Irreversible Enzyme Inhibitors LXXXVII

Hydrophobic Bonding to Dihydrofolic Reductase IX. Mode of Binding of *m*-Aryloxyalkyl Groups on 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazine

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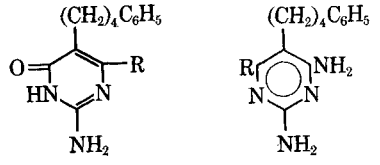
The relative binding to dihydrofolic reductase by 16 *m*-substituted 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazines—nine of which are newly synthesized—has been measured. Strong evidence is presented to indicate that the increased binding by terminal phenyl groups on a *m*-substituent is due to complexing to a relatively hydrophilic area since terminal benzyl and terminal phenoxy groups give nearly the same increments in binding. The possible conversion of such compounds to candidate active-site-directed irreversible inhibitors of dihydrofolic reductase is discussed.

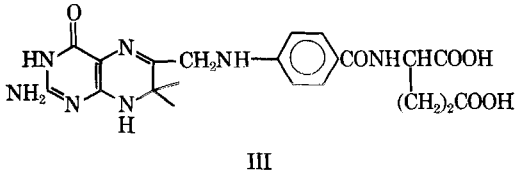
A HYDROPHOBIC BONDING area on an enzyme can have considerable utility in the design of potent and selective inhibitors, particularly if this area is adjacent to the active site (1). Since a hydrophobic bonding area on an enzyme that uses a polar substrate, such as dihydrofolic reductase, is probably nonfunctional with respect to binding of the substrate (1), large differences in the hydrophobic bonding area of this enzyme from different species would be expected to occur by evolution (2); such differences in the hydrophobic bonding area between pigeon liver and *E. coli* B dihydrofolic reductases (3), or between T_2-phage induced and *E. coli* B dihydrofolic reductases (4), or in other species (5) have been detected. In the case of dihydrofolic reductase, binding to the hydrophobic region can lead to complications; for example, if hydrophobic bonding is sufficiently strong, different rotomers of pyrimidine-type inhibitors may be bound where the hydrophobic group on the inhibitor is determinate for the rotomer (6).

The evidence for rotomers was initially based on differences in reversible binding with a variety of inhibitors (6, 7), then further verified with candidate irreversible inhibitors (8, 9). It was noted in the 2-amino-5-phenylbutyl-4-pyrimidinol series (I) that the reversible binding varied considerably with the R-substituent at the 6-position (6); the 6-methyl derivative (I*a*) was about 30 times more effective than the 6-propyl derivative (I*b*) (6), and the 6-phenylethyl derivative (I*d*) was in between in effectiveness (10). In contrast, in the 2,4-diamino series (II), the binding was independent of the 6-R-group; R = CH_3 (II*a*) (11), R = *n*-propyl (II*b*) (6), and R = the phenethyl derivative (II*d*) (8). All showed nearly identical reversible inhibition of the dihydrofolic reductase from pigeon liver. These results were rationalized on the basis that (*a*) the hydrophobic region is not between the pyrimidyl and phenyl moieties of dihydrofolate (III) (12, 13), but is near where the 4-position (or 8-position) of III resides on the enzyme (6)—as indicated by rotomer I; (*b*) the 4-pyrimidinols projected the 6-R group back toward the active site as indicated in rotomer I; and (*c*) the 2,4-diaminopyrimidines projected the 6-R group away from the active site (6) with the R-group probably not even in contact with the enzyme. That the alkylating function of I*d* and II*d* was not positioned the same way within their complexes with dihydrofolic reductase was indicated by the facts that the 4-pyrimidinol (I*d*) was an effective irreversible inhibitor of the enzyme (10), but the



