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THE SYNTHESIS OF OLIGODEOXYPYRIMIDINES ON A POLYMER SUPPORT

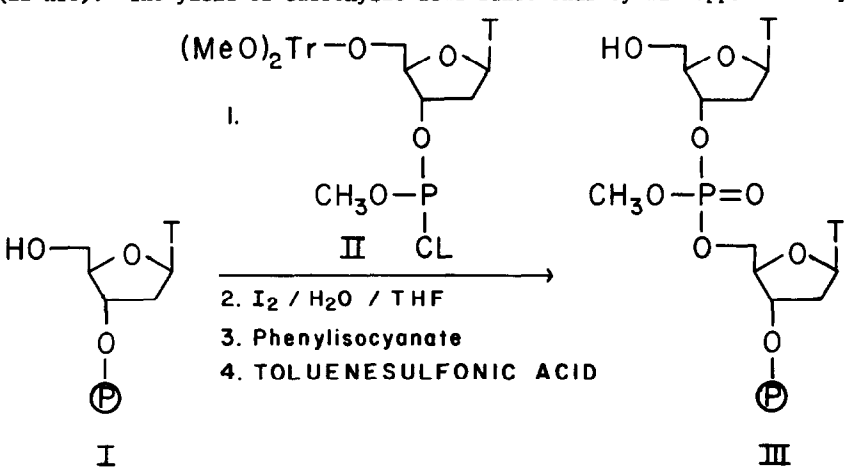
M. D. Matteucci and M. H. Caruthers*

Department of Chemistry, University of Colorado
Boulder, Colorado 80309

A polymer supported method for synthesizing deoxypolynucleotides is described. Two nonanucleotides were synthesized. Yields exceeding 90% were obtained for each condensation. The time per nucleotide addition was four hours.

Attempts to develop a successful methodology for synthesizing sequence defined oligonucleotides have been numerous[1,2]. However despite these achievements the stepwise synthesis of oligonucleotides still remains a difficult and time consuming task. We are developing procedures which should solve this problem. This communication outlines our initial results on adapting the phosphite coupling procedure[3] to polymer supported polynucleotide synthesis.

Classically a major problem with polymer supported synthetic strategies has been the polymer support. Various polymers have been shown to be inadequate for reasons such as slow diffusion rates into the support, excessive swelling of various macroporous, low crosslinked polymers and irreversible adsorption of reagents onto the polymer[2]. We selected macroporous, high performance liquid chromatography (hplc) silica gel because this material should be free of these problems. Other polymers consisting of polydimethylacrylamide[4], polyacrylmorpholide[5], or polystyrene grafted on the surface of polytetrafluoroethylene[6] also appear promising. The functionalized, insoluble support was prepared from a Separation Group silica gel (Vydak TP silica, 20 μm particle size, 300 Å pore size) using a three step procedure. The initial step involved refluxing 3-aminopropyltriethoxysilane (2.3 g, 0.01 M) with silica gel (2.6 g) in dry toluene for 3 hours[7]. Succinic anhydride (2.5 g, 0.025 M) in water was next reacted with the amino silica gel. The pH was maintained between 4 and 6 by addition of 2 N NaOH[8]. Excess silicic acid functionality was eliminated by treatment of the matrix with $(CH_3)_3SiCl$ (1.09 g, 0.01 M) in anhydrous pyridine (12 hrs). The yield of carboxylic acid functionality was approximately 250 μmole/g.



Thymidine was linked through the 3'-OH via an ester bond to the carboxylic acid support. Compound I was prepared by condensing 5'-O-dimethoxytritylthymidine [$(MeO)_2Tr$-T, 1.17 g 0.002 M] with the derivatized silica gel (4 g, 0.001 mole carboxyl groups) in anhydrous pyridine using dicyclohexylcarbodiimide (2.06 g, 0.01 M) as condensing agent. After 40 hours, residual acid groups were converted to an inert amide by addition first of p-nitrophenol and then piperidine[9]. After removal of the dimethoxytrityl group using 0.1 N p-toluenesulfonic acid in acetonitrile[9], the yield of thymidine attached to the support (compound I, dT-Ⓟ) was 40 μmole/g).

