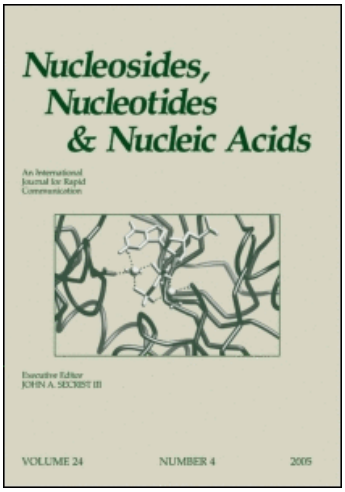

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Ajay Kumar[a]
[a] National Institute Of Immunology, New Delhi, India

To cite this Article Kumar, Ajay(1993) 'A New Solid Phase Method for the Synthesis of Oligonucleotides with Terminal -3'-Phosphate', Nucleosides, Nucleotides and Nucleic Acids, 12: 5, 441 — 447
To link to this Article: DOI: 10.1080/07328319308021214
URL: http://dx.doi.org/10.1080/07328319308021214

NUCLEOSIDES & NUCLEOTIDES, 12(5), 441-447 (1993)

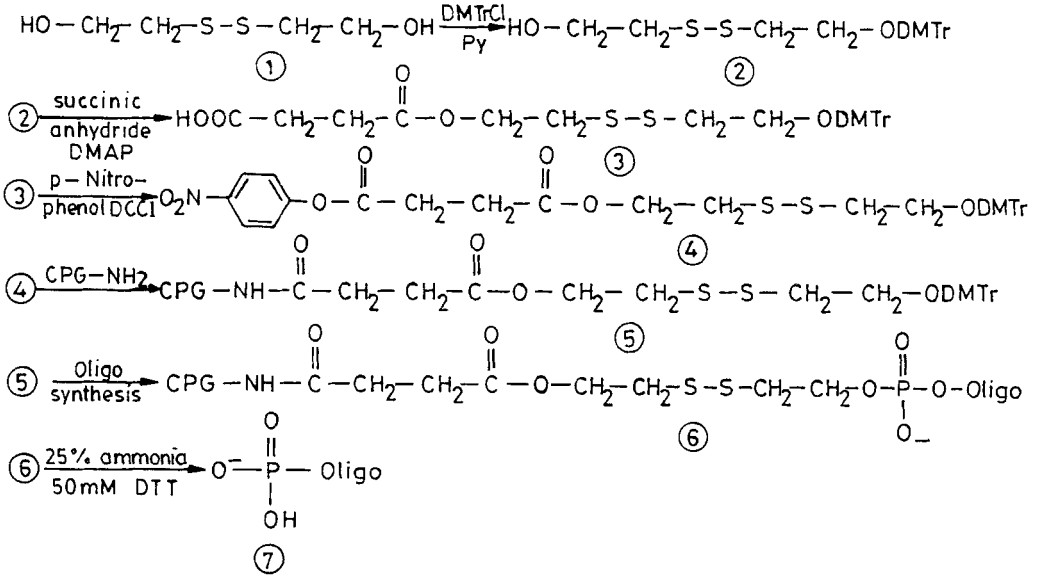
A NEW SOLID PHASE METHOD FOR THE SYNTHESIS OF OLIGONUCLEOTIDES WITH TERMINAL -3'-PHOSPHATE

AJAY KUMAR

National Institute Of Immunology
Shahid Jeet Singh Marg
New Delhi-110067,India

ABSTRACT: A novel method for the synthesis of oligonucleotides with terminal-3'-phosphate using universal CPG polymer support is described.

Recently there has been a great interest in the development of new methods for the introduction of amino, thiol, carboxyl or phosphate group at 3' or 5' end of the oligonucleotides. In some cases like chemical ligation (1), introduction of modified internucleotidic bonds (2) and structural studies (3),oligonucleotides with terminal 3'-phosphate group are needed. The methods for obtaining oligonucleotide 3'-phosphate have been described either in solution (4) or by solid phase synthesis (5,6). In our view methods described so far are unsatisfactory difficult for several reasons. The solid supports used were teflon (7) polystyrene (8,9),polyacrylamide (6) or silica gel (10). These are not compatible with phosphoramidite chemistry,which is mostly used in the automatic synthesiser. Recently CPG based polymer supports have been employed (11,12) for the synthesis of the oligonucleotide-3'-phosphate. The method (12) makes use of thiol derivatised CPG support. We here, describe a simple method for the preparation of CPG support using commercially



SCHEME-1

available amino propyl derivatised CPG-500^{o}A. This support is compatible with the established phosphoramidite chemistry of the internucleotide bond formation that give rise to oligonucleotide-3'-phosphate during final deprotection.

