Irreversible Enzyme Inhibitors. CXXXVI.[1,2] 2,4-Diamino-5-(3,4-dichlorophenyl)-6-[p-(m-fluorosulfonylbenzamido)phenoxymethyl]pyrimidine, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase. Effect of Structure on Isozyme Specificity

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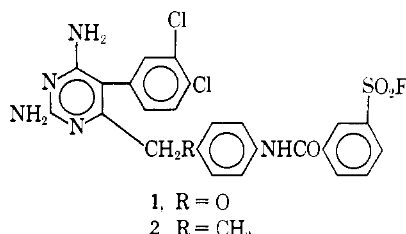
The title compound (1) was previously observed to be an active-site-directed irreversible inhibitor of dihydrofolic reductase from L1210 mouse leukemia, but not the enzyme from mouse liver, spleen, or intestine. Replacement of the CH_2O bridge with $(CH_2)_2$ (2) enhanced reversible binding 15-fold, thus decreasing by this factor the concentration needed for irreversible inhibition.[9] However, this structural change also led to loss of specificity since the liver dihydrofolic reductase could now be inactivated by 2. Similarly, replacement of the NHCO bridge of 1 by $NHSO_2$ (8) resulted in a 50-fold enhancement of reversible inhibition, but specificity was again lost. Substitution on one or both of the benzene rings of the 6 side chain of 1 with Cl or Me gave only two- to fourfold enhancement of reversible binding, but specificity was maintained in some cases (7, 10, 11, 17).

Three classes of active-site-directed irreversible inhibitors[3] of dihydrofolic reductase have been found.[4-7] A member (1) of one of these classes showed irreversible



inhibition of dihydrofolic reductase from L1210 mouse leukemia with no irreversible inhibition of the mouse liver, spleen, or intestine enzymes.[7,8] Even though 1 showed selective irreversible inhibition of the tumor enzyme, its $I_{50} \simeq 6K_i$ of 0.8 μM (Table I) was considered too high for 1 to be able to operate *in vivo*.[8] Therefore a study has been made on compounds related to 1 where (a) the fluorosulfonylbenzoyl moiety has been modified, or (b) the CH_2O bridge of 1 and related compounds was replaced by CH_2CH_2[5,7] to see if better reversible binding could be achieved with maintenance of specificity; an arbitrary standard of $I_{50} \leq 0.1$ μM has been set.[8,9] Two additional arbitrary standards set[8] for *in vivo* evaluation were that the compound at a K_i concentration should give >70% irreversible inhibition of the tumor enzyme, but at $12K_i$ should give <20% irreversible inhibition of the liver enzyme. The effects of these substitutions on enzyme specificity is the subject of this paper.

Enzyme Results.—Conversion of the oxymethylene bridge of 1 to ethylene (2) gave about a 15-fold increment in binding;[10] that such a structural change would give an increment in binding was expected based on previous observation.[5,7] However, 2 was not as good an irreversible inhibitor of the L1210 enzymes when measured at a concentration giving equal amounts of reversible EI complex, the rate-determining species;[9] furthermore, specificity was lost since 2 could now inactivate the liver enzyme in contrast to 1. When the SO_2F group of 2 was moved to the *para* position (3), the extent of reversible inhibition was maintained, but irreversible inhibition was not improved over 2. Conversion of the NHCO bridge of 3 to NHCONH (4) resulted in a 22-fold loss in reversible binding; 4 was not any better an irreversible inhibitor[9] than 3, nor did 4 show selectivity of action toward the liver enzyme. Insertion of a Cl atom (5) *ortho* to the SO_2F group of 4 also failed to give a better reversible or more selective irreversible inhibitor. Insertion of a p-NHCONH bridge and Cl atom (6) on 2 gave only a twofold loss in reversible binding; unfortunately, 6 still showed insufficient specificity.

The loss in specificity with the five derivatives with a 6-phenethyl bridge (2–6) compared to 1 is noteworthy. As discussed in detail in a following paper,[11] these results have been rationalized on the basis of the allowable ground-state conformations of the 6 side chain of 1 *vs.* 2.

