Irreversible Enzyme Inhibitors. 196.[†,‡] Active-Site-Directed Irreversible Inhibitors of Dihydrofolate Reductase Derived from 1-(4-Benzyloxy-3-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine and Bearing a Terminal Phenyl Sulfonate Group

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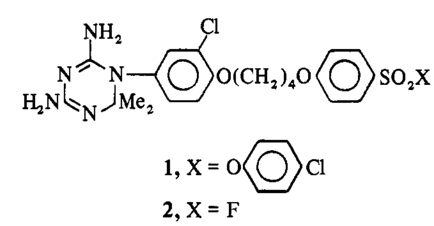
Twenty derivatives of the title compound containing a terminal, substituted phenyl sulfonate group in place of the previously studied sulfonyl fluoride were prepared and evaluated as active-site-directed irreversible inhibitors of dihydrofolate reductase. All of these sulfonate esters proved to be excellent reversible inhibitors of the enzyme, and several were at least as active against L1210 leukemic cell culture as the corresponding sulfonyl fluoride. However, none of the compounds showed the desired potency or tissue selectivity of irreversible inhibition.

Previous work from this laboratory has demonstrated that diaminodihydrotriazines and diaminopyrimidines bridged to a terminal sulfonyl fluoride are capable of irreversibly inactivating dihydrofolate reductase, sometimes in a tissue-specific manner.[1b-3] Because of the apparent metabolic hydrolysis of sulfonyl fluorides *in vitro*[4,5] and *in vivo*,[5] we undertook an investigation of other reactive groups capable of forming a covalent bond with a nucleophilic group on the enzyme. Ideally, the functional group should be reactive enough to inactivate the enzyme at a reasonable rate but sufficiently stable to reach its site of action *in vivo* without metabolic degradation.

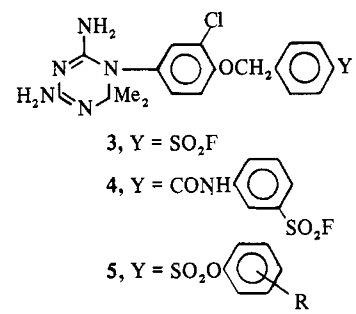
It has already been shown that 1[6] is a potent, though nonselective, irreversible inhibitor of dihydrofolate reductase. However, the apparent membrane transport of 1 (ED_{50}/I_{50} = 7)[#] was relatively poor compared to that of the corresponding sulfonyl fluoride **2** (ED_{50}/I_{50} = 0.02).[6]



Compound 3[8] was chosen as the model for designing sulfonate ester analogs. This triazine was known to be a good irreversible inhibitor of dihydrofolate reductase with some selectivity and moderately good transport.[8] Furthermore, the excellent transport shown by **4** (ED_{50}/I_{50} = 0.05)[9] suggested that the structurally similar **5**, with appropriate R substituents, might also be transported effectively.

Consequently, a series of triazines of type **5** was prepared for evaluation. The effect of substituents on the phenoxy moiety with respect to irreversible inhibition and membrane transport was of particular interest. The results of this study are presented below.

Biological Results. A comparison of the inhibition of dihydrofolate reductase and activity against L1210 mouse



leukemia cells in culture by the sulfonate esters of type **5** (**6–25**) and the corresponding sulfonyl fluoride (**3**) is presented in Table I. All of the sulfonate esters were excellent reversible inhibitors; the observed I_{50} was generally less than 0.01 *μM*.

In the case of a few compounds, no meaningful value could be assigned for irreversible inhibition of the rat liver enzyme, even after a considerable number of runs. The reasons for this observed variability are not understood.

None of the sulfonate esters gave consistently greater than 75% inactivation of the Walker 256 dihydrofolate reductase, nor did any show significantly selective irreversible inhibition of the tumor enzyme compared to the liver enzyme. In general, compounds having the most hydrophobic substituents on the phenoxy group (*e.g.*, **10**, **14–16**) tended to give the lowest amount of irreversible inhibition. No correlation was observed between the electron-withdrawing or -donating effects of the substituents and the extent of irreversible inhibition.

