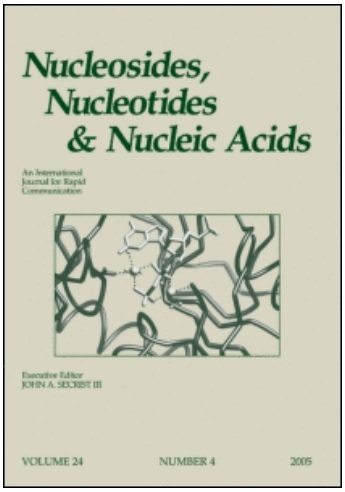

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INVESTIGATION OF THE KINETICS OF DEGRADATION OF HEXOPYRANOSYLATED CYTOSINE NUCLEOSIDES USING LIQUID CHROMATOGRAPHY

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Dedicated to the memory of Gertrude B. Elion

Abstract. Liquid chromatography was used to follow the degradation of hexopyranosylated cytosine nucleosides in buffers of acid, neutral and alkaline pH and of constant ionic strength. The compounds were found to degrade by hydrolysis to cytosine and/or by deamination to the corresponding uracil nucleosides. Degradation in acid is influenced by the number of sugar hydroxyl groups, presence of sugar double bonds and the type of anomer. Stability of some of the compounds was compared with that of related thymine nucleosides. Temperature studies support a unimolecular mechanism of hydrolysis at pH 1.22.

Introduction

The challenge to synthesize oligonucleotides which are enzymatically stable, but still apt to form stable duplexes has prompted, amongst others, modifications on the sugar moiety. Nucleosides with a hexopyranose sugar moiety instead of the naturally occurring pentofuranose sugar have been synthesized for incorporation into oligonucleotides. Oligonucleotides containing 1-(2,3-dideoxy-β-D-*erythro*-hexopyranosyl)thymine, 1-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine and 1-(3,4-

complement[1-4]. The structure of 2,4-dideoxy-β-D-*erythro*-hexopyranose is particularly interesting because it closely resembles that of the natural deoxyribose. Compound **1** (Figure 1) was synthesized as part of a series of 2,4-dideoxy-β-D-*erythro*-hexopyranosyl nucleosides for incorporation into oligonucleotides[5]. Synthesis of this series was also important from an antiviral point of view since the discovery of anhydrohexitol nucleosides as potent antivirals[6]. Compounds **2-6** (Figure 1) were synthesized as part of a structure-activity relationship study of nucleoside analogues active against human immunodeficiency virus (HIV), but on evaluation none of them displayed any antiviral activity, probably due to poor intracellular phosphorylation[7-9]. They are not recognized as substrates by cellular or viral kinases and hence are devoid of antiviral activity.

Nevertheless, for future research in this field it can be important to have some idea about the structure stability relationship in such nucleosides. Therefore, the aim of this work was to investigate the degradation of hexopyranosylated cytosine nucleosides in buffers of acid, neutral and alkaline pH and of constant ionic strength and hence evaluate characteristics of hydrolysis, structure-stability relationships and temperature-rate profiles as well as to compare the stability with that of related nucleosides.

Experimental

Samples, solvents and reagents

The synthesis of compounds **1**[5], **2**[7,9], **3**[7], **4**[7,9], **5**[8] and **6**[8,9] has been described. Cytosine (Cyto), cytidine (Cyd), 2'-deoxycytidine (dCyd), uracil (Ura), uridine (Urd), 2'-deoxyuridine (dUrd) and sodium octanesulphonate were obtained from Acros Organics (Geel, Belgium). Methanol was distilled before use. LC-grade acetonitrile was from Rathburn (Walkerburn, Scotland). Water was distilled twice in glass apparatus. Other reagents were of pro-analysi quality (Acros Organics).

