# The Effect of 5-Alkyl Modification on the Biological Activity of Pyrrolo[2,3-*d*]pyrimidine Containing Classical and Nonclassical Antifolates as Inhibitors of Dihydrofolate Reductase and as Antitumor and/or Antiopportunistic Infection Agents[1a–e]

Aleem Gangjee,*,† Hiteshkumar D. Jain,||,† Sherry F. Queener,‡ and Roy L. Kisliuk§

*Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282, Department of Pharmacology and Toxicology, School of Medicine, Indiana University, Indianapolis, Indiana 46202, Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts 02111*

*Received March 6, 2008*

Novel classical antifolates (**3** and **4**) and 17 nonclassical antifolates (**11**−**27**) were synthesized as antitumor and/or antiopportunistic infection agents. Intermediates for the synthesis of **3**, **4**, and **11**−**27** were 2,4-diamino-5-alkylsubstituted-7*H*-pyrrolo[2,3-*d*]pyrimidines, **31** and **38**, prepared by a ring transformation/ring annulation sequence of 2-amino-3-cyano-4-alkyl furans to which various aryl thiols were attached at the 6-position via an oxidative addition reaction using $I_2$. The condensation of α-hydroxy ketones with malonodinitrile afforded the furans. For the classical analogues **3** and **4**, the ester precursors were deprotected, coupled with diethyl-L-glutamate, and saponified. Compounds **3** ($IC_{50}$ = 60 nM) and **4** ($IC_{50}$ = 90 nM) were potent inhibitors of human DHFR. Compound **3** inhibited tumor cells in culture with $GI_{50} \leq 10^{-7}$ M. Nonclassical **17** ($IC_{50}$ = 58 nM) was a potent inhibitor of *Toxoplasma gondii* (*T. gondii*) DHFR with >500-fold selectivity over human DHFR. Analogue **17** was 50-fold more potent than trimethoprim and about twice as selective against *T. gondii* DHFR.

## Introduction

Dihydrofolate reductase (DHFR) along with thymidylate synthase (TS) forms part of the system responsible for the synthesis of 2′-deoxythymidine-5′-monophosphate (dTMP), a key component in DNA biosynthesis and cell replication. TS catalyzes the de novo synthesis of dTMP from 2′-deoxyuridine-5′-monophosphate (dUMP). The cofactor, $N^5$,$N^{10}$-methylenetetrahydrofolate ($N^5$,$N^{10}$-$CH_2$-THF), serves as the donor of the methyl group as well as the reductant for this step and is itself oxidized to 7,8-dihydrofolate (7,8-DHF). The recyclization of 7,8-DHF to 5,6,7,8-tetrahydrofolate (5,6,7,8-THF) is catalyzed by DHFR[2] for which NADPH acts as the source of the reductant. Thus inhibition of DHFR and/or TS leads to "thymineless death".[a]

*Pneumocystis jirovecii* (*P. jirovecii*) previously known as *Pneumocystis carinii* (*P. carinii*)[3,4] [Note: *P. jirovecii* is the strain that infects humans, while *P. carinii* refers to the strain that infects rats] and *Toxoplasma gondii* (*T. gondii*)[3,4] are often fatal opportunistic infections in AIDS patients. *Mycobacterium avium* (*M. avium*) complex (MAC),[3,4] a group of organisms that is responsible for disseminated infections in AIDS patients, additionally decreases the quality of life of patients with AIDS. Several DHFR and TS inhibitors have found clinical utility as antitumor and antiopportunistic agents.[5] Classical antifolates like methotrexate[6] (MTX) (Figure 1), raltitrexed,[7] and pemetrexed[8] are clinically used as antitumor agents. Nonclassical antifolates like trimetrexate (TMQ), pyrimethamine, and trimethoprim (TMP) are clinically used as antiopportunistic infection agents.[4]

The combination of a weak DHFR inhibitor (TMP, pyrimethamine), along with a potent dihydropteroate synthase (DHPS) inhibitor (sulfamethoxazole), is currently used to treat infections caused by opportunistic pathogens in AIDS patients.[9] However, the combination therapy is successful in only 50−75% of the AIDS population; up to 60% are unable to tolerate the combination therapy due to severe, adverse drug reactions.[10] Trimetrexate is coadministered with leucovorin, the classical folate cofactor (6*R*,6*S*)-5-formyl-5,6,7,8-THF, which selectively rescues the host cell from the toxicity caused by nonselective TMQ.[11]

Gangjee et al.[12] recently reported **1** (Figure 2) as a dual inhibitor of human DHFR ($IC_{50}$ = 0.21 μM) and human TS ($IC_{50}$ = 0.54 μM). The 5-$CH_3$ moiety of **1** was incorporated to provide hydrophobic interaction with Val115 in human DHFR. Compound **1** was designed as a nonpolyglutamylatable DHFR inhibitor. However, unexpectedly, **1** had reasonable folyl poly-γ-glutamate synthetase (FPGS) substrate activity. Molecular modeling using SYBYL 6.8[13] suggested that **1** binds to human DHFR in the normal 2,4-diamino mode (Figure 3) while it could bind to human TS in the flipped mode. In addition, molecular modeling also indicated that the 5-$CH_3$ group in **1** could provide hydrophobic interaction with Trp109 in human TS. Compound **1** was a reasonably potent inhibitor of the growth of human CCRF-CEM leukemia cells in culture with an $EC_{50}$ value of 190 nM as compared with MTX ($EC_{50}$ = 12.5 nM). In 11 of the 60 tumor cell lines evaluated at the National Cancer Institute (NCI) preclinical screening program, compound **1** showed $GI_{50}$ values of $\leq 10^{-7}$ M (Table 3). Homologation of the 5-methyl group in **1** to a 5-ethyl group as in **2** (Figure 2) afforded a 3-fold more potent human DHFR inhibitor ($IC_{50}$ = 0.066 μM).[14] Surprisingly, compound **2** was devoid of any significant TS

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

† Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University.

‡ Department of Pharmacology and Toxicology, School of Medicine, Indiana University.

§ Department of Biochemistry, Tufts University School of Medicine.

|| Current address: Department of Chemistry, University of Wisconsin—Milwaukee, Milwaukee, WI 53211.

[a] Abbreviations: *T. gondii, Toxoplasma gondii*; DHFR, Dihydrofolate reductase; TS, thymidylate synthase; *P. jirovecii, Pneumocystis jirovecii*; *M. avium, Mycobacterium avium*; MTX, methotrexate; TMQ, trimetrexate; TMP, trimethoprim; DHPS, dihydropteroate synthase; FPGS, folyl poly-γ-glutamate synthetase.

10.1021/jm800244v CCC: $40.75 © 2008 American Chemical Society
Published on Web 07/08/2008

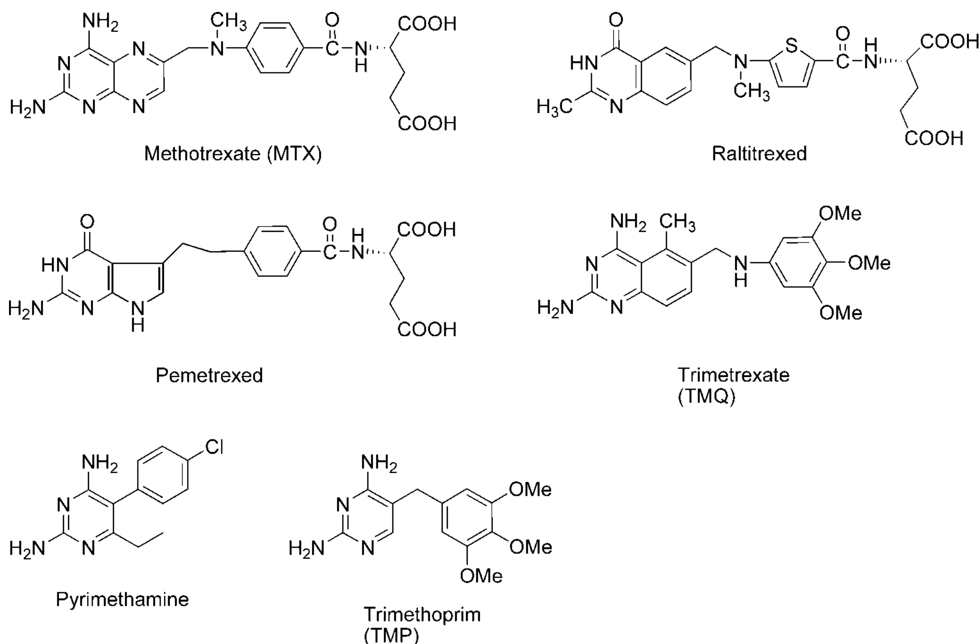



**Figure 1**

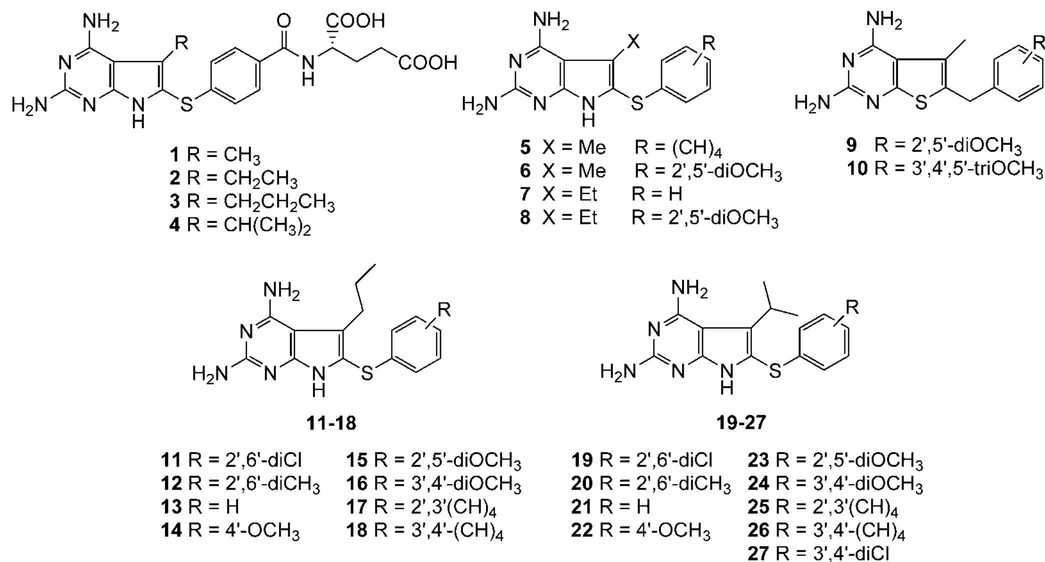



**Figure 2**

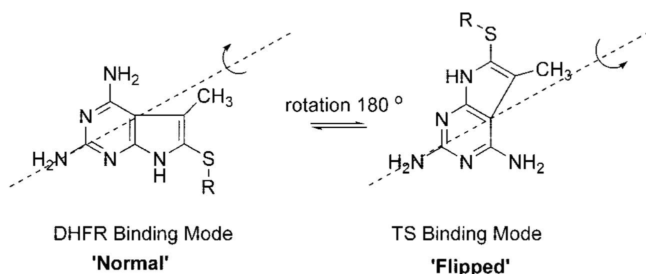



**Figure 3.** The "Normal" and "Flipped" modes of pyrrolo[2,3-*d*]pyrimidine.

inhibitory activity (37% inhibition @ >17 $\mu$M). However, compound **2** demonstrated increased tumor cell growth inhibitory activities against certain tumor cell lines compared to **1** in the NCI preclinical screening program (Table 3). Thus the size of the alkyl group attached to the 5-position of the 2,4-diamino pyrrolo[2,3-*d*]pyrimidine scaffold dictates the activity against DHFR and/or TS as well as tumor cell growth inhibitory potency.

Molecular modeling (SYBYL 6.91)[13] further indicated that the 5-alkyl group could be homologated to a 5-propyl or 5-isopropyl. This homologation could further enhance the van der Waals interaction with Val115 in human DHFR (Figure 4) in the normal DHFR binding mode. To determine the optimum size of the 5-alkyl group for DHFR and/or TS inhibitory activity as well as tumor cell growth inhibitory potency, the 2,4-diamino-5-propyl-6-arylthio-7*H*-pyrrolo[2,3-*d*]pyrimidine (**3**) and 2,4-diamino-5-isopropyl-6-arylthio-7*H*-pyrrolo[2,3-*d*]pyrimidine (**4**) classical analogues (Figure 2) were designed and synthesized.

