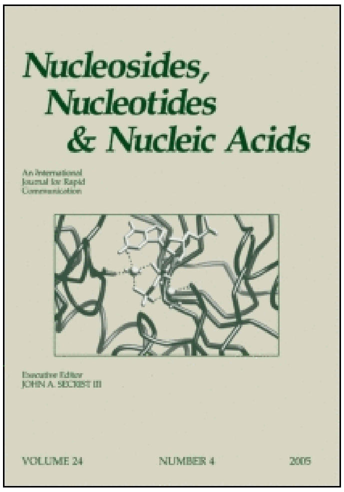

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CHEMICAL AND BIOCHEMICAL STUDIES WITH DITHIOATE DNA

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Abstract. Dithioate DNA was synthesized and used for various biochemical studies. Results from these studies indicate that dithioate DNA is a potent inhibitor of HIV Reverse Transcriptase, activates endogenous RNase H in Hela cell nuclear extracts, and is a useful probe for studying protein-DNA interactions.

INTRODUCTION

Although synthetic DNA having the natural 3'-5' phosphodiester linkage has proven to be extremely valuable for a large number of biochemical and biological applications,[1,2] certain key uses require modified synthetic polynucleotides. For these applications, DNA is needed that is nuclease stable, easily tagged with reporter groups, and achiral. Such analogs can then be used potentially for diagnosing disease, as therapeutic drugs, and for a large number of basic, biological investigations. For some time now, our research has focused on the development of derivatives that meet these criteria. Among the more potentially useful is a new compound called dithioate DNA; an achiral, anionic mimic of natural DNA that contains an internucleotide phosphodiester group having sulfur at the two nonlinking positions. In this report, the chemistry for synthesizing dithioate DNA will be outlined and experiments presented that indicate how this derivative may prove useful for a large array of biochemical and biological applications.

SYNTHESIS OF PHOSPHORODITHIOATE DNA

In order to use dithioate DNA for a large number of different applications, chemical synthesis methodologies must be rapid, high yielding, and compatible with current approaches for preparing natural DNA. If these criteria are met, then this derivative can be incorporated into normal DNA in a pre-selected manner so as to generate a polynucleotide having any combination of natural and dithioate internucleotide linkages.

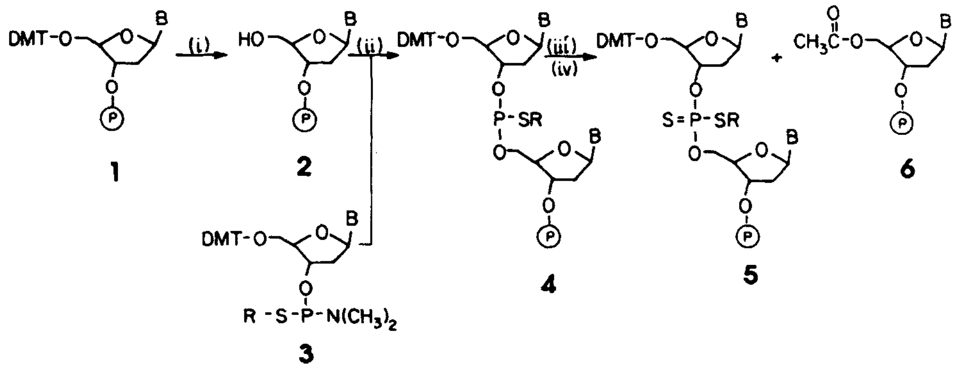


FIG. 1. Synthesis of Deoxyoligonucleotide Dithioates From Deoxynucleoside Phosphorothioamidites. (i) 3% trichloroacetic acid; (ii) tetrazole; (iii)sulfur; (iv) 1-methylimidazole + acetic anhydride. Abbreviations: R, 2,3-dichlorobenzyl; DMT, di-p-methoxytrityl; B, N-benzoyladenine, N-benzoylcytosine, thymine, N-isobutyrylguanine; Ⓟ, silica gel support.

