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Kinetic solvent deuterium isotope effect in transesterification of RNA models

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ABSTRACT: 2-Methylbenzimidazole ribonucleoside arylphosphates (**1a**,**b**) and alkylphosphates (**2a**,**b**) were synthesized as RNA model compounds containing a minimised number of exchangeable protons. Intramolecular transesterification of these substrates was studied in H_2O and D_2O solutions over a wide p*L* range and apparent kinetic solvent deuterium isotope effects of the alkaline cleavage of both substrates and of the cleavage and isomerisation of **2a** under neutral and acidic conditions were determined. The observed k_{H_2O}/k_{D_2O} of 4.9 obtained for the alkaline cleavage of the arylphosphate **1b** can be primarily attributed to the ΔpK of the attacking nucleophile. The alkyl leaving group in **2a** brings about an additional 1.5-fold isotope effect (k_{H_2O}/k_{D_2O} of 7.1 observed), which, considering the p*L*–dependence of the reaction, can not be explained by a process involving a proton transfer. Differences in solvation of the transition state are tentatively suggested as a source of the difference. In contrast to alkaline cleavage, under neutral and acidic conditions the cleavage and isomerisation of **2a** showed no apparent solvent isotope effect. Several examples found in the literature show that intramolecular proton transfer from phosphate to the leaving group in pre-equilibria may not necessarily result in an observable solvent isotope effect. This may also explain the results obtained in the present work, since intramolecular proton transfer processes take place in transesterification reactions of **2a** under neutral and acidic conditions. Relevance of the results obtained in the base catalysed cleavage to hammerhead ribozyme reaction is briefly discussed. Copyright © 2004 John Wiley & Sons, Ltd.
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KEYWORDS: RNA; phosphodiester bonds; transesterification; solvent isotope effect

INTRODUCTION

The mechanism of the cleavage of phosphodiesters has been extensively studied[1] ever since it was discovered that DNA and RNA are polymers made up of nucleoside units linked by phosphodiester bonds. Studies on the cleavage of simple phosphodiesters *in vitro* have been carried out to help to understand the mechanisms utilized by enzymes involved in phosphate transfer processes. More recently, the mechanism of autocatalytic cleavage observed with ribozymes has attracted wide interest.[2] Studies with RNA models have also been carried out to define the parameters required of efficient catalysts for RNA cleavage to develop artificial nucleases,[3] chemical catalysts that are hoped to permit the selective and efficient cleavage of intracellular RNA molecules.

The phosphodiester bonds in RNA react via an intramolecular transesterification, where the 2′-OH group attacks the phosphate, resulting in the formation of a pentacoordinated species.[1c–e] The status of this species depends on the reaction conditions. Under acidic and neutral conditions, a phosphorane intermediate is formed, and isomerization of the natural 3′,5′-phosphodiester bond to a 2′,5′-bond competes with cleavage (Scheme 1). Under alkaline conditions the situation is not as clear. Base-catalysed isomerization of the phosphodiester bonds is not observed, so the reaction is believed to proceed via a dianionic phosphorane-like transition state or a marginally stable intermediate (Scheme 2). However, a stepwise mechanism has also been recently proposed.[4] Irrespective of the exact status of the dianionic species, the departure of the alkyl leaving group takes place in the rate-limiting step of the reaction.

The kinetic solvent deuterium isotope effect is a tool utilized in mechanistic studies,[5] which has been widely applied to study phosphate transfer.[6–8] Information on the proton transfer processes in or before the rate-limiting step of the reaction can be obtained, and the role of solvent molecules as nucleophiles or general acid–base catalysts can be deduced by comparing the rates of the reaction in H_2O and D_2O solutions. Medium effects can also contribute to the observed $k(H_2O)/k(D_2O)$ values.[5] Results of kinetic solvent deuterium isotope effect experiments carried out with simple phosphate esters[6] and with ribozymes[7] have been reported over the last few decades. The mechanism of the metal ion-promoted

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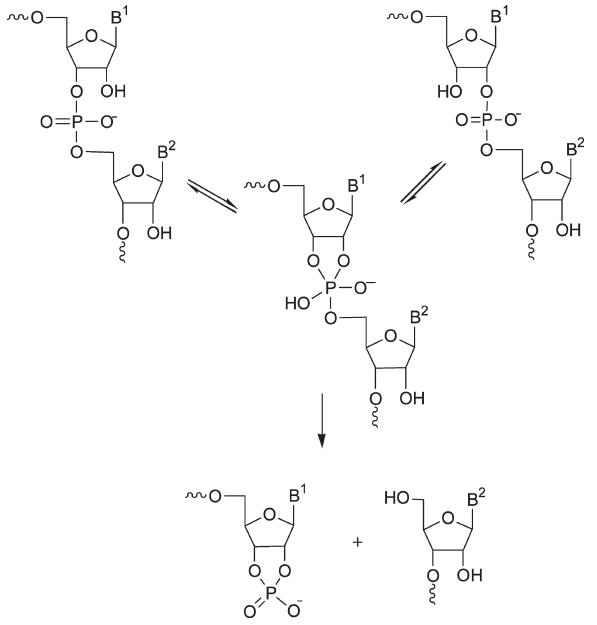
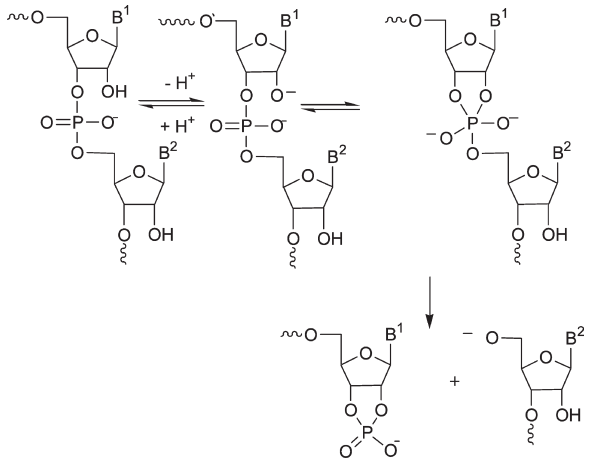


Scheme 1



Scheme 2

cleavage of phosphodiesters has also been studied by applying kinetic solvent deuterium isotope effects.[8]

