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Solid phase synthesis of polynucleotides. VI. Further studies on polystyrene copolymers for the solid support

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ABSTRACT

A simple solid phase method for the synthesis of oligodeoxyribonucleotides has been developed using the phosphotriester approach. Mononucleotide coupling units are sequentially added to the polystyrene copolymer with 1% divinylbenzene and two kinds of oligonucleotides, d(CACGACCCCTCCACGT) and d(AACTGGTATTACTGGGCG), are synthesized in a relatively high yield. One cycle of the mononucleotide addition is about 70 minutes, and this method is particularly suitable for the automation of the synthesis upon availability of an automatic synthesizer.

INTRODUCTION

A number of solid supports have been recommended for use with the phosphotriester approach to synthesize oligodeoxyribonucleotides: polydimethylacrylamide (1, 2), polyacrylmorpholide (3, 4) and cellulose (5). Although the coupling reactions on these polyamide resins are consistently efficient (4,6) and a hentriacontanucleotide was synthesized on the polyacrylmorpholide resin (7), these polyamides have two inherent disadvantages. The high affinity nature of polyamides to protic solvents may require a coevaporation or extensive washing step prior to the coupling reaction in order to obtain a high coupling yield in the formation of a phosphotriester linkage (4, 6, 7). Needless to say, a small amount of protic solvents, such as water, remaining in the resin inhibits coupling reactions on a small scale. Incorporation of the coevaporation step, particularly for the automation of the synthetic process, is not practical. Secondly, deblocking of the dimethoxytrityl (DMT) groups on the polyamide resins using zinc bromide ($ZnBr_2$) in place of a protic acid, such as benzenesulfonic acid (BSA), is too slow to be of practical use (8). While these problems inherent to polyamide resins may be improved by the methods reported by Gait and coworkers (9, 10), we have searched for a better polystyrene copolymer which could have some advantages over polyamide resins.

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Previous papers in this series (11, 12) have shown the practical usage of a polystyrene copolymer with 2% divinylbenzene for the solid support in the synthesis of oligonucleotides. We have used two strategies for the synthesis: 1) coupling of appropriate mononucleotides and dimer or trimer nucleotide blocks to the support in pyridine in the presence of a coupling reagent (11), and 2) addition of deoxynucleoside 3'-o-chlorophenylphosphorotriazolides to the support in tetrahydrofuran (THF) in the presence of N,N-dimethylaminopyridine (DMAP) (12). Good coupling yields were consistently obtained in these synthesis except in the first coupling reaction using the first strategy. In order to circumvent this difficulty and to replace BSA by $ZnBr_2$ for removal of the DMT group, a few derivatives of the polystyrene copolymer with 1% divinylbenzene were examined.

In this paper we describe some significant improvements to our strategy, including a better polystyrene copolymer and a simple and economical solid phase method for the synthesis of oligodeoxyribonucleotides.

RESULTS AND DISCUSSION

The basic phosphotriester approach to synthesize oligodeoxyribonucleotides on solid supports has been described previously (11). The outline of the strategy includes the following steps (Figure 1):

1. Attachment of a 5'-O-dimethoxytrityl-2'-deoxynucleosides to the support via 3'-O-succinate linkage.
2. Removal of the dimethoxytrityl group under acidic conditions to give a 5'-hydroxyl group.
3. Condensation of the liberated 5'-hydroxyl group with a 5'-O-dimethoxytrityl nucleotide in the presence of a coupling reagent.
4. Capping on any unreacted hydroxyl group.

These synthetic cycles are repeated using various 5'-O-dimethoxytrityl nucleotides until the desired sequence is assembled. All of the protecting groups, dimethoxytrityl for 5'-hydroxyl, acyl for amino and o-chlorophenyl for phosphate functions are removed and the linkage between a support and an oligonucleotide is cleaved. Then the product is purified by high performance liquid chromatography (HPLC) or gel electrophoresis. Several recent modifications and improvements in each step described above will be discussed in this section.

