Design, Synthesis, and Biological Evaluation of Classical and Nonclassical 2-Amino-4-oxo-5-substituted-6-methylpyrrolo[3,2-*d*]pyrimidines as Dual Thymidylate Synthase and Dihydrofolate Reductase Inhibitors

Aleem Gangjee,*[,†] Wei Li,[†] Jie Yang,[†] and Roy L. Kisliuk[‡]

Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, 600 Forbes Avenue, Pittsburgh, Pennsylvania 15282, and Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111

Received August 24, 2007

We designed and synthesized a classical antifolate *N*-{4-[(2-amino-6-methyl-4-oxo-3,4-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)methyl]benzoyl}-L-glutamic acid **4** and 11 nonclassical analogues **5–15** as potential dual thymidylate synthase (TS) and dihydrofolate reductase (DHFR) inhibitors. The key intermediate in the synthesis was *N*-(4-chloro-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl)-2,2-dimethylpropanamide, **29**, to which various 5-benzyl substituents were attached. For the classical analogue **4**, the ester obtained from the N-benzylation reaction was deprotected and coupled with diethyl L-glutamate followed by saponification. Compound **4** was a potent dual inhibitor of human TS (IC_{50} = 46 nM, about 206-fold more potent than pemetrexed) and DHFR (IC_{50} = 120 nM, about 55-fold more potent than pemetrexed). The nonclassical analogues were marginal inhibitors of human TS, but four analogues showed potent *T. gondii* DHFR inhibition along with >100-fold selectivity compared to human DHFR.

Introduction

Folate metabolism has long been recognized as an attractive target for chemotherapy because of its crucial role in the biosynthesis of nucleic acid precursors.[1,2] Inhibitors of folate-dependent enzymes in cancer, microbial, and protozoan cells provide compounds that have found clinical utility as antitumor, antimicrobial, and antiprotozoal agents.[3,4] Thymidylate synthase (TS[a]),which catalyzes the reductive methylation of deoxyuridylate (dUMP) to deoxythymidylate (dTMP), has been of particular interest.[5] This reaction affords 7,8-dihydrofolate (7,8-DHF), which is reduced by dihydrofolate reductase (DHFR)[6] to tetrahydrofolate (THF).[7] Thus, TS and DHFR are crucial for the synthesis of dTMP in dividing cells. Several TS and DHFR inhibitors, as separate entities, have found clinical utility as antitumor agents. Usually a 2,4-diamino-substituted pyrimidine ring is considered important for potent DHFR inhibitory activity, while a 2-amino-4-oxopyrimidine or 2-methyl-4-oxopyrimidine ring is considered important for potent TS inhibitory activity. Examples of clinically used TS and DHFR inhibitors are raltitrexed[8] (ZD1694), pemetrexed[9] (Alimta, LY231514), and methotrexate[10] (MTX) illustrated in Figure 1. Raltitrexed is a quinazoline analogue that can be transported into cells via the reduced folate carrier (RFC)[11] and undergoes rapid polyglutamylation by the enzyme folylpolyglutamate synthetase (FPGS).[12–15] Raltitrexed is approved as a first-line agent for advanced colorectal cancer in several European countries, Australia, Canada, and Japan. Pemetrexed is an antifolate in which a pyrrole ring replaces the pyrazine of folic acid and a methylene group replaces the N10 nitrogen in the bridge. Pemetrexed contains a 6-5 pyrrolo[2,3-*d*]pyrimidine system and is designated a multitargeted antifolate (MTA). Pemetrexed and its polyglutamylated metabolites are reported to be inhibitors of several important folate-dependent enzymes including TS, DHFR, glycinamide ribonucleotide formyltransferase (GARFT), and aminoimidazole carboxamideribonucleotide formyltransferase (AICARFT).[16,17]

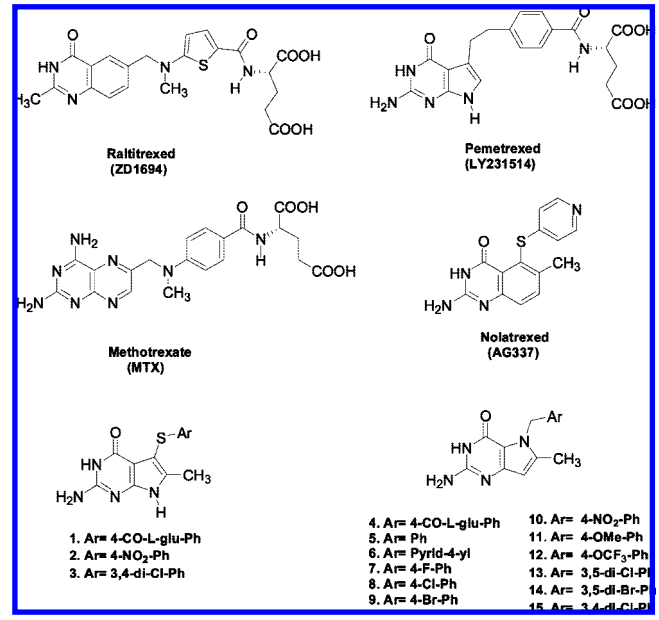


Figure 1

However, the primary locus of action of pemetrexed is the inhibition of TS, and this is responsible for its cytotoxic effects. Inhibition of other folate-dependent enzymes may also be important, since pemetrexed is cytotoxic to human cancer cell lines that are resistant to raltitrexed and 5-FU as a result of TS amplification.[18] Pemetrexed, in combination with cisplatin,

* To whom correspondence should be addressed. Phone: 412-396-6070. Fax: 412-396-5593. E-mail: gangjee@duq.edu.

† Duquesne University.

‡ Tufts University School of Medicine.

[a]Abbreviations: TS, thymidylate synthase; dUMP, deoxyuridylate; dTMP, deoxythymidylate; DHFR, dihydrofolate reductase; THF, tetrahydrofolate; MTX, methotrexate; RFC, reduced folate carrier; FPGS, folylpolyglutamate synthetase; GARFT, glycinamide ribonucleotide formyltransferase; AICARFT, aminoimidazole carboxamideribonucleotide formyltransferase; 5-FU, fluorouracil; *E. coli*, *Escherichia coli*; tg, *Toxoplasma gondii*; NCI, National Cancer Institute.

10.1021/jm701052u CCC: $40.75
Published on Web 12/12/2007

has been recently approved for the treatment of malignant pleural mesothelioma and also for non-small-cell lung cancer.[19]

Classical antifolates that have an *N*-benzoyl-L-glutamic acid side chain such as raltitrexed and pemetrexed are able to function as substrates for FPGS. FPGS catalyzes the formation of poly-γ-glutamates, which leads to high intracellular concentrations of these antitumor agents and increases TS inhibitory activity for some antifolates (raltitrexed 60-fold[8] and pemetrexed 130-fold[9]) compared to their monoglutamate forms. Although polyglutamylation of certain antifolates is necessary for their cytotoxic activity, it has also been implicated in toxicity to host cells because of cellular retention of the polyanionic poly-γ-glutamate metabolites which also do not efflux from normal cells.[20] Additionally, tumor cells develop resistance to antifolates that depend on polyglutamylation for their antitumor effects by producing low or defective FPGS and thereby limiting their utility. Another problem associated with classical antifolates is their dependence on the carrier systems for their uptake into tumor cells. The RFC is the most important carrier, and impairment of the RFC system in tumor cells also leads to drug resistance.[21–25]

In general, the problem of resistance in tumors, due to low or defective FPGS, has placed limitations on the use of classical antifolates, which depend on polyglutamylation for their antitumor effects. To overcome these problems associated with classical antifolates, lipophilic nonclassical antifolates were also designed and synthesized. These lipophilic nonclassical antifolates lack the polar glutamate found in classical antifolates and hence do not depend on FPGS for their inhibitory activity of the target enzymes. Additionally, they also do not require the RFC system for active uptake into the cell because they are lipophilic and are passively transported into cells. Nolatrexed (Figure 1) is the first antitumor nonclassical TS inhibitor to reach clinical trials.[26,27]

It has been demonstrated with both clinically used antifolates as well as in preclinical studies that TS inhibitors and DHFR inhibitors, when used separately or in combination with other agents, can provide successful antitumor therapy. In addition, synergism of two separate antifolates that inhibit TS and DHFR has been demonstrated in growth inhibitory studies against *Lactobacillus casei*,[28,29] rat hepatoma cells,[30,31] and human lymphoma cells.[32,33] Thus, one of our goals has been to design potent dual TS and DHFR inhibitory activity in single agents. Such dual inhibitors could act at two different sites (TS and DHFR) and might be capable of providing "combination chemotherapy" in single agents without the pharmacokinetic disadvantages of two separate agents. This strategy may also lead to an improved toxicity profile. Gangjee et al.[34] reported a classical antifolate *N*-{4-[(2-amino-6-methyl-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]benzoyl}-L-glutamic acid, **1** (Figure 1), as a potent inhibitor of human TS ($IC_{50} = 54$ nM) and a marginal inhibitor of human DHFR ($IC_{50} = 2.2$ μM) in its monoglutamate form, thus providing a dual inhibitor of human TS and human DHFR. Compound **1** was not a substrate for human FPGS at concentrations up to 1045 μM, indicating that **1** does not require polyglutamylation for its potent inhibition of human TS. Using molecular modeling (SYBYL 6.91),[35] Gangjee et al.[36] suggested that the 6-methyl group in **1** makes important hydrophobic contacts with Trp109 in human TS and also sterically restricts the rotation of the 5-position side chain so that it adopts a favorable conformation for binding to human TS. In addition, molecular modeling indicated that the 6-methyl group of **1** also makes hydrophobic contact with Val115 in human DHFR. This potential hydrophobic interaction with Val115 is absent in pemetrexed, which does not have a 6-methyl group. Thus, compound **1** is a promising lead compound that can be further structurally modified to optimize dual human TS and human DHFR inhibitory activities. Though compound **1** was a dual TS/DHFR inhibitor, its human DHFR inhibitory activity was marginal. One of the principal goals of this study was to maintain (or improve) human TS inhibitory activity of **1** while substantially increasing the human DHFR inhibitory activity.

