Synthesis of Phosphorodithioate DNA via Sulfur-Linked, Base-Labile Protecting Groups[1]

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Phosphorodithioate DNA, a new and potentially useful DNA analog with a deoxynucleoside-OPS_2O-deoxynucleoside internucleotide linkage, was synthesized from deoxynucleoside 3′-phosphorothioamidites having a variety of thioesters and thiocarbonates as base-labile phosphorus protecting groups. The major challenge in the synthesis of this DNA analog was to derive a reaction pathway whereby activation of deoxynucleoside 3′-phosphorothioamidites occurred rapidly and in high yield under conditions that minimize Arbuzov rearrangements, exchange reactions, unwanted oxidation to phosphorothioates, and several other side reactions. Of the various phosphorus protecting groups examined for this purpose, a thorough evaluation of these parameters led to the conclusion that β-(benzoylmercapto)ethyl was preferred. Synthesis of phosphorodithioate DNA began by preparing deoxynucleoside 3′-phosphorothioamidites from the appropriately protected deoxynucleoside, tris-(pyrrolidino)phosphine, and ethanedithiol monobenzoate via a one-flask synthesis procedure. These synthons were activated with tetrazole and condensed with a deoxynucleoside on a polymer support to yield the deoxynucleoside thiophosphite. Subsequent steps involved oxidation with sulfur to generate the completely protected phosphorodithioate triester, acylation of unreacted deoxynucleoside, and removal of the 5′-protecting group. Yields per cycle were usually 97−98% with 2−5% phosphorothioate contamination as determined by ^{31}P NMR. By using deoxynucleoside 3′-phosphorothioamidites and deoxynucleoside 3′-phosphoroamidites, deoxyoligonucleotides having phosphorodithioate and the natural phosphate internucleotide linkages in any predetermined order can also be synthesized.

Introduction

Deoxyoligonucleotide analogs bearing modified phosphodiester linkages have been the focus of considerable interest in the antisense field because of their nuclease resistance and thus prolonged biological half-lives.[2−7] Methyl phosphonates and phosphorothioates are the most extensively studied variations to date. The substitution of a single nonbridging oxygen atom with either a methyl group or a sulfur atom renders the internucleotide linkage nuclease resistant, but unlike natural DNA, the modified phosphorus center is chiral which leads to a mixture of unresolvable diastereomeric oligomers having variable biochemical, biophysical, and biological properties. While stereocontrolled synthesis of P-chiral phosphorothioates or methylphosphonates represents one possible solution to this problem,[8,9] another lies in the synthesis of modifications which are achiral at phosphorus.

The substitution of both nonbridging oxygen atoms with sulfur gives rise to a phosphorodithioate linkage, which, like natural DNA, is achiral at phosphorus. This linkage has been found to be highly stable toward both chemical and enzymatic hydrolysis.[10] Recent studies have found that phosphorodithioate DNA oligomers have biophysical and biological properties which make them ideally suited for antisense applications.[11,12] For example, of the many analogs synthesized to date, only two, the phosphorodithioate and phosphorothioate derivatives, have been shown to activate RNase H which appears to be crucial for demonstrating antisense activity *in vivo*.[7b] Phosphorodithioate deoxyoligonucleotides have also been found to be potent inhibitors of HIV-1 reverse tran-

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(1) This is paper 42 in a series on Nucleotide Chemistry. For paper 41, see: Fischer, R. W.; Caruthers, M. *Tetrahedron Lett.* **1995**, *36*, 6807. This research was supported by the National Institutes of Health (Grant GM25680) and a Postdoctoral Fellowship from the American Cancer Society (PF-3465) to W.T.W. Abbreviations: DMT, 4,4′-dimethoxytrityl; CPG, controlled pore glass; T, thymine; CBz, 4-*N*-benzoylcytosine; C-Isob, 4-*N*-isobutyrylcytosine; ABz, 6-*N*-benzoyladenine; G-Isob, 2-*N*-isobutyrylguanine; G-Pac, 2-*N*-phenylacetylguanine; Ph, phenyl; Ac, acetyl; Prop, propyl; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Nuc, 2′-deoxynucleoside; B(prot), N-protected 2′-deoxynucleoside pyrimidine or purine base; FMOC, 9-fluorenylmethyloxycarbonyl; DMAP, 4-(*N*,*N*-dimethylamino)-pyridine; TEA, triethylamine.

(2) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543−584.

(3) Stein, C. A.; Tonkinson, J. L.; Yakubov, L. *Pharmacol. Ther.* **1991**, *52*, 365−371.

(4) Agrawal, S. *TIBTECH* **1992**, *10*, 152−155.

(5) Goodchild, J. *Bioconjugate Chem.* **1990**, *2*, 165−186.

(6) Cohen, J. S., Ed. *Oligonucleotides: Antisense Inhibitors of Gene Expression*; Topics in Molecular and Structural Biology; MacMillan Press: London, 1989; Vol. 12.

(7) (a) Stein, C. A.; Cohen, J. S. *Cancer Res.* **1988**, *48*, 2659−2668. (b) Stein, C. A.; Cheng, Y.-C. *Science* **1993**, *261*, 1004−1012.

(8) Stec, W. J.; Grajkowski, A.; Koziolkiewicz, M.; Uznanski, B. *Nucleic Acids Res.* **1991**, *19*, 5883−5888.

(9) Stec, W. J.; Lesnikowski, Z. J. in *Methods in Molecular Biology, Protocols for Oligonucleotides and Their Analogs, Synthesis and Properties*; Agarwal, S., Ed.; Humana Press: Clifton, NJ, 1993; pp 285−313.

(10) (a) Nielsen, J.; Brill, W. K.-D.; Caruthers, M. H. *Tetrahedron Lett.* **1988**, *29*, 2911−2914. (b) Grandas, A.; Marshall, W. S.; Nielsen, J.; Caruthers, M. H. *Tetrahedron Lett.* **1989**, *30*, 543−546. (c) Porritt, G. M.; Reese, C. B. *Tetrahedron Lett.* **1989**, *30*, 4713−4716. (d) Okruszek, A.; Sierzchala, A.; Feason, K. L.; Stec, W. J. *J. Org. Chem.* **1995**, *60*, 6998−7005.

(11) (a) Caruthers, M. H.; Beaton, G.; Cummins, L.; Dellinger, D.; Graff, D. Ma, Y.-X.; Marshall, W. S.; Sasmor, H.; Shankland, P.; Wu, J. V.; Yau, E. K. *Nucleosides Nucleotides* **1991**, *10*, 47−59. (b) Marshall, W. S.; Beaton, G.; Stein, C. A.; Matsukura, M.; Caruthers, M. H. *Proc. Nat. Acad. Sci. U.S.*., **1992**, *89*, 6265−6269. (c) Marshall, W. S.; Caruthers, M. H. *Science* **1993**, *259*, 1564−1570.

(12) (a) Dahl, B. H.; Bjergårde, K.; Sommer, V. B.; Dahl, O. *Acta Chem. Scand.* **1989**, *43*, 896−901. (b) Bjergårde, K. Thesis, University of Copenhagen, 1990. (c) Dahl, B. H.; Bjergårde, K.; Nielsen, J.; Dahl, O. *Tetrahedron Lett.* **1990**, *31*, 3489−3492. (d) Bjergårde, K.; Dahl, O. *Nucleic Acids Res.* **1991**, *19*, 5843−5850. (e) Bjergårde, K. Oligonucleoside Phosphorodithioates, Cand. Scient. Thesis, Kobenhauns Universitet, 1990, pp 61−63.

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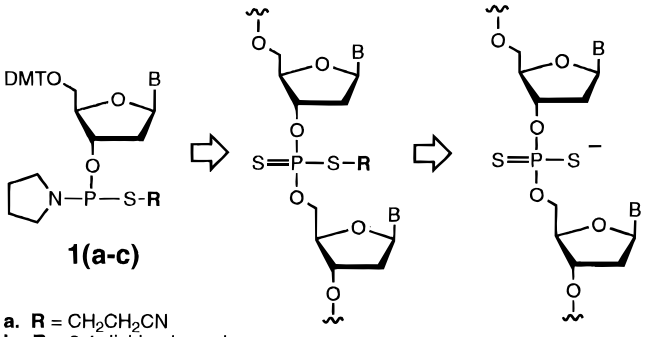
scriptase and of HIV-1 viral replication and thus represent an additional class of potential therapeutic agents against acquired immunodeficiency syndrome.[11b,c]

Since the initial reports on the solution[10a] and solid-phase[13] synthesis of phosphorodithioate DNA, there have been numerous synthetic efforts directed toward the development of this chemistry.[10b−d,12,14] The solid-phase synthesis of phosphorodithioate DNA oligomers is similar to conventional methods for synthesizing deoxyoligonucleotides,[15] but there are several essential modifications since two sulfur atoms are introduced into the phosphate linkage. The first sulfur is incorporated through phosphorothioamidite synthon **1** and the second via sulfur oxidation to give phosphorodithiotriester **2**.



Upon completion of automated synthesis, the protecting groups on sulfur and the deoxynucleoside bases are removed and the phosphorodithioate oligomers are detached from the support to yield **3**. The successful synthesis of this analog requires a thorough investigation of various deoxynucleoside phosphorothioamidite synthons, sulfurization parameters, and the conditions associated with removal of protecting groups.

