A simple and efficient procedure for the synthesis of 5'-aminoalkyl oligodeoxynucleotides

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ABSTRACT

Synthetic deoxyoligonucleotides have been 5'-aminoalkylated at the end of step-wise synthesis on the polymer support. This was achieved through the activation of the 5'-hydroxyl group as its 5'-imidazolyl derivative using carbonyldiimidazole, which was subsequently displaced with hexamethylene diamine to yield the title compound. The alkyl carbamate linkage thus generated withstands the deprotection conditions used in oligonucleotide synthesis. Purification by gel electrophoresis and further derivatization at the 5'-amino group with N-hydroxysuccinimidobiotin is described.

INTRODUCTION

Derivatization of synthetic oligodeoxynucleotides at the 5'-terminus has been difficult due to the unreactive nature of the primary hydroxyl group. Thus far there have been only a few reports for the preparation of deoxyoligonucleotides having a 5'-aminoalkyl derivative. Hood and coworkers (1) recently prepared synthetic oligomers containing a 5'-amino group through the introduction of suitably protected 5'-amino-5'-deoxythymidine as its reactive phosphoramidite derivative. This was done at the end of the synthesis cycle. However, this methodology required the synthesis of a 5'-amino-5'-deoxythymidine monomer. The second method, as developed by two groups (2, 3), reported the preparation of 5'-aminoalkyl phos-phoramite derivatives of unprotected oligonucleotides by reaction of diamines with a 5'-phosphorimidazolide. However, this method suffers from the disadvantage of introducing a phosphoramide linkage between the 5'-end of the oligomer and the diamine which is prone to ready acid-catalyzed hydrolysis. The third method of Kemp, et al. (4) introduced 2-(biotinylamide)ethanol by reaction with a 5'-phosphorylated

polymer-supported nucleotide. Recently, Connolly and Rider
have introduced a thiol group at the 5'-terminus of a synthetic
oligomer using S-trityl-6-mercaptohexanol as its morpholino-
phosphite derivative and subsequent deprotection of the S-
trityl group (5).

In the present report we wish to communicate a simple and
efficient procedure, using commercially available reagents, for
the synthesis of 5'-aminoalkyl derivatives of synthetic
oligomers on a Controlled Pore Glass solid support. The
present procedure utilizes the highly reactive carb-
onyldiimidazole (CDI) to activate the 5'-hydroxyl group as its
5'-imidazolyl derivative which undergoes a clean nucleophilic
displacement reaction in the presence of an alkylamino
compound.

CDI has been used for the preparation and isolation of
stable steroidal imidazole-1-carboxylic acid esters (6).
Recently, Bethell and coworkers, in a series of papers (7),
investigated the use of CDI for the activation of polysac-
charides for subsequent use in affinity chromatography of
proteins. These workers observed that the CDI-activated
polysaccharides were reasonably stable to aqueous hydrolysis
(30 hr at pH 8.5 for complete hydrolysis at room temperature)
but were extremely susceptible to nucleophilic attack.
Further, they found that the N-alkylcarbamate linkage, formed
between an amino group and the CDI-activated polysaccharides,
were stable at room temperature over a wide pH range (2.5-12).
Before the successful extension of this method for preparation
of 5'-aminoalkyl derivatives of synthetic oligomers, the fol-
lowing criteria must be met. Since CDI is highly moisture
sensitive, it was reasoned that the activation of the 5'-
hydroxyl group has to be carried out at the end of the step-
wise synthesis of oligonucleotides on the polymer support.
Further displacement reaction of the activated oligomer with an
aminoalkyl compound should be performed prior to treatment with
concentrated ammonium hydroxide which is used for cleavage and
deblocking. Also, the alkylcarbamate linkage thus generated
should be stable enough to withstand the conc. NH_4OH at 55° for
5 hr. Recently, Sproat and Brown have shown the cleavage of
3'-arylcarbamate linkage with conc. NH_4OH over a 24-48 hr
period at 55° (8). Finally, the reactions had to be clean and

quantitative for ease in separation and isolation of the final product.

