Nucleic Acids Research, 1994, Vol. 22, No. 12 2209–2216

Use of the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) and related protecting groups in oligoribonucleotide synthesis: stability of internucleotide linkages to aqueous acid

Daniel C.Capaldi and Colin B.Reese*
Department of Chemistry, King's College London, Strand, London WC2R 2LS, UK

Received April 20, 1994; Revised and Accepted May 20, 1994

ABSTRACT

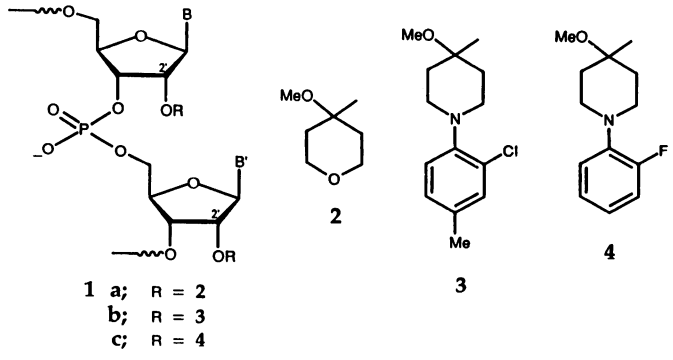
The internucleotide linkage of uridylyl-(3′→5′)-uridine (r[UpU]) does not undergo detectable hydrolytic cleavage or migration in *ca.* 24 hr in 0.01 mol dm^{-3} hydrochloric acid (pH 2.0) at 25°C. However, unlike r[UpU] and previously examined relatively high molecular weight oligoribonucleotides, oligouridylic acids are very sensitive to aqueous acid under the latter conditions (pH 2.0, 25°C). Thus when the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group is used to protect the 2′-hydroxy functions in the synthesis of r[(Up)$_9$U] and r[(Up)$_{19}$U], the final unblocking process must be carried out above pH 3 if hydrolytic cleavage and migration are to be avoided. It is demonstrated that the rate of acid-catalyzed hydrolysis of the internucleotide linkages of oligoribonucleotides is sequence dependent. As Fpmp groups may be virtually completely removed from average partially-protected oligoribonucleotides within *ca.* 24 hr at pH 3 and 25°C, it is concluded that Fpmp is a suitable 2′-protecting group even in the synthesis of particularly acid-sensitive sequences.

INTRODUCTION

The most crucial decision to be made in the design of a chemical synthesis of oligo- and poly-ribonucleotides (i.e. RNA sequences), either in solution or on a solid support, is the choice of the protecting group for the 2′-hydroxy functions. Many years ago, we formed the opinion that certain acetal groups most fully met the requirements for 2′-protection, and we have recently discussed this matter in a review on oligo- and poly-ribonucleotide synthesis (1). In the 1960s, we introduced the achiral 4-methoxytetrahydropyran-4-yl (Mthp **2**, as in **1a**) group (2) for the protection of 2′-hydroxy functions in oligoribonucleotide synthesis in solution and, in the 1980s, we introduced the related 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp **3**, as in **1b**) (3) and 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp **4**, as in **1c**) (4) groups for the protection of 2′-hydroxy functions in oligoribonucleotide synthesis on a solid support. The Ctmp **3** and Fpmp **4** acetal protecting groups combine all the favourable properties of the Mthp group **2**; in addition, these 1-arylpiperidin-4-one acetal protecting groups are also relatively stable (5) under more drastic conditions of acidic hydrolysis and would be expected to remain intact during the 'detritylation' steps in solid phase oligo- and poly-ribonucleotide synthesis.

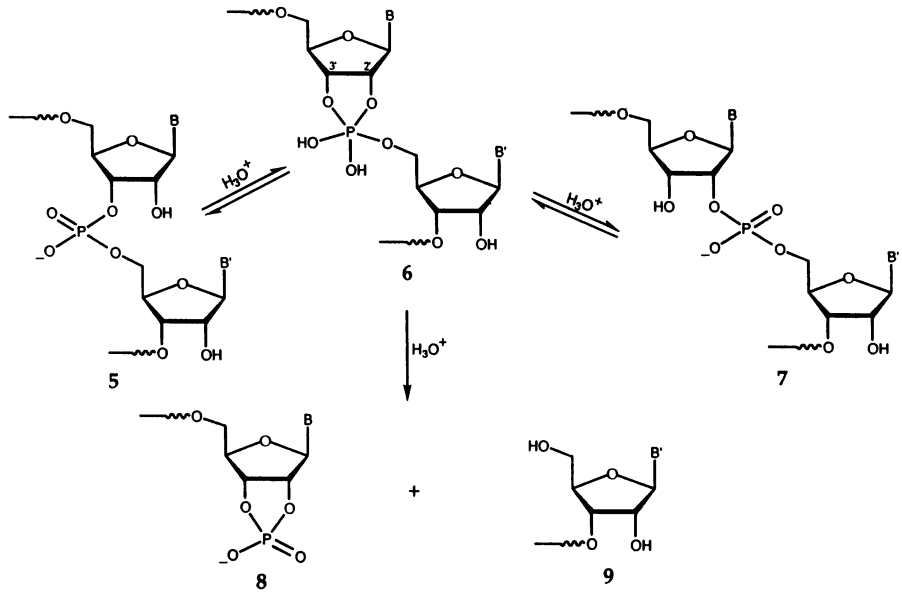
It has long been realized (6) that the internucleotide linkages of RNA sequences can both migrate (as in **5**→**6**→**7**) and undergo cleavage (as in **5**→**6**→**8** + **9**) under acidic conditions, and the mechanism involved is believed (6,7) to be that suggested in outline in Scheme 1. While synthetic oligo- and poly-ribonucleotides can usually be separated from cleavage products and other truncated sequences by liquid chromatography (HPLC) or by polyacrylamide gel electrophoresis (PAGE), it is anticipated that freeing the RNA sequences containing only natural internucleotide linkages from contaminating material of similar molecular weight containing randomly-sited 2′→5′-internucleotide linkages would present a truly formidable problem. Thus, if an acid labile group is used to protect the 2′-hydroxy functions in oligo- and poly-ribonucleotide synthesis, it must be removable under conditions under which migration of the internucleotide linkages is completely avoided.

