Mutual isomerization of uridine 2′- and 3′-alkylphosphates and cleavage to a 2′,3′-cyclic phosphate: the effect of the alkyl group on the hydronium- and hydroxide-ion-catalyzed reactions

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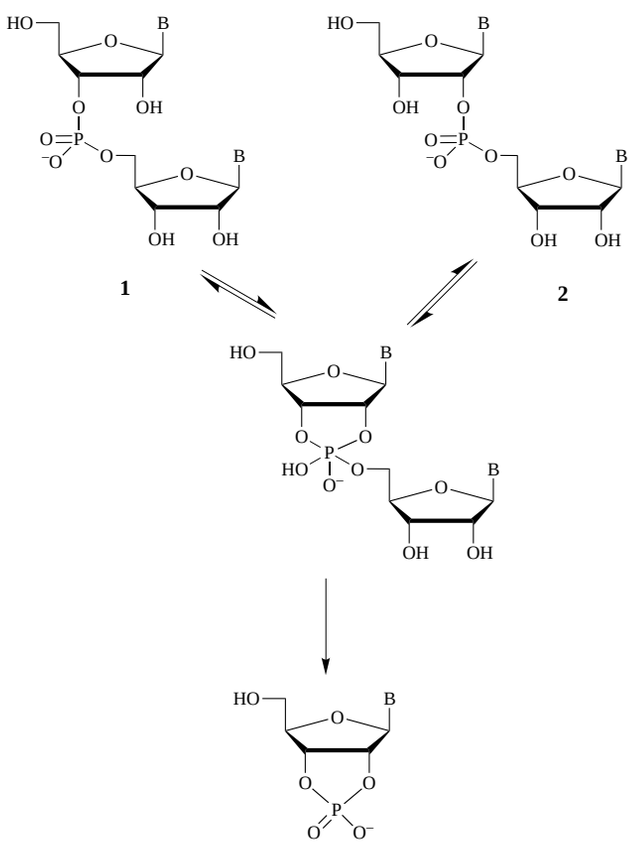
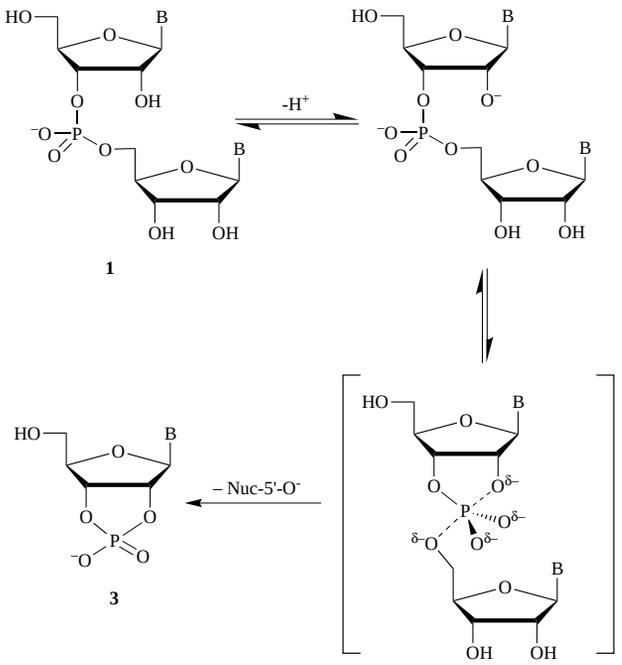
Isopropyl, ethyl, 2-ethoxyethyl, 2-chloroethyl, 2,2-dichloroethyl and 2,2,2-trichloroethyl esters of uridine 3′-phosphate have been prepared. In aqueous acid the compounds undergo concurrent isomerization to 2′-alkylphosphates and cleavage to uridine 2′,3′-cyclic phosphate, but in aqueous alkali only cleavage to the cyclic phosphate takes place. Buffer-independent rate constants for these reactions have been determined. The hydroxide-ion-catalyzed reaction to the 2′,3′-cyclic monophosphate is exceptionally susceptible to the polar nature of the leaving group, the β_{lg} value being −1.28 ± 0.05. By contrast, the hydronium-ion-catalyzed isomerization and cleavage are both rather insensitive to the electron-withdrawing ability of the alkyl group, the β and β_{lg} values being −0.18 ± 0.02 and −0.12 ± 0.05, respectively. The transition state structures of the reactions are discussed on the basis of these structural effects.

