2,4-Diamino-5-deaza-6-Substituted Pyrido[2,3-*d*]pyrimidine Antifolates as Potent and Selective Nonclassical Inhibitors of Dihydrofolate Reductases[1,2]

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Fifteen novel nonclassical and two classical 2,4-diamino-6-(benzylamino)pyrido[2,3-*d*]pyrimidine antifolates were synthesized as potential inhibitors of *Pneumocystis carinii*, (pc) *Toxoplasma gondii*, (tg) rat liver (rl), and human (h) recombinant dihydrofolate reductases (DHFR). These analogues lack a 5-methyl substitution which has been shown to be important for increased hDHFR inhibitory activity. In addition, they contain a reversal of the C9−N10 bridge present in folates and most antifolates. The synthesis of the compounds involved the reaction of 2,4,6-triaminopyrimidine with the sodium salt of nitromalonaldehyde to afford the key intermediate 2,4-diamino-6-nitropyrido[2,3-*d*]pyrimidine (**7**), in a single step. Reduction of **7** to the 2,4,6-triaminopyrido[2,3-*d*]pyrimidine (**8**), followed by reductive amination with the appropriate benzaldehydes or phenylacetaldehydes afforded the target compounds. N9 methylation of these analogues was carried out using formaldehyde and sodium cyanoborohydride. The analogues demonstrated significant inhibition of pcDHFR and tgDHFR. N9 methylation significantly increased DHFR inhibitory potency. Compound **11**, the 3′,4′,5′-trimethoxy-substituted analogue with a selectivity ratio of 9.4 for tgDHFR (compared to rlDHFR) was the most selective analogue of the nonclassical series. Compound **22**, the N9 methyl 2′,5′-dimethoxy-substituted analogue was the most potent analogue against tgDHFR (IC_{50} = 6.3 nM) and was the second most selective analogue for tgDHFR (compared to rlDHFR) in the nonclassical series. The naphthyl-substituted analogues **23**−**25** were generally more potent against rlDHFR than against pcDHFR and tgDHFR. Selected analogues were also evaluated against *Streptococcus faecium* (sf) DHFR, *Escherichia coli* (ec) DHFR, *Lactobacillus casei* (lc) DHFR and tgDHFR with hDHFR as the mammalian reference, under slightly different assay conditions than those employed for rlDHFR. Analogues **11** and **22** had selectivity ratios of greater than 100 for tgDHFR (compared to hDHFR). Analogue **22** in particular, was the most selective analogue of the nonclassical series against tgDHFR (selectivity ratio = 303.5) with excellent potency (28 nM). Analogue **11**, also displayed significant selectivity for sfDHFR (selectivity ratio = 4902). Compound **22** was evaluated *in vivo* for the inhibition of the growth of *T. gondii* trophozoites in mice, where at 50 mg/kg orally, it demonstrated distinct prolongation of survival without toxicity. Compounds **11**, **12**, and **21**−**23** were evaluated as antitumor agents in the National Cancer Institutes preclinical *in vitro* screening program. Compounds **12**, **22**, and **23** showed GI_{50}s for tumor growth inhibition in the 10^{-6}−10^{-7} M range.

Infections with *Pneumocystis carinii* and *Toxoplasma gondii* are the principal cause of death in patients with acquired immunodeficiency syndrome (AIDS).[3] One approach to treat infections with these organisms is the inhibition of the enzyme, dihydrofolate reductase (DHFR).[4] Trimetrexate (TMQ) and piritrexim (PTX) are potent nonclassical DHFR inhibitors.[5] TMQ has recently been approved for the treatment of *P. carinii* infections,[6] while PTX has been evaluated in clinical trials for the treatment of these opportunistic infections. However, neither of these drugs are selective for *P. carinii* (pc) or *T. gondii* (tg) DHFR, and hence their use alone is associated with considerable host toxicity. Thus, TMQ is administered with the reduced folate leucovorin[7] to rescue host cells. Since *P. carinii* and *T. gondii* lack the carrier mediated uptake mechanism(s) necessary for classical folates, they do not benefit from the coadministration of leucovorin, which is selectively taken up by the host cells. Recently,[8] epiroprim, a trimethoprim analogue, has been reported to be 200-fold selective for pcDHFR (against human DHFR). However, its potency is low and in the micromolar range.

We[9,10] along with others[11] have reported the syntheses and biological activities of a number of nonclassical pyrido[2,3-*d*]pyrimidine antifolates of general structure **1** as inhibitors of pcDHFR and tgDHFR. These analogues contain variations of both the substituents on the side chain as well as the N10 nitrogen to optimize hydrophobic interactions with the enzyme(s). These studies afforded several analogues which display high selectivity or high potency against pcDHFR and/or tgDHFR. However, compounds with high selectivity and potency that are able to significantly penetrate *P. carinii* and *T. gondii* cells have remained a challenge.

The 5-methyl moiety of 5-deaza folates has been shown to be important for potent inhibition of human DHFR and of tumors and contributes favorably to the

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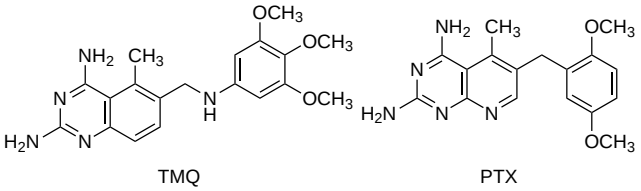
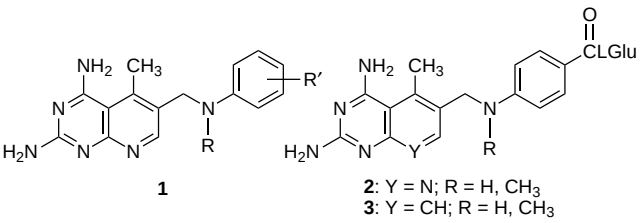
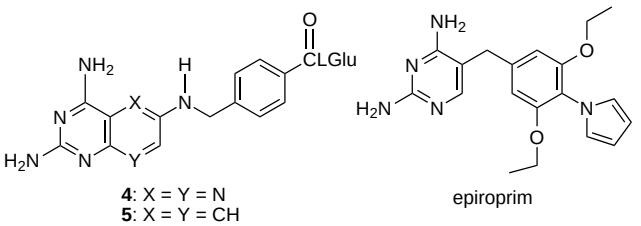






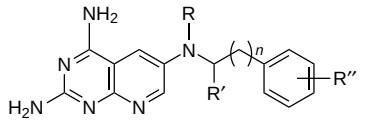
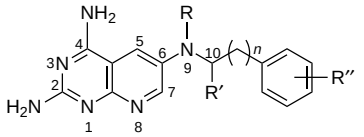


Table 1. Structures of 2,4-Diamino-6-(benzylamino)pyrido[2,3-*d*]pyrimidines

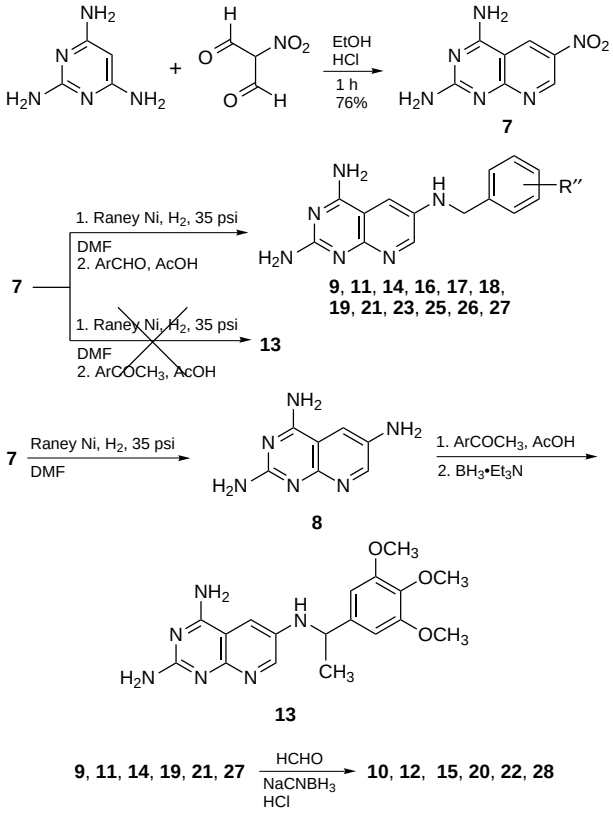


analogue no.	*n*	R	R′	R″
9	0	H	H	H
10	0	CH_3	H	H
11	0	H	H	3′,4′,5′-OMe
12	0	CH_3	H	3′,4′,5′-OMe
13	0	H	CH_3	3′,4′,5′-OMe
14	0	H	H	2′,3′,4′-OMe
15	0	CH_3	H	2′,3′,4′-OMe
16	0	H	H	2′,4′,6′-OMe
17	0	H	H	2′,4′,5′-OMe
18	0	H	H	3′,4′-OMe
19	0	H	H	3′,5′-OMe
20	0	CH_3	H	3′,5′-OMe
21	0	H	H	2′,5′-OMe
22	0	CH_3	H	2′,5′-OMe
23	0	H	H	2′,3′-C_4H_4
24	0	H	H	4′-OMe, 2′,3′-C_4H_4
25	0	H	H	6′-OMe, 2′,3′-C_4H_4
26	0	H	H	4′-O-C_6H_5
27	1	H	H	H
28	1	CH_3	H	H
29	0	H	H	4′-CONH-L-glutamic acid
30	0	CH_3	H	4′-CONH-L-glutamic acid

