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Transesterification of an RNA model in buffer solutions in H_2O and D_2O

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ABSTRACT: Transesterification of a phosphodiester bond of RNA models has been studied in various buffer solutions, under neutral and slightly alkaline conditions in H_2O and D_2O. The results show that imidazole is the only buffer system where a clear buffer catalysis on the cleavage of a phosphodiester bond is observed. The rate enhancement in sulphonic acid buffers is smaller, and a sulphonate base, particularly, is inactive as a catalyst. The rate-enhancing effect of imidazole is, however, catalytic, and the catalytic inactivity of sulphonate buffers can be attributed to their structure and/or charge. The catalysis by imidazole is a complex system which, in addition to first-order reactions, involves a process that shows a second-order dependence in imidazole concentration. The latter reaction becomes significant in acidic imidazole buffers ($pH < pK_a$), as the buffer concentration increases. The kinetic solvent deuterium isotope effect k_H/k_D, referring to first-order catalysis by imidazole base, is 2.3 ± 0.3. That referring to second-order catalysis is most probably much larger, but an accurate value could not be obtained. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: cleavage of RNA; general acid base catalysis; kinetic solvent deuterium isotope effect; buffer solutions

INTRODUCTION

We have recently started a project to determine kinetic solvent deuterium isotope effects, typical for RNA transesterification reactions, under various conditions.[1] The underlying idea of these studies is to start from reactions of simplest possible substrates with a limited number of exchangeable protons. Results obtained with such simplified substrates are easier to interpret, and proceeding systematically from simple towards more complex systems, reliable reference values for studies with biologically more relevant compounds, can be obtained.

The aim of the present work is to determine the kinetic solvent deuterium isotope effects of general acid and base-catalyzed cleavage and isomerization of phosphodiester bonds of RNA. The project has two aims. One is to provide k_H/k_D values, as a reference for mechanistic studies with more complex systems. Catalysis by RNase A, for example, involves a general acid/base catalysis by two histidine moieties, and the cleavage of phosphodiesters in imidazole has been extensively studied to model the reaction.[2] More recently, general acid/base catalysis has been identified as one of the factors that may contribute to the efficient catalysis by ribozymes, where nucleic acid bases are believed to act as proton donors/acceptors.[3]

The other aim is to study further the details of the mechanism of general acid/base catalysis, which despite the extensive research, is not fully understood. The general acid/base-catalyzed reaction in neutral solutions has proved to be a surprisingly complex system, which probably involves at least two parallel buffer-dependent reactions, the proportion of which depends on reaction conditions.[2e] The interpretation of results is further complicated by the fact that the buffer catalysis is very modest, and in the concentrated buffer solutions required, the reaction rates are affected by medium effects.

The substrate chosen for the studies is a 2-methylbenzimidazole nucleoside alkylphosphate (**1a** in Scheme 1), where the number of exchangeable protons has been reduced by substituting a natural nucleic acid base with 2-methylbenzimidazole. Corresponding aryl ester **5** has been used for comparative purposes, since the transesterification is simpler and the results are more easily interpreted.

RESULTS

Transesterification of 2-methylbenzimidazole alkyl- and aryl phosphates was studied in various buffer solutions, including imidazole, MOPSO, and glycine buffers

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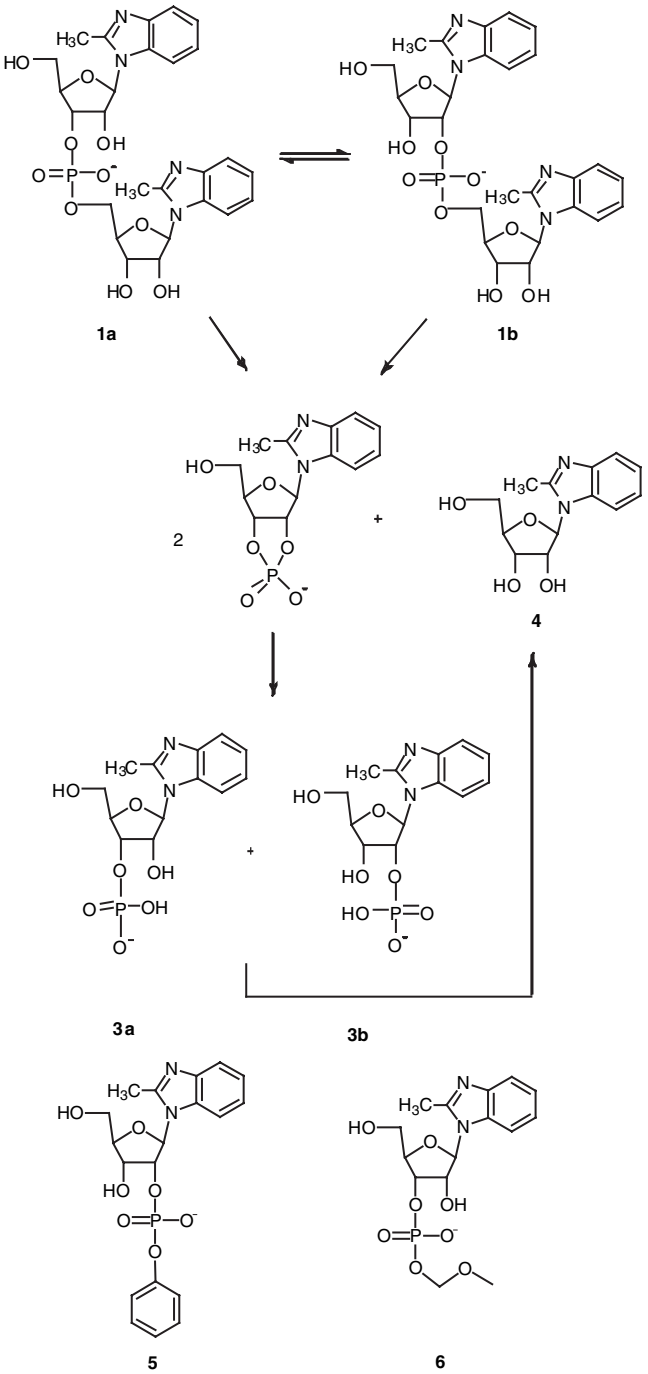

Scheme 1

in H_2O and D_2O. Preparation of buffers is described in the Experimental section. Rate constants were determined at four buffer concentrations ranging from 0.1 to 0.7 M. Such high buffer concentrations were required, since the rate enhancements are modest. The experiments were generally carried out at two buffer ratios; one on either side of the buffer pK_a. Buffer ratio is given in following as the percentage of buffer base, and buffers containing 20% and 80% of buffer base are referred to as acidic and basic, respectively.

