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Hydrolytic stability of 2′,3′-*O*-methyleneadenos-5′-yl 2′,5′-di-*O*-methylurid-3′-yl 5′-*O*-methylurid-3′(2′)-yl phosphate: implications to feasibility of existence of phosphate-branched RNA under physiological conditions†

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Hydrolytic reactions of 2′,3′-*O*-methyleneadenos-5′-yl 2′,5′-di-*O*-methylurid-3′-yl 5′-*O*-methylurid-3′(2′)-yl phosphate (**1a,b**) have been followed by RP-HPLC over a wide pH range to evaluate the feasibility of occurrence of phosphate-branched RNA under physiological conditions. At pH <2, where the decomposition of **1a,b** is first order in [H_3O^+], the P–O5′ bond is cleaved 1.5 times as rapidly as the P–O3′ bond. Under these conditions, the reaction probably proceeds by an attack of the 2′-OH on the phosphotriester monocation. Over a relatively wide range from pH 2 to 5, the hydrolysis is pH-independent, referring to rapid initial deprotonation of the attacking 2′-OH followed by general acid catalyzed departure of the leaving nucleoside. The P–O5′ bond is cleaved 3 times as rapidly as the P–O3′ bond. At pH 6, the reaction becomes first order in [HO^-], consistent with an attack of the 2′-oxyanion on neutral phosphate. The product distribution is gradually inversed: in 10 mmol L^{-1} aqueous sodium hydroxide, cleavage of the P–O3′ bond is favored over P–O5′ by a factor of 7.3. The results of the present study suggest that the half-life for the cleavage of **1a,b** under physiological conditions is only 100 s. Even at pH 2, where **1a,b** is most stable, the half-life for its cleavage is less than one hour and the isomerization between **1a** and **1b** is even more rapid than cleavage. The mechanisms of the partial reactions are discussed.

Introduction

It has been suggested that two small nuclear RNAs, *viz.* U2 and U6 snRNA of human spliceosome, form a phosphotriester structure by an attack of the 2′-OH group of adenosine 21 of U2 snRNA on an A53pG54 phosphodiester bond of U6 snRNA.[1,2] This finding is somewhat unexpected because ribonucleoside 3′-dialkylphosphates are known to undergo a facile nucleophilic attack of the neighboring 2′-OH function on the phosphotriester group.[3,4] To evaluate the hydrolytic stability of the proposed species by a model compound that mimics phosphate-branched RNA (RNA X) more closely than simple 3′-dialkylphosphates, a trinucleoside-2′,3′,5′-monophosphate, 2′,3′-*O*-methyleneadenos-5′-yl 2′,5′-di-*O*-methylurid-3′-yl 5′-*O*-methylurid-3′-yl phosphate (**1a**), has been synthesized and its decomposition studied over a wide pH range. While in RNA X (Fig. 1) the 5′-OH of A21, 3′-and 5′-OH of A53 and 3′-OH of G54 are involved in phosphodiester linkages, in the model compound (**1a**) the respective sites are alkylated. The base moieties of **1a** differ from those present in the RNA X structure, but this in all likelihood is of minor importance, since the reactions of small molecule ribonucleoside 3′-phosphodiesters and triesters are not sensitive to the identity of base moieties. The results also shed some light on the transesterification reaction catalyzed by the self-splicing group II introns, which occurs by a mechanism similar to that proposed for the RNA X formation.

† Electronic supplementary information (ESI) available: ^{13}C NMR spectra of compounds **2a**, **2b**, **4a**, **4b**, **10a** and **10b**. See http://www.rsc.org/suppdata/ob/b5/b500054h/

Results

Preparation of fully protected trinucleoside 3′(2′),3′,5′-monophosphates (2a,b)

N^6-Benzoyl-2′,3′-*O*-methyleneadenos-5′-yl 2′,5′-di-*O*-methylurid-3′-yl 2′(3′)-*O*-(4,4′-dimethoxytrityl)-5′-*O*-methylurid-3′(2′)-yl phosphates (**3a,b**) were obtained by 4,5-dicyanoimidazole-(**3a**) or tetrazole-(**3b**) promoted stepwise displacement of the dimethylamino groups from tris(dimethylamino)phosphine with the appropriately protected nucleosides (**4**, **5**, **6**) (Schemes 1 and 2). The nucleosides were prepared as described in the literature.[5–9] Removal of the N^6-benzoyl protection with methanolic ammonia, followed by RP-HPLC purification, then gave the desired triesters **2a,b**. Attempts to separate the two diastereomers of **2a** were unsuccessful. In contrast, a good separation of the diastereomers of **2b** was achieved by RP-HPLC.

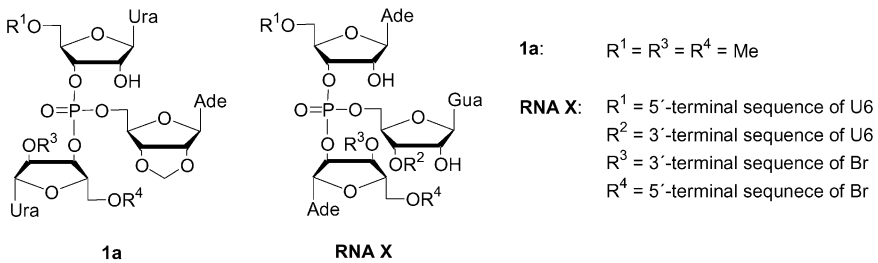

Fig. 1 Trinucleoside monophosphate **1a** as a mimic for the proposed RNA X structure.

