Crystallography, Quantitative Structure–Activity Relationships, and Molecular Graphics in a Comparative Analysis of the Inhibition of Dihydrofolate Reductase from Chicken Liver and *Lactobacillus casei* by 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(substituted-phenyl)-*s*-triazines[1]

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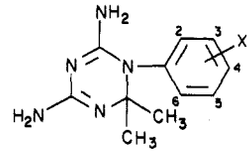
The inhibition of dihydrofolate reductase from chicken liver and from *Lactobacillus casei* has been studied with 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(substituted-phenyl)-*s*-triazines. It was found that for the chicken enzyme, inhibitor potency for 101 triazines was correlated by the following equation: $\log 1/K_{i\,app} = 0.85\sum\pi' - 1.04 \log (\beta \cdot 10^{\sum\pi'} + 1) + 0.57\sigma + 6.36$. The parameter π' indicates that for certain substituents, $\pi = 0$. In the case of the *L. casei* DHFR results, meta and para derivatives could not be included in the same equation. For 38 meta-substituted compounds, it was found that $\log 1/K_{i\,app} = 0.38\pi'_3 - 0.91 \log (\beta \cdot 10^{\pi'_3} + 1) + 0.71I + 4.60$ and for 32 para-substituted phenyltriazines $\log 1/K_{i\,app} = 0.44\pi'_4 - 0.65 \log (\beta^{\pi'_4} + 1) - 0.90\nu + 0.69I + 4.67$. In the *L. casei* equation, I is an indicator variable for substituents of the type $CH_2ZC_6H_4$-Y and $ZCH_2C_6H_4$-Y, where Z = O, NH, S, or Se. The parameter ν is Charton's steric parameter, which is similar to Taft's E_s. The mathematical models obtained from correlation analysis are compared with stereo color graphics models.

The rapid advances in biochemistry and the elucidation of the structures of many macromolecules using X-ray crystallography should make possible the design of effective enzyme inhibitors by rational means. An important concurrent development of help in such problems is the formulation of quantitative structure–activity relationships (QSAR).[3] The advantage of QSAR is that by probing an enzyme with a well-designed set of congeners, one can obtain information about the active-site region in terms of its hydrophobic, steric, and electronic requirements for ligand interaction.

The QSAR approach is very sensitive in that one can quantify differences in the free energy of binding of less than 0.5 kcal/mol. Also, QSAR is powerful in that various physiochemical interactions of differing parts of the ligand can be separated. Moreover, one can derive QSAR for the enzyme–ligand effect on cell cultures with the enzyme in its natural environment.[4,5] Practicing QSAR is somewhat like a blind man defining an object or a room by exploring it with his hands: eventually, a good image can be developed. The use of crystallography is somewhat like giving sight to the blind man: one can immediately "see" what sort of ligands would be foolish to make from, say, the steric or hydrophobic point of view and which ligands might be good bets to test.

Recent developments in three-dimensional computer graphics[6,7] and especially the technique of placing color-coded van der Waals surfaces on those parts of a macromolecule and/or ligand in which there is special interest have greatly augmented our ability to study ligand–macromolecule interactions. It is now possible to check QSAR-drawn conclusions by studying relevant crystallographic structures. Results with QSAR from the papain[8] hydrolysis of esters and the inhibition of bacterial dihydrofolate reductase (DHFR)[9–11] were correlated with stereochemical arguments based on molecular models constructed from crystallographic coordinates. In the present report we discuss the inhibition of DHFR from chicken liver and *L. casei* cells by triazines I, combining



I

QSAR and models constructed from X-ray diffraction data on the avian DHFR, the structure of which has been recently reported.[12]

The biochemistry of DHFR from various sources has been extensively studied,[13–15] and crystallographic structures have been established for enzymes from *Escherichia*

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Table I. Parameters Used to Derive Equations 3, 6, and 7 for the Inhibition of Chicken Liver Dihydrofolate Reductase by Triazines I

no.	X	log $1/K_i$ obsd[e]	log $1/K_i$ calcd	log $1/K_i$ calcd[c]	ν	σ	π'
1	H	6.69 (±0.04)	6.29[a] 6.49[b]	6.34	0.00	0.00	0.00
2	3-SO_2NH_2	5.00 (±0.03)	4.90[a]	5.08	0.00	0.46	−1.82
3	3-$CONH_2$	5.07 (±0.03)	5.07[a]	5.26	0.00	0.28	−1.49
4	3-$COCH_3$	5.56 (±0.01)	6.09[a]	6.11	0.00	0.38	−0.55
5	3-$COOCH_2CH_3$[d]	5.20 (±0.02)	7.04[a]	6.95	0.00	0.37	0.51
6	3-OH	5.57 (±0.01)	5.75[a]	5.86	0.00	0.12	−0.67
7	3-CF_3	7.01 (±0.05)	7.34[a]	7.23	0.00	0.43	0.88
8	3-F	6.79 (±0.08)	6.71[a]	6.65	0.00	0.34	0.14
9	3-Cl	7.36 (±0.11)	7.19[a]	7.09	0.00	0.37	0.71
10	3-BR	7.35 (±0.07)	7.30[a]	7.19	0.00	0.39	0.86
11	3-I	7.44 (±0.05)	7.39[a]	7.31	0.00	0.35	1.12
12	3-NO_2	6.95 (±0.03)	6.64[a]	6.52	0.00	0.71	−0.28
13	3-CN[d]	6.94 (±0.03)	6.23[a]	6.19	0.00	0.56	−0.57
14	3-$CH_2N(CH_3)_3{}^+Cl^-$[d]	3.79 (±0.04)	1.14[a]	1.93	0.00	0.40	−5.50
15	3-CH_3	7.08 (±0.07)	6.70[a]	6.73	0.00	−0.07	0.56
16	3-CH_2CH_3	7.00 (±0.10)	6.99[a]	7.03	0.00	−0.07	1.03
17	3-$(CH_2)_5CH_3$	7.12 (±0.07)	7.02[a]	7.13	0.00	−0.08	3.21
18	3-$(CH_2)_8CH_3$	6.53 (±0.05)	6.77[a]	6.83	0.00	−0.08	4.83
19	3-$(CH_2)_{11}CH_3$	6.38 (±0.05)	6.52[a]	6.52	0.00	−0.08	6.45
20	3-$C(CH_3)_3$	6.75 (±0.02)	7.14[a]	7.26	0.00	−0.10	1.98
21	3-DL-$CH(OH)C_6H_5$[d]	5.73 (±0.02)	6.71[a]	6.74	0.00	−0.04	0.54
22	3-OCH_3	6.41 (±0.02)	6.40[a]	6.41	0.00	0.12	0.00
23	3-OCH_2CH_3	6.47 (±0.07)	6.38[a]	6.40	0.00	0.10	0.00
24	3-$O(CH_2)_2CH_3$	5.92 (±0.03)	6.38[a]	6.40	0.00	0.10	0.00
25	3-$O(CH_2)_3CH_3$	6.20 (±0.03)	6.38[a]	6.40	0.00	0.10	0.00
26	3-$O(CH_2)_4CH_3$	6.28 (±0.03)	6.38[a]	6.40	0.00	0.10	0.00
27	3-$O(CH_2)_5CH_3$	6.30 (±0.04)	6.38[a]	6.40	0.00	0.10	0.00
28	3-$O(CH_2)_8CH_3$	6.55 (±0.05)	6.38[a]	6.40	0.00	0.10	0.00
29	3-$O(CH_2)_{10}CH_3$	6.56 (±0.03)	6.38[a]	6.40	0.00	0.10	0.00
30	3-$O(CH_2)_{11}CH_3$	6.38 (±0.03)	6.38[a]	6.40	0.00	0.10	0.00
31	3-$O(CH_2)_{12}CH_3$	6.48 (±0.04)	6.38[a]	6.40	0.00	0.10	0.00
32	3-$O(CH_2)_{13}CH_3$	6.50 (±0.05)	6.38[a]	6.40	0.00	0.10	0.00
33	3-$O(CH_2)_2OC_6H_5$	7.15 (±0.04)	7.30[a]	7.35	0.00	0.10	1.68
34	3-$O(CH_2)_2OC_6H_4$-3'-CH_3	7.02 (±0.05)	7.26[a]	7.33	0.00	0.10	2.56
35	3-$O(CH_2)_4OC_6H_5$	7.20 (±0.03)	7.24[a]	7.31	0.00	0.10	2.71
36	3-$O(CH_2)_4OC_6H_4$-3'-CF_3	7.54 (±0.03)	7.12[a]	7.16	0.00	0.10	3.59
37	3-$OCH_2C_6H_5$	6.93 (±0.02)	7.30[a]	7.35	0.00	0.10	1.66
38	3-$OCH_2C_6H_3$-3',4'-Cl_2	6.78 (±0.02)	7.30[a]	7.35	0.00	0.10	1.66
39	3-$OCH_2C_6H_4$-4'-$CONH_2$	7.05 (±0.03)	7.30[a]	7.35	0.00	0.10	1.66
40	3-OCH_2-1-adamantyl[d]	6.13 (±0.02)	7.11[a]	7.16	0.00	0.10	3.61
41	3-CH_2O-c-C_6H_{11}	7.19 (±0.04)	7.23[a]	7.27	0.00	0.06	1.43
42	3-$CH_2NHC_6H_3$-3',5'-$(CONH_2)_2$	6.98 (±0.04)	7.09[a]	7.09	0.00	0.06	1.00
43	3-$CH_2NHC_6H_4$-4'-SO_2NH_2	7.18 (±0.08)	7.09[a]	7.09	0.00	0.06	1.00
44	3-$CH_2OC_6H_5$	7.28 (±0.05)	7.27[a]	7.32	0.00	0.06	1.66
45	3-$CH_2OC_6H_4$-3'-Cl	7.18 (±0.04)	7.27[a]	7.32	0.00	0.06	1.66
46	3-$CH_2OC_6H_4$-3'-CN	7.59 (±0.05)	7.27[a]	7.32	0.00	0.06	1.66
47	3-$CH_2OC_6H_4$-3'-OCH_3	7.29 (±0.04)	7.27[a]	7.32	0.00	0.06	1.66
48	3-$CH_2OC_6H_4$-3'-CH_2OH	7.10 (±0.03)	7.27[a]	7.32	0.00	0.06	1.66
49	3-$CH_2OC_6H_4$-3'-CH_3	7.14 (±0.04)	7.27[a]	7.32	0.00	0.06	1.66
50	3-$CH_2OC_6H_4$-3'-CH_2CH_3	7.27 (±0.04)	7.27[a]	7.32	0.00	0.06	1.66
51	3-$CH_2OC_6H_4$-3'-$CH(CH_3)_2$	7.47 (±0.04)	7.27[a]	7.32	0.00	0.06	1.66
52	3-$CH_2OC_6H_4$-3'-$C(CH_3)_3$	7.24 (±0.06)	7.27[a]	7.32	0.00	0.06	1.66
53	3-$CH_2OC_6H_4$-3'-C_6H_5	6.79 (±0.08)	7.27[a]	7.32	0.00	0.06	1.66
54	3-$CH_2OC_6H_4$-3'-$NHCOCH_3$	7.64 (±0.05)	7.27[a]	7.32	0.00	0.06	1.66
55	3-$CH_2OC_6H_4$-3'-$NHCSNH_2$	7.22 (±0.03)	7.27[a]	7.32	0.00	0.06	1.66
56	3-$CH_2OC_6H_4$-3'-$NHCONH_2$	7.46 (±0.03)	7.27[a]	7.32	0.00	0.06	1.66
57	3-$CH_2OC_6H_4$-4'-$(CH_2)_4CH_3$	6.71 (±0.08)	7.27[a]	7.32	0.00	0.06	1.66
58	3-CH_2O-2-naphthyl	7.50 (±0.04)	7.27[a]	7.32	0.00	0.06	1.66
59	3-CH_2O-1-naphthyl	7.15 (±0.01)	7.27[a]	7.32	0.00	0.06	1.66
60	3-$CH_2SC_6H_5$	7.47 (±0.08)	7.26[a]	7.34	0.00	0.06	2.30
61	3-$CH_2SC_6H_4$-3'-CH_3	7.70 (±0.04)	7.26[a]	7.34	0.00	0.06	2.30
62	3-$CH_2SeC_6H_5$	7.70 (±0.10)	7.25[a]	7.33	0.00	0.06	2.37
63	3-$SCH_2C_6H_5$	7.52 (±0.04)	7.23[a]	7.32	0.00	0.03	2.30
64	3-$SCH_2C_6H_4$-4'-Cl	7.55 (±0.08)	7.23[a]	7.32	0.00	0.03	2.30
65	4-SO_2NH_2	4.70 (±0.03)	4.88[b]	4.82	0.99	0.00	−1.82
66	4-SO_2CH_3	5.25 (±0.02)	5.02[b]	4.98	0.99	0.00	−1.63
67	4-$CONH_2$	4.95 (±0.03)	5.20[b]	5.10	0.72	0.00	−1.49
68	4-$COCH_3$	5.69 (±0.04)	5.88[b]	5.89	0.72	0.00	−0.55
69	4-$COOCH_3$[d]	4.75 (±0.03)	6.05[b]	6.33	1.51	0.00	−0.01
70	4-$COOCH_2CH_3$[d]	4.45 (±0.05)	6.42[b]	6.74	1.51	0.00	0.51
71	4-OH	5.70 (±0.02)	5.91[b]	5.79	0.32	0.00	−0.67
72	4-NH_2	5.67 (±0.03)	5.49[b]	5.32	0.35	0.00	−1.23
73	4-$NHCOCH_3$[d]	4.69 (±0.02)	5.67[b]	5.54	0.39	0.00	−0.97

