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THE EFFECT OF PROTECTING GROUPS OF THE NUCLEOBASE AND THE SUGAR MOIETIES ON THE ACIDIC HYDROLYSIS OF THE GLYCOSIDIC BOND OF 2′-DEOXYADENOSINE: A KINETIC AND ^{15}N NMR SPECTROSCOPIC STUDY

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Abstract - The rate constants for the hydrolysis of several N^6-substituted 2′-deoxyadenosines were measured at different concentrations of oxonium ion in order to assess the role of various N^6 and sugar protecting groups in depurination reaction encountered in nucleic acid synthesis. The site of protonation was established by recording the ^{15}N NMR spectra in DMSO-d_6 both in the absence and presence of trifluoroacetic acid. The exceptional lability of the monocations of N^6-acyl-2′-deoxyadenosines has been accounted for by a preferred N7 protonation.

Studies over last ca 30 years have shown[1-8] the success in oligodeoxynucleotide synthesis by phosphodiester,[8] phosphotriester[1] and phosphite-triester[5] approaches to depend , among other factors, on a successful design of efficient N^6 and 5′-protected building block for 2′-deoxyadenosine. This is mainly due to the fact that the N-glycosidic linkage of N^6-benzoyl-2′-deoxyadenosine is appreciably more labile, by almost one order of magnitude, to acidic hydrolysis than the N-glycosidic linkage of 2′-deoxyadenosine itself.[1,9] Therefore, a considerable reduction of yield of 5′-hydroxy block is observed during acidic removal of 4,4′-dimethoxytriphenylmethyl (dimethoxytrityl)[8] or 9-phenylxanthen-9-yl (pixyl)[10] group from the 5′-end of a fully protected oligodeoxynucleotide. Several approaches have been suggested to remeady this problem, such as (1) the use of mild Lewis acids ($ZnBr_2$)[11] and different protic acids in mixed solvents[12] to remove 5′-dimethoxytrityl or pixyl group; (2) structural modifications of the dimethoxytrityl group which increased its lability under acidic[13] or basic conditions;[14] (3) other 5′-protecting groups[28] which are removable under nonacidic conditions; and (4) several N^6-protecting groups that reduce the depurination during the acidic removal of the 5′-protecting group. A few N^6-protecting groups are routinely used today as a partial solution to the depurination problem: N-methyl-2-pyrrolidineamidine,[2] 9-fluorenylmethoxycarbonyl,[3] 2-nitrophenylsulfenyl,[4] dialkylformamidine,[5] phthaloyl,[6] and 4,4′,4″-tris(benzoyloxy)trityl groups[7] have all been reported to stabilize the N-glycosidic bond. However, little is known about the mechanistic details of their actions.

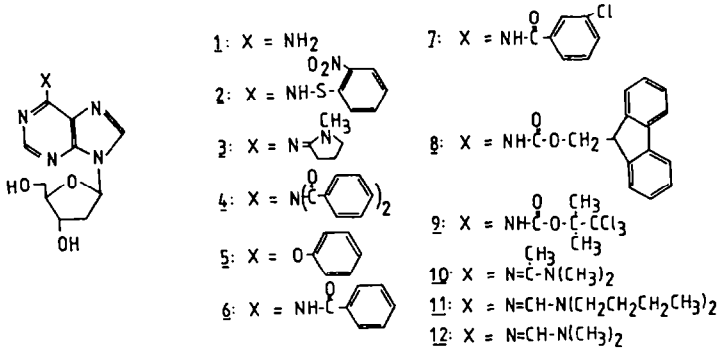
The acidic hydrolysis of 6-substituted 9-(2-deoxy-β-D-erythro-pentofuranosyl)-purines has been shown to proceed by a rate-limiting departure of the protonated base moiety with a concomitant formation of a cyclic glycosyl oxocarbenium ion.[15,16] In consistence with this mechanism, the polar nature of the 6-substituent has only a moderate effect on the rate of hydrolysis. Electron-withdrawing groups, for example,

decrease the standing concentration of the protonated substrate, but simultaneously they weaken the N-glycosidic bond. These opposite influences usually cancel each other almost completely. Accordingly, 9-(2-deoxy-β-D-erythro-pentofuranosyl)-6-methoxypurine decomposes only 1.5 times more rapidly than 2′-deoxyadenosine, although the basicity difference between these compounds is 2 pK units.[16] On these bases it is rather surprising that N^6-benzoyl group accelerates the hydrolysis of 2′-deoxyadenosine by one order of magnitude.[1]

