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Inhibition of Mammalian Dihydrofolate Reductase by Selected 2,4-Diaminoquinazolines and Related Compounds

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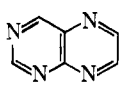
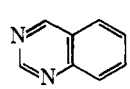
A variety of quinazolines, pyrido[2,3-*d*]pyrimidines, and pteridines were evaluated as inhibitors of dihydrofolate reductase obtained from rat liver and L1210 mouse leukemia cells. Certain of the 2,4-diaminoquinazolines bearing benzylamino or anilinomethyl substituents at position 6 proved to be capable of inhibiting enzyme activity, from both sources, to a degree (ID_{50} 10^{-8}–10^{-9} *M*) comparable to that observed for the antineoplastic agent methotrexate. In the quinazoline series, the presence of primary amino groups at positions 2 and 4 was essential for high inhibitory potency. Neither introduction of halogen, methyl, or hydroxyl substituents anywhere into the phenyl ring of 2,4-diamino-6-benzylaminoquinazoline (1) nor replacement of the phenyl substituent with heteroaromatic functions (pyridyl, furyl, thienyl) markedly affected inhibitory potency. On the other hand, the presence of a chloro substituent at position 5 of 1 was associated with a pronounced increase in inhibitory potency. Interestingly, 2,4-diamino-6-piperidinoquinazoline was found to possess modest activity (ID_{50} = 2×10^{-6} *M*) as an inhibitor of both enzyme systems studied, and insertion of a 2-ethyl function into the piperidine ring of this compound produced an approximately 50-fold increase in inhibitory potency. 2,4-Diaminopyrido[2,3-*d*]pyrimidines investigated were as potent as the analogous quinazolines as inhibitors of dihydrofolate reductase activity from both sources. A series of 2,4-diaminopteridines characterized by a progressive increase in the size of an alicyclic ring fused to positions 6 and 7 of the pteridine nucleus was evaluated against both enzyme systems. A consistent trend of increased inhibitory potency with increasing ring size was observed and is exemplified by the lower ID_{50} observed for the compound bearing a 12-membered ring (ID_{50} = 2.1×10^{-7} *M*) compared with that for the compound bearing a six-membered ring (ID_{50} = 1.9×10^{-4} *M*).

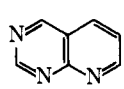
Many compounds possessing the 2,4-diaminopyrimidine nucleus, including a variety of pteridines, can function as inhibitors of dihydrofolate reductase [5,6,7,8-tetrahydrofolate:NAD(P) oxidoreductase E.C. 1.5.1.3.] from bacterial,[1-3] protozoal,[4-7] and mammalian sources.[8-10] The types of compounds which have been synthesized as potential inhibitors of this enzyme system include quinazolines and pyrido[2,3-*d*]pyrimidines, which differ from pteridine by, respectively, the absence of two and one nitrogen atoms in the ring fused to the pyrimidine nucleus.



pteridine



quinazoline



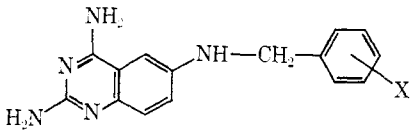
pyrido[2,3-*d*]pyrimidine

Such "deaza" pteridines have been found to exhibit a variety of important pharmacological actions which frequently can be ascribed to their interference with folic acid metabolism.[1,11-18] Elslager and his colleagues have reported recently[19-22] on the antiprotozoal and antibacterial activity of a series of selected 2,4-diaminoquinazolines and pyrido[2,3-*d*]pyrimidine analogs. We have been interested, for several years, in investigating the efficacy of a variety of 2,4-diamino heterocyclic compounds as inhibitors of dihydrofolate reductase from different sources in an effort to establish relationships between chemical structure and inhibitory potency. It is hoped that studies of this type may provide guidelines for synthesis of new antifolate agents and may identify compounds which merit examination as antineoplastic, antibacterial, and antiprotozoal agents of potential clinical utility. The present report describes results of studies of the inhibitory potency, evaluated against dihydrofolate reductase obtained from rat liver and L1210 mouse leukemia cells, of a series of 2,4-diaminoquinazolines, some related pyrido[2,3-*d*]pyrimidines, and a series of 2,4-diaminopteridines characterized by the presence of an alicyclic ring fused to positions 6 and 7 of pteridine nucleus.

