Synthesis of Classical and a Nonclassical 2-Amino-4-oxo-6-methyl-5-substituted Pyrrolo[2,3-*d*]pyrimidine Antifolate Inhibitors of Thymidylate Synthase[1]

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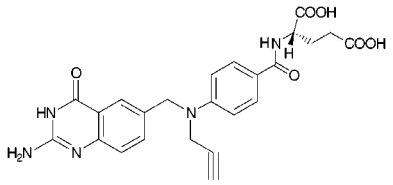
Compounds **2**−**5** were designed as potential antifolate nonpolyglutamatable inhibitors of thymidylate synthase (TS). These analogues are structurally related to 2-amino-4-oxo-5-substituted quinazolines and 2-amino-4-oxo-5-substituted pyrrolo[2,3-*d*]pyrimidines which have shown excellent inhibition of TS and, for the quinazoline, significant promise as clinically useful antitumor agents. Compounds **2**−**4** were synthesized by appropriate amine exchange reactions on pivaloyl-protected 5-dimethylaminomethyl-substituted 6-methyl pyrrolo[2,3-*d*]pyrimidine **7** which in turn was obtained from the Mannich reaction of pivaloylated-6-methyl pyrrolo[2,3-*d*]pyrimidine **6**. In instances where the amine exchange reaction was sluggish, the Mannich base was quaternized with methyl iodide which afforded much faster exchange reaction with improved yields. For compound **5**, 4-mercaptopyridine was used as the nucleophile and reacted with **7**. The analogues **2**−**4** inhibited *Lactobacillus casei* (lc) TS and recombinant human (h) TS with IC_{50} in the 10^{-4} to 10^{-5} M range. Compound **5** inhibited lcTS and hTS 20% at 26 and 25 μM, respectively. In addition, compound **5** inhibited the growth of *Pneumocystis carinii* and *Toxoplasma gondii* cells in culture by 76% at 32×10^{-6} M and 50% at 831×10^{-6} M, respectively.

Introduction

Thymidylate synthase (TS) is a crucial enzyme that catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) utilizing 5,10-methylenetetrahydrofolate, a cofactor which acts as the source of the methyl group as well as the reductant.[2] This is the exclusive de novo source of dTMP, hence inhibition of TS activity, in the absence of salvage, leads to "thymineless death". Thus inhibition of TS has long been an attractive goal for the development of antitumor agents.[3,4]

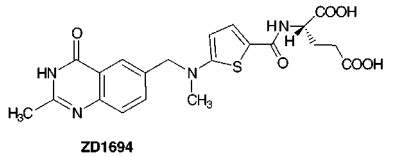
5-Fluorouracil (5-FU), a mechanism-based inhibitor of TS, is metabolically transformed to 5-fluorodeoxyuridinemonophosphate (FdUMP) and is the only TS inhibitor antitumor agent FDA-approved for clinical use in the United States. Since several types of cancer either do not respond to 5-FU or develop resistance to it, effort continues to design folate analogues as potential TS inhibitors and as antitumor agents.[5]

The first antifolate TS inhibitor to enter clinical trials was 10-propargyl-5,8-dideazafolate (CB3717, PDDF) synthesized by Jones et al.[6] PDDF inhibited human TS with a $K_i \approx 10$ nM and showed in vivo antitumor activity. However, PDDF displayed nephrotoxicity as well as hepatotoxicity, and clinical trials were discontinued. The N10-propargyl moiety of PDDF provides a 10-fold increase in potency against TS as compared to its N10-methyl analogue. The crystal structure of the ternary complex PDDF with *Escherichia coli* (ec) TS and dUMP[7] shows that the N10-propargyl moiety is in



CB3717, PDDF

hydrophobic interaction with Phe176 and the backbone of Gly173. The propargyl moiety was thus determined to be essential for the potent activity of PDDF against TS. Following the unsuccessful clinical trials of PDDF, a series of 2-desamino modified analogues were reported which improved the solubility characteristics.[8] From these studies emerged the 2-desamino-2-methyl-N10-methyl-5,8-dideaza analogue ZD1694 (Tomudex).[9] This compound is a weaker inhibitor of TS ($K_i = 670$ nM), as compared to PDDF, however the 2-methyl moiety affords significantly improved solubility characteristics compared to the 2-amino analogues. ZD1694 has been approved in Europe as an anticancer agent and is currently undergoing clinical trials in the United States. Recent X-ray crystallographic studies of the ternary complex of ecTS-dUMP-ZD1694[10] show its structure to be similar to that of ecTS-dUMP-PDDF.



