Tetrahedron Letters, Vol.30, No.5, pp 543-546, 1989 0040-4039/89 $3.00 + .00
Printed in Great Britain Pergamon Press plc

SYNTHESIS OF DEOXYCYTIDINE OLIGOMERS CONTAINING PHOSPHORODITHIOATE LINKAGES

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Deoxydicytidine phosphoramidite, 4-chlorobenzylmercaptan and tetrazole reacted to form dinucleoside thiophosphite. Oxidation with sulfur yields phosphorodithioates which were used to synthesize pentadecadeoxyoligonucleotides containing nuclease resistant phosphorodithioate internucleotide linkages.

Recent reports have documented how internucleotide phosphate analogs may have therapeutic value.[1,2,3] Because of these results and other potential applications, we have focused our interest on phosphorodithioates since they are achiral, nuclease resistant, and readily alkylated.[4] So far two different approaches have been developed for synthesizing dinucleoside phosphorodithioates.[4,5] We now report an additional method whereby dinucleoside phosphorodithioates are synthesized from dinucleoside phosphoramidites by reaction with a mercaptan in the presence of tetrazole followed by oxidation with elemental sulfur (see footnote 20 of reference 5 for a brief description of this procedure). The method is illustrated by the synthesis of deoxycytidine oligomers having phosphorodithioate linkages at various positions.[6]

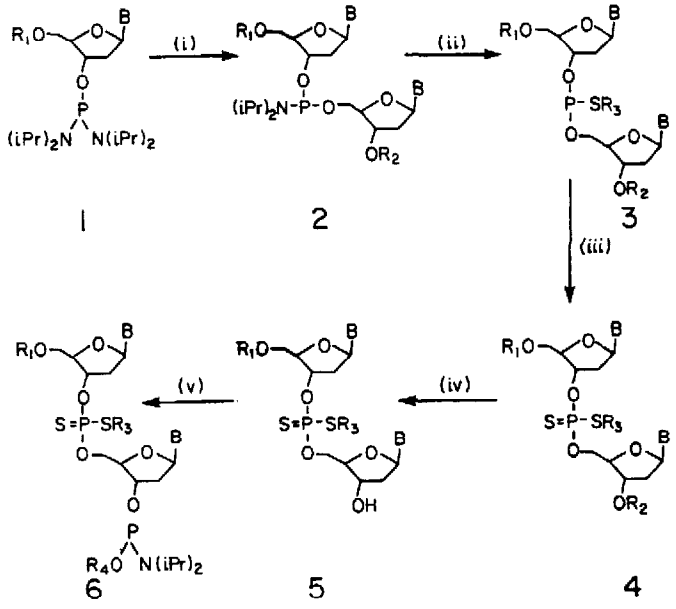

Figure 1. Synthesis of a Dinucleoside Phosphorodithioate. (i) 3'-O-phenoxyacetyl-N4-toluoyldeoxycytidine + tetrazole; (ii) 4-chlorobenzylmercaptan + tetrazole; (iii) sulfur; (iv) *t*-butylamine; (v) bis(diisopropylamino)-2-cyanoethoxyphosphine + tetrazole. Abbreviations: R_1, dimethoxytrityl, DMT; R_2, phenoxyacetyl, phac; R_3, 4-chlorobenzyl, cb; R_4, 2-cyanoethyl; B, N4-toluoylcytosine, Ctol; iPr, isopropyl.

