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The Synthesis and Xanthine Oxidase Inhibitory Activity of Pyrazolo[3,4-*d*]pyrimidines

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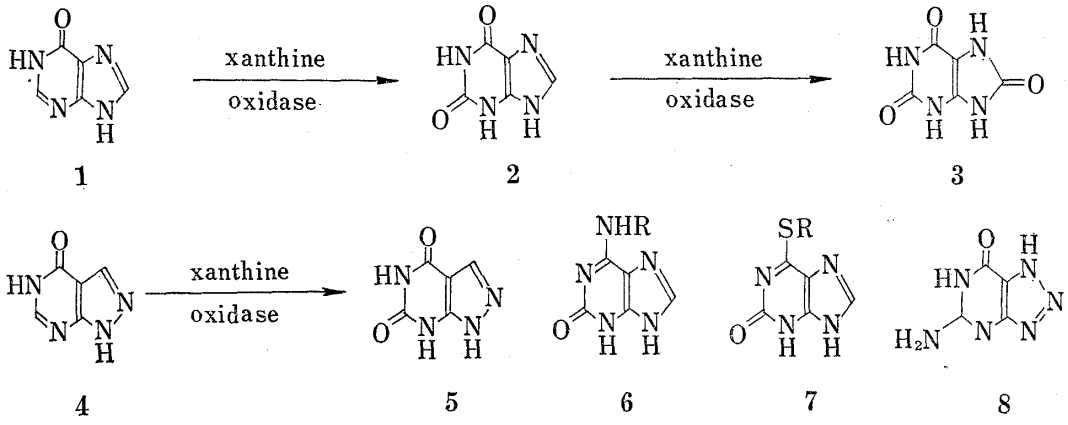
A series of compounds possessing the pyrazolo[3,4-*d*]pyrimidine nucleus were synthesized, characterized and screened for the ability to inhibit xanthine oxidase activity. The compounds were compared with 4-hydroxypyrazolo[3,4-*d*]pyrimidine (4), which showed 100% inhibition at a 33 μmole concentration. The structure-activity relationships of the synthesized compounds were discussed. 4-Amino-6-hydroxy-3-phenylpyrazolo[3,4-*d*]pyrimidine (**30b**) and 4-amino-3-(*p*-chlorophenyl)-6-hydroxypyrazolo[3,4-*d*]pyrimidine (**30c**) were found to be as potent inhibitors as allopurinol.

Xanthine oxidase is a catabolic enzyme that oxidizes hypoxanthine (**1**) and xanthine (**2**) to uric acid (**3**).[2,3] A variety of purine and azapurine analogs have been shown to serve as substrates for and inhibitors of xanthine oxidase.[4] Thus, besides their main metabolic pathways in the body, 2-hydroxypurine,[5] 8-hydroxypurine,[5] and adenine[6] can be oxidized by xanthine oxidase. 4-Hydroxypyrazolo[3,4-*d*]pyrimidine (**4**, Allopurinol[7]) is also slowly oxidized to 4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine (**5**) and both **4** and **5** act as potent inhibitors of xanthine oxidase.[8] An assortment of N^6-substituted 2-hydroxyadenines



1) Location: *Juso, Higashiyodogawa-ku, Osaka, 532, Japan.*
2) M. Dixon and S. Thurlow, *Biochem. J.*, **18**, 971 (1924).
3) E.C. DeRenzo, "Advances in Enzymology," Vol. 17, ed. by F.F. Nord, Interscience Publishers, Inc., New York, N.Y., 1956, p. 293.
4) G.B. Elion, S. Callahan, H. Nathan, S. Bieber, R.W. Rundles, and G.H. Hitchings, *Biochem. Pharmacol.*, **12**, 85 (1963), and references cited therein; B.R. Baker and W.F. Wood, *J. Med. Chem.*, **11**, 644 (1968); *idem, ibid.*, **12**, 211, 214 (1969); S.S. Parmar, C. Diwivedi, and B. Ali, *J. Pharm. Sci.*, **61**, 179 (1972).
5) F. Bergmann, G. Levin, H. Kwietny-Gourin, and H. Unger, *Biochem. Biophys. Acta*, **47**, 1 (1961).
6) J.B. Wyngaarden and J.T. Dunn, *Arch. Biochem. Biophys.*, **70**, 150 (1957).
7) Trade name Zyloprim, Borroughs Wellcome Co.
8) G.B. Elion, A. Kovensky, G.H. Hitchings, E. Metz, and R.W. Rundles, *Biochem. Pharmacol.*, **15**, 863 (1966).

(**6**)[9] and S[6]-substituted 2-hydroxy-6-mercaptopurines (**7**),[9] as well as 8-azaguanine (**8**),[10] also have been reported to be inhibitors of xanthine oxidase.

4-Hydroxypyrazolo[3,4-*d*]pyrimidine (**4**) has been marketed as a gout-relieving drug because it prevents the formation of uric acid for two reasons. First, it inhibits xanthine oxidase activity and second, it exercises a negative feedback regulation on the purine biosynthesis.[11] We have synthesized a series of compounds with the pyrazolo[3,4-*d*]pyrimidine nucleus and screened the compounds for inhibition of xanthine oxidase activity to find more powerful inhibitors which can be used as possible therapeutic agents for treatment of gouty patients.

Experiments were conducted with the isolated enzyme for avoiding complication such as transport through membranes and possible metabolic inactivation in the body.

This paper reports the synthesis and preliminary enzymic assessment of several series of pyrazolo[3,4-*d*]pyrimidines and also discusses the structure-activity relationship of the compounds synthesized.

