Synthesis of Classical and Nonclassical, Partially Restricted, Linear, Tricyclic 5-Deaza Antifolates[1]

Aleem Gangjee,*[,†] Yibin Zeng,[†] John J. McGuire,[‡] and Roy L. Kisliuk[§]

Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282, Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263, and Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Seven novel 2,4-diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine derivatives **3**−**9** with different benzyl and a benzoyl substitution at the N7 position were designed and synthesized, as classical and nonclassical, partially restricted, linear tricyclic 5-deaza antifolates. The purpose was to investigate the effect of conformational restriction of the C6−C9 (τ_1) and C9−N10 (τ_2) bonds via an ethyl bridge from the N10 to the C7 position of 5-deaza methotrexate (MTX) on the inhibitory potency against dihydrofolate reductase (DHFR) from different sources and on antitumor activity. The synthetic methodology for most of the target compounds was a concise five-step total synthesis to construct the tricyclic nucleus, 2,4-diamino-5-deaza-7*H*-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (**23**), followed by regioselective alkylation of the N7 nitrogen. Biological results indicated that this partial conformational modification for the classical analogue *N*-[4-[(2,4-diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridin-7-yl)methyl]benzoyl]-L-glutamic acid **3** was detrimental to DHFR inhibitory activity as well as to antitumor activity compared to MTX or 5-deaza MTX. However, the classical analogue **3** was a better substrate for folypolyglutamate synthetase (FPGS) than MTX. These results show that a classical 5-deaza folate partially restricted via a bridge between the N10 and C7 positions retains FPGS substrate activity and that the antitumor activity of classical tricyclic analogues such as **3** would be influenced by FPGS levels in tumor systems. Interestingly, the nonclassical analogues **4**−**9** showed moderate to good selectivity against DHFR from pathogenic microbes compared to recombinant human DHFR. These results support the idea that removal of the 5-methyl group of piritrexim along with restriction of τ_1 and τ_2 can translate into selectivity for DHFR from pathogens.

Introduction

Folate metabolism has been an attractive chemotherapeutic target because of its crucial role in the biosynthesis of nucleic acid precursors.[2] Tetrahydrofolate (FH₄), the key component of folate metabolism, serves as a cofactor to carry one-carbon units for the biosynthesis of purines and thymidylate (dTMP). During the synthesis of dTMP, FH₄ is oxidized to 7,8-dihydrofolate (FH₂) and must be converted back to the reduced form by dihydrofolate reductase (DHFR) to continue DNA synthesis.[3] Inhibition of DHFR depletes the FH₄ pool and indirectly inhibits DNA synthesis. Thus, DHFR inhibitors have found clinical utility as antitumor, antibacterial, and antiprotozoan agents.[4,5]

Methotrexate (MTX, Figure 1) is a well-known clinically used DHFR inhibitor. Because of the frequent occurrence of tumor resistance and ineffectiveness against many solid tumors, extensive structural modifications of MTX have been reported to improve its antitumor spectrum of activity and to circumvent tumor resistance.[4−7] One area of structural modification that may afford increased biological activity is conformational restriction of flexible parts of a lead molecule.

Studies of previous conformational restricted analogues of MTX and 5-deaza MTX have concentrated on tethering the bridge N10 to the N5 or C5 position, which afforded angular tricyclic analogues.[8−10] However, none of these modified analogues showed better DHFR inhibitory or antitumor activity than MTX. Linear, conformationally restricted, tricyclic analogues of MTX or its pyrido[2,3-*d*]pyrimidine analogues have not been explored to any significant extent in the literature as potential antifolate or antitumor agents. It was therefore of interest to evaluate the biological effects of conformational restriction of the C6−C9 (τ_1) and C9−N10 (τ_2) bonds of 5-deaza MTX via an ethyl bridge from the N10 to the C7 position. The additional carbon between the tricyclic heterocycle and the phenyl ring was added to allow conformational flexibility of the phenyl ring to adopt an optimal orientation for DHFR inhibitory and antitumor activity. Thus, *N*-[4-[(2,4-diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridin-7-yl)methyl]benzoyl]-L-glutamic acid (**3**), a novel, classical, conformationally restricted, linear, tricyclic 5-deaza analogue of MTX was designed as an antifolate.

Polyglutamylation via folylpolyglutamate synthetase (FPGS) is an important mechanism for trapping classical folates and antifolates within the cell, thus maintaining high intracellular concentrations and in some instances increasing binding affinity for folate-dependent enzymes.[11a,b] However, reduced levels of FPGS in

---

* To whom correspondence should be addressed. Phone: 412 396-6070. Fax: 412 396-5593. E-mail: gangjee@duq.edu.
† Duquesne University.
‡ Roswell Park Cancer Institute.
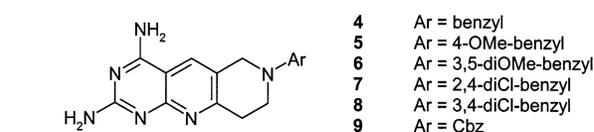§ Tufts University School of Medicine.
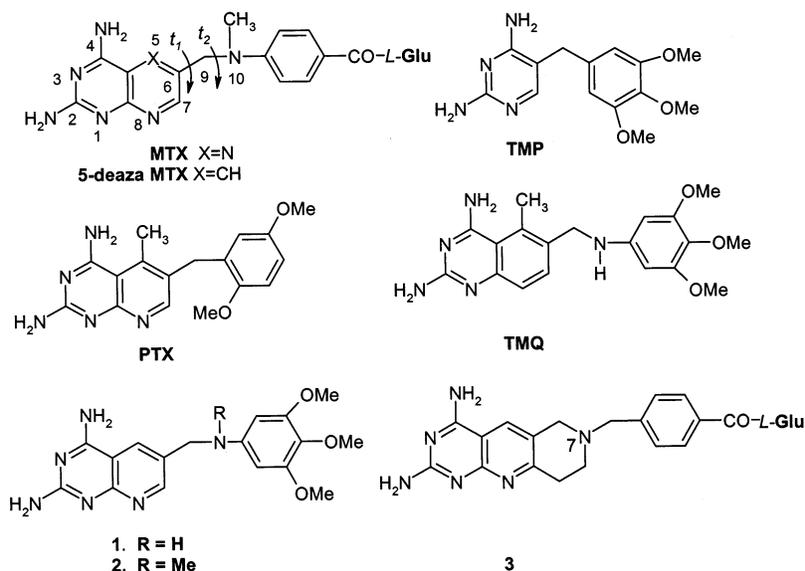
Figure 1.

tumor cells is a well-defined mechanism of resistance to classical antifolates such as MTX, which may be circumvented by antifolates that are not substrates for FPGS.[12,13] Substitutions at the C7 position of deaza folates with small alkyl groups such as methyl have been reported to abolish FPGS substrate activity.[14] Thus, it was anticipated that compound **3**, which is a 7-substituted analogue, would not be an FPGS substrate and might circumvent resistance in tumor cells that arise as a result of decreased FPGS activity.

A disadvantage of classical antifolates as antitumor agents is that they require an active transport mechanism to enter cells, which, when impaired, causes resistance. In addition, cells that lack these transport mechanisms, such as bacterial and protozoan cells, are not susceptible to the action of classical antifolates. In an attempt to overcome these potential drawbacks, nonclassical lipophilic antifolates have been developed as antitumor agents that do not require the folate transport system(s) and enter cells via diffusion.[15]

Several lipophilic DHFR inhibitors such as trimethoprim (TMP), trimetrexate (TMQ), and piritrexim (PTX) have also been used in the treatment of opportunistic infections caused by *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg).[16−19] These infections are the principal cause of death in patients with acquired immunodeficiency syndrome (AIDS). TMP is a weak inhibitor of pcDHFR and tgDHFR, whereas TMQ and PTX are highly potent pcDHFR and tgDHFR inhibitors, with IC$_{50}$ of 20 nM (TMQ against pcDHFR),[17] 19.3 nM (PTX against pcDHFR), and 17.0 nM (PTX against tgDHFR).[16] Unfortunately, both TMQ and PTX inhibit mammalian DHFR to an equal or greater extent. Therefore, TMQ and PTX have potential toxicity when used as single agents to treat opportunistic infections.
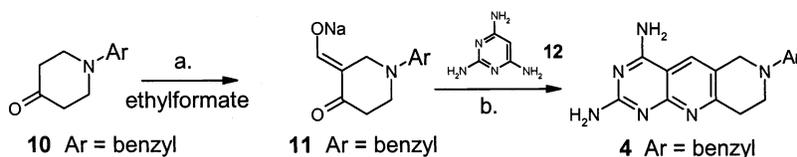
The clinical use of nonclassical antifolates to treat *P. carinii* and *T. gondii* infections capitalizes on the fact that a carrier-mediated active transport system for the uptake of classical folates and antifolates with polar glutamate side chains is present in mammalian cells but is absent in the opportunistic organisms. Lipophilic nonclassical antifolates penetrate these pathogens by passive diffusion.[17] Thus, the toxic effects of nonselec-

