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Kinetics of azathioprine degradation in aqueous solution

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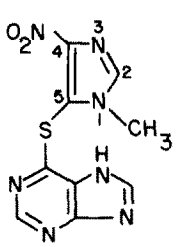
Summary

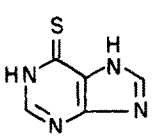
The kinetics of decomposition of azathioprine to 6-mercaptopurine was studied as a model reaction to assess the suitability of thioether derivatives as potential prodrugs for compounds containing thiol functionality. The rates of decomposition yielding 6-mercaptopurine and 1-methyl-4-nitro-5-hydroxyimidazole in stoichiometric amounts were determined at 73°C over the pH range of 1–13 at an ionic strength of 0.5. The pH–rate profile was accounted for by the specific acid- and base-catalyzed reactions and also by assuming spontaneous or water-catalyzed decomposition of both undissociated and dissociated azathioprine. Maximum stability occurred at pH 5.5–6.5 in which region water was the principal catalyst in the decomposition pathway. Various buffer substances were found to exhibit general acid and base catalysis of the degradation. The effects of temperature and ionic strength on the reaction rates were also evaluated. The principal mode of azathioprine degradation may be the cleavage of the carbon sulfur bond by direct nucleophilic attack of the water molecule or the hydroxide ion on the carbon atom at the 5-position of the methyl-nitro-imidazole ring.

Introduction

Azathioprine, 6-[(1-methyl-4-nitro-5-imidazolyl)thio]purine (I) was developed as part of a program designed to present the thiopurines in a masked but therapeutically active form (Clarke et al., 1958; Elion et al; 1960). The usefulness of 6-mercaptopurine (II) and thioguanine in the treatment of human leukemia led to the syntheses and investigation of a large number of related purines as potential chemotherapeutic agents in neoplastic diseases (Goodman et al., 1955; Elion et al., 1956, 1959, 1960). The principal aim of these studies was to find a derivative with an improved chemotherapeutic index rather than one with a new mechanism of action.



I



II

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It was expected that S-substitution would decrease the rate of inactivation of II by enzymatic S-methylation, non-enzymatic oxidation or conversion to thiourate by xanthine oxidase. It was also hoped that the substituent might influence the tissue distribution and perhaps result in a preferential liberation of the parent thiopurine in the tumor. Tests on adenocarcinoma 755 revealed that I had a chemotherapeutic index superior to that of the parent thiopurine when given by the oral route (Elion et al., 1960).

In the course of studies with I, further interesting biological properties were revealed. It was found to have a chemotherapeutic index superior to that of II in suppressing the formation of haem agglutinating antibodies in mice following the injection of sheep red blood cells, and in suppressing the homograft reaction to renal transplants in the dog as well (Elion et al., 1925). These findings led to its use as an immunosuppressive agent and it was found to be less toxic and more effective than II in preventing rejection of renal homografts in humans and laboratory animals. It is currently used as an adjunct for the prevention of renal transplant rejection, and in the treatment of conditions that are thought to have an auto-immune basis such as severe active rheumatoid arthritis. The immunosuppressive action of I is attributed to its prior conversion into II.

Little is reported in the literature on the kinetics of degradation of azathioprine in an aqueous solution. A detailed kinetic study to find how azathioprine is converted to 6-mercaptopurine under various conditions is pertinent to a better understanding of its mode of action and may explain some of its differences with 6-mercaptopurine as an immunosuppressive agent. A detailed investigation of the effects of pH, buffers, ionic strength and temperature on the degradation of I in aqueous solution is described in this article.

Experimental

Materials

Azathioprine (I), 6-mercaptopurine (II) and 6-methyl thiopurine were supplied by Burroughs Wellcome Co., Research Triangle Park, NC, and were used as received. Buffer substances and all other chemicals were of reagent grade. For the preparation of HPLC mobile phase, spectral grade acetonitrile (Burdick and Jackson, Muskegon, MI) was used. Distilled, deionized water was used for the preparation of buffer solutions as well as mobile phases.

Analytical methods

The reactions were followed by a high-pressure liquid chromatographic method with ultraviolet detection at 254 nm. Apparatus and instruments used have been described in a previous article (Mitra and Narurkar, 1986).

The following buffers were used: at pH < 2.4, hydrochloric acid; at pH 3.5–5.0, acetate; at pH 5.5–8.0, phosphate; at pH 9.0–10.4, borate and at pH > 11.5, sodium hydroxide. A constant ionic strength was maintained for each buffer by adding an appropriate amount of sodium chloride.

