Irreversible Enzyme Inhibitors. CXIX.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase with Tissue Specificity Derived from 2,4,6-Triaminopyrimidine with a Terminal Sulfonyl Fluoride at the 5 Position

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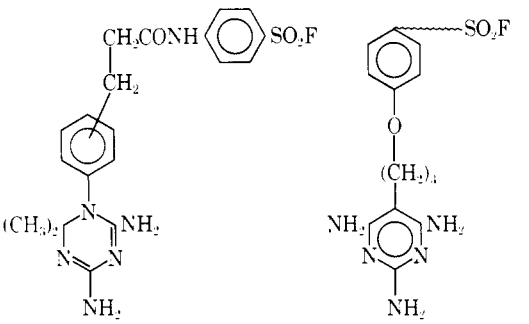
Four derivatives of 5-phenoxypropyl-2,4,6-triaminopyrimidine with the following substituents on the *para* position were synthesized as candidate active-site-directed irreversible inhibitors of dihydrofolic reductase: *m*-fluorosulfonylbenzamido (**10**), *p*-fluorosulfonylbenzamido (**11**), *m*-fluorosulfonylphenylureido (**12**), and *p*-fluorosulfonylphenylureido (**13**). At a concentration near 1 μM, **10** could rapidly inactivate the dihydrofolic reductase from Walker 256 rat tumor and L1210/FR8 mouse leukemia, but showed little irreversible inhibition of the enzyme from rat liver, mouse liver, or L1210/0. This specificity for rat tumor enzyme over rat liver enzyme was considerably decreased when the carboxamido group of **10** was lengthened to ureido (**12**), the tumor enzyme being inactivated about fourfold faster than the rat liver enzyme; however, specificity of inactivation of the L1210/FR8 enzyme by **12** with no irreversible inhibition of the mouse liver enzyme was maintained. Movement of the sulfonyl fluoride from the *meta* position of **10** and **12** to give **11** and **13** resulted in considerably slower irreversible inhibitors; since the enzyme could apparently catalyze hydrolysis of the sulfonyl fluoride of **11** and **13** to the irreversibly ineffective sulfonic acid more rapidly, the amount of irreversible inhibition was also decreased.

In two previous papers[2,3] a comparison of binding to the hydrophobic bonding region of the dihydrofolic reductases from Walker 256 tumor and the liver of the rat with 40 selected compounds was made; the maximum difference in binding observed with the compounds available was only 100-fold, a far sufficient spread to be useful for chemotherapy. The magnitude of this difference was actually more than might be expected from an evolutionary standpoint;[4,5] if a single amino acid had been changed in the hydrophobic bonding region, the latter being adjacent to and not part of the active site, such a change would be greatly magnified with an appropriately designed active-site-directed irreversible inhibitor.[4] It was recently predicted[6] that "probably the greatest difference in irreversible inhibition of dihydrofolic reductase from different tissues and species would arise if the group on the inhibitor can be branched (from the hydrophobic bonding moiety) toward a hydrophilic area; if a covalent forming group is then attached to this branch, its ability to form a covalent bond is also dependent upon the effect of the hydrophobic bonding region on the bridging to the nucleophilic site." Schematically, this can be represented in Figure 1.

The first example of this principle for species specificity for dihydrofolic reductase was observed with **1** where little difference in reversible binding occurred between the enzyme from pigeon liver, rat liver, Walker 256 rat tumor, and L1210/FR8; of these four sources of enzyme, however, only the pigeon liver enzyme was irreversibly inhibited.[7] When the propionanilide moiety of **1** was moved to the *para* position (**2**), then the enzyme from all four sources could be rapidly inactivated. If the sulfonyl fluoride group of **2** was moved to the *meta* position, the enzyme from Walker 256 was still irreversibly inhibited, but the enzyme from L1210/FR8 was not,[8] another species specificity. In this paper are reported the studies on compounds of type **3** where the hydrophobic bonding group has been changed from the phenylethyl group of **1** to propoxyphenyl; some of the compounds of type **3** now show irreversible specificity of action on the two tumor enzymes with little effect on the liver enzyme of the corresponding animals.



1, *meta* isomer
2, *para* isomer
3

Two bromoacetamido derivatives (**4**, **5**) (Table I) of 5-phenoxypropyl-2,4,6-triaminopyrimidine were previously studied as irreversible inhibitors of pigeon liver dihydrofolic reductase, but neither compound inactivated the enzyme.[9] As shown in Table I, neither **4** nor **5** could inactivate the dihydrofolic reductase from Walker 256 or L1210/FR8. Two similar candidate irreversible inhibitors derived from 2,4-diamino-6-phenylpyrimidine, namely **6**[10] and **7**,[11] also had failed to inactivate the pigeon liver enzyme; further investigation (Table I) showed these two com-

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series see B. R. Baker and M. A. Johnson, *J. Med. Chem.*, **11**, 486 (1968).

(3) B. R. Baker, *ibid.*, **11**, 483 (1968), paper CXVII of this series.

(4) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter IX.

