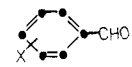
Table IV. Benzaldehydes Prepared for the Synthesis of Pyrimidines I



no.	X	no. from Table I	bp (mmHg) or mp, °C obsd	bp (mmHg) or mp, °C lit.	yield, %	method of synthesis[a]
1	3,5-$(CH_2OH)_2$	1	112–113		14.3	A
2	3-$O(CH_2)_7CH_3$	9	130–132 (0.45)	166–168 (0.05)[b]	100.0	B
3	3-CH_2OH	4	95–96 (2)		57.6	C
4	3-$CH_2O(CH_2)_3CH_3$	10	110 (0.7)		74.5	D
5	3-CH_2OCH_3	5	75–78 (1.1)	76.5 (0.4)[c]	86.7	D
6	3-$O(CH_2)_3CH_3$	13	110–113 (2.5)	142–143 (14)[d]	53.4	B
7	3-$O(CH_2)_5CH_3$	12	128–129 (0.7)	110 (0.01)[b]	33.2	B
8	4-$O(CH_2)_3CH_3$	8	125–128 (1.6)	148–149 (10)[e]	78.4	B
9	3-I	11	59.5–61	58[f]	12.8	E

[a] A: The diethyl ester of 5-nitrophthalic acid was reduced by $LiAlH_4$ in THF to give 5-amino-1,3-bis(hydroxymethyl)benzene (mp 99–100 °C). The amine was diazotized and treated with $Cu_2(CN)_2$ to produce the 5-cyano alcohol (mp 141.5–143.5 °C), which was treated with Raney Ni in 75% formic acid to produce 3,5-bis(hydroxymethyl)benzaldehyde.[14] B: Hydroxybenzaldehyde was refluxed with appropriate alkyl bromide in ethanolic KOH solution. C: α-Bromo-*m*-tolunitrile was hydrolyzed by $AgNO_3$ in 50% acetone (H_2O) to give *m*-(hydroxymethyl)benzonitrile, which was then reduced by Raney Ni in 75% formic acid to give *m*-(hydroxymethyl)benzaldehyde. D: α-Bromo-*m*-tolunitrile reacted with appropriate sodium alkoxide to give the corresponding *m*-(alkoxymethyl)benzonitrile. The benzonitrile was reduced by Raney Ni (Al-Ni) in 75% formic acid to give the corresponding benzaldehyde.[14] E: Direct iodination of benzaldehyde.[9] [b] Reference 12. [c] Reference 10. [d] Reference 11. [e] Reference 13. [f] Reference 9.

(9.6 mmol) of CH_3SO_2Cl in 5 mL of anhydrous benzene with cooling in an ice bath. The crude product (compound 10 in Table III) was removed by filtration and washed with 2 N KOH and then with water, mp 169–178 °C. This crude product was then recrystallized twice from methanol.

The benzaldehydes prepared for the synthesis of the pyrimidines are listed in Table IV.

Enzymatic Assay. The procedure for determining $K_{i\,app}$ and its confidence interval is that given in our recent publication.[3]

Acknowledgment. This research was supported by Grant CA 11110 from the National Cancer Institute. We thank P. Y. C. Jow for the determination of the log *P* values for trimethoprim and tetroxoprim. We are indebted to Dr. Martin Poe of the Merck Institute for a generous sample of *E. coli* DHFR. This material is based in part upon work supported by the National Science Foundation under Grant SPI-7914805; S.W.D., NSF National Needs Postdoctoral Fellow. We also thank Dr. H. Kubinyi for calling our attention to the importance of tetroxoprim and supplying us with a sample.

Inhibition of Bovine and Rat Liver Dihydrofolate Reductase by 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(4-substituted-phenyl)-*s*-triazines

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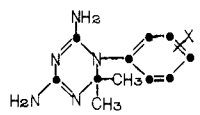
Quantitative structure–activity relationships (QSAR) have been formulated for the inhibition of purified bovine liver and rat liver dihydrofolate reductase (DHFR) by a series of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(4-X-phenyl)-*s*-triazines. The derived QSAR equations indicate that the interactions of the smaller 4-X substituents with both enzymes are hydrophobic, although size-limited, in nature. Further studies are suggested for elucidation of the specific interactions (hydrophobic or otherwise) of larger 4-X substituents with DHFR from mammalian sources.