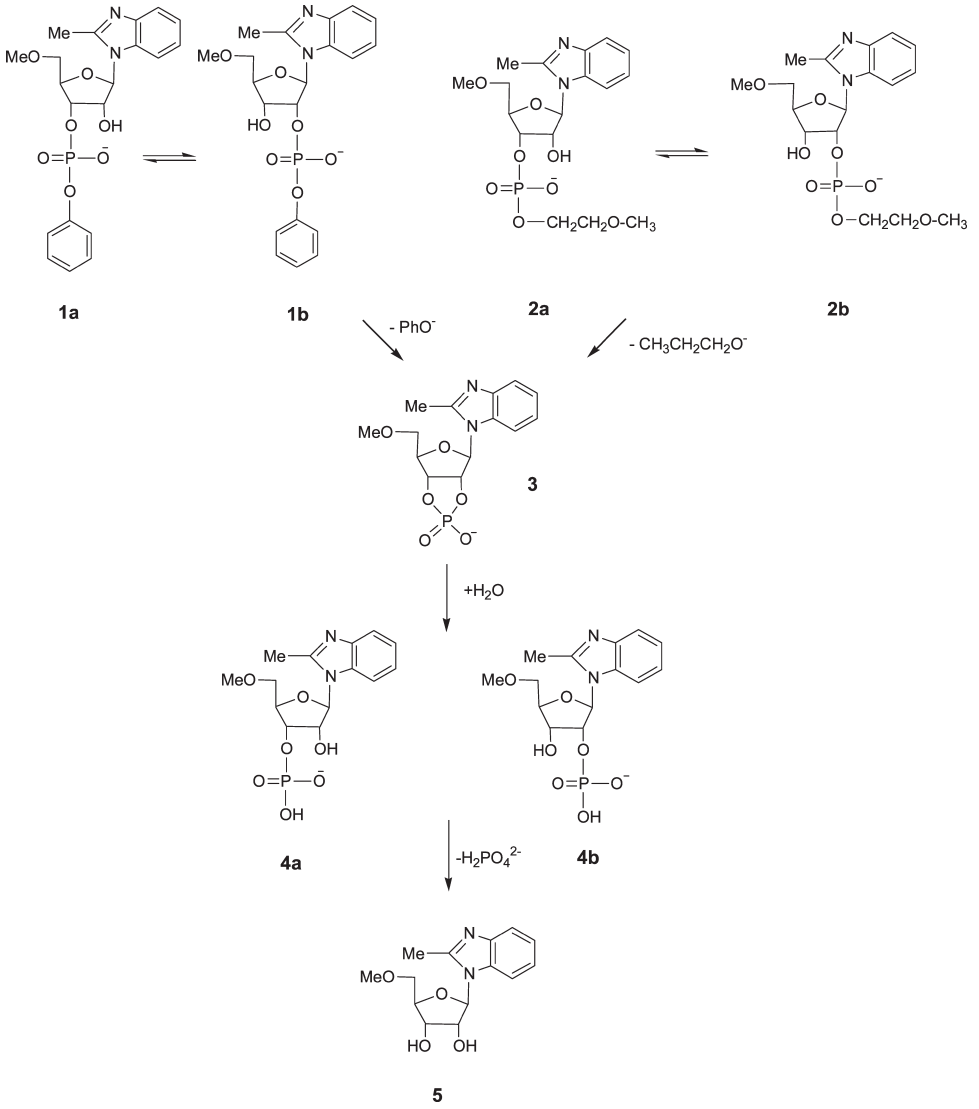
Although the solvent isotope effect is a powerful tool in mechanistic studies, the results may be difficult to interpret. With a simple well-defined system the interpretation is often straightforward and unambiguous, but as the systems studied grow more complicated, the number of plausible mechanistic alternatives increase. The cleavage of phosphodiester bonds of RNA is a very complicated system owing to the number of potential ionic forms of the ground and transition states. There are also a number of exchangeable protons in the heterocyclic nucleic acid bases, which may contribute to the overall isotope effect observed. The situation becomes even more complicated when reactions are carried out in buffer solutions and/or in the presence of metal ions, which add to the number of protolytic equilibria and proton transfer processes in the reaction system.

We have therefore started a systematic study on the kinetic solvent deuterium isotope effects in the transesterification of phosphodiester bonds of RNA. The underlying idea is to start the experiments using model systems where the number of exchangeable protons has been minimized so that any effects observed can be attributed to only a few proton transfer processes, and then increase the number of possible protolytic processes. As the first part of the project, we report here the synthesis of model compounds **1a**,**b** [1-(5′-*O*-methyl-β-D-ribofuranosyl)-2-methylbenzimidazole 2′- and 3′-phenylphosphates] and **2a**,**b** [1-(5′-*O*-methyl-β-D-ribofuranosyl)-2-methylbenzimidazole 2′-and 3′-(2-methoxyethyl)phosphates] and the results of studies on their intramolecular transesterification reactions (Scheme 3) in H_2O and D_2O solutions over a wide p*L* range. The 2-methylbenzimidazole base moiety in **1** and **2** contains no exchangeable protons and the proton transfer between the 5′-OH and solvent has been prevented by methylation. Compound **1** contains a good leaving group, which is known to depart as an aryloxy ion under neutral and alkaline conditions[9] and, therefore, the deprotonation of the 2′-OH group is the only proton transfer process involved in the alkaline cleavage. The leaving group in **2** is poorer and its departure clearly limits the observed reactivity.[10] Under neutral and acidic conditions, the alkyloxy leaving group of **2** becomes protonated on departure. Furthermore, **2** also isomerises under neutral and acidic conditions (Scheme 3), and the kinetic solvent deuterium isotope effect on this reaction was also determined. The mechanisms of the intramolecular transesterification reactions of RNA model compounds are known and, therefore, the solvent isotope effects obtained can be attributed to different acid- and base-dependent reaction steps. The data obtained therefore provide useful background information to interpret solvent isotope effects obtained with more complicated systems such as metal ion-promoted, ribozyme and enzyme-catalysed reactions.

RESULTS

Synthesis of 1a,b and 2a,b

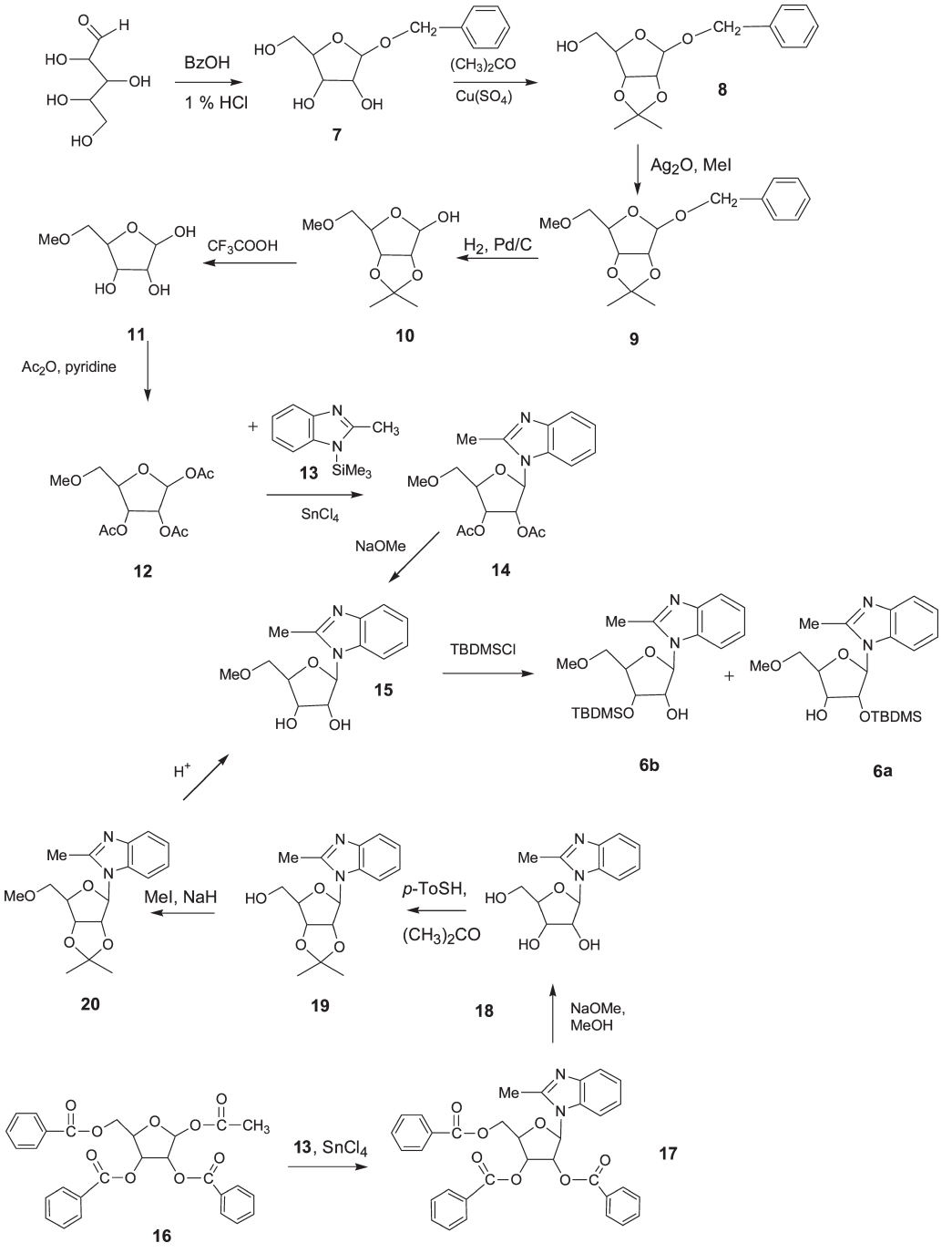
Compounds **1a**,**b** and **2a**,**b** were synthesized by phosphorylation of protected 2-methylbenzimidazole nucleosides **6a**,**b.** The synthetic routes are shown in Schemes 4–6 and a detailed description of the synthetic procedures is given as electronic supplementary material (available in Wiley Interscience). Two different routes were followed. Initially, **6a**,**b** were prepared from D-(−) ribose, which was cyclized in a reaction with benzyl alcohol in 1% HCl solution to give **7**. NMR analysis showed that the product was predominantly in the β-configuration. The 2- and 3-hydroxyl groups were protected with an isopropylidene group and the 5-hydroxyl was methylated with methyl iodide. The benzyl and 2,3-isopropylidene groups were



