The mechanism of the metal ion promoted cleavage of RNA phosphodiester bonds involves a general acid catalysis by the metal aquo ion on the departure of the leaving group

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A series of uridine 3′-alkyl phosphates and 3′-aryl phosphates were synthesised and their cleavage was studied in the presence of Zn^{2+} aquo ions. A β_{lg} value was determined for the Zn^{2+} promoted cleavage of both types of compounds. Comparison of the results obtained to those reported previously for the cleavage of the same substrates in the absence of metal ion catalysts suggests that the alkyl leaving group departs as an alcohol in the presence of metal ion catalysts. Furthermore, metal ion catalysts seem to enhance the departure. The aryl leaving group, in contrast, departs as an oxyanion.

Introduction

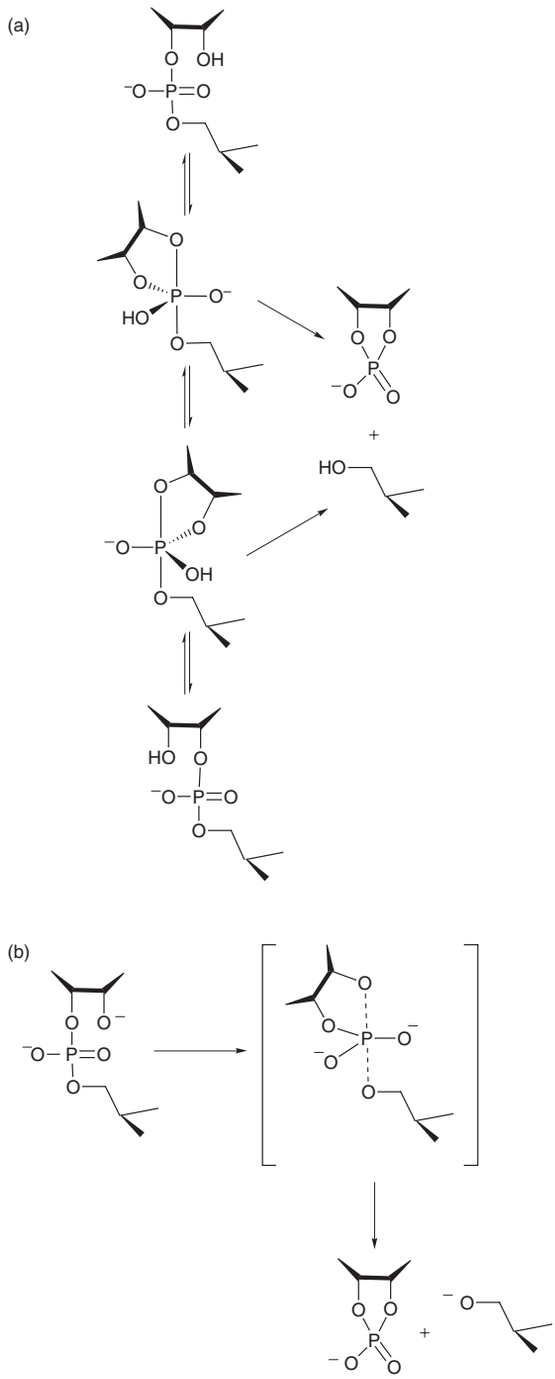
Metal ion promoted cleavage of phosphodiester bonds of RNA and the mechanism of this reaction have been extensively studied during the last few decades.[1–4] One of the aims of such studies is to find efficient catalysts that could be utilised as a catalytically active function in artificial restriction enzymes. Detailed understanding of the mechanism of the rate-enhancement by metal ions is hoped to enable a rational design of such catalysts. Studies on model systems could also help to elucidate the role of metal ions in nucleic acid modifying metallo enzymes.

Numerous mechanistic studies on the metal ion promoted cleavage of different phosphodiester compounds have been reported. The model systems employed include dinucleoside monophosphates[5–12] and diphosphates,[13–16] short oligonucleotides[17–22] and structurally simpler model compounds, such as aryl and alkyl esters of nucleotides[23,24] or aryl esters of 2-hydroxypropyl phosphate.[25–30] The rate of the cleavage reaction has been shown to exhibit a first-order dependence on the catalyst concentration, and the rate of the metal ion promoted cleavage increases as the pH increases. More importantly, the catalytic activity of the metal ion species seems to depend on the acidity of the aquo ligand of the catalyst: metal ions and their complexes with acidic aquo ligands usually are efficient catalysts.

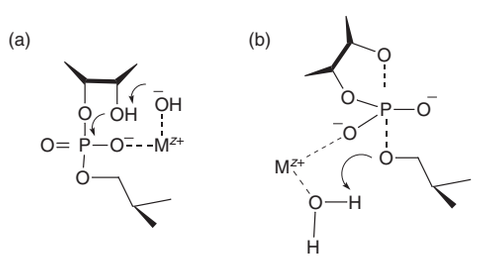
The phosphodiester bonds of RNA are cleaved by intramolecular transesterification: the 2′-hydroxy function attacks the phosphate group and a pentaco-ordinated phosphorane intermediate is formed (Scheme 1a).[31–33] Under neutral and acidic conditions the phosphorane formed is sufficiently stable to undergo two different reactions. A cleavage of the exocyclic P–O-bond results in the formation of a 2′,3′-cyclic phosphate group and a release of the 5′-linked nucleoside. Alternatively, a pseudorotation around the phosphorus atom followed by a cleavage of the endocyclic 3′-O–P yields the 2′,5′-isomer of the starting material. The departure of the leaving group is the rate-limiting step of the uncatalysed cleavage, which is clearly slower than the uncatalysed phosphate migration.[32,33] Under alkaline conditions, the attacking nucleophile is deprotonated and a dianionic phosphorane species is formed (Scheme 1b). The intermediate does not have a sufficiently long life-time to permit the pseudorotation, and no base-catalysed phosphate migration has ever been observed.