Kinetic studies

All kinetic studies were performed in aqueous buffer solutions ($\mu = 0.5$) at 73°C. The choice of 73°C was made because the reaction proceeded at a reasonable rate for all pH values at this temperature. The buffer solutions were kept in a thermostatically controlled Dubnoff metabolic shaking (± 0.5°C) incubator.

Studies were initiated by adding 0.1 ml of a 1×10^{-3} M stock solution of I in dimethyl acetamide to 4.9 ml of buffer solution to produce an initial concentration of 5×10^{-5} M. Stock solutions of azathioprine in DMA showed no observable degradation over the time course of the study. Thirty μl samples were taken at appropriate intervals and analyzed for undecomposed I by high-pressure liquid chromatographic assay. The final concentration of 6-methyl thiopurine, an internal standard, was 1×10^{-5} M. A typical baseline separation was achieved with capacity factors of 5.2 and 7.4 for the internal standard and the drug, respectively, which generated a selectivity factor of 1.4 between them.

Results and Discussion

Kinetics of hydrolysis

The kinetics of decomposition of I was examined in aqueous solution of 0.5 ionic strength at 73°C over the pH range of 1–13. At constant values of pH, temperature and ionic strength, the overall loss of I displayed strict first-order kinetics for > 3 half-lives when followed by the HPLC method. In all kinetic runs, II was found to be the primary degradation product with HPLC retention time corresponding to pure 6-mercaptopurine. The reformation of II from I was found to be on an equimolar basis.

The observed pseudo-first-order rate constant (k_{obs}) for the overall degradation of I was calculated from the slopes of the straight lines obtained by plotting the logarithm of the residual amount of I against time.

General acid–base catalysis

The degradation of I was found to be subject to general acid–base catalysis by most of the buffer species utilized in the present study. The buffer catalytic effect could be determined by measuring the rates of degradation at constant pH, ionic strength and temperature, varying only the buffer concentration at a specific pH. The studies were repeated at several pH values within the effective range of the buffers studied.

Typical plots for the catalytic effect of acetate buffer at various pH's are shown in Fig. 1, exhibiting reasonably linear relationships at constant pH in all cases. Extrapolation of such plots to zero buffer concentration provides, as intercepts, the pseudo-first-order rate constants, k_{H^+}, corresponding to the non-buffer catalyzed decomposition process. The observed rate constant, k_{obs}, may be expressed mathematically in the acetate buffer system as:

$$k_{obs} = k_{H^+} + k_{CH_3COOH}[CH_3COOH] + k_{CH_3COO^-}[CH_3COO^-] \quad (1)$$

where k_{CH_3COOH} and $k_{CH_3COO^-}$ are second-order catalytic rate constants associated with the unionized acetic acid and ionized acetate species, respectively. Eqn. 1 can be rewritten in terms of total acetate concentration $[A]_T$

$$k_{obs} = k_{H^+} + \left[k_{CH_3COO^-} + \left(k_{CH_3COO^-} - k_{CH_3COOH}\right) f_{CH_3COOH}\right][A]_T \quad (2)$$

where f_{CH_3COOH} refers to the fraction of acetic acid buffer species, $f_{CH_3COOH} = a_{H^+}/(a_{H^+} + k_a)$, a_{H^+} being the hydrogen ion activity. Slopes of the linear plots shown in Fig. 1 and described by Eqn. 2 were calculated for each pH and plotted against

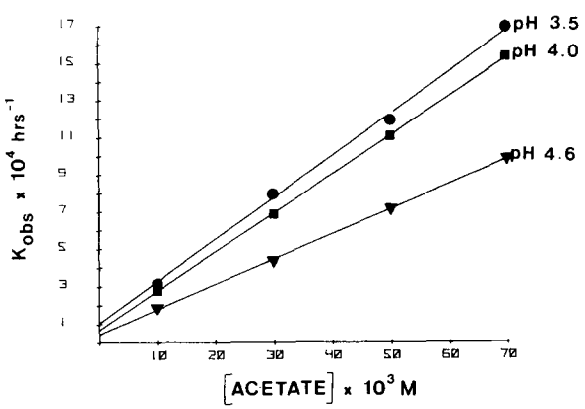


Fig. 1. The effect of acetate buffer concentration on the observed rate constant for the degradation of azathioprine at various pH values (73°C, $\mu = 0.5$).