The approach that meets these criteria[3,4] is outlined in FIG. 1 and TABLE 1. Briefly deoxynucleoside 3'-phosphorothioamidite synthons (3) are condensed in any desired sequence with a growing polynucleotide on a polymer support. These synthons are prepared by condensing a suitably protected deoxynucleoside with tris-pyrrolidinylphosphine and tetrazole to yield the diamidite which is converted without isolation to the phosphorothioamidite by addition of 2,4-dichlorobenzylmercaptan. As a specific example, tris-pyrrolidinylphosphine (1.02 ml, 4.7 mmole) and tetrazole (1 ml of a 0.5 M solution in acetonitrile) are added to 5'-O-dimethoxytrityl-N-benzoyldeoxycytidine (2.53 g, 4 mmole) dissolved in dichloromethane:acetonitrile (2:1; 48 ml) and the reaction mixture stirred for 15 min. 2,4-Dichlorobenzylmercaptan (1.60 ml, 6.9 mmole) is added and after 15 min, the extent of reaction to the deoxynucleoside 3'-phosphorothioamidite is monitored by ^{31}P NMR (162, 160 ppm). The reaction mixture is then diluted with ethylacetate (200 ml containing 1% triethylamine) and extracted sequentially with 10% sodium carbonate (2 X 200 ml) and brine (200 ml). After drying the resulting organic solution over anhydrous sodium sulfate and removal of solids by filtration, the product is isolated by first concentrating the organic solution to 20 ml, precipitation from degassed heptane containing 1% triethylamine (500 ml), filtration, and finally drying *in vacuo* (3.3 g, 89%). These synthons are then stored as dry powders under an inert gas atmosphere without decomposition.

Table 1. Chemical Steps for Synthesis of Dithioate DNA on a Solid Support*

Step		Reagent or Solvent**	Purpose	Time (min)
(i)	a.	Trichloroacetic acid in CH_2Cl_2 (3%, w/v)	Detritylation	0.50
	b.	CH_2Cl_2	Wash	0.50
	c.	Acetonitrile	Wash	0.50
	d.	Dry Acetonitrile	Wash	0.50
(ii)	a.	Activated nucleotide in acetonitrile¶	Add nucleotide	0.75
	b.	Repeat step a	Complete nucleotide addition	1.50
	c.	CS_2	Wash	0.25
(iii)	a.	Sulfur in CS_2:pyridine: TEA (95:95:10; v/v/v)†	Oxidation	1.00
	b.	CS_2	Wash	0.50
	c.	CH_3OH	Wash	0.50
	d.	CH_2Cl_2	Wash	0.50
(iv)	a.	NMI:THF (30:70; v/v)‡ acetic anhydride:lutidine: THF (2:2:15; v/v/v)	Capping reaction	0.50
	b.	CH_2Cl_2	Wash	0.50

* See Figure 1 for an explanation of the various steps i-iv.

** Multiple washes with the same solvent are possible.

¶ For each micromole of deoxynucleoside attached to silica, 0.48 M tetrazole (0.125 ml) and 0.15 M deoxynucleoside phosphorothioamidite (0.125 ml) are premixed in acetonitrile.

† 1.56 M sulfur in CS_2:pyridine:TEA (95:95:10; v/v/v); TEA, triethylamine.

‡ NMI, N-methylimidazole; THF, tetrahydrofuran.

