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THE PROTECTION OF 2'-HYDROXY FUNCTIONS IN OLIGORIBONUCLEOTIDE SYNTHESIS

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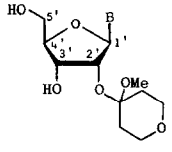
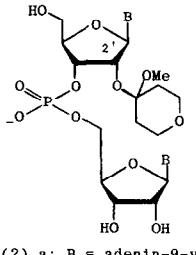
Summary: The suitability of the 4-methoxytetrahydropyran-4-yl group for the protection of 2' (or 3')-hydroxy functions in oligoribonucleotide synthesis is confirmed; the latter protecting group is removed in 0.01*M* - hydrochloric acid at room temperature under conditions which, contrary to a recent report, lead to no detectable cleavage or migration of the internucleotide phosphodiester linkages.

Although there has recently been significant progress in the methodology of oligoribonucleotide synthesis[1], the results obtained have been less striking than in the deoxy-series. This is partly due to the fact that the problem of hydroxy function protection[2] is necessarily more complex in the ribose series. It is of particular importance that the 2'-hydroxy functions should remain protected throughout the assembly of the desired nucleotide sequence and then be released only in the final unblocking step. We first investigated the use of the acid-labile tetrahydropyranyl group[3] for the protection of 2'-hydroxy functions and subsequently introduced the achiral 4-methoxytetrahydropyran-4-yl [methoxytetrahydropyranyl, as in 2'-O-methoxytetrahydropyranyladenosine (1a)] group[4] for this purpose. Although we have investigated the possibility of using other protecting groups[5], we have, in recent years[1,9], used exclusively the methoxytetrahydropyranyl group for the protection of 2'-hydroxy functions in oligoribonucleotide synthesis.



(1) a; B = adenin-9-yl
b; B = uracil-1-yl
c; B = cytosin-1-yl
d; B = guanin-9-yl



(2) a; B = adenin-9-yl
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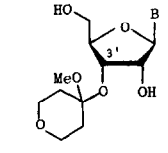
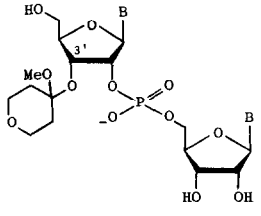


(3) a; B = adenin-9-yl
b; B = uracil-1-yl



(4) a; B = adenin-9-yl
b; B = uracil-1-yl

As it seems very likely that much more effort will, in the future, be put into the chemical synthesis of relatively high molecular weight oligo- and poly-ribonucleotides, it is a matter of some urgency that any group selected to protect the 2'-hydroxy functions should be subjected to a most thorough investigation. A particularly critical requirement for a 2'-hydroxy protecting group is that it should be easily and virtually quantitatively removable under conditions under which unprotected oligo- and poly-ribonucleotides are completely

stable. In this paper, we present new evidence which confirms that the methoxytetrahydropyranyl group fully meets this requirement.

We have generally carried out the final unblocking step in oligoribonucleotide synthesis, that is the removal of the 2'-O-methoxytetrahydropyranyl[4] and terminal 2',3'-O-methoxymethylene[10] protecting groups, by treatment with 0.01*M* - hydrochloric acid (pH 2) at room temperature for 6 hr. It can be seen from Table 1 that the half-times ($t_{\frac{1}{2}}$) for the hydrolysis of the 2'-O-methoxytetrahydropyranyl derivatives [(1a)-(1d)] of the four common ribonucleosides in 0.01*M* - hydrochloric acid at 22°C are 34, 18.7, 40 and 35 min, respectively [Table 1, entries nos. 1-4]. From these data, it can be seen that the 2'-O-methoxytetrahydropyranyl protecting group will be 99% removed from the least easily hydrolyzed of the nucleoside derivatives [i.e. (1c)] after *ca.* 4.5 hr and 99.9% removed from it in under 7 hr [$10t_{\frac{1}{2}}$ for (1c) = 400 min] under the latter conditions. However, as was observed a number of years ago in the case of 2'-O-tetrahydropyranyl derivatives[3b], the presence of a vicinal 3'→5'-phosphodiester internucleotide linkage appears to facilitate the hydrolysis of a 2'-O-methoxytetrahydropyranyl group. Thus (2a) undergoes hydrolysis at pH 2.0 at a rate *ca.* 1.7 times as fast as that of (1a) [Table 1, entries nos. 5 and 1, respectively] and (2b) undergoes hydrolysis under the same conditions at a rate which is more than three times as fast as that of (1b) [entries nos. 6 and 2, respectively]. If the smaller phosphodiester rate enhancement effect observed in the case of adenosine [entries nos. 1 and 5] were assumed also to obtain for cytidine and guanosine [entries nos. 3 and 4, respectively] and if it is assumed that the rate of unblocking of methoxytetrahydropyranyl groups does not decrease with increasing oligonucleotide size, then the time required for 99.9% removal of all of the 2'-O-methoxytetrahydropyranyl protecting groups in 0.01*M* - hydrochloric acid at 22°C from an otherwise unprotected[11] oligoribonucleotide would be *ca.* 4 hr. It would therefore appear that the reaction conditions which we have normally used[1,9] (see above) in the final unblocking step of oligoribonucleotide synthesis are quite satisfactory. It can also be seen from Table 1 [entries nos. 7-10] that the removal of the methoxytetrahydropyranyl protecting group from 3'-O-methoxytetrahydropyranyladenosine (3a), 3'-O-methoxytetrahydropyranyluridine (3b), and from the corresponding dinucleoside phosphates [(4a) and (4b)] occurs at rates which are between *ca.* 3.0 and 5.4 times faster than the rates of hydrolysis of the corresponding 2'-protected isomers [(1a), (1b), (2a) and (2b); entries nos. 1, 2, 5 and 6, respectively]. These data are of importance in connection with the synthesis[12] of 2-5A and its analogues.

