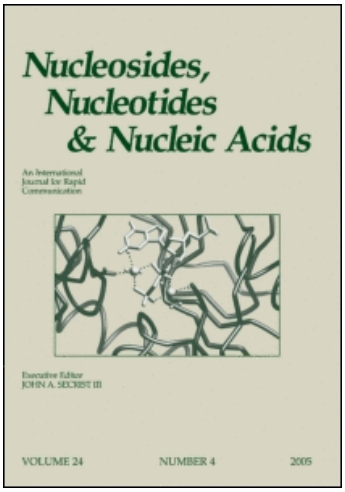

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EFFECTS OF 3'-*C*-METHYLATION ON THE HYDROLYTIC STABILITY AND HYDROXYL pK_a VALUES OF DINUCLEOSIDE 2',5'- AND 3',5'-MONOPHOSPHATES

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Abstract The first-order rate constants for hydrolysis of 3'-*C*-methyluridylyl(2',5')- and -(3',5')adenosine and the corresponding native dinucleoside monophosphates (2',5'- and 3',5'-UpA) have been determined as a function of hydroxide-ion concentration (0.025 - 7 M) at 25 °C. In addition to the effects on the hydrolytic stability of the compounds, the effects of the 3'-*C*-methyl substitution on the kinetically determined pK_a values for the sugar hydroxyls of the uridine moiety are discussed.

The 2'- and 3'-*C*-methylated,[1] as well as 5'-*C*-methylated analogs[2] of dinucleoside monophosphates have been employed as models for elucidating the nucleotide conformations in the productive enzyme-substrate complexes of RNA-cleaving enzymes. The substituent effects on the enzymic cleavage of the diester linkage were discussed in relation to the effects the same substituents have on the conformation of the nucleotides.[1,2] However, usage of modified nucleotides as mechanistic probes in enzymic studies requires as background information also good knowledge of the possible variations in chemical (non-enzymatic) reactivity. We have earlier compared the reactivities of the two isomeric cytidylyl-(3',5')-5'-*C*-methyluridines with those of the native dinucleoside monophosphates in hydrolysis catalyzed by acids, bases and metal ions.[3] In the present communication we report on the kinetics of the alkaline hydrolysis of 3'-*C*-methyluridylyl(3',5')- and -(2',5')adenosine (**1a** and **2a**, respectively) in comparison with the "native" 3',5'- and 2',5'-UpA (**1b** and **2b**, respectively). In addition to the hydrolytic stabilities, the present kinetic data also allows us to compare the ionization constants of the hydroxyl functions adjacent to the phosphodiester linkage. This is one of the crucial physico-chemical factors to be taken into account in enzymatic studies. In analogy to the chemical reaction, also the RNase catalyzed hydrolysis involves in its first step attack of the ionized 2'-hydroxyl on phosphorus atom.

RESULTS AND DISCUSSION

The hydroxide-ion-catalyzed hydrolysis of the internucleosidic phosphodiester bond of an RNA-sequence proceeds by intramolecular nucleophilic attack of the deprotonated neighbouring hydroxyl group on phosphorus