inhibition of bacterial DHFRs as well. Piper *et al.*[12] have synthesized 5-alkyl-5-deaza analogues of aminopterin (AMT) and methotrexate (MTX) represented by the general structure **2** and reported that the introduction of a 5-methyl substituent resulted in a decrease in the K_i (3.7 to 2.9 nM) for inhibition of DHFR from L1210 cells, indicating that a methyl group at the 5-position was beneficial to binding to DHFR. The 5-methyl-substituted analogues (IC_{50} 0.1−0.3 nM) of AMT were also reported to be superior to AMT (IC_{50} 0.72 nM) against the growth of L1210 cells in culture. The K_m values for unidirectional influx of these 5-deaza folates were identical to those of AMT and MTX. This, along with the fact that the 5-deaza analogues were poor substrates for polyglutamylation, and hence have no improved cellular retention over MTX or AMT, indicated that the sole reason for the high potency of the 5-methyl analogues is their increased ability to inhibit DHFR. Hynes *et al.*[13] have synthesized 5-methyl-5,8-dideaza analogues of aminopterin and methotrexate of general structure **3**. These workers similarly reported that 5-methyl-5,8-dideaza-AMT is 4 times as potent as the 5-desmethyl analogue against rat liver (rl) DHFR. Bertino *et al.*[14] also reported nonclassical 2,4-diaminoquinazolines as DHFR inhibitors and of MTX resistant murine leukemia cell lines and found that 2,4-diamino-5-methyl-6-[*N*-(9-phenanthryl)amino]quinazoline (IC_{50} = 1.5 nM) was 60 times more potent than the 5-desmethyl analogue (IC_{50} = 94 nM). Similarly, 2,4-diamino-5-methyl-6-[*N*-(2-fluoryl)amino] quinazoline (IC_{50} = 1.7 nM) was 88 times more potent than its 5-desmethyl congener (IC_{50} = 150 nM), demonstrating further the importance of the 5-methyl group for potency against mammalian DHFR. We[9,10,15] have also reported that 5-methyl-substituted nonclassical antifolates are more potent as inhibitors of rlDHFR compared to their 5-desmethyl analogues.

On the basis of the demonstrated importance of the 5-methyl group for inhibition of DHFR from mammalian sources, we decided to synthesize compounds of general structure **6** which lack the 5-methyl group. The design of these analogues was based on the premise that since the 5-methyl moiety was important for the inhibition of rlDHFR, L1210 DHFR, and hDHFR and its absence, in certain instances, resulted in analogues with significantly *decreased* potency for mammalian DHFRs, such 5-desmethyl analogues would be less toxic to mammalian cells. Removal of the 5-methyl group was expected to also result in a decrease in potency against pcDHFR and tgDHFR. However, if this decrease in potency against pcDHFR and tgDHFR was less than the decrease in the inhibition of mammalian DHFR, selective and nontoxic inhibitors of pcDHFR and/or tgDHFR would be at hand. To investigate this hypothesis, we synthesized a series of 5-desmethyl-6-substituted, 2,4-diaminopyrido[2,3-*d*]pyrimidines **9**−**28** (Table 1), in which the usual C9−N10 bridge of folates and most antifolates was reversed to also determine the effect of substitutions at the 9-position of 5-deazapyrido-[2,3-*d*]pyrimidines, a feature which has not been investigated in the 5-deaza series. There is precedence in the literature for enhanced DHFR inhibition of 9-substituted antifolates. DeGraw *et al.*[16] reported the synthesis and biological activity of 9-methyl-10-deaza AMT, which was as potent as MTX against DHFR. Interestingly, it was 20 times more potent than MTX against the growth of L1210 cells in culture. The choice of the side chain substitutents of compounds **9**−**28** was based, in part, on the results obtained from our previous studies with other nonclassical analogues.[9,10,17−19] The nonclassical analogues **9**−**28** were evaluated as inhibitors of pcDHFR, tgDHFR, and rlDHFR.[5] Selected analogues were also evaluated[33] against *Streptococcus faecium* (sf) DHFR, *Escherichia coli* (ec) DHFR, *Lactobacillus casei* (lc) DHFR, and recombinant tgDHFR and hDHFR under slightly different assay conditions. Davoll *et al.*[20] have reported 2,4-diamino-6-(benzylamino)-pyrido[2,3-*d*]pyrimidine, 2,4-diamino-6-[(3′,4′-dichlo-

Scheme 1



robenzyl)amino]pyrido[2,3-*d*]pyrimidine, and its N9 nitroso derivative as antimalarial agents. However, no DHFR inhibitory activity was reported for these analogues nor were they evaluated against pcDHFR or tgDHFR. Hence, we resynthesized one of these analogues **9** and evaluated it along with all the other analogues against pcDHFR, tgDHFR, rlDHFR, and hDHFR.

The synthesis and DHFR inhibitory activity of the classical analogues **29** and **30** (Table 1) of the 5-deaza series with a N9−C10 bridge were of particular interest in light of the potent activity of isoaminopterin **4** against L1210 DHFR reported by Nair and Baugh[21] and of the N9 methyl analogue of 5,8-dideazaisoaminopterin **5**, synthesized by Hynes *et al.*,[22] which was not only a potent inhibitor of DHFR (IC_{50} = 4.6 nM) but also a potent inhibitor of thymidylate synthase (IC_{50} = 33 nM).

