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A SIMPLE METHOD FOR THE DERIVATISATION OF LONG CHAIN ALKYLAMINE-CONTROLLED PORE GLASS (LCAA-CPG) FOR SOLID PHASE SYNTHESIS OF OLIGONUCLEOTIDES

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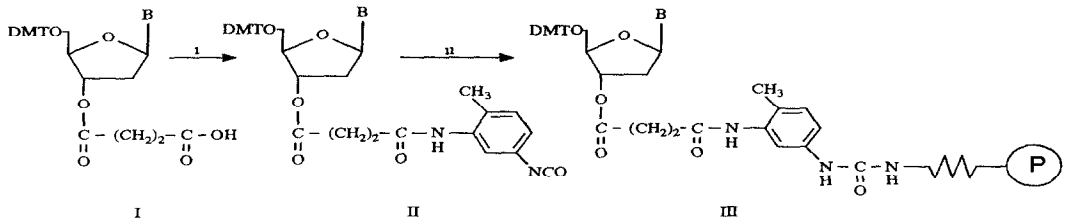
Summary: A simple and rapid method for the derivatisation of LCAA-CPG for the solid phase synthesis of oligonucleotides is described. The appropriately protected 2'-deoxynucleoside-3'-O-succinate **I** is reacted with an analogous amount of a bifunctional reagent, tolylene-2,4-diisocyanate in the presence of 4-dimethylaminopyridine to generate monoisocyanate **II** which, in the subsequent reaction with LCAA-CPG in the presence of N-ethyldiisopropylamine, generated the fully functionalised support **III** with excellent nucleoside loadings.

The nature of solid supports and the type of linkages used for anchoring the first nucleoside to the support play a vital role in solid phase oligonucleotide synthesis. The current methodology[1] for oligonucleotide synthesis utilizes nucleosides anchored on the long chain alkylamine controlled pore glass (LCAA-CPG) by a succinyl linker. A number of other linkages such as ester[2], urethane[3] and those cleaved under mild basic conditions[4], are known for synthesizing oligonucleotides and modified oligonucleotides. However, the succinate linkage remains the most commonly used linkage for solid phase oligonucleotide synthesis by phosphotriester and phosphoramidite approaches. The conventional method[5] of derivatising LCAA-CPG involves the dicyclohexylcarbodiimide (DCC) mediated synthesis of pentachlorophenyl or p-nitrophenyl active ester of appropriately protected deoxynucleoside-3'-O-succinates which in the subsequent condensation reaction with primary amino groups of LCAA-CPG gives the desired functionalised support. However, this method generally yields moderate loading of the nucleosides on the LCAA-CPG (10-25 μmol/g). and takes considerable time (30h) for derivatisation of the supports. Pon et al.[6] have proposed the use of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) as an alternative condensing reagent for obtaining better nucleoside loadings. However, the longer time (24h) required for the functionalisation of LCAA-CPG has been the main limitation of this approach.

We describe herein a versatile and rapid approach for the derivatisation of LCAA-CPG in which appropriately protected 2'-deoxynucleoside-3'-O-succinates are coupled to the LCAA-CPG, using a commonlly available bifunctional reagent, tolylene-2,4-diisocyanate (TDIC) (Fluka, Switzerland, Cat No., 89870). The derivatisation of the support is very rapid (8h) and provides supports with a moderately high degree of nucleoside loadings (38-40 μmol/g support). The derivatised support **III** was found to be compatible with the established phosphoramidite approach of oligonucleotide synthesis.

