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SOLID-PHASE 'PHOSPHITE' SYNTHESIS OF OLIGONUCLEOTIDES

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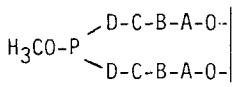
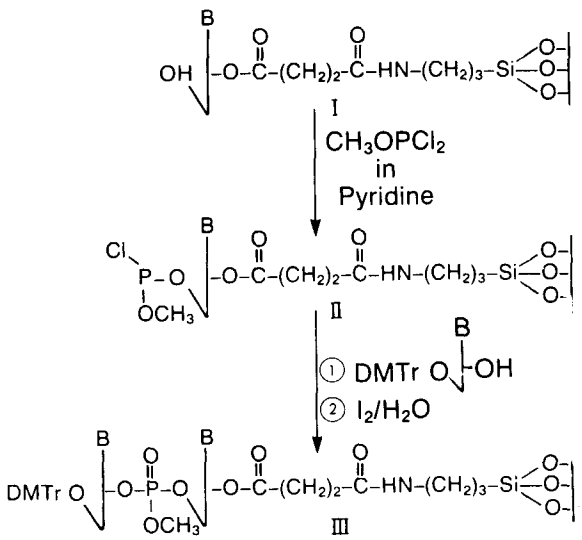
Abstract: A simple modification of the Letsinger's phosphite approach is shown to have a very good potential for fast, efficient and economical synthesis of oligonucleotides on silica gel support.

The 'phosphite' approach introduced by Letsinger et. al (1) has been adapted to the synthesis of oligonucleotides on silica gel support (2-4). The basic principle in this solid-phase approach is that a suitably protected nucleoside is reacted in solution with a bi-functional phosphitylating agent such as methoxydichlorophosphine and the resulting nucleoside phosphomonochloridite is then reacted with a second protected nucleoside attached to the support. Subsequent oxidation with iodine converts the 'dinucleoside phosphite' to the 'dinucleoside phosphate'. The main disadvantage of this procedure is the need to carry out the preparation of nucleoside phosphomonochloridite at -78°C due to its instability at higher temperatures. In addition, the formation of 3'-3' isomer during this preparation is quite considerable even at -78°C and even when the ratio of nucleoside to phosphitylating agent is carefully chosen. To overcome these problems Caruthers et. al. (5,6) prepared the 'tetrazolide' and 'phosphoramidite' derivatives of the nucleoside. The 'tetrazolide' derivative is not very stable and the preparation of 'phosphoramidite' is time consuming and hence we were interested in a simpler modification of the method. In this report we show that a simple rearrangement of the sequence of reactions can lead to significant improvements.

In our approach, derivatized silica carrying a nucleoside with a free 5'-hydroxyl group (I) is treated with methoxydichlorophosphine at 0°C or room temperature. This treatment yielded a nucleoside phosphomonochloridite attached to silica (II). Further treatment of the immobilized nucleoside phosphomonochloridite at room temperature with a second protected nucleoside followed by oxidation with iodine yielded a fully protected dimer (III). (See scheme I)

The advantages of this approach are: 1) It only requires the easily available starting materials such as fully protected nucleosides and methoxydichlorophosphine 2) Reactions can be done at 0°C or at room temperature which makes the method more easily adaptable for automation and 3) The excess nucleoside can be recovered by extraction procedures and can be reused. A potential disadvantage of this method is the formation of a 5'-5' cross-linking between two adjacent oligonucleotide chains during treatment with methoxydichlorophosphine as shown below.





Scheme I

We hope that this will not be a serious problem if the incorporation of nucleotides on the support is moderately small (~30 μmole/g). We can also circumvent this problem by converting the methoxydichlorophosphine to a tetrazole derivative.

To test the validity of our approach the following experiments were carried out. Derivatized silica (Vydak TP, Chem Genes Corporation, 100mg) carrying 4 μmole of deoxythymidine was treated with methoxydichlorophosphine (40-60 μmole) in anhydrous pyridine (0.2ml) at 0°C or at room temperature. The reaction mixture was shaken immediately and centrifuged (the total reaction time including centrifugation being 10' at 0°C or 2' at room temperature). The supernatant was removed and a 10 fold excess of 5'-O-(Dimethoxytrityl)-N-benzoyldeoxycytidine (40 μmole, 25mg) was added in 0.2ml pyridine. After 10' at room temperature, the reaction mixture was oxidized with iodine in a mixture of tetrahydrofuran/lutidine/water (2:1:1 v/v/v). The yield of the product was estimated by the amount of trityl group released (O.D at 498nm) and by HPLC after the product was deblocked. The yield was found to be 90% for this and several other dimer preparations. To eliminate concern for the introduction of moisture into the reaction during centrifugation, we examined the product formation without removing the excess methoxydichlorophosphine. In this experiment dT-silica (100mg, 4 μmole thymidine) was treated with methoxydichlorophosphine (40-60 μmole) in pyridine (0.2ml) for 10' at 0°C or 2' at room temperature. 5'-O' (Dimethoxytrityl)-N-benzoyldeoxycytidine (130-200 μmole) in pyridine (0.25ml) was then added to the reaction mixture. After 15' at room temperature, oxidation with iodine in a mixture of tetrahydrofuran/lutidine/water, (2:1:1 v/v/v) was carried out. The silica was then sequentially washed with 50% aqueous tetrahydrofuran, tetrahydrofuran and ether. The yield of the product was estimated to be 95% by the absorption of trityl cation at 498nm. The yield was also confirmed by reverse phase HPLC analysis after deblocking for 12-16 hours at 50°C with concentrated ammonium hydroxide followed by treatment with 80% acetic acid. The dimer peak was collected during HPLC analysis

