Volume 9 Number 7 1981 Nucleic Acids Research

Rapid synthesis of oligodeoxyribonucleotides VI. Efficient, mechanised synthesis of heptadecadeoxyribonucleotides by an improved solid phase phosphotriester route

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Received 11 February 1981

ABSTRACT

Efficient mechanised synthesis of heptadecadeoxyribonucleotides has been achieved on an economically small scale by an improved solid phase phosphotriester method on a polydimethylacrylamide resin. Improvements were made in the preparation of dinucleotide building blocks, reaction conditions for oligonucleotide assembly and in purification of deprotected oligonucleotides by h.p.l.c. Several milligrams of pure heptadecamers were obtained. Two of the heptadecamers were designed for sequencing in opposite directions of DNA cloned in phage M13mp2.

INTRODUCTION

We have previously demonstrated the practical utility of polydimethylacrylamide resins for solid phase synthesis of oligodeoxyribonucleotides[1-3]. Our recent phosphotriester assembly strategy involved the coupling of appropriate monomer, dimer or trimer blocks to the support in pyridine in the presence of a coupling agent[2,3]. By this route we were able to rapidly prepare oligomers up to 12 units long containing all four bases.

A number of other polymers have also been recommended for use with a phosphotriester strategy, viz. polyacryloylmorpholide[4,5], cellulose[6] and polystyrene copolymers[7]. It was shown that the use of polyamide resins is preferable to polystyrene in that the yield in the first coupling step to the support is much better[7]. None of these other polymers has yet been used in mechanised oligonucleotide assembly systems. Alternative synthetic routes have been prepared that do not involve use of coupling agents. Good coupling yields were obtained by the addition of deoxynucleoside-3'O-chlorophenylphosphotriazolides to a polystyrene support suspended in tetrahydrofuran in the presence of N,N-dimethylaminopyridine[8]. Similarly good results were obtained using a 'phosphite-triester' method with silica gel as support[9]. As yet only monomer units have been coupled to supports by these last two methods and the longest oligonucleotide containing all four bases so far reported made is 13

long.

Recently Markham et al. have described the synthesis of oligodeoxyribonucleotides up to 21 long on a polydimethylacrylamide resin using our original phosphotriester route[10]. We now describe a number of significant improvements to our strategy and the mechanised assembly and rapid isolation of heptadecadeoxyribonucleotides on an economically small scale and in substantially better yields than hitherto. The oligonucleotides d(G-T-A-A-A-C-G-A-C-G-G-C-C-A-G-T) and d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C) were designed as primers for the sequencing of DNA cloned in bacteriophage M13mp2[11,12]; the heptadecamer d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T) corresponds to part of the 5' end of tobacco mosaic virus RNA and the heptadecamer d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T) was designed as a probe for the cDNA corresponding to the mRNA of the histocompatibility antigen HLA.B8.

DISCUSSION

The basic phosphotriester assembly strategy using a beaded polydimethylacrylamide resin[13] has been outlined previously[2,3]. Synthesis is carried out in a 3'-5' direction and the 3'-terminal residue is attached to the support via an alkali-labile 3'-O-succinate linkage. We have found that resin functionalised with each of the four 5'-O-dimethoxytrityl-2'-deoxynucleoside derivatives may be conveniently prepared on a 1 g scale as previously described[2] and stored as dry beads at -20° for at least nine months without noticeable decomposition. Each resin contains 0.22-0.28 mmole g^{-1} of deoxynucleoside derivative and a trityl/glycine ratio of 0.90-0.99 (the resin contains an internal glycine marker as previously described[2,3]).

The resin is swollen in pyridine and treated with 10% phenylisocyanate in pyridine as a precaution to ensure the absence of water. Oligonucleotide assembly is carried out by successive cycles of synthesis that involve (1) acidic removal of terminal 5'-O-dimethoxytrityl groups and (2) reaction of the liberated hydroxyl groups with 5'-O-dimethoxytrityl-2'-deoxynucleoside- or oligonucleotide-3'-O-p-chlorophenylphosphates in the presence of a coupling agent (Fig. 1). These synthetic cycles are carried out in a totally enclosed 'oscillating' glass reaction vessel fitted with a sintered glass disc[1,2], using either a bench-top valve/solvent bottle system[1] or a modified Beckman 990.B Solid Phase Peptide Synthesiser[1,2]. Although equally good oligonucleotide assemblies may be obtained using either system, we have concentrated particularly on the use of the mechanised Synthesiser, since its use reduces both the time and labour of assembly. Also other 'oscillating vessel' Synthesisers are

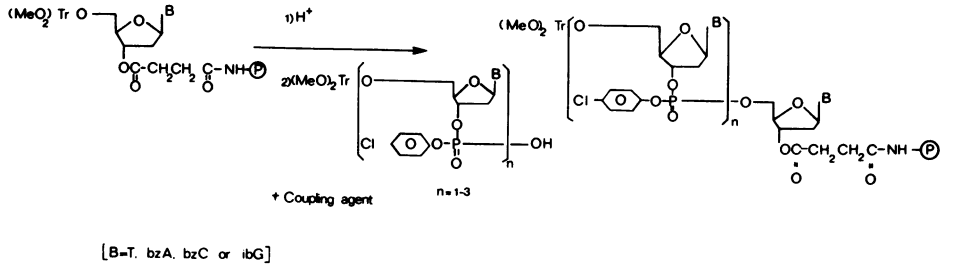


Figure 1

now commercially available [14].

