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Irreversible Enzyme Inhibitors. 101. A Modified Synthesis of 2-Substituted-4,6-diamino-1,2-dihydro-2-methyl-1-phenyl-*s*-triazines (1,2)

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One of the objectives in this laboratory is the design and synthesis of active-site-directed irreversible inhibitors (3,4) of dihydrofolic reductase that can operate at concentrations less than 10^{-6} *M* (5,6). Although active-site-directed irreversible inhibitors of dihydrofolic reductase have been found (5-8), these require a concentration of 10-40 x 10^{-6} *M* to operate efficiently. Since the rate of active-site-directed irreversible enzyme inhibition is dependent upon the concentration of the reversible enzyme-inhibitor complex, then the better the inhibitor reversibly complexes with the enzyme, the lower is the required concentration for irreversible inhibition (9,10). Diamino derivatives of such heterocyclic systems as pyrimidine and 1,2-dihydro-*s*-triazine are 300-3000 fold better inhibitors of dihydrofolic reductase than the corresponding 2-amino-4-hydroxypyrimidines (5); it follows that an irreversible inhibitor derived from such a 2,4-diamino heterocycle should be capable of operating at the desired low concentration (11,12).

Of potential use are candidate irreversible inhibitors derived from 4,6-diamino-1,2-dihydro-1-phenyl-*s*-triazines, which bear a leaving group on the 2-side-chain, such as I. Since Modest (13) had reported that methyl ketones capable of forming a sodium bisulfite derivative can be expected to condense with phenylbiguanide (II) to give the dihydro-*s*-triazines (I), no difficulty was anticipated in this synthesis. Attempts to prepare Ia from cyanoguanidine or phenylbiguanide hydrochloride (II) by the three or two component methods, respectively, of Modest (13) failed to give a crystalline product even though the cuprammonium test (13) for II appeared negative. The difficulty was eventually traced with chromatography to the fact that the cuprammonium test is rather insensitive and actually mixtures of Ia and II were obtained. In a previous study (14), it was noted that compounds such as I and II were too polar to be moved from the origin with TLC on silica gel, but paper chromatography was satisfactory; the more recent availability of polyamide resin TLC for separation of polar compounds was then tried and proved to be a rapid method for separation of Ia and II.

Since the reaction of phenylbiguanide hydrochloride (II) with phenoxyacetone to Ia could not be driven to completion by the usual methods (13), the formation of water as a reaction product was then considered as the possible source of difficulty. Therefore, the two-component condensation of II with phenoxyacetone was investigated in the presence of ethyl orthoformate as a water scavenger. The reaction was performed in boiling methanol and was readily monitored by TLC on polyamide and was found to go to completion; the product (Ia) was then readily crystallized from the reaction mixture.

Without ethyl orthoformate, the reaction of phenylbiguanide hydrochloride (II) with either phenylacetone or 4-phenyl-2-butanone to give Ib and Ic also could not be driven to completion and neither product could be crystallized. However, in the presence of triethyl orthoformate the reaction could be driven to completion and the products Ib and Ic were readily crystallized.

Three mechanisms can be envisioned for the beneficial effect of ethyl orthoformate in the two-component synthesis, (a) simple water scavenging, (b) the reaction proceeds via the triazine, III, or (c) the reaction proceeds via the ketal, IV. That the reaction did not proceed via III or IV was shown in the following manner:

Omission of the ketone led to direct reaction of phenylbiguanide (II) with ethyl orthoformate to the aromatic triazine (III), a previously known compound (15). That III was not an intermediate in the formation of the dihydro-*s*-triazines (I) was indicated by the fact that III failed to react with phenoxyacetone in methanol. Similarly, after phenoxyacetone was converted to its diethyl ketal (IV) with ethyl orthoformate (22), IV failed to form the dihydro-*s*-triazine (Ia) when treated with phenylbiguanide hydrochloride (II) in methanol. Thus, three possible reactions can occur with ethyl orthoformate when phenylbiguanide hydrochloride (II) is reacted with phenoxyacetone, (a) formation of the diethylketal (IV), (b) formation of the aro-