Continuing our studies[1-5] of the inhibition of DHFR from various sources by triazines of type I, we report now



I

on the inhibition of this enzyme from two mammalian sources (bovine and rat liver) by a series of 4-X substituted I. The impetus for this work is our desire to gain a general understanding of the parameters of importance (and hence the physical and chemical properties they model) for the interaction of ligands with enzymes and especially to develop the techniques for designing inhibitors which would be selective for enzyme from one source. We formulated eq 1 and 2 in an initial investigation[5] of inhibitors of type

Inhibition of Bovine Liver DHFR

$$\log (1/C) = 1.05\ (\pm 0.14)\ \pi_3 - 1.21\ (\pm 0.20) \log (\beta \cdot 10^{\pi_3} + 1) + 6.64\ (\pm 0.11) \quad (1)$$

$n = 28$; $r = 0.955$; $s = 0.210$; $\pi_0 = 1.56$; $\log \beta = -0.736$

Inhibition of Rat Liver DHFR

$$\log (1/C) = 1.12\ (\pm 0.15)\ \pi_3 - 1.34\ (\pm 0.26) \log (\beta \cdot 10^{\pi_3} + 1) + 6.28\ (\pm 0.12) \quad (2)$$

$n = 18$; $r = 0.977$; $s = 0.171$; $\pi_0 = 1.68$; $\log \beta = -0.978$

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Table I. Inhibition Constants and Physicochemical Parameters Used for Deriving Equations 4-11 for Inhibition of Bovine Liver and Rat Liver DHFR by Triazines of Type I, 4-X

		log (1/C)[a]								
		bovine liver DHFR			rat liver DHFR					
no.	4-X	obsd[b]	calcd[c]	|Δ|[d]	obsd[b]	calcd[e]	|Δ|[d]	π_4	MR_4	$\mathcal{R}^-_4$
1[f,g]	$COOC_2H_5$	4.76 ± 0.08	6.97	2.21	4.44 ± 0.03	6.29	1.85	0.51	1.75	0.31
2	$CONH_2$	4.81 ± 0.03	5.33	0.52		4.81		−1.49	0.98	0.39
3	SO_2NH_2	5.05 ± 0.05	5.01	0.04	4.54 ± 0.03	4.49	0.05	−1.82	1.23	0.53
4[f,g]	$COOCH_3$	5.13 ± 0.08	6.66	1.53	4.26 ± 0.06	6.02	1.76	−0.01	1.29	0.31
5	SO_2CH_3	5.71 ± 0.10	5.20	0.51		4.67		−1.63	1.35	0.44
6	NH_2	5.73 ± 0.03	5.58	0.15		5.05		−1.23	0.54	−0.17
7	OH	5.83 ± 0.04	6.11	0.28		5.55		−0.67	0.28	−0.45
8	$COCH_3$	5.93 ± 0.06	6.22	0.29	5.25 ± 0.04	5.64	0.39	−0.55	1.12	0.55
9	H	6.33 ± 0.06[h]	6.67	0.34	5.99 ± 0.06[h]	6.03	0.04	0.00	0.10	0.00
10[f]	$OCH_2CON(CH_2CH_2)_2O$	6.82 ± 0.04	5.43	1.39		4.90		−1.39	3.42	−0.42
11	OCH_3	6.89 ± 0.05	6.65	0.24	6.26 ± 0.04	6.01	0.25	−0.02	0.79	−0.42
12	$C(CH_3)_3$	6.91 ± 0.07	7.24	0.33	6.40 ± 0.05	6.86	0.46	1.98	1.96	−0.06
13	I	6.97 ± 0.06	7.16	0.19	6.28 ± 0.05	6.54	0.26	1.12	1.39	−0.10
14	Br	7.02 ± 0.05	7.10	0.08	6.24 ± 0.04	6.44	0.20	0.86	0.89	−0.16
15	CF_3	7.06 ± 0.07	7.11	0.05	6.06 ± 0.06	6.45	0.39	0.88	0.50	0.27
16	CH_3	7.07 ± 0.08	7.00	0.07	6.41 ± 0.04	6.31	0.10	0.56	0.56	−0.11
17	$O(CH_2)_2OC_6H_4$-4'-NH_2	7.23 ± 0.06	6.94	0.29		6.26		0.45	4.34	−0.42
18	F	7.24 ± 0.05	6.76	0.48	6.67 ± 0.02	6.11	0.56	0.14	0.09	−0.38
19	$OCH_2C_6H_5$	7.33 ± 0.09	7.23	0.10	7.27 ± 0.07	6.74	0.53	1.66	3.22	−0.42
20	$(CH_2)_3CH_3$	7.37 ± 0.04	7.25	0.12	7.14 ± 0.04	6.91	0.23	2.13	1.96	−0.09
21	$OCH_2C_6H_3$-3',4'-Cl_2	7.37 ± 0.09	7.27	0.10	7.22 ± 0.09	7.18	0.04	2.91	4.22	−0.42

[a] Log (1/C) = log ($1/K_{i\,app}$). [b] This study, unless otherwise noted; uncertainty units are for construction of 95% confidence intervals; see Experimental Section. [c] Calculated using eq 5. [d] $|\Delta| = |\log (1/C)_{obsd} - \log (1/C)_{calcd}|$. [e] Calculated using eq 9. [f] Omitted in the calculation of eq 4-7. [g] Omitted in the calculation of eq 8-10. [h] Data from ref 5.

