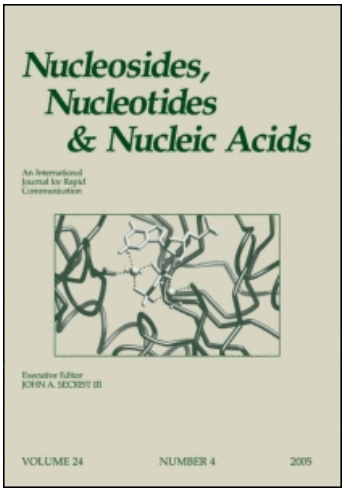

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SYNTHESIS OF PEPTIDE-OLIGONUCLEOTIDE PHOSPHOROTHIOATE CONJUGATES BY CONVERGENT OR STEPWISE SOLID-PHASE STRATEGIES

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ABSTRACT

A convergent strategy was employed to link eight 10–27-mer peptides to oligonucleotide phosphorothioates, resulting in twenty-six various conjugates. A stepwise synthesis strategy for the preparation of peptide-oligonucleotide phosphorothioate conjugates, employing Fmoc peptide chemistry, was developed and applied to the synthesis of four conjugates. Three of these conjugates contained either a 10 or 16-mer peptide, incorporating either 2 or 3 arginine residues, respectively.

INTRODUCTION

Three strategies for peptide-oligonucleotide conjugation have been reported so far: The convergent strategy (CS)—a post-synthetic conjugation of pre-purified oligonucleotide (ON) and a peptide, both bearing appropriate reactive groups (1–12); the stepwise synthesis strategy (SSS)—a stepwise solid-phase assembling of conjugates, requiring only one purification step upon final deprotection (13–15); and the mixed type strategy (MTS)—a fragment coupling of a pre-purified peptide, bearing an appropriate reactive group and the protected ON, also bearing an appropriate reactive group and attached to a solid support (16–18).

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While CS and MTS were applied for the preparation of relatively long peptides and ONs, the stepwise strategy has only a few examples, mainly with conjugates of very short peptides and short oligo-sequences. Moreover only a few amino-acid residues may be found in these short peptide sequences (13–15).

Undoubtedly, all three strategies have certain advantages and disadvantages. CS only needs a few changes in standard peptide and/or ON synthesis technologies. However, the typically modest conjugation yield is strongly dependant on the varying solubility of the peptides. This method always requires several purification steps of both peptides and ONs, and finally of the target conjugates. Conversly, SSS is typically characterized by a very high conjugation yield and a single purification step. Nevertheless, several changes in standard peptide synthesis technology are required. A limited choice of protecting groups for amino-acid derivatives that are suitable for the SSS and compatible with ON stability is a consideration. MTS, as with CS, also requires few changes in standard peptide and ON synthesis technologies. Moderate conjugation yields, the inevitable peptide purification procedures and varying solubilities of peptides may also be considered as disadvantages of this technology.

The objectives of our study were: in the area of CS—to develop a reliable procedure based on standard solid phase synthesis protocols that leads to conjugates of ONs with any peptide in reasonable yields; in the area of SSS—to develop appropriate protection groups for solid phase peptide synthesis (SPPS), that are compatible with ON stability.

RESULTS AND DISCUSSION

Convergent Strategy

A number of different procedures for convergent conjugation of peptides and ONs have been published (1–12,19,20). An approach utilizing a disulfide bond formation (19,20) is one of the possibilities used to achieve reasonable yields in the final synthetic step. In order to develop a method of cross-linking that is relatively independent of the molecular structures of peptides, the composition of the solvent mixture and concentrations of the reacting components must be optimized. After performing a number of experiments, we have found that the best yields were obtained when the resulting conjugation mixture contained about 90% of formamide, with the concentration of the peptides being about 0.1 mg/mL and the ONs about 0.6 OD/mL. In principle, there are at least three direct routes to generate a disulfide bond between an ON phosphorothioate 3′-tethered with a linker bearing a mercapto group and a synthetic peptide incorporating a thiol function at either the peptide's C-terminal cysteine or the mercaptoalkyl tether at the N-terminus:

