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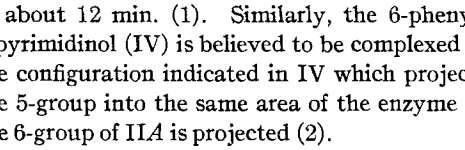
Irreversible Enzyme Inhibitors LXXXIV

Candidate Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase IX. Derivatives of 2,4-Diaminopyrimidine III

By B. R. BAKER and RICH B. MEYER, JR.

The candidate active-site-directed irreversible inhibitor, 5-(*p*-bromoacetamidophenoxypropyl)-2,4-diamino-6-phenylpyrimidine (VI) has been synthesized *via* the key intermediate, 2-amino-5-(*p*-nitrophenoxypropyl)-6-phenyl-4-pyrimidinol (XI*c*). Although VI was a better reversible inhibitor than the corresponding 4-pyrimidinol, 2-amino-5-(*p*-bromoacetamidophenoxypropyl)-6-phenyl-4-pyrimidinol (IV), VI failed to inactivate dihydrofolic reductase when incubated in the presence or absence of TPNH. The fact that IV in the absence of TPNH could inactivate the enzyme, but VI at the same concentration showed no inactivation of the enzyme, clearly demonstrated that IV and VI had different conformations when complexed to dihydrofolic reductase. Evidence for the possible binding conformations of IV and VI is presented.

THE SUCCESS of the 5-phenylbutyl-4-pyrimidinol (II) (1) and the 6-phenyl-4-pyrimidinol (IV) (2) as active-site-directed irreversible inhibitors (3, 4) of dihydrofolic reductase was predicated on a knowledge of (*a*) the strength and location of the hydrophobic bonding region on the enzyme (5–10), and (*b*) the probability that one of several possible rotomers of the pyrimidine ring could be complexed in the active-site (7) which was determined by the position of the hydrophobic group on the pyrimidine carrier (10). The substrate, dihydrofolate (I), must have some given conformation of the pteridine ring when complexed to the enzyme, which is arbitrarily depicted as indicated in I; other rotomeric conformations of the pyrimidines to be discussed below are in relation to the conformation depicted in I. Thus, the active-site-directed irreversible inhibitor of dihydrofolic reductase derived from a 4-pyrimidinol with a hydrophobic 5-phenylbutyl group (II) probably has its hydrophobic group projected vertically as indicated; the 4-pyrimidinol (II) is believed to complex in configuration II*A* which projected the 6-group into a hydrophilic region where covalent bond formation to the enzyme rapidly occurred within the enzyme-inhibitor complex with a half-life of about 12 min. (1). Similarly, the 6-phenyl-4-pyrimidinol (IV) is believed to be complexed in the configuration indicated in IV which projects the 5-group into the same area of the enzyme as the 6-group of II*A* is projected (2).



I

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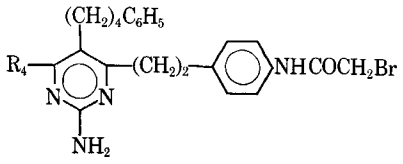
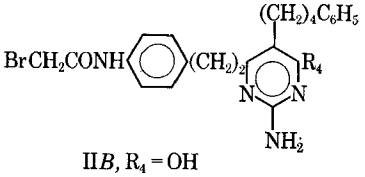
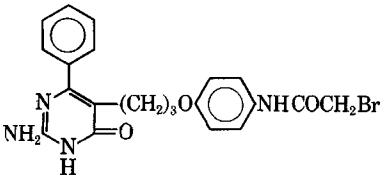


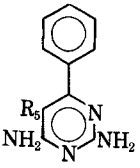
IIA, $R_4 = OH$
IIIA, $R_4 = NH_2$



IIB, $R_4 = OH$
IIIB, $R_4 = NH_2$



IV



V, $R_5 = BrCH_2CONH$-⟨⟩-$(CH_2)_3NH$-

VI, $R_5 = BrCH_2CONH$-⟨⟩-$O(CH_2)_3$-

Although success with these two active-site-directed irreversible inhibitors of dihydrofolic reductase was greatly encouraging in the chemotherapeutic approach, these two compounds suffered from the drawback that too high a concentration was needed for reversible complexing of the enzyme, since the rate of the inactivation is dependent upon the fraction of the enzyme being in a reversible complex, which in turn is dependent upon the dissociation constant, K_i (11, 12).

