Volume 16 Number 19 1988 Nucleic Acids Research

# Studies on the t-butyldimethylsilyl group as 2'-O-protection in oligoribonucleotide synthesis via the H-phosphonate approach

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Received August 5, 1988; Accepted September 15, 1988

Abstract

Two model compounds, **1** and **2**, have been studied to test the stability of the t-butyldimethylsilyl (t-BDMSi) group towards conditions used during chemical synthesis of RNA fragments by the H-phosphonate approach. When **1** was treated with anhydrous acid for 16 h both the H-phosphonate diester and the t-BDMSi group remained intact. Removal of the t-BDMSi group from **2** with 1.0 M tetrabutylammonium fluoride (TBAF · $3H_2O$) in THF was complete within 4 h and neither concomitant cleavage nor migration of the phosphodiester linkage could be detected even after 24 h. The dimer **2** was not completely stable towards concentrated aqueous ammonia and both loss of the t-BDMSi group and concomitant cleavage of the phosphodiester linkage occurred upon prolonged treatment. These reactions were substantialy suppressed in ethanol containing ammonia solutions, however to alleviate this problem during oligoribonucleotide synthesis, more labile protecting groups for heterocyclic bases would be desired. In conclusion, these studies indicate that 2'-O-t-BDMSi can be considered as a convenient and safe protecting group, which should secure synthesis of oligoribonucleotides with exclusively 3'-5' internucleotidic linkages.

## INTRODUCTION

In the last two years the use of hydrogenphosphonates (H-phosphonates) for synthesis of internucleotidic linkages has attracted much attention. This concept was first investigated by Todd et al.[1] but it was soon abandoned in favour of the phosphorodiester and phosphorotriester methods which appeared more promising at that time. However, our recent studies on the chemistry of nucleoside H-phosphonates[2,3] showed that under different experimental conditions, nucleoside H-phosphonates can be valuable intermediates in oligonucleotide synthesis.

Since these initial studies on the H-phosphonate approach to oligonucleotide synthesis, the method has been further improved and applied to the automated synthesis of both DNA[4-6] and RNA[7,8] fragments. Synthesis of medium size (19-43 mers) oligoribonucleotides using phosphoramidite chemistry have also recently been reported[9-11].

We have continued to explore the H-phosphonate approach to be able to produce longer oligoribonucleotides, e. g. of t-RNA size. In order to do so, we have carried out a number of studies on the chemistry of nucleoside H-phosphonates. These include studies on activation and condensation using chlorophosphates[12], sulfonylchlorides[13] and acyl chlorides[14], as well as investigations on oxidation[15] of H-phosphonates, and on new potential condensing agents[16]. These studies may provide a basis for further optimization of condensation and oxidation steps. However, other parts to optimize, when aiming at longer RNA fragments, are deprotection conditions and/or the choice of protecting groups.

In the most recent approaches[7-11] to automated RNA synthesis three different 2′-O-protecting groups have been successfully employed. These are the o-nitrobenzyl[8,10], the 1-[(2-chloro-4-methyl)phenyl]-4-methoxypiperidin-4-yl (Ctmp)[9] and the t-butyldimethylsilyl (t-BDMSi)[7,11] groups. The disadvantage of using o-nitrobenzyl protecting group is its photochemical deprotection which may pose severe problems with longer oligoribonucleotides[10]. The Ctmp group seems quite promising but the reported coupling efficiency (93%) is still insufficient for synthesis of long fragments. This may perhaps be improved by optimization or by combining the Ctmp group with H-phosphonate chemistry.

It has been demonstrated, particularly by Ogilvie et al.[11], that the t-butyldimethylsilyl (t-BDMSi) group can be successfully used for 2′-protection of the ribose residues during oligoribonucleotide synthesis. We have shown that the same protection for RNA synthesis can be used also in conjunction with H-phosphonate chemistry[7]. However, in order to optimize the deprotection scheme, quantitative studies on the stability and removal of the t-BDMSi group were necessary.

In the method developed by us[7], the synthesis of oligoribonucleotides is based on the use of ribonucleoside 3′-H-phosphonates (e. g. **3**) carrying t-butyldimethylsilyl (t-BDMSi) protection for the 2′-OH function. A protected H-phosphonate (e.g. **3**) is coupled in the presence of a condensing agent to a nucleoside attached to the solid support *via* its 3′-OH function. The 5′-O-dimethoxytrityl group (DMT) of the resulting H-phosphonate diester is then removed with anhydrous acid to generate a free 5′-OH function in the growing oligonucleotidic chain. These two steps are then repeated (with washing in between) until the desired chain is assembled. The oligoribonucleoside H-phosphonate chain is then oxidized with $I_2$ in pyridine/$H_2O$ to produce the protected RNA fragment. The deprotection is accomplished in two steps. First, treatment with ammonia to cleave the oligonucleotidic chain from the support

and to remove acyl protecting groups from the heterocyclic bases, and then tetrabutylammonium fluoride in THF is used to remove the t-BDMSi groups.

