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On-line solid phase synthesis of oligonucleotide-peptide hybrids using silica supports

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Abstracts: A procedure for the preparation of oligonucleotide-peptide hybrid molecules by means of automated synthesis is described. The conjugates have been assembled on silica supports including CPG (Controlled Pore Glass) and Fractosil supports. The novel N^{ε}-lysine protecting group, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl (Dde) was used.

Conjugates containing both oligonucleotide and peptide moieties represent new tools for molecular biology. Oligonucleotide polyamide hybrid molecules have been used as non-radioactive labels[1] and as PCR primers[2]. These conjugates appear also to be interesting potential "antiviral"[3] and "antigene"[4] inhibitors of gene expression. The synthesis of such compounds becomes even more eagerly anticipated since nuclear transport signal peptide[5], hydrophobic peptide[6] or polylysine[7] have been shown to increase markedly the cellular uptake of oligonucleotides.

With peptide exhibiting hydrolytic activity, it becomes possible to generate specific artificial nucleases. Along this line, we conjugate a peptide that acts as an endonuclease to oligodeoxyribonucleotide sequences. Alternating sequences of hydrophobic and basic aminoacids such as $(Leu\text{-}Lys)_n$ or $(Leu\text{-}Lys\text{-}Lys\text{-}Leu)_n$ accelerate the hydrolysis of oligoribonucleotides[8]. The phosphodiester bond cleavage requires the presence of basic groups arranged in a well defined geometry, like β-sheet or α-helical conformations. Although the highest hydrolytic activity was measured with $(Leu\text{-}Lys)_n$, we decided to start with $(Leu\text{-}Lys\text{-}Lys\text{-}Leu)_n$, because it adopts an intramolecular helical structure while poly(Leu-lys) consists in a multichain aggregate. $(Leu\text{-}Lys\text{-}Lys\text{-}Leu)_3$ has been shown to be long enough to adopt an α-helix conformation and to exhibit a ribonuclease-like activity[9]. We assumed that both features will be preserved in the hybrid molecule.

Haralambidis and coworkers have described a general approach to synthesize peptide-oligonucleotide conjugates on CPG supports[10]. A strategy using teflon polymers was published by Juby[6]. In both approaches, the peptide synthesis was carried out manually on a peptide synthesizer. In this paper, we present a completly automatized general procedure which allows the preparation of 3'-oligonucleotide-peptide conjugates.

The feasibility of the synthesis was first tested by taking a dodecathymidylate as oligonucleotide moiety. The strategy has been extented to the 12-mer $^{5'}$d-(CACCGACGGCGC)$^{3'}$ complementary to a Ha-ras mRNA sequence[11]. Conjugates on-line synthesis were achieved on CPG and Fractosil supports, usually employed in solid phase oligonucleotide synthesis. Some authors reported that silica supports show mechanical instability under the vigorous stirring conditions[12]. In our hands, these solid supports appeared to be suitable for the on-line synthesis of both peptide and oligonucleotide moieties on an automated synthesizer. No loss of peptide chains was observed during vortexing on the peptide synthesizer.

Haralambidis and al[10] have used the Fmoc-Lys(Boc) strategy for the peptide moiety synthesis. However, when Boc groups are used to protect N^{ε}-lysine amino functions, an additional 90% TFA treatment is needed which is harmful for the purine bases. The standard Boc-Lys(Fmoc) strategy afforded, in our hands, the desired conjugates. However, we observed side-reactions during the final cleavage. This difficulty was overcome by a preliminary Fmoc deprotection with piperidine, when the conjugates were still on the solid supports. The most satisfactory strategy included the use of Fmoc-Lys(Dde)-OH[13], a new aminoacid derivative marketted by Novabiochem. The Dde side-chain protecting group is particularly well adapted because it is stable in 20% piperidine used for Fmoc group cleavage but can, nevertheless, be cleaved under basic conditions with ethanolamine or hydrazine solutions. The strategy which was finally chosen for the synthesis is illustrated in figure 1.

