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Evaluation of 2′-hydroxyl protection in RNA-synthesis using the H-phosphonate approach

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ABSTRACT

A number of different protecting groups were compared with respect to their usefulness for protection of 2′-hydroxyl functions during synthesis of oligoribonucleotides using the H-phosphonate approach. The comparison was between the t-butyldimethylsilyl (t-BDMSi), the *o*-chlorobenzoyl (*o*-ClBz), the tetrahydropyranyl (THP), the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp), the 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp), and the 1-(2-chloroethoxy)ethyl (Cee) protecting groups. All these groups were tested in synthesis of dodecamers, $(Up)_{11}U$ and $(Up)_{11}A$, using 5′-O-(4-monomethoxytrityl) or (4,4′-dimethoxytrityl) uridine H-phosphonate building blocks carrying the respective 2′-protection. The performance of the t-BDMSi and *o*-ClBz derivatives were also compared in synthesis of $(Up)_{19}U$. The most successful syntheses were clearly those where the t-butyldimethylsilyl group was used. The *o*-chlorobenzoyl group also gave satisfactory results but seems somewhat limited with respect to synthesis of longer oligomers. The results with all tested acetal derivatives (Fpmp, Ctmp, Cee, THP) were much less successful due to some accompanying cleavage of internucleotidic H-phosphonate functions during removal of 5′-O-protection (DMT).

INTRODUCTION

The protecting group strategy can often be limiting for the efficiency of an approach to synthesis of oligoribonucleotides. Many current methods differ mainly in their choice of protection for the 2′-OH of the ribose moieties. When using phosphoroamidite building blocks for assembly of the oligonucleotidic chain the t-butyldimethylsilyl (TBDMS)[1–6] group or acetal derivatives (several N-substituted piperidin-4-yl groups[7–9] and the chloroethoxyethyl group[10]) have been among the most successful protections for the 2′-hydroxyls. As an alternative to phosphoroamidite methodology the H-phosphonate approach has gained more attention and RNA-synthesis is the area where the popularity of this approach has increased most. We have for some time worked on improvements of RNA-synthesis using H-phosphonates. An important part of these studies has been connected to reaction conditions during the condensation step. Other important parts to optimise are deprotection conditions and/or choice of protecting groups.

In the first report on oligoribonucleotide synthesis using H-phosphonates[11] the t-butyldimethylsilyl (TBDMS) group was used as protection for the 2′-hydroxyl functions. Later reports have included use of the photolabile o-nitrobenzyl group[12], the acid sensitive tetrahydropyranyl (THP)[13] and 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp)[14] groups as well as base labile benzoyl derivatives[15–18]. Evaluation of the different strategies can be a difficult task without having direct comparative studies as a basis. Consequently, it seemed important to compare some of the more promising alternatives in order to find out which advantages each respective group could offer. The different 2′-O-protecting groups that we have evaluated for oligoribonucleotide synthesis using H-phosphonate methodology are: the t-butyldimethylsilyl (TBDMS), the o-chlorobenzoyl (o-ClBz), the tetrahydropyranyl (THP), the 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl (Ctmp), the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) and the 1-(2-chloroethoxy)ethyl (Cee) groups. The two latter ones have not previously been used in oligonucleotide synthesis together with H-phosphonate methodology but both have been claimed to offer some advantages over the Ctmp group when using the phosphoroamidite approach[9,10,19]. The different protecting groups have their respective advantages and disadvantages, e.g. concerning efficiency in preparation of starting materials, availability of reagents, requirements of strict conditions during deprotection in order to avoid cleavage or migration of phosphodiester linkages etc. Various problems related to preparation of starting materials are not treated here but we are mainly comparing the outcome of oligonucleotide synthesis with the respective building blocks. All these can be obtained in reasonable yields although the preparation may require more effort in some cases. We are also not comparing different deprotection procedures but we have only used established conditions for

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removal of the different 2'-hydroxyl protections that have been thoroughly investigated and found not to cause any detectable migration. In 0.01 M HCl Reese *et al.*[20] could not see any migration after 24 h and only about 1% after 216 h. It is well established that basic conditions, e.g., as used for deprotection of acyl groups, does not cause migration but only cleavage[21,22]. It has also been reported, by independent workers, that migration could not be detected after tetrabutylammonium fluoride treatment for 12[23] or 24 h[24]. What this comparison of 2'-hydroxyl protection then boils down to is to their different compatibilities with the synthetic cycles and efficiencies of condensation when using the H-phosphonate approach as well as how much cleavage of the produced oligomers that occur during the different deprotection steps.

