Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo

Analogs of Tetrahydrofolic Acid. XIII. On the Mode of Binding of the Anilino Group of 2-Amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol to Dihydrofolic Reductase and Thymidylate Synthetase (1,2)

B. R. Baker, Beng-Thong Ho, and Girish B. Chheda

Six analogs of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (II) with modifications in the anilino group have been synthesized in order to shed light on the mode of binding of the anilino group to dihydrofolic reductase and thymidylate synthetase. Replacement of the anilino group of II with *n*-butylamino (XI) led to great decrease in binding, indicating that the anilino group did not bind to these enzymes in the protonated form. Replacement of the anilino group of II with benzyl gave a compound (XIIb) which was a 26-fold and 1.5-fold better inhibitor of dihydrofolic reductase and thymidylate synthetase, respectively; these results indicated that the NH portion of the anilino group contributed less to the enzyme binding than did the benzene ring, and that the latter probably was bound as an electron acceptor in a charge-transfer complex to an electron-rich locus on the enzyme. Further evidence was also obtained that the benzene ring was complexing as an electron acceptor by placement of electron-donating or electron-withdrawing groups on the *p*-positions of anilino group, and by N-acetylation of the anilino group.

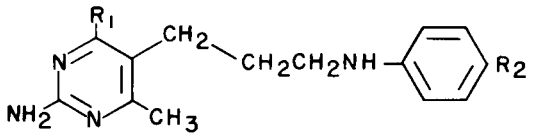
Inhibition of rat liver folic reductase by the pyrimidyl analog (I) of tetrahydrofolic acid has been previously reported (3); I was bound more tightly to this folic reductase than the substrate, folic acid. A further study on the contribution of the *p*-aminobenzoyl-L-glutamate moiety to binding to the folic reductase showed that the *p*-aminophenyl group contributed more to the enzymic binding than did the carboxy-L-glutamate moiety; II was bound one-sixth as well as substrate to folic reductase (4).

In the accompanying previous paper of this series (2), compounds II-IV and others were investigated as inhibitors of pigeon liver dihydrofolic reductase and *E. Coli* B thymidylate synthetase. The carboxy-L-glutamate moiety of III (5) made only a minor contribution to binding to the thymidylate synthetase since IV (5) was bound almost as well; IV was bound to thymidylate synthetase one-tenth as well as the substrate, 5,10-methylene-tetrahydrofolate, and one-seventh as well as the substrate (dihydrofolate) to the dihydrofolic reductase. Replacement of the 4-hydroxyl group of II by mercapto (IV) increased binding to thymidylate synthetase by 5-fold and to dihydrofolic reductase by 18-fold; the loss in binding by removal of the carboxy-L-glutamate moiety of I was more than compensated by the increased binding of the mercapto group in IV, that is, IV was a considerably better inhibitor of the two enzymes than was the prototype tetrahydrofolate analog, I.

We then posed the following series of questions: What is the mode of binding of the anilino group to the two enzymes? If it is the NH group that binds, is it by hydrogen bonding or is it by cationic binding of a protonated species? If it is benzene binding, does the benzene ring bind to an electron-poor center on the enzyme or to an electron-rich center (6)? Is the mode of binding the same for the two enzymes? If the mode of binding were different for the two enzymes, then greater selectivity in inhibition of the two enzymes should be possible by applying the answers to the above questions to synthesis of more selective compounds.

Selected modifications of II and IV were synthesized and evaluated as inhibitors of dihydrofolic reductase and thymidylate synthetase to help shed light on these questions; the results are the subject of this paper.

The synthesis of 2-amino-5-(3-anilinopropyl)-4-mercapto-6-methylpyrimidine (VIII, R = H) by condensation of the thiapyrano-pyrimidine V with aniline to give VI (R = H), followed by sodium borohydride reduction has been previously described (5). Further exploration has now shown that this method is not general. For example, *p*-dimethylaminoaniline condensed smoothly

