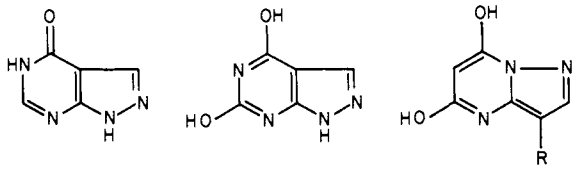
Synthesis and Enzymic Activity of Some Novel Xanthine Oxidase Inhibitors. 3-Substituted 5,7-Dihydroxypyrazolo[1,5-a]pyrimidines[1]

Robert H. Springer, Mary K. Dimmitt, Thomas Novinson, Darrell E. O'Brien, Roland K. Robins, Lionel N. Simon, and Jon P. Miller*

ICN Pharmaceuticals, Inc., Nucleic Acid Research Institute, Irvine, California 92664. *Received July 28, 1975*

A series of 3-substituted 5,7-dihydroxypyrazolo[1,5-a]pyrimidines containing various aromatic [phenyl- (**3e**), 3-pyridyl- (**3f**), *p*-bromophenyl- (**3g**), *p*-chlorophenyl- (**3h**), *p*-acetamidophenyl- (**3i**), *p*-tolyl- (**3j**), *m*-tolyl- (**3k**), 3,4-methylenedioxyphenyl- (**3m**), or naphthyl- (**3n**)] or nonaromatic [hydrogen- (**3a**), nitro- (**3b**), bromo- (**3c**), or chloro- (**3d**)] substituents in the 3 position was synthesized and tested as inhibitors of xanthine oxidase. The compounds (**3a–m**) were synthesized by condensation of the appropriate 3-amino-4-substituted pyrazole with diethyl malonate in alcoholic sodium methoxide and neutralization of the resulting enol sodium salts. As inhibitors of xanthine oxidase, **3e–n** > **3a,c,d** ≃ allopurinol > **3b**. The 3-aryl-substituted compounds **3e–n** were 30–160 times better xanthine oxidase inhibitors than allopurinol using hypoxanthine as substrate and 10–80 times better using xanthine as substrate, as evidenced by a comparison of K_i values. The inhibition by all compounds (**3a–n**) was totally reversible and of the noncompetitive or mixed type. A study of the pH dependence of xanthine oxidase inhibition by **3a,e,g** and allopurinol indicated that the 3-aryl substituents facilitated binding to the enzyme. These and the above results show that the compounds reported here inhibit xanthine oxidase by a mechanism which is significantly different from that of allopurinol.

Allopurinol[3] (1), despite some disadvantages,[4] is the most widely used drug for the treatment of gout. The effectiveness of this drug is due to its inhibition of xanthine oxidase, which in turn prevents the formation of excess quantities of uric acid in the body. The present study was



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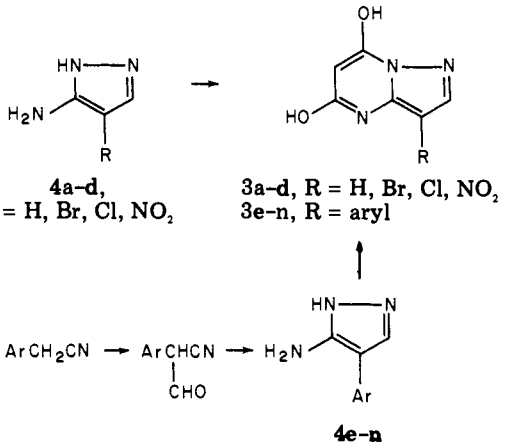
initiated in an attempt to design other compounds capable of inhibiting xanthine oxidase. Since allopurinol is rapidly metabolized in vivo to 4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine (2),[5,6] which also inhibits xanthine oxidase,[7] one logical approach was to synthesize certain 3-substituted 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines (3).

The rationale for synthesizing these compounds as potential xanthine oxidase inhibitors included the following: (1) the 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines (3) already contained the two hydroxyl groups in the same spatial arrangement as oxypurinol (2), which has been shown to be a "stoichiometric" inhibitor of xanthine oxidase that is not competitive with substrate;[8,9] (2) enzymatic hydroxylation of the pyrazolo[1,5-*a*]pyrimidine ring system probably would not take place, based on the chemical properties of this ring system;[10,11] (3) the lack of a nitrogen in the 3 position and the presence of a bridgehead nitrogen in the pyrazolo[1,5-*a*]pyrimidines should exclude the possibility that these compounds would be converted to unnatural nucleotides as is allopurinol;[12,13] and (4) extensive studies by Baker and Wood[14] have established that purines containing 9-aryl groups are excellent xanthine oxidase inhibitors due to hydrophobic bonding of the 9-substituent. Since the 3 position of the pyrazolo[1,5-*a*]pyrimidines (3) is spatially equivalent to the 9 position of purine, a hydrophobic group in the 3 position might give increased binding due to hydrophobic interactions. Derivatives of 3 were therefore synthesized with R = H, Cl, Br, NO_2, or various aryl groups.[15]

