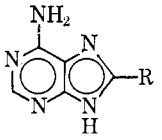
TABLE V

PHYSICAL PROPERTIES OF



No.	R	Method	% yield	Mp, °C dec	Formula	Analyses	λ_{max}, mμ[a] pH 1	λ_{max}, mμ[a] pH 13
7	$CH_2C_6H_4(NHCOC_6H_4SO_2F\text{-}p)\text{-}m$	F[b]	62[c]	250–252	$C_{19}H_{15}FN_6O_3S\cdot H_2O$	C, H, N	270	272
8	$C_6H_4(NHCOC_6H_4SO_2F\text{-}p)\text{-}m$	F[b]	58[c]	>340	$C_{18}H_{13}FN_6O_3S\cdot 0.5H_2O$	C, H, N	226, 294	237,[d] 305
9	$C_6H_4(NHCOC_6H_4SO_2F\text{-}m)\text{-}p$	F[b]	59[c]	>340	$C_{18}H_{13}FN_6O_3S\cdot 0.5H_2O$	C, H, N	320	252,[d] 323
10	$C_6H_4(NHCOC_6H_4SO_2F\text{-}p)\text{-}p$	F[b]	65[e]	>340	$C_{18}H_{13}FN_6O_3S\cdot H_2O$	C, H; F[f]	322	251,[d] 325
40	$C_6H_4NO_2\text{-}m$	E	78[c]	>350	$C_{11}H_8N_6O_2$	C, H, N	224, 288	237, 304
41	$C_6H_4NO_2\text{-}p$	E	47[c]	>350	$C_{11}H_8N_6O_2$	C. H, N	247, 265, 326	259, 369
42	$CH_2C_6H_4NO_2\text{-}m$	E	70[c]	307–309	$C_{12}H_{10}N_6O_2$	C, H, N	266	272
43	$C_6H_4NH_2\text{-}m$	C	85[g]	>340	$C_{11}H_{10}N_6\cdot 0.5H_2O$	C, H, N	226, 294	239,[d] 306
44	$C_6H_4NH_2\text{-}p$	C	76[c]	>340	$C_{11}H_{10}N_6\cdot 0.25H_2O$	C, H, N	226, 295	254, 318
45	$CH_2C_6H_4NH_2\text{-}m$	C	53[g]	242–245	$C_{12}H_{12}N_6\cdot H_2O$	C, H	267	276

[a] In 10% EtOH. [b] See method A, ref 2. [c] Recrystallized from MeOEtOH–H_2O. [d] Inflection. [e] Recrystallized from EtOH–H_2O. [f] F: calcd, 4.42; found, 3.86. [g] Recrystallized from DMSO–H_2O.

4,6-Diamino-5-(*m*-nitrobenzamido)pyrimidine (34) (Method D).—To a stirred mixture of 4.82 g (20 mmoles) of **31** sulfate on 40 ml of 1 *N* NaOH cooled in an ice bath was added dropwise a solution of 3.70 g (20 mmoles) of *m*-nitrobenzoyl chloride in 5 ml of dioxane over a period of 1 hr. The pH was maintained at 10–11 by addition of 1 *N* NaOH as needed. After being stirred for an additional 4 hr, the mixture was filtered and the product was washed with H_2O. Recrystallization from DMF–H_2O gave 1.75 g (32%) of pure product, mp >350°. See Table IV for additional data and other compounds prepared by this method.

8-(*m*-Nitrophenyl)adenine (40) (Method E).—To a mixture of 1.20 g of **34** and 12.5 g of P_2O_5 cooled in an ice bath was added 9 ml of 85% H_3PO_4. The mixture was heated in a bath at 165–170° for 1.5 hr, then cooled and poured into 20 ml of iced H_2O with stirring. The solution was adjusted to pH 8–9 with 4 *N* NaOH. The product was collected on a filter and washed with H_2O, then MeOH. Recrystallization from DMF–H_2O gave 0.60 g (55%) of pure product, mp >350°. See Table V for additional data and other compounds prepared by this method.

4-5-Diamino-6-(*o*-nitroanilino)pyrimidine (38).—A mixture of 500 mg (1.65 mmoles) of **32**, and 15 ml of 4 *N* NaOH was refluxed for 10 hr. The cooled suspension was filtered and the product was washed with H_2O. The solid was dissolved in 3 *N* H_2SO_4, then spin evaporated to a syrup *in vacuo*. The sulfate salt was collected on a filter and washed with Et_2O; yield 410 mg, mp 225–226°, that moved as a single spot on tlc. The salt was dissolved in H_2O and the free base precipitated by addition of 2 *N* NaOH. The product was collected on a filter and thoroughly washed with H_2O, then MeOH, and finally Et_2O; yield 260 mg (67%); mp 249–252° dec; λ_{max} (mμ) pH 1, 267, 401; pH 13, 280 (infl), 409. *Anal.* ($C_{10}H_{10}N_6O_2$) C, H, N.

The *para* isomer (**39**) was prepared similarly except that the sulfate salt was insoluble in cold 3 *N* H_2SO_4. The sulfate salt was collected by filtration and recrystallized from DMF–Et_2O; yield 270 mg (93%), mp 303–305° dec. *Anal.* ($C_{10}H_{10}N_6O_2\cdot 0.5H_2SO_4$) C, H. The free base was recrystallized from MeOEtOH–H_2O; yield 156 mg (64%); mp 328–331° dec; λ_{max} (mμ) pH 1, 261, 368; pH 13, 256, 382. *Anal.* ($C_{10}H_{10}N_6O_2$) C, H; N: calcd, 34.1; found, 33.5.