In the past, we have recommended that Mthp groups **2** should be removed from the 2′-hydroxy functions of otherwise



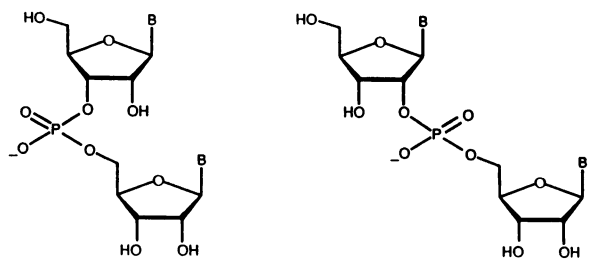
1 a; R = 2
b; R = 3
c; R = 4

*To whom correspondence should be addressed



Scheme 1. Acid-catalyzed migration and cleavage of inter-ribonucleotide linkages.

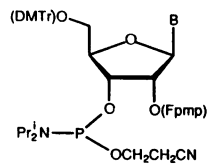
unprotected RNA sequences by treatment (8) with 0.01 mol dm^{-3} hydrochloric acid (pH 2.0) at room temperature for 6 hr, and have successfully used these unblocking conditions in the synthesis (9) of the 3'-terminal heptatriacontamer (37-mer) sequence of yeast $tRNA^{Ala}$. More recently, we have successfully removed Fpmp groups **4** from 2'-protected RNA sequences at pH 2.0–2.3 (20°C, 20 hr) (10), and other workers have effected unblocking at pH 2.0–2.5 under very similar conditions (11,12). These unblocking conditions are based on a careful examination of the acid-stability of uridylyl-(3'→5')-uridine **10a** (13), adenylyl-(3'→5')-adenosine **10b** (14) and adenylyl-(2'→5')-adenosine **11b** (14). These studies (13,14) clearly showed that while acid-catalyzed hydrolysis and migration of the internucleotide linkage proceeded at a negligible rate at pH 2.0 (0.01 mol dm^{-3} hydrochloric acid) and room temperature, hydrolysis and migration both occurred (13) to a significant extent at pH 1.0 (0.1 mol dm^{-3} hydrochloric acid) and room temperature.



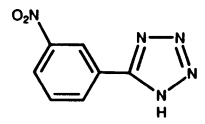
10

a; B = uracil-1-yl; b; B = adenin-9-yl

11



12



13

RESULTS AND DISCUSSION

Since HPLC was not available when we carried out our original studies (13) on the action of acid on uridylyl-(3'→5')-uridine **10a**, we have re-examined the stability of **10a** both in 0.1 and 0.01 mol dm^{-3} hydrochloric acid at 25°C. The HPLC profiles obtained are illustrated in Figure 1. It can be seen (Figure 1a) that while uridylyl-(3'→5')-uridine **10a** (R_t 8.65 min) undergoes a 4.1% conversion to uridylyl-(2'→5')-uridine **11a** (R_t 7.89 min) and *ca.* 8.4% hydrolysis to uridine (R_t 3.79 min) and uridine 2'(3')-phosphates (R_t 5.09 min) in 0.1 mol dm^{-3} acid (pH 1.01) in *ca.* 24 hr, it remains unchanged in 0.01 mol dm^{-3} acid (pH 2.0) solution after almost 24 hr (Figure 1b). Previous experiments involving the unblocking of RNA sequences with 2'-acetal (i.e. Mthp **2** (9), Ctmp **3** (15) and Fpmp **4** (10)) protecting groups by aqueous acids had, in accordance with the observed behaviour of uridylyl-(3'→5')-uridine **10a** (Figure 1b) led us to believe that, if unblocking could be completed within 24 hr at pH 2.0–2.3 and room temperature, no significant concomitant cleavage and migration of the internucleotide linkages would result. We now report that this is not a valid generalization, and that the rates of hydrolytic cleavage and migration of the internucleotide linkages of an oligoribonucleotide depend on the base sequences and possibly also the number of nucleotide residues, as well as on pH and temperature.

Oligoribonucleotides were prepared from commercially available 2'-*O*-Fpmp protected phosphoramidite building blocks **12** in an automatic synthesizer using 5-(3-nitrophenyl)-1*H*-tetrazole **13** (10,16) as activator. The crude 5'-*O*-DMTr-2'-*O*-Fpmp oligonucleotides, following release from the solid support and partial unblocking by ammonolysis, were purified by

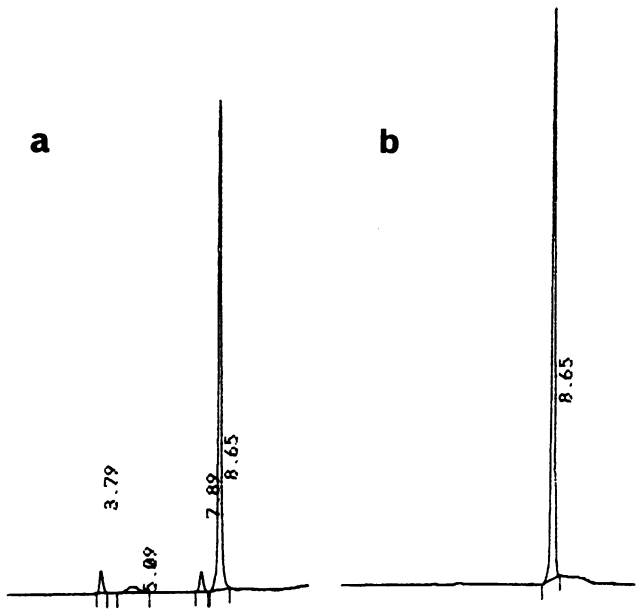


Figure 1. Reverse phase HPLC profiles of the products obtained by allowing uridylyl-(3′→5′)-uridine **10a** to stand (**a**) in 0.1 mol dm^{-3} hydrochloric acid solution (pH 1.01) at 25°C for 24.1 hr, (**b**) in 0.01 mol dm^{-3} hydrochloric acid solution (pH 2.0) at 25°C for 23.65 hr.

preparative HPLC. The HPLC profiles of crude DMTr-$(U'p)_9U$, purified DMTr-$(U'p)_9U$, crude DMTr-$(U'p)_{19}U$ and purified DMTr-$(U'p)_{19}U$ [A′, C′, G′ and U′ represent (10) nucleoside residues in which the 2′-hydroxy functions are protected with Fpmp groups] are illustrated in Figures 2a–d, respectively. One of the particular merits of the Fpmp **4** and indeed of other acetal (Mthp **2** and Ctmp **3**) protecting groups is that they are stable to ammonolysis, and such 2′-protected RNA sequences are completely stable to endonuclease attack. These protected oligonucleotides can be isolated in a pure state (Figures 2b and 2d) and stored.