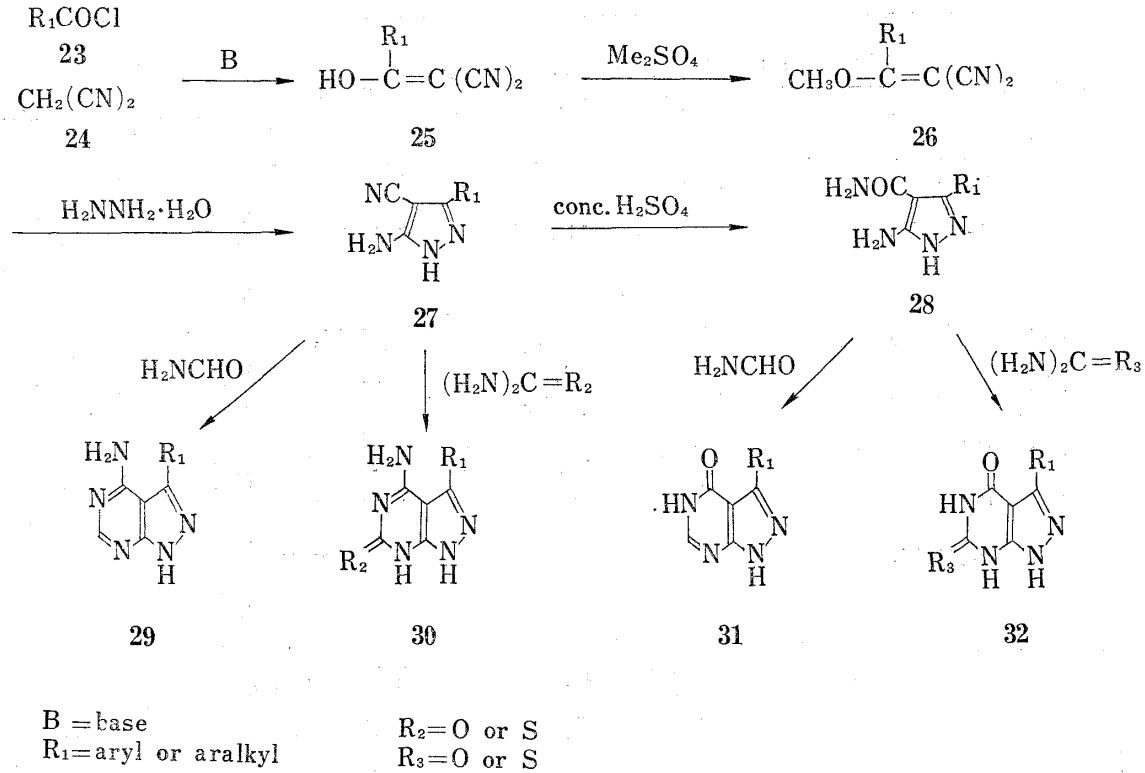
Synthesis

4-Substituted and 4,6-disubstituted pyrazolo[3,4-*d*]pyrimidines (**4**, **9**—**18**)[12] were synthesized by known methods.[13] Syntheses of 3,4-disubstituted and 3,4,6-trisubstituted pyrazolo-[3,4-*d*]pyrimidines (**29**—**32**) were carried out by the procedures of Libis and Fleury[14] and of Robins[13a] as outlined in Chart 1. Malononitrile (**24**) was treated with acid chloride (**23**) in the presence of a base to yield acylmalononitrile (**25**), which were subsequently methylated with dimethyl sulfate to give the substituted methoxymethylenemalononitrile (**26**). This compound (**26**) was then reacted with hydrazine hydrate in boiling alcohol to give the 3-substituted-5-aminopyrazole-4-carbonitrile (**27**) in good yield. Treatment of the 3-substituted 5-amino-pyrazole-4-carbonitrile with cold concentrated sulfuric acid gave the 3-substituted 5-amino-pyrazole-4-carboxamide (**28**) (Table I). Syntheses of pyrazolo[3,4-*d*]pyrimidines were accomplished from these two pyrazole intermediates, **27** and **28**. Thus, 3,4-disubstituted pyrazolo-[3,4-*d*]pyrimidines (**29** and **31**) were obtained by treating the corresponding **27** and **28** with boiling formamide. Similarly, 3,4,6-trisubstituted pyrazolo[3,4-*d*]pyrimidines (**30** and **32**) were obtained by fusing the corresponding **27** and **28** with urea and thiourea (Table II).

To prepare the amino acid conjugates of types **21** and **22**, 4-mercaptopyrazolo[3,4-*d*]-pyrimidine (**11**) and 4-hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidine (**10**) were subjected to the reaction with chloroacetyl derivatives of the corresponding amino acids[15] by the method of Kaverzneva, *et al.*[16] as shown in Chart 2.

Reactions were conducted in an aqueous medium in the presence of triethylamine with heating for a short period at about 80° or with stirring at room temperature for 12 hr. The reaction product separated out as a solid on acidification to pH 2—3. In all cases, there was little difficulty in purifying the products, in contrast to the difficulty met in purifying purine derivatives[16] (Tables III and IV).

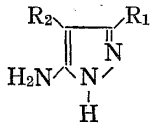
9) E.O. Leonard, W.H. Orme-Johnson, R.R. MacMurtray, C.G. Skinner, and W. Shive, *Arch. Biochem. Biophys.*, **99**, 16 (1962).
10) B.R. Baker and J.L. Hendrickson, *J. Pharm. Sci.*, **56**, 955 (1967).
11) R. McCollister, W.R. Gilbert, D.M. Ashton, and J.B. Wyngaarden, *J. Biol. Chem.*, **239**, 1560 (1964).
12) Nomenclature problems around with these derivatives are difficult since 1-methyl-4-hydroxypyrazolo-[3,4-*d*]pyrimidine, for example, exists mainly in the oxo tautomer (1-methyl-5H-pyrazolo[3,4-*d*]pyrimid-4-one) in neutral solution [C.C. Cheng and R.K. Robins, *J. Org. Chem.*, **21**, 1240 (1956)]. In an effort to reduce this difficulty the policy adopted in this paper is to name compounds as simple derivatives of the parent pyrazolo[3,4-*d*]pyrimidine (Table V, footnote).
13) *a*) R.K. Robins, *J. Am. Chem. Soc.*, **78**, 784 (1956); *b*) *Idem*, *ibid.*, **79**, 6407 (1957).
14) B. Libis and J.-P. Fleury, *Bull. Soc. Chim. France*, **1965**, 3323.
15) E. Ronwin, *J. Org. Chem.*, **18**, 127 (1953).
16) E.D. Kaverzneva, V.K. Zvorykina, and V.V. Kiseleva, *Bull. Acad. Sci. USSR*, **1966**, 1153.