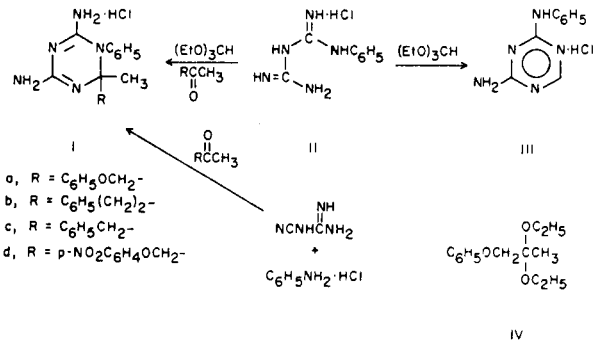
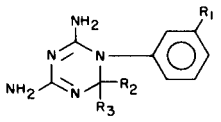


TABLE I

Inhibition of Dihydrofolic Reductase (a,b) By



Compound	R_1	R_2	R_3	μM Conc. for 50% Inhibition
Ia	H	CH_3	$C_6H_5OCH_2$-	2.5
Ib	H	CH_3	$C_6H_5(CH_2)_2$-	2.3
V	H	CH_3	CH_3	0.11 (c)
VI	Cl	CH_3	CH_3	0.012 (d)
VII	Cl	H	C_6H_5-	5.5 (d)
VIII	Cl	H	$C_6H_5CH_2$-	1.1 (c)
IX	Cl	H	$C_6H_5(CH_2)_2$-	0.070 (c)

(a) The technical assistance of Barbara Baine with these assays is acknowledged. (b) The dihydrofolic reductase was a 45-90% saturated ammonium sulfate fraction from pigeon liver that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in *p*H 7.4 Tris buffer as previously described (20). (c) Data from reference 18. (d) Data from reference 17.

matic triazine (III), or (c) formation of the dihydro-*s*-triazine (Ia) where the evolved water is scavenged by the ethyl orthoformate. Since (a) and (b) are dead-ends, it seems clear that (c) is probably the fastest reaction of the three. Furthermore, if III and IV are formed, they are readily separated from Ia; in contrast Ia is separated only with difficulty from II.

With the less reactive *p*-nitrophenoxyacetone, no dihydro-*s*-triazine (Id) formation occurred with the usual amount of triethyl orthoformate; with larger amounts of triethyl orthoformate, the aromatic *s*-triazine (III) was obtained and no Id could be detected by TLC, thus supporting the contention that Ia, Ib, and Ic are formed directly in the presence of triethyl orthoformate since their rate of formation is more rapid than that of III. Furthermore, the dihydro-*s*-triazine (Ia) was stable in aqueous solution at room temperature even in the presence of excess acid; therefore, an equilibrium between Ia plus water on the one hand and II plus phenoxyacetone on the other is not the reason the reaction of II with phenoxyacetone in the presence of water does not go to completion.

2,2-Dimethoxypropane has been used as a water scavenger for preparation of 1-alkyl-2,2-dimethyl-1,2-dihydro-*s*-triazines from an alkylbiguanide and acetone (18) where reaction does not take place without water removal. It is obvious that 2,2-dimethoxypropane cannot be used as a water scavenger for condensation of ketones other than acetone. However, it may prove to be useful at times with sluggishly reacting ketones to use a mixture of the higher ketone and its diethyl ketal for preparation of the dihydro-*s*-triazines where the ketal can act as a water scavenger.

Finally, the three-component method for synthesis of Ia from aniline hydrochloride, cyanoguanidine, and phenoxyacetone in the presence of ethyl orthoformate was investigated. Although some Ia could be detected by TLC on polyamide, the reaction was not nearly as clean as the two-component method in the presence of ethyl orthoformate, and Ia could not be isolated by crystallization from this three-component reaction mixture; since phenylbiguanide hydrochloride (II) is usually prepared in aqueous medium (16), it is possible that this first step was sluggish under these anhydrous conditions.

ENZYME RESULTS

The reversible inhibition of dihydrofolic reductase by Ia and Ib are recorded in Table I. About a 25-fold loss in binding occurred by increasing the size of the 2-methyl group of V to phenoxymethyl (Ia) or phenethyl (Ib). Groups larger than methyl at the 2-position have been previously studied with R_2 = H (17,18). Note that the 2-phenyl group of VII caused a 450-fold loss in binding compared to the 2,2-dimethyl derivative (VI). However, when the chain was extended to benzyl (VIII) the loss in binding was reduced to 90-fold and with phenethyl (IX) to 6-fold. Compounds such as I, VIII and IX are sufficiently good reversible inhibitors to warrant conversion to irreversible inhibitors.