Experimental Section

Acetone powders prepared from rat liver (Sigma Chemical Co., St. Louis, Mo.) and L1210 mouse leukemia cells (kindly provided by Dr. J. A. R. Mead, National Cancer Institute, Bethesda, Md.) were extracted with pH 7.0 phosphate buffer (Na_2HPO_4 and KH_2PO_4 in a molar ratio of 6:4); centrifugation of the mixture yielded a supernatant solution which was used as the enzyme source. The assay system used was a slight modification of that described by Burchall and Hitchings[1] involving measurement of the decrease in absorbance at 340 nm catalyzed by dihydrofolate reductase in a system (1.0 ml total volume) composed of NADPH (1×10^{-4} *M*; PL Biochemicals, Milwaukee, Wis.), 2-mercap-

Table I. Inhibition of Dihydrofolate Reduction by 2,4-Diamino-6-(benzylamino)quinazolines



No.	X	ID_{50}, M[a] Rat liver	L1210
1	Hydrogen	1.5×10^{-6}	1.1×10^{-6}
2	2-Methyl	1.3×10^{-6}	1.7×10^{-6}
3	3-Methyl	2.6×10^{-6}	4.6×10^{-6}
4	4-Methyl	4.8×10^{-6}	3.0×10^{-6}
5	2-Hydroxyl	1.9×10^{-6}	1.7×10^{-6}
6	3-Hydroxyl	3.4×10^{-6}	3.0×10^{-6}
7	4-Hydroxyl	2.9×10^{-6}	4.4×10^{-6}
8	4-Hydroxymethyl	9.2×10^{-6}	1.0×10^{-5}
9	4-Fluoro	1.1×10^{-6}	1.5×10^{-6}
10	4-Chloro	2.2×10^{-6}	1.4×10^{-6}
11	4-Bromo	1.9×10^{-6}	1.5×10^{-6}
12	3-Bromo	6.0×10^{-7}	6.4×10^{-7}
13[b]	3,4-Dichloro	8.0×10^{-7}	5.2×10^{-7}
14	4-Carboxy	1.4×10^{-5}	4.5×10^{-6}
15	4-Carboxyethyl ester	2.7×10^{-6}	4.6×10^{-6}
16	4-Dimethylamino	$>10^{-5}$	$>10^{-5}$
17	2-Amino	2.5×10^{-6}	1.1×10^{-6}
18	2-Nitro	3.3×10^{-7}	3.2×10^{-7}
19[c]	Hydrogen (5-chloro)	3.8×10^{-8}	3.5×10^{-8}
20[d]	3,4-Dichloro (5-chloro)	1.2×10^{-8}	5.8×10^{-9}
21	3,4-Dichloro (5-methyl)	1.1×10^{-7}	5.6×10^{-8}
22	Hydrogen (7-methyl)	4.0×10^{-7}	1.1×10^{-6}

[a] ID_{50} values in all tables represent average values from at least four determinations. The variation in ID_{50} values between determinations was approximately ±10%. [b] Ashton, *et al.*,[26] reported an ID_{50} of 1.8×10^{-8} M for compound **13** in the rat liver system using 9 μmol of dihydrofolate and 30 μmol of NADPH. [c] Substituents in parentheses are attached directly to the quinazoline nucleus. [d] Anilinomethyl rather than benzylamino.