The reaction promoted by metal ions resembles the base catalysed cleavage in the sense that the phosphate migration is not enhanced.[5,6] Since the rate of cleavage in the presence of metal ions increases on increasing the pH,[6,18,27,28,34] and is obviously dependent on the acidity of the metal aquo ion[6] and on the strength of the metal ion–substrate complex,[19,20,22] it can be concluded that metal ions act as intracomplex base catalysts (Scheme 2a). Whether the rate-enhancement by metal ion hydroxides is a general or a specific base catalysis cannot be concluded on the basis of existing data. Since the difference between these is only the position of a proton transfer, distinguishing between the two options is difficult in a complex system involving several proton transfer equilibria. In either case, however, the mechanism of the metal ion promoted cleavage is similar to the base-catalysed reaction in that the reaction has no stable intermediate. One question concerning the metal ion promoted cleavage of RNA, yet to be answered, is the possible assistance of the departure of the leaving group by intracomplex general acid catalysis (Scheme 2b). If a base catalysis on the nucleophilic attack is involved, the intermediate/transition state formed will be dianionic, and the leaving group will have to depart as an oxyanion, as is the case under alkaline conditions. This kind of reaction could be expected to be facilitated by the protonation of the leaving group, as the 5′-oxyanion of a nucleoside is an unstable species under neutral conditions, where the metal ion catalysed reactions take place. A protonation of a leaving group departing from a dianionic intermediate has been suggested to take place in buffer solutions under neutral conditions.[31,35] Co^{3+} complexes have been suggested to promote the cleavage of a dinucleoside monophosphate as general acid catalysts only.[36] A general acid catalysis of the departure of a nucleoside leaving group by a heterogeneous bimetallic system has also been proposed.[7]

We believe on the basis of our kinetic results that metal ions



Scheme 1

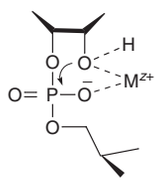


Scheme 2

act as general base catalysts.[6,34,37] General base catalysis by a metal bound hydroxide in different systems has also been suggested by several other research groups.[15,16,18,30,38,39] A mechanism where a metal ion assists the deprotonation of the nucleophile by direct co-ordination (Scheme 3) cannot, however, be strictly excluded on the basis of the existing data. This

Table 1 First-order rate constants of the cleavage of uridine 3′-alkyl phosphates at 90 °C and pH 5.6 in the presence of 10 mM $Zn(NO_3)_2$. $I = 0.1$ M ($NaNO_3$)

Substrate	$k/10^{-5}$ s^{-1}	pK_a of alkyl group[47]
1a	0.316 ± 0.005	17.1
1b	1.6 ± 0.2	16.0
1c	3.5 ± 0.3	14.8
1d	7.6 ± 0.1	12.9
1e	19.0 ± 0.4	12.3



Scheme 3

kind of mechanism has been shown to be involved in Co^{3+} promoted cleavage of phosphoesters,[40] and a similar mechanism has also been suggested for the cleavage of RNA models by other metal ions.[41] As the basis of the catalysis is the same in both cases—the inductive effect of the metal ion polarises an O–H bond—the experimental evidence obtained by kinetic techniques also applies to both mechanisms. Analogously to the catalysis on the first step, a direct co-ordination of the metal ion to the leaving group oxyanion could also enhance the second step of the cleavage. This kind of mechanism has been suggested to be involved in ribozyme catalysis.[42,43]

In the work we report in this paper, we have set out to study the question of the catalysis on the departure of the leaving group by determining β_{lg} values of the metal ion promoted cleavage of uridine 3′-alkyl phosphates. Previous studies from our laboratory have shown that the β_{lg} values for the cleavage proceeding by the departure of an alcohol, as in the acid catalysed cleavage, or an oxyanion, as in the base-catalysed reaction, are clearly different.[44] Hence the results of studies on the effect of the acidity of the leaving group in the presence of metal ion catalysts should be mechanistically indicative. The cleavage of corresponding uridine 3′-aryl phosphates has been studied for comparative purposes. These molecules contain a good leaving group the departure of which most probably takes place concerted with the attack of the 2′-hydroxy function.[45,46] Hence their metal ion promoted cleavage may also be expected to proceed as a one-step reaction, the departure of the aryloxy group not being assisted by general acid catalysis. The results obtained in the presence of metal ion catalysts will be compared to those obtained for the hydroxide and hydronium ion catalysed cleavage.

Results

The cleavage of uridine 3′-alkyl phosphates **1a**–**e**, promoted by Zn^{2+} aquo ions was followed at pH 5.6. The rate constants obtained at 90 °C, in the presence of 10 mM catalyst are collected in Table 1. Under these conditions, only the cleavage of phosphodiester bonds took place to any significant extent. Isomerisation was observed more clearly at a lower metal ion concentration (1 mM). The rate constants of isomerisation determined under these conditions show that, consistent with earlier reports,[5,6] only the cleavage of phosphodiester bonds is promoted by metal ions.

Comparison of the rate constants collected in Table 1 to those of the metal ion independent cleavage of the same substrates reported before,[44] shows that, similarly to the situation with dinucleoside monophosphates,[6] the rate enhancement by

Table 2 First-order rate constants of the cleavage of uridine 3′-aryl phosphates at 25 °C in the absence and in the presence of 10 mM Zn^{2+} aquo ions. Conditions: (a) No metal ion catalyst, pH 5.9, 0.1 M MES buffer, (b) 10 mM $Zn(NO_3)_2$ at pH 5.9. $I = 0.1$ M ($NaNO_3$)

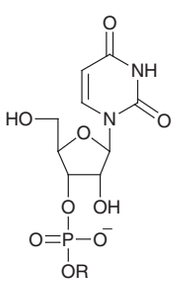
Substrate	k(a)/10^{-6} s^{-1}	k(b)[a]/10^{-6} s^{-1}	pK_a of aryl group[45]
2a	0.25 ± 0.02	0.71 ± 0.02	9.95
2b	0.68 ± 0.02	2.4 ± 0.2	9.38
2c	0.95 ± 0.01	4.35 ± 0.03	8.48
2d	5.06 ± 0.04	58.6 ± 0.8	7.51
2e	18.91 ± 0.09	620 ± 20	7.14

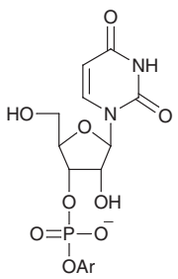
[a] The rate constants of the cleavage in the absence of the metal ion catalyst have been subtracted.

