2 H, $CH_2C=$), 2.3 (s, 6 H, 2 $CH_3C=$), 1.7-2.1 (m, 6 H, 3 CH_2). Mass spectra: m/e 396 (M^+).

Ethyl 4-[[7-(3-Methyl-5-isoxazolyl)heptyl]oxy]benzimidate Hydrochloride (7). A solution of 12.7 g (0.043 mol) of **5** in 40 mL of EtOH and 80 mL of Et_2O was saturated at -70 °C with HCl gas, then allowed to warm to 0 °C, and left for 3 h. Finally the solution was left overnight at room temperature. Removal of the solvent in vacuo gave a solid residue that was triturated with ether and filtered to afford 15.6 g (95%) of **7**, mp 112-114 °C. Anal. ($C_{20}H_{28}N_2O_3$·HCl) C, H, N. NMR ($CDCl_3$): δ 5.8 (s, 1 H, =CH), 4.3 (m, 2 H, $N=COCH_2CH_3$).

5-[7-[4-(4,5-Dihydro-2-oxazolyl)phenoxy]heptyl]-3-methylisoxazole (14). To a solution of 2.40 g (0.0063 mol) of **7** in 250 mL of CH_2Cl_2 was added 7 g of TEA in 20 mL of CH_2Cl_2. After stirring for 1 h, the solution was washed with water and dried. The solvent was removed, leaving a solid. To the solid was added 0.432 g (0.007 mol) of ethanolamine, and the mixture heated in an oil bath with stirring at 120 °C at which point gas evolution (NH_3) was observed. After $1^1/_2$ h the melt was cooled and dissolved in hot *i*-ProAc (100 mL) and chilled. Solid formed and was collected: 1.7 g (79%) of **14** was obtained; mp 86-89 °C. Anal. ($C_{20}H_{26}N_2O_3$) C, H, N. NMR ($CDCl_3$): δ 5.79 (s, 1 H, =CH), 3.8-4.6 (m, 6 H, 2 OCH_2, NCH_2), 2.71 (t, 2 H, $CH_2C=$), 2.25 (s, 3 H, $CH_3C=$), 1.2-2.0 (m, 10 H, 5 CH_2).

Method B. 4-(4,5-Dihydro-2-oxazolyl)phenol (9a). To a slurry of 61.8 g (0.34 mol) of 4-hydroxy-*N*-(2-hydroxyethyl)-benzamide[10] in 500 mL of *i*-PrOAc was added dropwise 40 mL (0.54 mol) of $SOCl_2$. After stirring for 2 h, the mixture was filtered to give 65.2 g (96%) of **9a**, mp 160-162 °C. Anal. ($C_9H_9NO_2$·HCl) C, H, N.

5-(4-Bromobutyl)-3-methylisoxazole (10, *n* = 4). To a solution of 28 mL (0.2 mol) of diiscpropylamine in THF was added at -5 °C and under nitrogen 77 mL of 2.6 M *n*-butyllithium in hexane (0.2 mol). After the addition was complete, the solution was cooled to -60 °C and 19.6 mL of 3,5-dimethylisoxazole (0.2 mol) in 50 mL of THF was added dropwise. The mixture was stirred for an additional 1 h at -60 °C, then added, via a nitrogen purge, to 250 g (1.2 mol) of 1,3-dibromopropane in 100 mL of THF, and chilled to -60 °C with stirring. The mixture was allowed to gradually warm to room temperature and then stirred overnight. After quenching with 20 mL of saturated NH_4Cl solution, the mixture was extracted with 250 mL of *i*-ProAc and the extract washed with water and dried. Removal of the solvent and excess dibromopropane gave 33.2 g of brown oil, which was purified by HPLC using 1:1 EtOAc-cyclohexane to give 23.4 g (54%) of **10**. NMR ($CDCl_3$): δ 2.77 (t, 2 H, $BrCH_2$), 2.27 (s, 3 H, $CH_3C=$), 1.7-2.2 (m, 4 H, CH_2CH_2). Mass spectra: m/e 217 (M^+, 1 Br).

5-[4-[4-(4,5-Dihydro-2-oxazolyl)phenoxy]butyl]-3-methylisoxazole (11). A mixture of 6.0 g (0.037 mol) of **10** (*n* = 4) 8.1 g (0.041 mol) of **9a**, 25 g (0.18 mol) of milled K_2CO_3, and 5 g (0.031 mol) of NaI in 200 mL of CH_3CN was heated to reflux with stirring for 21 h. The mixture was filtered, the filtrate concentrated in vacuo, and the residue partitioned between CH_2Cl_2 and H_2O. The organic layer was washed with 5% KOH solution and water and dried. Removal of the solvent gave a solid that was recrystallized from *i*-PrOAc to give 8.5 g of **11** (75%), mp 93-94 °C. Anal. ($C_{17}H_{20}N_2O_3$) C, H, N. NMR ($CDCl_3$): δ 7.9 (d, 2 H, aromatic), 6.9 (d, 2 H, aromatic), 5.8 (s, 1 H, =CH), 4.2-4.5 (m, 2 H, NCH_2), 3.8-4.2 (m, 4 H, 2 OCH_2), 2.8 (t, 2 H, $CH_2C=$), 2.3 (s, 3 H, $CH_3C=$), 1.7-2.1 (m, 4 H, CH_2CH_2).

5-[7-[4-[4,5-Dihydro-5-(methoxymethyl)-2-oxazolyl]phenoxy]heptyl]-3-methylisoxazole (21). To a suspension of 1.08 g (0.027 mol) of a 60% NaH dispersion in 50 mL of dry THF was added dropwise at 30 °C 6.6 g (.0177 mol) of **20** in 50 mL of THF at 30 °C. The resulting mixture was heated at gentle reflux for 15 min and then cooled to room temperature, and 4.3 g (0.03 mol) of CH_3I in 25 mL of THF was added dropwise during a 10-min period. The mixture was stirred at room temperature overnight. The solvent was removed, leaving a solid that was washed with pentane. The residual material was dissolved in EtOAc, washed with H_2O, and dried. After removal of the EtOAc an oil remained that solidified on standing. Recrystallization from hexane gave 5.3 g of **21** (80%), mp 60-61 °C. Anal. ($C_{22}H_{30}N_2O_4$) C, H, N. NMR ($CDCl_3$): δ 3.49 (s, 3 H, OCH_3), 2.77 (t, 2 H, $CH_2C=$), 2.31 (s, 3 H, $CH_3C=$), 1.3-2.1 (m, 10 H, 5 CH_2).

