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Chemical and enzymatic properties of bridging 5′-*S*-phosphorothioester linkages in DNA

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ABSTRACT

We describe physicochemical and enzymatic properties of 5′ bridging phosphorothioester linkages at specific sites in DNA oligonucleotides. The susceptibility to hydrolysis at various pH values is examined and no measurable hydrolysis is observed at pH 5–9 after 4 days at 25°C. The abilities of three 3′- and 5′-exonuclease enzymes to hydrolyze the DNA past this linkage are examined and it is found that the linkage causes significant pauses at the sulfur linkage for T4 DNA polymerase and calf spleen phosphodiesterase, but not for snake venom phosphodiesterase. Restriction endonuclease (*NsiI*) cleavage is also attempted at a 5′-thioester junction and strong resistance to cleavage is observed. Also tested is the ability of polymerase enzymes to utilize templates containing single 5′-*S*-thioester linkages; both Klenow DNA polymerase and T7 RNA polymerase are found to synthesize complementary strands successfully without any apparent pause at the sulfur linkage. Finally, the thermal stabilities of duplexes containing such linkages are measured; results show that T_m values are lowered by a small amount (2°C) when one or two thioester linkages are present in an otherwise unmodified duplex. The chemical stability and surprisingly small perturbation by the 5′ bridging sulfur make it a good candidate as a physical and mechanistic probe for specific protein or metal interactions involving this position in DNA.

INTRODUCTION

Strategically placed sulfur atoms have found widespread utility in the probing of specific interactions of proteins, enzymes and metals with nucleic acids. Replacement of sulfur for oxygen in the sugar–phosphate backbone of DNA and RNA has been central to many mechanistic studies of bond cleavage reactions. For example, much work has been carried out with sulfur replacing specific non-bridging phosphate oxygens in DNA and RNA (1–6), which has led to important insights into enzymatic and RNA-catalyzed cleavage of phosphodiesters in several classes of molecules. Sulfur replacement for oxygen has also been carried out at the 2′ position of RNA (7–9) and in the 3′ and 5′ positions of RNA (10–15) and of DNA (16–25). These last two positions are termed ‘bridging’ positions in the phosphate linkage and these positions are important because they can make specific interactions with proteins or metals and because they act as leaving groups in various catalyzed RNA or DNA cleavage reactions.

Recent work has focused on the properties of a 5′ bridging sulfur in the context of RNA, in part because of its relevance to enzymatic and RNA-catalyzed cleavage reactions (10–15). This sulfur replacement is useful as a mechanistic probe and it is especially labile to hydrolysis because it presents the already labile RNA linkage with a better leaving group. This property results in an ~10^6-fold increase in its ease of hydrolysis at neutral pH (12).

The analogous 5′ bridging sulfur in DNA is considerably less well studied as a mechanistic probe. While a number of synthetic methods have been investigated for creating such a linkage (16–19,22), very few studies of chemoenzymatic properties of this structure exist. Although it has been shown that silver nitrate and other chemical reagents can cleave this linkage specifically (22), we are aware of only two studies which investigate enzyme–DNA interactions. One report studied a dinucleotide containing a 5′ bridging sulfur and it was reported that phosphodiesterases from calf spleen and snake venom were able to cleave this linkage, but without reference to rate or comparison with the natural DNA linkage (16). A second study investigated dT oligomers completely substituted with 5′ bridging sulfurs (18) and it was found that the exonuclease of T4 DNA polymerase and snake venom phosphodiesterase (SVPD) showed significantly reduced ability to cleave such oligomers. Thus, little detailed work has been focused on this linkage and, importantly, no studies have examined the hydrolytic stability of this linkage, the effect of isolated 5′-S linkages on duplex stability or whether this linkage can act as a substrate for either restriction endonucleases or polymerase enzymes.