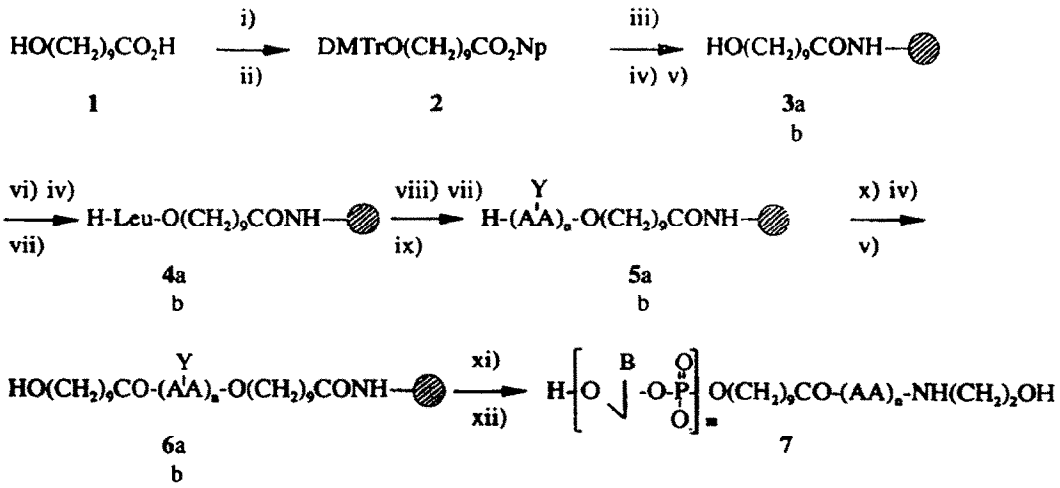

Figure 1: Synthesis of peptide-oligonucleotide.

H_2N-◍ = Support: (a) Aminopropyl CPG_{500}, (b) Aminopropyl $Fractosil_{500}$; $\overset{Y}{A'A}$ = Lys(Dde) or Leu; AA = Lys or Leu; B = Nucleic Base. i) DMTr-Cl,pyr. ii) p.nitrophenol,DCC,dioxane. iii) H_2N-◍ ,Et_3N,NMP/CH_2Cl_2. iv) Ac_2O,pyr.,DMAP. v) Cl_2CHCO_2H. vi) Fmoc-Leu-OH,DCC/DMAP. vii) piperidine/CH_2Cl_2 20%. viii) Fmoc-Lys(Dde)-OH or Fmoc-Leu-OH,HOBt/DCC. ix) repeat step viii) and vii) n-1 times. x) 2/HOBt. xi) Oligodeoxyribonucleotide synthesis. xii) ethanolamine/EtOH, 60C, 24h.

Ratio used to obtain:

3a	75 µmoles/gm resin (trityl assay),100-150eq. of 2				
3b	33	"	"	"	
4a	55 µmoles/gm resin (Fmoc assay),130eq. of Fmoc-Leu-OH				
4b	32	"	"	"	"
5a	33 µmoles/gm resin (Fmoc assay),30eq./cycle of Fmoc-Lys(Dde)-OH or Fmoc-Leu-OH				
5b	28	"	"	"	"
6a	32 µmoles/gm resin (trityl assay),100eq. of 2				
6b	27	"	"		

The solid supports were derivatized with the 10-hydroxydecanoïc acid derivative **2** to introduce a spacer arm with a terminal hydroxyl group **3**. A large excess of activated ester **2** was necessary (100-150 eq.) to achieve the coupling reaction. After the attachment of the first aminoacid residue via an ester linkage, which required 130 eq. of aminoacid and 24 hour coupling time, subsequent couplings were carried out on an Applied Biosystem model 431A peptide synthesizer. Synthesis were run with standard programs but increased coupling times.

After the peptide chain elongation, the supported peptides were treated with the spacer reagent **2**. Quantitative yields were obtained with 100 eq. of activated ester for both CPG and Fractosil supports. Then, these substrates were transfered to a Pharmacia Gene Assembler and oligonucleotide chain elongation via usual cyanoethylphosphoramidite chemistry[14] was used.