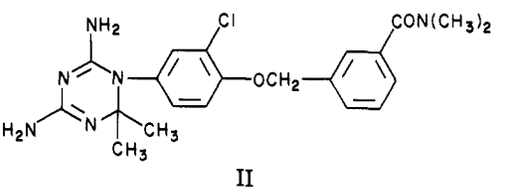
Table I (*Continued*)

no.	X	log $1/K_i$ obsd[e]	log $1/K_i$ calcd	log $1/K_i$ calcd[c]	ν	σ	π'
74	4-CF_3	6.77 (±0.03)	6.85[b]	6.98	0.91	0.00	0.88
75	4-F	6.89 (±0.05)	6.51[b]	6.45	0.27	0.00	0.14
76	4-Cl	6.95 (±0.04)	6.84[b]	6.88	0.55	0.00	0.71
77	4-Br	7.12 (±0.04)	6.92[b]	6.97	0.65	0.00	0.86
78	4-I	6.93 (±0.04)	7.05[b]	7.11	0.78	0.00	1.12
79	4-CN[d]	4.95 (±0.03)	5.96[b]	5.87	0.40	0.00	−0.57
80	4-OCH_2CO-c-$N(CH_2CH_2)_2O$[d]	6.77 (±0.05)	5.30[b]	5.18	0.62	0.00	−1.39
81	4-$O(CH_2)_2OC_6H_4$-4′-NH_2	6.76 (±0.03)	6.64[b]	6.69	0.61	0.00	0.45
82	4-CH_3	7.09 (±0.05)	6.74[b]	6.77	0.52	0.00	0.56
83	4-$(CH_2)_3CH_3$	7.38 (±0.05)	7.59[b]	7.31	0.68	0.00	2.13
84	4-$(CH_2)_8CH_3$	6.41 (±0.04)	6.42[b]	6.87	0.68	0.00	4.83
85	4-$C(CH_3)_3$	6.71 (±0.04)	7.38[b]	7.32	1.24	0.00	1.98
86	4-CCC_6H_5[d]	5.57 (±0.03)	7.64[b]	7.27	0.58[f]	0.00	2.65
87	4-OCH_3	6.48 (±0.04)	6.39[b]	6.34	0.36	0.00	0.00
88	4-$O(CH_2)_2CH_3$	5.90 (±0.02)	6.32[b]	6.34	0.58	0.00	0.00
89	4-$O(CH_2)_5CH_3$	6.46 (±0.04)	6.31[b]	6.34	0.61	0.00	0.00
90	4-$O(CH_2)_{10}CH_3$	6.03 (±0.07)	6.30[b]	6.34	0.65	0.00	0.00
91	4-$O(CH_2)_{11}CH_3$	6.50 (±0.11)	6.30[b]	6.34	0.65	0.00	0.00
92	4-$OCH_2C_6H_5$	7.53 (±0.05)	7.41[b]	7.29	0.65	0.00	1.66
93	4-$OCH_2C_6H_3$-3′,4′-Cl_2	7.14 (±0.08)	7.41[b]	7.29	0.65	0.00	1.66
94	4-$OCH_2C_6H_4$-4′-SO_2NH_2	7.49 (±0.07)	7.41[b]	7.29	0.65	0.00	1.66
95	4-$OCH_2C_6H_4$-4′-$CONH_2$	7.30 (±0.07)	7.41[b]	7.29	0.65	0.00	1.66
96	4-$OCH_2C_6H_4$-4′-CH_2OH	7.35 (±0.07)	7.41[b]	7.29	0.65	0.00	1.66
97	4-$CH_2SC_6H_5$	8.17 (±0.10)	7.58[b]	7.30	0.82	0.00	2.30
98	4-$CH_2SC_6H_4$-2′-CH_3	7.37 (±0.02)	7.58[b]	7.30	0.82	0.00	2.30
99	4-$CH_2SC_6H_4$-3′-CH_3	7.40 (±0.04)	7.58[b]	7.30	0.82	0.00	2.30
100	4-$SCH_2C_6H_5$	7.71 (±0.09)	7.48[b]	7.30	1.15	0.00	2.30
101	4-$SCH_2C_6H_4$-4′-Cl	7.13 (±0.06)	6.89[b]	7.30	1.15	0.00	2.30
102	4-C≡CH[d]	6.05 (±0.03)	6.61[b]	6.66	0.58[f]	0.00	0.40
103	4-$C{\equiv}CSi(CH_3)_3$[d]	5.99 (±0.03)	7.60[b]	7.32	0.58[f]	0.00	2.06
104	3-Cl,4-$OCH_2C_6H_4$-$CON(CH_3)_2$	7.01 (±0.05)	0.0	7.30	0.65	0.00	2.37
105	3-Cl,4-$(CH_2)_4C_6H_3$-2′-Cl,4-SO_2F	7.55 (±0.08)	0.0	6.81	0.70	0.00	5.13
106	3-SO_2NH_2, 4-Cl	5.66 (±0.04)	0.0	5.42	0.55	0.00	−1.11
107	3-OCH_3, 4-OCH_3	6.01 (±0.04)	0.0	6.34	0.36	0.00	0.00
108	3-NH_2, 4-C_2H_5	6.50 (±0.05)	0.0	6.17	0.56	0.00	−0.21
109	3-$CH_2SC_6H_5$, 4-Cl	7.58 (±0.08)	0.0	7.21	0.55	0.00	3.01
110	3-Cl, 4-$SCH_2C_6H_5$	7.40 (±0.07)	0.0	7.21	1.15	0.00	3.01
111	3-Cl, 4-$CH_2SC_6H_5$	7.33 (±0.02)	0.0	7.21	1.15	0.00	3.01
112	3-Cl, 4-OC_9H_{19}	6.46 (±0.06)	0.0	6.84	0.61	0.00	5.00
113	3,4-$(CH_2)_4$	7.72 (±0.08)	0.0	7.20	0.56	0.00	1.32
114	3,5-Cl_2	7.03 (±0.07)	0.0	7.23	0.00	0.00	1.42

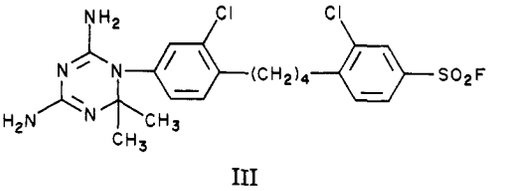
[a] Calculated by eq 3. [b] Calculated by eq 4. [c] Calculated by eq 7. [d] Not used in formulating eq 1–7. [e] Values in parentheses are for the construction of 95% confidence intervals. [f] ν for only the C≡CH group has been used. Since all three acetylenic congeners have almost the same log $1/K_i$ values, only the C≡C moiety must be involved in the steric effect.

coli[16–19] and *L. casei*[16,18,19] bacteria, as well as from chicken liver.[12] We have found that DHFR from a variety of sources yields good correlations with the QSAR method.[10,20–23] In addition to the theoretical interest in the mechanism of inhibitory action of I on DHFR, there is great practical interest in congeners I, since Baker's antifols (II and III) are active against a variety of tumors and are undergoing clinical studies. In fact, our present interest in triazines I stems in part from a study[23] of Baker's effort to find suitable derivatives of I for cancer chemotherapy.

QSAR for Chicken Liver DHFR. Dihydrofolate reductase is easily isolated from chicken liver, and this enzyme has been well characterized.[24] Using the parameters in Table I, we have derived eq 1–7. We initially factored the data in two sets: 3- and 4-substituted I. Then



II



III

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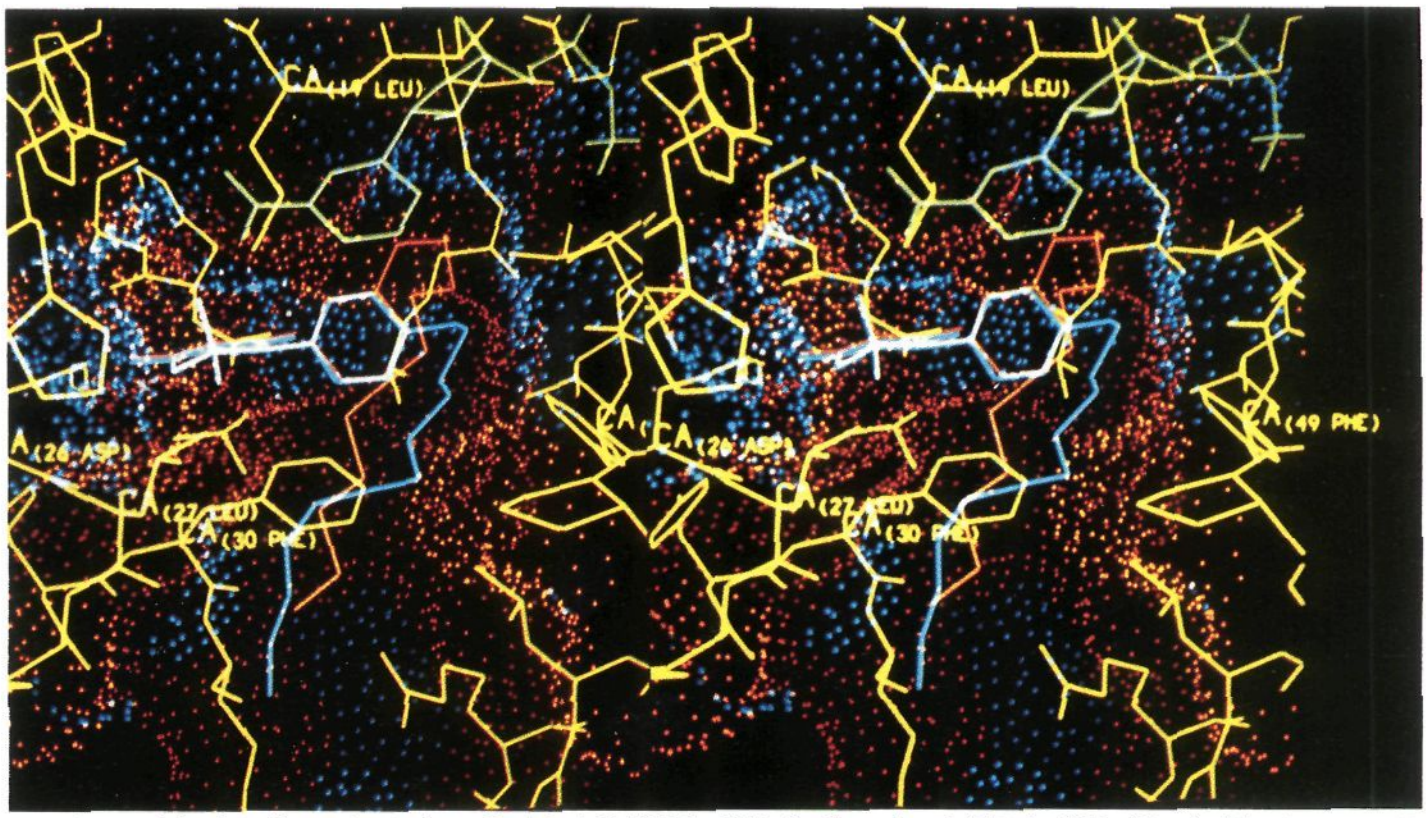

Figure 1. Comparison of the binding of triazines I with 3-O$(CH_2)_{10}CH_3$ (red) and 4-O$(CH_2)_{10}CH_3$ (blue) side chains to *L. casei* DHFR. The red dots indicate hydrophobic surface (carbon) and the blue dots indicate polar surface (oxygen and nitrogen). Part of the cofactor NADPH is shown in green.

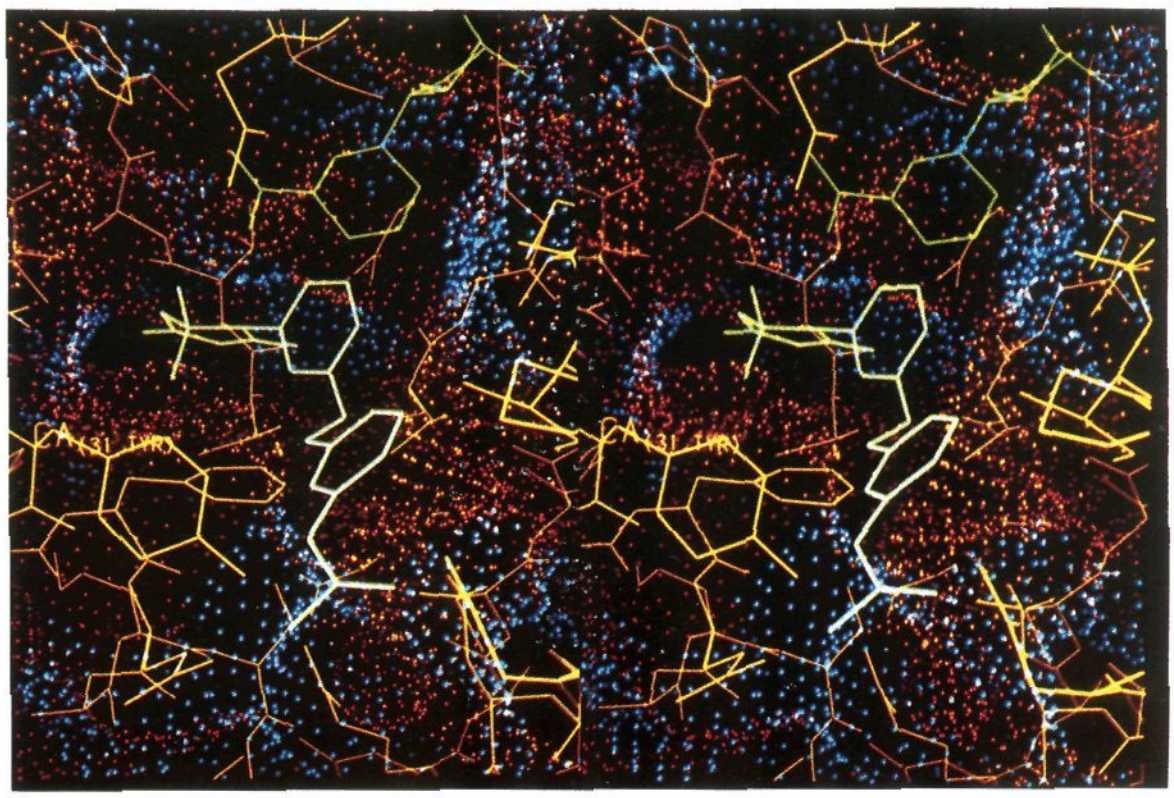

Figure 2. Triazine I, with 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$ in "wire model" form, binding to chicken liver DHFR. The color coding of the surface-defining dots is the same as in Figure 1. The pyridinyl moiety of NADPH is shown in green.

these two sets were combined along with some 3,4-disubstituted I to obtain a more general equation.

