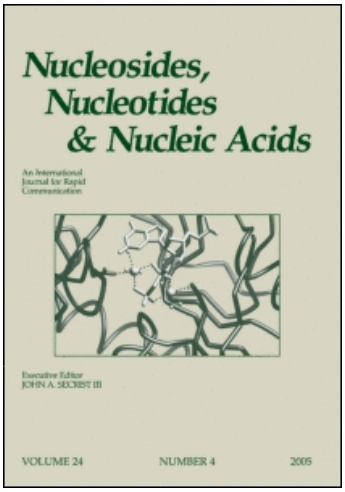

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The Problem of 2'-Protection in Rapid Oligoribonucleotide Synthesis

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THE PROBLEM OF 2'-PROTECTION IN RAPID OLIGORIBONUCLEOTIDE SYNTHESIS

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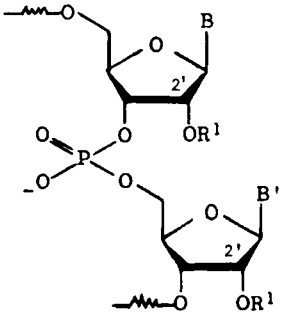
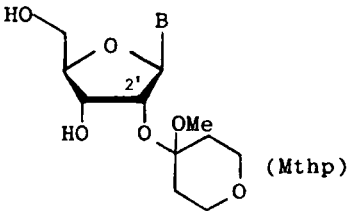
Abstract. An acetal protecting group derived from 1-(2-chloro-4-methylphenyl)piperidin-4-one (12a) has been developed for the blocking of 2'-hydroxy functions in rapid oligoribonucleotide synthesis.

The development of effective and convenient methods for the rapid synthesis of oligo- and poly-deoxyribonucleotides of defined sequence[1,2] undoubtedly represents one of the most significant achievements in nucleic acid chemistry in recent years. The problem in this field that arguably most merits attention at the present time is the development of equally effective and convenient methods for the rapid synthesis of oligo- and poly-ribonucleotides.



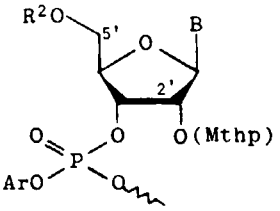
(1)



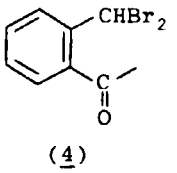
(2)

The most crucial decision that has to be made in the synthesis of oligoribonucleotides (e.g. 1; R^1=H) is the choice of the protecting group (R^1) for the 2'-hydroxy functions[3]. It is essential that this protecting group should remain intact until the final unblocking step at the end of the synthesis, and then be removable under very mild conditions such that the released 2'-hydroxy functions do not attack vicinal phosphodiester groups and thereby promote cleavage or migration of the internucleotide linkages. In 1967, we introduced[4] the achiral 4-methoxytetra-

hydropyran-4-yl [Mthp, as in (2)] group specifically for 2'-protection and, since then, we and other workers have used it for the synthesis of oligoribonucleotides by the phosphotriester approach in solution. For example, we have very recently used[5] the latter protecting group in the successful synthesis of the 3'-terminal nonadecamer and heptatriacontamer (37-mer) sequences of yeast $tRNA^{Ala}$. The Mthp group is relatively easy to introduce and it cannot migrate (see below); furthermore, it may be removed at the end of the synthesis under very mild conditions of acidic hydrolysis that do not affect the internucleotide phosphodiester linkages[6]. An important part of our strategy is that, in order to avoid possible degradation by traces of contaminating phosphorylytic enzymes (e.g. ribonucleases), the synthetic oligo- and poly-ribonucleotides are purified and stored with their 2'-hydroxy functions protected. It must then be possible to unblock the latter hydroxy functions cleanly and quantitatively, and thereby obtain pure, fully unprotected products. When all of the latter considerations are taken into account, the Mthp group emerges as being particularly suitable for the protection of 2'-hydroxy functions in oligoribonucleotide synthesis.