The existing regimen used to treat opportunistic infections in AIDS and other immunocompromised patients is suppressive rather than curative and the therapy must be continued indefinitely.[3,4] Thus, it is of considerable interest to design single agents that have both the desired selectivity of TMP and the

**Table 1.** Inhibitory Concentration ($IC_{50}$, $\mu$M) and Selectivity Ratios[j] against Isolated TS and DHFR[a]

| cmpd | TS | | | DHFR | | | Selectivity ratios[j] | |
|---|---|---|---|---|---|---|---|---|
| | human[b] | *E. coli*[b] | *T. gondii*[c] | human[d] | *E. coli*[e] | *T. gondii*[c] | *h/ec*[j] | *h/tg*[j] |
| **1**[f] | 0.54 | >180 | 1.8 | 0.21 | 0.016 | 0.17 | 13 | 1.2 |
| **2**[g] | >17 (37)[h] | | | 0.066 | 0.002 | | 33 | |
| **11** | >22 (0) | >22 (0) | ND | 2.6 | 1.3 | ND | 2 | |
| **12** | >24 (0) | >24 (0) | ND | 3.0 | 1.5 | ND | 2 | |
| **13** | >14 (0) | >14 (0) | >1.4 (0) | 33 | 1.3 | 0.12 | 25 | 275 |
| **14** | >13 (0) | >13 (0) | >13 (12) | >30 (15) | 0.61 | 0.23 | >49 | >130 |
| **15** | >12 (0) | >12 (0) | >12 (0) | >28 (13) | 1.4 | 1.4 | >20 | >20 |
| **16** | >11 (0) | >11 (0) | >11 (16) | >26 (35) | 0.5 | 1.0 | >52 | >26 |
| **17** | >12 (0) | >12 (0) | >12 (16) | >29 (17) | 1.5 | 0.058 | >19 | >500 |
| **18** | >12 (0) | >12 (0) | >15 (12) | >15 (30) | 0.60 | 0.15 | >25 | >100 |
| **19** | >22 (18) | >2.2 (0) | >2.2 (0) | >26 (37) | 2.1 | 0.52 | >12 | >50 |
| **20** | >13 (0) | >13 (0) | >13 (0) | >30 (10) | 3.0 | 0.30 | >10 | >100 |
| **21** | >27 (0) | >2.7 (0) | >2.7 (0) | 16 | 0.13 | 0.064 | 123 | 250 |
| **22** | >25 (0) | >2.5 (0) | >2.5 (0) | 30 | 0.15 | 0.60 | 200 | 50 |
| **23** | >23 (0) | >2.3 (0) | >2.3 (0) | >28 (0) | 0.11 | 1.4 | >254 | >20 |
| **24** | >23 (0) | >2.3 (0) | >2.3 (0) | 27 | 0.11 | 2.7 | 245 | 10 |
| **25** | >12 (0) | >12 (0) | >12 (0) | >29 (27) | 0.15 | 0.12 | >193 | >241 |
| **26** | >24 (39) | >2.4 (0) | >2.4 (0) | >29 (36) | 0.29 | 1.5 | >100 | >19 |
| **27** | >23 (14) | >2.3 (0) | >2.3 (0) | >27 (0) | 0.14 | 1.4 | >193 | >19 |
| MTX | 29 | 90 | 18 | 0.022 | 0.0066 | 0.011 | 3.3 | 2 |
| pemetrexed[i] | 9.5 | 76 | 2.8 | 1.5 | 230 | 0.46 | 0.006 | 3.26 |
| TMP | | | | 680 | 0.020 | 2.9 | 34000 | 234 |
| pyrimethamine | | | | 6.0 | 2.0 | 0.080 | 3 | 75 |

[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. [b] Kindly provided by Dr. Frank Maley, New York State Department of Health, Albany, NY. [c] Kindly provided by Dr. K. Anderson, Yale University. [d] Kindly provided by Dr. J. H. Freisheim, Medical College of Ohio, Toledo, OH. [e] Kindly provided by Dr. R. L. Blakley, St. Jude Children's Hospital, Memphis, TN. [f] Data taken from ref 12. [g] Data taken from ref 14. [h] Number in parenthesis indicated % inhibition at that concentration. [i] Kindly provided by Dr. Chuan Shih, Eli Lilly & Co., Indianapolis IN. [j] Selectivity ratios, *h/ec* = $IC_{50}$ human dihydrofolate reductase/$IC_{50}$ *E. coli* dihydrofolate reductase; *h/tg* = $IC_{50}$ human dihydrofolate reductase/$IC_{50}$ *T. gondii* dihydrofolate reductase.

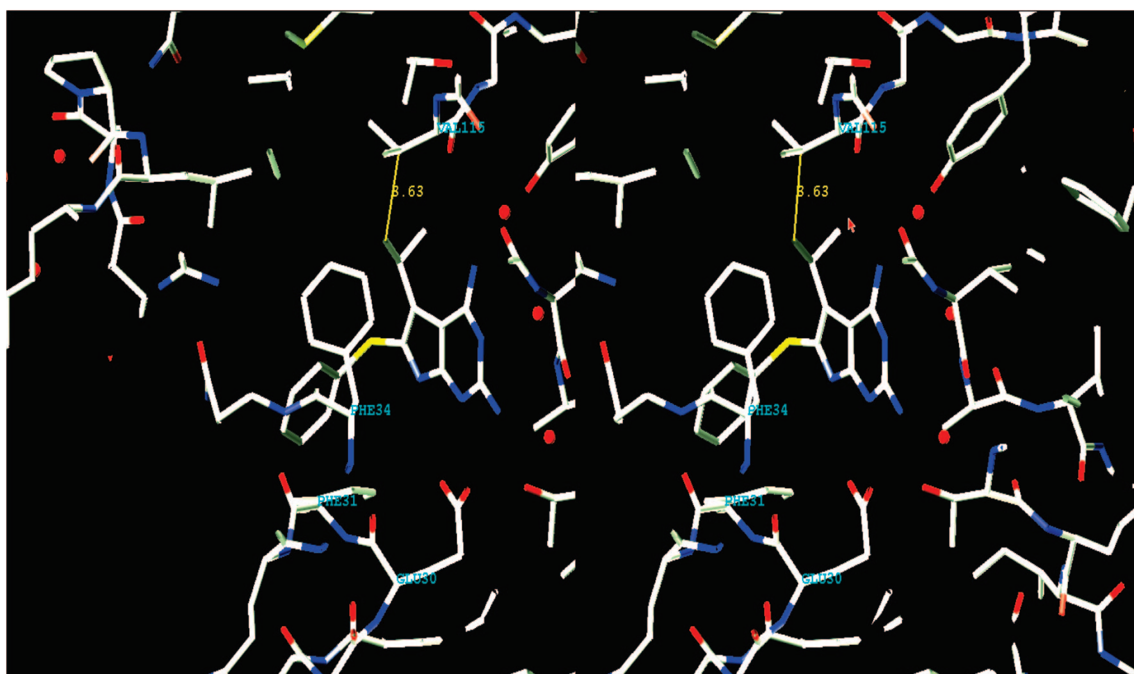



**Figure 4.** Stereo view of compound **4** in human DHFR (PDB code 1U72).[30] The hydrophobic interaction between 5-isopropyl and Val 115 is shown.

potency of TMQ. Such agents could be used as single agents to treat opportunistic infections in immunocompromised patients to decrease cost and increase patient compliance. Because patients with AIDS are often infected with multiple opportunistic infections, it is highly desirable to develop single agents that simultaneously target two or more opportunistic pathogen DHFR.

Gangjee et al.[15] also reported nonclassical analogues of **1**, including **5** and **6** (Figure 2) as inhibitors of DHFR from opportunistic pathogens. The 5-$CH_3$ moiety was designed to afford hydrophobic interaction with Ile123 in *P. carinii* DHFR, Val151 in *T. gondii* DHFR, and Ile102 in *M. avium* DHFR on the basis of X-ray crystal structure,[16,17] multiple sequence alignment,[18,19] and molecular modeling (SYBYL 6.8[13]) studies, respectively. The 5-$CH_3$ group was also suggested to influence the conformations of the 6-arylthio side chain in these inhibitors, thus limiting its flexibility and contributing to the potency of these compounds. Several compounds, including **5** and **6** (Table 2), displayed 10-fold or higher selectivity ratios for *T. gondii* DHFR and/or *M. avium* DHFR compared to rat liver (rl)

**Table 2.** Inhibitory Concentration ($IC_{50}$, $\mu$M) against Isolated DHFR[a] and Selectivity Ratios[b]

| cmpd | *P. carinii* | rat liver | rl/pc[b] | *M. avium* | rl/ma[b] |
|---|---|---|---|---|---|
| **3** | 0.00311 | 0.06 | 19.3 | 0.000737 | 81.4 |
| **4** | 0.0048 | 0.186 | 38.8 | 0.000391 | 475.7 |
| **5** | 37.3 | 4.57 | 0.12 | 0.7 | 6.5 |
| **6** | 14.6 | 7.8 | 0.5 | 0.1 | 78 |
| **7** | 21.8 | 5.6 | 0.3 | 0.88 | 6.36 |
| **8** | 6.04 | 4.05 | 0.7 | 0.1043 | 38.8 |
| **11** | 5.35 | 5.95 | 1.1 | 12.3 | 0.5 |
| **12** | 5.32 | 2.43 | 0.5 | 10.4 | 0.23 |
| **13** | 4.21 | 1.25 | 0.3 | 1.31 | 1.0 |
| **14** | 4.21 | 1.25 | 0.3 | 0.63 | 2.0 |
| **15** | 10.4 | 8.9 | 0.9 | 0.6 | 14.8 |
| **16** | 8.45 | 3.45 | 0.4 | 0.21 | 16.4 |
| **17** | 8.6 | 6.5 | 0.8 | 4.1 | 1.59 |
| **18** | 3.5(16%)[c] | 3.8 | ND | 7.8 | 0.5 |
| **19** | 22(14%) | 2.4 | ND[d] | 2.7 | 0.9 |
| **20** | 27.2 | 14.8 | 0.5 | 28.6 | 0.52 |
| **21** | 11.2 | 2 | 0.2 | 0.3687 | 5.42 |
| **22** | ND | 1.9 | ND | ND | ND |
| **23** | 35.6 | 50.3 | 1.4 | 2.1 | 24 |
| **24** | 28.2 | 4.6 | 0.2 | 3.4 | 1.3 |
| **25** | 0.68 | 0.91 | 1.3 | 0.24 | 3.8 |
| **26** | 7.1 | 2.1 | 0.3 | 2.54 | 0.83 |
| **27** | 10.9 | 13.5 | 1.2 | 1.14 | 11.84 |
| TMQ | 0.042 | 0.003 | 0.07 | 0.0015 | 2.0 |
| TMP | 12 | 180 | 15 | 0.3 | 600 |

[a] These assays were carried out at 37 °C under conditions of substrate (90 $\mu$M dihydrofolic acid) and cofactor (119 $\mu$M NADPH) in the presence of 150 mM KCl. [b] Selectivity ratios, rl/pc = $IC_{50}$ rat liver dihydrofolate reductase/$IC_{50}$ *P. carinii* dihydrofolate reductase; rl/ma = $IC_{50}$ rat liver dihydrofolate reductase/ $IC_{50}$ *M. avium* dihydrofolate reductase. [c] Number in parenthesis indicate the percentage inhibition at the given concentration. [d] ND = not determined.

