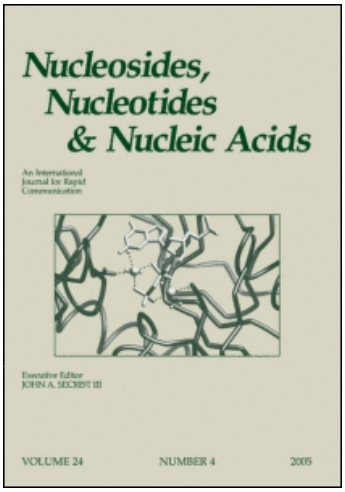

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Synthesis of Oligodeoxynlicleotides Containing Specific Methylphosphonothioate and Dithioate Moieties

W. K. -D. Brill[a]; M. H. Caruthers[a]
[a] Department of Chemistry & Biochemistry, University of Colorado, Boulder, CO, USA

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SYNTHESIS OF OLIGODEOXYNUCLEOTIDES CONTAINING SPECIFIC METHYLPHOSPHONOTHIOATE AND DITHIOATE MOIETIES

W. K.-D. Brill and M. H. Caruthers*
University of Colorado, Department of Chemistry & Biochemistry,
Boulder, CO 80309-0215, USA

Abstract. Deoxynucleoside thiophosphoramidites and methyl phosphonothioates were synthesized and used to prepare dinucleotides containing phosphorodithioates and methylphosphonothioate internucleotide linkages.

The introduction of chiral methylphosphonothioate or non-chiral phosphorodithioate functionalities into oligonucleotides are of potential interest for biochemical applications. The former may help locate interactions between protein side-chains and the phosphate backbone of DNA. These O,S diesters are also hydrolyzable under conditions where unmodified DNA is stable which leads to selective introduction of nicking sites. Phosphorodithioates are hydrolytically more stable than the parent phosphodiesters, sterically very similar to natural DNA, possess the same charge, and are alkylated under milder conditions than natural internucleotide linkages.

Previously we described a pathway to internucleotide phosphorodithioates[1] which has successfully been used to introduce single phosphorodithioate moieties into medium sized oligonucleotides. Since extension to the synthesis of deoxyoligonucleotides with several modified linkages is not straight forward, we have focused on another route (Figure 1).

The synthons are nucleotide 4-chlorobenzyl thiophosphoramidites bearing base and 5'-O-protecting groups. This phosphorus protecting group was chosen as it represents a compromise between facil removal ($t_{1/2}$:16 min in thiophenol:triethylamine:dioxane, 1:1:2) and reactivity of the phosphoramidite. The thiophosphoramidite moiety is introduced by

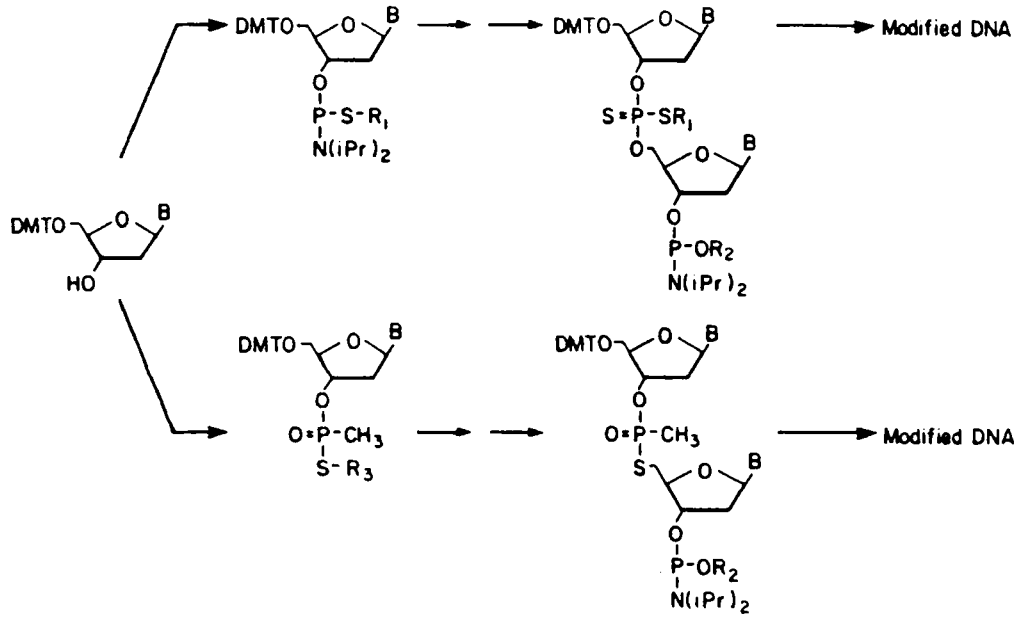

Figure 1. DMT, dimethoxytrityl; B, appropriately protected purine or pyrimidine base; R_1, 4-chlorobenzyl; R_2, β-cyanoethyl; R_3, 2,4-dichlorobenzyl; iPr, isopropyl.