Chemistry. The condensation of various 3-amino-4-arylpyrazoles (4) with diethyl malonate in alcoholic sodium ethoxide (method A, Experimental Section) gave the respective enol sodium salts. Neutralization of the aqueous solutions of these salts gave the corresponding 3-aryl-5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines (**3e–n**). The derivatives of 4 (R = aryl) were prepared according to a similar literature method[16] (method B, Experimental Section). Arylacetonitriles were formylated and then cyclized with hydrazine in acetic acid to give the 3-amino-4-arylpyrazoles.

The condensation of 3-aminopyrazole with diethyl malonate has been reported to have given 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidine[17a] (**4a**, R = H). In a similar fashion, condensation of the known[17b] 4-bromo-, 4-chloro-, or 4-nitro-3-aminopyrazoles with the malonate ester gave the corresponding pyrazolo[1,5-*a*]pyrimidines substituted in the 3 position with these groups (**4b–d**).

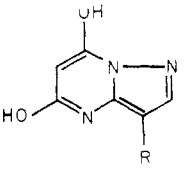


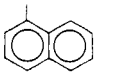
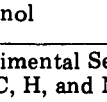
4a–d, R = H, Br, Cl, NO_2 — **3a–d**, R = H, Br, Cl, NO_2; **3e–n**, R = aryl

4e–n

Enzyme Assays. The assay for the reduction of electron acceptors[18] consisted of a 3-ml reaction mixture containing (in μmol) Tris–HCl, pH 7.8, 150; EDTA, 0.1; substrate, 1.0; bovine milk xanthine oxidase (0.13 units per 1 mg, ICN Life Sciences Group), 175 μg; and either 2,-6-dichlorophenol–indophenol, 0.3; or cytochrome *c*, 0.24, plus phenozine methosulfate, 0.24; plus bovine liver catalase (40000 units per 1 mg, ICN Life Sciences Group), 25 units. The reduction of 2,4-dichlorophenol–indophenol was followed at 600 nm and that of cytochrome *c* at 550 nm. The control reactions lacked substrate. The xanthine oxidase assay[19] consisted of a 3-ml reaction mixture containing (in μmol) Tris–HCl, pH 7.5, 150; EDTA, 0.1; xanthine oxidase, 20–40 μg; and either xanthine, 0.013, or hypoxanthine, 0.042. Enzyme assays were carried out aerobically at 25° at a single wavelength (290 nm for xanthine and 278 nm for hypoxanthine) with a Cary 15

Table I. Physical, Chemical, and Enzymatic Data for 3-Substituted 5,7-Dihydroxypyrazolo[1,5-a]pyrimidines



No.	R	Mp, °C	Yield, %	Method[a] of prepn	I_{50}, $M \times 10^{8}$ [b]	% inhibn after incubation[b] Trial 1[c]	Trial 2[d]	Emp formula[e]
3a	-H[f]	[f]	[f]	A	500.0	47	49	$C_6H_4N_4O_2$
3b	$-NO_2$	226-228 dec	77	A, B	1500.0	51	53	$C_6H_3N_5O_4$
3c	-Br	330-332 dec	45	A, B	120.0	50	52	$C_6H_3N_4O_2Br$
3d	-Cl	330-331 dec	64	A, B	140.0	45	44	$C_6H_3N_4O_2Cl$
3e	$-C_6H_5$	315-317 dec	48	A	3.8	47	49	$C_{12}H_8N_4O_2$
3f		275-277	25	A	4.3	50	53	$C_{11}H_7N_5O_2$
3g	$-C_6H_4$-*p*-Br	330-332 dec	36	A	3.3	52	53	$C_{12}H_7N_4O_2Br$
3h	$-C_6H_4$-*p*-Cl	330-331 dec	57	A	4.3	53	50	$C_{12}H_7N_4O_2Cl$
3i	$-C_6H_4$-*p*-NHCOCH$_3$	272-274 dec	54	A	8.9	47	48	$C_{14}H_{12}N_4O_3 \cdot 2H_2O$
3j	$-C_6H_4$-*p*-CH_3	255-257 dec	41	A	9.6	55	52	$C_{13}H_{10}N_4O_2$
3k	$-C_6H_4$-*m*-CH_3	284-285 dec	35	A	2.5	50	48	$C_{13}H_{10}N_4O_2$
3m		245-246 dec	59	A	2.9	49	46	$C_{13}H_8N_4O_4$
3n		219-221 dec	23	A	2.5	46	45	$C_{16}H_{10}N_4O_2$
Allopurinol					500.0	100	100	

[a] See Experimental Section. [b] See Enzyme Assays section. [c] For 1 hr at 4°. [d] For 20 min at 25°. [e] All compounds analyzed for C, H, and N within 0.4% of calculated theoretical value. [f] Reference 17a.

recording spectrophotometer using the 0–0.1 o.d. slide wire in the synchronous drive mode. Reactions were started by adding enzyme.