I II

a, R = CH_3
b, R = $-C_3H_7$-*n*
c, R = $-(CH_2)_4-C_6H_4-NHCOCH_2Br$
d, R = $-(CH_2)_2-C_6H_4-NHCOCH_2Br$



III

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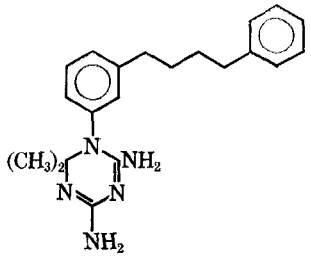
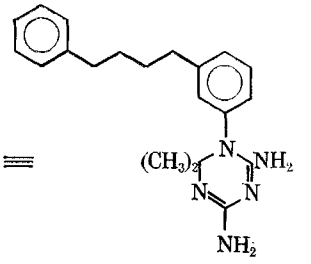


IV*A*



IV*B*

corresponding 2,4-diaminopyrimidine (II*a*) was not (8); although this result with II*d* was not unanticipated, it did create a serious problem in the design of irreversible inhibitors effective at low physiological concentration (8).

The effectiveness of an active-site-directed irreversible enzyme inhibitor is dependent upon two major factors: (*a*) the rate of inactivation is dependent upon the concentration of reversible enzyme–inhibitor complex which is in turn dependent upon the concentration of the inhibitor and the binding constant of the inhibitor, and (*b*) the rate of reaction of the neighboring group reaction between the leaving group on the inhibitor juxtaposed to a nucleophilic site on the enzyme (14, 15). Thus, in order to have an irreversible inhibitor effective at a physiological concentration of 10^{-8} *M*, it would be necessary that the inhibitor have a reversible binding constant of 10^{-7} *M* or less (8, 15). The 2-amino-4-pyrimidinol (I*d*) meets one of the two criteria for an effective irreversible inhibitor: I*d* rapidly alkylates a nucleophilic site on the enzyme with a half-life of about 12 min. at a concentration sufficient to convert 50% of the enzyme to a complex, but this concentration (4×10^{-5} *M*) is much too high for *in vivo* use. The 2,4-diaminopyrimidine (II*d*) also meets only one of the two criteria for an effective irreversible inhibitor since at 4×10^{-9} *M* it can complex 50% of the enzyme, but unfortunately does not inactivate the enzyme in a rotomeric conformation believed to be II. One possible solution to this enigma has been previously explored (16); the exploration of another possible solution is the subject of this paper.

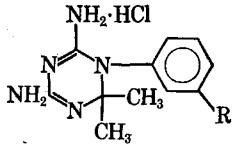
DISCUSSION

The 4,6-diamino-1-phenyl-*s*-triazines are believed to complex to dihydrofolic reductase in a rotomeric configuration similar to the 2,4-diamino-5-phenylbutylpyrimidines (II). It was recently observed that additional binding to the enzyme can occur with aralkyl groups that are substituted on the *m*- or *p*-position of the 1-phenyl moiety (17) (see Table I). Since the 1-phenyl group does not have a restricted rotation and must be nearly coplanar with the triazine ring when complexed to the dihydrofolic reductase from most species (5, 7, 18–21), a substituent such as the phenylbutyl group of IV can be pointed to the left (IV*B*) away from the active site or pointed to the right (IV*A*) toward the enzyme active site when complexed to the enzyme. Since the area on the right approaches the area that binds the polar dihydrofolate (III) it should be more polar than the hydrophobic region and be more apt to have polar nucleophilic groups present, which could be subject to attack by active-site-directed irreversible inhibitors; therefore, the mode of binding of the *m*-phenylalkyl substituents were investigated, and the results are listed in Table I.

Two series of side chains were investigated with terminal aryl or aryloxy groups—namely, (*a*) *m*-alkyl substituents and (*b*) *m*-alkoxy substituents. The latter class has the advantage that there is a greater variety of ground-state conformations for the bridge between the two phenyl rings; this is of considerable importance in the ability of a leaving group of the inhibitor to bridge to a nucleophilic site on the enzyme within the enzyme–inhibitor reversible complex.