R-SH	+	R′-SH	→	R-SS-R′	(Route A)
R-SS-Py	+	R′-SH	→	R-SS-R′	(Route B)
R-SH	+	R′-SS-Py	→	R-SS-R′	(Route C)

R = Oligo; R′ = Peptide; Py = pyridyl

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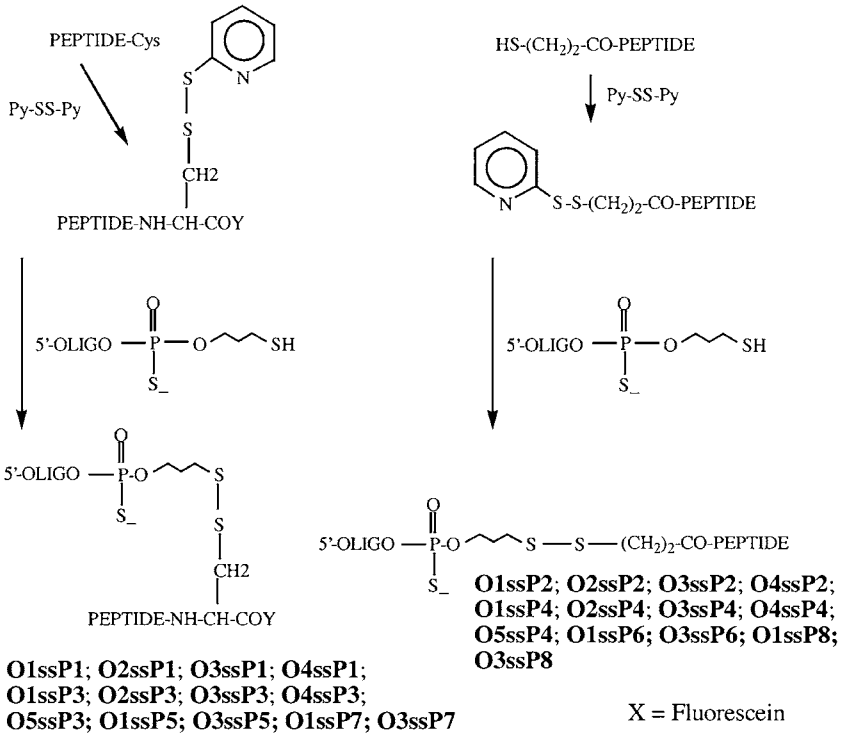
In order to compare these three methods we performed a relatively large number of conjugations. In the first set of experiments 15-mer ON phosphorothioate, bearing a mercaptoalkyl function at the 3′-terminus, was conjugated to 10–26mer peptides having either a mercaptopropionic acid at the N-end or a free thiol group of cystein at the C-end (Route A, typical yields 0.4–23%). In the second set, the same ON phosphorothioate, bearing a pyridyl disulfide tether, was reacted with the same peptides (Route B, yields typically 7–25%). And, finally, the third set, ON phosphorothioate, bearing a mercaptoalkyl function at the 3′-terminus, was linked to peptides having a pyridyl disulfide group at either the N or C-terminus (Route C, yields typically 36–88%). Route C proved to be most efficient (data not shown) and therefore we used this procedure for the preparation of a number of conjugates (Scheme 1).



OLIGO = 5'-TGGCGTCTTCCATTT-3 ' (**O1**) OLIGO = 5'-TTTACCTTCTGCGGT-3' (**O2**)

OLIGO = 5'-ACCGCAGAAGGTAAA-3' (**O3**) OLIGO = 5'-XTGGCGTCTTCCATTT-3 ' (**O4**)

OLIGO = 5'-TTTTGGCGTCTTCCATTTTACCAAC-3 ' (**O5**)

All oligonucleotides are phosphorothioates

Scheme 1.

