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Chemical synthesis of d(G-C)$_4$, d(G-C)$_5$ and d(GGTGGACCTC) by continuous flow solid phase phosphotriester method

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Abstract. A simplified protocol for the synthesis of oligodeoxyribonucleotides by phosphotriester approach on controlled pore glass resins using a manual DNA synthesiser is presented. The main features of this method are: (i) a single system of solvents (acetonitrile : dichloromethane, 8 : 2) is used in the assembly procedure reducing the number of mechanical manipulations, (ii) dichloroacetic acid is used as a good compromise between the efficiency of deprotection and minimal depurination and (iii) it competes effectively with the phosphite method in terms of speed, efficiency and ease. All the required protected mononucleotides and functionalised resins were home-made and detailed procedures are reported. The utility of the procedure is demonstrated by the actual synthesis of sequences d(G-C)$_4$, d(G-C)$_5$ and d(GGTGGACCTC) required for biophysical studies in our laboratory. The oligonucleotides were purified by the recently introduced method of fast protein liquid chromatography which gives good resolutions in shorter time periods as compared to the high performance liquid chromatography technique.

Keywords. Oligodeoxyribonucleotide synthesis: solid phase; fast protein liquid chromatography.

1. Introduction

The technique of solid phase synthesis has now become a versatile method for routine chemical synthesis of oligodeoxyribonucleotides (Gassen and Lang 1982; Gait 1984). This success is mainly due to constant improvements in the properties of solid supports, the efficiency of condensing agents and the amenability of the method for either partial or complete automation. Automation is achieved by a continuous-flow process in which the support is sequentially treated with the coupling mixture, solvents and the deprotecting agent for pre-determined time intervals. In such a set-up, the concentrations of the monomers typically used are about 4–5 equivalents over the resin loading and the volumes of solvents and reagents are about 10 times the bed volume for each washing. These aspects of continuous-flow synthesis amplify the effect of impurities present even in trace amounts and hence demand the use of very high purity solvents, monomers and reagents for good results. Taking these factors into consideration, one of us (Ganesh 1984) recently presented a simplified protocol for the synthesis of oligodeoxynucleotides by a continuous-flow phosphotriester approach on controlled pore glass resins. One of the features of this method is that all the required monomers were prepared in our laboratory starting from deoxynucleosides using

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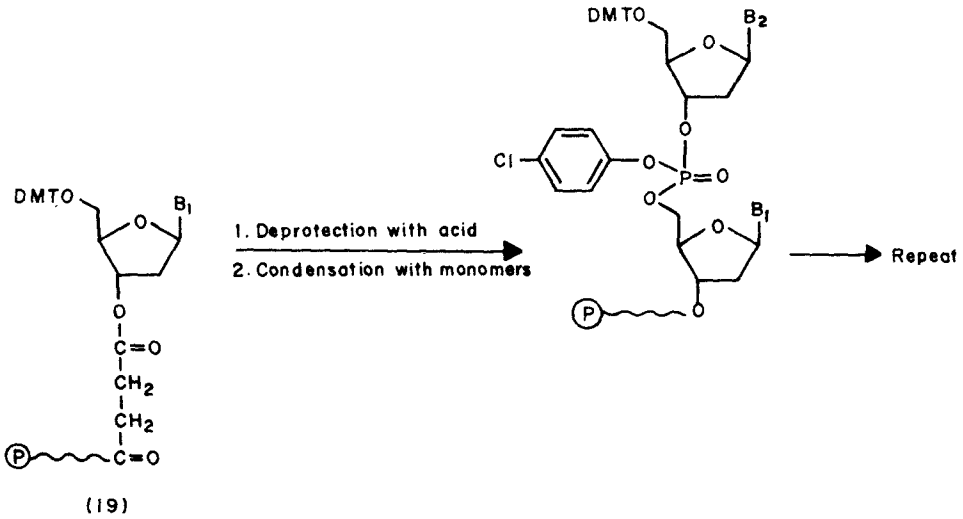
indigenous reagents and solvents. In this paper, we present the detailed methodology for the synthesis of monomers, functionalisation of resins and the application of the reported method for the successful synthesis of DNA fragments $d(G\text{-}C)_4$, $d(G\text{-}C)_5$ and d(GGTGGACCTC).

