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Solid phase phosphotriester synthesis of large oligodeoxyribonucleotides on a polyamide support

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ABSTRACT

Phosphotriester solid phase methodology on a polyamide support [(1980) Nucleic Acids Research, 8, 1081-1096] has been extended for the rapid synthesis of the tetradecanucleotide, d(AGTTGTTTGTAGTT), the octadecanucleotide, d(GTGGGTTTGGGGCAGGTC), and the heneicosanucleotide, d(GTGCTCTTATCCTCTTGGCTC). Thus, oligodeoxyribonucleotides comparable in size to those obtainable by solution synthesis are readily accessible using solid phase techniques. An approach to the purification of the synthetic octadecanucleotide without recourse to high performance liquid chromatography is described.

INTRODUCTION

Recent months have seen a major renaissance of interest in the synthesis of oligodeoxyribonucleotides on solid supports (Ref. 1-6). This development owes much to studies by Gait and Sheppard[7] who have demonstrated in a series of publications that oligodeoxyribonucleotides can be assembled on polydimethylacrylamide resins using a phosphodiester approach. The high polarity of growing chains in phosphodiester synthesis and the consequent need for polar solvents have been invoked as reasons for the use of such materials[7,8]. Other supports which are less permeable to polar solvents have not proved so valuable in solid phase phosphodiester synthesis (but see Ref. 9).

The situation with solid phase phosphotriester synthesis is however less well-defined. Although the original publications which showed that such an approach was practical exploited polyamide resins[1,3] a number of recent reports suggest that a variety of solid supports will be useful for phosphotriester assembly of oligodeoxyribonucleotides, at least to the 13-mer level [4,5,6]. Thus the solid support of choice remains to be established.

In this publication we demonstrate that oligodeoxyribonucleotides up to 21-units long can be assembled on a polydimethylacrylamide support by ten cycles of dimer additions. Thus, this type of support merits consideration for general use in phosphotriester oligonucleotide assembly. In the synthesis of the octadecanucleotide d(GTGGGTTTGGGGCAGGTC) we have observed that oligo-

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nucleotides above the octamer [d(GGCAGGTC)] could not be resolved by ion exchange high performance liquid chromatography (hplc) in 5% ethanol systems. A method of fractionation is described to overcome this difficulty using polyacrylamide gel electrophoresis in 7M urea after ^{32}P-labelling the mixture of oligonucleotides cleaved from resin after each chain assembly stage.

DISCUSSION

Methodology for the synthesis of functionalised polydimethylacrylamide resin and the rationale for the choice of resin-oligonucleotide linkage have been described previously[1]. In brief, beaded resin was obtained by the co-polymerisation of acryloylsarcosine methyl ester with dimethylacrylamide and ethylene bisacrylamide[10]. Functionalisation involved reaction with ethylene diamine followed by the symmetrical anhydride of t-butyloxycarbonylglycine. After assaying the resin to determine the level of glycine functionality (in the examples reported here 0.28 mmole g^{-1}) the t-butyloxycarbonyl groups were removed and the 3'-residue of the target oligonucleotide introduced by reaction with the symmetrical anhydride of the relevant 5'-O-dimethoxytrityl-2'-deoxy-nucleoside-3'-O-succinate (Fig. 1). Although in the examples described here only those resins where X=T or bzC have been used, resins where X=bzA or ibG have been prepared in identical fashion and function equally well in oligo-nucleotide assemblies (Ref. 2 and Markham A.F., et al, unpublished results).

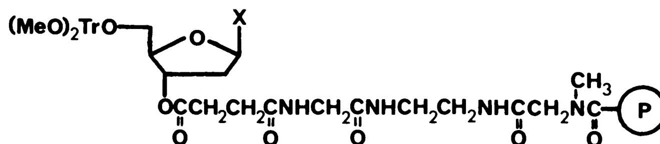

Fig.1 (X = T, bzC, bzA, ibG) **1**

4,4'-Dimethoxytrityl and glycine content of resin samples were assayed as described[1] then a precautionary treatment with phenyl isocyanate performed[7] although incorporations of the 3'-O-succinate residues were essentially quantitative (see Experimental Section).

