Tetrahedron Vol. 47, No 14/15, pp. 2377-2388, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00

SYNTHESIS OF OLIGONUCLEOTIDE PHOSPHORODITHIOATES

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(*Received in USA* 23 *October* 1990)

ABSTRACT The synthesis of DNA containing sulfur at the two nonbonding internucleotide valencies is reported. Several different routes using either tervalent or pentavalent mononucleotide synthons are described.

INTRODUCTION

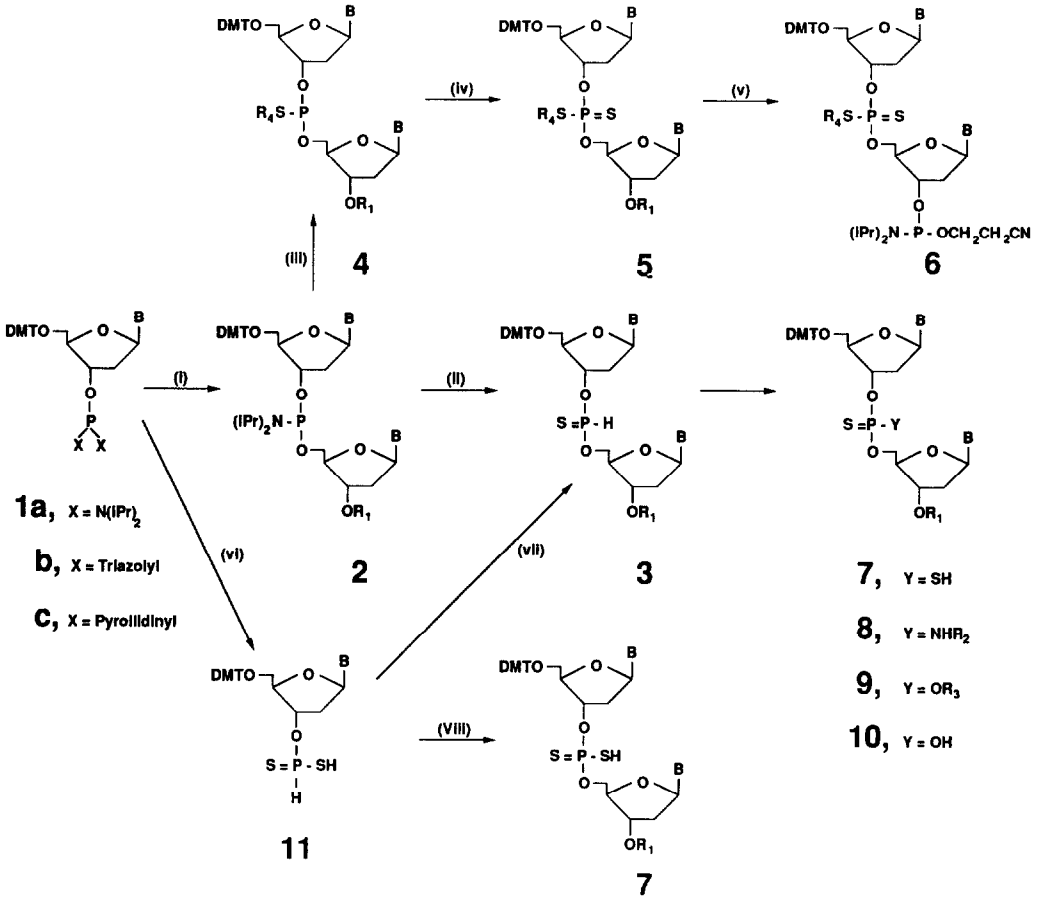
Over the past several years, the development of high yielding, rapid methods for synthesizing DNA[1,2] have led to a large number of applications for oligonucleotides.[3] These include its use for cloning and synthesizing genes,[4] as primers for sequencing DNA and various PCR applications,[5] mutagenesis of genes in a site specific manner,[6] examination of how nucleic acids interact with proteins,[7] and for studies on nucleic acid structure.[8] Some of the exciting results from these studies have further stimulated the development of DNA analogs for additional applications in basic research, DNA probe diagnostics, and the use of DNA potentially as a therapeutic drug. Among these analogs are DNA derivatives with internucleotide methylphosphonate, phosphoramidate, and phosphorothioate linkages.[9] These derivatives are all phosphorus chiral which inevitably leads to a large number of nonresolvable diastereomeric oligomers having variable biophysical, biochemical and biological properties.

Recent research in nucleic acid chemistry has therefore focused on the development of stereoselective approaches for synthesizing these derivatives;[10] or, alternatively, the development of achiral analogs.[11] A particularly attractive analog in the latter category is the deoxyoligonucleotide phosphorodithioate (dithioate DNA) which has deoxynucleoside-OPS_2O-deoxynucleoside internucleotide linkages. It is isostructural and isopolar with the natural phosphate diester linkage, stable toward enzymatic and chemical hydrolysis, and has other biochemical properties similar to unmodified DNA. Although the initial synthetic pathway for preparing this analog has only recently been published,[12] its attractiveness for a large number of applications has stimulated considerable additional effort.[13-24] In this laboratory several methods have been developed for its synthesis. These include the use of deoxynucleoside diamidites, thioamidites, H-phosphonodithioates, and dithiophosphate triesters as synthons. The chemical synthesis of DNA dithioates from these synthons will be described.