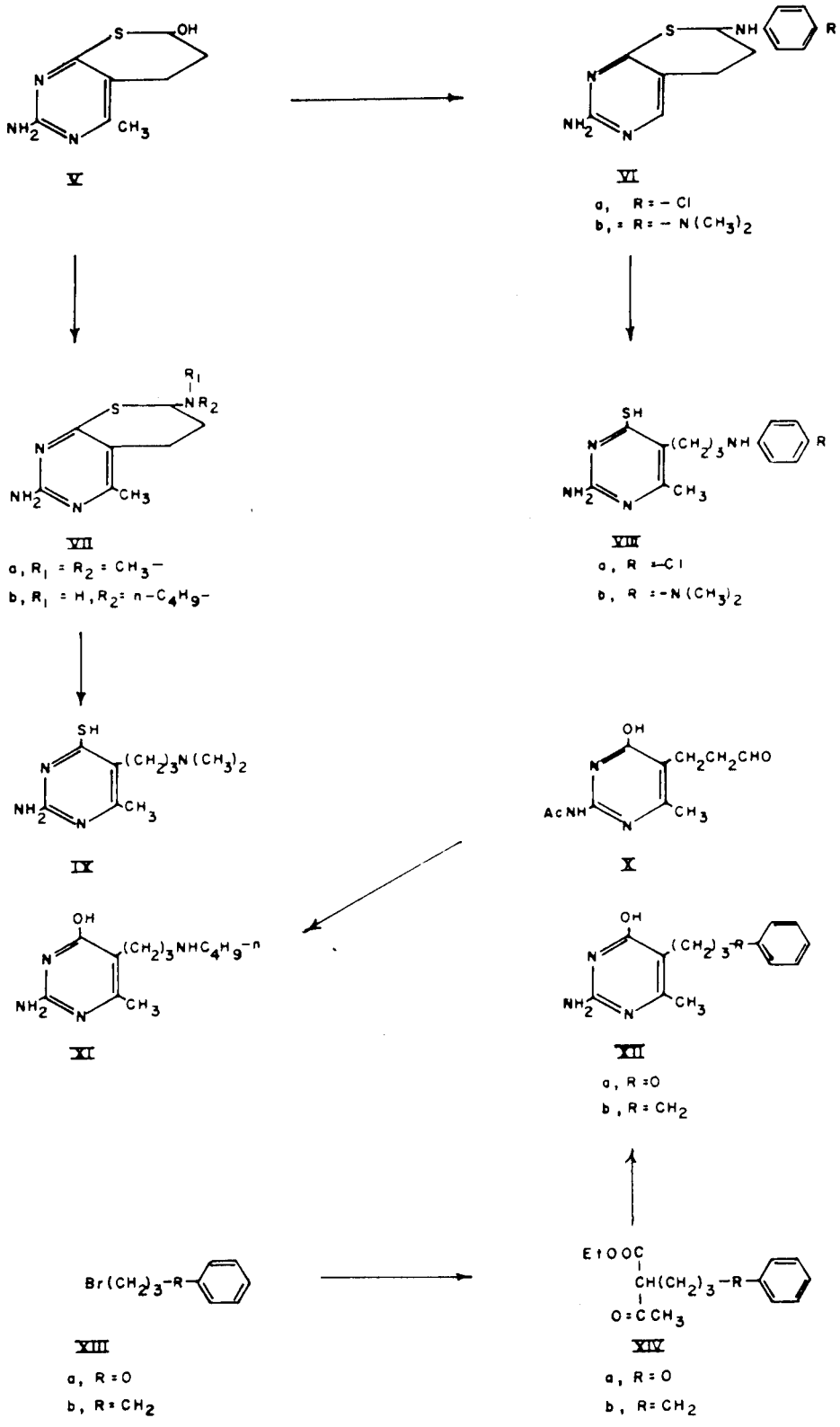


I, R_1 = OH, R_2 = — CONHCHCOOH
                        |
                        CH_2CH_2COOH

II, R_1 = OH, R_2 = H

III, R_1 = SH, R_2 = — CONHCHCOOH
                         |
                         CH_2CH_2COOH

IV, R_1 = SH, R_2 = H


V
VI
a, R = -Cl
b, R = -N(CH3)2
VII
a, R1 = R2 = CH3-
b, R1 = H, R2 = n-C4H9-
VIII
a, R = -Cl
b, R = -N(CH3)2
IX
X
XI
XII
a, R = O
b, R = CH2
XIII
a, R = O
b, R = CH2
XIV
a, R = O
b, R = CH2

with V to give VIb which could then be reduced with sodium borohydride to pure VIIIb. *p*-Chloroaniline behaved differently; the condensation product (VIa) could not be obtained pure, but borohydride reduction of the crude VIa gave pure VIIIa. N-Methylaniline and *p*-anisidine gave condensation products (VI) that could neither be purified, nor reduced to pure VIII. Aliphatic amines behaved still differently; dimethylamine reacted smoothly with V to give VIIa which was readily purified, but appeared to dissociate when put into basic solution for reduction, and IX was not formed. With *n*-butylamine, no condensation product could be isolated before (VIIb) or after reduction. Since an aliphatic amino side chain was deemed necessary for enzymological evaluation, 2-amino-5-(*n*-butylaminopropyl)-6-methyl-4-pyrimidinol (XI) was synthesized by reductive condensation of the pyrimidine-5-propionaldehyde (X) (3) with *n*-butylamine, using sodium borohydride as the reducing agent. The product (XI) was extremely water soluble, but could be isolated *via* its water-insoluble, crystalline dipicrate. The dipicrate was then converted to the dihydrochloride of XI with aqueous hydrochloric acid.

