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Degradation of 4′-azidothymidine in aqueous solution

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Summary

The kinetics of the degradation of 4′-azidothymidine (ADRT) in aqueous solution from pH 1 to 11 were studied by reverse phase and ion exchange HPLC. The pH-rate profiles at 50, 60 and 80°C were U-shaped with the rate law of $k_{obs} = k_H a_H + k_{H_2O} + k_{OH} a_{OH}$. The pH-rate profile of ADRT differs from that of 3′-azido-3′-deoxythymidine, AZT, which exhibits acid and water catalyzed degradation 340 and 130 times slower, respectively than ADRT and did not display base catalyzed degradation. Thymine and azide were identified by co-injection of authentic samples as major degradation products of ADRT at all pH values studied. In acidic solutions, 2-deoxy-4-hydroxy-pentose (**1**) which is the stable form of 2-deoxy-4-pentulose (**2**) was identified as an additional degradation product by mass spectral and ^{1}H- and ^{13}C-NMR analysis. The results suggest that stable aqueous formulations of ADRT can be prepared and that formation of azide by non-enzymatic routes may not be pharmacologically significant.

Introduction

The thymidine analog, 4′-azidothymidine (ADRT) is an anti-viral agent being developed for potential treatment of acquired human immune deficiency syndrome (Maag et al., 1992). It is the first 4′-substituted nucleoside with potent activity against human immunodeficiency virus (HIV) in vitro and it retains its activity against HIV strains that are resistant to 3′-azido-3′-deoxythymidine, AZT. Although ADRT possesses a hydroxyl group at the 3′-position its mechanism of action still involves DNA chain termination (Chen et al., 1992).

In order to ensure the development of a chemically stable dosage form, it was necessary to define the pH-rate profile for the degradation of ADRT in aqueous solution. Degradation product studies were also required since azide ion, a potential degradation product, has known pharmacological effects (Sax and Lewis, 1989). Therefore, the kinetics and products from the decomposition of ADRT in aqueous solution were measured. Comparisons in the reactivity were made to nucleosides not substituted at the 4′-position, AZT and thymidine, and α-azido ethers in order

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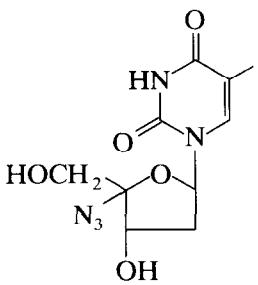
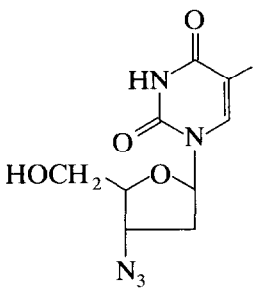
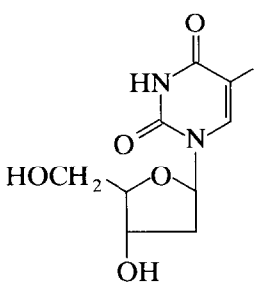
to determine the effect of having a leaving group and electron withdrawing group at the 4′-position of ADRT.



ADRT



AZT



thymidine

Experimental

Materials

ADRT (Maag et al., 1992) and AZT were obtained from the Institute of Organic Chemistry or the Institute of Bioorganic Chemistry, Syntex Research. Distilled deionized water, potassium hydroxide solutions (Aldrich) and hydrochloric acid volumetric solutions (Aldrich) were used for the preparation of the buffer solutions. All other chemicals used were of reagent grade.

Instrumentation

pH measurements were made using a Radiometer Model PHM64 Research pH meter equipped with a Sensorex Model SG900C combination electrode. The HPLC system consisted of a Hewlett Packard HP 1090 pump and autosampler connected to a UV/VIS detector from Applied Biosystems or a SP8430 RI detector from Spectra Physics. The detectors were interfaced to a Macintosh SE computer and the data were analyzed using Dynamax® software from Rainin.