Scheme 3

removed with trifluoroacetic acid. The free hydroxyl groups were then acetylated with acetic anhydride in pyridine and the fully protected sugar was reacted with trimethylsilyl-protected 2-methylbenzimidazole (**13**) using $SnCl_4$ as a catalyst. After removal of the 2′- and 3′-acetyl protecting groups with NaOMe in methanol, the nucleoside was reacted with TBDMSCl in pyridine with diaminopyridine as a catalyst, which gave a mixture of 2′- and 3′- protected nucleosides **6a**,**b**. This route was initially chosen because all the reagents are inexpensive and the methods well documented in the literature. However, it is tedious owing to the large number of steps and the overall yield was low. Therefore, an alternative route was used to prepare another batch of the protected nucleoside, and **6a**,**b** were synthesized starting from 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**16**), which was reacted with silylated 2-methylbenzimidazole base **13** to prepare the corresponding nucleoside **17**. Hydrolysis of **17** in 0.4 M sodium methoxide in methanol yielded **18**, which was protected with a 2′,3′-isopropylidene group and methylated at 5′-OH. Acid-catalysed hydrolysis of the isopropylidene group of **20** gave nucleoside **15**, which was reacted with TBDMSCl using Ag_2NO_3 as a catalyst to give **6a**,**b**.

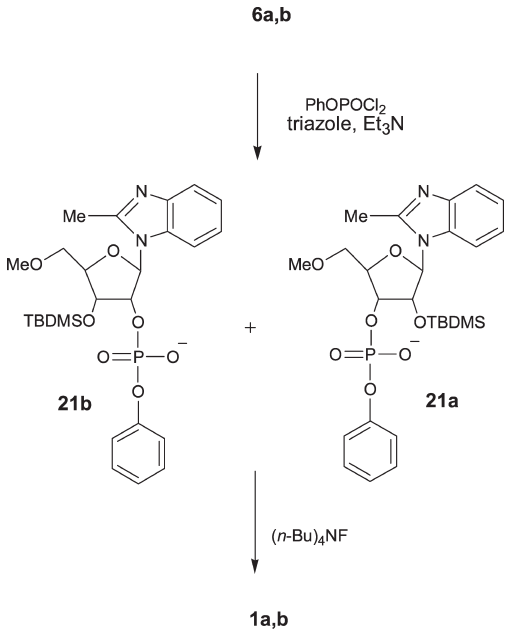
Isomers **6a** and **6b** could not be separated by silica gel chromatography so the phosphorylation reactions were carried out with the isomer mixture. Compounds **1a**,**b** were prepared by phosphorylating **6a**,**b** with phenyl phosphorodichloridate using triazole as an activator (Scheme 5). The TBDMS protecting group was removed with tetrabutylammonium fluoride in anhydrous THF. The deprotected phosphodiesters were purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Compounds **2a**,**b** were prepared by phosphorylation of **6a**,**b** with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite in anhydrous acetonitrile with tetrazole as an activator (Scheme 6). Compounds **22a**,**b** were reacted with anhydrous 2-methoxyethanol in the presence of tetrazole under an N_2 atmosphere to give **23a**,**b**. The phosphoramidite group



Scheme 4

was oxidized with iodine in a mixture of lutidine, water and THF, the cyanoethyl group removed in methanolic ammonia and the TBDMS group removed with tetrabutylammonium fluoride. The products were purified by RP-HPLC.

The final products were analysed by high-resolution mass spectrometry (HRMS) and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. Assignation of signals was supported by proton–proton and proton–carbon correlation spectra. The spectral data showed that in both cases the 2′-isomer was the predominant product. Clear splitting of the 2′-H signal due to the coupling with phosphate was observed in the ^{1}H NMR spectra. In the case of **1b** this was also verified by the proton–phosphorus correlation spectrum that showed coupling between the 2′-H and phosphate. As both isomers of the TBDMS protected nucleoside **6a**,**b** were present, it appears that the 2′-isomer **6a** is less reactive in phosphorylation reactions than its 3′-isomer **6b**. In fact, practically no 3′-isomer **2a** was formed at all and the yield of **1a** was very low. Compound **2a** was prepared by isomerizing **2b** in 0.1 M MES buffer at pH 5.5. Under these conditions the isomerization is the predominant reaction of ribonucleoside alkylphosphates,[1d] and the 3′-isomer accumulates to a significant



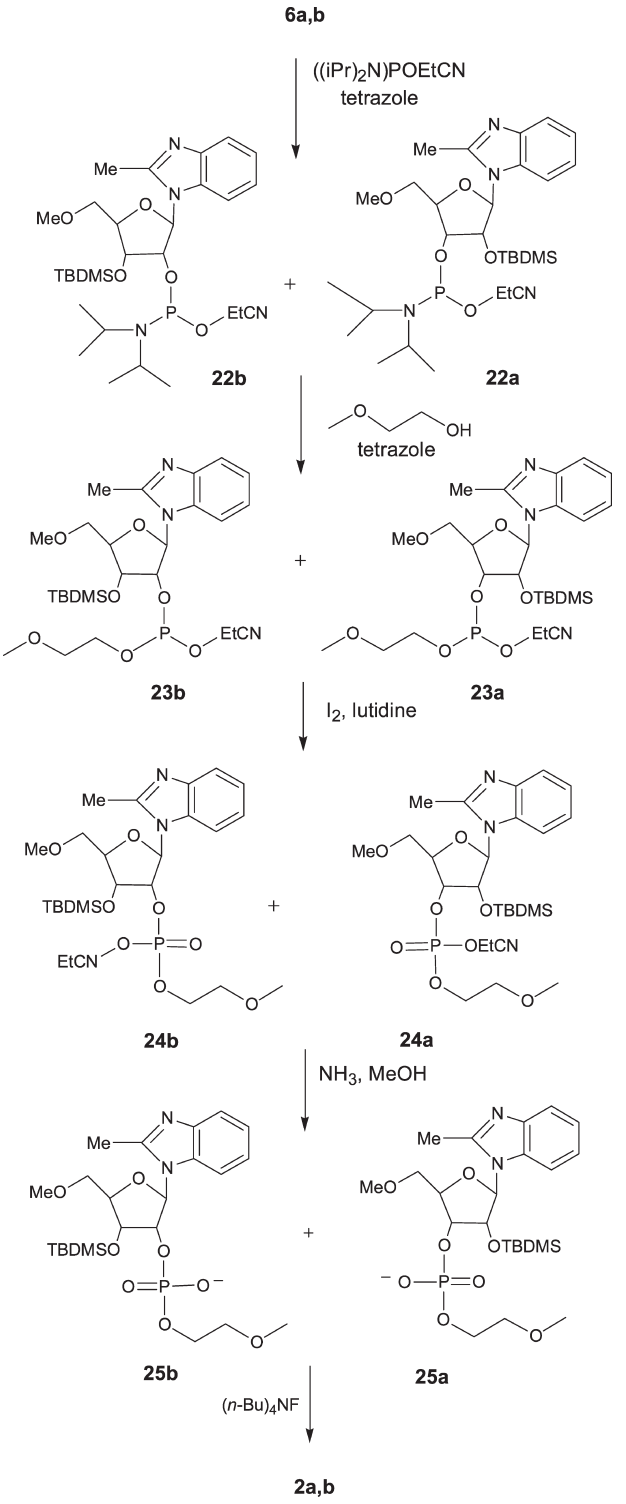
Scheme 5

extent. This method could not be used to obtain **1a**, since the cleavage of ribonucleoside arylphosphates predominates over a wide pH range, and isomerization is only observed under acidic conditions.[11] For this reason, the cleavage of 2-methylbenzimidazole arylphosphates was studied with the 2′-isomer **1b** as the substrate, whereas the 3′-isomer **2a** was used in kinetic studies of the reactions of 2-methylbenzimidazole alkylphosphates.

