2.7 g (0.01 mol) of 1-(methoxyphenyl)cyclohexylpiperidine dissolved in 60 mL of CH_2Cl_2 was slowly added to an ice-cold solution of 3 mL of BBr_3 (0.03 mol) in 20 mL of CH_2Cl_2 while stirring continuously. Then the solution was stirred overnight at room temperature. With an efficient cooling, cold water was cautiously added until no more gas evolved (HBr) and a precipitate occurred. After 5 h in the cold (0–5 °C), the precipitate was collected and crystallized from alcohol to give the hydrobromide of the phenolic PCP derivative. In the case of **3**, the hydrobromide is mostly concentrated in the organic layer where it precipitates slowly (48 h). **1**: yield 1.7 g (50%); mp (HBr) 122–124 °C. Anal. ($C_{17}H_{26}NOBr$) C, H, N. **2**: yield 1.5 g (45%); mp (HBr) 192–195 °C. Anal. ($C_{17}H_{26}NOBr$) C, H, N. **3**: yield 1.9 g (55%); mp (HBr) = 154–157 °C. Anal. ($C_{17}H_{26}NOBr$) C, H, N.

4-Phenyl-4-(1-piperidinyl)cyclohexanols 4a,b. Phenylmagnesium bromide was added to 4-hydroxycyclohexanone to obtain 5.1 g of 1-phenyl-4-hydroxycyclohexanol. The substitution of the tertiary OH by N_3^- was made using an already published procedure[18,19] with slight modifications: 5.1 g (0.027 mol) of the diol was added at 0 °C to a vigorously stirred suspension of 3.51 g (0.054 mol) of NaN_3 and 4.59 (0.027 mol) of CCl_3COOH in 30 mL of $CHCl_3$. After a 5-h reaction, neutralization with NH_4OH, and extraction with $CHCl_3$, the organic layer was dried on Na_2CO_3. After evaporation in vacuo, the residue containing the two isomeric azides weighed 5.48 g. These crude azides were refluxed in ether overnight with 1.5 g of $LiAlH_4$. $CHCl_3$ (20 mL) was added after decomposition by H_2O, and the complex was filtered. The chloroform filtrate was extracted with 10% HCl; then the acidic solution, after neutralization with NH_4OH, extraction by $CHCl_3$, drying (Na_2SO_4), and evaporation in vacuo, gave a residue of primary amines (2.3 g). The crude amines in a solution of 1 equiv of 1,5-dibromopentane in 20 mL of anhydrous acetone were refluxed for 48 h. Then, 1 equiv of K_2CO_3 was added, and the reflux was maintained for an additional 48 h. After filtration, evaporation, dissolution in 10% HCl, and ether extraction, the resulting aqueous phase was neutralized (NH_4OH) and extracted ($CHCl_3$). Drying and evaporation of the solvent gave a crude residue in a yield of 2.2 g. Column chromatography on silica gel gave 0.8 g of **4b**, eluted with 15% MeOH in ether, and 1.1 g of **4a**, eluted with 20% MeOH in ether. **4a**: mp (base) 152–153 °C; mp (HCl) 190 °C dec (lit.[11] 200–201 °C). **4b**: mp (base) 170–171 °C; mp (HCl) 205 °C dec (lit.[11] 201–202 °C). Anal. ($C_{17}H_{26}NOCl$) C, H, N.

3-Phenyl-3-(1-piperidinyl)cyclohexanols 5a,b. Compounds **5a** and **5b** were prepared as described for the 4-hydroxy derivatives: 2.1 g of the crude isomeric mixture was obtained from 8.3 g of 1-phenyl-3-hydroxycyclohexanol. Column chromatography on silica gel gave 1 g of **5b**, eluted with 30% of petroleum ether in ether, and 0.7 g of **5a**, eluted with 1% MeOH in ether. **5a**: base, oily; HCl salt, hygroscopic; **5b**: mp (base) 117–121 °C; mp (HCl) 175 °C dec. Anal. ($C_{17}H_{25}NO$) C, H, N.

1-(1-Phenylcyclohexyl)-4-piperidinol (6). The α-aminonitrile was prepared in an organic medium according to a published method[21] from cyclohexanone, 4-piperidinol, and KCN with a yield of 77% from crude material. The Bruylants reaction was performed as usual[12,22] on 2.1 g of the α-aminonitrile crystallized in petroleum ether and gave 2.2 g of crude material. After a chromatography on aluminum oxide in pure ether, we obtained 1.5 g (58% yield) of **6**: mp (base) 116–117 °C (lit.[12,19,21] 116–118 °C); mp (HCl) 223–224 °C. Anal. ($C_{17}H_{26}NOCl$) C, H, N.

Binding Assays. Brain tissue preparation and binding experiments were carried out as described by Vincent et al.[8,9] ([^{3}H]phencyclidine binding), Yamamura and Snyder[29] (muscarinic cholinergic receptor), and Pert and Snyder[30] (opiate receptor).

Radioactively labeled compounds were obtained as follows: [^{3}H]phencyclidine (48 Ci/mmol) from New England Nuclear; [^{3}H]quinuclidinyl benzylate (QNB; 5 Ci/mmol) and [^{3}H]morphine (30 Ci/mmol) from Amersham.

