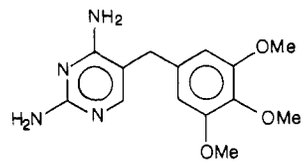
2,4-Diamino-5-benzylpyrimidines as Antibacterial Agents. 7. Analysis of the Effect of 3,5-Dialkyl Substituent Size and Shape on Binding to Four Different Dihydrofolate Reductase Enzymes

Barbara Roth,*,† Barbara S. Rauckman,† Robert Ferone,† David P. Baccanari,† John N. Champness,‡ and Richard M. Hyde‡

Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709, and Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, England. *Received June 6, 1986*

A group of trimethoprim (TMP) analogues containing 3,5-dialkyl(or halo)-4-alkoxy, -hydroxy, or -amino substitution were analyzed in terms of their inhibitory activities against four dihydrofolate reductase (DHFR) isozymes. Although selectivities were lower than with TMP, the activities against vertebrate DHFR were usually at least 2 orders of magnitude less than against enzymes from microbial sources. However, the *profiles* of activity were remarkably similar for rat, *Neisseria gonorrhoeae*, and *Plasmodium berghei* enzymes in all three series, although somewhat different for *Escherichia coli* DHFR, leading to the conclusion that the hydrophobic pockets are similar for the first three isozymes. Optimal substitution was reached with 3,5-di-*n*-propyl or 3-ethyl-5-*n*-propyl groups. Branching of chains at the α-carbon, which resulted in increased substituent thickness, was detrimental to *E. coli* DHFR inhibition in particular. MR is an inadequate parameter for use in correlating such substituent effects. Conformational changes of the more bulky inhibitors can be invoked to explain some differences in inhibitory pattern. Although log *P* explains simple substituent effects with the vertebrate DHFRs very well, it is insufficient in the more complex cases described here, where shape is clearly involved as well. Solvent-accessible surface areas were measured for TMP in *E. coli* and chicken DHFRs, where the coordinates are now known. The environment is more hydrophobic in the latter case; this can also be postulated for rat DHFR, which has a very similar activity profile. As with the mammalian isozymes, *N. gonorrhoeae* DHFR contains an active site phenylalanine replacing Leu-28 of *E. coli* DHFR, thus creating a more hydrophobic pocket. A similar replacement may also occur in the *P. berghei* isozyme. Selectivity for bacterial DHFR is dependent on the nature of the 4-substituent, being low for polar 4-hydroxy compounds but high for polar 4-amino analogues, possibly as a result of solvation differences. With complex substituents, the environment of each atom in the active site must be taken into account to adequately explain structure–activity relationships.

This paper compares and analyzes the inhibitory activities of a series of 3,5-dialkyl-4-substituted-benzyl analogues of trimethoprim (TMP, **1a**),[1] 3,5-dihalo derivatives, and related compounds against dihydrofolate reductase (DHFR, E.C. 1.5.1.3) enzymes from *Escherichia coli*, *Neisseria gonorrhoeae*, *Plasmodium berghei*, and rat liver, as well as describing their antimicrobial activities. The synthesis of the majority of these compounds is described in papers 2, 4, and 6 of this series.[2-4]



1a (TMP)

The alkyl substituents discussed here include both straight and branched chains totaling up to eight carbon atoms for the combined 3,5-substituents. The problem of analyzing such substituent effects in terms of minimum-energy conformations in enzyme complexes is formidable when one considers the many degrees of freedom for such side chains, the possible torsional angles about the methylene group joining the two rings, and the fact that the 3- and 5-substitutions may be dissimilar. However, an analysis of minimum-energy conformations for the side chains alone of the small molecules showed only small energy differences among the various conformers.

For this initial analysis we base our qualitative arguments for substituent effects on the known conformations of trimethoprim in ternary complex with NADPH in *E. coli* and chicken liver DHFRs.[5,6] The only DHFR enzymes for which the three-dimensional structures are known by X-ray crystallography are those above mentioned, plus *Lactobacillus casei* DHFR.[7] A preliminary report has appeared on the three-dimensional structure of the isozyme from mouse L1210 lymphoma,[8] which suggests that its structure resembles that of chicken DHFR. There is quite high homology among the vertebrate DHFR enzymes, which contain approximately 189 residues and an active site glutamate, as opposed to approximately 160 residues and an active site aspartate for the known bacterial DHFRs. The latter are quite low in homology. We use the chicken DHFR coordinates, therefore, as a model for interpretation of the rat DHFR data.

Chemistry

Most of the new compounds reported here were prepared by simple alkylation of phenolic benzylpyrimidines or precursors. The 3,5-diiodophenol (**26**) was prepared by iodination with ICl. The ether derivatives of this compound were quite unstable in boiling ethanol, readily losing part of their iodine. Details of these preparations are found

† Research Triangle Park, NC.
‡ Beckenham, England.

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(3) Roth, B.; Aig, E.; Lane, K.; Rauckman, B. S. *J. Med. Chem.* **1980**, *23*, 535.
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(5) Champness, J. N.; Stammers, D. K.; Beddell, C. A. *FEBS Lett.* **1986**, *199*, 61.
(6) Matthews, D. A.; Bolin, J. T.; Burridge, J. M.; Filman, D. J.; Volz, K. W.; Kaufman, B. T.; Beddell, C. R.; Champness, J. N.; Stammers, D. K.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 381.
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Table I. Inhibitory Activities of Three Series of 2,4-Diamino-5-(3,5-dialkylbenzyl)pyrimidines with 4-Methoxy-, 4-Hydroxy-, or 4-Aminobenzyl Substituents against Dihydrofolate Reductase Enzymes Compared to Their 3,5-Dimethoxybenzyl Counterparts and 4-Methoxybenzyl Analogues

no.	benzene substituents 3	5	$I_{50} \times 10^8$ M vs. DHFR enzymes: *E. coli*	rat liver	*N. gonorrhoea*	*P. berghei*
			I. 4-Methoxybenzyl Series			
2[a]	CH_3	CH_3	15	25000	50	12
3[b]	CH_3	C_2H_5	5.3	8300	31	12
4[a]	C_2H_5	C_2H_5	1.4	5800	15.5	1.2
5[b]	C_2H_5	C_3H_7-*n*	1.2	3200	4.4	1.0
6[b]	C_3H_7-*n*	C_3H_7-*n*	4.4	2100	6	2.0
7[a]	C_3H_7-*i*	C_3H_7-*i*	16	1300	6.3	2.3
8[b]	CH_3	C_4H_9-*t*	20	13000	24	7.7
9[a]	C_4H_9-*t*	C_4H_9-*t*	44	3100	5.8	1.3
1a (TMP)[c]	OCH_3	OCH_3	0.7 (av)	37000 (av)	45	12
1d[d]	OCH_3		6.2	8600	53	
			II. 4-Hydroxybenzyl Series			
10[b]	CH_3	CH_3	7.0	7100	52.6	
11[b]	CH_3	C_2H_5	1.9	3900	35.1	
12[b]	C_2H_5	C_2H_5	1.6	1600	12	2.8
13[b]	C_2H_5	C_3H_7-*n*	0.6	445	4.9	1.5
14[b]	C_3H_7-*n*	C_3H_7-*n*	0.7	200	2.0	0.57
15[b]	C_3H_7-*i*	C_3H_7-*i*	3.2	890	2.9	11
16[b]	CH_3	C_4H_9-*t*	11	12000	25.9	
17	C_4H_9-*t*	C_4H_9-*t*	17	3450	6.1	14
1b[c]	OCH_3	OCH_3	1.1	9200	33	30
1e[e]	OCH_3		6.6	3500		
			III. 4-Aminobenzyl Series			
18[f]	CH_3	CH_3	18	38000 (42%)	48.5	
19[f]	CH_3	C_2H_5	4.8	14400	56	
20[f]	C_2H_5	C_2H_5	0.32	3800	10.7	
21[f]	C_2H_5	C_4H_9-*sec*	2.1, 1.9	2700	7.5	
22[f]	C_3H_7-*i*	C_3H_7-*i*	1.7, 1.4	3400	13.7	
23[f]	CH_3	C_3H_7-*i*	6.4, 10	11000	26.1	
24[f]	CH_3	C_4H_9-*t*	9.0, 9.1	37000	39	
1c[g]	OCH_3	OCH_3	1.0	52000	81	
1f	OCH_3		14	47000		

[a] Reference 3. [b] Reference 4. [c] Reference 2. [d] Falco, E. A.; DeBreuil, S.; Hitchings, G. H. *J. Am. Chem. Soc.* **1951**, *73*, 3758. [e] Reference 1. [f] Reference 9. [g] Kompis, I.; Rey-Bellet, G.; Zanetti, G. Ger. Offen. 2 443 682, 1975; *Chem. Abstr.* **1975**, *83*, 43376*h*.

