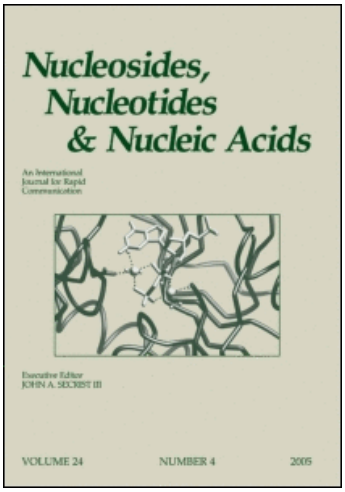

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

A versatile Solid Phase Method For the Synthesis of Masked 3'-Thiol Group Containing Oligonucleotides

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To cite this Article Kumar, Ajar(1993) 'A versatile Solid Phase Method For the Synthesis of Masked 3'-Thiol Group Containing Oligonucleotides', Nucleosides, Nucleotides and Nucleic Acids, 12: 10, 1047 — 1059
To link to this Article: DOI: 10.1080/07328319308016203
URL: http://dx.doi.org/10.1080/07328319308016203

NUCLEOSIDES & NUCLEOTIDES, 12(10), 1047-1059 (1993)

A Versatile Solid Phase Method For the Synthesis of Masked 3'-Thiol Group Containing Oligonucleotides

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ABSTRACT:

A new solid phase method for the introduction of masked thiol group on to the 3'-end of the oligonucleotide is reported. The modified oligonucleotides thus obtained can be better resolved by reverse phase column chromatography.

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-radioisotope labels 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-radioisotopic methods. In an effort to couple peptides and other fluorescent dyes 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 (11,14). The methods for the synthesis of free 3'-thiol group containing oligonucleotide has been reported (13,27).

A general method for selectively modifying the 3'-terminus of the oligonucleotides is developed. A universal controlled pore glass (CPG) functionalized with a masked thiol group is first prepared, on which the oligonucleotide can be synthesized by following the standard phosphoramidite chemistry (28). Deprotection is carried out by treating the CPG with 25% ammonia solution for 16 hrs at 60^0 C in the absence of dithiothreitol (DTT). The synthesis of polymer support involves coupling of the S-trityl-3-(4,4'-dimehtoxytrityl)-2-propanol with aminopropyl CPG support via succinylation. The main advantage of our method is that it provides a Tr- group at 3'-end of the oligonucleotide, which being lipophilic, simplifies the purification by reverse phase chromatography.

MATERIALS AND METHODS

Synthesis of 3-S-trityl-1-(4,4'-dimethoxytrityl)-2-propanol(3)

3-bromo-1,2-propanediol, triphenylmethylmercaptan and dimethoxytritylchloride were purchased from Aldrich and use without further purification.CPG(amino propyl derivatised controlled pore size glass ,pore size 500^{o}A) was purchased from Fluka. Solvents used were from local chemical company and were dried befor use. DNA synthesis materials were from Applied Biosystems.

Synthesis of S-trityl-1,2-propanediol

3-bromo-1,2-propanediol 1 was added to a solution of ethanolic triphenylmethylmercaptan (30 mM) and aq. sodium hydroxide (33 mM, 7.5 ml). After the completion of the reaction (TLC,90 min), the reaction mixture was evaporated to dryness, residue dissolved in $CHCl_3$ (50 ml) and washed with water (3X10 ml). The organic phase after drying over sodium sulfate and evaporation yielded crude 2,which was purified over silica gel column using $CHCl_3$+CH_3OH (4%) as eluent. Yield 8.2 g, 84.24%. ^{1}H NMR ($CDCl_3$,S TMS=0.00) 7.7-7.2 (m,aromatic,15 H),3.6-3.3,(m,-CH_2OH,CHOH), 2.55-2.3 (d,-S-CH_2).

