yield 3.00 g (43%), mp 139–140°. *Anal.* ($C_{14}H_{11}FN_2O_6S$) C, H, N. See Table III for other compounds prepared by this method.

N-(4-Fluorosulfonyl-3-methylphenyl)-N′-(4-nitrobenzyl)urea (12c) (Method B).—To a stirred mixture of 0.95 g (5 mmoles) of **14**·HCl,[16] 1.55 g (5 mmoles) of **11c** (Table III), and 4 ml of DMF was added 0.51 g (5 mmoles) of Et_3N. After 17 hr the mixture was diluted with 15 ml of H_2O. The product was collected on a filter, washed with 50% MeOH, then recrystallized from MeOEtOH–H_2O; yield 1.47 g (80%), mp 205–206°; tlc in 1:1 EtOAc–petroleum ether showed one spot. See Table IV for additional compounds prepared by this method.

Irreversible Enzyme Inhibitors. CXLII.[1,2] Further Studies on Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 5-(*p*-Aminophenoxypropyl)-2,4,6-triaminopyrimidine Bearing a Terminal Sulfonyl Fluoride

B. R. BAKER AND RICH B. MEYER, JR.

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

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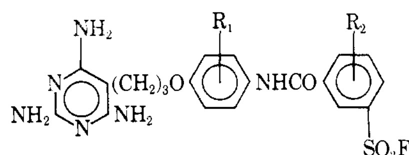
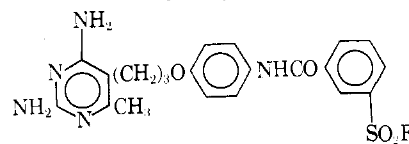
5-[*p*-(*m*-Fluorosulfonylbenzamido)phenoxypropyl]-2,4,6-triaminopyrimidine (**1**) at a K_i concentration is an active-site-directed irreversible inhibitor of the dihydrofolic reductase from three strains of L1210 mouse leukemia; furthermore, **1** at $40K_i$ concentration showed no irreversible inhibition of enzyme from mouse liver. The lack of effective *in vivo* action of **1** has been attributed to its relatively high $K_i \simeq 1\ \mu M$. The maximum enhancement in reversible binding that could be achieved by substitution of chloro, methyl, or isopropyl on one of the two benzene rings was only about twofold, as seen with the 2-chlorophenoxy analog (**9**) of **1**; this was still insufficient for effective *in vivo* activity. The specificity patterns of irreversible inhibition with the four analogs of **1** are presented and discussed.

The 5-phenoxypropylpyrimidine **1** was observed[3,4] to be an active-site-directed irreversible inhibitor[5] of dihydrofolic reductase from Walker 256 rat tumor as well as three strains of L1210 mouse leukemia; furthermore, **1** showed good tissue specificity since it failed to inactivate the enzyme from rat liver or mouse liver. However, **1** failed to show activity against



1, $R_1 = R_2 = H$
2, R_1 or $R_2 \neq H$



3

L1210 *in vivo*;[4] the failure of **1** to work *in vivo* could be attributed to a combination of poor solubility of the sulfate salt of **1** used in the assay and the relatively poor reversible complexing of **1** to dihydrofolic reductase. The latter is an important factor since the rate of active-site-directed irreversible inhibition of an enzyme is dependent upon the concentration of the reversible enzyme–inhibitor complex which in turn is dependent upon the binding constant (K_i) and the concentration of inhibitor;[6] that is, the lower the K_i, the less inhibitor is needed to convert 50% of the enzyme to the rate-limiting reversible complex. Furthermore, the lower the K_i, the less soluble a compound must be to be effective. Therefore two approaches were investigated to seek compounds with a better $I_{50} \simeq 6K_i$. In this paper is described the effect of substitution (**2**) on the benzene rings of **1** on the I_{50}; in the paper that follows[7] is described the synthesis of **3** which would be expected to show a 10–150-fold increment in reversible binding,[8] but may or may not still be an irreversible inhibitor.

