Table II. Jackknifing Log $[1/K_{i(app)}]$: Competitive Enzyme Inhibition[a]

no.	$[I_t]$ ($\times 10^{-7}$ M)	V_i/V_0	log $[1/K_{i(app)}]$ $-i$th ($\hat{\theta}_{-i}$)	log $[1/K_{i(app)}]$ ith pseudo ($\tilde{\theta}_i$)
1	0.365	0.9868	6.1765	6.1556
2	0.953	0.8265	6.1692	6.2287
3	1.827	0.8070	6.1784	6.1358
4	1.907	0.7287	6.1655	6.2650
5	3.654	0.5852	6.1588	6.3327
6	3.813	0.6566	6.1797	6.1230
7	5.481	0.5155	6.1648	6.2727
8	5.720	0.5569	6.1797	6.1233
9	7.308	0.4597	6.1693	6.2276
10	11.440	0.4170	6.1871	6.0494
11	19.067	0.3392	6.1907	6.0128

[a] Log $[1/K_{i(app)}]$ for all 11 data points ($\hat{\theta}$) = 6.1746.

activity; and (3) have accurately determined dependent and independent variable values.

One additional and very valuable aspect of the jackknife technique is that it permits one to examine the influence of each of the individual members of a data set on the estimates of the parameters of the equation (QSAR) as the data points are dropped one at a time. While certain cases of instability of the estimates may be quite obvious (e.g., only a single data point with log $P >$ log P_0), such is not always the situation. The $-i$th values (i.e., $\hat{\theta}_{-i}$) and jackknife estimates (i.e., θ) can be calculated for jackknifing not only log P_0 (or π_0) but also the regression coefficients for a QSAR equation. Examination of these values makes it possible to determine which, if any, data points are critical in determining the form of the derived QSAR equation (i.e., spotting of potential outliers). Similarly, the $-i$th values obtained by jackknifing log $[1/K_{i(app)}]$ for competitive enzyme inhibition data can be used to determine which, if any, data points are critical for the estimation of log $[1/K_{i(app)}]$.

In conclusion, the jackknife technique does appear to be a useful statistical tool for constructing confidence intervals for parameters which are estimated by linear or nonlinear regression techniques. In particular, the method appears well suited to confidence interval estimation of the independent variable value associated with maximum activity in the bilinear QSAR model, for log $[1/K_{i(app)}]$ from competitive enzyme inhibition data, and (perhaps) also for the parabolic QSAR model maximum activity independent variable value. Application of this technique is by no means limited to these three cases; extension to confidence intervals for other parameters which are complicated functions of other variables is possible and of general utility.

It should also be noted that a number of other authors (e.g., ref 15–17) have also examined the use of the jackknife technique for parameter and confidence interval estimation in the examination of enzyme kinetic data.

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(15) Dammkoehler, R. A. *J. Biol. Chem.* **1966**, *241*, 1955.
(16) Cornish-Bowden, A.; Wong, J. T. *Biochem. J.* **1978**, *175*, 969.
(17) Duggleby, R. G. *Biochem. J.* **1979**, *181*, 255.
(18) Lien, E. J.; Hansch, C.; Anderson, S. M. *J. Med. Chem.* **1968**, *11*, 430.
(19) Hansch, C.; Glave, W. R. *Mol. Pharmacol.* **1971**, *7*, 337.

Quantitative Structure–Selectivity Relationships. Comparison of the Inhibition of *Escherichia coli* and Bovine Liver Dihydrofolate Reductase by 5-(Substituted-benzyl)-2,4-diaminopyrimidines

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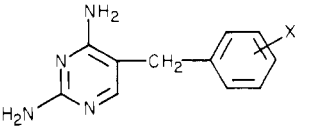
A quantitative structure–activity relationship (QSAR) has been formulated for the inhibition of purified *E. coli* dihydrofolate reductase by 23 5-(substituted benzyl)-2,4-diaminopyrimidines: log $1/C = 1.14MR'_{3,4,5} + 5.73$; $r = 0.887$; $s = 0.285$. In this expression, $MR'_{3,4,5}$ refers to the sum of MR values for X in the 3, 4, and 5 positions of the phenyl moiety. MR′ signifies that the effective value of MR is limited to 0.79. Comparison of the QSAR for *E. coli* enzyme inhibition with that previously obtained for bovine enzyme offers the first general explanation for the great selectivity of the important antibacterial agent trimethoprim. Such QSSR promise to be of value in devising more selective drugs.

This report continues our analysis of the interaction of dihydrofolate reductase (DHFR; EC 1.5.1.3) from various species with substituted pyrimidines and triazines.[1] In particular, we discuss the inhibition of DHFR from *E. coli* by benzylpyrimidines of type I.



I

Since DHFR shows such wide variation from organism to organism in its sensitivity to inhibitors, it offers an exceptional opportunity for selective inhibition of a pathogen with respect to the host. Inhibitors of DHFR have proved to be of great value as antimicrobial agents, as well as in cancer chemotherapy. It therefore is important to gain a clearer understanding of the molecular forces which determine the relative inhibitory activities of these in-

(1) (a) Blaney, J. M.; Dietrich, S. W.; Reynolds, M. A.; Hansch, C. *J. Med. Chem.* **1979**, *22*, 614. (b) Dietrich, S. W.; Smith, R. N.; Fukunaga, J. Y.; Olney, M.; Hansch, C. *Arch. Biochem. Biophys.* **1979**, *194*, 600. (c) Dietrich, S. W.; Smith, R. N.; Brendler, S.; Hansch, C. *Arch. Biochem. Biophys.* **1979**, *194*, 612. (d) Hansch, C.; Dietrich, S. W.; Smith, R. N. in "Chemistry and Biology of Pteridines"; Kisliuk, R. L.; Brown, G. M., Eds.; Elsevier: New York, 1979; Vol. 4, p 425. (e) Silipo, C.; Hansch, C. *J. Am. Chem. Soc.* **1975**, *97*, 6849.

0022-2623/80/1823-1205$01.00/0

Table I. Parameters for the Formulation of QSAR Equations for Pyrimidines of Type I Inhibiting Dihydrofolate Reductase

no.	X	bovine DHFR: log 1/C, obsd[a]	E. coli DHFR: log 1/C obsd[b]	E. coli DHFR: log 1/C calcd[c]	|Δ|	$MR_{3,4,5}$
1	H	5.19	6.18 (0.03)	6.13	0.05	0.31
2	4-NO_2	5.02	6.20 (0.04)	6.65	0.45	0.94
3	3-F	5.33	6.23 (0.02)	6.11	0.12	0.30
4	4-NH_2	4.57	6.30 (0.01)	6.49	0.19	0.75
5	4-F	5.18	6.35 (0.03)	6.12	0.23	0.30
6	4-Cl	5.10	6.45 (0.03)	6.53	0.08	0.81
7	3,4-$(OH)_2$	4.30	6.46 (0.06)	6.52	0.06	0.67
8	4-CH_3	4.80	6.48 (0.04)	6.50	0.02	0.77
9	4-OCF_3	4.99	6.57 (0.03)	6.69	0.12	0.99
10	3-Cl	5.47	6.65 (0.03)	6.82	0.17	0.81
11	3-CH_3	5.22	6.70 (0.02)	6.76	0.06	0.77
12	4-$N(CH_3)_2$	4.76	6.78 (0.03)	6.69	0.09	1.76
13	4-Br	5.17	6.82 (0.05)	6.69	0.13	1.09
14	4-OCH_3	4.92	6.82 (0.02)	6.69	0.13	0.99
15	4-$NHCOCH_3$	5.09	6.89 (0.02)	6.69	0.20	1.70
16	3-OCH_3	5.02	6.93 (0.03)	7.08	0.15	0.99
17	3-Br	5.54	6.96 (0.02)	7.08	0.12	1.09
18	3-NO_2, 4-$NHCOCH_3$	5.16	6.97 (0.02)	7.57	0.60	2.33
19	3-$OCH_2C_6H_5$	6.10	6.99 (0.03)	7.08	0.09	3.42
20	3-CF_3	5.53	7.02 (0.05)	6.68	0.34	0.71
21	3-CF_3, 4-OCH_3	5.79	7.69 (0.07)	7.24	0.45	1.39
22	3,4-$(OCH_3)_2$	5.15	7.72 (0.07)	7.64	0.08	1.68
23[d]	3,4,5-$(OCH_3)_3$	5.10	8.87 (0.05)	8.59	0.28	2.36

