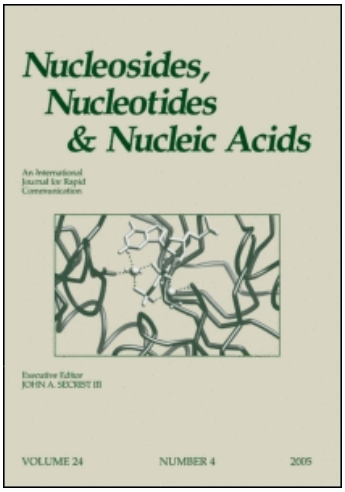

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EVALUATING COUPLING PROCEDURES FOR SYNTHESIS OF LNA-PEPTIDE CONJUGATES

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□ *As initial studies of block coupling of peptides with C-terminal glycines, we have evaluated of coupling of glycine building blocks under various conditions to a preassembled solid support bound LNA-fragment. Potentially competing side reaction have been studied and we have worked out a procedure for trifluoroacetyl protection of peptides.*

Keywords Oligonucleotides; peptides; conjugates; protection; coupling

Peptide conjugates of oligonucleotides (POCs) are essential tools in biomedicine.[1] We have earlier developed a method for on-line synthesis of POCs[2] and are now in the process of looking at strategies for block couplings. Here we present our recent investigations of potential coupling strategies for the synthesis of POCs using stepwise or block-coupling[3] of a peptide fragment to a preassembled solid support bound oligonucleotide.

Before starting to look at coupling procedures we first wished to develop a convenient way to convert a commercially available peptide into a protected derivative that could be readily used in block coupling. A peptide containing one proline four lysines, one arginine and a C-terminal glycine with sequence PKKKRKVG was purchased and protected using ethyl trifluoroacetate in methanol.[4] Mass analysis showed that five trifluoroacetates were incorporated into the peptide, suggesting that all lysines and the N-terminal proline was protected. It is reasonable to assume that the guanidinyl of arginine remains unprotected and submitting an arginine

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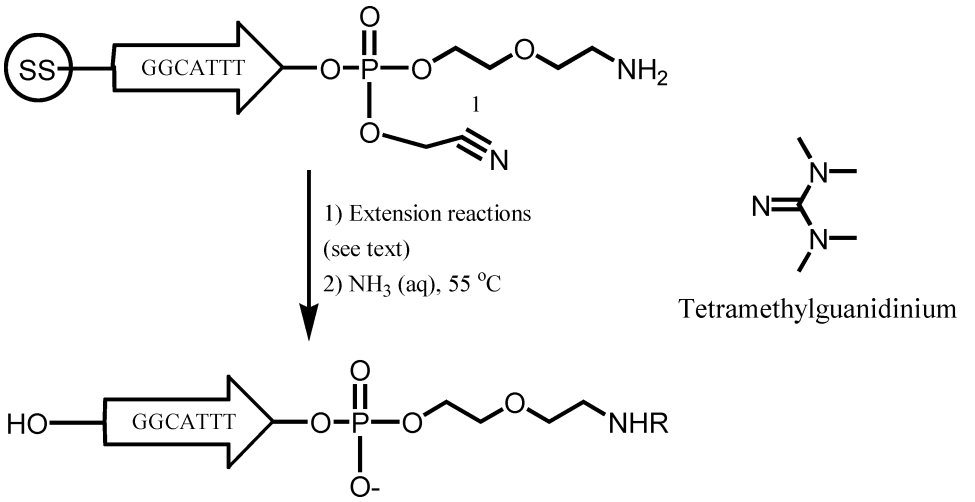

SCHEME 1 Formation of different LNA-conjugates. **2**, **R** = H (Retention time (Rt): 30.5 minutes, mass found (M): 2487.2); **3**, **R** = Ac (Rt: 34.1 minutes, M: 2529.3); **4**, **R** = Gly (Rt: 30.2 minutes, M: 2545.1); **5**, **R** = CO-NH-CH_2-CH_2-NH-Gly; (Rt: 33.6 minutes, M: 2620.8). **6**, **R** = CO-NH_2 (Rt: 32.1 minutes, M: 2530.6); **7**, **R** = CO-NH-CH_2-CH_2-NH_2; (Rt: 32.1 minutes, M: 2551.8) **8**, **R** = Cys(Trt); (Rt: 60.1 minutes, M: 2810.3). **9**, **R** = Tetramethylguanidinium (Rt: 36.4 minutes, M: 2584.7).

