XXXVIII in 200 ml. of 2-methoxyethanol was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide catalyst; reduction was complete in 90 min. The filtered solution was spin-evaporated *in vacuo* and the residue was recrystallized from aqueous 2-methoxyethanol; yield, 2.04 Gm. (84%) of buff-colored crystals, m.p. 193–194°. Recrystallization from aqueous 2-methoxyethanol gave nearly white crystals with unchanged m.p. $\nu_{max.}$ 3550 (NH); 1680 (carboxyl C=O); 1620, 1550, 1505 (C=C, NH); 825 (*p*-$C_6H_4$); 765 (*m*-$C_6H_4$); no $NO_2$ near 1520 or 1340 cm.$^{-1}$.

*Anal.*—Calcd. for $C_{14}H_{14}N_2O_2$: C, 69.4; H, 5.82; N, 11.5. Found: C, 69.5; H, 6.00; N, 11.3.

**N - Acetyl - N - (m - aminobenzyl) - p - aminobenzoic Acid (XXVII).**—A solution of 942 mg. (3 mmoles) of XXXIX in 100 ml. of ethanol was shaken with hydrogen at 2–3 Atm. in the presence of 60 mg. of platinum oxide catalyst; reduction was complete in about 15 min. The filtered solution was spin-evaporated *in vacuo* leaving 849 mg. (99%) of a glassy residue which showed 2 spots on TLC in methanol. After separation by preparative TLC, the material still could not be crystallized.

To a solution of 400 mg. of the crude product in 5 ml. of absolute ethanol was added 172 mg. (25% excess) of cyclohexylamine. Addition of 20 ml. of ether caused the separation of a gum (228 mg.). The supernatant liquid was decanted and deposited 127 mg. of crystals on standing which had m.p. 170–174°. Two recrystallizations from absolute alcohol–ether gave 90 mg. of pure cyclohexylammonium salt, as white crystals, m.p. 168–170°. $\nu_{max.}$ 3450, 3400 (NH); 2200, 2150 ($NH^+$); 1660 (amide C=O), 1600 ($COO^-$); 1625, 1540, 1500 cm.$^{-1}$ (NH, C=C).

*Anal.*—Calcd. for $C_{16}H_{16}N_2O_3 \cdot C_6H_{11}NH_2$: C, 68.9; H, 7.62; N, 11.0. Found: C, 68.7; H, 7.80; N, 10.8.

The free acid could be obtained as a glass free of other organic matter in 73% over-all yield by preparative thin-layer chromatography on Silica Gel $HF_{254}$ with methanol as solvent. Since the compound would not dissolve in acetone or chloroform, methanol was used for elution; the resultant product had a C/N ratio of 6.89 (calcd. 6.86), but could not be freed of about 10% of extracted silica.

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# Analogs of Tetrahydrofolic Acid XXXIII

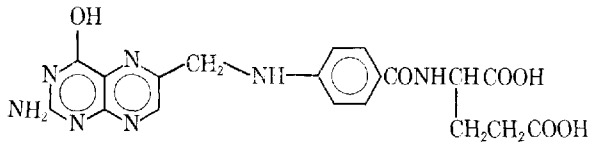
## Hydrophobic Bonding to Dihydrofolic Reductase V. Inhibition by Some Pyrimidines Bridged to Benzoic Acid

By B. R. BAKER*, BENG-THONG HO, JAMES K. COWARD, and DANIEL V. SANTI

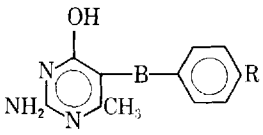
**Folic acid (I), pteroic acid (IV), and a series of 2-amino-6-methyl-4-pyrimidinols bridged from its 5-position to the *p*-position of benzoic acid with aminopropyl (VII), butyl (X), carbamoylpropyl (XIII), sulfonamidopropyl (XV), and *N*-acetylaminopropyl (XVI) were compared as inhibitors of dihydrofolic reductase. Evidence was presented that VII and X probably had their side chains off of the 5-position of the pyrimidine complexed to the hydrophobic region of the enzyme, whereas XIII, XV, and XVI probably had their side chains complexed to the locus on the enzyme that normally binds the *p*-aminobenzoyl moiety of folic acid (I).**

In the preceding paper of this series (1), experimental evidence was presented that the hydrophobic bonding region of dihydrofolic reductase (2) is probably not between the binding regions for the pyrimidyl and *p*-aminobenzoyl-L-glutamate moieties of folic acid (I) or dihydrofolic acid. If the hydrophobic bonding area is in some other region, then of the hundreds of compounds, such as type II, evaluated as dihydrofolic reductase inhibitors and presented in previous

Received November 8, 1965, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo.
Accepted for publication December 22, 1965.
This work was generously supported by grants CA-05867 and CA-06624 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
Previous paper: Baker, B. R., Schwan, T. S., Novotny, J., and Ho B.-T., *J. Pharm. Sci.*, **55**, 295(1966).
* Address inquiries to the Department of Chemistry, University of California, Santa Barbara.



