Irreversible Enzyme Inhibitors. 187.[1,2] Studies on Bulk Tolerance with Inhibitors of Guanosine Phosphorylase

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Purine nucleoside phosphorylase, which can use inosine, guanosine, thioinosine, and thioguanosine as substrates, is, since the reaction is reversible, inhibited by respective products hypoxanthine, guanine, 6-mercaptopurine, and thioguanine. Forty-seven derivatives of these 4 purines, substituted on the 1, 2, 3, 7, 8, or 9 position with large groups such as Ph, benzyl, or benzylthio, were investigated as inhibitors of the enzyme; the loss in binding was 0- to 5-fold indicating bulk tolerance within the enzyme–inhibitor complex at these 6 positions. Hydrophobic bonding could be detected only at the 9 position, but it was too weak (3-fold increment) to be useful. No polar point interaction with the enzyme could be detected with 9 derivatives of hypoxanthine or 6-mercaptopurine containing substituents on position 9 containing a polar group.

Purine nucleoside phosphorylase is an enzyme that can cleave guanosine or inosine to guanine and hypothanine, respectively;[3] it is therefore sometimes called guanosine phosphorylase or inosine phosphorylase. This enzyme is probably involved in the catabolic detoxification of the anticancer agent thioguanosine by cleavage to thioguanine[4] followed by the guanine deaminase mediated conversion to thioxanthine which is further catabolized to the nontoxic thiouric acid by xanthine oxidase.[5] The enzyme does not attack adenosine,[3,6] and its phosphorolysis of xanthosine[7] is considerably slower than inosine; however, deoxyguanosine and deoxyinosine are rapidly cleaved.[3] Thioguanosine can be converted to the cytoxic thioguanylate[8] by a kinase.[9] Thus the growth of any cell line containing this kinase will be inhibited by thioguanosine providing the cell line has a low enough level of the phosphorylase not to cleave all of the nucleoside and a low enough level of nucleotide pyrophosphorylase[10] not to cleave all of the nucleotide to thioguanine; if the level of either catabolic enzyme is too high, cytoxicity will be reduced due to a lowered level of nucleotide. Tumor lines containing the kinase and the phosphorylase, but little or no pyrophosphorylase could be made susceptible to thioguanosine toxicity if the phosphorylase could be blocked; however, a non-tissue-specific inhibitor of the phosphorylase would also block detoxification in normal cells in an animal. Therefore, a tissue-specific inhibitor[11,12] of guanosine phosphorylase would be needed; such tissue-specific irreversible inhibitors are achievable with active-site-directed irreversible inhibitors.[12] The subject of this paper is a study of the first phase of the *modus operandi*[13] for construction of an active-site-directed irreversible inhibitor of guanosine phosphorylase. Namely, where can large groups be placed on an inhibitor without interference with the formation of the enzyme–inhibitor complex and can hydrophobic bonding be found which can greatly increase the binding of an inhibitor?

Enzyme Results.—The equilibrium in the guanosine phosphorylase catalyzed phosphorolysis of guanosine to guanine and ribose 1-phosphate favors the nucleoside;[14] this results in rapid curvature in the rate of phosphorolysis making the determination of the initial rate difficult. However arsenolysis at pH 5.9 favors cleavage due to the rapid solvent hydrolysis of the ribose 1-arsenate to ribose.[15] Thus curvature in the rate of arsenolysis occurs later due to inhibition by guanine; inhibition is therefore more accurately measured by arsenolysis as described in the Experimental Section. Guanosine was used at 0.04 m*M* and the rate of change of OD at 260 mμ was followed.[16]

Since inosine, guanosine, thioinosine, and thioguanosine are substrates, it would be expected that the products hypoxanthine (**1**), guanine (**2**), 6-mercaptopurine (**3**), and thioguanine (**4**) (Table I) would be inhibitors of this reversible reaction. Hypoxanthine (**1**) was complexed almost as well as the substrate guanosine. Guanine (**2**) was complexed 0.5 as well as hypoxanthine;[17] replacement of the 6-oxo function of hypoxanthine or guanine with thione led to a 2-fold loss in binding in both cases (**3**, **4**).[17]

Introduction of a 1-Me group (**5**) on guanine led to a 3-fold loss in binding which indicates the 1-H group of guanine makes a very minor contribution to binding. When a 1-benzyl group (**6**)[18] was introduced on hypoxanthine (**1**) a 5-fold loss in binding occurred; thus it appeared that there was reasonable bulk tolerance for the large benzyl group at the 1 position, but there was no hydrophobic interaction.

