Irreversible Enzyme Inhibitors. CXX.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 6-Substituted 2,4-Diamino-5-phenylpyrimidines[3] with Tissue Specificity

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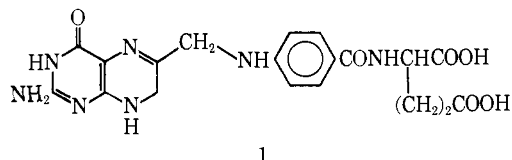
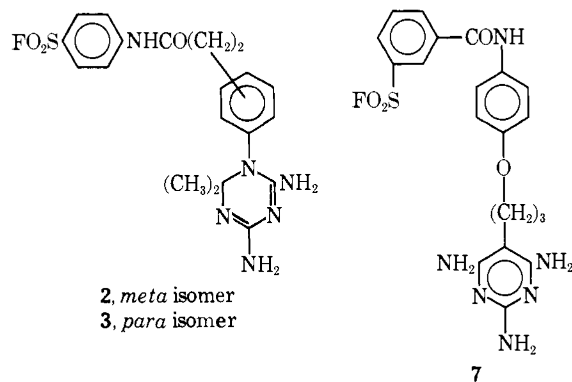
Twelve candidate irreversible inhibitors of dihydrofolic reductase derived from 2,4-diamino-5-(4-chlorophenyl)pyrimidine and its 3′-chloro derivative that are bridged from the 6 position of the pyrimidine to a sulfonyl fluoride leaving group have been synthesized and evaluated for tissue specificity. The most effective and selective of the twelve was 2,4-diamino-5-(3,4-dichlorophenyl)-6-[*p*-(*m*-fluorosulfonylbenzamidomethyl)phenoxymethyl]pyrimidine (**15**). At 1.2×10^{-7} *M*, **15** gave 90% inactivation of the dihydrofolic reductase from L1210/FR8 mouse leukemia with a half-life of 6 min; at 35×10^{-7} *M*, **15** gave barely perceptible inactivation of the mouse liver enzyme. The selective inhibition of the Walker 256 enzyme compared to rat liver by **15** was not as selective as in the case of the mouse enzymes, but was the most selective of the twelve candidate irreversible inhibitors.

Two important factors have emerged for the design of irreversible inhibitors that have the potential to inactivate the dihydrofolic reductase of cancer cells with less effect on this same enzyme from normal tissues.[4] Both of these factors utilize parts of the enzyme that are adjacent to the active site of the enzyme. A hydrophobic bonding region near where the 4-oxo group (or 8-N) of the substrate, dihydrofolate (**1**), resides on the enzyme has been found.[4-6] For example, the phenyl group of **2** and **3** and the adjacent two methylenes are complexed to the hydrophobic region on the enzyme, but this region then becomes more polar where the carboxanilide moiety resides.[4,7] Similarly, the phenoxypropyl group of **7** complexes to the hydrophobic region of the enzyme, but the *p*-benzamido moiety resides in a polar region of the enzyme.[8] A third type (**5**) has the chlorophenyl and phenoxymethyl groups complexed to the hydrophobic bonding region where the 3-chloro group of **5** projects toward the active site on the right side of the inhibitor.[3,9]

Extremely large differences in bonding to the hydrophobic regions of the dihydrofolic reductases from bacteria and mammalian liver have been found;[10] these 50,000-fold differences are adequate for a selective chemotherapeutic effect. In contrast, the largest difference in binding to the hydrophobic region of the enzyme from Walker 256 tumor and the liver of the rat was only 100-fold,[11] which is insufficient for chemotherapeutic use. These small differences in the hydrophobic bonding region can be greatly magnified by utilization of the bridge principle of specificity with irreversible inhibitors,[12] particularly if the group on the inhibitor forming a covalent bond with the enzyme is influenced in its juxtaposition to an attacking enzymic

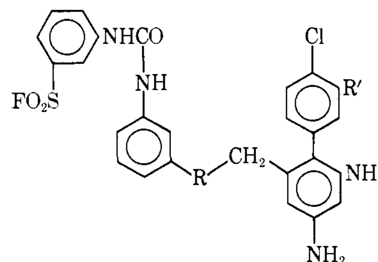


1



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

7



4, R = O; R′ = H
5, R = O; R′ = Cl
6, R = CH_2; R = H

(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 R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 489 (1968).

(3) Paper IV of this subseries; for paper III see B. R. Baker, P. C. Huang, and R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 475 (1968).

(4) For a review on the mode of binding to dihydrofolic reductase, see 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 X.

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

(6) (a) B. R. Baker, T. J. Schwan, J. Novotny, and B.-T. Ho, *ibid.*, **55**, 295 (1966); (b) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966).