Irreversible Enzyme Inhibitors. CXXVI.[1,2] Hydrocarbon Interaction with Xanthine Oxidase by Phenyl Substituents on Purines and Pyrazolo[3,4-*d*]pyrimidines

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A hydrophobic bonding region exists on xanthine oxidase just adjacent to the active site that can complex aryl groups attached to purines and pyrazolo[3,4-*d*]pyrimidines. Inhibition by 57 purines and pyrazolo[3,4-*d*]-pyrimidines bearing polar groups or both polar and hydrophobic bonding groups was measured; no unifying theory emerged on the mode of binding of these heterocycles to xanthine oxidase, although it was established by several parameters that the heterocycles could bind in one of a number of rotomeric configurations depending upon the positions of polar and phenyl groups on the heterocycle. The three best reversible inhibitors of xanthine oxidase found in this study were 8-phenylhypoxanthine (**8**), 8-(*m*-nitrophenyl)adenine (**15**), and 6-(*m*-nitrophenyl)pyrazolo[3,4-*d*]pyrimidine (**42**), which were complexed 100–500-fold better than the substrate hypoxanthine (**5**) and 12–54-fold better than 4-hydroxypyrazolo[3,4-*d*]pyrimidine.

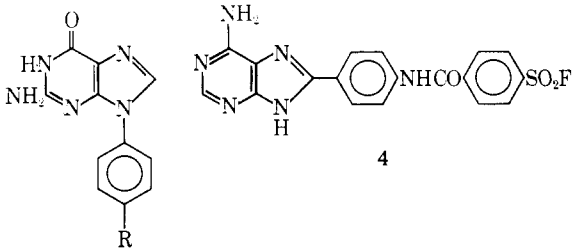
The Bergmann school has made extensive studies on the influence of substituents on the xanthine oxidase catalyzed oxidation of purines in order to elucidate the mode of binding of purines and the mechanism of action of the enzyme.[3] From their studies it was apparent that there were multiple modes of binding of purines to the enzyme depending upon the purine substituents. For example, hypoxanthine and 8-hydroxypurine were oxidized at the 2 position but 2-hydroxypurine was oxidized at the 8 position; in contrast, adenine was oxidized at the 8 position, but 2-amino- and 8-amino-

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series see B. R. Baker and J. A. Kozma, *J. Med. Chem.*, **11**, 656 (1968).

(3) F. Bergmann, G. Levin, H. Kwietny-Gorvin, and H. Ungar, *Biochim. Biophys. Acta*, **47**, 1 (1961), and references therein.

purines were oxidized at the 6 position,[3,4] Two additional binding parameters for xanthine oxidase have been uncovered in our laboratory: (a) the strong hydrophobic bonding to xanthine oxidase by a 9-phenyl substituent (**1**) on guanine,[5-7] and (b) insertion of a bromoacetamido group (**2**)[8] or *p*-fluorosulfonylbenzamido group (**3**)[9] on the *para* position gave active-site-directed irreversible inhibitors[10] of xanthine oxidase.



4

1, R = H
2, R = $NHCOCH_2Br$
3, R = $NHCOC_6H_4SO_2F$-*p*

It was envisioned that these three parameters might be combined to try to rationalize the mode of purine binding to xanthine oxidase in the following manner. (a) If one purine such as hypoxanthine is oxidized at the 2 position, but another purine such as adenine is oxidized at the 8 position, then the modes of binding of the two purines are different; such a statement requires the reasonable assumption[11] that there is only one catalytic subsite per active site on xanthine oxidase. (b) If a phenyl group on one position of a purine such as guanine (**1**) gives a strong hydrophobic interaction, but does not give hydrophobic interaction if the purine substituents other than phenyl are changed, such as 9-(*p*-chlorophenyl)xanthine (**34**), then the two purines do not bind in the same fashion. (c) If one active-site-directed irreversible inhibitor such as **3** inactivates the enzyme, but does not inactivate the enzyme when the other substituents on the purine or the purine or both are changed, such as in the case of **4**, then it can be equivocally stated that the sulfonyl fluoride group does not reside in an identical position within the respective inhibitor–enzyme reversible complexes.[10]

Numerous attempts to use these three parameters to rationalize the mode of binding of purines to xanthine oxidase still failed even though more compounds were added in each attempt to clarify the situation; part of the difficulty in interpretation may be due to the observation of Hofstee[12a] that excess substrate can inhibit xanthine oxidase, the kinetics and interpretation of which are in themselves complicated since xanthine oxidase has two FAD's and presumabaly two active sites. Even though these attempts to rationalize the mode of binding were failures, a number of new and potent reversible inhibitors emerged from which several new classes of candidate active-site-directed irreversible inhibitors can be made; therefore, the main emphasis in the discussion in this paper will be on new types of candidate irreversible inhibitors for xanthine oxidase.[13]

Enzyme Results.—The relative abilities of 57 purines and pyrazolo[3,4-*d*]pyrimidines to inhibit xanthine oxidase are collated in Table I. Since hypoxanthine (**5**) is used as the substrate, the derivatives of hypoxanthine (**6**-**13**) will be discussed first to set a baseline. Introduction of a benzylthio group at the 2 position (**6**) or 8 position (**7**) gives up to a tenfold enhancement in binding over hypoxanthine,[11] presumably due to a hydrophobic interaction of the benzyl group with the enzyme.