Table II. Inhibitory Activities of 2,4-Diamino-5-(3,5-dihalo-4-substituted-benzyl)pyrimidines against Dihydrofolate Reductase Enzymes

no.	benzene substituents 3	4	5	$I_{50} \times 10^8$ M vs. DHFR enzymes: *E. coli*	rat liver	*N. gonorrhoeae*	*P. berghei*
25	I	OCH_3	I	1.0	1600	2.8	3.1
26	I	OH	I	1.0	990	2.8	5.3
27	I	$O(CH_2)_3OPh$	I	2.9	158	4.3	
28[a]	Br	NH_2	Br	2.8	5700	9.3	
29[a,b]	Cl	NH_2	Cl	11	13000	31.8	
30[a]	Cl	NH_2	CH_3	13	16000		

[a] Reference 9. [b] Perun, T. J.; Rasmussen, R. J.; Horrum, B. W. U.S. Patent 4 087 528, 1978.

Table III. Effect of Chain Variations in the Benzene 4-Substituent of 2,4-Diamino-5-(3,5-dialkylbenzyl)pyrimidines and Analogues on Dihydrofolate Reductase Inhibition

no.	benzene substituents 2	3	4	5	6	$I_{50} \times 10^8$ M vs. DHFR enzymes: *E. coli*	rat liver	*N. gonorrhoeae*	*P. berghei*
31[a]	CH_3	CH_3	OCH_3	CH_3	CH_3	31	0% @ 10^{-6} M		
32[a]	CH_3	CH_3	OC_2H_5	CH_3	CH_3	38	0% @ 10^{-6}		
33[a]		C_2H_5	OC_2H_5	C_2H_5		2.0	5200	35.6	3.5
34		C_2H_5	OCH_2CH_2OH	C_2H_5		1.6	12000		4.5
35		C_2H_5	$OCH_2CH_2OCH_2Ph$	C_2H_5		1.5	2600	4.8	3.4
36		C_2H_5	$O(CH_2)_3OPh$	C_2H_5		3.2	875	6.4	1.0
37		C_2H_5	$OCH_2CH(C_2H_5)C_4H_9$-*n*	C_2H_5		15	0% @ 2×10^{-5}	31.9	4.2
38		C_2H_5	$OC_{12}H_{25}$-*n*	C_2H_5		175	6400	350	35
39[a]		C_3H_7-*n*	OC_4H_9-*n*	C_3H_7-*n*		1.7	1500	13.2	0.58
40[b]		OC_2H_5	OC_2H_5	OC_2H_5		0.3	3000	22	0.75

[a] Reference 4. [b] Reference 28.

in the Experimental Section.

DHFR Inhibitory Activities and Data Analysis

Table I presents a listing of 3,5-dialkyl-4-methoxy, -4-hydroxy, and -4-aminobenzylpyrimidines and their I_{50} values for four DHFR enzymes, compared with their 3,5-dimethoxy and monomethoxy counterparts. The first two series have directly comparable substituents, but the third, which was synthesized by other investigators in our laboratories,[9] does not. Table II lists several 3,5-dihalo or

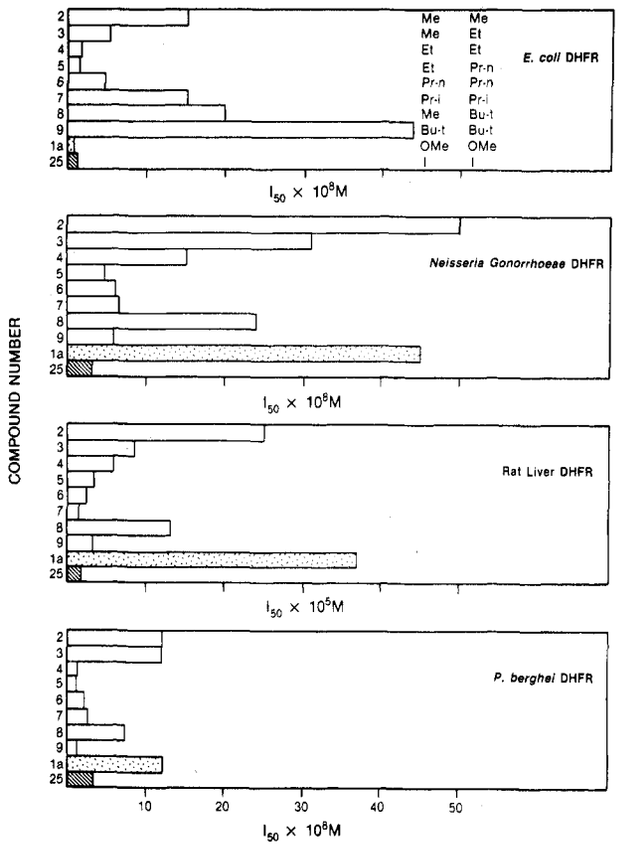

Figure 1. Activity profiles for 3,5-dialkyl-4-(methoxybenzyl)-pyrimidines against DHFR enzymes compared to trimethoprim (**1a**) and its 3,5-diiodo analogue (**25**). Note that scale differs 1000-fold for rat liver DHFR.

3-halo-5-alkyl derivatives with I_{50} data, and Table III presents alkoxy variations in the 4-position of 3,5-dialkyl derivatives.

The activity profiles for compounds **2–9** of Table I, compared to TMP (**1a**) and the 3,5-diiodo derivative **25**, are shown in Figure 1 for the four DHFR enzymes. It is readily apparent that in all cases the activity is related to the nature of the 3,5-dialkyl groups. This is consistent with very early deductions about the importance of 3- and 5-substituents.[1] With *E. coli* DHFR optimal activity is reached with the diethyl and ethyl *n*-propyl analogues **4** and **5**; the least active compound is the bulky 3,5-di-*tert*-butyl derivative **9**. In comparison, TMP (**1a**) and the diiodo analogue (**25**) are even more active than **4** and **5**.

In comparing rat DHFR data with those from microbial sources, a rather remarkable effect should be noted. Although the activities with the mammalian enzyme are lower by 2–3 orders of magnitude than those from bacterial DHFR, the *profiles* of relative activity are very similar to those from the *N. gonorrhoeae* and *P. berghei* enzymes, especially so in the former case. However, these three profiles differ in several important respects from that observed for *E. coli* DHFR. With the first three enzymes, the unsymmetrical methyl *tert*-butyl derivative **8** is significantly less active than the di-*tert*-butyl analogue **9**, whereas the least active dialkyl compound is the dimethyl derivative **2**. Optimal activity resides with compounds **5–7** and **9**, in contrast with the *E. coli* case. Here the relatively bulky diisopropyl compound **7** is considerably less active than **4** and **5**, but more active in turn than the di-*tert*-butyl derivative **9**. It then appears that shape, as influenced by thickness of the side chains in the plane of the benzene ring, may be an important factor in restricting binding to the *E. coli* enzyme. Compression of bulk in this direction would then seem advantageous. The current data will be examined quantitatively for consistency with this hypothesis in a paper to follow.