The question naturally arose as to whether the failure of the inhibitors to give a high degree of irreversible inhibition of dihydrofolate reductase was due simply to a slow rate of reaction with the enzyme while bound at the active site or tc destruction of the inhibitor during the course of the incubation. Time-course studies on the inactivation of Walker 256 dihydrofolate reductase by **15**, **17**, and **20** indicated that nearly the maximum extent of inactivation (even if only 30%) had occurred within 8 to 15 min after the start of incubation; very little additional irreversible inhibition occurred between 15 and 60 min. This suggests that the bulk of the fraction of inhibitor which had not covalently linked with the enzyme during the first 15 min had been destroyed, presumably by hydrolysis of the sulfonate ester. The stability of the sulfonate ester group in neutral solution noted during the preparation of the inhibitors does not, of course, rule out the possibility of enzyme-catalyzed hydrolysis.

For inhibitors containing a terminal sulfonyl fluoride

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‡For the previous paper in this series see Baker and Neenan.[1a]

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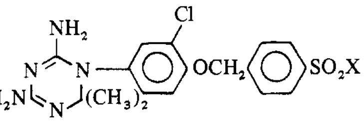
#For a discussion of the use of the ED_{50}/I_{50} ratio as an approximation of membrane transport, see ref 7.

Table I. Inhibition[a] of Dihydrofolate Reductase by



No.	X	Enzyme source[b]	I_{50},[c] μM	Inhibitor, μM	% inactvn[d]	ED_{50},[e] μM	ED_{50}/I_{50}
3[f]	F	L1210/DF8	0.026	0.052	91	0.08	3
		Mouse liver		0.078	8		
		W256		0.052	98		
		Rat liver		0.078	92		
6	OC_6H_5	W256	0.0063	0.050	54	0.006	1
		Rat liver		0.050	69		
7	OC_6H_4Cl-*p*	W256	0.017	0.050	39	0.2	10
		Rat liver		0.050	39		
8	OC_6H_4Cl-*m*	W256	0.019	0.050	41	0.01	0.5
		Rat liver		0.050	49		
9	OC_6H_4Cl-*o*	W256	0.0024	0.050	48	0.4	200
		Rat liver		0.050	45		
10	$OC_6H_3Cl_2$-3,4	W256	0.0056	0.050	22	0.15	30
		Rat liver		0.050	11		
11	OC_6H_4F-*p*	W256	0.0040	0.050	45	0.06	15
		Rat liver		0.050	89		
12	OC_6H_4F-*m*	W256	0.0035	0.050	46	0.09	30
		Rat liver		0.050	53		
13	OC_6H_4F-*o*	W256	0.0018	0.050	56	0.03	20
		Rat liver		0.050	*g*		
14	OC_6H_4Me-*m*	W256	0.0036	0.050	26	0.005	1
15	$OC_6H_4CF_3$-*m*	W256	0.0082	0.050	22	0.2	20
		Rat liver		0.050	38		
16	$OC_6H_4CF_3$-*o*	W256	0.0047	0.050	37	0.03	6
		Rat liver		0.050	39		
17	OC_6H_4OMe-*p*	W256	0.0040	0.050	75	0.04	10
		Rat liver		0.050	*g*		
18	OC_6H_4OMe-*m*	W256	0.0030	0.050	38	0.05	20
		Rat liver		0.050	89		
19	OC_6H_4OMe-*o*	W256	0.0040	0.050	55	0.1	25
		Rat liver		0.050	33		
20	OC_6H_4CN-*p*	W256	0.0041	0.050	71	0.07	20
		Rat liver		0.050	*g*		
21	OC_6H_4CN-*m*	W256	0.0057	0.050	59	0.08	10
		Rat liver		0.050	*g*		
22	OC_6H_4CN-*o*	W256	0.0020	0.050	54	0.02	10
		Rat liver		0.050	73		
23	$OC_6H_4CONMe_2$-*p*	W256	0.0024	0.050	58	0.08	30
		Rat liver		0.050	73		
24	$OC_6H_4CONMe_2$-*m*	W256	0.0017	0.050	55	0.2	100
		Rat liver		0.050	63		
25	$OC_6H_4CONMe_2$-*o*	W256	0.0023	0.050	71	3	1000
		Rat liver		0.050	72		

