Rapid synthesis of oligodeoxyribonucleotides VII. Solid phase synthesis of oligodeoxyribonucleotides by a continuous flow phosphotriester method on a kieselguhr-polyamide support

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ABSTRACT

A new kieselguhr-polydimethylacrylamide support has been used in a continuous flow, column system for solid phase synthesis of oligodeoxyribonucleotides by a phosphotriester procedure. Using only protected mononucleotides a 14-mer, 20-mer and 27-mer were assembled in high repetitive yield using a simple manually operated, bench top apparatus.

INTRODUCTION

In previous papers in this series we have developed the use of swellable polydimethylacrylamide gel copolymers as solid supports for oligodeoxyribonucleotide synthesis. In the most recent paper [1] we showed that 17-mers could be efficiently prepared using phosphotriester chemistry and dimer building blocks. Several other phosphotriester routes have also been described recently involving a diversity of solid phases, viz: polyacryloylmorpholide [2], polystyrene [3] and cellulose [4]. Such non-rigid materials (including polydimethylacrylamide gels) require gentle handling with solvents and reagents being added batchwise followed by gentle agitation and filtration. More efficient washing can be achieved by packing the solid phase into a column and passing solvents through continuously. This has been used recently with silica gel in conjunction with phosphite [5] and phosphoramidite [6] chemistry.

Although attempts are presently being made to use polystyrene [7], silica gel [8] and glass beads [9], especially on a small scale in columns with phosphotriester chemistry, conventional polyamide gels are too swollen to be handled in this way [10]. Recently the preparation and use in peptide synthesis of a new kieselguhr-polydimethylacrylamide composite was described, specifically designed for use in continuous flow systems [11]. In a preliminary communication we showed [12] that this material was particularly suitable for oligodeoxyribonucleotide synthesis. The methods are now

presented in more detail and in particular we describe some significant improvements to apparatus, monomer purification and oligonucleotide isolation conditions. The efficient synthesis of a 14-mer, 20-mer and 27-mer is described in each case using only monomer building blocks.

DISCUSSION AND RESULTS

The solid phase

The composite support, which is commercially available, consists of particles of fabricated, inert kieselguhr having extremely large pores of several thousand Å diameter, in which the polydimethylacrylamide gel has been prepared *in situ*. The macroporous support packed in a glass column exerts negligible back-pressure during solvent flow. Thus far only one loading of functional group has been investigated ($0.108 \text{ mmole g}^{-1}$.) These are sarcosine methyl ester groups and attachment of the first deoxynucleoside residue is achieved by methods similar to those previously described [13] (Figure 1). Essentially two rounds of peptide synthesis are carried out to obtain a double glycine spacer, followed by formation of a 3'-O-succinamido linkage to the first deoxynucleoside residue by an analogous coupling step. All reactions can be followed by the ninhydrin test, and final resin quantitated for glycine and dimethoxytrityl content. Reproducible loadings of deoxynucleoside of $80\text{--}90 \text{ } \mu\text{mole g}^{-1}$ of final support are achieved.

Figure 1

Oligonucleotide assembly

For the assembly reactions we have constructed a simple apparatus from low cost, commercially available components, consisting principally of solvent bottles, a six-way rotary solvent selector valve, small glass column and assorted Teflon tubes and connectors. Originally solvent delivery was by a pump [12] but we have now replaced this by a more convenient gas pressure delivery (Figure 2). Once assembled the only manipulations required are operation of the single six-way valve for solvent selection and injection of activated nucleotides via a septum port.

The basic phosphotriester route as applied to solid phase synthesis (Figure 3) has been described several times before [1,13]. Only two chemical reactions are necessary per synthesis cycle. The first is removal of 5'-O-dimethoxytrityl groups using 10% trichloroacetic acid in chloroform [14]. Deprotection is reproducibly complete within three minutes and very little unwanted depurination is observed. During deprotection a bright orange colour (the dimethoxytrityl cation) is released and serves as a useful qualitative guide to the efficiency of assembly.

