Rapid synthesis of oligodeoxyribonucleotides IV. Improved solid phase synthesis of oligodeoxyribonucleotides through phosphotriester intermediates

M.J.Gait*, M.Singh and R.C.Sheppard
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

M.D.Edge, A.R.Greene, G.R.Heathcliffe, T.C.Atkinson, C.R.Newton and A.F.Markham*
I.C.I. Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Received 9 January 1980

ABSTRACT

A phosphotriester solid phase method on a polyamide support has been used to prepare oligodeoxyribonucleotides up to 12 units long. Compared to solid phase phosphodiester synthesis the new methodology is quicker, more flexible and gives 10-60-fold better overall yields.

INTRODUCTION

Previous papers in this series[1-3] have demonstrated the practical value of polydimethylacrylamide resins for the solid-phase synthesis of oligodeoxyribonucleotides. In these syntheses appropriate monomer units were coupled to the resin using a phosphodiester approach. Good coupling yields could be maintained provided that highly purified monomer units were used[3]. However, isolated yields of deprotected oligonucleotides decreased rapidly as a function of chain length owing to substantial accumulation of by-products. These we attribute mostly to the susceptibility of each phosphodiester bond to reaction with active phosphorylating agent during successive coupling steps[4].

For some time we have felt that a phosphotriester approach applied to our polyamide resins might hold distinct advantages in terms of overall yields, speed of synthesis and length of oligonucleotide attainable, but only recently has sufficiently reliable solution methodology emerged[5], particularly with regard to deprotection of phosphotriesters at the end of the synthesis[6]. Although a phosphotriester approach on solid phase has been attempted several times in the past[7], only the very recently described synthesis of oligothymidylates on a polyacryloylmorpholide resin has been at all encouraging[8]. We now describe the solid phase synthesis of the octanucleotides, d(T-C-T-G-G-T-T-T), d(C-C-T-C-C-T-G-C), d(T-T-C-C-C-A-C-C) and d(C-T-C-C-C-A-C-C) and the dodecanucleotides, d(C-T-C-C-C-A-C-C-A-T-T-T) and d(T-T-C-C-C-A-C-C-A-T-T-T), obtained in good overall isolated yields using a phosphotriester approach.

DISCUSSION

The Polymeric Support. The principles behind the choice of polydimethylacrylamide resins have been discussed at length[1,9]. Recently we introduced a new functional monomer, acryloylsarcosine methyl ester, which when copolymerised with dimethylacrylamide and ethylene bisacrylamide gave beaded resins in easily handlable form[10]. In peptide synthesis on polydimethylacrylamide resins it has been standard practice to incorporate a reference amino acid on the resin before starting chain assembly[11]. Yields may then be estimated by measurement of amino acid content of resin-bound peptides normalised to the value of the reference, hence eliminating errors associated with increase in resin weight. In oligonucleotide synthesis we now find that use of a reference amino acid allows easy measurement of overall yields of oligonucleotides. In addition functional groups (such as 4,4-dimethoxytrityl) can be assayed with respect to the amino acid content of the resin to give a useful guide to the efficiency of coupling reactions.

The new resin is functionalised by treatment with ethylene diamine at room temperature and the resultant amino groups then reacted with the symmetrical anhydride of t-butyloxycarbonylglycine in DMF (Fig. 1). After washing the resin (gly 0.28-0.35 mmole g^{-1}) may be dried and stored.

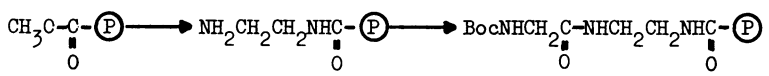
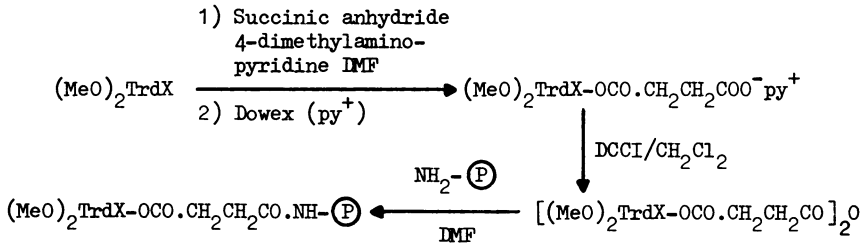


Fig. 1.

The Resin-Polymer Linkage. In our phosphodiester approach a reversible linkage was obtained by reaction of a suitably protected 2'-deoxynucleoside-5'-phosphate with a resin-bound β-hydroxyethylthiophenol derivative[1]. The resultant phosphodiester could be cleaved by oxidation to the corresponding sulphone followed by base-catalysed elimination. Whereas this linkage was stable indefinitely in the presence of pyridine an analogous 3'-phosphotriester linkage formed by coupling of the hydroxy-resin with $(MeO)_2$TrdT-(ClPh) was ca 30% cleaved in one week at room temperature in pyridine and was therefore judged unsuitable.