Introduction

The mechanistic details of the hydrolysis of the phosphodiester bonds of RNA have recently received considerable interest for several reasons: to get a better insight into the action of ribonucleases, above all RNaseA, to understand the action of catalytic ribonucleic acids and to provide a rational basis for the development of artificial nucleases. It is well known that dinucleoside 3′,5′-monophosphates (**1**), the dimeric fragments of RNA, undergo in aqueous solution two competing intramolecular transesterification reactions, *viz.* isomerization to a 2′,5′-phosphodiester (**2**) and cleavage to a cyclic 2′,3′-phosphodiester (**3**) with concomitant release of the 5′-linked nucleoside (Scheme 1). [1–8] Studies with homopolymers have shown that the situation remains similar for polyribonucleotides.[9,10] Under acidic conditions, both reactions proceed in all likelihood *via* a common pentacoordinated intermediate obtained by an attack of the 2′-hydroxy function on the protonated monocationic phosphate group.[4,5] This intermediate, when deprotonated to a neutral species, is sufficiently stable to be able to pseudorotate.[11] Accordingly, any of the bridging phosphate oxygens, *viz.* O2′, O3′ and O5′, may take an apical position, and hence leave after protonation as an alcohol. The breakdown of the intermediate is symmetrical, since formation of the 2′,3′-cyclic monophosphate and mutual isomerization of the 2′,5′- and 3′,5′-monophosphates occur at approximately the same time.[4,5] In contrast, formation of the 2′,3′-cyclic monophosphate is the only reaction detected under alkaline conditions.[4,5,12,13] The deprotonated 2′-oxyanion now attacks at the monoanionic phosphate, and the resulting dianionic pentacoordinated species appears to be too unstable to be able to pseudorotate (Scheme 2). Consistent with this kind of in-line displacement, the R_p and S_p diastereomers of dinucleoside 3′,5′-phosphoromonothioates have been observed to undergo hydroxide-ion-catalyzed cyclization to 2′,3′-cyclic phosphoromonothioates by complete inversion of configuration at phosphorus.[14] The lifetime of the dianionic phosphorane is still being debated. Perreault and Anslyn[15] have recently reviewed the relevant literature and concluded that a dianionic phosphorane may possibly exist but it cannot be kinetically distinguished from a transition state. In particular, it cannot undergo protonation to a monoanionic phosphorane which is known to pseudorotate. The *ab initio* calculations of Taira and co-workers, however, suggest that dianionic phos-



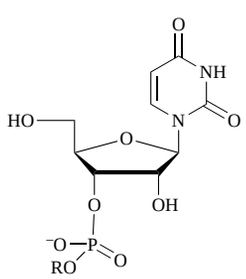
Scheme 1



Scheme 2

phorane is sufficiently stable in aqueous solution to be regarded as an intermediate.[16,17]

To learn more about the transition state structures of the reactions indicated above, especially that proceeding *via* a dianionic phosphorane, we now report the results of kinetic studies carried out on a number of monoalkyl esters of uridine 3′-phosphate (**4a**–**f**). The pK_a values of the esterified alcohols



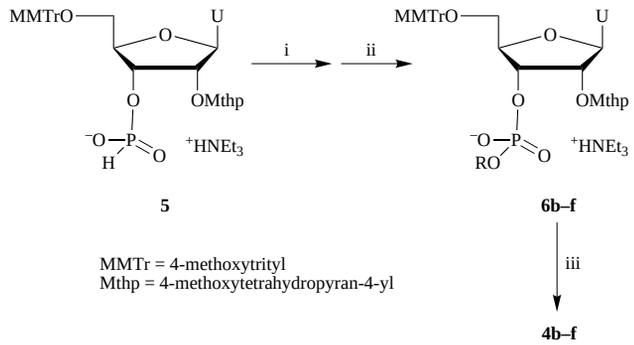
4a R = $CH(CH_3)_2$
b R = CH_2CH_3
c R = $CH_2CH_2OCH_2CH_3$
d R = CH_2CH_2Cl
e R = CH_2CHCl_2
f R = CH_2CCl_3

cover a range of 5 units (12–17). These studies are also relevant because simple alkyl and aryl esters of nucleoside 3′-phosphates, as well as their non-nucleosidic analogues, are often used as mimics of dinucleoside 3′,5′-monophosphates. Quantitative knowledge of the effects that this kind of structural simplification may exert on the rate of various transesterification reactions helps in assessing the general applicability of the mechanistic conclusions drawn on the basis of the behaviour of model compounds.

Results

Preparation of the model compounds

Uridine 3′-isopropylphosphate (**4a**) apart, the alkyl esters of uridine 3′-phosphate (**4b**–**f**) were obtained by coupling 5′-*O*-(4-methoxytrityl)-2′-(4-methoxytetrahydropyran-4-yl)uridine 3′-*H*-phosphonate (**5**) with appropriate alcohols, and oxidizing the resulting *H*-phosphonate diester *in situ* with iodine in aqueous pyridine (Scheme 3). The sugar moiety protecting groups were removed by acid-catalyzed hydrolysis in aqueous THF,