The most crucial points to investigate were:

i) whether the silyl groups are stable towards the repeated acid treatments used for removal of the 5′-O-dimethoxytrityl group,

ii) if any loss of the t-BDMSi groups occur during deprotection of the heterocyclic bases and if so, how much cleavage of the RNA chain may be the result,

iii) whether any isomerization or cleavage of phosphodiester linkages can be detected when removing the t-BDMSi groups with TBAF in THF.

## RESULTS AND DISCUSSION

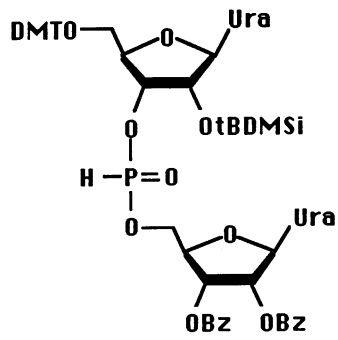
It is of utmost importance that the fully protected H-phosphonate monoesters (e. g. **3**) are isomerically pure, since traces of the undesired 3′-O-t-butyldimethylsilyl 2′-H-phosphonate (e.g. **4**) inevitably would give oligomers contaminated with 2′-5′ linkages.

2′-O-t-BDMSi protected nucleosides (e.g. **5**) may isomerize rapidly to **6** in alcoholic or aqueous solutions [17-19] but this should not occur to any appreciable extent in neutral or weakly basic dry solvents[18,19]. In agreement with this we could not detect any migration of t-BDMSi group even after 24 h when 5′-O-dimethoxytrityl-2′-O-t-butyldimethylsilyluridine **5** was left in the solvent used for chromatography (toluene/ethyl acetate) or in the solvent used for the phosphitylation (acetonitrile). The $^{31}P$ n.m.r. spectra of the crude product from the reaction of **5** with the $PCl_3$/imidazole/triethylamine system did not show any contamination with the unwanted isomer **4**[20]. However, when a crude reaction product was checked by t.l.c. (overloaded application) traces (less then 0.5%) of compound with the chromatographic mobility of **4** could be detected. This presumed isomerization can be explained as a result of salt or/and base (triethylamine, imidazole) catalyzed migration of the t-BDMSi group under the phosphitylation conditions. However, during the chromatographic purification, routinely used by us, such traces of contaminant are easily removed.

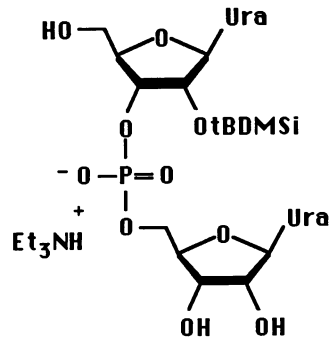
### Stability of the t-BDMSi group under detritylation conditions

Treatment of the H-phosphonate diester **1** with 2.5% dichloroacetic acid (DCA) in dichloromethane was followed by both $^{31}P$ n.m.r. spectroscopy and by thin layer chromatography (t.l.c.) on a silica gel.

When **1** ($\delta$=8.5 ppm) was dissolved in 2.5% DCA in dichloromethane the characteristic deep orange colour from the trityl cation developed immediately




1

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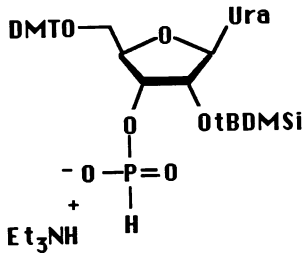


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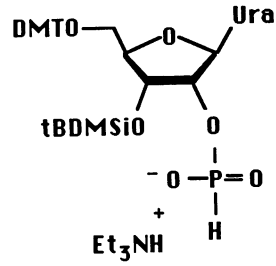


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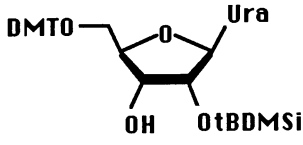


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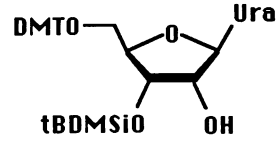



6

**Scheme 1**

but the pattern in the $^{31}P$ n.m.r. spectrum remained very close to that of 1 ($\delta$=8.6 ppm). No further changes in the $^{31}P$ n.m.r. spectrum were observed within 16 h. When the same reaction was analysed by t.l.c., only detritylated product and the tritanol were detected. The compound obtained after a 16 h treatment with DCA was also isolated by preparative t.l.c. and its $^{1}H$ n.m.r. spectrum revealed that the silyl group remained intact.

A 16 h acid treatment corresponds to 480 detritylation times in automated solid phase synthesis. The results thus indicate that both the H-phosphonate

Table 1.
Ammonia treatment at 55°C and at room temperature (R.T.)