Reactions were followed by withdrawing aliquots at appropriate intervals and analyzing their composition by RP-HPLC, as described in the Experimental section. Products were identified by spiking with authentic samples. In the case of nucleoside aryl phosphate **5,** the cleavage of the phosphodiester bond was the only reaction observed under the experimental conditions and the only products observed were 2′,3′-cyclic monophosphate **2** and phenol. No subsequent processes were observed, since the hydrolysis of the cyclic monophosphate is much slower than the cleavage of the aryl substrate, containing a good leaving group.[1]

The reaction of dialkylphosphate **1a** in glycine and CHES buffers (pH 8.9 and 8.7, respectively, at 90 °C) resembles that of the aryl phosphate in that the cleavage is the only reaction observed. Because of the poor leaving group, the cleavage of **1a** is slow and therefore, the hydrolysis of 2′,3-cyclic monophosphate **2** to a mixture of nucleoside 3′- and 2′- monophosphates **3a** and **3b** was observed. The hydrolysis of **2** is faster than the cleavage of a phosphodiester bond and the mole fraction of **2** remained low throughout the reaction. Dephosphorylation of monophosphates, which unlike the hydrolysis of the cyclic monophosphate, is not base-catalyzed,[4] is slow, and monophosphates **3a** and **3b** accumulate to a significant extent.

The reaction system in imidazole and MOPSO buffers (pH 5.5–7.0 at 90 °C) was more complex. Under these conditions, isomerization of phosphodiester bonds competes with the cleavage and in acidic buffers (20% base), the hydrolysis of the *N*-glycosidic bond significantly contributed to the disappearance of **1a**. Hydrolysis of the *N*-glycosidic bond releases a 2-methylbenzimidazole base, and since nucleosides generally depurinate faster than dinucleoside monophosphates,[5] the proportion of the base among the reaction products increased throughout the reaction. Under neutral and slightly acidic conditions, the hydrolysis of **2** and the dephosphorylation of **3a,b** are significantly faster than the cleavage of the phosphodiester bond, and, hence, nucleoside **4** was the predominant cleavage product.

First-order rate constants for the cleavage of the phosphodiester bond were calculated from the decrease of the mole fraction of the starting material. In the case of **1a,** where cleavage takes place in parallel with isomerization, the 2′- and 3′- isomers were assumed to be cleaved at the same rate, and the sum of their mole fractions was used as the basis of calculations. Previous studies[2e,,6] have shown that the reactivity difference between the isomers is small and does not affect the results to any significant extent. Consistent with this, plots $\ln x(\mathbf{1a}) + x(\mathbf{1b})]$ *versus* reaction time were linear, even in cases where the rates of isomerization and cleavage were comparable. In cases, where the depurination contributed to the disappearance of the starting material, the rate constant for depurination was subtracted from k_{obs} values to give the rate constant for the cleavage of the phosphodiester bond (k_c). First-order rate

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constants for the isomerization of **1a** to **1b** (k_i) were calculated, as described previously.[2e] Rate constants for the hydrolysis of the cyclic monophosphate were calculated by a non-linear fit of the mole fraction of the cyclic product **2** against reaction time, as described in the Experimental section.

The first-order rate constants for the cleavage of phosphodiesters (k_c) were plotted against the buffer concentration and extrapolated to zero concentration to obtain the rate constants for the buffer-independent reaction (k_0). In cases where the plot was linear, the extrapolation was made by a linear least-square fit, and first-order rate constants for the buffer-independent (k_0) and second-order rate constants for the buffer-dependent cleavage (k_2) were obtained, as intercepts and slopes of the plots, respectively. These values are collected in Table 1. Plots referring to acidic imidazole buffers (20% base) were curved and a similar treatment of the data could not be employed. In those cases, the data were fitted according to an equation, taking into account a second-order dependence on imidazole concentration, as explained in the Experimental section, and k_0 values obtained are included in Table 1. Rate constants for the cleavage of **5** are collected in Table 2, rate constants for the hydrolysis of **2** in Table 3, and rate constants for the isomerization of **1a** in Table 4.

The k_0 values in Tables 1–4 form a set of values which is consistent with the existing information on the reactions of nucleoside phosphodiesters. Buffer-independent cleavage of phosphodiesters **1a** and **5** and buffer-independent hydrolysis of **2** become base-catalyzed, approximately at pH 7, while the rate of the isomerization of **1a** is pH-independent.[4] Apparent k_H/k_D values, referring to base-catalyzed cleavage reactions are large, but under the conditions where a pH-independent reaction predominates, the reactions are only slightly faster in H_2O.[1] The rate constants for the depurination (data not shown) are also consistent with those previously reported;[1] the rate decreases as the pH increases and the reaction is faster in D_2O than in H_2O solutions.

The data on the buffer-catalyzed cleavage of **1a** are shown in Figure 1, where rate constants, relative to those for the uncatalyzed reaction are shown as a function of the buffer concentration. It can be seen, that the rate enhancements are very modest: 0.7 M imidazole buffers promote the cleavage approximately by a factor of five. The value obtained in acidic imidazole buffers is slightly uncertain, due to the large error limit of the k_0 value obtained by the non-linear fit, but in no case, the rate enhancement exceeds the value of 10. A rate enhancement of this magnitude is consistent with other reports on imidazole catalysis.[2] The rate enhancement in imidazole is, however, clear, whereas other buffers tested promote the cleavage of a phosphodiester bond only very slightly, if at all. Acidic MOPSO (0.7 M, 20% base; pH 5.6) promoted the cleavage by a factor of 2, in comparison to the uncatalyzed reaction and in basic MOPSO (80% base; pH 6.8), no rate enhancement was observed. Similarly, basic CHES buffers (80% base; pH 8.7) did not promote the cleavage of phosphodiester bonds, whereas in 0.7 M basic glycine (80% base; pH 8.9), a 1.5-fold rate enhancement was observed. Two other buffers of different type were tested for their catalytic ability under neutral conditions: phosphate (50% base) and citrate buffers (65% base), both promoted the cleavage of **1a**, even if the rate enhancements were modest. Rate enhancements in 0.25 M citrate and in 0.7 M phosphate were 1.5- and 2.2-fold, respectively.

The results obtained with aryl phosphate **5** are shown in Fig. 2. As can be seen, the rate enhancements with a substrate with a good leaving group, are even more modest. Similar to the situation with **1a**, there is a difference between imidazole and sulphonic acid buffers. Both acidic and basic imidazole promote the cleavage;

Table 1. Kinetic parameters of the buffer-catalyzed cleavage of **1a** at 90 °C and $I = 1.0$ (adjusted with NaCl)

Buffer	Solvent	pL[a]	(k_0) $s^{-1(b)}$	(k_2) $dm^3\,mol^{-1}\,s^{-1(b)}$
MOPSO, 20% base	H_2O	5.5	$(1.13 \pm 0.04) \times 10^{-7}$	$(7.0 \pm 0.7) \times 10^{-8}$
	D_2O	6.1	$(7.3 \pm 0.7) \times 10^{-8}$	$(6 \pm 2) \times 10^{-8}$
MOPSO, 80% base	H_2O	6.7	$(5.5 \pm 0.2) \times 10^{-7}$	$(1.6 \pm 0.3) \times 10^{-7}$
	D_2O	7.3	$(1.0 \pm 0.4) \times 10^{-7}$	$(1.1 \pm 0.8) \times 10^{-7}$
Imidazole, 20% base	H_2O	5.6	$(4.5 \pm 1.5) \times 10^{-7}$[c]	
	D_2O	6.1	$(1.0 \pm 0.4) \times 10^{-7}$	$(1.1 \pm 0.1) \times 10^{-6}$
Imidazole, 80% base	H_2O	6.8	$(6.3 \pm 0.6) \times 10^{-7}$	$(4.1 \pm 0.2) \times 10^{-6}$
	D_2O	7.3	$(4.0 \pm 0.8) \times 10^{-7}$	$(1.8 \pm 0.2) \times 10^{-6}$
Glycine, 80% base	H_2O	8.9	$(7.9 \pm 0.5) \times 10^{-5}$	$(6 \pm 1) \times 10^{-5}$
	D_2O	9.5	$(6.7 \pm 0.2) \times 10^{-5}$	$(4.1 \pm 0.3) \times 10^{-5}$
CHES, 80% base	H_2O	8.7	$(6.4 \pm 0.2) \times 10^{-5}$	[d]
	D_2O	9.4	$(3.8 \pm 0.2) \times 10^{-5}$	[d]