Kinetic experiments

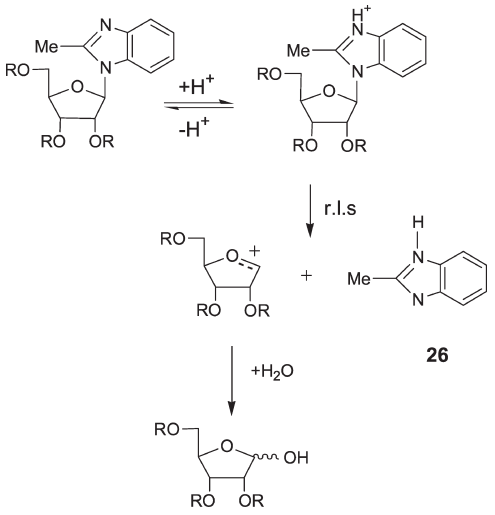
Cleavage of **1b** was followed over a p*L* range of 7–13 and the reactions of **2a** from p*L* 1 to 13. Reactions of **2a** and the cleavage of **1b** below p*L* 11 were followed by taking aliquots from a reaction solution that was thermostated to the appropriate temperature using a water bath. As is explained in more detail in the Experimental section, the aliquots were analysed by using RP-HPLC with a mixture of acetic acid buffer and acetonitrile as an eluent. UV detection at 245 nm was employed. At p$L > 11$ the cleavage of **1b** was too fast for HPLC analysis, and the reaction was followed by UV spectrophotometry. The reaction was carried out in the spectrophotometer cell thermostated at 25.0 °C and the absorbance at 287 nm was recorded. At this wavelength the 2-methylbenzimidazole base absorbs weakly, and the increase in absorbance due to the release of the phenolate product is readily detected.

The cleavage of **1b** resulted in the formation of the 2′,3′-cyclic monophosphate of 2-methylbenzimidazole nucleoside (**3**) and phenol (Scheme 3). Both products were detected by HPLC and identified by spiking with authentic samples. The cleavage of **2a** resulted in the



Scheme 6

formation of **3** and methoxyethanol, which, being non-chromophoric, was not detected in the HPLC analysis. Consistent with previous reports on the cleavage of nucleoside alkyl phosphates,[10–13] the cleavage of **2a** is slower than the subsequent hydrolysis of the cyclic phosphate product **3**, and 2′- and 3′-monophosphates of 2-methylbenzimidazole nucleoside (**4a**,**b**) were detected



Scheme 7

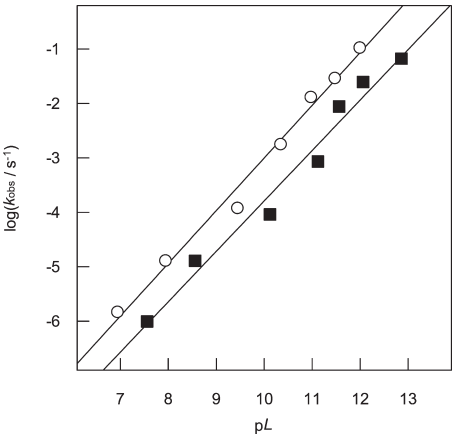


Figure 1. p*L*–rate profiles of the cleavage of **1b** to **3** and phenolate at 25 °C and $I = 1.0$ M. Open circles, cleavage in H_2O solutions; closed circles, cleavage in D_2O solutions

as the reaction products. Under neutral and acidic conditions the monophosphate products were further dephosphorylated to yield the corresponding nucleoside **5**. Below p*L* 9, isomerization of **2a** to its 2′-isomer **2b** competes with the cleavage, and between p*L* 5 and 7 it is the faster process of the two, similarly to the reaction of other ribonucleoside alkylphosphates.[10–13] Compound **1b** does not isomerize under neutral or slightly acidic conditions. As was mentioned above, isomerization of aryl phosphates has only been observed below pH 2.[14] Acid-catalysed depurination of the 2-methylbenzimidazole nucleoside in **2a** was detected between p*L* 2 and 7, and 2-methylbenzimidazole **26** was formed as a product (Scheme 7), consistent with results obtained previously with benzimidazole nucleosides.[15]

Calculation of rate constants

Rate constants for the cleavage of **1b** between p*L* 7 and 11 were calculated from the decrease of the signal area of **1b** in the chromatograms, and above p*L* 11 from the increase of the absorbance at 287 nm detected spectrophotometrically, by applying the integrated first-order rate law. In the spectrophotometric analysis, the reaction was followed for 5–10 half-lives to obtain the final absorbance value. Rate constants for the cleavage and isomerization of **2a** were calculated on the basis of mole fraction of the substrate remaining in the reaction solution using the Ufit program as described previously.[16] In cases where depurination was observed, the rate constants of cleavage obtained from Ufit were divided into contributions from phosphate cleavage and depurination by using the ratio of the products formed. The mole fractions based on signal areas were used as the basis of the calculations, since the chromophoric group is the same in the substrate and products, and the molar absorptivity is not expected to vary significantly. This assumption was verified by following the reaction spectrophotometrically at pH 3, where all the three processes contribute to the overall disappearance of the starting material. Less than 10% decrease in the total absorbance at 250 nm was observed during three half-lives of the reaction. As discussed below, the rate constants for cleavage, isomerization and depurination match those reported for similar substrates, which shows that the calculation methods are correct.

The observed rate constants for the cleavage of **1b** and the cleavage and isomerization of **2a** as a function of p*L* are shown in Figs 1, 2(a) and 2(b), respectively. The data in Fig. 1 were determined at 25 °C and those in Fig. 2(a) and (b) at 90 °C. The observed rate constants of the cleavage and isomerization of **2a** in Fig. 2(a) and (b) were fitted to Eqns (1) and (2), respectively.[12]

$$kc_{obs} = (k_a[H^+]^2/K_{a,1} + k_b[H^+]/K_{a,1} + k_c + k_d K_{a,2}/[H^+])/([H^+]/K_{a,1} + 1 + K_{a,2}/[H^+]) \quad (1)$$

$$ki_{obs} = (k_e[H^+]^2/K_{a,1} + k_f[H^+]/K_{a,1} + k_g)/(1 + [H^+]/K_{a,1}) \quad (2)$$

In Eqn (1), kc_{obs}, k_a, k_b, k_c and k_d are the observed first-order rate constant for the cleavage of **2a**, the second-order rate constant for the acid-catalysed cleavage of neutral **2a** and the first-order rate constants for the cleavage of neutral, monoanionic and dianionic form of **2a**. In Eqn (2), ki_{obs}, k_e, k_f and k_g are the observed first-order rate constant for isomerization of **2a** to **2b**, the second-order rate constant for the acid-catalysed isomerization of neutral **2a** and the first-order rate constants for isomerization of neutral and monoanionic form of **2a**. $K_{a,1}$ and $K_{a,2}$ are the equilibrium constants for deprotonation of the phosphate and 2′-hydroxy groups, respectively.

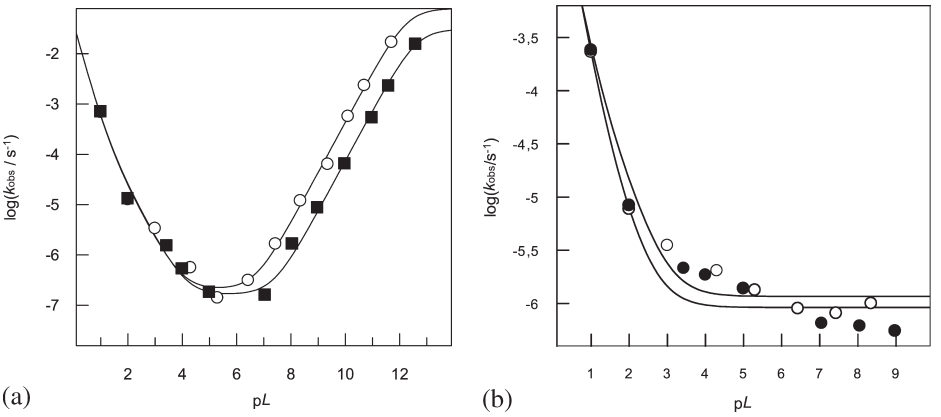


Figure 2. (a) p*L*–rate profiles of the cleavage of **2a** to **3** and methoxyethanol at 90 °C and $I = 1.0$ M. Open circles, cleavage in H_2O solutions; closed circles, cleavage in D_2O solutions. (b). p*L*–rate profiles of the isomerization of **2a** to **2b** at 90 °C and $I = 1.0$ M. Open circles, isomerization in H_2O solutions; closed circles, isomerization in D_2O solutions