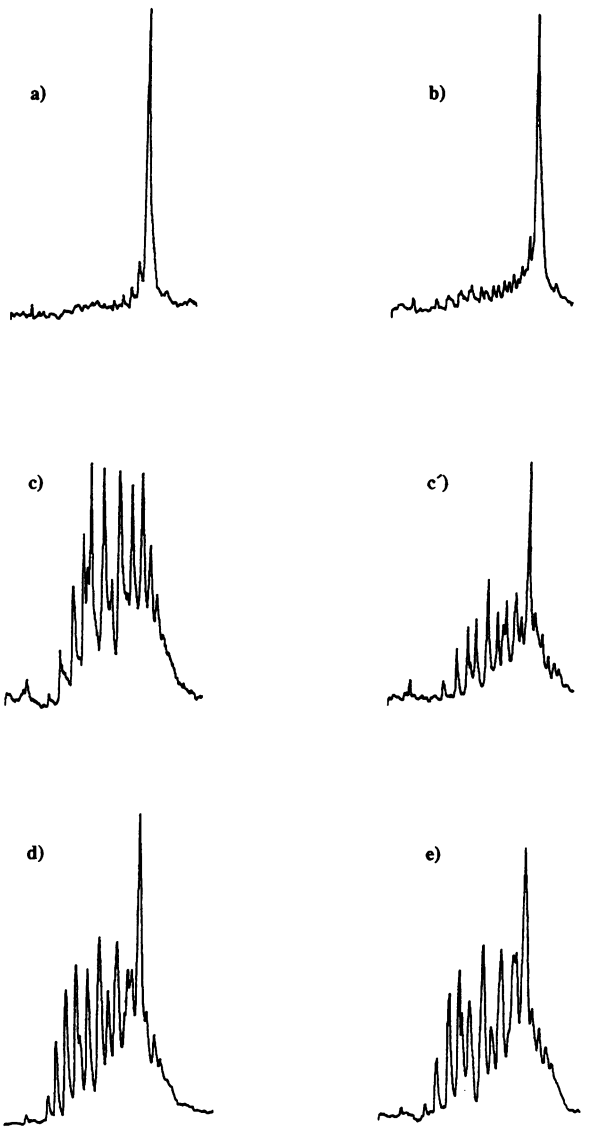


Figure 1. HPLC profiles (anion exchange on Bakerbond Widepore PEI, 5 μm, 4.6×250 mm using a linear gradient from 1.0 mM to 0.3 M phosphate buffer (pH = 6.5) in MeCN-water (3:7), during 40min.) of crude oligoribonucleotide mixtures from synthesis of dodecamers using the differently protected H-phosphonates a–e. (**a**) result from synthesis of $(Up)_{11}U$ using the TBDMS building block 1a. (**b**) result from synthesis of $(Up)_{11}U$ using the 2'-O-*o*-ClBz building block 1b. (**c**) result from synthesis of $(Up)_{11}U$ using the 2'-O-Ctmp building block 1c. (**c'**) result from synthesis of $(Up)_{11}A$ using the 2'-O-Ctmp building block 1c. (**d**) result from synthesis of $(Up)_{11}U$ using the 2'-O-Fpmp building block 1d. (**e**) result from synthesis of $(Up)_{11}U$ using the 2'-O-Cee building block 1e. Detritylation conditions when making the oligomers shown in 1a–1c were 1% trifluoroacetic acid in dichloroethane for 1 min and for the oligomers shown in 1c'–1e 1% DCA in dichloroethane for 1.5 min.

