# Irreversible Enzyme Inhibitors LXXI

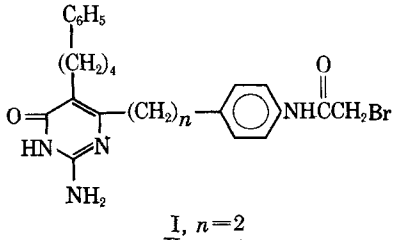
## Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase V. Derivatives of 6-Phenylpyrimidine

By B. R. BAKER and HOWARD S. SHAPIRO

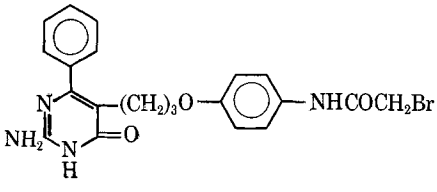
A series of 2-amino-5-anilinopropyl-6-phenyl-4-pyrimidinols bearing *p*-chloroacetyl (V*a*), *p*-(4-chloro-3-butanon-1-yl) (V*b*), *p*-(4-chloro-1-buten-3-one-1-yl) (V*c*), and *p*-bromoacetamidoethyl (IV*d*) groups on the anilino moiety were synthesized as candidate active-site-directed irreversible inhibitors of dihydrofolic reductase. Only V*b* inactivated dihydrofolic reductase when they were incubated together at 37°; the half-life was about 60 min. when sufficient V*b* was utilized to convert 45 per cent of the enzyme to a reversible complex. That this inactivation by V*b* proceeded through a reversible enzyme–inhibitor complex and not by a random bimolecular mechanism was indicated by the lack of inactivation of dihydrofolic reductase by chloroacetone at 2.5 times the concentration of V*b*. The failure of IV*d* and V*e* to inactivate the enzyme at a concentration sufficient to convert 50–60 per cent of the enzyme to the respective reversible complexes indicated that the alkylating function of IV*d* and V*e* could not bridge to a nucleophilic site on the enzyme within the enzyme–inhibitor complex.

IN ORDER for a compound to be an active-site-directed irreversible enzyme inhibitor the following requirements must be met (1, 2): (*a*) a reversible complex between the compound and the enzyme must form; (*b*) the proper positioning of a leaving group on the inhibitor must be attained that does not interfere with reversible complex formation; (*c*) the leaving group on the inhibitor—when complexed to the enzyme—must be able to bridge to a nucleophilic group on the enzyme surface with resultant covalent bond formation *via* a neighboring group reaction.

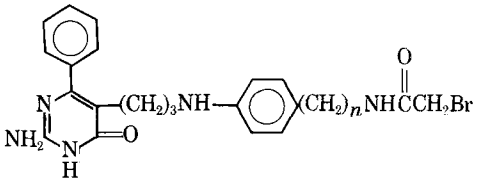
An unusual amount of difficulty was encountered for the successful design of an active-site-directed irreversible inhibitor of dihydrofolic reductase; although studies on requirements (*a*) and (*b*) appeared to follow the normal course of the *modus operandi* for the design of these irreversible inhibitors (1, 2), factor (*c*) was particularly difficult until the strong hydrophobic bonding region of dihydrofolic reductase was discovered (3–8) and utilized properly (9, 10).



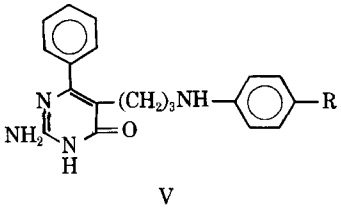
I, $n=2$
II, $n=4$



III



IV
series *d*, $n=2$



V

*a*, R = —$COCH_2Cl$
*b*, R = —$(CH_2)_2COCH_2Cl$
*c*, R = —$CH=CH-COCH_2Cl$

### DISCUSSION

Successful candidates for active-site-directed irreversible inhibitors were achieved by complexing the hydrophobic region of the enzyme by the 5-phenylbutyl group of I and II (9) or the 6-phenyl group of III (10) which then positioned their bromoacetamido groups in a nucleophilic area of the enzyme. In order for I–III to irreversibly

