# Irreversible Enzyme Inhibitors. CIII. 9-(*p*-Bromoacetamidophenyl)guanine, an Irreversible Inhibitor of Xanthine Oxidase[1,2]

B. R. BAKER AND WILLIAM F. WOOD

*Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106*

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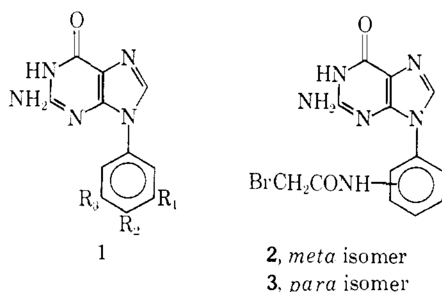
9-(*p*-Bromoacetamidophenyl)guanine (**3**) and its *meta* isomer (**2**) were synthesized from 2-amino-6-chloro-5-phenylazo-4-pyrimidinol (**9**) and the appropriate aminoacetanilide *via* the key intermediate, 9-(*m*- or -*p*-aminophenyl)guanine. The *para* isomer (**3**) at a concentration of $5 \times 10^{-6}$ *M* irreversibly inhibited xanthine oxidase with a half-life of about 40 min at 37°; this inactivation gave kinetic parameters in agreement with the active-site-directed mechanism of irreversible inhibition. In contrast, **3** failed to show inactivation of guanine deaminase at a concentration of $16 \times 10^{-6}$ *M*. The *meta* isomer (**2**) failed to inactivate either guanine deaminase or xanthine oxidase.

In the previous paper of this series a study on the mode of phenyl binding of some 9-phenylguanines to guanine deaminase and xanthine oxidase was reported;[2]




**2**, *meta* isomer
**3**, *para* isomer

earlier we had found that 9-phenylguanine inhibited guanine deaminase and xanthine oxidase 28 and 140 times, respectively, more effectively than 9-methylguanine.[3,4] Strong evidence was presented[2] that the benzene ring of **1** was bound to the enzymes by a hydrophobic interaction. Furthermore, still more enhanced binding to guanine deaminase was obtained with **1** when it was substituted with nonpolar groups at $R_2$, $R_3$, or both; xanthine oxidase differed in that further hydrophobic bonding by nonpolar groups was obtained at $R_3$, and only at $R_2$ when the nonpolar chain was at least three atoms long. Further evidence indicated that $R_1$ was not in a hydrophobic region with either enzyme, but only xanthine oxidase could tolerate a polar electron-rich group at $R_2$. Therefore it was suggested[2] that a leaving group could be placed at either $R_1$ or $R_2$ to convert **1** into a candidate active-site-directed irreversible inhibitor[5,6] of xanthine oxidase, but only at $R_1$ for a candidate irreversible inhibitor of guanine deaminase. The synthesis and enzymic evaluation of the candidate irreversible inhibitors, **2** and **3**, are the subjects of this paper.

**Enzyme Results.**—The data on reversible and irreversible inhibition of xanthine oxidase and guanine deaminase by the candidate irreversible inhibitors, **2** and **3**, are presented in Table I; some appropriate reversible inhibitors are also listed for comparative purposes. The *m*-bromoacetamido derivative (**2**) was a better reversible inhibitor of both enzymes than the *para* isomer (**3**); however, the *meta* isomer (**2**) failed to irreversibly inhibit either enzyme when incubated with the enzyme at a concentration 5–12 times that necessary to give 50% reversible inhibition.

At a concentration of $5 \times 10^{-6}$ *M*, the *para* isomer (**3**) showed nearly complete inactivation of xanthine oxidase in 2 hr at 37°. A time study showed that the half-life of the reaction was about 40 min. Considerable inactivation by **3** at $0.5 \times 10^{-6}$ *M* in 30 min was observed. In contrast, the *para* isomer (**3**) showed no inactivation of guanine deaminase at $16 \times 10^{-6}$ *M*, about the same concentration as needed for 50% reversible inhibition.