Two analogs of II, which had the anilino NH replaced by O or CH_2 (XII), were synthesized. Condensation of the alkyl bromides (XIII) with ethyl acetoacetate gave XIV which were oils, but gave proper IR and u.v. spectra for their assigned structures. Condensation of XIVa and b with guanidine carbonate in *t*-butyl alcohol gave the two crystalline pyrimidines (XII) in good yields.

EXPERIMENTAL

Melting points were taken on a Mel-temp block in capillary tubes or on a Fischer-Johns apparatus and those below 230° are corrected. Infrared spectra were determined in KBr disk with a Perkin and Elmer Model 137B recording spectrophotometer. Ultraviolet spectra were determined with a Perkin and Elmer Model 202 spectrophotometer.

2-Amino-5,6-dihydro-7-(*p*-dimethylaminoanilino)-4-methyl-7H-thiapyrano[2,3-d]pyrimidine (VIb).

A solution of 148 mg. (0.75 mole) of V (5) and 119 mg. (0.87 mole) of N,N-dimethyl-*p*-phenylendiamine in 25 ml. of absolute ethanol was refluxed one hr., then spin evaporated to dryness *in vacuo* leaving 257 mg. of a greenish-yellow solid, m.p. 160-165°. Recrystallization from toluene gave 131 mg. (55%) of pure product, m.p. 175-177°; ν max (KBr) 3320, 3180 (NH); 1630, 1550, 1520 cm^{-1} (NH, C=C, C=N); λ max (*p*H 1) 220 (ϵ, 22,200); 309 mμ (ϵ, 11,100); λ max (*p*H 7) 237 (ϵ, 22,200); 309 mμ (ϵ, 11,100); λ max (*p*H 13) 316 (ϵ, 15,700); shoulder at 242 mμ.

Anal. Calcd. for $C_{16}H_{21}N_5S$: C, 60.9; H, 6.71; N, 22.2. Found: C, 60.6; H, 6.83; N, 22.0.

2-Amino-7-(*p*-chloroanilino)-5,6-dihydro-4-methyl-7H-thiopyrano[2,3-d]pyrimidine (VIa).

Similarly, *p*-chloroaniline gave 2-amino-7-(*p*-chloroanilino)-5,6-dihydro-4-methyl-7H-thiopyrano[2,3-d]pyrimidine (VIa) in 84% yield, m.p. 190-192°. Recrystallization from toluene gave crystals of unchanged m.p. that failed to give satisfactory combustion values and may have been impure, but could be converted to pure VIIIa.

Anal. Calcd. for $C_{14}H_{15}ClN_4S$: C, 54.8; H, 4.93; N, 18.3; Cl, 11.6. Found: C, 55.9, 56.1; H, 5.69, 5.49; N, 19.2; Cl, 11.0, 11.3.

Unsatisfactory products were obtained from V by reaction with N-methylaniline, *p*-anisidine or *n*-butylamine.

2-Amino-5,6-dihydro-7-dimethylamino-4-methyl-7H-thiopyrano[2,3-d]pyrimidine (VIIa).

To a solution of 148 mg. (0.75 mole) of V in 25 ml. of boiling absolute ethanol was added 0.5 ml. of 20% dimethylamine in absolute alcohol. After being refluxed for one hr., the solution was spin-evaporated *in vacuo*; yield, 177 mg. of nearly white solid, m.p. 182-183°. Recrystallization from ethyl acetate gave 120 mg. (71%) of white crystals m.p. 184-185°; ν max (KBr) 3360, 3200 (NH); 1650, 1560, 1540 cm^{-1} (NH, pyrimidine); λ max (*p*H 1) 217 (ϵ, 16,200); 270 (ϵ, 9,100); 317 mμ (ϵ, 9,100); λ max (*p*H 7) 232 (ϵ, 14,000); 308 mμ (ϵ, 8,700); λ max (*p*H 13) 317 (ϵ, 14,400); inflection centering at 261 mμ (ϵ, 7,400).

Anal. Calcd. for $C_{10}H_{16}N_4S$: C, 53.5; H, 7.19; N, 25.0. Found: C, 53.2; H, 7.19; N, 25.1.

2-Amino-5-[3-*p*-dimethylaminoanilino)propyl]-6-methyl-4-pyrimidinethiol (VIIIb).

