Irreversible Enzyme Inhibitors. CLIX.[1,2] Effect of Substitution on Transport and Isozyme Specificity of *p*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)-*o*-chlorophenoxyacetamidobenzenesulfonyl Fluoride, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

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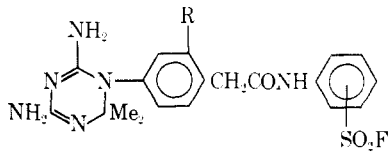
Twelve analogs of the title compound (1) were evaluated as irreversible inhibitors of the dihydrofolic reductase from L1210 mouse leukemia and mouse liver; these thirteen compounds were also evaluated for ability to kill L1210 cells in culture, an approximation to cell-wall transport. None of the compounds was more effective than 1. The amide bridge of 1 is believed to be the major deterrent to good transport.

The selective irreversible inhibition of dihydrofolic reductase from L1210 mouse leukemia by the active-site-directed irreversible inhibitor[3] **1** with no irreversible



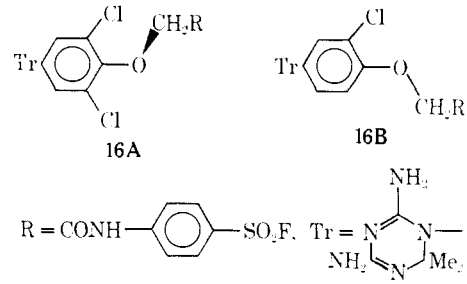
1, *para*, R = Cl
2, *para*, R = H
3, *meta*, R = Cl
4, *meta*, R = H

inhibition of this enzyme from normal mouse liver has been reported from this laboratory;[4] that the chlorine atom was necessary for irreversible inhibition was shown by the failure of **2** to inactivate the enzyme for conformational reasons previously discussed.[4] The *meta* isomer **3** was a somewhat less effective irreversible inhibitor of the L1210 dihydrofolic reductase, but again a chlorine atom was necessary for inactivation as shown with **4**.[4] It has now been observed (Table I) that the concentrations for 50% inhibition (ED_{50}) of L1210 cell culture are 0.4 and 2 μM for **1** and **3**, respectively; very little difference was seen with **1** *vs.* **2** or **3** *vs.* **4**. A study has now been made to see if the ED_{50} and the extent of inactivation by **1** or **3** could be improved by substitution on the benzenesulfonyl fluoride moiety; the results are the subject of this paper.

Enzyme Results.[5]—The first group of analogs of the parent **1** contained a methyl or chloro substituent on the benzenesulfonyl fluoride moiety (**5**–**8**) (Table I). In all four cases, irreversible inhibition of L1210 dihydrofolic reductase was maintained, but selectivity was reduced since 30–60% irreversible inhibition of the mouse liver enzyme was observed. The most potent irreversible inhibition of the L1210 enzyme was seen with the 2-chloro-substituted derivative (**8**).

Replacement of the 3-chloro group on the phenoxy moiety of **1** by bromo (**9**) enhanced reversible inhibition of the L1210 enzyme about fivefold, but the amount of irreversible inhibition was unchanged; however, 21% irreversible inhibition of the mouse liver enzyme was observed.

Introduction of a second chloro group on the 5 position of **1** gave **10**; the latter showed essentially the same reversible and irreversible inhibition of the L1210 and mouse liver enzymes as **1**. This result allows an important deduction regarding the binding conformation of the oxyacetamido bridge of **1**. The oxyacetamido bridge of the dichloro analog (**10**) would be out of the plane of the phenoxy group due to the two *o*-chloro substituents as shown in **16A**. The two extremes in ground-state conformation of the bridge of the monochloro derivative **1** could be coplanar as in **16B** or in perpendicular planes as in **16A**. Since **1** and **10** have the same irreversible inhibition pattern, it is highly probable that both inactivate the enzyme by complexing in the same conformation, namely **16A**.



Five analogs of the *m*-sulfonyl flouride (**3**) were then synthesized for enzymic evaluation; these analogs had methyl, chloro, or methoxy substituents either at the 4 position of the benzene-3-SO_2F moiety or *para* to the SO_2F moiety (**11**–**15**). Four (**11**, **12**, **14**, **15**) showed essentially the same irreversible inhibition pattern of the L1210 and liver enzymes as the parent **3**; the remaining analog (**13**) showed a decreased amount of irreversible inhibition of the L1210 enzyme and no irreversible inhibition of the liver enzyme. That the analog **13** with a 2-methyl substituent was less effective than the corresponding analogs with a 2-chloro (**11**) or 2-methoxy (**12**) substituent indicates that this effect was not just steric.