[a] pL values have been calculated using pK_a values extrapolated to experimental conditions, as explained in the Experimental section.
[b] k_0 and k_2 values have been obtained as the intercept and the slope of a plot of k_c versus c (buffer), unless otherwise mentioned.
[c] The plot k_c versus c was curved (Fig. 3). The value has been obtained by a non-linear fit according to Eqn (2). The parameters k_2 and k_3 referring to first-order and second-order catalysis by imidazole are $(3 \pm 4) \times 10^{-6}\,dm^3\,mol^{-1}\,s^{-1}$ and $(2.1 \pm 0.7) \times 10^{-5}\,dm^6\,mol^{-2}\,s^{-1}$.
[d] Rate constants k_c do not depend on the concentration of the buffer. Relative rate constants k_c/k_0, obtained in H_2O solutions are shown in Fig. 1.

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Table 2. Kinetic parameters of the cleavage of **5** in buffer solutions at 60 °C and $I = 1.0$ M (adjusted with NaCl)

Buffer	Solvent	pL[a]	(k_0) s^{-1}[b]	(k_2) $dm^3\,mol^{-1}\,s^{-1}$[b]
MOPSO, 20% base	H_2O	5.8	$(5.6 \pm 0.1) \times 10^{-6}$	$(3.2 \pm 0.3) \times 10^{-6}$
	D_2O	6.4	$(4.7 \pm 0.2) \times 10^{-6}$	$(2.6 \pm 0.4) \times 10^{-6}$
MOPSO, 80% base	H_2O	7.0	$(7.18 \pm 0.08) \times 10^{-5}$	[c]
Imidazole, 20% base	H_2O	6.1	$(1.00 \pm 0.03) \times 10^{-5}$[d]	
	D_2O	6.6	$(3.1 \pm 0.1) \times 10^{-6}$[e]	
Imidazole, 80% base	H_2O	7.3	$(1.02 \pm 0.02) \times 10^{-4}$[f]	
	D_2O	7.8	$(5.2 \pm 0.1) \times 10^{-5}$	$(2.7 \pm 0.2) \times 10^{-5}$

[a] pL values have been calculated using pK_a values extrapolated to experimental conditions, as explained in the Experimental section.
[b] k_0 and k_2 values have been obtained as the intercept and the slope of a plot of k_c *versus* c (buffer), unless otherwise mentioned.
[c] Rate constants k_c decrease as the buffer concentration increases. Relative rate constants k_c/k_0 are shown in Fig. 2.
[d] The plot k_c *versus* c was curved (Fig. 4). The value has been obtained by a non-linear fit according to Eqn (2). The parameters k_2 and k_3, referring to first-order and second-order catalysis by imidazole are $(1 \pm 1) \times 10^{-5}\,dm^3\,mol^{-1}\,s^{-1}$ and $(1.4 \pm 0.2) \times 10^{-5}\,dm^6\,mol^{-2}\,s^{-1}$.
[e] The plot k_c *versus* c was curved (Fig. 4). The value has been obtained by a non-linear fit according to Eqn (2). The parameters k_2 and k_3, referring to first-order and second-order catalysis by imidazole are $(3.4 \pm 0.3) \times 10^{-5}\,dm^3\,mol^{-1}\,s^{-1}$ and $(1.9 \pm 0.4) \times 10^{-5}\,dm^6\,mol^{-2}\,s^{-1}$.
[f] The plot k_c *versus* c was curved (Fig. 4). The value has been obtained by a non-linear fit according to Eqn (2). The parameters k_2 and k_3, referring to first-order and second-order catalysis by imidazole are $(4 \pm 1) \times 10^{-5}\,dm^3\,mol^{-1}\,s^{-1}$ and $(1.9 \pm 0.9) \times 10^{-5}\,dm^6\,mol^{-2}\,s^{-1}$.

relative rate constants in 0.7 M buffer solutions are 2.2 and 1.3, in acidic (20% base; pH 6.1 at 60 °C) and basic (80% base; pH 7.3) buffers, respectively. These values are consistent with previous reports on the buffer catalysis on the cleavage of nucleoside aryl phosphates.[7] Acidic MOPSO buffers (20% base; pH 5.8 at 60 °C) also slightly enhance the cleavage, but in basic MOPSO (80% base; pH 7.0 at 60 °C), the rate constants slightly decrease as the buffer concentration increases.

The data in Figs 1 and 2 show clearly the curious feature observed in imidazole buffers: rate constants do not depend linearly on the buffer concentration, but the plots show an upward curvature. Figures 3 and 4 show the rate constants obtained in imidazole in H_2O and D_2O. It can be seen, that in the case of the alkylphosphate **1a**, the curvature is only observed in acidic imidazole buffers in H_2O, whereas the plots referring to basic buffers or to experiments carried out in D_2O solutions are linear. In the case of aryl phosphate **5,** the deviation is more pronounced than with alkylphosphate **1a** and a slight curvature is observed also in basic imidazole in H_2O and in acidic imidazole in D_2O.

It could be argued that the curvature of plots, consisting of only four data points is just a coincidence, and therefore, the experiments were repeated several times using fresh reaction solutions and different substrates. The results obtained in acidic imidazole (20% base) with 2′,5′- isomer **1b** and 2-methylbenzimidazole alkylphosphate **6** showed that the curved shape of the k_c *versus* c plots is reproducible.

The difference in the catalytic activity between imidazole and other buffers was seen also in results obtained with the cyclic monophosphate **2**. In this case, the difference is not as pronounced: increasing MOPSO concentration enhances the cleavage of **2**, although the rate enhancement is not as significant as with imidazole: 0.7 M imidazole buffer promotes the hydrolysis by a factor of 10, whereas in 0.7 M MOPSO, a three- to fourfold rate enhancement was observed. Under more alkaline conditions, the catalysis is more modest. The

Table 3. Kinetic parameters of the cleavage of **2** in buffer solutions at 90 °C and $I = 1.0$ M

