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Syntheses of Oligonucleotide-Amino Acid Conjugates: Using TentaGel and CPG Matrices for the Synthesis of 3′-Phosphoryltyrosine-Terminated Oligonucleotides

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Abstract: 3′-Phosphoryl-O-tyrosine terminated oligonucleotides were synthesized using controlled-pore glass (CPG) and TentaGel solid matrices. For the synthesis on CPG, the tyrosine hydroxyl was protected with the levulinoyl group which was removed by sodium borohydride. Copyright © 1996 Elsevier Science Ltd

Conservative site-specific recombinases and topoisomerases cleave DNA via the nucleophilic attack of a hydroxy group of a hydroxylated amino acid (usually tyrosine) upon an internucleotide phosphodiester bond.[1,2] As a result, two shorter DNA strands are formed, one with a free 5′-OH and the other bound to the enzyme through a 3′-phosphodiester linkage involving the hydroxyl of the amino acid. The amino acid acts as the leaving group in the nucleophilic substitution by a primary hydroxy group of another oligonucleotide. As a result, the internucleotide phosphodiester bond is resealed (Scheme 1).

Oligonucleotides that are terminated in the 3′-phosphoryltyrosine have been used as substrates for the study of the strand joining reaction catalyzed by conservative site-specific recombinases such as the Flp recombinase and by mammalian topoisomerase I.[1-3] We previously reported the synthesis of 3′-phosphoryltyrosine-terminated oligonucleotides using a Teflon fibre-based solid support (Scheme 2; upper portion). When this support was no longer available, we were obliged to explore the use of other matrices.

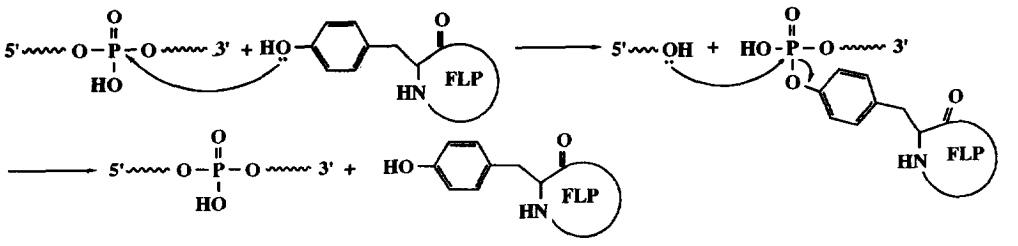
As shown in Scheme 2, the original synthesis began with tyrosine whose hydroxyl was protected by O-t-butyl.[1,2] After linking the protected tyrosine to the Teflon support, the t-butyl group was removed by the treatment with trifluoroacetic acid. The oligonucleotide synthesis started on the hydroxyl group of tyrosine, and it used the standard phosphoramidite chemistry with an ABI automatic oligonucleotide synthesizer. At the completion of the oligonucleotide synthesis, Fmoc was removed by the treatment with 30% aqueous NH_3. The 3′-phosphoryltyrosine terminated oligonucleotide was released from the Teflon support at the same time through the ammonolysis of the ester bond between the support and tyrosine resulting in the formation of a new carboxylic amide.[2]

Since the functionalized Teflon support recently became unavailable, we sought an alternative support. We first investigated the use of a TentaGel support (Rapp Polymere, Tübingen, Germany). TentaGels are polymers of ethylene glycol grafted onto polystyrene. While they are used primarily for polypeptide syntheses,[4] they were recently utilized for oligonucleotide syntheses as well[5] and their high loading capacity was cited[5] as their primary advantage over CPG. Tentagels seemed to be sufficiently stable under the conditions of the Teflon-based synthesis. The synthetic protocol with which the automatic synthesizer operates on both CPG and Teflon supports has been modified for oligonucleotide synthesis on TentaGels.[6] Although the higher loading capacity of TentaGels over CPG was noticeable in the synthesis of shorter (<20 nucleotides), the lower yields per cycle resulted in generally lower yields of tyrosine-oligonucleotide conjugates when synthesis of longer oligonucleotides was attempted (see below).