(7) (a) B. R. Baker and B.-T. Ho, *J. Heterocycl. Chem.*, **2**, 335 (1965); (b) B. R. Baker, B.-T. Ho, and G. J. Lourens, *J. Pharm. Sci.*, **56**, 737 (1967), paper LXXXVI of this series; (c) B. R. Baker and G. J. Lourens, *ibid.*, **56**, 871 (1967).

(8) B. R. Baker, B.-T. Ho, J. K. Coward, and D. V. Santi, *ibid.*, **55**, 302 (1966).

(9) B. R. Baker and J. H. Jordaan, *J. Heterocycl. Chem.*, **4**, 31 (1967), paper LXXXIII of this series.

(10) (a) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965); (b) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, **1**, 126 (1965).

(11) (a) B. R. Baker, *J. Med. Chem.*, **11**, 483 (1968), paper CXVII of this series; (b) B. R. Baker and M. A. Johnson, *ibid.*, **11**, 486 (1968), paper CXVIII of this series.

(12) See ref 4, pp 172–184.

nucleophilic group by the hydrophobic bonding region.[13]

The sulfonyl fluoride group of **3** can form a covalent bond within the enzyme–inhibitor complex with the dihydrofolic reductase from Walker 256 rat tumor, rat liver, L1210/FR8 mouse leukemia, and pigeon liver. However, when the bridging group is moved to *meta* position (**2**) only the pigeon liver enzyme (of this group of four) is inactivated; this was the first example of selective irreversible inhibition of dihydrofolic reductase among vertebrate species.[14,15] When the position *ortho* to the sulfonyl fluoride group of **3** is substituted by methyl, tissue specificity is observed; this methyl derivative still rapidly inactivates the enzyme from L1210/FR8 mouse leukemia but has a barely perceptible effect on the mouse liver enzyme.[16] Another example is the sulfonyl fluoride **7**, which can rapidly inactive the dihydrofolic reductase from Walker 256 or L1210/FR8 with little effect on the enzyme from mouse or rat liver.[2] Similar observations have now been made with **4–6** and nine of their analogs (**7–15**); these results are the subject of this paper.

Enzyme Results.—The reversible and irreversible inhibition of dihydrofolic reductase from Walker 256, L1210/FR8, rat liver, and mouse liver are collated in Table I. The rate of irreversible inhibition (V) by an active-site-directed irreversible inhibitor is dependent upon the amount of the total enzyme (E_t) reversibly complexed (EI) by the inhibitor (I), *i.e.*, $V = k[EI]$;[17] thus, the relative amounts of [EI], rather than [I], must be considered when comparing inactivation rates by two different compounds or one compound at two different concentrations. Second, two different reactions between the enzyme and a sulfonyl fluoride can occur within the enzyme-inhibitor reversible complex; covalent bond formation may occur or the enzyme can catalyze the hydrolysis of the sulfonyl fluoide to the corresponding sulfonic acid which is no longer capable of irreversible inhibition.[18] If only covalent bond formation occurs, then a plot of time against the log of remaining enzyme is linear throughout 80–90% inactivation; in contrast, if enzyme-catalyzed hydrolysis of the sulfonyl fluoride occurs simultaneously, then line curvature will be seen where the rate of inactivation decreases with time due to the decreasing concentration of the irreversible inhibitor.[18]

The results with the Walker 256 and rat liver enzymes in Table I will be discussed first. With sufficient **4** to give 95% [EI], curvature was observed in the inactivation rate; above 40 min was required for 50% inactivation and the inactivation stopped at about 70%. Even more curvature was shown with the rat liver enzyme and sufficient **4** to give 95% [EI], the inactivation stopping at about 40%; thus, even though the tumor enzyme was inactivated to a greater extent than the liver enzyme, the difference was insufficient to be useful. Substitution of a 3′-chloro group (**5**) on **4** gave a better reversible inhibitor as expected;[4,19] however, the amount of hydrolysis of the sulfonyl fluoride, compared to the amount of inactivation, was increased with the Walker 256 enzyme to the point where **5** was almost ineffective as an irreversible inhibitor. In contrast, replacement of the phenoxy oxygen of **4** by CH_2 resulted in a compound (**6**) that gave better inactivation of the Walker 256 enzyme with less enzyme-catalyzed hydrolysis of the sulfonyl fluoride; however, little specificity was seen when compared to the inactivation of the rat liver enzyme by **6**.

When the *m*-ureidophenylsulfonyl fluoride moiety of **4** was moved to the *para* position, the resultant **7** had about the same rate curve as **4** for the inactivation of the Walker 256 enzyme. In contrast, **7** was superior to **4** as an irreversible inhibitor of the rat liver enzyme; thus, **7** was more effective on the rat liver enzyme than the Walker 256 enzyme. The latter effect of **7** was reversed when the 3′-chloro substituent (**8**) was introduced, *i.e.*, **8** was a considerably better irreversible inhibitor of the Walker 256 than liver enzyme; similar results were observed when the phenoxy group of **7** was changed to benzyl (**9**).

