Polymer supported synthesis of oligonucleotides by a phosphotriester method

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ABSTRACT

A new polymer supported synthesis of deoxyribooligonucleotides which can be adapted to automation is described. The method is based on the elongation of an oligomer chain in the 5'- to 3'-end direction using the modified phosphotriester chemistry. The approach is exemplified by the synthesis of a nonanucleotide d(TTCGTCTTG).

INTRODUCTION:

Synthetic oligonucleotides of biologically important DNA sequences are in great demand not only for genetic engineering purposes but also for various studies in molecular biology (1,2). A number of methods have been developed in several laboratories for the synthesis of oligonucleotides of specified sequence (3,4,5,6,7). Among these methods the phosphotriester (2,3,6) and the phosphite triester syntheses (8,9) are the most widely used methods for the rapid preparation of oligonucleotides. In the last couple of years we have been using the phosphotriester method in solution to synthesize oligonucleotides as probes, primers, linkers and gene fragments. During these studies we developed a new, rapid and simple procedure for the synthesis of oligonucleotides in the 5' to 3'-end direction on a polymer support using the modified phosphotriester method. In this paper, we report on the synthesis of a nonanucleotide d(T-T-C-G-T-C-T-G) as an example to demonstrate the feasibility and efficiency of this method.

RESULTS AND DISCUSSION:

In the last few years, several methods for the polymer supported synthesis of oligonucleotides in the 3'- to 5'-end direction have been described by a number of laboratories (10,11,12,12,14). In this approach, the 3'-hydroxyl of a nucleotide was attached to the polymer support and the oligonucleotide chain extended towards the 5'-end by the addition of

suitably 5'-protected nucleotide components containing either 3'-phosphate or phosphite moiety. These methods were also successfully applied to the development of automated DNA synthesizers. However, despite these achievements, there still remain a few problems, such as the instability of reagents and failure to produce the desired final compounds. In order to improve the overall methodology for automation, we have investigated the extension of the oligonucleotide chain in the other direction, viz, the 5'-to 3'-end direction as shown in Figure 1. The outline of our strategy includes the following steps:

- (i) Attachment of 5'-hydroxyl 3'-fully protected phosphate nucleotides (II) to the polymer support via 5'-O-trityl linkage or 5'-O-succinate linkage.
- (ii) Removal of a 3'-phosphate blocking group such as β -cyanoethyl under mild basic conditions (triethylamine or diisopropylamine in pyridine) to give a 3'-phosphodiester component (III).
- (iii) Condensation of the liberated 3'-phosphodiester component (III) with a 5'-hydroxyl-3'-fully protected phosphate nucleotide (II) in the presence of a coupling reagent.
- (iv) Capping any unreacted phosphate group (not studied in the present work).

These synthetic cycles are repeated using different 5'-hydroxyl 3'-protected nucleotides until the desired sequence is constructed. At the end, the linkage between the support and the oligonucleotide chain is cleaved and all of the protecting groups are removed. Finally, the product is purified by high performance liquid chromatography (HPLC).

Various polymers consisting of polystyrene copolymer with 1 or 2 percent divinylbenzene (10), polydimethylacrylamide (11), polyacrylmorpholide (12), cellulose (13) or hplc silica gel (14) have appeared promising as polymer supports. However, Itakura, et al, studied in detail (10) the practical usage of a polystyrene copolymer for the solid support in the synthesis of oligonucleotides for 3'-to 5'-end assembly and found that this polymer swelled better in organic solvents, giving higher coupling yields in the internucleotide phosphotriester bond formation. Therefore, we selected polystyrene-copolymer with 1 or 2 percent divinylbenzene as the solid support for the present work.

The readily available starting material, chloromethylpolystyrene crosslinked with 1 or 2 percent divinylbenzene, was derivatized to form a trityl-containing polymer (I) by following essentially the published

Figure 1. Functionalization of Polymer and Scheme for the Synthesis of the Oligonucleotide.

procedure (15) and then was anchored to the 5'-hydroxyl of the first nucleotide. (Figure 1).

Thus, starting from chloromethylated 1 or 2 percent divinyl-benzene-styrene copolymer containing 700 μ moles of Cl/gm, the trityl polymer, I, (510 μ mole of Cl/gm) was obtained (72 percent yield). This polymer (I) was then reacted with the 5'-hydroxyl -3'phosphate protected thymidine (II, B=thymidine) in pyridine: toluene (2:1,v/v) at room temperature for 60 hrs to give a polymer, III, (B=thymidine) into which 280 μ mole (58 percent) of nucleotide had been incorporated. The 3'-fully protected phosphate in the polymer III was converted into 3'-phosphodiester component, IV (B=thymidine), by shaking it with the excess of diisopropylamine in pyridine at room temperature for 30 minutes.

