Solid-phase synthesis of polynucleotides. II. Synthesis of polythymidylic acids by the block coupling phosphotriester method

Ken-ichi Miyoshi, Tetsuo Miyake, Toyoharu Hozumi and Keiichi Itakura

Division of Biology, Molecular Genetics Section, City of Hope Research Institute, Duarte, CA 91010, USA

Received 25 July 1980

ABSTRACT

Synthesis of two oligothymidylic acids, tridecamer and nonadecamer, is described by a rapid and simple solid-phase method on two kinds of polyacrylamide supports derivatized from commercially available Enzacryl Gel K-2. The syntheses were performed by the phosphotriester method using di- and tri-thymidylic acid blocks as the incoming 3'-phosphodiester component. High coupling yields were consistently obtained and the final product was isolated very easily by high performance liquid chromatography on Permaphase AAX.

INTRODUCTION

Solid-phase techniques appear quite attractive to rapidly synthesize oligonucleotides, since the situation in the peptide field has been very successful.¹ However, much investigation has thus far made little practical impact in the oligonucleotide field. There may be three reasons for this relative lack of success. 1) The attention has focused almost entirely on the phosphodiester method,² with its inherent disadvantages, such as low yields in coupling reaction. 2) The commonly used supports have been polystyrene and its derivatives. These non-polar polymers do not swell very well in the polar solvent required for oligonucleotide synthesis; and 3) the stepwise coupling approach³ gives a series of oligonucleotides with one nucleotide difference and, therefore, it is very difficult to isolate the desired product from the final product mixture. In a preliminary report, we outlined the synthesis of nonadecathymidylic acid by the block coupling phosphotriester approach on a carboxyl polyacrylmorpholide support (IX)⁴. In this and the following paper⁵, full details of these experiments and further

improvements to establish a general methodology for a rapid and simple solid-phase synthesis of polydeoxyribonucleotides are described.

RESULTS AND DISCUSSION

At the beginning of this work, we were concerned with two major criteria of a solid-phase method for the synthesis of oligonucleotides: 1) The method should be very simple, rapid and reliable in operation for future consideration of automation by machine; and 2) the final desired oligomers should be easily purifiable in high yields. To meet these criteria, important requirements must be taken into account:

- 1) assembly of polynucleotides on a support,
- 2) polymer support and nucleotide-polymer support linkage,
- 3) purification method of polynucleotides.

Assembly of Polynucleotides on a Support

Although the phosphodiester approach of oligonucleotide synthesis is powerful and accurate⁶, it has some inherent disadvantages caused by unprotected phosphate charges, such as low yields of coupling reactions and side-reactions. To overcome these problems of the phosphodiester approach, many groups have extensively worked on the improvement of the phosphotriester approach and the latter method has now become the method choice for the synthesis of oligonucleotides.⁷ However, no extensive study on the solid-phase method using the advantages of the phosphotriester approach has been reported.⁸

In solution synthesis, the extension of an oligomer chain can be performed to either direction, the 3'- to 5'-end or vice versa. Contrarily, in solid-phase synthesis only one direction of the chain elongation is feasible since either the 3'- or 5'-end of the chain is attached to a polymer support.⁹ In this work, we investigated only one direction, the 3'- to 5'-end (Figure 1b), because this direction seems to have advantages over the other direction (Figure 1a) on the phosphotriester bond formation. In the 5'- to 3'-end assembly, the 5'-hydroxyl group is used for the attachment on a polymer support (1a), and the oligomer chain is extended toward the 3'-end by the addition

B = protected base

(P) = polymer support

 R_1, R_2 and R_3 = protecting group

Figure 1 Direction of chain elongation

of a 5'-hydroxyl-3'-protected nucleotide (IIa) to the activated 3'-phosphodiester component (Ia) on a resin. One disadvantage of this approach might be that both the activation of the 3'-phosphodiester (Ia) and the coupling reaction is carried out on a polymer support. On the other hand, in the 3'- to 5'-end assembly, the activation of the 3'-phosphodiester (IIb) is performed in solution and only the coupling reaction is carried out on a polymer support. Therefore, the latter method seems to be more efficient to form phosphotriester bonds on a solid-phase, even though it has the disadvantage that one of the by-products, sulfonylation of the 5'-hydroxyl group by the coupling reagent¹⁰,

can not be removed during synthesis. Since this disadvantage can be overcome by using the block coupling strategy (see below), the 3'- to 5'-end assembly is the method choice for the synthesis of polynucleotides on a polymer support in this article.