Buffer	Solvent	pL[a]	(k_0) s^{-1}[b]	(k_2) $dm^3\,mol^{-1}\,s^{-1}$[b]
MOPSO, 20% base	H_2O	5.5	$(2.7 \pm 0.5) \times 10^{-6}$	$(1.5 \pm 0.1) \times 10^{-5}$
	D_2O	6.1	$(2.0 \pm 0.2) \times 10^{-6}$	$(7.1 \pm 0.3) \times 10^{-6}$
MOPSO, 80% base	H_2O	6.7	$(5 \pm 2) \times 10^{-6}$	$(9 \pm 4) \times 10^{-6}$
	D_2O	7.3	$(2.0 \pm 0.4) \times 10^{-6}$	$(6.7 \pm 0.8) \times 10^{-6}$
Imidazole, 20% base	D_2O	6.2	$(3.3 \pm 0.5) \times 10^{-6}$	$(6 \pm 1) \times 10^{-6}$
Imidazole, 80% base	H_2O	6.8	$(3.6 \pm 0.3) \times 10^{-6}$	$(2.7 \pm 0.1) \times 10^{-5}$
	D_2O	7.3	$(3.1 \pm 0.8) \times 10^{-6}$	$(7 \pm 2) \times 10^{-6}$
Glycine, 80% base	H_2O	8.9	$(3.0 \pm 0.2) \times 10^{-4}$	$(5.9 \pm 0.4) \times 10^{-4}$
	D_2O	9.5	$(2.7 \pm 0.2) \times 10^{-4}$	$(1.8 \pm 0.3) \times 10^{-4}$
CHES, 80% base	H_2O	8.7	$(3.4 \pm 0.4) \times 10^{-4}$	[c]
	D_2O	9.4	$(1.2 \pm 0.3) \times 10^{-4}$	[c]

[a] pL values have been calculated using pK_a values extrapolated to experimental conditions, as explained in the Experimental section.
[b] k_0 and k_2 values have been obtained as the intercept and the slope of a plot of k_c *versus* c (buffer).
[c] Rate constants k_c decrease as the buffer concentration increases.

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Table 4. Kinetic parameters of the isomerization of **1a** to **1b** in buffer solutions at 90 °C and $I = 1.0$ M (adjusted with NaCl)

Buffer	Solvent	pL[a]	(k_0) $s^{-1(b)}$	(k_2) $mol\,s^{-1}\,dm^{-3(b)}$
20% fb Imidazole	H_2O	5.6	$(8 \pm 2) \times 10^{-7}$	$(1.2 \pm 0.3) \times 10^{-6}$
	D_2O	6.1	$(6 \pm 2) \times 10^{-7}$	$(7 \pm 3) \times 10^{-7}$
80% fb Imidazole	H_2O	6.8	$(6.1 \pm 0.4) \times 10^{-7}$	$(2.4 \pm 0.8) \times 10^{-7}$
	D_2O	7.3	$(4.5 \pm 0.4) \times 10^{-7}$	$(1.2 \pm 0.8) \times 10^{-7}$
20% fb MOPSO	H_2O	5.5	$(6.6 \pm 0.2) \times 10^{-7}$	$(8 \pm 3) \times 10^{-8}$
20% fb MOPSO	D_2O	6.1	$(4.5 \pm 0.1) \times 10^{-7}$	$(6.5 \pm 0.9) \times 10^{-8}$
80% fb MOPSO	H_2O	6.7	$(5.0 \pm 0.5) \times 10^{-7}$	$(1.4 \pm 0.9) \times 10^{-7}$
80% fb MOPSO	D_2O	7.3	$(3.8 \pm 0.3) \times 10^{-7}$	$(1.2 \pm 0.6) \times 10^{-7}$

[a] pL values have been calculated using pK_a values extrapolated to experimental conditions, as explained in the Experimental section.
[b] k_0 and k_2 values have been obtained as the intercept and the slope of a plot of k_c *versus* c (buffer).

hydrolysis of **2** is promoted by a factor of 2.2 in 0.7 M glycine buffer, and in CHES buffers, the rate of the cleavage rather decreases as the buffer concentration increases.

No curvature of the k_c *versus* c plots was observed in the case of **2**, even though such a phenomenon in the imidazole promoted hydrolysis of cytidine 2′,3′-cyclic monophosphate has been previously reported.[8] This may result from different reaction conditions or from the inaccuracy of the data on the hydrolysis of **2**. The non-linear fit of the mole fraction of **2,** that remained low during the reaction, *versus* reaction time, is more likely to be prone to errors than the more direct analysis methods, employed in the cases of cytidine 2′,3′-cyclic monophosphate or **1a**. Despite the relative inaccuracy, there is no doubt that MOPSO buffers also promote the hydrolysis. As an evidence of this, the maximal mole fraction of the cyclic product formed by the cleavage of the phosphodiester bond, decreased as the buffer concentration increased.

The rate enhancement of the isomerization of **1a** in buffer solutions is even more modest than that of the cleavage. Consistent with previous reports,[2,9] on the effect of imidazole concentration on the isomerization of phosphodiesters bonds, 0.7 M acidic imidazole (20% base) promotes the isomerization by a factor of two, and approximately, a 20–30% rate enhancement is observed in basic imidazole buffer (80% base) at 0.7 M concentration. The difference between the catalytic activity of

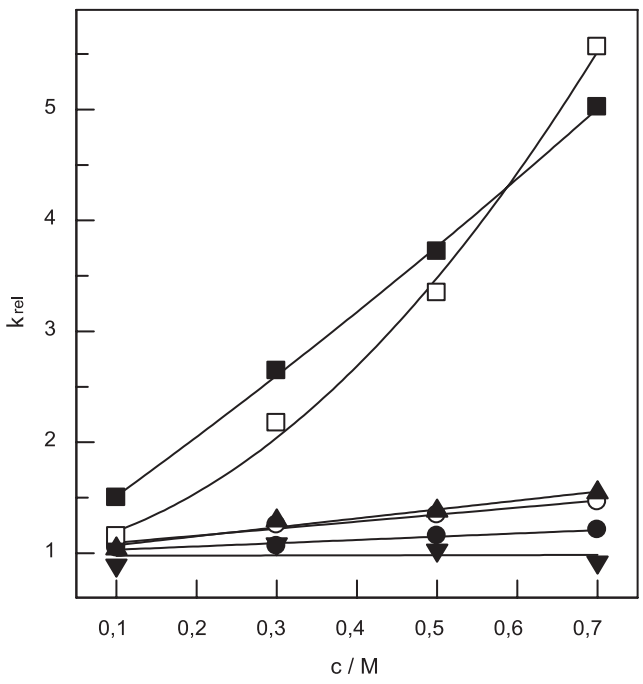

Figure 1. Relative rate constants for the cleavage of **1a** in buffer solutions at 90 °C and $I = 1.0$ M. Notation: □, imidazole 20% base; ■, imidazole 80% base; ▲, glycine 80% base; ○, MOPSO 20% base; ●, MOPSO 80% base; ▼, CHES 80% bas

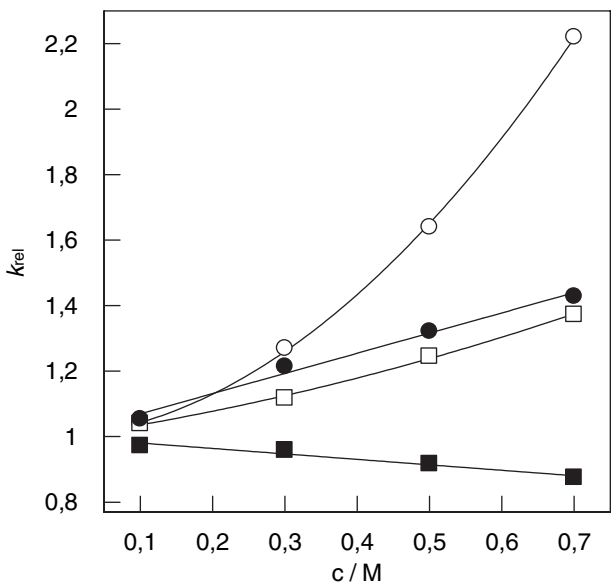

Figure 2. Relative rate constants for the cleavage of **5** in buffer solutions at 60 °C. Notation: ○, imidazole 20% base; □, imidazole 80% base; ●, MOPSO 20% base; ■, MOPSO 80% base

