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A NOVEL METHOD FOR THE INTRODUCTION OF AN ALIPHATIC PRIMARY AMINO GROUP AT THE 5' TERMINUS OF SYNTHETIC OLIGONUCLEOTIDES

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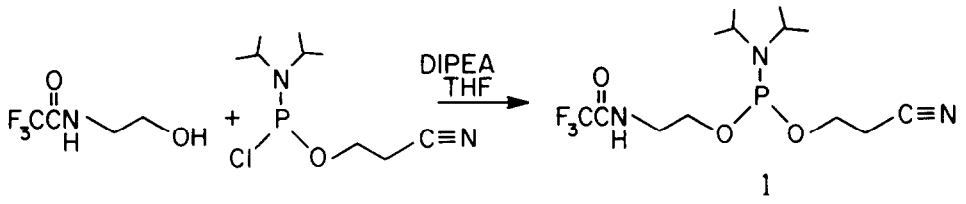
Summary: Reaction of the 5' hydroxyl group of a support bound synthetic oligonucleotide with the new reagent N-trifluoroacetyl-2-aminoethyl-(2-cyanoethyl)-N,N-diisopropylaminophosphoramidite yields, upon deprotection, an oligonucleotide having a 5' primary amino terminus. The amino derivatized oligomer can then be specifically reacted with the N-hydroxysuccinimide ester of d-biotin to give a stable oligonucleotide-biotin adduct.

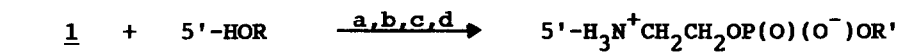
Synthetic oligonucleotides covalently attached to d-biotin (1,2), fluorescent labels (3), and biologically active molecules (4) are becoming important tools of molecular biology. They have been used for the detection of nucleic acids (1,2), for DNA sequence analysis (3) and to inhibit the translation of mRNA (4). A variety of enzymatic and chemical procedures have been developed for their synthesis (1-3,5-7). Central to some of these procedures was the introduction of a single primary amino group into the oligonucleotide (1,3,6,7). The amino group was reacted with an electrophile such as an N-hydroxysuccinimide-carboxylic acid ester or an isothiocyanate to yield the desired oligodeoxyribonucleotide derivative. Attachment of the amine to the oligonucleotide was accomplished either by incorporation of an amine-containing modified nucleoside during the oligomer synthesis (3,7) or by a combination of enzymatic and chemical reactions on the purified DNA molecule (1,6). Since both methods require the chemical synthesis of an oligonucleotide, the former has the advantage that the amine is easily introduced in high yield during the oligonucleotide synthesis. However, it requires the synthesis of a modified nucleoside and its conversion into a phosphoramidite or phosphodiester monomer.

We have prepared a new N-protected aliphatic amino phosphoramidite (8) reagent that facilitates the introduction of a primary amino group into an oligonucleotide during its synthesis. The compound is used directly in a phosphoramidite solid-phase oligonucleotide synthesis protocol. A slight modification of the standard deprotection conditions (9) minimizes the formation of a minor side product. Reagent 1 is readily obtained in high yield from previously described starting materials.

When N-trifluoroacetyl-2-aminoethanol (10) was reacted with N,N-diisopropylamino-2-cyanoethoxychlorophosphine (9) in the presence of diisopropylethylamine, compound 1 was obtained in 100% crude yield. Short column chromatography on silica gel (11) removed traces of a phosphonic acid impurity to give a pure colorless oil in 72% yield (12). Compound 1 is stable for at least a month at -20°C.

To test the efficacy of 1 for amine group introduction, 80 mg of an improved controlled substitution aminopropyl derivatized silica (13) containing 4.0 μmol of 5'-O-dimethoxytrityl-thymidine was reacted five times with the 3'-N,N-diisopropylamino-2-cyanoethylphosphoramidite





