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Thermal stabilisation of RNA·RNA duplexes and G-quadruplexes by phosphorothiolate linkages†‡

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The effect of 3′-*S*-phosphorothiolate linkages on the stability of RNA·RNA duplexes and G-quadruplex structures has been studied. 3′-Thio-2′-deoxyuridine was incorporated into RNA duplexes and thermal melting studies revealed that the resulting 3′-*S*-phosphorothiolate linkages increased the stability of the duplex to thermal denaturation. Additionally, and contrary to expectation, a similar effect on duplex stability was observed when the same thionucleoside was incorporated into the RNA strand of a RNA·DNA duplex. A suitably protected derivative of 3′-thio-2′-deoxyguanosine was prepared using an oxidation–reduction strategy and this residue also increased the thermal stability the $[d(TGGGGT)]_4$ G-quadruplex when positioned centrally. The results are discussed in terms of the influence that the sulfur atom has on the conformation of the furanose ring and imply that the previously noted high thermal stability of parallel RNA quadruplexes is not derived from H-bonding interactions of the 2′-hydroxyl group, but can be attributed to conformational effects.

Introduction

The 3′-phosphorothiolate linkage (3′-SP, **1**, Fig. 1), in which the 3′-oxygen atom of the phosphodiester bond is replaced by sulfur, can formerly be considered as the result of incorporating a 3′-thionucleoside into nucleic acids.[1] This modification has proved to be an extremely useful probe for studying metal ion-dependent phosphoryl-transfer. In particular it has contributed considerably to our understanding of the mechanism of RNA cleavage by the group I[2] and group II[3] introns and the splicesome.[4]

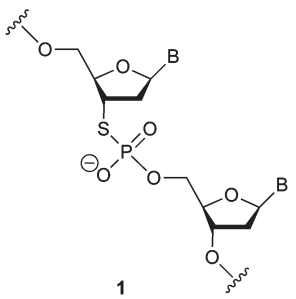

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Fig. 1 3′-*S*-Phosphorothiolate linkage.

When incorporated into DNA this modification has also been valuable in characterizing the mechanism of action of nucleic acid processing enzymes as exemplified by restriction endonuclease BfiI,[5] and the Klenow fragment of DNA polymerase.[6] Interestingly, incorporating a 3′-SP linkage into DNA shifts the conformation of the sugar to which the sulfur is attached to the north pucker (C3′-*endo*), a conformation that is usually associated with standard A-form RNA.[7] As a consequence of this conformational effect, the incorporation of 3′-SP linkages into a DNA strand increases the thermal stability of a DNA·RNA duplexes, but destabilises DNA·DNA duplexes.[8] The conformational preference of this modification to adopt the C3′-*endo* sugar pucker also appears to be responsible for its ability to stabilise the 4-stranded i-motif structure,[9] which is found in a number of gene promoter sequences.[10]

It is evident that 3′-SP linkage acts as a RNA mimic even when it is associated with a deoxyribose sugars; it thus raises the question as to whether this modification could also stabilise other nucleic acids structures which have a preference to adopt the C3′-*endo* sugar pucker. In particular, two structures were of interest: RNA·RNA duplexes and the G-quadruplex. The stabilisation of RNA duplexes (both thermally and in terms of improving resistance to nucleases) is important in generating highly active short interfering RNAs (siRNA). However, in order to achieve efficient processing of the siRNA into the

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†Electronic supplementary information (ESI) available: Details for the synthesis of 5′-*O*-dimethoxytrityl-2′-deoxy-3′-thiouridine phosphor-amidite; oligonucleotide synthesis; HPLC and thermal melting studies. See DOI: 10.1039/c2ob26940f

‡In memory of Professor Har Gobind Khorana who was a PhD graduate (1948) at the University of Liverpool.

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ribonucleoprotein complex, known as the RNA-induced silencing complex (RISC), stabilisation has to be achieved with minimum perturbation to the standard A-form RNA duplex.[11] In this respect, the advantageous conformational properties of the 3′-SP modification are achieved without the introduction of any steric encumbrance that is likely to interfere with either the assembly or function of the RISC complex. In addition, as the 3′-SP linkage is used in a DNA context, it is likely to prove resistant to most RNases.

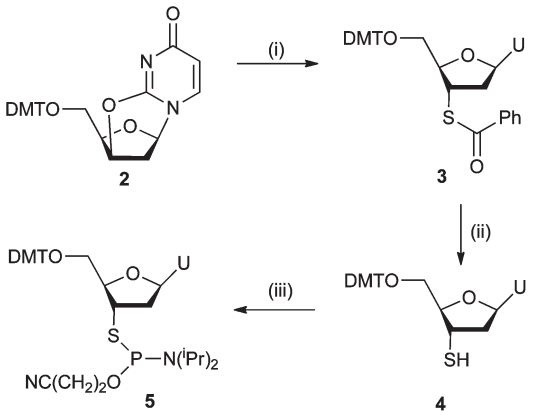
G-quadruplexes are four stranded structures and are formed because of the capacity of guanine bases to self-associate through Hoogsteen hydrogen bonds, resulting in stacked G-quartets.[12] Potential quadruplex structures have been associated with the G-rich sequences of telomeres[13] and are also linked with gene promoter regions.[14] Interestingly, parallel tetrameric G-quadruplexes derived from ribonucleosides [*e.g.* r(UGGGGU)$_4$] have been shown to be thermally more stable than those composed of 2′-deoxyribonucleosides [d(TGGGGT)$_4$],[15,16] although it is not entirely clear as to whether the stabilisation stems solely from conformational effects or whether hydrogen bonding interactions involving the 2′-hydroxyl groups also contribute.[17]

Previous work on the incorporation of phosphorothiolate linkages into DNA has been limited to the pyrimidine nucleosides.[18] In order to synthesise G-quadruplex sequences, it is necessary to prepare a suitably protected derivative of 3′-thio-2′-deoxyguanosine. We now report the synthesis of RNA duplexes containing 3′-thio-2′-deoxyuridine and show that this modification thermally stabilises RNA duplexes. In addition 3′-thio-2′-deoxyguanosine has been incorporated into DNA strands and is demonstrated to stabilise G-quadruplex structures.

Results and discussion

Synthesis of 3′-thio-2′-deoxyuridine for incorporation into RNA

As outlined above, the incorporation of 3′-SP linkages derived from deoxyribose sugars was expected to confer favourable conformational properties and nuclease resistance to RNA with minimal alteration to the overall structure. 3′-Thio-2′-deoxyuridine is the synthetically most convenient "RNA-compatible" deoxyribonucleoside for this purpose. Although this nucleoside has previously been incorporated into RNA substrates to study RNA splicing,[2a,4] no details of the synthesis of the required deoxyuridine monomer have previously been reported. The synthesis of the 5′-*O*-dimethoxytrityl-2′-deoxy-3′-thiouridine phosphoramidite (**5**) is shown in Scheme 1 and it follows the route previously described for the analogous thymidine derivative.[18] (Full experimental details are available in the ESI.†) Interestingly, opening of the anhydronucleoside (**2**) to give the benzothioate ester (**3**) can be done either using pre-prepared sodium thiobenzoate (40–74% yield), as previously reported,[18] or by using thiobenzoic acid in pyridine (48%). The latter procedure is more straightforward and gives much more consistent yields.