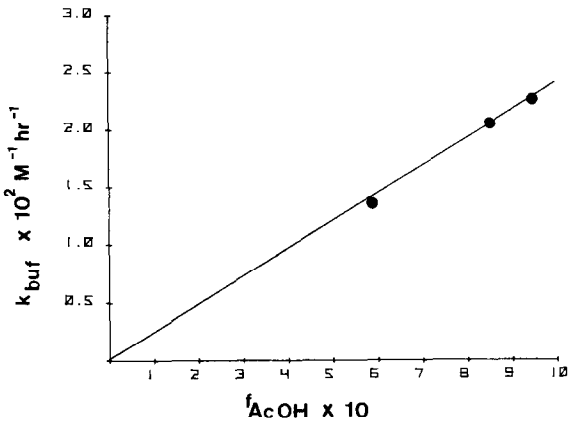


Fig. 2. Dependence of the apparent second-order rate constant for acetate buffer-catalyzed degradation of azathioprine at 73°C on the fraction of acetic acid in the buffers.

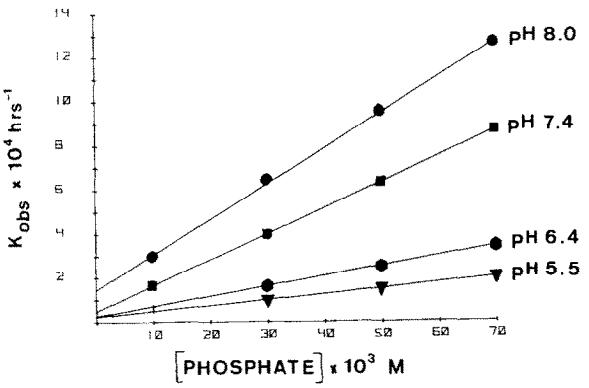


Fig. 3. The effect of phosphate buffer concentration on the observed rate constants for the degradation of azathioprine at various pH values (73°C, $\mu = 0.5$).

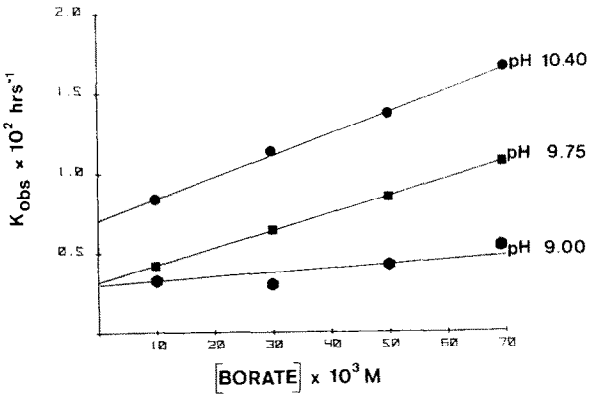


Fig. 5. The effect of borate buffer concentration on the observed rate constants for the degradation of azathioprine at various pH values (73°C, $\mu = 0.5$).

the fraction of undissociated acetic acid at that pH. Such a plot is linear, shown in Fig. 2, with intercepts $k_{CH_3COO^-}$ when f_{CH_3COOH} is zero, and k_{CH_3COOH} when f_{CH_3COOH} is unity. The plot in Fig. 2 provides a second-order catalytic rate constant of $2.4 \times 10^{-2}\ M^{-1} \cdot h^{-1}$ for the undissociated acetic acid species. No significant catalytic effect can be assigned to the acetate ion.

The catalytic effect of phosphate buffers (pH 5.5–8.0 at 73°C) on the degradation of I is shown in Fig. 3. Treatment of the rate data in a manner analogous to that used in the interpretation of the acetate buffer catalysis data generated the plot shown in Fig. 4 which resulted in the following values of the catalytic rate constants for monohydrate phosphate ion and dihydrogen phosphate ion:

$$k_{HPO_4^{2-}} = 1.82 \times 10^{-2}\ M^{-1} \cdot h^{-1},$$

$$k_{H_2PO_4^-} = 2.25 \times 10^{-3}\ M^{-1} \cdot h^{-1}$$

Fig. 5 shows the effect of borate buffers (pH 9.0–10.4) on the rate of degradation of I at 73°C. As can be seen, only marginal catalysis was observed in the borate buffers and no attempt was made to evaluate the catalytic constants for the buffer components.