That there was reasonable bulk tolerance for large

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(2) For the previous paper in this series see B. R. Baker and M. Cory *J. Med. Chem.*, **14**, 805 (1971).

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(13) See ref 12, Chapters 9–12.

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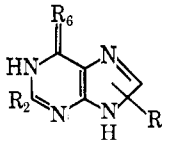
TABLE I

INHIBITION[a] OF GUANOSINE PHOSPHORYLASE BY HYPOXANTHINE, GUANINE, AND 6-MERCAPTOPURINE CONTAINING HYDROPHOBIC SUBSTITUENTS



No.	R_2	R_6	R	I_{50},[b] mM	No.	R_2	R_6	R	I_{50},[b] mM
1[c]	H	O	H	0.065	26	H	O	9-$C_6H_5(CH_2)_3$	>0.40
2[c]	NH_2	O	H	0.10	27	H	S	9-$C_6H_5(CH_2)_3$	>0.30
3[c]	H	S	H	0.11	28	H	O	9-$C_6H_5(CH_2)_4$	>0.60
4[c]	NH_2	S	H	0.24	29	H	S	9-$C_6H_5(CH_2)_4$	>0.20
5[d]	NH_2	O	1-Me	0.31	30[d]	NH_2	O	9-C_5H_{11}-*n*	0.24
6[e]	H	O	1-C_6H_5-CH_2	0.34	31[d]	NH_2	O	9-Cyclohexyl	0.34
7[f]	$C_6H_5CH_2S$	O	H	0.30	32[d]	NH_2	O	9-$C_{11}H_{23}$-*n*	>0.20
8[g]	C_6H_5	O	H	0.44	33[d]	NH_2	O	9-(α-Furyl)methyl	0.10
9[h]	H	O	3-$C_6H_5CH_2$	0.25	34	H	O	9-$C_6H_5O(CH_2)_2$	0.41
10[h]	H	O	7-$C_6H_5CH_2$	0.33	35	H	O	9-$C_6H_5O(CH_2)_3$	0.37
11[h]	H	S	7-$C_6H_5CH_2$	>0.20	36	H	O	9-$CH_2C_6H_4Cl$-*p*	0.32
12[f]	H	O	8-$C_6H_5CH_2S$	0.33	37[d]	NH_2	O	9-$CH_2C_6H_4Cl$-*p*	0.25
13[i]	H	O	8-C_6H_5	0.35	38	H	O	9-$CH_2C_6H_4Cl$-*m*	0.26
14[i]	NH_2	O	8-C_6H_5	0.25	39	H	O	9-$CH_2C_6H_4Cl$-*o*	0.33
15[i]	NH_2	O	8-$C_6H_5(CH_2)_2$	0.26	40	H	O	9-$CH_2C_6H_3Cl_2$-3,4	>0.20
16[i]	NH_2	O	8-$C_6H_5(CH_2)_3$	0.34	41	NH_2	O	9-$CH_2C_6H_3Cl_2$-3,4	0.22
17[k]	H	O	9-Me	0.57	42	H	S	9-$CH_2C_6H_3Cl_2$-3,4	>0.40
18[d]	NH_2	O	9-Me	0.29	43	H	O	9-$CH_2C_6H_3Cl_2$-2,6	0.38
19[k]	H	O	9-C_6H_5	>1.2	44	H	O	9-$CH_2C_6H_4OMe$-*p*	0.29
20[d]	NH_2	O	9-C_6H_5	0.25	45	H	S	9-$CH_2C_6H_4OMe$-*p*	>0.80
21[l]	H	O	9-$C_6H_5CH_2$	0.19	46	H	O	9-$CH_2C_6H_4Me$-*p*	0.20
22[d]	NH_2	O	9-$C_6H_5CH_2$	0.071	47	H	O	9-$CH_2C_6H_3Me_2$-2,5	0.38
23[i]	H	S	9-$C_6H_5CH_2$	0.061	48	H	S	9-$CH_2C_6H_3Me_2$-2,5	>0.40
24	H	O	9-$C_6H_5(CH_2)_2$	0.22	49	H	O	9-$CH_2C_{10}H_7$-α	0.33
25[m]	NH_2	O	9-$C_6H_5(CH_2)_2$	0.18	50	H	O	9-$CH(CH_3)C_6H_5$	0.38