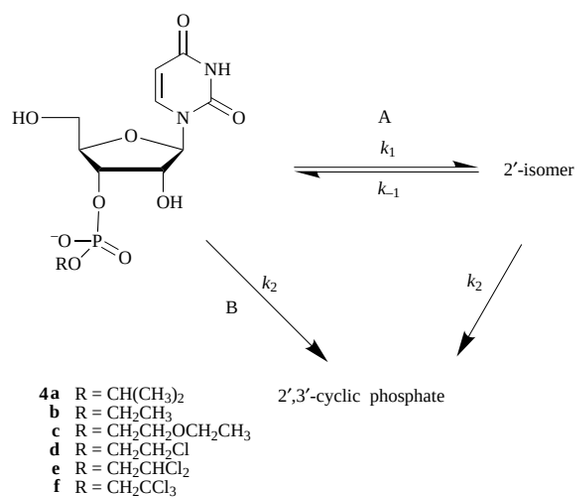


Scheme 3 (i) bis(2-oxo-oxazolidin-3-yl)phosphoric chloride and ROH in Py; (ii) I_2–H_2O–Py; (iii) HCl–H_2O–THF

and the products were purified by RPHPLC. The syntheses were not optimized, but they were considered to be adequate for the purpose of obtaining pure material for kinetic investigations. **5** was prepared from 3′,5′-*O*-tetraisopropyldisiloxanediyluridine[18] *via* appropriate acetalization of the 2′-hydroxy function,[19] removal of the disiloxanediyl group,[18] 5′-*O*-monomethoxytritylation and 3′-phosphonylation.[20,21] The isopropyl ester of uridine 3′-phosphate (**4a**) was obtained by phosphotriester approach.[22] Accordingly, 5′-*O*-(4-methoxytrityl)-2′-*O*-(tetrahydropyran-2-yl)uridine was converted to its 2-chlorophenyl isopropyl triester, hydrolyzed to 3′-isopropylphosphate in aqueous alkali and deprotected with aqueous acid.

Kinetic measurements

The concurrent intramolecular transesterifications of the alkyl esters of uridine 3′-phosphate (**4a**–**f**) to uridine 2′,3′-cyclic phosphate (reaction B in Scheme 4) and uridine 2′-alkyl-



4a R = $CH(CH_3)_2$
b R = CH_2CH_3
c R = $CH_2CH_2OCH_2CH_3$
d R = CH_2CH_2Cl
e R = CH_2CHCl_2
f R = CH_2CCl_3

Scheme 4

phosphates (reaction A in Scheme 4) were followed in 0.5 mol dm^{-3} aqueous hydrogen chloride at 298.2 K by determining the composition of the aliquots withdrawn at appropriate intervals by RPHPLC. Uridine 2′,3′-cyclic phosphate is not actually accumulated under these conditions, but it is hydrolyzed to a 1:2 mixture of uridine 2′- and 3′-phosphates, and subsequent dephosphorylation to uridine is several orders of magnitude slower than their formation.[23–27] Accordingly, in addition to the intermediate formation of uridine 2′-alkylphosphate, accumulation of uridine 2′/3′-phosphates as final products was observed in each case. Table 1 records the pseudo-first-order rate constants obtained for reactions A and B. The dependence of the logarithmic values of these rate constants on the pK_a value of the esterified alcohol is shown in Fig. 1. The values of the reaction constants are -0.12 ± 0.05 and -0.18 ± 0.02 for the cleavage (reaction B) and isomerization (reaction A), respectively.

Table 1 First-order rate constants for the interconversion of uridine 2′- and 3′-alkylphosphates ($k_1 + k_{-1}$) and their transesterification to uridine 2′,3′-cyclic monophosphate (k_2) in aqueous hydrogen chloride (0.5 mol dm^{-3}) at 298.2 K[a]

Compound	R	$k_2/10^{-5}$ s^{-1}	$(k_1 + k_{-1})/10^{-5}$ s^{-1}
4a	$CH(CH_3)_2$	0.636 ± 0.009	0.47 ± 0.03
4b	CH_2CH_3	2.00 ± 0.05	0.72 ± 0.02
4c	CH_2CH_2OEt	2.93 ± 0.06	1.02 ± 0.01
4d	CH_2CH_2Cl	3.12 ± 0.02	1.05 ± 0.08
4e	CH_2CHCl_2	4.05 ± 0.19	2.23 ± 0.05
4f	CH_2CCl_3	2.61 ± 0.10	3.87 ± 0.21

[a] k_1/k_{-1} ranged from 0.75 to 0.88 at pH 4.2 and 363.2 K.

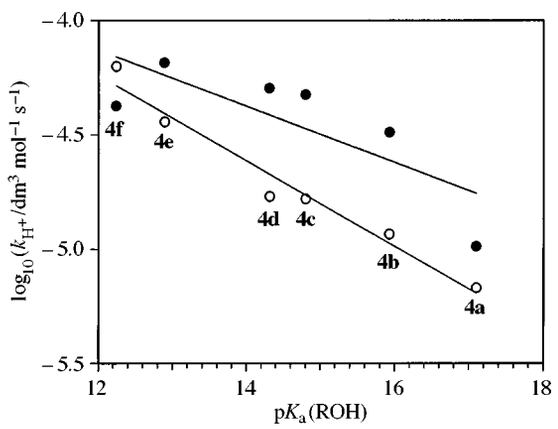


Fig. 1 Logarithmic first-order rate constants for the hydronium-ion-catalyzed transesterification of uridine 3′-alkylphosphates to uridine 2′-alkylphosphates (○) and uridine 2′,3′-cyclic phosphate (●) plotted against the pK_a values of the esterified alcohols.[35] The data refer to [HCl] = 0.5 mol dm^{-3} at T = 298.2 K.