RESULTS AND DISCUSSION

The exploration of several methods for synthesizing oligonucleotide dithioates was considered necessary for several reasons. First the synthesis of dithioate DNA had not been reported prior to our initial work. Thus it was important that several methods be investigated to ascertain the advantages and problems of different approaches. Another desirable feature was to develop methods that had considerable versatility so that further derivatization at phosphorus could be achieved by relatively simple methods. This could very well be important for modifying a dithioate linkage by introducing certain reporter groups (fluors, spin-labels, antigens, etc.) or other desirable functional moieties such as steroids, sugars, fatty acids, or peptides. Finally, if these

molecules prove to be effective as anticancer or antiviral drugs, then methods for the large scale synthesis of dithioate DNA will become important. These various considerations led us to investigate both tervalent and pentavalent chemistries.



Scheme 1. Synthesis of Dithioate DNA From Diamidites. B = thymine, N-benzoyladenine, N-benzoylcytosine, N-isobutyrylguanine; DMT, di-*p*-methoxytrityl; R_1, acetyl or phenoxyacetyl; R_2, alkyl or aryl; R_3, alkyl or aryl; R_4, 4-chlorobenzyl or 2,4-dichlorobenzyl. (i) Tetrazole, (ii) H_2S, (iii) 4-chlorobenzylmercaptan, (iv) sulfur, (v) phosphitylation, (vi) H_2S, (vii) deoxynucleoside + N-methyl-1-chloropyridinium iodide, (viii) deoxynucleoside + iodine.

A particularly versatile series of methods starts with phosphordiamidites (Scheme 1), a class of compounds that have been used in the polynucleotide field for the preparation of deoxynucleoside phosphoramidites[25,26] and, as deoxynucleoside phosphordiamidites, for the synthesis of deoxydinucleoside phosphoramidites.[27,28] Thus a deoxynucleoside phosphordiamidite (**1a**) can react with a 3′-protected deoxynucleoside to yield the deoxydinucleoside phosphoramidite (**2**). This compound is then activated with tetrazole and reacted with H_2S or a mercaptan to yield, respectively, the hydrogen phosphonothioate (**3**) or the phosphorothioite triester (**4**). Further sulfurization of **4** yields the deoxydinucleoside phosphorodithioate triester (**5**) which, after removal of the 3′-protecting group and conversion to the 3′-diisopropylamidite (**6**), can be used as

a deoxydinucleotide synthon. For example, **6** (B = N4-toluoylcytosine; R_1 = phenoxyacetyl) can be condensed repeatedly on a silica support[29] to generate an oligodeoxycytidine pentadecamer having alternating dithioate and phosphate internucleotide linkages. The resulting compound has essentially the same antiviral activity as the all phosphorothioate homologue.[30] The major advantages of this approach are that **5** can be generated from **2** by a one pot procedure and then freed of side-products such as H-phosphonates and phosphorothioates through column chromatography prior to use in polynucleotide synthesis. A challenging problem is that tetrazole further activates **4** which leads to several uncharacterized side-products. Additionally, water contamination generates the H-phosphonate from either **2** or tetrazole activated **4** and, as a consequence, the phosphorothioate forms after sulfurization. Further studies on activation of **2** should eliminate or drastically reduce these problems.

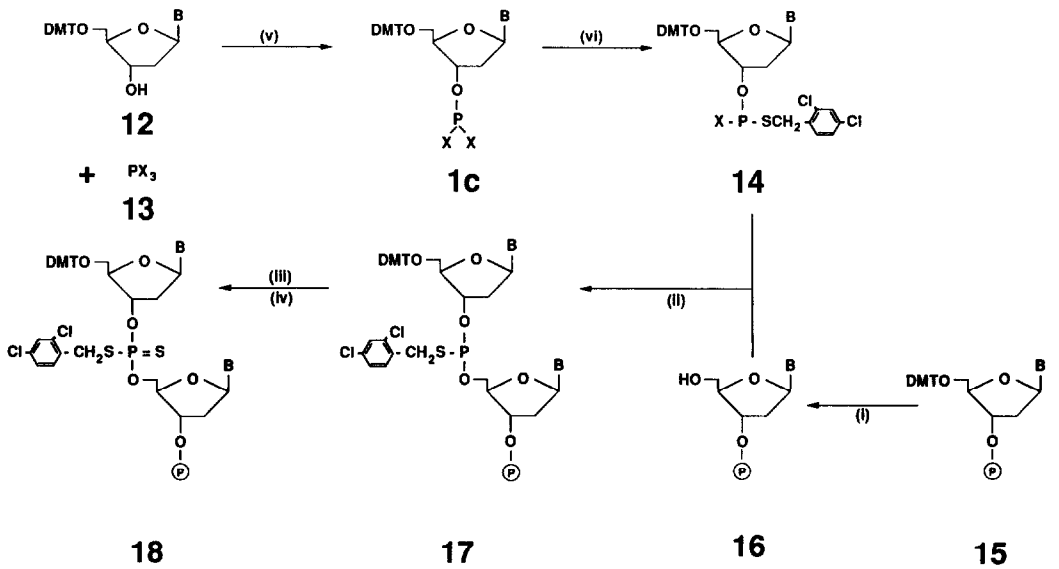
The deoxydinucleoside H-phosphonothioate (**3**) can serve as a key intermediate for synthesizing the phosphorodithioate and, additionally, several other phosphorus modified internucleotide linkages. When treated with sulfur in 2,6-lutidine and toluene, sulfurization yields the deoxydinucleoside phosphorodithioate (**7**). This compound can then be converted to **6** (R_4 = 2,4-dichlorobenzyl) by sulfur protection with α,2,4-trichlorotoluene, removal of the acyl group with *t*-butylamine in methanol, and phosphitylation to the phosphoramidite. Dimer synthons prepared via this route have been used to synthesize several *lac* operators with dithioate linkages at defined sites in an otherwise unmodified DNA segment. Results from these studies show that dithioate linkages are extremely site specific relative to their effect on *lac* repressor recognition.[30] By iodine oxidation of **3** with amines, alcohols, or water, the corresponding thioamidates (**8**), thiophosphate triesters (**9**), and thiophosphate diesters (**10**) can be formed in very high yield.[12] The flexibility of this pathway for generating multiple analogs, including the dithioate derivative, for use in DNA synthesis is perhaps its key advantage. It is, however, a rather lengthy route with the maximum possible synthetic steps.