I_{50} values were determined using this standard xanthine oxidase reaction using xanthine as substrate. Three to five concentrations of inhibitor producing between 30 and 70% inhibition of the reaction were used, and the I_{50} value was obtained from a plot of the percent inhibition vs. the log of the concentrations of inhibitor. The I_{50} is the concentration of compound that produces a 50% inhibition of the uninhibited control reaction. The 0.3-ml preincubation reaction contained (in μmol) Tris–HCl, pH 7.5, 12; EDTA, 0.045; xanthine oxidase, 32 μg; and the pyrazolo[1,5-a]pyrimidine inhibitor at a concentration ten times its I_{50} value. After preincubation as indicated the mixture was added to the remaining components of the standard assay mixture with xanthine as substrate and the rate of the reaction determined. This resulted in a tenfold dilution of the inhibitor such that its final concentration was equal to its I_{50} value. Control reactions were preincubated in the same manner in the absence of inhibitor.

Results

Ability of 3-Substituted 5,7-Dihydroxypyrazolo[1,5-a]pyrimidines to Serve as Substrates for Xanthine Oxidase. Each of the compounds (see Table I for structures) was tested as described in the Experimental Section for its ability to act as a substrate for xanthine oxidase by following the rate of reduction of known artificial electron acceptors for the enzyme.[18] Using either cytochrome *c* plus phenazine methosulfate or 2,6-dichlorophenol–indophenol no reduction of either electron acceptor by xanthine oxidase could be detected in the presence of any of the compounds under study. Under the same conditions in the presence of xanthine or allopurinol, these electron acceptors were both reduced at significant rates.

The ultraviolet spectra of allopurinol or the pyrazolo[1,5-a]pyrimidines were observed for possible changes after exposure of these compounds to xanthine oxidase and oxygen. It was found that oxygen itself was reduced by allopurinol under the assay conditions used, as evidenced by the slow conversion from an allopurinol spectrum to an oxipurinol spectrum. The rate was only about 1/100th the rate of hypoxanthine oxidation. There were no changes in the spectrum of the pyrazolo[1,5-a]pyrimidines under these same conditions, which is consistent with the chemical properties of this ring system.[10,11]

Affect of Preincubating Xanthine Oxidase with Inhibitor Prior to Addition of Substrate. Table I gives the I_{50} values of all the compounds studied using 1.75×10^{-5} *M* xanthine as substrate. In a separate experiment a concentration of each compound equal to ten times its I_{50} was preincubated with a concentration of xanthine oxidase equal to ten times that used in the standard assay in the presence of 40 m*M* Tris–HCl, pH 7.5, and 0.15 m*M* EDTA for either 1 hr at 4° or 20 min at 25°. Each compound–xanthine oxidase preincubation mixture was then diluted tenfold into a standard reaction mixture containing xanthine as substrate. Controls containing no inhibitor were treated the same way. The percent inhibition compared to control was then determined for each inhibitor. The results, shown in Table I, demonstrate that for all compounds the percent inhibition after either preincubation condition was very close to 50%. This is what would be expected of a simple reversible inhibitor. The inhibition by allopurinol was not reversible. It has been previously shown that this is because it is converted to oxipurinol, which only slowly dissociates from the enzyme.[8,9]

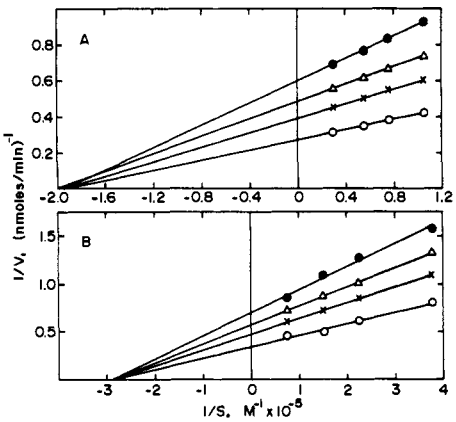


Figure 1. Kinetics of inhibition of xanthine oxidase by 3-phenyl-5,7-dihydroxypyrazolo[1,5-a]pyrimidine. The xanthine oxidase assay was performed as described in the Enzyme Assays section. In A hypoxanthine was used as substrate and the inhibitor concentrations used were either 65 n*M* (●—●), 39 n*M* (△—△), 26 n*M* (×—×), or no inhibitor (○—○). In B xanthine was used as substrate and the inhibitor concentrations used were either 50 n*M* (●—●), 30 n*M* (△—△), 20 n*M* (×—×), or no inhibitor (○—○).