The analogs, **10–14**, have one NH of the ureido bridge removed; none of these were good irreversible inhibitors of the Walker 256 enzyme due to the increased ratio of rate of hydrolysis of the sulfonyl fluoride to the rate of irreversible inhibition. In two cases (**11, 12**), the compounds were better irreversible inhibitors of the liver enzyme than the tumor enzyme.

The most effective irreversible inhibitor in Table I of the Walker 256 enzyme was **15** where the two rings on the 6 position are bridged by $-CH_2NHCO-$. The latter bridge has two extra degrees of rotation compared to the $-NHCONH-$ bridge of **8**; the $-NHCONH-$ bridge is held coplanar to the two benzene rings by the π-orbital overlap of the carbonyl with the two rings, but both of the $-CH_2-N$ bonds are free to rotate. Thus **15** at an I_{50} concentration can give 87% inactivation of the Walker 256 enzyme, whereas **8** at an I_{50} concentration proceeds only to 33% inactivation. However, when the concentration of **15** was reduced to 0.03 μM, which is sufficient to form 50% [EI], then inactivation stops at 35% due to the simultaneous enzyme-catalyzed hydrolysis of the sulfonyl fluoride.[18] Furthermore, **15** is a considerably more effective irreversible inhibitor of the Walker 256 than the rat liver enzyme; at a concentration of 0.18 μM, **15** gives 87 and 41% inactivation of the Walker 256 and rat liver enzymes, respectively, in 60 min.

Although this separation in irreversible inhibition of Walker 256 and rat liver enzymes by **15** is encouraging, the separation is still insufficient. A similar separation in irreversible inhibition is seen with **8** and **9**, but a $5I_{50}$ concentration is required; at I_{50} concentration, **8** and **9** are rather ineffective irreversible inhibitors of the Walker 256 enzyme due to the competitive enzyme-catalyzed hydrolysis[18] of the sulfonyl fluoride group. Further studies on analogs of **8**, **9**, and **15** are underway (a) to separate further the relative effectiveness on Walker 256 enzyme compared to rat liver enzyme and (b) to increase the ratio of the rate of enzyme inactiva-

(13) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **55**, 1417 (1966), paper LXVII of this series.

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

(15) An earlier example of species specificity in irreversible inhibition of the dihydrofolic reductase from pigeon liver and *E. coli* B was reported by B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **56**, 660 (1967), paper LXXXVIII of this series.

(16) B. R. Baker and G. J. Lourens, manuscript in preparation.

(17) For the kinetic parameters of irreversible inhibition see (a) ref 4, pp 122–129; (b) B. R. Baker, W. W. Lee, and E. Tong, *J. Theor. Biol.*, **3**, 459 (1962).

(18) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968), paper CXIII of this series; (b) B. R. Baker and E. H. Erickson, *ibid.*, **11**, 245 (1968), paper CXV of this series.

(19) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **53**, 1137 (1964).

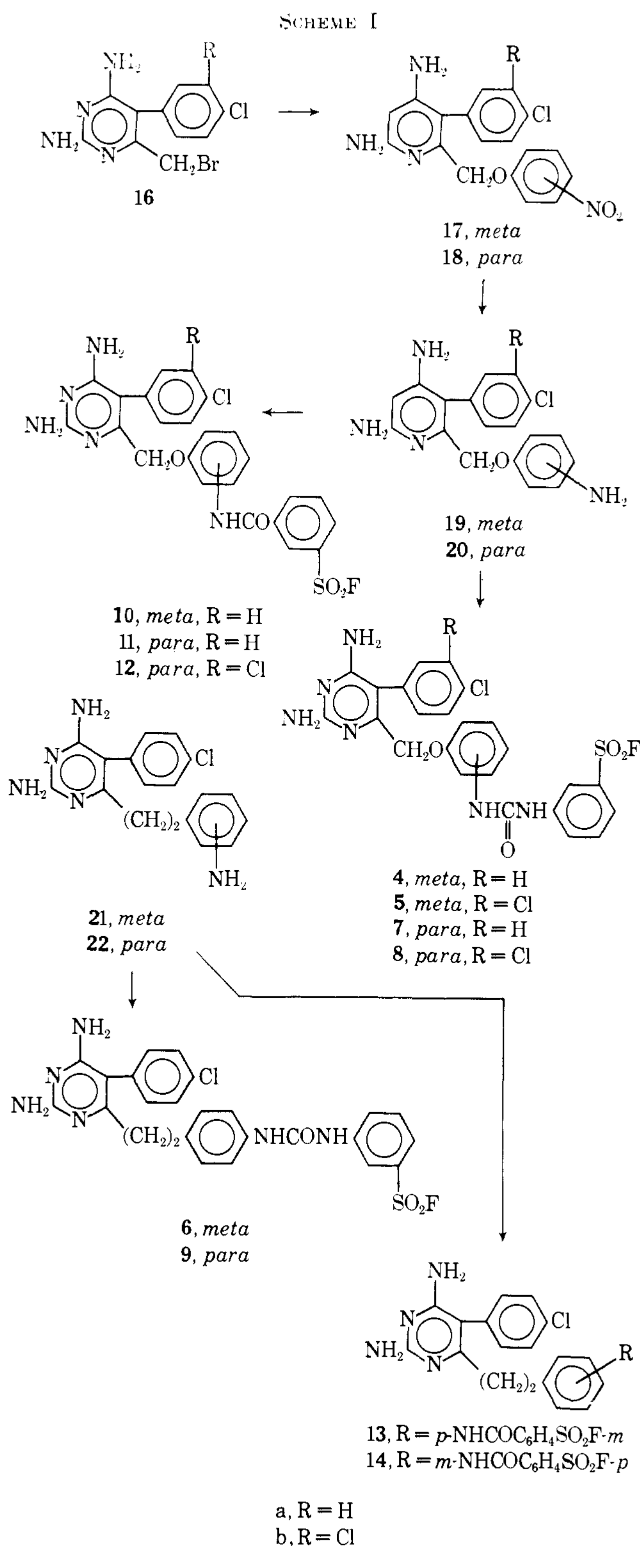
TABLE I: INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY

(2,4-diaminopyrimidine: 4-NH₂ replaced in structure; 5-(3-R₁-4-chlorophenyl)-6-CH₂R₂)

No.	R_1	R_2	Enzyme source	Reversible: I_{50},[b] µM	Reversible: Estd K_i $\times 10^6$ M[c]	Irreversible: Inhib concn, µM	Irreversible: E···I,[d] %	Irreversible: Time, min	Irreversible: Inactn,[e] %
4	H	$-O-C_6H_4-NHCONH-C_6H_4-SO_2F$	Walker 256[f]	3.5	0.58	16	95	120	68
						16	95	40[g]	50
			Rat liver	8.8	1.4	25	95	5, 60[g]	34, 39
			L1210/FR8[f]	3.3	0.55	16	96	13, 60[g]	50, 90
						3.3	87	20, 60[g]	50, 71
			Mouse liver			3.3		60[g]	<10
5	Cl	$-O-C_6H_4-NHCONH-C_6H_4-SO_2F$	Walker 256[f]	0.56	0.093	2.8	97	60	30
			Rat liver	0.70	0.12	3.5	97	60	0
			L1210/FR8[f]	1.4	0.23	1.4	87	6, 30[g]	50, 80
						0.23	50	30, 60[g]	50, 60
			Mouse liver			1.4		60	0
						7.0		3, 60[g]	52, 52
6	H	$-CH_2-C_6H_4-NHCONH-C_6H_4-SO_2F$	Walker 256[f]	0.77	0.13	3.8	97	5, 60[g]	50, 95
			Rat liver	0.35	0.060	1.7	97	60	67
			L1210/FR8[f]	1.6	0.27	1.6	87	4, 30[g]	50, 92
			Mouse liver			1.6		8, 60[g]	15, 15
						8.0		<1, 60[g]	60, 60
7	H	$-O-C_6H_4-NHCONH-C_6H_4-SO_2F$	Walker 256	1.2	0.20	5.8	97	5, 60[g]	50, 60
			Rat liver	1.6	0.26	8.2	97	9, 30[g]	50, 86
			L1210/FR8	6.0	1.0	6.0	87	7, 30[g]	50, 84
						1.0	50	18, 60	50, 75
			Mouse liver			6.0		60[g]	0
						25		3, 60[g]	33, 33
8	Cl	$-O-C_6H_4-NHCONH-C_6H_4-SO_2F$	Walker 256	0.15	0.025	0.73	97	7, 60[g]	50, 86
						0.15	87	9, 60[g]	33, 33
			Rat liver	0.16	0.025	0.80	97	5, 60[g]	28, 28
			L1210/FR8	1.0	0.16	1.0	87	6, 30[g]	50, 85
						0.20	55	13, 18, 60[g]	50, 61, 61
			Mouse liver			1.0		6, 60[g]	43, 43
9	H	$-CH_2-C_6H_4-NHCONH-C_6H_4-SO_2F$	Walker 256	0.24	0.040	1.2	97	9, 60[g]	50, 90
						0.24	87	20, 60[g]	40, 40
			Rat liver	0.36	0.060	1.8	97	60	33
			L1210/FR8	0.71	0.12	0.71	87	24, 60[g]	50, 82
						0.12	50	22, 60[g]	50, 65
			Mouse liver			0.71		60	0
						3.5		5, 60[g]	50, 50
10	H	$-O-C_6H_4-NHCOC_6H_4SO_2F$-*m*	Walker 256	32	5.3	25	83	60	0
			L1210/FR8	>50	>8	25	<76	60	36
11	H	$-O-C_6H_4-NHCO-C_6H_4-SO_2F$	Walker 256	4.7	0.80	25	97	30[g]	50
			Rat liver	4.1	0.70	25	97	9, 60[g]	50, 93
			L1210/FR8	50	8.3	25	75	2, 30[g]	50, 91
			Mouse liver			25		60[g]	0
12	Cl	$-CH_2-C_6H_4-NHCO-C_6H_4-SO_2F$	Walker 256	0.26	0.043	1.2	97	10, 60[g]	50, 50
			L1210/FR8	0.87	0.14	1.0	93	16, 60[g]	50, 78
			Mouse liver			1.0		60[g]	0
						5.0		60[g]	<10
13	H	$-O-C_6H_4-CH_2NHCO-C_6H_4-SO_2F$	Walker 256	0.38	0.063	2.0	97	4, 60[g]	30, 30
			Rat liver	0.34	0.057	1.8	97	60[g]	0
			L1210/FR8	0.58	0.097	2.9	97	5, 60[g]	42, 42
14	H	$-CH_2-C_6H_4-NHCOC_6H_4SO_2F$-*p*	Walker 256	1.3	0.22	6.4	97	60	0
			L1210/FR8	5.4	0.90	5.4	87	25, 60[g]	50, 68
			Mouse liver			5.4		2, 60[g]	17, 17
15	Cl	$-O-C_6H_4-NHCO-C_6H_4-SO_2F$	Walker 256	0.18	0.03	0.18	87	6, 60[g]	50, 87
						0.030	50	10, 60[g]	34, 35
			Rat liver			0.18		4, 60[g]	21, 41
			L1210/FR8	0.70	0.12	0.70	87	2, 60[g]	50, 97
						0.12	50	8, 60[g]	50, 90
			Mouse liver			0.70		60[g]	0
						3.5		2, 60[g]	12, 12