B = base
R_1 = aryl or aralkyl
R_2 = O or S
R_3 = O or S

Chart 1. Reaction Scheme for Synthesis of 3,4- and 3,4,6-Substituted Pyrazolo[3,4-*d*]pyrimidines

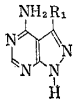
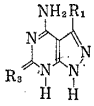
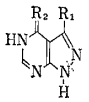
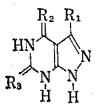
TABLE I. 5-Amino-3-aryl(aralkyl)pyrazole-4-carboxamides(carbonitriles)



Compd.	Substituents R_1	Substituents R_2	mp (°C)	Recrystn. solvent	Yield (%)	IR ν_{max}^{Nujol} (cm^{-1})	Formula	Analysis (%) Calcd. (Found) C	H	N	Cl
27a	C_6H_5	CN	200[a]	H_2O	86	2220 (CN)	$C_{10}H_8N$	65.20 (65.32)	4.38 (4.52)	30.42 (30.39)	— —
27b	*p*-ClC_6H_4	CN	212	H_2O	95	2220 (CN)	$C_{10}H_7N_4Cl$	54.93 (55.01)	3.23 (3.14)	25.62 (25.44)	16.22 (16.03)
27c	*p*-$O_2NC_6H_4$	CN	293 (decomp.)	50% EtOH	90	2220 (CN)	$C_{10}H_7O_2N_5$	52.40 (52.33)	3.08 (3.29)	30.56 (30.72)	— —
27d	$C_6H_5CH_2$	CN	142	H_2O	94	2220 (CN)	$C_{11}H_{10}N_4$	66.65 (66.46)	5.09 (5.25)	28.27 (28.02)	— —
27e	$C_6H_5CH_2CH_2$	CN	130	H_2O	70	2210 (CN)	$C_{12}H_{12}N_4$	67.90 (68.15)	5.70 (5.73)	26.40 (26.71)	— —
28a	C_6H_5	$CONH_2$	202—203	H_2O	86	1655 ($CONH_2$)	$C_{10}H_{10}ON_4$	59.39 (59.65)	4.98 (4.99)	27.71 (27.71)	— —
28b	*p*-ClC_6H_4	$CONH_2$	219—220	H_2O	82	1635 ($CONH_2$)	$C_{10}H_9ON_4Cl$	50.71 (50.61)	3.83 (3.73)	23.68 (23.45)	14.98 (14.93)
28c	*p*-$O_2NC_6H_4$	$CONH_2$	285	*b*)	88	1655 ($CONH_2$)	$C_{10}H_9O_3N_5$	48.58 (48.79)	3.65 (3.85)	28.33 (28.37)	— —

a) lit.[14] mp 200°
b) Reprecipitation from 1N NaOH solution with acetic acid.

Table II. 3-Substituted Pyrazolo[3,4-*d*]pyrimidines

Hetero-cycle	Compd.	Substituents R_1	R_2	R_3	mp (°C)	Recrystn. solvent	Yield (%)	Formula	Analysis (%) Calcd. (Found) C	H	N	S(Cl)
	**29a**	C_6H_5	—	—	256—268	H_2O	64	$C_{11}H_9N_5$	62.55 (62.32)	4.30 (4.30)	33.16 (33.22)	— —
	29b	*p*-ClC_6H_4	—	—	>300	H_2O	59	$C_{11}H_8N_5Cl$	53.78 (53.63)	3.28 (3.41)	28.51 (28.48)	— (14.43) — (14.33)
	29c	$C_6H_5CH_2$	—	—	260—261	50% EtOH	59	$C_{12}H_{11}N_5$	63.98 (64.14)	4.92 (4.78)	31.09 (31.12)	— —
	29d	$C_6H_5CH_2CH_2$	—	—	229—230	50% EtOH	59	$C_{13}H_{13}N_5$	65.25 (65.10)	5.48 (5.50)	29.27 (29.48)	— —
	**30a**	C_6H_5	—	S	>300	*a*)	36	$C_{11}H_9N_5S$	54.32 (54.35)	3.73 (3.71)	28.80 (28.50)	13.16 (13.11)
	30b	C_6H_5	—	O	>300	*a*)	84	$C_{11}H_9ON_5$	58.14 (58.25)	3.99 (3.89)	30.82 (30.68)	— —
	30c	*p*-ClC_6H_4	—	O	>300	*a*)	70	$C_{11}H_8ON_5Cl$	50.49 (50.59)	3.08 (2.99)	13.55 (13.51)	— (6.11) — (6.21)
	**31a**	C_6H_5	O	—	>300	H_2O	89	$C_{11}H_8ON_4$	62.25 (62.24)	3.80 (3.67)	26.40 (26.54)	— —
	31b	*p*-ClC_6H_4	O	—	>300	H_2O	80	$C_{11}H_7ON_4Cl$	53.56 (53.42)	2.86 (2.92)	22.72 (22.68)	— (14.38) — (14.40)
	31c	*p*-$O_2NC_6H_4$	O	—	>300	H_2O	77	$C_{11}H_7O_3N_5$	51.36 (51.38)	2.74 (2.95)	27.23 (27.11)	— —
	31d	$C_6H_5CH_2$	O	—	264	50% EtOH	59	$C_{12}H_{10}ON_4$	63.70 (63.77)	4.46 (4.64)	24.77 (24.62)	— —
	33	C_6H_5	S	—	>300	H_2O	56	$C_{11}H_8N_4S$	57.89 (57.88)	3.53 (3.71)	24.55 (24.99)	14.22 (13.88)
	**32a**	C_6H_5	O	S	>300	*a*)	100	$C_{11}H_8ON_4S$	54.10 (54.03)	3.30 (3.41)	22.94 (22.43)	13.11 (13.30)
	32b	*p*-ClC_6H_4	O	S	>300	50% EtOH	83	$C_{11}H_7ON_4SCl$	47.40 (47.35)	2.53 (2.25)	20.10 (19.89)	11.50 (12.72) (11.61) (12.89)
	32c	C_6H_5	O	O	>300	*a*)	95	$C_{11}H_8O_2N_4$	57.89 (57.87)	3.53 (3.56)	24.55 (24.70)	— —
	32d	*p*-ClC_6H_5	O	O	>300	*a*)	58	$C_{11}H_7O_2N_4Cl$	50.49 (50.52)	2.70 (2.49)	21.41 (20.91)	— (13.17) — (13.27)

a) Reprecipitation from a hot 2N NaOH solution with acetic acid.

Biochemical Studies

Xanthine oxidase activity has been found in various animal tissues. Another well-known source is cow's milk. Though little work has been done with mammalian and avian tissue enzymes, available evidence indicates that these enzymes are capable of affecting the substrates for the milk enzyme.[2)] This implies that results of the experiments on the milk enzyme would parallel the results with other tissue enzymes, which are generally more tedious to prepare in a purified state. Thus, a partially-purified enzyme was prepared from milk by the method of Horecker and Heppel[17)] without conducting the gel adsorption procedure.

