five 3′-SP modifications. The phosphorothioamidite monomer is known to be relatively unreactive in comparison to the standard phosphoramidites and this explains the low coupling yields.[7g,13a] Attempts to introduce more modifications resulted in complex mixtures of failure sequences, from which the desired oligomer could not be adequately purified. All oligodeoxyribonucleotides were purified by reverse-phase C-18 HPLC with the trityl group present. Following detritylation, oligodeoxyribonucleotides were characterised by electrospray (ES) mass spectrometry (see Table 3 in Experimental section).

Thermal melting studies

T_m values for DNA·RNA duplexes formed between the phosphorothiolate-substituted sequence d(GCGTTTTTTTTTTGCG) and the complementary RNA strand are shown in Table 1. As can be seen, in comparison to the unmodified duplex (duplex 1), incorporation of increasing numbers of phosphorothiolate linkages considerably stabilises the duplex. Thus, the increase in T_m (ΔT_m) observed for 5 modifications (duplexes 9 and 10) is almost 7 °C, equivalent to a ΔT_m modification of 1.4 °C.

As noted above, the unmodified DNA·RNA duplex (duplex 1) has the lowest T_m when compared to the 3′-SP-modified DNA·RNA duplexes (duplexes 2–6), and is therefore the least thermodynamically stable. By comparison with the helical conformations adopted by other unmodified DNA·RNA duplexes, it is expected that the DNA strand of this duplex would adopt a conformation intermediate between a B- and A-type helix, with a large proportion of the furanose sugars assuming a predominantly south pucker; whilst the RNA strand would adopt an A-type conformation with all of its sugars existing in a predominantly north pucker.[8,9,14] Based on our NMR study on the DNA·RNA duplex 5′-d(CCTAAATTTGCC)·3′-r(GGATTTAAACGG) (T = a thymidine associated with a 3′-SP linkage),[8,9] the incorporation of phosphorothiolate linkages in the DNA strand would be expected to steer the conformation of the sugar to which the sulfur is directly attached (*n* sugar), and the adjacent 3′-sugar (*n* + 1 sugar), towards a pure north and a predominantly north conformation, respectively. In addition, all of the remaining sugars in the DNA strand would be expected to adopt a predominantly south conformation, whilst the sugars in the RNA complement would exist in a predominantly north pucker. In this situation, the DNA strand of the duplex would adopt a more A-like conformation at the phosphorothiolate modification sites than would be observed for the equivalent region in the unmodified DNA·RNA duplex. As there is more extensive base–base overlap in the A-form helix,[15] improved intrastrand base stacking would most likely account for the enhanced stability of this duplex compared to the unmodified DNA·RNA duplex. This result is also expected based on a comparative study of the duplex stability of RNA·RNA and RNA·DNA duplexes.[16]

Comparisons can also be drawn between our current studies and those conducted with the more conformationally constrained 'locked nucleic acids' (LNA) system. When incorporated into a DNA strand, LNA residues increase thermal duplex stability with a complementary RNA strand and have a similar effect on the conformation of the furanose rings as the 3′-SP linkage.[17] A feature of the LNA–modified (in DNA strand)-RNA hybrids is a 'saturation' limit in the measured T_ms. That is, there is a reduction in the increase in T_m after a specific number of modifications have been introduced.[18,19] The helical thermostability per LNA residue was found to reach a maximum for sequences containing <50% LNA monomers.[19] Nonamers containing just three spaced LNAs (with their RNA complement) were found to adopt a near canonical A-type duplex hence the advantage of further

Table 1 T_m data for DNA·RNA duplexes formed with r(CGCAAAAAAAAAACGC). T indicates a thymidine associated with a 3′-SP linkage. See Experimental section for details

Duplex number	Number of modifications	Oligodeoxynucleotide	T_m/°C	ΔT_m/°C
1	0	GCGTTTTTTTTTTGCG	39.7	—
2	1	GCGTTTTTTTTTTGCG	40.7	1.0
3	2 alternating	GCGTTTTTTTTTTGCG	42.5	2.8
4	2 contiguous	GCGTTTTTTTTTTGCG	41.8	2.1
5	3 alternating	GCGTTTTTTTTTTGCG	43.6	3.9
6	3 contiguous	GCGTTTTTTTTTTGCG	43.0	3.3
7	4 alternating	GCGTTTTTTTTTTGCG	45.1	5.4
8	4 contiguous	GCGTTTTTTTTTTGCG	45.1	5.4
9	5 alternating	GCGTTTTTTTTTTGCG	46.5	6.8
10	5 contiguous	GCGTTTTTTTTTTGCG	46.5	6.8

