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# FACILE PREPARATION OF 3'OLIGONUCLEOTIDE-PEPTIDE CONJUGATES

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**Summary:** The solid phase synthesis of 3' deoxyoligonucleotide (17 mer)-peptide conjugates is described. The oligonucleotide is complementary to the template strand of the measles virus in the region of the nucleocapsid protein on the 60 nucleotide leader RNA The conjugates were synthesized on teflon support using a combination of solid phase FMOC peptide chemistry and phosphoramidite chemistry linked by a branched modifier.

The concept of specific control of gene expression through the use of oligonucleotides promises rational therapeutic approaches to viral[1] diseases as well as to certain cancers[2]. Several distinct modes of action for inhibition of gene expression[3] have been demonstrated; they all have in common a requirement that oligonucleotides be readily incorporated into living cells There is currently intense research activity focussed on the synthesis of oligonucleotide conjugates capable of enhanced cellular uptake

In attempts to increase the level of cellular incorporation, oligonucleotides have been covalently linked to a variety of hydrophobic groups such as cholesterol[4], or lipids[5] or to positively charged groups such as poly-L-lysine[6] In this context, the synthesis of peptide-oligonucleotide conjugates offers great potential in terms of increased uptake and also through the possibility of targeting the antisense construct to certain types of cells using specific peptides A generally applicable approach has been recently published[7], this approach however requires several preliminary steps to modify the commercially available solid support before synthesis of the conjugate proper can begin

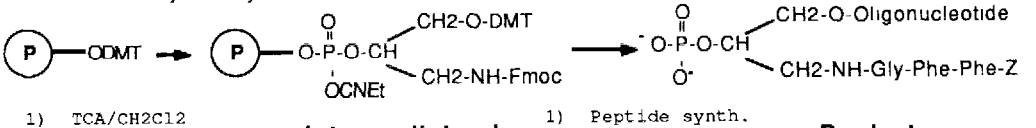
In this paper, we describe a synthetic method which is rapid and simple to carry out and which allows preparation of peptide-oligonucleotide conjugates starting directly from commercially available starting materials.

The peptide (Z-D-Phe-L-Phe-Gly, Z=benzyloxycarbonyl) was found to be a specific inhibitor of cell fusion and hemolysis[8]. The oligonucleotide part of the conjugate is a 17-mer with the sequence 5'CAAAGTTGGGTAAGGAT. It was designed as a positive (mRNA) sense oligomer hybridizing with the genomic, negative strand of this paramyoxvirus in the leader RNA region, starting 50 bases upstream of the mRNA cap site Complexes which bind the viral genome in this region have shown ability to inhibit transcription The hydrophobic conjugate $(Trp)_5$ and the positively charged conjugate $(Lys)_5$ are expected to provide enhanced uptake of the oligonucleotide moiety

The strategy is outlined in Figure 1, it is based on a commercially available teflon resin which contains a

**Figure 1**

DMT= Dimethoxytrityl, Fmoc = Fluorenylmethoxycarbonyl, TCA= Trichloroacetic acid
CNEt= 2-cyanoethyl




1) TCA/CH2Cl2
2) Branched modifier/ tetrazole

**Intermediate I**

1) Peptide synth.
2) Oligo synth.
3) Deprotection

**Product**

25 atom spacer arm terminating in a Dimethoxytrityl (DMT) protected hydroxyl To this hydroxyl group is added the bifunctional branched modifier to create Intermediate I This intermediate bears both a DMT-protected hydroxyl group and an Fmoc-protected amino group and therefore can be used directly both for solid phase Fmoc peptide synthesis and for subsequent oligonucleotide synthesis by the solid phase phosphoramidite method

