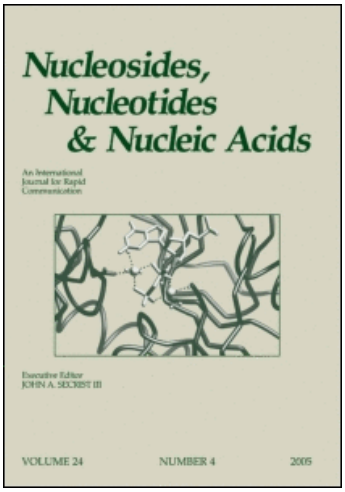

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Introduction of 5'-Terminal Amino and Thiol Groups into Synthetic Oligonucleotides

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INTRODUCTION OF 5'-TERMINAL AMINO AND THIOL GROUPS INTO SYNTHETIC OLIGONUCLEOTIDES

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ABSTRACT

Oligonucleotides terminating in a 5'-primary amine group are synthesized using solid phase phosphoramidite chemistry. The 5'-terminal amine group in the deprotected oligonucleotide is further derivatized with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) followed by treatment with dithiothreitol (DTT) to produce 5'-thiol terminated oligonucleotides. Introduction of 5'-thiol group is further confirmed by reading the absorbance of the released chromophore, pyridine-2-thione at 343 nm; ε_{343}=8080/M.

INTRODUCTION

Oligonucleotides modified at either exocyclic bases or at 5'-end are becoming important tools in biochemistry and molecular biology. Oligonucleotides containing modified bases have been used to characterize the mode of action of various restriction enzymes[1]. 5'-End modified[2] oligonucleotides can conveniently be attached to d-biotin[3,4], fluorescent labels[5] and biologically active molecules[6] in order to prepare probes or primers for DNA sequencing[5], for the detection of nucleic acids[3,4], and to inhibit the translation of mRNA[6]. Usually, these approaches have aimed to produce an oligomer that contains a 5'-amino or thiol group. A number of chemical and enzymatic methods[3,5,7-15] have been developed for the preparation of oligonucleotides with amino group at their 5'-end. However, methods for the selective introduction of a thiol group at the 5'-end of oligonucleotides are limited[16,17] and require multistep synthesis. Recently, Sproat et al[18,19] have introduced a

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thiol group at the 5'-end of synthetic oligonucleotides using protected 5'- (S-triphenylmethyl) mercapto-2',5'-dideoxyribonucleoside-3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidites. However, the method requires the synthesis of four different appropriately protected nucleoside phosphoramidites and preparation of each nucleoside phosphoramidite involves at least three different steps.

In the present report, I wish to describe a simple and versatile one step method to introduce a thiol group into the 5'-termini of deprotected synthetic oligonucleotides. The method is based upon the specific derivatization of a 5'-terminal primary amine group with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), in the presence of an efficient acylating catalyst[20], 4-dimethylaminopyridine (DMAP). This led to a 5'-thiol protected oligonucleotide which in the subsequent reaction with DTT resulted in a free 5'-thiol containing oligonucleotide and pyridine-2-thione. A new N-protected aliphatic aminophos phoramidite reagent viz., 5-N-(4,4'-dimethoxytrityl) aminopentan-1-O-(methyl-N,N-diisopropylamino) phosphoramidite (2) for the selective introduction of a primary amino group at the 5'-termini of synthetic oligonucleotides was prepared. Compound 2 was used directly in solid phase phosphoramidite oligonucleotide synthesis. Furthermore, the 5'-primary aliphatic amino group containing oligonucleotides have been successfully coupled with d-biotin for the preparation of labelled probes. The chemical reaction involved in derivatizing the amine are easily and rapidly performed in aqueous conditions and do not involve hazardous reagents.

MATERIALS AND METHODS

d-Biotin, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), dithiothreitol (DTT), (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), dichloroacetic acid (DCA), avidin-agarose and Tris-HCl were the products of Sigma (St. Louis, USA); sym. collidine, diisopropylethylamine, 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCCI), N-hydroxysuccinimide, 5-aminopentan-1-ol were obtained from Fluka (Buchs, Switzerland); silica gel 60 (70-230 mesh) was procured from Merck (Darmstadt, FRG); spleen

phosphodiesterase was purchased from Boehringer (Mannheim, FRG). Protected deoxynucleoside-3'-O-succinate linked CPG supports and tetrazole were obtained from CSIR Centre for Biochemicals (Delhi, India). Solvents used were duly purified prior to use.

