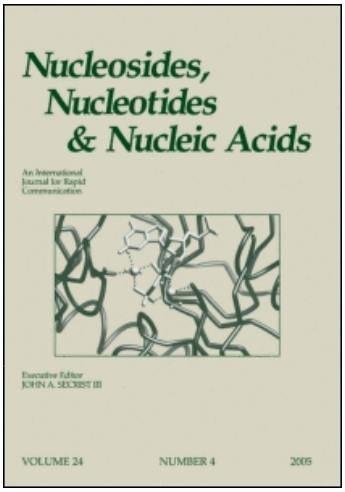

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Evaluation of the Kinetics of Hydrolysis of Monoamino Analogues of 2'- or 3'-Deoxyadenosine and of 9-(2-Deoxy-β-D-*Threo*-pentofuranosyl)Adenine or 9-(3-Deoxy-β-D-*threo*-pentofuranosyl)Adenine by Liquid Chromatography

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To cite this Article Thoithi, Grace , Van Schepdael, Ann , Busson, Roger , Herdewijn, Piet , Roets, Eugène and Hoogmartens, Jos(1995) 'Evaluation of the Kinetics of Hydrolysis of Monoamino Analogues of 2'- or 3'-Deoxyadenosine and of 9-(2-Deoxy-β-D-*Threo*-pentofuranosyl)Adenine or 9-(3-Deoxy-β-D-*threo*-pentofuranosyl)Adenine by Liquid Chromatography', Nucleosides, Nucleotides and Nucleic Acids, 14: 7, 1559 — 1579
To link to this Article: DOI: 10.1080/15257779508009492
URL: http://dx.doi.org/10.1080/15257779508009492

NUCLEOSIDES & NUCLEOTIDES, 14(7), 1559-1579 (1995)

EVALUATION OF THE KINETICS OF HYDROLYSIS OF MONOAMINO ANALOGUES OF 2'- OR 3'-DEOXYADENOSINE AND OF 9-(2-DEOXY-ß-D-*THREO*-PENTOFURANOSYL)ADENINE OR 9-(3-DEOXY-ß-D-*THREO*-PENTOFURANOSYL)ADENINE BY LIQUID CHROMATOGRAPHY

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Abstract. Liquid chromatography was used to follow the degradation of monoamino analogues of 2'- or 3'-deoxyadenosine and of 9-(2-deoxy-ß-D-*threo*-pentofuranosyl)adenine or 9-(3-deoxy-ß-D-*threo*-pentofuranosyl)adenine in buffers of different pH and constant ionic strength (μ). Comparison of stabilities of some of the compounds under study with those of corresponding hydroxyl analogues showed that at acid pH the aminated compounds are more stable than the corresponding hydroxyl compounds. The higher stability associated with the presence of an amino group in the sugar is explained in function of pK_a values, which were determined by ^{13}C NMR.

Introduction

Nucleoside analogues are important because of their antitumour (cytarabine, floxuridine, tioguanine analogues) and antiviral (aciclovir, ganciclovir, idoxuridine, ribavirin, trifluridine, vidarabine) activity. Zidovudine, zalcitabine, stavudine and didanosine are the only antiviral agents with activity against human immunodeficiency virus (HIV) that have been released for general use. Intensive research is focused on new drugs for treatment of acquired immunodeficiency syndrome (AIDS) and a variety of nucleosides with activity against HIV have been discovered. Fig. 1 shows

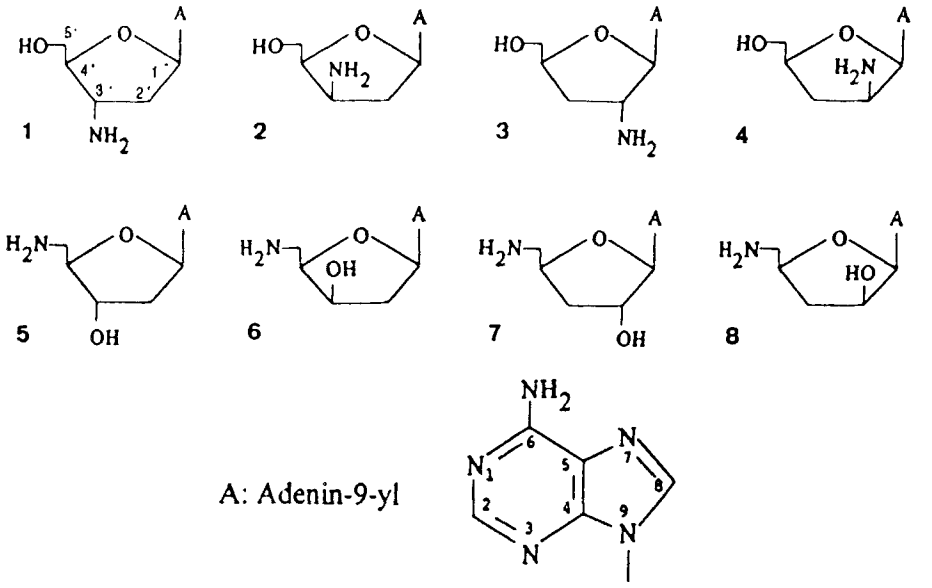


FIG. 1. Structures of monoamino analogues of 2'- or 3'-deoxyadenosine and 9-(2-deoxy-β-D-*threo*-pentofuranosyl)adenine or 9-(3-deoxy-β-D-*threo*-pentofuranosyl)adenine

the structures of monoamino analogues of 2′- or 3′-deoxyadenosine and of 9-(2-deoxy-ß-D-*threo*-pentofuranosyl)-adenine or 9-(3-deoxy-ß-D-*threo*-pentofuranosyl)adenine (**1-8**). Compounds **1-8** were synthesized as part of a structure-activity relationship study of analogues of 2′- or 3′-deoxyadenosine with potential activity against HIV, but none of them showed substantial antiretroviral or cytostatic activity[1].