Since relatively small quantities of oligonucleotides are sufficient for most biological applications we decided for reasons of economy to reduce the scale of synthesis to a minimum. The smallest quantity of resin that can be conveniently handled in practice is 50-60 mg (14 µmole of functional group) and we have, therefore, adapted our methods to suit this scale.

Preparation of protected mono- and dinucleotides

For the synthesis of oligonucleotides up to about 20 long the maximum use of dimers (rather than trimers) represents to us the best strategy. This keeps the number of reaction cycles to 10 or less and only 20 units (four monomers, 16 dimers) are necessary in order to be able to prepare any desired sequence. One advantage of small scale oligonucleotide assembly is that 1 g of a fully protected dimer is sufficient for at least 10 reaction cycles. We have therefore prepared on a 1-2 g scale all 16 dinucleotides of the form, $(MeO)_2TrdX^{O}X'^{O}_{-}(CE)$ where X and X' represent 2'-deoxyribonucleosides, viz. 2'deoxythymidine, 6-N-benzoyl-2'-deoxyadenosine, 4-N-benzoyl-2'-deoxycytidine or 2-N-isobutyryl-2'-deoxyguanosine, and $^{O}_{-}$ represents a p-chlorophenyl protected 3'-5' phosphodiester. To this end we have utilised the 'barium salt' route of Gough et al.[15], but with a number of important improvements (Fig. 2).

(1) The nucleotide barium salts (I) may be conveniently prepared on a 10 mmole scale by phosphorylation of the 5'-O-dimethoxytrityl-2'-deoxynucleoside derivatives in pyridine with a prereacted solution of 1.85 equivalents of p-chlorophenylphosphodichloridate and eight equivalents of triazole in pyridine. No triethylamine is used in the prereaction and hence no filtration step is required for removal of triethylammonium chloride. The reaction is complete within 10 min and in contrast to the observations of Broka et al.[16]

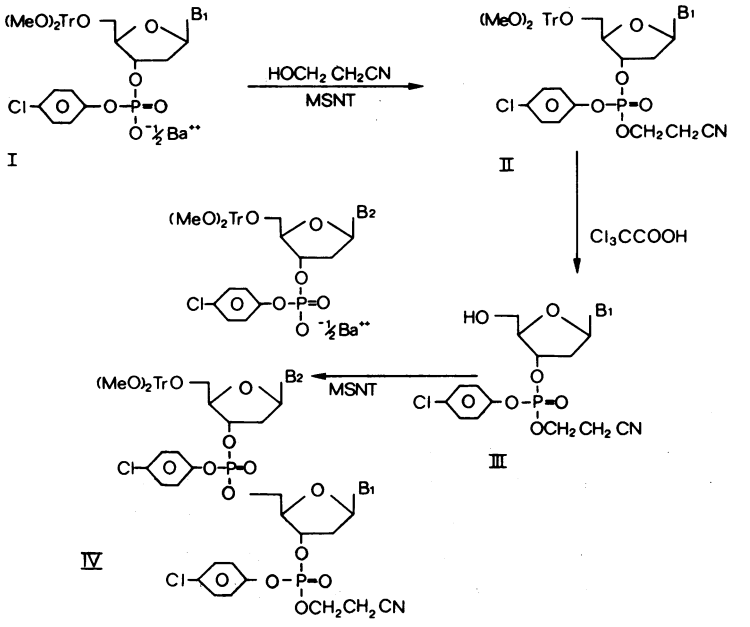


Figure 2

no side reactions involving the guanine moiety or other bases are seen under these conditions[17]. Subsequent hydrolysis and precipitation of the barium salt is carried out essentially as described by Gough et al.[15].

(2) The coupling agent 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT)[18] is used for both the preparation of the fully protected phosphotriester II and for coupling to form the dimer IV. In general these reactions are complete within 1 h and a minimum of side products is observed[19].

(3) Removal of 5'-O-dimethoxytrityl groups is accomplished by reaction with three equivalents of trichloroacetic acid in chloroform at 0°. There is less danger of loss of N-benzoyladenine from 6-N-benzoyl-2'-deoxyadenosine derivatives using this reagent rather than benzenesulphonic acid[3].

(4) After neutralisation of trichloroacetic acid with pyridine all four monomer units of type III may be efficiently extracted into chloroform from 0.1 M sodium bicarbonate solution (cf. Gough et al. found it impossible to completely extract the deoxyguanosine derivative into ethyl acetate and hence a more complicated protocol was needed for these derivatives[15]).

(5) The use of short column chromatography[20] using silica gel H we find

gives much better separations than our previous system. 1-2 g of dimer may be separated on a 35 g silica column in 1-2 h using 800-1000 ml of solvent (ethanol/chloroform) under slight nitrogen pressure.

