Irreversible Enzyme Inhibitors. CX.[1,2] Candidate Irreversible Inhibitors of Dihydrofolic Reductase Derived from 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-s-triazine. IV[2,3]

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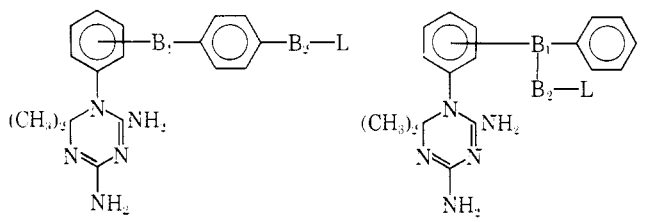
Four new candidate active-site-directed irreversible inhibitors for dihydrofolic reductase of the 1-phenyl-*s*-triazine type with branched chains have been synthesized. Three of the compounds had a bromoacetamidomethyl group on the alkane bridge of a 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(phenylalkylphenyl)-*s*-triazine; the fourth compound was 1-[3-bromoacetamido-4-phenylpropyloxyphenyl)-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine. The four compounds showed considerable loss in reversible binding to dihydrofolic reductase compared to the parent compounds without the bromoacetamido branch; even though these reversible binding studies indicated that the bromoacetamido group of the four compounds was in contact with the enzyme surface, none of the four showed irreversible inhibition of the dihydrofolic reductases from pigeon liver, Walker 256 rat tumor, or L1210/FR8 mouse leukemia.

In the previous paper of this series,[2] dihydro-*s*-triazines of type **1** were synthesized and evaluated as



active-site-directed irreversible inhibitors[5,6] of dihydrofolic reductase; B_1 is a bridge between the two benzene rings, B_2 is a bridge between the outside phenyl group and a leaving group, L, of the bromoacetamide, chloromethyl ketone, or maleamic acid types.[7] Although compounds of type **1** are excellent reversible inhibitors of dihydrofolic reductase, none showed irreversible inhibition of the enzyme from Walker 256 rat tumor, L1210/FR8 mouse leukemia, or pigeon liver. That these compounds of type **1** were not irreversible inhibitors could be rationalized in one of two ways: (1) the leaving group, L, was not juxtaposed to a nucleophilic group on the enzyme within the reversible enzyme-inhibitor complex,[5] or (2) the juxtaposed enzymic nucleophilic group did not have the proper character to react at a detectable rate with leaving groups, L, of the halomethylcarbonyl or α,β-unsaturated carbonyl types.[5]

Two approaches were investigated to solve this enigma, the first of which is the subject of this paper. The bromoacetamido group, L, was placed on a branch, B_2, as shown in **2**. This could project the leaving group into a different area of the enzyme than does type **1**. The second approach was to change the leaving group on **1** to SO_2F; these results have been reported.[3a]

Enzyme Results.—The concentration of the inhibitors necessary for 50% reversible inhibition (I_{50}) of the dihydrofolic reductase from pigeon liver, Walker 256 rat tumor, and L1210/FR8 mouse leukemia are listed in Table I. The following correlations can be made.

(1) Chain branching to give structures of type **2** leads to a loss in binding to the enzyme. Note that introduction of the bromoacetamidomethyl group of **4** on the ethane bridge of **3** results in a 12-fold loss in binding to the pigeon liver enzyme. Even larger losses in binding occur when the butyl bridge is branched. Note that introduction of the bromoacetamidomethyl group of **6** on **5** in the *meta* series results in a 350-fold loss in binding; a similar 160-fold loss in binding occurs in the *para* series (**8** *vs.* **7**).

(2) Two relatively large substituents on the 1-phenyl group of the 1-phenyl-*s*-triazine system also lead to a large loss in binding. Comparison of the *m*-phenylpropyloxy derivative (**9**)[8] with the *p*-phenylpropyloxy-*m*-bromoacetamido derivative (**10**) shows the latter is 60-fold less effective in binding; since *meta* and *para* substituents of the phenylalkyl type on the 1-phenyl-*s*-triazine give similar binding,[5] this comparison is not too unreasonable.

