Volume 6 Number 4 April 1979

Nucleic Acids Research

Rapid synthesis of oligodeoxyribonucleotides III. Effect of added carboxylate ion on the efficiency of internucleotide bond formation. Solid-phase synthesis of the dodecanucleotide, d(pT-A-A-C-T-G-C-T-C-A-C-T)

M.J.Gait and R.C.Sheppard

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK

Received 14 February 1979

ABSTRACT

In solid-phase oligodeoxyribonucleotide synthesis the addition of benzoic acid to internucleotide phosphodiester bond-forming reactions lowers coupling yields by a far greater amount than anticipated on a simple nucleotide-carboxylate molar ratio. High coupling yields were consistently obtained in synthesis of the dodecanucleotide, d(pT-A-A-C-T-G-C-T-C-A-C-T), using rigorously purified nucleotide starting materials.

INTRODUCTION

We have previously described a practical method for solid-phase synthesis of oligodeoxyribonucleotides up to 9 units long by a sequential phosphodiester approach on a polyamide resin.[1,2] In contrast to syntheses using other supports, good coupling yields were maintained, such that the major component of the final product mixture was the desired oligonucleotide. The maintenance of high yields is a crucial factor in determining the length of oligonucleotide attainable by this approach. We now report the synthesis of the dodecanucleotide, d(pT-A-A-C-T-G-C-T-C-A-C-T) (designed as a sequencing primer for the mouse immunoglobulin light chain mRNA[3]), in which high coupling yields were consistently obtained by use of nucleotide derivatives of the highest purity.

DISCUSSION

The methodology for one cycle of nucleotide addition involves reaction of the polydimethylacrylamide resin (prefunctionalised to provide a reversible linkage for oligonucleotide chains) with a ca. 10 fold excess of the appropriately N-protected 3'-O-acetyl nucleoside-5'-phosphate preactivated with triisopropylbenzenesulphonylchloride (TPS). After mandatory treatment with aqueous pyridine the resin is dried with a 10% solution of phenyl isocyanate in pyridine and acetyl groups removed by reaction with sodium methoxide

in pyridine/methanol (1:1). The resin is then ready for the next cycle of nucleotide addition[1,2] (Fig. 1).

Previously nucleotide monomers had been 3'-O-acetylated in ca. 1 mmole quantities just prior to activation. When prepared on a 5-10 mmole scale for multiple use it was now observed that coupling yields became highly variable, dropping on occasion well below 50%. No correlation was found with nucleotide storage time but yields were self-consistent within a particular batch. Although UV and chromatographic data did not differ markedly between batches of the same nucleotide or between those prepared on different scale, the possibility existed of the presence of highly reactive impurities in varying amounts. Because of the use of necessarily large excesses of monomers these impurities might cause a much larger reduction in coupling yield than their relative molar proportion would indicate.

Carboxylic acids or their derivatives were likely candidates, which might acylate hydroxyl groups on the resin in the presence of TPS. Hydroxyl groups would be regenerated from the resultant esters by subsequent sodium methoxide treatment and could give rise to the formation of failure sequences (sequences missing an internal nucleotide residue) during further coupling reactions. Strong evidence for this had come from a study of chromatography patterns of products obtained during solid-phase synthesis. Moreover, phenyl isocyanate treatment was seemingly ineffective in preventing formation of failure sequences.

Acidic impurities (acetate, benzoate, anisate and isobutyrate) are well-

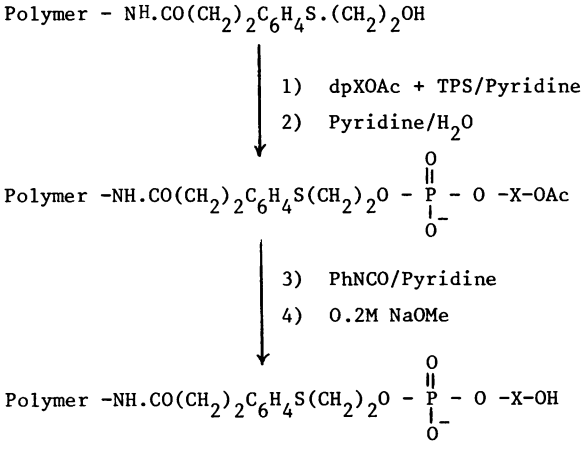

Fig. 1. (X = T, bzA, ibG or anC)