Here we present data to show that all the above criteria have been met and that 5'-aminoalkyl oligomers could be successfully synthesized in high yields. Also that these were further derivatized at the 5'-amino group to obtain a 5'-biotinylated oligomer reacting with N-hydroxysuccinimido biotin.

EXPERIMENTAL PROCEDURES
Materials
    Carbonyldiimidazole was purchased from Pierce Chemical Company and Sigma Chemical Company. HPLC grade dioxane was obtained from Caledon Laboratories (Georgetown, Ontario, Canada). Biotin-N-hydroxysuccinimide ester was from Calbiochem. Deoxyoligonucleotides were synthesized according to published procedure (9, 10) on an Applied Biosystems 380A DNA synthesizer. Fully protected nucleoside O-methylphosphoramidite monomers were purchased from Applied Biosystems (Foster City, California). The HRP Color Development Reagent was obtained from Biorad.
Methods
    Synthesis of deoxynucleotides. Oligonucleotides were synthesized starting with 0.10-0.30 μM of 3'-base attached to controlled pore glass solid support. The 380A synthesizer was programmed for DMTr-off, Manual as the ending method.
    Synthesis of 5'-aminoalkyl oligonucleotides. The solid support, with the DNA still attached to it, was taken up in a small reaction vessel having a medium porosity frit and a stopcock. The resin was washed well under anhydrous conditions with HPLC grade dioxane (3x3 ml). CDI (50 mg) was dissolved in 1 ml of dioxane and this solution was introduced into the reaction vessel with the use of a syringe. The activation was allowed to proceed for 30 min at room temperature with occasional shaking. After this time, the solid support was washed well with dry dioxane (5x3 ml) and argon-dried.
    The activated oligomer was then allowed to react with a 1.0 ml solution of 0.2 M hexamethylenediamine dissolved in 9:1 dioxane:water. The reaction was allowed to proceed for 20 min at room temperature. At the end of this time, the reagents

were washed off with dioxane, methanol, and air-dried.

Cleavage, deprotection, and purification of derivatized
oligomers. The solid support was treated with a 1.0 ml solu-
tion of thiophenol : triethylamine : dioxane (1:2:2) for 30 min
to remove the phosphate protecting groups. The oligomer was
then cleaved from the support with conc. NH_4OH for 1 hour and
the ammonium hydroxide solution (~3 ml) was then heated at 55°
for ~5 hours to remove base protecting groups. The ammonium
hydroxide solution was removed under vacuum. The oligomer was
taken up in water and lyophilized to dryness. It was then
purified on a 1.5 mm thick denaturing polyacrylamide gel (for
the 15-mer and 21-mer a 20% gel was used whereas for the 30-mer
a 15% gel was used). The oligomer was visualized under short-
wave UV light and the prominent DNA band was cut out and eluted
off the gel pieces using 100 mM triethylammonium bicarbonate
buffer (pH 7.0) for 4 hr at 60°.

The oligomer present in the eluent was then desalted on a
1 ml disposable C_{18} column (Baker) and was eluted off the
column with 30% $MeOH/H_2O$. The DNA content was measured at A_{260}
in a 1 ml diluted aliquot.

Preparation and purification of 5'-biotinylated oligomer.
Biotinylation was performed after removal of protecting groups
with conc. NH_4OH, then removing the ammonia by repeated
lyophilization with water (2x300 μl). The crude oligomer was
then suspended in 200 μl 0.2 M HEPES buffer (pH 7.7). To this
was added a solution of N-hydroxysuccinimido-biotin (2 mg in
100 μl DMF). The reaction was allowed to proceed for 2-4 hr
with occasional shaking. At the end of this time, the reaction
mixture was applied to a 25 ml G25 Sephadex column and was
eluted with 10 mM triethylammonium bicarbonate (pH 7.0). There
were 25 fractions of ~.60 ml collected, the void volume frac-
tions showing A_{260} absorption were pooled (usually 4 tubes) and
lyophilized. The oligomer was further purified on a denaturing
polyacrylamide gel and desalted as before.