Dissociation constants ($K_{0.5}$) and Hill coefficients (n_H) were computed using a Wang 2200 calculator as previously described.[8]

Rotarod Test. This test, involving the ability of mice to remain on a rotating rod, was carried out as previously described.[34]

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A Comparison of the Inhibitory Action of 5-(Substituted-benzyl)-2,4-diaminopyrimidines on Dihydrofolate Reductase from Chicken Liver with That from Bovine Liver†

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Forty-four 5-(substituted-benzyl)-2,4-diaminopyrimidines have been tested as inhibitors of chicken and bovine liver dihydrofolate reductase. The chicken enzyme is, on the average, about 10 times less easily inhibited than bovine enzyme. Substituents which show the greatest selectivity are 4-$NHCOCH_3$, 3-OC_4H_9, 3-I, 3-CF_3-4-OCH_3, and 3,4,5-$(OCH_3)_3$. The inhibition constants have been used to formulate quantitative structure–activity relationships for comparative purposes.

One approach to the development of new drugs, when the biochemistry is known, is to find inhibitors that are selective for a crucial enzyme from a pathogen that is relatively nontoxic to the enzyme from the host. When the enzymes can be readily obtained, this allows one to establish an intrinsic therapeutic index before one commences the study of the inhibitors under extremely complex conditions in animals. An outstanding success story based on such a concept is the antibacterial trimethoprim [I, X = 3,4,5-$(OCH_3)_3$] developed by Roth et al.[1,2] of the

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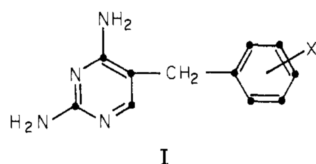

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Wellcome Laboratories. This drug is thousands of times more active against bacterial dihydrofolate reductase (DHFR) than mammalian enzyme. DHFR would seem to be an enzyme holding great potential for medicine, since enzyme from different sources shows greatly varying activity with a variety of inhibitors.[3,4] The reasons for this selectivity of trimethoprim (as well as other inhibitors) for DHFR from varous sources is a subject of intense interest with which we have become fascinated.[5-8] In a recent report[5] we compared the quantitative structure-selectivity relationship (QSSR) for 5-(substituted-benzyl)-2,4-diaminopyrimidines (I) acting on DHFR from *Escherichia coli* and bovine liver. In this paper we have extended the study of bovine DHFR and now compare it with reductase from chicken liver. It is our belief that the best way to get a firm understanding of how ligands react with enzymes and other receptors in general is to study one particular enzyme in depth. DHFR is particularly attractive because such a large effort is being made to elucidate the structure of this substance from various sources.

The amino acid sequence is known for both chicken liver,[9,10] and bovine liver[11] DHFR, and there are numerous differences in these two enzymes as well as in DHFR from other sources.[4,9,10] Chicken DHFR contains a preponderance of basic amino acid residues and has an isoelectric point at pH 8.4, while bovine has a p*I* of 6.8. Chicken DHFR contains Lys at positions 32, 106, and 154, while bovine DHFR has Gln, Thr, and Glu at the corresponding positions. The avian molecule has His at positions 42, 131, and 140, while in the bovine enzyme Ser, Val, and Gln occur at the corresponding locations. Other changes are (avian → bovine): Ala → Glu at position 98, Ser → Asp at position 102. The chicken DHFR is activated 12- to 13-fold by a stoichiometric amount of methylmercuric hyroxide (CH_3HgOH), and a 2-fold excess of *p*-(hydromercuric)benzoate (pHMB) yields an 8-fold increase in activity. In contrast, the bovine enzyme is *inhibited* by CH_3HgOH. Comparison of the avian enzyme with the bovine enzyme shows 75% identities in the amino acid sequence, while its comparison with *Lactobacillus casei* or *E. coli* reductase shows only 24 and 22% identities, respectively. These differences between chicken and bovine reductase and others discussed by Kumar et al.[9] lead one to expect differences in the way the two enzymes would react with a set of inhibitors.

Besides the large amount of biochemical work that has been carried out on the avian enzyme, Matthews and Kraut have now completed the X-ray crystallographic structure of this enzyme.[7]

Results

The log $(1/K_{i\,app})$ values for the action of variations of I on the two forms of DHFR are given in Table I for assay at pH 7.2. In our earlier report[5] on the action of I on the bovine reductase, a number of the measurements were made at pH 6.25. The activity of these has now been determined at pH 7.2; in addition, we have also synthesized eight new congeners.

Equations 1–5 for the inhibition of chicken liver DHFR

$$\log (1/K_{i\,app}) = 0.38\ (\pm 0.10)\ \pi_{3,4,5} + 4.46\ (\pm 0.10) \quad (1)$$

$$n = 39;\ r = 0.794;\ s = 0.317;\ F_{1,37} = 63.1$$

$$\log (1/K_{i\,app}) = 0.34\ (\pm 0.09)\ \pi_{3,4,5} + 0.18\ (\pm 0.11)\ \mathrm{MR}_3 + 4.32\ (\pm 0.12) \quad (2)$$

$$n = 39;\ r = 0.849;\ s = 0.280;\ F_{1,36} = 11.6$$

$$\log (1/K_{i\,app}) = 0.39\ (\pm 0.08)\ \pi_{3,4,5} + 0.23 (\pm 0.10)\ \mathrm{MR}_3 - 0.083\ (\pm 0.06)\ \pi^2_{3,4,5} + 4.38\ (\pm 0.12) \quad (3)$$