ZD1694

Taylor et al.[11] reported the 2-amino-4-oxo-5-substituted classical pyrrolo[2,3-*d*]pyrimidine LY231514 as a multitarget antifolate which inhibits TS as well as

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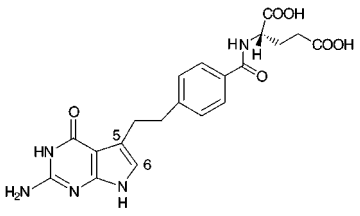
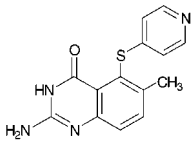
dihydrofolate reductase (DHFR).[12] This compound is currently in phase II clinical trials as an antitumor agent and differs from PDDF and ZD1694 in that it contains a 6-5-pyrrolo[2,3-*d*]pyrimidine ring system rather than the 6-6-quinazoline ring system of PDDF and ZD1694. LY231514 is a moderate TS inhibitor with a K_i = 340 nM (recombinant mouse); however, conversion to the pentaglutamate increased its TS inhibitory potency by 100-fold.[12] Interestingly, LY231514 is also a powerful DHFR inhibitor with a K_i = 7.0 nM as its monoglutamate.[12]



LY231514

AG337 (Thymitaq) is a nonclassical, 5-thiopyridine-substituted 2-amino-4-oxo-6-methylquinazoline antifolate which was designed as a TS inhibitor using the X-ray crystal structure of *E. coli* TS with PDDF and 5FdUMP by Webber et al.[13] This was the first nonclassical antifolate TS inhibitor to reach clinical trials.[14] AG337 is currently being developed by Zarix. AG337 had reached phase II/III clinical trials as an antitumor agent for the treatment of solid tumors of the liver and head and neck cancer by Agouron Pharmaceuticals.

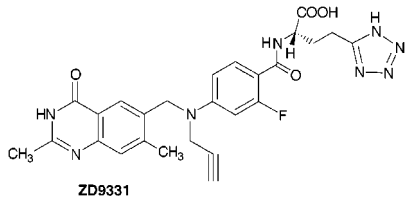


AG337

An important determinant of the antitumor activity of classical antifolates is their ability to function as a substrate for the enzyme folylpolyglutamate synthetase (FPGS) which converts classical antifolates into long chain noneffluxing poly-γ-glutamates within the cell. The ineffectiveness of many classical antifolates against resistant tumor cells has been attributed, in part, to both reduced uptake[15−18] and/or inefficient polyglutamylation.[19−22] Several examples exist where inefficient polyglutamylation contributes to poor antitumor activity, particularly with methotrexate (MTX), in solid tumors such as soft-tissue sarcomas,[23] cervical squamous cell carcinomas,[24] and head and neck squamous cell carcinomas.[25] In addition to solid tumors, certain forms of leukemias also respond ineffectively to MTX due to insufficient polyglutamylation.

