REFERENCES

(1) Cavallini, G., Massarani, E., Nardi, D., and D'Ambrosio, R., *J. Am. Chem. Soc.*, **79**, 3514(1957).
(2) Milla, E., and Grumelli, E., *Farmaco Pavia Ed. Sci.*, **14**, 714(1959).
(3) Crescenzi, E., Marazzi Uberti, E., and Coppi, G., *Arch. Ital. Sci. Farmacol.*, to be published.
(4) Gold-Aubert, P., and Locher, A., *Helv. Chim. Acta*, **42**, 1156(1959).
(5) Takeichi, K., *Hakko Kogaku Zasshi*, **38**, 167(1960); through *Chem. Abstr.*, **55**, 14374*i*(1961).
(6) Vaidya, M. G., and Cannon, J. G., *J. Med. Pharm. Chem.*, **5**, 389(1962).
(7) Foo, P., and Wang, T., *J. Chinese Chem. Soc.*, **8**, 374, (1961); through *Chem. Abstr.*, **58**, 13881*b*(1963).
(8) Brisou, J., and de Rautlin de la Roy, J., *Compt. Rend. Soc. Biol.*, **158**, 642(1964).
(9) Cannon, J. G., *Div. Med. Chem. Am. Chem. Soc.*, Abstracts of papers presented at Chicago, Ill., September 3–8, 12–0(1961).
(10) Crescenzi, E., Coppi, G., and Bietti, G., *Farmaco Pavia Ed. Sci.*, **20**, 134(1965).
(11) Merz, K. W., and Stolte, H., *Arch. Pharm.*, **292**, 496 (1959).
(12) Cavallini, G., and Massarani, E., *Farmaco Pavia Ed. Sci.*, **11**, 167(1956).
(13) Wilson, E., and Tishler, M., *J. Am. Chem. Soc.*, **73**, 3635(1951).
(14) Janssen, P. A. S., U. S. pat. 2,997,472(1961).
(15) Hromatka, O., and Engel, E., *Ber.*, **76B**, 712(1943).
(16) Irwin, S., Communication at the Gordon Research Conference on Medicinal Chemistry, August 3–7, 1959, New London, N. H.
(17) Wilhelmi, G., *Medizinische*, **50**, 1591(1952).
(18) Adami, E., and Marazzi, E., *Arch. Intern. Pharmacodyn.*, **107**, 322(1956).
(19) Domenjoz, R., *Ann. N.Y. Acad. Sci.*, **86**, 263(1960).
(20) Magnus, R., *Pflügers Arch. Ges. Physiol.*, **102**, 123 (1904).

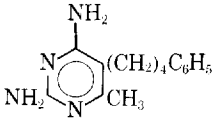
Analogs of Tetrahydrofolic Acid XXXII

Hydrophobic Bonding to Dihydrofolic Reductase IV. Inhibition by *p*-Substituted Benzoic and Benzoyl-L-glutamic Acids

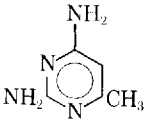
By B. R. BAKER*, THOMAS J. SCHWAN, JAROSLAV NOVOTNY, and BENG-THONG HO

A series of *p*-substituted benzoic acids and benzoyl-L-glutamic acids were synthesized and evaluated as inhibitors of dihydrofolic reductase in order to gain information on the position of the hydrophobic bonding region of the enzyme with respect to the position of the substrate, dihydrofolate, when the latter is complexed to the enzyme. Hydrophobic bonding by the *p*-substituted benzoyl-L-glutamic acids was reached 4–8 atoms from the *p*-position, thus indicating that the hydrophobic bonding region was not between the pyrimidyl and *p*-aminobenzoyl moieties of the substrate, dihydrofolate; in contrast, hydrophobic bonding with *p*-substituted benzoic acids was reached 1–4 atoms from the *p*-position, thus indicating that the *p*-substituted benzoic acids were complexed in a different region of the enzyme than the *p*-substituted benzoyl-L-glutamic acids.

THE DISCOVERY of strong hydrophobic bonding to dihydrofolic reductance with alkyl pyrimidines and 1,2-dihydro-*s*-triazines (1) has led to a major program in this laboratory on the nature, stereochemistry, and position of this hydrophobic bonding. That the aryl group of 1-aryl-1,2-dihydro-*s*-triazines and 5-arylpyrimidines of the pyrimethamine[1] type is also most probably complexed to dihydrofolic reductase by hydrophobic bonding has received strong experimental support (2). Furthermore, the 5-alkyl group of 5-alkyl-2,4-diamino-6-pyrimidines had maximum hydrophobic bonding with the 3-methylbutyl group (3); less binding was observed with 2-methylbutyl, butyl, and 1-methylbutyl, in that order. Furthermore, cyclohexyl was as good as *n*-butyl, but cyclopentyl was considerably poorer; thus, there are definite conformational requirements for alkyl groups to give maximum hydrophobic bonding (3). The magnitude of hydrophobic bonding to dihydrofolic reductase can be enormous; the phenylbutyl group of I alone had a free energy of binding of 6.0 Kcal./mole, equivalent to 80% of the total binding of the substrate, dihydrofolate. This binding by the phenylbutyl group could be calculated from the increment of 40,000 observed between I and II in their relative ability to inhibit dihydrofolic reductase.



I



II

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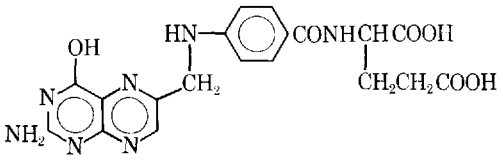
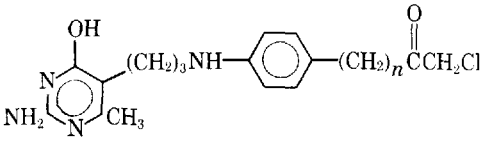
[1] Marketed as Daraprim by Burroughs Wellcome Co.