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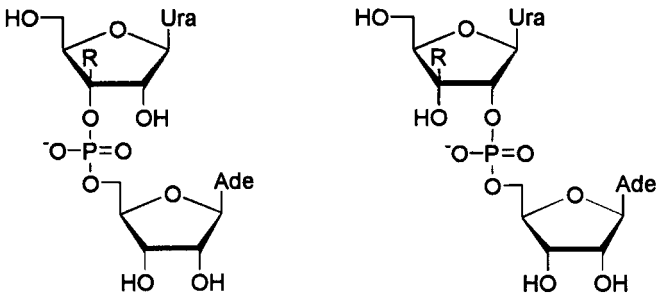
and concomitant departure of the 5'-esterified nucleoside (Scheme 1).[4,5] The 2',3'-cyclic monophosphate formed as an initial product does not accumulate, but is rapidly hydrolyzed to a mixture of 2'- and 3'-monophosphates. In consistence with this mechanism, the rate of hydrolysis of dinucleoside monophosphates[6] (as well as that of polyuridylic acid[7]) exhibits a first-order dependence on hydroxide-ion concentration over a wide pH-range (pH > 6), but levels off to a constant value under very alkaline conditions, where the hydroxyl group is completely deprotonated.[6] Accordingly, the acidity constant of the hydroxyl group adjacent to the internucleosidic phosphodiester linkage may be determined on the basis of the curvature of the pH-rate profile of hydrolysis.[6] We have now applied this method to compare the acidities of the 2'/3'-hydroxyl functions of **1a** and **2a** to those of 2',5'- and 3',5'-UpA (**1b**, **2b**). In order to do this, the hydrolyses of the compounds in sodium hydroxide solutions ([NaOH] = 0.025 - 7 mol dm^{-3}) at 298.2 K were followed by HPLC. The product analysis showed that the release of adenosine is accompanied by the accumulation of a mixture of 3'-*C*-methyluridine 2'- and 3'-monophosphates in a 3/7 mutual molar ratio [x(2'-P)/x(3'-P)], which is equal to that obtained[8] in the alkaline hydrolysis of 3'-*C*-methyluridine 2',3'-cyclic monophosphate. Figure 1 shows the logarithmic first-order rate constants obtained as a function of the basicity value $H\_$ of the solutions.[9] The rate profiles drawn for hydrolysis of 3'-*C*-MeU(3',5')A (**1a**) and for 3',5'- and 2',5'-UpA were obtained by a least-squares fitting of the observed first-order rate constants of hydrolysis to eq 1. Here, K_a stands for the acidity constant of the 2'- or 3'-hydroxyl group and k_b is the first-

$$k_{obs} = \frac{k_b}{\frac{1}{K_a a_{OH^-}} + 1} \qquad (1)$$

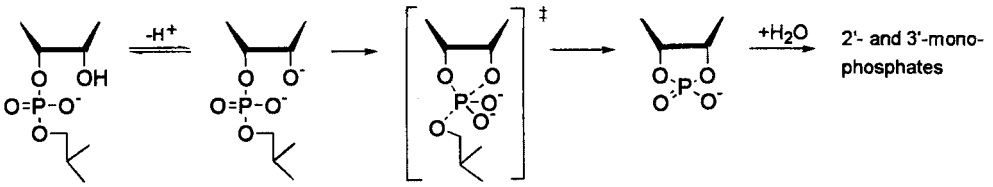
order rate constant of hydrolysis of the ionized species. The hydroxide ion activity (a_{OH^-}) is based on the $H\_$ scale.[9] The values obtained for the parameters are shown in Table 1. As expected, the 2'-hydroxyl of **1a** showed to be slightly, 0.4 pK_a units, less acidic than the corresponding function of the natural dinucleoside monophosphate (3',5'-UpA), but it is still nearly 0.5 units more acidic than the 3'-OH of 2',5'-UpA.

The 3'-*C*-methyl substitution increases the rate of hydrolysis of 3',5'-UpA by more than one order of magnitude. In principle, both inductive and steric effects of the substituent may affect the reactivity, but relative importance of the various factors is difficult to determine. However, the effects of the methyl substituent on sugar ring conformation appear to be rather moderate. 3'-*C*-Methyluridine has been shown[11] to slightly favor S-type puckering (x_S = 0.76 and 0.48 for 3'-*C*-MeUrd and uridine, respectively). The value of coupling constant $J_{1',2'}$ = 7.9 Hz for **1a** is comparable to those[11] obtained with 3'-*C*-methyl nucleosides, suggesting a same type of sugar ring puckering. Thus, the methyl group does not strictly "lock" the conformation in any form, and it is unlikely that the small conversion of conformational equilibrium could markedly affect hydrolytic stability. Another structural effect to be considered is that methylation of the 3'-carbon is known[10] to slightly lengthen the 3'-C-OH bond.[10] An analogous effect may take place even with dinucleoside monophosphates in solution, but the net effect of this on hydrolysis rate is difficult to propose. A possible rate enhancing effect could be reduced ring strain in the cyclic transition state, due to increased flexibility of the phosphate moiety. For comparison, however, neither 3'- nor 2'-*C*-methyl substitution affects markedly the rate of alkaline hydrolysis of 2',3'-cyclic



