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SOLID PHASE SYNTHESIS OF 5'-PHOSPHATE LABELLED POLYNUCLEOTIDES

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Abstract - The solid phase synthesis of non-radioactive 5'-phosphate labelled polydeoxyribonucleotides is described, based on a single polymeric support prefunctionalized with a marking molecule.

Synthetic polynucleotides of biologically interesting DNA sequences are becoming important tools not only in molecular biology, but also in applied fields like diagnostic medicine. This has created a demand for stable, non-radioactive probes, onto which reporter groups, such as biotin or a fluorophore, are introduced[1].

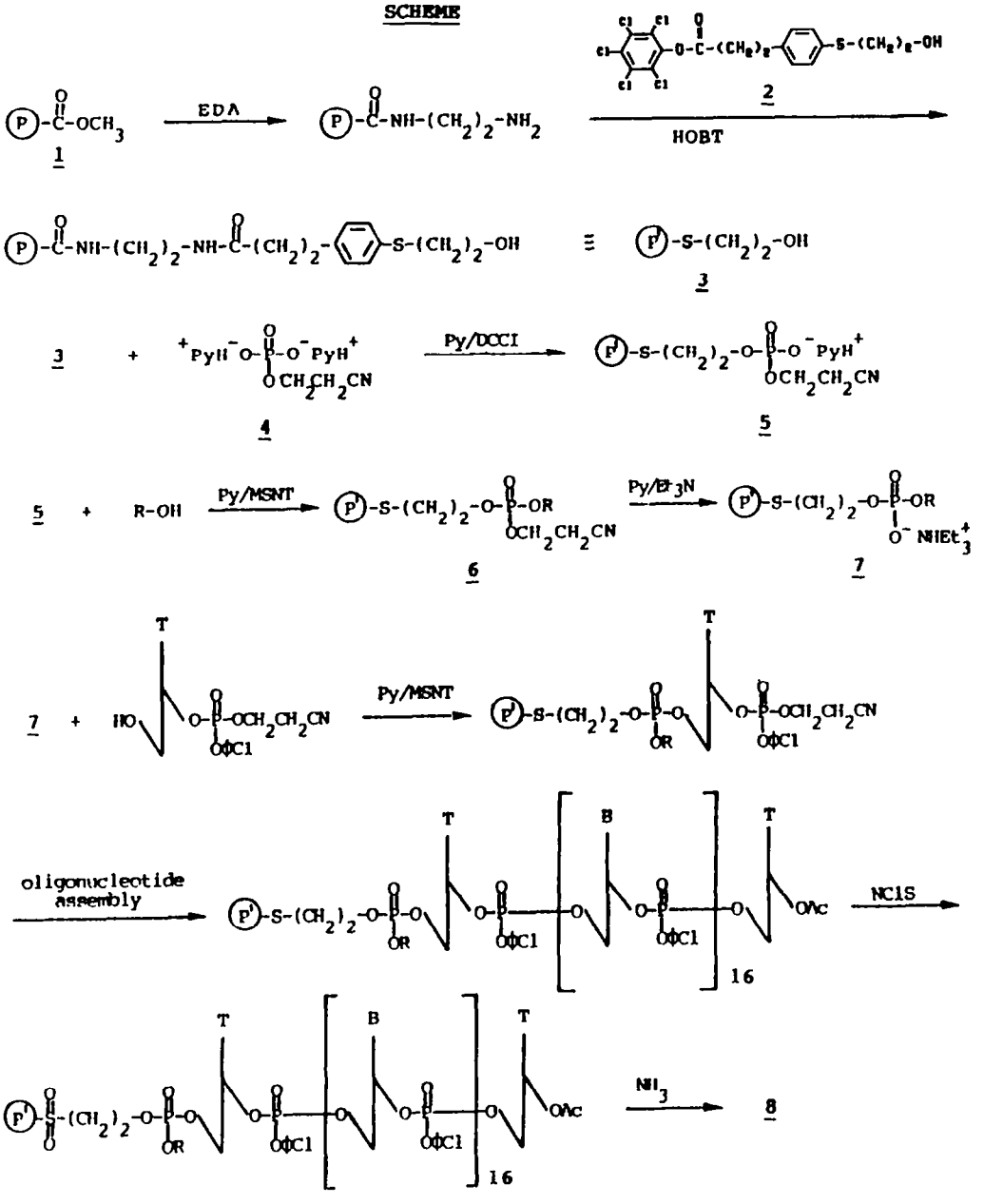
Methods have been described for the preparation of non-radioactive probes whose hybridization properties are not effected and the attachment of the markers was achieved *via* a reaction involving the 5'-termini of previously synthesized oligonucleotides[2].