Biotinyl-N-hydroxysuccinimide ester (BNHS) was prepared by the published method[21]. Chloro-N,N-diisopropylamino-methoxyphosphine was synthesized according to the method reported by Adams[22]. Base protected nucleosides were prepared by the transient protection procedure[23], followed by dimethoxytritylation and phosphitylation with chloro-N,N-diisopropylaminomethoxyphosphine.

Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck, Darmstadt, FRG) with the solvent system (A) CH_2Cl_2-EtOAc-triethylamine (TEA) (45:45:10) and compounds detected by spraying perchloric acid solution. ^1H NMR spectrum was recorded on Hitachi FT-60B instrument with tetramethylsilane as an internal standard. ^{31}P NMR spectrum was recorded on a Bruker 500 MHz instrument, sample was dissolved in $CDCl_3$ and 5% aqueous H_3PO_4 was used as reference. UV and visible measurements were done with a Gilford response spectrophotometer.

High pressure liquid chromatography (HPLC) was performed on a LC-4A system (Shimadzu Corporation, Kyoto, Japan) consisting of a UV detector (SPD-2AS) and a data processor (C-R3A). Zorbax ODS column (250 x 4.6 mm I.D., 5 μm) from DuPont (Wilmington, DE, USA) and μ Bondapak C_{18} column (300 x 3.9 mm I.D., 10 μ m) from Waters (Milford, MA, USA) were used for HPLC purifications.

5-N-(4,4'-Dimethoxytrityl)aminopentan-1-ol(1)

To a stirred solution of 4,4'-dimethoxytrityl chloride (2.03 g, 6.0 mmol) and TEA (1.3 ml, 9.0 mmol) in anhydrous pyridine (30 ml), 5-aminopentan-1-ol (2.48 g, 24.0 mmol) was added. The reaction mixture was allowed to stir under argon at room temperature and the progress of the reaction was monitored on TLC. After 3 h of stirring TLC showed completion of reaction and the reaction mixture was concentrated in vacuo to a syrup. The residual syrup was dissolved in dichloromethane (50 ml) and was extracted with

5% $NaHCO_3$ (2 x 50 ml) and aqueous NaCl (2 x 50 ml). The organic phase was dried (Na_2SO_4) and evaporated in vacuo to an oil. The oil was redissolved in a small amount of dichloromethane-TEA (9.5:0.5, v/v) and subjected to column chromatography on Merck silica gel (70-230 mesh) packed in the same solvent system. Fractions containig the desired product were pooled together and concentrated in vacuo to afford compound **1** as an oil. The product obtained above could not be crystallized even at - 20°C. Yield (1.7 g, 70%); R_f (A) 0.59; ^1H NMR ($CDCl_3$): δ 7.5-6.7 (m, 13 H, Ar), 3.77 (s, 6 H, 2 x OCH_3), 3.6 (t, 2 H, $-CH_2OH$), 2.3 (br, 2 H, $-NH-CH_2-$), 1.6 (br, 4 H, aliphatic).

5-N-(4,4'-Dimethoxytrityl) aminopentan-1-O-(methyl-N,N-diisopropylamino) phosphoramidite (2)

Compound **1** (1.0 g, 2.5 mmol) was dried in vacuo over silica gel and dissolved in dry dichloromethane (10 ml) (freshly passed over a column of basic alumina) under argon. Diisopropylethylamine (0.8 ml, 4.8 mmol) was added and the mixture was cooled in an ice-bath. To the above reaction mixture chloro-N,N-diisopropylaminomethoxyphosphine (0.6 ml, 3.0 mmol) was added slowly over 5 min. After 30 min of stirring at room temperature, TLC on silica gel showed completion of reaction (R_f of starting compound 0.59, R_f of product 0.75) and the reaction was arrested with methanol (0.2 ml). Next, the reaction mixture was poured into a solution of ethyl acetate-TEA (20:1, v/v, 50 ml) followed by extraction with cold 10% aqueous Na_2CO_3 (2 x 50 ml) and saturated solution of NaCl (2 x 50 ml). The organic phase was dried (Na_2SO_4), filtered, and evaporated in vacuo to an oil. The oil was redissolved in a small volume of dichloromethane-TEA (9.5:0.5, v/v) and purified on a short column of Merck silica gel (70-230 mesh), packed in the same solvent system. Fractions containing the desired compound were pooled together, and concentrated in vacuo to give the phosphoramidite **2** as a pale yellow oil. The phosphoramidite obtained above could not be crystallized but was pure on TLC. Yield (1.09 g, 78%); R_f(A) 0.75; ^{31}P NMR ($CDCl_3$): δ 148. The product was stored under argon in a sealed vial at -20°.