(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**, 684 (1969).

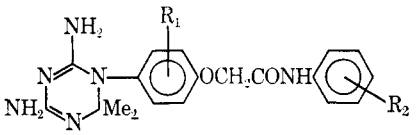
(3) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley and Sons, Inc., New York, N. Y., 1967.

(4) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **12**, 95 (1969), paper CXL of this series.

(5) See B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), for the enzyme assay methods.

TABLE I

INHIBITION[a] OF DIHYDROFOLIC REDUCTASE AND L1210 CELL CULTURE BY



No.	R_1[b]	R_2	Mouse tissue	I_{50},[c] μM	Inhib, μM	Time, min	% inactvn[d]	ED_{50},[e] μM	ED_{50}/I_{50}
1	3-Cl	4-SO_2F	L1210/DF8	0.037	0.074	60	76[f]	0.4	10
					0.12	2, 30	73, 79[g]		
			Liver		0.070	60	0[f]		
3	3-Cl	3-SO_2F	L1210/DF8	0.019	0.095	60	61[f]	2	100
					0.095	2, 30	53, 68[g]		
			Liver		0.095	60	13[f]		
5	3-Cl	3-Me-4-SO_2F	L1210/DF8		0.066	2, 30	72, 78[g]	0.2	10
			Liver	0.022	0.066	60	50		
6	3-Cl	3-Cl-4-SO_2F	L1210/DF8		0.063	2, 30	62, 62[g]	0.6	30
			Liver	0.021	0.063	60	31		
7	3-Cl	2-Me-4-SO_2F	L1210/DF8		0.064	2, 30	48, 70[g]	2	70
			Liver	0.032	0.096	60	38		
8	3-Cl	2-Cl-4-SO_2F	L1210/DF8		0.050	60	95	0.08	4
			Liver	0.018	0.050	60	58		
9	3-Br	4-SO_2F	L1210/DF8	0.0073	0.050	60	64	0.3	40
			Liver		0.050	60	21		
10	3,5-Cl_2	4-SO_2F	L1210/DF8	0.018	0.050	60	65	2	100
			Liver		0.050	60	0		
11	3-Cl	2-Cl-5-SO_2F	L1210/DF8		0.068	2, 30	64, 70[g]	2	100
			Liver	0.017	0.051	60	36		
12	3-Cl	2-MeO-5-SO_2F	L1210/DF8		0.045	60	70	1	70
			Liver	0.015	0.045	60	33		
13	3-Cl	2-Me-5-SO_2F	L1210/DF8		0.069	60	47	4	200
			Liver	0.023	0.069	60	0		
14	3-Cl	4-Cl-3-SO_2F	L1210/DF8		0.050	60	75	0.6	40
			Liver	0.016	0.050	60	28		
15	3-Cl	4-Me-3-SO_2F	L1210/DF8		0.054	60	60	6	200
			Liver	0.027	0.081	60	17		

[a] The technical assistance of Diane Shea and Sharon Lafler with these assays is acknowledged. [b] Numbered from triazinyl position = 1. [c] I_{50} = concentration for 50% inhibition when measured with 6 μM dihydrofolate and 0.15 M KCl at pH 7.4 as previously described.[5] [d] Enzyme incubated with inhibitor at 37° in pH 7.4 Tris buffer containing 60 μM TPNH, then the remaining enzyme was assayed as previously described.[5] [e] Concentration for 50% inhibition of L1210 cell culture. [f] Data from ref 4. [g] From six-point time study.[5]

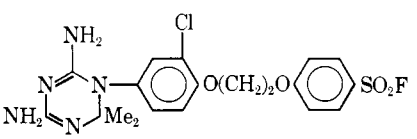
L1210 Cell Culture Assays.[6]—The concentration for 50% cell kill (ED_{50}) (Table I) varied about 70-fold from 0.08 to 6 μM. A similar 50-fold spread was observed when the results were compared as ED_{50}/I_{50} where the small differences in I_{50} are normalized.

With the analogs **5–10** of the 4-SO_2F inhibitor (**1**), the ED_{50} varied about 25-fold; with the analogs **11–15** of the 3-SO_2F inhibitor (**3**), the ED_{50} varied only about sevenfold.