[a] The technical assistance of Maureen Baker is acknowledged. [b] I_{50} = concn necessary for 50% inhibition when assayed with 0.04 mM guanosine as described in the Experimental Section. [c] Commercial. [d] A gift from Dr. Roland K. Robins. [e] Synth from inosine as described in ref 18. [f] See ref 19 for synth. [g] Synth according to ref 20. [h] Synth according to ref 21. [i] A gift from Dr. Harry B. Wood, Jr., CCNSC. [j] See ref 22 for synth. [k] See ref 23 for synth. [l] Synth as described in ref 26. [m] See ref 27 for synth.

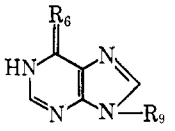
groups at the 2 position of hypoxanthine was indicated by introduction of a 2-benzylthio (**7**)[19] or 2-Ph (**8**)[20] group; the loss in binding was 5- to 6-fold, but no hydrophobic bonding occurred. Similarly, reasonable bulk tolerance to a 3-benzyl (**9**)[21] or 7-benzyl (**10**)[21] was shown, but with no hydrophobic bonding.

At the 8 position of hypoxanthine there was reasonable bulk tolerance for a benzylthio (**12**)[19] or Ph (**13**) group, the loss in binding being about 5-fold; no hydrophobic bonding was observed. Similar results were seen by substituting guanine on the 8 position[22] by Ph (**14**), phenethyl (**15**), or phenylpropyl (**16**), the loss in binding being about 3-fold.

Methylation of the 9 position of hypoxanthine to **17**[23,24] gave a 9-fold loss in binding; similarly, the 9-Me (**18**) on guanine gave a 3-fold loss in binding. Replacement of the 9-Me group on hypoxanthine with C_6H_5 (**19**)[23,25] led to a > 2-fold loss in binding; however, a similar replacement on guanine by C_6H_5 (**20**) gave no change in binding. Replacement of the 9-Me group on hypoxanthine by $C_6H_5CH_2$ (**21**)[26] gave a 3-fold enhancement in binding and a similar replacement in the guanine series (**22**) gave a 4-fold increase in binding; it appeared that the Ph group of the benzyl moiety was

TABLE II

INHIBITION[a] OF GUANOSINE PHOSPHORYLASE BY HYPOXANTHINE AND 6-MERCAPTOPURINE WITH POLAR SUBSTITUENTS ON POSITION 9



No.	R_6	R_9	I_{50},[b] μM
51[c]	S	CH_2COOH	>2.4
52[d]	O	$(CH_2)_2COOH$	0.37
53[e]	O	$(CH_2)_4COOH$	0.47
54[f]	S	$(CH_2)_4CONH_2$	>2.4
55[g]	S	$(CH_2)_6COOH$	>2.4
56[f]	S	$(CH_2)_3NHCOCHCl_2$	>2.4
57[e]	O	$CH_2C_6H_4COOH$-*p*	0.64
58[e]	S	$CH_2C_6H_4COOH$-*p*	1.9
59[h]	O	$CH_2C_6H_4NO_2$-*p*	0.22

[a,b] See Table I. [c] See ref 26 and 31 for synth. [d] See ref 28 for synth. [e] See ref 29 for synth. [f] See ref 32 for synth. [g] See ref 31 for synth. [h] Synth according to ref 30.