RESULTS AND DISCUSSION

Oligonucleotide synthesis

We decided to first compare the performance of the various protecting groups by employing them in synthesis of some short oligoribonucleotides. Homooligomers are more sensitive as probes for inefficient condensation or cleavage of internucleotic linkages since the shorter fragments are distinct and well defined as opposed to mixed sequences where the larger number of smaller peaks can be smeared out into the baseline of the chromatogram. Oligouridylic acids should be the most sensitive probes for cleavage of internucleotidic linkages (e.g., during ammonolysis) since phosphodiester functions in dinucleoside phosphates are least stable next to uridine residues[25]. Taking the above into account, and that uridine building blocks require no base protection, it seemed most appropriate to carry out this study on such oligomers. Consequently the fragments $(Up)_{11}U$ and $(Up)_{11}A$ were synthesized using the appropriate building blocks and a standard protocol for oligoribonucleotide synthesis with the H-phosphonate approach. The idea was then to compare synthesis of longer oligomers if there was not a clear difference at this level. Possible sources of such differences could be in the efficiency of condensation, and in cleavage of formed linkages during the elongation cycles or during removal of the 2'-protection. 4-Monomethoxytrityl (MMT) protection was used for the 5'-position of the uridine H-phosphonate building blocks carrying 2'-O-t-butyldimethylsilyl or *o*-chlorobenzoyl groups, whereas the 4,4'-dimethoxytrityl (DMT) group was used for the 2'-acetal derivatives in order to allow a milder acid treatment in the detritylation step of the oligonucleotide synthesis. The differently protected uridine H-phosphonates used in the present study are presented in Scheme 1. The corresponding nucleoside derivatives were prepared using published methods and subsequently phosphonylated with either the salicylchlorophosphite[26] or the PCl_3/imidazole/triethylamine[27] procedure.

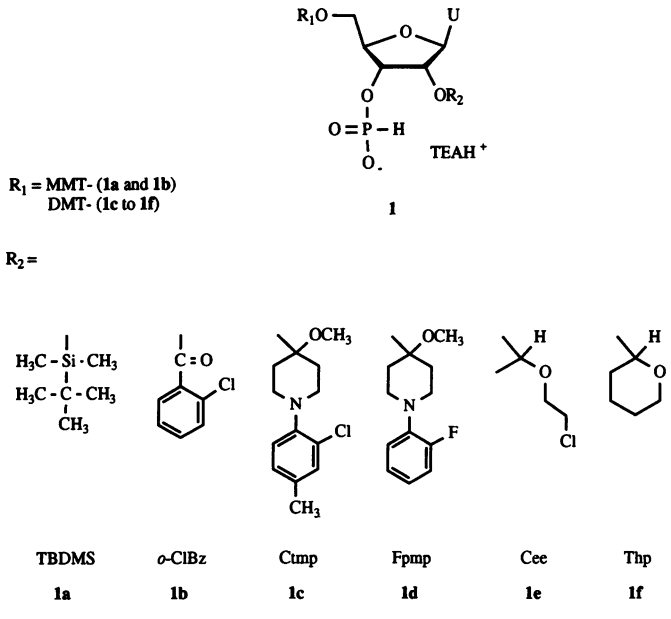
Automated solid support synthesis was carried out using identical cycles for all these derivatives. The support was always controlled pore glass beads (Pierce LCAA-CPG, 500 Å). The standard detritylation conditions used were 1% trifluoroacetic acid (TFA) in dichloroethane for 1 minute. All oligomers were cleaved from the support using identical conditions (32% NH_3 (aq)-EtOH, 3:1), deprotected in the respective ways and subsequently treated in an identical fashion (i.e. change of counterion to sodium and desalting). The condensation yields as estimated from removal of trityl-groups were similar in all synthesis (98,5–99%). Major differences in the resulting oligomers would then most likely originate from some other step in the synthesis. The oligonucleotides were analysed using anion exchange HPLC. The results from analysis of the products from the synthesis of dodecauridylic acids using the various building blocks can be seen in Fig 1. Analyses are of crude oligomers that have not been pretreated or prepurified in any way (contrary to the quite common practice of separating trityl-containing oligomers on RP-18 columns, HPLC or Sep-Pak from Waters, before the final

detritylation and/or ethanol or isopropanol precipitation of the crude oligomers).

The TBDMS derivative 1a gives the most satisfactory result (Fig. 1a) and the *o*-ClBz protection also works quite well (Fig. 1b). However, the syntheses when utilising acetal derivatives 1c–f are strikingly less successful (Fig. 1c–e, results from the THP derivative 1f not shown but these are qualitatively similar or worse). The desired oligomer is produced but is not even the major product. Suspecting a connection with the detritylation step of the synthetic cycles during oligonucleotide synthesis we changed the conditions for this reaction. When a weaker acid is used in this step some improvement can be seen (Fig 1c′) and the desired oligomer is then the major product but the results are still clearly inferior compared to when using the TBDMS or the *o*-ClBz derivatives.

Since moisture could be the cause of the relatively poor results we carried out syntheses with the acetal derivatives using different conditions (1–3 % dichloroacetic, trichloroacetic and trifluoroacetic acid in dichloroethane) for the detritylation step and took special care to make and keep the acid solutions dry[28]. The results were qualitatively similar, which convinced us that the reason for the less successful oligonucleotide synthesis with acetal protection must be sought after elsewhere.