Few specific generalizations on the effect of substituents on ED_{50} can be made. The comparison of ED_{50}/I_{50} ratios gives a reasonable first approximation of the ability of the compounds to penetrate the cell wall.[7] With the analogs **11–15** of **3**, the ED_{50}/I_{50} ratio varied only about fivefold which is probably within experimental error; thus small substituents on the benzene-3-SO_2F moiety of **3** did not effect transport. In contrast, substituents **5–8** on the benzene-4-SO_2F moiety of **1** varied the penetration by 25-fold; however, there was no consistency in these effects as can be seen by comparing a 2-chloro (**8**) with a 2-methyl (**7**) substituent.

Only three compounds were available for comparison of the effects substitution on the phenoxy moiety had on cell wall penetration. The most pronounced effect was seen with the 3,5-dichloro-substituted derivative **10**, with its limited conformation of the bridge; note that **1** was 20-fold more effective in cell-wall penetration (ED_{50}/I_{50}) than **10**. This result can be interpreted that the **16A** conformation, which has more three-dimensional bulk than **16B**, does not penetrate as well as the more planar **16B** conformation. This proposal is also supported by the fourfold difference in ED_{50}/I_{50} between the more bulky bromo derivative (**9**) and **1**.

The relatively poor transport of compounds in Table I could be attributed to the amide bridge; an amide slows transport since its hydrogen-bond structure to water must be broken as it transfers to a lipid phase,[8] an energy-requiring process. When the CONH bridge of **1** was replaced by CH_2O to give **17**,[4] transport



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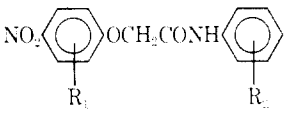
(6) We wish to thank Dr. Florence White of the CCNSC for these results obtained by Dr. Philip Himmelfarb of Arthur D. Little, Inc.

(7) For a more detailed discussion see (a) ref 5; (b) B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **12**, 668 (1969), paper CLIV of this series.

(8) W. D. Stein, "The Movement of Molecules across Cell Membranes," Academic Press Inc., New York, N. Y., 1967.

TABLE II
PHYSICAL CONSTANTS OF



No.	R_1[a]	R_2	Yield,[b] %	Mp, °C	Formula[c]
20a	3-Cl	3-Me-4-SO_2F[d]	52[e]	175	$C_{15}H_{12}ClFN_2O_6S$
20b	3-Cl	3-Cl-4-SO_2F[f]	43[e]	176–181	$C_{14}H_9Cl_2FN_2O_6S$
20c	3-Cl	2-Me-4-SO_2F[d]	30[g]	220–222	$C_{15}H_{12}ClFN_2O_6S$
20d	3-Cl	2-Cl-4-SO_2F[h]	52[g]	219–220	$C_{14}H_9Cl_2FN_2O_6S$
20e	3-Br	4-SO_2F[i]	65[g]	214–215	$C_{14}H_{10}BrFN_2O_6S$
20f	3,5-Cl_2	4-SO_2F[i]	66[f]	203–204	$C_{14}H_9Cl_2FN_2O_6S$
20g	3-Cl	2-Cl-5-SO_2F[h]	29[g]	223–224	$C_{14}H_9Cl_2FN_2O_6S$
20h	3-Cl	2-MeO-5-SO_2F[i]	28[g]	248–249	$C_{15}H_{12}ClFN_2O_7S$
20i	3-Cl	2-Me-5-SO_2F[j]	69[g]	221–223	$C_{15}H_{12}ClFN_2O_6S$
20j	3-Cl	4-Cl-3-SO_2F[h]	69[g]	225–226	$C_{14}H_9Cl_2FN_2O_6S$
20k	3-Cl	4-Me-3-SO_2F[d]	62[g]	203–204	$C_{15}H_{12}ClFN_2O_6S$

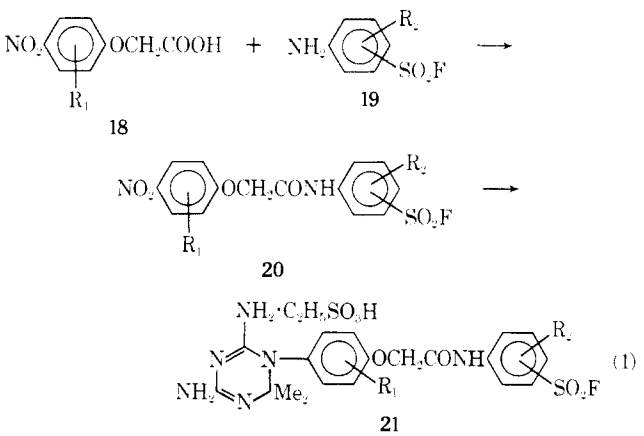
[a] Numbered from NO_2 at position 1. [b] By condensation of the appropriate acid chloride and aminobenzenesulfonyl fluoride by the previously described method;[4] yield of analytically pure material. [c] Analyzed for C, H, and N. [d] See ref 10 for starting amine. [e] Recrystallized from EtOH. [f] See ref 12 for starting amine. [g] Recrystallized from MeOEtOH–H_2O. [h] See ref 11 for starting amine. [i] Starting amine commercially available. [j] See ref 13 for starting amine.