Although polyglutamylation of certain classical antifolates appears necessary for cytotoxicity it has also been implicated as a possible cause of toxicity to host cells due to the prolonged retention of polyanionic forms of polyglutamates. Potent inhibitors of TS which do not require polyglutamylation would not only avoid drug resistance due to inefficient polyglutamylation but could also reduce the side effects associated with retention in host cells via polyglutamylation. Further lipophilic, nonclassical TS inhibitors such as AG337 do not require the reduced folate transport systems to gain access to tumor cells and hence could be useful where resistance is attributable to inefficient uptake systems.[13]

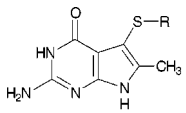
In an attempt to circumvent the drawbacks of polyglutamylation, Rosowsky et al.,[26a−c] Webber et al.,[13] Jackman et al.,[27] and Bavetsias et al.[28a−c] have designed and synthesized a variety of nonpolyglutamatable TS inhibitors as antitumor agents. The most successful of these is ZD9331 which is a 2,7-dimethyl-3′-fluoro-γ-tetrazole analogue of PDDF and was reported by Marsham et al.[29] This compound is a potent TS inhibitor



ZD9331

(K_i = 0.44 nM) as well as a potent inhibitor of L1210 cell growth, utilizes the reduced folate carrier system, and is currently in phase II clinical trials as an antitumor agent. The presence of the 7-methyl moiety as well as the tetrazole in ZD9331 prevents its polyglutamylation. Thus ZD9331 is currently the most successful nonpolyglutamatable antifolate and attests to the importance of nonpolygutamatable TS inhibitors as potential antitumor agents.

Gangjee et al.[30, 31] reported both classical and nonclassical 5-substituted 2-amino-4-oxo-6-methylpyrrolo-[2,3-*d*]pyrimidines which are hybrid analogues of AG337 and LY231514 and possess significant TS inhibitory activity. For example, compounds **1a**−**e** inhibited human TS with IC_{50} = 340,[30] 42,[30] 1000,[31] 130,[31] and 150 nM,[31] respectively, compared to PDDF IC_{50} = 180 nM[31] and ZD1694 IC_{50} = 880 nM.[31] Interestingly compound



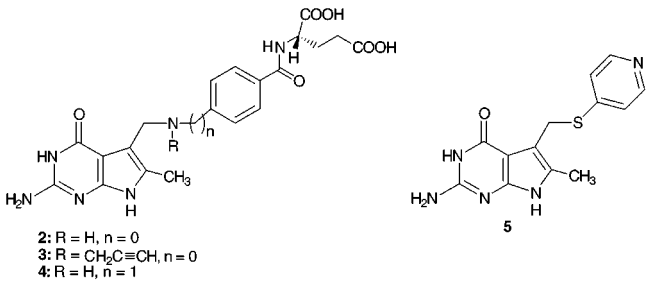
1a R = 4-pyridine
1b R = *p*-benzoyl-L-glutamate
1c R = 4′-ClPh
1d R = 3′,4′-diClPh
1e R = 4′-NO_2Ph

1b is not a substrate for FPGS.[30] Thus **1b** was different from PDDF, ZD1694, and LY231514 in that all three of these antitumor agents are substrates for FPGS and indeed require polyglutamylation for potent inhibition of TS and antitumor activity. Compounds such as **1b** might therefore be utilized in instances where the tumor is resistant to ZD1694 or LY231514 due to inefficient FPGS activity in the tumor system. We were therefore interested in providing additional analogues that were potent TS inhibitors which would not require polyglutamylation for antitumor activity.

Molecular modeling using SYBYL 6.4[32] and superimposing the 6-5-pyrrolo[2,3-*d*]pyrimidine on to the 6-6-quinazoline (PDDF and ZD1694) indicates that the smaller 6-5 system has its 5- and 6-atoms closer to the pyrimidine ring than the 6- and 7-atoms of the quinazoline. In compounds **1a**−**e** not only was the bridge attached to the 5-position of the pyrrolo[2,3-*d*]pyrimidine, thus shortening the distance between the pyrimidine ring and the side chain substituent as compared

to the quinazolines, but the bridge was also truncated to a single atom as compared with PDDF, ZD1694, and LY231514.