| | 25% $NH_4OH$ | | | 35% $NH_4OH$ | | | 25% $NH_4OH$/ EtOH 3/1 | | | 35% $NH_4OH$/ EtOH 3/1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 55°C | | | | | | |
| Time (h) | U(Si)pU | UpU | U | U(Si)pU | UpU | U | U(Si)pU | UpU | U | U(Si)pU | UpU | U |
| 0 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| 1 | 94.7 | 5.3 | 0.0 | 96.5 | 3.5 | 0.0 | 99.1 | 0.9 | 0.0 | 99.9 | 0.1 | 0.0 |
| 2 | 92.6 | 7.4 | 0.0 | 93.4 | 6.6 | 0.0 | 98.6 | 1.4 | 0.0 | 99.3 | 0.7 | 0.0 |
| 4 | 86.4 | 12.8 | 0.8 | 89.0 | 10.8 | 0.2 | 97.2 | 2.8 | 0.0 | 98.5 | 1.5 | 0.0 |
| 6 | 79.0 | 19.0 | 2.0 | 82.8 | 15.5 | 1.7 | 96.2 | 3.8 | 0.0 | 97.4 | 2.6 | 0.0 |
| 8 | 71.6 | 23.8 | 4.6 | 77.3 | 19.4 | 3.3 | 95.2 | 4.7 | 0.1 | 96.1 | 3.9 | 0.0 |
| 12 | 62.5 | 27.3 | 10.2 | 72.4 | 23.2 | 4.4 | 93.2 | 6.3 | 0.5 | 94.8 | 5.2 | 0.0 |
| 24 | 27.5 | 32.4 | 40.1 | 35.5 | 33.5 | 30.9 | 87.9 | 9.9 | 2.2 | 87.9 | 11.1 | 1.0 |
| | | | | | | R.T. | | | | | | |
| 0 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| 1 | 99.6 | 0.4 | 0.0 | 99.9 | 0.1 | 0.0 | 99.9 | 0.1 | 0.0 | 99.9 | 0.1 | 0.0 |
| 2 | 99.2 | 0.8 | 0.0 | 99.6 | 0.4 | 0.0 | 99.8 | 0.2 | 0.0 | 99.9 | 0.1 | 0.0 |
| 4 | 98.5 | 1.5 | 0.0 | 99.2 | 0.8 | 0.0 | 99.7 | 0.3 | 0.0 | 99.8 | 0.2 | 0.0 |
| 6 | 97.8 | 2.2 | 0.0 | 99.0 | 1.0 | 0.0 | 99.6 | 0.4 | 0.0 | 99.7 | 0.3 | 0.0 |
| 8 | 97.0 | 3.0 | 0.0 | 98.5 | 1.5 | 0.0 | 99.5 | 0.5 | 0.0 | 99.6 | 0.4 | 0.0 |
| 12 | 96.4 | 3.6 | 0.0 | 97.3 | 2.7 | 0.0 | 99.3 | 0.7 | 0.0 | 99.4 | 0.6 | 0.0 |
| 24 | 89.5 | 10.5 | <0.1 | 94.3 | 5.7 | <0.1 | 98.5 | 1.5 | 0.0 | 98.7 | 1.3 | 0.0 |

diester function and the 2′-O-t-BDMSi group should survive detritylation conditions when repeated hundreds of times.

Using the same analytical tools it could be seen that **1** was completely stable in the solvents used for washings in machine-assisted synthesis, and also that the t-BDMSi group was stable during oxidation of H-phosphonate diester **1** with iodine in aqueous pyridine even after a prolonged time (9 h).

Stability of the 2′-O-t-BDMSi group in dimer **2** towards ammonia treatment

The stability of **2** towards conditions used for the removal of base protecting groups was monitored using a reversed phase h.p.l.c. system (Fig. 3).

Four different ammonia solutions at 55 °C and at room temperature were investigated: 25% ammonia (aq.), 35% ammonia (aq.), 25% ammonia (aq.)/absolute ethanol (3:1, v/v) and 35% ammonia (aq.)/absolute ethanol (3:1, v/v).

The rate of removal of acyl protecting groups from the heterocyclic bases does not differ much under these various ammonia conditions[21]. However, as can be seen from Table 1, there is a significant difference in the stability of the t-BDMSi group depending on the ammonia concentration. This is even more profound when comparing systems with and without ethanol. For example, in 25% ammonia at 55 °C ca 28 % of the silyl groups from the dimer **2** were lost after 8 h and ca 72% after 24 h. In 35% ammonia/ethanol solution at 55 °C,

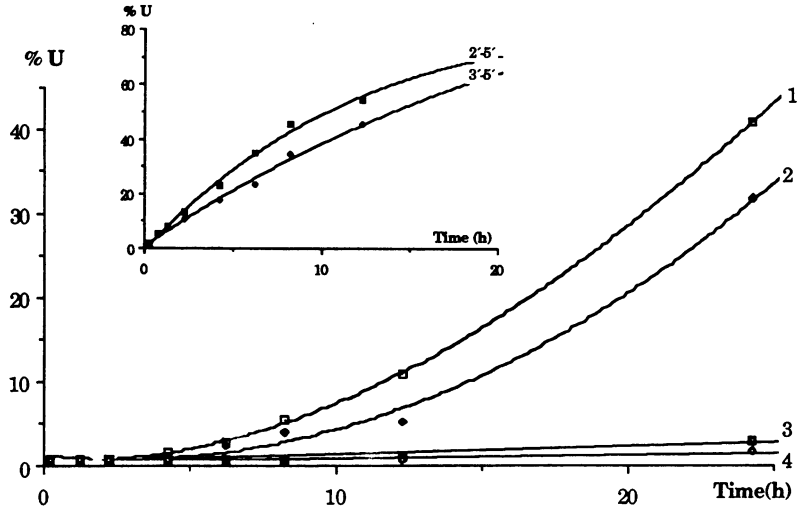



**Fig. 1**: Graph showing degradation of the 2′-O- silylated dimer **2** ( due to loss of the t-BDMSi group) as calculated from the amount of uridine released at 55°C in different ammonia (aq) solutions. Curve 1, 25% ammonia; Curve 2, 35% ammonia; Curve 3, 25% ammonia/ EtOH (3:1, v/v); Curve 4, 35% ammonia/EtOH (3:1, v/v). Insert shows cleavage of U(2′-5′)U and U(3′-5′)U at 55 °C in 25% ammonia.