For the oligonucleotide assemblies described here (Fig. 2) mononucleotides [$(MeO)_2$TrdX-(ClPh)] and dinucleotide blocks [$(MeO)_2$TrdX$\overset{o}{-}$X-(ClPh)] as the triethylammonium salts were prepared by standard procedures[1]. In the synthesis of the heneicosanucleotide, five-fold excesses of nucleotide component with respect to resin functionality were used throughout. In the tetradeca-nucleotide and octadecanucleotide syntheses, the excess of nucleotide com-ponent at each stage was reduced to four-fold. In all cases two equivalents

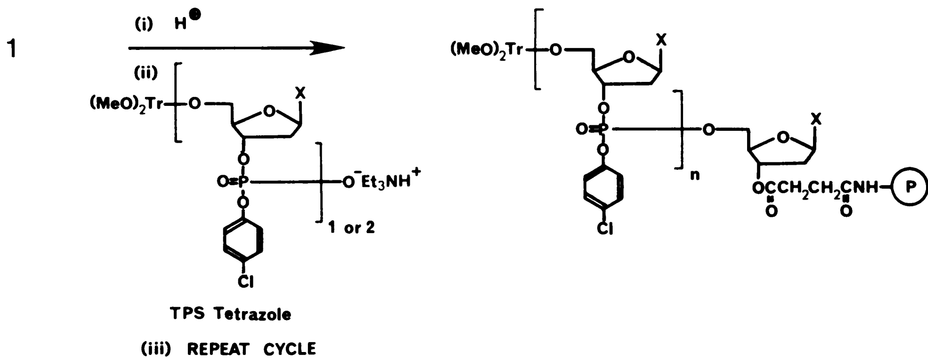

Fig. 2. Oligonucleotide Synthesis Method.

The Tetradecanucleotide : AG TT GT TT GT AG T T-Resin

The Octadecanucleotide : GT GG GT TT GG GG CA GG T C-Resin

The Heneicosanucleotide : GT GC TC TT AT CC TC TT GG CT C-Resin

Fig. 3. Schematic Representation of Oligonucleotide Assembly Strategies.

of triisopropylbenzenesulphonyl tetrazole with respect to nucleotide component were used.

Fig. 3 illustrates the strategy for the synthesis of the three oligonucleotides. 4,4'-Dimethoxytrityl and glycine contents of resins were assayed after every synthetic cycle (see Experimental Section). At the hexamer levels and above in the assembly of the tetradecanucleotide and octadecanucleotide and at the pentamer level and above in the assembly of the heneicosanucleotide, the product oligonucleotide mixtures were cleaved from the resin, fully deprotected, and examined by ion-exchange hplc on Partisil 10SAX. The series of chromatographic patterns obtained in the assembly of d(AGTTGTTTGTAGTT) is shown in Fig. 4 and the yields at various stages are presented in Table 1. In the light of a series of chromatograms of this type, identification of the final 14-mer product peak is unequivocal. The isolated yield of tetradecanucleotide was 2.0%, equivalent to an average 58% yield per coupling step. Figs. 5 and 6 illustrate the similar series of chromatographic patterns obtained in the assembly of d(GTGCTCTTATCCTCTTGGCTC). Other criteria are presented in Table 2. The relatively poor reaction going from trimer to pentamer in this synthesis (as judged by the amount of 4,4'-dimethoxytrityl group incorporated) is reflected in the appearance of a second