Solid Support

Recent improvement in the coupling method used to form internucleotidic phosphate bonds between nucleosides (or nucleotides) by both the phospho-

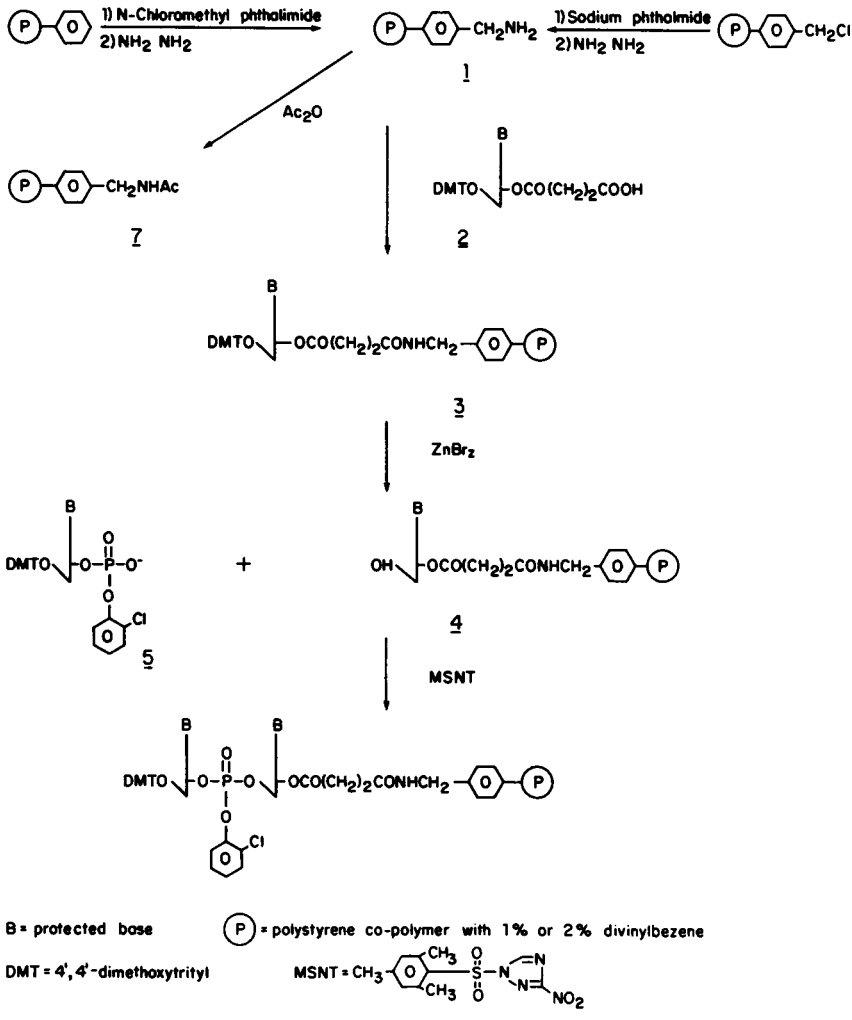

Figure 1. OUTLINE OF THE SOLID-PHASE SYNTHESIS

triester (13) and phosphite-triester (14) approaches permit us to synthesize oligodeoxyribonucleotides in a very short time on various solid supports (1-7, 10-12, 15, 16, 17). It is likely that one of the most important studies on the solid phase method in the future will be to find better solid supports. We have investigated various resins for the supports (4,11) and found that none of them are completely satisfactory for one of our goals in the synthesis ---automation of all synthetic operations.

Indeed, polyamide supports have a high affinity to protic solvents which

are inhibitors of the coupling reaction, and deblocking of the DMT groups by $ZnBr_2$ on the resins is too inefficient (8). The yield of the first coupling reaction on the 2% cross-linked polystyrene with divinylbenzene is usually lower than those of successive coupling reactions (11). Polystyrene copolymer with 1% divinylbenzene swells better in various organic solvents and gives higher coupling yields in peptide synthesis than the 2% counterpart (18). Subsequently, coupling efficiency to form internucleotidic phosphotriester bonds on these two resins were compared. Commercially available 1% and 2% polystyrene resins were derivatized as described in the experimental section. The nucleoside succinate ester 2 was activated by N',N'-dicyclohexyldicarboimide (DCC) in the presence of DMAP, reacted with the amino resins 1 and followed by $ZnBr_2$ treatment to give the 5'-hydroxyl nucleoside resin 4 (Figure 1). Figure 2 shows the coupling yields with various reaction time using the mononucleotide as a coupling unit. On the 1% resin, the reaction proceeds much faster than on the 2% resin. The first coupling yield on the 2% resin is about 15% lower than that of the second resin (11). However, on the 1% resin the first and following coupling yields are equally very high. These results clearly demonstrate that the 1% resin is superior to the 2% counterpart. One disadvantage of the 1% resin may be mechanical instability. However it was found that, by using a high concentration of the reagents, mechanical shaking or stirring was not required for all synthetic operations. Therefore, the 1% resin was used exclusively for the following experiments.