Enzyme Results.—5-Phenoxypropyl-2,4,6-triaminopyrimidine (**4**) was synthesized and evaluated as a reversible inhibitor of the dihydrofolic reductase from L1210/0 in order to establish a base line. Note that **4** had $I_{50} = 31 \mu M$ (Table I) which is about tenfold less than the parent irreversible inhibitor **1** (Table II); thus when the *m*-fluorosulfonylbenzamido moiety (**1**) is attached to **4** about a tenfold increment in binding emerged. The positions open for study on the phenoxy ring of **1** in order to increase binding were 2, 3, 5, and 6.

Insertion of a 2-chloro (**5**) or 3-chloro (**6**) atom gave a twofold increment in binding over the parent **1**. When a 3-chloro atom was inserted on 2-chlorophenoxy group of **5** to give **7**, another twofold gain in binding was observed indicating that the effect of the 2- and 3-chloro atoms was additive. No change in binding occurred when a 5-chloro atom (**8**) was inserted on **5**. Whether or not 2,6-dichloro substituents would have given better binding was not investigated.

(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 G. J. Lourens, *J. Med. Chem.*, **12**, 101 (1969).

(3) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **11**, 489 (1968), paper CXIX of this series.

(4) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), paper CXXXIII of this series.

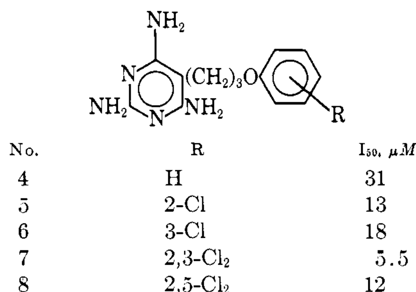
(5) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

(6) For the kinetics of irreversible inhibition see (a) ref 5, Chapter 8; (b) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(7) B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **12**, 108 (1969), paper CXLIII of this series.

(8) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965).

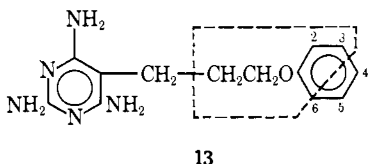
TABLE I
INHIBITION[a,b] OF DIHYDROFOLIC REDUCTASE BY



No.	R	I_{50}, μM
4	H	31
5	2-Cl	13
6	3-Cl	18
7	2,3-Cl_2	5.5
8	2,5-Cl_2	12

[a] The technical assistance of Diane Shea with these assays is acknowledged. [b] Assayed with 6 μM dihydrofolate in pH 7.4 Tris buffer containing 0.15 M KCl and 10% DMF as previously described;[4] dihydrofolic reductase was a 45–90% $(NH_4)_2SO_4$ fraction from L1210/0.[4]

These results indicate a weak hydrophobic interaction on only one side of the phenoxy ring as shown in **13**. Position 4 is not likely in a hydrophobic area



13

since the polar carboxamide function of **1** can reside in this area. Position 5 is not in a hydrophobic area since **8** gives no increment in binding over **5**. Whether or not position 6 should be included in the hydrophobic region is not known. The second two methylenes and the phenoxy oxygen were previously shown to reside in a hydrophobic area on the enzyme.[8]

When a chloro atom was inserted (**9**) on the parent irreversible inhibitor (**1**), a twofold increment in binding to the dihydrofolic reductase from L1210/0, L1210/DF8, and liver was noted (Table II); a fivefold increment to the enzyme from L1210/FR8 was observed.[9] The irreversible inhibition pattern with **9** was similar to **1**. Good inactivation of dihydrofolic reductase from the three L1210 strains was seen with **9** at a K_i concentration;[6] neither **1** nor **9** at $>20K_i$ concentration showed inactivation of the mouse liver enzyme.