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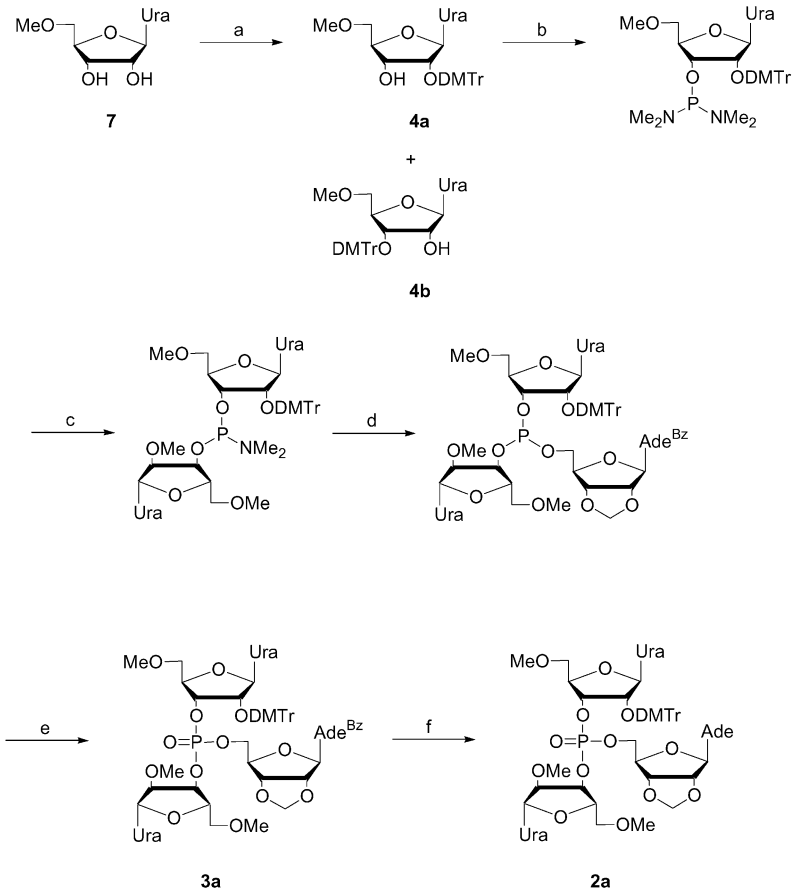
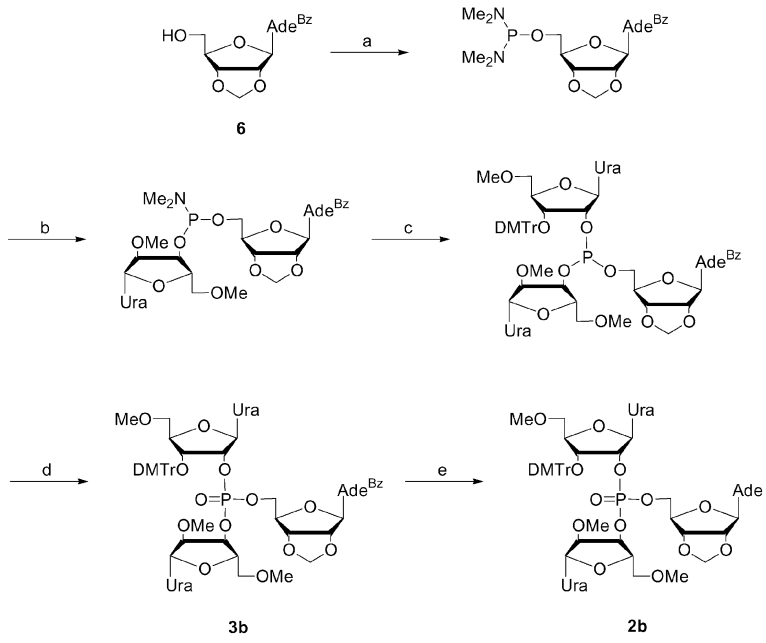


Scheme 1 Preparation of the trinucleoside 3′,3′,5′-monophosphate **2a**‡.



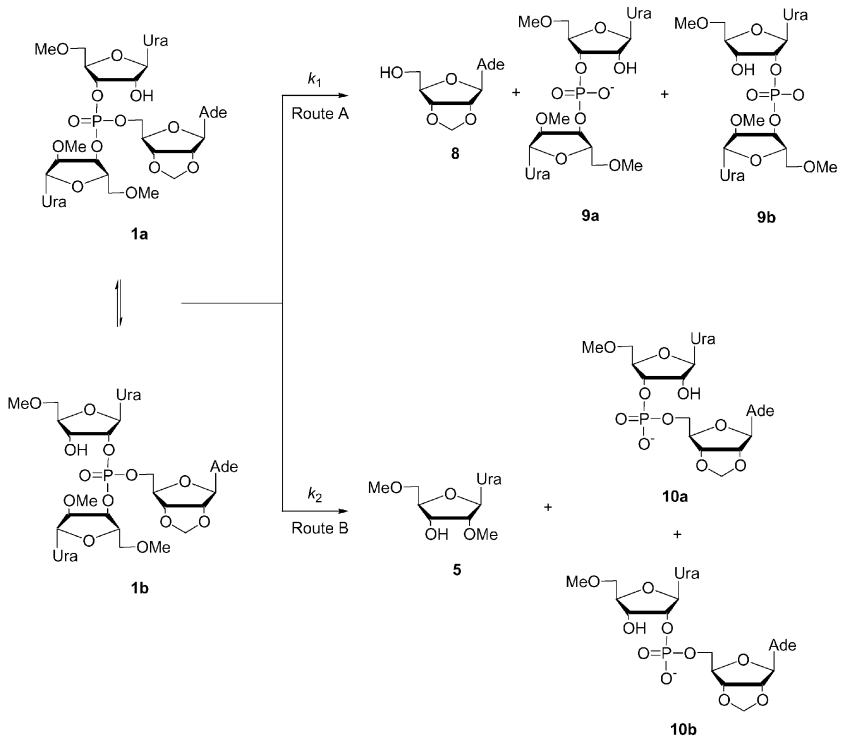
Scheme 2 Preparation of the trinucleoside 2′,3′,5′-monophosphate **2b**§.

Product distribution and reaction pathways

The hydrolysis of **1a,b** was followed over a wide pH range (0–9) at 25 °C by analyzing the composition of the aliquots withdrawn from the reaction mixture at appropriate time intervals by RP-HPLC. The 2′-*O*-(4,4′-dimethoxytrityl) group of **2a,b** was first removed with 99 mmol L^{-1} HCl in 1,4-dioxane to give **1a,b**, after which the appropriate aqueous solution was created in the reaction vessel so that the final amount of 1,4-dioxane was

‡ Reagents and conditions: (a) DMTrCl, Py, (b) chromatographic separation, $P(NMe_2)_3$, DCI, MeCN, (c) 2′,5′-di-*O*-methyluridine (**5**), DCI, MeCN, (d) **6**, DCI, MeCN, (e) I_2, H_2O, THF, 2,6-lutidine, (f) NH_3, MeOH.

§ Reagents and conditions: (a) $P(NMe_2)_3$, tetrazole, MeCN, (b) **5**, tetrazole, MeCN, (c) **4b**, tetrazole, MeCN, (d) I_2, H_2O, THF, 2,6-lutidine, (e) NH_3, MeOH.



Scheme 3 Hydrolytic reaction pathways of **1a,b**.

5% (v/v). The aliquots were quenched by cooling to −16 °C and adjusting their pH to approximately 3 with a formic acid buffer when necessary. The products were identified by spiking with authentic samples and the peak areas were converted to relative concentrations by calibrating the system with uridine and adenosine solutions of known concentration.

Two distinct sets of products were observed in the entire pH range (Scheme 3). Release of 2′,3′-*O*-methyleneadenosine (m < A, **8**) by P–O5′ bond fission produced the isomeric diesters 5′-*O*-methyluridylyl-3′,3′-(2′,5′-di-*O*-methyluridine) [mU(3′,3′)m_2U, **9a**] and 5′-*O*-methyluridylyl-2′,3′-(2′,5′-di-*O*-methyluridine) [mU(2′,3′)m_2U, **9b**]. Cleavage of one of the P–O3′ bonds, in turn, resulted in the formation of 2′,5′-di-*O*-methyluridine (m_2U, **5**) and the isomeric diesters 5′-*O*-methyluridylyl-3′,5′-(2′,3′-*O*-methyleneadenosine) [mU(3′,3′)m < A, **10a**] and 5′-*O*-methyluridylyl-2′,5′-(2′,3′-*O*-methyleneadenosine) [mU(2′,3′)m < A, **10b**]. In acidic solutions, formation of the former set predominated, in alkaline solutions the latter. The product distribution was not dependent on the regioisomer (**1a,b**) or the diastereomer of **1b** used as a starting material. No nucleoside monophosphates were formed regardless of pH, indicating that the diester products were not hydrolysed under the experimental conditions.