Table 3 First-order rate constants of the hydronium ion catalysed and pH independent cleavage of uridine 3′-aryl phosphates at 90 °C. Conditions: (a) 0.1 M HCl and (b) formic acid buffer, pH 3.5, $I = 0.1$ M ($NaNO_3$)

Substrate	k(a)/10^{-3} s^{-1}	k(b)[a]/10^{-4} s^{-1}
2a	0.77 ± 0.02	0.7 ± 0.1
2b	1.08 ± 0.04	1.2 ± 0.1
2c	1.23 ± 0.08	1.4 ± 0.1
2d	3.0 ± 0.1	2.5 ± 0.1
2e	5.0 ± 0.1	2.9 ± 0.1

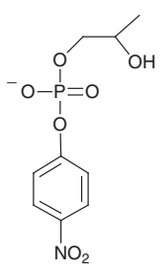
[a] Rate constants extrapolated to zero buffer concentration.





1a: R=$CH(CH_3)_2$
1b: R=CH_2CH_3
1c: R=$CH_2CH_2OCH_2CH_3$
1d: R=CH_2CHCl_2
1e: R=CH_2CCl_3

2a: Ar=Phenyl
2b: Ar=2-Chlorophenyl
2c: Ar=4-Chlorophenyl
2d: Ar=2,5-Dichlorophenyl
2e: Ar=4-Nitrophenyl



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Zn^{2+} aquo ions is rather modest. The cleavage of the most reactive of the compounds studied, **1e**, is enhanced by a factor of only 17 in the presence of 10 mM Zn^{2+} at pH 5.6. The rate-enhancement increases as the leaving group becomes poorer, and under the same conditions the cleavage of **1a** is enhanced 500-fold.

The effect of pH on the rate constants of the cleavage was studied with **1a** and **1e**, *i.e.* the least and the most reactive of the alkyl phosphate substrates employed in the present study. In the presence of 10 mM Zn^{2+} aquo ions, the rate of the cleavage of both of these substrates increases as the pH increases, similarly to the situation with 3′,5′-UpU[6] or poly-U[34] as reported before. The slopes of the plots log(k/s^{-1}) *vs.* pH were (1.0 ± 0.2) and (0.8 ± 0.1) for **1a** and **1e**, respectively.

The metal ion promoted cleavage of uridine 3′-aryl phosphates **2a**–**e**, which are clearly more reactive than the alkyl esters, was studied at 25 °C. The rate constants of the cleavage were determined in the presence of 10 mM Zn^{2+} at pH 5.9. Consistent with previous reports,[46] no isomerisation of phosphodiesters was observed under these conditions. Rate constants of the cleavage in the absence of the metal ion catalysts were determined under the same conditions. All the rate constants obtained under neutral conditions are collected in Table 2.

Comparison between the rate constants of the cleavage of **2a**–**e** obtained in the absence and in the presence of metal ion catalysts shows that the rate-enhancement by Zn^{2+} aquo ions is even more modest than it is in the case of alkyl esters. This is in contrast to the results of previous studies[5,26] on the metal ion promoted cleavage of the 4-nitrophenyl phosphate ester of propylene glycol (**3**), where a more significant rate enhancement has been observed. Another significant difference observed in this study between the alkyl phosphates and aryl phosphates is that, while with alkyl esters the rate-enhancement by metal ion catalysts is the higher the poorer the leaving group is, with aryl esters an opposite situation is observed. With 10 mM Zn^{2+} aquo ion as a catalyst at pH 5.9, the cleavage of the least reactive of the aryl esters, **2a**, is enhanced by only two-fold, whereas with the most reactive compound, **2e**, the cleavage is promoted by a factor of 32.

To obtain a complete set of β_{lg} values, the cleavage of aryl esters **2a**–**e** was also studied under acidic conditions, in the absence of metal ion catalysts. β_{lg} of the acid catalysed reaction was determined in 0.1 M HCl at 90 °C. The first-order rate constants obtained are collected in Table 3. Isomerisation of the phosphodiester bonds of **2a**–**e** was not observed to any significant extent under these conditions. The pH-independent cleavage was studied in formic acid buffers at pH 3.5 at 90 °C. This pH was chosen on the basis of a pH–rate profile determined for the cleavage of the most reactive of the aryl esters, **2e**, in buffer solutions within a limited pH-range. The rate of the reaction showed only a modest dependence on pH between pH 3 and 4, with a minimal reactivity observed at pH 3.5. The rate of the cleavage of **2c** has been reported to be independent of pH from pH 2 to 5.5.[46] It is hence to be expected that the less reactive compounds are also cleaved by the pH-independent mechanism at pH 3.5. The rate constants were determined at two different buffer concentrations, and they showed only a very modest dependence on the buffer concentration: the rate constants obtained in 0.1 M buffer were only 10–15% larger than those in 0.02 M buffer solutions. Rate constants extrapolated to a buffer concentration of zero are collected in Table 3.

The β_{lg} values of the cleavage of aryl and alkyl esters of 3′-UMP in the presence of Zn^{2+} aquo ions were determined as slopes of plots log(k/s^{-1}) *vs.* pK_a of the leaving group. The pK_a values of the leaving groups were found in the literature,[45,47] and are included in Tables 1 and 2. In case of the aryl esters, where the catalysis by Zn^{2+} was very modest, the rate constant of the uncatalysed reaction obtained under the same conditions was subtracted to obtain the rate constants of the cleavage dependent on the metal ion catalysts only. With the alkyl esters exhibiting more marked rate-enhancements, this was not necessary, since the contribution of the uncatalysed reaction was insignificant. In the case of the pH-independent cleavage of aryl esters **2a**–**e**, the rate constants extrapolated to zero buffer concentration were used for calculations. The plot for the Zn^{2+} promoted cleavage of the alkyl esters of 3′-UMP is shown in Fig. 1. The Brønsted plots of the cleavage of the aryl esters under different conditions are shown in Fig. 2.

