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A rapid and convenient synthesis of poly-thymidylic acid by the modified triester approach

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ABSTRACT

By using anhydrous triethylamine-pyridine to selectively remove the cyanoethyl group from the fully protected oligonucleotide, a substantial improvement has been achieved in yields and the rates of condensation by the modified triester approach from the 5'→3' end. The unreacted oligonucleotide containing the 5'-hydroxy group was removed by treatment with *bis*(triazolyl)-*p*-chlorophenyl phosphate after each condensation *in situ*. These modifications, as exemplified by the synthesis of fully protected T_{12}, T_{18}, T_{24} and T_{38} in 80%, 77%, 70% and 50% yields respectively, should allow the ready synthesis of polynucleotides of even longer chain lengths by purely chemical methods.

INTRODUCTION

We have reported previously on the synthesis of deoxyribooligonucleotides with demonstrated biological activities[2] by the modified[3] triester[4] method. In this communication we wish to report a rapid and highly efficient triester approach for the synthesis of polythymidylic acid containing 38 base units.

RESULTS AND DISCUSSION

Our earlier attempts[5] to extend the growing chain from the 5'→3' end were unsuccessful beyond hexamer because of the failure to remove selectively the cyanoethyl group from the 3'-phosphotriester function on treatment with sodium hydroxide-dioxane. Obviously a non-hydrolytic base was required. Potassium *tert.*-butoxide in tetrahydrofuran/*tert.*-butanol was found to be satisfactory, but as with the sodium hydroxide treatment, work-up such as treatment with Dowex-50 resin (pyridinium form) followed by repeated coevaporation with pyridine before the next coupling step was essential. We have now solved this problem by treating the fully protected oligonucleotide with an excess of a volatile

base such as triethylamine[6] (20-100 molar equivalents) in anhydrous pyridine for 4-6 hr. at room temperature. This treatment resulted only in quantitative β-elimination of the cyanoethyl group while other base-labile groups such as N-acyl[7] and *p*-chlorophenyl protecting groups were untouched. After evaporation of excess triethylamine, pyridine and acrylonitrile *in vacuo*, the coupling reaction was performed *in situ* with the incoming fragment containing a 5'-hydroxyl group and mesitylenesulfonyl tetrazole[8] in anhydrous pyridine as condensing agent. After completion of the reaction as judged by tlc on silica-gel, an excess of *bis*(triazolyl)-*p*-chlorophenylphosphate[9] was added to phosphorylate any unreacted material containing the 5'-hydroxyl group (Figure 1). This step was important since, in several cases, the mobilities of the product and unreacted 5'-hydroxyl containing fragment were very similar on silica-gel. After work up, the fully protected pure product could be isolated by column chromatography on silica-gel or preparative tlc in the case of dodecamer and longer oligomers. The reaction conditions and

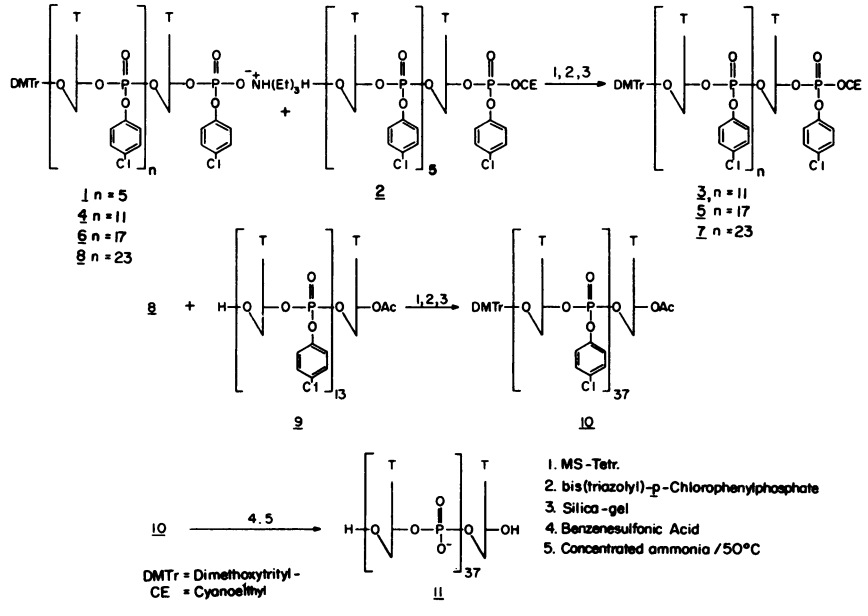

Figure 1. Modified triester approach for the synthesis of oligonucleotides from 5'→3' ends.