Needless to say, the coupling reaction to form phosphate bonds, even by the phosphotriester method, would never go to completion. Thus, the stepwise method using mononucleotides as a coupling unit gave a mixture of oligonucleotides with one base difference at the end of the synthesis and encountered the difficulty of making the longer oligonucleotide. The strategy involving the coupling of purified, protected oligonucleotide blocks (di and trinucleotides) to an oligonucleotide chain bound on a solid support could overcome the purification problem. This approach is similar to the stepwise method except that the soluble components are protected oligomers rather than protected mononucleotides. The major advantage lies in the isolation of the final product. The desired products are easier to separate from the by-products lacking an oligomer fragment than those from the by-products lacking only a mononucleotide residue. Indeed, this concept materialized in the synthesis of trideca and nonadecathymidylic acids using di and trinucleotide blocks. The principles of our block solid-phase polynucleotide synthesis by the phosphotriester method are very simple (Figure 2). Essentially, two main reactions are involved: 1) condensation of a 5'-O-dimethoxytrityl-3'-phosphodiester block (IV) with 5'-hydroxyl groups on the polymer support (Ib) followed by 2) removal of the 5'-O-dimethoxytrityl group to liberate a new 5'-hydroxyl function (VI).

Polymer Support and Nucleotide-Polymer Linkage

The commonly used polymer supports, polystyrene or its derivatives, for the solid-phase synthesis of oligonucleotides do not give high yields in the formation of phosphate bonds even by the phosphotriester method.¹¹ For the reaction involving polar intermediates, such as peptide and oligonucleotide synthesis, polar polymer supports might be superior to non-polar polymers.¹²

Cross-linked polyacrylmorpholide¹³ (Enzacryl Gel K-2, VII) is known to swell very well in polar organic solvents, such as

Figure 2 Block coupling strategy

pyridine, as required for oligonucleotide synthesis. The commercially available carboxyl derivative of the polyacrylmorpholide (VIII) was first examined as follows (Figure 3). The polymer (VIII, 1 g, 1 mmole equivalent of the carboxyl group) was functionalized by reaction with 5'-O-dimethoxytrityl thymidine and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS) in a mixture of acetonitrile and pyridine (1:1 v/v) for 24 hours. The dimethoxytrityl group of the resulting polymer was removed by treatment with a 2% solution of benzenesulfonic acid (BSA) in chloroform-methanol (7:3 v/v)¹⁴ to give the starting polymer (IX) containing about 0.14 mmole of thymidine per 1 g of the polymer. The 3'-hydroxyl group of 5'-O-dimethoxytrityl thymidine was linked to the polymer by the formation of an ester bond and, therefore, it is easily cleaved with concentrated ammonium hydroxide after making the desired oligonucleotide chain. The trinucleotide block (IV, n = 3) was repeatedly coupled to the polymer six times. The nonadecathymidylic acid was synthesized with a fairly high yield and isolated very easily by high performance liquid chromatography (HPLC) on a Permaphase AAX column

(see below for details).