In our method first one hydroxy group of 2-di(hydroxyethyl)disulphide 1 was reacted with 4,4'-dimethoxytritylchloride in pyridine to give 2(R_f.77,TLC Silica,$CHCl_3$:MeOH,95:5)(13). The p-nitrophenol ester 4 (R_f 0.9,TLC Silica,$CHCl_3$:MeOH,95:5) was prepared as described for 5-dimethoxytrityldeoxynucleoside (14) derivatives by succinylation of the hydroxyl function of 2 with succinic anhydride followed by coupling the carboxylic group of 3 with p-nitrophenol in the presence of DCCI. The reaction of the activated ester 4 with amino propyl function of the derivatized CPG-500 ^{o}A gave the support 5. The reactions for the synthesis of 5 are shown in scheme -1.

The amount of dimethoxytrityl cation released by perchloric acid treatment showed a loading of 44 μmole/g. The underivatized amino function of the support were capped by

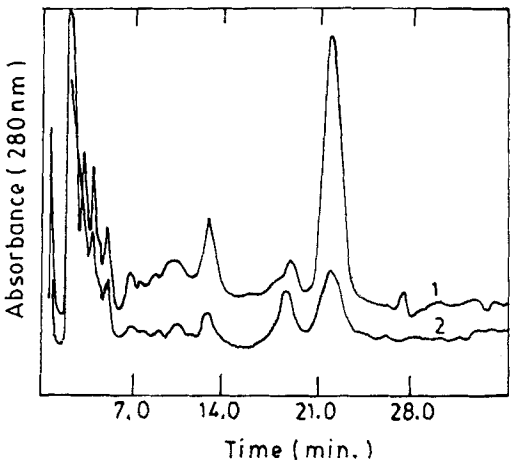


FIG.-1 : FPLC profile of the crude d(ACGTACGT$_p$) synthsised by present method (peak-1) and by Kumar et.al method (peak-2).

acetylation with acetic anhydride (14) In order to test utility of the synthesised support 5 the oligonucleotide d(ACGTACGT$_p$) was synthesised at 0.2 μ mole scale on Pharmacia Gene Assembler(15),following standard protocol. The coupling efficiency for each reaction cycle during the the synthesis was exceeded 98 %. This demonstrate that the newly synthesised support was stable during deprotection,coupling ,oxidation and capping conditions use in the solid phase phosphoramidite chemistry method. For comparison the oligonucleotide d(ACGTACGT$_p$) was synthesised as described by Kumar et.al.(12).

Both the oligonucleotides were deblocked from the polymer support by treating with a solution (2 ml) of 50 mM dithiothreitol in aq ammonia (25%) at 55^{0}C for 16 hrs(11,12). The deblocking solution was chilled and dried in a speed vac concentrator. A portion of both the oligonucleotides were passed through a G-25 Sephadex columns. The fraction containing oligomers were pooled and dried. The desalted oligomers (0.5 OD A_{260} each) were dephosphorylated by treating with one unit of alkaline phosphatase (17).The crude oligonucleotides were analysed over FPLC , Mono Q column. Figure-1 shows the Mono Q, anion exchange profile of

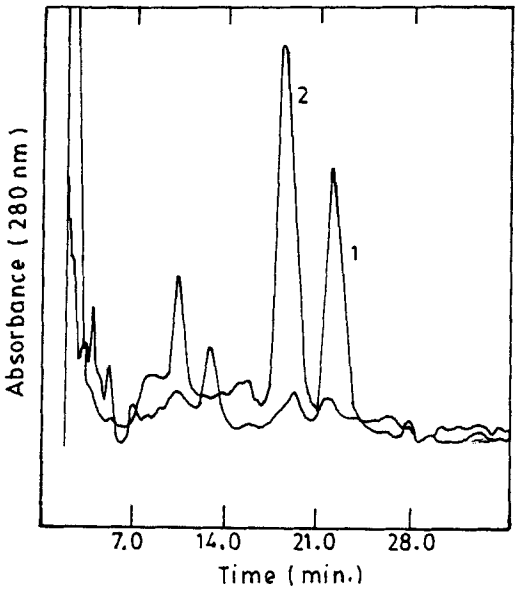


FIG.-2 : FPLC profile of crude d(ACGTACGT$_p$) (peak-1) and dephosphorylated d(ACGTACGT$_p$) (peak-2) synthsised by present method.

