Synthesis of Oligodeoxyribonucleotides by a Continuous-flow, Solid-phase Method using Phosphotriester Intermediates

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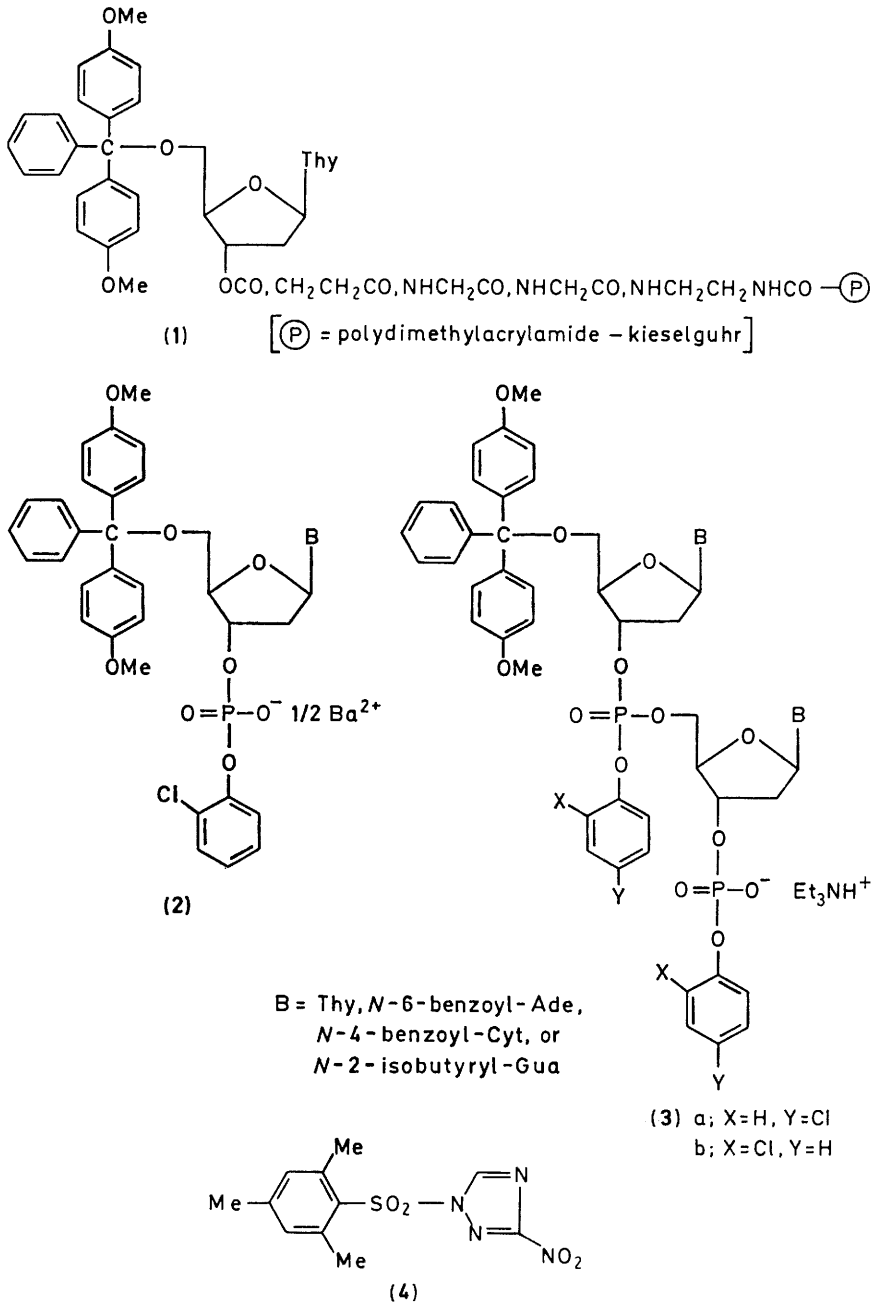
A new composite polydimethylacrylamide–kieselguhr support has been used in a continuous-flow assembly apparatus for the efficient solid-phase synthesis of an undeca- and a heptadeca-deoxyribonucleotide using phosphotriester intermediates.