Apparatus and operating conditions

Liquid chromatography (LC): LC apparatus consisted of an SP 8700 XR solvent delivery system (Thermo Separation Products, Fremont, CA, U.S.A.), used at a flow rate of 1 ml/min; an injector Model CV-6UH-Pa-N-60 (Valco, Houston, TX, U.S.A.) equipped with a 20 µl loop; a Merck-Hitachi (Darmstadt, Germany) Model L-4000 variable UV detector set at 270 nm and a Hewlett-Packard Model 3396 integrator (Avondale, PA, U.S.A.). The stationary phase was PLRP-S 100 Å 8 µm (Polymer

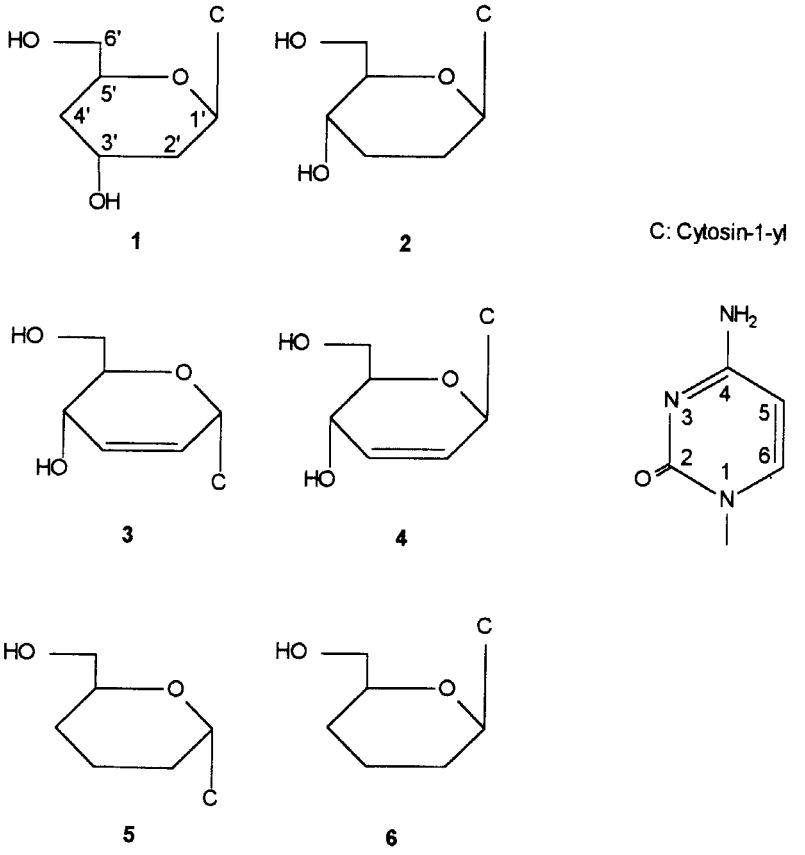


FIG. 1. Structures of compounds **1-6**

1: 1-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)cytosine

2: 1-(2,3-dideoxy-β-D-*erythro*-hexopyranosyl)cytosine

3: 1-(2,3-didehydro-2,3-dideoxy-α-D-*erythro*-hexopyranosyl)cytosine

4: 1-(2,3-didehydro-2,3-dideoxy-β-D-*erythro*-hexopyranosyl)cytosine

5: 1-(2,3,4-trideoxy-α-D-*glycero*-hexopyranosyl)cytosine

6: 1-(2,3,4-trideoxy-β-D-*glycero*-hexopyranosyl)cytosine

Laboratories, Church Stretton, Shropshire, U.K.), in a 250 mm x 4.6 mm I.D. column, whose temperature was equilibrated by means of a water bath. The mobile phase consisted of acetonitrile - 0.02 M sodium octanesulphonate pH 2.5 - 0.2 M potassium phosphate buffer pH 2.5 - water (X:25:50:25-X, v/v, where X is 1 for cytidine, 5 for **1-4**

and 10 for **5-6**). Development of the liquid chromatographic system is described elsewhere[10].

Other apparatus: Samples for kinetic studies were stored in a Memmert (Schwabach, Germany) oven, fitted with a calibrated thermometer. A Waters Model 990 Photodiode Array (PDA) detector (Milford, MA, U.S.A.) was used to record on-line UV spectra. pH was measured at room temperature with a Consort P 514 pH meter (Turnhout, Belgium) using a Schott pH electrode (Mainz, Germany). ^{1}H NMR spectroscopy was done on a Gemini 200 (Varian, Palo Alto, CA, U.S.A.) spectrometer. Mass spectra were recorded on a Kratos 1H mass spectrometer (Manchester, U.K.).