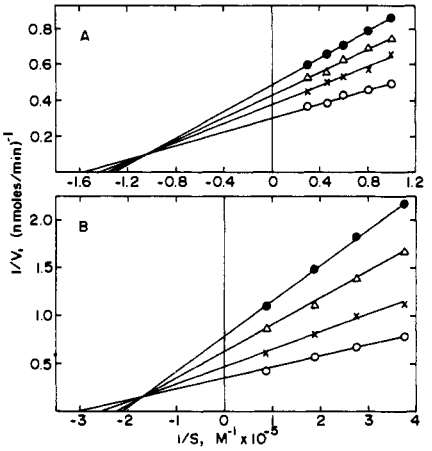
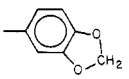
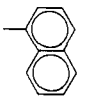


Figure 2. Kinetics of inhibition of xanthine oxidase by 3-(4-bromophenyl)-5,7-dihydroxypyrazolo[1,5-a]pyrimidine. The xanthine oxidase assay was performed as described in the Enzyme Assays section. In A hypoxanthine was used as substrate and the inhibitor concentrations used were either 3.0 n*M* (●—●), 2.4 n*M* (△—△), 1.8 n*M* (×—×), or no inhibitor (○—○). In B xanthine was used as substrate and the inhibitor concentrations used were either 2.8 n*M* (●—●), 2.0 n*M* (△—△), 1.2 n*M* (×—×), or no inhibitor (○—○).

Kinetics of Inhibition of Xanthine Oxidase by 3-Substituted 5,7-Dihydroxypyrazolo[1,5-a]pyrimidines (3a–n). The type of inhibition displayed by compounds **3a–n** was determined from Lineweaver–Burk plots[20] (Figures 1 and 2). The experiments were performed with three concentrations of inhibitor and at least four different concentrations of hypoxanthine or xanthine as substrate. For example, Figure 1 demonstrates the profile of **3e**, which is a noncompetitive inhibitor[21] with either substrate. Figure 2 illustrates a similar example in which **3g** demonstrates "mixed type" inhibition[21] with either substrate.[22] The same kinetic analysis was performed on each of the compounds and the K_i values,[23] determined from the same data by the method of Dixon,[26] as well as the type of inhibition, determined from Lineweaver–Burk plots, are presented in Table II. A similar analysis of allopurinol is included for comparison. For every compound, the K_i values are consistent with the I_{50} values presented in Table I. Each compound demonstrated the same type of inhibition and a similar K_i value with either hypoxanthine or xanthine as substrate.

Table II. K_i Values and Type of Inhibition of Xanthine Oxidase of Various 3-Substituted 5,7-Dihydroxypyrazolo[1,5-a]pyrimidines

		Hypoxanthine as substrate		Xanthine as substrate	
No.	R	K_i, $M \times 10^5$	Type of inhibn[a]	K_i, $M \times 10^5$	Type of inhibn[a]
3a	-H	420	N	540	N
3b	$-NO_2$	3100	M	3700	M
3c	-Br	120	N	150	N
3d	-Cl	180	N	200	N
3e	$-C_6H_5$	6.0	N	5.3	N
3f		3.1	N	2.5	N
3g	$-C_6H_4$-*p*-Br	2.3	M	2.6	M
3h	$-C_6H_4$-*p*-Cl	1.9	M	2.3	M
3i	$-C_6H_4$-*p*-NH-$COCH_3$	7.7	M	8.5	M
3j	$-C_6H_4$-*p*-CH_3	2.4	M	1.7	M
3k	$-C_6H_4$-*m*-CH_3	1.9	M	2.0	M
3m		1.8	M	2.3	M
3n		1.6	N	1.3	N
Allopurinol		260	M	100	M

[a] "N" refers to noncompetitive inhibition and "M" refers to "mixed-type" inhibition.