I with X in position 3. C in these equations is the molar concentration of triazine equivalent to $K_{i\,app}$ (usually equal to I_{50}, the concentration of inhibitor which inhibits the enzyme by 50%), π_3 is the hydrophobic constant[6] for substituents in position 3, β is a disposable parameter obtained by an iterative procedure, the values in parentheses are for the construction of 95% confidence intervals, and π_0 is the optimum value of π_3 for this bilinear[7] model relating activity to a nonlinear dependence on the hydrophobicity of 3-X. Equations 1 and 2 describe two straight lines joined by a short section of parabola. For eq 1, the left ascending side of the bilinear curve has a slope of +1.05, while the right descending side has a slope of 1.05 − 1.21 = −0.16. Equation 2 has essentially the same form as eq 1. Thus, for 3-X analogues of I, inhibitory potency increases linearly with π_3 up to $\pi_0 \approx 1.6$, and activity then remains almost constant (slope −0.16 or −0.21) as π_3 takes values $>\pi_0$. This was interpreted to mean that substituents with large π_3 values (>1.6) projected beyond the enzyme into the circumambient aqueous phase and, hence, that portion of inhibitor extending out of the hydrophobic pocket of the enzyme has essentially no effect on the binding interaction. For three of these 3-X congeners [i.e., X = 3-$CH_2NHC_6H_3$-3',5'-$(CONH_2)_2$, 3-$CH_2NHC_6H_4$-4'-SO_2NH_2, and 3-$CH_2OC_6H_4$-3'-$NHCONH_2$], π_3 was assigned the value of π for $CH_2NHC_6H_5$ or $CH_2OC_6H_5$; that is, the assumption was made that the polar part of the substituents [$\pi_{3,5\text{-}(CONH_2)_2} = -2.35$; $\pi_{SO_2NH_2} = -1.82$; $\pi_{NHCONH_2} = -1.30$] projects out of the hydrophobic pocket into the aqueous phase and, hence, has no effect on the inhibitory power of 3-X. Such correction is not needed for substituents like X = $-O(CH_2)_{11}CH_3$ which have large positive π_3 values, since these are taken care of by the negative bilinear term in eq 1 and 2.

The correlations with eq 1 and 2 show that rat liver and bovine liver DHFR interact in much the same way with triazine inhibitors. The rather good correlations suggest that 3-X is binding in a typically hydrophobic pocket, and the positive slope of about 1 for the linear π_3 term suggests that binding in this pocket of a wide variety of substituents directly parallels the way these substituents partition between octanol and water. It is almost impossible to rationalize such results without assuming a good deal of flexibility in the enzyme. That such fluidity does exist has long[8,9] been apparent in Koshland's idea of induced fit. More recently, others have developed evidence that enzymes are more flexible than many had heretofore been willing to concede; in fact, most of the QSAR we have formulated make no sense unless enzymes are considered to have rather large flexible pockets, some composed of mostly apolar residues and some composed of mostly polar residues.[10-12]

The results of eq 1 and 2 confirmed our earlier conclusion[1] (eq 3) that 3-X groups bind in hydrophobic space of

Inhibition of Walker 256 Rat Tumor and Mouse L-1210 Leukemia DHFR

$$\log (1/C) = 0.68\pi_3 - 0.12(\pi_3)^2 + 0.23MR_4 - 0.024(MR_4)^2 + 0.24I_1 - 2.53I_2 - 1.99I_3 + 0.88I_4 + 0.69I_5 + 0.70I_6 + 6.49 \quad (3)$$

$n = 244$; $r = 0.923$; $s = 0.377$; ideal π_3 = 2.9 (2.6–3.3); ideal MR_4 = 4.7 (4.2–5.6)

mammalian DHFRs. Equation 3 contains six indicator

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variables for special, rather complex structural features with which we are not much concerned at present. Rather, what is of interest for this study are the terms in π_3 and MR_4. Attempts to fit the data on which eq 3 is based to the bilinear model, or even a double bilinear model for both π_3 and MR_4, did not give as good a correlation as eq 3. The data for eq 3 were obtained by B. R. Baker and his students using very crude enzyme; in addition, they did not make allowance for very tight binding (in effect, enzyme titration by inhibitor) or another type of inhibition in which there is rapid initial binding of inhibitor and NADPH to the DHFR, followed by a slow conformational change in the DHFR–NADPH–inhibitor complex.[13] Moreover, the structural changes in X employed by Baker were enormous and difficult to parameterize. The indicator variable I_1 (eq 3) takes the value of 1 for Walker tumor enzyme and 0 for the leukemia enzyme. Despite these not insignificant problems and shortcomings in the data, a *large* amount of structure–activity information is compactly stored and reasonably rationalized by eq 3; in particular, eq 3 correctly characterizes π_3 space (the region of the enzyme into which 3-X falls), although the π_0 value is, for reasons which are not yet clear, higher than those of eq 1 and 2.