Xanthine oxidase showed little inactivation by $5 \times 10^{-6}$ *M* iodoacetamide, indicating that **3** did not inactivate the enzyme by a random bimolecular process.[7] The fact that **3** at $5 \times 10^{-7}$ *M* showed inactivation, but **2** at $4 \times 10^{-7}$ *M* did not, also supports the concept that the inactivation by **3** proceeds through a facile neighboring-group reaction within an enzyme–inhibitor complex, where the leaving group of **3** is juxtaposed to a nucleophilic site on the enzyme, the so-called active-site-directed mechanism of irreversible inhibition.[7]

It has previously been reported from this laboratory[8] that 8-(*m*-bromoacetamidobenzylthio)hypoxanthine (**7**) was an irreversible inhibitor of xanthine oxidase, but that the *ortho* isomer **8** was not. Both **3** and **7** showed a half-life of inactivation of 40–50 min at about twice the concentration necessary for 50% reversible inhibition. Examination of molecular models of **3** and **7** showed that the bromomethyl groups were practically in the same position with respect to the guanine moiety as indicated in conformations **3a** and **7**; it is therefore possible that the same amino acid is attacked by both **3a** and **7**. There are two possible ground-state conformations each for the acetamide functions of **3** and **7** still allowing the orbital overlap between the benzene ring and the amide carbonyl, but only conformations **3a** and **7** have the bromoacetamide positioned in the same area.

(1) This work was supported in part 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 W. F. Wood, *J. Med. Chem.*, **10**, 1101 (1967).

(3) B. R. Baker and D. V. Santi, *ibid.*, **10**, 62 (1967); paper LXXIV of this series.

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

(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.

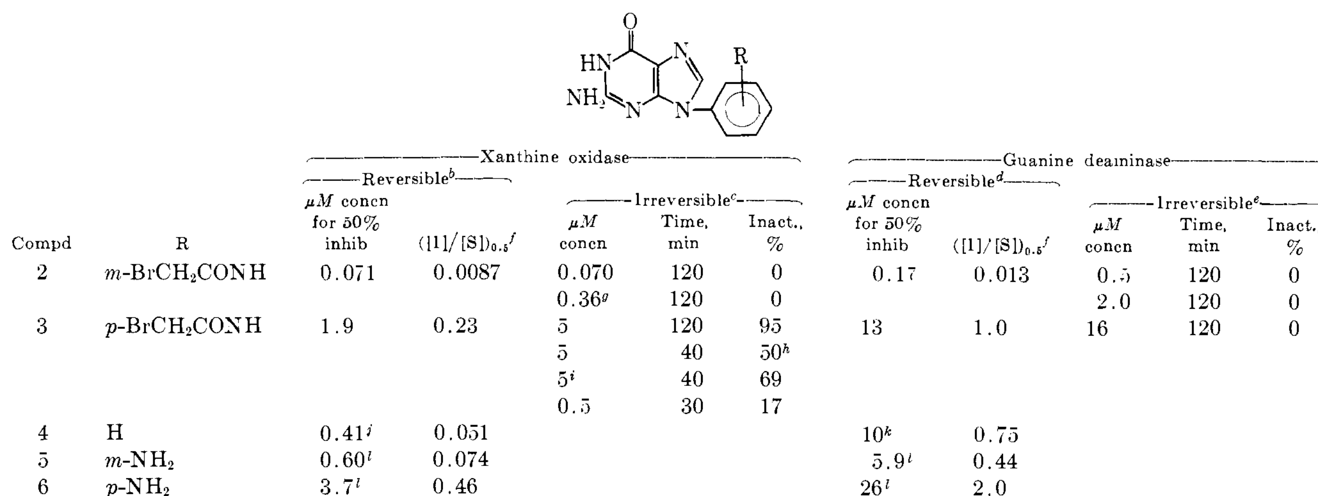
(6) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

(7) For a discussion of the kinetics and types of irreversible inhibition see (a) ref 5, Chapter VIII; (b) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(8) B. R. Baker and J. Kozma, *J. Med. Chem.*, **10**, 682 (1967); paper XCV of this series.