The present paper is aimed to compare the effects of various base moiety protecting groups on the stability of the N-glycosidic bond, and to explain mechanistically the large destabilizing effect of the N^6-acyl groups. For this purpose, the rate constants for the partial reactions via mono- and diprotonated substrates have been calculated from the pH-rate profiles with the aid of spectrophotometrically determined acidity constants. The results are compared to the information that ^{15}N NMR spectroscopy gives on the relative basicities of the potential protonation sites. The influences of O′-acyl groups on the cleavage of the N-glycosidic bond have been discussed.

RESULTS AND DISCUSSION

Fig. 1 shows the first-order rate constants obtained at different concentrations of oxonium ion for the hydrolysis of the N-glycosidic bond of several protected 2′-deoxyadenosines (1-9). The pH-rate profiles fall in two distinct groups. With unprotected 2′-deoxyadenosine (1) and its N^6-(2-nitrophenylsulfenyl) (2), N^6-(N-methyl-2-pyrrolidineamidine) (3) and N^6-dibenzoyl (4) derivatives, as well as with 9-(2-deoxy-β-D-erythro-pentofuranosyl)-6-phenoxypurine (5), the reaction is apparently of first-order with respect to oxonium ion over the whole acidity range



studied. This kind of behavior has earlier been reported to be characteristic for the hydrolysis of 6-substituted purine nucleosides.[15-17] The linear rate profiles result from the fact that the rate constants, k_1/K_1 and k_2/K_2, referring to the partial reactions via the mono- and di-protonated species (Scheme 1) are almost equal. Consequently, the observed rate constant, expressed by eqn. (1), is approx-

$$k(\text{obs.}) = \frac{\frac{k_1}{K_1}[H^+] + \frac{k_2}{K_1K_2}[H^+]^2}{1 + \frac{[H^+]}{K_1} + \frac{[H^+]^2}{K_1K_2}} \qquad (1)$$

imately proportional to the concentration of oxonium ion over the whole acidity range.[17] In contrast, this appears not to be the case with N^6-monoacylated 2′-

