Volume 8 Number 22 1980 Nucleic Acids Research

Solid-phase synthesis of polynucleotides. III. Synthesis of polynucleotides with defined sequences by the block coupling phosphotriester method

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Received 25 July 1980

ABSTRACT

Preparation of the three hexadecanucleotides, dGpTpApTpCpApCpGpApGpGpCpCpCpTpT, dCpGpApCpGpApGpCpGpTpGpApCpApCpC and cTpGpCpCpGpGpCpCpApCpGpApTpGpCpG, is described by a rapid and simple solid-phase method on polyacrylamide supports. The syntheses were performed by the extension of the method described in the previous paper using di and trinucleotides of defined sequences as an incoming 3'-phosphodiester unit. Although the coupling yields to form phosphotriester bonds are slightly lower than those for the homothymidylic acid series, pure polydeoxyribonucleotides of defined sequences can be synthesized without any major difficulty.

INTRODUCTION

In the previous paper[1], we reported a rapid and simple solid-phase method for the synthesis of polythymidylic acids. Generally speaking, the yields of the coupling reaction to make internucleotidic phosphotriester bonds between thymidine derivatives are always higher than those among other bases, including guanine, adenine and cytosine, by the solution method. Therefore, the true value of a new approach for the synthesis must always be tested with the synthesis of polynucleotides with defined sequences.

Sequence-specific oligodeoxyribonucleotides are an essential requirement in the various studies of molecular biology; such as gene synthesis for the production of peptide hormones in *E. coli*[2,3], DNA and RNA sequencing[4,5], specific probes for hybridization[6], and so on[7]. All of these studies require the rapid synthesis of oligonucleotides ($\sim$ pentadecanucleotide) with defined sequences. The current phosphotriester approach in solution is reasonably rapid and accurate for the synthesis of those oligo-

nucleotides. However, there still remains some problems in the synthesis. One of these problems is the time-consuming chromatographical purification step. In this article, we will report a rapid and accurate solid-phase method for the synthesis of sequence-specific polydeoxyribonucleotides.

RESULTS AND DISCUSSION

The chemical principles involved in the synthesis of sequence-specific polydeoxyribonucleotides have been reported in the previous paper[1]. The essential features of the approach are a) sequential addition of di and trinucleotides as coupling units instead of mononucleotides, b) the use of polyacrylamide resin, well-swollen in various solvents and c) the extension of the oligomer chain from the 3'- to the 5'-end. In addition to these features, several modifications have been introduced in this study:

1) The unreacted 5'-hydroxyl groups on a solid-phase were completely masked before the next step.

2) To avoid depurination of the N-benzoyl deoxyadenosine, the reaction time of the acid treatment to remove dimethoxytrityl groups was shortened to 1 minute at room temperature.

3) In addition to the Permaphase AAX column[3], a μBondapak C_{18} column[8] was used for the final analysis and purification of the products.

4) As a protecting group for internucleotidic phosphate bonds, an o-chlorophenyl group was used in place of p-chlorophenyl because the former gave less by-products during the deblocking[9].

Polymer Support and Nucleotide-Polymer Linkage

As described in the accompanying paper[1], the polymer-support (II) derivatized from the commercially available Enzacryl Gel K-2 (I) has essential advantages over the carboxyl polymer (III) and, therefore, II was used exclusively in this study (Figure 1). The activates esters (IV bcd) were coupled with the resin (II) to afford the dimethoxytrityl resin (V) with a similar yield as in the case of the thymidine base (IVa, 0.15∿0.18 mmole/g of nucleoside). The amount of nucleosides attached on the polymer (V) were estimated by the quantitative analysis of dimethoxytrityl group and nucleosides liberated from the resin.