(5) V. Bryson and H. J. Vogel, Ed., "Evolving Genes and Proteins," Academic Press Inc., New York, N. Y., 1965.

(6) Reference 4, p 248.

(7) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

(8) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 39 (1968), paper CXII of this series.

(9) B. R. Baker and D. V. Santi, *J. Pharm. Sci.*, **56**, 380 (1967), paper LXIX of this series.

(10) B. R. Baker, G. D. F. Jackson, and R. B. Meyer, Jr., *ibid.*, **56**, 566 (1967), paper LXXXII of this series.

(11) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **56**, 570 (1967), paper LXXXIV of this series.

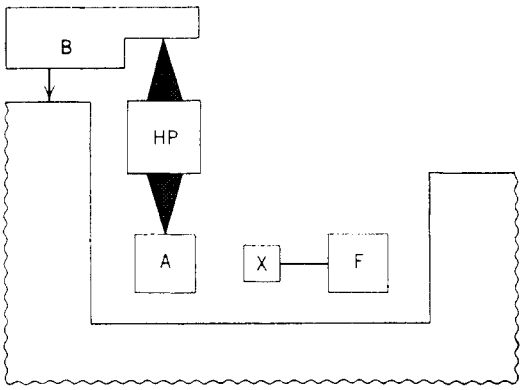


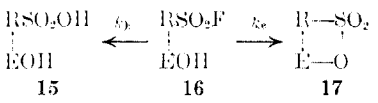
Figure 1.—A schematic representation of the bridge principle of specificity with dependence on the mode of hydrophobic bonding. A is the pyrimidine binding area, HP is the hydrophobic bonding area, B is a group in a noncontact area bearing a covalent-forming group represented by the arrow, and X–F is the coenzyme or cosubstrate (from ref 6).

pounds were not irreversible inhibitors of the two tumor enzymes.

Since the 6-phenyl group of compounds such as **6** was probably complexed with the hydrophobic bonding region of dihydrofolic reductase and the 5 group must be projected into a hydrophilic region,[11,12] two variants of **6** were synthesized for evaluation with a shorter chain at 5 position (**8**, **9**). Considerable loss in reversible binding to the enzyme occurred with **8** and **9** compared to **6**; furthermore, neither **8** nor **9** showed irreversible inhibition of the two tumor enzymes.

The discovery of irreversible inhibitors of type **1** and **2**[7] suggested that the terminal sulfonyl fluoride type of leaving group be investigated with 5-phenoxypropyl-2,4,6-triaminopyrimidine (**3**). These compounds (**10**–**13**) were synthesized from the same 5-(*p*-aminophenoxypropyl)-2,4,6-triaminopyrimidine used for the synthesis of **4** and **5**.[9] The first compound synthesized (**10**) showed irreversible inhibition of both the Walker 256 and L1210/FR8 enzymes.

The velocity, V, of active-site-directed irreversible inhibition is dependent upon the amount [EI] of total enzyme [E_t] reversibly complexed by the equations $V = k[EI]$ and $[EI] = [E_t]/(1 + K_i/[I])$.[13,14] Therefore comparisons of the rates of inactivation by two compounds or two concentrations of one compound should be made by comparing [EI] and not [I]. Furthermore, two reactions involving the enzyme and a sulfonyl fluoride type of inhibitor can occur:[15,16] (a) covalent bond formation (**17**) between a juxtaposed enzymic nucleophilic group such as an hydroxyl and the sulfonyl fluoride (**16**) can occur which leads to irreversible inhibition, and (b) the juxtaposed nucleophilic group (**16**), if a hydroxyl, can catalyze hydrolysis of the sulfonyl fluoride to a sulfonic acid (**15**), thus destroying the irreversible properties of the inhibitor.



The rates of these two reactions within the reversible complex, [EI] (**16**), are dependent upon their relative rate constants, k_c and k_b. These two rate constants are in turn dependent upon the relative positioning of the hydroxyl group to the sulfonyl fluoride, which can vary up to 3 Å and still give one reaction or the other. This relative positioning is in turn dependent upon the enzymic environment between the triaminopyrimidine moiety complexing in the active site and the enzymic nucleophilic group being covalently linked, the so-called bridge principle of specificity.[4,17] Thus, the positioning of the sulfonyl fluoride moiety in **16** could be influenced by small differences in the hydrophobic bonding region (see Figure 1) that could result in the enzyme from one source being inactivated (**17**) and the enzyme from another source catalyzing hydrolysis of the sulfonyl fluoride (**15**). Even more favorable for specificity, one enzyme may have evolved with the nucleophilic serine of **16** having been replaced by another amino acid such as alanine which would undergo neither reaction.

