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HIGH-YIELD SYNTHESIS OF OLIGORIBONUCLEOTIDES USING _o_-NITROBENZYL PROTECTION OF 2'-HYDROXYLS

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Summary: Use of published procedures for photolytic removal of 2'-_O_-(_o_-nitrobenzyl) substituents from model oligoribonucleotides results in low yields of fully-deprotected products accompanied by significant amounts of oligomers carrying altered, ultraviolet light-resistant residues derived from the 2'-blocking groups. The efficiency of the deprotection has been found to depend on pH; the side-reactions are avoided when the photolysis is carried out in solution buffered at pH 3.5.

The failure of systems for oligoribonucleotide synthesis to keep pace with advances made in the construction of oligodeoxyribonucleotides in recent years can be attributed primarily to the lack of a completely satisfactory protection mode for ribonucleoside 2'-hydroxyls. Nevertheless, a number of diverse blocking groups have been employed, with varying degrees of success, to prevent reaction at these sites. The most useful constitute ketals and acetals in the form of methoxytetrahydropyranyl[1], tetrahydropyranyl[2], and tetrahydrofuranyl[3] substituents, or ethers such as the _t_-butyldimethylsilyl[4], _o_-nitrobenzyl[5], and _p_-methoxybenzyl[6] derivatives. For our own oligoribonucleotide syntheses, we favor the photolabile _o_-nitrobenzyl group, first adopted for ribonucleoside protection in 1974[7,8] and used extensively for preparation of oligomers by Ohtsuka, Ikehara and their colleagues. In our opinion, this function possesses a wider range of desirable characteristics than any of the others mentioned above. Specifically, the nitrobenzyl ether linkage is resistant to both acid and base, and therefore satisfies an important requirement of oligoribonucleotide synthesis by remaining intact throughout all of the commonly used construction and deprotection steps, until its final cleavage by irradiation with long-wave UV light. Moreover, the nitrobenzyl group shows no tendency to migrate from the 2'- to the 3'-hydroxyl, nor does it possess a chiral center. It should therefore be less trouble to synthesize pure 2'-_O_-substituted nucleosides in the absence of complications associated with the formation of position isomers[9] and diastereomers. However, despite these significant advantages, it is evident from reports in the literature that, when this method of 2'-protection is used, variable yields of oligoribonucleotides are obtained. We decided to investigate this drawback, using a previously described scheme for oligoribonucleotide synthesis[10] based on the one we have employed successfully for several years to construct defined sequence oligodeoxyribonucleotides in solution[11,12]. By these methods, we were able to

prepare fully protected ribo-oligomers without difficulty, but by following the published procedures for photolytic removal of o-nitrobenzyl groups in the last stage of the deblocking process, we consistently obtained low yields of the desired products. The problem is exemplified by the deprotection of [$(MeO)_2$Tr]bzA(NBzl)≗bzA(NBzl)≗bzA(NBzl)≗bzC$(Bz)_2$[13]. For preparation of this tetranucleotide, the 5'-O-dimethoxytrityl derivative of 2'-O-(o-nitrobenzyl)-6-N-benzoyladenosine[14,15] was synthesized and converted by a rapid phosphorylation technique[16] into its 3'-(p-chlorophenyl phosphate); the product, [$(MeO)_2$Tr]bzA(NBzl)-(ClPh), was isolated as the analytically pure barium salt[11] and used in turn to construct the dimer [$(MeO)_2$Tr]bzA(NBzl)≗bzA(NBzl)≗(CNEt) by the methods we employ to synthesize the analogous deoxyribo-dinucleotides[11]. The dimer, obtained in 89% yield, was detritylated and condensed with [$(MeO)_2$Tr]bzA(NBzl)-(ClPh) barium salt under the action of toluenesulfonyl nitrotriazole (TSNT) to give [$(MeO)_2$Tr]bzA(NBzl)≗bzA(NBzl)≗bzA(NBzl)≗(CNEt) in 77% yield. This trinucleotide was then decyanoethylated and allowed to react with $N^4,O^{2'},O^{3'}$-tribenzoylcytidine and TSNT, giving the tetramer [$(MeO)_2$Tr]bzA(NBzl)≗bzA(NBzl)≗bzA(NBzl)≗bzC$(Bz)_2$ in 88% yield.

A sample of the tetramer (5.5 μmol) was treated with 0.2 ml of 1 M tetramethylguanidinium pyridine-2-aldoximate in dioxane-water (1:1 v/v) for 48 h at 25°. Pyridine (2 ml) and concentrated ammonia (20 ml) were then added. The mixture was allowed to stand for a further 48 h, then evaporated to an oil. Traces of pyridine were removed by coevaporation with water and the residue was dissolved in 40% ethanol (10 ml). An aliquot (2 ml) of this solution was concentrated to dryness and treated with 10 ml of acetic acid-water (4:1 v/v) for 20 min. The