Yields of dimers were 54-84% based on the hydroxy component III and products were homogeneous on silica gel tlc. Prior to oligonucleotide assembly 75-80 μmole quantities of the appropriate dimers are treated with anhydrous triethylamine/acetonitrile (1:1) for 1 h to remove terminal cyanoethyl groups[3] and the dimers isolated by precipitation into diethyl ether.

For the preparation of oligonucleotides containing an even number of bases a single monomer unit is required in the first assembly cycle. We have confirmed the findings of Norris et al.[5] that efficient couplings to the resin may be obtained using the barium salt of the appropriate nucleotide derivative I.

Improvements to the assembly strategy

(1) Removal of terminal dimethoxytrityl groups. We have already shown that the use of trichloroacetic acid is preferable to benzene sulphonic acid for removal of dimethoxytrityl groups, especially when 6-N-benzoyl-2'-deoxyadenosine derivatives are present[3]. When our previously described conditions, 10% trichloroacetic acid in chloroform/methanol (98:2) were used on small scale, intermittent failures in coupling reactions were observed. It was found that small droplets of water were slowly formed on storage of the solution of acid, presumably due to esterification of trichloroacetic acid by methanol. This water was retained by the resin during deprotection and then inhibited subsequent coupling reactions. This problem was eliminated by use of 10% trichloroacetic acid in chloroform alone. 2 x 2 treatments at room temperature are sufficient for complete reaction in all cases which is then quenched by addition of dimethylformamide.

(2) Internucleotide coupling reactions. In a model reaction in solution it was shown that the use of MSNT led to faster coupling rates than TPS tetrazole, which we had previously used, and that unwanted 5'-O-sulphonation was only ca. 1% when the concentration of coupling agent was 0.1 M[21]. We have now found that MSNT is a convenient coupling agent for use in solid phase synthesis. Time for coupling is reduced to 90 min and the overall cycle time is reduced to 3-3½ h. (N.B. The cycle time has not been totally optimised and a substantial reduction may be possible in times for intermediate washings.)

Many other solid phase routes described recently have incorporated a co-evaporation step prior to coupling to remove traces of water[4-7]. In

mechanised assembly this technique is cumbersome and slow. However, relatively small amounts of water (2-3 µl) could totally inhibit coupling reactions on small scale. We have found that good coupling reactions are consistently achieved by addition of an extra quantity of coupling agent in the coupling reaction (five equivalents of dimer and 15 equivalents of MSNT per equivalent of resin funtionality). The use of excess coupling agent is not detrimental provided that oximate deprotection is used at the end of assembly to reverse any modifications on guanine residues[19].

The synthetic cycle (Experimental section) is fully programmed on the Synthesiser and manual intervention is required only when activated monomer or dimer is added (step 8) or to obtain resin samples after each cycle for trityl analysis[2,3].

(3) Deprotection and isolation of oligonucleotides. The resin is reacted with 1,1,4,4-tetramethylguanidinium syn-p-nitrobenzaldoxime in dioxan/water (1:1)[22] for 60 h. We have found that for long oligonucleotides a shorter time of reaction can give rise to incomplete removal of p-chlorophenyl groups and/or incomplete reversal of base-modifications. This leads to internucleotide cleavage and irreversible base-modification respectively during subsequent steps. The oligonucleotide is further deprotected using concentrated ammonia at 50° for 5 h followed by 80% aqueous acetic acid for 30 min at room temperature[2,3].

Initial purification is achieved by ion exchange h.p.l.c. on Partisil 10 SAX. Markham et al. have reported that resolution of long oligonucleotides was not obtained using 5% ethanol in aqueous buffer systems[10]. We have now found that excellent resolution is obtained at least up to the 17-mer level by use of gradients of potassium phosphate (pH 6.2) in the presence of 30% ethanol and at 50-60°. After desalting on Biogel P2 the oligonucleotide is further chromatographed on µ-Bondapak C18. We confirm the observations of Markham et al. that recovery yields are sometimes worse than expected[10], but in our experience there is no direct correlation with oligonucleotide length.

The significant improvements to the methodology of solid phase synthesis are exemplified below.

Synthesis of heptadecanucleotides

Primers for sequencing of cloned DNA in phage M13mp2; d(G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T) and d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C). Anderson et al. have shown the utility of the cloned primer psp14 for sequencing cloned DNA in phage M13mp2[23]. One strand of this primer (primer 1) had 24 bases complementary to the region just prior to the EcoRI site (Fig. 3). However, a