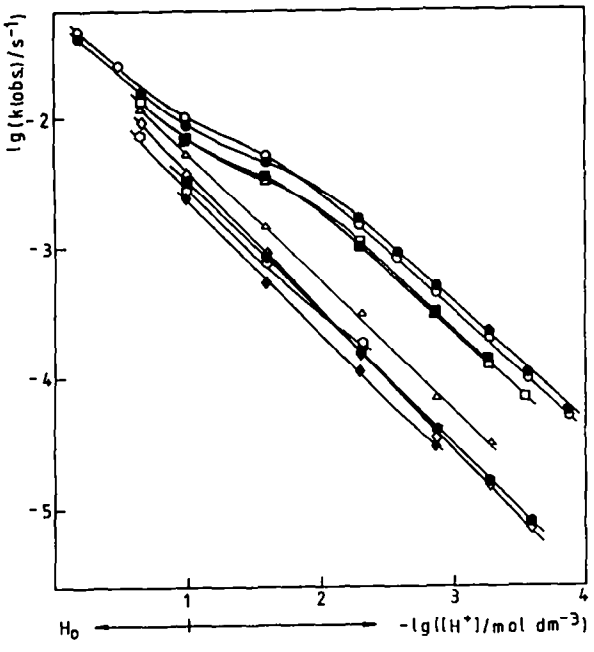


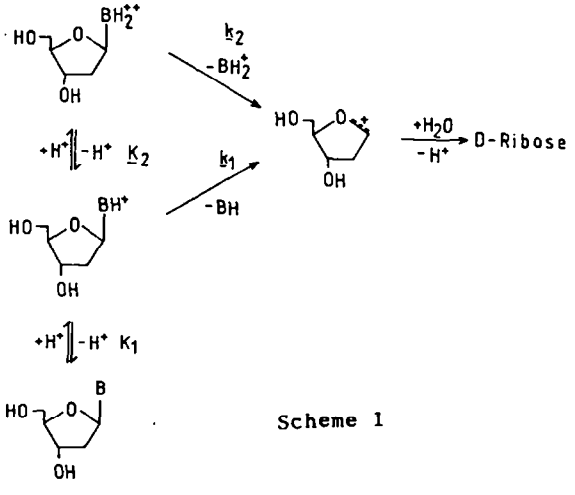
Fig. 1: Rate profiles for the hydrolysis of protected 2'-deoxyadenosines at 323.2 K. The ionic strength was adjusted to 0.1 mol dm^{-3} at $[H^+] < 0.1$ mol dm^{-3}. The values of H_0 were taken from Ref. 18. Notation: 1 (⬢), 2 (◇), 3 (◆), 4 (⬡), 5 (△), 6 (●), 7 (○), 8 (□) and 9 (■).

deoxyadenosines. N^6-Benzoyl (6), N^6-(3-chlorobenzoyl) (7), N^6-(9-fluorenylmethoxycarbonyl) (8) and N^6-(2,2,2-trichloro-tert.-butyloxycarbonyl) (9) derivatives all exhibit curvilinear rate profiles passing through an inflection point at pH = pK_1. The shape of the curves strongly suggests that with these compounds k_1/K_1 is considerably larger than k_2/K_2.

As shown previously, eqn. (1) may be approximated by eqn. (2) at $[H^+] < 10 \times K_1$, i. e. under conditions where the concentration of the diprotonated substrate is negligible compared to the sum concentration of the neutral and monoprotonated

$$k(\text{obs.}) = \frac{k_1[H^+] + \frac{k_2}{K_2}[H^+]^2}{K_1 + [H^+]} \qquad (2)$$

species.[17] The partial rate constants, k_1/K_1 and k_2/K_2, calculated by the latter



Scheme 1

Table 1: Rate and equilibrium constants for the partial reactions involved in the acidic hydrolysis of N^6-protected 2'-deoxyadenosines at 323.2 K.[a]

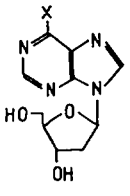
Compound	$k(obs.)/10^{-3}$ s^{-1} [b]		[c]	$-lg(K_1/mol\ dm^{-3})$	k_1/K_1 / $dm^3\ mol^{-1}\ s^{-1}$	k_2/K_2 / $dm^3\ mol^{-1}\ s^{-1}$
1	3.16	±0.03		3.67	0.027	0.025
2	3.63	0.03		1.38	0.025	0.043
3	2.34	0.03	0.034	4.77	[d]	
4	2.70	0.05		[e]		
5	5.15	0.10		[e]		
6	8.65	0.05		1.76	0.39	0.035
7	9.37	0.13		1.62	0.34	0.042
8	6.90	0.18		1.65	0.24	0.031
9	6.91	0.09		1.84	0.25	0.046
10	2.13	0.05	0.029		[d]	
11	1.49	0.02	0.023		[d]	
12	1.47	0.04	0.190		[d]	

[a] The rate and equilibrium constants are defined in Scheme 1. The ionic strength was adjusted to 0.10 mol dm^{-3}. [b] Observed rate constant for the rupture of the N-glycosidic bond in aqueous hydrogen chloride (0.10 mol dm^{-3}). [c] Observed rate constant for the cleavage of the N^6-protecting group at $[H^+] = 2.9\times10^{-4}$ mol dm^{-3}. [d] Could not be determined due to the cleavage of the N^6-protecting group. [e] The value of K_1 too small to be determined accurately.

equation from the observed rate constants and the spectrophotometrically determined acidity constants are collected in Table 1. For the N^6-amidine protected compounds (3,10-12) the partial rate constants could not be obtained, since the protecting group was cleaved under the conditions where the reaction through the monocation prevailed. The acidity constants of the N^6-dibenzoyl (4) and 6-phenoxy (5) derivative are in turn too small to be measured accurately.

As seen from Table 1, the values of k_2/K_2 are of the same order of magnitude with all the compounds studied, in consistence with the previous observation that the polar influences on the pre-equilibrium protonation and rate-limiting heterolysis cancel each other.[16] In contrast, the rate constants k_1/K_1 obtained with the N^6-monoacyl-2'-deoxyadenosines (6-9) are from 8 to 15 times larger than those for the hydrolysis of the other derivatives of 2'-deoxyadenosine. N^6-Acyl groups thus accelerate the hydrolysis via the monocations, but not the hydrolysis via dications. To understand this, the relative basicities of the potential protonation sites, viz. the N1 and N7 atoms, are considered in the following.