was considerably enhanced, the ED_{50} of **17** for 50% cell kill being 0.01 μM and $ED_{50}/I_{50} = 0.2$

Since **17** does show some selectivity in irreversible inhibition of the dihydrofolic reductase from L1120 over irreversible inhibition of this enzyme from liver,[4] the synthesis and evaluation of compounds related to **17** are being vigorously pursued to determine whether further improvement in transport and selectivity can be achieved.

Chemistry.—The triazines in Table I were prepared by the general route previously used;[4,9] the irreversible inhibitors in Tables I and III have the general structure **21**.

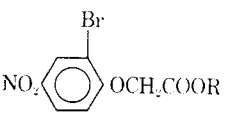
Condensation of the acid chloride of the appropriate *p*-nitrophenoxyacetic acid (**18**) with the appropriate aminobenzenesulfonyl fluoride (**19**)[10-13] gave the intermediates **20** (eq 1) (Table II). Catalytic reduction of



(1)

the NO_2 group with a PtO_2 catalyst to minimize hydrogenolysis of halogen gave the corresponding amines; these were not purified but were condensed with cyanoguanidine in acetone[14] in the presence of ethanesulfonic acid[4] to give the dihydro-*s*-triazines (**21**) (Table III).

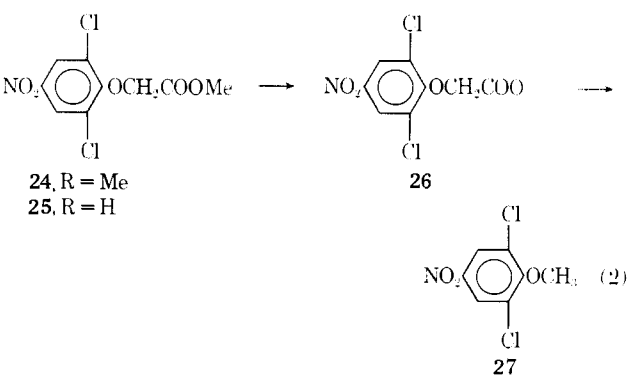
The synthesis of 2-chloro-4-nitrophenoxyacetic acid by condensation of 2-chloro-4-nitrophenol with *t*-butyl chloroacetate followed by acid-catalyzed elimination of isobutene was previously described from this laboratory;[4] the same sequence has now been used with 2-bromo-4-nitrophenol to form **23** *via* **22**. The more



22, R = *t*-Bu
23, R = H

sterically hindered 2,6-dichloro-4-nitrophenol failed to undergo alkylation with *t*-butyl chloroacetate, but could be slowly alkylated with methyl chloroacetate in the presence of NaI to give **24**.

When **24** was saponified with 0.45 *N* NaOH in 90% methanol by reaction at room temperature for 2.5 hr, the product was the anisole derivative **27** obtained in 74% yield (eq 2); it was established by tlc that this



(2)

facile decarboxylation of the carboxylate anion **26** was occurring in alkaline solution. When the reaction

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

(10) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 677 (1968), paper CXXIX of this series.

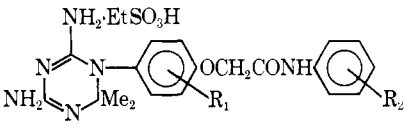
(11) A. H. deCat and R. K. vanPoucke, *J. Org. Chem.*, **28**, 3426 (1963).

(12) G. J. Lourens, Ph.D. thesis, University of California at Santa Barbara, 1968.

(13) I. Kageyama and S. Nakanishi, Japanese Patent 16 (60) (1960); *Chem. Abstr.*, **54**, P18985*f* (1960).

(14) E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).