I



II, R = H
III, R = COOH

papers of this series, which have side chains on the heterocycles that complex with this hydrophobic region and which have side chains that complex with the *p*-aminobenzoyl locus? As a side chain is made more and more polar or has added groups that can complex at one or more of the points of the glutamate locus, at what stage does the side chain shift from the hydrophobic bonding region to the more polar *p*-aminobenzoyl-L-glutamate binding region? Such information is considered crucial for rational design of active-site-directed irreversible inhibitors (3), since the covalent bond-forming group on the inhibitor must be juxtapositioned to a nucleophilic group on the enzyme in order to operate; such a nucleophilic group is polar by definition and is less apt to be present in a hydrophobic region of the enzyme. One possible approach to the problem is to compare effects of binding to dihydrofolic reductase when a given pyrimidine bridged to a benzene ring such as II is substituted on the phenyl ring by a *p*-carboxyl group as in III. The results of such a study are the subject of this paper.

## DISCUSSION

In a previous study (4) it was noted that the pyrimidyl prototype inhibitor (VI) of dihydrofolic reductase with the intact *p*-aminobenzoyl-L-glutamate moiety (5) was consistently slightly less effective than the corresponding inhibitor (VII) derived from *p*-aminobenzoic acid (Table I). In contrast, folic acid (I) was complexed about eightyfold more effectively than pteroic acid (V) as inhibitors of dihydrofolic reductase (6). These conflicting data on removal of the L-glutamate moiety from the 2 types of inhibitors were in part resolved in the preceding paper of this series (1); that is, it appeared that the propylaminobenzoate side chain of VII was complexed in the hydrophobic region of dihydrofolic reductase rather than at the *p*-aminobenzoyl locus normally complexing this moiety of folic acid (I). The prototype inhibitor (VI) appeared to be complexed in the same manner as folic acid (I). It should also be noted that removal of the carboxyl group of VII gave an inhibitor (VIII) that was only tenfold less effective than VII.

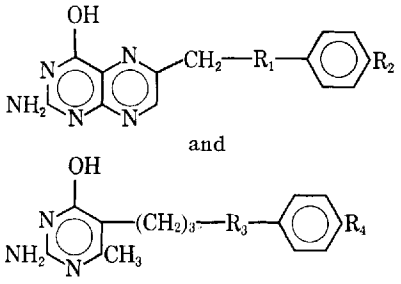
Since both VII and VIII are believed to have their pyrimidyl side chains complexed in the hydrophobic region, 3 other types of side chains previously studied were converted to their *p*-carboxy derivatives to determine if a similar increase in magnitude of binding observed with VII and VIII would be obtained. If a similar increment were obtained, then this increment could be used as evidence for the hypothesis that the side chain in question was complexed to the hydrophobic region of the enzyme. The following 3 side chains were selected: (*a*) the phenylbutyl side chain (IX), since this side chain gave 27-fold better inhibition than the anilinopropyl side chain (VIII) (7); (*b*) the benzamidopropyl side chain (XI), since this side chain gave an inhibitor about as effective as VIII (8); and (*c*) the *p*-tolylsulfonamidopropyl side chain (XIV), since this side chain gave an inhibitor less effective than VIII (8).

Introduction of the *p*-carboxyl (X) into the phenylbutyl side chain (IX) gave a twelvefold enhancement in binding (Table I), thus indicating that the phenylbutyl side chains (IX and X) and the anilinopropyl side chain (VII and VIII) were complexed in the same region of the enzyme, presumably the hydrophobic region. In contrast, introduction of the carboxyl group on the benzamidopropyl side chain (XII) to give XIII or on the benzenesulfonamidopropyl side chain (XIV) to give XV showed no enhancement in binding; these data would indicate that these two side chains of XII and XIV are not complexed in the hydrophobic region, and could be presumed to be complexed to the *p*-aminobenzoyl locus where folic acid (I) is normally complexed.[1] However, such an interpretation would require considerable substantiation since other explanations are possible, for example, (*a*) the chain length between the pyrimidyl and benzene moieties of XII and XIV are 1 atom longer than in the case of the anilinopropyl (VIII) or phenylbutyl series (X); (*b*) the ground-state staggered conformations about the $C_6H_5CH_2CH_2$ group of X or the $C_6H_5NHCH_2$— group of VIII are different than the ground-state planar conformations of the $C_6H_5CONCH_2$ group of XII or conformation of the $C_6H_5SO_2NCH_2$ group of XIV which is not quite so near planarity as the amide.