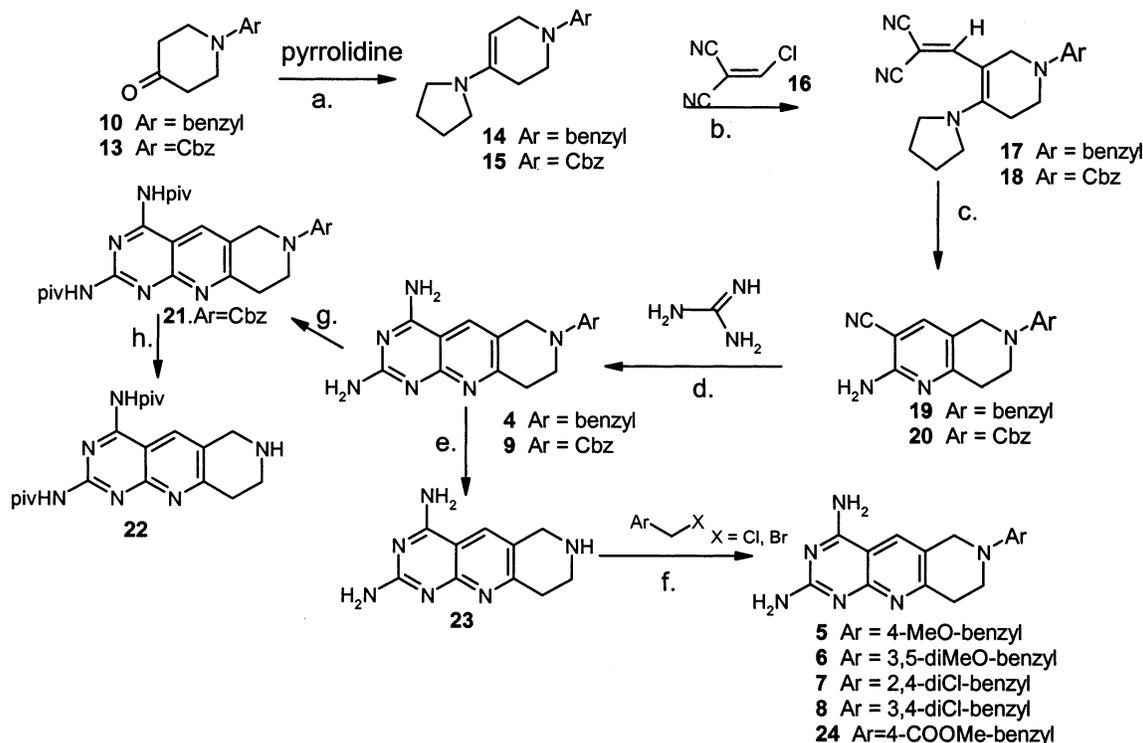| **4** | Ar = benzyl |
5	Ar = 4-OMe-benzyl
6	Ar = 3,5-diOMe-benzyl
7	Ar = 2,4-diCl-benzyl
8	Ar = 3,4-diCl-benzyl
9	Ar = Cbz

Figure 2.

tive, lipophilic antifolates such as TMQ can be selectively circumvented by coadminstration of a reduced folate, typically leucovorin, which is taken up only by host cells and hence bypasses DHFR inhibition and reverses toxicity selectively in host cells. The other advantage of these lipophilic agents is their penetration into the central nervous system where *T. gondii* infections usually occur.[19] The host rescue with leucovorin has disadvantages that include the fact that leucovorin is expensive and the occurrence of severe side effects resulting from ineffective rescue, which sometimes requires cessation of therapy. Thus, the development of selective and potent pcDHFR and tgDHFR inhibitors is of interest.

The rationale for the synthesis of the nonclassical, partially restricted, linear, tricyclic 5-deaza antifolates, compounds **4−9** (Figure 2), was based on a comparison of the X-ray crystallographic structures of human DHFR and pcDHFR complexed with PTX and TMP. Two major differences were reported in the active site.[20] In the upper pocket, the amino acid residue responsible for hydrophobic interaction with the 5-methyl group in PTX is Val115 in hDHFR, while in pcDHFR it is Ile123. Thus, the 5-methyl moiety interacts with both hDHFR as well as pcDHFR. TMQ, which contains a similar 5-methyl moiety, perhaps also interacts with hDHFR and pcDHFR in a similar fashion. Thus, TMQ and PTX display much higher DHFR inhibitory activity (against both hDHFR and pcDHFR) than TMP, which lacks the 5-methyl group. TMP, however, is selective for pcDHFR but is not potent.

Gangjee et al.[21] reported a 5-desmethyl analogue of PTX, **1**, which caused a decrease in potency against both bacterial DHFR and mammalian DHFR. The decrease in potency against mammalian DHFR was somewhat greater and translated into higher selectivity against pcDHFR and tgDHFR.

Scheme 1[a]

10 Ar = benzyl   **11** Ar = benzyl   **4** Ar = benzyl

[a] Reagents and conditions: (a) Na, EtOH reflux; (b) 75% AcOH, reflux, N_2, 5−6 h.

Scheme 2[a]

10 Ar = benzyl
13 Ar = Cbz

14 Ar = benzyl
15 Ar = Cbz

17 Ar = benzyl
18 Ar = Cbz

19 Ar = benzyl
20 Ar = Cbz

4 Ar = benzyl
9 Ar = Cbz

21. Ar = Cbz

22

23

5 Ar = 4-MeO-benzyl
6 Ar = 3,5-diMeO-benzyl
7 Ar = 2,4-diCl-benzyl
8 Ar = 3,4-diCl-benzyl
24 Ar = 4-COOMe-benzyl

[a] Reagents and conditions: (a) benzene/*p*-TSOH, reflux; (b) THF, −20 °C, 2 h; (c) NH_3/MeOH, overnight; (d) DMF, N_2, 100 °C; (e) 30% KOH/MeOH, N_2, reflux; (f) DMF, Et_3N, DMAP, N_2, 60−80 °C, 3−4 h; (g) DMF, piv_2O, Et_3N, DMAP, N_2, 60−80 °C, 1.5 h; (h) Me_3Sil/CH_3Cl.

In addition, small substitutents such as a methyl group when placed at the N10 position, as in compound **2**, caused some conformational restriction to the flexibility of the C9−N10 bridge, thus decreasing the number of accessible low-energy conformations compared with **1**. Such N10 methyl moieties also increase hydrophobic interactions, which could increase the DHFR inhibitory potency while maintaining the selectivity against pcDHFR and tgDHFR.

Like the classical analogue, the nonclassical, partially restricted linear, tricyclic antifolates have also not been explored in the literature as potential antitumor or antibacterial agents. It was thus important to determine the effects of conformational restriction of τ_1 and τ_2 in compounds **1** and **2** with respect to DHFR inhibitory activity. The absence of the 5-methyl could afford selectivity against pcDHFR and/or tgDHFR as observed for **1**, while the C7−N10 ethyl bridge provides conformational restriction of the side chain, which may increase DHFR inhibitory activity and/or selectivity. Thus, nonclassical 7-substituted 2,4-diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine derivatives **4**−**9** with different benzyl and a benzoyl substitution at the N7 position were designed and synthesized. Different conformations of the phenyl ring in TMP have been implicated in its selectivity for bacterial DHFR.[22] Thus,

the additional carbon between the tricyclic heterocycle and the phenyl ring was added to allow conformational flexibility of this part of the molecule to adopt different orientations.

Chemistry

The overall strategy was to synthesize the 2,4-diamino-5-deaza-7*H*-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine tricyclic system **23** (Scheme 2) from which all the target compounds could be obtained by regiospecific N7 alkylation.

It was initially anticipated that the condensation of 2,4,6-triaminopyrimidine **12** (Scheme 1) with a biselectrophile such as 3-hydroxymethylene-1-benzyl-4-piperidone **11** (generated in situ from the 1-benzyl-4-piperidone **10** and ethyl formate in the presence of sodium metal and ethyl ether as previously described[23]) could afford the desired tricyclic nucleus in target compounds such as **4**. This, followed by catalytic hydrogenation, would afford the common intermediate **23** (Scheme 2). However, in model reactions it was found that purification of the annulated product was problematic. Several attempts to modify the reaction conditions resulted in poor yields of compound **4** (≤30%).

An alternative method (Scheme 2) using modifications of procedures reported by DeGraw et al.[24] and Taylor

Scheme 3
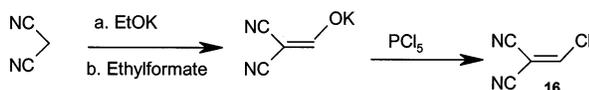
et al.,[25] where a total synthesis of the tricyclic ring system was involved, proved to be successful. Thus, the 4-pyrrolidinoenamines of 1-aryl-4-piperidones were converted to 2-amino-3-cyano-6-(4-aryl)-5,6,7,8-tetrahydropyrido[4,3-*b*]pyridines (**19**, **20**), which proceeded by the sequence of reactions described in Scheme 2. This method involved alkylation of an enamine with (chloromethylene)malononitrile to generate the 5-substituted 2-amino-3-cyano-5,6,7,8-tetrahydropyrido[4,2-*b*]pyridines. In the model reaction, the requisite enamine **15** was prepared from 1-benzyl-4-piperidone (**10**) and pyrrolidine via quantitative removal of water by a Dean–Stark apparatus and evaporation of the solvent.[26] The crude enamine was dissolved in THF and directly alkylated (without purification) with (chloromethylene)-malononitrile in THF at −40 °C followed (without isolation of the intermediate) by treatment with methanolic ammonia to afford 2-amino-3-cyanopyrido[4,3-*b*]-pyridine **19** in 45% overall yield (Ar = unsubstituted benzyl) starting from the piperidone. Annulation to generate the 2,4-diaminopyrimidine ring was then carried out with guanidine free base in DMF at 100 °C under N_2 and afforded **4** in 63% yield. Freshly prepared (chloromethylene)malononitrile **16** was used and was obtained[27] as outlined in Scheme 3 in 40% overall yield.