Two different sulfur protecting groups, the β-cyanoethyl and 2,4-dichlorobenzyl, have been used in previous approaches with good but limited success. The β-cyanoethyl group can be removed under the standard ammonium hydroxide treatment that is used to deblock the base protecting groups; however, high levels (8−9%) of contaminating phosphoromonothioate are incorporated into the deoxyoligonucleotide products.[12] This contamination is reduced to more acceptable levels (2−4%) when the 2,4-dichlorobenzyl group is used to block sulfur.[14g] Deprotection of the resulting phosphorodithiotriester linkage **2b** can be cleanly carried out by a post-synthesis treatment with either thiophenolate or an odorless substitute.[16] Unfortunately, the phosphorothioamidite synthons **1b** containing this group are relatively unstable to oxidation which necessitates changing solutions of **1b** on the DNA synthesizer every few hours.

Here we describe investigations with deoxynucleoside 3′-phosphorothioamidites that have a series of sulfur protecting groups. These studies include an analysis of reaction pathways and a characterization of side products resulting from the synthesis of the deoxynucleoside 3′-phosphorothioamidites, sulfurization of protected thiophosphite triesters, and deprotection of dithioate oligomers. These studies led to the development of a novel base-labile β-(benzoylmercapto)ethyl protecting group, its incorporation into deoxynucleoside 3′-phosphorothioamidites (**1c**), and the use of this synthon in the preparation of phosphorodithioate DNA. This protecting group combines the best features of the two previously employed for phosphorodithioate synthesis as it has a stability toward oxidation similar to phosphorothioamidites containing a β-cyanoethyl group and its removal can be conveniently carried out during a simple base-deprotection treatment to give phosphorodithioate deoxyoligonucleotides with relatively low levels of phosphorothioate contamination.

Results and Discussion

Model Studies. Previous studies had shown that protection of sulfur with the 2,4-dichlorobenzyl group gave rise to lower levels of phosphorothioate impurity than those attained using β-cyanoethyl protection.[14f] While a variety of mechanisms have been proposed to account for the generation of the phosphorothioate impurity,[12d,e] selectivity of deprotection would appear to be an important factor. In particular, nucleophilic displacement with thiophenolate of the 2,4-dichlorobenzyl protecting group should be a more selective process than removal of the β-cyanoethyl group with ammonium hydroxide.

As a consequence of these considerations, a protecting group for sulfur was sought which could be eliminated via intramolecular nucleophilic displacement. Use of the β-mercaptoethyl group in deoxyoligonucleotide synthesis has been previously reported. In these studies, the thiol is masked as either a trityl thioether[17] or a disulfide.[18,19] Release of the free thiol and treatment with base results in elimination of ethylene sulfide to give the desired phosphate product. We adapted this "protected" protecting group for blocking sulfur in phosphorodithioates. The strategy involved masking the β-mercaptoethyl group with a variety of thioesters or thiocarbonates that could be readily eliminated under ammonia conditions.

Protection of phosphorodithioates with β-(acylmercapto)ethyl groups was initially evaluated on O,O-diethyl *S*-(β-(acylmercapto)ethyl) phosphorodithioate model compounds **4a**−**d** where the acyl groups were varied from acetate (**4a**) to pivaloate (**4d**). These compounds were easily obtained from two independent routes: alkylation of *O,O*-diethyl dithiophosphoric acid (**6**) with esters of bromoethanethiol or acylation of *O,O*-diethyl S-(β-mercaptoethyl) phosphorodithioate (**5**). Deprotection under basic conditions to the desired phosphorodithioate (**6**) was

(13) Brill, W. K.-D.; Tang, J.-Y.; Ma, Y.-X.; Caruthers, M. H. *J. Am. Chem. Soc.* **1989**, *111*, 2321−2322.

(14) (a) Farschtschi, N.; Gorenstein, D. G. *Tetrahedron Lett.* **1988**, *29*, 6843−6846. (b) Brill, W. K.-D.; Yau, E. K.; Caruthers, M. H. *Tetrahedron Lett.* **1989**, *30*, 6621−6624. (c) Stawinski, J.; Thelin, M.; Zain, R. *Tetrahedron Lett.* **1989**, *30*, 2157−2160. (d) Yau, E. K.; Ma, Y.-X.; Caruthers, M. H. *Tetrahedron Lett.* **1990**, *31*, 1953−1956. (e) Porritt, G. M.; Reese, C. B. *Tetrahedron Lett.* **1990**, *31*, 1319−1322. (f) Beaton, G.; Brill, W. K.-D.; Grandas, A.; Ma, Y.-X.; Nielsen, J.; Yau, E.; Caruthers, M. H. *Tetrahedron* **1991**, *47*, 2377−2388. (g) Beaton, G.; Dellinger, D.; Marshall, W. S.; Caruthers, M. H. In *Oligonucleotides and Analogues: A Practical Approach*;, Eckstein, F., Ed.; IRL Press: 1991; pp 109−135. (h) Piotto, M. E.; Granger, J. N.; Cho, Y.; Farschtschi, N.; Gorenstein, D. G. *Tetrahedron* **1991**, *47*, 2449−2461. (i) Caruthers, M. H. Beaton, G.; Wu, J. V.; Wiesler, W. *Methods Enzymol.* **1992**, *211*, 3−20.

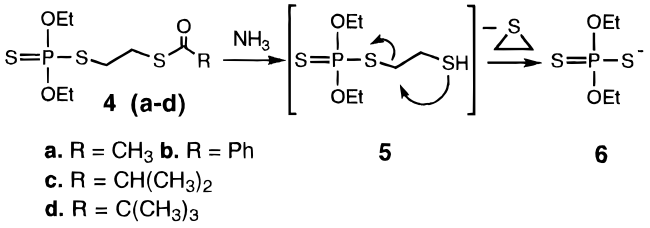
(15) Caruthers, M. H. *Science* **1985**, *230*, 281−285.

(16) Dahl, B. H.; Bjergårde, K.; Henriksen, L.; Dahl, O. *Acta Chem. Scand.* **1990**, *44*, 639−641.

(17) Conolly, B. A. *Tetrahedron Lett.* **1987**, *28*, 463−466.

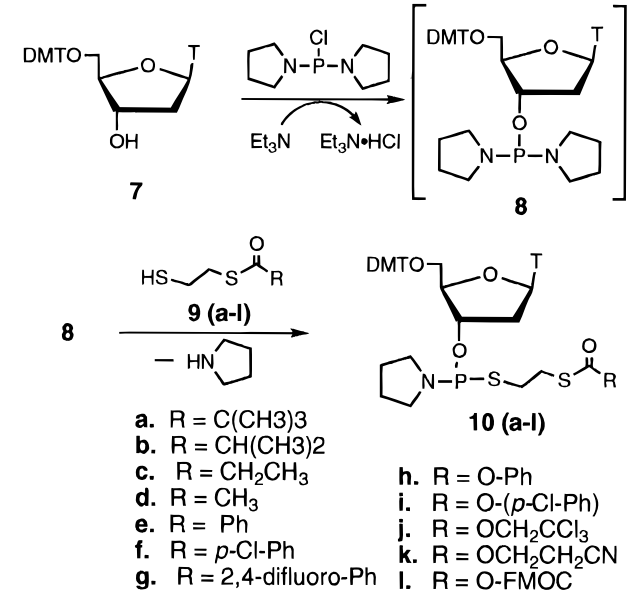
(18) (a) Asseline, U.; Bonfils, E.; Kurfürst, R.; Chassignol, M.; Roig, V.; Thuong, N. T. *Tetrahedron* **1992**, *48*, 1233−1254. (b) Asseline, U.; Thuong, N. T. *Tetrahedron Lett.* **1989**, *30*, 2521−2524.

(19) (a) Kumar, P.; Bose, N. K.; Gupta, K. C. *Tetrahedron Lett.* **1991**, *32*, 967−970. (b) Gupta, K. C.; Sharma, P.; Kumar, P.; Sathyanarayana, S. *Nucleic Acids Res.* **1991**, *19*, 3019−3025.



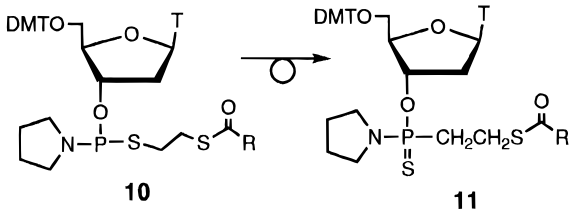
confirmed by ^{31}P NMR. The rates of deprotection were dependent upon the lability of the acyl group as well as the amine base used. A variety of primary and secondary amines were examined in addition to ammonia with pyrrolidine giving the fastest deprotection (seconds with acetate **4a**, minutes with benzoate **4b**). While the acetate and benzoate compounds were fully deprotected within an hour regardless of the amine used, deprotection of the pivaloyl derivative **4d** required several hours. The model compounds were found to be stable to tetrazole, iodine/water oxidation, sulfur oxidation, and acetic anhydride capping conditions which indicated that the acyl-protected β-mercaptoethyl group was compatible with DNA synthesis protocols.

