Irreversible Enzyme Inhibitors. CXXXVIII.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 6-(*p*-Aminomethylphenethyl)-2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine with a Terminal Sulfonyl Fluoride

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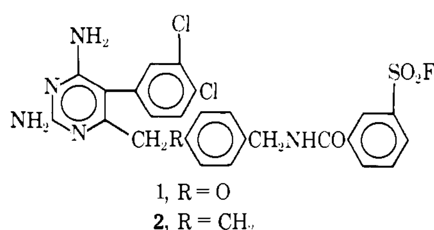
Six candidate irreversible inhibitors derived from the title compound were synthesized and evaluated with the dihydrofolic reductase from L1210/DF8 and L1210/0 mouse leukemias and from mouse liver. The six compounds at $<6 \times 10^{-8}$ *M* were good irreversible inhibitors of the enzyme from the three sources, but no specificity in irreversible inhibition of the enzyme from the three sources was seen. In contrast, 2,4-diamino-5-(3,4-dichlorophenyl)-6-[*p*-(*m*-fluorosulfonylbenzamidomethyl)phenoxymethyl]pyrimidine (1) showed excellent selectivity, being able to inactivate the L1210 mouse leukemia enzyme, but not the mouse liver enzyme;[5] however, the concentration of 1 required for irreversible inhibition was too high. The difference in selectivity with 1 and and its phenethyl analog (2) is believed to be due to the relatively fixed ground-state conformation of the 6 side chain of 2.

One of the major objectives of this laboratory has been the design, synthesis, and enzymic evaluation of candidate active-site-directed irreversible inhibitors[3] of dihydrofolic reductase which might show sufficient selectivity to inactivate the enzyme from a tumor, but not normal tissues. That **1** could irreversibly inhibit



the dihydrofolic reductase from L1210/FR8, L1210/DF8, and the parent L1210/0 line of mouse leukemia with no irreversible inhibition of the dihydrofolic reductase from mouse liver has been reported.[4,5] Although **1** showed this high tissue specificity,[6] it did not meet the arbitrary criteria[5] set for a compound to be a likely candidate for effective *in vivo* chemotherapy of L1210. Since **1** had $I_{50} = 0.37$ μM on the enzyme from L1210/DF8, it did not meet the first criterion; however, **1** did meet the second and third criteria. Although it is fairly easy to increase reversible binding by a factor of 4–10 by simple substitution, such substitution might lead to loss of irreversible inhibition or loss of specificity of irreversible inhibition.[7]

Two approaches were initially investigated to increase binding. The first approach was substitution on one or both benzene rings on the 6 side chain; although a good I_{50} was achieved, specificity was lost.[2] The second approach was based on the observation that replacement of the phenoxy oxygen of a compound related to **1** by CH_2 gave a satisfactory increase in reversible binding.[8] Therefore, **2** and some related compounds were synthesized for enzymic evaluation; the results are the subject of this paper.

Enzyme Results.—Evaluation of **2** (Table I) on the dihydrofolic reductase from L1210/DF8 showed that **2** was a 15-fold better reversible inhibitor than **1**; furthermore, at $2I_{50}$, **2** was an excellent irreversible inhibitor. In contrast to **1**, specificity was lost since **2** was now an excellent irreversible inhibitor of the enzyme from mouse liver. The position of the sulfonyl fluoride moiety (**3**) and the bridge between the benzenesulfonyl fluoride and phenyl moieties (**4**, **5**) were then modified. All three compounds (**3–5**) showed good I_{50}'s and could inactivate dihydrofolic reductase; unfortunately, no specificity was observed, the liver enzyme also being inactivated.

Introduction of a chlorine atom (**6**) *para* to the SO_2F moiety of **2** or a CH_3 (**7**) *ortho* to this SO_2F moiety of **2** gave little change in I_{50}. Furthermore, these changes failed to increase the specificity of **2**, both L1210 enzymes and the mouse liver enzyme still being inactivated by **6** and **7**.