17) B.L. Horecker and L.A. Heppel, "Method in Enzymology," Vol. 2, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, Inc., New York, N. Y., 1955, p. 482.

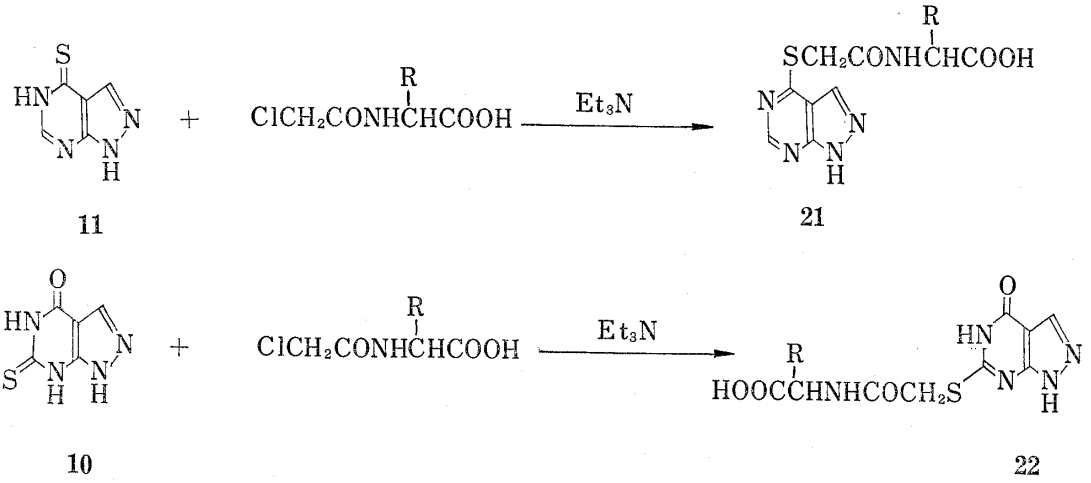
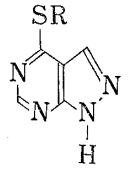


Chart 2. Reaction Scheme for Synthesis of Amino Acid Conjugates

Table III. S^4-Substituted 4-Mercaptopyrazolo[3,4-*d*]pyrimidines

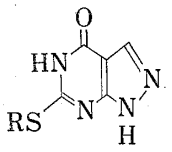


Compd.	Substituent R	mp (°C)	Recrystn. solvent	Yield (%)	Formula	Analysis (%) Calcd. (Found) C	H	N	S	Percent inhibition[a]
19	$CH_2C_6H_5$	173—174	50% MeOH	78	$C_{12}H_{10}N_4S$	59.50 (59.70)	4.16 (3.98)	23.13 (23.44)	13.21 (13.35)	14
20		192—193	70% MeOH	86	$C_{10}H_8N_4S_2$	48.39 (48.68)	3.25 (3.34)	22.58 (22.57)	25.79 (25.81)	12
21a	CH_2CO-Gly-OH	198—200	EtOH	58	$C_9H_9O_3N_5S$	40.45 (40.75)	3.40 (3.49)	26.21 (26.25)	11.98 (11.69)	82
21b	CH_2CO-Ala-OH	205 (decomp.)	EtOH	21	$C_{10}H_{11}O_3N_5S$	42.71 (42.47)	3.94 (4.14)	24.90 (25.16)	11.38 (11.72)	50
21c	CH_2CO-Val-OH	183 (decomp.)	EtOH	62	$C_{12}H_{15}O_3N_5S$	46.60 (46.73)	4.89 (4.81)	22.65 (22.76)	10.35 (10.16)	82
21d	CH_2CO-Leu-OH	163—164	EtOH	37	$C_{13}H_{17}O_3N_5S$	48.29 (48.20)	5.30 (5.59)	21.66 (21.76)	9.90 (9.77)	45
21e	CH_2CO-Met-OH	167—169 (decomp.)	EtOH	38	$C_{12}H_{15}O_3N_5S_2$	42.43 (42.29)	4.43 (4.62)	20.53 (20.36)	18.76 (18.68)	70
21f	CH_2CO-Tyr-OH	204—205 (decomp.)	EtOH	32	$C_{16}H_{15}O_4N_5S$	51.47 (51.37)	4.05 (4.01)	18.76 (18.59)	8.57 (8.47)	54
21g	CH_2CO-Phe-OH	171—174 (decomp.)	20% EtOH	29	$C_{16}H_{15}O_3N_5S$	53.78 (53.33)	4.23 (4.50)	19.60 (19.66)	8.79 (8.98)	27
21h	CH_2CO-Phe-OH[b]	186—189 (decomp.)	20% EtOH	28	$C_{16}H_{15}O_3N_5S$	53.78 (53.68)	4.23 (4.21)	19.60 (19.77)	8.79 (8.59)	26

a) concentration of inhibitor $=3.3\times10^{-5}$M *b*) $[\alpha]_D^{29}$ −37.0° (c=0.47, 50% EtOH)

For the comparison of the inhibitory activity, we have undertaken the assessment at a 33 μM concentration, at which allopurinol showed 100% inhibition in our test system (Fig. 1).

Table IV. S^6-Substituted 4-Hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidines



Compd.	Substituent R	mp (°C)	Recrystn. solvent	Yield (%)	Formula	Analysis (%) Calcd. (Found) C	H	N	S	Percent inhibition[a]
22a	CH_2CO-Ala-OH	225 (decomp.)	50% EtOH	61	$C_{19}H_{11}O_4N_5S$	40.41 (40.49)	3.73 (3.75)	23.56 (23.76)	10.77 (10.53)	31
22b	CH_2CO-Val-OH	153 (decomp.)	50% EtOH	40	$C_{12}H_{15}O_4N_5S$	44.31 (44.33)	4.65 (5.01)	21.53 (21.43)	9.84 (9.73)	18
22c	CH_2CO-Met-OH	208 (decomp.)	50% EtOH	24	$C_{12}H_{15}O_4N_5S_2$	40.34 (40.12)	4.23 (4.19)	19.60 (19.88)	17.92 (17.70)	30
22d	CH_2CO-Phe-OH	105 (decomp.)	50% EtOH	39	$C_{16}H_{15}O_4N_5S$	51.47 (51.69)	4.05 (4.15)	18.76 (18.92)	8.57 (8.17)	32