A comparison of the K_i values of compounds in Table II demonstrates that **3e–n** inhibit xanthine oxidase to a much greater extent than those compounds **3a–d**, which do not have aryl substituents. This observation shows that an aryl group in the 3 position of pyrazolo[1,5-a]pyrimidines makes the resulting compounds better able to interact with xanthine oxidase in a way that results in the inhibition of the enzyme activity. This is consistent with the results of Baker and Wood[14] on the inhibition of xanthine oxidase by 9-aryl-substituted purines. The difference in the type of inhibition among the compounds cannot be understood with just the data presented. Both the halogen (**3b–d**) and aryl (**3e–n**) derivatives were noncompetitive inhibitors, but the nitro compound (**3b**) was a mixed inhibitor. No mechanism is postulated, but a visual inspection of the Hammett π and σ differences between the nitro group and the other substituents suggests that the electronic ground state[27] of the nitro group plays an important role in this context.

Effect of pH on the I_{50} Value of Some 3-Substituted 5,7-Dihydroxypyrazolo[1,5-a]pyrimidines. Figure 3, A, shows the results of a study in which the I_{50} values for some of the compounds were determined at various pH values between 6.6 and 9.0. All I_{50} values are expressed relative to the value at pH 7.5, which is set equal to 1.0. Figure 3, B, gives the pH dependence of xanthine oxidase using either hypoxanthine or xanthine as substrate at the reaction conditions used in the study shown in Figure 3, A. The pH profile is essentially the same regardless of which substrate is used. 5,7-Dihydroxypyrazolo[1,5-a]-pyrimidine (**3a**) shows an essentially proportional increase in I_{50} with increasing pH from pH 6.6 to 7.6. Between pH 7.5 and 7.6 the slope of the line changes and a proportional increase in I_{50} with increasing pH is again seen between pH 7.6 and 7.9. With the 3-phenyl- (**3e**) or 3-(4-bromophenyl)-5,7-dihydroxypyrazolo[1,5-a]pyrimidine (**3g**), a

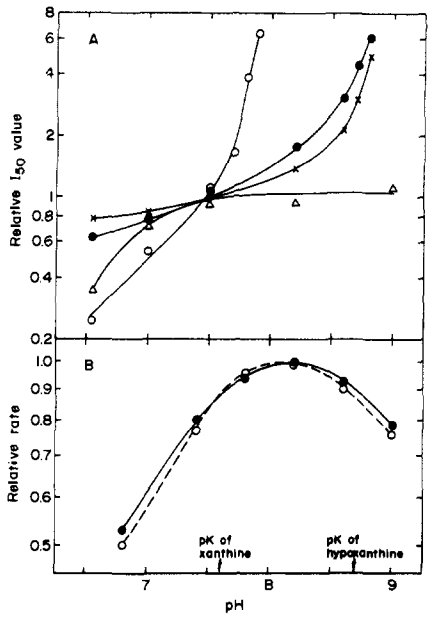


Figure 3. Effect of pH on the activity and inhibition of xanthine oxidase. Xanthine oxidase assays and determination of I_{50} values using xanthine or hypoxanthine as substrate were performed as described in the Enzyme Assays section with the exception that the 50 m*M* Tris-HCl, pH 7.5, was replaced by 50 m*M* Tris-malate at the pH values indicated on Figure 3. In A the compounds used as inhibitors were allopurinol (△—△), 3-(4-bromophenyl)-5,7-dihydroxypyrazolo[1,5-*a*]pyrimidine (**3g**) (×—×), 3-phenyl-5,7-dihydroxypyrazolo[1,5-*a*]pyrimidine (3e) (●—●), and 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidine (3a) (○—○). In B the activity of xanthine oxidase at varying pH values was determined using either xanthine (●—●) or hypoxanthine (○—○) as substrate.

linear increase in I_{50} with increasing pH is seen between pH 6.6 and ~8.2, a transition between pH 8.2 and 8.6, and a linear relationship again between pH 8.6 and 8.8. Allopurinol, by comparison, shows an increase in I_{50} between 6.6 and 7.5 but no significant change above pH 7.5.

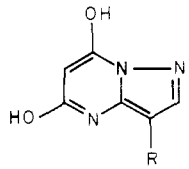
p*K* Values of Some 3-Substituted 5,7-Dihydroxypyrazolo[1,5-*a*]pyrimidines and Related Compounds. In order to gain further insight into the mechanism of inhibition of xanthine oxidase by these compounds, their p*K* values were determined and are compared in Table III with the values for allopurinol, hypoxanthine, and xanthine obtained in parallel determinations. The pH values for the 3-substituted 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines are significantly lower than those values for allopurinol, xanthine, and hypoxanthine and far below the lowest pH value (pH 6.6) used in the experiment in Figure 3, A.

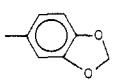
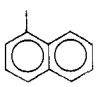
Discussion

The results presented here show that a number of 3-substituted 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines are potent inhibitors of xanthine oxidase in vitro. Those compounds containing a 3-aryl substituent were significantly better inhibitors than those compounds containing nonaromatic groups. In addition, many of the compounds demonstrated K_i values two orders of magnitude lower than that for allopurinol.