Assembly of Oligonucleotides by the Addition of Mononucleotides

For the synthesis of oligonucleotides up to about 30 bases long, di and trinucleotides were exclusively used in our strategy (7). Therefore, these oligonucleotiodes must be synthesized in advance in a solution before the oligonucleotide chain is assembled on a solid support. Particularly, 64 units of trimers are necessary in order to synthesize any desired sequence. Even with the improved method for synthesis of trimers (19), it is still very time consuming. Consequently, we reexamined the assembly strategy by using mononucleotide coupling units. One of the stumbling blocks in the mononucleotide assembly strategy is purification of the final product. If the yield of each coupling reaction on a solid support is only 80-90% (as we observed in the block coupling approach), the final product should be a series of oligonucleotides with one nucleotide difference. Therefore, the desired product would not be a major product in the mixture and the purification could be tedious.

In a previous paper (12), we reported a mononucleotide coupling approach

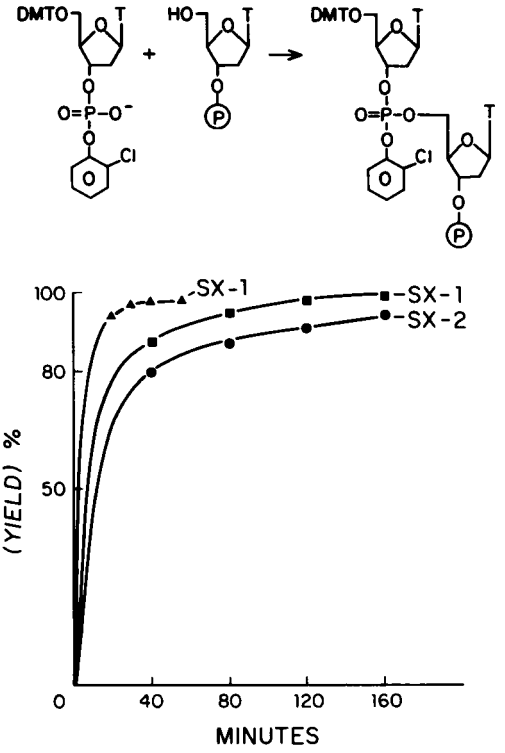

FIGURE 2. Comparison of the coupling yields on 1% and 2% polystyrene resins. The coupling reactions were carried out on the polystyrene copolymers with 1% (SX-1) and 2% (SX-2) divinylbenzene. The loading of thymidine on the copolymers were 0.16 mmole/g and 0.17 mmole/g for 1% and 2% copolymers, respectively. The thymidine supports (100 mg) were reacted with 5'-O-dimethoxytrityl thymidine-3-O-chlorophenylphosphate (0.1 Molar solution, -●- and -■-, 0.15 Molar solution -▲-) in pyridine (1 ml) by the presence of three times excess of the coupling reagent (MSTe) without shaking. The aliquots were taken at various times and the yields were estimated by measurement of the dimethoxytrityl functionalities liberated from the supports as described in the text.

using deoxynucleoside-3'-o-chlorophenylphosphorotriazolides as coupling units. Although this approach gives a high coupling yield (approx. 95%), it may not be a general approach because the reagents are not completely stable during the storage. Lower coupling yields in the block coupling approach could be due partly to the low concentration of reagents (0.05 Molar solution of nucleotides). Indeed, with a higher concentration of the mononucleotide (0.1 M and 0.15 M) in the coupling reaction mixture, the coupling reactions went almost to completion on the solid support (Figure 2). For these preliminary

studies, the coupling reagent 1-(mesitylene-2-sulfonyl)-tetrazole (MSTe) was used. However, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) was exclusively used for the synthesis of oligonucleotides of defined sequences, since the reaction was complete in a similar time as by MSTe and a minimum of side reactions was reported (20, 21).

For the preparation of 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-o-chlorophenylphosphate 5 we examined a simplified procedure (10), phosphorylation by o-chlorophenylphosphodichloridate and triazole in pyridine gave colored products. In the case of the deoxyguanosine, the color was especially extensive and, therefore, triethylamine in tetrahydrofuran was used in the preparation of o-chlorophenylphosphoroditriazolide (22). An excess of the ditriazolide (1.5 equivalent) permit the phosphorylation of 3'-hydroxyl nucleosides to complete in 30 minutes. Subsequent hydrolysis and precipitation of the product was carried out as described in the experimental section. The resultant colorless 3'-o-chlorophenylphosphates 5 were used for the assembly of oligonucleotides on the polystyrene resin.