As is seen in Table 4, the β_{lg} value for the Zn^{2+} aquo ion dependent cleavage of alkyl esters is only slightly negative,

Table 4 Brønsted β_{lg} values of the cleavage of uridine 3′-aryl phosphates and 3′-alkyl phosphates under different conditions

Substrate	Conditions	β_{lg}
1a–**1e**	10 mM $Zn(NO_3)_2$, pH 5.6, 90 °C	−0.32 ± 0.04
2a–**2e**	10 mM $Zn(NO_3)_2$, pH 5.9, 25 °C	−0.9 ± 0.2
2a–**2e**	0.1 M HCl, 90 °C	−0.27 ± 0.05
2a–**2e**	Formic acid buffer, pH 3.5, 90 °C	−0.20 ± 0.02

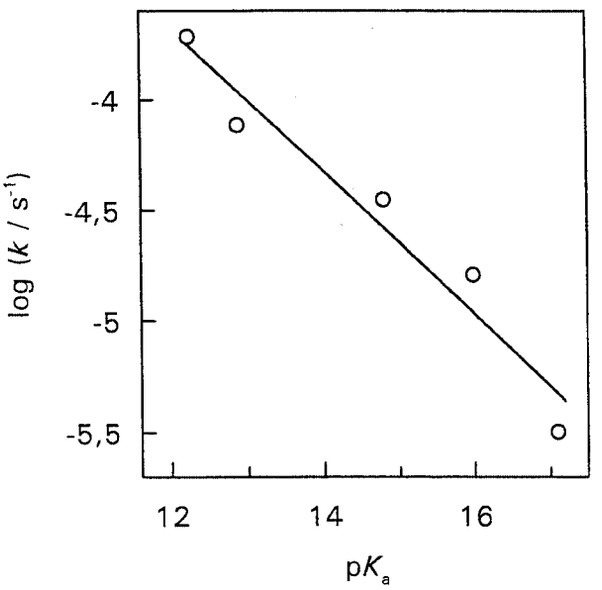

Fig. 1 The Brønsted plot of the Zn^{2+} promoted cleavage of uridine 3′-alkyl phosphates at pH 5.6 and 90 °C. I = 0.1 M ($NaNO_3$) and [$Zn(NO_3)_2$] = 10 mM.

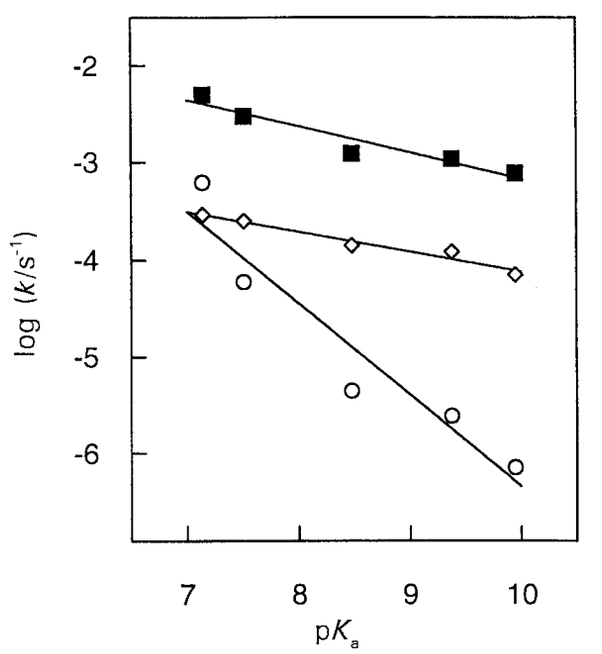

Fig. 2 The Brønsted plots of the cleavage of uridine 3′-aryl phosphates. Notation: ◇ Buffer and pH independent cleavage in formic acid buffer at pH 3.5 and 90 °C, ■ acid catalysed cleavage in 0.1 M HCl and 90 °C, and ○ Zn^{2+} dependent cleavage at pH 5.9 and 25 °C (10 mM $Zn(NO_3)_2$). I = 0.1 M ($NaNO_3$).

−0.32. β_{lg} values of −1.28, −0.12 and −0.59 have been reported previously for the cleavage of the alkyl esters promoted by hydroxide ions,[44] cleavage promoted by hydronium ions[44] and for the pH-independent cleavage,[48] respectively. At pH 5.6, both the pH-independent and hydroxide ion-dependent cleavages operate, and at this pH, β_{lg} of the background reaction could be expected to fall between those reported for these two reactions. The β_{lg} value obtained for the cleavage promoted by Zn^{2+} aquo ions is hence clearly less negative than the value expected in the absence of Zn^{2+}, and significantly less negative than that of a base catalysed reaction.

The situation with uridine 3′-aryl phosphates is rather different. Firstly, the β_{lg} value of the cleavage promoted by Zn^{2+} is clearly more negative than in the case of the uridine 3′-alkyl phosphates. The value obtained was −0.9. Secondly, in contrast to the situation with the alkyl phosphates, the value for the Zn^{2+} promoted cleavage of the aryl esters is more negative than that in the absence of metal ion catalysts. β_{lg} values reported earlier for the hydroxide ion catalysed and buffer dependent cleavage of nucleoside 3′-aryl phosphates are −0.54 and −0.59, respectively.[45] Consistent with these values, the β_{lg} value of the background cleavage obtained in this work at pH 5.9 was (−0.6 ± 0.1). β_{lg} values of the cleavage of the uridine 3′-aryl phosphates obtained under acidic conditions, in the absence of metal ion catalysts, are all less negative than those discussed above: values of −0.27 for the acid catalysed cleavage in 0.1 M HCl, and −0.20 for the pH and buffer independent cleavage at pH 3.5 were obtained in this work.