[a] Data from ref 1a. [b] This study; values in parentheses are for the construction of the 95% confidence intervals; see Experimental Section. [c] Calculated using eq 6. [d] Trimethoprim.

hibitors against enzyme from difference sources. Our interest is not in increased potency but rather in discovering the factors that are associated with selective interaction (QSSR). While the object of this research is to obtain better antitumor drugs, we are also interested in the general principles of drug design at the molecular level. The elucidation of the relative importance of specific inhibitor–enzyme interactions through the use of QSAR will be of value in all types of drug research. Specifically, the information and experience that we gain from examining the interactions of inhibitors with mammalian and bacterial DHFR's will help to direct and will make much more efficient the QSAR study of inhibition of DHFR from normal and cancerous human cells.

Our first study[1a] of inhibitors of type I against DHFR from bovine liver resulted in the formulation of eq 1 in

$$\log 1/C = 0.62(\pm 0.13)\pi_3 + 0.33(\pm 0.18)\Sigma\sigma + 4.99 \quad (1)$$

$$n = 23;\ r = 0.931;\ s = 0.146$$

which C is the molar concentration of inhibitor producing 50% inhibition [$C = I_{50} = K_{i(app)}$; see calculation of log $[1/K_{i(app)}]$ values under Experimental Section; see Table I for listing of the log $1/C$ values used in ref 1a for derivation of eq 1]; π_3 refers only to the hydrophobicity of 3-substituents, while $\Sigma\sigma$ refers to substituents in positions 3, 4, and 5. For this and subsequent QSAR regression equations, n = number of data points, r = correlation coefficient, s = standard deviation of the regression equation, and values in parentheses are for the construction of the 95% confidence intervals; see ref 2 for discussion, sources, values, and calculation of the substituent constants. The most important term is π_3 (accounting for 76.4% of the variance in log $1/C$), while $\Sigma\sigma$ accounts for only 10.3%. For the set of 23 congeners, $\Sigma\pi$ and ΣMR (molar refractivity) of the substituents are reasonably orthogonal; hence, it is the hydrophobic character of the substituents that is of paramount importance. Substituents in the 4 position show no detectable nonspecific interactions (π or MR related). However, large substituents in the 4 position were not studied; the largest considered was 4-$NHCOCH_3$.

Our motivation for studying congeners of I stemmed from an analysis[3] of the work by Hitchings et al. from which we derived eq 2 for the inhibition of *E. coli* DHFR.

$$\log 1/C = -1.12\Sigma\sigma_R^+ + 5.54 \quad (2)$$

$$n = 10;\ r = 0.986;\ s = 0.182$$

Although there were a variety of ways of correlating the activity of the 10 congeners on which eq 2 is based, we selected the single-variable equation because it gave the highest correlation and also seemed to make mechanistic sense. The great importance of through resonance (σ_R^+ is Taft's parameter[2]) brought out by eq 2 was of course most interesting, especially so since σ^+ was chosen with respect to the ortho position and not to the point of the CH_2 joining phenyl to pyrimidine. The great difference between eq 1 and 2 suggested that electronic effects of substituents might be responsible for the selective inhibitory power of congeners of type I against bacterial DHFR compared with human DHFR.

We have now tested the same congeners upon which eq 1 is based against DHFR from *E. coli*.

Results and Discussion

After a rather extensive analysis of the data in Table I which considered MR, π, σ^+, $\mathcal{F}$, and $\mathcal{R}$ in combination (e.g., $\Sigma\pi$) or singly (e.g., $\pi_3 + \pi_4 + \pi_5$), it became apparent that if one omits four of the compounds of Table I having substituents with MR > 1 (**12**, $MR_{N(CH_3)_2} = 1.56$; **15** and **18**, $MR_{NHCOCH_3} = 1.49$; **19**, $MR_{OCH_2C_6H_5} = 3.22$), the reasonable correlation of eq 3 could be derived. $MR_{3,4,5}$ is the

$$\log 1/C = 1.25(\pm 0.28)MR_{3,4,5} + 5.64(\pm 0.29) \quad (3)$$

$$n = 19;\ r = 0.918;\ s = 0.272$$

(2) Hansch, C.; Leo, A. "Substituent Constants for Correlation Analysis in Chemistry and Biology"; Wiley-Interscience: New York, 1979.

(3) Hansch, C.; Fukunaga, J. Y.; Jow, P. Y. C.; Hynes, J. B. *J. Med. Chem.* **1977**, *20*, 96.

sum of MR for all three positions (MR is scaled by 0.1). Using $\pi_{3,4,5}$ in place of $MR_{3,4,5}$ in eq 3 gives an equation with $r = 0.016$. Since $\pi_{3,4,5}$ and $MR_{3,4,5}$ are highly orthogonal ($r^2 = 0.002$), eq 3 indicates that classical hydrophobic interaction is not correlated with inhibiting power. A slightly better correlation can be obtained by factoring MR as shown in eq 4. Of the thousands of other equations

$$\log 1/C = 1.50(\pm0.30)MR_{3,5} + 0.88(\pm0.35)MR_4 + 5.68(\pm0.25) \quad (4)$$

$$n = 19;\ r = 0.945;\ s = 0.231$$

generated (via our usual approach),[4] none was significantly better than eq 4. In particular, no role could be found for electronic or hydrophobic terms.

If one arbitrarily sets the maximum value that MR may take for any substituent to be 0.79 ($MR_{OCH_3} = 0.79$) and calls the resulting independent variable MR′, eq 5 and 6

$$\log 1/C = 1.14(\pm0.27)MR'_{3,4,5} + 5.73(\pm0.29) \quad (5)$$

$$n = 23;\ r = 0.887;\ s = 0.285$$

$$\log 1/C = 1.38(\pm0.30)MR'_{3,5} + 0.82(\pm0.35)MR'_4 + 5.77(\pm0.25) \quad (6)$$

$$n = 23;\ r = 0.918;\ s = 0.250$$

are obtained for all 23 data points. Including all 23 data points in eq 3 or 4 gives quite poor correlations (respectively for eq 3 and 4: $r = 0.599$ and 0.598; $s = 0.495$ and 0.508). Further addition of various squared terms in MR to eq 3 or 4 for all 23 data points yielded consistently poorer correlations (than obtained by the modeling with MR′ in eq 5 and 6). Similar attempts at modeling with bilinear terms failed.