HPLC-MS analyses were performed on a TSQ-70 mass spectrometer equipped with a thermospray (TSP) ionization source from Finnigan-MAT Corporation. The mass spectrometer was connected in series with a UV-visible detector from Applied Biosystems. Eluent was pumped with a Rainin Rabbit-HP solvent delivery system.

NMR measurements were made on a Bruker 500 MHz NMR spectrometer. Peaks were referenced to sodium 3-(trimethylsilyl)-1-propanesulfonate.

pK_a determination

The dissociation constant of ADRT was determined by potentiometric titration at a drug concentration of 10 mg/ml. The pH of the drug solution (7 ml) was adjusted by adding 25 μl aliquots of 1.0 N KOH (0.1 equivalents) and recording the pH at the desired temperature. pK_a values were determined by fitting the data to the Henderson-Hasselbach equation (Zubay, 1983).

Kinetic methods

Aqueous solutions of ADRT and AZT were prepared so that the final solution contained 50–250 μg/ml drug in 0.01 M buffer. Solutions were filtered through 0.22 μm cellulose acetate Corning filters after adding the drug. The drug solutions were flame-sealed in 3 ml clear ampoules. Several samples were placed at −18°C to serve as 'zero time' solutions at the time of assay. At selected time intervals, individual samples were removed from the elevated temperature ovens and stored at −18°C until time of assay. Samples were allowed to warm to room temperature before analysis of HPLC Method I. All samples were assayed against a zero time sample to determine the percent drug remaining.

HPLC methods

Two different HPLC methods were used. Method I is a reverse phase method which was used to quantitate both the drug and thymine. It used a Zorbax C8 column (5 μm, 4.6 mm × 250

mm) and UV detection at 266 nm. The mobile phase consisted of methanol : water (15 : 85). Method II used an Aminex HPX column (7.8 mm × 300 mm) from BioRad with a mobile phase of formic acid (pH 2.7) and refractive index detection. This method was used to quantitate the drug and thymine and monitor the appearance of azide and **1**.

HPLC-MS analysis

HPLC eluent (method II) was passed through a heated vaporizer (0.15 mm internal diameter tubing) into the high vacuum of the mass spectrometer. The vaporizer temperature was 75°C and the jet temperature was 230°C. The collector voltage was 150 V.

Isolation of 2-deoxy-4-hydroxy-pentose

2-Deoxy-4-hydroxy-pentose (**1**) was prepared by reacting ADRT (10 mg/ml) in 0.001 M HCl for 40 h at 80°C. For NMR studies the preparation was performed in D_2O/DCl so that isolation would not be required. Compound **1** was separated from the drug and thymine by passing the acidic solution through a Waters Sep Pak®, C18, which had been pretreated with acetonitrile and water or D_2O. Fractions (0.75 ml) were collected and **1** was identified in the second fraction by HPLC method II. The solution was concentrated by lyopholization but was not carried out to dryness because this resulted in poor recovery of **1**. HPLC, ^{1}H- and ^{13}C-NMR confirmed the sample purity. ^{1}H-NMR δ 5.25 (t, $J = 4$ Hz, H1), δ 4.90 (d of d, $J = 6$ Hz and $J = 2.5$ Hz, H1), δ 3.93 (m, H3), δ 3.84 (d of d, $J = 5$ Hz and $J = 6$ Hz, H3), δ 3.38–3.80 (m, H5), δ 2.22–1.69 (m, H2). ^{13}C-NMR δ 94.5 (C1), δ 92.5 (C1), δ 92.3 (C4), δ 92.0 (C4), δ 70.6 (C3), δ 69.1 (C5), δ 67.9 (C3), δ 65.96 (C5), δ 37.5 (C2), δ 36.5 (C2).