[a] The technical assistance of Barbara Baine, Jean Reeder, and Diane Shea is acknowledged. [b] I_{50} = concentration necessary for 50% inhibition when assayed with 6 µM dihydrofolate and 30 µM TPNH at pH 7.4 as previously described.[14] [c] Calcd from $K_i = K_m \cdot [I_{50}]/[S]$ where $[S] = 6 \times 10^{-6}$ M and $K_m = 1 \times 10^{-6}$ M.[4] [d] [EI] = the per cent of total enzyme, $[E_t]$, complexed by [I]; calculated from $[EI] = [E_t]/(1 + K_i/[I])$.[17] [e] Enzyme remaining after incubation at 37° with [I] and 60 µM TPNH at pH 7.4; inactivation performed as previously described.[14] [f] Data previously reported.[3] [g] From a six-point time study.[14]

SCHEME I



SCHEME II

16b → 23 (CH_2O–C_6H_4–CN) → 24 (CH_2O–C_6H_4–$CH_2NH_2 \cdot 2HCl$) → 15 (CH_2O–C_6H_4–CH_2NH–C(=O)–C_6H_4–SO_2F)

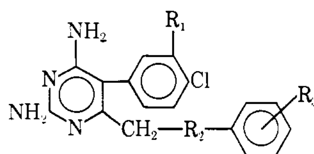
tion to the rate of enzyme-catalyzed hydrolysis of the inhibitor; such studies on 1-phenyl-1,2-dihydro-*s*-triazine involving substitution on the phenyl group bearing the sulfonyl fluoride moiety have been effective.[16]

The most effective irreversible inhibitors of the L1210/FR8 enzyme were **15** and **6**–**8**. Four parameters should be considered with sulfonyl fluorides in discussing their relative effectiveness: (a) the sulfonyl fluoride at a K_i concentration (50% [EI] complex) should be able to inactivate the enzyme >80%, thus showing a favorable ratio of enzyme inactivation to enzyme-catalyzed hydrolysis of the sulfonyl fluoride; (b) the faster the inactivation, the more effective is the irreversible inhibitor; (c) the lower the K_i of the sulfonyl fluoride, the lower will be the concentration necessary for 50% [EI] complex; and (d) the compound should be more effective on the tumor than the liver enzyme. For example, **11** meets parameters a, b, and d on the L1210/FR8 enzyme, but has the second highest K_i of the compounds in Table I. In contrast, **15** is one of the best reversible inhibitors in Table I, can inactivate the L1210/FR8 enzyme at a K_i concentration with a half-life of 8 min, and has little effect on the mouse liver enzyme at 30 times the concentration that is effective on the L1210/FR8 enzyme. In order for **15** to be effective *in vivo* the following additional requirements must be met: (a) the compound should dissolve with sufficient rapidity to give an intracellular concentration of 1×10^{-7} *M* and (b) the compound should have minimal effects on the dihydrofolic reductases from other normal tissues in addition to the liver. The second requirement is best investigated by assay in the intact animal. The first requirement can be improved by using a more soluble salt, making structural changes that lower the K_i so that the required concentration for 50% [EI] is lowered, and finally making structural changes that will increase solubility. For example, ethanesulfonate and hydroxyethanesulfonate (isethionate) salts are considerably more soluble than sulfates; replacement of the phenoxy group by benzyl (compare **4** and **6**) lowers the I_{50} by five- to tenfold; removal of the 4-chloro group of **15** would have no effect on the I_{50}[19] but should increase solubility tenfold.[18a] Further studies on these requirements are underway.