In order to convert I, VIII or IX to a candidate irreversible inhibitor it is necessary to functionize the phenyl group in order to introduce a leaving group (5). The synthesis of VIII and IX require phenylacetaldehyde and phenylpropionaldehyde as starting materials; the synthesis of these aldehydes with functionalized phenyl groups is difficult and laborious; in contrast, the synthesis of the methyl ketones with functionalized phenyl groups for preparation of analogs of I is much more straight forward. In fact, this modification of the Modest two-component synthesis (13) utilizing triethyl orthoformate has led to a variety of analogs of I bearing leaving on the phenyl group of 2-side-chain (19); these are currently being investigated as active-site-directed irreversible inhibitors (3) of dihydrofolic reductase (5).

EXPERIMENTAL

Melting points were taken in capillary tubes on a Mel-temp block and those below 230° are corrected. Ultraviolet spectra were determined in 10% aqueous ethanol with a Perkin-Elmer 202 spectrophotometer. Infrared spectra were determined in potassium

bromide discs with a Perkin-Elmer 137B or 337 spectrophotometer. Thin layer chromatograms (TLC) were run on Brinkmann MN-polyamide UV_{254} with 1:1 ethanol-chloroform (unless otherwise indicated) and spots were detected by visual examination under ultraviolet light; in this system phenylbiguanide hydrochloride (II) had an R_f about 0.6, the dihydro-*s*-triazine salts (I) and R_f of about 0.9 and the ketones at the solvent front.

4,6-Diamino-1,2-dihydro-2-methyl-2-phenoxymethyl-1-phenyl-*s*-triazine Hydrochloride (Ia).

A solution of 165 mg. (1.1 mmoles) of phenoxyacetone, 213 mg. (1 mmole) of phenylbiguanide hydrochloride (II), 0.50 ml. of methanol, 0.50 ml. of ethyl orthoformate, and 0.04 ml. of 12 *N* aqueous hydrochloric acid was refluxed for 30 hours with magnetic stirring; at this time TLC showed only a trace of II. The cooled reaction mixture was diluted with ether; the separated gum was titurated with several portions of fresh ether. The gum was crystallized from ethanol-ether; yield, 226 mg. (65%). The product was recrystallized from ethanol-ether, then recrystallized from ethanol-petroleum ether (b.p. 30-60°) to give a 54% recovery of white crystals, m.p. 181-182°, that moved as a single spot on TLC. An analytical sample was prepared by a further recrystallization from ethanol-ether as white crystals, m.p. 195-196°; λ max 244, 265, 275 mμ (inflection); ν max 3320, 3120 (NH); 1670, 1640, 1600, 1550 (NH, C=N, C=C); 1250, 1170 (C-O-C); 752, 697 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{17}H_{19}N_5O \cdot HCl$: C, 59.0; H, 5.84; N, 20.2. Found: C, 59.2; H, 5.88; N, 20.4.

When the reaction was run at room temperature for 9 days, considerable II still remained.

4,6-Diamino-1,2-dihydro-2-methyl-2-phenethyl-1-phenyl-*s*-triazine Hydrochloride (Ib) and Picrate.

A mixture of 2.13 g. (10 mmoles) of phenylbiguanide hydrochloride (II), 2.00 g. (13 mmoles) of benzylacetone, 5 ml. of methanol, 5 ml. of ethyl orthoformate and 0.40 ml. of 12 *N* aqueous hydrochloric acid was magnetically stirred in a stoppered flask for 7 days at ambient temperature; at this time only a trace of II could be detected by TLC. The mixture was chilled overnight at -5°. The crude product was collected on a filter, then washed with ether; weight, 3.22 g. The crude product was suspended in hot ethanol, then dissolved by dropwise addition of water. Ether was then added until turbidity appeared, then the mixture was warmed to boiling when solution reoccurred. After being chilled at -5° overnight, the mixture was filtered and the white crystals were washed with ether; yield, 3.21 g. (94%), m.p. 160-165° dec., that moved as a single spot on TLC and showed the absence of II. For analysis a sample was recrystallized once more in the same fashion: white crystals, m.p. 189-190° dec.; λ max 245 mμ; ν max 3115, 3130 (NH); 1650, 1600, 1550 (NH, C=C, C=N); 759, 700 cm^{-1} (monosubstituted phenyl). This hydrochloride was an ether solvate that gave variable analysis depending upon the amount of drying, but could not be obtained solvent free. A typical analysis was the following.