The pseudo first-order rate constants for the decomposition of **1** were obtained by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material. Between pH 3 and 6, considerable buffer catalysis was observed (Table 1). Buffer-independent rate constants were obtained by varying the buffer concentration from 0.04 to 0.24 mol L^{-1} and extrapolating the observed pseudo first-order rate constants to zero buffer concentration by linear regression.

Table 1 Second-order rate constants for the buffer-catalyzed cleavage of **1a,b**

Buffer acid	[HA]/[A^-]	$k_{cl}^{buf}/10^{-4}$ L mol^{-1} s^{-1}
Formic acid	3 : 1	6.3 ± 0.8
	1 : 3	21 ± 2
Acetic acid	1 : 1	29 ± 3
	1 : 3	47 ± 3

pH–Rate profiles

Fig. 2 shows the pH–rate profile for the overall disappearance of **1**. At pH <2, the overall reaction is first-order in [H_3O^+]. The predominant reaction is cleavage of the P–O5′ bond (Scheme 3, route A), accounting for 60% of the hydrolysis of **1**. Over a relatively wide range from pH 2 to 5, the overall reaction is pH-independent and cleavage of the P–O5′ bond (route A) predominates, accounting for 75% of the overall hydrolysis. At pH 6, the overall reaction becomes first-order in [HO^-] and cleavage of the P–O3′ bond (route B) gradually starts to take over (Fig. 3). In 10 mmol L^{-1} aqueous sodium hydroxide, 88% of the overall hydrolysis results from cleavage of the P–O3′ bond.

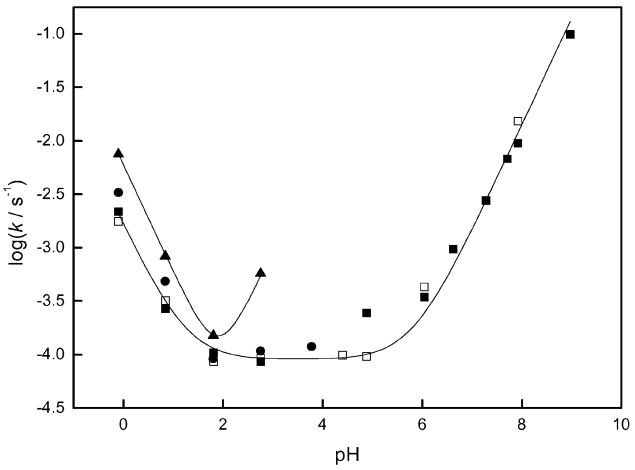


Fig. 2 pH–Rate profile for the decomposition (eqn. (1)) and mutual isomerization (eqn. (2)) of **1a,b** at 25 °C, $I(NaNO_3)$ = 1.0 mol L^{-1}: (●) cleavage of the diastereomeric mixture of **1a**, (■) cleavage of the first diastereomer of **1b**, (□) cleavage of the second isomer of **1b**, (▲) mutual isomerization of **1a** and **1b**.

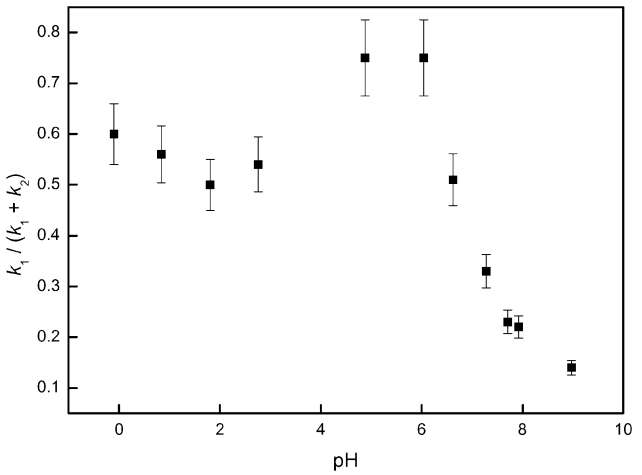


Fig. 3 Ratio of k_1 (route A) to $k_1 + k_2$ (overall cleavage) as a function of pH ($T = 25$ °C, $I(NaNO_3) = 1.0$ mol L^{-1}).

Mutual isomerization of **1a** and **1b** is rapid and it can only be followed at pH <3.[3,4] At this pH, the reaction is already first-order in [HO^-]. In other words, isomers **1a** and **1b** are rapidly equilibrated except under markedly acidic conditions. Essentially identical rate constants were observed regardless of the regioisomer (**1a,b**) or the diastereomer of **1b** used as a starting material.

Under conditions where the overall cleavage is pH-independent, considerable buffer catalysis was observed. Only the basic buffer constituent, *viz.* the carboxylate anion, contributed to the catalysis. Table 1 summarizes the second-order rate constants for the catalysis by formic and acetic acid buffers at two different ratios of [HA]/[A^-].

The observed rate constant for the cleavage of **1**, k_{cl}^{obs}, may be expressed by eqn. (1). A similar equation may be written for the isomerization (eqn. (2)).

$$k_{cl}^{obs} = k_{cl}^{H} a_{H^+} + k_{cl}^{W} + \frac{k_{cl}^{OH} K_W}{a_{H^+}} \quad (1)$$

$$k_{is}^{obs} = k_{is}^{H} a_{H^+} + k_{is}^{W} + \frac{k_{is}^{OH} K_W}{a_{H^+}} \quad (2)$$

k_{cl}^{H}, k_{cl}^{W} and k_{cl}^{OH} are the rate constants for the hydronium-ion catalyzed, pH-independent and hydroxide-ion catalyzed cleavage, respectively. Similarly, k_{is}^{H}, k_{is}^{W} and k_{is}^{OH} are the respective rate constants for isomerization. These rate constants were obtained by fitting the experimental values to eqns. (1) and (2) by a nonlinear least-squares method. The rate constants for the overall cleavage of **1**, k_{cl}, can then be bisected to the rate constants for the partial reactions, *viz.* cleavage of P–O5′ (route A) and P–O3′ (route B) bonds, on the basis of the relative concentrations of the monomeric products of these competing reactions (eqns. (3) and (4)). The calculated rate constants are presented in Table 2.