In addition to the inhibition of human TS and DHFR we are also interested in selective inhibition of TS and/or DHFR from pathogens that cause opportunistic infections in AIDS patients such as *Toxoplasma gondii* (tg).[37]

The pyrrolo[3,2-*d*]pyrimidine scaffold is a regioisomer of the pyrrolo[2,3-*d*]pyrimidine found in pemetrexed. This pyrrolo[3,2-*d*]pyrimidine modification maintains the overall size of the molecule intact but removes a potential hydrogen bond donor from the N7 position. In addition, quinazolines such as ralitrexed and nolatrexed, which have a carbon atom in the 8-position rather than a nitrogen, are more potent inhibitors of human TS than the corresponding pyrido[2,3-*d*]pyrimidines and pteridines perhaps because of a more favorable hydrophobic interaction with Trp109 in human TS.[26]

Molecular modeling using SYBYL 7.0[35] and superimposing **4** on raltitrexed crystallized with human TS (PDB code 1KMV) indicated that, like compound **1**, the 6-methyl moiety of compound **4** interacts with Trp109 as do the C6 and C7 atoms of **4**. Similarly molecular modeling (SYBYL 7.0[35]) and superimposing the pyrimidine ring of compound **4** onto the 2,4-diaminopyrimidine ring of SRI 9662 in human DHFR X-ray crystal structure (PDB 1KMV[38]) indicate that the C7 of the pyrrolo[3,2-*d*]pyrimidine ring of **4** makes hydrophobic contact with Phe31 of human DHFR, as shown in Figure 2. This hydrophobic interaction would be absent with the N7 of **1** and could translate into a better DHFR inhibitory potency for **4** compared to **1**. The Glu30 that makes contact with the 2-NH_2 and N1 positions of the pyrimidine ring and the second Phe34 that makes contact with the side chain phenyl ring of **4** are also indicated in Figure 2. Thus, it was anticipated that compound **4** would have better human DHFR inhibitory activity compared to **1**. It was therefore of interest to synthesize the classical analogue *N*-{4-[(2-amino-4-oxo-6-methylpyrrolo[3,2-*d*]pyrimidin-5-ylmethyl)benzoyl}-L-glutamic acid, **4**, to explore the effects of N5 substitution of the pyrrolo[3,2-*d*]pyrimidine scaffold on human TS and human DHFR inhibitory activity.

Gangjee et al.[39] showed that nonclassical analogues of **1** with electron-withdrawing groups in the phenyl ring of the side chain also enhance human TS inhibitory activity. SAR studies indicated that analogues with electron-withdrawing groups at the 3- and/or 4-position of the phenyl side chain provide optimum inhibitory potency against human TS. In addition, nonclassical analogues such as **2** ($IC_{50} = 0.15$ μM) and **3** ($IC_{50} = 0.13$ μM) were more potent inhibitors of human TS than the clinically used raltitrexed and pemetrexed. In contrast to the requirements for human TS inhibition, electron-donating substituents such as methoxy and methyl and bulky substituents such as naphthyl are conducive for DHFR inhibition;[39] hence, analogues containing these substituents were also synthesized. As indicated above for the classical analogue **4**, it was

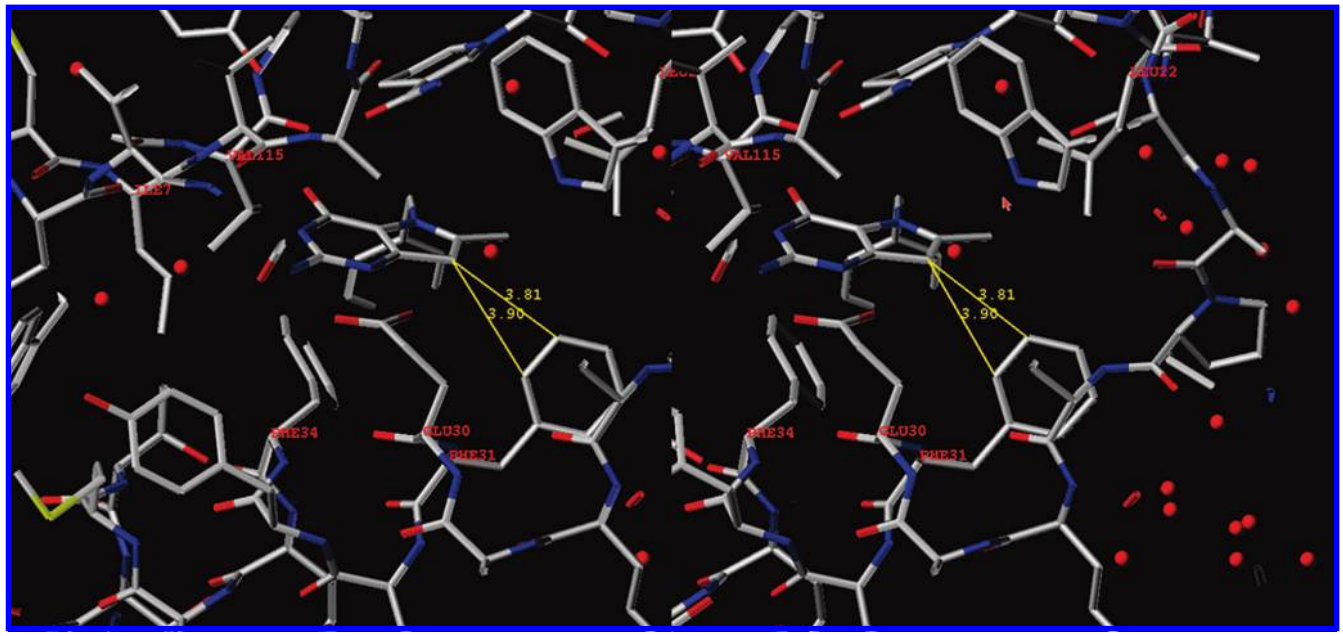
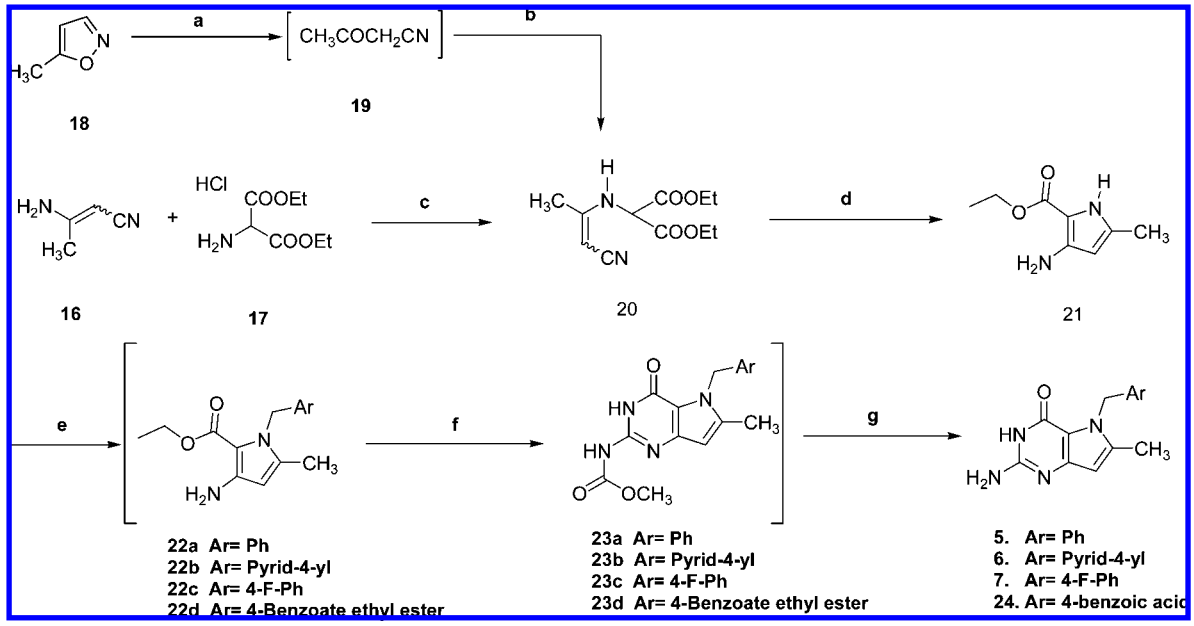


Figure 2. Stereoview of compound **4** superimposed on SRI 9662 in human DHFR (PDB code 1KMV).[38] The hydrophobic interaction between C7 and Phe31 is shown.

Scheme 1[a]



[a] Reagents and conditions: (a) NaOEt, EtOH, 0 °C, 1 h; (b) AcOH, diethyl aminomalonate hydrochloride, room temp, 24 h; (c) MeOH, room temp, 5 h; (d) NaOEt, EtOH, 60 °C, 6 h; (e) NaH, DMF, benzyl bromides, room temp, 4 h; (f) (1) 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea, AcOH, MeOH, room temp, 12 h; (2) NaOMe, MeOH, room temp, 12 h; (g) 1 N NaOH, 55 °C, 3 h.

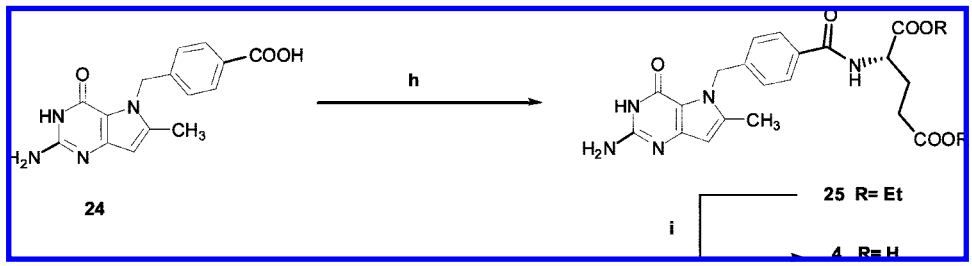
anticipated that the nonclassical analogues **5**–**15** would also provide dual inhibitory activity against human TS and human DHFR.