Synthesis of DNA begins by treating a 5'-dimethoxytrityldeoxynucleoside linked to a silica support (**1**) with 3% trichloroacetic acid to yield **2**, a compound having a free 5'-hydroxyl group accessible for polynucleotide synthesis (see Table 1 for a more complete outline of these steps). Deoxynucleoside 3'-phosphorothioamidites (**3**) are then activated with tetrazole and condensed with **2** to yield a thiophosphite triester (**4**). The challenge with this step is to achieve high yields rapidly using a synthon stable toward storage but yet highly reactive so that relatively weak acids such as tetrazole can be used to activate the deoxynucleoside thioamidite. Otherwise with stronger acids, **3** and **4** will rearrange to Arbuzov products and also be readily oxidized to thiophosphoramidates and thiophosphates, respectively. These considerations have led to the choice of N,N-dimethylamino or pyrollidino as the reactive amidites and 2,4-dichlorobenzyl as the sulfur protecting group. This step is followed by sulfurization using elemental sulfur to yield the protected phosphorodithioate derivative (**5**), capping or acylating unreactive silica-linked nucleoside with acetic anhydride (**6**), and detritylation with trichloroacetic acid. Further repetitions of this cycle using either deoxynucleoside phosphorothioamidites or deoxynucleoside phosphoramidites as synthons and tetrazole as an activator yield oligodeoxynucleotides having normal phosphate diester and phosphorodithioate diester linkages in any combination. So far segments containing up to 28 dithioate linkages have been prepared by this approach. Additionally yields of 96-98% per cycle (based upon dimethoxytrityl cation released during detritylation) and 97-98% dithioate per linkage have been obtained (the remaining 2-3% internucleotide linkages are phosphorothioate). When sulfur protecting groups other than 2,4-dichlorobenzyl, i.e. β-cyanoethyl, are used they lead to higher yields of the phosphorothioate (5-10%). Therefore, even though 2,4-dichlorobenzyl suffers from the need to use thiophenol to remove it after completion of the synthesis, it is currently the protecting group of choice as high yields of phosphorodithioate are observed. Obviously additional research on sulfur protecting groups is needed.

Although the method outlined in FIG. 1 leads to the rapid synthesis of a large variety of phosphorodithioate containing oligonucleotides, it is perhaps most useful for preparing small quantities of DNA (1 μmole or less). For certain applications such as therapeutic or biophysical (NMR and X-ray crystallography) studies, considerably larger quantities of phosphorodithioate DNA will be needed and it is not obvious that a polymer support approach will satisfy these needs. These considerations have led to the development of several approaches that, potentially, may be of greater value as non-polymer support methods for preparing dithioate DNA. One of these methods[5] is outlined in FIG. 2. The first step is preparing the fully protected phosphorodithioate triesters (**7a,b**) from commercially available deoxynucleoside phosphoramidites by treatment first with 4-chloro or 2,4-dichlorobenzylmercaptan and tetrazole and then, without isolation, a saturated sulfur solution. Intermediates useful for DNA synthesis (**8a,b**; **9b**) can be generated from **7a,b** by removal of the β-cyanoethyl (Et_3N:CH_3CN, 1:1, at r.t. for 2-3 h) or the dimethoxytrityl (3% trichloroacetic acid at 0°C for 30 min) groups. Further condensation of **8b** (1 eq) with **9b** (1.2 eq) using triisopropylbenzenesulfonylchloride (3 eq) and 1-methylimidazole (5 eq) in CH_2Cl_2 yields the completely protected dinucleoside phosphorodithioate (**10a,b,c**) in 95% yield contaminated with 1% of **11c**, the dinucleoside phosphorothioate. The latter compound can be removed by silica gel column chromatography. So far this chemistry has been used to synthesize a deoxy-

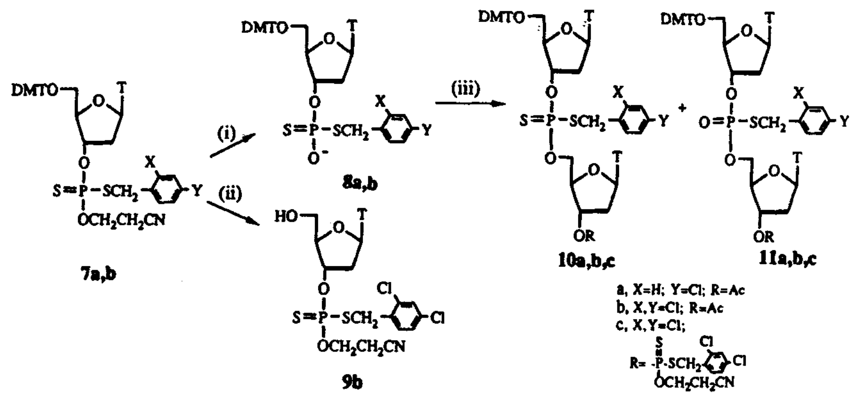


FIG. 2. Synthesis of Deoxydinucleotide Dithioates From a Solution Phosphotriester Approach. (i) Et3N:CH3CN (1:1, v:v); (ii) 2% p-toluenesulfonic acid; (iii) triisopropylbenzenesulfonyl chloride. Abbreviation: Ac, acetyl.