The first step leading to the synthesis of **4**, the completely protected deoxydinucleoside phosphorodithioate, was preparation of the deoxydinucleoside amidite (**2**) following the method described by Marugg et al.[7] Thus 5'-O-dimethoxytrityl-N4-toluoyldeoxycytidine was condensed with bis(diisopropylamino)chlorophosphine to form the deoxynucleoside phosphorodiamidite (**1**) which was then reacted without isolation with 3'-O-phenoxyacetyl-N4-toluoyldeoxycytidine to yield **2** (67%) after silica gel column chromatography (5% triethylamine in hexanes:dichloromethane, 1:1, v/v).[8,9] Synthesis of the deoxydinucleoside phosphorodithioate proceeded by adding 4-chlorobenzylmercaptan (0.5 ml, 3.7 mmol) and tetrazole (190 mg, 2.7 mmol) to a solution of **2** (1.40 g, 1.12 mmol) in anhydrous acetonitrile (purged with helium so as to avoid oxidation of **3** to the phosphorothioate). After stirring the reaction mixture under argon for 30 min, the resulting thiophosphite (**3**)[10] was oxidized to the corresponding deoxydinucleoside phosphorodithioate triester (**4**) by addition of sulfur dissolved in toluene:2,6-lutidine (19:1, v/v) (5 ml, 0.4 M in sulfur). Following addition of ethylacetate, extraction with 5% aqueous sodium bicarbonate, and precipitation into hexanes, the fully protected phosphorodithioate was purified by column chromatography (40 g silica, elution with dichloromethane:hexanes:triethylamine, 66:33:0.03 and dichloromethane:triethylamine, 100:0.03, v/v) and isolated by precipitation into pentane (68%).[11,12]

Conversion of **4** to a synthon useful for DNA synthesis (**6**) involved removal of the 3'-protecting group followed by phosphitylation to the corresponding dinucleotide 3'-phosphoramidite. The special requirements associated with removal of this group under conditions where the other blocking groups were completely stable guided us to choose O-toluoyl[13,14] and phenoxyacetyl[15,16] for protection of the exocyclic amino and 3'-hydroxyl, respectively, of deoxycytidine. Using this combination of protecting groups, removal of the 3'-phenoxyacetyl could be carried out selectively by adding a solution of *t*-butylamine in methanol (12 ml, 0.3 M) to a chilled solution of **4** (355 mg, 0.26 mol) in 12 ml acetonitrile:methanol (1:3, v/v). After stirring the solution for 90 min in an ice bath, the reaction mixture was concentrated to dryness. **5** was then isolated in 95% yield by column chromatography (30 g silica using a gradient of dichloromethane and dichloromethane:methanol, 97:3, with each solvent containing 0.03% triethylamine) and precipitation into pentane.[17] Further conversion to **6** via phosphitylation with bis(diisopropylamino)-2-cyanoethoxyphosphine (1.6 eq) and tetrazole (1.1 eq) for 2 hr at r.t. followed standard procedures.[4,18]

Deoxycytidine pentadecamers containing phosphorodithioate internucleotide linkages at selected sites were synthesized using **6** and 5'-O-dimethoxytrityl-N-benzoyldeoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite with the standard phosphoramidite synthesis methodology.[19-21] The average coupling efficiency was 99% (3 min coupling time, 0.2 μmol deoxycytidine on CPG, Applied Biosystems model 380A DNA synthesizer). The products were first freed of protecting groups by treatment with a solution of thiophenol: triethylamine:dioxane (1:1:2, v/v/v) at room temperature[22] and concentrated ammonium hydroxide at 55°C (15 hr) and then purified by polyacrylamide gel electrophoresis. Three pentadecamers having phosphorodithioate linkages at positions described in the legend to Figure 2 and one unmodified deoxyoligonucleotide of the same length were synthesized (I, II, III, IV). After labeling the pentadecamers at either the 5' or 3'-hydroxyl with ^{32}P-phosphate, each compound was tested for resistance to spleen and snake venom phosphodiesterase. Using conditions where the natural deoxycytidine oligonucleotide was completely degraded with spleen phosphodiesterase, all three phosphorodithioate containing oligonucleotides were degraded exonucleolytically only until the first phosphorodithioate linkage was encountered. Similarly, the phosphorodithioate linkage was resistant to snake venom phosphodiesterase.[23,24]