Since 2,4-diaminopyrimidines are complexed as much as 3000 times more strongly to dihydrofolic reductase than 4-pyrimidinols (5, 13, 14), the synthesis and evaluation of candidate active-site-directed irreversible inhibitors from this system was undertaken. That such an undertaking might be more difficult than merely converting a successful 4-pyrimidinol-type irreversible inhibitor such as II or IV to III or VI, respectively, was indicated by earlier studies (10) on the mode of reversible binding by pyrimidines; 4-pyrimidinols were believed to have conformations II*A* or IV, whereas their 2,4-diamino counterparts would probably have conformation III*B* and VI, which would project the alkylating function in the opposite direction. Unfortunately, these earlier fears were borne out with the candidate 2,4-diaminopyrimidine irreversible inhibitors, III*B* (15) and V (16), which failed to inactivate dihydrofolic reductase, although the two compounds were much better reversible inhibitors of dihydrofolic reductase than II or IV. Since V had a slightly different side-chain than IV which may have made V ineffective as an irreversible inhibitor due to differences in allowable ground-state conformations of the 5-side-chain, the synthesis and evaluation of the exact 2,4-diamino analog (VI) of the 4-pyrimidinol (IV) was undertaken; the results are the subject of this paper.

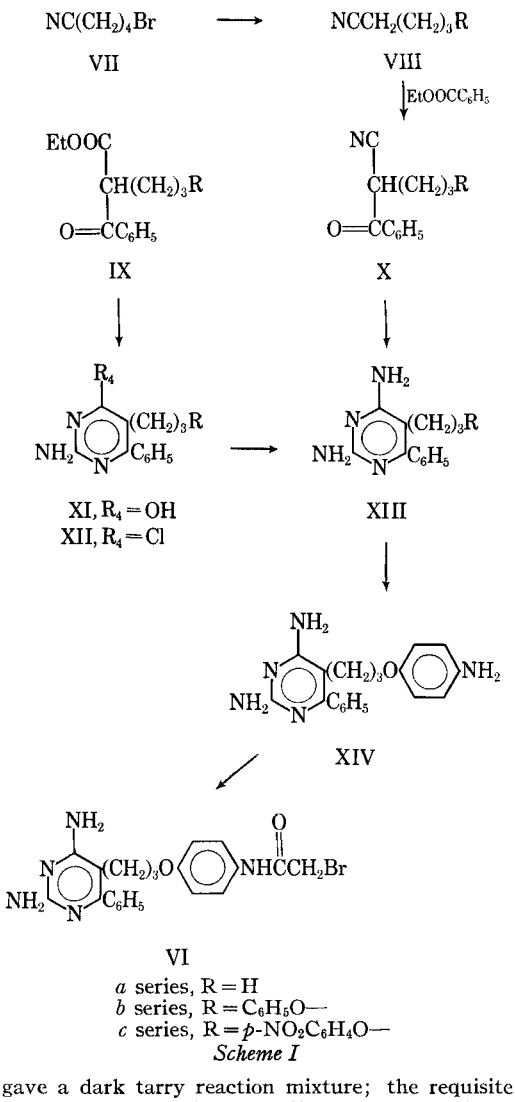
CHEMISTRY

Methods

Several routes to the key 2,4-diamino-5-(*p*-nitrophenoxypropyl)-6-phenylpyrimidine (XIII*c*) were investigated (Scheme I); the successful route will be discussed first. The previously described 5-(*p*-nitrophenoxypropyl)-4-pyrimidinol (XI*c*) (2) is readily synthesized by alkylation of ethyl benzoylacetate with bromopropyl *p*-nitrophenyl ether to IX*c*, followed by reaction with guanidine. Considerable difficulty was initially encountered in conversion of XI*c* to the 4-chloropyrimidine (XII*c*), then further amination to XIII*c*; the intermediate chloropyrimidine (XII*c*) could not be crystallized, and the diaminopyrimidine (XIII*c*) was less soluble in 10% aqueous acetic acid than previously encountered with 2,4-diamino-6-phenylpyrimidines (10, 17). By suitable modifications of the general procedures, as described under *Experimental*, XIII*c* was obtained pure as its hemisulfate salt in 15% yield for the two steps.

Hydrogenation of the nitro group of XIII*c* to give XIV in the presence of platinum oxide catalyst proceeded satisfactorily in aqueous ethanol only if excess hydrochloric acid was present; without the added acid, gummy products were obtained. Reaction of XIV with excess bromoacetic anhydride in acetone—in the presence of acetic acid to protonate the 2,4-diaminopyrimidine ring system (18)—afforded the candidate active-site-directed irreversible inhibitor (VI) isolated as its hemisulfate salt.

The 2,4-diamino-5-(phenoxypropyl)-6-phenylpyrimidine (XIII*b*), which was needed for reversible binding studies with dihydrofolic reductase, was synthesized by two routes previously used for related compounds (10, 15, 16). Reaction of 5-bromovaleronitrile (VII) with phenol and potassium carbonate in boiling acetone afforded the phenyl ether (VIII*b*) as an oil. Claisen condensation of VIII*b* with ethyl benzoate and sodium hydride in xylene afforded the keto nitrile (X*b*) as an oil that was estimated to be about 60% pure by thin-layer chromatography. Fusion of the crude keto ester (X*b*) with guanidine carbonate at 180° (10, 16)



a series, R = H
b series, R = C_6H_5O—
c series, R = *p*-$NO_2C_6H_4O$—

Scheme I

gave a dark tarry reaction mixture; the requisite 2,4-diaminopyrimidine (XIII*b*) was readily isolated by extraction with 10% aqueous acetic acid, albeit in low over-all yield.