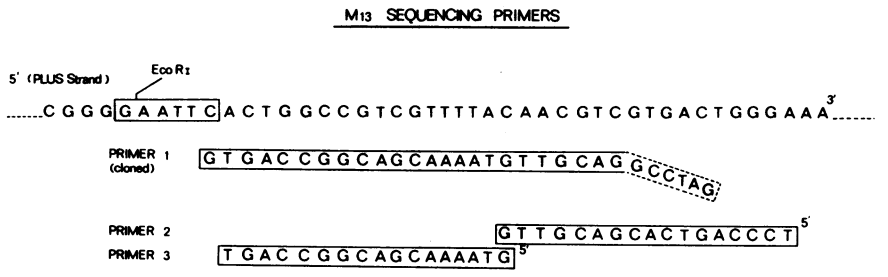


Figure 3

synthetic primer has the advantage that it may be prepared on considerably larger scale. Hence, the oligonucleotide d(T-C-C-C-A-G-T-C-A-C-G-A-C-G-T-T-G), primer 2, was synthesised by the methods described in our previous paper[3,24]. Unfortunately this primer had a secondary binding site on M13 that resulted in the presence of artifact bands on autoradiographs of sequencing gels. The artifacts could not be completely eliminated in all cases even under conditions of template excess[25]. Analysis of the M13mp2 (+) strand sequence[26] using the computer program SEQFIT[27] showed 13 out of 17 bases exactly complementary at the secondary site. A new primer was chosen by comparing all possible 17-mers with the M13mp2 sequence and eliminating all those in which more than 11 nucleotides were complementary at a second site. Of the remaining sequences, all of which scored a match of 11, the 17-mer, d(G-T-A-A-A-C-G-A-C-G-G-C-C-A-G-T), primer 3, was chosen as this minimised any complementarity at the 3' end of the primer[28]. The 17-mer has now been shown to be excellent for sequencing DNA in phage M13mp2[11,12,25].

The oligonucleotide d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C) was designed as a primer for sequencing in the reverse direction DNA cloned in phage M13mp2. The primer has been successful both to confirm sequences derived from sequencing in the normal direction and to obtain the sequences of much longer pieces of DNA. Full details of the use of this primer are published separately[11].

The syntheses were carried out as described above using solely dinucleotides as basic units, except that in the case of the first oligonucleotide only 10 equivalents of MSNT were used in the coupling step. Thus a larger number of shorter oligonucleotides were observed in the h.p.l.c. elution patterns (Fig. 4) due to incomplete couplings and the overall yield was only 1.3% (Table 1). The second 17-mer was isolated in 5.0% yield and the h.p.l.c.

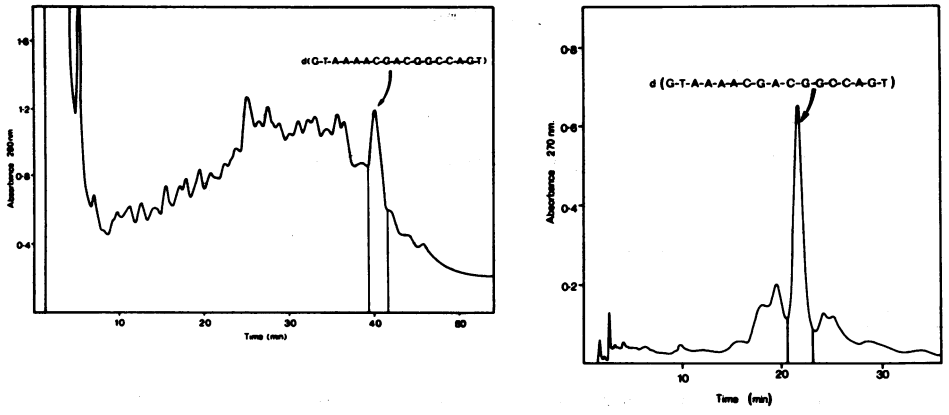


Figure 4 (a) Ion exchange (b) Reversed phase

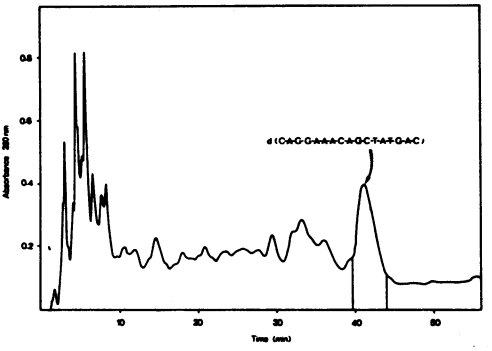
elution patterns were much better (Fig. 5).

The heptadecanucleotides, d(G-T-A-T-T-T-T-A-C-A-A-C-A-A-T-T) and d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T). The 17-mer, d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T), corresponds to part of the 5' end of tobacco mosaic virus RNA and has been used as an aid in cloning of cDNA[29]. The 17-mer, d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T), was designed as a probe for the cDNA corresponding to the mRNA of the histocompatibility antigen HLA.B8[30]. The syntheses were carried out as described above, once again using dimers as basic units, and the 17-mer isolated in 7.5% and 9.2% yields respectively (Table 1). H.p.l.c. elution patterns are shown in Figures 6 and 7.

The sequences of all oligonucleotides have been confirmed by standard analysis of ^{32}P-labelled samples.