No isomerization to 2′-alkylphosphates could be observed in aqueous sodium hydroxide. Uridine 3′-(2,2,2-trichloroethyl)-phosphate (**4f**) hydrolyzed so readily that the rate constant could not be determined in aqueous sodium hydroxide. With this compound, the measurements were carried out at a lower pH in an ammonia–ammonium chloride buffer (pH 9.54). The rate constant slightly increased with the increasing buffer concentration, in spite of the fact that the ionic strength was kept constant. Whether this modest rate-acceleration results from medium effects or from buffer catalysis cannot be confidently concluded. The buffer-independent second-order rate constant was calculated from the first-order rate constant obtained by extrapolation to zero buffer concentration. Table 2 summarizes the kinetic data under alkaline conditions. In Fig. 2 the logarithmic values of the second-order rate constants are plotted against the pK_a values of the alcohols corresponding to the displaced alkoxide ions. The β_{1g} value obtained in this manner is −1.28 ± 0.05.

Discussion

As discussed in the Introduction, the hydroxide-ion-catalyzed transesterification of ribonucleoside 3′-phosphodiesters to ribonucleoside 2′,3′-cyclic monophosphates is generally assumed to proceed by a simple 'in line' mechanism: the attacking 2′-oxyanion and the departing 5′-oxyanion occupy the apical positions within a phosphorane-type structure (*cf.* Scheme 2 for NpN, **1**). The β_{1g} value for the cleavage of the phenyl esters of uridine 3′-phosphate has been observed to be −0.54, suggesting that both the P–O2′ bond formation and P–O5′ bond cleavage are only weakly advanced in the transition state.[28] In other words, the reaction appears to be a concerted process proceeding *via* a pentacoordinated transition state. The situation is, however, different with the alkyl esters. As can be seen from Fig. 2, this reaction is quite susceptible to the basicity

Table 2 Second-order rate constants for the transesterification of uridine 3′-alkylphosphates to uridine 2′,3′-cyclic monophosphate in aqueous alkali at 298.2 K. The ionic strength was adjusted to 1.0 mol dm^{-3} with sodium chloride.

Compound	R	$k_2/10^{-5}$ dm^3 mol^{-1} s^{-1}[a]
4a	$CH(CH_3)_2$	0.053 ± 0.001[b]
4b	CH_2CH_3	4.6 ± 0.8[c]
4c	CH_2CH_2OEt	41.1 ± 0.5[c]
4d	CH_2CH_2Cl	459 ± 6[c]
4e	CH_2CHCl_2	20 300 ± 200[d]
4f	CH_2CCl_3	114 000 ± 3000[e]

[a] The pseudo-first-order rate constant divided by the concentration of hydroxide ion. [b] The pseudo-first-order rate constant was obtained in 1.00 mol dm^{-3} aqueous sodium hydroxide. [c] The pseudo-first-order rate constant was obtained in 0.10 mol dm^{-3} aqueous sodium hydroxide. [d] The pseudo-first-order rate constant was obtained in 0.010 mol dm^{-3} aqueous sodium hydroxide. [e] The pseudo-first-order rate constants were determined in aqueous ammonia–ammonium chloride buffers (buffer ratio 1:1). The values obtained at the total concentrations of 0.1, 0.2, 0.3 and 0.4 mol dm^{-3} were 7.89, 8.66, 9.29 and 10.4 × 10^{-5} s^{-1}, respectively. The value extrapolated to buffer concentration zero was used to calculate the second-order rate constant.