However, this polymer support has a disadvantage; the coupling reaction between the support and the nucleoside, 5'-O-dimethoxytrityl thymidine, was slow and inefficient. The activation of carboxyl groups on the solid-phase by TPS to form an ester bond with the 3'-hydroxyl group of nucleosides was not practical for the attachment of the nucleosides, other than thymidine, on the support. Other coupling reagents, including dicyclohexylcarbodiimide (DCC), 2,4,6-triisopropylbenzenesulfonyl tetrazolide (TPST), and thionyl chloride, was found to be less efficient than TPS. To overcome this problem, a new resin was derivatized from the polyacrylmorpholide (VII, Figure 3). 5'-O-Dimethoxytrityl thymidine was reacted with succinic anhydride to give the monosuccinate (XI) which was further activated by pentachlorophenol. The polyacrylmorpholide resin (VII) was functionalized by reaction with ethylene diamine in ethylene glycol¹³ and the resulting amino-polymer (X, 0.18 mmole/g) was coupled with the activated ester (XII) to give the desired polymer (XIV, containing 0.16 mmole of thymidine per 1 g of the resin). This polymer (XIV) has certain advantages over the carboxyl-polymer (IX); 1) the attachment of the first nucleoside can be performed under very mild conditions so that nucleosides other than thymidine can be attached without difficulty, 2) a long spacer between the resin and the coupling site, 5'-hydroxyl function, might give higher coupling yields. Indeed, the coupling efficiency of oligonucleotide blocks in each cycle was very high (~90%) (Table 2) and, therefore, the resin (XIV) was used exclusively for the synthesis of oligonucleotides with defined sequences in the following paper.

Purification Method of Oligonucleotides

Resulting from the polymer-support synthesis, the final product is most likely a mixture of oligonucleotides, the desired oligomer and a series of shorter oligomers. In addition to the block coupling approach discussed above, the rapid and efficient separation method is of great importance in obtaining satisfactory purity of the desired oligomers. An ion exchange chromatography has been the most widely used technique for the purification. Although diethylaminoethyl (DEAE) cell-

ulose in the presence of urea is powerful and gives good resolution of chains up to 20 residues long, it takes a long time to purify.¹⁵ The chromatography on a Permaphase AAX column under high pressure has shown to be a rapid and highly resolutional method for the analysis and isolation of oligonucleotides.^{16,17} This ion exchange material was used extensively for the analysis of reaction intermediates and the purification of the final products after the removal of all protecting groups from the fully protected oligonucleotides.

General Operation of the Coupling Reaction

For the synthesis of nonadecathymidylic acid, trimer block (IV, $n = 3, 5$ equivalents) was coupled to the polymer with the aid of a coupling reagent, TPST (10 equivalents), for 3 hours (Step 4 and 5). The reagents were washed from the polymer with solvents (Step 6 and 7) and dimethoxytrityl group was removed by treatment with a 2% solution of BSA (Step 1). After washing (Step 2 and 3) and evaporation with dry pyridine (Step 4), the new coupling reaction was resumed (Table 1). In each coupling cycle, the yield was estimated in two ways: 1) HPLC analysis of the phosphodiester products after removal of all protecting groups, and 2) visible light spectroscopic analysis of dimethoxytrityl function liberated from phosphotriester products.

1) HPLC Analysis

A sample of the polymer (after Step 7) was subjected to

TABLE 1.

STEP	SOLVENT or REAGENT	AMOUNT	SHAKING TIME (Minutes)	NUMBER OF OPERATIONS
1	2% BSA	10ml	3	1
2	CHCl ₃ -MeOH (7:3 v/v)	10ml	1	2
3	Pyridine	10ml	1	2
4	Dimer or Trimer in Pyridine	5 equivalent	180	3
		co-evaporation		
5	TPST	10 equivalent	180	1
	Pyridine	5ml		
6	Pyridine	10ml	1	2
7	CHCl ₃ -MeOH (7:3 v/v)	10ml	1	3

hydrolysis with a concentrated ammonium hydroxide solution to remove the p-chlorophenyl protecting groups and also to cleave the oligonucleotides from the polymer. The reaction mixture was filtrated and, after concentration, the residue was treated with 80% aqueous acetic acid to give each oligomer ($n = 3, 6, 9, 12, 15$ and 18). A sample of each oligomer was analyzed by chromatography on Permaphase AAX and the HPLC profile of the nonadecamer synthesized on two different resins was shown in Figure 4. The apparent conversion yields were reported as the ratio of the areas under the oligonucleotide peaks (chain length n) to those corresponding to chain length $n-3$ (Table 2).