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Table 1. Peptides Employed in the Convergent Strategy

For Conjugation of 3′-Terminus of ON and C-end of Peptide		For Conjugation of 3′-Terminus of ON and N-end of Peptide	
MPM-Cys-NH2	(**P1**)	HS(CH2)2CO- **MPM-NH2**	(**P2**)
MPM-NLS -Cys-OH	(**P3**)	HS(CH2)2CO- **MPM-NLS**	(**P4**)
NLS-Cys-OH	(**P5**)	HS(CH2)2CO- **NLS**	(**P6**)
TAT (free C-terminal Cys)	(**P7**)	HS(CH2)2CO- **TAT**(Acm) [C-terminal Cys(Acm)]	(**P8**)

MPM = 17-mer membrane permeable motif—the hydrophobic region of signal peptide sequence of the Kaposi fibroblast growth factor, known to interact with lipid bilayers—H-AlaAlaValAlaLeuLeuProAlaValLeuLeuAlaLeuLeuAlaPro-OH (21); **NLS** = 10-mer nuclear localization sequence of the transcription nuclear factor kB –H-ValGln-ArgLysArgGlnLys-LeuMetPro-OH (21); **MPM-NLS** = 27-mer hybrid peptide—H-AlaAlaValAlaLeuLeuProAla-ValLeuLeuAlaLeuLeuAlaProValGln-ArgLysArgGlnLys-LeuMetPro-OH (21); **TAT** = 14-mer truncated HIV-1 Tat protein basic domain-H-GlyArgLysLysArgArgGlnArgArgArgProPro-Gln-Cys-OH (22).
Acm = acetamidomethyl.

A total of 26 different conjugates (Scheme 1) of various 15–25-mer ON phosphorothioates (Scheme 1) and peptides (Table 1, Scheme 1) were synthesized. The isolated yields of conjugates were between 25–60%.

Our results (19,20) allow us to make the following conclusion: CS, based on our procedure to synthesize disulfide cross-linked conjugates, employs the standard solid phase synthesis protocols and leads to conjugates of ON phosphorothioates with virtually any peptide in reasonable yields. Nevertheless, this method demands several purification steps of both ON phosphorothioate and peptide derivatives before conjugation, as well as a final purification of conjugates after cross-linking. These multiple HPLC purifications seriously limit the number of conjugates available for biological testing and therefore, it appears that a fast and reliable stepwise synthesis strategy remains to be discovered. Such a procedure should allow the opportunity to synthesize a greater number of conjugates faster and easier.

Stepwise Synthesis Strategy

Before starting to develop any type of stepwise strategy one has to decide what type of synthetic approach (Fmoc-peptide chemistry *vs* Boc-peptide chemistry) is most appropriate for achieving this goal. Several considerations have to be taken into account:

– most of peptide synthesizers produced at present are designed for Fmoc-chemistry rather than Boc-chemistry;