The stepwise synthesis of Z-D-Phe-L-Phe-Gly on Teflon was performed as follows· the commercially available Teflon support (Molecular Biosystems, San Diego, CA, 1.0 μmole) was placed in a DNA synthesis column The DMT group was removed on the machine using 3% trichloroacetic acid (TCA) A non-standard synthesis cycle was run introducing the branched modifier I (Glen Research, Herndon, VA., 0.1M) phosphoramidite with the amidite reaction time extended to 110 seconds The DMT group on the branched modifier was left on The teflon packing was removed from the column and treated with 20% piperidine/DMF for 15 minutes to effect removal of the FMOC group. The teflon was washed with DMF and dry pyridine. Fmoc-Gly-OPFp (23.2 mg, 50.0 μmole, Milligen, Cambridge, U.K.) was added to the teflon and dissolved in 700 μl of dry pyridine. The reaction mixture was stirred overnight at room temperature After washing with DMF, 20% piperidine/DMF was added and stirred at room temperature for 15 minutes to effect removal of the Fmoc group The solution was removed and the support was washed with dry pyridine The previous step was repeated using Fmoc-L-Phe-OPFp (27.65 mg, 50 0 μmole) in 700 μl dry pyridine Finally, Z-D-Phe-OH (29.9 mg, 100.0 μmole) was reacted with the growing peptide chain using HOBT (15 3 mg, 100 0 μmole) and DCC (22.7 mg, 100 0 μmole) in 700 μl of dry pyridine. Within 5 minutes a precipitate was formed and the mixture was left stirring at room temperature overnight

After the peptide coupling, the teflon was placed on the Applied Biosystems Model 380A and DNA synthesis was carried out using the cyanoethyl phosphoramidite chemistry with extended reaction and detritylation times (110 sec. each) as specified by the manufacturer of the teflon resin The trityl cation release was assayed and gave an average stepwise yield of 98.9%. The deprotection of the oligonucleotide was not carried out using $NH_4OH$ because of the lability of the peptide bonds to strong base For this reason, the alternative procedure originally reported by Miller and coworkers[9] for the deprotection of methyl phosphonate oligonucleotides was employed. The method involves treating the derivatized teflon with ethylenediamine (EDA) in absolute ethanol (1 1 $^{v}/_{v}$) for 1 hour at 55 °C. The conjugate was removed from the support[10] by treating with a 1 ml solution of 100 mM sodium periodate, 100 mM sodium phosphate buffer, pH 7 2, in acetonitrile/water (1:4 $^{v}/_{v}$). The mixture was agitated at room temperature with exclusion of light After 4 hours the solution was removed and the support was washed with excess water and methanol The solution was removed and the teflon was treated with n-propylamine (50 ul), acetonitrile (100 ul), and water (400 ul) for 3 hours at 55 °C. The solution was evaporated to dryness in vacuo The product was purified by reverse phase HPLC to yield pure conjugate (14.8 O D., 10.4% for 1 step synthesis or 15.1 O.D., 10.7% for stepwise synthesis).

HPLC conditions were as follows: reverse phase C-18 column 5 μm particle size, LKB type Pep-S analytical, using a linear gradient between Solvent A, 0 1 M triethylamine acetate (TEAA) in water and Solvent B, 75% acetonitrile in 0.1 M TEAA. The gradient was run from 0-100% in 100 minutes The conjugate elutes between the oligonucleotide (23 5 min ) and the peptide (56.8 min ) giving a sharp single peak at 42.4 minutes

The $^1H$ NMR spectrum of the conjugate shows a phenyl absorption at 7 1-7 8 ppm, while the peptide (Z-D-Phe-L-Phe-Gly) shows a phenyl absorption at 7 1-7 3 ppm This peak broadening of the phenyl groups is consistent with previous results[11]

U V data ($H_2O$) is as follows ($\lambda_{max}$ 262 6 nm., $\epsilon$ = 150,000 (estimate), $\lambda_{min}$ 260.6 nm., $\lambda_{max}$ 256.6 nm., $\lambda_{min}$ 253 0 nm., $\lambda_{max}$ 250 4 nm., $\lambda_{min}$ 247.4 nm.) and peptide, Z-D-Phe-L-Phe-Gly ( $\lambda_{max}$ 263 9 nm , $\lambda_{min}$ 261.8 nm., $\lambda_{max}$ 258.1 nm , $\epsilon$ = 840, $\lambda_{min}$ 255.4 nm , $\lambda_{max}$ 252.3 nm ). These three maxima are indicative of the peptide.

Stepwise synthesis of $(Lys)_5$ and $(Trp)_5$ on teflon The procedure described above was applied to the synthesis of the $(Lys)_5$- and $(Trp)_5$- oligonucleotide conjugates Fmoc-Lys-Boc-OPFp (31 8 mg, 50 0 $\mu$mole) or Fmoc-Trp-OPFp (29 6 mg, 50.0 $\mu$mole) was added to the teflon and dissolved in 700 $\mu$l of dry pyridine. The reaction mixture was stirred for at least 4 hours at room temperature The solution was pipetted off and the support was washed with DMF. Following, 20% piperidine/DMF was added and stirred at room temperature for 15 minutes to effect removal of the Fmoc group. The solution was removed and the support was washed with dry pyridine The previous addition step was repeated four times The final Fmoc group was left on.