QSAR for 3-Substituted Triazines Inhibiting Chicken DHFR

$$\log 1/K_{i\,app} = 0.25\ (\pm 0.10)\ \pi'_3 + 6.58\ (\pm 0.18) \quad (1)$$

$$n = 59,\ r = 0.560,\ s = 0.508,\ F_{1,57} = 26.1$$

$$\log 1/K_{i\,app} = 0.91\ (\pm 0.13)\ \pi'_3 - 1.10\ (\pm 0.20) \log (\beta \cdot 10^{\pi'_3} + 1) + 6.48\ (\pm 0.10) \quad (2)$$

$$n = 59,\ r = 0.889,\ s = 0.286,\ \log \beta = -1.12,\ \pi_0 = 1.80\ (\pm 0.34),\ F_{2,55} = 62.5$$

$$\log 1/K_{i\,app} = 1.01\ (\pm 0.14)\ \pi'_3 - 1.16\ (\pm 0.19) \log (\beta \cdot 10^{\pi'_3} + 1) + 0.86\ (\pm 0.57)\ \sigma + 6.33\ (\pm 0.14) \quad (3)$$

$$n = 59,\ r = 0.906,\ s = 0.267,\ \log \beta = -1.08,\ \pi_0 = 1.89\ (\pm 0.36),\ F_{1,54} = 9.16$$

QSAR for 4-Substituted Triazines Inhibiting Chicken DHFR

$$\log 1/K_{i\,app} = 0.43\ (\pm 0.14)\ \pi'_4 + 6.32\ (\pm 0.23) \quad (4)$$

$$n = 32,\ r = 0.763,\ s = 0.542,\ F_{1,30} = 41.9$$

$$\log 1/K_{i\,app} = 0.81\ (\pm 0.12)\ \pi'_4 - 1.23\ (\pm 0.30) \log (\beta \cdot 10^{\pi'_4} + 1) + 6.37\ (\pm 0.13) \quad (5)$$

$$n = 32,\ r = 0.938,\ s = 0.301,\ \log \beta = -1.74,\ \pi_0 = 2.02,\ F_{2,28} = 34.7$$

$$\log 1/K_{i\,app} = 0.73\ (\pm 0.10)\ \pi'_4 - 1.40\ (\pm 0.31) \log (\beta \cdot 10^{\pi'_4} + 1) - 0.29\ (\pm 0.21)\ \nu + 6.49\ (\pm 0.18) \quad (6)$$

$$n = 32,\ r = 0.949,\ s = 0.280,\ \log \beta = -2.40,\ \pi_0 = 2.44,\ F_{1,27} = 5.26$$

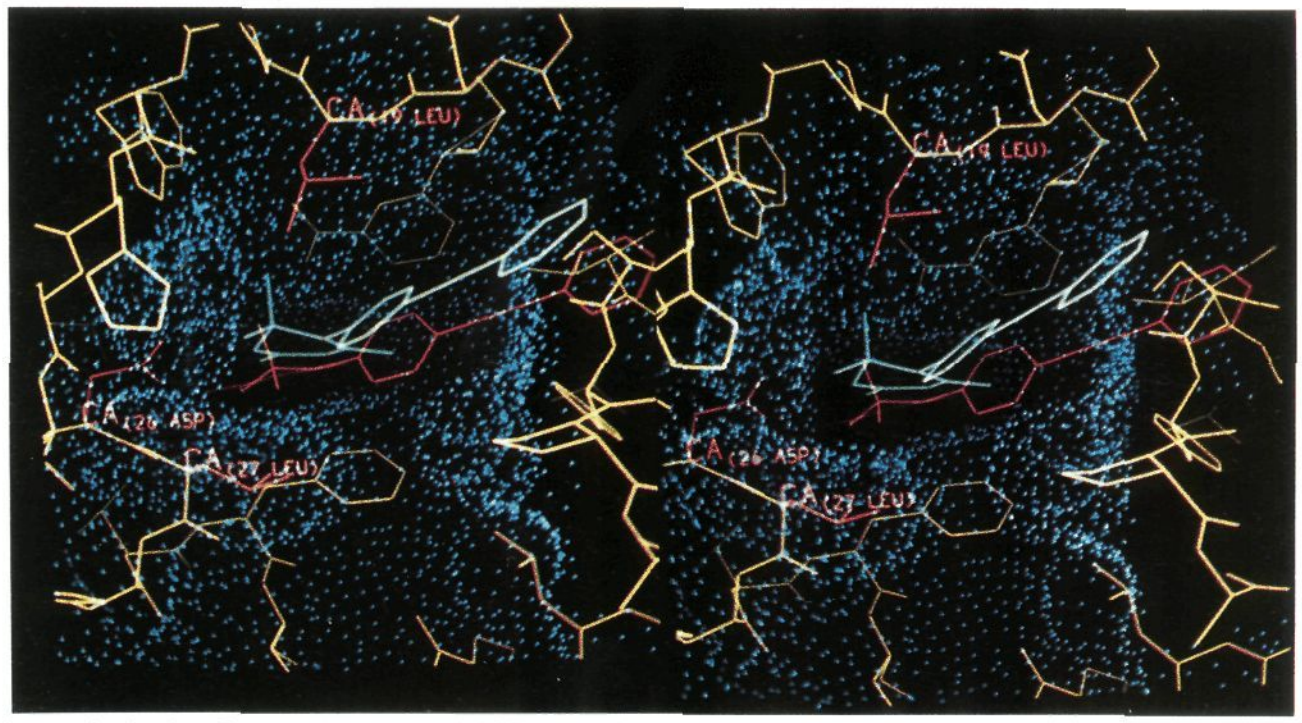

Figure 3. Two views of triazine I with the 4-C≡CC_6H_5 substituent bound to *L. casei* DHFR. The red version has its 2,4-diamino groups fitted by placing them in the X-ray crystallographic established positions for the corresponding groups in methotrexate. This forces the substituent through the blue surface of the enzyme. The blue version has had its 2,4-diamino groups pulled away from their "normal" binding position so as to avoid bad contact with the enzyme surface.

QSAR for 3- and 4-Substituted Triazines Inhibiting Chicken DHFR

$$\log 1/K_{i\,app} = 0.85\ (\pm 0.08)\ \Sigma\pi' - 1.04\ (\pm 0.14)\ \log\ (\beta \cdot 10^{\Sigma\pi'} + 1) + 0.57\ (\pm 0.49)\ \sigma + 6.36\ (\pm 0.09) \quad (7)$$

$$n = 101,\ r = 0.910,\ s = 0.294,\ \log \beta = -1.38,\ \pi_0 = 2.03\ (\pm 0.29)$$

$$\log 1/K_{i\,app} = 0.63\ (\pm 0.10)\ \Sigma\pi' - 0.88\ (\pm 0.21)\ \log\ (\beta \cdot 10^{\Sigma\pi'} + 1) + 0.88\ (\pm 0.78)\ \sigma + 6.21\ (\pm 0.14) \quad (7a)$$

$$n = 114,\ r = 0.791,\ s = 0.525,\ \pi_0 = 2.42\ (\pm 0.52),\ \log \beta = -2.02$$

In these equations n represents the number of data points used to derive the equation, r is the correlation coefficient, s is the standard deviation from the regression, and the figures in parentheses are for construction of the 95% confidence intervals. The disposable parameter β is obtained by an iterative procedure for the bilinear structure–activity model.[25] π' is the normal hydrophobic constant,[26] except for two types of substituents. For all 3-$O(CH_2)_nCH_3$ and 4-$O(CH_2)_nCH_3$, π is set equal to zero. This was done when it was discovered that regardless of the length of the alkoxy group, the effect on log $1/K_i$ was essentially constant. We initially assumed that this was the result of OR not making hydrophobic contact with the DHFR. Of course, the first member in this series, OCH_3, has a π constant of essentially zero (–0.02) and, therefore, is not expected to show a hydrophobic effect. For substituents of the type $CH_2ZC_6H_4$-Y, where Z = O, NH, S, or Se, π_Y is set equal to zero (i.e., $\pi_{CH_2ZC_6H_4\text{-}Y} = \pi_{CH_2ZC_6H_5}$). This same parameterization was also applied to groups of the type $ZCH_2C_6H_4$-Y, where Z = O, S. This technique was initiated when it was discovered that log $1/K_i$ was essentially constant for these congeners, regardless of whether Y was hydrophobic, hydrophilic, large, or small. It was thus assumed that Y did not make significant contact with the enzyme. We have encountered other examples of this kind in enzymic structure–activity studies[8,27] and found it necessary to set π_Y = O for correlation equations derived for DHFR from bovine, human, and *L. casei* sources. Evidence is now available from X-ray diffraction studies showing that our conclusion of Y not contacting the enzyme is valid (see below).

Equations 1–3 show the stepwise development of the QSAR of eq 3, and eq 4–6 show the corresponding development for eq 6. The coefficients with the π terms for the two classes of substituents are similar, although the coefficient with π in eq 6 is significantly smaller than that of eq 3. The major differences are that eq 3 contains a term in σ that is not significant for eq 6, and eq 6 contains a steric term (ν) that does not occur in eq 3. The σ term has been a source of concern to us, since it seems illogical to find this for 3-substituents but not for 4-substituents. To our knowledge there is no precedent for such a finding from the extensive studies that have been made of the Hammett equation and its many extended forms. If such a finding was limited to the present data set, one might be tempted to regard it as an artifact. However, in support of the finding of eq 3, we found σ terms in QSAR for 3-substituted triazines acting on DHFR from human, bovine, and murine sources. A possible explanation for electron withdrawal favoring inhibition by 3-substituents and having no effect with 4-substituents could be that the phenyl ring of 4-substituted compounds is in a slightly different type of enzymic space, although there is no direct evidence of this in the crystallographic results. Another explanation is that the correlation with σ reflects the negative electrostatic potential for those 3-substituents that are electron withdrawing. Localization of the σ effect at the 3-position may thus be due to a direct dipolar interaction with a region of positive electrostatic potential in the protein.

In eq 6 we find a small coefficient with Charton's steric parameter ν.[28] While this is not a major effect, it is significant (note F value), and from a study of the graphics

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it is apparent that 4-substituents contact Ile-60. We have also observed this effect with DHFR from other sources (see eq 14).

Since eq 3 and 6 are rather similar, we have combined the two data sets and included as well a number of 3,4-disubstituted congeners to obtain eq 7. In this combined equation, the significance of the ν term is lost because of the noise resulting from the less than perfect fit of the two data sets, which obscures the relatively small steric effect. The σ term applies only to 3-substituted triazines. It is assumed that the presence of a 4-substituent perturbs binding, so that the σ effect is not apparent for 3,4-disubstituted congeners. As expected, eq 7 is approximately an average of eq 3 and 6, with a slightly higher standard deviation than eq 6. Nevertheless, as can be observed in Table I, it does fit the disubstituted compounds reasonably well, even though it is based largely on monosubstituted congeners. The negative slope of the right-hand side of the bilinear model is greatest for eq 6 (0.73–1.40 = –0.67). Not much weight can be placed on this because there is only one data point with a π value greater than π_0 of 2.44. For this reason we cannot place confidence limits on π_0.

Equation 7a is based on all of the triazines in Table I.

While the alkyl groups show the expected rise in activity and then drop off with increasing chain length, congeners with alkoxy groups in both the 3- and 4-positions have essentially constant log $1/K_i$ values, regardless of chain length (up to $n = 13$).

The π_0 value of about 2 is essentially equivalent to π for four CH_2 units. X-ray crystallographic analysis of the ternary complex of NADPH–DHFR–3-$O(CH_2)_{10}CH_3$-I reveals that oxygen and the first three atoms in this side chain are tightly held by the DHFR and that the next two are loosely held. Electron density for the remaining seven atoms is not discernible, indicating that they are disordered and probably not in tight contact with the enzyme. All of the alkoxy side chains, both 3 and 4, have essentially the same log $1/K_i$ values. The mean value and standard deviation for the 11 3-OR congeners is 6.37 ± 0.19; for the five 4-OR congeners, the mean value and standard deviation for log $1/K_i$ is 6.27 ± 0.29. There is no trend for long OR groups to have slightly higher log $1/K_i$ values than short groups.

There is no doubt that at least the first four to five atoms of the OR groups make contact with the enzymes, since this is established for the 3-$O(CH_2)_{10}CH_3$ congener. However, all of the OR-containing congeners are a little less active than the parent compound (X = H), whose log $1/K_i$ is 6.7. Somehow the free energy of hydrophobic binding by the first atoms of these chains must be compensated by a steric effect or the increase in free energy caused by twisting the ether oxygen out of coplanarity with the phenyl ring. The pronounced differences in behavior of the alkyl groups (CH_3CH_2 > CH_3 > H) points to oxygen as being the source of the anomaly. Also, the forced desolvation of the ether oxygen upon binding to the hydrophobic pocket may increase the free energy of binding.

The following data points, which are poorly fit, were not included in the formulation of eq 1–7: 3-CN, 3-$COOC_2H_5$, 3-$CH(OH)C_6H_5$, 3-$CH_2N(CH_3)_3{}^+Cl^-$, 3-OCH_2-adamantyl, 4-CN, 4-$COOCH_3$, 4-$COOC_2H_5$, 4-$NHCOCH_3$, 4-C≡CH, 4-C≡CC_6H_5, 4-C≡$CSi(CH_3)_3$, and 4-OCH_2CO-c-N-$(CH_2CH_2)_2O$. Of these 13 derivatives, all except 3-CN, 4-OCH_2CO-c-$N(CH_2CH_2)_2O$, and 3-$CH_2N(CH_3)_3{}^+Cl^-$ are less active than expected. Two of these more active than expected congeners are interesting starting points for the development of more potent inhibitors. The 3-$CH_2N(CH_3)_3{}^+Cl^-$ is too inactive to be of interest.

Earlier[23] it was found in an analysis of Baker's study of the inhibition of tumor DHFR with triazines I that substituents with branching in X on the atom attached to the phenyl group of the parent structure (I) were less effective as inhibitors than one would expect from their π values alone. Five of the above badly fit congeners appear to be in this class—the three esters, 3-$CH(OH)C_6H_5$, and 4-$NHCOCH_3$. The 3-CN congener is about 10 times more active than expected and the 4-CN analogue is about 10 times less active than expected. This poor fit occurs with DHFR from other sources too.

In making the 3-$CH_2N(CH_3)_3{}^+Cl^-$ analogue it was not expected that it would be well fit by our correlation equations, since the partition coefficient of the ionic species depends heavily on the ionic strength of the solution. For comparative purposes, we have found it necessary in measuring P for ions to extrapolate to infinite dilution. Hence, the π value we have used for $CH_2N(CH_3)_3{}^+Cl^-$ is undoubtedly too negative. This congener is about 100 times more active than our equation projects. The derivative was prepared and tested out of curiosity to see if the charged group would completely destroy activity or if some unusual interaction might produce unexpectedly high activity. The observed log $1/K_i$ value (3.79) does not make this an interesting substituent for further study.

From a graphics study of the fit of the 3-methoxyadamantyltriazine to chicken DHFR, it was clear that bad contacts between this large group and the active site could not be avoided without some conformational adjustment in the protein. Nevertheless, the derivative was made and tested in order to see just how badly its K_i would be affected. The analogue is still quite active (6.1), although it is about 10 times less active than eq 7 predicts. This reduced activity suggests that the enzyme behaves in solution much like one would expect from the structure of the active site provided by X-ray crystallography obtained in the solid state; however, it also shows that there is enough flexibility in the enzyme in solution to accommodate the poor steric interactions within the "walls" of the active site. Other substituents (e.g., 3- and 4-COOR) also show that steric effects are not yes or no propositions.

Substituents of unusual interest are 4-C≡CC_6H_5 (log $1/K_i$ = 5.57), 4-C≡$CSi(CH_3)_3$ (log $1/K_i$ = 5.99), and 4-C≡CH (log $1/K_i$ = 6.05), which were not expected to be well predicted by the correlation equations. Although they are less active than eq 7 predicts, they are still quite active inhibitors. In the case of the chicken DHFR, it is clear from the graphics (see below) that if this congener binds with its amino groups occupying the positions established by X-ray crystallography for six different triazines bound to DHFR, the 4-C≡C groups would collide drastically with the enzyme in the region around Ile-60. This enzyme region must distort or the inhibitor must move from its position observed for other triazines.

Much evidence has accumulated in recent years suggesting that enzymes are more flexibile than the early concept of "lock and key fit" of enzyme and ligand led one to expect.[29-30] In fact, the coupling of enzymic chemical transformations to the thermal properties of the surrounding solution as proposed by current theories of biochemical transduction rests on the premise that a high degree of flexibility is present.[31] The idea of a lock and key fit with the lock made out of metal is a poor analogy. The lock (active site) would seem to be rather spongy.

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The poor fit of the 3-CN compound is a bothersome point, since this group has such modest steric demands. It has occurred to us that π for CN derived from the octanol/water system might not be a good parameter to correlate binding by enzymes. However, we have obtained good results using π_{CN} in other enzymic QSAR.[27,32] It is conceivable that the strongly polarized CN reacts with bound water, which does not show up at the present resolution of the crystallography.