Synthesis of 5'-amino group containing oligonucleotides

Oligonucleotides were synthesized on a Pharmacia Gene Assembler[24] using methyl phosphoramidite approach[25,26]. The synthesis was carried out on 1.3 μmol scale of bound first nucleoside. After the synthesis of the required sequence, an extra round of coupling was performed using 25 μmol of phosphoramidite **2** and 75 μmol of tetrazole. This step was exactly analogous to the coupling of a normal protected nucleoside phosphoramidite. Following this final coupling, the amino protecting dimethoxytrityl group was removed in the machine itself using an extra detritylation step (3% DCA in ethylene dichloride). The solid support was treated with ammonium thiophenoxide[27] for the removal of internucleotide phosphate protecting groups. The removal of the exocyclic base protecting groups together with the cleavage of the oligonucleotide from the support was achieved with aqueous ammonia treatment (25% aq. ammonia for 16 h at 60°C). The crude oligonucleotide was then purified on reverse-phase HPLC using 0.1 M ammonium acetate buffer, pH 7.0 with acetonitrile as gradient.

Generation of thiol group at the 5'-terminus

5'-Amino group containing oligonucleotide **3**, $H_2N(CH_2)_5$-O-d(pACTTTCG) (5.0 A_{260} units) in 1.0 ml of 0.05 M sodium bicarbonate buffer, pH 8.0 was reacted with 1.5 ml of 10 mM SPDP solution in dry acetonitrile containing 10 mg DMAP at room temperature. After 30 min, an additional 1 ml of 10 mM SPDP and 10 mg DMAP in acetonitrile were added and the reaction was left for a further period of 30 min. The reaction mixture was then concentrated *in vacuo* and desalted on Bio-Gel P-2 column using 0.1 M TEAA buffer, pH 7.0 as an eluent and was analyzed on reverse-phase HPLC. The reaction was quantitative as evident by the disappearance of the starting material and appearance of a single new peak. The pure thiol protected oligonucleotide **5** (4.2 A_{260}, 84%) was collected and concentrated (Savant Speed Vac).

Thiol protected oligonucleotide **5** (4.0 A_{260} units) was treated in 2 ml of 0.05 M Tris-HCl buffer, pH 9 with 1 ml of 50 mM aqueous DTT solution for 15 min at room temperature

resulting in a free 5'-thiol containing oligonucleotide **7** as shown by the HPLC analysis.

Further, the yellow colour of the liberated pyridine-2-thione **9** (λ_{max} =343; ε_{343}=8080/M) in the reduction of **5** with DTT was monitored spectrophotometrically at 343 nm against DTT reagent to determine the formation of free 5'-thiol containing oligonucleotide **7**. In a similar fashion thiol group was generated on $H_2N(CH_2)_5$-O-d(pT_{20}).

Reaction of 5'-amino group containing oligonucleotide with biotinyl-N-hydroxysuccinimide ester (BNHS)

5'-Primary amino group containing oligonucleotide **3**, $H_2N(CH_2)_5$-O-d (pACTTTCG) (1.25 A_{260} units) was dissolved in 150 µl of 0.05 M HEPES buffer pH 7.7, 150 µl of 15 mM BNHS (**10**) in DMF and the mixture was left at room temperature for 24 h. Next, the reaction mixture was concentrated (Savant Speed Vac), desalted on Bio-Gel P-2 using 0.1 M TEAA buffer, pH 7.0 as an eluent. The desalted product was again concentrated and purified on reverse-phase HPLC. The pure product peak was collected, concentrated and desalted on Bio-Gel P-2 column to yield 0.98 A_{260} units (79%) of the biotinylated oligonucleotide.

