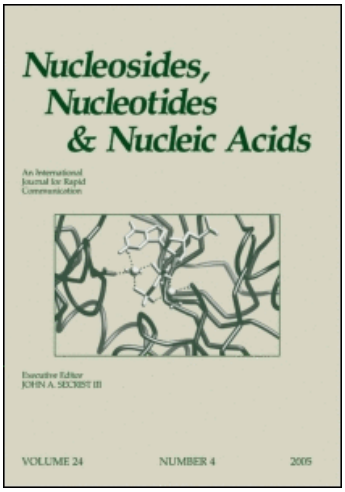

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SOLID-PHASE SYNTHESIS OF OLIGODEOXYNUCLEOTIDES CONTAINING 3′-*S*-PHOSPHOROTHIOLATE LINKAGES

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ABSTRACT

For the first time a fully automated procedure has been developed for the incorporation of a 3′-*S*-phosphorothiolate linkage into DNA, using phosphorothioamidite monomers. Coupling yields with either of the activators 5-ethylthiotetrazole or 4,5-dicyanoimidazole were in the range of 80–90%. Coupling yields were equally good when performed on either a 0.2 or 1 μmole reaction column, thus facilitating large scale synthesis.

In recent years DNA and RNA analogues have been extensively investigated as potential chemotherapeutic agents (1) and have become essential tools for probing structural and mechanistic aspects of nucleic acid biochemistry (2). The 3′-*S*-phosphorothiolate linkage (Fig. 1), in which the 3′-oxygen is replaced by sulfur, has attracted increasing attention and been constructed using a variety of approaches including phosphoramidite chemistry (3–6) and methods based on a Michaelis-Arbusov reaction (7–10) Very recently NMR studies on the phosphorothiolate dimer TspT have shown that 3′-sulfur substitution results in a sugar conformation that is predominately 3′-*C*-endo making these analogues interesting as potential antisense agents (11).

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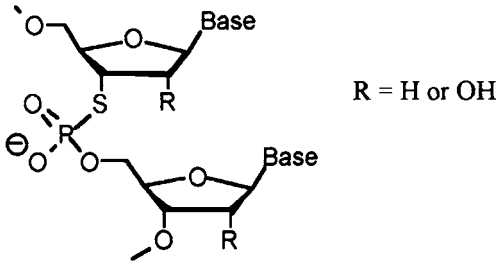

Figure 1.

Despite the increasingly wide spread use of oligonucleotides containing a single 3′-*S*-phosphorothiolate linkage, there is no report of a fully automated synthesis protocol for incorporation of these analogues. We now wish to report significant developments in the synthesis of oligodeoxynucleotides containing a 3′-*S*-phosphorothiolate linkage including: a shortened procedure for the synthesis of phosphorothioamidite derived from 3′-thiothymidine (**4**) and a fully automated synthesis protocol for incorporation of this thioamidite based on commercially available activators and reagents.

Synthesis of 3′-thiothiothymidine. The previously reported synthesis of 2′-deoxy-3′-thiothymidine (3,4) (**3**) started from the xylo–configured nucleoside involving displacement of the sulfonate ester with sodium thiobenzoate. We now report a more immediate route to 2′-deoxy-3′-thiothymidine through direct ring-opening of a 2,3′-anhydrothymidine with thiobenzoate. Synthesis started from DMT-protected thymidine; this is in contrast to the originally reported work on 3′-thiothymidine that used the more robust MMT group (4). Thus, DMT-protected 2,3′-anhydrothymidine (**1**) was converted through to the corresponding 3′-thiothymidine (**3**) as shown in Figure 2. The thionucleoside was converted to its phosphorothioamidite, (**4**) as previously described (4).

Oligonucleotide synthesis. In formerly described procedures for the synthesis of oligonucleotides containing 3′-*S*-phosphorothiolate linkages the key phosphorothioamidite coupling step has been performed by removing the reaction column from the synthesizer and manually introducing the thioamidite and activator [5-(*p*-nitrophenyl)tetrazole] by syringe (6,12). This manual coupling procedure made the rigorous exclusion of moisture difficult and the result was a time consuming and intricate operation that also gave inconsistent yields. Thus, there was an obvious requirement for a fully automated and relatively simple procedure that would make these analogues more accessible to the nucleic acid community.

