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Action of acid on oligoribonucleotide phosphotriester intermediates. Effect of released vicinal hydroxy functions

Colin B.Reese and Philip A.Skone

Department of Chemistry, King's College, Strand, London WC2R 2LS, UK

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ABSTRACT

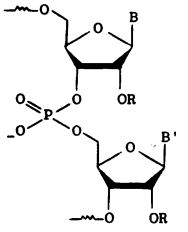
When 2'-O-methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (9) is treated with zinc bromide in dichloromethane-propan-2-ol (85:15 v/v) at room temperature, under stringently anhydrous conditions, the corresponding 5'-unblocked dinucleoside phosphate (10) is obtained in 86% isolated yield; however, when no special precautions are taken to exclude moisture, (10) is obtained in only 72% yield. The removal of the 5'-O-(9-phenylxanthen-9-yl) protecting group from (10) with a protic acid (phenyl dihydrogen phosphate) appears to be much less selective and efficient. 80% Acetic acid promoted removal of the methoxytetrahydropyranyl protecting group from the isomeric fully-protected uridylyl-(3'→5')- and uridylyl-(2'→5')-uridine derivatives [(11) and (21c), respectively] leads to virtually identical mixtures [Figures 1a and 1b, respectively] of the partially-protected dinucleoside phosphates [(14) and (15)], 2',3'-di-O-acetyluridine (8), 5'-O-acetyluridine 2',3'-cyclic phosphate (16), and 5'-O-acetyluridine 2'(3')-phosphates [(18) and (17)].

INTRODUCTION

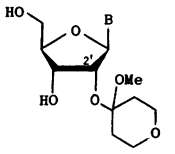
In recent years, remarkable progress has been made in the development of rapid methods for the chemical synthesis of oligo- and poly-deoxyribonucleotides of defined sequence[1,2]. Advances in this area have been stimulated by the considerable need for synthetic oligodeoxyribonucleotides in biology, and have been made possible by the development of the phosphotriester[3] and phosphite triester[2] approaches to oligonucleotide synthesis. Rapid synthesis of relatively high molecular weight polydeoxyribonucleotides has been carried out successfully on solid supports by means of both of the latter approaches[1,2]. Recently, the possibility of carrying out the rapid synthesis of oligodeoxyribonucleotides by the phosphotriester approach in solution has also been investigated[4].

Progress in the chemical synthesis of oligoribonucleotides[5,6] has been less rapid. Although it may be due partly to the fact that, at present, the requirements in biological research for oligodeoxyribo- are much greater than for oligoribo-nucleotides, the main reason for this situation is the increased

synthetic complexity resulting from the need to protect the additional 2'-hydroxy functions throughout the synthesis of oligoribonucleotides. Arguably the most crucial decision that has to be made in the synthesis of oligoribonucleotides (e.g. 1; R=H) is the choice of the protecting group (R) for the 2'-hydroxy functions. This protecting group must remain intact until the final unblocking step at the end of the synthesis, and it must then be removable under conditions which are mild enough to prevent subsequent attack of the released 2'-hydroxy functions on vicinal phosphodiester groups with consequent cleavage or migration of the internucleotide linkages.

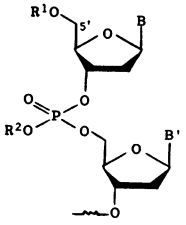


(1)

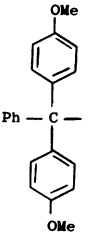


(2) a; B = uracil-1-yl

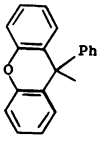
Initially, we investigated the use of the acid-labile tetrahydropyranyl group[7,8] for the protection of the 2'-hydroxy functions in oligoribonucleotide synthesis but, in recent years, we have exclusively used the somewhat more acid-labile, achiral methoxytetrahydropyranyl [Mthp, as in (2)] group[9]. The latter protecting group has proved[5,6] to be particularly suitable for this purpose in that its use permits the isolation of partially-protected oligoribonucleotides [e.g. 1; R = 4-methoxytetrahydropyran-4-yl] that are stable to alkaline hydrolysis and more particularly to digestion by ribonucleases, and which are converted, under very mild conditions of acidic hydrolysis, into completely unblocked products without the occurrence of concomitant side reactions[8,10].



(3) a; R^1 = (4)
b; R^1 = (5)



(4)



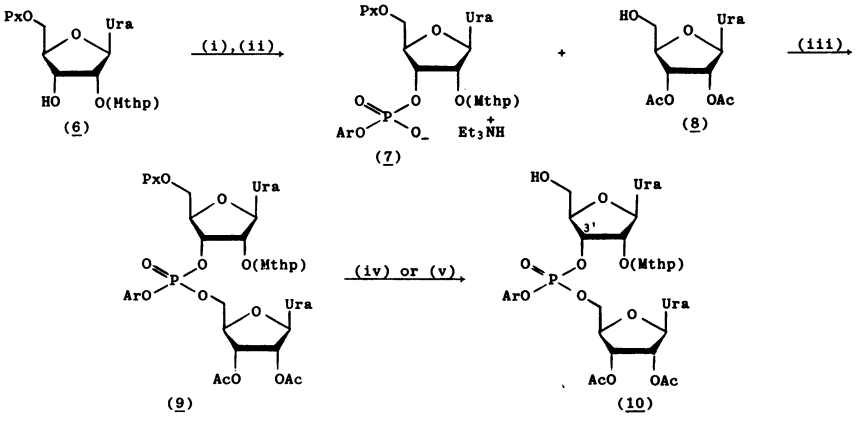
(5)

