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RECOVERY AND RECYCLING OF SYNTHETIC UNITS IN THE CONSTRUCTION OF OLIGODEOXYRIBONUCLEOTIDES ON SOLID SUPPORTS

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Summary: Barium salts of protected mono- and dinucleotides have been employed in a scheme for the construction of oligodeoxyribonucleotides on a glass support. The excesses of these synthetic units were recovered simply by precipitation, and reused in subsequent cycles.

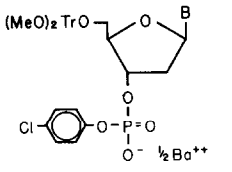
A number of practically useful systems for solid phase synthesis of deoxyribo- and ribooligonucleotides have recently been introduced[1-6]. They represent successful adaptations of both phosphotriester[1-4] and phosphite[5,6] methodologies to the construction of oligomers on a variety of supports such as polyacrylamide, polystyrene and silica gel. Whatever the chemistry involved, the basic strategy is the same in all these systems, namely addition of a protected nucleotide monomer or oligonucleotide block to the free 5'-hydroxyl group of a support-linked nucleoside, followed by a deprotection step which, by exposing a new hydroxyl function, allows the cycle to be repeated. The reduction in number of manipulations that results from carrying out these operations on a support rather than in solution is an important advantage, but one significant drawback remains. Since the solid matrix unavoidably "dilutes" the immobilized oligonucleotide chain, larger excesses of the incoming units are required to maintain effective concentrations, and hence reaction rates, relative to the same coupling step in solution. Any method for recovering the unused portions of these units, which are themselves the end-products of multi-step syntheses, would be of considerable value.

We have previously reported[7] the preparation of the barium salts of N-protected 5'-O-dimethoxytrityldeoxyribonucleoside-3' p-chlorophenyl phosphates $[(MeO)_2Tr]dN'\text{-}(ClPh)$[8], (1), which can be isolated in high yield and purity by precipitation from aqueous barium chloride. These monomers, together with the sixteen dinucleotides $[(MeO)_2Tr]dN'^{p}dN'^{p}(CNEt)$[8] are basic building units for our oligonucleotide syntheses carried out in solution. We noted[7] that the fully protected dinucleotides, after decyanoethylation, can also be isolated as the pure barium salts $[(MeO)_2Tr]dN'^{p}dN'\text{-}(ClPh).\frac{1}{2}Ba^{++}$ (2).

It occurred to us that the water insolubility of these heavy metal salts might permit their direct recovery from spent reaction mixtures in solid support syntheses simply by pouring them into dilute aqueous barium chloride. However, in the phosphotriester method, such mixtures also contain a condensing agent, and this must be removable in order to isolate the nucleotide salt in pure form. The coupling reagent that we are currently using, 1-(p-toluenesulfonyl)-3-nitro-1,2,4-triazole[9], hydrolyzes rapidly in aqueous pyridine (< 1 hr) to p-toluenesulfonate and nitrotriazole, both of which are soluble in dilute barium chloride.

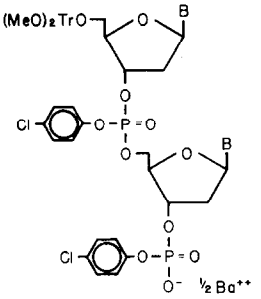
In order to study the feasibility of the recovery procedure we decided to prepare the two ribonucleoside terminated[10] oligomers d(A-T-G-C-A-T)-rU and $dC\text{-}dT_{13}\text{-}rU$ on a glass support. As

the first step, 5'-O-dimethoxytrityl-2'-O-(o-nitrobenzyl)uridine[11,12] was converted to its 3'-O-(pentachlorophenyl succinate) derivative by the method of Miyoshi et al.[13] One gram of long-chain alkylamine controlled pore glass (Pierce Chemical Co., 0.1 mmol amine/g) was then treated with the activated uridine derivative (0.3 mmol) in anhydrous pyridine (3 ml). After degassing, triethylamine (0.43 mmol) was added and the mixture was allowed to stand for 3 days at 25°.