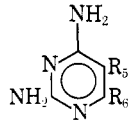
Whether or not inactivation of the enzyme is accompanied by hydrolysis of the sulfonyl fluoride can be detected by the shape of the inactivation curve when the log of the remaining active enzyme is plotted against time.[15] If the rate curve is linear though greater than 80–90% inactivation, little or no enzyme-catalyzed hydrolysis of the inhibitor has occurred; if rate curvature due to decreasing rate of inactivation is observed, then both reactions are occurring, the more curvature being observed the more favorable is the rate of hydrolysis *vs.* inactivation.[15] If no inactivation occurs, this could be due to either a rapid enzyme-catalyzed hydrolysis (**15**) or no reaction of either type; these two alternatives can frequently be separated by operating at a concentration considerably higher than K_i.[15]

The sulfonyl fluoride (**10**) inactivated the Walker 256 enzyme with a rate curve showing increased curvature as the initial concentration of **10** was decreased. There was little curvature at 5.3 μM (97% [EI]) and inactivation proceeded to 95%. At 0.2 μM (50% [EI]) there was considerable curvature, but the inactivation proceeded to 95% at a considerably slower rate. Since the rate curves in both cases were essentially linear through the first 50% of reaction, the half-life at 97% [EI] can be compared with the half-life at 50% [EI]; the two half-lives differed by approximately the theoretical factor of 2 (97/50).

The sulfonyl fluoride (**10**) also showed some inactivation of the liver enzyme from the same rats bearing the Walker 256 tumor, but the rate curves were considerably different. At 2.9 μM, where **10** gives 97% [EI] complex, 46% inactivation occurred over the first 15 min, then did not proceed further over 60 min. Similarly at 1.1 μM, where **10** forms 90% [EI] complex with the rat liver enzyme, only 18% inactivation occurred over 8 min, then no further inactivation occurred. Results of this type have been previously shown to be due to enzyme-catalyzed hydrolysis of the sulfonyl fluoride in **16** to the sulfonic acid (**15**), no further inactivation of the enzyme occurring when all of the sulfonyl fluoride has been hydrolyzed.[15,16] Thus a specificity with 1.1 μM **10** in the inactivation of the Walker 256 tumor enzyme over rat liver enzyme

(12) B. R. Baker and H. S. Shapiro, *J. Pharm. Sci.*, **55**, 308 (1966).
(13) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).
(14) See ref 4, Chapter VIII.
(15) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968), paper CXIII of this series.
(16) B. R. Baker and E. H. Erickson, **11**, *ibid.*, 245 (1968), paper CXV of this series.
(17) (a) B. R. Baker, *ibid.*, **5**, 651 (1962); (b) B. R. Baker, *Biochem. Pharmacol.*, **12**, 293 (1963).

TABLE I
INHIBITION[a] OF DIHYDROFOLIC REDUCTASES BY



				Reversible		Irreversible			
No.	R_5	R_6	Enzyme source	I_{50},[b] μM	Estd[c] K_i $\times 10^6$ M	[I], μM	[EI],[d] %	Time, min	% inactn[e]
4[f]	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*p*	NH_2	Walker 256	2.9	0.5	3	87	120	0
			L1210/FR8	8.8	1	1.4	50	120	0
5[f]	$-(CH_2)_3OC_6H_4NHCO(CH_2)_3C_6H_4NHCOCH_2Br$-*p*,*p*	NH_2	Walker 256	1.2	0.2	1.2	50	120	0
			L1210/FR8	14	2	14	87	60	0
6[g]	$-NH(CH_2)_3C_6H_4NHCOCH_2Br$-*p*	C_6H_5	Walker 256	0.052	0.009	0.29	97	120	0
			L1210/FR8	0.21	0.03	1.05	97	60	0
7[h]	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*p*	C_5H_5	Walker 256	0.17	0.03	0.83	97	120	0
			L1210/FR8	0.34	0.06	1.7	97	120	0
8	$-NHCH_2C_6H_4NHCOCH_2Br$-*m*	C_6H_5	Walker 256	23	4	4	50	120	0
			L1210/FR8	55	10	270	97	60	0
9	$-NHCH_2C_6H_4NHCOCH_2Br$-*p*	C_6H_5	Walker 256	18	3	4	57	120	0
			L1210/FR8	160	30	40	57	60	0
10	$-(CH_2)_3O$-C$_6$H$_4$-$NHCOC_6H_4SO_2F$-*m*	NH_2	Walker 256	1.1	0.2	5.3	97	2, 30	50, 95[i]
						1.1	87	3, 60	50, 94[i]
						0.2	50	5, 60	50, 95[i]
			Rat liver	0.58	0.1	2.9	97	15, 60	46, 46[i]
						1.1	90	8, 60	18, 18[i]
			L1210/FR8	6.5	1	6.5	87	2, 20	67, 92[i]
						1.3	57	2, 60	50, 96[i]
			Mouse liver			1.3		60	0[i]
						25		1, 60	~20[i]
			L1210/0	1.8	0.3	1.3	83	60	15
11	$-(CH_2)_3O$-C$_6$H$_4$-$NHCOC_6H_4SO_2F$-*p*	NH_2	Walker 256	0.45	0.07	1.8	97	60	35
			L1210/FR8	3.8	0.6	3.8	87	30, 60	50, 73[i]
			Mouse liver			3.8		60	~0
12	$-(CH_2)_3O$-C$_6$H$_4$-$NHCONHC_6H_4SO_2F$-*m*	NH_2	Walker 256	0.65	0.1	0.65	87	6, 60	50, 87[i]
			Rat liver	0.60	0.1	0.65	87	23, 60	50, 67[i]
			L1210/FR8	3.8	0.6	3.8	87	8, 40	50, 87[i]
			Mouse liver			3.8		60	0[i]
13	$-(CH_2)_3O$-C$_6$H$_4$-$NHCONHC_6H_4SO_2F$-*p*	NH_2	Walker 256	1.6	0.3	1.6	87	20, 60	50, 78[i]
			Rat liver			1.6		24, 60	50, 75[i]
			L1210/FR8	3.9	0.6	19.4	97	60	62
14	$-(CH_2)_3O$-C$_6$H$_4$-$NHCOC_6H_4SO_3H$-*m*	NH_2	Walker 256	0.60					
			Rat liver	0.58					
			L1210/FR8	0.85					
			Mouse liver	2.7					