We reasoned that increasing the length of the bridge of compound **1b** by one atom would provide analogues of LY231514. Further increasing the bridge length of **1b** by an additional atom, providing a three-atom bridge, would compensate for the smaller 6-5 ring system as compared to the 6-6 ring system and would place the *p*-aminobenzoyl-L-glutamate side chain at approximately the same distance from the pyrimidine ring as in the 6-6 ring system analogues, PDDF and ZD1694. We thus designed compounds **2**−**5** as potential antitumor agents. In the classical antifolate compounds **2**−**3**, the two-atom bridge is a C8−N9 chain. Compound **3** contains the N9-propargyl moiety similar to PDDF. Compound **4** is a three-atom bridged analogue. Compound **5** is a bridge homologue of the significantly potent compound **1a** and was designed to compensate for the contraction of the B-ring of **5** compared to the 6-6 ring system of AG337.

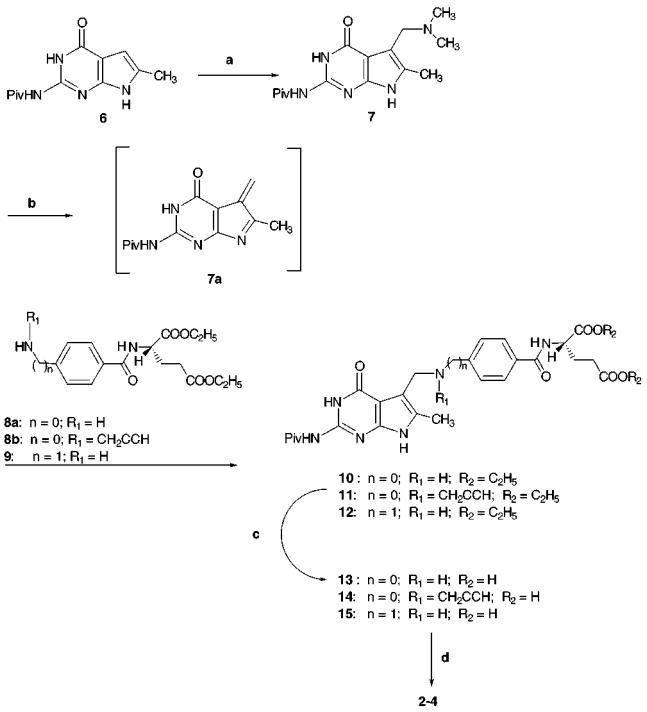


Webber et al.[13] in their design of the nonclassical TS inhibitor AG337 using the crystal structure of the ternary complex of ecTS-5FdUMP-PDDF suggested that the 6-methyl moiety of AG337 serves two important functions. In AG337 it makes important hydrophobic contacts with the Trp80 residue of ecTS and also conformationally restricts the 5-position side chain of AG337 into a favorable, low-energy conformation for attachment to TS. Molecular modeling using Sybyl 6.4 and superimposition of compounds **2**−**5** on to the ternary complex of ecTS-FdUMP-AG337[33] indicates that the pyrrolo[2,3-*d*]pyrimidine with a 6-methyl moiety mimics the 6-methyl quinazoline of AG337 and, as reported previously by Gangjee et al.[30,31] for compounds **1a**−**e**, provides for potent TS inhibition. In addition, metabolite protection studies of **1b** with FaDU squamous cell carcinoma cells indicated that the primary intracellular target of **1b** is TS. In addition to the role of the 6-methyl moiety to interact with Trp80 of ecTS and to conformationally restrict the 5-substituted side chain, Gangjee et al.[30] have reported that the 6-methyl group in classical 5-substituted pyrrolo[2,3-*d*]pyrimidines as in **1b** also prevents the molecule from functioning as a substrate for FPGS, and hence polyglutamylation of **1b** does not play a role in the cytotoxicity of **1b**. Thus we elected to retain the 6-methyl group in the design of compounds **2**−**5** as inhibitors of TS.