The second route to XIII*b* started with ethyl benzoylacetate which was alkylated with 3-bromopropyl phenyl ether to IX*b*; further reaction with guanidine carbonate in *tert*-butyl alcohol (19) afforded the 4-pyrimidinol (XI*b*) in 17% over-all yield for the two steps. Reaction of XI*b* with boiling phosphorus oxychloride gave the chloropyrimidine (XII*b*) as a gum which could not be crystallized; further reaction of the crude XII*b* with methanolic ammonia at 155° afforded the 2,4-diaminopyrimidine (XIII*b*).

Numerous attempts to prepare X*c* by condensation of 5-(*p*-nitrophenoxy)-valeronitrile (VIII*c*) with ethyl benzoate and sodium hydride in dimethylsulfoxide, benzene, or xylene gave dark tars; apparently the nitrophenyl group is incompatible with these Claisen conditions, as previously observed with *p*-nitrophenylacetonitrile (20). Although conversion of XIII*b* to XIII*c* by nitration might be a feasible reaction, it was not studied since the synthesis of XIII*c* *via* XI*c* was successful.

Synthesis

Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B or 337 spectrophotometer. Ultraviolet spectra were determined in 10% alcohol, unless otherwise indicated, with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms were run on Brinkmann silica gel GF and spots were detected by visual examination under ultraviolet light.

2-Amino-5-phenoxypropyl-6-phenyl-4-pyrimidinol (XI*b*)—To a stirred solution of 15.5 Gm. (81 mmoles) of ethyl benzoylacetate and 17.5 Gm. (81 mmoles) of 3-bromopropyl phenyl ether in 75 ml. of reagent dimethylsulfoxide was added 4.0 Gm. (81 mmoles) of sodium hydride (as a 50% dispersion in mineral oil) over a period of 45 min. (21). After being stirred for 24 hr., the mixture was acidified with glacial acetic acid, then poured into a stirred mixture of 150 ml. of benzene and 200 ml. of water. The separated aqueous layer was extracted with two 50-ml. portions of benzene. The combined benzene solutions were washed with two 100-ml. portions of water, dried with magnesium sulfate, then evaporated *in vacuo;* yield, 28 Gm. of crude IX*b* which showed two spots on TLC in 5:1 petroleum ether (b.p. 60–110°)–ethyl acetate, neither of which were starting materials.

To a solution of this crude IX*b* in 100 ml. of *tert*-butyl alcohol was added 6.5 Gm. (36 mmoles) of guanidine carbonate. After being refluxed with magnetic stirring for 72 hr., during which time the product separated, the mixture was cooled and filtered (21). The product was washed with ethanol; yield, 4.0 Gm. (17%), m.p. 264–267°, suitable for further transformation. A sample was recrystallized three times from aqueous 2-methoxyethanol: white crystals, m.p. 270–274°, that showed one spot on TLC in 4:1 ethanol–chloroform. The compound had $\lambda_{max.}$ (pH 1, 7): 279 mμ; (pH 13): 286 mμ; $\nu_{max.}$ 3350, 3100 (NH); 1640, 1580, 1480 (C=O, C=N, C=C); 755, 697, 688 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{19}N_3O_2$: C, 71.0; H, 5.96; N, 13.1. Found: C, 70.9; H, 5.88; N, 12.9.

2,4-Diamino-5-phenoxypropyl-6-phenylpyrimidine (XIII*b*) Hemisulfate—*Preparation A*—A mixture of 2.4 Gm. (15 mmoles) of 5-bromovaleronitrile (VII), 1.40 Gm. (15 mmoles) of phenol, 2.0 Gm. (15 mmoles) of anhydrous potassium carbonate, and 5 ml. of reagent acetone was refluxed with magnetic stirring for 8 hr. The mixture was spin-evaporated *in vacuo*, then the residue was extracted with 20 ml. of benzene. The benzene solution was washed with two 20-ml. portions of 0.1 *N* aqueous sodium hydroxide, then three 20-ml. portions of water. Dried with magnesium sulfate, the solution was evaporated to residue *in vacuo;* yield, 2.40 Gm. (89%) of crude VIII*b;* $\nu^{film}_{max.}$ 2260 (C≡N); 1600, 1490 (C=C); 1240 (C—O—C); 755, 690 cm.$^{-1}$ (C_6H_5).