Table 2 Rate constants for the partial reactions of cleavage and isomerization of **1a,b**

	$k^H/10^{-3}$ L mol^{-1} s^{-1}	$k^W/10^{-5}$ s^{-1}	$k^{OH}/10^3$ L mol^{-1} s^{-1}
k_{cl}	1.6 ± 0.3	9.0 ± 0.9	7.5 ± 0.9
k_{is}	5.96 ± 0.07	—	50 000 ± 10 000
k_1	1.0 ± 0.2	6.8 ± 0.7	0.9 ± 0.1
k_2	0.6 ± 0.1	2.3 ± 0.2	6.6 ± 0.8

$$k_1 = \frac{[m < A]}{[m < A] + [m_2U]} k_{cl} \quad (3)$$

$$k_2 = \frac{[m_2U]}{[m < A] + [m_2U]} k_{cl} \quad (4)$$

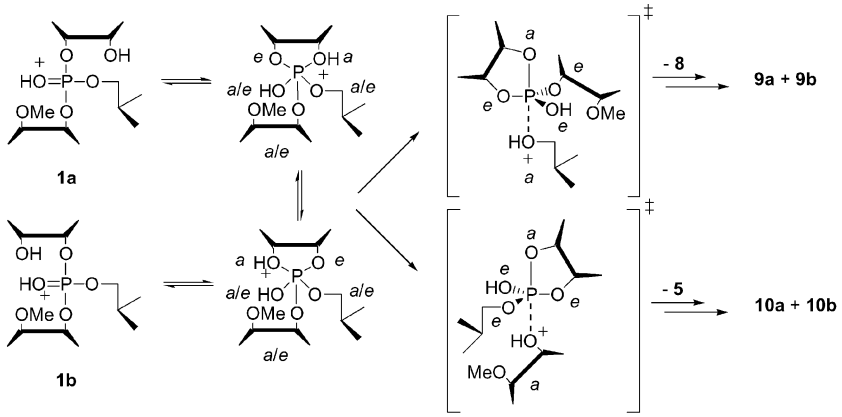
Discussion

Hydronium-ion catalyzed cleavage

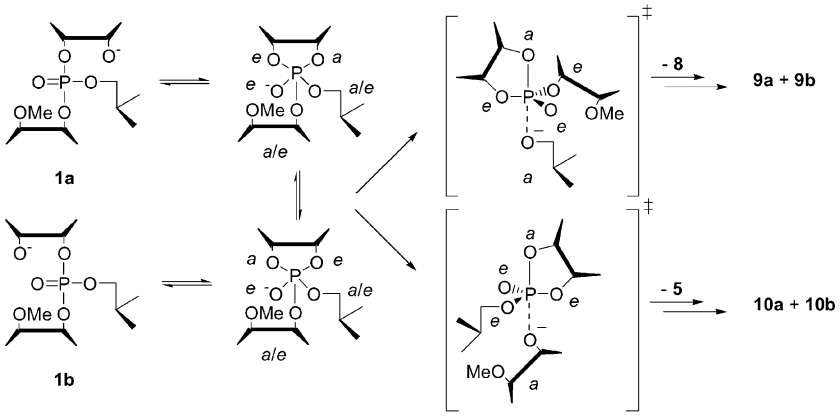
At pH <2, the overall disappearance of **1** is first-order in [H^+]. In this acid-catalyzed region, the leaving group departs as an alcohol (Scheme 4) and the rate of the reaction is only moderately sensitive to the acidity of the leaving group, the β_{lg} value being −0.40.[3] The ratio of the second-order rate constant for the hydronium-ion catalyzed cleavage of the P–O5′ bond (route A), k_1^H (0.96×10^{-3} L mol^{-1} s^{-1}), to the respective rate constant for the cleavage of the P–O3′ bond (route B), k_2^H (0.64×10^{-3} L mol^{-1} s^{-1}), is 1.5, whereas the previously reported β_{lg} value of −0.40 and the approximate ΔpK_a of 1.0 between **8** and **5**[10] would predict a ratio of 0.4. This discrepancy might be attributed to the fact that the 2′,5′-di-*O*-methylurid-3′-yl group as a sterically demanding substituent prefers an equatorial position in the pentacoordinated phosphorane intermediate, thus making its departure less probable.[11] For comparison, a similar, albeit somewhat smaller, effect has been observed with the hydronium-ion catalyzed hydrolysis of uridine-3′-alkylphosphates: the rate constant for the isopropyl derivative is only 40% of the value predicted by the results obtained with other alkyl groups.[12] Since 2′,5′-di-*O*-methylurid-3′-yl is even bulkier than isopropyl, the observed rate retardation is of expected magnitude. Taking this steric effect into account, the ratio of rate constants k_1^H and k_2^H agrees fairly well with the previously reported β_{lg} value of −0.40.

Hydroxide-ion catalyzed cleavage

At pH >6, the overall disappearance of **1** is first-order in [OH^-] and the departure of **5** (route B) is favored over that of **8** (route A). This change in the product distribution is expected, since in



Scheme 4 Mechanism of the hydronium-ion catalyzed cleavage of **1a,b**.

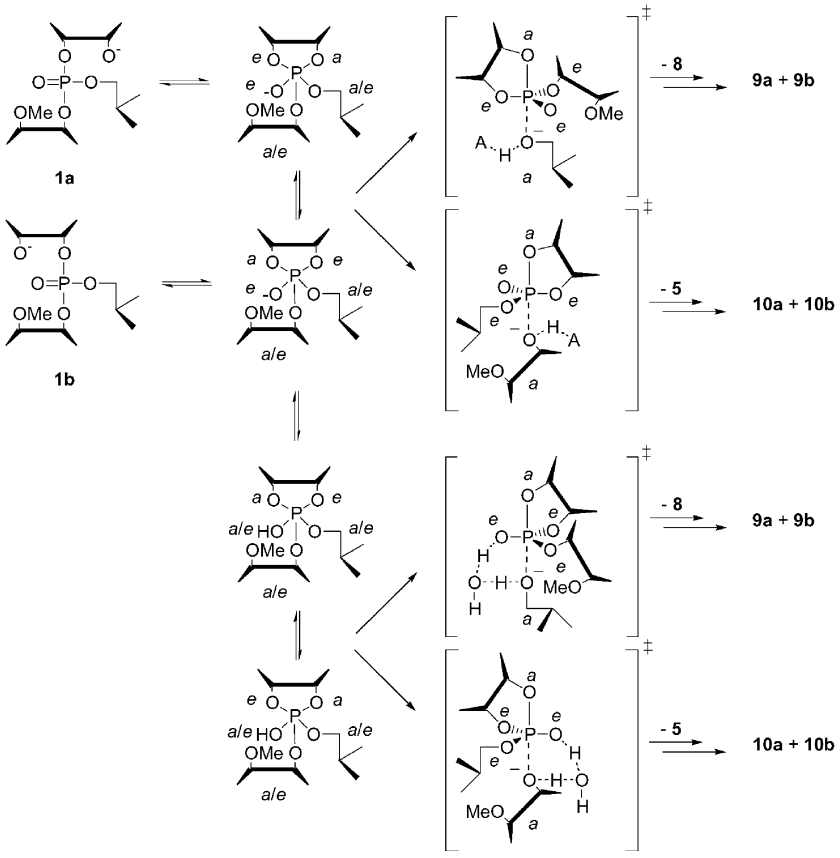


Scheme 5 Mechanism of the hydroxide-ion catalyzed cleavage of **1a,b**.