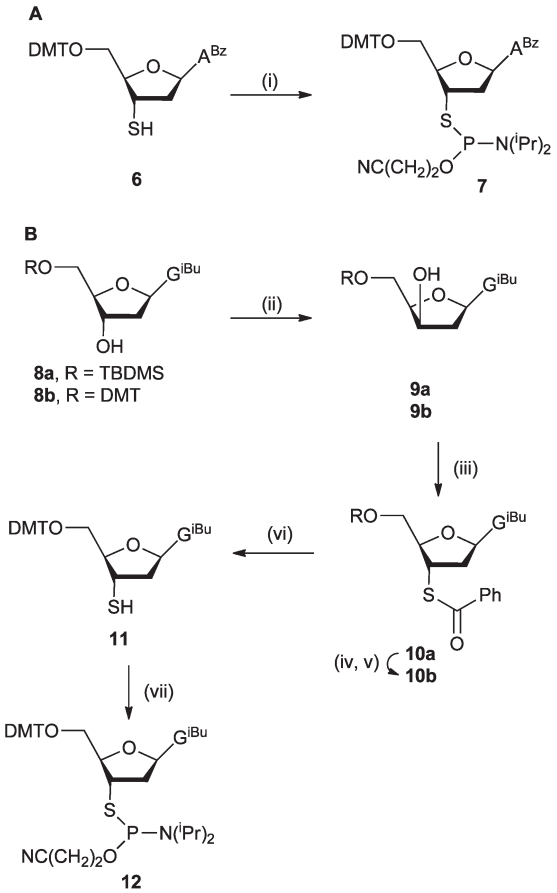


Scheme 1 Synthesis of 3′-thio-2′-deoxyuridine monomer. *Reagents and conditions*: (i) NaSBz, DMAc, 110 °C, 40–74% or BzSH, pyridine, 110 °C, 48%; (ii) NaOH, H_2O/EtOH, 92%; (iii) P(NiPr$_2$)$_2$(OCH$_2$CH$_2$CN), 1*H*-tetrazole, MeCN, 72%. Full experimental details are available in the ESI.†

Synthesis of 3′-thio-2′-deoxyguanosine for incorporation into DNA

Whilst the synthesis of pyrimidine 3′-thionucleosides is readily achieved through the use of anhydronucleoside intermediates to control stereochemistry at the C3′ position, this route is not available for their purine counterparts. There are a number of methods for epimerising the C3′ of purine nucleosides and we have previously used Herdewijn's method for preparing protected derivatives of 2′-deoxy-3′-thioadenosine (**6**, Scheme 2A).[19] However, we were attracted to the recently reported *in situ* oxidation–reduction procedure of Eisenhuth and Richert for the preparation of *xylo*-purines.[20] Thus, a 5′-TBDMS-protected derivative of deoxyguanosine (**8a**, Scheme 2B) was converted to the corresponding *xylo* derivative (**9a**, 79% yield) using this oxidation–reduction strategy. Introduction of the masked thiol was accomplished in 65% through a Mitsunobu reaction using thiobenzoic acid to give the benzothioate ester and exchange of the TBDMS group for the dimethoxytrityl group was achieved in 85% for these two further steps.

In an attempt to avoid the protecting group exchange, the oxidation–reduction procedure was also performed directly on the dimethoxytritylated substrate (**8b**), but in this case the *xylo* derivative (**9b**) was obtained in only 20% yield. Likewise, a Mitsunobu reaction on **9b** gave the DMT-protected benzothioate ester (**10b**) in very low yield (5%). In both cases the yields were reduced largely due to loss of the DMT group. The DMT-protected thioester (**10b**) was subsequently hydrolysed to give the protected 3′-thio-2′-deoxyguanosine **11** under conditions similar to those previously used for hydrolysis of the corresponding cytidine derivative.[18] Finally, phosphitylation of **11** gave the required 3′-thio-2′-deoxyguanosine phosphorothioamidite (**12**) in an overall yield of 21% starting from deoxyguanosine. In addition, the phosphitylation 2′-deoxy-3′-thioadenosine[19] (**6**) was also accomplished under similar conditions to give the phosphorothioamidite (**7**).



Scheme 2 Synthesis of 3′-thio-2′-deoxyguanosine and 3′-thio-2′-deoxyadenosine monomers. *Reagents and conditions*: (i) $P(NiPr_2)_2(OCH_2CH_2CN)$, 5-ethylmercapto-1*H*-tetrazole, CH_2Cl_2, 82%; (ii) Dess–Martin periodinane, CH_2Cl_2, 0 °C to 25 °C, then $NaBH_4$, −60 °C, 79% and 20% yield for **9a** and **9b**, respectively; (iii) PPh_3, diisopropyl azodicarboxylate, BzSH, THF, 0 °C, 65% and 5% yield for **10a** and **10b**, respectively; (iv) AcOH, THF, TBAF; (v) DMTCl, py, 85% over (iv) and (v); (vi) NaOH, THF/MeOH/H_2O; (vii) $P(N^iPr_2)_2(OCH_2CH_2CN)$, 5-ethylmercapto-1*H*-tetrazole, CH_2Cl_2, 81% yield over (vi) and (vii).

Table 1 List of RNA sequences, together with their theoretical and experimental average masses

		Average masses	
ORN sequence	ORN no	Theoretical	Expt
r(GCGUUUUUUUUUGCG)	1	4990.93	4990.65
r(GCGUUUUUsUUUUUGCG)	2	4990.99	4991.03
r(GCGUUUUsUsUUUUUGCG)	3	4991.06	4990.77
r(GCG UUUUsUsUsUUUUGCG)	4	4991.13	4990.77
r(CGCAAAAAAAAAACGC)	—	5141.28	5141.29

All sequences written 5′–3′. Us = indicates a 3′-SP linkage associated with a 2′-deoxy-3′-thiouridine residue.

Synthesis and characterisation of oligoribonucleotides

The sequence of the RNA strand chosen for incorporation of the 2′-deoxy-3′-thiouridine is shown in Table 1. It is based on an equivalent DNA sequence that has previously been used to study the incorporation of phosphorothiolate linkages into DNA and in that context was previously shown to give smooth thermal melting curves.[8] Oligoribonucleotides were prepared using 2′-*O*-TBDMS-protected monomers and 5-ethylmercapto-1*H*-tetrazole (ETT) (0.25 M) as the activator. For incorporation of the 2′-deoxy-3′-thiouridine phosphorothioamidite (**5**) the concentration of ETT was increased to 1 M and a 15 min coupling was used.[18] Oligoribonucleotides were synthesised without the 5′-terminal DMT group attached and following removal of the 2′-*O*-TBDMS groups, the oligoribonucleotides were purified by ion-exchange HPLC (see Table 1 for sequences prepared).

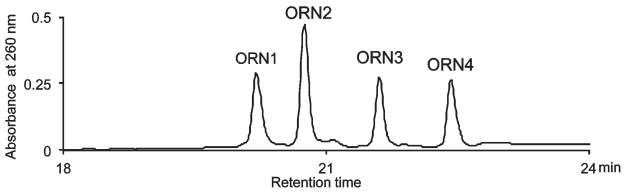


Fig. 2 Reverse-phase HPLC trace for a mixture of ORNs 1–4 (see Table 1) showing the retention time increasing with increasing number of phosphorothiolate linkages. HPLC elution gradient = 0–16% MeCN over 30 min in 0.1 M triethylammonium bicarbonate (pH 7.6).

The oligoribonucleotides (ORNs) were characterised by electrospray mass spectrometry and their purity established by reverse-phase HPLC. The incorporation of 3′-thio-2′-deoxyuridine into the oligoribonucleotides increased hydrophobicity as clearly indicated by the increasing retention times on reverse-phase HPLC as more phosphorothiolate linkages are incorporated (Fig. 2).

Synthesis and characterisation of oligodeoxynucleotides

Oligodeoxyribonucleotides containing phosphorothiolate linkages were prepared as previously described, with the 5′-terminal DMT group attached and they were purified by reverse-phase HPLC (see Table 2 for sequences prepared).[18] As previously noted, the HPLC traces for the hexamer sequences all showed slower eluting peaks corresponding to the G-quadruplex structure.[21]

Table 2 List of DNA sequences, together with the theoretical and experimental average masses

	Average masses	
ODN sequence	Theoretical	Experimental
5′-d(CGCAAAAAAAAAACGC)	4885.24	4884.72
5′-d(CGCAAAAAsAsAsAAACGC)	4933.44	4932.88
5′-d(TGGGGT)	1863.35	1862.42
5′-d(TGsGGGT)	1879.31	1878.42
5′-d(TGGsGGT)	1879.31	1878.42
5′-d(TGGGsGT)	1879.31	1878.37
5′-d(TGGGGsT)	1879.31	1878.27

As or Gs indicates a 3′-SP linkage associated with either 2′-deoxy-3′-thioadenosine or 2′-deoxy-3′-thioguanosine, respectively.