That the 9-H of hypoxanthine (**5**) was needed for binding to the enzyme was indicated by the 17-fold loss in binding when this 9-H was replaced by methyl (**9**). A 9-phenyl substituent (**10**) gave a hydrocarbon interaction with the enzyme with an 11-fold increment in binding over the 9-CH_3 (**9**); thus the loss in binding by removal of the 9-H is essentially recouped by addition of the 9-phenyl group of **10**. Introduction of a nitro group on **10** was detrimental to binding, a 2.4-fold and fivefold loss occuring with a *m*-NO_2 (**11**) or *p*-NO_2 (**12**), respectively. A twofold increment in increased binding occurred with the *p*-OCH_3 substituent (**13**).

A strong hydrophobic interaction with xanthine oxidase occurred when an 8-phenyl substituent (**8**) was introduced; **8** was one of three best inhibitors in Table I, being complexed 130-fold better than hypoxanthine. The effect of substituents on the binding of the phenyl group of **8** is yet to be studied.

Although adenine (**14**) is only a slow substrate,[3] it is complexed to xanthine oxidase as effectively as hypoxanthine (**5**);[11] however, the mode of binding of derivatives of adenine is different than some of the corresponding derivatives of hypoxanthine. Replacement of the 9-H of adenine (**14**) with methyl (**18**) gave a huge 500-fold loss in binding; since the corresponding change on hypoxanthine (**5** *vs.* **9**) gave only a 17-fold loss in binding, it is clear that the 9-H is less essential for binding of hypoxanthine than adenine, perhaps because the 9-methylhypoxanthine (**9**) can bind in a different rotomeric conformation where its 1-H supplies the necessary acidic hydrogen for binding. Replacement of the 9-methyl (**18**) by 9-phenyl (**19**) on adenine gave only a threefold increment in binding, in contrast to hypoxanthine where an 11-fold increment was observed with **9** *vs.* **10**; furthermore, introduction of a nitro group (**20**) was beneficial to binding, but the same change (**12**) on hypoxanthine was detrimental to binding.

Enhanced binding to xanthine oxidase was observed with derivatives of 8-phenyladenine, the amount of enhancement being extremely subject to substituent effects. The 8-(*m*-nitrophenyl) substituent (**15**) gave a huge 350-fold increment in binding compared to adenine (**14**); in fact, **15** was the best reversible in-

(1) (a) F. Bergmann, G. Levin, and H. Kwietny, *Biochim. Biophys. Acta*, **30**, 509 (1958); (b) F. Bergmann, H. Kwietny, G. Levin, and H. Engelberg, *ibid.*, **37**, 433 (1960); (c) J. B. Wyngaarden and J. T. Dunn, *Arch. Biochem. Biophys.*, **70**, 150 (1957).

(5) B. R. Baker, *J. Pharm. Sci.*, **56**, 959 (1967), paper XCIII of this series.

(6) B. R. Baker and W. F. Wood, *J. Med. Chem.*, **10**, 1106 (1967), paper CII of this series.

(7) B. R. Baker and W. F. Wood, *ibid.*, **11**, 644 (1968), paper CXXII of this series.

(8) B. R. Baker and W. F. Wood, *ibid.*, **10**, 1112 (1967), paper CIII of this series.

(9) B. R. Baker and W. F. Wood, *ibid.*, **11**, 650 (1968), paper CXXIII of this series.

(10) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

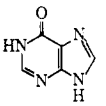
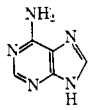
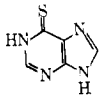
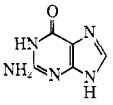
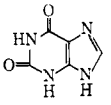
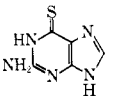
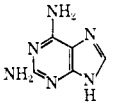
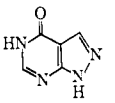
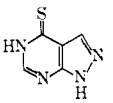
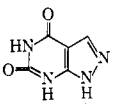
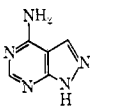
(11) B. R. Baker and J. L. Hendrickson, *J. Pharm. Sci.*, **56**, 955 (1967), paper XCII of this series.

(12) (a) B. H. J. Hofstee, *J. Biol. Chem.*, **216**, 235 (1955); (b) J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. I, Academic Press Inc., New York, N. Y., 1963, p 120.

(13) See ref 11 for the chemotherapeutic utility of a tissue-specific inhibitor of xanthine oxidase.