RESULTS
Synthesis of 5'-aminoalkyl Derivatives of Oligonucleotides
    Oligonucleotides of different length and composition have
been 5'-aminoalkylated according to the reaction scheme in Fig-
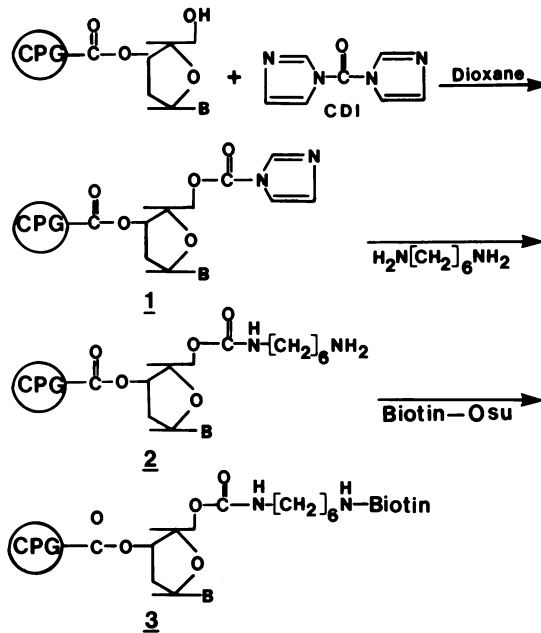1. The most critical reaction is the initial activation with

Fig.1   A general reaction scheme for the synthesis of 5'-aminoalkyl oligomer and its further derivatization to 5'-biotinylated oligomer. B = A, G, C, or T.

CDI.   We have done this in a reaction vessel such as that used for manual synthesis or in a semi-automated mode on the 380A DNA Synthesizer.   We observed no difference in efficiency in either   procedure,   provided   that   moisture   is   thoroughly excluded.   Even though in the manual synthesis we have used a large   excess   of   CDI   for   activation, this was reduced to at least 1/3 in the automated mode with similar end results.   We also found that the activation reaction was complete in 30-45 minutes.

     Once the activation of the oligomers was complete, the solid support was thoroughly washed and the displacement reac-tion with an excess of hexamethylenediamine was done manually for 15-20 minutes. It was then taken through the deprotection cycle as described under Experimental Procedures. We used hexamethylenediamine due to the previously observed fact (3, 11) that a spacer between the oligomer and a reporter molecule marginally increases the sensitivity of detection without undue
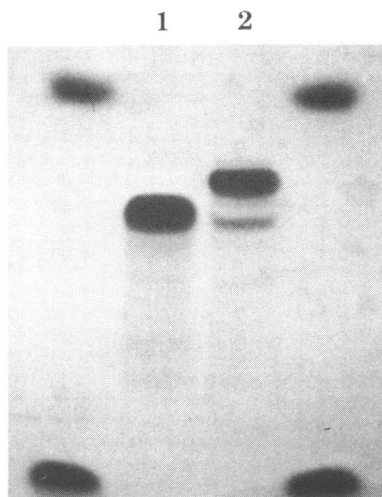
Fig.2   Electrophoretic mobility pattern of crude 5'-hydroxyl-dT$_{15}$ (lane 1) and that of crude 5'-aminoalkyl-dT$_{15}$ (lane 2) on a 20% bisacrylamide/7M urea gel.   Approximately 20% of the crude reaction product of each was loaded per lane and was visualized by UV shadowing. Lane 2 also shows the presence of a small amount of underivatized 5'-hydroxyl-dT$_{15}$.   In this case, the activation reaction with CDI was done using a semi-automated mode on a 380A DNA synthesizer and the reaction time was 30 min.   The displacement reaction with the diamine was done manually.