Among other base-labile linkages nucleoside derivatives have been attached to succinylated polystyrene resins as 5'-O-esters[12]. In line with our proposed chain extension procedure we wished to attach the first nucleoside residue via its 3' position[13]. To avoid the possibility of unsubstituted and potentially deleterious carboxylic acids[3] remaining on the resin appropriate 2'-deoxynucleoside derivatives were themselves succinylated and then coupled

to the resin. Accordingly $(MeO)_2$TrdT, $(MeO)_2$TrdbzA, $(MeO)_2$TrdbzC and $(MeO)_2$TrdibG were each treated with succinic anhydride in the presence of 4-dimethylaminopyridine in DMF for 20 h at room temperature and the corresponding 3'-O-succinates isolated as their pyridinium salts following preparative layer chromatography. In the presence of dicyclohexylcarbodiimide in dichloromethane the succinates readily formed their respective symmetrical anhydrides which, after removal of dicyclohexylurea, were reacted in DMF with amino groups on the resin liberated by acidic cleavage of t-butyloxycarbonyl protecting groups and neutralisation (Fig. 2). Completion of reaction was



[X = T, bzA, bzC or ibG]

Fig. 2.

judged by the ninhydrin test[14]. An advantage of this procedure is that the succinates are the only by-products remaining in solution and can be recovered for re-use. The linkage is stable in the presence of pyridine and under acidic conditions [5% benzene sulphonic acid in chloroform/methanol (7:3)] but is rapidly cleaved by aqueous sodium hydroxide or ammonia treatment.

Oligonucleotide Assembly. For the most part the phosphotriester approach as modified by Stawinski et al.[5] has been used. Basic units are $(MeO)_2$TrdT-(ClPh), $(MeO)_2$TrdbzA-(ClPh), $(MeO)_2$TrdbzC-(ClPh) and $(MeO)_2$TrdibG-(ClPh) and corresponding di- and trinucleotide blocks[15], which are coupled to the resin using triisopropylbenzenesulphonyltetrazole (Fig. 3). These reactions alter-

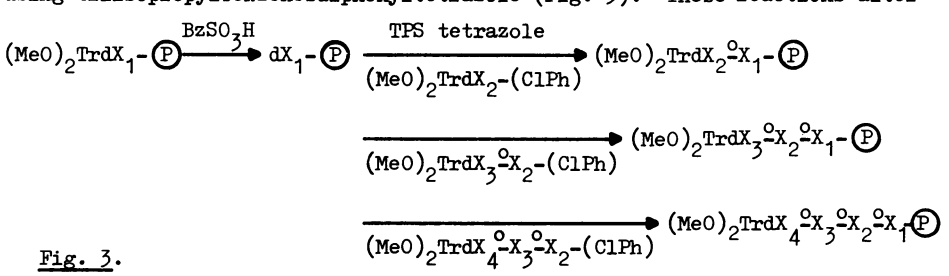


Fig. 3.

nate with terminal 5'-deprotection using 5% benzene sulphonic acid in chloroform/methanol (7:3)[16]. Each complete synthetic cycle can be carried out in 5-6 h using a bench-top manual solvent delivery system[1] or a modified Beckman 990B Solid Phase Peptide Synthesiser[2,17] using essentially similar operations (Table 1, Experimental Section). For oligonucleotides containing N-benzoyl adenine the minimum of acidic treatment is vital to prevent depurination. In the syntheses described below two 30s treatments followed by rapid resin washing were sufficient for complete deprotection without concomittant depurination, but the precise limits of this procedure in the case of adenine-rich oligonucleotides have yet to be determined. In contrast to the phosphodiester approach resin drying with phenyl isocyanate[1,2] is not necessary during synthesis, although a precautionary 30m treatment is given at the start of the synthesis.

Oligonucleotide Cleavage and Isolation. Aqueous ammonia has most commonly been used for cleavage of aryl groups from internucleotide linkages[8,18]. This procedure also liberates oligonucleotides from the resin and removes all base-protecting groups. Dimethoxytrityl groups may then be removed with acetic acid (Fig. 4, Procedure A). However, the use of ammonia for removal

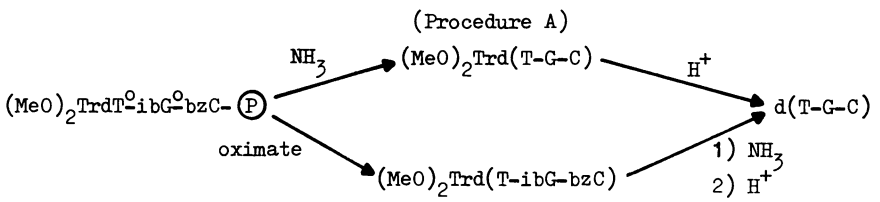


Fig. 4.

of aroyl groups has been shown to cause considerable chain degradation[6]. We have confirmed these observations and shown that alternative use of 0.3 M tetramethylguanidinium p-nitrobenzaldoximate[6] leads to higher yields of the desired oligonucleotide. The reagent also cleaves the oligonucleotide-resin linkage. Further deprotection of the detached oligonucleotide with ammonia and acetic acid proceeds as before (Procedure B).

The completely deprotected oligonucleotide is purified by ion-exchange hplc[2,3]. This is particularly advantageous in that in contrast to reversed phase hplc the desired oligonucleotide is always eluted later than any truncated or failure sequence and can be readily identified. After desalting the product is then assayed for purity by reversed phase hplc on μ-Bondapak C18[10,19] and by sequencing of ^{32}P-labelled samples.