To a magnetically stirred solution of 4.8 Gm. (27 mmoles) of similarly prepared crude VIII*b* in 50 ml. of xylene was added 1.20 Gm. (26 mmoles) of sodium hydride (as a 50% dispersion in mineral oil) followed by 3.75 Gm. (25 mmoles) of ethyl benzoate. The mixture, protected from moisture, was care-

fully brought to the b.p., then refluxed for 24 hr. The mixture, acidified with glacial acetic acid, was poured into a mixture of 100 ml. of benzene and 150 ml. of water. The separated organic layer was washed with three 100-ml. portions of water, then spin-evaporated *in vacuo;* yield, 9.0 Gm. of crude X*b* which showed one major spot and several minor spots on TLC in 1:10 ethyl acetate–petroleum ether (b.p. 60–110°).

A mixture of the above crude X*b* and 4.5 Gm. (25 mmoles) of guanidine carbonate was placed in a bath preheated to 180°; after 30 min. at 180°, gas evolution was essentially complete. The cooled melt was stirred with 100 ml. of 10% aqueous acetic acid for about 30 min., then the mixture was filtered through a diatomaceous earth[1] pad. To the filtrate was added 10 ml. of 20% aqueous sulfuric acid. After several hours, the mixture was filtered and the product was recrystallized from aqueous 2-methoxyethanol; yield, 0.68 Gm. (5.9% over-all based on VII), m.p. 235–240°; $\lambda_{max.}$ (pH 1): 287 mμ; (pH 13): 297 mμ; $\nu_{max.}$ 3350, 3200 (NH); 1630, 1490 (NH, C=C, C=N); 1240 (C—O—C); 755, 685 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{20}N_4O \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 59.2; H, 5.97; N, 14.6. Found: C, 59.2; H, 6.11; N, 14.6.

Preparation B—A magnetically stirred mixture of 1.00 Gm. (3 mmoles) of XI*b* and 10 ml. of phosphorus oxychloride under a reflux condenser and protected from moisture was placed in a bath preheated to 110° and maintained at 110° for 1 hr. The cooled solution was poured into 60 ml. of petroleum ether (b.p. 60–110°), then allowed to stand until the supernatant liquid had cleared (1 hr.). The supernatant was then decanted from the gum. The gum was dissolved in chloroform and the solution washed with two 50-ml. portions of ice-cold 10% aqueous sodium acetate, then two 50-ml. portions of water; the layers readily emulsified, and separation was slow. Dried with magnesium sulfate, the chloroform solution was spin-evaporated *in vacuo* leaving about 1 Gm. of crude XII*b* which had $\lambda_{max.}$ (pH 1): 323 mμ; (pH 13): 308 mμ.

The crude XII*b* was heated with 40 ml. of methanol previously saturated with ammonia in a Parr bomb at 155° for 10 hr. The cooled mixture was spin-evaporated *in vacuo*. The residue was extracted with 50 ml. of 10% aqueous acetic acid on a steam bath. The decanted solution was clarified with carbon, then diluted with 20 ml. of 20% aqueous sulfuric acid. The cooled mixture was filtered and the product washed with water; yield, 0.223 Gm. (18%), m.p. 200–210°, that was identical with *Preparation A* as shown by U.V., I.R., and TLC in 5:1 chloroform–ethanol.

2,4-Diamino-5-(p-nitrophenoxypropyl)-6-phenylpyrimidine (XIIIc) Hemisulfate—The crude chloropyrimidine (XII*c*), prepared from 1.92 Gm. (5.11 mmoles) of XI*c* (10), as described for the preparation of XII*b*, was dissolved in 40 ml. of methanol previously saturated with ammonia at −10°. The solution was heated in a Parr bomb at 150–155° for 20 hr., then spin-evaporated *in vacuo*. The residue was extracted with 300 ml. of 10% aqueous acetic acid on a steam bath. The solution was clarified with decolorizing carbon by filtration through a diatomaceous earth pad. To the filtrate was added 20 ml. of 20% sulfuric acid. The cooled mixture was filtered and the product was washed with water; yield 1.15 Gm. (52%) of crystalline product, m.p. 239–256°, which showed two spots on TLC in 4:1 chloroform–ethanol. Six recrystallizations from aqueous 2-methoxyethanol gave pure product in 30% recovery, m.p. 261–263°; $\lambda_{max.}$ (pH 1): 293 mμ; (pH 13): 305 mμ; $\nu_{max.}$ 3300, 3100 (NH); 1660, 1620, 1500 (NH, C=C, C=N); 1250 (C—O—C); 1105 ($SO_4^=$); 890 (*p*-C_6H_4); 750, 695 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{19}N_5O_3 \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 52.8; H, 5.15; N, 16.2. Found: C, 52.7; H, 5.47; N, 16.0.