To a suspension of 788 mg. (2.5 moles) of VIb in 78 ml. of reagent methanol was added 2.7 ml. of N methanolic sodium methoxide. After being stirred for 30 min., 1.0 g. of sodium borohydride was added in portions, then the mixture was refluxed with stirring for 1.5 hrs. The solution was spin-evaporated *in vacuo*. The residue was suspended in 75 ml. of water and brought to *p*H 3 with hydrochloric acid, then the filtered solution was adjusted to *p*H 7 with 10% aqueous sodium hydroxide. The product was collected on a filter and washed with water; yield, 636 mg. (80%), m.p. 176-178°. For further purification, the product was dissolved in 60 ml. of water by adding hydrochloric acid to *p*H 3, and the filtered solution again brought to *p*H 7; yield, 383 mg. (48%) of pure product, m.p. 180-182°; ν max (KBr) 3450, 3350, 3050 (NH); 1660, 1600, 1570, 1520, (NH, C=C, C=N); 825 cm^{-1} (*p*-C_6H_4-); λ max (*p*H 1) 328 (ϵ, 11,700); inflection centering at 258 mμ (ϵ, 11,700); λ max (*p*H 7) 252 (ϵ, 18,900); 317 (ϵ, 8,500); 350 mμ (ϵ, 8,800); λ max (*p*H 13) 317 (ϵ, 13,900); 248 mμ (inflection, ϵ, 21,600).

Anal. Calcd. for $C_{16}H_{23}N_5S$: C, 60.5; H, 7.30; N, 22.0. Found: C, 60.3; H, 7.27; N, 21.9.

2-Amino-5-[3-(*p*-chloroanilino)propyl]-6-methyl-4-pyrimidinethiol (VIIIa).

Reduction of 307 mg. (1 mmole) of VIa was performed as described for VIb. After removal of the methanol, the residue was dissolved in 30 ml. of water and acidified to about *p*H 8. The product was collected on a filter and washed with water; yield, 286 mg. (93%), m.p. 190-192° dec. Recrystallization from absolute ethanol gave 206 mg. of light yellow crystals, m.p. 194-196° dec.; ν max (KBr) 3450, 3310, 3050, (NH); 1660, 1600, 1560 (NH, C=C, C=N); 815 cm^{-1} (*p*-C_6H_4); λ max (*p*H 1) 260 (ϵ, 5,600); 338 mμ (ϵ, 15,800); λ max (*p*H 7) 251 (ϵ, 18,300); 350 mμ (ϵ, 15,600); λ max (*p*H 13) 248 (ϵ, 22,500); 318 mμ (ϵ, 14,900).

Anal. Calcd. for $C_{14}H_{17}ClN_4S$: C, 54.4; H, 5.55; N, 18.1; Cl, 11.5; Found: C, 54.2; H, 5.64; N, 18.1; Cl, 11.5.

2-Amino-5-[3-(*n*-butylamino)propyl]-6-methyl-4-pyrimidinol (XI).

A solution of 560 mg. (2.5 mmoles) of X (3) and 0.91 g. (12.4 mmoles) of *n*-butylamine in 10 ml. of reagent N,N-dimethylformamide was allowed to stand for thirty min., then diluted with 50 ml. of reagent methanol. Then 1.0 g. of sodium borohydride was added to the stirred solution in portions over a period of 25 min. After being stirred for 18 hrs. more, the mixture was diluted with 25 ml. of 0.1 N aqueous sodium hydroxide, then spin-evaporated *in vacuo*. The residue was dissolved in 25 ml. of 3N hydrochloric acid, the solution was clarified by filtration, then adjusted to *p*H 9 with aqueous sodium hydroxide. The solution was spin-evaporated *in vacuo* and the residue was extracted with hot chloroform (5 x 20 ml.). The combined chloroform extracts were dried with magnesium sulfate, then spin-evaporated *in vacuo* leaving 0.65 g. of crude XI as a glass that could not be crystallized.

To a solution of the crude XI in 6 ml. of 95% ethanol was added a solution of 1.26 g. (5.5 mmoles) of picric acid in 16 ml. of 95% ethanol. The XI dipicrate was collected on a filter and washed with ethanol; yield, 1.25 g. (72%), m.p. 240-242°. Two recrystallizations from 50% ethanol gave 1.00 g. (58%) of yellow crystals of unchanged m.p.

Anal. Calcd. for $C_{24}H_{28}N_{10}O_{15}$: C, 41.4; H, 4.05; N, 20.1. Found: C, 41.5; H, 4.15; N, 19.9.