$$n = 39;\ r = 0.880;\ s = 0.254;\ \pi_0 = 2.33\ (1.4\text{–}6.9);\ F_{1,35} = 8.53$$

$$\log (1/K_{i\,app}) = 0.38\ (\pm 0.08)\ \pi_{3,4,5} + 0.20\ (\pm 0.10)\ \mathrm{MR}_3 - 0.069\ (\pm 0.06)\ \pi^2_{3,4,5} + 0.32\ (\pm 0.26) \sum\sigma + 4.36\ (\pm 0.11) \quad (4)$$

$$n = 39;\ r = 0.900;\ s = 0.237;\ \pi_0 = 2.76\ (1.6\text{–}13);\ F_{1,34} = 6.31$$

$$\log (1/K_{i\,app}) = 0.55\ (\pm 0.19)\ \pi_{3,4,5} + 0.20\ (\pm 0.10)\ \mathrm{MR}_3 - 0.42\ (\pm 0.35)\ \log (\beta \cdot 10^{\pi_{3,4,5}} + 1) + 0.32\ (\pm 0.26) \sum\sigma + 4.46\ (\pm 0.16) \quad (5)$$

$$n = 39;\ r = 0.900;\ s = 0.241;\ \log \beta = -0.222$$

have been derived from the data in Table I. In these equations $\pi_{3,4,5}$ refers to the $\sum\pi$ (relative hydrophobicity[12]) of substituents of these positions of I, MR_3 (scaled by 0.1) is the molar refractivity[12] of the 3-substituent, and σ is the Hammett substituent constant.[12] The figures in parentheses are for the construction of 95% confidence limits. The number of data points upon which the correlation is based is represented by n, r is the correlation coefficient, and s is the standard deviation from the regression equation. Equations 1–4 show the stepwise development of the so-called parabolic QSAR model[13] with validation of each term by the F test. The most marginal term, σ, is justified at $\alpha = 0.05$ ($F_{1,30(\alpha=0.05)} = 4.17$). This small dependence of inhibitory power of the benzylpyrimidines on σ turns up in other QSAR for DHFR.

Equation 5 is the corresponding bilinear model of Kubinyi[14,15] for comparison with eq 4. Note that the corre-

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lation with eq 5 is slightly poorer (compare values of s) than eq 4. The right-hand side of the bilinear portion of eq 5 has a positive slope of 0.13 (0.55 − 0.42); hence, using our present data set, we cannot derive π_0 from this equation. Attempts to test more lipophilic congeners to establish π_0 were frustrated by the low water solubility of such molecules. The chicken liver DHFR is about 10 times less sensitive to the benzylpyrimidines than bovine liver DHFR and even less sensitive than the bacterial DHFR. As of the present, we shall have to be satisfied with the rough estimate of π_0 from eq 4.

The MR_3 term has the same coefficient in both eq 4 and 5, and its positive coefficient suggests that the volume of the 3-substituent plays a special role in increasing the inhibitory power of I. This would seem to us to occur via 3-substituents interacting at a sensitive site in the active cavity, the nature of which will have to await detailed study of the X-ray crystallographic structure of the enzyme.

The following squared correlation matrix shows that π, MR, and σ are reasonably independent vectors. Therefore, MR_3 and σ do seem to make small independent contributions to the SAR.

	$\pi_{3,4,5}$	MR_3	$\Sigma\sigma$
$\pi_{3,4,5}$	1.00	0.05	0.00
MR_3		1.00	0.03
$\Sigma\sigma$			1.00

Even though eq 5 is a slightly less good correlation than eq 4, we prefer to discuss the SAR in terms of this model, since we have in general found the bilinear model to be most applicable to describe the SAR of antifolates with isolated DHFR as well as with some of the cell culture studies.

Turning now to the results with bovine liver DHFR, we have formulated eq 6–9. Because of the greater activity

$$\log (1/K_{i\,app}) = 0.27\ (\pm 0.10)\ \pi_{3,5} + 5.49\ (\pm 0.11) \quad (6)$$

$$n = 42;\ r = 0.663;\ s = 0.333;\ F_{1,40} = 30.5$$

$$\log (1/K_{i\,app}) = 0.50\ (\pm 0.12)\ \pi_{3,5} - 1.29\ (\pm 0.49) \log (\beta \cdot 10^{\pi_{3,5}} + 1) + 5.53\ (\pm 0.08) \quad (7)$$

$$n = 42;\ r = 0.825;\ s = 0.258;\ \pi_0 = 2.14;\ F_{1,38} = 14.8;\ \log \beta = -2.34$$

$$\log (1/K_{i\,app}) = 0.50\ (\pm 0.12)\ \pi_{3,5} - 1.28\ (\pm 0.40) \log (\beta \cdot 10^{\pi_{3,5}} + 1) + 0.15\ (\pm 0.10)\ MR_3 + 5.43\ (\pm 0.11) \quad (8)$$

$$n = 42;\ r = 0.858;\ s = 0.237;\ \pi_0 = 1.74\ (0.70\text{–}2.78);\ F_{1,37} = 7.88;\ \log \beta = -1.93$$

$$\log (1/K_{i\,app}) = 0.48\ (\pm 0.11)\ \pi_{3,5} - 1.25\ (\pm 0.40) \log (\beta \cdot 10^{\pi_{3,5}} + 1) + 0.13\ (\pm 0.10)\ MR_3 + 0.24\ (\pm 0.24)\ \sigma + 5.43\ (\pm 0.10) \quad (9)$$