Binding of biotinylated oligonucleotide on avidin-agarose

The presence of biotin moiety at the 5'-terminus of oligonucleotide **11** was checked by its selective retention on an avidin-agarose column[28]. A column containing 0.2 ml of avidin-agarose (Sigma Chem. Co. 50 U/ml) was washed successively with 2 ml of buffer A , 0.5 ml of buffer A saturated with biotin at a flow rate of 2 ml/h, 0.6 ml of buffer A, 0.6 ml buffer B (2 M urea in buffer A) and 1 ml buffer C (6 M guanidinium hydrochloride, pH 2.5). The column was regenerated by washing with 1 ml buffer A. The biotinylated oligonucleotide about 1 nmol was loaded in buffer A at 2 ml/h flow. The column was washed with 0.6 ml buffer A and 0.6 ml buffer B. The biotinylated material was eluted off the column with 0.9 ml buffer C, desalted on Bio-Gel P-2 column using TEAA buffer, pH 7.0 as an eluent and lyophilized. The biotinylated oligonucleotide was again analyzed on HPLC.

Enzymatic analysis of amino containing oligonucleotides

The 5'-amino group containing oligonucleotide 3 or 4 (1-2 A_{260} units) was digested with spleen phosphodiesterase in 0.1 M ammonium acetate, pH 6.5 (20 µl), for 30 min in an eppendorf tube at 37°C. The enzyme was deactivated by heating at 100°C for 2 min and the reaction mixture was desalted on Bio-Gel P-2 using 0.1 M TEAA buffer, pH 7.0 as an eluent. The enzyme digested oligonucleotide was then subjected to HPLC analysis under the identical conditions used for the purification of 5'-amino containing oligonucleotides.

RESULTS AND DISCUSSION

A large number of reagents[11,12,14] have been proposed for the selective protection of amino groups of amino alcohols in the recent past. Among the known protecting groups, N-tritylation offers several advantages, since N-tritylation is selective in presence of hydroxyl functionalities and the lipophilic character of trityl group usually facilitates the easy and rapid separation of 5'-tritylated oligonucleotides by reverse-phase (C_{18}) HPLC. Selective N-tritylation of amino groups in the presence of hydroxyl functionalities has already been reported in the case of amino acids and amino alcohols in aqueous conditions[29-31]. Recently, Connolly[15] has demonstrated the use of trityl and monomethoxytrityl groups for the selective blocking of amino group of 3-aminopropan-1-ol. The method, however, suffers from the drawback that the cleavage of N-monomethoxytrityl linkage requires considerable time (2 h in 80% aqueous acetic acid), while the N-trityl linkage does not cleave completely even after 24 h in 80% acetic acid at room temperature. I decided therefore, to investigate the use of 4,4'-dimethoxytrityl group for the protection of amino functionalities of amino alcohols. DMTr group can be introduced selectively in an analogous way to trityl and monomethoxytrityl and can be removed under very mild conditions compatible to DNA synthesis. Dimethoxytritylation of 5-aminopentan-1-ol (4 equiv.) with dimethoxytrityl chloride was carried out in dry pyridine. Almost exclusive N-tritylation as opposed to O-tritylation was obtained.