2. Results and discussion

2.1 *Preparation of the monomers*

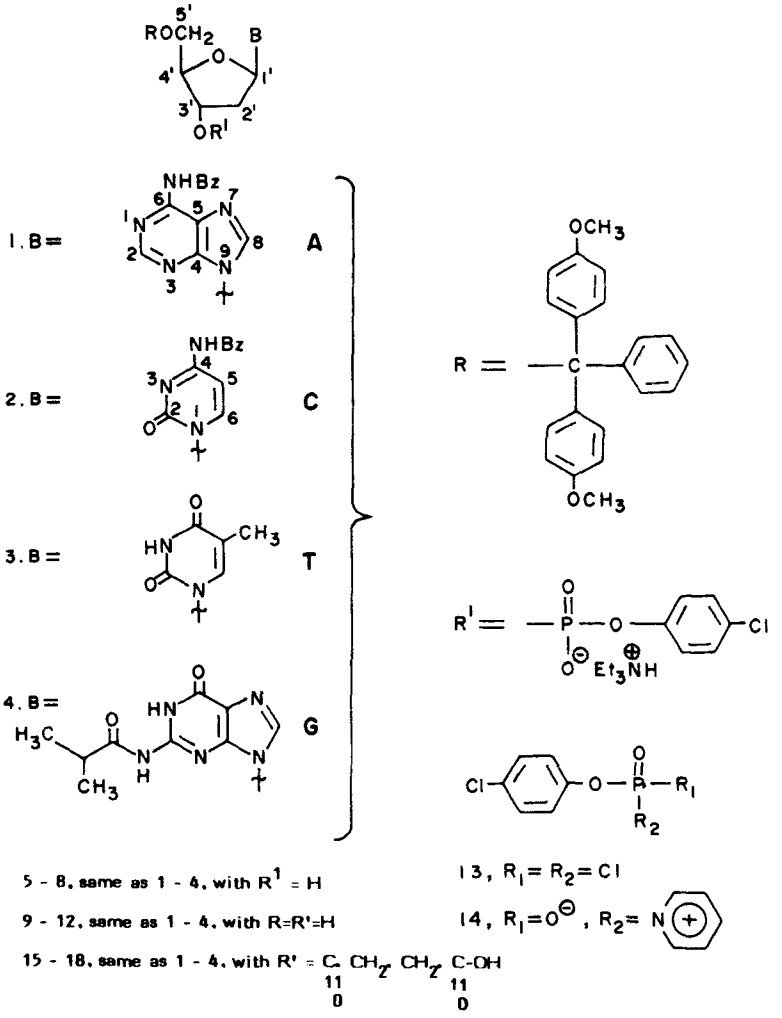
The monomers required for the solid phase synthesis (scheme 1) are the protected deoxynucleotides (1–4, scheme 2) derived from the protected nucleosides (5–8). The latter were prepared by the one-pot transient protection method of Ti *et al* (1982). The unprotected deoxynucleosides (dA, dC and dG) were first treated with trimethylchlorosilane in pyridine to mask the hydroxyl groups, immediately followed by reaction with benzoyl chloride (for dA and dC) and isobutyryl chloride (for dG) to obtain N-acyl-3,5-bis trimethylsilyl deoxynucleosides. The hydrolysis of the silyl groups was then effected in a few minutes with aqueous ammonia to obtain the N-acyl-2′-deoxynucleosides (9–12) in 85–90% yields. These were converted into respective N-acyl-5′-O-(4,4′-dimethoxytrityl)-2′-deoxynucleosides (5–8) by treatment with 4,4′-dimethoxytrityl chloride in pyridine and the products were purified by flash chromatography on silica gel H. This one-flask procedure of preparing the N-acyl-5′-O-protected nucleosides is less cumbersome and much quicker than conventional methods (Narang *et al* 1980).

The N,O-protected deoxynucleosides (5–8) were phosphorylated at the 3′-hydroxyl group by the procedure of Effimov *et al* (1982). Here the phosphorylating agent is the reactive 4-chlorophenyl phosphoryl pyridinium derivative (14) which is generated *in situ* by simple addition of an equimolar amount of water to 4-chlorophenyl phosphorodichloridate (13) in pyridine. The latter reagent was prepared by reaction of 4-chlorophenol with phosphorous oxychloride (Owen *et al* 1974) and purified by



(19)

Scheme 1.