2) Visible Light Spectroscopic Analysis

The filtrates from Step 1 and Step 2 were combined and the optical density of the dimethoxytrityl function liberated from the resin was measured at 507 nm in 1% BSA in CHCl_3 solution. The yield was reported as the total amount of the function released from the resin after each coupling reaction. However, the estimation of the yield was complicated by many factors, such as the breakdown of the internucleotidic bonds during de-blocking the phosphate protecting groups with concentrated NH_4OH (Method 1) and no attempt to mask the unreacted 5'-

TABLE 2.
Yield of Each Step Coupling Estimated by Two Methods

NUCLEOTIDE	YIELD (%)			
	On Carboxyl Resin (VIII)		On Amino Resin (XIV)	
	DMTr Analysis	HPLC Analysis	DMTr Analysis	HPLC Analysis
dT ₄	77	a	97	a
dT ₇	90	75	99	80
dT ₁₀	89	79	97	77
dT ₁₃	95	71	91	67
dT ₁₆	92	58	92	63
dT ₁₉	70	48	93	57

a It was technically difficult to estimate the yield of the first coupling by HPLC analysis on a Permaphase AAX column.

hydroxyl group from the previous coupling cycle (Method 1 and 2). Neither of the two methods gave the exact yield of the coupling reaction to form phosphoate bonds and the actual coupling yield should be between these two figures.

The analysis of dimethoxytrityl function by spectroscopic method in 1% BSA solution is very simple, and removal of the function is an essential step for the synthetic operation. Accordingly, if the unreacted 5'-hydroxyl function of the previous coupling is blocked with acetic anhydride or phenyl isocyanate before removal of the dimethoxytrityl group, the accurate monitoring of the coupling reaction in each cycle can be done without any extra operations (see the following papers). For the synthesis of tridecathymidylic acid, the dimer block (IV, $n=2$) was coupled on the resin (XIV) by essentially the same method as described for nonadecathymidylic acid. The HPLC profile of the tridecamer was shown in Figure 4.

EXPERIMENTAL SECTION

Materials and General Methods

Enzacryl Gel K-2 and its carboxyl form, succinic anhydride, DMAP, pentachlorophenol, ethylene diamine and ethylene glycohol were purchased from Aldrich Chemical. Thymidyl oligonucleotides (dimer and trimer) were synthesized as described in the previous paper.¹⁸ High performance liquid chromatography on Permaphase AAX was carried out exactly as published.¹⁷ Partial venom phosphodiesterase digestion of the oligothymidyl acids was performed by the same method as published.¹⁹ All other reagents and chemicals were prepared or purchased as described in previous papers.²⁰

Attachment of 5'-O-Dimethoxytrityl Thymidine onto the Enzacryl Gel K-2 Carboxyl Form (VIII)

The commercially available polymer (1 g of VIII, 1 mmole equivalent of carboxyl group) was shaken with 5'-O-dimethoxytrityl thymidine (4 mmole) and TPS (8 mmole) for 24 hours at room temperature in a mixture of dry acetonitrile (6 ml) and pyridine (6 ml). The mixture was shaken further for 2 hours with methanol (3 ml) to block the unreacted activated carboxyl

FIGURE 4. ANALYTICAL FRACTIONATION OF OLIGOTHYMYDYLIC ACIDS BY HPLC.

The HPLC analysis was carried out on a Permaphase AAX column with a linear gradient of buffer B (0.05 M KH_2PO_4 /1.0 M KCl, pH 4.5) in buffer A (0.05 M KH_2PO_4 , pH 4.5). The gradient was performed by starting with buffer A and applying 3% of buffer B per minute. The elution was at 55°C with a flow rate of 2 ml/minute. At A, dT₁₉ synthesized on the carboxyl resin (VIII); B, dT₁₉ on the amino resin (X); and C, dT₁₃ on X.

groups, and filtered. The resulting resin was washed with pyridine (2 X 10 ml) and methanol (2 X 10 ml), and dried *in vacuo* (yield = 1.1 g). The amount of thymidine attached onto the resin was estimated in two ways: the quantitative analysis of the dimethoxytrityl function and thymidine liberated from the resin with acid and base treatment.

