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Towards a general method for the stepwise solid-phase synthesis of peptide–oligonucleotide conjugates

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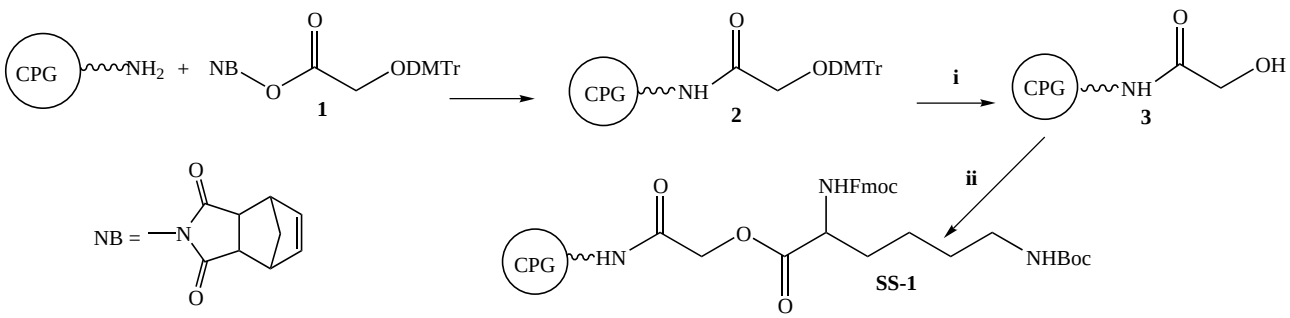
Abstract—Three peptide–oligonucleotide phosphorothioate hybrids were synthesized using a new approach for stepwise solid-phase synthesis of conjugates. This method utilizes commercially available N_α-Fmoc amino acids for peptide synthesis and a new solid support. Three specific modifications of the solid-phase were made after the peptide and before the oligonucleotide assembly.

No general method for the stepwise synthesis of any peptide–oligonucleotide phosphorothioate conjugate (POPC) on a single solid support has been reported so far.[1] The sequential assembly leads to a limited number of POPCs due to the incompatibility of the peptide (P) and the oligonucleotide (ON) protecting groups. As a result, the use of various base-labile peptide side-chain protecting groups have been reported.[2–7]

Previously we[8] described the synthesis of three conjugates containing 10- or 16-mer Ps, which incorporated two or three arginine residues. The required Ps were assembled on the support by using common N_α-Fmoc amino acids, but with Fmoc-Orn(Mtt)-OH as a precursor of -Arg-. Solid phases were then modified to offer doubly protected guanidine functions of arginines. Finally, ONs were assembled and POPCs were cleaved from the support and deprotected with aqueous ammonia.

Described herein, is a procedure for further developing a general method for the stepwise solid-phase synthesis of POPCs. This procedure also uses commercially available N_α-Fmoc amino acids for P synthesis along with certain specific modifications after the P and before the ON assembly.

In this work a suitable solid support was prepared, containing a Fmoc-protected amino-function for easy standard peptide chain elongation, a Boc-protected amino function, which was stable during the process of peptide synthesis but easily removable for subsequent modification and, finally, a base-labile bridge to a solid matrix–long chain alkylamino controlled pore glass (lcaa-CPG). The synthesis of solid support **SS-1** is outlined in Scheme 1. Initially, a long chain alkylamino controlled pore glass (CPG) was derivatized by the *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboxamide ester of 2-(4′,4″-dimethoxytriphenylmethyloxy) acetic acid



Scheme 1. (i) DCA; (ii) Fmoc-Lys(Boc)-OH/TPS-Cl.