1a: R = CH_3
1b: R = H

2a: R = CH_3
2b: R = H



Scheme 1

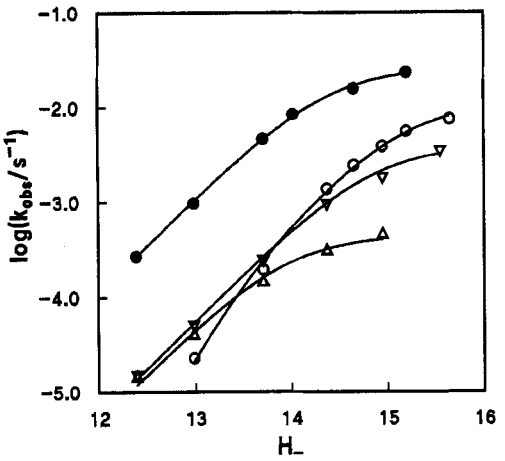


FIG. 1: Rate profiles for hydrolysis of 3'-*C*-MeU(3',5')pA (**1a**; ●), 3'-*C*-MeU(2',5')pA (**2a**; ○), 3',5'-UpA (**1b**; Δ) and 2',5'-UpA (**2b**; ▽) in sodium hydroxide solutions at 298.2 K.

TABLE 1. Kinetically determined pK_a values[a] for the 2'- or 3'-OH of 3'-*C*-MeU(3',5')A (**1a**), U(3',5')A (**1b**), and U(2',5')A (**2b**), and the first-order rate constant for the hydrolysis of the ionized species at 298.2 K.

compd	pK_a	$k_b/10^{-3}$ s^{-1}
3'-C-MeU(3',5')A (**1a**)	14.38 ± 0.05	26 ± 2
U(3',5')A (**1b**)	13.96 ± 0.09	0.44 ± 0.06
U(2',5')A (**2b**)	14.84 ± 0.08	3.8 ± 0.5

[a]The pK_a values refer to basicity function H_- (ref. 9).

uridine phosphate, but result only in 30 to 40 % rate decrease.[8] This suggests that the affects of the substituents on ring strain are not very dramatic. Furthermore, it may be good to note that 3'-*C*-methylation accelerates by more than one order of magnitude even the intramolecular transesterification of uridine 3'-monophosphate under acidic conditions, and that this effect was shown to result mainly from electronic effects making the tertiary phosphate more susceptible to intramolecular nucleophilic attack.[8] Anyway, whatever is the reason for the increased hydrolysis rate of **1a** compared to **1b**, it may be noted that the slight rate *decrease* due to the 3'-*C*-methyl substitution in RNase catalyzed hydrolyses does not correlate with the difference in chemical reactivities.[1]

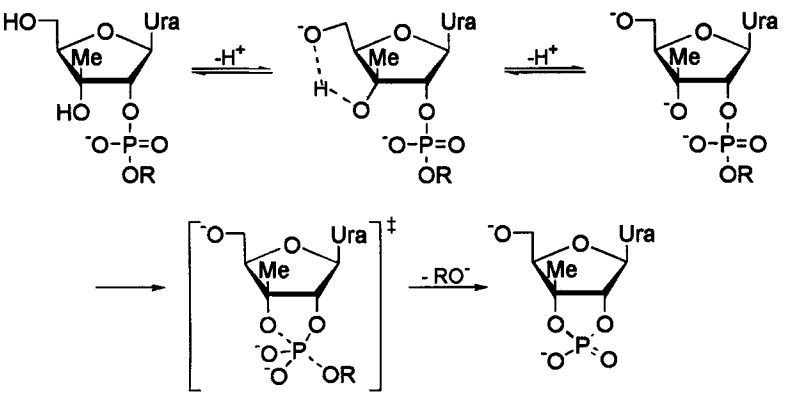
The hydrolytic behavior of the 2',5'-isomer of **1a**, *viz.* 3'-*C*-MeU(2',5')pA (**2a**), somewhat differs from that of either **1a** or the UpA's (**1b**, **2b**). The rate constants of hydrolysis of **2a** do not fit to eq 1, since the reaction order with respect to the hydroxide-ion seems to exceed unity at H_- < 14.5. Accordingly, a better fit was obtained with eq 2, where equilibrium constants for two subsequent pre-equilibrium deprotonations are included,