It became clear from studies relating to the action of acid on purified DMTr-$(U'p)_9U$ and DMTr-$(U'p)_{19}U$ (Figure 3) that, below pH 3, the internucleotide linkages of r[$(Up)_9U$] and r[$(Up)_{19}U$] are considerably more susceptible to hydrolytic cleavage than that of the simple dinucleoside phosphate, r[UpU] **10a**. Thus when DMTr-$(U'p)_9U$ was allowed to stand in 0.01 mol dm^{-3} hydrochloric acid (pH 2.0) for 20.95 hr at 25°C (Figure 3a), it underwent almost total degradation; when this substrate was allowed to stand at 25°C in aqueous acetic acid solution at pH 2.24 for 26.1 hr (Figure 3b), pH 2.42 for 28.3 hr (Figure 3c) and pH 2.57 for 24.1 hr (Figure 3d), the estimated extent of hydrolytic cleavage was *ca.* 64, 41 and 24%, respectively. If hydrolytic cleavage is observed, it can safely be assumed that the internucleotide linkages of the remaining

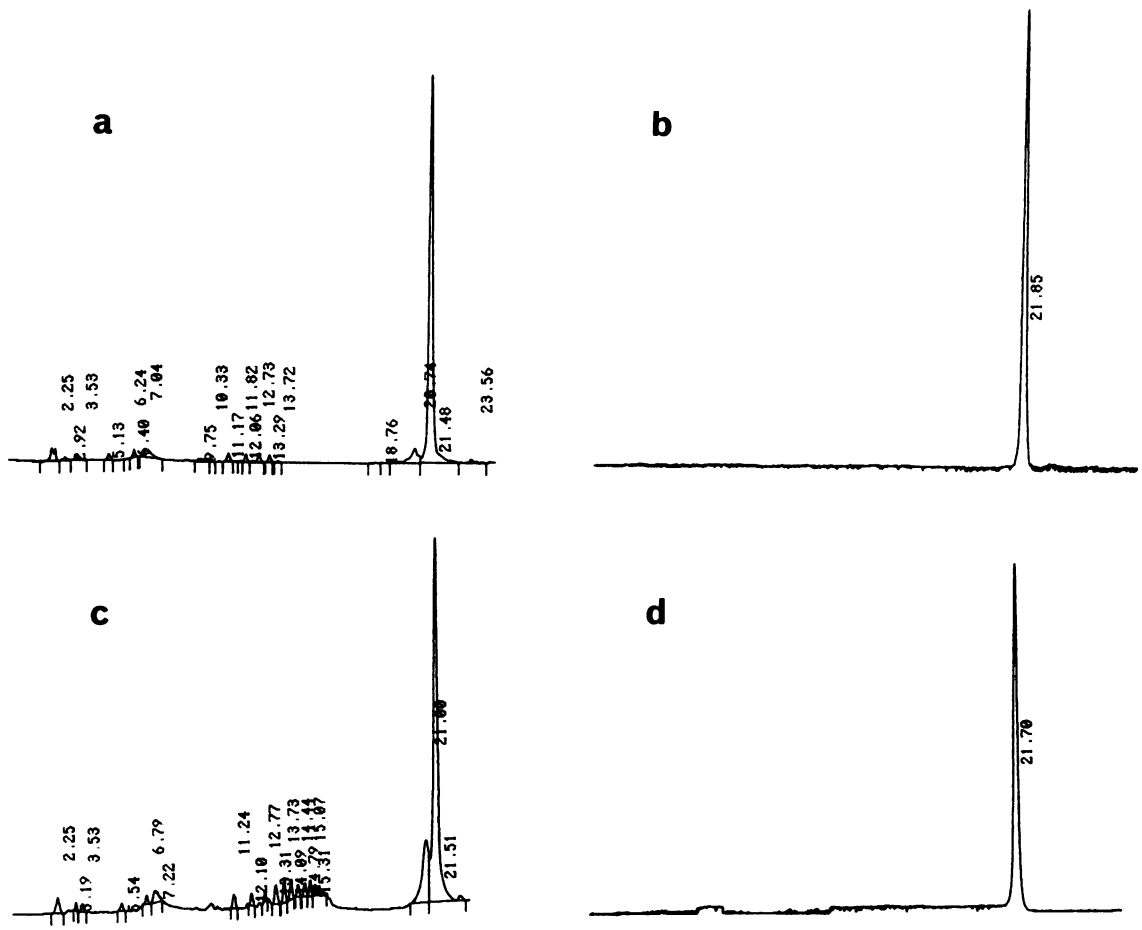


Figure 2. Reverse phase HPLC profiles of (**a**) crude DMTr-$(U'p)_9U$, (**b**) purified DMTr-$(U'p)_9U$, (**c**) crude DMTr-$(U'p)_{19}U$, and (**d**) purified DMTr-$(U'p)_{19}U$. A′, C′, G′ and U′ represent (10) nucleoside residues in which the 2′-hydroxy functions are protected with Fpmp groups.