In this paper we describe a simple and rapid method for the synthesis of labelled, non-radioactive probes, which makes use of a pre-functionalized solid support suitable for any sequence of the oligonucleotide to be prepared. This procedure, which allows the preparation of a DNA fragment covalently bonded through a 5'-phosphate group to any of a large variety of organic molecules, is based on the elongation of the chain in the 5'- to 3'-end direction and on a phosphate solid phase link already utilized for the synthesis of unprotected polynucleotides anchored to a solid support[3].

The efficiency of the method was checked by the synthesis of the mixture of labelled 18-mers 5'-acridinylphosphate-dTCATCAT$^{T}_{C}$TG$^{A}_{G}$TT$^{A}_{G}$CA$^{A}_{G}$T (**8**) useful for detecting mRNA of human pancreatic RNAase.

Resin (commercially available polyacrylamido acryloylsarcosinemethylester, **1**, 0.35 meq/g) was prefunctionalized with ethylenediamine and then was reacted with pentachlorophenyl 4-(2-hydroxyethylthio)dihydrocinnamate (**2**, synthesized according to the procedure of Gait and Sheppard[4]) in the presence of 1-hydroxybenzotriazole (see scheme). The polymer thus obtained (**3**) was treated with cyanoethylphosphate, pyridinium salt[5] (**4**) in dry pyridine, using N,N'-dicyclohexylcarbodiimide as a condensing agent. After three days at room temp the resin (**5**) was washed with

SCHEME



Ⓟ = Polyacrylamide resin
DCCI = N,N'-dicyclohexylcarbodiimide
EDA = ethylenediamine
MSNT = 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole
NClS = N-chlorosuccinimide
OHBT = 1-hydroxybenzotriazole
Py = pyridine
R-OH = 4-hydroxyacridine
T = thymine
B = protected base
ΦCl = o-chlorophenyl

pyridine, ethanol and then exhaustively with dry pyridine.

At this stage any organic molecule capable of reacting with an activated phosphodiester function to give a derivative stable during the chain assembly and the final deprotection stage, can be introduced by leaving it in contact with the resin in the presence of a coupling agent in dry pyridine.

Starting from a resin containing 0.25 meq/g of phosphate group (measured by standard procedure[6]), we obtained a polymer which showed a nearly quantitative incorporation of 4-hydroxyacridine (determined spectrophotometrically by detaching acridine phosphate from a weighted sample of the resin by a basic treatment after a mild oxidizing step).

This functionalized support (6) can be dried, stored and used as required for the synthesis of labelled polydeoxyribonucleotides of any sequence, as illustrated here.

After swelling the resin in dry pyridine the synthesis of the oligonucleotide can start and will continue until the chain reaches the desired length. Each cycle comprises only two reactions, deprotection and coupling, performed in the same solvent[7]. The phosphate protecting ß-cyanoethyl group is removed by a mild basic treatment with Et_3N/Pyridine. In the coupling step the solution of a N-protected-2'-deoxynucleoside-3'-O-(2-chlorophenyl-2-cyanoethyl) phosphate is left in contact with the resin in the presence of 1-(mesitylenesulfonyl) -3-nitro-1,2,4-triazole. Appropriate washing steps with pyridine have to be included.

The removal of the polynucleotide from the resin is achieved by a two steps reaction sequence: i) oxidation of the thioether to sulfone by reaction with N-chlorosuccinimide in a phosphate buffer for 30 min, followed by ii) a ß-elimination catalyzed by conc. ammonia for 1 h at room temp. Then the filtrate is kept at 50°C for 5 h to achieve the complete removal of the protecting groups from the polynucleotide.

For the synthesis of the above mixture of 18-mers we used 100 mg of functionalized support (6) and performed 18 cycles using the appropriate nucleotide or equimolecular mixture of two nucleotides (T/C or A/G) when an ambiguity is present. Since there is no significant difference of reactivity between T and C and between A and G[8], the synthetic mixture of 18-mers will contain almost equimolecular amounts of each sequence.

The chain assembly was monitored by FPLC analysis[9] of the nucleotidic material cleaved from a small sample (1-2 mg) of the resin taken at particular intermediate stages[10]. The coupling yields, measured by the chromatographic profiles, were consistently in the order of 92-95%.