The fact that a methyl group, as in compound **2**, is apparently too small for effective binding is interesting when one considers that its radius (2.0 Å) is only slightly less than that of an iodine atom (2.15 Å),[10] yet the diiodo derivative **25** retains high activity. Explanations in terms of polarizability, or the role of d orbitals, or a more refined interpretation of substituent shape may provide the answer.

Hansch and co-workers have contrasted the inhibition of *E. coli*, *L. casei*, and bovine and chicken DHFRs by 3,4-disubstituted benzylpyrimidines and TMP quantitatively in terms of MR and π and have drawn the following conclusions: (a) that activity is in all cases dependent on the properties of the meta substituents in the benzene ring, (b) that the dominant properties are substituent bulk and substituent hydrophobicity, as expressed by MR and π, respectively, (c) that mammalian DHFR inhibitory activity is better correlated with π_3 and that bacterial DHFR inhibition is better correlated with MR, with hydrophilicity in the meta position being associated with selectivity for the bacterial enzyme.[11-14]

Roth and co-workers have concluded that an important role of the 4-substituent is to constrain unsymmetrical 3- and 5-functions, such as methoxy groups, to a favorable conformation for binding to the *E. coli* enzyme.[15] Similarly, Hyde and Roth have suggested that the shortcomings of a simple correlation between activity and MR are suggestive of substituent shape rather than bulk and that MR is not capable of describing shape at the required level of resolution.[16] It is a fact that all three methoxy groups of TMP are in contact with the enzyme in the ternary complex with *E. coli* DHFR and NADPH as discussed below.[5] All in all, it may be concluded that the 3,4,5-trisubstituted benzylpyrimidines, as represented by TMP and analogues, comprise a much better series for accurate quantitation of substituent effects than do the mono- or disubstituted derivatives. Such effects are discussed descriptively in this paper.

Figure 2 presents graphically a comparison of the inhibitory activities of 3,5-dialkyl-4-(hydroxybenzyl)pyrimidines against the same four DHFR enzymes, along with data on the 3,5-dimethoxy-4-(hydroxybenzyl)pyrimidine **1b** and the iodo derivative **26**. The profiles of Figure 2 should first be compared with those of the 4-methoxy series of Figure 1. The relative activities for the two series will be observed to resemble each other very closely. The major difference is in the absolute activities, which are greater

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(10) Pauling, L. *Nature of the Chemical Bond* 3rd ed.; Cornell University: Ithaca, NY, 1960.
(11) Li, R.; Dietrich, S. W.; Hansch, C. *J. Med. Chem.* **1981**, *24*, 538.
(12) Hansch, C.; Li, R.; Blaney, J. M.; Langridge, R. *J. Med. Chem.* **1982**, *25*, 777.
(13) Li, R.; Hansch, C.; Matthews, D.; Blaney, J. M.; Langridge, R.; Delcamp, R. J.; Susten, D. D.; Freisheim, J. H. *Quant. Struct.-Act. Relat. Pharmacol., Chem., Biol.* **1982**, *1*, 1.
(14) Blaney, J. M.; Hansch, C.; Silipo, C.; Villon, A. *Chem. Rev.* **1984**, *84*, 333.
(15) Roth, B.; Aig, E.; Rauckman, B. S.; Strelitz, J. Z.; Phillips, A. P.; Ferone, R.; Bushby, S. R. M.; Sigel, C. W. *J. Med. Chem.* **1981**, *24*, 933.
(16) Hyde, R. M.; Roth, B. In *Strategy in Drug Research*; Buisman, J. A. K., Ed.; Elsevier: Amsterdam, 1982; p 385.

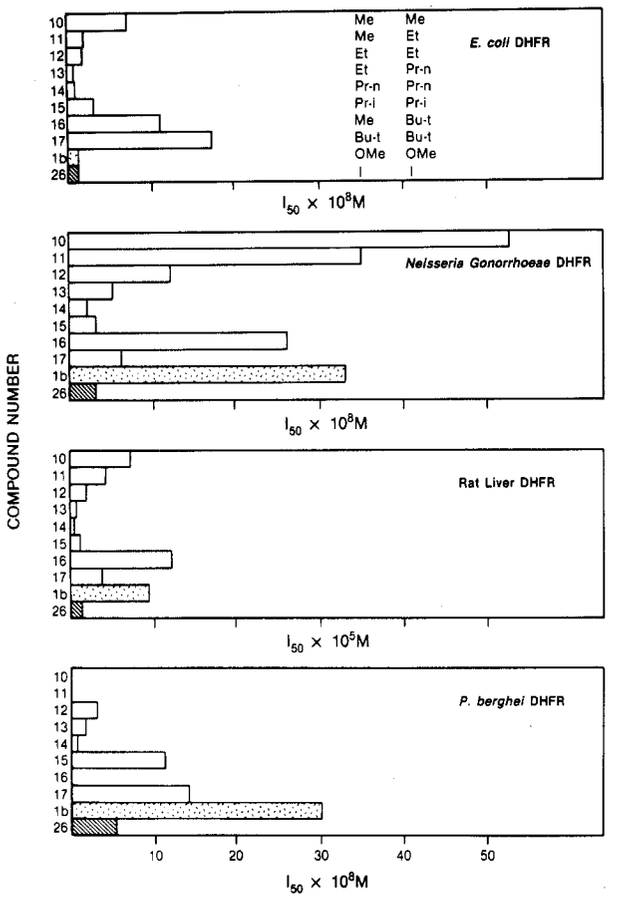

Figure 2. Activity profiles for 3,5-dialkyl-4-(hydroxybenzyl)-pyrimidines vs. DHFR compared to **1b** and **26**.

for the 4-hydroxy compounds of Figure 2 than for their 4-methoxy counterparts (Figure 1) for the first three enzymes cited.[17] The only exception is the iodo compound **26**, which retains similarly high activity for all four enzymes.

Figure 3 shows profiles of activity for several 3,5-dialkyl-4-amino derivatives against three DHFR enzymes, compared to the 3,5-dimethoxy, 3,5-dibromo, and 3,5-dichloro counterparts. Unfortunately, these are only partially analogous with the derivatives of Figures 1 and 2, since appropriate intermediates were not always available. Once more the profile for *E. coli* DHFR is different from that for the other two enzymes. Very high optimal activity is achieved with the diethyl analogue **20**. Compound **21**, the unsymmetrical ethyl *sec*-butyl derivative, is optimal for the other two enzymes. The very skewed methyl *tert*-butyl derivative **24** is again poor for *N. gonorrhoeae* and rat DHFRs, but we have no di-*tert*-butyl derivative for comparison. Most interesting is the very low activity of **1c** for the latter two enzymes, but high activity for *E. coli* DHFR; thus, it is highly selective. Here we again have a small hydrophilic substituent in the 4-position with no out-of-plane atom larger than hydrogen, but it has an effect opposite to the Figure 2 compounds on *N. gonorrhoeae* and rat enzymes. There are clearly complexities in this picture

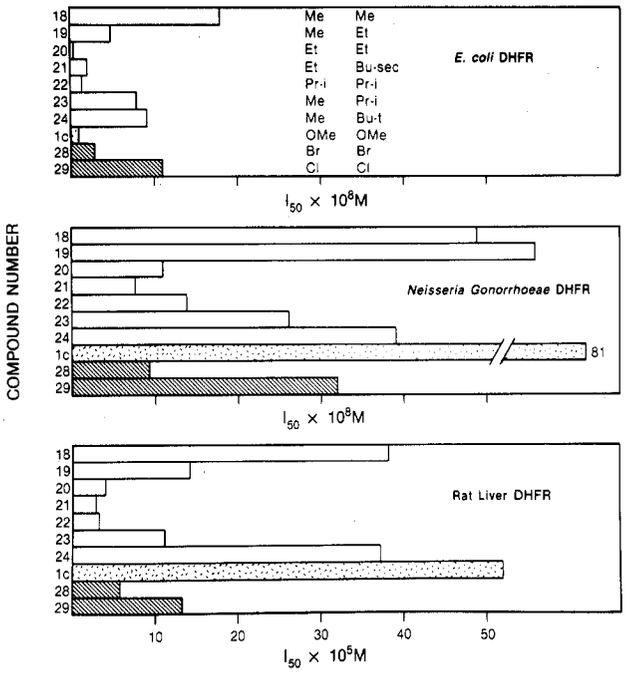

Figure 3. Activity profiles for 3,5-dialkyl-4-(aminobenzyl)pyrimidines against DHFR enzymes compared to 3,5-dimethoxy (**1c**) and dihalo analogues (**28** and **29**).

not satisfied by normal QSAR methods. The bromo derivatives are 2–4 times more active than their smaller chloro counterparts against all three enzymes. The π values for these two substituents are 0.86 and 0.71, respectively.