In rapid oligodeoxyribonucleotide synthesis both on solid supports and in solution, it is essential that the 5'-terminal protecting group (R^1) of the growing fully-protected oligonucleotide (3) should easily be 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[11] [DMTr (4), as in (3a)] or the 9-phenylxanthen-9-yl[12] [Px (5), as in (3b)] group to protect the 5'-terminal hydroxy functions. The latter protecting groups are both readily removable under mild acidic conditions. If a successful rapid synthesis of oligoribonucleotides is to be developed, there is, at present, no obvious alternative to the use of such modified trityl groups [i.e. DMTr (4) and Px (5)] for the protection of 5'-terminal hydroxy functions. It is therefore a matter of considerable importance to establish whether or not the use of 4,4'-dimethoxytrityl (4) and 9-phenylxanthen-9-yl (5) protecting groups is fully compatible with the use of methoxytetrahydropyranyl or related acetal groups for the protection of 2'-hydroxy functions.

RESULTS AND DISCUSSION

The present investigation has two specific purposes. The first purpose is to attempt to find conditions for the conversion of fully-protected dinucleoside phosphates [such as (9), Scheme 1] into the corresponding 5'-unprotected derivatives [such as (10)] with the minimum concomitant loss of the 2'-O-Mthp group, and thereby to determine whether the use of 5'-O-Px (or 5'-O-

Scheme 1



Ura = uracil-1-yl; Ar = 2-chlorophenyl

Reagents: (i) 2-chlorophenyl phosphorodi-(1,2,4-triazolide), 1-methylimidazole/tetrahydrofuran; (ii) triethylamine, water; (iii) 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole(MSNT)/pyridine; (iv) anhydrous zinc bromide/dichloromethane-propan-2-ol (85:15 v/v); (v) phenyl dihydrogen phosphate/chloroform-ethanol (95:5 v/v).

DMTr) and 2'-O-Mthp protecting groups are mutually compatible in oligoribonucleotide synthesis. The second purpose is to determine what reactions subsequently take place if loss of the 2'-O-Mthp group does indeed occur.

Treatment of 2'-O-methoxytetrahydropyranyluridine (2a)[9,13] with a slight excess of 9-chloro-9-phenylxanthene[14] in pyridine solution gave 2'-O-methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridine (6) as a colourless solid in 94% isolated yield. The latter compound (6) was readily converted in the usual way[5] (see Scheme 1 and Experimental) into the triethylammonium salt of its 3'-(2-chlorophenyl) phosphate (7) in 95% isolated yield. When (7) and 2',3'-di-O-acetyluridine[15] (8) were allowed to react together in the presence of 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT)[5,16] in pyridine solution, the fully-protected dinucleoside phosphate (9) was obtained in 81% isolated yield.

The compatibility of the 5'-O-Px and 2'-O-Mthp protecting groups was tested first (Scheme 1) by treating (9) with zinc bromide in dichloromethane-propan-2-ol solution[17] at room temperature, and then isolating the resulting 5'-unprotected dinucleoside phosphate (10) by silica gel chromatography. When (9) was treated with ca. 50 mol. equiv. of zinc bromide (1.0 M) in dichloromethane-propan-2-ol (85:15 v/v), complete unblocking occurred within ca. 15 min at room temperature. Treatment of (9) with zinc bromide under these conditions, with special precautions being taken to exclude traces of moisture (see Experimental), followed by short column chromatography of the products gave the partially-protected dinucleoside phosphate (10) in 86% isolated yield. When this experiment was repeated without such special precautions being taken, (10) was obtained in only 72% isolated yield after 15 min, and in zero yield after 17 hr reaction time. However, as it is uncertain that zinc bromide is an effective catalyst for the removal of 5'-O-Px (and 5'-O-DMTr) protecting groups from relatively high molecular weight oligo- and poly-nucleotides, protic acids are often used for this purpose[18]. When (9) was treated with an eightfold excess of phenyl dihydrogen phosphate (0.32 M) in chloroform-ethanol (95:5 v/v) at room temperature[4], 5'-deprotection was complete after 5 min but, following work-up and short column chromatography of the products, the desired partially-protected dinucleoside phosphate (10) was obtained in only 54% isolated yield. It seems likely (see below) that the relatively low yield of (10) was due to partial loss of the 2'-O-Mthp protecting group.

It is reasonable to conclude from the above studies that (a) it is probably safe to use 1.0 M- zinc bromide in dichloromethane-propan-2-ol (85:15 v/v) to remove 5'-O-Px (or 5'-O-DMTr) protecting groups from oligoribonucleotide

phosphotriester intermediates in which the 2'-hydroxy functions are protected with Mthp (or tetrahydropyranyl) groups provided that stringent precautions are taken to exclude traces of moisture, and (b) that it is not safe to use phenyl dihydrogen phosphate (pK_a = 1.25) and acids of similar (e.g. dichloroacetic acid) or greater (e.g. toluene-p-sulphonic acid) strengths for this purpose. Ohtsuka et al.[19] have used zinc bromide under the above conditions to remove 5'-O-DMTr in the presence of 2'-O-tetrahydrofuranyl protecting groups, and a suspension of zinc bromide in dichloromethane has been used[20] to remove 5'-O-Px in the presence of 2'-O-Mthp protecting groups. Other workers, however, have reported the use of protic acids for the removal of 5'-O-DMTr in the presence of 2'-O-Mthp[21] (and 2'-O-tetrahydropyranyl[22]) protecting groups in oligoribonucleotide synthesis. Notwithstanding the apparent relative safety of using zinc bromide when stringently anhydrous reaction conditions are observed, doubts relating to its efficacy in the unblocking of high molecular weight oligo- and poly-ribonucleotides lead us to the general conclusion that the Mthp group should be replaced by a protecting group which is fully stable under the protic acid conditions required for the rapid and complete removal of the 5'-O-Px (or 5'-O-DMTr) group. Work on the development of such a protecting group is now in progress.