In contrast to the many studies published[2-14] on stability of normal deoxynucleosides and dideoxynucleosides, there are no reports about stability of amino substituted nucleoside analogues. Therefore, we carried out stability studies on **1-8**. This knowledge might be useful when carrying out derivatisation reactions with these nucleosides. Besides, compounds **1-8** are also potential candidates for incorporation into oligodeoxynucleotides, serving as enzymatically stable substitutes for the natural deoxynucleosides. These

oligodeoxynucleotides can be targeted either at mRNA (antisense approach) or at dsDNA (antigene approach) functioning as potential therapeutics for the treatment of various kinds of infectious diseases or cancers.

The purpose of this study was to investigate the characteristics of hydrolysis of compounds **1-8** in acid and alkaline media and hence evaluate structure-stability relationships, pH-rate profiles and temperature-rate profiles.

Experimental

Samples and reagents

The synthesis of compounds **1-8**, 3′-deoxyadenosine (cordycepin) and 9-(3-deoxy-ß-D-*threo*-pentofuranosyl)adenine has been described elsewhere[1] and so has that of 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)uracil (αpdUrd) and 1-(2-deoxy-ß-D-*erythro*-pentopyranosyl)uracil (ßpdUrd)[15]. 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)uracil (αfdUrd) was synthesized according to a previously published procedure[16]. Adenine, 2′-deoxyadenosine (dAdo), 1-(2-deoxy-ß-D-*erythro*-pentofuranosyl)uracil (2′-deoxyuridine, dUrd) and 4,5,6-triaminopyrimidinesulphate hydrate were purchased from Acros Chimica (Geel, Belgium) and 4,6-diamino-5-formamidopyrimidine from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Buffers for the calibration of pH measurements were prepared following the European Pharmacopoeia[17]. Glycine hydrochloride (0.1 M) and 0.1 M potassium phosphate buffers of different pH values were used for the kinetic studies. In each case the ionic strength was adjusted to 0.4 with potassium chloride (Acros Chimica) before measuring the final pH. For each of the buffers at acid or alkaline pH, a KOH or HCl solution was prepared at a concentration that neutralized the buffer in equal volumes (about 0.1 M).

Apparatus

Liquid chromatography (LC) was carried out with a Milton Roy minipump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Marathon autosampler (Spark Holland, Emmen, The

Netherlands) equipped with a 20 μl loop, a Waters Model 441 Absorbance detector (Milford, MA, U.S.A.) set at 254 nm and a Hewlett Packard Model 3393 A integrator (Hewlett Packard, Avondale, PA, U.S.A.). A Waters Model 990 photodiode array (PDA) detector was used to record on-line UV spectra. A Spherisorb ODS1 10 μm column (250 x 4.6 mm I.D.) maintained at 25 °C by means of a water-jacket was used with a mobile phase containing acetonitrile - 0.2 M potassium phosphate buffer pH 6.0 - 0.2 M tetramethylammonium phosphate (TMA) pH 6.0 - water (5:5:0.5:89.5, v/v) at a flow rate of 1 ml/min. TMA (0.2 M) was prepared from a 20 % m/v solution of tetramethylammonium hydroxide in methanol and the pH was adjusted to 6.0 with 85 % phosphoric acid before the solution was brought to the final volume. pH was measured at room temperature with a Consort P 514 pH-meter (Turnhout, Belgium) using a Schott pH electrode (Mainz, Germany) for all measurements except for pK_a determinations, where a Hamilton Minitrode pH electrode (Bonaduz, Switzerland) was used on a CG 822 Schott pH meter. pK_a values were determined by ^{13}C NMR spectroscopy on a Jeol FX90Q (Tokyo, Japan) spectrometer for compounds **2**,**4** and **5** and a Gemini 200 (Varian, Palo Alto, CA, U.S.A.) spectrometer for compounds **1** and **3**.

Procedure

For the kinetic studies, vials containing 0.5 ml solution of the sample (approximately 1 x 10^{-4} M) in the buffer of appropriate pH were stored in a Memmert (Schwabach, Germany) oven. Vials were removed from the oven at regular intervals and quenched with an equal volume of corresponding neutralizing solution, mixed thoroughly and stored at -19 °C until they could all be analyzed as a series by LC. Since there is a linear relationship (for example : r = 0.9999 for **8** tested in a range of 0.05 x 10^{-4} to 5 x 10^{-4} M) between concentration of sample and detector response, the chromatographic peak area was used to represent the sample concentration. Rate constants were calculated from first-order plots of disappearance of compounds **1-8**.

For determination of pK_a values, a ^{13}C NMR spectrum of a nearly saturated solution of the sample in deuterium oxide, whose pH had previously been measured, was taken using dimethyl sulphoxide in a capillary as the external reference standard. pH adjustments were performed with 5 N HCl and 17.2 N NH_4OH.

Results and Discussion

Characteristics of hydrolysis

The degradation of compounds **1-8** exhibits pseudo-first order kinetics and Table 1 gives the observed rate constants of degradation, k.

Liquid chromatograms of the partially degraded samples of **1-8** at pH 1.15 indicated there is only one UV-absorbing degradation product, which corresponds to adenine. Mass balance calculations performed on the concentrations of adenine and compounds **1-6** at pH 1.15, 2.89, 4.77 and 6.87 and of **7** and **8** at pH 1.15, in partially degraded samples revealed that adenine is the only UV-absorbing compound formed and thus indicated that the only reaction occurring is hydrolysis of the glycosyl-purine bond. The homogeneity of the peaks corresponding to compounds **1-8** and the identity of adenine were further investigated and confirmed using a PDA detector. Formation of anomers and ring-isomers of compounds **1-8** could not be definitely excluded because the α- and β-pyranosyl and α-furanosyl forms of **1-8** were not available. However, deductions can be made by comparing the results with those for other nucleosides. Ring-isomers and anomers of 2′-deoxyuridine have been previously separated by reversed-phase LC[18]. Using the LC system developed for analysis of compounds **1-8**[19], partial separation of the α- and β-pyranosyl forms of 2′-deoxyuridine (αpdUrd, βpdUrd) was effected, but the α- and β-furanosyl (αfdUrd, dUrd) forms were not separated (capacity factors were 0.7, 0.9, 0.9 and 1.1 for αpdUrd, αfdUrd, dUrd and βpdUrd, respectively). If isomers of compounds **1-8** had been present in the samples, it is very likely that they would have been separated on the LC system because **1-8** are

TABLE 1. Pseudo-first order rate constants (± standard error) observed for degradation of **1-8** at 100°C and ionic strength 0.4 as a function of pH. Glycine hydrochloride buffer was used at pH 1.15 and potassium phosphate buffer at all other pH values.