The data of Table III answer a number of additional questions. Compounds **31** and **32**, tetramethyl compounds, have slightly unfavorable spatial interactions in the ortho positions for *E. coli* DHFR (cf. **2**, Table I) and apparently totally incompatible interactions with the rat enzyme. Clearly their additive π values have no useful effect. Compound **34**, with a hydrophilic CH_2OH component added to a hydrophilic out-of-plane- 4-methoxy moiety, has a log *P* value of 1.55 (octanol/0.01 N NaOH) compared with 2.96 for the 4-methoxy analogue (**4**). This additional substitution has little, if any, effect on *E. coli* DHFR and decreases rat liver activity by a factor of 2. The extended ring-chain 4-substituents of **35** and **36** have little effect on *E. coli* DHFR, suggesting minimal enzyme contact, but do add activity to rat DHFR in a manner dependent on the locus of the chain ether substituent. As would be expected, the atoms have individual effects dependent on their locus in a partially hydrophobic environment. Comparisons of compounds **36** (Table III) and **27** (Table II) with their 4-methoxy counterparts **4** and **25** show 6.6- and 10-fold enhancements in binding, respectively, to rat DHFR provided by the additional CH_2CH_2OPh moiety. That this enhancement is provided almost solely by the aromatic ring is suggested by comparisons with compounds **33**, **38**, and **39**, all of which contain straight alkyl chains at the 4-position. Only the 3,5-di-*n*-propyl compound **39** shows increased binding to the rat enzyme compared to **4**, and this can be ascribed to the increased chain length at the 3,5-positions (cf. **6**, Table I). For *N. gonorrhoeae* and *P. berghei* DHFR, none of the 4-substituents of Table III has a marked positive effect on binding. The aralkyl groups of **35** and **36** both give about a 3-fold enhancement for the former enzyme, but straight and branched chains have a deleterious effect if anything.

The in vitro antibacterial activities of these TMP analogues will be reported in a separate paper, which also

(17) Although the data of this paper were obtained as I_{50} values, the use of K_i values is the preferred method of representing inhibitory affinity, because it is a true kinetic constant, which at saturating NADPH equals the dissociation constant of I from the E–I–NADPH ternary complex. However, the use of I_{50} values for a given series will provide the relative activities and selectivities, as discussed here. See ref 36 of Kuyper et al. (*J. Med. Chem.* **1985**, *28*, 3) for further discussion.

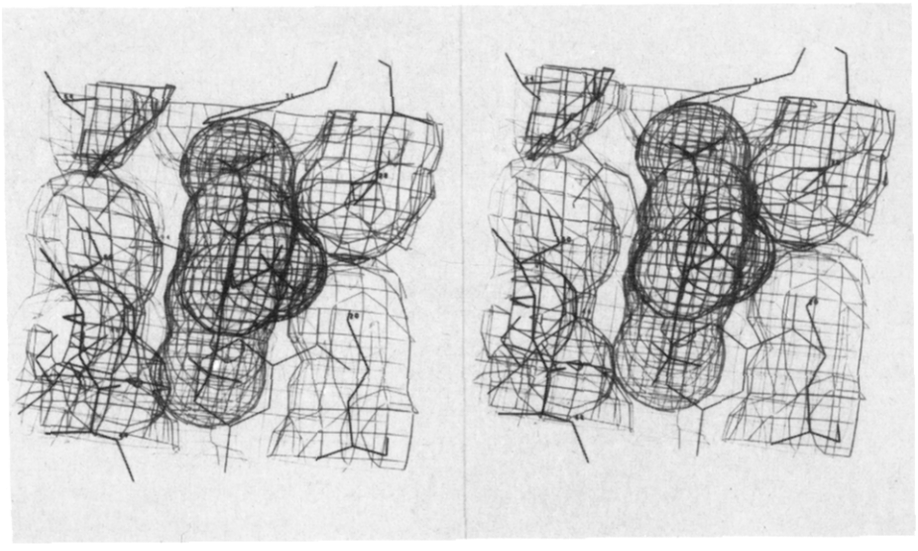

Figure 4. Stereo view of the active site of *E. coli* DHFR with TMP and NADPH as determined by X-ray studies.[5] The diagram shows the skeletal model with the envelope of the van der Waals radii of the enzyme active site and NADPH atoms; the van der Waals surface of the trimethoxybenzene of TMP is also shown, contoured finer to distinguish it from the rest of the active site. No surface is displayed for the diaminopyrimidine of TMP.

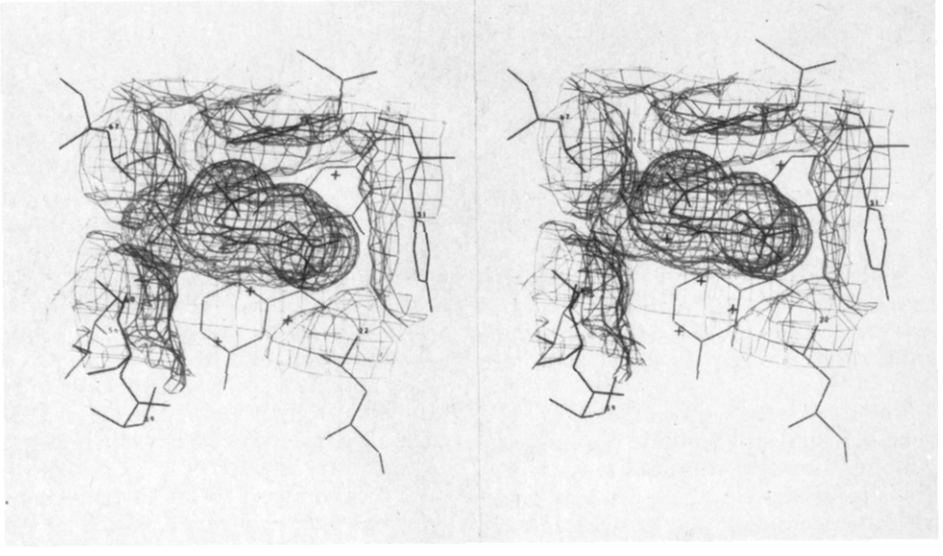

Figure 5. Stereo view of the active site of chicken DHFR with TMP and NADPH, analogous to Figure 4, as determined by X-ray studies.[6] Here certain solvent molecules, as discerned by crystallographic refinement, are also included.

discusses the pharmacokinetics of one of the compounds.

Discussion

The fact that the three-dimensional X-ray structure of *E. coli* DHFR in combination with TMP and NADPH has now been solved makes it possible to discuss the probable interactions of the TMP analogues of this paper with the enzyme at the molecular level.[5] In making use of the known structural data on DHFR, we make the assumption that the pyrimidine ring of each of our analogues retains the same locus as that of trimethoprim in ternary complex with the *E. coli* or chicken DHFR. This assumption is based on the fact that several hydrogen bonds are involved in the interaction of the pyrimidine with the enzyme.[7] We cannot always assume that the torsional angles between the two rings remain essentially as observed in the two TMP complexes, since some severe spatial constraints may develop, as will be discussed below. These torsional angles are, in fact, quite different for TMP in *E. coli* and chicken DHFR.[5,6] In the case of chicken DHFR, the conformations of several ligands were determined, and it was found that the aromatic moieties of each remained essentially superimposed in the center of a hydrophobic pocket.[18] However, there is a second lower hydrophobic pocket in this enzyme, as discussed by Matthews et al.[6,18] This pocket could easily accommodate the aromatic ring of TMP, but the upper pocket is no doubt preferred because of better van der Waals contacts in a situation where the cleft is wider than it is in the *E. coli* enzyme. This can actually be observed with computer graphics, using van der Waals surfaces for the lower pocket. In the *E. coli* case the narrower cleft width does not permit a good fit of TMP in the conformation observed in the chicken enzyme.