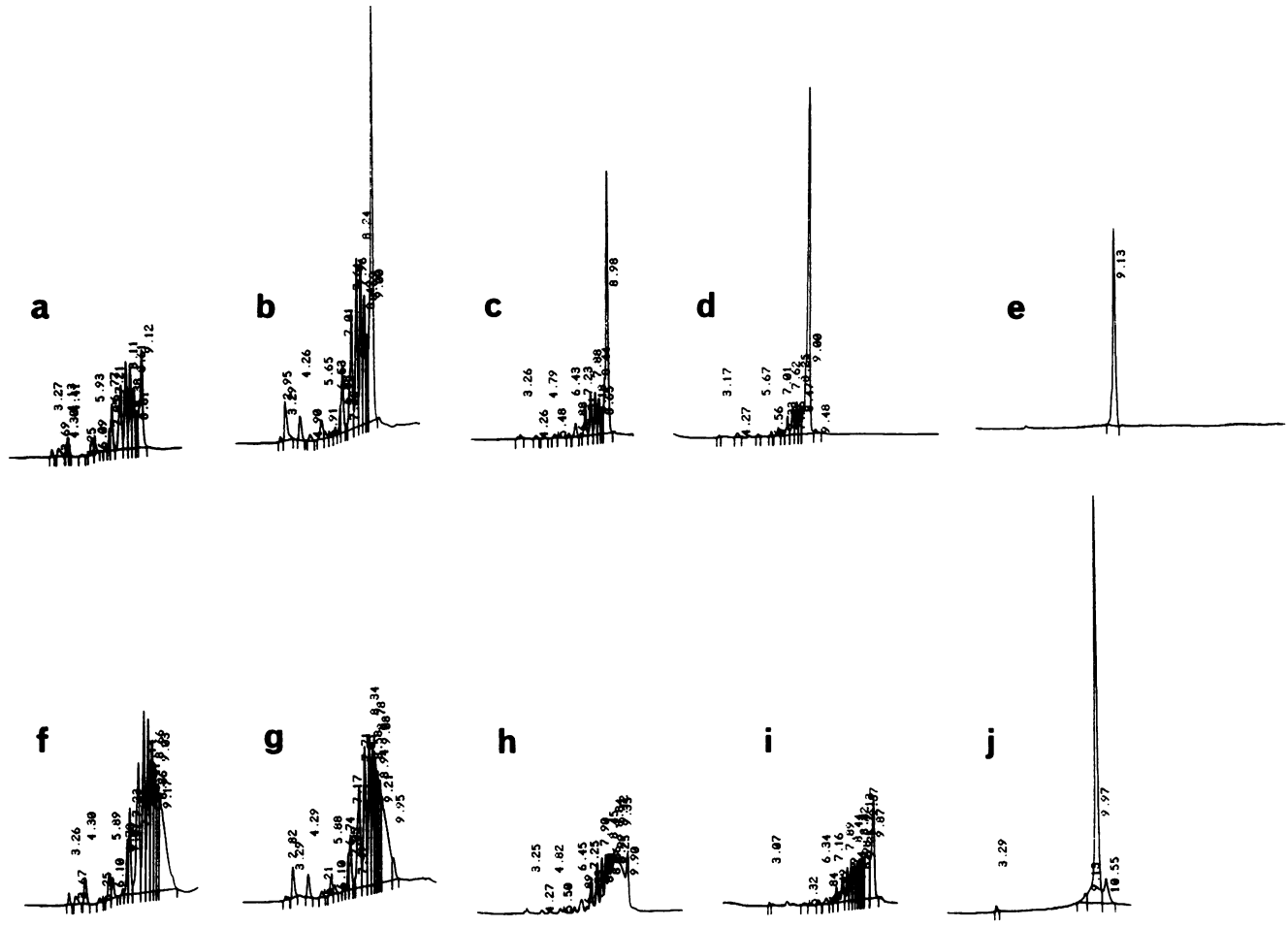


Figure 3. Reverse phase HPLC profiles for the products obtained by allowing purified DMTr-(U'p)$_9$U to stand in aqueous solution at 25°C for (**a**) 20.95 hr at pH 2.0, (**b**) 26.1 hr at pH 2.24, (**c**) 28.3 hr at pH 2.42, (**d**) 24.1 hr at pH 2.57, (**e**) 23.75 hr at pH 3.03, and by allowing purified DMTr-(U'p)$_{19}$U to stand in aqueous solution at 25°C for (**f**) 21.25 hr at pH 2.0, (**g**) 25.5 hr at pH 2.24, (**h**) 28.55 hr at pH 2.42, (**i**) 24.5 hr at pH 2.57, (**j**) 24.5 hr at pH 3.03.

unhydrolyzed material have partially migrated and, if migration is observed, it can be assumed that it is accompanied by hydrolysis. Furthermore, the extent of acid-catalyzed hydrolysis generally appears (13) to exceed the extent of acid-catalyzed migration by a factor of approximately 2. In a separate experiment, DMTr-(U'p)$_9$U was allowed to stand at pH 2.57 in acetic acid–water (98.5:1.5 v/v) solution at 25°C for 22.2 hr, and the products were then digested in the presence of ribonuclease A and bacterial alkaline phosphatase to give uridine and uridylyl-(2'→5')-uridine **11a** in the relative molar proportions of 98.9:1.1. This indicates an average migration of 1.1% per internucleotide linkage, and thereby suggests that statistically *ca.* 10% of the unblocked r[(Up)$_9$U] obtained would be expected to contain one 2'→5' internucleotide linkage. When DMTr-(U'p)$_9$U was allowed to stand in 0.5 mol dm^{-3} tris acetate (pH 3.03) buffer at 25°C for 23.75 hr (Figure 3e), complete unblocking was observed and no internucleotide cleavage or migration could be detected. The action of aqueous acid 25°C on DmTr-(U'p)$_{19}$U below pH 2.5 (Figures 3f–h) led to virtually total degradation. Unblocking at pH 2.57 (Figure 3i, 25°C, 24.5 hr) led to *ca.* 75% hydrolytic cleavage and it was estimated that *ca.* 40% of the unhydrolyzed r[(Up)$_{19}$U] contained an average of one 2'→5'-internucleotide linkage. Again a very much better result was obtained when unblocking was carried out at pH 3.03 (Figure 3j, 0.5 mol dm^{-3} tris acetate, 25°C, 24.5 hr). Disregarding the small high R_t (10.55 min), probably higher molecular weight impurity peak in the latter HPLC profile, the main peak (R_t 9.97 min), relating to r[(Up)$_{19}$U], represents *ca.* 97% of the total remaining absorbance. There does appear to be *ca.* 3% internucleotide cleavage. Although we believe that such hydrolytic cleavage is inevitably accompanied by migration, the extent of migration was too low for us to detect.