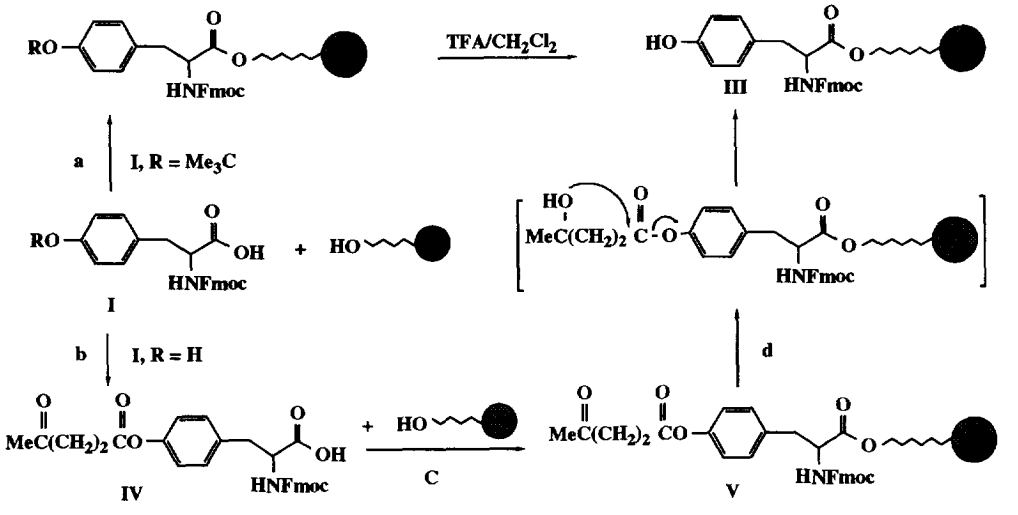
Consequently we have turned our attention to synthesis with CPG.[7] Since the CPG solid support was not sufficiently acid stable we had to avoid treatment with trifluoroacetic acid. We sought a protective group which could be removed from the tyrosine hydroxyl under non-acidic conditions.[8] The levulinoyl[9] ester group satisfied this requirement as $NaBH_4$ is the most suitable deprotecting reagent.[9,10] Hydrazine[9b] cannot be used as a deprotection agent in the presence of ester groups.

The complete synthesis is portrayed in Scheme 2. Briefly, the starting Fmoc tyrosine was treated with levulinic anhydride in pyridine at room temperature.[11] The O-levulinoyl tyrosine which was formed in excellent yield was subsequently linked with a functionalized alkylamine CPG column[11b] using dicyclohexyl carbodiimide.[1,2] This heterogeneous condensation as well as the subsequent removal of the levulinoyl group with sodium borohydride[12] proceeded smoothly. The free hydroxyl of tyrosine then assumed the role of the first 5′-hydroxyl in the standard oligonucleotide synthetic procedure.

Comparing efficiency of the oligonucleotide synthesis on all three supports, we noted that the yields per cycle were comparable when CPG and Teflon were used (above 98%). The yields per cycle on Tentagel were lower (90-91%) so that we were not able to synthesize a 36-mer on Tentagel. The products also differed in purity. After cartridge purification, the 3′-phosphoryl tyrosine-terminated 18-oligonucleotide conjugates were labelled with ^{32}P at their 5′-termini with polynucleotide kinase[13], annealed with the two unlabelled oligonucleotides to form a synthetic ligation substrate analyzed by electrophoresis (Fig.1; odd-numbered lanes).[1] 18nt conjugates made on Teflon and CPG both contained approximately 35% of impurities as measured by phosphoimage analysis (see Fig. 1, Panel A, lanes 1 and 5). The same conjugate made on Tentagel was free of these impurities (Panel A, lane 3), while the impurities could be removed from the conjugates by electrophoresis[12] (cf.Panel B, lane 1). We further compared the effectiveness of the three oligonucleotide conjugates in the Flp-mediated ligation assay. The products formed after incubation with Flp were analyzed on a denaturing polyacrylamide gel (Fig.1; even-numbered lanes). As seen in Fig.1,[13] each of the synthetic oligonucleotides gave equally efficient ligation as evidenced by the appearance of a ^{32}P-labelled, 40-nucleotide product. The gels show that the impurities do not inhibit the ligation reaction.



Scheme 1



[a] DCC/HOBT/Py, Teflon or TentaGel support; [b] Levulinic anhydride/Py; [c] DCC/HOBT/Py, CPG support; [d] $NaBH_4$/EtOH/H_2O.

