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SOLID PHASE SYNTHESIS AND PURIFICATION OF 5'-MERCAPTOALKYLATED OLIGONUCLEOTIDES

P. Kumar, D. Bhatia, R.C. Rastogi[+] and K.C. Gupta[*]
Nucleic Acids Research Laboratory, Centre for Biochemical Technology, Mall Road, Delhi and
[+]Department of Chemistry, University of Delhi, Delhi -110 007, India

ABSTRACT

A novel strategy for the synthesis of thiol link phosphoramidites having base labile S-acyl type protecting groups is described. The method involves the reaction of 6-bromohexanol with potassium thioacetate or thiobenzoate in the presence of 18-crown-6 as a phase transfer catalyst to obtain the compounds **3(a,b)**, *which were then converted to their corresponding phosphoramidites* **4(a,b)** *and used for solid phase synthesis of 5'-mercaptoalkylated oligonucleotides. The thiolated oligomers were purified by single step covalent chromatography using an activated sulfhydryl group containing support.*

Non-radiolabelled oligonucleotide conjugates have become an indispensable tool for various applications in modern molecular biology. A large number of protocols[1] have been described for their synthesis. Basically, two types of approaches[1-6] are currently being followed. In one approach, a reactive functional group (aminoalkyl or mercaptoalkyl) is introduced in the protected form at the 5'-termini of asynthetic oligonucleotide with the help of a suitable linker arm. After deprotection, the fully deprotected oligonucleotide bearing the reactive functional group is reacted with an appropriate activated ligand to obtain the desired oligonucleotide-conjugate. In the second approach, the ligand is firstconverted to its phosphoramidite via the use of a linker or not, and then used in the last cycle of the synthesis. Both of the approaches are in use. For the synthesis of 5'-mercaptoalkylated oligonucleotides, Connolly[3] and Sinha et al.[4] have reported the use of a S-trityl protecting group that requires reductive conditions for its cleavage, i.e. silver nitrate treatment followed by dithiothreitol (DTT) complex formation, which causes considerable loss of the desired material during work-up. Moreover, this protecting group poses some problems during oxidation step with aqueous iodine. These problems have been circumvented by the introduction of base labile protecting groups for thiolfunction by Eritja et al.[7] and Kuijpers and van Boeckel[8]. However, the synthesis of their reagents is a multistep protocol. In this communication, we wish to report a simple and general method for the synthesis of thiol link reagents having a base labile protecting group involving commercially available reagents. The efficiency of the proposed reagents was established with synthesiz ing a number of 5'-mercaptoalkylated oligonucleotides in an automated DNA synthesizer and the desired 5'-mercaptoalkylated oligomers were purified by single step affinity chromatography using an activated thiol group bearing polymer support. Truncated sequences were removed by washing steps and the desired 5'-thiolated oligomer was released from the support.

The synthesis of thiol link reagents 4(a,b) was carried out as shown in figure 1. The synthesis of the reagents involves the reaction of potassium thioacetate or thiobenzoate with 6 - bromohexanol in the presence of 18-crown-6 to obtain 3(a,b) in just 15 min in 92% yields. The compounds 3(a,b), after purification, were phosphitylated using 2-cyanoethyl-N,N,N',N'- tetraisopropylphosphoramidite to yield the desired thiollink reagents 4(a,b).