Synthesis of Oligonucleotides. Three alternative strategies were investi-

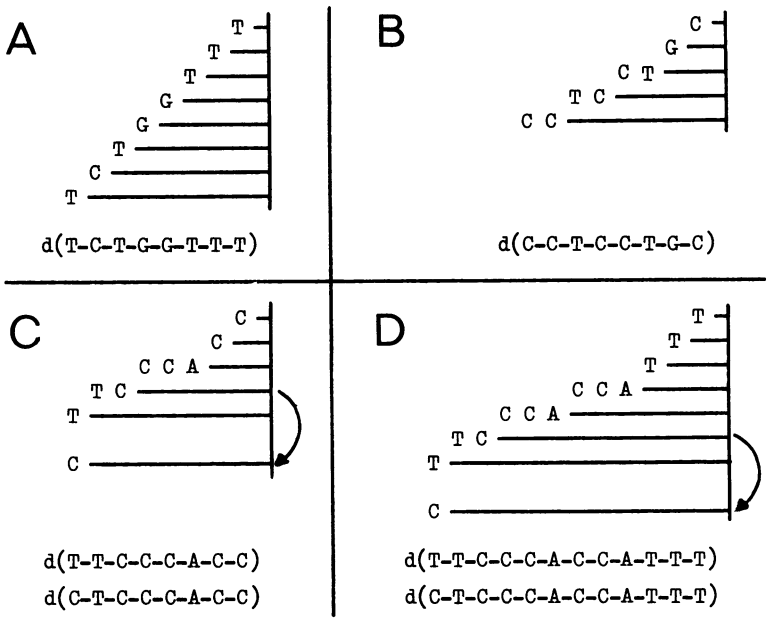


Fig. 5. Schematic plan for assembly of oligonucleotides.

gated for the synthesis of octanucleotides (Fig. 5, A-C). The octanucleotide d(T-C-T-G-G-T-T-T) was assembled purely by the addition of monomer units, the octanucleotide d(C-C-T-C-C-T-G-C) by successive addition of a monomer and three dimers, the octanucleotides d(T-T-C-C-C-A-C-C) and d(C-T-C-C-C-A-C-C) by addition of monomers, one dimer and one trimer. A nearly four-fold increase in overall yield of the first octanucleotide (Table 2) was obtained by use of deprotection procedure B rather than A. The improvement can also be seen by comparison of the chromatographic patterns obtained (Fig. 6) where both the product peak and peaks corresponding to shorter chain intermediates are substantially larger. The purity of the octanucleotide as judged by μ-Bondapak C18 hplc[20] was also increased.

In the synthesis of the other three octanucleotides (Table 2) deprotection procedure B was used throughout and the chromatographic patterns (Figs. 7-9) all show clear product peaks (yields 4.1-7.8%).

In the synthesis of the dodecanucleotides d(T-T-C-C-C-A-C-C-A-T-T-T) and d(C-T-C-C-C-A-C-C-A-T-T-T) the assembly strategy involved use of both monomers and preformed blocks (Fig. 5, D). Whereas deprotection procedure A was adequate for shorter chain intermediates (Table 3) overall yields of dodecamers dropped to 1.7 and 1.2% respectively. In the latter case use of

TABLE 2

Sequence	Resin sample (mg)	Glycine (μmole)	Cleavage method	Product isolated		Overall yield %	Purity % (μ Bondapak C18)
				(A_{260})	(μmole)		
d(T-C-T-G-G-T-T-T)	44.53	7.19	A	8.0	0.107[1]	1.8	87
d(T-C-T-G-G-T-T-T)	66.20	10.43	B	36.3	0.486[2]	6.8	93
d(C-C-T-C-C-T-G-C)	16.59	2.58	B	7.0	0.106	4.1	90
d(T-T-C-C-C-A-C-C)	9.76	1.92	B	9.9	0.142	7.4	94
d(C-T-C-C-C-A-C-C)	26.75	5.19	B	27.6	0.406	7.8	95

Note: [1] Only 85% of cleavage product purified; [2] Only 69% of cleavage product purified

deprotection procedure B doubled the yield (2.5%) and the product purity was increased. Chromatographic patterns of the two dodecanucleotides (Figs. 10 and 11) deprotected by different routes also reflect this improvement.

Certain direct comparisons can be made between the present phosphotriester syntheses and our original phosphodiester approach[1-3]. Individual coupling yields assessed by ratio of peak areas on hplc chromatograms are apparently similar (80-90% for monomer addition, 60-80% for blocks) but give a misleading impression. Instead the overall isolated yield of octanucleotides (4.1-

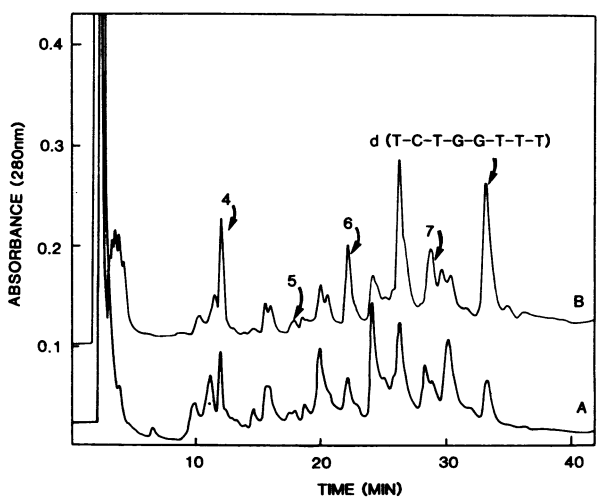


Fig. 6. Chromatographic patterns of the octanucleotide d(T-C-T-G-G-T-T-T) following deprotection procedures A and B.