TABLE 1. Removal of 2'- and 3'-O-Methoxytetrahydropyranyl Protecting Groups[a]

Entry No.	Substrate	pH	$t_{\frac{1}{2}}$(min)[b]	$t_{0.99}$(min)[c]	Entry No.	Substrate	pH	$t_{\frac{1}{2}}$(min)[b]	$t_{0.99}$(min)[c]
1	(1a)	2.0	34	226	6	(2b)	2.0	6.1	41
2	(1b)	2.0	18.7	124	7	(3a)	2.0	7.4	49
3	(1c)	2.0	40	266	8	(3b)	2.0	6.3	44
4	(1d)	2.0	35	233	9	(4a)	2.0	3.7	25
5	(2a)	2.0	19.9	132	10	(4b)	2.0	1.7	11

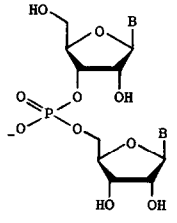
[a] Hydrolysis reactions were carried out in 0.01*M* - hydrochloric acid (pH 2) at 22°C. Substrates (*ca.* 0.5 - 1.0 mg) were dissolved in hydrochloric acid (3.0 ml). Aliquots (0.2 ml) of the reaction solutions were removed after suitable intervals of time, neutralised with triethylammonium bicarbonate buffer and analyzed by h.p.l.c. [Zorbax ODS column, isocratic elution with methanol-water].

[b] Pseudo first order kinetics were observed for all reactions ($t_{\frac{1}{2}}$ = half-time): straight lines were obtained when logarithms of the percentages of substrates remaining were plotted against time.

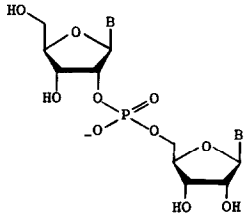
[c] $t_{0.99}$ represents the time required for 99% removal of the methoxytetrahydropyranyl group; the time required for 99.9% removal is *ca.* $10t_{\frac{1}{2}}$.

As the action of aqueous acid on oligoribonucleotides can lead both to internucleotide cleavage and phosphoryl migration, we had previously carried out a very careful study and had

come to the conclusion[3b] that, while uridylyl-(3'→5')-uridine (5b) underwent both internucleotide cleavage and isomerization [to give uridylyl-(2'→5')-uridine (6b)] at measurable rates in 0.1*M*-hydrochloric acid (pH 1) at 25°C, both of these reactions proceeded at negligibly slow rates in 0.01*M*-hydrochloric acid (pH 2) under the same conditions. We were therefore surprised by the recent claims[13] that treatment of adenylyl-(3'→5')-adenosine (5a) with 0.01*M*-hydrochloric acid at 20°C for 6 hr leads to almost 2% internucleotide cleavage[14] and *ca.* 0.08% isomerization to adenylyl-(2'→5')-adenosine (6a) and that, under the same conditions [0.01*M*-hydrochloric acid, 6 hr, 20°C], adenylyl-(2'→5')-adenosine (6a) undergoes *ca.* 4% internucleotide cleavage[14] and *ca.* 0.6% isomerization to adenylyl-(3'→5')-adenosine (5a). We now report the results of our own studies with the latter substrates.



(5) a; B = adenin-9-yl
b; B = uracil-1-yl



(6) a; B = adenin-9-yl
b; B = uracil-1-yl

The course of the reaction between adenylyl-(3'→5')-adenosine (5a) and 0.01*M*-hydrochloric acid (pH 2) at 23°C is illustrated in Fig. 1: after 6 hr (Fig. 1a), only one component (R_T 12.5 min), corresponding to (5a) was observed by liquid chromatography; after 96 hr (Fig. 1b), (5a) (R_T 11.7 min)[15] and adenosine (R_T 17.5 min, <5%) were detected and, after 240 hr (Fig. 1c), (5a) (R_T 10 min)[15] had been substantially converted into adenosine (R_T 16.8 min, *ca.* 45%). Trace (≯1%) components with R_T's 2.5 and 6.0 min could also be detected. We believe that the observed conversion of (5a) into adenosine at pH 2 is due to contamination with a microorganism or with enzymes rather than to the action of acid. The course of the reaction between adenylyl-(2'→5')-adenosine (6a) and 0.01*M*-hydrochloric acid (pH 2) at 23°C is illustrated in Fig. 2. Virtually no hydrolysis or isomerization can be detected after 6 and 200 hr (Figs. 2a and 2b) and, indeed, even after 504 hr (Fig. 2c).