TABLE 1

Sequence	Product isolated		Overall yield %	Yield after μ-Bondapak	
	A_{260}	μmole		A_{260}	% recovery
d(GTAAAACGACGGCCAGT)	35.0	0.180	1.3	8.6	25
d(CAGGAAACAGCTATGAC)	142.0	0.705	5.0	53.0	37
d(GTATTTTTACAACAATT)	198.0	1.047	7.5	88.0	44
d(GCAGCCTGAGAGTAGCT)	240.0	1.280	9.2	137.0	57



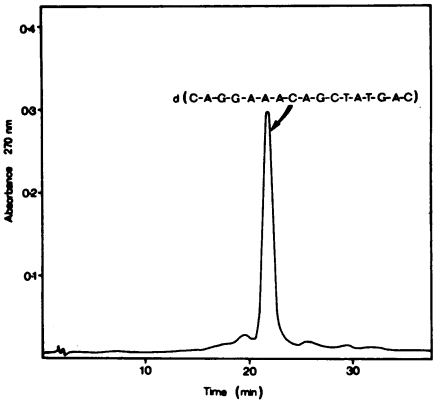
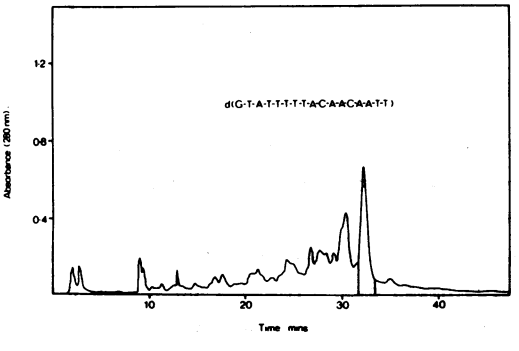


Figure 5 (a) Ion exchange (b) Reversed phase

CONCLUSIONS

We have shown that oligodeoxyribonucleotides of 17 residues may be prepared on an economically small scale, in good overall yields (5-9%) and in sufficient quantities for most biological purposes (1-10 mg). Average overall yields per step are 70-75%, but it is clear from h.p.l.c. patterns that coupling yields are substantially higher than this. Use of a mechanised synthesiser allows 2-3 reaction cycles to be readily carried out per day and oligonucleotides may be routinely prepared and isolated in pure form within two weeks. No direct comparison is possible with results obtained with phosphotriester syntheses on other supports, since in no cases have overall



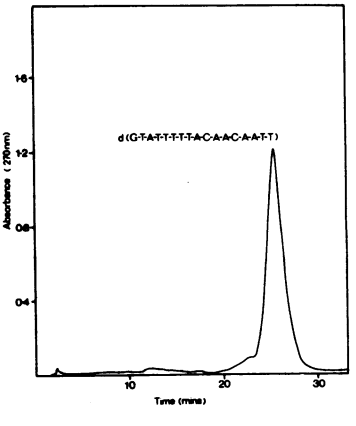


Figure 6 (a) Ion exchange (b) Reversed phase

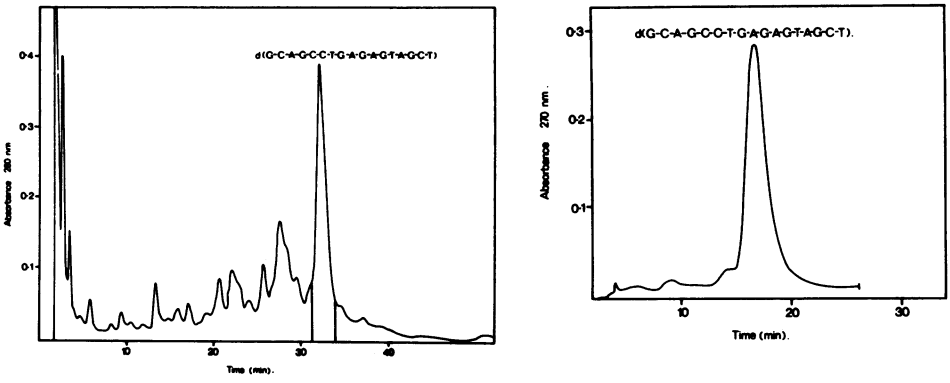


Figure 7 (a) Ion exchange (b) Reversed phase

isolated yields of final oligonucleotide been quoted[4-9]. It is noteworthy, however, that our overall yields compare favourably with available solution methodologies[31].

Future work is aimed at further increasing yields and extending the range of oligonucleotides attainable. Analysis of resin eluates after acidic deprotection steps in synthesis of the 17-mer, d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T), still showed a total of 27% loss of N-benzoyladenine during the complete synthesis (i.e. ~1% depurination per adenine per deprotection step). Very recently we have found that depurination can be reduced still further if trichloroacetic acid treatment is carried out at 0°[32]. The use of O-chlorophenyl as protecting group for phosphodiesters[4,8,18] is also under investigation. We have already confirmed that dinucleotide blocks incorporating this protecting group may be readily prepared by the route described herein[33].

EXPERIMENTAL SECTION

Unless otherwise mentioned, materials and methods are as previously described[1-3]. Silica gel chromatography was carried out on Kieselgel 60H (Merck 7736) by the short column method[20]. Typically 1-2 g of product dissolved in 10-20 ml of chloroform/0.1% pyridine was applied to 35 g silica in a column fitted with a flat sintered glass disc (diameter 60 mm) and eluted under slight nitrogen pressure with ethanol/chloroform containing 0.1% pyridine. The silica was used for one separation only. Ion exchange h.p.l.c. was carried out as previously described[2] except that buffer A was 1 mM potassium phosphate (pH 6.2)/30% ethanol and buffer B was 0.3 M potassium

phosphate (pH 6.2)/30% ethanol. Columns of Partisil 10 SAX were operated at 50-60° at flow rates of 2 ml min^{-1} (analytical) or 7 ml min^{-1} (preparative). Reverse phase chromatography was carried out on μ-Bondapak C18 (Waters) at ambient temperature as previously described[2,3]. Samples were warmed to 50° before injection. Buffer A was 0.1 M NH_4OAc, buffer B was 0.1 M NH_4OAc/CH_3CN (2:8).