[a] The technical assistance of Barbara Baine, Jean Reeder, and Diane Shea is acknowledged. [b] I_{50} = concentration necessary for 50% inhibition in the presence of 6 μM dihydrofolate which was measured in pH 7.4 Tris buffer as previously described.[7] [c] Estimated from $K_i = [I_{50}] \times K_m/[S]$ where $K_m \simeq 1 \times 10^{-6}$ M and $[S] = 6$ μM; this equation is valid when $[S] > 4K_m$ (ref 4, p 202). [d] [EI] = per cent of enzyme reversibly complexed, which is calculated from $[EI] = [E_t]/(1 + K_i/[I])$ where $[E_t]$ = total enzyme concentration.[13,14] [e] Assayed at 37° at pH 7.4 in the presence of 60 μM TPNH as previously described.[7] [f] Synthesis and failure to inactivate pigeon liver enzyme previously reported.[9] [g] Synthesis and failure to inactivate pigeon liver enzyme previously reported.[10] [h] Synthesis and failure to inactivate pigeon liver enzyme previously reported.[11] [i] Taken from a time study at 0, 2, 4, 8, 30, and 60 min; see ref 7 for procedure.

is seen, due in part to the difference in rates of hydrolysis *vs.* inactivation in the complex **16**; furthermore, the tumor enzyme is still inactivated with 0.2 μM **10**.

A similar specificity with **10** was seen with the enzymes from L1210/FR8 mouse leukemia and the liver from the same mice. The L1210/FR8 enzyme was 67% inactivated in 2 min and 92% inactivated in 20 min with 6.5 μM **10** (87% EI) with some curvature in the rate curve; curvature was increased at 1.3 μM **10** (57% EI), but 96% inactivation still occurred in 60 min. In contrast, the mouse liver enzyme showed no inactivation with 1.3 μM **10**; that this lack of inactivation was due to a fast hydrolysis of **10** by the mouse liver enzyme was indicated by the near 20% inactivation of this enzyme with 25 μM **10**.

The inactivation curves could also be explained by the possibility that the sulfonic acid (**14**) formed by enzyme-catalyzed hydrolysis of **10** might be a powerful reversible inhibitor of the tumor enzymes, but a much weaker inhibitor of the liver enzymes. For example, if **14** were a tenfold better reversible inhibitor than **10**, then an I_{50} concentration (1.1 μM) of **10** would result in 50% reversible inhibition by **14** after all the sulfonyl fluoride had been hydrolyzed since the assay concentration of inhibitor is one-tenth the incubation concentration.[7] That such was not the case was proven by synthesis of **14** and its evaluation as a reversible inhibitor (Table I); there was less than a twofold difference in binding between **10** and **14** on the two rat enzymes and fourfold better binding of **14** than **10** to the L1210/FR8 enzyme.

When the sulfonyl fluoride group of **10** was moved to the *para* position to give **11**, reversible binding to the tumor enzymes changed by only a factor of 2; however, the rate of inactivation of the two enzymes was considerably slowed. With 1.8 μM **11**, sufficient to give 97% [EI] complex, only 35% inactivation of the tumor enzyme was seen after 60 min in the two-point (0 and 60 min) screen;[7] we have arbitrarily used >70% inactivation at 97% [EI] in the screen to separate interesting from noninteresting candidate irreversible inhibitors, since these lower inactivations when measured in a time study usually show curvature with no further inactivation after 60 min due to enzyme-catalyzed hydrolysis of the sulfonyl fluoride (**15**).

The rate of inactivation of the L1210/FR8 enzyme by **11** at 97% [EI] complex is considerably slower than by **10** at 97% [EI] complex, 50% inactivation requiring 2 and 30 min, respectively. However, no inactivation of the mouse liver enzyme was seen in 60 min with the same concentration of **11**, another example of tissue specificity.