A third pathway involves conversion of **1b** to the H-phosphonodithioate (**11**) by sulfhydrolysis with H_2S. Compound **11** can then be converted to **7** by two different routes. In one case, activation of **11** by N-methyl-2-chloropyridinium iodide in the presence of a 3′-O-protected deoxynucleoside generates **3** which can then be converted to **7** with elementary sulfur. The reaction to yield **3** is somewhat slow (15-30 min) and several side-products are present. An alternative synthetic route involves oxidative activation of **11** with iodine to generate **7** directly. The main use for this route could be as a method for synthesizing dinucleoside dithioates or mononucleotide dithioate diesters because it is very fast and easily monitored by decolorization of iodine. In order to use **7** and iodine activation for polynucleotide synthesis, it must be further converted to the triester. Otherwise iodine in the presence of contaminating water will lead to desulfurization and the formation of thioate and phosphate internucleotide linkages.

Although chemical synthesis procedures using deoxynucleoside diamidites for generating dithioate DNA appear promising for certain applications, their use as a general method suffers from several potential limitations. For example, deoxynucleoside diamidites that are sufficiently reactive for the rapid synthesis of dithioate DNA are unstable. This means that they cannot be stored or purified and must be used immediately after synthesis. One can, however, prepare all 16 deoxydinucleoside phosphorodithioates (**5**) and use these synthons for introducing the phosphorodithioate internucleotide linkage at any specific site in DNA. This procedure would require considerable preparation and storage of precursors and lacks the flexibility needed for a general synthesis method. For these reasons and because of success using deoxynucleoside 3′-phosphoramidites to synthesize unmodified DNA,[3] deoxynucleoside 3′-phosphorothioamidites (**14**) were examined as

intermediates for preparing phosphorodithioate DNA (Scheme 2). The objective was to develop a stable, highly reactive deoxynucleoside 3′-phosphorothioamidite that could be used to introduce the phosphorodithioate linkage rapidly and quantitatively into any predetermined position in a deoxyoliognucleotide.

Although the chemistry for synthesizing phosphorodithioate DNA from deoxynucleoside 3′-phosphorothioamidites continues to evolve, our current methodology is summarized in Scheme 2 and Table 1. Briefly deoxynucleoside 3′-phosphorothioamidite synthons (**14**) are prepared via a one pot, two step procedure from a suitably protected deoxynucleoside, tris-pyrrolidinylphosphine, and tetrazole. The resulting deoxynucleoside diamidite (**1c**) is converted without isolation to the deoxynucleoside phosphorothioamidite by addition of 2,4-dichlorobenzylmercaptan. Following an aqueous work-up and drying over sodium sulfate, these synthons are isolated by precipitation and stored as dry powders under an inert gas atmosphere without decomposition. Several alternative phosphines have been tried, with varying degrees of success, for synthesizing **14**. In some cases, such as with bis(pyrrolidino)-S-(4-chlorobenzyl)phosphine or bis(N,N-dimethylamino)-S-(4-chlorobenzyl)phosphine, the phosphine could not be purified by fractional distillation and thus complex mixtures of reaction products were obtained. Other bifunctional phosphitylating agents such as the



Scheme 2. Synthesis of Dithioate DNA from Thioamidites. X = pyrrolidino; Ⓟ, silica support. (i) Trichloroacetic acid, (ii) tetrazole, (iii) sulfur, (iv) acetic anhydride, (v) tetrazole, (vi) 2,4-dichlorobenzylmercaptan.