known by-products in the acylation reactions involved in preparation of the nucleotide derivatives, but their competitive effect in coupling reactions has not to our knowledge been studied. Accordingly in a model experiment one complete cycle of nucleotide addition was carried out on functionalised resin using pyridinium 3'-O-acetylthymidine-5'-phosphate (dpT-OAc) as monomer. After de-acetylation the resin was treated with 10 equivalents of a mixture of dpT-OAc and (carboxyl-^{14}C)-benzoic acid (93:7) pre-activated as usual with TPS. After extensive resin washing with pyridine and aqueous pyridine only 73% of the radioactivity was found in the filtrate. Treatment of the resin with 0.2 M sodium methoxide in methanol/pyridine (1:1) liberated most of the rest of the radioactivity (22%). Further analysis of the nucleotidic products on the resin showed a coupling yield of only 50% in the presence of benzoate compared with yields of 76 and 80% obtained using the same batch of nucleotide derivative in control reactions.

The experiment demonstrates the disproportionately large inhibition of coupling caused by added benzoate. Thus far no free benzoate has been found in dpbzA-OAc by hplc analysis on μBondapak C18[4], but the possibility of the presence of impurities containing reactive, covalently bound benzoyl groups (e.g. on the phosphate moeity) is now under investigation. The most likely major impurity is pyridinium acetate, formed during 3'-O-acetylation of the nucleotide derivatives. Although we have as yet no reliable assay system for this or other reactive impurities we have observed that they are effectively removed by extraction of an aqueous pyridine solution of the nucleotide derivatives with methylene chloride/n-butanol (3:1) prior to final precipitation with ether. Nucleotides purified in this way gave consistently high yields in the dodecanucleotide synthesis described below.

The dodecanucleotide, d(pT-A-A-C-T-G-C-T-C-A-C-T)

Twelve cycles of nucleotide addition were carried out on prefunctionalised polyamide resin with the aid of a modified Beckman Solid Phase Peptide Synthesiser[2] at a rate of one unit per day. After each cycle samples of resin were taken for analysis or product isolation. Oligonucleotides were cleaved from the resin as described previously[1,2] and analysed by hplc on Partisil 10SAX. An estimate of coupling yield was thus obtainable within 5 hours of resin sampling and did not delay the synthesis. The first five coupling yields were 92, 90, 87, 92 and 84% respectively. After cleavage from samples of resin taken after 6, 8, 10 and 12 cycles hexa, octa, deca, and dodecanucleotides respectively were isolated by 1) preparative hplc on Partisil 10SAX at pH 6.8, 2) desalting on Biogel P2, 3) deprotection with

TABLE

Cycle	Resin taken (mg)	Total A_{270} units cleaved	A_{270} units isolated protected oligonucleotide	%	A_{260} units isolated deprotected oligonucleotide	%
6	17.0	47.5	11.6	24	5.1	49
8	24.5	54.6	7.7	14	2.9	44
10	56.6	172.5	10.3	6	2.7	36
12	75.6	150.0	8.3	5	0.7	11

concentrated ammonia solution at 50° for 4.5 h in a sealed tube, 4) preparative hplc on Partisil 10SAX at pH 4.0 at 50° in the presence of 7M urea and 5) final desalting on Biogel P2. Amounts obtained and yields are summarised in the Table.

The chromatographic pattern of the base-protected octanucleotide (Fig. 2) illustrates the effect of the higher yields obtained here compared to the earlier synthesis of the protected octanucleotide d(pT-ibG-ibG-anC-anC--anC-anC-bzA-T) (cf. Fig. 12 in ref. 2). Moreover, reduction of the ethanol content of the elutant to 5% enhanced resolution of closely related side-products. These impurities increase drastically as the chain is extended (Fig. 3 and 4).