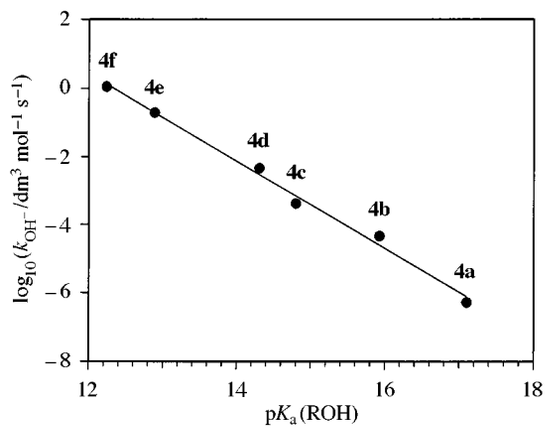
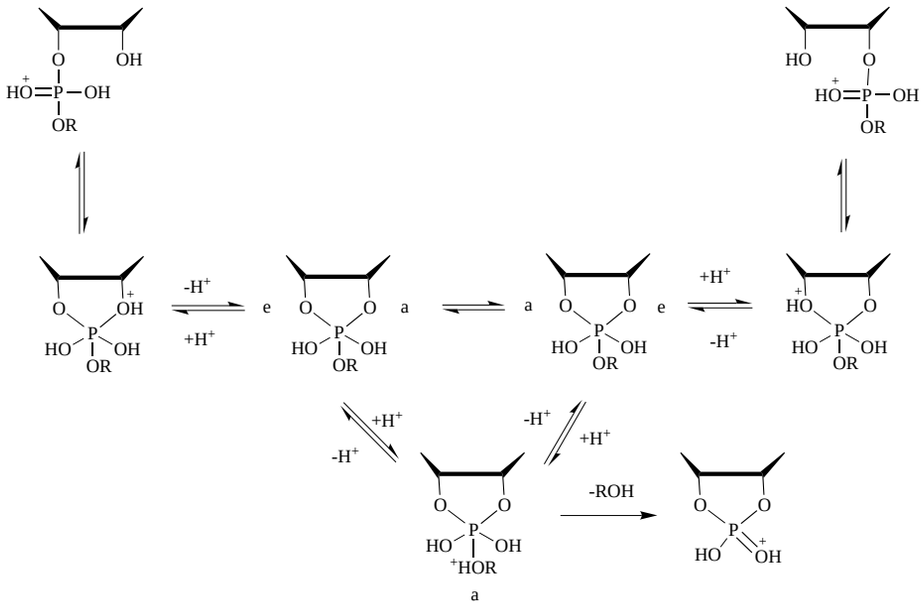


Fig. 2 Logarithimic second-order rate constants for the hydroxide-ion-catalyzed transesterification of uridine 3′-alkylphosphates to uridine 2′,3′-cyclic phosphate plotted against the pK_a values of the esterified alcohols.[35] The data refer to T = 298.2 K and I = 1.0 mol dm^{-3}.

of the alkoxy group being displaced (OR in Scheme 4); the β_{1g} value is −1.28 ± 0.05. The electronegativity of the alkyl group probably does not markedly affect the pre-equilibrium deprotonation of the 2′-hydroxy function, since the inductive effect of the alkoxy group on the 2′-oxygen is transmitted *via* five σ-bonds. The highly negative β_{1g} suggests that the alkoxy ion character of the leaving-group is well developed in the transition state. Comparable highly negative β_{1g} values have also been reported for other related intramolecular displacement reactions of phosphodiesters: −0.9 for the cleavage of substituted guanosine 3′-benzylphosphates,[29] −1.13 for the cyclization of the dianion of alkyl α,α-bis(trifluoromethyl)-2-hydroxybenzyl phosphates,[30] and −1.26 for the cyclization of the dianion of aryl 2-carboxyphenyl phosphate.[31] Actually, the β_{1g} value of −1.28 is sufficiently negative to be taken as an indication of a clearly stepwise mechanism, *i.e.* pre-equilibrium formation of a dianionic phosphorane followed by its unimolecular heterolysis to an alkoxide ion and 2′,3′-cyclic phosphate. The fact that no hydroxide-ion-catalyzed isomerization has ever been detected, however, argues against this alternative. Evidently the dianionic phosphorane is too short-lived to pseudorotate as a dianion or *via* a kinetically invisible intermediary protonation to the monoanionic phosphorane. Note that protonation to monoanionic phosphorane is thermodynamically favoured under mildly alkaline conditions (pK_a is estimated to range from 13 to 15[15]), and it hence should be a diffusion controlled process. Perhaps the most conclusive evidence against the hydroxide-ion-catalyzed isomerization has been obtained with the phos-



Scheme 5

phonate analog of GpU, having the O5′ of the 5′-linked nucleoside replaced with a methylene group.[32] Since the P–C bond cannot be cleaved, isomerization is the only reaction taking place under alkaline conditions. The isomerization, however, remains strictly pH-independent even at pH 10. At higher pH the uracil base starts to degrade. Under these conditions dinucleoside 3′,5′-monophosphates undergo rapid hydroxide-ion-catalyzed cleavage, indicating that the 2′-oxyanion attacks the phosphate diester monoanion. It is probable that the 2′-oxyanion also attacks the phosphonate ester monoanion, but this attack does not lead to a detectable reaction. The dianionic intermediate simply reforms the starting material. Protonation to a monoanion would undoubtedly lead to isomerization, since the pH-independent phosphate migration *via* a monoanionic phosphorane is even faster with the phosphonate analog than with an unmodified dinucleoside 3′,5′-monophosphate. Accordingly, the cleavage of ribonucleoside 3′-alkylphosphates appears to proceed *via* a very late transition state but without formation of a fully developed dianionic phosphorane intermediate that could be stabilized by protonation. The mechanism most likely lies on the borderline between a concerted and a stepwise mechanism.