The small amount of existing work on enzymatic processing of the 5′ bridging linkage in DNA left a number of uninvestigated questions and we felt that a number of possible potential uses justified more detailed study. Recent methods for reagent-free ligation of DNA result in formation of this 5′ bridging thioester structure (26–28) and so if this methodology is to be useful for the assembly of DNA sequences for further applications (27), significant chemical and enzymatic stability may be necessary. Also important, this specific atomic position could possibly act as a leaving group in metal-catalyzed hydrolytic cleavage of DNA (29–31) as well as in enzyme-mediated cleavage, such as occurs with 5′-exonucleases (32), and so 5′ bridging sulfur atoms could be particularly useful in mechanistic studies related to those activities. In addition, recent RNA-catalyzed and DNA-catalyzed cleavage reactions on DNA substrates (33–35) engender added interest in mechanistic investigations involving this position. Finally, the 5′ bridging position in DNA may also interact with proteins directly where no cleavage chemistry is involved. Thus,

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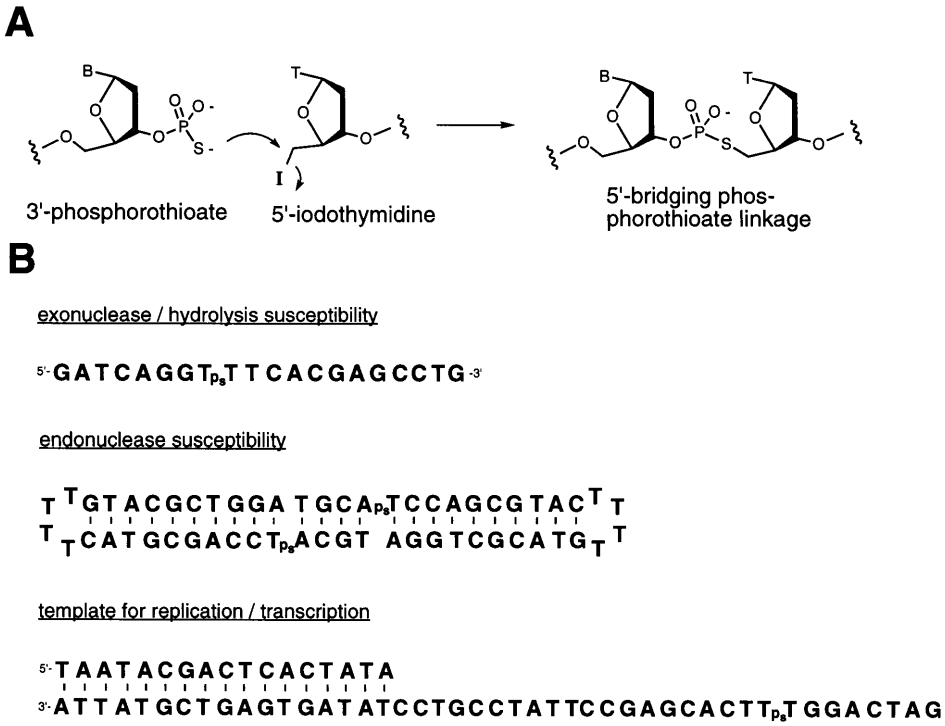

Figure 1. Structures and sequences in this study. (**A**) Mechanism of iodothymidine-mediated autoligation, resulting in a 5′ bridging phosphorothioate linkage. (**B**) Sequences of autoligation precursor DNAs and products obtained after ligation, where p_s denotes 5′ bridging phosphorothioester linkage.

a survey of physicochemical and enzymatic properties of this linkage is warranted.

We now describe such studies carried out on short, sequence-defined oligodeoxynucleotides. The 5′-S linkages are found to be stable for extended periods in aqueous buffers and to be resistant to some, but not all, exonuclease enzymes. A restriction endonuclease is strongly inhibited in cleaving bonds adjacent to the P-S bonds, although singly placed 5′ bridging sulfurs are found to cause only small thermal destabilization of duplexes. Surprisingly, DNAs with 5′-S linkages are found to act as normal templates for DNA and RNA polymerases.

MATERIALS AND METHODS

Preparation of 5′-iodinated oligodeoxyribonucleotides and 3′-phosphorothioate oligodeoxyribonucleotides

Phosphorylation at the 3′-end of DNA strands was carried out with a phosphoramidite reagent (36) purchased from Glen Research. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems (ABI) 392 synthesizer using standard β-cyanoethylphosphoramidite chemistry, except that for 3′-phosphorothioate sequences the first nucleotide added after the phosphorylation reagent was sulfurized by the sulfurizing reagent from ABI (37). 5′-Iodo-oligonucleotides were synthesized by the standard protocol (16,27).