5-(p-Aminophenoxypropyl)-2,4-diamino-6-phenylpyrimidine (XIV)—A solution of 300 mg. (0.70 mmole) of XIII*c* in 100 ml. of 85% aqueous ethanol and 0.20 ml. of 12 *N* hydrochloric acid was shaken with hydrogen at 2–3 Atm. in the presence of 50 mg. of platinum oxide for 30 min. when hydrogen uptake was complete. The solution was filtered through a diatomaceous earth pad, then evaporated *in vacuo*. The residual amine salt was dissolved in 20 ml. of boiling 50% aqueous ethanol; the solution was made alkaline with 2 ml. of 1 *N* aqueous sodium hydroxide, then water was added to turbidity. On cooling, the solution deposited 190 mg. (78%) of crystals that analyzed for a monohydrate, m.p. 158–159° dec.; $\nu_{max.}$ 3400 (H_2O); 3300, 3180–3050 (NH); 1620, 1550 (NH, C=C, C=N); 1245 (C—O—C); 820 (*p*-C_6H_4); 710 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1): 283 mμ; (pH 13): 297 mμ. After being dried at 80° for 1 day in high vacuum, the compound was still partially hydrated.

Anal.—Calcd. for $C_{19}H_{21}N_5O \cdot {}^1/_2H_2O$: C, 66.3; H, 6.44; N, 20.3. Found: C, 66.3; H, 6.65; N, 20.0.

3-(p-Bromoacetamidophenoxypropyl)-2,4-diamino-6-phenylpyrimidine (VI) Hemisulfate—To a magnetically stirred mixture of 17 mg. (0.050 mmole) of XIV, 2 ml. of acetone, and 0.165 ml. (0.055 mmole) of 3.3 m*M* aqueous acetic acid cooled in an ice bath was added a solution of 16 mg. (0.06 mmole) of bromoacetic anhydride in 0.5 ml. of acetone. After 30 min., the solution was treated with 0.5 ml. of 0.2 *N* aqueous sulfuric acid. The solution was concentrated to about 1 ml. *in vacuo*. The mixture was warmed on a steam bath, then just sufficient alcohol was added to give a clear solution. Water was added to turbidity, then the solution was allowed to cool; yield 15 mg. (58%), that was uniform on TLC in 5:1 chloroform–ethanol. Recrystallization from aqueous 2-methoxyethanol gave the analytical sample, m.p. 171–172° dec.; $\lambda_{max.}$ (pH 1, 7): 280 mμ; (pH 13): 291 mμ.

Anal.—Calcd. for $C_{21}H_{22}BrN_5O_2 \cdot {}^1/_2H_2SO_4 \cdot H_2O$: C, 48.1; H, 4.81; N, 13.4. Found: C, 48.2; H, 4.85; N, 13.3.

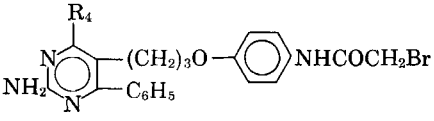
The compound gave a positive test for a reactive halogen with *p*-nitrobenzylpyridine (18) and negative Bratton-Marshall test for aromatic amine (18).

RESULTS AND DISCUSSION

Table I presents the results of the candidate 2,4-diamino active-site-directed irreversible inhibitor (VI) on inactivation of dihydrofolic reductase; the previous results with the corresponding 2-amino-4-pyrimidinol (IV) (2) are also presented. Note that

[1] Marketed as Celite by the Johns-Manville Corp., New York, N. Y.

TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_4	Estimated $K_i \times 10^8$ M[a]	Irreversible[b] Expt.	μM Concn. Inhibitor	μM Concn. TPNH	% $E \cdot \cdot \cdot I$[c]	Time, min.	% Inactivation
IV[d]	OH	130	*A*	40	0	25	50	50
VI	NH_2	1.7	*B*	2	12	54	60	0
IV	OH	130	*C*	20	0	13	120	37
VI	NH_2	1.7		20	0	>13[e]	120	0

The technical assistance of Barbara Baine, Maureen Baker, and Susan Lakatos with the assays of Tables I and II is acknowledged. [a] The dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol, as previously described (22). The K_i was estimated from $K_i = I \times K_m/S$, where I = inhibitor concentration giving 50% inhibition, $K_m = 1 \times 10^{-6}$ M, and $S = 6 \times 10^{-6}$ M; this equation is usually valid when $S > 4\ K_m$ (6, 23). [b] Dihydrofolic reductase was incubated with inhibitor at 37° in 0.05 M Tris buffer (pH 7.4) containing *no* mercaptoethanol as previously described (24); in each case an enzyme control was run and the per cent inactivation by inhibitor is corrected for the small amount of thermal inactivation in the enzyme control. [c] Calculated from $[EI] = E_t/[1 + K_i/I]$, where E_t = the concentration of total active enzyme, $[EI]$ = the fraction of E_t reversibly complexed by I, and I = the inhibitor concentration (3, 12). [d] Data from *Reference 2*. [e] The $[EI]$ concentration is unknown in the absence of TPNH, due to the ternary binding requirement for 2,4-diaminopyrimidines; see *Results and Discussion*.