The peptide synthesis mixture was pipetted off and the support was washed with dry acetonitrile. The teflon was placed in a DNA synthesis column The oligonucleotide was synthesised as described above. The terminal Fmoc group was removed by treating with 20% piperidine/DMF for 15 minutes The oligonucleotide was deprotected by treating with ethylenediamine/abs ethanol, 1/1 at 55°C for 1 hour. The Boc groups on the lysine conjugate were removed by treating with 90% TFA/ethanedithiol for 5 minutes[12]. The teflon was washed with acetonitrile and 20% $Et_3N$/DCM then again with acetonitrile. The conjugates were removed from the support by treating with sodium periodate as described above.

The DNA-$(Trp)_5$ product was purified by reverse phase HPLC to yield pure conjugate (1.8 O.D., 1.3% overall yield). HPLC conditions Conjugate retention time of 47 2 min , DNA retention time of 24 6 min., using the same HPLC column and conditions as above. U.V. data ($H_2O$) was obtained for the Trp conjugate $\lambda_{max}$ 256.0 nm with a shoulder at 280 nm $\lambda_{min}$ 235 nm

The DNA-$(Lys)_5$ product was purified by DEAE ion exchange HPLC to yield pure conjugate ( 12 O.D , 8.5% overall yield ) HPLC conditions: Conjugate retention time of 38 4 min , DNA retention time of 42 5 min , column, Protein-Pak, DEAE 5PW, Waters, analytical, gradient 0-100% of B in 100 minutes at a flow rate of 1 0 ml/min. Solvent A = 25 mM Tris pH 7 5, Solvent B = 20% acetonitrile in 25 mM Tris, 1 M NaCl pH 7.5. The conjugate was desalted on a sephadex G-25 Fine column U V. data ($H_2O$) was obtained for the Lys conjugate $\lambda_{max}$ 256 nm , $\lambda_{min}$ 237 nm. Amino acid analysis of this conjugate gave lysine and glycine peaks The glycine comes from hydrolysis of the oligonucleotide.

The DNA peptide conjugates were run beside the oligonucleotide itself on a 15% polyacrylamide gel containing 7 M urea (Figure 2) The Z-D-Phe-L-Phe-Gly conjugate and $(Trp)_5$ conjugate run slower than the oligonucleotide. The $(Lys)_5$ conjugate does not migrate on the gel due to the positively charged lysine residues. The DNA was visualized on the gel by U V. shadowing.

The conjugate was shown to be resistant to snake venom phosphodiesterase under conditions (0.8 O.D of oligonucleotide, 0.1 unit enzyme, 0.1M Tris-HCl pH 8 9, 0 1 M NaCl, 0.014 M $MgCl_2$, 37 °C, 1 hour) which severely degraded the unconjugated oligonucleotide (data not shown)

In conclusion the results describe a facile synthesis starting directly with commercially available reagents of oligonucleotide-peptide conjugates with potential biological activity against measles virus.

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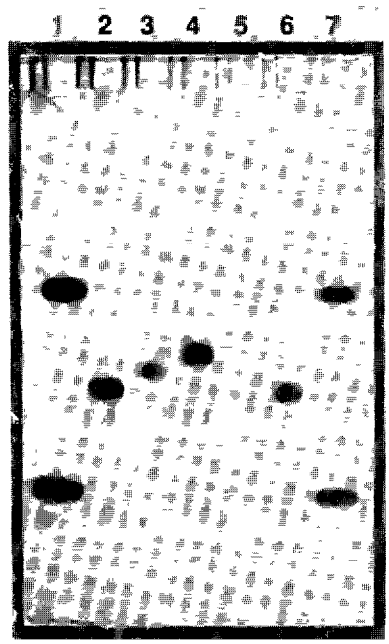


Figure 2.
Lane 1 + 7, Bromphenol Blue and Xylene Cyanol. Lane 2 + 6 oligonucleotide 17-mer. Lane 3 Z-D-Phe-L-Phe-Gly conjugate. Lane 4 $(Trp)_5$ conjugate. Lane 5 $(Lys)_5$ conjugate. 0.8 O.D. of sample loaded per well.

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