$$n = 42;\ r = 0.875;\ s = 0.227;\ \pi_0 = 1.52\ (0.47\text{–}2.57);\ F_{1,36} = 4.32;\ \log \beta = -1.98$$

of the bovine enzyme, we were able to test three of the more lipophilic compounds too insoluble for testing with the avian enzyme. As a consequence, π_0 for the bovine reductase has been established as about 1.8, a value somewhat lower than that obtained for our initial study[5] (2.2). In the present investigation, 44 congeners were tested on the bovine enzyme, but two [1 and 5, Table I: I, X = 3,5-$(OH)_2$ and 3,5-$(OCH_3)_2$-4-$O(CH_2)_2OCH_3$] were badly fit and not included in the development of eq 6–9. If these two badly fit points are included in the analysis, it is found that σ is no longer significant and that the best equation is eq 10.

$$\log (1/K_{i\,app}) = 0.64\ (\pm 0.16)\ \pi_{3,5} - 1.29\ (\pm 0.47) \log (\beta \cdot 10^{\pi_{3,5}} + 1) + 0.20\ (\pm 0.15)\ MR_3 + 5.39\ (\pm 0.14) \quad (10)$$

$$n = 44;\ r = 0.822;\ s = 0.328;\ \pi_0 = 1.46\ (-0.06\text{–}2.98);\ \log \beta = -1.47$$

Considering the confidence limits on the parameters of the two equations, eq 10 cannot be said to be significantly different from eq 8 except for the σ term. This term is of such marginal importance that the additional noise introduced by the two bad points obscures the role of σ.

Whether π_0 for eq 3 and 4 is significantly different from eq 8 is not clear because of the large confidence limits on this parameter. Even the fact that we have used $\pi_{3,5}$ in eq 6–10 and $\pi_{3,4,5}$ in eq 1–5 cannot be taken as an important difference in the two enzymes. This can be shown by splitting the data into a set of 4-substituted congeners and a set of 3,5-disubstituted derivatives. It is not possible to derive a QSAR of much significance from the 4-substituted congeners. The best equation is eq 11.

$$\log (1/K_{i\,app}) = 0.09\ (\pm 0.08)\ \pi + 0.21\ (\pm 0.24)\ \sigma + 5.39\ (\pm 0.11) \quad (11)$$

$$n = 14;\ r = 0.664;\ s = 0.162;\ F_{2,11} = 4.34$$

One of the main reasons for this poor correlation is the lack of variance in K_i. Note that the standard deviation of eq 11 is considerably lower than that of eq 8; that is, it is lower than our level of resolution set by eq 8. The 4-$NHCOCH_3$ congener—the most active of the 4-X-I group—has been omitted in the formulation of eq 11. Including this analogue results in an even poorer result. It seems likely that through some specific polar or weak hydrogen bonding with the bovine reductase it achieves about 5-fold more activity than eq 11 predicts.

The wide variance in structure of the 4-substituents simply does not result in a wide variation in activity. For example, 4-F, 4-NO_2, and 4-$O(CH_2)_6CH_3$ (16, 17, and 19, Table I) all have almost identical inhibitory effects.

In particular, hydrophobicity of the 4-substituent does not appear to play any role in the inhibitory process. The π term in eq 11 has a coefficient too low for any mechanistic significance. Although the σ term alone is not significant, its coefficient in eq 11 is about the same as in eq 8. Again we are forced to the same conclusion of our earlier study[5] that 4-substituents make little or no contact with the enzyme.

The equation comparable to eq 11 for chicken DHFR is eq 12. The correlation with eq 12 is better than eq 11

$$\log (1/K_{i\,app}) = 0.19\ (\pm 0.15)\ \pi + 0.34\ (\pm 0.36)\ \sigma + 4.41\ (\pm 0.15) \quad (12)$$

$$n = 14;\ r = 0.740;\ s = 0.242;\ F_{2,11} = 6.66$$

in terms of F, r, and the confidence limits on the π term. The standard deviation is comparable to that of eq 3 or 4. The 4-$NHCOCH_3$ analogue is well behaved with this equation and is used in its formulation. The 4-$O(CH_2)_6CH_3$ congener was too insoluble to be tested with the more weakly active chicken liver DHFR; hence, eq 12 is based on only 14 derivatives. Although the case for a true hydrophobic effect for the 4-substituents is weak, we have elected for the present to use $\pi_{3,4,5}$ in eq 1–5 rather than $\pi_{3,5}$; so doing yields a slightly better correlation.

These results and the results of our earlier work[5] are similar to the qualitative observations by Roth et al.[2] obtained from a set of 48 congeners of I having the substituent arrangement 3,5-$(OCH_3)_2$-4-X. These benzylpyrimidines were assayed against *E. coli* CN314. Except