(3) a; R^2 = H
b; R^2 = (4)
c; R^2 = (5)



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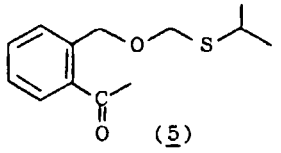
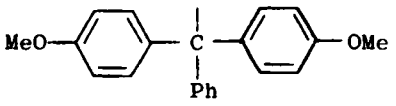


(5)

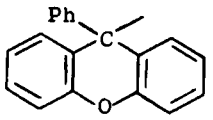
Ar = 2-chlorophenyl

In a stepwise synthesis either on a solid support or in solution, it must be possible selectively to remove the protecting group (R^2) from the 5'-terminal hydroxy function of the growing oligoribonucleotide [e.g. (3)]. If the 2'-hydroxy functions are protected with acid-labile (Mthp) groups, it would not at first sight seem sensible also to use an acid-labile group for the protection of the 5'-terminal hydroxy function. Indeed, in our previous work on the synthesis of oligo- and poly-ribonucleotides[7,8] in solution, we successfully used the 2-dibromomethylbenzoyl[9] [Dbmb, as in (3b)] and 2-(isopropylthiomethoxymethyl)benzoyl[5,10] [Ptmt, as in (3c)] 'protected' protecting groups[11], which are removable essentially under very mildly basic conditions, to block the 5'-terminal

hydroxy function. For example, in our synthesis of the 3'-terminal heptatriacontamer sequence of yeast $tRNA^{Ala}$, we were able[5] selectively to remove the Ptmt group [as in (3c)] from a fully-protected nonadecaribonucleoside octadecaphosphate containing eighteen Mthp groups and eighteen 2-chlorophenyl protected internucleotide linkages, and isolate the product in 88% yield. The first step of the removal of 'protected' protecting groups[11], such as Dbmb[9] [as in (3b)], requires the use of heavy metal ions and can involve heterogeneous reaction media which, in turn, can lead to slow reactions. Mainly for this reason, the Dbmb group was found to be unsuitable for 5'-protection in the rapid synthesis of oligodeoxyribonucleotides in solution by the 'filtration' method[12], and it is very likely that it would be even more unsuitable for this purpose in solid phase synthesis.



(6)



(7)

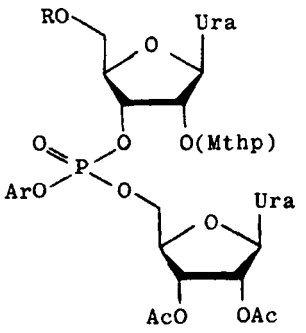
An essential requirement in solid phase synthesis is that the 5'-terminal protecting group (R^2) of the growing oligonucleotide (3) should be rapidly and quantitatively removable at the end of a synthetic cycle. In order to ensure this, virtually every worker in this field has used either the 4,4'-dimethoxytrityl[13] [DMTr (6), as in (3; R^2 = (6))] or the 9-phenylxanthen-9-yl[14] [Px (7); as in (3; R^2 = (7))] group to protect the 5'-terminal hydroxy functions. The latter protecting groups are both readily and quantitatively removable under relatively mild acidic conditions. If a successful rapid synthesis of oligoribonucleotides on a solid support is to be developed, there does not, at present, appear to be any satisfactory alternative to these modified trityl groups [e.g. DMTr (6) and Px (7)] for the protection of 5'-terminal hydroxy functions. It was therefore a matter of considerable importance to determine whether or not the use of 4,4'-dimethoxytrityl (6) and 9-phenylxanthen-9-yl (7) is compatible with the use of Mthp or related acetal groups (such as tetrahydropyranyl) for the protection of 2'-hydroxy functions. Recent studies carried out both by ourselves[15] and other workers[16] unfortunately have clearly indicated that 5'-O-DMTr and 5'-O-Px groups are unlikely to be compatible with 2'-O-Mthp (or tetrahydropyranyl) protecting groups in solid phase oligoribonucleotide synthesis. Therefore, if DMTr (or Px)

is to be used as the 5'-protecting group, an alternative to the Mthp (or tetrahydropyranyl) group must be found for the protection of the 2'-hydroxy functions.