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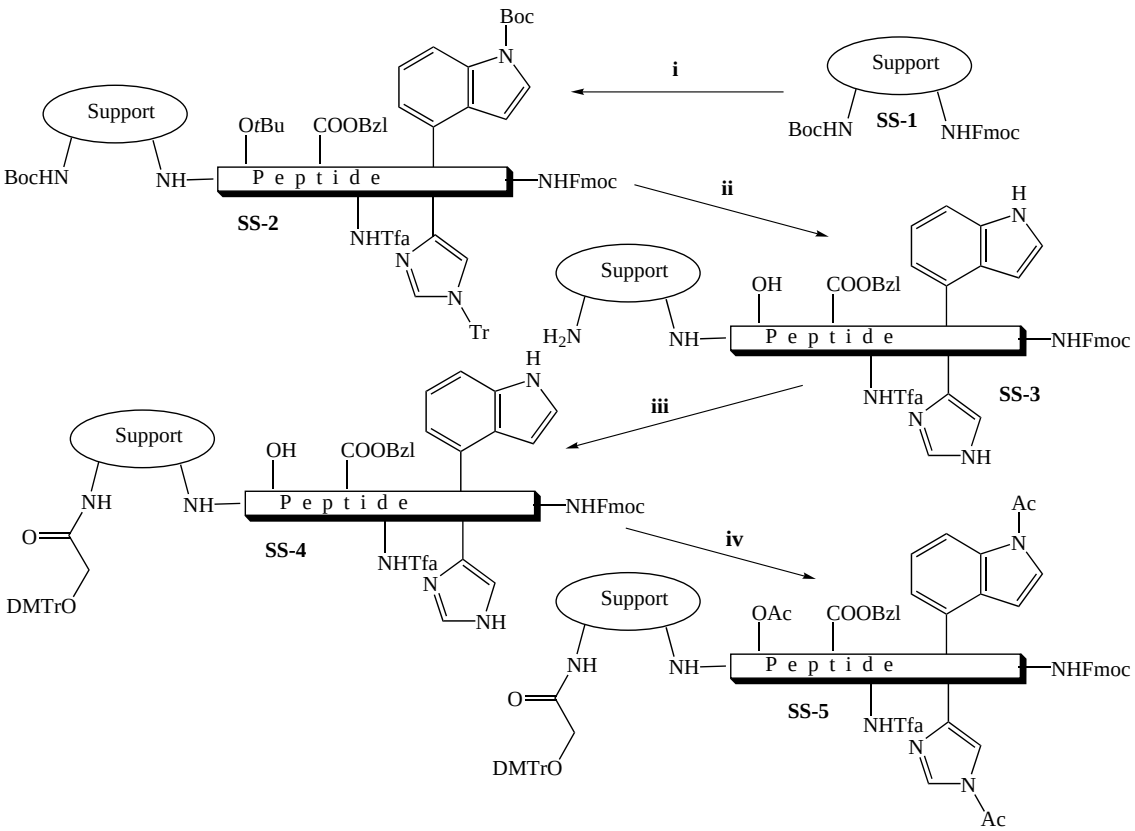
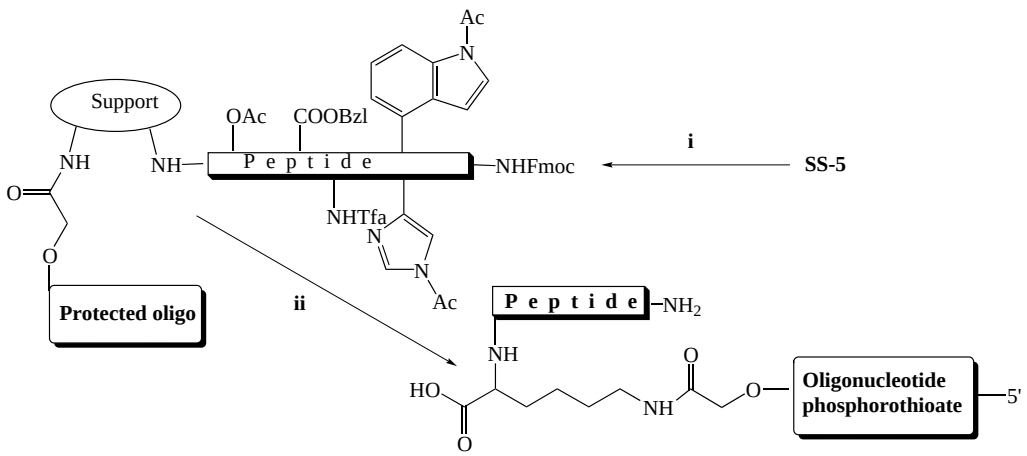
(**1**)[9] to give matrix **2**. After the removal of the DMTr group with 2% dichloroacetic acid (DCA) in dichloromethane, Fmoc-Lys(Boc)-OH was linked to the matrix **3** in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) to give solid support **SS-1**. The resulting **SS-1** contained 35 μmol of Fmoc-groups per gram of CPG.[10]

Scheme 2 demonstrates the preparation of solid support **SS-5**, incorporating the required P with base-labile side-chain protecting groups and a linker ready for ON assembly. In this procedure, standard Fmoc-peptide chemistry and commercially available N_α-Fmoc amino acids[11] were employed to assemble two Ps on our initial **SS-1** matrix to give phases **SS-2**. These Ps were either H-Met-Tyr-Ile-Glu-Ala-Leu-Asp-Lys-Tyr-Ala-Cys-(Acm)-OH (**P-1**) or (H-Met-His-Ile-Glu-Ser-Leu-Asp-Ser-Tyr-Thr-Cys(Acm)-OH (**P-2**). After the P assembly, all acid-labile protecting groups were removed from Ps, attached to the solid-phase, which resulted in **SS-3** supports.



Scheme 2. (i) Standard Fmoc-peptide synthesis; (ii) 40% TFA; (iii) **1**; (iv) Ac_2O/*N*-methylimidazole/2,6-lutidine.



Scheme 3. (i) Standard oligonucleotide phosphorothioate synthesis; (ii) 0.4 M NaOH, rt, 17 h.

