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SYNTHESIS AND CHARACTERIZATION OF DINUCLEOSIDE PHOSPHORODITHIOATES

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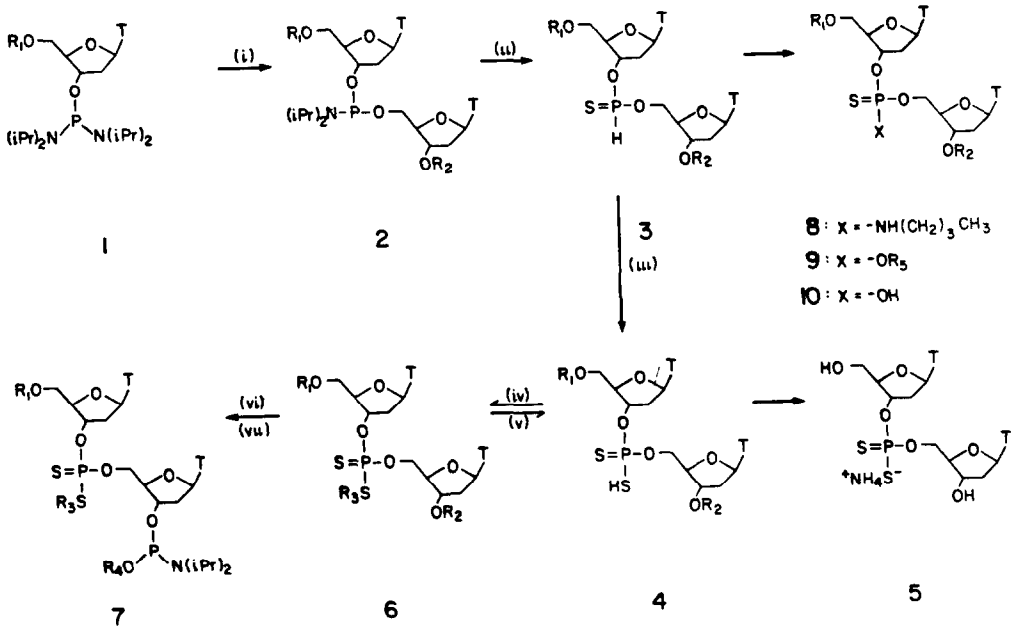
Dinucleoside phosphoramidites, H_2S, and tetrazole react to form dinucleoside H-phosphonothioates. Oxidation with sulfur yields phosphorodithioates. Iodine oxidation in the presence of amines, alcohols, or water yields phosphorothioamidates, thiotriesters or thiodiesters.

Polynucleotide analogs having modifications at phosphorus are receiving increasing attention as potential therapeutic reagents[1,2,3] and for studies on the interactions of nucleic acids with enzymes[4,5] or repressors.[6] These reports have demonstrated that versatile procedures are needed for the synthesis of both chiral and achiral phosphorus analogs. Here we report a route to the synthesis of dideoxynucleoside phosphorodithioates - an attractive new group of nucleotide analogs which are achiral, conserve the ionic character of the internucleotide linkage, and are resistant to nucleases. We also demonstrate that the synthetic method for preparing these analogs can be extended to generate dideoxynucleoside phosphorothioamidates, alkyl phosphorothioates and phosphorothioates.



Synthesis of Pentavalent Dinucleotide Sulfur Derivatives. (i) 3'-O-acetylthymidine; (ii) tetrazole + H_2S; (iii) sulfur; (iv) α,2,4-trichlorotoluene; (v) triethylammonium thiophenolate; (vi) *t*-butylamine; (vii) tetrazole + bis(diisopropylamino)-2-cyanoethoxy phosphine. Abbreviations: R_1, dimethoxytrityl, DMT; R_2, acetyl,Ac; R_3, 2,4-dichlorobenzyl, dcb; R_4, 2-cyanoethyl; R_5, 9-anthracenylmethyl; iPr, isopropyl; T, thymine.