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Registry No. 1, 91944-93-3; **2**, 91945-23-2; 4, 91944-99-9; **5**, 91944-98-8; **6**, 98466-34-3; **7**, 98360-44-2; **9a**, 81428-58-2; **10** (*n* = 4), 98033-94-4; **11**, 98033-95-5; **12**, 98034-30-1; **13**, 98033-84-2; **14**, 87495-31-6; **15**, 98033-91-1; **16**, 98034-32-3; **17**, 98524-87-9; **18**, 98033-99-9; **19**, 98054-14-9; **20**, 98033-97-7; **21**, 98034-10-7; **22**, 98033-63-7; ethanolamine, 141-43-5; 4-hydroxy-*N*-(2-hydroxyethyl)benzamide, 75268-14-3; 3,5-dimethylisoxazole, 300-87-8; 1,3-dibromopropane, 109-64-8.

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Quantitative Structure-Activity Relationship of Antifolate Inhibition of Bacteria Cell Cultures Resistant and Sensitive to Methotrexate

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Sets of 5-(substituted benzyl)-2,4-diaminopyrimidines and 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted phenyl)-*s*-triazines as well as several other antifolates were tested as inhibitors of *Escherichia coli* dihydrofolate reductase and *E. coli* cell cultures both sensitive and resistant to methotrexate. From the results quantitative structure-activity relationships (QSAR) were formulated. The triazines were found to inhibit sensitive and resistant cell cultures to the same degree, but the benzylpyrimidines showed marked differences against the two types of cells. Increased hydrophobicity produced benzylpyrimidines more active against the resistant *E. coli* cell. Metroprine did not discriminate between the two types of cells cultures, but pyrimethamine and 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine (BW 301U) did. The results are compared with triazines and benzylpyrimidines acting on *Lactobacillus casei* and murine tumor cells sensitive and resistant to methotrexate. QSAR is shown to be an effective means for detecting receptor differences.

A most serious problem in drug research is the development of the means for making drugs effective against pathogenic cells which have become drug resistant. While there are a variety of ways one might approach such problems, we have been trying to understand the differences in the structure-activity relationships of antifolates acting on sensitive and resistant cells of bacterial and tumor origin.[1,2] Apart from metabolism there are several

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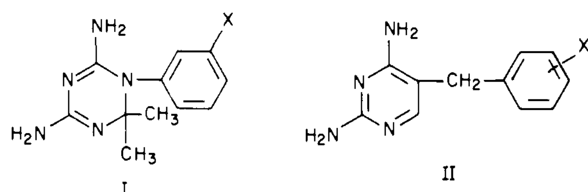
recognized mechanisms which organisms can utilize to protect themselves from xenobiotics which inhibit the enzyme dihydrofolate reductase (DHFR).[3,4] Via gene amplification[3] hundreds of times the normal amount of DHFR can be formed by resistant cells which helps protect them from inhibitors. In mammalian cells and certain others (*Lactobacillus casei*) there is an active transport system for folic acid which transports methotrexate as well as other antifolates. In resistant cells this transport system appears to be greatly impaired. Further evidence also suggests that the conformation and/or the structure of the DHFR in vivo differs in sensitive and resistant cells. Finally, the least well documented mechanism involves an alteration in the composition of the cell membranes in resistant cells. This might affect the passive transport of xenobiotics directly or by affecting the structure of the transport system it might influence transport indirectly.

We have been investigating two classes of inhibitors, triazines I and benzylpyrimidines II, on various types of sensitive and resistant cells.



In a first study[1] quantitative structure–activity relationships (QSAR) were formulated for the inhibition of purified *L. casei* DHFR by triazines and the results compared with the inhibition of *L. casei* cell cultures sensitive and resistant to methotrexate. The results are contained in eq 1–3. In these expressions K_i is the apparent inhibition constant of purified enzyme and C is the molar concentration causing 50% inhibition of the cell culture. The number of data points used to derive the equation is represented by n, r is the correlation coefficient, and s is the standard deviation from the regression equation. π is the hydrophobic parameter for X of I.[5]

Inhibition of *L. casei* DHFR by Triazines I

$$\log 1/K_i = 0.53\pi' - 0.64 \log (\beta\cdot 10^{\pi'} + 1) + 1.49I + 0.70\sigma + 2.93 \quad (1)$$

$$n = 44,\ r = 0.953,\ s = 0.319,\ \pi_0 = 4.31$$

50% Inhibition of *L. casei* Cells Sensitive to Methotrexate by Triazines I

$$\log 1/C = 0.80\pi' - 1.06 \log (\beta\cdot 10^{\pi'} + 1) - 0.94MR_Y + 0.80I + 4.37 \quad (2)$$

$$n = 34,\ r = 0.929,\ s = 0.371,\ \pi_0 = 2.94$$

50% Inhibition of *L. casei* Cells Resistant to Methotrexate by Triazines I

$$\log 1/C = 0.45\pi + 1.05I - 0.48MR_Y + 3.37 \quad (3)$$

$$n = 38,\ r = 0.965,\ s = 0.259$$

The prime with π signifies that for substituents where $X = -CH_2ZC_6H_4-Y$ (Z = O or NH) π for Y is set equal to 0. That is, π of $-CH_2ZC_6H_4-Y = \pi$ for $-CH_2ZC_6H_5$. It was found from a study of K_i values that Y has essentially no effect on K_i. Later it was discovered from X-ray crystallographic analysis of triazines bound to DHFR that Y projects beyond the enzyme surface.[6]