VI gives no inactivation of the enzyme in the absence of TPNH or in the presence of 12 μM TPNH; in contrast, IV shows inactivation of the enzyme even at a concentration of 20 μM where IV reversibly complexes with only 13% of the total available dihydrofolic reductase.

Before discussion of these results, the recent experiments by Bertino *et al.* (25, 26) on measurement of binding to dihydrofolic reductase by tryptophan fluorescence spectra should be considered. They observed (25) that the 2,4-diamino-type inhibitor—2,4,7-triamino-6-phenylpteridine—could complex to dihydrofolic reductase in the absence of TPNH (binary binding), but binding was sixtyfold tighter in the presence of TPNH (ternary binding). In Table II are presented some of their results (26) on our 6-phenylpyrimidines.

Note that the 2,4-diamino-6-phenylpyrimidines with a 5-phenylbutyl (XXII) or 5-anilinopropyl (XXIII) show tighter binding in the presence of TPNH (ternary binding) than in the absence of TPNH (binary binding); the extent of this increased binding could not be measured due to the limit of detection of the ternary K_D, but the increase in binding was >fortyfold with XXII and >100-fold with XXIII. In contrast, a much less dramatic effect by TPNH was seen with the 2-amino-6-phenyl-4-pyrimidinols; with the 5-anilinopropyl (XXVII) and the 5-phenylbutyl side-chain (XXVI), only a 2.5-fold and twelvefold increase in ternary binding over binary binding being observed. Also note that the 2,4-diaminopyrimidines gave stronger binary binding than the corresponding 2-amino-4-pyrimidinols; with the 5-phenylbutyl side-chain, XXII was complexed sixtyfold better than XXVI and with the 5-anilinopropyl side-chain, XXIII was complexed thirtyfold better than XXVII.

Now let us return to experiment *C* in Table I. At a concentration of 20 μM, the diaminopyrimidine (VI) should complex more of the enzyme than the 2-amino-4-hydroxypyrimidine (IV) when TPNH is absent—although this exact number cannot be determined kinetically since TPNH is required for a kinetic experiment (25). The fact that 20 μM IV still shows inactivation of the enzyme, but 20 μM VI shows none, demonstrates unequivocally that IV and VI form complexes with dihydrofolic reductase that are conformationally different, since the terminal alkylating function is juxtaposed to a nucleophilic group on the enzyme in the case of IV, but not in the case of VI.

How can these conformations within the enzyme–inhibitor reversible complex be different? In Table II are listed reversible binding constants for five classes of compounds—determined kinetically in the presence of TPNH. There are at least three distinct modes of binding with respect to the side-chains.

In class *A* are listed the 5-(*p*-chlorophenyl)-2,4-diaminopyrimidines with varying substituents at the 6-position. It was previously noted that the *p*-chlorophenyl group gave a 5500-fold increment in binding due to hydrophobic bonding (6); this tremendous increase in binding was believed to be determinant (3, 10) on which rotomer (7) of the pyrimidine would complex to the enzyme. Note that there is less than a threefold difference in binding with small and large chains at the 6-position in class *A;* these results are best interpreted as indicating that the 6-side-chain is not in contact with the enzyme, but is projected into space.

Similar observations were made with the class *B* compounds containing a 5-phenylbutyl side-chain (except XX); the 5-phenylbutyl side-chain gives a 40,000-fold increment in binding (6), and presumably determines which rotomer (7) will complex to the enzyme (3,10); note again that small or large 6-side-chains have no more than a two fold effect on binding.

In class *E* are listed some 2-amino-4-pyrimidinols; note that with the 5-phenylbutyl side-chain a loss in binding occurs in proceeding from a 6-methyl (XXVIII) to a 6-*n*-propyl side-chain (XXIX); this result has been attributed to a repulsion of the propyl group from a hydrophilic area on the enzyme (10). Thus, the propyl group of XXIX would have to be in contact with the enzyme in order for this repulsion to occur, in contrast to the noncontact of the 6-group in series *A* and *B*.

