Griffin, Robert G. Pendleton, James F. Kerwin, Allen Misher, and Harry Green for helpful discussions. We are grateful to Miss Roberta Carraine for technical assistance with the bioassay, Miss Margaret Carroll and staff for elemental analyses, and Mr. Walter Hamill and staff for optical rotations.

Potential Folic Acid Antagonists. 4. Synthesis and Dihydrofolate Reductase Inhibitory Activities of 2,4,6-Triamino-5-arylazopyrimidines

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Received January 14, 1971

A series of 49 2,4,6-triamino-5-arylazopyrimidines have been synthesized and examined for their inhibitory activity toward chicken liver dihydrofolate reductase. This activity was comparatively independent of the substituent character of the 5-aryl group; however, para substituents (almost without exception) reduced activity, meta substituents maintained activity, and small *o*-alkyl substituents increased activity. The most active compound in the series was found to be 2,4,6-triamino-5-*o*-ethylphenylazopyrimidine.

Previous studies in this laboratory[1-3] and elsewhere[4-7] have revealed that 5-arylazopyrimidines exhibit significant inhibitory activity towards folate–dihydrofolate reductase. In continuation of our studies of the structural requirements for this activity we report the synthesis and inhibitory activities of a series of 2,4,6-triamino-5-arylazopyrimidines.

Experimental Section[8]

Synthetic Procedure.—2,4,6-Triamino-5-arylazopyrimidines (Table I) were prepd by the general method described by Timmis and his coworkers.[4] The diazotized amine (0.1 mole) free from HNO_2 was added to a vigorously stirred soln of 2,4,6-triaminopyrimidine (0.1 mole) in H_2O (350 ml) at 0° with sufficient NaOAc to maintain the pH at 6–7. When the addn was complete, the mixt was stirred at 0–5° for 10 hr and then at 10° for 2–3 days. The product was collected and washed well with H_2O and air-dried: crude yields ranged from 70 to 100%. The compds were recrystd from *i*-PrOH which caused considerable losses but had the advantage of giving analytically pure specimens with the minimum of manipulation.

Enzyme Procedure.—Dihydrofolate reductase was partially purified from chicken liver according to Kaufman and Gardiner:[9] their procedure was followed to step IV, yielding a prepn with an increase in specific activity of 125- to 150-fold over that of the starting supernatant.

The standard assay mixt contained 5×10^{-5} *M* TPNH and 6×10^{-5} *M* dihydrofolate[10] in 50 m*M* phosphate buffer at pH 7.9. The velocity was detd spectrophotometrically at 340 n*M* using a cell of 1-cm path length. Inhibitors were added as aq solns if sol, or if insol in H_2O, in DMSO to give a final concn of 3.3% DMSO. When DMSO was employed the controls contained an equal amt of the solvent. Experiments with 10 water-sol compds selected at random showed that this concn of DMSO had little effect on the extent of inhibition produced. Initial velocities were detd over the first 2 min of the reaction in the presence of at least 6 inhibitor concns which were chosen to give a range of inhibition from 20 to 80%. From these data the $[I]/[S]_{0.5}$ values listed in Table I were determined. The [I]/[S] ratios for 50% inhibition provide a convenient numerical manner for denoting changes in the affinity of an antagonist[1] with structure since when $[S] > 5K_m$, $[I]/[S]_{0.5} = K_i/K_m$.

Results and Discussion

It is quite apparent from the data of Table I that many of the substituents have but modest effects on the inhibitory activity of the parent compound, 2,4,6-triamino-5-arylazopyrimidine (**1**). In general, the introduction of para substituents (**4**,**7**,**12**,**15**,**17**,**21**,**23**, **26**,**28**,**31**,**34**) reduces activity relative to the parent compound: this effect is most marked with hydrocarbon substituents (**4**,**7**,**9**,**12**,**15**,**17**) although there is no obvious correlation with substituent size. In contrast, introduction of the more polar *p*-Cl, *p*-Br, *p*-I, *p*-MeO, and *p*-CO_2Et substituents produces a smaller reduction in activity; 2,4,6-triamino-5-*p*-iodophenylazopyrimidine (**26**) appears to be the sole example in which a para substituent increases activity above that of the parent unsubstituted compound (**1**).