Removal of Terminal Dimethoxytrityl Groups

Dimethoxytrityl group has been exclusively used for the protection of 5'-hydroxyl functions in the chemical synthesis of oligodeoxyribonucleotides both by the liquid phase and solid phase methods. The dimethoxytrityl group must be removed at each cycle of the synthesis for the elongation of oligonucleotide chains. Deblocking by benzenesulfonic acid (BSA) under mild conditions (at 0°C) generally gave satisfactory results (23) although a side reaction, cleavage of glycosidic bonds on N-benzolydeoxyadenosine, was observed to some extent (9). Two laboratories, Caruthers and Koster, independently developed very mild conditions using zinc bromide to selectively remove the dimethoxytrityl group (24, 25). As previously reported, we examined and modified these conditions and found that the deblocking reaction using 1 Molar solution of $ZnBr_2$ in a mixed solvent of dichloromethane-isopropanol (85:15 v/v) was rapid and quantitative without any side reactions (26). When these conditions were applied to our strategy for the solid phase method using the commercially available polystyrene (1.25 mmole/g Cl function) copolymer with 1% divinylbenzene, the reaction rate of removal of the dimethoxytrityl groups decreased gradually with the extension of oligonucleotide chains for the synthesis of a hexadecanucleotide. This effect could be due to a lower concentration of $ZnBr_2$ inside the resin caused by the interaction of $ZnBr_2$ with amide groups, including acetoamide on the polystyrene resin 7, benzamide on adenine and cytidine bases, and iso-butyrylamide on guanine bases in the oli-

gonucleotides. Indeed, the fact that detritylation on polyamide resins by $ZnBr_2$ under various conditions is very slow (8) supports our speculation.

Consequently, the polystyrene copolymer with 1% divinylbenzene (no functional group) was derivatized to a resin containing lower amounts of the aminomethyl group (1, 0.15-0.30 mmole/g) by the published procedure (27) and then a dimethoxytrityl nucleoside was attached to the resin (0.1 mmole/g). It was observed that the rate of detritylation on this resin did not decrease during the synthesis of an octadecanucleotide. Therefore, we could conclude that a certain number of amide groups in the matrix (resin and protected bases) will hinder the detritylation. Obviously, the detritylation by $ZnBr_2$ would limit the length of oligonucleotides synthesized on the solid supports. It should be noted that there is no significant difference in the coupling yields between these two resins (Table I).

Synthesis of Oligodeoxyribonucleotides

The hexadecamer d(CACGACCCCTCCACGT) was designed for screening a mouse gene library in order to isolate H-2K^b gene (28). The comercially available chloromethyl polystyrene resin (1.25 mmole/g) was derivatized to the aminomethyl resin and then the first nucleoside thymidine loaded on the resin (0.17 mmole/g, T-resin) as described in Figure 1. The sequence of the octadecamer

TABLE I. Synthesis of Hexa- and Octadecanucleotides

Sequence	d(CACGACCCCTCCACGT)	d(AACTGGTATTACTGGGCG)
Amount of NH_2 Group on Resin	1.50 mmole/g	0.15 mmole/g
Nucleoside Loading	0.17 mmole/g (T)	0.10 mmole/g (G)
Coupling Times	15	17
Overall Coupling Yield (%)[a]	38.1	43.5
Overall Isolation Yield (%)[b]	13.5	16.4
Removal of DMT Group (Minutes)	20-50	5-10

[a] The yields were estimated by the ratio of dimethoxytrityl functionalities liberated from resins of the first nucleoside and the last coupling reaction.

[b] The yields were based on the amount of oligonucleotides isolated by HPLC on a Permaphase AAX column (Figure 4).

d(AACTGGTATTACTGGGCG) was designed for making a deletion mutant of the signal peptide for *E. coli* lipoprotein. The aminomethyl resin (0.15 mmole/g) was obtained from the commercially available 1% cross-linked polystyrene resin (no functionality) by the method described in the experimental section. The first nucleoside, deoxyguanosine, (0.10 mmole/g, G-resin) was attached to the resin by the method described in Figure 1. For the synthesis of both sequences, 50 mg of the T-resin (7.5 μmole) and G-resin (5 μmole) was used. The synthesis was performed solely using mononucleotides (0.15 M solution in pyridine) as coupling units and the operation was carried out in a simple sintered glass filter equipped with a stopcock (Figure 3) without any shaking. It took about 70 minutes to complete one cycle of the operation (Table II).