R = protected oligonucleotide, R' = deprotected oligonucleotide
a) 1-H-tetrazole b) aqu. I_2 c) DBU d) conc. NH_4OH, 60°C

of 5'-O-dimethoxytritylthymidine using our standard synthetic cycle (14). The synthesis was conducted in a manually operated syringe apparatus (15). Following the last detritylation step, 25% of the support was removed and exposed to 5.0 mL of concentrated aqueous ammonia for 20 hours at 60°C to yield 43.7 A_{260} units of the hexamer T-T_4-T. The remaining support was reacted for 10 minutes with 0.03 mmol (10 fold excess) of reagent 1 in 0.3 mL of dry acetonitrile containing 0.170 mmol of sublimed 1-H-tetrazole. After oxidation of the phosphite triester with aqueous iodine solution, 33% of the resin (1 μmol of initial thymidine) was subjected to ammonolysis as above to yield 43.1 A_{260} units of the 5' amino derivatized hexamer $H_2NCH_2CH_2O$-(pT-T_4-T). The final two thirds of the support (2 μmol of initial thymidine) was treated with 3.0 mL of dry pyridine containing 0.83 mmol of 1,8-diazabicyclo[5.4.0]undecene (DBU) for 24 hours at 20°C. After removal of the DBU-pyridine solution the silica was washed with acetonitrile, dried, and exposed to ammonia as described above to produce a further 89.1 A_{260} units of the amino derivatized hexamer.

Figures 1A and 1B show the anion exchange HPLC (16) profiles of the crude material obtained from the ammonia and DBU-ammonia treatments of the support bound T-T_4-T reacted with reagent 1. Peak I was shown by fast atom bombardment mass spectrometry (17) to be the desired amino derivatized oligonucleotide. Peak II in figure 1A is a side product which can be avoided by the DBU-ammonia treatment (see figure 1B). It may arise by competitive hydrolysis of the phosphate triester prior to beta-elimination of the cyanoethyl group. This type of side reaction has recently been observed by others (18) and may be due to decreased steric hindrance of the phosphorus atom in the cyanoethyl phosphate triesters of primary alcohols. The chromatographic properties of both products were unchanged after exposure to alkaline phosphatase and spleen phosphodiesterase. Furthermore, both products were completely degraded by snake venom phosphodiesterase.

$H_2NCH_2CH_2O$-(pT-T_4-T) (2.0 A_{260} units, 4 nmol) was reacted with the N-hydroxysuccinimide activated ester of d-biotin (19) (0.4 μmol) in 40 μL of 50% aqueous dimethylformamide, 0.05 M HEPES, pH 7.7. The reaction was quantitative in 1 hour at 20°C as evidenced by the disappearance of the starting material and appearance of a single new peak as shown in figure 1C. The isolated oligonucleotide-biotin adduct was completely bound by streptavidin-agarose (20) while $H_2NCH_2CH_2O$-(pT-T_4-T) was not. No reaction products were observed from the exposure of T-T_4-T or peak II (figure 1A) to the activated ester of d-biotin under the above conditions, indicating that the material in peak II does not contain a primary amino group.

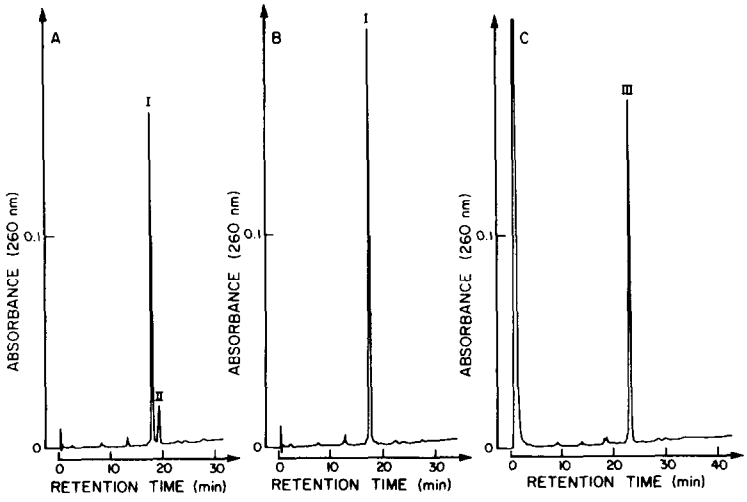


FIGURE 1:
Anion exchange HPLC profiles
A. $H_2NCH_2CH_2O$-(pT-T_4-T) from the conc. NH_4OH treatment
B. $H_2NCH_2CH_2O$-(pT-T_4-T) from the DBU and conc. NH_4OH treatment
C. Reaction of peak I with the N-hydroxysuccinimide-ester of d-biotin
For details on the HPLC conditions see reference 16.

