Since 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine (biopterin) is a growth-promoting agent for the trypanosomatid flagellate, *Crithidia fasciculata*,[46] we decided to determine whether this agent was capable of serving as a substrate for the reductase obtained from *T. equiperdum;* biopterin (at 10^{-4} *M*) was not a substrate for the trypanosomal reductase, nor was it capable (again at 10^{-4} *M*) of inhibiting the reduction of dihydrofolate by this preparation. Biopterin has also been found by Roberts and Hall[47] to be ineffective as an inhibitor of rat liver dihydrofolate reductase. The related 2-amino-4-hydroxypteridines, xanthopterin (2-amino-4,6-dihydroxypteridine) and isoxanthopterin (2-amino-4,7-dihydroxypteridine), also proved ineffective both as substrates and inhibitors of the trypanosomal reductase. A number of 4-substituted 2,6-diaminopyridines can inhibit the growth of such protozoa as *Tetrahymena pyriformis* and *C. fasciculata*.[48] In the case of 4-alkoxy-2,6-diaminopyridines, inhibitory activity has been ascribed to interference with biopterin function in *C. fasciculata*.[49] We found that 4-butoxy-2,6-diaminopyridine[50] had no appreciable activity as an inhibitor of trypanosomal dihydrofolate reductase, and this observation seems in accord with the lack of activity of biopterin itself in the protozoal reductase system. Recent experiments[51] with dihydrofolate reductase obtained from *C. fasciculata* indicated that biopterin shows no activity in this system. Folic acid was found previously[2] to be essentially inactive as a substrate for trypanosomal dihydrofolate reductase, but it was found to be a reasonably effective inhibitor of the enzyme (ID_{50} *ca.* 10^{-5} *M*); similar results were obtained with the triglutamate derivative[52] of folic acid which proved to be ineffective as a substrate while possessing inhibitory potency comparable to that of folic acid itself.

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Irreversible Enzyme Inhibitors. CLIV.[1,2] Some Factors in Cell Wall Transport of Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase[3] Derived from 5-Substituted 2,4-Diaminopyrimidines

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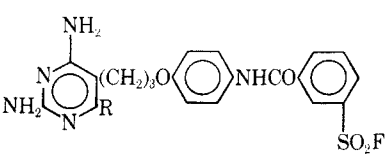
5-[*p*-(*m*-Fluorosulfonylbenzamido)phenoxypropyl]-2,4,6-triaminopyrimidine (**1a**) was previously observed to be an isozyme-specific active-site-directed irreversible inhibitor of dihydrofolic reductase from L1210 mouse leukemia that showed no irreversible inhibition of the enzyme from normal mouse liver; however, **1a** was ineffective *in vivo* due to poor penetration of the L1210 cell wall. Replacement of the ether bridge of **1a** by thioether (**2**) or methylene (**3**) gave irreversible inhibitors of similar isozyme specificity that could operate at much lower concentration due to their better reversible binding than **1a**: cell wall transport was not improved with **2** or **3**. This difficulty in cell wall transport of **1a**, **2**, and **3** compared to 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (**5**) was traced to two factors: (a) replacement of the 3,4-dichlorophenyl group of **5** by phenylbutyl (**7**) or *p*-aminophenoxypropyl (**6**) was extremely detrimental to transport, and (b) further smaller losses in transport occurred when **6** or **7** were converted to the *m*-fluorosulfonylbenzamido type of irreversible inhibitor (**1b**, **8**). In contrast to **7**, 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-phenylbutyl-2-triazine (**16**) was transported as well as **5**.

5-Phenoxypropylpyrimidines of type **1**[4,5] were found to be excellent active-site-directed inhibitors[6] of the dihydrofolic reductase from L1210 mouse leukemia, but showed no inactivation of this enzyme from normal mouse liver, intestine, or spleen.[7] However, **1a** and **1b**



1a, R = NH_2
b, R = CH_3

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

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were ineffective *in vivo* due to poor penetration of the L1210 cell wall, presumably by passive diffusion.[5,7] Similar results were seen when the phenoxy oxygen of **1b** was replaced by CH_2.[3] Therefore, a more detailed study has now been performed to determine the structural characteristics of **1** responsible for poor membrane transport; the results are the subject of this paper.