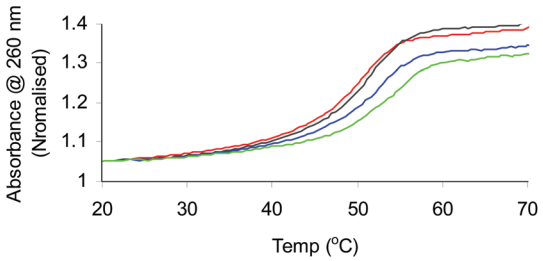


Fig. 3 Melting curves for RNA duplexes 1–4 (see Table 3). Melting studies were performed on samples containing 1.5 μM duplex in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl. Duplex 1 = red, duplex 2 = black, duplex 3 = blue, duplex 4 = green.

Table 3 T_m data for RNA·RNA duplexes (duplexes 1–4) and DNA·RNA duplexes (duplexes 5–8)

Duplex number	Sequence	T_m (°C)	ΔT_m (°C)
1	5′-r(GCG UUU UUU UUU UGC G) 3′-r(CGC AAA AAA AAA ACG C)	50.9	—
2	5′-r(GCG UUU UUsU UUU UGC G) 3′-r(CGC AAA AA A AAA ACG C)	51.9	1.0[a]
3	5′-r(GCG UUU UsUsU UUU UGC G) 3′-r(CGC AAA A A A AAA ACG C)	53.2	2.3
4	5′-r(GCG UUU UsUsUs UUU UGC G) 3′-r(CGC AAA A A A AAA ACG C)	54.3	3.4
5	5′-r(GCG UUU UUU UUU UGC G) 3′-d(CGC AAA AAA AAA ACG C)	30.0	—
6	5′-r(GCG UUU U U U UUU UGC G) 3′-d(CGC AAA AsAsAs AAA ACG C)	35.9	5.9
7	5′-r(GCG UUU UsUsUs UUU UGC G) 3′-d(CGC AAA A A A AAA ACG C)	36.5	6.5
8	5′-r(GCG UUU UsUsUs UUU UGC G) 3′-d(CGC AAA AsAsAs AAA ACG C)	41.1	11.1

Us or As indicates a 3′-SP linkage associated with either 2′-deoxy-3′-thiouridine or 2′-deoxy-3′-thioadenosine, respectively. Samples were prepared as described in the legend to Fig. 3. [a] Whilst a ΔT_m of 1 °C is at the limits of experimental error, it is consistent with the general trend, which is revealed more clearly in the more substituted duplexes.

Thermal melting studies on RNA·RNA and RNA·DNA duplexes

All RNA·RNA duplexes produced melting curves with sharp monophasic transitions (Fig. 3) and show a shift to higher T_m values for increasing numbers of 3′-SP linkages incorporated (approximately 1 °C increase per modification, see Table 3). NMR studies on simple dinucleotides have previously shown[22] that 3′-thio-2′-deoxyribose has a greater tendency to adopt the 3′-*endo* conformation than the corresponding ribose sugar and in this respect incorporation of the 3′-SP linkages would be expected to provide greater preorganisation of the modified strand for binding to its RNA complement; this is likely to be the cause of the increased T_m values for duplexes 2, 3 and 4. An interesting observation is the trend in decreasing hypochromicity with increasing number of 3′-SP modifications (Fig. 3). A drop in hypochromicity implies a reduction in base stacking interactions which is an important factor in the overall stability of the duplex. As noted above, it is expected that the deoxyribose sugars associated with the 3′-SP linkages should favour the 3′-*endo* conformation, thus increasing base stacking interactions. However, other factors are likely to affect the structure. For example in RNA duplexes, the 2′-hydroxyl groups line the minor groove and show a hydration pattern similar to that of the negatively charged phosphate group.[23] This significantly contributes to the thermodynamic stability of RNA. By increasing the number of 3′-thio-2′-deoxyuridine residues in the RNA duplex, the number of 2′-OH groups in the minor groove is correspondingly reduced resulting in a drop of hydration. In the absence of additional information it is difficult to predict the how the reduced hydration at the central base pairs will affect the duplex structure and stability.

Interesting results were obtained for the thermal melting studies performed on DNA·RNA duplexes between the 5′-r(GCGUUUUUUUUUGCG) and the complementary DNA strand (duplexes 5–8, Table 3). In DNA·RNA duplexes, the RNA strand has been shown to adopt the A-type conformation, whilst in the DNA strand the nucleotide conformations are closer to that of B-DNA and globally, these hybrid duplexes show closer resemblance to the A-form rather than the B-form.[24] As expected, based on the previously discussed conformational preference of the 3′-thiosugar, incorporation of 3′-SP linkages into the DNA strand of an RNA·DNA duplex increased the T_m by about 2.0 °C per modification (duplex 6, Table 3). More surprisingly, a similar increase in T_m was observed when the 3′-SP linkages were incorporated into the RNA strand (duplex 7). This was contrary to expectation as in this case the 3′-SP modification was predicted to enhance the mismatch in sugar conformations between the two strands and thereby decrease the T_m. When 3′-SP linkages were incorporated into both strands and in direct opposition (duplex 8), the T_m was increased by just over 11 °C (ΔT_m = 1.8 °C per modification). Thus, the 3′-SP linkages increase the thermal stability of a RNA·DNA duplex independent of which strand is modified. The reason for this is unclear, although previous studies have shown that the global structure of a RNA·DNA duplex in which the RNA strand is pyrimidine rich (as in the case of these duplexes) has less A-type helical character and is thermodynamically less stable than when the RNA strand is purine rich.[25] This may mean that for the duplex under investigation here, the incorporation of 3′-SP linkages into either strand globally increases the A-type character of the duplex and this results in the higher T_m values. In this respect it would be interesting to examine phosphorothiolate substitution into an analogous duplex in which the RNA strand was purine rich.

Spectrophotomeric studies on G-quadruplex structures

Tetrameric G-quadruplex structures are well suited to analogue replacement studies and in particular [5′-d(TGGGGT)]$_4$ and simple variants of this quadruplex, have previously been investigated.[15–17] For this study, formation of G-quadruplexes was confirmed by a thermal difference spectrum (TDS), which is simply the difference between UV spectra recorded for the unfolded and folded states. The base stacking interactions

Table 4 $T_{1/2}$ data for G-quadruplexes. Gs indicates a 3′-SP linkage associated with 2′-deoxy-3′-thioguanosine

Quadruplex no	G-quadruplex	$T_{1/2}$/°C	$\Delta T_{1/2}$/°C
9	$[5'\text{-d(TGGGGT)}]_4$	61.1	N/A
10	$[5'\text{-d(TGsGGGT)}]_4$	60.1	−1.0
11	$[5'\text{-d(TGGsGGT)}]_4$	70.0	8.9
12	$[5'\text{-d(TGGGsGT)}]_4$	68.7	7.6
13	$[5'\text{-d(TGGGGsT)}]_4$	61.2	0.1

Melting studies were performed on samples containing 10 μM single strand in sodium cacodylate buffer (10 mM, pH 7.0) containing 100 mM NaCl. The heating rate was 0.5 °C min^{-1}.

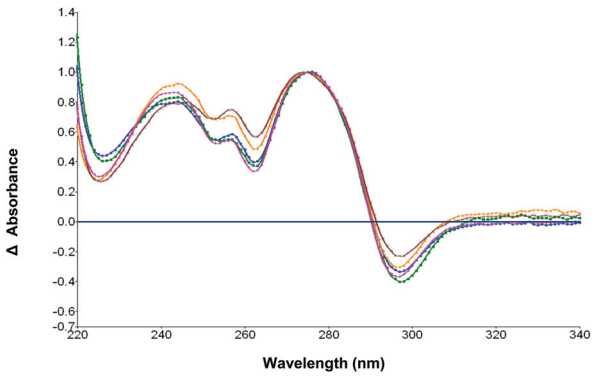


Fig. 4 Thermal difference spectra in the 220–340 nm region for the five quadruplexes studied (9–13, Table 4). Pink = quadruplex 9; green = quadruplex 10; brown = quadruplex 11; blue = quadruplex 12 and orange = quadruplex 13. Thermal difference spectra were performed on samples containing 10 μM single strand in sodium cacodylate buffer (10 mM, pH 7.0) containing 100 mM NaCl.

present in each type of nucleic acid structure is reflected in the TDS and it thus has a specific shape which is unique to that particular structure.[26] The TDS for the unmodified and the 3′-SP substituted quadruplexes (sequences shown in Table 4) all showed positive (~243 and 273 nm) and negative (~295 nm) peaks that are characteristic of a G-quadruplex and the global shape of all the spectra was very similar (Fig. 4).[26]

The results of thermal melting studies performed on the $[5'\text{-d(TGGGGT)}]_4$ quadruplexes containing 3′-SP linkages are shown in Table 4. The measured T_m for a G-quadruplex generally depends on the heating rate and is not therefore the true thermodynamic T_m; this apparent T_m is often referred to as $T_{1/2}$.[16,27] A sequence specific effect on the $T_{1/2}$ of the modified quadruplexes is evident from the data and a significant enhancement in the $T_{1/2}$ is observed when the single modified residue is located at either of the central positions (Table 4, quadruplexes 11 and 12). In comparison, the effect is much smaller when either of the terminal dG residues is modified and a marginal destabilising effect results from incorporation at the 5′-dG residue (quadruplex 10).