Ligations to produce 5′-*S*-thioester linkages

Oligonucleotides containing 5′-iodo- and 3′-phosphorothioate groups (20 µM) were incubated with 22 µM complementary splint oligomer in a pH 7.0 buffer (50 mM Tris–borate) containing 10 mM $MgCl_2$ at room temperature for 24 h (27). The splint sequence used for the 20mer and 45mer DNAs was 5′-d(CTA GTC CAA AGT GCT CGG); for the hairpin sequence no splint was needed. Ligation products were isolated on preparative denaturing polyacrylamide gels.

Exonuclease cleavage

Snake venom phosphodiesterase digestion. 5′-^{32}P-labeled oligonucleotides (500 000 c.p.m.) and 0.12 mU SVPD (Boehringer Mannheim) were incubated at room temperature in a pH 7.5 buffer (70 mM Tris–borate) containing 10 mM $MgCl_2$, in a total volume of 50 µl. Aliquots (8 µl) were removed at the desired time points and stopped by addition of 8 µl stop solution (30 mM EDTA, 8 M urea).

T4 polymerase digestion. 5′-^{32}P-labeled oligonucleotides and 0.2 U T4 DNA polymerase (US Biochemical) were incubated at room temperature in a pH 8.8 buffer (33 mM Tris–HCl) containing 10 mM $MgCl_2$, 66 mM KOAc, 5 mM dithiothreitol, 0.01% bovine serum albumin (BSA), in a total volume of 50 µl. Aliquots (8 µl) were removed at the desired time points and stopped by addition of 8 µl stop solution.

Calf spleen phosphodiesterase digestion. 20mer oligodeoxynucleotides (Fig. 1B) were 3′-end-labeled with [α-^{32}P]ddATP and terminal deoxynucleotidyltransferase (US Biochemical). They were incubated with 0.2 U calf spleen phosphodiesterase (CSPD; US Biochemical) at room temperature in a pH 6.0 buffer (30 mM NaOAc), in a total volume of 30 µl. Aliquots (4 µl) were removed at the desired time points and stopped by addition of 8 µl stop solution.

Endonuclease cleavage

Aliquots of 0.05 nmol dumbbell DNA (Fig. 1B) and 50 U *Nsi*I (Gibco BRL) were incubated at 37°C for 1.5 h in a pH 8.0 buffer (50 mM Tris–HCl) containing 10 mM $MgCl_2$, 100 mM NaCl, in a total volume of 50 µl. Reactions were stopped by heating at 68°C for 20 min. After phenol/chloroform extraction and ethanol precipitation, pellets were brought up in 5 µl H_2O prior to loading on an analytical PAGE gel.

Polymerase studies

Conditions for the primer extension experiment were as follows: 10 nM template DNA strand, 10 nM primer strand (Fig. 1B), 1 mM each dATP, dTTP, dCTP and dGTP (Boehringer Mannheim) and 3 U Klenow fragment of DNA polymerase I (exo$^-$; US Biochemical) were incubated in a pH 7.5 buffer (50 mM Tris–HCl) containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 µg/ml BSA at 37°C, in a total reaction volume of 20 µl. Reactions were stopped by addition of 10 µl stop solution and heated to 90°C for 2 min, followed by chilling on ice prior to loading on the gel.

Conditions for the run-off transcription reactions were as follows: 1 µM template, 50 U T7 RNA polymerase (New England Biolabs), 0.5 mM each ATP, GTP and CTP, 60 µM UTP and 0.27 µCi [α-^{32}P]UTP were incubated in a pH 7.9 buffer (40 mM Tris–HCl) containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol at 37°C, in a total volume of 15 µl. Reactions were stopped by addition of 15 µl stop solution and heated to 90°C for 2 min, followed by chilling on ice prior to loading on the gel.

Thermal melting studies

Solutions for thermal denaturation studies contained a 1:1 ratio of the complementary strands (1.0 µM each). The solution for the 20mer duplexes contained 100 mM NaCl and 10 mM $MgCl_2$ buffered with 10 mM Na · PIPES at pH 7.0. The solutions for the dumbbell duplexes contained 10 mM Na · PIPES at pH 7.0 and 1 mM EDTA. Solutions were heated to 90°C and allowed to cool slowly to room temperature prior to the melting experiments. Melting studies were carried out in Teflon-stopped 1 cm pathlength quartz cells under a nitrogen atmosphere on a Varian Cary 1 UV-vis spectrophotometer equipped with a thermoprogrammer. Absorbance was monitored at 260 nm while the temperature was raised from 10 to 95°C at a rate of 0.5°C/min. Melting temperatures were determined by computer fitting using a two-state approximation with linear sloping baselines. Error in T_m is estimated at ±0.5°C or less.