Chemistry

Our initial methods for the synthesis of the classical compound **4** and the nonclassical compounds **5**–**7** are outlined in Schemes 1 and 2. The key intermediate in the synthesis of **4**–**7** was the ethyl 3-amino-5-methyl-1*H*-pyrrole-2-carboxylate, **21** (Scheme 1), which could be further condensed with a guanylating agent to form the pyrrolo[3,2-*d*]pyrimidine ring system. A search of the literature revealed that there was no synthesis or other report for **21**. However, two methods[40,41] for the synthesis of the corresponding desmethyl analogue of **21** from diethyl[(2-cyanovinyl)amino]malonate, the desmethyl analogue of **20**, were reported.

Thus, it appeared attractive to adopt the reported methodology for the synthesis of the intermediates **20** and **21**. The conversion of 5-methylisoxazole **18** to 3-oxobutanenitrile **19** was catalyzed by sodium ethoxide. Compound **19** was condensed with diethyl aminomalonate to afford **20**, which was further cyclized under basic conditions at room temperature to afford the desired pyrrole **21**. The total yield, over three steps, from **18** to **21** was a mere 12%. In addition, the purification of the desired product **21** was difficult because the byproduct had close R_f values (TLC) to **21**. Therefore, it was necessary to explore alternative

Scheme 2[a]



[a] Reagents and conditions: (h) L-glutamic acid diethyl ester hydrochloride, 2-chloro-4,6-dimethoxy-1,3,5-triazine, *N*-methylmorpholine, DMF, room temp, 5 h; (i) (1) 1 N NaOH, room temp, 4 h; (2) 3 N HCl.

procedures for the synthesis of **21**. A further search of the literature afforded a method that allowed a conjugate addition–elimination of **16** (Scheme 1) with a variety of nucleophiles.[42] Initial attempts to react **16** with diethyl aminomalonate free base with variation of time (up to 8 h) and temperature (at reflux) were unsuccessful. However, the crucial intermediate **20** (*E*/*Z*-mixture) was readily obtained from **16** (*E*/*Z*-mixture) and diethyl aminomalonate hydrochloride, **17** (rather than the free base), in methanol at room temperature for 5 h. A possible explanation is that the imine is a stronger base than the amine. Once the hydrochloride salt was added, the imine is protonated and the carbon of the imine is more susceptible to nucleophilic attack by the amine. In the absence of the amine salt, 3-aminobut-2-enenitrile **16** exists predominantly as the enamine and is less susceptible to nucleophilic attack by the amino group. With **20** in hand, use of reaction conditions similar to those reported by Furneaux and Tyler,[40] which involved heating a mixture of **20** and sodium ethoxide in ethanol under N_2 for 12 h at 55–60 °C, afforded the pyrrole **21** in 65% yield following chromatographic separation (Scheme 1). This reaction was incomplete (TLC) even after 12 h. Longer reaction times resulted in decomposition of the product (TLC). However, intermediate **21** could be isolated and different aryl substituents were introduced at the N1 position using appropriate benzyl bromides under basic conditions to afford **22a**−**c**. The isolation of pure **22a**−**c** was tedious and required extensive column chromatography. Longer reaction times gave additional products (TLC), further complicating the purification. Thus, the crude benzylic substituted pyrroles **22a**−**c** were directly subjected to condensation with 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea with 5 equiv of acetic acid as catalyst and MeOH to afford **23a**−**c**. Compounds **23a**−**c** were used for the next step without further purification. Hydrolysis of the carbamate group with aqueous sodium hydroxide at 55 °C afforded the 2-amino-4-oxo-6-methylpyrrolo[3,2-*d*]pyrimidines **5** and **6** and the corresponding acid **24** in yields that ranged from 30% to 41% (over three steps). The yield for the 4-fluorophenyl substituted compound **7** was 20% (over three steps).

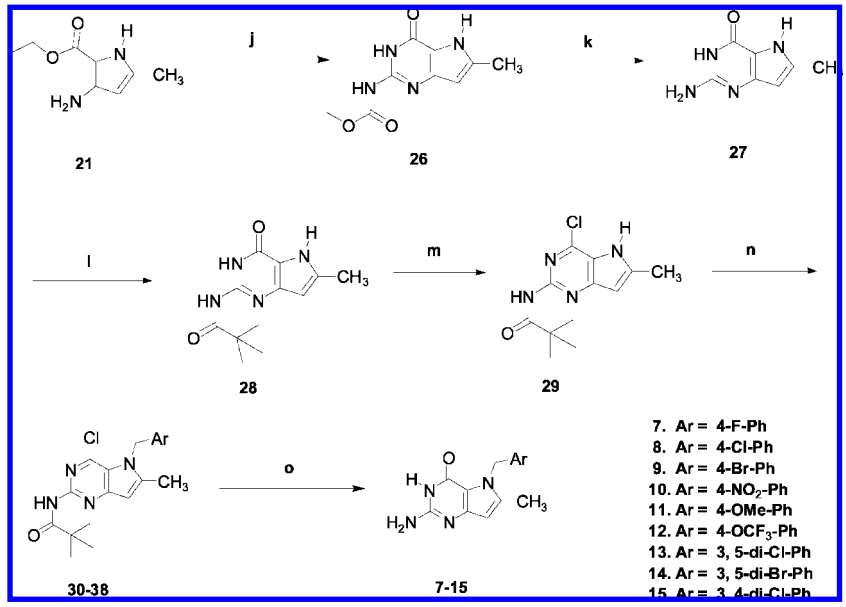
Conversion of free acid **24** (Scheme 2) to the corresponding L-glutamic acid diethyl ester **25** involved conventional peptide coupling with L-glutamic acid diethyl ester hydrochloride using 2-chloro-4,6-dimethoxy-l,3,5-triazine[43] followed by chromatographic purification to afford the coupled product **25** in 73% yield. The ^{1}H NMR of **25** revealed the newly formed peptide NH proton at 8.40 ppm as a doublet, which exchanged on addition of D_2O. Hydrolysis of **25** with aqueous NaOH at room temperature, followed by acidification with 3 N HCl in the cold, afforded compound **4** in 60% yield. Once again, because of the tedious separation and the inability to characterize intermediates **22a**−**d** and **23a**−**d** (Scheme 1), it was necessary to devise an alternative synthetic strategy for the target compounds **7**–**15**, and this is shown in Scheme 3. Elliott et al. reported the synthesis of pyrrolo[3,2-*d*]pyrimidines. This method was modified to afford intermediate **27** via cyclocondensation of the pyrrole **21** with 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea followed by removal of the carbamate moiety with aqueous NaOH. This afforded an efficient synthesis of the key intermediate **27** in 63% yields (over two steps).

We initially attempted a direct N-benzylation in the N5 position of pyrrolo[3,2-*d*]pyrimidine **27** using the appropriate benzyl bromides under basic conditions. Unfortunately direct benzylation of **27** gave a mixture of the desired product contaminated with a several side products (TLC). Chromatographic and recrystallization attempts to purify the product were fruitless. Failure of the above method led us to explore a new alternative strategy where the pyrrolo[3,2-*d*]pyrimidine **27** could be converted to its 2-protected 4-chloro derivative **29**, which could undergo N-benzylation and depivaloylation to afford target compounds **7**–**15**. The 2-amino group of **27** was successfully protected with pivaloyl chloride to afford **28**. Compound **28** was converted to the 4-chloro derivative **29** by treatment with phosphorus oxychloride in 86% yield. The N-benzylated pyrrolo[3,2-*d*]pyrimidines **30**–**38** were synthesized from **29** with appropriately substituted benzyl bromides in yields ranging from 65% to 74%. Hydrolysis of the benzylic substituted pyrrolo[3,2-*d*]pyrimidines **30**–**38** under basic conditions gave the target compounds **7**–**15** in 68−75% yields. The structures of target compounds **4**–**15** and all intermediates were confirmed by ^{1}H NMR and elemental analysis. The total yield for **7** in Scheme 3 from **21** to **7** was 19% (over six steps), which is comparable to 20% (over three steps) in Scheme 1. Though there is no difference in the total yield, there are three additional steps in Scheme 3, which allow the purification and characterization of all intermediates, involved in the synthesis of target compounds **7**–**15**.

Biological Evaluation and Discussion

The classical and nonclassical analogues **4**–**15** were evaluated as inhibitors of human, *Escherichia coli* (*E. coli*) and *Toxoplasma gondii* (*T. gondii*) DHFR[45] and TS.[46] The inhibitory potencies (IC_{50}) are listed in Table 1 and compared with PDDF, MTX, and trimethoprim. The classical analogue **4** was a potent dual inhibitor of human TS (IC_{50} = 46 nM) and human DHFR (IC_{50} = 120 nM). Against human TS, **4** was similar in potency to the previously reported **1** and about 2-fold more potent than PDDF and 206-fold more potent than pemetrexed. This result indicates that isomeric structural modification of the pyrrolo[2,3-*d*]pyrimidine scaffold to a pyrrolo[3,2-*d*]pyrimidine maintains high potency against human TS and increases human DHFR inhibition by 18-fold for **4** compared with lead compound **1**. Thus the principal goal to increase the human DHFR inhibitory activity of **1** without compromising the potent human TS inhibitory activity was accomplished.

Scheme 3[a]



[a] Reagents and conditions: (j) (1) 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea, AcOH, MeOH, room temp, 12 h; (2) NaOMe, MeOH, room temp, 2 h; (k) 1 N NaOH, 55 °C, 3 h; (l) PivCl, DMAP, TEA, dichloroethane, 50 °C, 12 h; (m) $POCl_3$, reflux, 3 h; (n) NaH, benzyl bromides, DMF, 0 °C, 3 h; (o) 2 N NaOH, 1,4-dioxane, reflux, 24 h.

