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2'(3')-O-BENZOYLURIDINE 5' LINKED TO GLASS: AN ALL-PURPOSE SUPPORT FOR SOLID PHASE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES

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Summary: Porous glass containing 2'(3')-O-benzoyluridine 5'-succinyl ligands serves as a universal support for construction of oligodeoxyribonucleotides of any sequence, using either the phosphotriester or the phosphite approach. At the conclusion of a synthesis, the released and deprotected oligomer bears a terminal uridine residue which can be readily removed as its 2',3'-cyclic phosphate by treatment with Pb^{++} ion.

Most current strategies for solid phase synthesis of oligodeoxyribonucleotides entail the preparation of four separate supports, each one derivatized with a different deoxyribonucleoside destined to be the 3' terminus of the completed chain. This nucleoside is generally attached via a dicarboxylic acid so that, upon completion of synthesis, an oligonucleotide with a free 3' hydroxyl end can be released from the support by alkaline treatment. We have described a new 3'-terminal protection system for oligodeoxyribonucleotides consisting of a ribonucleoside linked by its 2'- or 3'-hydroxyl group to a 3' phosphate at the end of the chain and subsequently removed as its 2',3'-cyclic phosphate by Pb^{++} ion[1]. Since this procedure also generates oligomers with 3' hydroxyl termini, we decided to incorporate it into our solid phase strategy, which employs a support-bound ribonucleoside as a removable linker for the construction of any defined sequence oligonucleotide[2]. Controlled pore glass, which we introduced in an earlier synthetic system[3], was chosen as the support. The effectiveness of this material in solid phase work has been recently confirmed by use in a number of syntheses in our laboratory, and by its application to the preparation of two 51-base polynucleotides using the phosphite method[4].

The plan was to obtain the glass substituted with ribonucleoside residues possessing free 2' and 3' hydroxyl groups[5]. One or other of these positions could then be blocked with any desired acyl function by selection of an appropriate 2',3'-orthoester intermediate[7], while the remaining hydroxyl would serve as the initiation point for the oligomer chain. From our experience with the analogous system in solution[1] we considered that benzoyl might be a suitable choice for this role as 2'(3')-blocking group.

First, an activated 5'-O-succinyl derivative of uridine[8] was prepared using a modification of a previously reported procedure[9]. 2',3'-O-Methoxymethyleneuridine[10] (430 mg, 1.5 mmol) was treated in anhydrous pyridine (4 ml) with 4-dimethylaminopyridine (122 mg, 1 mmol) and succinic anhydride (100 mg, 1 mmol). After 18 hr at 25°, 4,4'-dimethoxytrityl chloride (678 mg, 2 mmol) was added to derivatize the unreacted 5' hydroxyl groups of the excess methoxymethyleneuridine, facilitating subsequent chromatographic isolation of the 5'-O-succinate ester. After a further

18 hr the reaction mixture was treated with water (50 μl) for 15 min, then evaporated to remove most of the pyridine. The oily residue was dissolved in $CHCl_3$ (10 ml) and chromatographed on a column of silica gel (Mallinckrodt CC-7, 2.5 X 30 cm) at 4°. The column was washed with $CHCl_3$ (400 ml) and $CHCl_3$:MeOH (97:3 v/v, 300 ml) to remove the 5'-O-dimethoxytrityl-2',3'-O-methoxymethyleneuridine, then with $CHCl_3$:MeOH (94:6 v/v, 300 ml) to elute the 5'-O-succinate derivative. The fractions containing the product were evaporated to ca. 8 ml and added dropwise to n-pentane (200 ml) with vigorous stirring. The resulting white solid was collected by filtration and dried (338 mg, 87%)[11]. For the activation of its carboxyl group, the material was allowed to react with pentachlorophenol in dimethylformamide under the action of dicyclohexylcarbodiimide[9]. The pentachlorophenyl ester (Figure 1) was obtained in 93% yield and used without further purification.

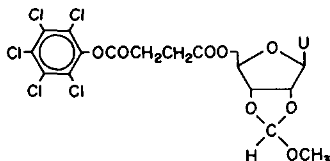

FIGURE 1.