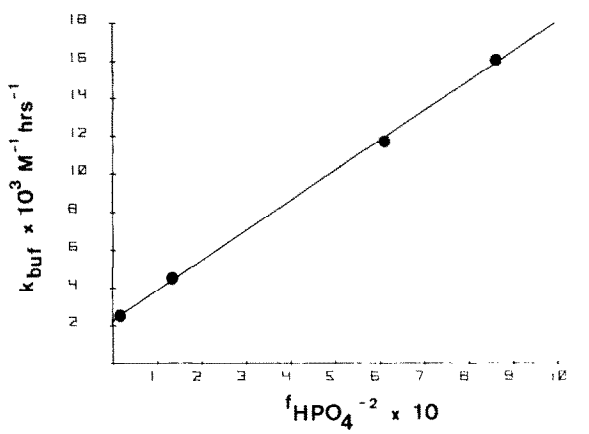


Fig. 4. Dependence of the apparent second-order rate constant for the phosphate buffer-catalyzed degradation of azathioprine at 73°C on the fraction of phosphate dianion in the buffers.

Primary salt effect

The primary salt effect on the degradation of I was studied at constant pH and temperature, but the ionic strength was varied with the addition of sodium chloride. Studies were conducted at pH 1.2 and 11.6 where general base catalysis was absent and secondary salt-effect would be unimportant. The data at these pH values for ionic strength, μ, 0.1–0.5 are given in Table 1.

Within restricted ranges of μ, plots of log k_{obs} versus $\sqrt{\mu}$ should yield theoretical slopes equal to $2 \cdot A \cdot Z_A \cdot Z_B$, where A is a constant for the solvent at a given temperature and Z_A and Z_B are the charges on the reaction species A and B, respectively. The data from Table 1 were used to prepare

TABLE 1

Effect of ionic strength (μ) on pseudo-first-order rate constants for I degradation at pH 1.2 and 11.6

μ	$\sqrt{\mu} \times 10$	$k_{obs} \times 10^3$ (h^{-1})	
		pH 1.2	pH 11.6
0.1	3.16	26.71	1.75
0.3	5.48	26.68	2.89
0.4	6.32	26.69	3.74
0.5	7.07	26.70	4.467

the log k_{obs} versus plots in Fig. 6. Linear regression of the data at pH 11.6 resulted in a correlation coefficient of 0.99, a slope of 1.04 and the rate constant at infinite dilution ($\mu \rightarrow 0$) of 8.2×10^{-3} h^{-1}.

This result can be used as evidence for the reaction of two negatively charged species, i.e. anion of I and hydroxide ion. By contrast, a kinetic salt effect was not observed at pH 1.2, suggesting that at least one of the reactants was electrically neutral. This would be true since at pH 1.2, I is present entirely as undissociated species.

Effect of temperature

The effect of temperature on the decomposition of I was determined by measuring the degradation rate at 55, 65, 73 and 85°C at pH 1.2 and $\mu = 0.5$. The logarithm of the observed rate constant can be plotted against the reciprocal of the absolute

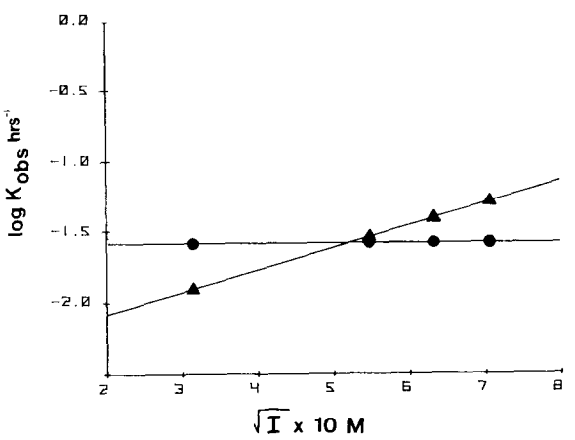


Fig. 6. Plot of log k_{obs} versus $\sqrt{\mu}$ for the degradation of azathioprine at 73°C. Key: ▲, pH 11.6; ●, pH 1.2.

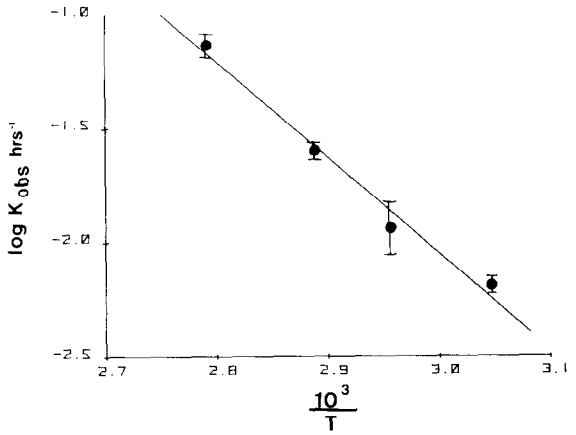


Fig. 7. Arrhenius-type plot of the logarithm of the observed pseudo-first-order rate constants against the reciprocal of absolute temperature for the overall degradation of azathioprine at pH 1.2, $\mu = 0.5$.