(3) The greatest differences in binding to the different dihydrofolic reductases caused by substitution on the bridge are seen with **8** and **10**; these differences are relatively small, being less than tenfold. These results again emphasize that differences in reversible binding among mammalian enzymes will most probably be insufficient for use in cancer chemotherapy,[9,10] but these small differences should be exploitable with properly constructed active-site-directed irreversible inhibitors.[5a,9]

None of the candidate irreversible inhibitors in Table I showed irreversible inhibition of the three dihydrofolic reductases when the enzyme was incubated at 37° with a $5I_{50}$ concentration of inhibitor by the

(1) This work was generously supported by Grant CA-08035 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series, see B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 26 (1968).

(3) For the earlier papers on candidate irreversible inhibitors derived from 1-phenyl-*s*-triazine see (a) B. R. Baker and G. J. Lourens, *ibid.*, **10**, 1113 (1967), paper CV of this series; (b) B. R. Baker and R.-T. Ho, *J. Pharm. Sci.*, **56**, 28 (1967), paper LXX of this series.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

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

(6) B. R. Baker, *J. Pharm. Sci.*, **53**, 347 (1964).

(7) The reasons for selection of compounds of type **1** are discussed in ref 2.

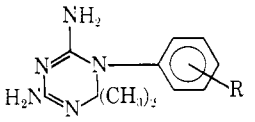
(8) B. R. Baker and G. J. Lourens, *J. Pharm. Sci.*, **56**, 871 (1967).

(9) See ref 5, Chapter X.

(10) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967), paper XCVII of this series.

TABLE I

INHIBITION OF DIHYDROFOLIC[a,b] REDUCTASES BY

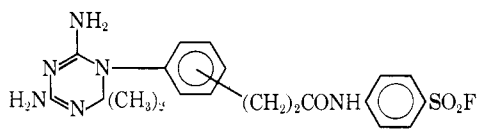


No.	R	I_{50}[c] μM Pigeon liver	Walker 256	L1210/FR8
3	*m*-$CH_2ClI_2C_6H_5$	0.024[d]		
4	*m*-$CH_2CHC_6H_5$ (CH$_2$NHCOCH$_2$Br)	0.29	0.19	0.22
5	*m*-$(ClI_2)_4C_6H_6$	0.0027[e]		
6	*m*-$CH(CH_2)_3C_6H_5$ (CH$_2$NHCOCH$_2$Br)	0.95	0.43	3.1
7	*p*-$(CH_2)_4C_6H_5$	0.010[e]		
8	*p*-$CH(CH_2)_3C_6H_5$ (CH$_2$NHCOCH$_2$Br)	1.6	0.30	0.33
9	*m*-$O(CH_2)_3C_6H_5$	0.088[f]		
10	*m*-NHCOCH$_2$Br-*p*-$O(CH_2)_3C_6H_5$	5.8	0.78	0.50

[a] The technical assistance of Barbara Baine and Jean Reeder with these assays is acknowledged. [b] Assayed with 6 μM dihydrofolate at pH 7.4 as previously described;[3a] 12 μM TPNH was used with the pigeon liver enzyme and 30 μM TPNH with the other two. [c] I_{50} = concentration of inhibitor required for 50% inhibition. [d] Data from B. R. Baker and B.-T. Ho, *J. Heterocyclic Chem.*, **2**, 72 (1965). [e] Data from B. R. Baker, B.-T. Ho, and G. J. Loureus, *J. Pharm. Sci.*, **56**, 737 (1967). [f] Data from ref 8.

methods previously described.[3a] Even though the reversible inhibition data indicated that the bromoacetamido groups of the candidate compounds were in contact with the enzyme surface, it is probable that no amino acid on the surface in contact with the bromoacetamide has the proper type of nucleophilic group required for reaction with the leaving group.

The results from this and the previous study[2] on compounds of type **1** bearing the halomethyl carbonyl type of leaving group suggested that a leaving group be explored that could attack a serine or threonine hydroxyl. Such a compound is **11** which rapidly in-