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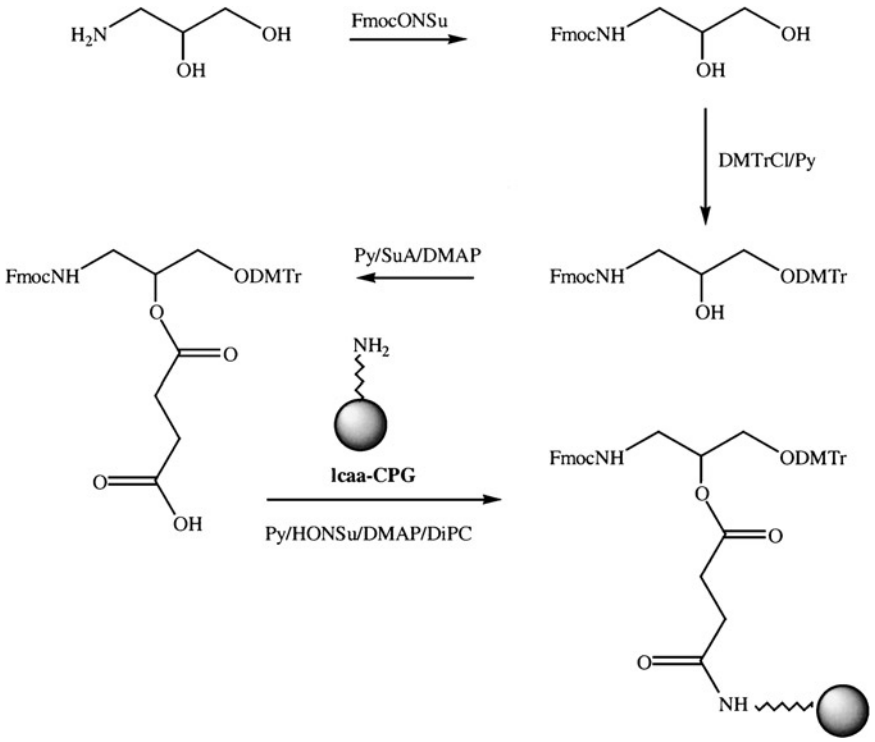
– some functional amino acid derivatives (e.g. BocArg(Ts)OH, BocSer-(Bn)OH, BocThr(Bn)OH, BocLys(Cbz)OH) in Boc-SPPS require a final HF deprotection, which is absolutely unacceptable for ONs;
– in Fmoc-SPPS (unlike Boc-SPPS), several amino acid derivatives with unprotected side chains may be employed.

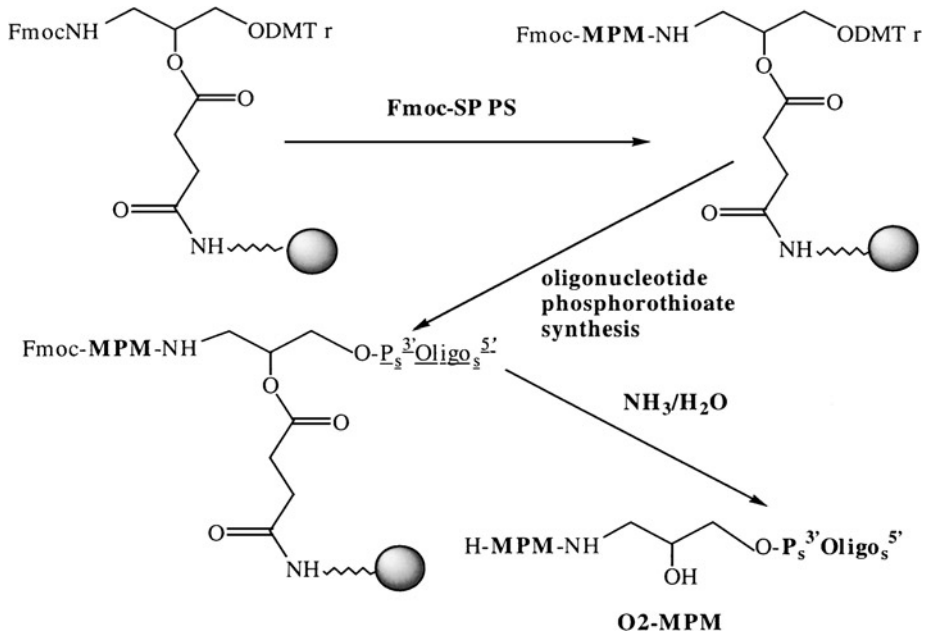
At present, Fmoc-SPPS seems to be more favorable. Nevertheless, a considerable number of synthetic manipulations are required to make Fmoc chemistry applicable for conjugate preparation. Standard Fmoc-SPPS involves a final acidic cleavage/deprotection step, which is unacceptable for ONs. A general method for the stepwise solid phase synthesis of conjugates requires:

– new solid supports, allowing a final basic (i.e. aqueous ammonia) cleavage/deprotection reaction;
– replacement of all amino-acid derivatives that incorporate acid-labile side chain protecting groups by their corresponding unprotected derivatives, or derivatives incorporating aqueous base-labile or silyl functional groups.