When the amide bridge between the two benzene rings of **10** was changed to ureido to give **12**, little change in reversible binding occurred. The rate of inactivation of the Walker 256 enzyme by **12** was about one-half the speed of **10** ($t_{1/2}$ = 6 and 3 min, respectively) when both were measured at 87% [EI] complex and the total inactivation after 60 min was 87 and 94%, respectively. Although the irreversible inhibition of the Walker 256 enzyme by **10** and **12** was quite similar, the inactivation of the rat liver enzyme by the two compounds was quite different. At a concentration to give 87% [EI] complex, **12** showed 50% inactivation in 23 min and 67% inactivation in 60 min with inactivation still occurring; in contrast, **10** showed only a maximum of 18% inactivation in 8 min which was not further increased with time. Thus, the rate of enzyme-catalyzed hydrolysis of **12** by the rat liver enzyme is considerably slower than the hydrolysis of **10** and selectivity of inactivation between the Walker 256 and rat liver enzymes is considerably reduced with **12** compared to **10**.

Even though **12** inactivates the L1210/FR8 enzyme at about one-fourth the rate shown by **10** when both are measured at 87% [EI] complex, **12** still shows selectivity of inactivation between the mouse leukemia and liver enzymes; **12** shows no inactivation of the mouse liver enzyme.

When the sulfonyl fluoride group of **12** is moved to the *para* position to give **13**, the rate of inactivation of the Walker 256 enzyme is slowed; in addition, **13** shows practically identical rate curves for inactivation of the enzymes from Walker 256 and rat liver, specificity being completely lost. At a concentration sufficient to give 97% [EI] complex, **13** only gave 62% inactivation of the L1210/FR8 enzyme in 60 min, thus failing the two-point screen.[7]

Although the specificity of irreversible inhibition shown by **10** with the tumor and liver enzymes can be considered a "quantum-jump" in the design of active-site-directed irreversible inhibitors for chemotherapy of cancer, this progress is at best only a milestone on the long road to chemotherapeutic control. It was stated[18] earlier that "the studies described to this point have dealt only with irreversible inhibition of isolated enzymes—a far cry from chemotherapy in a host system. It is obvious that there are a variety of other factors—other than selective irreversible inhibition of isolated enzyme systems—that play important roles in whether the inhibitor would be effective in a host system, among them being the following: (a) transport to and into the desired target cell from the site of administration of the inhibitor must be achieved, (b) the inhibitor must be reactive enough to give a reasonable rate of inactivation of the target enzyme but not so reactive that insufficient inhibitor reaches the desired cell containing the target enzyme, (c) there must be selectivity of inhibition of the enzyme in the target cell."

(18) (a) Reference 4, p 190; (b) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

Assay of **10** as its sulfate salt on Walker 256 and L1210/0 mouse leukemia *in vivo* gave the following preliminary results.[19a] At 500 mg/kg, **10** was toxic; at 100 mg/kg, **10** showed a slight beneficial effect that may not have been statistically significant; at 0.8–20 mg/kg no toxicity or beneficial effect was seen. A check of the irreversible inhibition of dihydrofolic reductase from L1210/0, the parent strain, by **10** showed only 15% inactivation in 60 min. Clearly the dihydrofolic reductases from the parent L1210/0 and the amethopterin-resistant L1210/FR8 strain are different,[19b] which could account for the ineffectiveness of **10** on L1210/0 *in vivo*, but not Walker 256.

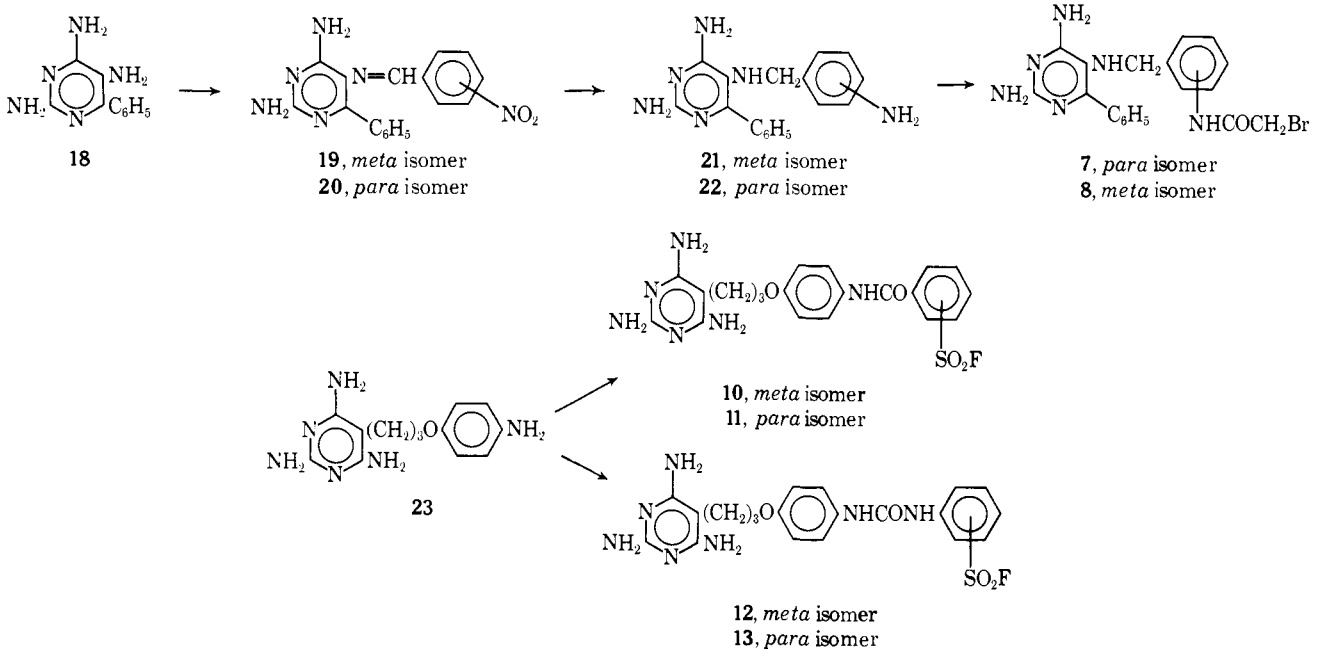
These near negative results with Walker 256 merely emphasize again the problems quoted above that remain to be solved with irreversible inhibitors; furthermore, the unique chemistry and biochemistry of the sulfonyl fluorides give additional parameters to be considered.