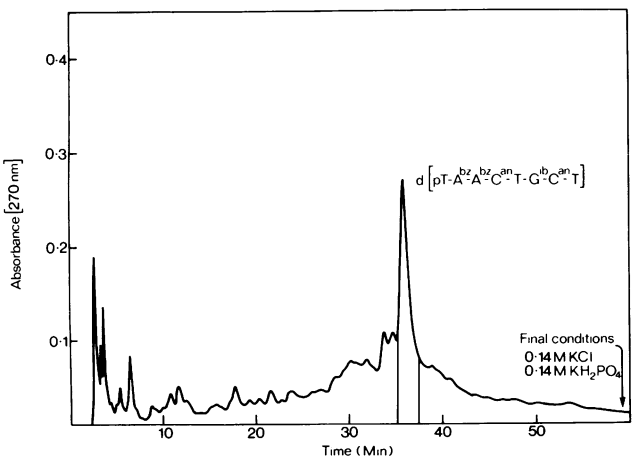

Fig. 2 Chromatographic pattern of the protected octanucleotide.

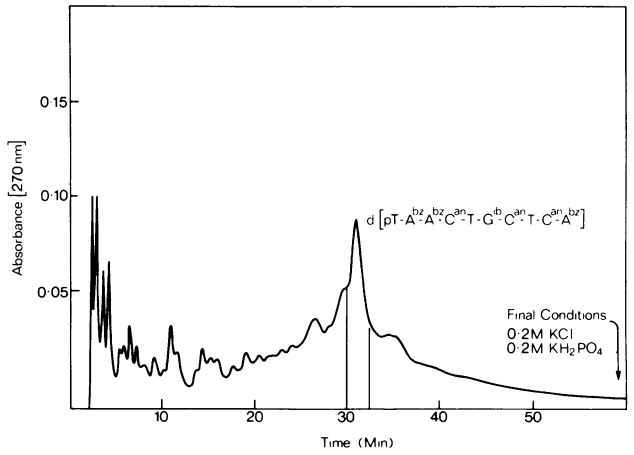

Fig. 3 Chromatographic pattern of the protected decanucleotide.

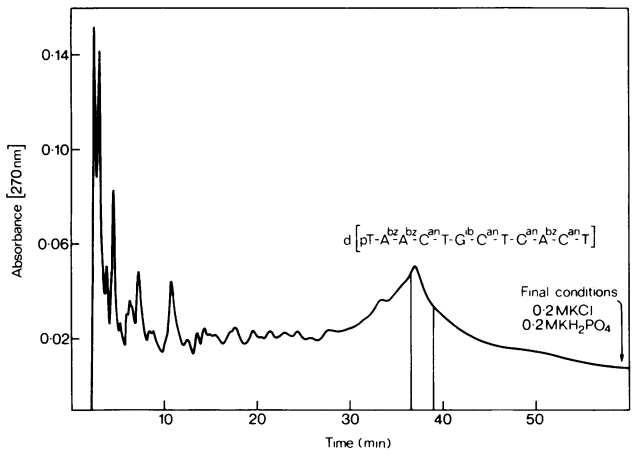

Fig. 4 Chromatographic pattern of the protected dodecanucleotide.

Deprotected oligonucleotides were isolated by preparative ion-exchange hplc under denaturing conditions (Figs. 5-7). Although extremely rapid the resolving power deteriorates at longer chain lengths.

A ^{32}P-labelled sample of dodecanucleotide was found to consist of two components. The major product (60 - 70% of radioactivity) had the correct sequence. The minor product ran slower on gel electrophoresis (pH 8.3) but faster on cellulose acetate electrophoresis (pH 3.5). It appeared to consist of a mixture of sequences of structure, dpT-A(-----)T-G-C-T-C-A-C-T, where the section in parenthesis could not be unequivocally determined. Another sample of deprotected dodecanucleotide was purified by hplc on μBondapak C18[4] (instead of ion exchange) and showed after ^{32}P-labelling only

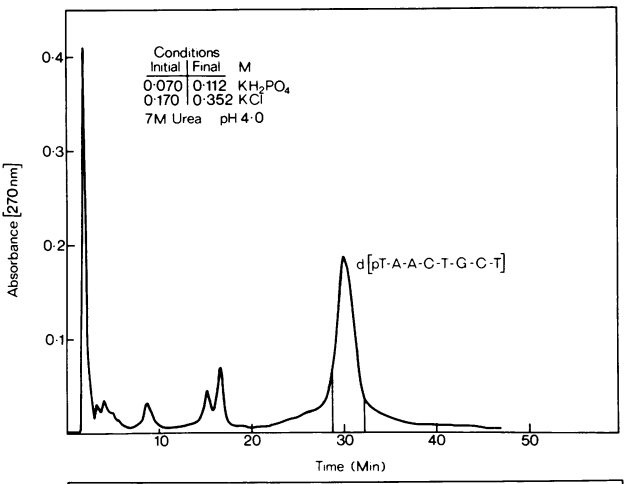

Fig. 5 Chromatographic pattern of the deprotected octanucleotide.