Scheme 2.

vacuum distillation. This procedure of phosphorylation of nucleosides is more convenient as compared to the use of bifunctional phosphorylating agents such as tri- or tetraazole activated 4-chlorophenyl phosphates (Narang *et al* 1980) and the yields of phosphodiesters isolated as triethylammonium salts (1–4) are satisfactory. These crude nucleotide monomers were purified on a short column of silica gel H under a nitrogen atmosphere and were homogeneous on silica gel TLC plates as detected by UV and trityl positive spots.

2.2 *Functionalisation of the resin*

The controlled pore glass resin supplied by Pierce already carries a long chain alkyl amino function which was functionalised by treatment with N-acyl-5'-O-dimethoxyltrityl-3'-succinyl deoxynucleosides (15–18) by the active anhydride pro-

cedure (Gait *et al* 1982). The latter were prepared by the reaction of N-acyl-5'-O dimethoxytrityl deoxynucleosides (5–8) with succinic anhydride and 4-dimethylamino-pyridine in pyridine. The symmetrical anhydride of the 3'-O-succinyl nucleosides was obtained using DCC as the condensing agent and the functionalisation of the resin was smooth in dimethylformamide. The resin (9), functionalised by linking to the first nucleoside through 3'-hydroxyl, was finally capped by acetylation to block the remaining free amino groups and at this stage the resin showed a negative test with ninhydrin test solutions (Kaiser *et al* 1970). The nucleoside loadings on the resins as estimated by trityl analyses of the resins after treatment with 60% perchloric acid-ethanol were about 30–35 μ mole g^{-1}.

2.3 *Assembly and deprotection of the oligonucleotide chains*

Oligonucleotides were synthesised on the functionalised support in the 3' to 5' direction by a cyclical method involving only two chemical reactions per cycle (scheme 1). The first consists of the removal of the 5'-O-trityl protecting group by a suitable protic acid, followed by solvent washing to remove residual acid on the resin. During the second reaction, the appropriate nucleotide monomer (1–4) in the form of triethylammonium salts is condensed with the acid liberated 5'-hydroxyl groups on the resin in presence of an activating agent and a nucleophilic catalyst. These reactions are easily manipulated with an OMNIFIT manual DNA synthesiser by the continuous-flow method (Gait *et al* 1980, figure 1). The resin is contained in a small glass column fitted with a 6-way rotary valve which allows the selection of solvents and reagents flowing through the column. The coupling mixture consisting of the monomer, the condensing agent and the catalyst is injected into the column at appropriate points during the cycle through a septum fitted onto its top.

Based on the idea suggested by Effimov *et al* (1982), we have used a single solvent system acetonitrile : dichloromethane (8 : 2 v/v) for washing the resin, the condensations

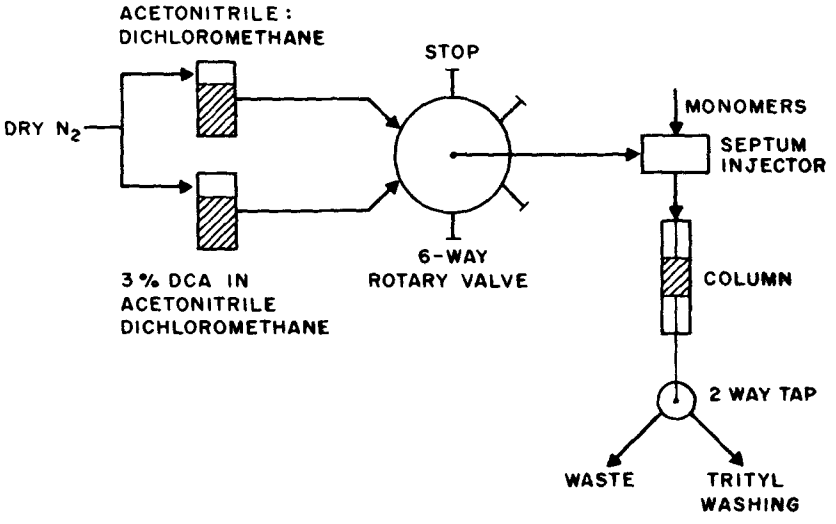


Figure 1. Diagram of assembly apparatus. The arrow indicates flow direction of solvents.

