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RAPID OLIGODEOXYRIBONUCLEOTIDE SYNTHESIS BY THE "FILTRATION" METHOD

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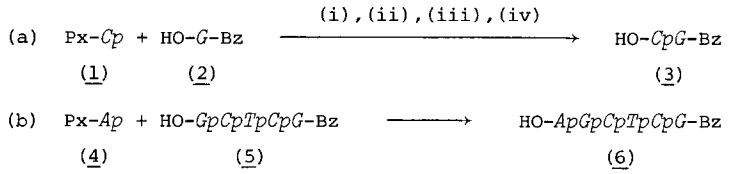
Summary: A rapid method for the synthesis of oligodeoxyribonucleotides by the phosphotriester approach in solution is described; the method is suitable for the preparation of relatively large quantities of pure compounds.

In the past few years, there has been remarkable progress in the development of rapid methods for the synthesis of relatively long-chain oligodeoxyribonucleotides on solid supports[1,2]. While synthesis on a solid support leads, on the whole, to much smaller quantities of far less pure products than synthesis in solution, it nevertheless appears to meet most of the present needs of molecular geneticists. However, when relatively large quantities of especially pure oligonucleotides are required either for molecular biological or physicochemical studies, the solid phase approach may well prove to be unsatisfactory.

In both the phosphotriester[3] and phosphite triester[2] approaches to oligonucleotide synthesis, a growing oligonucleotide is essentially a relatively lipophilic, uncharged intermediate which is soluble in certain organic solvents. However, when it is attached to a solid support, it is effectively kept out of solution while the often large excess of unreacted monomeric or dimeric phosphodiester building block, which is negatively charged and hence more polar, remains in solution. It seemed to us that a more logical approach to rapid synthesis would be to retain the growing oligonucleotide in solution and then to remove any remaining phosphodiester building block from that solution by virtue of its relatively greater polarity. We now report that 3'-(2-chlorophenyl) phosphate esters of 2'-deoxyribonucleosides (and their base-protected derivatives) can be removed from solution by rapid chromatography on silica gel[4]. Indeed, as the chromatographic process involved is hardly more than filtration, we have termed the synthetic procedure based on it the "filtration" method of rapid oligonucleotide synthesis.

Scheme 1



The synthetic cycle of the "filtration" method consists essentially of four main steps [(i)-(iv)] which may conveniently be illustrated by considering the first such cycle, *i.e.* the

preparation of the dinucleoside phosphate [HO-*CpG*-Bz][5] (Scheme 1a), in the synthesis of the hexanucleoside pentaphosphate, d[AGCTCG].

Step (i). The appropriate phosphodiester block [Px-*Cp* (1), 0.24g, 0.275 mmol] and the 3'-terminal nucleoside derivative [HO-*G*-Bz (2), 0.12g, 0.25 mmol] were dried by evaporation, under reduced pressure, from pyridine solution [time required, 15 min], and were then condensed together in the presence of 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole[8] (MSNT; 0.25g, 0.825 mmol) in pyridine (2 ml) solution [time required, 15 min; total time required for step (i), 30 min].

Step (ii). The remaining MSNT was destroyed by treatment with aqueous $NaHCO_3$, and the products were partitioned between chloroform and 0.2*M* - aqueous triethylammonium bicarbonate (pH 7.5). The organic layer was then extracted with *ca.* 2*M* - acidic (pH 2) sodium phosphate buffer to remove the remaining pyridine. The dried ($MgSO_4$) organic layer was then evaporated under reduced pressure [total time required for step (ii), 25 min].

Step (iii). The material obtained above was dissolved in $CHCl_3$-EtOH (95:5, v/v; 8 ml) and solid phenyl dihydrogen phosphate[9] (PDHP; 0.38g, 2.2 mmol) was added. A yellow coloration was obtained instantly. After 5 min[10], the products were diluted with chloroform and extracted with aqueous $NaHCO_3$. The organic layer was evaporated to a glass [time required for step (iii), 15 min].

Step (iv) ["Filtration" Step]. The above material was applied in chloroform (2 × 1 ml) solution to a short column (*ca.* 2 cm diameter) containing Merck Kieselgel 60 [Art. 9385] (8g). The column was eluted first with chloroform (20 ml) to remove non-nucleotide impurities and then with anhydrous tetrahydrofuran-pyridine (3:1 v/v; 50 ml). The desired product (3) was contained in the latter eluate[11] which was evaporated under reduced pressure at 25°C. [time required for "filtration", 10 min; time required for evaporation, 30 min; total time required for step (iv), 40 min].