Table 1. Inhibitory Concentrations (IC_{50} in μM) against TS and DHFR[a]

	TS (μM)			DHFR (μM)			
compd	human[b]	*E. coli*[b]	*T. gondii*[e]	human[c]	*E. coli*[d]	*T. gondii*[e]	rh/tg[j]
1	0.054	0.27	nd	2.1	21	nd	nd
2[f]	0.13	45	nd	>54	nd	nd	nd
3[f]	0.15	13	nd	>62 (0)[g]	nd	nd	nd
4	0.046	0.069	0.23	0.12	>23 (23)	0.023	5.2
5	20	>39 (27)	>39 (0)	>39	>39	3.9	>10
6	2.3	12	15	>37	39	20	>1.8
7	3.7	31	22	>37 (18)	>37 (0)	1.8	>20.5
8	2.9	1.5	17	>35 (32)	>35 (0)	0.35	>100
9	1.3	7.5	18	>35 (31)	>30 (10)	0.27	>129.6
10	1.4	14	28	33	>33 (0)	0.33	100
11	1.5	15	17	>35 (10)	>35 (0)	3.5	>10
12	1.3	7.5	13	>35 (16)	>35 (0)	0.3	>116.6
13	>26 (0)	>26 (15)	>26 (13)	>31 (0)	>31 (0)	15	>2.1
14	10	>20 (36)	>20 (24)	>24 (0)	>24 (0)	24	>1
15	5.4	27	27	>32 (0)	>32 (0)	1.6	>20
pemetrexed[h]	9.5	76	2.8	6.6	230	0.43	15.35
PDDF[i]	0.085	0.019	0.43	1.9	23	0.22	8.6
MTX	nd	nd	nd	0.02	0.0088	0.033	0.6
trimethoprim[f]	nd	nd	nd	>340 (22)	0.01	6.8	>50

[a] The percent inhibition was determined at a minimum of four inhibitor concentrations within 20% of the 50% point. The standard deviations for determination of 50% points were within ±10% of the value given. nd = not determined. [b] Kindly provided by Dr. Frank Maley, New York State Department of Health. [c] Kindly provided by Dr. Andre Rosowsky, Dana-Farber Cancer Institute, Boston, MA. [d] Kindly provided by Dr. R. L. Blakley, St. Jude Children's Hospital, Memphis, TN. [e] Kindly provided by Dr. Karen Anderson, Yale Univerisy, New Haven, CT. [f] Data derived from ref 39. [g] Numbers in parentheses indicate the percent inhibition at the stated concentration. [h] Kindly provided by Dr. Chuan Shih, Eli Lilly and Co. [i] Kindly provided by Dr. M. G. Nair, University of South Alabama. [j] rh/tg is the selectivity ratio for *T. gondii* DHFR and is the (IC_{50} against rhDHFR)/(IC_{50} against *T. gondii* DHFR).

Usually a 2-amino-4-oxo substitution on the pyrimidine ring of bicyclic systems such as quinazolines and pyrrolo[2,3-*d*]pyrimidines is considered to be important for potent TS inhibitory activity. Compound **4** is a 2-amino-4-oxo system, and its TS inhibitory activity is as anticipated. However, the human DHFR and *T. gondii* DHFR inhibitory activities of **4** are not as easily explained. 2,4-Diamino substitution on the pyrimidine ring of bicyclic systems is usually important for DHFR inhibitory activity. Compound **4** is not a 2,4-diamino substituted system. The DHFR inhibitory activity of 2-amino-4-oxo-pyrrolo[2,3-*d*]pyrimidines such as **1** can be explained by rotating the NH_2−C2 bond by 180° such that the N7 functions as the 4-amino moiety.[47] Since analogue **4** is a pyrrolo[3,2-*d*]pyrimidine, we cannot explain the increased DHFR inhibitory activity on the basis of a NH_2−C2 bond rotation of 180°. The increase in activity against human DHFR of **4** over **1** can be explained on the basis of our molecular modeling where the C7 of **4** interacts with Phe31 of human DHFR and the N7 of **1** would not have the same interaction. Similar modeling with *E. coli* DHFR in which a Leu28 replaces Phe31 in human DHFR indicates that the C7 of **4** is >4.0 Å away from the Leu28 and would not have a productive interaction. This lack of a hydrophobic interaction would explain the poor *E. coli* DHFR inhibitory result with **4**. There are no X-ray crystal structures available for *T. gondii* DHFR/TS. However, recent homology modeling[48] indicates that *T. gondii* DHFR contains two phenylalanine residues, Phe32 and Phe35, in its active site, similar to Phe31 and Phe34 in the active site of human DHFR. Thus,

the potent inhibitory activity of **4** and the nonclassical analogues against *T. gondii* could be due, in part, to the interaction of the C7 of the pyrrolo[3,2-*d*]pyrimidine with Phe32 of *T. gondii* DHFR. Nonclassical compounds **8**–**10** and **12** are also reasonably potent against *T. gondii* DHFR and have 100- to >100-fold selectivity over human DHFR. The IC_{50} values against *T. gondii* DHFR are about 20-fold better than the clinically used trimethoprim.

In summary, the 2-amino-4-oxo-5,6-disubstituted pyrrolo[3,2-*d*]-pyrimidine classical antifolate **4** and 11 nonclassical analogues **5**–**15** were synthesized as potential dual TS and DHFR inhibitors. Biological studies indicated that **4** was a potent dual TS−DHFR inhibitor with nanomolar inhibition for both enzymes. Compound **4** maintained the TS inhibitory activity of **1** but increased the human DHFR inhibition by 18-fold. To our knowledge this is the most potent dual TS−DHFR inhibitor reported. Currently, classical analogue **4** is under further evaluation by the National Cancer Institute (NCI) as an antitumor agent. The nonclassical analogues were marginally active against TS and less active against DHFR except compounds **8**–**10** and **12**, which showed high potency and selectivity against *T. gondii* DHFR comparable to trimethoprim.

Experimental Section

Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P_2O_5 at 80 °C. 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 (^{1}H 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 an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with a 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 a 230–400 mesh silica gel (Fisher, Somerville, NJ) column. 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 in spite of 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. or Fisher Scientific and were used as received.

Diethyl {[(*E*/*Z*)-2-Cyano-1-methylvinyl]amino}malonate (20). To a suspension of an *E*/*Z* mixture of 3-aminobut-2-enenitrile **16** (3 g, 35.1 mmol) in MeOH (60 mL) was added diethyl aminomalonate hydrochloride **17** (7.9 g, 36.8 mmol). The resulting mixture was stirred at room temperature for 5 h. TLC showed the disappearance of the starting materials and the formation of one major spot at $R_f = 0.26$ (ethyl acetate/*n*-hexane, 1:2). The reaction solvent was diluted with ethyl acetate (50 mL), washed with brine (30 mL × 2), and dried over $MgSO_4$. To the organic solvent 15 g of silica gel was added, and the mixture was evaporated to dryness under reduced pressure. This silica gel plug was loaded on a dry silica gel column (2 cm × 15 cm) and flash-chromatographed initially with *n*-hexane (200 mL) and then sequentially with 500 mL of 5% ethyl acetate in *n*-hexane, 500 mL of 10% ethyl acetate in *n*-hexane, and 500 mL of 15% ethyl acetate in *n*-hexane. Fractions containing the desired product (TLC) were pooled and evaporated to afford 6.74 g (80%) of **20** as an off-white solid: mp 50–52 °C; ^{1}H NMR (DMSO-d_6) δ 1.18–1.23 (t, 3 H, $J = 6.9$ Hz, CH_3), 2.05 (s, 6 H, CH_3), *E* isomer 3.94 (s, 1 H, CH), 4.18–4.24 (m, 4 H, CH_2), 4.96 (d, 1 H, $J = 7.8$ Hz, CH), *Z* isomer 5.32 (s, 1 H, CH), 7.51–7.56 (d, 2 H, $J = 7.8$ Hz, NH). Anal. ($C_{11}H_{16}N_2O_4$) C, H, N.

Ethyl 3-Amino-5-methyl-1*H*-pyrrole-2-carboxylate (21). A solution of NaOEt in EtOH (0.5 M, 120 mL) was added slowly to a stirred solution of **20** (1 g, 4 mmol) in 20 mL of EtOH. The reaction mixture was stirred for 6 h at 60 °C and cooled to room temperature. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel with 10% ethyl acetate/*n*-hexane as the eluent to yield **21** (0.45 g, 65%) as an off-white solid: mp 85–87 °C; $R_f = 0.36$ (ethyl acetate/*n*-hexane, 1:1); ^{1}H NMR (DMSO-d_6) δ 1.24 (t, 3 H, $J = 6.4$ Hz, CH_3), 2.03 (s, 3 H, CH_3), 4.12 (q, 2 H, $J = 6.4$ Hz, CH_2), 4.91 (s, 2 H, NH_2), 5.26 (s, 1 H, CH), 10.21 (s, 1 H, NH). Anal. ($C_8H_{12}N_2O_2$) C, H, N.