Assay Results.—It has been demonstrated that the folic acid antagonist, 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (pyrimethamine), penetrates cell walls by passive diffusion in contrast to close analogs of folic acid such as aminopterin (4-amino-4-deoxyfolic acid) which penetrate by active transport.[8] The direct assay of passive diffusion[8a] is quite difficult and would be extremely time consuming if a hundred or more inhibitors were evaluated. As a first approximation, the rate of diffusion of potent enzyme inhibitors can be evaluated by cell kill; it is then only necessary to measure the effectiveness of enzyme inhibition in a broken cell system (I_{50}) and then compare with the concentration necessary for cell kill (ED_{50}). The interpretation of such data contains the assumption that a sufficiently potent enzyme inhibitor leads to cell death by inhibition of the target enzyme and that secondary effects on other enzymes are negligible. With these assumptions it could be calculated that structural change of some inhibitors of the dihydrofolic reductase from *E. coli* could change diffusion by a factor as large as 2700-fold;[9] comparison was made of the relative ED_{50}/I_{50} ratios.[3]

The first compound synthesized for evaluation replaced the ether bridge of **1a** by a thioether (**2**); such a structural change could be expected to lead to a more potent irreversible inhibitor due to a further reduced K_i,[10] since the ether linkage of **1a** resides on a hydrophobic bonding region of the enzyme[11,12] and sulfur is considerably less polar than oxygen.[13] Furthermore, the thioether of **2** should allow as much conformational freedom as the ether of **1a**, but should give the binding equivalent to the more conformationally rigid methylene group of **3**. In Table I is shown that **2** is a 200 times better reversible inhibitor than **1a**; **2** or **3** are equivalent reversible inhibitors. Furthermore **2** and **3** showed the same specificity pattern as **1a**, that is, they could inactivate the L1210 mouse leukemia enzyme with little or no inactivation of the mouse liver enzyme.

When **2** or **3** were assayed for inhibition of L1210 in cell culture,[14] they had the same order of potency (ED_{50}) as **1a**; when these results are normalized by comparison of the ED_{50}/I_{50} ratios, they would indicate that cell penetration is even less effective with **2** and **3** than with **1a**.

Since the benzanilide moiety of **1–3** is relatively rigid in a ground-state planar conformation, the corresponding benzylaniline derivative (**4**) was synthesized to determine if this freely rotating benzylaniline moiety would allow better cell wall penetration. The benzyl derivative (**4**) showed about the same reversible inhibition as **1a** and also showed isozyme specificity; however, **4** was not as good an irreversible inhibitor of L1210 dihydrofolic reductase as **1a** since the extent of inactivation was lower, presumably due to more enzyme-catalyzed hydrolysis of the SO_2F moiety[15] of **4**. Unfortunately, **4** also showed poor penetration of the L1210 cell wall as estimated from its $ED_{50}/I_{50} = 37$.

The 3,4-dichlorophenyl analog (**5**) (Table II) of pyrimethamine shows high toxicity to mammals,[16] but also shows selective inhibition of the growth of Walker 256 in the rat due to the poor ability of this tumor to assimilate folic acid by active transport.[8c,17] It has now been found that **5** shows potent cytoxicity to L1210 cell culture with an ED_{50} of 2×10^{-11} *M* and an ED_{50}/I_{50} ratio of 0.002. Thus, **5** was selected as a base line to determine why compounds **1–4** showed such poor cell wall penetration with ED_{50}/I_{50} ratios in the range of 3–460.