[a]The technical assistance of Julie Beardslee, Pauline Minton, and Janet Wood is acknowledged. [b]L1210/DF8 = mouse leukemia resistant to amethopterin; W256 = Walker 256 rat tumor. [c]Concn for 50% reversible inhibn when assayed with 6 μM dihydrofolate, 30 μM NADPH, and 0.15 M KCl in pH 7.4 Tris buffer as previously described.[7] [d]Extent of inactivation of enzyme after 60-min incubation with inhibitor at 37°. [e]Concn for 50% inhibition of L1210 cell culture; these data were supplied by Dr. Florence White of CCNSC. [f]Data from ref 8. [g]Highly variable.

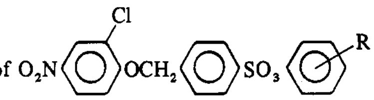
group, it has been proposed[10,11] that an enzymic hydroxyl group could either form a covalent bond with the SO_2F group, or catalyze the hydrolysis of the SO_2F group, or both, depending on subtle differences in the positioning of the sulfonyl fluoride while in the enzyme-inhibitor complex. Such a mechanism could also be operative for inhibitors of type **5** bound to dihydrofolate reductase. Hydrophobic substituents may cause the phenoxy group to reside on the enzyme in a conformation which favors enzyme-catalyzed hydrolysis, whereas polar or semipolar substituents allow a binding conformation leading to a greater extent of covalent bond formation with the enzyme and a lesser extent of hydrolysis. This would account for the observed substituent effects.

Alternatively, the sulfonate ester might be hydrolyzed by some other enzyme in the 45-90% $(NH_4)_2SO_4$ fraction from Walker 256 or rat liver, analogous to the "sulfonyl fluoridase" believed to be present in the crude extract from rat liver but not in that from Walker 256.[5,12]

A number of the inhibitors of type **5** were as effective as or more effective than the corresponding sulfonyl fluoride (**3**) against L1210 cell culture. The most potent in this respect were the unsubstituted compound (**6**) and the *m*-Cl (**8**) and *m*-Me (**14**) analogs. The poor transport of the *o*-$CONMe_2$ derivative (**25**) may be the result of unfavorable steric effects. In general, there was no clear correlation between the ED_{50} or ED_{50}/I_{50} and the nature of the substituent or the ring position substituted. It is apparent, at least in this series of compounds, that replacement of sulfonyl fluoride by sulfonate ester with an appropriate phenoxide leaving group is not necessarily an unfavorable modification from the standpoint of membrane transport.

Chemistry. The nitro intermediates (**27**) (Table II) for the synthesis of the inhibitors in Table I were prepared by