Synthesis of *S*-(β-(Acylmercapto)ethyl) Phosphorothioamidites. Preliminary studies focused on the preparation of various thymidine phosphorothioamidites for subsequent comparison in automated phosphorodithioate oligothymidylate synthesis on a solid support. These synthons, in which the sulfur was protected with various β-(acylmercapto)ethyl groups, were prepared via a modified version of previously reported syntheses.[12c,13] The 5′-dimethoxytrityl protected thymidine **7** was first phosphitylated with bis(pyrrolidinyl)chlorophosphine to give thymidine bis(pyrrolidino) diamidite **8**. In the same pot,



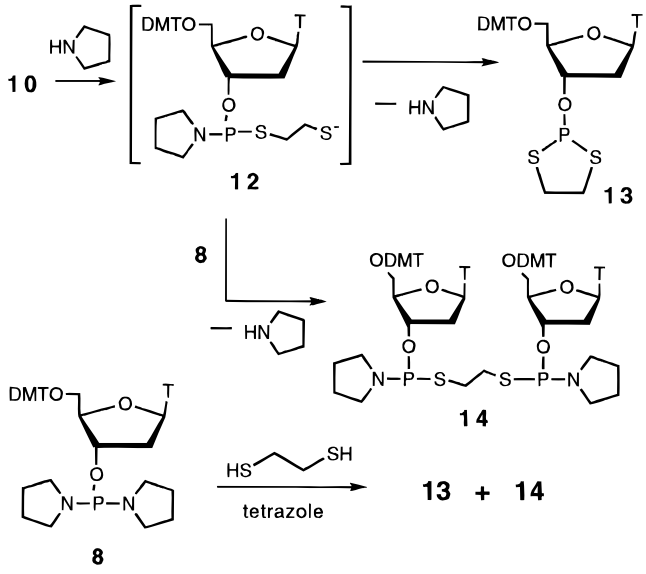
this intermediate was converted to the desired thymidine phosphorothioamidites **10** upon addition of ethanedithiol monoacylates **9**. Following an aqueous workup and drying, the products were isolated by precipitation from heptane to give stable powders. Purity of the phosphorothioamidites (159, 163 ppm) as analyzed by ^{31}P NMR was typically 85−90%.

Attempts to purify the phosphorothioamidites resulted in degradation and rearrangement, most notably to the Arbuzov-type product **11** (89 ppm). Thus the phosphorothioamidites were used in deoxyoligonucleotide synthesis without further purification even though a number



of penta- and trivalent phosphorus impurities were detected at low levels. Two of these, the oxidation (30 ppm) and hydrolysis (13 ppm) products, each typically at the 2−4% level, were not considered to adversely affect deoxyoligonucleotide synthesis, as these pentavalent phosphorus compounds were unreactive under coupling conditions. However, the participation of trivalent phosphorus impurities in side reactions could negatively affect the quality of deoxyoligonucleotide products. One of these reactive impurities, the bis(pyrrolidino) phosphorodiamidite **8** (133 ppm), was initially observed at the level of 1%, but was found to be eliminated by increasing the reaction time.

Three other trivalent phosphorus impurities were not as easily minimized. Two of these were by-products from the undesired deacylation of the β-mercaptoethyl group during phosphorothioamidite synthesis. One equivalent of pyrrolidine was released upon conversion of phosphorodiamidite **8** to phosphorothioamidite **10**. Pyrrolidine was found to be a very effective sulfur deprotection agent, particularly with the more labile (acetyl and benzoyl) thioesters.[20] Thus during phosphorothioamidite synthesis, the released pyrrolidine caused formation of deacylated product **12**, which upon intramolecular cyclization

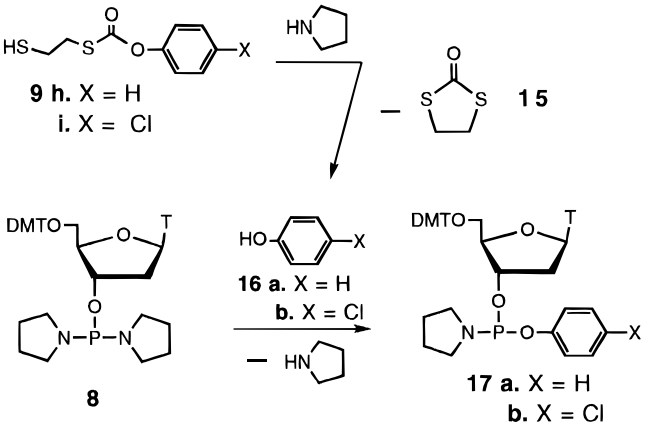


gave 2−6% dithiophosphite byproduct **13** (149 ppm). To a lesser extent, the deacylated intermediate **12** participated in an intermolecular condensation with the phosphorodiamidite **8** to yield the phosphorothioamidite dimer **14** (157.6, 157.9, 160.9 and 161.2 ppm). Reaction of the phosphorodiamidite **8** with ethanedithiol gave these two products exclusively (**13** and **14**), which confirmed that

(20) Previous work had shown that it was essential to retain in the deoxynucleoside phosphorothioamidite an amine with properties similar to pyrrolidine if these synthons were to be reactive in DNA synthesis.[21]

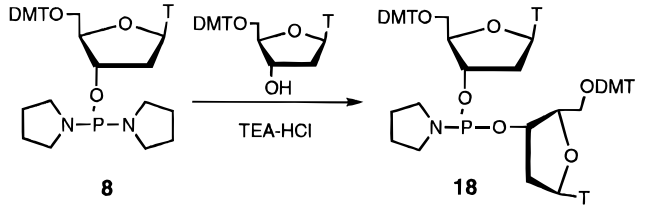
they were indeed deprotection byproducts. While phosphorothioamidite dimer **14** was expected to result in unwanted side reactions during deoxyoligonucleotide synthesis, the major byproduct, dithiophosphite **13**, was found to be unreactive under standard coupling conditions.

Synthesis of phosphorothioamidites with carbonate protection of the β-mercaptoethyl group was plagued by formation of an additional deprotection byproduct. In the presence of released pyrrolidine, phenyl thiocarbonates **9h** and **9i** degraded to give dithiocarbonate **15** and phenols **16a,b**. The addition of tetrazole to the reaction (to increase the rate and buffer released pyrolidine) reduced but did not eliminate this problem. Condensation of phenols with phosphorodiamidite **8** gave rise to phosphoroamidites **17** (140, 141 ppm). These byproducts



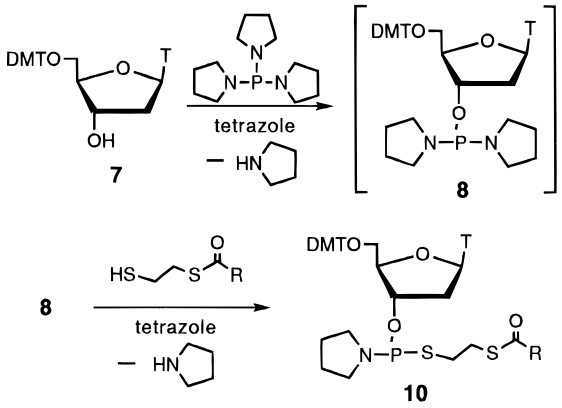
(**17**) were more reactive than phosphorothioamidites under deoxyoligonucleotide coupling conditions and form phosphorothioates upon oxidation of this internucleotide phosphite triester with sulfur. Thus the quality of phosphorodithioate deoxyoligonucleotides made from phosphorothioamidites having carbonate protection was adversely affected due to the increase in phosphorothioate contamination.

Phosphorothioamidites prepared via phosphitylation with bis(pyrrolidinyl)chlorophosphine were routinely contaminated with 1% dithymidine phosphoroamidite **18** (142 ppm). It presumably formed as the phosphitylation neared completion, at which point the triethylamine hydrochloride salt catalyzed condensation of **8** with unreacted starting material. The resulting dinucleoside

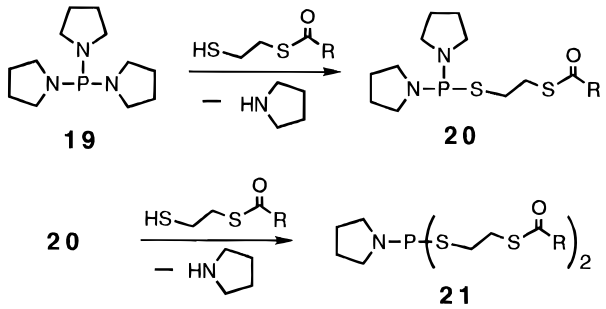


phosphoroamidite was more reactive using tetrazole-catalyzed coupling conditions than **10** and thus gave rise to greater than 1% side reactions under deoxyoligonucleotide synthesis conditions where the thioamidite synthon was used in excess. The resulting adducts introduced branching points in deoxyoligonucleotides and yielded unwanted higher molecular weight products (see below).

Efforts to prevent formation of the reactive contaminant **18** by varying base, solvent, temperature, and order of addition were not successful. However, it was found that an alternative phosphitylation procedure using tris(pyrrolidino)phosphine under tetrazole catalysis did not generate this byproduct. While this approach offered a

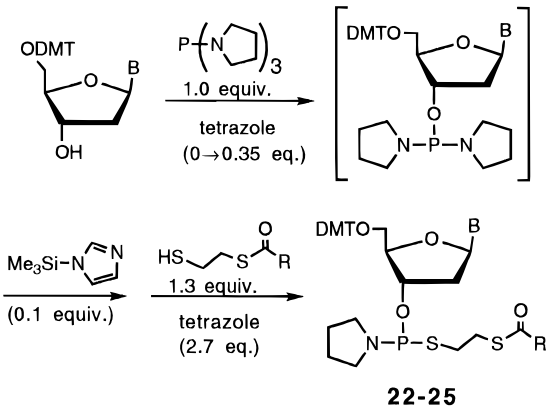


means of eliminating the dinucleoside phosphoroamidite contamination, it gave rise to increased levels of deprotection byproducts **13** and **14** because large amounts of pyrrolidine were generated. Not only were 2 equiv formed during the reaction sequence but additional quantities of pyrrolidine were released from reaction of excess phosphine **19** and thiol to yield phosphine adducts **20** and **21**. This adverse effect of pyrrolidine was