destabilization of DNA-DNA hybrid when the oligomer was used for hybridization studies. Further, the introduction of a six-carbon chain at the 5'-end decreases the mobility of the derivatized oligomer compared to an underivatized one thereby making it easier to isolate the product on a denaturing polyacrylamide gel. (Figure 2 shows the mobility pattern of crude derivatized and underivatized oligomer dT$_{15}$.) This is also true for other oligomers of different length and composi-tion, although to a lesser extent (Fig-3). This fact is also reflected in the retention time of the oligomers on a HPLC, reverse phase C$_{18}$ column. Thus, although the 5'OH-dT$_{15}$ and 5'-aminoalkylated-dT$_{15}$ gave base-line separation from each other (Fig-4), the derivatized 21-mer and 30-mer had nearly identical retention times compared to the 5'-hydroxyl oligomers (data not shown).   However, we believe that the use of ion-pairing reagents during the HPLC-mode of separation should discriminate between 5'-aminoalkyl and 5'-hydroxyl oligomers sufficiently so
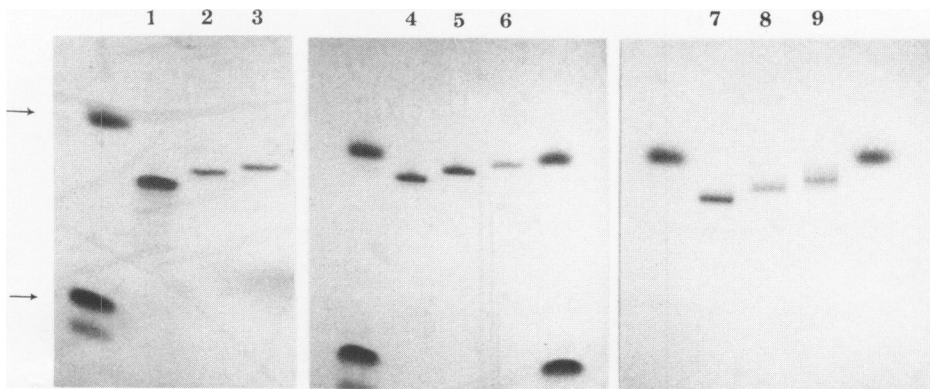
Fig.3   Electrophoretic mobility patterns of the 5′-hydroxyl, 5′-aminoalkyl, and the 5′-biotinylated samples of a 15-mer $(dT_{15},)$ 21 mer (5′-CTCCTGGGCACAGCGGGACGA-3′), and 30-mer (5′-CACGCTGCTCTCCTGGGCACAGCGGGACGA-3′). Lane 1: 5′-hydroxyl-dT_{15}, Lane 2: 5′-aminoalkyl-dT_{15}, Lane 3: 5′-biotinylated-dT_{15}, Lane 4: 5′-hydroxyl-21-mer, Lane 5: 5′-aminoalkyl-21-mer, Lane 6: 5′-biotinylated-21-mer, Lane 7: 5′-hydroxyl-30-mer, Lane 8: 5′-aminoalkyl-30-mer, Lane 9: 5′-biotinylated-30-mer. The 15-mer and 21-mer are on a 20% bisacrylamide/7M urea gel. The 30-mer is on a similar 15% gel. Approximately 0.20 A_{260} units are loaded per lane. Visualization is by UV shadowing. Arrows show the position of the dyes, xylene cyanol (upper) and bromophenol blue (lower).

as to make it an alternative method of separation irrespective of length and composition.

One of the potential side reactions that could take place during nucleophilic displacement with the diamine, was the cleavage of the succinoyl ester bond present between the oligomer and the solid support thereby releasing the oligomer prematurely. We have excluded this side reaction by collecting the effluent after treatment with diamine, lyophilizing to dryness, taking the residue through the deprotection cycle with conc. NH_4OH, and electrophoresing the residue on a denaturing polyacrylamide gel. On UV visualization, we were unable to detect any oligomer band. The absence of any such side reaction may be partly due to the minimal reaction time that we have used and partly due to steric effects.