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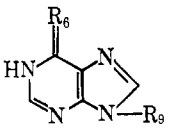
TABLE III
PHYSICAL CONSTANTS OF



No.[a]	R_6	R_9	Method	Yield,[b] %	Mp,[c] °C	Formula[d]
24	O	$C_6H_5(CH_2)_2$	A, C	19[e]	259–263	$C_{13}H_{12}N_4O$
26	O	$C_6H_5(CH_2)_3$	A, C	19[f]	252–255	$C_{14}H_{14}N_4O$
27	S	$C_6H_5(CH_2)_3$	A, D	60[g]	>290[h]	$C_{14}H_{14}N_4S$
28	O	$C_6H_5(CH_2)_4$	A, C	23[f]	>215[h]	$C_{15}H_{16}N_4O$
29	S	$C_6H_5(CH_2)_4$	A, D	76[g]	>290[h]	$C_{15}H_{16}N_4S$
34	O	$C_6H_5O(CH_2)_2$	B, C	15[i]	250–255	$C_{13}H_{12}N_4O_2$
35	O	$C_6H_5O(CH_2)_3$	B, C	18[i]	>215[h]	$C_{14}H_{14}N_4O_2$
36	O	$CH_2C_6H_4Cl$-*p*	A, C	47[i]	>215[h]	$C_{12}H_9ClN_4O$
38	O	$CH_2C_6H_4Cl$-*m*	A, C	18[f]	>215[h]	$C_{12}H_9ClN_4O$
39	O	$CH_2C_6H_4Cl$-*o*	A, C	7[i,k]	>215[h]	$C_{12}H_9ClN_4O$
40	O	$CH_2C_6H_3Cl_2$-3,4	A, C	12[e]	>215[h]	$C_{12}H_8Cl_2N_4O$
42	S	$CH_2C_6H_3Cl_2$-3,4	A, D	32[l]	>300	$C_{12}H_8Cl_2N_4S$
43	O	$CH_2C_6H_3Cl_2$-2,6	B, C		>290[i]	$C_{12}H_8Cl_2N_4O$
44	O	$CH_2C_6H_4OMe$-*p*	A, C	11[f]	251–255	$C_{13}H_{12}N_4O_2$
45	S	$CH_2C_6H_4OMe$-*p*	A, D	71[g]	298–301	$C_{13}H_{12}N_4OS$
46	O	$CH_2C_6H_4Me$-*p*	A, C	22[i]	292–295	$C_{13}H_{12}N_4O$
47	O	$CH_2C_6H_4Me_2$-2,5	A, C	12[i]	253–257	$C_{14}H_{14}N_4O$
48	S	$CH_2C_6H_4Me_2$-2,5	A, D	60[g]	>300	$C_{14}H_{14}N_4S$
49	O	$CH_2C_{10}H_7$-α	B, C		245–250[i]	$C_{16}H_{12}N_4O$
50	O	$CH(CH_3)C_6H_5$	A, C	37[m]	195–199	$C_{13}H_{12}N_4O$

[a] All anal. samples had proper uv spectra at pH 1, 7, and 14, proper ir spectra, and moved as a single spot on tlc with Brinkmann silica gel GF. [b] Overall yield of anal. pure material from 6-chloropurine or 5-amino-4,6-dichloropyrimidine. [b] Mp's taken in capillary tubes on a Mel-Temp block and are uncor. [d] Anal. for C, H, and N agreed within 0.3% of theory. [e] Recrystd from 2-$MeOC_2H_4OH$. [f] Recrystd from CH_3CN. [g] Triturated with EtOH. [h] Gradually decomp above this temp. [i] Recrystd from EtOH. [j] Recrystd from DMF. [k] Yield 18% by method B, C. [l] Dissolved in 0.1 *N* NaOH and repptd with 12 *N* HCl. [m] Triturated with Me_2CO.

interacting weakly with the enzyme and the interaction of a 9-benzyl (**23**) group on 6-mercaptopurine also appeared to be about 4-fold, although the 9-Me derivative was not available for comparison.