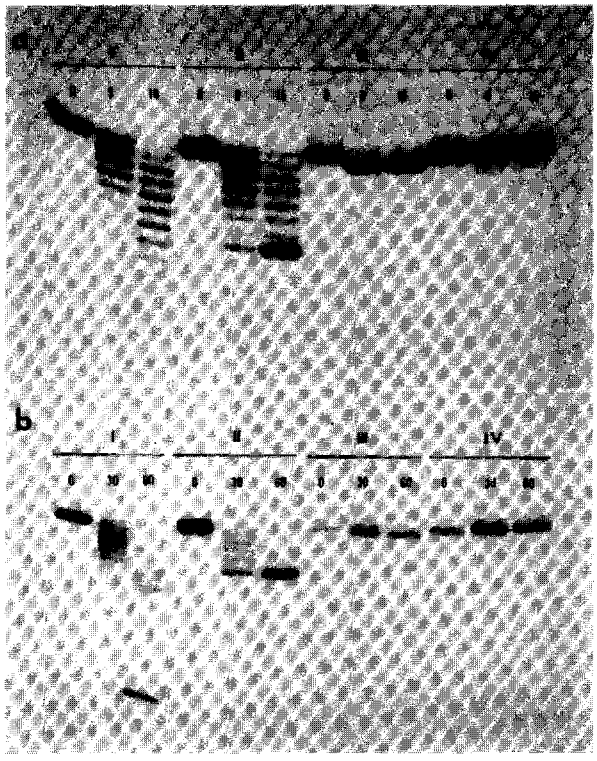

Figure 2. Enzymatic Characterization of Pentadecadeoxynucleotides of Deoxycytidine Having Variable Numbers and Positions of Phosphorodithioate Internucleotide Linkages. 5'- and 3'end labelings of compounds I-IV were with T4-polynucleotide kinase and terminal deoxynucleotidyl transferase using γ-^{32}P ATP and α-^{32}PddATP, respectively.[25] Equal quantities of labeled compounds I-IV were treated with the same number of snake venom or spleen phosphodiesterase units, quenched (after the times (min) listed on the gels) with formamide and heating, and then analyzed. Panel a, analysis with snake venom phosphodiesterase. Panel b, analysis with spleen phosphodiesterase. I, dC15 unmodified; II, d(C-C-C-C-C-C-CpC-C-C-C-C-C-C-C); III, d(C-CpC-C-C-C-C-C-C-C-C-CpC-C); IV, d(CpC-CpC-CpC-CpC-CpC-CpC-CpC-C). The symbols p and - refer to phosphorodithioate and natural phosphate diester internucleotide linkages, respectively.

These results provide insights on the nuclease resistance of phosphorodithioate internucleotide linkages and outline a new route for incorporating this analog into synthetic DNA. Thus under conditions where both snake venom and spleen phosphodiesterases degrade natural DNA, phosphorodithioate moieties appear to be completely resistant. If these observations can be extended to other exo- and endonucleases as well, phosphorodithioate analogs may be very useful for various diagnostic and therapeutic applications, for studying protein-DNA interactions *in situ*, and for co-crystallization experiments involving nucleases with polynucleotides. Moreover the synthetic method outlined here leads to the preparation of a protected phosphorodithioate directly and does not require, as in a previously published procedure,[4] the isolation of intermediates. Further research directed toward the development of phosphorodiamidite mononucleotides which are stable to purification but still highly reactive under coupling conditions may lead to a methodology for incorporating these synthons into an automated polymer support procedure.

Acknowledgements

We wish to thank P. Sadecky and R. Barkley for the FAB Mass spectral analysis and M. Ashley for his help with the ^{31}P-NMR. This work was supported by NIH (GM25680) and a NATO fellowship to A.G. This is paper XXVI on Nucleotide Chemistry. Paper XXV is reference 5. Current addresses: A. Grandas, University of Barcelona, Barcelona, Spain; J. Nielsen, University of Copenhagen, Copenhagen, Denmark.