Attempts at debenzylation[28] of **4**, which included catalytic hydrogenation, catalytic hydrogen transfer, and acid/base, were unsuccessful. Thus, the *N*-Cbz-protected 4-piperidone **13** was used in place of benzyl 4-piperidone. Following the same synthetic sequence, the 2-amino-3-cyanopyrido[4,3-*b*]pyridine **20** was isolated in 75% yield. Subsequent annulation with guanidine afforded **9** in 78% yield.

It was anticipated that protection of the 2,4-diamino groups of **9** prior to the removal of the N7-Cbz protecting group would minimize side product formation in the subsequent alkylation step. Thus, 7-Cbz-2,4-dipivaloyl-amino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine **21** was prepared from **9** in 92% yield using 4 equiv of pivaloyl anhydride in DMF, in the presence of a catalytic amount of DMAP, at 80 °C under N_2. The Cbz protecting group was most effectively removed with trimethylsilane iodide.[29] During the workup process, however, most of the intermediate was transformed to the N7-benzyl byproduct (60%) and the desired intermediate **22** was isolated in only 30% yield. Thus, the direct deprotection of the Cbz group from **9** followed by

regiospecific N7 alkylation became an attractive route. Several different methods were attempted for removal of the Cbz group, which included catalytic hydrogenation, catalytic hydrogen transfer, TMSI, acid, and base.[29–33] The most effective method for deprotection was 30% KOH (aqueous) in methanol. The crucial common intermediate **23** was isolated in 82% yield. Regiospecific alkylation of the N7 nitrogen with the appropriately substituted benzyl halides was carried out under optimized conditions that included 1.2 equiv of the benzyl halide, 1.5 equiv of triethylamine, and DMF in the presence of a catalytic amount of DMAP at 60–80 °C for 1.5 h. The desired compounds **5**–**8** and the precursor to the classical analogue **24** were isolated in 45–63% yield.

Hydrolysis of **24** afforded the corresponding acid **25** (Scheme 4), which was coupled, using a modification of a previous report,[34] to diethyl-L-glutamate followed by saponification[34] to give the target compound **3**.

Biological Evaluation and Discussion

Compounds **3**–**9** were evaluated as inhibitors of recombinant human (rh), *Pneumocystis carinii*, *Toxoplasma gondii*, and *Escherichia coli* (ec) DHFRs.[35–38] The inhibitory potencies (IC_{50}) are listed in Table 1 and compared with MTX, TMP, and TMQ. The classical compound **3** only marginally inhibited rhDHFR. Against pcDHFR and ecDHFR, **3** was also much less potent than MTX. In contrast to MTX, which was equipotent against tgDHFR and rhDHFR, compound **3** was 6-fold more potent against tgDHFR compared to rhDHFR. In the nonclassical series **4**–**9**, except for the ortho-substituted analogue **7**, the other analogues showed some selectivity against pathogenic DHFR compared to rhDHFR. This validates the idea that the removal of the 5-methyl group of PTX and conformational restriction about τ_1 and τ_2 does afford selectivity against DHFR from pathogenic microbes. The most potent and selective tgDHFR inhibitor in this nonclassical series was the unsubstituted benzyl analogue **4** with an IC_{50} of 1.4 μM and a rhDHFR/tgDHFR ratio of 25.6. Substitution on the phenyl ring by electron-withdrawing Cl or electron-donating OMe groups resulted in decreased tgDHFR inhibitory potency as well as selectivity. The most selective pcDHFR inhibitor in this series was the 3,4-diCl-benzyl analogue **8** with a rhDHFR/pcDHFR ratio of >5.2. For ecDHFR, the most potent and selective inhibitor was the N7-Cbz analogue **9**. For rhDHFR inhibition, both the nature and position(s) of substitution were important. Thus, the 2,4-diCl analogue **7** was the most potent. Compared to the benzyl substitution (**4**), the benzoyl substituent (**9**) led to an increase in
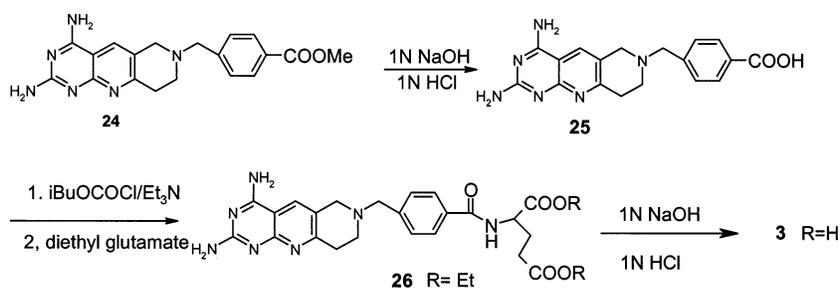
Scheme 4

Table 1. Inhibitory Concentration (IC$_{50}$, μM) and Selectivity Ratios against ecDHFR, pcDHFR, and tgDHFR vs rhDHFR[a]

compd	Ar	ec	rh	rh/ec	pc	rh/pc	tg	rh/tg
3	4-benzoyl−L-glu	0.9	5.4	6	1.8	3	0.9	6
4	Bn	10	32	3.2	16	2	1.4	25.6
5	4-OMe−Bn	8	27	3.4	>15 (35%)	nd	2.9	9.3
6	3,5-diOMe−Bn	3	22	7.3	14	1.48	2.7	8.2
7	2,4-diCl−Bn	3	1.3	0.43	4	0.32	2.1	0.62
8	3,4-diCl−Bn	3	>26 (33%)	>8.6	5	>5.2	5.1	>5
9	Cbz	3	30	10	>15 (35%)	nd	22	1.3
TMP		0.02	340	17000	15	22.4	3.4	100
MTX		0.006	0.022	3.7	0.0011	20	0.022	1
TMQ		0.007	0.018	2.6	0.01	1.8	0.0051	3.6

[a] All enzymes were assayed spectroscopically in a solution containing 50 μM dihydrofolate, 80 μM NADPH, 0.05 μM 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 OD at 340 nM of 0.015/min.

Table 2. Activity of **3** as a Substrate for Human FPGS[a]

substrate	K_m (μM)	V_{max}, rel	V_{max}/K_m	n
aminopterin	2.15 ± 0.55	1.00 ± 0.00	0.50 ± 0.13	2
3	27.30 ± 0.8	1.07 ± 0.03	0.04 ± 0.00	2
MTX	49^b	0.82^c	0.02^c	

[a] 1000 units of FPGS activity; one unit of FPGS catalyzes the incorporation of 1 pmol of [^{3}H]Glu/h. Values presented are average ± range. V_{max} values are calculated relative to aminopterin within the same experiment. [b] Data derived from ref 12. [c] Data derived from ref 13.

inhibitory potency and selectivity against ecDHFR. In contrast, against tgDHFR, the benzyl analogue **4** was more potent and selective. These data suggest that the basicity of the N7 nitrogen, along with the conformational flexibility of the phenyl side chain, may play a role in the inhibitory potency against DHFR from different pathogenic microbes and that the effects are quite different against different DHFR. Whether this difference in activity and selectivity of the benzoyl-substituted analogue compared to the benzyl analogue is due to the basicity of N7 or to the presence of the amide carbonyl and/or its ability to restrict the conformation of the side chain or other factors is not known at present.

Since polyglutamylation plays a role in the mechanism of action of some classical antifolates,[11a,b] it was of interest to evaluate the classical analogue **3** as a substrate for human FPGS (Table 2). Compound **3** was a reasonable substrate for FPGS, albeit less so than aminopterin, primarily because of a high K_m. This suggests that catalysis by FPGS is relatively insensitive to conformational restriction in the bridge region. Since compound **3** is substituted at the C7 position, its ability to function as a substrate for hFPGS was surprising, since 7-CH$_3$-substituted folate analogues are generally not substrates for FPGS.[14,39a,b]

Compound **3** was also evaluated as an inhibitor of the growth of CCRF-CEM human leukemia cells and its MTX-resistant sublines in culture during continuous exposure (Table 3).[40−43] Compound **3** was a weak inhibitor of CCRF-CEM growth in continuous (120 h) exposure; it was 60-fold less potent than MTX. Against R30dm, a subline expressing decreased FPGS activity, compound **3** was 3-fold cross-resistant in continuous exposure, which suggests that polyglutamylation plays a role in its mechanism of action. The data are consistent with the data in Table 2 showing that **3** is a substrate for FPGS. Although DHFR is often insensitive to the polyglutamylation status of substrates and inhibitors,[44] enhanced potency of polyglutamates of a few

Table 3. Growth Inhibition of CCRF-CEM Human Leukemia Cells and Its Methotrexate-Resistant Sublines (EC$_{50}$, nM) during Continuous Exposure (0−120 h)[a]

cell line	resistance mechanism	MTX	3
CCRF-CEM	sensitive	12.0 ± 3.1 ($n = 4$)	771 ± 111 ($n = 3$)
R1	increased DHFR	595 ± 85 ($n = 2$)	>10000 ($n = 2$)
R2	decreased in transport	1700 ± 0 ($n = 2$)	>10000 ($n = 2$)
R30dm	decreased FPGS	16 ± 0 ($n = 2$)	1900 ± 0 ($n = 2$)

[a] EC$_{50}$ is the concentration of drug required to decrease cell growth by 50% relative to untreated control after 5 days of treatment.