acid-catalyzed hydrolysis the leaving group departs as an alcohol and in base-catalyzed hydrolysis as an alkoxide ion (Scheme 5). The base-catalyzed cleavage is thus considerably more sensitive to the pK_a of the conjugate acid of the leaving group, the β_{lg} value being −1.38.[3] Evidently, isomerization is also first-order in [OH^-] and too rapid to be followed. The ratio of the second-order rate constant for the hydroxide-ion catalyzed cleavage of the P–O5′ bond (route A), k_1^{OH} (0.9×10^3 L mol^{-1} s^{-1}), to the respective rate constant for the cleavage of the P–O3′ bond (route B), k_2^{OH} (6.6×10^3 L mol^{-1} s^{-1}), is 0.14, while a ratio of 0.042 would be expected on the basis of the previously reported β_{lg} value and the difference between pK_a values of **8** and **5**. As in the case of the hydronium-ion catalyzed cleavage, this probably results from the fact that the bulky 2′,5′-di-*O*-methylurid-3′-yl group prefers equatorial orientation in the pentacoordinated phosphorane intermediate. Taking this into account, the ratio of the observed rate constants k_1^{OH} and k_2^{OH} agrees reasonably well with the known β_{lg} value of −1.38.

pH-Independent and buffer catalyzed cleavage

In the relatively wide range from pH 2 to 5, where isomerization is much faster than cleavage, the cleavage is pH-independent but susceptible to general base catalysis by carboxylate buffer anions. The latter reaction may be interpreted to be a sequential specific base/general acid catalysis, where the attacking 2′-OH is deprotonated in a pre-equilibrium step and proton transfer from the general acid to the leaving oxyanion occurs concerted with the rate-limiting P–O bond scission (Scheme 6).[3] The second-order rate constant for the buffer catalyzed cleavage is nearly independent of the leaving group (**8** *vs.* **5**), probably because the polar effect of the departing nucleoside on the rate of proton transfer and bond cleavage are opposite. In striking contrast, the first-order rate constants for the pH- and buffer-independent cleavage differ considerably, the departure of **8** being favored over that of **5** by a factor of 3. This behavior, albeit surprising, may tentatively be explained as follows. In all likelihood the



Scheme 6 Mechanism of the pH-independent and buffer-catalyzed cleavage of **1a,b**.

reaction involves a rate-limiting water-mediated proton transfer from the phosphorane hydroxyl ligand to the departing oxygen (Scheme 6).[4,13] The ease of this kind of intramolecular proton transfer is possibly sensitive to the structure of the solvation sphere around the reaction center, and this, in turn, depends on the detailed geometry of the phosphorane intermediate. The Brønsted β value for the observed general base catalysis is approximately 0.5, which means that the a value for the general acid catalyzed step of the sequential specific base/general acid catalysis is 0.5. The previously reported β_{lg} value for the reaction is −0.73. Taking these results together, the proton transfer and P–O bond fission appear to be more or less equally advanced in the transition state.

On the basis of these results one may estimate the half-life for the cleavage of **1a,b** under physiological conditions to be only 100 s. Even at pH 2, where **1a,b** is most stable, the half-life for its cleavage is less than one hour and the isomerization between **1a** and **1b** is even more rapid than cleavage. The existence of RNA phosphotriester linkages such as the one in RNA X in biological systems seems, hence, doubtful at best. It should be noted, though, that while the observations on RNA X formation refer to *in vitro* transcribed snRNAs composed entirely of the four native ribonucleotides,[1,2] in nature, the two residues flanking the proposed phosphotriester linkage, A53 and G54, are 2′-*O*-methylated[14], which undoubtedly stabilizes the phosphotriester toward hydrolysis. On the basis of our previous studies on the hydrolytic stability of a fully alkylated trinucleoside-3′,3′,5′-monophosphate, 2′,3′-*O*-methyleneadenos-5′-yl bis(2′,5′-di-*O*-methylurid-3′-yl) phosphate, this stabilization may be estimated to be at least three orders of magnitude at neutral pH, resulting in a half-life of more than one day.[10] The possible shielding of the phosphotriester linkage from solvent by the folded RNA strands remains a topic for further experiments. Alternatively, the conformation of the folded RNA structure might cause an unfavorable orientation of the 2′-OH nucleophile with respect to the phosphate moiety, thus inhibiting the cleavage.[11,15] It is also evident on the basis of this study that steric demands of the pentacoordinated phosphorane intermediate can be a significant factor in determining the course of splicing reactions catalyzed by large ribozymes.

Experimental

Materials

Nucleosides were commercial products and they were used as received after checking the purity by HPLC. The buffer constituents were of reagent grade.

2′(3′)-*O*-(4,4′-Dimethoxytrityl)-5′-*O*-methyluridine (4a,b). 5′-*O*-Methyluridine[5] (**7**, 1.93 mmol, 0.4977 g) was coevaporated three times from anhydrous pyridine. The residue was dissolved in anhydrous pyridine (50 mL) and 4,4′-dimethoxytrityl chloride (2.62 mmol, 0.8844 g) was added. After being stirred for 15 h at room temperature and 24 h at 45 °C, the reaction mixture was evaporated to dryness. A conventional CH_2Cl_2–aq. $NaHCO_3$ workup was carried out and the product was purified on a silica gel column eluting with a mixture of MeOH, CH_2Cl_2 and Et_3N (2 : 97 : 1, v/v). Yields were 51% (0.55 g) and 13% (0.14 g) for **4a** and **4b**, respectively. ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6, **4a**) 11.29 (s, 1H), 7.16 (d, 1H, J = 8.1 Hz), 6.7–7.5 (m, 13H), 6.06 (d, 1H, J = 7.5 Hz), 5.38 (d, 1H, J = 8.1 Hz), 5.00 (d, 1H, J = 5.3 Hz), 4.13 (dd, 1H, J_1 = 4.8 Hz, J_2 = 7.4 Hz), 3.86 (m, 1H), 3.71 (s, 3H), 3.70 (s, 3H), 3.32 (m, 1H), 3.18 (m, 2H). ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6, **4b**) 11.37 (s, 1H), 7.25 (d, 1H, J = 8.1 Hz), 6.8–7.7 (m, 13H), 5.99 (d, 1H, J = 7.0 Hz), 5.84 (d, 1H, J = 6.5 Hz) 5.69 (d, 1H, J = 8.1 Hz), 4.05 (dd, 1H, J_1 = 4.8 Hz, J_2 = 7.4 Hz), 3.93 (m, 1H), 3.73 (m, 1H), 3.35 (s, 3H), 3.10 (s, 3H), 2.89 (m, 2H). HRMS (FAB, **4a**) M^- calcd 559.2080, obsd 559.2089. HRMS (FAB, **4b**) M^- calcd 559.2080, obsd 559.2074.