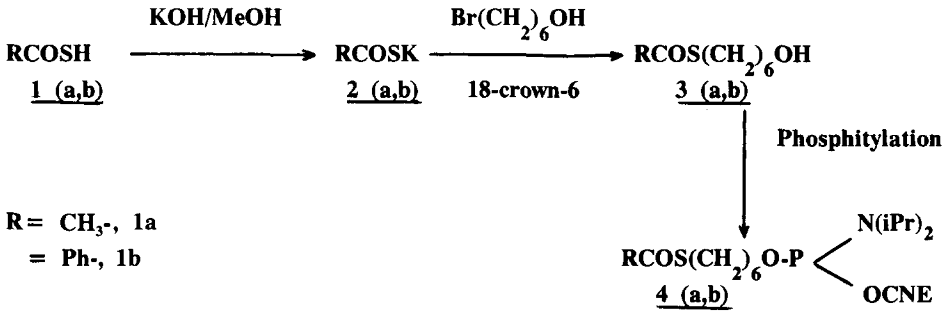

Fig. 1 Preparation of reagents for 5'-mercaptoalkylation

In order to establish the utility of these reagents for the synthesis of 5'-mercaptoalkylated oligonucleotides, a large number of oligonucleotides were assembled on solid support in an automated DNA synthesizer (Pharmacia LKB Gene Assembler Plus)[9] at 0.2 µmol scale and the last cycle was performed with either oneof the two reagents 4(a,b) in the synthesizer itself. The normal nucleoside-phosphoramidites were used in an analogous manner.

The deprotection of 5'-mercaptoalkylated oligonucleotides was carried out by single step treatment with aq. ammonia (29%) containing 50 mM DTT at 55°C for 16h. This one step deprotection process allows the removal of classical protecting groups (2 - cyanoethyl from phosphates and acyl from nucleic bases) and the presence of DTT serves two purposes, viz., (i) it prevents the unwanted reaction of free 5'-mercaptoalkyl group of oligonucleotides with acrylonitrile resulting from the deprotection of 2-cyanoethyl group[8] and (ii) it keeps the 5'- mercaptoalkyl groups in reduced form.

5'-Mercaptoalkylated oligonucleotides were purified by single step affinity chromatographic method employing activated mercaptoalkylated-fractosil support[10]. Since only the desired oligomer with the mercaptoalkyl group undergoes disulfide exchange reaction with the activated mercaptoalkyl groups present on the affinity matrix, i.e. covalently binds with the affinity matrix by disulfide bond, the truncated or the capped sequences devoid of mercaptoalkyl groups come out of the matrix without retention. The covalently bound mercaptoalkylated oligomer was later on eluted from the affinity matrix under reductive conditions (50 mM DTT in elution buffer). The purity of the eluted oligomer was analysed on RP-HPLC (Fig. 2).