The two terms in MR_4 characterize the enzyme space into which 4-X falls. The MR_4 values have been scaled by 0.1 to make them somewhat equiscalar with respect to π (especially the apolar groups). The large value for ideal MR_4 indicates a much larger binding region off the 4 position. For the substituents on which eq 3 is based, π_4 and MR_4 were rather collinear and unusually large, spanning a considerable portion of enzymatic space. The purpose of this study was to investigate a set of 4-X-I with more reasonable substituents using highly purified enzymes and avoiding problems of unusually tight binding to better characterize 4-space.

Results and Discussion

We have derived eq 4–11 from the data in Table I. Equations 4–6 show that 4-space for substituents no larger than those in Table I is hydrophobic. If MR_4 is used in eq 4 in place of π_4, an equation with $r = 0.392$ is found. If the same substitution is made in eq 5, the equation obtained has $r = 0.492$. For the 18 substituents upon which eq 4–6 are based, π_4 and MR_4 are reasonably orthogonal vectors ($r^2_{\pi,MR} = 0.28$). While eq 4 and 5 clearly define enzymatic space near position 4 of I as being hydrophobic, work with larger substituents is needed to get a clearer picture of enzymatic space further removed from the 4 position of the phenyl moiety. There are only a few large substituents ($\pi \geq 2$) in Table I so that, in fact, eq 5 does not define an optimum π_4 (0.98 − 0.96 = 0.02); that is, as π_4 is increased, activity increases rapidly until $\pi_4 \approx 1.5$. As π_4 is increased further, activity remains essentially constant.

Inhibition of Bovine Liver DHFR by 4-X-I at pH 6.25

$$\log (1/C) = 0.52\ (\pm 0.16)\ \pi_4 + 6.39\ (\pm 0.22) \quad (4)$$

$$n = 18;\ r = 0.865;\ s = 0.428$$

$$\log (1/C) = 0.98\ (\pm 0.27)\ \pi_4 - 0.96\ (\pm 0.52) \log (\beta \cdot 10^{\pi_4} + 1) + 6.79\ (\pm 0.27) \quad (5)$$

$$n = 18;\ r = 0.938;\ s = 0.316;\ \log \beta = -0.454$$

$$\log (1/C) = 0.83\ (\pm 0.28)\ \pi_4 - 0.86\ (\pm 0.54) \log (\beta \cdot 10^{\pi_4} + 1) - 0.38\ (\pm 0.56)\ \mathcal{R}^-_4 + 6.68\ (\pm 0.24) \quad (6)$$

$$n = 18;\ r = 0.947;\ s = 0.305;\ \text{ideal } \pi_4 = 2.01;\ \log \beta = -0.682$$

The use of the bilinear eq 5 is actually not statistically justified (as judged by a partial F test[14]) as compared to the corresponding parabolic eq 7. We favor the form of

$$\log (1/C) = 0.63\ (\pm 0.14)\ \pi_4 - 0.15\ (\pm 0.09)\ \pi_4^2 + 6.64\ (\pm 0.22) \quad (7)$$

$$n = 18;\ r = 0.931;\ s = 0.322;\ \text{ideal } \pi_4 = 2.15\ (1.42\text{–}4.72)$$

the bilinear equation because of its consistency with eq 1 and 2 and with the model of a size-limited hydrophobic pocket which opens up to solvent at its end. With further testing of larger 4-X now in progress, we shall be able to clearly define the best type of equation (parabola or bilinear) for 4-X groups.

Equation 6 contains an additional term in $\mathcal{R}_4^-$. This parameter is the "through resonance" parameter from which the inductive effect has been removed (i.e., $\mathcal{R}^- = \sigma^- - \mathcal{F}$). The parameters $\mathcal{F}$ and $\mathcal{R}$ are Swain and Lupton's electronic parameters.[15] Although the correlation coefficient and the standard deviation of eq 6 suggest that it is a better correlation than eq 5, the F statistic does not ($F_{1,13} = 1.98$). If the 4-OH congener is dropped and eq 4–6 are rederived with the remaining 17 data points, then the $\mathcal{R}^-_4$ term is statistically justified. At best, there may be a small resonance effect from 4-substituents; however, further work on this point is needed before we can make a firm statement either for or against a resonance effect from 4-substituents.

Three data points in Table I have not been employed in the derivation of eq 4–6: 4-$COOCH_2CH_3$, 4-$COOCH_3$, and 4-$OCH_2CON(CH_2CH_2)_2O$. In this connection, it is of interest to note that the 3-$COOCH_2CH_3$ analogue is badly fit by eq 1 [log $(1/C)$ mispredicted by 1.2]. The indicator variable I_3 in eq 3 was used to parameterize what were termed rigidly attached substituents in both the 3 and 4 positions of I. These substituents included nine examples of: 4-$CONHC_6H_4$, 4-C_6H_5, 4-$CH{=}CHCONHC_6H_4$, 4-$CH(C_6H_5)CH_2$, 3-$CONHC_6H_4$, and 3-C_6H_5. What most of these substituents appear to have in common is branching at an sp^2 carbon attached to the phenyl moiety of I. If we use the coefficient of I_3 in eq 3 to correct the fit of the two esters of Table I, a reasonable fit would be obtained. Another congener in Table I similar to the esters is 4-$CONH_2$. This data point is also not well fit. Further work on the type of structures which need I_3 of eq 3 is now in progress.