Previous studies have shown that DNA tetrameric quadruplexes such as $[d(TGGGGT)]_4$ and its RNA equivalent $[r(UGGGGU)]_4$ adopt similar structures; both have a parallel arrangement of the four strands with all nucleosides in the anti conformation.[15,28] There are however, some differences with regard to the sugar conformations, with the DNA quadruplexes adopting the C2′-*endo* pucker whereas the RNA equivalent adopts a mixture of C2′ and C3′-*endo* conformations.[15,29] Parallel RNA tetrameric quadruplexes are known to be considerably more thermally stable than their DNA counterparts. This is a result of both a much faster association and slower dissociation for the RNA quadruplexes.[16] A potential explanation for the difference between these RNA and DNA quadruplexes relates to the effect that the sugar conformation has on the *syn*/*anti* equilibrium; the *anti*-conformation, which favours parallel quadruplexes, is more accessible from the C3′-*endo* pucker.[30] The results presented here are also consistent with this interpretation, given that a 3′-SP linkage acts as a conformational mimic of a ribonucleoside. Additionally, it has been established that the analogous, fully LNA-modified tetramolecular quadruplexes, in which the sugar is locked into the C3′-*endo* pucker, also shows a similar stabilisation effect.[17] There has been speculation that stabilisation of the tetrameric G-quadruplexes by 2′-OH and 2′-OMe substituents,[31] as well as LNA residues,[17] could be due to the ability of these modifications to participate in H-bonding interactions. However, the results reported here, with a modification that has no H-bonding functionality at the 2′-position, suggest that H-bonding interactions do not play a major role in stabilising the structure.

As already noted above, the current results show that incorporation of the 3′-SP linkage into the terminal dG residues has only a small effect on quadruplex stability (quadruplexes 10 and 13). This positional effect is consistent with NMR studies which show that these outer tetrads are more flexible than those in the centre and are therefore less likely to contribute to the stability of the structure as a whole.[15,32] The marginal drop in $T_{1/2}$ observed here for incorporation of the phosphorothiolate linkage at the 5′-terminal G residue (quadruplex 10, Table 4) can also be rationalised based on previous studies. It has been shown that $T_{1/2}$ is increased when guanosine analogues that preferentially adopt the *syn*-conformation are incorporated at the 5′-terminal G residue of a tetrameric quadruplex, suggesting that a 5′-*syn* residue might, in fact, stabilise the structure.[33] In this case the lower $T_{1/2}$ for quadruplex 10 is likely to result from the *syn* conformation being less accessible due to the C3′-*endo* sugar pucker induced by the 3′-SP linkage.

Conclusions

Previous studies have shown that 3′-*S*-phosphorothiolate linkages are able to stabilise RNA·DNA duplexes[8] (when the modification is in the DNA strand) and the I-motif.[9] The current study now reveals that both RNA·RNA and G-quadruplex structures are also thermally stabilised by the 3′-SP linkage. In addition, it is now revealed that the 3′-SP linkage stabilises RNA·DNA duplexes irrespective of which strand contains the modification. Thus of all the structures so far investigated it is only DNA·DNA duplexes that are thermally destabilised by

3′-SP linkages.[8] In all cases this stabilisation appears to stem from the influence that the sulfur atom has on the conformation of the furanose ring. In particular, results presented here for the G-quadruplexes imply that the high thermal stability of parallel RNA quadruplexes is not derived from H-bonding interactions of the 2′-hydroxyl group, but can be attributed to conformational effects.

Experimental

General

Unless otherwise stated, all general reagents were purchased from either Aldrich or BDH and used as supplied. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite was purchased from Chemgenes. Solvents were purchased from BDH or Fisher. HPLC grade acetonitrile and triethylamine were used to prepare RP-HPLC buffers. *Milli-Q* water and HPLC methanol were used to prepare ion-exchange HPLC buffers. 5-Ethylmercapto-1*H*-tetrazole and all reagents for DNA/RNA synthesis were supplied by Link Technologies. DNA synthesis grade acetonitrile was used to prepare phosphoroamidite and activator solutions. Analytical thin layer chromatography was performed on UV_{254} sensitive, silica gel 60 coated, aluminium tlc plates purchased from Merck. Ellman's reagent was used to distinguish compounds containing a 3′-thiol group.[19] Flash column chromatography was performed using silica (particle size 40–63 μM, supplied by BDH).

All NMR spectra were recorded on a Bruker 400 MHz spectrometer; operating at 400 MHz for ^{1}H, 101 MHz for ^{13}C NMR and 162 MHz for ^{31}P. All spectra were recorded in either d_6-DMSO or $CDCl_3$, relative to an internal standard of tetramethylsilane (^{1}H NMR and ^{13}C) or an external standard of 85% H_3PO_4 (^{31}P). All chemical shifts are reported in ppm and coupling constants (*J*) in Hertz. ^{13}C and ^{31}P spectra were ^{1}H decoupled and were singlets unless otherwise stated. Standard nucleoside numbering systems have been used with additional abbreviations as follows: Ar_2 = 4-methoxyphenyl on DMT; Ph = phenyl on DMT, OCH_2 = cyanoethyl. Mass spectra were recorded on a MicroMass LCT mass spectrometer using electrospray ionisation (EI) and direct infusion syringe pump sampling. All mass spectra were visualised as a series of multicharged ions.

For details regarding DNA/RNA synthesis, HPLC chromatography and thermal melting studies see ESI.† Details of the sample preparation for thermal melting studies are given in the legends to the figures and tables.

***N*2-Isobutyryl-5′-*O*-*tert*-butyldimethylsilyl-2′-deoxyxylo-guanosine (9a).** A solution of *N*2-isobutyryl-5′-*O*-*tert*-butyldimethylsilyl-2′-deoxyguanosine[20] (**8a**, 4.81 g, 10.65 mmol) dissolved in anhydrous CH_2Cl_2 (20 mL) was slowly added over 10 min to a chilled to 0 °C solution of Dess–Martin periodinane in CH_2Cl_2 (15% wt, 45.2 mL, 15.98 mmol) under N_2. The reaction was allowed to stir at 0 °C for 1 hour. The cooling bath was then removed and the reaction let stir at room temperature for 4 further hours. 2-Propanol (35 mL) was next added and the resulting white slurry cooled to −60 °C. Freshly powdered $NaBH_4$ (0.81 g, 21.3 mmol) was subsequently added and the mixture allowed to stir at −60 °C overnight. Acetone (35 mL) was next added and the slurry was allowed to warm to room temperature. Half of the volume of solvent was removed by evaporation before the mixture was taken up in EtOAc (200 mL) and washed successively with equal volumes of H_2O, $NaHCO_3$ and brine. The collected organic layer was then dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude product as an off-white solid. Purification by flash column chromatography, eluting with a gradient of 1% MeOH in $CHCl_3$–3% MeOH in $CHCl_3$, yielded the title compound as a white amorphous solid (3.80 g, 8.41 mmol, 79%).