When **9** was assayed *in vivo* against L1210/DF8 in the mouse,[12] it showed toxicity at 250 mg/kg and no life extension at 125 or 62.5 mg/kg. Among the factors which could account for this lack of *in vivo* activity, the most likely is the poor I_{50} which requires too high a dose of **9** to be effective; a second factor is the toxicity noted with **1** that is not reversed with citrovorum factor and may be due to some other toxicity of the 2,4,6-triaminopyrimidine system.[4] For comparison, note that amethopterin has $I_{50} \simeq 0.001\ \mu M$[13] and is effective against L1210/0 at a dose of 1 mg/kg; thus it can be calculated that a dose of 1400 mg/kg of **9** would be required if it were as effective as amethopterin in blocking dihydrofolic reductase.

The possibility of obtaining better reversible inhibition by substitution on the benzenesulfonyl fluoride moiety of **1** was then investigated. Insertion of a 4-methyl (**10**), 6-methyl (**11**), or 4-isopropyl group (**12**) on **1** gave little change in reversible binding, except for **12** which gave a two- to threefold increment. The effects of these substitutions on the pattern of irreversible inhibition compared to **1** was investigated. All three compounds still failed to show inactivation of the mouse liver dihydrofolic reductase. The 4-methyl derivative (**10**) was about as effective as **1** at a $6K_i$ concentration on the L1210 enzymes. In contrast, the 4-isopropyl derivative (**12**) was less effective on the L1210 enzymes.

Since the substitutions on the irreversible inhibitor (**1**) listed in Table II failed to give a sufficiently good I_{50} for consideration of *in vivo* testing, three other structural changes were considered for more effective reversible inhibition.[6] Based on previous studies,[8,14] replacement of the phenoxy oxygen of **1** by CH_2 could be expected to give a 30–80-fold increment in reversible binding; however, such a structural change might lead to loss of irreversible inhibition[15] or loss in specificity of irreversible inhibition.[16] A second approach would be to replace the phenoxy oxygen of **1** by sulfur. Since sulfur is more hydrophobic than oxygen and since a $-CH_2S-$ moiety has the same freedom to rotate as the $-CH_2O-$ moiety, in contrast to the $-CH_2CH_2-$ moiety, it could be expected that such an isosteric replacement would still give a selective irreversible inhibitor with a pattern similar to **1**, but perhaps with better reversible inhibition. The third approach would be to replace the 6-amino group of **1** by a methyl (**3**) which would be expected to give a 10–150-fold increment in reversible binding,[8] but perhaps with little change in the specificity pattern; the results of this third approach are presented in the paper that follows.[7]

Chemistry.—The substituted 5-phenoxypropyl-2,4,6-triaminopyrimidines (**16**) needed for this enzyme study were prepared by the general route described earlier.[17] The appropriate phenol (**14**) was alkylated with 1,3-dibromopropane to **15**, then condensed with malononitrile in DMSO–NaH to **17** (Scheme I). Reaction of **17** with guanidine afforded the required 2,4,6-triaminopyrimidines (**16**).

The irreversible inhibitors described in Table II can be generalized by **20** and **21**. Those of structure **20** were prepared by acylation of **18**[17] with the appropriate *m*-fluorosulfonylbenzoyl chloride in DMF;[3] **21** (= **9**) was prepared similarly from **19**.

The required derivatives of *m*-fluorosulfonylbenzoic acid (**24**) were prepared by chlorosulfonation of the appropriate benzoic acid (**22**) to **23**[18] followed by treatment with KF in dioxane.[19] That the sulfonyl group

(9) L1210/0 is the parent wild strain of mouse leukemia; L1210/FR8[10] and L1210/DF8[11] are relatively stable mutants of L1210/0 resistant to amethopterin.

(10) E. R. Kashket, E. J. Crawford, M. Friedkin, J. R. Humphreys, and A. Goldin, *Biochemistry*, **3**, 1928 (1964).

(11) This strain was isolated in the laboratory of Dr. A. Goldin, National Cancer Institute.

(12) We wish to thank Dr. Florence White of the CCNSC, National Cancer Institute, for expediting these assays.