Figures 4 and 5 depict the active sites of *E. coli* and chicken liver DHFR, respectively.[5,6] The trimethoxyphenyl moiety of TMP is shown in each case with the stick model

(18) Matthews, D. A.; Bolin, J. T.; Burridge, J. M.; Filman, D. J.; Volz, K. W.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 392.

Table IV. Solvent Accessible Surface Areas[a] (Å^2) for Methoxy Groups of Trimethoprim in Ternary Complex with *E. coli* DHFR and NADPH[b] Compared to the Corresponding Accessibility in Chicken DHFR Ternary Complex[c,d]

substit atoms	solvent accessible surface area, Å^2			percent accessible	
	TMP alone[e]	*E. coli* ternary complex	chicken ternary complex	*E. coli* complex	chicken complex
3-methoxy					
O	6.9	1.8	0.0	26	0
CH_3	84.4	19.4	0.0	23	0
4-methoxy					
O	13.4	8.7	1.5	65	11
CH_3	77.3	27.0	18.5	35	24
5-methoxy					
O	6.4	2.9	0.0	45	0
CH_3	82.0	0.3	34.1	0.4	42

[a] Lee, B.; Richards, F. M. *J. Mol. Biol.* **1971**, *55*, 379. [b] The coordinates used are those of Champness et al. (ref 5). [c] We are most grateful to Dr. David Matthews for these coordinates. [d] The aromatic ring is almost completely buried in both complexes. [e] Phillips, T.; Bryan, R. F. *Acta Crystallogr., Sect. A.* **1969**, *A25*, S200.

surrounded by a van der Waals radius surface. This enables one to see readily the environment of these atoms. It should be noted that the *E. coli* DHFR coordinates are from X-ray data collected at 3.0-Å resolution.[5] These data are currently undergoing refinement, which may provide information about water structure in the vicinity of the inhibitor. The chicken DHFR coordinates are from a partly refined 2.2-Å resolution set of data.

Let us first study Figure 4, that for the *E. coli* enzyme. The milieu of the three methoxy groups provides key information for this study. The aromatic moiety lies almost vertically in the cleft as shown, with the three methoxy groups at the edge of the cleft, partially exposed to solvent. The oxygen atom of the 3-methoxy group (at the top) is in contact with Leu-28 to one side. The methyl group is in contact with the side chains of Leu-28 and Phe-31, with Ile-50 and Leu-54 side chains lying close by. The oxygen of the 4-methoxy group has Ile-50 main chain and Met-20 side chain nearby, but appears largely exposed to solvent. The methyl function, while fairly close to Leu-28 and Met-20 side chains, seems similarly exposed. The 5-methoxy oxygen atom has peptide 49/50 and Met-20 side chain nearby, but again faces solvent. The methyl moiety is largely buried in a hydrophobic pocket, comprising residues Met-20, Thr-46, Ser-49, and the nicotinamide moiety of NADPH. It is clear then that when a substituent contains more than one atom, its effect in the system we have described will be dependent on the particular atoms of the substituent and their location. If the atoms of the substituent are switched, for example by converting a methoxy group to a hydroxymethyl function, one might expect the inhibitory activities to be altered in the above-described situation. This is indeed the case with the 3,4-dimethoxy-5-hydroxymethyl analogue of TMP, which is 24-fold less active than TMP as an inhibitor of *E. coli* DHFR.[19] The methylene moiety will be largely in contact with solvent, but the solvated hydroxyl function, particularly if in the 5- (down) position, will require desolvation to interact favorably with the hydrophobic side chains of the enzyme, which costs energy.

Figure 5 shows a stereo view of the active site of chicken liver DHFR complexed with TMP and NADPH.[6] TMP adopts an entirely different conformation in this enzyme from that observed in Figure 4 and is rather less exposed to solvent. The inner *m*-methoxy group (3-methoxy) is totally buried. The 4- and 5-methoxy groups are positioned such that the methyl groups are both facing toward solvent, with the oxygen atoms of each now partially buried. The oxygen of the 4-methoxy group has side chains of Phe-34, Ile-60, and Leu-67 close by. The methyl group is in contact with Phe-34 and Leu-67 side chains, while the side chain of Gln-35 lies nearby. The whole of the methoxy group is accessible to solvent on the side not in contact with the above residues. The oxygen of the 5-methoxy group lies close to Phe-34, and the methyl function is in van der Waals contact with Tyr-31. As with the 4-substituent, one side of this methoxy group is accessible to solvent.

We measured the exposed surface areas for the oxygen and methyl moieties of the methoxy groups of TMP alone and in ternary complex with *E. coli* and chicken DHFRs. The numerical results are shown in Table IV, expressed as percent solvent accessibility of each moiety, as influenced by the presence of the protein and coenzyme. In the *E. coli* DHFR complex, all oxygen atoms are partially exposed, with the para oxygen being the most accessible to solvent. However, the methyl groups are less available. The 5-methyl, near the nicotinamide moiety of NADPH, is almost completely buried, as seems evident by inspection of Figure 4. In the chicken DHFR complex, measurements confirm the observation that the inner *m*-methoxy group is buried. As observed in Figure 5, the other two oxygen atoms are also largely buried, but the methyl groups now have more contact with solvent. In another paper we will compare these results with those for the 3,4,5-triethyl analogue of TMP in complex with chicken DHFR.

Most of our inhibitory data on vertebrate DHFR is against that from rat liver, which unfortunately has not been sequenced. However, sequences are known for DHFRs from mouse L1210 and S180,[20,21] from human K/B and 6A3 cells,[22,23] and from bovine,[24] porcine,[25] and chicken[26] DHFRs. The fact that all of these sequences are very similar in the region of the active site makes it reasonable to draw conclusions on the rat data, based on the chicken DHFR structure.

Replacement of the two *m*-methoxy groups of TMP by

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ethyl functions, while not providing precisely the same enzyme fit due to small differences in bond angles and lack of resonance energy supplied by the ether oxygen to hold the substituent in the plane of the ring, nevertheless will constitute a near isostere. Binding to *E. coli* DHFR should not be, and is not, altered appreciably, since, as discussed above, the methylene functions replace oxygen atoms that are partially in contact with solvent. However, in the vertebrate enzyme the methylene moieties, especially that of the 3-substituent, will occupy a largely hydrophobic environment, and this should, and does, increase binding by about 5-fold over the oxygen-containing analogue. The net result of these binding differences is that while shape appears important for *E. coli* DHFR, hydrophobicity is more significant for binding to the vertebrate enzyme, as proposed by Hansch using MR in QSAR calculations.[11-14] However, this result is clearly dependent on the nature and locus of atoms comprising the substituent. A suitable 4-substituent will furthermore affect the shape of the meta-X-methyl substituents by forcing them to bend from the 4-position toward the side chains with which they interact in the enzyme. It will thus influence binding indirectly.