octathymidine having phosphorodithioate internucleotide linkages (approximately 200 mgs) and a large assortment of di-and trinucleotide dithioates (0.5 to 1.0 mmole amounts) having several different sequences. As is the case with the classical triester approach however, the isolated yields of guanine rich dimers and trimers containing dithioate linkages are considerably lower (50-60%) than with the pyrimidines (80-90%). Clearly the approach, especially relative to protecting groups, needs additional research but it may prove useful for the large scale synthesis of dithioate oligonucleotides.

Additional approaches, whereby deoxynucleoside 3'-phosphorodiamidites,[6,7] 3'-H-phosphonothioates,[8,9] and 3'-H-phosphonodithioates[9,10] are used to synthesize large quantities of dithioate DNA, have also been examined. The most extensively studied of these methods so far, which uses deoxynucleoside 3'-phosphorodiamidites, is shown in FIG. 3. The first step leading to **15** is condensation of a protected deoxynucleoside with bis(diisopropylamino)chlorophosphine in dioxane containing triethylamine.[7] The resulting 3'-phosphorodiamidite is reacted without isolation with a 3'-O-phenoxyacetyl protected deoxynucleoside to yield **12** (60-70%) after silica gel column chromatography. Synthesis of **15** can then proceed by (i) treatment with H_2S and tetrazole to yield **13**, (ii) oxidation with elemental sulfur, and (iii) alkylation with α,2,4-trichlorotoluene[7] or 2,4-dinitrobenzylchloride.[11] Alternatively,[6] **12** can be treated with (iv) 2,4-dichlorobenzylmercaptan and (v) elementary sulfur which again yields **15**. After removal of the 3'-protecting group from **15** and conversion to the 3'-phosphoramidite or 3'-phosphorothioamidite, the dimers can be incorporated into polynucleotides via a non-polymer

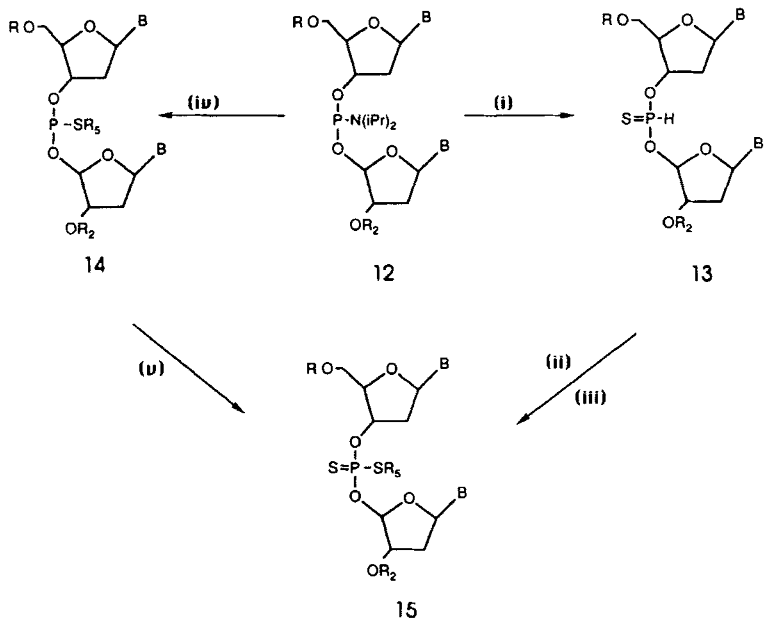


FIG. 3. Synthesis of Deoxydinucleotide Dithioates Via Deoxynucleoside Phosphorodiamidites. (i) H_2S + tetrazole; (ii) sulfur; (iii) α,2,4-trichlorotoluene or 2,4-dinitrobenzylbromide; (iv) 2,4-dichlorobenzylmercaptan; (v) sulfur. R_1, dimethoxytrityl; R_2, phenoxyacetyl; R_3, 2,4-dichlorobenzyl; iPr, isopropyl; B, protected nucleoside base.