11, *para* isomer
12, *meta* isomer

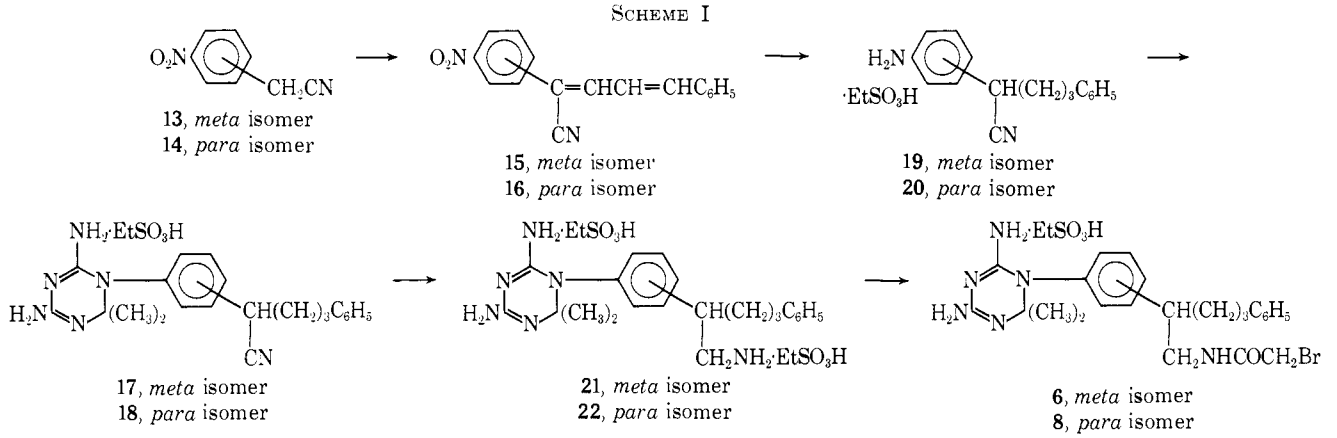
activates the dihydrofolic reductase from Walker 256 rat tumor, L1210/FR8 mouse leukemia, and pigeon liver; only the latter enzyme was inactivated by the *meta* isomer (**12**). These studies, on **11** and **12**, although completed after those on the bromoacetamide leaving group reported here and earlier,[2] were published first in order to establish that the methodology of the enzyme assays was correct.

Chemistry.—The key reaction for synthesis of compounds of type **2** was the condensation of a substituted phenylacetonitrile such as **13** or **25** with a substituted benzaldehyde or cinnamaldehyde.[11] Condensation of cinnamaldehyde with *m*-nitrophenylacetonitrile (**13**) in the presence of methanolic KOH afforded the diene (**15**) in 24% yield (Scheme I); the *para* isomer (**16**)[11b] was prepared similarly in 75% yield. Hydrogenation of **16** with a Pd catalyst reduced the nitro group and the butadiene system, but not the nitrile group as anticipated, to afford **20** isolated as its crystalline ethanesulfonate salt in 86% yield; the *meta* isomer (**19**) was prepared similarly but was obtained as an oil. Condensation of **19** or **20** with cyanoguanidine and Me_2CO by the three-component method of Modest[12] afforded the crystalline *s*-triazines **17** and **18**. Hydrogenation of the CN group of **17** and **18** with a Pt catalyst proceeded in the presence of ethanesulfonic acid to give the aminomethyl derivatives **21** and **22**; these were not further purified, but were treated[13] with bromoacetic anhydride in DMF in the presence of triethylamine to give the crystalline bromoacetamides **6** and **8**.

Condensation of *m*-nitrobenzaldehyde (**23**) with phenylacetonitrile (**25**) in methanolic KOH afforded the stilbene (**26**) in 56% yield (Scheme II). A similar condensation of **25** with *m*-nitrocinnamaldehyde (**24**) afforded the butadiene (**27**)[11c] as a mixture of geometric isomers. Hydrogenation of **27** with a Pd catalyst reduced the nitro group and the double bonds to give **28** which was directly converted to the crystalline *s*-triazine ethanesulfonate (**30**). In contrast, hydrogenation of the stilbene **26** with a Pd catalyst reduced the nitro group but not the double bond; the presence of the latter in **29** was shown by a uv maximum at 315 mμ characteristic of its conjugated system. Conversion of **29** to the *s*-triazine hydrochloride (**31**) proceeded smoothly.