Smaller substituents in the meta position, such as methyl, would be expected from Figure 4 to interact less well with *E. coli* DHFR, since they will not be in close contact with the side chains of Phe-31, Leu-54, and Ile-50 unless these side chains move, and indeed **2** and **3** are less active than **4**. This is also true of **29** compared to **28**. However, the larger iodo atom is still far from reaching the combined major radius of a methoxy or ethyl substituent and is indeed only slightly larger than a methyl group, as stated earlier. However, compound **25** is nearly as active as TMP. The π value for iodo, 1.12, is nearly equal to that of ethyl (1.02). Thus lipophilicity, rather than shape, could be a major factor influencing the effects of this type of substituent on *E. coli* DHFR. However, one must also consider the polarizability of an iodo atom. The parameter MR, which includes polarizability as well as bulk, is 5.65 for methyl but 13.94 for I. On the other hand, **25** is about 4 times as effective as **4**, and **26** about 1.5 times as effective as **12**, against the rat enzyme, suggesting that the symmetry of the halogen atom may assist in improved vertebrate DHFR binding.

Additional thickness of substituents, as in **7–9** and **15–17**, may require side-chain adjustments, for example, of Met-20, Ser-49, and Leu-28 in *E. coli* DHFR to accommodate the inhibitor (see Figure 4), which will increase binding energy, as noted in Table I. The data with rat DHFR are less obvious, since they follow a different pattern. The hydrophobic pocket associated with the inner (3-) methoxy group of TMP in chicken DHFR (Figure 5) appears to just fit this moiety. A *tert*-butyl substituent would cause considerable distortion of the enzyme. However, a conformational change for such compounds might occasion a better fit to the enzyme, for example if the the benzyl moieties now occupy the lower pocket in the approximate conformation observed for *E. coli* DHFR. This pocket, about 1 Å wider in chicken than in *E. coli* DHFR, should now make good van der Waals contacts with an isopropyl or *tert*-butyl substituent. The asymmetric compounds **8** and **16** would make poor contacts at the *m*-methyl position, however, causing them to have considerably lower activity than **9** and **17**. This hypothesis of course requires crystallographic proof. If large conformational changes were to occur within a series, such that the enzyme contacts were changed, as described here, it could render a normal QSAR analysis meaningless. In any event, shape, as well as lipophilicity, is clearly a factor in the binding of such compounds to vertebrate DHFR.

E. coli	Met^{20}	Pro^{21}	Trp^{22}	Asn^{23}		Leu^{24}	Pro^{25}	Ala^{26}	Asp^{27}	Leu^{28}	Ala^{29}
N. gonorrhoeae	Met^{22}	Pro^{23}	Trp^{24}	His^{25}		Ile^{26}	Pro^{27}	Glu^{28}	Asp^{29}	Phe^{30}	Ala^{31}
Mouse L1210	Leu^{22}	Pro^{23}	Trp^{24}	Pro^{25}	Pro^{26}	Leu^{27}	Arg^{28}	Asn^{29}	Glu^{30}	Phe^{31}	Lys^{32}

Figure 6. Comparison of partial sequences for three DHFR enzymes.

One intriguing observation is the effect of three types of 4-substituent on binding. We have seen that a 4-hydroxy group produces 2- to 3-fold increased binding to *E. coli* DHFR over the 4-methoxy series in the case of 3,5-dialkyl substitution, and as much as 10-fold improvement for rat DHFR (cf. **6** vs. **14**). That a hydrophilic substituent should produce this effect in a largely hydrophobic environment seems surprising. On the contrary, a 4-amino substituent, while causing modest improvements in binding to *E. coli* DHFR, has a very marked deleterious effect on binding to the rat enzyme, thus producing considerably greater selectivity. A possible explanation is that a 4-hydroxy group with two adjacent alkyl substituents cannot accept or donate a hydrogen bond to water, particularly if the hydrogen faces away from solvent. This seems plausible, since it is extremely difficult to dissolve compounds **10–17** in alkali. If on the other hand an amino substituent, which has two hydrogen atoms available for H bonding, can donate a hydrogen bond to water to produce a solvated aromatic moiety, this would certainly have a deleterious effect on binding to vertebrate DHFR, but might actually favor binding to that from *E. coli*, since the solvated amino function would remain in the aqueous phase, but with increased bulk would assist in forcing the alkyl substituents away from it toward the enzyme contacts. We have noted previously that an out-of-plane 4-substituent, as in TMP, favors high selectivity, probably by creating a poorer van der Waals fit of the meta substituents than a totally planar structure.[15]

We have included three disubstituted derivatives of TMP (**1d–f**) in Table I for a further consideration of the above phenomena. All three of these compounds are 7–14 times less active than their trisubstituted congeners against the *E. coli* enzyme, as expected, since the second meta contact to the enzyme is missing. However, in the case of both **1d** and **1e**, rat DHFR binding is increased 3- to 4-fold by omission of the second methoxy substituent, possibly because of deletion of an ether oxygen from a hydrophobic environment. On the other hand, **1f** retains low activity similar to **1c**, possibly as a result of 4-amino solvation, which would be more readily accomplished in the absence of the second flanking function.

The similarity in binding patterns between *N. gonorrhoeae*, *P. berghei*, and rat DHFRs suggests that the TMP analogues lie in a more hydrophobic environment in these enzymes than is the case with *E. coli* DHFR and furthermore that the three hydrophobic domains are similar. Although we do not know the three-dimensional structure of any of these enzymes, the *N. gonorrhoeae* enzyme has been sequenced.[27] A partial sequence comparison in the active site region between *E. coli*, *N. gonorrhoeae*, and mouse L1210 enzymes (Figure 6) illustrates a single important difference at position 28 (*E. coli* numbering), and that is the replacement of Leu by Phe. This residue becomes Tyr in chicken DHFR. This side chain is very important for hydrophobic contacts with the various lig-

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ands in each enzyme, and it will be appreciated that an aromatic residue is much more bulky than is Leu, thus creating a more completely hydrophobic environment at the mouth of the cleft. We would therefore predict that the methoxy groups of TMP in *N. gonorrhoeae* DHFR would be largely buried and inaccessible to solvent, in contrast to the case with *E. coli* DHFR. The result would be that the more hydrophobic analogues of Table I would be more active than TMP. A similar substitution is predicted for the malarial enzyme.

In conclusion, we may state that increased knowledge of ligand–enzyme–solvent interactions at the molecular level has produced a much greater awareness of the complexities of such interactions. Without this knowledge, empirical QSAR methods have proven to be generally predictive of trends. Recent efforts by Hansch and coworkers to explain QSAR parameters with the aid of molecular graphics have produced a realization that very complex mathematical equations may be required, as is the case with TMP analogues bound to chicken liver DHFR.[28] Here we have found that MR as a QSAR parameter fails to deal effectively with substituent shape of alkylbenzylpyrimidines, which represents a major barrier to fuller interpretation using QSAR. Alternative quantitative approaches are to be discussed in a future paper.

Experimental Section

Melting points were determined with a Hoover melting point apparatus and are uncorrected. Where analyses are indicated only by symbols of elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. Nuclear magnetic resonance (NMR) spectra were recorded on Varian XL-100 and FT-80 spectrophotometers; chemical shifts are reported in parts per million (δ) from internal tetramethylsilane. Ultraviolet spectra were recorded on a Cary 118 spectrophotometer.

2,4-Diamino-5-[4-(benzyloxy)benzyl]pyrimidine (41). To a stirred solution of 4-(benzyloxy)benzaldehyde (200 g, 0.94 mol) and β-anilinopropionitrile (138.8 g, 0.95 mol) in Me_2SO (600 mL) was added a solution of NaOMe (50.8 g, 0.94 mol) in 250 mL of MeOH. The mixture was warmed to 85 °C for 15 min and then cooled to 60 °C, and 1250 mL of water was added. After the mixture was cooled to 10 °C, the product, **3-anilino-2-[4-(benzyloxy)benzyl]acrylonitrile**, was filtered, washed with water, and used directly in the next reaction without purification. This product (320 g, 0.94 mol) was dissolved in 800 mL of EtOH with 191 g (2.0 mol) of guanidine hydrochloride, followed by the addition of 108 g (2.0 mol) of NaOMe. The mixture was heated under reflux for 2 h, diluted with 1.5 L of EtOH, and chilled to 10 °C. The product was washed with EtOH and water and dried; wt, 159 g (57%); mp 200–201.5 °C (absolute EtOH). Anal. ($C_{18}H_{18}N_4O$) C, H, N.