being carried out in acetonitrile. This is a very convenient solvent system as compared to pyridine used in other phosphotriester approaches (Sproat and Bannworth 1983) because of its ease of purification and its being less unpleasant to work with. The efficiency of condensation in acetonitrile is as good and in fact slightly faster than that in pyridine. We have used a mixture of triisopropylbenzene sulphonyl chloride and N-methyl imidazole as the coupling agent and the kinetic studies of Effimov *et al* (1982) have indicated that the condensation reaction on the resin with these reagents is almost complete in 15 min. The capping reaction at the end of every monomer addition was found to be unnecessary. The detritylating agent was a 3% solution of dichloroacetic acid in acetonitrile: dichloromethane and the deprotections were essentially over within 5 min for A, T and G additions whereas with C it was a little longer. Dichloroacetic acid is found to be a better agent, than either trifluoro or tirchloroacetic acids as this effects less depurinations (Adams *et al* 1983). All the acid washings were diluted to a known volume with 60% perchloric acid-ethanol and estimated spectrophotometrically by monitoring the absorbance at 495 nm. These analyses showed only a 2–3% drop in trityl values with consecutive couplings. Though this is not direct evidence of a successful synthesis, it does indicate a negligible cleavage of oligonucleotide chains from the resin during the synthesis. After the last coupling step, the trityl group was retained on the 5'-terminal nucleotide.

At the end of the assembly, the resin was treated with the oximate reagent (Reese and Zard 1981) which consists of a mixture of 2-nitrobenzaldoxime and 1,1,3,3-tetramethyl-guanidine in dioxane : water (1 : 1, v/v). This cleaves the oligonucleotides from the resin with the simultaneous deprotection of the phosphate moiety. The product was then treated with concentrated ammonia in a sealed tube at 60°C for 20 hrs to deacylate the side chain amido groups on base residues. After removal of excess ammonia, the product was reacted with 80% aqueous acetic acid for 30 min to detritylate the 5'-terminal nucleotide.

2.4 *Purification of oligonucleotides*

The initial purification of crude mixtures of synthetic oligonucleotides is best carried out by ion-exchange HPLC (high performance liquid chromatography) which separates oligonucleotides principally on the basis of their length. The most likely impurities in solid phase synthesis are either truncated or failure sequences which being shorter in lengths are eluted earlier and the identification of the desired peak on chromatogram is easy since it elutes as a large peak at the end. We have used the recent technique of FPLC (fast protein liquid chromatography) to purify the oligonucleotide mixtures. Compared to HPLC, this technique offers higher resolution in shorter times and preparative runs are possible with sample loads of a few milligrams. Using polyanion SI columns (Pharmacia) and phosphate buffer gradient (see experimental for details) excellent separations of oligonucleotides were obtained (figure 2). The broad peaks observed in low intensities beyond the desired product peak may arise either from the incompletely deprotected components or from the presence of secondary structures. The latter may result from the fact that the eluting conditions are not completely denaturing and the columns are run at room temperature. The desired peak was collected and the combined fractions from several runs were desalted on a gel filtration HPLC column which showed a single peak. Alternatively, the desalting can be achieved by passing the FPLC eluant over a Bio-gel P2 column and eluting with 20% aqueous ethanol. The

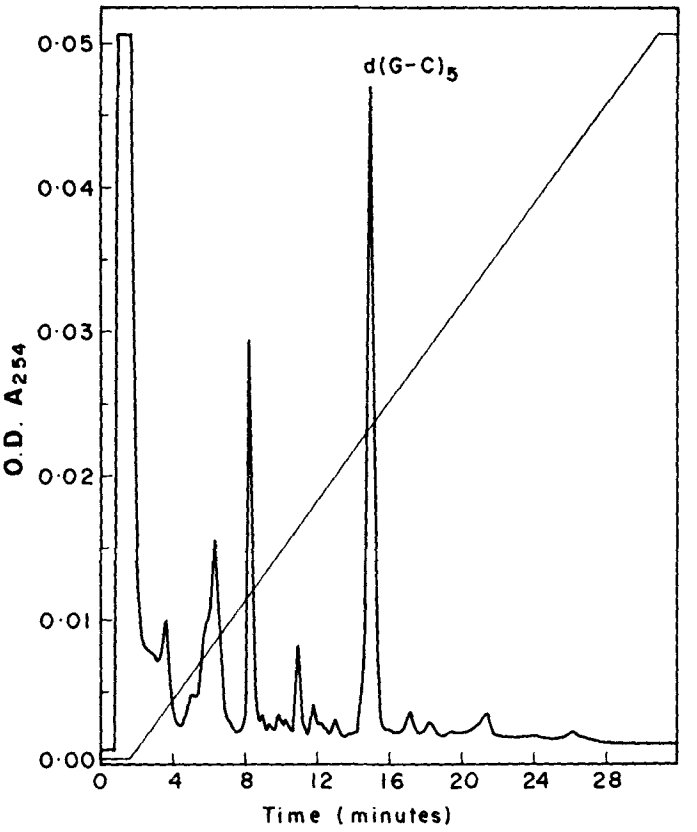


Figure 2. FPLC ion-exchange elution profile of crude product (See § 3 for details).