The indicator variable I is assigned the value of 1 for substituents of the type $-CH_2ZC_6H_4-Y$. Congeners containing this group are about 30 times more active than one would predict from π' and σ alone. This class of substituent appears to bind unusually tightly which may be the result of it occupying approximately the same position on DHFR as that taken by the *p*-aminobenzoyl moiety of folic acid. The Hammett σ term in eq 1 shows that electron withdrawal by X increases inhibitory potency slightly. The hydrophobic terms are in the form of the bilinear model. That is, activity first increases as π' increases with a slope of 0.53 until the point where $\pi = 4.31$ (π_0). Then the slope changes to −0.11 (0.53–0.64). This indicates that large substituents having $\pi > 4.31$ project beyond hydrophobic space into the surrounding solution and this fact has been confirmed by X-ray crystallographic studies.[6] In comparing eq 2 for sensitive cells to eq 1 for DHFR there are three distinct differences. Most important is the new term in MR_Y. MR represents the molar refractivity of a substituent and it is primarily a measure of the volume of the substituent.[5] Hence the negative coefficient with MR_Y means that bulky groups prevent binding of the triazines to DHFR, presumably because Y encounters some kind of steric effect from DHFR or more likely a nearby macromolecule. To our knowledge this is the first instance of a demonstration of a specific difference in the behavior between an enzyme in vitro and in vivo. This of course depends on the assumption that it is, for practical purposes, only the interaction of the triazine with DHFR in the living cell which limits cell growth.

The coefficients with π are somewhat different in eq 2 compared to eq 1 which results in a lower π_0. In the case of the cells the initial increase in activity with increase in hydrophobicity is greater (slope of 0.80 vs. 0.53), but activity then decreases (0.80 − 1.06 = −0.26). The difference in the hydrophobic terms in eq 1 and 2 may be the result of membrane penetration and the partitioning among the various lipophilic phases.[7] It may also be influenced by interactions with protein carrier molecules involved in active transport.

Another significant difference between eq 1 and 2 is the smaller coefficient with I in the QSAR from the cell culture study. This difference would suggest a slight change in that portion of the active site where the $CH_2ZC_6H_4-Y$ group binds.

Finally no term in σ appears in eq 2, but since this is the least important variable in eq 1, its effect may simply be masked by noise in the data. While there are these discrete differences between eq 1 and 2, the general similarity between these equations as well the similarity in potency convince us that the enzyme in vitro behaves much like that in wild type *L. casei* cells. However it must be emphasized that the purified DHFR was obtained from

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Table I. Parameters Used To Derive Equations 4–7 for the Inhibition of *E. coli* DHFR by Triazines I[a]

no.	X	log $1/K_{i\,app}$ obsd	log $1/K_{i\,app}$ calcd[b]	Δ log $1/K_{i\,app}$	σ	I	π'
1	H	4.51	4.93	−0.42	0.00	0.00	0.00
2	3-SO_2NH_2	3.69	3.60	0.09	0.46	0.00	−1.82
3	3-$CONH_2$	3.48	3.73	−0.25	0.28	0.00	−1.49
4	3-CF_3	5.69	6.05	−0.36	0.43	0.00	0.88
5	3-F	5.85	5.51	0.34	0.34	0.00	0.14
6	3-Cl	5.87	5.90	−0.03	0.37	0.00	0.71
7	3-I	5.58	6.01	−0.43	0.35	0.00	1.12
8	3-CN	5.51	5.13	0.38	0.56	0.00	−0.57
9	3-CH_3	5.42	5.23	0.19	−0.07	0.00	0.56
10	3-$(CH_2)_5CH_3$	5.75	5.63	0.12	−0.08	0.00	3.21
11	3-$C(CH_3)_3$[c]	4.72	5.52	−0.80	−0.10	0.00	1.98
12	3-$O(CH_2)_3CH_3$	6.05	5.75	0.30	0.10	0.00	1.55
13	3-$O(CH_2)_{10}CH_3$	6.19	6.00	0.19	0.10	0.00	5.37
14	3-$O(CH_2)_{11}CH_3$	5.88	6.03	−0.15	0.10	0.00	5.91
15	3-$O(CH_2)_{13}CH_3$	5.85	6.09	−0.24	0.10	0.00	6.99
16	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	6.04	5.84	0.20	0.10	0.00	2.56
17	3-$OCH_2C_6H_5$	5.31	5.76	−0.45	0.10	0.00	1.66
18	3-$OCH_2C_6H_3$-3′,4′-Cl_2	6.15	5.76	0.39	0.10	0.00	1.66
19	3-OCH_2-1-adamantyl	6.01	5.90	0.11	0.10	0.00	3.61
20	3-$CH_2OC_6H_5$	5.92	6.12	−0.20	0.06	1.00	1.66
21	3-$CH_2OC_6H_4$-3′-Cl	6.01	6.12	−0.11	0.06	1.00	1.66
22	3-$CH_2OC_6H_4$-3′-CN	6.05	6.12	−0.07	0.06	1.00	1.66
23	3-$CH_2OC_6H_4$-3′-OCH_3	6.02	6.12	−0.10	0.06	1.00	1.66
24	3-$CH_2OC_6H_4$-3′-CH_2OH	6.19	6.12	0.07	0.06	1.00	1.66
25	3-$CH_2OC_6H_4$-3′-CH_3	6.27	6.12	0.15	0.06	1.00	1.66
26	3-$CH_2OC_6H_4$-3′-CH_2CH_3	5.95	6.12	−0.17	0.06	1.00	1.66
27	3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$	6.06	6.12	−0.06	0.06	1.00	1.66
28	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	5.99	6.12	−0.13	0.06	1.00	1.66
29	3-$CH_2OC_6H_4$-3′-$NHCSNH_2$	6.17	6.12	0.05	0.06	1.00	1.66
30	3-$CH_2OC_6H_4$-3′-$NHCONH_2$	5.91	6.12	−0.21	0.06	1.00	1.66
31	3-$CH_2SC_6H_5$	6.53	6.17	0.36	0.06	1.00	2.30
32	3-$CH_2SC_6H_4$-3′-CH_3	6.57	6.17	0.40	0.06	1.00	2.30

[a] Squared correlation matrix:

	π'	I	σ
π'	1	0.00	0.21
I		1	0.18

[b] Calculated by eq 7. [c] Not used in the derivation of equations.

the resistant cells and since sequencing has not been done on DHFR from both sources they cannot, with complete assurance, be said to be identical.

Equation 3 for the inhibition of resistant cells is radically different from eq 1 and 2 in that it is linear in π; hence, it is not possible to calculate π_0 although it is estimated to be around 5.9.[1] In eq 3 π is used rather than π' because the data is better correlated by thus taking into account the hydrophobicity of Y. One would expect Y to interact hydrophobically with cells membranes and other lipophilic parts of the cells. As in eq 2 terms are found for I and MR but not for σ.