The second reaction is coupling of appropriately N-protected 5'-O-dimethoxytrityl-2'-deoxynucleoside-3'-O-2-chlorophenyl phosphates to the support. In the past we have used dinucleotides rather than mononucleotide units in the coupling reactions in order to reduce the number of synthesis cycles [13]. However, the use of monomers has the advantage that only four (rather than 16) coupling units are necessary for any synthesis. In further examination of the use of mononucleotides we noticed large variations in

Figure 2

Figure 3

coupling yields when different batches of the same deoxynucleotide derivative were used. We concluded that small amounts of reactive impurities were being carried through in preparation of the monomers by standard methods. Such impurities become magnified by virtue of the use of large excesses of nucleotide in solid phase reactions and presumably react preferentially to cap off the oligonucleotide chains at their 5'-hydroxyl groups [15]. After investigating alternative purification techniques we have found that consistently high purity can be obtained by chromatographing the monomers as their triethylammonium salts on columns of Kieselgel 60H by the short column method. Monomers purified in this way give significantly improved results in solid phase synthesis.

Hence for 60 mg of composite support (5 μ mole) a solution of monomer (40 μ mole) in anhydrous pyridine (0.35 ml) is pre-activated by addition to mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) (200 μ mole), injected on to the column and left in contact with the support for 45 min. A large excess of coupling agent is used partly as an *in situ* drying agent, ensures reproducible coupling and obviates the need for any physical drying step. No 'capping' step is necessary [16] and the complete cycle (Table 1) takes about 60 min.

Deprotection and purification

After chain assembly oligonucleotides are deprotected by (1) treatment of

TABLE 1. Deprotection and wash cycle

Solvent		Time (min)
1.	Pyridine	5
2.	Chloroform	3
3.	10% Trichloroacetic acid/chloroform	3
4.	DMF	2
5.	Pyridine	5
6.	Coupling (stop flow)	40

the support with 1,1,3,3-tetramethylguanidinium-2-nitrobenzaldoximate [17] for 16 h at room temperature to remove 2-chlorophenyl groups and liberate oligonucleotides into solution, (2) concentrated ammonia at 50° for 5 h to remove N-protecting groups, and (3) acetic acid/water (8:2) for 30 min at room temperature to remove terminal dimethoxytrityl groups [1,13]. Non-nucleotidic material is then removed by extraction with diethyl ether. Ion exchange high performance liquid chromatography (hplc) is particularly useful for initial purification. Most likely impurities are shorter and hence less strongly retained. Thus the desired product is readily identified as the most delayed peak. In addition resolution of all the chain lengths gives useful information as to the efficiency of each coupling reaction. Hitherto separation of long, purine-rich or self-complementary chains has been difficult, because the high strength phosphate buffers cause aggregation leading to insufficient peak resolution and anomalous elution behaviour. The use of the disaggregant, formamide, in the buffers recently suggested to us [18] significantly improves peak resolution and has the added benefit of drastically increasing column lifetime. The product peak is desalted on Biogel P2 and purity estimated by reversed phase hplc.

Examples of oligonucleotide synthesis

The above methods were applied to the synthesis of a 14-mer and a 20-mer both designed as primers for direct sequencing of bacteriophage λ [19]. The 14-mer, d(G-T-A-A-C-T-C-A-C-G-G-G-T), was prepared from 60 mg of T-support (5.1 μ mole) and the 20-mer (d(A-C-T-G-G-C-A-G-A-T-T-A-G-G-C-C-G-T-G), from 60 mg of G-support (4.6 μ mole). Analytical ion exchange chromatography patterns (Figures 4 and 5) showed clear product peaks. A single injection of 1-2% of the synthetic product is sufficient for the isolation of about 1 A₂₆₀