RESULTS AND DISCUSSION

The 5′ bridging linkage has been previously described in the DNA context (16–19,22) and until recently has been constructed by single nucleoside addition methods or by oligomerization of bis-reactive monomers. More recently, this same bridging sulfur linkage has been produced at specific sites in unmodified DNA by non-enzymatic ligation reactions. Studies by Letsinger have shown that oligonucleotides can undergo efficient ligation when a 3′-phosphorothioate displaces a 5′-end tosylate on thymidine (17,26,28). We have recently shown that a 5′-end iodide (16) is a convenient alternative to tosylate as a leaving group (Fig. 1A), since the lower reactivity of iodide allows it to survive automated DNA synthesis and deprotection virtually intact (27). Figure 2 shows a time course study for two ligations (one enzymatic and one involving self-ligation), generating duplex dumbbell DNAs from two half-length hairpins. Although the non-enzymatic ligation clearly proceeds more slowly than the enzymatic one, the two cases proceed with comparable yields, as judged by gel analysis of the products after 24 h. Interestingly, in both the enzymatic and non-enzymatic cases there are apparently two conformations observed for the hairpins; these converge to a single band in some gels and with high temperature denaturation (not shown).

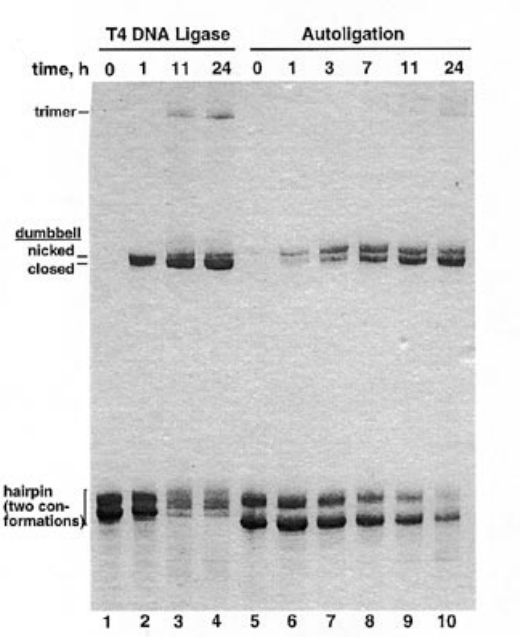

Figure 2. Time course of autoligation of a hairpin DNA to closed circular dumbbell form at 25°C (lanes 5–10), with comparison to ligation mediated by T4 DNA ligase in the same sequence (lanes 1–4). Complete reaction requires two ligations, the first of which joins two hairpins (creating a nicked dumbbell) and the second of which closes the dumbbell into circular form. The sequence of the autoligating hairpin is 5′-I-d(TCCAGCGTACTTTTGTACGCTGGATGCA)-p_s-3′ and that of the comparison hairpin is d(pTCCAGCGTACTTTTG-TACGCTGGATGCA).

To test the ability of enzymes and varied buffer conditions to cleave P-S bonds in 5′ bridging phosphorothioates, a 20mer 5′-S-containing oligonucleotide (Fig. 1B) was constructed by ligation of an octamer carrying a 3′-phosphorothioate with a 12mer carrying a 5′-iodide in the presence of an 18mer complementary strand. This produces a single sulfur at the 5′-carbon of thymidine in the ninth position of this sequence. We have previously shown that this ligation is carried out efficiently on a preparative scale and proceeds to near completion after 24 h with no side products (27).