There is an enormous difference in the action of methotrexate on the two types of cells. The log $1/C$ for sensitive cells is 10.9 while that for resistant cells is 2.85; thus one needs an increase of 10^8 in the methotrexate concentration necessary to elicit a 50% decrease in resistant cell growth. Although part of this increased resistance is no doubt due to the large increase in DHFR (100–200-fold), and some may be attributed to a conformational change in structure of the enzyme, we believe that the greatest difference is probably due to an impaired transport system. This suggests that in the case of the resistant cells entry of the inhibitors is primarily by passive diffusion.

In order to check out this hypothesis it was necessary to ascertain the effects of the triazines on some organism which does not have an active transport system. For this reason we have chosen to test the triazines on two types of *E. coli* cell systems: one sensitive and the other resistant to methotrexate.

Results

From the data in Table I we have derived eq 4–7 for the inhibition of purified DHFR from *E. coli* cells resistant to methotrexate by triazines I. In the derivation of these

Inhibition of *E. coli* DHFR by Triazines I

$$\log 1/K_i = 0.21\ (\pm 0.12)\ \pi'_3 + 5.38\ (\pm 0.30) \quad (4)$$

$$n = 31,\ r = 0.562,\ s = 0.582,\ F_{1,29} = 13.4$$

$$\log 1/K_i = 0.88\ (\pm 0.19)\ \pi'_3 - 0.92\ (\pm 0.24) \log (\beta \cdot 10^{\pi'_3} + 1) + 5.22\ (\pm 0.18) \quad (5)$$

$$n = 31,\ r = 0.888,\ s = 0.336,\ F_{2,27} = 30.1,\ \pi_0 = 2.41$$

$$\log 1/K_i = 0.89\ (\pm 0.18)\ \pi'_3 - 0.98\ (\pm 0.23) \log (\beta \cdot 10^{\pi'_3} + 1) + 1.34\ (\pm 0.96)\ \sigma + 4.76\ (\pm 0.29) \quad (6)$$

$$n = 31,\ r = 0.913,\ s = 0.303,\ F_{1,26} = 7.23,\ \pi_0 = 2.62$$

$$\log 1/K_i = 1.16\ (\pm 0.25)\ \pi'_3 - 1.10\ (\pm 0.29) \log (\beta \cdot 10^{\pi'_3} + 1) + 1.36\ (\pm 0.90)\ \sigma + 0.41\ (\pm 0.25)\ I + 5.08\ (\pm 0.28) \quad (7)$$

$$n = 31,\ r = 0.930,\ s = 0.280,\ F_{1,25} = 5.52$$

equations one point [3-C(Me)$_3$] has been omitted since it is rather poorly fit (see Table I). This derivative is 6.4 times less active than eq 7 predicts. Including this point yields essentially the same equation with a lower correlation ($r = 0.917$) and higher standard deviation ($s = 0.307$). It is of interest to note that in the bilinear eq 5–7 the slope of the right-hand portion is essentially zero, making π_0 difficult to evaluate. It can be established for eq 5 and 6 but not for eq 7. Although eq 1 and 7 contain the same

Table II. Parameters Used To Derive Equations 8 and 9 for the Inhibition of Growth of *E. coli* Cells by Triazines I

		MB1417			MB1428			
		log 1/C		Δ log	log 1/C		Δ log	
no.	X	obsd	calcd[a]	1/C	obsd	calcd[b]	1/C	π
1	3-SO_2NH_2	1.52	1.43	0.09	1.09	1.30	−0.21	−1.82
2	3-SO_2F	2.50	2.38	0.12	2.00	1.32	−0.32	0.05
3	3-CF_3	2.31	2.80	−0.49	2.30	2.77	−0.47	0.88
4	3-CN	2.25	2.06	0.19	2.15	1.98	0.17	−0.57
5	3-$O(CH_2)_3CH_3$	3.29	3.14	0.15	3.49	3.13	0.36	1.55
6	3-$O(CH_2)_{10}CH_3$	5.50	5.08	0.42	5.50	5.22	0.28	5.37
7	3-$O(CH_2)_{13}CH_3$	5.50	5.90	−0.40	5.50	6.10	−0.60	6.99
8	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	4.01	3.65	0.36	4.14	3.68	0.46	2.56
9	3-$OCH_2C_6H_3$-3′,4′-Cl_2	4.13	4.03	0.10	4.01	4.09	−0.08	3.30
10	3-OCH_2-1-adamantyl	4.49	4.18	0.31	4.63	4.26	0.37	3.61
11	3-$CH_2OC_6H_4$-3′-CN	2.74	2.91	−0.17	3.02	2.88	0.14	1.09
12	3-$CH_2OC_6H_4$-3′-OCH_3	3.21	3.19	0.02	3.26	3.18	0.08	1.64
13	3-$CH_2OC_6H_4$-3′-CH_2OH	2.38	2.67	−0.29	2.51	2.63	−0.12	0.63
14	3-$CH_2OC_6H_4$-3′-CH_3	3.27	3.48	−0.21	3.41	3.50	−0.09	2.22
15	3-$CH_2OC_6H_4$-3′-CH_2CH_3	3.67	3.71	−0.04	3.98	3.75	0.23	2.68
16	3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$	4.10	4.20	−0.10	4.19	4.27	−0.08	3.64
17	3-$CH_2SC_6H_5$	3.39	3.52	−0.13	3.52	3.54	−0.02	2.30
18	3-$CH_2SC_6H_4$-3′-CH_3	3.87	3.80	0.07	3.76	3.85	−0.09	2.86
19	4-Cl[c]	2.47			2.50			

[a] Calculated by using eq 8. [b] Calculated by using eq 9. [c] Not used in the derivation of eq 8 and 9.

terms, the coefficients with the π terms are different and there is a large difference in the intercepts showing *E. coli* DHFR to be intrinsically more sensitive to the triazines. The collinearity among the variables of eq 7 is low as seen in the squared correlation matrix in Table I (footnote).