Adjusting the p*L* of the reaction solutions

Under acidic conditions, p*L* was adjusted with HCl or DCl, and under alkaline conditions with NaOH or NaOD. Between p*L* 3 and 10 the lyonium ion concentration was maintained with an appropriate buffer. Varying the buffer concentration did not have any effect on the observed rate constants, and the rate constants refer to a total buffer concentration of 0.1 M. The ionic strength was maintained at 1.0 M with NaCl or $NaNO_3$. The p*L* values under the experimental conditions were calculated using the data available for the effects of temperature and ionic strength on the pK_a values of the buffer acids and on the water autoprotolysis constant pK_w in H_2O and D_2O. The calculations performed are explained in detail in the Experimental section and the pK_a and pK_w values used are given in Table 1. Even though in some cases the calculation of the p*K* values is based on rather extensive extrapolation, the facts that the rate constants obtained in different buffers are consistent with each other, both in H_2O and D_2O, with those reported before for other ribonucleoside alkylphosphates, and that the solvent isotope effects calculated under acidic and neutral conditions are independent of the buffer used, suggest that the p*K* values calculated are reliable.

Table 1. pK_a values of buffers and values of autoprotolysis constants of water used to calculate the p*L* values of reaction solution (all values refer to $I = 1.0$ M)

Parameter	H_2O, 25 °C[a]	H_2O, 90 °C[a]	D_2O, 25 °C[a]	D_2O, 90 °C[a]
pK_a formate		3.74		4.13
pK_a acetate		4.67		5.19
pK_a MOBS		6.82		7.44
pK_a HEPES	7.45		8.07	
pK_a CHES	9.75		10.43	
pK_a glycine		8.33		8.96
pK_a Et_3N	10.99		11.58	
pK_w	13.727	12.102	14.683	12.984

[a] References and detailed descriptions of the calculations performed can be found in the text.

DISCUSSION

The rate constants shown in Figs 1, 2(a) and 2(b) are consistent with previous data on the reactivity of ribonucleoside aryl and alkyl phosphates. The p*L*–rate profiles of the cleavage and isomerization of **2a** are similar to those obtained previously with 3′,5′-UpU[12] and with adenosine[11] and uridine[10,13] 3′-alkylphosphates. The cleavage of **2a** shows a first-order dependence on hydroxide ion concentration above p*L* 7 and on hydronium ion concentration below p*L* 4. Between p*L* 1 and 2, a second-order dependence on hydronium ion concentration is observed. Consistent with previous reports, isomerization is not base-catalysed, but this reaction is independent of p*L* above p*L* 5. Above p*L* 9, the cleavage is much faster than isomerization and the rate constants for isomerisation cannot be obtained. The cleavage of **1b** is much faster owing to the presence of a good aryloxy leaving

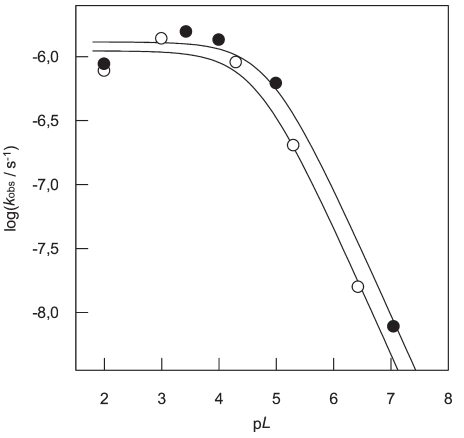


Figure 3. p*L*–rate profiles of the depurination of **2a** at 90 °C and $I = 1.0$ M. Open circles, depurination in H_2O solutions; closed circles, depurination in D_2O solutions

group, and isomerization of this compound cannot be detected under the experimental conditions.[9,14,17] The rate constants of acid-catalysed depurination of **2a** (Fig. 3) are fully consistent with data on the depurination of other benzimidazole nucleosides.[15] The observed rate constants can be fitted to a reaction scheme where the only active ionic form is the protonated base. The kinetic pK_a is 4.6, which in good agreement with the pK_a of 4.0 at 393 K.[15]

Solvent isotope effects on the alkaline cleavage of 1b and 2a

The solvent isotope effects on the alkaline cleavage of **1b** and **2a** were first calculated on the basis of the second-order rate constants of hydroxide/deuteroxide ion-catalysed cleavage (k_{OL}) obtained from the slopes of k_{obs} vs [LO^-] plots using the linear part of the p*L*–rate profiles above p*L* 7. The $k_{OH}(HO^-)$ and $k_{OD}(DO^-)$ values for the cleavage of **1b** were 10.4 ± 0.2 and 2.1 ± 0.1 mol dm^{-3} s^{-1}; for the cleavage of **2a**, $k_{OH} = 1.61 \pm 0.03$ and $k_{OD} = 0.22 \pm 0.01$ mol dm^{-3} s^{-1}. These data give solvent isotope effects of 4.9 ± 0.2 for **1b** and 7.2 ± 0.2 for **2a**. The value obtained with **1b** is reasonably consistent with the value of 4.01 reported for the alkaline cleavage of 2-hydroxypropyl-*p*-nitrophenyl phosphate.[8d] As the alkaline cleavage of RNA involves deprotonation of the nucleophilic OH group in a pre-equilibrium before attack at the phosphate, the values obtained consist of contributions from solvent isotope effects on the pK_a of the attacking nucleophile and on the cleavage reaction of the dianionic substrate.

pK_a values for similar OH nucleophiles have been determined kinetically by measuring the rate constants for the cleavage at pHs where the deprotonation of the hydroxy group approaches completion.[12] Saturation is not observed with either of the substrates studied in the present work, suggesting that the 2′/3′-OH groups of the benzimidazole nucleoside are more basic than those in natural nucleosides, and consequently the ΔpK_a [$pK_a(D_2O) - pK_a(H_2O)$] could not be reliably determined in the present case. An estimate was obtained from the non-linear fit according to Eqn (1) as discussed below. The $k_{OH}(HO^-)/k_{OH}(DO^-)$ value of 4.9 obtained with **1b** is probably entirely due to this difference in the deprotonation of the attacking OH group of the 2′-methylbenzimidazole nucleoside. This suggestion is based on the fact that the cleavage of nucleoside arylphosphates under neutral and alkaline conditions results in a release of aryl oxyanion.[9] Once the attacking OH group is deprotonated, there is no other proton transfer between the solvent and the substrate and, consequently, no other source of primary isotope effect. The solvent isotope effect for the attack of a fully ionized oxyanion on a diester is 0.9 with the *p*-nitrophenyl ester of 3′-TMP,[6i] 0.8 with *cis*-4-hydroxytetrahydrofuran 3-phenylphosphate[6c] and 1.04 with dialkyl 2-carboxyphenyl phosphates. If the $k_{OH}(HO^-)/k_{OH}(DO^-)$ value of 4.9 is completely attributed to the deprotonation of the attacking nucleophile, a ΔpK_a of 0.69 can be calculated, which is consistent with the ΔpK_a value of 0.63 estimated for the *cis*-hydroxyl group of *cis*-4-hydroxytetrahydrofuran 3-phosphate.[6c]

The alkaline cleavage of nucleoside alkylphosphates differs from the cleavage of corresponding arylphosphates in that the departure of the leaving group more clearly determines the rate of the reaction. The β_{lg} values of the alkaline cleavage of uridine 3′-aryl and alkylphosphates are -0.54[9] and -1.10,[10] respectively. The highly negative value obtained with alkylphosphates has been suggested to support a mechanism where the departure of the alkyloxy leaving group is far advanced in the transition state.[1e,10] In contrast, the modestly negative β values obtained with uridine 3′-arylphosphates are consistent with a reaction proceeding through a symmetric transition state with bond formation to the incoming nucleophile and the bond cleavage of departing aryloxyion being similarly advanced.[9] Alternatively, these data have been interpreted in terms of a change in rate-limiting step, with the formation of a dianionic phosphorane rate limiting for aryloxy leaving groups and its breakdown for alkoxy leaving groups.[4] In either case, the transition states will be substantially different and the mechanistic interpretation does not change the conclusions about the origin of the solvent isotope effects observed. As the solvent isotope effect on the pre-equilibrium deprotonation of the attacking OH group can be assumed to be similar in **1b** and **2a**, the difference in the apparent $k_{OH}(HO^-)/k_{OH}(DO^-)$ values show that a reaction involving a release of an alkyloxy leaving group is more sensitive to the isotopic composition of the solvent and an additional isotope effect of 1.5 needs to be accounted for in the reaction involving an alkyl leaving group.

