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SITE-SPECIFIC FUNCTIONALIZATION OF OLIGODEOXYNUCLEOTIDES FOR NON-RADIOACTIVE LABELLING

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Abstract: For site specific functionalization of oligonucleotides, a suitably protected diamine, N-1-trifluoroacetylhexanediamine has been synthesized and used to incorporate primary aliphatic amines at internucleoside phosphate as phosphoramidates at specific sites into synthetic oligonucleotides. Its preparation and use in automated solid phase oligonucleotide synthesis is described.

There is at present growing interest in non-radioactively labelled modified oligodeoxynucleotides. Biotin (1,2), fluorophores (3,4), intercalating (5) and chelating (6) reagents attached to synthetic oligonucleotides are becoming important tools of molecular biology. A variety of enzymatic and chemical procedures have been developed for their synthesis (7, review). Central to some of these procedures are (a) the introduction of a reactive group at either the 3'- or 5'-terminus of the oligonucleotide (1,2,8,9) or (b) the synthesis of modified nucleosides containing the masked reactive group and then incorporating it into the nucleic acid (8, and references there in).

Here we wish to report the synthesis of oligonucleotides, in which one or more specific internucleoside phosphates are modified to give aminoalkylphosphoramidate residues. The amino group of these oligonucleotides may then be further reacted inter alia with the N-hydroxysuccinimide ester of biotin and N-caproyl amidobiotin and a variety of fluorophore isothiocyanates.

The strategy is based on oxidizing an H-phosphonate internucleoside linkage with N-1-trifluoroacetylhexanediamine, $CF_3CO\ NH(CH_2)_6NH_2$ (I) in presence of carbon tetrachloride, to give a phosphoramidate internucleoside linkage (10-14). This phosphoramidate linkage is stable under oligonucleotide assembly conditions using phosphoramidite chemistry (15) and to subsequent deprotection steps.

N-1-trifluoroacetylhexanediamine was prepared by adding ethyltrifluoroacetate (1.2 ml, 10 mmol) dropwise over one hour to a stirred mixture of hexanediamine (1.16g; 10 mmol) and triethylamine (1 ml; 7 mmol) in 20 ml methanol. The solution was stirred overnight. After removal of solvents, the reaction mixture was flash chromatographed on silica using 0-25% methanol in dichloromethane. The fractions containing the desired product were pooled and concentrated to give a colorless powder (1.1 gm, yield-42.6%), M.pt. 52°. ^1H NMR ($CDCl_3$, d, TMS=0.00) 7.1-7.2 (m, 3H, NH_2, NH) 3.2-3.3 (m, 2H, $CO-NH-CH_2$), 2.8-2.9 (m 2H, CH_2-NH_2) 1.2-1.6 (m, 8H,$-CH_2-(CH_2)_2-CH_2-$).

To test the efficacy of (I) for amino group introduction at specific sites of oligonucleotides, a 17-mer sequence, GTA AAA CGA CGG CCA GT 1, was made together with 2-5 carrying aminohexyl residues at different sites, as shown by (ϒ).

1 GTA AAA CGA CGG CCA GT
2 GTA AAA CGA CGG CCA G^{ϒ}T
3 GTA AAA CG^{ϒ}A CGG CCA GT
4 G^{ϒ}TA AAA CGA CGG CCA GT
5 G^{ϒ}TA AAA CGA CGG CCA G^{ϒ}T

The steps involved for labelling, for sequence 2 are shown in figure 1. The first coupling was carried out using H-phosphonate chemistry. The support bound dinucleoside H-phosphonate (II) was then oxidized with 4% N-1-trifluoroacetyldiaminohexane in carbon tetrachloride-dioxane (8:2, v/v) for 30 minutes to give (III). After oxidation, the assembly of the rest of the sequence was carried out using phosphoramidite or H-phosphonate chemistry. After the synthesis, the oligonucleotide (IV) was deprotected in aqueous ammonia for 6 hours at 55° forming the aminohexyl oligomer (V).