Table 4. Protection of CCRF-CEM Cells from the Growth Inhibitory Effects of MTX and **3** by Leucovorin (LV)[a]

drug	conc	relative growth (% of control)			
		[LV] = 0	[LV] = 0.1 μM	[LV] = 1 μM	[LV] = 10 μM
MTX	30 nM	2.8	95	92	91
	40 nM	3.7	74	75	77
3	2 μM	9.5	101	107	101
	5 μM	4.3	109	112	105

[a] LV up to 10 μM itself had no effect on growth of CCRF-CEM cells. Values are a range of closely agreeing duplicates. The entire experiment was repeated with similar results.

novel antifolates against DHFR has been reported.[45] A DHFR-overexpressing subline (R1) and a subline with reduced MTX transport (R2) were >14-fold cross-resistant to compound **3**. Despite its relatively lower potency (compared to MTX) as an inhibitor of purified rhDHFR (Table 1), the data are consistent with some need for polyglutamylation, transport via the reduced folate carrier, and inhibition of DHFR.

Leucovorin protection studies were carried out with **3** in order to further elucidate its mechanism of action. Both **3** (at 2 or 5 μM) and MTX (at 30 and 40 nM), at drug levels that inhibited growth by 92−96%, were fully protected by as little as 0.1 μM leucovorin, indicating that **3** acts as an antifolate (Table 4), which is consistent with the cell culture results and its DHFR inhibitory activity.

Compounds **3**, **4**, and **9** were evaluated as inhibitors of the growth of tumor cells in culture in the National Cancer Institute in vitro preclinical antitumor screening program.[46] In the leukemia cell lines, these analogues showed inhibitory activities with GI$_{50}$ values of 10^{-6}− 10^{-7} M, while in all the other tumor cell lines the compounds did not exhibit significant cytotoxic effect at 10^{-5} M.

In summary, seven novel 2,4-diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine derivatives with a varied benzyl and a benzoyl substitution at the N7 position (**3**−**9**) were designed and synthesized as classical and nonclassical, conformationally restricted, linear, tricyclic 5-deaza antifolates to investigate the effect of the conformational restriction, of C6−C9 (τ_1) and C9−N10 (τ_2) bonds via an ethyl bridge from the N10 to the C7 position of 5-deaza MTX, with respect to the inhibitory potency against different pathogenic DHFR and antitumor activity. Biological studies indicated that this conformational modification was detrimental to DHFR inhibitory activity as well as to antitumor activity compared to MTX or 5-deaza MTX. Surprisingly, the classical compound **3** was a better substrate than MTX for FPGS. These results indicate that the classical 5-deaza folate, tethered at the N10 and C7 positions, retains FPGS substrate activity and that the antitumor activity of tricyclic analogues such as **3** could be influenced by FPGS levels in tumor systems. Interestingly, the nonclassical analogues **4**−**9** showed moderate to good selectivity against pathogenic DHFR compared to rhDHFR. These results further support the idea that removal of the 5-methyl group of PTX along with τ_1 and τ_2 restriction can afford selectivity against pathogenic DHFR vs rhDHFR.

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in an Abderhalden drying apparatus over P_2O_5 and refluxing ethanol. Melting points were determined on a MEL-TEMP II melting point apparatus with a FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton NMR were recorded on a Bruker WH-300 (300 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, t = triplet, q = quartet, m = mutiplet, br = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on 230−400 mesh silica gel purchased from Aldrich, Milwaukee, WI. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within ±0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates could not be prevented despite 24−48 h of drying in vacuo and were confirmed, where possible, by their presence in the ^{1}H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. and Fisher Scientific and were used as received.

N-[4-[(2,4-**Diamino-5-deaza-6,7,8,9-tetrahydropyrido-[3,4-*g*]pteridin-7-yl)methyl]benzoyl]-L-glutamic Acid (3).** To a solution of 4-(2,4-diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridin-7-yl)methyl benzoic acid (**25**) (130 mg, 0.37 mmol) in anhydrous DMF (9 mL) was added triethylamine (130 μL), and the mixture was stirred under N_2 at room temperature for 5 min. The resulting solution was cooled to 0 °C, isobutyl chloroformate (130 μL, 0.96 mmol) was added, and the mixture was stirred at 0 °C for 30 min. At this time TLC (MeOH/CHCl$_3$, 2:5) indicated the formation of the activated intermediate at R_f = 0.40 and the disappearance of the starting acid at R_f = 0.28. Diethyl-L-glutamate hydrochloride (240 mg, 0.96 mmol) was added to the reaction mixture followed immediately by triethylamine (130 μL, 0.96 mmol). The reaction mixture was slowly allowed to warm to room tem-

perature and was stirred under N_2 for 6 h. The reaction mixture was then subjected to another cycle of activation and coupling using half the quantities listed above. The reaction mixture was slowly allowed to warm to room temperature, was stirred under nitrogen for 24 h, and then was subjected to a third round of activation and coupling using the same the quantities as the second round and was stirred for an additional 24 h. TLC showed the formation of one major spot at R_f = 0.38 (MeOH/CHCl$_3$, 1:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of CH$_3$Cl/MeOH, 4:1, and chromatographed on a silica gel column (2 cm × 15 cm) and with 4% MeOH in CHCl$_3$ as the eluent. Fractions that showed the desired single spot at R_f = 0.38 were pooled and evaporated to afford **26** (120 mg) as a syrup. Attempts at crystallization of this syrup were unsuccessful, and it was used directly for the next step.

To a solution of the diester **26** (120 mg, 0.2 mmol) in MeOH (10 mL) was added 1 N NaOH (6 mL), and the mixture was stirred under nitrogen at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f = 0.38) and one major spot at the origin (MeOH/CHCl$_3$, 2:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3 with dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice/acetone bath, thawed in the refrigerator at 4−5 °C, and filtered. The residue was washed with a small amount of cold water and ethyl acetate and was dried in vacuo over P_2O_5 to afford 100 mg (56%) of **3** as a brown powder: mp >300 °C (dec); TLC R_f = 0.22 (MeOH/CHCl$_3$, 2:5); ^{1}H NMR (DMSO-d_6) δ 2.11−2.16 (2 sets of t, 2 H, Glu β-CH$_2$), 2.32−2.37 (t, 2 H, Glu γ-CH$_2$), 2.81−2.83 (t, 2 H, C9−CH$_2$), 2.88−2.90 (t, 2 H, C8−CH$_2$), 3.54 (s, 2 H, C6−CH$_2$), 3.76 (s, 2 H, benzylic-CH$_2$), 4.41 (m, 1 H, Glu α-CH), 6.17 (s, 2 H, 2-NH$_2$), 7.29 (s, 2 H, 4-NH$_2$), 7.52−7.55 (d, 2 H, C$_6$H$_4$), 7.93−7.96 (d, 2 H, C$_6$H$_4$), 8.00 (s, 1 H, 5-CH), 8.59−8.61 (d, 1 H, −CONH−), 12.14 (br, 2 H, 2 × COOH). Anal. (C$_{23}$H$_{25}$N$_7$O$_5$·1.5HCl·0.3CH$_3$COOEt) C, H, N, Cl.

2,4-Diamino-7-benzyl-5-deaza-6,7,8,9-tetrahydropyrido-[3,4-*g*]pteridine.[4] Method A. Guanidine hydrochloride (1.50 g, 12 mmol) was added in one portion to a slurry of 1.5 g (13.6 mmol) of potassium *tert*-butoxide in 6 mL of anhydrous DMF under N_2, and the mixture was stirred at room temperature for 1 h. To this solution was added 2-amino-3-cyano-6-4−benzyl-5,6,7,8-tetrahydropyrido[4,3-*b*]pyridine **19** (1.4 g, 4 mmol) in one portion, and the resulting mixture was heated at 100 °C under N_2 with stirring overnight. The reaction mixture was cooled and filtered, the yellow residue was washed with hot water (20 mL) followed by methanol (10 mL × 2), acetone (20 mL), and ethyl acetate (20 mL) and was dried in vacuo to give 1 g (63%) of **4** as a light-yellow powder: mp 290.5−292 °C; R_f = 0.30 (MeOH/CHCl$_3$, 2:5); ^{1}H NMR (DMSO-d_6) δ 2.80−2.82 (t, 2 H, C8−CH$_2$), 2.87−2.89 (t, 2 H, C9−CH$_2$), 3.51 (s, 2 H, C6−CH$_2$), 3.68 (s, 2 H, benzylic-CH$_2$), 6.15 (s, 2H, 2-NH$_2$), 7.29 (s, 2H, 4-NH$_2$), 7.30−7.37 (m, 5 H, C$_6$H$_5$), 7.99 (s, 1 H, C5−CH). Anal. (C$_{18}$H$_{18}$N$_6$O$_2$·0.4H$_2$O) C, H, N.