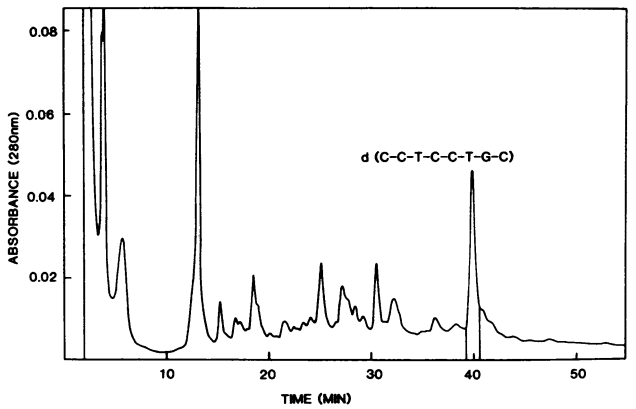


Fig. 7. Chromatographic pattern of the octanucleotide d(C-C-T-C-C-T-G-C)

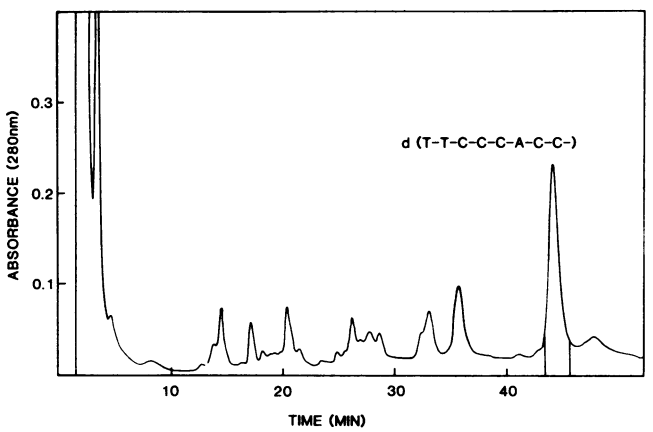


Fig. 8. Chromatographic pattern of the octanucleotide d(T-T-C-C-C-A-C-C)

7.8%) and dodecanucleotides (1.7-2.5%) should be compared with approximate values of 0.5%[2] and 0.04%[3] obtained respectively for an octanucleotide and a dodecanucleotide synthesised by the phosphodiester approach. This 10-60-fold increase in overall yield is attributable to a substantial reduction in formation of resin-bound by-products and to improved efficiency of cleavage of oligonucleotides from the resin. Chain assembly is faster and the procedure more flexible; equally good yields are obtained in routes involving both monomer and block addition. Moreover, oligonucleotides of satisfactory purity can be isolated by a single chromatographic step[21]. We intend to extend this

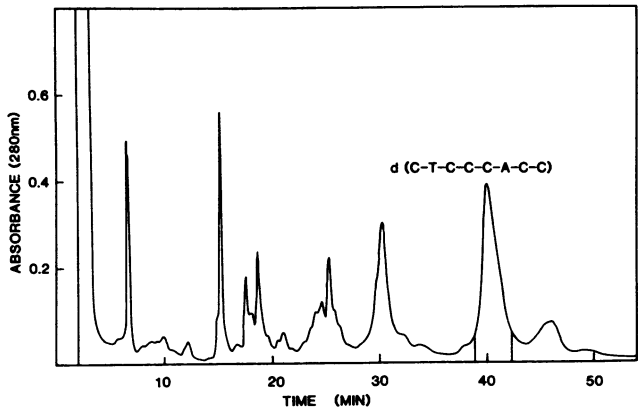


Fig. 9. Chromatographic pattern of the octanucleotide d(C-T-C-C-C-A-C-C)

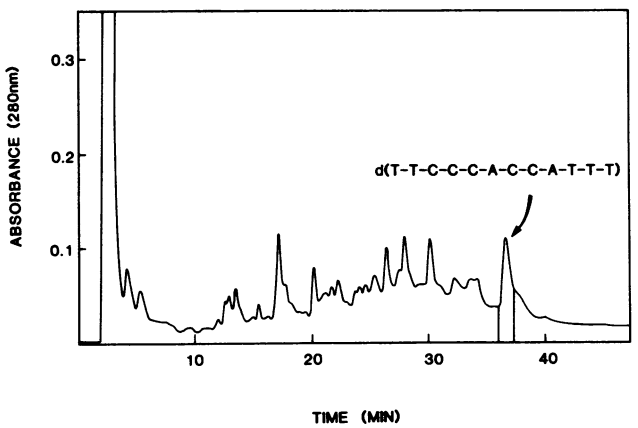


Fig. 10. Chromatographic pattern of the dodecanucleotide d(T-T-C-C-C-A-C-C-A-T-T-T); deprotection procedure A

