Inhibition of Uricase by Substituted Pyrimidines

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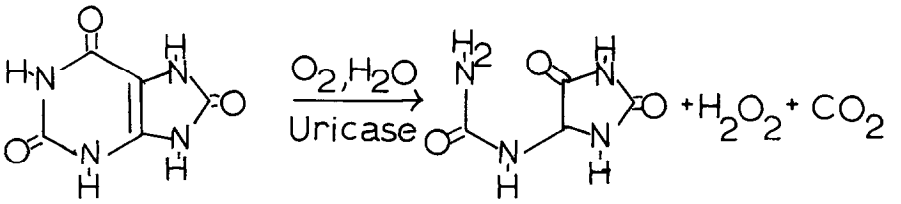
Summary: Twenty-eight substituted pyrimidine derivatives were tested for their ability in inhibit uricase at pH 8.5. Half of the compounds competitively inhibited the enzyme with K_i values which ranged from 4.4×10^{-4} to 4.2×10^{-6} M. Qualitatively, there is a relationship between the degree of electron withdrawing ability of substituents at C-5 of the pyrimidine ring system and the magnitude of inhibitor interaction with the enzyme, a result which is interpreted as being due to the binding of pyrimidine anions rather than the binding of the protonated species.

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) functions in the purine degradative pathway of mammals except man and higher apes. In the presence of molecular oxygen and water, the copper containing enzyme catalyzes the conversion of uric acid to yield allantoin, hydrogen peroxide and carbon dioxide (Eq. 1).



(1)

The initial enzyme reaction results in an intermediate product (1)

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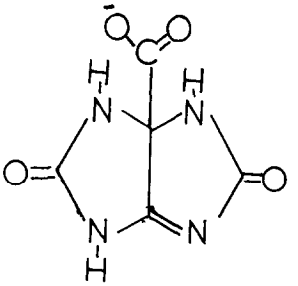
presumed to be 1-carboxy-2,4,5,6-tetraazabicyclo [3.3.0] octa-4-ene-3,7-dione (I). This contraction



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of a 5,6 disubstituted pyrimidine ring system to yield a five-membered ring coupled with reports of potent uricase inhibition by oxonate and similar s-triazines led us to test various substituted pyrimidines as potential uricase inhibitors.

The objectives of this report are to show that certain substituted pyrimidines are effective competitive inhibitors of uricase.

Materials and Methods

Uricase (type 1, from hog liver) was obtained as a suspension in ammonium sulfate from Sigma Chemical Company and was used without purification.

The various substituted pyrimidines and dihydropyrimidines tested as potential inhibitors of uricase were obtained as follows. 5-Bromo-5,6-dihydrouracil (3), 5-iodo-5,6-dihydrouracil (4), and 5-bromo-6-methoxy-5,6-dihydrouracil (4) were prepared by chemical synthesis using published methods. Imidazolidinone-2-carboxylic acid, 5-fluoro-orotic acid and boric acid (reagent grade) were from Aldrich Chemical Co., Biochemical Laboratories Inc., and Matheson, Coleman, Beall; respectively. All other compounds were from Sigma Chemical Co. All commercially available compounds were used as received. Glass distilled water was used to prepare all solutions and reaction mixtures. Uric acid solutions were freshly prepared prior to use.

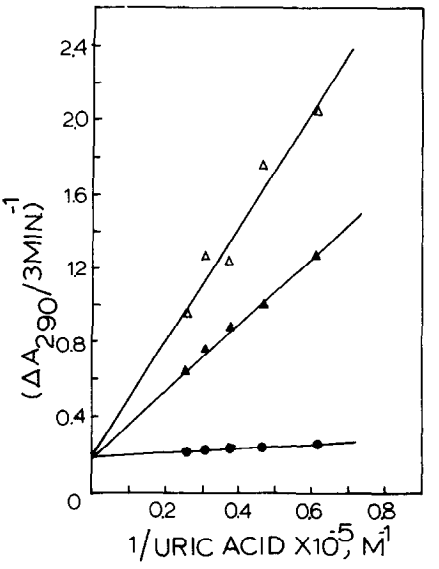


Fig. 1. Competitive inhibition of uricase by 5-nitrouracil at 25°, pH 8.5. (●), no inhibitor; (Δ), 3.0 x 10^{-4} M 5-NO_2-uracil; (▲), 1.5 x 10^{-4} M 5-NO_2-uracil.