2-Amino-5-benzyl-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (5). To a solution of ethyl 3-amino-5-methyl-1*H*-pyrrole-2-carboxylate **21** (0.5 g, 3 mmol) in dry DMF (10 mL) was added slowly NaH (0.09 g, 3.6 mmol) under nitrogen at room temperature. The resulting mixture was stirred for about 15 min when there was no more gas produced, and then (bromomethyl)-benzene (0.52 g, 3 mmol) in THF (3 mL) was added dropwise. The reaction mixture was stirred at room temperature for 4 h, and 20 mL of water was added carefully to quench the reaction. The sample was extracted with $CHCl_3$ (25 mL × 4), and the organic phases were combined and dried (Na_2SO_4). Evaporation of the solvent offered a gummy residue, which was used for the next step without purification. The gummy residue (~3 mmol) was dissolved in MeOH (10 mL), and 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea (0.7 g, 3.3 mmol) was added followed by AcOH (1.0 g, 15 mmol). The mixture was stirred at room temperature overnight and became a thick paste. NaOMe in MeOH (25%) (7 mL, 22 mmol) was added, and stirring was continued at room temperature overnight. The mixture was neutralized with AcOH, and the methanol was removed under reduced pressure. To the residue was added water (20 mL), and the pH value was adjusted to 10–11 by adding $NH_3 \cdot H_2O$. The solid was collected by filtration and washed well with water. The resulting solid was added to 1 N NaOH (2 mL), and the mixture was heated at 55 °C for 3 h. The mixture was cooled and neutralized with acetic acid, and then the pH of the mixture was adjusted to 10–11 by adding $NH_3 \cdot H_2O$. The solid was collected by filtration. The solid was dissolved in MeOH and $CHCl_3$ (1:1, 5 mL), and 1 g of silica gel was added. The solvent was removed under reduced pressure to form a plug, which was loaded at the top of a silica gel column (15 mm × 150 mm) and eluted with 5% MeOH in $CHCl_3$ (containing 0.5% $NH_3 \cdot H_2O$). Fractions containing the desired compound (TLC) were pooled and evaporated to afford **5** (35% for three steps) as an off-white solid: mp >250 °C; $R_f = 0.46$ (MeOH/$CHCl_3$, 1:10); ^{1}H NMR (DMSO-d_6) δ 2.14 (s, 3 H, CH_3), 5.54 (s, 2 H, CH_2), 5.75 (br s, 2 H, NH_2), 5.80 (s, 1 H, CH), 7.00–7.32 (m, 5 H, C_6H_5), 10.35 (s, 1 H, NH). Anal. ($C_{14}H_{14}N_4O$) C, H, N.

2-Amino-6-methyl-5-(pyridin-4-ylmethyl)-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (6). Compound **6** was synthesized as described for **5**: yield 31%, mp >250 °C; $R_f = 0.31$ (MeOH/$CHCl_3$, 1:10); ^{1}H NMR (DMSO-d_6) δ 2.13 (s, 3 H, CH_3), 5.57 (s, 2 H, CH_2), 5.79 (br s, 2 H, NH_2), 5.85 (s, 1 H, CH), 6.94 (d, 2 H, $J = 7.2$ Hz, C_5NH_4), 8.49 (d, 2 H, $J = 7.2$ Hz, C_5NH_4), 10.39 (s, 1 H, NH). Anal. ($C_{13}H_{13}N_5O \cdot 0.2H_2O$) C, H, N.

2-Amino-5-(4-fluorobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (7). Compound **7** was synthesized as described for **5**: yield 20%, mp 185–186 °C; $R_f = 0.40$ (MeOH/$CHCl_3$, 1:5); ^{1}H NMR (DMSO-d_6) δ 2.14 (s, 3 H, CH_3), 5.50 (s, 2 H, CH_2), 5.75 (s, 1 H, CH), 5.60 (s, 2 H, NH_2), 7.07–7.10 (m, 4 H, C_6H_4), 10.36 (s, 1 H, NH). Anal. ($C_{14}H_{13}FN_4O \cdot 0.11CH_3COOH$) C, H, N, F.

4-[(2-Amino-6-methyl-4-oxo-3,4-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)methyl]benzoic Acid (24). Compound **24** was synthesized as described for **5**: yield 41%; mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 2.15 (s, 3 H, CH_3), 5.62 (s, 2 H, CH_2), 5.87 (s, 1 H, CH), 6.05 (br s, 2 H, NH_2), 7.12 (d, 2 H, $J = 6.9$ Hz, C_6H_4), 7.89 (d, 2 H, $J = 6.9$ Hz, C_6H_4), 11.15 (s, 1 H, NH). Anal. ($C_{15}H_{14}N_4O_3$) C, H, N.

Diethyl *N*-{4-[(2-Amino-6-methyl-4-oxo-3,4-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)methyl]benzoyl}-L-glutamate (25). To a suspension of benzoic acid **24** (150 mg, 0.5 mmol) in DMF (10 mL) at 25 °C was added *N*-methylmorpholine (NMM, 0.062 mL, 0.57 mmol) followed by 2-chloro-4,6-dimethoxy-1,3,5-triazine (100 mg, 0.57 mmol), and the resulting solution was stirred at 25 °C for 2 h. NMM (0.062 mL, 0.57 mmol) was added to the solution followed by L-glutamic acid diethyl ester hydrochloride (140 mg, 0.59 mmol). The resulting mixture was stirred at 25 °C for 4 h and concentrated in vacuo (0.5 mmHg), and the residue was dissolved in CH_2Cl_2 (50 mL). The CH_2Cl_2 solution was washed with 5% $NaHCO_3$, dried (Na_2SO_4), and concentrated in vacuo. The residue was chromatographed on silica gel with 5% MeOH in CH_2Cl_2 as the eluent. Fractions containing the product (TLC) were combined and evaporated to give **25** as a white solid (73%): mp 86–88 °C; $R_f = 0.75$ (MeOH/$CHCl_3$, 1:10); ^{1}H NMR (DMSO-d_6) δ 1.10–1.21 (m, 6 H, 2 × OCH_2CH_3), 1.90–2.15 (m, 2 H, CH_2), 2.15 (s, 3 H, CH_3), 2.40 (t, 2 H, CH_2), 4.00–4.18 (m, 4 H, 2 × OCH_2CH_3), 4.40 (m, 1 H, CH), 5.60 (s, 2 H, CH_2), 5.78 (br s, 2 H, NH_2), 5.82 (s, 1 H, CH), 7.12 (d, 2 H,, $J = 6.9$ Hz, C_6H_4), 7.78 (d, 2 H,, $J = 6.9$ Hz, C_6H_4), 8.40 (d, 1 H, $J = 7.4$ Hz, NH), 10.38 (s, 1 H, NH). Anal. ($C_{24}H_{29}N_5O_6 \cdot 0.1CH_3OH$) C, H, N.

***N*-{4-[(2-Amino-6-methyl-4-oxo-3,4-dihydro-5*H*-pyrrolo[3,2-*d*]pyrimidin-5-yl)methyl]benzoyl}-L-glutamic Acid (4).** To a solution of **25** (193 mg, 0.4 mmol) in methanol (4 mL) was added 1 N NaOH (2 mL) at 0 °C. After the reaction mixture was stirred at room temperature for 3–4 h, the TLC showed the disappearance of the starting material and a new spot appeared at the original position (MeOH/$CHCl_3$, 1:10). The methanol was evaporated under reduced pressure, the residue was dissolved in water (4 mL), and then the solution was cooled to 0 °C and acidified carefully to pH 4 with dropwise addition of 3 N HCl. The suspension was left at 5 °C for 24 h and filtered. The residue was washed well with water and ether and dried to offer **4** (102 mg, 60%) as an off-white solid: mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 1.82–2.14 (m, 2 H, $-CH_2$), 2.14 (s, 3 H, CH_3), 2.38 (t, 2 H, $J = 6.9$ Hz, CH_2), 4.38 (m, 1 H, CH), 5.61 (s, 2 H, CH_2), 5.80 (br s, 2 H, NH_2), 5.82 (s, 1 H, CH), 7.16 (d, 2 H, $J = 6.9$ Hz, C_6H_4), 7.82 (d, 2 H, $J = 6.9$ Hz, C_6H_4), 8.54 (d, 1 H, $J = 7.4$ Hz, NH), 10.50 (s, 1 H, NH), 12.40 (s, br, 2 H, 2 × COOH). Anal. ($C_{20}H_{21}N_5O_6 \cdot 0.6H_2O$) C, H, N.

Methyl (6-Methyl-4-oxo-4,5-dihydro-3*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl)carbamate (26). The pyrrole **21** (2.68 g, 16 mmol) was dissolved in MeOH (40 mL), and 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea (3.74 g, 18 mmol) was added followed by AcOH (4.6 mL). The mixture was stirred at room temperature overnight and became a thick paste. To the reaction mixture was added 45 mL of NaOMe in MeOH (25%), and stirring was continued at room temperature for 2 h. The mixture was neutralized with AcOH, and the solid was collected by filtration and washed well with water. After drying, **26** (2.44 g, 69%) was obtained as an off-white powder: mp 234–236 °C; TLC $R_f = 0.22$ (MeOH/$CHCl_3$, 1:5); ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 3 H, CH_3), 3.73 (s, 3 H, OCH_3), 5.95 (s, 1 H, CH), 10.90 (s, 1 H, NH), 11.10 (s, 1 H, NH), 11.76 (s, 1 H, NH). Anal. ($C_9H_{10}N_4O_3 \cdot 0.79C_6H_6 \cdot 0.55C_7H_8O_3S$) C, H, N.

2-Amino-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (27). To a 200 mL round-bottomed flask was added **26** (1 g, 4.5 mmol) suspended in 1 N NaOH (35 mL). The reaction mixture was heated at 55 °C for 3 h. The resulting solution was cooled in an ice bath and neutralized with AcOH. The precipitated solid was collected by filtration, washed with brine, and dried in vacuo to afford 0.67 g (92%) of **27** as a white solid: mp 252–254 °C; TLC $R_f = 0.15$ (MeOH/$CHCl_3$, 1:5); ^{1}H NMR (DMSO-d_6) δ 2.20 (s, 3 H, CH_3), 5.65 (s, 1 H, CH), 5.65 (s, 2 H, NH_2), 10.21 (s, 1 H, NH), 11.15 (s, 1 H, NH). Anal. ($C_7H_8N_4O \cdot 0.73H_2O$) C, H, N.

2,2-Dimethyl-*N*-(6-methyl-4-oxo-4,5-dihydro-3*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl)propanamide (28). To a 250 mL round-bottomed flask was added **27** (1.37 g, 8 mmol) suspended in 40 mL of dichloroethane. Then trimethylacetyl chloride (1.99 mL, 16 mmol), DMAP (0.13 g, 1 mmol), and triethylamine (2.68 mL) were added. The mixture was stirred overnight at 50 °C. The resulting mixture was cooled, diluted with dichloromethane (50 mL), washed with brine (40 mL × 2), dried over Na_2SO_4, and concentrated in vacuo. To this solution were added methylene chloride (30 mL) and silica gel (5 g), and the solvent evaporated. The silica gel plug obtained was loaded onto a silica gel column and eluted with 9:1 ethyl acetate/*n*-hexane The fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 1.33 g (67%) of **28** as a white solid: TLC $R_f = 0.47$ (MeOH/$CHCl_3$, 1:10); mp 156–157 °C; ^{1}H NMR (DMSO-d_6) δ 1.19 (s, 9 H, CH_3), 2.29 (s, 3 H, CH_3), 5.97 (s, 1 H, CH), 10.72 (s, 1 H, NH), 11.78 (s, 1 H, NH), 11.87 (s, 1H, NH). Anal. ($C_{12}H_{16}N_4O_2 \cdot 0.15CH_3COCH_3$) C, H, N.