Figure 4. Analytical ion exchange hplc of 14-mer, d(G-T-A-A-C-T-C-A-C-G-G-G-G-T), gradient 0-70% buffer B, 45 min, temperature 20°. Inset: Reversed phase hplc of 14-mer, gradient 0-45% buffer B, 40 min.

units (50 μ g). In these cases overall isolated yields were 10.0 and 9.3%, respectively (Table 2). On preparative columns recovery yields drop somewhat, but with multiple injections about 2 mg of oligonucleotide can be purified from the total product on this scale of synthesis. Both 14-mer and 20-mer showed ca. 90% of the UV activity in a single peak when analysed by reversed phase hplc. (Figures 4 and 5 insets).

The 27-mer, d(G-G-G-T-C-T-A-G-A-A-T-T-C-A-A-G-C-T-T-G-G-A-T-C-C-T-C), wanted for use in the construction of a poly-linker in cloning experiments, was prepared from 60 mg of C-support (5.9 μ mole). Ion exchange hplc of the product (Figure 6) showed the most delayed peak as the major product which was isolated in 2.9% yield. Once again reversed phase hplc showed at least 90% in a single peak. In the three syntheses [20] isolated yields expressed on a per assembly cycle basis were 84-88%.

Figure 5. Analytical ion exchange hplc of 20-mer, d(A-C-T-G-G-C-A-G-A-T-T-A-C-G-C-C-C-G-T-G), gradient 0-100% buffer B, 50 min, temperature 20°. Inset: Reversed phase hplc of 20-mer, gradient 14-24% buffer B, 30 min.

EXPERIMENTAL PROCEDURES

Materials and methods not mentioned below are as described previously [1, 13]. A laboratory manual designed for use by a non-specialist is also available [21]. Kieselguhr-polydimethylacrylamide composite support is

TABLE 2. Yields of oligonucleotides prepared

Oligonucleotide	Ion exchange column size	Amount purified %	Overall yield		
			A ₂₆₀ units	%	% per cycle
14-mer: d(GTAACTCA CGGGGT)	Anal. Prep.	1.0 70	0.78 30.7	10.0 5.6	84 80
20-mer: d(CTGGCAG ATTACGCCGTG)	Anal. Prep.	2.0 20	1.8 9.0	9.3 4.6	88 85
27-mer: d(GGGTCTAG AATTCAAGCTTGGATCCT C)	Anal.	1.6	0.76	2.9	87

Figure 6. Analytical ion exchange hplc of 27-mer, d(G-G-G-T-C-T-A-G-A-A-T-T-C-A-A-G-C-T-T-G-G-A-T-C-C-T-C), gradient 0-100% buffer B, 50 min, temperature 41°. Inset: Reversed phase hplc of 27-mer, gradient 10-25% buffer B, 45 min.

manufactured by Victor Wolf, Ltd., Clayton, Manchester, U.K. and is available also through other distributors.

Functionalisation of support

1 g of composite support ($0.108 \text{ mmol g}^{-1}$ nominal loading) was placed in a small glass reaction vessel fitted with sintered glass disc and stopcock drain valve at the bottom. Solvents and reagents were added manually. Filtration was by application of slight nitrogen pressure to the top of the vessel. Anhydrous ethylene diamine (Fluka) (5 ml) was added, the vessel stoppered, and after swirling briefly the mixture was allowed to stand overnight at room temperature. After filtration the support was washed 10-15 times with DMF (5 ml) until the eluate was negative in the ninhydrin test [13]. The support was washed with 10% diisopropylethylamine/DMF (5 ml) and 5 x DMF (5 ml). The support was then reacted with five equivalents of freshly prepared [22] fluorenylmethoxycarbonyl glycine anhydride in DMF (5 ml) and then treated with piperidine/DMF (2:8) (5 ml) to remove terminal protecting groups. The support was washed with 5 x DMF (5 ml), reacted once again with fluorenylmethoxycarbonyl glycine anhydride as before, washed with