Cross-linked polydimethylacrylamide resins have been successfully used for solid-phase synthesis of oligodeoxyribonucleotides. Phosphodiester[1] and more recently phosphotriester[2] routes have been used to assemble oligonucleotide chains, and the synthesis of several heptadecanucleotides has been described.[3] In these and similar[4] methods conventional washing of the gel-like resin in batches was necessary. Clearly much greater efficiency of washing would be achieved if the resin could be packed into a column and solvent passed through continuously, but lack of rigidity precluded this in the case of these gel resins.

Polystyrene grafted on to Teflon beads was the first support to be used in a flow system for oligonucleotide synthesis using phosphodiester chemistry.[5] Recently porous silica has proved useful with a phosphite-triester route of oligonucleotide assembly.[6] Hitherto no support has been described for oligonucleotide synthesis using a continuous-flow system in conjunction with phosphotriester chemistry. A recent communica-



tion[7] described the preparation and use in peptide synthesis of a new composite support having cross-linked polydimethylacrylamide resin contained in the pores of a rigid, macroporous, kieselguhr matrix. The support, packed in a glass column, exerted very little back pressure during solvent flow. We now describe the use of this support for the efficient synthesis of oligodeoxyribonucleotides *via* phosphotriester intermediates.

The support (containing *ca.* 108 μmol g^{-1} of functional methyl ester groups) was treated for 16 h with anhydrous ethylenediamine. The resultant amino polymer was washed in a conventional batch fashion with *N*,*N*-dimethylformamide (DMF) and treated with five equivalents of the symmetrical anhydride derivative of fluorenylmethoxycarbonylglycine in DMF for 1.5 h.[8] The support was washed with DMF, treated with piperidine–DMF (2:8 v/v) for 10 min to deprotect the amino groups[8] and the support washed again with DMF. A second glycine residue was coupled to the support in an analogous way and amino groups were deprotected as before. The support was then treated with five equivalents of the symmetrical anhydride derivative of pyridinium 5′-*O*-α,α-bis(*p*-methoxyphenyl)benzyl-2′-deoxythymidine-3′-*O*-succinate in DMF for 2 h,[2] washed with DMF, dichloromethane, dioxan, and diethyl ether, and dried *in vacuo*.

Support† (**1**) [120 mg, 82 μmol g^{-1} of the α,α-bis(*p*-methoxyphenyl)benzyl group[2]] was slurry-packed in pyridine into a glass column [6.5 × 50 mm (Omnifit Ltd., Cambridge)] fitted with a variable length plunger and 20 μ Teflon frit. On the top of the column was a septum injector which was connected *via* Teflon tubing to a bubble trap, Milton Roy minipump (operated at 1.1 ml min^{-1}) and thence to a six-way rotary valve which enabled solvents to be selected. The column effluent was monitored by a Gilson spectrochrom M u.v. spectrophotometer at 300 nm. The void volume of the column was *ca.* 0.4 ml.

† Supports functionalised with the other three nucleotide bases are currently being prepared. No particular problems are envisaged here based on previous experience with conventional polydimethylacrylamide resins.[2,3]

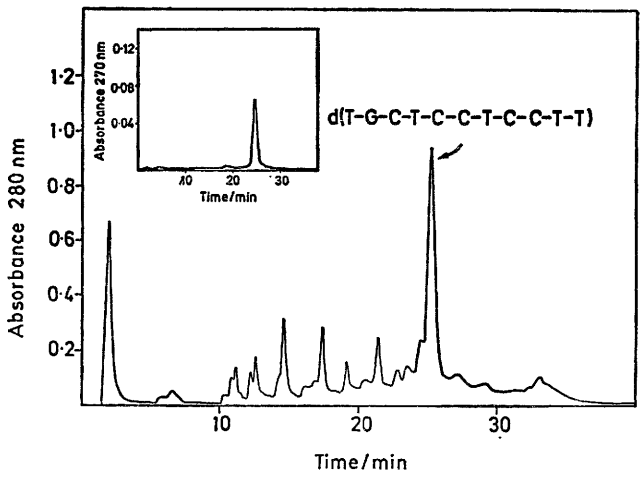


Figure 1. Ion exchange chromatographic pattern of the undecanucleotide. d(T–G–C–T–C–C–T–C–C–T–T), monomer route of synthesis. Conditions: buffer A, 0.001 M; buffer B, 0.3 M KH_2PO_4, pH 6.3, 30% ethanol; 0% B, 4 min, linear gradient 0—55% B, 45 min, column temperature 40 °C, flow rate 2 ml min^{-1}. *Inset.* Reversed phase chromatographic pattern, buffer A, 0.1 M ammonium acetate; buffer B, 0.1 M ammonium acetate–acetonitrile (2:8); 11% B, 4 min, linear gradient 11—17% B, 45 min.