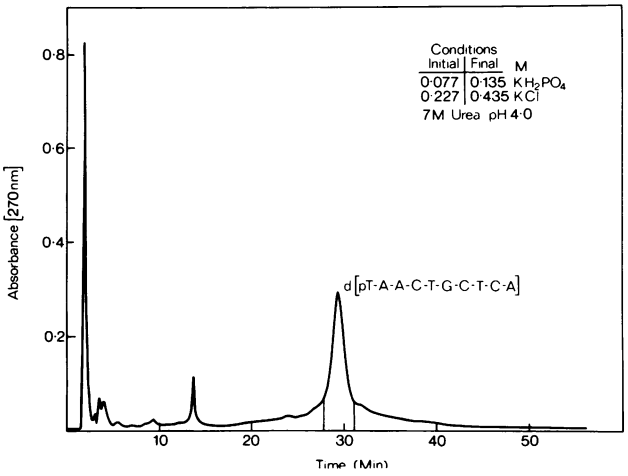

Fig. 6 Chromatographic pattern of the deprotected decanucleotide.

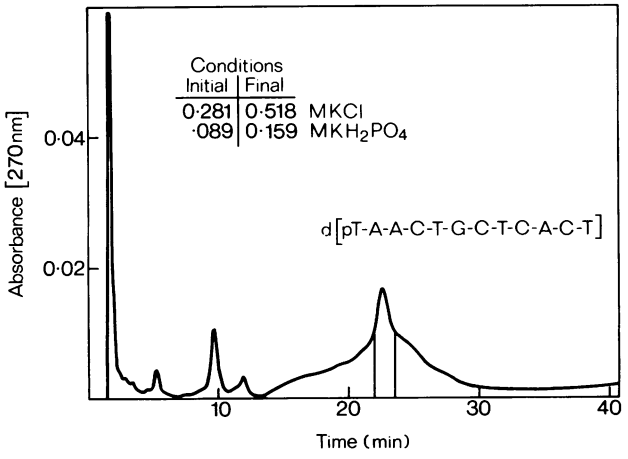

Fig. 7 Chromatographic pattern of the deprotected dodecanucleotide.

one component. Our initial experience suggests that reversed phase hplc is superior to ion exchange for the separation of other deprotected oligonucleotides.

The first solid-phase synthesis of a dodecanucleotide described above was made possible by the maintenance of consistently high coupling yields resultant from the use of highly purified nucleotide derivatives. This observation may have general relevance to phosphodiester approaches in solution where large excesses of oligonucleotide blocks are also customarily used. In our experience, however, the present solid-phase methodology is best suited to oligonucleotides not exceeding 10 residues, because of accumulation of side products and increased isolation problems at longer chain lengths. The use of block addition, perhaps involving a phosphotriester methodology, may well extend the synthetic range available to the solid-phase approach, and is under investigation.

Experimental Section

Materials and methods not otherwise mentioned here are as described previously.[1,2]

Functionalisation of the resin

This was carried out as described previously[1] except that a time of 75 min was found to be sufficient for reaction of activated ester, $HO(CH_2)_2S.C_6H_4.(CH_2)_2$-$COOC_6Cl_5$, with the resin.

General procedure for 3'-acetylation of N-protected 2'-deoxy nucleotides

The nucleotide derivative as its pyridinium salt (2 g) was dissolved in dry pyridine (300 ml) and the volume reduced to 150 ml by rotary evaporation. The solution was cooled in an ice bath, acetic anhydride (40 ml) added and the mixture left at room temperature in the dark for 4 h in the absence of moisture. The mixture was cooled to -30^{o}, methanol (80 ml) added and the mixture allowed to warm carefully to room temperature. After 30 m water (100 ml) was added and the mixture left in the dark for 16 h. The mixture was evaporated with repeated addition of dry pyridine to ca. 50 ml and product precipitated by dropwise addition to dry ether (1 ℓ). The collected precipitate was dissolved in pyridine/water (1:4) (150 ml) and extracted with 4 x 100 ml CH_2Cl_2 - - n-butanol (3:1). The aqueous layer was evaporated as before to an anhydrous pyridine solution (50 ml) and product precipitated with ether (1ℓ). The precipitate was collected and dried in vacuo. Yield 1.7 - 2.0 g. Tlc homogeneous on cellulose in ethanol/1M ammonium acetate (pH 7.5) (7:3).