In the last cycle 3'-O-acetyl-2'-deoxythymidine was added in order to obtain a mixture of 3'-OH-free oligodeoxyribonucleotides. After the detachment and the deprotection of the mixture of polynucleotides, the crude product was chromatographed by FPLC (Fig. 1) and the product peak was collected, desalted on biogel P2 and analyzed by gel electrophoresis and enzymatic digestion with S1 nuclease (Fig. 2).

8 (calcd yield 11.0% of isolated product) showed a fluorescence spectrum simi-

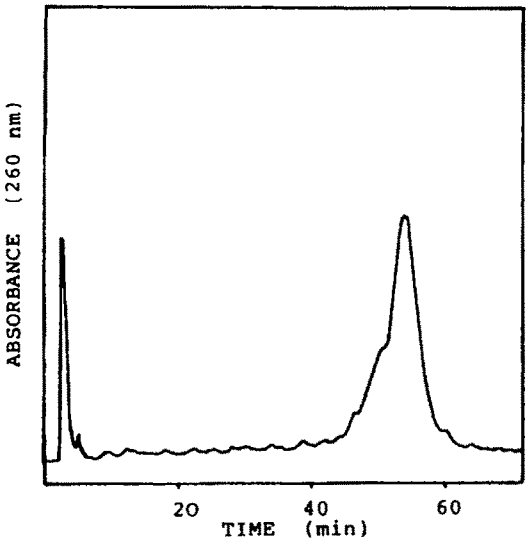


Fig. 1. FPLC profile of crude 8 on a Mono Q R 515 column.

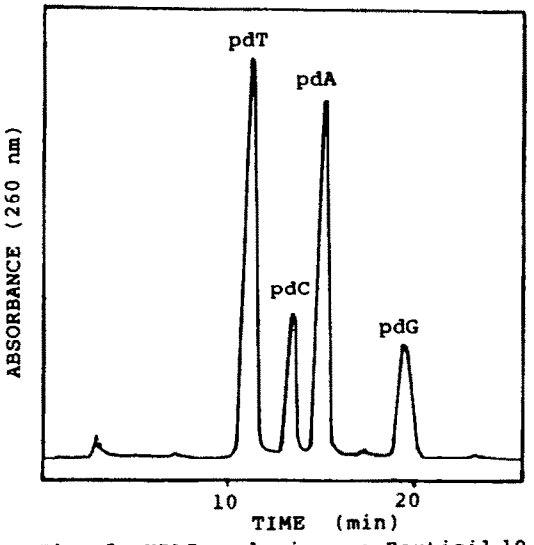


Fig. 2. HPLC analysis on a Partisil 10 SAX column of the product of enzymatic digestion of 8 with S1 nuclease.

lar to that of acridinylated deoxythymidilic acid[10]. Preliminary results suggest that this non-radioactive probe will be useful for the screening of m-RNA colonies.

In a different experiment we allowed the phosphorylated resin (5) to react with 2-(biotinylamido)ethanol and performed a complete coupling cycle utilizing 2'-deoxythymidine-3'-O-(2-chlorophenyl-2-cyanoethyl) phosphate. The analysis of the detached product revealed it to be a biotinylated nucleotide. Spectral data did not enable us to unambiguously determine the oxidation state of the sulfur. However this should be irrelevant to the standard avidin test[11]. Further experiments are in progress.

EXPERIMENTAL

UV spectra were recorded on a Perkin-Elmer 550 S spectrophotometer. ^{1}H NMR spectra were measured on a Bruker WM-250 spectrometer in CD_3OD solutions. All chemical shifts are reported with respect to the residual methanol signal (δ 3.34). Fluorescence spectra were recorded on a Perkin-Elmer LS5 instrument connected with a Perkin-Elmer Data station. Two chromatographic systems were used: (a) a Pharmacia fast protein liquid chromatography (FPLC) system consisting of a gradient programmer with two p-500 pumps equipped with a LKB S UV monitor; and (b) a Varian 5000 liquid chromatograph equipped with a Varichrom UV detector.

All the operations on the resin and the oligonucleotides assembly were carried out in a short glass column equipped with a sintered-glass filter and a stopcok.

Functionalization of the resin.

