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IMPROVED SYNTHESIS OF OLIGODEOXYNUCLEOTIDES ON CONTROLLED PORE GLASS USING PHOSPHOTRIESTER CHEMISTRY AND A FLOW SYSTEM

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Abstract. A very fast and efficient method for assembling oligodeoxynucleotides on controlled pore glass using monomer or dimer components and a minimum of reagents has been devised.

Until recently the main disadvantage of the phosphotriester method of oligodeoxynucleotide synthesis as compared to the phosphite method has been the slowness of the coupling step. It was recently shown by Efimov et al.[1] that the coupling time can be considerably reduced if one uses mesitylenesulphonyl chloride and N-methylimidazole as the condensing agent, giving a cycle time of ca. 35 min when a controlled pore glass support is used. It has also been shown that N-methylimidazole activates stable condensing agents such as 2,4,6-triisopropylbenzenesulphonyl-4-nitroimidazolide and mesitylene-4-nitro-imidazolide, giving coupling times in solution of about 2 h.[2] We wish to report that the use of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) which is easier to handle than mesitylenesulphonyl chloride, plus N-methylimidazole effects rapid and efficient coupling, 15 min for monomer addition and 20 min for dimer addition in pyridine at room temperature with a phosphodiester concentration of 0.13 M. This and several other modifications have now been incorporated into the solid phase phosphotriester method as described by Gait et al.[3]

We have found that controlled pore glass/long chain alkylamine (500 Å pore diameter, particle size 125-177 μ, supplied by Pierce) is an ideal support for oligonucleotide synthesis using our method. This support offers several advantages compared to the polydimethylacrylamide-Kieselguhr composite support:[3]

(i) there is no swelling or contraction with change of solvent;

(ii) it is very easily functionalised with the required base protected nucleoside-3'-O-succinate;

(iii) since it is less polar, the strength of the protic acid used for the detritylation step can be reduced considerably with no loss of speed or efficiency.

However, we recommend that the polydimethylacrylamide-Kieselguhr composite support is

used for those applications which require large quantities of oligonucleotides, e.g. n.m.r. and crystallographic studies, since the much higher functionalisation of the support (ca. 200 μmol g^{-1} as compared with ca. 25 μmol g^{-1} for the glass support) is a considerable advantage.

One further advantage of controlled pore glass is that Lewis acids such as $ZnBr_2$ can be used for the detritylation step. However, since the reaction time increases considerably with increasing chain length we favour the use of a protic acid for this step, although there is then some depurination of N^6-benzoyl protected adenosine. A 10% solution of trichloroacetic acid in 1,2-dichloroethane effects loss of 5'-dimethoxytrityl within 20-40 sec with a flow rate of 1-2 ml min^{-1}. However, it does undoubtedly cause some depurination. We therefore prefer to use the weaker dichloroacetic acid; a 3% solution of this reagent in 1,2-dichloroethane effects loss of the 5'-dimethoxytrityl group within 40 sec for 5'-A or G and within 75 sec for 5'-C or T irrespective of the oligonucleotide chain length. These times can be reduced by about 30% if the slightly more acid labile 9-phenylxanthen-9-yl (pixyl) protecting group[4] is used. Adams et al.[5] have independently shown that dichloroacetic acid causes only a minimum of depurination, and utilised this reagent in their recent synthesis of two 51 mers by the phosphite method.

The system that we have now developed can compete very effectively with the phosphite method of oligonucleotide synthesis in terms of speed and efficiency, but has the added advantage that one can couple monomers, dimers, trimers or whatever blocks are available. Using this methodology it is quite easy to simultaneously assemble several oligonucleotides at the 20 mer size in one day using the Omnifit system[3] and several columns. The assembly procedure is as follows: the support (generally 25 mg or 50 mg for dimer addition, loading ca. 10-25 μmol nucleoside-3'-O-succinate g^{-1}) is first dehydrated with 10% phenyl isocyanate in pyridine (150 μl) for 15 min, and then the following cycle is carried out:

Pyridine wash	3 min
1,2-dichloroethane wash	2 min
3% dichloroacetic acid in 1,2-dichloroethane wash	40-75 sec depending on 5'-base
1,2-dichloroethane wash	1 min
Pyridine wash	4 min
Coupling*	15 min for monomers 20 min for dimers

*The coupling mixture for monomer addition consists of 13.3 μmol monomer (as the triethylammonium salt of the phosphodiester[3]), MSNT (20 mg, 67.5 μmol), and N-methylimidazole (10 μl, 126 μmol) in 100 μl pyridine, and is made up just prior to injection onto the support. For dimer addition using 50 mg of support increase the quantities above by 50%.