Chemistry

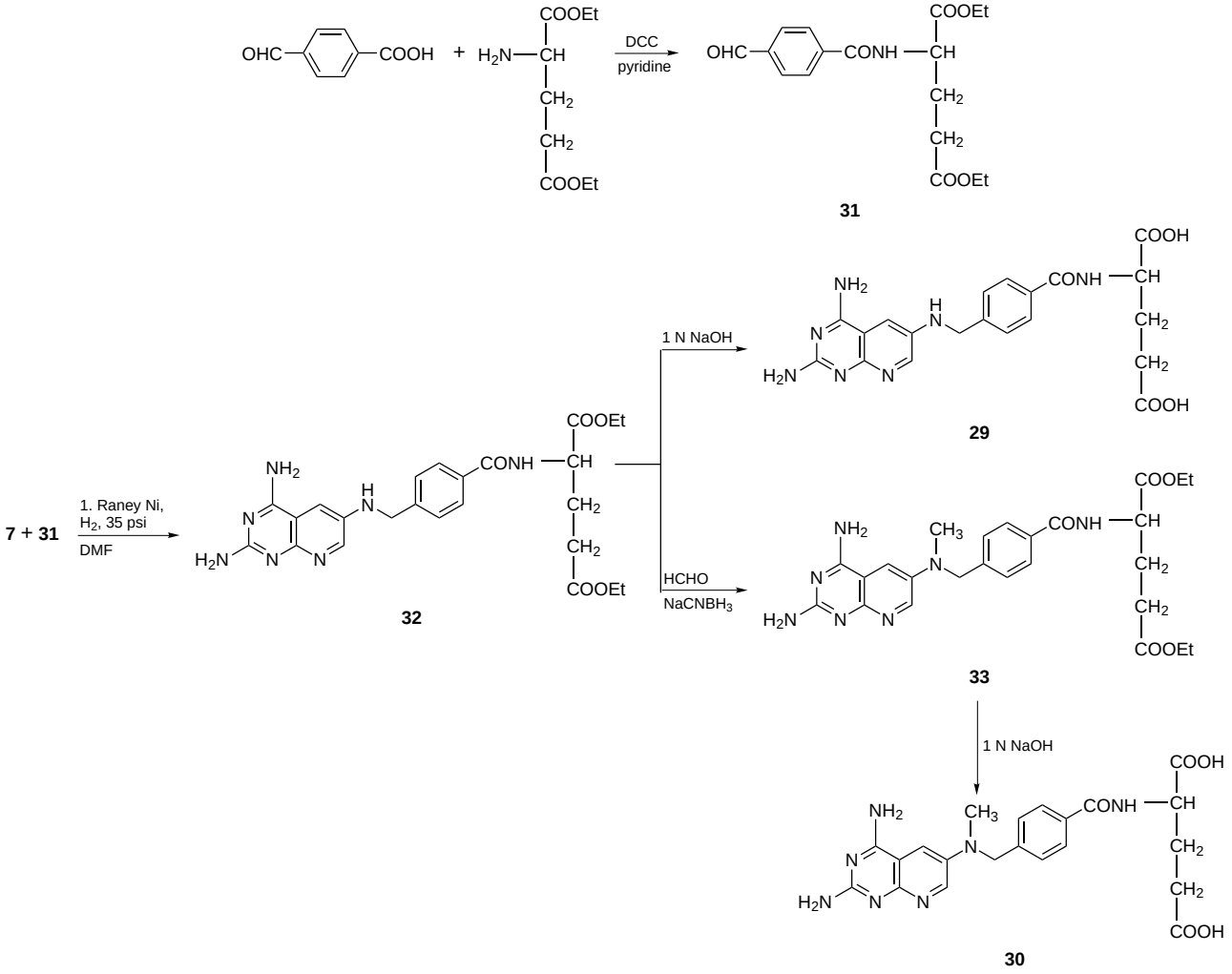
The synthesis of the desired compounds was achieved by the reaction of 2,4,6-triaminopyrido[2,3-*d*]pyrimidine (**8**), with appropriately substituted benzaldehydes or phenylacetaldehyde. The precursor to this compound, 2,4-diamino-6-nitropyrido[2,3-*d*]pyrimidine **7** (Scheme 1), had been reported by Davoll[20] in 1972 *via* a three-step procedure which involved the reaction of nitromalonaldehyde with cyanoacetamide followed by chlorination and cyclization with guanidine (62% overall yield). Since **7** was to serve as the key intermediate for the entire series, it was important to devise a more efficient synthesis of **7**. We, along with others, have previously reported the reaction of a variety of bis-electrophiles such as *β*-keto esters,[23,24] *β*-keto aldehydes,[25] and *β*-dialdehydes[26,27] with 2,4,6-triaminopyrimidine to yield pyrido[2,3-*d*]pyrimidines. Thus, we envisioned that **7** could similarly be synthesized *via* the condensation of nitromalonaldehyde (bis-electrophile) with 2,4,6-triaminopyrimidine. The nitromalonaldehyde was synthesized according to the method of Fanta[28] by the reaction of mucobromic acid and sodium nitrite to afford the desired *β*-dialdehyde in 65% yield as a sodium salt which was used immediately following its preparation. Thus, condensation of nitromalonaldehyde and 2,4,6-triaminopyrimidine in absolute ethanol at reflux, containing sufficient concentrated HCl to solubilize the pyrimidine, afforded a thick red suspension (Scheme 1). Due to the susceptibility of the 4-amino group of folates to hydrolysis under similar conditions,[29] the cyclocondensation reaction was closely monitored by TLC. The ^{1}H NMR of the product corresponded to that expected of **7**. Thus, **7** was obtained in a single step starting from 2,4,6-triaminopyrimidine in 76% yield. A similar methodology, involving base-catalyzed cyclization of 2,6-diamino-4-hydroxypyrimidine and nitromalonaldehyde, has been reported by Price *et al.*[30] for the synthesis of the 2-amino-4-hydroxy-6-nitropyrido[2,3-*d*]pyrimidine.

Compound **7** was reduced to the corresponding 6-amino analogue **8** using Raney Ni in DMF at 35 psi. Complete reduction was obtained in 3 h, as indicated by TLC. This intermediate was not isolated but reacted directly with the appropriately substituted benzaldehydes or phenylacetaldehyde in acetic acid and hydrogenated at 35 psi. The duration of the reductive aminations varied from 3 to 6 h and was predicated on the nature of the substitution on the aldehyde. This procedure afforded analogues **9**, **11**, **14**, **16**−**19**, **21**, **23**, and **25**−**28** in 60−70% yield following chromatographic purification. These analogues displayed an intense green fluorescence at 254 nM, probably due to the presence of the exocyclic amino moiety. Similar attempts with 3,4,5-trimethoxyacetophenone and **7** for the synthesis of **13** did not afford the desired C10 methyl analogue. Prolonged reaction times and increased pressure conditions resulted in recovery of unreacted **8**. As a result, **8** had to be isolated, and reductive amination with 3,4,5-trimethoxyacetophenone was carried out in acetonitrile using a borane−triethylamine complex to reduce the intermediate Schiff base which afforded **13**.

N-Methylation of these analogues was carried out by reductive methylation, using HCHO and $NaCNBH_3$ as reported by us earlier.[9] It was observed that carrying out the methylations in acetonitrile using sufficient concentrated HCl to effect solution resulted in high isolated yields of the pure N9 methylated products.[9] The position of the alkylation was confirmed by the ^{1}H NMR in deuterated dimethyl sulfoxide which indicated the absence of the N9 H and the presence of a sharp singlet at around *δ* 2.8 ppm corresponding to the N9 CH_3 group.

For the synthesis of the classical analogues **29** and **30**, 4-carboxybenzaldehyde was coupled with diethyl L-glutamate in anhydrous pyridine using diethylcarbodiimide by the method of Hynes and Garrett[31] to afford (4-formylbenzoyl)-L-glutamate, **31** (Scheme 2). Compound **7** was reduced to **8** using Raney Ni and then reductively aminated with **31**, as described above for the nonclassical analogues, to afford the diester **32**. N9 methylation of **32** with HCHO, $NaCNBH_3$, and HCl afforded **33** in 72% yield. Both **32** and **33** were saponified with 1 N NaOH to afford the desired classical analogues **29** and **30**.

Scheme 2



Biological Acitivity and Discussion

The nonclassical analogues were evaluated[5,32] as inhibitors of pcDHFR, tgDHFR, and rl DHFR, and the results (IC_{50}) are reported in Table 2. Selectivity ratios (IC_{50} rlDHFR/IC_{50} pcDHFR and IC_{50} rlDHFR/IC_{50} tgDHFR) determined using rlDHFR as the mammalian source are also reported in Table 2. The classical analogues along with selected nonclassical analogues were also evaluated against ecDHFR, lcDHFR, sfDHFR, and hDHFR[33] as well as recombinant tgDHFR under slightly different assay conditions, and the results are reported in Table 3. In the nonclassical analogues evaluated, N9 methylation consistently resulted in a substantial increase in inhibition of pcDHFR, tgDHFR, rlDHFR, and hDHFR. In some cases, N9 methylation also provided for increased selectivity against pcDHFR and tgDHFR compared to the N9 H analogues. As indicated in Table 2, comparison of the unsubstituted phenyl analogues **9** and **10** indicates that N9 methylation increases potency 15−40-fold against all three DHFRs and also increases selectivity for both pcDHFR and tgDHFR (vs rlDHFR). This increase on N9 methylation in both potency and selectivity is significantly greater for pcDHFR than tgDHFR. For the N9 H trimethoxy-substituted analogues (**11**, **14**, **16**, and **17**), the effect was predicated on the position of the methoxy groups. For the 3′,4′,5′- and 2′,3′,4′-trimethoxy analogues **11** and **14**, potency decreased compared to **9**, the phenyl-unsubstituted analogue, for all DHFRs except tgDHFR. Selectivity for pcDHFR in all of the trimethoxy N9 H analogues was less than for **9**. However for tgDHFR, the selectivity increases with trimethoxy substitution (except for **17**) with compound **11** being the