Compound	pH	$k(h^{-1})$		$t_{½}(h)$	
1	1.15	35.69 ± 3.01	(n=18,x=8,y=5,z=2)	0.02 ±	0.002
	2.89	0.73 ± 0.02	(n=16,x=8,y=4,z=1)	0.95 ±	0.02
	4.77	0.05 ± 0.02	(n=30,x=8,y=2,z=2)	13.85 ±	0.51
	6.87	0.01 ± 0.001	(n=24,x=8,y=2,z=2)	65.13 ±	6.13
	8.85	0.004 ± 0.0001	(n=24,x=7,y=3,z=1)	170.97 ±	2.53
	11.93	0.06 ± 0.009	(n=26,x=9,y=4,z=1)	11.39 ±	0.17
2	1.15	24.65 ± 2.02	(n=26,x=8,y=5,z=2)	0.03 ±	0.002
	2.89	0.38 ± 0.02	(n=20,x=7,y=2,z=2)	1.82 ±	0.11
	4.77	0.03 ± 0.002	(n=16,x=4,y=3,z=2)	20.88 ±	1.18
	6.87	0.02 ± 0.0009	(n=27,x=8,y=1,z=2)	37.33 ±	1.83
	8.85	0.007 ± 0.0002	(n=14,x=5,y=3,z=1)	98.34 ±	3.28
	11.93	0.07 ± 0.001	(n=24,x=8,y=3,z=1)	10.29 ±	0.21
3	1.15	0.77 ± 0.02	(n=16,x=9,y=3,z=1)	0.90 ±	0.03
	2.89	0.06 ± 0.001	(n=15,x=6,y=2,z=1)	11.00 ±	0.25
	4.77	0.03 ± 0.003	(n=17,x=5,y=4,z=2)	21.10 ±	1.96
	6.87	0.003 ± 0.0002	(n=12,x=8,y=1,z=1)	227.96 ±	16.58
	8.85	0.002 ± 0.0001	(n=21,x=7,y=2,z=1)	327.08 ±	8.89
	11.93	0.06 ± 0.0006	(n=15,x=7,y=2,z=1)	12.38 ±	0.13
4	1.15	8.62 ± 0.49	(n=22,x=8,y=5,z=2)	0.08 ±	0.005
	2.89	0.78 ± 0.06	(n=17,x=6,y=6,z=2)	0.89 ±	0.07
	4.77	0.13 ± 0.03	(n=26,x=8,y=4,z=2)	5.41 ±	0.11
	6.87	0.01 ± 0 0004	(n=29,x=8,y=3,z=2)	63.96 ±	2.54
	8.85	0.003 ± 0.0001	(n=14,x=6,y=3,z=1)	206.10 ±	6.78
	11.93	0.02 ± 0.0007	(n=19,x=8,y=2,z=1)	31.54 ±	0.95
5	1.15	40.00 ± 3.24	(n=24,x=8,y=6,z=2)	0.02 ±	0.001
	2.89	0.51 ± 0.02	(n=28,x=8,y=3,z=2)	1.35 ±	0.06
	4.77	0.01 ± 0.0002	(n=15,x=8,y=4,z=1)	57.10 ±	1.14
	6.87	0.006 ± 0.0005	(n=18,x=6,y=5,z=2)	116.63 ±	10.19
	8.85	0.004 ± 0.0001	(n=21,x=7,y=3,z=1)	195.40 ±	2.92
	11.93	0.05 ± 0.0009	(n=16,x=8,y=3,z=1)	14.86 ±	0.28
6	1.15	13.54 ± 1.35	(n= 8,x=5,y=2,z=1)	0.05 ±	0.005
	2.89	0.90 ± 0.04	(n=30,x=8,y=3,z=2)	0.77 ±	0.03
	4.77	0.02 ± 0.0008	(n=23,x=6,y=4,z=2)	33.30 ±	0.33
	6.87	0.006 ± 0.0004	(n=22,x=6,y=4,z=2)	109.68 ±	7.39
	8.85	0.005 ± 0.0001	(n=17,x=6,y=3,z=1)	127.51 ±	2.32
	11.93	0.08 ± 0.002	(n=15,x=6,y=3,z=1)	9.03 ±	0.24
7	1.15	0.28 ± 0.02	(n=29,x=8,y=2,z=2)	2.50 ±	0.20
	2.89	0.01 ± 0.0005	(n=24,x=7,y=2,z=2)	49.21 ±	1.74
	4.77	0.003 ± 0.0003	(n=16,x=6,y=2,z=2)	257.19 ±	28.14
	6.87	0.005 ± 0.0004	(n=22,x=7,y=4,z=2)	151.59 ±	13.62
	8.85	0.004 ± 0.0001	(n=21,x=7,y=3,z=1)	196.67 ±	6.81
	11.93	0.14 ± 0.004	(n=15,x=5,y=4,z=1)	4.89 ±	0.13
8	1.15	1.40 ± 0.08	(n=22,x=6,y=6,z=2)	0.49 ±	0.03
	2.89	0.04 ± 0.001	(n=32,x=8,y=4,z=2)	17.91 ±	0.55
	4.77	0.003 ± 0.0002	(n=23,x=6,y=2,z=2)	222.07 ±	12.55
	6.87	0.005 ± 0.0003	(n=21,x=6,y=4,z=2)	131.69 ±	7.10
	8.85	0.004 ± 0.0002	(n=16,x=7,y=3,z=1)	172.94 ±	6.46
	11.93	0.05 ± 0.0008	(n=24,x=8,y=3,z=1)	14.78 ±	0.09

n: Total number of chromatographic observations, x: points on the time axis, y: amount of half-lives tested and z: number of independent experiments done.

eluted more slowly than dUrd isomers, but separation of any isomers was not observed. It therefore seems that isomerization of **1-8** did not occur at pH 1.15 and consequently the C_1,-O_4, bond most likely did not open during degradation.