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Previous paper: Baker, B. R., and Ho B.-T., *J. Pharm. Sci.*, **56**, 28(1967).

inhibit dihydrofolic reductase, it was apparent that the leaving bromo group was properly juxtaposed to a nucleophilic site on the enzyme, thus forming a covalent bond within the enzyme–inhibitor complex. That this bridge distance (1, 2, 11, 12) between the binding locus for the 6-phenylpyrimidine and the leaving group on the inhibitor was an important factor in active-site-directed irreversible inhibition of dihydrofolic reductase was noted with compounds of types IV and V; three of the four compounds of type IV and V were at least as good reversible inhibitors as III, but only one inactivated the enzyme *via* a neighboring group reaction within the enzyme–inhibitor complex. Of the compounds in series IV, only the synthesis of $n = 2$ was completed—for reasons that will be discussed later. Three compounds of series V were synthesized.

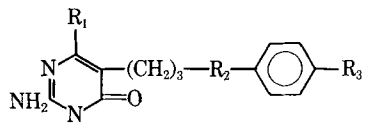
The enzymic evaluation of these compounds are summarized in Table I. Compounds IV*d* and V*c*, at a concentration sufficient to convert 50–60% of the enzyme to a reversible complex, failed to show inactivation of dihydrofolic reductase in 60 min. at 37°; in contrast, III showed 50% inactivation in 45 min. at 37° with sufficient inhibitor to convert only 25% of the enzyme to the reversible complex (10). The chloromethyl ketones have about the same chemical reactivity toward thiosulfate as bromoacetamides and iodoacetamides (14); furthermore, the chloromethyl ketones can attack the same nucleophilic groups on a protein that are attacked by bromoacetamides. Therefore, the failure of IV*d* and V*c* to inactivate the enzyme is most probably due to the lack of juxtaposition of their respective leaving group with an enzymic nucleophilic group within the enzyme–inhibitor reversible complex. Stated another way, the respective leaving group of the inhibitors apparently cannot bridge to an enzymic nucleophilic group within the reversible complex (1, 2, 11, 12, 18).

Due to lack of solubility, the amount of reversible inhibition by the chloroketone (V*a*) could not be determined; nevertheless, V*a* was investigated at its limit of solubility (20 $\mu M$) as an irreversible inhibitor, but with negative results that could be due to either a lack of reversible complex formation or a lack of proper bridging.

Only one of the 6-phenyl-4-pyrimidinols in Table I showed inactivation of the enzyme in addition to the previously examined III (10); the chlorobutanone derivative (V*b*), when incubated at 37° with dihydrofolic reductase at a concentration to convert 45% of the enzyme to the reversible complex, showed inactivation of the enzyme with a half-life of about 60 min. This result was first attributed to a random bimolecular inactivation of the enzyme without complex formation (2, 16), since 1-chloro-4-phenyl-3-*p*-tolylsulfonamido-2-butanone (VII, Table I) (19, 20) showed no reversible inhibition of the enzyme, but could inactivate the enzyme even more rapidly than V*b*. The fallacy of this experiment became apparent when the lack of reversible inhibition by VII ($K_i > 13 \times 10^{-5} M$) was converted to the maximum amount of reversible complex that could be formed at a concentration of 40 $\mu M$; note that as much as 25% of the enzyme could have been reversibly complexed with VII.

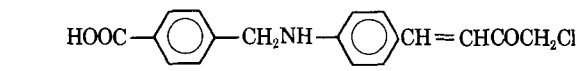
In a later study on inhibitors that might complex only with the hydrophobic region of dihydrofolic reductase then bridge to and alkylate a nucleophilic group on the enzyme, the benzoic acid derivative