Introduction of an ether linkage such as methoxy (VIII) on the 1-phenyl moiety leads to a fivefold loss in binding (Table I); such a loss could be

TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R	Concn. × 10^9 *M* for 50% Inhib.[a]	Inhibition Ratio: V/ Compd.	Inhibition Ratio: VI/ Compd.
V	—C_4H_9-*n*	30[b]	1	
VI	—OC_3H_7-*n*	1500[b]		1
VII	H	110[c]		
VIII	—OCH_3	540		
IX	—$(CH_2)_2C_6H_5$	24[c]	8.8[d]	
X	—$CH_2OC_6H_5$	55	3.8[d]	
XI	—CH_2OCH_3	210		
XII	—$(CH_2)_3C_6H_5$	5.9[b]	5.1	
XIII	—$O(CH_2)_2C_6H_5$	280		5.3
IV	—$(CH_2)_4C_6H_5$	2.7[b]	11	
XIV	—$O(CH_2)_3C_6H_5$	88		17
XV	—$O(CH_2)_2OC_6H_5$	75		20
XVI	—$(CH_2)_5C_6H_5$	7.1[b]	4.7	
XVII	—$(CH_2)_4OC_6H_5$	3.2	10	
XVIII	—$O(CH_2)_4C_6H_5$	74		20
XIX	—$O(CH_2)_3OC_6H_5$	110		14

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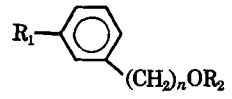
[a] Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction prepared from pigeon liver and assayed with 6 μM dihydrofolate and 12 μM TPNH at pH 7.4 as previously described (27). [b] Data from *Reference 17*. [c] Data from *Reference 18*. [d] Ratio of XI to compound.

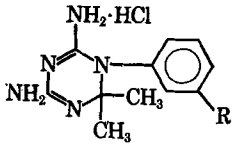
TABLE II—PHYSICAL CONSTANTS FOR



Compd.[a]	R_1	R_2	n	Method	% Yield	M.p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
XXII*b*	NO_2	$C_6H_5(CH_2)_2-$	0	*A*	23	46–48[b]						
XXII*c*	NO_2	$C_6H_5(CH_2)_3-$	0	*A*	62	27–28[c]	70.2	5.65	5.60	70.0	5.87	5.44
XXII*d*	NO_2	$C_6H_5O(CH_2)_2-$	0	*B*	64	94–95[d]	64.9	5.05	5.40	65.0	5.11	5.44
XXII*e*	NO_2	$C_6H_5(CH_2)_4-$	0	*B*	64	Oil						
XXII*f*	NO_2	$C_6H_5O(CH_2)_3-$	0	*B*	73	Oil						
XXIV*b*	$NH_2\cdot HCl$	$C_6H_5(CH_2)_2-$	0	*C*	56	126–128[e]	67.3	6.46	5.61	67.4	6.48	5.47
XXIV*c*	$NH_2\cdot HCl$	$C_6H_5(CH_2)_3-$	0	*C*	95	140–143[f]	68.3	6.88	5.31	68.2	7.02	5.39
XXIV*d*	$NH_2\cdot HCl$	$C_6H_5O(CH_2)_2-$	0	*C*	57	167–170[e]	63.3	6.07	5.27	63.2	6.29	5.13
XXIV*e*	$NH_2\cdot HCl$	$C_6H_5(CH_2)_4-$	0	*C*	22[g]	110–111[e]	69.2	7.26	5.04	68.9	7.36	4.83
XXIV*f*	$NH_2\cdot HCl$	$C_6H_5O(CH_2)_3-$	0	*C*	43[g]	161–164[e]	64.4	6.49	5.01	64.2	6.70	4.98
XXVII*a*	NO_2	CH_3-	1	*B*[h]		Oil						
XXVII*b*	NO_2	C_6H_5-	1	*B*	82	Oil[i]						
XXIX*a*	$NH_2\cdot HCl$	CH_3-	1	*C*	98[j]	111–112[e]	55.3	6.97	8.07	55.1	7.10	8.27
XXIX*b*	$NH_2\cdot HCl$	C_6H_5-	1	*C*	41[j]	143–146[e]	66.2	5.99	5.94	66.0	6.27	6.13
XXXIII	$NH_2\cdot HCl$	C_6H_5-	4	*D*	47	150–152[e]	69.2	7.26	5.04	69.2	7.38	5.14