Procedure

Kinetic studies: Sodium dihydrogen citrate buffer (0.01 M) was adjusted to pH 1.22 using 1.0 N HCl. Appropriate amounts of 0.01 M sodium dihydrogen phosphate and 0.01 M disodium hydrogen phosphate solutions were mixed to give 0.01 M sodium phosphate buffer of pH 6.76. Similarly, sodium phosphate buffer of pH 11.99 was prepared from 0.01 M disodium hydrogen phosphate and 0.01 M trisodium phosphate solutions. The ionic strength of the buffers at pH 6.76 and 11.99 was adjusted to 0.07 (to match that of the buffer at pH 1.22) with potassium chloride before measuring the final pH. Vials containing 0.5 ml solution of the sample (approximately 10^{-4} M) in the buffer of appropriate pH were stored in an oven. Vials were removed at regular intervals and quenched with an equal volume of the corresponding neutralizing solution (0.1 N KOH, water, or 0.01 N HCl), mixed thoroughly and immediately frozen at -20 °C till they could all be analyzed as a series by LC. Samples were stored in the freezer for a maximum of 1 month. The maximum length of time between thawing and LC analysis of a sample was 10 h. For determination of temperature dependence of the kinetics, the procedure was repeated at pH 1.22 and 11.99 and at supplementary temperatures.

Results and Discussion

Characteristics of degradation

The degradation of compounds **1-6** in acidic, neutral and alkaline buffers exhibited pseudo-first order kinetics. The kinetics were compared with those of cytidine and dCyd. Compounds **1-6**, Cyd and dCyd degraded by hydrolysis to Cyto and/or by deamination to the corresponding Ura nucleosides. Nonchromophoric compounds were also formed.

Degradation of Ura, Cyto, Urd and dUrd was also carried out in order to determine the fate of the degradation products of the cytosine nucleosides. Table 1 shows the observed rate constants, k_{obs}, at various temperatures.

At pH 1.22, LC and PDA analyses showed that hydrolysis to cytosine was the only degradation process for compounds **2**, **5**, dCyd and dUrd and the main process for compounds **1**, **3**, **4** and **6**. No deamination of compounds **1-6** was detected. The minor products of **1**, **3**, **4** and **6** could not result from degradation of Cyto because a pure sample of Cyto was stable under the conditions of the experiments. Cyd degraded mainly by deamination and only slightly by hydrolysis. The hydrolysis of **1-6** most likely proceeds by the classical unimolecular mechanism, in which the base is protonated and then the nucleoside dissociates in the rate determining step to a glycosyl oxocarbenium ion. The latter reacts with water to yield a sugar. Lönnberg[11] showed this to be the mechanism for hydrolysis of Cyd in acid. Deoxygenation of the sugar moiety allows hydrolysis to become the preferential process. Sugar hydroxyl groups, especially at $C_{2'}$, tend to retard hydrolysis by their negative inductive effect which destabilizes formation of an oxocarbenium ion. Lönnberg and Käppi[12] have reported that in aqueous acid both hydrolysis and deamination of Cyd occur. Earlier, other researchers[13-15] had shown that deamination prevails in acidic buffer solutions. Shapiro and Klein[13] suggested that in acidic buffer, deamination involves saturation of the C_5, C_6 double bond by attack of a buffer anion on C_6 with subsequent deamination by water attack on C_4, followed by release of buffer anion to give the Ura derivative. Mass balance calculations were done for partially degraded samples of compounds **1-6**, but there was no formation of non-chromophoric compounds at pH 1.22.

At pH 6.76 hydrolysis was the only degradation process for **3**, **4**, **5** and dUrd and the main process for **6**. Deamination occurred to a small extent for **6** and to about the same extent as hydrolysis for **1**, **2** and dCyd. Mass balance calculations indicated that compound **1** degraded largely (60%) by formation of nonchromophoric compounds. The molar absorbtivities of the deamination products of **1**, **2**, and **6** are not known and they were assumed to be the same as that of Urd. Cyd degraded solely by deamination, and Ura and Urd were found to be stable. An isolated sample of the deamination product of **1** was degraded at pH 6.76 and it was found to degrade by hydrolysis to Ura. Therefore, while the hydrolytic product of **1-6** (Cyto) was deaminated to Ura, it seems that the

TABLE 1. Pseudo-first order rate constants (± standard errors) observed for degradation of **1-6**, cytosine, cytidine, 2'-deoxycytidine, uracil, uridine and 2'-deoxyuridine