2,4-Diamino-5-(4-hydroxybenzyl)pyrimidine (42). Compound **41** (29.4 g, 0.1 mol) in 150 mL of glacial AcOH was hydrogenated at 90–100 °C on a Parr hydrogenator at about 45 psi with 5% Pd/C (10 g). The filtered solution was concentrated, and the resultant product was washed with 1 N NaOH (100 mL), followed by crystallization from DMF; yield, 12.2 g (56%); mp 303–308 °C. Anal. ($C_{11}H_{12}N_4O$) C, H, N.

2,4-Diamino-5-(3,5-diiodo-4-hydroxybenzyl)pyrimidine Hydrochloride (26). Compound **42** (2.1g, 9.7 mmol) was dissolved in 75 mL of glacial AcOH and heated to 40 °C. Then 3.4 g (21 mmol) of ICl in 10 mL of glacial AcOH was added dropwise to the reaction, followed by 70 mL of H_2O. The mixture was heated at 80 °C for 0.5 h, cooled, and diluted with 150 mL of H_2O, followed by the addition of 141 mL of concentrated NH_4OH to pH 8.5, while the temperature was maintained between 20 and 40 °C. A brown precipitate separated; 3.72 g (82%). Two recrystallizations from 85% EtOH plus 1 equiv of concentrated hydrochloric acid gave 1.15 g of **26**, mp 237–241 °C. Anal. ($C_{11}H_{10}I_2N_4O\cdot HCl$) C, H, Cl, I, N.

2,4-Diamino-5-(3,5-diiodo-4-methoxybenzyl)pyrimidine Hydrochloride (25). Compound **26** (0.95 g, 1.9 mmol) was dissolved in 10 mL of Me_2SO under N_2. Then 0.25 g (3.8 mmol) of KOH in 1 mL of H_2O was added to the reaction, followed by 0.12 mL (1.9 mmol) of MeI. The reaction stood overnight in a stoppered flask at room temperature, after which the solvent was removed in vacuo. The residue was dissolved in a mixture of 2:1 $CHCl_3$/MeOH plus 0.5 N NaOH. The organic layer was separated, dried, and evaporated to dryness. The residue (0.79 g; 86% as **25**) was recrystallized from absolute EtOH plus 1 equiv of concentrated hydrochloric acid; mp 236–238 °C. Anal. ($C_{12}H_{12}I_2N_4O\cdot HCl$) C, H, Cl, I, N.

2,4-Diamino-5-[3,5-diiodo-4-(3-phenoxypropoxy)benzyl]pyrimidine Hydrochloride (27). Compound **26** was alkylated with 3-phenoxypropyl bromide in Me_2SO with KOBu-*t* as the base. The mixture was heated at 105 °C for 1.5 h, followed by removal of the solvent in vacuo, slurrying the residue in 0.5 N NaOH, and extracting the product with $CHCl_3$. The solvent was removed, and the product was recrystallized as the hydrochloride salt from EtOH; mp 276–278 °C; 37%; NMR (Me_2SO-d_6) δ 2.27 (quintet, 2 CH_2), 3.65 (s, 2, CH_2 bridge), 4.07 (tr, 2, CH_2), 4.24 (tr, 2, CH_2), 6.8–7.5 (m, 5, Ar), 7.65 (s, 1, pyr-6-H), 7.70 (br, 2, NH_2), 7.78 (s, 2, Ar), 7.8–8.4 (br d, 2, NH_2), 12 (br, 1, NH); MS, 602, 510, 468. Anal. ($C_{20}H_{20}I_2N_4O_2\cdot HCl$) H, N; C: calcd, 37.61; found, 38.27.

2,4-Diamino-5-[3,5-diethyl-4-(β-hydroxyethyl)benzyl]pyrimidine (34). 2,4-Diamino-5-(3,5-diethyl-4-hydroxybenzyl)pyrimidine[4] (**12**) was alkylated with 2 equiv of 2-bromoethanol and 2 equiv of NaOMe, which were added 1 equiv at a time to a Me_2SO solution of the phenol. The product was worked up as for **27** and recrystallized from EtOH; mp 190.5–193 °C; 24%; NMR (Me_2SO-d_6) δ 1.13 (tr, 6, $(CH_2CH_3)_2$), 2.60 (q, 4, $(CH_2CH_3)_2$), 3.53 (s, 2, CH_2), 3.73 (s, 4, OCH_2CH_2), 4.80 (br, 1, OH), 5.67 (br s, 2, NH_2), 6.05 (br s, 2, NH_2), 6.92 (s, 2, Ar), 7.52 (s, 1, pyr-6-H); UV (cation, 0.1 N HCl) λ_{max} 271 nm (ϵ 5700), sh 218 (32 000), (free base, 0.01 N NaOH) λ_{max} 287 (7350), sh 237 (13 000); log *P* (octanol/0.01 N NaOH) 1.55. Anal. ($C_{17}H_{24}N_4O_2$) C, H, N.

2,4-Diamino-5-[3,5-diethyl-4-[2-(benzyloxy)ethoxy]benzyl]pyrimidine Hydrochloride (35). Compound **12** was alkylated with 2-(benzyloxy)ethyl *p*-toluenesulfonate[29] on a 25-mmol scale in Me_2SO plus NaOMe and following the procedure for **27**. The product was crystallized twice from EtOH and then converted to the hydrochloride salt; mp 234–238 °C (EtOH); 49%; NMR (Me_2SO-d_6) δ 1.13 (tr, 6, $(CH_2CH_3)_2$), 2.60 (q, 4, $(CH_2CH_3)_2$), 3.65 (s, 2, CH_2), 3.83 (br s, 4, CH_2CH_2), 4.60 (s, 2, OCH_2 Ar), 6.98 (s, 2, Ar), 7.37 (s, 5, Ar), 7.52 (s, 1, pyr-6-H), 7.67 (br s, 2, NH_2), 8.03 (br, 2 NH_2), 12.5 (v br, 1, NH). Anal. ($C_{24}H_{30}N_4O_2\cdot HCl$) C, H, N.

2,4-Diamino-5-[3,5-diethyl-4-(3-phenoxypropoxy)benzyl]pyrimidine Hydrochloride (36). Compound **12** was alkylated with 3-phenoxypropyl bromide in EtOH with KOH dissolved in a minimum of water as the base. The solution was heated under reflux for 5 h and then worked up as for **27**; mp 111–113 °C; 62%. The product was converted to the hydrochloride salt in absolute EtOH; mp 244–247 °C; NMR (free base) ($CDCl_3$) δ 1.13 (tr, 6, $(CH_2CH_3)_2$), 2.25 (quintet, 2, $CH_2CH_2CH_2$), 2.58 (quartet, 4, $(CH_2CH_3)_2$), 3.47 (s, 2, CH_2), 3.95 (tr, 2, CH_2), 4.25 (tr, 2, CH_2), 4.66 (br s, 2, NH_2), 4.85 (br s, 2, NH_2), 6.87 (s, 2, Ar), 6.9–7.5 (m, 5, Ar), 7.79 (s, 1, pyr-6-H). Anal. ($C_{24}H_{30}N_4O_2\cdot HCl$) C, H, N.

2,4-Diamino-5-[3,5-diethyl-4-[(2-ethylhexyl)oxy]benzyl]pyrimidine (37). Compound **12** was alkylated with 2-ethylhexyl iodide by the method of **36**; mp 122–123 °C (EtOH); 55%. Anal. ($C_{23}H_{36}N_4O$) C, H, N.

2,4-Diamino-5-[3,5-diethyl-4-(dodecyloxy)benzyl]pyrimidine (38). Compound **12** was alkylated with *n*-dodecyl iodide in absolute EtOH, plus KOH, and purified as in the previous examples; mp 140–142 °C (EtOH); 75%. Anal. ($C_{27}H_{44}N_4O$) C, H, N.