Hydrogenation of **31** with a PtO_2 catalyst in the presence of HCl reduced both the cyano and the stilbene double bond to give **32**. Bromoacetylation of **32**

SCHEME I



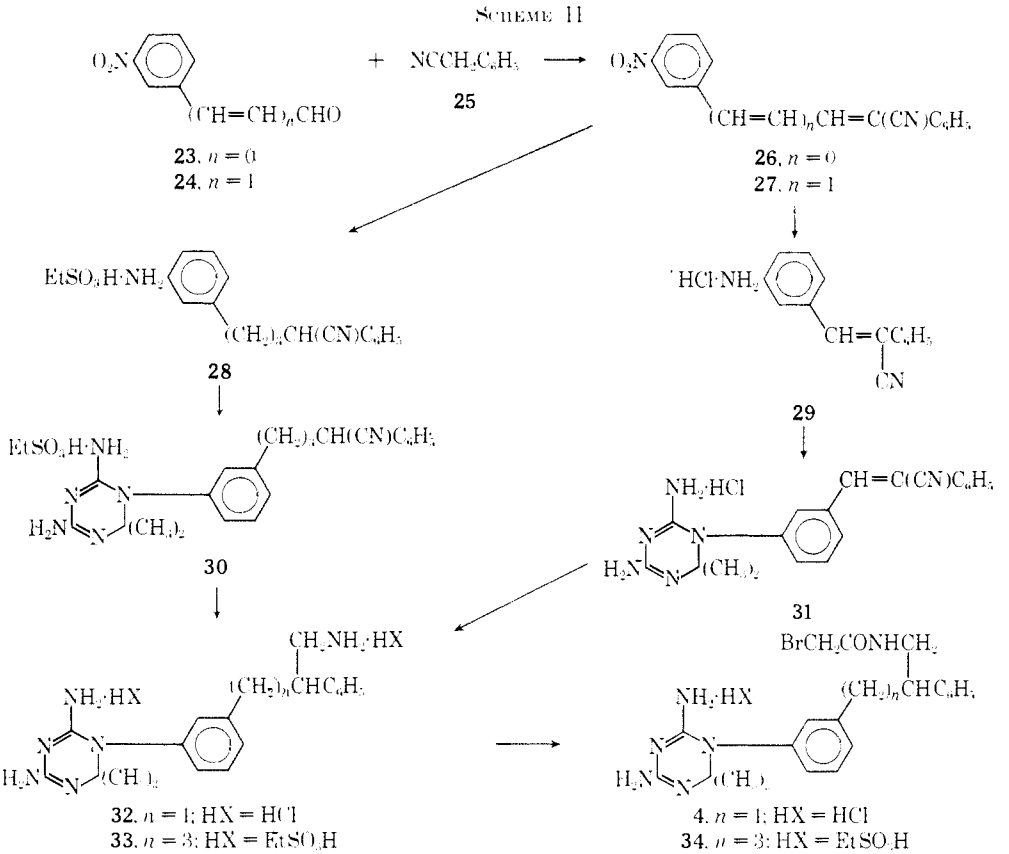
13, *meta* isomer
14, *para* isomer
15, *meta* isomer
16, *para* isomer
19, *meta* isomer
20, *para* isomer
17, *meta* isomer
18, *para* isomer
21, *meta* isomer
22, *para* isomer
6, *meta* isomer
8, *para* isomer

(11) (a) H. Lettré, W. Haede, and L. Schäfer, *Z. Physiol. Chem.*, **289**, 298 (1952); (b) P. Remse, *Ber.*, **23**, 3133 (1890). (c) J. Colonge and P. Frank, *Bull. Soc. Chim. France*, 3090 (1964).

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

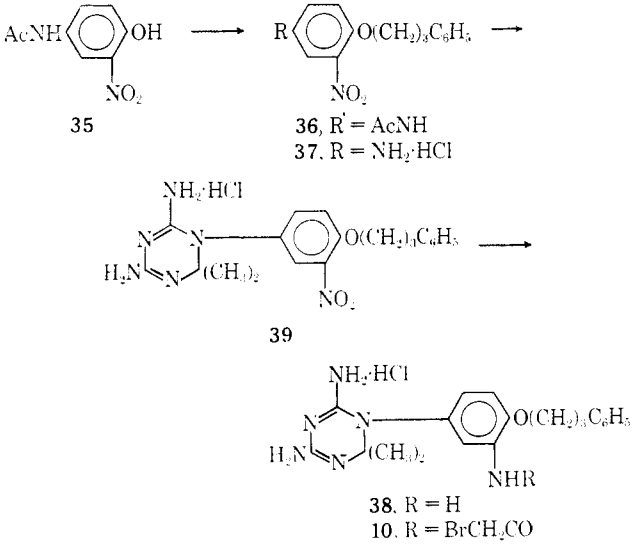
(13) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocyclic Chem.*, **3**, 425 (1966).