Compound	pH	Temp (°C)	$k_{obs}(h^{-1}) \times 10^2$		$t_{1/2}(h)$	
1	1.22	81	6.07 ±	0.46 (n=18, x=6, y=1)	11.42 ±	0.86
	1.22	90	18.59 ±	0.74 (n=30, x=9, y=1)	3.73 ±	0.15
	1.22	101	76.14 ±	24.39 (n=25, x=8, y=3)	0.91 ±	0.29
	1.22	108	130.89 ±	3.60 (n=31, x=9, y=2)	0.53 ±	0.01
	6.76	100	0.21 ±	0.01 (n=32, x=8, y=1)	325.31 ±	16.14
	11.99	101	1.50 ±	0.07 (n=28, x=9, y=1)	46.29 ±	2.03
	11.99	110	3.61 ±	0.10 (n=26, x=7, y=1)	19.19 ±	0.51
	11.99	120	5.81 ±	0.13 (n=29, x=9, y=2)	11.92 ±	0.26
	11.99	129	14.55 ±	0.58 (n=18, x=5, y=1)	4.76 ±	0.19
2	1.22	81	4.04 ±	0.35 (n=22, x=7, y=1)	17.15 ±	1.49
	1.22	90	11.23 ±	0.30 (n=36, x=9, y=1)	6.17 ±	0.16
	1.22	101	53.89 ±	1.76 (n=29, x=9, y=2)	1.29 ±	0.04
	1.22	108	94.38 ±	5.07 (n=34, x=9, y=1)	0.73 ±	0.04
	6.76	100	0.16 ±	0.01 (n=24, x=7, y=1)	432.97 ±	21.62
	11.99	101	1.33 ±	0.04 (n=41, x=7, y=2)	51.88 ±	1.61
	11.99	110	3.53 ±	0.04 (n=48, x=9, y=2)	19.64 ±	0.25
	11.99	120	7.68 ±	0.07 (n=22, x=5, y=2)	9.03 ±	0.09
	11.99	129	15.59 ±	0.77 (n=19, x=5, y=1)	4.44 ±	0.22
3	1.22	50	4.93 ±	2.84 (n=15, x=4, y=1)	14.05 ±	8.08
	1.22	60	12.94 ±	0.90 (n=26, x=8, y=1)	5.36 ±	0.37
	1.22	70	32.24 ±	1.16 (n=28, x=7, y=1)	2.15 ±	0.08
	1.22	80	126.71 ±	7.70 (n=19, x=7, y=1)	0.55 ±	0.03
	6.76	100	1.30 ±	0.03 (n=31, x=6, y=2)	53.40 ±	1.38
	11.99	101	1.60 ±	0.09 (n=20, x=5, y=2)	43.23 ±	2.47
	11.99	110	4.34 ±	0.09 (n=47, x=8, y=3)	15.95 ±	0.32
	11.99	120	10.38 ±	0.33 (n=32, x=6, y=3)	6.68 ±	0.21
	11.99	129	21.92 ±	0.64 (n=20, x=5, y=2)	3.16 ±	0.09
4	1.22	50	2.47 ±	0.30 (n=16, x=7, y=1)	28.11 ±	3.42
	1.22	60	8.84 ±	0.57 (n=29, x=8, y=1)	7.84 ±	0.50
	1.22	70	32.61 ±	1.29 (n=21, x=7, y=1)	2.12 ±	0.08
	1.22	80	126.43 ±	11.49 (n=28, x=8, y=1)	0.55 ±	0.05