The design and synthesis of a new solid support, suitable for SSS, appears to be a simple task (23). Scheme 2 shows a route for preparing one of these supports,



Scheme 2.



Scheme 3. $\underline{^{3'}\text{Oligo}^{5'}}_{s}$ = protected oligonucleotide phosphorothioate; $^{3'}\mathbf{Oligo}_{s}^{5'}$ = deprotected oligonucleotide phosphorothioate.

starting from readily available and inexpensive (±)-3-amino-1,2-propanediol. In order to demonstrate its applicability for SSS, we have prepared the conjugate of a 15-mer oligonucleotide phosphorothioate with 16-mer **MPM** (Scheme 3). **MPM** was chosen because it is mostly composed of hydrophobic amino acids, which do not require side protection. Thus, **MPM** was first assembled following the standard Fmoc-SPPS protocol, followed by ON phosphorothioate synthesis. The desired conjugate was finally cleaved with aqueous ammonia and isolated by ion exchange HPLC, with a conjugate yield of 26% (23).

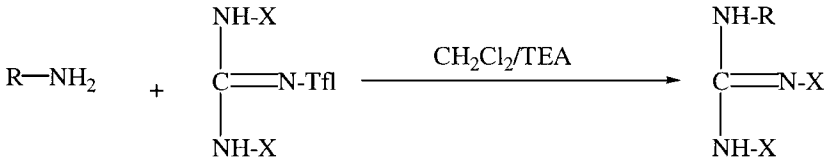
The second task—replacement of all acid labile side chain protecting groups appeared to be a more complex problem. Standard Fmoc-SPPS utilizes 10 building blocks, which have acid-labile protection groups on the side chains. SSS requires the substitution of these building blocks for the corresponding unprotected derivatives or building blocks incorporating aqueous base-labile or silyl functional groups. It is noteworthy that three of the required blocks are commercially available (FmocLys(Tfa)OH, FmocAsnOPfp, FmocGlnOPfp) and six are relatively easy to synthesize. A more significant problem exists with using arginine as a building block. In contrast to other amino-acid building blocks, a suitable protected arginine derivative is neither commercially available nor easy to prepare. An alternative solution would incorporate a suitable precursor building block that would be easily transformed to the corresponding arginine

derivative upon assembly of peptide. The building block to generate–Arg-residues must:

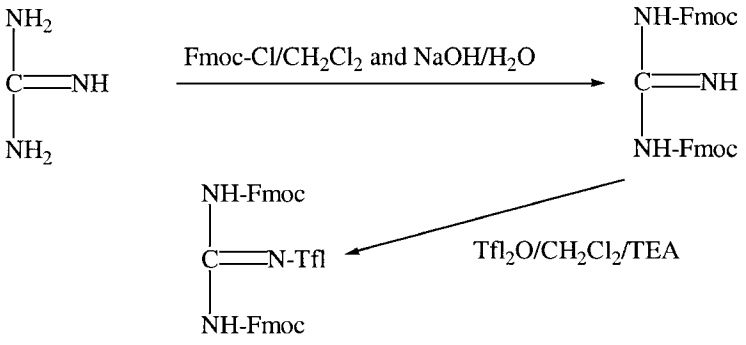
- give rise to a peptide having doubly protected guanidine functions of arginines in order to avoid by-products in the process of ON synthesis;
- result in arginine, doubly protected, that is easily cleavable upon the final treatment with aqueous ammonia.