The results obtained at pH 2.0, even in the unblocking of DMTr-(U'p)$_9$U (Figure 3a), are reminiscent of the results recently reported by Rozners *et al.* (17) in the preparation of r[(Up)$_{11}$U] by the *H*-phosphonate approach using both the Ctmp **3** and Fpmp **4** groups for the protection of the 2'-hydroxy functions and very similar unblocking conditions (i.e. 0.01 mol dm^{-3} hydrochloric acid, 24 hr). Although the octadecamer, r[ApGpUpApUpApApGpApGpGpApApCpApApUpG] had been successfully prepared (18) by the *H*-phosphonate approach using the Ctmp **3** protecting group, Rozners *et al.* concluded (17) that the t-butyldimethylsilyl and 2-chlorobenzoyl but not the Ctmp **3** and Fpmp **4** groups were suitable for the protection of the 2'-hydroxy functions in solid phase oligoribonucleotide synthesis

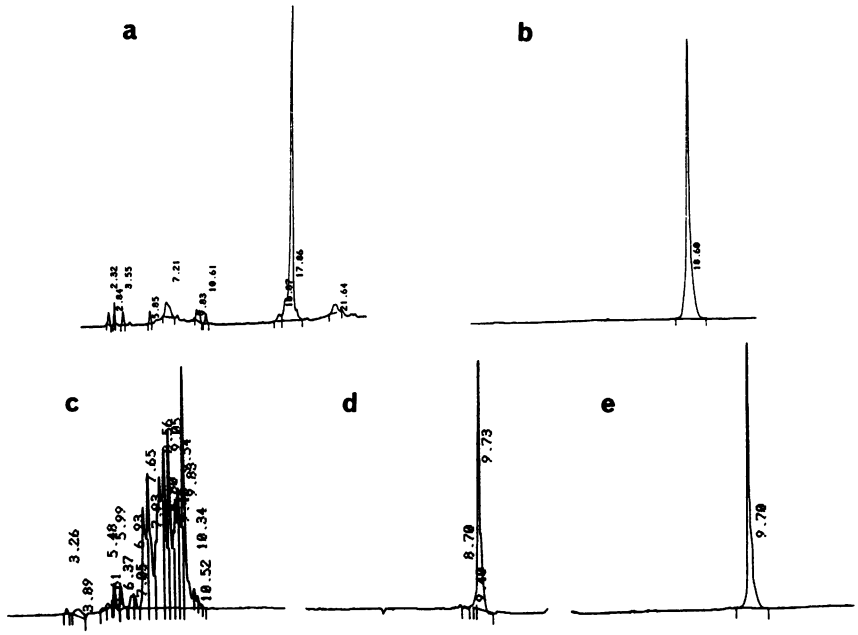


Figure 4. Reverse phase HPLC profiles of (a) crude DMTr-A'pU'pG'pA'pU'pC'pA'pU'pA'pC'pA, (b) purified DMTr-A'pU'pG'pA'pU'pC'pA'pU'pA'pC'pA and the products obtained by allowing purified DMTr-A'pU'pG'pA'pU'pC'pA'pU'pA'pC'pA to stand in aqueous solution at 25°C for (c) 23.5 hr at pH 1.01, (d) 24.9 hr at pH 2.0, (e) 26.5 hr at pH 2.42.

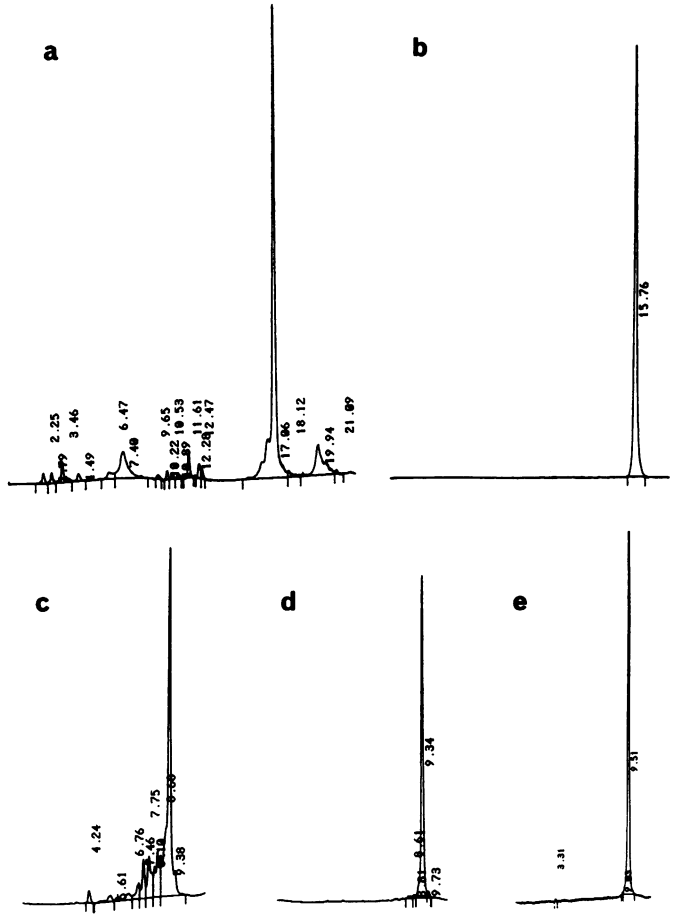


Figure 5. Reverse phase HPLC profiles of (a) crude DMTr-G'pG'pA'pU'pU'pA'pG'pU'pU'pC'pA'pC'pC'pA, (b) purified DMTr-G'pG'pA'pU'pU'pA'-pG'pU'pU'pC'pA'pC'pC'pA, and the products obtained by allowing purified DMTr-G'pG'pA'pU'pU'pA'pG'pU'pU'pC'pA'pC'pC'pA to stand in aqueous solution at 25°C for (c) 25.2 hr at pH 1.51, (d) 28.8 hr at 2.42, (e) 25.65 hr at pH 3.03.