It must be noted that the attacking nucleophiles in **1b** and **2a** are not in the same position and their pK_a values are expected to be different.[12] When the reactivities of **1a** and **1b** are compared, it is observed that the cleavage of **1a** is approximately four times faster than that of **1b** (data not shown), suggesting that the pK_a of the 2′-OH is lower than that of 3′-OH by $\sim$0.6 pK_a units (assuming that the fully ionized forms have identical reactivity). Differences of the same magnitude have also been obtained with dinucleoside monophosphates.[12] However, as the reactivity difference between **1a** and **1b** is the same in H_2O and D_2O, the observed solvent isotope effects must also be the same. Therefore, even though different absolute values of $k_{OH}(HO^-)$ and $k_{OH}(DO^-)$ would have been obtained with **1a**, the ΔpK_a value obtained by the equivalent analysis would have been the same as those obtained with **1b**.

The rate constants obtained for the reaction of dianion **2a** from the non-linear fits are not sufficiently accurately defined owing to the lack of data at very high p*L* to draw firm conclusions, but the same 1.5-fold isotope effect on

the cleavage is observed. Similarly, the ΔpK_a value (0.54) obtained from the fits is consistent with the value obtained as the ratio of the second-order rate constants of the alkaline cleavage of **1b** (0.69). The $pK_{a,2}$ values obtained are 12.06 and 12.60 in H_2O and D_2O, respectively, and as discussed above, the pK_a value of the 2′-OH group of **2a** in H_2O is slightly higher than the corresponding pK_a value in 3′,5′-UpU (11.5 at 90 °C[12]).

The origin of the additional solvent isotope effect of 1.5 observed in the cleavage of **2a** is not clear. As shown in Fig. 2(a) and reported by several authors previously, the cleavage of nucleoside 3′-alkylphosphates shows a first-order dependence on hydroxide ion concentration. No kinetically significant protonation/deprotonation step, other than the deprotonation of the attacking nucleophile, can take place in the cleavage reaction, since this would result in a different dependence on hydroxide ion concentration. Therefore, the additional effect has to be attributed to the properties of the phosphorane intermediate/transition state. Medium effects, such as differences in solvation, can contribute significantly to observed solvent isotope effect[5,18] and the 1.5-fold difference could result from a poorer hydrogen bond stabilization of the polar transition state in D_2O than in H_2O. Consistent with this suggestion, $k(H_2O)/k(D_2O)$ values from 1.1 to 1.8, which have been attributed to medium effects, have been reported for the nucleophilic reactions of carboxylic acid esters.[18] The fact that such an effect is reduced in the cleavage of the arylphosphate **1b** can be explained by the lower polarity of the transition state: as the charge on the leaving group in the transition state for the cleavage of an alkylphosphate is more developed, the charge is less dispersed than in the case of arylphosphates, and so hydrogen bonding with solvent may be more important than in the case of the arylphosphate **1b**. The solvent isotope effect values reported for the cleavage of arylphosphates[6c,d,i] by fully ionized intramolecular nucleophiles are close to unity, which supports the suggestion that these medium effects play a minor role in the case of arylphosphates.

The apparent solvent isotope effects obtained with **1b** and **2a** in this work are very similar to those obtained in hammerhead ribozyme reaction in the presence of Mg^{2+} and NH_4^+ ions (4.4[7a] and 7.69,[7e] respectively). Therefore, it is tempting to speculate about the mechanism of the hammerhead cleavage on the basis of the present results. Since the $k_{obs}(H_2O)/k_{obs}(D_2O)$ value is similar to that obtained with **1b**, it may be that Mg^{2+} ions promote the hammerhead reaction mainly by stabilizing the phosphorane and/or the leaving group, thereby making the phosphorane resemble that in the cleavage of an arylphosphate. The solvent isotope effects obtained do not need to be explained by catalysis of the deprotonation of the 2′-OH by Mg^{2+}, but could simply reflect the spontaneous deprotonation of the attacking 2′-nucleophile. As the $k_{obs}(H_2O)/k_{obs}(D_2O)$ value obtained in the presence of NH_4^+ ions is very close to that obtained in this work with **2a**, the NH_4^+ ion might not enhance the hammerhead reaction by proton transfer to the leaving group, but let it depart without catalysis.

Solvent isotope effects under neutral and acidic conditions

In contrast to the alkaline cleavage, no significant solvent isotope effect was observed in the cleavage and isomerization of **2a** under acidic conditions, as is shown by the results in Fig. 2(a) and (b) and Table 2. The second-order rate constants of the acid-catalysed cleavage (kc_H in Table 2) and isomerization (ki_H) of **2a** give isotope effects of 0.9 and 1.0, respectively. Similar values are obtained from the rate constants of different partial reactions obtained from the non-linear fit according to Eqns (1) and (2) collected in Table 2.

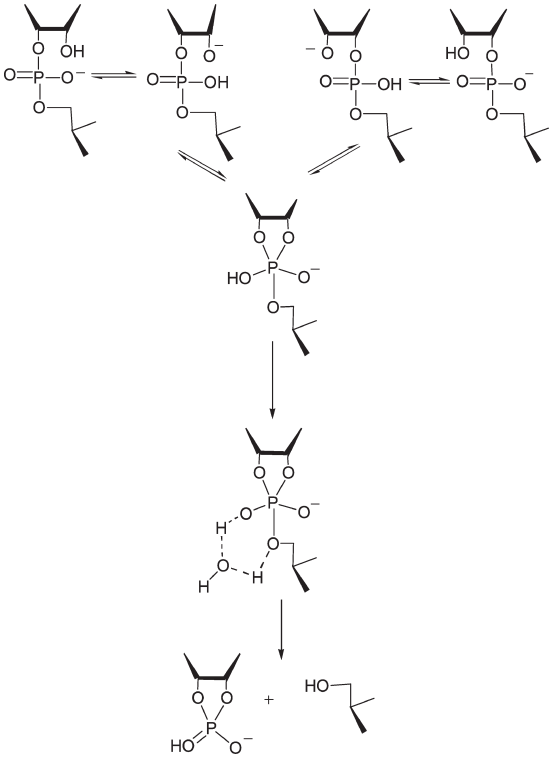
Practically no solvent isotope effect on the spontaneous cleavage and isomerization of monoanionic **2a** was observed either. The rate constants of spontaneous reactions (k_c and k_g in Table 2) obtained by the non-linear fits of Eqns (1) and (2) give solvent isotope effects of 1.3 and 1.2 for cleavage and isomerization, respectively.

The similarity of the $k(H_2O)/k(D_2O)$ values for the cleavage and isomerization reactions obtained under neutral conditions is slightly surprising, since the mechanisms of these reactions are different. Both reactions take place via the same monoanionic phosphorane intermediate,[1d,e] which is formed via a minor tautomer with a deprotonated 2′-oxy group and a protonated phosphate (Scheme 8).[14] However, the second steps, which are rate-limiting, at least in the cleavage reaction, involve differing proton transfers. The second step of the isomerization does not require protonation of the leaving group as the endocyclic leaving group can depart as an oxyanion, whereas exocyclic cleavage requires protonation of the leaving group oxygen (Scheme 8).[1e] Therefore, the cleavage reaction involves a proton transfer from the phosphorane to the leaving group, which probably takes place simultaneously with the departure of the leaving group, while isomerization involves no such protonation. Hence, different solvent isotope effects may be expected. It is possible that the intramolecular proton transfer proposed to take place in the second step of the cleavage reaction, even if it is concerted with the departure of the leaving group, need not result in an observable solvent isotope effect, but that the effects of proton transfer from the phosphate and to the leaving group compensate each other. Supporting this contention are several reports of $k(H_2O)/k(D_2O)$ values close to zero for the dephosphorylation of various different phosphate esters.[19] All these reactions involve a proton transfer from a monoanionic phosphate to the leaving group in a pre-equilibrium or concertedly to form a zwitterionic species with a dianionic phosphate and a protonated leaving group. Therefore, the similar and small $k(H_2O)/k(D_2O)$ values of the