dichloro(pyrrolidino)phosphine or dichloro-N,N-dimethylaminophosphine proved unsuccessful because symmetrical deoxydinucleoside thiophosphites were the predominant product under all reaction conditions tested (low temperatures, order of additions and mole ratios of reagents). A more successful route[31] was based upon the use of bis-pyrrolidinochlorophosphine and bis-(N,N-dimethylamino)chlorophosphine. The synthesis of these phosphines in pure form was possible and their use for preparing **14** involved a one pot, two step procedure analogous to the pathway shown in Scheme 2. In this case, the amine hydrochloride from the first step catalyzed the formation of **14** in the presence of a mercaptan. However, certain problems with this approach have led us to the tris-aminophosphines as phosphitylating reagents. For example, if amine

Table 1. Chemical Steps for Synthesis of Dithioate DNA on a Solid Support*

Step		Reagent or Solvent**	Purpose	Time (min)
(i)	a.	Trichloroacetic acid in CH_2Cl_2 (3%, w/v)	Detritylation	0.50
	b.	CH_2Cl_2	Wash	0.50
	c.	Acetonitrile	Wash	0.50
	d.	Dry Acetonitrile	Wash	0.50
(ii)	a.	Activated nucleotide in acetonitrile¶	Add nucleotide	0.75
	b.	Repeat step a	Complete nucleotide addition	1.50
(iii)	a.	Sulfur in CS_2:pyridine: TEA (95:95:10; v/v/v)†	Oxidation	1.00
	b.	CS_2	Wash	0.50
	c.	CH_3OH	Wash	0.50
	d.	CH_2Cl_2	Wash	0.50
(iv)	a.	NMI:THF (30:70; v/v)‡ acetic anhydride:lutidine: THF (2:2:15; v/v/v)	Capping reaction	0.50
	b.	CH_2Cl_2	Wash	0.50

*See Scheme 2 for an explanation of the various steps i-iv.

**Multiple washes with the same solvent are possible.

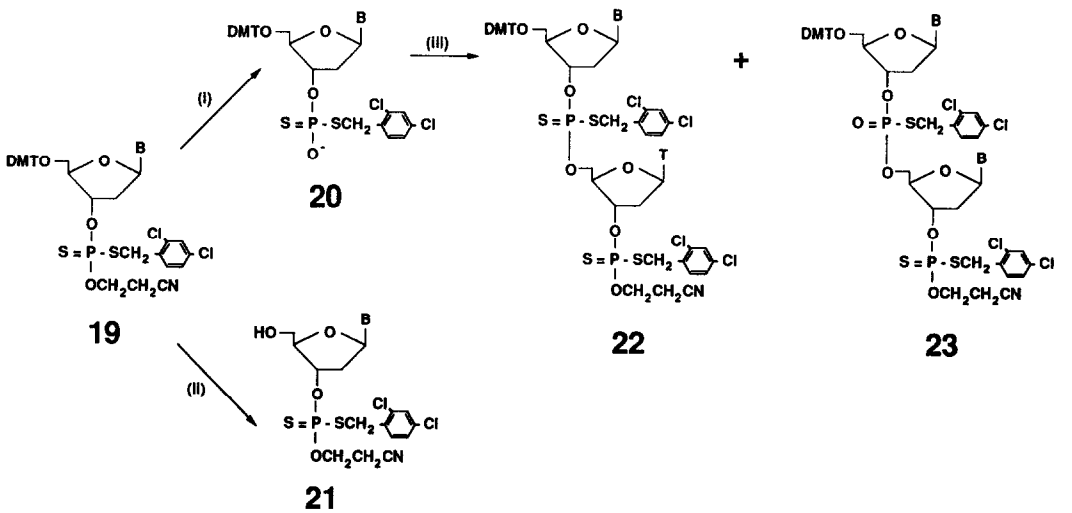
¶For each micromole of deoxynucleoside attached to silica, 0.48 M tetrazole (0.125 ml) and 0.15 M deoxynucleoside phosphorothioamidite (0.125 ml) are premixed in acetonitrile. During this coupling step, activated nucleotide and tetrazole are flushed from the lines leading into the reaction chamber. This procedure reduces contaminating phosphorothioate internucleotide linkages.

†5% sulfur by weight in CS_2:pyridine:TEA (95:95:10; v/v/v); TEA, triethylamine.

‡NMI, N-methylimidazole; THF, tetrahydrofuran.

hydrochloride is not removed entirely from the reaction product, decomposition occurs and **14** hydrolyzes or disproportionates to **1c** and the bis-mercaptylphosphite upon dissolution in solvent. Several side-products, including the phosphorothioate can then be detected during oligonucleotide synthesis.

