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Analogs of Tetrahydrofolic Acid. XXX. Inhibition of Dihydrofolic Reductase by Some 6-Substituted 2,4-Diamino-*s*-triazines (1,2)

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Four different routes to 6-alkyl-, 6-aryl-, and 6-aralkyl-2,4-diamino-*s*-triazines were investigated. The most convenient was reaction of an ester with biguanide in methanol at room temperature. Seven of these 6-substituted 2,4-diamino-*s*-triazines were investigated as inhibitors of dihydrofolic reductase. As a class they were weaker inhibitors than the corresponding 2,4-diamino-6-substituted pyrimidines, this difference being attributed to the weaker basicity of the former class. Although hydrophobic bonding by 6-alkyl and 6-aralkyl groups on the 2,4-diamino-*s*-triazine system could be detected, the extent of hydrophobic bonding was much weaker than that previously seen with 2,4-diaminopyrimidine containing a 5-alkyl or 5-aralkyl group or 1-alkyl- and 1-aralkyl-2,4-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazines.

A variety of 2,4-diaminoheterocycles have been reported to be inhibitors of dihydrofolic reductase and the field has been recently reviewed (3). Since 2,4-diamino-*s*-triazines with a 6-substituent are readily synthesized in one step from biguanide and an ester or cyanoguanidine and a nitrile (4), it was considered of value to investigate this type of 2,4-diamino heterocycle as inhibitors of dihydrofolic reductase in more detail; if good reversible binding to the enzyme could be obtained, then the 2,4-diamino-6-substituted-*s*-triazines system might be worthy of further investigation towards synthesis of possible active-site-directed irreversible inhibitors (5).

2,4-Diamino-6-methyl-*s*-triazine (II) and 2,4-diamino-6-phenyl-*s*-triazine (IV) showed 50% inhibition of dihydrofolic reductase at concentrations of 7.0 and 2.5 m*M*, respectively (Table I). The corresponding 2,4-diamino-6-methyl- and 6-phenylpyrimidines (I and III) showed 50% inhibition at 1.1 and 0.16 m*M*, respectively. Thus, in the methyl series, the pyrimidine, I, is only a 7-fold better inhibitor than the *s*-triazine series, II, and in the 6-phenyl series, III is only a 15-fold better inhibitor than IV. That a pyrimidine would be a better inhibitor than the corresponding *s*-triazine can now be rationalized on their differences in basicity. For example, 2,4-diamino-6-methylpyrimidine (I) has a pK_a = 7.7 (6), but 2,4-diamino-6-methyl-*s*-triazine (II) has a pK_a = 4.6 (7). In an earlier study from this laboratory, data were presented which showed that the basicity of the inhibitor was important in binding to dihydrofolic reductase; for example 5-(*p*-chlorophenyl)-2,4-diamino-6-methylpyrimidine (V) with a pK_a of 7.7 was a 250-fold better inhibitor than the corresponding 6-trifluoromethylpyrimidine (VI) with a pK_a of 2.5 (8). That basicity was an important factor in binding was further confirmed by *p*H profile studies which showed that a weakly basic pyrimidine such as VI was bound to the enzyme best at acidic *p*H to a protonated form of the enzyme, but that V was bound poorly at *p*H 5 and 9 compared to binding at *p*H 7.4 (9). The fact that I and III are better inhibitors than the corresponding triazines, II and IV, further confirm the hyopthesis that basicity is indeed an important factor.

In an accompanying paper (10), strong evidence is presented that the aryl group of 1-aryl-2,4-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazines is complexed to dihydrofolic reductase by hydrophobic bonding; the 1-phenyl-*s*-dihydrotriazine was bound to the enzyme nearly 800-times better than the corresponding 1-methyl triazine (11). Although it is noteworthy that 2,4-diamino-6-phenylpyrimidine (III) is a 7-fold better inhibitor than the corresponding 6-methylpyrimidine (I) and 2,4-diamino-6-phenyl-*s*-triazine (IV) is a 3-fold better inhibitor that the corresponding 6-methyl-*s*-triazine, a bigger difference might have been anticipated in view of the much larger increment in binding observed with 1-phenyl-*s*-dihydrotriazines and 5-phenylpyrimidines when compared to the corresponding methyl derivatives (11). However, hydrophobic bonding was also less than expected with some 2,4,6-triamino-5-alkylpyrimidines compared to the parent 2,4,6-triaminopyrimidine (11); these results were rationalized on the basis that the 4(6)-amino group of the 5-alkyl-2,4,6-triaminopyrimidine was forced into the hydrophobic region resulting in some repulsion of this amino group which is reflected in poorer binding (11,12). Such an explanation could also be used for the 6-phenyl-*s*-triazine (IV), that is, the phenyl is complexed hydrophobically, but there is some hydrophobic repulsion of a basic group; strong evidence that the 6-phenyl group of III is also complexed to the hydrophobic region has been obtained recently (13).