temperature according to the following Arrhenius equation:

$$\log k_{obs} = \log A - \frac{E_a}{2.303 \cdot R \cdot T} \quad (3)$$

where log A, the intercept of the plot, is the logarithm of the collision frequency and E_a is known as the activation energy required for the reaction. Fig. 7 illustrates such an Arrhenius plot. The activation energy calculated from the slope is 19.24 kcal/mol.

pH–rate profile

The pH dependence of the overall rate of degradation of I at 73°C and ionic strength of 0.5 is shown in Fig. 8. The rate constants used in the construction of the graph have been obtained from the intercepts of the graphs of k_{obs} versus total buffer concentrations at various pH values (Figs. 1, 3 and 5). Specific acid/base rate constants have been corrected for the activity coefficients of H^+ at 73°C. Results from runs performed in dilute hydrochloric acid (pH < 3) and sodium hydroxide (pH > 11.5) solutions have been incorporated.

In the pH-range studied, I exists as the undissociated (AH) and anionic (A^-) form, the apparent pK_a being 7.87 and 7.99 determined at 25°C by spectrophotometric and solubility methods respec-

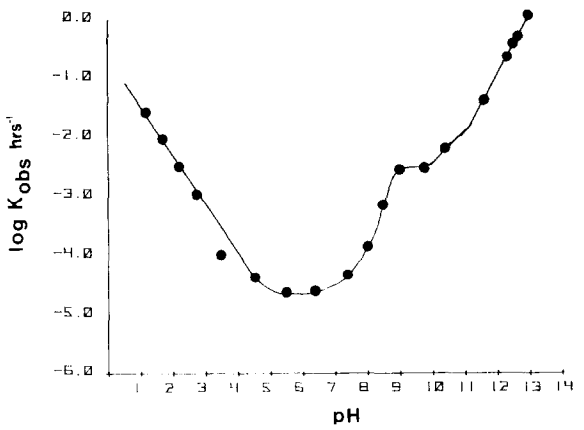


Fig. 8. Log k_{obs}–pH profile for the degradation of azathioprine in aqueous solution at 73°C ($\mu = 0.5$) where k_{obs} is the pseudo-first-order rate constant for decomposition in buffer-free solutions. The points are experimental and the line theoretical, calculated by employing Eqns. 5, 6 and 8.

tively (Newton et al., 1972). The shape of the pH–rate profile suggests that the following reactions contribute to the overall velocity of I degradation.

Reactions	Rate constants
(1) $AH + H^+ \rightarrow$ Products	k_H
(2) $AH + H_2O \rightarrow$ Products	k_{H_2O}
(3) $A^- + H_2O \rightarrow$ Products	k_{H_2O}'
(4) $A^- + OH^- \rightarrow$ Products	k_{OH^-}

The overall velocity of the degradation of I is expressed by;

$$V = -\frac{d[A]_T}{dt} = k_{obs} \cdot [A]_T \qquad (4)$$

where $[A]_T$ is the total concentration of I.

At pH < 3.0, I exists essentially as undissociated species and the rate of degradation can be attributed exclusively to a proton-catalyzed reaction of undissociated I (reaction 1):

$$V = k_H[H^+][AH] \qquad (5)$$

The specific rate constant k_H calculated according to Eqn 5 is 0.36 $M^{-1} \cdot h^{-1}$. At pH 4–10, the overall degradation consists predominantly of the water-catalyzed spontaneous reactions 2 and 3, although reaction 1 and the hydroxide attack on AH seems to affect the pH–rate profile. At pH $\approx$ pK_a of I, the shoulder-type break of the pH–rate profile indicates an increased spontaneous hydrolysis of the anionic species relative to the undissociated species. Within this pH range, the overall rate of decomposition may be described by the sum of the rates of these reactions (2 and 3):

$$V = \frac{d[A]_T}{dt} = \left(k''_{H_2O} \cdot f_{AH} + k''_{H_2O}' \cdot f_{A^-}\right) \cdot [A]_T \qquad (6)$$

where $f_{AH} = a_{H^+}/(a_{H^+} + k_a)$ and $f_{A^-} = k_a/(a_{H^+} + k_a)$ and $k''_{H_2O} = k_{H_2O}[H_2O]$ and $k''_{H_2O}' = k'_{H_2O}[H_2O]$.