Stability of S-trityl-1,2-propanediol

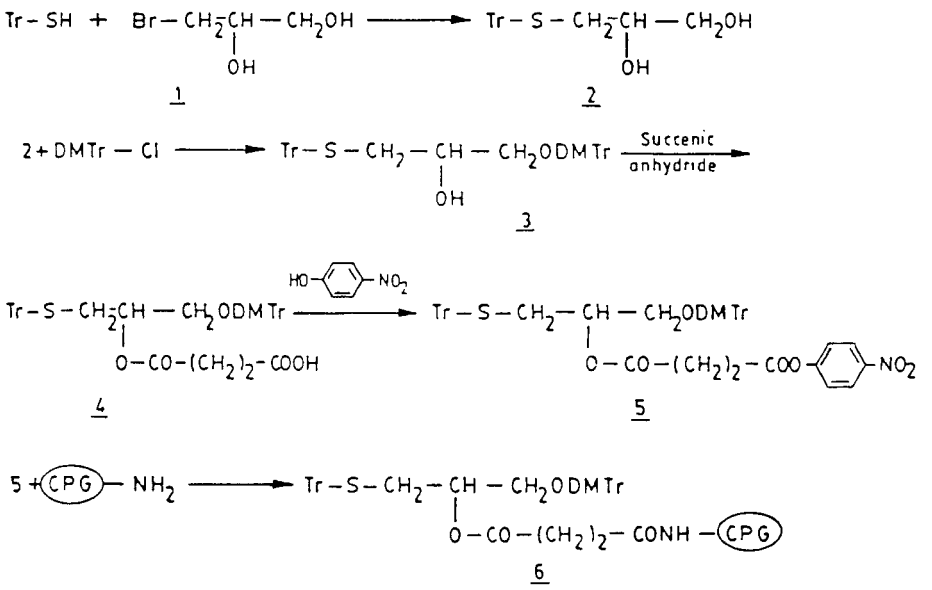
About 5.0 mg of S-trityl-1,2-propanediol was dissolved in 1.0 ml each of 2% dichloroacetic acid in dichloroethane and oxidation solution and left for 2.0 hrs at room temperature. The stability of S-trityl-1,2-propanediol was monitored by TLC.

Preparation of 3-S-trityl-1-(4,4'-dimethoxytrityl)-2-propanol

2 on reaction with dimethoxytrityl chloride in presence of catalytic amount of dimethylaminopyridine in dry pyridine yielded 3 in 2 hrs. The pyridine was evaporated and finally coevaporated with dry toluene (3X 10 ml) under reduced pressure to get the crude material. Purification over silica gel column using $CHCl_3$ as eluent provided pure 3 in 73% yield (11.2 g). TLC ($CHCl_3$) R_f0.67. ^{1}H NMR ($CDCl_3$,S TMS =0.00),7.66-6.8 (m,aromatic,28H),3.8 (m,-OCH_3 ,-CHOH),3.1 (d,CH_2OH),2.4 (d,-S-CH_2).

Functionalization of polymer support

The synthesis of 3-S-trityl-1-(4,4'-dimethoxytrityl)-2-propanol succinate 4 derivatised CPG polymer support 6 was



Scheme 1

carried following the published procedure (23). The reactions for the synthesis of polymer support are shown in Scheme-1. The loading capacity of the polymer support was assayed by determining the amount of dimethoxytrityl cation released by perchloric acid treatment (23).

Utility of the polymer support

In order to test the utility of the synthesized support, oligonucleotide d(TTTTTTTT-STr) was synthesized at 1.3 μM scale on Pharmacia Gene Assembler (24) following standard protocol. The coupling efficiency of each cycle during synthesis exceeded 98%. This demonstrates that the newly synthesized support was stable during deprotection, coupling, oxidation and capping conditions used in solid phase phosphoramidite chemistry method. The oligonucleotide d(TTTTTTTT) and d(TTTTTTTTT) were also synthesized using the standard polymer support. All the three oligonucleotides