When the 3,4-dichlorophenyl group of **5** was replaced by *p*-aminophenoxypropyl (**6**), a 10^6 loss in inhibition of L1210 cell culture was observed; since about 30-fold of this can be attributed to the difference in reversible inhibition of dihydrofolic reductase by **5** and **6**, a closer approximation to cell wall penetration can be achieved by comparison of the relative ED_{50}/I_{50} ratios of **5** and **6** where a 24,000-fold difference is observed. A further small loss in penetration occurs when **6** is converted to the irreversible inhibitor **1b**.

Replacement of the dichlorophenyl moiety of **5** by phenylbutyl (**7**) gave a 10^4-fold loss in ability to inhibit L1210 cell culture and a 6000-fold less effective ED_{50}/I_{50}; a further fivefold loss in ED_{50}/I_{50} ratio occurred with **7** when the fluorosulfonylbenzamido moiety was introduced (**8**).

Since the 6-methyl group of **5** and related compounds contributes little to binding to dihydrofolic reductase (compare **1b** and **10**), one can estimate the loss in cell wall penetration when the dichlorophenyl moiety of **5** is replaced by *p*-aminophenylbutyl (**9**); a 13,000-fold increase in the ED_{50}/I_{50} ratio was observed. A further 28-fold loss in penetration occurred when **9** was converted to the irreversible inhibitor **10**.

Replacement of the 6-Me group of the 2,4-diaminopyrimidine type of inhibitor by 6-NH_2 has been previously observed[11] to decrease the effectiveness of reversible inhibition of dihydrofolic reductase. Thus, one can make even less valid use of the ED_{50} for comparison of cell wall penetration of 2,4,6-triaminopyrimidines with 2,4-diamino-6-methylpyrimidines. Replacement of the 3,4-dichlorophenyl and 6-methyl moieties of **5** with *p*-aminophenylthiopropyl and 6-amino (**11**) gave a 4300-fold less favorable ED_{50}/I_{50} ratio. A further loss in penetration was observed when **11** was converted to the irreversible inhibitor **2**; part of this loss was accounted for by conversion of the *p*-amino

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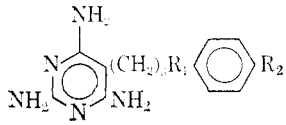
Table I

Inhibition[a] of Dihydrofolic Reductase by

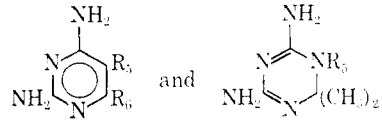


No.	R_1	R_2	Enzyme source	I_{50},[b] μM	Inhib, μM	Time, min	% inactvn[c]	ED_{50},[d] μM	ED_{50}/I_{50}
1a	O	$NHCOC_6H_4SO_2F$-*m*	L1210/0	2.7	1.3	60	93[e]	7.9	2.9
			L1210/DF8	3.1	1.3	60	100[e]		
			Liver	3.6	1.3	60	0[e]		
2	S	$NHCOC_6H_4SO_2F$-*m*	L1210/DF8	0.013	0.12	60	97	6.0	460
					0.060	60	72		
			Liver	0.060	0.18	60	0		
3	CH_2	$NHCOC_6H_4SO_2F$-*m*	L1210/DF8	0.010	0.042	60	89	1.2	120
			Liver	0.042	0.13	60	9		
4	O	$NHCH_2C_6H_4SO_2F$-*m*	L1210/DF8	1.7	2.8	60	52	62	37
			Liver	2.4	8.3	60	0		

[a] The technical assistance of Diane Shea and Sharon Lafler with these assays is acknowledged. [b] Concentration necessary for 50% inhibition when assayed with 6 μM dihydrofolate in 0.05 M Tris buffer (pH 7.4) containing 0.15 M KCl as previously described.[7] [c] Incubated at 37°, then the concentration of remaining enzyme assayed as previously described.[7] [d] Concentration for 50% inhibition of L1210/0 cell culture. [e] Data from ref 7.

