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Stepwise solid-phase synthesis of peptide–oligonucleotide phosphorothioate conjugates employing Fmoc peptide chemistry

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Abstract

A straightforward stepwise method for the preparation of peptide–oligonucleotide phosphorothioate conjugates, was developed, based on the highly efficient Fmoc peptide solid phase synthesis, followed by oligonucleotide phosphorothiate chain assembly. The three conjugates synthesized contained 15- or 17-mer oligonucleotide phosphorothioates and 10- or 16-mer peptides, incorporating two or three arginine residues.

Recently we[1] described the synthesis of a peptide–oligonucleotide phosphorothioate conjugate (POPC) on a solid support, designed to link the 3′-terminus of the 15-mer oligonucleotide phosphorothioate to the C-terminus of a 16-mer membrane permeable motif (H-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-OH, **MPM**) via a phosphorothioate bond. The Fmoc solid-phase peptide synthesis (Fmoc-SPPS) was followed by the standard oligonucleotide phosphorothioate assembly on the same solid support. The final cleavage, deprotection and purification gave the resulting POPC. This demonstrated that a facile method for the preparation of peptide–oligonucleotide phosphorothioate conjugates (POPCs) might be based on the highly efficient Fmoc-SPPS followed by oligonucleotide (ON) chain assembly–stepwise solid-phase peptide–oligonucleotide conjugate synthesis (SSPP-OCS).

In the case of **MPM**, the peptide sequence contained amino-acid residues that do not require side chain protection in the process of assembly. Moreover, peptides incorporating building blocks that require such protection must be assembled by using N_{α}-Fmoc amino acids with base labile protection of the side chains. Unfortunately, the Fmoc-SPPS involves a final acidic deprotection step, which is unacceptable for ONs. Therefore, in order to develop a general method for the preparation of POPCs, all acid labile side chain protection groups must be replaced by their corresponding base labile functions. Such base labile protection groups must

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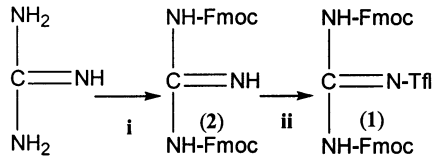
be either stable in the process of the Fmoc-SPPS or introduced right after completion of peptide assembling and before ON synthesis. In contrast to several other amino-acid building blocks (e.g. base labile protection groups for the side chains of lysine, aspartic acid, glutamic acid, etc.), which must be adopted for SSPP-OCS, a suitable protected arginine derivative is neither commercially available nor easy to prepare. The building block in question must give rise to a peptide containing doubly protected guanine functions of arginines. Such specific base labile groups are needed in order to avoid by-products in the process of ON synthesis. In addition, these groups must be reasonably stable in the process of ON assembly and easily cleaved upon final deprotection with aqueous ammonia.

The objective of the present work was to employ SSPP-OCS for the synthesis of POPCs containing 2 or 3 arginine residues.

Previously Goodman and co-workers[2] reported on the preparation of *N*,*N*′-di-Boc-guanidine-*N*″-triflate and *N*,*N*′-di-Cbz-guanidine-*N*″-triflate in the transformation of ornithine residues into arginine within short peptides. Similarly we prepared *N*,*N*′-di-Fmoc-guanidine-*N*″-triflate (**1**) starting from guanidine (Scheme 1). Shaking a solution of guanidine nitrate (1 equiv.) and sodium hydroxide (25 equiv.) in water with a solution of Fmoc chloride (3 equiv.) in dichloromethane for 3 h at 20°C gave *N*,*N*′-di-Fmoc-guanidine (**2**).[3] Finally, treatment of **2** with triflic anhydride (1.1 equiv.) in the presence of triethylamine (1 equiv.) in dichloromethane (10 min at –40°C followed by 3 h at 20°C) gave compound **1**[4] after purification by silica gel chromatography and crystallization from hexane.