The studies of Dalby *et al.*[30] on cyclization of methyl α,α-bis(trifluoromethyl)-2-hydroxybenzyl phosphate by the displacement of methoxide ion with the neighbouring phenoxide ion suggest that the departure of the methoxide ion is susceptible to general acid catalysis by ammonium ions. The modest rate-accelerating effect of NH_3–NH_4^+ buffers on the cleavage of uridine 3′-(2,2,2-trichloroethyl)phosphate may tentatively be accounted for in a similar way.

The hydronium-ion-catalyzed cleavage and isomerization (Scheme 5) proceed *via* a pseudorotating pentacoordinated intermediate obtained by the attack of 2′-OH on the monocationic phosphodiester bond. According to *ab initio* calculations, the pseudorotation barrier of a neutral phosphorane is sufficiently low to allow rapid equilibration between the forms having either O2′ or O3′ apical. The leaving group is now an alcohol, not an alkoxide ion.[4,5] The rate of cleavage (reaction B) is, as expected, rather insensitive to the polar nature of the departing alcohol ($\beta_{lg} = -0.12 \pm 0.05$). Electron-withdrawal by a polar substituent, for example, weakens the P–OR bond by increasing its polarity and facilitates the attack of the 2′-OH on phosphorus, but simultaneously the pre-equilibrium protonation of the starting material and the protonation of the leaving oxygen are retarded. These partial effects cannot be quantified on the basis of the data available, but evidently they cancel each other almost entirely. A similar observation has been made in the hydronium-ion-catalyzed cyclization of the neutral ionic form of alkyl α,α-bis(trifluoromethyl)-2-hydroxybenzyl phosphates: the β_{lg} value is only −0.15.[30]

The isomerization (reaction A) also is rather insensitive to the electronegativity of the alkyl group ($\beta = -0.18 \pm 0.02$). Tentatively one may assume that an electron-withdrawing alkyl group facilitates the nucleophilic attack on phosphorus, and hence increases the equilibrium concentration of the phosphorane intermediate. The increasing electronegativity of the alkyl group also seems to favour isomerization over cleavage, although the effect is small. Since both reactions proceed *via* a common intermediate, this effect has to be attributed to pseudorotation/breakdown of this intermediate. In other words, the increasing electronegativity of the alkyl group favours the cleavage of the P–O3′ bond over cleavage of the P–OR bond. The origin of this influence is difficult to define. The rate-limiting step of both reactions is assumed to be a unimolecular rupture of the P–OH^+ bond. The increasing electronegativity of the alkyl group retards the pre-equilibrium protonation of the P–OR bond and accelerates the departure of the protonated alkoxy ligand. In contrast, the protonation and subsequent cleavage of the P–O3′ bond may be expected to be rather insensitive to electron-withdrawal by the alkyl group. Perhaps the effect on the basicity of the alkoxy oxygen is so dominant that the P–OR bond cleavage becomes retarded compared to that of P–O2′.

Experimental

General

Pyridine, acetonitrile, ethanol, 2-chloroethanol, 2,2-dichloroethanol and 2,2,2-trichloroethanol were dried by storage over molecular sieves. 2-Ethoxyethanol and phosphorus trichloride were distilled prior to use. Triethylamine and THF were distilled over calcium hydride and lithium aluminium hydride, respectively. Uridine, 4-methoxy-5,6-dihydro-2*H*-pyran, tetrabutylammonium fluoride, 4-methoxytrityl chloride, bis(2-oxo-oxazolidin-3-yl)phosphonic chloride, 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane and iodine were used as received. ^{1}H NMR spectra were recorded at 270 MHz, and all signals were assigned by ^{1}H–^{1}H homocoupling experiments to verify that phosphoric acid was esterified to the 3′-hydroxy function of uridine.

Table 3 ^{1}H and ^{31}P NMR chemical shifts for uridine 3′-alkylphosphates (**4a**–**f**) in $^{2}H_2O$ at 25 °C[a]

	δ								
Compound	H1′	H2′	H3′	H4′	H5′,H5″	H5	H6	OR	P
4a	5.79	4.28	4.39	4.15	3.74; 3.65	5.73	7.71	1.10; 4.28	+3.00
4b	5.88	4.38	4.58	4.21	3.72; 3.80	5.85	7.88	3.88	−0.85
4c	5.84	4.34	4.49	4.18	3.70; 3.77	5.74	7.74	1.16; 3.47; 3.59; 3.93	−0.96
4d	5.88	4.38	4.52	4.29	3.74; 3.84	5.82	7.81	3.79; 4.08	−1.04
4e	5.90	4.46	4.62	4.32	3.82; 3.90	5.88	7.86	4.25; 6.08	−0.50
4f	5.96	4.49	4.68	4.37	3.85; 3.94	5.91	7.89	4.56	−1.36

[a] The ^{1}H and ^{31}P chemical shifts are given in ppm from external TMS and phosphoric acid, respectively.

