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A VERSATILE SOLID PHASE METHOD FOR THE SYNTHESIS OF OLIGONUCLEOTIDE-3'-PHOSPHATES

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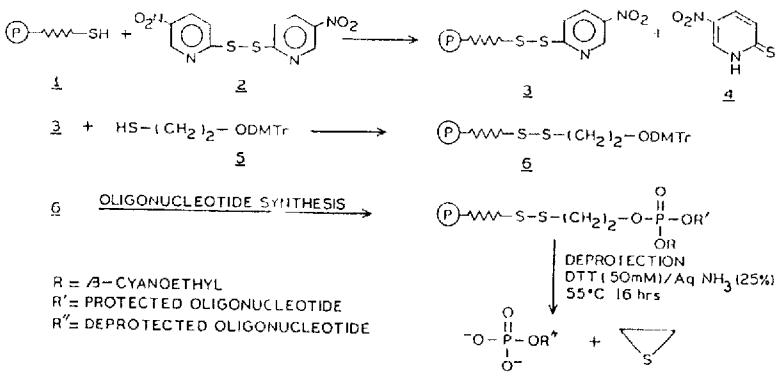
Abstract : The polymer support 6 with 4,4'-dimethoxytrityloxy-2-mercaptoethyl group allows the automated oligonucleotide synthesis giving rise to oligonucleotide-3'-phosphate during final deprotection with ammonical DTT at 55°C.

Currently there is a great interest in the chemical synthesis of oligonucleotides bearing amino, thiol, carboxyl or phosphate group at their 3'- or 5'-positions. In some cases like chemical ligation (1),modifications at internucleotidic linkages (2) and structural studies (3), oligonucleotides with 3'-phosphate group are required. The methods reported (4-6) for the synthesis of oligonucleotide-3'-phosphates via solution or solid phase chemistry, in our opinion, are unsatisfactory for several reasons. The solid supports employed,viz., polystyrene, polyacrylamide, teflon or silica gel are not compatible with phosphoramidite chemistry. Recently, a CPG-based support has also been reported (7) for the synthesis of oligonucleotide-3'-phosphates. However, the functionalization of polymer support is tedious and time consuming.

We describe here a general method involving the use of a universal Controlled-Pore Glass (CPG)- based support compatible with established phosphoramidite approach of DNA synthesis to give rise to oligonucleotide-3'-phosphates during final deprotection.

The functionalization of CPG-based support begins with the reaction of 3-mercaptopropylated-CPG 1 (500 mg, -SH loading 55 µmol / g support) (8) (Scheme I) with five fold excess of 2,2'-dithiobis(5-nitropyridine) (DTNP) 2 in isopropanol (10 ml) for 2h at room temperature. The excess DTNP was removed by washing with isopropanol(2x10 ml) and the polymer support 3 was then allowed to react with five fold excess of 4,4'-dimethoxytrityloxyethyl-2-mercaptan 5 (9) in dichloromethane (10 ml) for 2h at room temperature to obtain the derivatised support 6. The excess reagent 5 was removed by filtration followed by washing with dichloromethane, methanol and acetone (10 ml each). The contents of 4,4'-dimethoxytrityl groups on the support 6 was determined spectrophotometrically after reacting a weighed amount of the support 6 with perchloric acid (10). The loading was found to be 55 µmol/g polymer support. This clearly indicates



SCHEME - 1

that no free sulfhydryl groups are left on the polymer support 6. This was further confirmed by DTNP negative test of the polymer support 6. In a separate experiment the polymer support 6 on exposure to 50 mM DTT solution, pH 8.5 for 2h at 40°C resulted in a complete loss of 4,4'-dimethoxytrityl groups on the support due to the cleavage of disulfide linkage.

In order to test the utility of the polymer support 6 for the synthesis of oligomer-3'-phosphates, two sequences,viz., d(TTT TT-PO_4) X and d(CTC TCT CTC T-PO_4) Y were synthesized, using 1.3 μmol scale on support 6 and 12 μmol of nucleosidephosphoramidites per cycle with a cycle time of 7.5 min following standard protocol (11) on a Pharmacia Gene Assembler Plus. Oligo d(TTT TT) and d(TTT TTT) were also synthesized using the standard 5'-DMTrthymidine support with O-succinate linkage. The coupling efficiency per cycle based upon the released 4,4'-dimethoxytrityl cation exceeded 98.4 % and was found to be identical within experimental error for the oligomers X and Y synthesized using the polymer support 6. It was also demonstrated that the disulfide linkage containing polymer support 6 was stable to the coupling, deprotection, capping and oxidative conditions used in the solid phase phosphoramidite chemistry.