Why the activity of the 4-$OCH_2CON(CH_2CH_2)_2O$ analogue is so much greater than its π_4 value predicts is most interesting. This is a rather long substituent, and oxygen of the morpholino moiety may extend beyond the hydrophobic pocket into a region of polar space.

If all 21 data points in Table I are fit to the form of eq 5, eq 5a is obtained. Not only is eq 5a a poor correlation,

$$\log (1/C) = 0.53\ (\pm 0.37)\ \pi_4 - 0.52\ (\pm 0.21) \log (\beta \cdot 10^{\pi_4} + 1) + 6.35\ (\pm 0.37) \quad (5a)$$

$$n = 21;\ r = 0.676;\ s = 0.727;\ \log \beta = -1.931$$

the three points are so badly fit that the shape of eq 5 is significantly changed. A different mechanism of inhibition is involved with these three points.

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(15) See ref 6, page 5.

Table II. Triazine Inhibitors (I, 4-X)[a]

no.	4-X	mp, °C obsd	mp, °C lit.	formula[b]
1	$COOC_2H_5$	182-185	186-188[c]	$C_{14}H_{19}N_5O_2 \cdot HCl$
2	$CONH_2$	230-231	210-212[d]	$C_{12}H_{16}N_6O_1 \cdot HCl$
3	SO_2NH_2	207-209[e]	206-208[f]	$C_{11}H_{16}N_6O_2S_1 \cdot HCl$
4	$COOCH_3$	191-192[e]		$C_{13}H_{17}N_5O_2 \cdot HCl$
5[g]	SO_2CH_3	226-228		$C_{12}H_{17}N_5O_2S_1 \cdot HCl$
6[g]	NH_2	248-252 dec		$C_{11}H_{16}N_6 \cdot HCl$
7	OH	>240 dec[e]		$C_{11}H_{15}N_5O_1 \cdot HCl$
8	$COCH_3$	213-214.5[e]	210.0-211.6[h]	$C_{13}H_{17}N_5O_1 \cdot HCl$
9	H	203-206	*i*	
10[g,j]	$OCH_2CON(CH_2CH_2)_2O$	207.5-208.5	219-220[k]	$C_{17}H_{24}N_6O_3 \cdot C_2H_5SO_3H$
11	OCH_3	214-218	212-213[l]	$C_{12}H_{17}N_5O_1 \cdot HCl$
12[m]	$C(CH_3)_3$	242-245		$C_{15}H_{23}N_5 \cdot HCl$
13	I	200-202	205-207[l]	$C_{11}H_{14}I_1N_5 \cdot HCl$
14	Br	200.5-202.5	208-209[l]	$C_{11}H_{14}Br_1N_5 \cdot HCl$
15	CF_3	210-214	210-211[l]	$C_{12}H_{14}F_3N_5 \cdot HCl$
16	CH_3	202.5-203.5	209-210[l]	$C_{12}H_{17}N_5 \cdot HCl$
17[g]	$O(CH_2)_2OC_6H_4$-4'-NH_2	177-178 dec		$C_{19}H_{24}N_6O_2 \cdot HCl$
18	F	205-207.5		$C_{11}H_{14}F_1N_5 \cdot HCl$
19[n]	$OCH_2C_6H_5$	187-190	213-218[o,l]	$C_{18}H_{21}N_5O_1 \cdot HCl$
20[g]	$(CH_2)_3CH_3$	197.5-199.5	198-200[l]	$C_{15}H_{23}N_5 \cdot HCl$
21[p]	$OCH_2C_6H_3$-3',4'-Cl_2	221.5-222.5[e]		$C_{18}H_{19}Cl_2N_5O_1 \cdot HCl$

[a] Unless otherwise noted, prepared by method A, ref 5, from a commercial sample of the appropriately substituted aniline (II) and recrystallized from H_2O; 46-89% yield. [b] Analyzed for C and H. [c] Reference 18. [d] Reference 16. [e] Recrystallized from EtOH/H_2O. [f] Reference 19. [g] See Acknowledgments for source. [h] Reference 20. [i] Reported in our earlier study.[5] [j] Ethane sulfonate salt. [k] Reference 21. [l] Reference 19. [m] Prepared from 5-*tert*-butylaniline hydrochloride (method L, ref 5). [n] Prepared from intermediate 3, Table III. [o] Monohydrate. [p] Prepared from intermediate 4, Table III.

Equations 4–6 for inhibition of bovine liver DHFR can be compared with eq 8–10 for inhibition of rat liver.