[a] All compounds had infrared spectra in agreement with their assigned structures. [b] Melting point of 50° and 29% yield previously reported (25) for this compound prepared by a different method. [c] Recrystallized from *n*-propanol. [d] Recrystallized from isopropyl alcohol. [e] Recrystallized from isopropyl alcohol–petroleum ether (b.p. 40–60°). [f] Recrystallized from isopropyl alcohol–ether. [g] Over-all yield for two steps from *m*-nitrophenol. [h] See Mann, F. G., and Stewart, F. H. C., *J. Chem. Soc.*, **1954**, 4127. [i] A b.p. of 182°/3 mm. for this oil has been reported (26). [j] Over-all yield for two steps from *m*-nitrobenzyl chloride.

TABLE III—PHYSICAL CONSTANTS OF



Compd.[a]	R	% Yield	M.p., °C.	Anal. Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
VIII	$-OCH_3$	77	189–190[b]	50.8	6.39	24.7	50.5	6.52	24.7
X	$-CH_2OC_6H_5$	76	136–138[c]	60.1	6.16	19.5	59.9	6.27	19.3
XI	$-CH_2OCH_3$	88	192–193[c]	52.4	6.77	23.5	52.4	6.75	23.6
XIII	$-O(CH_2)_2C_6H_5$	83	175–176[d]	61.0	6.47	18.7	61.1	6.70	18.5
XIV	$-O(CH_2)_3C_6H_5$	60	175–178[c]	61.9	6.75	18.0	62.1	6.68	18.0
XV	$-O(CH_2)_2OC_6H_5$	65	173–174[d]	58.5	6.20	18.0	58.3	5.91	17.7
XVII	$-(CH_2)_4OC_6H_5$	88	174–175[c]	62.8	7.02	17.4	62.6	6.99	17.3
XVIII	$-O(CH_2)_4C_6H_5$	80	170–171[d]	62.8	7.02	17.4	62.5	7.21	17.6
XIX	$-O(CH_2)_5OC_6H_5$	88	171–172[d]	59.5	6.49	17.3	59.4	6.66	17.3

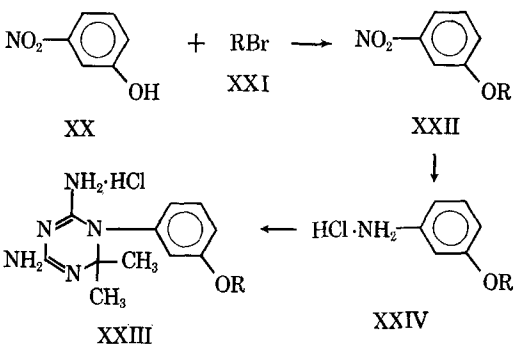
[a] All compounds were prepared by method *E* and had infrared and ultraviolet spectra in agreement with their assigned structures. [b] Recrystallized from ethanol. [c] Recrystallized from ethanol–ether. [d] Recrystallized from ethanol–petroleum ether (b.p. 40–60°).

anticipated since introduction of other polar groups on the 1-phenyl moiety led to decreased binding, presumably due to repulsion from the hydrophobic region on the enzyme (22). Surprisingly, another threefold loss occurred in binding when the chain was extended to *n*-propoxy (VI). Of more importance was the fact that introduction of a terminal phenyl on the *n*-propoxy group, as in XIV, gave a seventeenfold increase in binding; this result compares favorably with the elevenfold increase in binding when a terminal phenyl group is introduced on the *n*-butyl group as in IV (17). Similar increments in terminal phenyl binding were observed in IX *versus* X and XII *versus* XIII.