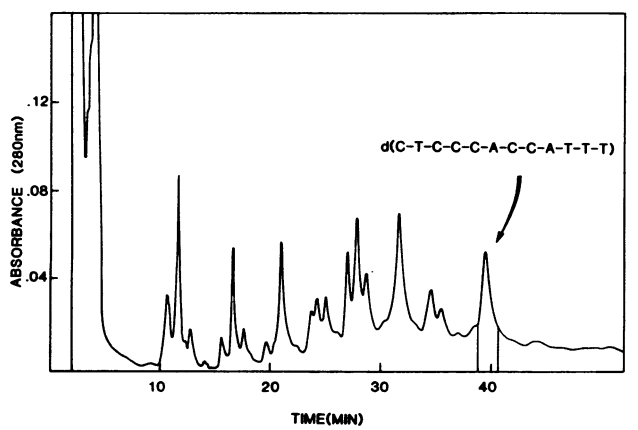


Fig. 11. Chromatographic pattern of the dodecanucleotide d(C-T-C-C-C-A-C-C-A-T-T-T); deprotection procedure B

TABLE 3 :

Sequence	Resin sample (mg)	Glycine (μmole)	Cleavage method	Product isolated (A_{260})	Product isolated (μmole)	Overall yield %	Purity % (μ Bondapak C18)
d(C-C-A-T-T-T)	13.57	3.148	A	23.8	0.422	13.4	93
d(C-C-A-C-C-A-T-T-T)	7.43	1.687	A	9.0	0.104	6.2	93
d(T-C-C-C-A-C-C-A-T-T-T)	24 14	5.180	A	9.6	0.094	1.8	86
d(T-T-C-C-C-A-C-C-A-T-T-T)	29.92	5.592	A	10.8	0.097	1.7	75
d(C-T-C-C-C-A-C-C-A-T-T-T)	18.20	3.432	A	4.6	0.042	1.2	74
d(C-T-C-C-C-A-C-C-A-T-T-T)	7.47	1.409	B	3.8	0.035	2.5	88

approach to the synthesis of both adenine-rich and longer oligonucleotides and believe that this rapid methodology will find wide application in the preparation of biologically useful oligonucleotides.

EXPERIMENTAL SECTION

Unless otherwise mentioned materials and methods are as previously described[1-3]. 4,4-Dimethoxytrityl chloride and p-chlorophenylphosphorodichloridate were obtained from Lancaster Synthesis, 1,2,4-triazole, 1-H tetrazole, 3-hydroxypropionitrile and 4-dimethylaminopyridine from Aldrich, succinic anhydride from BDH, benzenesulphonic acid, electronic grade, from Eastman, thymidine from Sigma, deoxyadenosine from Calbiochem, deoxyguanosine and deoxycytidine hydrochloride from Leon Laboratories, St. Louis. Koch-Light were suppliers of 1,1,3,3,-tetramethylguanidine and p-nitrobenzyl alcohol from which p-nitrobenzaldehyde[22] and then p-nitrobenzaldoxime[23] were prepared. Fully protected nucleoside and oligonucleotide phosphotriester building blocks were prepared essentially as previously described[5,24]. Silica gel column chromatography was carried out on a Waters prep. LC/system 500 using methanol/dichloromethane solvent mixtures or on a Merck Lobar column, size C, using methanol/chloroform-0.5% pyridine eluants. Removal of cyanoethyl protecting groups followed the procedure of Sood and Narang[25] except that the phosphodiester products were freed from cyanoethanol by partition between chloroform and saturated brine. The organic phase was evaporated and product precipitated with diethyl ether/n-pentane (3:2). Hplc was carried out using an Altex modular system consisting of two 110 pumps, 420 system controller and 210 deluxe injection valve. Column effluents were monitored by a Cecil 212A UV photometer linked to a Tekman TE 220 recorder. Partisil 10SAX columns (PXS-analytical or M9-preparative, Whatman) were eluted with gradients of potassium phosphate (pH 6.5)/5% ethanol from 1 mM (pump A) to

0.2 M (pump B) at ambient temperature. These columns appear to have limited lifetime (30-50 injections) due to voiding caused by dissolving of the packing material. Small voids can be rectified by filling the space with glass beads, but we are informed by the manufacturer that a small precolumn of silica before the injector reduces this problem by presaturating the eluant. Eluant pH should not be dropped as this results in considerable loss of resolution and peak delay. μ-Bondapak C18 (Waters) columns were eluted with 0.1 M ammonium acetate/acetonitrile solvent mixtures.