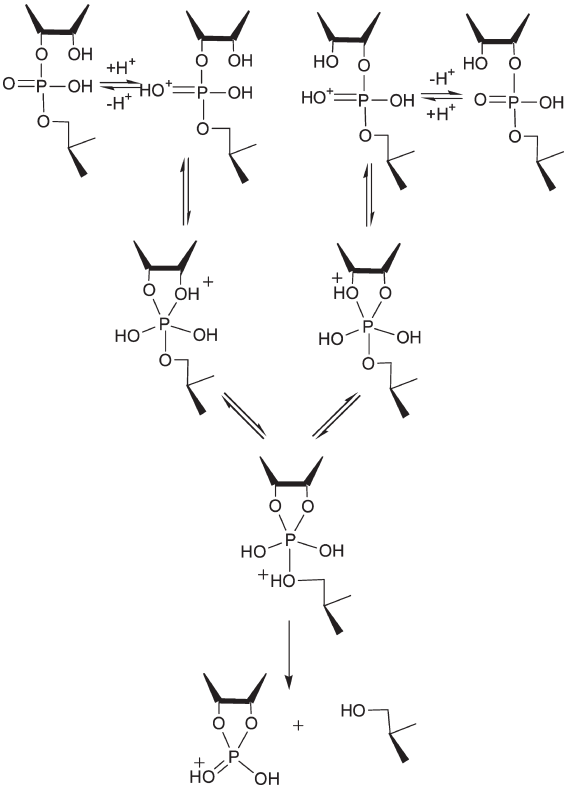
Table 2. Kinetic solvent deuterium isotope effects of intramolecular transesterification of the phosphodiester bond of **1b** and **2a**

Parameter	In H_2O	In D_2O	$k(H_2O)/k(D_2O)$
k_{HO}(**1b**) ($dm^3\,mol^{-1}\,s^{-1}$)[a]	10.4 ± 0.2	2.1 ± 0.1	4.94
k_{HO}(**2a**) ($dm^3\,mol^{-1}\,s^{-1}$)[b]	1.61 ± 0.03	0.223 ± 0.005	7.24
kc_H(**2a**) ($dm^3\,mol^{-1}\,s^{-1}$)[c]	$(6.7 \pm 0.4) \times 10^{-3}$	$(7.3 \pm 0.4) \times 10^{-3}$	0.92
ki_H(**2a**) ($dm^3\,mol^{-1}\,s^{-1}$)[d]	$(2.30 \pm 0.07) \times 10^{-3}$	$(2.40 \pm 0.07) \times 10^{-3}$	1.00
k_d(**2a**, dianion) (s^{-1})[e]	0.045 ± 0.037	0.030 ± 0.034	1.5
k_c(**2a**, monoanion) (s^{-1})[f]	$(1.7 \pm 0.8) \times 10^{-7}$	$(1.3 \pm 0.8) \times 10^{-7}$	1.3
k_b(**2a**, neutral) (s^{-1})[g]	0.16 ± 0.06	0.16 ± 0.06	1.0
k_a(**2a**, neutral) ($dm^3\,mol^{-1}\,s^{-1}$)[h]	3 ± 2	3 ± 2	1.0
k_g(**2a**, monoanion) (s^{-1})[i]	$(1.2 \pm 0.3) \times 10^{-6}$	$(1.0 \pm 0.3) \times 10^{-6}$	1.2
k_f(**2a**, neutral) (s^{-1})[j]	0.1 ± 0.1	0.1 ± 0.1	1.0
k_e(**2a**, neutral) ($dm^3\,mol^{-1}\,s^{-1}$)[k]	1 ± 2	2 ± 4	1.0

[a] Second-order rate constant of base-catalysed cleavage of **1b** at 25 °C. Obtained as a slope of plot k_{obs} vs $[LO^-]$.
[b] Second-order rate constant of base-catalysed cleavage of **2a** at 90 °C. Obtained as a slope of plot k_{obs} vs $[LO^-]$.
[c] Second-order rate constant of acid-catalysed cleavage of **2a** at 90 °C. Obtained as a slope of plot k_{obs} vs $[L_3O^+]$.
[d] Second-order rate constant of acid-catalysed isomerization of **2a** at 90 °C. Obtained as a slope of plot k_{obs} vs $[L_3O^+]$.
[e] Rate constant of the cleavage of dianionic **2a** at 90 °C. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (1).
[f] Rate constant of the spontaneous cleavage of monoanionic **2a** at 90 °C. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (1).
[g] Rate constant of the cleavage of neutral **2a** at 90 °C. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (1).
[h] Second-order rate constant of acid-catalysed cleavage of neutral **2a**. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (1).
[i] Rate constant of the spontaneous isomerization of monoanionic **2a** at 90 °C. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (2).
[j] Rate constant of the isomerization of neutral **2a** at 90 °C. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (2).
[k] Second-order rate constant of the acid-catalysed isomerization of neutral **2a** at 90 °C. Obtained by a non-linear fit of k_{obs} vs $[H^+]$ according to Eqn (2).



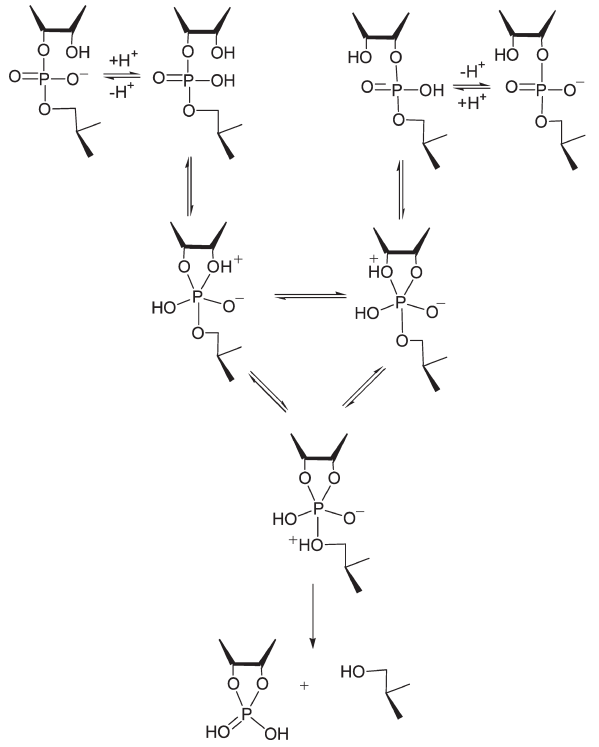
Scheme 8



Scheme 9

cleavage and isomerization observed under neutral conditions can be explained most plausibly by suggesting that neither step of the two reactions is characterized by any significant solvent isotope effect.

The results obtained under acidic conditions are slightly more difficult to explain. The cleavage and isomerization occur via two different routes, showing second- and first-order dependences on hydronium ion concentration. The second-order dependence refers to the reaction that proceeds via a cationic phosphorane formed by a nucleophilic attack of a neutral 2′-OH on the doubly protonated phosphate (rate constants k_a for cleavage and k_e for isomerization in Table 2) (Scheme 9). In the reaction showing a first-order dependence, the phosphate



Scheme 10

and the phosphorane are neutral (k_b and k_f) (Scheme 10). In both cases, a prototropic arrangement that places a proton on the leaving group oxygen takes place in the second step of the reaction, and the leaving group departs as an alcohol. As can be seen in Schemes 9 and 10, the reactions involve protonation of the phosphate. As the pK_a of the phosphate is higher in D_2O than in H_2O,[20] the proportion of the protonated form of the phosphate is higher and, therefore, D_2O solution favours the formation of the intermediate. If, as above, the intramolecular proton transfer processes taking place in the second step of isomerization and cleavage do not result in an observable solvent isotope effect, then an inverse solvent isotope effect would have been expected. No such effects are observed and the $k(H_2O)/k(D_2O)$ values obtained under acidic conditions were close to unity. However, the suggestion that the intramolecular proton transfer does not result in a solvent isotope effect is based on observations obtained with phosphomonoesters, where a species with a dianionic phosphate and a protonated leaving group is formed. A similar species is formed with **2a** under neutral conditions, but it may be that these results cannot be extrapolated to reactions where the proton transfer takes place between initially neutral species. Such a proton transfer might be characterized by a $k(H_2O)/k(D_2O)$ value higher than unity which compensates for any effects on ground-state protonation.

