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CONVERGENT SOLUTION-PHASE SYNTHESIS OF A NUCLEOPEPTIDE USING A PROTECTED OLIGONUCLEOTIDE

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Abstract: A nucleopeptide was prepared in a convergent manner via segmental coupling of the protected biopolymers in solution. The resulting nucleopeptide (**4b**, 72%) containing the binding site of λ repressor and a peptide containing the consensus sequence of the DNA binding helix of the helix turn-helix-proteins was obtained using only five equivalents of the peptide relative to the oligonucleotide. This demonstrates that the recently developed method for the solution phase coupling of protected oligonucleotides is amenable to the convergent synthesis of larger nucleopeptides that are potentially capable of adopting secondary structure.

Nucleopeptides are a class of oligonucleotide conjugates that are potentially useful in the design of therapeutic agents. The peptide component can facilitate membrane transport of antisense molecules, serve as a cell signaling entity, or function as a nuclease.[1-7] Nucleopeptides are also potentially useful in diagnostic applications and for the construction of supramolecular assemblies.[8] Consequently, both linear and convergent methods have been developed for nucleopeptide synthesis. Linear methods typically involve carrying out conventional solid-phase peptide (carboxy to amino) and oligonucleotide (3'- to 5'-) syntheses sequentially on a conventional solid-phase support, or on a specially designed branched solid-phase support.[1,6,9,10] Post-synthetic conjugation of oligonucleotides functionalized at their 3'-termini with a suitable functional group in solution benefits from being a convergent process, and is compatible with coupling to peptides and enzymes. Yields of nucleopeptides obtained via a variety of bond forming reactions can be very high using unprotected biopolymers.[3,4,7] However, the use of deprotected biopolymers can also present limitations with respect to the sequence of peptides employed, and side reactions can result in the formation of heterogeneous products.[7,11] Post-synthetic solution-phase synthesis of nucleopeptides using protected biopolymers offers potential advantages that include convergency, chemoselectivity, and elimination of problems associated with aggregation of deprotected peptides. Herein, we describe the synthesis of a nucleopeptide containing a modified DNA binding helix of the helix-turn-helix protein, λ repressor. The bioconjugate was prepared using a recently developed general method for 3'-oligonucleotide conjugate synthesis that involves coupling protected biopolymers in solution (Scheme 1).[12]

Success in the area of segmental peptide synthesis suggested that efficient conjugation of 3'-alkylamine containing oligonucleotides via amide bond formation to a peptide long enough to potentially adopt secondary structure should be viable.[13] In the present case the approach was tested by attempting to synthesize 3'-oligo-

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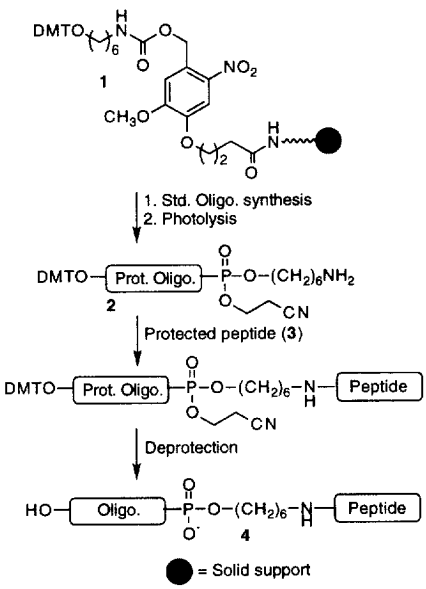
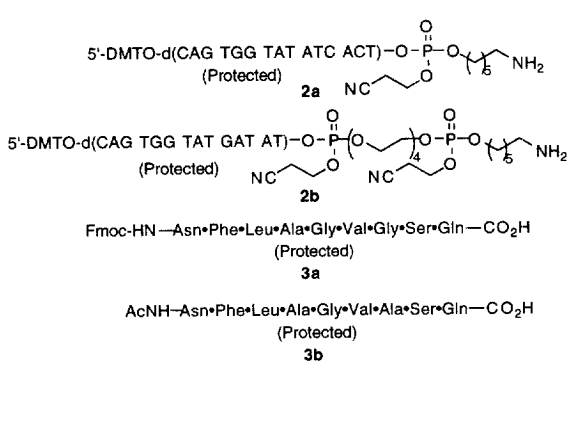
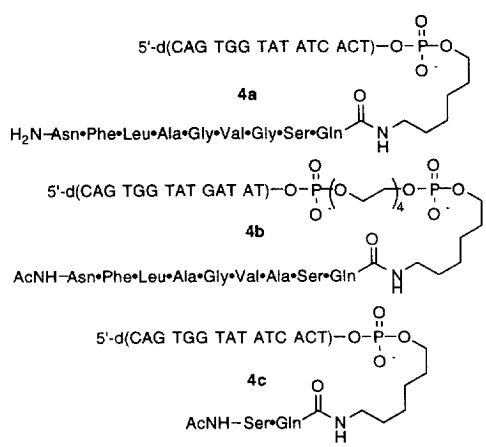
Scheme 1