however, the loss of the t-BDMSi groups amounts to only ca 4% and 10% after 8 and 24 h respectively. Even if the silyl groups are to be removed in the next deprotection step, loss of protecting groups from the 2′-OH functions of oligoribonucleotides under basic conditions may result in subsequent cleavage of the phosphodiester linkages. This was indeed observed, especially in non-alcohol containing ammonia solutions at 55 °C. For example, in 25% ammonia, 4.6% cleavage of the dimer **2** was observed after 8 h and ca 40% after 24 h (Fig. 1). The extent of cleavage of **2** in 35% ammonia/ ethanol solution at 55 °C was considerably smaller, being undetectable after 12 h and consisted of ca 1% after 24 h (Fig. 1). These results together with the rates of hydrolysis of U(3′-5′)U and U(2′-5′)U (see insert in Fig. 1) also indicate that cleavage of **2** proceeds *via* U(3′-5′)U as an intermediate and not *via* an immediate attack of an alkoxy ion formed *in situ* during desilylation.

Since the deprotection time for acyl groups at the nucleoside level in 35% ammonia/ethanol at 55 °C is ca 2 h for $N^4$-benzoylcytidine, ca 6 h for $N^6$-benzoyl-adenosine and ca 8 h for $N^2$-isobutyrylguanosine (t.l.c. experiments)[21], it seems that this ammonia/ethanol system should be suitable for deprotection of medium-size oligomers (20-40 mers). However, in the synthesis of longer RNA

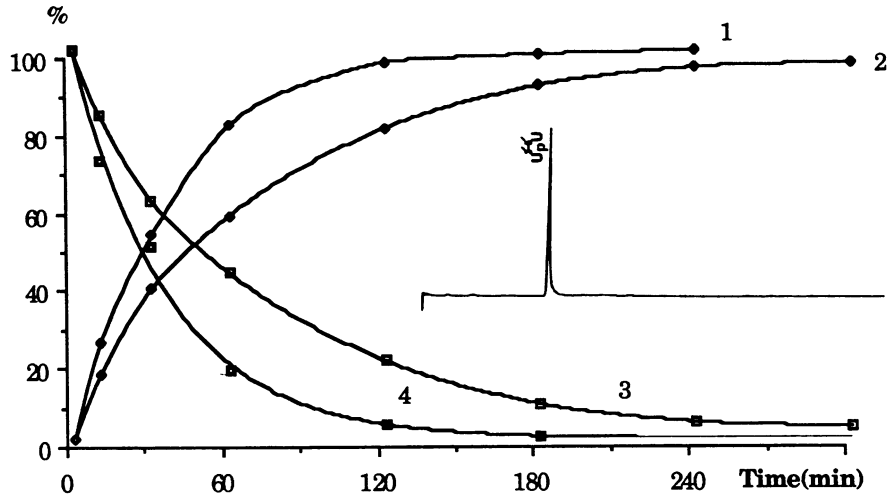


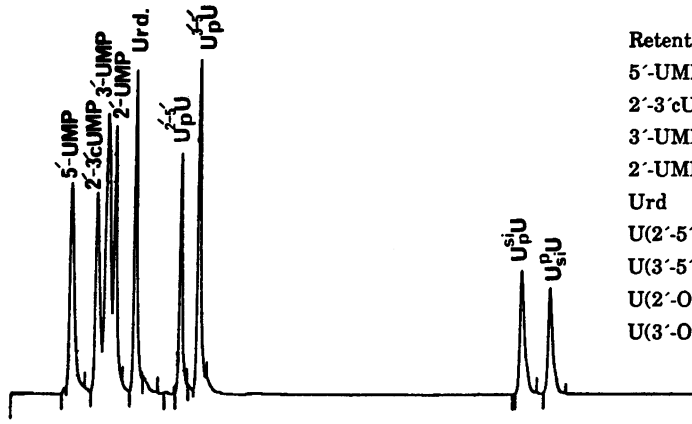

**Fig. 2**: Graph showing decrease of **2** and increase of U(3′-5′)U with time during treatment with TBAF in THF. Curves 1 and 4, 1.0 M TBAF; curves 2 and 3, 0.1 M TBAF. Insert shows h.p.l.c. profile after 24 hours treatment of **2** with 1.0 M TBAF.

fragments, especially when an extended deprotection time would be required, even small cleavage of phosphodiester linkages due to the loss of t-BDMSi groups, will strongly influence the outcome of synthesis. Thus, keeping the t-BDMSi groups intact during heterocyclic bases deprotection is most important. In addition, it would also secure the stability of RNA fragments during storage.