substitutions is reduced. Thus it appears that once the target geometry has been achieved with its associated thermal stability subsequent additional chemical changes will have a diminishing effect. This feature seems to be present in the 3′-SP sequences studied here, whether the substitutions are alternating or contiguous; although the saturation point does not occur at the same substitution number. The difference between alternating and contiguous substitution patterns on T_m is most apparent at low levels of incorporation. When 2 or 3 phosphorothiolate linkages are present, the T_m for the alternating spacing shows a small increase (0.6–0.7 °C) over that of the contiguous arrangement (Table 1, compare duplexes 3 with 4 and 5 with 6). This is before the DNA strand adopts the fully A-helical conformation, and where a significant advantage is gained from spacing the modifications due to the occurrence of the 3′-transmitted conformational shift. Recently we have shown that it is actually disadvantageous to have contiguous modifications at low substitution levels.[20] NMR studies of dTTT (with contiguous 3′-SP linkages) indicated that the 3′-conformational shift was not transmitted to the third sugar ring, moreover base stacking was disrupted; whereas in dTTTG (with alternating modifications) all four sugars have significant north character.[21] The difference between alternating and contiguous at these low levels may therefore be attributed to the differing levels of 3′-transmission. Once 4 or 5 modifications are introduced no advantage is gained from the alternating arrangement (Table 1, compare duplexes 7 with 8 and 9 with 10). This may be a manifestation of the 'saturation' phenomenon noted above. Consequently in designing DNA-based systems to interact with RNA maximum benefit of the modified linkages is derived from having fewer modifications and having them situated alternately.

It is well established that nucleic acid duplexes are stabilised in the presence of salts due to cations masking the repulsive Coulombic interactions between the phosphates.[22] Three successful polyelectrolyte models have been developed to explain this behaviour; the counterion condensation theory,[23] the Poisson–Boltzmann theory[24] and the more recently proposed tightly bound ion model.[25] The effect of salt concentration on the stability of the DNA·RNA duplex was assessed by measuring T_m values over a range of salt concentrations and plotting T_m as a function of NaCl concentration (Fig. 2). No plot of T_m *versus* ln [Na^+] is presented as it has recently been shown that this relationship is nonlinear between 70 mM and 1.0 M.[26] As can be seen from the plot, increasing the salt concentration stabilises both the unmodified and phosphorothiolate duplexes to a similar extent at salt concentrations up to 0.1 M. At higher salt concentrations (0.5 M) the phosphorothiolate containing duplex is stabilised to a greater extent. This may reflect the fact that duplexes associated with the north sugar pucker (C3′-*endo*, A-type helix) have closer interphosphate distances (5.9 Å) than those that adopt the south pucker (C2′-*endo*, B-type helix) which is about ~7 Å,[27] and thus the shielding effect of Na^+ is likely to be greater for the phosphorothiolate containing duplex which has greater A-form helical character.

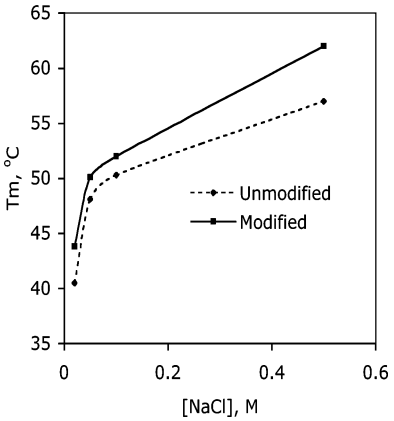

Fig. 2 T_m *versus* [NaCl] plotted for duplex 3 (see Table 2).

T_m values for DNA·DNA duplexes formed between the phosphorothiolate-substituted d(GCGTTTTTTTTTTGCG) strand and its unmodified DNA complement are shown in Table 2. In this case, increasing the number of phosphorothiolate linkages leads to a destabilisation of the duplex with an average ΔT_m of almost −1 °C per modification. The decrease in T_m is once again consistent with the phosphorothiolate linkages imparting A-type helical structure to the modified strand. The duplex therefore becomes conformationally similar to a RNA·DNA heteroduplex which have generally been shown to have a lower thermal stability than the analogous DNA·DNA B-form structure.[16]

It is also of interest to compare the effect of the 3′-*S*-phosphorothiolate linkage with that of the 3′-*N*-phosphoramidite (3′-NP) linkage, to which it is most closely related.[4] Both these modifications induce a predominantly N-type pucker of the furanose ring and in this respect can be considered to be conformational mimics of RNA. Previous studies have shown that the incorporation of 3′-NP linkage into the DNA strand of a DNA·RNA duplex enhanced the thermal stability by 2.3–2.6 °C per modification, in comparison to their phosphodiester counterparts.[28] The magnitude of this stabilising effect is greater than that observed here for the 3′-SP linkage, but a direct comparison is difficult due to differences in sequence and concentration.