Inhibition of Rat Liver DHFR 4-X-I at pH 6.25

$$\log (1/C) = 0.53\ (\pm 0.20)\ \pi_4 + 5.89\ (\pm 0.28) \quad (8)$$

$$n = 13;\ r = 0.869;\ s = 0.395$$

$$\log (1/C) = 0.98\ (\pm 0.47)\ \pi_4 - 0.63\ (\pm 0.91) \log (\beta \cdot 10^{\pi_4} + 1) + 6.28\ (\pm 0.63) \quad (9)$$

$$n = 13;\ r = 0.900;\ s = 0.387;\ \log \beta = 0.180$$

$$\log (1/C) = 0.35\ (\pm 0.15)\ \pi_4 - 1.16\ (\pm 0.57)\ \mathcal{R}^-_4 + 5.96\ (\pm 0.18) \quad (10)$$

$$n = 13;\ r = 0.959;\ s = 0.237$$

Adding a term in π_4^2 to eq 8 or a bilinear or π_4^2 term to eq 10 does not result in a significant improvement in correlation. The bilinear term in π_4 of eq 9 is not statistically justified, although the form of this tentative equation is quite similar to that of eq 5. The failure to obtain significant bilinear or parabolic terms in π_4 for eq 8–10 is due to the limited size of the data set as well as the limited number of larger substituents. We report the results with the rat enzyme at this time because we plan no further work with it, since the results are so similar to those from bovine enzyme.

Replacing π_4 by MR_4 in eq 8 gives a much poorer correlation ($r = 0.503$), again emphasizing the interactions of the 4-X substituents as being hydrophobic. The activities of two esters, X = 4-$COOCH_3$ and X = 4-$COOC_2H_5$, were omitted in the derivation of eq 8–10 and, as for the bovine enzyme, are much lower than predicted by the equations.

Including the two compounds of Table I which were dropped in the derivation of eq 10 and rederiving it, we obtain eq 10a.

$$\log (1/C) = 0.29\ (\pm 0.31)\ \pi_4 - 1.81\ (\pm 1.1)\ \mathcal{R}^-_4 + 5.80\ (\pm 0.36) \quad (10a)$$

$$n = 15;\ r = 0.877;\ s = 0.511$$

That the specific interactions between 4-X-I and these two mammalian enzymes are similar can be seen from eq 11, showing correlation between log $(1/C)$ for the 15 congeners which were tested against both enzymes.

$$\log (1/C)_{rat} = 1.04\ (\pm 0.18) \log (1/C)_{bovine} - 0.82\ (\pm 1.2) \quad (11)$$

$$n = 13;\ r = 0.963;\ s = 0.276$$

In conclusion, it can be said with a certainty not possible with eq 3 that the enzymatic space in which 4-X-I substituents interact is typically hydrophobic in both bovine and rat liver DHFR. Exactly how large this pocket is cannot be sharply defined, but π_0 seems to be near 2. This would not be very large and is of course much smaller than one would anticipate from the ideal MR_4 value of 4.7 from eq 3. It is clear from Baker's studies, as well as the studies with methotrexate analogues, that there is a large region of enzyme off of the 4 position of the phenyl moiety of I with which substituents can interact. Studies with a large number of molecular probes (inhibitors) must be made before we can more clearly define this region. At present, we feel that there is a region in 4-space up to a π of 2–3 which will prove to be hydrophobic and a region beyond this which will prove to be generally polar in character.

As the X-ray coordinates become available for the structures of mammalian DHFR, we plan to correlate our findings with the character of the amino acid residues of 4-space.

Experimental Section

Synthesis of Triazine Inhibitors. The 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(4-X-phenyl)-*s*-triazine hydrochloride salts (4-X-I·HCl) were prepared, as in our previous study,[5] by the three-component synthetic method of Modest[16] from the appropriate para-substituted anilines (II) and HCl (or the para-substituted aniline hydrochloride salt), dicyandiamide (III), and acetone; see Scheme I and Table II. Preparation and physical properties of synthetic intermediates are presented in Table III.

Melting points (Buchi capillary apparatus) are uncorrected. Microanalyses were performed by C. F. Geiger, Ontario, CA, or Galbraith Laboratories Inc., Knoxville, TN, and are within ±0.4% of the theoretical values. TLC (precoated qualitative silica gel

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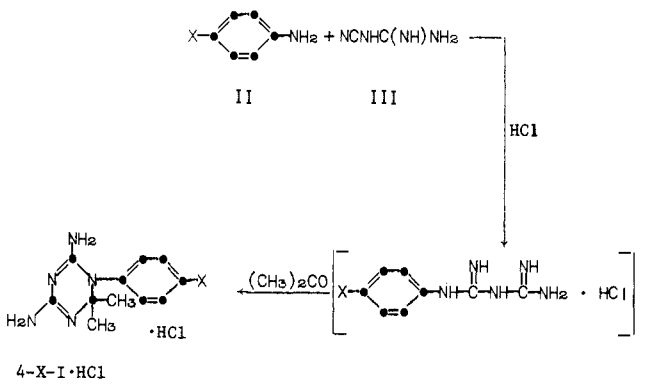
Table III. Synthetic Intermediates

no.	R_1	R_4	mp, °C	method[a]	formula[b]
1	NO_2	$OCH_2C_6H_5$	106.5-107.5[c]	K	
2	$NHCOCH_3$	$OCH_2C_6H_3$-3',4'-Cl_2	182-183[d]	O	$C_{15}H_{13}Cl_2N_1O_2$
3	NH_2·HCl	$OCH_2C_6H_5$	225-226 dec[e]	K	
4	NH_2·HCl	$OCH_2C_6H_3$-3',4'-Cl_2	>210 dec[e]	Q	$C_{13}H_{11}Cl_2N_1O_2$·HCl