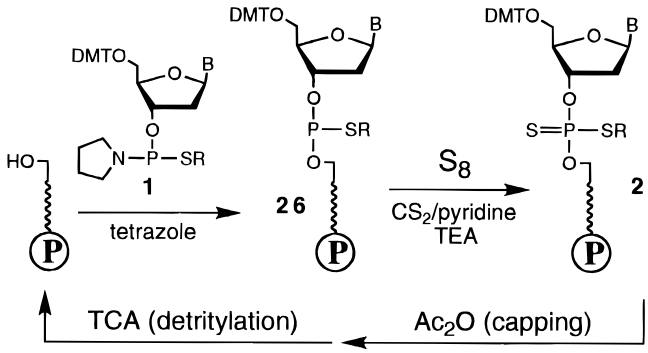
countered in two ways. First, the amount of tetrazole present in the reaction mixture was increased to 3 equiv upon addition of thiol, as it buffered the pyrrolidine produced and increased the rate of reaction. Reducing the excess phosphine (1.3 to 1.0 equiv) and thiol (2 to 1.3 equiv) resulted in a further decrease of the deprotection byproducts. However, without using excess phosphine, the dinucleoside phosphoroamidite contaminant **18** reappeared at levels of 1−2%, presumably due to underphosphitylation of deoxynucleoside. Unreacted deoxynucleoside subsequently condensed with bis(pyrrolidino) phosphorodiamidite **8** upon addition of the large tetrazole excess. This problem was solved by addition of 0.1 equiv of (trimethylsilyl)imidazole to convert unphosphitylated deoxynucleoside to its silyl ether. In this manner, phosphorothioamidites **22**−**25** were prepared completely free from contaminating dinucleoside phosphoroamidite **18** and contained very low levels (1−4%) of deprotection byproducts **13** and **14**. All four bases with a variety of protecting groups were conveniently prepared in multigram quantities by this approach.

Deoxyoligonucleotide Synthesis. The synthesis of phosphorodithioate containing deoxyoligonucleotides from phosphorothioamidite synthons **1**, **10**, and **22**−**25** followed previously reported procedures.[14i] After conventional detritylation of a deoxynucleoside linked to CPG, the phosphorothioamidites were coupled to the support-linked, growing deoxyoligonucleotide via conventional



22 a. B = T, R = Ph
b. B = T, R = Ac
23 a. B = C^{bz}, R = Ph
b. B = C^{bz}, R = Ac
c. B = C^{bz}, R = Prop
d. B = C^{ib}, R = Ph
24 a. B = A^{bz}, R = Ph
b. B = A^{bz}, R = Ac
25 a. B = G^{ib}, R = Ph
b. B = G^{ib}, R = Ac
c. B = G^{pac}, R = Ph

tetrazole activation in two 45 s pulses. The resulting thiophosphite triester **26** was oxidized with elemental sulfur to the desired phosphorodithioate triester. Unreacted CPG-bound deoxynucleoside was then capped with acetic anhydride and the cycle continued.

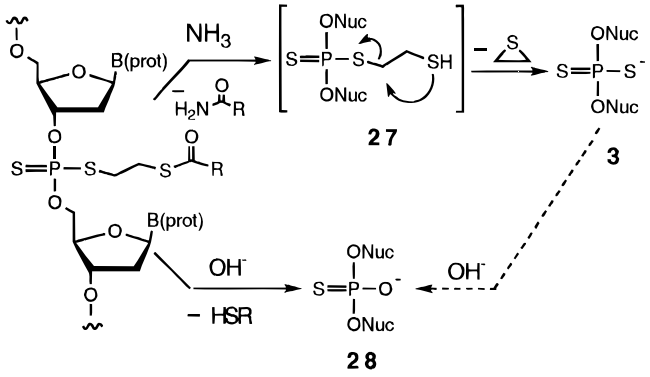


Initial ^{31}P NMR studies to evaluate the phosphorothioamidites having varied acyl protection of the β-mercaptoethyl group focused upon side-by-side comparisons of oligothymidylate products. In the first study, the level of phosphorothioate impurity (55 ppm) in octathymidylates having phosphorodithioates at all seven linkages was found to be directly related to the lability of the thioester group. Pivaloyl protection gave rise to a high level of thioate (11.5%, Table 1, entry 1), which was reduced using the more labile acetate (8.0%, entry 4) or benzoate (4.8%, entry 6). These differences can be attributed to the relative rates of two competing processes: deacylation and subsequent elimination of ethylene sulfide to yield the desired phosphorodithioate **3** versus hydroxide attack at phosphorus to displace thiol and generate the phosphorothioate impurity **28**. By treating the support with pyrrolidine prior to the standard ammonia deprotection, the levels of phosphorothioate were further reduced (entries 5 and 7), thus supporting the rationale that phosphorothioate derives largely from hydroxide attack at phosphorus. However, as has been shown previously, the phosphorothioate impurity was only 1% in model experiments on the synthesis of phosphorodithioate dimers in solution with β-cyanoethyl,[12] 2,4-dichlorobenzyl,[21a] or β-(benzoylmercapto)-ethyl[22] protection on sulfur. Thus the higher level of phosphorothioate observed when preparing deoxyoligonucleotides on a support was peculiar to this mode of synthesis.

Table 1. Synthesis of Oligothymidylate Phosphorodithioates[a]

entry	deoxyoligo-thymidylate	phosphoro-thioamidite	deprotection[b]	phosphoro-thioate, %
1	$T(sT)_7$	**10a**	NH_4OH	11.5
2	$T(sT)_7$	**10b**	pyrrolidine	9.5[c]
3	$T(sT)_7$	**10c**	NH_4OH	9.2
4	$T(sT)_7$	**10d**	NH_4OH	8.0
5	$T(sT)_7$	**10d**	pyrrolidine/NH_4OH	5.2[d]
6	$T(sT)_7$	**10e**	NH_4OH	4.8
7	$T(sT)_7$	**10e**	pyrrolidine/NH_4OH	3.7[d]
8	$T(sT)_7$	**10e**	NH_3·MeOH/NH_4OH	3.8[e]
9	$T(sT)_7$	**10e**	NH_3·EtOH, 24 h, rt	3.7[e]
10	$T(sT)_7$	**10e**	NH_3·MeOH, 24 h, rt	4.0[e]
11	$T(sT)_7(pT)_7$	**10e**	NH_3·MeOH, 24 h, rt	3.4[e]
12	$T(pT)_7(sT)_7$	**10e**	NH_3·MeOH, 24 h, rt	1.5[e]
13	$T(pT)_7(sT)_7$	**10e**	NH_3·MeOH	2.0[e]
14	$T(pT)_7(sT)_7$	**10e**	NH_4OH/EtOH (1:1)	2.3
15	(same oligo as entry 14 re-treated with NH_4OH/EtOH 15 h, 55 °C)			4.9

[a] Percentage of phosphorothioate was determined by ^{31}P NMR (see Experimental Section). s = phosphorodithioate; p = phosphate diester. [b] Unless otherwise indicated, deprotection was completed at 55 °C in a sealed vial for 15 h. [c] 500 μL pyrrolidine, 11 h at 55 °C; workup by addition of 500 μL of water, removal of liquid, and then washing beads with 500 μL of water. [d] 500 μL pyrrolidine, 55 °C for 1 h, addition of 500 μL of concd ammonium hydroxide, proceed at 55 °C, 15 h. [e] Ammoniacal methanol and ethanol were prepared by bubbling anhydrous ammonia through these anhydrous alcohols.



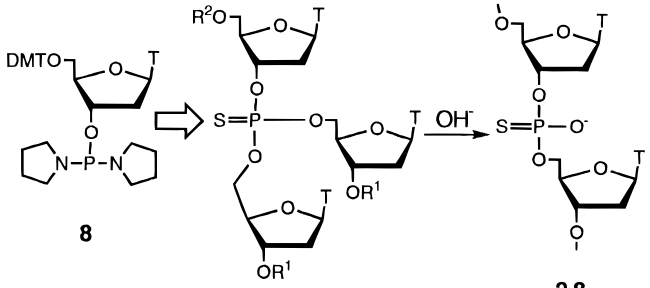
Because previous studies had not implicated deprotection procedures as a major cause of phosphorothioate impurities,[12] alternative sources of this contaminant were also investigated. Variation of sulfurization conditions and reaction time gave essentially the same levels of phosphorothioate. While incomplete sulfurization of thiophosphite triester **26** can lead to phosphorothioate formation (via oxidation of **26** upon subsequent exposure to TCA), it did not appear to be significant. Doubling the coupling time, sulfurization time, or both had no affect upon the level of phosphorothioate impurity.

In a previous study using β-cyanoethyl phosphorothioamidites, the high levels (8−9%) of phosphorothioate impurity were attributed in part to the tetrazole-catalyzed dismutation of phosphorothioamidites to bisamidites of type **8**. Under synthesis conditions this

(21) (a) Brill, W. K.-D.; Nielsen, J.; Caruthers, M. H. *J. Am. Chem. Soc.* **1991**, *113*, 3972−3980. (b) Beaton, G.; Caruthers, M. H., unpublished results.

(22) Goodman, B.; Wiesler, W. T.; Caruthers, M. H., unpublished results.

reactive impurity couples to hydroxyls of two growing, support-linked oligomers to give the branched thiotriester linkage **29a**, which during deprotection slowly hydrolyzes to phosphorothioate **28** and its 5′-5′ isomer.[12e] The ^{31}P

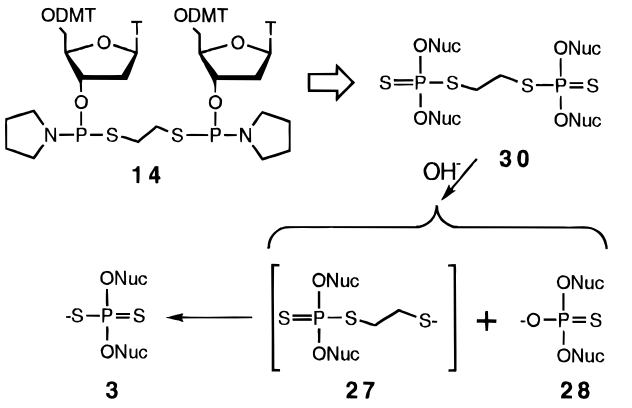


29 **a**. R^1, R^2 = PS_2ONuc
b. R^1= Ac, R^2= DMT

NMR of model compound **29b** (66.6 ppm) confirmed that the thiotriester **29a** (64.9, 66.4 ppm) was a minor impurity in phosphorodithioate deoxyoligonucleotides as synthesized on CPG. Experiments on supports[22] where phosphorothioamidites were spiked with high levels of bisamidite **8** demonstrated that this impurity could be the causative agent of thiotriester **29a** and the resulting increased level of phosphorothioate contamination in phosphorodithioate deoxyoligonucleotides.