TABLE I

INHIBITION[a] OF XANTHINE OXIDASE BY PURINES AND PYRAZOLO[3,4-*d*]PYRIMIDINES WITH HYDROCARBON SUBSTITUENTS

Heterocycle	Compd no.	Position	Substituent	I_{50},[b] μM	Source[p]
	5		None	8.1[c]	NBC
	6	2	$C_6H_5CH_2S$	0.75[d]	
	7	8	$C_6H_5CH_2S$	2.8[d]	
	8	8	C_6H_5	0.062	CCNSC[e]
	9	9	CH_3	140	*f*
	10	9	C_6H_5	13	*g*
	11	9	*m*-$NO_2C_6H_4$	31	Exptl
	12	9	*p*-$NO_2C_6H_4$	63	Exptl
	13	9	*p*-$CH_3OC_6H_4$	6.3	Exptl
	14		None	5.6[d]	NBC
	15	8	*m*-$NO_2C_6H_4$	0.016	*h*
	16	8	*p*-$NO_2C_6H_4$	0.21	*h*
	17	8	*p*-$NH_2C_6H_4$	2.3	*h*
	18	9	CH_3	2800	*f*
	19	9	C_6H_5	900	*g*
	20	9	*p*-$NO_2C_6H_4$	450	Exptl
	21		None	10[i]	Sigma
	22	9	C_6H_5	69	CCNSC[i]
	23		None	40[d]	NBC
	24	8	C_6H_5	7.4[k]	
	25	8	$C_6H_5(CH_2)_3$	8.4[k]	
	26	9	CH_3	58[k]	Robins
	27	9	C_6H_5	0.41[k]	Robins
	28	9	$C_6H_5CH_2$	23[k]	Robins
	29	9	*p*-ClC_6H_4	1.8[k]	Robins
	30	9	*m*-$NH_2C_6H_4$	0.60[l]	
	31	9	*p*-$NH_2C_6H_4$	3.7[l]	
	32	9	*p*-$CH_3OC_6H_4$	0.50[l]	
	33		None	2.5[m]	
	34	9	*p*-ClC_6H_4	240	Robins
	35		None	7.7[d]	Sigma
	36	9	C_6H_5	1.1	Robins
	37		None	34[d]	NBC
	38	9	C_6H_5	460	Robins
	39	N^6	C_6H_5	25[d]	Robins
	40		None	0.87[n]	
	41	6	C_6H_5	6.5	*h*
	42	6	*m*-$NO_2C_6H_4$	0.70	*h*
	43	6	*p*-$NO_2C_6H_4$	0.83	*h*
	44	6	*m*-$NH_2C_6H_4$	18	*h*
	45	6	*p*-$NH_2C_6H_4$	4.0	*h*
	46	1	C_6H_6	260	Robins
	47		None	0.20	Robins
	48	6	CH_3	130[k]	Taylor
	49	6	*p*-$NO_2C_6H_4$	3.3[k]	Taylor
	50	6	*p*-$CH_3OC_6H_4$	2.5[k]	Taylor
	51	6	*p*-$NH_2C_6H_4$	32[k]	Taylor
	52	1	CH_3	1100	Robins
	53	1	C_6H_5	88	Robins
	54		None	2.3[n]	Robins
	55	1	CH_3	370	Robins
	56	1	*p*-ClC_6H_4	0.57	Robins
	57		None	70	Robins
	58	1	CH_3	330	Robins
	59	1	C_6H_5	12	Robins
	60	6	*m*-$NO_2C_6H_4$	0.58	*o*
	61	6	*m*-$NH_2C_6H_4$	14	*o*

[a] The technical assistance of Maureen Baker and Pepper Caseria with these assays is acknowledged. [b] I_{50} = concentration of inhibitor necessary for 50% inhibition when assayed with 8.1 μM hypoxanthine in pH 7.4 Tris buffer containing 10% DMSO as previously described;[11] K_m = 8.5 μM [J. B. Wyngaarden, *J. Biol. Chem.*, **224**, 453 (1957)]. [c] Substrate concentration. [d] Data from ref 11. [e] NSC-88412. [f] Prepared according to R. K. Robins and H. H. Lin, *J. Am. Chem. Soc.*, **79**, 490 (1957). [g] Prepared according to S. M. Greenberg, L. O. Ross, and R. K. Robins, *J. Org. Chem.*, **24**, 1314 (1959). [h] See ref 2 for synthesis. [i] Previously reported to be an inhibitor with K_i = 18 μM.[15] [j] NSC-26293. [k] Data from ref 5. [l] Data from ref 8. [m] Calcd from K_m = 20 μM; H. Gutfriend and J. M. Sturtevant, *Biochem. J.*, **73**, 1 (1959). [n] Previously reported as an inhibitor.[20,21] [o] See ref 2. [p] NBC: Nutritional Biochemical Corp.; CCNSC: Cancer Chemotherapy National Service Center; Sigma: Sigma Chemical Co.; Robins: Professor R. K. Robins; Taylor: Professor E. C. Taylor.

hibitor in Table I. When the nitro group was moved to *para* position (**16**), the increment in binding was considerably less, **16** being complexed 27-fold better than adenine; reduction of the nitro group of **16** to amino gave a compound (**17**) which was complexed only twofold better than adenine (**14**). Since the NH_2 group is both more polar and more electron donating than NO_2, further studies would be worthwhile to see if these substituent effects are due to the relative hydrophobicity of the groups or to their electronegativity or both,[14] as previously explored with the binding of 9-phenylguanine to xanthine oxidase.[6,7]

Little can be said about hydrophobic bonding to xanthine oxidase by 9-phenyl-6-mercaptopurine (**22**) other than a sevenfold loss in binding occurs compared to 6-mercaptopurine (**21**); the latter complexes to xanthine oxidase nearly as well as hypoxanthine,[15] and is a slow substrate.[16] More compounds in this series would be needed to state whether or not some hydrophobic bonding occurs by the phenyl group of **22** as was done in the hypoxanthine series (**5**, **9**, **10**); such an undertaking is probably not worthwhile.

From other studies in this laboratory on substituted 9-phenylguanines as inhibitors of guanine deaminase,[6-9,17] a large number of these compounds were available. Since 9-phenylguanine was also a good inhibitor of xanthine oxidase,[5] these compounds were also investigated as inhibitors of this enzyme.[6-9] Selected examples are shown in Table I that clearly indicate a difference in binding of derivatives of guanine and hypoxanthine to xanthine oxidase.

