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BEHAVIOR OF N-METHYLATED ALLOPURINOLS AND RELATED 4-THIOXOPYRAZOLO [3,4-*d*] PYRIMIDINES TOWARDS BOVINE MILK XANTHINE OXIDASE

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Summary

1. All available *N*-mono- and *N,N'*-dimethylallopurinols and the corresponding 4-thioxo derivatives have been tested as substrates or inhibitors of bovine milk xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2).

2. None of the compounds tested revealed any inhibitory activity towards the enzyme.

3. All compounds were resistant to enzymic oxidation, with the exception of 7-methylallopurinol and its 4-thioxo analog. Both these compounds were attacked at position 6. 7-Methylallopurinol was oxidised nearly ten times faster than the isomeric 3-methylhypoxanthine.

4. These observations can be explained by assuming that for attack at C-6, the enzyme must bind both to N-1 and N-2 in the pyrazole ring and causes tautomerisation, which places a double bond at position 5,6 in the pyrimidine ring. This activation process resembles the activation of hypoxanthine.

Allopurinol (pyrazolo[3,4-*d*]pyrimidin-4-one) and the corresponding 4-thioxo derivative are highly efficient inhibitors of milk xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2) [1]. Allopurinol is used clinically to reduce the formation of uric acid, e.g. in gout [2—4] or during antitumor chemotherapy, which is accompanied by massive lysis of cells. In such processes, large amounts of purines are liberated which can serve as substrates of xanthine oxidase [5]. Furthermore purine antimetabolites, used in chemotherapy, may be inactivated by enzymic oxidation. Thus 6-mercaptopurine

is converted into 8-hydroxy-6-mercaptopurine and subsequently to 6-thiouric acid [6]; also 6-chloropurine is attacked by xanthine oxidase [7]. Combined application of allopurinol and these antimetabolites extends their biological half-life considerably [8].

In view of the clinical applications of allopurinol, it was of interest to study its methylated derivatives, which have become available recently [9], as substrates or inhibitors of xanthine oxidase.

Most of the pyrazolo[3,4-*d*]pyrimidines, used in the present experiments, have been described recently [9]. The pertinent physical properties of 7-methylallopurinol 1 and the corresponding 4-thioxo derivative 3 are given in Table II.

7-Methylalloxanthine 2 and the corresponding 4-thioxo derivative 4 are new compounds. They were isolated from the enzymic reaction mixtures by paper chromatography (see Table II) and were identified by comparing their ultraviolet spectra with those of the non-methylated mother substances. Thus the neutral form of 2 (λ_{max} 248 nm) resembles alloxanthine (λ_{max} 254 nm), and the neutral form of 4 (λ_{max} 323 nm) is very similar to 6-oxo-4-thioxo-pyrazolo[3,4-*d*]pyrimidine (λ_{max} 325 nm).

For paper chromatography on Whatman paper No. 1 by the descending method, the following solvents were used: A, *n*-butanol/5 M acetic acid (2:1, v/v); B, isopropanol/dimethylformamide/ammonia (d = 0.88) (13:5:2, v/v). Spots were localised with the aid of a Desaga MinUvis lamp ($\lambda \sim 254$ or 366 nm).

Highly purified xanthine oxidase (30 000 units/ml; 115 mg of protein/ml) was a gift of Prof. R.C. Bray, School of Molecular Sciences, University of Sussex, England; ratio of activity/A_{450} at 25° C about 120. At pH 8.0 and 29° C, one unit of xanthine oxidase converts 1 μmol/min of xanthine to uric acid, when the concentration of the substrate is 5×10^{-5} M.