In order to investigate what subsequent reaction or reactions ensue when loss of a Mthp group actually occurs and a hydroxy function vicinal to a phosphotriester group is liberated under acidic conditions, the fully-protected dinucleoside phosphate (11) was prepared by treating (10) with an excess of acetic anhydride in pyridine solution. This substrate (11) was dissolved in acetic acid-water (4:1 v/v) at room temperature and its decomposition was monitored by t.l.c. on silica gel coated plates. Development of the latter in chloroform-methanol (9:1 v/v) [solvent system A, see Experimental] revealed that the disappearance of starting material [(11), R_F 0.36] was accompanied by the formation of only two product components [R_F's 0.00, 0.34], the less intense of which had the same R_F (0.34) as 2',3'-di-O-acetyluridine (8). After 140 min, when no starting material (11) remained, the products were carefully concentrated under reduced pressure at room temperature. Liquid chromatographic analysis of the residue [Figure 1a] revealed five main components with R_T's 2.5, 2.8, 8.1, 14.2, and 17.9 min, which have been identified (see below and Experimental) as 5'-O-acetyluridine 2'(3')-phosphates [(18) and (17)], 5'-O-acetyluridine 2',3'-cyclic phosphate (16), 2',3'-di-O-acetyluridine (8), 5'-O-acetyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) (15), and 5'-O-acetyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) (14), respectively. Integration

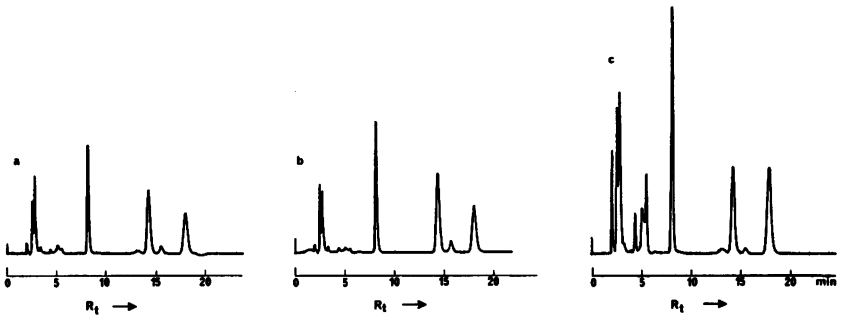
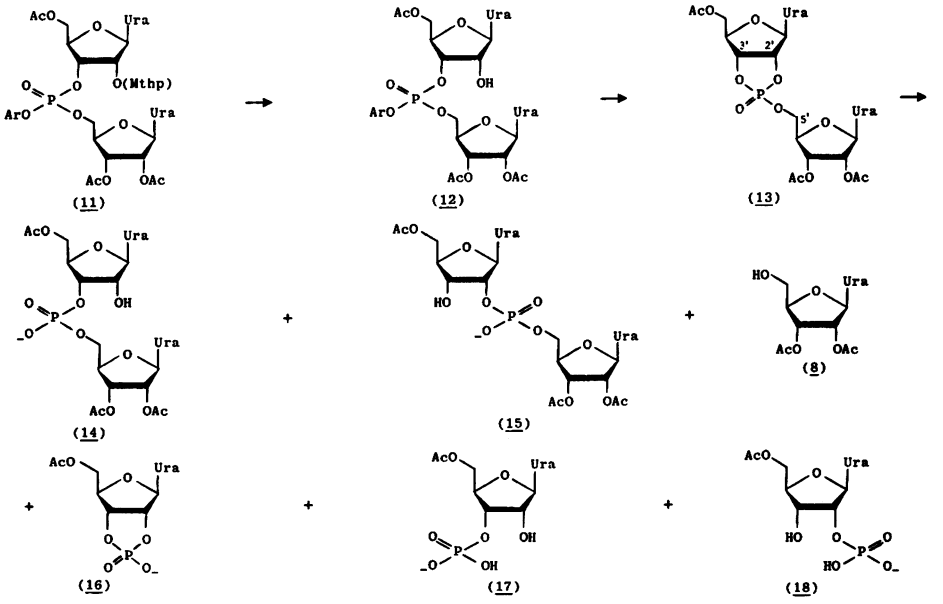


Figure 1. Liquid chromatograms [Jones APEX ODS column, 0.1*M* - triethylammonium acetate-acetonitrile (88:12 v/v)] of hydrolysis products obtained in 0.02*M* - solutions of (a) (11) in acetic acid-water (4:1 v/v), (b) (21c) in acetic acid-water (4:1 v/v), and (c) (11) in 0.1*M* - hydrochloric acid-dioxan (1:1 v/v).

of the peaks in Figure 1a reveals that (8), (15) and (14) account for 26, 24 and 19%, respectively, of the total absorbance at 254 nm, and that (16), (17) and (18) combined account for a further 21%. Thus 90% of the total absorbance at 254 nm is accounted for by the six products indicated above.