**Table 3.** Cytotoxicity Evaluation ($GI_{50}$, M) of Compounds **1**-**4** against Selected Tumor Cell Lines[29]

| cell line | R = Me (**1**) | R = Et (**2**) | R = Pr (**3**) | R = $^{i}$Pr (**4**) |
|---|---|---|---|---|
| | | Leukemia | | |
| CCRF-CEM | $2.21 \times 10^{-7}$ | | | $5.36 \times 10^{-7}$ |
| HL−60 × (TB) | $4.35 \times 10^{-8}$ | $3.61 \times 10^{-8}$ | $1.28 \times 10^{-6}$ | $5.65 \times 10^{-6}$ |
| K-562 | $2.95 \times 10^{-8}$ | | $9.52 \times 10^{-7}$ | $9.32 \times 10^{-7}$ |
| MOLT-4 | $<1.00 \times 10^{-8}$ | | $1.94 \times 10^{-5}$ | $1.31 \times 10^{-6}$ |
| RPMI-8226 | $<1.00 \times 10^{-8}$ | $1.4 \times 10^{-7}$ | | $7.74 \times 10^{-6}$ |
| | | Nonsmall Cell Lung Cancer | | |
| NCI-H460 | $6.31 \times 10^{-7}$ | $2.44 \times 10^{-7}$ | $2.67 \times 10^{-7}$ | $6.45 \times 10^{-6}$ |
| | | Colon Cancer | | |
| HT29 | $1.60 \times 10^{-7}$ | $5.4 \times 10^{-8}$ | $9.58 \times 10^{-6}$ | $5.76 \times 10^{-6}$ |
| SW-620 | $1.24 \times 10^{-7}$ | $9.0 \times 10^{-8}$ | $1.21 \times 10^{-6}$ | $4.71 \times 10^{-6}$ |
| HCT 15 | $5.95 \times 10^{-5}$ | $6.7 \times 10^{-7}$ | $>1.0 \times 10^{-4}$ | $3.48 \times 10^{-5}$ |
| | | Central Nervous System Cancer | | |
| U251 | $8.24 \times 10^{-7}$ | $1.6 \times 10^{-6}$ | | $7.24 \times 10^{-6}$ |
| SF268 | $7.47 \times 10^{-5}$ | $2.2 \times 10^{-7}$ | $1.61 \times 10^{-6}$ | $1.78 \times 10^{-5}$ |
| | | Melanoma | | |
| LOX IMVI | $1.71 \times 10^{-7}$ | $4.9 \times 10^{-8}$ | $<1.00 \times 10^{-8}$ | $1.14 \times 10^{-6}$ |
| | | Prostrate Cancer | | |
| PC-3 | $4.36 \times 10^{-8}$ | $>1.0 \times 10^{-4}$ | $>1.0 \times 10^{-4}$ | |
| | | Breast Cancer | | |
| MCF-7 | $>1 \times 10^{-4}$ | $5.9 \times 10^{-7}$ | $1.64 \times 10^{-7}$ | |
| | | Renal Cancer | | |
| ACHN | $2.42 \times 10^{-5}$ | $4.5 \times 10^{-7}$ | $1.52 \times 10^{-5}$ | $7.39 \times 10^{-6}$ |
| CAKI-1 | $>1 \times 10^{-4}$ | $1.25 \times 10^{-7}$ | $3.76 \times 10^{-7}$ | $4.25 \times 10^{-6}$ |
| 786-0 | | | $1.28 \times 10^{-8}$ | $5.58 \times 10^{-6}$ |

DHFR.[15] Compound **6** with a 2′,5′-$(OCH_3)_2$ substitution was 16-fold more potent and equally selective compared to TMP against *T. gondii* DHFR.

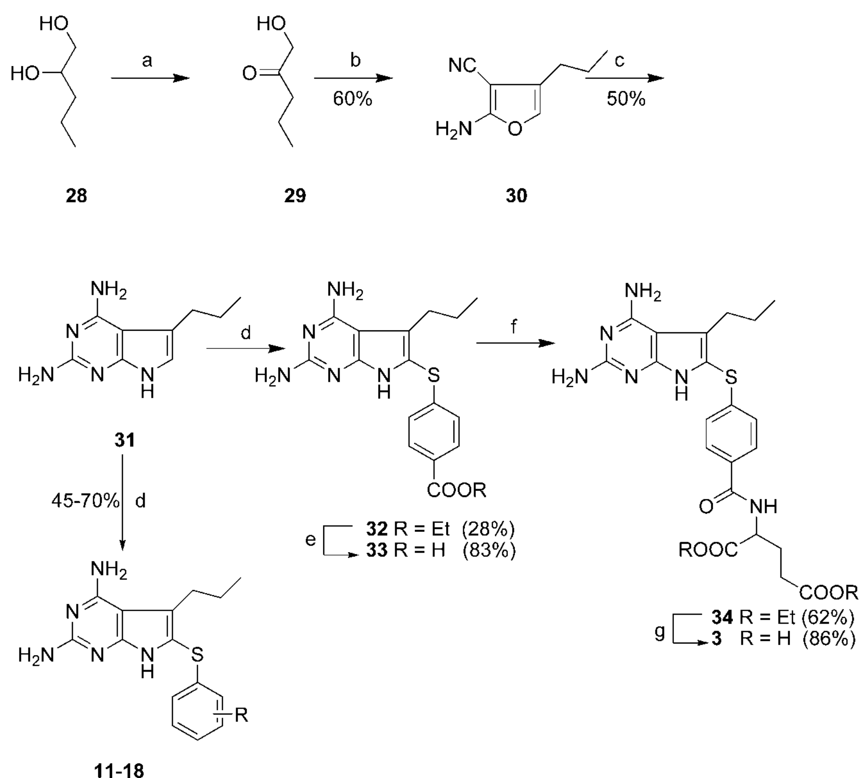
Rosowsky et al.,[20] using a different approach, reported compounds **9** and **10** (Figure 2) with a single carbon atom bridge that displayed fair *T. gondii* DHFR potency and good selectivity. Analogue **9** ($IC_{50}$ = 0.07 $\mu$M) was the most potent in this series against *T. gondii* DHFR, while analogue **10** was the most selective for *T. gondii* DHFR compared to rlDHFR with a selectivity ratio of 81.

A sulfur atom was incorporated in compounds **5** and **6** rather than a carbon atom, as in compounds **9** and **10**, to increase the proximity of the 6-arylthio ring to the hydrophobic residues on the pathogen DHFR due to the increased atomic size of the sulfur atom as well as a decrease in the C−S−C angle (98°) compared to a C−C−C angle (109°).[15] Compound **6** was 19-fold more potent and nearly one-half as selective as the most selective compound (**10**) of the 6-carbon-bridged analogues. The biological activity of compounds **5** and **6** supported the hypothesis that the 6-arylthio side chain of these compounds indeed interacts more favorably with Phe91 in *T. gondii* DHFR and Val158 in *M. avium* DHFR and that the sulfur bridge increased activity and selectivity. Gangjee et al.[14] have also synthesized the ethyl homologues of **5** and **6** with the goal of further increasing the potency and selectivity. Compound **8** (Figure 2), the ethyl homologue of **6**, was found to have increased potency and/or selectivity against *P. carinii* and *T. gondii* DHFR compared to rlDHFR (Table 2). Similar to their methyl counterparts, the ethyl homologues including **7** and **8** were found to have increased potency and/or selectivity against *T. gondii* and/or *M. avium* DHFR. In most instances, the ethyl homologues tested were found to be more active and/or selective against two or more pathogen DHFR. In an attempt to optimize the size of the 5-alkyl substitution on the potency and selectivity for *P. carinii* DHFR, *T. gondii* DHFR, and *M. avium* DHFR compounds **11**−**27** (Figure 2) were also designed and synthesized. Compounds **11**−**18** contain a 5-propyl group, while compounds **19**−**27** contain a 5-isopropyl group.

## Chemistry

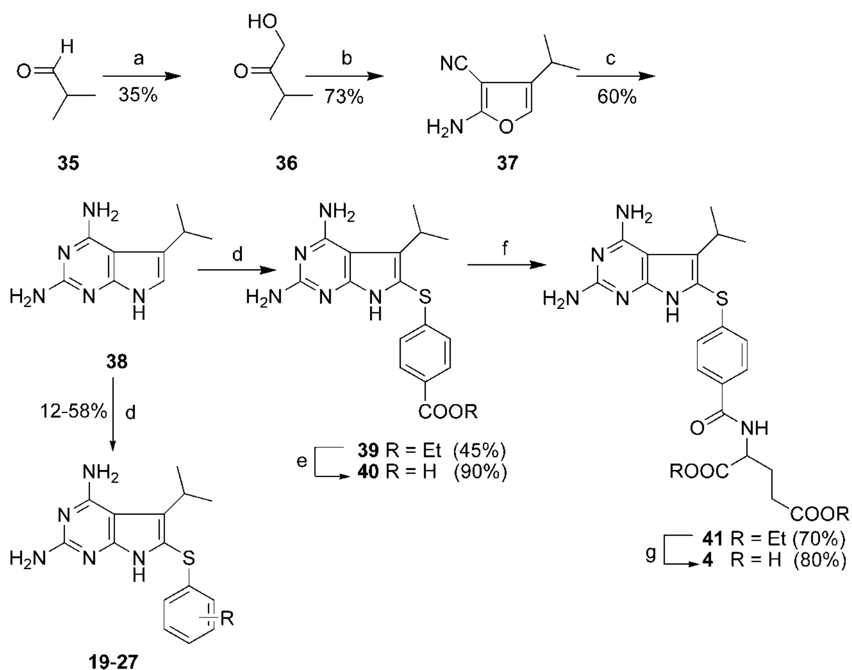
The syntheses of compounds **3** and **11**−**18** required the synthesis of 2,4-diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidine, **31** (Scheme 1), while the synthesis of **4** and **19**−**27** required the synthesis of 2,4-diamino-5-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidine, **38** (Scheme 2). Taylor et al.[21] have reported the synthesis of various 2,4-diamino-5-alkyl-7*H*-pyrrolo[2,3-*d*]pyrimidines by a ring transformation/ring annulation sequence of 2-amino-3-cyano-4-alkyl furans. These furans were in turn obtained by the condensation of suitable α-hydroxy ketones with malonodinitrile in the presence of a suitable base such as triethylamine. Gangjee et al.[12,14,15,22] and Rosowsky et al.[23,24] have also successfully adopted this methodology in their synthesis of pyrrolo[2,3-*d*]pyrimidine containing antifolates. Extending this general methodology to the synthesis of **31** required the synthesis of 1-hydroxy-2-pentanone, **29** (Scheme 1). Compound **29** was in turn obtained from the commercially available 1,2-pentanediol, **28**, by regiospecific oxidation of the secondary alcohol using hexabutyldistannoxane (HBD) and $Br_2$ (Scheme 1).[25] Two methods were attempted for the separation of the 1-hydroxy-2-pentanone, **29**, from the reaction mixture. The first involved silica gel chromatography on the crude reaction mixture and the second was a direct distillation of the crude reaction mixture. In general, distillation was found to be superior to column chromatography. Condensation of **29** with malonodinitrile using triethylamine as base afforded the 2-amino-4-propyl-furan-3-carbonitrile, **30**, in 60% yield. Further, condensation of **30** with guanidine (liberated from guanidine

**Scheme 1**[a]




[a] Conditions: (a) $O(SnBu_3)_2$, $Br_2$, $CH_2Cl_2$; (b) malonodinitrile, $NEt_3$, MeOH, 24 h; (c) guanidine hydrochloride, NaOMe, overnight; (d) ArSH, $I_2$, EtOH/$H_2O$ (2:1), 100−110 °C; (e) 1N NaOH, 80 °C, 24 h; (f) 2-chloro-4,6-dimethoxy-1,3,5-triazine, *N*-methylmorpholine, diethyl-L-glutamate hydrochloride, 0 °C to r.t.; (g) 1N NaOH, 0 °C to r.t.

**Scheme 2**[a]




[a] Conditions: (a) HCHO, 3-ethylbenzothiazolium bromide, $Et_3N$, EtOH, 60 °C, 72 h; (b) malonodinitrile, $NEt_3$, MeOH, 24 h; (c) guanidine hydrochloride, NaOMe, 96 h; (d) ArSH, $I_2$, EtOH/$H_2O$ (2:1), 100−110 °C; (e) 1N NaOH, 80 °C, 24 h. (f) 2-chloro-4,6-dimethoxy-1,3,5-triazine, *N*-methylmorpholine, diethyl-L-glutamate hydrochloride, 0 °C to r.t.; (g) 1N NaOH, 0 °C to r.t.

hydrochloride and NaOMe) afforded **31** in 50% yield. Reaction of 2,4-diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidine, **31**, and ethyl 4,4′-bismercaptobenzoate in EtOH/$H_2O$ followed by the addition of $I_2$ at reflux afforded compound **32** in 28% yield.[26,27] The disappearance of the 6-vinyl proton at $\delta$ 6.38 and the appearance of the characteristic AA′XX′ pattern for the 6-aryl protons in the $^1H$ NMR spectrum of **32** in DMSO ($d_6$) indicated the success of the oxidative addition reaction.