toethanol (1×10^{-2} M), and dihydrofolate (1×10^{-4} M; prepared according to the method of Futterman[23]) in pH 7.0 phosphate buffer (0.1 M). The quantity of enzyme solution used for each assay was sufficient to produce an absorbance change of 0.20 units during an 11-min assay period. Enzyme assays were performed using a Perkin-Elmer 202 recording spectrophotometer equipped with a thermostated sample chamber (34 ± 1°) and multiple sample analysis system; a reference blank containing buffer, enzyme, and 2-mercaptoethanol was employed throughout. Stock solutions of compounds tested were prepared with dimethyl sulfoxide (DMSO) as solvent; all subsequent dilutions also were made using DMSO. Compounds being tested as inhibitors were added (in a volume of 10 μl) to a solution of buffer, enzyme, and 2-mercaptoethanol and, after a 2-min preincubation period, first NADPH and then dihydrofolate were added to the cuvettes to initiate the enzymatic reaction. Absorbance changes were measured every 100 sec for a period of 11 min, and the concentration of inhibitor required to reduce the reaction velocity to 50% of the control (unhibited) reaction was determined graphically from a plot of enzyme activity *vs.* inhibitor concentration. Methotrexate was used as a "standard" for these inhibition studies and, under the conditions described, produced 50% inhibition of enzyme activity at a concentration of 8.0×10^{-9} M in the rat liver system and 2.0×10^{-9} M in the L1210 system.

All quinazoline derivatives and pyrido[2,3-*d*]pyrimidines used in this study were kindly provided by Dr. E. F. Elslager of Parke-Davis & Co., Ann Arbor, Mich. 2,4-Diaminopteridines were synthesized by Professor E. C. Taylor and his colleagues at Princeton University, Princeton, N. J.

Results and Discussion

2,4-Diamino-6-benzylaminoquinazoline (1) was found to be a moderately effective inhibitor of dihydrofolate reductase from rat liver and no significant difference in the susceptibility of the rat liver enzyme ($ID_{50} = 1.5 \times 10^{-6}$ M) and of the L1210 system ($ID_{50} = 1.1 \times 10^{-6}$ M) to the inhibitory effects of this agent was noted (Table I). A variety of compounds which differed from compound 1 only by addition of a single substituent to the phenyl ring of the 6-benzylamino side chain was examined for inhibitory potency against the enzymes studied. As shown in Table I, the insertion of methyl groups or hydroxyl groups at positions 2, 3, or 4 of the phenyl substituent produced relatively minor changes in inhibitory potency in either enzyme system. The observation that the addition of methyl groups or hydroxyl groups to the phenyl substituent of 1 is not associated with marked changes in ability to inhibit the reductase system is paralleled by observations concerning the antibacterial activity of these compounds.[19] The presence of halogen atoms at position 4 of the phenyl substituent of 1 did not produce a marked alteration in inhibitory potency, and neither the 3-bromophenyl nor the 3,4-dichlorophenyl analog of 1 differed greatly from 1 with respect to inhibitory activity. Again there was a similarity between our observations concerning the inhibitory action of the halogenated derivatives of 1 evaluated against the reductases and the antibacterial actions of these compounds reported previously.[19] Examination of the inhibitory activity of compounds with a 4-carboxyl or a 4-hydroxymethyl function on the phenyl substituent of 1 revealed that the presence of these substituents is associated with a reduction in inhibitory potency; esterification of the 4-carboxyl function on the phenyl ring resulted in a smaller loss of potency than was observed for the carboxylic acid itself. The derivative of 1 which possessed a dimethylamino function at position 4 of the phenyl ring was without effect on either enzyme when evaluated at a concentration of 1×10^{-5} M. This relative lack of activity parallels the observation that the compound is about 100 times less potent an inhibitor of the growth of *Streptococcus fecalis* than is 1.[19] We wish to stress that we are not implying, in our comparisons of the enzyme inhibitory activity we have observed and the antibacterial activity reported by others, that a direct relationship exists between our data and the antibacterial data but rather we simply wish to point out that some similarities may exist in the structure-activity patterns which emerge from different types of studies.