[a] Prepared as for the meta isomer by the method of ref 5. [b] Analyzed for C and H. [c] Recrystallized from EtOH/H_2O; literature[22] mp 106 °C. [d] Recrystallized from EtOH. [e] Literature[23] mp 230-231 °C.

Scheme I



or alumina plates; UV visualization) was routinely used to check the purity of intermediates and the final triazines.

Inhibition Assays. Assays were performed as described in our previous study:[5] Durrum D-110 stopped-flow spectrophotometer, 2-cm cell path length; assays were initiated by mixing equal volumes of a solution containing dihydrofolic acid (FAH_2), NADPH, and inhibitor and of a solution containing DHFR; final assay solution = 14 μM FAH_2, 100 μM NADPH, 100 mM phosphate buffer, pH 6.25, 50 mM 2-mercaptoethanol, 25 °C. Sources of the DHFRs are also described in our previous study:[5] bovine liver DHFR, sp act. $\approx$ 8 μmol min^{-1} mg^{-1}; rat liver DHFR, sp act. = 1–2 μmol min^{-1} mg^{-1}.

Calculation of Log $(1/K_{i\,app})$ Values. (See ref 4 and references cited therein.) A rapid-equilibrium bireactant system, inhibitor competitive with FAH_2 but allowing NADPH to bind, and saturating [NADPH] were assumed. For such a system it can be shown that:

$$\frac{[I_t]}{1-(V_i/V_0)} = [E_t] + K_{i\,app}(V_0/V_i) \tag{12}$$

where $K_{i\,app}$ = apparent inhibition constant.

$$K_{i\,app} = \beta K_i\left(1 + \frac{[FAH_2]}{\alpha K_{FAH_2}}\right)$$

$[E_t] \ll K_{i\,app}(V_0/V_i)$ for a nonstoichiometric inhibitor (i.e., binding of inhibitor to the enzyme does not cause a significant decrease in the free [I]). Then, elimination of the $[E_t]$ term from eq 12 and rearrangement provides

$$\frac{V_i}{V_0} = \frac{K_{i\,app}}{K_{i\,app} + [I_t]} \tag{13}$$

$[E_t] \approx K_{i\,app}(V_0/V_i)$ when binding of the inhibitor to the enzyme does cause a significant decrease in the free $[I_t]$. Then, eq 12 can be rearranged to

$$[E_t](V_i/V_0)^2 + ([I_t] + K_{i\,app} + [E_t])(V_i/V_0) - K_{i\,app} = 0$$

The quadratic root

$$V_i/V_0 = -([I_t] + K_{i\,app} - [E_t]) + [([I_t] + K_{i\,app} - [E_t])^2 - 4[E_t](-K_{i\,app})]^{1/2} \times 2[E_t] \tag{14}$$

then provides for solution of V_i/V_0 as a function of $[I_t]$, $[E_t]$, and $K_{i\,app}$, where $0 < V_i/V_0 < 1$.

$[E_t] \gg K_{i\,app}(V_0/V_i)$ for a stoichiometric inhibitor. Then, elimination of the $K_{i\,app}(V_0/V_i)$ term from eq 12 and rearrangement provides eq 15.

$$\frac{V_i}{V_0} = 1 - \frac{[I_t]}{[E_t]} \tag{15}$$

$[E_t]$ for use in eq 14 was calculated as follows. Methotrexate (MTX) is an essentially stoichiometric inhibitor of DHFR when MTX, NADPH, and DHFR are preincubated for ~5 min and the assay is then initiated by an addition of FAH_2. A least-squares fit of the assay data to eq 15 should provide an estimate for $[E_t]$; however, it is experimentally difficult to obtain exactly the same $[E_t]$ in every assay solution. To compensate for this, each V_i/V_0 value (for the MTX inhibition) was converted to the value that would be observed if $[E_t] = [E_t]' =$ that $[E_t]$ which would give $V_0 = 0.1000\ \Delta A/min$:

$$\left(\frac{V_i}{V_0}\right)' = \frac{0.1000\ \Delta A/min - (V_0 - V_i)}{0.1000\ \Delta A/min}$$