Commercially available polyacrylamido acryloylsarcosinemethylester (1, 0.35 meq/g, 200 mg) was treated with ethylenediamine (12 ml) overnight under shaking . After filtration the resin was washed with DMF until the eluate gave negative blue colour test with ninhydrine and then was left in contact with a solution of the activated ester 2[4] and 1-hydroxybenzotriazole (both reagents in a 10-fold molar excess over the initial functionality of the resin) in dry DMF (9 ml) for 16 h at room temp under shaking. The resulting polymer (3) was exhaustively washed with DMF, then with anhydrous $CHCl_3$ (5 x 2 min), with dry Et_2O (2 min) and dried *in vacuo*. The reaction with 2 was quantitative as resulted from negative Kaiser test[12] performed on a sample (1-2 mg) of the resin 3.

Phosphorylation of the resin 3.

180 mg of 3 were swollen in dry pyridine and then treated with cyanoethylphosphate pyridinium salt (4, 0.7 mmol)[5] in the presence of N, N'-dicyclohexylcarbodiimide (1.0 mmol) in dry pyridine, under shaking at room temp for 72 h. After washing with pyridine (10 x 2 min), ethanol (5 x 2 min), anhydrous chloroform (5 x 2 min) and anhydrous diethylether (5 x 2 min) the polymer was dried in vacuo.

The incorporation of ß-cyanoethylphosphate (0.25 meq/g) was determined by treating a weighted sample of resin with conc. HNO_3 and then measuring the released orthophosphoric acid by the modified stannous chloride reagent method[6].

Attachment of the marking molecules

100 mg of 5 (0.025 meq), swollen in dry pyridine, were treated with a soln of 4-hydroxyacridine (0.25 mmol) and 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (0.5 mmol) in dry pyridine (2.5 ml) under shaking for 4 h at room temp. After washing with pyridine (10 x 2 min), anhydrous chloroform (5 x 2 min) and anhydrous diethylether (5 x 2 min) the resin was dried in vacuo and stored at -10°C.

The incorporation of 4-hydroxyacridine was estimated spectrophotometrically after detachment of 4-hydroxyacridine phosphate (λ_{max} = 356 nm, ϵ = 4100) as described below for the detachment of oligonucleotides and resulted to be almost quantitative.

The attachment of 2-(biotinylamido)ethanol, prepared from biotin-N-hydroxy-succinimide ester and aminoethanol according to Kempe et al[2], was analogous to that of 4-hydroxyacridine. Also in this case the incorporation should be almost quantitative as judged from the amount of biotinylated nucleotide released in the experimental conditions described below after the coupling reaction with 2'-deoxythymidine-3'-O-(2-chlorophenyl-2-cyanoethyl) phosphate.

Chain assembly.

The acridinylated support 6 (100 mg, 0.023 meq) was swollen by addition of dry pyridine in the column and then treated for 20 min with Et_3N/Py (1:1) at 50°C. After washing with pyridine (10 x 0.5 min) the resin was left in contact with a soln of 2'-deoxythymidine-3'-O-(2-chlorophenyl-2-cyanoethyl) phosphate (0.18 mmol) and 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (0.35 mmol) in dry pyridine (2.5 ml) for 20 min at 50°C. After washing with dry pyridine (10 x 0.5 min) the second cycle was begun. Cycles were continued using the pertinent N-protected 2'-deoxynucleoside-3'-O-(2-chlorophenyl-2-cyanoethyl) phosphate until the required lenght was reached. During the 8th, 11th, 14th and 17th cycles, in the coupling step equimolecular mixtures of T/C, A/G, A/G and A/G components, respectively, were used. In the last cycle 3'-O-acetyl-2'-deoxythymidine was added.

In a different experiment, one cycle was performed on the above biotinylated polymer (50 mg, 0.011 meq) using 2'-deoxythymidine-3'-O-(2-chlorophenyl--2-cyanoethyl) phosphate (0.09 mmol) in the coupling step.

Cleavage from the resin, deprotection and purification of labelled oligonucleotides.