The functionalisation of LCAA-CPG is shown in Scheme-1. The appropriately protected 2'-deoxynucleoside-3'-O-succinate (0.2 mmol) was dried in a septum sealed vial fitted with a needle over phosphorous pentoxide under high vacuum for several hours at room temperature and then reacted with one equivalent of tolylene-2,4-diisocyanate (0.2 mmol, 28.7 μl) in the presence of 4-dimethylaminopyridine (0.2 mmol, 24.4 mg)[7] in dry dichloromethane (5 ml) at room temperature for 10 min. The monoisocyanate **II** was not isolated but directly added to a septum sealed vial (10 ml) containing LCAA-CPG (1.0 g, ca. 0.1 mmol amino groups; pore size 500 A^{o} and particle size 80-100 mesh) (Sigma



Scheme-1 Functionalisation of LCAA-CPG. Reagents, i = Tolylene-2,4-diisocyanate; 4-dimethylaminopyridine; dichloromethane. ii = LCAA-CPG; N-ethyldiisopropylamine DMT = 4,4'-dimethoxytrityl and B = an appropriately protected nucleic base.

Chem. Co., USA) along with N-ethyldiisopropylamine (0.2 mmol, 34.2 µl). The reaction vial was briefly agitated to ensure complete mixing of the reaction mixture and then left at room temperature for 6h with occasional shaking. The polymer support **III** was recovered by filtration in a sintered glass funnel, washed with dichloromethane (3 x 10 ml) and dry diethyl ether (3 x 10 ml). The support was suspended in pyridine / water (10 ml, 8:2,v/v) at room temperature for 2h in order to hydrolyse any residual isocyanate groups on the support **III**. The polymer support was filtered in a sintered glass funnel, washed with dichloromethane (3 x 10 ml) and dry diethyl ether (3 x 10 ml). The residual amino groups on the polymer support **III** were then capped following the reported method[8]. The support was then recovered by filtration in a sintered glass funnel, washed with dichloromethane (3 x 10 ml) and dry diethyl ether (3 x 10 ml). The dried supports were stored at -20^{0}C until required.

At this stage the support **III** gave negative ninhydrin test clearly indicating the absence of any free amino groups present on the support **III**. The nucleoside loading of the derivatised support **III** was determined spectrophotometrically after reacting a small weighed amount of the support **III** with perchloric acid[1]. In a typical experiment the following loadings were obtained:

T-support = 39.2 µmol / g support; C-support = 38.5 µmol / g support

A-support = 38.3 µmol / g support; G-support = 40.0 µmol / g support.

In order to check the effect of TDIC on nucleic bases modification during the functionalisation of polymer support, the following experiment was carried out. The four functionalised polymer supports **III** (100 mg) were subjected to aq. ammonia (25%) treatment for 10 min at room temperature. The ammonical solution was concentrated (Savant) and the cleaved 5'-O-(4,4'-dimethoxytrityl)-N-protected-2'-deoxynucleosides were extracted in diethyl ether (2 x 5 ml). Diethyl ether was removed and a portion of each of the cleaved nucleosides was taken in methanol / ethanol and subjected to UV analysis. The other portion of the nucleosides were subjected to HPLC analysis. THe UV and HPLC analysis[9] of each of the nucleosides released from the polymer supports **III** were found to be identical to the standard 5'-O-(4,4'-dimethoxytrityl)-N-protected-2'-deoxynucleosides[10], clearly indicating that no modification of nucleic bases occurred with DTIC during the functionalisation of polymer supports. In addition, Sproat and Brown[3] have also observed, while anchoring the nucleosides to the LCAA-CPG via urethane linkage, that no modification of nucleic bases resulted with aryldiisocyanates.

To test the utility of derivatised polymer supports **III** in routine oligonucleotide synthesis, a number of oligomers were synthesised using the supports **III**. In order to compare the derivatised polymer supports **III** with the standard LCAA-