For compounds **7** and **8** at pH 2.89-6.87 a side-product was formed which eluted slightly faster than adenine and amounted to about 10 % of total chromatographic area at most. The UV spectrum of these side-products was identical to that of compound **8B** (discussed below) suggesting that the process by which **8B** is formed may be present at pH 2.89 - 6.87 though at a lower rate than at basic pH. Its low retention indicates that it does not have a sugar moiety, but it is unlikely to be a degradation product of adenine because adenine is stable under the conditions used. Therefore, it seems that for **7** and **8**, another reaction, besides the cleavage of the glycosyl-purine bond, is occurring in the said acid pH region.

At pH 8.85 and 11.93 LC analysis and on-line UV spectra of samples indicated that the main degradation product of compounds **1-8** is adenine. Other minor degradation products that eluted before adenine, such as **6B** and **8B** in Figure 2, were formed for **1-8**. In addition, compound **6** gave a product (**6C** in Fig. 2) that eluted between adenine and **6**. The UV spectra of these minor degradation products are very different from those of the starting compounds and this suggests that they are not ring-isomers or anomers of **6** and **8**. The UV spectra are also different from those of 4,6-diamino-5-formamidopyrimidine and 4,5,6-triaminopyrimidine, which are some of the expected intermediates in alkaline hydrolysis of adenine nucleosides[10].

pK_a values

In order to understand structure-stability relationships and pH-rate profiles better, a study of the basicity of compounds **1-5** was carried out. That of compounds **6-8** could not be done because of lack of sufficient material, but it is expected that the pK_a values of **6-8** would be similar to those of **5**. Table 2 shows ^{13}C NMR chemical shifts

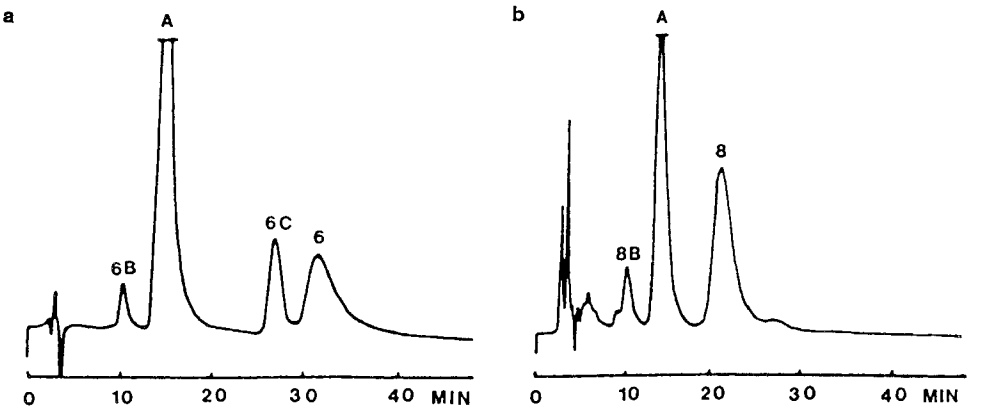


FIG. 2. Liquid chromatogram of a) compound **6** degraded at pH 11.93 and 100°C for 22.5 hours b) compound **8** degraded at pH 11.93 and 100°C for 24 hours. Column: Spherisorb ODS1 10 μm, 250 mm x 4.6 mm I.D. Mobile phase acetonitrile-0.2 M potassium phosphate buffer pH 6.0-0.2 M tetramethylammonium phosphate pH 6.0-water (5:5:0.5:89.5, v/v). Flow: 1 ml/min. Temperature: 25°C. Detection: UV at 254 nm. A: adenine. 6B, 6C and 8B: other degradation products of compounds **6** and **8**, respectively.

TABLE 2. ^{13}C NMR chemical shifts (ppm) of various carbon atoms and pK_a values of N_1 and sugar amino groups of compounds **1-5**

	C_2	C_6	C_8	$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$
Compound **1**								
pH 1.02	144.9	150.3	143.9	85.1	36.0	51.03	84.1	61.4
pH 2.66	146.1	151.0	143.0	b	36.0	b	84.1	b
pH 3.27	147.6	152.0	142.4	b	36.0	b	84.0	b
pH 4.18	151.2	154.4	140.9	b	35.9	b	83.9	b
pH 4.97	152.3	155.2	140.4	b	35.9	b	83.9	b
pH 5.72	152.4	155.2	140.3	b	36.0	b	84.0	b
pH 6.29	152.5	155.3	140.3	b	36.3	b	84.2	b
pH 6.77	152.4	155.2	140.2	b	36.6	b	84.4	b
pH 7.15	152.4	155.2	140.1	b	37.1	b	85.0	b
pH 7.82	152.4	155.2	140.1	b	38.8	b	86.6	b
pH 8.10	152.3	155.0	139.9	b	39.2	b	87.0	b
pH 9.48	152.3	155.0	139.8	b	40.1	b	87.8	b
pH 10.07	152.3	155.0	139.8	b	40.1	b	87.9	b
pK_a	3.5	3.5	3.5		7.4		7.4	
	mean pK_a for N_1=3.5				mean pK_a for $C_{3'}$ NH_2=7.4			

Table 2 (continued)

