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Oxidation of 7-aminothiadiazolo(3,4-*d*)pyrimidines and 7-aminofurazano(3,4-*d*)pyrimidines by xanthine oxidase and aldehyde oxidase*

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We have been investigating, for several years, the behavior of pteridines in flavoprotein oxidase systems, specifically the xanthine oxidase and aldehyde oxidase systems. 1,2,5-Thiadiazolo(3,4-*d*)pyrimidines (Fig. 1, compound a, X = S) have been found [1] to exhibit properties, both spectroscopic and with regard to chemical reactivity, which are similar to those reported for analogous pteridines; for example, the ultraviolet absorption spectrum of 7-aminothiadiazolo(3,4-*d*)pyrimidine closely resembles that of 4-aminopteridine (Fig. 1, compound c). We, therefore, were interested in determining whether thiadiazolopyrimidines such as compound a would react with xanthine oxidase and aldehyde oxidase in a fashion similar to that observed with pteridines. 7-Aminofurazano(3,4-*d*)pyrimidine (Fig. 1, compound b, X = O) has been reported [2], on the basis of X-ray analysis, to differ substantially from the analogous thiadiazolopyrimidine (compound a) with respect to electron delocalization in the pyrimidine portion of the molecule, and the great ease with which compound b undergoes nucleophilic reactions has been attributed to its unusual delocalization pattern. In several instances, the ease of oxidation of heterocyclic substrates by xanthine oxidase may be correlated with the abilities of these compounds to undergo nucleophilic reactions. For example, the nucleophilic addition of water, in acid solution, occurs readily with 2-amino-6,7-dimethylpteridine, but the isomeric 4-amino derivative resists hydration under comparable conditions. Xanthine oxidase readily converts 2-amino-6,7-dimethylpteridine to its 4-hydroxy derivative, but 4-amino-6,7-dimethylpteridine is not oxidized to a detectable extent by the enzyme [3]. We wished to determine whether compound b can serve as a substrate for xanthine oxidase and aldehyde oxidase and, if so, whether the susceptibility of this furazanopyrimidine to enzymatic oxidation is markedly different from that observed for the analogous thiadiazolopyrimidine. We also wished to determine whether suitably substituted analogs of compound b could serve as inhibitors of either xanthine oxidase or aldehyde oxidase.