III



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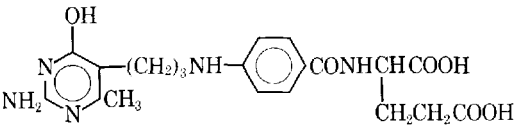
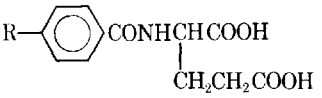


V



VI, R = NH_2
VII, R = H

The discovery of this hydrophobic bonding creates a serious problem from the standpoint of design of an active-site-directed irreversible inhibitor (4) of dihydrofolic reductase—namely, where is the hydrophobic region with respect to the binding region for the *p*-aminobenzoyl moiety of folic acid (III) on dihydrofolic reductase? Is this hydrophobic region between the *p*-aminobenzoyl and the pyrimidine moieties or is it elsewhere, such as near the region where the 4-oxo group of folic acid is in the enzyme–inhibitor complex? If the hydrophobic region is not between these 2 moieties, then the anilino group of IV would probably not be complexed with the *p*-aminobenzoyl locus. If the anilino group of IV is complexed with a different locus, then an inhibitor of type IV (5) would not have its alkylating function sufficiently neighboring to the glutamate binding points of folic acid (III) to alkylate irreversibly such a binding point. Furthermore, if the anilino group of IV is complexed to a hydrophobic region, then by definition there is apt not to be a nucleophilic group in this region of the enzyme. A variety of approaches to answer this important question were initiated, since it could be expected to be difficult to obtain an unequivocal answer; one approach is the subject of this paper.

DISCUSSION

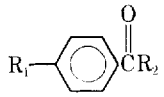
p-Aminobenzoyl-L-glutamic acid (VI) was measured as an inhibitor of dihydrofolic reductase. With the consideration that VI did not have the pyrimidyl moiety of the prototype inhibitor, V (6), the 12 m*M* concentration of VI needed for 50% inhibition (Table I) compared favorably with V, where 0.10 m*M* was needed for 50% inhibition (5). The 120-fold difference in binding between V and VI is a difference of 2.9 Kcal./mole. Since it can be calculated that 2-amino-6-methyl-4-pyrimidinol has a free energy of binding to dihydrofolic reductase of 3.0 Kcal./mole (1), the agreement is fairly reasonable for the amount of inhibition that can be expected when the pyrimidyl moiety is removed from V to give VI; these results support the suggestion that *p*-aminobenzoyl-L-glutamate (VI) binds to the same region of dihydrofolic reductase that complexes this moiety of V.

With the now reasonable assumption that *p*-aminobenzoyl-L-glutamic acid (VI) binds at the same locus as the *p*-aminobenzoyl-L-glutamate moiety of folic acid (III) then hydrophobic bonding by alkyl, aryl, or aralkyl groups substituted at the *p*-position of VII should be observed if the hydrophobic bonding region were between the pyrimidyl and *p*-aminobenzoyl-L-glutamate moieties of folic acid (III). Note that the *p*-amino group of VI contributed little to inhibition since benzoyl-L-glutamic acid (VII) was about as good an inhibitor (Table I). Little, if any, hydrophobic bonding occurred when a 3-bromopropyl (X) or a phenyl group (XIV) was introduced into the *p*-position. However, when the *p*-substitutent was lengthened to *n*-octyl (XII), about a ninetyfold increase in binding occurred (Table I). These data clearly show that the hydrophobic region begins at least 3 atoms away from the *p*-position of benzoyl-L-glutamic acid (VII). Since the distance between the pyrimidyl and benzoyl-L-glutamate moieties of folic acid (III) consists of a 4-atom chain, it is clear that the hydrophobic region is elsewhere than between these 2 moieties. It should again be emphasized that this interpretation contains the assumption that VII and the *p*-aminobenzoyl-L-glutamate moiety of folic acid (III) are complexed to the same region on the enzyme. Although this interpretation is quite reasonable, it is not unequivocal.

A previous study on the relative contribution of the functional groups (XXIII–XXVI) of the carboxy-L-glutamate moiety of the prototype inhibitor (V) to folic reductase (7), is now open to question on the validity of the interpretations, since it was made prior to the discovery of the strong hydrophobic bonding to dihydrofolic reductase (1). A particularly plaguing inconsistency was the fact that pteroic acid was eightyfold less effective than folic acid (III) as an inhibitor of dihydrofolic reductase (8), whereas XXVI was as good or better an inhibitor than V (5, 7). Although V may be complexed in the same manner as folic acid (III) to dihydrofolic reductase, the possibility existed that the less polar relatives (XXIII–XXVI) were complexed with the hydrophobic region rather than the *p*-amino benzoyl-L-glutamate region. Furthermore, it was previously noted that XXIV was almost as effective as V when assayed with the dihydrofolic reductase system (5); in the folic reductase system, XXIV was one-sixth as effective as V and XXV was about one-half as effective as V (7).

