Synthesis of Oligodeoxynucleoside Phosphorodithioates via Thioamidites

Wolfgang K.-D. Brill, Jin-Yan Tang, Yun-Xi Ma, and Marvin H. Caruthers*

Department of Chemistry and Biochemistry
University of Colorado
Boulder, Colorado 80309-0215
Received November 10, 1988

Oligonucleotide analogues having modifications in the phosphate diester linkage are generating increasing interest as potential therapeutic or diagnostic reagents[1-4] and as novel tools for studying various biochemical processes.[5-7] One especially attractive analogue is the phosphorodithioate internucleotide linkage (nucleoside–OPS_2O–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 groups[10] 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 ^{31}P NMR (Figure 2A) indicate essentially complete conversion to the phosphorothioamidite without formation of the deoxynucleoside 3′-phosphorodithioite or bis(deoxynucleoside 3′)-phosphorothioite. These results as well as those reported previously during studies on the formation of deoxynucleoside phosphoramidites[12] 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 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 ^{31}P NMR data).

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

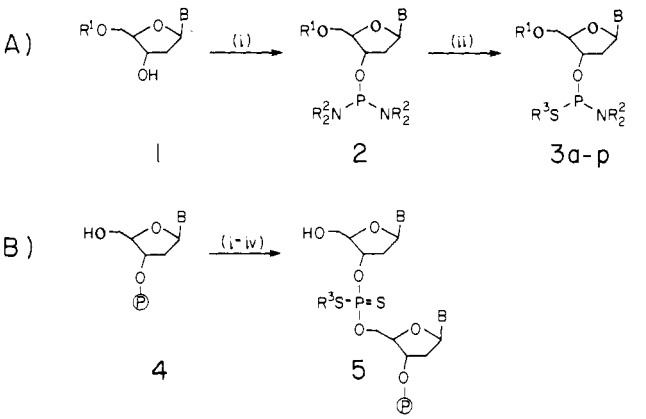

Figure 1. Synthesis of dithioate DNA. Part A, synthesis of deoxynucleoside phosphorothioamidites: (i) dipyrrolidinylchlorophosphine or bis(dimethylamino)chlorophosphine; (ii) 4-chlorobenzyl or 2,4-dichlorobenzylmercaptan. Part B, synthesis of dithioate DNA on a polymer support: (i) compound **3a-p** + tetrazole; (ii) sulfur; (iii) acetic anhydride; (iv) trichloroacetic acid. Abbreviations: R^1, dimethoxytrityl; R^2, $(CH_2)_4$ or $(CH_3)_2$; R^3, 4-chlorobenzyl or 2,4-dichlorobenzyl; B, thymine (T), *N*-benzoylcytosine (C^{Bz}), *N*-benzoyladenine (A^{Bz}), or *N*-isobutyrylguanine (G^{iB}); Ⓟ silica support; compounds **3a-p** are defined in Table I.

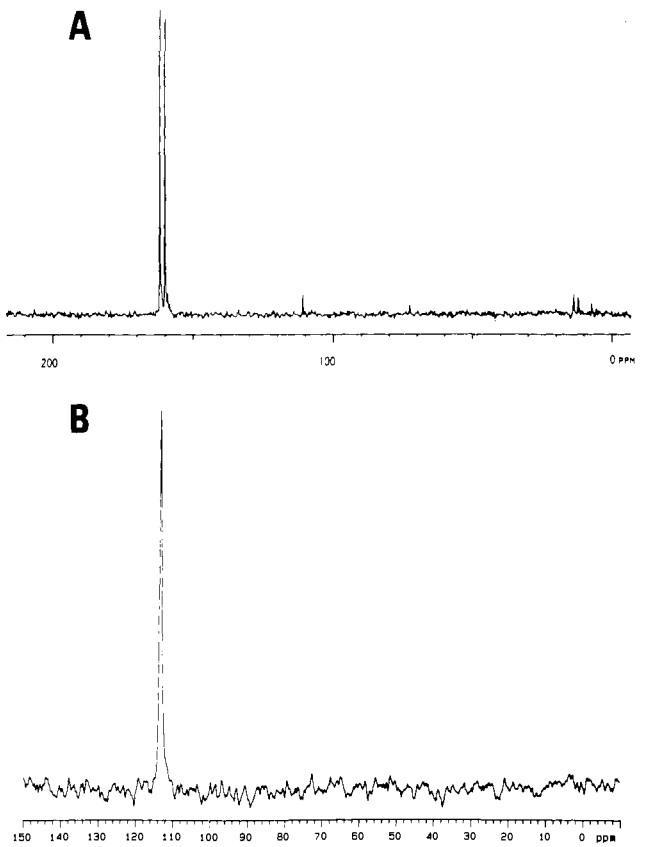

Figure 2. ^{31}P NMR spectra of nucleoside and polynucleotide derivatives: panel A, spectra of compound **3f** prior to aqueous extraction; panel B, spectra of $d(C)_{15}$ containing exclusively phosphorodithioate internucleotide linkages (113 ppm in D_2O). ^{31}P NMR spectra were recorded on a Brucker WM-250 (panel A) and a Varian VXR-500S (panel B) Aqueous 85% H_3PO_4 was the external standard.