The first step leading to **3**, a key intermediate useful for the synthesis of various phosphorothioate analogs, is condensation of 5'-O-dimethoxytritylthymidine with bis(diisopropylamino)chlorophosphine in dioxane containing triethylamine.[7] The resulting phosphorodiamidite (**1**) is reacted without isolation with 3'-O-acetylthymidine to yield homogeneous **2** in 62% yield after silica gel chromatography (5% triethylamine in ethylacetate). Synthesis of **3** proceeds by dissolving **2** (470 mg, 0.5 mmol) in acetonitrile (5 ml), bubbling H_2S through the solution for 1 min, adding tetrazole (35 mg, 0.5 mmol in 1 ml acetonitrile), and finally stirring the sealed reaction flask for 16 h.[8] The reaction mixture was concentrated to a gum on a rotary evaporator, redissolved in ethylacetate (50 ml) and extracted twice with 2 M triethylammonium bicarbonate (pH 7.4, 20 ml each). After concentrating *in vacuo* to a gum, compound **3** was dissolved in dichloromethane (5 ml) and isolated by precipitation into pentane (400 mg, 90%).[9,10]

Dithymidine phosphorodithioate in protected form (**4**) was synthesized by stirring **3** (104 mg, 0.1 mmol in 1 ml dichloromethane) with elementary sulfur (1 mmol in 2 ml toluene:2,6-lutidine, 19:1, v/v) for 0.5 h. Purification via silica gel column chromatography (0-12% methanol in dichloromethane and 0.5% triethylamine) afforded 70% isolated yield.[11] So far all attempts to synthesize **3** or **4** from nucleoside monoester H-phosphonothioate have failed.[12] The dinucleoside phosphorodithioate was deprotected by standard procedures[13] and isolated in 86% yield after ether extractions (3x), sephadex G10 gel filtration (H_2O), and lyophilization as the ammonium salt (compound **5**).[14] When **5** was phosphorylated with T4-polynucleotide kinase and [γ-^{32}P]ATP, the rate of kination was approximately one-half that of unmodified 3'-5' dithymidine phosphate under identical conditions. Further testing of **5** with snake venom phosphodiesterase (*Crotalus adamanteus* venom, Sigma) indicated that the phosphorodithioate was stable using conditions where the natural dinucleotide was completely hydrolyzed (assayed by reverse phase HPLC). Compound **5** was also observed to be stable to conc. ammonium hydroxide at 55°C (16 h), as no degradation or isomerization was observed (^{31}P NMR, TLC). The chemical stability of a dinucleoside phosphorodithioate is not surprising considering the wide industrial applications of phosphorodithioates which range from oil additives[15] to pesticides.[16]

In order to introduce the phosphorodithioate linkage into oligonucleotides, a protection/deprotection scheme for the phosphorodithioate internucleotide linkage was developed. Based upon earlier research involving protection of the P-S bond in methylphosphonothioate deoxynucleoside derivatives,[17] the 2,4-dichlorobenzyl group was tested and found to be completely satisfactory for this purpose. Thus, **4** (57 mg, 0.06 mmol) was alkylated with α,2,4-trichlorotoluene (50 µl, 1 h, 55°C) in acetonitrile to yield **6** quantitatively.[18] Further testing of **6** revealed that it was completely stable to reagents used in DNA synthesis (1% trifluoroacetic acid in dichloromethane and iodine in aqueous lutidine/THF) and that the phosphorodithioate triester was specifically S-dealkylated by treatment with thiophenolate (thiophenol:triethylamine:dioxane, 1:1:2, v/v/v, $t_{1/2}$ = 3 min at rt). Conversion of **6** to a synthon useful for DNA synthesis (**7**) was a two step process. **6** was first deacylated using 0.15 M *tert*-butylamine in methanol (0°C, 10 h) and purified by silica gel chromatography. Less than 5% cleavage of the internucleotide linkage (^{31}P NMR, TLC) was observed.[19] The deacylated compound was then reacted with bis(diisopropylamino)-2-cyanoethoxy phosphine (1.5 eq) in the presence of tetrazole (1 eq, 1 h at rt)[20] to yield **7** (76%).[21] The resulting dinucleotide phosphoramidite has been used successfully in com-

bination with unmodified mononucleotide phosphoramidites[22] for the synthesis of a 26-mer *lac* operator fragment containing the phosphorodithioate linkage between positions 8-9 (98.2% coupling efficiency).[23]

The dinucleoside H-phosphonothioate was also found to be useful as a versatile synthon for preparing several analogs (**8-10**) rapidly (5 min) in quantitative yield (^{31}P NMR). Thus, when oxidized with iodine/n-butylamine the phosphorothioamidate (**8**) was isolated in 92% yield[24] and similarly the triester phosphorothioate (**9**) was formed with iodine/9-anthracenyl methanol (10 eq) under anhydrous conditions.[25] Treatment with an aqueous solution of iodine/pyridine[13] gave the dinucleoside phosphorothioate (**10**) in 87% yield.[26]

These results outline methods for synthesizing dithymidine phosphorodithioate and several additional sulfur containing internucleotide linkages. This synthetic route can now be extended to the other deoxynucleosides. Because the dinucleoside phosphorodithioate linkage is electronically and sterically similar to natural DNA and appears to be chemically and enzymatically stable, we anticipate that oligonucleotides containing this linkage may be useful as hybridons[1] for inhibiting biochemical processes *in vivo* and as potential antivirals. By oxidation in the presence of various amines or alcohols, several new classes of polynucleotides having intercalators, spin labels, or site specific reactive residues for various biophysical and biochemical applications are also possible. Since phosphorodithioates are alkylated under facile conditions, chemically labile reporter groups can also be introduced into deprotected, synthetic DNA via mild S-alkylation at these linkages when they are located at specific sites.