TABLE I

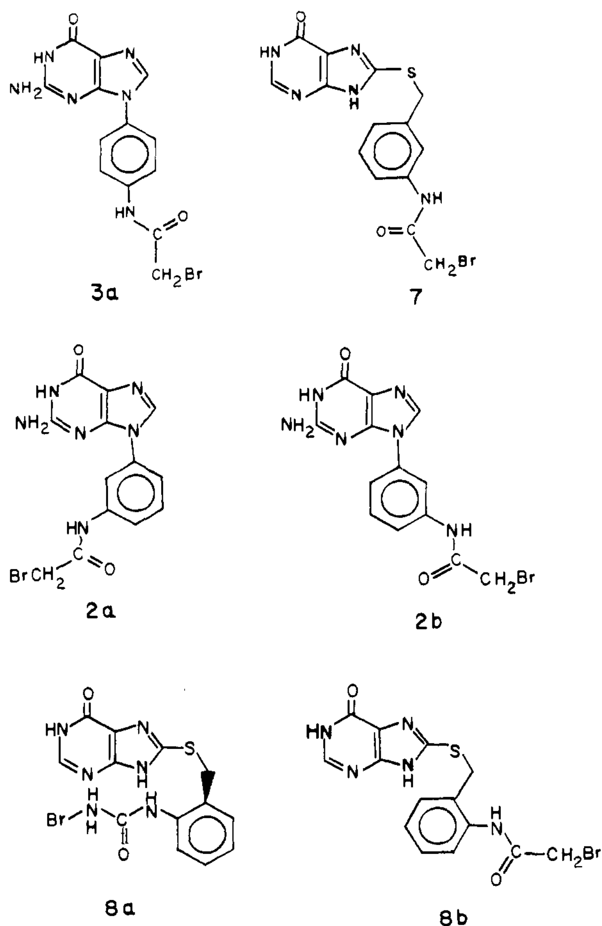
INHIBITION[a] OF XANTHINE OXIDASE AND GUANINE DEAMINASE BY



| | | Xanthine oxidase | | | | | Guanine deaminase | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Reversible[b] | | Irreversible[c] | | | Reversible[d] | | Irreversible[e] | | |
| Compd | R | μM concn for 50% inhib | ([I]/[S])$_{0.5}$[f] | μM concn | Time, min | Inact., % | μM concn for 50% inhib | ([I]/[S])$_{0.5}$[f] | μM concn | Time, min | Inact., % |
| 2 | m-$BrCH_2CONH$ | 0.071 | 0.0087 | 0.070 | 120 | 0 | 0.17 | 0.013 | 0.5 | 120 | 0 |
| | | | | 0.36[g] | 120 | 0 | | | 2.0 | 120 | 0 |
| 3 | p-$BrCH_2CONH$ | 1.9 | 0.23 | 5 | 120 | 95 | 13 | 1.0 | 16 | 120 | 0 |
| | | | | 5 | 40 | 50[h] | | | | | |
| | | | | 5[i] | 40 | 69 | | | | | |
| | | | | 0.5 | 30 | 17 | | | | | |
| 4 | H | 0.41[j] | 0.051 | | | | 10[k] | 0.75 | | | |
| 5 | m-$NH_2$ | 0.60[l] | 0.074 | | | | 5.9[l] | 0.44 | | | |
| 6 | p-$NH_2$ | 3.7[l] | 0.46 | | | | 26[l] | 2.0 | | | |

[a] The technical assistance of Pepper Caseria with these assays is acknowledged. [b] Commercial xanthine oxidase from bovine milk was assayed with 8.1 μM hypoxanthine in Tris buffer (pH 7.4) containing 10% DMSO as previously described in paper XCII of this series: B. R. Baker and J. L. Hendrickson, *J. Pharm. Sci.*, **56**, 955 (1967). [c] Inactivation of xanthine oxidase was performed as previously described[8] in pH 7.4 Tris buffer at 37°. [d] Commercial guanine deaminase from rabbit liver was assayed with 13.3 μM guanine in Tris buffer (pH 7.4) diluted with 10% DMSO as previously described.[13] [e] See Experimental Section. [f] Ratio of concentration of inhibitor to substrate giving 50% inhibition. [g] This same concentration of iodoacetamide was incubated simultaneously in another tube and showed no inactivation. [h] Half-life of reaction study; see Experimental Section. [i] Iodoacetamide at this concentration showed barely perceptible inactivation. [j] Data previously reported.[4] [k] Data previously reported.[3] [l] Data previously reported.[2]