Chemistry

Gangjee et al.[31] recently reported the synthesis of the pivaloyl-protected pyrrolo[2,3-*d*]pyrimidine **6** (Scheme 1). 2-Acylamino pyrrolo[2,3-*d*]pyrimidines have been reported[31] to afford the 5-substituted aminomethyl compounds. The 2-acylamino group was proposed to increase the nucleophilicity of the β-carbon of the pyrrole ring compared to that of the unacylated analogue, thus favoring the 5-substituted product. Hence, a Mannich reaction was carried out on the pivaloylated pyrrolo-[2,3-*d*]pyrimidine **6** utilizing the method described by Benghiat and Crooks.[34,35] The Mannich reagent was prepared by the slow addition of 37% aqueous formaldehyde to a cold (0 °C) solution of *N*,*N*-dimethylamine in glacial acetic acid. Compound **6** was added to the cold reagent solution, and the mixture was heated to 70 °C for 5 h until the disappearance of **6** on TLC. The product **7** was isolated as a white solid in 82% yield. Compound **7** was homogeneous on TLC and was characterized by its ^{1}H NMR and high-resolution mass spectrum.

Scheme 1[a]

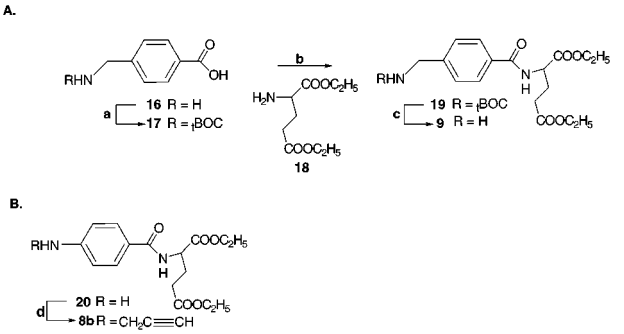


[a] Reagents and conditions: (a) dimethylamine/37% HCHO/AcOH; (b) DMF/Δ; (c) 1 N NaOH/sonicate/15 min; (d) 1 N NaOH/sonicate/1 h/50 °C.

The amine exchange reaction of 3-dimethylaminomethylindole and its related compounds with an amine (primary or secondary) was shown to proceed by an elimination−addition mechanism to afford indoles with a variety of aminomethyl side chains at the 3-position. Akimoto et al.[36] have used this technique to synthesize the nucleoside, Queuosine, analogues. The 5-(substituted aminomethyl)pyrrolo[2,3-*d*]pyrimidine **7** was found to undergo facile amine exchange reaction on warming a solution of the compound with an excess of the primary or secondary amine. The introduction of a new amino function into the pyrrolo[2,3-*d*]pyrimidine ring was presumed to have progressed via the formation of a conjugated unsaturated ring system **7a** by amine elimination from **7** followed by the addition of the added amine (Scheme 1).

The analogues **2** and **3** were synthesized by a similar amine exchange reaction between the intermediate **7**

Scheme 2[a]



[a] (a) Reagents and conditions: (a) DBDC; (b) isobutylchloroformate/Et_3N; (c) TFA/Me_2Cl_2/rt/10 min; (d) propargyl bromide/Hunig's base/toluene/90 °C/18−20 h.

and the appropriate L-glutamate diethyl esters **8a**,[37] **8b**,[6] and **9**[38] (Scheme 1). For compound **2** a mixture of *p*-aminobenzoyl L-glutamate diethyl ester **8a** and **7** was heated at 100 °C in DMF for 53 h. Workup and purification using silica gel column chromatography and gradient elution (0−10% MeOH/$CHCl_3$) afforded **10** in 47% yield. Compound **10** had a mp of 195 °C and was homogeneous on TLC. Its mass spectrum showed an M^+ peak at 582 and its ^{1}H NMR spectrum showed the 8-CH_2 at 4.31 ppm and the NH triplet at 6.45 ppm, along with the appropriate protons from both the glutamate and the pyrrolo[2,3-*d*]pyrimidine moieties, corroborating the structure. The ester groups were cleaved on sonicating a suspension of **10** in 1 N NaOH until a clear brown solution was formed (15 min). The product obtained upon acidification of this solution was identified, by ^{1}H NMR and elemental analysis, as the pivaloyl protected acid, **13**. The 2-pivaloyl group was cleaved on stirring **13** in 1 N NaOH for 1 h at 50 °C. Neutralization with glacial acetic acid afforded the target compound **2**, in 88% yield.