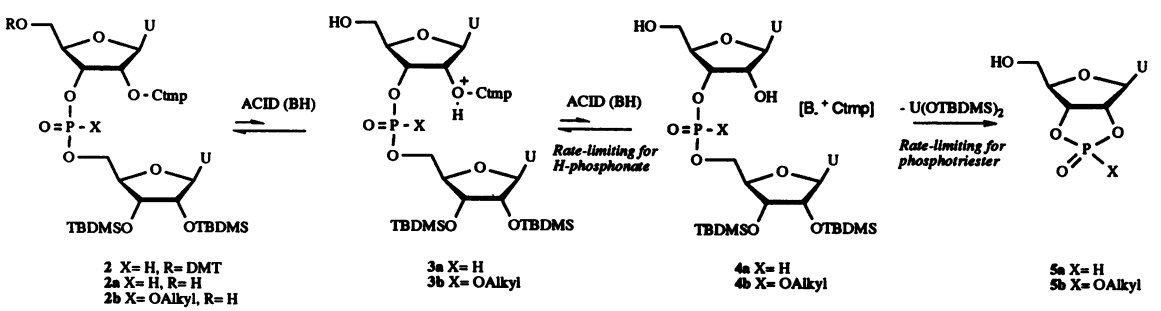
In order to get a clearer distinction between the two most successful approaches, *i. e.*, utilising the TBDMS or the *o*-ClBz derivatives, we decided to compare the outcome in synthesis of longer oligouridylic acids. The results from synthesis of 20-mers ($(Up)_{19}U$) are shown in Fig 2. It is quite clear that 1a gives better results (Fig. 2a) than 1b (Fig. 2b). The probable cause is cleavage of internucleotidic phosphodiesters during the ammonolysis step where removal of the *o*-ClBz group bares the vicinal hydroxyl under basic conditions. The result is, in our opinion, still quite agreeable.



Scheme 1. The differently protected uridine H-phosphonates used in oligoribonucleotide synthesis.

Investigation of problems related to 2′-acetal protection

The discouraging results from oligonucleotide synthesis with the acetal protected derivatives prompted us to synthesize dimer 2 (Scheme 2) and investigate the behaviour of this compound under anhydrous acidic conditions. When monitoring these mixtures with ^{31}P NMR spectroscopy signals at 24–30 ppm (25.6 ppm and 29.4 ppm in 2% TCA in dichloromethane) appeared and also some at 5–5.5 ppm. The area of these signals relative to 2a increased with time to constitute from 35–60 % of the material after 50 min., the amount depending on the acid used. Signals 16–21 ppm downfield from the diuridine 3′,5′-H-phosphonate 2a are consistent with the cyclic 5-membered ring H-phosphonate diester 5a[29] and those at around 5 ppm must be the 2′ and 3′-monoesters. The monoesters are probably formed by reaction of 5a with moisture. It was difficult to avoid this completely but it was clear that the presence of trace amounts of water only affected the ratio of 5a to monoesters and not the rate with which the dinucleoside H-phosphonate was cleaved. With drying agents such as pivaloyl chloride present only trace amounts of monoester could be detected together with 2a and 5a. This also did not alter the rate with which 2a was cleaved. Experiments where water was added to the reaction mixture were also performed. The rate of reaction was essentially the same irrespective of if the reaction was virtually anhydrous or if one or several equivalents of water was present ($t_{1/2}$ for 2a is 40–45 min in 2% TCA in dichloromethane and somewhat slower with the weaker acid 1.5% DCA in dichloroethane, $t_{1/2} > 1h$). With even as little as one equivalent of added water the cyclic H-phosphonate 5a can not be detected but only the 2′ and 3′-monoesters. These observations show that the hydrolysis of the cyclic H-phosphonate must be considerably faster than its formation. To take this further this also means that under conditions where 5a is detected the solution



Scheme 2. Proposed mechanism for cleavage of internucleotidic linkages during the detritylation step of oligoribonucleotide synthesis on solid support.