All thiadiazolopyrimidines and furazanopyrimidines evaluated as substrates and/or inhibitors in the enzyme systems were synthesized at Princeton University [1, 4]. 7-Amino-5-hydroxythiadiazolopyrimidine was prepared by the method of Timmis [5], in which 2-hydroxy-4,5,6-triaminopyrimidine was condensed with thionyl chloride. 2-Amino-4-hydroxy-6-pteridine aldehyde was also synthesized at Princeton. Milk xanthine oxidase was a commercial product obtained from Worthington Biochemical Corp., Freehold, N.J. and P-L Biochemicals, Milwaukee, Wis. Rat liver xanthine oxidase was obtained by the method outlined by Kielley [6], and aldehyde oxidase was prepared by a modification of the method described by Rajagopalan *et al.* [7]; the modification consisted in use of ammonium sulfate fractionations [8] rather than chromatographic procedures to separate xanthine oxidase from aldehyde oxidase activity. Evaluation of compounds as substrates for xanthine oxidase and aldehyde oxidase was carried out spectrophotometrically using a Perkin–Elmer model 202 recording spectrophotometer with a thermostated sample chamber held at 34 ± 1°. Absorption spectra of reaction mixtures were recorded serially in the range of 270–390 nm. Evaluations of compounds as substrates for xanthine oxidase were carried out routinely in a 1-ml system using pH 7·0 phosphate buffer (0·1 M), the candidate substrate (1×10^{-4} M), and xanthine oxidase sufficient to produce an absorbance increase of 0·60 over 10 min with hypoxanthine (5×10^{-5} M) as the standard substrate. To ensure that spectroscopic changes observed on incubation of a given compound with xanthine oxidase were, in fact, enzyme mediated, all compounds judged to be substrates were incubated with xanthine oxidase in the presence of a high concentration (1×10^{-4} M) of 4-hydroxypyrazolo(3,4-*d*)pyrimidine (allopurinol: Aldrich Chemical Co., Milwaukee, Wis.), a potent inhibitor of xanthine oxidase. Studies using aldehyde oxidase were performed using Tris buffer (0·05 M; pH 7·8, containing 0·005% EDTA), the candidate substrate (1×10^{-4} M), catalase (1500 units/ml; obtained from Worthington Biochemical Co.), and sufficient aldehyde oxidase to produce an absorbance increase of 0·30 over 10 min at 300 nm using *N*-1-methyl nicotinamide (3×10^{-3} M) as the standard substrate. For inhibition studies with aldehyde oxidase, 2-methyl-1,4-naphthoquinone (menadione) was added to the reaction mixture at a concentration of 1×10^{-5} M. Studies of the xanthine oxidase-mediated oxidations, using an indirect method based on ferricyanide reduction, were performed at 420 nm, in the standard xanthine oxidase system to which potassium ferricyanide (1×10^{-3} M) was added. Thin-layer chromatographic identification of the product of the oxidation of compound a by xanthine oxidase was carried out using a reaction mixture composed of compound a (5×10^{-4} M), phosphate buffer (2·0 ml; pH 7·0), and xanthine oxidase (0·05 units) which was incubated overnight at 25°; 5 μl of the reaction mixture was applied to a 5 × 20 cm MN cellulose plate (Analtech, Newark, Del.), and the mobility of the reaction product was compared in an ethanol–water system (3:1) with those of compound a (blue fluorescence: R_f 0·69) and the 5-oxo derivative of compound a (yellow fluorescence: R_f 0·56). Estimation of K_m values and maximum velocities was done spectroscopically by measuring the increase in absorbancy at 320 nm (compound a) and the decrease in absorbancy at 340 nm (compound b) mediated by xanthine oxidase and aldehyde oxidase.

When compound a is incubated with xanthine oxidase, pronounced spectroscopic changes are observed; the characteristic absorption maximum of compound a at 340 nm is lost, and a new maximum appears at 328 nm. We

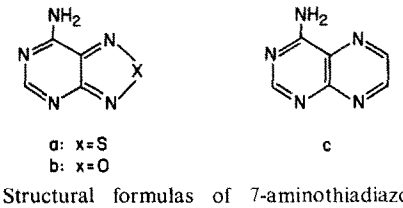

Fig. 1. Structural formulas of 7-aminothiadiazolo(3,4-*d*)pyrimidine, 7-aminofurazano(3,4-*d*)pyrimidine and 4-aminopteridine.

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expected that enzymatic oxidation of compound a would occur at the unsubstituted 5-position of the heterocyclic ring, yielding 7-amino-5-hydroxythiadiazolopyrimidine; this expectation was confirmed by our observation that the spectroscopic and chromatographic properties of the product of the xanthine oxidase-mediated reaction were identical to those of 7-amino-5-hydroxythiadiazolo(3,4-*d*)pyrimidine prepared chemically. The absorption maximum of the neutral molecule of the reaction product (328 nm) is similar to that reported [1] for the cation of the analogous 5,7-diaminothiadiazolopyrimidine (λ_{max} 323 nm); a similar relationship between the spectroscopic properties of the cation of 2,4-diaminopteridine and the neutral molecule of 4-amino-2-hydroxypteridine has been described recently by Albert and Taguchi [9]. Compound a has been reported previously [10] to be a substrate for xanthine oxidase in a study in which an indirect assay procedure (involving ferricyanide reduction) was employed, but no product identification or sensitivity of the reaction to inhibition was described. When compound a was incubated with xanthine oxidase in the presence of allopurinol, complete inhibition of the oxidation was observed (Table 1). We also found that 2-amino-4-hydroxy-6-pteridine aldehyde, another potent inhibitor of xanthine oxidase [11], was able to suppress the xanthine oxidase-mediated oxidation of compound a. Concomitant reduction of cytochrome *c* is normally detectable during xanthine oxidase-mediated oxidation of suitable substrates [12]; when cytochrome *c* was added to the reaction mixture containing compound a and xanthine oxidase, reduction of cytochrome *c* was readily demonstrable, as evidenced by the appearance of a sharp absorption maximum at 550 nm. The effect of varying the concentration of compound a on the rate of oxidation of the substrate by xanthine oxidase was investigated over a concentration range of 10^{-5} to 10^{-4} M; the apparent K_m value for compound a was determined to be 5×10^{-5} M. This K_m value is higher than those reported for purine derivatives such as xanthine and hypoxanthine but is closely similar to those reported by Krenitsky *et al.* [10] for several pteridines and related fused pyrimidines. The addition of an acetyl function to the 7-amino group of compound a was associated with a loss of substrate activity for xanthine oxidase; no oxidation of the acetylated derivative (at 1×10^{-4} M) was observable under conditions in which compound a was rapidly and completely oxidized.