In Table I it can be noted that *p*-aminobenzoyl-L-glutamic acid (VI) was a greater than seventeenfold better inhibitor than *p*-aminohippuric acid (XXI)

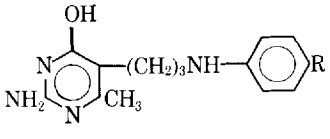
TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_1	R_2[a]	mM Concn.	% Inhibition	Estimated mM Concn. for 50% Inhibition[b]
VI	NH_2	GL	12	50	12
VII	H	GL	16	50	16
VIII	H	OH	75	0	>300[c]
IX	$Br(CH_2)_3$—	OH	17	50	17
X	$Br(CH_2)_8$—	GL	4.5	50	4.5
XI	n-C_8H_{17}—	OH	1.0[d,e]	15	5.6
XII	n-C_8H_{17}—	GL	0.17[d]	50	0.17
XIII[f]	C_6H_5—	OH	6.7	50	6.7
XIV	C_6H_5—	GL	6.5[d]	50	6.5
XV	$C_6H_5CH_2$—	OH	10	50	10
XVI	$Br(CH_2)_4$—	OH	6.2	50	6.2
XVII	$Cl(CH_2)_5$—	OH	6.0	50	6.0
XVIII[f]	C_6H_5CO—	OH	13[d]	50	13
XIX	$CONHC_6H_4$—$(CH_2)_2CH(CH_3)_2$-p—	OH	0.20[d,e]	0	>0.80[c]
XX	$CONHC_6H_4CH_2$-p—$(CH_2)_2CH(CH_3)_2$-p—	OH	0.60[d,e]	0	>2.4[c]
XXI[f]	NH_2	—$NHCH_2COOH$	50	0	>200[c]
XXII[g]	NH_2	—$NH(CH_2)_3COOH$	75	0	>300[c]
XXVI	m-$NH_2C_6H_4CH_2NH$-p—	OH	0.76	50	0.76
XXVII	m-$NH_2C_6H_4CH_2N$-p—$COCH_3$	OH	9	0	>36[c]
XXXVIII	m-$NO_2C_6H_4CH_2NH$-p—	OH	1.8	50	1.8

Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction 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 and 1 mM Versene as previously described (20). Solutions of inhibitors were prepared in the 0.05 M Tris buffer by adjustment of the pH to 7.4 with 0.1 N KOH, unless otherwise indicated. [a] GL = L-glutamate. [b] The concentration for 50% inhibition was determined by plotting V_0/VI against I for several concentrations of I that would give 30–70% inhibition, where V_0 = velocity without inhibitor, VI = velocity with inhibitor, and I = concentration of inhibitor; the concentration for 50% inhibition was obtained where $V_0/VI = 2$ (21, 22). When 50% inhibition could not be reached due to lack of solubility, the line was extended to the 50% inhibition point; the less the maximum inhibition, the greater is the error in the estimated concentration for 50% inhibition. [c] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is greater than 4 times the concentration measured. [d] Solution of inhibitor prepared in 1:1 N,N-dimethylformamide–Tris buffer by adjusting the pH to 7.4 with Tris base in 50% aqueous N,N-dimethylformamide; the assay was run in 10% N,N-dimethylformamide. [e] Maximum solubility in cell in 10% N,N-dimethylformamide. [f] Commercial sample. [g] See *Reference 7* for preparation.

and greater than a 33-fold better inhibitor than γ-(*p*-aminobenzoyl)butyric acid (XXII). If it is again assumed that *p*-amino benzoyl-L-glutamate *per se* as well as this moiety of V are complexed to the binding locus for the *p*-aminobenzoyl-L-glutamate moiety of folic acid (III), it follows that XXI–XXVI are complexed elsewhere, perhaps in the hydrophobic region.



V, R = —CONHCH(CH$_2$CH$_2$COOH)COOH

XXIII, R = H

XXIV, R = CONHCH$_2$COOH

XXV, R = CONHCH(CH$_2$CH$_2$COOH)CH$_2$

XXVI, R = COOH

In order to obtain experimental evidence for or against this hypothesis, some *p*-substituted benzoic acids were investigated for their ability to inhibit dihydrofolic reductase.

Note that benzoic acid (VIII) showed no inhibition at a concentration of 75 mM, but that *p*-substitution with a bromopropyl gave a compound (IX) that showed 50% inhibition at 17 mM. Lengthening the chain to bromobutyl (XVI) or chloropentyl (XVII) gave still better inhibitors, with 50% inhibition at about 6 mM. Furthermore, *p*-phenyl-(XIII), *p*-benzyl-(XV), and *p*-benzoylbenzoic (XVIII) acids showed 50% inhibition in the 6–13 mM range. Thus, these hydrophobic groups could give as much as a greater than fiftyfold increase in binding compared to benzoic acid (VIII). Whether there were limitations on the length of this *p*-group for hydrophobic bonding could not be shown by XI, XIX, or XX due to insolubility; however, it was apparent that longer groups in XI and XX could not have given much further increment in hydrophobic bonding. Thus the hydrophobic region begins soon after the first atom in the *p*-position of benzoic acid, when these benzoic acids are complexed to dihydrofolic reductase; in contrast, the *p*-position of benzoyl-L-glutamate must have greater than a 4-

atom chain before hydrophobic bonding is detected.

That hydrophobic bonding can occur also with *N*-substituents on *p*-aminobenzoic acid is shown with the *m*-aminobenzyl and the *m*-nitrobenzyl derivatives, XXVI and XXXVIII (Table I); XXVI is actually the best benzoate-type inhibitor in Table I. Surprisingly, *N*-acetylation on the *p*-amino group led to a greater than 47-fold loss in binding to dihydrofolic reductase. A possible interpretation of this result will be discussed (9).

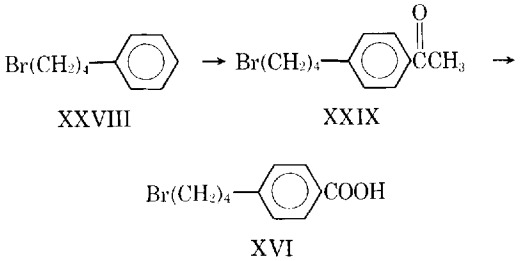
Regardless of the mode of binding of benzoyl-L-glutamic acid (VII) and the substituted benzoic acids, it is clear that the benzoyl group of the 2 classes of inhibitors are complexed to different regions of the enzyme. Whether the benzoyl-L-glutamates are complexed to the normal region for this moiety in folic acid, and the benzoic acids are complexed in hydrophobic region cannot yet be proven unequivocally. However, as a working hypothesis, such modes of binding have led to useful results which could not have been accrued otherwise. These results on the nature and position of hydrophobic bonding are the subjects of additional papers to be submitted in the near future.