Bertino *et al.* (6) recently noted that 10-formyl folic acid (IV) was a twentyfold better inhibitor of dihydrofolic reductase than folic acid (I) (Table I). It was interesting to observe that acetylation of VII on the "10-position" corresponding to folic acid gave an inhibitor (XVI) that was less than twofold better than VII and as an inhibitor. A similar small increment has been observed previously on acetylation of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinethiol (9). This lack of appreciable increase in binding after acylation could be interpreted to indicate that folic acid (I) and presumably pteroic acid (V) are complexing differently to dihydrofolic reductase than are the 5-anilinopropyl-pyrimidine types (VII and VIII).

[1] Since the *N*-butyl benzamidopropyl and *N*-butyl *p*-tolylsulfonamidopropyl side chains previously studied (8) are less polar, these may be complexed by hydrophobic bonding to the hydrophobic region.

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY

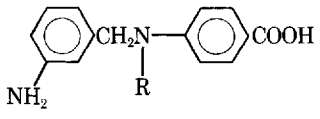


| Compd. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $\mu M$ Concn. for 50% Inhibition[a] | $K_i \times 10^6$[b] |
|---|---|---|---|---|---|---|
| I | —NH— | —CONHCHCOOH<br>$(CH_2)_2COOH$ | ... | ... | 3[c] | 0.11 |
| IV | O=CH<br>—N— | —CONHCHCOOH<br>$(CH_2)_2COOH$ | ... | ... | | 0.0061 |
| V | —NH— | —COOH | ... | ... | | 8 |
| VI | ... | ... | —NH— | —CONHCHCOOH<br>$(CH_2)_2COOH$ | 100[d,e] | |
| VII | ... | ... | —NH— | —COOH | 77[d,e] | |
| VIII | ... | ... | —NH— | H | 800[f,g] | |
| IX | ... | ... | $—CH_2—$ | H | 30[f,h] | |
| X | ... | ... | $—CH_2—$ | —COOH | 2.5[e] | |
| XI | ... | ... | $—CH_2—$ | $—COOC_2H_5$ | 13[g] | |
| XII | ... | ... | —HNCO— | H | 480[g,i] | |
| XIII | ... | ... | —NHCO— | —COOH | 480[e] | |
| XIV | ... | ... | $—NHSO_2—$ | $CH_3—$ | 2400[g,i] | |
| XV | ... | ... | $—NHSO_2—$ | —COOH | 2800[e] | |
| XVI | ... | ... | O=CCH<sub>3</sub><br>—N— | COOH | 53[e] | |

[a] Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver which was prepared and assayed 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 1 m$M$ Versene as previously described (12). The technical assistance of Mrs. Shirley Humphrey, Miss Karen Smith, and Mrs. Gail Salomon is acknowledged. [b] Data of Bertino *et al.* (6). They used the dihydrofolic reductase from Ehrlich ascites carcinoma cells with dihydrofolate as substrate at pH 7.5; dihydrofolate had apparent $K_m$ $1.3 \times 10^{-6}$. [c] Data from *Reference 15*. [d] Data from *Reference 4*. [e] For assay the inhibitor was dissolved in 0.05 $M$ Tris buffer containing 10 m$M$ mercaptoethanol and 1 m$M$ Versene, then the pH was readjusted to 7.4 with 0.1 $N$ KOH. [f] Data from *Reference 7*. [g] The inhibitor was dissolved in $N,N$-dimethylformamide and the assay was run in the presence of 10% $N,N$-dimethylformamide. [h] The same results were obtained with or without 10% $N,N$-dimethylformamide in the assay. [i] Data from *Reference 8*.

That 10-formyl folic acid (IV) is a twentyfold better inhibitor of dihydrofolic reductase than folic acid (I) (6) has considerable bearing on the mode of binding of folic acid to dihydrofolic reductase. In the $—C_6H_4—\underset{|}{N}—CH_2—$ moiety of folic acid (I) the bond angles about the nitrogen are pyramidal, whereas the same bonds are nearly planar in the 10-formyl folic acid (IV) due to the amide resonance. Therefore, it is probable that either folic acid or the enzyme or both undergo an energetically unfavorable conformational change to accommodate the nonplanarity of the $N_{10}$ bonds in folic acid (1); this energy formation of the complex between the enzyme and folic acid (I), being reflected in less net binding energy. Then the planar $N_{10}$ of 10-formyl folic acid is presumably in a more favorable ground-state conformation for complexing with the enzyme, being reflected in better net energy of binding than in the case of folic acid (1).