homogeneity of the product at this stage was demonstrated by a second analytical HPLC run under reverse phase mode on a C18 column.

2.5 *The target sequences*

The target oligonucleotide sequences required for biophysical studies in our laboratory were $d(G\text{-}C)_4$, $d(G\text{-}C)_5$ and d(GGTGGACCTC) and these have been prepared by the above procedure. The $d(G\text{-}C)_n$ sequences are known to exhibit the left handed *Z* conformation under certain specific conditions and hence the above sequences of defined lengths are useful in a systematic study of the $B \rightarrow Z$ transitions under a variety of conditions, our particular interest being those transition induced by metal ions. The last decamer sequence is part of a 17 base pair operator region on P22 DNA involved in the interaction with *mnt* repressor. This was required for our studies directed towards understanding the DNA-protein recognition in this system; the *mnt* repressor is being isolated in our laboratory using the cloned plasmids.

We have recently been able to synthesise a 17-mer using a slight modification of our above procedure. Here the condensations were done in pyridine and rest of the cycle remained same. The product was initially purified by DEAE sephacel ion-exchange chromatography (figure 3) and the purity of the product shown by FPLC analysis of the

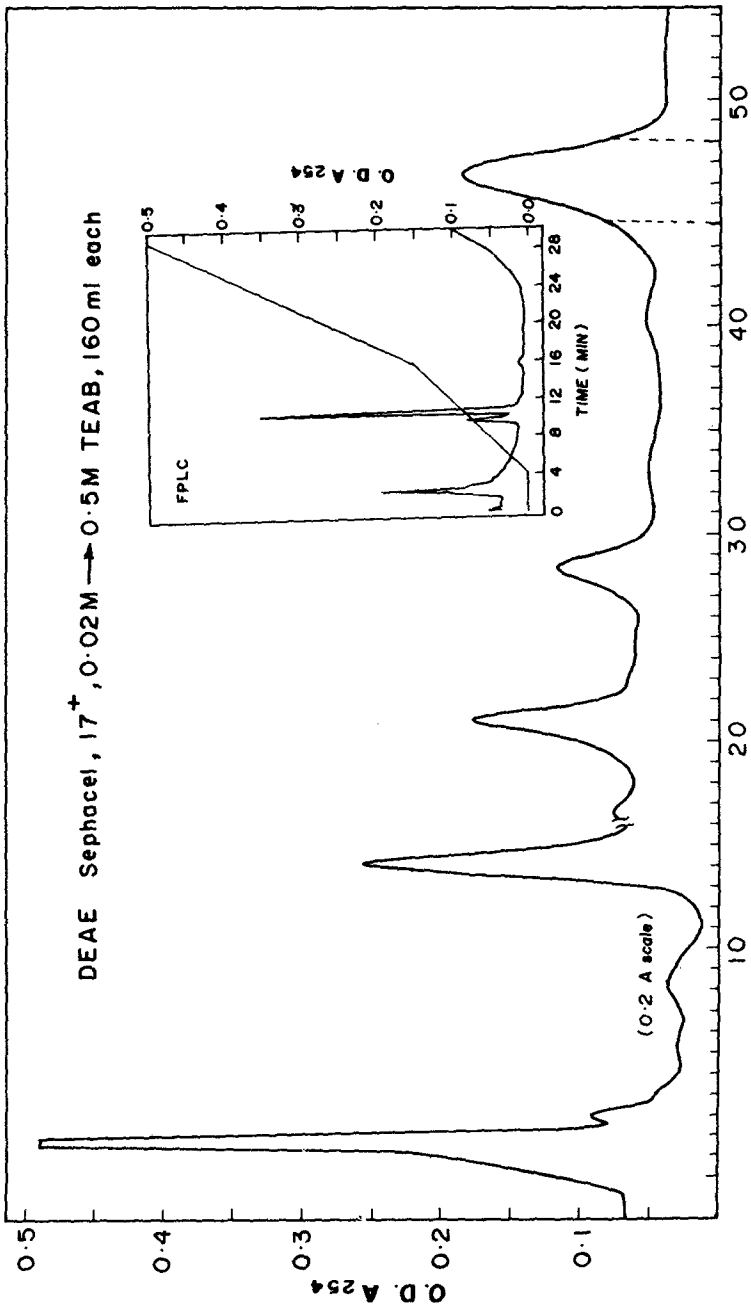


Figure 3. Ion-exchange chromatography of 17^+ (AGGTCCACGGTGGACCT) on DEAE Sephacel. Inset: FPLC analysis of the end peak (enclosed within dotted lines) from DEAE Sephacel chromatography.

final peak which is also the major product of the synthesis. The detailed procedures will be reported elsewhere.

3. Experimental

The 2'-deoxynucleosides, 4,4'-dimethoxytritylchloride, 2,4,6-triisopropyl benzenesulphonyl chloride, 4-dimethylamino pyridine and triazole were obtained from Sigma, USA. Trimethylchlorosilane, N-methyl imidazole and dichloroacetic acid (DCA) were procured from Fluka, Switzerland. Pyridine GR (E Merck, India) was refluxed and distilled over ninhydrin followed by distillation over calcium hydride. Acetonitrile and dichloromethane (Extra pure, E Merck India) were distilled over phosphorous pentoxide followed by distillation over calcium hydride. 2,4,6-triisopropyl benzene sulphonyl chloride was recrystallized from hexane (BDH, India), triazole from dioxane and 4-dimethylaminopyridine from diethyl ether. All column chromatographic purifications were done over silica gel H (without binder, BDH, India) by short column method (Still *et al* 1978) and monitored by TLC over silica gel G (BDH, India) coated plates. The spots were visualized by spraying with 60% perchloric and ethanol (3 : 2) for trityl derivatives (1–8, 15–18). Compounds without trityl group showed black spots on spraying with the above reagent followed by heating.