A suspension of 0.90 g. of recrystallized dipicrate in 120 ml. of 1N hydrochloric acid was heated on a steam bath until solution was obtained. The cooled solution was washed with chloroform until the washings (10 x 20 ml.) were colorless. The aqueous solution was spin-evaporated *in vacuo* and the residue triturated with acetone; yield, 0.39 g. (97% recovery) of XI dihydrochloride, m.p. 275-278° dec. Recrystallization from absolute ethanol-acetone gave 0.22 g. (55% recovery) of white crystals, m.p. 277-280° dec.; ν max (KBr) 3350, 3050 (broad OH, NH); 2950, 2760 (broad NH, NH+); 1680 (C=NH+); 1650, 1540 cm^{-1} (NH, pyrimidine); λ max (*p*H 1) 229 (ϵ, 10,600); 267 mμ (ϵ, 9,700); λ max (*p*H 6) 272 (ϵ, 5,600); inflection centering at 295 mμ; λ max (*p*H 13) 280 (ϵ, 7,600); shoulder at 233 mμ (ϵ, 8,900).

Anal. Calcd. for $C_{12}H_{24}Cl_2N_4O$: C, 46.3; H, 7.77; N, 18.0. Found: C, 46.4; H, 8.01; N, 18.3.

5-(N-Acetyl-3-anilinopropyl)-2-amino-6-methyl-4-pyrimidinethiol (XV).

A suspension of 500 mg. (1.82 mmoles) of IV (5) in 5 ml. of acetic anhydride was stirred at room temperature for 2.5 hrs. The solid was collected on a filter and washed with a small amount of acetic anhydride, then ethanol; yield, 419 mg. (73%), m.p. 246-247°. Recrystallization from methanol gave 332 mg. (58%) of analytically pure material as yellowish-white crystals, m.p. 250-252°; ν max (KBr) 3450, 3350, 3200, 3070 (NH); 1640-1625, 1570 cm^{-1} (amide C=O, pyrimidine, NH, C=C); λ max (*p*H 1) 340 (ϵ, 16,200); 261 mμ (shoulder, ϵ, 6,000); λ max (*p*H 7) 350, (ϵ, 17,000), 262 mμ (shoulder, ϵ, 7,700); λ max (*p*H 13) 319 (ϵ, 14,700), inflection centering at 264 mμ (ϵ, 7,700).

Anal. Calcd. for $C_{16}H_{20}N_4OS$: C, 60.7; H, 6.37; N, 17.7. Found: C, 60.7; H, 6.49; N, 17.5.

2-Amino-5-(4-phenylbutyl)-6-methyl-4-pyrimidinol (XIIb).

To a solution of 3.25 g. (25 mmoles) of ethyl acetoacetate in 35 ml. of *t*-butyl alcohol was added in portions 1.12 g. (25 mmoles) of a 53.5% dispersion of sodium hydride in mineral oil. When the sodium hydride had dissolved, 4.26 g. (20 mmoles) of 4-phenylbutyl bromide (XIIIb) (21) was added. After being refluxed with magnetic stirring for 20 hrs., the mixture was neutralized with acetic acid and spin-evaporated *in vacuo*. The residue was partitioned between 40 ml. of chloroform and 20 ml. of water. The chloroform layer, dried with magnesium sulfate, was spin-evaporated *in vacuo*, finally at 1 mm. (bath 90°) to remove ethyl acetoacetate; yield, 4.43 g. (85%) of crude XIVb; ν max (film) 1735, 1710 (C=O); 745 cm^{-1} (C_6H_5); λ max (1N NaOMe) 283 mμ (ϵ, 13,800).

A mixture of 1.35 g. (7.5 mmoles) guanidine carbonate, 4.33 g. (16.5 mmoles) of crude XIVb, and 20 ml. of *t*-butyl alcohol was refluxed with magnetic stirring

for 60 hrs. The mixture was cooled, then the product was collected on a filter and washed with *t*-butyl alcohol and water, yield, 1.48 g. (38%), m.p. 209-211°. The combined *t*-butyl alcohol filtrate and *t*-butyl alcohol washings were neutralized with acetic acid, then spin-evaporated *in vacuo*. The solid residue was triturated with 50% aqueous acetone to give an additional 1.13 g. (total 67%) m.p. 205-206°. Recrystallization of a sample of the first crop from 2-methoxyethanol by addition of water gave white crystals, m.p. 210-211°; ν max (KBr) 3390, 3100 (NH, OH); 1650, 1540 (NH, C=C, C=N); 745, 695 cm^{-1} (C_6H_5); λ max (pH 1) 266 mμ (ϵ, 10,600); λ max (pH 13) 280 mμ (ϵ, 9,350).

Anal. Calcd. for $C_{15}H_{19}N_3O$: C, 70.0; H, 7.44; N, 16.3. Found: C, 70.2; H, 7.63; N, 16.3.

2-Amino-5-(3-phenoxypropyl)-6-methyl-4-pyrimidinol (XIIa).