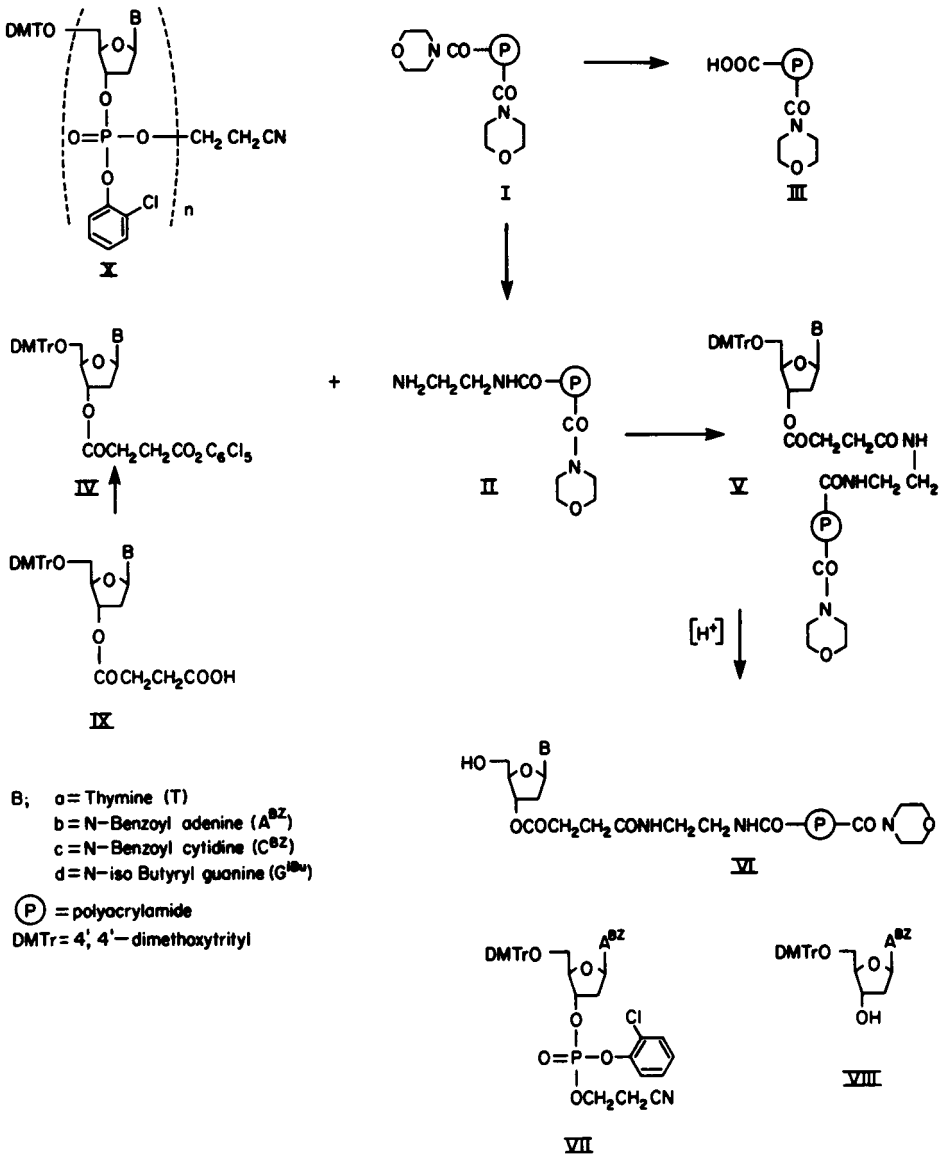

FIGURE 1. Preparation of Resin with the First Nucleoside.

Detritylation of Vb (B = N-benzoyl adenine) under the conditions of a 2% solution of benzenesulfonic acid in chloroform-methanol (7:3 v/v) at room temperature for 1 minute, caused the