TABLE III: PHYSICAL CONSTANTS OF

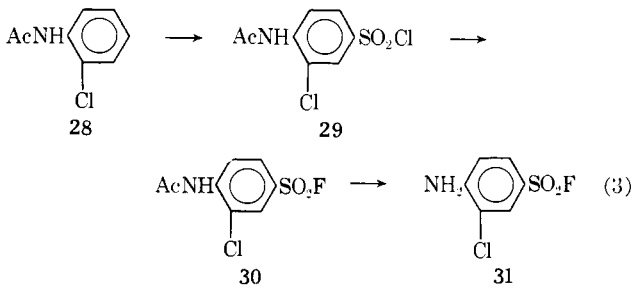


No.	R_1[a]	R_2	Yield,[b] %	Mp, °C dec	Formula[c]
5	3-Cl	3-Me-4-SO_2F	47[d]	222–224	$C_{22}H_{28}ClFN_6O_7S_2$
6	3-Cl	3-Cl-4-SO_2F	17[e]	217–219	$C_{21}H_{25}Cl_2FN_6O_7S_2$
7	3-Cl	2-Me-4-SO_2F	33[d]	218–220	$C_{22}H_{28}ClFN_6O_7S_2$
8	3-Cl	2-Cl-4-SO_2F	45[d]	226–227	$C_{21}H_{25}Cl_2FN_6O_7S_2$
9	3-Br	4-SO_2F	4[d,f]	224–225	$C_{21}H_{26}BrFN_6O_7S_2$
10	3,5-Cl_2	4-SO_2F	46[d]	200–202	$C_{21}H_{25}Cl_2FN_6O_7S_2$
11	3-Cl	2-Cl-5-SO_2F	15[d]	239–242	$C_{21}H_{25}Cl_2FN_6O_7S_2$
12	3-Cl	2-MeO-5-SO_2F	24[d]	239–241	$C_{22}H_{28}ClFN_6O_8S_2$
13	3-Cl	2-Me-5-SO_2F	42[d]	231–233	$C_{22}H_{28}ClFN_6O_7S_2$
14	3-Cl	4-Cl-3-SO_2F	52[d]	205–207	$C_{21}H_{25}Cl_2FN_6O_7S_2$
15	3-Cl	4-Me-3-SO_2F	35[d]	211–213	$C_{22}H_{28}ClFN_6O_7S_2$

[a] Numbered from triazinyl at 1. [b] Prepared from the compounds in Table II by Pt-catalyzed reduction followed by condensation of $EtSO_3H$ salt with cyanoguanidine in acetone as previously described;[4] yield of analytically pure material. [c] Analyzed for C, H, and F. [d] Recrystallized from *i*-PrOH–H_2O. [e] Amorphous product obtained by trituration of crude product with hot *i*-PrOH. [f] The intermediate was contaminated with debrominated amine, perhaps accounting for low over-all yield.

time was shortened to 5 min, the desired acid **25** was isolated in 62% yield.[15]

Although the synthesis of 3-chlorosulfanilyl fluoride (**31**) from 3-chloro-4-nitroaniline has been described,[11] the required aniline is obtainable only in by-product quantities.[16] Therefore the chlorosulfonation of *o*-chloroacetanilide was investigated (eq 3); the major



product was **29** which was readily purified. The remainder of the sequence to **31** was performed by the general method of deCat and vanPoucke.[11]

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. All analytical samples had ir spectra in agreement with their assigned structures; each gave combustion values for C, H, and N or F within 0.3% of theoretical. All analytical samples of intermediates gave a single spot on tlc with Brinkmann silica gel GF.

***t*-Butyl 2-Bromo-4-nitrophenoxyacetate (22).**—A mixture of 5.05 g (23 mmoles) of 2-bromo-4-nitrophenol, 4.20 g (28 mmoles) of *t*-butyl chloroacetate, 3.45 g (25 mmoles) of K_2CO_3, 0.75 g (1 mmole) of NaI, and 25 ml of DMF was stirred at about 70° for 22 hr. The cooled mixture was poured into a mixture of 5% NaOH, 25 ml of petroleum ether (bp 60–110°), and excess ice. The product was collected on a filter and washed with H_2O, then petroleum ether. Recrystallization from petroleum ether (bp 60–110°) gave 4.16 g (54%) of light yellow crystals, mp 106–108° (tlc in C_6H_6). *Anal.* ($C_{12}H_{14}BrNO_5$) C, H, N.

2-Bromo-4-nitrophenoxyacetic acid (23) was prepared from **22** as described for the corresponding 2-chloro derivative.[4] The crude product was dissolved in excess 5% $NaHCO_3$, then the solution was washed with $CHCl_3$. Acidification gave 1.97 g (60%) of light yellow crystals, mp 178–179°. *Anal.* ($C_8H_6BrNO_5$) C, H, N.