Catalase (Worthington) had an activity of 50 000 units/ml and contained

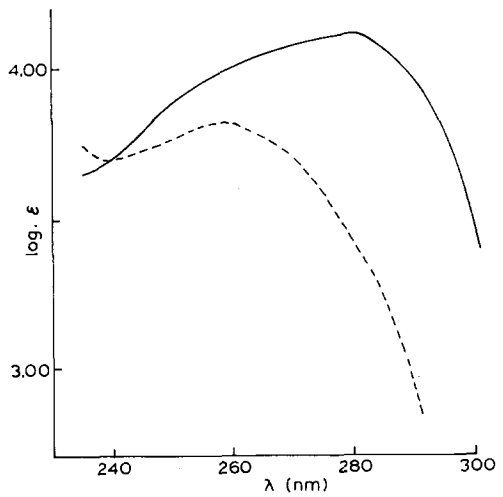
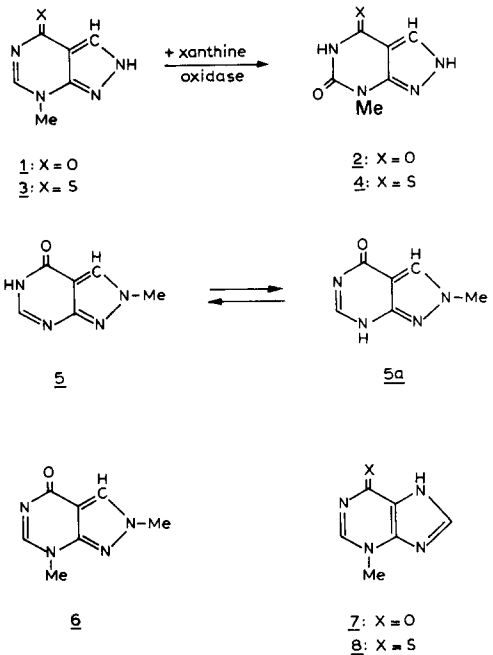


Fig. 1. Ultraviolet absorption spectra at pH 8. ——, 7-methylallopurinol 1; - - -, 7-methylalloxanthine 2. Note the marked hypsochromic and hypochromic shift, when 1 is converted into 2.

3 μg/ml protein. At a concentration of 0.06 M H_2O_2, 1 unit of catalase decomposes 1 μmol/min of H_2O_2 at 25° C and pH 7.

All enzymic reactions were carried out at 29° C in 0.01 M phosphate buffer of pH 8.0. Substrate (5×10^{-5} M) and catalase (10 units/ml) were incubated together for 3 min. Xanthine oxidase was added at zero time, and readings were taken at the wave lengths, specified in Table I. Rates were derived from the initial linear portions of the curves, showing ΔA as function of time. Relative rates were calculated by assuming a linear relation between enzyme concentration and rate of oxidation. A value of 100 was always assigned to the rate of xanthine at any given concentration of xanthine oxidase.

Xanthine oxidase underwent pre-incubation with the inhibitor for 30 min, before the substrate plus catalase were added at zero time. The highest concentration of potential inhibitors was 5×10^{-4} M, against 5×10^{-5} M xanthine as substrate.



1: X = O
3: X = S

2: X = O
4: X = S

5

5a

6

7: X = O
8: X = S

(1) N-Methyl derivatives of allopurinol as inhibitors of xanthine oxidase. None of the compounds tested, including 1-, 2-(5), 5- and 7-methylallopurinol (1) and the 1,5-, 2,5-, 1,7- and 2,7-dimethyl (6) derivatives, inhibited xanthine oxidase, although their concentration was 10 times higher than that of the standard substrate xanthine. Likewise the corresponding 4-thioxo derivatives were devoid of any inhibitory effect.

(2) N-Methyl derivatives of allopurinol as substrates of xanthine oxidase. None of these compounds served as substrate, with the exception of 7-methylallopurinol 1, which was oxidised to 7-methylalloxanthine 2 at a relative rate of 5.5 (Table I). Thus 1 is attacked about 10 times faster than 3-methylhypoxanthine 7 (rate 0.6) [10]. Similarly the 7-methyl-4-thioxo derivative 3 is

TABLE I

ENZYMIC OXIDATION OF 7-METHYLALLOPURINOL 1 AND ITS 4-THIOXO ANALOG

Reaction conditions: 0.01 M phosphate buffer of pH 8.0; temperature 29°C. Substrate and catalase (10 units/ml) were incubated together for 3 min, and the reaction was started by adding xanthine oxidase at zero time. Total volume was 3 ml. See Fig. 1 for spectral changes at pH 8 during the conversion of 1 into 2.

Compound No.	Wave lengths (nm) used for rate determinations	Concentration of		Relative rate (xanthine = 100)
		Substrate (M)	Xanthine oxidase (units per ml)	
1	280 290	5×10^{-5}	2.7	5.5
3	345 355	4.5×10^{-5}.	5.4	1.2

the only member of this series that is oxidised by xanthine oxidase to 4 (rate = 1.2). It should be recalled that the corresponding 3-methyl-6-thioxopurine 8 was found refractory [11].

It is noteworthy that the reactions of 1 and 3 go to completion. Conversion of 1 to 2 at pH 8 is accompanied by a marked hypsochromic shift of λ_{max} from 280 to 257 nm (Fig. 1). Similarly oxidation of 3 to 4 at pH 8 leads to a displacement of λ_{max} from 345 to 330 nm. These observations contrast with the spectral properties of the corresponding purines (see Table II) [11].