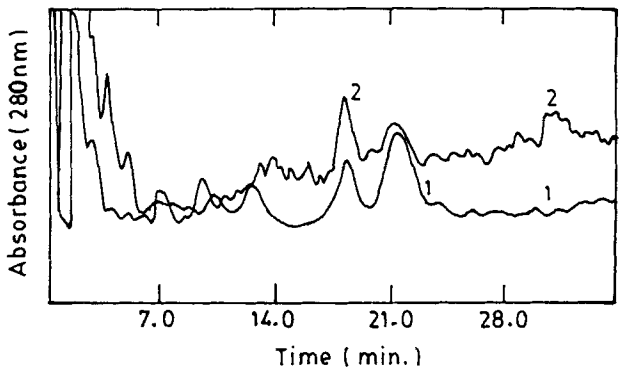


FIG.-3 :FPLC profile crude d(ACGTACGT$_p$) (peak-1) and dephosphorylated d(ACGTACGT$_p$) (peak-2) synthesised by Kumar et.al.method.

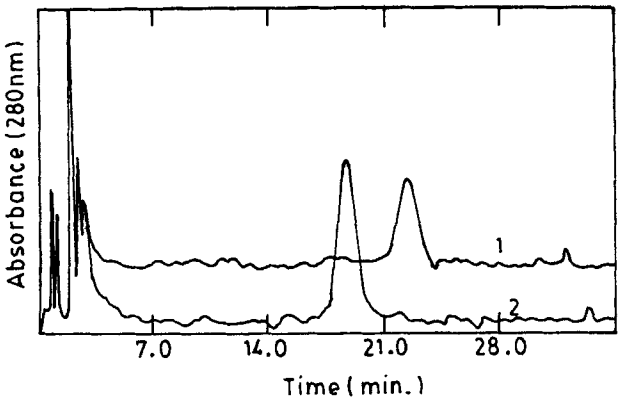


FIG.-4 : FPLC profile of purified d(ACGTACGT$_p$) (peak-1) and dephosphorylated d(ACGTACGT$_p$) (peak-2) synthsised by present method.

the crude d(ACGTACGT$_p$) (peak 1) and crude d(ACGTACGT$_p$)(peak-2) synthesised by present method and Kumar et.al.method respectively. Figure-2 shows the anion exchange profile of crude d(ACGTACGT$_p$)(peak 1) and dephosphorylated (ACGTACGT$_p$) (peak-2) synthesised by present method. The column,buffer and gradient for FPLC anaylsis are described in reference 18. The FPLC profiles of the crude d(ACGTACGT$_p$) and dephosphorylated d(ACGTACGT$_p$) synthesised by Kumar et.al.method are shown in Figure-3. Figure-4 shows the FPLC profile of purified d(ACGTACGT$_p$) and dephosphorylated d(ACGTACGT$_p$) synthsised by present method.

Conclusion : A comparison of the FPLC data clearly shows that our method gives not only better 3'-phosphate oligonucleotides but the 3'-phosphate label is in 100% monoester form. In case of Kumar et.al. the dephosphorylation was incomplete. This shows that some amonut of 3'-phosphate is not present in monoester form.

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13.Synthesis Of 2

Di-(2-hydroxyethyl)disulphide 1 (1.54 g, 10 m moles) was dried by repeated evaporation with dry pyridine. The dried 1 was taken in pyridine (15 ml) and 4'4'-dimethoxytritylchloride (2.23 g,6.6 m mole) was added. The mixture was left for stirring overnight. The reaction mixture was then concentrated in vacuum. The last trace of pyridine was removed by coevaporation with toluene. The residue dissolved in ethylacetate (50 ml) was extracted with 5% aq $NaHCO_3$ and 80% saturated aq.NaCl and organic phase was dried over anhydrous Na_2SO_4. After removal of solvents the product was purified by silica gel column chromatography to give (1.2 g) of

pure 2 (R_F=.77) ,TLC,Silica $CHCl_3$:MeOH,95:5). ^1H NMR ($CDCl_3$-TMS δ=0),δ,2.2-2.7 (m, 4H, 2X-S CH_2), 3.45 (br S 3H,-CH_2OH),3.8 (br S 8 H, 2X-OCH_3 +-CH_2ODMTr),6.8-7.1 (m, 4H,Ar),7.25-7.5 (m,9H, Ar).

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18. Column Mono Q HR5/5
Buffer A= 10 mM NaOH,0.4 M NaCl pH=12.
B= 10 mM NaOH,0.7 M NaCl pH=12.
Gradient 0-40% B in 35 min. Flow rate 1.5 ml/min

Received 4/15/92
Accepted 3/9/93