At the end of the 18th cycle of chain assembly, an aliquot of the resin (50 mg) was washed (5 x 2 min), with a soln of 0.1 M potassium phosphate (pH 7.0) in dioxane water (1:1) (buffer A) and then treated (2 x 15 min) with a soln of 0.2 M N-chlorosuccinimide in buffer A (5 ml). After washing steps (5 x 2 min) with buffer A and, successively, with dioxane/water (1:1) (5 x 2 min) the resin was treated with conc. ammonia (5 ml) for 1 h at room temp. After filtration the resin was washed with ethanol/water (2:8) (2 x 2 min). The filtrate and the washings were dried in vacuo and kept at 50°C for 5 h in conc. ammonia (4 ml). After cooling the soln diluted with water (6 ml) was washed with Et_2O (x3) and $CHCl_3$ (x3) and taken to dryness under red press. The released mixture of 18-mers was chromatographed by FPLC using a Pharmacia mono Q R 515 (50 x 5 mm i.d., 10 µm, flow rate of 0.5 ml/min) eluted with a linear gradient from 0 to 80% in 60 min of 0.02 M piperazine-1 M KCl (pH 9.7)-20% acetonitrile (solvent B) in 0.02 M piperazine (pH 9.7)-20% acetonitrile (solvent A). The product peak was desalted on a Biogel P2 (120 x 2 cm) column (eluting solvent: EtOH/H_2O, 2:8). The chain lenght of the purified mixture of 18-mers (calcd yield 11.0%) was confirmed by a polyacrylamide gel electrophoresis in comparison with oligomers of known size whose bands were visualized under UV light by transfering the gel to a 20x20 cm silica gel TLC plate containing a fluorescent indicator (the product band is fluorescent itself when irradiated).

The same procedure was carried out at the end of some intermediate stage (1st,

3rd, 5th, 8th, 13th, 15th)[10] of the synthesis of 8 and for the detachment and deptrotection of the biotinylated deoxythymidilic acid. The resulting nucleotidic material was analyzed by FPLC using the above column (flow rate 0.5 ml/min) eluted with the following linear gradients of solvent B in solvent A: from 0 to 25% in 30 min for the analyses of the 1-ers, 3-er and 5-er; from 0 to 60% in 30 min for the 8-ers, 13-ers and 15-ers. The separation on a preparative scale of the labelled monomers to allow their spectral analyses was performed by reversed phase HPLC [column RP-18 Merck (250 x 10 mm i.d., 7 μm, flow rate 1.0 ml/min) eluted with a linear gradient from 0 to 30% of acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) in 30 min].

Enzymatic digestion of 8.

The purified mixture of 18-mers (2 A_{260} units) was dissolved in 200 l of a soln 280 mM NaCl, 50 mM NaOAc (pH 4.6), 4.5 mM $ZnSO_4$ containing 20 μg/ml of DNA carrier and 200 units of S1 nuclease were added. The mixture was incubated at 37°C for 30 min and heated at 60°C for 5 min.

The direct analysis by HPLC (Fig. 2) [column Whatman Partisil 10 SAX, 250 x 4.6 mm i.d., 10 μm, flow rate 1.0 ml/min; eluted with a linear gradient from 0 to 60% of 0.2 M KH_2PO_4–0.2 M KCl (pH 6.8)–5% EtOH in 0.007 M KH_2PO_4–0.007 M KCl (pH 6.8)–5% EtOH in 60 min] showed that the product was completely digested to give pdT, pdC, pdA and pdG in a ratio (3.00, 1.45, 1.86 and 1.00, calculated by using molar absorption coefficients at 260 nm as 8.8×10^3, 7.3×10^3, 1.54×10^4 and 1.17×10^4, respectively) which is in good agreement with that required by the base composition of 8 (3.00, 1.40, 1.80 and 1.00).

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10. At the level of the first nucleotide (T) the procedure was carried out on a larger amount of the resin to allow ^{1}H-NMR measurements on the detached material. The ^{1}H-NMR spectrum of the purified product showed it to be, as expected, 2'-deoxythymidine-5'-O-(4-acridinyl) phosphate-3'-O-(2-chlorophenyl) phosphate. UV (H_2O): λ max 356nm (ε = 4000); fluorescence (H_2O): λ max (excitation) 246nm, λ max (emission) 442nm; ^{1}HNMR (250 MHz, CD_3OD); nucleotide protons: δ 7.84 (bs, 6-H), 6.32 (dd, J= 6.0 Hz and 6.5 Hz, 1'-H), 5.18 (m, 3'-H), 4.50-4.40 (m, 4'-H and 5'-H_2), 2.42 (m, 2'-H_2) and 1.66 (bs, Me-5); phenyl ring protons: δ 7.57 (bd, J= 7.5 Hz, 6-H), 7.31 (bd, J= 7.5 Hz, 3-H), 7.19 (bt, J= 7.5 Hz, 5-H) and 7.00 (bt, J= 7.5 Hz, 4-H); acridine protons: δ 9.04 (s, 9-H), 8.30 (bd, J= 9.0 Hz, 8-H), 8.13 (bd, J= 9.0 Hz, 5-H), 7.90-7.80 (complex signal, 1-H, 2-H and 3-H), 7.60-7.50 (complex signal, 6-H and 7-H).
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