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SOLID PHASE SYNTHESIS OF DIRECTLY LINKED PEPTIDE-OLIGODEOXYNUCLEOTIDE HYBRIDS USING STANDARD SYNTHESIS PROTOCOLS

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Abstract: We describe a general methodology for the solid phase synthesis of directly linked peptide-oligonucleotide hybrids using a sarcosine-modified controlled pore glass (CPG) support and employing phosphoramidite chemistry for the oligonucleotide synthesis and Fmoc strategy for the peptide assembly. Using this approach a special linker is not needed and the resultant peptide-oligonucleotide hybrid can be further elongated with an oligonucleotide sequence.

Hybrid molecules composed of peptides and nucleic acids have found use in several applications such as non-radioactive labels,[1] as PCR primers[2] and in encoded combinatorial chemistry.[3,4] Furthermore, they may become important in antisense inhibition of gene expression.[5-8]

Two different strategies are mainly used to synthesize such hybrid molecules. In one approach, the peptide part is assembled first on a solid support using standard peptide chemistry. This is followed by the introduction of a linker molecule usually containing both a carboxylic acid and a protected hydroxyl function, i.e. derivatives of an α,ω-hydroxycarboxylic acid, to form a stable phosphodiester linkage on continuation with the oligonucleotide elongation via the phosphoramidite method.[7-10] In the other approach, the solid support is first modified with a bifunctional linker molecule carrying both an Fmoc-protected amino group to start the peptide assembly using Fmoc chemistry and a hydroxy function blocked as the DMTr ether from where the oligonucleotide synthesis is later initiated.[4,11] In both strategies a linker unit is needed to connect the two biomolecule moieties.

Syntheses describing directly linked oligonucleotide-peptide hybrids have only been reported for naturally occurring nucleopeptides in which the 5'-hydroxy group of an oligonucleotide is covalently linked via a phosphodiester bond to the hydroxyl function of a serine, threonine or tyrosine in the peptide sequence.[12-17]

Here we present a new strategy to synthesize peptide-oligonucleotide hybrids starting with the oligonucleotide assembly. Incorporating 5'-amino-5'-deoxythymidine[18] as the last nucleotide building block yields a free amino function for the further extension with amino acid building blocks. Applying this methodology the insertion of a linker molecule is not needed. Since phosphoramidites have been found to couple with high efficiency to amino functions[18] the resulting peptide-oligonucleotide hybrids could also be further elongated with a DNA fragment to yield DNA-peptide-DNA hybrids.

The synthesis of the peptide-oligonucleotide hybrids using thymidine and L-alanine as building blocks was performed according to Figure 1. The procedure is generally applicable for other nucleotide building blocks and amino acids. First, the oligonucleotide synthesis was carried out using standard technology[19] on a DBU-stable sarcosine modified CPG support[20] which was derivatized with thymidine (**1**) using phosphoramidite chemistry. For the first four couplings the allyl phosphoramidite **2** [21,22] was applied, whereas 5'-N-MMTr-5'-amino-5'-deoxythymidine-3'-[allyl N,N-diisopropyl phosphoramidite] (**3**) [23] was used for the last coupling step to give **4a**. The allyl phosphate protecting group was chosen since it is compatible with

Fmoc peptide chemistry when DBU is used for the removal of the Fmoc group.[22] Intermediate **4a** was then subsequently extended with 6 alanine molecules using standard Fmoc peptide chemistry yielding **6a**. High coupling yields were obtained with Fmoc-Ala-OH (**5**) and HBTU [24] or TATU [25] whereas, DIC / HOBt or HOAt gave no detectable coupling with the 5'-amino terminus of oligonucleotide **4a**.