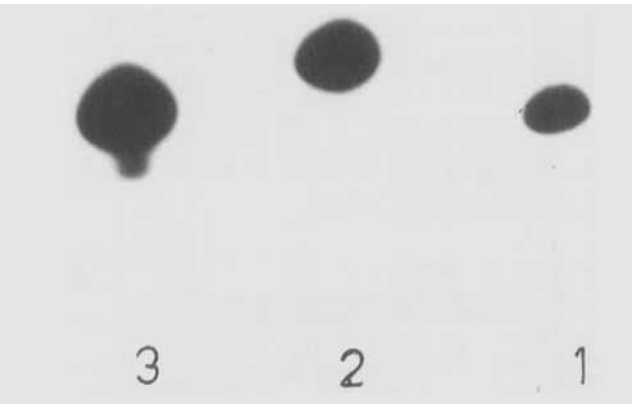


FIG.-1 Autoradiogram of d(TTTTTTTT) (Lane 1),d(TTTTTTTT-CH_2-CH(OH)-CH_2-STr) (Lane 2) and d(TTTTTTTTT) (Lane 3).

were treated with 25% ammonia solution at 60^0C for 16 hrs. A portion of all the three oligonucleotides was purified on PAGE containing 7M urea (25). All the three oligonucleotides were phosphorylated using T-4 polynucleotide kinase and ^{32}Pγ ATP and their electrophoretic mobility was checked by running a analytical PAGE containing 7M urea. Autoradiogram is shown in Fig.-1

A portion of d(TTTTTTTT-CH_2-CH(OH)-CH_2-STr) was treated with DTT/$AgNO_3$ to obtain d(TTTTTTTT-CH_2-CH(OH)-CH_2-SH), which gave a positive Ellman's (22) reagent test showing quantitative incorporation of the thiol group at 3'-end of the oligonucleotide. Finally the oligonucleotides were subjected to the FPLC C_{18} reverse phase column. The FPLC profile of crude d(TTTTTTTT)1 ,d(TTTTTTTT-CH_2-CH(OH)CH_2-STr)2 and d(TTTTTTTT-CH_2-CH(OH)-CH_2-SH) 3 are shown in Fig.-2

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

The 2.0 OD A_{260} of purified d(TTTTTTTT-CH_2-CH(OH)-CH_2SH) was treated with 2,2'-Dithio-bis- (5-nitropyridine) (DTNP) (11).

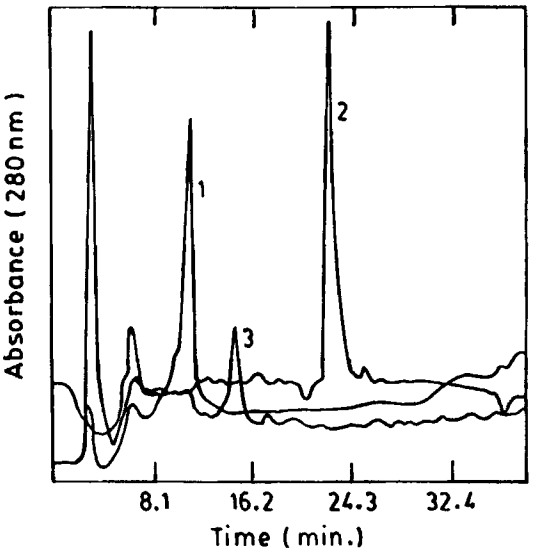


FIG.-2 FPLC profile of crude d(TTTTTTTT) (peak 1),d(TTTTTTTT-CH_2-CH(OH)-CH_2-STr) (peak 2) and d(TTTTTTTT-CH_2-CH(OH)-CH_2-SH)(peak 3) Column,Pro RPC.Flow rate 1 ml/min.;gradient 0-8% B in 5 min,8-28% B in 30 min.A= 0.1 M ammonium acetate buffer pH =7,B=80% acetonitrile in 0.1 M ammonium acetate.

The excess of DTNP and released 5-nitropyridyl-2-thione was removed by passing the reaction mixture through a Sephadex G-25 column. Fig.-3 shows the FPLC profile of (1) purified d(TTTTTTTT-CH_2-CH(OH)-CH_2SH) A coinjected with d(TTTTTTTT-CH_2-CH(OH)CH_2S-S-PY-NO_2) B,(2) purified d(TTTTTTTT-CH_2-CH(OH)-CH_2STr).