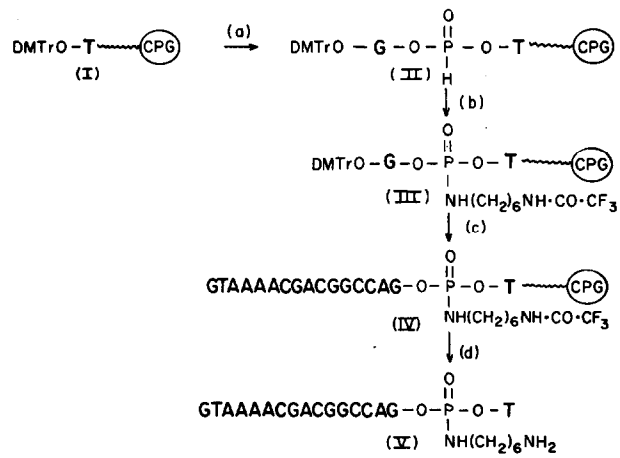

Figure 1(a) Coupling using H-phosphonate chemistry (b) oxidation with N-1-trifluoroacetylhexanediamine (I) in carbon tetrachloride (c) assembly of the rest of the sequence by using phosphoramidite chemistry (d) deprotection in aquous ammonia.

Analytical ion exchange HPLC of oligomer 2 showed the major peak eluting earlier than that of oligomer 1 with the same gradient (figure 2a and 2b), confirming that in 2, one of the internucleoside linkages is phosphoramidate which is non-ionic. Sequences 3 and 4 which are functionalized at different sites also showed similar HPLC profile as oligomer 2. Oligomer 5, which is functionalized at two sites, was eluted even earlier (figure 2c).

When ion exchange HPLC purified 2 was checked on reversed phase HPLC, it gave a doublet peak in ratio of 1:2 (figure 2e) compared to 1 (figure 2d). This results from the diastereoisomeric nature of phosphoramidate internucleoside linkage. Similarly, 5 eluted as a broad peak because of two such diastereoisomeric linkages (figure 2f). Both oligomers 2 and 5 had retention time longer than the oligomer 1 because of the hydrophobic nature of the alkyl chain.

Reaction of 2 was carried out with biotin N-hydroxysuccinimide using reported conditions (1). The reaction mixture after gel filtration (sephadex G-25) showed two new peaks of the diastereomeric biotin adducts (figure 3b). Similarly, reaction of 5 gave a broad peak as a doublet eluting later than the unreacted material (figure 3c and 3d).

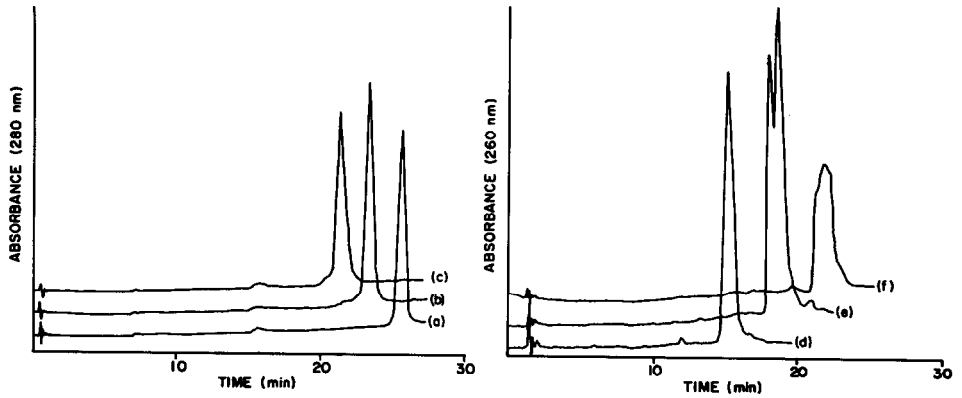

Figure 2 Ion exchange HPLC traces of (a) oligomer 1 (b) 2 and (c) 5. HPLC traces (d) (e) and (f) are on reversed phase of oligomers 1, 2 and 5 respectively (for conditions see reference 22).