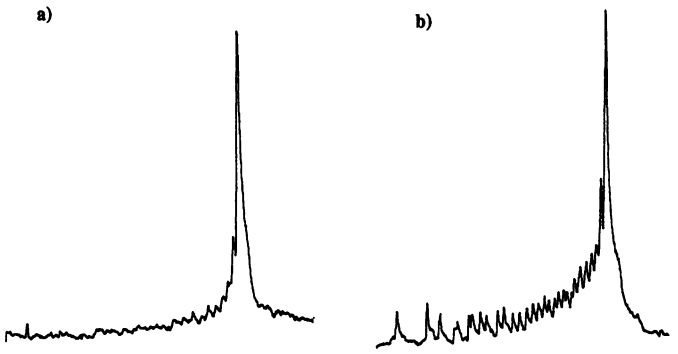


Figure 2. HPLC profiles (anion exchange on Bakerbond Widepore PEI, 5 μm, 4.6×250 mm using a linear gradient from 1.0 mM to 0.3 M phosphate buffer (pH = 6.5) in MeCN-water (3:7), during 1h.) of crude oligomer mixtures from synthesis of $(Up)_{19}U$ using the t-BDMSi or *o*-ClBz protected building blocks 1a or 1b. (**a**) 2′-O-t-BDMSi, (**b**) 2′-O-*o*-ClBz.

must be anhydrous, but the dimer still cleaves under these conditions.

We would like to suggest the following (Scheme 2): The acetal 2a is in equilibrium with the protonated form 3a which loses the oxycarbonium ion in a rate-limiting step to give 4a that undergoes fast cyclisation to form 5a (equivalent to what happens in acetal hydrolysis only that the subsequent step is not attack by water but trapping of the hydroxyl function by cyclisation). The same kind of event could in principle occur with an acetal group vicinal to a phosphotriester (2b → 5b) but should be much less significant since the cyclisation 4b to 5b is bound to be much slower than 4a to 5a. Comparing data for hydrolysis of the 2′-(1-(2-chloro-4-methylphenyl))-4-methoxypiperidin-4-yluridine ($t_{1/2}$ 52 min at pH= 2.5 => $k \approx 2.2\times10^{-4}$ s^{-1})[6,7] used in this study with cyclisation of a ribonucleoside 3′-(or 2′) (dimethylphosphate) ($t_{1/2}$ 30−32h at pH= 2.5 => $k \approx 6\times10^{-6}$ s^{-1})[30] shows that cyclisation of a 3′-phosphotriester in the ribofuranose system under acidic conditions can indeed be considerably slower than the rate-limiting step for hydrolysis of the investigated acetals. This also explains why the use of acetal groups is more successful in the phosphoroamidite or phosphotriester approaches to oligonucleotide synthesis. The difference found points towards the different reactivities of H-phosphonate diesters and phosphotriesters. Acid-catalysed hydrolysis of diethyl H-phosphonate[31] has a second-order rate-constant of about 1.2×10^{-4} M^{-1} s^{-1} at 25°C whereas the corresponding value for triethyl phosphate[32] can be estimated to around 1.9×10^{-9} M^{-1} s^{-1} at 25°C. One would expect that this difference of almost 10^5 in the rate-constants for hydrolysis should be of the same order also for an intramolecular transesterification. The above presented data then implies that one should expect cyclisation of the H-phosphonate diester 4a to be much faster than cleavage of the acetal group which in turn is faster than cyclisation of a ribonucleoside 3′-(dialkyl phosphate). For analogous reactions involving either an H-phosphonate (2a → 5a) or a phosphotriester (2b → 5b) different steps would be rate-limiting. For the H-phosphonate loss of the protecting group is rate-limiting (3a → 4a, as in acetal hydrolysis) whereas the cyclisation step (4b → 5b) is rate-limiting in the case of a phosphotriester. In view of this it is hardly surprising to find that oligoribonucleotide synthesis using the 2′-acetal protected building blocks (1c−f) is not a particularly successful approach.

CONCLUSIONS

Out of those compared here, the t-butyldimethylsilyl group is clearly the 2′-hydroxyl protection that gives the best results in oligoribonucleotide synthesis when using the H-phosphonate approach. Attempts to improve the RNA-synthesis are likely to be more effective elsewhere, e.g., by increasing coupling efficiency.

The *o*-chlorobenzoyl group is unlikely to be successful in synthesis of longer RNA-fragments due to inevitable partial degradation of the oligoribonucleotides under the basic conditions required to remove the protection. The group can, however, be most useful in synthesis where the desired oligomer is still the main product and possible to isolate. It could even be considered a first choice for some applications since the monomeric building blocks can be prepared in fewer steps and from inexpensive reagents.