Introduction of meta substituents (**3**,**6**,**14**,**18**,**20**,**22**, **25**,**27**,**30**,**33**,**35**) has only modest effects on the inhibitory activity. In general, activity is maintained or increased slightly and this is most marked with *m*-I (**25**) which shows a 4-fold increase. These effects of meta substituents appear to be relatively independent of substituent character.

The effect of nonpolar ortho substituents reaches a maximum at *o*-Et (**5**) which shows a 5.5-fold increase in activity: further increases in size of the substituent does not produce any further increase in activity. Other substituents (Cl, CF_3, MeO_2C, CF_3) do not increase activity and it is interesting that the iodo sub-

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TABLE I

PHYSICAL, ANALYTICAL, AND ENZYME INHIBITORY DATA FOR 2,4,6-TRIAMINO-5-ARYLAZOPYRIMIDINES

No.	5 Substituent	Mp, °C	Formula	Analyses	[I] $[S]_{50}$
1	C_6H_5	261	$C_{10}H_{11}N_7$	*a*	0.31
2	2-$CH_3C_6H_4$	254–255	$C_{11}H_{13}N_7$	C, H, N	0.12
3	3-$CH_3C_6H_4$	256	$C_{11}H_{37}N_7$	C, H, N	0.30
4	4-$CH_3C_6H_4$	263–264	$C_{11}H_{13}N_7$	C, H, N	5.8
5	2-$C_2H_5C_6H_4$	257–258	$C_{12}H_{15}N_7$	C, H, N	0.058
6	3-$C_2H_5C_6H_4$	232	$C_{12}H_{15}N_7$	C, H, N	0.20
7	4-$C_2H_5C_6H_4$	255	$C_{12}H_{15}N_7$	C, H, N	1.68
8	2-$CH_3(CH_2)_2C_6H_4$	181	$C_{13}H_{17}N_7$	C, H, N	0.14
9	4-$CH_3(CH_2)_2C_6H_4$	256–257	$C_{13}H_{17}N_7$	C, H, N	13.3
10	2-$(CH_3)_2CH$ C_6H_4	225	$C_{13}H_{17}N_7$	C, H, N	0.103
11	2-$CH_3(CH_2)_3C_6H_4$	176–178	$C_{14}H_{19}N_7$	C, H, N	0.158
12	4-$CH_3(CH_2)_3C_6H_4$	259	$C_{14}H_{19}N_7$	C, H, N	>4.0
13	2-$C_6H_5C_6H_4$	252–254	$C_{16}H_{15}N_7$	C, H, N	0.93
14	3-$C_6H_5C_6H_4$	284–285	$C_{16}H_{15}N_7$	C, H, N	0.13
15	4-$C_6H_5C_6H_4$	295–296	$C_{16}H_{15}N_7$	C, H, N	>4.0
16	2-$CH_3(CH_2)_6C_6H_4$	97–98	$C_{17}H_{25}N_7$	C, H, N	0.83
17	4-$CH_3(CH_2)_8C_6H_4$	226–228	$C_{19}H_{29}N_7$	C, H, N	3.67
18	3-F C_6H_4	277	$C_{10}H_{10}F$ N_7	C, H, N	0.20
19	2-ClC_6H_4	298	$C_{10}H_{10}ClN_7$	*b*	0.33
20	3-ClC_6H_4	264–265	$C_{10}H_{10}ClN_7$	*c*	0.18
21	4-ClC_6H_4	259	$C_{10}H_{10}ClN_7$	*d*	1.66
22	3-BrC_6H_4	269	$C_{10}H_{10}BrN_7$	C, H, Br, N	0.12
23	4-BrC_6H_4	261–262	$C_{10}H_{10}BrN_7$	*e*	1.42
24	2-I C_6H_4	306 dec	$C_{10}H_{10}I$ N_7	C, H, I, N	0.67
25	3-I C_6H_4	265	$C_{10}H_{10}I$ N_7	C, H, I, N	0.09
26	4-I C_6H_4	273–274	$C_{10}H_{10}I$ N_7	C, H, I, N	0.12