Attachment of the uridine derivative to the support was carried out by adding 0.3 mmol of pentachlorophenyl 2',3'-O-methoxymethyleneuridine 5'-succinate to 1.0 g of long chain alkylamine controlled pore glass (CPG/LCAA, Pierce Chemical Co.; pore diam., 50 nm; particle size, 125-177 μm; ca. 0.1 meq amine/g) in dry pyridine (3 ml). The suspension was degassed and treated with triethylamine (60 μl, 0.43 mmol). After 2-3 days at 25°, the glass was washed with pyridine (9 X 5 ml) and $CHCl_3$ (9 X 5 ml), then dried. Residual amino groups were acylated by treatment with acetic anhydride:pyridine (1:3 v/v, 4 ml) for 24 hr, and the support was washed and dried as before. The support-bound uridine residues were then modified using previously published techniques[10,12]. The glass was carried through the four reaction steps listed below and depicted in Figure 2. At the beginning of each step the glass support was degassed in the reaction solvent and, at the end of each treatment period, it was subjected to 6-10 washes with 5 ml portions of MeOH followed by an equal number of 5 ml washes with $CHCl_3$ before being dried in vacuo.

(i) AcOH:H_2O (4:1 v/v, 5 ml) for 24 hr at 25° to open the cyclic orthoester function.

(ii) Morpholine (20 μl) in n-PrOH (3 ml) for 2 hr at 25° to remove 2'(3')-O-formate groups.

(iii) Trimethyl orthobenzoate (3 ml) containing p-toluenesulfonic acid (300 mg) for 24 hr at 25°.

(iv) AcOH:H_2O (4:1 v/v, 5 ml) for 6 hr at 25° to open the 2',3'-benzoic acid orthoester to a mixture of the 2'- and 3'-O-benzoates.

In order to monitor the changes occurring on the support throughout this procedure, 10 mg amounts of the derivatized glass were withdrawn before step (i) and after steps (ii), (iii), and (iv). A suspension of each sample in n-PrOH:H_2O (1:2 v/v, 1.5 ml) was degassed, then

treated with conc. NH_4OH (2 ml) for 18 hr at 25°, and the hydrolysis products were identified by chromatography (TLC, Eastman Chromagram silica gel plates developed with $CHCl_3$:MeOH (85:15 v/v)). As expected, methoxymethyleneuridine was the nucleoside found in the sample taken before step (i), methoxybenzylideneuridine in that taken after step (iii), and uridine in the samples removed after steps (ii) and (iv). Spectrophotometric analysis of the supernatant of the sample withdrawn after step (iv) indicated that the amount of uridine released was 0.036 mmol/gram of support.

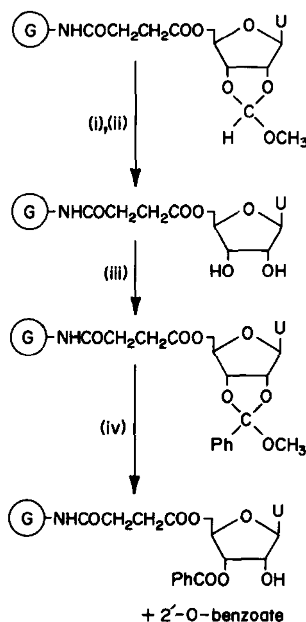

FIGURE 2.

The derivatized glass was used to construct the model oligonucleotide dA-dA_4-dA, using methods similar to those we have previously described[3,13]. After deprotection and release, the oligomer dA-dA_4-dA3'-2'(3')rU was treated with Pb^{++} ion and the product was subjected to ion-exchange chromatography as outlined in the accompanying paper. Pure dA-dA_4-dA was isolated in 42% yield[15], a value comparable to that previously obtained[3] for a hexanucleotide using glass with uridine linked to it through the 3' position in the conventional manner. In a separate experiment, the new support was also successfully employed to build up the same deoxyadenosine hexamer by the phosphite procedure, using six cycles of 5'-O-dimethoxytrityl-6-N-benzoyldeoxyadenosine 3'-(N,N-diisopropyl-O-methyl)phosphoramidite[4] addition, followed by release, deprotection and Pb^{++} treatment.

Controlled pore glass bearing 2'(3')-O-benzoyluridine ligands is therefore a versatile support for solid phase oligodeoxyribonucleotide synthesis employing either the phosphotriester or phosphite methodologies[17]. It renders unnecessary the preparation of four separate supports for synthesis of oligomers with differing 3'-terminal nucleosides and, as we have noted elsewhere[1], its use in the construction of chains that end in deoxyadenosine residues is expected to help protect the terminus against depurination.