In order to achieve this, it seems that more labile base protecting groups are needed. It can be seen from Table 1, that groups requiring deprotection with ammonia /ethanol solution at room temperature for 8-12 h (or preferable less) should be a suitable choice, since during that time less than 1% of the silyl groups are lost from **2** without detectable cleavage of the internucleotidic bond. Such criteria are met by e.g. the phenoxyacetyl group as protection for adenine and guanine, and the isobutyryl group for cytosine, as shown by Teoule et al.[22] in DNA synthesis. These groups (in the deoxy series) can be removed in less than 4 h in 29% ammonia at room temperature and the deprotection time for oligoribonucleotides should be of a similar magnitude.

Treatment of the 2′-O-silylated dimer **2** with TBAF · $3H_2O$ in THF

The deprotection of **2** with TBAF · $3H_2O$ in THF was followed by h.p.l.c. analysis on a reversed phase column (Fig. 3). The rate of reaction was determined in 0.1 M and 1.0 M TBAF solutions (Fig. 2). Approximate half-




| Retention time | (min) |
|---|---|
| 5'-UMP | 2.0 |
| 2'-3'cUMP | 2.8 |
| 3'-UMP | 3.2 |
| 2'-UMP | 3.5 |
| Urd | 4,4 |
| U(2'-5')U | 5.9 |
| U(3'-5')U | 6.7 |
| U(2'-OSi)(3'-5')U | 18.1 |
| U(3'-OSi)(2'-5')U | 19.1 |

**Fig. 3**: H.p.l.c. chromatogram showing separation of all reference compounds (see Experimental for details).

times of the removal of t-BDMSi group were found to be ca 30 and 45 min. for the reactions in 1.0 M TBAF and 0.1 M TBAF respectively. No starting material **2** could be detected after 4 h (1.0 M TBAF) and after 7 h (0.1 M TBAF). This is in agreement with some protocols for medium size oligoribonucleotide synthesis[11]. However, it seems likely, that treatment with 1.0 M TBAF for a longer time then 4 h should have a beneficial effect on the yield of deprotection, especially for longer RNA fragments.

The solubility of the oligomers in THF may be considered a problem, but since they carry many tetrabutylammonium counterions one may anticipate that the oligomers will remain in solution also after removal of the silyl groups.

The most important finding from these experiments is that deprotection of **2** with TBAF in THF gives nothing but the correct product , U(3'-5')U. Thus neither cleavage nor isomerization of the phosphodiester occurs even after 24 h (h.p.l.c., Fig. 2, detection limit estimated to be less than 0.1%). Fig. 3 shows that all relevant compounds separate in the h.p.l.c. system used.

Summarizing the above results, it seems that the t-BDMSi group is completely stable towards detritylation conditions, washings, and towards the oxidizing reagent used during RNA synthesis *via* the H-phosphonate approach. Furthermore, the t-BDMSi group can be completely removed within 4 h in 1.0 M TBAF in THF without any concomitant cleavage of the internucleotidic bond. Most important is, that no migration of the phosphodiester linkage occurs during the fluoride treatment, thus ensuring that the synthesized RNA fragments will contain exclusively the desired 3'-5' linkages.

For removal of base protecting groups with ammonia, the right conditions must be chosen. From the deprotection systems discussed above, 35% ammonia (aq) /absolute ethanol (3:1, v/v) is the preferred choice. However, using more labile base protecting groups will be even more advantageous. That should minimize (or eliminate) cleavage of internucleotidic bonds due to premature loss of the t-BDMSi groups and this is of prime importance, irrespective of the condensation procedure, in the synthesis of long RNA fragments. If these conditions will be fulfilled, then the yield of the desired oligoribonucleotide should depend mainly on the efficiency of the condensation step.

## EXPERIMENTAL

### Materials and Methods

$^{1}H$, $^{13}C$ and $^{31}P$ n.m.r. spectra were recorded on a Jeol FX-400 FT spectrometer. $^{1}H$ and $^{13}C$ n.m.r. spectra were referenced to the internal solvent signal and for $^{31}P$ n.m.r. spectra 1% $H_3PO_4$ in $D_2O$ was used as an external standard (coaxial inner tube). T.l.c. was carried out on Merck silica gel 60 $F_{254}$ pre-coated plates using the following eluents: isopropanol/ammonia/water 16:1:2 (v/v)(system A); chloroform/methanol 9:1 (v/v)(system B); toluene/ethyl acetate 1:2 (v/v) (system C).

Analytical h.p.l.c. was carried out on a Waters Resolve™ 5μm spherical C-18 columns, using a 20 min linear gradient of acetonitrile /water (1:3, v/v) in 0.05 M $KH_2PO_4$ (aq) (0 to 100%) (System D) with flow rate of 1.0 ml/min. Detection was carried out by measuring absorbance at 254 nm and the relative areas were obtained using a Hewlett Packard 3340A integrator. All reported areas were corrected by calibration using reference compounds.