It was previously observed that the binding of a 5-phenylbutyl group on the pyrimidine (I*a*) was thirtyfold better than the 5-phenoxypropyl group (23); this result has been attributed to the increased hydrophobic bonding of a terminal benzyl group compared to a terminal phenoxy group plus some possible repulsion of the ether oxygen from the hydrophobic region (7). A similar repulsion of the ether group of VIII has just been discussed. Therefore, the replacement of a methylene group by an ether oxygen can be used as a test for the relative hydrophobic character of a binding region. Note that exchange of benzyl in XIV for phenoxy in XV gave no appreciable change in binding; similar comparisons are XVI *versus* XVII and XVIII *versus* XIX. Therefore, the nearly identical binding by phenoxy and benzyl indicates that the terminal phenyl moiety may well be complexed to a relatively hydrophilic region on the enzyme surface, as suggested earlier for the conformation of IV*B*. If indeed these terminal phenyl groups of molecules such as IV and XV are complexed in a relatively polar region of the enzyme, then introduction of a bromoacetamido group on the terminal phenyl group should make logical candidate active-site-directed irreversible inhibitors of dihydrofolic reductase that could be operational in the 10^{-7}–10^{-8} *M* region; such studies are being vigorously pursued.

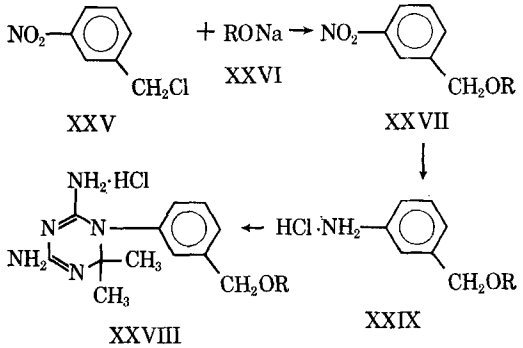
CHEMISTRY

The 1,2-dihydro-1-phenyl-*s*-triazines in Tables I and III were made from the appropriate aryl amine hydrochloride and cyanoguanidine in acetone—the three-component method of Modest (20). The synthetic methods for the required amines could be divided into three classes. The first class contained an ether linkage to the 1-phenyl moiety of type XXIII. *m*-Nitrophenol (XX) was converted to the sodium salt with sodium hydride then alkylated with the appropriate bromide (XXI), except for XXII*e* where 4-phenylbutyl chloride was employed. The nitro groups were reduced catalytically with a palladium-charcoal catalyst in ethanol in the presence of a slight excess of hydrochloric acid; the amine hydrochlorides (XXIV) were isolated in good yield. Condensation of XXIV with cyanoguanidine in acetone afforded the triazines (XXIII); of course, commercial XXIV*a* base was employed. (Scheme I.)



Series *a*, R = CH_3
b, R = $C_6H_5(CH_2)_2$—
c, R = $C_6H_5(CH_2)_3$—
d, R = $C_6H_5O(CH_2)_2$—
e, R = $C_6H_5(CH_2)_4$—
f, R = $C_6H_5O(CH_2)_3$—