2′,3′-*O*-Methyleneadenos-5′-yl 2′,5′-di-*O*-methylurid-3′-yl 2′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-methylurid-3′-yl phosphate (2a). 2′-*O*-(4,4′-Dimethoxytrityl)-5′-*O*-methyluridine (**4a**, 0.299 mmol, 0.1678 g) was coevaporated twice from anhydrous pyridine and once from anhydrous MeCN. The residue was dissolved in anhydrous MeCN (10 mL) and tris(dimethylamino)phosphine (0.33 mmol, 0.06 mL) was added. A solution of 4,5-dicyanoimidazole (0.36 mmol, 0.0425 g) in MeCN (1.44 mL) was added and the reaction mixture was stirred at room temperature for 20 h. 2′,5′-Di-*O*-methyluridine[5–7] (**5**, 0.3012 mmol, 0.082 g) was coevaporated twice from anhydrous pyridine and once from anhydrous MeCN, and the residue was added to the reaction mixture. A solution of 4,5-dicyanoimidazole (0.36 mmol, 0.0425 g) in MeCN (1.44 mL) was added and the reaction mixture was stirred at room temperature for 24 h. N^6-Benzoyl-2′,3′-*O*-methyleneadenosine[8,9] (**6**, 0.334 mmol, 0.1282 g) was coevaporated twice from anhydrous pyridine and once from anhydrous MeCN, and the residue was added to the reaction mixture. A solution of 4,5-dicyanoimidazole (0.36 mmol, 0.0425 g) in MeCN (1.44 mL) was added and the reaction mixture was stirred at room temperature for 24 h, followed by an additional 2 h at 45 °C. Iodine (0.47 mmol, 0.12 g) in a mixture of water (2.1 mL), THF (4.2 mL) and 2,6-lutidine (1.1 mL) was added and the reaction mixture was stirred for 90 min at room temperature. CH_2Cl_2 (70 mL) was added and the resulting mixture was washed with saturated aqueous $NaHSO_3$. The aqueous phase was back extracted with CH_2Cl_2 and the product was purified on a silica gel column eluting with a mixture of MeOH, CH_2Cl_2 and Et_3N (5 : 94 : 1, v/v). The crude product thus obtained was dissolved in saturated methanolic ammonia (20 mL). After being stirred for 5 h at room temperature, the reaction mixture was evaporated to dryness. The product was purified by HPLC on a LiChrospher RP-18 column (10 × 250 mm, 5 μm) eluting with a mixture of 0.05 mol L^{-1} of aqueous NH_4OAc and MeCN (53 : 47, v/v). Finally the buffer salts were removed on the same column by eluting with a mixture of water and MeCN. Compound **2a** was obtained as two diastereomers in an approximately 1 : 2 ratio. The overall yield starting from **4a** was 4.3% (15 mg). Attempts to separate the two diastereomers of **2a** were unsuccessful but because of the biased product distribution, the ^{1}H NMR could nevertheless be resolved. ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6, major diastereomer) 8.13 (s, 1H), 8.10 (s, 1H), 7.68 (d, 1H, J = 8.2 Hz), 7.60–6.60 (m, 13H), 7.15 (d, 1H, J = 8.2 Hz), 6.25 (d, 1H, J = 8.2 Hz), 6.13 (d, 1H, J = 2.4 Hz), 5.94 (d, 1H, J = 6.1 Hz), 5.55 (d, 1H, J = 8.2 Hz), 5.33 (dd, 1H, J_1 = 2.4 Hz, J_2 = 6.4 Hz), 5.25 (d, 1H, J = 8.2 Hz), 5.13 (s, 1H), 5.07 (s, 1H), 5.06 (dd, 1H, J_1 = 3.7 Hz, J_2 = 6.6 Hz), 4.87 (m, 1H), 4.33 (m, 1H), 4.23 (m, 2H), 4.08 (m, 1H), 3.98 (m, 1H), 3.64 (s, 3H), 3.63 (s, 3H), 3.57 (m, 1H), 3.39 (m, 2H), 3.32–3.10 (m, 4H), 3.25 (s, 3H), 3.18 (s, 3H), 3.15 (s, 3H). ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6, minor diastereomer) 8.15 (s, 1H), 8.10 (s, 1H), 7.64 (d, 1H, J = 8.2 Hz), 7.60–6.60 (m, 13H), 7.20 (d, 1H, J = 8.2 Hz), 6.33 (d, 1H, J = 8.1 Hz), 6.14 (d, 1H, J = 2.4 Hz), 5.86 (d, 1H, J = 6.3 Hz), 5.55 (d, 1H, J = 8.2 Hz), 5.34 (dd, 1H, J_1 = 2.4 Hz, J_2 = 6.4 Hz), 5.25 (d, 1H, J = 8.2 Hz), 5.17 (s, 1H), 5.09 (s, 1H), 5.08 (dd, 1H, J_1 = 3.1 Hz, J_2 = 6.6 Hz), 4.84 (m, 1H), 4.36 (m, 1H), 4.23 (m, 2H), 4.08 (m, 1H), 3.95 (m, 1H), 3.62 (s, 3H), 3.61 (s, 3H), 3.60 (m, 1H), 3.40 (m, 2H), 3.32–3.10 (m, 4H), 3.26 (s, 3H), 3.23 (s, 3H), 3.14 (s, 3H). ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, major diastereomer) −2.97. ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, minor diastereomer) −2.07. HRMS (FAB) M^- calcd 1154.3508, obsd 1154.3497.

2′,3′-*O*-Methyleneadenos-5′-yl 2′,5′-di-*O*-methylurid-3′-yl 3′-*O*-(4,4′-dimethoxytrityl)-5′-*O*-methylurid-2′-yl phosphate (2b). N^6-Benzoyl-2′,3′-*O*-methyleneadenosine (**6**, 0.81 mmol, 0.31 g) was coevaporated three times from anhydrous pyridine and once from anhydrous MeCN. The residue was dissolved in anhydrous MeCN (3.0 mL) and tris(dimethylamino)phosphine (0.99 mmol, 0.180 mL), and tetrazole (0.97 mmol, 0.068 g) were