Initial rates of the uricase catalyzed degradation of uric acid were spectrophotometrically measured at 290 nm with a Gilford 2000 recording spectrophotometer equipped with a cell compartment thermostated at 25 ± 0.1°. An extinction coefficient (5) for uric acid at 290 nm of 1.22 x 10^4 M^{-1} cm^{-1} was used in all calculations. All reaction mixtures contained 0.20 moles borate buffer (pH 8.5), increasing amounts of uric acid, fixed amounts of inhibitor and enzyme in a final volume of 3.0 ml. After 5 minutes preincubation, reactions were initiated by the addition of 50 µl of the stock enzyme solution which had been previously diluted 10 fold with 0.10 M borate buffer (pH 8.5). Inhibition constants (K_i) and the mode of inhibition were evaluated graphically using standard methods (6). The pK_a of 5-azauracil was determined spectrophotometrically (250 nm) in 0.10 M sodium acetate, sodium phosphate, and tris(hydroxymethyl)aminomethane

TABLE I

Competitive Inhibition Constants for Uricase
Inhibition by Substituted Pyrimidines, 25°, pH 8.5

Pyrimidine	$10^6 K_i$, M
5-azauracil (allantoxaidine)	4.2[1]
6-azauracil	16
5-nitrouracil	13
5-fluorouracil	89
5-chlorouracil	72
5-bromouracil	680
5-iodouracil	680
5-hydroxyuracil (isobarbituric acid)	640
6-hydroxyuracil (barbituric acid)	1100
5-nitro-6-hydroxyuracil (diluturic acid)	360
5-carboxyuracil (iso-orotic acid)	140
5-fluoroorotic acid	360
5-nitroorotic acid	130
5-aminoorotic acid	440

[1]Reported as 1.7×10^{-6} M by Fridovich as a compound named allantoxaidine

buffers (25°, ionic strength 1.0 M) using a Cary 17 recording spectrophotometer equipped with a thermostated cell compartment.

Results and Discussion

Based on analysis of double-reciprocal plots of initial velocity against uric acid concentration all of the various pyrimidine derivatives which inhibited uricase exhibited competitive inhibition. Fig. 1 shows typical results for uricase inhibition by 5-nitrouracil. The intercepts of the double reciprocal plots at $1/v_i = 0$ were used to evaluate inhibition constants (K_i). For each inhibitor tested, a value of K_m was determined in the absence of added inhibitor. The

average value of K_m was equal to 0.78 ± 0.16 x 10^{-5} M, a value which compares favorably with the value of 0.50 x 10^{-5} M previously reported by Fridovich (2).

Of the 28 pyrimidine derivatives tested as uricase inhibitors, 14 competitively inhibited the enzyme sufficiently at experimentally feasible concentrations so that reliable inhibition constants (K_i) could be evaluated. These compounds and K_i values are summarized in Table I.

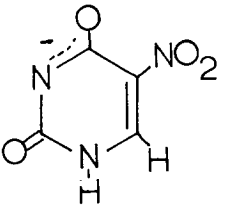
Another group of 4 pyrimidine derivatives only weakly inhibited uricase (10-15%) at the upper limits of concentrations which were experimentally feasible due to the background absorption of the inhibitors at 290 nm. Under these conditions, it was not possible to rigorously evaluate K_i values, however, estimates could be made. These estimated K_i values are: 6-aminouracil, 6 x 10^{-4} M; orotic acid, 2 x 10^{-3} M; 2-thioorotic acid, 1 x 10^{-3} M; and 5-bromo-6-methoxy-5,6-dihydrothymine, 3-4 x 10^{-3} M.