Guanine (**23**) binds fivefold less effectively than hypoxanthine (**5**) in our assay method; guanine has also been reported to be a slow substrate of xanthine oxidase.[18] Introduction of the 9-methyl group (**26**) on guanine (**23**) gave little change in binding, in contrast to hypoxanthine (**5** *vs.* **9**). However, introduction of the 9-phenyl group (**27**) gave a 140-fold increment in binding over 9-methyl (**26**), considerably larger than the increment in the hypoxanthine series (**9** *vs.* **10**). Further studies showed the 9-phenyl group (**27**) on guanine was hydrophobically bonded and substituent effects were correlatable with hydrophobicity;[6,7] how-

(14) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964).

(15) H. R. Silberman and J. B. Wyngaarden, *Biochim. Biophys. Acta*, **47**, 178 (1961).

(16) (a) G. B. Elion, S. Bieber, and G. H. Hitchings, *Ann. N. Y. Acad. Sci.*, **60**, 297 (1954); (b) T. L. Loo, M. E. Michael, A. J. Garceau, and J. C. Reid, *J. Am. Chem. Soc.*, **81**, 3039 (1959).

(17) (a) B. R. Baker and D. V. Santi, *J. Med. Chem.*, **10**, 62 (1967), paper LXXIV of this series; (b) ref 10, pp 101–109.

(18) J. B. Wyngaarden, *J. Biol. Chem.*, **224**, 453 (1957).

ever, compounds corresponding to **29–32** were not available in the hypoxanthine series for comparison.

Introduction of an 8-phenyl group (**24**) on guanine (**23**) gave only a sixfold increment in binding, in contrast to the hypoxanthine series where a 130-fold increment was observed (**5** *vs.* 8).

That a 9-arylxanthine did not complex the same as a 9-arylhypoxanthine or a 9-arylguanine was seen in the comparison of 9-(*p*-chlorophenyl)xanthine (**34**): the latter was complexed 100-fold less effectively than xanthine (**33**) in contrast to a 122-fold gain in binding in the guanine series (**23** *vs.* **29**) and little change in binding in the hypoxanthine series (**5** *vs.* **10**).

9-Phenyl-6-thioguanine (**35**) was about half as effective as 9-phenylguanine (**27**) as an inhibitor; however, the increments in binding were not strictly comparable since thioguanine (**35**) was a fivefold better inhibitor than guanine (**23**), but the increment in phenyl binding was 14 times stronger in the guanine series (compared **35** *vs.* **36** with **25** *vs.* **27**).

The last member of the purine series investigated was 2,6-diaminopurine (**37**); this was similar to the adenine series where a loss in binding occurred when the 9-phenyl substituent (**38**) was introduced.

4-Hydroxypyrazolo[3,4-*d*]pyrimidine (**40**) (allopurinol)[19] is reported to be an excellent inhibitor of xanthine oxidase[20] that is active in man for decreasing uric acid excretion.[21] Therefore, a series of pyrazolo[3,4-*d*]pyrimidines were investigated for hydrophobic bonding. In our test system, allopurinol (**40**) was complexed about tenfold better than hypoxanthine (**5**). However, in contrast to hypoxanthine (**5** *vs.* **10**), introduction of a 1-phenyl group (**46**) on allopurinol gave a 300-fold loss in binding; thus 9-phenylhypoxanthine (**10**) and the corresponding pyrazolo derivative (**46**) cannot bind to xanthine oxidase in the same fashion.

Introduction of 6-phenyl group (**41**) on allopurinol (**40**) gave a sevenfold loss in binding. However, it is still probable that this phenyl group interacts hydrophobically with the enzyme with the heterocyclic ring taking a different rotomeric binding conformation than **40** for two reasons. First, the ability of the 6-phenylallopurinol (**41**) to bind is influenced strongly by substituents; the *m*-nitrophenyl derivative (**42**) is a 12-fold better inhibitor than allopurinol (**40**) and the *p*-nitrophenyl derivative (**43**) is the same as allopurinol. However, reduction to amino leads to a 260-fold loss in binding in the *meta* series (**42** *vs.* **44**) and a fivefold loss in binding in the *para* series (**43** *vs.* **45**). The second line of evidence is based on the binding of derivatives of the thio analog (**47**) to be discussed later. The nitro and amino pattern of **42–45** is similar to that observed in the 4-amino series (**60**, **61**), the 4-thio series (**49–51**), and the 8-phenyladenine series (**15–17**), but quite different from the pattern observed in the 9-phenylhypoxanthine (**10–13**) or 9-phenylguanine series (**27**, **29–32**).

4-Mercaptopyrazolo[3,4-*d*]pyrimidine (**47**) was a fourfold better inhibitor than allopurinol (**40**) and the substitution effects were similar in the two systems. Introduction of a 1-phenyl group (**53**) gave a 400-fold loss in binding, but **53** was still a 12-fold better inhibitor than the 1-methyl analog (**52**), indicating a hydrophobic interaction of the phenyl group of **53**; a similar loss in binding was noted in the 4-hydroxy series (**40** *vs.* **46**).

The 650-fold loss in binding when a 6-methyl group (**48**) is introduced on **47** is particularly striking.[5] Since no acidic hydrogen was replaced which might be a binding point, the loss in binding with **48** can only be steric due to a close fit to the enzyme at the 6 position of enzyme. When the 6-methyl group of **48** was replaced by *p*-nitrophenyl (**49**), binding was enhanced 39-fold. It is clear that **49** must bind in a different rotomeric configuration than the parent **47**, since the smaller 6-methyl of **47** was not tolerated by the enzyme; this different rotomer then allows a hydrocarbon interaction of the 6-nitrophenyl group with the enzyme. Replacement of the *p*-nitro group of **49** by *p*-methoxyl (**50**) gave little change in binding, but replacement by *p*-amino (**51**) gave a tenfold loss in binding, a pattern seen with other hydrophobically bonded phenyl groups discussed earlier.