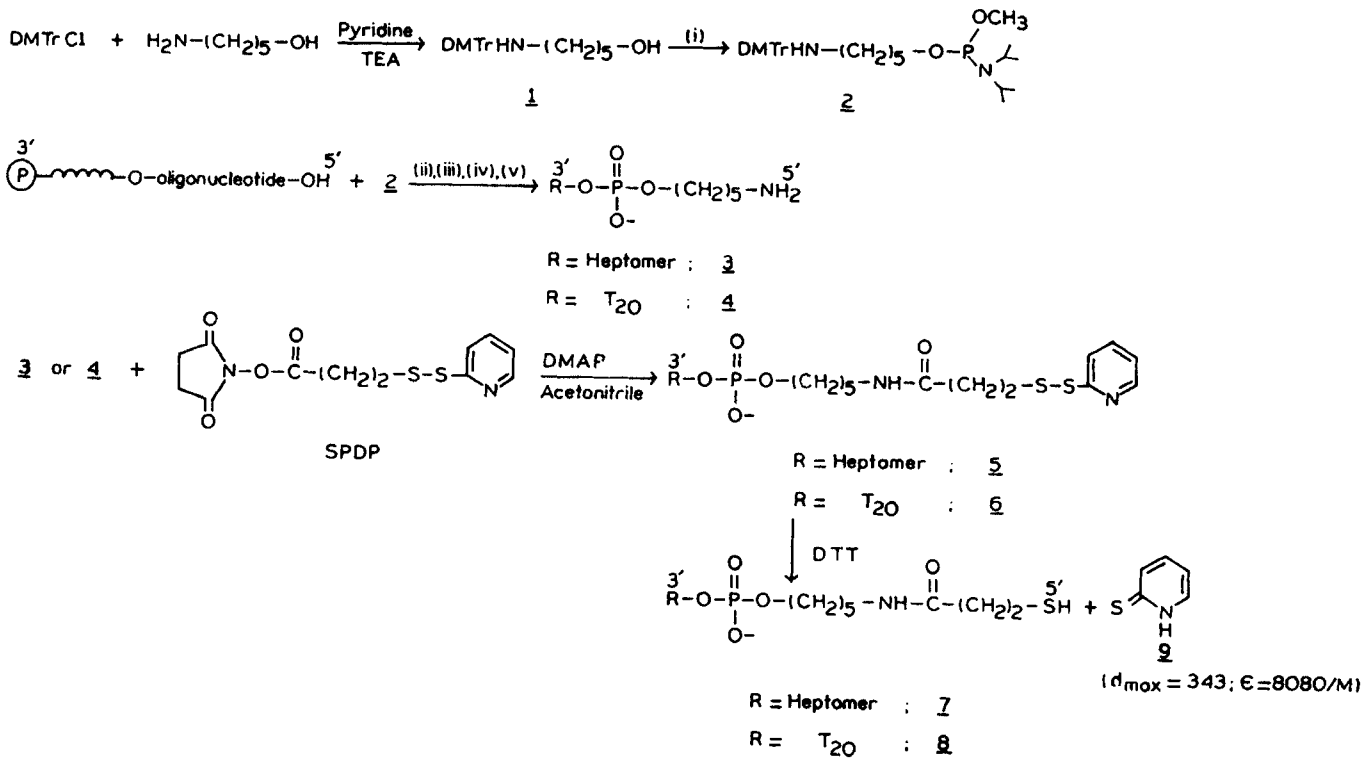

FIG. 1. Reaction scheme for the synthesis of 5'-amino and 5'-thiol containing oligonucleotides; i)= dichloromethane/diisopropylethylamine/chloro-N,N-diisopropylaminomethoxyphosphine; ii)= tetrazole; iii)= aq. iodine solution; iv)= 3% DCA in EDC; v)= 25% aq. ammonia, 60° C for 16 h; R = deprotected oligonucleotide.