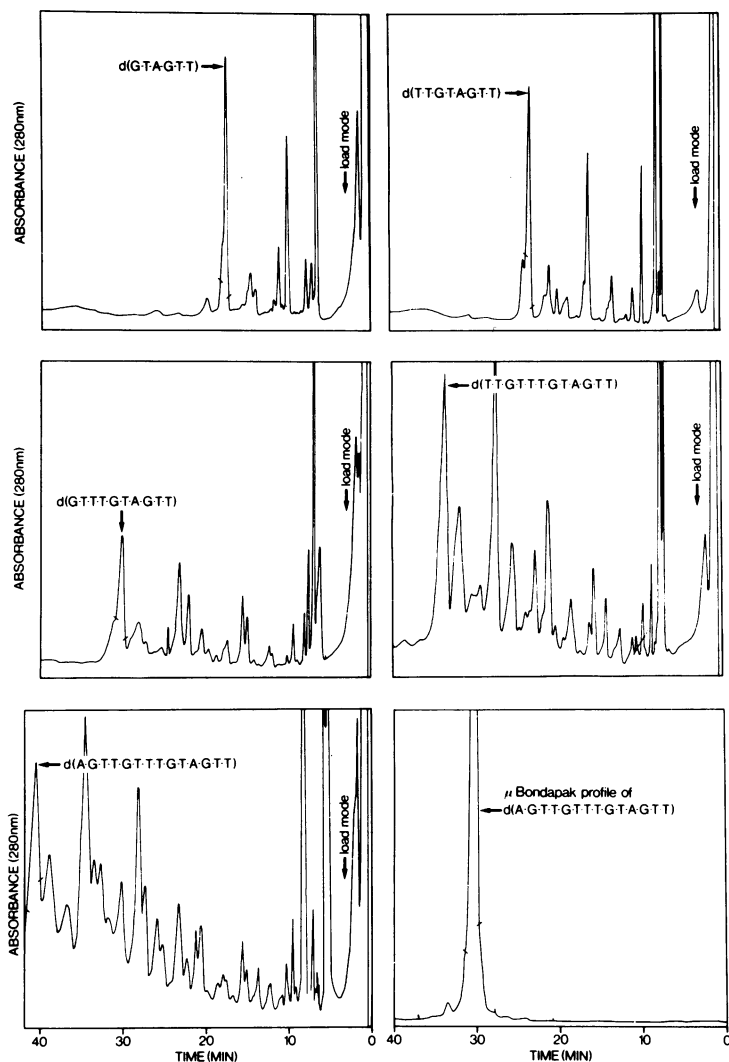

Fig. 4. Hplc profiles at various stages in the synthesis of d(AGTTGTTTGTAGTT).

major peak in the chromatographic profile which we attribute to unreacted trimer. As no procedure is included to block unreacted 5'-hydroxyl groups after coupling cycles, both these chains are elongated through further cycles of assembly. Although the yield of 21-mer is somewhat reduced (0.4%, 58% yield per coupling step, Table 2) identification of the required product peak is again straightforward. In all cases the deprotection procedure entailed treatment with: (1) 0.3M tetramethylguanidinium syn-p-nitrobenzaldoximate in

TABLE 1

Sequence	Glycine µmole/mg	Trityl A_{498}/mg	Trityl µmole/mg	Product Isolated A_{260}/mg	Product Isolated µmole/mg	Overall yield %	% Recovery (µ-Bondapak C_{18})	λ max (nm)
d(GTAGTT)	0.278[a]	8.58	0.119	1.738	0.0269	9.7	-[b]	258
d(TTGTAGTT)	0.203	13.66	0.190	1.152	0.0141	6.9	72.9	259
d(GTTTGTAGTT)	0.282[a]	6.38	0.089	1.033	0.0101	(3.6)[a]	62.1	258
d(TTGTTTGTAGTT)	0.153	8.33	0.116	1.217	0.0102	6.7	53.3	258
d(AGTTGTTTGTAGTT)	0.197	7.24	0.101	0.587	0.0040	2.0	47.0	258

[a] Values measured on <1mg quantities of resin and hence subject to error.

[b] Overall yield quoted is after purification on µ-Bondapak.