Synthesis of dithioate DNA begins by treating a 5′-O-dimethoxytrityldeoxynucleoside linked to a silica support (**15**) with 3% trichloroacetic acid to yield **16**, a compound having a free 5′-hydroxyl group accessible for polynucleotide synthesis (see Table 1 for a more complete outline of these steps). Deoxynucleoside 3′-phosphorothioamidites are then activated with tetrazole and condensed with **16** to yield a thiophosphite triester (**17**). This step is followed by sulfurization using elemental sulfur to yield the protected phosphorodithioate derivative (**18**), capping or acylating unreactive silica-linked deoxynucleoside with acetic anhydride, and detritylation with trichloroacetic acid. Further repetitions of this cycle using either deoxynucleoside phosphorothioamidites or deoxynucleoside phosphoramidites and tetrazole as an activator yield oligodeoxynucleotides having normal phosphate diester and phosphorodithioate diester linkages in any combination. Condensation yields of 96-98% per cycle (based upon dimethoxytrityl cation released during detritylation) and 97-98% dithioate per linkage (the remaining 2-3% is phosphorothioate) have been obtained. The major challenge with this approach is to generate high yields of the thiophosphite and then the phosphorodithioate linkage under conditions where side-products do not form. These criteria translate into finding the right balance from among



Scheme 3. Synthesis of Dithioate DNA From Mononucleotide Dithioate Triesters. (i) Triethylamine, (ii) trichloroacetic acid, (iii) triisopropylbenzenesulfonyl chloride.

the protecting group on sulfur, the amino group that is part of the thioamidite, and the activating reagent. Thus although the 2,4-dichlorobenzyl group suffers from a requirement for thiophenol during deprotection, it is currently preferred as others such as β-cyanoethyl and 4-chlorobenzyl lead to a higher percentage of phosphorothioate internucleotide linkages. Similarly when X is N,N-diisopropylamino, activation with tetrazole occurs

very slowly and stronger acids, which lead to side products, must be used. Currently, the use of deoxynucleoside phosphorothioamidites in the synthesis of dithioate DNA has been extremely promising as we have synthesized a large number of oligonucleotides. The approach requires only the four deoxynucleoside phosphorothioamidites for complete synthetic versatility and can be used in combination with deoxynucleoside phosphoramidites to generate polynucleotides of any sequence and having all combinations of phosphate and phosphorodithioate internucleotide linkages.

For certain applications of dithioate DNA such as therapeutic or biophysical studies, significantly larger quantities (at least gram amounts) will be needed and it is not obvious that a polymer support approach will satisfy these needs. Such considerations have led us[23] to examine the classical phosphate triester approach[32-35] for DNA synthesis where dimers are synthesized in gram quantities, converted first to trimers, then hexamers, and finally dodecamers or larger oligomers. All reactions are completed in solution and intermediates purified by column chromatography. The comparable phosphorodithioate DNA strategy is summarized in Scheme 3. The first step is preparing the fully protected phosphorodithioate triesters (**19**) from commercially available deoxynucleoside phosphoramidites by treatment first with 2,4-dichlorobenzylmercaptan and tetrazole and then, without isolation, a saturated sulfur solution. Intermediates useful for DNA synthesis (**20** and **21**) can be generated from **19** by removal of the β-cyanoethyl or dimethoxytrityl groups. Further condensation of **20** with **21** using triisopropylbenzenesuphonylchloride and 1-methylimidazole yields the completely protected dinucleoside phosphorodithioate (**22**) in 95% yield contaminated with 1% of **23**, the dinucleoside phosphorodithioate. The latter compound can be removed by silica gel column chromatography. So far this approach has been used to synthesize a deoxyoctathymidine having phosphorodithioate internucleotide linkages (0.198 g, 0.046 mmol) and an assortment of di- and trinucleotide dithioates (0.5 to 1.0 mmol) with several different sequences. As is the case with the triester approach for DNA synthesis, the isolated yields of guanine rich dimers and trimers containing dithioate linkages are considerably lower (50-60%) than with pyrimidines (80-90%). Clearly the approach, especially relative to protecting groups, needs additional research. Recently 3,4-dihydro-3-hydroxy-4-oxobenzotriazine has been shown to be useful for synthesizing dithioate DNA via the triester approach as well.[24] Perhaps one of these two procedures may prove to be the method of choice for the large scale synthesis of dithioate oligonucleotides.

EPILOGUE

Access to these compounds has led us to investigate several potential biochemical, biological, and therapeutic applications for dithioate DNA.[37] Because this derivative has been shown to be completely resistant to nucleases, including those found in Hela cell nuclear and cytoplasmic extracts, we anticipate that dithioate DNA will be useful for many *in situ* experiments. As a first step in this direction, we have examined the ability of dithioate DNA to stimulate RNase H activity in Hela cell nuclear extracts. The results indicate that indeed deoxyoligonucleotides containing combinations of dithioate and normal linkages (also all dithioate links) are active in stimulating RNase H and, in contrast to normal DNA, survive attack by endogenous nucleases. These results suggest that dithioate DNA may be useful as an antisense DNA therapeutic and for a large number of basic, biological experiments that need to be completed *in situ*. Other experiments completed in this laboratory show that dithioate DNA can be used to probe protein-DNA interactions and to inhibit HIV reverse transcriptase. Because our results also demonstrate that dithioate DNA binds heavy metals and, through

alkylation, various reporter groups such as fluorescein and biotin, we anticipate that this derivative will find many additional uses in x-ray crystallography and other analytical applications. Thus these rather diverse observations already suggest that dithioate DNA will become an extremely valuable oligonucleotide analog in the years to come.