Table II

Inhibition of L1210 Cell Culture by



No.	Ring system[a]	R_5	R_6	I_{50},[a,b,d] μM	ED_{50},[c] μM	ED_{50}/I_{50}
1b[f]	A	$(CH_2)_3OC_6H_4$-*p*-$NHCOC_6H_4SO_2F$-*m*	CH_3	0.016	2.2	140
5	A	$C_6H_3Cl_2$-3,4	CH_3	0.010	0.00002	0.002
6[g]	A	$(CH_2)_3OC_6H_4NH_2$-*p*	CH_3	0.28	14	49
7	A	$(CH_2)_4C_6H_5$	CH_3	0.027[f,h]	0.34	12
8	A	$(CH_2)_4C_6H_4$-*p*-$NHCOC_6H_4SO_2F$-*m*	CH_3	0.011[f,i]	0.68	62
9	A	$(CH_2)_4C_6H_4NH_2$-*p*	H	0.19[k]	4.9	26
10	A	$(CH_2)_4C_6H_4$-*p*-$NHCOC_6H_4SO_2F$-*m*	H	0.010	7.3	730
11	A	$(CH_2)_3SC_6H_4NH_2$-*p*	NH_2	0.58	5.0	8.7
12	A	$(CH_2)_3SC_6H_4NHAc$-*p*	NH_2	0.10	3.2	32
13	A	$(CH_2)_4C_6H_5$	NH_2	8.4[h]	5.4	0.64
14	A	$(CH_2)_3OC_6H_3$-2-Cl-4-$NHCOC_6H_4SO_2F$-*m*	NH_2	1.2[f]	17	14
15	B	$C_6H_3Cl_2$-3,4		0.015[f,h]	0.00019	0.013
16	B	$(CH_2)_4C_6H_5$		0.041[f,h]	0.00008	0.002

[a–c] See corresponding footnotes in Table I. [d] Assayed with dihydrofolic reductase from mouse liver unless otherwise indicated. [e] A, 2,4-diaminopyrimidine; B, 4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine. [f] Data from ref 11. [g] See ref 5 for synthesis. [h] Pigeon liver enzyme. [i] Data from ref 3. [j] L1210/0 enzyme. [k] See ref 3 for synthesis. [l] Data from ref 7.

group of **11** to an amide (**12**). Similarly, the phenylbutyl group of **13** gave a 320-fold loss in penetration compared to **5** when estimated by ED_{50}/I_{50} ratios; again a further 200-fold loss occurred when **13** was converted to the irreversible inhibitor **3**.

In summary, the poor penetration of the L1210 cell by **1–4** is due to two factors: (a) the phenylalkyl side chains give a 300–24,000 loss in cell wall penetration compared to **5** and (b) introduction of the fluorosulfonylbenzamido moiety gives a smaller (3–200-fold) loss in penetration, both estimated by comparative ED_{50}/I_{50} ratios. Thus, it is unlikely that further modification of the 5 side chains of **1–4** would lead to compounds useful for *in vivo* treatment of L1210 mouse leukemia.

Two possible solutions to the above dilemma have emerged. Bridging of the terminal sulfonyl fluoride moiety to the phenyl group of a 2,4-diamino-5-phenylpyrimidine should be explored for cell wall penetration and isozyme specificity. The second possible solution emerged by comparison of **5** with its dihydro-*s*-triazine counterpart (**15**) and **16** with **7**; **15** is nearly as good an inhibitor of L1210 cell culture as **5** within a factor of 10. Quite surprising was the observation that the 1-phenylbutyl-*s*-triazine (**6**) was an even better inhibitor in cell culture than **15** and, in fact, **6** was as effective as **5** when normalized by comparison of their ED_{50}/I_{50} ratios; the effectiveness of **6** should be compared with the relative ineffectiveness of **13** and **7** in cell culture. Since the side chains in **1–4** impart high isozyme specificity, but poor cell wall penetration, these side chains should be placed on the 1 position of 4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine to take advantage of the good cell wall penetration of **16**. Even if as much as a 200-fold loss in penetration occurs by insertion of the fluorosulfonylbenzamido moiety on **16**, but if isozyme specificity can be maintained with these dihydro-*s*-triazines, then useful compounds for cancer chemotherapy could emerge; such studies are being pursued vigorously.

