

Synthesis of Oligodeoxynucleoside Phosphorodithioates via Thioamidites

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Received November 10, 1988

Oligonucleotide analogues having modifications in the phosphate diester linkage are generating increasing interest as potential therapeutic or diagnostic reagents¹⁻⁴ and as novel tools for studying various biochemical processes.⁵⁻⁷ One especially attractive analogue is the phosphorodithioate internucleotide linkage (nucleoside-OPS₂O-nucleoside) because it is isosteric and isopolar with the normal phosphodiester and should have other biochemical and biophysical properties similar to natural DNA. It is also nuclease resistant^{8,9} and easily derivatized with reporter groups¹⁰ which are useful chemical properties for a large number of biochemical and biological applications. Here we outline a procedure for the synthesis of oligodeoxynucleotides bearing any predetermined combination of phosphorodithioate and natural phosphate linkages or exclusively phosphorodithioate moieties.

The synthons for accomplishing these goals are the deoxynucleoside 3'-pyrrolidinyl- or deoxynucleoside 3'-(dimethylamino)phosphorothioamidites (Figure 1, part A, compounds **3a-p**). Attempts to prepare these compounds by condensation of a protected deoxynucleoside with either *S*-(4-chlorobenzyl)-*N,N,N'*,*N'*-tetramethylphosphorothiodiamidite or *S*-(4-chlorobenzyl)dipyrrolidinylphosphorothiodiamidite failed because these diamidites were unstable toward vacuum distillation and, unlike *S*-(4-chlorobenzyl)-*N,N,N',N'*-tetraisopropylphosphorothiodiamidite,¹¹ could not be purified by crystallization. Therefore a new, extremely simple procedure has been developed which also appears quite promising for preparing deoxynucleoside phosphoramidites. In a typical experiment, dipyrrolidinylchlorophosphine or bis(dimethylamino)chlorophosphine (0.6 mmol) is added to the appropriately protected deoxynucleoside (0.5 mmol) in 4 mL of acetonitrile:triethylamine (2:1, v/v). Immediately a precipitate appears. After 5 min, either 4-chlorobenzyl- or 2,4-dichlorobenzylmercaptan (1 mmol) is added, and the reaction mixture is evaporated to a glass in vacuo. After dissolving the reaction products in 4 mL of acetonitrile:triethylamine (2:1, v/v), TLC and ³¹P NMR (Figure 2A) indicate essentially complete conversion to the phosphorothioamidite without formation of the deoxynucleoside 3'-phosphorodithioate or bis(deoxynucleoside 3')-phosphorothioate. These results as well as those reported previously during studies on the formation of deoxynucleoside phosphoramidites¹² indicate that selective activation of a single phosphorus-nitrogen bond in phosphorodiamidites is possible with the appropriate choice of an amine salt as the acid catalyst. Following further dilution with 30 mL of ethyl acetate, sequential extraction

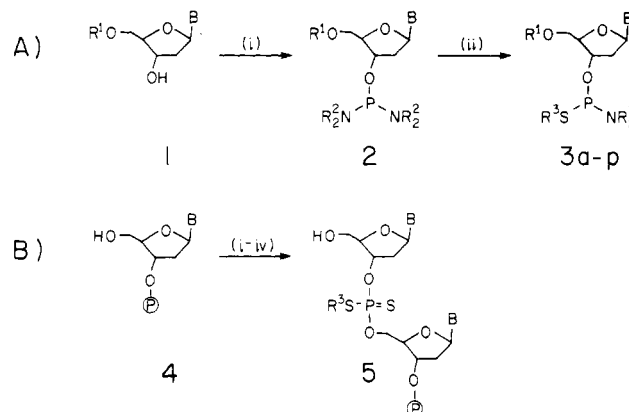


Figure 1. Synthesis of dithioate DNA. Part A, synthesis of deoxynucleoside phosphorothioamidites: (i) dipyrrolidinylchlorophosphine or bis(dimethylamino)chlorophosphine; (ii) 4-chlorobenzyl or 2,4-dichlorobenzylmercaptan. Part B, synthesis of dithioate DNA on a polymer support: (i) compound **3a-p** + tetrazole; (ii) sulfur; (iii) acetic anhydride; (iv) trichloroacetic acid. Abbreviations: R¹, dimethoxytrityl; R², (CH₂)₄ or (CH₃)₂; R³, 4-chlorobenzyl or 2,4-dichlorobenzyl; B, thymine (T), *N*-benzoylcytosine (C^{Bz}), *N*-benzoyladenine (A^{Bz}), or *N*-isobutyl-tyr-guanine (G^{IB}); ⊕, silica support; compounds **3a-p** are defined in Table I.