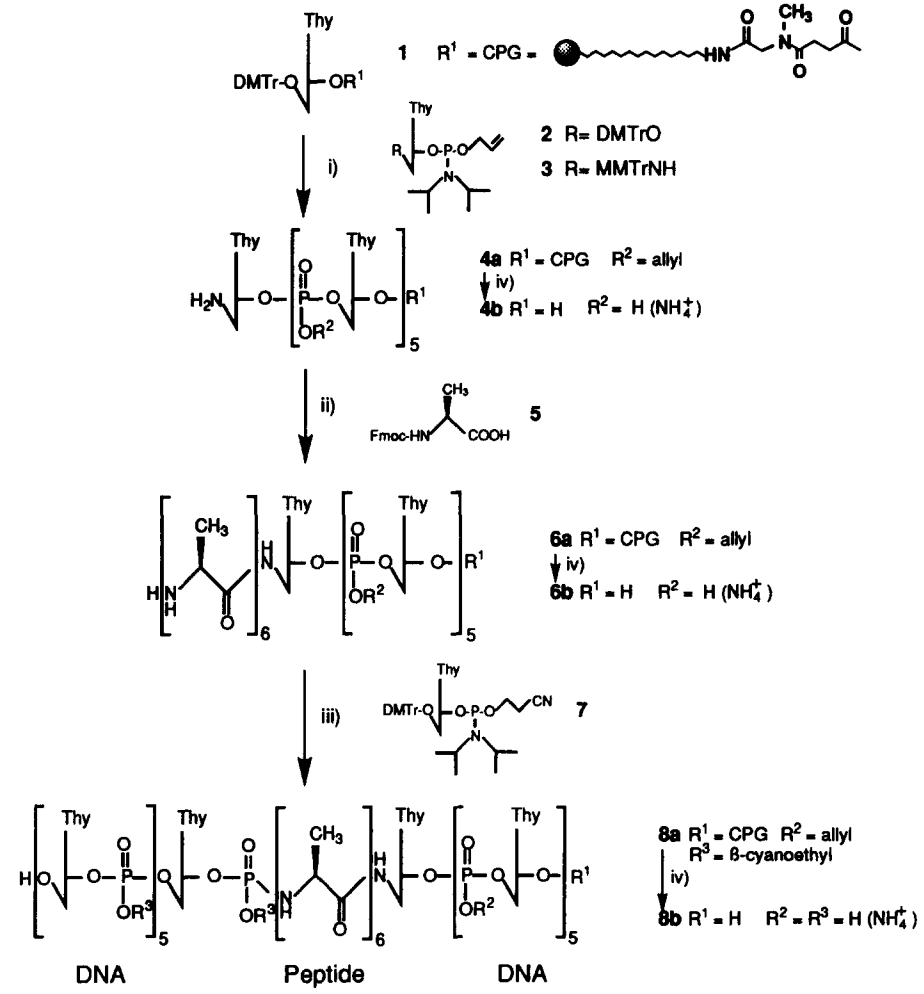
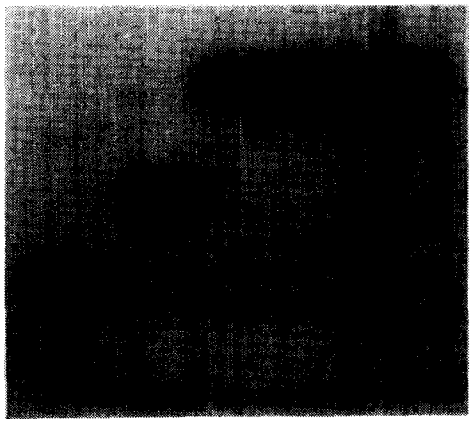


Figure 1: Stepwise synthesis of oligodeoxyribonucleotide-peptide hybrids

i) oligodeoxyribonucleotide synthesis applying allyl phosphoramidites **2** resp. **3** for the last coupling step. ii) peptide synthesis applying Fmoc-Ala-OH (**5**). iii) oligodeoxyribonucleotide synthesis using 2-cyanoethyl phosphoramidite **7**. iv) cleavage from support and protecting group deprotection: conc. NH_3 (2h at 70°C) or $Pd^{(0)}[P(C_6H_5)_3]_4$ / morpholine in DMSO/THF/dioxane/0.5M HCl 2:2:2:1 (3h at 60°C) followed by treatment with conc. NH_3 (2h at r.t.).

Oligonucleotide chain elongation of **6a** was possible by using 2-cyanoethyl phosphoramidite **7** to give **8a** wherein the peptide terminus is bound to the second oligonucleotide moiety via a phosphoramidate linkage which is stable to common deprotection conditions.[18] The cleavage of protecting groups and removal from the support was achieved by treatment of **4a**, **6a** and **8a** with 30% aqueous ammonia for 2h at 70°C [22] to yield **4b**, **6b** and **8b**, respectively. Treatment of the support material **4a**, **6a** and **8a** with $Pd^{(0)}[P(C_6H_5)_3]_4$ / morpholine in DMSO/THF/dioxane/0.5M HCl 2:2:2:1 (3h at 60°C)[26-28] followed by 30% aqueous ammonia (2h at rt.) gave identical products as shown by PAGE (Figure 2).[29]



Lane 1: crude d (T_5) : NH_4OH
Lane 2: crude d (T_5) : $Pd^{(0)}$; NH_4OH
Lane 3: crude d ($^{H2N}TT_5$) : NH_4OH
Lane 4: crude d ($^{H2N}TT_5$) : $Pd^{(0)}$; NH_4OH
Lane 5: crude Ala_6 d ($^{HN}TT_5$) : NH_4OH ; coupling with TATU
Lane 6: crude Ala_6 d ($^{HN}TT_5$) : NH_4OH ; coupling with HBTU
Lane 7: crude Ala_6 d ($^{HN}TT_5$) : $Pd^{(0)}$; NH_4OH ; coupling with HBTU
Lane 8: crude d (T_6) Ala_6 d ($^{HN}TT_5$) : NH_4OH
Lane 9: crude d (T_6) Ala_6 d ($^{HN}TT_5$) : $Pd^{(0)}$; NH_4OH
Lane 10: d (T_6) Ala_6 d ($^{HN}TT_5$) : HPLC purified