5′-*O*-(4-Methoxytrityl)-2′-*O*-(4-methoxytetrahydropyran-4-yl)-uridine 3′-*H*-phosphonate triethylammonium salt (5)

Uridine was converted to 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine,[18] and this to 2′-*O*-(4-methoxytetrahydropyran-4-yl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine,[19] as described previously. The silyl protection was removed with tetrabutylammonium fluoride in acetonitrile,[18] and the product was purified by silica gel chromatography, using a stepwise gradient of methanol (1–10%, v/v) in chloroform containing 1% triethylamine. 2′-*O*-(4-Methoxytetrahydropyran-4-yl)uridine obtained (2.44 g, 7.21 mmol) was treated with 4-methoxytrityl chloride (3.34 g, 10.8 mmol) in dry pyridine (69 ml). Conventional sodium bicarbonate workup and subsequent purification on silica gel, using a mixture of chloroform and methanol (19:1) containing 1% triethylamine as an eluent, gave 2′-*O*-(4-methoxytetrahydropyran-4-yl)-5′-*O*-(4-methoxytrityl)uridine in 58% yield. This compound (0.88 g, 1.44 mmol) was dried by coevaporations from pyridine and acetonitrile, and dissolved in acetonitrile (36 ml). The solution was added dropwise (over a period of 1 h) to a mixture of imidazole (1.05 g, 15.5 mmol), phosphorus trichloride (5.04 g, 3.65 mmol) and triethylamine (2.25 ml, 16.2 mmol) in acetonitrile (1.8 ml) in an ice bath.[20,21] After an additional 15 min mixing at room temperature, aqueous triethylammonium bicarbonate (5 ml, 0.1 mol dm^{-3}) was added, and the mixture was evaporated to dryness. The residue was coevaporated from a mixture of pyridine and triethylamine (4:1), and then from toluene. The crude product was equilibrated between chloroform and aqueous triethylammonium bicarbonate (0.1 mol dm^{-3}). The aqueous phase was extracted twice with chloroform, and the combined organic phases were evaporated to dryness. Silica gel chromatography using a stepwise gradient of methanol (1–10%) in chloroform containing 1% triethylamine gave **5** in 89% yield.

5′-*O*-(4-Methoxytrityl)-2′-*O*-(4-methoxytetrahydropyran-4-yl)-uridine 3′-monoalkylphosphate triethylammonium salts (6b–f)

5 (0.20 g, 0.25 mmol) was dissolved in dry pyridine (10 ml) and the appropriate alcohol (0.28 mmol) was added. To the stirred reaction mixture, bis(2-oxo-oxazolidin-3-yl)phosphonic chloride (0.13 g, 0.50 mmol) was then added. After the reaction was completed, iodine in a mixture of pyridine and water (4% w/v, 96:4 v/v, 10 ml) was added. The oxidation of *H*-phosphonate diester to phosphate diester took place in 5 min. The reaction mixture was diluted with dichloromethane and evaporated to near dryness. The evaporation was repeated several times from the same solvent. The residue was dissolved in dichloromethane and washed with aqueous sodium thiosulfate (10%, w/v) and then with saturated aqueous sodium bicarbonate. The crude product was purified by silica gel chromatography using a stepwise gradient of methanol (1–10%) in chloroform containing 1% triethylamine. The yields of **6b**–**f** varied from 70 to 95%.

Uridine 3′-monoalkylphosphate triethylammonium salts (4a–f)

The protected phosphodiesters (**6b**,**c**,**e**,**f**) were dissolved in a mixture of THF and water (9:1, 10 ml) containing hydrogen chloride (0.01 mol dm^{-3}) to remove the acid labile protecting groups. With **6d**, a more concentrated acid solution was used ([HCl] = 0.038 mol dm^{-3}, THF–water 6:4). The progress of deprotection was followed by TLC (propan-2-ol–conc. aq. ammonia–water 8:1:1). After complete deprotection, the pH was adjusted to 6 with aqueous sodium hydroxide, and the solutions were washed with diethyl ether. The products (**4b**–**f**) were purified by RPHPLC (Lichrosorb RP-18, 25 × 250 mm, 7 μm) using a linear gradient from 20 to 100% of buffer B in buffer A over 30 min [buffer A: 25 mmol dm^{-3} aqueous triethylammonium acetate (pH 6.5); buffer B: 25 mmol dm^{-3} triethylammonium acetate in 50% aqueous acetonitrile]. The retention times were: **4b**, 18 min; **4c**, 17 min; **4d**, 13 min; **4e**, 15 min; **4f**, 17 min. The ^{1}H and ^{31}P NMR chemical shifts are listed in Table 3, and the RPHPLC retention times and electrospray mass spectroscopy (ESMS) signals in Table 4.