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Each analytical sample gave proper uv and ir spectra, moved as a single spot on tlc on Brinkmann silica gel GF, and gave combustion values for C, H, and N or F within 0.4% of theoretical.

Pyrimidine precursors for the irreversible inhibitors (**2**, **3**) were synthesized by the previously described general method[18] from malononitrile and the appropriate bromide, then acylated with *m*-fluorosulfonylbenzoyl chloride. Condensation of 5-(*p*-aminophenoxypropyl)-2,4,6-triaminopyrimidine[19] with *m*-bromomethylbenzenesulfonyl fluoride[20] gave **4**.

5-(*p*-Nitrophenylbutyl)-2,4,6-triaminopyrimidine (17).—Alkylation of 1.98 g (30 mmoles) of malononitrile with 7.74 g (30 mmoles) of *p*-nitrophenylbutyl bromide[21] in DMSO with NaH, then condensation with guanidine[18] gave a crude product that was recrystallized from MeOEtOH–H_2O; yield 2.4 g (27%), mp 266–269°. *Anal.* ($C_{14}H_{18}N_6O_2$) C, H, N.

5-(*p*-Acetamidophenylthiopropyl)-2,4,6-triaminopyrimidine (12) Diacetate.—A solution of 25 g (0.15 mole) of *p*-acetamidothiophenol and 8.27 g (0.153 mole) of NaOMe in 150 ml of MeOH was spin evaporated *in vacuo*. The residual Na salt, dissolved in 75 ml of DMSO, was added dropwise to a vigorously stirred solution of 100 g of $Br(CH_2)_3Br$ in 50 ml of DMSO. After being stirred for 30 min more, the mixture was partitioned between 300 ml of $CHCl_3$ and 100 ml of 1 *N* NaOH. The organic layer was washed successively with 200 ml of 1 *N* NaOH and two 200-ml portions of H_2O. Dried with $MgSO_4$, the solution was evaporated *in vacuo*; excess $Br(CH_2)_3Br$ was then removed under high vacuum at 80–90°. The residue was heated to boiling with C_6H_6, then filtered from 1,3-bis(*p*-acetamidophenylthio)propane. The hot filtrate was diluted with petroleum ether (bp 60–110°), then chilled. The crude *p*-acetamidophenylthiopropyl bromide was collected by filtration; yield 25 g, mp 74–83°. Tlc in EtOAc showed about 70% bromide and 30% bis-substituted propane. If the $Br(CH_2)_3Br$ was added last, mostly bis-substituted propane was obtained.

The crude bromide was condensed with malonitrile and then with guanidine.[18] The crude product was recrystallized from 10% HOAc until a single spot on tlc in EtOH was obtained; yield 10% of white crystals, mp 178–190°. *Anal.* ($C_{15}H_{20}N_6OS \cdot 2CH_3CO_2H$) C, H, N.

5-(*p*-Aminophenylthiopropyl)-2,4,6-triaminopyrimidine (11) Disulfate.—A stirred mixture of 1.37 mmoles of **12**, 10 ml of EtOH, and 4 ml of 25% NaOH was refluxed for 10 hr, then evaporated *in vacuo*. The residue was dissolved in hot H_2O by addition of 12 *N* HCl to about pH 2. The solution was clarified with charcoal, then 3 ml of 6 *N* H_2SO_4 was added. The product that separated on cooling was collected by filtration and recrystallized from dilute H_2SO_4 by addition of HOAc; yield 0.42 g (84%), mp 194–195°, that moved as one spot on tlc in EtOH–$CHCl_3$ (1:4). *Anal.* ($C_{13}H_{18}N_6S \cdot 2H_2SO_4 \cdot H_2O$) C, H, N.