9-(*p*-Chlorophenyl)xanthine (**34**) and its pyrazolo analog (**56**) were distinctly different in their ability to complex to xanthine oxidase; in the xanthine case (**33** *vs.* **34**), a 100-fold loss in binding occurred, but in the pyrazolo case (**54** *vs.* **56**) a fourfold gain in binding occurred. That the phenyl group of **56** gave a strong interaction with the enzyme was shown by the 650-fold gain in binding of **56** over the 1-methyl derivative (**55**); furthermore, the 1-H of **54** was essential to binding since replacement by a methyl (**55**) gave a 160-fold loss in binding. Thus the pattern of binding of **54–56** is similar to the 9-substituted hypoxanthine series (**5** *vs.* **9** *vs.* **10**).

4-Aminopyrazolo[3,4-*d*]pyrimidine (**57**) was a rather poor inhibitor of xanthine oxidase, being 350-fold less effective than the allopurinol (**40**). However, the 6-phenyl analogs gave superior hydrophobic bonding in the 4-amino series. Introduction of the 6-(*m*-nitrophenyl) group (**60**) gave a 120-fold increment in binding, compared to the 12-fold increment in the allopurinol series (**40** *vs.* **42**); however, **42** was still a sevenfold better inhibitor than **60**. In both systems, reduction of the nitro group to amino led to a loss in binding (**42** *vs.* **44** and **60** *vs.* **61**).

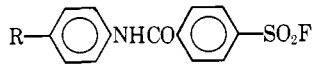
The mode of binding of 9-phenyladenine (**19**) was apparently different than the corresponding 1-phenylpyrazole (**59**). In the latter case the 1-phenyl (**59**) gave a 28-fold increment in binding over 1-methyl (**58**), but in the adenine series the increment was only threefold. Furthermore the acidic hydrogen was more important to the binding of adenine (**14**) than the corresponding pyrazole (**57**); note that introduction of a 9-methyl (**18**) on adenine gave a 500-fold loss in binding, but introduction of a 1-methyl (**58**) on **57** gave only a fivefold loss in binding.

From the preceding discussion it is clear that there are multiple modes of binding of these purines and pyrazolo[3,4-*d*]pyrimidines in Table I to xanthine oxidase; the mode of binding is dependent upon the position and nature of both polar and hydrophobic substituents. That multiple modes of binding of the rotomeric type takes place is further supported by three series of irreversible inhibitors. If two heterocycles

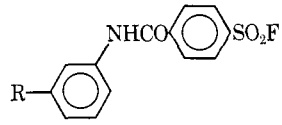
(19) Trademark of Burroughs Wellcome Co.

(20) G. B. Elion, A. Kovensky, G. H. Hitchings, E. Metz, and R. W. Rundles, *Biochem. Pharmacol.*, **15**, 863 (1966).

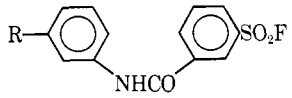
(21) R. W. Rundles, J. B. Wyngaarden, G. H. Hitchings, G. B. Elion, and H. R. Silberman, *Trans. Assoc. Am. Physicians*, **76**, 126 (1963).



3, R = guanin-9-yl
4, R = adenin-8-yl



62, R = guanin-9-yl
63, R = adenin-8-yl
64, R = 4-hydroxypyrazolo[3,4-*d*]-pyrimidin-6-yl
65, R = 4-aminopyrazolo[3,4-*d*]-pyrimidin-6-yl



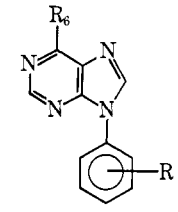
66, R = guanin-9-yl
67, R = adenin-8-yl
68, R = 4-hydroxypyrazolo[3,4-*d*]-pyrimidin-6-yl
69, R = 4-aminopyrazolo[3,4-*d*]-pyrimidin-6-yl

bearing the identical hydrophobic and leaving groups such as **3** and **4** are complexed to an enzyme, but only one such as **3** can inactivate the enzyme by a neighboring-group reaction within the enzyme–inhibitor complex, it can be stated unequivocally that the leaving group on the two molecules is positioned differently within the complex.[22] Since the guanine derivative (**3**) can rapidly inactivate the enzyme,[9] but the adenine derivative (**4**) does not,[2] it is clear that the phenyl groups of two molecules are not complexed to xanthine oxidase in the same way. Similarly, the 4-hydroxypyrazolo-[3,4-*d*]pyrimidine (**64**) can inactivate xanthine oxidase,[2] but the corresponding derivatives of guanine (**62**),[9] adenine (**63**),[2] and 4-aminopyrazolo[3,4-*d*]-pyrimidine (**65**)[2] do not inactivate the enzyme; therefore **64** complexes to the enzyme in a different mode than **62**, **63**, and **65**. The same conclusion can be reached by comparing the irreversible inhibitor **68** with **66**, **67**, and **69** which are not irreversible inhibitors.[2,9]