Table I. Parameters Used in the Derivation of Equations 1-12

		$\log (1/K_{i\,\mathrm{app}})$									
		bovine			chicken						
no.	X	obsd	calcd [a]	$\lvert\Delta\rvert$	obsd	calcd [b]	$\lvert\Delta\rvert$	$\pi_{3,4,5}$	$\pi_{3,5}$	MR_3	$\Sigma\sigma$
1	$3,5\text{-}(OH)_2$ [c]	3.39	4.88	1.49				−1.34	−1.34	0.27	0.24
2	$3,5\text{-}(CH_2OH)_2$	4.30	4.53	0.23	3.23	3.52	0.29	−2.06	−2.06	0.72	0.0
3	$3,4\text{-}(OH)_2$	4.59	5.08	0.49	3.59	3.77	0.18	−1.34	−0.67	0.29	−0.28
4	3-OH	4.88	5.17	0.29	3.87	4.25	0.38	−0.67	−0.67	0.29	0.12
5	$3,5\text{-}(OCH_3)_2$, $4\text{-}O(CH_2)_2OCH_3$ [c]	4.88	5.51	0.63	3.64	4.15	0.51	−0.72	−0.04	0.79	0.0
6	$4\text{-}NH_2$	4.96	5.28	0.32	3.73	3.66	0.07	−1.23	0.0	0.10	−0.66
7	$3,4\text{-}(OCH_2CH_2OCH_3)_2$	5.17	5.46	0.29	3.91	4.08	0.17	−0.80	−0.40	1.93	−0.14
8	$3\text{-}CH_2OH$	5.20	5.03	0.17	4.31	4.03	0.28	−1.03	−1.03	0.72	0.0
9	$4\text{-}N(CH_3)_2$	5.21	5.24	0.03	4.01	4.19	0.18	0.18	0.0	0.10	−0.83
10	$4\text{-}CH_3$	5.27	5.40	0.13	4.56	4.61	0.05	0.56	0.0	0.10	−0.17
11	$3\text{-}OCH_2CONH_2$	5.27	5.01	0.25	4.27	3.92	0.35	−1.37	−1.37	1.60	0.12 [d]
12	$4\text{-}OCH_2CH_2OCH_3$	5.31	5.38	0.07	4.26	4.21	0.05	−0.40	0.0	0.10	−0.24 [d]
13	$4\text{-}OCH_3$	5.35	5.37	0.02	4.29	4.35	0.06	−0.02	0.0	0.10	−0.27
14	4-Br	5.42	5.50	0.08	4.79	4.88	0.09	0.86	0.0	0.10	0.23
15	$4\text{-}OCF_3$	5.42	5.52	0.10	4.26	4.99	0.73	1.04	0.0	0.10	0.35
16	$4\text{-}NO_2$	5.43	5.63	0.20	4.37	4.69	0.32	−0.28	0.0	0.10	0.78
17	$4\text{-}O(CH_2)_6CH_3$	5.45	5.36	0.08				3.23	0.0	0.10	−0.32 [d]
18	$3\text{-}CH_2OCH_3$	5.49	5.22	0.26	4.37	4.16	0.21	−0.78	−0.78	1.21	0.02
19	4-F	5.51	5.45	0.05	4.79	4.55	0.24	0.14	0.0	0.10	0.06
20	$3,5\text{-}(OCH_3)_2$	5.51	5.63	0.12	4.12	4.61	0.49	0.08	0.08	0.79	0.24
21	$3,4,5\text{-}(OCH_3)_3$	5.51	5.53	0.02	3.98	4.26	0.28	−0.60	−0.04	0.79	0.07
22	$4\text{-}O(CH_2)_5CH_3$	5.55	5.36	0.18	4.71	4.47	0.24	−1.25	−0.28	0.74	0.71 [d]
23	$3\text{-}NO_2$, $4\text{-}NHCOCH_3$	5.55	5.57	0.02	4.34	4.23	0.11	2.67	0.0	0.10	−0.32
24	$3\text{-}O(CH_2)_6CH_3$ [c]	5.57	5.94	0.37	4.54	5.42	0.88	3.23	3.23	3.52	0.10 [d]
25	$3\text{-}OCH_2CH_2OCH_3$	5.58	5.52	0.06	4.83	4.35	0.48	−0.40	−0.40	1.93	0.10 [d]
26	$3\text{-}OSO_2CH_3$	5.58	5.33	0.25	4.33	4.27	0.06	−0.88	−0.88	1.70	0.39
27	4-Cl	5.60	5.50	0.10	4.83	4.83	0.00	0.71	0.0	0.10	0.23
28	$3\text{-}OCH_3$	5.61	5.55	0.05	4.45	4.52	0.07	−0.02	−0.02	0.79	0.12
29	$3,4\text{-}(OCH_3)_2$	5.66	5.52	0.13	4.46	4.45	0.01	0.08	0.04	0.79	−0.12
30	H	5.67	5.44	0.23	4.71	4.47	0.24	0.14	0.14	0.09	0.34
31	3-F	5.67	5.59	0.08	4.70	4.67	0.03	0.0	0.0	0.10	0.0
32	$3\text{-}CH_3$	5.71	5.74	0.03	4.72	4.65	0.07	0.56	0.56	0.57	−0.07
33	$4\text{-}O(CH_2)_3CH_3$	5.74	5.36	0.37	4.67	4.86	0.19	1.55	0.0	0.10	−0.32
34	$3\text{-}O(CH_2)_7CH_3$	5.78	5.61	0.17				3.79	3.79	3.97	0.10 [d]
35	$3\text{-}CF_3$	5.78	5.99	0.21	4.92	4.97	0.05	0.88	0.88	0.50	0.43
36	3-Cl	5.80	5.92	0.12	5.01	4.89	0.12	0.71	0.71	0.60	0.37
37	3-Br	5.81	6.02	0.21	5.03	4.95	0.08	0.86	0.86	0.89	0.39
38	$4\text{-}NHCOCH_3$	5.83	5.44	0.39	4.26	4.06	0.20	−0.97	0.0	0.10	0.0
39	$3\text{-}CH_2O(CH_2)_3CH_3$	5.86	6.15	0.29	5.17	4.79	0.38	0.84	0.84	2.60	0.02 [d]
40	3-I	6.15	6.17	0.02	4.79	5.02	0.23	1.12	1.12	1.39	0.35
41	$3\text{-}CF_3$, $4\text{-}OCH_3$	6.27	5.92	0.35	4.99	4.85	0.14	0.86	0.88	0.50	0.16
42	$3\text{-}O(CH_2)_5CH_3$	6.39	6.21	0.17	5.67	5.32	0.35	2.67	2.67	3.07	0.10 [d]
43	$3\text{-}O(CH_2)_3CH_3$	6.48	6.32	0.15	5.22	5.04	0.18	1.55	1.55	2.17	0.10
44	$3\text{-}OCH_2C_6H_5$	6.53	6.47	0.05	5.63	5.08	0.35	1.66	1.66	3.17	0.12 [d]