All resin treatments were carried out in an all-glass reaction vessel as previously described using a bench-top valve system[1,2] or a modified Beckman 990B solid phase peptide synthesiser[2,16]. Resin prefunctionalisation was carried out in a 200 ml capacity vessel using 140 ml solvent per wash. Resin (5 g) was treated with anhydrous ethylene diamine (Fluka, 170 ml) for 16 h and the resin washed with DMF until the eluate gave no blue colour in the ninhydrin test[14] (10-15 washes). The resin was then treated with (1) 3 x 10% diisopropylethylamine/DMF, 5 min, (2) 5 x DMF, 2 min, (3) 1 x 4 equivalents of the symmetrical anhydride of Boc gly, 90 min [Boc gly (12 mmole) and DCCI (6 mmole) in the minimum volume of dichloromethane were stirred for 15 min at room temperature, the mixture filtered, evaporated to dryness and dissolved in DMF (100 ml) just prior to use], (4) 5 x DMF, 2 min, (5) 1 x 10% diisopropylethylamine/DMF, 5 min, (6) 3 x DMF, 2 min, (7) 5 x dichloromethane, 5 min, (8) 5 x dioxan, 5 min, (9) 5 x diethyl ether, 5 min. The resin was dried in vacuo and assayed for glycine[26] using a Beckman 119C amino acid analyser with α-amino-β-guanidinopropionic acid as internal standard.

General Procedure for Preparation of Pyridinium 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-O-succinates. The 5'-O-dimethoxytrityl deoxynucleoside (dT,dbzA,dbzC or dibG) (3.35 mmole) was dissolved in DMF (15 ml) and succinic anhydride (6.7 mmole) and 4-dimethylaminopyridine (6.7 mmole) added. After 20 h at room temperature the mixture was evaporated to dryness and dissolved in pyridine/water (2:3). The solution was passed slowly through a column of Dowex 50-X8 (pyridinium). The eluate and column washings were evaporated to dryness and co-evaporated with pyridine to a foam, which was dissolved in chloroform/0.1% pyridine and applied to 10 preparative silica plates (20 x 20 x 2 mm, Merck 5717). The plates were eluted with chloroform/ethanol/pyridine (100:10:0.1) and the major band (UV and trityl positive) in each case scraped off and together eluted batchwise with chloroform/ethanol/pyridine (80:20:0.5). The eluate was evaporated to dryness and product precipitated with diethyl ether/pentane (3:2). Tlc analysis of the product on silica in chloro-

form/ethanol/pyridine (90:10:0.1) is essential to ensure the absence of pyridinium succinate (Rf 0.1-0.2) which will seriously interfere with coupling of the nucleoside derivative to the resin. Yields 30-60% - as partial pyridinium salts:-

5'-O-Dimethoxytrityl-2'-deoxythymidine-3'-O-succinate; λ max (ethanol) 234, 266, λ min 254 nm; ir 1735 cm^{-1} (C=O stretch succinate); nmr δ 9.97 (s,1H,NH-3) 7.7-6.7 (m,14H,Ar+H-6) 6.38 (tr,1H,H-1') 5.45 (m,1H,H-3') 4.16 (m,1H,H-4') 3.80 (s,6H,CH_3O-) 3.45 (m,2H,H-2') 2.65 (s+m,6H,H-5'+ succinate) 1.38 (s,3H,CH_3-5).

5'O-Dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine-3'-O-succinate; λ max 233, 278 (br), λ min 256; ir 1740; nmr δ 8.67 (s,1H,H-2) 8.22) (s,1H,H-8) 8.1-6.6 (m,18H,Ar) 6.45 (tr,1H,H-1') 5.50 (m,1H,H-3') 4.28 (m,1H,H-4') 3.76 (s,6H,CH_3O-) 3.40 (m,2H,H-2') 2.64 (s+m,6H,H-5'+ succinate).

5'-O-Dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine-3'-O-succinate; λ max 236, 258,284 (sh), 304, λ min 250,290; ir 1740; nmr δ 8.16 (d,1H,H-6) 8.04-6.70 (m,19H,H-5+Ar) 6.26 (tr+s,2H,H-1+NH-4) 5.41 (m,1H,H-3') 4.24 (m,1H,H-4') 3.80 (s,6H,CH_3O-) 3.43 (m,2H,H-2') 2.68 (s+m,6H,H-5'+ succinate).

5'-O-Dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine-3'-O-succinate; λ max 236,252 (sh), 260 (sh), 274,280, λ min 270; ir 1735; nmr δ 9.65 (s,1H,NH-1) 7.76 (s,1H,H-8) 7.4-6.6 (m,13H,Ar) 5.96 (tr,1H,H-1') 5.44 (m+s,2H,H-3'+NH-2) 4.22 (m,1H,H-4') 3.77 (s,6H,CH_3O-) 3.30 (m,2H,H-2') 2.69 (s+m,7H,H-5'+succinate+CO-CH) 1.10 (tr or d.d.,6H,CH_3-C).

(N.B. All UV spectra were identical to the corresponding nucleoside starting materials.)