$$k_{obs} = \frac{k_b}{\frac{1}{K_{a1}K_{a2}(a_{OH^-})^2} + \frac{1}{K_{a2}a_{OH^-}} + 1} \qquad (2)$$

namely K_{a1} referring to dissociation of the 5'-*O*- and K_{a2} to dissociation of the 3'-*O*-proton of the uridine moiety. The values obtained for the parameters of eq 2 by the least-squares fitting are: k_b = (0.011 ± 0.002) s^{-1}, pK_{a1} = 13.3 ± 0.2, and pK_{a2} = 15.2 ± 0.2. According to this interpretation, the tertiary 3'-hydroxyl group of **2a** is about 0.4 pK_a units less acidic than the secondary 3'-hydroxyl of 2',5'-UpA (Table 1).

Tentatively, it may be thought that when either the 3'- or 5'-hydroxyl group of the uridine moiety of **2a** is deprotonated, an intramolecular hydrogen bond could be formed between the oxyanion and the remaining hydroxyl function (Scheme 2). The 3'-oxyanion could then act as a nucleophile towards the neighbouring phosphodiester group only, when also the 5'-hydroxyl is simultaneously deprotonated. This kind of behavior could be expected to bring about a second-order dependence of the hydrolysis rate on hydroxide ion activity.

It is not so obvious, however, why **2a** behaves in this respect differently from 2',5'-UpA (**2b**), which has exactly the same kind of rate profile of hydrolysis as the 3',5'-isomer. As mentioned above, 3'-*C*-methyl substitution both restricts the conformations of the nucleotide and lengthens the 3'C-OH bond.[10] Although these changes are not



Scheme 2

very marked, their affection may not be excluded. Of course, one has to bear in mind that the present kinetic data refer to reactions in sufficiently concentrated sodium hydroxide solutions, where several factors may contribute. Even specific salt and solvation effects may reflect somewhat differently on kinetics of the reactions involving either a tertiary or a secondary alkoxide ion. Nevertheless, the difference in rate profiles of hydrolysis of 2',5'-UpA and its 3'-*C*-methylated analog **2a** is clearly beyond experimental error and reveals some kind of variation in the reaction mechanisms to exist.

EXPERIMENTAL SECTION

General. NMR spectra were recorded on a Bruker AMX 400 spectrometer at 300 K in D_2O. The chemical shifts were related to the water signal at 4.60 ppm. The signals were assigned by the aid of phosphorus decoupling measurements. HPLC separations were carried out on a Hypersil ODS column (4 x 250 mm, 5 μm) using as eluent an acetic acid/sodium acetate buffer (pH 4.2) including 0.05 M ammonium chloride and 5 % (*v/v*) acetonitrile. The flow rate was 1 mL/min. The TLC-chromatography was performed on Kieselgel 60 F_{254} plates developed in 2-propanol/NH_3(aq, conc.)/water 7:1:2 (system B) or on PI-cellulose plates in 0.15 M NH_4HCO_3 (system C).

Materials. 2',5'- and 3',5'-UpA, as well as uridine 2'- and 3'-monophosphates and adenosine used as reference materials, were purchased from Sigma. Preparation of 3'-*C*-methyluridine 2',3'-cyclic monophosphate and characterization of its hydrolysis products (2'- and 3'-monophosphates) have been described earlier.[8]

3'-C-Methyluridylyl-(3',5')-adenosine (**1a**) and *3'-C-methyluridylyl-(2',5')-adenosine* (**2a**). The mixture of 5'-*O*-benzoyl-3'-*C*-methyluridine[12] (0.25 mmol) and N^6-acetyl-2',3'-di-*O*-acetyladenosine 5'-monophosphate (pyridinium salt, 0.38 mmol) was dried by coevaporations with dry pyridine and dissolved in 3 mL of the same solvent. *N,N'*-Dicyclohexylcarbodiimide (DCC, 1.1 mmol) was added and