9-(m-Bromoacetamidophenyl)guanine (**2**) has four possible ground state conformations, all of which are planar. The bromoacetamide can project into either the $R_1$ or $R_3$ area of **1**, as in conformations **2a** and **2b**, and in both cases can flip toward the guanine moiety or away from it. With the **2b** conformation it is not unreasonable to expect **2** to irreversibly inhibit the enzyme since the position of the bromomethyl group in **2b** and **3a** are fairly close. Not only does **2** not irreversibly inhibit xanthine oxidase but **2** is a 27-fold better reversible inhibitor than **3**; therefore, the bromoacetamido group of **2** exerts some reversible binding to the enzyme. Since conformation **2b** is unlikely for the reason given above, the **2a** conformation would place the bromomethyl group in an area previously shown[2] to be hydrophobic; thus a 27-fold increment between **2** and **3**, as well as the fivefold increment between **2** and 9-phenylguanine (**4**) (Table I) can be readily accounted for by hydrophobic bonding by the bromomethyl group of **2a**. Furthermore, the actual complexing to the enzyme by the bromoacetamido group of **2** in conformation **2a** would disallow binding in conformation **2b** with an energy difference of at least 1 kcal and possibly as large as 4 kcal/mole.




It was previously observed[8] that the o-bromoacetamido isomer (**8**) of the irreversible inhibitor **7** showed no irreversible inhibition of xanthine oxidase, but **8** was reversibly complexed to the enzyme sevenfold better than **7**. The *ortho* isomer (**8**) has only two allowable ground-state formations for the acetanilide moiety as indicated in **8a** or **8b**; however, in contrast to **2** and **3** which are planar, **8** has free rotation around the $-S-CH_2$ bonds. A near planar conformation like **8a** would explain why the o-bromoacetamido isomer shows sevenfold more reversible binding than the *meta* isomer (**7**); the bromomethyl group in a conformation like **8a** projects into the proposed hydrophobic area[2] which could readily account for this binding difference.

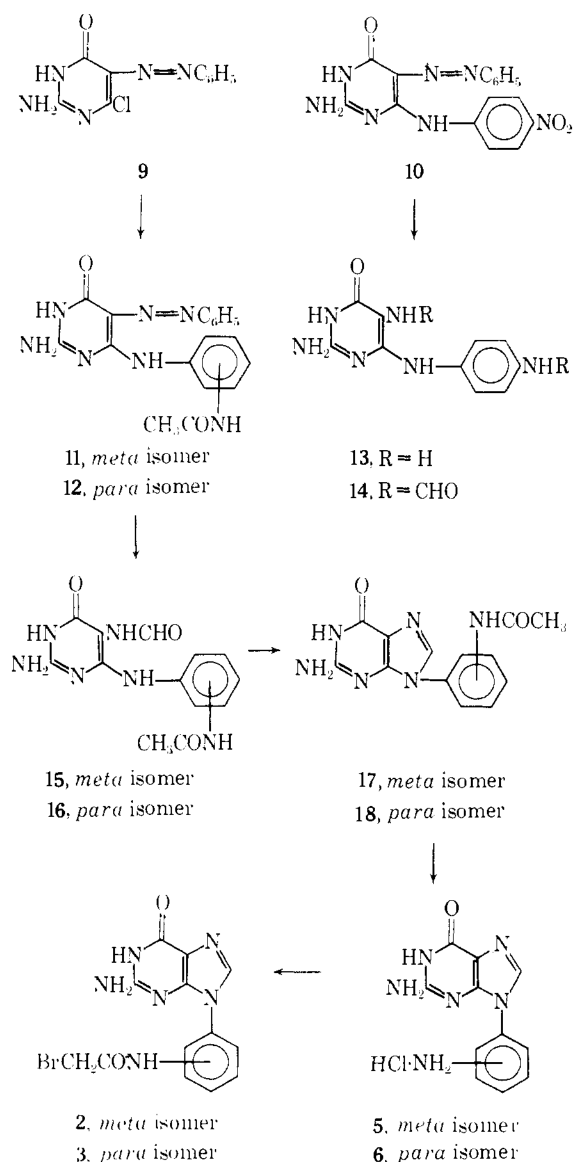
Guanine deaminase also has a hydrophobic bonding region larger than that encompassed by the benzene ring of 9-phenylguanine; this hydrophobic region extends into both the $R_2$ and $R_3$ area of **1**. Thus the 60-fold increment in binding by the m-bromoacetamido group of **2** compared to **4** (Table I) can be accounted for by

hydrophobic bonding of the bromomethyl group in the **2a** conformation. The *p*-bromoacetamido group in conformation **3a** or its alternate ground state conformer is considerably less apt to project into the proposed[2] hydrophobic area.