Hplc of oligonucleotides

Hplc was carried out using two M6000A pumps, a 660 solvent programmer and a U6K injector (Waters Associates) and a Cecil 212 variable wavelength spectrophotometer. Base-protected oligonucleotides were chromatographed on

Partisil 10SAX prepacked columns (Whatman) of type PXS (analytical) or M9 (preparative) at a flow rate of 2 ml min^{-1}. Linear, convex or concave gradients could be generated using any initial and final mixture of two buffers (A: 0.007 M KCl, 0.007 M KH_2PO_4 (pH 6.8) - 5% ethanol; B: 0.2 M KCl, 0.2 M KH_2PO_4 (pH 6.8) - 5% ethanol). Generally 0.2 - 2.0 A_{270} of oligonucleotide mixture was injected on analytical separations. Preparative columns could handle up to 150 A_{270} units of an oligonucleotide mixture per injection but best results were obtained at 50 - 100 A_{270} units. 5 - 7 injections were usually enough to complete most preparative separations. Deprotected oligonucleotides (up to 5 A_{270} per injection) were chromatographed on Partisil 10SAX using a PXS column jacketed at 50° (buffer A: 0.02 M KCl, 0.02 M KH_2PO_4, 7 M urea (pH 4.0); buffer B: 0.85 M KCl, 0.25 M KH_2PO_4, 7 M urea (pH 4.0)). Reversed phase chromatography was carried out on a μ-Bondapak C18 column (3.9 x 300 mm) with gradients of acetonitrile in 0.1 M aqueous ammonium acetate.[4]

An experiment to determine the effect of benzoic acid addition on a nucleotide coupling reaction.

0.25 g of polyamide resin (0.338 meqg^{-1} of amino groups) was functionalised as described previously[1] and one cycle of nucleotide addition carried out (beginning step 9, table 1, reference 2) using pyridinium 3'-O-acetylthymidine-5'--phosphate (0.80 mmole) preactivated with TPS (1.35 mmole) in the coupling step. (NB. This nucleotide batch had not been purified by extraction with organic solvent and had given 76 and 80% yields in control coupling). A manual bench synthesis apparatus was used in this experiment.[1] In the second cycle the same batch of nucleotide derivative (0.825 mmole) and (carboxyl-^{14}C) benzoic acid (0.06 mmole, specific activity 2.94 x 10^9 cpm $mmole^{-1}$) preactivated with TPS (1.35 mmole) were used in the coupling step. Nine per cent of this mixture (1.6 x 10^7 cpm) was held up in the delivery system and did not enter the reaction vessel. After coupling the resin was washed with 1) 5x pyridine for 2', 2) 2x pyridine/water (4:1) for 5', 3) 1x pyridine/water for 2 days, 4) 5x pyridine for 2', 5) 5x pyridine/methanol (1:1) for 2', 6) 2x 0.2 M sodium methoxide in pyridine/methanol for 5', and 7) 5x pyridine/methanol for 2'. Steps 1 - 4 eluted 11.59 x 10^7 cpm (73%). Step 5 eluted only background cpm. Steps 6 and 7 eluted 3.36 x 10^7 cpm (22%). Further alkaline treatment and washing eluted only background cpm. Analysis of a sample of resin showed dpT/d(pT-T) of 49.7:50.3 (hplc conditions: 60 min linear gradient, 0-35% buffer).

Preparation of the dodecanucleotide, d(pT-A-A-C-T-G-C-T-C-A-C-T) and synthetic intermediates.