This compound was synthesized from 3-bromopropyl phenyl ether (XIIIa), via XIVa (83% crude yield), as described for XIIb; Yield, 2.24 g. (58%) m.p. 226-227°, that separated from *t*-butyl alcohol and 0.63 g., m.p. 214-218°, that was obtained from the mother liquor. Recrystallization of the latter from 2-methoxyethanol-water gave 0.34 g. (total 66%) of pure product, m.p. 225-226°. Further recrystallization gave white crystals of unchanged m.p.; ν max (KBr) 3450, 3100, (NH, OH); 1675, 1645, 1610, 1500 (NH, C=N, C=C); 1255, 1040 (ether C-O-C); 748, 675 cm^{-1} (C_6H_5-); λ max (pH 1) 267 mμ (ϵ, 10,300); λ max (pH 7) 271 mμ (ϵ, 7,600); λ max (pH 13) 277 mμ (ϵ, 9,600).

Anal. Calcd. for $C_{14}H_{17}N_3O_2$: C, 64.8; H, 6.61; N, 16.2. Found: C, 65.0; H, 6.54; N, 16.4.

Methods of Assay (7).

The partial purification of dihydrofolic reductase from pigeon liver and thymidylate synthetase from *E. Coli* B have been described in the accompanying previous paper (2). The dihydrofolic reductase assays were run with dihydrofolate and TPNH in 0.05M Tris Buffer (pH 7.4) containing 1 mM Versene and 10 mM mercaptoethanol, the velocity being determined by the rate of decrease of optical density at 340 mμ, as previously described (2). The thymidylate synthetase assays were performed with tetrahydrofolate, formaldehyde, magnesium chloride and 2'-deoxyuridylate, the velocity being determined by the rate of increase in optical density at 338 mμ (2).

RESULTS AND DISCUSSION

By plotting V_O/V_I against I for several concentrations of I, the concentration of I necessary to give 50% inhibition ($V_O/V_I = 2$) was obtained, where V_O = velocity of the enzymic reaction without inhibitor, V_I = velocity of the reaction in the presence of the inhibitor, and I = concentration. These 50% inhibition values are listed in Table I. In some cases it was necessary to use 3-10% N,N-dimethylformamide in order to increase the solubility of the inhibitor. These exceptions are noted in Table I; for slight modifications of the assays in these circumstances see reference 2.

The relative effects of the inhibitors are best compared in the column of inhibitor: substrate ratios necessary for 50% inhibition. With these comparisons it is possible to shed light on some of the questions posed at the beginning of this paper.

1. Does a protonated species of the anilino group account for the total binding of the anilino group to the two enzymes?

N-Methylaniline is a weak base with pK$_a$ in the region of 4; in contrast secondary aliphatic amines are much stronger bases with pK$_a$'s in the order of 9. Therefore, the anilino group of II (Table I) would be less than 0.1% in the protonated form at the pH of the enzyme assays (pH 7.4) whereas the *n*-butylaminopropyl pyrimidine (XI) would be protonated in the side chain greater than 99.9%. As a result, XI should be a more effective inhibitor than II if the anilino group binds only through a protonated species; if binding of the anilino group to the two enzymes is through a neutral species, then II should be a better inhibitor than XI. That the binding of the anilino group is through a neutral species is clearly indicated by the poor inhibition of the two enzymes by the butylaminopropyl pyrimidine (XI); the order of inhibition of thymidylate synthetase by XI would be compatible with only pyrimidine binding and no appreciable side-chain binding.

2. Does the anilino group bind to the enzymes (E) through a hydrogen bond of the type E: $\rightarrow$ H-NH_6H_5? or E-H $\leftarrow$: N-C_6H_5?

The phenoxypropyl pyrimidine (XIIa) could bind to the enzyme by a modification of the second type of hydrogen bond, E-H $\leftarrow$: OC_6H_5, but not the first type. Since the phenoxypropyl pyrimidine (XIIa) binds to dihydrofolic reductase as well as the anilinopropyl pyrimidine (II) (Table I), the E: $\rightarrow$ H-NC_6H_5 type of hydrogen bond is eliminated. The phenoxypropyl pyrimidine (XIIa) binds less effectively to thymidylate synthetase than the anilinopropyl pyrimidine (II); unfortunately due to the insolubility of XIIa in the assay system, it could not be determined how much less XIIa was bound to thymidylate synthetase than II. Other evidence that the hydrogen of the anilino-NH group does not bind to dihydrofolic reductase is that both aminopterin and N^{10}-methylaminopterin (amethopterin) inhibit the enzyme to the same magnitude (8). Furthermore, the same is true of tetrahydro-aminopterin and tetrahydroamethopterin on thymidylate synthetase (12).

3. Does the anilino group of II bind to either enzyme by a hydrogen bond of the type E-H $\leftarrow$: NHC_6H_5 or does the benzene ring bind through its electrons, or both?