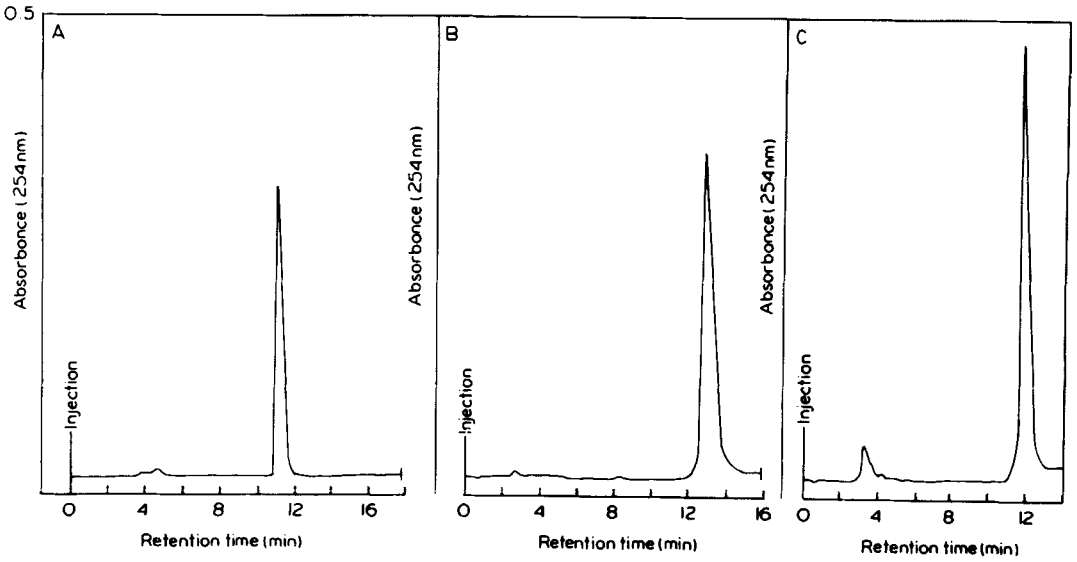

FIG. 2. Reverse phase HPLC profile of : A, $H_2N-(CH_2)_5-O-(pACTTTCG)$; B, analysis of the reaction mixture after reacting the 5'-amino terminated oligonucleotide 3 with SPDP ; C, analysis of the mixture after reacting the thiol protected oligonucleotide 5 with DTT, column; Zorbax ODS (4.6 x 250 mm); flow 1ml/min; gradient 0% B in 2 min, 0-100% B in 20 min; solvent A, 5 % acetonitrile in 0.1 M ammonium acetate buffer pH 7.0; solvent B, 100% acetonitrile.

Compound 1 was obtained as a yellow oil in 70% yield after column chromatography and was treated with chloro N,N-diisopropylaminomethoxyphosphine in dry dichloromethane in the presence of diisopropylethylamine to give crude phosphoramidite (2) which was purified by column chromatography on silica gel. The phosphoramidite 2 was found to be homogeneous by TLC and ^{31}P NMR spectroscopy. Unfortunately, it could not be obtained in solid form but could be stored as an oil under anhydrous conditions at -20°C for months. The reaction scheme for the synthesis of phosphoramidite 2 is depicted in FIG. 1.

Phosphoramidite 2 was used for the preparation of a model sequence $DMTrHN-(CH_2)_5O-d(pACTTTCG)$ using methyl phosphoramidite chemistry by solid phase approach. The above sequence was detritylated by 3% DCA in the machine itself

to obtain free 5'-amino group containing oligonucleotide $H_2N(CH_2)_5$ O-d(pACTTTCG) (3). The crude primary amino group containing oligonucleotide $H_2N(CH_2)_5$ O-d(pACTTTCG) (3) was then purified by reverse-phase (C_{18}) HPLC. FIG. 2 A shows a reverse-phase HPLC chromatogram of the oligonucleotide $H_2N(CH_2)_5$ O-d(pACTTTCG)(3). The peak eluting at 11.Ø4 min is characteristic of the desired product. The HPLC purified sequence was digested with spleen phosphodiesterase enzyme, and after desalting it was subjected to reverse-phase HPLC analysis. The chromatographic behaviour of the enzyme digested oligonucleotide **3** was found to be unchanged, i.e., eluted with the same retention time. This, clearly demonstrated that the 5'-amino group containing sequence was not accepted as a substrate by the spleen phosphodiesterase enzyme. This confirms that the 5'-position of the oligonucleotide is not hydroxyl but has been substituted with an alkyl amino chain. The oligonucleotide H_2N-$(CH_2)_5$ O-d($pT_{2Ø}$) (4, FIG. 1) was also prepared and purified in an analogous fashion to heptamer sequence. FIG. 3 A shows the HPLC purification of H_2N-$(CH_2)_5$O-d($pT_{2Ø}$) on C_{18} column. Synthesis scheme of biotinyl-N-hydroxysuccinimidyl ester **1Ø** is depicted in FIG. 4 and was carried out according to the reported method[21] in 58% yield by reacting d-biotin with N-hydroxysuccinimide in aqueous DMF.

As an example of the use of amino containing oligonucleotide **3**, its reaction with biotinyl-N-hydroxysuccinimidyl ester (BNHS) was investigated. A Large excess of BNHS (1Ø) was required to obtain high product yield. The reaction was quantitative under the experimental conditions as evident by the disappearance of the starting material and the appearance of a single new peak as shown in FIG. 5. The isolated oligonucleotide-biotin adduct was completely bound by an avidin-agarose column while H_2N-$(CH_2)_5$ O-d(p ACTTTCG) was not. Recovery of the biotin labeled heptamer **11** (FIG. 4) was 65%. No reaction products were observed from the exposure of d(ACTTTCG) to the activated ester of d-biotin under the identical conditions, indicating that the oligonucleotide d(ACTTTCG) does not contain a primary aliphatic amino group.