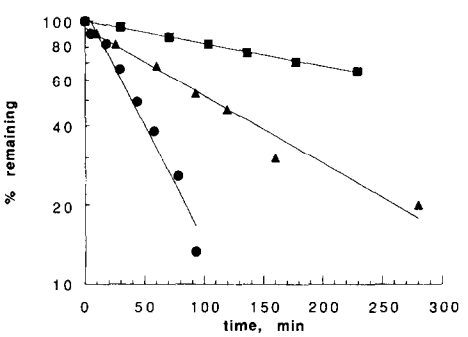


Fig. 1. First-order plots for the decomposition of ADRT at 80°C and pH 1.0 (▲), 8.0 (■) and 9.7 (●).

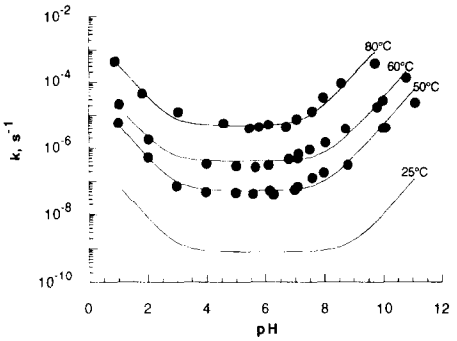


Fig. 2. pH-rate profile for the decomposition of ADRT in aqueous solution at 50, 60 and 80°C. The data were fitted using Eqn. 1 with pK_w values of 13.26, 13.02 and 12.60 at 50, 60 and 80°C, respectively. The lower curve was obtained by extrapolating the data to 25°C using the activation parameters in Table 1.

Results and Discussion

Effect of pH on the degradation of ADRT

The aqueous stability of ADRT was studied in buffer solutions from pH 1 to 11 at 50, 60 and 80°C. Fig. 1 shows typical first-order plots at 80°C and pH 1.0, 8.0 and 9.7. The pH dependence of the degradation rate at 50, 60 and 80°C is shown in Fig. 2. The U-shaped pH-rate profile can be fitted by a kinetic equation that has specific-acid (k_H), water (k_{H_2O}) and specific-base (k_{OH}) catalyzed terms.

$$k_{obs} = k_H a_H + k_{H_2O} + k_{OH} a_{OH} \quad (1)$$

In Eqn 1, a_H and a_{OH} are, respectively, the hydrogen and hydroxide ion activities at the reaction temperature. Values for the apparent kinetic parameters (k_H, k_{H_2O}, k_{OH}) at different temperatures were determined according to Eqn 1 using a non-linear regression method. The solid curves drawn in Fig. 2 were constructed from these apparent kinetic parameters which are summa-

TABLE 1

Calculated rate constants and activation energies for the decomposition of ADRT in aqueous solution

Rate constant	50°C	60°C	80°C	E_a (kcal/mol)
k_{H_2O} (s^{-1})	5.3×10^{-8}	4.1×10^{-7}	4.8×10^{-6}	33
k_{OH} ($M^{-1}\,s^{-1}$)	7.5×10^{-3}	3.1×10^{-2}	5.9×10^{-1}	33
k_H ($M^{-1}\,s^{-1}$)	4.8×10^{-5}	1.8×10^{-4}	3.1×10^{-3}	32

rized in Table 1. When the data are extrapolated to 25°C using the Arrhenius activation energies (Table 1), a t_{90} of 4 years is predicted in the range pH 4–8 (Fig. 2).

Curvature in the pH-rate profile may occur near the pK_a of ADRT (Table 2) if the reactivity of the neutral and ionized forms of ADRT were significantly different. However, Eqn 1 appears to adequately fit the data, suggesting that differences in reactivity of ADRT due to changes in ionization state are negligible.