Method B. In a 250 mL three-neck flask (freshly dried) equipped with a drying tube was placed sodium metal (0.56 g, 25.5 mmol) that had been cut into small pieces (<1 cm^3). Anhydrous ether (50 mL) was added followed by ethyl formate (2.8 g, 38 mmol). To this mixture was added 1-benzyl-4-piperidone **10** (4.8 g, 25.5 mmol). The mixture was then cooled to 5 °C using an ice bath. The reaction was initiated by the addition of 0.2 mL of absolute ethanol, and the mixture was stirred for 6 h. The yellow solid formed was filtered and washed with ethyl ether (100 mL) and dried in vacuo under P_2O_5 overnight to give 5.2 g (88%) of **11**[23] as a yellow powder. This powder was used directly for the next step. ^{1}H NMR (D$_2$O) δ 2.42−2.45 (t, 2 H, C−CH$_2$−N), 2.57−2.60 (t, 2 H, O=C−CH$_2$), 3.20 (s, 2 H, C=C−CH$_2$), 3.58 (s, 2 H, benzylic-CH$_2$), 7.47 (br, 5 H, C$_6$H$_5$), 9.17 (s, 1 H, =CH−ONa).

2,4,6-Triaminopyrimidine **12** (2.5 g, 20 mmol) in 100 mL of 75% AcOH (aqueous, w/w) was heated to 100 °C, the resulting

clear solution was cooled to 70 °C, and **11** (4.8 g, 20 mmol) was added in portions over 30 min. The resulting mixture was refluxed at 130 °C (oil bath) for 5 h. Distilled water (50 mL) was added, and the resulting mixture was cooled overnight in a refrigerator. The pH was adjusted to 7 using an ice bath with concentrated NH$_4$OH. The precipitate formed was collected and washed with water and MeOH and was dried in vacuo. The solid obtained was flash-chromatographed through a silica gel column (4 cm × 20 cm) using 10% MeOH (saturated with ammonia) in CHCl$_3$ as the eluent. The desired fractions (TLC) was pooled and evaporated. The residue was triturated with MeOH and filtered to afford 1 g (17%) of **4** as a yellow power that was identical in every respect to the sample prepared in method A.

General Procedure for the S**ynthesis of 2,4-Diamino-7-(substituted benzyl)-5-deaza-6,7,8,9-tetrahydropyrido-[3,4-*g*]pteridines (5−8 and 24).** Freshly dried 2,4-diamino-7*H*-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine **23** (110 mg, 0.5 mmol), DMF (5 mL), 4-*N*,*N*-dimethylaminopyridine (DMAP) (20 mg), triethylamine (0.2 mL), and the corresponding benzyl halide (0.6 mmol) were added in one portion to a 100 mL flask. The resulting mixture was stirred under N$_2$ at 60−80 °C (oil bath) for 2−3 h until the starting material disappeared (TLC). After evaporation of the solvent under reduced pressure, silica gel (5 g) and methanol (20 mL) were added and the resulting suspension was evaporated to dryness under reduced pressure. This silica gel plug was loaded on a dry silica gel column (3 cm × 15 cm) and was flash-chromatographed initially with CHCl$_3$ (100 mL), then sequentially with 200 mL of 5% MeOH (saturated with ammonia) in CHCl$_3$ and 200 mL of 10% and 15% MeOH (saturated with ammonia) in CHCl$_3$. Fractions that showed the desired product spot were pooled and evaporated to dryness. The residue was recrystallized from ethyl acetate and MeOH to afford the desired 2,4-diamino-7-(substituted benzyl)-5-deaza-6,7,8,9-tetrahydropyrido-[3,4-*g*]pteridines (**5−8**, **24**) in 45−64% yield.

2,4-Diamino-7-(4-methoxybenzyl)-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (5). Compound **23** (110 mg, 0.5 mmol) and 4-methoxylbenzyl chloride (95 mg, 0.6 mmol) afforded 80 mg (48%) of **5** as a light-yellowish solid: mp 257.2−259.6 °C; TLC R_f = 0.32 (CHCl$_3$/MeOH, 5:2); ^{1}H NMR (DMSO-d_6) δ 2.80 (t, 2 H, C9−CH$_2$), 2.88 (t, 2 H, C8−CH$_2$), 3.48 (s, 2 H, C6−CH$_2$), 3.60 (s, 2 H, benzylic-CH$_2$), 3.74 (s, 3 H, OCH$_3$), 6.25 (s, 2 H, 2-NH$_2$), 6.88−6.91 (d, 2 H, C$_6$H$_4$), 7.26−7.29 (d, 2 H, C$_6$H$_4$), 7.34 (s, 2 H, 4-NH$_2$), 8.00 (s, 1 H, 5-CH). Anal. (C$_{18}$H$_{20}$N$_6$O·0.3H$_2$O) C, H, N.

2,4-Diamino-7-(3,5−dimethoxybenzyl)-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (6). Compound **23** (110 mg, 0.5 mmol) and 3,5-dimethoxybenzyl chloride (110 mg, 0.6 mmol) afforded 105 mg (57%) of **6** as a light-yellow solid: mp 263.2−265.7 °C; TLC R_f = 0.35 (CHCl$_3$/MeOH, 5:2); ^{1}H NMR (DMSO-d_6) δ 2.79−2.81 (t, 2 H, C9−CH$_2$), 2.88−2.90 (t, 2 H, C8−CH$_2$), 3.53 (s, 2 H, C6−CH$_2$), 3.60 (s, 2 H, benzylic-CH$_2$), 3.73 (s, 6 H, OCH$_3$), 6.23 (s, 2 H, 2-NH$_2$), 6.40 (s, 2 H, C$_6$H$_3$), 6.54 (s, 2 H, C$_6$H$_3$), 7.33 (s, 2 H, 4-NH$_2$), 8.02 (s, 1 H, 5-CH). Anal. (C$_{19}$H$_{22}$N$_6$O$_2$·0.3 MeOH) C, H, N.

2,4-Diamino-7-(2,4−dichlorobenzyl)-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (7). Compound **23** (110 mg, 0.5 mmol) and 2,4-dichlorobenzyl chloride (115 mg, 0.6 mmol) afforded 120 mg (64%) of **7** as a yellow solid: mp 268.1−270 °C (dec); TLC R_f = 0.39 (CHCl$_3$/MeOH, 5:2); ^{1}H NMR (DMSO-d_6) δ 2.84 (t, 2 H, C9−CH$_2$), 2.90 (t, 2 H, C8−CH$_2$), 3.61 (s, 2 H, C6−CH$_2$), 3.76 (s, 2 H, benzylic-CH$_2$), 6.24 (s, 2 H, 2-NH$_2$), 7.37 (s, 2 H, 4-NH$_2$), 7.43−7.46 (m, 1 H, C$_6$H$_3$), 7.57−7.63 (m, 2 H, C$_6$H$_3$), 8.03 (s, 1 H, 4-CH). Anal. (C$_{17}$H$_{16}$N$_6$Cl$_2$·0.2H$_2$O) C, H, Cl.

2,4-Diamino-7-(3,4−dichlorobenzyl)-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (8). Compound **23** (110 mg, 0.5 mmol) and 3,4-dichlorobenzyl chloride (115 mg, 0.6 mmol) afforded 85 mg (45%) of **8** as a light-yellow solid: mp 258.1−261 °C (dec); TLC R_f = 0.40 (CHCl$_3$/MeOH, 5:2); ^{1}H NMR (DMSO-d_6) δ 2.83 (t, 2 H, C9−CH$_2$), 2.90 (t, 2 H, C8−CH$_2$), 3.53 (s, 2 H, C6−CH$_2$), 3.69 (s, 2 H, benzylic-CH$_2$), 6.22 (s, 2 H, 2-NH$_2$), 7.33 (s, 2 H, 4-NH$_2$), 7.37−7.40 (m, 1 H, C$_6$H$_3$),

7.59−7.64 (m, 2 H, C$_6$H$_3$), 8.02 (s, 1 H, 5-CH). Anal. (C$_{17}$H$_{16}$N$_6$-Cl$_2$·0.3H$_2$O) C, H, N, Cl.

2,4-Diamino-7-carbobenzyloxy-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (9). Guanidine hydrochloride (1.50 g, 12 mmol) was added in one portion to a slurry of potassium *tert*-butoxide (1.5 g, 13.6 mmol) in anhydrous DMF (6 mL) under N$_2$, and the mixture was stirred at room temperature for 1 h. To this solution was added 2-amino-3-cyano-6-(4-Cbz)-5,6,7,8-terahydropyrido[4,3-*b*]pyridine **20** (1.4 g, 4 mmol) in one portion, and the resulting mixture was heated at 100 °C under N$_2$ with stirring overnight. The reaction mixture was cooled and filtered, and the yellow residue was washed with hot water (20 mL) followed by methanol (10 mL × 2), acetone (20 mL), and ethyl acetate (20 mL) to give 1.1 g (78%) of **9** as a light-yellow powder: mp 290.5−292 °C; TLC R_f = 0.28 (MeOH/CHCl$_3$, 1:7); ^{1}H NMR (DMSO-d_6) δ 2.80−2.82 (br, 2 H, C9−CH$_2$), 3.74 (br, 2 H, C8−CH$_2$), 4.60 (s, 2 H, C6−CH$_2$), 5.14 (s, 2 H, benzylic-CH$_2$), 6.25 (s, 2 H, 4-NH$_2$), 7.32−7.39 (m, 7 H, C$_6$H$_5$ and 2-NH$_2$), 8.16 (s, 1 H, 5-CH). Anal. (C$_{18}$H$_{18}$N$_6$O$_2$) C, H, N.