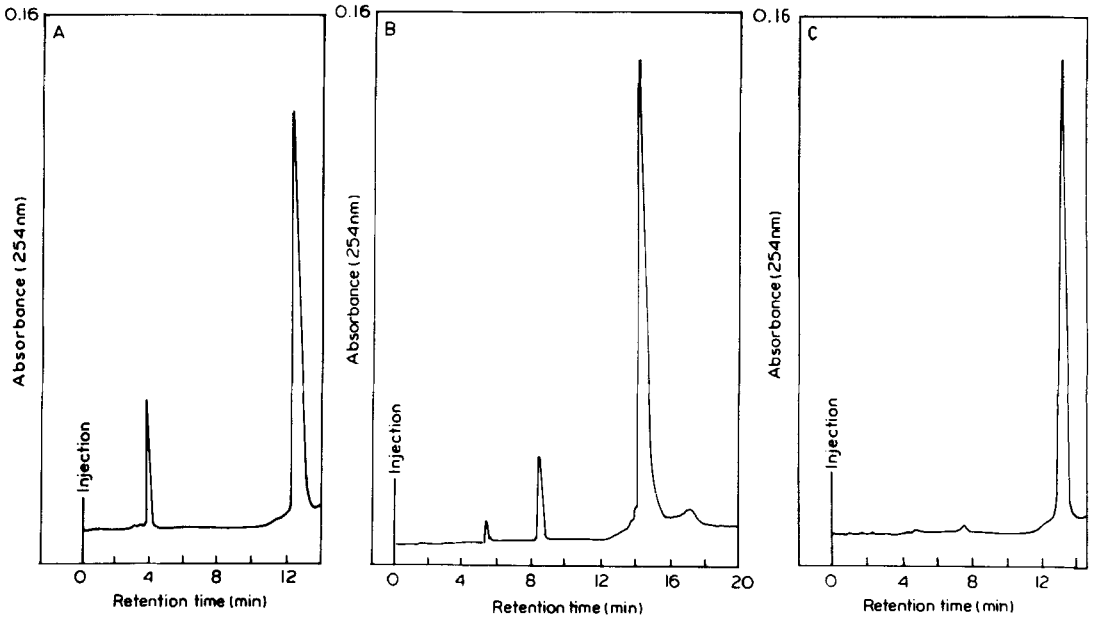

FIG. 3. Reverse phase HPLC profile of: A, $H_2N-(CH_2)_5-O-(pT_{20})$; B, analysis of the reaction mixture after reacting 5'-amino terminated oligonucleotide 4 with SPDP; C, analysis of the mixture after reacting the thiol protected oligomer 6 with DTT ; elution conditions same as described in Fig. 2.

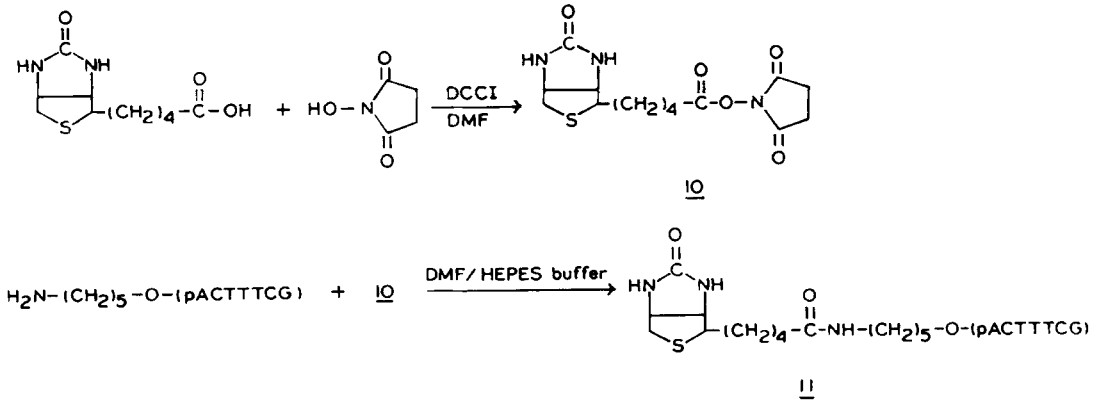

FIG. 4. Reaction scheme for synthesis of biotinyl-N-hydroxysuccinimide ester and its further derivatization to a 5'-amino group containing oligonucleotide 3.

