Department of Chemistry, University of California at Santa Barbara

Irreversible Enzyme Inhibitors. CXI. Candidate Active-site-directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 4,6-Diamino-1,2-dihydro-1-phenyl-*s*-triazines. V. (1,2)

B. R. Baker and Morris A. Johnson

Condensation of 5-(*p*-nitrophenyl)-2-pentanone with phenylbiguanide hydrochloride (V) gave a 2-methyl-2-(*p*-nitrophenylpropyl)-1,2-dihydro-*s*-triazine (IX); hydrogenation of the nitro group to amino followed by bromoacetylation afforded the candidate irreversible inhibitor of dihydrofolic reductase, namely, 2-(*p*-bromoacetamidophenylpropyl)-4,6-diamino-1,2-dihydro-2-methyl-*s*-triazine hydrochloride (VIII). Similarly, the *o*, *m*, and *p*-isomers of 5-nitrophenoxy-2-pentanone were converted to the corresponding 2-(bromoacetamidophenoxypropyl)-1,2-dihydro-*s*-triazines (XI). The four candidate irreversible inhibitors were evaluated on the dihydrofolic reductases from pigeon liver, Walker-256 rat tumor, and L-1210/FR8 mouse leukemia. Only VIII was an irreversible inhibitor; VIII slowly inactivated the L-121-/FR8 mouse leukemia enzyme with a half-life of 2-3 hours at 37°, but VIII showed no inactivation of the other two dihydrofolic reductases—a species specific inactivation.

4,6-Diamino-1,2-dihydro-1-phenyl-*s*-triazines such as I are excellent reversible inhibitors of dihydrofolic reductase, I being complexed to the enzyme 60-fold better than the substrate, dihydrofolate (3). A molecule such as I is readily synthesized from cyanoguanidine, aniline hydrochloride, and acetone or from phenylbiguanide hydrochloride (V) and acetone; these are known as the "three-component" and "two-component" methods, respectively, of Modest (4). In order to convert a molecule such as I to an active-site-directed irreversible inhibitor (3,5) of dihydrofolic reductase, the following two additional factors must be considered: (a) an area on the inhibitor should be found where relatively large groups can be substituted without interfering with reversible complex formation with the enzyme, a so-called bulk-tolerance area (3,5); and (b) a leaving group should be positioned on the bulk-tolerance area of the inhibitor so that it is juxtaposed to a nucleophilic site on the enzyme surface, thus allowing rapid covalent bond formation to take place by an anchimerically assisted reaction which inactivates the enzyme (3,5).

In a previous paper of this series (6), a method was devised for condensation of V with higher ketones to give dihydro-*s*-triazines with larger substituents at the 2-position such as phenethyl (II) and phenoxymethyl (III). Both II and III were complexed to dihydrofolic reductase about 25-fold less effectively than the parent 2-methyl-*s*-triazine (I) (Table I). Since II and III were still good reversible inhibitors that complexed about 3-fold more effectively than the substrate, the terminal phenyl group on the 2-position was therefore a logical place to introduce a leaving group in order to convert these molecules to candidate active-site-directed irreversible inhibitors of dihydrofolic reductase; the results are the subject of this paper.

Although III could be prepared by condensation of phenoxyacetone with phenylbiguanide hydrochloride (V) in the presence of ethyl orthoformate as a water scavenger (6), no reaction took place with *p*-nitrophenoxyacetone (7) under the same conditions. When the reaction conditions were forced, none of the desired dihydro-*s*-triazine (IV, R = NO_2) was formed; instead the product was the aromatic triazine, XII, which was also formed from triethyl orthoformate and V in the absence of a ketone (6).

The failure of *p*-nitrophenoxyacetone to condense with V can be attributed to the deactivation of the ketone by the electron-withdrawing *p*-nitro group. Therefore, the nitro ketone was reductively acetylated to *p*-acetamidophenoxyacetone in good yield. The latter appeared to condense with V to give IV (R′ = AcNH) and no aromatic triazine (XII) was formed; several products were observed on TLC and the desired IV (R′ = AcNH) could not be isolated.