This data set contains only one 5-substituted analogue (**23**, Table I), which is also the most active compound. To ensure that this data point (being a potential outlier and also potentially acting by a somewhat different binding mechanism; see Experimental Section) was not exerting an undue influence on the formulation of the regression equations, eq 7–10 (analogous to eq 3–6) were derived,

$$\log 1/C = 1.06(\pm0.38)MR_{3,4} + 5.91(\pm0.32) \quad (7)$$

$$n = 18;\ r = 0.826;\ s = 0.263$$

$$\log 1/C = 1.35(\pm0.43)MR_3 + 0.81(\pm0.39)MR_4 + 5.91(\pm0.27) \quad (8)$$

$$n = 18;\ r = 0.879;\ s = 0.230$$

$$\log 1/C = 0.91(\pm0.33)MR'_{3,4} + 6.01(\pm0.29) \quad (9)$$

$$n = 22;\ r = 0.790;\ s = 0.262$$

$$\log 1/C = 1.17(\pm0.39)MR'_3 + 0.71(\pm0.36)MR'_4 + 6.01(\pm0.26) \quad (10)$$

$$n = 22;\ r = 0.836;\ s = 0.240$$

omitting it. Although their r values have decreased slightly, the s values are comparable and the overall forms of the regression equation remain essentially unchanged upon omitting data point **23**.

Consistently better correlations could be obtained for eq 3–10 by dropping the two analogues with an NO_2 substituent (**2** and **18**, Table I); e.g., eq 6 gives $n = 21$, $r = 0.962$, and $s = 0.178$. The reason for the deviations for these two analogues containing an NO_2 substituent is unclear at present.

At this point in the development of the QSAR of congeners of type I we do not have a large enough group or a wide enough selection of large groups in both the 3 and 4 positions to *make any solid statements about the role of large groups*. The nonlinear dependence on MR of the inhibitory activity of these analogues vs. the *E. coli* enzyme (as modeled by MR′ in eq 5 and 6) obviously requires further investigation in order to more precisely define the origin and form of this dependence. Our primary objective in selecting the congeners of Table I was to assess the electronic role of substituents; eq 2 had stimulated this effort. It is now clear that the electronic role of substituents in the inhibition by inhibitors of type I of both bovine and *E. coli* DHFR is marginal at best. A most important byproduct of this effort is the finding that 3-substituents of I encounter a hydrophobic pocket in bovine DHFR but not in *E. coli* DHFR. This offers a concrete lead for understanding the great selective toxicity of trimethoprim for bacterial enzyme rather than mammalian enzyme which makes trimethoprim such a safe drug. In the case of eq 1, π models a partitioning-like process whereby 3-substituents of the inhibitor bind to bovine DHFR.

The *E. coli* DHFR inhibition is brought about by a different mechanism in which the molar refractivity of the substituent is crucial. Molar refractivity is an ambivalent parameter in that it is a measure of the volume of a substituent as well as its polarizability:

$$MR = \left(\frac{n^2 - 1}{n^2 + 2}\right)\left(\frac{MW}{d}\right) \quad (11)$$

$$= \frac{4}{3}\pi N\alpha \quad (12)$$

In eq 11, n is the index of refraction, MW is the molecular weight, and d is the density. In eq 12, N is Avogadro's number and α is the polarizability. Since the range in n is small, eq 11 shows that MR is a kind of corrected molar volume. The degree of correction depends on how loosely the electrons are held as measured by the index of refraction. The cohesive potential energy (E) between two molecular moieties a and b is:

$$E = \left(\frac{-3\alpha_a\alpha_b}{2r^6}\right)\left(\frac{I_aI_b}{I_a + I_b}\right) \quad (13)$$

where r refers to the distance between a and b and I refers to their ionization potential. Hence, eq 3 and 4 show that molar volume and/or dispersion forces between substituent and enzyme produce the inhibitory effect. Desolvation (as measured by π) does not seem to be of importance; this suggests a quite different kind of enzymatic space through which X of I operates.

This last point is of extreme interest in view of the published X-ray crystallographic studies of a binary *E. coli* DHFR–methotrexate complex[5] and of a ternary *L. casei* DHFR–methotrexate–NADPH complex.[6] Preliminary computer graphic studies (by these authors at the Computer Graphics Laboratory, Department of Pharmaceutical

(4) Our general approach to QSAR is discussed in *J. Med. Chem.* **1976**, *19*, 605.

(5) Matthews, D. A.; Alden, R. A.; Bolin, J. T.; Freer, S. T.; Hamlin, R.; Xuong, N.; Kraut, J.; Poe, M.; Williams, M.; Hoogsteen, K. *Science*, **1977**, *197*, 452.

(6) Matthews, D. A.; Alden, R. A.; Bolin, J. T.; Filman, D. J.; Freer, S. T.; Hamlin, R.; Hol, W. G. J.; Kisliuk, R. L.; Pastore, E. J.; Plante, L. T.; Xuong, N.; Kraut, J. *J. Biol. Chem.* **1978**, *253*, 6946.

Chemistry, School of Pharmacy, University of California, San Francisco, Calif.) of these X-ray structures, especially with various DHFR inhibitors inserted in place of methotrexate, indicate that: (a) It is possible for benzylpyrimidines of type I to place the benzyl ring and its substituents (if small) into the same hydrophobic pocket of the enzyme where the phenyl ring of methotrexate binds. (b) This would be a reasonable type of interaction for the small hydrophobic substituents (e.g., 3-CF_3, π = 0.88) but not for the small hydrophilic substituents [e.g., 3,4,5-$(OCH_3)_3$, π = −0.60]. (c) The hydrophilic substituents (at least) may be interacting in some unusual, nonhydrophobic manner within the hydrophobic pocket or, perhaps more likely, may be interacting with a portion of the enzyme outside of the hydrophobic region of the FAH_2 binding pocket.

Compounds **12**, **15**, **18** and **19** of Table I have not been included in the formulation of eq 3 and 4. It is interesting to note that these compounds all have essentially the same activity; this suggests that only a certain fraction of the larger substituents in the 3 and 4 positions contact the enzyme. The rest of the substituent would appear to project into the surrounding aqueous phase and to cause no effect on the inhibitory power of the compound. This is further supported by the MR′ modeling we have used in the derivation of eq 5 and 6. Possible nonequivalence of the 3-, 4-, and 5-substituents with respect to the strength of their interactions with the *E. coli* enzyme is further suggested upon comparison of eq 3 with 4 and of eq 5 with 6.

The forms of eq 3 and 4 are not what we had expected from eq 2; however, the data which were used to obtain eq 2 are not inconsistent with eq 3. Fitting the data used to derive eq 2 to eq 3 yields eq 14. Considering the 95%

$$\log 1/C = 1.38(\pm 0.35)MR_{3,4,5} + 4.83(\pm 0.50) \quad (14)$$

$$n = 10;\ r = 0.954;\ s = 0.332$$

confidence limits on the slopes of eq 3 and 14, the agreement between the two equations is reasonable. The difference in the intercepts of the two equations is most likely due to differences in assay conditions and/or in specific enzyme sources and purities.