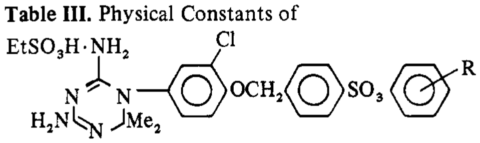
Table II. Physical Constants of



No.	R	Yield,[a] %	Mp, °C	Formula[b]
27a	H	84[c]	151	$C_{19}H_{14}ClNO_6S$
27b	4-Cl	76[c]	143-144	$C_{19}H_{13}Cl_2NO_6S$
27c	3-Cl	80[d]	115	$C_{19}H_{13}Cl_2NO_6S$
27d	2-Cl	75[e]	116	$C_{19}H_{13}Cl_2NO_6S$
27e	3,4-Cl_2	71[c]	158-159	$C_{19}H_{12}Cl_3NO_6S$
27f	4-F	81[c]	159-161	$C_{19}H_{13}ClFNO_6S$
27g	3-F	75[e]	105-107	$C_{19}H_{13}ClFNO_6S$
27h	2-F	90[c]	144-145	$C_{19}H_{13}ClFNO_6S$
27i	3-Me	83[d]	113-114	$C_{20}H_{16}ClNO_6S$
27j	3-CF_3	72[e]	96	$C_{20}H_{13}ClF_3NO_6S$
27k	2-CF_3	80[c]	145-146	$C_{20}H_{13}ClF_3NO_6S$
27l	4-OMe	78[c]	151-152	$C_{20}H_{16}ClNO_7S$
27m	3-OMe	43[f]	128-129	$C_{20}H_{16}ClNO_7S$
27n	2-OMe	59[g]	167-168	$C_{20}H_{16}ClNO_7S$
27o	4-CN	81[c]	163-164	$C_{20}H_{13}ClN_2O_6S$
27p'	3-CN	80[c]	145-146	$C_{20}H_{13}ClN_2O_6S$
27q	2-CN	61[c]	148-149	$C_{20}H_{13}ClN_2O_6S$
27r	4-$CONMe_2$	75[h]	222-223	$C_{22}H_{19}ClN_2O_7S$
27s	3-$CONMe_2$	64[i]	183-184	$C_{22}H_{19}ClN_2O_7S$
27t	2-$CONMe_2$	83[c]	172-174	$C_{22}H_{19}ClN_2O_7S$

[a]Yield of analytically pure material. [b]*Anal.* C, H, N. [c]Recrystd from 2-methoxyethanol-H_2O. [d]Recrystd from EtOH-H_2O. [e]Recrystd from EtOH. [f]Recrystd from MeOH-MeCN. [g]Recrystd from toluene. [h]Recrystd from 2-methoxyethanol-DMF. [i]Recrystd from MeCN.