Of the three factors (a–c) that must be met to have a useful chemotherapeutic agent, only part of factor c is met by **10**. The latter shows specificity of inhibition of a tumor enzyme over the liver enzyme in the same animal, but the effect of **10** on the dihydrofolic reductase from some other tissue, such as bone marrow or gastrointestinal tract, may also be pronounced; such a barrier can be attacked by pathological examination of the drug-killed animal to determine the normal tissue most sensitive to the drug, followed by investigation of the irreversible inhibition of the enzyme from this tissue. However, there are more immediate problems that should be solved first.

With the molecular weight of **10** being in the 600 range, the theoretical dose to achieve a 1 μM concentration of **10** would be 0.6 mg/kg. Since no effect on the L1210/0 or Walker 256 lines *in vivo* was seen below 100 mg/kg, it is clear that the *in vivo* concentration achieved with 100-mg/kg injection was not even 0.6 mg/kg = 1 μM. In order to achieve a 1 μM tissue concentration with an 0.6-mg/kg injection, an aqueous solution at far higher concentration would be needed. In actuality, **10** was isolated as its highly water-insoluble hemisulfate for the convenience of purification

(19) (a) We wish to thank Dr. Florence White of the CCNSC, National Cancer Institute, for her aid in the design and expedition of these assays. (b) L1210/FR8 is a stable mutant resistant to amethopterin. E. R. Kashket, E. J. Crawford, M. Friedkin, S. R. Humphreys, and A. Goldin [*Biochemistry*, **3**, 1928 (1964)] reached the conclusion that dihydrofolic reductase from L1210/0 and L1210/FR8 were probably identical. However, their studies could also be explained by the identity of the active site of the dihydrofolic reductase from the two sources; since **10** presumably partially complexes within the active site, but forms a covalent bond outside of the active site, it is clear that these are not identical enzymes in some area adjacent to the active site.

SCHEME I



and sufficient solubility for enzyme assay; but sufficient solubility for animal assay did not need consideration prior to enzyme assay. An insufficient concentration of a sulfonyl fluoride type of irreversible inhibitor is detrimental on two counts; first, the inactivation rate of the enzyme will be one-tenth of the maxmum achievable rate of the inhibitor present at a concentration of 0.2 μM which is sufficient to give only 10% [EI] reversible complex, and second, at concentrations below the K_i concentration (0.2 μM) destruction of the inhibitor by enzyme-catalyzed hydrolysis of the sulfonyl fluoride (**16**, **15**) may be the prevalent reaction.[15,16]

Furthermore, the liver enzyme apparently can rapidly hydrolyze **10**; thus, at too low a concentration, insufficient inactivation of the tumor enzyme may have occurred before the liver enzyme hydrolyzed all of the circulating sulfonyl fluoride. There are three obvious approaches to solve this enigma: (a) utilize a more water-soluble salt of **10**; (b) replace the 6-amino group of **10** with a methyl which will increase solubility and decrease the concentration of inhibitor needed since 5-substituted 2,4-diamino-6-methylpyrimidines are considerably more potent reversible inhibitors than the corresponding 2,4,6-triaminopyrimidines of type **10**;[20] and (c) search for a compound that still inactivates the tumor enzyme, but is not hydrolyzed by the liver enzyme and does not appreciably inactivate the liver enzyme. Finally, it is possible that a compound such as **10** cannot penetrate a cell wall by passive diffusion due to its molecular weight of 460. Since the antimalarial, 5-(*p*-chlorophenyl)-2,4-diamino-6-ethylpyrimidine, with its pK_a near 7 and molecular weight of 248 can penetrate a cell wall by passive diffusion,[21] it seems reasonable that the free base of **10** with a similar pK_a and a molecular weight of 460 should also be able to enter by passive diffusion. Cell wall penetration can be determined by toxicity to L1210 in tissue culture when a sufficiently soluble salt of **10** has been prepared.