support, block synthesis approach. For example re-cycling **15** to the 3'-phosphorodiamidite, condensation with a protected deoxynucleoside, and steps (iv)/(v) should yield a trimer which can then be converted to hexamers and dodecamers having any combination of sequence and dithioate phosphate linkages. The approaches outlined in FIG. 3 have at least two potential advantages over the triester method. (1) The reductions in yields with purines, especially guanine, that are observed with sulfonylchlorides, triazolides and tetrazolides should not occur via these procedures. (2) Compound **13** is a highly versatile synthon[7] as it can also be used to generate thioamidates and thioates for introduction into polynucleotides. This may be of interest for inserting, via a solution approach, various reporter groups into large quantities of DNA. These speculations, however, must still be examined carefully as there is only minimal data currently available that supports the pathways outlined in FIG. 3.[6,11]

BIOCHEMICAL STUDIES WITH DITHIOATE DNA

Of particular interest is whether dithioate DNA is nuclease stable and capable of forming duplexes. So far results with snake venom and calf spleen phosphodiesterase[6] and nuclease P1[11] indicate that this derivative is not degraded by nucleases. More recently, results have also shown that dithioate DNA cannot be degraded by the 3'-5' exonuclease activity of T4-DNA polymerase (L. Gold & M. Caruthers, unpublished results) and the nucleases present in Hela cell nuclear extracts (L. Cummins & M. Caruthers, unpublished results). Recent data (FIG. 4) also demonstrates that oligonucleotides containing dithioate linkages readily form stable duplexes with somewhat reduced melting temperatures (Tm) relative to unmodified DNA. With a 20mer having 58% dithioate linkages (11 of 19), the Tm is reduced by 5°C. With lesser numbers of these linkages (4 of 19, 21%; 7 of 19, 37%), Tm reductions of 1°C and 3°C, respectively, are observed. Similarly with a 15mer having two and four dithioates per segment, Tm reductions of 0.4°C and 4.4°C suggest that the same trend continues with perhaps a more significant change in duplex stability for shorter segments. Relative to phosphorothioate oligomers of comparable size and percent guanine-cytosine base pairs,[12] these results are surprising as similar reductions in duplex stabilities are observed for both analogs. This is the case even though, on a mole basis, dithioate DNA contains twice the sulfur as thioate DNA. Perhaps the large number of diastereomers with thioates destabilizes the duplex to a greater extent than would be expected from substitution of sulfur for an oxygen. In any case, the results clearly indicate that stable duplexes can be formed with dithioate DNA.

Initial labeling experiments suggest that phosphorodithioate internucleotide linkages are significantly more reactive than phosphorothioates. Using a model system comparable to previous studies,[13] an oligonucleotide, d(GAAGATCT*TACGGCCGGC-A) having one dithioate or phosphorothioate linkage at the marked (*) position, was tested for reactivity with excess monobromobimane and the results monitored by HPLC. Under conditions where the dithioate linkage was almost completely (95%) converted to the triester in 5 hours, the phosphorothioate oligomer was alkylated only to 60% after 24 hours. These results and others with iodoacetylfluorescein demonstrate that dithioate DNA can be tagged more rapidly and efficiently than comparable thioate oligonucleotides. Since the dithioate linkage is sequence independent but easily controlled as to relative position in a polynucleotide, it should prove very useful for specific labeling of DNA with various reporter groups.

Oligonucleotide dithioates have also been shown to induce RNase H activity in Hela cell nuclear extracts. The 20mer as listed in FIG. 4 having 58% dithioate linkages is complementary to the 5'-oligonucleotide sequence of U2 snRNA. When placed in a Hela nuclear extract, this compound stimulates endogenous RNase H to degrade U2 RNA (L. Cummins, data not shown) in a manner identical to earlier results with the same DNA segment having natural internucleotide linkages.[14,15] However unlike the normal oligonucleotide, the dithioate derivative stimulates complete degradation of U2 RNA.