Product studies

The products from the degradation of ADRT were studied by HPLC, HPLC-MS, ^{1}H- and ^{13}C-NMR. An HPLC chromatogram (method I) of a partially degradaded solution (pH 1.0) of ADRT is shown in Fig. 3. Thymine, a fragmentation product, was identified as a degradation product by co-injection of an authentic sample. HPLC method II which employed a BioRad ion exchange column and an RI detector was developed to detect degradation products from the remaining fragment. Typical HPLC chromatograms of degraded samples of ADRT using HPLC method II are shown in Fig. 4. In addition to thymine, azide was identified as a degradation product at

TABLE 2

Dissociation constants for ADRT measured by potentiometric titration

Temperature (°C)	pK_a
25	9.72
50	9.33
60	9.23

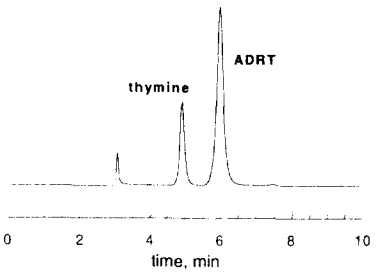


Fig. 3. HPLC chromatogram (method I) of ADRT degraded at pH 1.0 for 40 h at 50°C.

all pH conditions by co-injection of sodium azide. Sodium azide eluted in the solvent front of HPLC method I.

An additional peak was detected in the chromatograms of samples hydrolyzed in acidic (pH 2

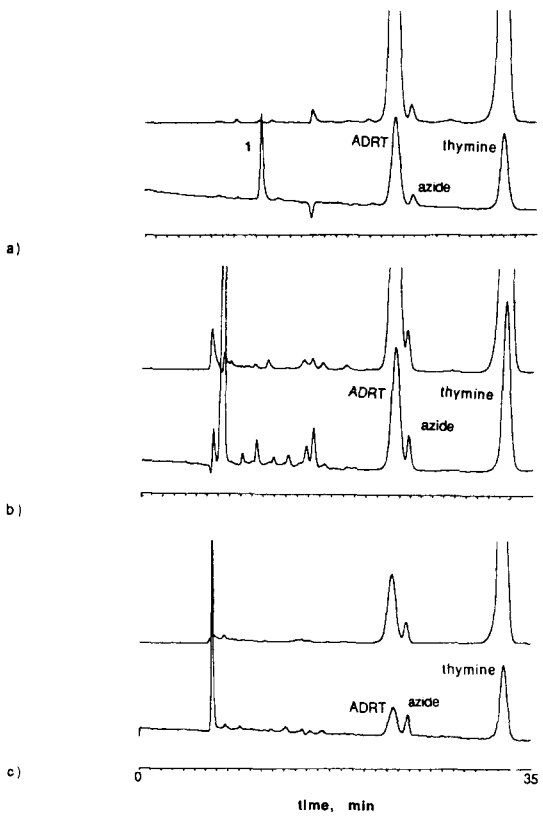
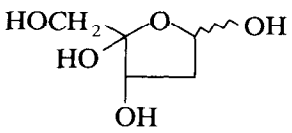
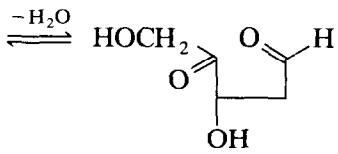


Fig. 4. HPLC chromatogram (method II) of ADRT degraded at (a) pH 3.0, 40% remaining; (b) pH 7, 33% remaining; (c) pH 10.4, 18% remaining. The top and bottom chromatograms were taken with UV (210 nm) and RI detection, respectively.

and 3) but not in basic conditions (pH 11). HPLC-MS analysis of an eluted peak using HPLC method II showed an $M+1$ peak of 133. This suggests that the molecular formula of the degradation product is $C_5H_8O_4$ and the structure is tentatively assigned to be 2-deoxy-4-pentulose, **2** (Dizdaroglu et al., 1975; Rabow et al., 1990).