We also investigated the ability of compound a to serve as a substrate for xanthine oxidase obtained from rat liver. As shown in Table 1, this mammalian liver oxidase is capable of oxidizing compound a; the spectral characteristics of the product of this reaction are identical to those of the product observed with the milk enzyme.

Aldehyde oxidase resembles xanthine oxidase both in structure and in catalytic function. A variety of simple pteridines are oxidized by aldehyde oxidase [10, 13] and the oxidation of compound a by this enzyme has been reported previously, but no data on product identification or susceptibility of the reaction to inhibition were provided. We find that compound a is converted by aldehyde oxidase to the same product as observed in the xanthine oxidase system. The oxidation of compound a mediated by aldehyde oxidase is inhibited virtually completely by menadione (Table 1). The apparent K_m established for compound a in the aldehyde oxidase system was $1{\cdot}2 \times 10^{-4}$ M, a value close to that observed for this compound as a substrate for xanthine oxidase. The similarity in the K_m values estimated for the oxidation of compound a by both xanthine oxidase and aldehyde oxidase is of interest in

Table 1. Inhibition studies and cytochrome *c* reduction in the xanthine oxidase and aldehyde oxidase systems*

Experiment	Absorbancy change	Cytochrome *c* reduction†
7-Aminothiadiazolo(3,4-*d*)pyrimidine and xanthine oxidase	0·120‡ (0·110)§	0·100
7-Aminothiadiazolo(3,4-*d*)pyrimidine and xanthine oxidase (plus allopurinol)	0·000‡ (0·000)	0·010
7-Aminofurazano(3,4-*d*)pyrimidine and xanthine oxidase	0·150‖ (0·110)	0·160
7-Aminofurazano(3,4-*d*)pyrimidine and xanthine oxidase (plus allopurinol)	0·005‖ (0·010)	0·010
7-Aminothiadiazolo(3,4-*d*)pyrimidine and aldehyde oxidase	0·180‡	0·220
7-Aminothiadiazolo(3,4-*d*)pyrimidine and aldehyde oxidase (plus menadione)	0·008‡	0·010
7-Aminofurazano(3,4-*d*)pyrimidine and aldehyde oxidase	0·075‖	0·100
7-Aminofurazano(3,4-*d*)pyrimidine and aldehyde oxidase (plus menadione)	0·010‖	0·010

* Xanthine oxidase studies were carried out at pH 7·0 with the substrate at 7×10^{-5} M and sufficient xanthine oxidase to give an absorbancy change of approximately 0·600 over 10 min at 290 nm with hypoxanthine at 7×10^{-5} M. Aldehyde oxidase studies were carried out at pH 7·8 with the substrates at 7×10^{-5} M and sufficient aldehyde oxidase to give an absorbancy change of approximately 0·300 at 300 nm using *N*-methylnicotinamide at 1×10^{-3} M. For inhibition studies, allopurinol (1×10^{-4} M) and menadione (1×10^{-5} M) were added to the reaction solution just before addition of the substrates. Cytochrome *c* was added to the reaction vessels at a final concentration of 1 mg/ml. Catalase (1500 units) was added to all aldehyde oxidase reaction mixtures and to xanthine oxidase reaction mixtures containing cytochrome *c*.

