Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 18, pp. 2247-2252, 1996
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0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00408-8

UNIVERSAL POLYMER SUPPORT FOR THE SYNTHESIS OF 3'-END MODIFIED OLIGONUCLEOTIDES

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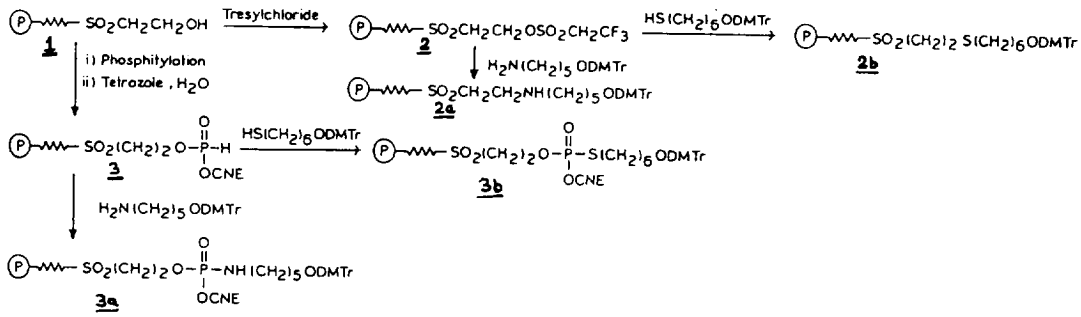
Abstract: **Functionalization of universal polymer supports is described for the synthesis of oligonucleotides bearing different functionalities (mercaptoalkyl or aminoalkyl) at 3'-end during final deprotection**. Copyright © 1996 Elsevier Science Ltd

Non-radiolabelled oligonucleotides have become vital tools in molecular biological studies. Looking to the increasing importance of these molecules, a large number of chemical methods have been developed for their preparation. 5'-Modified oligos are relatively simple to synthesize either by first introducing a suitable functional group to the oligonucleotide, which is then coupled to a ligand of choice or ligand itself is first modified and incorporated in oligonucleotide chain as last coupling.

Unlike 5'-end modifications, 3'-end modifications are not that straightforward. Since 3'-hydroxyl group of the leader nucleoside is not available for manipulation during solid phase synthesis of oligonucleotides, as it is attached to the polymer support via its 3'-hydroxyl group. Therefore, modified supports have been designed to incorporate modifications at the 3'-termini of oligonucleotides. A number of such supports have been described from various research groups (1-3). However, one still requires different polymer supports to introduce different functionalities at 3'-termini of oligonucleotides. In the present investigation (4), universal polymeric support has been designed which, upon little modification, leads to polymer supports required for the synthesis of oligonucleotides bearing different functionalities at their 3'-termini. The derivatized supports are found to be fully compatible with the established phosphoramidite chemistry of oligonucleotide synthesis, the method of choice nowadays. The modified oligos synthesized using these supports and their appropriate fluorescent-conjugates were characterized by reversed phase HPLC.

The functionalization of universal polymer supports is described in scheme-1. The support 1 having terminal hydroxyethyl groups (~35 μmol/g) was functionalized following the published procedure (5). The activation of polymer support 1 was carried out in two ways, viz., tresyl chloride method (6) and phosphonate approach (7), as shown in the scheme-1. In tresyl chloride method, the support 1 was treated with a large excess of tresyl chloride in acetonitrile in the presence of pyridine. The excess reagent was removed and the activated support 2 was reacted with a large excess of 1-O-(4,4'-dimethoxytrityl)-5-aminopentanol (8a) or 1-O-(4,4'-dimethoxytrityl)-3-mercaptopropanol (8b) to obtain the derivatized support 2a (26μmol /g support) or support 2b (24 μmol / g support). The resultant support



Scheme : Preparation of Polymer Supports for 3'-Amino and Mercaptoalkylated Oligonucleotides

was washed, dried under high vacuum and the residual hydroxyethyl groups, if any, were capped following standard procedure (8). The support **1** was activated by an alternative procedure, phosphonate method. In this approach the support **1** was treated with a large excess of 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite in acetonitrile in the presence of 0.5 equivalent of tetrazole and water to give rise to polymer supported H-phosphonate **3**, which in the subsequent reaction with 1-O-(4,4'-dimethoxytrityl)-5-aminopentanol or 1-O-(4,4'-dimethoxytrityl)-3-mercaptopropanol in carbontetra-chloride generated the fully functionalized polymer support **3a** (27 μmol/g support) or **3b** (25μmol/g support). Other steps, viz., washings and capping were performed as described in case of tresyl chloride method.