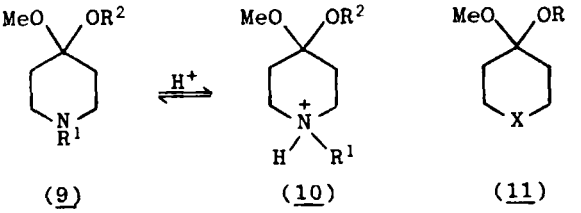
Acyl groups[17] and the t-butyldimethylsilyl (TBDMS) group[18] readily migrate from the 2'- to the 3'-hydroxy functions (and *vice versa*) of ribonucleoside derivatives under mildly basic conditions. Nevertheless, the TBDMS group has been used for the protection of the 2'-hydroxy functions in oligoribonucleotide synthesis both in solution[19] and on a solid support[20]. It is, however, essential to ensure that the internucleotide linkages are exclusively 3'→5', and for this reason we believe that it is highly desirable to base a synthesis on nucleoside building blocks that are protected on their 2'-hydroxy functions with groups that cannot migrate. Ikehara and his co-workers[21] have used the photo-labile 2-nitrobenzyl group for the protection of the 2'-hydroxy functions. Although the latter protecting group has recently been reported[22] to undergo light-promoted removal readily at pH 3.5, it is not clear that the unblocking of high molecular weight 2-nitrobenzyl protected oligo- and poly-nucleotides would go to completion under these conditions and, in any case, it would be difficult to monitor the unblocking process. For the reason indicated above, it is advisable to store oligoribonucleotides with their 2'-hydroxy functions protected, and it is then important that the unblocking conditions required should be such that the 2'-protecting groups can be removed swiftly and cleanly, and without any damage to the resulting products. While we are confident that the conditions of acidic hydrolysis required[6] to remove Mthp groups fully meet the latter requirements, it is by no means clear from the literature that this is the case for the removal of 2'-O-(t-butyldimethylsilyl)[19,20] and 2'-O-(2-nitrobenzyl)[22] groups from the corresponding partially-protected oligoribonucleotides. We therefore decided to attempt to find an acid-labile protecting group for the 2'-hydroxy functions that could be used in conjunction with a 5'-O-Px (or DMTr) group in rapid oligoribonucleotide synthesis.

In the final unblocking step of oligoribonucleotide synthesis, the 2'-O-Mthp (or tetrahydropyranyl) protecting group is generally removed[6] under relatively mild conditions, for example, by treatment with 0.01 M-hydrochloric acid at room temperature. In solid phase oligodeoxyribonucleotide synthesis, the 5'-O-DMTr (or Px) protecting group is usually removed by treatment with a relatively strong protic acid (e.g. dichloroacetic acid[23], pK_a 1.48). Unless very great care is taken to exclude traces of moisture, some concomitant removal of the Mthp groups is likely

to occur during each 5'-unblocking step. Recent studies[15,24] have indicated that the release, under acidic conditions, of a 2'-hydroxy function vicinal to a 2-chlorophenyl protected internucleotide linkage leads to a series of very fast reactions that result largely in the cleavage and migration of that internucleotide linkage. This problem could become very serious as the chain length and the number of Mthp groups increases. The difficulty in selective 5'-unblocking was clearly demonstrated[15] by treating the fully-protected dinucleoside phosphate (8a) with an eightfold excess of phenyl dihydrogen phosphate (pK_a 1.25) in chloroform-ethanol (95:5 v/v) solution for the minimum time required (5 min) to remove the 5'-O-Px group. Following work-up and chromatography of the products, (8b) was isolated only in 54% yield.