When the SO_2F group of 1 was moved to the *para* position (7), reversible inhibition was improved about fourfold; the specificity pattern was maintained, 7 being essentially uneffective as an irreversible inhibitor of the liver enzyme.

Replacement of the NHCO bridge of 1 by $NHSO_2$ (8) resulted in about a 50-fold increment in binding, a result attributed to the change in bond angle from planarity; however, specificity was lost as shown by

(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**, 79 (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 G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

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

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

(7) B. R. Baker and P. C. Huang, *ibid.*, **11**, 495 (1968), paper CXX of this series.

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

(9) The rate of inactivation of an enzyme by an active-site-directed irreversible inhibitor is dependent upon the concentration of the rate-limiting enzyme–inhibitor reversible complex [EI] which in turn is dependent upon K_i and the concentration of the inhibitor; see ref 3, Chapter 8, for the kinetics of irreversible inhibition.

(10) Use of I_{50} values for inhibition of the dihydrofolic reductase from either L1210/0 or L1210/DF8 are reasonably accurate since they have not differed more than twofold.[8]

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

the ability of **8** to inactivate the liver enzyme. Similar results were observed when the SO_2F group of **8** was moved to the *para* position (**9**).

Insertion of a 2-chloro (**10**) on the phenoxy moiety of **1** gave little change in reversible binding. The resultant **10** was just as selective toward the liver enzyme as 1; however, both **1** and **10** gave only 40–50% irreversible inhibition at near K_i concentration, thus failing the criterion of >70% inactivation at this concentration. When the SO_2F group of **10** was moved to the *para* position (**11**), little change in reversible inhibition took place; **11** was still not an irreversible inhibitor of the liver enzyme, but was not quite as effective an irreversible inhibitor[9] of the L1210 enzymes as **1**.

Insertion of a 3-methyl (**12**) of the phenoxy moiety of **1** gave a small increment in reversible binding; although **12** was a somewhat better irreversible inhibitor than **1** when compared at a K_i concentration,[9] specificity toward the liver enzyme was lost with **12**. Similar results were observed with the p-SO_2F isomer (**13**) of **12**.

The effect of substituents on the benzenesulfonyl fluoride moiety of **1** was then studied. Introduction of a Cl atom *para* to the SO_2F group (**14**) gave about a tenfold increment in binding, but **14** was a poorer and less specific irreversible inhibitor than **1**. Introduction of an *o*-Me group (**15**) gave about a fivefold increment in reversible binding, but was somewhat detrimental to irreversible inhibition of the L1210 enzyme.