Pyridine, acetonitrile and triethylamine were refluxed with $CaH_2$ overnight and then distilled and stored over molecular sieves (4Å) or $CaH_2$. Tetrahydrofuran (THF) was distilled just before use from lithium aluminium hydride. Dichloroacetic acid (DCA), imidazole and tetrabutylammonium fluoride (TBAF · $3H_2O$) were Aldrich commercial grade. 25% and 35% ammonia (p.a.) were from BDH. Reference samples of U(2′-5′)U, U(3′-5′)U, 2′-UMP, 3′-UMP, 5′-UMP, 2′,3′-cUMP were purchased from Sigma. Compound **5** and **6** were synthesized according to Ref. 23 with the exception that toluene/ethyl acetate (9:2, v/v) was used for column chromatography.

### Synthesis of 5′-O-(4,4′-dimethoxytrityl)-2′-O-t-butyldimethylsilyluridin-3′-yl hydrogenphosphonate triethylammonium salt, **3**

Imidazole (5.8 g, 86 mmol, coevaporated twice with acetonitrile) was dissolved in acetonitrile (100 ml) and then kept in an ice-water bath with

stirring. $PCl_3$ (2.45 ml, 28 mmol) was then added dropwise to the solution followed by triethylamine (12.5 ml, 90 mmol) in acetonitrile (10ml).

A solution of **5** [23] (5.3 g, 8 mmol, coevaporated first with pyridine and then with acetonitrile) in acetonitrile (200 ml) was added dropwise to the stirred reaction mixture during 30 min. After the addition was complete, the ice-salt bath was removed and the mixture was stirred for an additional 15 min at ambient temperature, whereafter 0.1 M triethylammonium bicarbonate (TEAB) (20 ml) was added. The reaction mixture was evaporated to near dryness, coevaporated with pyridine/triethylamine (4:1, v/v) and then coevaporated with toluene to dryness. The residue was partitioned between chloroform and 1.0 M TEAB. The aqueous phase was extracted twice with chloroform. The combined organic phases were then washed with 1.0 M TEAB and evaporated. The crude product was purified using short column chromatography with a stepwise gradient of MeOH in $CH_2Cl_2$ (containing 0.1% $Et_3N$) (0 to 10%). After evaporation of pure fractions, a white foam was obtained. This was dissolved in $CH_2Cl_2$ and precipitated from hexane/ether mixture (1:1, v/v) to give a white powder. Yield: 7.3 g, 92%. $R_f$=0.8 (system A).

**$^{31}$P n.m.r.** (pyridine): δ=2.8 ppm, $^1J_{PH}$=622 Hz, $^3J_{PH}$=10.2 Hz

**$^1$H n.m.r.** ($CDCl_3$, δ in ppm): 0.12 and 0.15 (2s, 3+3H, Me-Si), 0.87 (s, 9H, t-Bu-Si), 1.25 (t, 9H, $C\underline{H_3}CH_2$-N), 3.07 (q, 6H, $CH_3C\underline{H_2}$-N), 3.42 and 3.48 (m, 1+1H, 5′-H and 5′′), 3.74 (s, 6H, MeO), 4.26 (m, 1H, 4′-H), 4.44 (t, 1H, 2′-H), 4.81 (m, 1H, 3′-H), 5.14 (d, 1H, 5-H), 5.87 (d, 1H, 1′-H), 6.83 (d, 1H, P-H, $^1J_{PH}$=609 Hz), 6.85 - 7.44 (m, 13H, Ar-H),7.86 (d, 1H, 6-H).

**$^{13}$C n.m.r.** ($CDCl_3$, δ in ppm): -4.28 and -4.53 (Me-Si), 9.00 and 46.66 (Et-N), 18.75 ($Me_3C$-Si), 26.14 ($Me_3$C-Si), 55.91 (MeO), 63.47 (C-5′), 73.08 (C-3′), 76.10 (C-2′), 83.63 (C-4′), 87.80 (C-1′), 89.53 ($Ar_3C$), 102.70 (C-5), 140.90 (C-6), 151.70 (C-2), 164.26 (C-4).

Synthesis of 2′,3′-di-O-benzoyluridine

Uridine (12.2 g, 50 mmol) was coevaporated with pyridine and then dissolved in the same solvent (100 ml). After cooling in an ice bath, 4,4′-dimethoxytrityl chloride (18.6 g, 55 mmol) was added and the mixture was left with stirring at room temperature overnight. Benzoyl chloride (13 ml, 110 mmol) was then added dropwise and after the reaction was over (t.l.c.), the mixture was quenched with MeOH ( 5 ml) and evaporated. The residue was partitioned between chloroform and 5% sodium bicarbonate (aq.). The organic phase was evaporated and then coevaporated with toluene to remove traces of pyridine. The crude product was then dissolved in 1% p-toluenesulfonic acid in chloroform/methanol (95:5, v/v) ( 200 ml) and after 5 min. the solution was