The lack of the effect of preincubation of the enzyme with high concentrations of these inhibitors indicates that the compounds are interacting with xanthine oxidase in a reversible manner. This is in contrast to the allopurinol which is a "stoichiometric" inhibitor that is only reversible by dialysis.[8,9] In the latter case the enzyme oxidizes allopurinol to oxipurinol, the latter being the "stoichiometric" inhibitor. Studies reported here show

Table III. Ultraviolet Spectra and p*K* Values of 3-Aryl-5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines and Related Purines[a]



No.	R	Uv (MeOH) λ_{max} ($\epsilon_{max} \times 10^4$) pH 1	pH 11	p*K*
3a	-H			3.7
3e	$-C_6H_5$	203 (2.34), 238 (1.82)	234 (1.95), 297 (1.66)	3.8
3f		205 (2.745), 215 (2.85), 242 (2.05), 280 (1.95)	232 (3.175), 275 (2.50)	
3g	$-C_6H_4$-*p*-Br	202 (3.19), 247 (2.75)	234 (4.14), 308 (3.71)	3.8
3h	$-C_6H_4$-*p*-Cl	204 (2.65), 245 (2.37)	233 (2.40), 305 (2.15)	
3i	$-C_6H_4$-*p*-NHCOCH$_3$	204 (3.85), 262 (3.11)	232 (3.60), 311 (3.20)	
3j	$-C_6H_4$-*p*-CH$_3$	203 (3.06), 243 (2.05)		
3k	$-C_6H_4$-*m*-CH$_3$	207 (2.92), 240 (2.05)	230 (2.64), 286 (1.88)	
3m		305 (2.99), 295 (1.045)	230 (1.73), 275 (1.41)	
3n		205 (3.25), 256 (1.57), 290 (1.21)	230 (1.94), 275 (1.57)	
Allopurinol				9.0
Xanthine				7.6[b]
Hypoxanthine				8.7[c]

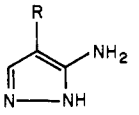
[a] Uv spectra and p*K* values were determined as described in the Experimental Section. [b] p*K* values of ~0.8, 7.4, and 11.1 have been reported.[28] [c] p*K* values of 2.0, 8.9, and 12.1 have been reported.[28]

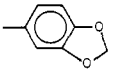
that the 3-substituted 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines do not serve as substrates for xanthine oxidase, a fact important for the interpretation of the kinetic data.

The data show that, although these compounds are not utilized as substrates and interact reversibly with the enzyme, they do not demonstrate competitive inhibition. A few of the compounds are noncompetitive inhibitors but the majority demonstrate a "mixed-type" of inhibition. It cannot be determined from the data if the compounds are interacting with the active site, a site other than the active site, or both. The structural similarity of these compounds to xanthine suggests that they may be involved with the active site in some way. In addition, enhanced binding to the enzyme by compounds containing a 3-aryl substituent suggests the presence of a hydrophobic region immediately adjacent to the active site of xanthine oxidase which can bind these aromatic substituents, thus reinforcing the conclusions of Baker and Wood[14] from studies on various 9-substituted purines as inhibitors of xanthine oxidase.

The results on the effect of pH on the I_{50} values of some of these compounds show, in agreement with the above, that this class of compounds differs greatly from allopurinol in the manner in which they interact with xanthine oxidase. The results also show that those compounds containing a 3-aryl substituent (**3e–n**) differ from **3a** in

Table IV. Physical Data of 3-Amino-3-arylpyrazoles



No.	R	Mp or bp, °C	Recrystn solvent	Yield, %	Emp formula
4e	$-C_6H_5$	174–176	a	a	$C_9H_9N_3$
4f		162–163	EtOH–H_2O	45	$C_8H_8N_4$
4g	$-C_6H_4$-*p*-Br	149–151	EtOH–H_2O	62	$C_8H_8BrN_3$
4h	$-C_6H_4$-*p*-Cl	141–143	a	a	$C_8H_8ClN_3$
4i	$-C_6H_4$-*p*-NH-$COCH_3$	192–193	EtOH–H_2O	27	$C_{11}H_{12}N_4O$
4j	$-C_6H_4$-*p*-CH_3	174–175	a	a	$C_{10}H_{11}N_3$
4k	$-C_6H_4$-*m*-CH_3	120–121	a	a	$C_{10}H_{11}N_3$
4m		165–167	EtOH–H_2O	38	$C_{10}H_{10}N_3O_2$
4n		109–110	a	a	$C_{13}H_{11}N_3$

