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THE SOLID PHASE SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING A 3'-PEPTIDE MOIETY

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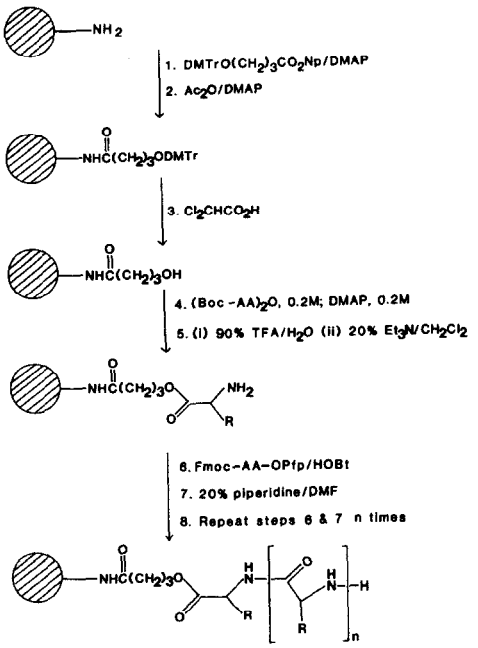
Summary: A hybrid comprising an oligodeoxyribonucleotide linked via the 3'-hydroxyl to the amino terminus of a peptide has been prepared using a combination of solid-phase FMOC peptide synthesis methodology and phosphoramidite chemistry.

Synthetic oligonucleotides have important utility in molecular biology as probes for specific nucleic acids[1]. They are particularly useful in the technique of hybridization histochemistry[2] where the oligonucleotide is radioactively labelled at the 5' end and used to locate the sites of gene expression in tissue sections. Detection of the probe requires autoradiography which may take several days. Non-radioactive probes with, for example, fluorescent tags, offer significant advantages, including greater safety, indefinite shelf-life and the ability to directly detect hybridization using the microscope. A major problem in the development of non-radioactive probes has been the lack of suitable procedures for the attachment of a sufficient number of tags to the oligonucleotide to provide the necessary sensitivity. We have approached this problem in a number of ways. One procedure reported by us recently[7] attaches fluorophores directly to the C-5 of deoxyuridines through a linker arm. Another novel approach, described here, has been to prepare oligonucleotides with a peptide attached to the 3'-terminal hydroxyl. The peptide sequence is designed to incorporate multiple lysine residues for the subsequent attachment, via the side chain ε-amino groups, of suitable non-radioactive markers. Using a combination of solid-phase FMOC peptide synthesis and phosphoramidite chemistry we have assembled a peptide segment followed by a specific oligonucleotide sequence. In this way the 3'-hydroxyl of the oligonucleotide is linked to the terminal amino group of the peptide.

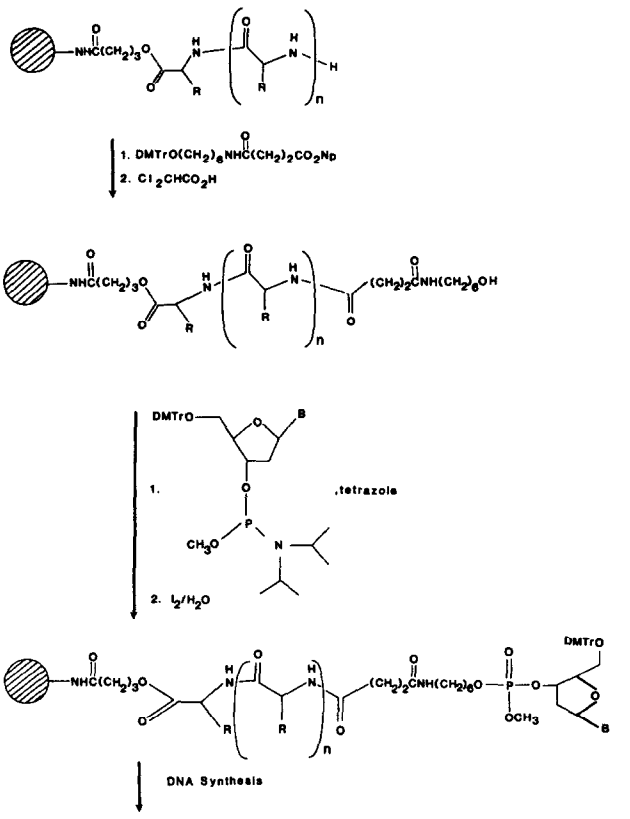
The solid support was Aminopropyl Controlled Pore Glass (CPG), pore size 500 A (Fluka). The CPG was further derivatized to provide a spacer arm with a terminal hydroxyl group. To CPG (0.5 g, containing ~20 μmole of amino functionality) was added p-nitrophenyl 4-(4,4'-dimethoxytrityloxy)butyrate[3] (130 mg, 250 μmole) and dimethylaminopyridine (DMAP, 30.5 mg, 250 μmole) in 2 mL of DMF (Scheme I). The reaction mixture was shaken for 3 hr. Residual amino groups (~6 μmole) were acetylated with acetic anhydride (0.5 mL, 2.5 mmole) and DMAP (50 mg, 0.4 mole) in pyridine (2 mL) for 15 min. No significant residual amino group was detected. Treatment of this substrate with 3% dichloroacetic acid in CH_2Cl_2 (2 x 5 min)



SCHEME I

removed the dimethoxytrityl (DMTr) group (27 μmole of functionality per gram of solid support).