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5. W. K.-D. Brill, J. Nielsen, and M. H. Caruthers, *Tetrahedron Lett.*, in press.
6. Deoxycytidine oligomers were selected for synthesis because these compounds as phosphorothioates have been shown to inhibit HIV (ref. 3). Biochemical results with deoxycytidine oligomers containing phosphorodithioates will be published separately.
7. J. E. Marugg, A. Burik, M. Tromp, G. A. van der Marel, and J. H. van Boom, *Tetrahedron Lett.* **27**, 2271 (1986).
8. ^{1}H- and ^{31}P-NMR were recorded in $CDCl_3$ unless otherwise specified with tetramethylsilane and 85% H_3PO_4 as reference standards, respectively. The matrix used for FAB MS was magic bullet. R_f values refer to separations on silica gel plates using solvent A (dichloromethane:methanol, 90:10) or solvent B (chloroform:ethylacetate:triethylamine, 45:45:10).
9. 5'-O-Dimethoxytrityl-N4-toluoyldeoxycytidinyl-3'-O-(5'-O-N4-toluoyldeoxycytidinyl-3'-O-phenoxyacetyl) diisopropylphosphoramidite (**2**): ^{31}P-NMR δ 149.3 and 149.1. R_f(B) = 0.48.
10. Conversion to **3** was complete in 15 min (^{31}P-NMR δ 193.4 in the reaction mixture.)
11. 5'-O-Dimethoxytrityl-N4-toluoyldeoxycytidine-3'-O-(5'-O-N4-toluoyldeoxycytidinyl-3'-O-phenoxyacetyl)-S-(4-chlorobenzyl)phosphorodithioate (**4**): ^{31}P-NMR δ 97.5 and 96.7; FAB^- mass spectrum, 882 $(DMTdCtolPS_2\ R3O)^-$, 714 $(dCtolphacPS_2\ R3O)^-$. R_f (A) = 0.79.
12. A completely protected dithymidine phosphorodithioate also was synthesized using the same procedure (91% yield after column chromatography).
13. H. Koster, K. Kulinowski, T. Liese, W. Heikens, and V. Kohli, *Tetrahedron* **37**, 363 (1981).
14. 5'-O-Dimethoxytrityl-N4-toluoyldeoxycytidine: FAB^+ mass spectrum, 648 $(M + H)^+$, 303 $(DMT)^+$, 230 $(dCtol + 2H)^+$; FAB^- mass spectrum, 646 $(M - H)^-$, 228 $(dCtol)^-$. R_f (A) = 0.60.
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16. 3'-O-Phenoxyacetyl-N4-toluoyldeoxycytidine: FAB^+ mass spectrum, 480 $(M + H)^+$, 230 $(dCtol + 2H)^+$; FAB^- mass spectrum, 478 $(M - H)^-$, 228 $(dCtol)^-$. R_f (A) = 0.67.
17. 5'-O-Dimethoxytrityl-N4-toluoyldeoxycytidine-3'-O-(5'-O-N4-toluoyldeoxycytidinyl)-S-(4-chlorobenzyl)phosphorodithioate (**5**). ^{31}P-NMR δ 96.5 and 96.2; FAB^- mass spectrum, 1211 $(M - H)^-$, 882 $(DMTdCtolPS_2\ R3O)^-$, 580 $(dCtolPS_2\ R3O)^-$. R_f (A) = 0.62.
18. 5'-O-Dimethoxytrityl-N4-toluoyldeoxycytidine-3'-O-(5'-O-N4-toluoyldeoxycytidine-3'-O-β-cyanoethyl-N,N-diisopropylaminophosphine)-S-(4-chlorobenzyl)phosphorodithioate (**6**). ^{31}P-NMR δ 149.5, 149.2 and 149.0 (amidite) and δ 96.5 and 96.0 (dithioate). R_f (B) = 0.47.
19. S. L. Beaucage and M. H. Caruthers, *Tetrahedron Lett.* **22**, 1859 (1981).
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22. Although the 4-chlorobenzyl was found to be removed in solution with a $t_{1/2}$ = 10 min with dinucleoside phosphorodithioates, some S-protected dithioate still remained after 1.5-6 hr with the pentadecamers. Current studies involve investigations using the 2,4-dichlorobenzyl protecting group.
23. A dinucleoside phosphorodithioate was previously shown to be resistant to snake venom phosphodiesterase under conditions where the natural linkage was completely degraded (reference 4).
24. Since all commercial snake venom phosphodiesterase preparations have variable amounts of endonuclease, extensive treatment with these enzyme samples yields further degradation. For example with III, further degradation yields ^{32}P-labeled 14mer, trimer, and monomer.
25. $MgCl_2$ was used rather than $CoCl_2$ in the 3'-labeling experiment as theformation of stable cobalt-dithioate complexes would have unduly complicated the results.

(Received in USA 26 October 1988)