5-[*p*-(*m*-Fluorosulfonylbenzamido)phenylthiopropyl]-2,4,6-triaminopyrimidine (2) Hemisulfate.—To a stirred solution of 200 mg (0.40 mmole) of **11** and 250 mg of 1,4-diazabicyclo[4.3.0]non-5-ene (DBN) in 1.5 ml of 85% DMF cooled to −10° was added a solution of 220 mg (1 mmole) of *m*-fluorosulfonylbenzoyl chloride in 0.5 ml of DMF over 5 min. After an additional 15 min, the solution was poured into a stirred mixture of 30 ml each of 1 *N* H_2SO_4 and $CHCl_3$. The product was collected and washed with hot $CHCl_3$. Recrystallization from EtOH–H_2O gave 140 mg (63%) of product, mp 151° dec, that moved as a single spot on tlc in EtOH–$CHCl_3$ (1:4). *Anal.* ($C_{20}H_{21}FN_6O_3S_2 \cdot 0.5H_2SO_4 \cdot 2H_2O$) C, H, F.

5-[*p*-(*m*-Fluorosulfonylbenzamido)phenylbutyl-2,4,6-triaminopyrimidine (3) Hemisulfate.—A mixture of 0.906 g (3 mmoles) of **17**, 100 ml of MeOEtOH, and 0.25 g of 10% Pd–C was shaken with H_2 at 2–3 atm for 2 hr when reduction was complete. The filtered solution was evaporated *in vacuo* and the residue dissolved in 50 ml of 2% HOAc. After addition of 3 ml of 6 *N* H_2SO_4, the solution was concentrated *in vacuo* to about 5 ml, then the hot solution was diluted with HOAc to turbidity. On cooling, the solution deposited 0.94 g (75% calculated as a $1.5H_2SO_4$ salt) of 5-(*p*-aminophenylbutyl)-2,4,6-triaminopyrimidine, mp 242–246° dec. Although the compound moved as a single spot on tlc in EtOH, satisfactory combustion values were not obtained.

Acylation of 419 mg (1 mmole) of the preceding sesquisulfate with 333 mg (1.5 mmoles) of *m*-fluorosulfonylbenzoyl chloride, as described for the preparation of **2**, gave a crude sulfate salt. Recrystallization from dilute H_2SO_4 by addition of HOAc gave 240 mg (44%) of white crystals, mp 196–199° dec, that moved as a single spot on tlc in EtOH–$CHCl_3$ (1:4). *Anal.* ($C_{21}H_{23}FN_6O_3S \cdot 0.5H_2SO_4 \cdot 2H_2O$) C, H, F.

5-[*p*-(*m*-Fluorosulfonylbenzylamino)phenoxypropyl]-2,4,6-triaminopyrimidine (4) Sulfate.—A mixture of 815 mg (3 mmoles) of 5-(*p*-aminophenoxypropyl)-2,4,6-triaminopyrimidine,[19] 10 ml of DMF, and 1.0 g (4 mmoles) of α-bromo-*m*-toluenesulfonyl fluoride[20] was stirred for 30 min, then added to 100 ml of HOAc and 5 ml of 6 *N* H_2SO_4. Me_2CO was added to the hot solution to turbidity. On cooling the solution deposited crystals that were a mixture of product and starting amine sulfates. One recrystallization from dilute H_2SO_4–HOAc–Me_2CO and two from dilute H_2SO_4–dilute HOAc gave 51 mg (3%) of product that was free of starting amine as shown by tlc in EtOH–$CHCl_3$ (1:4) and that gradually decomposed over 240°. No attempt was made to recover more product from the filtrates or to obtain optimum yields. *Anal.* ($C_{20}H_{23}FN_6O_3S \cdot H_2SO_4$) C, H, F.

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