luted with the same; the remainder of the inhibitors were dissolved in 10 mM aqueous sodium hydroxide and diluted with water.

In a 3-ml. cell were placed 2.70 ml. of buffer, 0.30 ml. of dimethylsulfoxide, and 50 μl. of 0.5 mM hypoxanthine. The solution was vigorously shaken for about 20 sec. to absorb air. Then 50 μl. of diluted enzyme solution was added and the rate of increase in absorbance at 290 mμ was noted with a Gilford recording spectrophotometer; sufficient enzyme was employed to give about 0.01 absorbance units change per minute. If the inhibitor was dissolved in water, up to 0.3 ml. could be used in the cell by decreasing the buffer by the corresponding amount. If the inhibitor was dissolved in dimethylsulfoxide, up to 0.30 ml. could be used, keeping the total volume of dimethylsulfoxide at 0.30 ml. Sufficient inhibitor was used to give 30–70% inhibition; the μM concentration of inhibitor necessary for 50% inhibition ($V_0/V_I = 2$) was determined by plotting V_0/V_I against I, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = inhibitor concentration (33).

The cell concentration of hypoxanthine was 8.1 μM; the velocity was the same in the presence or absence of 10% dimethylsulfoxide.

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Irreversible Enzyme Inhibitors XCIII

Hydrophobic Bonding to Xanthine Oxidase by Some Phenylpurines

By B. R. BAKER

Nine derivatives of guanine with alkyl, aryl, or aralkyl groups at the 8- or 9-position were investigated as inhibitors of xanthine oxidase. Maximum hydrocarbon interaction with the enzyme was observed with the in-plane 9-phenyl substituent, a 100-fold increment in binding being observed. The "2"-position of purines was also investigated with phenyl substituents on the 6-position of 4-mercaptopyrazolo[3,4-*d*]pyrimidine; a thirtyfold increment in binding was observed with either a *p*-nitrophenyl or a *p*-methoxyphenyl substituent at the 6-position of the pyrazolopyrimidine.

THE PREVIOUS PAPER of this series discussed the possible chemotherapeutic use for a blockade of xanthine oxidase (1): since 6-mercaptopurine can be detoxified by oxidation to the nontoxic 6-thiouric, an active-site-directed irreversible inhibitor that is sufficiently tissue specific on a given tumor line would be a useful adjunct to 6-mercaptopurine therapy. The normal substrates of xanthine oxidase, hypoxanthine and xanthine, are highly polar molecules; therefore, if a hydrophobic region could be detected by purines substituted by hydrocarbon groups, this hydrophobic region would most probably be just adjacent to the active site, but not part of

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the active site. It is outside the active site where evolutionary changes of amino acids could more easily occur and still maintain the integrity of the active site (2). Such hydrophobic regions adjacent to the active site have been discovered on dihydrofolic reductase (3–5), guanase (6, 7), and thymidine phosphorylase (8, 9); the exploitation of hydrophobic regions adjacent to the active site for the design of reversible (5, 10) and irreversible inhibitors (5, 11) has been discussed. Therefore, a search for a hydrophobic bonding region on xanthine oxidase was initiated and the results are the subject of this paper.

DISCUSSION

It was noted in the previous paper that 8-azaguanine (II) was complexed as well as the substrate, hypoxanthine, and guanine (I) was complexed one-fifth as well (Table I). Since substitution of the 9-hydrogen of guanine (I) by methyl (III) gave less than a twofold loss in binding, variation of the 9-substituent of guanine was investigated for detection of hydrophobic bonding; these compounds were kindly supplied by Professor Roland K. Robins (12).

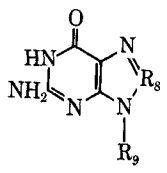
The 9-(*n*-amyl)-substituent of IV and the cyclohexyl substituent of V gave no appreciable increase in binding over guanine, and the 9-benzyl substituent (VI) gave only about twofold increase in binding. A large increment in binding was observed with the in-plane 9-phenyl substituent of VII, VII being complexed 100-fold better than guanine (I) and 140-fold better than 9-methylguanine (III). 9-(*p*-Chlorophenyl)guanine (VIII) also showed hydrophobic bonding to the enzyme with a 22-fold increment in binding over that of guanine (I), but which was less than that noted with 9-phenylguanine (VI). Similarly, the 9-(*p*-chlorophenyl) substituent (IX) of 8-azaguanine showed a 31-fold increment in binding over the parent 8-azaguanine (II). Thus, IX is the most potent inhibitor in Tables I and II, being complexed 33-fold better than the substrate, hypoxanthine which has $K_m = 7.8 \times 10^{-6}$ *M* (13).