(13) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **54**, 1740 (1965).

(14) B. R. Baker, B.-T. Ho, and G. B. Chheda, *J. Heterocycl. Chem.*, **1**, 88 (1964).

(15) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 666 (1968), paper CXXVII of this series.

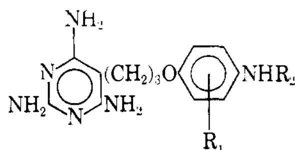
(16) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 89 (1969), paper CXXXVIII of this series.

(17) (a) B. R. Baker and D. V. Santi, *J. Pharm. Sci.*, **56**, 380 (1967), paper LXIX of this series; (b) B. R. Baker and D. V. Santi, *ibid.*, **54**, 1252 (1965).

(18) G. B. Jackman, V. Petrow, O. Stephenson, and A. M. Wild, *J. Pharm. Pharmacol.*, **14**, 679 (1962).

(19) A. H. deCat and R. K. van Poucke, *J. Org. Chem.*, **28**, 3426 (1963).

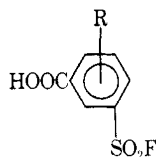
TABLE II
INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



				Reversible[b]		Irreversible[c]			
No.	R_1	R_2	Enzyme source	I_{50},[d] μM	Estd $K_i \times 10^6$ M[e]	Inhib, μM	% EI[f]	Time, min	% inactvn
1[g]	H	$COC_6H_4SO_2F$-*m*	L1210/FR8	6.5	1.1	1.3	55	2, 60	50, 96[h]
			L1210/0	2.7	0.45	1.3	71	60	93[i]
						0.4	48	60	85[i]
			L1210/DF8	3.1	0.51	1.3	71	60	100
						0.4	44	60	97
			Liver	3.6	0.60	2.5	98	60	20
						1.3	67	60	0
9[g]	2-Cl	$COC_6H_4SO_2F$-*m*	L1210/FR8	1.2	0.20	0.2	50	4, 60	50, 90[h]
			L1210/0	1.4	0.23	2.4	91	60	98[i]
						0.23	50	60	76[i]
			L1210/DF8	1.2	0.20	0.2	50	60	96[i]
			Liver	1.5	0.25	6.0	95	60	0[i]
			Spleen			2.0		60	0[i]
			Intestine			2.0		20	5[i]
10	H	COC_6H_3-3-SO_2F-4-CH_3	L1210/FR8	1.4	0.23	1.4	87	8, 60	50, 96[h]
			L1210/0	2.0	0.33	2.0	87	10, 30, 60	30, 51, 62[h]
			L1210/DF8	3.2	0.53	0.3	35	60	87
			Liver	5.0	0.83	6.0	87	60	0
11	H	COC_6H_3-2-CH_3-5-SO_2F	L1210/DF8	3.5	0.58	7.0	92	60	92
			L1210/0			7.0		60	98[i]
			Liver			7.0		60	0[i]
12	H	COC_6H_3-3-SO_2F-4-$CH(CH_3)_2$	L1210/DF8	0.85	0.14	1.7	92	60	51[i]
			L1210/0	1.2	0.20	1.7	85	60	58[i]
						0.20	50	60	0
			Liver			6.0		60	0

[a] The technical assistance of Diane Shea and Sharon Lafler with these assays is acknowledged. [b] Assayed with 6 μM dihydrofolate and 30 μM TPNH in pH 7.4 Tris buffer containing 0.15 M KCl as previously described.[4] [c] Incubated at 37° in pH 7.4 Tris buffer in the presence of 60 μM TPNH as previously described.[4] [d] I_{50} = concentration for 50% inhibition. [e] Estimated from $K_i = K_m[I_{50}]/[S]$ which is valid since $[S] = 6K_m = 6\ \mu M$ dihydrofolate; see ref 5, Chapter 10. [f] Estimated from $[EI] = [E_t]/(1 + K_i/[I])$ where [EI] is the amount of total enzyme (E_t) reversibly complexed.[6] [g] Data from ref 4. [h] From a six-point time study; see B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967). [i] Zero point determined by adding inhibitor to assay cuvette.[4]