	C_2	C_6	C_8	$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$
Compound **2**								
pH 0.78	145.3	150.7	145.3	84.9	36.5	52.3	79.8	59.6
pH 2.52	146.3	151.4	144.9	b	36.5	b	79.8	59.6
pH 3.44	149.5	153.8	143.8	b	36.5	b	79.8	59.7
pH 4.50	152.1	155.8	143.0	b	36.5	b	79.9	59.9
pH 5.80	152.5	156.0	142.8	b	36.4	b	79.8	59.8
pH 6.88	152.5	156.0	142.6	b	36.5	b	79.9	59.8
pH 7.97	152.5	a	142.4	b	36.9	b	80.2	60.0
pH 9.32	152.6	155.8	141.2	b	39.2	b	81.9	61.3
pH 11.77	152.7	155.8	141.0	b	39.8	b	82.4	61.6
pK_a	3.1	3.1	3.1		9.0		9.0	9.0
	mean pK_a for N_1=3.1				mean pK_a for $C_{3'}$ NH_2=9.0			
Compound **3**								
pH 1.09	145.1	150.4	143.1	88.2	56.0	30.3	81.9	62.7
pH 2.00	145.5	150.7	143.0	88.1	b	30.3	b	b
pH 2.92	147.3	151.9	142.4	88.0	b	30.3	b	b
pH 3.67	150.6	154.1	141.2	87.8	b	30.2	b	b
pH 4.25	152.1	155.1	140.6	87.7	b	30.3	b	b
pH 5.04	152.8	155.6	140.4	87.8	b	30.3	b	b
pH 5.89	152.9	155.7	140.3	88.2	b	30.7	b	b
pH 6.62	152.8	155.7	140.3	89.5	b	31.9	b	b
pH 7.57	152.7	155.6	140.4	91.1	b	33.3	b	b
pH 8.48	152.7	155.7	140.4	91.7	b	33.8	b	b
pH 9.66	152.7	155.7	140.4	91.9	b	33.9	b	b
pH 11.61	152.7	155.7	140.4	91.9	b	33.9	b	b
pK_a	3.6	3.5	3.5	6.6		6.6		
	mean pK_a for N_1=3.5				mean pK_a for $C_{2'}$ NH_2=6.6			

(continued)

Table 2 (continued)

	C_2	C_6	C_8	$C_{1'}$	$C_{2'}$	$C_{3'}$	$C_{4'}$	$C_{5'}$
Compound **4**								
pH 0.74	145.5	150.6	143.5	85.0	53.3	29.8	80.0	62.1
pH 1.75	145.9	150.7	143.5	85.3	53.2	29.9	b	b
pH 2.59	147.0	151.5	143.1	85.2	53.2	30.0	b	b
pH 3.74	151.3	154.4	141.5	85.1	53.1	30.2	b	b
pH 5.33	153.3	155.8	140.8	85.2	53.3	30.6	b	b
pH 6.50	153.3	156.0	140.9	86.1	54.2	31.7	b	b
pH 7.74	152.8	155.7	140.8	86.8	54.9	32.6	b	b
pH 12.12	152.8	155.7	140.8	87.0	55.1	32.8	b	b
pK_a	3.3	3.3	3.3	6.2	6.2	6.2		
	mean pK_a for N_1=3.3				mean pK_a for C_2, NH_2=6.2			
Compound **5**								
pH 0.80	144.9	a	144.2	86.4	39.0	72.4	83.4	42.1
pH 2.00	145.1	150.6	144.1	86.3	b	b	83.3	41.9
pH 3.13	146.1	151.3	144.0	86.5	b	b	83.5	42.2
pH 4.47	152.6	155.4	141.2	85.6	b	b	83.1	42.0
pH 7.10	152.9	155.8	141.0	85.6	b	b	83.1	42.0
pH 7.27	152.9	a	140.9	85.5	b	b	83.2	42.0
pH 8.74	152.9	155.6	140.5	85.0	b	b	85.0	42.5
pH 9.23	153.1	155.9	140.5	84.7	b	b	86.3	42.9
pH 10.16	153.1	a	140.3	84.2	b	b	87.8	43.4
pH 13.03	153.3	156.0	140.4	84.1	b	b	88.5	43.7
pK_a	4.1	4.1	4.2	9.0			8.9	9.0
	mean pK_a for N_1=4.1				mean pK_a for C_5, NH_2=9.0			

a: Chemical shift could not be determined because the signal was too small to be distinguished and b: chemical shift showed negligible change with respect to pH.

of some selected atoms of **1-5** at different pH values, from which pK_a values were calculated.

Upon protonation of the sugar amino groups, the most diagnostic change was that of the carbon atoms in ß position to the amino groups, which showed an upfield shift. When N_1 was protonated, sp^2 carbons C_2 and C_6 (both α to N_1) had an upfield shift and C_8 showed a downfield shift. The pK_a values were determined by calculation of the second derivative of the changes in chemical shifts of various carbon atoms against pH. The pH at which the second derivative of the chemical shifts was equal to zero corresponded with the inflexion point in the titration curve and thus gave the pK_a value. Each final pK_a value is a mean of the pK_a values calculated for the respective sugar or base carbons.

pK_a values indicate that the C_2, NH_2 of compounds **3** and **4** is the least basic of the amino groups and this is easily explained by its proximity to the strongly electron withdrawing imidazole group (N_9). The C_5, NH_2 of **5** is the most basic because it is farthest from the base moiety. It is not clear how the different spatial positions of the sugar amino groups of **1** and **2** can explain their different basicity or why the pK_a of **2** is as high as that of **5**. For **1-4**, pK_a of N_1 is almost the same and about 1 unit lower than that of N_1 of adenine (pK_a = 4.18[20]). This is probably because of strong electron withdrawal of the protonated sugar amino groups which reduces basicity of N_1. In **5**, the amino group is far from the base and has the least effect on the base and so basicity of **5** is similar to that of adenine.

Structure-stability relationships

At pH 1.15, comparison of the rates of hydrolysis (Table 1) of **7** vs **5** and **8** vs **6** shows that the presence of a hydroxyl group on $C_{2'}$ stabilizes the compounds more than the presence of the same group on $C_{3'}$. This is consistent with the report of other workers[4,9] that the 2′-hydroxyl group has a stronger stabilizing effect against acid hydrolysis than the 3′-hydroxyl group in adenine nucleosides. Garrett and Mehta[4] worked on, amongst other compounds, adenosine, dAdo, 3′-

deoxyadenosine and adeninyl ß-arabinoside and ß-xyloside. Some of the compounds that York[9] worked on include adenosine, dAdo, 3′-deoxyadenosine, 2′,3′-dideoxyadenosine and adeninyl ß-xyloside, arabinoside and lyxoside. The stabilisation against hydrolysis associated with the hydroxyl group substitution is likely to be primarily due to an inductive, electron withdrawing effect which diminishes the electron density at the C_1,-O_4, region and hence destabilizes the intermediate cyclic oxocarbenium ion which is formed in the rate-determining step[12]. Since inductive effects are effected through bonds, a C_2, hydroxyl group has a greater stabilizing effect against hydrolysis than the more remote C_3, hydroxyl group.