Compound **6** is not quite as effective an irreversible inhibitor of the L1210/FR8 enzyme as **15**; a higher concentration of **6** is required and it is not as selective with respect to the mouse liver enzyme. Compound **8**

TABLE II

PHYSICAL PROPERTIES OF



No.	R_1	R_2	R_3	Method	Yield, %	Mp, °C dec	Formula	Analyses
7	H	O	*p*-$NHCONHC_6H_4SO_2F$-*m*	A	81[a]	150–152	$C_{24}H_{20}ClFN_6O_4S \cdot 0.5H_2O$	C, H; N[b]
8	Cl	O	*p*-$NHCONHC_6H_4SO_2F$-*m*	B	52[c]	217–219	$C_{24}H_{19}Cl_2FN_6O_4S \cdot 0.5H_2SO_4 \cdot 0.5H_2O$	C, H, N
9	H	CH_2	*p*-$NHCONHC_6H_4SO_2F$-*m*	B	52[c]	213–215	$C_{25}H_{22}ClFN_6O_3S \cdot 0.5H_2SO_4 \cdot 1.5H_2O$	C, H, N
10	H	O	*m*-$NHCOC_6H_4SO_2F$-*m*	C	55[a]	184–188	$C_{24}H_{19}ClFN_5O_4S \cdot 0.5H_2SO_4$	C, H, N
11	H	O	*p*-$NHCOC_6H_4SO_2F$-*m*	C	56[a]	220–225	$C_{24}H_{19}ClFN_5O_4S \cdot 0.5H_2SO_4 \cdot H_2O$	C, H, N
11	H	O	*p*-$NHCOC_6H_4SO_2F$-*m*	C	56[a]	220–225	$C_{24}H_{19}ClFN_5O_4S \cdot 0.5H_2SO_4 \cdot H_2O$	C, H, N
12	Cl	O	*p*-$NHCOC_6H_4SO_2F$-*m*	C	58[b]	199–202	$C_{24}H_{18}Cl_2FN_5O_4S \cdot 0.5H_2SO_4 \cdot 1.5H_2O$	C, H; N[d]
13	H	CH_2	*p*-$NHCOC_6H_4SO_2F$-*m*	C	67[b]	196–200	$C_{25}H_{21}ClFN_5O_3S \cdot 0.5H_2SO_4 \cdot H_2O$	C, H, N
14	H	CH_2	*m*-$NHCOC_6H_4SO_2F$-*p*	C	60[b]	235–238	$C_{25}H_{21}ClFN_5O_3S \cdot 0.5H_2SO_4$	H, N; C[e]
15	Cl	O	*p*-$CH_2NHCOC_6H_4SO_2F$-*m*	C[f]	50[b]	175–178	$C_{25}H_{20}Cl_2FN_5O_4S \cdot 0.5H_2SO_4 \cdot 0.5H_2O$	C, H, F

[a] Recrystallized from EtOH–H_2O. [b] N: calcd, 15.2; found, 14.7. [c] Recrystallized from MeOEtOH–H_2O. [d] N: calcd, 10.9; found, 10.2. [e] C: calcd, 52.2; found, 52.8. [f] Two equivalents of Et_3N was added to neutralize the dihydrochloride salt of **24**.

is almost as effective a reversible inhibitor of the L1210/FR8 enzyme as **15** but is not as selective as **15**. Conversely, **7** is as selective as **15**, but requires a fivefold higher concentration than **15** to give the same amount of [EI].

Chemistry.—The synthesis of three (**4–6**) of the twelve compounds in Table I have been previously described.[3] For synthesis of the remaining nine compounds (**7–15**), six intermediate amines (**19a**, **20a**, **20b**, **21**, **22**, **24**) were required; of these, the synthesis of **19a** and **20a** from **16**,[3] and **21**[3] and **22**[9] from 5-(*p*-chlorophenyl)-2,4-diaminopyrimidine-6-carboxaldehyde has been previously described. The two remaining amines were synthesized as follows.

Condensation of the 6-bromomethylpyrimidine (**16b**) with sodium *p*-nitrophenolate in DMF afforded the *p*-nitrophenyl ether (**18b**), which was catalytically reduced with a PtO_2 catalyst to **20b** (Scheme I). Similarly, alkylation of *p*-cyanophenol with **16b** afforded the *p*-cyanophenyl ether (**23**) which was catalytically reduced in acid solution to the benzylamine derivative (**24**) (Scheme II).