Table 2 T_m data for DNA·DNA duplexes formed with d(CGCAAAAAAAAAACGC). T indicates a thymidine associated with a 3′-SP linkage. See Experimental section for details

Duplex number	Number of modifications	Complementary oligodeoxynucleotide	T_m/°C	ΔT_m/°C
11	0	GCGTTTTTTTTTTGCG	43.3	—
12	1	GCGTTTTTTTTTTGCG	42.6	−0.7
13	2	GCGTTTTTTTTTTGCG	41.9	−1.4
14	3	GCGTTTTTTTTTTGCG	40.0	−3.3
15	4	GCGTTTTTTTTTTGCG	39.0	−4.3
16	4	GCGTTTTTTTTTTGCG	39.9	−3.4
17	5	GCGTTTTTTTTTTGCG	38.6	−4.7

In the case of DNA·DNA duplexes, the effect of introducing 3′-NP linkages into one of the strands varied, depending on the pattern of substitution. Oligodeoxynucleotides with contiguous 3′-NP modifications stabilised duplex formation in comparison to the natural linkage, whilst an alternating arrangement had a destabilising effect.[28] In contrast, both arrangements of the 3′-SP linkages destabilised a DNA·DNA duplex, although the alternating pattern (duplex 15, Table 2) was slightly more destabilising than the contiguous arrangement (duplex 16, Table 2). Subtle variation in the properties of the phosphoramidate and phosphorothiolate oligonucleotides would be expected based on the fact that the two backbones are likely to differ in terms of rigidity and hydration. Structural analysis of a fully substituted 3′-NP duplex has revealed a conformational uniformity that is consistent with substantially increased backbone rigidity and is attributed to a strong anomeric effect between the 3′-nitrogen lone pair and the σ* orbital of the P–O5′ bond.[4] In contrast ^{31}P NMR measurements made as part of the conformational analyses of 3′-SP modified di- and tri-nucleotides are supportive of little or no alteration in the phosphate backbone (conformation or conformational stability) following the introduction of the sulfur modification.[19,29]

Conclusions

The incorporation of 3′-SP linkages into the DNA strand of a DNA·RNA hybrid duplex has been shown to thermodynamically stabilise duplex formation. For up to three modifications the stabilising effect is slightly greater for an alternating arrangement of SP linkages over contiguous pattern, but this advantage is lost once four or more modifications are introduced. The T_m studies presented here on the DNA·RNA duplexes, when taken in conjunction with our previous studies,[9] suggest that duplex stabilisation by the 3′-SP linkage is independent of sequence. However, studies on more heterogeneous systems will be required to confirm this.

In the case of a DNA·DNA duplex, phosphorothiolate substitution reduces duplex stability and, unlike the 3′-NP modification, this effect is independent of the pattern of substitution. Whilst the 3′-SP linkage clearly has some similarities with the 3′-NP linkage, there are subtle variations which probably result from differences between the two analogues in hydration and rigidity. In terms of its effect on duplex stability and sugar conformation, DNA containing phosphorothiolate linkages is a very good mimic of RNA and suggests that this modification may be of interest in RNA interference.

Experimental

General

Oligodeoxynucleotide synthesis was performed on an Expedite™ 8909 DNA synthesizer equipped with standard Expedite™ Workstation Software. RNA was prepared on an ABI 391 PCR-mate oligonucleotide synthesiser. All standard reagents for oligonucleotide synthesis were purchased from either Applied Biosystems or Glen Research. 5-Ethyl-1*H*-thiotetrazole was purchased from Glen Research. Reverse-phase HPLC was performed on a Gilson HPLC system equipped with an autoinjector, a photodiode array detector and a dual hydraulic pump. Chromatographic data were handled using UniPoint software version 3.0. Separations were carried out using a 250 mm × 4.60 mm, 5 μ Gemini C18 column supplied by Phenomenex®. Oligonucleotides were characterised on a Micromass LCT mass spectrometer using negative mode electrospray ionization and direct infusion syringe pump sampling.