Figure 2

The mobility of **4b** is clearly slower in comparison to T_5 due to the compensation of one negative phosphodiester charge by the terminal 5'-amino group. Introduction of $(Ala)_6$ (**6b**) leads to a further decrease in mobility due to the increased molecular mass of **6b** by unchanged number of phosphodiester residues. Compound **8b** has a similar mobility shift as **6b** indicating that the effect of the additional phosphodiester charges is compensated by increase in size of the molecule. Purification was achieved by preparative PAGE and consecutive desalting by Sephadex gel filtration or by RP18-HPLC (Figure 3). All structures were confirmed by MALDI TOF-MS.

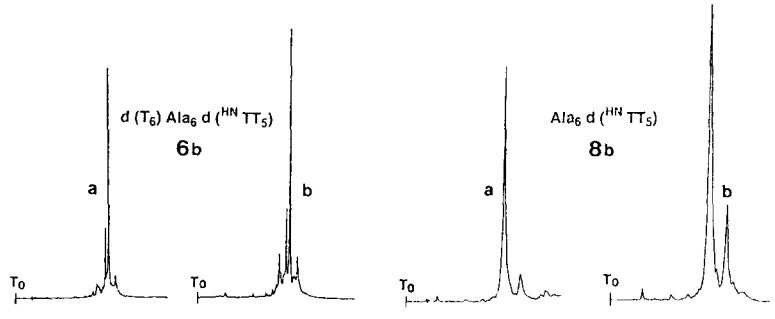


Figure 3: HPLC of crude 6b and 8b : a: $Pd^{(0)}$; NH_4OH b: NH_4OH

In summary, we have developed a general method to synthesize directly linked peptide-oligonucleotide hybrids starting first with the oligonucleotide assembly and omitting a linker

between the two moieties. Furthermore, we could show that these hybrid molecules can easily be further elongated with an oligonucleotide chain to form DNA-peptide-DNA hybrids.

The developed methodology can also be applied to the synthesis of directly linked PNA -DNA hybrids which should be "in phase" since the amino-terminus of the PNA molecule corresponds to the 5'-end of the oligonucleotide part, thus allowing base pairing to a complementary DNA or RNA strand. The synthesis of such DNA-PNA hybrids and the evaluation of their hybridization properties are under investigation and will be reported in due course.

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23. Preparation of **3**: 5'-Amino-5'-deoxythymidine (1.54 g; 3 mmol) was 3x co-evaporated with dry MeCN and dried for 2h at high vacuum. Diisopropylammonium tetrazolide (0.26 g; 1.5 mmol; dried overnight at high vacuum) and dry CH_2Cl_2 (30 ml) were added and kept under Ar. Then allyloxy-bis-(diisopropylamino)phosphane (1.4 g; 4.75 mmol) was added dropwise to the solution. Stirring was continued for 1.5h, then the mixture was poured into sat. $NaHCO_3$ soln. and extracted with CH_2Cl_2 (3x). The org. phase was dried (Na_2SO_4) and evaporated. Purification by flash chromatography (30 g SiO_2; elution with n-hexane/acetone 1:1 / 2% NEt_3 (500 ml)), followed by precipitation of **3** in CH_2Cl_2 with n-pentane at -70°C to give a colorless powder of **3** (1.3 g; 62%). ^{31}P-NMR ($CDCl_3$): 148.15 / 148.03 ppm (2 diastereoisomers). The structure was also confirmed by ^{1}H-NMR, UV and elemental analysis.
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29. Abbreviations: DBU: 1,8-Diazabicyclo[5.4.0]undec-7-ene; DIC: 1,3-Diisopropylcarbodiimide; DIPEA: Diisopropylethylamine; DMTr: 4,4'-Dimethoxytrityl; Fmoc: 9-Fluorenylmethoxycarbonyl; HBTU: 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt: 7-Aza-1-hydroxybenzotriazole; HOBt: 1-Hydroxybenzotriazole; LCAA-CPG: Long chain alkyl amine controlled pore glass; MALDI TOF-MS: Matrix assisted laser desorption ionization time-of-flight mass spectrometry; MMTr: 4-Methoxytrityl; PAGE: Polyacrylamide gel electrophoresis; PCR: Polymerase chain reaction; RP18-HPLC: Reversed phase (C18) high performance liquid chromatography; TATU: 2-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate;

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