(8) a; R = Px
b; R = H



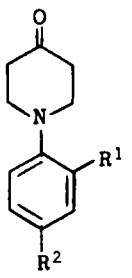
(9) (10) (11)

Ar = 2-chlorophenyl

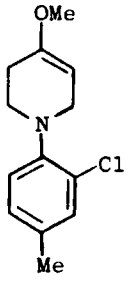
Acetal hydrolysis is subject to specific acid catalysis[25], and its rate is dependent on the concentration of hydrogen ions. Thus, as expected, the rate of removal of the protecting group from a 2'-O-Mthp derivative of a ribonucleoside [e.g. the uridine derivative (2; B = uracil-1-yl)] increases sharply[26] with decreasing pH. It occurred to us that, if we could design an acetal system that had hydrolysis properties similar to that of the Mthp protecting group in the region of pH 2 but which did not undergo hydrolysis at an increased rate at lower pH, we should have found a solution to the problem of the selective removal of a 5'-terminal Px (or DMTr) protecting group in rapid oligoribonucleotide synthesis. It seemed possible that an acetal system (9) derived from a 1-substituted piperidin-4-one would have the desired properties if R^1 were chosen in such a way that the tertiary amino function were weakly basic. If, for example, the latter function had pK_a = 2.0, at pH 2.0 the nitrogen atom would be half protonated [as in (10)] and half

unprotonated [as in (9)]. Depending, of course, on the nature of R^1, it seemed likely that (9) would undergo hydrolysis at pH 2 at a rate that was of the same order of magnitude as the corresponding Mthp derivative (11; X = O, R = R^2). However, due to the large inductive effect of the positively charged nitrogen, (10) would be expected to undergo hydrolysis at a rate several orders of magnitude slower than that of (9). The sensitivity of these acetal systems to such inductive effects is clearly illustrated by the observation[27] that the thioether system (11; X = S) undergoes acid-catalyzed hydrolysis at a rate more than three orders of magnitude faster than that of the corresponding sulphone (11; X = SO_2). If the tertiary amino function of (9) had, say, pK_a = 2.0 and the pH were decreased from 2.0 to 1.0, its rate of hydrolysis would increase by a factor of 10 but the concentration of (9) would decrease by the same factor. If it can be assumed that the much stabler piperidinium acetal (10) undergoes hydrolysis at negligible rates both at pH 1.0 and 2.0, the rate of hydrolysis of the acetal system would then be independent of pH.

We soon found[26] that the acetal system derived from 1-(2-chloro-4-methylphenyl)piperidin-4-one (12a) had almost precisely the hydrolysis properties that we were seeking. The latter compound (12a) which was



(12) a; R^1 = Cl, R^2 = Me
b; R^1 = H, R^2 = Me
c; R^1 = R^2 = H



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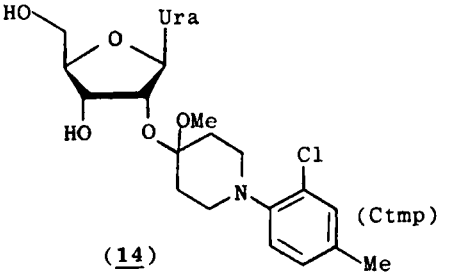
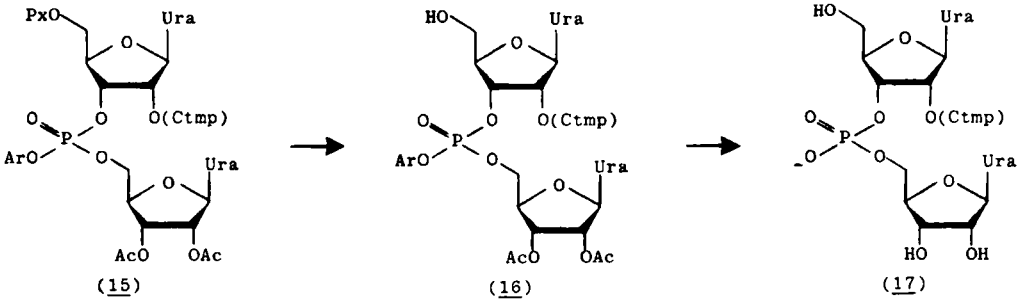