Table 2. Inhibition Concentrations (IC_{50}, in nM) of Dihydrofolate Reductases from *P. carinii*, *T. gondii*, and Rat Liver and Selectivity Ratios[a]

no.	pcDHFR	tgDHFR	rlDHFR	rl/pc	rl/tg
9	2700	520	2100	0.8	4.0
10	68	32	140	2.1	4.4
11	14100	350	3300	0.23	9.4
12	61	14	33	0.5	2.4
13	9200	194	1270	0.14	6.5
14	15300	670	3240	0.2	4.8
15	79	26	30	0.4	1.2
16	20700	230	1200	0.06	5.2
17	5500	480	1100	0.2	2.3
18	4800	730	1500	0.3	2.1
19	5700	1200	3400	0.6	2.8
20	76	31	72	0.9	2.3
21	3800	310	350	0.09	1.1
22	84	6.3	57	0.7	9.0
23	3900	980	240	0.06	0.2
24	8200	380	430	0.05	1.1
25	15400	710	370	0.02	0.5
26	24300	3700	2900	0.12	0.8
27	1940	4450	280	0.14	0.06
28	1800	920	1400	0.8	1.5
TMP	12000	2700	133000	11.1	49
TMQ	42	10	3.0	0.07	0.3
epir.	2600	470	73000	28.1	155
MTX	1.3	14	2.5	1.9	0.2

[a] These assays were carried out at 37 °C under conditions of substrate (90 μM dihydrofolic acid) and cofactor (119 μM NADPH) in the presence of 150 mM KCl.[5,32]

Table 3. Inhibition Concentrations (IC_{50}, in nM) and Selectivity Ratios of Selected Analogues against DHFRs from Various Sources[a]

no.	hDHFR	tgDHFR	h/tg	ecDHFR	h/ec	sfDHFR	h/sf	lcDHFR	h/lc
9	6200	nd	nd	nd	nd	nd	nd	nd	nd
10	700	35	20	nd	nd	nd	nd	nd	nd
11	76000	1300	**192**	760	329	51	4902	15000	17
15	530	26	20.4	nd	nd	nd	nd	nd	nd
19	160000	nd	nd	1400	114.2	86	1860	17000	9.4
20	2900	29	**100**	nd	nd	nd	nd	nd	nd
21	13000	500	26	130	100	15	867	5100	2.5
22	8500	28	**304**	140	60.7	28	304	5700	1.5
29	320	nd	nd	13	24.6	10	32	67	4.8
30	650	nd	nd	13	50	2.2	295	170	3.8
MTX	38	22	1.4	2.2	17	2.2	17	55	0.7

[a] Methotrexate was purchased from Sigma, St. Louis, MO. Recombinant hDHFR was provided by Dr. J. H. Freisheim. Recombinant tgDHFR was provided by Dr. D. V. Santi. Recombinant ecDHFR was provided by Dr. R. L. Blakley. DHFR assay conditions:[33] all enzymes were assayed spectrophotometrically in a solution containing 50 μM dihydrofolate, 80 μM NADPH, 0.05 M Tris HCl, 0.001 M 2-mercaptoethanol, and 0.001 M EDTA at pH 7.4 and 30 °C. The reaction was initiated with an amount of enzyme yielding a change in o.d. at 340 nM of 0.015/min.

most selective tgDHFR inhibitor in the entire series. N9 methylation of **11** and **14** to afford **12** and **15**, respectively, significantly increased potency from 25- to 231-fold and from 25- to 194-fold, respectively, against all three DHFRs and was similar to that observed for **9** and **10**. Thus, while the 2′,4′,5′-trimethoxy analogue **17** was the most potent of the N9 H trimethoxy series against pcDHFR, the 2′,4′,6′-trimethoxy-substituted analogue **16** was the most potent against tgDHFR. The 3′,4′,5′-trimethoxy analogue **11** on the other hand was the most selective against tgDHFR. It was 31 times more selective than TMQ against tgDHFR and was the most tgDHFR selective compound (vs rlDHFR). N9 methylation of **11** (analogue **12**) resulted in a 230-fold increase in potency against pcDHFR and a 25-fold increase against tgDHFR inhibition. However the selectivity ratio for tgDHFR decreased 4-fold. These differential inhibitory effects of N9 methylation on pcDHFR and tgDHFR underscore the fact that the active site of pcDHFR and tgDHFR are different and that separate structure−activity/selectivity relationships need to be developed for each enzyme. C10 methylation of **11** to afford **13** provided unexpected results. This analogue though more potent than the nonmethylated analogue **11** is still a poor inhibitor of pcDHFR, tgDHFR, and rlDHFR with IC_{50}s in the micromolar range. This was surprising since in the C9−N10 series previously reported by us,[15] methylation of N10 afforded an extremely potent inhibitor of pcDHFR, tgDHFR, and rlDHFR, with IC_{50}s in the 10^{-8} M range. The low inhibitory activity of **13** could be attributed, in part, to the intolerance of the enzyme(s) to the N9 H as a H-bond donor or a hydrophilic substitution at the 9-position and is reflected in the low activity of all the N9 H analogues. Further studies are currently underway in our laboratory to determine the cause of this apparent anomaly in the SAR. The N9 methyl 2′,3′,4′-trimethoxy analogue **15** displayed increased activity against all the three DHFRs to a similar extent as did **12**. Thus, **15** was 195-fold and 25-fold more active than the N9 H analogue **14** against pcDHFR and tgDHFR, respectively. As a result, the pcDHFR selectivity for **15** increased 2-fold, and decreased 4-fold for tgDHFR.

In the dimethoxy-substituted N9 H analogues **18**, **19** and **21**, the compounds have decreased potency compared to the phenyl unsubstituted analogue **9** against all three DHFRs (except **21** for rlDHFR). Moving the 2′-methoxy group of **21** to the 3′-position as in **19**, results in a 10-fold decrease in inhibition of rlDHFR. N9 methylation in the dimethoxy series to give compounds **20** and **22** allows for a significant increase in potency for all three DHFRs. N9 methylation of **19** results in a 75-fold increase in pcDHFR inhibition and a 47-fold increase in tgDHFR inhibition. Selectivity for pcDHFR is somewhat increased for **20** with a slight decrease in selectivity for tgDHFR. The 2′,5′-dimethoxy substitution pattern, as we have previously reported for the 5-CH_3, C9−N10 analogues,[9] afforded the highest potency against all three DHFRs. N9 methylation of **21** to afford the N9 methyl 2′,5′-dimethoxy analogue **22** resulted in the most potent inhibitor of tgDFHR of all the analogues tested. Compound **22** is 429 times more potent than TMP against tgDHFR. It is also more potent than TMQ against tgDHFR and is 30-fold more selective. Analogue **22** afforded a 10-fold increase in selectivity relative to TMQ against both pcDHFR and tgDHFR. With its high potency of 6.3 nM (better than TMQ) against tgDHFR along with the second highest selectivity against tgDHFR of the nonclassical series, **22** was an excellent lead analogue. Its potency and selectivity against pcDHFR was noteworthy and ranked it among the most potent and selective analogues of the nonclassical series. The significantly different effects of N9 methylation on pcDHFR and tgDHFR inhibition observed for the trimethoxy analogues was absent in the dimethoxy analogues. Thus, deletion of a methoxy group in the trimethoxy series provides for an increase in potency and selectivity against both pcDHFR and tgDHFR.

Analogues **23**−**25** were designed to evaluate the role of a naphthalene moiety on the side chain in place of a phenyl ring. Piper *et al.*[34] have shown that the active site of human DHFR is large enough to accommodate such a substitution. Further, we[9] have shown that naphthalene substitution provides analogues which have excellent cell penetration, which is an important attribute for clinical viablility. Comparison of the activities of **23** and **9** indicate that the additional hydrophobic contacts provided by the naphthalene ring are conducive to rlDHFR inhibition, but are detrimental to pcDHFR and tgDHFR inhibition. Compound **23** is 10 times more potent than **9** against rlDHFR and is the most potent of all the N9 H analogues against rlDHFR. Incorporation of a methoxy group at either the *ortho* or the *para* position of the naphthalene ring results in analogues with decreased affinity for pcDHFR and rlDHFR and a slight increase in tgDHFR inhibition. Substitution of a phenoxy group at the *para* position of the phenyl ring of compound **9** affords **26** which is an

extremely poor inhibitor of all three DHFRs reflecting a lack of bulk tolerance at the 4′-position.

Insertion of an extra methylene between the N9 and the side chain phenyl ring affords the three-atom-bridged analogue **27**. A similar bridge extension has been reported by us in the tetrahydroquinazoline series.[19] This analogue **27** is more potent than **9** against pcDHFR and rlDHFR but is 10-fold less active against tgDHFR. Interestingly N9 methylation of **27** to afford **28** results in a significant increase in potency against tgDHFR and a slight increase in inhibition of pcDHFR, but the rlDHFR inhibition is decreased 5-fold.