Although ribonucleoside H-phosphonates with a 2′-acetal protection can be used in synthesis of shorter oligomers (provided a not too strong acid is used for detritylation) the results are only moderately successful. In view of the present results it can only be concluded that the use of 2′-acetal protection in combination with H-phosphonates is of limited value in oligoribonucleotide synthesis.

EXPERIMENTAL PART

Materials and methods

Pyridine, acetonitrile and triethylamine were refluxed with CaH_2 and then distilled and stored over molecular sieves (4Å) or CaH_2 (TEA). Dichloroethane and dichloromethane were distilled from P_2O_5 and stored over molecular sieves (4Å). Pivaloyl chloride was distilled under atmospheric pressure and stored at −20°C in a sealed flask. The regularly used trifluoroacetic acid (TFA) was distilled under atmospheric pressure and stored in sealed bottles at −20°C. Dichloroacetic and trichloroacetic acid were distilled under reduced pressure and stored in a sealed flask at −20°C. One batch of TFA was also distilled from trifluoroacetic anhydride and trichloroacetic acid was also prepared according to Reese *et al.*[28]. All the reagents used to introduce the acetal groups that are not commercially available were prepared as published[33,34]. All other reagents for synthesis of the protected uridine H-phosphonate building blocks were of commercial grade (TBDMS-Cl, 2-chloroethyl vinyl ether and 2,3-dihydropyran, Aldrich; MMT-Cl and DMT-Cl, Fluka; uridine, Sigma). TLC was performed on Merck silica gel 60 plates using solvents A (chloroform-methanol, 9:1 v/v), B (isopropanol-32% aqueous ammonia-water 85:5:10 v/v/v) and C (toluene-ethyl acetate 1:2 v/v). Reactions monitored by ^{31}P NMR were carried out in 10 mm NMR tubes. ^{31}P NMR chemical shifts are relative to 2% H_3PO_4 in D_2O as the external standard (coaxial inner tube). All NMR spectra were recorded on a Jeol GSX-270 FT spectrometer. The water used when handling oligonucleotides was doubly distilled in a glass apparatus (acid washed every 6 months) but not further treated or sterilised.

Synthesis of oligonucleotides

Oligonucleotide synthesis was done on a long chain alkylamine controlled pore glass carrier (Pierce, LCAA-CPG, loaded with 2′-O-*o*-ClBz protected nucleoside derivatives using standard procedures[35]) with the help of a modified Gene Assembler (Pharmacia). The condensation step was carried out for 2 min

using 30 mM of the H-phosphonate building block and 90 mM pivaloyl chloride in pyridine-acetonitrile (1:3 v/v). Detritylation was done with 1% trifluoroacetic acid in dichloroethane when using the TBDMS- and the *o*-ClBz-protection and various conditions (1−3 % of dichloroacetic, trichloroacetic or trifluoroacetic acid in dichloroethane) when using the acetal derivatives. A 30 min treatment with 2% Iodine in pyridine-water (98:2) was used for oxidation in all cases.

All oligomers were cleaved from the support using 32 % NH_3/EtOH, 3:1 for 12−14 h at room temperature. The ammonia solutions were evaporated and the oligomers arising from the *o*-ClBz building blocks were simply redissolved in water while the other ones were deprotected as follows. The TBDMS-protected oligomers were treated with 1.0 M tetrabutylammonium fluoride (TBAF) in THF for 12−14 h. Water was then added and the THF was evaporated under reduced pressure. The acetal derivatives were deprotected with 0.01 M HCl for 24 h and neutralised using either dilute aqueous ammonia[7,9,10,14] or 2 M triethylammonium bicarbonate buffer (with no detectable difference in the outcome). All oligomers were then passed through a cation exchange resin (SP-Sephadex C-25, 9×100 mm), lyophilised, redissolved in water, subsequently desalted using a Pharmacia NAP-25 column (Sephadex G-25) and finally lyophilised again before analysis by anion exchange HPLC (see figure texts).