It can be seen that the total time required for the first synthetic cycle [Scheme 1a] in the preparation of d[AGCTCG] was 110 min. Step (iii) sometimes requires 25 min[10] and, in practice, it is wise to allow a total time of 135 min for each cycle. The four other synthetic cycles, which involved the appropriate phosphodiester (0.3 mmol) and MSNT (0.9 mmol), were carried out in the same way. The final cycle is indicated in Scheme 1b. After the 5 cycles had been completed, the material obtained was subjected to rapid chromatography (10 - 15 min) on a very short column (*ca.* 3 cm diameter) of Merck Kieselgel 60 [Art. 7729] (1.5g). The column was eluted first with chloroform and then with chloroform containing increasing quantities of ethanol (up to 10%). The hexanucleoside pentaphosphate [(6), 0.24g, 0.080 mmol, 32% overall yield; see Table 1, entry no. 1] was isolated as a precipitated solid following evaporation of the appropriate fractions.

A part (0.04g) of the HO-*ApGpCpTpCpG*-Bz (6) obtained was unblocked to give d[AGCTCG] by a three step procedure [(i) acetic anhydride (0.2 ml), pyridine (0.5 ml), 20°C, 2 hr; (ii) *syn*-2-nitrobenzaldoxime[12] (0.134g, 0.80 mmol), N^1,N^1,N^3,N^3-tetramethylguanidine (0.091 ml, 0.72 mmol), dioxan (2.68 ml), water (0.27 ml, added after 1.5 hr), 20°C, 14 hr; (iii) aqueous ammonia (*d* 0.88, 10 ml), 20°C, 8 hr]. The unblocked material was passed through a Sephadex G-10 column and was then chromatographed on DEAE Sephadex A25 [linear gradient from 0.001 - 1.0*M* - triethylammonium bicarbonate (pH 7.5)]. The nearly symmetrical elution profile obtained is illustrated in Figure 1. The desired material [450 A_{257} units], which was eluted with an

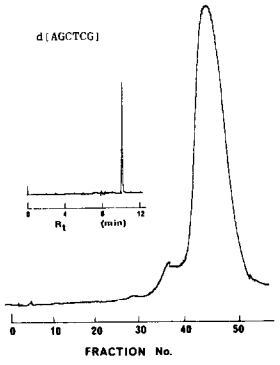


FIGURE 1 : DEAE Sephadex A25 Chromatography [linear gradient of triethylammonium bicarbonate (pH 7.5) from 0.001-1.0 M] of d[AGCTCG]. Inset: h.p.l.c. (Jones APEX ODS column) of main peak.

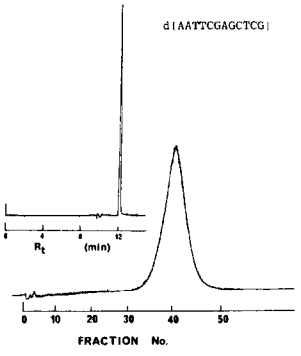


FIGURE 2 : DEAE Sephadex A25 Chromatography [linear gradient of triethylammonium bicarbonate (pH 7.5) from 0.001-1.3 M] of d[AATTCGAGCTCG]. Inset: h.p.l.c. (Jones APEX ODS column) of main peak.

average buffer concentration of 0.76*M*, was homogeneous on h.p.l.c. (see Fig. 1 inset) and gave rise to the expected products on enzymatic digestion[13].

It can be seen from Table 1 that substantial quantities of other protected and partially-protected hexamers were readily prepared by the "filtration" process. On the average, the total time required for the preparation and isolation of these hexamers was 13 hr. All of these products except the last two (entries nos. 8 and 9) were unblocked by the above three step procedure and all the expected oligonucleotides were obtained, perhaps rather surprisingly, in at least as pure a state as would be expected if they had been prepared by the much more laborious conventional solution procedure. Yields varied in the range 76-84% per synthetic cycle, and it would appear (entries nos. 6 and 7) that double protection of guanine residues (as 2-*N*-phenylacetyl-6-*O*-(2-nitrophenyl)guanine][14] is advantageous. However, it is not yet clear whether protection of thymine residues [as 4-*O*-phenylthymine][14] (entries nos. 1 and 3) is also worthwhile.