It was previously noted that XVII was a relatively good inhibitor of dihydrofolic reductase showing 50% inhibition at 0.76 m$M$ (1), about the same as the 5-anilinopropyl pyrimidine (VIII) (1). The aminobenzyl group and part of the benzoate group of XVII were believed to be complexed to the hydrophobic region, but the carboxyl group was probably not in contact with the hydrophobic region of the enzyme surface (1). It was noted that $N$-acetylation of XVII on the $p$-amino group gave a compound (XVIII) that failed to inhibit dihydrofolic reductase at 12 times the concentration required for XVII to show 50% inhibition. The planarity about the "$N_{10}$" (*para*)nitrogen of XVIII is apparently unfavorable for complexing to the hydrophobic region



XVII, R = H
XVIII, R = $CH_3CO$

of enzyme particularly when a terminal carboxylate is present.

Since acylation of folic acid (I) gives an inhibitor (IV) that is 20 times more effective than I, but acylation of XVII gives an inhibitor (XVIII) that is less than one-fortieth as effective as XVII, it is quite reasonable that XVI has its side chain complexed at the *p*-aminobenzoyl locus; this is in contrast to VII, which probably has its side chain complexed to the hydrophobic region. Again this deduction has the tenuous assumption that VII and XVII have the same conformation in the complex.

A problem that still remains is how a carboxyl group can increase the phenyl binding to the hydrophobic region since the carboxyl is ionized at the pH of the assay to the highly polar carboxylate anion; it is therefore obvious that the carboxylate cannot be in direct contact with the hydrophobic region. That the carboxyl of a compound such as X does not exert an inductive effect on binding of the phenyl is clear from the relative binding of X and its ester, XI. The ester (XI) is only one-fifth as effective as the carboxylate (X), but the ester is still 2–3 times as effective as the parent phenyl compound, IX. It was previously noted that an *n*-octyl group gave no more hydrophobic bonding than an *n*-butyl group, but placement of a phenyl on the terminus of the *n*-butyl group did give an increment in binding (2); these results were interpreted to indicate that a flat interaction between the phenyl and the enzyme was required which an aliphatic group could not duplicate. If the hydrophobic region ends at the end of the phenylbutyl group of IX, then an additional carboxylate may form a hydrogen bond with the enzyme in this region; the ester group of XI could then form a somewhat weaker hydrogen bond to account for the relative order of activity of X > XI > IX. Such a hypothesis might be verified by replacing the carboxyl group of X by a bromomethyl to determine if an active-site-directed irreversible inhibitor (3) would be obtained that would presumably operate by alkylation of this carboxylate binding point to the enzyme.

To put these working hypotheses presented here on a firmer basis would require considerably more investigation of related compounds. However, these working hypotheses in this and in the accompanying papers appeared attractive enough to warrant pursuit of the design of active-site-directed irreversible inhibitors (3) that can bind to both the hydrophobic and the *p*-aminobenzoyl loci of the enzyme. Such studies are being pursued as direct evidence for these 2 binding loci. Initial results with several potential active-site-directed irreversible inhibitors built on the hypothesis have been successful; when the results are on completely firm ground, they will be presented in future papers.

Although it cannot be unequivocally stated at this time that a molecule such as 2-amino-5-(*p*-carboxyphenylbutyl)-6-methyl-4-pyrimidinol (X) has its side chain complexed to the hydrophobic region of the enzyme, the unequivocal result remains that X is the most effective 2-amino-4-pyrimidinol type of dihydrofolic reductase inhibitor yet observed.

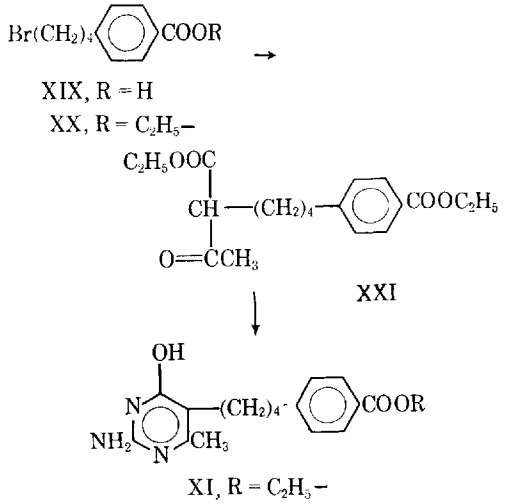
## EXPERIMENTAL

**Synthesis.**—Ethyl *p*-(4-bromobutyl)benzoate (XIX) was prepared by esterification of the corresponding acid (XX) (1) in 92% yield. Alkylation of ethyl acetoacetate with XX was performed with sodium hydride in *tert*-butyl alcohol to avoid alcoholysis of the alkylated keto ester (XXI) (9). The crude keto ester (XXI) was condensed with guanidine carbonate in *tert*-butyl alcohol (9) to give a 21% over-all yield of the crystalline pyrimidine ester (XI). Saponification of XI with aqueous 1 *N* sodium hydroxide proceeded in 97% yield to the desired *p*-carboxyphenylbutyl pyrimidine (X). (Scheme I.)

