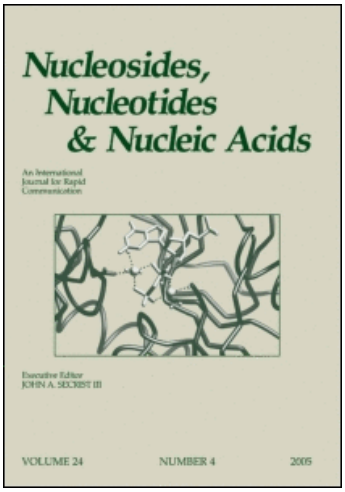

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RAPID SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES VIII.[1] AN IMPROVED PROCEDURE FOR SOLID-PHASE OLIGODEOXYRIBONUCLEOTIDE SYNTHESIS USING A CONTROLLED PORE GLASS SUPPORT AND PHOSPHOTRIESTER CHEMISTRY

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Summary. An improved method for solid phase oligodeoxyribonucleotide synthesis is described that uses phosphotriester chemistry, controlled pore glass as support, a new and more stable linkage agent and a better protecting group combination.

Recently we described the use of long chain alkylamine controlled pore glass (LCAA-CPG) as an improved support for rapid synthesis of oligodeoxyribonucleotides by a phosphotriester procedure.[2] Internucleotide coupling times were reduced to 15 minutes when 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) was used as coupling agent in the presence of N-methyl imidazole and pyridine. An 18-mer was assembled using a semi-manual flow system[1] and isolated in overall yield of 14.7% .

Further improvements might be expected by reducing the potential for side reactions. To lessen the risk of loss of adenyl moieties during 5'-terminal acidic deprotection, we have investigated the use of 9-phenylxanthen-9-yl (pixyl)[3] as 5' protecting group for deoxyribonucleotide monomers. Using 3% dichloroacetic acid in 1,2-dichloroethane, the pixyl group is removed from CPG-bound oligonucleotides faster than the dimethoxytrityl group previously used. We have also found that the phthaloyl[4] and the di(n-butylamino)methylene[5] groups, recently suggested for the N^6-protection of adenines, are equally useful in preference to the benzoyl group, because of the increased stability to acid of the corresponding N^6-protected deoxyadenosine derivatives.

In addition, we report on a new and convenient type of linkage between support and oligonucleotide. The 'TDIC' linkage (Figure 1) is formed by reaction of 0.2 mmol of 5'-O-pixyl-thymidine, -N^6-di(n-butylamino) methylene deoxyadenosine, - N^4-benzoyldeoxycytidine or -N^2-isobutyryldeoxyguanosine with 0.2mmol each of N,N-diisopropylethylamine and tolylene 2,6-diisocyanate in 1ml of anhydrous 1,2-dichloroethane/pyridine (1:1V/V) for 6h at room temperature in the dark, followed by direct reaction for 24h with LCAA-CPG (2g, 0.2mmol) suspended in dry 1,2-dichloroethane (4.5 ml). After washing, capping with pyridine/acetic anhydride /1-methyl imidazole (8:1:1 V/V) for 30 min and further washing steps, the support loadings of deoxynucleoside obtained were 22-30 $\mu mol\ g^{-1}$. The TDIC

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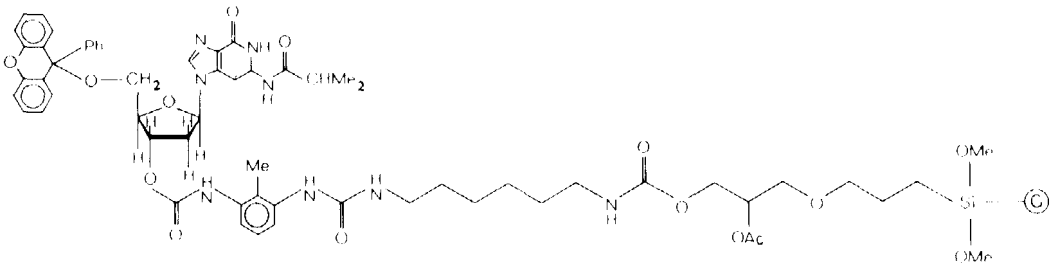

FIG 1. 5'-O-pixyl-N^2-isobutyryldeoxyguanosine joined to CPG-LCAA via the TDIC linkage.

linkage is more stable to basic hydrolysis than the previously used succinate linkage[2], and is not prone to cleavage by amino impurities in basic solvents such as pyridine. Also the need for preparation of deoxynucleoside-3' O-succinates is obviated. The TDIC linkage is cleaved by concentrated aqueous ammonia at 56° for 36-48h, conditions also required for complete removal of di(n-butylamino)methylene protecting groups from adenines[5].