Scheme I



Series *a*, R = CH_3
b, R = C_6H_5

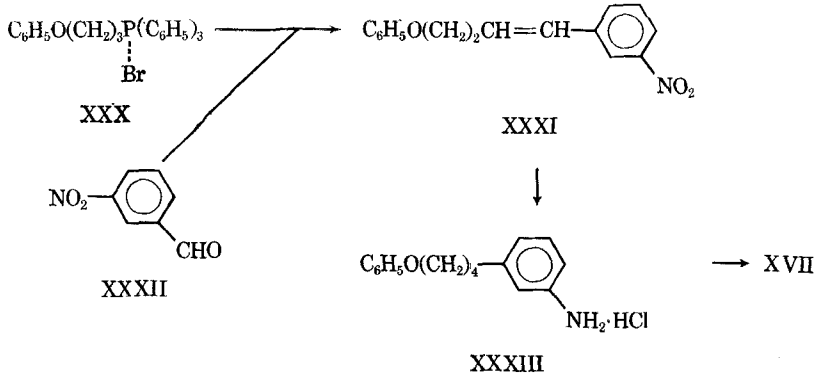
Scheme II

The second series contained the oxymethylene bridge as in XXVIII; these were prepared *via* *m*-nitrobenzyl chloride (XXV). Reaction of XXV with phenol in acetone in the presence of potassium carbonate afforded XXVII*b*. The methyl ether (XXVII*a*) was prepared from XXV with sodium methoxide. The remainder of the sequence for XXVIII was the same as for XXIII. (Scheme II.)

The third type is represented by XVII (Table III). Condensation of the Wittig reagent (XXX) from phenoxypropyl bromide (24) with *m*-nitrobenzaldehyde (XXXII) in tetrahydrofuran in the presence of potassium *tert*-butoxide afforded XXXI as an oil. Hydrogenation with platinum oxide catalyst gave crystalline XXXIII in 40% over-all yield from XXXII. Condensation with cyanoguanidine afforded the desired dihydro-*s*-triazine (XVII). (Scheme III.)

Methods—Melting points were determined in capillary tubes with a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in water with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel GF and spots were detected by iodine vapor or by visual examination under ultraviolet light.

m-Nitrophenyl γ-Phenylpropyl Ether (XXIIc)—*Method A*—A solution of 3.83 Gm. (27.5 mmoles) of *m*-nitrophenol in 10 ml. of dimethylsulfoxide was added dropwise over a period of about 10 min. to a magnetically stirred mixture of 10 ml. of dimethylsulfoxide and 1.27 Gm. (26 mmoles) of a 53.5% dispersion of sodium hydride in mineral oil, protected from moisture. The red solution was warmed to 80°, then 4.98 Gm. (25 mmoles) of 3-



Scheme III

phenylpropyl bromide was added; the temperature rose to 95°. After being stirred in a bath at 80–85° for 90 min., the mixture was cooled, diluted with 75 ml. of benzene, and washed successively with 100 ml. of water, 25 ml. of water, 5% aqueous sodium hydroxide (3 × 15 ml.), and finally water (2 × 25 ml.). The benzene solution, dried with magnesium sulfate, was evaporated *in vacuo*. Crystallization from *n*-propanol gave 4.02 Gm. (62%) of product, m.p. 27–28°. $\nu_{max.}$ (film): 1610, 1580 (C=C); 1530, 1345 (NO_2); 1245, 1030 (C—O—C); 810, 795 (*m*-C_6H_4); 735, 698 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data and for other compounds prepared by Method *A*.)

m-Nitrophenyl Phenoxyethyl Ether (XXIId)—*Method B*—To a solution of 1.41 Gm. (26 mmoles) of sodium methoxide in 10 ml. of reagent methanol was added 3.83 Gm. (27.5 mmoles) of *m*-nitrophenol (XX), followed by 5.03 Gm. (25 moles) of β-bromophenetole. After being refluxed with magnetic stirring for 18–22 hr., the mixture was spin-evaporated *in vacuo*. The residue was partitioned between 50 ml. of ether and 30 ml. of water. The separated ether layer was washed with 5% aqueous sodium hydroxide (2 × 10 ml.), then water (3 × 10 ml.). Dried with magnesium sulfate, the solution was evaporated *in vacuo*. Recrystallization of the residue from isopropyl alcohol gave 4.13 Gm. (64%) of product, m.p. 94–95°. $\nu_{max.}$ 1605, 1600, 1580, 1500 (C=C); 1520, 1350 (NO_2); 1250, 1060 (C—O—C); 805, 800 (*m*-C_6H_4); 762, 742, 700 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data and for other compounds prepared by this method.)

