

EFFICIENT SYNTHESIS OF OLIGONUCLEOTIDE- PEPTIDE CONJUGATES ON LARGE SCALE

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ABSTRACT

The conjugation of oligonucleotide phosphorothioates with antennapedia peptide was studied in detail to allow efficient preparation of the conjugates on up to 15 μmol scale. Under optimized conditions, the use of oligonucleotides and the peptide in an equimolecular ratio gave the desired conjugates in more than 60% isolated yield.

Oligonucleotides and their analogs have proven their value as sequence specific, potent reagents for modulation of translation of mRNA in living cells (1). However, realization of the full potential of efficacy of oligonucleotides using this paradigm *in vivo* is often hampered by limited cellular uptake of oligonucleotides followed by subsequent entrapment and degradation in lysosomal and endosomal compartments. Many chemical conjugation approaches have been undertaken to overcome these critical issues (2). An emerging approach to address this problem consists in conjugation of antisense oligonucleotides with peptides that possess cell membrane translocation and/or nuclear localization properties. The peptide derived from Antennapedia homeodomain protein is considered among the most promising “delivery vehicles”. Oligonucleotide conjugates with this peptides has been shown to improve the cellular uptake of oligonucleotides thus increasing their potency as antisense agents (3,4).

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Table 1. Gapmer Oligonucleotides Synthesized^a

Sequence, 5' → 3'	Backbone
TCC GTCATCGCT CCTCAGGG	P=S
AGCTTC TTTGCACA TGTAAA	P=S

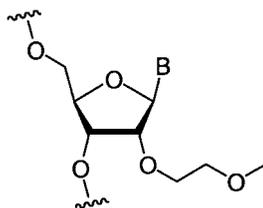
^a2'-*O*-MOE nucleoside residues are depicted in bold italicized letters.

Typically, conjugates of oligonucleotides with peptides are synthesized on 1 μ mol or a smaller scale. Thus, the conjugates are isolated in quantities considerably lower than those required for advanced animal studies *in vivo*. To meet these needs, we studied in detail the conjugation reaction. This allowed us to scale up the conjugation of oligonucleotide phosphorothioates with antennapedia peptide (pAntp) and synthesize the desired compounds on 15 μ mol scale.

The desired conjugates **6** and **7** of oligonucleotide phosphorothioates and pAntp were prepared as depicted in Figure 2. Starting from a commercial solid support (**5**), the 20-mer 3'-derivatized oligonucleotide phosphorothioates **1** and **2** were synthesized first (Table 1). In order to improve their binding affinity and nuclease stability, **1** and **2** bore three to eight 2'-*O*-(2-methoxyethyl)(2'-*O*-MOE) modified ribonucleoside residues at each termini (Fig. 1). These were reduced with 0.25 M dithiothreitol (DTT) at pH 9. The resultant 3'-mercaptopropyl oligonucleotides were isolated by reverse phase (RP) HPLC and treated with 2,2'-dipyridyl disulfide to give **3** and **4**, which were purified by RP HPLC. The activated oligonucleotides **3** and **4** were analyzed by HPLC and LC-MS and revealed no side products related to the activation of the phosphorothioate groups.

For the conjugation reaction, **3** and **4** were treated with the antennapedia peptide **5** synthetically modified at the *N*-terminus (**6**). Initially, extremely poor yields of conjugates were obtained, which was attributed to an extensive precipitation that occurred on mixing the components. In order to disrupt the ionic interactions between negatively charged oligonucleotides and positively charged **5**, the effect of different denaturing agents on solubility of **3** and **5** was studied on a small scale.

Under optimized conditions (20–30% aq MeCN, 0.1 M KCl, 1 M urea, 0.1 M NH₄OAc, 1 μ M DIEA), mixing **5** with **3** or **4** resulted in formation of stable and transparent solutions where the conjugation occurred rapidly. Indeed, as judged

**Figure 1.** General structure of 2'-*O*-MOE nucleoside residues.

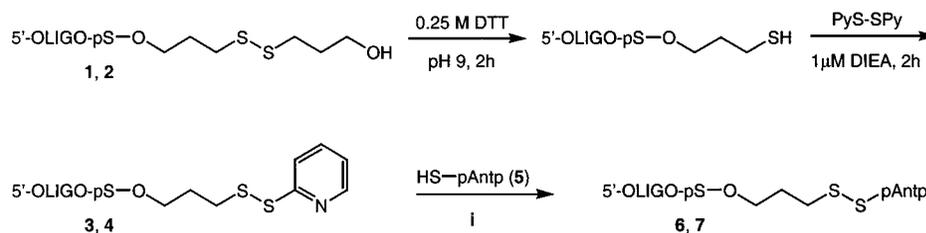


Figure 2. Preparation of oligonucleotide-peptide conjugates. (i) 20–30% aq MeCN, 0.1 M KCl, 1 M urea, 0.1 M NH_4OAc , 1 μM DIEA.

Table 2. Characterization of Oligonucleotide Conjugates **6** and **7**

Conjugate	Scale (μmol)	Yield based on HPLC	Isolated yield	Purity (HPLC)	ESMS	
					Found	expected
6	2.3	75%	60%	95%	9988.2	9988.8
7	13.2	82%	64%	98%	10070.4	10070.8

by ion-exchange HPLC, the reaction of equimolecular amounts of **5** and oligonucleotides **3** and **4** gave the conjugates **6** and **7** in 70–80% yield irrespectively to the scale employed. The reaction mixtures were then separated by the RP HPLC on a C4 column to furnish **6** and **7** (Table 2).

Addition of excess **5** (0.2 eq) resulted in nearly quantitative conversion of **3** and **4** to **6** and **7**, respectively. However, attempted separation of the reaction mixtures that contained any unconjugated peptide gave very poorly reproducible results on the RP HPLC and substantially reduced the lifetime of columns. In contrast, isolation by ion-exchange HPLC gave the desired conjugates in the expected yields (*ca.* 70–80%). However, the tendency of **6** and **7** to form aggregates on the column severely limited the loading of the material on ion-exchange columns. Although not important on a small scale, this feature made purification on a larger scale very labor intensive.

In conclusion, a reliable method for the preparation and isolation of the conjugates **6** and **7** on large scale were developed. The conjugates were synthesized in more than 60% isolated yield employing equimolecular amounts of oligonucleotides and the peptide. Their high purity (>95%) was confirmed by HPLC, ES-MS, and capillary gel electrophoresis.

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5. 3'-Thiol-modifier C3 S-S CPG 500 (Glen Research) was used to introduce a disulfide group at the 3'-terminus of oligonucleotides. On deprotection, oligonucleotides were purified by a "DMT-On" RP-HPLC.
6. A modified antennapedia peptide, (*N*-Acetyl)C-(γ -aminobutyric acid)-GGRQIKI-WFQNRMRMKWKK-NH₂, (**5**) was provided by Commonwealth Biotechnologies, Richmond, VA, USA.