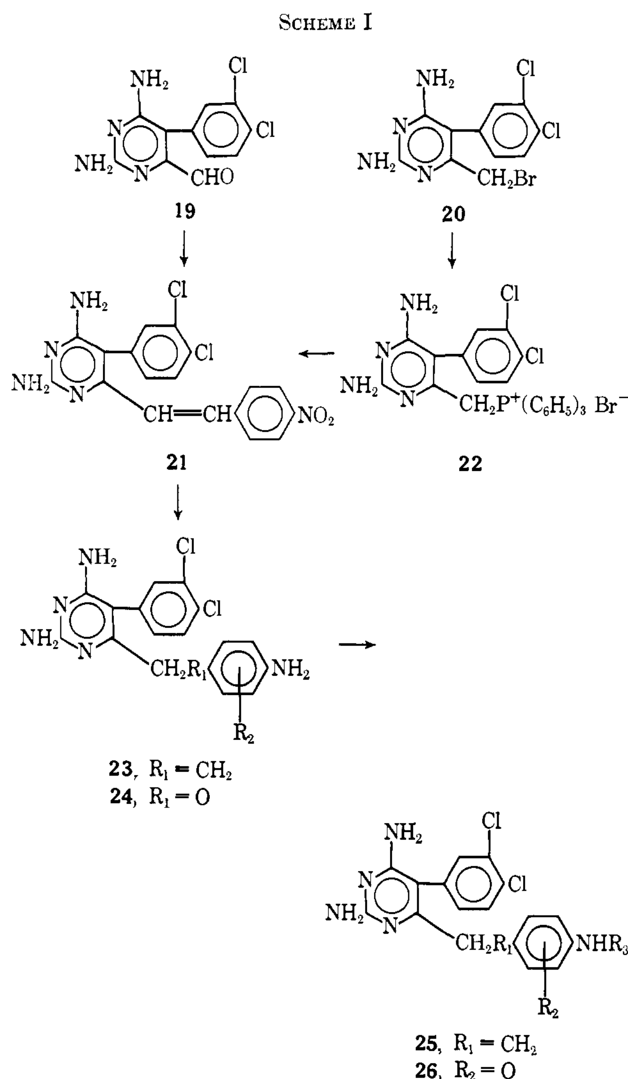
Substitutents in both phenyl groups on the 6 side chain were then studied. Insertion of Me *ortho* to the SO_2F (**16**) of **10** decreased the inhibitory properties. Insertion of a 3-Me group (**18**) on **14** gave little change in reversible inhibition of the L1210 enzyme; irreversible inhibition of the liver and L1210 enzymes was improved, but specificity was not achieved. Insertion of a 2-chlorine atom (**17**) on **14** decreased reversible binding about twofold; however, irreversible inhibition of the L1210 enzyme and selectivity were increased with **17**.

All of the compounds in Table I meeting the first criterion of an $I_{50} \leq 0.1\ \mu M$ failed to show specificity. Of the compounds (**1**, **7**, **10**, **11**, **17**) that show little or no inactivation of the liver enzyme and good inactivation of the tumor enzyme at a concentration of $2I_{50}$, none met the first criterion of $I_{50} \leq 0.1\ \mu M$ and none met the second criterion of >70% inactivation of the tumor enzyme at a concentration of $K_i = I_{50}/6$. However, *in vivo* testing of one or more of these five compounds could be helpful to establish whether or not these criteria are valid or should be changed.

Chemistry.—The candidate irreversible inhibitors in Table I can be generalized by structures **25** and **26** which were made by acylation of the amines **23** and **24** with the appropriate fluorosulfonylbenzoyl chloride. The synthesis of **24** has been previously described.[7,12] The second amine (**23**) was synthesized in the following manner (Scheme I).

The 6-bromomethylpyrimidine (**20**)[5] was converted in 90% yield to the Wittig reagent (**22**) with triphenylphosphine in THF. Condensation of **22** with p-nitrobenzaldehyde in DMF using 1,5-diazabicyclo[4,3,0]-nonene[13] (DBN) afforded the 6-(p-nitrostyryl)pyrimidine (**21**) in 65% yield of pure product; an alternate synthesis of **21** from the pyrimidine-6-carboxaldehyde (**19**)[5] and p-nitrobenzyltriphenylphosphonium bromide in DMF with KOBu-*t* or DBN as the base proceeded in similar yield. Catalytic reduction of **21** with PtO_2 catalyst proceeded to **23**.

SCHEME I



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

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

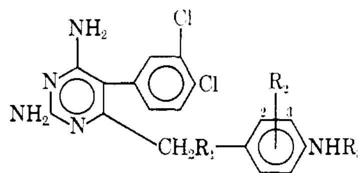
Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Each analytical sample moved as a single spot on tlc on Brinkmann silica gel GF, each had appropriate ir and uv spectra, and each gave combustion values for C, H, and N or F within 0.4% of theoretical. The physical properties of **2–8** are listed in Table II.

2,4-Diamino-5-(3,4-dichlorophenyl)-6-pyrimidylmethyltriphenylphosphonium Bromide (**22**).—A stirred mixture of 2.0 g (5.2 mmoles) of **20**,[5] 1.7 g (5.2 mmoles) of triphenylphosphine, and 50 ml of THF was refluxed for 1 hr, then cooled. The product was collected on a filter and washed with C_6H_6; yield, 2.9 g (90%) that showed one spot on tlc in 1:4 EtOH–$CHCl_3$ and was suitable for the next step. Recrystallization of a sample from EtOH gave white crystals, mp 275–287° dec. *Anal.* ($C_{29}H_{24}BrCl_2N_4P$) C, H, N.