Scheme II



afforded **4** as an amorphous solid. A similar reduction and bromoacetylation of **30** gave **34** as a gum which could not be obtained sufficiently pure for enzyme assay.

Alkylation of 4-acetamido-2-nitrophenol (**35**)[14] with phenylpropyl bromide in DMF in the presence of K_2CO_3 at 60–65° afforded **36** in 79% yield. Treatment of **35** with hot HCl in H_2O–EtOH gave the amine



hydrochloride (**37**) in 75% yield. Conversion of **37** to the *s*-triazine (**39**) proceeded smoothly. Hydrogenation of the nitro group of **39** to **38** followed by bromoacetylation in DMF afforded the desired candidate irreversible inhibitor (**10**).

(14) P. Friedländer and M. Zeitlin, *Ber.*, **27**, 192 (1894).

Experimental Section[15]

***m*-Nitro-α-phenylcinnamonitrile (26). Method A.**—To a stirred mixture of 6.04 g (40 mmoles) of **23**, 5.08 ml (40 mmoles) of **25**, and 10 ml of MeOH cooled in an ice bath was added 1 ml of 25% KOH in H_2O. After being stirred 1 hr in the ice bath, during which time the product began to separate, the mixture was stirred at ambient temperature for 24 hr. The product was collected on a filter, washed with cold MeOH, then recrystallized from EtOH; yield 5.58 g (56%) of yellow crystals: mp 133–134°; λ_{max} 280 (infl), 310 mμ; ν_{max} 2200 (C≡N), 1600 (C=C), 1510, 1340 (NO_2), 770, 732, 690 cm^{-1} (phenyl CH). See Table II for additional data.

***p*-Amino-α-(phenylpropyl)phenylacetonitrile Ethanesulfonate (20). Method B.**—A mixture of 2.76 g (10 mmoles) of **16**, 0.25 g of 5% Pd–C, and 100 ml of EtOH was shaken with H_2 at 2–3 atm for 6 hr when 50 mmoles of H_2 was absorbed. The mixture was filtered through a Celite pad and the filtrate was treated with 1.11 g (10 mmoles) of $EtSO_3H$. Spin-evaporation *in vacuo* gave a solid that was recrystallized from *i*-PrOH; yield 3.10 g (86%): mp 184–185°; λ_{max} 247 mμ; ν_{max} 2920, 2630, 1900 (NH^+), 2230 (C≡N), 1625, 1600, 1550, 1510 (NH, C=C), 1210, 1040 (SO_3^-), 750, 700 cm^{-1} (phenyl CH). See Table II for additional data.

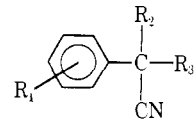
3-Nitro-4-(phenylpropoxy)acetanilide (36).—A mixture of 1.96 g (10 mmoles) of **35**,[14] 2.99 g (15 mmoles) of phenylpropyl bromide, 1.38 g (10 mmoles) of K_2CO_3, and 10 ml of DMF was stirred in a bath at 60–65° for 20 hr. The mixture was spin-evaporated *in vacuo*. The residue was heated on a steam bath with 50 ml of C_6H_6 and 25 ml of H_2O. After cooling, the mixture was filtered and the product was washed with H_2O and petroleum ether (bp 60–110°). Recrystallization from C_6H_6–petroleum ether with the aid of charcoal gave a 2.47-g (79%) yield of light yellow crystals, mp 119–120°. The compound moved as a single spot on tlc in EtOAc and had ν_{max} 3200 (NH), 1650 (amide C=O), 1580 (NH), 1520, 1330 (NO_2), 1250 (C–O–C), 880, 830 cm^{-1} (phenyl CH).

Anal. Calcd for $C_{17}H_{18}N_2O_4$: C, 64.9; H, 5.77; N, 8.91. Found: C, 64.8; H, 5.91; N, 8.76.