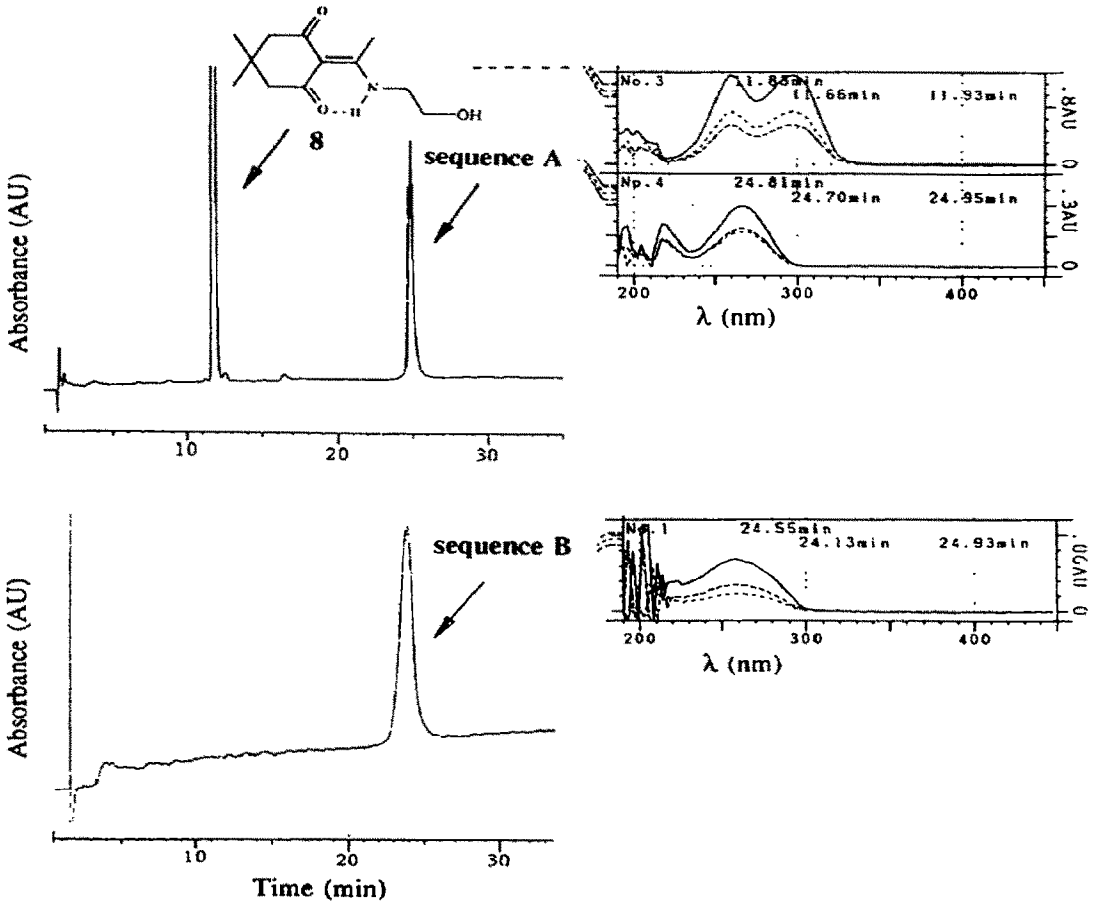

Figure 2: Reversed-phase analysis on a Lichrospher 100 RP18 (5µm) column (125x4mm) using a linear gradient of CH_3CN in 0.1M aqueous triethylammonium acetate buffer, pH 7, with a flow rate of 1 ml/min [0 to 100% CH_3CN in 50 min]. (top): Mixture of compound **8** and sequence A (both purified) and (bottom): Sequence B purified. The insets show the corresponding absorption spectra recorded between λ =200 nm and λ = 450 nm.

Sequence A: $^{5'}$d-[TTTTTTTTTTTT]$^{3'}$-O$(CH_2)_9$CO-[Leu-Lys-Lys-Leu]$_3$-NH$(CH_2)_2$OH

Sequence B: $^{5'}$d-[CACCGACGGCGC]$^{3'}$-O$(CH_2)_9$CO-[Leu-Lys-Lys-Leu]$_3$-NH$(CH_2)_2$OH

The deprotection of the conjugates was carried out using ethanolamine in absolute ethanol (1:1) under argon for 30 hours at 60C. This treatment simultaneously cleaves lysine Dde, the base and phosphate protection and the ester linkage to the support. In addition, ethanolamine delivers an ethanolamide peptide C-termini. Under the same conditions, concentrated aqueous ammonia affords a mixture of C-terminal amide and carboxylate[15]. The by-product formed under Dde-cleavage has been isolated by HPLC. The ^{1}H NMR gave the predicted amino 2-[(1-4,4 dimethyl-2,6 dioxocyclohexylidene)ethyl] ethanol **8**. The substrates were purified by reverse phase to yield the pure conjugates (27 O.D., 6.5% yield for sequence A with Fractosil$_{500}$, 5 µmoles scale; 14.3 O.D.,12.9% yield for sequence A with CPG$_{500}$,1.2 µmoles scale; 2.2 O.D., 1.5% yield for sequence B with CPG$_{500}$, 1.2 µmoles scale) (figure 2). Complete digestion with nucleases and HPLC of the digest gave the expected nucleotide composition.

The hydrolytic activity of these new peptide-oligonucleotide hybrid molecules and their ability to inhibit gene expression are presently under investigation.

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