† Increase in absorbancy at 550 nm over 10 min.

‡ Increase in absorbancy at 320 nm over 10 min.

§ Data in parentheses were obtained using rat liver xanthine oxidase.

‖ Decrease in absorbancy at 340 nm over 10 min.

view of the observations [11] that pteridine and its 4-hydroxy derivative exhibit similar K_m values when evaluated with both of these enzymes, while purine and 6-hydroxypurine (hypoxanthine) exhibit substantially lower K_m values with aldehyde oxidase than with xanthine oxidase. Compound a, therefore, appears to resemble pteridines rather than purines with respect to apparent affinity for these enzymes.

7-Aminofurazano(3,4-*d*)pyrimidine (compound b) is an effective substrate for xanthine oxidase. The reaction of compound b with xanthine oxidase is characterized by a surprisingly large hypsochromic shift of the absorption maximum of the reaction mixture from 345 to 282 nm. The oxidation of compound b by xanthine oxidase is completely inhibited by allopurinol and is accompanied by a concomitant reduction of cytochrome *c* (Table 1). We have not yet been able to prepare the 7-amino-5-hydroxyfurazopyrimidine which we anticipated to be the product of the oxidation of compound b by xanthine oxidase. However by analogy with the reaction observed with compound a and xanthine oxidase, we have formulated the product of enzymatic oxidation of compound b as the 7-amino-5-hydroxy derivative. The assignment of structure to this product receives some support from the observation that the relatively low absorption maximum of the product of oxidation of compound b by xanthine oxidase is similar to that (287 nm) which we determined for the cation of 5,7-diaminofurazanopyrimidine, and this similarity is consistent with the spectroscopic correlation reported by Albert and Taguchi [9]. The assignment of structure to the product of enzymatic oxidation of compound b is consistent with the observation that 7-amino-5-methylfurazano(3,4-*d*)pyrimidine is not susceptible to oxidation by xanthine oxidase, an observation which indicates that oxidation of compound b does not involve, for example, *N*-oxide formation in the heterocyclic nucleus. Replacement of a hydrogen atom in the 7-amino function of compound b by a methyl group did not result in any marked reduction in substrate activity evaluated with xanthine oxidase, since under identical reaction conditions, the 7-methylamino derivative was oxidized at a rate closely similar to that of the parent compound (b). The K_m value estimated for compound b in the xanthine oxidase system was 7×10^{-5} M; this value is close to that estimated for compound a in this system and indicates that the apparent affinities of compound a and its "oxa" analog for xanthine oxidase are virtually the same. The very large differences in the susceptibilities of compounds a and b to nucleophilic reaction in a simple chemical system were not reflected in a comparatively large difference in the susceptibilities of the compounds to enzymatic oxidation by xanthine oxidase. The maximum rate of oxidation of compound b under standardized conditions was found to be $1{\cdot}8 \times 10^{-5}$ moles/liter/min, a value only 2- to 3-fold greater than that ($0{\cdot}70 \times 10^{-5}$ moles/liter/min) found for the thia analog (compound a). It has been reported previously [10] that compound a is oxidized by xanthine oxidase at a rate some 40-fold less than that observed for hypoxanthine, when both compounds were evaluated at identical substrate concentrations. We did not find such a large difference in the rates of oxidation of compound a and hypoxanthine when both compounds were studied at a concentration of 7×10^{-5} M. For example, under these conditions, hypoxanthine was oxidized at a rate ($1{\cdot}1 \times 10^{-5}$ moles/liter/min) only 7-fold higher than that observed with compound a. We have been informed (Dr. Thomas A. Krenitsky, personal communication) that the previous value [10] for the oxidation of compound a in the xanthine oxidase system was incorrect; Dr. Krenitsky informs us that his data indicate that hypoxanthine is, in fact, oxidized 6·5 times more rapidly than compound a, in good agreement with our finding. Adenine (6-aminopurine) has been reported [10] to be oxidized by xanthine oxidase at a rate 2-fold greater than that observed for compound a. We found that compound a, in the system employed by us, was oxidized 10 times more rapidly than adenine; this discrepancy between our observations and those of other workers may be due to differences in the assay system used. That the rate of oxidation determined for compound a using ferricyanide as an "indicator" of enzymatic function differs from that determined by direct observation of the oxidation process in the absence of ferricyanide was established by our observations that analysis of the oxidation of compound a using the ferricyanide method results in a significant reduction in the apparent rate of hydroxylation of this compound (control rate, $0{\cdot}15 \times 10^{-5}$ moles/liter/min; rate with ferricyanide assay, $0{\cdot}04 \times 10^{-5}$ moles/liter/min); the rate of oxidation of hypoxanthine on the other hand was closely similar using both assays.