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8. The rate of reaction was dependent upon the tetrazole concentration (see B. H. Dahl, J. Nielsen and O. Dahl, *Nucleic Acids Res.* **15**, 1721, 1987). With 2 eq of tetrazole, the reaction was complete in 1 h (greater than 99%).
9. ^{1}H NMR and ^{31}P NMR were recorded in $CDCl_3$ unless otherwise specified. Trimethylsilane and 85% H_3PO_4 were external standards, respectively. R_f values are from TLC analysis on silica gel.
10. 5'-O-Dimethoxytritylthymidine-3'-O(5'-O-thymidylyl-3'-O-acetyl) H-phosphonothioate (**3**). FAB^+ mass spectrum, 527 (anhydro DMT dT); FAB^- mass spectrum, 890 (M^-), 623 (DMT dT-3'-PHO_2^-), 363 (M-527, 5'-PHO_2^--dT-3'-OAc); ^{31}P NMR δ 71.7 and 70.7 ($^{1}J_{HP}$ = 673.8 Hz and 676.3 Hz); ^{1}H NMR δ 7.81 and 7.80 (P-H, $^{1}J_{HP}$ = 671.4 Hz and 676.7 Hz), 7.55 and 7.53 (s, H_6),

7.37-7.20 (m, aromatic), 6.82 (d, J = 8.8 Hz, DMT), 6.49 and 6.26 (m, $H_{1'}$), 5.49 and 5.25 (m, $H_{3'}$), 4.35 (m, $H_{4'}$), 4.19 (m, $H_{5'}$), 4.07 (m, $H_{4'}$), 3.76 s, MeO-DMT), 3.42 (m, $H_{5'}$), 2.54-2.32 (m, $H_{2'}$), 2.08 and 2.07 (2 x s, CH_3-acetyl), 1.90 (m, CH_3-T), 1.43 (s, CH_3-T). R_f = 0.35 and 0.28 (methanol/dichloromethane, 1:9, v/v).