The utility of the supports **2a** and **3a** for the synthesis of 3'-aminoalkyl group containing oligos and **2b** and **3b** for the synthesis of 3'-mercaptoalkyl group containing oligos were established by synthesizing a number of oligonucleotides. In a typical experiment following sequences were synthesized on the supports shown against each sequence. d(CTC TTC CGC T) **3b**, d(ACT CTT CT) **2b**, d(CTC TCT CTC T) **3a**, d(TTT TT) **3a** , d(TTT TT) **2a**. All the syntheses were performed at 0.2 μmol scale following standard protocol supplied with the Pharmacia Gene Assembler Plus. The coupling efficiency per cycle based on released 4,4'-dimethoxytrityl was found to be >98% in each oligonucleotide synthesis.

In order to establish the deprotection conditions, the polymer supports **2a** and **3a** were subjected to aq. ammonia (29%) treatment in sealed vials at 55°C for 16h. The resultant polymer supports after washing with methanol and diethyl ether were subjected to loading analysis following standard procedure (9). Both of the supports were found to have lost aminopentyl linker as evident by trityl negative test. Because of the lability of sulfone ($-SO_2-$) linker under basic conditions (3c), aq. ammonia treatment was used for the deprotection of 3'-aminoalkyl containing oligonucleotides from these supports. In case of supports **2b** and **3b**, it was necessary to use ammonical DTT treatment to prevent

the reaction of mercaptoalkyl groups with acrylonitrile released from phosphate functions. Hence, 3'-modified-oligonucleotides were completely deprotected by a single step process.

The cleaved oligonucleotides were desalted and purified on reversed phase HPLC. In order to ascertain that the supports **2a** and **3a** generate the same sequence, d(TTT TT) was assembled on both the supports and deprotection was carried out under identical conditions. The deprotected oligomers were analyzed separately and finally co-injected on HPLC, they were found to be identical in their retention times. 3'-Modified oligomers were then suitably labelled with appropriate fluorephore and analyzed on HPLC. Purified aminoalkylated-d(TTT TT) synthesized on the support **2a** was labelled with dansyl chloride. Figure 1 shows the HPLC profile of a mixture of dansylated oligomer and the corresponding aminoalkylated oligomer. Similarly Fig. 2 shows the co-injection of FITC labelled d(CTC TCT CTC T) synthesized on support **3a** and the corresponding aminoalkylated oligomer. Aminoalkylated d(TTT TT) synthesized on **3a** was labelled with biotin and purified on HPLC and Fig. 3 shows the HPLC profile of biotinylated oligomer co-injected with the corresponding aminoalkylated oligomer. The oligomer d(CTC TTC CGC T) synthesized on the support **3b** was labelled with 1,5-I-AEDANS. Figure 4 shows the HPLC pattern of labelled and the corresponding mercaptoalkylated oligomers. Mercaptoalkylated oligomer d(ACT CTT CT) was synthesized on the support **2b**, labelled with 1,5-I-AEDANS and HPLC profile of the mixture of labelled and corresponding mercaptoalkylated oligomer is shown in Fig. 5.

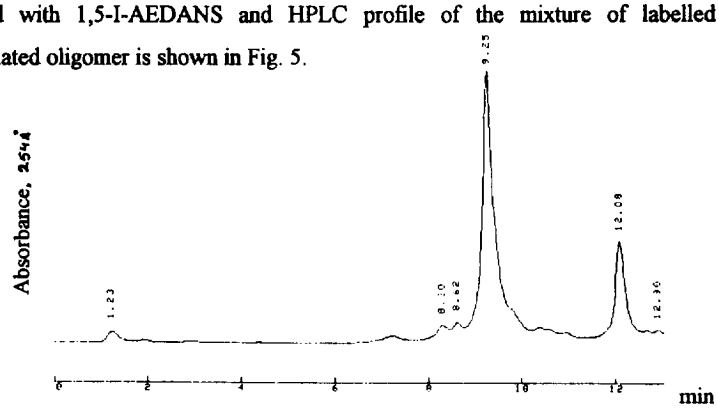


Fig. 1. HPLC profile of a mixture of 3'-aminoalkylated d(TTT TT) and dansylated d(TTT TT). Column : Lichrosphere RP-18; Buffer A = 0.1M ammonium acetate, solvent B = Acetonitrile; Gradient : 0-25%B in 15 min.