EXPERIMENTAL

General Procedures. Proton (^{1}H-NMR) and phosphorus (^{31}P-NMR) nuclear magnetic resonance spectra were recorded on a Brucker WM-250, Varian Gemini 300, or a Joel 90XQ in deuterated chloroform with tetramethylsilane as internal standard (^{1}H-NMR) and 85% phosphoric acid as external standard (^{31}P-NMR). Thin layer chromatography (tlc) was on aluminum backed sheets (silica gel 60 F, 0.2 mm, E. Merck). Preparative chromatography was by flash column chromatography on silica gel 60, 230-400 mesh (Macherey Nagel, Dueren, FRG). Concentrations of solutions were carried out in vacuo at 40 °C or lower using an aspirator or an oil vacuum pump. Solids were dried at rt in a desiccator over phosphorus pentoxide and potassium hydroxide unless otherwise specified. All reactions were carried out at rt unless reported differently.

Pyridine and dichloromethane were freshly distilled over calcium hydride. Triethylamine was distilled over toluenesulfonyl chloride and then calcium hydride. Anhydrous diethylether (ether, Merck or Mallinckrodt) was used directly. Acetonitrile was distilled over phosphorus pentoxide and then calcium hydride. Tetrahydrofuran (THF) was distilled over sodium metal in the presence of benzophenone.

Deoxyribonucleosides were obtained from Aldrich. 5′-O-Di-*p*-methoxytritylated and N-protected deoxynucleosides, 3′-O-acetylthymidine, and 3′-O-(4-chlorophenoxyacetyl)thymidine were prepared according to published procedures.[36] 1-H-Tetrazole (tetrazole, Aldrich) was sublimed before use. Synthetic oligonucleotides having dithioate linkages were isolated free of protecting groups by a two step protocol (thiophenol:triethylamine:dioxane, 1:1:2, v/v/v for at least 2 h followed by concentrated ammonium hydroxide for 15 h) and then purified to homogeneity by standard procedures (polyacrylamide gel electrophoresis and reverse phase HPLC).

Synthesis of O-5′-O-(Di-*p*-methoxytrityl)nucleosid-3′-yl, S-(4-chlorobenzyl), O-nucleosid-5′-yl-phosphorodithioate as well as the S-(2,4-dichlorobenzyl) derivative via 2′-Deoxynucleosid-3′-yl-phosphorodiamidites and their use in the synthesis of dithioate DNA. Two methods were developed. One method involved (1.) preparation of 2′-deoxynucleosid-3′-yl-phosphordiamidites, (2.) condensation of the 2′-deoxynucleosid-3′-yl phosphordiamidite with a 2′-deoxynucleoside to yield a 2′-deoxydinucleoside phosphoramidite, (3.) sulfhydrolysis with H_2S, (4.) oxidation with sulfur, (5.) protection of the resulting dithioate via alkylation, and (6.) conversion of the dinucleoside dithioate to the 3'-phosphoramidite. For the second method, the 2′-deoxydinucleoside phosphoramidite was condensed directly with a mercaptan and oxidized with sulfur to yield the product. Descriptions of both methods in sequential order are presented in this section.

The first method has the following six steps.

(1.) Synthesis of 5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl-N,N,N′,N′-tetraisopropylphosphordiamidite (**1a**). 5′-O-(Di-*p*-methoxytrityl)thymidine (2.21 mmol, 1.20 g) was dried by coevaporation with THF and then dissolved in THF (10 mL) containing triethylamine (3.3 mmol, 0.46 mL). Bis(diisopropylamino)-chlorophosphine (2.44 mmol, 0.650 g) was added with stirring. After 35 min, triethylammonium hydrochloride

was removed by filtration from the soluble reaction product and washed with THF (1 mL). The filtrates were combined and concentrated to dryness. The product was redissolved in acetonitrile (5 mL) and used without further purification.

(2.) Synthesis of O-5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl, O-3′-O-(acetyl)thymidin-5′-yl-N,N-diisopropylphosphoramidite (**2**). 3′-O-acetylthymidine (2.25 mmol, 0.639 g) and tetrazole (2.0 mmol, 0.142 g) were dried by coevaporation with anhydrous THF (10 mL) and dissolved in acetonitrile (5 mL). The resulting solution was mixed with **1a** (prepared as described above and added in an amount equimolar with 3′-O-acetylthymidine) and the reaction mixture stirred for 45 min. The products were diluted with dichloromethane (75 mL), extracted with saturated sodium bicarbonate and brine, and dried over sodium sulfate. After removal of salt by filtration, the product was purified by silica gel column chromatography using ethyl-acetate:chloroform: triethylamine (45:45:10, v:v:v) and obtained in 75% yield (1.59 g). ^{31}P-NMR (CH_3CN) δ 148.5, 148.1.

(3.) Synthesis of O-5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl, O-3′-O-(acetyl)thymidin-5′-yl-hydrogenphosphonothioate (**3**). Compound **2** (0.5 mmol, 0.470 g) and tetrazole (0.5 mmol, 0.035 g) were dissolved in acetonitrile (1 mL) and H_2S was bubbled through the solution for one min. The reaction flask was sealed and stored with stirring at rt for 16 h. Evaporation of the solvent (danger: H_2S) afforded a gum which was redissolved in ethylacetate and extracted twice (20 mL each) with 2 M triethylammonium bicarbonate (pH 7.4). The product was dried over sodium sulfate, concentrated to a gum, redissolved in dichloromethane (5 mL) and precipitated into *n*-pentane (250 mL). After filtration and desiccation over molecular sieves, the desired product was obtained in 90% yield (0.400 g). ^{31}P NMR δ 70.7, 71.7.