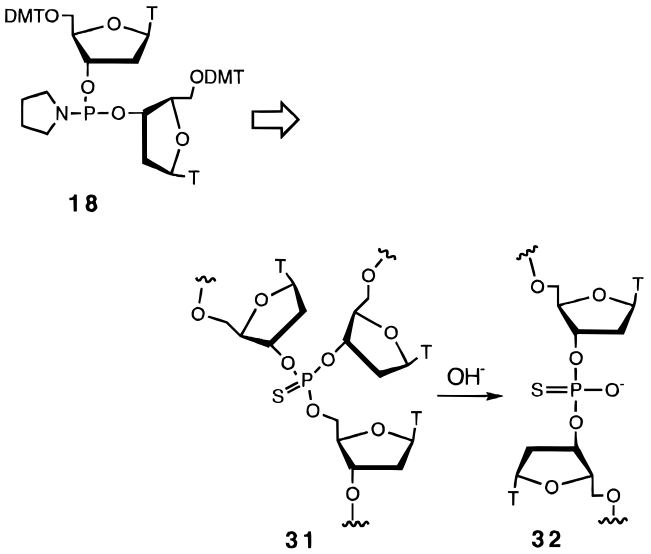
However, the extent to which dismutation contributed to phosphorothioate formation was unclear. Coupling is carried out with two deliveries of fresh phosphorothioamidite and tetrazole to the column, each followed by a 40 s wait, during which time dismutation may have occurred. Extension of this wait period (and thus delaying the delivery of fresh phosphorothioamidite) did not increase the level of phosphorothioate impurity which suggested that dismutation to bisamidite **8** was not a significant source of phosphorothioate. Thus, in our work on supports we attributed the presence of **29a** to bisamidite **8** which was present as an impurity in some of our deoxynucleoside phosphorothioamidite preparations.

Other reactive trivalent phosphorus impurities present in phosphorothioamidites may also have contributed to phosphorothioate formation. One of these impurities, deprotection byproduct **14**, is unique to β-(acylmercapto)-ethyl-protected phosphorothioamidites. Coupling of this dimer byproduct to one hydroxyl on the support would likely be followed by a second coupling to give **30**, in which two parallel deoxyoligonucleotides are linked.



Hydrolysis during ammonia deprotection would introduce phosphorothioate impurity **28** into one deoxyoligonucleotide and the desired phosphorodithioate linkage **3** into the other via β-mercaptoethyl dithiotriester **27**. While contamination of phosphorothioamidites with 2% of byproduct **14** could give rise to an additional 1% phosphorothioate contamination in deoxyoligonucleotides, **14** is sterically hindered relative to phosphorothioamidites **10** and is likely to be less reactive.

An additional minor contaminant observed in the ^{31}P NMR of phosphorodithioate deoxyoligonucleotides was identified as thiotriester **31** (65 ppm). The level of this contamination (typically 1% in initial studies) was directly correlated with the level of dinucleoside amidite **18** in the phosphorothioamidite preparations. Introduction of this branched triester linkage was also correlated to the presence of higher molecular weight bands in crude deoxyoligonucleotides as observed by PAGE. While hydrolysis of triester **31** could contribute to phosphorothioate **32**, introduction of this branched linkage has the potential of diverting significant quantities of deoxyoligonucleotide product to high molecular weight byproducts as **18** is more reactive than **10**. Thus elimination of this impurity was given priority, and an alternative route was later developed for this purpose (see above).



Because initial studies indicated that nonspecific deprotection was the major source of phosphorothioate impurity, additional variations were examined for protecting the β-mercaptoethyl group. Phosphorothioamidites were prepared with more labile benzoyl (**10f**,**g**) and carbonate (**10h**−**l**) groups. Low levels (3.6−5.6%) of phosphorothioate were observed with phenyl carbonate and β-cyanoethyl carbonate protection; however, the deoxyoligonucleotides contained an additional thiotriester contamination (61−63 ppm) owing to the presence of phosphoroamidite impurities of type **17** in the phosphorothioamidites **10h**−**l**. Interestingly, phosphoroamidites bearing the most labile protecting groups (difluorobenzoyl, **10g**; FMOC, **10l**) gave rise to the highest levels of phosphorothioate observed (>15%). These groups were the most hydrophobic of all the variations examined, which suggests that poor solubility of the protected deoxyoligonucleotides in ammonia may increase the extent of nonspecific hydrolysis to phosphorothioate.

In order to improve the solubility of phosphorodithioate deoxyoligonucleotides during deprotection, aqueous ammonia was replaced with various methanolic or ethanolic ammonia mixtures. Clear reductions in the levels of the phosphorothioate impurity were observed (Table 1, en-

Table 2. Synthesis of Deoxyoligonucleotide Phosphorodithioates[a]

entry	deoxyoligonucleotide	deprotection[b,c]	phosphorothioate, %
1	$C(sC)_{13}$	pyrrolidine/NH_4OH	7.0
2	$C(sC)_{13}$	NH_3·EtOH/benzene	2.6
3	$C(sC)_{20}$	NH_3·MeOH/NH_4OH	5.0
4	$C(sC)_{20}$	NH3·EtOH/benzene	3.2[d]
5	$C(sC)_{26}$	NH_3·EtOH/benzene	3.0
6	$C(sC)_{27}$	NH_3·EtOH/benzene (24 h, 4 °C)	3.0[e]
7	CsTsGsTsTsCsGsGsGsCsGsCsCsA	NH_3·EtOH	5.0[f]
8	CsTsGsTsTsCsGsGsGsCsGsCsCsA	NH_3·EtOH	4.2
9	CsTsGsTsTsCsGsGsGsCsGsCsCsA	NH_3·EtOH/benzene	2.9
10	CsTsGsTsTsCsGsGsGsCsGsCsCsA	NH_3·EtOH/benzene (12 h, rt)	3.6[g]
11	CsTsGsTsTsCsGsGsGsCsGsCsCsA	NH_4OH/pyridine/EtOH (1:1:2) 3 d, 4 °C	3.6[g]
12	GsAsCsAsGsAsTsGsCsAsCsCsAsTsTsCsTsG	NH_3·EtOH/benzene	5.0
13	GsAsCsApGsApTsGpCsApCsCpAsTpTsCsTsG	NH_3·EtOH/benzene	4.1
14	GsAsCsApGpAsTpGpCsApCpCsApTpTsCsTsG	NH_3·EtOH/benzene	3.6
15	GsApCsApGsApTsGpCsApCsCpAsTpTsCpTsG	NH_3·EtOH/benzene	3.9
16	GsGsCsAsGsGsAsAsGsAsAsGsCsGsGsAsGsAsCsAsGsCsGsAsCsGsA	NH_3·EtOH/benzene	6.0
17	TsCsGsTsCsTsTsGsTsCsCsCsGsTsCsTsCsGsTsTsGsCsCsCsCsTsC	NH_3·EtOH/benzene	3.7
18	CmCmAmApAsApGsGpCsCpGsApGsApAsGpCmGmAmT	NH_4OH/pyridine/EtOH (1:1:2) 3 d, 4 °C	1.0
19	CmCmAmApApAsGpGpCsCpGpAsGpApAsGpCmGmAmT	NH_4OH/pyridine/EtOH (1:1:2) 3 d, 4 °C	1.0
20	TmCmGmTmCpGsCpTsGpTsCpTsCpCsGpCmTmTmCmT	NH_4OH/pyridine/EtOH (1:1:2) 3 d, 4 °C	1.3
21	TmCmGmTmCpGpCsTpGpTsCpTpCsCpGpCmTmTmCmT	NH_4OH/pyridine/EtOH (1:1:2) 3 d, 4 °C	1.0

[a] Percentage of phosphorothioate was determined by ^{31}P NMR (see Experimental Section). s = phosphorodithioate; p = phosphodiester; m = methylphosphonate. [b] Unless otherwise indicated, deprotection was completed at 55 °C in a sealed vial for 15 h and deoxynucleoside phosphorothioamidites **22a**, **23a**, **24a**, and **25a** were used to prepare the phosphorodithioate deoxyoligonucleotides. [c] Ammoniacal methanol and ethanol were prepared by bubbling anhydrous ammonia through these anhydrous alcohols. Benzene (150 μL) and these ammoniacal solutions (850 μL) were then mixed in order to generate appropriate deprotection reagents. [d] Prepared from thioamidite **23c**. [e] Prepared from thioamidite **23d**. [f] Prepared from thioamidites **22b**, **23b**, **24b**, and **25b.** [g] Prepared from thioamidites **22a**, **23d**, and **25c**.

tries 8-15) where the lowest level (1.5%, entry 12) was with a 15-mer having seven phosphodiester linkages at the 5′-end. The analogous 15-mer with seven phosphodiester linkages at the 3′-end did not show any reduction in phosphorothioate (entry 11). It is difficult to explain these observations without more extensive studies. Because deprotection of normal internucleotide β-cyanoethyl phosphate triesters is very rapid (deoxynucleoside 3′-(β-cyanoethyl phosphoroamidites) are used for synthesizing normal DNA internucleotide linkages) perhaps the oligomer having this type of linkage at the 5′-end would be more accessible to solvent as the diester following deprotection. Solubilization in this manner might therefore lead to a lower phosphorothioate contamination in a manner similar to model studies in solution.