Even though the achievement of an irreversible inhibitor that is selective in action on dihydrofolic reductase from two tissues might be considered an important milestone in the chemotherapeutic control of cancer, the above problems (and perhaps some unseen ones) must be solved. Such solutions are being vigorously pursued.

Chemistry.—6-Phenyl-2,4,5-triaminopyrimidine (**18**), available from benzoylacetonitrile in three steps,[10] was condensed with *m*- and *p*-nitrobenzaldehyde in EtOH catalyzed by a trace of HOAc to give the anils (**19**, **20**) (Scheme I). Simultaneous reduction of the anil and nitro groups of **19** and **20** was accomplished by $NaBH_4$ in MeOH catalyzed by Pd;[22] the resultant amines (**21**, **22**) were difficult to purify completely, but could be obtained essentially homogeneous on tlc. Bromoacetylation in acetone[23] afforded the candidate irreversible inhibitors (**7**, **8**) which were isolated as their picrates and which gave a negative Bratton–Marshall test for aromatic amines and positive 4-(*p*-nitrobenzyl)pyridine test for activated halogen.[23]

The four-step synthesis of the key triaminopyrimidines bearing a *p*-aminophenoxy group (**23**) was previously described as an intermediate to **4** and **5**.[9] Acylation of **23** with *m*-fluorosulfonylbenzoyl chloride in DMF containing HOAc gave **10** which was isolated as its sulfate salt. Similarly, reaction of **23** with *p*-fluorosulfonylbenzoyl chloride in DMF–Me_2CO containing some HOAc to protonate the triaminopyrimidine ring system[23] afforded **11** isolated as its diacetate salt. Reaction of **23** diacetate[9] with *m*-fluorosulfonylphenyl isocyanate afforded the urea (**12**) which was isolated as its picrate since it failed to give a crystalline sulfate or acetate. The *para* isomer (**13**) was synthesized by condensation of **23** with N-carbophenoxysul-

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fanilyl fluoride[24] in DMSO, isolated as its ethanesulfonate salt.

Experimental Section[25]

2,4-Diamino-5-(*m*-nitrobenzalamino)-6-phenylpyrimidine (19).—A solution of 2.01 g (10 mmoles) of **18**,[10] 1.51 g (10 mmoles) of *m*-nitrobenzaldehyde, and 1 drop of glacial HOAc in 100 ml EtOH was warmed on a steam bath for 1 hr; after 10 min orange crystals began to separate. The cooled mixture was filtered and the product was washed with EtOH; yield 2.9 g (87%) suitable for the next step. Two recrystallizations of a sample from MeOEtOH–H_2O gave orange crystals, mp 187–188°. Nitrogen analyses were variable. *Anal.* ($C_{17}H_{14}N_6O_2$) C, H, O.

The *para* isomer (**20**) was prepared similarly in 78% yield. The analytical sample formed orange crystals, mp 331–333°. *Anal.* ($C_{17}H_{14}N_6O_2$) C, H, N.

5-(*m*-Bromoacetamidobenzylamino)-2,4-diamino-6-phenylpyrimidine (8) Picrate.—To a stirred solution of 600 mg (1.75 mmoles) of **19** in 175 ml of MeOH was added 100 mg of 10% Pd-C. Then 3.5 g of $NaBH_4$ was added in portions over 60 min with occasional cooling to maintain the temperature at 20–30°; tlc with 8:1 $CHCl_3$–EtOH showed one spot at this time. The filtered solution was evaporated *in vacuo*. The residue was extracted with three 25-ml portions of hot EtOAc. The combined extracts were evaporated *in vacuo* and the residue was recrystallized from MeOH–H_2O; yield 290 mg (54%) of **21**, mp 104–106°; this compound (**21**) gave C, H, N values just outside acceptable limits, but moved as a single spot on tlc.

To a solution of 50 mg (0.16 mmole) of **21** in 2 ml of Me_2CO and 0.015 ml (0.25 mmole) of HOAc at 0° was added 50 mg (0.19 mmole) of bromoacetic anhydride.[26] After 30 min at 0°, the solution was treated with 0.5 ml of 5% picric acid in EtOH. The solution was heated to boiling, then diluted with H_2O (5 ml) to turbidity. On cooling the solution deposited yellow crystals which were collected by filtration. Recrystallization from MeOEtOH–H_2O gave a near quantitative yield. A second recrystallization afforded the analytical sample as yellow crystals, mp 206° dec. *Anal.* ($C_{19}H_{19}BrN_6O \cdot C_6H_3N_3O_7 \cdot H_2O$) C, H, N.

Similarly, the *para* isomer (**7**) was prepared in 15% yield, mp 177–179°. The compound moved as a single spot on tlc with picric acid remaining at the origin and gave the proper color reactions,[23] but analyses were not quite acceptable. *Anal.* Calcd for $C_{19}H_{19}BrN_6O \cdot C_6H_3N_3O_7$: C, 45.6; H, 3.52; N, 19.1. Found: C, 46.1; H, 3.40; N, 18.5.

