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PREPARATION OF FUNCTIONALIZED OLIGONUCLEOSIDE METHYLPHOSPHONATE SUITABLE FOR NON-RADIOACTIVE LABEL ATTACHMENT

Sudhir Agrawal

Worcester Foundation for Experimental Biology,
222 Maple Avenue, Shrewsbury, Massachusetts 01545

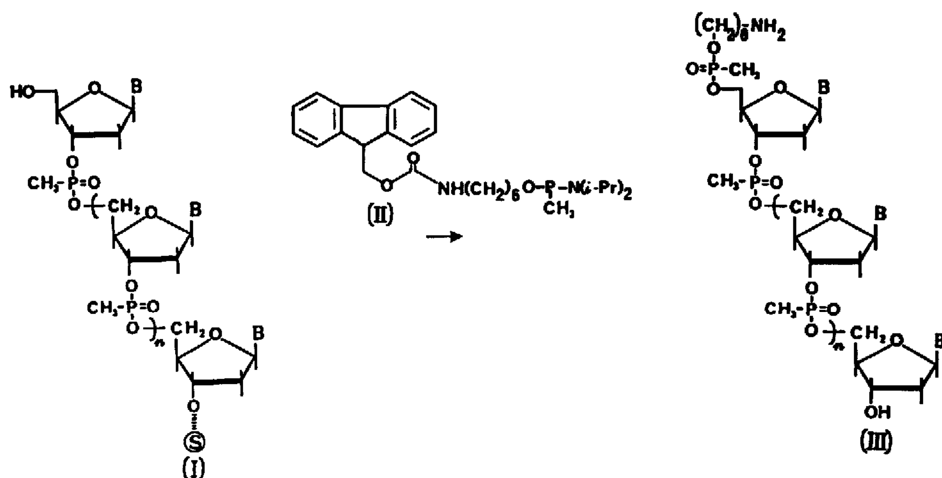
Abstract The synthesis is described of a linker molecule for the specific attachment of non-radioactive labels such as biotin and fluorophores to the 5'- or 3'-terminus of synthetic oligonucleoside methylphosphonates. Oligonucleoside methylphosphonates are nuclease resistant and can thus be used to detect and isolate nucleic acids in presence of nucleases.

Synthetic oligonucleotides covalently attached to d-biotin (1), fluorescent labels (2,3), and biologically active molecules (4) are becoming important tools of molecular biology. They have been used for the detections of nucleic acids (1-6) and for DNA sequence analysis (7,8). A variety of enzymatic and chemical procedures have been developed for their synthesis (9, review). Central to some of these procedures is the introduction of alkylamino group into the oligonucleotides (1,10,11), which can then be reacted with variety of non-radioactive reporter groups.

There are limitations on the use of non-radioactive oligonucleotide probes having normal phosphodiester backbones, since they are not stable towards most of the nucleases. Also, the duplex of a normal oligodeoxynucleotide with RNA is a substrate for RNase H which can lead to cleavage of the RNA component. For these reasons they cannot be used to detect or isolate nucleic acids in presence of such nucleases, e.g. from nuclear extracts under the mild conditions necessary to prevent protein denaturation.

Here I wish to report the synthesis of oligonucleoside methylphosphonate, functionalized at either the 5'- or 3'- terminus with an alkylamino group, which has been reacted with the N-hydroxysuccinimide esters of biotin, and N-caproylamidobiotin and with rhodamine isothiocyanate.

A pentadecamer oligonucleoside methylphosphonate, AAG CTT CCG GTC TCC, complementary to the 5' end of a human U 2 small nuclear RNA "maxi transcript" (12), was synthesized from nucleoside methylphosphonamidites (13) using an automated DNA synthesizer (Biosearch 8700). To functionalize the 5'- terminus of support bound oligonucleoside methylphosphonate (I) the last coupling was carried out with 2-(9- fluorenylmethoxycarbonyl) aminohexanol-(N,N-diisopropylmethyl) phosphonamidite (II).



The latter was prepared by reacting 2-(9-fluorenylmethoxycarbonyl) aminohexanol (1) with methylchloro-N,N-diisopropyl aminophosphine (13) in dichloromethane containing N,N-diisopropylethylamine at room temperature for 20 minutes. Aqueous work up and precipitation from ethylacetate using pentane at -40° gave an oil in 90% yield. The product was pure as judged by tlc, ^{1}H and ^{31}P NMR (14). To functionalize the 3'-terminus the assembly of the required sequence was carried out from 3' to 5' on N-fluorenylmethoxycarbonyl-O^{1}-dimethoxytrityl-3-amino-1,2-propanediol-(O^{2})-long chain alkylamine CPG support (Clontech Laboratories, Inc.).