EXPERIMENTAL

Functionalization of polymer support 1

The support **1** was functionalized following the procedure published earlier (5) with terminal hydroxyethyl groups loading ~35 μmol / g support.

Preparation of supports 2a and 2b

Support **1** (100 mg, containing 3.5 μmol hydroxyethyl groups) was suspended in dry acetonitrile (0.5 ml) and pyridine (100 μl) in a reaction vial sealed with a rubber septum. Tresyl chloride (50 μl) was taken in a glass

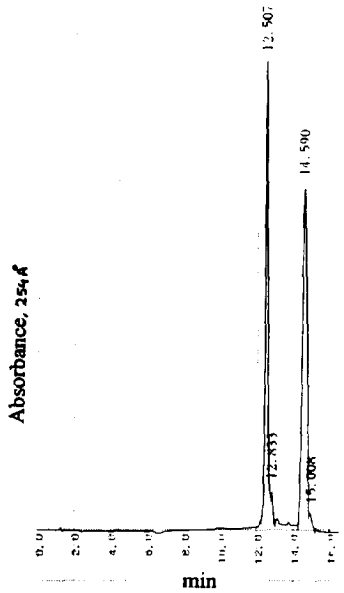


Fig. 2. HPLC profile of a mixture of 3'-aminoalkylated d(CTC TCT CTC T) and FITC labelled oligomer. Column: Lichrosphere RP-18; Buffer A = 0.1M ammonium acetate, solvent B = Acetonitrile; Gradient : 0-30%B in 20 min.

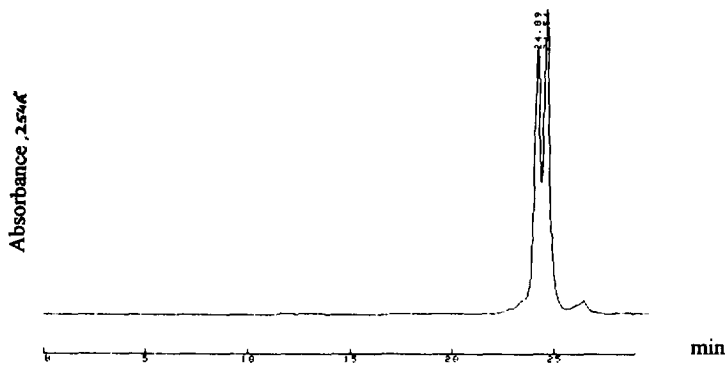


Fig. 3. HPLC profile of a mixture of 3'-aminoalkylated d(TTT TT) and biotinylated oligomer. Column : Lichrosphere RP-18; Buffer A = 0.1M ammonium acetate, solvent B = Acetonitrile; Gradient : 0-15%B in 30 min.

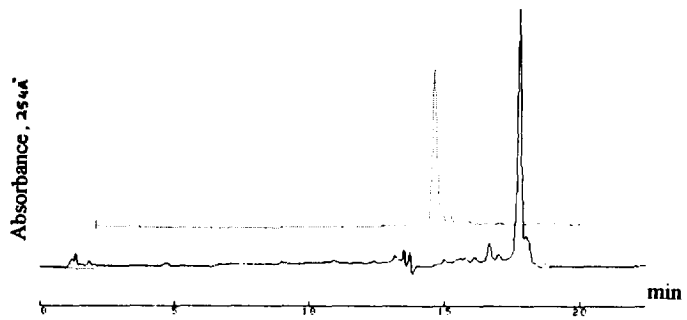


Fig. 4. HPLC profiles of (a) 3'-mercaptoalkylated d(CTC TTC CGC T) and (b) 1,5-I-AEDANS labelled d(CTC TTC CGC T). Column : Lichrosphere RP-18; Buffer A = 0.1M ammonium acetate, solvent B = Acetonitrile; Gradient : 0-30%B in 30 min.