(Note that for the MTX and the triazine inhibition assays, the same DHFR solution was used to determine any particular V_i value and its associated V_0 value, from which V_i/V_0 is calculated.) A least-squares fit of the MTX inhibition data to eq 16 then

$$\left(\frac{V_i}{V_0}\right)^2 = 1 - \frac{[I_t]}{[E_t]'} \tag{16}$$

provides an estimate for $[E_t]'$. In practice, before fitting the MTX inhibition data to eq 16, it was first determined that the 95% confidence interval for C contains the value of 1 for a least-squares fit of the data to

$$\left(\frac{V_i}{V_0}\right)' = C - \frac{[I_t]}{[E_t]'}$$

$[E_t]$ values for use in eq 14 were then calculated for each of the triazine inhibitor V_i/V_0 data points, using the transformation

$$[E_t] = [E_t]'\left(\frac{V_0}{0.1000\ \Delta A/min}\right)$$

where V_0 is the V_0 value associated with that particular V_i/V_0 data point.

For each of the triazine inhibitors, $K_{i\,app}$ values were calculated utilizing least-squares fit of the data to both eq 13 and 14, iterating on $K_{i\,app}$. Final estimates for each log $(1/K_{i\,app})$ and its 95% confidence interval were obtained in both cases by jackknifing log $(1/K_{i\,app})$. See ref 17 for a discussion of (a) our rationale concerning error distribution in the dependent variable of least-squares solutions of equations and, hence, for using these iterative fits of the experimental data to equations of the form

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of eq 13 or 14; (b) the jackknife procedure; and (c) details of the iteration procedure. Obviously, if $[E_t] \ll K_{i\,app}(V_0/V_i)$, then the log $(1/K_{i\,app})$ will be the same using eq 13 or 14. If $[E_t] \approx K_{i\,app}(V_0/V_i)$, eq 14 will always give the larger (and correct) estimate for log $(1/K_{i\,app})$; in such cases this was the equation finally used to calculate the log $(1/K_{i\,app})$ estimates.

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Inhibitors of Polyamine Biosynthesis. 9. Effects of *S*-Adenosyl-L-methionine Analogues on Mammalian Aminopropyltransferases in Vitro and Polyamine Biosynthesis in Transformed Lymphocytes

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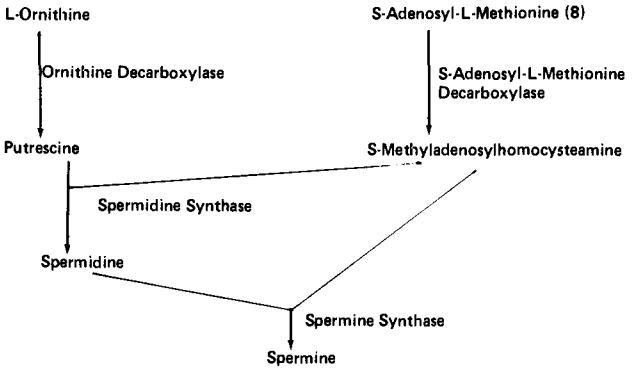
Seven analogues of *S*-adenosyl-L-methionine were studied as inhibitors or substrates for mammalian spermidine and spermine synthases. One of these, *S*-(5′-deoxy-5′-adenosyl)-(±)-1-methyl-3-(methylthio)propylamine (**5**), showed a unique spectrum of activities on the polyamine biosynthesis enzymes. It was an inhibitor of *S*-adenosyl-L-methionine decarboxylase from rat liver and spermine synthase from bovine brain and rat ventral prostate. This compound was a substrate for the spermidine synthases from bovine brain and rat ventral prostate but not a substrate for the spermine synthases from these same sources. At concentrations of 0.2 mM and higher, compound **5** blocked the increases in polyamine levels and in [^{3}H]thymidine incorporation induced by concanavalin A in cultured mouse lymphocytes. At approximately a 0.5 mM concentration of **5**, the cellular polyamine levels and the rate of thymidine incorporation were similar to those of the unstimulated lymphocytes. Lower concentrations of **5** (0.02–0.1 mM) produced a dose-dependent increase in thymidine incorporation. A dose-dependent decrease in the cellular polyamine levels was observed in the range of 0.05–0.5 mM of the inhibitor. These results suggest that the effects of **5** on transformed lymphocytes are complex and may not be solely due to the inhibition of polyamine biosynthesis by this compound.

The polyamines appear to play an essential role in cellular metabolism and regulation.[2] The development of specific inhibitors of polyamine biosynthesis has received considerable attention during the past decade. These inhibitors were developed as tools for the study of the function of the polyamines and as potential therapeutic agents. Four enzymes are known to be involved in the synthesis of polyamines in mammalian tissues (Scheme I). Many inhibitors of the first two enzymes in the pathway, L-ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase, have been developed.[3-5] However, only a few studies have been carried out on inhibitors of spermidine and spermine synthases.[6-12] Potent and specific inhibitors of these synthases may have some advantages over the currently available inhibitors of the decarboxylases. Specifically, such inhibitors may produce significant depletion of spermine. This has not been al-

Scheme I



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