Recently Goodman and co-workers have reported *N*,*N*′-di-Boc-*N*″-triflylguanidine and *N*,*N*′-di-Cbz-*N*″-triflylguanidine for the conversion of primary amines, amino-acids and short peptides into their corresponding guanidine-derivatives (24). In analogy to that we prepared di-Fmoc-guanidine-triflate (Scheme 4) (25).

This reagent was tested in the preparation of two model peptides, one having a single arginine residue and the second two residues (Scheme 5). In assembly of the peptides, an ornithine building block was used as a precursor to the arginine block. This commercial ornithine derivative was protected with 4-methyl-trityl group (Mtt) at the δ-amino group of side chain. After peptides were assembled on a commercial Wang resin using Fmoc chemistry, both resins were treated with 2% trifluoroacetic acid in dichloromethane to cleave the Mtt functions and the resulting amino-functions were modified with di-Fmoc-guanidine-triflate in the



X=Boc or Cbz; R=amino-acid derivative or short peptide with free NH_2 function



Scheme 4.

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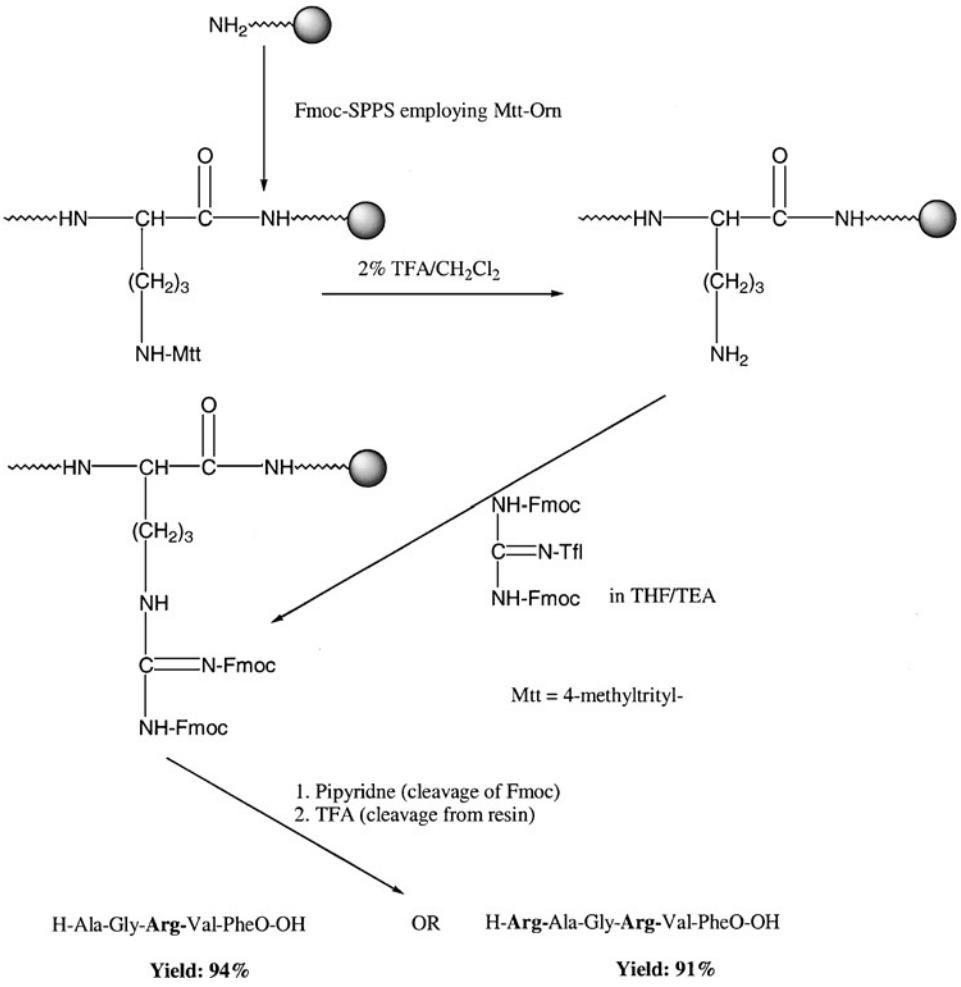
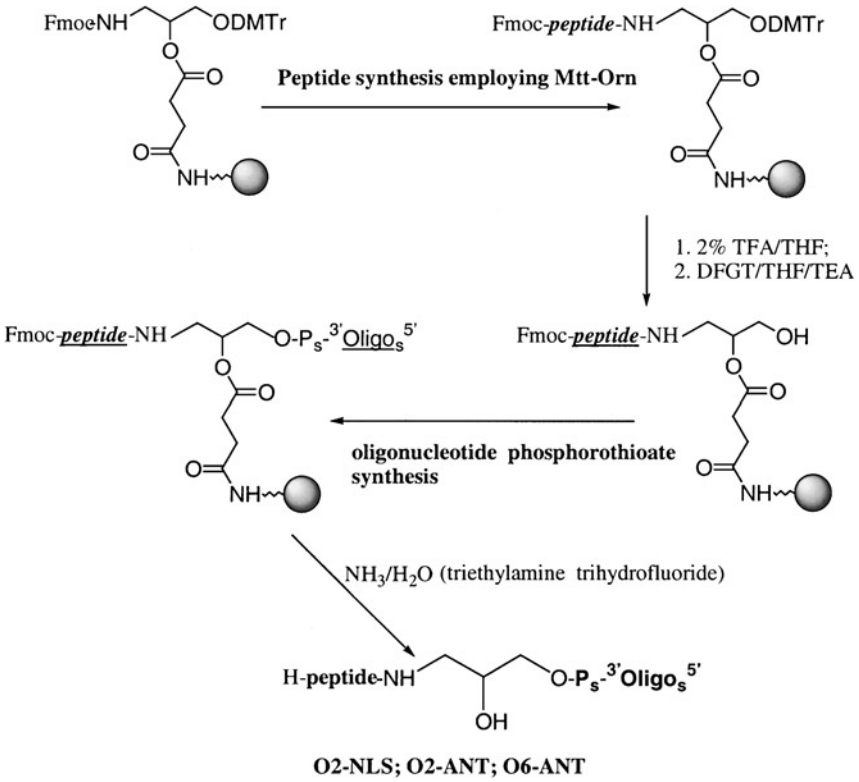