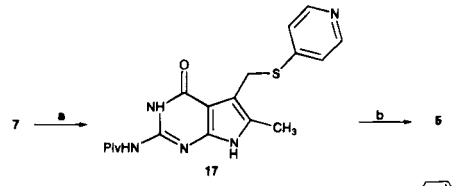
Compound **8b** was synthesized using a slight modification of the literature method.[6] Thus alkylation of *p*-aminobenzoyl-L-glutamate with propargyl bromide in the presence of Hunig's base and heating in toluene for 18−20 h afforded the monoalkylated product (Scheme 2B). Purification of the crude product using silica gel column chromatography with toluene/ethyl acetate (10:2) as eluent afforded **8b** which was identical to that reported in the literature.[6]

For the analogue **4**, *p*-aminomethylbenzoyl-L-glutamic acid diethyl ester (**9**) was used for the amine exchange reaction. The ester **9** was in turn obtained from 4-aminomethyl benzoic acid as previously described by Gangjee et al. (Scheme 2A).[38]

The amine exchange reaction between **7** and **8b** and **7** and **9** was extremely slow and occurred only when the dimethyl amino group of **7** was converted to a better leaving group via quaternization using methyl iodide.

A solution of the compound **7** in DMF was stirred with 0.9 equiv of methyl iodide. The quaternization of the dimethyl groups was assumed to have occurred when the DMF solution turned turbid after stirring for 2 h at room temperature. The amine **8b** or **9** was added to this turbid solution, and the mixture was sonicated until no more starting material was detected on TLC (48 h). Compounds **11** and **12** were isolated from the reaction with **8b** and **9**, respectively, after silica gel column

Scheme 3[a]



[a] Reagents and conditions: (a) (1) CH_3I, (2) HS— (**16**). (b) MeOH−1 N NaOH/sonicate/50 °C/1 h.

Table 1. Inhibitory Concentrations (IC_{50}, in μM) against TS

compd	*L. casei*[a,39]	human[39]
PDDF[31]	0.036	0.036
1a[30]	100	0.34
1b[30]	0.021	0.042
2	180	180
3	90	ND
4	360	ND
5	>26 (20)[a]	>25 (20)[a]

chromatography using gradient elution with 0−5% MeOH−$CHCl_3$. Compounds **11** and **12** were characterized by their ^{1}H NMR spectra and elemental analyses. The diethyl ester moiety and the 2-pivaloyl groups were cleaved by first dissolving the suspension of **11** or **12** in 1 N NaOH by sonication followed by heating at 50 °C for 1 h. Compounds **3** and **4** were obtained, as solids, upon acidification of this solution, to pH 2, with glacial acetic acid. Target compounds **3** and **4** were characterized by their ^{1}H NMR spectra and elemental analyses.

The nonclassical compound **5** was similarly synthesized. In this case a sulfur nucleophile was required to displace the dimethylamino moiety of **7** (Scheme 3). Benghiat and Crooks[34,35] have reported the preparation of *S*-(3-indolylmethyl) derivatives of cysteine from gramine, 2-methylgramine, and 3-(dimethylamino)-1*H*-pyrrolo[2,3-*b*]pyridine. The dimethylamino group of gramine was reported to be readily substituted by thiols in aqueous sodium hydroxide. An analogous displacement of the dimethylamino group of **7** with 4-mercaptopyridine (**16**) was expected to yield the analogue **17**. Thus a solution of **16** in DMF was first activated with NaH followed by the addition of 5-dimethylaminomethylpyrrolo[2,3-*d*]pyrimidine **7**. The reaction was allowed to proceed for 12 h at 50 °C after which the product **17** was isolated in 38% yield following workup. The pivaloyl group was cleaved by stirring a solution of **17** in a mixture of MeOH−1 N NaOH at 50 °C for 1 h, which afforded the target compound **5** in 51% yield. The product was homogeneous on TLC and was characterized by its ^{1}H NMR spectrum and elemental analysis.