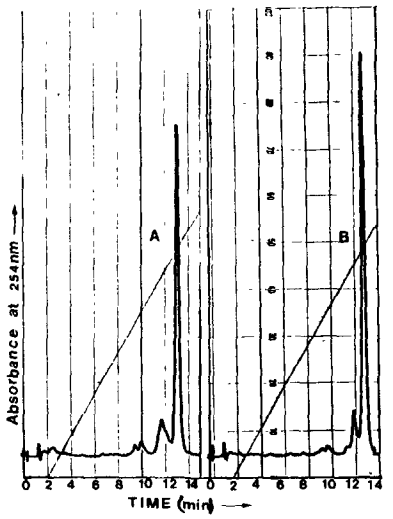


Fig.1 FPLC profiles of decamer d(CAT TAT TGC T), (A) synthesized, on support III, Retention time = 13.04 min and (B) on standard LCAA-CPG support, Retention time = 13 0 min Column Mono Q HR 5/5.. Buffers: A = 0.4M NaCl and 0.01M NaOH, pH 12.0; B = 1M NaCl and 0.01M NaOH, pH 12.0. Gradient: 0 % B for 2 min, 0-100% B in 23 min. Flow rate 1 ml / min, Auf 0.1

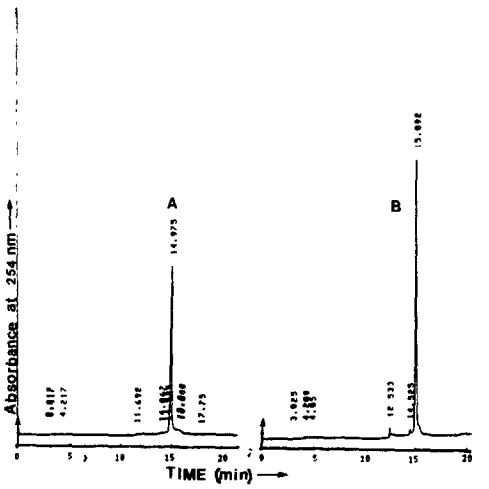


Fig. 2 HPLC profiles of FPLC purified decamers X and Y (A) oligomer X Retention time = 14.975 min and (B) oligomer Y, Retention time = 15.09min Column, Zorbax C18 (4.6 x 250 mm); Gradient, 0-35% B in 35 min, flow rate 1.0 ml / min. Buffers: A = 0.1M ammonium acetate buffer, pH 7.0, B = 100% acetonitrile, Auf 0.16.

CPG supports, a decamer oligonucleotide d(CAT TAT TGC T) X was synthesised, using 0.2 μmol scale on support III following standard protocol[11] on a Pharmacia-LKB Gene Assembler Plus. The same sequence d(CAT TAT TGC T) Y was also synthesised on a standard 5'-DMTr dT-LCAA-CPG support with O-succinate linkage. The coupling efficiency per cycle based upon the released 4,4'-dimethoxytrityl cation exceeded 99% and was found to be identical within the experimental error for the oligomer X synthesised using the support III. The oligomers were cleaved from the supports and deprotected following the standard protocol[11]. After being released from the support and full deprotection, the crude decamers X and Y were chromatographed on a Polyanion Mono Q HR 5/5 column (Fig. 1). The major product with the highest retention time was collected in each case, desalted on Bio-gel P2 column and analysed on a reverse phase C18 column under the identical conditions (Fig. 2). Both of the oligomers X and Y were found to be identical in respect to their elution time and gradient concentration required. This was further confirmed by injecting a mixture of purified X and Y under the conditions employed in Fig. 2. Both of the oligomers X and Y were eluted in a single peak with the same retention time.

The purified oligomer X was 5'-phosphorylated using [γ-^{32}P] ATP and T4 Polynucleotide Kinase[12] and subjected to analysis on a polyacrylamide (20%) slab gel electrophoresis along with 5'-phosphorylated oligomer Y. The oligomer X was found to be identical to oligomer Y (Fig. 3).

Conclusion: A rapid and economical procedure for the derivatization of long chain alkylamine-controlled pore glass (LCAA-CPG), a commonly used support for machine-aided DNA synthesis, has been developed. The method involves the use of commonly available reagents and is amenable for the functionalization of large quantities of polymer supports.

Fig.3 Autoradiograph of 20 % polyacrylamide / 7M urea gel electrophoresis of 5'-phosphorylated X and 5'-phosphorylated Y Lane 1, 5'-Phosphorylated Y; Lane 2, 5'-Phosphorylated X; Lane M, Oligo dT markers

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