5 x DMF (5 ml), piperidine/DMF (2:8) (5 ml) and 5 x DMF (5 ml) again. The support was now reacted with five equivalents of one of the four 5'-O-dimethoxytrityl-2'-deoxynucleoside succinate anhydrides (of dT, dbzA, dbzC or dibG) [13,21] in DMF (5 ml) for 1 h and then washed with 5 x DMF (5 ml), 5 x dichloromethane (5 ml), 5 x dioxan (5 ml) and 5 x diethyl ether (5 ml). The support was then dried in vacuo. The above procedures were monitored qualitatively for presence or absence of free amino groups on the support by the ninhydrin test. The final support was assayed for glycine content by amino acid analysis and for dimethoxytrityl content spectrophotometrically in 60% perchloric acid/ethanol (3:2) $\epsilon_{495} = 71700$. Typically glycine content was 170-180 $\mu\text{mole g}^{-1}$ and dimethoxytrityl content 80-90 $\mu\text{mole g}^{-1}$, thus showing quantitative functionalisation.

Preparation of nucleotide monomers

A mixture of 1,2,4-triazole (2.76 g, 40 mmole; recrystallised from anhydrous dioxan), dry triethylamine (4.87 ml, 35 mmole), freshly distilled 2-chlorophenylphosphodichloridate and dry THF (100 ml; distilled from sodium wire and benzophenone) was stirred for 30 min at room temperature with exclusion of moisture (a copious white precipitate of triethylammonium chloride formed immediately on addition of phosphorylating agent). The mixture was filtered in a dry box into predried (by evaporation of dry THF containing 1% triethylamine) 5'-O-dimethoxytrityl-2'-deoxynucleoside (10 mmole) [21] (N-protected in the case of Gua, Ade and Cyt) and the clear pale yellow solution left for 1 h at room temperature. Silica gel tlc in 10% ethanol/90% chloroform-0.1% pyridine showed reaction to be complete with a trityl positive spot near the baseline. 1 M triethylammonium bicarbonate (100 ml, pH 8) was added and the mixture extracted with chloroform (300 ml). The organic phase was washed with water (3 x 150 ml), any emulsions being separated by centrifugation, and evaporated in the presence of pyridine to a foam. The material was dissolved in dichloromethane/1% triethylamine and chromatographed on a short fat silica gel column (Kieselgel 60H, Merck 7736, 200 g), rapidly eluted with 2 L of 10% ethanol in dichloromethane/1% triethylamine. Pure fractions were pooled, evaporated in vacuo in the presence of pyridine to a glass, and precipitated from dichloromethane solution by dropwise addition into pentane/1% triethylamine. Yields 70-80%.

Assembly of oligonucleotides

The apparatus. Figure 2 shows a diagram of the apparatus. It consists simply of a pressure stat, four solvent bottles, a single rotary valve, a column assembly fitted with a septum injector, waste bottle, and Teflon

tubing. Solvent bottles are kept under slight pressure (5 psi) of argon or nitrogen, the back-pressure being obtained by use of 1 metre of thin (0.3 mm i.d.) tubing on the outlet side of the column. The apparatus is commercially available either as a kit or as individual components (Omnifit, Ltd., Cambridge, U.K.) and details of how to assemble the apparatus are given elsewhere [21].

Oligonucleotide assembly procedure. Functionalised support (~60 mg, 5 μ mole) is dry packed into the column and swollen by addition of pyridine. After 1 h all bubbles are removed and the column assembled for flow. 0.6 ml of phenylisocyanate/pyridine (1:9 v/v) is injected on to the column over 5 min and after a further 5 min the first deprotection and wash cycle is begun (Table 1). Flow rate is set for 1 ml min⁻¹ for pyridine flow. The flow is stopped and a solution of nucleotide monomer (40 μ mole) in anhydrous pyridine (0.35 ml) freshly activated with MSNT (60 mg, 200 μ mole) is injected on to the column over a 5-min period via a chemically inert Hamilton syringe (1750 RN-81230). The column is allowed to stand for 40 min further and the second wash cycle begun as above. Cycles of wash and nucleotide addition are continued until the required length is reached. Should it be necessary to leave an assembly overnight it is recommended that the support is left either dry or in contact with chloroform in the dark [23]. After the final coupling reaction the support is washed with pyridine and chloroform and then washed into a small sintered glass funnel using diethyl ether. The support is sucked dry and can be stored dry at -20° if desired.