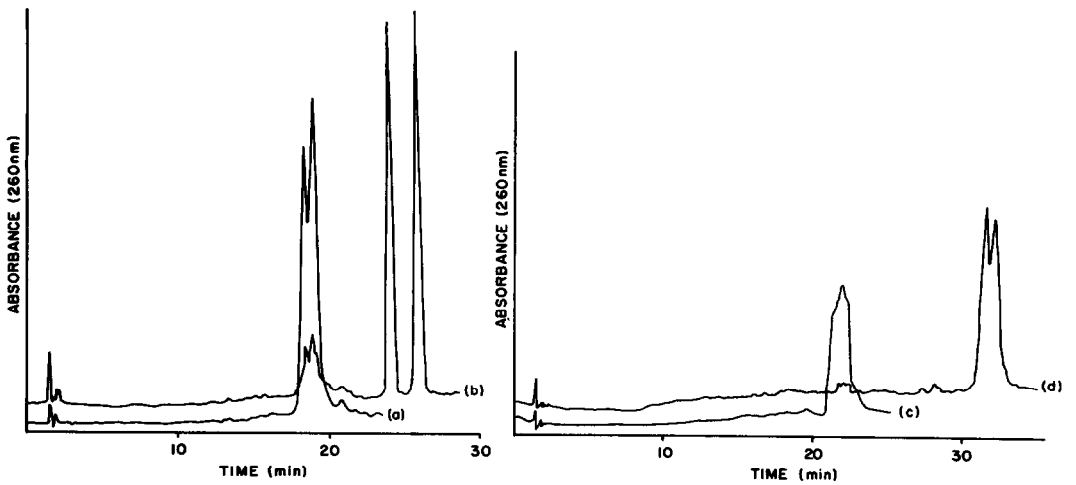

Figure 3 Reversed phase HPLC traces of : (a) oligomer 2 (b) reaction mixture of oligomer 2 with biotin N-hydroxysuccinimide (c) oligomer 5 and (d) reaction mixture of 5 with biotin N-hydroxysuccinimide (for conditions see reference 22).

We conclude that, for internal labelling or multilabelling at specific sites, the coupling at that site can be carried out using H-phosphonate chemistry, followed by oxidation with N-trifluoroacetylhexanediamine in carbon tetrachloride. The sequence before and after the functionalized site can be assembled using phosphoramidite chemistry.

This route provides a way for functionalizing oligonucleotides at one or more specified sites. We have illustrated the method of introducing reporter groups by reaction with biotin active ester. In other experiments (data not shown) the aminohexyl residue was reacted in high yield with fluorescein and rhodamine isothiocyanate to

generate a fluorescent hybridization probe. Multiple labelling may increase the sensitivity of detection in diagnostic assays. Using similar strategy phosphoramidate analogues of dinucleotides have been prepared and attached to various ligands (14,16), also moieties such as monofunctional alkyl or aryl amines and cholesterol have been attached to oligonucleotides at a specific site (12,17,18). There is a recent report of attachment of a reporter group at a specific site in oligodeoxynucleotides containing phosphorothioate linkages (8). However, that method has limitations as the subsequent iodine oxidation of phosphorothioate containing sequences causes loss of sulphur (19,20,21).

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22. The HPLC system consisted of a Waters 600E system controller, Water Lambda Max model 481 detector, Waters 745 data module ion exchange HPLC was carried out on analytical partisphere SAX column (Whatman) using gradient of buffer A: 1mM KH_2PO_4 (pH 6.3)/formamide (4:6) and buffer B: 300mM KH_2PO_4/formamide (4:6), 0-70% B in 30 min, flow rate 2mL min^{-1}. Reversed phase HPLC was carried out on Novapak C18 column (RCM-100), buffers were 100mM ammonium acetate containing (a) 0% CH_3CN and (b) 80% CH_3CN. Gradient was 0% B for 2 min, 0-8% B in 5 min, 8-18% B in 30 min, flow 1.5 mL min^{-1}.

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