TABLE I.—REVERSIBLE AND IRREVERSIBLE INHIBITION OF DIHYDROFOLIC REDUCTASE BY



| Compd. | $R_1$ | $R_2$ | $R_3$ | Reversible[a] $[I/S]_{0.5}$[c] | Reversible[a] $K_i \times 10^5$ $M$[d] | Irreversible[b] $\mu M$ Concn. | Irreversible[b] $EI$, %[e] | Irreversible[b] Time, min. | Irreversible[b] Inactivation, %[f] |
|---|---|---|---|---|---|---|---|---|---|
| III[g] | $C_6H_5$ | O | $-NHCOCH_2Br$ | 130 | 13 | 40 | 25 | 45 | 50 |
| IV*d* | $C_6H_5$ | NH | $-(CH_2)_2NHCOCH_2Br$ | 20 | 2.0 | 30 | 60 | 120 | 0 |
| V*a* | $C_6H_5$ | NH | $-COCH_2Cl$ | >13 | >1.3 | 20 | <60 | 60 | 0 |
| V*c* | $C_6H_5$ | NH | $-CH=CHCOCH_2Cl$ | 200[h] | 20 | 200 | 50 | 60 | 0 |
| V*b* | $C_6H_5$ | NH | $-(CH_2)_2COCH_2Cl$ | 50 | 5.0 | 40 | 45 | 60 | 50 |
| VI | $CH_3$ | NH | $-(CH_2)_2COCH_2Cl$ | 20[i] | 2.0 | 50 | 71 | 120 | <10 |
| VII | $C_6H_5CH_2CHCOCH_2Cl$ | | | >130[j] | >13 | 40 | <25 | 35 | 50 |
| | | | $NHSO_2C_6H_4CH_3-p$ | | | | | | |
| VIII | Chloroacetone | | | | | 100 | | 120 | 0 |

[a] Dihydrofolic reductase was a 45–90% ammonium sulfate fraction isolated from pigeon liver; the reversible inhibition studies were run with 6 $\mu M$ dihydrofolate and 12 $\mu M$ TPNH in 0.05 $M$ Tris buffer (pH 7.4) containing 10 m$M$ mercaptoethanol and 10% $N,N$-dimethylformamide as previously described (13). [b] The dihydrofolic reductase was incubated at 37° with the candidate irreversible inhibitor in 0.05 $M$ Tris buffer (pH 7.4) containing *no* mercaptoethanol and *no* TPNH, but diluted with 10% $N,N$-dimethylformamide as previously described (14). [c] Ratio of concentration of inhibitor to 6 $\mu M$ dihydrofolate giving 50% inhibition. [d] Calculated from $K_i = K_m \times [I/S]_{0.5}$ where $K_m = 1 \times 10^{-6} M$; this equation is valid if $S > 4\ K_m$ (3, 15). [e] Calculated from $[EI] = \frac{[E_t]}{1 + K_i + I}$, where $[EI]$ = the fraction of $[E_t]$ in a reversible enzyme–inhibitor complex and $E_t$ = the total amount of active enzyme (2, 16); the rate of active-site-directed inactivation is dependent upon the fraction of $[E_t]$ in the complex, $[EI]$ (2, 16). [f] In all cases an enzyme control with no inhibitor was run as a control and either II or III was run as a standard simultaneously; 0–4% thermal inactivation of the enzyme control occurred and the per cent inactivation by the inhibitor was corrected for thermal inactivation. [g] Data from *Reference 10*. [h] Estimated from 27% inhibition at 0.4 m$M$, the maximum concentration allowing full light transmission. [i] Data from *Reference 17*. [j] No inhibition was observed at 0.2 m$M$, the maximum solubility; since 20% inhibition is readily detectable, the concentration for 50% inhibition is >0.8 m$M$ (13).



IX

(IX) was investigated (21); at a concentration of IX sufficient to reversibly complex 50% of the dihydrofolic reductase, IX inactivated the enzyme, but some related compounds did not inactivate the enzyme. Therefore, chloroacetone was investigated for inactivation; at a concentration of 100 $\mu M$, chloroacetone showed no inactivation of dihydrofolic reductase in 120 min. at 37°, in contrast to V*b* and VII which showed 50% inactivation at 40 $\mu M$ in 50 and 35 min., respectively. If either had inactivated the enzyme by the bimolecular mechanism, then chloroacetone should have also; *ergo*, neither V*b* nor VII inactivate by the bimolecular mechanism, but presumably inactivate through a complex. For example, if the $K_i$ of the tosylamido ketone (VII) were $20 \times 10^{-5}$ $M$—which is greater than $13 \times 10^{-5}$ $M$ [Table I (VII)]—the amount of reversible complex with 40 $\mu M$ of VII would be 16% of the total enzyme; this could still give a reasonable rate of inactivation *via* the reversible complex.