depurination (about 10%). It is known that the rate of depurination is dependent upon the substitution group on the 3'-hydroxyl group of the sugar part[10]. Indeed, detritylation of the fully protected mononucleotide (VII), under the conditions described above, does not cause any detectable depurination. On the other hand, N-benzoyl-5'-O-dimethoxytrityl deoxyadenosine (VIII) gives about 15% of N-benzoyl adenine under the same acidic treatment[11]. Accordingly, the one limitation of our solid-phase synthesis using the acid labile protecting group, 4',4'-dimethoxytrityl function, is the synthesis of the polydeoxyribonucleotides with adenine base at the 3'-terminal position. In the solution approach, we observed the same problem and carefully avoided the synthesis of oligonucleotides whose 3'-terminal base sequence was adenine[12]. Actually, this limitation has not been at all serious for the studies of biological problems, because careful design of the sequence does not necessarily require an adenine base at the 3'-terminus position of oligonucleotides. The following methods are suggested to overcome this problem: 1) Development of selective conditions to remove the dimethoxytrityl group. Very recently, two groups (M.H. Caruthers and H. Köster) have found, independently, a new condition to selectively remove the dimethoxytrityl function.[13] 2) Utilization of a new protecting group[14] for the 5'-hydroxyl function, which can be removed under very mild conditions, in place of the dimethoxytrityl group. 3) Utilization of a 3'-phosphotriester bond, such as a phosphoamide, instead of an ester bond for the linkage between the 3'-terminus hydroxyl group of N-benzoyl deoxyadenosine and the polymer support.

Masking of the Unreacted 5'-Hydroxyl Group

Quantitative internucleotidic coupling is rarely achieved in oligonucleotide synthesis. By our solid-phase synthesis, chain elongation from the 3'- to the 5'-end, it is impossible to remove the unreacted 5'-hydroxyl oligomers. After repeating many cycles of the coupling reaction, the desired final product is not a major product if the unreacted oligomers are not masked at each cycle. Assuming 90% of the available 5'-hydroxyl groups on the solid-phase react with the 3'-phosphodiester component at each coupling (this is probably the maximum yield by our solid-phase

method), after six couplings the desired final product is only $(0.90)^6$ X 100 = 53% among other oligonucleotides. Thus, unmasking of the unreacted 5'-hydroxyl group caused some inconveniences. For example, the analysis of the dimethoxytrityl groups liberated from the resin after coupling reactions did not give exact information on the coupling yields and the incoming 3'-phosphodiester components were wasted to react with 5'-hydroxyl groups of undesired shorter oligonucleotides[1]. For the masking of the 5'-hydroxyl functions, two reagents were used, acetic anhydride and phenyl isocyanate. After every coupling reaction, the resin was treated with 10% solution of acetic anhydride or phenyl isocyanate in pyridine for 1 hour and then the dimethoxytrityl group was removed. Although the dimethoxytrityl group has some disadvantages for the protection of the 5'-hydroxyl group, as discussed above, this group makes it possible to immediately estimate the coupling yields without any special treatment of the resin.

Purification and Analysis of Polynucleotides

As described in the accompanying paper, the efficient separation of the final reaction mixture, including not only desired oligomers but also a series of shorter oligomers, was an essential requirement for a reliable synthesis. Permaphase AAX column chromatography, was quite satisfactory in the case of the $dT(pT)_{18}$ synthesis. The analysis of the product at each cycle, after removal of all the protecting groups, by high performance liquid chromatography (HPLC) on the Permaphase AAX colum revealed that the synthesis of polynucleotides with defined sequences on the solid-phase was also more complicated than those of polythymidylic acid as observed in the solution method. Consequently, the final product isolated by the ion-exchanger was further analyzed and purified by μBondapak C_{18} reverse-phase chromatography[8]. The separation was achieved with linear acetonitrile gradient at 55°C at pH 7.0 (Figures 2 and 3). The separation of the reverse-phase column is very sensitive to the lipophilicy of the products and the small peaks having longer retention time than the desired major peaks were always observed. These peaks probably contained not completely deblocked polynucleotides that still have protecting groups for amino functions