Substitution of a chlorine atom for a hydrogen atom in position 5 of the quinazoline ring of 1 results in a compound (**19**) which is considerably more potent than the parent compound 1. Compound **20** differs from compound **19** not only by the presence of chlorine atoms at positions

Table II. Inhibition of Dihydrofolate Reduction by 2,4-Disubstituted 6-(Benzylamino)quinazolines

No.		ID_{50}, M Rat liver	L1210
23	2,4-Bis(dimethylamino)-6-[(3,4-dichlorobenzyl)amino]quinazoline	$>10^{-5}$	$>10^{-5}$
24	2,4-Bis(butylamino)-6-[(3,4-dichlorobenzyl)amino]quinazoline	$>10^{-5}$	$>10^{-5}$
25	2,4-Bis(diethylamino)-6-[(3,4-dichlorobenzyl)amino]quinazoline	$>10^{-5}$	$>10^{-5}$
26	2,4-Dianilino-6-[(3,4-dichlorobenzyl)amino]quinazoline	$>10^{-5}$	$>10^{-5}$
27	2-Amino-6-benzylamino-4-quinazolinol	$>10^{-5}$	$>10^{-5}$
28	4-Amino-6-benzylamino-2-methylquinazoline	$>10^{-5}$	$>10^{-5}$

Table III. Inhibition of Dihydrofolate Reduction by 2,4-Diamino-6-[[(heterocyclic)methyl]amino]quinazolines and -6-(heterocyclic)quinazolines

No.		ID_{50}, *M*	
		Rat liver	L1210
29	2,4-Diamino-6-(2-thenylamino)quinazoline	6.6×10^{-7}	5.4×10^{-7}
30	2,4-Diamino-6-[(5-chloro-2-thenyl)amino]quinazoline	5.6×10^{-7}	9.8×10^{-7}
31	2,4-Diamino-6-furfurylaminoquinazoline	3.3×10^{-6}	1.2×10^{-6}
32	2,4-Diamino-6-(2-pyridylmethylamino)quinazoline	2.6×10^{-6}	4.6×10^{-6}
33	2,4-Diamino-6-(4-pyridylmethylamino)quinazoline	2.0×10^{-6}	6.2×10^{-7}
34	2,4-Diamino-6-piperidinoquinazoline	1.7×10^{-6}	2.3×10^{-6}
35	2,4-Diamino-6-(2-ethylpiperidino)quinazoline	3.7×10^{-8}	3.9×10^{-8}
36	2,4-Diamino-6-(1,2,3,4-tetrahydro-2-isoquinolyl)quinazoline	3.0×10^{-7}	2.9×10^{-7}
37	2,4-Diamino-6-(4-phenylpiperidino)quinazoline	2.9×10^{-6}	3.3×10^{-6}
38	2,4-Diamino-6-(2-phenyl-1-pyrrolidinyl)quinazoline	1.1×10^{-8}	1.7×10^{-8}
39	2,4-Diamino-6-[2-(*p*-chlorophenyl)-1-pyrrolidinyl]quinazoline	1.8×10^{-8}	1.4×10^{-8}
40	2,4-Diamino-6-[2-(3,4-dichlorophenyl)-1-pyrrolidinyl]quinazoline	2.0×10^{-8}	7.0×10^{-9}
41	2,4-Diamino-6-(2-methyl-3-phenyl-1-pyrrolidinyl)quinazoline	1.9×10^{-6}	1.1×10^{-6}
42	2,4-Diamino-6-(2-isoindolinyl)quinazoline	$>10^{-5}$	2.4×10^{-5}
43	*N*-(2,4-Diamino-6-quinazolinyl)phthalimide	$>10^{-5}$	$>10^{-5}$
44	2,4-Diamino-6-(pyrrol-1-yl)quinazoline	4.0×10^{-6}	3.5×10^{-6}
45	2,4-Diamino-6-(2,5-dimethylpyrrol-1-yl)quinazoline	3.5×10^{-6}	2.2×10^{-6}
46	2,4-Diamino-6-[(2,5-dimethylpyrrol-1-yl)methyl]quinazoline	2.7×10^{-7}	1.3×10^{-7}
47	2,4-Diamino-6-(3,5-dimethylpyrazol-1-yl)quinazoline	6.8×10^{-6}	6.6×10^{-6}