1



2

Sufficient quantities of this material were purified (see Experimental) to obtain ^{1}H- and ^{13}C-NMR spectra. The ^{1}H-NMR data show that the sample is a mixture of two isomers from the presence of two anomeric hydrogens at δ 5.25 and δ 4.90. The absence of a carbonyl carbon in the ^{13}C-NMR and the location of the C5 hydrogens between δ 3.4 and δ 3.8 suggest that in solution, water adds to 2-deoxy-4-pentulose, **2**, to form two anomers of 2-deoxy-4-hydroxy-pentose, **1**. In the mass spectrometer, **1** losses water to give an $M+1$ peak at 133. The presence of 10 signals in the ^{13}C-NMR suggests that only two stereoisomers exist in solution, therefore, the stereochemistry at the 4-position is fixed. The relative stereochemistry at this position is undetermined presently. At pH 7, small amounts of **1** and other apparent products can be detected by HPLC (Fig. 3b) but none of these products appear to accumulate with time.

TABLE 3

Rate constants for the decomposition of ADRT, AZT, thymidine and α-azido ethers in aqueous solutions

Compound	pH	Temperature (°C)	k_{obs} (s^{-1})
ADRT	1.0	60	2.1×10^{-5}
	2.0		1.8×10^{-6}
	5.0		2.6×10^{-7}
	7.1		8.7×10^{-7}
	10.8		1.2×10^{-4}
	5.0	25	1.0×10^{-9}
AZT	1.0	60	6.2×10^{-8}
	2		7.9×10^{-9}
	5.0		2.0×10^{-9}
	7.1		2.0×10^{-9}
	10.8		5.9×10^{-9}
Thymidine	1 N HCl [a]		1.1×10^{-6}
	6.5 [b]		8.8×10^{-9}
3 [c]	7	25	0.77
4 [c]	7	25	1.30×10^{-3}
5 [c]	10.3	25	2.18×10^{-2}

[a] Garrett et al. (1966).
[b] Extrapolated from Shapiro and Kang (1969).
[c] Amyes and Jencks (1989).

Comparison of the degradation of 1 with thymidine, 2 and α-azido ethers

Since ADRT is a thymidine nucleoside substituted at the 4'-position by azide, it is interesting to compare the reactivity of ADRT with other thymidine nucleosides and α-azido ethers (Table 3 and Fig. 5). The kinetics of degradation of thymidine to thymine and deoxyribose have been reported at neutral pH conditions (Shapiro and Kang, 1969) and in acidic solutions (Pfitzner and Moffatt, 1964; Garrett et al., 1966; Shapiro and

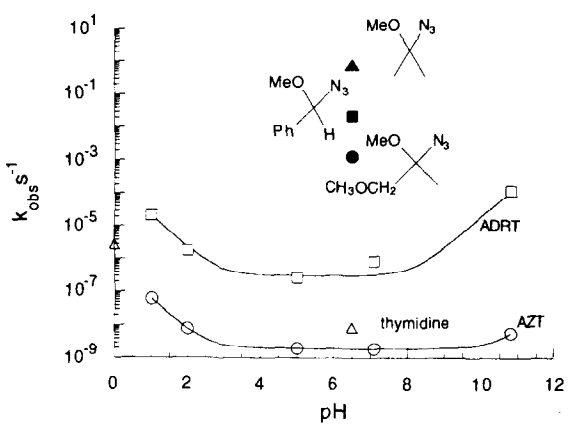


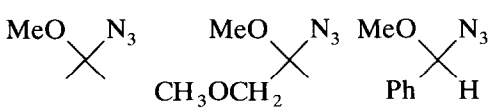
Fig. 5. Rate constants for the decomposition of ADRT, AZT, thymidine at 60°C and α-azido ethers at 25°C (Garrett et al. 1955; Shapiro and Kang, 1969; Amyes and Jencks, 1989).

Danzig, 1972). The rate constants for the degradation of thymidine are approx. 75- and 30-times slower than those for ADRT at 60°C at pH 1 and pH 6, respectively (see Table 3).