isolated yields of each step are documented in Table I. Two important observations have been made in the present studies: (i) Selective deblocking with anhydrous triethylamine-pyridine has been found to be quantitative and highly efficient even at longer-size oligonucleotides; (ii) The rate of the coupling reaction is substantially higher than reported previously.[8] This may be due to the increased nucleophilicity of the phosphate anion as a triethylammonium salt or the catalytic action of triethylamine on the mixed anhydrides giving a more reactive intermediate. It should be emphasized that these yields and rates (see Table I) are considerable improvements over any other reported chemical syntheses of similar sized polynucleotides and indicate it should now be possible to achieve a practical chemical synthesis of fragments of 30-50 units.

The characterization of T_{14}, T_{20} and T_{38} was achieved after removal of all protecting groups as described earlier.[10] The purity and size of these compounds were determined by labeling the 5'-hydroxyl group with polynucleotide kinase enzyme and [γ-^{32}P] ATP and measuring their relative mobilities on (i) homo-chromatography of $^{32}PT_{14}$, $^{32}PT_{20}$ and $^{32}PT_{38}$ in Homo-mix I[11] and (ii) 20%

Table I. Reaction conditions and the isolated yields of the various coupling reactions

5'-Protected containing 3'-phosphodiester group[a] (mmole)	5'-Hydroxyl component (mmole)	Mesitylenesulfonyl tetrazole (mmole)	Reaction time (hr.)	Product (fully protected)	Yield[b] (%)
Monomer (5.0)	Monomer (5.5)	12.5	0.75	Dimer	90-95
Dimer (4.0)	Monomer (4.4)	10.0	0.75	Trimer	90-95
Trimer (0.50)	Trimer (0.60)	1.5	1 hr.	Hexamer	85
Hexamer (0.05)	Hexamer (0.06)	0.125	2 hrs.	Dodecamer	80
Dodecamer (0.02)	Hexamer (0.03)	0.10	2 hrs.	Octadecamer	77
Octadecamer (0.0075)	Hexamer (0.015)	0.0375	6 hrs.	Tetracosamer	70
Tetracosamer (0.002)	Tetradecamer (OBz) (0.003)	0.015	18 hrs.	T_{38}	50
Dodecamer (0.01)	Dimer (OBz) (0.03)	0.05	3 hrs.	T_{14}	83
Octadecamer (0.0025)	Dimer (OBz) (0.0075)	(0.0125)	5 hrs.	T_{20}	61

a. Triethylammonium salt was used in each coupling reaction.

b. In a blank experiment, 2 g of silica-gel from the tlc plate was eluted with (2 × 60 ml) of chloroform-methanol (10:1 v/v). On evaporation to dryness, it gave (0.8 mg) of a residue.

polyacrylamide gel electrophoresis[12]. The results are shown in Figure 2 and 3 respectively. Each of the compounds ($^{32}PT_{14}$, $^{32}PT_{20}$ and $^{32}PT_{38}$) was found to be complete digested with snake venom phosphodiesterase enzyme.

EXPERIMENTAL SECTION

General Methods and Materials

The solvent systems for chromatography and other general methods and materials were as described previously.[10]

General Procedure of Deblocking Cyanoethyl Group from the Fully Protected Oligonucleotide

To a solution of fully protected hexamer (0.05 mmole, 144 mg) in anhydrous pyridine (2 ml) was added triethylamine (2 mmole, 202 mg). The reaction was complete in 2-3 hrs. as judged by silica-gel tlc (appearance of the trityl positive spot at the origin). The solution was then evaporated to a foam in order to remove excess of triethylamine and acrylonitrile liberated during the deblocking reaction. The foamy oligonucleotide material was used as such in the coupling reaction as described below.