Likewise, similar comparison of the rates of hydrolysis of **3** vs **1** and **4** vs **2** at pH 1.15 reveals that an amino group on C_2, stabilizes the compounds more than if this same group is present on C_3,. Again, a protonated amino group, like a hydroxyl group, causes rate retardation of nucleoside acid hydrolysis by its negative inductive effect.

Orientation of the amino or hydroxyl groups on C_2, has a distinct though small influence on the rate constants of the pairs of epimers **3,4** and **7,8** at pH 1.15. Our results are in agreement with those of Garrett and Mehta[4] and York[9], who found that the orientation of the hydroxyl group in the C_2, and C_3, position had some influence on acid hydrolysis of adenine nucleosides. The epimer with the hydroxyl group in the "up" position is less stable than the one in the "down" position. This can be explained in terms of steric strain. In the compounds which have the amino or hydroxyl group in the "up" position, these groups are in *cis* configuration to the N-glycosidic bond and the steric strain of the *cis* configuration accounts for the increased hydrolysis rate[9].

On the contrary, for the pairs **1,2** and **5,6** at pH 1.15, the epimer with a C_3, NH_2 or C_3, OH in the up position (**2** and **6**, respectively) is more stable than the one with a group in the down position (**1** and **5**, respectively). This may be due to other structural factors which are not known at the moment.

For example, intramolecular hydrogen bonding between N_3 of the base and proton donor groups on the sugar may reduce basicity of N_1, thus reducing hydrolysis, or it may lead to a conformation which stabilises or perhaps destabilises the more planar oxocarbenium ion intermediate.

At pH 2.89 and 4.77, N_1 of compounds **1-8** is not fully protonated. For example, at pH 2.89 compounds **1-4** are protonated 60-80 % and only 2-5 % at pH 4.77, whereas compound **5** is protonated 94 % at pH 2.89 and 18 % at pH 4.77. The N_1 non-protonated species which apparently should have a much lower hydrolysis rate may affect the structure-stability relationships discussed above.

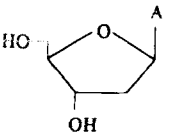
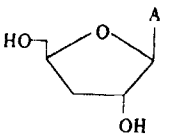
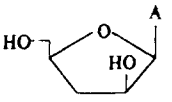
The stability of **1-8** in alkaline media has little or no relation to the position of the amino and hydroxyl groups on the sugar moiety and the configuration of these groups at C_2, and C_3, (Table 1). This is in agreement with the report of Garrett and Mehta[5], and Lehikoinen et al.[10], that the position and orientation of the sugar hydroxyl groups does not play an important role in the alkaline cleavage of adenine nucleosides.

Table 3 shows pseudo-first order rate constants for some hydroxyl analogues of **1-8** at pH 1.15 and 11.93, ionic strength 0.4 and at 100 °C. The rate constants at pH 1.15 were determined at 40 °C, because the compounds were too labile at 100 °C. For 2'-deoxyadenosine, the rate constant obtained at pH 1.15 and 40 °C was converted to pseudo-first order rate constant at 100 °C using the following formula derived from the Arrhenius equation :

$$\log \frac{k_1}{k_2} = \frac{-E_a}{2.303\ R} \times \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \qquad (1)$$

where E_a is the energy of activation, T is the absolute temperature and R is the gas constant. E_a of dAdo was obtained from literature[9] in which the experiments were carried out at pH 0.96 and 40 °C. Since the mechanism of reaction for acid hydrolysis at pH 0.96 and 1.15 is the same, E_a is expected to be the same at these two pH values.

TABLE 3. Pseudo-first order rate constants, k (h^{-1}), observed for degradation of some deoxynucleosides at ionic strength 0.4 and pH 1.15 and 11.93

Compound	pH	Temperature	k(h^{-1})	$t_{1/2}$(h)
	1.15	40°C	3.81	0.18
2'-Deoxyadenosine	11.93	100°C	0.06	11.33
	1.15	40°C	0.018	38.93
3'-Deoxyadenosine	11.93	100°C	0.25	2.74
	1.15	40°C	0.11	6.38
9-(3-deoxy-β-D-*threo*-pentofuranosyl)adenine	11.93	100°C	0.10	7.23

Therefore, at pH 1.15, 100 °C and μ = 0.4, dAdo has a rate constant of 1121 h^{-1}. It can be concluded that compounds **1,3, 4,5,7** and **8** are more stable than the corresponding hydroxyl analogues at pH 1.15, but at pH 11.93 there is little difference in stability.

The difference in acid stability can be explained in terms of protonation of the sugar amino group which takes place before protonation of the heterocyclic base. A protonated amino group has a stronger negative inductive effect and therefore lowers the electron density at $C_{1'}$-O_4, more than a corresponding hydroxyl group. In contrast to acid-catalyzed hydrolysis, substitution of the sugar hydroxyl

groups with amino groups has little or no influence on the rate of base-catalyzed hydrolysis. In alkaline hydrolysis, the hydroxide ion attacks C_8 of the base[5,10] and thus there is no significant difference expected for the effect of the sugar amino and hydroxyl groups on electron density at C_8.

pH-rate profiles

The rate constant, k, may be dependent on the ionic strength of the medium because of a primary salt effect on the activity coefficients of the reactants involved[21,22]. Due to this dependence of k on μ, μ was maintained at 0.4 by addition of KCl throughout the study. There was no general catalysis by either glycine hydrochloride or potassium phosphate buffers at pH 2.89, μ = 0.4 and 100 °C (concentration range : 0.05 M - 0.2 M) for degradation of compound **1**, and a literature survey[2-4,8,11,13] also indicates that purine nucleosides do not undergo general acid-base catalysis. Therefore, the results obtained with the two different kinds of buffers, indicated in Table 1, were pooled to establish pH-rate profiles. Fig. 3 shows the pH-rate profiles of compounds **1-8**. Compounds **1-8** exhibit a linear negative slope in the lower region of their pH-rate profiles.