the reaction of 1 mmol deoxynucleoside with 2 mmol of N,N,N',N'-tetraisopropyl-4-chlorobenzylthiodiamidite in acetonitrile and 2 mmol tetrazole (50% yield). In a typical internucleotide coupling reaction, 0.3 mmol of deoxynucleoside thiophosphoramidite and 0.1 mmol of the 3'-O- and base protected deoxynucleoside in acetonitrile were allowed to react using 0.6 mmol pyridinium tetrafluoroborate as a catalyst. Oxidation with 20 atomic equivalents of sulfur yields the dinucleotide phosphorodithoate (60%, characterized by NMR and FAB-MS).

For these coupling reactions, activating agents more acidic than tetrazole must be used to form the internucleotide linkage at a reasonable rate. With anilinium tosylate, trifluoroacetate and trifluoromethyltetrazolide, the thiophosphoramidite is rapidly activated to relatively stable intermediates (detected by tlc and ^{31}P-NMR) which react slowly with the 5'-hydroxyl of a nucleoside, present in the reaction solution, to form the thiophosphite triester. Using tetrazole

as activator, the intermediate thiophosphoryl-tetrazolide can only be detected (^{31}P-NMR, 157.1 ppm) in the absence of the coupling component which suggests that protonation of the thiophosphoramidite is the rate limiting step. We therefore examined pyridinium tetrafluoroborate as an activation catalyst and observed high coupling efficiency without detection of an intermediate (^{31}P-NMR and tlc). The formation of a major side product that consumes the thiophosphoramidite and does not interfere with the coupling reaction is currently under investigation.

In order to effectively prepare dinucleotide synthons, we chose the 4-chlorophenoxycarbonyl group for transient protection of the 3'-hydroxyl as it was stable toward the coupling conditions but was easily removed[2] using conditions that do not cause deprotection of the exocyclic amines (1 M imidazole/acetonitrile/2 h/r.t.). Conversion to dinucleotide 3'-phosphoramidites was then possible for use in DNA synthesis.

The synthesis of diastereomerically pure deoxyoligonucleotides containing methylphosphonothioates at defined sites begins by resolution using flash column chromatography of the diastereomers of 5'-O-dimethoxytritylated, base protected deoxynucleoside 3'-O-S-2,4-dichlorobenzylmethylphosphonothioates[3]. After removal of the 2,4-dichlorobenzyl group with retention of absolute configuration on phosphorus, S-alkylation was carried out with a 5'-tosyldeoxynucleoside. The rate of this alkylation reaction was enhanced by temperature, diisopropylethylamine, and tetrabutyl ammonium iodide. Thus in a typical coupling reaction, 1 mmol of diisopropylethylammonium deoxynucleoside 3'-O-methyl phosphonothioate is allowed to react at 55°C with 1 mmol 5'-O-tosylated deoxynucleoside in THF:diisopropylethylamine (4:1) in the presence of 0.1 equivalent of tetra-*n*-butylammonium iodide. After a 30 h reaction, the product was isolated without detectable racemization following an aqueous work-up and column chromatography. The dimers were then converted to the 3'-phosphoramidite and used in DNA synthesis following standard protocols.

The enhanced hydrolysability of methylphosphonothioate internucleotide linkages requires special precautions during deprotection. Thus after completion of a synthesis on silica the β-cyanoethyl protective groups were removed with diisopropylethylamine:dioxane (3:7) in 4 h at room temperature. The next step, removal of the synthetic DNA under

mild conditions (ethanol: aqueous ammonia, 3:7) from silica, had to be modified as well. We found that the diglycolate spacer satisfied our needs. By using standard synthesis procedures[4] where diglycolic anhydride is substituted for succinic anhydride, this linkage could be introduced first at the 3'-hydroxyl of deoxynucleosides and then incorporated into the spacer between DNA and silica. Finally deprotection of nucleoside bases was carried out according to Urdea and Horn[5]. The modified DNA was further purified by gel filtration and polyacrylamide gel electrophoresis. This procedure for synthesis and deprotection of diastereomerically pure methylphosphonothioate linked DNA has now been used to synthesize eight deoxyoligonucleotides containing from 14 to 28 mononucleotides. Extension of these procedures to automated machines is currently in progress.

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