27	3-CH_3O C_6H_4	230–231	$C_{11}H_{13}N_7O$	C, H, N	0.33
28	4-CH_3O C_6H_4	232	$C_{11}H_{13}N_7O$	*f*	0.83
29	2-$CF_3C_6H_4$	305 sub	$C_{11}H_{10}F_3N_7$	C, H, N	0.42
30	3-$CF_3C_6H_4$	270	$C_{11}H_{10}F_3N_7$	C, H, N	1.06
31	4-$CF_3C_6H_4$	271	$C_{11}H_{10}F_3N_7$	C, H, N	1.30
32	2-$CO_2MeC_6H_4$	282–284	$C_{12}H_{13}N_7O_2$	C, H, N	0.33
33	3-$CO_2EtC_6H_4$	231	$C_{13}H_{15}N_7O_2$	C, H, N	0.42
34	4-$CO_2EtC_6H_4$	256–258	$C_{13}H_{15}N_7O_2$	*g*	0.63
35	3-$NO_2C_6H_4$	289	$C_{10}H_{10}N_8O_2$	C, H, N	0.33
36	2,6-$(CH_3)_2C_6H_3$	199	$C_{12}H_{15}N_7$	C, H, N	0.11
37	3,5-$(CH_3)_2C_6H_3$	236–237	$C_{12}H_{15}N_7$	C, H, N	0.30
38	2,5-$(Cl)_2C_6H_3$	330–331	$C_{10}H_9Cl_2N_7$	*h*	1.33
39	2,4-$(Cl)_2C_6H_3$	311–313	$C_{10}H_9Cl_2N_7$	*i*	>4.0
40	2,6-$(Cl)_2C_6H_3$	245	$C_{10}H_9Cl_2N_7$	C, H, Cl, N	0.17
41	2,3-$(Cl)_2C_6H_3$	326–327	$C_{10}H_9Cl_2N_7$	*j*	3.1
42	3,4-$(Cl)_2C_6H_3$	262–263	$C_{10}H_9Cl_2N_7$	*k*	5.0
43	3,5-$(Cl)_2C_6H_3$	280–281	$C_{10}H_9Cl_2N_7$	C, H, Cl, N	0.10
44	2-CH_3, 3-ClC_6H_3	282–284	$C_{11}H_{12}ClN_6$	C, H, Cl, N	0.38
45	1-$C_{10}H_7$	266	$C_{14}H_{13}N_7$	*l*	0.20
46	2-$C_{10}H_7$	299 dec	$C_{14}H_{13}N_7$	*m*	>4.0
47	4-C_5H_4N (4-pyridyl)	270	$C_9H_{11}N_8$	C, H, N	>4.0
					>4.0
48	3-C_5H_4N (3-pyridyl)	>330	$C_9H_{11}N_8$	C, H, N	0.72
49	1-$C_{10}H_{11}$ (5,6,7,8-tetrahydro-1-naphthyl)	272	$C_{14}H_{17}N_7$	C, H, N	0.15

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stituent which increases activity in the meta and para positions actually decreases activity in the ortho position.

With disubstituted compounds no further increase in activity above that of the monosubstituted compounds was observed with two *o*-Me (**36**) or *m*-Me (**37**) substituents: the 2,6-dichloro (**40**) and 3,5-dichloro (**37**) substitutions did, however, produce activity above that of the corresponding monosubstitutions. However, 2,5-dichloro (**38**), 2,3-dichloro (**41**), 2,4-dichloro (**39**), 3,4-dichloro (**42**), and 2-Me, 3-Cl (**44**) substitutions decreased activity to a level below that of the corresponding monosubstitutions. The 1-naphthyl (**45**), 5,6,7,8-tetrahydro-1-naphthyl (**49**), and 2-naphthyl (**46**) derivatives may also be regarded as disubstituted derivatives and their activities are qualitatively consistent with the additive effects of *o*- and *m*- and *m*- and *p*-Et substitution.