The total lack of agreement between eq 2 and 3 and eq 14 is a good illustration of the danger of formulating a QSAR (*or an SAR*) from a set of congeners not having sufficient variation in substituents. The substituents upon which eq 2 and 14 are based are:

	X_3	X_4	X_5		X_3	X_4	X_5
1	H	H	H	6	OCH_3	H	H
2	H	CH_3	H	7	OCH_3	OCH_3	H
3	H	Cl	H	8	OCH_3	OCH_3	Cl
4	H	OH	H	9	OCH_3	OH	OCH_3
5	H	OCH_3	H	10	OCH_3	OCH_3	OCH_3

The kind of answers one gets is determined by the questions one asks; that is, the kind of substituents selected limit the information in the response and, hence, limit one's perspective. While the set of substituents of Table I is much more varied than those on which eq 2 is based, they are not completely adequate. Now that we have two working hypotheses for bacterial and mammalian enzyme inhibition, we can design and make congeners which should refine and strengthen the structure–activity relationship.

In conclusion, we can say that the QSSR of eq 1 and 3–6 offers an interesting rationalization for the selectivity of compounds like trimethoprim (**23**, Table I) for bacterial enzyme rather than mammalian enzyme. Our results show trimethoprim to be ~6000 times more effective against DHFR from *E. coli* than against DHFR from bovine liver. Trimethoprim with a log $1/C$ of 8.87 is approaching the point of irreversible binding with *E. coli* DHFR (see Experimental Section); hence, there is little or nothing to be gained by seeking a more potent inhibitor of *E. coli* DHFR in the quest for better antibacterial drugs. However, one should be able to make more selective drugs by increasing the hydrophilic character of the 3,5-substituents in order to inhibit binding to mammalian enzyme while maintaining an optimum ΣMR of about 2 to 3 in the 3, 4, and 5 positions. Experiments are in progress to check this hypothesis and to further investigate the mode of interaction with the enzyme of more large substituents.

Experimental Section

Inhibitors. Sources for the pyrimidines of type I are given in ref 1a.

Inhibition Assays. The assay procedures are a modification of our previous procedure[1b] and allow for more rapid, accurate, and reproducible determination of the activities of the DHFR inhibitors. These advantages, as compared to our earlier assay method, are the result of the following factors: (1) preparation of the solution under a N_2 atmosphere in a glove box is unnecessary if the solutions are rapidly and freshly made up and are stored on ice until used and if the concentrations of dihydrofolate (FAH_2) and NADPH in stock solutions are spectrophotometrically determined; (2) DHFR is usually stabilized by the presence of NADPH when dilute solutions of the enzyme are made up; and (3) assay procedure 1 described below minimizes the amounts of DHFR (often available in limited quantities) and expensive NADPH needed, due to the manner in which solutions are introduced into and flushed through the stopped-flow spectrophotometer.

Assays were performed utilizing a Durrum D-110 stopped-flow spectrophotometer at 25 °C with a 2-cm cell path length and a 67-μL cell volume. FAH_2 (Sigma Chemical Co.) and NADPH (Sigma Chemical Co.) samples were quickly weighed on a Cahn 25 Automatic Electrobalance and then stored at −20 °C, protected from moisture and light. Inhibitor samples were also weighed on the electrobalance. The following buffers were used: buffer A, 0.30 M NaCl, 50 mM Tris-HCl, pH 7.20, 1 mM dithiothreitol; buffer B, 50 mM Tris-HCl, pH 7.20, 50 mM 2-mercaptoethanol.

DHFR from *Escherichia coli* B (strain MB 1428), purified by methotrexate-affinity chromatography,[7] was obtained as a freeze-dried powder, lyophilized from 3.56 mL of buffer A. The lyophilized preparation, containing 100 units (2.5 mg, 140 nmol; sp act. = 40 μmol min^{-1} mg^{-1}) of the enzyme as a 1:1 complex with FAH_2, was dissolved in 3.65 mL of H_2O; 36.5-μL aliquots were pipetted into small vials and stored at −20 °C. For use, a single vial was thawed, diluted 10–50 times with buffer A, and stored at 0–5 °C.

All other solutions were freshly prepared using buffer B; FAH_2, NADPH, and DHFR solutions were kept on ice. Once prepared for final assaying, the solutions were protected from air in syringes equipped with stopcocks. Inhibitor solutions, except for methotrexate (MTX), were prepared by dissolving the inhibitor samples in a minimal volume of $(CH_3)_2SO$ and then diluting with buffer B, such that $[(CH_3)_2SO]$ in the final assay solution was 0–0.015%, v/v. $(CH_3)_2SO$ was not found to have any effect on the reaction velocity of the *E. coli* DHFR in this concentration range. MTX solutions were prepared by dissolving the inhibitor sample in buffer B and then diluting with the same buffer.

FAH_2 stock solution: ~1 mM; $[FAH_2]$ calibrated at 282 nm (ϵ 28 000 at pH 7.2)[8] for a 31× dilution of the stock solution. NADPH stock solution: ~2 mM; [NADPH] calibrated at 340

(7) (a) Poe, M.; Greenfield, N. J.; Hirshfield, J. M.; Williams, M. N.; Hoogsteen, K. *Biochemistry*, **1972**, *11*, 1023. (b) Williams, M. N.; Poe, M.; Greenfield, N. J.; Hirschfield, J. M.; Hoogsteen, K. *J. Biol. Chem.* **1973**, *248*, 6375.

(8) Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. in "Data for Biochemical Research", Oxford University Press: New York, 1969.

nm (ϵ 6220 at pH 7.2)[9] for a 21× dilution of the stock solution.

Assay procedure 1: solution no. 1 = 56 μM FAH_2; solution no. 2 = 200 μM NADPH and enough of the enzyme solution from one of the diluted vials (see above) so that the final assay solution with no inhibitor present would give a 0.05–0.10 change in absorbance/min; solution no. 3 = 1 mL of solution no. 1 + x mL of inhibitor solution + 1 − x mL of buffer = 28 μM FAH_2. Equal volumes of solutions no. 2 and 3 were mixed in the spectrophotometer to give the final assay solution.

Assay procedure 2: solution no. 4 = 28 μM FAH_2; solution no. 5 = 400 μM NADPH and enzyme (as for assay procedure 1, but amount of enzyme was varied); solution no. 6 = 1 mL of solution no. 5 + x mL of inhibitor solution + 1 − x mL of buffer = 200 μM NADPH; allowed to incubate ~5 min at 25 °C before assaying. Equal volumes of solutions no. 4 and 6 were mixed in the spectrophotometer to give the final assay solution.

For both assay procedures: final assay solution = 14 μM FAH_2 and 100 μM NADPH. (Saturating [NADPH], i.e., [NADPH] $\gg$ $K_{m(app)}$ for NADPH,[10] was used for reasons discussed under Experimental Section. In order that the initial reaction velocity would be constant for a reasonable length of time for more accurate measurement and so that any slow equilibria could be established before significant FAH_2 depletion, $[FAH_2] \approx 12 \times K_{m(app)}$ for FAH_2[10] was used.) The decrease in absorbance was followed for 2 min or 0.1 absorbance unit at 340 nm (tungsten lamp, grating, 1.5-nm wavelength bandwidth, 230–390 nm UV band-pass filter). Initial reaction rates were corrected for the decrease in absorbance observed with no enzyme present. Runs were repeated four times for each of eight to ten different concentrations of inhibitor and were evenly spaced over a concentration range giving 0–80% inhibition. The relative activity (V_i/V_0; see below) for each of the four runs for a given inhibitor concentration was based on the average reaction rate for four runs which contained no inhibitor and were run immediately before or after the four runs with the inhibitor present.