TABLE 1. Preparation of Fully- and Partially-Protected Oligodeoxyribonucleotides by the "Filtration" Method

Entry No.	Product[a]	No. of Cycles	Wt of Product Isolated (mg)	Overall Yield[b] (%)	Average % Yield/Cycle
1	HO-*Ap*G*p*C*p*T*p*C*p*G-Bz	5	240	32	80
2	HO-TpTpTpTpTpT-Ac	5	177	30	78.5
3	HO-*Gp*C*p*A*p*T*p*G*p*C-Bz	5	193	25	76
4	HO-*Gp*TpA*p*C*p*C*p*G-Bz	5	260	35	81
5	HO-*Cp*A*p*C*p*C*p*A*p*T-Ac	5	185	27	77
6	HO-*Gp*C*p*G*p*C*p*G*p*C-Bz	5	360	40	83
7	HO-*Gp*G*p*G*p*C*p*C*p*C-Bz	5	380	42	84
8	Px-TpTpTpTpTpTp-Dnb	5[c]	210	28	77.5
9	Px-*Ap*A*p*TpTp*C*p*G*p*-Dnb	5[c]	270	32	80

[a]See reference 5 for a key to the abbreviations. [b]This yield is based on 0.25 mmol of starting material (i.e. 3'-protected nucleoside derivative). [c]Step (iii) of the final synthetic cycle was omitted.

Although the yield of, for example, pure unblocked d[ACGTCG] was effectively 2,700 A_{257} units [450 A_{257} units were obtained (see above) by unblocking one-sixth of the material obtained (Table 1, entry no. 1)], there should be no difficulty in scaling up the "filtration" method. As well as leading to much larger quantities of purer oligonucleotides than any solid support procedure, the "filtration" method has the further advantage that it leads to

protected oligonucleotides which can be further extended by stepwise[15] or block condensation reactions. Thus, Px-*ApApTpTpCpGp* [0.20g, 0.06 mmol; obtained by removal[7] of the 3'-(2,4-dinitrobenzyl) (Dnb) protecting group from Px-*ApApTpTpCpGp*-Dnb (Table 1, entry no. 9), HO-*ApGpCpTpCpG*-Bz [(6), entry no. 1; 0.14g, 0.05 mmol] and MSNT (0.142g, 0.48 mmol) were allowed to react together in pyridine (2.0 ml) for 20 min to give Px-*ApApTpTpCpGpApGpCpTpCpG*-Bz. The products were then subjected to what are essentially steps (ii) and (iii) of the standard synthetic cycle (see above) to give HO-*ApApTpTpCpGpApGpCpTpCpG*-Bz, which was isolated as a precipitated solid (0.18g, 62%). Unblocking of this material (0.02g) by the above three step procedure gave d[AATTCGAGCTCG]. The latter dodecanucleoside undecaphosphate, which is an *Eco* RI $\rightarrow$ *Sst* I restriction site adaptor, was purified by chromatography on DEAE Sephadex A25 (elution profile illustrated in Figure 2): 175 A_{257} units of h.p.l.c. pure (see Fig. 2 inset) material, which gave rise to the expected products on enzymatic digestion[13], were obtained.

Acknowledgement. We thank the Science and Engineering Research Council for generous financial support.

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[4] Other workers [G.R. Gough, M.J. Brunden, J.G. Nadeau, and P.T. Gilham, *Tetrahedron Lett.*, 23, 3439 (1982)] have based a rapid synthesis of oligonucleotide blocks on the possibility of removing the excess of phosphodiester by aqueous extraction.

[5] 9-Phenylxanthen-9-yl[6] and 2,4-dinitrobenzyl[7] are abbreviated to Px and Dnb, respectively. 6-*N*-Pivaloyl-2'-deoxyadenosine, 4-*N*-benzoyl-2'-deoxycytidine, 2-*N*-phenylacetyl-2'-deoxyguanosine and thymidine residues are represented by *A*, *C*, *G* and T, respectively, 6-*O*-(2-nitrophenyl)-2-*N*-phenylacetyl-2'-deoxyguanosine and 4-*O*-phenylthymidine residues are represented by *G* and *T*, respectively. Phosphate residues which are protected by 2-chlorophenyl groups are represented by *p*.

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[9] G.R. Owen, C.J. Ransom, C.B. Reese, J.H. van Boom, and J.D.H. Herschied, *Synthesis*, 704 (1974).

[10] In cycles involving a Px-*Cp* building block, removal of the 9-phenylxanthen-9-yl protecting group can take as much as 15 min.

[11] Dimers, trimers and tetramers were eluted with *ca.* 30 ml of tetrahydrofuran-pyridine (3:1 v/v); pentamers and hexamers required 50 ml. None of the remaining phosphodiester was eluted from the column.

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[13] The unprotected oligodeoxyribonucleotide underwent complete digestion to give monomers both in the presence of calf spleen and snake venom phosphodiesterases. The expected nucleoside ratios were obtained when the snake venom phosphodiesterase digest was further treated with alkaline phosphatase.

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[15] Rapid chromatography on Merck Kieselgel 60 [Art. 7729] is recommended after 5 synthetic cycles; the stepwise addition of monomers can then be continued. Preliminary experiments suggest that 5 synthetic cycles based on dinucleotide phosphodiester building blocks, leading directly to at least undecamers, is also feasible.

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