Replacement of the NH of the anilino group by CH_2 gave an inhibitor (XIIb) that was 26-fold more effective on dihydrofolic reductase than II and about 1.5-fold more effective than II on thymidylate synthetase. At first glance, the tighter binding of XIIb to both enzymes would indicate that the anilino NH group of II is not involved at all in binding, and that all of the binding of the anilino group is due to the benzene ring. However, it is also possible that the potential loss of NH binding of II is more than compensated by increased binding of the benzene ring of XIIb.

If the benzene ring binds to an electron-rich locus of the enzyme, then the more electron-poor the benzene ring, the better the binding; the converse is true if binding is to an electron-poor locus of the enzyme. There are a variety of ways in which a complex between an aromatic ring and an enzyme might form, such as charge-transfer complex, dipole-dipole interaction, van der Vaals forces, intermolecular hydrogen bonding, or hydrophobic bonding (6). There are also several ways in which the electronegativity (electron density) of a substituted benzene ring (or other aromatic system) can be measured such as (a) the Hammett sigma constant of substituent groups on the ring (13,14), (b) the equilibrium constant (15-18) or bathochromic shift (19) of a charge-transfer complex or (c) ease of substitution in the ring by a reaction such as halogenation or the Friedel-Crafts (20).

Since the ethylamino and butylamino groups have sigma constants of -0.61 and -0.51, respectively, and the *n*-alkyl group has a sigma constant of -0.12 to -0.16, it is clear that the benzene ring of XIIb could be a better electron-acceptor than the benzene ring of II in a charge-transfer complex with an enzyme.

If the benzene ring of II or IV were bound as an electron acceptor to the enzyme in a charge-transfer complex, then substitution by *p*-chloro (VIIIa) or *p*-dimethylamino (VIIIb) should increase and decrease binding, respectively; as seen in Table I, the *p*-chloro-

TABLE I

Inhibition of Dihydrofolic Reductase and Thymidylate Synthetase by 4-R_1-5-(3-R_2-Propyl)-2-amino-6-methylpyrimidines

Compound No.	R_1	R_2	Dihydrofolic Reductase (a) μM Conc. FAH_2 (b)	mM Conc. Inhibitor	Percent Inhibition	Inhibitor: Substrate Ratio (c)	Thymidylate Synthetase (a) μM Conc. M-FAH_4 (d)	mM Conc. Inhibitor	Percent Inhibition	Inhibitor: Substrate Ratio (e)	Synthetase: Reductase Ratio (f)
II	OH	$-NHC_6H_5$	6	0.60 (g)	43	130	25.7	0.62 (h)	50	50	0.39
XI	OH	$-NHC_4H_9$-*n*	6	6.0 (i)	0	>4000	25.7	4.5 (i)	50	350	<0.09
IV	SH	$-NHC_6H_5$	3	0.022	50	7.3	25.7	0.08	38	11	1.5
VIIIa	SH	$-NHC_6H_4Cl(p)$	6	0.014	50	2.3	25.7	0.018	50	1.4	0.61
VIIIb	SH	$-NHC_6H_4NMe_2(p)$	3	0.030	50	10	25.7	0.08	40	8.6	0.86
XIIa	OH	$-OC_6H_5$	6	0.55 (g)	40	140	25.7	0.45 (h)	0	>140	>1
XIIb	OH	$-CH_2C_6H_5$	6	0.030 (g)	50	5.0	25.7	0.20 (h)	30	35	7.0
			6	0.030 (j)	50	5.0					
XV	SH	$-NC_6H_5$ O-CCH_3	6	0.02	50	3.3	25.7	0.040	50	3.9	1.2

(a) For assay methods see reference 2. (b) FAH_2 = dihydrofolate. (c) Ratio of concentrations of inhibitor to dihydrofolate required for 50% inhibition. (d) M-FAH_4 = 5,10-methylene-*dl*-tetrahydrofolate. (e) Ratio of concentrations of inhibitor to 5,10-methylene-*l*-tetrahydrofolate required for 50% inhibition. (f) Ratio of inhibitor: substrate for 50% inhibition of thymidylate synthetase to inhibitor: substrate for 50% inhibition of dihydrofolic reductase. (g) In 10% N,N-dimethylformamide. (h) In 3% N,N-dimethylformamide. (i) A 9.3 mM master solution of inhibitor in 0.05M Tris buffer containing 1 mM Versene and 10 mM mercaptoethanol adjusted to *p*H 7.4 with Tris base was employed. (j) In 1.6% N,N-dimethylformamide.

anilino pyrimidine (VIIIa) binds stronger than the anilinopropyl pyrimidine (IV), but there is little significant change when the *p*-dimethylamino group (VIIIb) is introduced. It cannot be assumed that the usual sigma plot (13) will be linear since both the anilino group and the pyrimidine moiety of IV contribute to binding; furthermore the effect having both a dimethylamino group and a propylamino group *para* to each other may not be additive.