The synthesis of nonanucleotide d(T-T-C-G-T-C-T-T-G) was then continued solely by using mononucleotides as coupling units as described in Figure 1. In the present work, we used 1-(4-toluenesulfonyl)-3-nitro-1,2,4-triazole (TsNT) as a coupling reagent because it was found to be more stable at room temperature and cheaper than the other condensing agents, viz. 2,4,6-triisopropylbenzenesulfonyl-1H-tetrazole (TPSt), or mesitylenesulfonyl-1H-tetrazole (MSt). The sequence of steps described in Figure (1) was carried out in a small fritted funnel equipped with a stopcock and a cap as follows:

- (1) Wash with pyridine, ether, and pyridine, 5 ml each.
- (2) Shake 30 minutes at room temperature with pyridine: diisopropylamine (2:1, v/v), 5 ml.
- (3) Wash with pyridine, ether, and pyridine, 10 ml each.
- (4) Wash with dry pyridine, ether, and dry pyridine, 5 ml each.
- (5) Add coupling reagent, TSNT (4 fold molar excess in 1.5 ml dry pyridine and mix briefly.
- (6) Add 5'-hydroxyl-3'-phosphate protected mononucleotide (2 fold molar excess, dried 2 times with dry pyridine). Shake for 30 minutes at room temperature.
- (7) Drain solvents.
- (8) Repeat steps 5, 6, and 7 described above.
- (9) Start cycle over at Step 1.

Finally, the protected nonanucleotide, d[T+T+bzC+ibG+T+bzC+T+T+ibG(Bz)] was removed from the polymer VII by shaking with saturated zinc bromide in nitromethane and the product was purified by thin layer chromatography on silica gel, then on KC₁₈ reverse phase plates. The desired major band was

isolated by extraction with methylene chloride/methanol (4:1, v/v). The protected nona-nucleotide thus obtained was treated first with N,N,N',N'-tetramethylguanidinium pyridine-2-carboxaldoximate, then concentrated ammonium hydroxide, as described in the experimental section. The HPLC elution profile on C₁₈-column of the desired nonanucleotide d(T-T-C-G-T-C-T-T-G) obtained in this way is shown in Figure 2A. The sequence of this oligonucleotide was confirmed by the standard analysis(16) of p³²-labelled sample (Figure 2B).

Analysis of the reaction products after removal from the polymer at several of the intermediate coupling steps revealed that the desired compound was the major product and that the coupling reaction forming phosphotriester bonds on the polymer support was efficient. Throughout the isolation process, cuts taken from chromatography plates and columns were rather narrow. The actual purification on a PEI plate was done with only a small portion of the total material. It is estimated from the analyses of the intermediate steps that each coupling was complete in the order of 80 percent.

We are currently attempting to synthesize a tetradecanucleotide by adapting this procedure to an automated Beckmann peptide synthesizer and the results will be published elsewhere. In order to further simplify the procedure for removal of polymer from the desired oligonucleotide chain and its final purification, we are also investigating a similar procedure to

Figure 2. (A) HPLC purification of d(TTCGTCTTG). The mobile phase was 0.1M aqueous TEAA containing 12 percent acetonitrile. (B) Two-dimensional finger-printing pattern of p³²(TTCGTCTTG) after partial digestion with snake venom phosphodiesterase.

synthesize a decanucleotide d(GGCGACCGTG) by using 5'-O-succinate linkage, $\text{P-NH-CO-CH}_2\text{-CH}_2\text{-COO-}$, instead of the 5'-O-trityl linkage.

CONCLUDING REMARKS:

Recently, the phosphite approach has been successfully applied to solid-phase synthesis and high coupling yields (8,18) have been reported. However, the method described in this work has the following advantages:

1. The reagents used are relatively cheap, stable, and commercially available.
2. The protected mononucleotides are stable in pyridine solution at room temperature.
3. The major impurity – the 5'-sulfonylated nucleotide formed by reaction of 5'-hydroxyl group with the coupling reagent—is washed away from the polymer.
4. The reactions involve no acidic or hydroxylic reagents or solvents.
5. The removal of the final product from the polymer is done aprotically, preventing depurination. The resulting oligomer, which is fully protected except at the 5'-end, may be purified by silica or reverse phase (C_{18}) TLC plates before treatment for final deprotection.
6. It is readily adaptable to automation, for the development of a DNA synthesizer.

EXPERIMENTAL

Materials and Methods:

Pyridine was treated with chlorosulfonic acid before distillation, redistilled from potassium hydroxide and stored over 4A molecular sieves. Diisopropylamine was distilled and stored over 4A molecular sieves. Other solvents viz. ether, toluene, and benzene used in this work were dried over 4A molecular sieves. Merck silica gel 60F-254 TLC plates (0.25 mm and 2 mm thickness) and Whatman KC_{18} -Reverse phase (RP-18) TLC plates with a fluorescent indicator were purchased commercially.