Although the $K_i$ of VII could not be determined due to its lack of solubility, it should be possible to determine $K_i$ by observing the effect of concentration of inhibitor on the rate of inactivation (2, 22); as a first approximation, one should also see a "rate-saturation" effect (2, 16) if the inactivation proceeds through a complex. If the tosylamido ketone (VII) is weakly complexed to dihydrofolic reductase, it is most likely through hydrophobic bonding as seen with IX. Even though such studies with VII could be of scientific interest, they were off the main stream of the research goals of this laboratory (1, 2), and were, therefore, not pursued further. Thus, the main evidence that the chlorobutanone (V*b*) inactivates dihydrofolic reductase by the active-site-directed mechanism (1, 2) is the fact that chloroacetone at over twice the concentration of V*b* shows no inactivation. Furthermore, the corresponding chlorobutanone attached to a 6-methylpyrimidinol (VI) (17) shows barely detectable inactivation at a concentration sufficient to convert 71% of the enzyme to a reversible complex. This result further supports the concept (2, 8–10) that the 6-phenyl group of V*b* is complexed to the hydrophobic region of dihydrofolic reductase which then projects the 5-side-chain bearing the alkylating function into a polar nucleophilic region of the enzyme; in contrast, the 6-methylpyrimidine (VI) probably has its 5-side-chain bearing the alkylating function complexed to the hydrophobic region of the enzyme which is not apt to have polar nucleophilic groups present. The results clearly eliminate the possibility of a random bimolecular mechanism (1, 2) for inactivation and give strong support to the concept that 6-phenylpyrimidines and 6-methylpyrimidines complex to the enzyme in different rotomeric configurations (2, 8, 23)—else both V*b* and VI should have inactivated the enzyme at the same rate for the same percentage of reversible E···I complex.

Similar to the effect of the phenylbutyl pyrimidine (II) as an irreversible inhibitor (9), the rate of inactivation by the chlorobutanone (V*b*) and the tosylamidoketone (VII) was slowed by the presence of TPNH; whether or not I, II, III, V*b*, and VII all attack the same amino acid—presumably in the TPNH binding region of the enzyme—must await sequence experiments after inactivation of pure enzyme, a problem for specialists in protein sequence research.

## CHEMISTRY

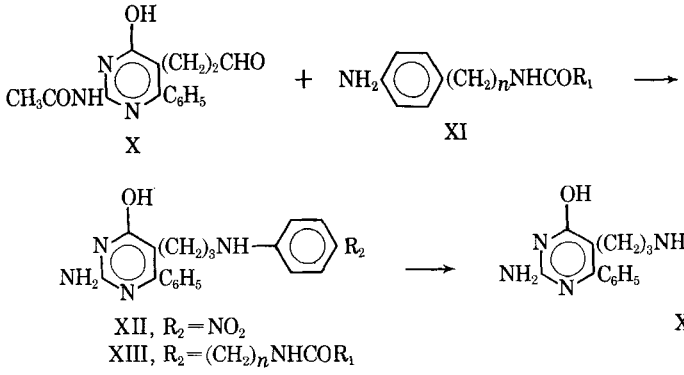
**Methods.**—Two general routes to 2-amino-6-R-4-pyrimidinols bearing a *p*-substituted anilinopropyl side chain at the 5-position such as IV and V have been developed (24–29). The first route involved reductive condensation of a substituted aniline with a pyrimidine-5-propionaldehyde such as X where the substituted anilino moiety is introduced last (24–26); the second route introduced the substituted anilinopropyl group at the first step (27–29). The first route was chosen for compounds such as IV and V since it is superior if a number of *p*-substituted anilino moieties are required.