Table 2 summarizes the effects that addition of trifluoroacetic acid in DMSO solutions of purine 2'-deoxyribonucleosides exerts on their ^{15}N NMR chemical shifts. With 2'-deoxyadenosine (1), its N^6,N^6-dimethyl derivative (13) and unsubstituted purine 2'-deoxyriboside (14) only the N1 shift is significantly influenced, in con-



13: X = $N(CH_3)_2$

14: X = H

sistence with the previous suggestions[19,20] that these compound undergo N1 protonation. Analogously, N^6-amidine protected 2'-deoxyadenosines (3,12) exhibit largest

Table 2: ^{15}N NMR chemical shifts of purine 2′-deoxyribonucleosides in the absence and presence of trifluoroacetic acid.[a]

Compound		δ(N1)	δ(N3)	δ(N7)	δ(N9)	δ(N^6)	d
1	b	-145.7	-158.5	-140.7	-208.3	-299.5	0
	c	-209.1	-157.3	-138.5	-201.1	-292.1	
2	b	-141.0	-148.3	-141.0	-206.9	-310.4	0.24
	c	-147.9	-148.8	-143.2	-205.9	-309.4	
3	b	-123.6	-148.4	-136.9	-209.2	-177.3	0
	c	-190.4	-150.3	-136.0	-201.3	-197.9	
4	b	-112.2	-130.6	-141.2	-205.7	-208.3	e
	c	-112.2	-130.6	-141.5	-205.7	-208.3	
5	b	-138.2	-138.2	-141.3	-205.5		e
	c	-138.2	-138.9	-141.8	-205.5		
6	b	-121.7	-136.8	-137.8	-207.6	-249.7	0.72
	c	-129.2	-137.2	-156.7	-204.8		
7	b	-122.9	-136.4	-136.9	-207.4	-247.3	0.81
	c	-124.9	-136.4	-145.7	-206.2		
8	b	-127.7	-139.5	-139.5	-207.1	-269.8	0.44
	c	-136.6	-139.9	-146.4	-205.4	-262.5	
9	b	-124.8	-138.4	-140.9	-207.0	-269.0	0.44
	c	-133.1	-139.1	-147.3	-205.5	-267.4	
12	b	-130.4	-147.7	-137.3	-208.9	-170.7	0
	c	-187.3	-148.9	-137.4	-201.6	-201.6	
13	b	-146.3	-160.8	-136.8	-208.7	-303.6	0
	c	-175.5	-164.0	-135.9	-205.4	-297.1	
14	b	-101.8	-130.6	-139.1	-208.1		0
	c	-122.6	-130.6	-139.0	-206.4		

[a]Taken as ppm from $CH_3{}^{15}NO_2$ in DMSO-d_6. [b]In the absence of trifluoroacetic acid. [c]In the presence of 1 equivalent of trifluoroacetic acid. [d]$\Delta\delta(N7)/[\Delta\delta(N1)+\Delta\delta(N7)]$. [e]No protonation under the conditions employed.

changes in the N1 resonance, althouh the N^6 resonance is also shifted upfield upon protonation. In contrast, with N^6-monoacyl derivatives (6-9) both the N1 and N7 atoms appear to become protonated. The observed change in the relative basicity of the ring-nitrogens may partly, but not completely, result from the electron-withdrawing effect of the acyl group that lowers the electron density of the pyrimidine ring to a larger extent than that of the imidazole ring. For comparison, N^6-(2-nitrophenylsulfenyl)-2′-deoxyadenosine (2), which is even less basic (Table 1) than the N^6-monoacyl derivatives, is still predominantly protonated at N1. One may tentatively assume that N^6-acyl groups suppress the amidine resonance in the pyrimidine ring, as suggested by Maki et al.,[21] and hence the relative basicity of the N1 and N7 sites is changed. 6-Dibenzoylamino (4) and 6-phenoxy (5) groups retard the proton attachment to the purine ring so efficiently that the site of protonation cannot be determined by the method employed.