Solubility of protected phosphorodithioate deoxyoligonucleotides having no phosphate internucleotide linkages was enhanced by the addition of benzene to ethanolic ammonia which further reduced the level of phosphorothioate impurity during deprotection. For example, with polydithioate deoxyoligocytidine 14-mers (Table 2, entries 1 and 2) and heterodeoxyoligomers (Table 2, entries 7−9), considerable reduction of phosphorothioate content was observed. In longer deoxyoligonucleotides, however, the level of phosphorothioate contamination was higher with an increasing number of purines. For example, the level of phosphorothioate in a purine rich 27-mer (6.0%, entry 16) was much higher than that for a pyrimidine rich oligomer of the same length (3.7%, entry 17). Again solubilization during deprotection most likely accounted for the difference. Relative to this interpretation of the results, it was interesting to note that the fully deprotected purine rich 27-mer (entry 16) showed limited solubility in water (pyrimidine rich polydithioates were very soluble in water).

Low levels of base-catalyzed desulfurization of phosphorodithioate DNA can also contribute to the generation of phosphorothioate impurity. When a fully deprotected phosphorodithioate deoxyoligonucleotide containing only 2.3% of phosphorothioate impurity was subjected to a second 15 h treatment with base, the level of phosphorothioate increased to 4.9% (Table 1, entry 15). Thus as much as 2−3% of the phosphorothioate contamination may be introduced via desulfurization under standard basic deprotection conditions. This observation quite possibly puts a limit on the extent to which the level of phosphorothioate may be reduced under these conditions.

These methods for synthesizing phosphorodithioate DNA were fully compatible with the procedures previously developed for preparing unmodified as well as phosphorothioate and methylphosphonate DNA. Relative to many biochemical and biological studies that utilize antisense deoxyoligonucleotides, several deoxyoligonucleotides were prepared that contained the phosphorodithioate analog in combination with other types of internucleotide linkages (Table 2, entries 13−15 and 18−21). The lowest levels of phosphorothioate impurity (1%) were observed in a series of 20-mer deoxyoligonucleotides containing four methylphosphonate linkages at each termini and 3-5 phosphorodithioate linkages in the interior of the oligomers (Table 2, entries 18−21). The mild deprotection conditions used for these oligomers (ammonium hydroxide−pyridine−ethanol, 1:1:2, v/v/v for 3 days) were initially considered to be the reason for this low level of contamination. In order to evaluate this possibility, phosphorothioamidites **23d** and **25c** were prepared from deoxynucleosides having more labile base-protecting groups.[23] However, deoxyoligonucleotides prepared from these synthons had the usual levels of phosphorothioate impurity (3.6%) using two different deprotection procedures that were significantly milder than the standard conditions (Table 2, entries 10 and 11). These results suggested that the low levels of phosphorothioate observed in certain examples were more a function of oligomer solubility than the temperature or duration of the deprotection treatment.

(23) Schulhof, J. C.; Molko, D.; Teoule, R. *Nuc. Acids Res.* **1987**, *15*, 397−416.

The hydrophobic properties of phosphorodithioate DNA were revealed not only by solubilization during deprotection but also by a variety of purification procedures.[24] Purification of this analog by HPLC can best be carried out using a divinylbenzene−polystyrene co-polymer reverse phase column (Hamilton PRP-1) where phosphorodithioate DNA oligomers were retained to a greater extent than either their unmodified or phosphorothioate counterparts. Another notable difference was the mobility of phosphorodithioate DNA by PAGE. Under denaturing conditions, phosphorodithioate DNA migrated with a mobility similar to unmodified DNA; however, in nondenaturing gels this analog as a single-stranded, noncomplementary oligomer migrated like natural DNA duplexes of the same length.[25] This observation suggests that single-stranded phosphorodithioate DNA may have some defined rodlike structure. It is possible that the phosphorodithioate internucleotide linkages allow for a greater degree of base stacking than is observed in natural DNA. This could account for the solubility differences observed between deoxyoligopyrimidines and various purine rich phosphorodithioate deoxyoligonucleotides.

Conclusion

A major challenge in the synthesis of phosphorodithioate DNA was to minimize contamination with phosphorothioate internucleotide linkages. A related challenge was to develop a deoxynucloside phosphorothioamidite synthon having chemical properties that lead to high yields of this analog while at the same time producing very few side products. Of the various synthons tested, these objectives were best achieved with the deoxynucleoside 3′-phosphorothioamidites having a β-(benzoylmercapto)ethyl protecting group on sulfur. Examination of the deprotection conditions further demonstrated that ethanolic ammonium hydroxide in benzene minimizes the generation of the phosphorothioate side product.

Experimental Section

General Methods. Protected deoxyribonucleosides were obtained from Cruachem (Sterling, VA). Suppliers for other reagents and solvents were as previously reported.[21a] 1*H*-Tetrazole was sublimed before use. ^{31}P NMR spectra were recorded in either acetonitrile (all phosphorothioamidites) or D_2O (deoxyoligonucleotides). Integrated percentage of phosphorothioate impurity in deoxyoligonucleotides was reproducible to within 0.2%. Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN). DNA synthesis was performed on an Applied Biosystems 380A automated DNA synthesizer using Applied Biosystems 1 μmol CPG columns.

***O,O*-Diethyl *S*-(*β*-(Acetylmercapto)ethyl) Phosphorodithioate (4a).** Diethyl dithiophosphate (3.02 g, 16.2 mmol) and 1-bromoethanethiol acetate[26] (2.97 g, 16.2 mmol) were refluxed in 10 mL of pyridine−dichloromethane (1:1, v/v) for 24 h, after which time starting material was converted to a product of lower R_f (ethyl acetate−hexane, 3:97, v/v). The reaction mixture was diluted with 50 mL of diethyl ether and washed with 4% HCl (50 mL), 5% aqueous sodium bicarbonate (50 mL), and brine (50 mL). After the solution was dried over Na_2SO_4, the diethyl ether layer was concentrated to 4.6 g of a yellow oil and distilled to give the product at 110−120 °C/0.15 mmHg (lit.[27] 135−137 °C/2.5 mmHg). ^{1}H NMR ($CDCl_3$): 4.15 (m, 4H), 3.15 (m, 2H), 2.98 (m, 2H), 2.30 (s, 3H, Me) 1.30 (t, 6H, Me's). ^{31}P NMR: 93.7 ppm. FAB+ MS: 288 (M).

***O,O*-Diethyl *S*-(*β*-(Benzoylmercapto)ethyl) Phosphorodithioate (4b).** The titled compound was prepared from 1-bromoethanethiol benzoate[26] and diethyl dithiophosphate following the procedure for **4a** with filtration of pyridine hydrobromide salt from the reaction mixture prior to aqueous workup. The product could not be distilled but was purified by flash chromatography (R_f = 0.23; ethyl acetate−hexane, 1:9, v/v). This compound was also prepared by treatment of *O,O*-diethyl *S*-(*β*-mercaptoethyl) phosphorodithioate (**5**) (3.3 g, 13.4 mmol) with benzoyl chloride (2.3 g, 16.3 mmol) in pyridine (1 h). Following the same aqueous workups, chromatography, and decolorization with activated charcoal, the intended product was obtained in 77% yield (3.7 g, 10.6 mmol). ^{1}H NMR ($CDCl_3$): 7.95 (m, 2H), 7.56 (m, 1H), 7.44 (m, 2H), 4.13 (m, 4H), 3.30 (dd, 2H), 3.10 (m, 2H), 1.34 (t, 6H, Me's). ^{31}P NMR: 92.6 ppm. FAB+ MS: 351 (M + 1), 245 (M − (benzoyl)).

***O,O*-Diethyl *S*-(*β*-(Isobutylmercapto)ethyl) Phosphorodithioate (4c).** *O,O*-Diethyl *S*-(*β*-mercaptoethyl) phosphorodithioate (**5**) (2.46 g, 10.0 mmol) was treated with isobutyrl chloride (1.3 g, 12.2 mmol) in the presence of Ag_2CO_3 (1.5 g) in dichloromethane (15 mL) to yield the desired product after 5 min when analyzed by TLC. The reaction mixture was diluted with diethyl ether (50 mL), treated with activated charcoal, and filtered through a small bed of Celite and silica, and excess acid chloride was removed *in vacuo*. The desired product was obtained without further purification. ^{1}H NMR ($CDCl_3$): 4.13 (m, 4H), 3.09 (m, 2H), 2.95 (m, 2H), 2.65 (m, 1H), 1.31 (t, 6H, Me's), 1.17 (m, 6H, Me's). ^{31}P NMR: 93.7 ppm.

***O,O*-Diethyl *S*-(*β*−(Pivaloylmercapto)ethyl) Phosphorodithioate (4d).** *O,O*-Diethyl *S*-(*β*-mercaptoethyl) phosphorodithioate (**5**) (2.8 g, 11.3 mmol) was treated with pivaloyl chloride (2.1 g, 17.4 mmol) in the presence of Ag_2CO_3 (3.0 g) in dichloromethane (15 mL). TLC showed the reaction was complete after 5 min. The workup procedure was the same as for **4c**. ^{1}H NMR ($CDCl_3$): 4.13 (m, 4H), 3.08 (m, 2H), 2.95 (m, 2H), 1.31 (t, 6H, Me's), 1.18 (s, 9H, Me's). ^{31}P NMR: 93.7 ppm. FAB+ MS: 331 (M + 1), FAB- MS: 301 (M − (ethyl)).