Hydrolysis of the P-S bond in the absence of enzymes is expected to be much more rapid than occurs with DNA phosphodiesters, which have a half-life of many thousands of years at neutral pH (38). To examine this we incubated the 20mer containing a sulfur linkage (Fig. 1B) at varied pH (pH 5.0, 7.0 and 9.0) over a 4 day period. Under these conditions no specific cleavage is visible (<1%) at any pH (data not shown). This allows us to set an upper limit for the hydrolysis rate constant for this P-S bond at $k_{obs} = 3 \times 10^{-8}$/s or less, and the half-life of the bond is therefore >1 year at pH values near neutral. The corresponding half-life for 5′ bridging thioesters in RNA is 3 days (12). It is

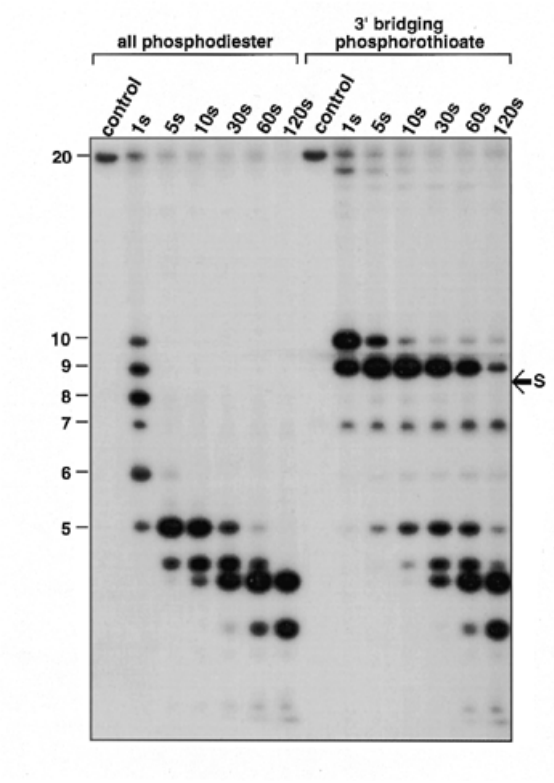

Figure 3. Susceptibility of a 5′ bridging phosphorothioate linkage to cleavage by the 3′-exonuclease activity of T4 DNA polymerase. The sequence is [5′-^{32}P]dGATCAGGTp$_s$TTCACGAGCCTG (s denotes the position of the sulfur in the phosphorothioate linkage). Shown for comparison is the cleavage profile for the same sequence with all phosphodiester linkages.

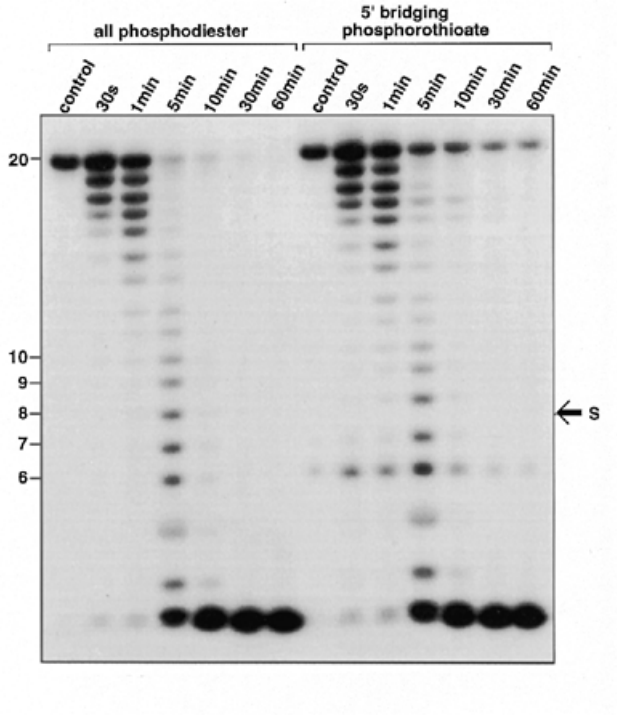

Figure 4. Susceptibility of a 5′ bridging phosphorothioate linkage to cleavage by the 3′-exonuclease activity of SVPD. The sequence is [5′-^{32}P]d(GATCAGGTp$_s$TTCACGAGCCTG) (s denotes the position of the sulfur in the phosphorothioate linkage). Shown for comparison is the cleavage profile for the same sequence with all phosphodiester linkages.

worth noting that such linkages in DNA can be cleaved rapidly, if desired, in the presence of silver nitrate (22).