The preceding discussion suggests that the exceptionally facile hydrolysis of N^6-acyl substituted 2′-deoxyadenosines (6-9) through monoprotonated species results from a change in the site of protonation. Fig. 2 shows the dependence of the rate constant k_1/K_1 on the mole fraction of the N7 protonated substrate among the monocations. The latter quantity has been calculated by assuming that an attachment of a proton to either the N1 or N7 site has similar influences on the shifts of these atoms. Although the correlation between k_1/K_1 and x(N7) is not a good one, it suggests that N7 protonated adenine ring is cleaved considerably faster than its N1 protonated counterpart. A perfect correlation cannot be expected, since the site of protonation is not the only factor affecting the value of k_1/K_1. The influences of

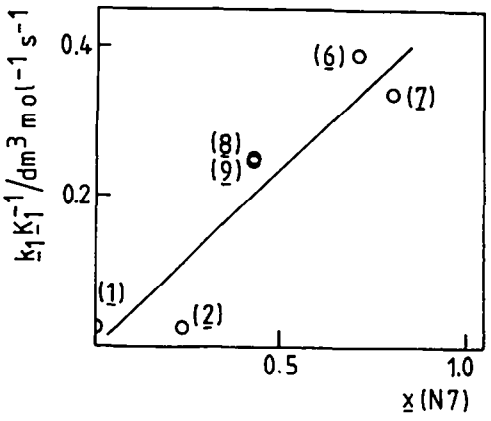


Fig. 2: Partial rate constant, k_1/K_1, for the hydrolysis of N^6-substituted 2′-deoxyadenosines plotted against the mole fraction, x(N7), of the N7 protonated species among the monocations. For k_1/K_1 see Scheme 1.

polar substituents on k_1 and K_1 are usually almost equal, and their ratio thus remains fairly constant. However, k_1/K_1 is not completely independent of the polar nature of the 6-substituent,[16] and hence rather large deviations from the correlation line depicted in Fig. 2 may be expected. The argument concerning the superiority of N7 protonated adenine ring as a leaving group is consistent with the results of the studies on the tautomeric equilibria of adenine. Several approaches have led to the conclusion that (7H)adenine is more stable than its 1H tautomer.[22] It should be noted that the preceding discussion refers only to the reaction via the monoprotonated substrate. At high oxonium ion concentrations the hydrolysis takes place mainly via the N1,N7-dication, and under such conditions all N^6-substituted 2′-deoxyadenosines are decomposed approximately as readily.

O′-Acyl groups, used frequently as protecting groups in oligonucleotide synthesis, are expected [1] to stabilize the N-glycosidic bond in three different manners: (1) by reducing the overall basicity of the base moiety, (2) by affecting the relative basicity of the potential protonation sites, and (3) by destabilizing the developing oxocarbenium ion. The data in Fig. 3 show that 3′-O-acetyl-2′-deoxyadeno-

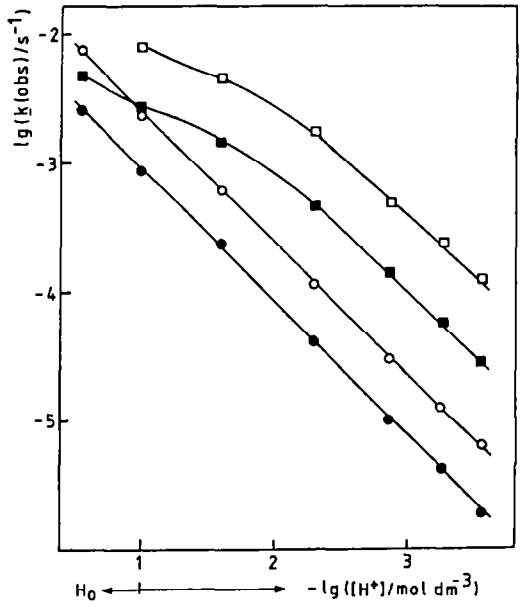
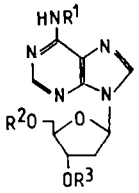


Fig. 3: Rate profiles for the hydrolysis of 3′-O-acetyl and 5′-O-benzoyl derivatives of 2′-deoxyadenosine and N^6-benzoyl-2′-deoxyadenosine at 323.2 K. The ionic strength was adjusted to 0.1 mol dm^{-3} at $[H^+] < 0.1$ mol dm^{-3}. The values of H_0 were taken from Ref. 18. Notation: 15 (●), 16 (■), 17 (○) and 18 (□).