Attachment of Deoxynucleoside-3'-O-succinate derivatives to the Resin. A resin batch containing 0.31 mmole g^{-1} of glycine was used in all experiments. Ca. 10 ml of solvent was used per wash per 0.35 g resin. Resin was swollen in DMF and treated with (1) 5 x t-amyl alcohol, 2 min, (2) 5 x acetic acid, 2 min, (3) 2 x 1.5 M hydrogen chloride in acetic acid, 5 + 25 min, (4) 5 x acetic acid, 2 min, (5) 10 x DMF, 2 min, (6) 3 x 10% diisopropylethylamine/DMF, 2 min, (7) 5 x DMF, 2 min, (8) 1 x 2 equivalents of the symmetrical anhydride of the succinate derivative (ir 1822 cm^{-1}, C=O stretch; formed from 4 equivalents of the succinate by the same procedure as for Boc gly), 120 min, (9) 5 x DMF, 2 min. A sample of resin was washed with 5 x dichloromethane, 2 min, and then 5 x diethylether, 2 min, and dried in vacuo. Three or four resin particles were assayed by the ninhydrin test[14] whilst 1-2 mg samples were analysed for glycine content and trityl incorporation respectively (determined spectrophotometrically in 5 ml of 60% perchloric acid/ethanol

[3:2], ε_{500} = 71,700). The nucleoside derivative should be quantitatively incorporated (ca. 0.25 mmole g^{-1} of final resin, trityl/gly 1.0).

Oligonucleotide Assembly. The resin is 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 1).

Deprotection and Cleavage from the Resin. Procedure A. The resin sample is treated with concentrated ammonia (1 ml per 10 mg resin) for 5 h at 50°, the liquid decanted and resin washed with ethanol/water (1:1) (3 x 1 ml). The decantate and washings are evaporated to dryness and the residue treated with acetic acid/water (4:1; 1 ml) for 30 min at room temperature. After

TABLE 1

Step	Reagent or solvent	Time of shaking (min)		No. of operations	
		Adenine absent	Adenine present	Adenine absent	Adenine present
1	$CHCl_3$/MeOH (7:3)	2		10	
2	$CHCl_3$/MeOH (7:3)	5		3[1]	
3	5% benzene sulphonic acid in $CHCl_3$/MeOH (7:3)	1	0.5	3	1[2]
4	$CHCl_3$/MeOH (7:3)	2	0.1	5	5[3]
5	DMF	2		5	
6	Pyridine	2		10	
7	Coupling mixture	180		1[4]	
8	Pyridine	2		5[5]	

[1] The extended washing procedure is necessary to ensure reproducible removal of pyridine, which would otherwise neutralise the acid subsequently added.

[2] An orange colour is immediately liberated into solution on addition of the acid.

[3] For oligonucleotides containing adenine steps 3 and 4 are repeated (usually once) until no further orange colour is seen. A sample of dried resin can also be assayed for trityl content.

[4] The nucleoside or oligonucleotide 3'-phosphate (5 equivs) is dried by co-evaporation three times with anhydrous pyridine and in a dry box triisopropylbenzene sulphonyltetrazole (8.75-10 equivs) added. After 5-10 min the mixture is introduced to the resin (1 equiv as glycine) via the Teflon feed lines[1,2] or by Pasteur pipette.

[5] 10 Washes are usually given in automatic operation. The programme also contains an instruction to hold until the operator is ready to begin again at step 1.

evaporation the product is dissolved in ethanol/water (1:1) ready for hplc.

Procedure B. The resin sample is shaken with 0.3 M tetramethylguanidinium p-nitrobenzaldoximate in dioxan/water (1:1) (1 ml per 10 mg resin) for 15-18 h, the liquid decanted and resin washed with dioxan/water (1:1) (3 x 1 ml). The decantate and washings are carefully neutralised with acetic acid, washed with chloroform (1 ml) and the aqueous phase evaporated to dryness. The residue is treated with concentrated ammonia (1 ml) at 50° for 5 h, evaporated to dryness and treated with acetic acid/water (4:1; 1 ml) for 30 min. After evaporation the product is dissolved in ethanol/water (1:1) ready for hplc.

The Octanucleotide d(T-C-T-G-G-T-T-T). Resin (0.354 g, 0.109 mmole glycine) was derivatised as described above to obtain the 5'-O-dimethoxytrityl-thymidine-3'-O-succinamido resin (trityl 0.252 mmole g^{-1}; trityl/gly 1.07). Seven cycles of nucleotide addition were carried out using the Beckman synthesiser and appropriate monomer units only. Resin samples at each stage gave trityl/gly values of 0.93, 0.91, 0.78, 0.82, 0.72, 1.11 and 0.95 respectively. A sample of final resin (44.53 mg, 7.19 μmole Gly) was treated according to cleavage procedure A. 85% of the product (λ max 269 nm, 311 A_{260} units) was chromatographed on Partisil 10SAX (Fig. 6A, 5 injections, elution 6 min 2% buffer B, 20 min 2-25% B, 25 min 25-40% B) to give 8.0 A_{260} units in the product peak (0.107 μmole, overall yield 1.75%). The material was desalted on Biogel P2 (λ max 266 nm, 260/280 1.408, calc. 1.351). μ-Bondapak C18 hplc assay showed 87% purity[20]. Another sample of final resin was treated according to cleavage procedure B. 69% of the product was chromatographed on Partisil 10SAX (Fig. 6B, 5 injections) to give 36.3 A_{260} units (0.486 μmole, overall yield 6.75%) in the product peak. The material was desalted on Biogel P2 (λ max 265 nm, 260/280 1.363, calc. 1.351). μ-Bondapak C18 hplc assay showed 93% purity. The sequence was confirmed by standard analysis of a ^{32}P-labelled sample.