1-(4-toluenesulfonyl)-3-nitro-1,2,4-triazole (TSNT) was prepared by the method of Jones, et al (17). The chloromethylpolystyrene polymer (1 percent or 2 percent cross linked with divinylbenzene) was obtained from Mr. Charles Campbell (MC797). The trityl containing polymer, I, was prepared according to the procedure of Melby and Strobach (15), with exception of the use of dimethylformamide instead of dimethylacetamide. All four protected mononucleotides were prepared by the method of Narang, et al (6) with some

modifications in the phosphorylation reaction.

General Procedure for the Attachment of Nucleotide (II) to the Polymer, I to give Polymer III.

The polymer I (1g, .00051mole Cl/gm) was shaken with 5'-hydroxyl-3' phosphate protected mononucleotide, II (B=thymidine, 0.74 g, .00153 mmole, which had been rendered anhydrous by coevaporation 3 times with pyridine) in 75 ml of 2:1 dry toluene/dry pyridine for 60 hrs. The resulting polymer (B=thymidine) was washed extensively with pyridine, methanol and then ether. Finally, it was vacuum dried. Incorporation of the protected thymidine phosphate was 0.000285 mole/gm by UV analysis, as described below:

A portion of the polymer III was agitated overnight in saturated zinc bromide/anhydrous nitromethane (1 percent water in CH_3NO_2 for thymidine) and filtered. The resin was washed with nitromethane, methylene chloride, and methanol successively. All the washings were combined and extracted twice with 1M ammonium acetate solution. The solvents were dried over sodium sulfate and evaporated. The residue was chromatographed on silica gel preparative TLC plates using 10-13 percent methanol/methylene chloride. The band containing the nucleotide material was cut, eluted and dissolved in dry methanol for UV determination. The extinction coefficient for each protected mononucleotide was determined previously by measurement of A_{260} of purified samples. All determinations were done in duplicate.

General Procedure for Condensation on the Polymer

The polymer III (B=Thymidine, 0.437 gms, 0.000124 mole) was shaken in 6 ml of 2:1 pyridine/diisopropylamine for 30 minutes to remove the cyanoethyl group. The resulting polymer IV (B=Thymidine) was washed 3 times with alternate 10 ml portions of Pyridine and dry ether. The polymer was washed with dry pyridine, dry ether and dry pyridine in a dry box. TSNT (0.13 g, 0.000485 mole) was added in 1.5 ml of dry pyridine and the mixture shaken for a few seconds. Then the 3'-phosphate protected nucleotide (B=thymidine, 0.14 gm, 0.000289 mole), which had been dried previously by coevaporation with dry pyridine two times, was added in 1.5 ml of dry pyridine. The reaction was shaken for 30 minutes. Then the solvent was drained and fresh TSNT and II(B=thymidine) were added. After another 30 minutes, the reactants were washed from the polymer with pyridine, followed by ether. The cycle was repeated starting with decyanoethylation. The final cycle used nucleotide VI (B=guanosine) as the chain termination step.

Purification and Isolation of the Final Product

The oligomer was removed from the Polymer VII as described above and the

residue was chromatographed on a silica gel plate (2 mm thickness) using 13 percent methanol/methylene chloride. The material of R_f 0.6 was cut, eluted and rechromatographed on KC₁₈ reverse phase TLC plate with 25 percent water/acetone. The major desired band was cut and eluted. The residue after evaporation was treated with 2 ml of N,N,N',N'-tetramethylguanidinium pyridine-2-carboxaldoximate (0.28M in 1:1 dioxane/water) for 26 hrs at room temperature. The solvents were evaporated and the residue dissolved in 8 ml of concentrated NH₄OH at 37°C for 18 hours and at 60°C for 6 hours. After removal of solvents, the residue was dissolved in 5 ml of 0.05M triethyl ammonium bicarbonate (TEAB) and passed through a small DEAE-52 column. The TEAB solvent was removed by evaporation and a portion of residue was purified on a PEI plate using 0.6M LiCl, 7M urea and 0.02M tris pH 7.6. The single band was cut and eluted with 2M TEAB. It was evaporated and the residue was dissolved in water. It was then purified on a C18 column by using 0.1M aqueous triethyl ammonium acetate containing 12 percent acetonitrile (Figure 2A). The major peak eluting at 9.1 min. was isolated. This was then lyophilized twice and dissolved in 1 ml of 10 mM tris pH 7.6. The yield was 1.95 A₂₆₀ units, as determined spectroscopically. A portion of this sample was phosphorylated by polynucleotide kinase using (γ-P³²) ATP and the sequence was determined by the two dimensional mobility shift method(16). The pattern obtained is shown in Fig. 2B.

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