In order to design active-site-directed irreversible inhibitors of guanine deaminase, it would be desirable to study further the size and conformational requirements of the hydrophobic bonding region; then perhaps more intelligent guesses can be made on proper placement of a leaving group in order to obtain an irreversible inhibitor.

**Chemistry.**—Condensation of the 6-chloropyrimidine (**9**)[9] with *p*-nitroaniline in alcohol proceeded smoothly to **10**. Attempted reduction of **10** to **13** with sodium hydrosulfite in aqueous alkali[10] was unsuccessful since **13** was extremely unstable to air. However, reductive formylation of the nitro and azo linkages of **10** with zinc in formic acid[2,11] proceeded smoothly to **14** which

Scheme I




was stable to air due to the protection by two N-formyl groups (Scheme I). Unfortunately, **14** could not be cyclized[2] to 9-(*p*-formamidophenyl)guanine due to insolubility. The difficulty was solved by using the more soluble acetamidophenyl derivatives.

Condensation of **9** with *p*-aminoacetanilide proceeded smoothly to **12**, which was reductively formylated to **16**, then cyclized to **18** by the previously described general method;[2] since crude **18** was tlc pure, it was directly hydrolyzed with 6 *N* HCl since the insoluble hydrochloride (**6**) separated from solution in pure form in 45% over-all yield from **9**. The *m*-amino isomer **5** was prepared similarly in 28% over-all yield from *m*-aminoacetanilide and **9**.

When a solution of **6** in DMF solubilized with triethylamine was treated with bromoacetic anhydride[12] at 0°, a monobromoacetamide was obtained in 70% yield; that the bromoacetyl group had reacted with *p*-amino group of **6** to give **3** was clearly demonstrated by the negative Bratton-Marshall test for aromatic amine and positive test for active halogen with 4-(*p*-nitrobenzyl)pyridine.[12] Similarly, the *m*-bromoacetamido isomer (**2**) was prepared from **5** in 61% yield.

## Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block; none of the compounds melted below 300°. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B or 337 spectrophotometer; all compounds had infrared spectra in agreement with their assigned structures. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer. Thin layer chromatograms were run on Brinkmann silica gel $GF_{254}$ and spots were detected by visual examination under ultraviolet light.

**2-Amino-5-formamido-6-(*p*-formamidoanilino)-4-pyrimidinol (14).**—A mixture of 2.0 g (8 mmoles) of **9**, 1.1 g (8 mmoles) of *p*-nitroaniline and 25 ml of absolute EtOH was refluxed with stirring for 5 hr during which time the product separated. The red-brown solid was collected by filtration and washed with EtOH until the washings were light yellow: yield of **10**, 1.5 g (54%).

To a solution of the crude **10** in 30 ml of hot 90% formic acid was added zinc dust in portions until the solution bleached in color. The mixture was heated on a steam bath for an additional 30 min, then filtered. The combined filtrate and washings were spin evaporated *in vacuo* to a syrup, then diluted with 30 ml of water. The product was collected on a filter and washed with water. Recrystallization from 1 l. of boiling water gave 1.0 g (82%) of white solid: $\lambda_{max}$ (pH 1), 254, 285 mμ; (pH 13), 261, 286 mμ. No suitable solvent for tlc could be found.

*Anal.* Calcd for $C_{12}H_{12}N_6O_3$: C, 50.0; H, 4.20; N, 29.2. Found: C, 50.1; H, 4.21; N, 29.3.

**9-(*p*-Aminophenyl)guanine Hydrochloride (6).**—Crude **18** was prepared from 1.85 g of **9** and *p*-aminoacetanilide by the general method previously described;[2] the product (**18**) that precipitated when the reaction mixture was diluted with water showed one spot on tlc in 3:5 EtOH–$CHCl_3$. A solution of the crude **18** in 20 ml of 6 *N* HCl was heated on a steam bath for 3 hr during which time the product crystallized from solution. The cooled mixture was filtered and the presumed dihydrochloride of the product was washed with 6 *N* HCl. Recrystallization from 0.1 *N* HCl gave 1.0 g (45% from **9**) of pure monohydrochloride: $\lambda_{max}$ (pH 1), 271 mμ; (pH 13), 252, 266 mμ (weak inflection).