added. The reaction mixture was stirred for 220 min at room temperature. 2′,5′-Di-*O*-methyluridine (**5**, 0.81 mmol, 0.22 g) was coevaporated three times from anhydrous pyridine and once from anhydrous MeCN, and the residue was added to the reaction mixture, together with anhydrous MeCN (1.0 mL) and tetrazole (1.43 mmol, 0.1 g). The reaction mixture was stirred for 25 h at 50 °C. 3′-*O*-(4,4′-Dimethoxytrityl)-5′-*O*-methyluridine (**4b**, 1.04 mmol, 0.58 g) was coevaporated three times from anhydrous pyridine and once from anhydrous MeCN, and the residue was added to the reaction mixture, together with tetrazole (1.14 mmol, 0.080 g). The reaction mixture was stirred for 24 h at 40 °C, after which iodine (0.51 mmol, 0.13 g) in a mixture of water (2.4 mL), THF (4.8 mL) and 2,6-lutidine (1.2 mL) was added. After being stirred for 90 min at room temperature the reaction mixture was concentrated under reduced pressure and the residue was dissolved in saturated aq. $NaHSO_3$ (40 mL). The mixture was extracted with CH_2Cl_2 and the organic phase was dried with Na_2SO_4 and evaporated to dryness. The product was purified on a silica gel column eluting with a mixture of MeOH, Et_3N and CH_2Cl_2 (2 : 1 : 97, v/v). The crude material thus obtained was dissolved in saturated methanolic ammonia (10 mL). The reaction mixture was stirred for 6 h at room temperature, after which it was evaporated to dryness. The product was purified first on a silica gel column eluting with a mixture of MeOH, Et_3N and CH_2Cl_2 (4 : 1 : 95, v/v), then by HPLC on a Supelcosil LC-18 column (25 cm × 21.2 mm, 12 μm) eluting with a mixture of 0.05 mol L^{-1} of aqueous NH_4OAc and MeCN (53 : 47, v/v). Finally the buffer salts were removed on the same column by eluting with a mixture of water and MeCN (45 : 55, v/v). Compound **2b** was obtained as two diastereomers in approximately 1 : 1 ratio. A good separation of the diastereomers was achieved by HPLC. The overall yield starting from **6** was 11% (103 mg). ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6, first diastereomer) 8.35 (s, 1H), 8.13 (s, 1H), 7.64 (d, 1H, $J = 8.2$ Hz), 7.54 (d, 1H, $J = 8.2$ Hz), 7.48–6.87 (m, 15H), 6.28 (d, 1H, $J = 7.0$ Hz), 6.21 (d, 1H, $J = 2.8$ Hz), 5.79 (d, 1H, $J = 6.5$ Hz), 5.73 (d, 1H, $J = 8.1$ Hz), 5.71 (d, 1H, $J = 8.1$ Hz), 5.28 (dd, 1H, $J_1 = 2.9$ Hz, $J_2 = 6.7$ Hz), 5.17 (s, 1H), 5.13 (s, 1H), 4.99 (dd, 1H, $J_1 = 4.0$ Hz, $J_2 = 6.6$ Hz), 4.85 (m, 1H), 4.78 (m, 1H), 4.35 (m, 1H), 4.27 (m, 2H), 4.12 (m, 1H), 4.04 (m, 1H), 3.73 (s, 3H), 3.72 (s, 3H), 3.45–3.30 (m, 6H), 3.29 (s, 3H), 3.24 (s, 3H), 3.03 (s, 3H). ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6, second diastereomer) 8.29 (s, 1H), 8.08 (s, 1H), 7.64 (d, 1H, $J = 8.2$ Hz), 7.56 (d, 1H, $J = 8.2$ Hz), 7.53–6.87 (m, 15H), 6.24 (d, 1H, $J = 2.6$ Hz), 6.20 (d, 1H, $J = 6.0$ Hz), 5.88 (d, 1H, $J = 6.4$ Hz), 5.73 (dd, 1H, $J_1 = 2.1$ Hz, $J_2 = 8.1$ Hz), 5.69 (dd, 1H, $J_1 = 2.0$ Hz, $J_2 = 8.0$ Hz), 5.27 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 6.5$ Hz), 5.17 (s, 1H), 5.14 (s, 1H), 5.03 (dd, 1H, $J_1 = 3.8$ Hz, $J_2 = 6.4$ Hz), 4.89 (m, 1H), 4.49 (m, 1H), 4.33 (m, 1H), 4.31–4.24 (m, 2H), 4.17 (m, 1H), 4.04 (m, 1H), 3.73 (s, 3H), 3.72 (s, 3H), 3.46–3.23 (m, 6H), 3.31 (s, 3H), 3.25 (s, 3H), 3.06 (s, 3H). ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, first diastereomer) −1.84. ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, second diastereomer) −2.07. HRMS (FAB) M^- calcd 1154.3508, obsd 1154.3540.

5′-*O*-Methyluridylyl-(3′,3′)-(2′,5′-di-*O*-methyluridine), 5′-*O*-methyluridylyl-(2′,3′)-(2′,5′-di-*O*-methyluridine) (9a,b). 5′-*O*-Methyluridine (**7**, 26.0 mmol, 6.7 g) was dissolved in anhydrous pyridine (100 mL) and chlorodimethylisopropylsilane (26.7 mmol, 4.2 L) was added. The reaction mixture was stirred for 2 h at 40 °C, after which it was evaporated to dryness. A conventional aq. $NaHCO_3$–CH_2Cl_2 workup was carried out and the product was purified on a silica gel column eluting with a mixture of MeOH, Et_3N and CH_2Cl_2 (5 : 1 : 94, v/v). The isomers were not separated at this point. The isomeric mixture (2.52 mmol, 0.9015 g) was coevaporated once from anhydrous pyridine. To the residue 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (3.15 mmol, 1.0 mL) and a solution of tetrazole (2.79 mmol, 0.195 g) in MeCN (6.2 mL) were added. After being stirred for 90 min at room temperature, the reaction mixture was evaporated to dryness. A conventional CH_2Cl_2–aq. $NaHCO_3$ workup was carried out and the combined organic phases were evaporated to dryness. Approximately half of the residue (1.12 mmol, 0.6264 g) and 2′,5′-di-*O*-methyluridine (**5**, 1.23 mmol, 0.3355 g) were coevaporated once from anhydrous MeCN and the residue was dissolved in anhydrous MeCN. A solution of tetrazole (1.26 mmol, 0.0882 g) in MeCN (2.8 mL) was added and the reaction mixture was stirred for 4 h at room temperature. Iodine (2.0 mmol, 0.52 g) in a mixture of THF (9.2 mL), water (4.6 mL) and 2,6-lutidine (2.3 mL) was added and the reaction mixture was stirred for an additional 2 h at room temperature, after which it was concentrated under reduced pressure. A CH_2Cl_2–aq. $NaHSO_3$ workup was carried out and the combined organic phases were evaporated to dryness. The product (a mixture of isomers) was purified on a silica gel column eluting with a mixture of CH_2Cl_2 and MeOH (95 : 5, v/v). The crude product mixture thus obtained was dissolved in saturated methanolic ammonia (15 mL). After being stirred for 4 h at room temperature, the reaction mixture was evaporated to dryness. The residue was dissolved in a mixture of acetic acid (6 mL), water (2 mL) and THF (6 mL) and the reaction mixture was stirred for 23 h at room temperature, after which it was reduced to dryness. The products were purified by HPLC on a Supelcosil LC-18 column (25 cm × 21.2 mm, 12 μm) eluting with a mixture of 0.06 mol L^{-1} of aqueous NaOAc and MeCN (89 : 11, v/v). Finally the buffer salts were removed on the same column by eluting with a mixture of water and MeCN (87 : 13, v/v). The overall yields starting from 2′(3′)-*O*-isopropyldimethylsilyl-5′-*O*-methyluridine 3′(2′)-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) were 2.4% (18.6 mg) and 4.5% (34.9 mg) for **9a** and **9b**, respectively. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6, **9a**) 7.72 (d, 1H, $J = 8.2$ Hz), 7.66 (d, 1H, $J = 8.1$ Hz), 5.85 (d, 1H, $J = 5.8$ Hz), 5.81 (d, 1H, $J = 6.9$ Hz), 5.70 (d, 1H, $J = 8.1$ Hz), 5.69 (d, 1H, $J = 8.1$ Hz), 4.56 (m, 1H), 4.54 (s, 1H), 4.38 (m, 1H), 4.18 (m, 1H), 4.13–4.08 (m, 2H), 3.90 (dd, 1H, $J_1 = 5.2$ Hz, $J_2 = 5.9$ Hz), 3.59–3.44 (m, 4H), 3.34 (s, 3H), 3.32 (s, 3H), 3.30 (s, 3H). ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6, **9b**) 7.68 (d, 1H, $J = 8.2$ Hz), 7.67 (d, 1H, $J = 8.2$ Hz), 5.86 (d, 1H, $J = 4.5$ Hz), 5.85 (d, 1H, $J = 6.3$ Hz), 5.71 (d, 1H, $J = 8.1$ Hz), 5.66 (d, 1H, $J = 8.0$ Hz), 4.54 (s, 1H), 4.52 (m, 1H), 4.41 (m, 1H), 4.18 (m, 1H), 4.10 (dd, 1H, $J_1 = 5.6$ Hz, $J_2 = 5.6$ Hz), 3.55–3.38 (m, 4H), 3.30 (s, 3H), 3.28 (s, 3H), 3.25 (s, 3H). ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, **9a**) −0.55. ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, **9b**) −0.82. ESI^--MS (**9a**): m/z 591.5 $[M - H]^-$. ESI^--MS (**9b**): m/z 591.2 $[M - H]^-$.