Except for trimethoprim (23, Table I), the pyrimidine inhibitors of type I (Table I) gave linear initial reaction velocities with the *E. coli* DHFR using either assay procedure. The log $[1/K_{i(app)}]$ values (calculated from the experimental data as described below) are the same for these inhibitors when determined using either assay procedure: e.g., for I, X = H (1, Table I), log $[1/K_{i(app)}]$ = 6.18 ± 0.03 and 6.21 ± 0.07 for assay procedures 1 and 2, respectively. Trimethoprim, however, shows nonlinear initial reaction velocities with the *E. coli* DHFR with assay procedure 1 but linear initial velocities with assay procedure 2. The nonlinear initial velocities observed with procedure 1 rapidly decrease, becoming more linear and approaching the initial velocities observed with procedure 2. Exactly the same effect was observed whether the DHFR was (1) preincubated with NADPH alone and then immediately assayed upon mixing with FAH_2 and trimethoprim (assay procedure 1), (2) preincubated with FAH_2 and trimethoprim and then immediately assayed upon mixing with NADPH, or (3) not preincubated but immediately assayed upon mixing with FAH_2 and NADPH and trimethoprim. It appears that trimethoprim, like methotrexate (MTX),[7b] may be involved in an equilibrium with DHFR, FAH_2, and NADPH that is slow relative to the time scale of the assay; therefore, the log $[1/K_{i(app)}]$ values were determined by assay procedure 1 for all of the pyrimidine inhibitors of type I (Table I) except for trimethoprim (23), for which it was necessary to use assay procedure 2. As had also been previously noted,[7b] MTX inhibition was found to be stoichiometric only when the enzyme is preincubated with both MTX and NADPH (assay procedure 2); when it was necessary to determine $[E_t]$ (see below), assay procedure 2 was therefore used for MTX inhibition of the enzyme.

Calculation of Log $[1/K_{i(app)}]$ Values. The FAH_2 + NADPH → FAH_4 + $NADP^+$ reaction catalyzed by *E. coli* MB 1428 DHFR appears to be a rapid equilibrium random bireactant system.[7b] If an inhibitor competes with the substrate, FAH_2, but allows the cofactor, NADPH, to bind (i.e., the inhibitor competes with the substrate and the enzyme–inhibitor–cofactor complex is catalytically inactive), then it can be shown[11] that:

$$\frac{V_i}{V_0} = \left[\alpha K_{FAH_2}\left(1 + \frac{K_{NADPH}}{[NADPH]}\right) + [FAH_2] \times \left(1 + \frac{\alpha K_{NADPH}}{[NADPH]}\right)\right] \Big/ \left[\alpha K_{FAH_2}\left(1 + \frac{K_{NADPH}}{[NADPH]} + \frac{[I]K_{NADPH}}{K_i[NADPH]} + \frac{[I]}{\beta K_i}\right) + [FAH_2]\left(1 + \frac{\alpha K_{NADPH}}{[NADPH]}\right)\right] \quad (15)$$

where V_0 = reaction velocity in the absence of inhibitor; V_i = reaction velocity in the presence of inhibitor; [I] = inhibitor concentration; $[FAH_2]$ = FAH_2 concentration; [NADPH] = NADPH concentration; K_{FAH_2} = the FAH_2–enzyme complex dissociation constant; K_{NADPH} = the NADPH–enzyme complex dissociation constant; K_i = the enzyme–inhibitor complex dissociation constant; α = the factor by which binding of FAH_2 changes K_{NADPH} and by which binding of NADPH changes K_{FAH_2}; and β = the factor by which the binding of I changes K_{NADPH} and by which the binding of NADPH changes K_i. However, if [NADPH] is saturating (i.e., if [NADPH] $\gg$ K_{NADPH}), then eq 15 reduces to:

$$\frac{V_i}{V_0} = \frac{\alpha K_{FAH_2} + [FAH_2]}{\alpha K_{FAH_2}\left(1 + \frac{[I]}{\beta K_i}\right) + [FAH_2]} \quad (16a)$$

$$= \frac{\beta K_i\left[1 + \frac{[FAH_2]}{\alpha K_{FAH_2}}\right]}{\beta K_i\left(1 + \frac{[FAH_2]}{\alpha K_{FAH_2}}\right) + [I]} \quad (16b)$$

$$= \frac{K_{i(app)}}{K_{i(app)} + [I]} \quad (16c)$$

where

$$K_{i(app)} = \beta K_i[1 + ([FAH_2]/\alpha K_{FAH_2})] \quad (16d)$$

Equations 16a–d are, of course, analogous to the equations which can be derived[11] for simple competitive inhibition of a unireactant system:

$$\frac{V_i}{V_0} = \frac{K_m + [S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (17a)$$

$$= \frac{K_i\left(1 + \frac{[S]}{K_m}\right)}{[I] + K_i\left(1 + \frac{[S]}{K_m}\right)} \quad (17b)$$

$$= \frac{K'_{i(app)}}{K'_{i(app)} + [I]} \quad (17c)$$

where

$$K'_{i(app)} = K_i[1 + ([s]/K_m)] \quad (17d)$$

[s] = substrate concentration

K_m = Michaelis-Menten constant for substrate–enzyme complex

With a rapid equilibrium random bireactant system, αK_{FAH_2} and αK_{NADPH} will be equal to $K_{m(app)}$ (the apparent Michaelis–Menten constant) for FAH_2 in the presence of saturating [NADPH] and for NADPH in the presence of saturating $[FAH_2]$, respectively. Hence, if an inhibitor competes with the substrate, FAH_2, but allows the cofactor, NADPH, to bind and if [NADPH] is saturating, then the inhibition can be treated as though the substrate

(9) Kornberg, A.; Horecker, R. L. *Biochem. Prep.* **1953**, *3*, 27.
(10) Greenfield, N. J. *Biochemistry* **1974**, *13*, 4494.
(11) Segel, I. H. "Enzyme Kinetics"; Wiley: New York, 1975; pp 22–24, 100–111, 150–159, 273–291.

of a unireactant system were being competitively inhibited (i.e., the saturating [NADPH] effectively drives all of the free enzyme to the E·NADPH complex).

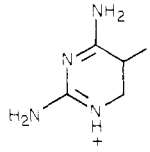
It is imperative to realize that the $K_{i(app)}$ values actually measured will be as described by eq 16d and not eq 17d. At saturating [NADPH] and fixed [FAH_2], the $1 + ([FAH_2]/\alpha K_{FAH_2})$ term will be constant for all inhibitors. For each inhibitor, however, relative inhibitory activity (proportional to the apparent, or overall, free energy of binding of I with the E·NADPH complex) will also be a function of both β and K_i:

$$\text{relative inhibitory act.} = \log [1/K_{i(app)}] = \log (1/\beta) + \log (1/K_i) + \text{constant} \quad (18)$$

From the QSAR perspective, it is obvious that (a) the physical and chemical properties of an inhibitor will not necessarily contribute equally to the log $(1/\beta)$ and log $(1/K_i)$ terms of eq 18, and (b) β may or may not be constant for different inhibitors.