3 and 4 of the phenyl substituent on the side chain but also in having a methylamino rather than aminomethyl bridge between the quinazoline ring and the phenyl substituent; these structural changes resulted in a slight increase (three- to sixfold) in the inhibitory potency of **20** relative to **19**. It is perhaps worth noting that the linkage *via* a methylamino function of the heterocyclic nucleus of **20** with the phenyl substituent is identical with the sequence between the pteridine ring and the benzoylglutamic acid moiety of folic acid and methotrexate. The potency of compound **20** as evaluated in both of the systems used by us is very similar to that of methotrexate. Since compounds such as **20** lack the ionizable carboxylic acid functions of methotrexate (and of quinazoline analogs of methotrexate[24,25]) such compounds may be worthy of investigation as antineoplastic agents, particularly in systems in which response to methotrexate is limited due to inadequate penetration of the drug into tumor cells or into "sanctuary" areas (such as the central nervous system) in which physiological barriers protect the tumor cells from conventional systemic chemotherapeutic agents.

Methyl substitution at position 5 of the quinazoline ring of **13** is associated with a lesser enhancement of potency than was observed when a chlorine function was introduced into the same position. A methyl group at position 7 of the ring (compound **22**) was observed to produce relatively little change in inhibitory potency as compared to that observed for the parent compound **1**.

Our observations, concerning the effects of substitution of chloro or methyl groups at position 5 of the quinazoline nucleus, on potency against dihydrofolate reductases correlate well with data reported by others. For example, it has been found[11,19] that substitution of small nonpolar groups at position 5 of 2,4-diaminoquinazoline derivatives leads to enhanced antibacterial activity. Ashton and his colleagues have recently reported[26] that 5-chloro and 5-methyl substituents substantially increase the inhibitory potency of other 2,4-diaminoquinazoline derivatives, evaluated against rat liver dihydrofolate reductase.

2,4-Diaminoquinazoline derivatives **23–26**, in which the amino groups were substituted with alkyl or aryl groups, were found to be completely ineffective as inhibitors of dihydrofolate reductase at the highest concentration tested (1×10^{-5} *M*). Replacement of a 4-amino group by a hydroxyl function (**27**) or of a 2-amino group by a methyl group (**28**) also sharply reduced inhibitory potency (Table II). The lack of activity of such compounds is, of course, not unexpected on the basis of similar findings in the pteridine series for example.[7,9,27,28] It is interesting to note that while the 2-amino-4-hydroxy derivative **27** tested by us was not an effective inhibitor of rat liver or L1210 enzymes, analogous quinazolines bearing *p*-aminobenzoylglutamic or aspartic acid moieties in the side chain at position 6 did exhibit a relatively high degree of activity as inhibitors of dihydrofolate reductase obtained from pigeon liver and *S. fecalis*.[29a] The activity of such 2-amino-4-hydroxyquinazolines, however, might be attributable to contamination with 2,4-diamino derivatives.[29b]

Several analogs of compound **1** in which the phenyl group was replaced by other aromatic functions were evaluated as inhibitors of the reductase systems in order to determine whether alterations in the nature of the aromatic ring at this position could significantly effect inhibitory potency. We found that the analogs (Table III) in which the phenyl group was replaced by pyridyl, furyl, or thienyl rings were similar in potency to the parent compound **1**.

Several compounds listed in Table III are characterized by the presence of a cyclic function directly linked to the quinazoline ring at position 6. The activity observed for the piperidine derivative **34** is equivalent to that observed for compound **1**. Surprisingly, the presence of a 2-ethyl function on the piperidine ring (compound **35**) is associated with a sharp increase (nearly 50-fold) in inhibitory potency, an observation which suggests important steric and/or hydrophobic influences on the ability of this type to "bind" to the enzyme systems under consideration.