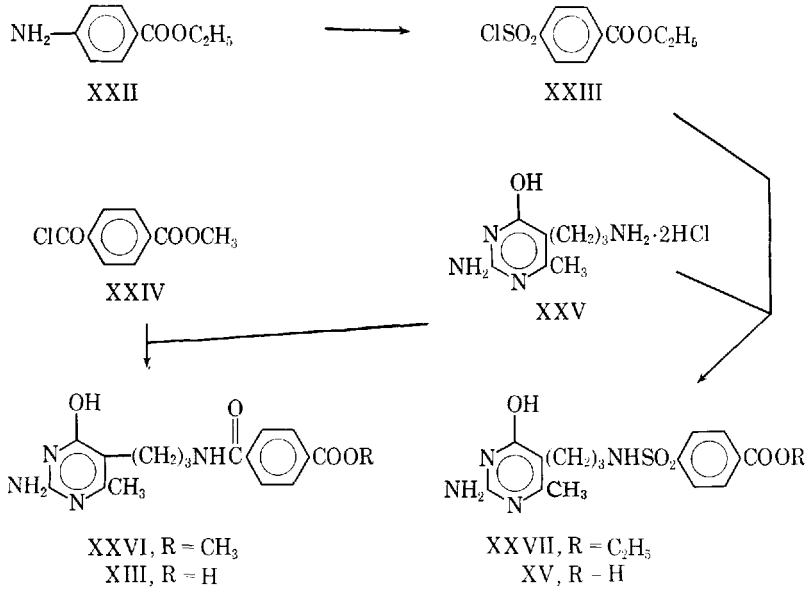
*p*-Carbomethoxybenzoyl chloride (XXIV) was prepared from dimethyl terephthalate by modification of the procedure of Williams *et al.* (10). Acylation of the pyrimidyl propylamine (XXV) (8) as its free base with XXIV in aqueous acetone proceeded smoothly to the pyrimidyl ester (XXVI) in 79% yield. Saponification of XXVI with 1 *N* aqueous sodium hydroxide at room temperature afforded XIII in 80% yield. (Scheme II.)

*p*-Carbethoxybenzenesulfonyl chloride (XXIII) was prepared in 81% yield by the Sandmeyer reaction (11); that is, the treatment of the diazonium salt derived from ethyl *p*-aminobenzoate (XXII) with sulfur dioxide catalyzed by cuprous chloride. Reaction of the sulfonyl chloride (XXIII) with the free base of XXV in aqueous acetone also proceeded smoothly in 81% yield. Saponification with 1 *N* aqueous sodium hydroxide gave the requisite carboxy pyrimidine (XV) in 80% yield. (Scheme II.)

Acetylation of the anilino NH-group of VII (14) with acetic anhydride in pyridine was sufficiently slowed by the electron-withdrawing *p*-carboxyl group so that acetylation of the 2-amino group (12) became competitive and selective acetylation of the anilino group was not feasible. In previous successful selective acylations of this type, an electron-withdrawing *p*-group was not present (7). Therefore, both amino groups were allowed to acetylate (13) giving XXVIII in 86% yield. Since the acetyl on the 2-amino group is considerably more labile than the acetyl group on the anilino moiety,




Scheme I




Scheme II

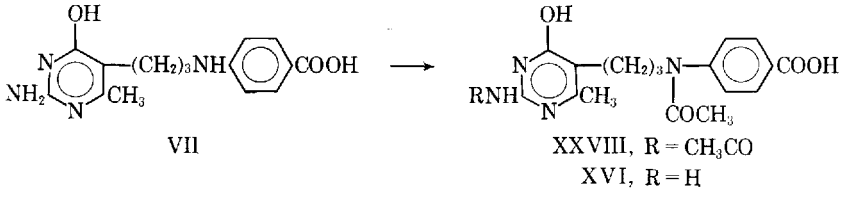
selective removal was achieved with *n*-butylamine in boiling methanol (13). The reaction was readily followed by the difference in ultraviolet spectra of XXVIII and XVI in 0.1 *N* acid, particularly at 247 mμ. After a 3-hr. treatment, XVI could be isolated analytically pure in 74% yield from XXVIII. (Scheme III.)

**Methods.**—Melting points were taken on a Mel-Temp block in capillary tubes and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in water, unless otherwise indicated, with a Perkin-Elmer 202 spectrophotometer.

**2 - Amino - 5 - (p - carbethoxyphenylbutyl) - 6-methyl-4-pyrimidinol (XI).**—A solution of 1.00 Gm. (3.89 mmoles) of XIX (1) in 10 ml. of ethanol and 1 ml. of ethanesulfonic acid was refluxed for 24 hr., then spin-evaporated *in vacuo*. The residue was dissolved in 20 ml. of chloroform, and the solution was washed with 3 *N* aqueous ammonia (2 × 10 ml.), then water (2 × 10 ml.). Dried with magnesium sulfate, the chloroform solution was spin-evaporated *in vacuo* leaving 1.02 Gm. (92%) of XX as an oil with $\nu_{max.}$(film) 1720 (ester C=O), 1270, 1100 cm.$^{-1}$ (ester C—O—C).