a) concentration of inhibitor $=3.3\times10^{-5}$ M

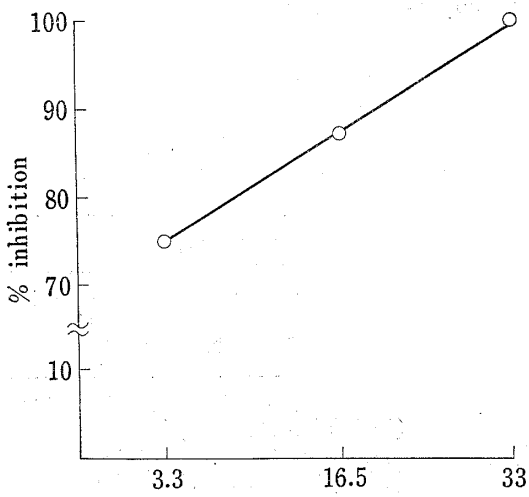


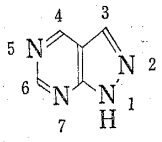
Fig. 1. Inhibition of Xanthine Oxidase by 4-Hydroxypyrazolo[3,4-*d*]pyrimidine

Table V. Inhibition of Xanthine Oxidase by Pyrazolo[3,4-*d*]pyrimidine Analogs[a] (**9**—**18**)

Substituents	Compd.	Percent inhibition[b]
4,6-Di-OH	**9**	69
4-OH, 6-SH	**10**	86
4-SH	**11**	98
4-SCH_3	**12**	68
4-OH, 6-SCH_3	**13**	74
4-NH_2, 6-OH	**14**	94
4-NH_2, 6-SH	**15**	86
4,6-Di-Cl	**16**	57
4-OH, 6-Cl	**17**	46
4-OH, 6-NH_2	**18**	78
4-OH	**4**	100

a) pyrazolo[3,4-*d*]pyrimidine

b) concentration of inhibitor $=3.3\times10^{-5}$ M



Enzyme assay was carried out by measuring the rate of the increase of optical density at 290 mμ, where uric acid has a maximum absorption and hypoxanthine a minimum,[18] as described in the Experimental section.

In the first stage of this work, several pyrazolo[3,4-*d*]pyrimidine analogs were assessed for inhibition of xanthine oxidase, in order to confirm the validity of the evaluation system employed. Table V summarizes the relative potencies of the compounds tested.

Result and Discussion

As shown in Table V, all compounds tested exhibited some degree of inhibitory activity. Among them, 4-mercaptopyrazolo[3,4-*d*]pyrimidine (**11**) and 4-amino-6-hydroxypyrazolo-

18) H.M. Kalcker, *J. Biol. Chem.*, **167**, 429 (1947).

[3,4-*d*]pyrimidine (**14**)[19] have been found to be an effective inhibitor of xanthine oxidase. Other compounds which definitely inhibited xanthine oxidase activity, were 4-hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidine (**10**) and 4-amino-6-mercaptopyrazolo[3,4-*d*]pyrimidine (**15**).

Elion, *et al.*[19b] and Baker, *et al.*[20] reported that substitution at the 6 position of pyrazolo-[3,4-*d*]pyrimidine by a hydroxy group results in a decreased affinity for the enzyme. Compound **9**, however, showed a fairly strong inhibitory activity. This, together with the fact that the compound has a structural analogy to allopurinol, prompted us to synthesize its homologs.

In general, introduction of a methylthio group at the 4 position to yield **12** and at the 6 position to yield **13**, and introduction of an amino group at the 6 position to yield **18**, decreased the inhibitory activity. Chlorine substitution (**16** and **17**) caused the greatest loss of activity, probably due to decreased binding with the enzyme. Compounds **19** and **20** suggest that a strong hydrophobic interaction of the aromatic substituent at the 6 position with xanthine oxidase might be a primary cause of the decreased inhibitory activity. While a lack of the activity of compounds **19** and **20** is due to a steric, the fact that compounds **12**, **13**, **16** and **17** are inactive might be better explained by an electronic effect of the substituent.

Purine derivatives containing amino acid residues in their molecules have received some attention as possible antimetabolites in the chemotherapy of cancer.[16,21] Also, many other workers reported that the introduction of residues of carboxylic acids into the mercapto group of purine-6-thiol (one of the most powerful antimetabolites used in clinical practice) does not reduce its biological activity.[22] In connection with this, it appeared worthwhile to synthesize and see whether compounds **21** and **22** would inhibit the xanthine oxidase. The relative inhibition potencies of these compounds are summarized in Tables III and IV. Thus far, none of the modified amino acid derivatives of the prototypes (**10** and **11**) have caused any great increase in inhibition. Only **21a** and **21c** showed moderate activity. Introduction of the phenylalanine moiety (**21g**) resulted in a drastic loss of activity. But interestingly enough, there was no definite difference between the activities of the *dl*- and *l*-forms (**21g** and **21h**), suggesting that the amino acid moiety does not play an important role in inhibition of the enzyme. Substitution at the 8 position (**22**) caused loss of activity in all cases tested (Table IV).

While this work was in progress, we also found a moderate inhibitory activity of **34** and a high inhibitory activity of **35** against xanthine oxidase.[23] Consequently, our efforts were directed to synthesizing **33** and the corresponding 4-oxo derivatives which bear a structural similarity to **35**. A strong hydrophobic interaction between inhibitors and xanthine oxidase has been argued with respect to some pyrazolo[3,4-*d*]pyrimidines.[20,24] Therefore, we have synthesized another pyrazolo[3,4-*d*]pyrimidines which bear a substituted phenyl group at the 3-position, and compared the effect of the structural changes on the inhibitory activity.

A series of compounds with an aromatic substituent at the 3 position of the ring prepared and evaluated are shown in Table VI. Introduction of a phenyl group (**30a**, **30b**, **31a**, **32a**,

19) *a*) P. Feigelson, J.D. Davidson, and R.K. Robins, *J. Biol. Chem.*, **226**, 993 (1957); *b*) G.B. Elion, F.M. Benezra, I. Carellas, L.O. Carrington, and G.H. Hitchings, *Israel J. Chem.*, **6**, 787 (1968) [*C. A.*, **70**, 86069 (1969)].

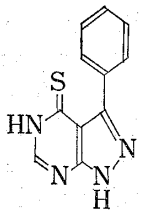
20) B.R. Baker, W.F. Wood, and J.A. Kozma, *J. Med. Chem.*, **11**, 661 (1968).