***N*-(4-Chloro-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl)-2,2-dimethylpropanamide (29).** To a 100mL round-bottomed flask was added **28** (1.16 g, 4.67 mmol) suspended in 30 mL of phosphorus oxychloride. The reaction mixture was heated at reflux with stirring in an anhydrous atmosphere for 3 h. The dark-orange solution was allowed to cool to room temperature and concentrated in vacuo. Water (20 mL) was then added to the residue at 0 °C with vigorous stirring to give an exothermic reaction. Concentrated aqueous ammonium hydroxide was added to attain pH 5 to give a precipitate, which was collected by filtration, washed with water (3 × 5 mL), and dried in vacuo. The crude product was purified by silica gel column chromatography with 2% MeOH/$CHCl_3$. Recrystallization from MeOH afforded 1.07 g (86%) of **29** as a white solid: TLC $R_f = 0.35$ (MeOH/$CHCl_3$, 1:10); mp 162–163 °C; ^{1}H NMR (DMSO-d_6) δ 1.19 (s, 9 H, CH_3), 2.47 (s, 3 H, CH_3), 6.33 (s, 1 H, CH), 9.85 (s, 1 H, NH), 12.07 (s, 1 H, NH). Anal. ($C_{12}H_{15}ClN_4O$) C, H, N, Cl.

***N*-[4-Chloro-5-(4-fluorobenzyl)-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (30).** To a mixture of NaH (33 mg, 1.3 mmol) and anhydrous DMF (10 mL) at 0 °C was added dropwise **29** (0.31 g, 1.08 mmol) in DMF (5 mL), and the mixture was stirred for 20 min. 1-(Bromomethyl)-4-fluorobenzene (0.27 g, 1.40 mmol) was added, and the mixture was stirred at 0 °C for 3 h. Analysis (TLC) of the product mixture revealed a complete conversion of starting material to product(s). The reaction mixture was removed under reduced pressure, and the crude product was purified by column chromatography on silica gel with 15% ethyl acetate/*n*-hexane as the eluent to afford 0.28 g (70%) of **30** as a white power: TLC $R_f = 0.30$ (MeOH/$CHCl_3$, 1:10); mp 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 1.20 (s, 9 H, CH_3), 2.42 (s, 3 H, CH_3), 5.68 (s, 2 H, CH_2), 6.54 (s, 1 H, CH), 6.94–7.14 (m, 4 H, C_6H_4), 9.94 (s, 1 H, NH). Anal. ($C_{19}H_{20}ClFN_4O \cdot 0.06H_2O$) C, H, N, F, Cl.

***N*-[4-Chloro-5-(4-chlorobenzyl)-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (31).** Compound **31** was synthesized as described for **30**: yield 65%; TLC $R_f = 0.35$ (MeOH/$CHCl_3$, 1:10); mp 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 1.19 (s, 9 H, CH_3), 2.40 (s, 3 H, CH_3), 5.67 (s, 2 H, CH_2), 6.53 (s, 1 H, CH), 6.88–7.35 (m, 4 H, C_6H_4), 9.94 (s, 1H, NH). Anal. ($C_{19}H_{20}Cl_2N_4O \cdot 0.12C_6H_{14}$) C, H, N, Cl.

***N*-[5-(4-Bromobenzyl)-4-chloro-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (32).** Compound **32** was synthesized as described for **30**: yield 72%; TLC $R_f = 0.38$ (MeOH/$CHCl_3$, 1:10); mp 182–183 °C; ^{1}H NMR (DMSO-d_6) δ 1.18 (s, 9 H, CH_3), 2.40 (s, 3 H, CH_3), 5.65 (s, 2 H, CH_2), 6.53 (s, 1 H, CH), 6.82–7.53 (m, 4 H, C_6H_4), 9.93 (s, 1 H, NH). Anal. ($C_{19}H_{20}BrClN_4O \cdot 0.01H_2O$) C, H, N, Br, Cl.

***N*-[4-Chloro-6-methyl-5-(4-nitrobenzyl)-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (33).** Compound **33** was synthesized as described for **30**: yield 71%; TLC $R_f = 0.37$ (MeOH/$CHCl_3$, 1:10); mp 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 1.11 (s, 9 H, CH_3), 2.33 (s, 3 H, CH_3), 5.75 (s, 2 H, CH_2), 6.48 (s, 1 H, CH), 7.07–8.09 (m, 4 H, C_6H_4), 9.92 (s, 1 H, NH). Anal. ($C_{19}H_{20}ClN_5O_3 \cdot 0.25CH_3COCH_3$) C, H, N, Cl.

***N*-[4-Chloro-5-(4-methoxybenzyl)-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (34).** Compound **34** was synthesized as described for **30**: yield 72%; TLC $R_f = 0.40$ (MeOH/$CHCl_3$, 1:10); mp 178–179 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (s, 9 H, CH_3), 2.43 (s, 3 H, CH_3), 5.63 (s, 2 H, CH_2), 6.53 (s,

1 H, CH), 6.86 (m, 4 H, C_6H_4), 9.94 (s, 1 H, NH). HRMS calcd for $C_{20}H_{23}N_4O_2NaCl$ 409.1407, found 409.1416.

***N*-{4-Chloro-6-methyl-5-[4-(trifluoromethoxy)benzyl]-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl}-2,2-dimethylpropanamide (35).** Compound **35** was synthesized as described for **30**: yield 70%; TLC R_f = 0.40 (MeOH/$CHCl_3$, 1:10); mp 182–183 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (s, 9 H, CH_3), 2.43 (s, 3 H, CH_3), 5.81 (s, 2 H, CH_2), 6.58 (s, 1 H, CH), 7.10–7.71 (m, 4 H, C_6H_4), 9.96 (s, 1 H, NH). Anal. ($C_{20}H_{20}ClF_3N_4O_2$) C, H, N, F, Cl.

***N*-[4-Chloro-5-(3,5-dichlorobenzyl)-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (36).** Compound **36** was synthesized as described for **30**: yield 72%; TLC R_f = 0.36 (MeOH/$CHCl_3$, 1:10); mp 182–183 °C; ^{1}H NMR (DMSO-d_6) δ 1.20 (s, 9 H, CH_3), 2.42 (s, 3 H, CH_3), 5.71 (s, 2 H, CH_2), 6.56 (s, 1 H, CH), 6.90–7.53 (m, 3 H, C_6H_3), 9.96 (s, 1 H, NH). Anal. ($C_{19}H_{19}Cl_3N_4O$) C, H, N, Cl.

***N*-[4-Chloro-5-(3,5-dibromobenzyl)-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (37).** Compound **37** was synthesized as described for **30**: yield 74%; TLC R_f = 0.35 (MeOH/$CHCl_3$, 1:10); mp 182–183 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (s, 9 H, CH_3), 2.43 (s, 3 H, CH_3), 5.72 (s, 2 H, CH_2), 6.58 (s, 1 H, CH), 7.08–7.77 (m, 3 H, C_6H_3), 9.97 (s, 1 H, NH). Anal. ($C_{19}H_{19}$ $Br_2ClN_4O \cdot 0.10C_6H_{14}$) C, H, N, Br, Cl.

***N*-[4-Chloro-5-(3,4-dichlorobenzyl)-6-methyl-5*H*-pyrrolo[3,2-*d*]pyrimidin-2-yl]-2,2-dimethylpropanamide (38).** Compound **38** was synthesized as described for **30**: yield 72%; TLC R_f = 0.31 (MeOH/$CHCl_3$, 1:10); mp 162–164 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (s, 9 H, CH_3), 2.44 (s, 3 H, CH_3), 5.73 (s, 2 H, CH_2), 6.58 (s, 1 H, CH), 6.92 (s, 1 H, CH), 7.36–7.54 (m, 3 H, C_6H_3), 9.97 (s, 1 H, NH). HRMS calcd for $C_{19}H_{20}N_4OCl_3$ 425.0703, found 425.0671.

2-Amino-5-(4-fluorobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (7). To a 100 mL round-bottomed flask was added **30** (50 mg, 0.13 mmol) suspended in 2 N NaOH (10 mL) and 1,4-dioxane (5 mL). The reaction mixture was stirred under reflux for 24 h. The solution was cooled to room temperature and neutralized with glacial AcOH. The resulting precipitate was collected by filtration and dried in vacuo. The crude product was purified by silica gel column chromatography, eluting with 5% MeOH/$CHCl_3$ to yield 25 mg (73%) of **7** as a white powder: TLC R_f = 0.40 (MeOH/$CHCl_3$, 1:5); mp 185–186 °C; ^{1}H NMR (DMSO-d_6) δ 2.14 (s, 3 H, CH_3), 5.50 (s, 2 H, CH_2), 5.75 (s, 1 H, CH), 5.60 (s, 2 H, NH_2), 7.07–7.10 (m, 4 H, C_6H_4), 10.36 (s, 1 H, NH). Anal. ($C_{14}H_{13}FN_4O \cdot 0.11CH_3COOH$) C, H, N, F.

2-Amino-5-(4-chlorobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (8). Compound **8** was synthesized as described for **7**: yield 74%; TLC R_f = 0.42 (MeOH/$CHCl_3$, 1:5); mp 182–183 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 3 H, CH_3), 5.48 (s, 2 H, CH_2), 5.71 (s, 2 H, NH_2), 5.77 (s, 1 H, CH), 6.98–7.32 (m, 4 H, C_6H_4), 10.32 (s, 1 H, NH). Anal. ($C_{14}H_{13}ClN_4O \cdot 0.27CH_3OH$) C, H, N, Cl.