^{1}H NMR (400 MHz, DMSO) δ 11.69 (br s, 2 H, NH), 8.14 (s, 1 H, H8), 6.12 (dd, J = 1.5, 8.0 Hz, 1 H, H1′), 5.35 (d, J = 3.8 Hz, 1 H, 3′-OH), 4.37–4.32 (m, 1 H, H3′), 4.00–3.88 (m, 2 H, H4′, H5′), 3.79–3.71 (m, 1 H, H5′), 2.80–2.62 (m, 2 H, C*H*-$(CH_3)_2$, H2′), 2.25–2.18 (m, 1 H, H2′), 1.09 (d, J = 6.8 Hz, 6 H, $CH(CH_3)_2$), 0.83 (s, 9 H, $C(CH_3)_3$), 0.00 (s, 6 H, $Si(CH_3)_2$. ^{13}C NMR (101 MHz, DMSO) δ 180.5 (NHibu C═O), 155.2 (C6), 148.6 and 148.4 (C2, C4), 138.4 (C8), 120.2 (C5), 85.8 (C4′), 82.9 (C1′), 69.2 (C3′), 62.5 (C5′), 41.4 (C2′), 35.1 ($CH(CH_3)_2$), 26.2 ($C(CH_3)_3$), 19.2 ($CH(CH_3)_2$), 18.4 ($C(CH_3)_3$), −4.9 ($SiCH_3$). ES-HRMS; $C_{20}H_{33}N_5O_5SiNa$ requires 474.2149; $[M + Na]^+$ = 474.2152 (0.7 ppm).

***N*2-Isobutyryl-5′-*O*-dimethoxytrityl-2′-deoxyxyloguanosine (9b).** Reaction conditions as for **9a**, performed on *N*2-isobutyryl-5′-*O*-dimethoxytrityl-2′-deoxyguanosine[34] (**8b**, 2.89 g, 4.52 mmol). Purification by flash column chromatography, eluting with a gradient of 2% MeOH in CH_2Cl_2–4% MeOH in CH_2Cl_2, yielded the title compound as a pale yellow amorphous solid (0.58 g, 0.91 mmol, 20%).

^{1}H NMR (400 MHz, DMSO) δ 11.77 (br s, 2 H, NH), 8.01 (s, 1 H, H8), 7.42–7.37 (m, 2 H, *m*-Ph), 7.29–7.18 (m, 7 H, *o*-Ph, *p*-Ph, *o*-Ar_2), 6.87–6.78 (m, 4 H, *m*-Ar_2), 6.24–6.18 (m, 1 H, H1′), 5.30 (d, J = 3.0 Hz, 1 H, 3′-OH), 4.34 (m, 1 H, H3′), 4.25–4.19 (m, 1 H, H4′), 3.73 (s, 3 H, OMe), 3.72 (s, 3 H, OMe), 3.39–3.35 (m, 1 H, H5′), 3.18 (dd, J = 2.7, 10.2 Hz, 1 H, H5′), 2.84–2.73 (m, 1 H, C*H*$(CH_3)_2$), 2.69 (ddd, J = 5.3, 8.4, 14.4 Hz, 1 H, H2′), 2.31–2.25 (m, 1 H, H2′), 1.13 (d, J = 1.9 Hz, 3 H, $CH(CH_3)_2$), 1.11 (d, J = 1.9 Hz, 3 H, $CH(CH_3)_2$). ^{13}C NMR (101 MHz, DMSO) δ 180.6 (NHibu C═O), 158.4 (*p*-Ar_2), 158.3 (C6), 148.6 and 148.3 (C2 and C4), 145.4 (*ipso*-Ph), 138.2 (C8), 136.1 (*ipso*-Ar), 130.1–127.0 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar_2), 121.0 (C5), 113.4 (*m*-Ar_2), 85.7 (CPh$(Ar)_2$), 84.4 (C4′), 83.4 (C1′), 69.6 (C3′), 63.5 (C5′), 55.3 (OMe), 41.1 (C2′), 35.1 ($CH(CH_3)_2$), 19.3 ($CH(CH_3)_2$). ES-HRMS: $C_{35}H_{37}N_5O_7Na$ requires 662.2591; $[M + Na]^+$ = 662.2598 (1.1 ppm).

***N*2-Isobutyryl-5′-*O*-*tert*-butyldimethylsilyl-3′-*S*-benzoyl-2′-deoxy-3′-thioguanosine (10a).** Diisopropyl azodicarboxylate (0.66 mL, 3.33 mmol) was added dropwise to a stirred solution of triphenylphosphine (0.87 g, 3.33 mmol) in anhydrous THF (8 mL) that had been chilled to 0 °C. The yellow mixture was allowed to stir at 0 °C for 30 min under N_2. A solution of the *xylo* nucleoside **9a** (0.5 g, 1.11 mmol) dissolved in anhydrous THF (3 mL) followed by a solution of thiobenzoic acid (0.46 mL,

3.86 mmol) dissolved in anhydrous THF (2 mL) were next added dropwise to the yellow mixture at 0 °C. The resulting reaction was left to stir at room temperature for 4 h under N_2. Solvent was then evaporated and the recovered yellow residue was dissolved in CH_2Cl_2 and washed successively with H_2O and $NaHCO_3$. The collected organic layer was then dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude product as a yellow oil. Purification by flash column chromatography, eluting with 1% MeOH in $CHCl_3$, yielded the title compound as a white amorphous solid (0.41 g, 0.72 mmol, 65%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.95 (br s, 1 H, NH), 9.06 (s, 1 H, NH), 7.98–7.95 (m, 2 H, *o*-SBz), 7.94 (s, 1 H, H8), 7.68–7.63 (m, 1 H, *p*-SBz), 7.55–7.48 (m, 2 H, *m*-SBz), 6.13 (dd, J = 2.8, 7.4 Hz, 1 H, H1′), 5.22–5.13 (m, 1 H, H3′), 4.18–4.11 (m, 1 H, H4′), 3.89 (dd, J = 3.0, 11.8 Hz, 1 H, H5′), 3.81 (dd, J = 3.8, 11.8 Hz, 1 H, H5′), 3.19 (ddd, J = 2.7, 7.6, 13.3 Hz, 1 H, H2′), 2.72 (spt, J = 7.2 Hz, 1 H, C*H*$(CH_3)_2$), 2.56 (ddd, J = 7.6, 9.9, 13.3 Hz, 1 H, H2′), 1.34 (d, J = 7.2 Hz, 6 H, CH(C*H*$_3)_2$), 0.81 (s, 9 H, C$(CH_3)_3$), 0.01–0.02 (m, 3 H, SiCH_3), −0.06 (s, 3 H, SiCH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.0 (SBz C═O), 180.0 (NHibu C═O), 156.0 (C6), 148.6 and 147.9 (C2 and C4), 138.8 (C8), 136.9 (*ipso*-SBz), 134.5 (*p*-SBz), 129.3 (*m*-SBz), 127.7 (*o*-SBz), 121.9 (C5), 85.4 (C4′), 84.8 (C1′), 62.9 (C5′), 40.3 (C3′), 39.6 (C2′), 37.1 (*C*H$(CH_3)_2$), 26.2 (C(*C*H$_3)_3$), 19.3 (CH(*C*H$_3)_2$), 18.8 (*C*$(CH_3)_3$), −5.2 (SiCH_3). ES-HRMS; $C_{27}H_{37}N_5O_5SSiNa$ requires 594.2182; $[M + Na]^+$ = 594.2160 (−3.8 ppm).