No unifying theory for the mode of binding to heterocycles to xanthine oxidase has emerged from this study, even though a number of practical objectives have been met. Although it would be aesthetically pleasing to have a unifying theory of binding such as that achieved with dihydrofolic reductase,[22c] such a theory is helpful, but not essential to the design of active-site-directed irreversible enzyme inhibitors.[10] From these studies on the mode of binding to xanthine oxidase have emerged a number of potent reversible inhibitors derived from purines and pyrazolo[3,4-*d*]pyrimidines that bear an oversized group tolerated within the enzyme–inhibitor complex. By placement of a leaving group on the oversized side chain a number of potent irreversible inhibitors such as **3**,[9] **64**, and **68**[2] have emerged. Similarly, irreversible inhibitors have been constructed

(22) (a) B. R. Baker and J. H. Jordaan, *J. Heterocycl. Chem.*, **4**, 31 (1967), paper LXXIII of this series; (b) B. R. Baker and R. B. Meyer, Jr., *J. Pharm. Sci.*, **56**, 570 (1967), paper LXXXIV of this series; (c) ref 10, pp 231–248.

TABLE II
PHYSICAL PROPERTIES OF



No.	R_6	R	Method[a]	% yield	Mp, °C dec	Formula[e]
11	OH	*m*-NO_2	B	73	>300[b]	$C_{11}H_7N_5O_4$
12	OH	*p*-NO_2	B	64	>300[c]	$C_{11}H_7N_5O_3 \cdot 0.25H_2O$
13	OH	*p*-OCH_3	B	75	>300[d]	$C_{12}H_{10}N_4O_2$
20	NH_2	*p*-NO_2	C	80	278–279	$C_{11}H_8N_6O_2$
73	Cl	*m*-NO_2	A	80	211–212	$C_{11}H_6ClN_5O_2$
74	Cl	*p*-NO_2	A	97	226–227	$C_{11}H_6ClN_5O_2$
75	Cl	*p*-OCH_3	A	64	201–202	$C_{12}H_9N_4O$

[a] All samples were crystallized from EtOH–H_2O. [b] λ_{max} (mμ) pH 1, 229, 250 (infl); pH 13, 233, 254 (infl). [c] λ_{max} (mμ) pH 1, 257; pH 13, 260, 290 (infl). [d] λ_{max} (mμ) pH 1, 238, 250 (infl); pH 13, 235. [e] All compounds were analyzed for C, H, N.

from **6** and **7**.[23] Further studies on variation of the length and stereochemical nature of the bridge between the heterocycle and sulfonyl fluoride of **3**, **4**, and **62–69** are under investigation in order to find additional types of irreversible inhibitors and to apply the bridge principle of specificity[24] for tissue-specific inhibitors of xanthine oxidase. New classes of candidate irreversible inhibitors of this enzyme derived from **8**, **10**, **17**, and **56** are worthy of investigation.

Chemistry.—All of the compounds in Table I except **11–13** and **20** have been previously described in earlier papers of this series, were in the literature, or were generously donated by Professor Roland K. Robins, Professor E. C. Taylor, or Dr. Harry B. Wood, Jr. (CCNSC). The remaining four new compounds were synthesized from 5-amino-4,6-dichloropyrimidine by the general methods of Montgomery.[25]

Experimental Section[26]

5-Amino-6-chloro-4-(*m*-nitroanilino)pyrimidine (70.)—A solution of 1.00 g (6.09 mmoles) of 5-amino-4,6-dichloropyrimidine, 0.84 g (6.1 mmoles) of *m*-nitroaniline, and 25 ml of EtOH–H_2O–12 *N* HCl (10:65:1) was refluxed with magnetic stirring for 8 hr during which time the product separated. The cooled mixture was filtered and the product was washed with 20 ml of H_2O; yield 1.50 g (91%), mp 235–239°, that was suitable for further transformation. For analysis a sample was recrystallized from EtOH–H_2O, then dried at 100° in high vacuum over P_2O_5: yellow crystals, mp 240–241° dec. *Anal.* ($C_{10}H_8ClN_5O_2$).

The *p*-nitro isomer (**71**) was prepared similarly in 80% yield and had mp 269° dec after recrystallization from EtOH–H_2O. *Anal.* ($C_{10}H_8ClN_5O_2$).

5-Amino-4-(*p*-anisidino)-6-chloropyrimidine (72) was prepared similarly in 87% yield from *p*-anisidine. Recrystallization from EtOH–H_2O gave white crystals, mp 197–199°. *Anal.* ($C_{11}H_{11}ClN_4O$).

(23) B. R. Baker and J. A. Kozma, *J. Med. Chem.*, **11**, 652 (1968), paper CXXIV of this series.

(24) Reference 10, pp 172–184.

(25) (a) C. Temple, Jr., C. L. Kussner, and J. A. Montgomery, *J. Med. Pharm. Chem.*, **5**, 866 (1962); (b) J. A. Montgomery and C. Temple, Jr., *J. Am. Chem. Soc.*, **83**, 630 (1961).

(26) Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Each analytical sample moved as a single spot on tlc in $CHCl_3$–EtOH and had ir and uv spectra compatible with its assigned structure. Combustion analyses (C, H, N) for each analytical sample agreed within 0.3% of theoretical.

6-Chloro-9-(*m*-nitrophenyl)purine (73) (Method A).—To a solution of 600 mg (2.26 mmoles) of **70** in 15 ml of ethyl orthoformate was added 1 ml of 12 *N* HCl. Within 1 min a product began to separate. After being stirred 24 hr at ambient temperature, the mixture was filtered and the product was washed with 5 ml of H_2O; yield 500 mg (80%), mp 206–209°, that was suitable for further transformation. See Table II for additional data and other compounds prepared by this method.