Biological Evaluation and Discussion

The analogues **2**−**5** were evaluated as inhibitors of *Lactobacillus casei* TS and recombinant human TS.[39,40a,b] The inhibitory potencies (IC_{50}) are listed in Table 1 along with that of PDDF, **1a**, and **1b**. The *N*-propargyl analogue **3** was 2-fold and 4-fold more active against *L. casei* TS than the corresponding N-unsubstituted analogues **2** and **4** respectively. This result is in accord with previous reports of increased TS inhibitory activity of *N*-propargyl analogues as compared with the N-unsubstituted compounds.[6] Compound **2** was also evaluated and found to be poorly active against human TS. The nonclassical analogue **5** inhibited both *L. casei* and

human TS by 20% at 2.6×10^{-4} M and 2.5×10^{-4} M, respectively. Higher concentrations of **5** could not be tested due to interference with absorbance at 340 nm used in the TS assay.

In view of our interest in obtaining selective agents for two prevalent opportunistic infections in AIDS patients, we also evaluated the nonclassical analogue **5** as an inhibitor of the growth of *Pneumocystis carinii* and *Toxoplasma gondii* cells in culture.[41,42] Compound **5** inhibited the growth of *P. carinii* cells in culture by 76% at 32 μM and *T. gondii* cells in culture by 50% at 831 μM. The assay for the inhibition of the growth of *P. carinii* cells in culture did not allow for a lower concentration of **5** to be tested.

The lack of potent inhibitory activity of the classical analogues **2**−**4** against TS was surprising, particularly in light of the high potencies of the classical analogue **1b** and LY231514 against TS. In addition, the low potency of the nonclassical analogue **5** against human TS compared to **1a** was also surprising. On the basis of the analogues reported in this study, along with the previous reports of Gangjee et al.,[30,31] it appears that for 5-substituted pyrrolo[2,3-*d*]pyrimidines the 6-methyl moiety is conducive for activity when the bridge at the 5-position is a single sulfur atom. This is true for the classical as well as the nonclassical analogues. However, if the bridge is homologated to a two-atom or three-atom chain with a 6-methyl substituent, the analogue loses activity as compared with a one-atom sulfur bridge (compare **1a** and **1b** with **2**−**5**). That homologation of the bridge to two atoms is conducive to TS inhibitory activity, at least in the classical pyrrolo[2,3-*d*]pyrimidine analogues, is illustrated by LY231514. Thus it would appear that the methyl moiety at the 6-position of compounds **2**−**5** could be responsible, in part, for the decrease in TS inhibitory activity. Taylor and Hu[43] have shown that placing a 6-methyl group on LY231514 substantially decreases the cytotoxicity of the molecule against the growth of CEM cells in culture; however, no TS inhibitory data was reported.

Molecular modeling indicated that the 6-methyl moiety of **2**−**5** occupies essentially the same position as the 6-methyl group of AG337 when bound to ecTS such that it interacts with Trp80 of ecTS. Further, since this interaction is conducive to potency against TS, it is unlikely that this attribute of the 6-methyl moiety could be responsible for the decreased inhibitory activity of compounds **2**−**5**. Webber et al.[13] suggested that the 6-methyl group of AG337 also functions to conformationally orient the side chain of AG337 for optimal interaction with TS. In compounds **2**−**5**, which contain a more flexible two- or three-atom bridge, the conformational restricting influence of the 6-methyl moiety is perhaps not as important as in **1a**, **1b**, and AG337 which contain a single sulfur atom bridge. It is conceivable that the 6-methyl group of analogues **2**−**5** may cause an unfavorable orientation of the side chain for interaction with TS, and any gain in interaction with Trp80 of the 6-methyl moiety is more than offset by the necessity of the side chain to reorient itself into a less stable conformation in order to interact with ecTS. The synthesis of analogues of compounds **2**−**5**, which lack the 6-methyl group, are currently in progress to determine the contribution of the 6-methyl moiety to TS inhibition and will be the subject of a future communication.