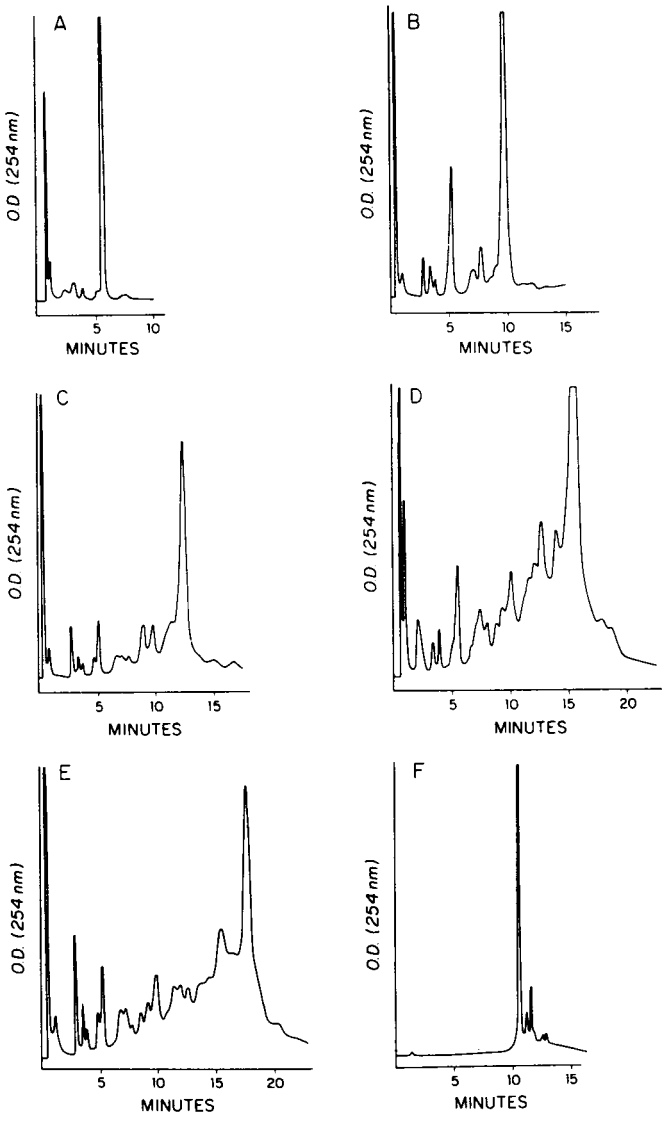

FIGURE 2. HPLC Analysis of the Synthesis of dTGCCGGCCACGATGCG.

After each coupling cycle, all the protecting groups were removed and the resultant oligonucleotides were fractionated on a Permaphase AAX column under the exact conditions previously reported.[1] At A: the first cycle (tetramer, dTGCG); B: the second cycle (heptamer, dCGATGCG); C: the third cycle (decamer, dCCACGAT GCG); D: the fourth cycle (tridecamer, dCGGCCACGATGCG); and E, the last cycle (hexadecamer, dTGCCGGCCACGATGCG). F: The last peak of E was further fractionated on μBondapak C_{18} after desalting under the conditions as described in the Experimental Section.