Baker's antifol (II) is well fit by eq 7, but his second antifol (III) is about 5 times more active than expected. This antifol contains an SO_2F function that has been shown by Kumar et al.[33] to react with Tyr-31 in chicken DHFR. Despite its potential for an irreversible substitution reaction, antifol (III) is not as potent an inhibitor as a number of other triazines in Table I, which do not have the potential for covalent bond formation. It was quite clear from our earlier QSAR on triazines[23] that the positioning of the SO_2F function plays an important role in the inhibitory potency. The SO_2F moiety in antifol III is probably not *ideally* located for nucleophilic attack by DHFR.

A number of triazines in Table I that are more potent than either of Baker's antifols could be prime candidates for cancer chemotherapy. However, before such testing is attempted, we must get a clear picture of the role of overall hydrophobicity (log P_0) in the penetration of triazines to the active sites in the cell, as well as some knowledge of the structural features that make some triazines more effective against cells resistant to methotrexate. We are in the process of getting such information.[5]

Equation 7 correlates rather well 10 examples where there is 3,4-disubstitution on the phenyl ring of I.

Another point that requires further discussion is the negative slope with the right side of the bilinear models. Why does one find this drop in activity with substituents having superoptimal π?

If a large hydrophobic group extends partly beyond the enzyme into aqueous space one would expect a flat slope (i.e., 0) for dependence on π for that portion of the substituent in the aqueous phase. This is, in fact, what occurs with Y of $CH_2ZC_6H_4$-Y. The slope of the right-hand side of the bilinear model is usually slightly negative, indicating that activity actually drops with the addition of CH_2 units. A possible explanation for this might be the so-called ponderal effect suggested by Ingold;[35] that is, the part of the substituent extending beyond the enzyme would be loosely held by the aqueous solvent, and the vibrations of this mass could loosen the binding of that part of the inhibitor in contact with the enzyme. We have no explanation why OR groups do not show this same effect.

An obvious reason for the drop in activity with increasing values of π is that the hydrophobic pocket has limited bulk tolerance and as the substituent size increases, steric effects begin to supervene. This would not seem to apply to the alkyltriazines and DHFR, since the crystallography shows a rather broad unrestricted binding region for such groups. It is also possible that "wrong-way" binding could be promoted by greatly increasing the hydrophobicity of one portion of a ligand. There is good evidence for this kind of effect in the QSAR for chymotrypsin.[36,37]

QSAR for *L. casei* DHFR. One of the most interesting aspects of dihydrofolate reductase is that enzyme from different sources reacts in strikingly different ways with various inhibitors.[38,39] Triazines I have been shown to be more effective inhibitors of mammalian DHFR than of bacterial DHFR.[20,38] Some of the structural features of the triazines that account for this selectivity were delineated in our preliminary study[20] comparing bovine liver DHFR with that from *L. casei*. Testing with *L. casei* DHFR has now been extended for comparison with that from chicken liver. Using the parameters of Table II, we have derived eq 8–14 for comparison with eq 1–6.

QSAR for 3-Substituted Triazines Inhibiting *L. casei* DHFR

$$\log 1/K_{i\,app} = 0.26\ (\pm 0.14)\ \pi'_3 + 5.12\ (\pm 0.33) \quad (8)$$

$$n = 38,\ r = 0.539,\ s = 0.710,\ F_{1,36} = 14.7$$

$$\log 1/K_{i\,app} = 1.02\ (\pm 0.18)\ \pi'_3 - 1.22\ (\pm 0.26) \log (\beta \cdot 10^{\pi'_3} + 1) + 4.70\ (\pm 0.20) \quad (9)$$

$$n = 38,\ r = 0.897,\ s = 0.384,\ \pi_0 = 2.37\ (\pm 0.32),\ \log \beta = -1.69,\ F_{2,34} = 44.7$$

$$\log 1/K_{i\,app} = 0.83\ (\pm 0.13)\ \pi'_3 - 0.91\ (\pm 0.19) \log (\beta \cdot 10^{\pi'_3} + 1) + 0.71\ (\pm 0.20)\ I + 4.60\ (\pm 0.13) \quad (10)$$

$$n = 38,\ r = 0.961,\ s = 0.244,\ \pi_0 = 2.69\ (\pm 0.61),\ \log \beta = -1.68,\ F_{1,33} = 51.1$$

$$\log 1/K_{i\,app} = 0.74\ (\pm 0.15)\ \pi'_3 - 0.82\ (\pm 0.23) \log (\beta \cdot 10^{\pi'_3} + 1) + 0.71\ (\pm 0.25)\ I + 4.68\ (\pm 0.16) \quad (10a)$$

$$n = 39,\ r = 0.935,\ s = 0.397,\ \pi_0 = 2.71\ (\pm 0.71),\ \log \beta = -1.76$$

QSAR for 4-Substituted Triazines Inhibiting *L. casei* DHFR

$$\log 1/K_{i\,app} = 0.33\ (\pm 0.10)\ \pi'_4 + 4.18\ (\pm 0.25) \quad (11)$$

$$n = 32,\ r = 0.765,\ s = 0.613,\ F_{1,30} = 42.4$$

$$\log 1/K_{i\,app} = 0.58\ (\pm 0.14)\ \pi'_4 - 0.76\ (\pm 0.35) \log (\beta \cdot 10^{\pi'_4} + 1) + 4.14\ (\pm 0.20) \quad (12)$$

$$n = 32,\ r = 0.869,\ s = 0.487,\ \pi_0 = 3.77\ (\pm 0.65),\ \log \beta = -3.27,\ F_{2,28} = 9.74$$

$$\log 1/K_{i\,app} = 0.59\ (\pm 0.12)\ \pi'_4 - 0.80\ (\pm 0.30) \log (\beta \cdot 10^{\pi'_4} + 1) - 0.83\ (\pm 0.50)\ \nu + 4.73\ (\pm 0.40) \quad (13)$$

$$n = 32,\ r = 0.910,\ s = 0.416,\ \pi_0 = 3.72\ (\pm 0.48),\ \log \beta = -3.27,\ F_{1,27} = 11.5$$

$$\log 1/K_{i\,app} = 0.44\ (\pm 0.11)\ \pi'_4 - 0.65\ (\pm 0.41) \log (\beta \cdot 10^{\pi'_4} + 1) - 0.90\ (\pm 0.42)\ \nu + 0.69\ (\pm 0.37)\ I + 4.67\ (\pm 0.33) \quad (14)$$

$$n = 32,\ r = 0.941,\ s = 0.348,\ \pi_0 = 4.53\ (\pm 0.71),\ \log \beta = -4.22,\ F_{1,26} = 12.6$$

$$\log 1/K_{i\,app} = 0.26\ (\pm 0.09)\ \pi'_4 - 0.99\ (\pm 0.51)\ \nu + 1.11\ (\pm 0.40)\ I + 4.67\ (\pm 0.40) \quad (14a)$$

$$n = 37,\ r = 0.874,\ s = 0.461$$

The noise with all data points included in eq 14a is so great that the bilinear model no longer fits the data.

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Table II. Parameters Used to Derive Equations 8-14 for the Inhibition of *L. casei* Dihydrofolate Reductase by Triazines I

no.	X	log $1/K_i$ obsd[a]	log $1/K_i$ calcd	ν	I	π'
1	H	4.70 (±0.03)	4.59[b] 4.67[c]	0.00	0.00	0.00
2	3-SO_2NH_2	2.93 (±0.03)	3.09[b]	0.00	0.00	−1.82
3	3-$COCH_3$	4.24 (±0.02)	4.14[b]	0.00	0.00	−0.55
4	3-OH	3.85 (±0.03)	4.04[b]	0.00	0.00	−0.67
5	3-CF_3	4.77 (±0.04)	5.27[b]	0.00	0.00	0.88
6	3-F	4.88 (±0.04)	4.70[b]	0.00	0.00	0.14
7	3-I	5.18 (±0.02)	5.43[b]	0.00	0.00	1.12
8	3-NO_2	4.74 (±0.03)	4.36[b]	0.00	0.00	−0.28
9	3-CN[d]	5.31 (±0.06)	4.12[b]	0.00	0.00	−0.57
10	3-CH_3	4.96 (±0.03)	5.03[b]	0.00	0.00	0.56
11	3-CH_2CH_3	5.40 (±0.04)	5.36[b]	0.0	0.00	1.02
12	3-$(CH_2)_6CH_3$	5.99 (±0.04)	5.85[b]	0.0	0.00	3.21
13	3-$(CH_2)_8CH_3$	6.27 (±0.04)	5.73[b]	0.0	0.00	4.83
14	3-$(CH_2)_{11}CH_3$	5.67 (±0.06)	5.60[b]	0.0	0.00	6.45
15	3-OCH_3	4.52 (±0.04)	4.57[b]	0.0	0.00	−0.02
16	3-OCH_2CH_3	5.19 (±0.02)	4.89[b]	0.0	0.00	0.38
17	3-$O(CH_2)_2CH_3$	5.58 (±0.03)	5.38[b]	0.0	0.00	1.05
18	3-$O(CH_2)_5CH_3$	5.69 (±0.02)	5.86[b]	0.00	0.00	2.62
19	3-$O(CH_2)_8CH_3$	5.64 (±0.04)	5.77[b]	0.00	0.00	4.29
20	3-$O(CH_2)_{11}CH_3$	5.39 (±0.03)	5.64[b]	0.00	0.00	5.91
21	3-OCH_2-1-adamantyl	5.29 (±0.06)	5.82[b]	0.00	0.00	3.61
22	3-$O(CH_2)_2OC_6H_4$-4′-CF_3	5.78 (±0.03)	5.86[b]	0.00	0.00	2.56
23	3-$O(CH_2)_4OC_6H_4$-3′-CF_3	5.87 (±0.03)	5.82[b]	0.00	0.00	3.59
24	3-$OCH_2C_6H_5$	5.68 (±0.02)	5.70[b]	0.00	0.00	1.66
25	3-$OCH_2C_6H_3$-3′,4′-Cl_2	5.57 (±0.04)	5.70[b]	0.00	0.00	1.66
26	3-$OCH_2C_6H_4$-4′-$CONH_2$	5.90 (±0.03)	5.70[b]	0.00	0.00	1.66
27	3-CH_2O-c-C_6H_{11}	5.69 (±0.04)	5.60[b]	0.00	0.00	1.43
28	3-$CH_2NHC_6H_4$-4′-SO_2NH_2	5.95 (±0.04)	6.06[b]	0.00	1.00	1.00
29	3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	5.68 (±0.02)	6.06[b]	0.00	1.00	1.00
30	3-$CH_2OC_6H_5$	6.57 (±0.02)	6.41[b]	0.00	1.00	1.66
31	3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$	6.45 (±0.03)	6.41[b]	0.00	1.00	1.66
32	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	6.61 (±0.03)	6.41[b]	0.00	1.00	1.66
33	3-$CH_2OC_6H_4$-3′-CN	6.44 (±0.02)	6.41[b]	0.00	1.00	1.66
34	3-$CH_2OC_6H_4$-3′-CH_2CH_3	6.33 (±0.02)	6.41[b]	0.00	1.00	1.66
35	3-$CH_2OC_6H_4$-3′-C_6H_5	6.69 (±0.06)	6.41[b]	0.00	1.00	1.66
36	3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$	6.09 (±0.03)	6.41[b]	0.00	1.00	1.66
37	3-$CH_2SC_6H_5$	6.55 (±0.03)	6.56[b]	0.00	1.00	2.30
38	3-$SCH_2C_6H_5$	6.00 (±0.04)	5.85[b]	0.00	0.00	2.30
39	3-$CH_2SeC_6H_5$	6.76 (±0.03)	6.56[b]	0.00	1.00	2.37
40	4-SO_2NH_2	2.97 (±0.01)	2.99[c]	0.99	0.00	−1.82
41	4-SO_2CH_3	2.71 (±0.03)	3.07[c]	0.99	0.00	−1.63
42	4-$CONH_2$	3.18 (±0.03)	3.37[c]	0.72	0.00	−1.49
43	4-$COCH_3$	3.52 (±0.04)	3.79[c]	0.72	0.00	−0.55
44	4-$COOCH_3$	3.39 (±0.02)	3.31[c]	1.51	0.00	−0.01
45	4-$COOCH_2CH_3$	3.41 (±0.02)	3.54[c]	1.51	0.00	0.51
46	4-OH[d]	4.91 (±0.02)	4.09[c]	0.32	0.00	−0.67
47	4-NH_2	3.94 (±0.02)	3.82[c]	0.35	0.00	−1.23
48	4-$NHCOCH_3$	3.90 (±0.03)	3.43[c]	0.91	0.00	−0.97
49	4-CF_3	3.68 (±0.03)	4.24[c]	0.91	0.00	0.88
50	4-F	4.65 (±0.04)	4.49[c]	0.27	0.00	0.14
51	4-Cl	4.76 (±0.03)	4.49[c]	0.55	0.00	0.71
52	4-Br	4.57 (±0.02)	4.46[c]	0.65	0.00	0.86
53	4-I	4.43 (±0.02)	4.46[c]	0.78	0.00	1.12
54	4-CN	3.30 (±0.04)	4.06[c]	0.40	0.00	−0.57
55	4-$OCH_2CON(CH_2CH_2)_2O$	4.27 (±0.03)	3.50[c]	0.62	0.00	−1.39
56	4-$O(CH_2)_2OC_6H_4$-4′-NH_2	5.12 (±0.03)	4.32[c]	0.61	0.00	0.45
57	4-CH_3	4.17 (±0.03)	4.45[c]	0.52	0.00	0.56
58	4-$(CH_2)_3CH_3$	5.05 (±0.04)	4.99[c]	0.68	0.00	2.13
59	4-$(CH_2)_8CH_3$	5.79 (±0.02)	5.71[c]	0.68	0.00	4.83
60	4-$C(CH_3)_3$[d]	3.56 (±0.02)	4.42[c]	1.24	0.00	1.98
61	4-CCC_6H_5[d]	3.86 (±0.04)	5.30[c]	0.58[e]	0.00	2.65
62	4-OCH_3	4.10 (±0.03)	4.34[c]	0.36	0.00	−0.02
63	4-$O(CH_2)_5CH_3$	5.25 (±0.03)	5.26[c]	0.61	0.00	2.62
64	4-$O(CH_2)_{10}CH_3$	5.57 (±0.03)	5.66[c]	0.65	0.00	5.37
65	4-$O(CH_2)_{11}CH_3$	5.58 (±0.03)	5.57[c]	0.65	0.00	5.91
66	4-$O(CH_2)_{13}CH_3$	5.36 (±0.05)	5.35[c]	0.65	0.00	6.95
67	4-$OCH_2C_6H_4$-4′-SO_2NH_2	5.35 (±0.04)	5.51[c]	0.65	1.00	1.66
68	4-$OCH_2C_6H_4$-4′-$CONH_2$	5.63 (±0.05)	5.51[c]	0.65	1.00	1.66
69	4-$OCH_2C_6H_5$	5.19 (±0.02)	5.51[c]	0.65	1.00	1.66
70	4-$OCH_2C_6H_3$-3′,4′-Cl_2	5.66 (±0.01)	5.51[c]	0.65	1.00	1.66
71	4-$CH_2SC_6H_5$	5.61 (±0.03)	5.63[c]	0.82	1.00	2.30
72	4-$CH_2SC_6H_4$-3′-CH_3	5.55 (±0.02)	5.63[c]	0.82	1.00	2.30
73	4-$SCH_2C_6H_5$	5.64 (±0.02)	5.34[c]	1.15	1.00	2.30

Table II. (*Continued*)

no.	X	log $1/K_i$ obsd[a]	log $1/K_i$ calcd	ν	I	π'
74	4-C≡CH[d]	3.88 (±0.02)	4.33[c]	0.58[e]	0.00	0.40
75	4-C≡CSi$(CH_3)_3$[d]	3.81 (±0.03)	5.05[c]	0.58[e]	0.00	2.06

[a] Values in parentheses are for the construction of the 95% confidence interval. [b] Calculated by eq 10. [c] Calculated by eq 14. [d] These points not used in the formulation of eq 10 or 14, as the case may be. [e] ν for only the C≡CH group has been used. Since all three acetylenic derivatives have almost idential log $1/K_i$ values, only the C≡C moiety must be involved in the steric effect.