It has been shown that various classes of inhibitors exhibit different modes of inhibition with respect to FAH_2 and NADPH for DHFR from different sources.[12] Ideally it would be desirable to experimentally demonstrate the mode of inhibition with respect to both FAH_2 and NADPH for each inhibitor against DHFR from every source examined. Realistically, however, this type of approach can lead to an excessively large drain on resources which could be better directed. This consideration is extremely important for the medicinal chemist, especially if he is involved in generating data for QSAR studies. Such a researcher is often testing large numbers of potential drug molecules in order to establish SAR's which can be used to better define the dependence of activity on structure and to direct the design of additional potential drug molecules. Such studies, therefore, necessitate that the researcher make certain *reasonable* assumptions in order to enable the rapid, economical, and yet accurate determination of the relative activities of a large number of molecules, often in more than one test system.

In the case of this and our previus studies[1] (and as also assumed by many other investigators, e.g., ref 13), we have assumed that (a) our inhibitors exhibit competitive inhibition with respect to FAH_2, but allow NADPH to bind (E·I and E·NADPH·I complexes), and (b) saturating [NADPH] drives all the free enzyme to the E·NADPH complex, for which I and FAH_2 compete. These assumptions are based on the following observations: (1) Our inhibitors all contain the same substructure



as MTX, a very tight binding (although competitive for FAH_2) inhibitor of DHFR which forms MTX·DHFR and MTX·NADPH·DHFR complexes by occupying the FAH_2 binding site.[5,6,13] (2) Such inhibitor classes have, in most instances, shown competitive inhibition with respect to FAH_2 (e.g., ref 12–15). Further complications can also arise, however, in that: (a) many inhibitors of various DHFR's exhibit varying degrees of competitive stoichiometric inhibition ($K_{i(app)} \ll [E_t]$ or $K_{i(app)} \approx [E_t]$) (that is, the inhibitor is so tight binding that the formation of E·I and E·NADPH·I complexes significantly changes the concentration of free inhibitor; e.g., see ref 13 and 16–18), and/or (b) The E·NADPH·I complex may be in slow (relative to the time scale of the assay) equilibrium between more than one conformer.[13,18] Both of these complications were observed for trimethoprim (23, Table I) but not for the other inhibitors. Our handling of the inhibition data in both instances is discussed further below.

In the following discussion for DHFR of the above two complications, a rapid equilibrium random bireactant system, competitive inhibition of FAH_2 but not NADPH, and a saturating [NADPH] are assumed. Hence, the system appears as a rapid equilibrium pseudounireactant system with competitive inhibition of FAH_2, and $K_{i(app)}$ corresponds to $K_{i(app)}$ of eq 16d. (A true unireactant system with competitive inhibition of substrate would yield analogous equations, but with $K_{i(app)}$ replaced by $K'_{i(app)}$ of eq 17d.)

If (a) no initial assumption is made with respect to the relative magnitudes of $K_{i(app)}$ and $[E_t]$ (i.e., as to whether the formation of E·I and E·NADPH·I complexes significantly changes the concentration of free I) and (b) the assumption of saturating [NADPH] is again made (i.e., $[NADPH] \gg K_{NADPH}$), then the procedure of Henderson[19] can be used to derive eq 19, where $[I_t]$

$$\frac{[I_t]}{1 - (V_i/V_0)} = [E_t] + K_{i(app)}(V_0/V_i) \quad (19)$$

= total inhibitor concentration; $[E_t]$ = total enzyme concentration; and $K_{i(app)}$ is as defined in eq 16d. Rearranging eq 19 provides eq 20 where $[I_{50}] = [I_t]$ which causes 50% inhibition of the enzyme

$$[I_{50}] = \frac{[E_t]}{2} + K_{i(app)} \quad (20)$$

(i.e., for $V_i = 0.5V_0$). For a competitive stoichiometric inhibitor (e.g., MTX with DHFR), $[I_t] \ll [E_t]$, and eq 19, after rearrangement, reduces to eq 21 and eq 20 reduces to eq 22. Hence,

$$\frac{V_i}{V_0} = 1 - \frac{[I_t]}{[E_t]} \quad (21)$$

$$[I_{50}] = [E_t]/2 \quad (22)$$

for MTX inhibiting DHFR, a plot of V_i/V_0 vs. $[I_t]$ (eq 21) provides a curve with slope $= -1/[E_t]$. For a single [MTX] and associated V_i/V_0 value, $[E_t]$ can be directly calculated from a rearrangement of eq 21 (eq 23). For a competitive nonstoichiometric inhibitor,

$$[E_t] = \frac{[I_t]}{1 - (V_i/V_0)} \quad (23)$$

$K_{i(app)} \gg [E_t]$ and eq 19 yields eq 24. Rearranging eq 24 provides

$$\frac{[I_t]}{1 - (V_i/V_0)} = K_{i(app)}\left(\frac{V_0}{V_i}\right) \quad (24)$$

eq 25.

$$\frac{V_0}{V_i} = 1 + \frac{[I_t]}{K_{i(app)}} \quad (25)$$

A plot of $[I_t]/[1 - (V_i/V_0)]$ vs. V_0/V_i (eq 24) should therefore provide a straight line with slope equal to $K_{i(app)}$, while a plot of V_0/V_i vs. $[I_t]$ (eq 25) should give a straight line with slope equal to $1/K_{i(app)}$. For reasons of error distribution discussed in the preceding paper,[20] however, it is preferable to rearrange eq 24 or 25 to give eq 16d (an expected result, for $K_{i(app)} \gg [E_t]$) and to then solve by iteration on $K_{i(app)}$, minimizing the sum of square deviations for V_i/V_0 as a function of $K_{i(app)}/(K_{i(app)} + [I_t])$. For each of the pyrimidine inhibitors of type I (Table I) (except trimethoprim, 23), this procedure was utilized, jackknifing log $[1/K_{i(app)}]$ to provide a final jackknife estimate of log $[1/K_{i(app)}]$ and its 95% confidence interval.[20]

The inhibition of the *E. coli* DHFR by trimethoprim (23, Table I) lies somewhere between these two extremes of competitive stoichiometric and nonstoichiometric inhibition, and normally one would then expect the full form of eq 19 to apply. As mentioned above, however, the E·NADPH·trimethoprim complex is

(12) Blakley, R. L.; Morrison, J. F. *Chem. Biol. Pteridines, Proc. Int. Symp., 4th, 1969* **1970**, 315–327.
(13) Williams, J. W.; Duggleby, R. G.; Cutler, R.; Morrison, J. F. *Biochem. Pharmacol.* **1980**, *29*, 589.
(14) Ho, Y. K.; Hakala, M. T.; Zakrzewski, S. F. *Cancer Res.* **1972**, *32*, 1023.
(15) Ho, Y. K.; Zakrzewski, S. F.; Mead, L. H. *Biochemistry* **1973**, *12*, 1003.
(16) Henderson, P. J. F. *Biochem. J.* **1973**, *135*, 101.
(17) Morrison, J. F. *Biochim. Biophys. Acta* **1969**, *185*, 269.
(18) Williams, J. W.; Morrison, J. F.; Duggleby, R. G. *Biochemistry* **1979**, *18*, 2567.
(19) Henderson, P. J. F. *Biochem. J.* **1972**, *127*, 321.
(20) Dietrich, S. W.; Dreyer, N. D.; Hansch, C.; Bentley, D. L. *J. Med. Chem.* **1980**, *23*, preceding paper in this issue.

apparently also in a relatively slow equilibrium between more than one conformer.