This modified solid support was then used for peptide synthesis. In order to couple the first amino acid (formation of the ester linkage), a high concentration of active amino acid was required. Thus, the derivatized CPG (100 mg, containing 2.7 μmole of hydroxy functionality) was reacted with a solution of N-BOC-alanine symmetrical anhydride and DMAP (0.2 M in each) in DMF (2 mL) for 20 hr. Residual hydroxyl groups were acetylated as before using Ac_2O/DMAP and the alanines deprotected to give the free amino group (25 μmole/g). Subsequent couplings were done using the FMOC peptide synthesis methodology[4]. The CPG substrate was reacted with N-α-FMOC-N-ε-BOC-Lys pentafluorophenyl ester (28 mg, 20 equiv.) in DMF (2 mL) in the presence of 1-hydroxybenzotriazole (4.3 mg, 20 equiv.) for 30 min. The reaction was quantitative by ninhydrin assay. The FMOC group was then removed with 20% piperidine in DMF (1 x 3 min, 1 x 7 min). Subsequent couplings were carried out in the same way, alternating the lysine residues with alanines (using FMOC-Ala pentafluorophenyl ester) to synthesize $(AlaLys)_5Ala$. Following deprotection of the last residue, the solid support was treated with the specially designed linkage reagent p-nitrophenyl 3-[6-(4,4'-dimethoxytrityloxy)hexylcarbamoyl] propanoate[5] (80 mg, 125 μmole) in 0.5 mL DMF for 16 hr (Scheme II). Residual amino groups were acetylated as before. This gave a trityl loading of 22 μmole/g. This substrate was then used for oligonucleotide synthesis, using methyl N,N-diisopropyl nucleoside phosphoramidites, on the ABI 380A Automated DNA Synthesizer[2,6]. The first phosphoramidite was coupled onto the terminal aliphatic hydroxyl group. This reaction was quantitative, as assayed on the trityl test. Oligonucleotide



SCHEME II

synthesis was continued and the 30mer oligodeoxyribonucleotide d(GGTCTTCACAACATCTGTGATGTCA-GCAGG) (KPIB), complementary to part of the mRNA for mouse kallikrein, was synthesized on this solid support. The average coupling yield, by trityl assay, was over 99%. Following chain assembly, the solid support was removed from the automated synthesizer and treated with $PhSH/Et_3N/CH_3CN$ 1:1:2 for 2 hr to remove the methyl protecting groups on the phosphotriesters. The BOC protecting groups on the lysine residues (and also the 5'-DMTr group) were removed with a 5 min treatment with 90% trifluoroacetic acid/10% ethanedithiol[7], followed by neutralization with 20% Et_3N/CH_2Cl_2. The ester group attaching the C-terminal amino acid to the solid support was then cleaved with conc. aq. NH_3 (4 hr), and the solution heated at 55°C for a further 16 hr to remove the nucleoside amino protecting groups.

Figure 1 shows the pattern obtained following 5'-^{32}P end labelling of the crude product with [γ-^{32}P] -ATP using polynucleotide kinase and electrophoresis on a 20% polyacrylamide gel containing 7 M urea. The oligonucleotide-peptide hybrid runs slower than the normal KPIB and is the major component of the product mixture. A similar pattern is seen when the unlabelled reaction mixture is run and the DNA visualized on the gel by UV shadowing. The pure hybrid product was obtained by preparative gel electrophoresis on a 10% gel.

Amino acid analysis of the product gave the expected ratio of 6 Ala:5 Lys, with 1 mole of $(AlaLys)_5Ala$ peptide per mole of KPIB. The product was resistant to snake venom

phosphodiesterase (blocked 3'-end) and was only partially digested (~10 nucleotides from the 5'-end) by spleen phosphodiesterase and P_1 nuclease (as assessed by HPLC analysis of the digests). It appears that the presence of the positively charged peptide at the 3'-end inhibits phosphodiesterase digestion in this region of the molecule.

These novel oligonucleotide-peptide hybrids should enable oligonucleotide probes with multiple fluorescent tags to be conveniently prepared, and such work is in progress.

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Figure 1. Electrophoresis of the crude (KPIB-(AlaLys)$_5$-Ala (Lane 1) and normal KPIB (Lane 2) on a denaturing 20% polyacrylamide gel.

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3. This was prepared by reacting sodium 4-hydroxybutyrate (1.26 g, 10 mmol) with 4,4'-dimethoxytrityl chloride (3.39 g, 10 mmol) in 30 mL of dry pyridine overnight, then adding p- nitrophenol (1.30 g, 10 mmol) and dicyclohexylcarbodiimide (DCC) (2.06 g, 10 mmol) and stirring for a further 2 days, filtering off the dicyclohexylurea (DCU) by-product, removing the solvent, and purifying the residue by flash chromatography (25% EtOAc/pet. ether 30-40°C as solvent) to give 5.0 g of the product (95%) as a light yellow oil. The structure was confirmed by ^1H and ^{13}C NMR.

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5. This was prepared by reacting succinic anhydride (1.0 g, 10 mmol) with 6-aminohexanol (1.17 g, 10 mmol) in dry pyridine for 4 days. 4,4'-Dimethoxytrityl chloride (3.39 g, 10 mmol) was then added, it was stirred for a further 4 hr, and p-nitrophenol (1.39 g, 10 mmol) and DCC (2.06 g, 10 mmol) added and was stirred for a further 2 days. DCU was filtered off, the solvent removed and the residue purified by flash chromatography (50% EtOAc/pet. ether 30-40°C) to give the product (4.09 g, 64% overall yield) as a clear light yellow oil. The structure was confirmed by ^1H and ^{13}C NMR.

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