With avian DHFR, results for all triazines could be combined into the single eq 7, but with the *L. casei* DHFR, inhibition by 3- and 4-substituted triazines is so different that they cannot be included in a single equation.

The use of π' in eq 8–14 is the same as in eq 1–7, except that OR groups are assigned normal P values. In the case of the 3-substituted triazines, we have found it necessary to use an indicator variable (I), which takes the value of 1 for all congeners containing the $CH_2ZC_6H_4$-Y (Z = O, NH, S, Se) moiety. All other substituents, including the type $ZCH_2C_6H_4$-Y (Z = O, S) are assigned the value of zero for this parameter. The positive coefficient with I brings out the fact that these derivatives bind 5 times more tightly than expected from the correlation equation. The $CH_2ZC_6H_4$ moiety may bind in much the same way as the *p*-aminobenzoyl moiety of folic acid or methotrexate and, therefore, be particularly effective. It is surprising that this indicator variable does not occur in the QSAR for chicken DHFR, since we have found it in QSAR for DHFR from all other sources (human, murine, and bovine).

Equations 10a and 14a have been derived by using all data points in Table II.

Only one data point (3-CN) was not included in the derivation of eq 8–10. As with chicken DHFR, the derivative is about 10 times more active than eq 10 predicts. The 3-methoxyadamantyl analogue, although better fit by the *L. casei* equation than the chicken equation, is misfit by about twice the standard deviation, which makes it three times less active than expected. The dependence on π of the 3-substituted triazines acting on *L. casei* DHFR is similar to that with the chicken DHFR. Although π_0 appears to be somewhat higher (2.7 vs. 2.0), the confidence limits on these values are rather large, so that one cannot say there is a significant difference between them.

One of the most striking aspects of the triazines is their rather high inhibitory activity against vertebrate DHFR compared to bacterial DHFR. The difference of ~2 log units in the intercepts between eq 6 and 10 is a rough measure of the intrinsic difference in activity.

The 3-OR groups interact normally with *L. casei* DHFR and fit well using the standard P values. The same is true for the alkyl groups.

Equations 11–14 show the stepwise development of the best QSAR for 4-substituted triazines acting on *L. casei* DHFR. In these expressions, π' carries the same connotation as for eq 8–10, and I takes the value of 1 for two types of substituents, 4-$ZCH_2C_6H_4$ and 4-$CH_2ZC_6H_4$, where Z = O or S. One of the major differences for the 4-substituted triazines is that the QSAR contains the same steric parameter (ν) observed in eq 6 for the avian reductase. It is readily apparent by simple inspection that small substituents, such as the halogens, CH_3, OCH_3, etc., are distinctly less active in position 4 compared to position 3. From the graphics model of *L. casei* DHFR, it is obvious that there is a bad steric contact between the first one to two atoms attached to the 4-position and the Phe-49. Again we note the surprising behavior of the 4-C≡CC_6H_5 substituent, which is only 20 times less active than expected from eq 14 but which the graphics show makes a direct collision with Phe-49, if we assume that the 2,4-diaminotriazine ring must bind analogously to the corresponding atoms on the pteridine ring of methotrexate. The three acetylenic substituents 4-C≡CH, 4-C≡CC_6H_5, and 4-C≡CSi$(CH_3)_3$ all have essentially the same log $1/K_i$ values, and, hence, they appear to contact the enzyme only via the C≡C moiety. The rest of the substituent must project beyond the enzyme. Hence, for these compounds we have used ν for C≡CH. The simplest one of this set, 4-C≡CH, is about 3 times less active than expected. The others appear to be much less active than expected because we have used π for the whole substituent. However, if we had used π for only C≡C portion, then all three congeners would have been mispredicted by the same amount (~0.5). Since the steric constant of C≡CH is small, it makes a rather small correction for this class of substituents. These results suggest that the enzyme somehow adjusts rather easily to these groups, which is a phenomenon we had not anticipated from our graphics models.

Although there are more ν constants available from E_s, it was necessary to make some estimates for some of our substituents (see Table III).

The F statistic shows that eq 13 is a highly significant improvement over eq 12 even though the steric constants are not ideal.

We have found molar refractivity to be useful in modeling bulk steric effects in enzyme–ligand interactions.[8] If MR of the first two atoms of the 4-substituent attached to the phenyl ring is used in place of ν, a slightly poorer correlation is obtained. Thus, both the molecular modeling and the QSAR are in agreement on the nature of the steric effect of small or flexible 4-substituents.

The other major difference between the QSAR for 3- and 4-substituted triazines is in the hydrophobic interactions. The coefficient with π' in eq 14 is only half that of eq 10, and π_0 for eq 14 is almost 2 log units larger than that of eq 10. Insight on these problems can be obtained from the graphics models (see below).

Five 4-substituted triazine data points were omitted in the derivation of eq 11–14: 4-C$(CH_3)_3$, 4-OH, 4-C≡CH, 4-C≡CC_6H_5, and 4-C≡CSi$(CH_3)_3$. Equation 14a is based on all data points. There is not enough flexibility in DHFR to accommodate the *tert*-butyl group even after parameterizing its steric effect by ν. It is about 6 times less active than expected. The OH group is surprisingly about 6 times more active than expected. There does not appear to be any obvious means of interaction for it unless this might be through water bound to the enzyme.

An interesting comparison of our QSAR on *L. casei* DHFR and the results of Woolridge[40] on triazines I inhibiting the growth of *Staphylococcus aureus* is provided by eq 15. Equation 15 is based on a good selection of 3-

$$\log 1/C = 0.60\pi - 1.89 \log (\beta \cdot 10^{\pi} + 1) + 2.84 \quad (15)$$

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

(40) Wooldridge, D. R. H. *Eur. J. Med. Chem.* **1980**, *15*, 63.

Table III

unknown ν	known substituent	ν values[a]
SO_2NH_2	SO_3^-	0.99
SO_2CH_3	SO_3^-	0.99
$CONH_2$	$COCH_3$	0.72
$COOC_2H_5$	$COOCH_3$	1.51
$NHCOCH_3$	$NHCH(CH_3)_2$	0.91
$OCH_2CON(CH_2CH_2)_2O$	$OCH_2CH(CH_3)CH_2CH_3$	0.62
$OCH_2C_6H_4$-Y	$OCH_2C_6H_{11}$	0.65
$SCH_2C_6H_5$	$SCH_2CH(CH_3)CH_2CH_3$	1.15
$OCH_2CH_2OC_6H_5$	OC_8H_{17}	0.61

[a] Reference 28.

and 4-substituted triazines, although the set did not contain any of the type $CH_2ZC_6H_4$-Y. In this expression, C is the minimum inhibitory molar concentration. Equation 15 is similar to eq 14 for the 4-substituted triazines and eq 10 for the 3-substituted triazines. 3-Substituted and 4-substituted triazines fit equally well, indicating one mode of binding to the *S. aureus* DHFR in vivo.

In our own studies of triazines acting on *L. casei* in cell culture, we observed that purified enzyme in vitro acted differently than enzyme in the living cell.[4] We have also noted from a graphics QSAR study of *E. coli* and *L. casei* DHFR interacting with benzylpyrimidines that *L. casei* had distinctly different features from *E. coli*.[9] Hence, at this point one can not be sure exactly why a QSAR on cells may differ from one obtained on isolated enzyme.

Crystallography and Computer Graphics Analysis. Both the amino acid sequence[24] and the X-ray crystallographic structure[12] of chicken DHFR have been determined. Ternary complexes containing avian DHFR, NADPH, and the following variations of I have been studied crystallographically: 3-I, 4-I, 4-OCH_3, 3-CN, 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$, and 3-$O(CH_2)_{10}CH_3$. The coordinates obtained from studies have enabled us to construct three-dimensional color models using these computerized graphics. Attempts can now be made to interpret major features of our QSAR in terms of the crystallographic models.

Modeling the interaction of a ligand or drug with its receptor site is a very complex problem; exact, quantitative methods for constructing and evaluating such interactions do not exist at this time. It is well accepted that the forces important for intermolecular association are hydrophobic, van der Waals, hydrogen bonding, and electrostatic (ion-pairing) interactions. Unfortunately, the immense number of degrees of freedom in a macromolecule–ligand complex and the lack of an adequate representation of solvent make the modeling of intermolecular association extremely difficult. Thus, it is still a major theoretical problem to determine the optimum fit and interaction energy of a ligand into a receptor site of known structure.

The solution of this problem is an essential first step to the solution of the more general problem of finding the optimum ligand for a receptor site of known structure and predicting the binding affinities of ligands for this receptor.

A major difficulty in modeling intermolecular interactions is the overwhelming amount of complex structural information present within a macromolecule. For example, in trying to model the fit of an inhibitor into an enzyme active site, one is faced with the difficult problems of determining which portions of the site are most likely to contact the inhibitor and to find the best fit of the inhibitor into the site in a reasonable conformation without contacting atoms of the site too closely. A display that combines the standard wire molecular model with the molecular surface of the molecule(s) provides a much better feeling for the three-dimensional shape, topography, and chemistry of the molecule and has proven to be extremely powerful in modeling complex intermolecular interactions.[6-10] This technique is based on the definition of a molecular surface proposed by Richards[41] and developed into a computer program for the calculation of molecular surfaces by Connolly.[42] Instead of calculating van der Waals spheres and solving the hidden surface problem, the program calculates the surface that corresponds to the solvent-accessible surface. A probe sphere with the radius of a water molecule (1.4 Å) traverses the surface of the molecule; a dot is placed at each point of contact of the sphere with the molecule (contact surface) or the inward-facing surface of the sphere when it is simultaneously in contact with more than one atom (reentrant surface). The resulting model resembles a transparent CPK model, except that interstices too small to accommodate the probe are eliminated, and clefts between atoms are smoothed over. Projecting each surface point of a receptor site 1.4 Å along a vector normal to the surface produces a new "extended" surface, which corresponds to the original solvent-accessible surface with the additional space of approximately 1 van der Waals radius added to it. This extended surface is conceptually and visually much easier to use than the original surface, since it retains all the information inherent in the original surface (i.e., the atom to which each surface point belongs), but is much simpler, since reentrant surface points are eliminated (all reentrant points project to the center of the probe sphere which created them). The extended surface removes the need for consideration of the molecular surface of the ligand, since the extended surface defines an approximate van der Waals boundary or shell that the ligand atoms must fit inside of. Simple visual inspection of the extended surface and the "wire model" representation of the ligand is now sufficient to monitor van der Waals contacts and is much easier than monitoring the intersection or interpenetration of two complex standard molecular surfaces. After the fit of the ligand has been made, the extended surface is deleted.

Molecular models were constructed and displayed by the programs MIDAS and CHEM. These programs allow the user to change the location of different molecules relative to each other, adjust torsion angles, monitor interatomic distances, and display both the "wire" molecular models and the molecular surfaces of each molecule in color and time-slice stereo on an Evans and Sutherland Picture System 2 run by a VAX 11/750 computer.

The molecular models are not intended to provide the definitive structure for the enzyme–ligand complex. The goal is rather to generate reasonable structural models consistent with experimental results, which can then provide experimentally testable predictions of the activity of new analogues.

For the present study we have constructed models of the active sites of DHFR from three sources. For construction of the chicken liver DHFR active site, the following residues were used: Ser-6 to Val-10, Ile-16 to Gly-17, Lys-18 to Ser-39, Asn-48 to Trp-57, Ser-59 to Pro-66, Leu-67 to Asn-72, Trp-113 to Tyr-121, Thr-136, Ile-138, Asp-145, and Thr-146. For the *L. casei* DHFR model, we used the following residues: Leu-4 to Ala-6, Gly-17 to Leu-19, Trp-21 to Arg-31, Val-41 to Ala-57, Ala-97, HOH-201. For the *E. coli* DHFR, the following residues were used: Ile-5 to Ala-7, Ile-14 to Gly-15, Met-16 to Met-20, Trp-22 to

(41) Richards, F. M.; *Annu. Rev. Biophys. Bioeng.* **1977**, *16*, 151.
(42) This program by M. C. Connolly is available from QCPE.

Pro-25, Asp-27 to Ala-29, Trp-30 to Lys-32, His-45 to Thr-46, Ser-49 to Ile-50, Arg 52, Leu-54, Pro-55, Arg-57, Ile-94 to Gly-97, Tyr-100, and Thr-113.

Figure 1 compares two possible binding modes for the 3- and 4-$O(CH_2)_{10}CH_3$ moieties with *L. casei* DHFR. This model has been constructed by using the recently refined[43] coordinates for the binary complex of *L. casei* DHFR–methotrexate. Since the X-ray coordinates of triazines bound to *L. casei* DHFR have not yet been determined, this model was constructed by placing the nitrogen atoms in the 1-, 2-, 3-, and 4-positions of the triazines in the same positions occupied by the corresponding atoms of the pteridine ring of methotrexate. The surface on the enzyme has been color-coded red for hydrophobic and blue for polar (oxygen and nitrogen) atoms. The triazine with the meta OC_{11} side chain is colored red, while the para analogue is blue. The first atoms of the *p*-alkoxy group make a bad steric contact with Phe-49. This accounts for the steric term in eq 13 and 14. The *m*-alkoxy group can avoid this problem as illustrated; hence, no steric term appears in eq 8–10. Some 4-substituents are sterically rather demanding (4-SO_2NH_2, 4-$COOCH_2CH_3$, 4-$NHCOCH_3$) but are well fit when the ν parameter is included. Even including the ν parameter, the branched 4-$COOCH_2CH_3$ group is badly fit by the chicken DHFR correlation equation, indicating that Ile-60 in chicken DHFR is more sterically inhibiting than Phe-49 in *L. casei* DHFR.

A difficult problem to explain is the difference in the coefficients, with π of 0.83 for meta substituents in eq 10 and 0.44 for para substituents in eq 14. In the chicken DHFR equations both slopes are high, suggesting rather complete desolvation and similar interactions of both classes of substituents. At first it was though that collinearity between π'_4 and ν in eq 14 might produce this difference, but these parameters are completely orthogonal ($r^2 = 0.00$). Running correlations on the substituents divided into two sets (OR and R and $CH_2ZC_6H_4$-Y) did not change the result significantly. Hence, the difference seems real. Along with this is the difference in π_0 of 2.7 (±0.61) for eq 10 and 4.5 (±0.71) for eq 14. Since the 95% confidence limits on these parameters are rather large, the difference between them may not in fact be as large as it appears. Inspection of the log $1/K_i$ values for the 3- and 4-OR and -R groups shows that they, in fact, have similar cut-off points.