(15) Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Ir spectra were determined in KBr pellet, uv spectra in EtOH. Tlc was performed on Brinkmann silica gel GF, except for the *s*-triazines where polyamide-MN was employed; spots were detected by visual examination under uv light.

TABLE II

PHYSICAL PROPERTIES[a] OF

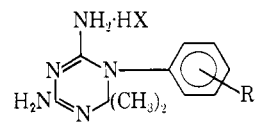


No.	R_1	R_2	R_3	Method	% yield	Mp, °C	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
15	*m*-NO_2	..	$C_6H_5CH{=}CHCH{=}$	A	24[b]	196–198	73.9	4.37	10.1	73.9	4.60	10.1
16	*p*-NO_2	..	$C_6H_5CH{=}CHCH{=}$	A	75[b]	208–209[c]						
19	*m*-NH_2[d]	H	$C_6H_5(CH_2)_3$	B		Oil[e]						
20	*p*-NH_2[d]	H	$C_6H_5(CH_2)_3$	B	86[f]	184–185	63.3[d]	6.68	7.74	63.1	6.82	7.77
26	H	..	*m*-$NO_2C_6H_4CH{=}$	A	56[g]	133–134	72.0	4.02	11.2	72.2	4.18	11.5
27	H	..	*m*-$NO_2C_6H_4CH{=}CHCH{=}$	A	42[b]	142–167[h,i]	73.9	4.37	10.1	74.0	4.50	10.3
28	H	H	*m*-$NH_2C_6H_4(CH_2)_3$[d]	B		Oil[e]						
29	H	..	*m*-$NH_2C_6H_4CH{=}$[j]	B	49[f,k]	182–185 dec	70.2[j]	5.10	10.9	70.3	5.27	11.0

[a] All analytical samples had ir and uv spectra in agreement with their assigned structures; each moved as a single spot on tlc unless otherwise indicated. [b] Recrystallized from 2-methoxyethanol. [c] Lit.[11b] mp 205–206°. [d] Ethanesulfonate. [e] Directly converted to *s*-triazine (see Table III). [f] Recrystallized from *i*-PrOH. [g] Recrystallized from EtOH. [h] Softens at 100°; a mixture of geometric isomers. Lit.[11c] mp 172°. [i] Tlc with 1:1 C_6H_6–petroleum ether (bp 30–60°) showed two spots. [j] Hydrochloride. [k] λ_{max} 233, 315 mμ.

TABLE III

PHYSICAL PROPERTIES[a] OF



No.	HX	R	Method	% yield	Mp, °C dec	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
17	$EtSO_3H$	*m*-$C_6H_5(CH_2)_3CH(CN)$	C	42[b,c]	175–176	59.5	6.65	17.3	59.6	6.72	17.5
18	$EtSO_3H$	*p*-$C_6H_5(CH_2)_3CH(CN)$	C	77[d]	196–197	59.5	6.65	17.3	59.5	6.72	17.3
30	$EtSO_3H$	*m*-$C_6H_5CH(CN)(CH_2)_3$	C	38[b,c]	168–169	59.5	6.65	17.3	59.2	6.76	17.1
31	HCl	*m*-$C_6H_5C(CN){=}CH$	C	83[e,f]	185–186	63.1	5.55	22.0	63.0	5.46	21.8
39	HCl	*m*-NO_2-*p*-$O(CH_2)_3C_6H_5$	C	77[g,h]	195–197	55.5	5.82	19.4	55.2	5.71	19.2
4	HCl	*m*-$C_6H_5CHCH_2$– $\lvert$ $CH_2NHCOCH_2Br$	D	99[i]	Amorphous[j]						
6	$EtSO_3H$	*m*-$C_6H_5(CH_2)_3CH$– $\lvert$ $NHCOCH_2Br$	D	32[k,d]	146–148	51.2	6.11	13.8	50.9	6.11	13.9
8	$EtSO_3H$	*p*-$C_6H_5(CH_2)_3CH$– $\lvert$ $NHCOCH_2Br$	D	41[d]	172–174	51.2	6.11	13.8	51.4	6.20	13.6
10	HCl	*m*-$BrCH_2CONH$-*p*-$O(CH_2)_3C_6H_5$	D[l]	69[d]	204–205	50.4	5.38	16.0	50.6	5.46	15.8