Hydrolysis of the ester **32** with aqueous 1N NaOH at 80 °C (24 h) followed by acidification gave the required acid, **33**, in 83% yield. Peptide coupling[21] of the acid **33** with diethyl-L-glutamate using 2,6-dimethoxy-4-chlorotriazine and *N*-methyl

morpholine, followed by chromatographic purification afforded the coupled product **34** in 62% yield. The $^1H$ NMR spectrum of **34** in DMSO ($d_6$) revealed the newly formed amide NH proton at $\delta$ 8.64–8.67 ppm as a doublet. Hydrolysis of the diester **34** with aqueous NaOH at 0 °C (4 h) and then at room temperature (24 h), followed by acidification, gave the desired compound **3** in 86% yield.

Similarly, reaction of **31** with appropriately substituted aryl thiols in a mixture of EtOH/$H_2O$ (2:1) followed by addition of $I_2$ at reflux as reported previously[27] afforded **11–18** in 45%–70% yields. The yields reveal no apparent correlation between the extent of pyrrolo[2,3-*d*]pyrimidine substitution and the electron-donating or -withdrawing effects of substituents in the thiophenol.

Analogous to **31**, the synthesis of **37** (Scheme 2) required the synthesis of 1-hydroxy-3-methyl-2-butanone, **36**. Thiazolium salt-catalyzed benzoin condensation of isopropyl aldehyde **35** with paraformaldehyde catalyzed by *N*-ethylbenzothiazolium bromide and triethylamine afforded the α-hydroxy ketone, **36**, after distillation, in 35% yield.[28] Compounds **4** and **19–27** were synthesized as shown in Scheme 2 starting with **36** in essentially the same way as described for **3** and **11–18** in Scheme 1.

The yields in Scheme 2, as before for Scheme 1, reveal no apparent correlation between the extent of pyrrolo[2,3-*d*]pyrimidine substitution, and the electronic nature of the substituents in the thiophenol. The lower yields of **18** (Scheme 1) and **19** (Scheme 2) may be a result of unfavorable steric interactions between the bulky 5-isopropyl group in **37** and the 2′,6′-disubstitution present on the thiophenols, which makes 6-substitution more difficult.

## Biological Evaluation and Discussion

Compounds **3**, **4**, and **11–27** were evaluated as inhibitors of human (h), *Escherichia coli* (*E. coli*), and *T. gondii* DHFR and TS. The inhibitory potency ($IC_{50}$) values are compared with MTX, pemetrexed, TMP, pyrimethamine, and the previously synthesized **1** and **2** (Table 1). Compounds **3** and **4** are good inhibitors of hDHFR with nanomolar $IC_{50}$ values and were about 3-fold and 4-fold less potent as hDHFR inhibitors, respectively, compared with MTX and about 25-fold and 17-fold more potent respectively than pemetrexed. Compound **3** was equipotent with the previously synthesized **2** and about 3.5-fold more potent than **1**. The biological data of **1–4** indicate that an ethyl, propyl, or isopropyl group at the 5-position are all conducive for potent hDHFR inhibition. The potent hDHFR activity of **2–4** compared to **1** could be attributed to increased hydrophobic interaction of the bulkier alkyl groups in **2–4** with Val115 in hDHFR. The increased activity of **2–4** may also result from favorable orientation of the 6-position thioaryl side chain when bound to hDHFR. Against hTS, **3** and **4** had similar inhibitory potency as MTX but were 4-fold less inhibitory than pemetrexed. Compounds **3** and **4** were about 63–74-fold less potent than **1** as inhibitors of hTS. These results indicate that homologation of the 5-methyl group in **1** to larger alkyl groups as in **2–4** is detrimental to hTS inhibition. This decrease in potency may be due to steric hindrance between the larger alkyl groups in **2–4** and Trp109 in hTS and/or due to unfavorable orientation of the 6-position side chains for interaction with the hTS in the presence of the bulkier 5-alkyl moiety.

Compound **3** was a poor inhibitor of hTS and *E. coli* TS (Table 1) but showed moderate inhibition against *T. gondii* TS (equipotent to pemetrexed). Compound **3** was a good inhibitor of all three DHFR tested. In addition, **3** is also a dual inhibitor of *T. gondii* DHFR and *T. gondii* TS. The nonclassical analogues **11–27** were all poor inhibitors of all three TS tested. They were however reasonably potent inhibitors of *E. coli* DHFR and *T. gondii* DHFR. Most of the analogues were weak or poor inhibitors of hDHFR.

*E. coli* DHFR (Table 1): In general the 5-isopropyl compounds (**19–27**) with the exceptions of **19** and **20** were more potent and selective against *E. coli* DHFR than the corresponding 5-propyl compounds (**11–18**). In the 5-propyl series, analogue **16** with a 3,4-dimethoxyphenyl side chain was the most potent and selective compound against *E. coli* DHFR. In the 5-isopropyl series, analogues **21–27** were potent against *E. coli* DHFR. A number of the nonclassical compounds in the 5-isopropyl series showed good selectivity for *E. coli* DHFR as compared to hDHFR. Compounds **21–27** were 100-fold to 254-fold more selective for *E. coli* DHFR than hDHFR. Thus the 5-isopropyl-6-substituted phenyl analogues were reasonably selective for bacterial DHFR.

*T. gondii* DHFR (Table 1): In general the 5-propyl compounds (**13–18**) with the exception of **13** were more potent and selective against *T. gondii* DHFR than the corresponding 5-isopropyl compounds (**21–26**). In the 5-propyl series, analogue **17** with a 1-naphthyl side chain was the most potent compound against *T. gondii* DHFR and was 50-fold more potent than TMP, 5-fold less potent than MTX and equipotent with pyrimethamine. Compound **17** was also the most selective compound against *T. gondii* DHFR with >500-fold selectivity over human DHFR. Thus **17** is 50-fold more potent than TMP and about twice as selective against *T. gondii* DHFR. Compound **13** with a phenyl side chain was 20-fold more potent than TMP and equally selective against *T. gondii* DHFR compared with human DHFR. In the 5-isopropyl series, analogue **21** with an unsubstituted phenyl side chain was the most potent analogue against *T. gondii* DHFR and was 45-fold more potent than TMP and equally selective. Analogue **25** with a 1-naphthyl side chain was 24-fold more potent than TMP and equally selective.

Compounds **3**, **4**, **11–27**, MTX, PYR, and TMP were also assayed against *T. gondii* DHFR using protocols described for Table 2 (data for *T. gondii* DHFR not shown), as well as under the conditions described for Table 1. Of the 20 compounds jointly assayed, MTX, **3**, and **4** were identified as the three most potent compounds by both protocols; both laboratories also placed TMP, **23**, **24**, and **27** as among the six least potent compounds. Selectivity could only be directly compared for the compounds in Table 1 that had defined selectivity values. Assays under both sets of conditions identified TMP, **13**, and **21** as the most selective compounds in this set of nine compounds and MTX as the least selective. Considering all compounds independently assayed as described in Table 2 for *T. gondii*, DHFR, the most selective compounds are TMP, **21**, **15**, **20**, **13**, **17**, and **23**; this list is consistent with the results in Table 1, except that the selectivity of compounds **15** and **20** is artificially depressed in Table 1 by the inability to generate full inhibition curves for the human reference enzyme.

Compounds **3**, **4**, and **11–27** were also evaluated as inhibitors of *P. carinii* DHFR, *M. avium* DHFR, and rlDHFR, which served as the mammalian surrogate under slightly different assay conditions. The inhibitory potency ($IC_{50}$) values are compared with TMQ, TMP, and the previously synthesized **5–8** (Table 2). Several compounds displayed 10-fold or higher selectivity ratios for *M. avium* DHFR. Against *P. carinii* DHFR, in general, the 5-propyl nonclassical analogues (**11–18**) were more potent and selective than the corresponding 5-isopropyl analogues (**19–27**). Against *M. avium* DHFR, the nature of the phenyl

substitution along with the 5-alkyl group determined the potency and selectivity of the compound.

*P. carinii* DHFR (Table 2): Against *P. carinii* DHFR the most potent analogues bore an unsubstituted phenyl (in **13**) or a 4′-methoxyphenyl substitution (in **14**) in the 5-propyl (**11**−**18**) series. Other substitutions such as a 2,6-dichloro **11**, 2,6-dimethyl **12**, 3,4-dimethoxy **16**, or 2,5-dimethoxy **15** caused a slight drop in activity. The 1-naphthyl substitution in **17** was found to display moderate DHFR inhibitory activity, while a 2-naphthyl substitution in **18** was found to be slightly detrimental for activity compared to **13**. In the 5-isopropyl (**19**−**27**) series, the most potent analogue contained a 1-naphthyl side chain **25** and displayed submicromolar inhibitory potency. All other substitutions in the side chains as in **19**−**27**, with the exception of **25**, displayed micromolar or higher inhibitory potency. Compounds **11**−**27** were not selective against *P. carinii* DHFR and increasing the size of the 5-alkyl group did not improve the selectivity, however, it increased the potency of the compounds against *P. carinii* DHFR compared to the corresponding methyl (compare **5** with **26** or **6** with **15**) and ethyl (compare **7** with **13** or **21**) analogues. The biological data of analogues **11**−**21** indicate that the homologation to a 5-propyl or 5-isopropyl group is conducive for potent inhibition of *P. carinii* DHFR, however, it does not improve the selectivity.

*Mycobacterium avium* DHFR (Table 2): Analogue **16** containing a 3′,4′-dimethoxy substitution in the phenyl ring was the most potent and selective analogue in the 5-propyl series. The second best analogue **15** had a 2′,5′-dimethoxy substitution in the side chain. Substitution of the phenyl ring with other substitutions as in **11**−**14**, **17**, and **18** resulted in analogues that were considerably less potent and selective. In the 5-isopropyl series, the most potent analogue **25** contains a 1-naphthyl side chain. The 2-naphthyl substituted analogue **26** was 10-fold less potent than **25**. The most selective analogue was **23**, with a 2′,5′-dimethoxy substitution in the side chain. The electron donating 3,4-dimethoxy substituted analogue **24** was found to be devoid of any selectivity. In sharp contrast, the analogue with electron withdrawing 3,4-dichloro substitution **27** had the second best selectivity. Again, the biological data of **11**−**27** indicate that both the alkyl group present at the 5-position as well as the substituents present on the 6-position thioaryl side chain play a role in determining the potency and selectivity of the analogues against *M. avium* DHFR.

Rat liver DHFR (Table 2): In the 5-propyl series, analogues **13** and **14** with an unsubstituted phenyl and a 4′-methoxy substitution, respectively, were the most potent. Dimethoxy substituted analogues **15** and **16** were 2-fold less potent than the monomethoxy **14**. Substitution of the phenyl ring with bulky groups such as 1-naphthyl **17** and 2-naphthyl **18** also resulted in 2-fold less potent compounds compared to **13**. However, substitution of the phenyl ring with either a 2′,6′-dichloro **11** or 2′,6′-dimethyl **12** substitution maintained activity compared to **13**. In the 5-isopropyl series, the 1-naphthyl substituted analogue **25** was the most potent. The 2-naphthyl substituted analogue **26** was 10-fold less potent than **25**. Replacing the 1-naphthyl substituent with an unsubstituted phenyl **21** or substitution of the phenyl ring with various electron donating methoxy (**23**, **24**), methyl (**20**) or electron withdrawing chloro (**19**, **27**) substitution also afforded analogues that were considerably less potent.

Compounds **3** and **4** were selected by the National Cancer Institute (NCI) for evaluation in its in vitro preclinical antitumor screening program.[29] The ability of compounds **3** and **4** to inhibit the growth of tumor cells was measured as $GI_{50}$ values, the concentration required to inhibit the growth of tumor cells in culture by 50% compared to a control. In 6 of the 60 tumor cell lines evaluated, compound **3** showed $GI_{50}$ values of $<1 \times 10^{-6}$ M (Table 3). While in only 2 of the 60 tumor cell lines evaluated, compound **4** showed $GI_{50}$ values of $<1 \times 10^{-6}$ M. It is noteworthy that compound **3** was not a general cell poison but showed selectivity both within a type of tumor cell line and across different tumor cell lines, with inhibitory values which in some instances differed by 10000-fold. In the melanoma LOX IMVI cell line and the renal cancer cell line 786-0, compound **3** displayed $GI_{50}$ values of $\leq 1 \times 10^{-8}$ M. It can be seen from the tumor cell growth inhibitory activity (Table 3) of compounds **1**−**4** that the tumor cell growth inhibitory potency, in certain instances, were more potent than either their human DHFR and/or human TS inhibitory activity alone (Table 1) and could be the result of a synergistic effect of dual inhibitory activities against TS and DHFR and/or that polyglutamylation increases inhibitory activity against TS and/or DHFR in tumor cell systems. Against the outgrowth of tumor cells in culture compound, **2** was in general the most potent compound followed by **3** then **1**, and the least potent is compound **4**. Though a strict structure−activity relationship cannot be considered for tumor cells in culture the 5-ethyl is clearly superior to a methyl, propyl or isopropyl.