The 2,4-diaminoquinazoline derivatives **38–40** possessing a 2-phenyl-1-pyrrolidinyl substituent at position 6 were found to be extremely effective inhibitors of dihydrofolate reductase; the potency of these compounds was very close to that observed in our systems for methotrexate. As was noted in the series of 6-benzylamino derivatives shown in Table I, the presence of chlorine substituents at positions 3 and 4 did not markedly influence the inhibitory potency of **38**.

Comparison of the ID_{50} estimated for compound **38** (Table III) with that of compound **1** reveals that the presence of the additional trimethylene "bridge" in compound **38** is associated with a 135-fold increase in inhibitory potency. The effect of this extra trimethylene bridge is evident also when the ID_{50} values for compounds **39** and **40**

Table IV. Inhibition of Dihydrofolate Reduction by Some Miscellaneous 2,4-Diaminoquinazolines

No.		ID_{50}, M	
		Rat liver	L1210
48	2,4-Diamino-6-[(*p*-chlorophenethyl)amino]quinazoline	1.9×10^{-7}	1.9×10^{-7}
49	2,4-Diamino-6-[(α-methylbenzyl)amino]quinazoline	5.8×10^{-7}	6.0×10^{-7}
50	2,4-Diamino-6-[(*p*-chloro-α-methylbenzyl)amino]quinazoline	6.0×10^{-7}	9.2×10^{-7}
51	2,4-Diamino-6-(2-naphthylmethylamino)quinazoline	8.0×10^{-7}	7.0×10^{-7}
52	2,4-Diamino-6-[[(2-chloro-1-naphthyl)methyl]amino]quinazoline	9.4×10^{-8}	9.6×10^{-8}
53	2,4-Diamino-8-(benzylamino)-5-methylquinazoline	$>10^{-5}$	$>10^{-5}$
54	2,4-Diamino-6,8-bis(2,5-dimethylpyrrol-1-yl)quinazoline	1.1×10^{-5}	1.0×10^{-6}
55[a]	6,6′-[*p*-Phenylenebis(methyleneimino)]bis[2,4-diaminoquinazoline]	5.2×10^{-5}	1.6×10^{-5}
56	6,6′-[Ethylenebis(oxy-*p*-phenylenemethyleneimino)]bis[2,4-diaminoquinazoline]	9.5×10^{-6}	1.3×10^{-5}
57	2,4-Diamino-6-[(3,4-dichlorobenzyl)methylamino]quinazoline	9.4×10^{-9}	1.4×10^{-8}

[a] J. B. Hynes, R. F. Gratz, and W. T. Ashton, *J. Med. Chem.*, **16**, 1332 (1973), reported an ID_{50} of 4.5×10^{-7} M for compound **55** in the rat liver system using 9 μmol of dihydrofolate and 30 μmol of NADPH.

Table V. Inhibition of Dihydrofolate Reduction by 2,4-Diamino-6-(benzylamino)pyrido[2,3-*d*]pyrimidines and by 2,4-Diamino-6,7-cycloalkenopteridines

No.		ID_{50}, M	
		Rat liver	L1210
58	2,4-Diamino-6-(benzylamino)pyrido[2,3-*d*]pyrimidine	5.4×10^{-7}	3.9×10^{-7}
59	2,4-Diamino-6-[(3,4-dichlorobenzyl)amino]pyrido[2,3-*d*]pyrimidine	5.2×10^{-7}	3.2×10^{-7}
60	2,4-Diamino-6-[(3,4-dichlorobenzyl)nitrosamino]pyrido[2,3-*d*]pyrimidine	6.6×10^{-7}	1.5×10^{-6}
61	2,4-Diamino-6,7-cyclohexenopteridine	1.9×10^{-4}	$>10^{-5}$
62	2,4-Diamino-6,7-cycloheptenopteridine	3.2×10^{-5}	4.2×10^{-5}
63	2,4-Diamino-6,7-cyclooctenopteridine	9.0×10^{-6}	6.6×10^{-6}
64	2,4-Diamino-6,7-cyclodecenopteridine	4.6×10^{-7}	3.0×10^{-7}
65	2,4-Diamino-6,7-cyclododecenopteridine	2.1×10^{-7}	1.9×10^{-7}