Methyl 2,6-Dichloro-4-nitrophenoxyacetate (24).—A solution of 41.6 g (0.20 mole) of 2,6-dichloro-4-nitrophenol, 23.8 g (0.22 mole) of methyl chloroacetate, 10.8 g (0.20 mole) of NaOMe, and 15 g (0.2 mole) of sodium iodide in 250 ml of MeOH was refluxed with stirring for 8 days, when tlc showed considerable phenol was still present. The mixture was poured into 1500 ml of ice water and 100 ml of petroleum ether (bp 60–110°). The crude product was collected on a filter and dissolved in 200 ml of C_6H_6, then the solution was clarified by filtration. The C_6H_6 solution was shaken with 250 ml of 10% Na_2CO_3, then filtered to remove the insoluble sodium phenolate. The benzene layer was washed with three 200-ml portions of 10% Na_2CO_3, then 500 ml of H_2O. Dried with $MgSO_4$, the solution was spin-evaporated *in vacuo*. Recrystallization from petroleum ether (bp 60–110°) gave 14.5 g (26%) of white crystals, mp 85–88° (tlc in C_6H_6). *Anal.* ($C_9H_7Cl_2NO_5$) C, H, N.

2,6-Dichloro-4-nitrophenoxyacetic Acid (25).—To 16 ml of 2 *N* NaOH (32 mmoles) in 80% MeOH was added 8.96 g (32 mmoles) of **24**. The stirred mixture rapidly formed a thick paste. After 5 min, the mixture was acidified with excess 5% HCl. The crude product was collected on a filter and purified as described for **23**; yield 5.28 g (62%) of white crystals, mp 189–190° (tlc in 1:1 EtOAc–C_6H_6), lit.[15] mp 185–186°.

N-Acetyl-3-chlorosulfanilyl Chloride (29).—A solution of 8.45 g (50 mmoles) of **28** in 16 ml of $ClSO_3H$ was heated for 3 hr with stirring protected from moisture in a bath at 75–80°. The cooled mixture was diluted with 100 ml of $CHCl_3$, then poured onto ice. The $CHCl_3$ layer was washed with H_2O, 100 ml of 5% $NaHCO_3$, and H_2O. Dried with $MgSO_4$, the solution was spin evaporated *in vacuo*. Recrystallization from C_6H_6–petroleum ether (bp 60–110°) gave 3.38 g (25%) of white crystals, mp 139–140° (tlc in 1:1 C_6H_6–EtOAc). *Anal.* ($C_8H_7Cl_2NO_3S$) C, H, N.

N-Acetyl-3-chlorosulfanilyl Fluoride (30).—To a refluxing and stirred solution of 2.95 g (11 mmoles) of **29** in 8 ml of dioxane (bath 125°) was added a solution of 1.45 g (25 mmoles) of KF in 2 ml of H_2O. After 5 hr at 120–125° (bath), the mixture was diluted with 3–5 ml of H_2O and cooled. The product was collected by filtration, washed with H_2O, and recrystallized from C_6H_6; yield 1.60 g (58%), mp 170° (tlc in C_6H_6). *Anal.* ($C_8H_7ClFNO_3S$) C, H, N.

3-Chlorosulfanilyl Fluoride (31).—A mixture of 1.51 g (6 mmoles) of **30**, 15 ml of EtOH, and 6 ml of 12 *N* HCl was refluxed for 100 min when tlc in C_6H_6 showed the reaction was complete. The cooled solution was poured into an ice-cold solution of 6.6 g of $NaHCO_3$ in 60 ml of H_2O. The product was collected on a filter, washed with H_2O, and dried *in vacuo;* yield 1.01 g (80%), mp 131–132° (tlc in 1:1 C_6H_6-EtOAc), lit.[11] mp 134°. The isomeric 3-amino-4-chlorobenzenesulfonyl fluoride has been reported[11] to have mp 70°.

(15) This acid has been prepared in unspecified yield from the corresponding ethyl ester by acid hydrolysis: J. K. Faulkner and D. Woodcock, *J. Chem. Soc.*, 1187 (1965).

(16) (a) R. Fuson, R. Bauman, E. Howard, and E. Marvell, *J. Org. Chem.*, **12**, 799 (1947); (b) H. Mayes and E. Turner, *J. Chem. Soc.*, 691 (1928).