Preparation of barium 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-p-chlorophosphates

Triazole (5.5 g, 80 mmole), recrystallised from anhydrous dioxan, was coevaporated twice with anhydrous pyridine and finally dissolved in pyridine (50 ml). p-Chlorophenylphosphodichloridate (3.7 ml, 18.5. mmole) was added and the mixture left at room temperature for 15 min. Meanwhile in a separate flask the 5'-O-dimethoxytrityldeoxynucleoside derivative of dT, dbzA, dibG or dbzC (10 mmole) was coevaporated three times with anhydrous pyridine leaving a final volume of 100 ml. To this was added the phosphorylating mixture and after 10 min the solution was added slowly to vigorously stirred water (500 ml). A clear solution soon resulted and after 30 min this was poured slowly into a vigorously stirred, ice-cold solution of barium chloride (20 g of the dihydrate in 2 ℓ of water). The mixture was stirred for 30 min. If a filterable precipitate had not been achieved the mixture was warmed to 30° with continual stirring and once more cooled to 0°. The precipitate was collected in a coarsely sintered glass funnel using gravity or very slight water pump pressure. After washing with a little iced water the precipitate was dried to constant weight over P_2O_5. Yields 80-90%. The product may still contain several moles of water.

General procedure for preparation of fully protected dimers (1.5 mmole scale)

(a) Preparation of the hydroxyl component. The barium 5'-O-$(MeO)_2$Tr-deoxynucleoside-3'-p-chlorophenylphosphate (6.6 mmole) was coevaporated three times with pyridine to give a final volume of 65 ml. 3-hydroxypropionitrile (Aldrich, stored over mol. sieve 4A)(2.2 ml, 32.2 mmole) was added followed by mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) (3.46 g, 13 mmole). After 1 h at room temperature silica gel tlc in 10% ethanol/chloroform showed almost complete conversion to a spot Rf 0.5-0.7. Ethyl acetate (450 ml) was added and the mixture washed with 1 M $NaHCO_3$ solution (3 x 150 ml) and then saturated sodium chloride solution (150 ml). The organic phase (and any necessary backwashings) was evaporated to an oil with pyridine and then coevaporated twice with toluene. The product was dissolved in chloroform (50 ml) and cooled in ice. A precooled solution of trichloroacetic acid (3.19

g, 19.5 mmole) in chloroform (50 ml) was added and the dark orange solution left at 0° (the reaction is followed by tlc in 10% ethanol/chloroform and is usually complete within 20 min). Pyridine (10 ml) was added and the solution diluted with chloroform (400 ml) and washed with 0.1 M $NaHCO_3$ solution (3 x 150 ml) followed by saturated sodium chloride solution (150 ml). The organic phase (and any necessary backwashings) was evaporated to an oil in the presence of pyridine and divided into four portions as pyridine solutions.

(b) Preparation of the dimer. To one of the four portions of 'hydroxyl component' (ca. 1.5 mmole) was added the appropriate barium 5'-O-$(MeO)_2$Tr-deoxynucleoside-3'-O-p-chlorophenylphosphate (2 mmole) and the mixture coevaporated three times with pyridine to give a final volume of 20 ml. MSNT (1.06 g, 4 mmole) was added and the solution left at room temperature for 1 h. Silica gel tlc in 10% ethanol/chloroform showed complete conversion of the hydroxyl component to higher Rf spot (or spots). Water (10 ml) was added and after 20-30 min chloroform (200 ml) was added and the mixture washed with 0.1 M $NaHCO_3$ solution (3 x 150 ml) followed by saturated sodium chloride solution (150 ml). On occasion emulsions were formed which were separated by centrifugation. The organic phase (and any necessary backwashings) was evaporated to an oil with pyridine, diluted with chloroform to ca. 15 ml and chromatographed on silica gel H (35 g) by the short column method. The column was eluted with 300 ml of chloroform/0.1% pyridine followed by a % of ethanol in chloroform/0.1% pyridine (see below) under ca. 5 psi pressure of nitrogen. The eluate was monitored by tlc and pure fractions pooled, evaporated to an oil with pyridine, dissolved in chloroform and product precipitated with ether/pentane (1:2). Yields 54-84% (based on hydroxyl component). Percentage of ethanol required in column elution: TT,TA-2.5%; TC,CC,CA,CT,AC,AA,AT-3%; TG,CG,GT,GA,GC-4%; AG,GG-5%.