The nine sulfonyl fluorides can be divided into two classes. The *m*-fluorosulfonylphenylureido types (**7–9**) were prepared by reaction of the appropriate arylamine with *m*-fluorosulfonylphenyl isocyanate in DMF. The fluorosulfonylbenzamido types (**10–14**) were prepared by reaction of the appropriate amine with *m*- or *p*-fluorosulfonylbenzoyl chloride in DMF with HOAc to weakly protonate the pyrimidine system[20] using the pyrimidine as the HCl acceptor; **15** was prepared similarly with Et_3N as an acid acceptor.

Experimental Section[21]

2,4-Diamino-5-(3,4-dichlorophenyl)-6-(*p*-nitrophenoxymethyl)pyrimidine (18b).—A mixture of 700 mg (2 mmoles) of **16b**,[3] 400 mg of sodium *p*-nitrophenolate·$2H_2O$, and 5 ml of DMF was stirred for 1 hr at ambient temperature, then at 60° for 5 hr. The cooled mixture was diluted with 15 ml of H_2O. The product was collected on a filter and washed with H_2O. Recrystallization from EtOH–H_2O gave 620 mg (77%) of nearly white crystals, mp 213–214°. *Anal.* ($C_{17}H_{13}Cl_2N_5O_3$)N.

6-(*p*-Aminophenoxymethyl)-2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine (20b) Dihydrochloride.—A solution of 550 mg (1.4 mmoles) of **18b** in 100 ml of MeOEtOH was shaken with H_2 at 2–3 atm in the presence of 50 mg of PtO_2 for 2 hr when reduction was complete. The filtered solution was evaporated *in vacuo* and the residue was recrystallized from EtOH–petroleum ether (bp 30–60°); yield 450 mg (85%) of **20b**, mp 216–218° dec, that was suitable for further transformations. For analysis a sample was converted to the dihydrochloride by solution in EtOH and addition of 1 *N* HCl; white crystals, mp 287–289° dec. *Anal.* ($C_{17}H_{15}Cl_2N_5O \cdot 2HCl \cdot 2H_2O$) C, H, N.

6-(*p*-Cyanophenoxymethyl)-2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine (23).—To a solution of 240 mg (2 mmoles) of *p*-cyanophenol and 110 mg (2 mmoles) of NaOMe in 5 ml of DMF was added 700 mg (2 mmoles) of **16b**.[3] The mixture was stirred in a bath at 60° for 6 hr, then cooled and diluted with 15 ml of H_2O. The product was collected on a filter, then washed with H_2O. Recrystallization from MeOEtOH–EtOH gave 620 mg (80%) of white needles, mp 221–222°. *Anal.* ($C_{18}H_{13}Cl_2N_5O \cdot 0.75H_2O$) C, H, N.

6-(*p*-Aminomethylphenoxymethyl)-2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine Dihydrochloride (24).—To a solution of 640 mg (1.6 mmoles) of **23** in 100 ml of MeOEtOH were added 0.60 ml of 6 *N* HCl and 50 mg of PtO_2. The mixture was shaken with H_2 at 2–3 atm for 10 hr when reduction was complete. The filtered solution was evaporated *in vacuo* and the residue was crystallized from EtOH. Recrystallization from EtOH gave 590 mg (79%) of white crystals, mp 230–232 dec. *Anal.* ($C_{18}H_{17}Cl_2N_5O \cdot 2HCl \cdot H_2O$) C, H, N.

5-(*p*-Chlorophenyl)-2,4-diamino-6-[*p*-(*m*-fluorosulfonylphenylureido)phenoxymethyl]pyrimidine (7) (Method A).—To a stirred solution of 171 mg (0.5 mmole) of **20a**[3] in 0.8 ml of dioxane cooled in an ice bath was added dropwise a solution of 101 mg (0.5 mmole) of *m*-fluorosulfonylphenyl isocyanate in 0.5 ml of

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(21) All analytical samples moved as a single spot on tlc, had ir and uv spectra in agreement with their assigned structures, and gave combustion values within 0.4% of theoretical unless otherwise indicated. Melting points were taken in capillary tubes on a Mel-Temp block and those below 230° are corrected.

dioxane over a period of about 5 min. After being stirred for 15 min in the ice bath and 20 min at ambient temperature, the solution was diluted with 5 ml of petroleum ether. An oil separated which solidified on trituration with fresh petroleum ether. Recrystallization from EtOH–H_2O gave 220 mg (81%) of white crystals, mp 150–152 dec. See Table II for additional data.