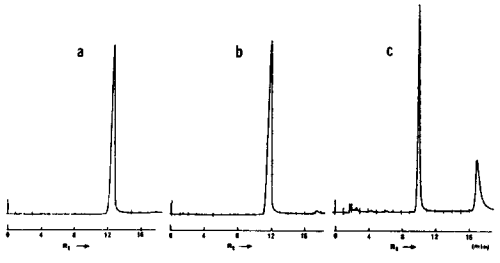


FIGURE 1. Liquid chromatograms [Zorbax ODS column, linear gradient, H_2O → MeOH: H_2O (1:1 v/v) over 20 min] obtained after a solution of adenylyl-(3' → 5')-adenosine (5a; 10 A_{260} units) in 0.01 M-hydrochloric acid (pH 2, 2.0 ml) had been allowed to stand at 23°C for (a) 6 hr, (b) 96 hr, and (c) 240 hr.

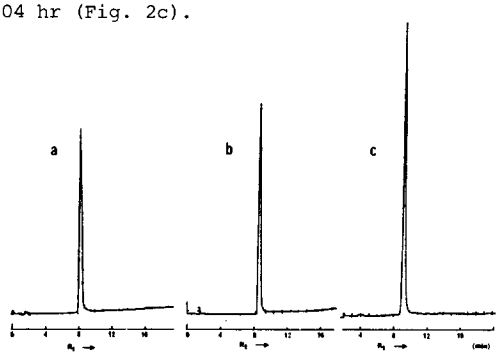


FIGURE 2. Liquid chromatograms [Zorbax ODS column, linear gradient, H_2O → MeOH: H_2O (1:1 v/v) over 20 min] obtained after a solution of adenylyl-(2' → 5')-adenosine (6a; 10 A_{260} units) in 0.01 M-hydrochloric acid (pH 2, 2.0 ml) had been allowed to stand at 23°C for (a) 6 hr, (b) 200 hr, and (c) 504 hr.

Apart from the observation of the putative enzyme-promoted digestion of adenylyl-(3'→5')-adenosine (5a) which appeared to occur after an initial induction period, we are therefore able to confirm our earlier conclusions[3b] regarding the relative stability of the inter-

nucleotide linkages of oligoribonucleotides in 0.01*M* - hydrochloric acid at room temperature. The results of both of our investigations are in complete contrast to those contained in the recent report[13] referred to above, and we can only conclude that the latter is based on incorrect experimental data. Finally, we believe that the present study fully confirms the particular suitability of the methoxytetrahydropyranyl group for the protection of 2'-hydroxy functions in oligoribonucleotide synthesis. We recommend that the synthetic oligonucleotides should be isolated and stored with their 2'-hydroxy functions protected, and that the exceptionally convenient unblocking procedure [0.01*M* - hydrochloric acid, room temperature, 6 hr] should be carried out, preferably under sterile conditions, only just before the free oligoribonucleotides are required. Possible digestion by contaminating enzymes should then be avoided.

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[5] We have investigated and rejected the possibility of using the benzyl ether protecting group[6] which can be removed, albeit with a degree of uncertainty, by catalytic hydrogenolysis; we also regard acyl[7] and t-butyldimethylsilyl[8] groups, which readily migrate under mildly basic conditions, as being unsatisfactory protecting groups for 2'-hydroxy functions.

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[11] That is, apart from the 2',3'-terminal methoxymethylene protecting group which undergoes acidic hydrolysis at a rate[10] similar to that of methoxytetrahydropyranyl.

[12] S.S. Jones and C.B. Reese, *J. Am. Chem. Soc.*, 101, 7399 (1979).

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[14] The authors[13] do not indicate whether or not they have allowed for adenosine in estimating the percentage yields of mononucleotide products. If they have not, their hydrolysis percentages for (5a) and (6a) are *ca.* 4 and 8, respectively, rather than *ca.* 2 and 4.

[15] Although the retention times of individual components varied from day to day, there is no doubt about their identity. The average retention times of mononucleotides [i.e. adenosine 2'(3')-phosphates], adenylyl-(2'→5')-adenosine (6a), adenylyl-(3'→5')-adenosine (5a), and adenosine were found to be *ca.* 2-3 min, *ca.* 8.5 min, *ca.* 11.5 min, and *ca.* 18 min, respectively. The retention time of (5a) was invariably at least 3 min longer than that of (6a).

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