Discussion

Comparison of the β_{lg} value of the cleavage promoted by Zn^{2+} aquo ion and those reported earlier for the cleavage in the absence of metal ion catalysts, strongly suggests that Zn^{2+} aquo ions assist the departure of the alkyl leaving groups. As was mentioned in the Introduction, the mechanism of the cleavage in the absence of metal ion catalysts changes on changing the pH, and the departure of either an alcohol leaving group or an alkoxide ion is observed depending on the pH.[32,33,44] Under acidic conditions the cleavage is a two-step process and the phosphorane formed upon the nucleophilic attack of the 2′-hydroxy group appears to be a true intermediate. Consistent with this, both isomerisation and cleavage of phosphodiester bonds are observed. These reactions are approximately as fast, which, considering that the second step of isomerisation can be regarded as a reverse of the nucleophilic attack, shows that under acidic conditions the two energy barriers of the cleavage are approximately as high. In the absence of any catalysts, the second step of the cleavage is clearly rate-limiting, and consequently the uncatalysed cleavage is up to two orders of magnitude slower than the isomerisation. Under alkaline conditions the mechanism of the cleavage is different: the attacking nucleophile is deprotonated and its attack on the monoanionic phosphate produces a dianionic phosphorane intermediate. Such species have been suggested to be too unstable to exist as a kinetically significant intermediate,[49,50] and the base catalysed cleavage most probably is a concerted process. Consistent with this, the isomerisation of phosphodiester bonds is not base-catalysed.

The cleavage of alkyl phosphates proceeding *via* the departure of an alcohol or an alkoxide ion are characterised by β_{lg} values clearly differing from each other. Under acidic conditions, where the leaving group departs as an alcohol, the rate of cleavage is quite independent of the acidity of the leaving group, and the β_{lg} value obtained is only −0.12.[44] Under alkaline conditions the leaving group departs as an alkoxy ion, and the β_{lg} value observed is very negative, −1.28.[44] The β_{lg} value of the Zn^{2+} promoted cleavage (−0.32) is hence more consistent with a reaction where the leaving group is an alcohol rather than an alkoxy ion.

Furthermore, a comparison to the results obtained with the same uridine 3′-alkyl phosphates in buffer solutions shows that Zn^{2+} aquo ions enhance the protonation of the leaving group. The moderately negative β_{lg} of the buffer independent cleavage under neutral conditions (−0.59) has been explained by a

mechanism which involves a solvent-mediated proton transfer from a phosphorane hydroxy ligand to the leaving group, which subsequently departs as an alcohol.[48] Since β_{lg} of the Zn^{2+} promoted cleavage (−0.32) is less negative than the value of the metal ion independent cleavage under the same conditions, it seems likely that the presence of Zn^{2+} ions enhances the degree of protonation of the alkyl leaving groups in comparison to the situation in the absence of metal ion catalysts.

It has to be emphasised that even though the β_{lg} value of the Zn^{2+} promoted cleavage is consistent with an acid catalysed reaction, the reaction does not appear to be catalysed by a general acid only, but the cleavage has characteristics of a base-catalysed reaction as well. The fact that metal ion catalysts do not promote the phosphate migration suggests that the attacking nucleophile has been deprotonated and a dianionic phosphorane has been formed. This, together with the fact that the rate of the Zn^{2+} promoted cleavage increases on increasing the pH, suggests that a base catalysis is involved. Furthermore, as will be discussed later, the results obtained with aryl esters suggest that the presence of metal ion catalysts enhances also the nucleophilic attack. Hence the present data are not inconsistent with previous reports on metal ion promoted cleavage of RNA phosphodiester bonds, but tend to show that both general acid and base catalysis are involved.

The observed difference in the behaviour of uridine 3′-alkyl phosphates and aryl phosphates is also consistent with the suggested general acid catalysis of the departure of alkyl leaving groups. The good leaving group of the aryl phosphates could be expected to depart as an oxyanion in the presence of metal ion catalysts, and only a general base catalysis by metal ion catalysts is, most likely, involved. This reaction seems to be characterised by a highly negative β_{lg} value. The clear difference between the β_{lg} values of the Zn^{2+} promoted cleavage of uridine 3′-aryl phosphates and alkyl phosphates also shows that the mechanism of the catalysis most probably is different.

As was mentioned in the Introduction, the cleavage of nucleoside 3′-aryl phosphates differs significantly from the cleavage of the corresponding alkyl esters. An aryloxy group, being less basic than an alkyl group, makes a better leaving group, and most probably the departure of an aryloxy group is no longer alone the rate-limiting step of the cleavage. Isomerisation of the phosphodiester bonds of nucleoside aryl phosphates has been observed only below pH 2, showing that under these conditions a stable phosphorane is formed. Consistent with the suggested rate-limiting nucleophilic attack, the β_{lg} value of the acid catalysed cleavage of the aryl esters obtained in this work was −0.27. The β_{lg} value of the cleavage independent of the buffer concentration and pH is −0.20, and at pH 3.5 no isomerisation is observed concomitant with the cleavage. This suggests that either the nucleophilic attack is the rate-limiting step or the cleavage is concerted and has an early transition state. Under alkaline conditions the nucleophile is negatively charged and the phosphorane formed is dianionic, similar to the situation of the alkyl phosphates. The β_{lg} values of the buffer dependent and base catalysed cleavage reported are −0.56 and −0.54, respectively.[45] These reactions have been shown to be concerted, with a leaving group departing as an anion and only a general base catalysis has been observed in buffer solution. The thorough analysis of Williams has shown that the bond formation between the incoming nucleophile and the phosphorus is only little advanced in the transition state.[45]

Under the conditions where the metal ion promoted cleavage of uridine 3′-aryl phosphates was studied, the background reaction most probably is similar to the buffer dependent and hydroxide ion catalysed reaction described above: a concerted process with the leaving group departing as an oxyion. This is shown by the β_{lg} values of the background reaction measured in this work (−0.6 at pH 5.9). The β_{lg} value of the Zn^{2+} promoted cleavage of the aryl esters is even more negative than that of the cleavage in the absence of metal ion catalysts or that of the base catalysed cleavage. Most probably the departure of an aromatic leaving group is not enhanced in the presence of metal ion catalysts, but it departs as an aryl oxyanion similarly to the situation in the absence of metal ion catalysts. Consistent with this, the transition state of the metal ion promoted cleavage seems to be later than in the acid catalysed cleavage.