A

A'

B

B'

In order to explain the differences in the rates of compounds 1 and 3 on one side and of their isomers, 3-methylhypoxanthine 7 and 3-methyl-6-thioxopurine 8 on the other, we may recall that the active form of hypoxanthine A can best be represented by structure A', which bears a double bond at position 1,2. Tautomerisation of A to A' is caused by the enzyme, which binds this substrate via the groupings 6-C = O, 7-NH and 3-NH, 9-N [11]. In 3-methylhypoxanthine 7, the imidazole moiety bears a 7-NH group [12], while a double bond is fixed in position 1,2, but the 3-methyl substituent prevents binding via position 3 and interferes sterically with the progress of the reaction at C-2. Thus the rate is reduced to a low level (0.6). In 6-mercaptopurine, the enzyme cannot bind effectively to the grouping 6-C = S, 7-NH due to the inability of the thiocarbonyl group to form sufficiently strong hy-

TABLE II

ULTRAVIOLET ABSORPTION MAXIMA OF THE NEUTRAL FORMS, pK AND R_f VALUES OF PURINES AND OF THE CORRESPONDING PYRAZOLO[3,4-d]PYRIMIDINES

The absorption maxima in this table refer to the neutral forms and thus may differ in part from the maxima, measured at pH 8, which is the optimum for xanthine oxidase.

Compound	λ_{max}(nm)	pK for mono-anion formation	R_f-values in solvent A	R_f-values in solvent B	Fluorescence*
3-Methylhypoxanthine 7	264	8.4			
3-Methylxanthine	272	7.5			
7-Methylallopurinol 1	277	8.5	0.43	0.50	violet
7-Methylalloxanthine 2	248	11.5	0.55	0.73	light violet
3-Methyl-6-thioxopurine 8	338	7.8			
3-Methyl-6-thioxanthine	345	7.9			
7-Methyl-4-thioxopyrazolo[3,4-d]pyrimidine 3	352	7.8	0.49	0.51	orange
7-Methyl-6-oxo-4-thioxopyrazolo[3,4-d]pyrimidine 4	323	12.5	0.80	0.74	yellow

*For spot detection see p. 216.

drogen bonds. Therefore tautomerisation cannot be promoted by the enzyme, and binding can take place only at the opposite pole of this substrate molecule, i.e. at 3-N, 9-NH. Thus the rate of oxidation of 6-mercaptopurine is only 1. In 3-methyl-6-thioxopurine 8, effective binding at either pole of this molecule is impossible; therefore this substrate is refractory.

The pyrazolo[3,4-*d*]pyrimidines lack nitrogen at position 3 (the equivalent of N-7 in purines, see formula 1) and thus must bind to xanthine oxidase in a different manner, presumably via 1-N, 2-NH or via 1-NH, 2-N. The ready oxidation of 1 indicates that attack at C-6 requires the presence of a 5,6-double bond, similar to the distribution of unsaturated bonds in the active structure A′ of hypoxanthine. Tautomerisation of allopurinol B to form B′ is facilitated if the enzyme can bind to positions 1 and 2, but this is impossible if a methyl substituent is present at either nitrogen of the pyrazole moiety.

The assumption that both N-1 and N-2 are involved in complex formation between pyrazolo[3,4-*d*]pyrimidines and xanthine oxidase, is supported by the following observations:

(a) The 7-methyl derivative 1 is attacked at a substantial rate, presumably because binding at positions 1 and 2 is only little impeded by the 7-methyl group. It is suggested that N-7 is not required for complexing with the enzyme.

(b) The 2-methyl group in 2-methyl- (5) and in 2,7-dimethylallopurinol 6 makes binding at positions 1 and 2 impossible, although the former derivative could in principle tautomerise to the *p*-quinoid form 5a, corresponding to B′, and although the latter derivative has already a fixed 5,6-double bond.

It is most remarkable that unlike the reaction of xanthine oxidase with allopurinol itself, oxidation of 1 and 3 goes to completion. This clearly shows that 7-methylalloxanthine 2 and its 4-thioxo analog 4 to not act as inhibitors. It appears that complex formation between alloxanthine and reduced xanthine oxidase [13] involves N-7 and 6-C = O, i.e. this type of binding differs from the mode of attachment of allopurinol to the oxidised form of the enzyme. As mentioned before, for the latter compound binding to N-7 appears not to be necessary. It would be of great interest to test the 6-oxo derivative of pyrazolo[3,4-*d*]pyrimidine as potential inhibitor of xanthine oxidase.

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