TABLE 1 Continued

	6.76	100	0.82 ±	0.02 (n=44, x=9, y=3)	84.41 ±	2.03
	11.99	101	2.30 ±	0.10 (n=17, x=5, y=2)	30.18 ±	1.37
	11.99	110	5.82 ±	0.13 (n=41, x=8, y=4)	11.92 ±	0.26
	11.99	120	12.15 ±	0.53 (n=16, x=5, y=1)	5.70 ±	0.25
	11.99	129	25.24 ±	0.61 (n=20, x=5, y=2)	2.75 ±	0.07
5	1.22	40	6.70 ±	0.29 (n=28, x=8, y=1)	10.35 ±	0.44
	1.22	46	18.69 ±	0.83 (n=24, x=8, y=1)	3.71 ±	0.17
	1.22	52	38.35 ±	2.54 (n=20, x=8, y=1)	1.81 ±	0.12
	1.22	60	110.68 ±	7.00 (n=21, x=6, y=1)	0.63 ±	0.04
	6.76	100	9.89 ±	0.59 (n=29, x=6, y=1)	7.00 ±	0.42
	11.99	101	2.30 ±	0.04 (n=31, x=9, y=2)	29.88 ±	0.47
	11.99	110	5.79 ±	0.13 (n=38, x=8, y=3)	1.97 ±	0.27
	11.99	120	12.90 ±	0.42 (n=28, x=6, y=1)	5.37 ±	0.18
	11.99	129	32.24 ±	1.80 (n= 9, x=4, y=1)	2.15 ±	0.12
6	1.22	70	12.06 ±	0.67 (n=20, x=8, y=1)	5.74 ±	0.32
	1.22	80	35.10 ±	2.55 (n=18, x=6, y=1)	1.97 ±	0.14
	1.22	90	104.11 ±	2.85 (n=21, x=6, y=2)	0.67 ±	0.02
	1.22	100	349.73 ±	20.30 (n=20, x=6, y=3)	0.20 ±	0.01
	6.76	100	0.45 ±	0.04 (n=21, x=6, y=1)	142.61 ±	11.79
	11.99	101	1.33 ±	0.06 (n=36, x=8, y=3)	51.93 ±	2.23
	11.99	110	2.48 ±	0.12 (n=19, x=4, y=3)	27.97 ±	1.32
	11.99	120	5.71 ±	0.13 (n=25, x=6, y=2)	12.15 ±	0.29
	11.99	129	10.33 ±	1.14 (n=17, x=5, y=3)	6.71 ±	0.73
Cytosine	1.22	100		a	a	
	6.76	100	0.15 ±	0.01 (n=14, x=7, y=1)	455.93 ±	18.65
	11.99	100	0.97 ±	0.04 (n=12, x=6, y=1)	71.14 ±	3.03
Cytidine	1.22	100	3.47 ±	0.07 (n=20, x=5, y=3)	19.97 ±	0.40
	6.76	100	0.17 ±	0.01 (n=28, x=7, y=1)	406.64 ±	14.40
	11.99	100	2.83 ±	0.04 (n=24, x=4, y=3)	24.49 ±	0.35
2'-Deoxycytidine*	1.22	100	112.89 ±	6.78 (n=12, x=4, y=1)	0.61 ±	0.04
	6.76	100	0.33 ±	0.01 (n=28, x=7, y=2)	208.97 ±	6.41
	11.99	100	2.06 ±	0.07 (n=16, x=5, y=2)	33.64 ±	1.14
Uracil*	1.22	100		a	a	
	6.76	100		b	b	
	11.99	100		c	c	

(continued)

TABLE 1. Continued

Compound	pH	Temp (°C)	$k_{obs}(h^{-1}) \times 10^2$	$t_{1/2}(h)$
Uridine*	1.22	100	a	a
	6.76	100	b	b
	11.99	100	1.26 ± 0.03 (n=12, x=6, y=1)	55.18 ± 1.28
2'-Deoxyuridine*	1.22	100	4.56 ± 0.23 (n=14, x=8, y=1)	15.21 ± 0.78
	6.76	100	0.67 ± 0.03 (n=14, x=7, y=1)	103.05 ± 4.55
	11.99	100	0.31 ± 0.01 (n=12, x=6, y=1)	152.75 ± 9.34

Sodium citrate buffer was used at pH 1.22 and sodium phosphate buffer at pH 6.76 and 11.99. a, b and c: no degradation after 2.5 h, 352 h and 70 h, respectively. n: total number of chromatographic observations. x: points on the time axis. y: number of half-lives tested. All experiments were done in duplicate except those of compounds marked *.

deamination products of **1**, **2** and **6** were hydrolyzed to Ura. A similar degradation pattern was observed by Lönnberg and Käppi[12] for degradation of Cyd in aqueous acid.