2-Amino-5-(4-bromobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (9). Compound **9** was synthesized as described for **7**: yield 72%; TLC R_f = 0.40 (MeOH/$CHCl_3$, 1:5); mp 191–192 °C; ^{1}H NMR (DMSO-d_6) δ 2.11 (s, 3 H, CH_3), 5.48 (s, 2 H, CH_2), 5.75 (s, 2 H, NH_2), 5.78 (s, 2 H, CH), 6.93–7.48 (m, 4 H, C_6H_4), 10.37 (s, 1 H, NH). Anal. ($C_{14}H_{13}BrN_4O \cdot 0.18C_2H_5OC_2H_5$) C, H, N, Br.

2-Amino-6-methyl-5-(4-nitrobenzyl)-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (10). Compound **10** was synthesized as described for **7**: yield 75%; TLC R_f = 0.44 (MeOH/$CHCl_3$, 1:5); mp 195–196 °C; ^{1}H NMR (DMSO-d_6) δ 2.15 (s, 3 H, CH_3), 5.68 (s, 2 H, CH_2), 5.78 (s, 2 H, NH_2), 5.86 (s, 2 H, CH), 7.22–7.20 (m, 4 H, C_6H_4), 10.42 (s, 1 H, NH). Anal. ($C_{14}H_{13}N_5O_3 \cdot 0.3CH_3OH$) C, H, N.

2-Amino-5-(4-methoxybenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (11). Compound **11** was synthesized as described for **7**: yield 73%; TLC R_f = 0.41 (MeOH/$CHCl_3$, 1:5); mp 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 2.15 (s, 3 H, CH_3), 5.45 (s, 2 H, CH_2), 5.74 (s, 2 H, NH_2), 5.74 (s, 2 H, CH), 6.83–7.02 (m, 4 H, C_6H_4), 10.35 (s, 1 H, NH). Anal. ($C_{15}H_{16}N_4O_2$) C, H, N.

2-Amino-6-methyl-5-[4-(trifluoromethoxy)benzyl]-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (12). Compound **12** was synthesized as described for **7**: yield 75%; TLC R_f = 0.45 (MeOH/$CHCl_3$, 1:5); mp 148–149 °C; ^{1}H NMR (DMSO-d_6) δ 2.14 (s, 3 H, CH_3), 5.63 (s, 2 H, CH_2), 5.83 (s, 2 H, NH_2), 5.83 (s, 2 H, CH), 7.17–7.69 (m, 4 H, C_6H_4), 10.42 (s, 1 H, NH). Anal. ($C_{15}H_{13}F_3N_4O_2$) C, H, N, F.

2-Amino-5-(3,5-dichlorobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (13). Compound **13** was synthesized as described for **7**: yield 74%; TLC R_f = 0.42 (MeOH/$CHCl_3$, 1:5); mp 188–189 °C; ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 3 H, CH_3), 5.53 (s, 2 H, CH_2), 5.81 (s, 2 H, NH_2), 5.84 (s, 2 H, CH), 7.02–7.49 (m, 4 H, C_6H_4), 10.42 (s, 1 H, NH). Anal. ($C_{14}H_{12}Cl_2N_4O \cdot 0.4CH_3COOH$) C, H, N, Cl.

2-Amino-5-(3,5-dibromobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (14). Compound **14** was synthesized as described for **7**: yield 72%; TLC R_f = 0.41 (MeOH/$CHCl_3$, 1:5); mp 188–189 °C; ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 3 H, CH_3), 5.53 (s, 2 H, CH_2), 5.83 (s, 2 H, NH_2), 5.83 (s, 2 H, CH), 7.20–7.72 (m, 3 H, C_6H_3), 10.44 (s, 1 H, NH). Anal. ($C_{14}H_{12}Br_2N_4O \cdot 0.1C_6H_{14}$) C, H, N, Br.

2-Amino-5-(3,4-dichlorobenzyl)-6-methyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (15). Compound **15** was synthesized as described for **7**: yield 68%; TLC R_f = 0.38 (MeOH/$CHCl_3$, 1:5); mp 195–196 °C; ^{1}H NMR (DMSO-d_6) δ 2.17 (s, 3 H, CH_3), 5.54 (s, 2 H, CH_2), 5.82 (s, 2 H, NH_2), 5.85 (s, 2 H, CH), 7.03 (s, 1 H, C_6H_3), 7.51 (d, 2 H, J = 6.9 Hz, C_6H_3), 10.40 (s, 1 H, NH). HRMS calcd for $C_{14}H_{12}ClN_4O$ m/z = 322.0382, found m/z = 322.0388.

Acknowledgment. This work was supported in part by a grant from the National Institutes of Health, NIAID, Grant AI069966 (A.G.).

Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chan, D. C. M.; Anderson, A.C. Towards Species-Specific Antifolates. *Curr. Med. Chem.* **2006**, *13*, 377–398.
(2) Hawser, S.; Lociuro, S.; Islam, K. Dihydrofolate Reductase Inhibitors as Antibacterial Agents. *Biochem. Pharmacol.* **2006**, *71*, 941–948.
(3) Gmeiner, H. W. Novel Chemical Strategies for Thymidylate Synthase Inhibition. *Curr. Med. Chem.* **2005**, *12*, 191–202.
(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) Rustum, Y. M. Thymidylate Synthase: A Critical Target in Cancer Therapy? *Front. Biosci.* **2004**, *9*, 2467–2473.
(6) Gangjee, A.; Kurup, S.; Namjoshi, O. Dihydrofolate Reductase as a Target for Chemotherapy in Parasites. *Curr. Pharm. Des.* **2007**, *13*, 609–639.
(7) Anderson, A. C. Targeting DHFR in Parasitic Protozoa. *Drug Discovery Today* **2005**, *10*, 121–128.
(8) Jackman, A. L.; Taylor, G. A.; Gibson, W.; Kimbell, R.; Brown, M.; Calvert, A. H.; Judson, I. R.; Hughes, L. R. ICI D1694, a Quinazoline Antifolate Thymidylate Synthase Inhibitor That Is a Potent Inhibitor of L1210 Tumour Cell Growth in Vitro and in Vivo: A New Agent for Clinical Study. *Cancer Res.* **1991**, *51*, 5579–5586.
(9) Taylor, E. C.; Kuhnt, D.; Shih, C.; Rinzel, S. M.; Grindey, G. B.; Barredo, J.; Jannatipour, M.; Moran, R. A Dideazatetrahydrofolate Analogue Lacking a Chiral Center at C-6, *N*-[4-[2-(2-Amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethylbenzoyl]-L-glutamic Acid, Is an Inhibitor of Thymidylate Synthase. *J. Med. Chem.* **1992**, *35*, 4450–4454.
(10) Bertino, J. R.; Kamen, B.; Romanini, A. Folate Antagonists. In *Cancer Medicine*; Holland, J. F., Frei, E., Bast, R. C., Kufe, D. W., Morton, D. L., Weichselbaum, R. R., Eds.; Williams and Wilkins: Baltimore, MD, 1997; Vol. 1, pp 907–921.
(11) Nair, M. G.; Galivan, J.; Maley, F.; Kisliuk, R. L.; Ferone, R. Transport, Inhibition of Tumor Cell Growth and Unambiguous Synthesis of 2-Desamino-2-methyl-*N*10-propargyl-5,8-dideazafolate (DMPDDF) and Related Compounds. *Proc. Am. Assoc. Cancer Res.* **1989**, *30*, 476.

(12) Gibson, W.; Bisset, G. M. F.; Marsham, P. R.; Kelland, L. R.; Judson, I. R.; Jackman, A. L. The Measurement of Polyglutamate Metabolites of the Thymidylate Synthase Inhibitor, ICI D1694, in Mouse and Human Cultured Cells. *Biochem. Pharmacol.* **1993**, *45*, 863–869.

(13) Sikora, E.; Jackman, A. L.; Newell, D. F.; Calvert, A. H. Formation and Retention and Biological Activity of N10-Propargyl-5,8-dideazafolic Acid (CB 3717) Polyglutamates in L1210 Cells in Vitro. *Biochem. Pharmacol.* **1988**, *37*, 4047–4054.

(14) Jackman, A. L.; Newell, D. R.; Gibson, W.; Jodrell, D. I.; Taylor, G. A.; Bishop, J. A.; Hughes, L. R.; Calvert, A. H. The Biochemical Pharmacology of the Thymidylate Synthase Inhibitor 2-Desamino-2-methyl-N10-propargyl-5,8-dideazafolic Acid (IC1 198583). *Biochem. Pharmacol.* **1991**, *41*, 1885–1895.

(15) Nair, M. G.; Abraham, A.; McGuire, J. J.; Kisliuk, R. L.; Galivan, J. Polyglutamylation as a Determinant of Cytotoxicity of Classical Folate Analogue Inhibitors of Thymidylate Synthase and Glycinamide Ribonucleotide Formyltransferase. *Cell. Pharmacol.* **1994**, *1*, 245–249.

(16) Mendelsohn, L. G.; Shih, C.; Chen, V. J.; Habeck, L. L.; Gates, S. B.; Shackelford, K. A. Enzyme Inhibition, Polyglutamation, and the Effect of LY231514 (MTA) on Purine Biosynthesis. *Semin. Oncol.* **1999**, *26*, 42–47.

(17) Shih, C.; Chen, V. J.; Gossett, L. S.; Gates, S. B.; Mackellar, W. C.; Habeck, L. L.; Shackelford, K. A.; Mendelsohn, L. G.; Soose, D. J.; et al. LY231514, a Pyrrolo[2,3-*d*]pyrimidine-Based Antifolate That Inhibits Multiple Folate-Requiring Enzymes *N*-[4-[2-(2-Amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)-ethyl]-benzoyl]-L-glutamic Acid (LY231514), Is a Novel Pyrrolo[2,3-*d*]pyrimidine-Based Antifolate Currently Undergoing Extensive Phase II Clinical Trials. *Cancer Res.* **1997**, *57*, 1116–1123.