A third type of binding is observed with the 2,4-diamino-6-phenylpyrimidines of class *C*. First, it should be noted that the 6-phenyl group of XXI gives only a sevenfold increment in binding (10) compared to the 6-methyl group of 2,4-diamino-6-methylpyrimidine (6)—much smaller than the 5500-fold increment observed with XV; thus the 6-phenyl group may not necessarily be determinant on which rotomer will bind even though most of the earlier data with 6-phenylpyrimidines could be rationalized on this basis (10). That the terminal phenyl group of a 5-side-chain in class *C* may not be complexed to the enzyme in the same place as a terminal phenyl group of class *B* is indicated by the following comparison; note that in class *B*, the phenylbutyl group of XVIII gives 85-fold tighter binding than the anilinopropyl group of XX, attributed to the terminal phenyl group being complexed in a hydrophobic region; in contrast, note in class *C* that the 5-phenylbutyl (XXII), 5-phenoxypropyl (XIII*b*), and 5-anilinopropyl (XXIII) side chains differ by no more than a factor of four in binding, indicating that the terminal phenyl group is in a more polar region. Similar results were previously observed in class *D* with XXVI and XXVII which should be compared with XXVIII, XXX, and XXXI in class *E*.

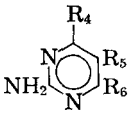
These reversible binding studies could indicate that the two candidate irreversible inhibitors, IV and VI (Table I), bind in a similar conformation; however, experiment *C* (Table I) indicates clearly that their conformations are different. A possible working hypothesis useful for further studies is the following.

The active-site-directed irreversible inhibitors of the 2-amino-4-pyrimidinol type such as II and XXXII are believed to have conformation *B* (Table III). This assignment is based on the decrease in binding when the 6-methyl group of class *E* compounds (XXVIII) (Table II) is extended to *n*-propyl (10), but the terminal phenyl group of XXXII gives a fourfold increase in binding (1); such a repulsion of an alkyl group by the enzyme is likely to take place if this alkyl group is complexed in the polar region that normally complexes the substrate, dihydrofolate, with assigned conformation I (10). Compound IV is believed to have conformation *A* (2, 10).

Since IV can inactivate dihydrofolic reductase, but the diaminopyrimidine (VI) does not, it follows that IV and VI must have different conformations when complexed to dihydrofolic reductase. That VI does not have a conformation *C* like that assigned to XVI is indicated by extra binding exerted by the phenoxyalkyl group of VI, whereas XVI shows no appreciable addition or loss of binding as the phenylalkyl group is varied (see class *A*, Table II).

A logical conformation for V and VI is *D* with the phenylalkyl group staggered to the right; it was pointed out above that the terminal phenyl group of class *B* compounds was complexed in a different area on the enzyme than class *C* compounds (Table II) since the side-chain binding of class *B* compounds such as XVIII and XX was considerably influenced when an NH group adjacent to the

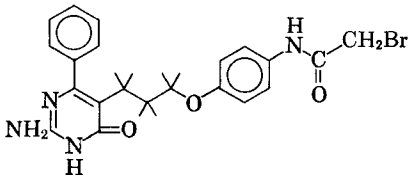
TABLE II—REVERSIBLE INHIBITION OF DIHYDROFOLIC REDUCTASE BY