At pH 11.99 deamination was the only degradation process for **1** and **2** and the main process for **6**. For **3** and **4** deamination occurred to about the same extent as hydrolysis. Compound **5** is the only one that degraded predominantly by hydrolysis at alkaline pH. The reason for this is unclear since neither **6**, which also has a trideoxygenated sugar, nor **3**, which is also an α anomer, behaved similarly. Like at pH 6.76, Cyto was deaminated to Ura, with the latter being stable. Cyd and dCyd degraded solely by deamination to Urd and dUrd, respectively, both of which fragmented to nonchromophoric compounds. For mass balance calculations, it was assumed that the molar absorbtivities of the deamination products of **1-6** were the same as the one for Urd. The calculations indicated that at pH 11.99 there was formation of nonchromophoric products (30-40%) during degradation of **1-6**. Neither Cyto, nor Ura, which are formed during alkaline degradation of the compounds under study, degrade to nonchromophoric products. An isolated sample of the deamination product of **1** also did not degrade further when stored at pH 11.99 and 100°C for 187 h. This shows that besides deamination, compound **1**, and possibly the other compounds under study, also degrade directly to nonchromophoric compounds at alkaline pH. Other workers have reported that cytidine[14] and arabinosyl

cytosine[16] degrade by deamination as well as formation of nonchromophoric compounds at alkaline pH. Lönnberg et al.[17] have proposed that deamination of Cyto nucleosides in alkaline media is due to direct displacement of the C_4 amino group by a hydroxide ion. Their argument was supported by the observation that methylation of the C_4 amino group results in a large rate retardation, but methylation of C_5 and C_6 has very little effect on deamination.

In alkaline medium the compounds under study were deaminated to different extents. Deamination was confirmed by isolation of the unknown degradation product of **2** at pH 11.99. NMR and mass spectra showed that the unknown compound is a deamination product of **2** with an intact sugar and Ura base. Compound **2** gave the following signals, referenced to TMS: ^{1}H NMR (DMSO-d_6) δ 1.52-2.02 (m, 4H, H-2', H-2", H-3', H-3"), 3.15-3.72 (m, 4H, H-4', H-5', H-6', H-6"), 4.52 (t, 1H, HO-6'), 4.88 (d, 1H, HO-4'), 5.56 (dd, 1H, H-1'), 5.71 (d, 1H, H-5), 7.10 (br d, 2H, H_2N-4), 7.62 (d, 1H, H-6); ^{13}C NMR (DMSO-d_6) δ 29.6, 31.6 (C-2', C-3'), 61.6 (C-6'), 64.3 (C-4'), 81.5 (C-1'), 83.4 (C-5'), 93.9 (C-5), 141.4 (C-6), 154.6 (C-2), 165.6 (C-4). The degradation product gave the following signals: ^{1}H NMR (DMSO-d_6) δ 1.46-2.16 (m, 4H, H-2', H-2", H-3', H-3"), 3.20-3.70 (m, 4H, H-4', H-5', H-6', H-6"), 5.49-5.57 (m, 2H, H-1', H-5), 7.57 (d, 1H, H-6). ^{13}C NMR (DMSO-d_6) δ 29.1, 31.6 (C-2', C-3'), 61.1 (C-6'), 64.1 (C-4'), 81.0 (C-1'), 83.5 (C-5'), 101.9 (C-5), 140.1 (C-6), 152.1 (C-2), 166.1 (C-4). The ^{13}C NMR signal for C_5 at 101.9 ppm was diagnostic of a Ura and not Cyto base, which gives a signal at 93.9 ppm. The coupling constants between C_5 and C_6 protons of the degradation product (J = 7.9 Hz) were typical of a Ura base as opposed to those of a Cyto base which are usually smaller (J= 7.4 Hz for C_5 and C_6 of compound **2**). The C_4 amino group protons of Cyto were clearly absent in the degradation product. Liquid secondary ion mass spectra (LSIMS) were recorded. The molecular ion of 243, $[M+H]^+$, supported the proposed structure. The collision-induced dissociation spectrum of the unknown was generated using helium as the collision gas in the first field-free region gas cell at a pressure causing a 50 % reduction in the parent $[M+H]^+$ ion (m/z 243) beam intensity. Ten daughter-ion linked scans (B/E is constant) were collected using the multichannel analyser mode. The spectra showed daughter ions at m/z 113 and m/z 131, corresponding to Ura+H and the 2,3-dideoxy-*β*-D-*erythro*-hexopyranosyl sugar moiety, respectively. LC analysis of partially degraded samples using acetonitrile - 0.01 M ammonium acetate

(2:98, v/v) and a column packed with PLRP-S 100 Å showed that the deamination products of **1-6** were different for each of the compounds because they had different retentions. PDA studies also showed that their spectra were slightly different, though they closely resembled that of Ura.