[a] All analytical samples had ir and uv spectra in agreement with their assigned structures; each moved as a single spot on tlc on polyamide-MN. [b] Over-all yield for two steps from nitro precursor. [c] Recrystallized from *i*-PrOH–EtOAc. [d] Recrystallized from *i*-PrOH. [e] Recrystallized from absolute EtOH–petroleum ether (bp 30–60°). [f] λ_{max} 243, 315 mμ. [g] Recrystallized from H_2O–EtOH. [h] λ_{max} 244, 320 mμ. [i] Reprecipitated from MeOH–Et_2O. [j] Gave positive 4-(*p*-nitrobenzyl)pyridine test[13] and moved as a single spot on polyamide-MN but contained about 20% solvent after drying at 50°. The compound decomposed at higher temperatures. [k] Recrystallized from *i*-PrOH–petroleum ether (bp 60–110°). [l] No extra acid added for hydrogenation and no Et_3N added for bromoacetylation.

3-Nitro-4-(phenylpropoxy)aniline Hydrochloride (37).—A mixture of 1.57 g (5 mmoles) of **36**, 10 ml of EtOH, and 10 ml of 12 *N* HCl was refluxed with stirring for 1 hr, during which time the product separated. The mixture was cooled, and the product was collected on a filter and washed with 1:1 *i*-PrOH–petroleum ether. Recrystallization from EtOH–H_2O gave 1.19 g (75%) of light yellow crystals, which gradually decomposed over 185°: ν_{max} 2800, 2550 (NH^+), 1610, 1590 (C=C, NH), 1525, 1330 (NO_2), 1260 (C–O–C), 840, 830, 740, 695 cm^{-1} (phenyl CH).

Anal. Calcd for $C_{15}H_{16}N_2O_3 \cdot HCl$: C, 58.3; H, 5.54; N, 9.07. Found: C, 58.2; H, 5.70; N, 8.83.

1-[*p*-(1-Cyano-4-phenylbutyl)phenyl]-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine Ethanesulfonate (18). Method C.—A mixture of 2.71 g (7.5 mmoles) of **20**, 0.65 g (7.7 mmoles) of cyanoguanidine, and 50 ml of reagent Me_2CO was refluxed with stirring for 20 hr. The cooled mixture was filtered and the product was washed with Me_2CO. Recrystallization from *i*-PrOH gave 2.81 g (77%) of white crystals: mp 196–197°; λ_{max} 246 mμ; ν_{max} 3370, 3325, 3150 (NH), 2230 (C≡N), 1660 (C=NH^+), 1650, 1560, 1510 (NH, C=C, C=N), 1190, 1040 (SO_3^-), 750, 700 cm^{-1} (phenyl CH). See Table III for additional data.

1-[*p*-(1-Bromoacetamidomethyl-4-phenylbutyl)phenyl]-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine Ethanesulfonate (8). Method D.—A mixture of 969 mg (2 mmoles) of **18**, 100 mg of PtO_2, 100 ml of EtOH, and 220 mg (2 mmoles) of $EtSO_3H$ was shaken with H_2 at 2–3 atm until 4 mmoles of H_2 were consumed (about 3 hr). The filtered solution was spin-evaporated *in vacuo* leaving 1.13 g (94%) of **22** as a hygroscopic white solid, λ_{max} 244 mμ.

To a solution of 449 mg (0.75 mmole) of **22** in 0.50 ml of DMF, stirred in an ice bath, was added 0.38 ml of 2 *M* Et_3N in MeOH (0.76 mmole) followed by 288 mg (1.13 mmoles) of bromoacetic anhydride. After 20 min in the ice bath, the mixture was diluted with 10 ml of Et_2O. The solution was decanted from the separated gum. The latter was triturated several times with fresh Et_2O (six 5-ml portions). Crystallization, then recrystallization from *i*-PrOH gave 200 mg (44%) of white crystals, mp 172–174°. The compound gave a positive 4-(*p*-nitrobenzyl)pyridine test[13] and had λ_{max} 245 mμ; ν_{max} 3300, 3150 (NH), 1680–1650 (amide C=O, C=NH^+), 1600, 1550, 1520 (NH, C=C, C=N), 1180, 1040 (SO_3^-), 740, 700 cm^{-1} (phenyl CH). See Table III for additional data.