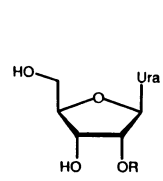
by the *H*-phosphonate approach. Although the conclusions (17) arrived at by Rozners *et al.* regarding the incompatibility of the Ctmp **3** and Fpmp **4** protecting groups with the *H*-phosphonate approach must now appear to be open to doubt, it is clearly important that the *H*-phosphonate synthesis of r[$(Up)_{11}U$] from 2′-*O*-Ctmp- and 2′-*O*-Fpmp- protected building blocks should be repeated and that the final unblocking step should then be carried out above pH 3.

The internucleotide linkages of oligouridylic acids (such as r[$(Up)_9U$] and r[$(Up)_{19}U$]) are considerably more susceptible to acid-promoted hydrolysis (and presumably also to migration) than the oligoribonucleotides of similar or even greater chain length that we have examined previously. It is not absolutely clear from the data obtained with r[$(Up)_9U$] and r[$(Up)_{19}U$] that the lability of the internucleotide linkages to acid increases with increasing chain length. This is difficult to estimate as the overall extent of fragmentation is bound to be greater as the number of internucleotide linkages is increased. However, the fact that some RNA sequences are stable for relatively long periods at *ca.* pH 2.0 and room temperature (10−12), clearly indicates that the acid-lability of inter-ribonucleotide linkages is dependent on base sequence. The increased acid-lability of, say, r[$(Up)_9U$] compared with that of uridylyl-(3′→5′)-uridine **10a** is very possibly due to distant nucleotide or even uracil residues in r[$(Up)_9U$] promoting the interaction between specific 2′-hydroxy functions and vicinal internucleotide linkages. From the point of view of their susceptibility to internucleotide cleavage, RNA sequences are extremely complex molecules. Thus it is well-known that certain RNA sequences are ribozyme substrates (19,20) and Kierzek has recently reported (21) that the rate of hydrolysis even of very short oligoribonucleotides in the presence of poly-(1-vinyl)pyrrolidine is sequence dependent. We have selected two additional RNA sequences (r[ApUpGpApUpCpApUpApCpA] and r[GpGpApUpUpApGpUpUpCpApCpCpA]) to illustrate further that the acid-lability of inter-ribonucleotide linkages is sequence dependent.

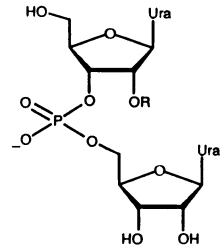
The first sequence, (r[ApUpGpApUpCpApUpApCpA]) was selected because Damha and Ogilvie (22) recently used it in the illustration of what they regarded to be typical examples of HPLC profiles obtained in solid phase oligoribonucleotide synthesis starting from 2′-*O*-(t-butyldimethylsilyl) protected phosphoramidite building blocks. If the latter are indeed typical, a comparison between the HPLC data in Figure 4 and those in the corresponding figure (23) in Damha and Ogilvie's article would appear to indicate the clear superiority of the Fpmp **4** over the t-butyldimethylsilyl group for the protection of 2′-hydroxy functions in the solid phase synthesis at least of moderately-sized oligoribonucleotides. The desired DMTr-A′pU′pG′pA′pU′pC′pA′pU′pA′pC′pA accounts for *ca.* 75% of the crude material (Figure 4a) released from the solid support by ammonolysis. When the HPLC-purified partially-protected material (Figure 4b) was unblocked at pH 1.01 and 25°C for 23.5 hr (Figure 4c), it was virtually totally destroyed. However, when unblocking was carried out at pH 2.0 for 24.9 hr (Figure 4d), r[ApUpGpApUpCpApUpApCpA] accounted for *ca.* 99% of the products obtained. A very slight improvement was observed when unblocking was carried out at pH 2.42 (Figure 4e). The latter sequence is clearly more stable to acid than r[$(Up)_9U$]. With regard to the second sequence, the crude partially-protected oligoribonucleotide, DMTr-G′pG′pA′pU′pU′pA′pG′pU′pU′pC′pA′pC′pC′pA (Figure 5a) was again purified by preparative HPLC (Figure 5b). Unblocking at pH 1.51 (25.2 hr, 25°C; Figure 5c) led to considerable degradation. A small amount (*ca.* 2%) of hydrolysis was observed at pH 2.42 (28.8 hr, 25°C; Figure 5d) but very much less than was observed for r[$(Up)_9U$] under similar unblocking conditions (Figure 3c). A very satisfactory result was obtained when unblocking was carried out at pH 3.03 (25.65 hr, 25°C, Figure 5e). It may be concluded that, below pH 3, r[GpGpApUpUpApGpUpUpCpApCpCpA] is more susceptible to acid hydrolysis (and presumably also to migration of the internucleotide linkages) than r[ApUpGpApUpCpApUpApCpA] but very much less susceptible than either r[$(Up)_9U$] or r[$(Up)_{19}U$].

Perhaps the most significant conclusion of this study is that the rates of acid-catalyzed hydrolysis and migration of the internucleotide linkages of an oligoribonucleotide are dependent on its base sequence. The other clearly important conclusion, especially with regard to the use of the Fpmp protecting group in oligoribonucleotide synthesis, is that the final unblocking step should be carried out at as high a pH as is convenient. Whereas numerous RNA sequences of varying chain lengths have been successfully unblocked at pH 2.0−2.5 and room temperature (9−12), other sequences (e.g. r[$(Up)_9U$] and r[$(Up)_{19}U$]) need to be unblocked above pH 3.0.