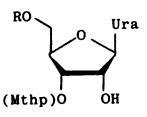
The proposed course of hydrolysis of (11) is indicated in outline in Scheme 2. As the starting material (11) and 2',3'-di-O-acetyluridine (8) are

Scheme 2

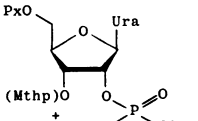


Ura = uracil-1-yl; Ar = 2-chlorophenyl

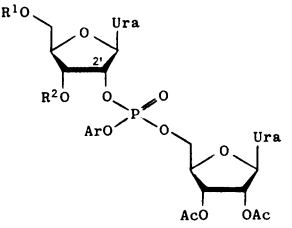
the only uncharged components detectable by t.l.c. (see above), it seems likely that the primary hydrolysis product (12) and the derived dinucleoside cyclic phosphate (13) are both very short-lived under the reaction conditions. The possibility, however, of R_F coincidences between the latter intermediates [(12) and (13)] and (8) and (11) cannot be excluded. It nevertheless seems certain (see below) that (13) is the key intermediate which then undergoes P-O(2') and P-O(3') hydrolytic cleavage to give (14) and (15), respectively, and P-O(5') cleavage to give (8) and (16). Under the reaction conditions, (16) undergoes further hydrolysis [see Experimental] to give (17) and (18). From the proportions of products obtained, it appears that hydrolysis of (13) involving cyclic phosphate cleavage [to give (14) and (15)] is marginally less favourable than hydrolysis involving P-O(5') cleavage. In a related study[23] involving the release of the 2'-hydroxy function vicinal to the phosphotriester group of a fully-protected dinucleoside phosphate under basic conditions, a cyclic phosphotriester [corresponding to (13)] was also proposed as the key intermediate, but the course of its subsequent decomposition was somewhat different.



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



(20)



(21) a; R^1 = Px, R^2 = Mthp
b; R^1 = H, R^2 = Mthp
c; R^1 = Ac, R^2 = Mthp
d; R^1 = Ac, R^2 = H

Ura = uracil-1-yl; Ar = 2-chlorophenyl

Very strong support for (13) being the key intermediate in the hydrolysis of (11) is provided by the fact that (21c) [the 2'→5'-isomer of (11)] decomposes in acetic acid-water (4:1 v/v) at room temperature to give [Figure 1b] virtually the same proportions of the same products that are obtained from (11) [Figure 1a]. Integration of the peaks in Figure 1b reveals that (8), (15) and (14) account for 26, 25 and 17.5%, respectively, of the total absorbance at 254 nm, and that (16), (17) and (18) combined account for a further 20%. The reaction, which was complete in 104 min, was somewhat faster than that involving (11). This would be expected if the loss of the 3'-O-Mthp group[10] from (21c) were the rate-determining step in its conversion, via (21d), to (13).

The required substrate (21c) was obtained by the acetylation of (21b) which, in turn, was prepared from (21a) [see Experimental]. The latter compound (21a) was prepared in 71% yield by allowing phosphodiester (20) to condense with 2',3'-di-O-acetyluridine (8) in the presence of MSNT in pyridine solution. The phosphodiester (20) itself was prepared from (19b) by the standard procedure [Scheme 1 and Experimental], and the latter compound (19b) was prepared from 3'-O-methoxytetrahydropyranyluridine[24] (19a) in 88% yield.

The decomposition of (11) was also examined in acidic media other than acetic acid-water (4:1 v/v). When a 0.02 M - solution of (11) in 0.1 M - hydrochloric acid-dioxan (1:1 v/v) was allowed to stand at room temperature, the starting material (11) was consumed and t.l.c. again revealed the presence of only one uncharged product, corresponding to 2',3'-di-O-acetyluridine (8). After 8 hr, no starting material remained, and the products were then carefully neutralized and analyzed by liquid chromatography. It can be seen from Figure 1c that while the distribution of products does not differ very significantly from that observed in the 80% acetic acid experiment [Figure 1a], the five main components [R_T's 2.5, 2.8, 8.1, 14.2 and 17.9 min] now account for only 77.5% of the total absorption at 254 nm. It is possible that more deacetylation occurs in the hydrochloric acid experiment. The decomposition of (11) in 0.24 M - trifluoracetic acid in chloroform-ethanol (95:5 v/v) was also investigated. The reaction was complete after 190 min in the latter anhydrous medium, and a liquid chromatographic analysis of the products revealed that while (14) and (15) together accounted for only 15.5% of the total absorbance at 254 nm, 2',3'-di-O-acetyluridine (8) alone accounted for 42%.

The decomposition products of (11) and (21c) were identified by liquid chromatographic comparison (using a co-injection technique) with what we believe to be authentic samples of the products. As, at the outset, only 2',3'-di-O-acetyluridine[15] (8) was available, it was necessary to synthesize the other five compounds. The two dinucleoside phosphates [(14) and (15)] were prepared by treating the corresponding phosphotriesters [(11) and (21c), respectively] with E-2-nitrobenzaldoxime[25] and N^1,N^1,N^3,N^3-tetramethylguanidine in anhydrous dioxan. Unfortunately, some deacetylation occurred during the work-up of these reactions, and reacetylation of the impure products was necessary before the Mthp protecting groups were removed by acidic hydrolysis. The cyclic phosphate (16) was prepared[26] by treating a mixture of the sodium salts of uridine 2'- and 3'-phosphates with acetic anhydride in pyridine-dimethylformamide. When (16) was digested with ribonuclease A, (17) was obtained; however, when (16) [R_T 2.8 min] was allowed to stand in acetic acid-

water (4:1 v/v) at room temperature, it was converted into a single component with an R_T (2.5 min) corresponding to that of (17). It was reasonable to assume that a mixture of (17) and (18) had been obtained. Further confirmation for the structures of the products obtained [Scheme 2] by treating (11) with acetic acid-water (4:1 v/v) was obtained by digesting the total hydrolysate with ribonuclease A. After 2 hr, the component with R_T 17.9 min [putative structure (14); see Scheme 2 and Figure 1a] was virtually consumed, the component with R_T 14.2 min [putative structure (15)] was unaffected[27], and the components with R_T's 2.5, 2.8 and 8.1 min [putative structures (17), (16) and (8), respectively] had increased in intensity. It was further noteworthy that the ratio of the integral of the component with R_T 2.5 min to that of the component with R_T 2.8 min increased with time.