The 9-phenethyl group (**24**) on hypoxanthine complexed as well as 9-benzyl (**21**), but a similar change (**25**)[27] on guanine led to a 2.5-fold loss in binding. The 9-phenylpropyl (**26**, **27**) and 9-phenylbutyl (**28**, **29**) were less effective inhibitors. Higher 9-alkyl groups (**30**, **31**) on guanine showed about the same inhibition as 9-methylguanine, as did 9-phenoxyethyl- (**34**) and 9-phenoxypropylhypoxanthine (**35**).

Since weak interaction between the Ph moiety of 9-benzylguanine (**22**), hypoxanthine (**21**), and 6-mercaptopurine (**23**) was observed with guanosine phosphorylase, a study of substituent effects on the binding was made; none of the compds (**36**–**50**) were better inhibitors than the corresponding 9-benzylpurines.

A number of purines bearing substituents on position 9 containing a polar group were available in this laboratory from other studies; these were investigated as inhibitors of guanosine phosphorylase to see if a polar point interaction with the enzyme could be detected (Table II). No polar interaction was seen with the COOH group of $(CH_2)COOH$ (**52**),[28] $(CH_2)_4COOH$ (**53**),[29] or $CH_2C_6H_4COOH$-*p* (**57**)[29] on hypoxanthine, the binding being about the same as 9-Me (**17**); similar results were observed with the 9-(*p*-nitrophenyl) substituent (**59**).[30] Conversion of **57** to the 6-mercapto derivative (**58**)[29] gave a 3-fold loss in binding, about the same as the difference between hypoxanthine (**1**) and 6-mercaptopurine (**3**). When 6-mercaptopurine was substituted in the 9 position with CH_2COOH (**51**)[26,31] $(CH_2)_4CONH_2$ (**54**),[32] $(CH_2)_6COOH$ (**55**),[31] or $(CH_2)_3NHCOCHCl_2$ (**56**)[32] groups, no inhibition was seen at 22 times the I_{50} concn of 6-mercaptopurine (**3**).

Although the search for bulk tolerance areas on hypoxanthine and guanine has been successful, no useful amount of hydrophobic bonding has been found; as a result, the compds show I_{50}'s in the high range of 0.07–0.5 m*M*. The large groups tolerated at the 1, 2, 3, 7, 8, or 9 positions could be converted to potential active-site-directed irreversible inhibitors by attachment of covalent forming groups such as SO_2F. Although such irreversible inhibitors may be useful for affinity labeling of pure purine nucleoside phosphorylase, these inhibitors probably would require too high a concentration to be effective *in vivo* for chemotherapeutic use.

Chemistry.—Many of the compds investigated in Tables I and II were available from other studies in this laboratory or were synthesized as described in the quoted literature. The new compds in Table I were all 9-substituted hypoxanthines or 6-mercaptopurines; the former were made by acid hydrolysis of the appropriate 6-chloropurine (method C)[26,33] while the latter were made by reaction of the appropriate 6-chloropurine with thiourea (method D).[26,33] The required 6-chloropurines were made either by alkylation of 6-chloropurine (method B)[26] or by reaction of 5-amino-4,6-dichloropyrimidine with the appropriate

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amine followed by acid-catalyzed ring closure with ethyl orthoformate (method A).[34] The intermediate 6-chloropurines and 6-substituted aminopyrimidines were not isolated. See Table III.

Experimental Section

Assay of Guanosine Phosphorylase.—Purine nucleoside phosphorylase (1 mg/ml) from calf spleen was purchased from Calbiochem; for assay 75 μl of the soln was dild with 5 ml of H_2O and kept at 0°. When assayed as described below the initial OD change of about 0.015 unit/min was followed on a Gilford recording spectrophotometer with a 0–0.1 OD slidewire at 260 mμ.