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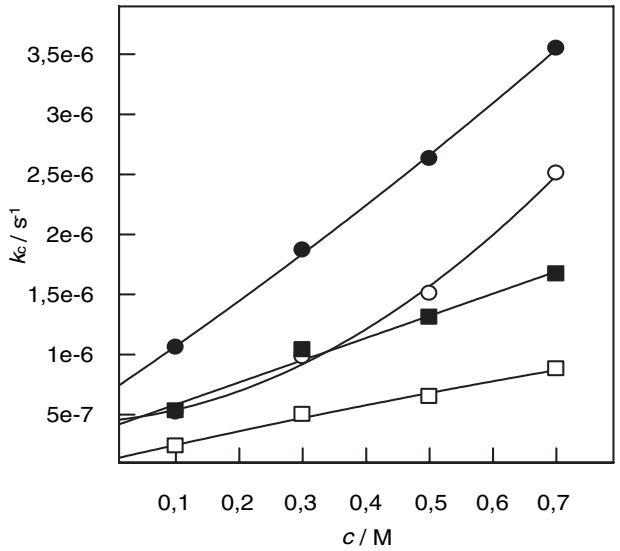

Figure 3. Rate constants for the cleavage of **1a** in imidazole buffers at 90 °C and $I = 1.0$ M. Notation: ●, 80% base imidazole in H_2O; ○, 20% base imidazole in H_2O; ■, 80% base imidazole in D_2O; □, 20% base imidazole in D_2O. $I = 1.0$ M (adjusted with NaCl). Data referring to acidic imidazole buffers have been fitted according to Eqn (2); other lines are linear least-square fits. Parameters of the fits are collected in Table 1

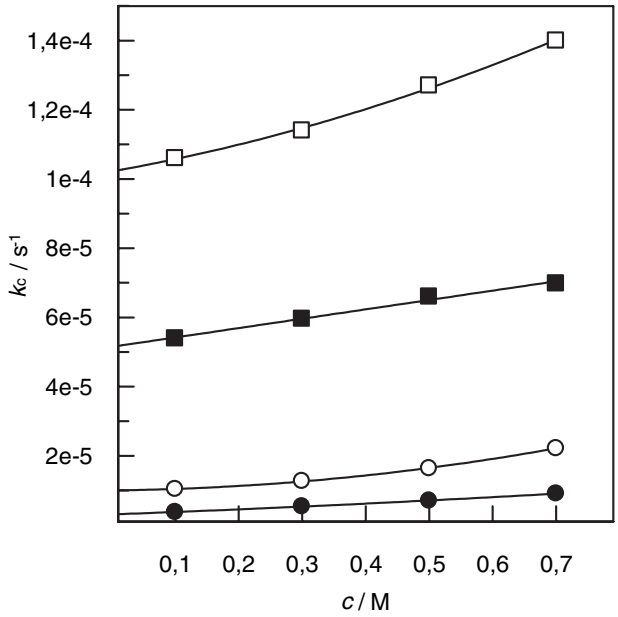

Figure 4. Rate constants for the cleavage of **5** in imidazole buffers at 60 °C and $I = 1.0$ M. Notation: □, 80% base imidazole in H_2O; ■, 80% base imidazole in D_2O; ○, 20% base imidazole in H_2O; ●, 20% base imidazole in D_2O. $I = 1.0$ M (Adjusted with NaCl). The line referring to basic imidazole in D_2O has been obtained by a linear least-square fit, other data have been fitted according to Eqn (2). Parameters of the fits are collected in Table 2

imidazole and MOPSO is observed also in the case of isomerization, and MOPSO buffers promote the isomerization only slightly, if at all.

DISCUSSION

Rate enhancing effect of buffers

The results presented above show that there is a clear difference between the catalytic ability of imidazole and sulphonic acid buffers in the cleavage of phosphodiesters. While imidazole promotes the cleavage of phosphodiester bonds and cyclic monophosphates, only the latter reaction is enhanced in sulphonic acid buffers, and even then, the rate enhancement is more modest than in imidazole. A slight rate enhancement of the cleavage of phosphodiester bonds of 2-methylbenzimidazole nucleoside alkyl and aryl phosphates is observed in acidic MOPSO (20% base), but MOPSO base is inactive as a catalyst. As the pK_a values of imidazole and MOPSO are nearly the same, the ionic form of the substrate is the same in both cases and, hence, the difference cannot be attributed to different background reactions taking place in these buffers.

The difference cannot be explained by significant medium effects that mask the modest catalysis by MOPSO or overemphasize the effect of imidazole, for results obtained in the present work and those reported previously suggest that these effects could be expected to work in an opposite direction. Under neutral conditions, the cleavage of a phosphodiester bond may proceed via a monoanionic or dianionic phosphorane species, and an increasing imidazole concentration has previously been shown to retard the latter reaction.[2c,2e] The effect of the sulphonate buffers seems to be the opposite: the cleavage of **1a** in 10 mM NaOH was slightly enhanced by an increasing HEPES concentration. Furthermore, mixed imidazole-MOPSO buffers (pH 6.2 at 90 °C) enhanced the cleavage of **1a**, under neutral conditions as efficiently as imidazole buffers do. It can, therefore, be concluded that sulphonic acid buffers do not exert medium effects that hinder the cleavage of a phosphodiester bond taking place, either via a dianionic or a monoanionic phosphorane species.

The catalytic inactivity of MOPSO could be tentatively attributed to its structure and charge. It could be speculated that the tertiary nitrogen that is a part of a morpholine ring cannot approach the substrate, close enough to abstract a proton from the 2′-hydroxyl of the ribose ring or act as a general acid catalyst, donating a proton to the substrate. Consistent with this suggestion, MOPSO buffers promote the hydrolysis of the cyclic monophosphate, where the general base can be suggested to abstract a proton from a water molecule.[10] The charge repulsion may further prevent the interaction between buffer species and a substrate. There is, however, no clear correlation between the charge of the buffer species and catalytic activity. CHES and glycine are both zwitterions/

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anions and yet, glycine buffers slightly promote the cleavage of **1a**, whereas CHES buffers do not. A high negative charge does not prevent the catalysis either; phosphate and citrate buffers also slightly enhanced the cleavage of **1a**, under neutral conditions.

Mechanism of imidazole catalysis

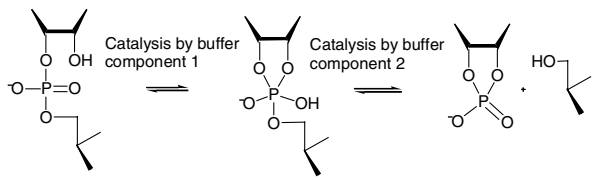
Previous studies have shown that under neutral conditions, the imidazole catalysis of the cleavage of phosphodiester bonds is a sequential process, where one buffer component promotes the formation of the phosphorane intermediate and the other one enhances the decomposition of the phosphorane to cleavage products (Scheme 2a).[2] A first-order dependence on buffer concentration is observed. A process, dependent on basic imidazole only, most probably predominates in more basic imidazole buffers (Scheme 2b).[2e] The results obtained in the present work show that a reaction that is second-order in imidazole concentration takes place, in parallel with these first-order reactions. As is shown by data in Figs 3 and 4, the plots of k_c *versus c* obtained in acidic imidazole (20% base) exhibit a clear upward curvature, and the data can be fitted, according to an equation that incorporates a second-order term (Eqn 2 in the Experimental section).