From Table 2, the most important criteria for high activity against pcDHFR and tgDHFR was N9 methylation as seen in compounds **10**, **12**, **15**, **20**, and **22**. The substitution on the side chain phenyl ring or their positions were relatively unimportant except for **12** and **22** with respect to tgDHFR. Compound **12** was also the most potent inhibitor of pcDHFR. Thus the 3′,4′,5′-trimethoxy analogue **12** and the 2′,5′-dimethoxy analogue **22** provided the most potent analogues of the series. In addition compounds **12** and **22** were the most potent analogues against tgDHFR, and **11** and **22** were the most selective. Thus in the dimethoxy-substituted compounds, N9 methylation was important for both pcDHFR and tgDHFR selectivity.

The nonclassical analogues **9**−**11**, **15**, and **19**−**22** were also evaluated against ecDHFR, lcDHFR, sfDHFR, recombinant tgDHFR, and recombinant hDHFR, and the results are listed in Table 3. These results were obtained under different assay conditions than that reported in Table 2. The tgDHFR inhibitory data in Tables 2 and 3 are remarkably similar despite different assay conditions. However, a significant difference in the inhibition of rlDHFR compared to hDHFR was observed. As a result, the selectivity ratios of these analogues for tgDHFR vs hDHFR were quite different and are also reported in Table 3. The corresponding selectivity ratios for MTX are also listed for comparison and are different for hDHFR compared to rlDHFR in Table 2. Thus **20**, which has a selectivity ratio of 2.3 (vs rlDHFR) for tgDHFR (Table 2), displays a 100-fold selectivity for tgDHFR compared to hDHFR (Table 3). Similarly, compound **11** which has a selectivity ratio of 9.4 (vs rlDHFR) for tgDHFR displays a 192-fold selectivity for tgDHFR compared to hDHFR. This analogue also displayed more than a 4000-fold selectivity for sfDHFR vs hDHFR. Compound **19** was also extremely selective for sfDHFR with a selectivity ratio of 1860. The N9 methyl analogue of **19**, compound **20**, showed an increase in inhibition of hDHFR by 2 orders of magnitude compared to the N9 H analogue and displayed excellent tgDHFR selectivity, compared to hDHFR, of 100.

The N9 methyl 2′,5′-dimethoxy-substituted analogue **22** showed astounding results with respect to potency and selectivity against tgDHFR. Compound **22** had a selectivity ratio of 300 for tgDHFR (Table 3) and is one of the most selective tgDHFR inhibitors reported to date. The high inhibitory potency against tgDHFR of **22** and its outstanding selectivity for tgDHFR suggests that this analogue could be a clinical candidate for the treatment of *T. gondii* infection.

The spectacular selectivity ratios obtained with **11**, **20**, and **22** for tgDHFR; **11**, **19**, and **21** for ecDHFR; and **11**, **19**, **21**, **22**, and **30** for sfDHFR (Table 3) validates

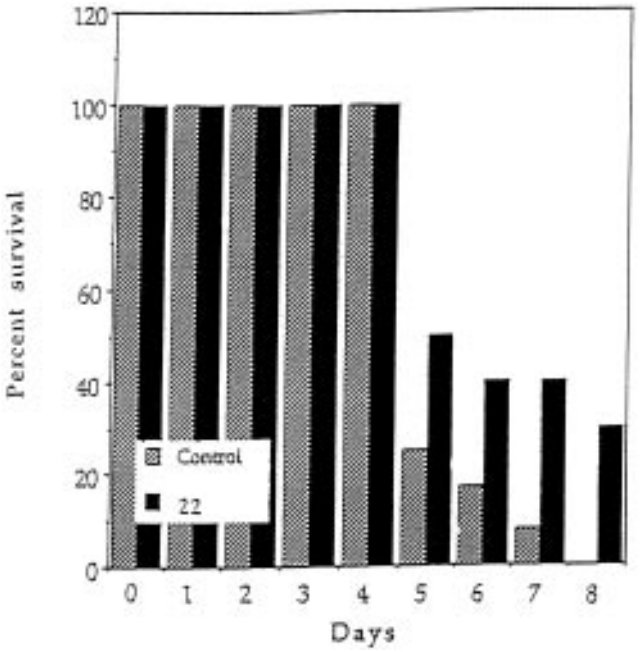


Figure 1. Percent survival of mice inoculated with *T. gondii* trophozoites. Each mouse received 5000 *T. gondii* trophozoites from culture (DCD13); the strain was derived from a clinical isolate from a patient at Indiana University Medical Center. The daily dose of drug was 50 mg/kg administered in food (compounded daily, 1.8 g of powdered rodent chow + 10 mg of drug + 3.2 g of peanut butter). There were 10 mice in this group. The control group consisted of 12 mice which received no drug. The amounts of drug were reduced proportionately as the mice died.

our initial premise that selective inhibitors of a variety of DHFRs can be generated by removal of the 5-methyl moiety and that this results in significantly greater decrease in potency against hDHFR, which translates into remarkably selective analogues against tgDHFR, ecDHFR, and sfDHFR.

Compound **22** on the basis of its potency and selectivity was identified for further development. A pilot study against *T. gondii* infection in the mouse model was carried out. The data for eight days is shown in Figure 1. The data indicates that after 7 days only one mouse survived in the control group (8.3% survival), while four mice survived in the drug group (40% survival), and after 8 days, all the mice in the control group had died and three mice were alive in the drug group, providing for a 30% survival. This data strongly indicates that **22** is active orally and allows a prolongation of life. Further, the arbitrarily chosen dose produced no obvious toxicity.

The classical analogues **29** and **30** were evaluated against lcDHFR, ecDHFR, sfDHFR, and hDHFR. These results are reported in Table 3. Both the N9 H and the N9 methyl analogues were moderate inhibitors of hDHFR with IC_{50}s in the 10^{-7} M range. N9 methylation decreased activity against hDHFR and lcDHFR, while there was no change against ecDHFR inhibition. Against sfDHFR, **30** was as potent as MTX.

Selected analogues were also evaluated as antitumor agents in the National Cancer Institute preclinical *in vitro* screening program.[35] The improved DHFR inhibition observed on N9 methylation was corroborated by the inhibitory activity observed against the growth of nonsmall lung cancer cell lines, colon cancer cell lines, melanoma cell lines, and breast cancer cell lines. Thus, while **11** was an extremely poor inhibitor of all the cell

lines evaluated with GI_{50}s > 10^{-4} M, the N9 methyl congener displayed GI_{50}s in the 10^{-6}−10^{-7} M range against several of the cell lines. Similarly, a 1−2 orders of increase in the inhibition of tumor growth was observed for **22** compared to **21**. The higher rlDHFR inhibition observed with **23**, the naphthyl analogue, was corroborated by its tumor growth inhibition (GI_{50}s in the 10^{-6}−10^{-7} M range), which was much better than any of the other N9 H analogues tested.

Remarkably potent and selective inhibitors of various DHFRs compared to hDHFR have been achieved by the removal of the 5-methyl moiety and the reversal of the C9−N10 bridge of nonclassical 6-substituted 2,4-diaminopyrido[2,3-*d*]pyrimidine antifolates. Whether the selectivities obtained are solely attributable to the removal of the 5-methyl moiety, the reversal of the C9−N10 bridge, or both factors will be the subject of future reports. Preliminary animal studies indicate that at 50 mg/kg orally, compound **22** is active in the prolongation of life in mice infected with *T. gondii* without toxicity. Analogue **22** with its impressive potency and selectivity against tgDHFR is currently undergoing further studies under the auspices of The National Institute of Allergy and Infectious Diseases, Division of AIDS. In addition, the crystal structures of this analogue with pcDHFR and hDHFR is in progress and will be the subject of future communications.

Experimental Section

Melting points were determined on a Mel-Temp II melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Perkin-Elmer Model 1430, in Nujol mulls. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz) with internal standard TMS; s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Low-resolution mass spectra were obtained on an LKB-9000 instrument. Thin layer chromatography was performed on silica gel plates with fluorescent indicator and were visualized with light at 254 and 366 nm. Column chromatography was performed with 230−400 mesh silica gel purchased from Aldrich Chemical Company, Milwaukee, WI. All anhydrous solvents were purchased from Aldrich Chemical Co. and were used without further purification. Samples for microanalysis were dried *in vacuo* over phosphorus pentoxide at 70 °C or 110 °C. Microanalyses were performed by Atlantic Microlabs, Norcoss, GA, and are within ±0.4% of the theoretical value.