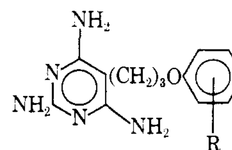
TABLE III
PHYSICAL PROPERTIES OF



No.	R[a]	% yield[b]	Mp, °C	Formula[d]
24a	4-Me	61	173–175	$C_8H_7FO_4S$
24b	4-C_3H_7-*i*	37	197–200	$C_{10}H_{11}FO_4S$
24c	4-Cl	60	193–197[c]	$C_7H_4ClFO_4S$
24d	2-Me	56	154–156	$C_8H_7FO_4S$
24e	2-Cl	51	147–150	$C_7H_4ClFO_4S$

[a] Position numbered from COOH at 1. [b] By method A from intermediate sulfonyl chlorides prepared by the method in ref 18; yield is for pure material recrystallized from EtOH–H_2O. Each compound showed an S-F band at 770–795 cm^{-1}. [c] Gavaert Photo-Producten N.V. [Belgian Patent 634,665 (1964)] reported mp 196°. [d] All compounds except **24c** were analyzed for C, H.

TABLE IV
PHYSICAL PROPERTIES OF



No.	R	% yield[a]	Mp, °C	Formula[g]
4	H	4[b,c]	146–151	$C_{13}H_{17}N_5O \cdot 2HOAc$
5	2-Cl	4[d]	179–180	$C_{13}H_{16}ClN_5O$
6	3-Cl	6[e]	253–257	$C_{13}H_{16}ClN_5O \cdot 0.5H_2SO_4 \cdot H_2O$[f]
7	2,3-Cl_2	11[e]	209–211	$C_{13}H_{15}Cl_2N_5O$
8	2,5-Cl_2	1.2[e]	262–279 dec	$C_{13}H_{15}Cl_2N_5O \cdot 0.5H_2SO_4$
16a	2-Cl-4-NO_2	21[b,c]	234–236	$C_{13}H_{15}ClN_6O_3$

[a] Over-all yield of pure material for three steps from **14** prepared by methods in ref 17. [b] Over-all yield from R-phenoxypropyl bromide. [c] Recrystallized from 10% HOAc in H_2O. [d] Recrystallized from C_6H_6. [e] Recrystallized from MeOEtOH–H_2O. [f] H: calcd, 5.31; found, H 4.73. [g] All compounds were analyzed for C, H, N.

had entered *para* to R_1 or *ortho* to R_2 was verified by their nmr spectra. For reaction with the amines (**20** and **21**), the benzoic acids (**24**) were converted to the acid chlorides with $SOCl_2$.

Experimental Section

All analytical samples had uv and ir spectra compatible with their assigned structures; each moved as a single spot on tlc on Brinkmann silica gel GF and gave combustion analyses for C, H, and N or F within 0.4% of theoretical. Melting points were