3.1 *6-N-benzoyl,2'-deoxyadenosine* (9)

2'-Deoxyadenosine 1·3 g, 5 m mol) dried by coevaporation with pyridine was suspended in 25 ml of dry pyridine and treated with 3·2 ml (25 m mol) of trimethylchlorosilane. The mixture was stirred for 15 min and to this was added 3·0 ml (25 m mol) of benzoyl chloride. The reaction mixture was maintained at room temperature for 2 hrs after which it was cooled in an ice bath and quenched with 10 ml of water. After 5 min it was treated with 10 ml of 29% aqueous ammonia at room temperature for 30 min, the mixture evaporated to dryness and the residue dissolved in 100 ml of water. It was washed once with diethyl ether and the aqueous layer on cooling yielded 1·5 gm (90%) of (9).

3.2 *4-N-benzoyl-2'-deoxy cytidine* (10)

2'-Deoxy cytidine (1·20 g, 5 m mol), in a reaction similar to the above with trimethylchlorosilane (3·2 ml) followed by benzoyl chloride (3·0 ml) in pyridine, (25 ml) yielded 1·5 g (92%) of (10) on work up.

3.3 *2-N-isobutyryl-2'-deoxyguanosine* (12)

This was prepared from 2'-deoxyguanosine (0·7 g, 5 m mol), trimethylchlorosilane (3·2 ml) and isobutyrylchloride (4·0 ml) by a procedure similar to that of above. The product (12) crystallised from water was obtained in 70% yield (1·0 g).

3.4 *General procedure for preparation of N-acyl-5'-O-dimethoxytrityl deoxynucleosides*

The N-acyl deoxynucleoside (5 m mol) was dissolved in anhydrous pyridine (15 ml) and the solution evaporated to dryness. The solid was dissolved in pyridine (10 ml) and

treated with 4,4'-dimethoxytritylchloride (1·7 g, 6 m mol). The mixture was shaken in the dark for 4 hrs in a sealed flask during which period pyridine hydrochloride separated out. TLC (silica gel) analysis in chloroform : ethanol (90 : 10 v/v) showed the reaction to be complete as trityl and UV tests showed one positive spot at higher R_f than the starting compound. Methanol (1 ml) was added to the mixture and extracted with chloroform (3 × 40 ml). The organic layer was washed with 1 M aqueous sodium bicarbonate (25 ml) and evaporated to dryness, resulting in a gummy mass. This was chromatographed by the short column method on silica gel H under N_2 atmosphere (1·5 p.s.i.), and eluted with dichloromethane containing 1 % triethylamine and increasing amounts of ethanol. The product started eluting around 5 % ethanol concentration as monitored by TLC and the appropriate fractions were combined and concentrated. The residue was dissolved in dichloromethane (10 ml) and precipitated by slow addition into a solution of 150 ml of ether:heptane (1:1 v/v) containing 1% triethylamine when the trityl derivatives separated out as amorphous white powder.