In a 3-ml quartz cuvette were placed 1.60 ml of 0.2 *M* arsenate–succinate buffer of pH 5.9, 0.60 ml of 0.2 m*M* aq guanosine, 0.40 ml of H_2O, and 0.30 ml of DMSO. The contents were thoroughly mixed, then a base line run; if the base line was not 0 due to inadequate mixing of the DMSO, further mixing was made until the base line was 0. Then 100 μl of enzyme soln was added, the contents were quickly mixed, and the initial OD change was noted by drawing a tangent to 0 time. Since curvature is fairly rapid it is inadvisable to run more than 1 cuvette at a time. Inhibitors were dissolved in DMSO. The cuvette concn of guanosine was 0.04 m*M*; its K_m was found to be 57 μ*M* by the reciprocal plot method.

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Irreversible Enzyme Inhibitors. 188.[1,2] Inhibition of Mammalian Thymidine Phosphorylase

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Eighty uracils containing hydrophobic substituents at the 1, 5, or 6 positions were investigated as inhibitors of rabbit liver thymidine phosphorylase to try to detect a hydrophobic bonding region adjacent to the active site. Such a region was best detected by uracils containing a 2,4-dimethylanilino, phenethylamino, or phenylbutylamino substituent at the 6 position. Comparison of the inhibition profiles of the thymidine phosphorylase from rabbit liver and *Escherichia coli* B showed that this hydrophobic bonding region had undergone immense evolutionary change between the 2 species. As a result several of the compds were far more effective on the *E. coli* B enzyme than the mammalian enzyme; for example, 6-(α-naphthylmethylamino)uracil was complexed 900-fold better to the bacterial enzyme than the mammalian enzyme.

There are two enzymes in mammalian tissues that can phosphorylyse 5-fluoro-2′-deoxyuridine (FUDR) to FU and 2-deoxy-D-ribose 1-phosphate; these are uridine–deoxyuridine phosphorylase (EC 2.4.2.4)[3-5] and thymidine phosphorylase (EC 2.4.2.3).[3,4,6] Some mammalian tissues such as rat liver contain both enzymes which can be separated by chromatography on DEAE-Sephadex.[5,7,8] Other tissues such as horse liver,[9] 14 human tumors,[10,11] human leukocytes,[12] human spleen,[10a] mouse liver,[4] and rabbit liver[13] apparently contain only the thymidine phosphorylase (EC 2.4.2.4.). In contrast, rodent tumors[10b] such as Walker 256 carcinoma,[8,10a] Dunning hepatoma,[10a] Novikoff hepatoma,[10a] Morris 5123 hepatoma,[11] Morris 3683 hepatoma,[11] and Butter yellow induced hepatoma[11] in the rat and Ehrlich ascites[11] and Ehrlich Lettre ascites[11] in the mouse apparently contain only the uridine phosphorylase (EC 2.4.2.3).

1-(2-Deoxy-β,D-glucopyranosyl)thymine is a good inhibitor of the uridine phosphorylase,[5,10a] but shows no inhibition of the thymidine phosphorylase;[5] thus the 2 enzymes can be readily differentiated with this inhibitor.[5] The glucosylthymine complexed to the uridine phosphorylase from Ehrlich ascites 20-times better than the substrate, thymidine.[5] 5-Benzyluracil has been reported from this laboratory to be an excellent inhibitor of the uridine phosphorylase from Walker 256 carcinoma, being complexed 80-fold better than the substrate FUDR; introduction of a *m*-EtO or *m*-$C_6H_5CH_2O$ substituent increased the potency to 300- and 800-fold, resp, better than the substrate.[8] These 5-benzyluracils can also be used to distinguish between uridine phosphorylase, which is strongly inhibited, and mammalian thymidine phosphorylase which is not (Table II).

Considerable effort was devoted to develop inhibitors of thymidine phosphorylase from *Escherichia coli* B before it was fully realized that a hydrophobic bonding region adjacent to an active site could have undergone considerable evolutionary change between species;[14-16] these evolutionary differences between species of a hydrophobic region are readily detected by inhibitors bearing appropriate hydrophobic groups and differential binding as large as 10^4 can be observed.[14-16] Systematic studies on the thymidine phosphorylase from

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