21) C.E. Carter, *J. Biol. Chem.*, **223**, 139 (1956); H. Lettlé and H. Ballweg, *Ann. Chem.*, **633**, 171 (1960); A. Ballio and V.D. Vittorio, *Gazz. Chim. Ital.*, **90**, 501 (1960); D.N. Ward, J. Wade, E.F. Wallborg, and T.S. Osdeue, *J. Org. Chem.*, **26**, 5000 (1961).

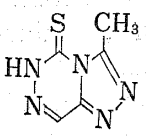
22) C. Huber, *Angew. Chem.*, **68**, 706 (1956); C.G. Skinner, J.R. Glaybrook, D.L. Ross, and W. Shive, *J. Org. Chem.*, **23**, 1223 (1958); G.B. Elion, I. Goodmann, W. Lange, and G.H. Hitchings, *J. Am. Chem. Soc.*, **81**, 1898 (1959); M. Semonsky, A. Cerny, and V. Jelinek, *Collection Czech. Chem. Commun.*, **92**, 287 (1959) [*C.A.*, **55**, 16576b (1961)].

23) T. Matsuo, Y. Shirakawa, and Z. Suzuoki, to be published.

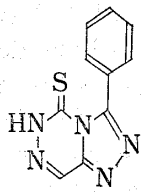
24) *a*) B.R. Baker, *J. Pharm. Sci.*, **56**, 959 (1967); *b*) B.R. Baker and J.A. Kozma, *J. Med. Chem.*, **11**, 656 (1968).



33



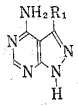
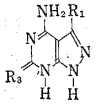
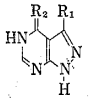
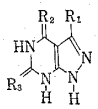
34



35

32c, and **33**) resulted either in decreased or increased activity when compared to the parent compounds (**15**, **14**, **4**, **10**, **9**, and **11**). Only **30b** showed an order of activity comparable to that of allopurinol. Introduction of a chlorine atom in the *para* position of a 3-phenyl group (**29b**, **30c**, **31b**, **32b**, and **32d**) resulted in increased activity in all compounds tested. Compound **30c** also showed 100% inhibition. Furthermore, introduction of a nitrophenyl group (**31c**) was beneficial to the activity, as was the case for introduction of the 6-phenyl substituent.[20,24b]

Table VI. Inhibition of Xanthine Oxidase Activity by 3,4- and 3,4,6-Substituted Pyrazolo[3,4-*d*]pyrimidines

Heterocycle	Compd.	Substituents			Percent inhibition[a]
		R_1	R_2	R_3	
	**29a**	C_6H_5	—	—	9
	29b	*p*-ClC_6H_5	—	—	23
	29c	$C_6H_5CH_2$	—	—	5
	29d	$C_6H_5CH_2CH_2$	—	—	11
	**15**	none	—	S	86
	30a	C_6H_5	—	S	62
	14	none	—	O	94
	30b	C_6H_5	—	O	100
	30c	*p*-ClC_6H_4	—	O	100
	**4**	none	O	—	100
	31a	C_6H_5	O	—	11
	31b	*p*-ClC_6H_4	O	—	23
	31c	*p*-$O_2NC_6H_4$	O	—	88
	31d	$C_6H_5CH_2$	O	—	40
	11	none	S	—	98
	33	C_6H_5	S	—	11
	**10**	none	O	S	86
	32a	C_6H_5	O	S	19
	32b	*p*-ClC_6H_4	O	S	58
	9	none	O	O	69
	32c	C_6H_5	O	O	55
	32d	*p*-ClC_6H_4	O	O	64

a) concentration of inhibitor = 3.3×10^{-5} M

4-Aminopyrazolo[3,4-*d*]pyrimidine has been reported to be a rather weak inhibitor of xanthine oxidase, being 350-fold less effective as allopurinol.[20,25] However, the 6-phenyl analog exhibited an enhanced activity due to superior hydrophobic properties.[20] In contrast, in our experiments, introduction of the 3-phenyl group (**29a**) did not result in an increase of activity. Introduction of a benzyl or phenethyl group (**29c** and **29d**) also did not increase activity.

From the above discussion it appears that **30b** and **30c** would be worthy of *in vivo* testings. Furthermore, since the NO_2 group of **31c** imparts more powerful inhibition than the Cl atom

25) P. Feigelson, J.D. Davidson, and R.K. Robins, *J. Biol. Chem.*, **226**, 993 (1957).

(**31b**), further investigation on the effects of small substituents on the phenyl moiety should be worthwhile. Details on enzymic studies, including the results of *in vivo* testing, will be reported elsewhere by the investigators of Biological Research Laboratories of this Division.[23]

Experimental[26]

N-(Pyrazolo[3,4-*d*]pyrimidin-4-ylthio)acetyl Glycine (21a)——To a solution of 0.30 g of 4-mercaptopyrazolo[3,4-*d*]pyrimidine (**11**) in 8 ml of water and 1 ml of triethylamine, 0.30 g of N-(chloroacetyl)glycine was added. The mixture was heated for 2 hr on a water-bath at 80°. After cooling, the solution was acidified with 2N HCl to pH 3 and refrigerated overnight. The precipitate formed was collected by filtration and recrystallized from ethanol to yield 0.31 g of the product; mp 198—200°; IR ν_{max}^{Nujol} cm^{-1}: 1705 (C=O), 1630 (CONH); NMR (d_6-DMSO) δ: 3.80 (2H, doublet, J=6 cps, NCH_2CO), 4.20 (2H, singlet, SCH_2CO), 8.29 (1H, singlet, 6-CH), 8.49 (1H, multiplet, CONH), 8.70 (1H, singlet, 3-CH), *ca.* 13.00 (2H, COOH and 1-NH). *Anal.* Calcd. for $C_9H_9O_3N_5S$: C, 40.45; H, 3.40; N, 26.21; S, 11.98. Found: C, 40.75; H, 3.49; N, 26.25; S, 11.69.