3.5 *General procedure for preparation of N-acyl-5'-O-(4,4'-dimethoxytrityl) 3'-O-(4-chlorophenyl) phosphate triethyl ammonium salts* (1–4)

N-acyl-5'-O-dimethoxytrityl-deoxynucleoside (1 m mol) was suspended in anhydrous pyridine (25 ml) and the mixture was evaporated to a final volume of 10 ml. 4-Chlorophenyl phosphorodichloridate (5 m mol) was added to pyridine (10 ml) contained in a glass reaction vessel fitted with a sintered disc and a stopcock and while cooling, water (5 m mol, 90 μl) was added into the reaction vessel. On leaving the mixture at room temperature for 10 min, pyridine hydrochloride separated out. It was filtered into the reaction flask containing the nucleosides in pyridine under N_2 atmosphere. The mixture was concentrated to 10 ml and after 30 min at room temperature, the phosphorylation was found to be complete as shown by TLC. The reaction was stopped by the addition of 1 M triethyl ammonium bicarbonate (TEAB, 15 ml) at 0°C. It was extracted into chloroform (2 × 75 ml), washed with 0·1 M TEAB and coevaporated to an oil with pyridine. This was then chromatographed over silica gel H by the short column method using 1 % triethylamine in dichloromethane and increasing amounts of ethanol. The phosphorylated product eluted as triethyl ammonium salt around 10 % ethanol in dichloromethane. The appropriate fractions were pooled and concentrated and the product was precipitated as amorphous white power from dichloromethane solution by adding slowly into ether : hexane (1 : 1, v/v).

3.6 *N-acyl-5'-O(4-4'-dimethoxytrityl)-3'O-succinates* (15–18)

The N-acyl-5'-O(4,4'-dimethoxytrityl) deoxy nucleosides (9–12, 4 m moles) was dissolved in dry pyridine (25 ml) to which was added succinic anhydride (0·99 g, 9·3 m mol) and stirred with 4-dimethylaminopyridine (1·18 g, 9 m mol). The solution was clear pale yellow within 5 minutes and it was kept stirred at room temperature for 24 hrs at the end of which TLC showed the completion of the reaction. Water (3 ml) was added and the excess pyridine was evaporated under vacuum to yield a gum. This was dissolved in dichloromethane (150 ml), washed twice with aqueous citric acid (810 mg in 100 ml) followed by water, and the dried organic layer concentrated to a gum. The gum on dissolving in dichloromethane (15 ml) and adding to ether : hexane (1 : 1, v/v) gave a white precipitate of the succinyl derivatives in 80 % yields.

3.7 *Functionalisation of controlled pore glass (CPG)*

The long chain alkylamine controlled pore glass (Pierce, 1 g) was dried under vacuum over phosphorous pentoxide and transferred to a 10 ml glass reaction vessel fitted with a G-1 sinter and a stopcock. The filtration is by slight nitrogen pressure on top and the sequence given in table 1 was used for functionalisation of the resin, with volumes of 5 ml per washings. The functionalised resin was dried and a trityl analysis done to get the loading value. The typical values obtained were A = 32·2 μmol g^{-1}, C = 34·7 μmol g^{-1}, G = 31·5 μmol g^{-1}.

3.8 *Solid phase assembly and deprotection of the oligonucleotide chains*

The assembly was done on an OMNIFIT (Cambridge, UK) manual DNA synthesiser equipped with a 6-way rotary valve and a glass column 5 mm × 60 mm). The resin (25 mg, 0·9 μm) was transferred into the column and allowed to equilibrate with acetonitrile : dichloromethane (8 : 2, v/v) after fitting the column end pieces. It was then capped with 20 μl of acetonitrile : acetic anhydride : N-methyl imidazole (8 : 1 : 1, v/v) for 10 minutes, followed by cyclical washings indicated below (with a flow rate of 1·5 ml/min driven by dry N_2 gas at 1·5 p.s.i.): (i) acetonitrile : dichloromethane (8 : 2, v/v) 2 min; (ii) 3% DCA acetonitrile : dichloromethane (8 : 2, v/v) 6–8 min; (iii) acetonitrile : dichloromethane (8 : 2, v/v) 2 min; (iv) coupling (stop flow) 15 min.