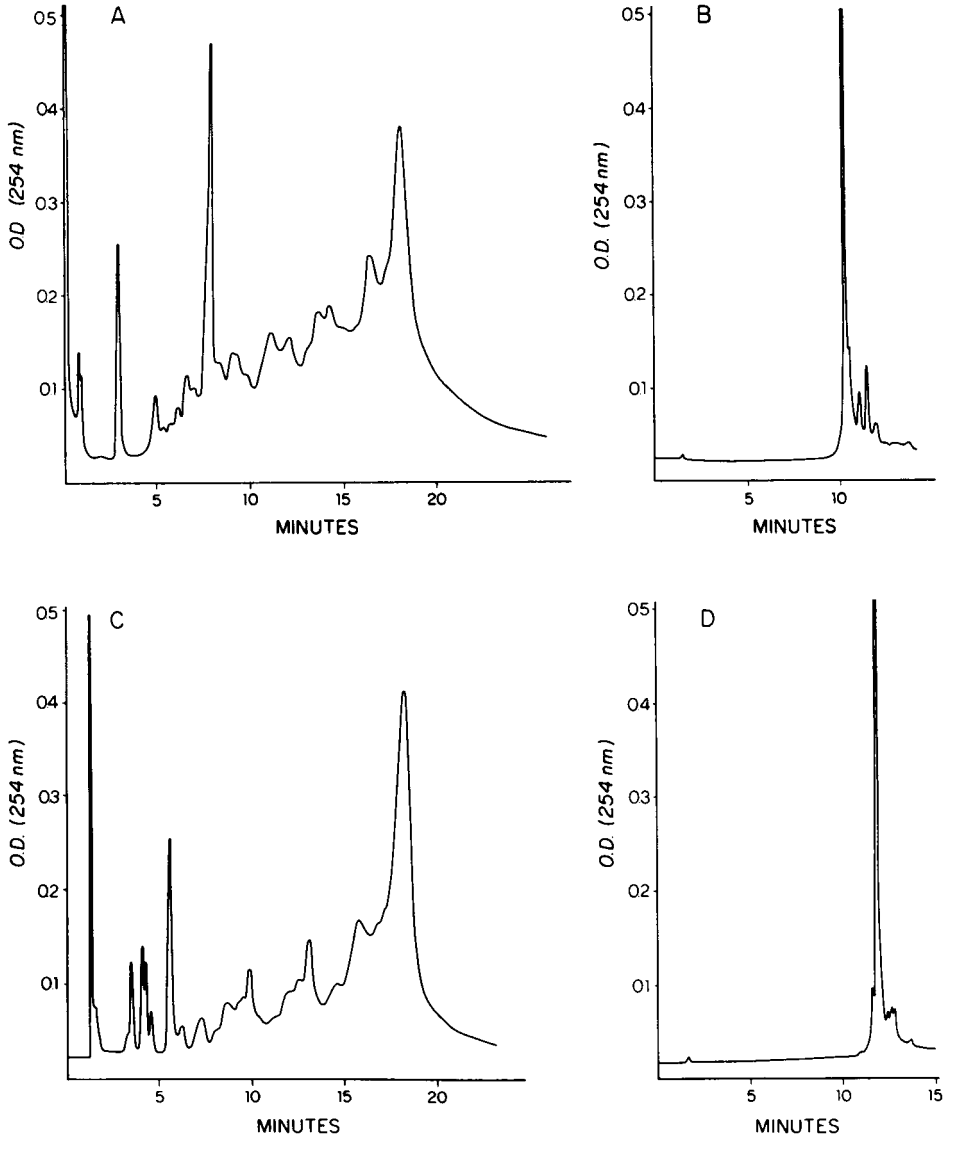

FIGURE 3. HPLC Analysis of Hexadecamers by Two Different Principles.

At A, the fractionation of the final cycle to synthesize dGTATCA CGAGGCCCTT on a Permaphase AAX column; B, further analysis of the last peak in A on μBondapak C_{18}. At C and D, the analysis of dCGACGAGCGTG ACACC on a Permaphase AAX and a μBondapak C_{18} column, respectively.

or phosphate groups. The analysis and purification of the desired product by these two different principles should be essential if the very pure polynucleotides are required. The desired product thus isolated was identified by the sequencing method[15].

General Procedure for the Synthesis

One cycle of the addition of an oligonucleotide unit to the solid-phase was essentially the same as described in the accompanying paper[1], except for the additional step to mask the unreacted 5'-hydroxyl groups and a shorter time of the acidic treatment to remove the dimethoxytrityl group (see Table 1). A part of the resin at each cycle was removed and treated successively with aqueous ammonia (d = 0.88) and 80% acetic acid and the reaction mixture of each coupling reaction was analyzed by high performance liquid chromatography on Permaphase AAX.

The typical HPLC profile of each cycle to synthesize the hexadecanucleotide, dTpGpCpCpGpGpCpCpApCpGpApTpGpCpG, was shown in Figure 2. The HPLC profiles of the other hexadecamers were shown in Figure 3. These polynucleotide sequences are being used as primers for the DNA sequencing developed by Sanger[16] and the results will be published elsewhere.

The above experiments demonstrated that the new solid-phase synthesis using oligonucleotide blocks as an additional unit at

TABLE 1.