2,4-Diamino-7-(4−carbomethoxybenzyl)-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (24). Compound **23** (110 mg, 0.5 mmol) and 4-carbomethoxybenzyl bromide (135 mg, 0.6 mmol) following the general procedure described earlier afforded 110 mg (60%) of **24** as a yellow solid: mp 278−281 °C (dec); TLC R_f = 0.42 (CHCl$_3$/MeOH, 5:2); ^{1}H NMR (DMSO-d_6) δ 2.81−2.83 (t, 2 H, C9−CH$_2$), 2.88−2.90 (t, 2 H, C8−CH$_2$), 3.54 (s, 2 H, C6−CH$_2$), 3.76 (s, 2 H, benzylic-CH$_2$), 3.85 (s, 3 H, OCH$_3$), 6.17 (s, 2 H, 2-NH$_2$), 7.29 (s, 2 H, 4-NH$_2$), 7.52−7.55 (d, 2 H, C$_6$H$_4$), 7.93−7.96 (d, 2 H, C$_6$H$_4$), 8.00 (s, 1 H, 5-CH). Anal. (C$_{19}$H$_{20}$N$_6$O$_2$·0.2EtOAc) C, H, N.

2-Amino-3-cyano-6-(4-benzyl)-5,6,7,8-terahydropyrido-[4,3-*b*]pyridine (19). To 1-benzyl-4-piperidone (**10**) (3.78 g, 20 mmol) and pyrrolidine (5 mL, 60 mmol) in 20 mL of anhydrous benzene was added 30 mg of *p*-toluenesulfonic acid, and the resulting mixture was stirred under reflux overnight using a Dean−Stark apparatus to remove the water (∼0.4 mL).[26] After evaporation of the solution, the residue, 1-benzyl-4-(*N*-pyrrolidino)-1,2,5,6-tetrahydropyridine **14**, was dissolved in 100 mL of THF and was used directly for the next step. To this solution was added triethylamine (3.5 mL), and the reaction mixture cooled to −40 °C in a dry ice/methanol bath. To this solution was added, dropwise, a solution of freshly prepared 1-chloro-2,2-dicyanoethylene (**16**)[27] (2.82 g, 25 mmol) in THF (5 mL) over a period of 2 min. The reaction mixture rapidly turned deep-red. The mixture was stirred at −40 °C for 30 min and then at room temperature for 1 h and was filtered through Celite, and the residue washed with copious amounts of THF and methylene chloride. Evaporation of the filtrate gave a deep-red residue (**17**), which was suspended in 100 mL of saturated methanolic ammonia. The mixture was stirred at room temperature overnight to give a pink suspension that was filtered, and to the filtrate was added silica gel (5 g). The solvent was evaporated to afford a plug. This plug was loaded onto a silica gel column (3 cm × 5 cm) and was eluted with hexane/ethyl acetate (2:1). The desired fractions (TLC) were pooled and evaporated and the residue was recrystallized from ethyl acetate to afford 2.4 g (45%) of **19** as a yellow powder: mp 211.3−213.2 °C; R_f = 0.33 (hexane/ethyl acetate, 1:1); MS *m*/*z* 264 (M$^+$); ^{1}H NMR (DMSO-d_6) δ 2.70 (t, 2 H, C8−CH$_2$), 3.31−3.36 (m, 4 H, C6−CH$_2$, C5−CH$_2$), 3.62 (s, 2 H, benzylic-CH$_2$), 6.63 (s, 2H, 2-NH$_2$), 7.27−7.34 (m, 5 H, C$_6$H$_5$), 7.56 (s, 1 H, 4-CH). Anal. (C$_{16}$H$_{16}$N$_4$) C, H, N.

2-Amino-3-cyano-6-(4-carbobenzyloxy)-5,6,7,8-terahydropyrido[4,3-*b*]pyridine (20). To benzyl 4-oxo-1-piperidinecarboxylate **13** (6 g, 20 mmol) and pyrrolidine (5 mL, 60 mmol) in 20 mL of anhydrous benzene was added 30 mg of *p*-toluenesulfonic acid, and the resulting mixture was stirred under reflux overnight using a Dean−Stark apparatus to remove the water (∼0.4 mL). After evaporation of the solution, the residue **15** was dissolve in 100 mL of THF and was directly used for the next step. To this solution was added triethylamine (3.5 mL), and the reaction mixture was cooled to −40 °C in a dry ice/methanol bath. To this solution was added,

dropwise, a solution of freshly prepared **16** (2.82 g, 25 mmol) in THF (5 mL) over a period of 2 min, followed by the same process as described for the synthesis of **19**. Evaporation of the filtrate gave a deep-red residue (**18**) that was suspended in 100 mL of saturated methanolic ammonia. The mixture was stirred at room temperature overnight to give a pink suspension. The solid collected by filtration was washed with a small amount of methanol followed by ethyl acetate to afford 4.6 g (75%) of **20** as a light-yellow powder: mp 167.3–169.1 °C; TLC R_f = 0.68 (MeOH/CHCl$_3$, 1:5); ^{1}H NMR (DMSO-d_6) δ 2.69–2.73 (t, 2 H, C8–CH$_2$), 3.67 (t, 2 H, C6–CH$_2$), 4.48 (s, 2 H, C5–CH$_2$), 5.11 (s, 2 H, benzylic-CH$_2$), 6.76 (s, 2 H, 2-NH$_2$), 7.30–7.38 (m, 5 H, C$_6$H$_5$), 7.75 (s, 1 H, 4-CH). Anal. (C$_{17}$H$_{16}$N$_4$O$_2$·0.3H$_2$O) C, H, N.

2,4-Diamino-7*H*-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridine (23). A suspension of **9** (540 mg, 1.5 mmol) and 40% KOH (20 mL) in MeOH (30 mL) was refluxed under N$_2$ for 2 h to give a clear solution. TLC indicated the disappearance of the starting material, R_f = 0.28 (MeOH/CHCl$_3$, 1:7), and a major spot near the origin. After evaporation of half the amount of solvent, the resulting precipitate was collected through filtration and washed with small amounts of ice water, MeOH, and ethyl acetate and was dried in vacuo over P$_2$O$_5$ to afford 270 mg (82%) of **23** as a white powder: mp 271.5–273.1 °C (dec); TLC R_f = 0.03 (MeOH/CHCl$_3$, 2:5) δ 2.76 (t, 2 H, C9–CH$_2$), 3.02 (t, 2 H, C8–CH$_2$), 3.84 (s, 2 H, C6–CH$_2$), 6.16 (s, 2 H, 2-NH$_2$), 7.38 (s, 2 H, 4-NH$_2$), 7.80 (s, 1 H, 7-NH), 8.00 (s, 1 H, 4-CH). Anal. (C$_{10}$H$_{12}$N$_6$·0.1H$_2$O) C, H, N.

4-(2,4-Diamino-5-deaza-6,7,8,9-tetrahydropyrido[3,4-*g*]pteridin-7-yl)methylbenzoic Acid (25). To a solution of **24** (180 mg, 0.5 mmol) in MeOH/DMSO (2:1, 25 mL) was added 2 N NaOH (5 mL), and the mixture was stirred under N$_2$ at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f = 0.42) and one major spot at the origin (MeOH/CHCl$_3$, 2:5). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in distilled water (8 mL), and the solution was filtered through Celite and washed with water (5 mL). The filtrate was cooled in an ice bath and the pH adjusted to 4 by dropwise addition of 2 N HCl. The resulting suspension was frozen in a dry ice/acetone bath, thawed in the refrigerator at 4–5 °C, and filtered. The residue was washed with a small amount of cold water and ethyl acetate and was dried in vacuo over P$_2$O$_5$ to afford 150 mg (85%) of **25** as a yellow powder: mp >300 °C (dec); TLC R_f = 0.28 (MeOH/CHCl$_3$, 2:5); ^{1}H NMR (DMSO-d_6) δ 2.82–2.84 (t, 2 H, C9–CH$_2$), 2.89–2.91 (t, 2 H, C8–CH$_2$), 3.56 (s, 2 H, C6–CH$_2$), 3.79 (s, 2 H, benzylic-CH$_2$), 6.18 (s, 2 H, 2-NH$_2$), 7.25 (s, 2 H, 4-NH$_2$), 7.54–7.57 (d, 2 H, C$_6$H$_4$), 7.95–7.98 (d, 2 H, C$_6$H$_4$), 8.01 (s, 1 H, 5-CH), 12.03 (br, 1 H, COOH). Anal. (C$_{18}$H$_{18}$N$_6$O$_2$·0.2H$_2$O) C, H, N.