[a] See ref 7 for physical data.

their respective interactions with xanthine oxidase. The changes in I_{50} with pH most likely represent changes in the physical and/or chemical nature of the enzyme. The pH vs. rate profiles for xanthine or hypoxanthine as substrate are identical in spite of the fact that the pK values for the two substrates vary by more than one pH unit and that they fall on opposite sides of the optimum pH for the enzyme. These data are consistent with other data[29] which also showed that the activity of xanthine oxidase is independent of the degree of ionization of the substrate. In addition, the pK values for the compounds show that they are not undergoing ionization in the pH range where the large changes in I_{50} values were observed. All of the 3-substituted 5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines studied demonstrated large increases in I_{50} at pH values greater than 7.5, while the I_{50} of allopurinol was unchanged between pH 7.5 and 9.0. Differences were seen also between the **3e** and **3g** as compared to **3a**. The changes in the properties of xanthine oxidase that result in increased I_{50} values require higher pH for the effects to be seen in the case of the **3e** and **3g** than **3a**. This indicates that the 3-aryl substituents can interact with the enzyme at higher pH value in such a way that the detrimental effect of increasing pH is at least partially overcome.

All of these results taken together indicate that these compounds inhibit xanthine oxidase by a mechanism which is significantly different from that of allopurinol and oxipurinol.

Experimental Section

Melting points were all taken on a Hoover-Thomas apparatus (capillary tubes) and are uncorrected. Analyses for C, H, and N were determined by the Heterocyclic Chemical Corp. of Harrisonville, Mo., and were all within ±0.4% of the calculated values. Uv spectra were recorded in MeOH on a Cary 15 spectrophotometer. Ir spectra of all solids were determined in KBr pellets (liquids as thin films on NaCl cells or in Nujol mull) on a Perkin-Elmer 257 instrument. ^1H NMR spectra were run in Me_2SO-d_6 (Me_4Si internal standard) or D_2O–NaOH (DDS internal standard), unless other solvents are specified, employing a Hitachi Perkin-Elmer spectrometer. Uv spectra are recorded (λ_{max}, ϵ_{max}) in Table III. pK determinations, also recorded in Table III, were made using a Radiometer auto burette titration assembly and recording pH meter. Each compound was titrated at three different concentrations with 25 mM NaOH ranging from 0.1 to 0.5 mM and the values obtained were averaged. The NaOH was standardized against potassium hydrophthalate. Allopurinol was synthesized as previously described.[3]

Method A. General Procedure for Condensation. 5,7-Dihydroxy-3-(3,4-methylenedioxyphenyl)pyrazolo[1,5-*a*]pyrimidine (3m). Diethyl malonate (3.21 g, 20 mmol) and 3-amino-4-(3,4-methylenedioxyphenyl)pyrazole (4 g, 20 mmol) were added to a solution of sodium ethoxide (1.78 g, 77 mmol) in ethanol (200 ml). The resultant solution was stirred at reflux for 16 hr. The sodium salt gradually formed and was filtered after cooling the mixture to room temperature. The sodium salt was washed with ethanol, dried in vacuo at 40° (10 mm), and then dissolved in 150 ml of water and acidified with 6 N hydrochloric acid (pH 2). The precipitated title compound was filtered, washed well with water, and dried at 100° (10 mm). Purification of the product was accomplished by reprecipitation from 6 N NaOH solution with 6 N HCl solution. Physical data for the title compound and the other 3-aryl-5,7-dihydroxypyrazolo[1,5-*a*]pyrimidines (prepared by an identical method) are recorded in Tables I and III.

Method B. General Procedure for Synthesis of 3-Amino-4-arylpyrazoles. 3-Amino-4-(3,4-methylenedioxy)phenylpyrazole (4m). A solution of NaOEt was prepared (22 g of Na metal, 300 ml of EtOH) and to this was added ethyl formate (100 g) and 3,4-methylenedioxyphenylacetonitrile (50 g, 0.3 mol). The mixture was stirred at reflux for 15 min, whereupon a white sodium enol salt precipitated. The salt was filtered, washed with EtOH, air-dried, and then dissolved in 1000 ml of ice–water and brought to pH 5 with ~100 ml of acetic acid. A white precipitate of 1-formyl-3,4-methylenedioxyphenylacetonitrile formed (ca. 46 g). This material was used in the next step without purification. To a solution of 19.0 g of this material in 80 ml of acetic acid was added 13 ml of 85% hydrazine hydrate via dropping funnel. The reaction mixture became blood red and was somewhat exothermic. The mixture was refluxed 2 hr and then poured into 500 ml of water and 2 ml of 12 N HCl was added. This mixture was refluxed for 10 min and then allowed to cool to room temperature. The mixture was filtered and the filtrate was diluted with an equal amount of ice–water to precipitate a tan solid. Filtration and recrystallization of this solid (EtOH–H_2O) gave 10 g (38%) of the title pyrazole: mp 165–167° (Table IV). A similar procedure was used to prepare the 4-(3-pyridyl)- (**4f**), 4-(*p*-bromophenyl)- (**4g**), and 4-(*p*-acetamidophenyl)-3-aminopyrazoles (**4i**) listed in Table IV. The 4-phenyl (**4e**), 4-(*p*-chlorophenyl) (**4h**), 4-(*p*-tolyl) (**4j**), 4-(*m*-tolyl) (**4k**), and 4-(naphthyl) (**4n**) analogues have been previously reported.[16]