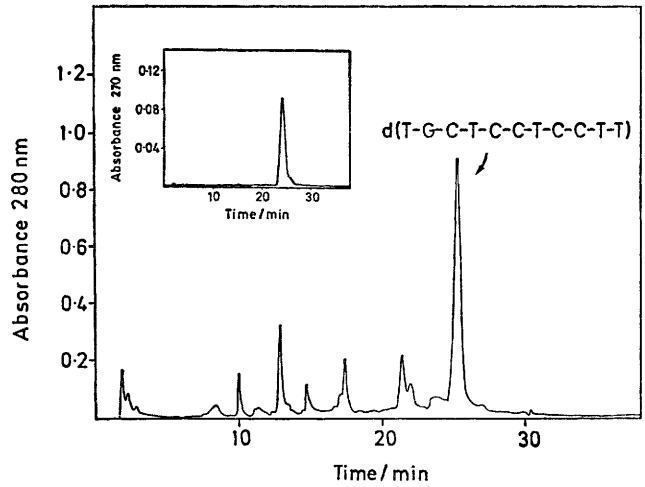


Figure 2. Ion exchange chromatographic pattern of the undecanucleotide, d(T–G–C–T–C–C–T–C–C–T–T), dimer route of synthesis. Conditions: as Figure 1. *Inset.* Reversed phase chromatographic pattern. Conditions as Figure 1.

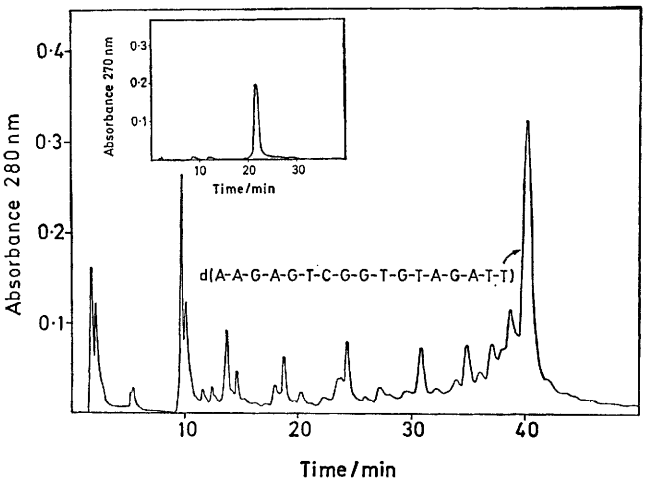


Figure 3. Ion exchange chromatographic pattern of the heptadecanucleotide, d(A–A–G–A–G–T–C–G–G–T–G–T–A–G–A–T–T). Conditions: as Figure 1 except linear gradient 0—70% B, 45 min. *Inset.* Reversed phase chromatographic pattern. Conditions as Figure 1 except 8% B, 4 min, linear gradient 8—15% B 45 min.

Phenyl isocyanate–pyridine (1:9, v/v) (0.6 ml) was injected on to the column over 5 min. After a further 5 min the support was washed by passage of pyridine (4 min). A cycle of oligonucleotide assembly then consisted of washing with (i) chloroform, 4 min; (ii) trichloroacetic acid–chloroform (1:9, v/v) 4 min, to remove terminal bis(*p*-methoxyphenyl)benzyl groups;[2] (iii) DMF, 4 min; and (iv) pyridine, 4 min. Solvent flow was stopped and a solution of the appropriate mononucleotide (**2**) or dinucleotide (**3**) (80 μmol) and the coupling agent, 1-(mesitylsulphonyl)-3-nitro-1,2,4-triazole (**4**) (180 μmol), in anhydrous pyridine (0.6 ml) injected over 5 min. After a further 55 min the column was washed with pyridine (4 min) to complete the assembly cycle. Following the requisite number of cycles the support was washed with chloroform and diethyl ether and dried briefly *in vacuo*. Oligonucleotides were cleaved from the support and deprotected by treatment with (i) 0.4 M 1,1,4,4-tetramethylguanidinium *syn*-2-nitrobenzaldoximate[9] in dioxan–water (1:1) for 16 h for 2-chlorophenyl protected chains or 24—36 h for 4-chlorophenyl protected chains; (ii) concentrated ammonia, 50 °C, 5 h; and (iii) acetic acid–water (4:1, v/v), 30 min.[2] Deprotected oligonucleotides were purified by anion-exchange high performance liquid chromatography on Partisil-10 SAX, the desired product being readily recognised as the most delayed of the product peaks.[2]