From the rate data obtained in the pH range 4–10, the value of k_{H_2O} and k'_{H_2O} were estimated to be 7.6×10^{-7} $M^{-1} \cdot h^{-1}$ and 6.6×10^{-5} $M^{-1} \cdot h^{-1}$, respectively. The kinetic pK_a of azathioprine at 73°C has been calculated to be 9.34. The increase in degradation rate with pH at pH > 6 can be explained by water attack on the anionic species (reaction 3). It can also be explained by kinetically equivalent hydroxide ion attack on the undissociated I species.

The observed rate constant for the degradation of I above pH 10 may be written as:

$$k_{obs} = k_{OH^-}[OH^-]f_{A^-} \qquad (7)$$

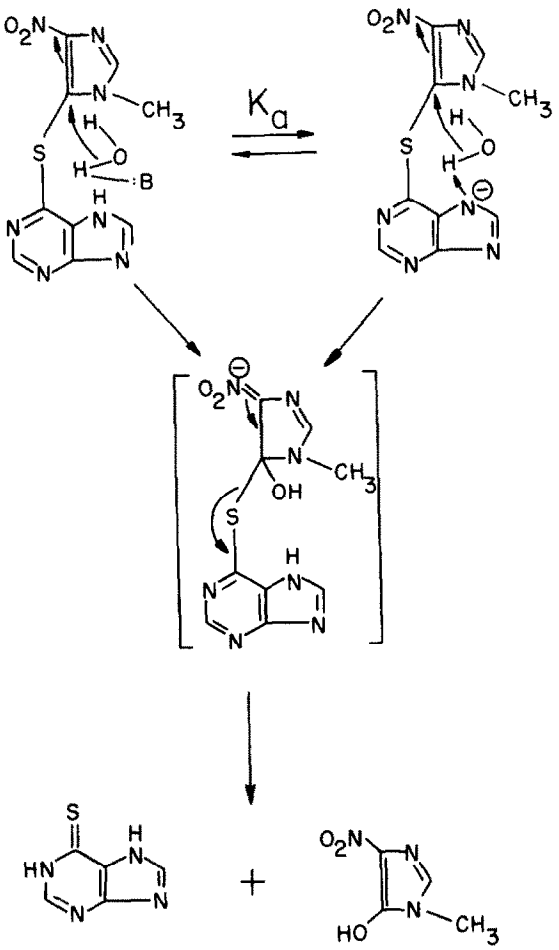
Eqn. 7 can be rewritten as a function of proton concentration as shown in Eqn. 8:

$$k_{obs} = k_{OH^-}\frac{K_w}{[H^+]}f_{A^-} \qquad (8)$$

The ion product of water, K_w, is a function of temperature. The value of K_w at 73°C can be calculated by Eqn. 9 (Ho; 1972):

$$-\log K_w = \frac{4470.99}{T} + 0.01706T - 6.0875 \qquad (9)$$

and is found to be 1.845×10^{-13}. The k_{OH} value has been determined from the portion of the



Scheme 1.

pH–rate profile above pH 12 and has been found to be 0.39 $M^{-1} \cdot h^{-1}$.

The possible mechanism of I decomposition in neutral to slightly alkaline solutions has been depicted in Scheme I. The degradation reaction may be initiated by the nucleophilic attack by the water molecule or by the hydroxide ion on the carbon atom at the 5-position of the methyl-nitro-imidazole ring of I with simultaneous delocalization of π-electrons between the C(4) and C(5) bond towards the electron-withdrawing nitro group. The water attack can be assisted by general base catalysis in the form of a proton abstraction from the attacking water molecule. In the case of the anionic form of I, the purine anion can itself assist in general base catalysis by intramolecular proton abstraction from the attacking water molecule. In the transition state, electronic rearrangements may occur leading to the double-bond formation between C(4) and C(5) with simultaneous breakdown of C(5)–S bond leading to II. As can be seen from the proposed mechanism α,β unsaturation or the presence of a carbonyl group (thioesters), α to the carbon–sulfur linkage is essential for electron delocalization. If the adjacent groups are saturated, the carbon–sulfur bond would probably be very stable. The presence of electron-withdrawing groups at the C(4) position of the methyl-nitro-imidazole ring facilitated the reaction whereas an opposite (stabilizing) effect might be observed from electron-donating groups.

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