Discussion

The fact that specificity is lost when the phenoxy oxygen of **1** is replaced by CH_2 (**2**) would at first glance seem incongruous. The difference in the number of allowable ground-state conformations between **1** and **2** is immense; to achieve rotation of the $-CH_2CH_2-$ moiety of **2** requires up to 2 kcal/mole due to proton interaction, whereas the $-CH_2O-$ moiety is free to rotate. Thus one might anticipate that **2** would have more specificity than **1** since there is considerably less freedom to rotate with **2**; therefore **1** might be expected to rotate easily to juxtapose the SO_2F moiety with the attacking nucleophilic group on the enzyme surface. However, the free rotation of the $-CH_2O-$ moiety of **1** has a second consequence. If the benzenesulfonyl

(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 N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 86 (1969).

(3) 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.

(4) B. R. Baker and P. C. Huang, *J. Med. Chem.*, **11**, 495 (1968), paper CXX of this series.

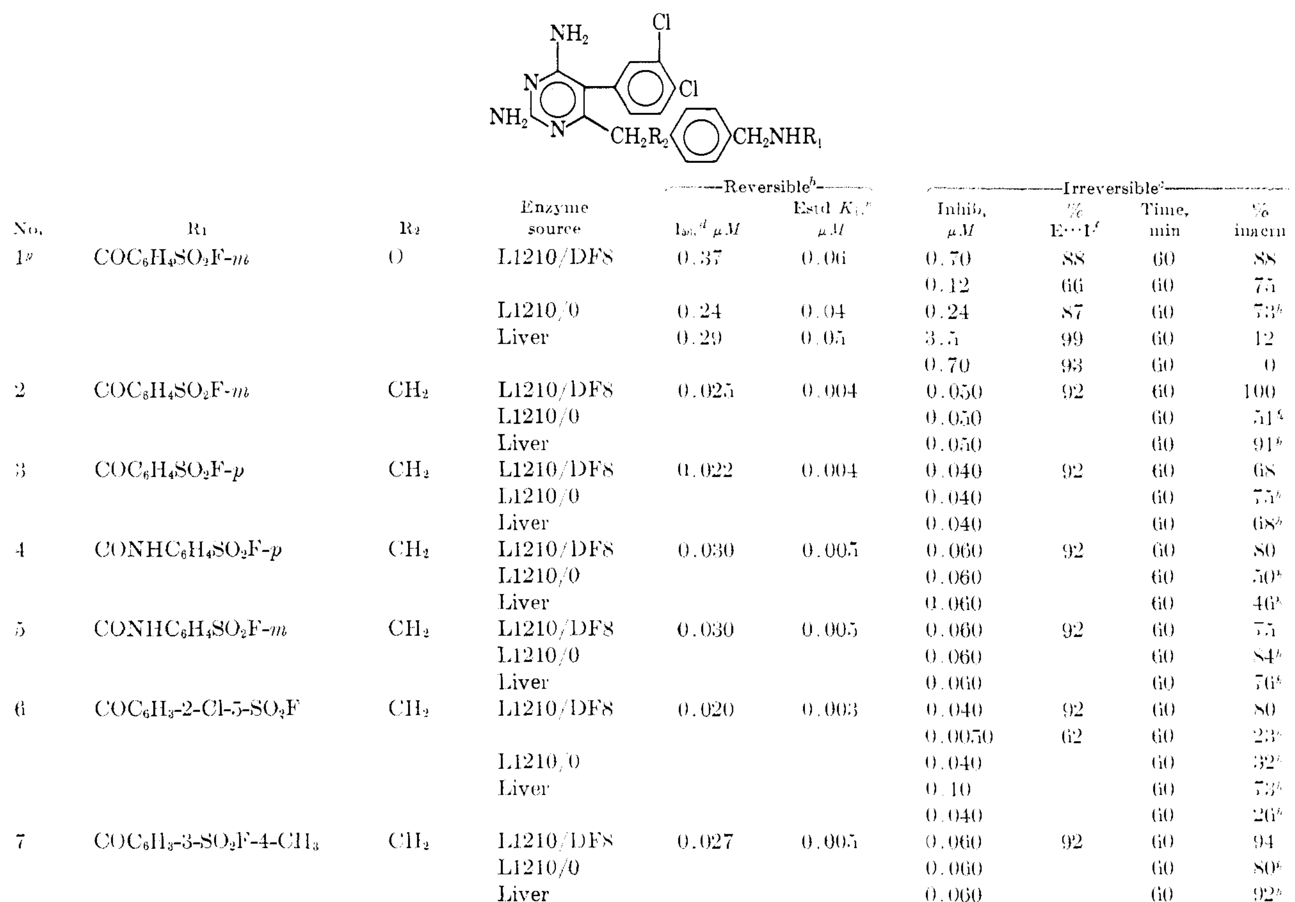
(5) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), paper CXXXIII of this series.