EXPERIMENTAL

Methods.—*p*-(4-Bromobutyl)benzoic acid (XVI) was synthesized from 4-(bromobutyl)benzene (XXVIII) by Friedel-Crafts acetylation to XXIX followed by hypobromite oxidation; similarly, XVII was synthesized. (Scheme I.) The synthesis of IX (10) and XI (11) by this route have been previously recorded.

The *p*-substituted benzoyl-L-glutamic acids (X, XII, and XIV) were synthesized by condensation of the appropriate acid chloride in an organic solution with an aqueous sodium carbonate solution of L-glutamic acid; the known benzoyl-L-glutamic acid (VII) (12) was also synthesized in this manner. All but XIV were isolated as the bis-cyclohexylammonium salts, which were readily crystallized and purified, in contrast to the free acids which were difficult to crystallize or purify, or both.

4′-Nitrodiphenic acid (XXXII) was synthesized by modification of a procedure in the patent literature (13, 14) by Friedel-Crafts acetylation of XXX in nitrobenzene to XXXI followed by sodium hypobromite oxidation to XXXII without isolation of XXXI. Catalytic reduction to XXXIII proceeded smoothly in a 50% ethanolic solution of the sodium salt in the presence of Raney nickel; this method was considered to be more convenient than the ammonium sulfide reduction described previously (15). Acylation with 4-methylvaleroyl chloride to XIX in acetone in the presence of potassium carbonate proceeded satisfactorily. Similarly, acylation of



XXVIII XXIX XVI

Scheme I

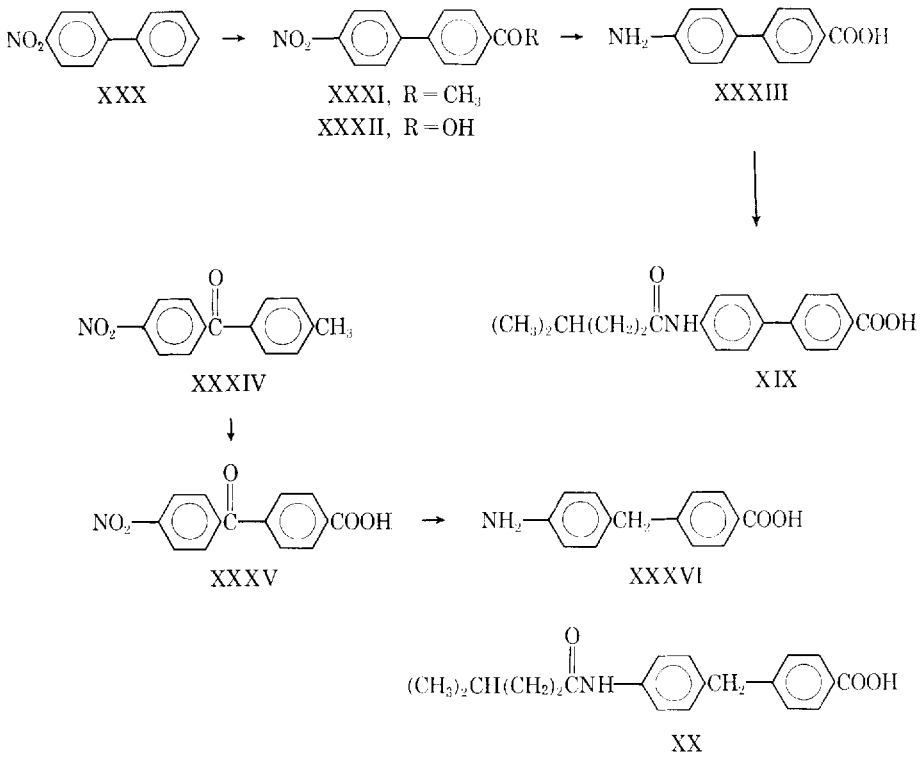
XXXVI to XX was performed. The required intermediate amino acid (XXXVI), although known in the literature (15), was prepared by an alternate route. Oxidation of 4-methyl-4′-nitrobenzophenone (XXXIV) (16) with chromium trioxide in acetic acid gave the acid (XXXV) in 55% yield of analytically pure material. Oxidation of XXXIV to XXXV with potassium permanganate in aqueous alkali or in acetone proceeded poorly due to overoxidation to water-soluble products. Huang-Minlon reduction of the carbonyl group of XXXV with hydrazine in diethyleneglycol containing potassium hydroxide also gave concomitant reduction of the nitro group; the desired amino acid (XXXVI) was obtained in 82% yield. (Scheme II.)

Condensation of *m*-nitrobenzaldehyde with *p*-aminobenzoic acid in ethanol gave the anil (XXXVII) in 96% yield. Reduction of a suspension of the anil in methanol with sodium borohydride gave the benzylamine (XXXVIII) in 98% yield. Further hydrogenation in 2-methoxyethanol with a platinum oxide catalyst afforded the desired amino acid (XXVI). Acetylation of XXXVIII with boiling acetic anhydride gave an anhydride of the desired *N*-acetyl derivative which was readily converted to XXXIX with 1 *N* sodium hydroxide in quantitative over-all yield. Catalytic reduction in ethanol afforded the requisite amino acid (XXVII) in good yield. (Scheme III.)