The results of the second part of the present study suggest that the loss, under acidic conditions, of protecting groups from the 2'-hydroxy functions in the phosphotriester approach to oligoribonucleotide synthesis is probably followed rapidly by the loss of the 2-chlorophenyl protecting group from the adjacent internucleotide linkages and the subsequent hydrolysis reactions indicated in Scheme 2. Thus this part of the study further emphasises the above conclusion that, if a rapid synthesis of oligo- and poly-ribonucleotides is to involve the use of the 5'-O-Px (or 5'-O-DMTr) protecting group, then it is absolutely essential that the 2'-protecting groups are completely stable under the acidic conditions required to release the 5'-hydroxy functions at the end of each synthetic cycle.

EXPERIMENTAL

U.v. absorption spectra were measured with a Perkin-Elmer 402 spectrometer. ^{1}H-N.m.r. spectra were measured at 90 and 250 MHz, respectively, with Bruker HFX 90 and WM 250 spectrometers; tetramethylsilane was used as an internal standard. ^{31}P N.m.r. spectra were measured at 36.4 MHz with a Bruker HFX 90 spectrometer; 85% orthophosphoric acid was used as an external standard.

T.l.c. was carried out on Merck silica gel 60 F_{254} pre-coated plates which were developed in solvent system A [$CHCl_3$-MeOH (9:1 v/v)]. H.p.l.c. was carried out on column (I) [a Jones APEX ODS column, which was eluted isocratically with 0.1 M - aqueous triethylammonium acetate-acetonitrile (88:12 v/v) unless otherwise stated] and on column (II) [a Partisil PXS 10/25 SAX column, which was eluted isocratically with 0.05 M - potassium phosphate buffer (pH 3.35)]. Merck Kieselgel 60H was used for short column chromatography[28]. Anion-exchange chromatography on DEAE-Sephadex A25 was carried out with linear

gradients of triethylammonium hydrogen carbonate buffer (pH 7.5).

Dioxan, acetonitrile and pyridine were dried by heating, under reflux, with CaH_2 for 3-5 hr; these solvents were then distilled at atmospheric pressure and stored over molecular sieves (no. 4A).

2'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridine (6).

A solution of 9-chloro-9-phenylxanthene (1.79g, 6.11 mmol) in anhydrous pyridine (50 ml) was added dropwise over a period of 1 hr to a stirred solution of 2'-O-methoxytetrahydropyranyluridine (1.76g, 4.91 mmol) in pyridine (50 ml) at room temperature. Water (0.5 ml) was added to the stirred products and, after a period of 10 min, the products were concentrated under reduced pressure. A solution of the residue in chloroform (60 ml) was extracted with saturated aqueous sodium hydrogen carbonate (60 ml) and the aqueous layer was back extracted with chloroform (2 x 60 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give a glass. The latter material was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with chloroform-ethanol (96:4 v/v), were combined, evaporated under reduced pressure, dissolved in chloroform (15 ml) and the solution added dropwise to petroleum ether (b.p. 30-40°C, 500 ml). The desired product was obtained as a colourless solid precipitate (2.86g, 94%), R_F 0.48 (system A); ^{1}H-n.m.r. [$(CD_3)_2SO$, 250 MHz]: δ 1.55 - 1.85 (4H, m), 3.06 (2H, m), 3.07 (3H, s), 3.35 - 3.85 (4H, m), 3.93 (1H, m), 3.99 (1H, m), 4.45 (1H, dd, J = 4.9, 7.3 Hz), 5.30 (1H, d, J = 4.9 Hz), 5.58 (1H, d, J = 8.0 Hz), 5.98 (1H, d, J = 7.4 Hz), 7.1 - 7.5 (13H, m), 7.70 (1H, d, J = 7.8 Hz), 11.41 (1H, br.s).

3'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridine (19b).

This compound (19b) was prepared from 3'-O-methoxytetrahydropyranyluridine (0.719g, 2.0 mmol) and 9-chloro-9-phenylxanthene (0.73g, 2.5 mmol) by the above procedure and was isolated as a colourless solid (1.09g, 88%); R_F 0.48 (system A); ^{1}H-n.m.r. [$(CD_3)_2SO$-D_2O, 250 MHz]: δ 1.5 - 1.8 (4H, m), 3.01 (3H, s), ca. 3.02 (1H, m), 3.21 (1H, dd, J = 2.6, 10.5 Hz), 3.38 (2H, m), 3.65 (2H, m), 4.03 (1H, m), 4.1 - 4.22 (2H, m), 5.47 (1H, d, J = 8.0 Hz), 5.68 (1H, d, J = 4.2 Hz), 7.1 - 7.5 (13H, m), 7.73 (1H, d, J = 8.1 Hz).

2'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (9).