(6) Other data from this laboratory showed that **1** also is not an irreversible inhibitor of dihydrofolic reductase from mouse spleen, but showed weak irreversible inhibition of the enzyme from mouse intestine.[5]

(7) B. R. Baker and N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 74 (1969), paper CXXXIV of this series.

(8) B. R. Baker, P. C. Huang, and R. B. Meyer, Jr., *ibid.*, **11**, 475 (1968), paper CXVI of this series.

TABLE I
INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



				Reversible[b]		Irreversible[c]			
No.	R_1	R_2	Enzyme source	I_{50},[d] μM	Estd K_i,[e] μM	Inhib, μM	% E···I[f]	Time, min	% inactn
1[g]	$COC_6H_4SO_2F$-*m*	O	L1210/DF8	0.37	0.06	0.70	88	60	88
						0.12	66	60	75
			L1210/0	0.24	0.04	0.24	87	60	73[h]
			Liver	0.29	0.05	3.5	99	60	12
						0.70	93	60	0
2	$COC_6H_4SO_2F$-*m*	CH_2	L1210/DF8	0.025	0.004	0.050	92	60	100
			L1210/0			0.050		60	51[h]
			Liver			0.050		60	91[h]
3	$COC_6H_4SO_2F$-*p*	CH_2	L1210/DF8	0.022	0.004	0.040	92	60	68
			L1210/0			0.040		60	75[h]
			Liver			0.040		60	68[h]
4	$CONHC_6H_4SO_2F$-*p*	CH_2	L1210/DF8	0.030	0.005	0.060	92	60	80
			L1210/0			0.060		60	50[h]
			Liver			0.060		60	46[h]
5	$CONHC_6H_4SO_2F$-*m*	CH_2	L1210/DF8	0.030	0.005	0.060	92	60	75
			L1210/0			0.060		60	84[h]
			Liver			0.060		60	76[h]
6	COC_6H_3-2-Cl-5-SO_2F	CH_2	L1210/DF8	0.020	0.003	0.040	92	60	80
						0.0050	62	60	23[h]
			L1210/0			0.040		60	32[h]
			Liver			0.10		60	73[h]
						0.040		60	26[h]
7	COC_6H_3-3-SO_2F-4-CH_3	CH_2	L1210/DF8	0.027	0.005	0.060	92	60	94
			L1210/0			0.060		60	80[h]
			Liver			0.060		60	92[h]

[a] The technical assistance of Sharon Lafler, Diane Shea, and Carolyn Wade 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.[5] [c] Incubated at 37° in pH 7.4 Tris buffer in the presence of 60 μM TPNH as previously described.[5] [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 3, p 202. [f] Estimated from $[EI] = [E_t]/(1 + K_i/[I])$ where [EI] is the amount of total enzyme (E_t) reversibly complexed; see ref 3, Chapter 8. [g] Data from ref 5. [h] Zero point obtained by adding inhibitor to assay cuvette prior to addition of enzyme aliquot.[5]

moiety is reversibly complexed to the enzyme, then a freely rotating moiety such as the $-CH_2O-$ of **1** will adopt the ground-state conformation that gives maximum reversible binding to the enzyme; this conformation for maximum reversible binding could remove the SO_2F group from proper juxtaposition to an enzymic nucleophilic group, thus giving more specificity than could be obtained with **2**.