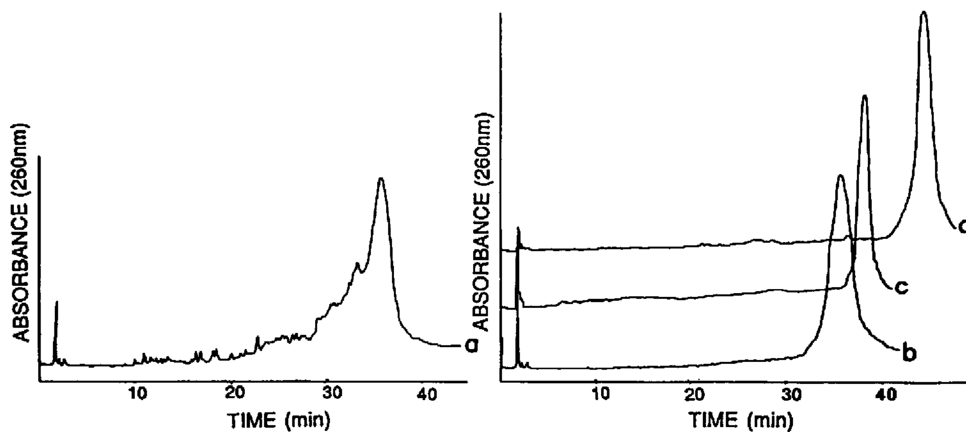


Figure 1 - Reversed phase HPLC traces of: (a) crude amino-15 mer; (b) purified amino-15 mer; (c) biotin-15 mer; and (d) N-caproylamido biotin-15 mer (for conditions - see reference - 19).

After the assembly of the functionalized sequence, deprotection was carried out at room temperature in aqueous ammonia for 2 hours followed by treatment with ethylendiamine-ethanol (1:1) for 6 hours (13,15). The crude amino-15 mer (III) was analyzed and purified on reverse phase HPLC [figure 1(a) and 2(b)], the elution time of amino-15 mer was later than that of the corresponding 15-mer without a terminal amino group. The purified amino-15-mer was then reacted with N-hydroxysuccinimide biotin and also with N-hydroxysuccinimide N-caproylamido biotin using the same conditions as described earlier (1). The analytical reverse phase HPLC analysis of the reaction mixture showed a new product, biotin-15-mer adduct [figure 1(c)] and caproylamido biotin-15-mer adduct [figure 1(d)] in quantitative yields. The products were purified by HPLC and were desalted by gel filtration.

Both the biotin 15-mer and caproylamido biotin-15-mer have been used in preliminary experiments to successfully isolate maxi U 2 RNA: ribonucleoprotein (RNP) complexes from nuclear extracts of cultured human cells by affinity chromatography of the oligomer: U 2 RNA hybrids on streptavidin agarose (Temsamani, J., Agrawal, S. and Pederson, T., unpublished data). In contrast, the use of biotinylated phosphodiester 15-mer in the same type of experiment did not lead to recovery of maxi-U 2 RNP complexes, due to cleavage of the DNA-RNA duplex by the endogeneous R Nase H activity present in the nuclear extract.

Recently, functionalization of oligonucleoside methylphosphonate with an alkylamino group has been reported (16) by using published procedures (17,18). The method described by Chu and Orgel (17) required three separate steps (enzymatic phosphorylation, imidazole treatment and reaction with 1,2-diaminoethane or 1,6-diaminohexane) and chromotographic purification. The other method reported by Draper et al., (18) of transamination of cytidine results in cleavage of methylphosphonate backbone of oligomer (16). The method described here can be used for automated synthesis, which after the routine deprotection step, gives functionalized oligonucleoside methylphosphonate.

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19. The HPLC system consisted of a Waters 600E system controller, Waters Lambda Max model U81 detector, Waters 745 data module and reverse phase column-Novapak C_{18} with RCM 100. Buffers were 0.1M ammonium acetate containing (a) 0% CH_3CN and (b) 80% CH_3CN. Gradient was 0% B 2', 0-60% B 35', flow 1.5 ml min^{-1}.

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