For the stepwise synthesis of oligodeoxythymidine, compound II was used. This phosphomonochloridite was synthesized by addition of 1.0 equivalent 5'-O-dimethoxytritylthymidine to 0.8 equivalent methyl phosphorodichloridite and 5 equivalents collidine in THF at -78°C. Compound II can be stored anhydrous in THF at -78°C under nitrogen for at least one week.

The use of compound II in oligonucleotide synthesis was tested by preparing $d(T)_9$. The silica gel resin containing thymidine was packed into a glass column. This column was attached through a series of valves and teflon tubing to a Milton Roy Minipump and an injector loop. The apparatus was organized so that reagents could be recycled through the column or flushed to waste or collect. The steps involved in synthesizing thymidylylthymidine attached to the support (compound III) are the following. (1) Recycle compound II in THF and collidine through the column containing dT-Ⓟ for 1 hr. (2) Oxidize the polymer supported dinucleoside phosphite to the phosphate using 0.01 M I_2 in water/2, 6 lutidine/THF (30 min). (3) Recycle phenylisocyanate in THF and 2, 6 lutidine through the column for 1.5 hr. This reagent protects against the formation of failure sequences by reacting with unphosphorylated nucleoside hydroxyl groups[10]. (4) Flush the column with toluenesulfonic acid in acetonitrile (2 min). The total time needed for addition of one nucleotide, including various wash cycles after each step was 4 hours. The same procedure was used for preparing d(T-C-T-C-T-C-T-T-T). The cytosine containing phosphomonochloridite was prepared from 5'-O-dimethoxytrityl-N-benzoyldeoxycytidine.

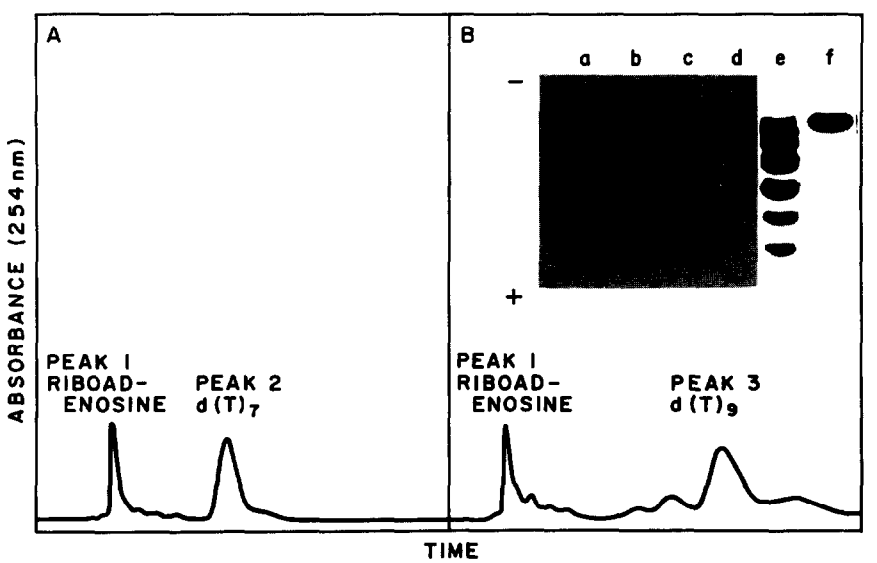


Figure 1. HPLC and Gel Electrophoresis Analysis of $d(T)_7$, $d(T)_9$ and d(T-C-T-C-T-C-T-T-T).