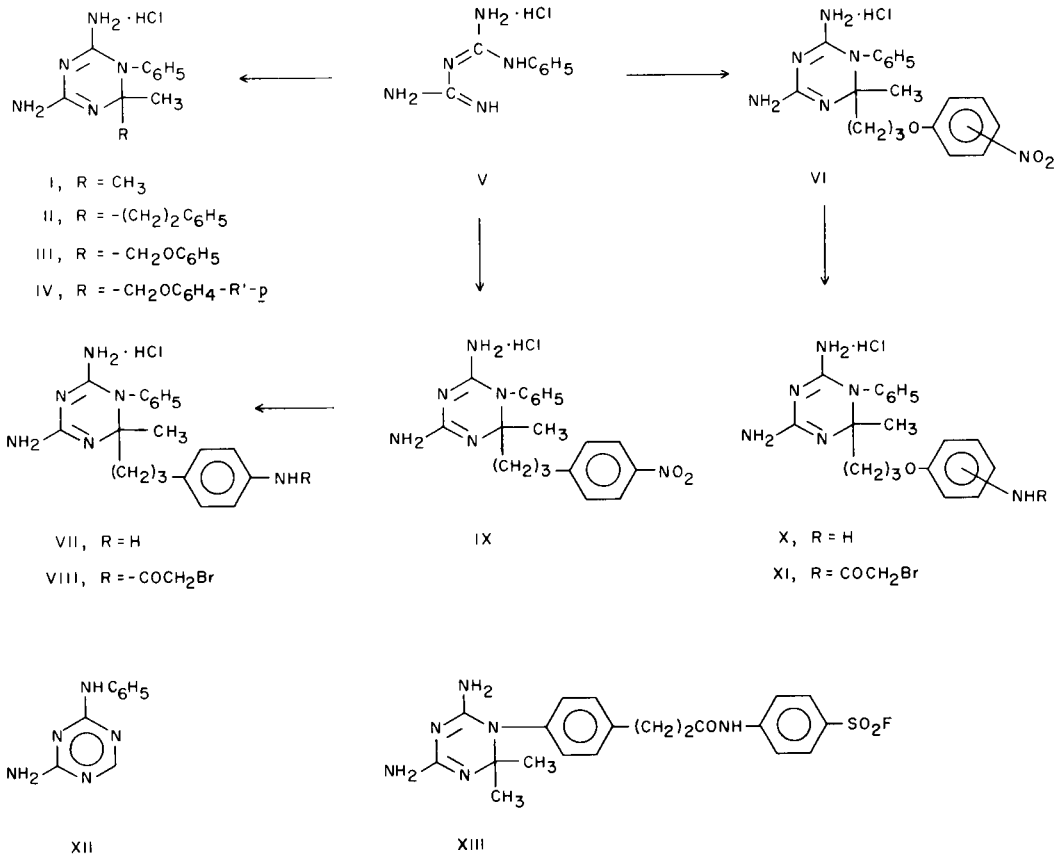
These difficulties were circumvented with the higher homologs derived from 5-phenoxy-2-pentanone. The desired *o*, *m*, and *p*-isomers of the dihydro-*s*-triazine (VI) were obtained by condensation of the appropriate 5-(nitrophenoxy)-2-pentanone with V in the presence of triethyl

CHART I

a series, ortho

b series, meta

c series, para



orthoformate as a water scavenger; in these cases, any possible effect of the nitrophenoxy group on the reactivity of the ketone function was reduced to nil by the added insulation from the two additional methylene groups. Catalytic hydrogenation of the nitro group to X followed by bromoacetylation (8) afforded the candidate irreversible inhibitors of structure XI. Similarly 5-(*p*-nitrophenyl)-2-pentanone (12) could be condensed with V to give the dihydro-*s*-triazine (IX) which was reduced to VII and bromoacetylated (8) to VIII.

Enzymic Evaluation.

The four candidate irreversible inhibitors (VIII, XIa, XIb, XIc) were evaluated on the dihydrofolic reductases from pigeon liver, Walker-256 rat tumor, and L-1210/FR8 mouse leukemia by the methods previously described (9); the reversible inhibition results are collated in Table I. The following correlations can be made:

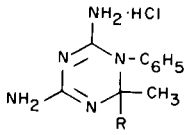
(a) The variability in binding ability of the compounds to the pigeon liver enzyme indicates the R group of the compounds in Table I is in contact with enzyme surface when the compounds are complexed to the enzyme; a similar conclusion can be drawn with the binding results to the enzyme from rat tumor and mouse leukemia, although the variation is less.

(b) Only a two-fold difference in binding of I is seen with the three enzymes; the greatest difference in binding is seen with VIII, but it is only 6-fold. These results support our earlier contention that there will not be sufficient differences in reversible binding to vertebrate dihydrofolic reductases to exploit for cancer chemotherapy (10).