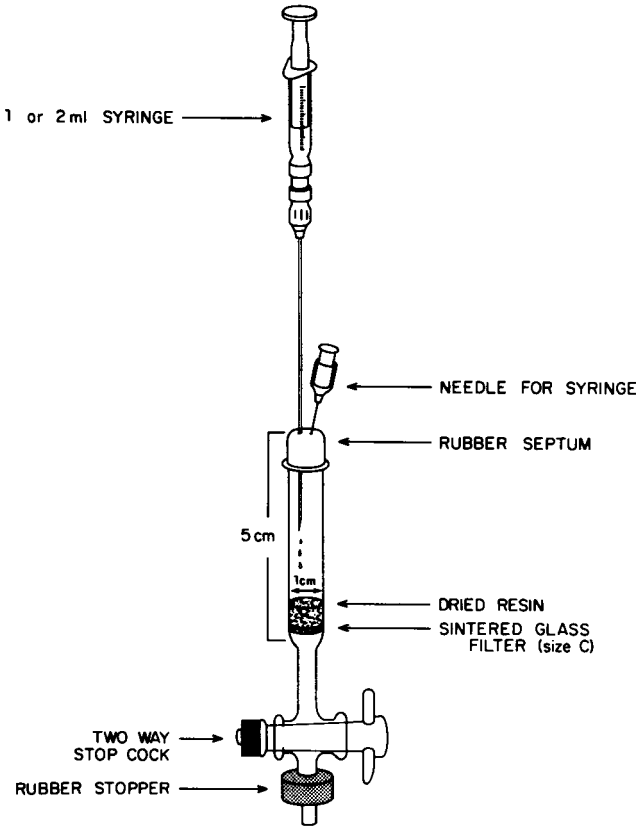

FIGURE 3. Reaction vessel. This picture shows the introduction of the coupling reaction mixture. The needle to release inside pressure is not necessary for other steps.

TABLE II. One Cycle of Operation

Step	Reagent or Solvent	Reaction Time (min.)	No. of Operations
1	1 M $ZnBr_2/CH_2Cl_2$-IPA	5 or 10[a]	1
2	0.5 M Et_3N^+H AcO^-/DMF	1	3
3	Pyridine	0.5	2
4	THF	0.5	2
5	Vacuum (oil pump)	5	1
6	Coupling Mixture[b]	40	1
7	Pyridine	0.5	1
8	Capping Mixture[c]	5	1
9	CH_2Cl_2-IPA	0.5	4

[a] Reaction time was dependent on the base at the 5'-end of an elongating oligonucleotide from which the DMT group was removed. Five minutes were enough for purines (guanine and adenine), but ten minutes was essential for the pyrimidines (thymine and cytosine).

[b] The deoxynucleoside-3'-o-chlorophenylphosphate (90 μmole) was dried by co-evaporation with pyridine twice to final volume of 0.6 ml (0.15 Molar solution). MSNT (270 μmole) was added and the mixture was added to the resin by means of a hypodermic syringe.

[c] THF-Ac_2O-pyridine (3 ml, 7:1:2 v/v) with DMAP (100 mg).

The HPLC elution profiles on Permaphase AAX and C_{18} columns of these two oligonucleotides after removal of all protecting groups were shown in Figure 4. They clearly demonstrated that the desired products were major peaks and that the coupling reaction forming the phosphotriester bonds were very efficient. Overall coupling and isolated yields are described in Table I. The sequence of these oligonucleotides was confirmed by standard analysis of ^{32}P-oligomers (Figure 5). These results demonstrate that oligodeoxyribonucleotides of 18 residues with defined sequences can be synthesized efficiently using monomer coupling units on a small scale. It should be noted that further scale-down of the synthesis using 20 mg of the support is more practical after one becomes familiar to the operations. Once a mechanized synthesizer was available, protected oligonucleotides could be easily prepared in a day by using the method described here and the purified products available in a few days.