extracted with 5% sodium bicarbonate (aq.) and then with water. The organic phase was evaporated and the residue washed with ether. The product was finally recrystallized from absolute ethanol. Yield: 17.8 g, 78%.
M.p. 195-197 °C, (lit.[24] m.p. 195-197 °C). $R_f$= 0.4 (system B)
**$^{13}$C n.m.r.** [$(CD_3)_2SO$, δ in ppm): 61.05 (C-5′), 72.22 (C-3′), 73.66 (C-2′), 83.38 (C-4′), 86.29 (C-1′), 102.76 (C-5), 128.47 (Ar tert-C), 128.82, 128.97, 129.38, 129.42 (Ar ortho- and metha-C), 134.01 (Ar para-C), 140.66 (C-6), 150.72 (C-2), 163.14 (C-4), 164.63 and 164.93 (C=O of 2′and 3′-O-benzoyl).
Synthesis of 5′-O-(4,4′-dimethoxytrityl-2′-O-t-butyldimethylsilyluridin-3′-yl 2′,3′-di-O-benzoyluridin-5′-yl hydrogenphosphonate **1**

Compound **3** (0.89 g, 1.1 mmol) and 2,3-di-O-benzoyluridine (0.45 g, 1 mmol) were coevaporated with pyridine and dissolved in the same solvent (10 ml). To the stirred reaction mixture bis(2-oxo-3-oxazolidinyl)phosphinic chloride[25] (OXP)(0.64 g, 2 mmol) was then added. After the reaction was complete, 0.5 M TEAB ( 50 ml) was added and the solution was evaporated. The residue was partitioned between methylene chloride and 1.0 M TEAB, the organic phase evaporated and the product was chromatographed on a short column using a three step gradient (toluene/ethyl acetate 2:1, 1:1 and 1:2, v/v). Yield: 1.1 g, 96%. Rf=0.81 (system B). $R_f$=0.46 and 0.35 (system C), two diastereoisomers.
**$^{31}$P n.m.r.** (pyridine): δ=9.5 ppm, $^1J_{PH}$=731 Hz, $^3J_{PH}$=8.5 Hz (diastereoisomer moving faster on t.l.c.); δ=8.8 ppm, $^1J_{PH}$= 719 Hz, $^3J_{PH}$=10.0 Hz (slower moving diastereoisomer).
**$^1$H n.m.r.** (CDCl3, δ in ppm) (faster moving diasteroisomer): 0.10 and 0.20 (2s, 3H + 3H, MeSi), 0.91 (s, 9H, t-Bu-Si), 3.53 (s, 2H, 5′-H), 3.78 and 3.79 (2s, 3+3H, MeO), 4.33-4.49 (2m, 4H, 3′-H*, 4′-H, 5′-H*), 4.61 (dd, 1H, 4′-H*), 5.16 (m, 1H, 3′-H), 5.27 (dd, 1H, 5-H), 5.59 (t, 1H, 2′-H), 5.76 (dd, 1H, 2′-H*), 5.81 (dd, 1H, 5-H*), 6.10 (d, 1H, 1′-H), 6.25 (d, 1H, 1′-H*), 6.85 (d, 4H, Ar-H, ortho to MeO on trityl), 7.15-7.39 (m, 13H, Ar-H), 7.22 (d, 1H, P-H, $^1J_{PH}$=735 Hz), 7.49 (d, 1H, 6-H*), 7.53-7.57 (m, 2H, Ar-H, para in benzoyl), 7.77 (d, 1H, 6-H), 7.90-7.94 (m, 4H, Ar-H, ortho in benzoyl), 8.21 and 8.30 (2d, 2H, N-H) (Symbol * indicates sugar and base protons in the 2′,3′-di-O-benzoyluridin-5′-yl unit of the dimer).
Synthesis of 2′-O-t-butyldimethylsilyluridin-3′-yl uridin-5′-yl phosphate, triethylammonium salt, **2**

A mixture of **3** (0.81 g, 1 mmol) and 2′,3′,-di-O-benzoyluridine (0.48 g, 1.1 mmol) was coevaporated with pyridine after which 10 ml of the same solvent was added. To the stirred reaction mixture, OXP (0.64 g, 2 mmol) was then added. After the reaction was complete, 4% $I_2$ in pyridine/water (96:4, v/v)(10

ml) was added and after 5 min the reaction mixture was diluted with methylene chloride, washed with 10% aqueous $Na_2S_2O_3$ and then with 1.0 M TEAB. The residue was chromatographed using the same system as for **3**. After evaporation of the desired fractions, a white solid was obtained. Yield: 1.13 g, 90%. $R_f$=0.64 (system A). **$^{31}$P n.m.r.** (pyridine) δ=0.51 ppm.

The product (1 g) was then dissolved in 35% ammonia/absolute ethanol (3:1)(50 ml). After 12 h the ammonia solution was evaporated and the residue was washed repeatedly with hot ether to give a white, chromatographically homogeneous solid.Yield: 0.75 g, 98%. $R_f$= 0.08 (system A).