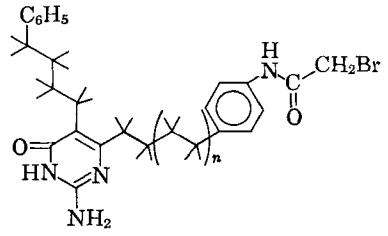
Compd.	Class	R_4	R_5	R_6	μM Concn. for 50% Inhibition[a]	$-K_D \times 10^9\ M$[b] Binary	Ternary
XV	*A*	NH_2	$-C_6H_4Cl$-*p*	CH_3	0.20[c]	...	...
XVI	*A*	NH_2	$-C_6H_4Cl$-*p*	$-(CH_2)_2C_6H_4NHCOCH_2Br$-*p*	0.53[d]	...	...
XVII	*A*	NH_2	$-C_6H_4Cl$-*p*	$-CH_2Br$	0.26[e]	...	...
XVIII	*B*	NH_2	$-(CH_2)_4C_6H_5$	CH_3	0.027[f]	...	...
XIX	*B*	NH_2	$-(CH_2)_4C_6H_5$	$-C_3H_7$-*n*	0.021[g]	...	...
III	*B*	NH_2	$-(CH_2)_4C_6H_5$	$-(CH_2)_2C_6H_4NHCOCH_2Br$-*p*	0.040[d]	...	...
XX	*B*	NH_2	$-(CH_2)_3NHC_6H_5$	CH_3	2.2[f]	...	...
XXI	*C*	NH_2	H	$-C_6H_5$	160[g]	...	...
XIII*a*	*C*	NH_2	$-C_4H_9$-*n*	$-C_6H_5$	29[g]	...	...
XXII	*C*	NH_2	$-(CH_2)_4C_6H_5$	$-C_6H_5$	1.1[g]	40	<1
XXIII	*C*	NH_2	$-(CH_2)_3NHC_6H_5$	$-C_6H_5$	0.88[g]	100	<1
XIII*b*	*C*	NH_2	$-(CH_2)_3OC_6H_5$	$-C_6H_5$	3.5	...	...
XIII*c*	*C*	NH_2	$-(CH_2)_3OC_6H_4NO_2$-*p*	$-C_6H_5$	3.5	...	...
XIV	*C*	NH_2	$-(CH_2)_3OC_6H_4NH_2$-*p*	$-C_6H_5$	8.4		
VI	*C*	NH_2	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*p*	$-C_6H_5$	8.1	...	...
XXIV	*C*	NH_2	NH_2	$-C_6H_5$	1100[h]	...	...
XXV	*C*	NH_2	$-NH(CH_2)_3C_6H_4NH_2$-*p*	$-C_6H_5$	0.42[h]	...	...
V	*C*	NH_2	$-NH(CH_2)_3C_6H_4NHCOCH_2Br$-*p*	$-C_6H_5$	0.33[h]	...	...
IV	*D*	OH	$-(CH_2)_3OC_6H_4NHCOCH_2Br$	$-C_6H_5$	800[i]	...	...
XXVI	*D*	OH	$-(CH_2)_4C_6H_5$	$-C_6H_5$	0.54	2400	200
XXVII	*D*	OH	$-(CH_2)_3NHC_6H_5$	$-C_6H_5$	1.8[i]	3000	1200
XXVIII	*E*	OH	$-(CH_2)_4C_6H_5$	$-CH_3$	30[k]	...	...
XXIX	*E*	OH	$-(CH_2)_4C_6H_5$	$-C_3H_7$-*n*	900[g]	...	...
XXX	*E*	OH	$-(CH_2)_3NHC_6H_5$	$-CH_3$	800[l]	...	...
XXXI	*E*	OH	$-(CH_2)_3OC_6H_5$	$-CH_3$	840[k]	...	...
XXXII	*E*	OH	$-(CH_2)_4C_6H_5$	$-(CH_2)_4C_6H_4NHCOCH_2Br$-*p*	240[m]	...	...

[a] See *Footnote a*, Table I. [b] Data from *Reference 26*; K_D = dissociation constant determined by quenching of fluorescence spectra of pure enzyme. Binary binding is determined in the absence of TPNH and ternary binding in the presence of TPNH. [c] Data from *Reference 7*. [d] Data from *Reference 15*. [e] Data from *Reference 24*. [f] Data from *Reference 6*. [g] Data from *Reference 10*. [h] Data from *Reference 16*. [i] Data from *Reference 2*. [j] $K_i \times 10^6\ M$ measured at pH 6 with folic acid as substrate; data from *Reference 17*. [k] Data from *Reference 27*. [l] Data from *Reference 22*. [m] Data from *Reference 1*.

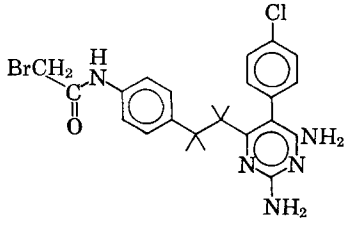
TABLE III—SOME POSSIBLE CONFORMATIONS FOR BINDING TO DIHYDROFOLIC REDUCTASE



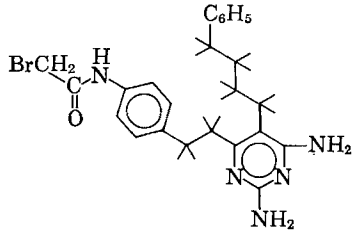
IV
Conformation *A*



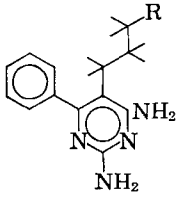
II, $n=0$
XXXII, $n=1$
Conformation *B*



XVI
Conformation *C*

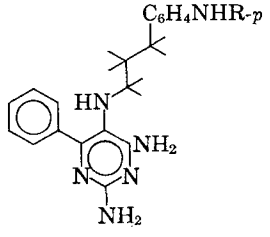


III
Conformation *C*



Conformation *D*

VI, R=—$OC_6H_4NHCOCH_2Br$-*p*
XIII*b*, R=—OC_6H_5
XXII, R=—$CH_2C_6H_5$
XXIII, R=—NHC_6H_5



Conformation *D*

V, R=—$COCH_2Br$
XXV, R=—H

phenyl of XX was replaced by methylene as in XVIII.

Therefore, the conformations outlined in Table III will be used as a working hypothesis. The synthesis of compounds V and XXV (16) is quite adaptable to variation of side chain in order to determine the ideal bridge distance for irreversible inhibition (3, 4); such studies are currently being pursued. Such active-site-directed irreversible inhibitors, if successful, would give another parameter to the assignment of binding conformations, as noted in the comparison of IV and VI.

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