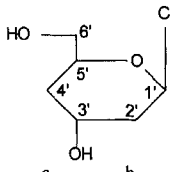
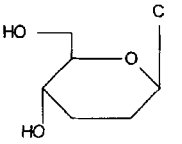
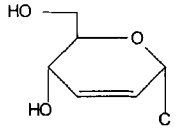
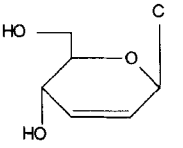
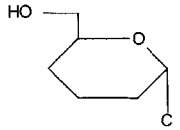
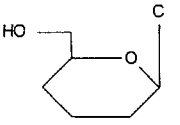
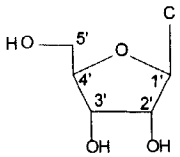
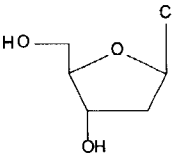
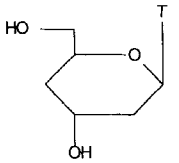
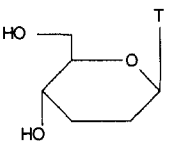
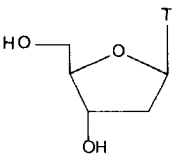
Structure-stability relationships

Pseudo-first order rate constants at 100°C and at different pH values, are summarized in Table 2 for hexopyranosyl cytosine nucleosides.

At pH 1.22 degradation of compounds **1-6** is influenced by the number of sugar hydroxyl groups, presence of sugar double bonds and the type of anomer. Compounds **1** and **2**, which have two sugar -OH groups, are more stable than compound **6**, which has only one sugar -OH group, and Cyd is 32 times more stable than dCyd. For pentofuranose nucleosides, it has been shown that the sugar hydroxyl groups retard acid hydrolysis by a negative inductive effect which reduces the electron density at $C_{1'}$-$O_{4'}$ region, thus destabilizing the oxocarbenium ion[18]. Oivanen et al.[18] and York[19] have shown that an -OH group at $C_{2'}$ has a higher influence on stability than one on $C_{3'}$, which in turn has more influence than one on $C_{5'}$, for natural (pentofuranose) nucleosides. A $C_{2'}$ sugar -OH group has a much higher influence on stability than other -OH groups because of its closeness to the $C_{1'}$-$O_{4'}$ region. The hexopyranose nucleosides with two -OH groups (**1**, **2**) are about twice as stable as dCyd. Presence of a $C_{2'}$-$C_{3'}$ double bond markedly reduces stability (**2** vs **4**). In compound **4** the oxocarbenium ion formed is stabilized by resonance[18], thus increasing hydrolysis. Compound **6** (β anomer) is much more stable than its α-anomer, **5**. At 50 °C and 60 °C, **4** (β anomer) is more stable than **3** (α anomer) and at 70 °C and 80 °C they have the same stability. These results are similar to those obtained by Van Schepdael et al.[20], who found that 1-(2,3-dideoxy-β-D-*erythro*-hexopyranosyl)thymine and 1-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine were 14-fold and 20-fold more stable than their α anomers at pH 1.22. This could be comparable with the hydrolysis of acetals. In this case the α-isomer seems to cleave more easily than the β-isomer because its conformation adapts better to a situation in which the ring oxygen lone pair is antiperiplanar with respect to the exocyclic bond[21,22].

The structure-stability relationships discussed above for pH 1.22 also hold at pH 6.76, but the differences in stability are less. At pH 11.99 the number of hydroxyl groups and their positions have little or no influence on degradation. Presence of a sugar double

TABLE 2. Pseudo-first order rate constants (k_{obs}) (h^{-1}) (in parentheses) of **1-6**, cytidine, 2'-deoxycytidine and thymidine nucleosides at 100 °C