The fit was performed to emphasize the trend observed, rather than for a quantitative analysis, since the accuracy of three parameters obtained from a fit of four points is dubious and error limits are large. It can, however, be estimated that at imidazole concentration of 0.7 M, the first-order and second-order processes are approximately equally important. The suggested second-order catalysis by imidazole is consistent also with the results obtained in D_2O solutions: A reaction that requires two proton transfers could be expected to be less favorable in D_2O, and while the proportion of the second-order process becomes significantly large in H_2O solutions, a second-order process is not observed to any significant extent in D_2O solutions.

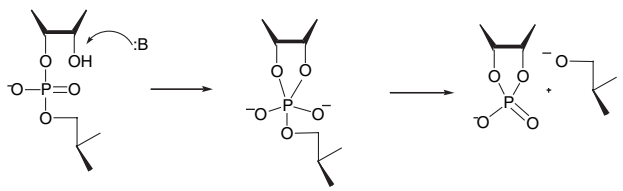
It could be argued that the curvature observed in imidazole buffers results from medium effects, operating in solutions of high buffer concentration, but this does not seem likely. As discussed above, imidazole has been shown to retard the buffer-independent reaction, which should result in a negative deviation of the k_c *versus c* plots from linearity. Furthermore, a previous study on the medium effects has shown that when the polarity of the reaction solution is kept constant with dioxane, the curvature of the *k versus c* plot is emphasized, while in the absence of dioxane, the curvature was barely observable.[2e] The authors have suggested as one potential explanation is that a medium effect by imidazole masks the second-order catalysis and, therefore, the second-order catalysis is usually barely observed, if at all. The results of the present work, particularly those obtained in D_2O solutions, strongly support the suggestion.

As was mentioned above, there most probably are two different imidazole-dependent first-order reactions: the sequential two-step process and the concerted reaction that is dependent on imidazole base only. The mechanism of the latter reaction is clear; an imidazole base deprotonates the 2′OH, a dianionic phosphorane is formed and this unstable species is cleaved to the cleavage products.[2e] In contrast, the exact mechanism of the sequential first-order process has been a subject of some disputation and different alternatives have been proposed.[2] Results obtained in the present work could be regarded as indirect support for a mechanism, where the first step, nucleophilic attack on the phosphate, is promoted by a general acid (or by specific acid and general base, which is the kinetic equivalent of general acid catalysis). This suggestion is based on the fact that the cleavage of **5** is promoted to any significant extent only in acidic imidazole buffers, whereas practically, no rate enhancement is observed in basic buffers. The cleavage of **5** involves a departure of a good aryl leaving group, which most probably is uncatalyzed. Therefore, the only catalysis required would be at the first step.

A detailed mechanism of the second-order catalysis cannot be proposed. The results obtained with the aryl phosphate **5** suggest, however, that at least in that case, the bifunctional catalysis operates on the first-step of the reaction. Nucleophilic attack of the 2′OH on the phosphate has been suggested to be the rate-limiting step of the cleavage of nucleoside aryl phosphates.[11] The departure of the leaving group takes place after the rate-limiting step and catalysis on this step would not be kinetically observed. One possible mechanism of the second-order catalysis of the first step of the cleavage reaction could be a protonation of the phosphate by imidazolium ion, and an imidazole promoted nucleophilic attack of the 2′OH on the phosphate group. Both of these elements have been proposed to be involved in the catalysis by the RNase A enzyme.[12]



Scheme 2a



Scheme 2b

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Kinetic solvent deuterium isotope effects

The original aim to determine the kinetic solvent deuterium isotope effects of buffer-dependent transesterification reactions of a phosphodiester bond of RNA was not fully achieved, due to the unexpected complexity of the reaction system. To complicate the matters, different reactions predominate in H_2O and D_2O solutions of imidazole. Although the data obtained in imidazole solutions could be fitted, according to an equation that takes the second-order catalysis by imidazole into account, the accuracy of the parameters does allow the calculation of accurate k_H/k_D values. Some values can, however, be obtained. The second-order catalysis plays a minor role in basic imidazole and k_2 values for the imidazole catalysis could be obtained as the slopes of the k_c *versus c* plots. The k_H/k_D value of 2.3 ± 0.3 obtained, most probably refers to a general base promoted cleavage that has been suggested[2e] to predominate in basic imidazole buffers. The kinetic solvent deuterium isotope effect for the second-order reaction in imidazole concentration is most probably much higher than that. The k_3 values obtained for the cleavage of the aryl ester **5** give a value of 7 ± 2, but this should be taken only as a rough approximation. Corresponding value for the cleavage the alkyl ester **1a** could not be obtained, because the second-order catalysis was not observed in D_2O solutions.

Rate enhancement of the isomeration of phosphodiester bonds in imidazole buffers was very modest and no kinetic solvent isotope effect could be observed. The absence of an isotope effect cannot, however, be taken as definite evidence against general acid/base catalysis in the isomerization, for a previous study[1] has shown that k_H/k_D values for isomerization generally are of the order of 1.0–1.5, even though the reaction can be expected to involve proton transfer processes between solvent and substrate.

k_H/k_D values of the cleavage of **2**, calculated from the second-order rate constants obtained in imidazole and glycine buffers are of the order of 3–3.5. These values are well consistent with the value of 3.8, reported previously[8] for the cleavage of 2′,3′-cyclic monophosphates of uridine and cytidine, promoted by imidazole base. k_H/k_D values obtained in MOPSO buffers are smaller, which may well reflect the sterical hindrance of catalysis by sulphonate buffers proposed above.

EXPERIMENTAL

Synthesis of the phosphodiesters

The synthesis of substrates **5** and **6** has been described before[1] and **1a** was synthesized, following the procedure reported previously for **6**. A key intermediate in the synthesis is nucleoside **7**, which is needed to prepare building blocks **8** and **9a**. Phosphoramidate chemistry was employed to link the building blocks to prepare phosphodiester **1a** (Scheme 3). The syntheses of the two nucleoside building blocks, **8** and **9a,** and of the phosphodiester **1a** are described below.

1-(β-D-Ribofuranosyl)-2-methylbenzimidazole (7)

Nucleoside **7** was synthesized, according to a procedure of Vorbruggen[12] from silyl protected 2-methylbenzimidazole and commercially available 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl- β-D-ribofuranoside (product of Sigma), with $SnCl_4$ as a catalyst. After the synthesis of the *N*-glycosidic bond, the benzoyl protections were removed with Na-methoxide in methanol. The product was characterized by ^{1}H NMR spectroscopy.