That an oxymethylene bridge with free rotation can eradicate irreversible inhibition when it replaces an ethylene bridge was previously seen in several cases.[5,9,10] It follows that if the number of allowable ground-state conformations were limited, then an irreversible inhibitor might emerge; such a prediction was reduced to practice.[11]

A second major point has solidified from the data in Table I. Introduction of CH_3 (**7**) or Cl (**6**) on the benzenesulfonyl fluoride moiety did not lead to specificity; in fact such changes on **1** led to a loss of specificity.[2] Therefore, the data on about 50 candidate irreversible inhibitors for dihydrofolic reductase with a substituent on the benzenesulfonyl moiety were reexamined.[2,7,12-17] In only four cases was the specificity for the tumor enzyme over liver enzyme beneficial,[14,15] a rather low return but worthwhile if other approaches fail. In the other cases, either specificity was lost or the irreversible effect on the enzyme was decreased. The only reason left for substitution on the benzensulfonyl moiety resides in the initial search for the first active-site-directed irreversible inhibitor of a given enzyme. A compound failing to show irreversible inhibition may show irreversible inhibition when the benzenesulfonyl fluoride ring is substituted; in the case of the search for the first irreversible inhibitor for an enzyme, selectivity is not the consideration of import.

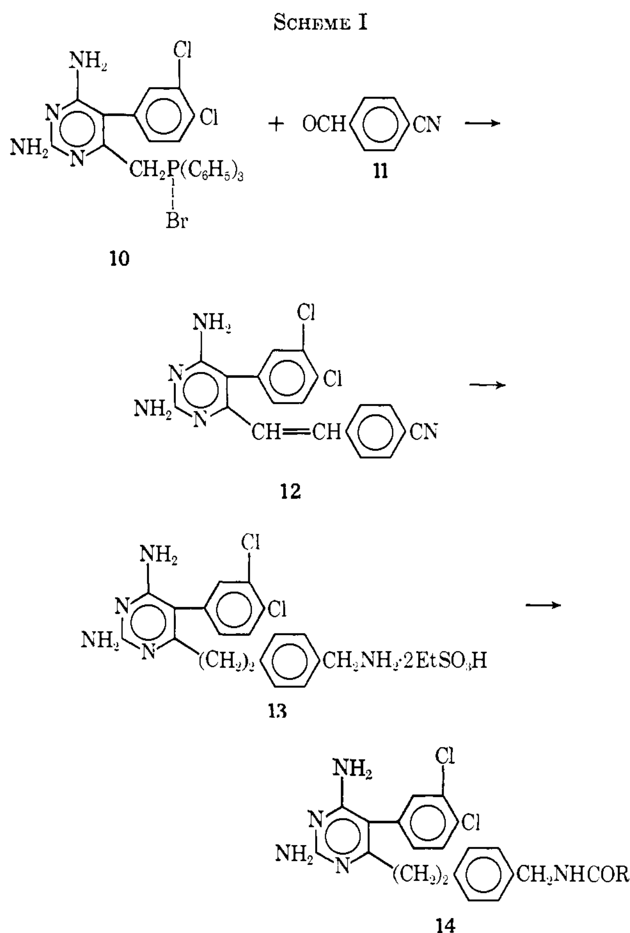
(9) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.
(10) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 666 (1968), paper CXXVII of this series.
(11) B. R. Baker and G. J. Lourens, *ibid.*, **12**, 95 (1969), paper CXL of this series.
(12) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 82 (1969), paper CXXXVI of this series.
(13) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 79 (1969), paper CXXXV of this series.
(14) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 677 (1968), paper CXXIX of this series.
(15) B. R. Baker and G. J. Lourens, *ibid.*, **12**, 101 (1969), paper CXLI of this series.
(16) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 104 (1969), paper CXLII of this series.
(17) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 108 (1969), paper CXLIII of this series.

From these considerations, it would appear at this time that the best chance for design of specificity would be in modification of the bridge between the benzenesulfonyl fluoride moiety and the pyrimidine portion of the inhibitor. Such studies with 6-substituted 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidines related to **1** and **2** and 1-phenyl-1,2-dihydrotriazines are continuing.

Chemistry.—The new candidate irreversible inhibitors in Table I can be generalized by structure **14**; the key intermediate was therefore the aliphatic amine **13**. Wittig condensation of **10**[12] with *p*-cyanobenzaldehyde (**11**) in DMF in the presence of 1 equiv of 1,5-diazabicyclo[4,3,0]nonene[18] afforded a 52% yield of pure **12** (Scheme I). Hydrogenation of **12** with a PtO_2 catalyst in the presence of 2 equiv of $EtSO_3H$ gave **13** as an amorphous powder which was uniform on tlc, but could not be further purified.