monomer to the same conditions also gave no adduct on the guanidinyl function. After HPLC purification we attempted to couple[2] the protected peptide to a solid supported LNA carrying a 5′-aminoethoxyethyl phosphate **1** (Scheme 1) using HBTU as condensing agent. After cleavage from support and deprotection (32% aq. ammonia for 12 hours at 55°C) we observed three main product peaks during HPLC analysis, of which two were identified as the unreacted LNA with 5′-aminolinker (**2**) and the LNA with acetylated linker (**3**). The acetylated product is probably formed prior to the peptide coupling step, as direct cleavage of the LNA from the solid support gave this product in the same relative amount (it is likely that acetylation arises from capping in the last amidite coupling cycle—omitting the last capping should remove this problem). The third product was also not the desired LNA-peptide conjugate but instead a compound with a mass corresponding to a group adding a mass of around 97 higher than the for **2**. Two type of side reactions adding that weight (within possible MS error) came to mind. Either direct reaction with the coupling agent giving a tetramethylguanidylated[5,6] linker or the trifluoroacetylated linker (from coupling of the trifluoroacetate counterion). This product accounted for approximately one third of the material which we found rather surprising. Direct reaction with uronium coupling agents in peptide coupling is known, but these typically occur at a very low level. Although trifluoroacetate can be coupled to amines,[7] trifluoroacetamides would not be expected to survive the ammonolysis step. We nevertheless decided to investigate both potential

side reaction but also to perform coupling of glycine building blocks to the same linker in order to evaluate a couple of alternative methods.

Coupling[2] of Fmoc-glycine to **1** using the same conditions as for the peptide gave after deprotection and cleavage from support the desired glycine oligonucleotide conjugate **4** as main product (and ca. 30% **3** as this present already in the purchased LNA sample). Coupling with HBTU was performed also with *N*-(2-biphenyl-2-propoxy)carbonyl glycine (Bpoc-glycine) and reaction of **1** with Fmoc-glycine pentfluorophenyl ester was also done. Both these procedures gave results similar to coupling with Fmoc-glycine, i.e., reaction with available (non-acetylated) aminolinker was virtually quantitative. Worth noting is that the Bpoc protection did not survive workup and ammonolysis all the couplings with different glycine derivatives gave the fully deprotected glycine-LNA conjugate. We have not investigated the Bpoc cleavage in detail at this stage.

We also carried out experiments with carbonyldiimidazole/ethylenediamine extension[8] before peptide (glycine) coupling. This can provide a convenient approach for large variation of the linker since other amines can be used and the cycle can be repeated to extend multiple times. First the linker is elongated by carbonyldiimide (CDI) for 120 minutes followed by reaction with ethylenediamine (EDA) for 12 hours. Subsequent coupling with Fmoc-glycine gave the desired glycine conjugate **5** as the main product. (less than 5% of **6** + **7**). Into extend the linker/linking possibilities further to include a "native ligation"[9] possibility we coupled Fmoc-(Trt)-S-Cysteine to **1**. The cystein conjugate **8** was formed as readily as with the glycine building blocks. The substantial increase in steric hindrance with the Cys derivative did not give any noticeable increase in side products.

When investigating the possibility of competing side reactions we first investigated trifluoroacetate coupling to **1** with HBTU and did obtain a product with the same mass (+97) and HPLC retention time as in the attempted block coupling. However, the relative amount of this product did not diminish upon additional ammonia treatment. Furthermore the outcome of the HBTU mediated coupling with Fmoc-glycine was not affected by added trifluoroacetate (1 or 5 equivalents), but only gave the glycine conjugate. Reaction of **1** with HBTU in the absence of any carboxyl component also gave an adduct with a similar mass (about 97 units higher than for **1)** and HPLC retention time as when either trifluoroacetate or peptide was present. From these experiments the picture now seems fairly clear. The undesired product formed when carrying out the peptide coupling must be the tetramethylguanidium adduct **9** originating from direct reaction of the linker with HBTU. The same product also is formed when attempting to couple trifluoroacetate to the linker and of course when reaction HBTU directly with the linker, indicating that TFA coupling is substantially slower than formation of the tetrametylguanidinium adduct. It is also clear that neither of these two

potential side reactions can compete to any larger extent (minor traces of **9** are visible in the chromatograms though) when coupling the glycine and cysteine bulding blocks to **1**. One may speculate that the coupling reaction of the protected peptide for various reasons could be even slower than for the cysteine building block and that direct reaction of HBTU would compete more sucessfully. It does, however, seem that there is more to it than that since only one third of the linker is converted to **9** and the rest remain unreacted, while not even a trace of peptide conjugate is formed. More studies are clearly needed to satisfactorily explain why no desired product was observed in the peptide block coupling.

Here we report on investigative studies towards solid phase stepwise or block coupling in synthesis of peptide-LNA conjugates. We have worked out a simple scheme for protection of the aminofunctions which also renders the peptide soluble in organic solvents used for coupling reactions. Couplings with different glycine and cysteine derivatives have been investigated and all led in all cases to the desired conjugates with high conversion. Block coupling of the TFA-protected peptide to the aminolinker-LNA require more in depth studies to sort out conditions for coupling

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