nucleotide conjugates containing modifications of helix 3 of the λ repressor and the sequence of this protein's binding site (**4a**,**b**).[14] The peptides (**3a**, **3b**) were chemically synthesized using *N*-Fmoc protected amino acids on Rink resin. In order to facilitate deprotection of all protecting groups of the bioconjugate in one step via concentrated aqueous ammonia, the hydroxymethyl group of serine was protected as its acetate. Following cleavage of the peptides from their solid supports under mildly acidic conditions, they were purified by reverse-phase HPLC and characterized by electrospray mass spectrometry.

When designing conjugate **4a** the alkylamine tether was linked directly to the 3'-terminal nucleotide of the oligonucleotide component of the bioconjugate. The *N*-Fmoc protected peptide (**3a**, 8.5 equiv.) was coupled to **2a** using PyBOP as activating agent. Following concentrated aqueous ammonia deprotection at 55 °C (6 h) and dedimethoxytritylation, the major conjugation product was purified in 96% yield by denaturing gel electrophoresis. However, electrospray mass spectral analysis revealed that the isolated conjugate contained only the two C-terminal amino acids of the original peptide (**4c**). Fragmentation is believed to have resulted from *N*- to *O*-transacylation via nucleophilic attack of the deprotonated hydroxymethyl group on the amide linkage between the serine and glycine residues in the peptide, and subsequent cleavage under the oligonucleotide deprotection conditions.[15] A slower migrating product, believed to be the intact conjugate (**4a**) was also observed, but in too low a yield to permit characterization by electrospray mass spectrometry. Efforts aimed at decreasing the amount of fragmented product (**4c**) using milder deprotection conditions (e.g., 0.4 M methanolic sodium hydroxide (4:1 by volume), 17 h) were unsuccessful. Consequently, nucleopeptide **4b** was designed in order to alleviate the problem of transacylation by increasing steric hindrance at the postulated site of nucleophilic attack. Other modifications included the incorporation of a





tetraethylene glycol linker between the alkylamine terminus and the 3'-nucleotide, and acetylation of the peptide's amino terminus. It was anticipated that the latter two modifications might facilitate the formation of secondary structure in the nucleopeptide when hybridized to its complement. Conjugation of **3b** (5 equiv.) to **2b** proceeded in 72% yield. The conjugate (**4b**) was characterized by electrospray mass spectrometry, which indicated the presence of a single compound (Calculated mass: 5682.0; Observed mass: 5683.0).