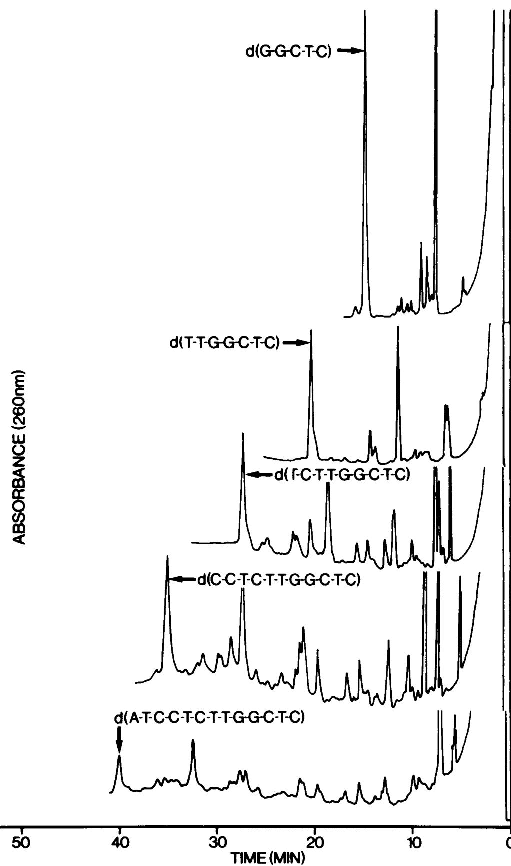

Fig. 5. Ion-exchange hplc profiles to the 13-mer stage in the synthesis of d(GTGCTCTTATCCTCTTGGCTC).

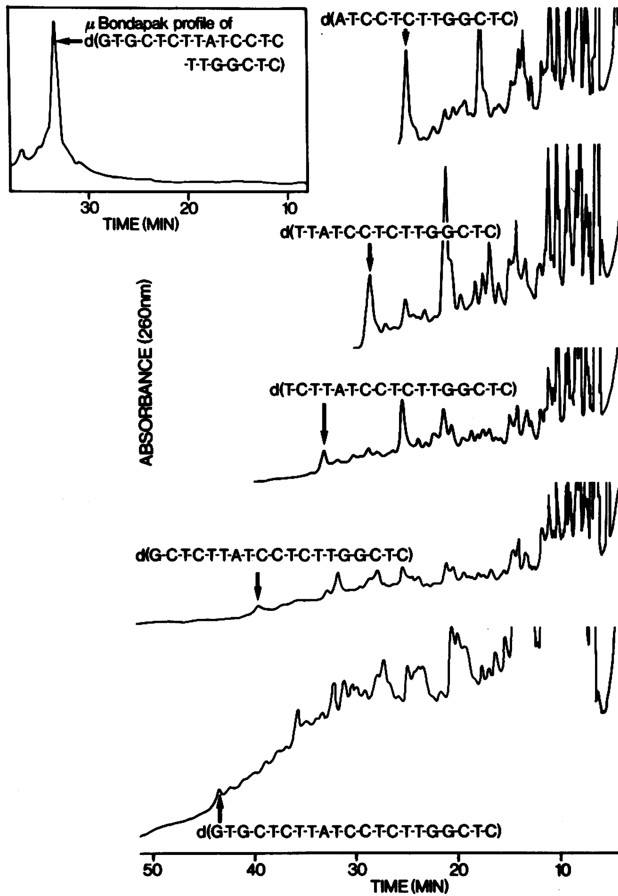

Fig. 6. Ion-exchange hplc profiles for the 13-mer to 21-mer in the synthesis of d(GTGCTCTTATCCTCTTGGCTC).