To a solution of 800 mg. (6.1 mmoles) of ethyl acetoacetate in 15 ml. of *tert*-butyl alcohol was added portionwise 273 mg. (6.1 mmoles) of a 53.5% dispersion of sodium hydride in mineral oil. When solution was complete, 1.51 Gm. (5.3 mmoles) of XX was added, then the solution was refluxed gently with magnetic stirring for 20 hr. The mixture, acidified with glacial acetic acid, was spin-evaporated *in vacuo*. The residue was partitioned between 20 ml. of chloroform and 10 ml. of water. The separated aqueous layer was extracted with 5 ml. of chloroform. Dried with magnesium sulfate, the combined extracts were spin-evaporated *in vacuo*, finally at less than 1 mm. (bath 90°) to remove the excess ethyl acetoacetate; yield, 872 mg. of crude XXI with $\lambda_{max.}$ (1 *N* methanolic sodium methoxide) 244, 284 mμ.

A mixture of 870 mg. (2.6 mmoles) of crude XXI, 234 mg. (1.3 mmoles) of guanidine carbonate, and 10 ml. of *tert*-butyl alcohol (9) was refluxed gently with magnetic stirring for 60 hr. The solution was clarified by filtration, neutralized with glacial acetic acid, then spin-evaporated *in vacuo*. The residue was partitioned between 25 ml. of chloroform and 10 ml. of water. The product which separated was collected on a filter and washed with water; yield, 341 mg., m.p. 163–165°. An additional 23 mg. (total 21% based on XX), m.p. 156–159°, was obtained by concentration of the chloroform layer.




Scheme III

Recrystallization of 290 mg. from aqueous ethanol gave 227 mg. (16%), m.p. 148–150°. A second recrystallization afforded 201 mg. (14%) of white crystals, m.p. 150–152°. $\nu_{max.}$ 3400, 3200 (NH); 1715 (ester C=O); 1650, 1630, 1600, 1530, 1510 (C=O, C=N, C=C, NH); 1280, 1110 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (ethanol) 290 mμ.

*Anal.*—Calcd. for $C_{15}H_{23}N_3O_3$: C, 65.6; H, 7.04; N, 12.8. Found: C, 65.4; H, 6.92; N, 12.9.

**2 - Amino - 5 - (p - carboxyphenylbutyl) - 6-methyl-4-pyrimidinol (X).**—A solution of 164 mg. (0.5 mmole) of XI in 3 ml. of 1 *N* aqueous sodium hydroxide was heated at 100° for 30 min. The cooled solution was clarified by filtration, then adjusted to pH 4–5 with acetic acid. The product was collected on a filter and washed with water; yield, 146 mg. (97%), m.p. 295–297°. Recrystallization from aqueous 2-methoxyethanol afforded 108 mg. (72%) of white crystals, m.p. 311–312°, unchanged on further recrystallization. $\nu_{max.}$ 3350, 3150 (NH); 1690, 1670, 1600, 1545–1500 (C=O, NH, C=N, C=C); no ester band near 1280 cm.$^{-1}$; $\lambda_{max.}$ (pH 1) 243, 274 mμ (inflection); (pH 13) 235, 280 mμ.

*Anal.*—Calcd. for $C_{16}H_{19}N_3O_3$: C, 63.8; H, 6.35; N, 13.9. Found: C, 63.6; H, 6.55; N, 13.7.

**p-Carbomethoxybenzoyl Chloride (XXIV).**—Dimethyl terephthalate was partially hydrolyzed as described by Williams *et al.* (10); they record a crude yield of 66%, but no melting point is given until after purification. The crude product contained considerable terephthalic acid. Solution in acetone at ambient temperature was more satisfactory than water (10) for removing the insoluble terephthalic acid.

A suspension of 270 mg. (1.5 mmoles) of *p*-carbomethoxybenzoic acid in 7 ml. of chloroform, 0.29 ml. (6 mmoles) of thionyl chloride, and a drop of pyridine was refluxed for 3 hr., solution being complete at that time. Spin-evaporation *in vacuo* gave the acid chloride as a crystalline solid with $\nu_{max.}$ (Nujol) 1780 cm.$^{-1}$ (acid chloride C=O) that was used immediately in the next step.

