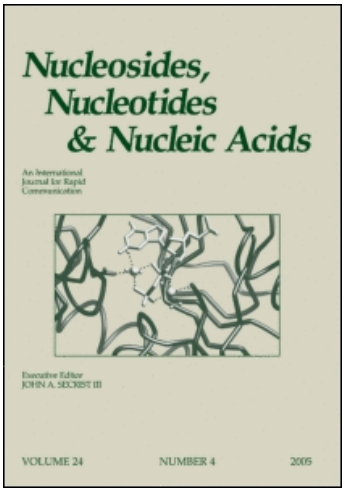

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

A Rapid Solid Phase Method for the Synthesis of 3'-Thiol Group Containing Oligonucleotides

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To cite this Article Kumar, Ajay(1993) 'A Rapid Solid Phase Method for the Synthesis of 3'-Thiol Group Containing Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 12: 7, 729 — 736
To link to this Article: DOI: 10.1080/07328319308021506
URL: http://dx.doi.org/10.1080/07328319308021506

NUCLEOSIDES & NUCLEOTIDES, 12(7), 729-736 (1993)

A RAPID SOLID PHASE METHOD FOR THE SYNTHESIS OF 3'-THIOL GROUP CONTAINING OLIGONUCLEOTIDES

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Abstract: A rapid solid phase method for the synthesis of 3'-thiol group containing oligonucleotides is described.

Introduction

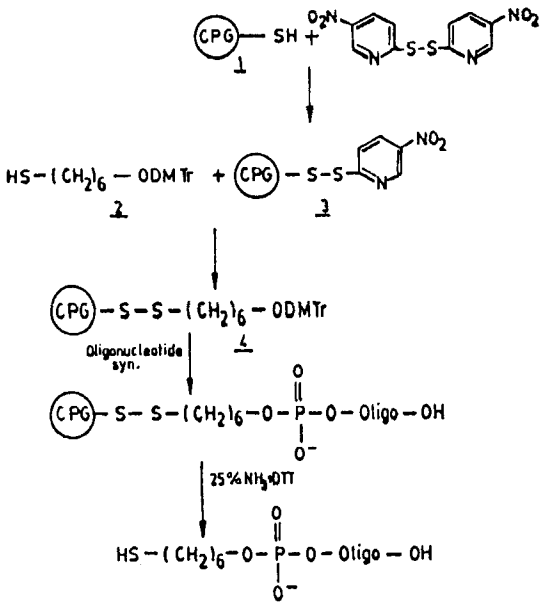
There has been great interest in developing easily workable methods for labeling oligonucleotides with non-radioactive labels. A number of chemical and enzymatic methods have recently been described for the selective incorporation of non-radioisotopes into synthetic oligonucleotides. The chemical method involves the introduction of amino (1-6) or thiol (7-17) group at 5' or 3' end of the synthetic oligonucleotides. These functionalized oligonucleotides are then derivatized with suitable fluorescent dye in order to prepare hybridization probes or primers for DNA sequencing (18-22) which can be detected by non-radioactive methods. In an effort to couple protein and other fluorescent dye at 3'-end of the synthetic oligonucleotides, we, require oligonucleotides containing a 3' -thiol group. Methods

developed so far suffer from some potential drawbacks. (i) Four different polymer supports are required (12). (ii) The methods used for the functionalization of polymer support are laborious (11,13,14).

We report here, a rapid method for selectively modifying the 3' -terminus of oligonucleotides in which the label is introduced in high yield in one step by reacting a thiol specific reagent. Our method describes the preparation of a universal controlled pore glass support modified with a disulfide containing spacer that allows the synthesis of oligonucleotides extended at their 3'-end by mercaptohexyl phosphate residue. This preparation is compatible with the established phosphoramidite chemistry (23). The deprotection was carried out by published method (11) i.e. by treating with 25% ammonia,containing 50 mM dithiothreitol for 6 hrs at 60°C.