The Fpmp **4** and other 1-arylpiperidin-4-one acetal systems were designed in such a way (1,5) as to make their rates of hydrolysis as pH-independent as possible in the pH range 0.5−2.5. An additional consideration in their design was that they should readily undergo hydrolysis at *ca.* pH 2.0−2.5 and room temperature. Fortunately, the Fpmp protecting group **4** is also readily susceptible to hydrolysis at pH 3.0 and 25°C. Thus the half time ($t_{1/2}$) for the removal (5) of the Fpmp group from the uridine derivative **14a** is 166 min and the estimated $t_{1/2}$ for the unblocking of the r[UpU] derivative **15a** is 75 min at pH 3.0 and 25°C. Acceleration of the rate of hydrolysis of 2′-acetal functions by the participation of vicinal phosphodiester internucleotide linkages appears to be a general phenomenon (5,13,14) and all the Fpmp groups in the partially-protected oligoribonucleotides described above [e.g. DMTr-$(U'p)_9U$ and DMTr-$(U'p)_{19}U$] are vicinal to such phosphodiester groups. A relatively high molecular weight Fpmp-protected oligo- or poly-uridylic acid should be virtually completely unblocked after *ca.* 12.5 hr (i.e. 10 $t_{1/2}$ at pH 3.0 and 25°C) if it can be assumed that $t_{1/2}$ for the removal of each Fpmp group is the same as $t_{1/2}$ for the unblocking of the dinucleoside phosphate **15a**. Earlier studies had shown (14) that the 2′-*O*-Mthp derivative (**14**; R = **2**) of uridine was *ca.* twice as labile to acidic hydrolysis as the corresponding derivatives of adenosine, cytidine and guanosine at pH 2.0. Taking this base-dependence into account, a reasonable



14



15

a; R = Fpmp **4** b; R = Ctmp **3**

estimate for the unblocking of an average Fpmp-protected hetero-RNA sequence would be *ca.* 24 hr at pH 3.0 and 25°C. If, in the case of a particularly acid-sensitive sequence, it is desirable to remove the Fpmp protecting groups at pH 3.5 ($t_{1/2}$ for the unblocking of **14a** at pH 3.5 and 25°C has been found to be 334 min), unblocking at 25°C may take as much as 48 hr. Indeed, if further studies should indicate that unblocking at or above pH 3.5 would be beneficial, this process could be speeded up by replacing the Fpmp by another 1-aryl-4-methoxypiperidin-4-yl acetal system. For example, the 2′-*O*-Ctmp derivative **14b** of uridine has been found (5) to undergo acid-catalyzed hydrolysis more rapidly than the corresponding Fpmp derivative **14a**. It is clearly important that the properties of the Ctmp **3** and other alternative 1-arylpiperidin-4-yl protecting groups should now be investigated more thoroughly. However, it seems clear that the use of the Fpmp protecting group will lead to the successful synthesis of the vast majority of oligo- and poly-ribonucleotides that are likely to be required for biological and other studies.

EXPERIMENTAL

Solid phase oligoribonucleotide synthesis

Automated oligoribonucleotide synthesis was carried out on a 1.0 μmolar scale in an Applied Biosystems 381A DNA Synthesizer as previously described (10) using Cruachem 5′-*O*-(DMTr)-2′-*O*-(Fpmp) ribonucleoside phosphoramidites **12** and 1.0 μmole disposable [2′(3′)-*O*-benzoyl] columns. Solvents and other reagents were purified as previously described (10). Coupling times were uniformly 3.0 min and the average coupling efficiency, as estimated by standard trityl assay (24), was 98.5%. The crude 5′-*O*-(DMTr)-2′-*O*-(Fpmp)-protected oligoribonucleotides were released from the solid support by digestion with concentrated aqueous ammonia (*d* 0.88, 2.0 ml) at 55°C for 14 hr. Partially-protected oligoribonucleotides were analyzed by HPLC [Jones APEX ODS 5 μ column (25 cm×4.6 mm), eluted with 0.1 mol dm^{-3} aqueous triethylammonium acetate (pH 7.0)−acetonitrile (75:25−50:50 v/v) mixtures] and by PA-GE (Raven vertical slab gel apparatus connected to a LKB Bromma 2197 power supply). The crude partially-protected oligoribonucleotides were purified by HPLC (same column and solvent system). The HPLC profiles obtained both with the crude and purified oligoribonucleotides are illustrated in Figures 2a−d, 4a−b and 5a−b.

Acid-catalyzed hydrolysis and isomerization of uridylyl-(3′→5′)-uridine

(a) Uridylyl-(3′→5′)-uridine **10a** (NH_4^+ salt, *ca.* 0.0002 g, purchased from Pharma-Waldhof GmbH) was dissolved in 0.1 mol dm^{-3} hydrochloric acid (5.0 ml) at 25°C. The pH (meter calibrated both at pH 2.0 and 4.0) of the solution was 1.01. After 24.1 hr at 25°C, the reaction solution was analyzed by HPLC [Jones APEX ODS column, eluted with 0.1 mol dm^{-3} triethylammonium acetate−acetonitrile (97:3−93:7 v/v), Figure 1a] and found to contain uridylyl-(3′→5′)-uridine **10a** (R_t 8.65 min, 87.5% of total absorbance), uridylyl-(2′→5′)-uridine **11a** (R_t 7.89 min, 4.1%), uridine 2′(3′)-phosphates (R_t 5.09 min, 4.1%) and uridine (R_t 3.79 min, 4.3%).

(b) Uridylyl-(3′→5′)-uridine **10a** (NH_4^+ salt, *ca.* 0.0002 g) was dissolved in 0.01 mol dm^{-3} hydrochloric acid (5.0 ml) at 25°C. The pH of the solution was 2.0. After 23.65 hr at 25°C, the reaction solution was analyzed as above by HPLC (Figure 1b).

Unblocking of partially-protected 2′-*O*-(Fpmp)-5′-*O*-(DMTr)-oligoribonucleotides

The following aqueous acid and buffer solutions were used to unblock partially-protected RNA sequences: 0.10 dm^{-3} hydrochloric acid (pH 1.01), 0.03 mol dm^{-3} hydrochloric acid (pH 1.51), 0.01 mol dm^{-3} hydrochloric acid (pH 2.0), 1.05 mol dm^{-3} acetic acid (pH 2.24), 0.52 mol dm^{-3} acetic acid (pH 2.42), 0.26 mol dm^{-3} acetic acid (pH 2.57) and 0.5 mol dm^{-3} tris acetate (pH 3.03). The following general unblocking procedure was adopted. The partially-protected oligoribonucleotide (*ca.* 0.25−0.5 A_{260} unit) and sterile water (0.25 ml) were placed in an Eppendorf tube and the resulting solution was evaporated under reduced pressure. More sterile water (0.25 ml) was added and the process was repeated. The residue was then dissolved in the appropriate aqueous acid or buffer solution (0.2 ml), and the reaction solution was maintained at 25°C. After appropriate reaction times the products were then analyzed by HPLC (Jones APEX ODS column eluted with 0.1 mol dm^{-3} triethylammonium acetate−acetonitrile (95:5−80:20 v/v). The HPLC profiles obtained are illustrated in Figures 3a−j, 4c−e and 5c−e.