Two guanine derivatives with an 8-phenyl (X) and 8-phenylpropyl (XI) substituent (6) were investigated for hydrophobic bonding; both showed about a fivefold increment in binding over guanine (I), but this increment was considerably less than that observed with the 9-phenyl substituent (VII).

Through a generous gift from Professor E. C. Taylor, the 4-mercaptopyrazolo[3,4-*d*]pyrimidines with hydrocarbon groups at the 2-position (14), were studied; these were of interest since 4-hydroxypyrazolo[3,4-*d*]pyrimidine is a known substrate and inhibitor (15) of xanthine oxidase and since hydrophobic bonding had been detected with 9-phenylguanine (Table I).

The 2-methyl derivative (XII) (Table II) was complexed fifteenfold less than hypoxanthine, the substrate. When the 2-methyl group of XII was replaced by the in-plane *p*-nitrophenyl group (XIII), a 38-fold increment in binding was observed; this is similar to the 31-fold increment observed in replacing the 9-methyl group (III) on guanine by *p*-chlorophenyl (VIII), but not so good as the increment in 9-phenylguanine (VII).

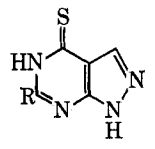
Table I—Inhibition of Xanthine Oxidase by



Compd.	R_8	R_9	μM Concn. for 50% Inhibition[a]	$[I/S]_{0.5}$[b]	Source
I	CH	H	40[d]	4.9	NBC
II	N	H	7.5[d]	0.93	NBC
III	CH	CH_3	58[d]	7.1	Robins
IV	CH	*n*-C_5H_{11}-	35	4.3	Robins
V	CH	Cyclohexyl-	55	6.8	Robins
VI	CH	$C_6H_5CH_2$-	23	2.9	Robins
VII	CH	C_6H_5-	0.41	0.051	Robins
VIII	CH	*p*-ClC_6H_4-	1.8	0.22	Robins
IX	N	*p*-ClC_6H_4-	0.25	0.030	Robins
X	CC_6H_5	H	7.4	0.92	(6)
XI	$C(CH_2)_2C_6H_5$	H	8.4	1.0	(6)

[a] Xanthine oxidase from bovine milk (Nutritional Biochemicals Corp.) was assayed with 8.1 μM hypoxanthine in Tris buffer (pH 7.4) containing 10% dimethylsulfoxide as previously described (1). [b] The ratio of the concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition. [c] Sources: NBC, Nutritional Biochemicals Corp.; Robins, a gift from Professor Roland K. Robins (12). [d] Data from *Reference 1*.

Table II—Inhibition of Xanthine Oxidase by



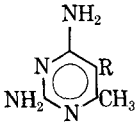
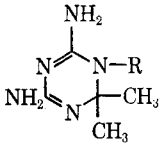
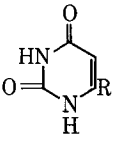
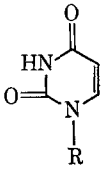
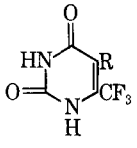
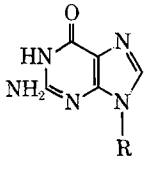
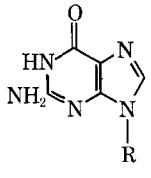
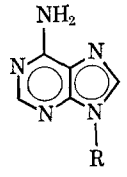
Compd.	R	μM Concn. for 50% Inhibition[a]	$[I/S]_{0.5}$[b]	Hansch π-Constant for φ Substituent[c]
XII	CH_3	120	15	
XIII	*p*-$NO_2C_6H_4$-	3.3	0.39	−0.28
XIV	*p*-$NH_2C_6H_4$-	32	4.0	−1.23
XV	*p*-$CH_3OC_6H_4$-	2.5	0.31	−0.02

[a] See *Footnote a*, Table I; these compounds were a gift from Professor E. C. Taylor (14). [b] Ratio of concentration of inhibitor to 8.1 μM hypoxanthine giving 50% inhibition. [c] A measurement of hydrophobic character on a log scale, a minus value being more polar than hydrogen; data from *Reference 24*.

The *p*-methoxyphenyl substituent (XV) was complexed as well as the *p*-nitrophenyl substituent (XIII); these results indicate that there is no donor-acceptor character in the mode of complexing of the phenyl group in this series to the enzyme, else considerable difference in binding should have been seen due to the strong electron-donating methoxyl group and the strong electron-withdrawing nitro group. Replacement of the *p*-methoxy group of XV with *p*-amino (XIV) gave a thirteenfold loss in binding. This result could be due to repulsion of the weakly basic amino group from an electron-donor site on the enzyme or due to the difference in polarity between amino on one hand and methoxy and nitro on the other; note in Table II that the amino group is by far the most polar of the three groups.