(14)

prepared[26] from p-toluidine by a four-step procedure in 37% overall yield was converted into its enol ether (13) in 67% yield. When 3',5'-di-O-acetyluridine was treated with an excess of enol ether (13) in the presence of trifluoroacetic acid in dioxane solution and the products then treated with methanolic ammonia, 2'-O-[1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl] uridine (14) was obtained[26] as a crystalline solid, m.p. 185-186°C, in 85% overall yield. At 25°C, the half-times ($t_{\frac{1}{2}}$) of hydrolysis of (14) were found[26] to be 33.5, 35.5, 35, 41, 52 and

80 min at pH 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0, respectively. It is evident from these data that the rate of removal of the 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp) protecting group hardly changes between pH 2.0 and 0.5, and that it is only *ca.* 1.5 times faster at pH 0.5 than it is at pH 2.5. Furthermore, in the pH range 2.0 - 2.5, the rate of hydrolysis of the Ctmp is similar to that of the Mthp group [at 25°C, $t_{\frac{1}{2}}$ = 20.5 and 126 min at pH 2.0 and 3.0, respectively][26]; it is, in fact, somewhat more stable at pH 2.0 and probably more labile at pH 2.5.



(15) (16) (17)

The 2'-O-Ctmp derivative of uridine (14) was converted[26] into the fully-protected dinucleoside phosphate (15) by standard procedures and in good yield. As anticipated, it was possible to remove the 5'-O-Px protecting group from (15) with a high degree of selectivity; when a 0.025 M - solution of (15) in dichloromethane was treated with trifluoroacetic acid (5.5 mol. equiv.) and pyrrole[26] (16.5 mol. equiv.) for 30 seconds at room temperature, no starting material remained and (16) was isolated from the products in 95.5% yield[26]. This contrasts with the relatively poor yield obtained[15] in the corresponding conversion of (8a) into (8b) (see above). Furthermore, when the unblocking reaction of (15) was allowed to proceed for 30 minutes (i.e. sixty times the time required for complete removal of the Px group), (16) was isolated from the products in 85% yield[26]. The latter yield was increased to 90% when[28] stringent precautions were taken to exclude traces of moisture. Unblocking of (16) in the usual way gave (17) which was converted[26] into uridylyl-(3'→5')-uridine by treatment with 0.01 M - hydrochloric acid at 25°C ($t_{\frac{1}{2}}$ = 21.5 and 23.5 min at pH 2.0 and 2.5, respectively).

We are at present engaged in the solid phase synthesis of oligoribonucleotides, starting with nucleoside building blocks that are protected on their 2'- and 5'-hydroxy functions with Ctmp and Px protect-

ing groups, respectively, and we intend to use the same building blocks in synthesis by the 'filtration' method[12]. We believe that the Ctmp group, and possibly acetal systems derived from other halogenated 1-aryl-piperidin-4-ones, will prove to be the protecting groups of choice for the rapid synthesis of oligoribonucleotides. For this reason, we are actively engaged in an attempt to simplify the preparation of the enol ether reagents [e.g. (13)] required for the synthesis of the necessary building blocks.

Acknowledgements

I should like to acknowledge the outstanding contributions that a number of my co-workers have made to our studies in the synthesis of oligo- and poly-ribonucleotides. Some of their names appear in the references below. I should also like to thank the Science and Engineering Research Council for generous financial support.

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