Synthesis of polymer support

The synthesis of polymer support involves coupling of activated CPG polymer support with 4,4'-dimethoxytrityl-6-mercaptohexane 2 via a disulfide linkage. The activation of CPG-based support begins with the reaction with pre synthesized 3'-thiol-CPG 1 (500 mg) (15) (-SH loading 40 μM/g support) with five fold excess of 2,2' -dithio-bis(-5-nitro pyridine) (DTNP) in dichloroethane (10ml) for 3 hrs at 37°C. The unreacted 2,2'-dithio-bis-(5- nitro pyridine) and 5-nitropyridine-thione was removed by washing with dimethylformamide (2x10ml),methanol (3x10ml) and dichloroethane(3x10ml). The DTNP activated polymer support 3 was reacted with five fold excess of pre synthesized 4,4'-dimethoxytrityloxy-6-mercaptohexane 2 (11) in dichloroethane (5ml) at 37°C for 3hrs. The unreacted 4,4'-dimethoxytrityloxy-6-mercaptohexane was removed by filtration followed by washing with dichloroethane (2x10ml),methanol (50ml) and finally with ether(25ml). The derivatized polymer



Scheme-1

support 4 was dried overnight under vacuum. The loading of 4,4'-dimethoxytrityl groups on the functionalized support was determined spectrophotometrically after reacting a weighed amount of the support with perchloric acid (24). The loading capacity was 40 μM/g polymer support. The Ellman's (22) reagent test carried out with functionalized polymer support was negative. This shows that there was no free thiol group left on the CPG-polymer support. Finally the unreacted amino groups on the polymer support were capped as described elsewhere(24). The reactions involved in the synthesis of 3'-thiol-CPG polymer support 4 are shown in Scheme-1.

Utility of the polymer support

In order to test the utility of synthesized support the oligonucleotide d(TTTTTTTT$_{SH}$) was synthesized at 1.3 μM scale on Pharmacia Gene Assembler (25) following phosphoramidite

chemistry. The coupling efficiency of each reaction cycle during synthesis exceeded 98%. This demonstrates that the newly synthesized support was stable during deprotection,coupling, oxidation and capping conditions used in the solid phase phosphoramidite chemistry method. The oligonucleotide d(TTTTTTTT) was also synthesized using the standard thymidine support. The thymidine oligomers were synthesized for correct comparison of gel electrophoretic mobility and retention time. Number of other oligonucleotides containing d(A,C,G) were also synthesized over newly developed polymer support and have been linked with proteins.

The oligonucleotide synthesized on the 3'-thiol on CPG support 4 was deblocked by treatment with 2ml aqueous ammonia (25%) containing 50 mM of dithiothreitol at 55°C for 16 hrs. The oligonucleotides containing other bases were also cleaved by ammonia/DTT treatment and no base modification was observed. The ammonia solution was concentrated and crude oligomer was passed through a G-25 Sephadex column. The oligonucleotide d(TTTTTTTT) synthesized on thymidine polymer support was treated with 1.5 ml of 25% ammonia solution at 60°C for 6 hrs. The volume of ammonia solutions were reduced to 100 µl and passed through a G-25 Sephadex column. Finally both the oligonucleotides were purified on PAGE gel containing 7M urea (26). The PAGE showed that 3'-thiol oligonucleotide electrophoresed slower than T-8 mer. Finally both the Oligonucleotides were phosphorylated using polynucleotide kinase and ^{32} PγATP and run on analytical 20% polyacrylamide-7M urea gel. The autoradiogram shown in Fig.-1 shows that the electrophoretic mobility of 3'-thiol labeled oligonucleotide is retarded due to the presence of 6-mercaptohexane. Ellman's reagent (22) test carried out for the purified 3'-thiol oligonucleotide showed 97% incorporation of the thiol groups. Finally the oligonucleotides were analyzed by FPLC on C_{18} reverse phase column. The reverse phase FPLC profile of the crude d(TTTTTTTT) (peak 1) and d(TTTTTTTT-$(CH_2)_6$-SH) (peak-2) are shown in Fig.-2. The FPLC profile of the crude oligonucleotides (Fig.-2) demonstrates that

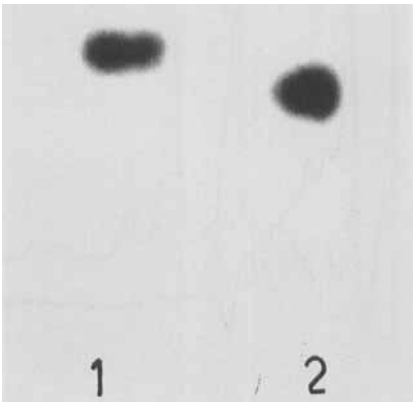


FIG.-1:Autoradiogram of d(TTTTTTTT-$(CH_2)_6$-SH) (Lane-1) and d(TTTTTTTT) (Lane-2).

mercaptohexylphosphate containing $d(T)_8$ was well resolved over C-18 reverse phase column (retention time 20.5). The normal $d(T)_8$ was eluted at 15.5 min. This suggests that mercaptohexylphosphate residue label oligonucleotides can be better resolved by reverse phase column chromatography.