TABLE 2

Sequence	Glycine µmole/mg	Trityl A_{498}/mg	Trityl µmole/mg	Product Isolated A_{260}/mg	Product Isolated µmole/mg	Overall yield %	% Recovery (µ-Bondapak C_{18})	λ max (nm)
d(C)	0.228	18.4	0.257	-	-	-	-	-
d(CTC)	0.191	12.9	0.180	-	-	-	-	-
d(GGCTC)	0.201	7.7	0.107	1.869	0.0404	20.1	-	260
d(TTGGCTC)	0.177	12.2	0 170	1.132	0.0178	10.1	-	262
d(TCTTGGCTC)	0.119	9.2	0.128	0.742	0.0093	7.8	62.4	264
d(CCTCTTGGCTC)	0.138	6.7	0.093	0.530	0.0056	4.1	62.3	264
d(ATCCTCTTGGCTC)	0.148	6.5	0.091	0.419	0.0035	2.4	44.2	263
d(TTATCCTCTTGGCTC)	0.154	4.0	0.056	0.298	0.0022	1.4	40.3	263
d(TCTTATCCTCTTGGCTC)	0.142	4.3	0.060	0.188	0.0012	0.9	25.6	263
d(GCTCTTATCCTCTTGGCTC)	0.143	3.9	0.054	0.179	0.0010	0.7	14.2	263
d(GTGCTCTTATCCTCTTGGCTC)	0.141	3.2	0.045	0.118	0.0006	0.4	13.1	262

dioxan/water (1:1), 48 hr[12]; (2) concentrated ammonia, 50°, 5h; and (3) 80% aqueous acetic acid, room temperature, 30 min. as previously described [1].

Previously[1] we have measured the purity of oligonucleotide samples from ion-exchange hplc by comparison of the peak size of the product on μ-Bondapak C_{18} hplc with that of other impurities present in the reverse phase chromatographic profile. Here we have taken known amounts of product oligonucleotides after ion-exchange hplc and desalting then isolated the product peaks from μ-Bondapak C_{18} hplc After lyophilisation the A_{260} recovered in the product peaks was measured. Comparison of the actual μ-Bondapak profiles (see Fig. 4 and Fig. 6, μ-Bondapak profiles of the other intermediate oligonucleotides (data not shown) all suggest >90% purity) with the amounts of material recovered (Tables 1 and 2) suggest that losses of oligonucleotides on μ-Bondapak C_{18} columns are considerable and may increase with increasing chain length. This effect has been noted previously[13] and is under continuing investigation. Products from μ-Bondapak hplc were ^{32}P-labelled by standard methods. They were homogeneous on 20% acrylamide gel electrophoresis and their sequences were confirmed by chemical degradation[14] (see Appendix).

The value of careful examination of products after each cycle of nucleotide addition is apparent in the synthesis of d(GTGGGTTTGGGGCAGGTC) (Fig. 7). Thus, although a satisfactory yield of product was obtained at the octamer level, no product peak eluting in the position expected for the decamer d(GGGGCAGGTC) was observed. Similar chromatographic patterns were obtained at the 12-mer, 14-mer, 16-mer and 18-mer levels (data not shown). However, analysis of 4,4'-dimethoxytrityl incorporation at these various stages suggested that coupling reactions were proceeding in reasonable yield (see Experimental Section). Aliquots of the mixtures of oligonucleotides liberated from the resin at various stages of assembly were therefore labelled with γ-[^{32}P]-ATP and polynucleotide kinase and the resulting solutions examined on 20% polyacrylamide gel electrophoresis in the presence of 7M urea (Fig. 8). A sample of the intermediate octanucleotide was purified by ion-exchange hplc then μ-Bondapak C_{18} hplc (Fig. 7) and similarly ^{32}P-labelled as a size marker. A number of other ^{32}P-labelled oligonucleotide mixtures are also included. Given the mobility of the octanucleotide (Fig. 8, lane D) the bands corresponding to 10-mer (lane F, arrowed), 12-mer (lane G), 14-mer (lane H), 16-mer (lane I) and 18-mer (lane J) could be identified. These assignments were confirmed for the 8-mer, 14-mer and 18-mer by eluting the oligonucleotide and sequencing[14] (see Appendix). Thus microgram quantities