the mixture was stored at 20 °C for 4 days. After addition of water (5 mL), the precipitating dicyclohexyl urea was filtered off and washed with 20 mL of 20 % aqueous pyridine. The combined filtrates were washed with ether (2 x 10 mL) and concentrated in vacuo. The residue was coevaporated with abs. toluene and methanol, and dissolved in 5 M methanolic ammonia (10 mL). After 2 days the solution was evaporated to dryness. The residue was dissolved in water (30 mL) and extracted with chloroform (2 x 15 mL). The aqueous layer was concentrated in vacuo to remove the traces of chloroform, and then applied to a column of DEAE-Toyopearl 650 M (200 mL, HCO_3^--form). The column was washed with water (500 mL) and eluted with concentration gradient of NH_4HCO_3 (from 0.02 M to 0.06 M, total volume 6 L). Fractions absorbing in the UV and eluted at a concentration of 0.027 M NH_4HCO_3 were combined, evaporated in vacuo, coevaporated with water (5 x 10 mL). Yield of **2a**: 0.055 mmol (22%). t_R (HPLC) 4.3 min (A). R_f 0.53 (B); 0.52 (C). ^{1}H NMR (400.13 MHz, D_2O): 8.20 s (1H, H-8 Ado), 8.10 s (1H, H-2 Ado), 7.69 d (1H, $J_{6,5}$ = 8.1 Hz, H-6 Urd), 5.99 d (1H, $J_{1',2'}$ = 7.8 Hz, H-1' Urd), 5.89 d (1H, $J_{1',2'}$ = 4.5 Hz, H-1' Ado), 5.18 d (1H, H-5 Urd), 4.50 dd (1H, $J_{2',P}$ = 9.8 Hz, H-2' Urd), 4.38 dd (1H, $J_{2',3'}$ = 5.0 Hz, H-2' Ado), 4.26 t (1H, $J_{3',4'}$ = 5.0 Hz, H-3' Ado), 4.06 m (1H, H-4' Ado), 3.89 m (3H, H-5'a, 5'b Ado, H-4' Urd), 3.62 dd (1H, $J_{5'a,4'}$ = 3.4 Hz, $J_{5'a,5'b}$ = -12.8 Hz, H-5'a Urd), 3.58 dd (1H, $J_{5'b,4'}$ = 4.0 Hz, H-5'b Urd), 1.25 s (3H, Me). ^{31}P NMR (161.98 MHz, D_2O) chemical shift in ppm from 80% phosphoric acid: -2.84

Compound **1a** was eluted at a concentration of 0.031 M NH_4HCO_3. Fractions were combined, evaporated in vacuo, coevaporated with water (5 x 10 mL). The yield of **1a** was 0.019 mmol (8%). t_R (HPLC) 7.4 min (A). R_f 0.53 (B); 0.41 (C). ^{1}H-NMR (D_2O): 8.26 s (1H, H-8 Ado), 8.08 s (1H, H-2 Ado), 7.69 d (1H, $J_{6,5}$ = 8.1 Hz, H-6 Urd), 5.94 d (1H, $J_{1',2'}$ = 5.2 Hz, H-1' Ado), 5.70 d (1H, $J_{1',2'}$ = 7.9 Hz, H-1' Urd), 5.68 d (1H, H-5 Urd), 4.61 dd (1H, $J_{2',3'}$ = 5.0 Hz, H-2' Ado), 4.42 dd (1H, $J_{4,5'a}$ = 3.5 Hz, $J_{4,5'b}$ = 4.0 Hz, H-4' Urd), 4.36 dd (1H, $J_{3',4'}$ = 4.7 Hz, H-3' Ado), 4.22 m (1H, H-4' Ado), 4.01 m (2H, H-5' Ado), 3.92 dd (1H, $J_{2',P}$ = 3.7 Hz, H-2' Urd), 3.57 dd (1H, $J_{5'a,5'b}$ = -11.8 Hz, H-5'a Urd), 3.55 dd (1H, H-5'b Urd), 1.36 s (3H, Me). ^{31}P NMR (161.98 MHz, D_2O) chemical shift in ppm from 80% phosphoric acid: -2.79

Kinetic Measurements. The hydrolyses of the dinucleoside monophosphate analogs were followed by the HPLC-method described previously.[6] The integrated first-order rate equation was applied to the diminution of the integrated peak area of the starting material.

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