The two nonanucleotides and one intermediate, $d(T)_7$, were freed from protecting groups, isolated and characterized. The methyl group was removed from phosphotriesters using triethylammonium thiophenoxide in dioxane[11]. This step was followed by treatment with concentrated NH_4OH which removes the N-benzoyl group from cytosine and frees the oligonucleotides from the support. The hplc profiles (reverse phase, C_{18}) for reaction mixtures containing $d(T)_7$ and $d(T)_9$ are shown in Figure 1. In each case the major product was the heptamer (Part A) or the nonamer (Part B). Peak 1 in each profile was a riboadenosine calibration standard and thus was not part of the reaction mixture. Similarly the hplc pattern for the reaction mixture containing d(T-C-T-C-T-C-T-T-T) is reproduced in Figure 2. Once again the major peak was the nonamer. Based on the amount of thymidine initially linked to the support, the isolated yield of $d(T)_9$ was 25%. The corresponding yield of d(T-C-T-C-T-C-T-T-T) was 23%. Both nonamers and the heptamer have been biochemically characterized. All three compounds were completely degraded by Snake Venom Phosphodiesterase (reverse phase hplc analyses are not shown). The oligonucleotides isolated from each nonamer synthesis (peak 3, Figure 1 and peak 2, Figure 2) were phosphorylated using [γ-^{32}P]ATP and T4-kinase. The inset to Figure 1, Part B displays electrophoretic profiles for these [5'-^{32}P] labeled oligonucleotides after partial degradation with Snake Venom Phosphodiesterase. The material isolated from peak 3 (Figure 1, Part B) is shown before (channel a) and after (channel b) partial degradation. As can be seen by counting the number of radioactive bands in channel b, this sample corresponds to [5'-^{32}P]$d(pT)_9$. The material isolated from peak 2 (Figure 2) was analyzed in a similar manner before (channels d and f) and after (channels c and e) partial enzymic degradation. Once again the analysis indicates that the product is homogeneous and contains nine nucleotides. In order to confirm the sequence of [5'-^{32}P]d(pT-C-T-C-T-C-T-T-T), the sample was analyzed by two dimension homochromatography[12]. The results are displayed as an inset to Figure 2. The sequence profile is consistent with the results expected for

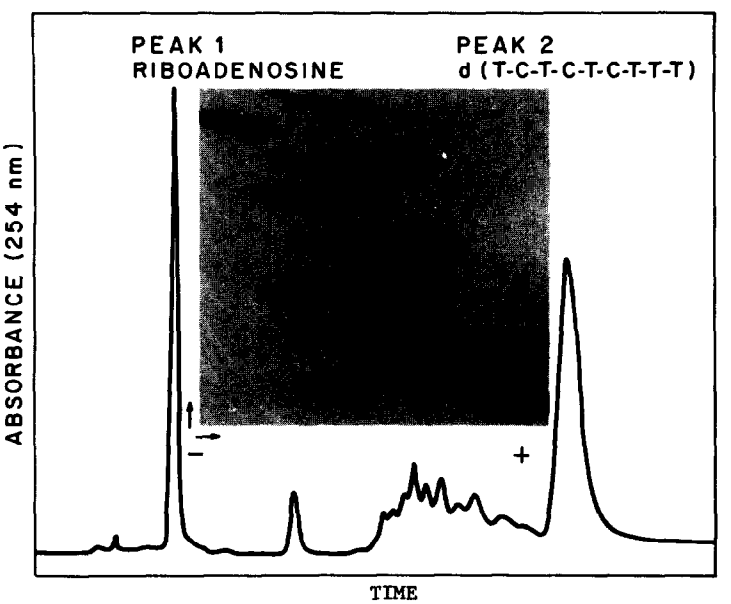


Figure 2. HPLC and Sequence Analysis of d(T-C-T-C-T-C-T-T-T).

[5'-^{32}P]d(pT-C-T-C-T-C-T-T-T). Finally [5'-^{32}P]d(pT)$_9$ was shown to polymerize in the presence of T4-ligase and polydeoxyadenosine indicating that [5'-^{32}P]d(pT)$_9$ forms a duplex with polydeoxyadenosine and that this duplex is recognized by T4-ligase. Therefore d(T)$_9$ and d(T-C-T-C-T-C-T-T-T) were biochemically active by every criteria so far tested. This synthetic methodology can be used for the rapid synthesis of deoxyoligopyrimidines in high yield. We also anticipate that this method can be applied to the synthesis of deoxyoligopurines as well.

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