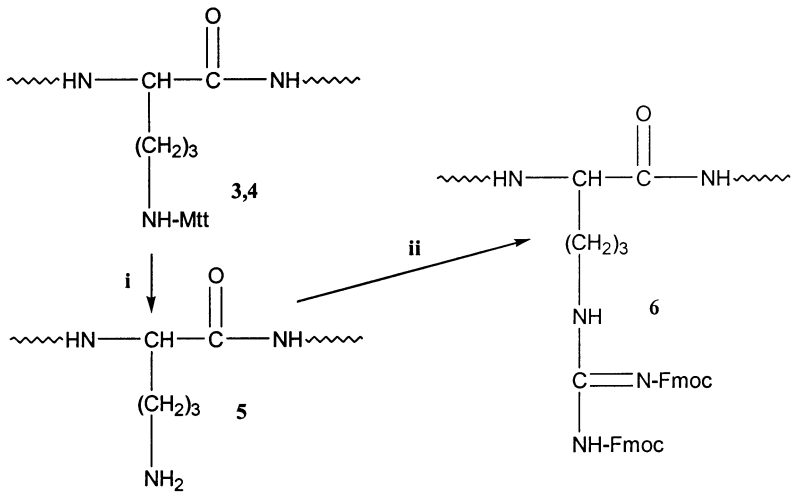


Scheme 1. (i) Fmoc-Cl/CH_2Cl_2 and NaOH/H_2O; (ii) Tfl_2O/CH_2Cl_2/TEA

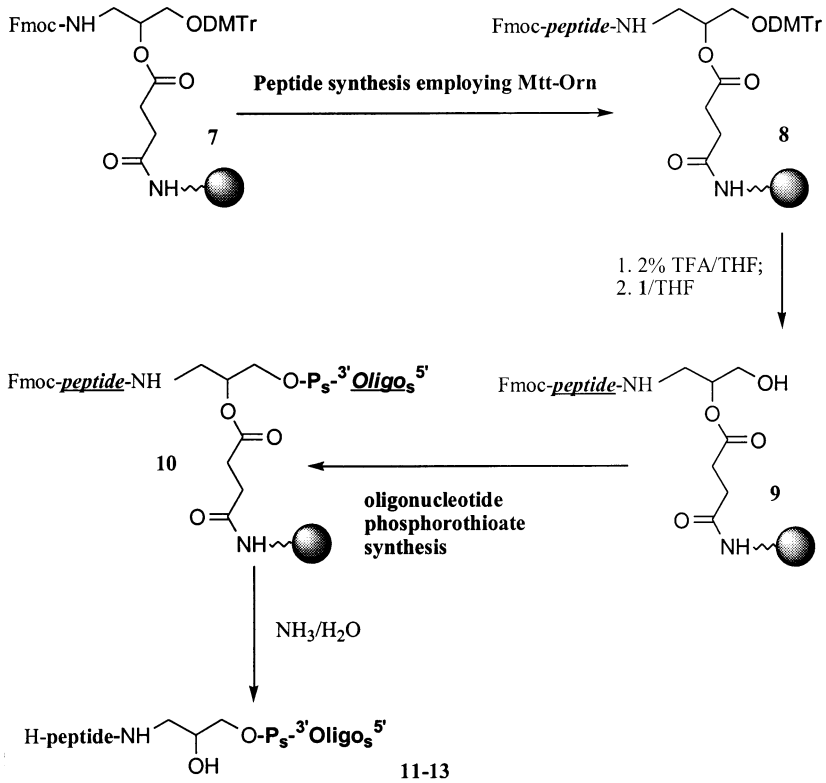
In order to demonstrate the applicability of **1** in the post-SPPS transformation of -Orn- residues to -Arg-, the two model peptide resins, -Fmoc-AlaGlyOrn(Mtt)ValPhe-O- resin (**3**) and Fmoc-Orn(Mtt)AlaGlyOrn(Mtt)ValPhe-O- resin (**4**) were assembled on the Wang solid support by using standard Fmoc/2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/triethylamine (TEA) chemistry. Peptide resins **3** and **4** were treated with 2% TFA in dichloromethane for 5 min to cleave Mtt groups (Scheme 2, structure **5**), washed with dichloromethane and 3% TEA in dichloromethane, then dried and agitated overnight in a solution of **1** in THF (10 equiv./1 equiv. amino-group) in the presence of TEA (1 equiv.) to give peptide resins **6** (Scheme 2).

Finally, both resins were filtered, α-amino groups were deprotected with 20% piperidine in DMF, and the resulting peptides were cleaved with TFA and purified by RP HPLC. The yield of these peptides was in the range 90–95%. They appeared to be identical to peptides H-AlaGlyArgValPhe-OH and H-ArgAlaGlyArgValPhe-OH obtained by standard SPPS. Their structures were confirmed by ESI-MS.