Folylpolyglutamate Synthetase (FPGS) Purification and Assay. Recombinant human FPGS was expressed in *E. coli* JM109 (λDE3) cells from a pET3A expression vector containing the entire open reading frame of the human cytosolic FPGS.[48] This expression vector was constructed in, and was a generous gift of, the laboratory of Dr. Barry Shane, Department of Nutrition, University of California, Berkeley. The expressed protein was purified by Affi-Gel Blue and size exclusion chromatography.[48] Substrate activity of **3** was determined as previously described[49–51] except that the DEAE cellulose columns used to separate ^{3}H-radiolabeled polyglutamate products from unreacted [^{3}H]glutamate were eluted with 10 mM Tris-HCl (pH 7.5)/25 mM 2-mercaptoethanol containing 95 mM NaCl (instead of the standard 110 mM NaCl). This change was required to ensure the quantitative retention of all polyglutamates of **3**. Kinetic constants were determined for **3** and aminopterin in the same experiment by nonlinear curve-fitting to the rectangular hyperbola function (SigmaPlot); initial estimates of parameters were based on visual inspection of kinetic data.

Cell Lines and Methods for Measuring Growth Inhibitory Potency (Table 3). Solutions used in cell culture studies were standardized using extinction coefficients. Extinction coefficients were determined for (pH 1, $\lambda_{\text{max}-1}$ = 224

nm (45 500), $\lambda_{\text{max}-2}$ = 322 nm (11 600); pH 7, λ_{max} = 227 nm (37 600); pH 13, $\lambda_{\text{max}-1}$ = 229 nm (37 500), $\lambda_{\text{max}-2}$ = 243 nm (38 500)). Extinction coefficients for methotrexate (MTX), a generous gift of Immunex (Seattle, WA), were from the literature.[47]

All cell lines were verified to be negative for Mycoplasma contamination using the GenProbe test kit. The human T-lymphoblastic leukemia cell line CCRF-CEM[40] and its methotrexate-resistant sublines R1,[41] R2 (Bos),[42] and R30dm[43] used in these studies were cultured as described.[43] R1 expresses 20-fold elevated levels of dihydrofolate reductase (DHRF), the target enzyme of MTX. R2 has dramatically reduced MTX uptake. R30dm expresses only 1% of the FPGS activity of CCRF-CEM and is resistant to short-term, but not continuous, MTX exposure; however, R30dm is generally cross-resistant in continuous exposure to antifolates requiring polyglutamylation to form potent inhibitors. EC$_{50}$ values were interpolated from plots of percent control growth versus logarithm of drug concentration.

Protection against growth inhibition of CCRF-CEM cells was assayed by including leucovorin (0.1–10 μM) simultaneously with a concentration of drug previously determined to inhibit growth by 90–95%; the remainder of the assay was as described. Growth inhibition was measured relative to the appropriate metabolite-treated control; metabolites caused no growth inhibition in the absence of drug, however.

References

(1) Presented in part at the 219th National Meeting of the American Chemical Society, San Francisco, CA, April 2000; Paper MEDI 89. Presented in part at the 92nd National Meeting of the American Association for Cancer Research, New Orleans, LA, April 2001; Abstract 1568.
(2) Berman, E. M.; Werbel, L. M. The Renewed Potential for Folate Antagonists in Contemporary Cancer Chemotherapy. *J. Med. Chem.* **1991**, *34*, 479–485.
(3) Kisliuk, R. L. The Biochemistry of Folates. In *Folate Antagonists as Therapeutic Agents*; Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., Montgomery, J. A., Eds.; Academic Press: New York, 1984; pp 1–68.
(4) Gangjee, A.; Elzein, E.; Kothare, M.; Vasudevan, A. Classical and Nonclassical Antifolates as Potential Antitumor, Antipneumocystis and Antitoxoplasma Agents. *Curr. Pharm. Des.* **1996**, *2*, 263–280.
(5) Kisliuk, R. L.; Gaumont, Y.; Powers, J. F.; Thorndike, J.; Nair, M. G.; Piper, J. R. Synergistic Growth Inhibition by Combination of Antifolates. In *Evaluation of Folate Metabolism in Health and Disease*; Picciano, M. F., Stokstad, E. L. R., Gregory, J. F., III, Eds.; Alan R. Liss: New York, 1990; pp 79–89.
(6) Rosowsky, A. Chemistry and Biological Activity of Antifolates. In *Progress in Medicinal Chemistry*; Ellis, G. P., West, G. B., Eds.; Elsevier Science Publishers: Amsterdam, 1989; pp 1–252.
(7) Slavik, K.; Slavikova, V.; Kolma, Z. Metabolism of Folic Acid. VI. Preparation of Intermediary Antimetabolites of Folic Acid. *Collect. Czech. Chem. Commun.* **1960**, *25*, 1929–1937.
(8) DeGraw, J. I.; Colwell, W. T.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. Synthesis and Antifolate Properties of 5,10-Methylenetetrahydro-8,10-dideazaaminopterin. *J. Med. Chem.* **1986**, *29*, 1786–1789.
(9) Su, T. L.; Yang, Y. K.; Huang, J. T.; Ren, W. Y.; Watanabe, K. A.; Chou, T. C. Synthesis of 4-[(1,3-Diaminopyrrolo[3′,4′:4′,5′]pyrido[2,3-*d*]pyrimidin-8-yl)-benzoyl]-L-glutamic Acid as Potential Antifolates. *J. Heterocycl. Chem.* **1993**, *30*, 1437–1443.
(10) DeGraw, J. I.; Christie, P. H.; Colwell, W. T.; Sirotnak, F. M. Synthesis and Antifolate Properties of 5,10-Ethano-5,10-dideazaaminopterin. *J. Med. Chem.* **1992**, *35*, 320–324.
(11) (a) Kisliuk, R. L.; Gaumont, Y.; Kumar, P.; Coutts, M.; Nair, M. G.; Nanavati, N. T.; Kalman, T. I. The Effect of Polyglutamylation on the Inhibitory Activity of Folate Analogs. In *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates*; Goldman, I. D., Ed.; Praeger: New York, 1985; pp 319–328. (b)

McGuire, J. J. Antifolate Polyglutamylation in Preclinical and Clinical Antifolate Resistance. In *Antifolate Drugs: Basic Research and Clinical Practice*; Jackman, A. L., Ed.; Humana Press: Totowa, NJ, 1999; pp 339−363.

(12) Tsukamoto, T.; Haile, W. H.; McGuire, J. J.; Coward, J. K. Mechanism-Based Inhibition of Human Folypolyglutamate Synthetase: Design, Synthesis, and Biomedical Characterization of Phosphopeptide Mimic of the Tetrahedral Intermediate. *Arch. Biochem. Biophys.* **1998**, *355*, 109−118.

(13) McGuire, J. J.; Hsieh, P.; Franco, C. T.; Piper, J. R. Folypolyglutamate Synthesis Inhibition and Cytotoxic Effects of Methotrexate Analogs Containing 2,omega-Diaminoalkanoic Acids. *Biochem. Pharmacol.* **1986**, *35*, 2607−2613.

(14) Sanghani, P. C.; Jackman, A.; Evans, V. R.; Thornton, T.; Hughes, L.; Calvert, A. H.; Moran, R. A Strategy for the Design of Membrane-Permeable Folypoly-γ-glutamate Synthetase Inhibitors: "Bay-Region"-Substituted 2-Desamino-2-methyl-5,8-dideazafolate Analogs. *Mol. Pharmacol.* **1994**, *45*, 341−351.

(15) Werbel, L. M. In *Folate Antagonists as Therapeutic Agents*; Sirotnak, F. M., Burchall, J. J., Ensminger, W., Montgomery, J. A., Eds.; Academic Press: Orlando, FL, 1984; Vol. 1, pp 261.

(16) Allegra, C. J.; Kovcas, J. A.; Drake, J. C.; Swan, J. C.; Chabner, B. A.; Masur, H. Activity of Antifolates against the *Pneumocystis carinii* Dihydrofolate Reductase and Identification of a Potent New Agent. *J. Exp. Med.* **1987**, *165*, 926−927.

(17) Kovcas, J. A.; Allegra, B. A.; Chabner, B. A.; Swan, J. C.; Drake, M.; Lunde, M.; Parrillo, J. E.; Masur, H. Potent Effect of Trimetrexate, a Lipid-Soluble Antifolate, against *Toxoplasma gondii*. *J. Infect. Dis.* **1987**, *155*, 1027.

(18) Kovcas, J. A.; Allegra, J. C.; Swan, J. C.; Drake, J. C.; Parrillo, J. E.; Chabner, B. A.; Masur, H. Potent Antipneumocystis and Antitoxoplasm Activities of Piritrexim, a Lipid-Soluble Antifolate. *Antimicrob. Agents Chemother.* **1988**, *32*, 430−431.

(19) Masur, H.; Polis, M. A.; Tuazon, C. U.; Ogata-Arakaki, D.; Kovcas, J. A.; Katz; Hilt, D.; Simmons, T.; Feuerstein, I.; Lundgren, B.; Lane, H. C.; Chabner, B. A.; Allegra, J. C. Salvage Trial of Trimetrexate-Leucovorin for the Treatment of Cerebral Toxoplasmosis in Patients with AIDS. *J. Infect. Dis.* **1993**, *167*, 1422−1423.