Measurement of acid-catalyzed phosphoryl migration in oligouridylic acids

The modification of the procedure that we originally used (13) to measure the rate of the acid catalyzed interconversion of uridylyl-(3′→5′)-uridine **10a** and uridylyl-(2′→5′)-uridine **11a** was developed in Professor S.M.Hecht's laboratory (25). After appropriate times, solid tris was added to the acidic solution of unblocked r[$(Up)_9$U] or r[$(Up)_{19}$U], obtained as above, until the pH increased to 8.0. Stock solutions of ribonuclease A [5 μl, obtained by dissolving ribonuclease A (0.001 g) in 0.1 mol dm^{-3} tris hydrochloride buffer (pH 8.0; 1.0 ml)] and bacterial alkaline phosphatase (5 μl, obtained by dissolving *ca.* 2 units of enzyme in 1.0 ml of the same buffer) were added and the reactants were maintained at 37°C. After 18 hr, the products were analyzed by HPLC [Jones APEX Octyl 10 μ column (25 cm×4.6 mm), eluted with 0.1 mol dm^{-3} triethylammonium acetate - acetonitrile (97:3−93:7 v/v). Uridine and uridylyl-(2′→5′)-uridine **11a** had R_t = *ca.* 3.0 and 5.75 min, respectively.

ACKNOWLEDGEMENTS

We thank the Cancer Research Campaign for generous financial support, Cruachem Ltd for the gift of RNA phosphoramidite building blocks and disposable columns, and Mr Anthony Vandersteen for help with oligoribonucleotide synthesis. We also thank Professor S.M.Hecht for communicating his unpublished results relating to studies on the acid-catalyzed migration of the internucleotide linkages of polyuridylic acid.

REFERENCES

1. Reese, C. B. (1989) In *Nucleic Acids and Molecular Biology*, Eckstein, F. and Lilley, D.M.J., Eds, Springer, Berlin, pp. 164−181.
2. Reese, C. B., Saffhill, R. and Sulston, J. E. (1967) *J. Am. Chem. Soc.*, **89**, 3366−3368.
3. Reese, C. B., Serafinowska, H.T. and Zappia, G. (1986) *Tetrahedron Lett.*, **27**, 2291−2294.
4. Reese, C. B. and Thompson, E.A. (1988) *J. Chem. Soc., Perkin Trans 1*, 2881−2885.

5. Reese, C. B. (1991) *Nucleosides Nucleotides*, **10**, 81–97.
6. Brown, D. M., Magrath, D.I., Neilson, A.H. and Todd, A.R. (1956) *Nature*, **177**, 1124–1125.
7. Anslyn, E and Breslow, R. (1989) *J. Am. Chem. Soc.*, **111**, 4473–4482.
8. Jones, S. S., Rayner, B., Reese, C. B., Ubasawa, A. and Ubasawa, M. (1980) *Tetrahedron*, **36**, 3075–3085.
9. Brown, J. M., Christodoulou, C., Modak, A.S., Reese, C. B. and Serafinowska, H. T., (1989) *J. Chem Soc., Perkin Trans. 1*, 1751–1767.
10. Rao, M. V., Reese, C. B., Schehlmann, V. and Pak Sang Yu (1993) *J. Chem Soc., Perkin Trans. 1*, 43–55.
11. Beijer, B., Sulston, I., Sproat, B. S., Rider, P., Lamond, A. I. and Neumer, P. (1990) *Nucleic Acids Res.*, **18**, 5143–5151.
12. Rao, M. V., personal communication.
13. Griffin, B. E., Jarman, M. and Reese, C. B. (1968) *Tetrahedron*, **24**, 639–662.
14. Norman, D. G., Reese, C. B. and Serafinowska, H. T. (1984) *Tetrahedron Lett.*, **28**, 3015–3018.
15. Rao, T. S., Reese, C. B., Serafinowska, H. T., Takaku, H. and Zappia, G. (1987) *Tetrahedron Lett.*, **28**, 4897–4900.
16. Froehler, B. C. and Matteuchi, M. D. (1983) *Tetrahedron Lett.*, **24**, 3171–3174.
17. Rozners, E., Westman, E. and Strömberg, R. (1994) *Nucleic Acids Res.*, **22**, 94–99.
18. Sakatsume, O., Ohtsuki, M., Takaku, H. and Reese, C. B. (1989) *Nucleic Acids Res.*, **17**, 3689–3697.
19. Uhlenbeck, O.C. (1987) *Nature*, **328**, 596–600.
20. Jeffries, A. C. and Symons, R. H. (1989) *Nucleic Acids Res.*, **17**, 1371–1377.
21. Kierzek, R. (1992) *Nucleic Acids Res.*, **20**, 5073–5077, 5079–5084.
22. Damha, M. J. and Ogilvie, K. K. (1993) In *Methods in Molecular Biology, Vol. 20. Protocols for Oligonucleotides and Analogs*, Agrawal, S., Ed., Humana, Totowa, pp. 81–114.
23. Damha, M. J. and Ogilvie, K. K. (1993) In *Methods in Molecular Biology, Vol. 20. Protocols for Oligonucleotides and Analogs*, Agrawal, S., Ed., Humana, Totowa, p. 105.
24. Atkinson, T. and Smith, M. (1984) in *Oligonucleotide Synthesis. A Practical Approach*, Gait, M. J., Ed., IRL Press, Oxford, pp. 35–81.
25. Hecht, S. M. and Morgan, M., personal communication.