5-[*p*-(*m*-Fluorosulfonylbenzamido)phenoxypropyl]-2,4,6-triaminopyrimidine (10) Hemisulfate.—To a stirred solution of 5.48 g (20 mmoles) of **23**[9] in 35 ml of DMF, 5 ml of H_2O, and 1.32 g (22 mmoles) of HOAc cooled in an ice–salt bath and protected from moisture was added dropwise a solution of 4.88 g (22 moles) of *m*-fluorosulfonylbenzoyl chloride in 10 ml of DMF over a period of about 10 min. After being stirred in the ice bath for 30 min more, the mixture was poured into 200 ml of 0.5 *N* H_2SO_4. The product was collected on a filter and washed with EtOH. Two recrystallizations from MeOEtOH–H_2O gave 7.00 g (56%) of white powder, mp 213–218°. *Anal.* ($C_{20}H_{21}FN_6O_5S \cdot 0.5H_2SO_4 \cdot 1.5H_2O$) C, H, N.

5-[*p*-(*p*-Fluorosulfonylbenzamido)phenoxypropyl]-2,4,6-triaminopyrimidine (11) Diacetate.—A solution of 187 mg (0.5 mmole) of **23**[9] in 0.5 ml of DMF, 0.5 ml of Me_2CO, 0.10 ml of H_2O, and 60 mg of HOAc at −10° was treated with 120 mg (0.54 mmole) of *p*-fluorosulfonylbenzoyl chloride in one portion. After 15 min in the ice–salt bath, the mixture was poured into 10 ml of saturated aqueous $NaHCO_3$. The crude base was collected on a filter, washed with water, and recrystallized from HOAc by addition of H_2O. Further recrystallization from MeOH–H_2O gave 130 mg (45%) of a white powder, mp >280°. *Anal.* ($C_{20}H_{21}FN_6O_5S \cdot 2HOAc$) C, H, N.

5-[*p*-(*m*-Fluorosulfonylphenylureido)phenoxypropyl]-2,4,6-triaminopyrimidine (12) Picrate.—To a solution of 197 mg (0.5 mmole) of **23** diacetate[9] in 2 ml of DMF was added a solution of 100 mg (0.5 mmole) of *m*-fluorosulfonylphenyl isocyanate in 0.5 ml of DMF. After 30 min at ambient temperature, the mixture was warmed for 2 min on a steam bath, then spin evaporated *in vacuo*. The residue was dissolved in MeOEtOH, then 5 ml of 5% ethanolic picric acid was added followed by water. The picrate was collected on a filter and recrystallized from *i*-PrOH–H_2O to give 60 mg (17%) of yellow crystals, mp 219–222° dec. *Anal.* ($C_{20}H_{23}FN_7O_4S \cdot C_6H_3N_3O_7 \cdot 0.5H_2O$) C, H, N.

5-[*p*-(*p*-Fluorosulfonylphenylureido)phenoxypropyl]-2,4,6-triaminopyrimidine (13) Ethanesulfonate.—A solution of 139 mg (0.5 mmole) of **23**,[9] 161 mg (0.55 mmole) of N-carbophenoxysulfanilyl fluoride,[24] and 40 mg (0.66 mmole) of HOAc in 1 ml of DMSO was stirred at ambient temperature protected from moisture for 2 hr, then heated on a steam bath for 5 min. The solution was poured into a solution of 121 mg (1.1 mmoles) of $C_2H_5SO_3H$ in 20 ml of EtOAc. After several hours at −5°, the mixture was filtered and the product was washed with EtOAc. Three recrystallizations from *i*-PrOH–H_2O gave 110 mg (27%) of white crystals, mp 245–248°. *Anal.* ($C_{20}H_{22}FN_7O_5S \cdot C_2H_5SO_3H \cdot 0.5H_2O$) C, H, F.

5-[*p*-(*m*-Sulfobenzamido)phenoxypropyl]-2,4,6-triaminopyrimidine (14).—A mixture of 170 mg (0.32 mmole) of **10** and 25 ml of 6 *N* NaOH was stirred at about 70° for 30 min. The cooled mixture was filtered and the insoluble Na salt was washed with H_2O. The combined filtrate and washings were adjusted to about pH 5 and the zwitterion of **14** was collected on a filter and washed with H_2O. The Na salt was stirred for about 1 hr with 25 ml of 0.025 *N* HCl, then the zwitterion was collected on a filter. The combined fractions were recrystallized from DMF–H_2O; yield 90 mg (61%), mp 338–340°. *Anal.* ($C_{20}H_{22}N_6O_5 \cdot 0.75H_2O$) C, H, O.

(24) The preparation of N-carbophenoxysulfanilyl fluoride and its scope of reaction with aryl- and alkylamines was first explored by G. J. Lourens in this laboratory and will be described in a future paper.

(25) All analytical samples moved as a single spot on tlc with 1:1 $CHCl_3$–EtOH (unless otherwise indicated) on Brinkmann silica gel GF and had ir and uv spectra compatible with their assigned structures; each gave combustion values for CHN, CHF, or CHO within 0.4 of the theoretical percentage except **7**.