Synthesis of Fully Protected Dodecanucleotide *3*

To the above reaction flask containing hexamer oligonucleotide *1* in dry pyridine was added a hexamer component containing the 5'-hydroxyl group *2* (0.06 mmole, 154 mg) and the solution was evaporated to dryness *in vacuo**. The syrupy residue was redissolved in dry pyridine (1 ml), followed by addition of mesitylenesulfonyl tetrazole (0.15 mmole, 32 mg). The reaction was over in 2 hrs. at room temperature as judged by silica-gel tlc (movement of trityl positive out of the origin). *Bis*-(triazolyl)-*p*-chlorophenylphosphate (0.05 mmole, 15 mg) was then added to remove an excess of the hydroxyl component. After stirring at room temperature for another 10 min. the reaction mixture was decomposed with distilled water (0.2 ml) and the resultant solution was evaporated to a gum *in vacuo*. The gum was dissolved in ice cold chloroform (20 ml) followed by neutralization of mesitylenesulfonic acid with a 5% solution of sodium bicarbonate in an ice-water bath. The chloroform layer was separated and the

*The vacuum was replaced with dry nitrogen.

aqueous layer was washed twice with chloroform (15 ml each). The combined organic layer was washed once with brine (15 ml), dried over anhydrous sodium sulfate and evaporated to dryness. The crude mixture was applied on five silica-gel plates (20 × 20 cm) (1 mm thickness) and eluted with 10% methanol-chloroform solvent. The isolated yield of dodecamer *3* was (216 mg, 80%).

Synthesis of Fully Protected Octadecamer *5*

The fully protected dodecamer *3* (0.02 mmole, 106 mg) in anhydrous pyridine (2 ml) was treated with triethylamine (2 mmoles, 202 mg) at room temperature for 4 hrs. to deblock the cyanoethyl group. After evaporation to a foamy residue, it was condensed with the hexamer *2* (0.03 mmole, 76.8 mg) in the presence of mesitylenesulfonyl tetrazole (0.1 mmole, 25.4 mg) in anhydrous pyridine (1.5 ml) for 2 hrs. *Bis*(triazolyl)-*p*-chlorophenyl phosphate (0.03 mmole, 9 mg) was then added. After stirring at room temperature for 10 min. distilled water (0.2 ml) was added and the reaction was evaporated to a gum *in vacuo*. It was then dissolved in ice-cold chloroform:methanol (9:1, 10 ml) followed by neutralization of the mesitylenesulfonic acid with a 5% solution of sodium bicarbonate in an ice-water bath. Distilled water (2 ml) was added to separate the organic layer from aqueous. The aqueous layer was saturated with sodium chloride and extracted with chloroform:methanol (9:1, 2 × 10 ml). The combined organic layer was washed once with brine (10 ml) and then evaporated to dryness. The crude mixture was fractionated on silica-gel plates. The major band containing trityl positive color was eluted with chloroform:methanol (9:1) to give 120 mg of the desired octadecamer *5* in a 77% yield.

Synthesis of Fully Protected Tetracosamer *7*

The fully protected octadecamer *5* (0.0075 mmole, 60 mg) in anhydrous pyridine (2 ml) was deblocked with triethylamine (0.75 mmole, 75 mg) at room temperature for 4 hrs. After evaporation to dry, it was condensed with the hexamer *2* (0.015 mmole, 39 mg) in the presence of mesitylenesulfonyl tetrazole (0.0375 mmole, 9 mg) in pyridine (1 ml) for 6 hrs. After the usual treatment with *bis*(triazolyl)-*p*-chlorophenylphosphate (0.03 mmole, 9 mg) and the work-up as in the case of octadecamer *5* preparation, the tetracosamer *23* was isolated by preparative tlc on silica-gel in a 70% yield (55 mg).

Synthesis of Fully Protected Octatriacontamer *11*

The fully protected tetracosamer *7* (0.002 mmole, 20 mg) in anhydrous pyridine (1 ml) was treated with triethylamine (0.2 mmoles, 20 mg) for 4 hrs. After evaporation to a foam, it was condensed with tetradecamer (containing OBz group at 3'-end) (0.003 mmole, 17 mg) in the presence of mesitylenesulfonyl tetrazole (0.015 mmole, 2.54 mg) in anhydrous pyridine (0.5 ml) for 18 hrs. After the work-up as described for the octadecamer, the desired fully protected compound *11* was isolated by preparative tlc in a 50% yield (14 mg).