2,4-Diamino-5-(3,4-dichlorophenyl)-6-[*p*-(*m*-fluorosulfonylphenylureido)phenoxymethyl]pyrimidine (8) Hemisulfate (Method B).—To a stirred solution of 113 mg (0.3 mmole) of **20b** in 1 ml of DMF cooled in an ice bath was added a solution of 60 mg (0.3 mmole) of *m*-fluorosulfonylphenyl isocyanate in 0.5 ml of DMF. After 15 min at 0° and 15 min at ambient temperature, the solution was treated with 3 ml of 0.5 N H_2SO_4. The product was collected on a filter and washed with H_2O. Recrystallization from MeOEtOH–H_2O gave 100 mg (52%) of white powder, mp 217–219° dec. See Table II for additional data.

Method C was the same as method B except that 1.1 mmoles of HOAc was added to the DMF for each millimole of pyrimidine.

Potential Anticancer Agents. IV. Nitrogen Mustards of Methylbenzoic Acids

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The synthesis of the title compounds is described. Physical and biological data are presented. 3-[N,N-Bis-(2-chloroethyl)amino]-4-methylbenzoic acid exhibited the highest antitumor activity, together with a low hematopoietic toxicity and was submitted to therapeutic trials. The relationship between the hydrolysis rate of the nitrogen mustards and the basicity of the respective unsubstituted amines was studied and the effects of substituents *ortho* to the nitrogen mustard group are pointed out.

One of the main limiting factors in cancer chemotherapy with alkylating agents is their great hematopoietic toxicity. Our investigations started from the hypothesis[1] that one of the structural features that may contribute to improve the therapeutic index of the aromatic nitrogen mustards, is the presence in the molecule of a "biologically compatible" chemical function, which could exert a strong −M and/or −I effect. Enhancing of the selectivity would then result both from reducing the reactivity of the nitrogen mustard group and from increasing the possibilities of interaction between drug and receptors.

The choice of the carboxyl group as such a function was based on the following pharmacological considerations: (a) the nitrogen mustards of arylcarboxylic acids[2] and aralkylcarboxylic acids[3] (*e.g.*, 4-[*p*-bis(2-chloroethyl)aminophenyl]butyric acid) displayed strong activity against a series of experimental tumors and in therapeutic trials;[4] (b) the carboxyl group confers to 4-[*p*-N,N-bis(2-chloroethyl)aminophenyl]butyric acid an increased selectivity for proteins;[5] (c) diffusion through cellular membranes seems to be promoted by un-ionized carboxyl;[6] benzoic acids (pK_a ranges 5–6) partially fulfill this requirement at physiological pH.

The isomeric nitrogen mustards of benzoic acid,[2] compounds with low chemical reactivity [4% hydrolysis in 0.5 hr in 50% acetone at 66° for the *meta* isomer (ref 6b, p 153)], showed moderate inhibitions against Walker 256 carcinosarcoma (ref 6b, p 123). However, testing against Jensen sarcoma gave inhibitions exceeding 85%,[7] an unusually high activity for the low chemical reactivity of the compounds, which prompted us to ascribe special "carrier" properties to the N-substituted aminobenzoic acids and to continue the investigations in this structural area.

Since carboxyl groups induce too strong a decrease in the chemical reactivity of the nitrogen mustard function (pK_a of aniline, 4.57; pK_a of methyl *o*-, *m*-, and *p*-aminobenzoates, 2.32, 3.57, and 2.49, respectively[8]), we tried to restore it partially by introducing on the benzene nucleus a third, electron-repelling group, which could increase the basicity of the nitrogen atom (pK_a of methyl aminotoluic esters, 2.03–4.06; see Table II). Thus, the ten isomeric nitrogen mustards of methylbenzoic acids were synthesized.

The cancerostatic screening revealed strong antitumor properties for all ten isomers, the best results being obtained with 3-[N,N-bis(2-chloroethyl)amino]-4-methylbenzoic acid (IVj) whose pharmacological behavior was sufficiently promising for clinical trials. The preliminary results are in some respects superior to those of some alkylating drugs in use in cancer chemotherapy (melphalan, thioTEPA), mainly in view of the low leukopenic effect at therapeutic doses.

It is of interest to note, that in IVj, the nitrogen mustard group is *meta* to carboxyl. The advantage of IVj over the other isomers is in accord with some new data on the isomeric nitrogen mustards of benzoic acids,[7,9] the best therapeutic ratio being obtained with the *meta* isomer; a similar observation was made in the case of the *meta* and *para* isomers of melphalan[10a] and of the isomeric nitrogen mustards of β-phenylalanine[10b,c] and of phenylglycine.[10d]

Synthesis.—The general procedure (Scheme I) starts from the aminomethylbenzoic esters (I) which are hydroxyethylated with ethylene oxide in glacial acetic acid and then chlorinated by means of thionyl chloride or phosphorus oxychloride. Acid hydrolysis (concentrated HCl, reflux) of the esters (III) gave the

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