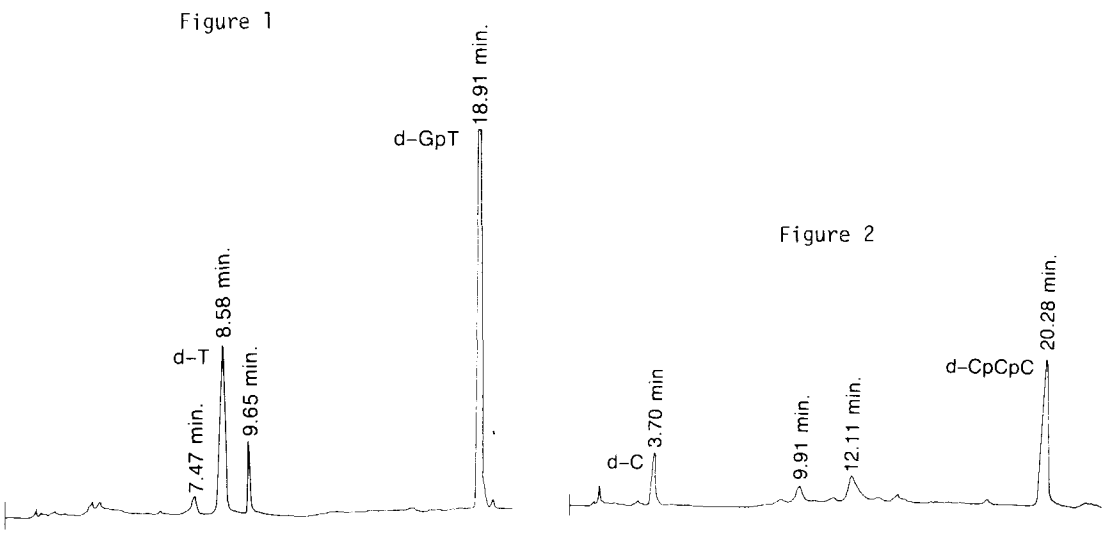
and the UV spectrum was found to agree very well with the computer generated spectrum. A few other dimers were similarly prepared. (Table 1).

Table 1

Compound	Isolated Yield (%)
d-CpC	96
d-GpT	87
d-CpT	95
d-GpG	98
d-CpG	84
d-GpA	81

Analysis of the reaction supernatants by TLC showed two spots in an approximate ratio of 80:20. The major spot corresponded to the unreacted nucleoside and the minor spot is presumed to be the 3'-3' isomer. This suggests that the excess nucleoside can be recovered if necessary. The effectiveness of this procedure was further tested by preparing the trimers, d-GpGpT, d-CpCpC, d-GpCpT, d-CpCpC, d-GpGpA and tetramers d-CpCpCpC and d-CpCpCpG in overall yields of 60-85%. The HPLC profiles of typical deblocked dimer and trimer reaction mixtures are shown in Figures 1 and 2.

Longer oligomers were also prepared successfully as exemplified by the synthesis of an octamer in Figure 3. The presence of tetrazole (2-3 fold molar excess over methoxydichlorophosphine) appears to enhance the yields for longer oligomers.



Figures 1 and 2. Reverse phase HPLC profiles on Partisil ODS-2 column of d-GpT (Figure 1) and d-CpCpC (Figure 2) preparations. A combined isocratic and gradient system of acetonitrile in 0.1M ammonium acetate, pH 5.8 was used. Isocratic run - 10 min with 1% CH_3CN followed by a gradient - 1-20% CH_3CN for 15'. Flow rate - 2.5ml/min.

Figure 3

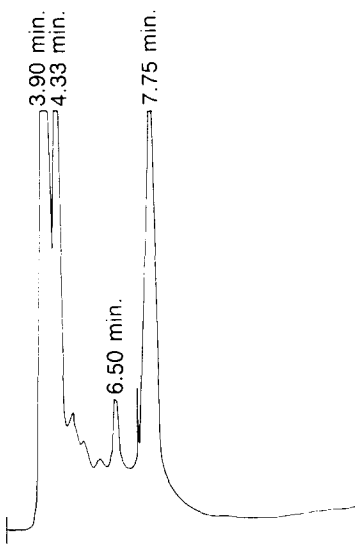


Figure 3. Ion-exchange chromatography of d-CAAGCTAG. The octamer peak (7.75 minutes) was isolated in 37% yield. The chain length of the oligomer was verified by analysis after labelling the 5'-end with γ-^{32}P-ATP using polynucleotide kinase.

Currently we have undertaken the synthesis of oligomers 10-17 residues long. The results will be presented in a future communication.

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