These improvements were tested in semi-manual flow synthesis[1,2] of oligonucleotides on an economical micro scale (15mg support) using an assembly cycle shown in the Table. Solvents and acidic deprotection media are delivered by argon pressure to a small glass column containing the support. Coupling reactions are carried out under stopped flow conditions by syringe injection through a septum. Triethylammonium 5'-O-pixyl-3'-O-chlorophenyl phosphates of thymidine, N^6-phthaloyl deoxyadenosine, N^2-isobutylryldeoxyguanosine and N^4-benzoyldeoxycytidine are used as monomer units. Final deprotection is achieved using 1)0.3M tetramethylguanidinium pyridine-2-carboxaldoximate, 38h, room temperature, 2) conc. ammonia, 42h, 56° and 3) acetic acid/water (8:2), 30 min room temperature. The use of an extended time for the oximate reaction is helpful in improving yields of guanine-rich oligonucleotides. This may be attributable to a slower reversal of O^6 modifications of guanine than had previously been thought[6].

OPERATION	REAGENT	FLOW TIME (MIN) (RATE 1 ml/min)
WASH	PYRIDINE	2
WASH	1,2-DICHLOROETHANE	2
DEPROTECTION	3% DICHLOROACETIC ACID/1,2-DICHLOROETHANE	0.5-0.75 (depending on 5' base deblocked)
WASH	1,2-DICHLOROETHANE	2
WASH	PYRIDINE	2
		STOP FLOW
COUPLING	5'-O-pixyl monomer: 8 µmole (7-8 mg) MSNT: 40 µmole (12 mg) N-methylimidazole: 6 µl (75 µmole) Pyridine: 80 µl	15

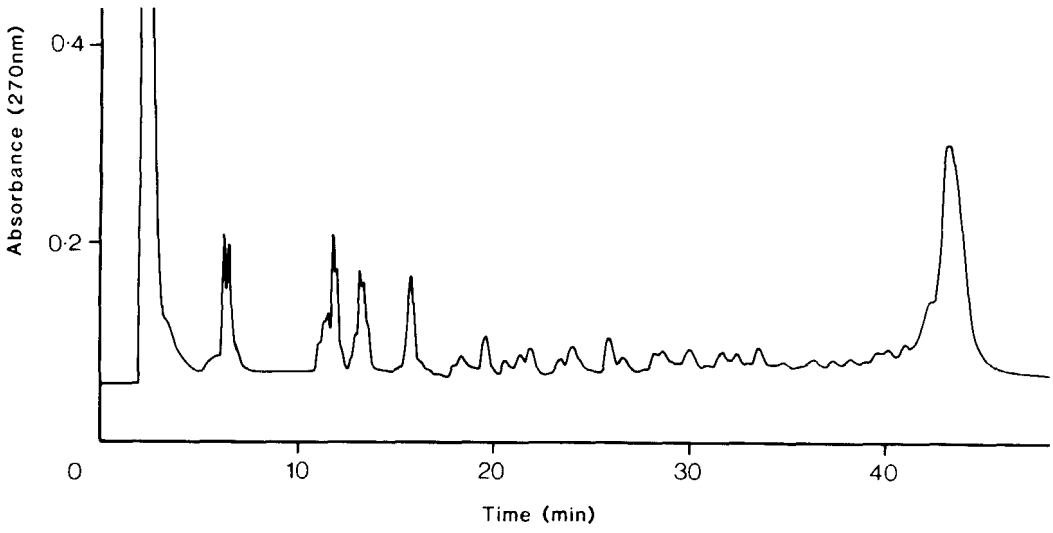

FIG 2. Ion exchange hplc of the 22-mer on Partisil 10-SAX using a gradient of KH_2PO_4 (pH 6.3) in formamide/water (6:4).

In the synthesis of the purine-rich 22-mer, d(AGGTCGCAAGAACCCAAGCAGC), for example, purification of one tenth of the crude product by ion exchange h.p.l.c. (Figure 2) yielded 0.89 A_{260} units from the material in the major peak (9% overall yield). Similar results were obtained for the synthesis of 23 chains of 10-22 residues long, required for assembly of a gene for somatomedin C.[7].

The method is also suitable for the preparation of larger quantities of oligodeoxyribonucleotides. Starting with 250mg of LCAA-CPG (ca 7.5 μmole) in each case, two 23-mers have been prepared corresponding to the DNA sequence of the O_R3 binding site of λ cro protein. The assembly cycle was similar to that used for micro synthesis except that the flow rate was ca 5 ml min^{-1}. In the coupling reactions 71 μmol of monomers (the adenine N^6 protecting group was di(n-butylamino)methylene), 250 μmole of MSNT, 40 μl of N-methyl imidazole and 0.7ml of pyridine were used. After deprotection d(ATTTATCCCTTGCGGTGATAGAT) and d(ATCTATCACCGCAAGGGATAAAT) were isolated in 11.2% (207 A_{260} units) and 4.9% (95 A_{260} units) yields respectively by ion-exchange h.p.l.c. The purity of each strand was 80-90% as judged by reversed phase h.p.l.c. The yields are similar to those obtained for the same oligonucleotides synthesised on a kieselguhr-polyamide support [1,8] except that the improved flow characteristics of CPG make it easier to handle in larger columns[9].

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