Reductive condensation of X with *p*-aminoacetanilide with sodium borohydride in a mixture of *N,N*-dimethylformamide and methanol gave XIII*a* in 21% yield of what appeared to be pure material; although XIII*a* had the proper spectral characteristics and was uniform on thin-layer chromatography, it gave erratic results on combustion analyses. Furthermore, attempts to acid hydrolyze XIII*a* to XIV*a* gave intractable, unstable materials that could not be purified due in part to the air sensitivity of the *p*-phenylenediamine moiety. The alternate route (27–29) was not investigated since catalytic reduction of XII or its 6-methyl analog (27) could also lead to unstable, intractable materials. This problem was ultimately solved by using the more stable 5-(*p*-aminophenoxypropyl) side chain (30) which subsequently resulted in the synthesis and enzymic evaluation of the active-site-directed irreversible inhibitor (III) (10).

The higher homologs of XIII having more parafinic character proved to be easier to purify and gave satisfactory combustion results. Reductive condensation of X with *N*-(*p*-aminobenzyl) acetamide (XI*b*) (32) using the sodium borohydride procedure gave a 55% yield of XIII*b*. Unfortunately, attempts to remove the *N*-acetyl blocking group of XIII*b* with acid or base under a variety of conditions failed to give any isolatable *p*-aminomethylanilinopropyl pyrimidinol (XIV*b*); the *p*-aminomethylanilino system was unstable to such extended treatment and appeared to undergo a reverse Mannich reaction with formation of ammonia (or ammonium chloride) and formaldehyde which led to polymerization (Scheme I).

Reductive condensation of benzyl *N*-(*p*-aminobenzyl)urethan (XI*c*) with X using sodium borohydride in methanol did proceed, but the carbobenzoxy group of XIII*c* was transesterified to carbomethoxy; whether such a condensation could be performed in neutral or slightly acidic medium to avoid transesterification was not ascertained. An intermediate such as XIII*c* could well lead to the desired XIV*c* by cleavage of the carbobenzoxy group with hydrogen bromide in acetic acid.[1]

[1] By this time the synthesis and enzymic evaluation of III had led to an active-site-directed irreversible inhibitor (10); therefore, this sequence was not pursued further.



X XI

XII, $R_2 = NO_2$
XIII, $R_2 = (CH_2)_nNHCOR_1$

XIV

IV

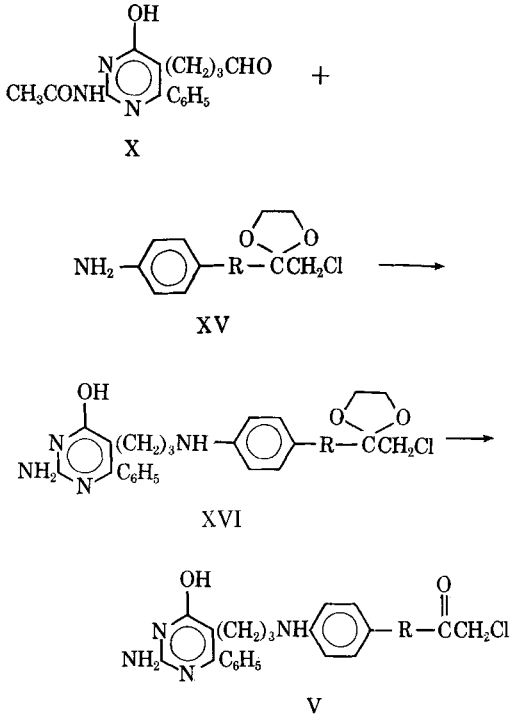
series *a*, $n=0$, $R_1=CH_3$
*b*, $n=1$, $R_1=CH_3$
*c*, $n=1$, $R_1=OCH_2C_6H_5$
*d*, $n=2$, $R_1=CH_3$
*e*, $n=4$, $R_1=C_6H_5$

Scheme I

The reductive condensation of *N* - (*p* - aminophenethyl)acetamide (XI*d*) (32) with X using sodium borohydride proceeded smoothly to an 83% yield of XIII*d*. Hydrolysis of XIII*d* with hot 10% sodium hydroxide gave a 70% yield of the *p*-aminoethylanilinopropyl-4-pyrimidinol (XIV*d*), isolated as its monohydrochloride. The bromoacetylation of XIV*d* to the candidate irreversible inhibitor (IV*d*) with bromoacetic anhydride has been described previously in a paper on selective methods for bromoacylation of polyfunctional amides (31).