MATERIALS AND METHODS

Unless otherwise mentioned, materials and methods are as previously described (4,11). Trifluoromethanesulfonic acid, trifluoroacetic acid, and 2-mesitylenesulfonyl chloride were purchased from Aldrich Chemicals. Chloromethylpolystyrene (1% and 2% cross-linked by divinylbenzene, 200-400

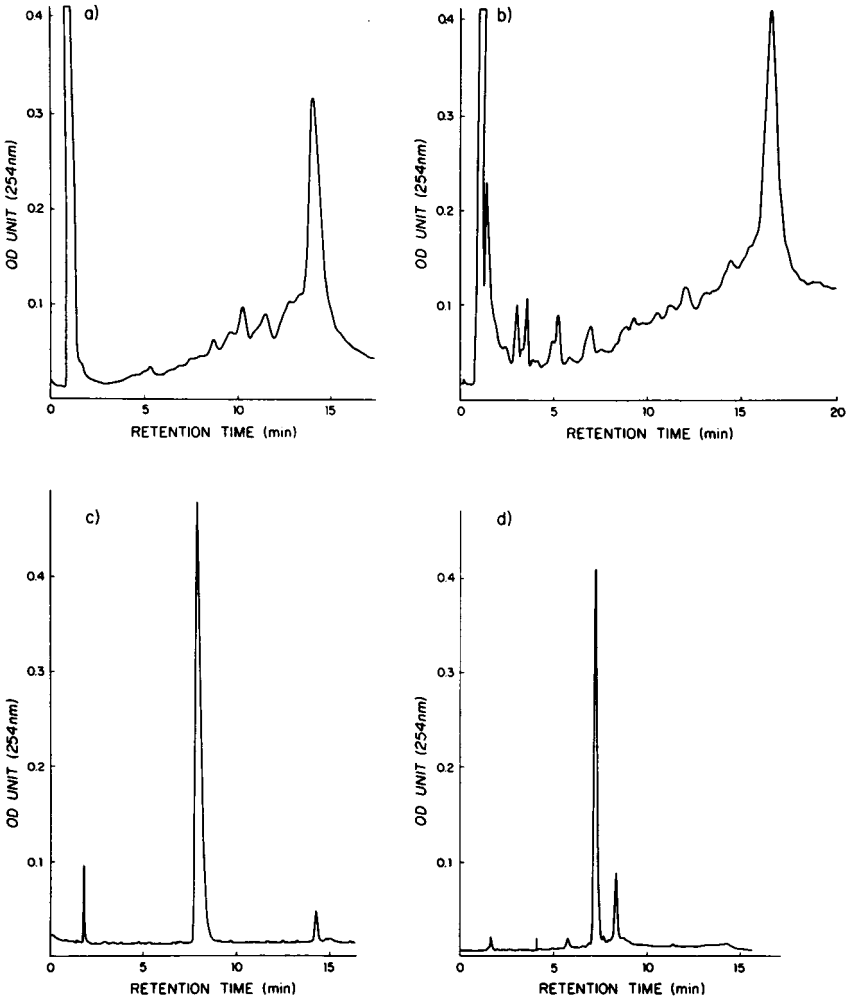

FIGURE 4. HPLC profiles of two oligonucleotides. The HPLC analysis of synthetic oligonucleotides was carried out successively on Permaphase AAX and μBondapak C_{18} columns as described previously (4, 11). Figures (a) and (b) show the analysis of the final reaction mixture of 16 mer and 18 mer on the Permaphase AAX column after removal of all protecting groups. The main peaks were isolated, dialyzed and analyzed further on the μBondapak C_{18} column, (c) is 16 mer and (d) the 18 mer.

mesh, 1.25 mmol/g, Cl function) and polystyrene (1% cross-linked by divinylbenzene, 200-400 mesh) were obtained from Biorad Laboratories. N-(Chloromethyl)phthalimide, $ZnBr_2$ and aminotriazole were purchased from Eastman Organic Chemicals. HPLC analysis on Permaphase AAX (DuPont) and