Biotinylation of 5′-aminoalkyl Oligomers

We have successfully derivatized the 5′-aminoalkyl oligomers with N-hydroxysuccinimido-biotin. This reaction could be done either before or after gel purification of the
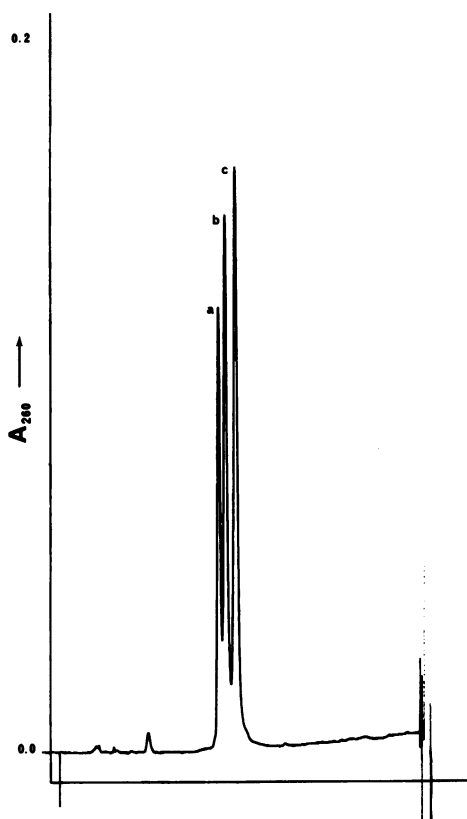
Fig. 4   HPLC C_{18} reverse phase elution profile of a mixture of approximately 0.1 A_{260} units of 5'-hydroxyl-dT_{15} (peak a, retention time 10 min.), 5'-aminoalkyl-dT_{15} (peak b, retention time 10.4 min.), and 5'-biotinylated-dT_{15} (peak c, retention time 10.9 min.). The initial conditions of the chromatogram shown are solvent A for 2 minutes and then a linear gradient of solvent A to 60% solvent B over 20 minutes at a flow rate of 1.4 ml/min (solvent A - 5% AN/95% 0.1 M NH_4Ac, pH 6.93; solvent B = 80% AN/20% 0.1 M NH_4Ac, pH 6.93.

oligomers. We have observed the reaction to be quantitative and complete in under 4 hours. The mobility of the biotiny-lated oligomers on a denaturing polyacrylamide gel was further decreased to a small extent and was well resolved from the 5'-aminoalkyl and 5'-biotinylated oligomers thereby facilitating the isolation of the product. The electrophoresis pattern of 5'-hydroxyl, 5'-aminoalkyl and 5'-biotinylated oligomers is presented in Fig-3. Again we observed a similar pattern, both

in electrophoretic mobility and retention time on a reverse phase C_{18} column, as was found for the 5'-aminoalkyl and 5'-hydroxyl oligomers. Thus on a reverse phase C_{18} column, we were able to get near base-line separation of 5'-aminoalkyl-dT_{15} and 5'-biotinylated-dT_{15}. However, under the conditions we have used, this separation was found to be difficult for the longer oligomers. The biotinylated oligomers appeared as a shoulder on the aminoalkyl oligomer peak for both the 21-mer and the 30-mer. Thus for long oligomers which do not resolve well on a polyacrylamide gel or by HPLC, an avidin column could be advantageous for further purification. It is necessary, however, to initially desalt the biotinylation reaction mixture on a Sephadex column.

Additional proof of the presence of biotin at the 5'-end is shown by a horseradish peroxidase color development reaction (data not shown). A nylon membrane was spotted with approximately 0.5 μg of the biotinylated oligomer. It was then blocked with 3% BSA and thoroughly rinsed before adding the streptavidin-biotinylated-horseradish peroxidase complex which allows for the amplification of the biotin signal in the color test. The color development reaction was consistent with the expected results thus reaffirming the presence of biotin at the 5'-end of the oligomer.

DISCUSSION

In this communication we report a simple and efficient synthesis of 5'-aminoalkyl derivatives of synthetic oligonucleotides using commercially available reagents. The fact that the carbamate linkage introduced between the oligomer and the aminoalkyl group is stable to the deprotection conditions normally used in oligonucleotide synthesis, raises the possibility of routinely synthesizing 5'-aminoalkyl oligomers on an automated mode. In addition, it would be possible to introduce a variety of reactive functional groups at the 5'-end. For example, reacting the CDI-activated oligomer with Σ-aminocaproic acid would furnish a carboxylic acid derivative. An -SH group could be introduced by reaction with a thiol containing aminoalkyl compound in which the -SH group is suitably protected. The oligomers thus prepared could be used for attaching a variety of reporter molecules at the 5'-end, or

in affinity chromatographic isolation of desired RNA molecules. Our study has also clearly shown that 5'-derivatization is possible irrespective of length and composition of the synthetic oligomer.

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