0.35 g of prefunctionalised polyamide resin (0.338 meqg^{-1} of amino groups)

was treated with twelve cycles of nucleotide addition using a modified 990 Beckman Solid-Phase Peptide Synthesiser.[2] 10 ml volumes of solvent per washing and 1.0 mmole quantities of the appropriate nucleotide derivative were used in each cycle. Each cycle took 24 hours and samples of resin (1 - 2 mg) were taken for analysis after every nucleotide addition. Larger samples of resin were taken after cycles 6, 8, 10 and 12 (Table) and oligonucleotides cleaved as described.[1,2] Hplc conditions for the base-protected oligonucleotides: cycles 2 and 3, 60' linear gradient, 0-35%; cycles 4 - 6, convex gradient (5), 0-35%; cycles 7 and 8, linear gradient, 5-35%; cycle 10, linear gradient, 10-50%; cycle 12, linear gradient, 17-50%. After preparative runs the pooled peaks were desalted on Biogel P2 and deprotected with concentrated ammonia solution at 50° for 4.5 hours in a sealed tube. After evaporation the product was dissolved in water ready for further hplc. (Conditions: hexa, 60' linear gradient, 20-35%; octa, linear gradient, 20-40%; deca, linear gradient 25-50%; dodeca, linear gradient, 30-60%). After peak pooling the oligonucleotides were desalted on Biogel P2 and stored frozen in aqueous solution.

A sample of dodecanucleotide was dephosphorylated using bacterial alkaline phosphatase, end-labelled using α-^{32}P-ATP and T4 DNA kinase and run on a thin 20% acrylamide gel at 1.5 kv for 3.5 h under denaturing conditions at pH 8.3.

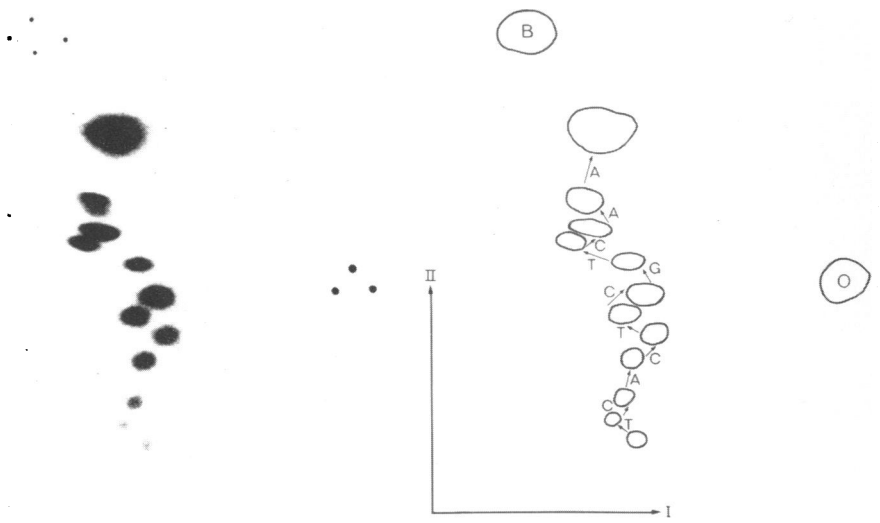

Fig. 8 Fluorograph from sequence analysis of the major band of d(pT-A-A-C-T-G-C-T-C-A-C-T). B = blue dye, O = orange dye.

Two bands were seen which were eluted and sequenced by partial snake venom phosphodiesterase treatment followed by 2-dimensional chromatography. The pattern of spots of the major band (Fig. 8) confirmed the expected sequence. The minor band was identical in sequence except for unassignable anomalies at the 3rd and 4th base from the 5' end. Dodecanucleotide isolated by reversed phase hplc showed after ^{32}P-labelling only one component having the expected sequence.

Acknowledgement

We thank Dr. Pamela Hamlyn for help with the sequence analysis of the synthetic dodecanucleotide.

REFERENCES

1. Gait, M.J. and Sheppard, R.C. (1977) Nucleic Acids Research, 4, 1135-1158.
2. Gait, M.J. and Sheppard, R.C. (1977) Nucleic Acids Research, 4, 4391-4410.
3. Hamlyn, P.H., Brownlee, G.G., Cheng, C-C., Gait, M.J., and Milstein, C. (1978) Cell, 15, 1067-1075.
4. Fritz, H-J., Belagaje, R., Brown, E.L., Fritz, R.H., Jones, R.A., Lees, R.G., and Khorana, H.G. (1978) Biochemistry, 17, 1257-1267.