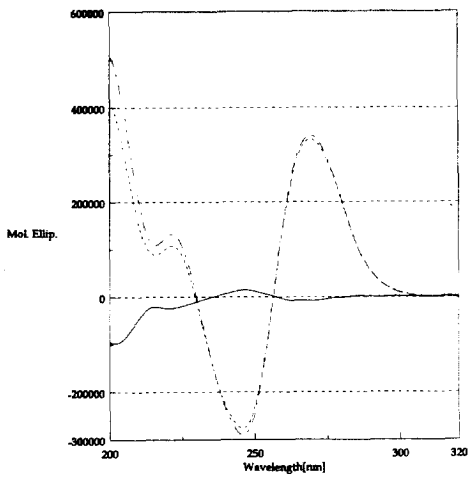
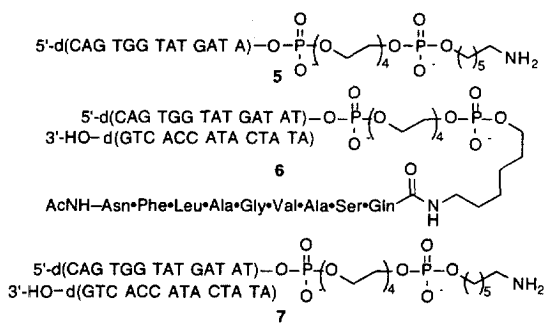


Figure 1. CD spectra of **6** (- - - -), **7**(— - — -), and difference spectrum of **6** - **7** (—).



Although exceptions exist, peptides as short as those employed in this study are generally incapable of α-helical formation.[16] However, there are examples in the literature where preorganization induces secondary structure formation in biopolymers.[17] Consequently, the possibility that conjugation between a peptide related to a portion of a protein known to adopt an α-helical structure and an oligonucleotide containing the respective protein's binding site would induce such an ensemble was considered. Nucleopeptide **4b** was examined by CD spectroscopy in search for possible α-helical peptide formation. For comparison purposes, unconjugated **5** was also analyzed. Strong absorbance below 200 nm (attributable to the phosphate buffer) precluded searching for the characteristic molar ellipticity maximum between 190 and 200 nm. Consequently, attention was focused on the region between 207 and 222 nm, where peptide α-helices exhibit a minimum. However, α-helix formation was not evident in **4b** or when this nucleopeptide was hybridized to its oligonucleotide complement (**6**, Figure 1). DNA duplex formation was confirmed by the presence of a minimum between 240 and 250 nm. Furthermore, addition of 50% trifluoroethanol, which is known to induce α-helix formation, had no effect on the structure of **4b**, or its respective duplex (**6**) (data not shown).

Summary. A nucleopeptide containing a nonapeptide was prepared in very good yield using protected biopolymers in solution. This convergent method should provide a general method for the synthesis of nucleopeptides capable of adopting secondary structures.

Sample coupling procedures:

Preparation of 4b. To a glass conical vial was added a 0.64 mM solution of **2b** (250 nmol in 391 μL of H_2O:CH_3CN; 1:1). The solvents were removed *in vacuo* on a speed vac, followed by drying the vial on a vacuum

line for 3-4 h. To an oven dried glass vial was added PyBOP (17 mg, 0.033 mmol), DMF (983 μL), and diisopropylethylamine (17.1 μL, 0.099 mmol). To the peptide (**3a**) was added DMF (7.7 μL) and the PyBOP solution (7.7 μL). The activated peptide solution was added to **2a** in a vacuum screw cap conical vial. The reaction was vortexed and then stirred for 2 h at 25 °C, at which time the reaction solution was frozen with liquid nitrogen and lyophilized at ambient temperature. The crude reaction was deprotected with 28% aqueous ammonia for 6 h at 55 °C. The solvents were removed *in vacuo* on a speed vac. Dedimethoxytritylation was performed following a standard commercial oligonucleotide purification cartidge protocol. Following removal of solvents *in vacuo*, **4c** was purified via 20% denaturing PAGE (96% yield). ESMS calcd: 5003.4, found 5003.0.

Preparation of 4b. The reaction of **2b** (100 nmol) with **3b** (5 equivalents) and activating reagents (5 equivalents) was performed as described above, with the exception that the 28% aqueous ammonia deprotection was performed at 25 °C for 12 h. Purification yielded **4b** (72%). ESMS calcd 5682.0, found 5683.0.

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