SCHEME I

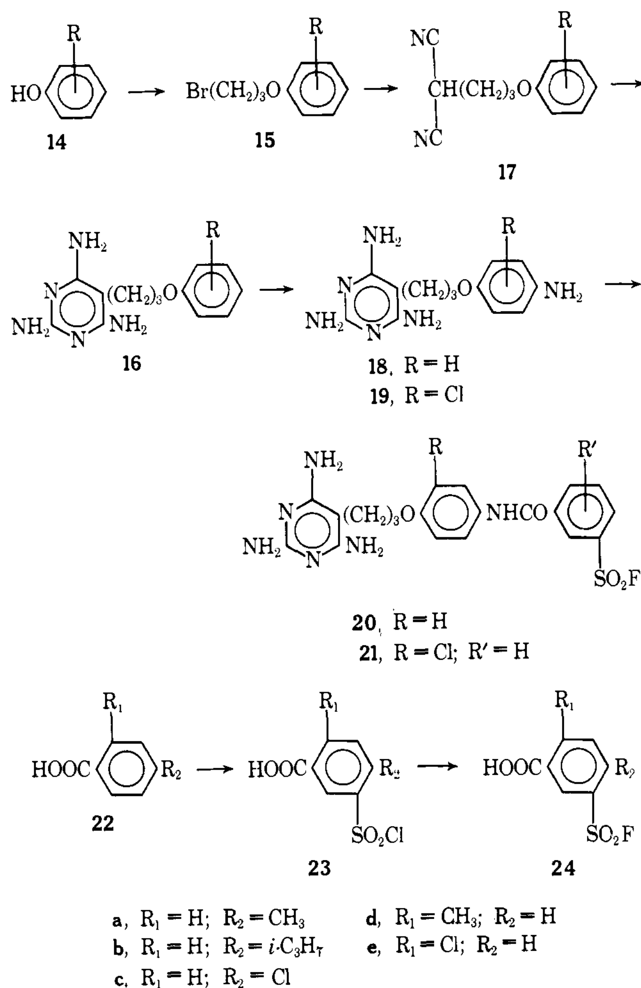
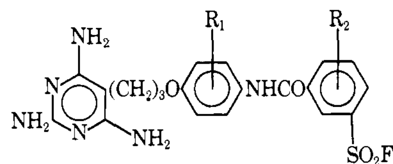


TABLE V

PHYSICAL PROPERTIES OF



No.	R_1[a]	R_2[b]	% yield[c]	Mp, °C	Formula[g]
9	2-Cl	H	50[d]	158–170	$C_{20}H_{20}ClFN_6O_4S \cdot EtSO_3H$
10	H	4-Me	46[e]	211–218	$C_{21}H_{23}FN_6O_4S \cdot 0.5H_2SO_4 \cdot 2H_2O$
11	H	2-Me	39[e]	[f]	$C_{21}H_{23}FN_6O_4S \cdot 0.5H_2SO_4 \cdot 2.5H_2O$
12	H	4-C_3H_7-i	33[e]	203–207	$C_{23}H_{27}FN_6O_4S \cdot 0.5H_2SO_4 \cdot 2H_2O$

[a] Numbering from phenoxy oxygen at 1. [b] Numbering from amide at 1. [c] Prepared by method described for **1**;[3] compound moved as a single spot on tlc with 1:4 EtOH–$CHCl_3$. [d] Recrystallized from EtOH–H_2O. [e] Recrystallized from MeOEtOH–H_2O. [f] Gradually decomposes over 150°. [g] All compounds were analyzed for C, H, F.

taken in capillary tubes on a Mel-Temp block and are uncorrected.

3-Fluorosulfonyl-4-methylbenzoic Acid (24a) (Method A).—To a stirred solution of 28.2 g (0.12 mole) of **23a**[18] in 40 ml of dioxane heated to reflux was added a solution of 10.4 g (0.18 mole) of KF in 11 ml of H_2O. After being refluxed with stirring for 40 min, the mixture was cooled and diluted with 200 ml of H_2O. The product was collected on a filter and washed with H_2O. Recrystallization from EtOH–H_2O gave 16 g (61%) of white needles, mp 173–175°. See Table III for additional data and compounds prepared by this method.

5-(4-Amino-2-chlorophenoxypropyl)-2,4,6-triaminopyrimidine (19).—A mixture of 3.5 g (10 mmoles) of **16a** (Table IV), 200 ml of 85% MeOEtOH–H_2O, and 0.14 g of PtO_2 was shaken with hydrogen at 2–3 atm for about 2 hr when reduction was complete. The filtered solution was evaporated *in vacuo*. Recrystallization from MeOEtOH–H_2O gave 2.5 g (78%) of nearly white crystals, mp 193–194°. *Anal.* ($C_{13}H_{17}ClN_6O$) C, H, N.

The physical properties of **9–12** are given in Table V.