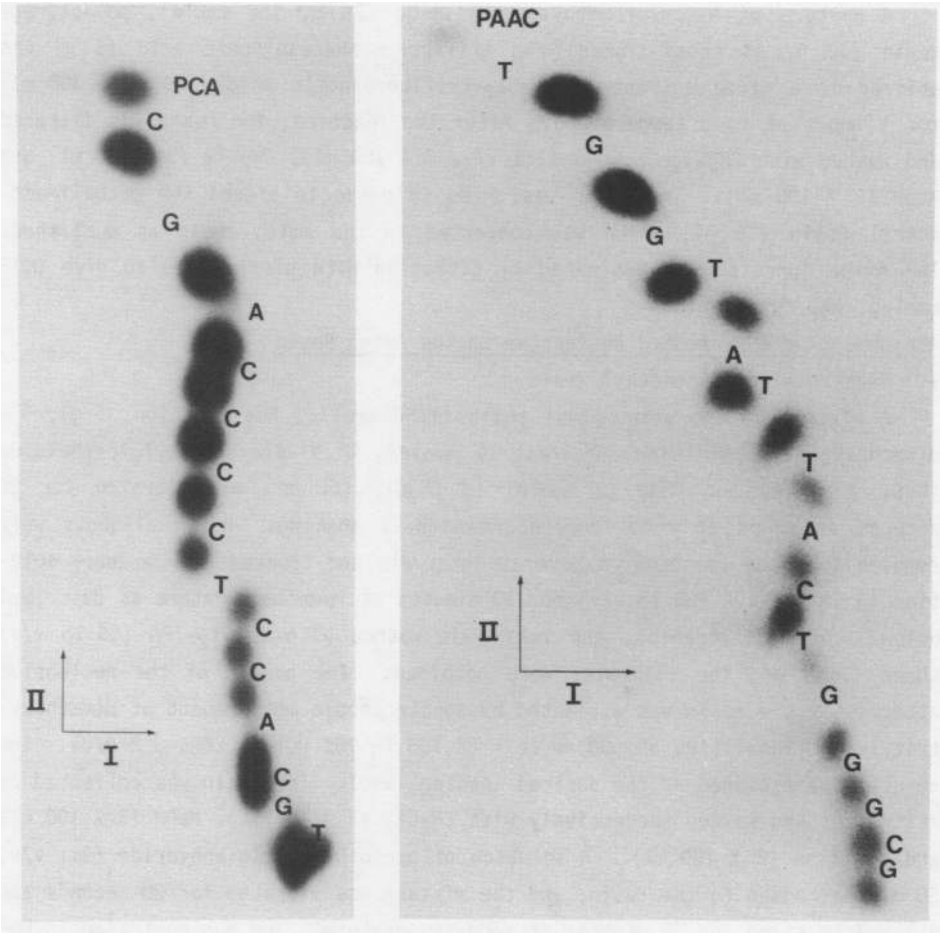

FIGURE 5. DNA sequence of two synthetic oligodeoxyribonucleotides. (a) shows the sequence analysis of d(CACGACCCCTCCACGT) and (b) shows that of d(AACTGGTATTACTGGGCG).

μBondapak C_{18} (Waters Associates) columns was carried out at 50°C as previously published (4).

Preparation of Aminomethyl Polystyrene

1. High-Loading Resin (1.50 mmole/g, NH_2 Function)

The commercially available chloromethylpolystyrene (1% and 2% cross-linked by divinylbenzene, 1.25 mmol/g, Cl function) was converted to the amino resin by the published procedures (29).

2. Low-Loading Resin (0.15 mmole/g, NH_2 Function)

A mixture of N-(chloromethyl)phthalimide (29 g, 150 mmole), polystyrene resin (30 g, 1% cross-linked) and trifluoromethanesulfonic acid (6 g) was stirred in a mixed solvent of CH_2Cl_2-trifluoroacetic acid (1:1 v/v, 300 ml) for 2 hours at room temperature. After the reaction, the resin was filtered and washed with CH_2Cl_2-CF_3COOH (1:1 v/v, 3 X 100 ml), CH_2Cl_2 (3 X 100 ml) and MeOH (2 X 100 ml). The resin was dried *in vacuo* to afford the phthalimadomethyl resin (36 g), which was converted to the amino resin as published. The amino function was estimated by titration with picric acid to give 0.15 mmol/g, NH_2 function.