STEP	SOLVENT or REAGENT	AMOUNT	SHAKING TIME (Minutes)	NUMBER OF OPERATIONS
1	2% BSA	10 ml	0.5	2
2	$CHCl_3$-MeOH (7:3 v/v)	10 ml	1	2
3	Pyridine	10 ml	1	2
4	Dimer or Trimer in Pyridine	5 equivalent	co-evaporation	3
5	TPST in Pyridine	10 equivalent /5 ml	180	1
6	Pyridine	10 ml	1	2
7	10% $(AcO)_2O$ or PhNCO in Pyridine	10 ml	60	1
8	Pyridine	10 ml	1	2
9	$CHCl_3$-MeOH (7:3 v/v)	10 ml	1	3

each cycle is very powerful for the rapid synthesis of polydeoxyribonucleotides. Furthermore, use of the rapid and efficient purification method by HPLC and the improved synthetic method for oligonucleotide blocks[17] have contributed significantly to increasing speed and accuracy of the synthesis. Although sequence-specific polydeoxyribonucleotides can be easily synthesized and isolated with a good yield by our method, there still remain some aspects to be investigated, including automation of all cycles by machine, depurination of N-benzoyl deoxyadenosine, and the length of polynucleotides to be synthesized on the solid-phase. These works are under continuing research.

EXPERIMENTAL SECTION

Material and Methods

Materials and methods not otherwise mentioned here have been described in previous papers.[1,17]

Synthesis of Monosuccinates (IX)

Anhydrous 5'-O-dimethoxytrityl nucleoside (10 mmole) in pyridine (40 ml) was reacted with succinic anhydride (1.50 g, 15 mmole) in the presence of dimethylaminopyridine (1.83 g, 15 mmole). The reaction mixture was stirred at room temperature overnight and concentrated to a gum, which was taken up in $CHCl_3$. The $CHCl_3$ solution was washed with 0.1 M NaH_2PO_4 solution, $NaHCO_3$ and water twice, dried (Na_2SO_4) and evaporated. The product was chromatographically purified on silica gel (120 g) eluted with $CHCl_3$-MeOH solution (0-5% v/v). The isolated product was dissolved in a small amount of $CHCl_3$, and added dropwise to pentane (2 ℓ). The precipitates were collected by filtration and dried. The yield was 83-90%.

Preparation of Pentachlorophenyl Succinate (IV)

A mixture of the monosuccinate (IX, 2 mmole), pentachlorophenol (590 mg, 2.2 mmole) and dicyclohexyldiimide (620 mg, 3 mmole) in 15 ml of dimethylforamide (DMF) was stirred at room temperature for 20 hours. After filtration, the filtrate was evaporated and the residue was dissolved in benzene to remove the urea derivative. The product was precipitated by the dropwise addition of the benzene solution to pentane (400 ml). The

yield was 80-85%.

Formation of Nucleoside-Polymer Linkage

The amino resin (5 g, 0.18 mmole/g) was reacted with the pentachloro ester (4.0 mmole) and triethylamine (0.44 g, 4.4 mmole) by shaking in 30 ml of DMF for 20 hours at room temperature. The mixture was filtered and the resin was washed with DMF and pyridine, and treated with phenylisocyanate (10% solution) in pyridine (35 ml) for 3 hours. The resin was filtered, washed with pyridine and MeOH, and dried. Both supports (V) had the same amount of nucleosides. The yield was: 0.15 mmole of deoxycytidine per 1 g of Vc and 0.15 mmole of deoxyguanosine per 1 g of Vd (see Table 2).

Typical Synthesis of a Hexadecanucleotide, dTpGpCpCpGpGpCpCpAp CpGpApTpGpCpG, using Trinucleotide Blocks

Each fully protected trinucleotide block (X, n=3, sequences of TGC-CGG-CCA-CGA-TGC) was synthesized as previously described[17], and decyanoethylated to give each trimer phosphodiester component. The nucleotide addition cycle (Table 1) was begun at Step 4 using a round-bottom flask, equipped with a sinister filter and two-way stopcock at the bottom, and manually operated system. A mixture of 300 mg of the hydroxyl resin (VId, 0.15 meq g^{-1} of N-isobutyryldeoxyguanosine), the first trimer phosphodiester component (TGC, 0.23 mmole) and pyridine (10 ml) was co-evaporated three times in the flask using a horizontal-type evaporator. To this mixture was added the coupling reagent, 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPST, 0.7 mmole),

TABLE 2.