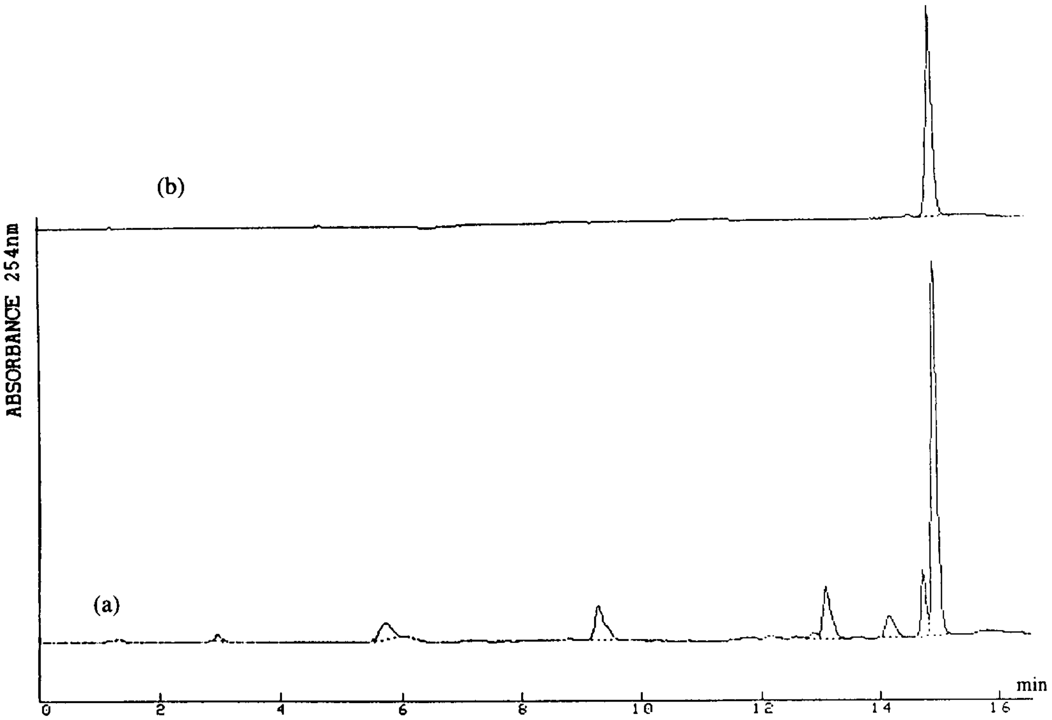

Fig. 2 Reverse phase hplc profiles of (a) crude 5'-thiolated oligomer d(TTT TT) and (b) 5'-thiolated d(TTT TT) eluted from affinity matrix (activated thiol-support). For hplc conditions, see ref. 11.

In a typical experiment, mercaptoalkylated oligomer d(TTT TT) was allowed to react with five equivalentsof fluorescent N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylenediamine (1,5-I-AEDANS) in the presence of DTT at room temperature for 1h. The resultant mixture was desalted and analysed on RP-HPLC (Fig. 3). Figure 4 shows the elution pattern of purified mercaptoalkylated oligomer d(CTA CCA CTG T) and its disulfide adduct obtained after the reaction with a thiol-specific reagent, 2,2'-dithiobis(5 - nitropyridine).

In conclusion, we have developed a rapid and efficient method for the solid phase synthesis of 5'-thiolated oligonucleotides and their one step affinity purification using activated mercaptoalkyl group containing support.

EXPERIMENTAL

Preparation of potassium thioacetate or thiobenzoate 2(a,b)

To a cold solution of thioacetic or thiobenzoic acid (10 mmol) in methanol was added a methanolic solution of potassium hydroxide (10 mmol) dropwise over a period of 5 min with continuous stirring. After complete addition, the mixture was stirred at room temperature for 15 min,

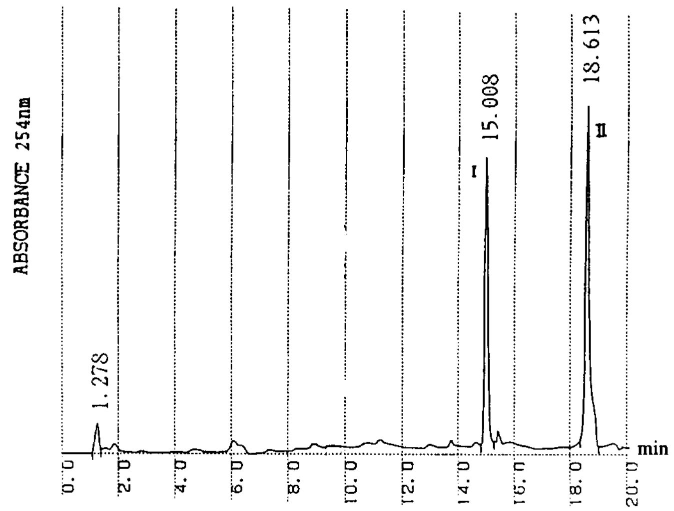

Fig. 3 Reverse phase hplc profiles of purified 5' - mercaptoalkylated d(TTT TT) (peak I) coinjected with AEDANS labelled d(TTT TT) (peak II).