Fully protected dimers bearing a 3'-(cyanoethyl, 2-chlorophenyl phospho) group are decyanoethylated by treatment with t-butylamine/pyridine (1:3 v/v)[6] for 15 min at room temperature. The solution is evaporated in vacuo and the residue dried by two evaporations of pyridine prior to use in a condensation reaction.

We have found that a capping step is totally unnecessary if good quality monomers and dimers are used. The overall cycle time using monomers is 26 min and using dimers is 31 min, which is considerably faster than that reported by Kohli et al.[7] for dimer addition on silica gel and faster than that reported by Köster et al.[8] for dimer addition on controlled pore

glass in the absence of any N-methylimidazole as catalyst (cycle time reported is about 2 h). At the end of the assembly the deprotection is as described previously, followed by purification by ion-exchange hplc, desalting, and finally reversed phase hplc to remove any base modified material.[3] Oligonucleotides greater than 30 bases in length are most easily purified by polyacrylamide gel electrophoresis as the first purification step. Alternatively the 5'-dimethoxytrityl protected oligonucleotide can be purified by reversed phase hplc on μ-Bondapak C_{18} using a gradient of 20-30% acetonitrile in 0.1 M triethylammonium acetate pH 7.[9] The dimethoxytrityl group is then removed in the normal way.

Using the methods outlined above a large number of oligodeoxynucleotides have now been prepared, and the following serve as representative examples: d[CCGGT($^{A}_{T}$)ACCGG] and d[GTAAAATCAAATATTTTG] were prepared by monomer addition and isolated in yields of 29% and 14.7% respectively after purification by ion-exchange hplc and desalting. Employing dimer addition a 37 mer, d[CCCAGGCGGTCTCCCATCCAAGTACTAACCAGGCCCG] has been synthesised and obtained in ca. 3% yield after several purification steps including finally, reversed phase hplc. Recently a purine rich 61 mer has been synthesised, and details of this will appear elsewhere.

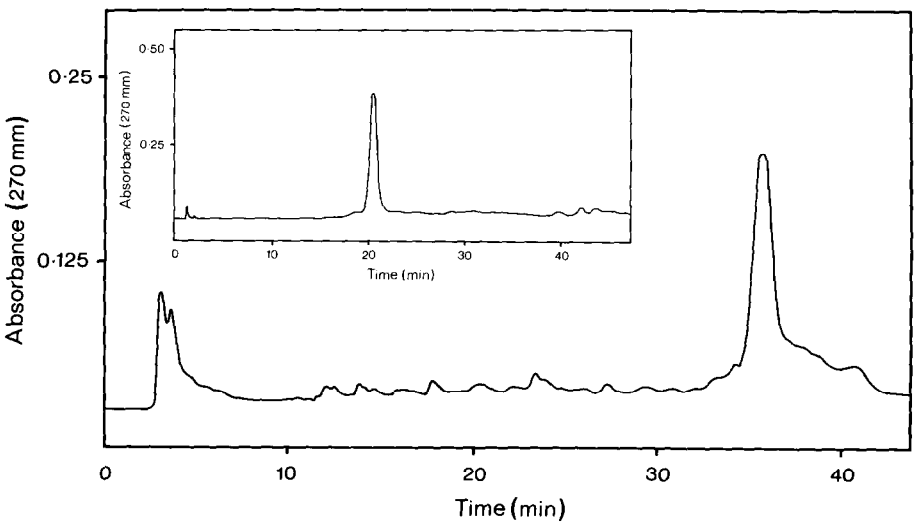

Figure: Analytical ion-exchange hplc of d[GTAAAATCAAATATTTTG] on Partisil 10 SAX using a gradient of 1 mM to 0.21 M KH_2PO_4 in 60% $HCONH_2$/40% H_2O over 45 min.[3] Inset shows reversed phase hplc of ion-exchange purified 18 mer on μ-Bondapak C_{18} using a gradient of 8%-12% acetonitrile in 0.1 M aqueous ammonium acetate over 45 min.

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