(18) Schultz, R. M.; Patel, V. F.; Worzalla, J. F.; Shih, C. Role of Thymidylate Synthase in the Antitumor Activity of the Multitargeted Antifolate, LY231514. *Anticancer Res.* **1999**, *19*, 437–443.

(19) Mesothelioma and safety information from Eli Lilly: http://www.alimta.com/.

(20) Bisset, G. M. F.; Bavetsias, V.; Thornton, T. J.; Pawelczak, K.; Calvert, A. H.; Hughes, L. R.; Jackman, A. L. The Synthesis and Thymidylate Synthase Inhibitory Activity of L-γ-L-Linked Dipeptide and L-γ-Amide Analogues of 2-Desamino-2-methy1-*N*10-propargyl-5,8-dideazafolic acid (IC1 198583). *J. Med. Chem.* **1994**, *37*, 3294–3302.

(21) Cao, W.; Matherly, L. H. Structural Determinants of Folate and Antifolate Membrane Transport by the Reduced Folate Carrier. In *Drug Metabolism and Transport*; Lash, L. H., Ed.; Humana Press: Totowa, NJ, 2005; pp 291–318.

(22) Fry, D. W.; Jackson, R. C. Membrane Transport Alterations as a Mechanism of Resistance to Anticancer Agents. *Cancer Surv.* **1986**, *5*, 47–49.

(23) Schornagel, J. H.; Pinard, M. F.; Westerhof, G. R.; Kathmann, I.; Molthoff, C. F. M.; Jolivet, J.; Jansen, G. Functional Aspects of Membrane Folate Receptors Expressed in Human Breast Cancer Lines with Inherent and Acquired Transport-Related Resistance to Methotrexate. *Proc. Am. Assoc. Cancer Res.* **1994**, *35*, 302.

(24) Wong, S. C.; Zhang, L.; Witt, T. L.; Proefke, S. A.; Bhushan, A.; Matherly, L. H. Impaired Membrane Transport in Methotrexate-Resistant CCRF-CEM Cells Involves Early Translation Termination and Increased Turnover of a Mutant Reduced Folate Carrier. *J. Biol. Chem.* **1999**, *274*, 10388–10394.

(25) Jackman, A. L.; Gibson, W.; Brown, M.; Kimbell, R.; Boyle, F. T. The Role of the Reduced Folate Carrier and Metabolism to Intracellular Polyglutamates for the Activity of ICI D1694. *Adv. Exp. Med. Biol.* **1993**, *339*, 265–276.

(26) Webber, S. E.; Bleckman, T. M.; Attard, J.; Deal, J. G.; Katherdekar, V.; Welsh, K. M.; Webber, S.; Janson, C. A.; Matthews, D. A.; Smith, W. W.; Freer, S. T.; Jordan, S. R.; Bacquet, R. J.; Howland, E. F.; Booth, C. J. L.; Ward, R. W.; Hermann, S. M.; White, J.; Morse, C. A.; Hilliard, J. A.; Bartlett, C. A. Design of Thymidylate Synthase Inhibitors Using Protein Crystal Structures: The Synthesis and Biological Evaluation of a Novel Class of 5-Substituted Quinazolines. *J. Med. Chem.* **1993**, *36*, 733–746.

(27) Hughes, A.; Calvert, A. H. Preclinical and Clinical Studies with the Novel Thymidylate Synthase Inhibitor Nolatrexed Dihydrochloride (Thymitaq, AG337). In *Antifolate Drugs in Cancer Therapy*; Jackman, A. L., Ed.; Humana Press: Totowa, NJ, 1999; pp 229–241.

(28) 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 Analogues. In *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates*; Goldman, I. D., Ed.; Praeger: New York, 1985; pp 319–328.

(29) 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.

(30) Galivan, J.; Nimec, Z.; Rhee, M. S. Synergistic Growth Inhibition of Rat Hepatoma Cells Exposed in Vitro to *N*10-Propargyl-5,8-dideazafolate with Methotrexate or the Lipophilic Antifolates Trimetrexate or Metoprine. *Cancer Res.* **1988**, *47*, 5256–5260.

(31) Galivan, J.; Rhee, M. S.; Johnson, T. B.; Dilwith, R.; Nair, M. G.; Bunni, M.; Priest, D. G. The Role of Cellular Folates in the Enhancement of Activity of the Thymidylate Synthase Inhibitor 10-Propargyl-5,8-dideazafolate against Hepatoma Cells in Vitro by Inhibitors of Dihydrofolate Reductase. *J. Biol. Chem.* **1989**, *264*, 10685–10692.

(32) Gaumont, Y.; Kisliuk, R. L.; Emkey, R.; Piper, J. R.; Nair, M. G. Folate Enhancement of Antifolate Synergism in Human Lymphoma Cells. In *Chemistry and Biology of Pteridines*; Curtius, H. C., Blau, N., Ghisla, S., Eds.; W. deGruyter: Berlin, 1990; pp 1132–1136.

(33) Gaumont, Y.; Kisliuk, R. L.; Parsons, J. C.; Greco, W. R. Quanatitation of Folic Acid Enhancement of Antifolate Synergism. *Cancer Res.* **1992**, *52*, 2228–2235.

(34) Gangjee, A.; Devraj, R.; McGuire, J. J.; Kisliuk, R. L. 5-Arylthio Substituted 2-Amino-4-oxo-6-methylpyrrolo[2,3-*d*]pyrimidine Antifolates as Thymidylate Synthase Inhibitors and Antitumor Agents. *J. Med. Chem.* **1995**, *38*, 4495–4502.

(35) Tripos Inc., 1699 South Hanley Road, St. Louis, MO 63144.

(36) Gangjee, A.; Jain, D. H.; McGuire, J. J.; Kisliuk, R. L. Benzoyl Ring Halogenated Classical 2-Amino-6-methyl-3,4-dihydro-4-oxo-5-substituted Thiobenzoyl-7*H*-pyrrolo[2,3-*d*]pyrimidine Antifolates as Inhibitors of Thymidylate Synthase and as Antitumor Agents. *J. Med. Chem.* **2004**, *47*, 6730–6739.

(37) Gangjee, A.; Guo, X.; Queener, S. F.; Cody, V.; Galitsky, N.; Luft, J. R.; Pangborn, W. Selective *Pneumocystis carinii* Dihydrofolate Reductase Inhibitors: Design, Synthesis, and Biological Evaluation of New 2,4-Diamino-5-substituted-furo[2,3-*d*]pyrimidines. *J. Med. Chem.* **1998**, *41*, 1263–1271.

(38) Klon, E. A.; Heroux, A.; Ross, L. J.; Pathak, V.; Johnson, A. C.; Piper, J. R.; Borhani, W. D. Atomic Structures of Human Dihydrofolate Reductase Complexed with NADPH and Two Lipophilic Antifolates at 1.09 Å and 1.05 Å Resolution. *J. Mol. Biol.* **2002**, *320*, 677–693.

(39) Gangjee, A.; Mavandadi, F.; Kisliuk, R. L.; McGuire, J. J.; Queener, S. F. 2-Amino-4-oxo-5-substituted-pyrrolo[2,3-*d*]pyrimidines as Nonclassical Antifolate Inhibitors of Thymidylate Synthase. *J. Med. Chem.* **1996**, *39*, 4563–4568.

(40) Furneaux, R. H.; Tyler, P. C. Improved Syntheses of 3*H*,5*H*-Pyrrolo[3,2-*d*]pyrimidines. *J. Org. Chem.* **1999**, *64*, 8411–8412.

(41) Lim, M.; Klein, R. S.; Fox, J. J. A New Synthesis of Pyrrolo[3,2-*d*]pyrimidines ("9-deazapurines") via 3-Amino-2-carboalkoxypyrroles. *J. Org. Chem.* **1979**, *44*, 3826–3829.

(42) Alberola, A.; Ortega, A. G.; Sadaba, M. L.; Sanudo, C. Versatility of Weinreb Amides in the Knorr Pyrrole Synthesis. *Tetrahedron* **1999**, *55*, 6555–6566.

(43) Kaminsky, Z. J. 2-Chloro-4,6-disubstituted-1,3,5-triazines, a Novel Group of Condensing Reagents. *Tetrahedron Lett.* **1985**, *26*, 2901–2904.

(44) Elliott, A. J.; Morris, P. E.; Petty, S. L.; Williams, C. H. An Improved Synthesis of 7-Substituted Pyrrolo[3,2-*d*]pyrimidines. *J. Org. Chem.* **1997**, *62*, 8071–8075.

(45) Kisliuk, R. L.; Strumpf, D.; Gaumont, Y.; Leary, R. P.; Plante, L. Diastereoisomers of 5,10-Methylene-5,6,7,8-tetrahydropteroyl-D-glutamic Acid. *J. Med. Chem.* **1977**, *20*, 1531–1533.

(46) Wahba, A. J.; Friedkin, M. The Enzymatic Synthesis of Thymidylate. Early Steps in the Purification of Thymidylate Synthetase of *Escherichia coli*. *J. Biol. Chem.* **1962**, *237*, 3794–3801.

(47) Gangjee, A.; Yu, J.; McGuire, J. J.; Cody, V.; Galitsky, N.; Kisliuk, R. L.; Queener, S. F. Design, Synthesis, and X-ray Crystal Structure of a Potent Dual Inhibitor of Thymidylate Synthase and Dihydrofolate Reductase as an Antitumor Agent. *J. Med. Chem.* **2000**, *43*, 3837–3851.

(48) Pelphrey, P. M.; Popov, V. M.; Joska, T. M.; Beierlein, J. M.; Bolstad, E. D.; Fillingham, Y. A.; Wright, D. L.; Anderson, A. C. Highly Efficient Ligands for Dihydrofolate Reductase *Toxoplasma gondii* Inspired by Structural Analysis. *J. Med. Chem.* **2007**, *50*, 940–950.

JM701052U