***O,O*-Diethyl *S*-(*β*-Mercaptoethyl) Phosphorodithioate (5).** Diethyl dithiophosphate (22.5 mL, 135 mmol) and ethylene sulfide (8.5 g, 142 mmol) were refluxed without solvent overnight. The mixture was distilled directly to yield the desired product which was collected at 80−90 °C/0.2 mmHg (lit.[26] 110−112 °C/1.5 mmHg) (13.7 g, 41%). ^{1}H NMR ($CDCl_3$): 4.10 (m, 4H), 3.00 (m, 2H), 2.73 (m, 2H), 1.64 (t, 1H, SH), 1.28 (t, 6H, Me's). ^{31}P NMR: 94.2 ppm.

Ethanedithiol Monopivaloate (9a). Ethanedithiol (5.7 g, 60.3 mmol) and pivaloyl chloride (7.3 g) were combined in pyridine−dichloromethane (1:3, v/v) and stirred overnight. The reaction mixture was filtered, concentrated in vacuum, and distilled to give the desired product at 120 °C/15−17 mmHg (lit.[27] 104−105 °C/12 mmHg). ^{1}H NMR ($CDCl_3$): 2.97 (m, 2H), 2.62 (m, 2H), 1.56 (t, 1H, SH), 1.17 (s, 9H, Me's).

Ethanedithiol Monoisobutyrate (9b). The title compound was prepared in the same manner as for **9a**. The product was collected in fractions 96−110 °C/10−12 mmHg. ^{1}H NMR ($CDCl_3$): 3.04 (m, 2H), 2.60−2.75 (m, 3H), 1.59 (t, 1H, SH), 1.17 (d, 6H, Me's).

Ethanedithiol Monoproprionate (9c). The title compound was prepared in the same manner as for **9a**. The product was collected in fractions 90−110 °C/12 mmHg. ^{1}H NMR ($CDCl_3$): 3.06 (m, 2H), 2.68 (m, 2H), 2.56 (q, 2H), 1.60 (t, 1H, SH), 1.15 (t, 3H, Me).

Ethanedithiol Monoacetate (9d). Ethanedithiol (7.0 mL, 83.5 mmol) and acetic anhydride (8.0 mL, 83.5 mmol) were stirred overnight in pyridine−dichloromethane (1:1, v/v). The reaction mixture was concentrated and distilled to give the product at 75 °C/10 mmHg (lit.[26] 92 °C/17 mmHg). ^{1}H NMR ($CDCl_3$): 3.04 (m, 2H), 2.64 (m, 2H), 2.30 (s, 3H, Me), 1.58 (t, 1H, SH).

(24) Wiesler, W. T.; Marshall, W. S.; Caruthers, M. H. In *Methods in Molecular Biology: Oligonucleotide Synthesis Protocols*; Agarwal, S., Ed.; Humana Press: Clifton, NJ, 1993; pp 191−206.

(25) Marshall, W. S.; Caruthers, M. H., unpublished results.

(26) Hansen, B. *Acta Chem. Scand.* **1957**, *11*, 537−540.

(27) Mastriukova, T. A.; Odnoralova, V. N.; Kabachnik, M. I. *Zh. Org. Khim. USSR* **1957**, *28*, 1563 (1613−1617, Engl. ed.).

Ethanedithiol Monobenzoate (9e). Ethanedithiol (100 g, 1.06 mol) was placed in a 2 L three-neck flask with 400 mL of anhydrous diethyl ether and 100 mL of anhydrous pyridine. The solution was cooled to 0 °C, and benzoyl chloride (123 mL, 1.06 mmol) was added dropwise from a dropping funnel over 2 h with mechanical stirring. The reaction was allowed to sit for an additional hour. The solution was then filtered to remove the large mass of pyridine hydrochloride salt which was washed with diethyl ether. The combined filtrates were concentrated to an oil, which was taken up in 500 mL of hot methanol and allowed to sit in a −5 °C freezer overnight. The white crystals of bisbenzoate byproduct were removed by filtration and washed with cold methanol. The combined filtrates were concentrated to approximately 150 mL of a yellow oil. Excess ethanedithiol starting material was removed under high vacuum by heating to 60 °C and collected in a −78 °C trap. The remaining oil was distilled with a microdistillation apparatus. The desired product was collected at 0.2 mmHg and 110 °C employing an oil bath at 160 °C. Typical yields were approximately 80 g (40%). The absence of any ethanedithiol contamination in the product was confirmed by ^{1}H NMR ($CDCl_3$): 7.95 ppm (d, 2H); 7.57 (t, 1H); 7.44 (m, 2H); 3.30 (t, 2H); 2.78 (m, 2H); 1.70 (t, 1H).

Ethanedithiol Mono(2,4-difluorobenzoate) (9g). The titled compound was prepared in the same manner as for **9a**. The product was collected at 110−120 °C/0.4 mmHg. ^{1}H NMR ($CDCl_3$): 7.85 ppm (m, 1H); 6.88 (m, 2H); 7.44 (m, 2H); 3.23 (dd, 2H); 2.76 (m, 2H); 1.66 (t, 1H).

Ethanedithiol Mono(phenyl carbonate) (9h). The titled compound was prepared in the same manner as for **9a** from phenyl chloroformate. The product was collected at 100 °C/0.3 mmHg. ^{1}H NMR ($CDCl_3$): 7.69 ppm (m, 2H); 7.51 (m, 2H); 7.31 (m, 4H); 4.43 (d, 2H); 4.20 (t, 1H); 3.00 (t, 2H); 2.64 (m, 2H); 1.56 (t, 1H, SH).

Ethanedithiol Mono(trichloroethyl carbonate) (9j). The titled compound was prepared in the same manner as for **9a** from trichloroethyl chloroformate. The product was collected at 100 °C/0.3 mmHg. ^{1}H NMR ($CDCl_3$): 4.86 (s, 2H), 3.17 (dd, 2H), 2.83 (m, 2H), 1.69 (t, 1H, SH).

Ethanedithiol Mono(β-cyanoethyl carbonate) (9k). Triphosgene (24.5 g, 83 mmol) was dissolved in anhydrous toluene (150 mL) and cooled to 0 °C. Dropwise addition of β-cyanoethanol (17.6 g, 248 mmol) was followed by dropwise addition of pyridine (20 mL) at 0 °C. The reaction mixture was then filtered to remove pyridine hydrochloride salt and concentrated to an oil. The crude β-cyanoethyl chlorocarbonate was dissolved in 50 mL of anhydrous ether, placed in a dropping funnel, and added dropwise to ethanedithiol (28 mL, 334 mmol) in anhydrous diethyl ether (200 mL) and pyridine (25 mL). The reaction was filtered, and the filtrate was washed with saturated bicarbonate (3 × 100 mL) and brine (100 mL) and dried over Na_2SO_4. The diethyl ether solution was concentrated to an oil and distilled. The product was collected at 140−150 °C/0.4 mmHg. ^{1}H NMR ($CDCl_3$): 4.43 (dd, 2H), 3.13 (dd, 2H), 2.79 (m, 4H), 1.70 (t, 1H, SH).

Ethanedithiol Mono(9-fluorenylmethyl carbonate) (9l). 9-Fluorenylmethyl chloroformate (15 g, 58 mmol) in anhydrous ether was added dropwise to an excess of ethanedithiol (15 mL, 178 mmol) in diethyl ether (50 mL) containing pyridine (6 ml). After 1 h, salts were removed by filtering and the filtrate was washed with bicarbonate (2 × 50 mL) and brine (50 mL). The ether solution was concentrated to a thick syrup under high vacuum, and the product was purified by flash chromatography (ethyl acetate−hexane, gradient from 1:99 to 1:9). R_f = 0.4 (ethyl acetate−hexane, 1:9, v/v). ^{1}H NMR ($CDCl_3$): 7.69 ppm (m, 2H); 7.51 (m, 2H); 7.31 (m, 4H); 4.43 (d, 2H); 4.20 (t, 1H); 3.00 (t, 2H); 2.64 (m, 2H); 1.56 (t, 1H, SH).

***O*-(5′-*O*-(Dimethoxytrityl)thymidin-3′-yl) *S*-(β-Acylmercapto)ethyl) Pyrrolidino Thiophosphoroamidites (10a−e). General Procedure I.** 5′-*O*-(Dimethoxytrityl)-deoxyribonucleoside (1 mmol) was dissolved in 2.5 mL of anhydrous THF and 0.5 mL of anhydrous triethylamine. Bis-(pyrrolidino)chlorophosphine[21a] (220 μL) was added via syringe and allowed to stir for 5 min. The reaction mixture was diluted with anhydrous dichloromethane (7 mL), and the ethanedithiol monoacylate was added (2 equiv). After 11 min, the reaction was diluted with dichloromethane (20 mL) and ethyl acetate (3 mL), washed with saturated bicarbonate (2 × 25 mL), 10% Na_2CO_3 (25 mL), and brine (25 mL), and dried over Na_2SO_4. The organic layer was concentrated to a syrup, dissolved in toluene (10 mL) with triethylamine (1 mL), and added dropwise to 400 mL of vigorously stirred heptane to precipitate the white product. The precipitate was collected by filtration through a medium frit glass funnel and dried in vacuo. Typical yield was 80%.