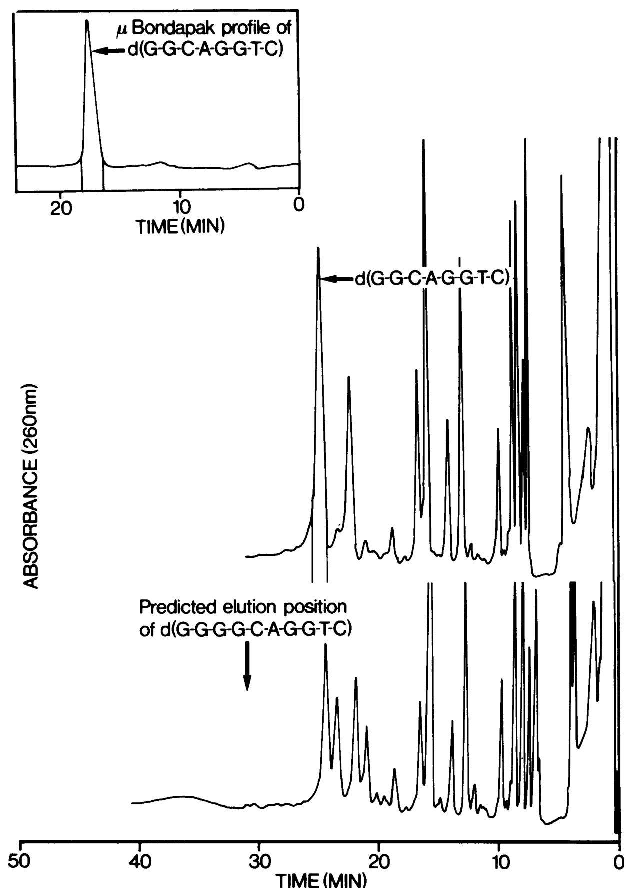

Fig. 7. Ion-exchange hplc profiles at the 8-mer and 10-mer stages in the synthesis of d(GTGGGTTTGGGGCAGGTC).

of ^{32}P-labelled oligonucleotides can be obtained directly by this approach. This type of behaviour on Partisil 10SAX in 5% ethanol of oligodeoxynucleotides containing four or more consecutive guanine residues seems to be general (unpublished results).

In a series of related studies[2], Gait et al have recently shown that a number of purine-rich oligodeoxyribonucleotides of 10-12 units could be prepared using this type of synthetic approach. Use of trichloroacetic acid as the reagent for removal of terminal 4,4'-dimethoxytrityl groups was shown to significantly reduce depurination during chain assembly. Given these results, those previously described[1] and those described herein, it appears

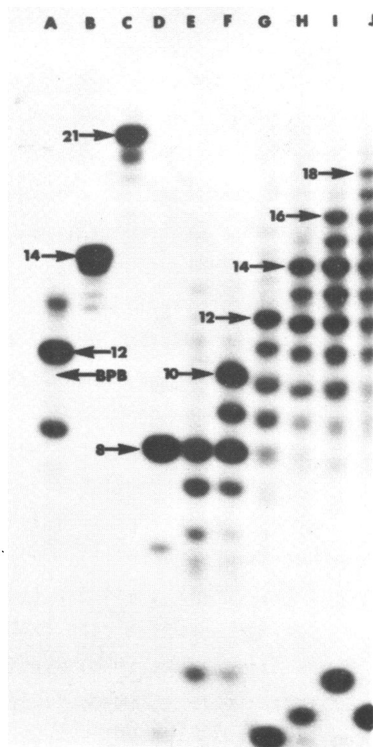

Fig. 8. Autoradiograph of 20% polyacrylamide gel electrophoretogram after ^{32}P-labelling of the oligonucletide mixtures at various stages in the synthesis of d(GTGGGTTTGGGGCAGGTC). Size Markers: Lane A impure d(CTCCCACCATTT); Lane B, d(AGTTGTTTGTAGTT) before μ-Bondapak hplc; Lane C, the 21-mer before μ-Bondapak hplc. Lane D, d(GGCAGGTC); Lane E, 8-mer mixture; Lane F, 10-mer mixture; Lane G, 12-mer mixture; Lane H, 14-mer mixture; Lane I, 16-mer mixture; Lane J, 18-mer mixture. BPB, bromophenol blue marker.

that oligodeoxyribonucleotides with any base sequence and of lengths at least as great as have been obtained by synthesis in solution[15] are rapidly accessible by solid phase approaches. Exploitation of such oligonucleotides to examine problems in molecular biology will be described elsewhere.