Compounds VIII, XIa, XIb, and XIc were then investigated at 5-times the concentration necessary for 50% reversible inhibition as irreversible inhibitors of the three enzymes by the methods previously described (9). All were negative except VIII; 6.5 μM of VIII inactivated the L-1210 enzyme at 37° with a half-life of 2-3 hours, but VIII showed no inactivation of the enzymes from pigeon liver or Walker-256 rat tumor. Thus, even though there is

TABLE I

Reversible Inhibition (a,b) of Dihydrofolic Reductase by

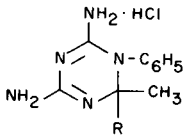


No.	R	μM Conc. for 50% Inhibition: Pigeon liver	Walker-256 rat tumor	L-1210/FR8 mouse leukemia
I	CH_3	0.11 (c)	0.12	0.21
II	$-(CH_2)_2C_6H_5$	2.3 (c)		
III	$-CH_2OC_6H_5$	2.5 (c)		
VIII	$-(CH_2)_3C_6H_4NHCOCH_2Br$-*p*	1.7	0.26	1.3
XIa	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*o*	2.5	0.90	1.4
XIb	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*m* (d)	0.44	0.18	0.34
XIc	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*p*	0.24	0.61	0.67

(a) The technical assistance of Barbara Baine and Jean Reeder with these assays is acknowledged. (b) Assayed at *p*H 7.4 7.4 with 6 μ*M* dihydrofolate as previously described (9). Data from reference 6. (d) Dipicrate.

TABLE II

Physical Constants of



No.	R	Method	% Yield	M.p. °C	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
VIa	$-(CH_2)_3OC_6H_4NO_2$-*o*	A	87	189-191 (a)	54.5	5.53	20.1	54.1	5.77	19.8
VIb	$-(CH_2)_3OC_6H_4NO_2$-*m*	A	14	184-186 (a)	54.5	5.53	20.1	54.4	5.66	20.0
VIc	$-(CH_2)_3OC_6H_4NO_2$-*p*	A	46	166-168 (a)	52.2 (b)	5.76	19.2	52.0	5.75	19.1
VIII	$-(CH_2)_3C_6H_4NHCOCH_2Br$-*p*	B	56	dec.>150 (c)	51.1	5.31	17.0	50.9	5.25	16.9
IX	$-(CH_2)_3C_6H_4NO_2$-*p*	A	51	181-185 (a)	54.4 (b)	5.98	19.9	54.4	6.15	19.7
XIa	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*o*	B	21	122-124 (c)	47.8 (b)	5.28	15.9	47.6	5.21	15.7
XIb (d)	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*m*	B	33	139-142 (a)	42.6	3.35	18.1	42.8	3.55	17.9
XIc	$-(CH_2)_3OC_6H_4NHCOCH_2Br$-*p*	B	4	dec.>150 (e)	47.8 (b)	5.28	15.9	47.9	5.07	16.0

(a) Recrystallized from ethanol. (b) Monohydrate. (c) Recrystallized from aqueous ethanol. (d) Dipicrate prepared with ethanolic picric acid. (e) Recrystallized from aqueous *N,N*-dimethylformamide.

no useful difference in reversible inhibition of the three enzymes by VIII, only the L-1210 enzyme is inactivated, albeit at too slow a rate to be useful. However, this example gives support to our earlier contention that irreversible inhibitors of dihydrofolic reductase could show more specificity than reversible inhibitors (3,5).

Fast active-site-directed irreversible inhibitors of dihydrofolic reductase such as XIII, which has the sulfonyl fluoride attacking group, have emerged from this laboratory (9). Therefore current work is being focused on the sulfonyl fluoride type of irreversible inhibitor where the carrier is a 2,4-diaminopyrimidine or a 4,6-diamino-1,2-dihydro-*s*-triazine.

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% ethanol and infrared spectra in potassium bromide pellets. Thin layer chromatograms (TLC) of the dihydro-*s*-triazines were run on Brinkmann MN-polyamide UV_{254} and spots were detected by visual examination under ultraviolet light. TLC of ketone precursors were run on Brinkmann silica gel GF. All analytical samples moved as a single spot on TLC and had spectra in agreement with their assigned structures.

p-Acetamidophenoxyacetone.

A solution of 3.00 g. (15 mmoles) of *p*-nitrophenoxyacetone (7) in 200 ml. of glacial acetic acid and 2.36 g. (23 mmoles) of acetic anhydride was shaken with hydrogen at 2-3 atmospheres in the presence of 50 mg. of platinum oxide until 45 mmoles of hydrogen was consumed (3 hours). The filtered solution was spin-evaporated *in vacuo* and the residue was recrystallized from ethanol, yield, 2.42 g. (72%), m.p. 162-164°.