BASE	NUCLEOSIDE ON RESIN*
Thymine (Va)	0.16 mmole/g
N-Benzoylcytosine (Vc)	0.15 mmole/g
N-isobutyrylguanine (Vd)	0.15 mmole/g

* The amount of nucleosides linked on the resin was established by the quantitative analysis of both the dimethoxytrityl group and the nucleosides liberated from the resin. The yield of N-benzoyl adenine was not reported because of depurination.

and anhydrous pyridine (10 ml) and the mixture was shaken for 3 hours. The mixture was filtered by the application of dry air pressure through the top of the flask. The resin was treated with phenylisocynate in pyridine (10% solution, 10 ml) under shaking for 1 hour to mask the unreacted 5'-hydroxyl groups. The reaction mixture was filtered and the resin was washed successively with pyridine (2 X 10 ml) and $CHCl_3$-MeOH (7:3 v/v, 3 X 10 ml). The dimethoxytrityl group was removed by treatment with a 2% benzenesulfonic acid solution (10 ml) in $CHCl_3$-MeOH (7:3 v/v) for 1 minute at room temperature. After washing the resin with $CHCl_3$-MeOH (7:3 v/v) two times, all the filtrates and washing solvents were pooled and the coupling yield was estimated by the spectroscopic analysis of the dimethoxytrityl function liberated from the resin. This coupling cycle was repeated five times and at the end of each cycle (Step 9 in Table 1) a sample of the resin (ca. 5 mg) was treated with concentrated NH_4OH (3 ml) for 20 hours at 50°C under shaking, and filtered. After evaporation of the filtrate, the residue was treated with 80% AcOH for 10 minutes at room temperature and evaporated. The HPLC profile of the resulting oligonucleotide at each cycle was reported in Figure 2 and the yield of each coupling cycle was 76, 90, 81, 85 and 95% for 1, 2, 3, 4 and 5 cycles, respectively.

The oligonucleotide chain was extended from the 3'-end to the 5'-end and Cycle 1 stands for the first coupling reaction between an oligonucleotide block (shown by a hyphen) and a nucleoside bound to the resin. Cycle 2 is a second coupling reaction of the resulting oligonucleotide chain bound on the resin with an incoming blocked nucleotide. For the synthesis of dGpTpApTpCpApCpGpApGpGpCpCpCpTpT, the exact same strategy described above was used. Each trimer block, GTA-TCA-CGA-GGC-CCT, was sequentially coupled to 300 mg of the resin (VIa, 0.16 meq g^{-1} of thymidine) and acetic anhydride was used for masking of the unreacted 5'-hydroxyl group. The yield of each coupling was 71, 76, 94, 78 and 90% for 1, 2, 3, 4 and 5 cycles, respectively. For the synthesis of dCpGpApCpGpApGpCpGpTpGpApCpApCpC, 300 mg of the starting resin (Vc, 0.15 meq g^{-1} of deoxycytidine) was coupled stepwise with each oligonucleotide block (CGA-CG-AG-CG-TGC-CAC). The yield of each step was 56, 98, 94, 87, 89 and

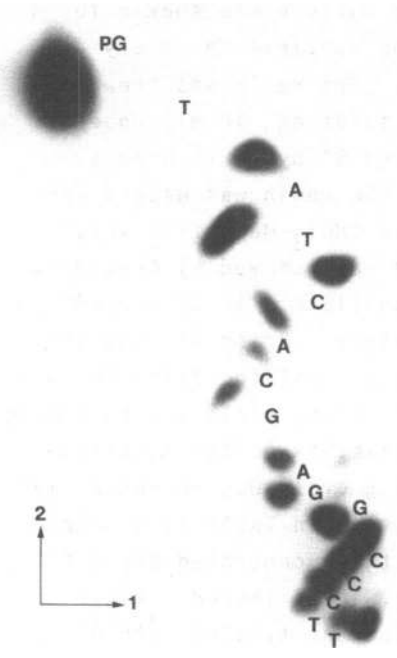

FIGURE 4a. Autoradiographs from Sequence Analysis of dG T A T C A C G A G G C C C T T.