To a stirred solution of 2'-O-methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridine (0.615g, 1.0 mmol) and 1-methylimidazole (0.32 ml, 4.0 mmol) in anhydrous tetrahydrofuran (10 ml) at room temperature was added 2-chlorophenyl phosphorodi-(1,2,4-triazolide) [3.0 mmol, prepared by adding 1,2,4-tria-

zole (0.414g, 6.0 mmol) and triethylamine (0.92 ml, 6.6 mmol) to a solution of 2-chlorophenyl phosphorodichloridate (0.736g, 3.0 mmol) in anhydrous tetrahydrofuran (20 ml) at room temperature and stirring the resulting mixture for 20 min]. After 20 min, triethylamine (2.5 ml, 17.9 mmol) and water (ca. 0.5 ml) was added. After 10 min, the resulting clear solution was concentrated under reduced pressure to ca. one-third volume and chloroform (50 ml) was added. The mixture obtained was extracted with 1.0 M - triethylammonium bicarbonate buffer (pH 7.5, 3 x 50 ml). The organic layer was separated, dried ($MgSO_4$) and concentrated under reduced pressure. When a solution of the residue in chloroform (5 ml) was added dropwise to petroleum ether (b.p. 30-40°C, 250 ml), the triethylammonium salt of 2'-O-methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)-3'-(2-chlorophenyl) phosphate was obtained as a colourless solid (0.86g, 95%); $\delta_p[(CD_3)_2SO]$: -6.74 p.p.m.

A solution of the above triethylammonium salt (0.82g, 0.90 mmol) and 2', 3'-di-O-acetyluridine (0.269g, 0.82 mmol) in anhydrous pyridine (15 ml) was evaporated under reduced pressure. After this procedure had been repeated once more, the residue was dissolved in anhydrous pyridine (12 ml) and 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT, 0.729g, 2.46 mmol) was added. The resulting solution was stirred at room temperature, with the exclusion of moisture, for 20 min and then saturated aqueous sodium hydrogen carbonate (1.5 ml) was added. After 10 min, chloroform (15 ml) was added and the products were washed with saturated aqueous sodium hydrogen carbonate (2 x 30 ml). The organic layer was separated, dried ($MgSO_4$), and concentrated under reduced pressure. Toluene (20 ml), ethanol (20 ml) and chloroform (20 ml) were added and then removed by evaporation to leave a glassy residue. The latter material was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with chloroform-ethanol (96:4 v/v), were combined and evaporated under reduced pressure. When a solution of the residue in chloroform (5 ml) was added to petroleum ether (b.p. 30-40°C, 250 ml), the desired fully-protected dinucleoside phosphate was obtained as a colourless precipitate (0.744g, 81%); R_F 0.38 (system A); δ_p $[(CD_3)_2SO]$: -7.55 (47%), -8.03 (57%) p.p.m.

3'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridylyl-(2'→5')-(2', 3'-di-O-acetyluridine) 2-chlorophenyl ester (21a).

3'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridine (0.615g, 1.0 mmol) was treated with 2-chlorophenyl phosphorodi-(1,2,4-triazolide) (3.0 mol. equiv.) according to the procedure described above to give the triethylammonium salt of 3'-O-methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)-3'-

(2-chlorophenyl) phosphate, which was isolated as a colourless solid (0.85g, 94%); δ_P [$(CD_3)_2SO$]: -6.22 p.p.m. The latter phosphodiester (0.794g, 0.88 mmol), 2',3'-di-O-acetyluridine (0.240g, 0.73 mmol) and MSNT (0.649g, 2.19 mmol) were allowed to react together in anhydrous pyridine (9 ml) solution at room temperature for 20 min, following the procedure described above. 3'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridylyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester was obtained and was isolated as a colourless solid (0.58g, 71%); R_F 0.40 (system A); δ_P [$(CD_3)_2SO$]: -7.39 (76%), -8.07 (24%).

Preparation of anhydrous stock solution of zinc bromide in dichloromethane-propan-2-ol (85:15 v/v).

Zinc bromide (33.78g, 150 mmol) was dissolved in propan-2-ol (50 ml) and the solution was evaporated under reduced pressure. This process was repeated two more times, and the residual zinc bromide was dissolved in anhydrous propan-2-ol (15 ml). Dichloromethane (85 ml) was added to give a 1.5 M - solution of zinc bromide in dichloromethane-propan-2-ol (85:15 v/v).

5'-Deprotection of 2'-O-methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)-uridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (9).

(a) Substrate (0.30g, 0.269 mmol) was dissolved in dichloromethane-propan-2-ol (85:15 v/v, 4.48 ml) and the above 1.5 M - anhydrous stock solution of zinc bromide (8.97 ml) was added. After 15 min, the products were poured into saturated aqueous sodium hydrogen carbonate (10 ml). The aqueous layer was back extracted with chloroform (3 x 15 ml). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with chloroform-ethanol (94:6 v/v) were combined and evaporated under reduced pressure. When a solution of the residue in chloroform (2 ml) was added to petroleum ether (b.p. 30-40°C, 100 ml), 2'-O-methoxytetrahydropyranyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (10) was obtained as a colourless solid (0.199g, 86%); R_F 0.28 (system A); δ_P [$(CD_3)_2SO$]: -7.67 p.p.m.

(b) Experiment (a) was repeated under the same conditions and on the same scale except that no special precautions were taken to ensure that the 1.5 M - zinc bromide solution was free from traces of moisture [i.e. the zinc bromide was not evaporated from dry propan-2-ol solution before it was dissolved in the appropriate quantities of propan-2-ol and dichloromethane]; yield of 5'-unblocked dinucleoside phosphate (10), 0.166g (72%).

(c) A 0.48 M - solution of phenyl dihydrogen phosphate in chloroform-

ethanol (95:5 v/v, 4.48 ml, 2.15 mmol) was added to a stirred solution of the substrate (0.30g, 0.269 mmol) in chloroform-ethanol (95:5 v/v, 4.48 ml) at room temperature. After 5 min, triethylamine (2.0 ml) was added and the products were poured into saturated aqueous sodium hydrogen carbonate (10 ml). The aqueous layer was separated and back extracted with chloroform (5 x 15 ml). The organic layers were combined, dried ($MgSO_4$), and evaporated under reduced pressure. The residual glass obtained was fractionated by short column chromatography and the desired 5'-unblocked dinucleoside phosphate (10) was isolated as above; yield, 0.125g (54%).