m-Aminophenyl β-Phenethyl Ether Hydrochloride (XXIVb)—*Method C*—A solution of 1.83 Gm. (7.5 mmoles) of XXII*b* in 100 ml. of ethanol containing 0.75 ml. of 12 *N* aqueous hydrochloric acid (9 mmoles) was shaken with hydrogen at 2–3 Atm. in the presence of 0.15 Gm. of 5% palladium-charcoal; reduction was complete in about 20 min. The filtered solution was evaporated *in vacuo*. Recrystallization of the residue from isopropyl alcohol–petroleum ether (b.p. 40–60°) gave 1.05 Gm. (56%) of white crystals, m.p. 126–128°. $\nu_{max.}$ 2870 (broad, NH); 2600, 1950 (NH_3^+); 1620, 1600, 1530, 1498 (NH, C=C); 1275, 1028 (C—O—C); 795, 785 (*m*-C_6H_5); 755, 702, 690 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data for other compounds prepared by this method.)

4-(m-Aminophenyl)butyl Phenyl Ether Hydrochloride (XXXIII)—*Method D*—To a magnetically stirred mixture of 1.51 Gm. (10 mmoles) of *m*-nitrobenzaldehyde, 5.25 Gm. (11 mmoles) of XXX (24), and 15 ml. of reagent tetrahydrofuran, cooled in an ice bath and protected from moisture, was added 1.12 Gm. (10 mmoles) of potassium *tert*-butoxide. After being stirred in an ice bath for 1 hr. and at ambient temperature for 18 hr., the mixture was filtered and the insolubles washed with tetrahydrofuran. The combined filtrate and washings were evaporated *in vacuo*. The residual oil was dissolved in benzene and passed through a 3-cm. diameter column containing 64 Gm. of silica gel to remove triphenylphosphine oxide. The first fraction of yellow colored solution was collected and evaporated *in vacuo;* yield, 1.83 Gm. (68%) of XXXI as a yellow oil. $\nu_{max.}$ (film) 1600, 1595, 1500 (C=C); 1525, 1350 (NO_2); 1245, 1040 (C—O—C); 800 (*m*-C_6H_4); 735, 690 cm.$^{-1}$ (C_6H_5).

A solution of 1.83 Gm. of XXXI in 100 ml. of ethanol containing 0.75 ml. of 12 *N* aqueous hydrochloric acid was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide catalyst; reduction was complete in 15 min. The reaction mixture was processed as described under *Method C;* yield, 1.10 Gm. (69%), m.p. 147–149°. A second recrystallization from isopropyl alcohol–petroleum ether (b.p. 40–60°) gave the analytical sample as white crystals, m.p. 150–152°. $\nu_{max.}$ 2850, 2600, 1950 (NH, NH_3^+); 1600, 1570, 1510, 1490 (NH, C=C); 1255, 1035 (C—O—C); 790 (*m*-C_6H_4); 758, 690 cm.$^{-1}$ (C_6H_5). (See Table II for analytical data.)

4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[m-(2-phenoxyethoxy)phenyl]-s-triazine Hydrochloride (XV)—*Method E*—A mixture of 664 mg. (2.5 mmoles) of XXIV*d*, 217 mg. (2.7 mmoles) of cyanoguanidine, and 4 ml. of reagent acetone was refluxed for 20 hr. with magnetic stirring. The cooled mixture was filtered and the product washed with acetone; yield, 631 mg. (65%), m.p. 168–170°. Recrystallization from ethanol–petroleum ether (b.p. 40–60°) gave 559 mg. (57%) of white crystals, m.p. 173–174°. $\lambda_{max.}$ 246, 268–280 (plateau) mμ; $\nu_{max.}$ 3300, 3150 (broad, NH); 1660 (C=NH^+); 1630, 1600, 1545, 1520 (NH, C=C, C=N); 1250, 1070 (C—O—C); 800, 785 (*m*-C_6H_4); 750, 705, 690 cm.$^{-1}$ (C_6H_5). (See Table III for analytical data and additional compounds made by this method).

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