are compared with values obtained for the analogous compounds (**10** and **13**) in Table I. Compound **38** was found to be highly active in suppressing *Plasmodium berghei* infections in mice and, in fact, compared well in this regard with pyrimethamine, a standard agent used clinically in antimalarial chemotherapy.[20] Compound **41** which differs from compound **38** in two ways, having a phenyl substituent at position 3 rather than position 2 of the pyrrolidine function and a methyl group at position 2, was found to be 175 times less potent an inhibitor than **38**. This observation suggests considerable structural specificity in determining the effectiveness of interaction of compound **38** with the enzymes. Compound **42** in which a benzene ring is fused to a pyrrolidine nucleus was found not to be an effective inhibitor of either dihydrofolate reductase. Compound **43** which differs from **42** by the addition of carbonyl functions to the pyrrolidine ring was also noninhibitory at the highest concentration (1×10^{-5} M) tested. Neither compound **42** nor compound **43** exhibited marked antimalarial activity against *P. berghei* in mice and these observations should be contrasted with the observation of a high degree of antimalarial activity for compound **38**.[20]

Three 2,4-diaminoquinazoline derivatives **44**–**46** bearing pyrrole substituents linked to position 6 were evaluated as inhibitors of the two mammalian reductases and relatively little difference in inhibitory potencies was noted in this group of compounds. The 6-(3,5-dimethylpyrazol-1-yl)quinazoline derivative **47** was found to be slightly less potent an inhibitor than any of the pyrrolyl derivatives investigated. It is worth pointing out that compound **47**, even though it was nearly 1000 times less potent than methotrexate as an inhibitor of mammalian dihydrofolate reductases, was found to be only 65 times less potent than methotrexate when evaluated in a *S. fecalis* test system *in vitro*.[20]

The ID_{50} values obtained for several miscellaneous 2,4-diaminoquinazolines are listed in Table IV. Compounds **49** and **50** which differ from compounds **1** and **10** by the addition of a methyl group to the methylene function of the side chain were only slightly more potent than the "parent" compounds. The presence of naphthyl substituents in the side chain (compounds **51** and **52**) was associated with retention of inhibitory activity against the reductases, and indeed compound **52** was found to be only tenfold less potent in the rat liver system used by us than was methotrexate. Both compound **51** and compound **52** have been found to exhibit significant activity in experimental malaria.[19]

When the 2,4-diaminoquinazoline nucleus is substituted with a benzylamino function at position 8 (**53**) rather than at position 6, inhibitory potency is sharply reduced. Thus, compound **53** was found to be virtually inactive as an inhibitor of the reductases when evaluated at a concentration as high as 2×10^{-5} M; compound **53** has been reported[19] to be devoid of antimalarial activity. The observation of the low degree of inhibitory activity of **53** suggests considerable positional specificity in the interaction between 2,4-diaminoquinazolines such as **21** and the enzymes. In this regard it is noteworthy that compound **54** which is characterized by having 2,5-dimethylpyrrole substituents at both positions 6 and 8 is only threefold less potent an inhibitor than the analogous compound **45** bearing a 2,5-dimethylpyrrole substituent only at position 6. It is interesting to note that compounds **45** and **54** each exhibited significant activity against *P. berghei* in mice but compound **54** was found to be over 3000 times less active as a growth inhibitor of *S. fecalis* than was **45**.[20] Compound **54** appeared to be significantly (tenfold) more potent as an inhibitor of L1210 reductase than of rat liver reductase; it will be interesting to determine whether larger differences might be observed in other quinazoline derivatives of this type.