5′-*O*-Methyluridylyl-(3′,5′)-(2′,3′-*O*-methyleneadenosine), 5′-*O*-methyluridylyl-(2′,5′)-(2′,3′-*O*-methyleneadenosine) (10a,b). A mixture of 2′-*O*-isopropyldimethylsilyl-5′-*O*-methyluridine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) and 3′-*O*-isopropyldimethylsilyl-5′-*O*-methyluridine 2′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (see the synthesis of **9a,b** above, 1.01 mmol, 0.6072 g) and N^6-benzoyl-2′,3′-*O*-methyleneadenosine (**6**, 1.21 mmol, 0.4671 g) were coevaporated once from anhydrous MeCN. To the residue, a solution of tetrazole (1.22 mmol, 0.085 g) in MeCN (5.7 mL) was added and the reaction mixture was stirred for 4 h at room temperature. Iodine (2.0 mmol, 0.52 g) in a mixture of THF (9.2 mL), water (4.6 mL) and 2,6-lutidine (2.3 mL) was added and the reaction mixture was stirred for an additional 2 h at room temperature, after which it was concentrated under reduced pressure. A CH_2Cl_2–aq. $NaHSO_3$ workup was carried out and the combined organic phases were evaporated to dryness. The product (a mixture of isomers) was purified on a silica gel column eluting with a mixture of CH_2Cl_2 and MeOH (95 : 5, v/v). The crude product mixture thus obtained was dissolved in saturated methanolic ammonia (15 mL). After being stirred for 5 h at room temperature the reaction mixture was evaporated to dryness. The residue was dissolved in a mixture of acetic

acid (6 mL), water (2 mL) and THF (6 mL) and the reaction mixture was stirred for 23 h at room temperature, after which it was evaporated to dryness. The products were purified by HPLC on a Supelcosil LC-18 column (25 cm × 21.2 mm, 12 μm) eluting with a mixture of 0.06 mol L^{-1} of aqueous NaOAc and MeCN (89 : 11, v/v). Finally the buffer salts were removed on the same column by eluting with a mixture of water and MeCN (87 : 13, v/v). The overall yields starting from 2′(3′)-*O*-isopropyldimethylsilyl-5′-*O*-methyluridine 3′(2′)-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) were 8.5% (26.5 mg) and 12.2% (38.1 mg) for **10a** and **10b**, respectively. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6, **10a**) 8.40 (s, 1H), 8.32 (s, 1H), 7.60 (d, 1H, J = 8.2 Hz), 7.35 (s, 2H), 6.15 (d, 1H, J = 3.2 Hz), 5.78 (d, 1H, J = 6.9 Hz), 5.67 (d, 1H, J = 8.0 Hz), 5.28 (dd, 1H, J_1 = 3.0 Hz, J_2 = 6.4 Hz), 5.17 (s, 1H), 5.14 (s, 1H), 4.94 (dd, 1H, J_1 = 3.6 Hz, J_2 = 6.6 Hz), 4.28 (m, 1H), 4.22 (m, H), 4.03 (dd, 1H, J_1 = 5.1 Hz, J_2 = 6.8 Hz), 3.91 (m, 1H), 3.90–3.76 (m, 2H), 3.35–3.17 (m, 2H), 3.18 (s, 3H). ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6, **10b**) 8.40 (s, 1H), 8.21 (s, 1H), 7.65 (d, 1H, J = 8.0 Hz), 7.33 (s, 2H), 6.11 (d, 1H, J = 3.0 Hz), 5.81 (d, 1H, J = 4.7 Hz), 5.60 (d, 1H, J = 8.1 Hz), 5.29 (dd, 1H, J_1 = 3.2 Hz, J_2 = 5.8 Hz), 5.13 (s, 1H), 5.11 (s, 1H), 4.88 (dd, 1H, J_1 = 2.9 Hz, J_2 = 6.5 Hz), 4.37 (m, 1H), 4.23 (m, 1H), 3.99 (m, 1H), 3.91 (m, 1H), 3.83 (m, 2H), 3.55–3.42 (m, 2H), 3.29 (s, 3H). ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, **10a**) 0.29. ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6, **10b**) −0.92. ESI$^-$-MS (**10a**): m/z 598.2 [M − H]$^-$. ESI$^-$-MS (**10b**): m/z 598.3 [M − H]$^-$.

Kinetic measurements. Reactions were carried out in sealed tubes immersed in a thermostated water bath, the temperature of which was adjusted to 25 °C within ±0.1 °C. The reactions were started by adding the starting material in DMSO (20 μL) to 80 μL of 99 mmol L^{-1} HCl in 1,4-dioxane. After 70 min, 1520 μL of the desired reaction solution (prethermostated to 25 °C) were added. The hydronium ion concentration of the reaction solutions was adjusted with nitric acid and formate, acetate, MES, HEPES, triethanolamine and glycine buffers. The pH values of the buffers were checked with a pH meter. The ionic strength of the solutions was adjusted to 1.0 mol L^{-1} with $NaNO_3$. Between pH 3 and 6, considerable buffer catalysis was observed. In the case of acetate, for example, the second-order rate constant for the buffer-catalyzed cleavage was *ca.* 1.8×10^{-3} L mol^{-1} s^{-1}. Buffer-independent rate constants were obtained by varying the buffer concentration from 0.05 to 0.25 mol L^{-1} and extrapolating the observed pseudo first-order rate constants to zero buffer concentration by linear regression.

The initial substrate concentration in the kinetic runs was *ca.* 10^{-4} mol L^{-1}. The composition of the samples withdrawn at appropriate intervals was analyzed by HPLC on a Hypersil-Keystone Aquasil C18 column (4 × 150 mm, 5 μm) using 0.06 mol L^{-1} formic acid buffer (pH = 3) and MeCN as an eluent. The amount of MeCN was 7% for the first 15 min, after which it was increased linearly to 27% during 20 min. The observed retention times (t_R, min) for the hydrolytic products of **1** (the flow rate was 1.0 mL min^{-1}) were as follows: 33.6 (**1**), 25.1 (**9a**), 23.1 (**9b**), 20.0 (**10a**), 14.5 (**10b**), 8.9 (**5**), 7.2 (**8**). The products were identified by spiking with authentic samples and the peak areas were converted to relative concentrations by calibrating the system with uridine and adenosine solutions of known concentration. The pseudo first-order rate constants for the decomposition of **1** were obtained by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material.

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