2,4-Diamino-6-nitropyrido[2,3-*d*]pyrimidine, **7.** To a suspension of 2,4,6-triaminopyrimidine (2.00 g, 0.02 mol) in refluxing ethanol was added concentrated HCl dropwise to effect solution. To this solution was added the sodium salt of nitromalonaldehyde (2.24 g, 0.02 mol) all at once, and the reaction mixture was stirred at reflux under nitrogen. Within 15 min, a thick red precipitate started to form. After 1 h, TLC analysis indicated trace amounts of starting material and a major spot for the product. The suspension was cooled, basified to a pH of 8, and filtered. The precipitate was washed with 100 mL of cold water to remove unreacted triaminopyrimidine and the precipitate dried to yield 2.51 g of pure **7** as a red solid (76%). A small amount was crystallized from water to yield analytically pure compound: mp >370 °C; ^{1}H NMR (Me_2SO-d_6) δ 7.25 (br s, 2H, 2-NH_2), 8.23 (br s, 2H, 4-NH_2), 9.32 (m, 2H, H-5 and H-7). Anal. Calcd for ($C_7H_6N_6O_2\cdot H_2O$) C, H, N.

General Procedure for the Synthesis of Compounds 9, 11, 14, 16−19, 21, and 23−27. To a solution of **7** in 50 mL of warm (40 °C) *N*,*N*-dimethylformamide (DMF) was added Raney Ni. The mixture was hydrogenated in a Parr shaker at 35 psi for 3 h. TLC analysis ($CHCl_3$:MeOH, 5:2) indicated the presence of a blue fluorescent spot (R_f = 0.27). At the end of this period, the appropriate aldehyde (substituted or unsubstituted benzaldehyde, substitued or unsubstituted naphthaldehyde, or phenylacetaldehyde) was added followed by 15 mL of acetic acid, and the mixture was hydrogenated for a further 3.5 h. The reaction mixture was treated with Norit and filtered through a Celite mat. Silical gel (1.0 g) was added to the filtrate which was then evaporated to dryness to afford a dry plug. This plug was chromatographed on a 1.05 in. × 23 in. column using $CHCl_3$:MeOH (10:1) as the eluant. Fractions containing the pure product, which showed an intense green fluorescence, were pooled and evaporated to yield a powder.

2,4-Diamino-6-(*N*-benzylamino)pyrido[2,3-*d*]pyrimidine, 9. Compound **9** was synthesized from **7** (0.49 g, 2.42 mmol), Raney Ni (3.0 g) and benzaldehyde (0.26 g, 2.42 mmol) to afford 0.39 g (61%) of **9**: mp 267 °C; ^{1}H NMR (Me_2SO-d_6) δ 4.28 (d, 2 H, CH_2), 6.09 (br s, 2 H, 2-NH_2), 6.29 (t, 1 H, NH), 7.23 (m, 1 H, 4′-H), 7.33 (m, 2 H, 3′-H and 5′-H), 7.39 (m, 2 H, 2′-H and 6′-H), 7.44 (d, 1 H, H-5), 8.33 (d, 1 H, H-7). Anal. Calcd for ($C_{14}H_{14}N_6\cdot H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(3′,4′,5′-trimethoxybenzyl)amino]pyrido[2,3-*d*]pyrimidine, 11. Compound **11** was synthesized from **7** (0.50 g, 2.43 mmol), Raney Ni (3.0 g), and 3,4,5-trimethoxybenzaldehyde (0.48 g, 2.43 mmol) to afford 0.54 g (62%) of **11**: mp 250 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.62 (s, 3 H, 4′-OCH_3), 3.76 (s, 6 H, 3′-OCH_3 and 5′-OCH_3), 4.20 (d, 2 H, CH_2), 6.31 (br s, 3H, 2-NH_2 and NH), 6.77 (s, 2 H, 2′H and 6′H), 7.50 (d, 1 H, 5-H), 7.63 (s, 2H, 4-NH_2), 8.32 (d, 1 H, 7-H). Anal. Calcd for ($C_{17}H_{20}N_6O_3\cdot 0.5H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(2′,3′,4′-trimethoxybenzyl)amino]pyrido[2,3-*d*]pyrimidine, 14. Compound **14** was synthesized from **7** (0.49 g, 2.42 mmol), Raney Ni (3.0 g), and 2,3,4-trimethoxybenzaldehyde (0.48 g, 2.42 mmol) to afford 0.52 g (61%) of **14**: mp 234 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.76 (s, 3 H, 2′-OCH_3), 3.77 (s, 3 H, 4′-OCH_3), 3.83 (s, 3 H, 3′-OCH_3), 4.18 (d, 2 H, CH_2), 5.933 (t, 1 H, NH), 6.02 (br s, 2H, 2-NH_2), 6.75 (d, 1 H, 5′-H), 7.05 (d, 1 H, 6′-H), 7.44 (m, 3 H, 4-NH_2 and 5-H), 8.34 (d, 1 H, 7-H). Anal. Calcd for ($C_{17}H_{20}N_6O_3\cdot H_2O$) C, H, N.

2,4-Diamino-6-[[*N*-(2′,4′,6′-trimethoxybenzyl)amino]methyl]pyrido[2,3-*d*]pyrimidine, 16. Compound **16** was synthesized from **7** (0.75 g, 3.64 mmol), Raney Ni (4.00 g), and 2,4,6-trimethoxybenzaldehyde (0.71 g, 3.64 mmol) to afford 0.82 g (63%) of **16**: mp 213 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.78 (s, 3 H, 4′-OCH_3), 3.79 (s, 6 H, 2′-OCH_3 and 6′-OCH_3), 4.04 (d, 2 H, CH_2), 5.39 (br s, 1H, NH), 6.12 (br s, 2 H, 2-NH_2), 6.27 (s, 2 H, 3′-H and 5′-H), 7.46 (br s, 3 H, 4-NH_2 and 5-H), 8.30 (d, 1 H, 7-H). Anal. Calcd for ($C_{17}H_{20}N_6O_3\cdot 0.5H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(2′,4′,5′-trimethoxybenzyl)amino]pyrido[2,3-*d*]pyrimidine, 17. Compound **17** was synthesized from **7** (0.45 g, 2.18 mmol), Raney Ni (3.0 g), and 2,4,5-trimethoxybenzaldehyde (0.43 g, 2.18 mmol) to afford 0.50 g (65%) of **17**: mp 235 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.66 (s, 3 H, 2′-OCH_3), 3.78 (s, 3 H, 4′-OCH_3), 3.81 (s, 3 H, 5′-OCH_3), 4.17 (d, 2 H, CH2), 6.00 (t, 1 H, NH), 6.49 (br s, 2 H, 2-NH_2), 6.71 (s, 1 H, 3′-H), 7.03 (s, 1 H, 6′-H), 7.55 (d, 1 H, 5-H), 7.79 (s, 2 H, 4-NH_2), 8.34 (d, 1 H, 7-H). Anal. Calcd for ($C_{17}H_{20}N_6O_3\cdot 0.75H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(3′,4′-dimethoxybenzyl)amino]pyrido-[2,3-*d*]pyrimidine, 18. Compound **18** was obtained from **7** (0.50 g, 2.43 mmol), Raney Ni (3.0 g), and 3,4-dimethoxybenzaldehyde (0.40 g, 0.50 mmol) to afford 0.46 g (58%) of **18**: mp 257 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.72 (s, 3 H, 4′-OCH_3), 3.75 (s, 3 H, 3′-OCH_3), 4.20 (d, 2 H, CH_2), 6.36 (br s, 3 H, 2 NH_2 and H), 6.91 (m, 2 H, 5′-H and 6′-H), 7.05 (s, 1 H, 2′-H), 7.51 (d, 1 H, H-5), 7.74 (br s, 2 H, 4-NH_2), 8.32 (d, 1 H, H-7). Anal. Calcd for ($C_{16}H_{18}N_6O_2\cdot H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(3′,5′-dimethoxybenzyl)amino]pyrido-[2,3-*d*]pyrimidine, 19. Compound **19** was obtained from **7** (0.35 g, 1.70 mmol), Raney Ni (1.50 g), and 3, 5-dimethoxybenzaldehyde (0.28 g, 1.70 mmol) to afford 0.33 mg (60%) of **19**: mp 264 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.71 (s, 6 H, 3′,5′-OCH_3), 4.24 (d, 2 H, CH_2), 5.72 (s, 1 H, 4′-H), 5.86 (s, 2H, 2′,6′-H), 6.01 (t, 1 H, NH), 6.14 (br s, 2 H, 2-NH_2), 7.14 (s, 2 H, 4-NH_2), 7.91 (d, 1 H, H-5), 8.31 (d, 1 H, H-7). Anal. Calcd for ($C_{16}H_{18}N_6O_2\cdot 0.5H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(2′,5′-dimethoxybenzyl)amino]pyrido-[2,3-*d*]pyrimidine, 21. Compound **21** was obtained from **7** (0.49 g, 2.42 mmol), Raney Ni (3.0 g), and 2,5-dimethoxy-