5'-O-Acetyl-2'-O-methoxytetrahydropyranyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (11).

2'-O-Methoxytetrahydropyranyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (0.162g, 0.188 mmol) was dried by evaporation from pyridine (2 x 1 ml) solution. The residue was dissolved in anhydrous pyridine (1.0 ml) and acetic anhydride (0.107 ml, 1.13 mmol) was added to the stirred solution at room temperature. After 2 hr, water (0.1 ml) was added and, after a further period of 30 min, chloroform (10 ml) was added. The products were extracted with saturated aqueous sodium hydrogen carbonate (15 ml), and the aqueous layer was back extracted with chloroform (3 x 10 ml). The organic layers were combined, dried ($MgSO_4$), and evaporated under reduced pressure. The residual glass obtained was fractionated by short column chromatography on silica gel. The appropriate fractions, which were eluted with chloroform-ethanol (94:6 v/v) were combined and evaporated under reduced pressure. When a solution of the residue in chloroform (1 ml) was added to petroleum ether (b.p. 30-40°C, 50 ml), the desired product was obtained as a colourless solid (0.160g, 94%); R_F 0.36 (system A); δ_p [$(CD_3)_2SO$]: -7.67 (54%), -7.87 (46%).

5'-O-Acetyl-3'-O-methoxytetrahydropyranyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (21c).

3'-O-Methoxytetrahydropyranyl-5'-O-(9-phenylxanthen-9-yl)uridylyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (21a, 0.30g, 0.269 mmol) was treated with zinc bromide in dichloromethane-propan-2-ol solution under the strictly anhydrous conditions described above to give 3'-O-methoxytetrahydropyranyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (0.185g, 80%); R_F 0.29 (system A); δ_p [$(CD_3)_2SO$]: -7.27 (75%), -8.03 (25%) p.p.m. The latter material (0.169g, 0.197 mmol) was treated with acetic anhydride (0.111 ml, 1.18 mmol) in anhydrous pyridine solution under the conditions described above, to give 5'-O-acetyl-3'-O-methoxytetrahydropyranyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (0.077g, 43%); R_F 0.37 (system A);

δ_P [$(CD_3)_2SO$]: -7.43 (71%), -8.15 (29%).

Action of aqueous acid on 5'-O-acetyl-2'-O-methoxytetrahydropyranyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (11).

(a) Acetic acid-water (4:1 v/v). The substrate (2.6 mg, 0.0029 mmol) was dissolved in acetic acid-water (4:1 v/v, 0.144 ml) at room temperature. The course of the reaction was monitored by t.l.c. (system A): the starting material (R_F 0.36) was gradually consumed and two product components only (R_F's 0.00 and 0.34) could be detected. At no time during the course of the reaction were any transitory components detectable by t.l.c. After 140 min, when t.l.c. revealed that no starting material remained, the products were concentrated under reduced pressure. The residue was analysed by h.p.l.c. on column (I) (see above): five main components were detected [see Figure 1a] with R_T's 2.5 and 2.8 (21% combined)†, 8.1 (26%), 14.2 (24%), and 17.9 (19%) min. The five components were identified as 5'-O-acetyluridine 2'(3')-phosphates, 5'-O-acetyluridine 2',3'-cyclic phosphate, 2',3'-di-O-acetyluridine, 5'-O-acetyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine), and 5'-O-acetyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine), respectively, by co-injecting the hydrolysis mixture with each authentic component (see below) in turn. These five components account for 90% of the total absorbance at 254 nm. The residue was also analyzed by h.p.l.c. on column (II) (see above): two main components (R_T's 2.7, 6.0 min) were detected and identified as 2',3'-di-O-acetyluridine and a mixture of 5'-O-acetyluridylyl-(2'→5')- and -(3'→5')-(2',3'-di-O-acetyluridine), respectively.

(b) 0.1 M - Hydrochloric acid-dioxan (1:1 v/v). The substrate (2.7 mg, 0.003 mmol) was dissolved in 0.1 M - hydrochloric acid-dioxan (1:1 v/v, 0.15 ml) at room temperature. After 8 hr, t.l.c. (system A) revealed two product components (R_F's 0.0 and 0.34), and no starting material (R_F 0.36). The products were then neutralized with aqueous ammonia and analysed by h.p.l.c. on column (I). The R_T's of the main components detected [see Figure 1c] were 2.0 (9%), 2.5 and 2.8 (23% combined), 4.3 (3%), 5.1 and 5.5 (13% combined), 8.1 (22%), 14.2 (17%), and 17.9 (15.5%) min. The components with R_T's 2.5, 2.8, 8.1, 14.2 and 17.9 min corresponded to those identified above in section (a); in total, they accounted for 77.5% of the total absorbance at 254 nm.

Action of acetic acid-water (4:1 v/v) on 5'-O-acetyl-3'-O-methoxytetrahydropyranyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (21c).

This experiment was conducted under the conditions described in experiment (a) above. The reaction was complete in 104 minutes. The main products obtained [see Figure 1b : R_T's 2.5 and 2.8 (20% combined), 8.1 (26%), 14.3

(25%) and 18.0 (17.5%) min] were identical to those obtained in experiment (a).

Action of ribonuclease A on acid hydrolysis products of 5'-O-acetyl-2'-O-methoxytetrahydropyranyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (11).