Removal of cyanoethanol groups from dimers

The dimer (75-80 µmole) was dissolved in triethylamine/acetonitrile (1:1, 2 ml) in a 10 ml flask. The reaction was followed by tlc in 10% ethanol/chloroform and was usually complete within 1 h to give one spot on the base-line. The solution was evaporated to an oil under high vacuum, dissolved in 2-3 ml chloroform/0.1% pyridine and product precipitated by dropwise addition to 200 ml anhydrous diethyl ether vigorously stirred in a centrifuge tube. The precipitate was separated by centrifugation and decantation, washed with 2 x 100 ml ether and dried in vacuo. Yields 90-95%.

Oligonucleotide assembly

The resin (14 µmole functionality) contained in a glass reaction

vessel[1,2], is swollen in pyridine overnight and washed with (1) 5 x pyridine, 2 min, (2) 1 x 10% phenylisocyanate/pyridine, 30 min, (3) 5 x pyridine, 2 min, followed by the appropriate number of synthetic coupling cycles (Table 2) using a modified Beckman 990B solid phase peptide synthesiser[1-3]. Delivery volume per wash is 5-6 ml.

Deprotection and cleavage from the resin

The resin is shaken with 0.3 M tetramethylguanidinium p-nitrobenzaldoximate in dioxan/water (1:1) (5 ml) for 60 h, the liquid decanted and resin washed with dioxan/water (3 x 5 ml). The decantate and washings are carefully neutralised with acetic acid, washed once with chloroform and the aqueous phase evaporated to dryness. The residue is treated with concentrated ammonia (10 ml) at 50° for 5 h in a sealed flask, evaporated to dryness and treated with acetic acid/water (4:1, 10 ml) for 30 min. The solution was washed three times with diethyl ether and evaporated to dryness. Product was dissolved in water ready for h.p.l.c.

Purification of oligonucleotides

1. d(G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T). Ion exchange: 89% injected in eight portions, 60°, 4' 30% B, 50' 30-90% B; desalted on Biogel P2; Reverse phase in

TABLE 2

Step	Reagent or Solvent	Time of Shaking (min)	No. of Operations
1	$CHCl_3$	2	10
2	$CHCl_3$	5	3
3	10% TCA/$CHCl_3$	2	2
4	DMF	2	1
5	$CHCl_3$	2	5
6	DMF	2	5
7	Pyridine	2	10
8	Coupling Mixture[/]	90	1
9	Pyridine	2	5

/ The deoxynucleoside- or oligonucleotide-3'-p-chlorophenylphosphate (70 μmole) is dried by coevaporation three times with anhydrous pyridine to a final volume of 1.5 ml. MSNT (210 μmole) is added and the resultant solution quickly added to the resin by means of a Pasteur pipette.

seven portions, 4' 8% B, 45' 8-15% B. Yield: 8.6 A_{260} units, 0.045 μmole - see Table 1.

2. d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C). Ion exchange: eight portions, 65°, 4' 40% B, 45' 40-80% B; desalted on Biogel P2; Reverse phase in seven portions, 4' 8%B, 45' 8-15% B. Yield: 53 A_{260} units, 0.263 μmole - see Table 1.

3. d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T). Ion exchange: eight portions, 54°, 4' 30% B, 30' 30-65% B (example shown: 4' 0% B, 45' 0-90% B); desalted on Biogel P2; Reverse phase: nine portions, 35°, 4' 10% B, 40' 10-13% B. Yield: 88 A_{260} units, 0.465 μmole - see Table 1.

4. d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T). Ion exchange: ten portions, 50°, 4' 30% B, 50' 30-90% B; desalted on Biogel P2; Reverse phase, ten portions, 4' 9% B, 45' 9-13% B. Yield: 137 A_{260} units, 1.280 μmole - see Table 1.

^{32}P-Labelling and sequencing

The four heptadecamers were ^{32}P-labelled using γ-^{32}P-ATP and T4 polynucleotide kinase under standard conditions and run on a 20% polyacrylamide gel (0.3 mm) in the presence of 7 M urea and 0.1 M Tris-Borate (pH 8.3), 2 mM EDTA (Fig. 8). All showed single bands which were eluted in water and sequenced by wandering spot analysis. All showed the expected pattern of spots[34].

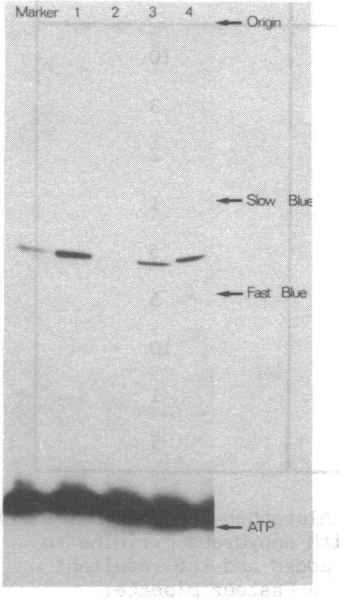


Figure 8

20% polyacrylamide gel of reaction mixture from ^{32}P-labelling of:

1) d(G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T),
2) d(C-A-G-G-A-A-A-C-A-G-C-T-A-T-G-A-C),
3) d(G-T-A-T-T-T-T-T-A-C-A-A-C-A-A-T-T),
4) d(G-C-A-G-C-C-T-G-A-G-A-G-T-A-G-C-T),
and MARKER: d(T-C-C-C-A-G-T-C-A-C-G-A-C-G-T-T-G)

ACKNOWLEDGEMENTS

The authors would like to thank Drs. R.C. Sheppard and H. Matthes for advice and encouragement and Drs. G. Winter, S. Anderson and F. Sanger for help in design, computer analysis, and testing of primers for sequencing with M13mp2. The award of a Research Fellowship to R.C.T. from I.C.I. Pharmaceuticals Division is gratefully appreciated.