2,4-Diamino-5-(3,4-dichlorophenyl)-6-(p-nitrostyryl)pyrimidine (**21**). **A.**—To a stirred solution of 3.05 g (5 mmoles) of **22** and 0.75 g (5 mmoles) of p-nitrobenzaldehyde in 20 ml of DMF protected from moisture was added 0.62 g (5 mmoles) of 1,5-diazabicyclo[4,3,0]nonene.[13] After 16 hr the mixture was diluted with 20 ml of H_2O. The product was collected on a filter and washed with EtOH. Recrystallization from MeOEtOH

TABLE I
INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



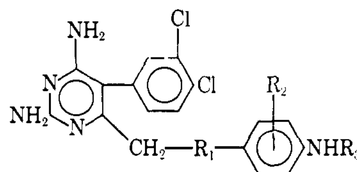
No.	R_1	R_2	R_3	Enzyme source	Reversible[b] I_{50},[d] μM	Reversible[b] K_i,[e] μM	Irreversible[c] Inhib. μM	Irreversible[c] % E·I[f]	Irreversible[c] Time, min	Irreversible[c] % inactvn
1[g]	O	H	$COC_6H_4SO_2F$-*m*	L1210/DFS	0.82	0.13	0.50	78	60	94
							0.13	50	60	43
				L1210/0			2.0		60	79[h]
							0.5		60	43[h]
				Liver			5.0		60	0[h]
				Spleen			1.0		60	0[h]
				Intestine			1.0		20	0[h]
2	CH_2	H	$COC_6H_4SO_2F$-*m*	L1210/0	0.057	0.0095	0.19	95	60	56[h]
				L1210/DFS			0.19		60	75[h]
				Liver	0.034	0.0057	0.19	97	60	31
3	CH_2	H	$COC_6H_4SO_2F$-*p*	L1210/0	0.041	0.0068	0.36	98	60	49[h]
				L1210/DFS			0.36		60	61[h]
4	CH_2	H	$CONHC_6H_4SO_2F$-*p*	L1210/DFS	0.89	0.15	1.8	93	60	76[h]
				L1210/0			1.8		60	77[h]
				Liver			1.8		60	90
5	CH_2	H	$CONHC_6H_3$-3-Cl-4-SO_2F	L1210/DFS	0.92	0.15	1.8	93	60	76[h]
				L1210/0			1.8		60	75[h]
				Liver			1.8		60	97[h]
6	CH_2	H	$CONHC_6H_3$-2-Cl-5-SO_2F	L1210/0	0.11	0.018	0.68	98	60	82[h]
							0.11	87	60	55[h]
				L1210/DFS			0.11		60	76[h]
							0.02		60	54
				Liver			0.68		60	69
							0.11		60	28
7	O	H	$COC_6H_4SO_2F$-*p*	L1210/0	0.18	0.030	0.36	93	60	33[h]
				L1210/DFS			0.36		60	69[h]
				Liver			0.36		60	6[h]
8	O	H	$SO_2C_6H_4SO_2F$-*m*	L1210/DFS	0.018	0.0030	0.036	93	60	42[h]
				L1210/0			0.036		60	41[h]
				Liver			0.090		60	58[h]
9	O	H	$SO_2C_6H_4SO_2F$-*p*	L1210/DFS	0.049	0.0082	0.098	93	60	93[h]
				L1210/0			0.098		60	54[h]
				Liver			0.098		60	73[h]
10	O	2-Cl	$COC_6H_4SO_2F$-*m*	L1210/DFS	0.53	0.088	0.53	87	60	82[h]
							0.10	53	60	62[h]
				L1210/0			1.1		60	70[h]
							0.16		60	41[h]
				Liver			1.1		60	0[h]
11	O	2-Cl	$COC_6H_4SO_2F$-*p*	L1210/0	0.31	0.052	0.62	93	60	39[h]
				L1210/DFS			0.62		60	77[h]
				Liver			0.62		60	10[h]
12	O	3-Me	$COC_6H_4SO_2F$-*m*	L1210/DFS	0.28	0.047	0.56	93	60	94
							0.050	51	60	73
				L1210/0			0.56		60	43
				Liver			0.56		60	29[h]
13	O	3-Me	$COC_6H_4SO_2F$-*p*	L1210/0	0.12	0.020	0.24	93	60	15[h]
				L1210/DFS			0.24		60	77[h]
				Liver			0.6			55[h]
14	O	H	COC_6H_3-2-Cl-5-SO_2F	L1210/DFS	0.085	0.014	0.17	95	60	58[h]
				L1210/0			0.17		60	27[h]
				Liver			0.17		60	33[h]
15	O	H	COC_6H_3-4-Me-3-SO_2F	L1210/0	0.15	0.025	0.30	93	60	35[h]
				L1210/DFS			0.30		60	58[h]
				Liver			0.30		60	0[h]
16	O	2-Cl	COC_6H_3-4-Me-3-SO_2F	L1210/DFS	0.50	0.063	0.50	87	60	51[h]
				L1210/0			1.0		60	34[h]
17	O	2-Cl	COC_6H_3-2-Cl-5-SO_2F	L1210/0	0.16	0.027	0.32	93	60	53[h]
				L1210/DFS			0.32		60	82[h]
							0.04		60	45[h]
				Liver			0.32		60	16[h]