Removal of protecting groups

The support, contained in a 2 ml Eppendorf tube, is treated with a solution of syn-2-nitrobenzaloxime (140 mg) in 2 ml of dioxan/water (1:1) to which 100 μ l of 1,1,3,3-tetramethylguanidine has been added. The mixture is left at room temperature for 16 h and filtered through a small sintered glass funnel and the support washed well with dioxan/water (1:1). The filtrate is evaporated to dryness and dissolved in concentrated ammonia solution (5 ml), the flask sealed well and heated at 50° for 5 h. The flask is cooled, ammonia blown off in an air stream and the solution evaporated to dryness. The residue is taken up in acetic acid/water (8:2) (5 ml) and left at room temperature for 30 min. The solution is diluted with water and extracted five times with diethyl ether. The aqueous phase is evaporated to dryness and taken up in water.

Purification by hplc

Ion exchange. A gradient hplc system is set up using a Partisil 10/25

SAX column (Whatman) (4.5 x 250 mm, analytical, or 7.0 x 250 mm semi-preparative) with buffers of 0.001 M and 0.3 M KH_2PO_4 pH 6.3 in formamide/water (6:4 v/v) as solvents A and B respectively and a column temperature of 20° or 40°. Collected fractions are desalted by passage down a Biogel P2 column (100 x 2.5 cm) in 50 ml batches, using ethanol/water (2:8) as eluant.

Reversed phase. A gradient hplc system is set up using a μ -Bondapak C_{18} column (Waters) and 0.1 M ammonium acetate as buffer A and 0.1 M ammonium acetate/acetonitrile (2:8 v/v) as buffer B. Samples are warmed to 50° before injection, but the column is operated at ambient temperature.

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14. Commercial grade chloroform contains 1% ethanol as stabiliser. Since completing this work we have discovered that if the chloroform is dried by distillation which also removes the ethanol [cf. Tanaka, T. and Letsinger, R.L. (1982) Nucleic Acids Res. 10, 3249-3260] the chloroform must be used within one to two days otherwise substantial degradation

of the final deprotected oligonucleotide is observed to have taken place. This is due to rapid formation of phosgene in the chloroform leading to modification and elimination of the heterocyclic bases and thence to internucleotide cleavage during deprotection. We have recently shown that 1,2-dichloroethane can replace chloroform in washing and deprotection steps and there is no tendency for phosgene formation or other side reactions.

15. A similar situation was observed in solid phase phosphodiester synthesis. Gait, M.J. and Sheppard, R.C. (1979) *Nucleic Acids Res.* 6, 1259-1268.
16. Several reports suggest the use of, for example, acetic anhydride in the presence of a basic catalyst for 'capping' of unreacted chains after coupling. We have observed almost no difference in the hplc chromatograms when a capping step is added to the assembly cycle and therefore suggest that in general truncated sequences are due to side reactions other than a failure of the hydroxyl groups to react. A similar conclusion has recently been drawn in 'phospite' solid phase reactions [Tanaka, T. and Letsinger, R.L. (1982) *Nucleic Acids Res.* 10, 3249-3260].
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23. Prolonged storage of resin in either pyridine or DMF is not recommended, since cleavage of the succinamido linkage takes place at a rate of 5-10% in 16 h at room temperature, even with redistilled solvents. Commercial grade pyridine and DMF may contain traces of primary and secondary amines that will catalyze an even faster cleavage.