If (a) the initial E·NADPH·I complex is in equilibrium with another conformer of the complex, (E·NADPH·I)*,[13]

$$K_i^* = \frac{[E\cdot NADPH\cdot I]}{[E\cdot NADPH\cdot I]^*}$$

(b) the equilibrium between these two complexes can be established before significant depletion of FAH_2, and (c) the restraints used to derive eq 19 are assumed, then the procedure of Henderson[19] can be used to derive eq 26, analogous to eq 19

$$\frac{[I_t]}{1-(V_i/V_0)} = [E_t] + K''_{i(app)}\left(\frac{V_0}{V_i}\right) \quad (26)$$

where

$$K''_{i(app)} = \beta K_i \frac{K^*}{K^*+1}\left(1 + \frac{[FAH_2]}{\alpha K_{FAH_2}}\right) \quad (27)$$

A plot of $[I_t]/[1-(V_i/V_0)]$ vs. V_0/V_i (eq 26) should yield a straight line with slope = $K''_{i(app)}$ and intercept = $[E_t]$. Again for reasons of error distribution described in the preceding paper[20] and because of the importance of the $[E_t]$ term in eq 26, the trimethoprim data were treated as follows. The same stock DHFR, NADPH, and FAH_2 solutions were used to obtain values for V_0 and V_i for MTX and trimethoprim. $[E_t]$ was calculated from V_0 and V_i for MTX and eq 23. $K''_{i(app)}$ for trimethoprim was then calculated from eq 26, rearranged to eq 28. Twenty-one experiments of this

$$K''_{i(app)} = \left(\frac{[I_t]}{1-(V_i/V_0)} - [E_t]\right)\left(\frac{V_i}{V_0}\right) \quad (28)$$

nature yielded, for trimethoprim, 21 values for log $[1/K''_{i(app)}]$ with mean = 8.87 ± 0.05. ($[E_t]$ for these 21 experiments ranged from 2.00 to 2.60 × 10^{-9} M.)

Obviously, $K''_{i(app)}$ values (eq 27) for inhibitors for which there is a slow (E·NADPH·I) ⇌ (E·NADPH·I)* equilibrium are not directly comparable to $K_{i(app)}$ values (eq 16d) for inhibitors for which there is only one E·NADPH·I complex; compare eq 18 for log $[1/K_{i(app)}]$ with eq 29. If, however, there is in the latter case

$$\log (1/K''_{i(app)}) = \log (1/\beta) + \log (1/K_i) + \log\left(\frac{K^*+1}{K^*}\right) + \text{constant} \quad (29)$$

a rapid (and hence unapparent) (E·NADPH·I) ⇌ (E·NADPH·I)* equilibrium, then the $K_{i(app)}$ values will actually be $K''_{i(app)}$ values and should be directly comparable with the $K''_{i(app)}$ values for inhibitors with slowly equilibrating E·NADPH·I complexes. [For these benzylpyrimidines this may actually be the case, since for two of these inhibitors (21 and 22, Table I) the initial reaction velocities (assay procedure 1) did show slight initial curvature, but with the reaction velocity quickly decreasing to a constant rate (which was taken as V_i). Hence, there may be a continuous gradation for the rate (and hence the ease) with which E·NADPH·I conformers can interconvert for different inhibitors.] Further studies are planned for investigation of the relative contributions of the physical and chemical properties of DHFR inhibitors to the various terms of log $(1/K''_{i(app)})$ (eq 29) for inhibitors for which the E·NADPH·I complex is in equilibrium with more than one conformer.

The procedures in this paper for calculation of the various log $[1/K_{i(app)}]$ values are a distinct improvement over the previous computational procedure for log $(1/I_{50})$ values from percent activity vs. [I] plots.[1b] These current procedures allow treatment of the experimental data with explicit relationships relating V_i, V_0, $[I_t]$, $[E_t]$, and $K_{i(app)}$ values for stoichiometric and/or nonstoichiometric competitive inhibition and permit consideration of equilibria between E·NADPH·I complex conformers.

Partition Coefficients. A number of new partition coefficients (P) were determined in the octanol/water system.[21] The hydrophobic parameter π is defined as $\pi_X = \log P_X - 2.13$, where 2.13 is the log P value for benzene and P_X is the partition coefficient for the X-substituted benzene.

Table II. Octanol/Water Partition Coefficients for Pyrimidines of Type I

X	aqueous phase	log P	$\pi_{X,obsd}$[a]	π_{X,C_6H_6}[b]
H	0.1 N HCl	−1.03 ± 0.02	0.00	0.00
4-$NHCOCH_3$	0.1 N HCl	−1.94 ± 0.04	−0.91	−0.97
3,4,5-$(OCH_3)_3$	0.1 N HCl	−1.55 ± 0.03	−0.52	−0.60
4-NO_2	0.1 N HCl	−1.03 ± 0.02	0.00	−0.28
3-OCH_3	0.1 N HCl	−0.92 ± 0.03	0.11	− 0.02
3-F	0.1 N HCl	−0.80 ± 0.01	0.23	0.14
3-CH_3	0.1 N HCl	−0.51 ± 0.02	0.52	0.56
3-Cl	0.1 N HCl	−0.36 ± 0.01	0.67	0.71
3-$OCH_2C_6H_5$	0.1 N HCl	0.53 ± 0.01	1.56	1.66
H	0.1 N NaOH	1.58 ± 0.02[c]	0.00	0.00
3,4,5-$(OCH_3)_3$	0.1 N NaOH	0.82 ± 0.01[c]	−0.76	−0.60
4-$N(CH_3)_2$	0.1 N NaOH	1.82 ± 0.03	0.24	0.18

[a] $\pi_{X,obsd} = \log P - \log P_{I,X=H}$ for the same aqueous phase. [b] From the X-substituted benzene system, ref 22 and 24. [c] Reference 25 gives octanol/water log P values for X = H and X = 3,4,5-$(OCH_3)_3$ of 1.60 and 0.89, respectively; specific aqueous phase not indicated, but pH probably >>7.2.

Log P = 1.00 ± 0.04 for o-nitroacetanilide; hence, $\Sigma\pi_{obsd} = \pi_{3\text{-}NH_2,4\text{-}NHCOCH_3} = 1.00 - 2.13 = -1.13$. This is in fairly good agreement with the value calculated by simple additivity: $\Sigma\pi_{calcd} = \pi_{NO_2} + \pi_{NHCOCH_3} = -0.28 + -0.97 = -1.25$. The slightly more positive value for $\Sigma\pi_{obsd}$ is not inconsistent with the electronic nature and/or potential shielding effects of the NO_2 and $NHCOCH_3$ substituents.[21,22] Simply dividing up $\pi_{3\text{-}NO_2,4\text{-}NHCOCH_3}$ according to the magnitudes of π_{NO_2} and π_{NHCOCH_3}, one obtains for 3-NO_2, 4-$NHCOCH_3$: $\pi_{3\text{-}NO_2} = -0.25$ and $\pi_{4\text{-}NHCOCH_3} = -0.88$. Since these values are, for all intents and purposes, equal to those from the monosubstituted benzenes, either set of values could be used for $\pi_{3\text{-}NO_2}$ and $\pi_{4\text{-}NHCOCH_3}$ for 3-NO_2, 4-$NHCOCH_3$.