^{1}H NMR (DMSO, 500 MHz) 7.83 (1*H*, *H*7); 7.52 (1*H*, *H*4); 7.17–7.10 (2*H*, *H*5, *H*6); 5.77 (1*H*, *H*1′); 5.46 (1*H*, 2′OH); 5.35 (1*H*, 3′OH); 5.19 (1*H*, 5′OH) 4.35 (1*H*, *H*2′); 4.12 (1*H*, *H*3′); 3.95 (1*H*, *H*4′); 3.69 (2*H*, *H*5′, *H*5″); 2.58 (3*H*, —CH_3).

1-(2′,3′-Di-*O*-acetyl-β-D-ribofuranosyl)-2-methylbenzimidazole (8)

5′OH group of nucleoside **7** was protected with dimethoxytrityl, and 2′- and 3′-hydroxyl groups with acetyl protection, following the standard procedures of protection of hydroxyl groups of nucleosides. The dimethoxytrityl protection was then removed by hydrolyzing in 75% CH_3COOH solution for 1 h at room temperature. The product was purified on silica gel, using 5% methanol in dichloromethane as an eluent and characterized by ^{1}H NMR spectroscopy.

^{1}H NMR (DMSO, 500 MHz): δ 7.96–7.92 (1*H*, *H*7); 7.56–7.52 (1*H*, *H*4); 7.21–7.15 (2*H*, *H*5, *H*6); 6.11 (1*H*, *H*1′); 5.52–5.45 (3*H*, *H*2′, *H*3′, O*H*5′); 4.23 (1*H*, *H*4′,); 3.82–3.73 (2*H*, *H*5′, *H*5″); 2.59 (3*H*, base —CH_3); 2.15 (3*H*, acetyl CH_3), 1.99 (3*H*, acetyl CH_3).

1-(2′,5′-Di-*O*-*tert*-butyldimethylsilyl-β-D-ribofuranosyl)-2-methylbenzimidazole and 1-(3′,5′-di-*O*-*tert*-butyldimethylsilyl-β-D-ribofuranosyl)-2-methylbenzimidazole (9a and 9b)

Nucleoside **7** was reacted with two equivalents of TBDMSCl in dry pyridine, with $AgNO_3$ as a catalyst for 6 h at room temperature. The product was purified on silica gel, using a gradient elution from 3 to 30% MeOH in CH_2Cl_2. The ^{1}H NMR analysis of the product showed that 2′-*O*- (**9a**) and 3′-*O*-TBDMS (**9b**) protected isomers had been formed in 1:1.65 ratio (2′:3′). The isomers could

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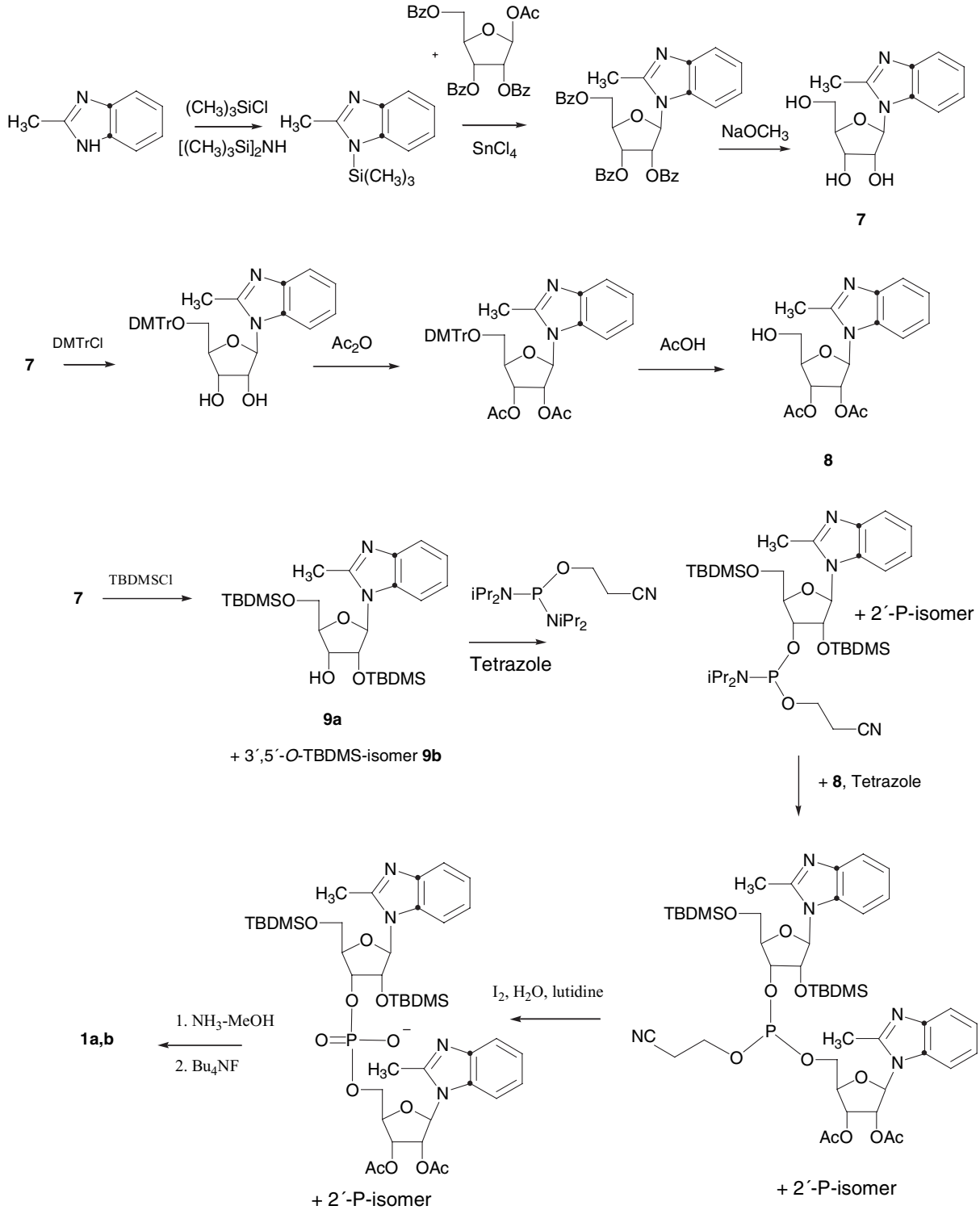

Scheme 3

not be separated, but an isomer mixture was used in subsequent steps.

^{1}H NMR (DMSO, 500 MHz) **9a**: δ 7.87 (1*H*, *H*7); 7.53 (1*H*, *H*4); 7.19–7.13 (1*H*, *H*5); 7.12–7.05 (1*H*, *H*6); 5.85 (1*H*, *H*1′); 5.19–5.14 (1*H*, 3′OH); 4.41 (1*H*, *H*2′); 4.10 (1*H*, *H*3′); 4.07 (1*H*, *H*3′); 3.9 (2*H*, *H*5′, *H*5″); 2.57 (3*H*, base —CH_3), 1.0–0.9 (9*H*, 5′-*O*—Si—C—CH_3); 0.66 (9*H*, 2′-*O*—Si—C—CH_3); 0.15 (6*H*, 5′-*O*—Si—CH_3), 0.27 (3*H*, 2′-*O*—Si—CH_3), -0.45 (3*H*, 2′-*O*—Si—CH_3).