Anal. Calcd. for $C_{11}H_{13}NO_3$: C, 63.8; H, 6.32; N, 6.76. Found: C, 63.7; H, 6.45; N, 6.59.

5-(*p*-Nitrophenoxy)-2-pentanone

A mixture of 9.85 g. (50 mmoles) of sodium *p*-nitrophenolate dihydrate, 6.60 g. (55 mmoles) of 5-chloro-2-pentanone, 0.15 g. of sodium iodide and 100 ml. of *N,N*-dimethylformamide was refluxed for 30 minutes, then diluted with several volumes of water and extracted with benzene. The benzene solution was washed with several portions of 1% aqueous sodium hydroxide until the washings were colorless. After being washed with water, the benzene solution was evaporated *in vacuo*. The residue was extracted with hot petroleum ether (b.p. 60-110°), decanting from some insoluble material. The combined hot extracts were clarified with decolorizing carbon then chilled at -15°. The product was collected on a filter and recrystallized twice more from petroleum ether (b.p. 60-110°); yield, 1.32 g. (12%), m.p. 61-64°. For analysis a sample was recrystallized once more from petroleum ether to give yellow crystals, m.p. 63-64°; λ max 228, 322 mμ.

Anal. Calcd. for $C_{11}H_{13}NO_4$: C, 59.2; H, 5.87; N, 6.27. Found: C, 59.4; H, 5.90; N, 6.09.

The meta-isomer was prepared similarly in 7% yield, m.p. 46-48°, λ max 275, 330 mμ.

Anal. Found: C, 59.4; H, 6.05; N, 6.29.

The *ortho*-isomer was obtained as an oil in 25% yield. It was characterized as its 2,4-dinitrophenylhydrazone, yellow crystals from ethanol, m.p. 113-114°; λ max 250, 282, 288, 343, 360 mμ.

Anal. Calcd. for $C_{17}H_{17}N_5O_7$: C, 50.6; H, 4.22; N, 17.3. Found: C, 50.6; H, 4.20; N, 17.3.

4,6-Diamino-1,2-dihydro-2-methyl-2-(*o*-nitrophenoxypropyl)-1-phenyl-*s*-triazine Hydrochloride (VIa) (Method A).

A mixture of 583 mg. of 5-(*o*-nitrophenoxy)-2-pentanone (2.67 mmoles), 475 mg. of V, 1.5 ml. of reagent methanol, 1.0 ml. of triethyl orthoformate and 0.10 ml. of 12 *N* aqueous hydrochloric acid was stirred in a stoppered flask. At the end of 5 days, TLC showed V was still present; 0.5 ml. of triethyl orthoformate was added and the mixture was stirred for an additional 6 days when TLC showed B was absent. The mixture was spin-evaporated *in vacuo*. The glassy residue was readily soluble in ethanol; the solution slowly deposited crystalline product at -15°; yield, 800 mg. (87%), m.p. 194-197° that moved as a single spot on TLC. For analysis a sample was recrystallized from ethanol. See Table II for additional data.

2-(*o*-Bromoacetamidophenoxypropyl)-4,6-diamino-1,2-dihydro-2-methyl-1-phenyl-*s*-triazine Hydrochloride (XIa) (Method B).

A mixture of 775 mg. (1.85 mmoles) of VIa, 100 ml. of ethanol, and 50 mg. of platinum oxide was shaken with hydrogen at 2-3 atmospheres until 5.5 mmoles ot hydrogen was absorbed. The filtered solution was spin-evaporated *in vacuo* leaving Xa as a glass.

The crude Xa was dissolved in 3 ml. of *N,N*-dimethylformamide, then treated at 0° with 609 mg. of bromoacetic anhydride with magnetic stirring (8). After 20 minutes at 0°, the mixture was diluted with 10 ml. of ether. The solvent was decanted from the gummy product. The latter was triturated three times with 10 ml. portions of ether; the amorphous product slowly crystallized from aqueous ethanol at 3°; yield, 154 mg. (21%), m.p. 122-124° dec.; λ max 243, 280 mμ (inflection). The compound gave a negative Bratton-Marshall test for aromatic amine and a positive 4-(*p*-nitrobenzyl)pyridine test for activated halogen (8). See Table II for additional data.

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