Equation 7 can be compared with eq 8 and 9 for cell culture studies derived from the data in Table II. The

50% Inhibition by Triazines of *E. coli* Cell Culture (MB 1417) Sensitive to Methotrexate

$$\log 1/C = 0.51 (\pm 0.06) \pi_3 + 2.35 (\pm 0.19) \quad (8)$$

$$n = 18, r = 0.973, s = 0.260, F_{1,16} = 281$$

50% Inhibition by Triazines of *E. coli* Cell Culture (MB 1428) Resistant to Methotrexate

$$\log 1/C = 0.54 (\pm 0.07) \pi_3 + 2.29 (\pm 0.22) \quad (9)$$

$$n = 18, r = 0.969, s = 0.299, F_{1,16} = 245$$

only significant difference between eq 8 and 9 is the poorer fit of the data to eq 9, which is best appreciated by comparison of values of *s*. Both sensitive and resistant cells respond to the triazines in much the same way.

It has been presumed that the triazines do not enter the *E. coli* cells by active transport, and hence, if our hypothesis is right about eq 3 primarily being the result of an impaired transport system, we would expect the same dependence on π for eq 3, 8, and 9. Indeed, the agreement is good.

The difference in sensitivity of the two types of *E. coli* cells to methotrexate is remarkably different from the *L. casei* case. Log 1/*C* for methotrexate inhibition of sensitive *E. coli* is 3.76 (3.70–3.81) while log 1/*C* for resistant cells is 2.70.

It is surprising that eq 8 and 9 for the cell cultures are so much simpler than eq 7 for the isolated enzyme. The results suggest that passive diffusion of the inhibitors to the DHFR is more rate limiting than interaction with the DHFR. The σ, *I*, and bilinear terms disappear in the cell culture equations. The standard deviation of eq 8 is essentially the same as that of eq 7 so that the quality of fit is just as good although it must be noted that a larger number of data points is encompassed by eq 7. There is more uncorrelated variance for eq 9, indicating small undefinable difference in the two types of cells. Log 1/*C* for methotrexate acting on the sensitive *E. coli* cells is 3.76, which is relatively close to that of 2.85 for inhibition of resistant *L. casei* cells. This would suggest similar penetration problems for the two systems. Setting aside different structural features in the DHFR, the two log 1/*C* values are close enough to suggest that when active transport is subtracted from the *L. casei* case, one sees relatively little difference in the inhibition of the two types of cells.

On the other hand, with the benzylpyrimidines II the following QSAR have been derived:

Inhibition of *E. coli* DHFR by Benzylpyrimidines[8]

$$\log 1/K_i = 1.33\ MR'_{3,5} + 0.94 MR'_4 + 5.69 \quad (10)$$

$$n = 34, r = 0.904, s = 0.281$$

The prime with MR signifies that MR has been truncated (in the 3-, 4-, and 5-positions) at the value of 0.79. That is, the maximum value for MR at any one position is 0.79 and for all three positions it is 2.37 (MR for H is 0.1). Only this fraction of the substituent appears to make effective contact with the enzyme. The smaller coefficient with MR'_4 indicates that contact of 4-substituents is less effective than contact by 3- and 5-substituents.

From the data in Table III eq 11–15 have been derived for inhibition of *E. coli* cell cultures. In this development of eq 13 it is clear that the most important variable is $MR'_{3,4,5}$, which accounts for 71% of the variance in log 1/*C*. Adding a term in π does not result in a significant improvement, but adding two terms in π (eq 13) yields a significantly better equation.

50% Inhibition of Growth of MB1417 by Pyrimidines II

$$\log 1/C = 1.06 (\pm 0.27)\ MR'_{3,4,5} + 3.75 (\pm 0.40) \quad (11)$$

$$n = 28, r = 0.845, s = 0.438, F_{1,26} = 74.7$$

$$\log 1/C = 1.09 (\pm 0.26)\ MR'_{3,4,5} - 0.10 (\pm 0.12) \pi_{3,4,5} + 3.79 (\pm 0.39) \quad (12)$$

$$n = 28, r = 0.863, s = 0.421, F_{1,25} = 3.10$$

$$\log 1/C = 1.15 (\pm 0.22)\ MR'_{3,4,5} + 0.27 (\pm 0.22) \pi_{3,4,5} - 0.14 (\pm 0.08) \pi^2_{3,4,5} + 3.79 (\pm 0.31) \quad (13)$$

$$n = 28, r = 0.916, s = 0.341, F_{1,24} = 14.1, \pi_0 = 0.94 (0.29\text{–}1.28)$$

The fact that eq 10 for the isolated enzyme does not contain a hydrophobic term while eq 12 and 13 do is presumed to be due to the interaction of the benzylpyrimidines with lipophilic molecules and membranes in the cell culture studies. This would also hold true for eq 15 as well. One must also remember that eq 10 accounts for only about 82% of the variance in log $1/K_i$ so that it is possible that we have not accounted for some hydrophobic effects with the isolated enzyme. The active site of this enzyme does contain hydrophobic space. However, the steric effects of MR seem to completely overshadow any discernible hydrophobic interaction.

It is not possible to obtain a true bilinear equation (with a positive left-hand and a negative right-hand slopes) with the data now in hand. With more supraoptimal congeners it should be possible to do so.

The striking fact about eq 13 is that the bulk parameter MR accounts for most of the variance. In eq 10 better results were obtained by factoring MR into two terms, but this difference in behavior of substituent position is nullified in the living cells. Collinearity between $MR'_{3,4,5}$ and $\pi_{3,4,5}$ is low ($r^2 = 0.04$), so that both characteristics can safely be considered in a single equation. Equation 15 for

50% Inhibition of Growth of MB1428 by Pyrimidines II

$$\log 1/C = 1.25\ (\pm 0.34)\ MR'_{3,4,5} + 2.45\ (\pm 0.47) \quad (14)$$

$$n = 26,\ r = 0.840,\ s = 0.510,\ F_{1,24} = 57.3$$

$$\log 1/C = 1.39\ (\pm 0.16)\ MR'_{3,4,5} + 0.35\ (\pm 0.08)\ \pi_{3,4,5} + 2.11\ (\pm 0.23) \quad (15)$$

$$n = 26,\ r = 0.969,\ s = 0.238,\ F_{1,23} = 87.3$$

resistant cells differs from eq 13 for sensitive cells in the size of the coefficient with the MR′ term and in the intercepts. However, the major difference is that π_0 can be established for eq 13 as 1.22, but for the resistant cells π_0 would seem to lie between 3.17 and 3.79. (Compare compounds 18 and 19 Table III.) Neither factoring MR nor adding a term in π^2 to eq 15 results in a significant reduction in the variance. Collinearity between $MR'_{3,4,5}$ and $\pi_{3,4,5}$ is low ($r^2 = 0.04$).