 **1** (0.761[a], 0.002[b], 0.015[c])	 **2** (0.539[a], 0.002[b], 0.013[c])
 **3** (7.053[a], 0.013[b], 0.016[c])	 **4** (12.122.[a], 0.008[b], 0.023[c])
 **5** (181.354[a], 0.099[b], 0.023[c])	 **6** (3.497[a], 0.005[b], 0.013[c])
 Cytidine (0.035[a], 0.002[b], 0.028[c])	 2'-Deoxycytidine (1.129[a], 0.003[b], 0.021[c])
 (0.008[a], 0.003[b], 0.007[c])*	 (0.007[a], 0.003[b], 0.005[c])*	 Thymidine (0.045[a], 0.004[b], 0.002[c])*

a: Sodium dihydrogen citrate buffer was used at pH 1.22 and μ=0.07. b and c: sodium phosphate buffer at pH 6.76 and 11.99, respectively and at ionic strength 0.07. * : results from reference 20.

bond halves stability (**2** vs **4**) and the type of anomer shows no relationship to stability. Thus, the influence of the number of sugar hydroxyl groups, their positions and the type of anomer is highest at acid pH, less at neutral pH and unimportant at alkaline pH. This is because the types of processes occurring are different in different media. At pH 1.22, acid catalyzed hydrolysis is the predominant process and the rate determining step is largely influenced by the inductive effect of the sugar functional groups. Towards neutral pH, hydrolysis is largely uncatalyzed and the sugar groups have less influence. At pH 11.99, deamination predominates and the sugar groups, which are too far away, have little or no influence on the displacement of the C_4 amino group by a hydroxide ion[17].

Compounds **1**, **2** and dCyd are all much less stable than the corresponding thymine nucleosides[20] in acid as well as alkaline media. At acid pH compounds **1-6** are degraded mainly by a unimolecular mechanism, in which protonation of the base (and thus pK_a) is important, whereas thymine nucleosides are also hydrolyzed via a bimolecular mechanism in which the sugar is protonated[20,23,24]. At alkaline pH compounds **1**, **2** and **6** are mainly deaminated while the thymine analogues are hydrolyzed to thymine. At neutral pH there is little difference in stability and this suggests that the process of uncatalyzed hydrolysis might be similar for both cytosine and thymine nucleosides.

Temperature-rate profiles

Regression analysis was performed on the data in Table 1 in accordance with the logarithmic form of the Arrhenius equation (Equation 1). The Arrhenius relationships and E_a values so obtained are given in Table 3. The enthalpy and entropy of activation, $\Delta H^{\neq}$ and $\Delta S^{\neq}$, were calculated from equations (2) and (3) respectively,

$$\log k = \log A - \frac{E_a}{2.303RT} \quad (1)$$

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

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

where A is a constant, $\tilde{k}$ is the Boltzmann constant (the gas constant per molecule) and h is Planck's constant.

At pH 1.22, $\Delta S^{\neq}$ values for **1-6** are positive or slightly negative and this supports a unimolecular mechanism of nucleoside hydrolysis, in which the base moiety is protonated and then the nucleoside dissociates into a glycosyl oxocarbenium ion which

TABLE 3. Arrhenius relationships, and energy of activation for **1-6** at pH 1.22

Compound	Arrhenius relationship	E_a (kcal.mol^{-1})	$\Delta H^{\neq}$ (kcal.mol^{-1})	$\Delta S^{\neq}$ (e.u.)
1	$\log k_1$=19.49-6881(1/T) r=0.9943	31.5	30.8	+9.6
2	$\log k_1$=19.99-7144(1/T) r=0.9969	32.7	32.0	+11.7
3	$\log k_1$=16.56-5387(1/T) r=0.9896	24.7	23.9	-9.4
4	$\log k_1$=20.13-6635(1/T) r=0.9974	30.4	29.6	+5.2
5	$\log k_1$=20.07-6257(1/T) r=0.9983	28.7	27.9	+2.1
6	$\log k_1$=18.23-6152(1/T) r=0.9989	28.2	27.4	+1.8

reacts with water to yield the sugar. Similarly, other workers[18,19,25-29] found positive or slightly negative $\Delta S^{\neq}$ values for nucleosides, and in conjunction with other criteria, concluded that the nucleosides hydrolyse by a unimolecular mechanism in acid.

Activation parameters at alkaline pH were not calculated because the reaction pathway is not clearly understood to allow use of the rate constant in the calculations. Besides, for **3-6** hydrolysis occurs to a significant extent and it would interfere with the values calculated for the deamination reaction.

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