N-(4-Hydroxypyrazolo[3,4-*d*]pyrimidin-6-ylthio)acetyl Alanine (22a)——To a solution of 0.50 g of 4-hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidine (**10**) in 15 ml of water and 1.5 ml of triethylamine, 0.50 g of N-(chloroacetyl)alanine[27] was added. The mixture was stirred at room temperature for 12 hr. The solution was acidified with 2N HCl to pH 3 and refrigerated overnight. The resulting precipitate was collected by filtration and recrystallized from 50% ethanol to give 0.54 g of the product; mp 225° (decomp.); NMR (d_6-DMSO) δ: 1.31 (3H, doublet, J=7 cps, CH_3), 4.01 (2H, singlet, SCH_2CO), 4.28 (1H, multiplet, J=7 cps, NCHCO), 8.06 (1H, singlet, 3-CH), 8.51 (1H, doublet, CONH), 12.44 and 12.96 (3H, COOH, 1- and 5-NH). *Anal.* Calcd. for $C_{10}H_{11}O_4N_5S$: C, 40.41; H, 3.73; N, 23.56; S, 10.77. Found: C, 40.49; H, 3.75; N, 23.76; S, 10.53.

5-Amino-3-(*p*-chlorophenyl)pyrazole-4-carbonitrile (27b)——*p*-Chlorobenzylmethoxymethylenemalononitrile (**26**, R_1=*p*-ClC_6H_4), 6.0 g, was added in small portions to 16.0 g of 80% hydrazine hydrate and the mixture was heated on a water bath for 1 hr to obtain a clear solution. After adding 100 ml of water, the solution was set aside in a refrigerator overnight. The resulting mushy solution was filtered and the solid was washed with 20 ml of cold water and dried. The crude yield of pale yellow material was 6.5 g, mp 208—210°. Recrystallization from water raised the mp to 212°; IR ν_{max}^{Nujol} cm^{-1}: 2220 (CN); NMR (d_6-DMSO) δ: 6.41 (2H, NH_2), 7.46 and 7.78 (4H, doublet, J=8 cps, C_6H_4), 12.16 (1H, NH). *Anal.* Calcd. for $C_{10}H_7N_4Cl$: C, 54.93; H, 3.23; N, 25.62; Cl, 16.22. Found: C, 55.01; H, 3.14; N, 25.44; Cl, 16.03.

5-Amino-3-phenylpyrazole-4-carboxamide (28a)——Concentrated sulfuric acid, 30 ml, was cooled to 20° and 9.0 g of finely powdered 5-amino-3-phenylpyrazole-4-carbonitrile (**27a**) was added with stirring so that the temperature did not rise above 40°. The solution was stirred at room temperature for 1 hr more to allow complete solution to take place. The sulfuric acid solution was next poured with stirring into a mixture of 120 ml of water and 60 g of ice and allowed to stand overnight in a refrigerator. The solution was then filtered and the product dissolved in 25 ml of water. This solution was adjusted to pH 9 with ammonium hydroxide. The precipitate was collected by filtration and recrystallized from water to yield 8.5 g of 5-amino-3-phenylpyrazole-4-carboxamide; mp 202—203°; IR ν_{max}^{Nujol} cm^{-1}: 1655 ($CONH_2$); NMR (d_6-DMSO) δ: 5.78 (2H, $CONH_2$), 6.12 (2H, NH_2), 7.49 (5H, singlet, C_6H_5), 11.90 (1H, NH). *Anal.* Calcd. for $C_{10}H_{10}ON_4$: C, 59.39; H, 4.98; N, 27.71. Found: C, 59.65; H, 4.99; N, 27.71.

4-Benzylmercaptopyrazolo[3,4-*d*]pyrimidine (19)——To a solution of 0.71 g of 4-mercaptopyrazolo-[3,4-*d*]pyrimidine (**11**) in 6 ml of 1N sodium methoxide, 0.70 g of benzylcholride was added with stirring. After thirty minutes, the precipitate gradually began to appear. Stirring was continued for an additional two hours and the precipitate removed by filtration. Recrystallization from 50% methanol yielded 0.94 g of the product; mp 173—174°. *Anal.* Calcd. for $C_{12}H_{10}N_4S$: C, 59.50; H, 4.16; N, 23.13; S, 13.21. Found: C, 59.84; H, 3.98; N, 23.41; S, 13.33.

Phenethylhydroxymethylenemalononitrile (25, R_1=$C_6H_5CH_2CH_2$)——To a solution of 1.5 g of sodium metal and 2.2 g of malononitrile, 5.4 g of 3-phenylpropionyl chloride in benzene was added in small portions with vigorous stirring. After one hour, the solvent was evaporated under reduced pressure. The residue was dissolved in 70 ml of 2N H_2SO_4 and the aqueous solution was extracted with ether, 3×50 ml portions. The ether solution was washed twice with 100 ml of water, then dried over anhydrous sodium sulfate. The solvent was evaporated and a brownish, oily substance crystallized on standing in a refrigerator overnight. Recrystallization from acetonitrile yielded 3.1 g of the product; mp 127—129°; IR ν_{max}^{Nujol} cm^{-1}: 2220 (CN).

26) Melting points were taken in open capillaries and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A-60 spectrophotometer (internal TMS standard). Infrared (IR) spectra were recorded on a Hitachi EPI-510 spectrometer.

27) The work was conducted with *dl*-amino acids unless otherwise stated.

Anal. Calcd. for $C_{12}H_{10}ON_2$: C, 72.71; H, 5.09; N, 14.13. Found: C, 72.80; H, 4.99; N, 13.94. Similarly, 4.7 g of phenylacetyl chloride yielded 3.5 g of benzylhydroxymethylenemalononitrile (**25**, $R_1=C_6H_5CH_2$); mp 76—77°. *Anal.* Calcd. for $C_{11}H_8ON_2$: C, 69.75; H, 4.68; N, 16.27. Found: C, 70.02; H, 4.48; N, 16.13.

Benzylmethoxymethylenemalononitrile (**26**, $R_1=C_6H_5CH_2$)——Benzylhydroxymethylenemalononitrile, 12 g, was dissolved in a mixture of 100 ml of dioxane and 16 ml of water. Next, 40 ml of dimethyl sulfate and 40 g of sodium bicarbonate were added and the mixture was heated on a water bath at 80—90° for two hours. Undissolved sodium bicarbonate was filtered off and the filtrate was diluted with 500 ml of water. This aqueous suspension was extracted with ether, 3×150 ml portions. The ether solution was washed with sodium bicarbonate solution and water, and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue was distilled under reduced pressure to yield 8.4 g of the product; bp_3 157°; IR ν_{max}^{Neat} cm^{-1}: 2210 (CN). *Anal.* Calcd. for $C_{12}H_{10}ON_2$: C, 72.71; H, 5.09; N, 14.13. Found: C, 72.56; H, 4.98; N, 14.01.