**2 - Amino - 5 - (p - carbomethoxybenzamidopropyl)-6-methyl-4-pyrimidinol (XXVI).**—To a magnetically stirred solution of 255 mg. (1 mmole) of XXV (8) and 0.67 ml. of 3 *N* aqueous sodium hydroxide and 0.5 ml. of water containing 159 mg. (1.5 mmoles) of sodium carbonate was added 1 ml. of acetone; then a solution of the crude XXIV from 270 mg. of acid in 2.5 ml. of acetone was added. After being stirred for 4 hr., during which time the product separated, the mixture was diluted with several volumes of water. The product was collected on a filter and washed with water; yield, 272 mg. (79%). Recrystallization from 2-methoxyethanol gave 210 mg. (61%) of white crystals, m.p. 240–242° dec. $\nu_{max.}$ 3300, 3100 (NH); 1720 (ester C=O); 1690, 1630 (sh), 1610, 1525 (C=O, NH, C=N, C=C), 1280 1100 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (pH 13) 276 mμ (sh).

*Anal.*—Calcd. for $C_{17}H_{20}N_4O_4 \cdot H_2O$: C, 56.3; H, 6.12; N, 15.5. Found: C, 56.0; H, 6.10; N, 15.9.

**2 - Amino - 5 - (p - carboxybenzamidopropyl) - 6-methyl-4-pyrimidinol (XIII).**—A suspension of 638 mg. (1.85 mmoles) of crude XXVI in 10 ml. of 1 *N* aqueous sodium hydroxide was magnetically stirred for 20 hr. at ambient temperature, during which time solution took place. Acidification to about pH 5 with 4 *N* aqueous hydrochloric acid precipitated the product which was collected on a filter and washed with water. The crude product was dissolved in 1 *N* aqueous sodium bicarbonate; the solution was clarified by filtration, then acidified to about pH 5. The product was collected by centrifugation and washed well with water; yield, 486 mg. (80%), of white solid, m.p. 290–295° dec. $\nu_{max.}$ 3450–3300 (NH); 1700 (carboxyl C=O); 1660, 1620, 1550 (C=O, C=N, C=C, N—H); no 1280 or 1100 cm.$^{-1}$ ester C—O—C bands; $\lambda_{max.}$ (pH 13) 276 mμ. The compound moved on paper as a single spot ($R_f$ 0.7) in 5% aqueous $K_2HPO_4$.

*Anal.*—Calcd. for $C_{16}H_{18}N_4O_4 \cdot H_2O$: C, 55.2; H, 5.78; N, 16.1. Found: C, 54.8; H, 5.79; N, 16.3.

**2 - Amino - 5 - (p - carbethoxybenzenesulfonamidopropyl) - 6 - methyl - 4 - pyrimidinol (XXVII).**—Treatment of 255 mg. (1 mmole) of XXV (8) with 372 mg. of XXIII (prepared in 81% yield as described for the methyl ester) (11) as described for preparation of XXVI, gave 369 mg. (94%) of crude product, m.p. 220–223°. Recrystallization from 85% ethanol afforded 319 mg. (81%) of white crystals, m.p. 223–224°. $\nu_{max.}$ 3300, 3100 (NH); 1710 (ester C=O); 1645, 1600 (sh), 1540 (NH, C=O, C=C, C=N); 1385, 1160 ($SO_2$); 1270, 1100 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (pH 13) 279 mμ.

*Anal.*—Calcd. for $C_{17}H_{22}N_4O_5S$: C, 51.8; H, 5.62; N, 14.2. Found: C, 51.5; H, 5.68; N, 14.1.

**2 - Amino - 5 - (p - carboxybenzenesulfonamidopropyl) - 6 - methyl - 4 - pyrimidinol (XV).**—A suspension of 394 mg. (1 mmole) of XXVII in 10 ml. of 1 *N* aqueous sodium hydroxide was magnetically stirred at room temperature for 2 days, during which time solution was complete. The product was isolated and reprecipitated from sodium bicarbonate solution as described for XIII; yield, 293 mg. (80%) of white solid, m.p. 310–315° dec.; $\nu_{max.}$ 3400, 3250 (NH); 1680, 1650 (C=N, C=C, C=O, NH); 1385, 1150 ($SO_2$); no 1280 or 1100 cm.$^{-1}$ ester C—O—C bands; $\lambda_{max.}$ (pH 13) 278 mμ.

*Anal.*—Calcd. for $C_{15}H_{18}N_4O_5S$: C, 49.2; H, 4.95; N, 15.3. Found: C, 49.1; H, 5.03; N, 15.4.

**2 - Acetamido - 5 - (N - acetyl - p - carboxyanilinopropyl) - 6 - methyl - 4 - pyrimidinol (XXVIII).**—A solution of 250 mg. (0.83 mmole) of VII (14) in 5 ml. of reagent pyridine and 5 ml. of acetic anhydride was heated in a bath at 95° for 2 hr., then spin-evaporated *in vacuo*. Crystallization from aqueous ethanol with the aid of decolorizing carbon gave 275 mg. (86%) of white crystals, m.p. 225–226°. $\lambda_{max.}$ (pH 1) 247, 265 mμ (inflect.) (*A* 260/280, 1.89); (pH 13) 245, 273 mμ (inflect.) (*A* 260/280, 1.45).