(4.) Synthesis of Triethylammonium O-5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl, O-3′-O-(acetyl)thymidin-5′-yl-phosphorodithioate (**7**). Compound **3** (0.1 mmol, 0.104 g) in dichloromethane (1 mL) was added to elemental sulfur (0.125 mmol elementary sulfur in toluene:2,6-lutidine (19:1, v:v)). After 0.5 h the reaction products were concentrated to a yellow gum, dissolved in dichloromethane and fractionated using silica gel column chromatography (0%-12% methanol in dichloromethane: triethylamine, 995:5, v:v). The product was isolated in 70% yield. ^{31}P NMR δ 112.7.

(5.) Alkylation of **7** with α,2,4-Trichlorotoluene. O-5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl, O-3′-O-(acetyl)thymidin-5′-yl-phosphorodithioate (0.057 g, 0.06 mmol) and α,2,4-trichlorotoluene (0.05 mL) were dissolved in acetonitrile (2 mL). After 1 h at 55 °C, tlc analysis indicated quantitative conversion to O-5′-O-(di-*p*-methoxytrityl)thymidin-3′-yl, S-(2,4-dichlorobenzyl), O-3′-O-(acetyl)thymidin-3′-yl-phosphorodithioate (**5**). The reaction product was concentrated to an oil, dissolved in dichloromethane, precipitated into *n*-pentane and dried in vacuo (90% yield). ^{31}P NMR δ 93.7, 94.4.

(6.) Conversion to Compound **6**. Conversion to a compound useful for DNA synthesis was a two step procedure. The dinucleoside phosphorodithioate triester (**5**) was first deacylated with 0.15 M *tert*-butylamine in methanol (O °C, 10 h) and purified by silica gel chromatography. Less than 5% cleavage of the internucleotide linkage (^{31}P, tlc) was observed. The deacylated compound was then reacted with bis(diisopropylamino)-2-cyanoethoxyphosphine (1.5 eq) in the presence of tetrazole (1 eq, 1 h at rt) to yield **6** (76%) after standard aqueous work-up and precipitation from hexanes. ^{31}P NMR δ 149.4, 149.2, 148.9, 97.2, 95.7, and 95.5.

The second method involved condensation of a dinucleoside phosphoramidite (**2**) with a mercaptan followed by sulfur oxidation. The method is illustrated by the synthesis of O-5′-O-(di-*p*-methoxytrityl)-thymidin-3′-yl, S-(4-chlorobenzyl), O-3′-O-(acetyl)thymidin-5′-yl-phosphorodithioate (**5**). Compound **2**

(1.66 mmol, 1.59 g), tetrazole (4.01 mmol, 0.281 g), and 4-chlorobenzylmercaptan (7.6 mmol, 1 mL, 1.2 g) were added to acetonitrile (7 mL) and the reaction mixture stirred for 30 min at rt After quenching (10 min) by addition of elemental sulfur (10 mL of a 0.05 M solution in toluene:2,6-lutidine, 19:1, v:v), the reaction products were diluted with ethylacetate (75 mL), extracted sequentially with aqueous saturated sodium bicarbonate and brine, dried over sodium sulfate, and filtered from the salt. The resulting product mixture was concentrated to an oil, diluted with ethylacetate (40 mL) and triturated with hexanes (200 mL) to give a white powder. Purification by silica gel column chromatography (2-12% methanol in dichloromethane) gave the completely protected product in 91% yield. ^{31}P NMR ($CHCl_3$) 97.9 and 96.4.

Synthesis of dithioate DNA from compound **6** and appropriately protected deoxynucleoside 3′-phosphoramidite was completed using published procedures on silica supports.[36] Removal of dithioate DNA from silica, elimination of protecting groups, and purification by reverse phase and ion exchange chromatography also was completed using published procedure.[36]

Synthesis of 5′-O-(Di-*p*-methoxytrityl)thymidin-3′-hydrogenphosphonodithioate (11). N-Methylmorpholine (0.25 mol, 27.5 ml) and PCl_3 (0.025 mol, 2.18 ml) were dissolved in dichloromethane. 1,2,4-Triazole (0.083 mol, 5.75 g) was added. The reaction was allowed to proceed with stirring at rt for 30 min and then it was cooled to 0 °C. During this process the reaction mixture became turbid and the ^{31}P NMR (48.1 ppm) indicated complete conversion to the bis-triazoylchlorophosphine. 5′-O-Di-*p*-methoxytritylthymidine (5 mmol, 2.73 g) in dry dichloromethane (66 ml) was added to this solution and the reaction mixture allowed to attain rt over 15 min. H_2S gas was then passed through the mixture for 15 min. During this sulfhydrolysis, the reaction mixture becomes clear. After removal of excess H_2S by passage of argon through the reaction solution, solvents were removed by evaporation *in vacuo*. The yellow reaction products were dissolved in dichloromethane, extracted with 1 M triethylammonium bicarbonate, and dried over sodium sulfate. After removing salt by filtration, the reaction products were fractionated by chromatography on silica gel (dichloromethane:ethylacetate:methanol:triethylamine; 60:30:5:5). The product fractions were combined and precipitated into *n*-pentane:ether (9:1) and dried *in vacuo* to yield 2.1 g (57%).