Scheme 5.

presence of triethylamine. Both peptides were obtained in good yield and were absolutely identical with authentic samples.

Our next step was to prepare conjugates containing either the 10-mer nuclear localization sequence peptide **NLS**, incorporating 2 arginines, 2 lysines and 1 glutamine or a 16-mer fragment (43–58) of homeodomain of Antennapedia (pAntennapedia) (**ANT**) (26), incorporating 3 arginines, 3 lysines, 2 glutamines and 1 asparagine. As with model peptides, we used an Mtt-ornitine building block in place of the arginines and assembled peptides on a solid support (Scheme 6). Mtt-groups were then cleaved and amino-groups of ornithine residues were converted to di-Fmoc guanidine derivatives of arginine residues by a reaction with di-Fmoc-guanidine-triflate in the presence of triethylamine. This step was then



Scheme 6. ***peptide*** = peptide containing Mtt-Orn residues; ***peptide*** = peptide containing $Fmoc_2$-Arg residues; **peptide** = peptide containing Arg residues; $^{3'}\underline{Oligo}_s^{5'}$ = protected oligonucleotide phosphoro thioate; $^{3'}\mathbf{Oligo}_s^{5'}$ = deprotected oligonucleotide phosphorothioate; **NLS**: H-Val-GlnArgLysArgGlnLy sLeuMetPro-OH;**ANT**:H-ArgGlnIleLysIleTrpPheGlnArgArgMetCysTrpLysLys-OH; $^{3'}\mathbf{Oligo}_s^{5'} = 3'$-TTTACCTTCTGCGGT-3′ phosphorothiate (**O2**); $^{3'}\mathbf{Oligo}_s^{5'} = 3'$-A<u>rU</u> TTTACCTTCTGCGGT-3′ phosphorothioate (**O6**); rU = ribouridine unit.