The Octanucleotide d(C-C-T-C-C-T-G-C). Resin (0.139 g, 0.043 mmole glycine) was derivatised as described above to obtain the 5'-O-dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine-3'-O-succinamido resin (trityl 0.270 mmole g^{-1}, trityl/gly 1.27). Using the manual solvent delivery system one cycle of assembly was carried out with $(MeO)_2$TrdibG-(ClPh) in the coupling step followed by three cycles using in order the dinucleotide blocks $(MeO)_2$TrdbzC$^{O}_{-}$T-(ClPh), $(MeO)_2$TrdT$^{O}_{-}$bzC-(ClPh) and $(MeO)_2$TrdbzC$^{O}_{-}$bzC-(ClPh). Trityl/gly values of resin samples at each stage were 1.35, 1.02, 0.95 and 0.54 respectively. A sample of final resin (16.59 mg, 2.578 μmole Gly) was treated

according to cleavage procedure B and the product chromatographed on Partisil 10SAX (Fig. 7, 3 injections, elution 6 min 8% B, 45 min 8-70% B). The material in the product peak was desalted on Biogel P2 to give 6.97 A_{260} units (0.106 μmole, overall yield 4.1%, λ max 270 nm, 260/280 1.200, calc. 1.148). μ-Bondapak C18 hplc assay showed 90% purity. The sequence was confirmed by standard analysis of a ^{32}P-labelled sample.

The Octanucleotides d(T-T-C-C-C-A-C-C) and d(C-T-C-C-C-A-C-C). Resin (0.154 g, 0.0476 mmole Glycine) was derivatised as described above to obtain the 5'-O-dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine-3'-O-succinamido resin (trityl 0.258 mmole g^{-1}, trityl/gly 1.05). Using the manual solvent delivery system one cycle of assembly was carried out with $(MeO)_2$TrdbzC-(ClPh), one with $(MeO)_2$TrdbzC^{O}bzC^{O}bzA-(ClPh) and one cycle with the dinucleotide block $(MeO)_2$ TrdT^{O}bzC-(ClPh). Trityl/gly values at each stage were 0.92, 0.64 and 0.61 respectively. The resin was divided into two approximately equal portions. With each portion one cycle of assembly was carried out using $(MeO)_2$TrdT-(Cl Ph) in the coupling step in one case and $(MeO)_2$TrdbzC-(ClPh) in the other. A sample of the former final resin (9.765 mg, 1.925 μmole Gly) was treated according to cleavage procedure B and the product chromatographed on Partisil 10SAX (Fig. 8, 2 injections, elution 6 min 5% B, 18 min 5-35% B, 27 min 35-45% B). The material in the product peak was desalted to give 9.9 A_{260} units (0.142 μmole, overall yield 7.4%, λ max 268 nm, 260/280 1.321 calc. 1.349). μ-Bondapak C18 hplc assay showed 94% purity. A sample of the latter final resin was treated according to cleavage procedure B and the product chromatographed on Partisil 10SAX (Fig. 9, 2 injections, elution 6 min 5% B, 18 min 5-35% B, 27 min 35-45% B). The material in the product peak was desalted to give 27.6 A_{260} units (0.406 μmole, overall yield 7.8%, λ max 268 nm, 260/280 1.265 calc. 1.298). μ-Bondapak C18 hplc assay showed 95% purity. The sequences of both octanucleotides were confirmed by standard analysis.

The Dodecanucleotides d(T-T-C-C-C-A-C-C-A-T-T-T) and d(C-T-C-C-C-A-C-C-A-T-T-T). Resin (0.354 g, 0.109 mmole, Glycine) was derivatised as described above to give the 5'-O-dimethoxytritylthymidine-3'-O-succinamido resin (trityl 0.250 mmole g^{-1}, trityl/gly 0.97). Using the manual solvent delivery system two cycles of assembly were carried out with $(MeO)_2$TrdT-(ClPh) in the coupling steps. Trityl/gly values at each stage were 0.82 and 0.81 respectively. Using part of the resin (0.237 g, 0.049 mmole) two further cycles of assembly were carried out with the trinucleotide block $(MeO)_2$TrdbzC^{O}bzC^{O}bzA-(ClPh) in the coupling steps followed by one cycle with the dinucleotide block $(MeO)_2$TrdT^{O}bzC-(ClPh). The resin was divided into two approximately

equal portions. With each portion one cycle of assembly was carried out using $(MeO)_2$TrdT-(ClPh) in the coupling step in one case and $(MeO)_2$TrdbzC-(Cl Ph) in the other. Isolation of oligonucleotides at various stages of synthesis is summarised in Table 3. Chromatography in all cases was carried out on Partisil 10SAX. Elution conditions for the dodecanucleotides (Figs. 10,11) were 6 min 10% B, 20 min 10-65% B, 25 min 65-90% B. The sequences of both dodecanucleotides were confirmed by standard analysis of ^{32}P-labelled samples.

Acknowledgement

We thank Dr. H.-J. Fritz for helpful discussions early in this work.

Appendix

Oligonucleotide sequencing data and μ-Bondapak C18 chromatography profiles were supplied to the referees but are omitted from the paper because of space limitation. The material is available on request.

* to either of whom reprint requests should be sent.

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