Plots of observed rate constants of depurination vs $[H^+]$ give second-order rate constants of 0.020 and 0.062 mol dm^{-3} s^{-1} in H_2O and D_2O, respectively. The apparent $k(H_2O)/k(D_2O)$ of 0.32 is consistent with the mechanism of the acid-catalysed hydrolysis of the *N*-glycosidic bond (Scheme 7). The rate-limiting step is the unimolecular bond rupture of the base-protonated substrate, which results in the formation of 2-methylbenzimidazole base and ribosyl carbocation.[14] Water attacks the carbocation in a subsequent step, and the nucleophilic attack does not contribute to the observed solvent isotope effect. The clear inverse solvent isotope effect is most simply attributed to the pre-equilibrium protonation of the substrate, which is favoured in D_2O.

CONCLUSIONS

The alkaline cleavage of nucleoside phosphodiesters is characterized by large apparent solvent deuterium isotope effect values. In the case of phosphodiesters with an aryl leaving group, the effect observed can be accounted for satisfactorily by the equilibrium effect on the ΔpK_a of the attacking nucleophile. When the leaving group is an alkyloxy function, an additional 1.5-fold isotope effect is observed, which may be due to the requirement for better solvation of the more highly polarized transition state. In contrast to alkaline cleavage, cleavage and isomerization under neutral and acidic conditions showed no significant solvent isotope effects which probably result from very small effects and from opposite effects involved in different steps of the reaction that compensate each other, respectively.

EXPERIMENTAL

General

Acetonitrile, pyridine and THF were refluxed with NaH, and acetone with Na_2SO_4, and then distilled. 1,2-Dichloroethane was refluxed with PO_5. DMF and xylene were dried with molecular sieves. Triazole was purified by recrystallization from ethanol. D_2O (99.97% D) and D_3COOD (99.5% D) were products of Eurisotop and DCOOD (98% D), NaOD (40 wt% in D_2O, 99% D) and DCl (20% in D_2O, 99.5% D) of Aldrich. Quarz-distilled water was used to prepare the reaction solutions. Buffer constituents were of analytical grade and other reagents of at least reagent grade. HPLC-grade acetonitrile was used in HPLC eluents. Details of the syntheses of **1a**,**b** and **2a**,**b** are given in the electronic supplementary material available in Wiley Interscience.

Preparation of buffers and calculation of pK_a values

All the reaction solutions were in freshly distilled water and buffers were prepared just before the experiments

to avoid CO_2 absorption. The pH of acidic solutions was adjusted with a known concentration of HCl or DCl. Under alkaline conditions, NaOH or NaOD was used. pH values between 2 and 10 were obtained with appropriate buffer systems. Formate, acetate, HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(ethanesulfonic acid), **27**], MOBS [4-(*N*-morpholine)butanesulfonic acid, **28**], CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid, **29**], glycine, triethylamine were employed. Their pK_a values under the experimental conditions were calculated as explained below. The values used are given in Table 1. The pH of the reaction solutions was checked with a pH meter, but the lyonium ion concentrations given are based on calculated pK_a values. Formate and acetate buffers were available in a deuterated form. In other cases, the deuterated form was prepared by evaporating the protiated form three times from D_2O. D_2O solutions were kept in a desiccator after preparation.

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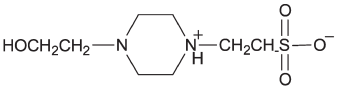

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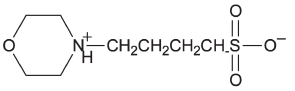

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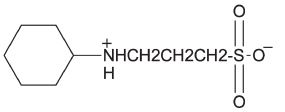

The pL values of reaction solution were calculated using pK_w and pK_a values referring to the experimental conditions. Using the data on the effects of temperature and ionic strength on the activity coefficients,[21] pK_w values of H_2O at $I = 1.0$ M of 13.727 and 12.102 were calculated at 25 and 90 °C. The ionic product of D_2O and its temperature dependence at $I = 0$ M are known.[22] Assuming that the ΔpK_w value $pK_w(D_2O) - pK_w(H_2O)$ is independent of ionic strength, pK_w values of D_2O at $I = 1.0$ M at 25 and 90 °C of 14.683 and 12.984, respectively, were calculated.

The effects of temperature[23]and ionic strength[24] on the pK_a of formic acid are known, and a value of 3.74 at 90 °C and at ionic strength of 1.0 M was calculated. A ΔpK_a $[pK_a(D_2O) - pK_a(H_2O)]$ of 0.394 has been reported[25] and assuming that the effects of temperature and I are the same as in H_2O, a pK_a of 4.13 can be calculated for DCOOD in D_2O at 90 °C and at ionic strength of 1.0 M. The pK_a values of acetic acid and CD_3COOD at 90 °C were calculated by using the known temperature dependence[26] and the effect of the ionic strength was calculated by using the effect reported at 20 °C,[27] assuming that it is the same in both cases. pK_a Values of CHES (9.75[28]) and HEPES (7.45[29]) at $I = 1.0$ M were calculated using data found in the literature. The effect of I on the pK_a value of MOBS was assumed to be similar to that on the pK_a of HEPES. This was considered a valid approximation, since HEPES (**27**) and MOBS (**28**) are structurally very similar. MOBS was the only sulfonic acid derivative used at 90 °C, and the pK_a value at 90 °C of 6.82 was calculated using the temperature dependence reported.[30] pK_a values of MOBS and CHES in D_2O were calculated by using ΔpK_a values reported for morpholine and cyclohexylammonium, respectively.[31] The approximation is assumed to be valid since the deprotonation equilibria in MOBS (**28**) and and CHES (**29**) take place at the nitrogen atom of the zwitterionic form. The ΔpK_a reported for morpholine was used also to calculate the pK_a of HEPES in D_2O. Values of 7.44 (90 °C), 10.43 (25 °C) and 8.07 (25 °C) were calculated for the pK_a of MOBS, CHES and HEPES, respectively, in D_2O at $I = 1.0$ M. The effects of temperature[32] and ionic strength[32,33] on the pK_a of glycine are known, and a value of 8.33 was calculated at 90 °C and $I = 1.0$ M. A ΔpK_a of 0.63 has been reported for glycine[34] and assuming that it is independent of temperature and ionic strength, a pK_a of 8.96 was calculated for glycine in D_2O at 90 °C and $I = 1.0$ M. The pK_a of triethylamine at $I = 1.0$ M at 25 °C has been reported (10.99[29]). The pK_a in D_2O at 25 °C (11.58) was calculated using ΔpK_a reported for trimethylammonium ion.[35]

HPLC and spectrophotometric methods

All reactions, except for those followed spectrophotometrically, were carried out in stoppered tubes that were thermostated to 90.0 ± 0.1 or 25.0 ± 0.1 °C in a waterbath. Reactions were initiated by adding a small volume of substrate stock solution to the thermostated reaction solution to make a final concentration of 0.1 mM or less. The 10–15 aliquots of 150 µl were withdrawn to cover 1–3 half-lives of the reaction, and the reactions were quenched by keeping the samples in an ice-bath. Acidic samples were neutralized with a small volume of sodium acetate solution and basic samples with acetic acid solution. In cases where the samples were not analysed immediately, they were kept at −20 °C until the analysis. The samples were analysed by RP-HPLC using a Hypersil ODS C18 (reactions of **1b**) or Aquasil C18 column (reactions of **2a**). Both columns were obtained from ThermoHypersil-Keystone. The dimensions of the columns were 150×4 mm i.d., 5 µm particle size. The eluents were mixtures of 0.06 M acetic acid buffer (pH 4.3, containing 0.1 M NH_4Cl) and acetonitrile. The acetonitrile content was 13 and 8% in the reactions of **1b** and **2a**, respectively. Signals were detected by a UV detector at 245 nm. Cleavage of **1b** at pH >11 was carried out in the spectrophotometer cell thermostated to 25.0 ± 0.1 °C. The absorbance at 287 was detected and the reaction was followed until the absorbance no longer increased.

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