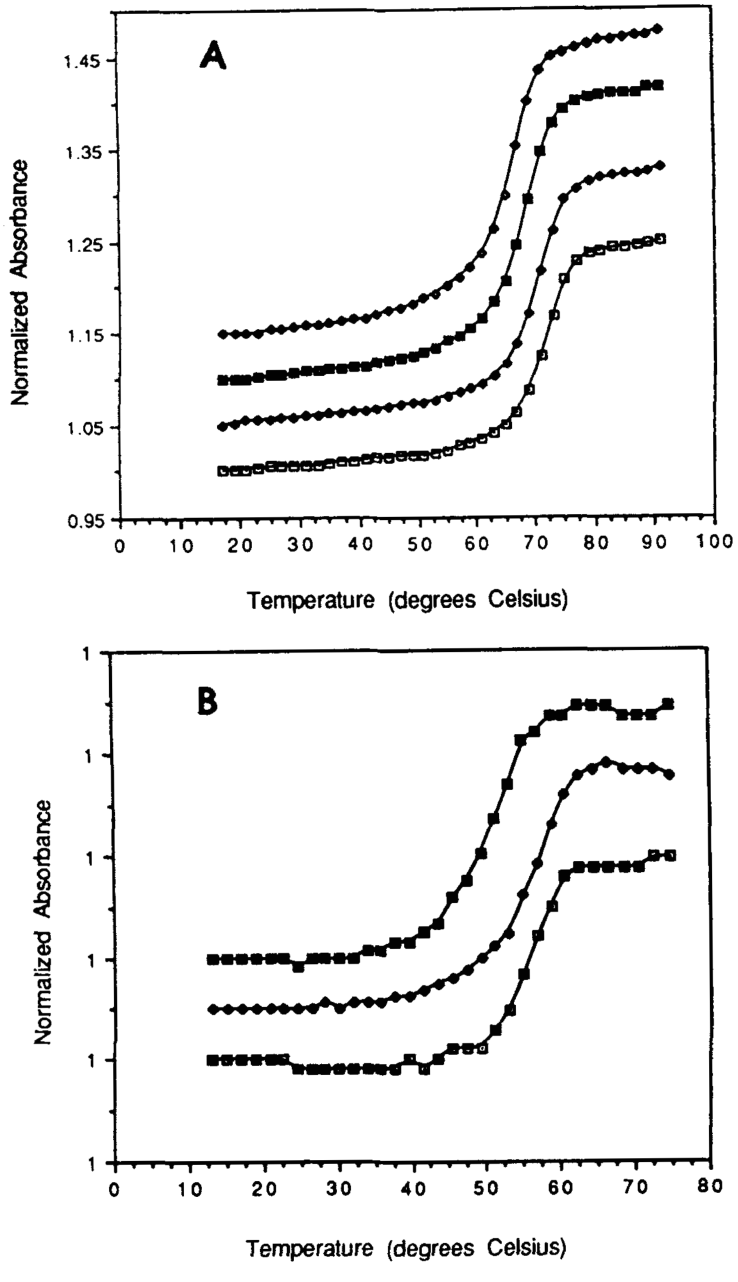
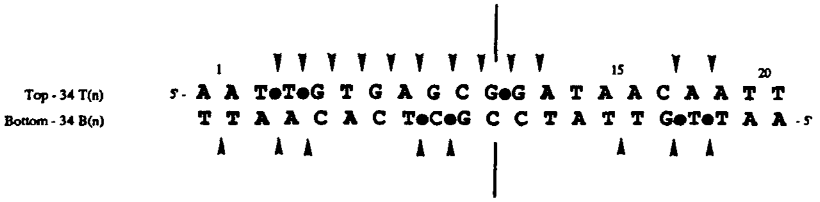