The relative reactivity of ADRT and AZT was studied also. The aqueous stability of AZT was briefly examined at 60°C and analyzed by HPLC method I and the rate constants for the decomposition of AZT at several pH values at 60°C are given in Table 3. The rate constants show that k_H and k_{H_2O} are approx. 340- and 130-times lower for AZT than ADRT, respectively. The difference in k_{OH} for AZT and ADRT can only be approximated since a significant k_{OH} term was not observed for the hydrolysis of AZT. However, assuming that the difference in the observed rate of hydrolysis of AZT between pH 7.1 and pH 10.8 is due to base catalysis,

$$\frac{k_{OH}(\text{ADRT})}{k_{OH}(\text{AZT})} = \frac{3.1 \times 10^{-2}}{6.4 \times 10^{-7}} = 48\,000$$

Although ADRT degraded considerably faster than other thymidine nucleosides, it degrades more slowly than some α-azido ethers recently studied (Amyes and Jencks, 1989). The reactivity of methoxymethyl-azides has been studied in neutral and basic pH regions in the presence of azide and added nucleophiles. All of the α-azido ethers that were studied are extremely reactive compared to ADRT (see Table 3); for instance, 2-methyl-2-methoxyethylazide (**3**) has a solvolysis rate constant approx. 10^9-fold larger than k_{H_2O} for hydrolysis of ADRT at 25°C (extrapolated from Table 1). The reactivity of 2-methyl-2,3-methyoxyethylazide (**4**) is reduced 590-times compared to **3** by the introduction of an electron withdrawing group. Interestingly, the methoxybenzylazide (**5**) which reacts 350-times slower than **3**, did not show an acceleration in rate in 0.01 N KOH. Acid catalyzed degradation of these compounds was not studied.