Table III. Physical Constants of

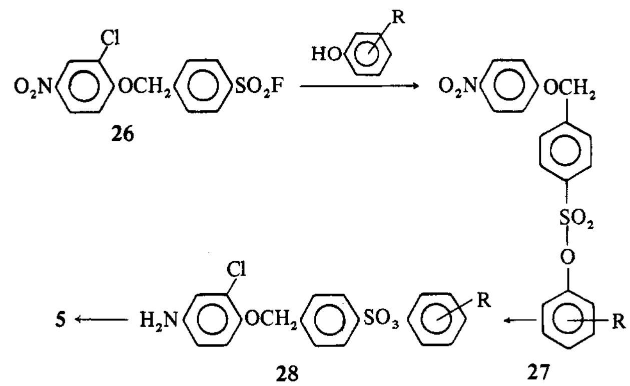


No.	R	Yield,[a-c] %	Mp, °C dec	Formula[d]
6	H	57	216-218	$C_{26}H_{30}ClN_5O_7S_2$
7	4-Cl	54	206-208	$C_{26}H_{29}Cl_2N_5O_7S_2$
8	3-Cl	68	204-205	$C_{26}H_{29}Cl_2N_5O_7S_2$
9	2-Cl	66	222-224	$C_{26}H_{29}Cl_2N_5O_7S_2$
10	3,4-Cl_2	63	210-212	$C_{26}H_{28}Cl_3N_5O_7S_2$
11	4-F	66	201-204	$C_{26}H_{29}ClFN_5O_7S_2$
12	3-F	66	210-211	$C_{26}H_{29}ClFN_5O_7S_2$
13	2-F	70	223-224	$C_{26}H_{29}ClFN_5O_7S_2$
14	3-Me	67	206-207	$C_{27}H_{32}ClN_5O_7S_2$
15	3-CF_3	60	208-210	$C_{27}H_{29}ClF_3N_5O_7S_2$
16	2-CF_3	69	216-218	$C_{27}H_{29}ClF_3N_5O_7S_2$
17	4-OMe	79	200-201	$C_{27}H_{32}ClN_5O_8S_2$
18	3-OMe	64	197-199	$C_{27}H_{32}ClN_5O_8S_2$
19	2-OMe	67	218-220	$C_{27}H_{32}ClN_5O_8S_2$
20	4-CN	22	201-202	$C_{27}H_{29}ClN_6O_7S_2$
21	3-CN	26	200-202	$C_{27}H_{29}ClN_6O_7S_2$
22	2-CN	39	214-216	$C_{27}H_{29}ClN_6O_7S_2$
23	4-$CONMe_2$	61	201-203	$C_{29}H_{35}ClN_6O_8S_2$
24	3-$CONMe_2$	67	205-206	$C_{29}H_{35}ClN_6O_8S_2$
25	2-$CONMe_2$	68	204-205	$C_{29}H_{35}ClN_6O_8S_2 \cdot H_2O$

[a]All compds prepd by method H in ref 9; 2-methoxyethanol was used as hydrogenation solvent. [b]All compds recrystd from *i*-PrOH-H_2O. [c]Yield of analytically pure material. [d]*Anal.* C, H, N.

reaction of the sulfonyl fluoride **26**[8] with the appropriate phenol in DMF in the presence of K_2CO_3. The Pt-catalyzed hydrogenation of **27** to **28** and the condensation of **28** with cyanoguanidine and acetone[13] in the presence of $EtSO_3H$ to give **5** were carried out as previously described.[9] The physical properties of the inhibitors are listed in Table III.

Only the phenols with dimethylcarbamoyl substituents were not commercially available. *N,N*-Dimethylsalicylamide was obtained in good yield by reaction of salicylic acid with refluxing DMF in the presence of P_2O_5 according to



the procedure of Schindlbauer.[14] *N,N*-Dimethyl-*p*-hydroxybenzamide[14] was prepared similarly, although less satisfactorily.** The meta isomer[15] was synthesized by another route.††

Experimental Section

Melting points (uncorrected) were taken in capillary tubes on a Mel-Temp block. All analytical samples had ir and uv spectra consistent with their assigned structures and were homogeneous on tlc using either Brinkmann silica gel GF (intermediates) or polyamide MN (triazines). Each analytical sample gave combustion values for C, H, and N (Galbraith Laboratories) within 0.4% of theoretical.

***p*-Chlorophenyl α-(2-Chloro-4-nitrophenoxy)-*p*-toluenesulfonate (27b).** A mixt of 450 mg (1.3 mmoles) of **26**,[11] 180 mg (1.4 mmoles) of *p*-chlorophenol, 180 mg (1.3 mmoles) of K_2CO_3, and 2.5 ml of DMF was stirred at 75-80° for 2 hr, then cooled, and added to 5 ml of pyridine. The solid product, which pptd upon gradual addn of 50 ml of H_2O, was isolated and washed with 10% Na_2CO_3, then H_2O. Recrystn from 2-methoxyethanol-H_2O gave 449 mg (76%) of cream-colored platelets, mp 143-144° (tlc in C_6H_6). *Anal.* ($C_{19}H_{13}Cl_2NO_6S$) C, H, N. Other compds prepd by this method are listed in Table II.

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**Schindlbauer[14] reported a melting point of 204-205.5° for this compound. In this case, however, two major products were obtained and separated by fractional recrystallization from MeCN. One product, mp 162-163°, had ir and nmr spectra as expected for *N,N*-dimethyl-*p*-hydroxybenzamide. The other compound, mp 202-203°, was identified by ir and nmr as the *p*-hydroxybenzoyl derivative of the desired product.

††Prepared in this laboratory by M. Cory, Ph.D. Thesis, University of California, Santa Barbara, Calif., 1971.