In summary, homologation of a 5-methyl (compound **1**) to a 5-propyl (compound **3**) or 5-isopropyl (compound **4**) in 2,4-diamino-6-thiobenzoyl-5-alkylpyrrolo[2,3-*d*]pyrimidines increases the human DHFR inhibitory activity but is detrimental to the human TS inhibitory activity. We have found that in classical *N*-[4-[(2,4-diamino-5-alkyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)-thio]-benzoyl]-L-glutamic acid containing analogues the size of the alkyl group at the 5-position dictates inhibition of TS and/or DHFR activity as well as tumor cell growth inhibitory activity. The fact that homologated 5-alkyl substituents such as ethyl, propyl, and isopropyl are not tolerated by human TS indicates that homologation of the 5-alkyl group beyond a methyl is not conducive for dual human TS-DHFR inhibition in classical 5-alkyl-6-arylthiosubstituted pyrrolo[2,3-*d*]pyrimidines. Homologation however maintains dual DHFR-TS inhibitory activity against the bifunctional enzyme derived from *T. gondii*. In the nonclassical series, homologation of the 5-alkyl group is highly conducive for potent inhibition of *P. carinii* DHFR; however, it does not improve the selectivity of the analogues. Homologation of the 5-alkyl group to a propyl or isopropyl is highly conducive for potent and selective inhibition of *T. gondii* DHFR compared to human DHFR. The size of the alkyl group present at the 5-position of the pyrrolo[2,3-*d*]pyrimidine ring system along with the nature of the lipophilic substituents present on the 6-arylthio side chain determines the potency and selectivity against *T. gondii* DHFR and *M. avium* DHFR.

## Experimental Section

All evaporations were carried out in vacuum with a rotary evaporator. Analytical samples were dried in vacuum (0.2 mmHg) in an Abderhalden drying apparatus over $P_2O_5$ at 70 °C. Thin-layer chromatography (TLC) was performed on silica gel plates (Whatman 250 $\mu$M PE SiLG/UV) with fluorescent indicator. Spots were visualized by UV light (254 and 365 nm) or by staining with a solution of $KMnO_4$ in EtOH. All analytical samples were homogeneous on TLC in at least two different solvent systems. Purification by column and flash chromatography was carried out using Merck silica gel 60 (200−400 mesh). The amount (weight) of silica gel for column chromatography was in the range of 50−100 times the amount (weight) of the crude compounds being separated.

Columns were dry-packed unless specified otherwise. Solvent systems are reported as volume percent of mixture. Melting points were determined on a Mel-Temp II melting point apparatus and are uncorrected. Proton nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a Bruker WH-300 (300 MHz) spectrometer. The chemical shift ($\delta$) values are reported as parts per million (ppm) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad singlet. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Elemental compositions were 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 removed despite 24 h of drying in vacuum and were confirmed, where possible, by their presence in the $^1$H NMR spectrum. High-resolution mass spectra (HRMS), using electron impact (EI), were recorded on a VG Autospec (Fisons Instruments) micromass (EBE Geometry) double-focusing mass spectrometer. All solvents and chemicals were used as received.

**1-Hydroxy-2-pentanone (29).** To a solution of 1,2 pentanediol, **28** (2.1 g, 20 mmol) and hexabutyldistannoxane (HBD) (15.5 g, 26 mmol) in anhydrous $CH_2Cl_2$ (100 mL), $Br_2$ (4.16 g, 26 mmol) solution in $CH_2Cl_2$ (10 mL) was added dropwise at room temperature with stirring under $N_2$ atmosphere. The mixture was stirred for 3 h at room temperature. The solvent was evaporated under reduced pressure, and the resulting oil was distilled under reduced pressure to give **29** as a colorless oil, bp = 68−70° (16 mmHg) [lit.[28] bp = 70° (20 mmHg)].

**2-Amino-4-propyl-furan-3-carbonitrile (30).** A mixture of malonodinitrile (2.65 g, 40 mmol) and $N(C_2H_5)_3$ (5.58 mL, 40 mmol) in anhydrous MeOH (120 mL) was added dropwise to a solution of the α-hydroxy ketone **29** (4 g, 40 mmol) in MeOH, and the resulting dark-red solution was stirred at room temperature for 24 h. To this solution was added silica gel (10 g), and the solvent was evaporated to dryness under reduced pressure to afford a dry silica gel plug, which was loaded on top of a wet (hexane) silica gel column and eluted first with hexane and then with 2:1 hexane/EtOAc to afford 1.88 g (60%) of the furan **30** as a red-cream solid: mp 58.8−62.5 °C; TLC $R_f$ = 0.51 (hexane/EtOAc, 2:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 0.85−0.94 (t, 3 H, 4-$CH_2CH_2$*$CH_3$*), 1.47−1.56 (m, 2 H, 4-$CH_2$*$CH_2$*$CH_3$), 2.21−2.26 (t, 2 H, 4-*$CH_2$*$CH_2CH_3$), 6.74 (s, 1 H, 5-CH), 7.20 (s, 2 H, 2-$NH_2$). Anal. calcd for ($C_8H_{10}N_2O$) C, H, N.

**2,4-Diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (31).** Amino nitrile furan **30** (1.8 g, 12 mmol) was added to a solution of guanidine hydrochloride (2.5 g, 26 mmol) and NaOMe (1.4 g, 26 mmol) in anhydrous EtOH (100 mL). The resulting dark-red solution was stirred under reflux overnight, during which time it became dark brown. To this solution was added silica gel (5 g), and the solvent was evaporated to dryness under reduced pressure to afford a dry silica gel plug, which was loaded on top of a wet ($CHCl_3$) column and eluted first with $CHCl_3$ and then with a gradient of 1−5% MeOH in $CHCl_3$ to afford 1.15 g (50%) of **31** as a dark-brown solid: mp 215−220 °C; TLC $R_f$ = 0.41 ($CHCl_3$/MeOH, 5:1, with 2 drops of conc $NH_4OH$). $^1$H NMR (DMSO-$d_6$): $\delta$ 0.83−0.89 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.50−1.60 (m, 2 H, $CH_2$*$CH_2$*$CH_3$), 2.57−2.62 (t, 2 H, *$CH_2$*$CH_2CH_3$), 5.36 (s, 2 H, 2/4-$NH_2$), 5.91 (s, 2 H, 2/4-$NH_2$), 6.38 (s, 1 H, 6-CH), 10.36 (s, 1 H, 7-NH). Anal. calcd for ($C_9H_{13}N_5 \cdot 0.1H_2O$) C, H, N.

**Ethyl 4-[2,4-Diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]benzoate (32).** To a suspension of **31** (1.1 g, 5.7 mmol) in a mixture of EtOH/$H_2O$ (2:1, 75 mL) was added diethyl 4,4′-dithiobis(benzoate) (2.2 g, 6 mmol) and the suspension was heated to 100−110 °C, then $I_2$ (3 g, 12 mmol) was added and the reaction was monitored (TLC) for completion (3 h). To this solution was added excess sodium thiosulfate and the solution was evaporated to dryness under reduced pressure and the resulting residue was washed with water and air-dried. This residue was then dissolved in MeOH (100 mL) and to this was added silica gel (15 g) and the resulting suspension was evaporated to dryness under reduced pressure to afford a dry silica gel plug that was loaded on top of a wet silica gel ($CHCl_3$) column and eluted first with $CHCl_3$ and then with a gradient of 1−5% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness to afford 610 mg (28%) of **32** as a white solid: mp = 260.6−261 °C; TLC $R_f$ = 0.54 ($CHCl_3$/MeOH, 5:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 0.82 (t, 3 H, $CH_2CH_2$*$CH_3$*), 1.29 (t, 3 H, $CH_2$*$CH_3$*), 1.41−1.43 (m, 2 H, $CH_2$*$CH_2$*$CH_3$), 2.76 (t, 2 H, *$CH_2$*$CH_2CH_3$), 4.27−4.29 (q, 2 H, *$CH_2$*$CH_3$), 7.09 (s, 2 H, 2/4-$NH_2$), 7.74 (s, 2 H, 2/4-$NH_2$), 7.13−7.16 (d, 2 H, $C_6H_4$), 7.84−7.87 (d, 2 H, $C_6H_4$), 12.08 (s, 1 H, 7NH). Anal. calcd for ($C_{18}H_{21}N_5O_2S$) C, H, N, S.

**4-[2,4-Diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]benzoic acid (33).** To a suspension of **32** (330 mg, 0.9 mmol) in EtOH (30 mL) was added aqueous 1N NaOH (12 mL) and the reaction mixture was stirred at 80 °C for 24 h. At this time, TLC indicated the disappearance of the starting ester at $R_f$ = 0.54 ($CHCl_3$/MeOH, 5:l) and formation of one major spot at the origin. The solvent was evaporated to dryness under reduced pressure, and the resulting sodium salt (yellow oil) was dissolved in water (15 mL) and carefully acidified to pH 4 by dropwise addition of 3N HCl. The resulting suspension was filtered and washed carefully with cold water and dried over $P_2O_5$ to afford 295 mg (83%) of **33** as a white solid. This was directly used in the next step without further characterization.

**Diethyl *N*-[4-[(2,4-Diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]-benzoyl]-L-glutamate (34).** To a suspension of the acid **33** (344 mg, 1 mmol) in anhydrous DMF (15 mL) under $N_2$ was added *N*-methyl morpholine (145 $\mu$L, 1.33 mmol) and the resulting suspension was cooled to 0 °C. At this point, 2-chloro-4,6-dimethoxy-1,3,5-benzotriazine (235 mg, 1.33 mmol) was added and the suspension was stirred for 2 h at 0 °C; during this time, it formed a solution. The reaction mixture was again cooled to 0 °C and diethyl-L-glutamic acid (317 mg, 1.33 mmol) was added followed by *N*-methyl morpholine (145 $\mu$L, 1.33 mmol). The solution was slowly allowed to warm to room temperature with stirring and left at room temperature for a total of 24 h. At this time, TLC indicated the formation of one major spot at $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:l). To the resulting solution was added silica gel (5 g), and the DMF was evaporated to dryness at room temperature using an oil pump. The silica gel plug was loaded on a wet ($CHCl_3$) silica gel column and eluted with a gradient of 1−3% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness under vacuum to afford 330 mg (62%) of **34** as a white solid: mp 260−260.5 °C; TLC $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 0.80−0.84 (t, 3 H, $CH_2CH_2$*$CH_3$*), 1.13−1.19 (m, 6 H, $CH_2$*$CH_3$*), 1.43−1.45 (m, 2 H, $CH_2$*$CH_2$*$CH_3$), 1.98−2.09 (m, 2 H, Glu $\beta$-$CH_2$), 2.42−2.50 (t, 2 H, Glu $\gamma$-$CH_2$), 2.72 (t, 2 H, *$CH_2$*$CH_2CH_3$), 4.00−4.10 (m, 4 H, *$CH_2$*$CH_3$), 4.40 (m, 1 H, Glu α-CH), 5.64 (s, 2 H, 2/4-$NH_2$), 6.22 (s, 2 H, 2/4-$NH_2$), 7.04−7.07 (d, 2 H, $C_6H_4$), 7.75−7.77 (d, 2 H, $C_6H_4$), 8.64−8.67 (d, 1 H, CONH), 11.06 (s, 1 H, 7-NH). Anal. calcd for ($C_{25}H_{32}N_6SO_5$) C, H, N, S.