Oligonucleotide synthesis

All oligodeoxynucleotides (ODNs) were prepared on a 1 μmol scale with the trityl group retained and the standard instrument protocols were used for the unmodified oligonucleotides. Phosphorothiolate linkages were introduced using the thymidine 3′-*S*-phosphorothioamidite[13] (0.15 M) loaded at position 5 of the synthesiser and the activator 5-ethyl-1*H*-thiotetrazole (1 M) supplied from position 7; the coupling time was 15 min. Standard reagents and protocols were used for the oxidation, capping and detritylation steps. Once synthesis was complete the reaction column was removed and dried by passage of nitrogen. The crude DMT-protected ODNs were obtained by treating the solid support with concentrated aqueous ammonia (1 mL) for 48 h at room temperature in a sealed Wheaton vial. DMT-protected ODNs were concentrated in a vacuum centrifuge, redissolved in triethylammonium bicarbonate (TEAB, 0.1 M) and purified by reverse-phase HPLC using a gradient of 0–40% acetonitrile in TEAB (0.1 M) over 25 min. The DMT group was removed as previously described.[13] ODNs that were less than 98% pure, as determined by HPLC, were repurified by reverse-phase HPLC using a gradient of 0–20% acetonitrile in TEAB (0.1 M) over 35 min. All oligonucleotides were characterised by mass spectrometry (see Table 3).

RNA synthesis followed a similar procedure to that used for the DNA sequences, however the coupling time was extended to 20 min. Deprotection of the support-bound oligonucleotide was achieved using freshly prepared saturated methanolic ammonia (1.5 mL) in a Wheaton screw top vial incubated at 30 °C for 24 h. Solutions were evaporated under a stream of nitrogen gas. Further deprotection steps to desilylate involved dissolving samples in 300 μL of anhydrous DMSO and 300 μL of $NEt_3 \cdot 3HF$ and incubating at 30 °C for a further 18 h. The solution was then quenched with a few drops of ddH_2O. Anion exchange chromatography was initially used to purify the RNA. A linear gradient of 0–80% 1 M NH_4Cl was employed for 20 min.

Table 3 ESI mass spectrometry data acquired for oligonucleotide sequences using negative mode ionisation. Samples were prepared in aqueous methanol + 0.1% diethylamine. T indicates 3′-thiothymidine

Oligonucleotide	Measured mass	Calculated Mass
d(GCGTTTTTTTTTGCG)	4875.091	4875.204
d(CGCAAAAAAAAAACGC)	4885.094	4885.290
d(GCGTTTTTTTTTGCG)	4891.169	4891.271
d(GCGTTTTTTTTTGCG)	4907.814	4907.337
d(GCGTTTTTTTTTGCG)	4922.509	4923.404
d(GCGTTTTTTTTTGCG)	4940.426	4939.470
d(GCGTTTTTTTTTGCG)	4955.542	4955.537
d(GCGTTTTTTTTTGCG)	4905.947	4907.337
d(GCGTTTTTTTTTGCG)	4922.625	4923.404
d(GCGTTTTTTTTTGCG)	4939.950	4939.470
d(GCGTTTTTTTTTGCG)	4955.598	4955.537
r(CGCAAAAAAAAAACGC)	5140.272	5141.280

Subsequently the material was further purified by reverse-phase HPLC with 100% ammonium acetate and 100% acetonitrile buffers; a gradient of 0–10% acetonitrile was used over 20 min. Samples were desalted using gel size exclusion columns.

UV thermal melting

UV thermal melting studies were performed using a Hewlett Packard 8452A diode array spectrophotometer equipped with a Peltier device. Both spectrophotometer and Peltier unit were controlled by a PC using software provided by HP. Absorbance was followed at 260 nm. The sample temperature was increased in half degree increments from 19 to 90 °C, with a five minute equilibration period before each reading. T_m Values were measured as the maximum of the first derivative of the melting curve (A_{260} *versus* temperature). Unless stated otherwise, samples were dissolved in 10 mM phosphate buffer, pH 7.0 and single strand concentrations were 1.5 μM.

Acknowledgements

The work was financially supported by the BBSRC (B18146) to JAB and an EPSRC studentship to JB. We would like to thank Alan Mills (Liverpool) for obtaining mass spectra.

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