We then investigated the susceptibility of this linkage to different exonuclease enzymes, specifically, the 3′-exonuclease activity of T4 DNA polymerase, SVPD (a different 3′-exonuclease) and CSPD, which is a 5′-exonuclease. Figures 3–5 display the results of the exonuclease studies with direct comparison with unmodified DNAs of the same sequence. The data presented in Figure 3 show that the sulfur causes a significant inhibition of T4 exonuclease activity. The pauses occur at sites 1 and 2 nt 3′ of (prior to) the thioester rather than during removal of the sulfur-containing nucleotide itself. We estimate the cleavage of the most resistant linkage to be inhibited by a factor of 5- to 10-fold. Since the S-P bond is not expected to be cleaved by this enzyme (products with this enzyme are normally 5′-monophosphates; 39), we surmise that this pause is due to unfavorable interaction of the enzyme with this sulfur, possibly because of the increased bond lengths or relatively poor hydrogen bond accepting ability of the sulfur relative to oxygen.

Interestingly, the snake venom 3′-exonuclease shows no visible inhibition by the presence of a bridging sulfur (Fig. 4). This enzyme also produces 5′-phosphates (40) and would not be expected to break the P-S bond in the thioester. It is clear that the interactions of this enzyme with DNA are significantly different to those in the exonuclease domain of T4 polymerase or that the bond length sensitivity is much lower for the snake venom enzyme. It is worth noting that the only previous report on SVPD cleavage of 5′-bridging sulfur linkages in DNA reported significant slowing of the enzyme (18). However, in that case all linkages were modified rather than a single one as in the present case, a fact which might explain the differences.

The final enzyme, CSPD, which is a 5′-exonuclease that produces 3′-phosphate products (32), was studied for its ability to digest a 21mer with sulfur between nt 8 and 9. This case is particularly noteworthy, since this enzyme would be expected to cleave the P-S bond in the 5′ bridging sulfur linkage. In this experiment a significant pause is observed (Fig. 5), although cleavage beyond this point clearly does occur, eventually yielding only very short products. The pause occurs at the position assigned to the nucleotide immediately before the linkage, which indicates that cleavage of the P-S bond by the enzyme is slower than processing of a normal P-O bond. Since sulfur is expected to be a much better leaving group than oxygen in the absence of enzyme, we surmise that this inhibition arises from less favorable protein (or protein-bound metal) electrophilic interactions with the leaving group. Alternatively, it is possible that the increased bond lengths cause a geometric problem at the active site. The P-S and S-C bonds in the diester linkage are each expected to be ~0.4 Å longer than the analogous P-O and O-C bonds in unmodified DNA (41).

We then tested the ability of a site-specific endonuclease to cleave a double-stranded sequence containing a 5′ bridging sulfur. For this study a hairpin-forming sequence with a self-complementary overhang was ligated with another copy to produce a dumbbell DNA consisting of a 24 bp duplex capped by 4 nt loops (Figs 1B and 2). The two sulfur linkages are present at the cleavage site of restriction endonuclease *Nsi*I in its 6 bp palindromic recognition sequence. An unmodified version of this sequence was constructed as a control. The results are shown in Figure 6. Although the unmodified sequence is completely