EXPERIMENTAL SECTION

Unless otherwise mentioned materials and methods are as previously described[1]. Protected nucleosides and dinucleotide phosphotriesters with 3'-p-chlorophenyl phosphodiester residues (as the triethylammonium salts) were prepared as described[1]. Hplc was carried out using a Pye Unicam system (LC-XPD pump, LC-XP gradient programmer, LC-UV detector and PM 8252 dual-pen recorder). Partisil 10SAX columns (PXS, Whatman) were eluted with gradients of potassium phosphate, pH6.8 in 5% ethanol from 1mM (buffer A) to 0.4M (buffer B) at ambient temperature. μ-Bondapak C_{18} reverse phase columns (Waters) were eluted with gradients of acetonitrile in 0.1M ammonium acetate from 0.1M ammonium acetate (buffer C) to 1:1 acetonitrile/ 0.1M ammonium acetate (buffer D). Preparation of functionalised resins (Fig. 1) and deprotection after oligonucleotide assembly were as already described[1]. Each cycle of nucleotide addition was as given in Table 1, reference 1. 4,4'-Dimethoxytrityl content was measured in 60% perchloric acid/ethanol (3:2) and glycine assayed as described[1].

The Tetradecanucleotide, d(AGTTGTTTGTAGTT). 5'-O-Dimethoxytrityl-thymidine-3'-O-succinamido resin (140mg, trityl 0.265 mmole g^{-1}; trityl/gly 1.11) was swollen by shaking in DMF (each operation involved a solvent volume of ca. 6ml). It was then treated with (1) 5 x pyridine, 2 min., (2) 1 x 10% phenylisocyanate/pyridine, 30 min., (3) 5 x pyridine, 2 min., and subjected to one cycle of nucleotide addition with $(MeO)_2$TrdT-(ClPh) followed by six cycles using the dinucleotide blocks $(MeO)_2$TrdbzA$\overset{o}{-}$ibG-(ClPh), $(MeO)_2$TrdibG$\overset{o}{-}$T-(ClPh), $(MeO)_2$TrdT$\overset{o}{-}$T-(ClPh), $(MeO)_2$TrdibG$\overset{o}{-}$T-(ClPh), $(MeO)_2$TrdT$\overset{o}{-}$T-(ClPh) and $(MeO)_2$TrdbzA$\overset{o}{-}$ibG-(ClPh) respectively (Fig. 3). After three cycles of addition and at each subsequent stage (i.e. at the hexamer level and above) resin samples were treated under the standard deprotection conditions and the liberated mixture of oligonucleotides was examined by ion-exchange hplc (Fig. 4). Elution was with gradients of 0-60% buffer B, 40 min. The yields of product oligonucleotides at the various stages of assembly are given in Table 1. For the tetradecanucleotide, $0.587A_{260}$ units were obtained per mg of resin deprotected. After μ-Bondapak C_{18} hplc (eluting with 10% buffer D, 2 min., 10-25% buffer D, 40 min.) the product peak (Fig. 4) was sequenced after standard ^{32}P-labelling (see Appendix).