1) Analysis of the Dimethoxytrityl Function

The above prepared resin (10~20 mg) was treated with a 2% solution of BSA in CHCl_3 -MeOH (2 ml, 70% v/v) for 3 minutes with vigorous shaking at room temperature. The mixture was filtered, and the residue was washed with a CHCl_3 -MeOH solution (3 ml, 70% v/v) two times. All washing solvents were combined and adjusted exactly to a total volume of 50 ml by the addition of CHCl_3 . After complete evaporation of a mixture of 1 ml of the above prepared solution and 2% BSA solution in CHCl_3 -MeOH (7:3 v/v, 1 ml), CHCl_3 (2 ml) was added to the residue and optical density was measured. The dimethoxytrityl function has a λ maximum of 507 nm and ϵ maximum of $92,100 \text{ M}^{-1}\text{cm}^{-1}$ in 1% BSA in CHCl_3 solution. The estimated yield was 0.141 mmole.

2) Analysis of Thymidine

The residue (XIII), after removal of dimethoxytrityl group, was treated with concentrated NH_4OH (3 ml) at 50°C for 16 hours, filtered and washed with distilled water (2 X 3 ml). The solvents were combined, evaporated, and dissolved in a small amount of water. The thymidine was purified by thin layer chromatography on silica gel in a mixed solvent, CHCl_3 -MeOH (4:1 v/v). After elution of thymidine was estimated by measurement of the optical density at 267 nm. The estimated yield was 0.144 mmole.

Attachment of 5'-O-Dimethoxytrityl Thymidine onto the Amino Resin (IX)

1) Derivatization of Polyacrylmorpholide by Ethylene Diamine (X).

A mixture of polyacrylmorpholide (Enzacryl Gel K-2, 21 g) in ethylene diamine (170 ml) and ethylene glycohol (170 ml) was heated at 175 – 180°C (bath temperature) for 18 hours with vigorous stirring. After the reaction mixture was cooled to room temperature, ice (50 g) was added, and the mixture was filtered.

The resin was washed successively with water (4 X 100 ml), 0.1 N HCl (4 X 100 ml), 0.1 N Na₂CO₃ (4 X 100 ml), water (4 X 100 ml), and MeOH (5 X 100 ml), and dried *in vacuo*. The yield was 19.0 g. When estimated by titration²⁰, the amount of amino functions was 0.18 mmole/g.

2) 3'-(5'-O-Dimethoxytrityl Thymidyl) Monosuccinate (X).

Anhydrous 5'-O-dimethoxytrityl thymidine (5.45 g, 10 mmole) was dissolved in pyridine (40 ml) containing dimethylaminopyridine (1.83 g, 15 mmole) and succinic anhydride (1.50 g, 15 mmole). The mixture was stirred at room temperature overnight and concentrated to a gum, which was taken up in CHCl₃. The CHCl₃ solution was washed with Na₂HPO₄ and H₂O, dried over Na₂SO₄ and evaporated. The product was purified by column chromatography on silica gel (120 g) eluted with CHCl₃-MeOH solution (0-5% v/v). Dissolved in a small amount of CHCl₃, the isolated product was added dropwise to pentane (2 l). The resultant precipitates were collected by filtration and dried. The yield was 4.48 g (70%); Anal calculated for C₃₅H₃₆O₁₀N₂, C, 65.20; H, 5.62; N, 4.35; found C, 64.96; H, 5.62; N, 4.41.

3) Pentachlorophenyl 3'-(5'-O-Dimethoxytrityl Thymidyl) Succinate (XII).

To the mixture of the monosuccinate (XI, 1.29 g, 2 mmole) and pentachlorophenol (590 mg, 2.2 mmole), in 15 ml of dimethyl formamide (DMF), was added dicyclohexylcarbodiimide (620 mg, 3 mmole); the reaction mixture 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 benzene solution was evaporated and the activated ester was dissolved in a small amount of benzene. The product was precipitated by the dropwise addition of the benzene solution to pentane (400 ml). The resulting precipitates were filtered, dried and used for the next step without further purification. The yield was 1.50 g (84%).