***N*2-Isobutyryl-5′-*O*-dimethoxytrityl-3′-*S*-benzoyl-2′-deoxy-3′-thioguanosine (10b).** Acetic acid (3.8 mL) was added slowly to a stirred solution of *N*2-isobutyryl-5′-*O*-*tert*-butyldimethylsilyl-3′-*S*-benzoyl-2′-deoxy-3′-thioguanosine (**10a**, 4.36 g, 7.62 mmol) in anhydrous THF (65 mL) at 0 °C. Next, a solution of tetrabutylammonium fluoride (1 M, 15.24 mL, 15.24 mmol) in THF was added dropwise over 5 min under N_2. The reaction was then allowed to stir at room temperature until it went to completion as confirmed by TLC. The resulting solution was then taken up in CH_2Cl_2 (100 mL) and washed successively with H_2O and $NaHCO_3$. The collected organic layer was then dried under Na_2SO_4, filtered and concentrated under reduced pressure to afford the title compound as an off-white amorphous solid (3.31 g, 7.23 mmol, 95%). The crude product was co-evaporated with anhydrous pyridine (3 × 15 mL) and subsequently dissolved in anhydrous pyridine (70 mL). The solution was next treated with 4,4′-dimethoxytrityl chloride (5.18 g, 15.32 mmol) and the reaction allowed to stir at room temperature under N_2 until it went to completion as indicated by TLC. The resulting solution was then quenched by addition of MeOH (10 mL) and the solvent was next evaporated to furnish an orange oil. The residue was dissolved in CH_2Cl_2 and washed successively with $NaHCO_3$ and brine. The collected organic layer was then dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude product as an orange solid. Purification by flash column chromatography, eluting with a gradient of 3% MeOH in CH_2Cl_2–5% MeOH in CH_2Cl_2, yielded the title compound as a white amorphous solid (4.92 g, 6.47 mmol, 85% over 2 steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.84 (s, 1 H, NH), 8.97 (s, 1 H, NH), 7.87–7.83 (m, 2 H, *o*-SBz), 7.74 (s, 1 H, H8), 7.61–7.57 (m, 1 H, *p*-SBz), 7.47–7.42 (m, 2 H, *m*-SBz), 7.23–7.20 (m, 2 H, *o*-Ph), 7.13–7.03 (m, 7 H, *p*-Ph, *m*-Ph, *o*-Ar$_2$), 6.61–6.55 (m, 4 H, *m*-Ar$_2$), 6.02 (dd, J = 2.2, 7.7 Hz, 1 H, H1′), 5.59–5.50 (m, 1 H, H3′), 4.09–4.03 (m, 1 H, H4′), 3.62 (s, 6 H, OMe), 3.33 (dd, J = 3.2, 10.6 Hz, 1 H, H5′), 3.26–3.17 (m, 2 H, H5′, H2′), 2.54–2.36 (m, 2 H, H2′, C*H*$(CH_3)_2$), 1.18 (d, J = 6.8 Hz, 3 H, CH-(C*H*$_3)_2$), 1.11 (d, J = 7.0 Hz, 3 H, CH(C*H*$_3)_2$).^{13}C NMR (101 MHz, $CDCl_3$) δ 192.2 (SBz C═O), 179.0 (NHibu C═O), 158.8 (*p*-Ar$_2$), 156.1 (C6), 147.8 and 147.7 (C2 and C4), 144.9 (*ipso*-Ph), 139.3 (C8), 136.9 (*ipso*-SBz), 136.0 (*ipso*-Ar$_2$), 134.6 (*p*-SBz), 130.4–127.2 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar$_2$, *m*-SBz, *o*-SBz), 122.4 (C5), 113.3 (*m*-Ar$_2$), 86.7 (CPh(Ar)$_2$), 84.9 (C1′), 83.8 (C4′), 62.4 (C5′), 55.5 (OMe), 40.5 (C3′), 39.1 (C2′), 36.9 (*C*H$(CH_3)_2$), 19.2 (CH(*C*H$_3)_2$). ES-HRMS: $C_{42}H_{41}N_5O_7SNa$ requires 782.2624; $[M + Na]^+$ = 782.2646 (2.8 ppm).

***N*2-Isobutyryl-5′-*O*-dimethoxytrityl-2′-deoxy-3′-thioguanosine (11).** N_2 was bubbled through an aqueous solution of NaOH (0.5 M, 20 mL) as well as a solution of MeOH–THF–H_2O (4 : 6 : 1, 230 mL) for 30 min. *N*2-Isobutyryl-5′-dimethoxytrityl-3′-*S*-benzoyl-2′-deoxy-3′-thioguanosine (**10b**, 2.5 g, 3.29 mmol) was added to the solution mixture of MeOH/THF/H_2O and the resulting solution was then cooled to 0 °C before the degassed NaOH was added dropwise over 5 min. The reaction was allowed to stir at 0 °C for 30 min under N_2. A solution of cold citric acid (1 M, 49.5 mL) was next added dropwise over 10 min to neutralise the base. The reaction solution was allowed to stir at 0 °C for 15 min, then taken up in EtOAc and washed once with an equal volume of $NaHCO_3$. The collected organic layer was then dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the crude product as an off-white amorphous solid (2.14 g, 3.26 mmol, 99%). The recovered material was carried on to the next reaction without any further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.95 (s, 1 H, NH), 8.32 (s, 1 H, NH), 7.81 (s, 1 H, H8), 7.36–7.31 (m, 2 H, *o*-Ph), 7.25–7.09 (m, 7 H, *m*-Ph, *p*-Ph, *o*-Ar$_2$), 6.75–6.70 (m, 4 H, *m*-Ar$_2$), 6.05 (dd, J = 3.4, 6.8 Hz, 1 H, H1′), 3.91–3.86 (m, 1 H, H4′), 3.71 (s, 6 H, OMe-H), 3.67–3.57 (m, 1 H, H3′), 3.41 (dd, J = 3.0, 10.8 Hz, 1 H, H5′), 3.26 (dd, J = 4.0, 10.8 Hz, 1 H, H5′), 2.83 (ddd, J = 3.4, 7.4, 13.7 Hz, 1 H, H2′), 2.43–2.24 (m, 2 H, C*H*$(CH_3)_2$, H2′), 1.60 (d, J = 6.6 Hz, 1 H, 3′-SH), 1.12 (d, J = 7.0 Hz, 3 H, CH(C*H*$_3)_2$), 1.07 (d, J = 6.8 Hz, 3 H, CH(C*H*$_3)_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.4 (NHibu C═O), 158.9 (*p*-Ar$_2$), 156.1 (C6), 148.2 and 148.1 (C2, C4), 144.8 (*ipso*-Ph), 137.6 (C8), 135.9 (*ipso*-Ar$_2$), 130.4–127.4 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar$_2$), 122.1 (C5), 113.6 (*m*-Ar$_2$), 88.6 (C4′), 87.0 (CPh(Ar)$_2$), 84.2 (C1′), 62.5 (C5′), 55.7 (OMe), 42.6 (C2′), 36.7 (C3′), 35.7 (*C*H$(CH_3)_2$), 19.3 (CH(*C*H$_3)_2$). ES-HRMS: $C_{35}H_{37}N_5O_6SNa$ requires 678.2362; $[M + Na]^+$ = 678.2393 (4.5 ppm).

***N*2-Isobutyryl-5′-*O*-dimethoxytrityl-2′-deoxy-3′-thio-guanosine-3′-*S*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (12).** A solution of 5-ethylmercapto-1*H*-tetrazole (0.25 M, 10 mL) in MeCN was added to a stirred solution of the thioguanosine derivative **11** (2.19 g, 3.34 mmol) in CH_2Cl_2 (20 mL)

under N_2. 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.59 mL, 5.01 mmol) was then added dropwise over 5 min to the stirred solution of nucleoside. The reaction was allowed to stir at room temperature for 3 hours under N_2. The resulting solution was subsequently quenched by addition of MeOH (6 mL) and then taken up in CH_2Cl_2 and washed once with $NaHCO_3$. The collected organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the crude product as a pale yellow solid. Purification by flash column chromatography, eluting with a gradient of 1 : 5 EtOAc : CH_2Cl_2–1 : 3 EtOAc : CH_2Cl_2, yielded the title compound as a white amorphous solid (2.34 g, 2.74 mmol, 82% for both diastereoisomers).

Fast eluting diastereoisomer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.02 (br s, 1 H, NH), 8.91 (s, 1 H, NH), 7.79 (s, 1 H, H8), 7.37–7.30 (m, 2 H, *m*-Ph), 7.26–7.07 (m, 7 H, *o*-Ph, *p*-Ph, *o*-Ar_2), 6.73–6.66 (m, 4 H, *m*-Ar_2), 6.12–6.04 (m, 1 H, H1′), 4.18–4.10 (m, 1 H, H4′), 3.69 (s, 6 H, OMe), 3.66–3.28 (m, 7 H, H3′, OCH_2, NC*H*$(CH_3)_2$, 2H5′), 2.90 (ddd, *J* = 3.8, 8.0, 13.9 Hz, 1 H, H2′), 2.58–2.31 (m, 4 H, H2′, CH_2CN, C*H*$(CH_3)_2$), 1.28–1.04 (m, 18 H, NCH(C*H*$_3)_2$, CH(C*H*$_3)_2$). ^{31}P NMR (162 MHz, $CDCl_3$) δ 161.9. ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.1 (NHibu C═O), 158.8 (*p*-Ar_2), 156.0 (C6), 148.0 and 147.7 (C2, C4), 145.5 (*ipso*-Ph), 137.9 (C8), 136.1 (*ipso*-Ar_2), 130.7–127.2 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar_2), 122.3 (C5), 117.8 (CN), 113.6 (*m*-Ar_2), 87.1 (CPh$(Ar)_2$), 86.7 (C4′), 85.4 (C1′), 64.1 (C5′), 60.4 (OCH_2), 55.6 (OMe), 47.3 (N*C*H$(CH_3)_2$), 42.1 (C2′), 41.1 (C3′), 36.5 (*C*H$(CH_3)_2$), 23.1 (NCH-(*C*H$_3)_2$), 20.5 (*C*H_2CN), 19.0 (CH(*C*H$_3)_2$).