It was quite surprising to find that sensitive and resistant *E. coli* cells interact in almost the same fashion with the triazines but in a quite different manner with the benzylpyrimidines. The pattern found with the benzylpyrimidines is reminiscent of that for triazines acting on sensitive and resistant *L. casei* cells. This same dependence of selectivity on hydrophobicity is also found for both triazines and benzylpyrimidines inhibiting sensitive and resistant murine tumor cells. (See Discussion.)

In all of these cases the cut-off point in the increase of activity with increasing lipophilicity of the parent compound arises much earlier with the sensitive cells. Hence one can design more effective drugs for resistant bacterial or cancer cells by making more lipophilic congeners. In doing so, one cannot of course, exceed log P_0, for the whole animal system.

At the bottom of Table III four other antifolates (methotrexate, BW 301U, pyrimethamine, and metoprine) are listed with the activities against both sensitive and resistant cells. Three of these show a pronounced difference between the two types of cells, but the fourth, metoprine, shows *no* selectivity and behaves like the triazines.

Discussion

The QSAR for the triazines acting on sensitive *E. coli* cells (eq 8) can be compared with other QSAR for triazines from work by Wooldridge and his colleagues.[9] The coefficients with π in eq 16 and 17 are essentially the same as for eq 8 and 9. The difference in the intercepts is due to differences in experimental conditions (MIC vs. 50% inhibition).

S. aureus[10]

$$\log 1/C = 0.59\pi - 1.52 \log (\beta \cdot 10^{\pi} + 1) + 2.83 \quad (16)$$

$$n = 23,\ r = 0.986,\ s = 0.218,\ \pi_0 = 5.79$$

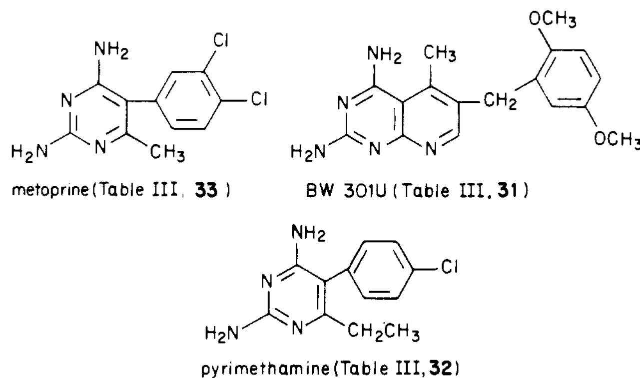
E. coli[9]

$$\log 1/C = 0.60\pi - 1.89 \log (\beta \cdot 10^{\pi} + 1) + 2.84 \quad (17)$$

$$n = 66,\ r = 0.963,\ s = 0.344,\ \pi_0 = 5.86$$

Since in the examples of *Staphylococcus aureus* and *E. coli* cells entrance of the triazines into the cells is not mediated by active transport, it can be concluded that passive diffusion of antifolates into cells is characterized by a coefficient of about 0.5 with the π or log P term. This is also true in the case of eq 3 for triazines acting on resistant *L. casei* cells (0.45π) and on benzylpyrimidines acting on resistant tumor cells (0.56π).[11] Triazines acting on resistant tumor cells also show this same dependency on hydrophobicity.[2,13] This supports our earlier contention that the major reason hydrophilic antifolates are so much less effective against resistant *L. casei* and mammalian cells is the grossly impaired transport system in these cells.

The essentially identical QSAR for triazines acting on resistant and sensitive *E. coli* cells is what we expected to find for cells not having an active transport system. The different QSAR for the benzylpyrimidines acting on the two types of cells was quite unexpected as were the results with BW 301U and pyrimethamine.



Since the triazines and metoprine show the same activity against sensitive and resistant cells, it does not seem possible to explain the difference in activity of the benzylpyrimidines, methotrexate, BW 301U, or pyrimethamine by postulating some change in the composition of the cellular membranes.

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Table III. Parameters Used in the Derivation of Equations 13 and 15 for the Inhibition of Growth of *E. coli* Cells by Pyrimidines