4) Formation of Nucleoside-Polymer Linkage (XIII).

A mixture of 5 g of the amino resin (X, 0.18 mmole/g), the activated ester (XII, 3.6 g, 4.0 mmole) and triethylamine (0.44 g, 4.4 mmole) in 30 ml of DMF was shaken at room temperature

for 20 hours. The reaction 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 to block free amino functions. The mixture was filtered, and the resin was washed with pyridine and MeOH, and dried. The yield was 5.6 g. The estimated amount of thymidine linked on the resin was 0.16 mmole/g by the spectroscopic measurement of the dimethoxytrityl function and the thymidine liberated from the resin.

General Procedure for the Synthesis of Oligothymidine on the Solid-Phase

All reactions were carried out in round-bottomed flasks and all steps for one cycle to link dimer or trimer thymidylic acid onto the resin are described below (also see Table 1).

Steps 1,2,3: Detritylation of the Dimethoxytrityl Group from the Resin.

A mixture of the resin (XIII, 0.45 g) and 2% BSA solution (10 ml) was shaken vigorously at room temperature for 3 minutes and filtered. The resulting resin was washed successively with a CHCl_3 -MeOH solution (10 ml, 7:3 v/v) twice. The dimethoxytrityl function liberated from the resin was analyzed at 507 nm in 1% BSA solution (0.072 mmole). The resin was further washed with anhydrous pyridine.

Step 4: Evaporation with Pyridine.

A mixture of the 5'-hydroxyl resin (0.072 mmole) and 5 equivalents of di- or tri-thymidylic acid (0.36 mmole) was co-evaporated with dry pyridine three times to remove a trace of water in the polyacrylamide backbone.

Steps 5,6,7: Coupling Reaction of Dimer or Trimer.

To this residue was added 10 equivalents of the coupling reagent (TPST, 245 mg) and dry pyridine (5 ml) and the mixture was shaken at room temperature for 3 hours. After the reaction, the mixture was filtered, and the resin was washed with pyridine (2 X 10 ml) and a CHCl_3 -MeOH solution (2 X 10 ml, 7:3 v/v), and dried (545 mg). A part of this dried resin (ca. 10 mg) was used for the HPLC analysis of the product on Permaphase AAX and the rest was submitted to further the elongation of the oligonucleotide chain. This cycle of the synthesis was repeated six times and trideca- and nonadeca-thymidylic acids were syn-

Figure 5

thesized using di and trithymidylic acids, respectively.

General Procedure for the Cleavage of Oligonucleotide from the Support

On an analytical scale (about 10 mg), the polymer sample (V) was treated with concentrated ammonia (3 ml) for 20 hours at 50°C under vigorous shaking. The mixture was filtered and the filtrate was concentrated under vacuum. The residue was treated with 80% acetic acid (1 ml) for 15 minutes and the solvent was evaporated, giving a white powder. The white residue, dissolved in 0.1 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5, 1 ml), was extracted with ether (3 X 1 ml) and the aqueous solution was evaporated. The product was isolated and analyzed by HPLC on Permaphase AAX, as published.

Identification of the Products dT(pT)₁₂ and dT(pT)₁₈

The identification of the products was carried out by following the published methods. The last peaks from the HPLC containing dT(pT)₁₂ and dT(pT)₁₈ were desalted by passing a Sephadex G-50 column (1.5 X 50 cm) eluted by 0.1 M TEAB buffer. After evaporation of the buffer, the oligonucleotides were phosphorylated by [γ -³²P]ATP with polynucleotide kinase and digested partially with snake venom phosphodiesterase. The partially digested oligonucleotides were fractionated by homochromatography on DEAE-cellulose plates.¹⁹ The d(pT)₁₃ gave 13 spots (data not shown) and the d(pT)₁₉ did 19 spots (see Figure 5).

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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