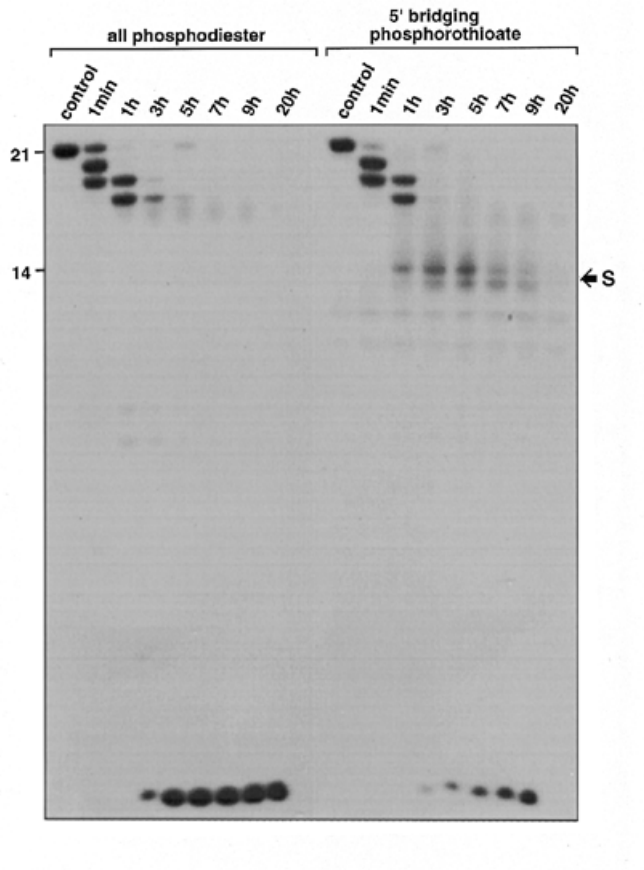

Figure 5. Susceptibility of a 5′ bridging phosphorothioate linkage to cleavage by the 5′-exonuclease activity of CSPD. The DNA is 3′-end-labeled. The sequence is d(GATCAGGTp$_s$TTCACGAGCCTG^{32}pA) (s denotes the position of the sulfur in the phosphorothioate linkage). Shown for comparison is the cleavage profile for the same sequence with all phosphodiester linkages.

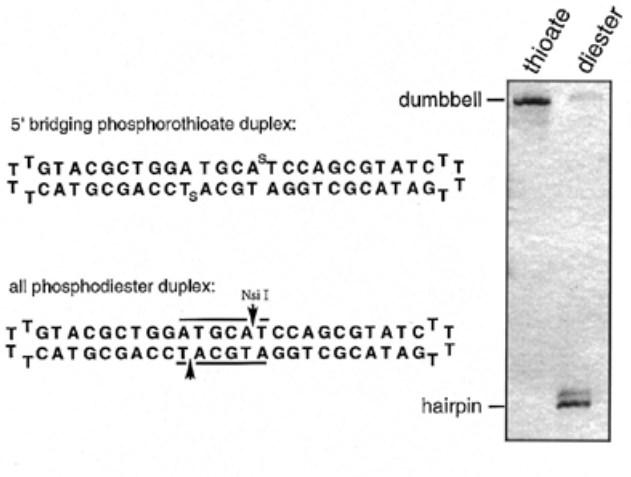

Figure 6. Attempted cleavage of a short duplex DNA containing 5′ bridging phosphorothioate linkages by the restriction endonuclease *Nsi*I. The sulfur linkage (denoted by s) occurs in both strands between the two nucleotides where cleavage is performed by the enzyme. For comparison, cleavage of the same duplex lacking the sulfur linkages is shown.

cleaved by the enzyme in 1.5 h, the sulfur-containing sequence shows no cleavage over this period. The enzyme would not be expected to cleave the P-S bond, since it normally leaves 5′-phosphate ends. This inhibition must therefore be due to

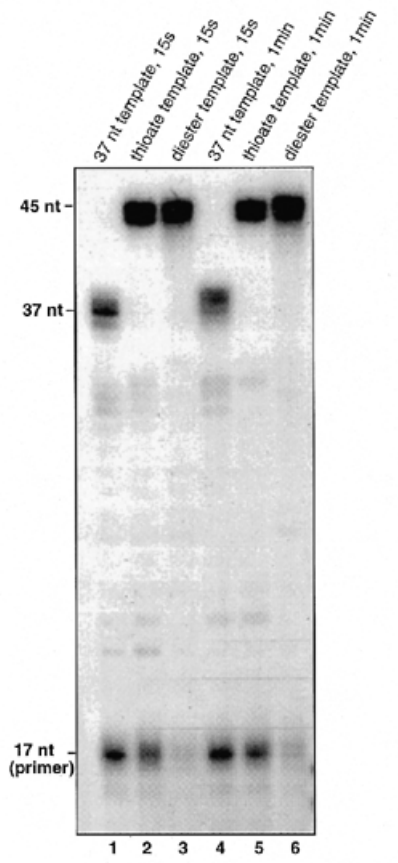

Figure 7. DNA synthesis on a template containing a 5′ bridging phosphorothioate linkage. The enzyme is the Klenow fragment of *E.coli* DNA pol I (exo$^-$) and the sulfur linkage is situated after base 20 downstream of the primer. Control lanes (1 and 4) show DNA synthesis on a template that ends at the position of the sulfur linkage in the longer templates.

unfavorable interactions between the enzyme and the 5′ bridging position or to altered geometry of the DNA arising from the longer P-S bonds.