		MB1417			MB1428				
		log $1/C$		Δ log $1/C$	log $1/C$		Δ log $1/C$		
no.	X	obsd	calcd[a]		obsd	calcd[b]		$\pi_{3,4,5}$	$MR'_{3,4,5}$
1	3,5-$(OCH_3)_2$,4-$OCH_2CH_2OCH_3$	5.83	6.24	−0.41	4.72	5.13	−0.41	−0.78	2.37
2	3,4,5-$(OCH_3)_3$	6.61	6.35	0.26	5.34	5.22	0.11	−0.52	2.37
3	3,5-$(OCH_3)_2$,4-SCH_3	6.97	6.53	0.44	5.66	5.41	0.25	0.02	2.37
4	3,5-$(OCH_3)_2$,4-Br	6.67	6.62	0.05	5.69	5.57	0.12	0.46	2.37
5	3,5-$(OCH_3)_2$,4-$C(CH_3){=}CH_2$	6.63	6.62	0.01	5.66	5.58	0.08	0.49	2.37
6	3,5-$(OCH_3)_2$,4-$O(CH_2)_7CH_3$	5.18	5.50	−0.32	*d*	*d*	*d*	3.80	2.37
7	3-OCH_2CH_3,4-$OCH_2C_6H_5$	5.04	5.69	−0.65	*d*	*d*	*d*	2.03	1.68
8	3-$O(CH_2)_7CH_3$,4-OCH_3	4.91	4.87	0.04	*d*	*d*	*d*	3.58	1.68
9	3,4-$(OCH_3)_2$	6.08	5.53	0.55	4.46	4.24	0.22	−0.58	1.68
10	3,5-$(OCH_3)_2$	6.08	5.74	0.34	4.55	4.45	0.10	0.02	1.68
11	3-OSO_2CH_3	4.59	4.59	0	3.35	3.18	0.17	−0.88	0.99
12	3-CH_2OCH_3	4.68	4.64	0.04	2.98	3.22	−0.24	−0.78	0.99
13	3-OH	4.35	4.12	0.23	2.94	2.56	0.38	−0.67	0.49
14	3-$OCH_2CH_2OCH_3$	4.33	4.84	−0.51	2.92	3.38	−0.46	−0.30	0.99
15	H	4.28	4.15	0.13	2.92	2.54	0.38	0	0.31
16	3-CH_3	4.91	4.78	0.13	3.29	3.36	−0.07	0.52	0.77
17	3-Cl	4.92	4.83	0.09	3.46	3.46	0.00	0.67	0.80
18	3-$O(CH_2)_5CH_3$	4.81	4.66	0.15	4.53	4.40	0.13	2.63	0.99
19	3-$O(CH_2)_6CH_3$	4.88	4.32	0.56	4.84	4.61	0.23	3.23	0.99
20	3-$O(CH_2)_7CH_3$	*d*	*d*	*d*	4.41	4.81	−0.40	3.79	0.99
21	4-$NHCOCH_3$	4.08	4.58	−0.50	3.03	3.17	−0.14	−0.91	0.99
22	4-$OCH_2CH_2OCH_3$	4.61	4.84	−0.23	3.11	3.38	−0.27	−0.30	0.99
23	4-NO_2	4.53	4.89	−0.36	3.43	3.43	0.00	0	0.95
24	4-OCH_3	5.24	4.88	0.36	3.46	3.42	0.04	−0.20	0.99
25	4-$N(CH_3)_2$	4.94	4.99	−0.05	3.46	3.57	−0.11	0.24	0.99
26	4-Br	5.12	5.06	0.06	3.69	3.79	−0.10	0.86	0.99
27	4-OCH_3	4.77	5.06	−0.29	3.70	3.85	−0.15	1.04	0.99
28	4-$O(CH_2)_3CH_3$	4.76	5.01	−0.25	4.01	4.03	−0.02	1.55	0.99
29	4-$O(CH_2)_5CH_3$	4.79	4.66	0.13	4.55	4.40	0.15	2.63	0.99
30	methotrexate	3.76			2.70				
31	BW 301U	5.95			4.56				
32	pyrimethamine	4.21			3.56				
33	metoprine	4.24			4.35				

[a] Calculated by using eq 13. [b] Calculated by using eq 15. [c] Not used in the derivation of eq 13. [d] I_{50} values could not be detd. due to solubility problems encountered with these congeners. [e] 2,4-Diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-*d*]pyrimidine

The difference in the activities of the pyrimidines might be attributed to a conformational change in the enzyme in the resistant cells. This could present a larger hydrophobic region for interaction of the large hydrophobic side chains. Also, it is conceivable that a slight change in the enzyme could result in a different binding mode of the pyrimidines by rotation around the axis formed by 3,6-positions. There is good evidence that methotrexate and folic acid, although very similar in structure, differ in binding by a similar rotation.[3,12] Whatever the cause, the evidence seems convincing that DHFR in the resistant cells has a geometry which is markedly different from that in sensitive cells or purified DHFR.

Yet another explanation for the different behavior of the triazines and the benzylpyrimidines is possible. It is conceivable that there are two different forms of DHFR present in the resistant *E. coli* cells. If for example the triazines showed equivalent potency against both forms, but the pyrimidines showed selectivity for the two forms, this could account for the difference in behavior of the two types of antifolates.

The structural features which determine whether or not an antifolate will recognize the difference in DHFR in methotrexate sensitive and resistant *E. coli* cells are not clear. Except for pyrimethamine, the antifolates which differentiate between the two systems all possess a flexible benzyl moiety. The rigid parent triazine structure as well as that of metoprine do not discriminate between the sensitive and resistant cells.

The major feature determining the inhibitory effect of the benzylpyrimidines is the steric effect reflected by MR′ which is quite similar in the isolated DHFR and in the sensitive and resistant cells. In the cell culture QSAR where interaction with membranes and other lipophilic sites in the cells become important, hydrophobic terms are essential. For the sensitive cells about 13% of the variance in log $1/C$ is rationalized by the two hydrophobic terms of eq 13, but for the resistant cells 23% of the variance is accounted for by the one term of eq 15. We should be able to capitalize on this characteristic of resistant cells and thus design more effective drugs for resistant cells.

In summary we can conclude that QSAR is an instrument of considerable importance in detecting differences between the modes of action of purified enzymes acting in vitro and enzymes acting in wild type or resistant cells. We believe that the insight gained via QSAR for antifolates will also be found with other types of drugs.

Experimental Section

Bacterial Cell Culture Growth Inhibition. The methotrexate sensitive (MB 1417) and methotrexate resistant (MB1428; ATCC 9637) strains of *Escherichia coli* B were obtained from Dr. Martin Poe of Merck & Co., Inc. The cultures were maintained on nutrient agar slants. The test inocula were prepared by twice transferring the cultures in test medium (a glucose, salts medium containing 0.6% vitamin free acid hydrolyzed casein), each transfer being incubated 24 h at 37 °C. A portion of the second transfer was centrifuged, washed twice with an equal quantity of test medium, and resuspended to a turbidity measuring 50 on a Klett photoelectric colorimeter (OD 0.1). This suspension was then diluted in test medium and 0.1 mL of a 10^{-5} dilution used to inoculate each tube of 10-mL final volume, thus providing an inoculum of approximately 15 cells/mL of test medium. Test compounds were dissolved in water or in 0.04 M HCl and diluted in sterile water. For inhibition studies, each tube contained 7.9 mL of basal test medium, 1 mL of separately sterilized 25% glucose solution, 1 mL of appropriately diluted inhibitor, and 0.1 mL of inoculum, giving the final volume of 10 mL. In some

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

no.	X	mp, °C	recryst solvent[a]	yield,[b] %	formula
6	3,5-$(OCH_3)_2$, 4-$O(CH_2)_7CH_3$	165–167[c]	A	17	$C_{21}H_{32}N_4O_3$
7	3-OCH_2CH_3, 4-$OCH_2C_6H_5$	170–172	A	20	$C_{20}H_{22}N_4O_2$[d]
8	3-$O(CH_2)_7CH_3$, 4-OCH_3	153–155	A	17	$C_{20}H_{30}N_4O_2$[d]

[a] A = 95% ethanol. [b] Yield based on starting benzaldehyde. [c] See ref 17, mp 163 °C. [d] Analyzed for C and H.

instances where amounts of inhibitor were limited, all components were adjusted to give final volumes of 3 mL.