Partition coefficients were also determined for a number of pyrimidines of type I in order to assess the validity of the assumption that additivity holds for

$$\log P_{I,X=X} = \log P_{I,X=H} + \pi_X \quad (30)$$

where $P_{I,X=X}$ and $P_{I,X=H}$ are the partition coefficients for I (X = X or H, respectively) and π_X is from the benzene system, as defined above. The pK_a of 2,4-diamino-5-benzylpyridine (I, X = H) is 7.27 and substitution on the phenyl ring has little affect on this value.[23] Hence, for the log P determinations, 0.1 N HCl or 0.1 N NaOH was used as the aqueous phase so that log P could be determined for either the fully protonated or the fully unprotonated species of I. The results of these determinations are presented in Table II. Except for some minor deviations, the $\pi_{X,obsd}$ values are in good agreement with the π_{X,C_6H_6} values. The assumption of eq 30 does appear to hold and, hence, the use of π values from the benzene system to parameterize the hydrophobicity of X in I is justified. Of special note are (1) protonation

(21) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525.
(22) Hansch, C.; Leo, A.; Unger, S. H.; Kim, K. H.; Nikaitani, D.; Lein, E. J. *J. Med. Chem.* **1973**, *16*, 1207.
(23) Roth, B.; Strelitz, J. Z. *J. Org. Chem.* **1969**, *34*, 821.
(24) Hansch, C.; Rockwell, S. D.; Jow, P. Y. C.; Leo, A.; Steller, E. E. *J. Med. Chem.* **1977**, *20*, 304.
(25) Roth, B.; Streltiz, J. Z.; Rauckman, B. S. *J. Med. Chem.* **1980**, *23*, 379.

of the pyrimidine nucleus of I decreases the log P of I by 2.37 [for X = 3,4,5-$(OCH_3)_3$] or 2.61 (for X = H), and (2) $\pi_{X,obsd}$ from the benzene system for 3,4,5-$(OCH_3)_3$ has an unusually low value of −0.60, as compared with $\Sigma\pi_{X,calcd} = 3\pi_{OCH_3} = -0.06$; this same effect is seen for I where X = 3,4,5-$(OCH_3)_3$.

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Structure-Activity Relationships in a Series of Newly Synthesized 1-Amino-Substituted Ellipticine Derivatives

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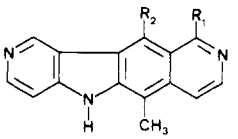
The synthesis of a series of 1-amino-substituted pyrido[4,3-*b*]carbazole derivatives, based on the substitution of corresponding 1-chloroellipticines, is reported. The cytotoxic properties on tumor cells grown in vitro, the in vivo acute toxicity of the most potent in vitro cytotoxic compounds, and the antitumor properties toward the L1210 leukemia system are described. No correlation between the apparent association constant to DNA and the in vitro cytotoxicity or the in vivo antitumor efficiency could be observed in this series. 9-Hydroxylated derivatives were more cytotoxic in vitro than the corresponding 9-methoxylated compounds. However, their antitumor efficiencies on the in vivo experimental systems do not confirm the advantage of demethylation. The presence of a [(dialkylamino)alkyl]amino side chain at the 1 position of ellipticines increases the antitumor potency: 1-[[3-(diethylamino)propyl]amino]-5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole (**5**) is a very potent antitumor compound (% ILS of 134 on the L1210 leukemia system).

Recently, we have described the synthesis and the antitumor properties of pyrido[3′,4′:4,5]pyrrolo[2,3-*g*]isoquinoline derivatives, which were formerly misnamed dipyrido[4,3-*b*][3,4-*f*]indoles. Amongst these compounds, those having a [(dialkylamino)alkyl]amino side chain at the 10 position of this new heterocyclic ring system (such as **1**) display a higher antitumor activity on the L1210



1, R_1 = $NH(CH_2)_3N(Et)_2$; R_2 = H
2, R_1 = H; R_2 = CH_3

leukemia system than the basic ellipticine analogue **2**.[1-5] Drugs in the ellipticine series are endowed with anticancer properties toward several experimental tumors. We then decided to examine whether various diamino side chains would also increase the biological activity in the ellipticine series, and an appropriate synthesis of required 1-chloropyrido[4,3-*b*]carbazoles, **3**, has been carried out in our laboratory.[6]

This report presents data concerning the preparation of new 1-[[(dialkylamino)alkyl]amino]ellipticine derivatives and the structure-activity relationships of these compounds studied in vitro on cultured tumor cells and in vivo on the L1210 leukemia system.

According to the intercalation model first described by Lerman,[7] the parent compounds **1** and **2** have been shown to bind to DNA in vitro with a high affinity.[4] Since biological activities of intercalating drugs were admitted to be related to their DNA affinity,[8,9] it was of interest to study if such a relation could also be shown with this new series of pyrido[4,3-*b*]carbazole derivatives. Thus, the apparent association constants for DNA of some compounds were also determined.

Chemistry. Compounds **4–9** were already described.[6] 1-Amino-substituted ellipticines **10–18** and **20–26** were obtained starting from chloroellipticines **3a–d**, which were substituted by their corresponding amines in an inert atmosphere. Substitutions were performed in boiling pure free amines, until complete disappearance of the starting material by monitoring with TLC on silica gel or alumina. 1-[[3-(Dimethylamino)propyl]amino]-5-methyl-9-hydroxypyrido[4,3-*b*]carbazole (**19**) was prepared by catalytic hydrogenation of benzylated derivative **18** as for

(1) C. Rivalle, C. Ducrocq, and E. Bisagni, *J. Chem. Soc., Perkin Trans. 1*, 138 (1979).
(2) C. Ducrocq, E. Bisagni, C. Rivalle, and J. M. Lhoste, *J. Chem. Soc., Perkin Trans. 1*, 142 (1979).
(3) J. C. Chermann, J. Gruest, L. Montagnier, F. Wendling, P. Tambourin, J. Perrin, F. Pochon, C. Ducrocq, C. Rivalle, and E. Bisagni, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **285**, 945 (1977).
(4) M. Tourbez-Perrin, F. Pochon, C. Ducrocq, C. Rivalle, and E. Bisagni, *Bull. Cancer*, **67**, 9 (1980).
(5) R. Lidereau, J. C. Chermann, J. Gruest, L. Montagnier, C. Ducrocq, C. Rivalle, and E. Bisagni, *Bull. Cancer*, **67**, 1 (1980).
(6) E. Bisagni, C. Ducrocq, J. M. Lhoste, C. Rivalle, and A. Civier, *J. Chem. Soc., Perkin Trans. 1*, 1706 (1979).
(7) L. S. Lerman, *J. Mol. Biol.*, **3**, 18 (1961).
(8) B. K. Bhuyan, I. J. Fraser, and L. H. Li, *Cancer Res.*, **32**, 2538 (1972).
(9) J. B. Le Pecq, N. D. Xuong, C. Gosse, and C. Paoletti, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 5078 (1974).

0022-2623/80/1823-1212$01.00/0