Synthesis of O-5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl, O-3′-O-(acetyl)thymidin-5′-yl-hydrogenphosphonothioate from 11. 3′-O-Acetylthymidine (0.1 mmol, 28 mg) and N-methyl-2-chloropyridinium iodide (0.15 mmol, 38 mg) were dissolved in 1 ml dry pyridine and **11** (0.1 mmol, 74 mg) was added. After 15 min, ^{31}P NMR analysis indicated formation of the product (60%) contaminated with side-products (mainly the deoxynucleoside 3′-hydrogenphosphonate) and unreacted starting material (16%). The reaction mixture was concentrated *in vacuo* to dryness, extracted successively with aqueous sodium bicarbonate and brine, and fractionated by column chromatography on silica gel (1,1,1-trichloroethane:methanol:triethylamine, 90:9.5:0.5). The product fractions were combined, concentrated to dryness, dissolved in chloroform, and precipitated into *n*-pentane. The product was collected and dried *in vacuo* to yield a white solid (40 mg, 45%). ^{31}P NMR ($CDCl_3$) δ 70.7 and 71.7.

Synthesis of Triethylammonium O-5′-O-(Di-*p*-methoxytrityl)thymidin-3′-yl, O-3′-O-(acetyl)thymidin-5′-yl-phosphorodithioate (7) from 11. 5′-O-(Di-*p*-methoxytrityl)thymidin-3′-hydrogenphosphonodithioate (0.1 mmol, 74 mg) and 3′-O-acetylthymidine (0.12 mmol, 28 mg) were dissolved in dry

pyridine (0.9 ml). Iodine (0.11 ml of a 1 M solution) was added dropwise. The reaction mixture decolorized instantly as the iodine was added to a yellow color that was not due to iodine. When the coupling was complete, excess iodine persisted in the reaction solution as a brown color. The ^{31}P NMR of the reaction mixture indicated two peaks. One corresponded to the desired product at 115.65 ppm and the other was a side-product at 116.7 ppm (10% of the ^{31}P NMR signal). After extraction with aqueous sodium bisulfite, the side-product peak disappeared. The reacion product was then isolated by standard silica gel column chromatography and precipitation of product fractions from *n*-pentane to yield 65 mg (57%). ^{31}P NMR ($CDCl_3$) δ 112.7.

Synthesis of 2′-Deoxynucleosid-3′-yl-S-(2,4-dichlorobenzyl) N,N-pyrrolidinophosphorothioamidite (14). A solution of protected deoxynucleoside (4 mmol) in dry dichloromethane (50 ml) was added to tris-pyrrolidinylphosphine (1.02 ml, 4.7 mmol) and tetrazole (1 ml of a 0.5 M solution in acetonitrile) and the reaction stirred for 15 min. Because of reduced solubility, 5′-O-(di-*p*-methoxytrityl)6-N-benzoyl-2′-deoxyadenosine was dissolved in 70 ml of dry dichloromethane. Analysis by tlc (ethyl acetate:dichloromethane:triethylamine, 45:45:10) confirmed the complete reaction of starting material. 2,4-Dichlorobenzylmercaptan (1.60 ml, 9.9 mmol) was added and the reaction stirred for a further 30 min. The extent of conversion to the deoxynucleoside 3′-phosphorothioamidite was monitored by ^{31}P NMR. The reaction mixture was then diluted with dichloromethane (200 ml containing 1% triethylamine) and extracted sequentially with saturated sodium bicarbonate (200 ml), 10% sodium carbonate (2 x 200 ml) and brine (200 ml). After drying the resulting organic solution over anhydrous sodium sulfate, removal of the solids by filtration and addition of toluene (20 ml), the product was isolated by concentration of the solution to 20 ml, precipitation from degassed heptane (500 ml) containing 1% triethylamine, filtration, and finally drying *in vacuo* (yields are typically 85-90%). These synthons were stored as dry powders under an inert gas atmosphere without decomposition.

Synthesis of Dithioate DNA from 14. The synthesis protocol is summarized in Table 1. The resulting oligonucleotides were deprotected and purified using published procedures.[36] ^{31}P NMR of phosphorodithioate DNA indicates that oligonucleotides synthesized by this procedure with the 2,4-dichlorobenzyl sulfur protecting group contain 2-3% phosphorothioate linkages. All other protecting groups yield a higher percent phosphorothioate.

ACKNOWLEDGMENTS

This is paper 34 in a series on Nucleotide Chemistry. Paper 33 is Brill, W. K.-D.; Nielsen, J.; Caruthers, M. H. *J. Am. Chem. Soc.*, submitted. This research was supported by the National Institutes of Health (Grant GM25680).

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