Tubes containing basal medium, glucose, and test inhibitor were thoroughly mixed both before and after addition of inoculum and then incubated at 37 °C for 22 h. The turbidities were then read in a Klett photoelectric colorimeter (filter 660 nm). Growth inhibitions by a minimum of eight inhibitor concentrations in duplicate were used to compute 50% inhibition values via jackknife regression.

Synthesis. The syntheses of most of the benzylpyrimidines and triazines used in this study have been previously reported.[6,14,15] Two pyrimidines 6 and 7 were synthesized by using the procedure of Poe et al.[16] Pyrimidine 8 was prepared by the method of Stenbuck et al.[19] The melting points are uncorrected (Büchi capillary apparatus). Microanalyses are within 0.4% of the theoretical values, and thin-layer chromatography (Analtech fluorescent alumina plates) was used to assess the purity of the final products. The yields are shown in Table IV, which lists the products obtained and their physical properties.

Enzyme Assay. Our previously described procedure for assaying inhibitors with DHFR and determining the $K_{i\,app}$ and confidence intervals has been used in this work.[18]

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Registry No. I (X = H), 4022-58-6; I (X = 3-SO_2NH_2), 70579-32-7; I (X = 3-$CONH_2$), 70579-33-8; I (X = 3-CF_3), 1492-81-5; I (X = 3-F), 3850-94-0; I (X = 3-Cl), 13351-02-5; I (X = 3-I), 51012-14-7; I (X = 3-CN), 70743-55-4; I (X = 3-CH_3), 4038-60-2; I (X = 3-$(CH_2)_5CH_3$), 70650-60-1; I (X = 3-$C(CH_3)_3$), 70579-36-1; I (X = 3-$O(CH_2)_3CH_3$), 70606-63-2; I (X = 3-$O(CH_2)_{10}CH_3$), 70579-55-4; I (X = 3-$O(CH_2)_{11}CH_3$), 70579-29-2; I (X = 3-O-$(CH_2)_{13}CH_3$), 70579-52-1; I (X = $O(CH_2)_2OC_6H_4$-3′-CF_3), 70579-37-2; I (X = $OCH_2C_6H_5$), 70579-38-3; I (X = 3-$OCH_2C_6H_3$-3′,4′-Cl_2), 70579-39-4; I (X = 3-OCH_2-1-adamantyl), 87871-38-3; I (X = 3-$CH_2OC_6H_5$), 79508-78-4; I (X = 3-$CH_2OC_6H_4$-3′-Cl), 79508-79-5; I (X = 3-$CH_2OC_6H_4$-3′-CN), 79519-97-4; I (X = 3-$CH_2OC_6H_4$-3′-OCH_3), 79508-80-8; I (X = 3-$CH_2OC_6H_4$-3′-CH_2OH), 79508-81-9; I (X = 3-$CH_2OC_6H_4$-3′-CH_3), 79508-82-0; I (X = 3-$CH_2OC_6H_4$-3′-CH_2CH_3), 79508-83-1; I (X = 3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$), 79508-85-3; I (X = 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$), 79508-86-4; I (X = 3-$CH_2OC_6H_4$-3′-$NHCSNH_2$), 79508-87-5; I (X = 3-$CH_2OC_6H_4$-3′-$NHCONH_2$), 70579-43-0; I (X = 3-$CH_2SC_6H_5$), 80239-83-4; I (X = 3-$CH_2SC_6H_4$-3′-CH_3), 87739-85-3; I (X = 3-SO_2F), 19160-13-5; I (X = 4-Cl), 13351-02-5; II (X = 3,5-$(OCH_3)_2$, 4-$O(CH_2)_2OCH_3$), 53808-87-0; II (X = 3,4,5-$(OCH_3)_3$), 738-70-5; II (X = 3,5-$(OCH_3)_2$, 4-SCH_3), 68902-57-8; II (X = 3,5-$(OCH_3)_2$, 4-Br), 56518-41-3, II (X = 3,5-$(OCH_3)_2$, 4-$C(CH_3)$=CH_2), 69194-91-8; II (X = 3,5-$(OCH_3)_2$, 4-$O(CH_2)_7CH_3$, 78025-72-6; II (X = 3-OCH_2CH_3, 4-$OCH_2C_6H_5$), 98612-08-9; II (X = 3-O-$(CH_2)_7CH_3$, 4-OCH_3), 98612-09-0; II (X = 3,4-$(OCH_3)_2$), 5355-16-8; II (X = 3,5-$(OCH_3)_2$), 20344-69-8; II (X = 3-OSO_2CH_3), 77113-58-7; II (X = 3-CH_2OCH_3), 77113-57-6; II (X = 3-OH), 77113-55-4; II (X = 3-$O(CH_2)_2OCH_3$), 80416-29-1; II (X = H), 7319-45-1; II (X = 3-CH_3), 69945-56-8; II (X = 3-Cl), 69945-58-0; II (X = 3-O-$(CH_2)_5CH_3$), 77113-62-3; II (X = 3-$O(CH_2)_6CH_3$), 80407-62-1; II (X = 3-$O(CH_2)_8CH_3$), 98612-10-3; II (X = 4-$NHCOCH_3$), 69945-53-5; II (X = 4-$O(CH_2)_2OCH_3$), 80407-59-6; II (x = 4-NO_2), 69945-52-4; II (X = 3-OCH_3), 59481-28-6; II (X = 4-$N(CH_3)_2$), 69945-51-3; II (X = 4-Br), 69945-55-7; II (X = 4-OCH_3), 20285-70-5; II (X = 4-$O(CH_2)_3CH_3$), 77113-59-8; II (X = 4-$O(CH_2)_5CH_3$), 80407-61-0; DHFR, 9002-03-3; methotrexate, 59-05-2; BW 301, 72732-56-0; pyrimethamine, 58-14-0; metoprine, 7761-45-7.

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