Synthesis.—Melting points were taken on a Fisher-Johns apparatus or a Mel-Temp block, and those below 230° are corrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer 137B spectrophotometer unless otherwise indicated; ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer.

p-(4-Bromobutyl)benzoic Acid (XVI).—To a stirred suspension of 5.6 Gm. (42 mmoles) of anhydrous aluminum chloride in 21 ml. of carbon disulfide, cooled in an ice bath and protected from moisture, was added 3.0 ml. (42 mmoles) of acetyl chloride over a period of 10 min. Then a mixture of 10.2 Gm. (48 mmoles) of 4-bromobutylbenzene (17) and 6.5 ml. (90 mmoles) of acetyl chloride was added as rapidly as reflux would allow. After the addition was complete, the mixture was stirred for 2.5 hr., then poured into a mixture of 50 Gm. of ice and 10 ml. of 12 *N* aqueous hydrochloric acid. To the mixture was added 40 ml. of benzene; an insoluble yellow solid was removed by filtration. The separated aqueous layer was extracted with three 10-ml. portions of benzene. The combined benzene extracts were washed successively with 10% hydrochloric acid, 2 *N* aqueous potassium hydroxide, and water. Dried with magnesium sulfate, the benzene solution was spin-evaporated *in vacuo* leaving 12.6 Gm. of the crude acetophenone, XXIX (18).

To an ice cold solution of 1.55 ml. (0.02 mmole) of bromine in 28 ml. of water containing 3.3 Gm. of sodium hydroxide was added 15 ml. of dioxane. Then a solution of 2.5 Gm. (10 mmoles) of crude XXIX in 5 ml. of dioxane was added over a period of 45 min. After being stirred for an additional 2 hr. in the ice bath, the solution was acidified with 10 ml. of 12 *N* aqueous hydrochloric acid. A brown oil separated that soon solidified. The product was collected on a filter and washed with water until the washings were colorless; yield, 1.21 Gm. (46% based on XXVIII), m.p. 135–137°.



Scheme II

Recrystallization from benzene–petroleum ether (b.p. 30–60°) gave white needles, m.p. 136–137°. $\nu_{max.}$ 2680–2550 (acidic OH); 1680 (C=O); 859 cm.$^{-1}$ (*p*-C_6H_4).

Anal.—Calcd. for $C_{11}H_{13}BrO_2$: C, 51.4; H, 5.08. Found: C, 51.2; H, 5.06.

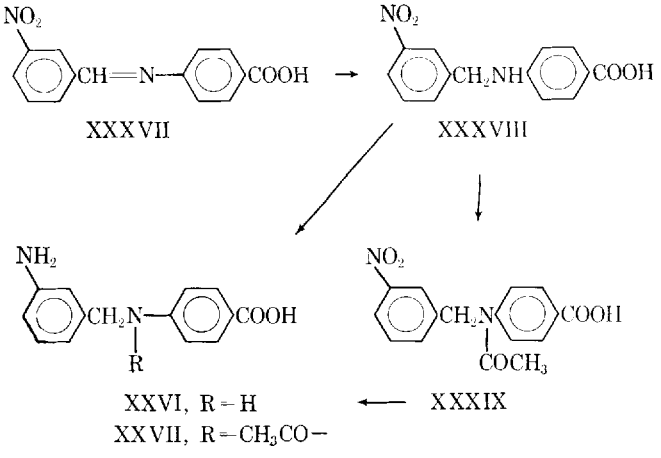
Similarly, IX (10) and XI (11) were prepared.

p-(5-Chloropentyl)benzoic Acid (XVII).—This compound was prepared in 46% over-all yield from 5-chloropentylbenzene as described for XVI. Recrystallization from benzene–petroleum ether (b.p. 30–60°) gave white crystals, m.p. 109–111°. $\nu_{max.}$ 1690 (C=O); 860, 840 cm.$^{-1}$ (*p*-C_6H_4).

Anal.—Calcd. for $C_{12}H_{15}ClO_2$: C, 63.6; H, 6.65. Found: C, 63.3; H, 6.49.

That this was a *p*-substituted benzoic acid, as indicated by the infrared spectrum, was further verified by oxidation to terephthalic acid.

p-(n-Octyl)benzoyl-L-glutamic Acid (XII) (Cyclohexylammonium Salt).—A solution of 351 mg. (1.5 mmoles) of XI, 10 ml. of chloroform, 0.9 Gm. of thionyl chloride, and a trace of pyridine was refluxed for 30 min. when gas evolution was complete. Solvent was removed by evaporation *in vacuo*. A solution of the residual acid chloride in 3 ml. of acetone was added in 1 portion to a magnetically



Scheme III

stirred solution of 147 mg. (1 mmole) of L-glutamic acid, in 0.67 ml. of 3 *N* sodium hydroxide (2 mmoles) and 5 ml. of water containing 160 mg. (1.5 mmoles) of sodium carbonate. After being stirred overnight at ambient temperature, the reaction mixture was spin-evaporated until the acetone was removed, then the solution was diluted with 50 ml. of water and adjusted to about pH 5 with dilute hydrochloric acid. After standing for a few hours, the mixture was filtered, and the recovered XI was washed with water. The filtrate was adjusted to about pH 1 with 3 *N* hydrochloric acid. The semisolid (XII) which separated (224 mg., 0.62 mmole) was dissolved in 5 ml. of methanol, and 155 mg. of cyclohexylamine (1.55 mmoles, 25% excess) was added. The solution was spin-evaporated *in vacuo* yield, 348 mg. (62%) of crude product. The residue was recrystallized twice from absolute ethanol–ether; yield, 71 mg. (13%) of analytically pure material, m.p. 170–175°. $\lambda_{max.}$ (pH 1, 13) 245 mμ; $\nu_{max.}$ (Nujol) 2200 (NH^+), 1670–1640, 1560–1500 cm.$^{-1}$ (COO^-, amide I and II, C=C).

Anal.—Calcd. for $C_{32}H_{55}N_3O_5$: C, 68.4; H, 9.87; N, 7.48. Found: C, 68.5; H, 9.96; N, 7.59.