It seems hence that while the Zn^{2+} ion enhances the cleavage of uridine 3′-alkyl phosphates both as general base and acid catalysts, only general base catalysis is observed with the corresponding aryl esters. The situation is actually similar to that of the buffer catalysed cleavage of nucleoside phosphoesters: both the acid and base forms of the buffer have been shown to be involved in the cleavage of nucleoside alkyl phosphates and internucleosidic phosphodiester bonds,[31,35,51] whereas the cleavage of aryl esters is catalysed by general bases only.[45,52]

Experimental

General

Acetonitrile was dried by refluxing on calcium hydride, and subsequent distillation. 2-Ethoxyethanol, 2,2-dichloroethanol and 2,2,2-trichloroethanol were distilled and stored over molecular sieves. Pyridine and ethanol were dried with molecular sieves. Pivaloyl chloride and triethylamine were distilled prior to use. 1,2,4-Triazole was recrystallized from toluene. Phenyl phosphorodichloridate and its 2-chloro, 4-chloro, 2,5-dichloro and 4-nitro derivatives were commercial products of Sigma, and they were used as received. Triethylamine trihydrofluoride (98%) was purchased from Lancaster.

Uridine 3′-alkyl phosphates (1a–e)

The synthesis and characterisation of compounds **1a**–**e** have been reported previously.[44] Compounds **1b**–**e** employed in the present study were, however, synthesised by a slightly modified route: *tert*-butyldimethylsilyl (TBDMS) group was used for the protection of the 2′-hydroxy function instead of the previously used tetrahydropyranyl protection. Accordingly, the following procedure was applied. The triethylammonium salt of 2′,5′-di-*O*-TBDMS-uridine 3′-H-phosphonate (0.2 g; 0.31 mmol), obtained by the standard phosphonylation of 2′,5′-di-*O*-TBDMS-uridine[53] with a phosphorus trichloride–imidazole reagent,[54] was dried by coevaporation with a mixture of acetonitrile and pyridine (3 : 1; v/v; 10 cm^3). The residue was dissolved in the same solvent mixture (10 cm^3) and the appropriate alcohol (0.94 mmol) was added, followed by pivaloyl chloride (0.62 mmol, 74 mg). After the reaction was complete (about 10 min, TLC analysis), iodine (2% w/v) in a mixture of pyridine and water (49 : 1; v/v; 5 cm^3) was added. After 30 min, the reaction mixture was diluted with dichloromethane (20 cm^3), washed with aqueous sodium thiosulfate (1 mol dm^{-3}; 2 × 10 cm^3) and triethylammonium acetate buffer (pH 7.7; 2 × 10 cm^3). The organic layer was evaporated to dryness, and the crude product was purified by silica gel chromatography using a stepwise gradient of methanol (1–10%) in chloroform containing 0.1% triethylamine. The yields of fully protected **1b**–**e** ranged from 70 to 89%. The compounds were characterised by ^1H and ^{13}C NMR spectroscopy (25 °C, $CDCl_3$, ppm from TMS). 2′,5′-Di-*O*-TBDMS-uridine 3′-ethyl phosphate: ^{13}C NMR δ −5.20, −5.00, −4.79, −4.44 (CH_3Si), 17.08 (CH_3CH_2-OP), 18.41, 18.70 [$(CH_3)_3CSi$], 25.96, 26.36 [$(CH_3)_3CSi$], 61.67 (*C*4′), 64.09 (CH_3CH_2OP), 75.35 (*C*3′), 75.82 (*C*2′), 85.48 (*C*5′), 87.36 (*C*5), 102.99 (*C*1′), 140.59 (*C*6), 151.28 (*C*2), 163.56 (*C*4). ^1H NMR δ −0.03–0.08 (12H; CH_3Si), 0.81–0.91 [18H; $(CH_3)_3CSi$], 1.22 (3H; CH_3CH_2OP), 3.86 (2H; *H*5′, *H*5″), 3.96 (2H; CH_3CH_2OP), 4.25 (1H; *H*4′), 4.46 (1H; *H*2′), 4.61 (1H; *H*3′), 5.70 (1H; *H*5), 6.08 (1H; *H*1′), 7.88 (1H; *H*6). 2′,5′-Di-*O*-TBDMS-uridine 3′-(2-ethoxyethyl phosphate): ^{13}C NMR δ −5.20, −5.00, −4.85, −4.45 (CH_3Si), 15.60 (CH_3CH_2O), 18.38, 18.66 [$(CH_3)_3CSi$], 25.95, 25.33 [$(CH_3)_3CSi$], 64.08 (*C*4′),

