POLYSYTYRENE-SUPPORTED SYNTHESIS BY THE PHOSPHITE TRIESTER APPROACH: AN ALTERNATIVE FOR THE LARGE SCALE SYNTHESIS OF SMALL OLIGODEOXYRIBONUCLEOTIDES.

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Abstract. The large scale synthesis of two octadeoxyribonucleotides has been successfully accomplished on polystyrene by the phosphite triester approach. Employing moderate excesses but high concentrations of phosphoramidites, the coupling yields are similar to those obtained in standard small scale syntheses.

There is an increasing need for large quantities of sequence-defined oligodeoxyribonucleotides for several biophysical and biochemical applications such as X-ray analysis (1) and NMR studies (2). Moreover, many clinical and diagnostic applications that require milligram amounts of synthetic oligodeoxyribonucleotides have recently been developed (3).

For many years, phosphotriester chemistry in solution has been the method of choice for undertaking such large scale syntheses since small excesses of nucleotide building blocks are employed and it allows the purification of the intermediates (4). Later on, the developement of solid-phase procedures resulted in an increase in speed and savings in labour. For large scale synthesis, the phosphotriester approach has been used in conjunction with several solid supports including polystyrene (5), controlled pore glass (CPG) (6,7) and Kieselguhr-polydimethylacrylamide (7), but nowadays the most efficient and widely employed method is the phosphite triester approach, developed by Caruthers (8), using silica gel (9) or CPG (10) as the solid matrix. More recently, the use of the H-phosphonate method with CPG has also been described (11).

Although many oligonucleotides are regularly obtained with such approaches, solid-phase procedures have been developed principally for the synthesis of microgram quantities of product and are based on the use of very large excess of the building blocks: activated phosphotriesters, phosphoramidites or H-phosphonates. When scaling up the synthesis to obtain milligram amounts , the method becomes prohibitive.

We believe that the key factor to reduce the cost of large scale solid-phase syntheses is to use a support with a higher degree of substitution, since it then becomes possible to employ higher concentrations but lower excesses of the monomer building blocks. Under such conditions, the problem of moisture in the solvents or the ambiental humidity is greatly reduced and the efficiency of the synthesis is improved. Crosslinked polystyrene-DVB is a well known solid phase support that fulfils this requirement since it is well suited to be functionalized the desired extent, and in order to maintain high coupling yields it offers the additional advantage that it can be stirred even when working with gram amounts of support.

We now describe the use of polystyrene for the large scale syntheses of two octadeoxyribonucleotides by the most effective and rapid synthetic method, the phosphite triester approach. The two octamers, synthesized for X-ray crystallographic studies, have the sequences CGTTTTCG (I) and CGAAAACG (II).

5'-O-dimethoxytrityl-β-cyanoethyl-N,N-diisopropyl-deoxynucleosidephosphoramidites (12) have been used as monomer synthons. The amino group of the bases C and G has been protected with benzoyl (Bz) and isobutyryl (iBu) groups respectively. In order to avoid unwanted depurination during the synthesis, the N^6 exocyclic amine of deoxyadenosine has been protected with the N,N-dimethylacetamidine group since it provides higher stability towards acidic conditions than the benzoyl group (13).

In both syntheses chain elongation was carried out manually over 250mg of functionalized support (DMT-G^{iBu}-resin, 0.14 mmol/g) (14) on an apparatus equipped with a 5 ml glass filter funnel (15), following the synthetic protocol described in Table 1. Some modifications in the usuall synthetic cycle have been made (16), since the support is only effective if it is well swollen and couplings with phosphoramidites/tetrazole are normally performed in acetonitrile which does not swell polystyrene . A 10 fold excess of phosphoramidites in dichloromethane and a 40 fold excess of a saturated solution of tetrazole in tetrahydrofuran/acetonitrile were used in each coupling step.

Table 1. Synthetic Protocol for Assembling of Phosphoramidites on a Polystyrene Support.

Step	Reagent or solvent[a]	Number of treatments	Time (min)
1	DCM	3	0.5
2	2% trichloroacetic /DCM (w/v)	6	0.5
3	DCM	3	0.5
4	5% triethylamine/DCM (v/v)	1	0.5
5	DCM	2	0.5
6	ACN (HPLC)	2	0.5
7	ACN (HPLC, dry)	2	0.5
8	Vacuum drying with an oil pump	1	3
9	1ml of 0.35M phosphoramidite in DCM followed by 1ml of 1.4M tetrazole in THF/ACN (7:3)	1	30
10	2ml of DMAP/THF/2,6-lutidine (6:90:10,w,v,v) then 0.2ml of acetic anhydride	1	5
11	0.1M I_2 in THF/lutidine/H_2O (35:25:5, v/v/v)	1	2
12	THF	2	0.5
13	DMF	4	0.5
14	DCM	1	0.5

a) each step volume was *ca.* 3 ml unless otherwise indicated.

In the synthesis of CGTTTTCG (I) the overall yield (17) was 82% , which corresponds to a 96.8% repetitive coupling yield. A slight increase in VIS absorption of the detritylation filtrates of II was observed after incorporation of the four consecutive adenosines (2% average on each one), suggesting that the abnormal increase is probably related to the adenine protecting group (18).

The products were cleaved from the resin by an overnight treatment with concentrated ammonia/ dioxane (1:1) at 60ºC. The use of dioxane seems to be necessary in order to swell the resin, thus increasing the accessibility and therefore the cleavage yields of the 3'-O-succinate linkage. A second overnight ammonia treatment of the filtrates from the cleavage reaction was performed with octamer II to ensure complete deprotection of the adenines, since it is known that amidine type protecting groups are more stable than benzoyl groups to ammonia (13).