No attempt was made to isolate additional material from the mother liquors.

p-(3-Bromopropyl)benzoyl-L-glutamic Acid (X) (Cyclohexylammonium Salt).—This compound was prepared as described for XII except that five 40-ml. extractions with ethyl acetate were employed since the product was fairly water soluble. Recrystallization from methanol–acetone afforded a 30% yield of the bis-cyclohexylammonium salt of X, m.p. 145–150°. A second recrystallization from the same solvent pair gave white crystals, m.p. 149–152°. $\nu_{max.}$ (Nujol) 2220 (NH^+); 1610–1640, 1590–1510 cm.$^{-1}$ (COO^-, amide I and II, C=C).

Anal.—Calcd. for $C_{27}H_{44}BrN_3O_5$: C, 56.8; H, 7.77; N, 7.36. Found: C, 56.6; H, 7.55; N, 7.12.

p-Phenylbenzoyl-L-glutamic Acid (XIV).—A mixture of 1.93 Gm. (10 mmoles) of 4-biphenylcarboxylic acid, 20 ml. of chloroform, 4 ml. (50 mmoles) of thionyl chloride, and a trace of pyridine was refluxed for 16 hr., then spin-evaporated *in vacuo*. The residual crystalline acid chloride was dissolved in 15 ml. of carbon tetrachloride, then added over a period of 1 hr. to a stirred solution of 1.47 Gm. (10 mmoles) of L-glutamic acid, and 3.41 Gm. (32.5 mmoles) of sodium carbonate in 15 ml. of water that was layered with 8 ml. of carbon tetrachloride. After being stirred for an additional 24 hr., the layers were separated; the aqueous layer was adjusted to pH 5.5 with 3 *N* hydrochloric acid, then washed with chloroform to remove 4-biphenylcarboxylic acid. The aqueous solution then was adjusted to pH 1.5. The white, difficultly filterable precipitate was collected by centrifugation, then washed with water. Recrystallization from aqueous acetone gave 1.08 Gm. (33%) of white crystals, m.p. 178–179°. The compound moved as a single spot on thin-layer chromatography on Silica Gel G with propanol–water (37:13) when viewed under ultraviolet light. The compound has $\nu_{max.}$ 3320 (amide NH), 3000, 2650, 2550 (broad acidic OH); 1700 (carboxyl C=O), 1630, 1600, 1580, 1520, 1500 (amide I and II, C=C); 850 (*p*-C_6H_4); 750, 685 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1) 273 mμ (ε 25,700); (pH 13) 272 mμ (ε 28,500).

Anal.—Calcd. for $C_{18}H_{17}NO_5$: C, 66.0; H, 5.23; N, 4.28. Found: C, 65.8; H, 4.99; N, 4.22.

Benzoyl-L-glutamic Acid (VII) (Cyclohexylammonium Salt).—A solution of L-glutamic acid (10 mmoles) in aqueous sodium carbonate was acylated with 1.42 ml. (15 mmoles) of benzoyl chloride in 8 ml. of carbon tetrachloride as described for the preparation of XIV. After removal of the benzoic acid at pH 1, the filtrate was spin-evaporated to dryness *in vacuo*. The residue was extracted with acetone. The filtered solution was spin-evaporated *in vacuo* leaving 2.20 Gm. (88%) of VII as an oil which solidified after several days, m.p. 125–130°. [Lit. m.p. 139–140° (12).]

To a solution of 638 mg. (2.5 mmoles) of VII in 5 ml. of ethanol was added 550 mg. (5.5 mmoles) of cyclohexylamine. Some of the cyclohexylammonium salt (449 mg., m.p. 186–190°) separated immediately, and an additional 600 mg. (total 94%), m.p. 180–190°, was isolated from the filtrate. Recrystallization from absolute ethanol–ether gave 849 mg. (75%) of white crystals, m.p. 191–193°. $\nu_{max.}$ 3250 (NH), 3000–2600, 2200 (NH^+); 1660, 1640, 1580–1520 (COO^-, amide I and II, C=C); 712, 692 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1, 7) 229 (ε 14,500), 245 mμ (sh) (ε 8000).

Anal.—Calcd. for $C_{24}H_{39}N_3O_5$: C, 64.4; H, 8.74; N, 9.35. Found: C, 64.4; H, 8.70; N, 9.40.

4′-Nitro-4-biphenylcarboxylic Acid (XXXII).—To a stirred solution of 30 Gm. (0.15 mole) of 4-nitrobiphenyl in 100 ml. of nitrobenzene protected from moisture was added 39 Gm. (0.29 mole) of anhydrous aluminum chloride. The mixture was heated to 50–53°, then a solution of 19.5 Gm. of acetyl chloride in 17 ml. nitrobenzene was added over a period of 8 hr. After being heated an additional 4 hr. at 62–64°, the mixture was stirred overnight at ambient temperature, then poured into 400 ml. of iced water. The separated nitrobenzene layer was washed with water (3 × 50 ml.), then poured in a thin stream into an ice-cooled stirred solution of 72 Gm. of bromine in 100 ml. of water containing 50 Gm. of sodium hydroxide and 300 Gm. of ice. The stirred mixture was gradually warmed to 70° over a period of about 30 min., then maintained at 60–70° for 1.5 hr.; during this time it was necessary to remove the heating bath occasionally to keep the temperature from rising above 70°. The mixture then was stirred under a reflux condensor in a bath at 100–105° for 2 hr. The orange sodium salt was collected by filtration after chilling at 15° for 3 hr. The sodium salt was extracted with three 800-ml. portions of boiling water, filtering the solution each time through glass wool to remove a brown insoluble by-product. The combined filtrates were reheated to dissolve the sodium salt, then acidified to pH 2–3 with 12 *N* hydrochloric acid; yield, 10.2 Gm. (28%), m.p. 338–342° dec. Recrystallization from 2-methoxyethanol gave 6.65 Gm. (18%), m.p. 345–348° dec. [Lit. m.p. 340°, 345° (13, 14).]