The furazanopyrimidine (compound b) also was found to be oxidized by aldehyde oxidase to a product exhibiting spectral characteristics similar to those observed for the product of the reactions of this compound with xanthine oxidase. Menadione strongly suppressed the oxidation of compound b by aldehyde oxidase; during the oxidation of compound b by aldehyde oxidase, cytochrome *c* reduction could be demonstrated. Neither the K_m values (compound a, $1{\cdot}2 \times 10^{-4}$ M; compound b, $1{\cdot}5 \times 10^{-4}$ M) nor the maximal velocities (compound a, $0{\cdot}9 \times 10^{-5}$ moles/liter/min; compound b, $0{\cdot}2 \times 10^{-5}$ moles/liter/min) estimated for compounds a and b in the aldehyde oxidase system showed marked differences. Our observations concerning the relative reactivities of compounds a and b in the xanthine oxidase and aldehyde oxidase systems, systems which appear [14] to function by a process involving enzyme-catalyzed hydration of an appropriate heterocyclic substrate, may be related to observations made with adenosine deaminase, an enzyme which also appears to function by hydration of heterocyclic substrates. Thus Wolfenden *et al.* [15] have reported that compound b is deaminated enzymatically at a lower rate than compound a by adenosine deaminase from both fungal and mammalian sources even though one might anticipate, on the basis of chemical reactivity, that compound b would be deaminated much more rapidly than compound a.

The 5-methyl, 5-amino, 5-dimethylamino and 5-methylthio derivatives of compound b were found not to be oxidized appreciably by either xanthine oxidase or aldehyde oxidase, and we, therefore, examined such compounds as potential inhibitors of these enzymes. None of the compounds tested exhibited significant inhibitory activity when evaluated at a high concentration (10^{-4} M) in both the xanthine oxidase and aldehyde oxidase systems.

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Digitoxin metabolism by rat liver microsomes

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Neither digitoxigenin and epidigitoxigenin nor their water soluble conjugation products can be found in the bile of rats within 4 hr after i.v. injection of digitoxin [1]. The major portion of conjugates consists of glucuronides and sulfates of digitoxigenin monodigitoxoside [1, 2]. Therefore it is necessary for the study of microsomal digitoxin metabolism *in vitro* to use the native glycoside as a substrate instead of digitoxigenin investigated previously [3, 4, 5].