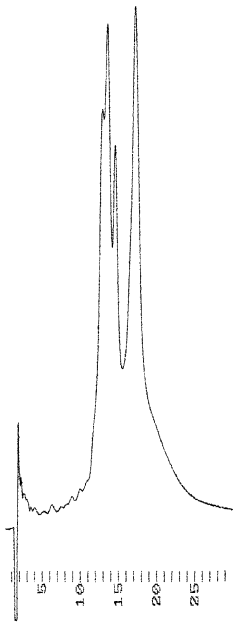


Figure 1. Ion-exchange HPLC traces of crude conjugate **O2-P1**.

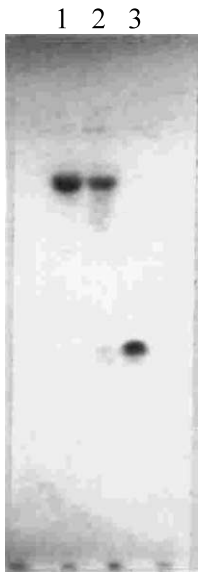


Figure 2. Photograph of 15% denaturing PAGE. Lane 1, **O2-P1**; lane 2, contaminated **O2-P1**; lane 3, **O2**.

Table 1. Measured and theoretically calculated average molecular masses of synthesized conjugates.

Conjugate	Calculated mass	Measured mass (M_r)
O1-P1	6404.8	6403.1
O2-P1	8067.1	8067.3
O2-P2	8046.0	8044.8

The primary amino group of **SS-3** was then selectively derivatized with compound **1** to give **SS-4**, incorporating a DMTr-oxy function. The remaining unprotected side-chain functional groups were finally acetylated to give rise to **SS-5** supports, ready for ON assembly. Both **SS-5** matrices contained 32 μmol of DMTr groups per gram of CPG.[12]

ON phosphorothioates (5′-TGGCGTCTTCCATTT-3′, **O-1** and 5′-TATGATCTGTCACAGCTTGA-3′, **O-2**) were assembled on **SS-5** supports using standard protocols (Scheme 3). Protected POPCs were cleaved from the support and deprotected with aqueous sodium hydroxide to give rise to the crude conjugates, **O-1-P-1**, **O-2-P-1** and **O-2-P-2**. The chromatographic profile of crude desalted **O-2-P-1** (product peak at t_R about 18 min) is shown in Fig. 1 as an illustrative example. After purification by IE HPLC[7] and desalting, the purity of the POPCs was higher than 95%, as determined by RP HPLC. Typically 20–30 AU of pure POPCs were obtained when starting from 1 μmol of **SS-5** solid supports. Fig. 2 demonstrates a typical 15% PAGE of conjugate **O-2-P-1** and oligomer **O-2** as an illustrative example. In all cases, POPCs migrated considerably slower than the corresponding ONs. Final characterization was made by ESI-MS (Table 1). The measured and calculated average molecular masses of the conjugates were in good agreement, with the difference between the calculated and measured M_r being less than 0.03%.

Acknowledgements

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9. Glycolic acid (22 mmol, 1.67 g) was dissolved in 100 ml of dry pyridine. DMTr-Cl (20 mmol, 6.76 g), dissolved in 50 ml of dry THF, was added and the mixture was stirred at room temperature overnight. The reaction was quenched by the addition of ice. The mixture was concentrated, dissolved in ethyl acetate and extracted with water. The organic layer was dried, concentrated and

finally purified by flash chromatography on silica gel. Concentration in vacuo gave 5.88 g (78%) of a pale yellow oil. This oil (11 mmol, 4.4 g) and *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboxamide (1.97 g, 11 mmol) were dissolved in 50 ml of dry acetonitrile, the solution was cooled down to −20°C and a solution of *N*,*N*′-dicyclohexylcarbodiimide (11 mmol, 2.31 g) in 10 ml of acetonitrile was added. The reaction mixture was left at +4°C overnight. Solids were filtered off and washed with cold acetonitrile. The filtrate and washings were combined and evaporated to dryness. Crystallization of the residue from 2-propanol gave 3.8 g (65%) of **1** as white crystals; mp 52–54°C; ^{1}H NMR (CD_3CN): δ 7.43–6.85 (m, 13H, arom.); 6.11 (s, 2H, CH_a=CH_b); 4.04 (s, 2H, Ch_aH_bCO); 3.75 (s, 6H, 2×$CH_3OC_6H_4$); 3.34 (s, 2H, 2×COC*H*); 3.32 (s, 2H, 2×C*H*-CH=CH); 1.66 (d, 1H, J=8.85, (CH-Ch_aH_b-CH); 1.51 (d, 1H, J=8.85, (CH-Ch_aH_b-CH). Anal. calcd for $C_{32}H_{29}NO_7$: C, 71.23; H, 5.42; N, 2.60; O, 20.76. Found: C, 71.49; H, 5.51; N, 2.32%.

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