Reductive condensation of *N*-(*p*-aminophenylbutyl)benzamide (XI*e*) (33) with X also proceeded satisfactorily, an 85% yield of XIII*e* being obtained.[1]

Three 6-phenylpyrimidinols (V) bearing a chloromethyl ketone group in the *p*-position of 5-anilinopropyl moiety were synthesized by the route used for the corresponding 6-methyl pyrimidines (17). Condensation of the appropriate *p*-substituted aniline bearing a *p*-chloromethylketone function blocked as a dioxolane (XV) were used to reductively aminate the pyrimidine-5-propionaldehyde (X); the blocked derivatives (XVI) were obtained in 47–80% yields. Hydrolysis of the dioxolane with hot 0.25 *N* 25% aqueous alcoholic hydrochloric acid gave the candidate active-site-directed irreversible inhibitors V*a–c* in 49–76% yields of pure material (Scheme II).



X

XV

XVI

V

series *a*, R = —
*b*, R = —$(CH_2)_2$—
*c*, R = —CH=CH—

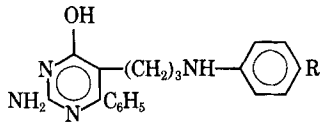
Scheme II

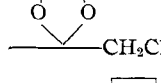
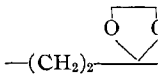
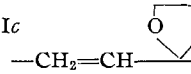
## EXPERIMENTAL

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in a KBr pellet with a Perkin-Elmer 137B recording spectrophotometer. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 recording spectrophotometer. Thin - layer chromatograms (TLC) were run on Brinkmann Silica Gel GF and spots were detected by visual examination under ultraviolet light.

**2 - Amino - 5 - (p - acetamidoethylanilinopropyl) - 6 - phenyl - 4 - pyrimidinol (XIIId).**—*Method A*.—A solution of 100 mg. (0.351 mmole) of X (25) and 264 mg. (1.40 mmoles) of XI*d* (32) in 1 ml. of *N*,*N*-dimethylformamide was magnetically stirred for 20 min., then diluted with 10 ml. of methanol and treated portionwise with 150 mg. of sodium borohydride over a period of about 15 min. (24–26). After being stirred for about 18 hr., the mixture was spin evaporated *in vacuo* to near dryness. The residue was dissolved in 10 ml.