benzaldehyde (0.40 g, 2.42 mmol) to afford 0.54 g (68%) of **21**: mp 216 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.66 (s, 3 H, 2′-OCH_3), 3.71 (s, 3 H, 5′-OCH_3), 4.22 (d, 2 H, CH_2), 5.97 (br s, 3H, 2-NH_2 and NH), 6.79 (m, 1H, 6′-H), 6.92 (m, 2H, 3′-H and 4′-H), 7.32 (s, 2 H, 4-NH_2), 7.43 (d, 1 H, 5-H), 8.35 (d, 1 H, 7-H). Anal. Calcd for ($C_{16}H_{18}N_6O_2$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-(1′-naphthylmethylene)amino]pyrido-[2,3-*d*]pyrimidine, 23. Compound **23** was synthesized from **7** (0.25 g, 1.21 mmol), Raney Ni (3.0 g), and 1-naphthaldehyde (0.19 g, 1.21 mmol) to afford 0.23 g (61%) of **23**: mp 259 °C; ^{1}H NMR (Me_2SO-d_6) δ 4.61 (d, 2 H, CH_2), 6.11 (t, 1 H, NH), 6.71 (br s, 2 H, 2-NH_2), 7.41 (m, 2 H, 2′-H and 3′-H), 7.56 (m, 2 H, 4′-H and 5′-H), 7.75 (d, 1 H, H-5), 7.90 (m, 5-H, 6′-H, 7′-H, 8′-H and 4-NH_2), 8.33 (d, 1 H, H-7). Anal. Calcd for ($C_{18}H_{16}N_6$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-[(4′-methoxy-1′-naphthyl)methylene]-amino]pyrido[2,3-*d*]pyrimidine, 24. Compound **24** was obtained from **7** (0.49 g, 2.42 mmol), Raney Ni (3.0 g), and 4-methoxy-1-naphthaldehyde (0.45 g, 2.42 mmol) to afford 0.49 g (59%) of **24**: mp 297 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.91 (s, 3 H, 4′-OCH_3), 4.61 (d, 2 H, CH_2), 6.11 (t, 1 H, NH), 6.60 (br s, 2 H, 2-NH_2), 7.31 (d, 1 H, 3′-H), 7.37 (d, 1 H, 2′-H), 7.56 (m, 1 H, 5′-H), 7.79 (d, 1 H, H-5), 7.96 (m, 5 H, 6′, 7′, 8′ H and 4-NH_2), 8.37 (d, 1 H, H-7). Anal. Calcd for ($C_{19}H_{18}N_6O$·$0.5H_2O$) C, H, N.

2,4-Diamino-6-[*N*-[(2′-methoxy-1′-naphthyl)methylene]-amino]pyrido[2,3-*d*]pyrimidine, 25. Compound **25** was obtained from **7** (0.60 g, 2.91 mmol), Raney Ni (3.0 g), and 2-methoxy-1-naphthaldehyde (0.54 g, 2.91 mmol) to afford 0.54 g (54%) of **25**: mp 288 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.98 (s, 3 H, 2′-OCH_3), 4.58 (d, 2 H, CH_2), 6.03 (t, 1 H, NH), 6.65 (br s, 2 H, 2-NH_2), 7.39 (m, 1 H, 3′-H), 7.52 (m, 2 H, 4′ and 5′-H), 7.75 (d, 1 H, H-5), 7.90 (m, 5 H, 6′, 7′, 8′-H and 4-NH_2), 8.33 (d, 1 H, H-7). Anal. Calcd for ($C_{19}H_{18}N_6O$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-(4′-phenoxybenzyl)amino]pyrido[2,3-*d*]pyrimidine, 26. Compound **26** was synthesized from **7** (0.40 g, 1.94 mmol), Raney Ni (3.0 g), and 4-phenoxybenzaldehyde (0.36 g, 1.94 mmol) to afford 0.38 g (55%) of **26**: mp 290 °C; ^{1}H NMR (Me_2SO-d_6) δ 4.26 (d, 2 H, CH_2), 5.87 (br s, 2 H, 2-NH_2), 6.23 (t, 1 H, NH), 6.38 (s, 2 H, 4-NH_2), 6.96−7.4 (m, 5 H), 7.95 (d, 2 H, 2′-H), 8.31 (d, 1 H, H-7), 8.69 (d, 1 H), 8.75 (d, 1 H). Anal. Calcd for ($C_{20}H_{18}N_6O$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-(2-phenylethyl)amino]pyrido[2,3-*d*]-pyrimidine, 27. Compound **27** was synthesized from **7** (0.60 g, 2.91 mmol), Raney Ni (3.0 g), and phenylacetaldehyde (0.35 g, 2.91 mmol) to afford 0.49 g (60%) of **27**: mp 251 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.86 (t, 2 H, CH_2CH_2), 3.24 (t, 2 H, CH_2CH_2), 5.80 (t, 1 H, NH), 5.87 (br s, 2 H, 2-NH_2), 7.32 (m, 5 H, C_6H_5), 7.37 (d, 1 H, H-5), 8.25 (d, 1 H, H-7). Anal. Calcd for ($C_{15}H_{16}N_6$·$0.5H_2O$) C, H, N.

General Procedure for the Synthesis of Compounds 10, 12, 15, 20, 22, and 28. To a suspension of the corresponding N9 H compound in 15 mL of acetonitrile were added HCHO and $NaCNBH_3$, followed by concentrated HCl dropwise to effect solution. TLC analyses after 1 h indicated the presence of a new spot corresponding to the product. The reaction mixture was stirred for 3 h, and the acetonitrile was evaporated. The residue was dissolved in water and brought to pH of 8 with concentrated NH_4OH. The resulting precipitate was filtered and washed with 25 mL of cold water to afford the pure N9 methyl compound.