***N*-[4-[(2,4-Diamino-5-propyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]benzoyl]-L-glutamic Acid (3).** To a suspension of **34** (200 mg, 0.4 mmol) in EtOH (15 mL) was added 1N NaOH (6 mL) at 0 °C and the resulting suspension was stirred at 0 °C (4 h) and then at room temperature for 24 h. At this point, TLC showed the disappearance of the starting ester at $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:l) and formation of one major spot at the origin. The solvent was evaporated to dryness under reduced pressure, and the sodium salt (yellow oil) was dissolved in water (5 mL) and the solution was cooled in an ice bath and acidified carefully to pH 4.0 with dropwise addition of 3N HCl. The resulting suspension was frozen using dry ice/acetone and the reaction flask was kept at 5 °C for 24 h and filtered. The residue was washed carefully with cold water and dried over $P_2O_5$ to afford 160 mg (86%) of **3** as a white solid: mp 259.5−260 °C. $^1$H NMR (DMSO-$d_6$): $\delta$ 0.74−0.82 (t, 3 H, $CH_2CH_2$*$CH_3$*), 1.43−1.45 (m, 2 H, $CH_2$*$CH_2$*$CH_3$), 1.93−2.08 (m, 2 H, Glu $\beta$-$CH_2$), 2.51 (t, 2 H, Glu $\gamma$-$CH_2$), 2.99 (t, 2 H, *$CH_2$*$CH_2CH_3$), 4.37 (m, 1 H, Glu α-CH), 5.67 (s, 2 H, 2/4-$NH_2$), 6.26 (s, 2 H, 2/4-$NH_2$), 7.04−7.07 (d, 2 H, $C_6H_4$), 7.76−7.78 (d, 2

H, $C_6H_4$), 8.51−8.53 (d, 1 H, CONH), 11.09 (s, 1 H, 7-NH), 12.37 (bs, 2 H, COOH). Anal. calcd for ($C_{21}H_{24}N_6SO_5 \cdot 1.0\ H_2O$) C, H, N, S.

**1-Hydroxy-3-methyl-2-butanone (36).** In a 1000 mL flask were placed paraformaldehyde (9.45 g, 0.3 mol), 3-ethylbenzothiazolium bromide (7.32 g, 0.03 mol), isobutraldehyde, **35**, (27.5 mL, 0.3 mol), anhydrous EtOH (300 mL), and $Et_3N$ (4.2 mL, 0.03 mol), and then dry $N_2$ gas was bubbled into the reaction mixture. The mixture was then heated in an oil bath at 60 °C for 72 h, during which time the color of the reaction mixture changed to dark reddish-brown. The solvent was evaporated to dryness under reduced pressure, and to the resulting residue was added EtOAc (20 mL). The resulting suspension was filtered, and the solid was washed repeatedly with EtOAc. The filtrate was evaporated under reduced pressure to a dark-brown oil, which was distilled under low pressure to afford 10.7 g (35%) of **36** as a colorless oil: bp = 65−68 °C (16 mmHg) [lit.[28] bp = 65 °C (20 mmHg)]. $^1$H NMR ($CDCl_3$-*d*): $\delta$ 1.15−1.17 (d, 6 H, CH($CH_3$)$_2$), 2.60−2.69 (m, 1 H, *CH*($CH_3$)$_2$), 3.13 (bs, 1 H, OH), 4.32 (s, 2 H, $CH_2$).

**2-Amino-4-isopropyl-furan-3-carbonitrile (37).** A mixture of malonodinitrile (12.55 g, 190 mmol) and $Et_3N$ (19.19 g, 190 mmol) in MeOH (220 mL) was added dropwise to a solution of the α-hydroxy ketone **36** (19.4 g, 190 mmol) in MeOH (10 mL) and the resulting solution was stirred at room temperature for 24 h. To this solution was added silica gel (50 g), and the solvent was evaporated under reduced pressure to afford a dry silica gel plug, which was loaded on top of a wet (hexane) silica gel column and eluted first with hexane and then with 2:1 hexane/EtOAc to afford the furan **37** (11.1 g, 73%) as a reddish-brown solid: mp 62−62.5 °C; TLC $R_f$ = 0.64 (hexane/EtOAc, 2:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 1.13−1.15 (d, 6 H, CH($CH_3$)$_2$), 2.59−2.65 (m, 1 H, *CH*($CH_3$)$_2$), 6.71 (s, 1 H, 5-CH), 7.23 (s, 2 H, 2-$NH_2$) Anal. calcd for ($C_8H_{10}N_2O$) C, H, N.

**2,4-Diamino-5-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidine (38).** Amino nitrile furan **37** (1.5 g, 10 mmol) was added to a solution prepared from guanidine hydrochloride (1.43 g, 15 mmol) and NaOMe (0.81 g, 15 mmol) in anhydrous EtOH (100 mL). The resulting dark-red reaction mixture was stirred under reflux for 96 h, during which time it turned dark-brown. To this solution was added silica gel (15 g), and the solvent was evaporated to dryness under reduced pressure to afford a silica gel plug, which was loaded on top of a wet ($CHCl_3$) silica gel column and eluted first with $CHCl_3$ and then with a gradient of 1−5% MeOH in $CHCl_3$ to give 1.15 g (60%) of **38** as a white solid: mp 221−222 °C; TLC $R_f$ = 0.38 ($CHCl_3$/MeOH, 5:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 1.17−1.19 (d, 6 H, CH($CH_3$)$_2$), 3.17 (m, 1 H, *CH*($CH_3$)$_2$), 5.33 (s, 2 H, 2/4-$NH_2$), 5.90 (s, 2 H, 2/4-$NH_2$), 6.39 (s, 1 H, 6-CH), 10.35 (s, 1 H, 7-NH). Anal. calcd for ($C_9H_{13}N_5$) C, H, N.

**Ethy l4-[2,4-Diamino-5-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]-benzoate (39).** To a suspension of **38** (1.0 g, 5.2 mmol) in a mixture of EtOH/$H_2O$ (2:1, 75 mL) was added diethyl 4,4′-dithiobis(benzoate) (2.2 g, 6 mmol) and the suspension was heated to 100−110 °C, then $I_2$ (3 g, 12 mmol) was added and the reaction was monitored for completion (3 h). To this solution was added excess sodium thiosulfate and the solution was evaporated to dryness under reduced pressure and the resulting residue was washed with water and air-dried. This residue was then dissolved in MeOH (100 mL) and to this was added silica gel (15 g), and the resulting suspension was evaporated to dryness under reduced pressure to afford a dry silica gel plug, which was loaded on top of a wet ($CHCl_3$) silica gel column and eluted first with $CHCl_3$ and then with a gradient of 1−5% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness to afford 890 mg (45%) of **39** as a white solid: mp = 263−264.7 °C; TLC $R_f$ = 0.61 ($CHCl_3$/MeOH, 5:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 1.23−1.29 (m, 9 H, $CH_2CH_3$ and CH($CH_3$)$_2$), 3.33−3.41 (m, 1 H, *CH*($CH_3$)$_2$), 4.23−4.29 (q, 2 H, $CH_2CH_3$), 5.65 (s, 2 H, 2/4-$NH_2$), 6.15 (s, 2 H, 2/4-$NH_2$), 7.05−7.08 (d, 2 H, $C_6H_4$), 7.82−7.84 (d, 2 H, $C_6H_4$), 11.03 (s, 1 H, 7-NH). Anal. calcd for ($C_{18}H_{21}N_5O_2S \cdot 0.4H_2O$) C, H, N, S.

**4-[2,4-Diamino-5-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)-sulfanyl]-benzoic acid (40).** To a suspension of **39** (530 mg, 1.43 mmol) in EtOH (50 mL) was added aqueous 1N NaOH (20 mL) and the reaction mixture was stirred at 80 °C for 24 h. At this point, TLC indicated the disappearance of the starting ester at $R_f$ = 0.54 ($CHCl_3$/MeOH, 5:l) and formation of one major spot at the origin. The solvent was evaporated to dryness, and the resulting sodium salt (yellow oil) was dissolved in water (15 mL) and carefully acidified to pH 4 by dropwise addition of 3N HCl. The resulting suspension was filtered and washed carefully with cold water and dried over $P_2O_5$ to afford 436 mg (90%) of **40** as a white solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 1.24−1.25 (d, 6 H, CH($CH_3$)$_2$), 3.35 (m, 1 H, *CH*($CH_3$)$_2$), 5.60 (s, 2 H, 2/4-$NH_2$), 6.07 (s, 2 H, 2/4-$NH_2$), 6.93−6.95 (d, 2 H, $C_6H_4$), 7.75−7.76 (d, 2 H, $C_6H_4$), 10.98 (s, 1 H, 7-NH). Anal. calcd for ($C_{16}H_{17}N_5O_2S$) MS (EI) calcd $m/z$ = 343.110297; found $m/z$ = 343.109307 ($M^+$).

**Diethyl *N*-[4-[(2,4-Diamino-5-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]-benzoyl]-L-glutamate (41).** To a suspension of the acid **40** (300 mg, 0.87 mmol) in anhydrous DMF (25 mL) under $N_2$ was added *N*-methylmorpholine (145 $\mu$L, 1.33 mmol) and the resulting suspension was cooled to 0 °C. At this point, 2-chloro-4,6-dimethoxy-1,3,5-triazine (235 mg, 1.34 mmol) was added and the suspension was stirred for 2 h, during which time it formed a solution. The reaction mixture was again cooled to 0 °C and diethyl-L-glutamate (317 mg, 1.33 mmol) was added followed by *N*-methylmorpholine (145 $\mu$L, 1.33 mmol). The solution was slowly allowed to warm to room temperature with stirring and left at room temperature for a total of 24 h. To the resulting solution was added silica gel (5 g) and the DMF was evaporated using an oil pump. The silica gel plug was loaded on a wet ($CHCl_3$) silica gel column and eluted with a gradient of 1−3% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness under vacuum to give 330 mg (70%) of **41** as a white solid: mp 217.6−218 °C; TLC $R_f$ = 0.53 ($CHCl_3$/MeOH, 5:1). $^1$H NMR (DMSO-$d_6$): $\delta$ 1.12−1.19 (m, 6 H, $CH_2CH_3$), 1.24−1.27 (d, 6 H, CH($CH_3$)$_2$), 1.97−2.07 (m, 2 H, Glu $\beta$-$CH_2$), 2.39−2.44 (t, 2 H, Glu $\gamma$-$CH_2$), 3.99−4.12 (m, 4 H, $CH_2CH_3$), 4.40 (m, 1 H, Glu α-CH), 5.63 (s, 2 H, 2/4-$NH_2$), 6.12 (s, 2 H, 2/4-$NH_2$), 7.02−7.05 (d, 2 H, $C_6H_4$), 7.74−7.77 (d, 2 H, $C_6H_4$), 8.63−8.65 (d, 1 H, CONH), 11.01 (s, 1 H, 7-NH). Anal. calcd for ($C_{25}H_{32}N_6O_5S \cdot 0.5H_2O$) C, H, N, S.

***N*-[4-[(2,4-Diamino-5-isopropyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)sulfanyl]-benzoyl]-L-glutamate (4).** To a suspension of **41** (200 mg, 0.37 mmol) in EtOH (15 mL) was added 1N NaOH (6 mL) and the suspension stirred at 0 °C (4 h) and then at room temperature for 24 h. The EtOH was evaporated to dryness under reduced pressure, the yellow oil was dissolved in water (5 mL), and the solution was cooled in an ice-bath and acidified carefully to pH 4.0 with dropwise addition of 3N HCl. This suspension was left at 5 °C for 24 h and filtered. The residue was washed well with water and $O(C_2H_5)_2$ and then dried over $P_2O_5$/vacuum to afford 165 mg (80%) of **4** as a white solid: mp 206.5−207 °C. $^1$H NMR (DMSO-$d_6$): $\delta$ 1.25−1.27 (d, 6 H, CH($CH_3$)$_2$), 1.93−2.06 (m, 2 H, Glu $\beta$-$CH_2$), 2.31−2.33 (t, 2 H, Glu $\gamma$-$CH_2$), 4.36 (m, 1 H, Glu α-CH), 5.78 (s, 2 H, 2/4-$NH_2$), 6.29 (s, 2 H, 2/4-$NH_2$), 7.03−7.06 (d, 2 H, $C_6H_4$), 7.76−7.79 (d, 2 H, $C_6H_4$), 8.51−8.54 (d, 1 H, CONH), 11.13 (s, 1 H, 7-NH), 12.4 (bs, 2 H, COOH). Anal. calcd for ($C_{21}H_{24}N_6O_5S \cdot 0.2H_2O \cdot 1.8C_4H_{10}O$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(2′,6′-dichlorophenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (11).** To a solution of **31** (300 mg, 1.57 mmol) in a mixture of EtOH/water (2:1, 30 mL) was added 2,6-dichlorophenylthiol (540 mg, 3.00 mmol) and the reaction mixture was heated to 100−110 °C, then $I_2$ (750 mg, 3.00 mmol) was added and the heating continued with stirring for a total of 3 h. To this mixture was added an excess of sodium thiosulfate and the reaction mixture concentrated under reduced pressure. To the resulting residue was added silica gel (10 g) and MeOH (50 mL) and the solution evaporated to dryness under reduced pressure to afford a dry silica gel plug, which was loaded on top of a wet ($CHCl_3$) silica gel column and eluted with a gradient of 1−3% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled

and evaporated to dryness. The resulting residue was washed with MeOH, filtered, and dried to yield 385 mg (67%) of **11**: mp 238−240 °C; TLC $R_f$ = 0.51 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.76 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.15 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.64 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 5.54 (s, 2 H, 2/4-$NH_2$), 6.08 (s, 2 H, 2/4-$NH_2$), 7.32−7.35 (m, 1 H, $C_6H_3$), 7.48−7.51 (d, 2 H, $C_6H_3$), 10.93 (s, 1 H, 7-NH). Anal. calcd for ($C_{15}H_{15}N_5Cl_2S \cdot 0.17CHCl_3$) C, H, N, Cl, S.