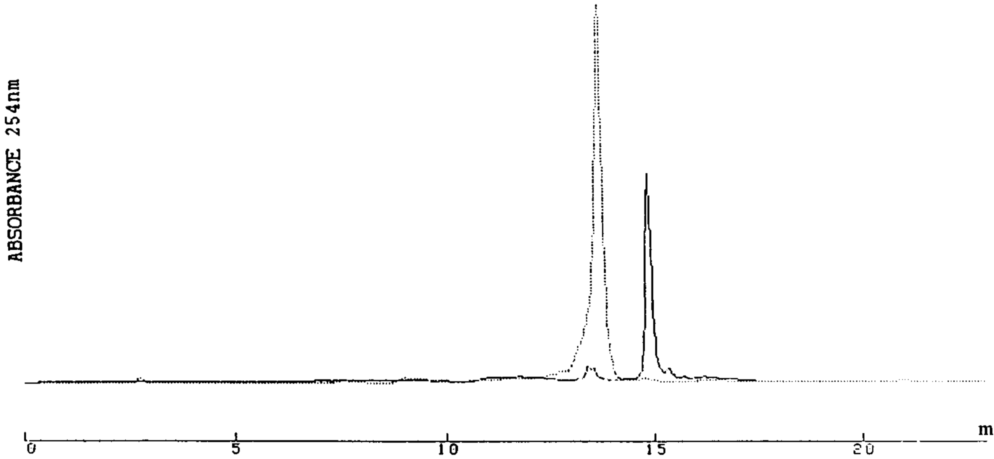

Fig. 4 Reverse phase hplc profile of purified 5'- mercaptoalkylated d(CTA CCA CTG) (dotted line) and 5-nitro-2 - thiopyridylated d(CTA CCA CTG) (solid line). See Ref. 11.

filtered to remove any insoluble material and the volume of the filtrate was reduced on a rotary evaporator to about 10 ml. This was subjected to trituration with enough amount of diethylether to precipitate the potassium salt. The material was filtered on a sintered glass funnel and dried under high vacuum in a desiccator.

Preparation of S-acyl-6-mercaptohexanol 3(a,b)

A mixture of potassium thioacetate (2.4 mmol), 6 - bromohexanol (2.0 mmol) and 18-crown-6 (0.2 mmol) was refluxed for 15 min in toluene (20 ml). The insoluble material from the resulting mixture was removed by filtration. The filtrate was diluted with toluene (30 ml) and washed successively with saturated aqueous sodium hydrogen carbonate (2 x 20 ml), sodium chloride (1 x 20 ml) and water (20 ml). The organic phase was dried over anhydrous sodium sulfate, concentrated and purified by silica gel column chromatography using petroleum ether : ethyl acetate (8 : 2, v/v) as an eluent. The fractions containing the desired material were pooled and concentrated to obtain the desired product as a yellowish oily material (92%). The compound was characterized by its NMR spectrum. S-Benzoyl-6 - mercaptohexanol was also synthesized in an analogous manner.

S-Acetyl-6-mercaptohexanol 3a:
Rf : 0.45 (hexane : ethyl acetate, 6:4)
^{1}H NMR ($CDCl_3$)δ:1.6 (m, 8H, -$(CH_2)_4$-), 2.4 (s, 3H, -$COCH_3$), 2.95 (t, 2H, -SCH_2-), 3.8 (m, 2H, -OCH_2-)

S-Benzoyl-6-mercaptohexanol 3b:
Rf : 0.52 (hexane : ethyl acetate, 6:4)
^{1}H NMR ($CDCl_3$)δ:1.54 (m, 8H, -$(CH_2)_4$-), 3.04 (t, 2H, -SCH_2-), 3.7 (m, 2H, -OCH_2-), 7.6 (m, 5H, Ar-H).

Preparation of S-acyl-6-mercaptohexyl-2-cyanoethyl-N,N-diisopropylphosphoramidite 4(a,b)

The compound 3(a,b) (1 mmol) was dissolved in anhydrous acetonitrile (10 ml) and N,N,N',N'-tetraisopropyl-2-cyanoethyl- phosphoramidite (1.5 mmol) was added. To this mixture was added tetrazole (0.75 mmol) dissolved in acetonitrile (1.5 ml) dropwise at room temperature and the resulting mixture was stirred for 2h. The progress of the reaction was monitored by tlc. After completion of the reaction, it was quenched by adding methanol (0.2 ml). The reaction mixture was concentrated under reduced pressure and the resulting syrupy mass taken up in ethyl acetate (20 ml). The organic phase was washed with saturated sodium chloride solution (2 x 10 ml) and dried over anhydrous sodium sulfate. After filtration, the solvent was removed and the material was purified over silica gel column. The desired material was eluted with hexane : ethylacetate : triethylamine (8:1:1). The fractions containing the desired material were pooled and concentrated in a rotary evaporator to obtain the title compounds 4(a,b) in 85% yield.