11. 5'-O-Dimethoxytritylthymidine-3'-O(5'-O-thymidylyl-3'-O-acetyl) phosphorodithioate (**4**). FAB^+ mass spectrum, 303 (DMT^+); FAB^- mass spectrum, 921 (M^-), 395 (5'-PS_2O^--dT-3'-OAc); ^{31}P NMR δ 112.7; ^1H NMR δ 8.12 (s, NH), 7.90 and 7.60 (2 x s, H_6), 7.40-7.24 (m, aromatic), 6.80 (d, J_{HP} = 8.8 Hz, DMT), 6.43 (m, $H_{1'}$), 5.46-5.36 (m, $H_{3'}$), 4.40 (m, $H_{4'}$), 4.16 (m, $H_{5'}$), 3.76 (s, MeO-DMT), 3.52 (m, $H_{5'}$), 2.28 (m, $H_{2'}$), 2.05 (CH_3-acetyl), 1.97 (CH_3-T), 1.58 (s, CH_3-T). R_f = 0.14 (methanol/dichloromethane, 1:9, v/v).
12. Analogously this reaction pathway has been used successfully for unmodified DNA and RNA synthesis (see P. S. Gregg, J. Lindh, T. Regberg, J. Stawinski and R. Stromberg, *Tetrahedron Lett.* **27**, 4051, 1987; B. C. Froehler, P. G. Ng, and M. D. Matteucci, *Nucleic Acids Res.* **14**, 5399, 1986).
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14. Thymidine 3'-O-(5'-O-thymidylyl)phosphorodithioate (**5**). FAB^+ mass spectrum, 579 (M); ^{31}P NMR (D_2O) δ 113.3; ^1H NMR δ 7.60 and 7.46 (2 x s, H_6), 6.11 and 5.99 (m, $H_{1'}$), 5.17 (m, $H_{3'}$), 4.85 (m, $H_{3'}$), 4.15 (m, $H_{4'}$), 4.03 and 3.62 (m, $H_{5'}$), 2.21 (m, $H_{2'}$), 1.88 m, CH_3-T). R_f = 0.25 (methanol/triethylamine/chloroform, 15:1:84, v/v/v).
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18. 5'-O-Dimethoxytritylthymidine S-(2,4-dichlorobenzyl)-3'-O-(5'-O-thymidylyl-3'-O-acetyl)-phosphorodithioate (**6**). FAB^+ mass spectrum, 527 (anhydro DMT dT); FAB^- mass spectrum, 922 (M + 1-dichlorobenzyl), 813 (DMT dT-3'-PSOS-dcb), 553 (5'-PSOS-dcb-dT-3'-OAc); ^{31}P NMR (CH_3CN, ext. lock) δ 94.4 and 93.7, ^1H NMR δ 7.55 and 7.52 (2 x s, H_6), 7.37-7.23 (m, aromatic), 6.81 (d, J = 4.6 Hz, DMT), 6.34 and 6.28 (m, $H_{1'}$), 5.38 and 5.01 (m, $H_{3'}$), 4.24-4.08 (m, CH_2-benzyl, $H_{5'}$ + $H_{4'}$), 3.76 (s, MeO-DMT), 3.42 (m, $H_{5'}$), 2.39 (m, $H_{2'}$), 2.08 (s, CH_3-acetyl), 1.89 and 1.87 (2 x s. CH_3-T), 1.43 and 1.42 (2 x s, CH_3-T). R_f = 0.74 (methanol/triethylamine/chloroform, 15:1:84, v/v/v).
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21. 3'-(Dinucleoside phosphorodithioate) N,N-diisopropyl-O-2-cyanoethylphosphoramidite (**7**). ^{31}P NMR δ 149.4, 149.2, 148.9 and 97.2, 95.7, 95.5. ^1H NMR δ 7.56 (s, H_6), 7.33-7.27 (m, aromatic), 6.84 (d, J = 8.5 Hz, DMT), 6.39-6.29 (m, $H_{1'}$), 5.44 (m, $H_{3'}$), 3.79 (s, MeO-DMT), 1.90 (s, CH_3-T), 1.45 (s, CH_3-T), 1.18 (d, J = 6.6 Hz, CH_3-iPr). R_f = 0.29 and 0.17 (chloroform/ethylacetate/triethylamine, 45:45:10, v/v/v).
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23. These results will be published separately. See reference 6 for the *lac* operator numbering scheme and a rationale for modification at this internucleotide phosphate.
24. 5'-O-Dimethoxytritylthymidine-3'-O-(5'-O-thymidylyl-3'-O-acetyl)-N-butylphosphorothioamidate (**8**). FAB^- mass spectrum, 961 (M^-), 695 (DMT dT-3'-POSNHBU), 434 (5'-POSNHBU-dT-3'-OAc); ^{31}P NMR δ 74.4 and 74.0; ^1H NMR δ 8.36 and 8.34 (2 x s, NH), 7.59 and 7.56 (2 x s, H_6), 7.44-7.24 (m, aromatic), 6.82 (d, J = 8.7 Hz, DMT), 6.41 and 6.28 (m, $H_{1'}$), 5.28 and 5.23 (m, $H_{3'}$), 4.21 and 4.13 (m, $H_{4'}$(2 x) + $H_{5'}$), 3.77 (s, MeO-DMT), 3.43 (m, $H_{5'}$), 2.94 (m, CH_2-N), 2.41 (m, $H_{2'}$), 2.09 and 2.07 (2 x s, CH_3-acetyl), 1.93 and 1.88 (2 x s, CH_3-T), 1.42 (s, CH_3-T), 1.39-1.23 (m, CH_2), 0.90 and 0.83 (2 x t, J = 7.2 Hz and 7.1 Hz, CH_3). R_f = 0.56 (methanol/dichloromethane, 1:9, v/v).
25. 5'-O-Dimethoxytritylthymidine-3'-O-(5'-O-thymidylyl-3'-O-acetyl)-O-(9-anthracenylmethey)-thiophosphate (**9**). FAB^+ mass spectrum, 527 (anhydro DMT dT); FAB^- mass spectrum, 906 (M-anthracenylmethyl), 639 (DMT dT-3'-PSO_2^-), 379 (5'-PSO_2^--dT-3'-OAc). ^{31}P NMR δ 51.7 and 51.0. R_f = 0.41 (methanol/dichloromethane, 1:9, v/v).
26. 5'-O-Dimethoxytritylthymidine-3'-O-(5'-O-thymidylyl-3'-O-acetyl)phosphorothioate (**10**). FAB^- mass spectrum, 906 (M^-), 603 (M-DMT), 379 (5'-PSO_2^--dT-3'-OAc) ^{31}P NMR δ 60.2 and 60.0.

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