(20) Champness, J. N.; Achari, A.; Ballantine, S. P.; Bryant, P. K.; Delves, C. J.; Stammers, D. K. The Structure of *Pneumocytis carinii* Dihydrofolate Reductase to 1.9 Å Resolution. *Structure* **1994**, *2*, 915−924.

(21) Gangjee, A.; Devraj, R.; Queener, S. Synthesis and Dihydrofolate Reductase Inhibitory Activities of 2,4-Diamino-5-deaza and 2,4-Diamino-5,10-dideaza Lipophilic Antifolates. *J. Med. Chem.* **1997**, *40*, 470−478.

(22) Mathews, D. A.; Bolin, J. T.; Burdge, J. M.; Filman, K. W.; Kaufman, B. T.; Bedell, C. R.; Champness, J. N.; Stammers, D. N.; Kraut, J. Dihydrofolate Reductase. The Stereochemistry of Inhibitor Selectivity. *J. Biol. Chem.* **1985**, *260*, 392−396.

(23) Gangjee, A.; Donkor, I. O. Synthesis of Substituted Tetrahydro-pyrimido[4,5-*b*][1,7]naphthyridines as Potential Antitumor Agents. *J. Heterocycl. Chem.* **1989**, *26*, 705−708.

(24) DeGraw, J. I.; Christie, P. H.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. Synthesis and Antifolate Properties of 10-Alkyl-5,10-dideaza Analogs of Methotrexate and Tetrahydrofolic Acid. *J. Med. Chem.* **1990**, *33*, 673−7.

(25) Taylor, E. C.; Skotnicki, J. S.; Fletcher, S. R. Synthesis of D,L-7,10-Ethano-5-deazaaminopterin and L-Ethano-5-deazafolic Acid. *J. Org. Chem.* **1985**, *50*, 1005−1010.

(26) McCabe, P. H.; Milne, N. J.; Sim, G. A. Conformational Study of Bridgehead Lactams. Preparation and X-ray Structural Analysis of 1-Azabicyclo[3.3.1]nonane-2,6-dione. *J. Chem. Soc., Perkin Trans. 2* **1989**, 1459−1461.

(27) Josey, A. D.; Dickinson, C. L., Jr.; Dewhirst, K. C.; McKusick, B. C. Trifluoromethylmalononitrile. The Reaction of 1,1-Dichloro-2,2-dicyanoethylene with Argentous Fluoride. *J. Org. Chem.* **1967**, *32*, 1941−1944.

(28) . *Protective Groups in Organic Synthesis*; Greene, T. W., Wuts, P. G. M., Eds.; John Wiley & Sons: New York, 1999; pp 579−580.

(29) Lott, R. S.; Chauhan, V. S.; Stammer, C. H. Trimethylsilyl Iodide as a Peptide Deblocking Agent. *J. Chem. Soc., Chem. Commun.* **1979**, 495−496.

(30) Angle, S. R.; Arnaiz, D. O. Stereoselective Synthesis of Substituted Pipecolic Acids. *Tetrehedron Lett.* **1989**, *5*, 515−518.

(31) Chelucci, G.; Falorni, M.; Giacomelli, G. Synthesis of 1-Substituted 2-[(2*S*)-2-Pyrrolidinyl]pyridine from L-Proline. *Synthesis* **1990**, 1121−1122.

(32) Anantharamaiah, G. M.; Sivanandaiah, K. M. Transfer Hydrogenation: A Convenient Method for Removal of Some Commonly Used Protecting Group in Peptide Synthesis. *J. Chem. Soc., Perkin Trans. 1* **1977**, 490−491.

(33) Ihara, M.; Taniguchi, N.; Noguchi, K.; Fukumoto, K. Total Synthesis of Hydrocinchonidine and Hydrocinchonine via Photo-oxygenation of an Indole Derivative. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1277−1281.

(34) Gangjee, A.; Zeng, Y.; McGuire, J. J.; Kisliuk, R. L. Effect of C9-Methyl Substitution and C8−C9 Conformational Restriction on Antifolate and Antitumor Activity of Classical 5-Substituted 2,4-Diaminofuro[2,3-*d*]pyrimidines. *J. Med. Chem.* **2000**, *43*, 3125−3133.

(35) . *E. coli* DHFR was kindly provided by Dr. R. L. Blakley, St. Jude Children's Research Hospital, Memphis, TN.

(36) *P. cariniii* DHFR was kindly supplied by Dr. D. Borhani, Southern Research Institute, Birmingham, AL.

(37) *T. gondii* DHFR was kindly supplied by Dr. D. V. Santi, University of California, San Francisco CA.

(38) Recombinant human DHFR was kindly supplied by Dr. J. H. Freisheim, Medical College of Ohio, Toledo, OH.

(39) (a) Marsham, P. R.; Wardleworth, J. M.; Boyl, F. T.; Hennequin, L. F.; Kimbell, R.; Brown, M.; Jackman, A. L. Design and Synthesis of Potent Non-Polyglutamatable Antifolate Thymidylate Synthase Inhibitors. *J. Med. Chem.* **1999**, *42*, 3809−3820. (b) Boyle, F. T.; Matusiak, Z. S.; Hughes, L. R.; Slater, A. M.; Stephens, T. C.; Smith, M. N.; Kimbell, R.; Jackman, A. L. Substituted 2-Desamino-2-methylquinazolines. A Series of Novel Antitumour Agents. *Chemistry and Biology of Pteridines and Folates, Advances in Experimental Medicine and Biology*; Ayling, J. E., Nair, M. G., Baugh, C. M., Eds.; Plenum Press: New York, 1995; pp 585−588.

(40) Foley, G. F.; Lazarus, H.; Farber, S.; Uzman, B. G.; Boone, B. A.; McCarthy, R. E. Continuous Culture of Lymphoblasts from Peripheral Blood of a Child with Acute Leukemia. *Cancer* **1965**, *18*, 522−529.

(41) Mini, E.; Srimatkandada, S.; Medina, W. D.; Moroson, B. A.; Carman, M. D.; Bertino, J. R. Molecular and Karyological Analysis of Methotrexate-Resistant and -Sensitive Human Leukemic CCRF-CEM Cells. *Cancer Res.* **1985**, *45*, 317−325.

(42) Rosowsky, A.; Lazarus, H.; Yuan, G. C.; Beltz, W. R.; Mangini, L.; Abelson, H. T.; Modest, E. J.; Frei, E., III. Effects of Methotrexate Esters and Other Lipophilic Antifolates on Methotrexate-Resistant Human Leukemic Lymphoblasts. *Biochem. Pharmacol.* **1980**, *29*, 648−652.

(43) McCloskey, D. E.; McGuire, J. J.; Russell, C. A.; Rowan, B. G.; Bertino, J. R.; Pizzorno, G.; Mini, E. Decreased Folylpolyglutamate Synthetase Activity as a Mechanism of Methotrexate Resistance in CCRF-CEM Human Leukemia Sublines. *J. Biol. Chem.* **1991**, *266*, 6181−6187.

(44) McGuire, J. J.; Coward, J. K. Pteroylpolyglutamates: Biosynthesis, Degradation, and Function. In *Folates and Pterins. Chemistry and Biochemistry of Folates*, 1st ed.; Blakley, R. L., Benkovic, S. J., Eds.; Wiley: New York, 1984; Vol. 1, pp 135−190.

(45) Rosowsky, A.; Galivan, J.; Beardsley, G. P.; Bader, H.; O'Connor, B. M.; Russelo, O.; Moroson, B. A.; DeYarman, M. T.; Kerwar, S. S.; Freisheim, J. H. Biochemical and Biological Studies on 2-Desamino-2-methylaminopterin, an Antifolate the Polyglutamates of Which Are More Potent than the Monoglutamate against Three Key Enzymes of Folate Metabolism. *Cancer Res.* **1992**, *52*, 2148−2155.

(46) We thank the Developmental Therapeutics Program of the National Cancer Institute for performing the in vitro anticancer evaluation.

(47) Blakley, R. L. *The Biochemistry of Folic Acid and Related Pteridines*; Elsevier North-Holland: Amsterdam, 1969; pp 92−94.

(48) Atkinson, I.; Garrow, T.; Brenner, A.; Shane, B. Human Cytosolic Folylpoly-Gamma-Glutamate Synthase; *Methods Enzymol.* **1997**, *281*, 134−140.

(49) McGuire, J. J.; Hsieh, P.; Coward, J. K.; Bertino, J. R. In Vitro Methotrexate Polyglutamate Synthesis by Rat Liver Folylpolyglutamate Synthetase and Its Inhibition by Bromosulfophthalein. *Adv. Exp. Med. Biol.* **1983**, *163*, 199−214.

(50) McGuire, J. J.; Hsieh, P.; Coward, J. K.; Bertino, J. R. Enzymatic Synthesis of Folylpolyglutamates. Characterization of the Reaction and Its Products. *J. Biol. Chem.* **1980**, *255*, 5776−5788.

(51) Bolanowska, W. E.; Russell, C. A.; McGuire, J. J. Activation of Mammalian Folylpolyglutamate Synthetase by Sodium Bicarbonate. *Arch. Biochem. Biophys.* **1990**, *281*, 198−203.