9-(*m*-Nitrophenyl)hypoxanthine (11) (Method B).—A mixture of 170 mg (0.62 mmole) of **73** and 5 ml of 6 *N* HCl was refluxed for 3 hr during which time solution occurred, then the product separated. The cooled mixture was filtered and the product was washed with 5 ml of H_2O. Recrystallization from 75 ml of 2:1 EtOH–H_2O gave 115 mg (73%) of white crystals, mp >300°. See Table II for additional data and other compounds prepared by this method.

9-(*p*-Nitrophenyl)adenine (20) (Method C).—To 15 ml of absolute EtOH saturated with NH_3 at 0° was added 233 mg (0.85 mmole) of **74**. After being heated in a steel bomb at 115° for 24 hr, the cooled mixture was concentrated until the product began to separate; yield 188 mg (80%), mp 278–279°. See Table II for additional data.

Irreversible Enzyme Inhibitors. CXXVII.[1-3] *p*-(2,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)phenylpropionylsulfanilyl Fluoride, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase. III. Effects of Modification of the Propionamide Bridge on Isozyme Specificity

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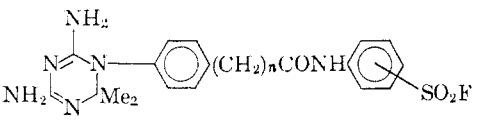
Received March 9, 1968

The title compound (1) is an active-site-directed irreversible inhibitor of the dihydrofolic reductase from Walker 256 rat tumor, rat liver, L1210/FR8 mouse leukemia, and mouse liver.[6] Six variants of the propionamide bridge, where the number of bridge atoms was held at four, were synthesized for enzymic evaluation, namely, acrylamido (**6**), methyleneureido (**8**), N-ethylsulfonamido (**10**), N-ethylcarboxamido (**12**), oxyacetamido (**13**), and oxyethyloxy (**15**). All but **6** were as good reversible inhibitors of dihydrofolic reductase as **1** with a K_i in the range of 1–6 × 10^{-9} *M* for Walker 256; **6** was 300-fold less effective. With the Walker 256 enzyme four showed no irreversible inhibition and two (**10, 12**) only poor irreversible inhibition. Of these six compounds, none showed irreversible inhibition of the L1210/FR8 enzyme. These results were rationalized on the basis of allowable ground-state conformations of the compounds *vs.* the conformation needed for juxtaposition of the sulfonyl fluoride to an enzymic nucleophilic group within the enzyme–inhibitor complex. The metanilyl isomer (**3**) of 1 can inactivate the dihydrofolic reductase from Walker 256 and rat liver, but not L1210/FR8.[7] When the propionamide bridge of **3** was changed to acrylamido (**7**), methyleneureido (**9**), N-ethylsulfonamido (11), or oxyacetamido (**14**), irreversible inhibition was again lost. These results show the sensitivity of active-site-directed irreversible inhibition to proper positioning of the inhibitor's leaving group with an enzymic nucleophilic group within the reversible complex.

The first active-site-directed irreversible inhibitor[5] of the terminal sulfonyl fluoride type found to be effective on the dihydrofolic reductase from Walker 256 rat tumor and L1210/FR8 mouse leukemia was the title compound **1**;[6] the compound could also inactivate the dihydrofolic reductase from rat liver[6] and mouse liver (Table I). The sulfonyl fluoride with a shorter bridge (**2**) could also inactivate the tumor enzymes, but a much higher concentration of **2** was required since it was a considerably less effective reversible inhibitor than **1**. When the sulfonyl fluoride group of **1** was moved to the *meta* position (**3**), a separation of activity on the enzymes of the two species was observed;[7] **3** failed to inactivate the dihydrofolic reductase from L1210/FR8 mouse leukemia but could still inactivate the two rat tissue enzymes. Conversely, a cross-over specificity



1, $n = 2$, *para* isomer
2, $n = 0$, *para* isomer
3, $n = 2$, *meta* isomer
4, $n = 3$, *para* isomer
5, $n = 0$, *meta* isomer

was noted with **5** which could inactivate the L1210/FR8 mouse leukemia enzyme, but not the Walker 256 rat tumor enzyme. Furthermore, when the bridge length was increased to give **4**, the Walker 256 enzyme was still inactivated, but the L1210/FR8 enzyme was not.[7] A study was then initiated on the effect that the changes in the bridge between the two benzene rings of **1–3** would have on the active-site-directed irreversible inhibition. Such a study might answer the following questions. (1) Can other effective bridges be found that would give additional flexibility in synthesis of active analogs? (2) What is the relationship of ground-state conformation of the bridge to effective irreversible inhibition? (3) Can activity be further separated[8] between tumor and liver enzymes? The

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper in this series see B. R. Baker, W. F. Wood, and J. A. Kozma, *J. Med. Chem.*, **11**, 661 (1968).

(3) For the previous paper on inhibitors of dihydrofolic reductase see B. R. Baker and P. C. Huang, *ibid.*, **11**, 495 (1968), paper CXX of the series.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

(5) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

(6) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

(7) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 38 (1968), paper CXII of this series.

(8) The effective inhibition of a tumor enzyme with little or no effect on the corresponding liver enzyme has been previously reported with 5-phenoxypropylpyrimidines[9] and 6-phenoxymethylpyrimidines[3] with a terminal fluorosulfonylbenzamido group on the benzene ring.

(9) B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 489 (1968), paper CXIX of this series.