*Anal.* Calcd for $C_{11}H_{10}N_6O \cdot HCl$: C, 47.4; H, 3.98; N, 30.1. Found: C, 47.2; H, 4.24; N, 30.0.

No suitable solvent system for tlc of this hydrochloride salt could be found, but the free base moved as a single spot.

**9-(*m*-Aminophenyl)guanine hydrochloride (5)** was prepared from **9** as described for **6** except that the final product did not separate from 6 *N* HCl solution until cooled; yield 0.70 g (28%); $\lambda_{max}$ (pH 1, 13), 270 mμ.

(9) W. R. Boon and T. Leigh, *J. Chem. Soc.*, 1499 (1951).

(10) H. C. Koppel, D. E. O'Brien, and R. K. Robins, *J. Am. Chem. Soc.*, **81**, 3046 (1959); C. W. Noell and R. K. Robins, *J. Med. Pharm. Chem.*, **5**, 558 (1962).

(11) B. R. Baker, J. P. Joseph, and R. E. Schaub, *J. Org. Chem.*, **19**, 631 (1954).

(12) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocyclic Chem.*, **3**, 425 (1966).

*Anal.* Calcd for $C_{11}H_{10}N_6O\cdot HCl$: C, 47.4; H, 3.98; N, 30.1. Found: C, 47.1; H, 4.21; N, 29.9.

**9-(*p*-Bromoacetamidophenyl)guanine (3).**—A mixture of 64 mg (0.63 mmole) of triethylamine, 5 ml of DMF, and 100 mg (0.32 mmole) of **6**·2HCl was warmed to complete solution, then cooled to 0° in an ice bath. To the stirred solution was added 125 mg (0.58 mmole) of bromoacetic anhydride. After 30 min in the ice bath, the reaction mixture was poured into 25 ml of $H_2O$ containing 53 mg of $NaHCO_3$. The crude product was collected on a filter and washed with water. A trace of the Bratton–Marshall-positive[12] **6** was removed by stirring the crude product in 0.1 *N* HCl for 30 min. The product was collected by filtration and washed with water. The compound now gave a negative Bratton–Marshall test for aromatic amine,[12] moved as a single spot on tlc with 5:3 $CHCl_3$–EtOH, and gave a positive 4-(*p*-nitrobenzyl)pyridine test for active halogen;[12] $\lambda_{max}$ (pH 1), 261 mμ; (pH 13), 271 mμ.

*Anal.* Calcd for $C_{13}H_{11}BrN_6O_2$: C, 43.0; H, 3.05; N, 23.1. Found: C, 43.0; H, 32.7; N, 22.9.

Similarly, the *meta* isomer (**2**) was prepared in 61% yield; it had the same properties as **3** except for $\lambda_{max}$ (pH 1), 254, 280 mμ (weak inflection); (pH 13), 268 mμ.

*Anal.* Found: C, 42.8; H, 3.34; N, 22.9.

**Inactivation of Guanine Deaminase.**—Guanine deaminase (guanase) from rabbit liver was purchased from Sigma Chemical Co. as a 1-mg/ml suspension; at this concentration it was reputed to deaminate 0.1 μmole of guanine/min. The inactivation experiments were performed as follows. The velocity of the enzyme reaction with 13.3 μ*M* guanine[13] was proportional to the enzyme concentration. The buffer employed was 0.05 *M* Tris (pH 7.4). The enzyme was stable at 37° for 2 hr. Bulk enzyme (1 mg/ml) (0.10 ml) as purchased was diluted with 1.90 ml of buffer. In two tubes were placed 0.95 ml of the diluted enzyme in a 37° bath. After 5 min, 50 μl of DMSO was added to tube 1 (enzyme control) and 50 μl of DMSO containing inhibitor was added to tube 2. The contents were mixed, the time was noted and an 0.5-ml aliquot was withdrawn from each tube as rapidly as possible and stored at 0° until ready for assay. The aliquot from the inhibitor tube was labeled $I_1$ and the aliquot from the enzyme control tube was labeled $C_1$. The remainder in the two tubes was then kept for 2 hr (or other chosen time) at 37°, then cooled in an ice bath until ready for assay and labeled $I_2$ and $C_2$. The amount of enzyme remaining was assayed as follows:

In a 1-ml cuvette was placed 0.70 ml of buffer and 200 μl of 66.7 μ*M* guanine in 70 μ*M* NaOH.[13] The enzyme reaction was then started by addition of 100 μl of $C_1$ (or other aliquot). The decrease in optical density at 245 mμ was followed with a Gilford 2000 recording spectrophotometer; the $C_1$ aliquot usually gave an OD change of about 0.008 unit/min. The velocities in OD/min were plotted on a log scale against time on a linear scale.[7b] This procedure is adequate for a routine screen for a plus or minus answer on irreversible inhibition. As many as three inhibitor tubes can be run with one enzyme control in 1 day.

With a positive compound, a larger amount of inhibitor–enzyme mixture can be set up, and then a number of aliquots can be removed at varying times in order to obtain the half-life of irreversible inhibition.

(13) B. R. Baker, *J. Med. Chem.*, **10**, 59 (1967); paper LXXIII of this series.

# Irreversible Enzyme Inhibitors. CIV. Inhibitors of Thymidine Phosphorylase. VIII. Further Studies on Hydrophobic Bonding with 6-Substituted Uracils[1,2]

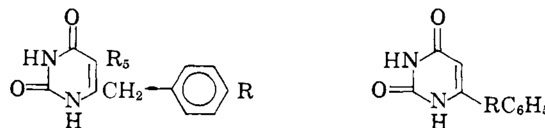
B. R. BAKER AND WACLAW RZESZOTARSKI[3]

*Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106*

*Received June 16, 1967*

6-Benzyluracil has been previously reported to be a good reversible inhibitor of thymidine phosphorylase; due to a hydrocarbon interaction of the benzene ring with the enzyme, this compound complexes to the enzyme about five times better than the substrate, 2′-deoxy-5-fluorouridine. Other bridges between the uracil and phenyl moieties are more easily synthesized than the methylene bridge of 6-benzyluracil and have now been shown also to have phenyl binding. 6-Anilinouracil, 6-phenoxyuracil, 6-phenylthiouracil, and 6-benzylaminouracil complex to the enzyme 10, 17, 100, and 65-fold better, respectively, than the substrate. In contrast, 6-benzoyluracil with its relatively fixed coplanar structure is a poorer inhibitor than 6-benzyluracil; the poor binding by 6-benzoyluracil compared to the other inhibitors suggests a likely optimal binding conformation for the inhibitors where the phenyl group is out-of-plane with the pyrimidine ring and approaches the 5 position of the pyrimidine in space.

Previous papers in this series have revealed that (a) 6-benzyluracil (**1**) is a good reversible inhibitor of thymidine phosphorylase due to a hydrophobic interaction between the benzyl group and the enzyme,[4] (b) the inhibition of **1** can be enhanced by introduction of a 5-bromine atom (**2**), which increases the acidity of the uracil,[5] and (c) 6-(*p*-bromoacetamidobenzyl)uracil (**3**) is an active-site-directed irreversible inhibitor,[6] though slow acting with a half-life of about 2–3 hr.[2b] These results posed the following questions. Can additional



**1**, R = H, R₅ = H
**2**, R = Br, R₅ = H
**3**, R = H, R₅ = BrCH₂CONH-

hydrophobic bonding be detected by appropriate substituents on the benzene ring? Can faster active-site-directed irreversible inhibitors with a half-life of 10 min or less[7] be synthesized by varying the position or elec-

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

(2) (a) For the previous paper in this series, see B. R. Baker and W. F. Wood, *J. Med. Chem.*, **10**, 1106 (1967); (b) for the previous paper on thymidine phosphorylase see B. R. Baker and M. Kawazu, *J. Pharm. Sci.*, in press; paper C of this series.

(3) On leave from the Department of Organic Chemistry, Pedagogical College, Opole, Poland.

(4) B. R. Baker and M. Kawazu, *J. Med. Chem.*, **10**, 311 (1967); paper LXXVIII of this series.

(5) B. R. Baker and M. Kawazu, *ibid.*, **10**, 316 (1967); paper LXXX of this series.

(6) 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.

(7) For the kinetic parameters of active-site-directed irreversible inhibition see ref 6, Chapter VIII.