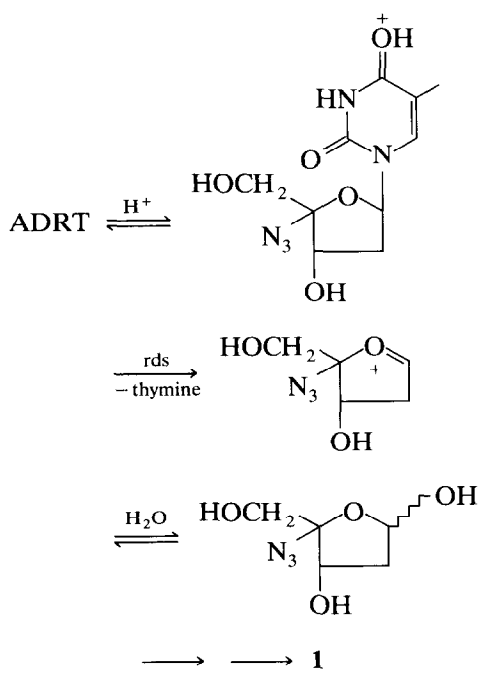


3 **4** **5**

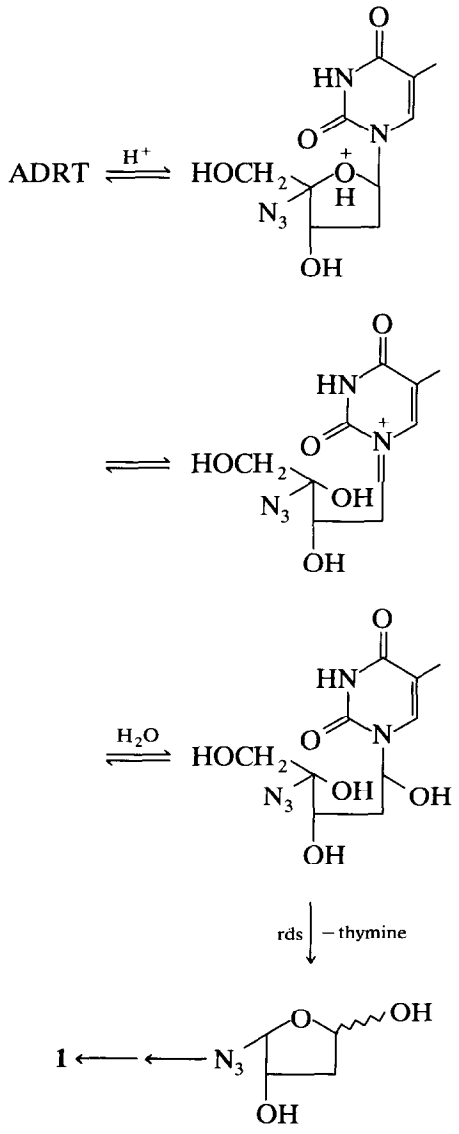
Mechanisms of degradation

Acidic conditions

The degradation of ADRT in the acidic solutions could potentially proceed through intermediates where the azide or nucleoside is protontated. Whereas, acid catalyzed hydrolysis of α-azido ethers has not been reported and alkylazides are protonated only in strongly acidic media (FSO_3H/SbF_5) (Mertens et al., 1983) or in the gas phase (Attina et al., 1989) acid catalyzed hydrolysis of thymidine nucleosides is well known. Two mechanisms have been proposed for the acid catalyzed degradation of thymidine nucleosides, Mechanism 1 (Garrett et al., 1966; Shapiro and Danzig, 1972; Lönnberg, 1982; Lönnberg and Kappi, 1985), and Mechanism 2 (Kenner, 1957; Dekker, 1960; Pfitzner and Moffatt, 1964; Garrett et al., 1966; Cadet and Teoule, 1974). Neither mechanism accounts for the increased reactivity of ADRT compared to thymine in the acidic solutions since the electron withdrawing nature of the azido-group would destabilize the protonated intermediates that exist on both pathways.

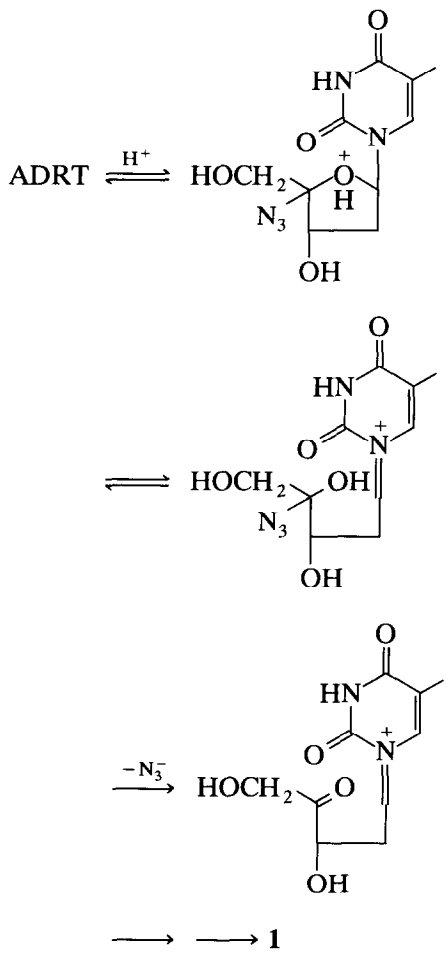


Mechanism 1



Mechanism 2

Mechanism 3, a variation of Mechanism 2, may provide a possible explanation for the enhanced reactivity of ADRT. It deviates from Mechanism 2 in that azide is expelled in the rate-determining step. Loss of azide ion in Mechanism 3 is potentially more rapid than the two step process of the equilibrium addition of water to the anomeric carbon and subsequent breakage of the C-N bond in Mechanism 2 resulting in an acceleration in the rate of decomposition of ADRT. The azide group would lower the pK_a of the ring oxygen and accelerate the breakage of the C-O bond resulting in little differences in rate for the initial two steps in the reaction.