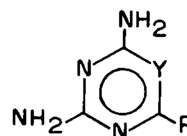
Attempts to increase the hydrophobic bonding by replacement of the phenyl group of 2,4-diamino-6-phenyl-*s*-triazine (IV) with *p*-chlorophenyl (VII), *m*-

chlorophenyl (VIII), benzyl (IX) or *n*-amyl (X) were unsuccessful. Benzyl (IX) was actually quite poor in hydrophobic bonding; that 1-benzyl-2,4-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine gives poorer hydrophobic bonding than the corresponding 1-phenyl dihydro-*s*-triazine has been previously noted (14). The *n*-amyl-*s*-triazine (X) was only complexed 2.4 times better than the *n*-methyl-*s*-triazine (II); note that the 5-aminopyrimidine (XII) was complexed 1100 times better than 2,4-diamino-6-methylpyrimidine (I) (11).

2,4-Diamino-6-(5'-phenylamyl)-*s*-triazine (XI) was the best inhibitor found in the *s*-triazine series among those investigated, being complexed 50-times better

TABLE I

Inhibition of Dihydrofolic Reductase by

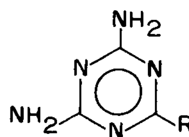


Compound Number	Y	R	m*M* Conc.	% Inhibition (a)	Estimated Conc. for 50% Inhibition (b)
I	CH	CH_3	1.1	50	1.1 (c, d)
II	N	CH_3	6.0	43	7.0 (c)
III	CH	C_6H_5	0.16	50	0.16 (c, e)
IV	N	C_6H_5	2.5	50	2.5 (c)
V	C-C_6H_4-Cl-*p*	CH_3	2×10^{-4}	50	2×10^{-4} (f)
VI	C-C_6H_4-Cl-*p*	CF_3	0.050	50	0.050 (f)
VII	N	*p*-C_6H_4Cl	2.2	50	2.2 (c)
VIII	N	*m*-C_6H_4Cl	8.4	43	11 (c)
IX	N	$C_6H_5CH_2$	6.0	23	18 (c)
X	N	*n*-C_5H_{11}-	2.9	50	2.9 (c)
XI	N	C_6H_5-$(CH_2)_5$-	0.15	50	0.15 (c)
XII	C-C_5H_{11}-*n*	CH_3	1.0×10^{-3}	50	1.0×10^{-3} (d)
XIII	C-$(CH_2)_4C_6H_5$	CH_3	2.7×10^{-5}	50	2.7×10^{-5} (d)
XIV	CH	NH_2	1.2	50	1.2 (d)
XV	C-$(CH_2)_4C_6H_5$	NH_2	3.5×10^{-3}	50	3.5×10^{-3} (d)

(a) Dihydrofolic reductase was a 45-90% saturated ammonium sulfate fraction from pigeon liver which was prepared and assayed with 6 μ*M* dihydrofolate and 12 μ*M* TPNH in 0.05 *M* Tris buffer at *p*H 7.4 as previously described (22). The technical assistance of Maureen Baker, Karen Smith, Shirley Humphrey, and Gail Westley with these assays is acknowledged. (b) The concentration for 50% inhibition was determined by plotting V_O/V_I against I for several concentrations of I that would give 30-70% inhibition, where V_O = velocity without inhibitor, V_I = velocity with inhibitor, and I = concentration of inhibitor; the concentration for 50% inhibition was obtained where the line crosses $V_O/V_I = 2$ (23). Where 50% inhibition could not be obtained due to lack of solubility, the line was extended to the 50% inhibition point; the less the maximum inhibition below 50% that can be obtained, the greater is the error in the estimated concentration for 50% inhibition. (c) Assay run in 10% aqueous N,N-dimethylformamide. (d) Data from reference 11. (e) This compound was synthesized according to the method of Russell (24). (f) Data from reference 8.

TABLE II

Physical Constants of



Compound Number	R	Method	% Yield	m.p. °C	λ max (ε)	Analyses Calcd. C	H	N	Found C	H	N
II	CH_3	A (a)	52	283-285 (b)	257 (3300)	38.4	5.64	56.0	38.5	5.67	55.8
		B (c)	53	276-278 (b)							
		C (d)	11	> 295							
VII	*p*-C_6H_4Cl	B	58	252-253 (e)	254 (22,200)	48.8	3.63	31.6	48.8	3.70	31.8
VIII	*m*-C_6H_4Cl	B	42	226-227 (f)	243 (19,300)	48.8	3.63	31.6	48.8	3.70	31.4
IX	$C_6H_5CH_2$-	D	57	246-247 (g, h)	258						
X	*n*-C_5H_{11}-	D	36 (i)	174-175 (g, j)	258	53.0	8.34	38.6	53.1	8.42	38.7
XI	$C_6H_5(CH_2)_5$-	D	52 (i)	169-170 (k)	258	65.3	7.44	27.2	65.2	7.50	27.3
XVI	$BrCH_2$-	D	57	258 dec. (g, l)	270 (3200)	23.5	2.96	34.3	23.7	3.03	34.0