**2,4-Diamino-5-propyl-6-(2′,6′-dimethylphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*] pyrimidine (12).** Compound **12** was synthesized as described for **11** using 2,6-dimethylphenylthiol (420 mg, 3.00 mmol) and **31** (300 mg, 1.57 mmol): yield 52%; mp 227−230 °C; TLC $R_f$ = 0.55 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.74−0.78 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.16−1.25 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.19−2.25 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 2.35 (s, 6 H, 2′,6′-di$CH_3$), 5.46 (s, 2 H, 2/4-$NH_2$), 6.00 (s, 2 H, 2/4-$NH_2$), 7.07 (m, 3 H, $C_6H_3$), 10.75 (s, 1 H, 7-NH). Anal. calcd for ($C_{17}H_{21}N_5S \cdot 0.6CH_3OH$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(phenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (13).** Compound **13** was synthesized as described for **11** using phenylthiol (280 mg, 2.00 mmol) and **31** (200 mg, 1.04 mmol): yield 65%; mp 252.2−252.7 °C; TLC $R_f$ = 0.53 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.81−0.86 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.43−1.45 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.70−2.75 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 5.59 (s, 2 H, 2/4-$NH_2$), 6.15 (s, 2 H, 2/4-$NH_2$), 7.00−7.02 (d, 2 H, $C_6H_5$), 7.12−7.14 (m, 1 H, $C_6H_5$), 7.24−7.29 (m, 2 H, $C_6H_5$), 10.98 (s, 1 H, 7-NH). Anal. calcd for ($C_{15}H_{17}N_5S \cdot 0.2H_2O$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(4′-methoxyphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (14).** Compound **14** was synthesized as described for **11** using 4-methoxyphenylthiol (280 mg, 2.00 mmol) and **31** (200 mg, 1.04 mmol): yield 50%; mp 247.9−248.2 °C; TLC $R_f$ = 0.63 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.82−0.87 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.41 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.74 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 3.69 (s, 3 H, 4′-$OCH_3$), 5.55 (s, 2 H, 2/4-$NH_2$), 6.11 (s, 2 H, 2/4-$NH_2$), 6.85−6.88 (d, 2 H, $C_6H_4$), 7.04−7.07 (d, 2 H, $C_6H_4$), 10.95 (s, 1 H, 7-NH). Anal. calcd for ($C_{16}H_{19}N_5OS \cdot 0.5H_2O$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(2′,5′-dimethoxyphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (15).** Compound **15** was synthesized as described for **11** using 2,5-dimethoxyphenylthiol (525 mg, 3.00 mmol) and **31** (300 mg, 1.57 mmol): yield 45%; mp 217−218 °C; TLC $R_f$ = 0.56 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.81−0.86 (t, 3 H, 5−$CH_2CH_2$*$CH_3$*), 1.40−1.47 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.66−2.71 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 3.53 (s, 3 H, 2′/5′-$OCH_3$), 3.80 (s, 3 H, 2′/5′-$OCH_3$), 5.59 (s, 2 H, 2/4-$NH_2$), 6.17 (s, 2 H, 2/4-$NH_2$), 5.96 (s, 1 H, $C_6H_3$), 6.64−6.67 (d, 1 H, $C_6H_3$), 6.89−6.92 (d, 1 H, $C_6H_3$), 10.91 (s, 1 H, 7-NH). Anal. calcd for ($C_{17}H_{21}N_5O_2S$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(3′,4′-dimethoxyphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (16).** Compound **16** was synthesized as described for **11** using 3,4-dimethoxyphenylthiol (350 mg, 2.00 mmol) and **31** (200 mg, 1.04 mmol): yield 65%; mp >230 °C (dec); TLC $R_f$ = 0.53 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.83−0.88 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.43−1.45 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.73−2.75 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 3.68 (s, 6 H, 3′,4′-di$OCH_3$), 5.59 (s, 2 H, 2/4-$NH_2$), 6.15 (s, 2 H, 2/4-$NH_2$), 6.59−6.62 (d, 1 H, $C_6H_3$), 6.82−6.88 (m, 2 H, $C_6H_3$), 10.98 (s, 1 H, 7-NH). Anal. calcd for ($C_{17}H_{21}N_5O_2S \cdot 0.2CHCl_3$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(1′-napthylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (17).** Compound **17** was synthesized as described for **11** using 1-naphthylthiol (320 mg, 2.00 mmol) and **31** (200 mg, 1.04 mmol): yield 58%; mp > 255 °C dec; TLC $R_f$ = 0.63 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.80−0.84 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.43−1.45 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.73−2.75 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 5.62 (s, 2 H, 2/4-$NH_2$), 6.19 (s, 2 H, 2/4-$NH_2$), 6.86 (d, 1 H, $C_{10}H_7$), 7.37 (t, 1 H, $C_{10}H_7$), 7.58−7.61 (m, 3 H, $C_{10}H_7$), 7.70 (d, 1 H, $C_{10}H_7$), 7.94 (d, 1 H, $C_{10}H_7$), 11.05 (s, 1 H, 7-NH). Anal. calcd for ($C_{19}H_{19}N_5S$) C, H, N, S.

**2,4-Diamino-5-propyl-6-(2′-napthylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (18).** Compound **18** was synthesized as described for **11** using 2-naphthylthiol (480 mg, 3.00 mmol) and **31** (300 mg, 1.57 mmol): yield 70%; mp > 250 °C (dec); TLC $R_f$ = 0.56 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 0.80−0.85 (t, 3 H, 5-$CH_2CH_2$*$CH_3$*), 1.42−1.49 (m, 2 H, 5-$CH_2$*$CH_2$*$CH_3$), 2.74−2.79 (t, 2 H, 5-*$CH_2$*$CH_2CH_3$), 5.60 (s, 2 H, 2/4-$NH_2$), 6.18 (s, 2 H, 2/4-$NH_2$), 7.17−7.20 (d, 1 H, $C_{10}H_7$), 7.40−7.50 (m, 3 H, $C_{10}H_7$), 7.73−7.76 (d, 1 H, $C_{10}H_7$), 7.81 (s, 1 H, $C_{10}H_7$), 7.84−7.85 (d, 1 H, $C_{10}H_7$), 11.06 (s, 1 H, 7-NH). Anal. calcd for ($C_{19}H_{19}N_5S \cdot 0.5H_2O$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(2′,6′-dichlorophenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (19).** To a solution of **38** (300 mg, 1.57 mmol) in a mixture of EtOH/water (2:1, 30 mL) was added 2,6-dichlorophenylthiol (540 mg, 3.00 mmol) and the reaction mixture was heated to 100−110 °C, then $I_2$ (750 mg, 3.0 mmol) was added and the heating continued with stirring for a total of 2 h. To this mixture was added an excess of sodium thiosulfate and the reaction mixture concentrated under reduced pressure. To the resulting residue was added silica gel (10 g) and MeOH (50 mL) and the solution evaporated to dryness under reduced pressure to afford a dry silica gel plug, which was loaded on top of a wet ($CHCl_3$) silica gel column and eluted with a gradient of 1−3% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness. The resulting residue was washed with MeOH, filtered, and dried to yield 70 mg (12%) of **19**: mp 230−231 °C; TLC $R_f$ = 0.60 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 1.10−1.12 (d, 6 H, CH(*$CH_3$*)$_2$), 5.54 (s, 2 H, 2/4-$NH_2$), 5.87 (s, 2 H, 2/4-$NH_2$), 7.31−7.33 (m, 1 H, $C_6H_3$), 7.46−7.49 (d, 2 H, $C_6H_3$), 10.90 (s, 1 H, 7-NH). Anal. calcd for ($C_{15}H_{15}N_5Cl_2S \cdot 0.3H_2O$) C, H, N, Cl, S.

**2,4-Diamino-5-isopropyl-6-(2′,6′-dimethylphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*] pyrimidine (20).** Compound **20** was synthesized as described for **19** using 2,6-dimethylphenylthiol (420 mg, 3.00 mmol) and **38** (300 mg, 1.57 mmol): yield 22%; mp 248−248.3 °C; TLC $R_f$ = 0.63 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 1.11−1.14 (d, 6 H, 5-CH(*$CH_3$*)$_2$), 2.30 (s, 6 H, 2′,6′-di$CH_3$), 5.46 (s, 2 H, 2/4-$NH_2$), 5.79 (s, 2 H, 2/4-$NH_2$), 7.08 (m, 3 H, $C_6H_3$), 10.66 (s, 1 H, 7-NH). Anal. calcd for ($C_{17}H_{21}N_5S \cdot 0.2H_2O$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(phenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (21).** Compound **21** was synthesized as described for **19** using phenylthiol (330 mg, 3.00 mmol) and **38** (300 mg, 1.57 mmol) except that the compound was washed with hexane and dried. Yield: 45%; mp 228.5−229 °C; TLC $R_f$ = 0.53 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 1.25−1.27 (d, 6 H, 5-CH(*$CH_3$*)$_2$), 5.39 (bs, 2 H, 2/4-$NH_2$), 5.89 (bs, 2 H, 2/4-$NH_2$), 6.97−7.29 (m, 5 H, $C_6H_5$), 10.97 (s, 1 H, 7-NH). Anal. calcd for ($C_{15}H_{17}N_5S \cdot 0.1C_6H_{14}$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(4′-methoxyphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (22).** Compound **22** was synthesized as described for **19** using 4-methoxyphenylthiol (280 mg, 2.00 mmol) and **38** (200 mg, 1.04 mmol): yield 40%; mp 288−289 °C; TLC $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 1.26−1.28 (d, 6 H, 5-CH(*$CH_3$*)$_2$), 3.41−3.48 (m, 1 H, 5-*CH*($CH_3$)$_2$), 3.70 (s, 3 H, 4-$OCH_3$), 5.56 (bs, 2 H, 2/4-$NH_2$), 6.02 (bs, 2 H, 2/4-$NH_2$), 6.86−6.89 (d, 2 H, $C_6H_4$), 7.02−7.04 (d, 2 H, $C_6H_4$), 10.93 (s, 1 H, 7-NH). Anal. calcd for ($C_{16}H_{19}N_5SO \cdot 0.1H_2O$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(2′,5′-dimethoxyphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (23).** Compound **23** was synthesized as described for **19** using 2,5-dimethoxyphenylthiol (800 mg, 4.00 mmol) and **38** (350 mg, 2.00 mmol): yield 53%; mp 243−244 °C; TLC $R_f$ = 0.60 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1$H NMR (DMSO-$d_6$): δ 1.24−1.26 (d, 6 H, 5-CH(*$CH_3$*)$_2$), 3.53 (s, 3 H, 2′/5′-$OCH_3$), 3.80 (s, 3 H, 2′/5′-$OCH_3$), 5.59 (bs, 2 H, 2/4-$NH_2$), 5.93 (s, 1 H, $C_6H_3$), 6.07 (bs, 2 H, 2/4-$NH_2$), 6.63−6.66 (d, 1 H, $C_6H_3$), 6.89−6.92 (d, 1 H, $C_6H_3$), 10.87 (s, 1 H, 7-NH). Anal. calcd for ($C_{17}H_{21}N_5SO_2$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(3′,4′-dimethoxyphenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (24).** Compound **24** was synthesized as described for **19** using 3,4-dimethoxyphenylthiol (520 mg, 3.00 mmol) and **38** (300 mg, 1.57 mmol): yield 58%; mp 293−293.5 °C; TLC $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1H$ NMR (DMSO-$d_6$): δ 1.27−1.29 (d, 6 H, 5-CH($CH_3$)$_2$), 3.67 (s, 3 H, 3′/4′-$OCH_3$), 3.69 (s, 3 H, 3′/4′-$OCH_3$), 5.56 (bs, 2 H, 2/4-$NH_2$), 6.01 (bs, 2 H, 2/4-$NH_2$), 6.56−6.59 (d, 1 H, $C_6H_3$), 6.79 (s, 1 H, $C_6H_3$), 6.87−6.89 (d, 1 H, $C_6H_3$), 10.93 (s, 1 H, 7-NH). Anal. calcd for ($C_{17}H_{21}N_5SO_2 \cdot 0.5H_2O$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(1′-napthylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (25).** Compound **25** was synthesized as described for **19** using 1-naphthylthiol (320 mg, 2.00 mmol) and **38** (200 mg, 1.04 mmol): yield 45%; mp 267−267.5 °C; TLC $R_f$ = 0.60 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1H$ NMR (DMSO-$d_6$): δ 1.26−1.28 (d, 6 H, 5-CH($CH_3$)$_2$), 5.63 (s, 2 H, 2/4-$NH_2$), 6.11 (s, 2 H, 2/4-$NH_2$), 6.76−6.78 (d, 1 H, $C_{10}H_7$), 7.37 (t, 1 H, $C_{10}H_7$), 7.59−7.72 (m, 3 H, $C_{10}H_7$), 7.94 (d, 1 H, $C_{10}H_7$), 8.2 (d, 1 H, $C_{10}H_7$), 11.02 (s, 1 H, 7-NH). Anal. calcd for ($C_{19}H_{19}N_5S$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(2′-napthylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (26).** Compound **26** was synthesized as described for **19** using 2-naphthylthiol (640 mg, 4.00 mmol) and **38** (350 mg, 1.83 mmol): yield 40%; mp 247−247.5 °C; TLC $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1H$ NMR (DMSO-$d_6$): δ 1.27−1.29 (d, 6 H, 5-CH($CH_3$)$_2$), 5.63 (s, 2 H, 2/4-$NH_2$), 6.12 (s, 2 H, 2/4-$NH_2$), 7.15−7.18 (d, 1 H, $C_{10}H_7$), 7.44−7.47 (m, 3 H, $C_{10}H_7$), 7.74−7.76 (d, 1 H, $C_{10}H_7$), 7.82−7.85 (d, 2 H, $C_{10}H_7$), 11.05 (s, 1 H, 7-NH). Anal. calcd for ($C_{19}H_{19}N_5S$) C, H, N, S.