TABLE II.—PHYSICAL AND ANALYTICAL DATA OF



| Compd. | R | Method | M.p., °C. | Yield, %[b] | Anal. Calcd. | Anal. Found | Ultraviolet Spectra[a] $\lambda_{max.}$ (mμ) | $\epsilon \times 10^{-3}$ | pH |
|---|---|---|---|---|---|---|---|---|---|
| XIII*a* | $NHCOCH_3$ | *A*[c] | >275 | 21[d-f] | | | 239 | 24.5 | 1 |
| | | | | | | | 275 | 11.7 | 1 |
| | | | | | N, 18.6[e] | N, 18.5 | 267 | 17.8 | 13 |
| XIII*b* | $-CH_2NHCOCH_3$ | *A*[g] | 243–246 | 55[h] | C, 67.5 | C, 67.7 | 276 | 10.3 | 1 |
| | | | | | H, 6.39 | H, 6.50 | 238[i] | 21.8 | 13 |
| | | | | | N, 17.9 | N, 17.9 | 289 | 8.3 | 13 |
| XIII*d* | $-(CH_2)_2NHCOCH_3$ | *A*[j] | 243–246 | 83[h] | C, 68.2 | C, 68.0 | 277 | 8.5 | 1 |
| | | | | | H, 6.67 | H, 6.62 | 293 | 6.8 | 13 |
| | | | | | N, 17.3 | N, 17.5 | | | 1 |
| XIII*e* | $-(CH_2)_4NHCOC_6H_5$ | *A*[g] | 223–226 | 85[h] | C, 72.7 | C, 72.6 | 275 | 8.5 | 1 |
| | | | | | H, 6.72 | H, 6.59 | 292 | 7.6 | 13 |
| | | | | | N, 14.1 | N, 14.2 | | | |
| XIV*d* | $-(CH_2)_2NH_2 \cdot HCl$[l] | *B*[m] | 285–288 dec. | 70[d] | C, 63.1 | C, 63.0 | | | |
| | | | | | H, 6.51 | H, 6.61 | | | |
| | | | | | N, 17.5[k] | N, 17.4 | | | |
| XVI*a* |  | *A*[g] | 189–191 | 51[h] | C, 62.6 | C, 62.3 | 280 | 13.7 | 1 |
| | | | | | H, 5.67 | H, 5.75 | 250 | 21.0 | 13 |
| | | | | | N, 12.7 | N, 12.2 | 285[i] | 8.7 | 13 |
| XVI*b* |  | *A*[g] | 222–224 dec. | 80[n] | C, 61.7[o] | C, 62.0 | 275 | 10.1 | 1 |
| | | | | | H, 6.37 | H, 6.20 | 290 | 10.1 | 13 |
| | | | | | N, 11.5 | N, 11.4 | | | |
| XVI*c* |  | *A*[g] | 218–221 dec. | 47[d] | C, 63.1[p] | C, 63.0 | 257 | 21.1 | 1 |
| | | | | | H, 5.88 | H, 5.51 | 280[i] | 14.8 | 1 |
| | | | | | N, 11.7 | N, 11.4 | 291 | 24.7 | 13 |
| V*a* | $-COCH_2Cl$ | *C* | 220 dec. | 76[d] | C, 63.6 | C, 63.8 | 282 | 19.6 | 0 |
| | | | | | H, 5.29 | H, 5.61 | 295 | 11.1 | 13 |
| | | | | | O, 8.07[q] | O, 8.10 | 352 | 23.8 | 13 |
| V*b* | $-(CH_2)_2COCH_2Cl$ | *C* | 201–203 dec. | 49[d] | C, 65.0 | C, 65.3 | 280 | 9.5 | 1 |
| | | | | | H, 5.89 | H, 5.83 | 245 | 19.0 | 13 |
| | | | | | O, 7.54[q] | O, 7.79[q] | 293 | 9.1 | 13 |
| V*c* | $-CH{=}CHCOCH_2Cl$ | *C* | 220–225 dec. | 60[h] | C, 65.3 | C, 65.3 | 285 | 21.6 | 1 |
| | | | | | H, 5.44 | H, 5.78 | 287 | 11.4 | 13 |
| | | | | | O, 7.57[q] | O, 7.76[q] | 412 | 16.5 | 13 |

[a] In 10% ethanol. [b] Yield of analytically pure material; all compounds had infrared spectra compatible with their structures. [c] Ratio of XI:X = 5. [d] After recrystallization from aqueous 2-methoxyethanol. [e] Erratic CH values were obtained presumably due to difficulty in combustion. [f] The compound moved as a single spot on TLC in 3:1 benzene–methanol. [g] Ratio of XI:X = 1. [h] After recrystallization from aqueous ethanol. [i] Inflection. [j] Ratio of XI:X = 4. [k] Cl, Calcd.: 8.89. Found: 8.75. [l] For conversion to IV*d* see *Reference 31*. [m] See under *Experimental*. [n] Crystallized directly from reaction mixture; collected and washed with methanol. [o] Calcd. for monohydrate. [p] Calcd. for hemihydrate. [q] Oxygen, the nitrogen could not be completely combusted.

of water, then adjusted to about pH 8 with 3 *N* aqueous hydrochloric acid. After being cooled for several hours at 0°, the mixture was filtered and the white solid was washed with water. Recrystallization from aqueous ethanol gave 118 mg. (83%) of white crystals, m.p. 243–246°; $\lambda_{max.}$ 2.89, 3.00 (NH); 6.05, 6.29, 6.60 (NH, C=C, C=N, C=O); 12.25 (*p*-$C_6H_4$); 13.20, 14.3 μ ($C_6H_5$). For analytical data see Table II.