Slower eluting diastereoisomer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.99 (br s, 1 H, NH), 8.86 (s, 1 H, NH), 7.79 (s, 1 H, H8), 7.37–7.31 (m, 2 H, *m*-Ph), 7.26–7.07 (m, 7 H, *o*-Ph, *p*-Ph, *o*-Ar_2), 6.73–6.66 (m, 4 H, *m*-Ar_2), 6.12–6.06 (m, 1 H, H1′), 4.19–4.10 (m, 1 H, H4′), 3.79–3.30 (m, 12 H, OCH_2, OMe, H3′, NC*H*-$(CH_3)_2$, H5′), 3.24 (dd, *J* = 5.1, 10.5 Hz, 1 H, H5′), 2.99 (ddd, *J* = 4.6, 7.6, 13.4 Hz, 1 H, H2′), 2.61–2.31 (m, 4 H, H2′, CH_2CN, C*H*-$(CH_3)_2$), 1.23–0.99 (m, 18 H, NCH(C*H*$_3)_2$, CH(C*H*$_3)_2$). ^{31}P NMR (162 MHz, $CDCl_3$) δ 160.7. ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.1 (NHibu C═O), 158.9 (*p*-Ar_2), 156.0 (C6), 148.0 and 147.7 (C2, C4), 145.4 (*ipso*-Ph), 137.9 (C8), 136.1 (*ipso*-Ar_2), 130.6–127.3 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar_2), 122.4 (C5), 117.9 (CN), 113.6 (*m*-Ar_2), 87.1 (CPh$(Ar)_2$), 86.7 (C4′), 85.4 (C1′), 64.1 (C5′), 60.6 (OCH_2), 55.6 (OMe), 47.3 (N*C*H$(CH_3)_2$), 42.1 (C2′), 41.1 (C3′), 36.5 (*C*H-$(CH_3)_2$), 23.0 (NCH(*C*H$_3)_2$), 20.5 (*C*H_2CN), 19.0 (CH(*C*H$_3)_2$). ES-HRMS: $C_{44}H_{54}N_7O_7PSNa$ requires 878.3441; $[M + Na]^+$ = 878.3467 (3.0 ppm).

***N*6-Benzoyl-5′-*O*-dimethoxytrityl-2′-deoxy-3′-thioadenosine-3′-*S*-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (7).** A solution of 5-ethylmercapto-1*H*-tetrazole (0.25 M, 4.62 mL,) in MeCN was added to a stirred solution of *N*6-benzoyl-5′-dimethoxytrityl-2′-deoxy-3′-thioadenosine (**6**, 1.04 g, 1.54 mmol) in CH_2Cl_2 (20 mL) under N_2. 2-Cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (0.73 mL, 2.31 mmol) was then added dropwise over 5 min to the stirred solution of nucleoside. The reaction was allowed to stir at room temperature for 2 hours under N_2. The resulting solution was subsequently quenched by addition of MeOH (3 mL) and then taken up in CH_2Cl_2 and washed once with $NaHCO_3$. The collected organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the crude product as a pale yellow solid. Purification by flash column chromatography, eluting with 3 : 1 EtOAc : hexane, yielded the title compound as a white amorphous solid (0.84 g, 0.96 mmol, 62% for both diastereoisomers).

Fast eluting diastereoisomer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.98 (s, 1 H, NH), 8.71 (s, 1 H, H2), 8.25 (s, 1 H, H8), 7.99–7.92 (m, 2 H, *o*-NBz), 7.58–7.51 (m, 1 H, *p*-NBz), 7.50–7.42 (m, 2 H, *m*-NBz), 7.37–7.31 (m, 2 H, *m*-Ph), 7.27–7.08 (m, 7 H, *o*-Ph, *p*-Ph, *o*-Ar_2), 6.74–6.70 (m, 4 H, *m*-Ph), 6.35 (dd, *J* = 2.3, 6.8 Hz, 1 H, H1′), 4.20–4.12 (m, 1 H, H4′), 3.79–3.73 (m, 1 H, H3′), 3.71 (s, 6 H, OMe), 3.63–3.50 (m, 5 H, OCH_2, NC*H*$(CH_3)_2$, H5′), 3.35 (dd, *J* = 4.6, 10.6 Hz, 1 H, H5′), 3.04 (ddd, *J* = 2.3, 7.2, 13.7 Hz, 1 H, H2′), 2.62 (ddd, *J* = 7.0, 10.2, 14.0 Hz, 1 H, H2′), 2.37 (t, *J* = 6.3 Hz, 2 H, CH_2CN), 1.13 (d, *J* = 6.8 Hz, 6 H, NCH-(C*H*$_3)_2$), 1.07 (d, *J* = 6.5 Hz, 6 H, NCH(C*H*$_3)_2$). ^{31}P NMR (162 MHz, $CDCl_3$) δ 164.5. ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.3 (NHBz C═O), 156.6 (*p*-Ar_2), 153.0 (C2), 150.6 (C6), 147.5 (C4), 144.9 (*ipso*-Ph), 142.1 (C8), 139.8 (*ipso*-Ar_2), 133.7 (*ipso*-NBz), 133.1 (*p*-NBz), 130.8–127.3 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar_2, *o*-NBz, *m*-NBz), 125.0 (C5), 115.1 (CN), 113.5 (*m*-Ar_2), 87.3 (CPh$(Ar)_2$), 84.5 (C4′), 82.9 (C1′), 62.7 (C5′), 60.9 (OCH_2), 55.6 (OMe), 47.0 (N*C*H$(CH_3)_2$), 42.9 (C2′), 39.4 (C3′), 24.6 (NCH(*C*H$_3)_2$), 20.5 (*C*H_2CN).

Slower eluting diastereoisomer. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.97 (s, 1 H, NH), 8.71 (s, 1 H, H2), 8.23 (s, 1 H, H8), 7.98–7.93 (m, 2 H, *o*-NBz), 7.55–7.52 (m, 1 H, *p*-NBz), 7.49–7.43 (m, 2 H, *m*-NBz), 7.34–7.30 (m, 2 H, *m*-Ph), 7.24–7.10 (m, 7 H, *o*-Ph, *p*-Ph, *o*-Ar_2), 6.72–6.68 (m, 4 H, *m*-Ph), 6.39 (dd, *J* = 2.4, 6.8 Hz, 1 H, H1′), 4.21–4.13 (m, 1 H, H4′), 3.77–3.71 (m, 3 H, OCH_2, H3′), 3.69 (s, 6 H, OMe), 3.59–3.44 (m, 3 H, NC*H*$(CH_3)_2$, H5′), 3.33 (dd, *J* = 4.6, 10.6 Hz, 1 H, H5′), 3.10 (ddd, *J* = 2.3, 7.2, 13.7 Hz, 1 H, H2′), 2.71–2.61 (m, 1 H, H2′), 2.53 (t, *J* = 6.1 Hz, 2 H, CH_2CN), 1.11 (d, *J* = 6.8 Hz, 6 H, NCH(C*H*$_3)_2$), 1.00 (d, *J* = 6.5 Hz, 6 H, NCH(C*H*$_3)_2$). ^{31}P NMR (162 MHz, $CDCl_3$) δ = 160.9. ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.3 (NHBz C═O), 156.6 (*p*-Ar_2), 153.1 (C2), 150.6 (C6), 147.7 (C4), 144.9 (*ipso*-Ph), 142.1 (C8), 139.9 (*ipso*-Ar_2), 133.7 (*ipso*-NBz), 133.1 (*p*-NBz), 130.8–127.3 (*o*-Ph, *m*-Ph, *p*-Ph, *o*-Ar_2, *o*-NBz, *m*-NBz), 125.0 (C5), 115.1 (CN), 113.5 (*m*-Ar_2), 94.9 (CPh$(Ar)_2$), 84.5 (C4′), 82.9 (C1′), 62.7 (C5′), 60.9 (OCH_2), 55.6 (OMe), 47.0 (N*C*H$(CH_3)_2$), 42.9 (C2′), 39.5 (C3′), 24.6 (NCH(*C*H$_3)_2$), 20.5 (*C*H_2CN). ES-HRMS: $C_{47}H_{52}N_7O_6PSNa$ requires 896.3335; $[M + Na]^+$ = 896.3337 (0.2 ppm).