Scheme 2

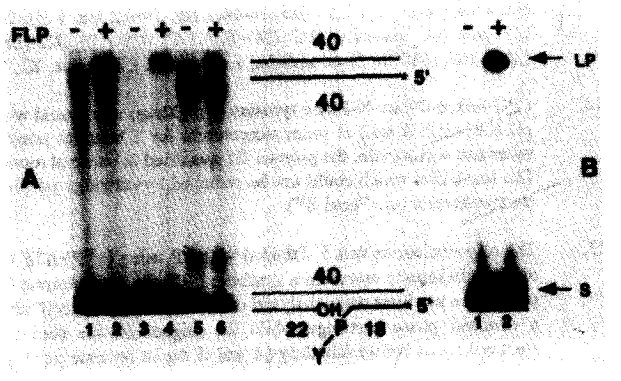


Fig. 1. Ligation of 3'-phosphotyrosyl oligonucleotides by the Flp recombinase. The 18 nucleotide oligomer was synthesized using a Teflon support (panel A. lanes 1 & 2), a TentaGel support (panel A, lanes 3 & 4), or the CPG support (panel A, lanes 5 & 6, and panel B).[13] The substrate for the Flp recombinase (S)[1,2] and the ligation product (LP) are indicated by the arrow.

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8. The O-t-butyl group is removed from O-t-Butyl-N-Fmoc-tyrosine linked to a support by treatment with 50% trifluoroacetic acid in dichloromethane at room temperature for 1 hour (cf. Ref.[1]), the conditions not compatible with CPG. Basic conditions lead to the loss of Fmoc protection and a scission of the ester linkage between tyrosine and the support.

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11. (a) N-Fmoc-tyrosine (I; 1.31 g; 3.28 mmol) on treatment with levulinic anhydride (1.39 g; 6.5 mmol) in dry pyridine (5.0 mL) at room temperature for 24 hours gave the levulinic ester IV. After the usual workup (cf. Ref.[9b]), IV crystallized from MeOH had m.p. 185-7 °C; ^{1}H NMR [$(CD_3)_2$ CO] [δ(ppm)]: 7.73 (d, J=7.50 Hz, 2H), 7.54 (dd, J=7.3 Hz, J=3 Hz, 2H), 7.23 (t,J=7.5 Hz, 2H), 7.20-7.15 (m, 4H), 6.91 (d, J=8.7 Hz, 2H), 4.38 (m, 1H), 3.15 (dd, J=14.1 Hz, J=4.7 Hz, 1H), 2.93 (dd, J=14.1 Hz, J=9.2 Hz, 1 H); 2.76 (t, J=6.8 Hz, 2H), 2.04 (s, 1.5H), 1.90 (s, 1.5H); MS, m/z (rel. intensity) 502 [$(M+H)^+$,1.5], 429 [10], 323 [15], 217 [100]; HRMS, For $C_{29}H_{27}NO_7$ calcd. 502,1866, found 502,1860.(b) Haralambidis, J.; Duncan, L.; Angus, K.; Treagar, G.W. *Nucleic Acids Res.* 1990, *18*, 493.

12. CPG-linked O-Lev-N-Fmoc-tyrosine (V; 100mg) was treated with a solution of $NaBH_4$ (5 mg) in EtOH-Tris buffer [1M; pH 6.8] (1:1; 3 mL) at room temperature for 2 hours to remove the levulinoyl group. After successive washing with water and acetonitrile, the product III was dried *in vacuo* at room temperature overnight before oligonucleotide synthesis. The impurities which could not be removed by cartridge purification (cf. Panel A, lane 5) were easily removed by gel electrophoresis (cf. Panel B[15]).

13. The oligonucleotide was 5'-labelled with ^{32}P using [γ^{32}P]-ATP and polynucleotide kinase(*), and annealed to two other oligonucleotides to assemble a synthetic FRT site, the substrate for the Flp recombinase (S).[1,2] After incubation with Flp protein the products were analyzed on a denaturing polyacrylamide gel. The oligonucleotides used in A were purified by a reversed phase cartridge while the oligonucleotide used in B was purified by preparative polyacrylamide gel electrophoresis before labelling (A and B depict separate gels).

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