That an in-plane phenyl group can give hydrophobic bonding on either the 9-position or "2" position of a purine or related compound agrees with the postulate of Bergmann *et al.* (16) that various rotomers of the purine can bind to the active site of xanthine oxidase. Strong hydrophobic bonding can be determinate on which rotomer binds to the enzyme, as previously postulated with binding to chymotrypsin (17, 18), dihydrofolic reductase (4, 5, 19, 20), some adenylate using enzymes (21), and adeno-

TABLE III—HYDROPHOBIC BONDING TO SOME SELECTED ENZYMES BY HETEROCYCLES WITH HYDROCARBON SUBSTITUENTS

Enzyme	Source	Heterocycle	R for Max. Hydrophobic Bonding	Binding Increment	Ref.
Dihydrofolic reductase	Pigeon liver		$C_6H_5(CH_2)_4$-	40,000	(3, 5)
			$C_6H_5(CH_2)_4$-	2000[a]	(3, 5)
			C_6H_5-	700[a]	(3, 5)
			i-C_5H_{11}-	1300[a]	(3, 5)
			m-$C_6H_4(CH_2)_4C_6H_5$	20,000	(29)
Thymidine phosphorylase	*E. coli* B		$C_6H_5CH_2$-	18	(8, 9)
			$C_6H_5(CH_2)_5$-	100[b]	(8, 9)
			$C_6H_5(CH_2)_4$-	9[b]	(30)
Guanase	Rabbit liver		C_6H_5-	28	(6, 7)
Xanthine oxidase	Bovine milk		C_6H_5-	100[c]	
Succinoadenylate kinosynthetase	*E. coli* B		$C_6H_5CH_2$-	11	(28)

[a] Included for comparison. [b] Higher phenylalkyl analogs not yet investigated, but lower analogs were less effective. [c] Data from Table I.

sine deaminase (22). By substitution of the phenyl group on various purines and related heterocycles, such as those in Tables I and II, it should then be possible to study the mode of binding of other substituents on the heterocyclic ring such as OH, NH, and SH; if good phenyl binding is observed compared to the corresponding methyl or benzyl purine, then it can be logically assumed that the phenyl group anchors one rotomer to the enzyme, but with two "flip" configurations (5, 20).

Also of interest would be to establish whether the phenyl group complexes to the enzyme only by hydrophobic bonding and the accompanying van der Waal's forces—as seen with dihydrofolic reductase (5, 23)—or if there is additional donor-acceptor character in the phenyl ring (unlikely). Furthermore, the use of these phenyl heterocycles for construction of active-site-directed irreversible inhibitors is worthy of pursuit.

Some seven enzymes using a heterocyclic substrate have been investigated in this laboratory for hydrophobic bonding by hydrocarbon groups attached to the heterocycle. Hydrophobic bonding has yet to be detected with thymidine kinase (9, 25, 26) and thymidylate synthetase (27) even though such a search was performed; weak hydrophobic bonding with 9-alkyl and 9-aralkyladenines to succinoadenylate kinosynthetase has been observed (28); strong hydrophobic bonding with 6-aralkyluracils to thymidine phosphorylase (8, 9), with 9-phenylguanines to guanase (6), and with 9-phenylpurines and related heterocycles to xanthine oxidase has been observed. The strongest hydrophobic bonding observed in this set of seven enzymes was with dihydrofolic reductase (3, 5, 23) where the in-plane phenyl group gives a 700-fold increment and the phenylbutyl group gives a 40,000-fold increment in binding.

These comparisons are summarized in Table III. Note that the type of hydrocarbon giving maximum hydrophobic bonding varies with the enzyme and with the position of the hydrophobic group on the inhibitor; for example, the in-plane phenyl group gives maximum hydrocarbon bonding with guanase and xanthine oxidase, but higher phenylalkyl groups are more effective on the other enzymes. Also note that the benzyl group on the 6-position of uracil gives maximum hydrocarbon interaction, but that higher phenylalkyl groups are more effective on the 1- and 5-positions of uracil. Although the 9-phenyl group on guanine is the most effective for hydrocarbon interaction with guanase and xanthine oxidase, substitution on the phenyl group has shown that the enzymic environment in the region of 9-phenyl bonding is not the same with the two enzymes; for example, a *p*-carboxylate on the 9-phenyl group aids binding to xanthine oxidase, but is extremely detrimental to binding to guanase (31).

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