Activation of sulfhydryl group of 3'-thiolated oligonucleotides with DTNP

The 2.0 OD A_{260} of the purified thiolated oligonucleotide was labeled with DTNP as described elsewhere (11). After the completion of the reaction the excess of DTNP and released 5-nitropyridyl-2-thione was removed by passing the reaction mixture through a Sephadex G-25 column. A Fig.-3 shows the FPLC profile of purified d(TTTTTTTT) 1 coinjected with d (TTTTTTTT-$(CH_2)_6$-SH) 2 and d(TTTTTTTT-$(CH_2)_6$-S-S-Py (NO_2)) 3. The DTNP activated oligonucleotides were conjugated with proteins.

Conjugation of sulfhydryl group of 3'-thiolated oliguncleotides with pyrenyl maleimide

The presence of free -SH group was confirmed by labeling with a thiol specific label, Pyrenyl maleimide(27). The fluorescence spectrum of the pyrenyl labeled oligonucleotide

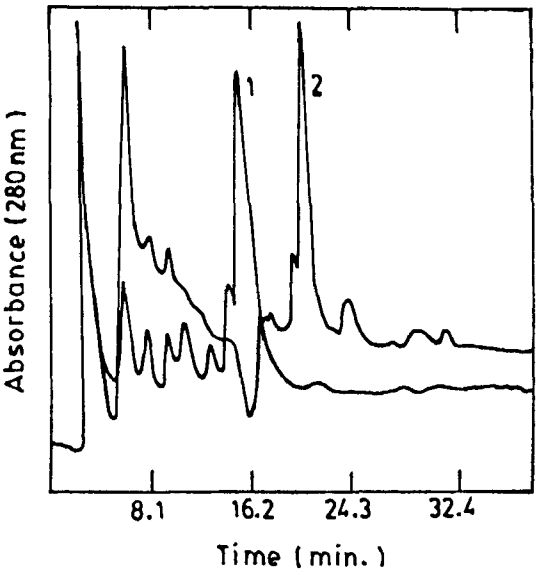


FIG.-2: FPLC profile of crude d(TTTTTTTT) (peak1)and d(TTTTTTTT-$(CH_2)_6$-SH(peak 2). Column, Pro RPC Flow rate 1 ml/min.; gradient 0-8% B in 5 min,8-18% B in 30 min.A=0.1 M ammonium acetate buffer pH=7.0 ,B=80% acetonitrile in 0.1 M ammonium acetate.

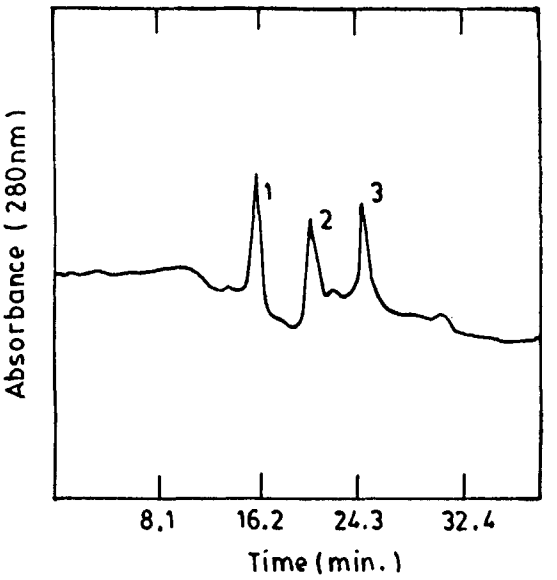


FIG.-3:FPLC profile of purified d(TTTTTTTT), (peak 1) coinjected with d(TTTTTTTT-$(CH_2)_6$-SH) (peak 2) and d(TTTTTTTT--(CH_2)-S-S-PY(NO_2). Column, Pro RPC, Flow rate 1 ml/min.; gradient,0-8%B in 5 min.,8-18%B in 30 min.A= 0.1M ammonium acetate pH=7,B= 80% acetonitrile in 0.1 M ammonium acetate.

obtained by excitation at 350 nm showed peaks at 380 nm and 397 nm (Fluorescence spectrum not shown).

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