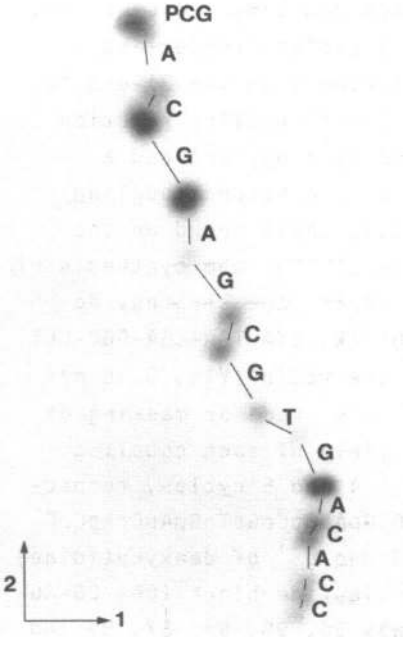

FIGURE 4b. Autoradiographs from Sequence Analysis of dC G A C G A G C G T G A C A C C.

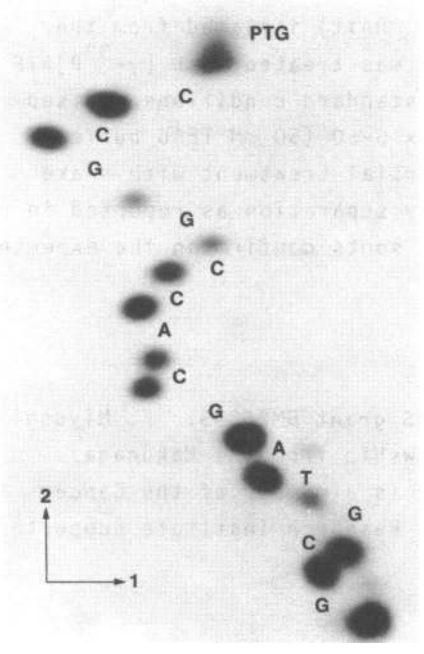

FIGURE 4c. Autoradiographs from Sequence Analysis of dT G C C G G C C A C G A T G C G.

81% for the sequential 6 cycle couplings. The HPLC profiles of these two hexadecanucleotides were shown in Figure 3.

HPLC Analysis and Purification of Oligonucleotides

Unprotected oligonucleotides were chromatographed on a Permaphase AAX column operated at 55°C under the exact conditions as reported.[1] The HPLC profiles were described in Figures 2 and 3. The major peak from the AAX column chromatography was desalted by passing through a Sephadex G-25 column (1.5 X 50 cm) eluted with a 50 mM triethylammonium bicarbonate (TEAB) solution. After evaporation of the buffer, the sample was analyzed and further purified on μBondapak C_{18} reverse-phase column chromatography (Waters Associates). Columns (0.3 X 30 cm) were operated at 55°C using an SP3500 HPLC equipment with an oven (Spectra-Physics). Linear gradients generated from each of 0% CH_3CN and 15% CH_3CN at pH 7.0 (0.01 M ethylenediammonium acetate buffer); flow rates: 2 ml min^{-1}; sweeping time: 15 minutes. The profiles of the hexadecamers were shown in Figures 2 and 3.

Sequencing of Oligonucleotides

The oligonucleotides (0.01 A_{260} unit) isolated from the reverse-phase column chromatography was treated with [γ-^{32}P]ATP and T4 polynucleotide kinase under standard conditions and separated by chromatography on Sephadex G-50 (50 mM TEAB buffer). After evaporation of the buffer, partial treatment with snake venom phosphodiesterase, followed by separation as reported in two dimensions, showed a pattern of spots confirming the expected sequences (Figure 4).[15]

ACKNOWLEDGEMENTS

This work is supported by USPHS grant GM24393. K. Miyoshi is supported by a Predoctoral Fellowship from the Wakunaga Pharmaceutical Company. K. Itakura is a member of the Cancer Research Center at the City of Hope Research Institute supported by USPHS grant CA16434.

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