To illustrate the use of this methodology the undecanucleotide d(T–G–C–T–C–C–T–C–C–T–T) was synthesised twice, first using in the coupling reactions mononucleotides containing 2-chlorophenyl-protected phosphates (**2**), and secondly using dinucleotides containing the alternative 4-chlorophenyl-protected phosphates (**3a**). In both syntheses the major product was the desired undecanucleotide (Figures 1 and 2) which was isolated in 12.5 and 21.5% overall yield,‡ respectively (Table 1). In both cases reversed phase chromatography of samples on μBondapak $C18^{2,3}$ (insets, Figures 1 and 2) showed single peaks and further purification was not required.§ The heptadecanucleotide, d(A–A–G–A–G–T–C–G–G–T–G–T–A–G–A–T–T) was also prepared. Here dinucleotides containing 2-chlorophenyl-protected phosphates (**3b**) were used in the coupling reactions. Once again the major product was the desired heptadecanucleotide (Figure 3) [11.7% overall yield (Table 1)]. Reversed phase chromatography of a sample (inset) showed >95% of the u.v. absorption at 270 nm in a single peak.‡

Yields obtained in the above syntheses are in line with those previously obtained using conventional polydimethylacryl-

Table 1.

Sequence	% of product purified	A_{260} units after ion exchange	Overall yield/%	Average yield per cycle
d(T–G–C–T–C–C–T–C–C–T–T) (monomer route)	20	22.7	12.5	81
d(T–G–C–T–C–C–T–C–C–T–T) (dimer route)	16	31.2	21.5	74
d(A–A–G–A–G–T–C–G–G–T–G–T–A–G–A–T–T)	17	38.7	11.7	76

‡ Yields are based on the amount of resin-bound 2′-deoxynucleoside.

§ The sequences of oligonucleotides were confirmed by full sequence analysis of radiolabelled samples.

amide resins in discontinuous processes.[2,3] Whereas mononucleotides can be used for the assembly of oligonucleotides, the use of dinucleotide blocks is to be recommended for the synthesis of long chains. The advantages of the above continuous-flow method of synthesis are increased speed and efficiency of assembly and economy of materials. The new composite resin has great potential also for use in mechanised systems of DNA synthesis.

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References

1 M. J. Gait and R. C. Sheppard, *Nucleic Acids Res.*, 1977, **4**, 1135, 4391; *ibid.*, 1979, **6**, 1259.
2 M. J. Gait, M. Singh, R. C. Sheppard, M. D. Edge, A. R. Greene, G. R. Heathcliffe, T. C. Atkinson, C. R. Newton, and A. F. Markham, *Nucleic Acids Res.*, 1980, **8**, 1081; M. J. Gait, S. G. Popov, M. Singh, and R. C. Titmas, *Nucleic Acids Res. Symp. Ser. No. 7*, 1980, 233.
3 M. L. Duckworth, M. J. Gait, P. Goelet, G. F. Hong, M. Singh, and R. C. Titmas, *Nucleic Acids Res.*, 1981, **9**, 1691.
4 K. Miyoshi, R. Arentzen, T. Huang, and K. Itakura, *Nucleic Acids Res.*, 1980, **8**, 5507.
5 V. K. Potapov, V. P. Veiko, O. N. Koroleva, and Z. A. Shabarova, *Nucleic Acids Res.*, 1979, **6**, 2041.
6 M. D. Matteucci and M. H. Caruthers, *Tetrahedron Lett.*, 1980, **21**, 719; K. K. Ogilvie and M. J. Nemer, *ibid.*, p. 4159.
7 E. Atherton, E. Brown, R. C. Sheppard, and A. Rosevear, *J. Chem. Soc., Chem. Commun.*, 1981, 1151.
8 E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1978, 537.
9 C. B. Reese and L. Sard, *Nucleic Acids Res.*, 1981, **9**, 4611.