Conjugation of Sulfhydryl group of 3'-thiolated oligonucleotides with pyrenyl maleimide

The 3'-thiol containing oligonucleotide was reacted with a thiol specific reagent pyrenyl maleimide (26). The fluorescence spectrum of the pyrenyl labeled oligonucleotide obtained by excitation at 350 nm showed peaks at 380 nm and

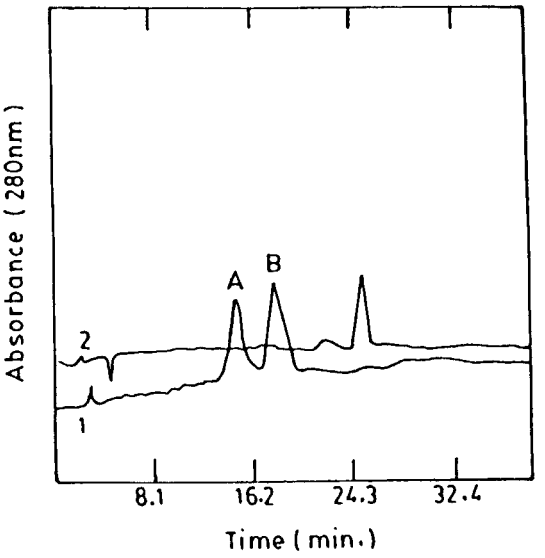


FIG.-3 FPLC profile of purified (1) d(TTTTTTTT-CH_2-CH(OH)-CH_2-SH) (peak A) coinjected with d(TTTTTTTT-CH_2-CH(OH)-CH_2S-S-$PyNO_2$ (peak B) (2) d(TTTTTTTT-CH_2-CH(OH)-CH_2-STr). Column,Pro RPC. Flow rate 1 ml/min.; gradient 0-8% B in 5 min.,8-28% B in 30 min.A= 0.1M ammonium acetate buffer pH=7,B=80% acetonitrile in 0.1 M ammonium acetate.

397 nm confirming the presence of a thiol group on to the oligonucleotide.

RESULTS AND DISCUSSION

In this communication we have attempted to derivatize CPG based polymer support for the synthesis of masked thiol group containing oligonucleotides. It has been shown that free thiol group containing oligonucleotides can not be store without suitable protection of thiol group for long time , as they undergo symmetrical disulfide formation due to aerial oxidation (12). It has also been demonstrated that oligonucleotides bearing a masked thiol group at 3'-end can

easily be purified and can be store for longer time (14). The methods for the introduction of partially protected sulfhydryl and free sulfhydryl group containing oligonucleotides have also been reported (13).

In the above mentioned methods some efforts have been made for resolving 3'-thiolated oligonucleotides from unthiolated oligonucleotides formed during synthesis and to prevent the aerial oxidation. However, so far the methods developed for the introduction of thiol group at 3'- end of the oligonucleotides have involved the use of disulfide linkage and the cleavage of oligonucleotides with ammonia/DTT. In our opinion a more suitable method is required for the synthesis of 3'-thiol group containing oligonucleotides which does not involve the use of disulfide linkage. Thus while planning for the derivatization of the polymer support we have following important considerations (i) the polymer support should be derivatize in such a way so that oligonucleotides contain protected thiol group even after deprotection from the support (ii) the protecting group used should be such that it simplifies the purification (iii) the derivatization procedure should be simple (iv) the protecting group used should be easily cleavable after purification of the oligonucleotides (v) the synthesis of oligonucleotides on derivatized polymer support and their deprotection condition should be compatible with the modern machine aided oligonucleotide synthesis via phosphoramidite approach, commonly used in oligonucleotide synthesis and involve as little alteration to these method as possible.

In order to satisfy these criteria, we undertook the preparation of CPG based polymer support, which on final deprotection give 3'-protected sulfhydryl containing oligonucleotides. The derivatization of polymer support was simple and deprotection of oligonucleotide from polymer support was compatible with procedure commonly used in oligonucleotides synthesis.