4′-Amino-4-biphenylcarboxylic Acid (XXXIII).—A solution of 243 mg. (1 mmole) of XXXII in 100 ml. of 50% ethanol and 2 ml. of 2 *N* sodium hydroxide was shaken with hydrogen at 2–3 Atm. in the presence of about 1 Gm. of Raney nickel; reduction was complete in 30 min. The filtered solution was adjusted to pH 6 with glacial acetic acid, then spin-evaporated *in vacuo* to about 50 ml. The product

was collected on a filter and washed with water. Yield, 170 mg. (80%), m.p. 245–248°. [Lit. m.p. 243–246° (15).]

4-(4′-Nitrobenzoyl)benzoic Acid (XXXV).—4-Methyl-4′-nitrobenzophenone (XXXIV) was prepared from *p*-nitrobenzoyl chloride, toluene, and aluminum chloride; the yield, after recrystallization from ethanol was 95%, m.p. 120–121° (16).

To a stirred solution of 9.05 Gm. (37.5 mmoles) of XXXIV in 100 ml. of glacial acetic acid was added 9.5 Gm. (95 mmoles) of chromium trioxide in portions over a period of 1 hr. The mixture was refluxed for 16 hr., then poured into 1 L. of ice water. The product was collected on a pad of Celite, then washed with water until the washings were colorless. The filter cake was stirred at about 90° with 300 ml. of 3 *N* aqueous sodium hydroxide, then filtered hot. The filtrate was rewarmed to dissolve the sodium salt, then the hot solution was acidified to about pH 1 with hydrochloric acid. The product was collected on a filter and washed with water; yield, 6.71 Gm. (66%). Recrystallization from methanol gave 5.86 Gm. (55%) of light yellow crystals, m.p. 257–258°. $\nu_{max.}$ 3000, 2650, 2550 (broad acidic OH); 1670 (carboxyl C=O); 1650 (ketone C=O); 1600 (C=C); 1520 cm.$^{-1}$ (NO_2); $\lambda_{max.}$ (pH 1) 227 (ϵ 13,300), 273 mμ (ϵ 26,100); (pH 13) 276 mμ (ϵ 24,200)

Anal.—Calcd. for $C_{14}H_{19}NO_5$: C, 62.0; H, 3.34; N, 5.16. Found: C, 61.8; H, 3.35; N, 4.96.

4-(4′-Aminobenzyl)benzoic Acid (XXXVI).—To a solution of 6.87 Gm. of potassium hydroxide in 120 ml. of diethyleneglycol was added 9.49 Gm. (35 mmoles) of XXXV and 7 ml. of hydrazine hydrate. After being refluxed for 2 hr., the solution was slowly distilled until 10 ml. of liquid was collected over 2 hr. After being refluxed 1 hr. more, the solution was diluted with several volumes of water, then acidified to pH 4.8 with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water; yield, 6.51 Gm. (82%), m.p. 220–223°, that was suitable for further transformations. Recrystallization of a sample from methanol with the aid of Norit gave slightly pink crystals, m.p. 227–228°. [Lit. m.p. 228° (15).]

p-Benzylbenzoic Acid (XV).—Reduction of *p*-benzoylbenzoic acid, as described for the preparation of XXXVI, gave (after recrystallization from aqueous methanol) a 60% yield of product, m.p. 159–160°; an additional 24%, m.p. 152–153°, was isolated from the filtrate. [Lit. m.p. 156–157° (19).]

4-[4′-(4-Methylvaleramido)benzyl]benzoic Acid (XX).—To a stirred mixture of 908 mg. (4 mmoles) of XXXVI and 4.16 Gm. (30 mmoles) of potassium carbonate and 20 ml. of acetone was added 0.94 ml. (7.2 mmoles) of 4-methylvaleroyl chloride. After being stirred for 3 hr. at 40–50° under a reflux condensor, the mixture was poured into 20 ml. of 1 *N* aqueous hydrochloric acid, then cooled. The product was collected on a filter and washed with water; yield, 982 mg. (75%), m.p. 229–231°. Recrystallization from ethanol gave 769 mg. (59%) of white prisms, m.p. 237–238°. $\nu_{max.}$ 3300 (NH); 2800, 2650, 2540 (broad acidic OH); 1680 (carboxyl C=O); 1650 (amide I); 1600, 1580, 1570 (C=C); 1520 (amide II); 830 cm.$^{-1}$ (*p*-C_6H_4); $\lambda_{max.}$ (pH 1) 258 mμ (ϵ 10,800); (pH 13) 247 mμ (ϵ 25,400).

Anal.—Calcd. for $C_{20}H_{23}NO_3$: C, 73.8; H, 7.12; N, 4.30. Found: C, 74.0; H, 7.26; N, 4.35.

4′-(4-Methylvaleramido)-4-biphenylcarboxylic Acid (XIX).—This compound was prepared in 60% yield from XXXIII as described for the preparation of XX. Recrystallization from ethanol with the aid of Norit gave white plates, m.p. 318–320°; the principal peaks in the infrared spectrum were similar to those of XX. $\lambda_{max.}$ (pH 1) 300 mμ (ϵ 19,000); (pH 13) 288 mμ (ϵ 38,500).

Anal.—Calcd. for $C_{19}H_{21}NO_3$: C, 73.3; H, 6.80; N, 4.50. Found: C, 73.0; H, 6.92; N, 4.27.

N-(m-Nitrobenzylidene)-p-aminobenzoic Acid (XXXVII).—To a stirred hot solution of 3.47 Gm. (20 mmoles) of *p*-aminobenzoic acid in 10 ml. of 95% ethanol was added a hot solution of 3.02 Gm. (20 mmoles) of *m*-nitrobenzaldehyde in 10 ml. of ethanol. The product rapidly separated. The mixture was heated to the b.p., then cooled to room temperature. The product was collected on a filter and washed with ethanol; yield, 5.19 Gm. (96%), m.p. 252–253°. Recrystallization from aqueous methanol gave yellow crystals, m.p. 251–257°. $\nu_{max.}$ 1680 (carboxyl C=O); 1640, 1600, 1575 (C=C, C=N); 1520, 1350 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_{14}H_{10}N_2O_4$: C, 62.2; H, 3.73; N, 10.4. Found: C, 62.1; H, 3.81; N, 10.4.