Similarly, 3.5 g of phenethylhydroxymethylenemalononitrile yielded 2.26 g of phenethylmethoxymethylenemalononitrile (**26**, $R_1=C_6H_5CH_2CH_2$); bp_5 127° (mp 54—55°). *Anal.* Calcd. for $C_{13}H_{12}ON_2$: C, 73.56; H, 5.70; N, 13.20. Found: C, 73.40; H, 5.99; N, 12.98.

4-Amino-3-phenylpyrazolo[3,4-*d*]pyrimidine (**29a**)——5-Amino-3-phenylpyrazole-4-carbonitrile (**27a**), 0.30 g, was dissolved in 3 ml of formamide and the solution was heated at 180—190° for 4 hr. The cooled solution was diluted with 10 ml of water. Filtration yielded 0.3 g of a crude tan material. Recrystallization from water gave 0.22 g of the product; mp 265—268°; NMR (d_6-DMSO) δ: 6.74 (2H, NH_2), 7.63 (5H, multiplet, C_6H_5), 8.26 (1H, singlet, 6-CH), 13.59 (1H, 1-NH). *Anal.* Calcd. for $C_{11}H_9N_5$: C, 62.55; H, 4.30; N, 33.16. Found: C, 62.32; H, 4.30; N, 33.22.

4-Amino-6-mercapto-3-phenylpyrazolo[3,4-*d*]pyrimidine (**30a**)——5-Amino-3-phenylpyrazole-4-carbonitrile (**27a**), 0.50 g, and 1.0 g of thiourea were heated at 180° for 30 min until the clear solution became mushy. Heating was then continued at 200° for an additional ten minutes. The cooled melt was dissolved in hot 2N KOH and the hot solution was acidified with glacial acetic acid to give 0.23 g of the product; mp>300°. *Anal.* Calcd. for $C_{11}H_9N_5S$: C, 54.32; H, 3.73; N, 28.80; S, 13.16. Found: C, 54.35; H, 3.71; N, 28.50; S, 13.11.

4-Hydroxy-3-phenylpyrazolo[3,4-*d*]pyrimidine (**31a**)——5-Amino-3-phenylpyrazole-4-carboxamide (**28a**), 3.4 g, and 15 ml of formamide were heated at 180—185° for 45 min. The cooled solution was diluted with 85 ml of cold water and filtered to give 3.3 g of the product. An analytical sample was obtained by recrystallizing the crude product from water; mp>300°; NMR (d_6-DMSO) δ: 7.46 and 8.34 (5H, multiplet, C_6H_5), 8.11 (1H, singlet, 6-CH), 12.14 (1H, 5-NH), 13.87 (1H, 1-NH). *Anal.* Calcd. for $C_{11}H_8ON_4$: C, 62.25; H, 3.80; N, 26.40. Found: C, 62.24; H, 3.67; N, 26.54.

3-Benzyl-4-hydroxypyrazolo[3,4-*d*]pyrimidine (**31d**)——5-Amino-3-benzylpyrazole-4-carbonitrile (**27d**), 0.3 g, was added to 1 ml of cooled concentrated sulfuric acid with stirring. The solution was stirred at room temperature for 1 hr more to allow complete solution to take place. The sulfuric acid solution was then poured onto ice. After several hours, the precipitate was collected by decanting and dried *in vacuo*. This brown precipitate was heated with 3 ml of formamide at 180—185° for 30 min. The cooled solution was diluted with 10 ml of water and filtered to give 0.2 g of crude product. Analytical sample was obtained by recrystallizing the crude sample from 50% ethanol; mp 264°; NMR (d_6-DMSO) δ: 4.23 (2H, singlet, CH_2), 7.31 (5H, C_6H_5), 7.97 (1H, 6-CH), 11.90 (1H, 5-NH), 13.40 (1H, 1-NH). *Anal.* Calcd. for $C_{12}H_{10}ON_4$: C, 63.70; H, 4.46; N, 24.77. Found: C, 63.77; H, 4.64; N, 24.62.

4,6-Dihydroxy-3-phenylpyrazolo[3,4-*d*]pyrimidine (**32c**)——5-Amino-3-phenylpyrazole-4-carboxamide (**28a**), 1.75 g, was heated with 3.5 g of urea at 160° for 10 min to obtain a clear solution. This solution became mushy and heating was continued for another 20 min at 190° until the mushy melt became solid. Next, the solid was dissolved in hot 2N KOH and the boiling basic solution was then carefully acidified with acetic acid. The solution was allowed to stand approximately 10 min and then filtered to give 1.88 g of the product; mp>300°. *Anal.* Calcd. for $C_{11}H_8O_2N_4$: C, 57.89; H, 3.53; N, 24.55. Found: C, 57.87; H, 3.56; N, 24.70.

4-Mercapto-3-phenylpyrazolo[3,4-*d*]pyrimidine (**33**)——4-Hydroxy-3-phenylpyrazolo[3,4-*d*]pyrimidine (**31a**), 0.5 g, and 2.5 g of phosphorus pentasulfide were added to 30 ml of tetraline. The solution was heated at reflux for 4 hr. The cooled solution was filtered and the solid material collected was recrystallized from water to give 0.3 g of the product; mp>300°. *Anal.* Calcd. for $C_{11}H_8N_4S$: C, 57.89; H, 3.53; N, 24.55; S, 14.22. Found: C, 57.88; H, 3.71; N, 24.29; S, 13.88.

Inhibition of Xanthine Oxidase (Xanthine-O_2 Oxidoreductase, E.C. 1.2.3.2)——An estimation of the amount of uric acid formed from the oxidation of hypoxanthine at 290 mμ, was used to determine xanthine oxidase activity. Xanthine oxidase from milk was obtained by the method of Horecker and Heppel without the gel adsorption. Prior to use, the enzyme preparation was diluted with water to 300 units suspension per 0.2 ml. A unit of activity was defined as the amount of enzyme causing an increase in absorbancy of 0.001/min.

A solution of 0.5 ml of 1.0M phosphate buffer, pH 7.4, 2.1 ml of water, and 0.2 ml of the diluted enzyme preparation, was put in a 3 ml cell and vigorously shaken for about 10 sec to cause absorption of air. Then

0.2 ml of 1.0 mM hypoxanthine solution was added and the rate of increase in absorbance recorded by a Gilford recording spectrophotometer. The inhibitor was dissolved in 0.04 ml of dimethylsulfoxide and added. Percent inhibition was calculated by the equation $100-(V_I/V_0\times 100)$, where V_0=velocity without inhibitor and V_I=velocity with inhibitor.

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