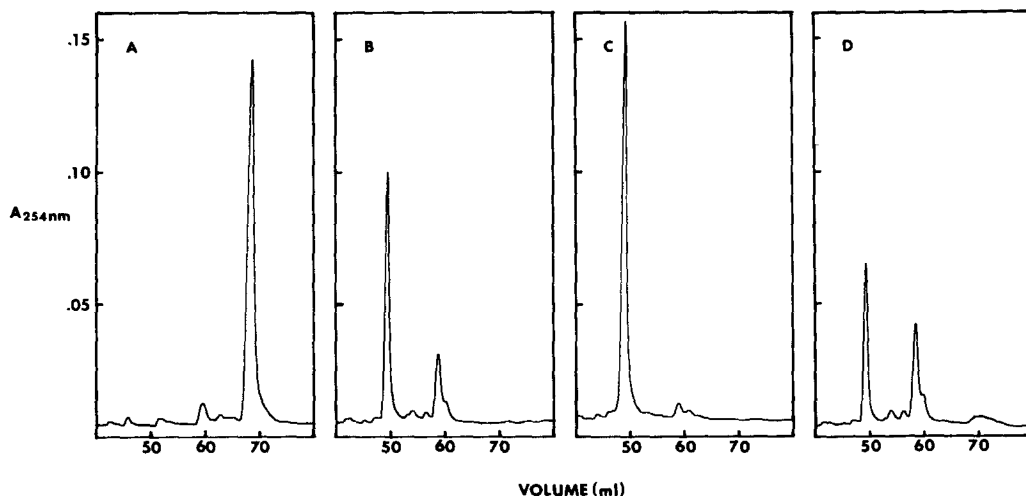

FIGURE 1. Chromatographic elution profiles of (A) the tetramer A(NBzl)-A(NBzl)-A(NBzl)-C; and the tetramer after exposure to long-wave ultraviolet light for 1 h in (B) water, (C) 0.1 M ammonium formate, pH 3.5, and (D) 0.2 M N-methylmorpholinium formate, pH 7.5. Column: cross-linked polyethyleneimine-silica[24] (1 X 25 cm). Solvent: 150 ml of aqueous 0.05 M KH_2PO_4 containing 30% (v/v) MeOH and a linear gradient of 0 - 0.4 M $(NH_4)_2SO_4$ at pH 6; flow rate: 2 ml/min.

acid was removed in vacuo and, after coevaporation with water, the residue was dissolved in 40% ethanol (2 ml). Half of this solution was desalted by passage through a column of Sephadex G-10, and A(NBzl)-A(NBzl)-A(NBzl)-C (20.5 A_{260} units) was obtained; its high purity at this stage is shown by its HPLC profile (Figure 1A). For removal of the nitrobenzyl groups, a small amount of the tetramer (0.31 A_{260} units) was subjected to photolysis following the method recommended in the literature[7,17], which calls for irradiation in water with long-wave ultraviolet light. The result, after 1 h, is shown in Figure 1B. Fully deprotected A-A-A-C, with a retention volume of 50 ml, is accompanied by significant amounts of side-products at 58-61 ml ; the latter material was unchanged after exposure to UV light for a further 1 h. We have observed similar side-products in irradiation experiments involving other sequences, when they are performed under these conditions.

For a detailed study of the deprotection in a simpler system, we synthesized a model dimer containing only one nitrobenzyl group, adjacent to the internucleotide linkage. This substance, A(NBzl)-U, was irradiated under a variety of conditions and it quickly became apparent that the pH of the photolysis reaction was a critical factor in the efficiency of the deblocking. The products of each reaction were analyzed by anion-exchange chromatography on a column (1 X 20 cm) of Polyanion SI-17 (Pharmacia Fine Chemicals) with 200 ml of 40% aqueous EtOH containing a linear gradient of 0.025 M NH_4OAc-0.0125 M AcOH to 0.1 M NH_4OAc-0.05 M AcOH as the eluting solvent. In this system, A(NBzl)-U and A-U have elution volumes of 68 ml and 94 ml, respectively. Irradiation at pH 3.5 cleanly converted the blocked dimer to A-U, whereas separate experiments at progressively higher pH generated increasing amounts of a side-product (elution volume: 68 ml) that was found to be resistant to ultraviolet cleavage. At pH 8.5 the yield of this material reached 35-40%, permitting its isolation in reasonable quantity by preparative paper chromatography with *n*-BuOH-AcOH-H_2O (5:2:3, v/v) as solvent system. Preliminary studies[18] on its structure indicate that it breaks down in alkaline solution giving A-U and *o*-nitrobenzoic acid as the major products.

The acidic conditions for deprotection were then used with the longer molecule A(NBzl)-A(NBzl)-A(NBzl)-C; a quantity of this material equal to that used in the unbuffered deblocking was irradiated for 1 h in 0.3 ml of 0.1 M ammonium formate at pH 3.5, and the resulting mixture was analyzed by HPLC. The deprotection was essentially complete (Figure 1C)[19,20]. On the other hand, a photolysis experiment carried out on a solution of the tetramer buffered at pH 7.5 with 0.2 M *N*-methylmorpholinium formate (Figure 1D) gave even larger amounts of side-products than were generated in water alone. With these results, we conclude that lack of awareness of the need for pH control during irradiation accounts for the less than optimal yields that have been reported for oligoribonucleotide syntheses employing *o*-nitrobenzyl blocking groups.

In light of the above, *o*-nitrobenzyl substitution now appears considerably more attractive as a protection system for 2'-hydroxyl functions. We plan to apply the new findings to the construction of ribo-oligonucleotides containing all four bases, both in solution and on solid supports.

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20. In the course of these studies, we also found that it was unnecessary to separate the oligomer from the deblocking reagents prior to photolysis: a sample of the crude deprotection mixture before acetic acid treatment was adjusted to pH 3.5 with formic acid, then irradiated for 1 h. The result was equivalent to that obtained with the desalted oligomer at pH 3.5. It was evident that the acidic conditions had removed the 5'-dimethoxytrityl group concomitantly with photolytic cleavage of the nitrobenzyls.
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