In Figure 1 we have shown two possible types of binding that the long alkyl chains might undergo. The red chain of 3-substituents has been placed rather high so that it goes between the two hydrophobic residues Leu-27 and Phe-49. The labels are placed on the α carbons of each of the residues. This makes a rather close fit, which would result in good desolvation of this part of the side chain. Thus, the coefficient of 0.83 with π' is reasonably near 1, the value we would expect for complete desolvation. In this model, part of the chain would project beyond the enzyme, and this could account for the lower π_0 value. The blue side chain of the 4-substituents after contact with Phe-49 has been constrained to drop down and follow the hydrophobic "floor" of the enzyme. This could account for the larger π_0 value for eq 14. At least the last part of the long 4-substituents would in this model be open to solvent on one side, and this less effective desolvation could account for the lower coefficient (0.44) with π in eq 14. It is conceivable that the steric interaction of the first atoms of the 4-substituents could cause a conformational change that would open up the channel so that more exposure of the complete side chain to solvent could occur. This would have to occur with both OR and $ZCH_2C_6H_4$-Y substituents, since both are fit by the same $0.44\pi'$ term plus the indicator variable for the $ZCH_2C_6H_4$-Y groups. The coefficient with this indicator variable is the same for eq 10 for 3-substituents, suggesting that this type of substituent has a similar and unusual interaction with the enzyme for both 3- and 4-substituents.

Although as yet we cannot give definitive answers to all of the questions raised by the QSAR and the graphics, the combined use of the two techniques is effective in spotting crucial molecules for X-ray crystallographic study when they are bound to the enzyme.

Gradually, cooperative studies should be able to greatly extend our understanding of ligand–enzyme interactions.

In Figure 2, the model of the 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$ inhibitor bound to chicken DHFR is shown. This model was built from coordinates obtained from the X-ray crystallography of the ternary complex of NADPH–DHFR–3-(methoxyphenyl)-3′-acetamidotriazine and, hence, gives us a definitive view of the region in which these substituents bind. The phenyl group is positioned between Tyr-31 and Ile-60, with the $NHCOCH_3$ group extending beyond hydrophobic space. Thus, crystallography confirms our QSAR finding that Y of $CH_2ZC_6H_4$-Y does indeed extend beyond the enzyme. The $NHCOCH_3$ group appears to be able to hydrogen bond with the OH of Tyr-31 which accounts for the fact that the activity of this congener is greater than any other member of the class 3-$CH_2OC_6H_4$-Y.

One of the most interesting substituents in the present study is 4-C≡C-X. These rigid groups were made and tested, even though they were expected to be poorly fit by our correlation equations, in order to obtain a better understanding of the fit of such substituents.

In Figure 3, two modes of binding to *L. casei* DHFR by the 4-C≡CC_6H_5-substituted triazine are shown. The red version was fitted by placing the amino groups of the triazine in the positions occupied by the amino groups of methotrexate, as established by X-ray crystallography. This forces the end phenyl group through the wall of the active site created by Phe-49. This, of course, would be a very unfavorable binding mode unless Phe-49 could move sufficiently to relieve the steric effect, which appears to be unlikely. The blue form of the 4-C≡CC_6H_5-substituted triazine has been fit into a channel opening to the aqueous phase so that there are no bad contacts between the ligand and the enzyme.

In order to achieve this, it is necessary to change considerably the position of the nitrogen atoms of the triazines from the methotrexate positions. The position of the side chains of two residues on the surface of the enzyme (Leu-17 and -27) were also adjusted, while the remainder of the enzyme was held rigid. Relaxation of the enzyme could allow the interaction of Asp-26 with the amino group of the inhibitor—only small motions in the peptide backbone and side chains appear to be necessary. This difference in fit of the two triazine units can be observed in Figure 3. The two models for binding of the triazine in Figure 3 represent extremes. In the blue model, the amino groups have been pulled away from their usual interaction with Asp-26.

Since the three examples of 4-C≡C-X all have the same log $1/K_i$ values, only the C≡C portion of the substituent contacts the enzyme; X projects into the aqueous phase. If we consider the difference between the observed and calculated log $1/K_i$ for C≡CH, we find the rather small

(43) (a) Bolin, J. T.; Filman, D. J.; Matthews, D. A.; Hamlin, R. C.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13650. (b) Filman, D. J.; Bolin, J. T.; Matthews, D. A.; Kraut, J. *Ibid.* **1982**, *257*, 13663.

value of 0.45, which is not much greater than the standard deviation of eq 14. The free-energy change in adjusting to the binding of the C≡C moiety is only about 0.5 kcal. Therefore, the enzyme easily accomodates the rigid ligand.

The great difference in the SAR of the 3- and 4-substituted triazines, which precludes the use of a single QSAR, is the most complex example of an enzyme–ligand interaction that we have so far encountered. Dihydrofolate reductase has a large and very complex active site and, in addition, has the added complexity of the need for a cofactor, which is known to enhance ligand binding. The relatively good binding of the rigid substituents C≡CR and the fact that 4-substituents do not show a huge drop in activity point to a flexibility in enzyme ligand interaction about which we have almost no understanding. The combination of QSAR and X-ray crystallography interpreted via molecular graphics show great promise for attacking such problems. It would appear that X-ray coordinates for a rather few well-selected examples of enzyme–inhibitor complexes would go far in defining the mode of binding for many others. Having a well-developed QSAR in hand before undertaking the selection of key cogeners for an X-ray crystallographic study would help avoid the collection of redundant information.

Conclusion

The present set of 114 triazines contains an extremely complex set of structural changes, so that there is no completely satisfactory way to parameterize them for the development of mathematical models. The flexibility of the substituents plus the adaptability of the enzyme for accepting them compound the problem. Thus, one cannot expect to obtain the sharp fit of data to correlation equations for macromolecules, which is currently possible for physical organic studies of reactions in homogeneous systems. Nevertheless, much valuable information can be obtained about the hydrophobic and steric requirements of an active site, which in most cases cannot as yet be defined by X-ray crystallography. It is interesting that the bad contact for the first atoms of the 4-substituents, which is clear from our graphics model, can be accounted for by a steric parameter as poorly designed for enzymes as ν. The regions of DHFR that we have deduced as being hydrophobic via QSAR are found to be so when models are constructed. The bilinear QSAR model works well to account for those portions of large substituents that extend beyond the enzyme.

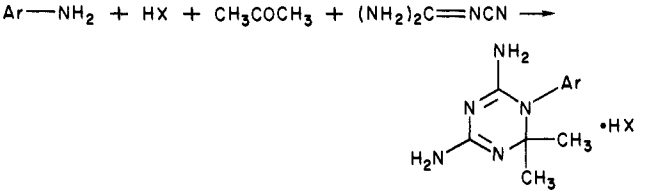
QSAR provides the chemist with a model and, in effect, a base line by which he can judge the general validity of his thinking with the results obtained from testing new congeners. QSAR models are built with full knowledge that sooner or later they can be shown to fail by simply making gross enough changes in the ligands. This does not mean that they are not valuable guides in conducting research for more effective ligands as well as characterizing the properties of the binding sites.

Once the character of the isolated macromolecular binding site is more or less established, then one can proceed with some confidence in attempting to deduce the nature of the receptor in the living cell.[4,5]

Experimental Section

Enzymatic Assay. The procedure for determining K_i and its confidence interval has been previously reported.[44]

Synthesis of Triazines. A number of the triazines used in our work have not been previously reported. The triazines were synthesized by the method of Modest,[45] by the following condensation:



The necessary aromatic amines generally were prepared from the corresponding nitro compounds, some of which have not been reported and are discussed below.

Aromatic Nitro Compounds. Method A. 3-[(Substituted-phenoxy)methyl]- or 3-[[(Substituted-phenyl)thio]methyl]nitrobenzenes. A suspension of the appropriate phenol or thiophenol (60 mmol), 3-nitrobenzyl chloride (60 mmol), and potassium carbonate (60 mmol) in acetone (200 mL) was heated at reflux for 24 h. The solids were filtered and the solvent was removed under reduced pressure. The residue was dissolved in ether (200 mL) and washed with water (2 × 100 mL), 10% sodium hydroxide (2 × 100 mL), and again with water (100 mL), and then dried ($MgSO_4$). The solvent was removed, and the crude products were either distilled or crystallized and then used in subsequent steps without further purification (Table IV).

Method B. A suspension of the appropriate phenols or thiophenols (60 mmol), the appropriate benzyl halides (60 mmol), and potassium carbonate (60 mmol) in acetone (200 mL) was heated at reflux for 24 h. The solvent was removed, and the residue was suspended in 100 mL of H_2O and acidified to pH 1 with concentrated HCl or H_2SO_4. The product was extracted with 2 × 200 mL of Et_2O. The ether layers were combined and dried ($MgSO_4$), and the solvent was removed to yield the crude products, which were recrystallized from an appropriate solvent and used without further purifcation (Table V).

4-[[4-(Hydroxymethyl)phenyl]methoxy]-1-nitrobenzene. The corresponding benzoic acid was reduced by the method of Brown and Subbarao:[46] yield 71%; mp 125–128 °C. Anal. ($C_{14}H_{13}NO_4$) C, H.

4-[(3-Nitrophenoxy)methyl]- and 4-[(4-Nitrophenoxy)methyl]benzamides. The corresponding benzoic acids (from method B, above; 20 mmol) were heated at reflux with 50 mL of $SOCl_2$ overnight. The excess $SOCl_2$ was removed under reduced pressure, and the crude acid chloride was dissolved in 30 mL of dioxane, and ice-cold concentrated NH_4OH (20 mL) was cautiously added with stirring. Stirring was continued for 1 h, and then the reaction added to 100 mL of water. The crude amides were filtered

Table IV

substituent	yield, %	mp (crystn solv) or bp (mmHg), °C
3-(4-*n*-pentyl-$C_6H_4OCH_2$)	51	220–225 (0.8)
3-(3-$C_6H_5C_6H_4OCH_2$)	81	133–135 (EtOH-Et_2O)
3-(3-$CH_3C_6H_4SCH_2$)	66	180–190 (0.3)

Table V

substituent	yield, %	mp (crystn solv), °C
3-(4′-$COOHC_6H_4CH_2O$)	92	215–220 (MeOH)
4-(4′-$COOHC_6H_4CH_2O$)	70	255–260 (EtOH-acetone)
4-(4′-$SO_2NH_2C_6H_4CH_2O$)	88	125–127 (EtOH-hexane)

Table VI

substituent	yield, %	mp, °C
4-[(3-nitrophenoxy)methyl]benzamide	79	168–172
4-[(4-nitrophenoxy)methyl]benzamide	74	220–223

(44) Dietrich, S. W.; Blaney, J. M.; Reynolds, M. A.; Jow, P. Y. C.; Hansch, C. *J. Med. Chem.* **1980**, *23*, 1205.

(45) Modest, E. J. *J. Org. Chem.* **1956**, *21*, 1.

(46) Brown, H. C.; Rao, B. C. S. *J. Am. Chem. Soc.* **1960**, *82*, 681.

Table VII

substituent	yield, %	mp, (crystn solv) °C
3-$SCH_2C_6H_5$	66	40-42 (EtOH-H_2O)
3-$SCH_2C_6H_4$-4'-Cl	74	71-73 (EtOH)
4-$SCH_2C_6H_4$-4'-Cl	58	119-121 (EtOH-acetone)

Table VIII

substituent	yield, %	bp (mmHg), °C
3-$CH{=}CH(CH_2)_6CH_3$	41	140-145 (0.3)
3-$CH{=}CH(CH_2)_5CH_3$	64	165-180 (0.1)

and crystallized from MeOH (Table VI).

Method C. 3- and 4-[[(Substituted-phenyl)methyl]-thio]-1-nitrobenzenes. Following the procedure of Overman et al.,[47] 3- or 4-nitrobenzenethiol was prepared from the appropriate disulfide (50 mmol). To the reaction solution were added potassium carbonate (100 mmol) and the appropriate benzyl halide (100 mmol), and the reaction mixture was heated at reflux overnight. The mixture was cooled to room temperature, poured into 100 mL of 10% NaOH, and then extracted with 2 × 400 mL of Et_2O. The Et_2O layers were combined and dried ($MgSO_4$), and the solvent was removed under reduced pressure. The residues were crystallized from an appropriate solvent, yielding the nitro compounds as yellow solids (Table VII).

Method D. 1-Nitro-3-[(phenylseleno)methyl]benzene. Diphenyl diselenide (12.0 g, 40 mmol) was dissolved in a mixture of EtOH (75 mL) and THF (75 mL). Sodium borohydride (3.07 g, 80 mmol) was added under an atmosphere of nitrogen to the well-stirred solution at a rate that kept the evolution of hydrogen gas from becoming too vigorous. After the solution was kept for 1 h at reflux temperature, *m*-nitrobenzyl chloride (12.0 g, 70 mmol) was added over a period of 15 min. After continuous heating at reflux temperature for 2 h, the solvent was removed under reduced pressure, and the oil was dissolved in ether (100 mL). The ethereal layer was washed with water (2 × 100 mL) and dried ($MgSO_4$). The solvent was removed to yield a crude yellow solid. The crude nitro compound was crystallized from MeOH–benzene and used without further purification: yield 12.6 g (61%); mp 59–60 °C.

Method E. 3- and 4-(1-Alkenyl)nitrobenzenes. A mixture of triphenylphosphine (26.3 g, 0.1 mol) and *m*-nitrobenzyl bromide (22.0 g 0.1 mol) was heated at reflux in dioxane for 1 h. The solution was cooled to room temperature and treated with a solution of NaOEt (0.1 mol) in EtOH (100 mL) dropwise. After the addition was complete, the dark reaction mixture was allowed to stir for 1 h and then treated with a solution of the appropriate aldehyde (octanal or undecanal, Aldrich Chemical Co., 0.1 mol) in 50 mL of dioxane. The reaction was allowed to stir overnight at room temperature. The solvent was removed, and the residue was redissolved in 1:1 $CHCl_3$–hexane and filtered through a pack of silica gel. The solvent was removed, the residue was suspended in pentane and then filtered, and the solids were washed with pentane. The pentane fractions were combined, the solvent was removed, and the residue was distilled under reduced pressure. The resulting products were not further purified, but directly used in the next step (Table VIII).

4-(1-Nonenyl)nitrobenzene was similarly prepared from triphenylphosphine, *p*-nitrobenzyl bromide, and octanal, using K_2CO_3 in MeOH as a base. The crude product from the workup was not distilled but used in the subsequent step without further purification: yield 57.4%.