(a) Prepared according to reference 15 where no m.p. has been given. (b) In a sealed capillary tube after recrystallization from water; if the m.p. was taken in the usual way on a Fischer-Johns apparatus, the compound did not melt up to 295°. (d) Prepared according to reference 16; m.p. 263° was recorded. (d) Prepared according to reference 17; a m.p. of 252-255° was recorded. (e) Recrystallized from aqueous 2-methoxyethanol. (f) Recrystallized from absolute alcohol-petroleum ether (b.p. 60-110°). (g) Recrystallized from water. (h) Literature m.p. 232-233° (19). (i) No effort was made to obtain more material from the mother liquors and this yield is a minimum value. (j) A m.p. of 168-170° (25a) and 174° (25b) has been recorded for this compound prepared by less convenient routes. (k) Recrystallized from aqueous ethanol. (l) Gradually darkens above 200° and decomposes to a black mass at 265°; lit. m.p. 185-200° (20), 226-229° (21).

than the corresponding 6-methyl-*s*-triazine (II); however this amount of hydrophobic bonding was still considerably less than the 3500-fold increment observed between XIV and XV and the 40,000-fold increment observed between XIII and I.

These studies again confirm the earlier observations that the magnitude of hydrophobic bonding that can be obtained is also dependent upon the position and nature of the other substituents on the heterocyclic ring system (11, 12). Furthermore, the relatively poor reversible binding of the aromatic *s*-triazines recorded in Table I make this class of compounds unlikely candidates for construction of active-site-directed irreversible inhibitors (5) that might operate *in vivo*, since the intracellular concentration of the *s*-triazine required to form sufficient reversible complex - the intermediate to active-site-directed irreversible inhibition - would be too high.

CHEMICAL METHODS

Synthesis.

Four routes to the *s*-triazines listed in Table II were employed. The 6-methyl-*s*-triazine (II) has been synthesized from biguanide and acetic anhydride in aqueous acetone (15) (Method A), cyanoguanidine and acetonitrile in a steel bomb at 192-194° (16) (Method B), or *via* fusion of N-acetyl guanidine (Method C) (17). The best method of the three for preparation of II was Method A. The route chosen for the remainder of the compounds in Table I was mainly predicated on availability of starting materials. Compounds VII and VIII were prepared by Method B in boiling 2-methoxyethanol (18). Compounds IX, X, XI, and XVI were prepared by the fourth route, the reaction of an ester with biguanide in methanol at room temperature (19) (Method D). Method D is considered to be the most useful and simplest of the four methods.

EXPERIMENTAL

Methods.

Melting points were taken on a Fischer-Johns apparatus unless otherwise indicated, and those below 230° are corrected. Infrared spectra were taken in KBr pellets with a Perkin-Elmer 137B spectrophotometer; all compounds gave infrared spectra in agreement with their assigned structures. Ultraviolet spectra were determined in methanol with a Perkin-Elmer 202 spectrophotometer.

6-(*p*-Chlorophenyl)-2,4-diamino-*s*-triazine (VII) (Method B).

A mixture of 525 mg. (6.25 mmoles) of cyanoguanide, 690 mg. (5 mmoles) of *p*-chlorobenzonitrile, 50 mg. of potassium hydroxide, and 2.5 ml. of 2-methoxyethanol was refluxed with magnetic stirring for 3 hours (18); solution occurred in 5 minutes, then the product began to separate after 10 minutes. After being cooled at 5° for 24 hours, the mixture was filtered. The product was washed successively with 2-methoxyethanol, petroleum ether (b.p. 30-60°), then hot water; yield, 644 mg. (58%), m.p. 252-253°. Recrystallization from aqueous 2-methoxyethanol gave 543 mg. of white crystals, m.p. 252-253°; ν max 3500, 3400, 3350 (NH); 1650, 1620, 1580, 1540-1530 (C=N, C=C, NH); 810 cm^{-1} (m-C_6H_4).

See Table II for analytical data; other compounds made by this Method B are listed in Table II.

2,4-Diamino-6-(5'-phenylamyl)-*s*-triazine (XI) (Method D).

A mixture of 240 mg. (2.37 mmoles) of biguanide, 3 ml. of reagent methanol, and 660 mg. (2.60 mmoles) of isopropyl 6-phenylhexanoate (8) was stirred at ambient temperature for 20 hours during which time the product separated. The product was collected on a filter and washed with ethyl acetate; yield, 328 mg. (52%), m.p. 170-171°; no attempt was made to obtain a second crop. Recrystallization from aqueous ethanol gave 294 mg. of white needles with unchanged m.p. and ν max 3500, 3350, 3150 (NH); 1680, 1650, 1550 (NH, C=N, C=C); 748, 692 cm^{-1} (C_6H_5).

For analytical data see Table II; other compounds prepared by this method D are listed in Table II.

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