*Anal.*—Calcd. for $C_{19}H_{22}N_4O_5$: C, 59.1; H, 5.74; N, 14.5. Found: C, 58.8; H, 5.61; N, 14.4.

**2 - Amino - 5 - (N - acetyl - p - carboxyanilinopropyl)-6-methyl-4-pyrimidinol (XVI).**—A solution of 230 mg. (0.6 mmole) of XXVIII and 0.1 ml. of *n*-butylamine in 8 ml. of methanol was refluxed for 3 hr. when an aliquot no longer showed any change in the absorbance at 247 mμ in 0.1 *N* hydrochloric acid. The solution was spin-evaporated *in vacuo*. The residue was dissolved in 4 ml. of hot methanol, 8.0 ml. of 0.2 *N* aqueous acetic acid was added, then the solution was chilled. The product was collected on a filter; yield, 148 mg. (74%), m.p.

239–240°. $\lambda_{max.}$ (pH 1) 234, 263 mμ (*A* 260/280, 1.80); (pH 13) 235, 278 mμ (*A* 260/280, 1.04).

*Anal.*—Calcd. for $C_{17}H_{20}N_4O_4$: C, 59.3; H, 5.85; N, 16.3. Found: C, 59.0; H, 5.98; N, 16.0.

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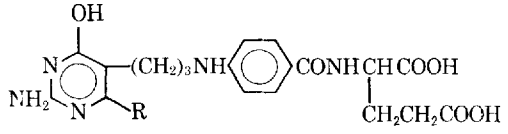
# Analogs of Tetrahydrofolic Acid XXXIV

## Hydrophobic Bonding to Dihydrofolic Reductase VI. Mode of Phenyl Binding of Some 6-Arylpyrimidines

By B. R. BAKER* and HOWARD S. SHAPIRO

**New data are presented which strongly support the concept that the increment in better binding observed by substituting a phenyl group on the 6-position of 4-pyrimidinol is due to hydrophobic bonding of the phenyl group. Furthermore, 11 6-phenylpyrimidines with various substituents at the 2,4, and 5-positions were compared with 18 5-aryl and 5-arylalkylpyrimidines as inhibitors of dihydrofolic reductase. The results cannot be explained by a single conformation of the pyrimidine being complexed to the enzyme; therefore, a number of rotational conformers for the pyrimidine ring are proposed where the strong hydrophobic bonding by the phenyl or phenylalkyl substituent is the determining factor for the particular preferred conformation of a given inhibitor. Such a hypothesis has previously been invoked to explain the inhibitor and substrate binding to chymotrypsin by Niemann *et al.***

THE 6-PHENYLPYRIMIDINE analog (I) (1) of tetrahydrofolic acid was observed to be a twelvefold better inhibitor of dihydrofolic reductase at pH 7.4 than the prototype 6-methylpyrimidine analog (II) (2, 3). When assayed



I, R = $C_6H_5$
II, R = $CH_3$

with folic acid as substrate at pH 6.1, I was found to be a twentyfold better inhibitor of the reductase than II (1, 4). Three possible explanations for this increased binding were proposed (1)—namely, (*a*) a charge-transfer complex, (*b*) the phenyl influences the binding of the pyrimidine ring, and (*c*) hydrophobic bonding. Since inductive effects by substitutents on the phenyl ring could not be completely correlated (5), explanations (*a*) and (*b*) can now be considered unlikely. The emergence of strong hydrophobic bonding by 5-alkylpyrimidines (6) and the further experimental evidence (7, 8) for hydrophobic bonding by the aryl group of 1-aryl-1,2-dihydro-*s*-triazines, 2-aryl-*s*-triazines, and 5-arylpyrimidines suggested that the mode of phenyl binding of 6-phenylpyrimidines be further investigated from the standpoint of hydrophobic bonding.

Rather than proceeding chronologically on the development of the possible hydrophobic bonding of the phenyl group of 6-phenylpyrimidines—as is customarily done with papers from this laboratory—it is somewhat easier to follow the evidence if the assumptions on binding are presented in logical order rather than by order arrived at from the tortuous trail of chronology.

## DISCUSSION

The following recent developments on binding of inhibitors to dihydrofolic reductase are pertinent to the arguments to follow.

Received November 8, 1965, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo.
Accepted for publication December 22, 1965.
This work was generously supported by grants CA-05867 and CA-06624 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
Previous paper: Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *J. Pharm. Sci.*, **55**, 302(1966).
* Address inquiries to the Department of Chemistry, University of California, Santa Barbara.