Attachment of a Protected Nucleoside to the Amino Resin

1. High-Loading Aminomethyl Resin

A mixture of the aminomethyl resin (1.50 mmol/g, NH_2 function, 5 g), 5'-dimethoxytrityl thymidine succinate (2 mmole), N',N'-dicyclohexyldicarboimide (DCC, 3 mmole) and DMAP (2 mmole) in CH_2Cl_2 (60 ml) was vibrated for 20 seconds and kept at room temperature without shaking. Resin aliquots were removed from the reaction mixture at intervals and treated with a $ZnBr_2$ solution in CH_2Cl_2-IPA (85:15 v/v) for 10 minutes at room temperature as described below. After filtration, the resin was washed with CH_2Cl_2-IPA (85:15 v/v) three times and the filtrates were combined. The amount of the nucleoside attached to the resin was estimated by spectroscopic measurement of dimethoxytrityl functionalities at 500 nm (ε = 88,700 in 70% $HClO_4$-EtOH, 3:2 v/v). The reaction was stopped at the desired loading level. The resin was collected by filtration and washed successively with CH_2Cl_2 (3 X 100 ml), MeOH (3 X 100 ml) and pyridine (2 X 100 ml). A solution of pyridine-acetic anhydride (9:1 v/v, 50 ml) was added to the resin, and the mixture was vibrated for 20 seconds and allowed to stand for 30 minutes at room temperature. The nucleoside-resin was washed with pyridine (3 X 100 ml) and CH_2Cl_2 (3 X 100 ml) and then dried *in vacuo*.

2. Low-Loading Aminomethyl Resin

A mixture of the low loading aminomethyl resin (0.15 mmole/g, NH_2 function, 5g), deoxyguanosine succinate 2 (1.5 mmole), DCC (2.25 mmole) and DMAP (2 mmole) in CH_2Cl_2 (60 ml) was reacted overnight. After filtration of the reaction mixture, the nucleoside resin was treated in the same way as described above. The yield was 0.10 mmole of the nucleoside loaded on 1 g of the polystyrene resin.

Removal of Dimethoxytrityl Group by Treatment with $ZnBr_2$

A mixture of resin (50 mg) and 1 Molar solution (3 ml) of $ZnBr_2$ in

CH_2Cl_2-isopropanol (85:15 v/v) was allowed to stand at room temperature 5-10 minutes. After the reaction, the mixture was filtered and washed successively with CH_2Cl_2-IPA (85:15 v/v, 2 X 5 ml), triethylamonium acetate buffer in DMF (0.5 M pH7.5, 3 X 5 ml), pyridine (2 X 5 ml) and THF (2 X 5 ml).

Preparation of Deoxynucleoside-3'-o-Chlorophenylphosphates

A mixture of o-chlorophenyldichlorophosphate (15 mmole), 1,2,4-triazole (40 mmole), triethylamine (35 mmole) and THF (100 ml) was shaken for 30 minutes. The mixture was filtered into a pre-dried 5'-dimethoxytrityl deoxynucleoside (10 mmole). The reaction mixture was kept for 30 minutes at room temperature and 1 M of triethylammonium bicarbonate (TEAB) buffer (10 ml, pH7.5) was added. $CHCl_3$ (300 ml) was added to the mixture, and the solution was washed three times with H_2O, dried over Na_2SO_4 and evaporated. The residue was coevaporated twice with dry pyridine and dissolved in CH_2Cl_2. The product was precipitated from pentane containing 1% triethylamine by the addition of a CH_2Cl_2 solution dropwise. The precipitates were collected by filtration and dried *in vacuo*. The yield was 85-95%.

Assembly of Oligonucleotides

All reactions were carried out in a small sintered glass filter without shaking (Figure 3). Table II describes the general procedure, using 50 mg of the nucleoside resin (0.10 mmole/g) prepared from the low-loading aminomethyl resin.

When absolute anhydrous conditions were required (Steps 3, 4, 5 and 6), a septum was put on top of the filter, and reagents and solvents were introduced by a hypodermic syringe (Figure 3). Otherwise, disposable pipets were used for the introduction without the septum. Removal of all reagents and solvents was done by application of vacuum.

Deprotection and Purification

After the coupling reaction, the resin (ca. 10 mg) was treated with 0.5 M tetramethylguanidine syn-p-nitrobenzaldoximate in dioxane-water (1:1 v/v, 0.3 ml) for 36 hours. The mixture was filtered and the resin washed twice with aqueous pyridine (50% v/v). The combined solution was evaporated and treated with NH_4OH (28%, 1 ml) at 55°C for several hours. After evaporation of NH_4OH, the residue was reacted with 80% AcOH (1 ml) for 20 minutes at room temperature and evaporated. The product was dissolved in water (1 ml), extracted with ether (3 X 1 ml) and evaporated. The residue was dissolved in water, ready for high pressure liquid chromatography (HPLC). HPLC purification was carried out successively on Permaphase AAX (DuPont) and μBondapak C_{18} (Waters) columns (Figure 4) at 55°C.

^{32}P-Labeling and Sequencing

The purified oligonucleotides were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and the sequencing was carried out by the standard method (29) (Figure 5).

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