Elslager and his colleagues have reported that linking of two diaminoquinazoline moieties at position 6 as exemplified by compounds **55** and **56** results in a sharp reduction of both antimalarial and antibacterial activity.[19] Our studies indicated that neither compound **55** nor **56** exhib-

its strong inhibitory potency against mammalian dihydrofolate reductase.

One of the most powerful inhibitors of dihydrofolate reductase among those compounds evaluated in this study was compound **57**. This compound, which was essentially equipotent with methotrexate as an inhibitor of rat liver reductase, differs from compound **13** only by the presence of a methyl group on the nitrogen atom attached to position 6 of the quinazoline ring. This relatively small change in the molecular structure of **57** was associated with an increase of nearly 100-fold in inhibitory potency as compared to the potency determined for compound **13**.

Several 2,4-diaminopyrido[2,3-*d*]pyrimidines have been found to be potent inhibitors of dihydrofolate reductase from bacterial and protozoal sources.[1,5,6] Compounds of this class, which closely resemble some quinazoline derivatives of interest to us, have been described recently. The pyrido[2,3-*d*]pyrimidines **58–60** were found to be much less active than analogous quinazolines as inhibitors of both *P. berghei* and *Trypanosoma cruzi in vivo*, but, on the other hand, the pyrido[2,3-*d*]pyrimidines were only slightly less potent than the related quinazolines as folic acid antagonists in a *S. fecalis* test system.[21] We found that the two pyrido[2,3-*d*]pyrimidines (**58** and **59**) tested by us were comparable with their analogous quinazoline derivatives (*e.g.*, **1** and **13**). The observed similarity of inhibitory potencies of analogous quinazolines and pyrido[2,3-*d*]pyrimidines is surprising and suggests that the alteration in electronic properties produced by successive insertion of nitrogen atoms in the benzenoid ring of 2,4-diaminoquinazolines does not play an important role in determining efficacy of interaction of these heterocyclic systems with the mammalian dihydrofolate reductases studied.

The presence of the nitroso function in the structure of **60** was not associated with a marked alteration in inhibitory potency compared with that determined for the compound **59** lacking this substituent. A similar observation for the analogous 2,4-diaminoquinazoline and its nitroso derivative has been reported previously.[30]

2,4-Diaminopteridines bearing lipophilic substituents attached to positions 6 and/or 7 of the pteridine ring system have been found to be capable of inhibiting dihydrofolate reductases and to exhibit cytotoxicity in mammalian and bacterial systems.[7,31-33] We were interested in examining the inhibitory activities of members of a series of 2,4-diaminopteridines which differed from each other only in the size of an alicyclic ring fused to positions 6 and 7 of the pteridine nucleus, hoping not only to establish a relationship between ring size, lipid solubility, and inhibitory potency in this series but also to identify 2,4-diaminopteridines of this type which might be worthy of investigation as antineoplastic agents *in vivo*. We observed a progressive increase in potency evaluated against both reductase systems, as the size of the ring fused to positions 6 and 7 increased. For example, as shown in Table V the 2,4-diaminopteridine derivative containing a six-membered ring is 1000 times less potent than the derivative containing a 12-membered alicyclic substituent. It may be that the increased tendency of the derivatives with larger rings to undergo "hydrophobic" interactions with appropriate sites on the enzyme is responsible, at least in part, for the observations shown here.

Our studies have identified some very potent inhibitors of mammalian dihydrofolate reductases in both the 2,4-diaminoquinazoline and 2,4-diaminopteridine series. Some of the structure–activity relationships established in the present study will be used as guidelines for the synthesis of new pteridine derivatives and other heterocyclic compounds which may have potential therapeutic value in neoplastic, protozoal, and bacterial diseases. None of the inhibitors studied during the course of this investigation showed any *marked* selectivity for neoplastic or "normal" mammalian reductase. The modest degrees of selectivity observed could be significant but their importance can in the final analysis be established only by *in vivo* studies.

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