The Octadecanucleotide, d(GTGGGTTTGGGGCAGGTC). 5'-O-Dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine-3'-O-succinamido resin (180mg, trityl 0.259 mmole g^{-1}; trityl/gly 1.11) was swollen in DMF (ca. 6ml), treated with (1) 5 x

pyridine, 2 min., (2) 1 x 10% phenylisocyanate/pyridine, 30 min., (3) 5 x pyridine, 2 min., and subjected to nine cycles of nucleotide addition (Fig. 3, 4-fold excess of nucleotide components). After four cycles of addition (i.e. the octamer level) and at each subsequent stage resin samples were treated under the standard deprotection conditions and the liberated mixtures of oligonucleotides were examined by ion-exchange hplc (Fig. 7). Elution was with gradients of 0-60% buffer B, 40 min. After desalting, the octanucleotide was chromatographed on μ-Bondapak C_{18} hplc (eluting with 10% buffer D, 2 min., 10-25% buffer D, 40 min.) to yield 1.075 A_{260} per mg of resin deprotected. (λ_{max} 252nm, see Table 1 for isolated yield of a comparable octamer). This product peak was ^{32}P-labelled as previously. Trityl values at A_{498} mg^{-1} of resin were 6.52 (8-mer), 5.63 (10-mer), 6.24 (12-mer), 6.70 (14-mer), 7.13 (16-mer)and 5.09 (18-mer). Aliquots of the reaction mixtures were ^{32}P-labelled as follows: after standard deprotection and evaporation, residues were dissolved in water (2ml), extracted with chloroform (2 x 2ml) and ether (2ml) then evaporated to dryness. Samples were dissolved in water (5ml) and the volumes given in Table 3 taken from these solutions for labelling. After evaporation the volumes of labelling mixture given in Table 3 were added: the labelling mixture comprised γ-[^{32}P]-ATP (5200 Ci/mmole, lmCi/ml, 500μL dried), (Radiochemical Centre); H_2O (50μL); lmM spermidine (10μL); 0.2M dithiothreitol (10μL); 0.1M $MgCl_2$ (10μL); 0.5M Tris.HCl (pH9.5), lmM EDTA (10μL); polynucleotide kinase (New England Biolabs) (1800 units/ml, 14μL). Incubation was for 45 min. at 37°. Samples were evaporated, dissolved in 80% deionised formamide 50mM Tris-borate, pH8.3, lmM MEDTA containing 0.1 (wt/vol) marker dyes (5μL), heat denatured and loaded on a 20% polyacrylamide gel[14]. Bands identified by autoradiography were eluted and sequenced by standard techniques (Fig. 8 and see Appendix).

The Heneicosanucleotide, d(GTGCTCTTATCCTCTTGGCTC). Resin (0.1815g, 0.0508 mmole glycine) was derivatised to obtain 5'-O-dimethoxytritylcytidine-

TABLE 3

Assembly Stage	Weight Resin Deprotected (mg)	Volume of 5ml Solution Taken (μL)	Volume Labelling Mixture (μL)
8	6.68	1	3.5
10	12.41	1	3.5
12	16.08	1	3.5
14	12.83	1	3.5
16	17.60	1	5.0
18	30.91	1.2	5.0

3'-O-succinamido resin (trityl 0.257 mmole g^{-1}; trityl/gly 1.13). Ten cycles of nucleotide addition were carried out with 5-fold excesses of the appropriate dimer blocks (Fig. 3). After two cycles of addition and at each subsequent stage resin samples were treated under the standard deprotection conditions and the liberated mixture of oligonucleotides was examined by ion-exchange hplc (Fig. 5,6). Elution was with gradients of 0-50% buffer B, 40 min. (Fig. 5) or with 0-25% buffer B, 5 min., then 25-75% buffer B, 40 min., (Fig. 6). For the heneicosanucleotide, 0.118 A_{260} units were obtained per mg of resin deprotected. After µ-Bondapak C_{18} hplc (eluting with 10% buffer D, 6 min., 10-25% buffer D, 40 min.) the product peak (Fig. 6) was sequenced after standard ^{32}P-labelling (see Appendix).

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Appendix

Oligonucleotide sequencing results were supplied to the referees but are omitted from the text because of space limitation. The material is available on request.

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