[a] Calculated using eq 9. [b] Calculated using eq 5. [c] Compounds 1 and 5 not used to derive eq 9 and compound 1 not used used to derive eq 5. [d] Estimated value.

for the $4\text{-}OCH_2COOH$ analogue, which had an I_{50} of 16–32 × 10^{-8} M, the other congeners were all highly active—falling in the narrow range of 0.4 to 6.2 × 10^{-8} M. Roth et al.[2] conclude that "The fact that such a diversity in side chains results in such similarity in effect leads one to conclude that the side chain beyond the $4\text{-}OCH_3$ functionality lies outside of the hydrophobic cleft of the DHFR and makes little useful contact with the enzyme."

Roth et al. call attention to the fact that the $3,4,5\text{-}(OCH_3)_3$ analogue is about 10 times as active as the $3,5\text{-}(OCH_3)_2$ congener. This illustrates the importance of having a small substituent in the 4-position (OH, OCH_3, or Cl) for increased activity against bacterial DHFR. This effect is missing in both avian and bovine DHFR, which have $\log (1/K_i)$ values of 5.51 [$3,5\text{-}(OCH_3)_2$] and 5.51 [$3,4,5\text{-}(OCH_3)_3$] for chicken DHFR. This factor contributes to the selectivity of 4-X congeners for bacterial enzyme compared to mammalian enzyme. There is clearly a hydrophobic pocket for 3-substituents with the chicken and bovine DHFR. In each series the most active congeners are those with large hydrophobic groups in the 3-position. We are much less sure of our ground about the 5-position. In the case of the bovine DHFR of the five $3,5\text{-}X_2$ congeners, only three have been used to derive eq 6–9. These three are well fit [**2**, **20**, and **21**, Table I: $3,5\text{-}(CH_2OH)_2$, $3,5\text{-}(OCH_3)_2$, $3,4,5\text{-}(OCH_3)_3$]. The $3,5\text{-}(OH)_2$ and $3,5\text{-}(OCH_3)_2\text{-}4\text{-}CH_2CH_2OCH_3$ (**1**, and **5**, Table I) congeners are poorly fit, especially the $3,5\text{-}(OH)_2$.

In the case of the chicken DHFR, it was not possible to test $3,5\text{-}(OH)_2$ because of its low activity and low solubility, and again tetroxoprim [$3,5\text{-}(OCH_3)_2\text{-}4\text{-}OCH_2CH_2OCH_3$] is poorly predicted, being less inhibitory than expected. This leaves three congeners with 3,5-groups, $3,5\text{-}(CH_2OH)_2$, $3,5\text{-}(OCH_3)_2$, and $3,4,5\text{-}(OCH_3)_3$, which are no more than a fair fit.

We can attack the comparison problem in a more direct way via eq 13. If all 41 data points common to both sets

$$\log [1/K_{i(\mathrm{bovine})}] = 0.73 (\pm 0.10) \log [1/K_{i(\mathrm{chick})}] + 2.18 (\pm 0.46) \quad (13)$$

$$n = 36;\ r = 0.930;\ s = 0.158$$

of data are correlated as in eq 13, a rather poor correlation is obtained ($r = 0.887$; $s = 0.213$). Dropping the five most