Part of this product (0.21 g) was dissolved in 80% acetic acid ( 50 ml). After 5 min. water (50 ml) was added, the aqueous solution was extracted with ether and after addition of triethylamine (1 ml) it was evaporated. The product was purified using semipreparative h.p.l.c. on a Merck LiChrosorb™ RP-18 (7 μm) column . The elution system was a 20 min. linear gradient of acetonitrile/water (1:3, v/v) in 0.01 M TEAB (0 to 100%). After lyophilization a white solid was obtained. Yield: 120 mg, 80%. H.p.l.c., r.t.=18.1 min (System D).
**$^1$H n.m.r.** (MeOD, δ in ppm): 0.20 and 0.23 (2s, 3H + 3H, Me-Si), 0.99 (s, 9H, t-Bu-Si), 3.95 (d, 2H, 5′-H), 4.32-4.39 (m, 3H, 3′-H* and 5′-H*), 4.30 (d, 1H, 2′-H*), 4.32 (m, 1H, 4′-H*), 4.42 (m, 1H, 4′-H), 4.54 (t, 1H, 2′-H), 4.61 (dt, 1H, 3′-H), 5.77 and 5.88 (2d, 1H+1 H, 5-H and 5-H*), 5.97 (d, 1H, 1′-H), 6.04 (d, 1H, 1′-H*), 8.06-8.21 (2d, 1+1H, 6-H and 6-H*) (Symbol * indicates protons in the uridin-5′-yl unit of the dimer).

Treatment of **2** with tetrabutylammonium fluoride (TBAF)

Compound **2** (0.5 - 1 mg) was dissolved in 1 ml of 0.1 M or 1.0 M TBAF · $3H_2O$ in THF. Aliquots of 100 ml were withdrawn at time intervals, evaporated and dissolved in 1 ml $H_2O$. The samples were passed through a small ion exchange column (Pharmacia S Sepharose™ Fast Flow, 1 ml ), evaporated, dissolved in water (1 ml) and then subjected to h.p.l.c. analysis.

Treatment of **2** with different ammonia solutions

Compound **2** (2-4 mg) was dissolved in 1 or 2 ml of the ammonia solution (25% ammonia, 35% ammonia, 25% ammonia/absolute ethanol (3:1, v/v) or 35% ammonia/absolute ethanol (3:1, v/v). Aliquots of 50 or 100 μl were withdrawn at time intervals, evaporated, dissolved in water (200 μl), and subjected to h.p.l.c. analysis.

Stability of the H-phosphonate diester **1** under acidic and neutral conditions

Compound **1** (1.0 mg, faster moving diastereoisomer) was dissolved in 1 ml 2.5% dichloroacetic acid (DCA) in methylene chloride. Aliquots were withdrawn at time intervals and analyzed by t.l.c.
$R_f$ of **1**=0.81 (system B); $R_f$ after detritylation = 0.37 (system B).

For the $^{31}P$ n.m.r. analysis, 30 mg of **1** (faster moving diastereoisomer) was dissolved in 2.5 ml of either methylene chloride, pyridine/acetonitrile or 2.5% DCA in methylene chloride in a 10 mm tube (with a coaxial inner tube containing 1% $H_3PO_4$ in $D_2O$). Spectra were then recorded at time intervals.

Preparative t.l.c. of an n.m.r. sample after 16 h treatment of **1** with 2.5% DCA was carried out on Merck PSC Kieselgel 60 $F_{254}$ (2 mm) plates using chloroform/methanol 9:1 (v/v) as eluent.

Synthesis of reference compounds.

The following compounds (not commercial available), used as references in $^{31}P$ n.m.r. experiments and/or in h.p.l.c. analysis were synthesized analogously to their isomers as described above.

5′-O-(4,4′-dimethoxytrityl)-3′-O-t-butyldimethysilyluridin-2′-yl H-phosphonate (**4**) was prepared from **6**. $R_f$=0.55 (system A). **$^{31}P$ n.m.r.** (pyridine): δ=2.9 ppm, ($^1J_{PH}$=628 Hz, $^3J_{PH}$=11.0 Hz).

5′-O-(4,4′-dimethoxytrityl)-3′-O-t-butyldimethylsilyluridin-2′-yl 2′,3′-di-O-benzoyluridin-5′-yl H-phosphonate was prepared from **4** and 2′,3′-di-O-benzoyluridine. $R_f$=0.59 (system B). **$^{31}P$ n.m.r.** (pyridine): δ=8.4 ppm, ($^1J_{PH}$=736 Hz, $^3J_{PH}$=8.5 Hz) and δ=9.5 ppm, ($^1J_{PH}$=719 Hz, $^3J_{PH}$=10.1 Hz).

5′-O-(4,4′-dimethoxytrityl)-3′-O-t-butyldimethylsilyluridin-2′yl 2′,3′-di-O-benzoyluridin-5′-yl phosphate was prepared by oxidation of the corresponding H-phosphonate. $R_f$=0.49 (system A). **$^{31}P$ n.m.r.** (pyridine): δ=0.71ppm.

3′-O-t-butyldimethysilyluridin-2′-yl uridin-5′-yl phosphate was prepared analogously to **2**. H.p.l.c., r.t.=19.1 min (System D).

ACKNOWLEDGEMENTS

We are indebted to Prof. Per J. Garegg for his interest and to Prof. Colin B. Reese for a fruitful discussion concerning parts of this study. Financial support from the Swedish Natural Science Research Council and the National Swedish Board for Technical Development is gratefully acknowledged.

*Dedicated to Prof. Maciej Wiewiorowski on the occasion of his 70th birthday .*

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