FIG. 4. Studies of Dithioate DNA Duplexes. Oligonucleotides containing dithioate linkages were annealed to the complementary, unmodified DNA segments and the absorbance monitored with temperature. Abbreviations: x, phosphorodithioate; p, phosphate. Panel A: □, d(CpCpApApApApGpGpCpCpGpApGpApApGpCpGpApT); ◆, d(CxCpApApApApGxGpCpCpGpApGxApApGpCpGpAxT); ■, d(CxCpApAxApApGxGpCpCxGpApGxApApGxCpGpAxT); ◊, d(CxCpAxApAxApGxGpCxCpGxApGxApAxGpCxGpAxT); Panel B: □, d(GpApTpTpCpApGpCpTpApGpTpCpCpA); ◆, d(GpApTpTpCxApGpCpTpAxGpTpCpCpA); ■, d(GpApTxTpCpAxGpCpTxApGpTxCpCpA).



duplex	relative affinity	duplex	relative affinity
34 T(3)	0.7	34 B(1)	0.9
T(4)	does not bind (+)	B(3)	does not bind (+)
T(5)	0.5	B(4)	0.9
T(6)	0.7	B(8)	1.2
T(7)	0.5	B(9)	0.3
T(8)	0.8	B(15)	0.8
T(9)	0.5	B(17)	does not bind (+)
T(10)	0.5	B(18)	0.5
T(11)	0.4		
T(12)	1.1		
T(17)	1.0		
T(18)	0.8		

FIG. 5. Studies of the *Lac* Repressor-*Lac* Operator Interaction. Assays of repressor-operator interactions were completed on nitrocellulose filters using standard procedures. The symbols T and B refer to the top and bottom strands as shown in the figure. Relative affinity is the ratio of equilibrium binding constants for modified, dithioate containing operators to unmodified operators. Although each oligonucleotide used in the binding assay was 34 nucleotides in length, only the central portion corresponding to *lac* operator is shown. The numbers 1-20 refer to internucleotide phosphate or phosphorodithioate linkages. The duplexes assayed are defined by the position of the phosphorodithioate linkage. For example T(3) and B(1) refer to two duplexes containing dithioate internucleotide linkages at positions 3 and 1 on the top (T) and bottom (B) strands, respectively. Both strands are numbered 1-20 for left to right as written. The symmetry axis of the operator is designated with a vertical line and the sites in operators that contain dithioate linkages are summarized by ▼.

This is because the natural segment is degraded by endogenous nuclease whereas the dithioate oligomer, even with only 58% modification, appears to be resistant toward degradation. These results are very encouraging for a potentially large number of experiments where the objective is to control endogenous RNA synthesis.

Also of considerable interest is whether oligonucleotides containing phosphorodithioate linkages alter significantly the conformation of DNA duplexes. In order to test this possibility and also to gain new insights on how these compounds may be used for various biochemical studies, the *lac* operator-*lac* repressor system was examined. The basic approach was to prepare a series of *lac* operators where each duplex contained one phosphorodithioate linkage and then to test how this modification altered the ability of *lac* repressor to recognize the operator and form a stable complex. A summary of these results is presented in FIG. 5. Generally minor reductions in binding affinity were observed (0 to 70%) although at two sites (T12 and B8) a slight enhancement in complex stability was seen. This information can then be used to introduce probes, reporter groups, and crosslinking agents such as fluorescein or heavy metals without altering the ability of *lac* repressor to recognize *lac* operator (H. Sasmor and G. Beaton, unpublished results). This approach should be quite general and provide new insights on protein-nucleic acid complexes.

There were, however, three sites (T4, B3 and B17) where insertion of a phosphorodithioate led to complete loss in affinity of repressor for operator when assayed by binding of the complex to nitrocellulose filters. These sites were either symmetric with one another (T4 and B17) or with the known nuclease sensitive site[16] associated with the formation of a stable *lac* repressor-*lac* operator complex (B3 and T18). This data may be telling us something about conformation changes associated with formation of a *lac* repressor-*lac* operator complex. Perhaps *lac* operator is in one conformation in the absence of *lac* repressor but altered in some way when it forms a complex with *lac* repressor. In this model, the key DNA sites involved in conformation changes would be T4, B3, T18 and B17. Insertion of a phosphorodithioate at sites T4, B3 and B17 would either prevent the desired conformation change or alter the operator structure in a way that reduces recognition by repressor. Since repressor-operator affinity was unaltered by insertion of a dithioate linkage at T18, this model would argue that a dithioate at T18 favorably aids the interaction. The nuclease sensitivity of this site (but not the symmetric B3 position) argues for different protein-DNA conformations with the two operator half-sites.