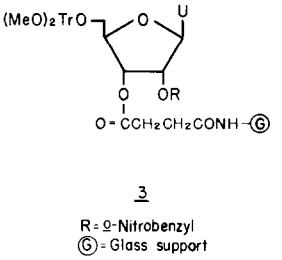


1

B = Thymin-1-yl
N^4-Benzoylcytosin-1-yl
N^6-Benzoyladenin-9-yl or
N^2-Isobutyrylguanin-9-yl



2



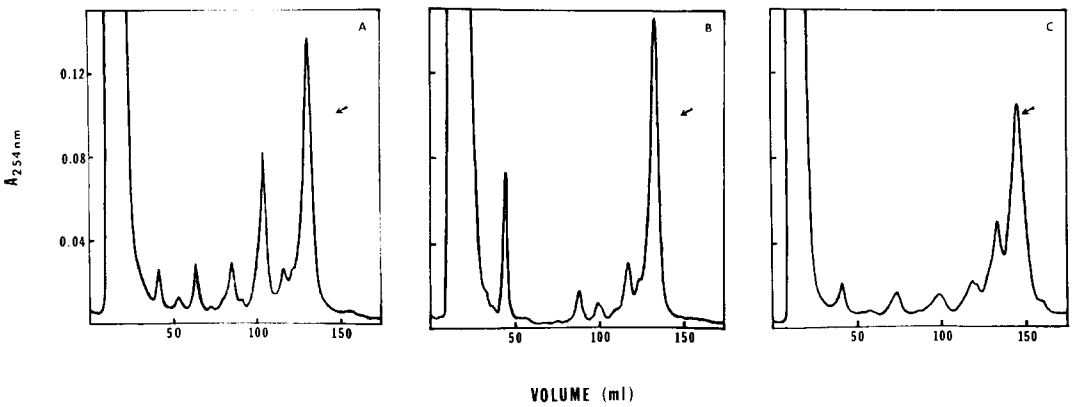
3

R = o-Nitrobenzyl
Ⓖ = Glass support

The glass was then washed thoroughly with pyridine and chloroform and dried (1.028 g of 3, R=o-nitrobenzyl). Treatment with isobutyl chloroformate (2 ml added at 0°) in pyridine (30 ml) for 3 days at 25° was carried out to derivatize residual free amino groups. The amount of uridine bound to the glass was determined as 0.041 mmol/g by detritylation and spectrophotometric measurement of the dimethoxytrityl cation[14]. Trityl assay also showed that 80% of the bound nucleoside could be released by 2 days' exposure to tetramethylguanidinium pyridine-2-aldoximate[15] (TMG-PAO, 0.33 M in 50% aqueous dioxane). In each of the syntheses outlined below a sample of the derivatized glass (3, 103 mg, 4.2 μmol of bound uridine) was detritylated, then treated with either 1 or 2 in the presence of p-toluenesulfonyl nitrotriazole[16]. When the reaction was complete, the excess reagent was removed, the nucleotide barium salt was recovered for reuse, and the glass was again detritylated ready for the next addition. Details of a complete cycle are given in footnote 17.

The preparation of d(A-T-G-C-A-T)-rU was accomplished in two ways. In the first, monomers (1) were used; the barium salts of $[(MeO)_2Tr]$dT-(ClPh) and $[(MeO)_2Tr]$dbzA-(ClPh), recovered from the first and second cycles respectively, were used again in the fifth and sixth. The addition yields at each step, estimated from dimethoxytrityl release and expressed as percentages of support-bound uridine, were 91, 89, 86, 64, 91 and 68%, the last being measured from a 5 mg sample of the glass. Barium salts of the monomers were recovered with an average yield of 96%. The oligomer was released from the glass and partially deprotected by 3 days' treatment with 0.33 M TMG-PAO. After a further 3 days in 14 M NH_4OH followed by removal of solvent, the product was treated with 80% AcOH for 25 min, then co-evaporated with water and neutralized. For analysis, 1.5% of the material was chromatographed on Pellionex SAX[18] (0.4x50 cm) using 200 ml of 40% EtOH containing a linear gradient of 0-0.5 M NH_4Cl (pH 8), and the result is shown in