The deblocking of oligomers was carried out by treatment with a solution (2 ml) of 50 mM dithiothreitol in aq. ammonia (25 %) at 55°C for 16h. This one step process allows the removal of the classical protecting groups (2- cyanoethyl from phosphate and acyl from nucleic bases) together with the cleavage of the disulfide bond by DTT (12) and the elimination of 2-mercaptoethyl group (13,14). The ammonia solution was concentrated and desalted on Sephadex G-50 column using 0.1M triethylammonium acetate buffer, pH 7.5. The crude oligomers were purified by anion - exchange chromatography using Mono Q HR 5/5 column on FPLC (Pharmacia). For both of

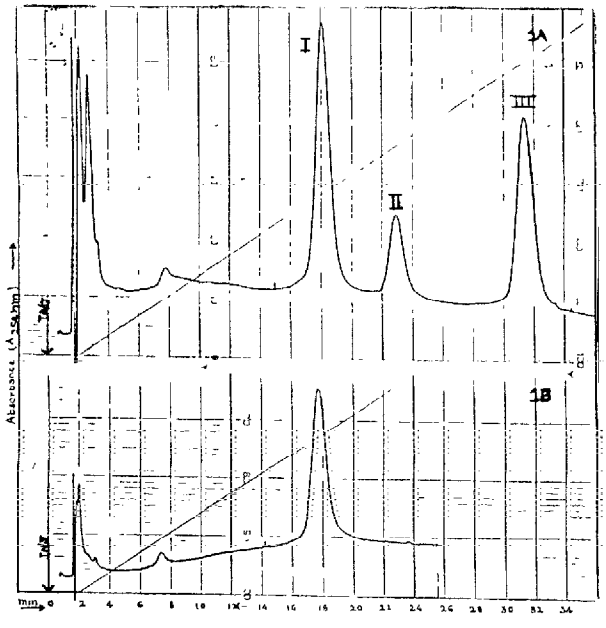


Fig. 1 Anion-exchange FPLC profile of (A) a mixture containing d(TTT TT), d(TTT TT-PO_4) and d(TTT TTT) on a Mono Q HR 5/5 column. Peak I = d(TTT TT); Peak II = d(TTT TT-PO_4); Peak III = d(TTT TTT) and (B) alkaline phosphatase treated d(TTT TT-PO_4). Buffers: A = 0.4M NaCl and 0.01M NaOH, pH 12; B = 0.6M NaCl and 0.01M NaOH, pH 12. Gradient 0 % B for 2 min, 0-100% B in 58 min. Flow rate 1 ml/min. Auf 0.1.

the oligomers X and Y , the major component with highest retention time was collected, concentrated and desalted. The desalted oligomers eluted with the same retention time on reanalysis under identical conditions.

The anion-exchange purified oligomers X and Y were found to be DTNP negative, clearly indicates the absence of 2- mercaptoethyl groups in the fully deprotected oligomers X and Y .

Fig. 1A. shows the anion-exchange FPLC profile of the purified d(TTT TT-PO_4) co-injected with a previously synthesized d(TTT TT) and d(TTT TTT). The oligomer X (0.5 O.D.units) was subjected to dephosphorylation with alkaline phosphatase (1 unit) (7) in 20 µl of digestion buffer (25 mM Tris-HCl, pH =8) at 37°C giving the expected results (Fig. 1B).

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8. Preparation of 3-mercaptopropylated-CPG : Unmodified CPG (Controlled Pore Glass) (5 g, 500 Å) was refluxed with 5 % solution of 3-mercaptopropyltrimethoxysilane in dry toluene (150 ml) for 4 hrs. The CPG was washed with toluene, methanol and acetone (50 ml each) and dried under reduced pressure. Unreacted silanol functionalities were capped following the standard protocol (10).
9. 4,4'-Dimethoxytrityloxyethyl-2-mercaptan was prepared by the reaction of 2-mercaptoethanol (5 mmol) with 4,4'-dimethoxytrityl chloride (12 mmol) in dry pyridine under the exclusion of moisture. The reaction mixture was concentrated and redissolved in chloroform . The organic phase was extracted twice with water and dried (anhydrous sodium sulphate). The S-DMTr group was selectively removed by silver nitrate/ DTT treatment (14). ^{1}H NMR ($CDCl_3$) δ : 2.4 (t, 2H, S-CH_2 -), 3.1 (t, 2H, O-CH_2-), 3.6 (s, 6H, 2xO-CH_3) and 6.7-7.5 (m, 13H, Ar-H).
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