sine (15) and its N^6-benzoyl derivative (16) are hydrolyzed about 4 times less readily than their 3′-hydroxy counterparts (1,6). The influence of 3′-O-acetyl



15: $R^1 = R^2 = H$, $R^3 = \overset{O}{\overset{\|}{C}}\text{-}CH_3$

16: $R^1 = \overset{O}{\overset{\|}{C}}$-Ph, $R^2 = H$, $R^3 = \overset{O}{\overset{\|}{C}}\text{-}CH_3$

17: $R^1 = R^3 = H$, $R^2 = \overset{O}{\overset{\|}{C}}$-Ph

18: $R^1 = R^2 = \overset{O}{\overset{\|}{C}}$-Ph, $R^3 = H$

19: $R^1 = R^3 = \overset{O}{\overset{\|}{C}}$-Ph, $R^2 = H$,

20: $R^1 = \overset{O}{\overset{\|}{C}}$-Ph, $R^2 = \overset{O}{\overset{\|}{C}}\text{-O-}CH_2$-(fluorenyl), $R^3 = H$

group on the overall basicity is small, the values of $-\lg(K_1/\text{mol dm}^{-3})$ obtained with 15 and 16 being 3.46 and 1.66, respectively. Similarly, the effect that 3′-O-acyl groups have on the relative basicity of the N1 and N7 atoms is hardly detectable. As seen from Table 3, 3′-O-acetyl (16) and 3′-O-benzoyl (19) groups decrease the x(N7) value of 6 from 0.72 to 0.71 and 0.68, respectively. The change of this magnitude is too small to be reflected to the reaction kinetics. As already mentioned, 3′-O-acetyl group does not retard the hydrolysis of 6 more than that of 1, although with the latter compound 3′-O-acetylation cannot cause any change in the site of protonation. Accordingly, the rate-retarding effect may be attributed to the electron-withdrawing influence of the 3′-O-acetyl group that lowers the electron density at C1′ and thus destabilizes the developing oxocarbenium ion.

As an electronegative substituent 5′-O-benzoyl group could also be expected to stabilize the N-glycosidic bond. Its influence on the overall basicity of 1 and 6 is very similar to that of 3′-O-acetyl group, the values of $-\lg(K_1/\text{mol dm}^{-3})$ obtained with 5′-O-benzoyl-2′-deoxyadenosine (17) and its N^6-benzoyl derivative (18) being 3.48 and 1.72, respectively. The relative basicity of the N1 and N7 atoms appears to be influenced even more markedly by 5′-O-acyl than by 3′-O-acyl groups (Table 3). Accordingly, it is rather surprising that 5′-O-benzoyl group has practically no effect on the hydrolysis rate of compounds 1 and 6 (Fig. 3). Since 5′-O-benzoyl group retards the pre-equilibrium protonation and decreases the mole fraction of the N7 protonated species, its influence on the rate-limiting heterolysis must be rate-accelerating. However, as an electronegative substituent

Table 3: ^{15}N NMR chemical shifts of O′-acyl derivatives of 2′-deoxyadenosine and N^6-benzoyl-2′-deoxyadenosine in the absence and presence of trifluoroacetic acid.[a]

Compound		δ(N1)	δ(N3)	δ(N7)	δ(N9)	δ(N^6)	d
16	b	-121.2	-137.3	-135.8	-210.0	-248.9	0.71
	c	-124.5	-137.5	-143.9	-209.0	-248.6	
17	b	-145.2	-156.5	-140.0	-208.5	-299.5	0
	c	-205.7	-156.4	-138.1	-202.1	-292.3	
18	b	-121.0	-135.7	-136.2	-208.5	-249.6	0.64
	c	-125.7	-136.1	-144.4	-207.3	-248.5	
19	b	-121.1	-137.3	-135.9	-210.2	-249.5	0.68
	c	-125.8	-137.6	-146.0	-208.7	-248.2	
20	b	-121.1	-136.0	-135.9	-208.6	-237.8	0.63
	c	-126.9	-136.5	-145.9	-207.2		

[a]For a - d see the footnote in Table 2.

it could be expected to retard the rupture of the N-glycosidic bond by decreasing inductively the stability of the oxocarbenium ion intermediate. One might tentatively assume that nonbonded interactions between the base moiety and the bulky 5′-substituent facilitate the bond rupture and thus cancel the rate-retarding inductive influences.