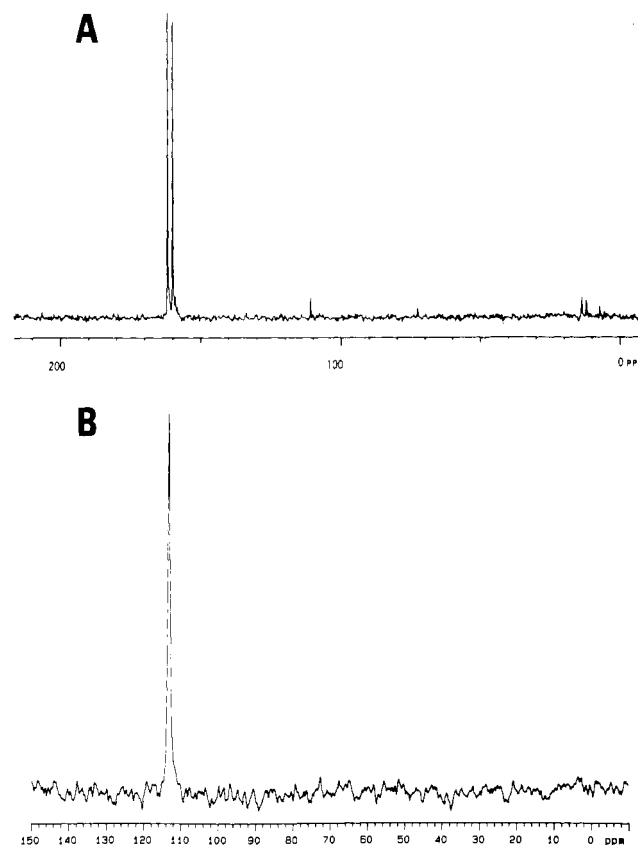


Figure 2. ³¹P NMR spectra of nucleoside and polynucleotide derivatives: panel A, spectra of compound **3f** prior to aqueous extraction; panel B, spectra of d(C)₁₅ containing exclusively phosphorodithioate internucleotide linkages (113 ppm in D₂O). ³¹P NMR spectra were recorded on a Bruker WM-250 (panel A) and a Varian VXR-500S (panel B). Aqueous 85% H₃PO₄ was the external standard.

with sodium bicarbonate and brine, drying over anhydrous sodium sulfate, and precipitation into pentane, the deoxynucleoside phosphorothioamidites are isolated in 80–85% yield (see Table I for ³¹P NMR data).

With use of a deoxynucleoside attached covalently to a silica support,¹³ synthesis of oligodeoxynucleotides containing phos-

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Table I. ^{31}P NMR Characterization of Deoxynucleoside Phosphorothioamidites

compd	base (B)	amidite	R ³	^{31}P NMR (δ) ^a
3a	T	pyrrolidinyl	2,4-dichlorobenzyl	164.8, 161.8
3b	T	pyrrolidinyl	4-chlorobenzyl	164.2, 161.0
3c	T	dimethylamino	4-chlorobenzyl	172.3, 170.5
3d	T	dimethylamino	2,4-dichlorobenzyl	172.1, 170.4
3e	C ^{Bz}	pyrrolidinyl	2,4-dichlorobenzyl	165.1, 162.6
3f	C ^{Bz}	pyrrolidinyl	4-chlorobenzyl	161.8, 159.9
3g	C ^{Bz}	dimethylamino	4-chlorobenzyl	171.9, 170.7
3h	C ^{Bz}	dimethylamino	2,4-dichlorobenzyl	172.0, 171.0
3i	A ^{Bz}	pyrrolidinyl	2,4-dichlorobenzyl	163.8, 162.7
3j	A ^{Bz}	pyrrolidinyl	4-chlorobenzyl	163.5, 162.3
3k	A ^{Bz}	dimethylamino	4-chlorobenzyl	171.8, 170.9
3l	A ^{Bz}	dimethylamino	2,4-dichlorobenzyl	171.7, 170.9
3m	G ^{IB}	pyrrolidinyl	2,4-dichlorobenzyl	163.9, 160.9
3n	G ^{IB}	pyrrolidinyl	4-chlorobenzyl	163.4, 161.6
3o	G ^{IB}	dimethylamino	4-chlorobenzyl	171.5, 169.5
3p	G ^{IB}	dimethylamino	2,4-dichlorobenzyl	171.9, 169.6

^a ^{31}P NMR were recorded in CDCl_3 on a Bruker WM-250 with 85% aqueous H_3PO_4 as external standard.