Because oligonucleotide analogs with modifications in the phosphate diester linkage have potential therapeutic applications,[17] phosphorodithioate DNA was also investigated in this regard. In our first experiments, the inhibition of retroviral reverse transcriptases by oligodeoxycytidine phosphorodithioates was examined. The results are summarized in TABLE 2. The assay involves carrying out repair synthesis with HIV reverse transcriptase using a primer (15mer) in complex with a template (30mer) in the presence or absence of the oligodeoxycytidine phosphorodithioate at increasing concentration. The assay was completed under saturating conditions of primer-template and deoxynucleotide triphosphates and the results analyzed by polyacrylamide

Table 2. A summary of ID_{50} Values for Phosphorodithioate DNA Inhibition of HIV Reverse Transcriptase*

	Compound	ID_{50}	
I	d(CpCpCpCpCpCpCpCpCpCpCpCpCpC)	36	μM
II	d(C-C-C-C-C-C-C-C-C-C-C-C-C-C-C)	1.7	μM
III	d(CxCxCxCxCxCxCxCxCxCxCxCxCxC)	60	nM
IV	d(CxCpCxCpCxCpCxCpCxCpCxCpCxCpC)	2.0	μM
V	d(TxTxTxTxTxTxTxTxTxTxTxTxTxT)	30	nM
VI	d(AxAxAxAxAxAxAxAxAxAxAxAxAxA)	75	nM
VII	d(CxCxCxCxCxCxCxCxCxCxCxCxCxCxCxCxCxCxCxC)	10	nM
VIII	d(CxCxCxCxCxCxCxCxCxC)	220	nM
IX	d(CxCxCxCxCxCxCxC)	1.2	μM
X	d(CxCxCxC)	20	μM

*Internucleotide linkages are represented by x for phosphorodithioate, p for phosphate, and - for phosphorothioate.

denaturing gel electrophoresis. The concentration of oligodeoxycytidine dithioate that inhibits this repair reaction to the 50% level is called the ID_{50}.

The results indicate that phosphorodithioate DNA is strongly inhibitory of HIV reverse transcriptase. An oligodeoxycytidine 14mer containing all phosphorodithioate linkages (III) has an ID_{50} value (50% inhibition) with HIV reverse transcriptase of 60 nM. The corresponding normal oligonucleotide (I) is 600 fold less inhibitory ($ID_{50} = 36$ mM) whereas the oligodeoxycytidine phosphorothioate 15mer (II) is still 28 fold less effective as an inhibitor ($ID_{50} = 1.7$ mM) than the phosphorodithioate (III). Also the extent of inhibition correlates with the content of phosphorodithioate. For example, compound IV with 50% each of phosphorodithioate and phosphate diester linkages is 33 fold less inhibitory ($ID_{50} = 2$ mM) than the phosphorodithioate 14mer (III). Equally of interest were observations that inhibition correlates with length (longer oligodeoxynucleotide dithioates are more inhibitory) and sequence of the homopolynucleotide (dT>dC>dA). Although the potential of these reagents as anti-HIV therapeutics is clear, future research must address the biochemistry (kinetics and the mechanism of complex formation), cell biology, and pharmacological utility of dithioate DNA.

CONCLUSIONS

These results demonstrate that dithioate DNA can be readily synthesized, is stable toward nucleases, forms duplexes with normal DNA, and has several potential biochemical applications. Perhaps as well, the discovery of this attractive new DNA derivative will stimulate new research in the polynucleotide field and lead to even more exciting analogs in the years ahead.

ACKNOWLEDGEMENTS

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