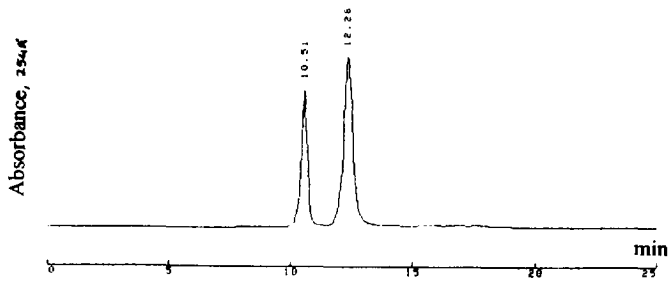


Fig. 5. HPLC profile of a mixture of 3'-mercaptoalkylated d(ACT CTT CT) and 1,5-I-AEDANS labelled oligomer. Column : Lichrosphere RP-18; Buffer A = 0.1M ammonium acetate containing 5% acetonitrile, solvent B = Acetonitrile; Gradient : 0-25%B in 20 min.

syringe and added to the reaction mixture under the exclusion of moisture. The contents of the vial were shaken for 15 min at room temperature followed by removal of the excess reagent and washing with acetonitrile (2x5 ml) in a sintered glass column under inert atmosphere. The activated support was then reacted with a large excess of 1-O-(4,4'-dimethoxytrityl)-5-aminopentanol (100 μmol) dissolved in anhydrous acetonitrile (500 μl). The reaction mixture was kept for 6h at room temperature with occasional swirling. The functionalized support was recovered on a sintered glass funnel and subjected to washings with acetonitrile, acetone and diethylether (5 ml of each). The residual hydroxyethyl functionalities, if any, on the support were capped following the standard procedure followed by washings as described above to obtain the fully functionalized support **2a**. The support was dried under vacuum and subjected to loading determination following standard procedure. The support **2b** was prepared following the same procedure except that 1-O-(4,4'-dimethoxytrityl)-3-mercaptopropanol (100 μmol) was used. The other steps were identical as described for the support **2a**.

Preparation of supports 3a and 3b

The polymer support 1 (100 mg, containing 3.5 μmol of hydroxyethyl groups) suspended in dry acetonitrile (0.5 ml) under inert atmosphere was treated with 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (200 μmol). 1H-tetrazole (100 μmol) dissolved in dry acetonitrile (0.5 ml) was added dropwise over a period of 2-3 min at room temperature under the exclusion of moisture and with continuous shaking. After 2h, a solution of tetrazole (0.5 mmol) in acetonitrile (1 ml) was added to the reaction mixture followed by the addition of water (0.5 ml). The activated support was recovered on a sintered disc glass funnel and subjected to washings with methanol, acetone and diethylether (5 ml of each). After drying, the polymer support was suspended in carbon tetrachloride (200 μl) and allowed to react with a solution of 1-O-(4,4'-dimethoxytrityl)-5-aminopentanol (0.5 mmol) in carbon tetrachloride (0.5 ml). The suspension was agitated at room temperature for 2h prior to filtration. Finally, the support was washed with acetonitrile and acetone (5 ml of each) followed by drying and capping of the residual hydroxyethyl groups as described in case of support **2a** to obtain the support **3a**. Similarly, polymer support **3b** was obtained on reacting 1-O-(4,4'-dimethoxytrityl)-3-mercaptopropanol (0.5 mmol) with activated support **1**. Other steps, viz., washings and capping were performed as outlined above in case of support **2a**.

Deprotection and purification of 3'-modified oligonucleotides were carried out following standard procedures. Labeling of 3'-aminoalkylated oligonucleotides with dansyl chloride, fluorescein isothiocyanate (FITC)

and biotin active ester was carried out as described in (10) and labeling of 3'-mercaptoalkylated oligomers with N-Iodoacetyl-N'-(5-sulpho-1-naphthyl)-ethylenediamine (1,5-I-AEDANS) was performed according to the procedure reported in (11).

ACKNOWLEDGEMENT: The financial support from the Department of Biotechnology, New Delhi-110 003 is gratefully acknowledged.

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(*Received in Belgium* 18 *June* 1996; *accepted* 26 *August* 1996)