followed by ON phosphorothioate synthesis. After completion of the ON assembly, conjugates were cleaved and deprotected with aqueous ammonia. In the case of conjugate **06-ANT**, the 2′-*O*-TBDMS protection of the ribouridine unit was removed with triethylamine trihydrofluoride. Conjugates **O2-NLS**, **O2-ANT** and **O6-ANT** (Scheme 6) were isolated by anion exchange HPLC at 11–17% yield (25).

The final characterization of all conjugates was achieved by electrospray ionization mass spectrometry. Mass spectra were acquired and molecular weights were reconstructed as reported earlier (19,20). Both the measured and theoretically calculated average molecular weights of compounds reported here were in excellent agreement (Table 2). The difference between the calculated and measured molecular weights was always less than 3.1 mass units (0.05%).

Table 2. Purity, Measured and Theoretically Calculated Average Molecular Weights of Conjugates Synthesized

Oligonucleotide or Conjugate	Purity[a], %	Calculated Mass	Measured Mass
O1ssP1	100.0	6536.4	6533.3
O1ssP2	96.1	6521,4	6519.7
O1ssP3	98.5	7803.0	7800.5
O1ssP4	99.2	7788.0	7787.3
O2ssP1	97.3	6536.4	6534.5
O2ssP2	98.1	6521.4	6518.6
O2ssP3	98.3	7803.0	7801.8
O2ssP4	99.1	7788.0	7787.7
O3ssP1	95.2	6630.5	6627.5
O3ssP2	98.2	6615.5	6613.9
O3ssP3	99.5	7897.1	7896.9
O3ssP4	100.0	7881.5	7880.7
O4ssP1	96.9	7151.5	7151.7
O4ssP2	96.1	7138.5	7137.2
O4ssP3	97.2	8418.1	8418.0
O4ssP4	99.6	8405.1	8403.2
O5ssP3	98.5	10987.7	10986.5
O5ssP4	96.9	10974.7	10973.3
O1ssP5	97.5	6305.1	6304.5
O1ssP6	98.3	6290.1	6289.3
O1ssP7	96.4	6399.2	6398.5
O1ssP8	97.3	6384.2	6383.3
O3ssP5	98.3	6740.5	6739.7
O3ssP6	96.9	6856.6	6859.0
O3ssP7	97.2	6900.9	6899.0
O3ssP8	96.1	6994.9	6993.6
O2-MPM	97.1	6416,9	6416,3
O2-NLS	98.7	6184.6	6183.1
O2-ANT	96.2	7147.7	7145.2
O6-ANT	95.4	7783.2	7783.5

[a]As assessed from PR HPLC by peak integration at 260 nm.

CONCLUSION

Convergent strategy, based on a disulfide exchange reaction between an oligonucleotide phosphorothioate tethered with a mercaptoalkyl group and a peptide bearing a pyridyl disulfide function in 90% formamide will give rise to virtually any conjugate with a reasonable yield. Nevertheless, several laborious purification procedures of modified ON, the reacting peptide and finally the desired conjugate appear to be inevitable. This fact seriously limits the possibility to conduct systematic studies on cell delivery of antisense ONs conjugated to certain peptides. The stepwise synthesis strategy seems to be an appropriate chemistry for the above-mentioned purpose. Our new procedure is a straightforward and fast method

to assemble conjugates and employs only a single purification step. Fine-tuning of this method is still needed in order to improve the yield and further simplify isolation of the target compounds.

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