The slope is unity (-1.01, r = 0.9992) between pH 1.15 and 4.77 for **5** and non-unity for **1-4**, **6-8**. All the slopes tend to flatten towards neutral pH and positive non-unity slopes are exhibited between pH 8.85 and 11.93. The negative slopes of **1-8** are due to acid catalysis of the compounds. pK_a values (Table 2) of **1-5** indicate that at pH 2.89 and 4.77 there are two different ionic species of the compounds in question, namely the mono- (sugar amino group) and diprotonated (sugar amino group and N_1) forms which are in equilibrium and are degraded at different rates resulting in non-unity slopes for **1-4**. It is not clear why compound **5** has a unity slope but it is worth noting that at pH 2.89 and 4.77 the base moiety of **5** is more protonated than those of **1-4**. Other factors that are known to cause non-unity slopes are general acid-base catalysis, variable ionic strength[22] and different processes being in operation[23]. However, in this

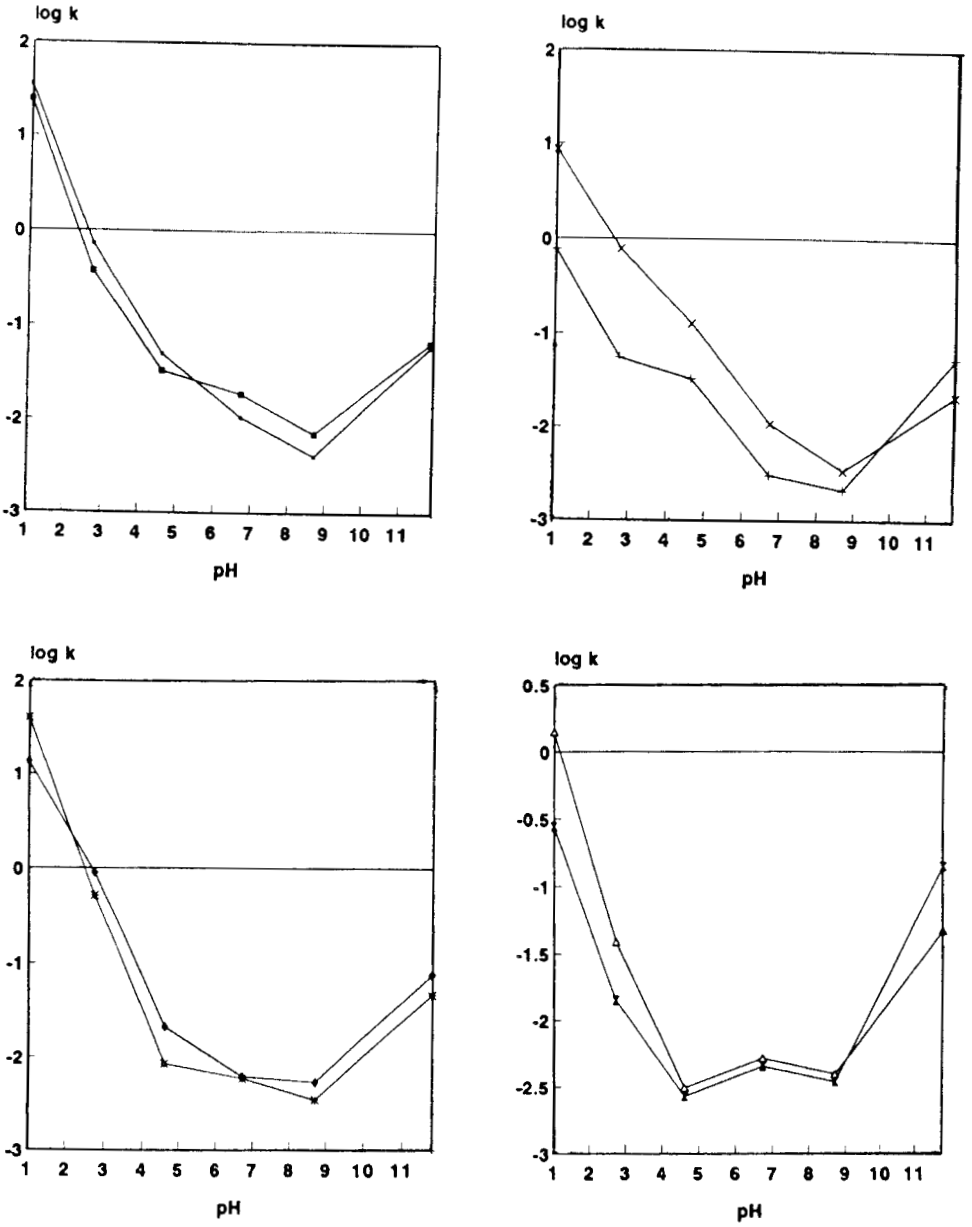


FIG. 3. pH-rate profiles of compounds 1-8.

—o— : compound 1, —■— : compound 2, —+— : compound 3, —✳— : compound 4,
—✳— : compound 5, —◆— : compound 6, —⧖— : compound 7, —△— : compound 8.

study the buffer and ionic strength were controlled. Examination of characteristics of hydrolysis showed that only one process was taking place, namely cleavage of the N-glycosidic bond, for **1-6** at all pH values examined below 6.87. For compounds **7** and **8**, in addition to hydrogen ion catalysis, there are several processes occurring at pH 2.89-6.87, as indicated by the presence of various degradation products which are similar to those shown in Fig. 2B. The flattening of the profile towards neutral pH is believed to be due to spontaneous hydrolysis through uncatalyzed reaction with water, which gains more and more importance as acidity is lowered. The positive non-unity slopes are due to hydroxide ion catalysis, which gives other by-products besides adenine.

Temperature-rate profiles

Table 4 shows rate constants for degradation of the compounds under study at pH 6.87 and at different temperatures. Regression analysis was performed on these data in accordance with the logarithmic form of the Arrhenius equation, from which E_a and k_{25}, the rate constant at pH 6.87 and 25 °C, were calculated. The enthalpy and entropy of activation at 25 °C, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, were calculated from equations (2) and (3) respectively.

$$\Delta H^{\neq} = E_a - RT \qquad (2)$$

$$\Delta S^{\neq} = \frac{\Delta H^{\neq}}{T} - R\ln\frac{T}{k} - R\ln\frac{k}{h} \qquad (3)$$

where k is the Boltzmann constant and h is Planck's constant. E_a, $\Delta H^{\neq}$, $\Delta S^{\neq}$ and k_{25} are given in Table 5.