**2,4-Diamino-5-isopropyl-6-(3′,4′-dichlorophenylsulfanyl)-7*H*-pyrrolo[2,3-*d*]pyrimidine (27).** Compound **27** was synthesized as described for **19** using 3,4-dichlorophenylthiol (540 mg, 3.00 mmol) and **38** (300 mg, 1.57 mmol): yield 37%; mp 244−244.5 °C; TLC $R_f$ = 0.58 ($CHCl_3$/MeOH, 5:1, with 2 drops of $NH_4OH$). $^1H$ NMR (DMSO-$d_6$): δ 1.24−1.27 (d, 6 H, 5-CH($CH_3$)$_2$), 5.67 (bs, 2 H, 2/4-$NH_2$), 6.17 (bs, 2 H, 2/4-$NH_2$), 6.96 (s, 2 H, $C_6H_3$), 7.37 (s, 1 H, $C_6H_3$), 11.04 (s, 1 H, 7-NH). Anal. calcd for ($C_{15}H_{15}N_5SCl_2$) C, H, N, S, Cl.

**Acknowledgment.** This work was supported in part by the National Institutes of Health, grants AI41743 (A.G.); AI44661 (A.G.), CA89300 (A.G.), and AI47759 (A.G.) from the National Institute of Allergy and Infectious Diseases.

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

## References


(1) (a) Taken in part from the dissertation submitted by H.J. to the Graduate School of Pharmaceutical Sciences, Duquesne University, in partial fulfillment of the requirements for the degree of Ph.D., July 2006. (b) Presented in part at the 229th American Chemical Society National Meeting, San Diego, CA, March 13−17, 2005; Abstract MEDI-491. (c) Presented in part at the 13th International Symposium on Chemistry and Biology of Pteridines and Folates, Egmond aan Zee, The Netherlands, June 20−24, 2005; Oral Presentation O04-02. (d) Presented in part at the 13th International Symposium on Chemistry and biology of Pteridines and Folates, Egmond aan Zee, The Netherlands, June 20−24, 2005; Poster number P-09. (e) Presented in part at the 231st American Chemical Society National Meeting, Atlanta, GA, March 26−30, 2006; Abstr. MEDI-348.

(2) Schnell, J. R.; Dyson, H. J.; Wright, P. E. Structure, Dynamics, and Catalytic Function of Dihydrofolate Reductase. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 119–140.

(3) Willemot, P.; Klein, M. B. Prevention of HIV-associated Opportunistic Infections and Diseases in the Age of Highly Active Antiretroviral Therapy. *Expert Rev. Anti-Infect. Ther.* **2004**, *2*, 521–532.

(4) Klepser, M. E.; Klepser, T. B. Drug Treatment of HIV-related Opportunistic Infections. *Drugs* **1997**, *53*, 40–73.

(5) Takimoto, C. H. Antifolates in Clinical Development. *Semin. Oncol.* **1997**, *24*, S18-40–S18-51.

(6) 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 & Wilkins: Baltimore, MD, 1997; Vol. 1, pp 907−921..

(7) 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.

(8) 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-y1)ethylbenzoyl]-L-glutamic Acid, Is an Inhibitor of Thymidylate Synthase. *J. Med. Chem.* **1992**, *35*, 4450–4454.

(9) Podzamczer, D.; Salazar, A.; Jimenez, J.; Consiglio, E.; Santin, M.; Casanova, A.; Rufi, G.; Gudiol, F. Intermittent Trimethoprim-Sulfamethoxazole compared with Dapsone-Pyrimethamine for the Simultaneous Primary Prophylaxis of *Pneumocystis* Pneumonia and Toxoplasmosis in Patients Infected with HIV. *Ann. Intern. Med.* **1995**, *122*, 755–761.

(10) Roudier, C.; Caumes, E.; Rogeaux, O.; Bricaire, F.; Gentilini, M. Adverse Cutaneous Reactions to Trimethoprim-Sulfamethoxazole in Patients with the Acquired Immunodeficiency Syndrome and *Pneumocystis carinii* Pneumonia. *Arch. Dermatol.* **1994**, *130*, 1383–1386.

(11) News. FDA Approve Trimetrexate as Second line Therapy of *Pneumocystis carinii* Pneumonia. *Am. J. Hosp. Pharm.* **1994**, *51*, 591−591.

(12) Gangjee, A.; Lin, X.; Kisliuk, R. L.; McGuire, J. J. Synthesis of *N*-{4-[(2,4-Diamino-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-L-glutamic Acid and *N*-{4-[(2-Amino-4-oxo-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-L-glutamic Acid as Dual Inhibitors of Dihydrofolate Reductase and Thymidylate Synthase and as Potential Antitumor Agents. *J. Med. Chem.* **2005**, *48*, 7215–7222.

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

(14) Gangjee, A.; Zeng, Y.; Talreja, T.; McGuire, J. J.; Kisliuk, R. L.; Queener, S. F. Design and Synthesis of Classical and Nonclassical 6-Arylthio-2,4-diamino-5-ethylpyrrolo[2,3-*d*]pyrimidines as Antifolates. *J. Med. Chem.* **2007**, *50*, 3046–3053.

(15) Gangjee, A.; Lin, X.; Queener, S. F. Design, Synthesis, and Biological Evaluation of 2,4-Diamino-5-methyl-6-substituted-pyrrolo[2,3-*d*]pyrimidines as Dihydrofolate Reductase Inhibitors. *J. Med. Chem.* **2004**, *47*, 3689–3692.

(16) Cody, V.; Wojtczak, A.; Kalman, T. I.; Friesheim, J. H.; Blakley, R. L. Conformational Analysis of Human Dihydrofolate Reductase Inhibitor Complexes: Crystal Structure Determination of Wild Type and F31 Mutant Binary and Ternary Inhibitor Complexes. *Adv. Exp. Med. Biol.* **1993**, *338*, 481–486.

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

(18) Roos, D. S. Primary Structure of the Dihydrofolate Reductase−Thymidylate Synthase Gene from *Toxoplasma gondii*. *J. Biol. Chem.* **1993**, *268*, 6269–6280.

(19) Ginkel, S. Z.; Dooley, T. P.; Suling, W. J.; Barrow, W. W. Identification and Cloning of the *Mycobacterium avium FolA* Gene, Required for Dihydrofolate Reductase Activity. *FEMS Microbiol. Lett.* **1997**, *156*, 69–78.

(20) Rosowsky, A.; Mota, C. E.; Wright, J. E.; Freisheim, J. H.; Huesner, J. J.; McCormack, J. J.; Queener, S. F. 2,4-Diaminothieno[2,3-*d*]pyrimidine. Analogs of Trimetrexate and Piritrexim as Potential Inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* Dihydrofolate Reductase. *J. Med. Chem.* **1993**, *36*, 3103–3112.

(21) Taylor, E. C.; Patel, H. H.; Jun, J.-G. A One-Step Ring Transformation/Ring Annulation Approach to Pyrrolo[2,3-*d*]pyrimidines. A New Synthesis of the Potent Dihydrofolate Reductase Inhibitor TNP-351. *J. Org. Chem.* **1995**, *60*, 6684–6687.

(22) Gangjee, A.; Ye, Z.; Queener, S. F. Synthesis of Three Carbon Atom Bridged 2,4-Diaminopyrrolo[2,3-*d*]pyrimidines as Nonclassical Dihydrofolate Reductase Inhibitors. *J. Heterocycl. Chem.* **2005**, *42*, 1127–1133.

(23) Rosowsky, A.; Chen, H.; Fu, H.; Queener, S. F. Synthesis of New 2,4-Diaminopyrido[2,3-*d*]pyrimidine and 2,4-Diaminopyrrolo[2,3-*d*]pyrimidine Inhibitors of *Pneumocystis carinii*, *Toxoplasma gondii*, and *Mycobacterium avium* Dihydrofolate Reductase. *Bioorg. Med. Chem.* **2003**, *11*, 59–67.

(24) Rosowsky, A.; Fu, H.; Queener, S. F. Synthesis of New 2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidines via the Taylor Ring Transformation/Ring Annulation Strategy. *J. Heterocycl. Chem.* **2001**, *38*, 1197–1202.

(25) Ueno, Y.; Okawara, M. Oxidation Using Distannoxane. I. Selective Oxidation of Alcohols. *Tetrahedron Lett.* **1976**, *50*, 4597–4600.

(26) Gangjee, A.; Jain, H. D.; 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.

(27) Gangjee, A.; Jain, H. D.; Phan, J.; Lin, X.; Song, X.; McGuire, J. J.; Kisliuk, R. L. Dual Inhibitors of Thymidylate Synthase and Dihydrofolate Reductase as Antitumor Agents: Design, Synthesis, and Biological Evaluation of Classical and Nonclassical Pyrrolo[2,3-*d*]pyrimidine Antifolates. *J. Med. Chem.* **2006**, *49*, 1055–1065.

(28) Matsumoto, T.; Ohishi, M.; Inoue, S. Selective Cross-Acyloin Condensation Catalyzed by Thiazolium Salt. Formation of 1-Hydroxy-2-one from Formaldehyde and Other Aldehydes. *J. Org. Chem.* **1985**, *50*, 603–606.

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

(30) Cody, V.; Luft, J. R.; Pangborn, W. Understanding the Role of Leu22 Variants in Methotrexate Resistance: Comparison of Wild-Type and Leu22Arg Variant Mouse and Human Dihydrofolate Reductase Ternary Crystal Complexes with Methotrexate and NADPH. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2005**, *61*, 147–155.

JM800244V