A completely satisfactory rationalization of these findings does not seem possible at the present time, and

attempts to subject the data to regression analysis[11] including such substituent constants as σ, π, E_s have not led to any significant correlations. However, since *p*-alkyl substituents decrease, *m*-alkyl substituents leave unchanged, and *o*-alkyl substituents increase inhibitory activity it may be that the 5-Ph group of **1** binds to a nonpolar area of restricted dimensions. The increased activity found with *o*-alkyl substituents may originate from increased binding in this area. Alternatively, binding of the azopyrimidines to a nonplanar enzyme surface may be facilitated by the *o*-alkyl substituents forcing the benzene and pyrimidine groups into a twisted configuration. The present data do not distinguish these possibilities.

The very extensive work of Baker and his colleagues[12] has already established the crucial importance to inhibitor binding of a nonpolar area on dihydrofolate reductase. The binding capacity of this area would appear to be distinctly larger than that revealed by this study; however, the well-established species differences in dihydrofolate reductase[12,13] may not make this a very definitive conclusion.

Acknowledgments.—This work was supported by grants from National Institutes of Health (CA 06645), NASA (NGR-33-015-016), and National Science Foundation (GU 1864).

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Medium Ring Homologs of Proline as Potential Amino Acid Antimetabolites[1]

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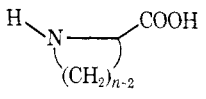
Received October 30, 1970

Hexahydro-1*H*-azepine-2-carboxylic acid (**1d**), octahydro-2-azocinecarboxylic acid (**1e**), octahydro-1*H*-azonine-2-carboxylic acid (**1f**), decahydro-2-azecinecarboxylic acid (**1g**), and azacycloundecane-2-carboxylic acid (**1h**), respectively, the 7-, 8-, 9-, 10-, and 11-membered α-imino acids that are ring homologs of the naturally occurring α-imino acid, L-proline, have been synthesized by rearrangement of α-halogenated-ω-aminolactams of ring sizes one larger than the corresponding imino acids. In addition, a number of derivatives of these imino acids which might be of use for detection of the latter in biological systems have been prepared, *viz.*, the methyl esters, phenylthiohydantoins, 1-nitroso-, 1-(2,4-dinitrophenyl)-, and 1-(6-dimethylaminonaphthalene-1-sulfonyl) derivatives. None of these α-imino acids showed growth inhibitory activities against the F-66 mouse mammary tumor in tissue culture or *Escherichia coli* in a glucose C medium, and only **1g** inhibited slightly the growth of the radicle of the mung bean, *Phaseolus aureus*. These imino acids showed no significant antitumor activity in the Cancer Chemotherapy National Service Center tumor screen, or antimalarial activity against *Plasmodium gallinaceum* or *P. berghei*.

The four-membered cyclic α-imino acid, L-azetidine-2-carboxylic acid (**1a**), the lower homolog of proline which occurs naturally in the plant kingdom as the



	n
1a	4
b	5
c	6
d	7
e	8
f	9
g	10
h	11

major nonprotein, nitrogenous component of the *Liliacae*,[2] has been shown to inhibit the growth of *Escherichia coli* in culture, as well as radicles of the mung bean, *Phaseolus aureus*.[3] This imino acid competitively substitutes for proline and is itself incorporated into the protein of *E. coli* and of *P. aureus*. The presence of **1a** also inhibits the incorporation of [^{14}C]proline into embryonic cartilage as well as the latter's conversion to [^{14}C]hydroxyproline, and results in the accumulation of abnormal protocollagen which contains incorporated **1a**.[4] When added to the culture media of *Streptomyces antibioticus* or of *S. chrysomallus*, **1a** (as well as the higher proline homolog, pipecolic acid, **1c**) is incorporated into the peptide side chain of the actinomycin molecule to produce new biosynthetic actinomycins.[5] The antimetabolic activities of proline homologs and analogs, as well as their comprehensive chemistry and biochemistry, have been the subject of

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