Method E. (3-Nitrobenzyl)trimethylammonium Chloride. To 100 mL of 25% trimethylamine in MeOH (0.42 mol, Eastman Kodak) was added 3-nitrobenzyl chloride (35.44 g, 0.2065 mol), and the resulting suspension was heated at 50 °C for 1 h. Another 75 mL of 25% trimethylamine in methanol was added, and the reaction was heated at 50 °C overnight. The volume of the reaction was reduced to 75 mL via distillation, and the remaining solution was cooled to room temperature. The solution was diluted with Et_2O with vigorous stirring to a volume of 1.0 L and then refrigerated overnight. Filtration of the precipitated product yielded 45.25 g (95.0%, mp 201–204 dec). Crystallization of an analytical sample from MeOH–Et_2O yielded material melting at 204–205 °C dec. Anal. ($C_{10}H_{15}ClN_2O_2$) C, H, N.

(47) Overman, L. E.; Smoot, J.; Orerman, J. D. *Synthesis* **1974**, 59.

Table IX

substituent	yield, %	mp (crystn solvent), °C
3-$(CH_2)_8CH_3$	75	118-121 (Et_2O-hexane)
3-$(CH_2)_{11}CH_3$	59	114-116 (Et_2O-benzene)
4-$(CH_2)_8CH_3$	81	111-114 (EtOH-Et_2O)
3-Cl, 4-$O(CH_2)_8CH_3$	22	210-212 (EtOH-Et_2O)

Table X

X	Y	yield, %	mp (crystn solv), °C
H	H	54	210 dec (EtOH)
2-CH_3	H	19	184-186 (*i*-PrOH)
3-CH_3	H	22	175-177 (EtOH)
H	Cl	10	70-72 (free base, EtOH)

Table XI

substituent	yield, %	mp (recrystn solvent), °C
3-$CH_2SeC_6H_5$	90	180-181 (EtOH)
3-$SCH_2C_6H_5$	49	131-133 (EtOH)
3-$SCH_2C_6H_4$-4'-Cl	77	164-166 (MeOH)
3-$CH_2SC_6H_4$-3'-CH_3	41	109-112 (benzene-Et_2O)
3-$OCH_2C_6H_4$-4'-$CONH_2$	58	143-147 (free base, crude)
4-$OCH_2C_6H_4$-4'-SO_2NH_2	68	239-241 (MeOH-EtOH)
4-$OCH_2C_6H_4$-4'-$CONH_2$	54	246-248 (MeOH)
4-$OCH_2C_6H_4$-4'-CH_2OH	57	250-252 (MeOH-EtOH)
3-$CH_2OC_6H_4$-3'-Ph	66	152-153 (acetone-Et_2O)
3-$CH_2OC_6H_4$-4'-$(CH_2)_4CH_3$	61	142-143 (EtOH)
3-Cl, 4-$SCH_2C_6H_5$	71	60-62 (free base, EtOH-Et_2O)
3-$SCH_2C_6H_4$-4'-Cl	77	164-166 (MeOH)
4-$SCH_2C_6H_4$-4'-Cl	62	209-212 (MeOH-EtOH)

Anilines. Method G. The nitro compound was dissolved in 50–100 mL of absolute ethanol and hydrogen on a Parr low-pressure apparatus at an initial pressure of 50 psi. Uptake was complete in 1–2 h. The catalyst was filtered, excess ethanolic HCl was added, the solvent was removed, and the crude products were crystallized (Table IX).

Method H. 4-[[(Substituted-phenyl)thio]methyl]-3-substituted-anilines and -aniline Hydrochlorides. The 3-substituted aniline (0.1 mol) was added to concentrated HCl (8.6 mL, 0.1 mol). The (substituted-phenyl)thiophenol (0.1 mol), 37% formaldehyde (0.1 mol), and 50–100 mL of absolute ethanol were mixed and slowly added to the 3-substituted aniline hydrochloride suspension. The solution was then heated at reflux for 1 h. While the solution was cooling, some products crystallized and were recrystallized from an appropriate solvent. In the case of X = H and Y = Cl, the product did not crystallize, so the solvent was removed, the residue was dissolved in water, and the aqueous solution was basified slowly to pH 10 with 10% NaOH. This solution was extracted with 2 × 100 mL of Et_2O. The Et_2O layers were dried ($MgSO_4$), and the solvent was removed. The residue was crystallized to yield the amine (Table X).

Method J. The substituted nitro compound (8 g) and iron powder (30 g) were suspended in H_2O (200 mL). Acetic acid (1 mL) was added, and the reaction was stirred and heated at 80–90 °C for 2–10 h. The reaction was cooled, and 10% Na_2CO_3 (100 mL) was added. The reaction was filtered, and the solids were washed with hot benzene or hot ethyl acetate. The filtrate was extracted with 200 mL of ethyl acetate, the organic layers were combined and then dried ($MgSO_4$), and the solvent was removed

Table XII

X	yield, %	mp, (crystn solv), °C	formula
3,4-$(OCH_3)_2$	34	240.5-241.5 ($EtOH-H_2O$)	$C_{13}H_{19}N_5O_2 \cdot HCl$
3-NH_2, 4-CH_2CH_3	76	224-226.5 ($EtOH-H_2O$)	$C_{13}H_{20}N_6 \cdot HCl$
3-SO_2NH_2, 4-Cl	82	216-217 ($EtOH-H_2O$)	$C_{11}H_{15}ClN_6O_2S \cdot HCl$
3-Cl, 4-$CH_2SC_6H_5$	10	206-207 (EtOH)	$C_{18}H_{20}ClN_5S \cdot HCl$
3-Cl, 4-$O(CH_2)_8CH_3$	43	218-220 (EtOH)	$C_{20}H_{32}ClN_5O \cdot HCl$
3-Cl, 4-$SCH_2C_6H_5$	46	224-226 (MeOH)	$C_{18}H_{20}ClN_5S \cdot HCl$
3,5-Cl_2	25	206-208 (MeOH)	$C_{11}H_{13}Cl_2N_5 \cdot HCl$
3-OC_2H_5	53	219-220 (MeOH)	$C_{13}H_{19}N_5O \cdot HCl$
3-C_2H_5	36	207-208 ($MeOH-Et_2O$)	$C_{13}H_{19}N_5 \cdot HCl$
3-$(CH_2)_8CH_3$	55	193-194 (acetone-EtOH)	$C_{20}H_{33}N_5 \cdot HCl$
3-$(CH_2)_{11}CH_3$	64	193-196 (acetone-MeOH)	$C_{23}H_{39}N_5 \cdot HCl$
3-$CH_2SeC_6H_5$	72	179-180 (EtOH)	$C_{18}H_{22}N_5Se \cdot HCl$
3-$CH_2OC_6H_4$-3'-C_6H_5	54	215-216.5 ($EtOH-Et_2O$)	$C_{24}H_{25}N_5O \cdot HCl$
3-$CH_2OC_6H_4$-4'-C_5H_{11}	35	149-151 ($MeOH-Et_2O$-acetone)	$C_{23}H_{31}N_5O \cdot HCl$
3-$OCH_2C_6H_4$-4'-$CONH_2$	13	170-172 (MeOH)	$C_{19}H_{22}N_6O_2 \cdot HCl$
3-$SCH_2C_6H_5$	50	212-214 (MeOH)	$C_{18}H_{21}N_5S \cdot HCl$
3-$SCH_2C_6H_4$-4'-Cl	60	211-213 ($EtOH-MeOH$)	$C_{18}H_{20}ClN_5S \cdot HCl$
3-CH_2S-C_6H_4-3'-CH_3	61	193-196 ($EtOH-Et_2O$)	$C_{19}H_{23}N_5S \cdot HCl$
3-$CH_2N(CH_3)_3{}^+Cl^-$	13	218-219.5 (EtOH-acetone)	$C_{15}H_{25}ClN_6 \cdot HCl \cdot H_2O$
4-Cl	31	233-235 (MeOH-EtOH)	$C_{11}H_{14}ClN_5 \cdot HCl$
4-$NHCOCH_3$	19	243-246 (MeOH)	$C_{13}H_{18}N_6O \cdot HCl$
4-$(CH_2)_8CH_3$	52	212-214 (EtOH)	$C_{20}H_{33}N_5 \cdot HCl$
4-$OCH_2C_6H_4$-4'-SO_2NH_2	30	224-226 (MeOH-EtOH)	$C_{18}H_{22}N_6O_3S \cdot HCl$
4-$OCH_2C_6H_4$-4'-$CONH_2$	50	260-262 (MeOH-EtOH)	$C_{19}H_{22}N_6O_2 \cdot HCl$
4-$OCH_2C_6H_4$-4'-CH_2OH	21	237-238.5 (MeOH-EtOH)	$C_{19}H_{23}N_5O_2 \cdot HCl$
4-$SCH_2C_6H_5$	51	212-214 (MeOH)	$C_{18}H_{21}N_5S \cdot HCl$
4-$SCH_2C_6H_4$-4'-Cl	36	219-222 (MeOH)	$C_{18}H_{20}ClN_5S \cdot HCl$
4-$CH_2SC_6H_5$	83	216-218 (EtOH)	$C_{18}H_{21}N_5S \cdot HCl$
4-$CH_2SC_6H_4$-2'-CH_3	43	207-209 (EtOH)	$C_{19}H_{23}N_5S \cdot HCl$
4-$CH_2SC_6H_4$-3'-CH_3	77	215-218 (MeOH-EtOH)	$C_{19}H_{23}N_5S \cdot HCl$
4-$O(CH_2)_2CH_3$	67	199-201 (MeOH)	$C_{14}H_{21}N_5O \cdot HCl$
4-$O(CH_2)_{10}CH_3$	92	161-163 (H_2O)	$C_{22}H_{37}N_5O \cdot HCl$
4-$O(CH_2)_{11}CH_3$	76	189-190 (H_2O)	$C_{23}H_{39}N_5O \cdot HCl$
4-$O(CH_2)_5CH_3$	85	202-204 (EtOH-acetone-H_2O)	$C_{17}H_{27}N_5O \cdot HCl$
3-$O(CH_2)_2CH_3$	31	200-202 (EtOH)	$C_{14}H_{21}N_5O \cdot HCl$
3-$O(CH_2)_4CH_3$	8	196-198 (EtOH)	$C_{16}H_{25}N_5O \cdot HCl$
3-$O(CH_2)_5CH_3$	18	187-189 (EtOH)	$C_{17}H_{27}N_5O \cdot HCl$
4-CCC_6H_5	20	223-226 (MeOH)	$C_{18}H_{19}N_5O \cdot HCl$
4-C≡CH	38	216-217 (EtOH)	$C_{13}H_{15}N_5 \cdot HCl$
4-C≡$CSi(CH_3)_3$	62	218-220 (EtOH)	$C_{16}H_{23}N_5Si \cdot HCl$

under pressure. The residue was dissolved in ether, and HCl gas was passed through the solution to form the hydrochloride salts, except as noted in Table XI.

Method K. (3-Aminobenzyl)trimethylammonium Chloride. To a solution of $SnCl_2$ (108.7 g, 0.482 mol) in 740 mL of concentrated HCl, cooled to 5 °C, was added over a 5-min period (3-nitrobenzyl)trimethylammonium chloride (37.05 g, 0.161 mol). The reaction mixture was heated to 75 °C for 1 h, then allowed to cool to 30 °C, and then cooled to 5 °C with an ice bath. The mixture was then filtered, and the solids were washed sequentially with cold, concentrated HCl, cold 1:1 acetone–ether, and then ether to yield a silvery-white solid. This solid was dissolved in 500 mL of H_2O, and H_2S was bubbled through the solution for 1 h to precipitate the tin salts as the sulfides. The solution was filtered through Celite, and the solvent was removed under reduced pressure to yield a yellow syrup. Trituration of the syrup with anhydrous ethanol produced a white solid: mp 253–256 dec; yield 22.64 g (59.4%). Crystallization of an analytical sample from EtOH gave material melting at 256–257 °C dec. Anal. ($C_{10}H_{17}ClN_2 \cdot HCl$) C, H, N.

Triazines. A mixture of the substituted aniline hydrochloride (1.0 equiv) and dicyandiamide (1.05 equiv) was heated at reflux in acetone for 24 h. The solvent was removed, and the residue was crystallized. In the case of aniline free base, 1.0 equiv of concentrated HCl was added to the reaction mixture. All compounds analyzed correctly for C and H (Table XII).

4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[3-[(cyclohexyloxy)methyl]phenyl]-*s*-triazine Hydrochloride. Mercuric oxide (4.3 g, 20.0 mmol) was suspended in $HClO_4$ (6 mL) and heated on a steam bath until it dissolved. Upon cooling to room temperature, cyclohexanol (25 mL), *m*-nitrobenzyl bromide (4.3 g, 20 mmol), and 1,2-dimethoxyethane (20 mL) were added in that order. The reaction was allowed to stir at room temperature for 12 h. The reaction was filtered through Celite and washed with Et_2O (200 mL). The Et_2O layer was extracted with 6 × 100 mL of H_2O and dried ($MgSO_4$), and the solvent was removed to yield an oil, which was 3-[(cyclohexyloxy)methyl]-1-nitrobenzene and cyclohexanol. The oil was dissolved in 100 mL of absolute EtOH and hydrogenated on a Parr apparatus, using 200 mg of 5% Pd/C. Uptake of H_2 was complete in 30 min. The catalyst was filtered, concentrated HCl (4 mL) was added, and the solvent was removed under reduced pressure to yield the aniline hydrochloride as a red oil, which resisted crystallization. Dicyandiamide (1.7 g, 20.0 mmol) and acetone (150 mL) were added to the oil, and the reaction was heated at reflux for 16 h. The solvent was removed, and the residue was chromatographed over silica gel. Elution with acetone removed less polar material, and the triazine was eluted with 1:1 acetone–MeOH. The appropriate fractions were combined, and the solvent was removed to yield a brown solid. Trituration of the solid with CH_3CN yielded the triazine as a tan solid: mp 174–177 °C; yield 2.0 g (27.3%). Anal. ($C_{18}H_{27}N_5O \cdot HCl$) C, H.

Acknowledgment. We thank Arleen Keller for the synthesis of triazine I, where X = 3-$SCH_2C_6H_5$.