Ten of the pyrimidine derivatives failed to inhibit uricase at concentrations which were experimentally reasonable. These compounds at the highest concentration tester are: uracil, 1 x 10^{-3} M; thymine, 1 x 10^{-3} M; 5-aminouracil, 2.5 x 10^{-4} M; 2-thiouracil, 1.7 x 10^{-4} M; isocytosine, 1.7 x 10^{-4} M; 5-iodoorotic acid, 3.3 x 10^{-4} M; 5-nitro-1,3-dimethyluracil, 3.5 x 10^{-4} M; imidazolidinone-2-carboxylic acid, 3.6 x 10^{-3} M; 5-iodo-5,6-dihydrouracil, 1.7 x 10^{-3} M; and 5-bromo-5,6-dihydrouracil, 1.7 x 10^{-3} M.

As can be seen from inspection of the K_i values, substitution of electron withdrawing groups at C-5 of the pyrimidine ring system tends to favor the degree of inhibitor binding to uricase; however, steric factors must also be considered. The uracil series exemplifies this observation. The parent compound, uracil, does not observably inhibit uricase at concentrations up to 1 x 10^{-3} M while substitution at C-5 with the strongly electron withdrawing nitro group yields a moderately

potent competitive inhibitor with a K_i value of 1.3×10^{-5} M. Substitution at C-5 with electronegative Cl- and F- atoms also yielded effective uricase inhibitors, however the Br- and I- derivatives were about 10-fold less effective, a result which may be caused by increased steric inhibition of binding due to the bulk of these halogen atoms. A similar but less dramatic correlation can be made for both the orotic acid and barbituric acid series.

It is perhaps of significance, to note the relationship between increased inhibitor binding to uricase and increased acidity of the N-3 proton of the pyrimidine ring system, both of which are functions of the degree of electron withdrawing ability of substituents at C-5. For example, uracil which fails to inhibit uricase under these conditions has a pK_a of 9.5 (7) while 5-nitrouracil which interacts strongly with uricase has a pK_a of 5.3 (8). The practical result of the increased acidity of the proton on N-3 is that at pH 8.5, the conditions of assay, the potent inhibitor 5 nitrouracil exists as an anion (II) while

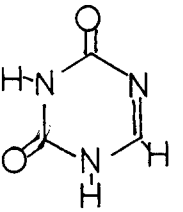


II

uracil which does not inhibit uricase is only about 10% deprotonated. It also should be noted that 1,3 dimethyl-5-nitrouracil, which cannot form an anionic species does not inhibit uricase although this result could be due to steric inhibition of binding due to the methyl groups at N-1 and N-3. In a similar way, the same correlation can be made for 5-fluorouracil, pK_a 8.0 (9) and 5-chlorouracil, pK_a 7.95 (9) each of which are about 75% ionized at pH 8.5.

The most potent inhibitor tested in this study was 5-azauracil (III), which exhibits a K_i equal to 4.2×10^{-6} M for the competitive

inhibition of uricase. This value agrees reasonably well with the value of 1.7×10^{-6} M previously determined by Fridovich (2). Again in the case of 5 azauracil, which has a pK_a of 6.55 (25°, μ = 1.0 M), an anion would be the dominant species present at pH 8.5. This inhibitor



III

might also have a statistically greater chance for interaction with uricase due to the substitution of a nitrogen for a carbon atom at position 5 of the ring system.

Currently, it is not possible to quantitatively relate the electron withdrawing ability of a C-5 substituent with the degree of inhibitor interaction with uricase probably because of steric interactions which also would be expected to influence the degree of inhibition. It is, however, possible to conclude that 5-substituted pyrimidines do inhibit uricase. Furthermore, it qualitatively appears that increased electron withdrawing ability influences inhibitor binding by increasing the acidity of protons on the pyrimidine ring system. Thus at pH 8.5, the conditions of assay, the most effective inhibitors would be in the anionic form. This is a reasonable hypothesis since uricase uses urate as the actual substance and is thought to have a cationic group at its active site (1).

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