^{1}H NMR (DMSO, 500 MHz) **9b**: 7.76 (1*H*, *H*7); 7.53 (1*H*, *H*4); 7.19–7.13 (1*H*, *H*5); 7.12–7.05 (1*H*, *H*6), 5.75 (1*H*, *H*1′); 5.35 (1*H*, 2′-\), 4.35 (1*H*, *H*2′); 4.27 (1*H*, *H*3′);

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4.02 (1*H*, *H*4′); 3.9 (2*H*, *H*5′, *H*5″); 2.57 (3*H*, base —CH_3); 1.0–0.9 (18*H*, 5′-and 3′-*O*—Si—C—CH_3); 0.15 (12*H*, 5′-and 3′-*O*—Si—CH_3).

[1-deoxy-1C-(2-methylbenzimidazol-1-yl)-β-D-ribofuranos-3-yl]-[1-deoxy-1C-(2-methylibenzimidazol-1-yl)-β-D-ribofuranos-5-yl] phosphate (1a)

Isomer mixture **9a,b**, tetrazole and 2-cyanoethyl tetraisopropylphosphoramidate were stirred at 40 °C for 4 h, after which nucleoside building block **8** was added as a CH_2Cl_2 solution. The reaction solution was stirred for additional 16 h. The product was oxidized to phosphate using a mixture of iodine and lutidine in THF–H_2O solution. The reaction mixture was stirred at room temperature for 4 h and then neutralized with $NaHSO_4$ solution. The product was purified on silica gel with 3–10% methanol in CH_2Cl_2 as an eluent.

Base-labile protecting groups were removed in methanolic ammonia by stirring the solution for 24 h at room temperature. Reaction mixture was then evaporated under reduced pressure and the residue was co-evaporated, three times from anhydrous MeCN. The TBDMS protection was removed with tetrabutylammonium fluoride by stirring overnight at room temperature in anhydrous MeCN. The product was purified by RP-HPLC with acetic acid buffer (0.1 M, pH 4.3), containing 11% MECN as an eluent. Fractions were evaporated and desalted using H_2O–MeCN (89:11) mixture as an eluent. Compounds were detected by a UV-detector at 254 nm. The product was characterized by ^{1}H NMR spectroscopy and HRMS. The assignment of the signals was verified by COSY. The subscripts a and b refer to 3′- and 5′-*O*-linked nucleosides, respectively.

^{1}H NMR (DMSO, 500 MHz): δ 7.74 (2*H*, *H*7$_{a,b}$); 7.54 (2*H*, *H*4$_{a,b}$); 7.0–7.2 (4*H*, *H*5$_{a,b}$, *H*6$_{a,b}$); 5.87 (2*H*, *H*1′$_{a,b}$); 4.58 (1*H*, *H*3′$_a$); 4.35–4.45 (2*H*, *H*2′$_{a,b}$); 4.20 (1*H*, *H*3′$_b$); 4.08 (2*H*, *H*4′$_{a,b}$), 3.95–4.05 (2*H*, *H*5′, *H*5″$_a$); 3.80–3.69 (2*H*, *H*5′, *H*5″$_b$); 2.55-2.60 (6*H*, —CH_3). HRMS: 589.1667 (found), 589.1700 (calculated for $C_{27}H_{32}N_4O_{10}P$).

Preparation of buffer solutions

Buffer systems used in the experiments were imidazole, MOPSO (3-(*N*-morpholino)-2-hydroxypropanesulphonic acid, CHES (2-(cyclohexylamino)ethanesulphonic acid), glycine, and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). All buffer solutions were prepared in freshly distilled water by diluting from a stock solution. NaOH was used to adjust the buffer ratio of CHES, MOPSO and glycine buffers, and HCl, in the case of imidazole buffers. Ionic strength was adjusted with NaCl. Deuterated buffer components were prepared by evaporating the protiated form three times from D_2O. The buffer ratio of deuterated buffers was adjusted with NaOD (40 wt % in D_2O, 99% D) or DCl (20 wt % in D_2O, 99.5% D. Deuterated solutions were stored in desiccator.

pK_a values of the buffer systems under the experimental conditions (T and I) were calculated, using the temperature and ionic effect dependence data found in the literature. pK_a values obtained are: imidazole: 6.2 at 90 °C and 6.7 at 60 °C;[13] MOPSO: 6.1 at 90 °C and 6.4 at 60 °C;[14] CHES: 8.2 at 90 °C;[15] and glycine: 8.3 at 90 °C.[16,17] pK_a values in D_2O were calculated using the ΔpK_a ((pK_a (H_2O) − pK_a (D_2O)) values: of 0.49[18] for imidazole and 0.63[19] for glycine. In the cases of MOPSO and CHES, values of 0.62[20] reported for morpholinium ion and 0.68[20] reported for cyclohexylammonium ion were used, respectively.

HPLC analysis

Aliquots withdrawn from the reaction solutions were cooled on an ice bath and kept in a freezer, until analyzed. The analysis was carried out using a Waters Atlantis^{TM} dC_{18} column (4.6 × 150 mM, particle size 5 μM). Samples of **1a** were analyzed by employing a step-wise eluation: first acetic acid buffer (0.06 M, pH 4.6, I = 0.1 M with $NaClO_4$) for 11 min, then acetic acid buffer containing 15% of acetonitrile for 10 min. In the case of **5**, the eluation was isocratic and the eluent contained 13% of acetonitrile. The flow rate was 1.5 ml min^{-1} and the reaction components were detected at 245 nm.

Calculation of rate constants

Observed first-order rate constants for the cleavage were calculated from the decrease of the signal area, as a function of time using the integrated first-order rate law. The proportion of the depurination was calculated on the basis of the signal area of 2-methylbenzimidazole base released. The rate constants for the depurination were subtracted from the k_{obs} values to give the rate constants for the cleavage of the phosphodiester bond (k_c). First-order rate constants for the isomerization were calculated, as has been reported before.[2e] First-order rate constants for the hydrolysis of the cyclic monophosphate **3** were calculated, using the rate law of consecutive first-order processes (Eqn 1),[21] where x is the mole fraction of the cyclic monophosphate product, a_1 the observed first-order rate constant for the cleavage of **1a** (k_c), and k_1 and k_2 the first-order rate constants for formation and hydrolysis of the cyclic monophosphate.

$$x = \frac{(\exp(-a_1 \cdot t) - \exp(-k_2 \cdot t)) \cdot k_1}{k_2 - a_1} \qquad (1)$$

Second-order rate constants for buffer-dependent reactions and first-order rate constants for the uncatalyzed reactions were obtained, as slopes and intercepts of k_c *versus* c (buffer) plots. The data obtained in imidazole

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buffers were fitted according to Eqn (2), which is adapted from that reported by Eftinkt and Biltonen.[8]

$$k_c = k_0 + \frac{k_2 \cdot c_T}{a} + \frac{k_3 \cdot b \cdot c_T^2}{a} \quad (2)$$

In Eqn (2), c_T is the concentration of imidazole buffer, $a = 1 + [H^+]/K_{im}$ and $b = ([H^+]/K_m)/(1 + [H^+]/K_m)$. k_c is the observed first-order rate constant for the cleavage and k_0 is the first-order rate constant for the uncatalyzed reaction. k_2 and k_3 are the rate constants for cleavage reactions, showing a first-order and second-order dependence on imidazole concentration.

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