Methyl (3,4,5-Triethoxybenzoyl)methyl Sulfone (43). A mixture of 15 mL of dry Me_2SO, 7.95 g of Me_2SO_2, and 4.1 g (57% real) of NaH was warmed to 50 °C in an atmosphere of N_2 for 90 min, after which a solution of methyl 3,4,5-triethoxybenzoate

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(11 g obtained as the crude ester from 3,4,5-triethoxybenzoic acid[30] plus ethanol and sulfuric acid) in Me_2SO (18 mL) was added dropwise at a rate such that foaming was not excessive. The mixture was then held at 60 °C for 90 min. After cooling it was poured onto flaked ice (155 g). The mixture was then acidified with 6 N hydrochloric acid (22 mL); a white precipitate separated, which was filtered, washed with water and ether, and dried; weight, 7.3 g. (54% crude product); mp 135–136 °C (EtOH). Anal. ($C_{15}H_{22}O_6S$) C, H.

Methyl β-Hydroxy-β-(3,4,5-triethoxyphenyl)ethyl Sulfone (44). Compound **43** (7.2 g), EtOH (10 mL), and water (20 mL) were cooled under N_2 to 15 °C, and a cold solution of $NaBH_4$ (0.32 g) in 12 mL of water added dropwise. The mixture was stirred at 25 °C for 3.5 h, after which it was chilled to 0 °C for 1 h, and the resultant white precipitate was isolated; 5.5 g (76%); mp 106–108 °C (EtOH). Anal. ($C_{15}H_{24}O_6S$) H; C: calcd, 54.20; found, 55.52.

β-Anilino-β-(3,4,5-triethoxybenzyl)acrylonitrile (45). A mixture of 5.4 g (0.016 mol) of **44**, 2.62 g (0.018 mol) of β-anilinopropionitrile, and 10 mL of dry Me_2SO was warmed to 40 °C under N_2 with stirring, and a solution of 1.82 g (0.016 mol) of KOBu-*t* in 13 mL of *t*-BuOH plus 5 mL of Me_2SO was added. The mixture was then maintained at 45 °C for 3.5 h. The solvent was then removed under vacuum and the residue poured into ice water. The resultant brown oil slowly crystallized upon cooling overnight; this was isolated and washed with cold water, followed by hexane; weight, 5.4 g (92%); mp 151–154 °C (EtOH). Anal. ($C_{22}H_{27}N_2O_3$) C, H, N.

2,4-Diamino-5-(3,4,5-triethoxybenzyl)pyrimidine (40).[31] To a solution of NaOMe (1.47 g, 0.0272 mol) in absolute EtOH (15 mL) was added 1.96 g (0.0205 mol) of guanidine hydrochloride. This mixture was warmed gently for about 10 min, and compound **45** (2.5 g, 0.0068 mol) was then added, followed by sufficient absolute EtOH to wash down the sides of the flask. The mixture was heated for 24 h under reflux, filtered hot to remove NaCl, concentrated, and chilled. The resultant precipitate was isolated and washed well with water; wt, 1.7 g; mp 171–171.5 °C (EtOH). Anal. ($C_{17}H_{24}N_4O_3$) C, H, N.

2,4-Diamino-5-(4-amino-3-methoxybenzyl)pyrimidine (46). *o*-Anisidine (37.8 g, 0.3 mol), 5-(hydroxymethyl)-2,4-diaminopyrimidine[4] (42 g, 0.3 mol), ethylene glycol (300 mL), and concentrated hydrochloric acid (25.1 mL) were stirred and heated at 110–115 °C for 6 h. The reaction was poured into 2 L of acetone and stirred for 3 h. The precipitate which formed was filtered and dried, giving 30 g 31%) of the dihydrochloride of **46**. This was converted to the free base by slurrying in ammonium hydroxide and water to give 16.5 g (22.4%) of product; mp 210–212 °C; NMR (Me_2SO-d_6) δ 3.45 (s, 2, CH_2), 3.65 (s, 3, OCH_3), 4.2 (br s, 2, NH_2-*p*), 5.5 (br s, 2, NH_2-pyr), 5.95 (s, 2, NH_2-pyr), 6.50 (d, 1, arom), 6.53 (d, 1, arom), 6.7 (s, 1, arom), 7.45 (s, 1, pyr-6H); MS, 245 (M^+) UV (neutral species, pH 12) λ_{max} 232 nm (ε 18900), 290.5 (11000), (cation, 0.01 N HCl) λ_{max} 273 (8200). Anal. ($C_{12}H_{15}N_5O$) C, H, N.

Enzyme Assays. Dihydrofolate reductase from strain T47 *Neisseria gonorrhoeae* was purified to homogeneity as previously described.[27] The standard assay was performed in 0.1 M imidazole chloride buffer, pH 6.4, with 12 mM mercaptoethanol, 60 μM NADPH, and 45 μM dihydrofolate in a final volume of 1 mL at 30 °C. One enzyme unit is defined as the amount of enzyme needed to reduce 1 μmol of dihydrofolate/min on the basis of an extinction coefficient of 12.3×10^3 M^{-1} cm^{-1} at 340 nM.[32] The concentration of inhibitor necessary to inhibit enzymic activity by 50% (IC_{50}) was determined by a modification of this assay. The enzyme, NADPH, and varying concentrations of inhibitor were preincubated for 2 min at 30 °C, and the reaction was initiated by the addition of dihydrofolate. Plots of the percentage inhibition vs. the logarithm of inhibitor concentration were used to estimated IC_{50} values.

Assays for *E. coli* and rat liver DHFR were carried out as previously described,[2] as were those for *P. berghei*.[33]

Acknowledgment. We are greatly indebted to Dr. Lee Kuyper and James Bentley for the calculations of solvent accessible surface areas of the inhibitor in the protein and to the former for many helpful discussions. We are grateful for the continued interest and support of Dr. David Henry and Dr. A. J. Everett.

Registry No. **1a**, 738-70-5; **1b**, 21253-58-7; **1c**, 56066-19-4; **1d**, 5355-16-8; **1e**, 73356-40-8; **1f**, 85544-45-2; **2**, 73576-30-4; **3**, 105639-85-8; **4**, 36821-38-2; **5**, 105639-86-9; **6**, 36821-94-0; **7**, 36821-90-6; **8**, 105639-87-0; **9**, 39667-06-6; **10**, 42310-36-1; **11**, 105639-88-1; **12**, 36821-97-3; **13**, 73554-79-7; **14**, 73554-78-6; **15**, 42310-33-8; **16**, 105639-89-2; **17**, 73554-80-0; **18**, 56066-80-9; **19**, 86519-43-9; **20**, 86519-42-8; **21**, 86519-45-1; **22**, 86519-37-1; **23**, 86519-44-0; **24**, 86519-46-2; **25**·HCl, 105639-90-5; **26**·HCl, 83322-30-9; **27**·HCl, 83534-26-3; **28**, 74674-60-5; **29**, 62909-61-9; **30**, 86519-47-3; **31**, 84876-41-5; **32**, 84876-42-6; **33**, 36821-98-4; **34**, 105639-91-6; **35**·HCl, 83322-33-2; **36**·HCl, 105639-92-7; **37**, 57506-38-4; **38**, 36821-99-5; **39**, 105639-93-8; **40**, 39711-86-9; **41**, 49873-11-2; **42**, 30077-67-9; **43**, 39666-74-5; **44**, 39666-75-6; **45**, 105639-94-9; **46**, 85544-45-2; **46**·HCl, 105639-95-0; 4-(benzyloxy)benzaldehyde, 4397-53-9; β-anilinopropionitrile, 1075-76-9; 3-anilino-2-[4-(benzyloxy)benzyl]acrylonitrile, 30077-74-8; *o*-anisidine, 90-04-0; 5-(hydroxymethyl)-2,4-diaminopyridine, 42310-45-2; dihydrofolate reductase, 9002-03-3.

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