Acknowledgements

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References and Footnotes

1. G. R. Gough, M. J. Brunden, and P. T. Gilham, Tetrahedron Letters, accompanying paper.
2. Our previously described approach[3] involved the use of a ribonucleoside bound to the support through its 3' position, with its 5'-hydroxyl acting as the linkage point for the projected oligomer. After completion of the synthesis, the released and deprotected chain contains the ribonucleoside linker which, by virtue of its free cis-hydroxyl system, can be removed by periodate oxidation and β-elimination. This procedure yields products that carry a 3' terminal phosphate.
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4. S. P. Adams, K. S. Kavka, E. J. Wykes, S. B. Holder, and G. R. Galluppi, J. Am. Chem. Soc. 105, 661 (1983).
5. A solid support containing a ribonucleoside linker attached through its 5' position was described some years ago[6]. In this case, the nucleoside was bound by a phosphate group to Sephadex LH 20 and was proposed as a system for anchoring the 5' ends of oligonucleotides to be constructed by the phosphodiester method. A similar concept has been exploited more recently using cellulose as the support (R. Crea and T. Horn, Nucleic Acids Res. 8, 2331 (1980); G. A. van der Marel, J. E. Marugg, E. de Vroom, G. Wille, M. Tromp, C. A. A. van Boeckel, and J. H. van Boom, Recl. Trav. Chim. Pays-Bas 101, 234 (1982)). Both groups made protected derivatives of the dinucleotides prU2'(3')-3'dN for use as linkers in their triester syntheses. However, this approach requires the preparation of a separate dimer for each kind of 3' terminus.
6. H. Koster and K. Heyns, Tetrahedron Letters, 1531 (1972).
7. C. B. Reese and J. E. Sulston, Proc. Chem. Soc., 214 (1964).
8. Our choice of uridine as the linker in solid phase work was dictated by the ready availability of the appropriately blocked precursors. We do not think that lack of protection at the uracil moiety of the linking nucleoside in our supports creates any problems during chain elongation reactions. Nevertheless, we are examining the blocking of this base with the anisoyl group, which we have been using effectively for uridine protection during syntheses of oligoribonucleotides in solution (S. H. Gray, unpublished experiments).
9. K. Miyoshi, T. Miyake, T. Hozumi, and K. Itakura, Nucleic Acids Res. 8, 5473 (1980).
10. B. E. Griffin, M. Jarman, C. B. Reese, and J. E. Sulston, Tetrahedron 23, 2301 (1967).
11. A sample of this compound gave a satisfactory elemental analysis and, upon exposure to NH_4OH overnight, yielded 2',3'-O-methoxymethyleneuridine as the only nucleoside product.
12. H. P. M. Fromageot, B. E. Griffin, C. B. Reese, and J. E. Sulston, Tetrahedron 23, 2315 (1967).
13. The hexamer was built up on the glass by three successive additions of the dinucleotide $[(MeO)_2Tr]$dbzA$\underline{p}$dbzA-(ClPh), ($\underline{p}$ represents a p-chlorophenyl phosphotriester linkage), using our previously described methodology[3]. Since the first addition was to a 2' or 3' secondary hydroxyl of the support-bound uridine, we allowed twice the normal reaction time for this initial condensation. The unreacted excess of dinucleotide from each cycle was recovered as its barium salt by our usual procedure[3]. Although barium salts are routinely employed in this laboratory for condensations carried out in solution and for recovery of excess nucleotides in solid phase syntheses, their tendency to form precipitates or gels is a drawback during addition reactions on solid supports, especially in automated systems. For this particular purpose, we therefore prefer to simply convert them to their tetraethylammonium forms which are then used without isolation. Oligonucleotide synthesis employing the latter salts has been described in an earlier publication[14].
14. G. R. Gough, C. K. Singleton, H. L. Weith, and P. T. Gilham, Nucleic Acids Res. 6, 1557 (1979).
15. The yield is based on the amount of the first dinucleotide added to the glass. Analysis using a spleen phosphodiesterase digestion method described earlier[16] gave a hyperchromicity of 48.6% at 260 nm (pH 6.5, 25°) and a base ratio of dAp:dA=5.0:1.0.
16. G. R. Gough, M. J. Brunden, J. G. Nadeau, and P. T. Gilham, Tetrahedron Letters 23, 3439 (1982).
17. We have also been able to attach uridine 5'-succinyl residues (at a level of 9 μmol/g) to non-porous glass, in the form of microfibre filter circles (Whatman GF/C, 2.4 cm) substituted with aminopropyl groups. Clearly, the general procedure outlined above should be adaptable to many other types of support.

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