Complete Deblocking of the Fully Protected Deoxyribooligonucleotides

The dimethoxy trityl group was removed by treating the fully protected compound (10 mg) with a 1% benzenesulfonic acid in chloroform:methanol (7:3) at 0°C for 10-30 min. After neutralizing with a 5% sodium bicarbonate solution, the reaction mixture was worked up as described for octadecamer. On removal of the solvent, the residue was purified by silica-gel tlc. The major band was eluted with chloroform-methanol (10:1 v/v) and the residue was treated with concentrated ammonium at 50° for 4-6 hrs. After removal of ammonia, the residue was washed with ether (2 × 2 ml). The compounds containing phosphodiester bonds were isolated by tlc on PEI-Cellulose at 60° eluting with lithium chloride-7 M urea solvents. Isolation of T_{14} was carried out using 0.5 M lithium chloride-7 M urea-0.025 M tris (pH 8.0) and it appeared as a major band with Rf of 0.8 with respect to yellow dye, T_{20}, Rf 0.5 in 0.6 M lithium chloride-7 M urea-tris (0.025 M) pH 8.0 and T_{38}, Rf 0.2 in 0.6 M lithium chloride-7 M urea-0.025 M tris (pH 8.0).

For recovery of oligonucleotides, the wet PEI-plates were washed three times in methanol and then dried. The compound was recovered from each band by eluting with 2 M triethylammonium bicarbonate (pH 9.5).

Characterization of T_{14}, T_{20} and T_{38} Containing 3'-5'-Phosphodiester Groups

Each of the compounds (200 pmol) was phosphorylated with T_4 polynucleotide kinase and [γ-^{32}P]ATP according to the published procedure.[10] The 5'-^{32}P labeled oligonucleotides were purified

and desalted on a Sephadex G-50 (Fine) column using 0.05 M triethylammonium bicarbonate (pH 8.0).

(i) Homo-chromatography

The homochromatography of $^{32}PT_{14}$, $^{32}PT_{20}$ and $^{32}T_{38}$ was carried out on DEAE-cellulose plate using homo-mix-I[11] and the results are shown in Figure 2. In each case, the compound was found to be more than 90% pure.