64.89 (CH_3CH_2O), 66.71 (OCH_2CH_2OP), 70.50 (OCH_2CH_2-OP), 75.49 (*C*3′), 75.73 (*C*2′), 85.31 (*C*5), 87.22 (*C*5′), 103.01 (*C*1′), 140.88 (*C*6), 151.49 (*C*4), 163.77 (*C*2). ^{1}H NMR δ −0.03–0.08 (12H; CH_3Si), 0.81–0.91 [18H; $(CH_3)_3CSi$], 1.10 (3H; CH_3CH_2O), 3.51 (2H; CH_3CH_2O), 3.56 (2H; OCH_2CH_2OP), 3.81 (2H; *H*5′), 3.95 (1H; *H*4′), 4.21 (1H; *H*2′), 4.45 (1H; *H*3′), 4.58 (2H; OCH_2CH_2OP), 5.65 (1H; *H*5), 6.08 (1H; *H*1′), 7.81 (1H; *H*6). 2′,5′-Di-*O*-TBDMS-uridine 3′-(2,2-dichloroethyl phosphate): ^{13}C NMR δ −5.23, −5.03, −4.76, −4.40 (CH_3Si), 18.40, 18.69 [$(CH_3)_3CSi$], 26.34, 26.45 [$(CH_3)_3CSi$], 27.68 (OCH_2CHCl_2), 64.00 (*C*4′), 70.76 (*C*3′), 71.23 (*C*2′), 71.30 (OCH_2CHCl_2), 85.18 (*C*5′), 87.27 (*C*5), 103.08 (*C*1′), 140.35 (*C*6), 151.54 (*C*2), 163.62 (*C*4). ^{1}H NMR δ −0.02–0.09 (12H; CH_3Si), 0.67–0.78 [18H; $(CH_3)_3CSi$], 3.85 (2H; *H*5′, *H*5″), 4.19 (2H; OCH_2CHCl_2), 4.25 (1H; *H*4′), 4.52 (1H; *H*2′), 4.79 (1H; *H*3′), 5.67 (1H; *H*5), 5.93 (1H; CH_2CHCl_2), 6.08 (1H; *H*1′), 7.88 (1H; *H*6). 2′,5′-Di-*O*-TBDMS-uridine 3′-(2,2,2-trichloroethyl phosphate): ^{13}C NMR δ −5.23, −5.03, −4.76, −4.40 (CH_3Si), 18.42, 18.71 [$(CH_3)_3CSi$], 26.34, 26.45 [$(CH_3)_3CSi$], 27.67 (OCH_2CCl_3), 64.06 (*C*4′), 75.72 (*C*3′), 75.80 (*C*2′), 85.23 (OCH_2CCl_3), 85.87 (*C*5′), 97.49 (*C*5), 103.07 (*C*1′), 140.43 (*C*6), 151.39 (*C*2), 163.50 (*C*4). ^{1}H NMR δ −0.02–0.09 (12H; CH_3Si), 0.67–0.78 [18H; $(CH_3)_3CSi$], 3.89 (2H; *H*5′, *H*5″), 4.28 (1H; *H*4′), 4.50 (2H; OCH_2CCl_3), 4.61 (1H; *H*2′), 4.62 (1H; *H*3′), 5.69 (1H; *H*5), 6.11 (1H; *H*1′), 7.80 (1H; *H*6).

The TBDMS-protected uridine 3′-alkyl phosphates (0.025 mmol) were dissolved in triethylammonium fluoride (1 cm^3). After *ca*. 2 h, the reaction was complete (TLC-analysis, $CHCl_3$–MeOH 8:2 v/v) and the mixture was diluted with water (10 cm^3) and washed with ethyl acetate (3 × 10 cm^3). The aqueous phase was lyophilised and redissolved in aqueous triethylammonium acetate (0.025 mol dm^{-3}, pH 6.5). The crude product was purified by RP HPLC (Hypersil RP-18, 10 × 250 mm) using aqueous triethylammonium acetate (0.025 mol dm^{-3}, pH 6.5) as the eluent buffer and applying a 30 min linear gradient from 0 to 12.5% acetonitrile, and then increasing the acetonitrile content to 50% in 5 min. The retention times were 15 min (**1b**), 19 min (**1c**), 25 min (**1d**) and 35 min (**1e**). The purified products were lyophilized. The yields ranged from 94 to 96%, and the compounds were found to be identical (by ^{1}H and ^{31}P NMR) with those previously synthesized[43] using 2′-*O*-tetrahydropyranyl protection. The preparation of the isopropyl ester (**1a**) has been described earlier.[44]

Uridine 3′-aryl phosphates (2a–e)