**2 - Amino - 5 - (p - aminoethylanilinopropyl)-6 - phenyl - 4 - pyrimidinol Hydrochloride (XIVd).**—*Method B.*—A solution of 1.00 Gm. (2.50 mmoles) of XIII*d* in 25 ml. of 10% aqueous sodium hydroxide was heated on a steam bath for 3 hr. The hot solution was clarified by filtration, then cooled in an ice-bath and adjusted to about pH 7 with 3 *N* hydrochloric acid. The white precipitate was collected on a filter, washed with water, then recrystallized from aqueous 2-methoxyethanol; yield, 0.636 Gm. (70%) of white crystals, m.p. 285–288° dec. $\lambda_{max.}$ 2.96 (NH); 6.01, 6.10, 6.30, 6.60 (NH, C=C, C=N, C=O); 12.24 (*p*-$C_6H_4$); 14.33 μ ($C_6H_5$). See Table II for analytical data.

**2 - Amino - 5 - [p - (4 - chloro - 3 - oxo - 1 - buten-1 - yl)anilinopropyl] - 6 - phenyl - 4 - pyrimidinol (Vc).**—*Method C.*—A solution of 200 mg. (0.428 mmole) of XVI*c* in 16 ml. of ethanol and 5.5 ml. of 1 *N* aqueous hydrochloric acid was refluxed for 1 hr., then diluted with 21 ml. of water. While being kept below 10° in an ice-bath, the stirred solution was made slightly alkaline by the dropwise addition of 10% aqueous sodium hydroxide. The product was collected on a filter and washed with water; yield, 162 mg. (90%) of orange solid, m.p. 220–225° dec. Recrystallization from warm ethanol by addition of water gave 108 mg. (62%) of orange crystals of unchanged m.p. $\lambda_{max.}$ 2.90 2.98 (NH); 6.05, 6.10, 6.30, 6.40, 6.59 (NH, C=O, C=C, C=N); 12.33 (*p*-$C_6H_4$); 14.3 μ ($C_6H_5$). See Table II for analytical data.

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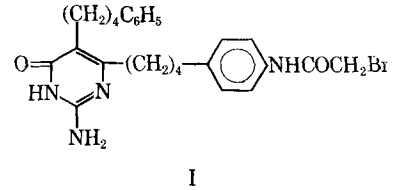
# Irreversible Enzyme Inhibitors LXXII

## Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase VI. Derivatives of Hydrophobically Bonded *p*-Alkyl and *p*-Aralkyl Benzoic Acids

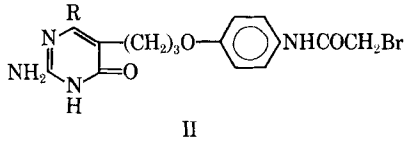
By B. R. BAKER, THOMAS J. SCHWAN, and BENG-THONG HO

**Since *p*-(bromobutyl)benzoic acid (IV), *N*-(*m*-aminobenzyl)-*p*-aminobenzoic acid (V), and *p*-benzylbenzoic acid (VI) showed good hydrophobic bonding to dihydrofolic reductase (6), four candidate active-site-directed irreversible inhibitors related to structures IV–VI were synthesized that contained a terminal alkylating function. Two of the candidates, *p*-(4-bromoacetamidobutyl)benzoic acid (IX) and *N*-(*m*-bromoacetamidobenzyl)-*p*-aminobenzoic acid (X), lost their ability to form a reversible complex with the enzyme. In contrast, α-(*p*-chloroacetylanilino)-*p*-toluic acid (XI) and α-[*p*-(4-chloro-1-buten-3-one-1-yl)anilino]-*p*-toluic acid (XII) formed reasonably good reversible complexes with dihydrofolic reductase and could inactivate the enzyme; chloroacetone under comparable conditions showed no inactivation, thus affording strong evidence that XI and XII inactivated the enzyme *via* a neighboring-group reaction within a reversible complex with the enzyme.**

THE DISCOVERY of hydrophobic bonding to dihydrofolic reductase (1) with its conformational implications on the mode of binding of pyrimidines (2–8) soon led to the proper design of active-site-directed irreversible inhibitors of this enzyme (9–11). These successful candidates, such as I (9) and II*a* (10), were designed on the principle that the 5-phenylbutyl group of I could complex to the hydrophobic region on dihydrofolic reductase which then projected the 6-side-



I



II

*a*, R = $C_6H_5$
*b*, R = $CH_3$

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