After cleavage and base deprotection, the products were detritylated with 80% acetic acid (30 min) and analyzed by reversed-phase HPLC(19). Crude octamer I was pure enough to be submitted to a single purification by semipreparative HPLC on a C18 column (Fig.1a). The purified product was shown to be homogeneous by analytical HPLC (Fig.1b) and was obtained in a 21% overall yield (cleavage and purification). Octamer II needed an additional purification step and was first submitted to a DEAE-Sephadex column chromatography (linear gradient from 0.05M to 1M of triethylammonium bicarbonate buffer) (Fig.2a) and then to semipreparative C18-HPLC as above (Fig.2b). The homogeneous product (Fig.2c) was isolated in an 8% overall yield. Purified products I and II were shown to be homogeneous by polyacrylamide gel electrophoresis and gave the expected ratios of deoxynucleosides after enzymatic digestion (20).
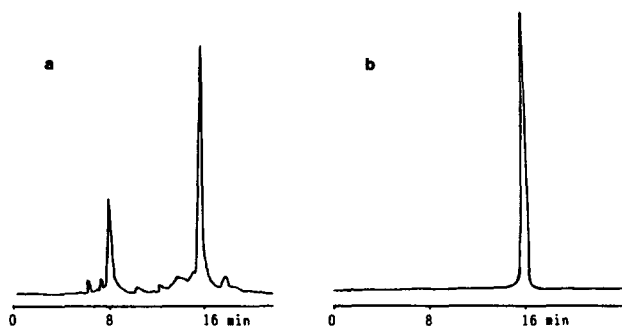
Figure 1: a) Semipreparative HPLC profile of d(CGTTTTCG) I (peak at 8.2 min does not correspond to oligonucleotidic material); b) analytical HPLC of purified product.
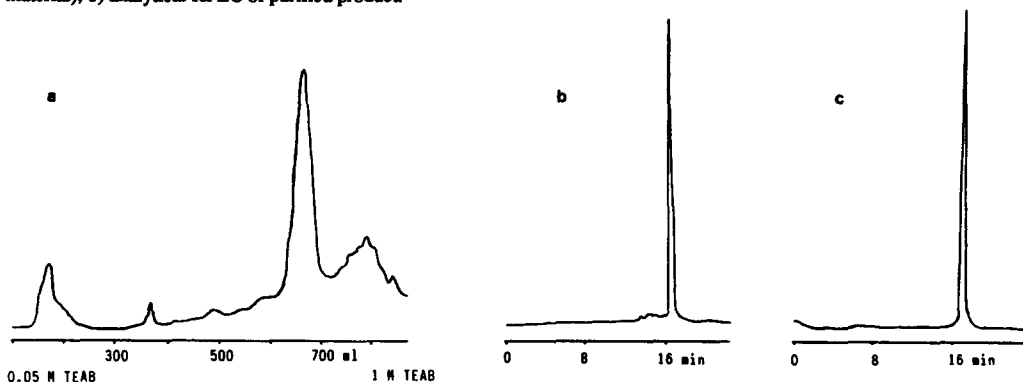
Figure 2: a) Chromatographic profile of d(CGAAAACG) II on DEAE-Sephadex; b) analytical HPLC after Shephadex purification c) analytical HPLC of completely purified II.

These results demonstrate that the combined use of polystyrene and the phosphite triester approach is feasible and that it can be a useful alternative for the large scale solid-phase synthesis of oligonucleotides. Employing moderate excesses but high concentrations of phosphoramidites, the coupling yields are similar to those obtained in standard small scale syntheses with CPG. Work is in progress to reduce further the excesses of monomers and the coupling times.

References and notes.

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14. 5'-O-DMT-G(iBu)-3'-O-succinate has been attached to aminomethylpolystyrene-1%-divinylbenzene (0.5-0.6 mmol/g,. Sigma) by means of dicyclohexylcarbodiimide/hydroxybenzotriazole/4-N',N'-dimethylaminopyridine (DMAP) (1:1:0.2 equivalents) activation. Capping of unreacted amino groups was performed with 2x30min treatment with a freshly prepared solution of acetic anhydride/pyridine/DMAP 5:80:0.25 (v,v,w).

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16. DMF washings (step 13) were included after oxidation treatment to remove the iodine that sticks to polystyrene. A triethylamine/DCM washing was performed after detritylation to eliminate traces of acid. Between steps 7 through 11 the reactor was capped with a rubber septum and the reagents and solvents added through appropiate syringes. During steps 1 to 6 and 12 to 14 the reactor was kept open and products added with Pasteur pipettes or wash bottles. A stream of dry argon was used to help the vacuum in the filtrations after each treatment when the reactor was closed and to allow a gentle stirring of the resin by countercourrent bubling during reactions in steps 9,10 and 11.

17. The filtrates of the detritylation reaction and the following DCM washings (steps 2 and 3) were collected, and their visible absorption was measured to determine the coupling yields.

18. The weight increase of the resins after the syntheses were 103 and 131mg for I and II respectively, in good accordance with the expected increase in weight (103 and 113 mg respectively).

19. Eluents: A: 0.05 M aqueous triethylammonium acetate; B: 0.005 M triethylammonium acetate in water/acetonitrile 1/1. Gradient: 15-30% B, 20 min. Detection wavelength: 260 nm.

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