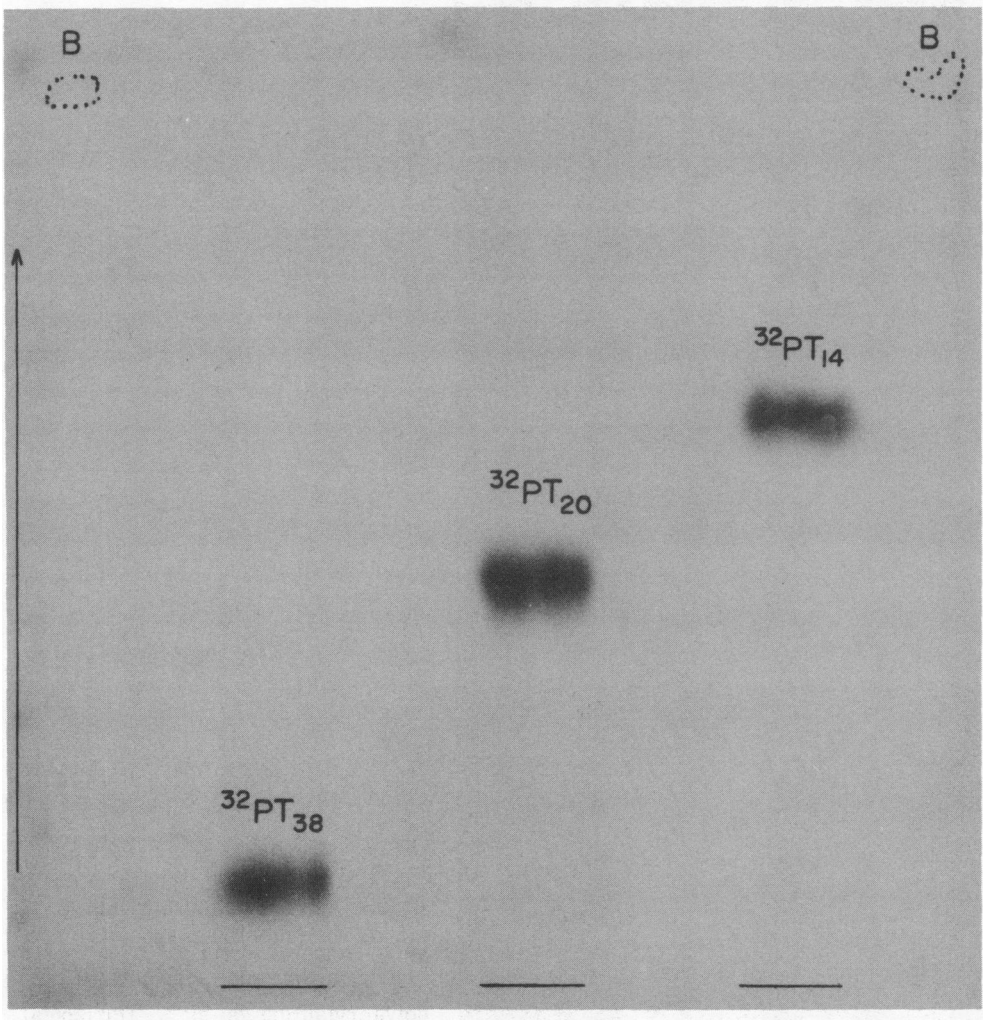

Figure 2. Autoradiogram showing the relative mobilities of $^{32}PT_{14}$, $^{32}PT_{20}$ and $^{32}PT_{38}$ on homo-chromatography in homo-mix-I solvent.

(ii) Polyacrylamide gel electrophoresis

Each of the labeled oligonucleotides was run on a 20% polyacrylamide gel slab (10 cm × 40.0 cm × 0.3 cm) containing Tris-borate (pH 8.3)-5 mM $MgCl_2$)[12] for 24 hrs. The same buffers were used in electrophoresis reservoirs. The results are shown in Figure 3.

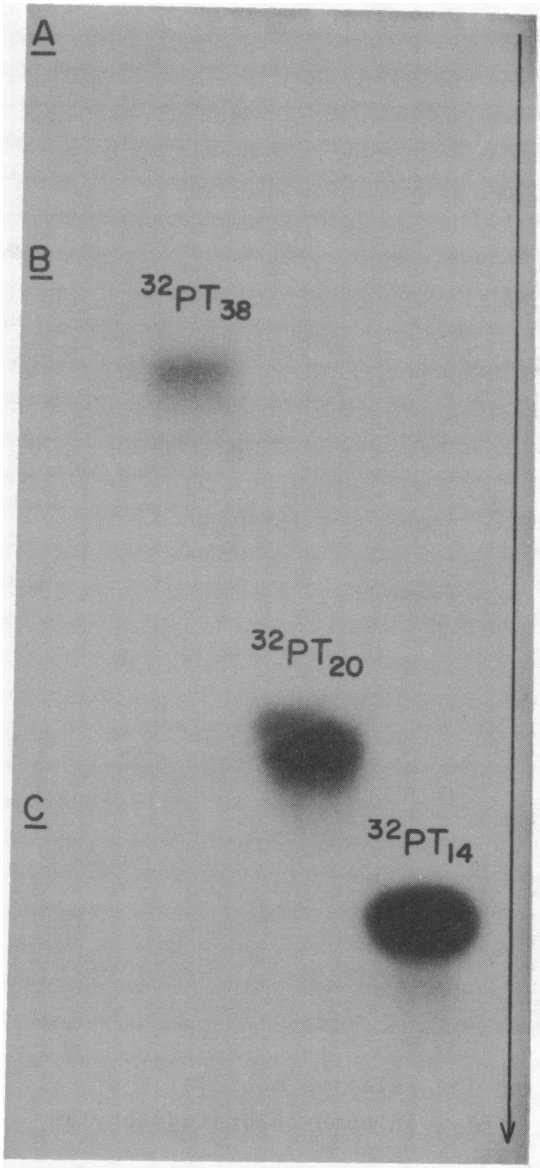

Figure 3. Autoradiogram showing the relative mobilities of $^{32}PT_{14}$, $^{32}T_{20}$ and $^{32}PT_{38}$ on 20% polyacrylamide gel slab. Horizontal lines indicate the position of the three dye markers, trypan red (A), xylene-cyanol FF (B) and bromophenol blue (C).

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