Liver microsomes of male Wistar rats (200 ± 10 g body wt) were prepared by the method of Kutt and Fouts [6]. Microsomal protein was determined by the method of Lowry *et al.* [7] with bovine serum albumin as the standard. For a complete system the incubation volume of 2·0 ml consisted of 0·15 M KCl, 50 mM $MgCl_2$, 10 mM succinate, 0·2 mg bovine serum albumin (Behring-Werke), 10 mM isocitrate, 1 mM NADP, 0·3 mM NADH, 200 mU isocitrate dehydrogenase, 3·5 mg microsomal protein, and 7 μM $^3H_{21-22}$-digitoxin (660 Ci/mole) prepared by the method of Haberland and Merten [8]. The mixtures were incubated at 37° with shaking. Controls were obtained by the omission of either pyridine nucleotides and NADPH regenerating system or microsomes or incubation at 37°. After the indicated incubation periods (see Table 1 and Fig. 2) 0·2 ml of the incubation mixture was transferred to 0·2 ml ice-cold methanol and centrifuged for 3 min at 5000 *g*. The pellet was washed with 0·5 ml methanol and centrifuged again. Reference compounds were added to the combined supernatants and subjected to thin-layer chromatography on precoated silicagel 60 F254 aluminium sheets (Merck). After development three times in solvent system I ($CHCl_3$–CH_3OH, 92:8, v/v) the plates were scanned for radioactivity (Dünnschicht-Scanner II, Berthold-Friesecke). Four zones of radioactivity were detected corresponding to the origin, digoxigenin glycosides, digitoxigenin glycosides, and an 'apolar' fraction (R_F relative to digitoxigenin 1·15). They were scraped off and eluted with $CHCl_3$–CH_3OH (1:2, v/v) for scintillation counting (Tricarb, Packard). Fractions containing the digoxigenin- and the digitoxigenin glycosides were rechromatographed in solvent system II (ethylacetate or ethylacetate–acetic acid–$CHCl_3$, 90:5:5, by vol., 3 developments) achieving a separation of glycosides according to the number of digitoxoses per molecule. The separated glycosides were scanned, eluted, and counted again. The recovery of radioactivity eluted from the plates amounted to more than 94% after development in system I. After chromatography in system II the recovery was in the range of 92–97% for the digitoxigenin glycosides and 87–94% for the digoxigenin glycosides.

To confirm the structure of the aglycone all fractions

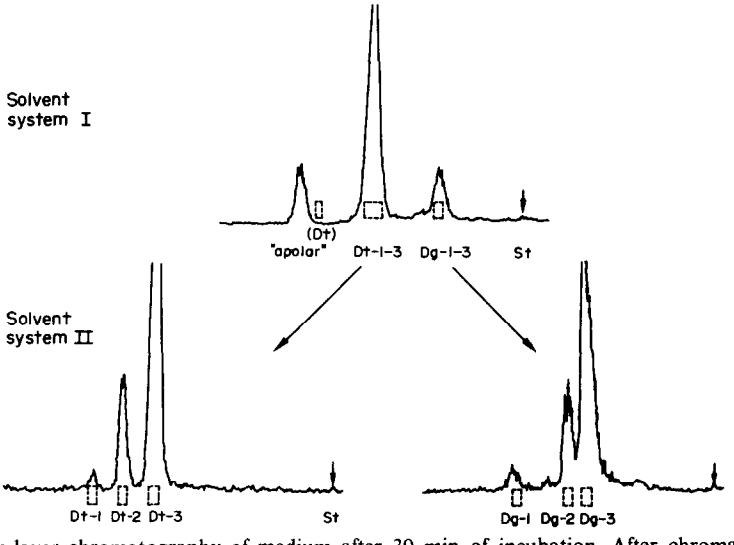

Fig. 1. Thin-layer chromatography of medium after 30 min of incubation. After chromatography in system I ($CHCl_3$–CH_3OH, 92:8, v/v) the zones corresponding to digitoxigenin- and digoxigenin digitoxosides were eluted and rechromatographed in system II (ethylacetate–$CHCl_3$–acetic acid, 90:5:5, by vol.) as described in the text. St = origin, Dg-1(2,3) = digoxigenin mono (bis, tri) digitoxoside, Dt-1(2,3) = digitoxigenin mono (bis, tri) digitoxoside, Dt = digitoxigenin.