Registry No. I (X = H), 1931-17-5; I (X = 3-SO_2NH_2), 10441-22-2; I (X = 3-$CONH_2$), 70743-45-2; I (X = 3-$COCH_3$), 70743-49-6; I (X = 3-$COOC_2H_5$), 70743-46-3; I (X = 3-OH), 70743-47-4; I (X = 3-CF_3), 1961-32-6; I (X = 3-F), 70743-51-0; I (X = 3-Cl), 1931-19-7; I (X = 3-Br), 4514-52-7; I (X = 3-I), 70743-57-6; I (X = 3-NO_2), 4514-45-8; I (X = 3-CN), 20315-54-2; I (X = 3-$CH_2N(CH_3)_3{}^+Cl^-$), 87739-86-4; I [X = 3-$CH_2N(CH_3)_3{}^+Cl^- \cdot HCl$], 87740-35-0; I (X = 3-$CH_3$), 4514-48-1; I (X = 3-$C_2H_5$), 87761-52-2; I (base; X) = (3-C_2H_5), 87739-76-2; I [X = 3-$(CH_2)_5CH_3$], 65972-40-9; I [X = 3-$(CH_2)_8CH_3$], 87761-53-3; I [base; X = 3-$(CH_2)_8CH_3$], 87739-77-3; I [X = 3-$(CH_2)_{11}CH_3$], 87761-54-4; I [base; X = 3-$(CH_2)_{11}CH_3$], 87739-78-4; I [X = 3-$C(CH_3)_3$], 70743-52-1; I [X = 3-*dl*-$CH(OH)C_6H_5$], 70743-48-5; I (X = 3-

OCH_3), 17399-10-9; I (X = 3-$O(C_2H_5)$, 87761-55-5; I (base; X = 3-OC_2H_5), 46985-98-2; I [X = 3-$O(CH_2)_2CH_3$], 14052-88-1; I [base; X = 3-$O(CH_2)_2CH_3$], 14052-49-4; I [X = 3-$O(CH_2)_3CH_3$], 70743-54-3; I [X = 3-$O(CH_2)_4CH_3$], 87761-56-6; I [base; X = 3-$O(CH_2)_4CH_3$], 87739-98-8; I [X = 3-$O(CH_2)_5CH_3$], 65972-33-0; I [base; X = 3-$O(CH_2)_5CH_3$], 74798-21-3; I [X = 3-$O(CH_2)_8CH_3$], 65972-41-0; I [X = 3-$O(CH_2)_{10}CH_3$], 65972-65-8; I [X = 3-$O(CH_2)_{11}CH_3$], 65972-35-2; I [X = 3-$O(CH_2)_{12}CH_3$], 87761-57-7; I [X = 3-$O(CH_2)_{13}CH_3$], 65972-43-2; I [X = 3-$O(CH_2)_2OC_6H_5$], 17399-15-4; I [X = 3-$O(CH_2)_2OC_6H_4$-3′-CH_3], 87761-58-8; I [X = 3-$O(CH_2)_4OC_6H_5$], 70743-60-1; I [X = 3-$O(CH_2)_4OC_6H_4$-3′-CF_3], 70743-53-2; I (X = 3-$OCH_2C_6H_5$, 70743-58-7; I (X = 3-$OCH_2C_6H_3$-3′,4′-Cl_2), 70743-61-2; I (X = 3-$OCH_2C_6H_4$-4′-$CONH_2$), 87761-59-9; I (base; X = 3-$OCH_2C_6H_4$-4′-$CONH_2$), 87739-82-0; I (X = 3-OCH_2-1-adamantyl), 87761-60-2; I (X = 3-CH_2O-c-C_6H_{11}), 87761-61-3; I [X = 3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$], 70743-50-9; I (X = 3-$CH_2NHC_6H_4$-4′-SO_2NH_2), 87761-62-4; I (X = 3-$CH_2OC_6H_5$), 17399-11-0; I (X = 3-$CH_2OC_6H_4$-3′-Cl), 80555-74-4; I (X = 3-$CH_2OC_6H_4$-3′-CN), 80555-75-5; I (X = 3-$CH_2OC_6H_4$-3′-OCH_3), 80555-76-6; I (X = 3-$CH_2OC_6H_4$-3′-CH_2OH), 80555-77-7; I (X = 3-$CH_2OC_6H_4$-3′-CH_3), 80555-78-8; I (X = 3-$CH_2OC_6H_4$-3′-C_2H_5), 80555-79-9; I [X = 3-$CH_2OC_6H_4$-3′-$CH(CH_3)_2$], 80555-80-2; I [X = 3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$], 80555-81-3; I (X = 3-$CH_2OC_6H_4$-3′-C_6H_5), 87739-36-4; I (base; X = 3-$CH_2OC_6H_4$-3′-C_6H_5), 87739-80-8; I (X = 3-$CH_2OC_6H_4$-3′-$NHCOCH_3$), 80555-82-4; I (X = 3-$CH_2OC_6H_4$-3′-$NHCSNH_2$), 80555-83-5; I (X = 3-$CH_2OC_6H_4$-3′-$NHCONH_2$), 70743-62-3; I [X = 3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$], 87739-37-5; I [base; X = 3-$CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$], 87739-81-9; I (X = 3-CH_2O-2-naphthyl), 87739-38-6; I (X = 3-CH_2O-1-naphthyl), 87739-39-7; I (X = 3-$CH_2SC_6H_5$), 87739-40-0; I (X = 3-$CH_2SC_6H_4$-3′-CH_3), 87739-41-1; I (base; X = 3-$CH_2SC_6H_4$-3′-CH_3), 87739-85-3; I (X = 3-$CH_2SeC_6H_5$), 87739-42-2; I (base; X = 3-$CH_2SeC_6H_5$), 87739-79-5; I (X = 3-$SCH_2C_6H_5$), 87739-43-3; I (base; X = 3-$SCH_2C_6H_5$), 87739-83-1; I (X = 3-$SCH_2C_6H_4$-4′-Cl), 87739-44-4; I (base; X = 3-$SCH_2C_6H_4$-4′-Cl), 87739-84-2; I (X = 4-SO_2NH_2), 77113-78-1; I (X = 4-SO_2CH_3), 77113-80-5; I (X = 4-$CONH_2$), 77113-77-0; I (X = 4-$COCH_3$), 77113-83-8; I (X = 4-$COOCH_3$), 77113-79-2; I (X = 4-$COOC_2H_5$), 4514-44-7; I (X = 4-OH), 77113-82-7; I (X = 4-NH_2), 77113-81-6; I (X = 4-$NHCOCH_3$), 87739-45-5; I (base; X = 4-$NHCOCH_3$), 74798-27-9; I (X = 4-CF_3), 5151-53-1; I (X = 4-F), 77113-85-0; I (X = 4-Cl), 152-53-4; I (base; X = 4-Cl), 516-21-2; I (X = 4-Br), 65972-46-5; I (X = 4-I), 65972-47-6; I (X = 4-CN), 87739-46-6; I (X = 4-$OCH_2CON(CH_2CH_2)_2O$), 87739-47-7; I [X = 4-$O(CH_2)_2OC_6H_4$-4′-NH_2], 77113-84-9; I (X = 4-CH_3), 65972-48-7; I [X = 4-$(CH_2)_3CH_3$], 4479-25-8; I [X = 4-$(CH_2)_8CH_3$], 87739-48-8; I [base; X = 4-$(CH_2)_8CH_3$], 87739-87-5; I [X = 4-$C(CH_3)_3$], 6828-14-4; I (X = 4-COC_6H_5), 87739-49-9; I (base; X = 4-COC_6H_5), 87739-99-9; I (X = 4-OCH_3), 2218-74-8; I [X = 4-$O(CH_2)_2CH_3$], 87739-50-2; I [base; X = 4-$O(CH_2)_2CH_3$], 87739-96-6; I [X = 4-$O(CH_2)_5CH_3$], 4479-28-1; I [base; X = 4-$O(CH_2)_5CH_3$], 4653-85-4; I [X = 4-$O(CH_2)_{10}CH_3$], 87739-51-3; I [base; X = 4-$O(CH_2)_{10}CH_3$], 87739-97-7; I [X = 4-$O(CH_2)_{11}CH_3$], 65972-55-6; I [base; X = 4-$O(CH_2)_{11}CH_3$], 79515-25-6; I (X = 4-$OCH_2C_6H_5$), 4022-83-7; I (X = 4-$OCH_2C_6H_3$-3′,4′-Cl_2), 77113-86-1; I (X = 4-$OCH_2C_6H_4$-4′-SO_2NH_2), 87739-52-4; I (base; X = 4-$OCH_2C_6H_4$-4′-SO_2NH_2, 87739-88-6; I (X = 4-$OCH_2C_6H_4$-4′-$CONH_2$, 87739-53-5; I (base; X = 4-$OCH_2C_6H_4$-4′-$CONH_2$), 87739-89-7; I (X = 4-$OCH_2C_6H_4$-4′-CH_2OH), 87739-54-6; I (base; X = 4-$OCH_2C_6H_4$-4′-CH_2OH), 87739-90-0; I (X = 4-$CH_2SC_6H_5$), 87739-55-7; I (base; X = 4-$CH_2SC_6H_5$), 87739-93-3; I (X = 4-$CH_2SC_6H_4$-2′-CH_3), 87739-56-8; I (base; X = 4-$CH_2SC_6H_4$-2′-CH_3), 87739-94-4; I (X = 4-$CH_2SC_6H_4$-3′-CH_3), 87739-57-9; I (base; X = 4-$CH_2SC_6H_4$-3′-CH_3), 87739-95-5; I (X = 4-$SCH_2C_6H_5$), 87739-58-0; I (base; X = 4-$SCH_2C_6H_5$), 87739-91-1; I (X = 4-$SCH_2C_6H_4$-4′-Cl), 87739-59-1; I (base; X = 4-$SCH_2C_6H_4$-4′-Cl), 87739-92-2; I (X = 4-C≡CH), 87739-60-4; I (base; X = 4-C≡CH), 87740-00-9; I [X = 4-C≡$CSi(CH_3)_3$], 87739-61-5; I [base; X = 4-C≡$CSi(CH_3)_3$], 87740-01-0; I (X = 3-Cl,4-$(CH_2)_4C_6H_3$-2′-Cl-4′-SO_2F), 87739-62-6; I (X = 3-SO_2NH_2,4-Cl), 87739-63-7; I (base; X = 3-SO_2NH_2,4-Cl), 87739-72-8; I [X = 3,4-$(OCH_3)_2$], 37550-96-2; I [base; X = 3,4-$(OCH_3)_2$], 87739-70-6; I (X = 3-NH_2,4-C_2H_5), 87739-64-8; I (base; X = 3-NH_2,4-C_2H_5), 87739-71-7; I (X = 3-$CH_2SC_6H_5$,4-Cl), 87739-65-9; I (X = 3-Cl,4-$SCH_2C_6H_5$), 87739-66-0; I (base; X = 3-Cl,4-$SCH_2C_6H_5$), 87739-75-1; I (X = 3-Cl,4-$CH_2SC_6H_5$), 87739-67-1; I (base; X = 3-Cl,4-$CH_2SC_6H_5$), 87739-73-9; I (X = 3-Cl,4-OC_9H_{19}, 87739-68-2; I (base; X = 3-Cl,4-OC_9H_{19}), 87739-74-0; I [X = 3,4-$(CH_2)_4$], 18914-88-0; I (X = 3,5-Cl_2), 3181-64-4; I (base; X = 3,5-Cl_2), 2727-10-8; I [X = 3-$O(CH_2)_2OC_6H_4$-4′-CF_3], 87739-69-3; I [X = 4-$O(CH_2)_{13}CH_3$], 65972-56-7; 3-(4-*n*-C_5H_{11}-$C_6H_4OCH_2$)$C_6H_4NO_2$, 87740-02-1; 3-(3-$C_6H_5C_6H_4OCH_2$)$C_6H_4NO_2$, 87740-03-2; 3-(3-$CH_3C_6H_4SCH_2$)$C_6H_4NO_2$, 71219-87-9; 3-(4′-$COOHC_6H_4CH_2O$)$C_6H_4NO_2$, 87740-04-3; 4-(4′-$COOHC_6H_4CH_2O$)$C_6H_4NO_2$, 87740-05-4; 4-(4′-$SO_2NH_2C_6H_4CH_2O$)$C_6H_4NO_2$, 87740-06-5; 3-$NO_2C_6H_4CH_2Cl$, 619-23-8; 4-(3-$NO_2C_6H_4OCH_2$)$C_6H_4CONH_2$, 87740-08-7; 4-(4-$NO_2C_6H_4OCH_2$)$C_6H_4CONH_2$, 87740-09-8; 4-(4-$HOCH_2C_6H_4CH_2O$)$C_6H_4NO_2$, 87740-07-6; 3-$C_6H_5CH_2SC_6H_4NO_2$, 87740-10-1; 3-(4′-Cl-$C_6H_4CH_2S$)$C_6H_4NO_2$, 87740-11-2; 4-(4′-Cl-$C_6H_4CH_2S$)$C_6H_4NO_2$, 87740-12-3; PhSeSePh, 1666-13-3; 3-$C_6H_5SeCH_2C_6H_4NO_2$, 59305-50-9; $P(C_6H_5)_3$, 603-35-0; 3-$NO_2C_6H_4CH_2Br$, 3958-57-4; $CH_3(CH_2)_8CHO$, 124-13-0; $CH_3(CH_2)_9CHO$, 112-44-7; 3-$NO_2C_6H_4CH$=$CH(CH_2)_6CH_3$, 87740-13-4; 3-$NO_2C_6H_4CH$=$CH(CH_2)_9CH_3$, 87740-14-5; 4-$NO_2C_6H_4CH$=$CH(CH_2)_6CH_3$, 87740-15-6; 4-$NO_2C_6H_4CH_2Br$, 100-11-8; $N(CH_3)_3$, 75-50-3; 3-$NO_2C_6H_4CH_2N(CH_3)_3^+Cl^-$, 5411-74-5; 3-$NH_2C_6H_4$-$(CH_2)_8CH_3$·HCl, 87740-16-7; 3-$NH_2C_6H_4(CH_2)_{11}CH_3$·HCl, 87740-17-8; 4-$NH_2C_6H_4(CH_2)_8CH_3$·HCl, 87740-18-9; 1-$NH_2$-3-Cl-$C_6H_3$-4-$O(CH_2)_8CH_3$·HCl, 87740-19-0; 4-$PhSCH_2C_6H_4NH_2$·HCl, 87740-20-3; 4-(2′-$CH_3C_6H_4SCH_2$)$C_6H_4NH_2$·HCl, 87740-21-4; 4-(3′-$CH_3C_6H_4SCH_2$)$C_6H_4NH_2$·HCl, 87740-22-5; 4-$PhSCH_2$-3-Cl-$C_6H_3NH_2$, 87740-23-6; 3-$NH_2C_6H_4CH_2SeC_6H_5$·HCl, 87740-24-7; 3-$NH_2C_6H_4SCH_2C_6H_5$·HCl, 20391-78-0; 3-$NH_2C_6H_4SCH_2C_6H_4$-4′-Cl·HCl, 87740-25-8; 3-$NH_2C_6H_4CH_2SC_6H_4$-3′-CH_3·HCl, 87740-26-9; 3-$NH_2C_6H_4OCH_2C_6H_4$-4′-$CONH_2$, 87740-27-0; 4-$NH_2C_6H_4OCH_2C_6H_4$-4′-SO_2NH_2·HCl, 87761-63-5; 4-$NH_2C_6H_4OCH_2C_6H_4$-4′-$CONH_2$·HCl, 87740-28-1; 4-$NH_2C_6H_4OCH_2C_6H_4$-4′-CH_2OH·HCl, 87740-29-2; 3-$NH_2C_6H_4CH_2OC_6H_4$-3′-Ph·HCl, 87740-30-5; 3-$NH_2C_6H_4CH_2OC_6H_4$-4′-$(CH_2)_4CH_3$·HCl, 87740-31-6; 4-$NH_2C_6H_4SCH_2C_6H_4$-4′-Cl·HCl, 87740-32-7; 3-$NH_2C_6H_4CH_2N(CH_3)^+Cl^-$·HCl, 87740-36-1; $(NH_2)_2C$=NCN, 461-58-5; *c*-$C_6H_{11}OH$, 108-93-0; 3-*c*-$C_6H_{11}OCH_2C_6H_4NO_2$, 87740-33-8; 3-*c*-$C_6H_{11}OCH_2C_6H_4NH_2$·HCl, 87740-34-9; DHFR, 9002-03-3.