elution pattern A. The overall yield[19] of d(A-T-G-C-A-T)-rU(NBzl), based on the first deoxyribonucleotide addition, was 27%. A second synthesis of the same molecule was carried out with dinucleotides 2; the recovered barium salt of [(MeO)$_2$Tr]dbzA^{Bz}dT-(ClPh) was reused in the third cycle. Yields in the three cycles were 64, 69, and 76% with respect to U; recovery of the dinucleotides was 99%. The oligomer was released, deprotected, and analyzed as described above (elution pattern B); the overall yield was 39%. The release mixtures from these two syntheses were combined and the d(A-T-G-C-A-T)-rU(NBzl) was isolated by chromatography on Dowex 1-X2 ion-exchange resin[18]. After essentially quantitative removal of the 3' nitrobenzyl group by irradiation with long-wave UV light[12], the sequence of the heptamer d(A-T-G-C-A-T)-rU was confirmed by mobility shift analysis[20]. Additionally, the terminal uridine residue was removed by treatment with periodate followed by β-elimination[21]. After phosphatase treatment, the resulting hexamer was chromatographically indistinguishable from d(A-T-G-C-A-T) previously synthesized in solution by well-established methods[7,20].



The oligomer dC-dT$_{13}$-rU was made by six consecutive additions of [(MeO)$_2$Tr]dT^{Bz}dT-(ClPh) to the glass, with recovery and reuse of the dinucleotide barium salt at each step. The addition yields were 64, 71, 57, 57, 57, and 52% and the average recovery of the dinucleotide was 97%. A final reaction with [(MeO)$_2$Tr]dbzC^{Bz}dT-(ClPh).½Ba^{++} went poorly (26% with respect to U), so the glass was given a second 3 hr treatment with this dimer before removal of the dimethoxytrityl group. Following release and deprotection, the pentadecamer dC-dT$_{13}$-rU(NBzl) was obtained in 32% yield (elution pattern C; chromatography was performed as described above except that 1.3% of the total product was used and the solvent was 200 ml of 40% EtOH containing a gradient of 0.1-0.7 M NH_4Cl). After isolation and removal of the nitrobenzyl group, the material gave the expected pattern on mobility shift analysis.

We conclude by noting that the barium salts of suitably protected mono- and dinucleotides should also be useful for syntheses in the ribo series[11], on other supports[4], and in automated systems. In these cases, as in the work described above, recovery and recycling operations can then be used for the conservation of valuable starting materials.

Acknowledgements

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References and Footnotes

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17. The glass was degassed in toluene (1 ml) in a 10 ml flask, then detritylated (4 X 5 ml of 2% benzenesulfonic acid in $CHCl_3$-MeOH(7:3), 30 min total at 0°). The combined supernatants were assayed for dimethoxytrityl, while the glass was neutralized by the addition of pyridine (0.1 ml). After washing with $CHCl_3$, the glass was dried by evaporation of pyridine (ca. 0.2 ml) in a desiccator over P_2O_5. It was treated with ca. 1 ml of a 0.1 M solution of 1 or 2 (dried by co-evaporation of anhydrous pyridine) and degassed. Toluenesulfonyl nitrotriazole (0.13 mmol) was then added. After 3 hr at 25° the liquid was removed and the glass was washed with pyridine (6 X 3 ml) and $CHCl_3$ (4 X 5 ml), then dried. The combined pyridine supernatant and washes, containing the excess nucleotide and condensing agent, were treated with water (3 ml) for 1 hr, then evaporated to ca. 1 ml and added dropwise to a stirred ice-cold solution of $BaCl_2.2H_2O$ (600 mg) in water (60 ml). The resulting precipitate was collected by centrifugation, washed with water (2 X 15 ml) and dried (P_2O_5), ready to be used again if necessary.
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