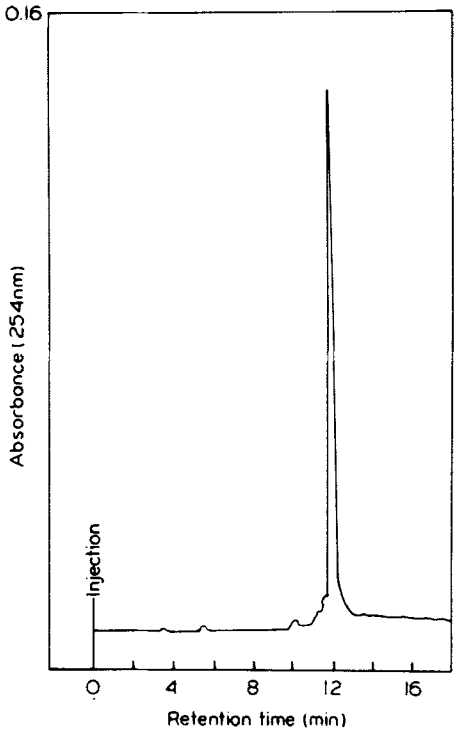

FIG. 5. Reverse phase HPLC profile of the reaction of a 5'-amino group containing oligonucleotide 3 with biotinyl-N-hydroxysuccinimide ester; column μ Bondapak C_{18} (3.9 x 300 mm); elution conditions same as described in Fig. 2.

It is well documented in the literature[13] and also confirmed from the present investigation that even with a large excess of BNHS (10) no side reaction with the exocyclic amino groups was observed. Recently, Bischoff et al[17] in a separate study also observed the same results with the reagent dithiobis (succinimidylpropionate) (DSP). Encouraged by the findings of Bischoff et al[17] and results obtained by the reaction of oligonucleotide 3 with biotinyl-N-hydroxysuccinimidyl ester, I decided to investigate the reaction of N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) with 5'-amino group containing oligonucleotides.

In order to generate a thiol group at the 5'-termini of synthetic oligonucleotides, H_2N-$(CH_2)_5$O-d(pACTTTCG) (3) was reacted with a commercially available reagent SPDP in the

presence of an acylating catalyst, 4-dimethylaminopyridine (DMAP). This led to a 5'-thiol protected oligonucleotide **5** which in the subsequent reaction with DTT resulted in a free 5'-thiol containing oligonucleotide **7** and pyridine-2-thione **9**. The reaction scheme is given in FIG. 1.

The acylation of 5'-amino containing oligonucleotide with SPDP was found to be quantitative in 1 h at room temperature as evident by the disappearence of the starting material and appearence of a single new peak as shown in the FIG. 2 B. The isolated 5'-thiol protected oligonucleotide was treated with DTT solution. The reaction was found to be complete in 30 min at room temperature as confirmed by the HPLC analysis of the desalted product (FIG. 2 C). The product peak appeared at 11.7 min as compared to the corresponding 5'-thiol protected oligonucleotide **5** (12.7 min). The decrease in retention time of the free 5'-thiol containing oligonucleotide **7** as compared to its corresponding 5'-thiol protected oligonucleotide **5** is attributed to the decrease in hydrophobicity of the free 5'-thiol containing oligonucleotide due to the release of the chromophoric group, pyridine-2-thione from the 5'-thiol protected oligonucleotide. Moreover, the yellow colour of the liberated pyridine-2-thione in the reaction of thiol protected oligonucleotide **5** with DTT also confirmed the generation of free thiol group. The formation of free 5'-thiol containing oligonucleotide was further confirmed and quantified by reading the absorbance of the released pyridine-2-thione (λ_{max}=343; ε_{343}=8080/M) at 343 nm against DTT solution as blank. Similarly thiol group was generated at the 5'-terminus of the oligonucleotide, H_2N-$(CH_2)_5$ O-d (pT_{20}). FIG. 3 (A-C) shows the HPLC profile of the oligonucleotides **4**, **6** and **8** respectively.

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