Original studies had shown that phosphorothioamidites are much less reactive than the standard amidites, presumably due to stabilization of the protonated phosphorus tautomer by sulfur, and that 5-(*p*-nitrophenyl)tetrazole was a superior to tetrazole for coupling phosphorothioamidites (4). Unfortunately however,

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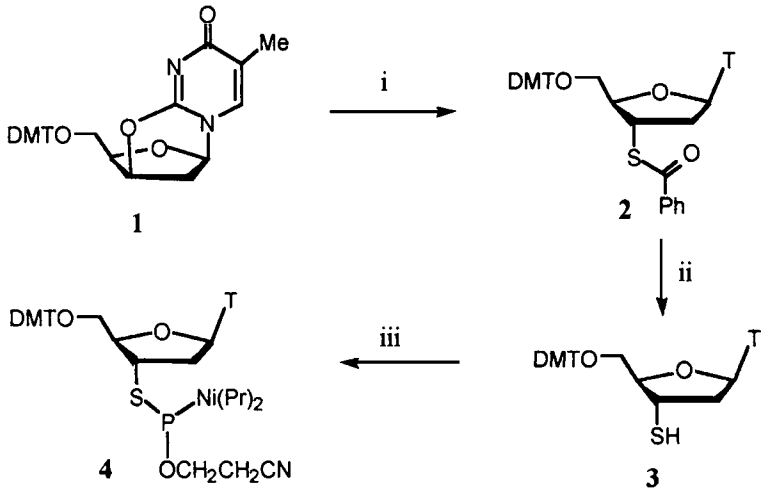

Figure 2. *Reagents and Conditions* i) CsBz, DMF, 110°C, 82%; ii) NaOH, EtOH, H_2O, 83%; iii) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, 1-methylimidazole, diisopropylethylamine, CH_2Cl_2, 87%.

5-(*p*-nitrophenyl)tetrazole is poorly soluble in acetonitrile and not ideally suited to automated synthesis. The activators 5-ethylthiotetrazole (13) (SET) and 4,5-dicyanoimidazole (14) (DCI) have been found to be more potent activators, although DCI is preferred due its reduced acidity.

To assess these commercially available activators and optimize the synthesis cycle two test sequences (5′-TTT TTsT TTT T and 5′-CCT AAA TTsT GCC, where Ts = 3′-thiothymidine) which contain a single phosphorothiolate residue were synthesized. When coupling times, activator and thioamidite concentrations were used at standard values coupling yields were disappointing. Under these conditions tetrazole failed to catalyze any incorporation of the thioamidite, as determined by the trityl release assay; whereas DCI and SET gave coupling yields that were detectable, but less than 10%. Using the activators at a concentration of 1M and with extended coupling times, good yields were obtained for the introduction of the thioamidite.

The activators SET and DCI were essentially equally effective both giving coupling yields of 80–90% with a 15 min coupling. Coupling yields were also equally good when performed on either a 0.2 or 1 μmole reaction column.

Notable in the synthesis protocol is the use of the standard iodine oxidation solution. There was some apprehension with regard to the use of this oxidant as aqueous iodine solutions have been shown to cleave the P−S bond in a phosphorothiolate diester (4). However, the trityl release assay (not shown) reveals that the coupling yields are consistently maintained subsequent to the introduction of the phosphorothiolate linkage and suggests that the iodine oxidant is not detrimental to the phosphorothiolate triester to any significant extent.

Oligonucleotides were deprotected using standard conditions, isolated by reverse-phase HPLC and characterised by electrospray mass spectrometry (15).

Experimental. All oligonucleotides were synthesised on an Expedite™ 8909 DNA synthesiser using standard protocols for the phosphoramidite-based coupling approach. The standard amidites were used at the recommended concentration (50 mg/mL), whilst the thymidine-3′-thioamidite was used at 100 mg/mL. Optimal coupling conditions were achieved using one of the activators SET or DCI at 1M concentration with coupling time of 15 min.

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15.

Sequence	Calculated mass	Measured mass
5′-TTT TTsT TTT T	2996.0	2995.9 ± 0.3
5′-CCT AAA TTsT GCC	3596.4	3597.1 ± 0.7

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