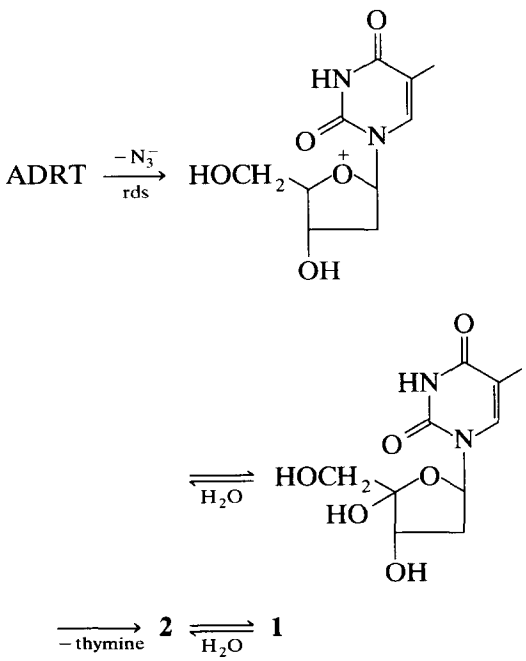


Mechanism 3

Neutral conditions

The water catalyzed reaction like the acid catalyzed reaction may occur with loss of azide or thymine. If loss of thymine is occurring in the rate-determining step, the rate of hydrolysis would be expected to be slower than thymidine and AZT due to the presence of the electron withdrawing group at the 4′-position. Since the reaction is faster, water catalyzed degradation of ADRT probably proceeds through an oxonium ion formed from the loss of the azide group as

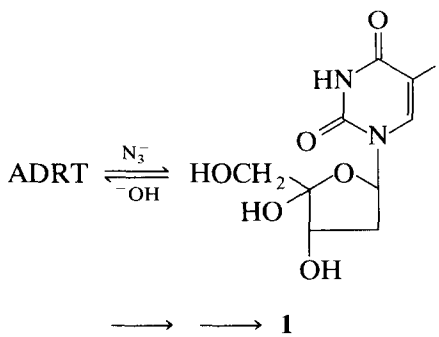
was seen for **3–5** (Amyes and Jencks, 1989) (Mechanism 4). The thymine and hydroxymethyl substituents due to their electrons withdrawing properties retard the rate of hydrolysis of ADRT relative to **3** by approx. 10^9-fold at neutral pH values if this is the mechanism occurring. A similar S_N1 mechanism is not possible for AZT since an α oxygen is not available to stabilize a carbocation intermediate if azide is the leaving group. An α oxygen is available to stabilize a thymine ion leaving group for both compounds, however, thymine ion is a much poorer leaving group than azide.



Mechanism 4

Basic conditions

Nucleosides are typically stable to base (Carey and Sundberg, 1977). One possible mechanism explaining the reactivity of ADRT in basic solution is a direct attack of hydroxide on the tertiary substituted C4′ carbon eliminating the azide group and forming 2-deoxy-4-hydroxythymidine (Mechanism 5). Since AZT does not contain an azide group at a position with an α oxygen, this mechanism accounts for the large difference in reactivity between ADRT and AZT. Further evidence that the reactivity of ADRT is a result of a reaction remote from the thymine moiety is a lack of curvature in the pH-rate profile at the pK_a of the thymine moiety (Table 2). The lack of curvature also suggests that the thymine group does not have an intramolecular effect on the rate of the hydrolysis reaction. Interestingly, solvolysis of the α-azido ether, **5**, was not accelerated in 0.01 N KOH (Amyes and Jencks, 1989). Compound **5**, however, is approx. 10^8-times more reactive than ADRT at neutral pH values and being more reactive than ADRT is less selective towards nucleophiles.



Mechanism 5

Conclusions

The kinetic results revealed in this study revealed that substitution of a leaving group at the 4′-position of a thymidine nucleoside causes unusual reactivity compared to other thymidine nucleosides and α-azido ethers. The presence of a 4′-azido group accelerates and alters the mechanism of decomposition of ADRT relative to thymidine nucleosides with the greatest effect occurring in the basic solutions. The cleavage of the carbon-azide bond is an important step in the rate and products of the degradation at all pH values studied. The presence of electron withdrawing groups in ADRT, greatly reduces the rate of degradation relative to other α-azido ethers. The results suggest that stable aqueous formulations of ADRT can be prepared and that

formation of azide by non-enzymatic routes should be low.

Acknowledgements

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