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

Leslie Wachter, Jo-Ann Jablonski and Kuzhal L. Ramachandran*

Department of Chemistry, Biogen Research Corp., 14 Cambridge Center, Cambridge, MA 02142, USA

Received 29 June 1986; Revised and Accepted 12 September 1986

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 phosphoramite 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-mercaptophexanol 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 carbonyldiimidazole (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 polysaccharides 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 following 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. NH₄OH at 55° for 5 hr. Recently, Sproat and Brown have shown the cleavage of 3'-arylcarmamate linkage with conc. NH₄OH over a 24-48 hr period at 55° (8). Finally, the reactions had to be clean and

7986
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 stop-cock. 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 solution 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. NH₄OH 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 C₁₈ column (Baker) and was eluted off the column with 30% MeOH/H₂O. The DNA content was measured at A₂₆₀ in a 1 ml diluted aliquot.

Preparation and purification of 5'-biotinylated oligomer. Biotinylation was performed after removal of protecting groups with conc. NH₄OH, 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 fractions showing A₂₆₀ 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
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 reaction 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
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 dT15.) This is also true for other oligomers of different length and composition, although to a lesser extent (Fig-3). This fact is also reflected in the retention time of the oligomers on a HPLC, reverse phase C18 column. Thus, although the 5'-OH-dT15 and 5'-aminoalkylated-dT15 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<sub>15</sub>) 21-mer (5′-CTCCTGGGCACAGGGAAGGA-3′), and 30-mer (5′-CACGCTGTCTCTGGCAGCAGGGAAGGA-3′). Lane 1: 5′-hydroxyl-dT<sub>15</sub>, Lane 2: 5′-aminoalkyl-dT<sub>15</sub>, Lane 3: 5′-biotinylated-dT<sub>15</sub>, 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<sub>260</sub> 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<sub>4</sub>OH, 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, reverse phase elution profile of a mixture of approximately 18.1 A260 units of 5'-hydroxyl-dT15 (peak a, retention time 10 min.), 25'-aminoalkyl-dT15 (peak b, retention time 10.4 min.), and 5'-biotinylated-dT15 (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 NH4Ac, pH 6.93; solvent B = 80% AN/20% 0.1 M NH4Ac, pH 6.93.

oligomers. We have observed the reaction to be quantitative and complete in under 4 hours. The mobility of the biotinylated 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 \( \mu g \) 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 E-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

7993
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.

*Address correspondence to this author.

REFERENCES
1. Smith, L.M., Fung, S., Hunkapillar, M.W., Hunkapillar, T.J. and Leroy Hood. (1985) Nucleic Acid Res. 13, 2399-2412
2. Chollet, A. and Kawashima, E.H. (1985) Nucleic Acid Res. 13, 1529-1541
3. Chu, B.C.F. and Orgel, L.E. (1985) DNA 4, 327-331
4. Kemp, T., Sundquist, W.I., Chow, F. and Hu, S.L. (1985) Nucleic Acid Res. 13, 45-57
5. Connolly, B.A. and Rider, P. (1985) Nucleic Acid Res. 13, 4485-4502
6. Farenholtz, K.E., Boris, A., Kennedy Jr., T.W. and Kierstead, R.W. (1974) J. Med. Chem. 17, 337-342
7. Bethell, G.S., Ayers, S.J., Hancock, W.S. and Hearn, M.T.W. (1979) J. Biol. Chem. 254, 2572-2574; Bethell, G.S., Ayers, J.S., Hearn, M.T.W. and Hancock, W.S. (1981) J. Chromatography 219, 353-359; Hearn, M.T.W., Harris, E.L., Bethell, G.S., Hancock, W.S. and Ayers, J.A. (1981) J. Chromatography 218, 509-518; and Hearn, M.T.W., Bethell, G.S., Ayers, J.S. and Hancock, W.S. (1979) J. Chromatography 185, 463-470
8. Sproat, B.S. and Brown, D.M. (1985) Nucleic Acid Res. 13, 2979-2987
9. McBride, L.J. and Caruthers, M.H. (1983) Tet. Lett. 24, 245-248
10. Adams, S.P., Kavka, K.S., Wykes, E.J., Holder, S.B. and Galluppi, G.R. (1983) J. Am. Chem. Soc. 105, 661
11. Leary, J.J., Brigati, D.J. and Ward, D.C. (1983) Proc. Natl. Sci. 80, 4045-4049