The aryl esters of uridine 3′-phosphate (**2a**–**e**) were obtained essentially as described previously by Davis *et al.*[45] Accordingly, the commercial substituted (or unsubstituted) phenyl phosphorodichloridate was converted in dry acetonitrile to the corresponding bis(1,2,4-triazolide) and reacted immediately in acetonitrile with 5′-*O*-(4-monomethoxytrityl)-2′-*O*-(tetrahydropyran-2-yl)uridine (5′-*O*-MMTr-2′-*O*-Thp-uridine) [prepared from the slower migrating diastereomer of 2′-*O*-(tetrahydropyran-2-yl)uridine]. The detailed procedure has previously been reported for 2-chlorophenyl phosphorodichloridate.[44] The progress of phosphorylation was followed by TLC (silica gel 60; CH_2Cl_2–EtOH 8:2). Upon completion of the reaction (1–3 h), aqueous triethylammonium acetate was added, and the mixture was stirred for 10 min to hydrolyse the remaining triazole ligand. After conventional workup, the products were purified by adsorption chromatography on a Silica gel column (3 × 26 cm) using a mixture of CH_2Cl_2, EtOH and Et_3N (96:3:1) as an eluent, and characterised by ^{1}H and ^{31}P NMR spectroscopy (20 °C, DMSO-d_6, ppm from TMS and H_3PO_4, respectively). 5′-*O*-MMTr-2′-*O*-Thp-uridine 3′-phenyl phosphate: ^{1}H NMR δ 1.44–1.65 (6H; Thp), 3.15–3.60 (4H; *H*5′, *H*5″, Thp), 3.73 (3H; MMTr), 4.20 (1H; *H*4′), 4.44 (1H; *H*2′), 4.70 (1H; *H*3′), 4.81 (1H; Thp), 5.26 (1H; *H*5), 5.96 (1H; *H*1′), 6.88 (3H; MMTr, Ph), 7.05 (2H; Ph), 7.12 (2H; Ph) 7.16–7.34 (12H; MMTr), 7.57 (1H, *H*6), 11.41 (1H, *H*3). ^{31}P NMR −5.96. 5′-*O*-MMTr-2′-*O*-Thp-uridine 3′-(2-chlorophenyl phosphate): ^{1}H NMR δ 1.44–1.65 (6H; Thp), 3.15–3.60 (4H; *H*5′, *H*5″, Thp), 3.73 (3H; MMTr), 4.29 (1H; *H*4′), 4.49 (1H; *H*2′), 4.79 (2H; *H*3′, Thp), 5.23 (1H; *H*5), 5.98 (1H; *H*1′), 6.87 (2H; MMTr), 6.91 (1H; ClPh), 7.10 (1H; ClPh), 7.17 (2H; MMTr), 7.22–7.34 (13H; MMTr, ClPh), 7.56 (1H, *H*6), 7.66 (1H; ClPh), 11.42 (1H, *H*3). ^{31}P NMR: −5.96. 5′-*O*-MMTr-2′-*O*-Thp-uridine 3′-(4-chlorophenyl phosphate): ^{1}H NMR δ 1.44–1.65 (6H; Thp), 3.15–3.60 (4H; *H*5′, *H*5″, Thp), 3.73 (3H; MMTr), 4.19 (1H; *H*4′), 4.43 (1H; *H*2′), 4.66 (1H; *H*3′), 4.79 (1H; Thp), 5.27 (1H; *H*5), 5.95 (1H; *H*1′), 6.87 (2H; MMTr), 7.06 (2H; ClPh), 7.16 (4H; MMTr, ClPh), 7.22–7.34 (12H; MMTr), 7.58 (1H; *H*6), 11.42 (1H; *H*3). ^{31}P NMR: −6.15. 5′-*O*-MMTr-2′-*O*-Thp-uridine 3′-(2,5-dichlorophenyl phosphate): ^{1}H NMR δ 1.44–1.65 (6H; Thp), 3.15–3.60 (4H; *H*5′, *H*5″, Thp), 3.73 (3H; MMTr), 4.31 (1H; *H*4′), 4.49 (1H; *H*2′), 4.71 (1H; Thp), 4.78 (1H; *H*3′), 5.25 (1H; *H*5), 5.98 (1H; *H*1′), 6.86 (2H; MMTr), 7.01 (1H; Cl_2Ph), 7.17 (2H; MMTr), 7.22–7.33 (12H; MMTr), 7.38 (1H; Cl_2Ph), 7.59 (1H; *H*6), 7.79 (1H; Cl_2Ph), 11.42 (1H; *H*3). ^{31}P NMR: −5.96. 5′-*O*-MMTr-2′-*O*-Thp-uridine 3′-(4-nitrophenyl phosphate): ^{1}H NMR δ 1.44–1.65 (6H; Thp), 3.15–3.60 (4H; *H*5′, *H*5″, Thp), 3.73 (3H; MMTr), 4.17 (1H; *H*4′), 4.42 (1H; *H*2′), 4.66 (1H; *H*3′), 4.80 (1H; Thp), 5.30 (1H; *H*5), 5.96 (1H; *H*1′), 6.86 (2H; MMTr), 7.15 (2H; MMTr), 7.22–7.30 (14H; MMTr, NO_2Ph), 7.59 (1H; *H*6), 8.09 (2H; NO_2Ph), 11.42 (1H; *H*3). ^{31}P NMR: −6.51.

The acid labile monomethoxytrityl and tetrahydropyranyl protecting groups were removed before kinetic runs with a 1:1 mixture of acetonitrile and aqueous hydrogen chloride (0.2 M) at room temperature (60 to 90 min). The solution was diluted with aqueous sodium acetate to adjust the pH at 4.7. The released 4-methoxytrityl alcohol was extracted into dichloromethane, and the aqueous phase was evaporated to dryness. The salts were removed by rapid RP HPLC on a semipreparative column (LiChrospher, 250 × 10 mm, 5 μm), using a 3:97 mixture of acetonitrile and water as an eluent.

Kinetic measurements

All the reaction solutions were prepared in sterilised water, and sterilised equipment was employed for handling them. The pH of the reaction solutions of metal ion promoted reactions was adjusted by MES [2-(*N*-morpholino)ethanesulfonic acid] or HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(ethanesulfonic acid)]. The pH of all reaction solutions was measured with a pH meter at room temperature, and extrapolated to the temperature of the kinetic measurements with the help of the known temperature dependence of the buffer system employed.[55]

The reactions of the alkyl esters were carried out in tightly stoppered glass tubes and those of the aryl esters in Eppendorf tubes. The temperature of the reaction solutions was controlled by a water bath thermostatted at 90.0 ± 0.1 °C or 25 ± 0.1 °C. Aliquots (10–12 for each reaction) were withdrawn at suitable intervals to cover at least one half-life of the cleavage. The reaction was quenched by adding an excess of EDTA to chelate the metal ion catalyst. Aliquots of the acid catalysed cleavage of the aryl esters were neutralised with a solution of K-salt of HEPES buffer. Samples were kept in an ice bath until the analysis on HPLC. Those from the reactions of the aryl esters were analysed immediately.

The aliquots were analysed by RP HPLC by using a Hypersil ODS column (250 × 5 mm, 5 μm particle size). Mixtures of acetic acid buffer (50 mM, pH 4.3, containing 0.1 M NH_4Cl) and acetonitrile were used as an eluent. A step-wise elution was employed for the analysis of the alkyl phosphates. The first step was an elution with acetic acid buffer for 8 minutes to elute the nucleoside monophosphate products. In the second step, the eluent contained 5–10% acetonitrile, depending on the sub-

strate. Isocratic elution using 11–15% acetonitrile was employed for the aryl esters. UV-detection at 260 nm was employed for the analysis.

Rate constants of the cleavage (and isomerisation) of the aryl esters were calculated as has been described before[51] by using a mole fraction of the substrate and its 2′-isomer for the calculation. The rate constants of the cleavage of the aryl esters were calculated by following the decrease of the signal of the substrate on a chromatogram as a function of time and applying the integrated rate-law of first-order processes.

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