Compounds **2**, **3**, **6**, **7** were prepared from **13** by acylation with the appropriate benzoyl chloride in DMF in the presence of 3 equiv of Et_3N.[12] Compounds **4** and **5** were prepared by reaction of **13** with the appropriate O-(*p*-nitrophenyl)urethan[7] in the presence of Et_3N to remove the $EtSO_3H$ from **13**.

SCHEME I



Experimental Section

All analytical samples had proper uv and ir spectra; each 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. Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. The physical properties of **2–7** are listed in Table II.

TABLE II

PHYSICAL PROPERTIES OF

[2,4-diamino-5-(3,4-dichlorophenyl)pyrimidin-6-yl]-$(CH_2)_2$-C_6H_4-$CH_2NHR \cdot 0.5H_2SO_4$

No.	R	Method[a]	% yield[b]	Mp, °C dec[d]	Formula[e]
2	$COC_6H_4SO_2F$-*m*	A	47	180	$C_{26}H_{22}Cl_2FN_5O_3S \cdot 0.5H_2SO_4 \cdot 0.5H_2O$
3	$COC_6H_4SO_2F$-*p*	A	46	192	$C_{26}H_{22}Cl_2FN_5O_3S \cdot 0.5H_2SO_4$
4	$CONHC_6H_4SO_2F$-*p*	B	56	206	$C_{26}H_{23}Cl_2FN_6O_3S \cdot 0.5H_2SO_4$
5	$CONHC_6H_4SO_2F$-*m*	B	42	200	$C_{26}H_{23}Cl_2FN_6O_3S \cdot 0.5H_2SO_4$
6	COC_6H_3-2-Cl-5-SO_2F	A[c]	67	157	$C_{26}H_{21}Cl_3FN_5O_3S \cdot 0.5H_2SO_4 \cdot H_2O$
7	COC_6H_3-4-Me-3-SO_2F	A[c]	59	170	$C_{27}H_{24}Cl_2FN_5O_3S \cdot 0.5H_2SO_4$

[a] Method A was the same as method C previously described.[4] Method B was the same as method E previously described,[7] except 2 equiv of Et_3N was added to neutralize the $EtSO_3H$ salt of **13**. [b] Yield after recrystallization from MeOEtOH–H_2O. [c] For the requisite fluorosulfonylbenzoic acid see ref 16. [d] Melting gradually occurred over a wide range at the temperature indicated. [e] All compounds showed corrected analyses for C, H, F.

6-(*p*-Cyanostyryl)-2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine (12).—To a stirred solution of 3.05 g (5 mmoles) of **10**[12] and 0.66 g (5 moles) of 11 in 20 ml of DMF cooled in an ice bath and protected from moisture was added 0.62 g (5 mmoles) of 1,5-diazabicyclo[4,3,0]nonene.[18] After being stirred for 20 hr at ambient temperature, the mixture was diluted with 20 ml of H_2O. The product was collected on a filter, washed (H_2O), and recrystallized from EtOH–THF; yield 1.00 g (52%), mp 264–268° dec (block preheated to 230°). *Anal.* ($C_{19}H_{13}Cl_2N_5$) C, H, N.

6-(*p*-Aminomethylphenethyl)-2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine Bisethanesulfonate (13).—A mixture of 2.32 g (6.1 mmoles) of **12**, 100 ml of MeOEtOH, 1.37 g (13 mmoles) of $EtSO_3H$, and 0.12 g of PtO_2 was shaken with H_2 at 2–3 atm for 3 hr when reduction was complete. The filtered solution was evaporated *in vacuo* leaving 3.20 g (85%) of **13** as an amorphous solid that moved as a single spot on tlc in 1:1 EtOH–$CHCl_3$; $\lambda_{max}^{pH\ 1}$ 275 mμ, $\lambda_{max}^{pH\ 13}$ 290 mμ.

(18) H. Oediger, H. Kabbe, F. Möller, and K. Eiter, *Chem. Ber.*, **99**, 2012 (1966).