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Notes and references

1 J. W. Gaynor and R. Cosstick, *Curr. Org. Chem.*, 2008, **12**, 291–308.
2 (*a*) J. A. Piccirilli, J. S. Vyle, M. H. Caruthers and T. R. Cech, *Nature*, 1993, **361**, 85–88; (*b*) L. B. Weinstein, B. C. M. N. Jones, R. Cosstick and T. R. Cech, *Nature*, 1997, **388**, 805–808; (*c*) S. Shan, A. V. Kravchuk, J. A. Piccirilli and D. Herschlag, *Biochemistry*, 2001, **40**, 5161–5171.
3 P. M. Gordon, R. Fong and J. A. Piccirilli, *Chem. Biol.*, 2007, **14**, 607–612.
4 E. J. Sontheimer, S. Sun and J. A. Piccirilli, *Nature*, 1997, **388**, 801–805.
5 G. Sasnauskas, L. Zakrys, M. Zaremba, R. Cosstick, J. W. Gaynor, S. E. Halford and V. Siksnys, *Nucleic Acids Res.*, 2010, **38**, 2399–2410.
6 J. F. Curley, C. M. Joyce and J. A. Piccirilli, *J. Am. Chem. Soc.*, 1997, **119**, 12691–12692.
7 (*a*) A. P. G. Beevers, K. J. Fettes, S. M. Roberts, I. A. O'Neil, J. R. P. Arnold, R. Cosstick and J. Fisher, *Chem. Commun.*, 2002, 1458–1459; (*b*) A. P. G. Beevers, K. J. Fettes, G. Sabbagh, F. K. Murad, J. R. P. Arnold, R. Cosstick and J. Fisher, *Org. Biomol. Chem.*, 2004, **2**, 114–119; (*c*) H. K. Jayakumar, J. L. Buckingham, J. A. Brazier, N. G. Berry, R. Cosstick and J. Fisher, *Magn. Reson. Chem.*, 2007, **45**, 340–345.
8 J. Bentley, J. A. Brazier, J. Fisher and R. Cosstick, *Org. Biomol. Chem.*, 2007, **5**, 3698–3702.
9 J. A. Brazier, J. Fisher and R. Cosstick, *Angew. Chem., Int. Ed.*, 2006, **45**, 114–117.
10 J. A. Brazier, A. Shah and G. D. Brown, *Chem. Commun.*, 2012, **48**, 10739–10741.
11 (*a*) J. W. Gaynor, B. J. Campbell and R. Cosstick, *Chem. Soc. Rev.*, 2010, **39**, 4169–4184; (*b*) R. E. Collins, X. Cheng and J. Cell, *Biochemistry*, 2006, **99**, 1251–1266.
12 S. Burge, G. N. Parkinson, P. Hazel, A. K. Todd and S. Neidle, *Nucleic Acids Res.*, 2006, **34**, 5402–5415.
13 (*a*) Y. Wang and D. J. Patel, *J. Mol. Biol.*, 1993, **234**, 1171–1183; (*b*) G. N. Parkinson, M. P. Lee and S. Neidle, *Nature*, 2002, **417**, 876–880; (*c*) A. T. Phan, K. N. Luu and D. J. Patel, *Nucleic Acids Res.*, 2006, **34**, 5715–5719.
14 (*a*) A. Siddiqui-Jain, C. L. Grand, D. J. Bearss and L. H. Hurley, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 11593–11598; (*b*) J. Dai, D. Chen, R. A. Jones, L. H. Hurley and D. Yang, *Nucleic Acids Res.*, 2006, **34**, 5133–5144; (*c*) V. Kuryavyi, A. T. Phan and D. J. Patel, *Nucleic Acids Res.*, 2010, **38**, 6757–6773.
15 C. Cheong and P. B. Moore, *Biochemistry*, 1992, **31**, 8406–8414.
16 J.-L. Mergny, A. De Cian, A. Ghelab, B. Saccà and L. Lacroix, *Nucleic Acids Res.*, 2005, **33**, 81–94.
17 A. Randazzo, V. Esposito, O. Ohlenschläger, R. Ramachandran and L. Mayol, *Nucleic Acids Res.*, 2004, **32**, 3083–3092.
18 G. Sabbagh, K. J. Fettes, R. Gossain, I. A. O'Neil and R. Cosstick, *Nucleic Acids Res.*, 2004, **32**, 495–501.
19 J. W. Gaynor, M. M. Piperakis, J. Fisher and R. Cosstick, *Org. Biomol. Chem.*, 2010, **8**, 1463–1470.
20 R. Eisenhuth and C. Richert, *J. Org. Chem.*, 2009, **74**, 26–37.
21 G. Di Fabio, J. D'Onofrio, M. Chiapparelli, B. Hoorelbeke, D. Montesarchio, J. Balzarini and L. De Napoli, *Chem. Commun.*, 2011, **47**, 2363–2365.
22 A. P. G. Beevers, E. Witch, B. C. N. M. Jones, R. Cosstick, J. R. P. Arnold and J. Fisher, *Magn. Reson. Chem.*, 1999, **37**, 814–820.
23 M. Egli, S. Portmann and N. Usman, *Biochemistry*, 1996, **35**, 8489–8494.
24 (*a*) O. Y. Fedoroff, M. Salazar and B. R. Reid, *J. Mol. Biol.*, 1993, **233**, 509–523; (*b*) A. N. Lane, S. Ebel and T. Brown, *Eur. J. Biochem.*, 1993, **215**, 297–306; (*c*) A. Noy, A. Pérez, M. Márquez, F. J. Luque and M. Orozco, *J. Am. Chem. Soc.*, 2005, **127**, 4910–4920.
25 (*a*) J. I. Gyi, G. L. Conn, A. N. Lane and T. Brown, *Biochemistry*, 1996, **35**, 12538–12548; (*b*) J. I. Gyi, A. N. Lane, G. L. Conn and T. Brown, *Biochemistry*, 1998, **37**, 73–80.
26 J.-L. Mergny, J. Li, L. Lacroix, S. Amrane and J. B. Chaires, *Nucleic Acids Res.*, 2005, **33**, e138.
27 E. E. Merkina and K. R. Fox, *Biophys. J.*, 2005, **89**, 365–373.
28 F. Aboul-ela, A. I. H. Murchie and D. M. J. Lilley, *Nature*, 1992, **360**, 280–282.
29 J. Deng, Y. Xiong and M. Sundaralingam, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 13665–13670.
30 (*a*) V. E. Marquez, T. Ben-Kasus, J. J. Barchi, K. M. Green, M. C. Nicklaus and R. Agbaria, *J. Am. Chem. Soc.*, 2004, **126**, 543–549; (*b*) H. Saneyoshi, S. Mazzini, A. Aviño, G. Portella, C. González, M. Orozco, V. E. Marquez and R. Eritja, *Nucleic Acids Res.*, 2009, **37**, 5589–5601.
31 B. Saccà, L. Lacroix and J.-L. Mergny, *Nucleic Acids Res.*, 2005, **33**, 1182–1192.
32 J.-L. Mergny, J. Gros, A. De Cian, A. Bourdoncle, F. Rosu, B. Saccà, L. Guittat, S. Amrane, M. Mills, P. Alberti, M. Takasugi and L. Lacroix, in *Quadruplex Nucleic Acids*, ed. S. Neidle and S. Balasubramanian, RSC Publishing, Cambridge, 2006, pp. 31–80.
33 J. Gros, F. Rosu, S. Amrane, A. De Cian, V. Gabelica, L. Lacroix and J.-L. Mergny, *Nucleic Acids Res.*, 2007, **35**, 3064–3075.
34 G. S. Ti, B. L. Gaffney and R. A. Jones, *J. Am. Chem. Soc.*, 1982, **104**, 1316–1319.