TABLE I (*Continued*)

No.	R_1	R_2	R_3	Enzyme source	Reversible[b] I_{50},[d] μM	Reversible[b] K_i,[e] μM	Irreversible[c] Inhib, μM	Irreversible[c] % E···I[f]	Irreversible[c] Time, min	Irreversible[c] % inactvn
18	O	3-Me	COC_6H_3-2-Cl-5-SO_2F	L1210/0	0.10	0.017	0.20	93	60	91[h]
				L1210/DF8			0.20		60	97[h]
				Liver			0.20		60	80[h]

[a] The technical assistance of Sharon Lafler and Diane Shea with these assays is acknowledged. [b] Assayed with 6 μM dihydrofolate and 30 μM TPNH in pH 7.4 Tris buffer containing 0.15 mM KCl as previously described.[8] [c] Incubated at 37° in pH 7.4 Tris buffer in the presence of 60 μM TPNH then assayed in the presence of 0.15 M KCl as previously described.[8] [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.[9] [g] Data from ref 8. [h] Zero point obtained by adding inhibitor to assay cuvette prior to addition of enzyme aliquot.[8]

TABLE II



No.	R_1	R_2[a]	R_3	Method[b]	% yield[c]	Mp, °C dec[h]	Formula[d]
2	CH_2	H	$COC_6H_4SO_2F$-*m*	D	54	191	$C_{25}H_{20}Cl_2FN_5O_3S \cdot 0.5H_2SO_4 \cdot 0.5H_2O$
3	CH_2	H	$COC_6H_4SO_2F$-*p*	D	61	232	$C_{25}H_{20}Cl_2FN_5O_3S \cdot 0.5H_2SO_4 \cdot 0.5H_2O$[e]
4	CH_2	H	$CONHC_6H_4SO_2F$-*p*	D	20	220	$C_{25}H_{21}Cl_2FN_6O_3S \cdot 0.5H_2SO_4$
5	CH_2	H	$CONHC_6H_3$-3-Cl-4-SO_2F	D[f]	19	225	$C_{25}H_{20}Cl_3FN_6O_3S \cdot 0.5H_2SO_4$
6	CH_2	H	$CONHC_6H_3$-2-Cl-5-SO_2F	D[g]	50	196	$C_{25}H_{20}Cl_3FN_6O_3S \cdot 0.5H_2SO_4$
7	O	H	$COC_6H_4SO_2F$-*p*	C	36	250	$C_{24}H_{18}Cl_2FN_5O_4S \cdot 0.5H_2SO_4 \cdot 0.5H_2O$
8	O	H	$SO_2C_6H_4SO_2F$-*m*	A	22	160	$C_{23}H_{18}Cl_2FN_5O_5S_2 \cdot 0.5H_2SO_4$
9	O	H	$SO_2C_6H_4SO_2F$-*p*	A	13	188	$C_{23}H_{18}Cl_2FN_5O_5S_2 \cdot 0.5H_2SO_4$
10	O	2-Cl	$COC_6H_4SO_2F$-*m*	C	33	195	$C_{24}H_{17}Cl_3FN_5O_4S \cdot 0.5H_2SO_4$
11	O	2-Cl	$COC_6H_4SO_2F$-*p*	C	33	195	$C_{24}H_{17}Cl_3FN_5O_4S \cdot 0.5H_2SO_4$
12	O	3-Me	$COC_6H_4SO_2F$-*m*	C	34	175	$C_{25}H_{20}Cl_2FN_5O_4S \cdot 0.5H_2SO_4$