The coupling mixture in acetonitrile : dichloromethane (8 : 2, v/v), consisting of the appropriate nucleotide monomer (12 mg, 13 μm). 2,4,6-triisopropyl benzene sulphonyl chloride (20 mg, 65 μm) and 10 μl of N-methyl imidazole, was injected onto the resin with a gas tight syringe through the septum at the top of the column. After the coupling and removal of excess reagents, the trityl washings were collected separately during every cycle for spectrophotometric estimation. This step was omitted after the last coupling to retain the trityl group on the 5′-terminal nucleotide. At the end the resin was thoroughly washed, dried and subjected to deprotection in three stages:

(i) The dry resin in a 2 ml eppendorf tube was treated with *syn*-2-nitrobenzaldoxime

Table 1. Sequence used for functionalisation of the resin.

Treatment	Number of washings	Time per wash (min)	Ninhydrin test
10% diisopropylethyl amine : DMF (v/v)	3	5	
DMF	5	2	Resin +
Nucleoside succinate anhydride[a]	1	300	
DMF	5	2	Resin –
Pyridine	5	2	Resin + (trityl test)
Acetic anhydride[b] pyridine (1 : 9, v/v)	1	60	
Pyridine	5	2	Resin –
CH_2Cl_2	5	2	
Ether	5	2	

[a] Prepared as follows: nucleoside succinate (10 N over the initial resin loading) dissolved in dichloromethane (5 ml) and stirred at room temperature with dicyclohexylcarbodiimide (DCC, 5 N) for 15 min. The mixture is evaporated to dryness, dissolved in 5 ml DMF and transferred immediately to the resin.
[b] Capping reagent

(35 mg) in 0·5 ml of dioxane : water (1 : 1, v/v) and 1,1,3,3-tetramethylguanidine (25 μl) for 20 hr shaking at room temperature. It was filtered, washed a few times with aqueous dioxane (1 : 1), and the combined filtrate and washings evaporated to an orange gum.
(ii) The gum was treated with 20 ml of concentrated NH_3 (BDH, specific gravity 0·91) in a sealed flask and heated at 60° for 15 hrs. The excess ammonia was evaporated and the aqueous layer was concentrated.
(iii) It was treated with 5 ml of acetic acid : water (80 : 20, v/v) for 30 min at room temperature and extracted with ether (2 × 5 ml). The aqueous layer was concentrated, co-evaporated twice with water (3 ml), and redissolved in 2 ml of water.

3.9 *General method for purification of oligonucleotide chains*

The oligonucleotides were purified using a Pharmacia FPLC system consisting of two P500 pumps, a GP 250 gradient programmer and a 5 MPa mixer. The samples (100 μl out of 2 ml for analytical runs) were injected through a V-7 (10 MPa) injector and the runs followed spectrophotometrically with Pharmacia (DP-2) dual wavelength monitor fixed at 254 nm. The FPLC column was polyanion SI (Pharmacia) and run with phosphate buffer gradient from 0·01 M KH_2PO_4 to 0·75 M KH_2PO_4 in 20% acetonitrile over 30 min with a flow rate of 0·5 ml/min. The product (decamers) eluted under these conditions at about the middle of the gradient (figure 2) and the peak fractions were collected from several runs. The combined fractions were desalted on a water HPLC system using a 160 gel filtration column and eluting with 25% aqueous methanol for 15 min during which a single peak appeared on the chromatogram.

4. Conclusions

In this paper we have demonstrated a convenient protocol for synthesising oligonucleotides by solid phase phosphotriester approach. The detailed methods for synthesis of monomers using indigenous reagents and solvents, functionalisation of the resin, assembly of the chains, deprotection and final purification are described. The paper also demonstrates the utility of the FPLC technique for fast separation of oligonucleotides. Using this procedure, we have synthesised $d(G\text{-}C)_4$, $(G\text{-}C)_4$ and d(GGTGGACCTC) sequences required for biophysical studies in our laboratory.

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