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REFERENCES AND NOTES

1. Gait, M.J. and Sheppard, R.C. (1977) Nucleic Acids Research 4, 1135-1158 and 4391-4410; idem. (1979) ibid, 6, 1259-1268.
2. Gait, M.J., Singh, M., Sheppard, R.C., Edge, M.D., Greene, A.R., Heathcliffe, G.R., Atkinson, T.C., Newton, C.R. and Markham, A.F. (1980) Nucleic Acids Research 8, 1081-1096.
3. Gait, M.J., Popov, S.G., Singh, M. and Titmas, R.C. (1980) Nucleic Acids Research Symposium Series No. 7, 243-257.
4. Miyoshi, K., Huang, T. and Itakura, K. (1980) Nucleic Acids Research 8, 5491-5505.
5. Norris, K.E., Norris, F. and Brunfeldt, K. (1980) Nucleic Acids Research Symposium Series No. 7, 233-241.
6. Crea, R. and Horn, T. (1980) Nucleic Acids Research 8, 2331-2348; Horn, T., Vassar, M.P., Struble, M.E. and Crea, R. (1980) Nucleic Acids Research Symposium Series No. 7, 225-232.
7. Miyoshi, K., Arentzen, R., Huang, T. and Itakura, K. (1980) Nucleic Acids Research 8, 5507-5517.
8. Miyoshi, K. and Itakura, K. (1980) Nucleic Acids Research Symposium Series No. 7, 281-291.
9. Caruthers, M.H., Beaucage, S.L., Efcavitch, J.W., Matteucci, M.D. and Stabinsky, Y. (1980) Nucleic Acids Research Symposium Series No. 7, 215-223.
10. Markham, A.F., Edge, M.D., Atkinson, T.C., Greene, A.R., Heathcliffe, G.R., Newton, C.R. and Scanlon, D. (1980) Nucleic Acids Research 8, 5193-5205.
11. Hong, G.F. (1981) Bioscience Reports, in the press.
12. Winter, G. and Fields, S., in preparation.
13. The resin is commercially available: U.C.B. Bioproducts, 68 Rue Berkendael, B-1060, Brussels, Belgium.
14. Vega Biochemicals, Tucson, Arizona, U.S.A.; Radiometer A/S, Copenhagen, Denmark.
15. Gough, G.R., Collier, K.J., Weith, H.L. and Gilham, P.T. (1979) Nucleic Acids Research 7, 1955-1964.
16. Broka, C., Hozumi, T., Arentzen, R. and Itakura, K. (1980) Nucleic Acids Research 8, 5461-5471.
17. We have already used these conditions for the preparation of fully protected deoxynucleoside-3'-phosphotriesters (Ref. 3).
18. Jones, S.S., Rayner, B., Reese, C.B., Ubasawa, A. and Ubasawa, M. (1980) Tetrahedron 36, 3075-3085.
19. Reese, C.B. and Ubasawa, A. (1980) Tetrahedron Letters 21, 2265-2268.
20. Hunt, B.J. and Rigby, W. (1967) Chem. and Ind., 1868-1869.
21. Gait, M.J. and Popov, S. (1980) Tetrahedron Letters 21, 2841-2842.
22. Reese, C.B., Titmas, R.C. and Yau, L. (1978) Tetrahedron Letters, 2727-2730.

23. Anderson, S., Gait, M.J., Mayol, L. and Young, I.G. (1980) Nucleic Acids Research 8, 1731-1743.
24. Gait, M.J., Popov, S.G. and Singh, M., unpublished results.
25. Anderson, S., unpublished results.
26. Van Wezenbeek, P., Hulsebos, Th. and Schoenmakers, J.G.G. (1980) Gene 11, 129-148.
27. Staden, R. (1977) Nucleic Acids Research 4, 4037-4051.
28. Synthesis of a 19-mer has been described identical to ours but with two extra bases on the 5' end: Narang, S.A. et al. (1980) Nucleic Acids Research Symposium Series No. 7, 377-385.
29. Goelet, P., unpublished results.
30. Duckworth, M. L., unpublished results.
31. Chattopadhyaya, J.B. and Reese, C.B. (1980) Nucleic Acids Research 8, 2039-2053; Hsiung, H.M., Sung, W.L., Brousseau, R., Wu, R. and Narang, S.A. (1980) Nucleic Acids Research 8, 5753-5765.
32. For a 3'-terminal A residue attached to the resin 12% depurination is observed in 1 x 2 min treatment with 10% trichloroacetic acid in chloroform at room temperature, but only 4% depurination at 0° in 1 x 4 min treatment (cf. Ref. 4).
33. Matthes, H., unpublished results.
34. Sequencing data was provided to the referees and is available on request.