phosphorodithioate linkages proceeds according to the reaction sequence outlined in Figure 1, part B. Synthesis begins by reacting a dry acetonitrile solution of compounds 3a-p (10 equiv) and tetrazole (50 equiv) with 1 μmol of 4 for 30 s (step i) followed by a 400-s oxidation with 5% sulfur in pyridine:carbon disulfide (1:1, v/v, step ii).¹⁴ Coupling is performed twice to ensure high yields (greater than 98%). Acylation of unreactive compound 4 (step iii), detritylation (step iv), and various washes are the same as those described previously for synthesizing natural DNA from deoxynucleoside phosphoramidites.^{15,16} Multiple repetitions of this cycle then lead to the synthesis of DNA containing exclusively phosphorodithioate linkages or, when used in combination with deoxynucleoside phosphoramidites, to oligodeoxynucleotides bearing both phosphorodithioate and phosphate internucleotide bonds.

Synthetic oligodeoxynucleotides are isolated free of protecting groups via a two-step protocol (thiophenol:triethylamine:dioxane, 1:1:2, v/v/v for 24 h followed by concentrated ammonium hydroxide for 15 h) and then purified to homogeneity by standard procedures (polyacrylamide gel electrophoresis and reverse phase HPLC).^{17,18} ^{31}P NMR spectra (Figure 2B) of phosphorodithioate DNA indicates that oligonucleotides synthesized by using this approach contain exclusively phosphorodithioate internucleotide linkages. No hydrolysis of these dithioates to phosphorothioates (^{31}P NMR δ 56) or phosphate is observed.

Although two methods have been reported for synthesizing DNA having phosphorodithioate internucleotide linkages, these procedures suffer from substantial limitations. In one case, deoxynucleoside 3'-O-bis(diisopropyl)amidites are used to synthesize phosphorodithioate dinucleotides which are then incorporated into DNA.^{8,9} The approach, however, lacks versatility as 16 synthons are required in order to introduce a dithioate linkage into any DNA sequence. Moreover deoxynucleoside diamidites having sufficient reactivity for efficient internucleotide bond formation are easily hydrolyzed and therefore difficult to incorporate into polynucleotide synthesis procedures. The other approach, which utilizes deoxynucleoside 3'-N,N-diisopropylphosphorothioamidites for preparing dinucleotides,¹¹ is also unsatisfactory for repetitive synthesis on supports. This is because these synthons are relatively inert and are only activated by strong acids such as pyridinium tetrafluoroborate—a reagent that leads to detritylation and side reactions due to long activation times.

The procedures outlined here are completely compatible with current methods for synthesizing natural DNA on silica supports.¹⁶ Thus relatively simple mononucleotide synthons can be used for introducing phosphorodithioate and natural internucleotide linkages in any predetermined sequence. So far pentadecamer homopolymers containing up to 14 dithioate linkages, lac and cro operators with multiple dithioates at defined sites, and a cro operator segment containing 17 contiguous dithioates have been synthesized.¹⁰ We anticipate that this approach using deoxynucleoside phosphorothioamidites as synthons will be the method of choice for preparing dithioate containing DNA.

Acknowledgment. This work was supported by NIH (GM25680). This is paper 28 on nucleotide chemistry. Paper 27 is Brill, et al., *Acta Crystallography*, in press.

Evidence for a Copper-Nitrosyl Intermediate in Denitrification by the Copper-Containing Nitrite Reductase of *Achromobacter cycloclastes*

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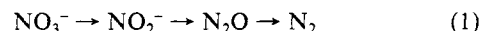
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Received July 25, 1988

Denitrification¹ is the microbial process by which nitrite is reduced to dinitrogen (eq 1). It is an important component of



the global nitrogen cycle that is responsible for regulating the amount of fixed nitrogen available for plant growth.² The key step in the pathway, the reduction of nitrite to gaseous nitrogen oxides, is catalyzed by two classes of nitrite reductase, containing either a heme *cd*₁ unit or copper. The mechanism of nitrite reduction has been extensively investigated in systems known to utilize heme *cd*₁-containing nitrite reductases. These investigations have demonstrated (i) that nitrite and nitrous oxide are free intermediates in the denitrification pathway¹ and (ii) in the *Pseudomonas stutzeri* enzyme at least (and by implication in the other *cd*₁ enzymes) that N_2O formation occurs via nucleophilic attack of a second nitrite molecule³⁻⁶ on an enzyme-bound nitrosyl intermediate^{7,8} (E-NO^+) derived from nitrite via a dehydration reaction. Inasmuch as hemes and heme enzymes readily form NO adducts under a variety of conditions,⁹⁻¹² while no well-

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