General Procedure II (10d−l). After the identical phosphitylation procedure as described above, the protected thiol (0.5 mL) and tetrazole (150 mg) were added together in a solution of anhydrous acetonitrile (7 mL). The reaction was shaken for 10−15 s and poured into dichloromethane (100 mL). The solution was washed with bicarbonate (2 × 100 mL), 10% Na_2CO_3 (100 mL), and brine (100 mL) and dried over Na_2SO_4. The product was precipitated from heptane (**10d**−**g**,**l**), hexane−cyclohexane (2:1, v/v) (**10h**,**i**), or cyclohexane−toluene (9:1, v/v) (**10j**,**k**) as described above.

Tris(pyrrolidino)phosphine (19). Phosphorous trichloride (50.4 g, 37 mmol) was placed in a 1 L flask with 400 mL of anhydrous ether. The flask was fitted with a 250 mL addition funnel containing 173.5 g of (trimethylsilyl)pyrrolidine (3.3 equiv). The flask was cooled to −10 °C (ice/brine) under argon with magnetic stirring, after which the TMS-pyrrolidine was added dropwise over the course of 1.5 h. Stirring was maintained for an additional 1 h, after which it was stopped to allow the small amount of precipitates to settle. The reaction mixture was filtered through a medium frit sintered glass funnel, and the salts were washed with an additional 50 mL of anhydrous ether. Ether and trimethylsilyl chloride were removed from the filtrate by rotary evaporation to give approximately 100 mL of crude phosphine. Distillation at reduced pressure (0.25 mmHg) afforded an initial fraction (40−103 °C, 5−10 g) which was discarded and the product (104−110 °C, 76.3 g) obtained in 86% yield as a clear colorless oil. ^{31}P NMR: 104.6 ppm (THF), 102.8 ppm ($CDCl_3$).

Deoxynucleosid-3′-yl *S*-(β-(Benzoylmercapto)ethyl) Pyrrolidino Thiophosphoroamidites (22−25). General Procedure. 5′-*O*-(Dimethoxytrityl)deoxyribonucleoside (2 mmol) was dissolved in 30 mL of anhydrous dichloromethane to which a spatula of 3 Å molecular sieves was added. Tris(pyrrolidino)-phosphine (485 mg, 470 μL) was added via syringe followed by seven 0.1 mmol aliquots of tetrazole (7 × 0.2 mL of a 0.5 M solution in anhydrous acetonitrile stored over sieves) at 2 min intervals. (Trimethylsilyl)imidazole (30 μL, 0.1 equiv) was then added to the reaction. After 5 min, tetrazole (10.8 mL of a 0.5 M solution in anhydrous acetonitrile) was added, immediately followed by the addition of ethanedithiol monobenzoate (520 mg, 440 μL). The reaction was allowed to proceed for 105 s (T), 120 s (dC, dA), or 150 s (dG). The reaction was quenched by pouring the solution into 75 mL of dichloromethane containing 5 mL of triethylamine. The mixture was immediately washed with saturated sodium bicarbonate (100 mL) followed by 10% sodium carbonate (2 × 100 mL) and saturated brine (100 mL). The organic layer was dried over Na_2SO_4. After 10−15 min the drying agent was removed by filtration. Triethylamine (5 mL) was added to the solution which was concentrated using a rotary evaporator to a syrup. The syrup was dissolved in toluene (25 mL) and triethylamine (5 mL), and this solution was pipetted into 900 mL of vigorously stirred heptane or pentane (**22a** only) to precipitate the fluffy white product. After most of the heptane was decanted, the white precipitate was collected by filtration through a medium sintered glass funnel and subsequently dried under vacuum to give the desired product in approximately 75−80% yield.

***O*-(5′-*O*-(Dimethoxytrityl)thymidin-3′-yl) *S*-(β-(Benzoylmercapto)ethyl) Pyrrolidino Thiophosphoroamidite (22a).** FAB+ MS: 841 (M), 857 (M + O), 527 (M − (OP(NC$_4$H$_8$)SCH$_2$CH$_2$C(O)Ph), 303 (DMT cation). Anal. Calcd for $C_{44}H_{48}N_3O_8PS_2$: C, 62.76; H, 5.75; N, 5.00; P, 3.68; S, 7.61. Found: C, 62.48; H, 6.04; N, 4.95; P, 3.57; S, 7.42. ^{31}P NMR: 162.7, 159.2 ppm. ^{1}H NMR (CD_3CN): 9.50 ppm (bs, 1H, NH),

7.87 (m, 2H), 7.60 (m, 1H), 7.43 (m, 4H), 7.3−7.2 (m, 8H), 6.81 (bd, 4H), 6.10 (m, 1H, H1′), 4.77 (m, 1H, H3′), 4.03 (m, 1H), 3.74 (s, 6H, OMe's), 3.2−3.0 (9H), 2.78 (m, 2H), 2.38 (m, 2H), 1.67 (m, 4H, CH_2's), 1.45 (bs, 3H, Me).

***O*-(5′-*O*-(Dimethoxytrityl)-2′-deoxycytidin-3′-yl) *S*-(*β*-(Benzoylmercapto)ethyl) Pyrrolidino Thiophosphoroamidite (23a).** FAB+ MS (2,4-di-*tert*-butylphenol matrix): 931 (M + 1), 303 (DMT cation). Anal. Calcd for $C_{50}H_{51}N_4O_8PS_2$: C, 64.50; H, 5.52; N, 6.02; P, 3.33; S, 6.89. Found: C, 64.04; H, 5.69; N, 5.99; P, 3.16; S, 6.92. ^{31}P NMR: 162.1, 159.2 ppm. ^{1}H NMR (CD_3CN): 9.40 ppm (bs, 1H, NH), 8.22 (m, 1H), 7.85 (m, 4H), 7.6−7.4 (m, 8H), 7.33−7.1 (m, 8H), 6.82 (bd, 4H), 6.09 (m, 1H, H1′), 4.74 (m, 1H, H3′), 4.12 (m, 1H), 3.73 (s, 6H, OMe's), 3.18 (m, 2H), 3.13−2.95 (5H), 2.78 (m, 2H), 2.58 (m, 1H), 2.15 (m, 1H), 1.68 (m, 4H, CH_2's).

***O*-(5′-*O*-(Dimethoxytrityl)-2′-deoxyadenosin-3′-yl) *S*-(*β*-(Benzoylmercapto)ethyl) Pyrrolidino Thiophosphoroamidite (24a).** FAB+ MS: 955 (M + 1), 886 (M − pyrrolidinyl), 303 (DMT cation). Anal. Calcd for $C_{51}H_{51}N_6O_7PS_2$: C, 64.13; H, 5.38; N, 8.80; P, 3.24; S, 6.71. Found: C, 63.75 H, 5.37; N, 8.93; P, 3.28; S, 6.29. ^{31}P NMR: 160.3, 159.2 ppm. ^{1}H NMR (CD_3CN): 9.58 ppm (bs, 1H, NH), 8.51 (s, 1H), 8.23 (s, 1H), 7.96 (d, 2H), 7.88 (d, 2H), 7.58 (dd, 2H), 7.46 (m, 4H), 7.33 (d, 2H), 7.2−7.1 (6H), 6.74 (5H), 6.40 (m, 1H, H1′), 4.98 (m, 1H, H3′), 4.19 (m, 1H), 3.68 (s, 6H, OMe's), 3.3−3.1 (9H), 2.80 (dd, 2H), 2.58 (m, 1H), 1.71 (m, 4H, CH_2's).

***O*-(5′-*O*-(Dimethoxytrityl)-2′-deoxyguanosin-3′-yl) *S*-(*β*-(Benzoylmercapto)ethyl) Pyrrolidino Thiophosphoroamidite (25a).** Anal. Calcd for $C_{48}H_{53}N_6O_8PS_2$: C, 61.52; H, 5.70; N, 8.97; P, 3.31; S, 6.84. Found: C, 61.17; H, 5.83; N, 8.85; P, 3.31; S, 6.60. ^{31}P NMR: 159.7, 158.6 ppm. ^{1}H NMR (CD_3CN): 10.80 ppm (bs, 1H, NH), 7.85 (m, 3H), 7.60 (m, 1H), 7.44 (m, 2H), 7.36 (m, 2H), 7.20 (m, 7H), 6.72 (m, 4H), 6.19 (m, 1H, H1′), 4.78 (m, 1H, H3′), 4.15 (m, 1H), 3.70 (s, 6H, OMe's), 3.3−3.0 (8H), 2.9−2.5 (5H), 1.69 (m, 4H, CH_2's), 1.12 (d. 6H, Me's).

Phosphorodithioate DNA Deprotection and Purification. After automated solid-phase synthesis,[14i] the CPG column was removed from the synthesizer and dried with argon. The CPG support was removed and placed in a dry, 1 dram vial to which ethanolic ammonia (850 μL) and benzene (150 μL) were added. The vial was sealed with a Teflon-lined screw cap, allowed to remain at rt for 1 h, and then placed in a 55 °C oven for 15 h. After the vial was removed from the oven and cooled to rt, toluene (2 mL) was added and the combined solution carefully removed from the CPG support by pipetting. This solution contained traces of DNA and nearly all of the benzamide. Water (1 mL) was added to the vial containing the CPG in order to extract the phosphorodithioate DNA from the support. In the case of purine-rich sequences, water/acetonitrile (30%) was used.

The resulting deoxyoligonucleotides were purified according to standard procedures.[28] For deoxyoligonucleotides with multiple phosphorodithioate internucleotide linkages (greater than 50%), the recommended approach was reverse-phase column chromatography on a PRP-1 column (Hamilton, Reno, NV) TRITYL-ON, removal of the dimethoxytrityl group with acid, and then purification by PAGE under denaturing conditions.[14i]

Supporting Information Available: Expanded versions of ^{31}P-NMR spectra and Tables 1 and 2 with data on 69 deoxyoligonucleotides (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(28) 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*, 287−313.