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(23) If K_i values are determined by suitable replotting of the slopes and intercepts of double-reciprocal plots vs. inhibitor concentration,[24] the inhibition constant for the slopes (K_{is}) is not equal to that for the intercepts (K_{ii}). However, we have chosen to follow the suggestion of Butterworth[25] and retain the use of Dixon plots for determining K_i values in conjunction with Lineweaver-Burk plots to determine the type of inhibition.
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Immobilized Glucuronosyltransferase for the Synthesis of Conjugates[1]

Indu Parikh,* Donald W. MacGlashan, and Catherine Fenselau

Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received April 21, 1975*

Partially purified rabbit liver UDPglucuronosyltransferase is immobilized on agarose by the cyanogen bromide activation method. Both soluble and matrix-bound enzyme preparations display very similar K_m and pH optimum. The storage stability of the immobilized enzyme at 4° is 5–10 times improved over the soluble preparations. The agarose-bound UDPglucuronosyltransferase is successfully used in the synthesis of *p*-nitrophenyl glucuronide in an overall yield of 50–70%. The matrix-bound enzyme is reusable over an extended period of time and offers an easy and convenient synthetic tool for various drug glucuronides.

The general progress in the understanding of drug metabolism has been hindered to a certain extent by the paucity of pure glucuronides of known structure available for use as standards. Isolation of drug glucuronides from dosed rabbits and from overdosed patients has been in certain cases only moderately successful.[2a] Attempts have also been made to synthesize selected drug glucuronides by in vitro enzymatic synthesis using crude liver homogenate or fresh liver slices.[2b] Direct chemical syntheses of glucuronides have experienced great difficulties especially in cases of polyfunctional drug molecules. The present study indicates that the use of immobilized UDPglucuronosyltransferase (E.C. 2.4.1.17) on an inert matrix offers good potential for the synthesis of glucuronides of various drugs.

The micrososomal enzyme, UDPglucuronosyltransferase, catalyzes the transfer of glucuronic acid from uridinediphosphoglucuronate to a wide variety of drugs and hormones.[3] It is not known whether the glucuronosyltransferase activity resides in one or several enzymes with overlapping specificities.[4] Although it is primarily a hepatic enzyme, it has been found in various other tissues of rat, rabbit, and guinea pig.[5] UDPglucuronosyltransferase is one of the least understood of the mammalian metabolic enzymes. The free enzyme is very sensitive to even moderately high temperature. Because of its limited stability, the earlier isolation methods,[6–8] requiring several days, resulted in great losses in enzymatic activity. A detailed comparative study of the stability and specific activity of the enzyme in different species has not been undertaken. The enzme has been solubilized and partially purified from various mammalian tissues.[6–8] Although the enzyme is not available in pure form, its various properties, including pH optimum, substrate specificities, gel filtration characteristics, and various kinetic parameters (in soluble form), have been extensively studied. The enzyme has never been immobilized on a solid support though a large number of methods are available.[9,10]

During recent years numerous applications of immobilized enzymes have been experimentally realized. It has been shown that immobilized enzyme reactors have various advantages compared to the corresponding enzymatic reactions in solution.[11] Immobilized preparations of glucose oxidase and urate oxidase have been elegantly used in the automated determination of glucose[12] and uric acid.[13] Proteolytic enzymes like trypsin and papain have been immobilized.[14] This paper reports covalent immobilization of UDPglucuronosyltransferase to agarose by the CNBr activation method and its application to the synthesis of model drug glucuronides.

Experimental Section

Materials and Methods. Agarose (Sepharose 4B) was purchased from Pharmacia. Uridinediphosphoglucuronic acid ammonium salt and *p*-nitrophenol glucuronide were obtained from Sigma. The solvents and reagents were analytical grade and were

* Address correspondence to this author at Burroughs-Wellcome Research Laboratories, Research Triangle Park, N.C. 27709.