Synthesis and deprotection of 5'-mercaptoalkylated oligonucleotides

In order to check the efficiency of the reagents 4(a,b) for the synthesis of 5'-mercaptoalkylated oligonucleotides, a number of oligonucleotides were assembled at 0.2 µmol scale using well established phosphoramidite chemistry on a Pharmacia Gene Assembler Plus. After synthesis of the desired oligonucleotides, the final coupling cycle was performed with either one of the phosphramidite reagents 4(a,b) in an analogous manner to the normal nucleoside phosphoramidites.

The deprotection of mercaptoalkylated oligonucleotides from the support and cleavage of protecting groups were carried out in a single step (50 mM DTT in 29% aq. ammonia) treatment at 55°C for 16h. The aqueous solution was divided into portions and concentrated in a Speed Vac concentrator.

Purification of 5'-mercaptoalkylated oligonucleotides by affinity chromatography

5'-Mercaptoalkylated oligonucleotides were purified by an affinity chromatographic method using 5-nitro-2-thiopyridyl activated 3-mercaptoalkylated-Fractosil support (having mercaptoalkyl loading

20 µmol/g of support, 500 A^{0} and 230-400 mesh). The activated mercaptoalkylated support (200 mg) was packed in a small column and equilibrated with 0.1M Tris-HCl buffer, pH 8.5 (10 ml). Crude mixture of mercaptoalkylated- oligonucleotides (1.95 O.D. A_{254}) was taken in 0.1M Tris-HCl buffer containing 20% acetonitrile and applied on to the column. The oligonucleotide solution was allowed to react with the column matrix for 1h followed by washing with the same buffer containing 20% acetonitrile (10 ml). After complete removal of truncated sequences, the desired mercaptoalkylated oligomer was eluted with 50 mM DTT solution in Tris-HCl buffer, pH 8.5 containing 20% acetonitrile. After concentration, the oligomer was desalted on Sephadex G-50 column using 0.1M triethylammonium acetate buffer, pH 7.1. The desalted oligomer (1.05 O.D. A_{254}) was analysed on RP-HPLC.

Labeling of 5'-mercaptoalkylated oligomer

(a) Purified 5'-mercaptoalkylated oligomer d(TTT TT) (1.0 O.D.) was taken in 100 µl of 5% aq. $NaHCO_3$ solution and reacted with 5 equivalents of 1,5-I-AEDANS dissolved in 100 µl of 2.5% aqueous sodium bicarbonate in the presence of one equivalent of DTT. The reaction mixture was agitated and kept in dark for 1h at room temperature with occasional shaking. After desalting and concentration, the labelled oligomer was obtained in 80% and analysed on RP-HPLC.

(b) In another experiment, purified 5'-mercaptoalkylated oligomer d(CTA CCA CTG T) (1.0 O.D.) was dissolved in 200 µl of 0.1M ammonium acetate buffer, pH 7.5 and treated with 2 equivalents of 2,2'-dithiobis(5-nitropyridine) (DTNP) dissolved in 300 µl of acetonitrile. The reaction mixture was agitated at room temperature with occasional shaking. After 1.5h, the reaction mixture was concentrated and desalted on Sephadex G-50. The resulting oligomer was obtained in 85% and analysed on RP-HPLC.

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11. HPLC conditions : column, Lichrosphere (Merck) RP-18; gradient, 0-30% solvent B in 30 minutes at a flow rate of 1.0 ml/min. Solvent A – 0.1M ammonium acetate, pH 7.2, solvent B = Acetonitrile.

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