Evidence that the anilino NH group of I and II may be involved in binding is that changing the propylamino group of I to propionamido led to a considerable decrease in binding to folic reductase (4) and dihydrofolic reductase. If the anilino NH group was bound to the enzyme by a hydrogen bond of the type EH ← : $N(C_6H_5)R$, then binding would be considerably decreased if R were changed from propyl to the electron-withdrawing propionyl group. However, since the acetamido group (14) has a sigma value of 0.0, binding by the benzene ring should have been increased even more than in the case of XIIb where the methylene group has a sigma value of about -0.13. It follows that some other explanation must be invoked for the lowered binding of the propionyl analog of I; steric or conformation hindrance to binding in the propionyl analog is easy to invoke, but hard to support experimentally.

The second point of evidence that the anilino NH group of II may be involved in binding is that the phenoxypropyl analog should have been more similar in binding to the phenylbutyl pyrimidine (XIIb) than to II, since the propoxy group (14) has a sigma value of -0.25. However, there is as yet no good evidence that the anilino NH group of II binds to dihydrofolic reductase since all of the results on binding (Table I) can most probably be accounted for by the benzene ring being an electron-acceptor in a charge-transfer complex.

In the case of inhibition of thymidylate synthetase, replacement of the anilino NH group of II by CH_2 (XIIb) increased binding 1.5-fold; this result indicated that the NH group is not necessary for binding, and that the benzene ring binds to the enzyme. Surprisingly, the phenoxypropyl pyrimidine (XIIa) does not bind as well as II. A further difference in the mode of binding of the anilino group to the two enzymes was noted; replacement of the propylamino group I by a propionamido group (4) gave a 4-fold decrease in binding to thymidylate synthetase, compared to an 80-fold decrease with rat liver folic reductase (4) and a 9-fold decrease with pigeon liver dihydrofolic reductase.

These differences suggested that the N-acetyl derivative of IV, 2-amino-5-(N-acetyl-3-anilinopropyl)-6-methyl-4-pyrimidinethiol (XV) might be a more selective inhibitor of thymidylate synthetase than dihydrofolic reductase when one considers the following additional facts: (a) acylation of the N^{10} group of folic acid leads to decreased binding to folic reductase (8,22) (b) conversion to an N-acetyl derivative should make the benzene ring a better electron-acceptor (sigma value of acetamido = 0.0), providing that acetylation does not give increased steric hindrance to binding. The N-acetyl derivative (XV) was found to bind to thymidylate synthetase about 3-fold better than the corresponding anilino compound (IV) (Table I). Surprisingly, binding to dihydrofolic reductase was not decreased; apparently the increased benzene binding was more than sufficient to off-set any hindrance to binding that the N-acetyl group of XV may have caused. It would be of interest to see if larger acyl will be unequally tolerated by the two enzymes, an application of the bulk principle of specificity (23). Furthermore, if larger acyl groups can be placed on the anilino group of II or IV or both that will still inhibit either or both of the enzymes, then use of acyl groups which carry groups that can potentially form covalent bonds with the enzyme could lead to active-site-directed irreversible inhibitors (24) with their extra dimension of specificity (25-28).

Of the compounds in Table I XI has the greatest specificity in favor of thymidylate synthetase, but is a weak inhibitor. The most selective compound in favor of dihydrofolic reductase in Table I is the 5-(phenylbutyl)pyrimidine (XIIb). We had previously reported (2) that 5-(3-anilinopropyl)-2,4-diamino-6-methylpyrimidine was 170-fold more effective on dihydrofolic reductase than thymidylate synthetase; it is not improbable that further variation of the anilino

group by other N-acyl groups could give a greater specificity for inhibition of thymidylate synthetase.

The synthesis of further modifications of the anilino group of II, such as the 3-pyridylamino group, should be made to see if these more potent electron accepting groups will give increased binding; these additional compounds might also shed further light on the important chemotherapeutic problem of how a benzene (or other aromatic) ring might bind to these two enzymes in particular and enzymes in general.

In a previous paper of this series (4), it was noted that placement of a *p*-carboxyl group on the anilino group of II increased binding to folic reductase by six-fold; it was concluded that this *p*-carboxyl group was an additional binding point in this compound and in I. In view of the results in this paper leading to the conclusion that the anilino group of II may be binding to (dihydro) folic reductase as an electron acceptor, it is possible that the *p*-carboxyl group does not actually bind *per se*, but increases binding of the anilino group due to the electron-withdrawing properties of the carboxyl group; this point should be reinvestigated by synthesis and enzymic evaluation of suitable candidate compounds.

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