Compound 1 was also used for the synthesis of the octamer $H_2NCH_2CH_2O$-(pC-A-T-T-C-T-G-T). HPLC and enzymatic analysis of the crude reaction product showed that compound 1 could be employed successfully in the high yield synthesis of 5' amino derivatized oligonucleotides containing all four bases. A small amount (<5%) of a side product was observed even when the DBU-ammonia deprotection conditions were used. This material was not susceptible to alkaline phosphatase or spleen phosphodiesterase, and was degraded by snake venom phosphodiesterase. Furthermore, this small amount of material is unreactive toward the N-hydroxysuccinimide ester of d-biotin and can be easily removed by anion exchange HPLC.

In conclusion, phosphoramidite 1 is a useful reagent for the convenient synthesis of oligonucleotides possessing 5' aliphatic amino termini. Future work will involve the synthesis and characterization of oligonucleotides covalently attached to a number of biochemically useful and biologically active small molecules. Compound 1 should also prove valuable for the synthesis of other compounds of therapeutic and biological interest such as phospholipid-analyte conjugates (21) and phosphosugar analogues (18 and ref. therein).

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12. N,N-Diisopropylamino-2-cyanoethoxychlorophosphine (13.5 mmol) was added slowly over 5 min. via syringe to a stirred solution of N-trifluoroacetyl-2-aminoethanol (15 mmol) and diisopropylethylamine (20 mmol) in 30 mL of dry tetrahydrofuran under argon. Within 5 min the amine hydrochloride precipitated. The reaction was stirred for an additional 15 min and filtered. The filtrate was concentrated to an oil, taken up in 150 mL of ethyl acetate, and extracted with three 50 mL portions of cold 5% aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated. The resulting oil was taken up in ethyl acetate/hexane/triethylamine, 25/70/5 (V/V), and applied to a short column of Merck silica gel 60G, 150 mL bed volume, packed in the same solvent mixture. The product fractions were identified by TLC on Merck Kieselgel 60 plates in the above solvent. After development the plates were sprayed with 5% percent aqu. $AgNO_3$ to reveal the compound as a brown spot, R_f= 0.3. 80 MHz ^{31}P NMR in $CDCl_3$: -149.0 ppm relative to 85% H_3PO_4 in H_2O as an external standard. 200 MHz ^1H NMR in $CDCl_3$ (δ, TMS= 0.00): 7.1 (br s, 1H, N-H) 4.0-3.7 (m, 4H, 2(C-H) + NCH_2) 3.7-3.5 (m, 4H, $POCH_2$ + $POCH_2$) 2.63 (t, 2H, CH_2CN, $J(CH_2-CH_2)$= 6.2 Hz) 1.16 (dd, 12H, 4(CH_3), $J(CH_3-CH)$= 6.8 Hz, $J(CH_3-P)$= 3.0 Hz)
13. The Fractosil (Merck, FRG) support is the subject of a future publication.
14. Synthesis cycle: 1.) Wash with CH_2Cl_2 2.) Detritylate with 3% TCA in CH_2Cl_2 3.) Wash with dry CH_3CN 4.) Dry with argon 5.) Coupling (per µmole of support bound 5' hydroxyl groups): 10 µmol of phosphoramidite in 100 µL of dry CH_3CN which is 0.57 M in sublimed 1-H-tetrazole for 10 min. 6.) Wash with CH_3CN 7.) Oxidize with 0.1 M I_2 in THF/lutidine/H_2O, 40/10/1 (V/V) 8.) Wash with CH_3CN
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e.) Column: 5cm x 0.4cm packed with 3µm, 120A pore size, Hypersil (Shandon, U. K.) which had been coated with 1% polyethyleneimine-6 (M.W 600), crosslinked with 1,3-diglycidylglycerol and quaternized with methyl iodide
Gradient: 0.01 M to 0.25 M NaH_xPO_4 pH 6.5 in 15% aqueous CH_3CN over 30 minutes
Flow rate: 1.0 mL per minute
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20. Streptavidin-agarose is available from Bethesda Research Laboratories, USA. The binding assays were performed in 0.125 M sodium phosphate, pH 6.5, containing 15% acetonitrile.
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