We also investigated the question of whether the increased bond lengths (S-C and S-P) in the modified DNA would be recognized as a template for common polymerase enzymes. This question has not previously been examined with this class of linkage. For this we constructed a 45mer template with sulfur after position 37 from the 3′-end. Thus, binding of a 17mer primer at the 3′-end allows DNA polymerase extension for 20 nt up to the sulfur position. This primer also acts as a promoter top strand for T7 RNA polymerase, thus allowing both DNA and RNA polymerase to be tested on the same template. Once again, an unmodified template was tested for comparison. The polymerases studied were the Klenow fragment of *Escherichia coli* DNA polymerase I (exo$^-$ mutant) and T7 RNA polymerase.

The results of the polymerase studies are shown in Figures 7 and 8. Interestingly, both the DNA and RNA polymerases proceeded beyond the sulfur-containing thymidines with no apparent pause seen before, at or after this residue. No difference is observed in the length of the products or in the amount of time necessary to reach this full length with these concentrations of enzyme and DNA. Thus these polymerases apparently recognize no difference between the oxygen- and sulfur-containing templates under these conditions, despite the longer bonds in the latter case.

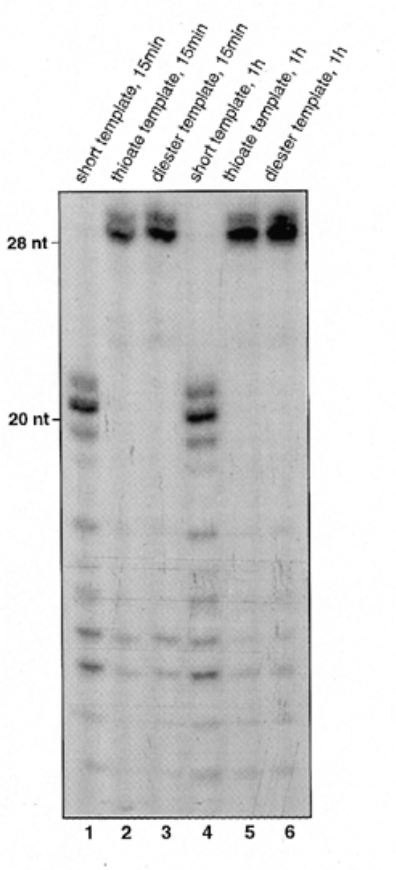

Figure 8. RNA synthesis on a template containing a 5′ bridging phosphorothioate linkage. The enzyme is T7 RNA polymerase and the sulfur linkage is situated after base 20 downstream of the promoter 3′-end (Fig. 1B). Control lanes (1 and 4) show RNA synthesis on a template that ends at the position of the sulfur linkage in the longer templates.

Finally, we examined the effects of one or two 5′ bridging sulfur linkages on the thermal stability of DNA–DNA duplexes. This was tested first in the context of a 20mer duplex carrying one sulfur linkage in one strand [d(GAT CAG GTp$_s$T TCA CGA GCC TG)] and its unmodified 20mer complement, then a completely unmodified duplex of the same sequence was examined for comparison. Thermal denaturation studies (100 mM Na^+, 10 mM Mg^{2+}) showed that both cases were well behaved, showing sharp transitions. T_m values were 68.8°C for the sulfur-containing duplex and 71.5°C for the unmodified duplex. A second case was then examined with 5′-S linkages in both strands of a duplex, this time using the dumbbell sequences shown in Figure 6. Because of their high stability, the denaturation studies were performed under low salt conditions (10 mM Na · PIPES, 1 mM EDTA). The results showed that the thermal stabilities of the modified duplex (T_m = 82.8°C) and the unmodified one (T_m = 83.3°C) are essentially the same. Thus, the 5′ bridging sulfur linkage causes very little destabilization of duplexes, at least for the cases studied here.

The results show overall that the 5′ bridging sulfur causes significant inhibition of some nuclease enzymes but no apparent inhibition of DNA or RNA polymerase enzymes. In terms of stability in the absence of enzymes, the 5′-thioester linkage is likely to be considerably more labile to hydrolysis than natural DNA phosphodiesters, but is stable at least for months at pH values of 5–9. This linkage is much more stable, therefore, than previously reported 5′ bridging thioesters in RNA. However, when specific cleavage is desired in thioester-linked DNA, treatment with silver produces cleavage under mild conditions (22). We anticipate that these properties will make the 5′ bridging phosphothioester quite useful as a biochemical probe in DNA.

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