13	O	3-Me	$COC_6H_4SO_2F$-*p*	C	47	190	$C_{25}H_{20}Cl_2FN_5O_4S \cdot 0.5H_2SO_4$
14	O	H	COC_6H_3-2-Cl-5-SO_2F	C[f]	26	188	$C_{24}H_{17}Cl_3FN_5O_4S \cdot 0.5H_2SO_4$
15	O	H	COC_6H_3-4-Me-3-SO_2F	C[f]	37	179	$C_{25}H_{20}Cl_2FN_5O_4S \cdot 0.5H_2SO_4$
16	O	2-Cl	COC_6H_3-4-Me-3-SO_2F	C[f]	41	187	$C_{25}H_{19}Cl_3FN_5O_4S \cdot 0.5H_2SO_4$
17	O	2-Cl	COC_6H_3-2-Cl-5-SO_2F	C[f]	36	184	$C_{24}H_{16}Cl_4FN_5O_4S \cdot 0.5H_2SO_4$
18	O	3-Me	COC_6H_3-2-Cl-5-SO_2F	C[f]	49	180	$C_{25}H_{19}Cl_3FN_5O_4S \cdot H_2SO_4$

[a] Numbered from R_1 at the 1 position. [b] For methods B and C, see ref 7; D is the same as B with an equivalent amount of Et_3N added and A with 1 equiv of pyridine. [c] Recrystallized from MeOEtOH–H_2O unless otherwise indicated. [d] Analyzed for C, H, and F. [e] H: calcd, 3.58; found, 4.37. [f] See B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **12**, 104 (1969), paper CXLII of this series, for preparation of the requisite benzoic acid derivative. [g] Recrystallized from MeOEtOH–EtOH. [h] Melting gradually occurred over a wide range starting at the temperature indicated.

with the aid of decolorizing carbon gave 1.3 g (65%) of yellow crystals, mp 309–313° dec (block preheated to 285°). *Anal.* ($C_{18}H_{13}Cl_2N_5O$) C, H, N.

B.—Condensation of **19**[5] and *p*-nitrobenzyltriphenylphosphonium bromide in the same manner gave 62% of **21**, mp 308–312° dec (block preheated to 285°).

6-(*p*-Aminophenethyl)-2,4-diamino-5-(3,4-dichlorophenyl)-pyrimidine (23) Bisethanesulfonate.—A mixture of 200 mg (0.50 mmole) of **21**, 110 mg (1 mmole) of ethanesulfonic acid, and 100 ml of MeOEtOH was shaken with H_2 at 2–3 atm in the presence of 60 mg of PtO_2 for 6 hr when reduction was complete. The filtered solution was evaporated *in vacuo* leaving 290 mg (97%) of product as a glass that was uniform on tlc in 1:9 EtOH–$CHCl_3$ and was suitable for further transformations.