Table II. Data Used in the Derivation of Equation 13

no.	X	log ($1/K_{i\,app}$) chicken	bovine obsd	bovine calcd	$\lvert\Delta\rvert$
1	3,5-(CH_2OH)$_2$	3.23 ± 0.03	4.30 ± 0.04	4.55	0.25
2	3,4-(OH)$_2$	3.59 ± 0.02	4.59 ± 0.03	4.81	0.22
3	3,5-(OCH_3)$_2$, 4-$OCH_2CH_2OCH_3$	3.64 ± 0.02	4.88 ± 0.03	4.85	0.03
4	3-OH	3.87 ± 0.03	4.88 ± 0.04	5.01	0.13
5	4-NH_2	3.73 ± 0.04	4.96 ± 0.02	4.92	0.04
6	3,4-($OCH_2CH_2OCH_3$)$_2$	3.91 ± 0.02	5.17 ± 0.03	5.05	0.12
7	3-CH_2OH	4.31 ± 0.04	5.20 ± 0.04	5.34	0.14
8	4-N(CH_3)$_2$	4.01 ± 0.02	5.21 ± 0.03	5.12	0.09
9	4-CH_3	4.56 ± 0.02	5.27 ± 0.04	5.52	0.25
10	3-OCH_2CONH_2	4.27 ± 0.02	5.27 ± 0.02	5.31	0.04
11	4-$OCH_2CH_2OCH_3$	4.26 ± 0.04	5.31 ± 0.03	5.30	0.01
12	4-OCH_3	4.29 ± 0.03	5.35 ± 0.03	5.33	0.02
13	4-Br	4.79 ± 0.03	5.42 ± 0.02	5.69	0.27
14	4-OCF_3	4.26 ± 0.04	5.42 ± 0.03	5.30	0.12
15	4-NO_2	4.37 ± 0.02	5.43 ± 0.03	5.39	0.04
16	3-CH_2OCH_3	4.37 ± 0.03	5.49 ± 0.03	5.39	0.10
17	4-F	4.79 ± 0.02	5.51 ± 0.03	5.69	0.18
18	3,5-(OCH_3)$_2$	4.12 ± 0.02	5.51 ± 0.04	5.20	0.31
19	3,4,5-(OCH_3)$_3$ [a]	3.98 ± 0.02	5.51 ± 0.02	5.10	0.41
20	4-O-n-C_6H_{13}	4.71 ± 0.05	5.55 ± 0.03	5.63	0.08
21	3-NO_2, 4-$NHCOCH_3$	4.34 ± 0.02	5.55 ± 0.02	5.36	0.19
22	3-O-n-C_7H_{15}	4.54 ± 0.04	5.57 ± 0.03	5.51	0.06
23	3-OSO_2CH_3	4.33 ± 0.06	5.58 ± 0.03	5.36	0.22
24	3-$OCH_2CH_2OCH_3$	4.83 ± 0.02	5.58 ± 0.03	5.72	0.14
25	4-Cl	4.83 ± 0.03	5.60 ± 0.04	5.72	0.12
26	3-OCH_3	4.45 ± 0.03	5.61 ± 0.02	5.44	0.17
27	3,4-(OCH_3)$_2$	4.46 ± 0.02	5.66 ± 0.04	5.45	0.21
28	H	4.71 ± 0.03	5.67 ± 0.02	5.63	0.04
29	3-F	4.70 ± 0.02	5.67 ± 0.02	5.63	0.04
30	3-CH_3	4.72 ± 0.03	5.71 ± 0.02	5.64	0.07
31	4-O-n-C_4H_9	4.67 ± 0.05	5.74 ± 0.04	5.60	0.14
32	3-CF_3	4.92 ± 0.05	5.78 ± 0.03	5.79	0.01
33	3-Cl	5.01 ± 0.02	5.80 ± 0.02	6.07	0.27
34	3-Br	5.03 ± 0.02	5.81 ± 0.03	5.86	0.05
35	4-$NHCOCH_3$ [a]	4.26 ± 0.02	5.83 ± 0.02	5.30	0.53
36	3-CH_2O-n-C_4H_9	5.17 ± 0.02	5.86 ± 0.03	5.97	0.11
37	3-I [a]	4.79 ± 0.03	6.15 ± 0.02	5.69	0.46
38	3-CF_3, 4-OCH_3 [a]	4.99 ± 0.04	6.27 ± 0.02	5.84	0.43
39	3-O-n-C_6H_{13}	5.67 ± 0.04	6.39 ± 0.04	6.34	0.05
40	3-O-n-C_4H_9 [a]	5.22 ± 0.04	6.48 ± 0.03	6.01	0.47
41	3-$OCH_2C_6H_5$	5.63 ± 0.03	6.53 ± 0.03	6.31	0.22

[a] These points not used in the formulation of eq 13.

Table III. 2,4-Diamino-5-(substituted-benzyl)pyrimidines

no.	X	mp, °C	yield, [a] %	formula [b]
1	3,5-(OH)$_2$	282-284 dec	25.8	$C_{11}H_{12}N_4O_2$
2	4-O(CH_2)$_6CH_3$	163-165	2.3	$C_{18}H_{26}N_4O$
3	3-O(CH_2)$_6CH_3$	119-120	14.6	$C_{18}H_{26}N_4O$
4	4-O(CH_2)$_5CH_3$ [b]	163.5-165	12.5	$C_{17}H_{24}N_4O$
5	3-OCH_2CONH_2 [b]	205-207	25.1	$C_{13}H_{15}N_5O_2$
6	4-$OCH_2CH_2OCH_3$	184-184.5	9.5	$C_{14}H_{18}N_4O_2$
7	3-$OCH_2CH_2OCH_3$	166-167	14.6	$C_{14}H_{18}N_4O_2$
8	3,4-($OCH_2CH_2OCH_3$)$_2$	145-146.5	5.2	$C_{17}H_{24}N_4O_4$

[a] Yield of pure material calculated on the amount of benzaldehyde used except for 1 and 5. [b] Analyzed for C and H.

poorly fit analogues [19, 35, 37, 38, and 40, Table II: 3-I, 4-$NHCOCH_3$, 3-CF_3-4-OCH_3, 3-OC_4H_9, 3,4,5-(OCH_3)$_3$] yields eq 13. A point by point comparison of the correlation can be seen in Table II. All of the poorly fit compounds are more active against bovine reductase than one would expect from studies with the avian enzyme, or one could say that they are less active against the chicken DHFR than expected from bovine DHFR studies. There is little in common among these substituents so that we