N-(m-Nitrobenzyl)-p-aminobenzoic Acid (XXXVIII).—To a stirred suspension of 4.05 Gm. (15 mmoles) of XXXVII in 80 ml. of methanol was added in portions over a period of about 30 min., 2.3 Gm. (60 mmoles) of sodium borohydride. The amber solution was refluxed for 15 min., then spin-evaporated *in vacuo*. The residual sodium salt was dissolved in 50 ml. of water, then the solution was acidified to pH 6. The product was collected on a filter and washed with water; yield, 4.01 Gm. (98%), m.p. 237–238°. Recrystallization from aqueous 2-methoxyethanol gave yellow crystals, m.p. 247–248°. $\nu_{max.}$ 3400 (NH); 1670 (carboxyl C=O); 1600, 1575 (C=C, NH); 1520, 1350 (NO_2); 838 (*p*-C_6H_4); 769 cm.$^{-1}$ (*m*-C_6H_4).

Anal.—Calcd. for $C_{14}H_{12}N_2O_4$: C, 61.7; H, 4.44; N, 10.3. Found: C, 61.7; H, 4.60; N, 10.4.

N-Acetyl-N-(m-nitrobenzyl)-p-aminobenzoic Acid (XXXIX).—A mixture of 2.50 Gm. (9.18 mmoles) of XXXVIII and 15 ml. of acetic anhydride was refluxed for 30 min., then poured into 30 Gm. of iced water. The oily product was extracted with chloroform (3 × 10 ml.). The combined extracts, dried with magnesium sulfate, were spin-evaporated *in vacuo*, leaving an oil which solidified on trituration with petroleum ether to a solid (3.16 Gm.), m.p. 91–94°; the infrared spectrum showed anhydride bands at 1800 and 1725 cm.$^{-1}$. The anhydride was warmed with 30 ml. of 1 *N* aqueous sodium hydroxide until solution was essentially complete. The solution was clarified by filtration, then acidified to pH 2 with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water; yield, 2.84 Gm. (99%), m.p. 209–211°. Recrystallization from aqueous ethanol afforded 2.69 Gm. (93%) of nearly white needles, m.p. 212–213°. $\nu_{max.}$ 2600–2500 (broad acidic OH); 1680 (carboxyl C=O); 1625 (amide C=O); 1625, 1580, 1525 (C=C); 1525, 1340 (NO_2); 870 (*p*-C_6H_4); 785 cm.$^{-1}$ (*m*-C_6H_4).

Anal.—Calcd. for $C_{16}H_{14}N_2O_5$: C, 61.1; H, 4.49; N, 8.92. Found: C, 61.1; H, 4.29; N, 8.81.

N-(m-Aminobenzyl)-p-aminobenzoic Acid (XXVI).—A solution of 2.72 Gm. (10 mmoles) of

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.

REFERENCES

(1) Baker, B. R., Ho, B.-T., and Santi, D. V., *J. Pharm. Sci.*, **54**, 1415(1965).
(2) Baker, B. R., and Ho, B.-T., *J. Heterocyclic Chem.*, **2**, 335(1965).
(3) Baker, B. R., and Lourens, G. J., *ibid.*, **2**, 344(1965).
(4) Baker, B. R., *J. Pharm. Sci.*, **53**, 347(1964).
(5) Baker, B. R., and Jordaan, J. H., *J. Med. Chem.*, **8**, 35(1965).
(6) Baker, B. R., and Morreal, C. E., *J. Pharm. Sci.*, **52**, 840(1963).
(7) Baker, B. R., *et al.*, *J. Med. Chem.*, **7**, 24(1964).
(8) Bertino, J. R., Perkins, J. P., and Johns, D. G., *Biochemistry*, **4**, 839(1965).
(9) Baker, B. R., Ho, B.-T., Coward, J. K., and Santi, D. V., *J. Pharm. Sci.*, **55**, 302(1966).
(10) Blicke, F. F., and Lilienfeld, W. M., *J. Am. Chem. Soc.*, **65**, 2281(1943).
(11) Zaki, A., and Fahim, H., *J. Chem. Soc.*, **1942**, 307.
(12) Ronwin, S., *J. Org. Chem.*, **22**, 1180(1957).
(13) Schroeder, H. E., U. S. pat. 2,567,126 (1951); through *Chem. Abstr.*, **45**, 10606(1951).
(14) Franklin, R. C., U.S. pat. 2,742,499 (1956); through *Chem. Abstr.*, **51**, 1280(1957).
(15) Cologne, J., and Fichet, E., *Bull. Soc. Chim. France*, **1955**, 412.
(16) Zonis, L. S. *et al.*, *Zh. Obshch. Khim.*, **33**, 3141(1963); through *Chem. Abstr.*, **60**, 53689(1964).
(17) Truce, W. E., and Milinois, J. P., *J. Am. Chem. Soc.*, **74**, 974(1952).
(18) Norman, J. J., Heggie, R. M., and Larose, J. B., *Can. J. Chem.*, **40**, 1547(1962).
(19) Akagi, S., and Iwashige, T., *J. Pharm. Soc. Japan*, **74**, 610(1954).
(20) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(21) Baker, B. R., and Sachdev, H. S., *J. Pharm. Sci.*, **52**, 933(1963).
(22) Baker, B. R., *et al.*, *J. Med. Pharm. Chem.*, **2**, 633 (1960).

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

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