In summary, the site of protonation in the base moiety appears to be a factor that merkedly affects the rate of the acidic hydrolysis of purine nucleosides, and has to be taken into account besides the overall basicity of the leaving group and the stability of the developing oxocarbenium ion.

EXPERIMENTAL

Preparation of Compounds. The protected derivatives of 2′-deoxyadenosine were prepared according to reported procedures: 2,[4] 3,[2] 4 and 6-7,[23] 8,[24] 10-12,[5] 15-20.[9] The preparation of 13 and 14 has been described earlier.[16] Compound 5 was obtained by the method described for the corresponding 6-methoxy derivative.[16]

Kinetic Measurements. Kinetic measurements were carried out by the HPLC technique described in detail previously.[16]

Acidity Constants. The acidity constants for the monocations of the protected derivatives of 2′-deoxyadenosine were determined spectrophotometrically (Cary 17D) as described earlier.[25]

^{15}N NMR Measurements. ^{15}N chemical shift determinations were made on a Jeol GX 270 spectrometer at 27.4 MHz. All ^{15}N NMR spectra were performed relative to $CH_3{}^{15}NO_2$ in CD_3NO_2 in a capillary. The probe temperature was around 30 °C. The assignments of ^{15}N resonances were done by fully proton decoupled condition (NOE) or under an inverse gated proton-noise decoupled mode (without NOE), or using the polarization-transfer pulse sequences INEPT or DEPT. Routinely 16 K data points were used for the acquisition, zero filled to 32 K and Fourier transformed with a broadening factor of 2-3 Hz. The samples were dissolved in distilled DMSO. A negative value for the chemical shift denotes an upfield shift.

Assignment of ^{15}N Resonances. In 6-substituted purine 2′-deoxyribonucleosides each nitrogen experiences a long range coupling with a proton: N7 and N9 with H8, and N1 and N3 with H2. Therefore transfer of magnetization from H8 or H2 using INEPT or DEPT pulse sequences allow (1) a reduction of the accumulation time by ca 10 times, and (2) an unambigous assignment of N7 and N9.

All assignments of the chemical shifts listed in Tables 2 and 3 were made according to previous studies on purines.[26] There are three kinds of nitrogens in 6-substituted purine nucleosides: (1) the amino or amido nitrogens at C6, (2) the "azine- or pyridine-like" nitrogen, and (3) the "pyrrole-like" nitrogen. Amino or amido nitrogens absorb at a higher field than the ring nitrogens and the "N-pyrrole" (N9) absorb at a lower frequency than the "N-azine" nitrogens (N1,N3,N7). These three categories of nitrogens also have different $J(^{15}N,^{1}H)$ coupling constants. Both the nitrogen atoms of the imidazole part have a scalar coupling with H8, but the coupling constant between N7 and H8 is always larger (10-12 Hz) than that of N9 and H8 (7-9 Hz). On the other hand it is difficult to distinguish N1 and N3 on the basis of $^{2}J(^{15}NH)$ coupling constant alone, since they have a comparable coupling constant (15-20 Hz). However, substituent increments can be used[26,27] to determine N1 and N3 chemical shifts. Recently some 6-substituted purine ribonucleosides were studied by ^{15}N NMR spectroscopy and therefore some rules can be drawn.[27] N3 chemical shifts are expected to experience a long range influence of the 6-substitution and to have values within a small range. For example, when the substituent is an amide function the N3 lies in a range of ca -130 ppm to ca -140 ppm, while N1 is subjected to a shift between ca -110 ppm to -130 ppm. The exact value depends on the electron-withdrawing effect of the substituent. We found that N3 is always at a higher field than N1. Ambiguities occur when the 6-substituent is an oxygen atom, which is shared between an electron-withdrawing effect and a donating mesomeric effect. In those cases, it is likely the π-electron rich imidazole ring that has the same electronic effect[27] to the pyrimidine moiety as opposed to the O^6 substituent. Therefore N3 and N1 have similar ^{15}N chemical shifts and their assignment may be reversed.

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