Table IV. Benzal Nitriles

no.	X	yield, %	bp, °C (mmHg)
2	4-O(CH_2)$_6CH_3$	25.6	180-210 (0.7)
3	3-O(CH_2)$_6CH_3$	71.4	195-225 (2.4)
4	4-O(CH_2)$_5CH_3$	51.3	160-170 (0.1)
6	4-$OCH_2CH_2OCH_3$	59.8	203-230 (3.5)
7	3-$OCH_2CH_2OCH_3$	47.8	155-190 (0.35)
8	3,4-($OCH_2CH_2OCH_3$)$_2$	46.3	220-245 (0.2)

Table V. Benzaldehyde Intermediates for Pyrimidine Synthesis

no.	X	bp, °C (mmHg) found	bp, °C (mmHg) lit.	yield, %	method of synthesis[a]
2	4-$O(CH_2)_6CH_3$	130-132 (0.1)	162-164 (7.0)[b]	78.3	A
3	3-$O(CH_2)_6CH_3$	120 (0.1)		80.1	A
4	4-$O(CH_2)_5CH_3$	130-132 (0.8)	154-155 (6.0)[c]	74	A
6	4-$OCH_2CH_2OCH_3$	140-142 (3.0)		60.5	B
7	3-$OCH_2CH_2OCH_3$	141 (1.3)		75.5	B
8	3,4-$(OCH_2CH_2OCH_3)_2$	162 (0.7)		72.6	B

[a] Method of synthesis: A, hydroxybenzaldehyde was refluxed with appropriate alkyl bromide in ethanolic KOH solution; B, hydroxybenzaldehyde was reacted with $ClCH_2CH_2OCH_3$ in DMF under refluxing using K_2CO_3 as an acid-neutralizing agent. [b] Reference 19. [c] Reference 20.

shall have to await the results from crystallographic studies to explain the differences in the two enzymes responsible for our findings.

A more important difference between the two DHFR in their perturbation pattern with the 41 benzylpyrimidines is the much greater intrinsic sensitivity of the bovine enzyme. The mean and standard deviation for the log $(1/K_i)$ for chicken DHFR inhibition is 4.50 (±0.53), while it is 5.54 (±0.46) for bovine DHFR. Thus, on the average, the bovine reductase is 10 times as active as the avian enzyme.

In summary, the differences in the amino acid sequence and, hence, in the structure for the two DHFR discussed in the introduction do not produce very different QSAR. In fact, the similarity of the QSAR surprised us. That there are differences is brought out by compounds **19**, **35**, **37**, **38**, and **40** in Table II. These differences mostly reside among the congeners most active against the bovine enzyme; however, the two different structures of the enzymes do show very different intrinsic sensitivities to the benzylpyrimidines. We hope further analysis will explain the 10-fold greater sensitivity of the bovine reductase.

Experimental Section

The assay procedures for the inhibition of the DHFR are the same as previously reported.[16]

Synthesis of Benzylpyrimidines. The new benzylpyrimidines were prepared using the general procedure of Stenbuck et al.[17] Melting points (Buchi capillary apparatus) are uncorrected. Microanalyses were made by C. F. Geiger of Ontario, CA, and are within ±0.4% of the theoretical values. TLC (precoated alumina plate, UV visualization) was always used to check the purity of the pyrimidines.

2,4-Diamino-3′,5′-dihydroxybenzylpyrimidine. This compound was prepared by ether cleavage of the corresponding 3,5-dimethoxybenzyl analogue using HBr.

2,4-Diamino-5-(3,5-dimethoxybenzyl)pyrimidine (0.005 mol) was refluxed with 48% HBr (40 mL) under nitrogen for 4.5 h. The HBr was evaporated under reduced pressure, the residue was neutralized with ammonium hydroxide, and the product was recrystallized three times from methanol: yield 25.8%; mp 282–284 °C.

2,4-Diamino-5-[3-[(aminocarbonyl)methoxy]benzyl]pyrimidine. 2,4-Diamino-5-(3-hydroxybenzyl)pyrimidine (0.005 mol) and finely powdered K_2CO_3 (0.006 mol) were refluxed in 15 mL of DMF, and a solution of chloroacetamide (0.006 mol) in 10 mL of DMF was added dropwise during the course of 30 min. After a total of 2 h refluxing, the reaction was cooled, and the solid formed was separated by filtration. After two recrystallizations from water, the product melted at 205–207 °C: yield 25.1%.

Estimation of Substituent Constants. Substituent constants are not available for all of the substituents of Table I, so we had to estimate values for some of them. Alkoxy groups larger than butoxy were calculated by adding a π of 0.54 for each additional CH_2 to the π for $O(CH_2)_3CH_3$. π values for 3,5-$(OCH_3)_2$-4-$OCH_2CH_2OCH_3$ and 3,4,5-$(OCH_3)_3$ were measured experimentally using the benzylpyrimidine system (other π values are from the benzene system).

The Hammett σ value for OCH_3 was used for all of the higher alkoxy groups as well as for OCH_2CONH_2. The σ value for OCH_2CH_3 was used for $OCH_2CH_2OCH_3$. The Hammett σ value for 3,4,5-$(OCH_3)_3$ was taken from Table II in ref 21. The σ value for 3,5-$(OCH_3)_2$-4-$OCH_2CH_2OCH_3$ is the sum of σ for OCH_3 and σ for OC_2H_5.

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