Anal. Calcd. for $C_{18}H_{21}N_5 \cdot HCl \cdot 1/2(C_2H_5)_2O$: C, 63.0; H, 7.14; N, 18.4. Found: C, 62.8, 62.9; H, 6.91, 6.92; N, 18.6, 18.7.

The compound was therefore converted to the picrate in ethanol; yield, 67% of yellow crystals, m.p. 190-192° dec.; λ max 247 (sh), 370 mμ; ν max 3300, 3200 (NH); 1650, 1615, 1600, 1580, 1540 (multiple peaks) (NH, C=C, C=N, NO_2); 750, 700 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{24}H_{24}N_8O_7$: C, 53.7; H, 4.52; N, 20.9. Found: C, 53.6; H, 4.50; N, 21.1.

2-Benzyl-4,6-diamino-1,2-dihydro-2-methyl-1-phenyl-*s*-triazine Hydrochloride (Ic).

This compound was prepared from 213 mg. (1 mmole) of phenylbiguanide hydrochloride (II) and 217 mg. (1.6 mmoles) of phenylacetone, essentially as described for Ib. When chilled at -5°, the mixture deposited a small amount of crystals which were primarily II as shown by TLC. The filtrate was diluted with several volumes of ether. The amorphous, hygroscopic product was collected on a filter and crystallized from 0.01 *N* aqueous hydrochloric acid; yield, 160 mg. (49%) of white crystals, m.p. 120-130° dec., which moved as a single spot on TLC. The analytical sample was obtained by a second recrystallization with 18% recovery from 0.01 *N* aqueous hydrochloric acid: white crystals, m.p. 147-159° dec.; λ max 246 mμ; ν max 3330, 3150 (NH); 1625, 1530 (NH, C=C, C=N); 760, 690 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{17}H_{19}N_5 \cdot HCl \cdot H_2O$: C, 58.8; H, 6.38; N, 20.2. Found: C, 58.4; H, 6.45; N, 20.6.

2-Amino-4-anilino-*s*-triazine and Hydrochloride (III).

(A) A mixture of 213 mg. (1 mmole) of phenylbiguanide hydrochloride (II), 0.50 ml. of methanol, 1.50 ml. of ethyl orthoformate, and 0.04 ml. of 12 *N* aqueous hydrochloric acid was magnetically stirred for 3 days at ambient temperature in a stoppered flask; at this time, TLC with 9:1 chloroform-ethanol showed the reaction was essentially complete. After being chilled at -5° overnight, the mixture was filtered and the product was washed with ether; weight, 214 mg. of crude product showing a trace of II.

Recrystallization from ethanol containing the minimum addition of water gave 117 mg. (50%) of white crystals, m.p. 251-254° dec. with softening at 228°; λ max 260 mμ; ν max 3165, 3100 (NH); 1660, 1600, 1550 (NH, C=C, C=N); 750, 688 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_9H_9N_5 \cdot HCl$: C, 48.4; H, 4.51; N, 31.3. Found: C, 48.8; H, 4.79; N, 30.9.

When the hydrochloride was stirred with 0.1 *N* aqueous sodium hydroxide, it was converted to the free base: white crystals, m.p. 234-236°. The free base has been reported (15a) to have m.p. 232-233° and the hydrochloride (III), m.p. 258-260° (15b); the compound had been prepared from II and formic acid.

(B) No reaction occurred when it was attempted to convert *p*-nitrophenoxyacetone (21) to Id by the methods described for Ia or Ib. When the amount of ethyl orthoformate was increased to the proportions in (A), reaction occurred, but the product was III—identical with preparation (A) as shown by TLC, UV, IR and mixture melting point.

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