Scheme 3 demonstrates the preparation of POPCs using our SSPP-OCS methodology. The required peptides, **NLS** (nuclear localization sequence)[5] and **ANT** (pAntennapedia 43–58)[6] were assembled on the support **7**[1] by using the Fmoc-SPPS and employing usual N_α-Fmoc amino-acids, but with Fmoc-Orn(Mtt)-OH as a precursor of -Arg- and Fmoc-Lys(Tfa)-OH for the

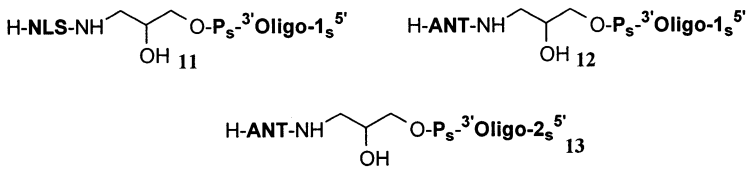


Scheme 2. (i) 2% TFA/CH_2Cl_2; (ii) **1**/THF/TEA



Scheme 3. ***peptide*** = peptide with Mtt-Orn residues; ***peptide*** = peptide with $Fmoc_2$-Arg residues; **peptide** = deprotected peptide with Arg residues; $^{3'}\underline{\mathbf{Oligo}}_{\mathbf{s}}{}^{5'}$ = protected oligonucleotide phosphorothioate; $^{3'}\mathbf{Oligo}_{\mathbf{s}}{}^{5'}$ = deprotected oligonucleotide phosphorothioate

introduction of the -Lys- residue. Solid supports of **8** containing assembled peptides, were deprotected and modified with triflate **1** (10 equiv./1 equiv. of amino-group resulting from -Orn-) as described above to give **9**. Finally, ON phosphorothioates were assembled (**10**), and POPCs **11**–**13** (Schemes 3 and 4) were cleaved from the support and deprotected with aqueous ammonia in the usual manner. In the case of conjugate **13**, the 2′-*O*-TBDMS protection of the *ribo*-uridine unit was removed by triethylamine trihydrofluoride. Conjugates **11**–**13** (Scheme 4) were isolated by anion exchange HPLC.[1] The chromatographic profile of crude **13** (product peak at t_R 13.92 min) is shown in Fig. 1 as an illustrative example. After purification by RP HPLC[1] and desalting the purity of POPCs was higher than 95%, as assessed by RP HPLC. Final characterization was made by ESI-MS (Table 1). The measured and calculated average molecular masses of conjugates were in good agreement, with the difference between the calculated and measured M_r being less than 0.03%.



Scheme 4. **NLS** = -ValGlnArgLysArgGlnLysLeuMetPro-; **ANT** = -ArgGlnIleLysIleTrpPheGlnAsnArgArgMetLys-TrpLysLys- ; $^{3'}$**Oligo-1**$_s^{5'}$ = oligonucleotide phosphorothioate 3′-TTTACCTTCTGCGGT-5′; $^{3'}$**Oligo-2**$_s^{5'}$ = oligonucleotide phosphorothioate 3′-ArUTTTACCTTCTGCGGT-5′

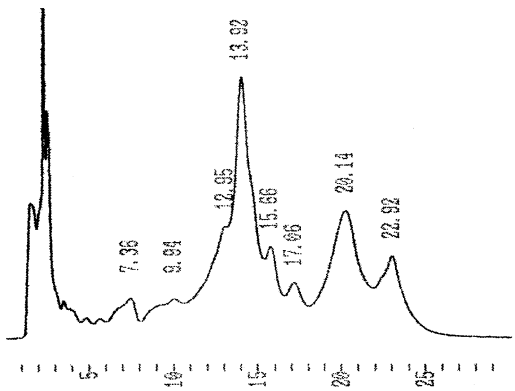


Figure 1. Ion-exchange HPLC traces of crude conjugate **13**

Table 1
Yields with measured and theoretically calculated average molecular masses of synthesized conjugates

Conjugate	Isolated yield (%)	Calculated mass	Measured mass (M_r)
11	16.1	6184.6	6183.1
12	13.2	7147.7	7145.2
13	11.2	7783.2	7783.5

Acknowledgements

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References

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2. Feichtinger, K.; Zapf, C.; Sings, H. L.; Goodman, M. *J. Org. Chem.* **1988**, *63*, 3804–3805.
3. Compound **2**: 64% as white crystals; mp 141–142°C; ^{1}H NMR ($CDCl_3$): δ 7.77–7.26 (m, 16H, arom.); 4.40 (d, $J=7.33$, 4H, CH_2); 4.23 (t, $J=7.24$, CH). Found: C, 74.51; H, 7.21; N, 7.15. Calcd for $C_{37}H_{43}N_3O_4$: C, 74.84; H, 7.30; N, 7.08; O, 10.78%.
4. Compound **1**: 71% as white crystals; mp 149–151°C; ^{1}H NMR ($CDCl_3$): δ 10.42 (br s, 2H, NH); 7.79–7.25 (m, 16H, arom.); 4.54 (d, $J=7.28$, 4H, CH_2); 4.28 (t, $J=7.23$, CH). Found: C, 62.55; H, 5.69; N, 5.86. Calcd for $C_{38}H_{42}F_3N_3O_6S$: C, 62.88; H, 5.83; F, 7.85; N, 5.79; O, 13.23; S, 4.42%.
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