5′-*O*-(2-Methoxytrityl)-4′-*O*-(tetrahydropyran-2-yl)uridine[33] was converted to its 3′-(2-chlorophenylisopropyl)phosphate by the method of Marugg *et al.*[22] 2-(Chlorophenoxy)phosphoryl-bis(1,2,4-triazole) was, however, used as a phosphorylating agent instead of 2-chlorophenyl-*O*,*O*-bis(benzotriazol-1-yl)-phosphate. The phosphotriester obtained (0.26 g, 0.31 mmol) was treated with a 1:4 mixture (100 ml) of aqueous sodium hydroxide (0.1 mol dm^{-3}) and dioxane overnight at room temperature. Aqueous hydrogen chloride (2 ml, 2 mol dm^{-3}) was added, and the mixture was kept overnight at room temperature. The solution was neutralized with triethylamine and evaporated to dryness. The residue was dissolved in water (20 ml), washed twice with an equal volume of dichloromethane, and the aqueous layer was evaporated to dryness. The product (**4a**) was purified by RPHPLC on a LiChrospher® 100 RP-18 column using a 93:7 (v/v) mixture of aqueous ammonium bicarbonate (0.05 mol dm^{-3}) and methanol as an eluent. The yield was 45%. The ^{1}H and ^{31}P NMR chemical shifts are listed in Table 3, and the RPHPLC retention times and ESMS signals in Table 4.

Kinetic measurements

The reactions were carried out in stoppered bottles immersed in a water bath, the temperature of which was adjusted to 298.2 K within 0.1 K. The initial concentration of the nucleotide esters was approximately 2×10^{-4} mol dm^{-3}. The progress of the reactions was followed by withdrawing 100 μl aliquots from the reaction mixture at appropriate intervals. The reactions were stopped by adjusting the pH to 4.5, and the composition of the aliquots was analyzed by RPHPLC on a Hypersil ODS column (250 × 4 mm, 5 μm) column. Isocratic elution with a mixture of acetic acid–sodium acetate buffer (0.045–0.015 mol dm^{-3}, pH 4.2, $[NH_4Cl]$ = 0.1 mol dm^{-3}) and acetonitrile was applied throughout. The acetonitrile content varied from 3.5 to 10% (v/v), depending on the hydrophobicity of the esterfied alcohol. Uridine 2′- and 3′-phosphates formed as hydrolysis products were identified by spiking with authentic samples. Isomerization of nucleoside 3′-alkylphosphates to their 2′-counterparts under acidic conditions has been established previously.[34] Table 4 records the acetonitrile contents and the retention times for the starting materials and their 2′-isomers.

The rate constants for the transesterification of **4a**–**f** to urid-

Table 4 Retention times of uridine 3′-alkylphosphates (**4a**–**f**) and their 2′-isomers on a Hypersil ODS column,[a] and the ESMS signals of the 3′-alkylphosphates

	t_R/min			
Alkyl group	3′-Isomer	2′-Isomer	Eluent[b]	*m*/*z*[c]
$CH(CH_3)_2$	6.9	4.1	5.0	365.4 (100%), 253.6 (20%)
CH_2CH_3	7.4	5.1	3.5	351.2 (100%), 239.3 (20%)
CH_2CH_2OEt	21.0	11.1	3.5	395.3 (100%), 283.4 (19%)
CH_2CH_2Cl	14.5	7.5	3.5	387.2 (33%), 385.3 (100%)
CH_2CHCl_2	23.8	10.2	5.0	421.1 (64%), 419.1 (100%), 347.3 (12%)
CH_2CCl_3	16.5	7.5	10.0	457.0 (28%), 455.0 (93%), 453.1 (100%)

[a] Column 4 × 250 mm, particle size 5 μm, flow rate 1 ml min^{-1}. [b] Acetonitrile content (%, v/v) in acetic acid–sodium acetate buffer (0.045/0.015 mol dm^{-3}), containing ammonium chloride (0.1 mol dm^{-3}). [c] Signals exhibiting the relative abundance >10% of that of the $[M-H]^-$ indicated.

ine 2′,3′-cyclic monophosphate (k_2; reaction B in Scheme 4) and the interconversion of **4a**–**f** and their 2′-isomers ($k_1 + k_{-1}$; reaction A) were calculated by rate laws of irreversible and reversible first-order reactions, as described previously in detail.[34] Calculation of the rate constant, $k_1 + k_{-1}$, of reversible first-order reactions is based on the equilibrium constant of the reaction. Since reaction B (Scheme 4) is, under the markedly acidic conditions employed in kinetic measurements (0.5 mol dm^{-3} aq. HCl) somewhat faster than the interconversion of nucleoside 2′- and 3-alkylphosphates (reaction A), these equilibrium constants could not be determined in the same solution, but were obtained at pH 4.2 and 363.2 K. However, our previous results[4,34] show that the equilibrium constant is not markedly sensitive to the hydronium ion concentration.

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