Entropy of activation has been used as a criterion for distinguishing between unimolecular, A_1, and bimolecular, A_2, mechanisms of hydrolysis. It has been suggested[24] that A_1 reactions should be expected to have small entropies of activation of either positive or negative sign and all A_2 reactions large negative entropies.

Entropy of activation values for compounds **1-6** at neutral pH range from -10.9 to +15.7 e.u. and this suggests that the rate-determining step is of unimolecular nature.

TABLE 4. Pseudo-first order rate constants (± standard error) for degradation of **1-8** in 0.1 M potassium phosphate buffer at pH 6.87 and at ionic strength 0.4 as a function of temperature

Compound	T(°C)	$k(h^{-1}x10^{-3})$			$t_{½}(h)$	
1	90.0	4.49	± 0.32	(n=29,x=8,y=4,z=2)	154.31	± 11.06
	100.0	10.64	± 1.00	(n=24,x=8,y=2,z=2)	65.13	± 6.13
	116.3	61.03	± 1.87	(n=31,x=8,y=2,z=2)	11.36	± 0.35
2	90.0	4.21	± 0.34	(n=18,x=7,y=4,z=2)	164.43	± 13.37
	100.0	18.56	± 0.91	(n=27,x=8,y=1,z=2)	37.33	± 1.83
	115.2	102.08	± 6.77	(n=14,x=4,y=4,z=2)	6.79	± 0.45
3	90.0	1.61	± 0.11	(n=23,x=7,y=1,z=2)	429.87	± 30.66
	100.0	3.04	± 0.22	(n=12,x=8,y=1,z=1)	227.96	± 16.58
	115.2	21.79	± 1.72	(n= 6,x=4,y=1,z=1)	31.81	± 2.52
4	90.0	3.65	± 0.23	(n=20,x=7,y=3,z=2)	189.86	± 11.74
	100.0	10.84	± 0.43	(n=29,x=8,y=3,z=2)	63.96	± 2.54
	115.2	47.56	± 1.99	(n=22,x=7,y=4,z=2)	14.57	± 0.61
5	90.0	1.36	± 0.09	(n=17,x=7,y=1,z=2)	510.01	± 35.60
	100.0	5.94	± 0.52	(n=18,x=6,y=5,z=2)	116.63	± 10.19
	115.2	35.71	± 2.16	(n=26,x=7,y=5,z=2)	19.41	± 1.17
6	90.0	1.09	± 0.10	(n=25,x=8,y=1,z=2)	663.51	± 57.95
	100.0	6.22	± 0.42	(n=22,x=6,y=4,z=2)	109.68	± 7.39
	115.2	36.10	± 3.46	(n=24,x=7,y=5,z=2)	19.20	± 1.84
7	90.0	1.15	± 0.08	(n=23,x=7,y=1,z=2)	601.82	± 40.39
	100.0	4.57	± 0.41	(n=22,x=7,y=4,z=2)	151.59	± 13.62
	115.2	11.61	± 0.75	(n=31,x=8,y=4,z=2)	59.70	± 3.87
	124.0	28.97	± 0.72	(n=12,x=6,y=2,z=2)	23.92	± 0.60
8	90.0	1.60	± 0.23	(n=22,x=7,y=1,z=2)	432.96	± 61.66
	100.0	5.26	± 0.28	(n=21,x=6,y=4,z=2)	131.69	± 7.10
	115.2	16.81	± 1.23	(n=15,x=6,y=2,z=2)	41.24	± 0.41
	124.0	27.34	± 1.14	(n=10,x=7,y=2,z=2)	25.35	± 1.06

n: Total number of chromatographic observations, x: points on the time axis, y: amount of half-lives tested and z: number of independent experiments done.

TABLE 5. Activation energy (E_a), enthalpy and entropy of activation ($\Delta H^{\neq}$ and $\Delta S^{\neq}$), and rate constants (k_{25}) at 25°C of **1-8** at pH 6.87 and ionic strength 0.4.

Compound	E_a(kcal.mol^{-1})	$\Delta H^{\neq}$(kcal.mol^{-1})	$\Delta S^{\neq}$(e.u.)	k_{25}(h^{-1})
1	28.1	27.4	-10.9	7.60×10^{-7}
2	35.2	34.4	+9.1	1.23×10^{-7}
3	29.5	28.7	-9.8	1.39×10^{-7}
4	28.5	27.7	-10.0	6.98×10^{-7}
5	36.1	35.4	+9.3	2.85×10^{-8}
6	38.5	37.7	+15.7	1.34×10^{-8}
7	25.6	24.9	-19.2	7.62×10^{-7}
8	23.7	23.0	-24.2	1.70×10^{-6}

This is consistent with the report of other investigators[3,4,6,7,9,12,14] who found similar slightly negative or positive $\Delta S^{\neq}$ (-11.3 to +12.6 e.u.) values for purine nucleosides' hydrolysis, and in conjunction with other criteria, concluded that these nucleosides hydrolyse by a unimolecular mechanism.

Compounds **7** and **8** have entropy of activation values of -19.2 and -24.2 e.u., respectively. These larger negative values of $\Delta S^{\neq}$ do not point to a rate determining step of unimolecular nature. Working on 2',3'-dideoxyadenosine, Anderson et al.[11] calculated the entropy of activation of the hydrogen ion-catalyzed hydrolysis of the protonated species, the uncatalyzed hydrolysis of the neutral species and the alkaline hydrolysis and obtained values of +12.2, -18.8 and -33.8 e.u., respectively. In this study, the thermodynamic parameters were obtained at neutral pH, at which uncatalyzed hydrolysis predominates. pH-rate profiles indicate that alkaline hydrolysis is superimposed on uncatalyzed hydrolysis for **7** and **8**. This, according to the results of Anderson et al., might explain the larger negative values of $\Delta S^{\neq}$.

Acknowledgements

G. Thoithi thanks the Belgian Administration for Development Cooperation (A.B.O.S.) for a scholarship. The donation of 3'-deoxyadenosine by Prof. W. Pfleiderer is gratefully acknowledged. The authors thank A. Decoux and I. Quintens for fine secretarial assistance.

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Received March 3, 1993
Accepted April 26, 1995