(1) Goodchild, J.; Agrawal, S.; Civeira, M. P.; Sarin, P. S.; Sun, D.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A* **1988**, *85*, 5507.
(2) Smith, C. C.; Aurelian, L.; Reddy, M. P.; Miller, P. S.; Ts'o, P. O. P *Proc. Natl. Acad.* Sci. *U.S.A.* **1986**, *83*, 2787.
(3) Matsukura, M.; Shinozuka, K.; Zon, G.; Mitsuya, H.; Reitz, M.; Cohen, J. S.; Broder, S. *Proc. Natl. Acad, Sci. U.S.A.* **1987**, *84*, 7706.
(4) Lemaitre, M.; Bayard, B.; Leblue, B. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 648.
(5) Noble, S. A.; Fisher, E. F.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 3387.
(6) Eckstein, F. *Ann. Rev. Biochem.* **1985**, *54*, 367.
(7) Letsinger, R. L.; Singman, C. N.; Histand, G.; Salunkhe, M. *J. Am. Chem. Soc.* **1988**, *110*, 4470.
(8) Nielsen, J.; Brill, W. K.-D.; Caruthers, M. H. *Tetrahedron Lett.* **1988**, *29*, 2911.
(9) Grandas, A.; Marshall, W.; Nielsen, J.; Caruthers, M. *Tetrahedron Lett.*, in press.
(10) Beaton, G.; Sasmor, H.; Marshall, W.; Caruthers, M. H., unpublished results.
(11) Brill, W. K.-D.; Nielsen, J.; Caruthers, M. H. *Tetrahedron Lett.* **1988**, *29*, 5517.
(12) Barone, A. D.; Tang, J.-Y.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 4051.
(13) Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3185.

0002-7863/89/1511-2321$01.50/0

Table I. ^{31}P NMR Characteriziation 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 Brucker WM-250 with 85% aqueous H_3PO_4 as external standard.

phorodithioate 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*

C. L. Hulse and B. A. Averill*

Department of Chemistry, University of Virginia Charlottesville, Virginia 22901

J. M. Tiedje

Department of Crop and Soil Sciences Michigan State University East Lansing, Michigan 48824

Received July 25, 1988

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

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2 \quad (1)$$

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 pathway[1] 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 molecule[3-6] on an enzyme-bound nitrosyl intermediate[7,8] ($E{\cdot}NO^+$) 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-

(1) Payne, W. J. *Denitrification*; John Wiley and Sons: New York, 1981.
(2) Knowles, R. In *Soil Biochemistry*; Paul, E. A., Ladd, J., Eds.; Marcel Dekker Inc.: New York, 1981, Vol. 5, pp 323-369.
(3) Aerssens, E.; Tiedje, J. M.; Averill, B. A. *J. Biol. Chem.* **1986**, *261*, 9652-9656.
(4) Weeg-Aerssens, E.; Tiedje, J. M.; Averill, B. A. *J. Am. Chem. Soc.* **1987**, *109*, 7214-7215.
(5) Weeg-Aerssens, E.; Tiedje, J. M.; Averill, B. A. *J. Am. Chem. Soc.* **1988**, *110*, 6851-6856.
(6) Shearer, G.; Kohl, D. H. *J. Biol. Chem.* **1988**, *263*, 13231-13245.
(7) Garber, E. A. E.; Hollocher, T. C. *J. Biol. Chem.* **1982**, *257*, 8091-8097.
(8) Kim, C.-H.; Hollocher, T. C. *J. Biol. Chem.* **1987**, *259*, 2092-2099.
(9) Yonetani, T.; Yamamoto, H.; Erman, J. E.; Leigh, J. S., Jr.; Reed G. H. *J. Biol. Chem.* **1972**, *247*, 2447-2455.
(10) Johnson, M. K.; Thomson, A. J.; Walsh, T. A.; Barber, D.; Greenwood, C. *Biochem. J.* **1980**, *189*, 285-294.
(11) Shimada, H.; Orii, Y. *J. Biochem. (Tokyo)* **1978**, *84*, 1553-1558.
(14) Ott, J.; Eckstein, F. *Biochemistry* **1987**, *26*, 8237.
(15) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859.
(16) Caruthers, M. H. *Science* **1985**, *230*, 281.
(17) Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteucci, M.; Stabinsky, Z.; Tang, J.-Y. *Methods Enzymol.* **1987**, *154*, 287.
(18) Currently we prefer the 2,4-dichlorobenzyl over the 4-chlorobenzyl protecting group as it is more easily removed with thiophenol.

0002-7863/89/1511-2322$01.50/0