In order to synthesis the polymer support we first synthesized 3-S-trityl-1-(4,4'-dimethoxy trityl)-2- propanol. The free secondary hydroxy group of 3 was succinylated , its p-nitrophenyl ester was synthesized and reacted with 3-aminopropylated CPG in the presence of triethylamine. The unreacted amino groups on the polymer support were then capped. The contents of DMTr groups on polymer supports were determined following standard procedure (23) and were found to be 43 μmole/g.

To demonstrate the suitability of this polymer support for the synthesis of 3'-sulfhydryl containing oligonucleotides, the oligo $d(T)_8$ was synthesized on this support. The $d(T)_8$ and $d(T)_9$ were synthesied on standard thymidine derivatized polymer support. The thymidine oligomers were synthesized for the correct comparison of the electrophoretic mobility and retention time of the thiolated oligonucleotide with normal oligonucleotides. The electrophoretic mobility of d(TTTTTTTT $(CH_2\text{-}CH(OH)\text{-}CH_2$ S Tr) 2 was found even less than that of d(TTTTTTTT)3. The retardation of electrophoretic mobility appears to be due to the presence of S-trityl-2-propanol moiety (Fig.-1). The FPLC profile of the crude oligonucleotides (Fig.-2) demonstrates that the Tr- group protected thiol containing $d(T)_8$ was well resolved (Retention time 24.8) over C-18 reverse phase column. The normal $d(T)_8$ was eluted much earlier at 8.5 min. Thus it suggest that thiol containing oligonucleotides synthesized using newly derivatized support can be well resolved from the unthiolated oligonucleotides. The resolution of thiolated oligonucleotide is due to the lipophilic nature of Tr-moiety. The trityl group from the oligonucleotide 2 was removed by $AgNO_3$/DTT treatment (7). The 100% detritylation was achieved as is indicated from the FPLC profile (Fig.-2). The FPLC profile (Fig.-2) also suggests that the S-trityl group was stable during the synthesis as no truncated oligonucleotides and undentified products were formed. The S-trityl linkage in S-trityl-1,2-propanediol was stable in 2% DCA in dichloroethane

and oxidation solution (0.01 M I_2, 26.0:2.4:12.0 AcCN:Py: H_2O). This stability of S-trityl linkage in S-trityl-1,2-propane diol is surprising in view of lability of S-trityl linkage towards iodine /pyridine under certain conditions (29) . However, there have been reports that S-trityl groups can only be cleaved by acids in presence of a trityl cation scanvenger (30). The stability of S-trityl function in acids have been shown by the fact that the compound trityl-S-$(CH_2)_2$ was only cleaved to a 50% extent after treatment with 10% trifluoroacetic acid for 20 hrs. Interestingly it has been demonstrated that S-trityl linkage is stable in oxidation solution during solid phase oligonucleotide synthesis. The S-trityl cleavage by iodine is known to involve intermolecular disulfide bond formation between two S-trityl compound and perhaps this is a steric impossibility while bound to the solid matrix. Surprisingly when trityl-S-$(CH_2)_n$-PO_4-ACGT, n = 3 or 6 was treated with iodine and dithiothreitol unidentified products were formed and no detritylation took place. In our opinion the stability or lability of S-trityl linkage towards iodine depends on the nature of the compound , concentration of iodine and the solvent in which the cleavage is carried out. The S-trityl linkage S-trityl-1,2,-propanediol was stable in oxidation solution used in solid phase synthesis of oligonucleotides because of low concentration of I_2 (0.01M) and low concentration of pyridine (6%) used for the preparation of oxidation solution.

CONCLUSIONS

We have provided a simple method for derivatizing the CPG polymer support for the introduction of masked thiol group at 3'-end of the oligonucleotides. The thiol containing oligonucleotides synthesized over new polymer support can well resolved unlike the earlier reported methods.

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Received 5/19/93
Accepted 9/8/93