2,4-Diamino-6-(*N*-benzyl-*N*-methylamino)pyrido[2,3-*d*]-pyrimidine, 10. Compound **10** was synthesized from **9** (0.10 g, 0.38 mmol), $NaCNBH_3$ (0.07 g, 1.12 mmol), and HCHO (0.10 mL) to afford 0.07 g (72%) of **10**: mp 255 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.97 (s, 3 H, N9 CH_3), 4.31 (s, 2 H, CH_2), 6.17 (br s, 2-H, 2-NH_2), 7.32 (m, 1 H, 4′-H), 7.36 (m, 2 H, 3′-H and 5′-H), 7.43 (m, 2 H, 2′-H and 6′-H), 7.59 (d, 1 H, H-5), 8.41 (d, 1 H, H-7). Anal. Calcd for ($C_{15}H_{16}N_6$·$0.5H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(3′,4′,5′-trimethoxybenzyl)-*N*-methylamino]pyrido[2,3-*d*]pyrimidine, 12. Compound **12** was synthesized from **11** (0.10 g, 0.28 mmol), HCHO (0.10 mL), and $NaCNBH_3$ (0.05 g, 0.84 mmol) to afford 0.08 g (75%): mp 279 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.03 (s, 3 H, N9 CH_3), 3.62 (s, 3 H, 4′-OCH_3), 3.71 (s, 6 H, 3′OCH_3 and 5′-OCH_3), 4.55 (s, 2 H, CH_2), 6.57 (s, 2 H, 2′H and 6′H), 6.88 (br s, 2 H, 2-NH_2), 7.82 (d, 1 H, 5-H), 8.25 (s, 2 H, 4-NH_2), 8.46 (d, 1 H, 7-H). Anal. Calcd for ($C_{18}H_{22}N_6O_3$·$0.5H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(2′,3′,4′-trimethoxybenzyl)-*N*-methylamino]pyrido[2,3-*d*]pyrimidine, 15. Compound **15** was obtained from **14** (0.09 g, 0.25 mmol), HCHO (0.10 mL), and $NaCNBH_3$ (0.05 g, 0.76 mmol) to afford 0.07 g (80%) of **15**: mp 236 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.81 (s, 3 H, CH_3), 3.75 (s, 3 H, 2′-OCH_3), 3.77 (s, 3 H, 4′-OCH_3), 3.81 (s, 3 H, 3′-OCH_3), 4.21 (s, 2 H, CH_2), 6.11 (br s, 3 H, 2 NH_2 and NH), 6.77 (d, 1 H, H-5), 7.11 (d, 1 H, 6′-H), 7.53 (m, 3 H, 4-NH_2 and H-5), 8.36 (d, 1 H, H-7). Anal. Calcd for ($C_{18}H_{22}N_6O_3$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-(3′,5′-dimethoxybenzyl)-*N*-methylamino]pyrido[2,3-*d*]pyrimidine, 20. Compound **20** was obtained from **19** (0.12 g, 0.37 mmol), $NaCNBH_3$ (0.06 g, 0.92 mmol), and HCHO (0.12 mL) to afford 0.09 g (79%) of **20**: mp 259 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.94 (s, 3 H, N9-CH_3), 3.71 (s, 6 H, 3′,5′-OCH_3), 4.26 (s, 2 H, CH_2), 5.75 (s, 1 H, 4′-H), 5.84 (s, 2 H, 2′,6′-H), 6.19 (br s, 2 H, 2-NH_2), 7.24 (s, 2 H, 4-NH_2), 7.95 (d, 1 H, H-5), 8.37 (d, 1 H, H-7). Anal. Calcd for ($C_{17}H_{20}N_6O_2$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-(2′,5′-dimethoxybenzyl)-*N*-methylamino]pyrido[2,3-*d*]pyrimidine, 22. Compound **22** was obtained from **21** (0.08 g, 0.25 mmol), $NaCNBH_3$ (0.05 g, 0.74 mmol), and HCHO (0.08 mL) to afford 0.06 g (69%) of **22**: mp 240 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.04 (s, 3 H, N9 CH_3), 4.60 (s, 2 H, CH_2), 6.54 (d, 1 H, 3′-H), 6.79 (d, 1 H, 4′-H), 6.96 (m, 3 H, 2-NH_2 and 6′-H), 7.30 (br s, 2 H, 4-NH_2), 7.87 (d, 1 H, H-5), 8.36 (d, 1 H, H-7). Anal. Calcd for ($C_{17}H_{20}N_6O_2$·$0.75H_2O$) C, H, N.

2,4-Diamino-6-[*N*-(2-phenylethyl)-*N*-methylamino]-pyrido[2,3-*d*]pyrimidine, 28. Compound **28** was synthesized from **27** (0.20 g, 0.71 mmol), $NaCNBH_3$ (0.14 g, 2.14 mmol), and HCHO (0.20 mL) to afford 0.14 g (66%) of **28**: mp 258 °C; ^{1}H NMR (Me_2SO-d_6) δ 2.86 (t, 2 H, CH_2CH_2), 2.92 (s, 3 H, CH_3), 3.21 (t, 2 H, CH_2CH_2), 5.89 (br s, 2 H, 2-NH_2), 7.39 (m, 5 H, C_6H_5), 7.47 (d, 1 H, H-5), 8.01 (br s, 2 H, 4-NH_2), 8.33 (d, 1 H, H-7). Anal. Calcd for ($C_{16}H_{18}N_6$·H_2O) C, H, N.

2,4-Diamino-6-[*N*-[(3′,4′,5′-trimethoxybenzyl)methyl]-amino]pyrido[2,3-*d*]pyrimidine, 13. Compound **7** (0.45 g, 2.18 mmol) was dissolved in 40 mL of warm DMF, Raney Ni (2.5 g) added, and the mixture hydrogenated for 4 h. The reaction mixture was filtered through a Celite mat and the DMF evaporated under reduced pressure (40 °C) to yield a brown solid. This solid was dissolved in 15 mL of methanol, and 3,4,5-trimethoxyacetophenone (0.47 g, 2.18 mmol) was added all at once. Following this, 2−3 mL of acetic acid was added followed by BH_3·Et_3N (0.48 g, 6.54 mmol). After 2 h, another 2−3 mL of acetic acid was added and the reaction mixture stirred overnight at room temperature. Water (15 mL) was then added, and all the solvents were evaporated. The residue was dissolved in methanol (30 mL), 1.0 g of silica gel added, and the methanol evaporated to yield a dry plug. This plug was eluted with $CHCl_3$:MeOH (5:1), and fractions containing the product were pooled and evaporated to dryness to yield pure **13** as a yellow powder (0.09 g, 12%): mp 283 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.3 (d, 3 H, CH_3), 3.62 (s, 3 H, 4′-OCH_3), 3.71 (s, 6 H, 3′ and 5′-OCH_3), 4.17 (m, 1 H, CH), 6.29 (m, 3 H, 2-NH_2 and NH), 6.61 (s, 2 H, 2′ H and 6′ H), 7.91 (d, 1 H, H-5), 8.12 (br s, 2 H, 4-NH_2), 8.41 (d, 1 H, H-7). Anal. Calcd for ($C_{18}H_{22}N_6O_3$·H_2O) C, H, N.

Diethyl 4-(Formylbenzoyl)-L-glutamate, 31. A solution of 4-carboxybenzaldehyde (3.38 g, 0.02 mol), diethyl L-glutamate ester hydrochloride (5.40 g, 0.02 mol), and DCC (4.64 g, 0.02 mol) in anhydrous pyridine was stirred for 7 days. At the end of this period, the pyridine was evaporated, and the oil was chromatographed on a silica gel column using hexanes:ethyl acetate (10:1) as the eluant. Fractions containing the desired product (TLC) were pooled and evaporated to yield 3.0 g (40%) of a white solid: mp 75 °C (lit.[31] mp 74−76 °C).

5-Deazaisoaminopterin, 29. To a solution of **7** (0.50 g, 2.42 mmol) in warm DMF was added 3.0 g of Raney Ni, and the mixture was hydrogenated for 3 h at 35 psi. At the end of this period, 4-(formylbenzoyl)-L-glutamate diethyl ester **31** (0.81 g, 2.42 mmol) was added followed by 15 mL of acetic acid, and the mixture was hydrogenated for a further 3.5 h. The

reaction mixture was filtered through Celite, and the filtrate was evaporated to dryness. Workup and purification was carried out as described above for **16** to yield the diethyl ester, **32** (0.69 g, 58%).

The diester was dissolved in 5 mL of DMSO and saponified using 10 mL of 1 N NaOH at room temperature over a period of 6 h to afford **29** as a yellow powder (0.47 g, 77%): mp 280 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.93−1.97 (m, 2 H, Glu β-CH_2), 1.99−2.03 (m, 2 H, Glu δ-CH_2), 4.36 (overlapping m, 3 H, CH_2 and Glu α-CH), 6.54 (br m, 3 H, 2-NH_2 and NH), 7.49 (m, 3 H, H-5 and 3′- and 5′-H), 7.67 (br s, 2 H, 4-NH_2), 7.83 (d, 2 H, 2′- and 6′-H), 8.32 (overlapping m, 2 H, H-7 and CONH). Anal. Calcd for ($C_{20}H_{21}N_7O_5 \cdot 1.5H_2O$) C, H, N.

9-Methyl-5-deazaisoaminopterin, 30. To a suspension of **32** (0.5 g, 1 mmole) in 30 mL of acetonitrile was added $NaCNBH_3$ (0.06 g, 3 mmol) and HCHO (0.5 mL) followed by concentrated HCl dropwise to effect solution. The reaction mixture was stirred for 3 h, and the acetonitrile was evaporated. The residue was dissolved in water and brought to pH 8 with concentrated NH_4OH. The resulting precipitate was filtered and washed with cold water to afford pure **33**. Saponification of **33** was carried out in a manner similar to that described for **32** (in the synthesis for **29** above) to yield **30** (0.24 g, 54%): mp 276 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.94−1.98 (m, 2 H, Glu β-CH_2), 1.99−2.03 (m, 2 H, Glu δ-CH_2), 2.89 (s, 3 H, CH_3), 4.41 (overlapping m, 3 H, CH_2 and Glu α-CH), 6.60 (br s, 2 H, 2-NH_2), 7.52 (m, 3 H, H-5 and 3′- and 5′-H), 7.71 (br s, 2 H, 4-NH_2), 7.87 (d, 2 H, 2′- and 6′-H), 8.34 (overlapping m, 2 H, H-7 and CONH). Anal. Calcd for ($C_{21}H_{23}N_7O_5 \cdot 2.0H_2O$) C, H, N.

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