The substrate was hydrolyzed with acetic acid-water (4:1 v/v) as above, and a solution of the total hydrolysis products (5.0 A_{260} units) in water (0.01 ml) was treated with 0.1 M - tris hydrochloride buffer (pH 8.0, 0.1 ml), followed by a solution of ribonuclease A (0.02 mg) and magnesium chloride (0.019g, 0.2 mmol) in 0.1 M - tris hydrochloride buffer (pH 8.0, 0.02 ml) at 37°C. The reaction was monitored by h.p.l.c. on column (I): after 2 hr, the components with R_T's 2.5, 2.8 and 8.4 min had increased in intensity, the component with R_T 15.2 min remained unchanged, and the component with R_T 19.2 min has virtually disappeared [the R_T's of the last three components were previously found to be 8.1, 14.2 (14.3) and 17.9 (18.0) min, respectively]. It was also found that the ratio of the integral of the component with R_T 2.5 min to that of the component with R_T 2.8 min increased with time.

5'-O-Acetyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) (14).

E-2-Nitrobenzaldoxime (0.037g, 0.22 mmol) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.025 ml, 0.20 mmol) was added to a stirred solution of 5'-O-acetyl-2'-O-methoxytetrahydropyranyluridylyl-(3'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (11, 0.020g, 0.022 mmol) in anhydrous dioxan (0.67 ml) at room temperature. After 110 min, the products were neutralized with a 1.0 M - solution of acetic acid in dioxan (0.177 ml, 0.177 mmol), and water (5 ml) was added. The resulting solution was extracted with chloroform (10 x 10 ml) and ether (10 x 10 ml), and was then evaporated under reduced pressure. The residue obtained was chromatographed on a column of DEAE-Sephadex A25 which was eluted with a linear gradient (0.001 - 0.20 M) of triethylammonium hydrogen carbonate buffer (pH 7.5). The appropriate fractions were combined, evaporated under reduced pressure and the residue dried by evaporation from anhydrous pyridine (3 x 2 ml) solution. The resulting material was dissolved in anhydrous pyridine (1.0 ml) and treated with acetic anhydride (0.02 ml, 0.2 mmol) at room temperature. After 2 hr, methanol (0.013 ml) was added and, after a further period of 30 min, pyridine (2 ml) was added and the solution was concentrated to ca. one-third its volume. Triethylamine (0.12 ml) was then added and, after 5 min, the products were evaporated under reduced pressure and triturated with ether (3 x 2 ml). A portion of the resulting material (2.0 mg), which was virtually homogeneous by h.p.l.c. [R_T 4.5 min, column (I) eluted isocratically with 0.1 M - triethylammonium acetate-acetonitrile (4:1

v/v)] was dissolved in 0.01 M - hydrochloric acid (3 ml) at room temperature. After 40 min, the solution which contained the desired product [R_T 3.1 min, column (I) eluted isocratically with 0.1 M - triethylammonium acetate-acetonitrile (4:1 v/v)] was carefully neutralized with aqueous triethylammonium bicarbonate. When this material was treated with ribonuclease A under the above conditions, it underwent digestion to give products with retention times [column (I)] corresponding to 5'-O-acetyluridine 3'-phosphate (17), 5'-O-acetyluridine 2',3'-cyclic phosphate (16) and 2',3'-di-O-acetyluridine (8). 5'-O-Acetyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) (15).

5'-O-Acetyl-3'-O-methoxytetrahydropyranyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) 2-chlorophenyl ester (21c, 0.020g, 0.022 mmol) was unblocked by the procedure used above for the corresponding (3'→5')-isomer (11). The R_T's [column (I), eluted isocratically with 0.1 M - aqueous triethylammonium acetate-acetonitrile (4:1 v/v)] of the desired product and its 3'-O-methoxytetrahydropyranyl derivative (i.e. the material obtained before treatment with 0.01 M - hydrochloric acid) were 5.5 and 2.8 min, respectively. 5'-O-Acetyluridylyl-(2'→5')-(2',3'-di-O-acetyluridine) (15) was unchanged after it had been treated with ribonuclease A, under the above conditions, for 2 hr. 5'-O-Acetyluridine 2',3'-cyclic phosphate (16) and 5'-O-acetyluridine 2'(3')-phosphates [(18) and (17), respectively].

The mixed disodium salts of uridine 2'- and 3'-phosphates (0.017g, 0.046 mmol) were dried by evaporation from pyridine (3 x 1 ml) solution, and were then dissolved in dimethylformamide (0.5 ml) and pyridine (1.0 ml). Acetic anhydride (0.047 ml, 0.5 mmol) was added to the stirred solution at room temperature. After 2 days, 1.5 M - triethylammonium hydrogen carbonate buffer (0.7 ml, 1.05 mmol) was added and, after a further period of 30 min, the solution was evaporated under reduced pressure. H.p.l.c. [column (I)] of the products revealed one principal component (ca. 90%) with R_T = 2.8 min. This material, which was assumed to be 5'-O-acetyluridine 2',3'-cyclic phosphate (16), slowly underwent digestion to a product [R_T = 2.5 min, assumed to be 5'-O-acetyluridine 3'-phosphate] when it was treated with ribonuclease A under the conditions described above. When the cyclic phosphate (16, 1.8 mg) was allowed to stand in acetic acid-water (4:1 v/v, 0.25 ml) at room temperature for 3 hr, it underwent ca. 40% conversion to a component with R_T = 2.5 min, assumed to be a mixture of 5'-O-acetyluridine 2'- and 3'-phosphates.

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†The numbers in parentheses indicate the percentages of the total absorbance at 254 nm accounted for by each component (or components).

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