Acid treatment of the H-phosphonate diester 2

The experiments were carried out in NMR-tubes (10 mm with a coaxial inner tube containing 2% H_3PO_4 in D_2O) and the reactions were monitored at three to four different times between 9 and 120 min. Compound 2 (0.05 mmol) was dissolved in 2 ml of either of the solutions: (a) 2 different solutions of 2% TCA in dichloromethane[28], (b) 1.5 % DCA in dichloroethane, (c) 2% TCA and 0.05 mmol H_2O in dichloromethane, (d) 2% TCA and 0.25 mmol H_2O in dichloromethane, (e) 2% TCA and 0.05 mmol pivaloyl chloride in dichloromethane or (f) 2% TCA and 0.05 mmol bis(2-oxo-3-oxazolidinyl)phosphinic chloride in dichloromethane. The ^{31}P chemical shifts for the two isomers of 2a in these acid solutions were 8.2−8.5 and 8.7−8.9 ppm respectively (a slight variation depending on the conditions). The ^{31}P chemical shift for the two isomers of the cyclic H-phosphonate 5a were 25.1−25.6 and 29.1−29.5 ppm. The uridine 3'- and 2'-H-phosphonate monoesters produced from 5a gave resonances at 4.9−5.3 and 5.0−5.5 ppm. The observed resonances were integrated and the natural logarithm of the values for the fraction of 2 still remaining were plotted against time. The half-lives were calculated from the equations of the obtained linear functions. Reactions under conditions (a) and (b) gave a mixture of 5a and uridine 3'- and 2'-H-phosphonates. The ratio of 5a to these monoesters under conditions (a) and (b) varied, depending on the moisture present, ranging from almost equal amounts to about 20 % monoesters. The ratio was always smaller at the early stages of the reactions suggesting that most of the moisture was present from the start. The cyclic H-phosphonate 5a could not be detected in reactions under conditions (c) and (d) but only the uridine 3'- and 2'-H-phosphonates. Reactions under conditions (e) and (f) gave the isomers of 5a as the main products with only trace amounts of uridine 2'- and 3'-H-phosphonates. The calculated half-lives were identical within the error limits (from 42 to 44 min) for all reactions except for conditions (b) that gave a half-life of about 62 min. Excess of water (0.25 mmol) was added after 2 h reaction under conditions (a) and (e) which in both cases resulted in complete conversion of 5a into the uridine 2'- and 3'-H-phosphonates in less than the time required to record another spectrum (about 2 min).

Synthesis of protected uridine H-phosphonates 1a−f

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(1-[2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl] uridine[14], 5'-O-(4,4'-dimethoxytrityl)-2'-O-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl] uridine[16] and 5'-O-(4,4'-dimethoxytrityl)-2'-O-[1-(2-chloroethoxy)ethyl] uridine[9] were prepared using published procedures except that the introduction of the acetal group was done with pyridinium hydrochloride as the acid catalyst according to Kumpins *et al.*[36]. The t-butyldimethylsilyl (TBDMS) H-phosphonate 1a[24], the *o*-chlorobenzoyl (*o*-ClBz) derivative 1b[18] and the tetrahydropyranyl (THP) compound 1f[13] were all synthesized according to published procedures. All protected nucleosides were phosphonylated using the PCl_3-imidazole-triethylamine reagent[27] and purified by chromatography on silica gel using gradient elution of 1−10 % of ethanol in $CHCl_3$. 1a and 1b were subsequently crystallised from acetonitrile.

5'-O-(4,4'-Dimethoxytrityl)-2'-O-[1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl] uridine 3'-H-phosphonate triethylammonium salt 1c. 4.40 g of protected nucleoside gave 4.25 g (79 %) of purified 1c, R_f= 0.19 (Solvent A) and 0.65 (Solvent B).

^1H NMR (25°C, $CDCl_3$) δ = 7.88 (d, J= 8.1 Hz, H6), 6.82−7.37 (m, Ar), 7.13 (d, J= 613.2 Hz, PH), 6.29 (d, J=7.7 Hz, H1'), 5.21 (d, H5), 4.86−4.97 (m, 2H, H2'+ H3'), 4.47 (m, H4'), 3.78 (s, 6H, OCH_3 in DMT), 3.42 and 3.56 (ABX system, 2H, H5'), 3.17 (s, 3H, OCH_3 in Ctmp), 2.75−3.17 (m, 4H, NCH_2 in Ctmp), 3.03 (q, J= 7.3 Hz, 6H, NCH_2), 2.24 (s, 3H, CH_3 in Ctmp), 1.75−2.15 (m, 4H, CH_2 in Ctmp), 1.32 (t, 9H, CH_3).

^{31}P NMR (25°C, pyridine) δ = 2.53.

5'-O-(4,4'-Dimethoxytrityl)-2'-O-[1-(2-fluorophenyl)-4-methoxypiperidin-4-yl] uridine 3'-H-phosphonate triethylammonium salt 1d. 2.20 g of protected nucleoside gave 1.65 g (81 %) of purified 1d, R_f= 0.18 (Solvent A) and 0.59 (Solvent B).

^1H NMR (25°C, $CDCl_3$) δ = 7.82 (d, J= 8.1 Hz, H6), 6.77−7.38 (m, Ar), 7.09 (d, J= 625 Hz, PH), 6.29 (d, J= 7.7 Hz, H1'), 5.17 (d, H5), 4.88−4.95 (m, 2H, H2'+ H3'), 4.45 (m, H4'), 3.79 (s, 6H, OCH_3 in DMT), 3.36 and 3.52 (ABX system, 2H, H5'), 3.19 (s, 3H, OCH_3 in Fpmp), 2.82−3.28 (m, 4H, NCH_2 in Fpmp), 3.04 (q, J= 7.3 Hz, 6H, NCH_2), 1.78−2.16 (m, 4H, CH_2 in Fpmp), 1.32 (t, 9H, CH_3).

^{31}P NMR (25°C, pyridine) δ = 2.51.

5'-O-(4,4'-Dimethoxytrityl)-2'-O-[1-(2-chloroethoxy)ethyl] uridine 3'-H-phosphonate triethylammonium salt 1e. 2.50 g of protected nucleoside gave 2.05 g (65 %) of purified 1e, R_f= 0.16 (Solvent A) and 0.53 (Solvent B).

^1H NMR (25°C, $CDCl_3$) δ = 7.85 and 7.90 (2d, J= 8.1 Hz, H6), 7.23−7.40 and 6.82−6.85 (m, Ar), 7.01 and 7.03 (2d, J= 629 Hz, PH), 6.02−6.05 (m, H1'), 5.17−5.27 (m, CH in Cee), 5.19 and 5.25 (2d, H5), 4.95−5.04 (m, H3'+ CH in Cee), 4.56−4.58 (m, H2'), 4.36 (m, H4'), 3.51−3.90 (m, 6H, H5'+ CH_2CH_2 in Cee), 3.78 (s, 6H, OCH_3), 3.01 (q, J= 7.3 Hz, 6H, NCH_2), 1.25−1.45 (m, 3H, CH_3 in Cee), 1.29 (t, 9H, CH_3).

^{31}P NMR (25°C, pyridine) δ = 2.38 and 2.47.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-O-[1-(2-chloro-4-methylphenyl)-4-methoxy-piperidin-4-yl] uridine 3'-(2',3'-di-O-t-butyldimethylsilyluridine 5'-H-phosphonate) 2. Compound 2 was prepared using the procedure published for synthesis of 5'-O-(4-,4'-dimethoxytrityl)-2'-O-t-butyldimethylsilyluridine 3'-(2',3'-di-O-benzoyluridine 5'-H-phosphonate)[24] by coupling of 1c with 2',3'-di-O-t-butyldimethylsilyluridine using pivaloyl chloride as condensing agent. Purification was by silica gel column chromatography using gradient elution of 10–50 % of ethyl acetate in toluene. R_f= 0.72 (Solvent C).

^{1}H NMR (25°C, $CDCl_3$, * indicates resonances from protons in the 2',3'-di-O-t-butyldimethylsilyluridine 5'- unit) δ = 8.65–8.68 (2s, 2H, NH), 7.76 (d, J= 8.1 Hz, H6), 7.57 (d, J= 8.1 Hz, H6*), 6.85–7.34 (m, Ar), 7.28 (d, J= 739 Hz, PH), 6.30 (d, J= 8.0 Hz, H1'), 5.78 (d, H5*), 5.68 (d, J= 3.7 Hz, H1'*), 5.05–5.22 (m, 3H, H2'+ H3'+ H5), 4.05–4.38 (m, 6H, H2'*+ H3'*+ H4'*+ H4'+ H5'*), 3.80 (s, 6H, OCH_3 in DMT), 3.47 (m, 2H, H5'), 3.30 (s, 3H, OCH_3 in Ctmp), 2.82–3.22 (m, 4H, NCH_2 in Ctmp), 2.26 (s, 3H, CH_3 in Ctmp), 1.77–2.22 (m, 4H, CH_2 in Ctmp), 0.89 and 0.90 (2s, 18H, Si-tBu), 0.08–0.10 (m, 12H, Si-CH_3).

^{31}P NMR (25°C,) δ = 8.08 & 8.23.

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