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in an Abderhalden drying apparatus over P_2O_5 and refluxing ethanol or toluene. Melting points were determined on a Fisher−Johns melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz) spectrometer. Data were accumulated by 16 K size with a 0.5 s delay time and 70° tip angle. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV_{254} silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Elemental analyses were performed by Atlantic Microlabs Inc., Norcoss, GA. Analytical results indicated by element symbols are within ±0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates were not removed despite 24−48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectrum. All solvents and chemicals were purchased from Aldrich Chemical Co. and Fisher Scientific and were used as received.

2-Pivaloylamino-6-methyl-5-[(dimethylamino)methyl]-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine (7). Glacial AcOH (6 mL) was added dropwise over a period of 0.5 h to ice-cold 40% w/v aqueous solution of dimethylamine (3 mL, 26.7 mmol). Aqueous HCHO (37%) (2.20 mL, 29.3 mmol) was then added followed by 2-pivaloylamino-6-methyl-4-oxo-7*H*-pyrrolo[2,3-*d*]-pyrimidine (**6**) (1.93 g, 7.80 mmol). The suspension was warmed to 70 °C, and the solution formed was stirred at 70 °C for 5 h, cooled to room temperature, and evaporated under reduced pressure, azeotroping first with water (3 × 5 mL) and then with MeOH (3 × 5 mL). Anhydrous ether (20 mL) was then added to the oily residue which was then cooled at 0 °C for 12 h. The white solid which separated was filtered, washed with excess anhydrous ether, and dried under vacuum for 12 h to yield 1.90 g (82%) of **7** as an off-white powder: mp 115−120 °C; TLC R_f = 0.14 (MeOH/1 drop NH_4OH, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.23 (s, 9 H, C $(CH_3)_3$), 2.12 (s, 6 H, $N(CH_3)_2$), 2.19 (s, 2 H, CH_2), 3.52 (s, 3 H, 6 CH_3), 11.28 (s, 1 H, NH); HRMS calculated for $C_{15}H_{23}N_5O_2$, m/z = 305.1851; found m/z = 305.1861.

***N*-{2-Pivaloylamino-4-oxo-6-methyl[(pyrrolo[2,3-*d*]-pyrimidin-5-yl)amin-methylene]benzoyl}-L-glutamic Acid Diethyl Ester (10).** A mixture of **6** (0.50 g, 1.64 mmol) and *p*-aminobenzoylglutamic acid diethyl ester (**8a**) (0.50 g, 1.55 mmol) in DMF (5 mL) was heated to 100 °C for 53 h. The reaction mixture was then cooled to room temperature, and the DMF evaporated under reduced pressure. The residue was dissolved in MeOH, and 1.00 g of silica gel was added to this solution which was evaporated to dryness to form a plug which was loaded onto a dry silica gel column (2.4 cm × 20 cm) and eluted with 0−10% MeOH:$CHCl_3$ gradient. Fractions containing the product eluted in 10% MeOH:$CHCl_3$ and were pooled together and evaporated to yield 0.45 g (47%) of **10** as a light brown powder: mp 195 °C; MS m/z 582 (M^+); TLC R_f = 0.74 ($CHCl_3$/MeOH/NH_4OH, 9:2:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.15 (m, 6 H, CH_2CH_3), 1.23 (s, 9 H, $C(CH_3)_3$), 2.00 (m, 2 H, Glu β-CH_2), 2.28 (s, 3 H, 6-CH_3), 2.40 (t, 2 H, Glu γ-CH_2), 4.05 (m, 4 H, OOC CH_2 CH_3), 4.31 (m, 3 H, N−CH_2, Glu α-CH), 6.45 (t, 1 H, 9-NH), 6.68 (d, 2 H, 3′,5′-CH), 7.61 (d, 2 H, 2′,6′-CH), 8.23 (d, 1 H, CONH), 10.75 (s, 1 H, NH), 11.45 (s, 1 H, NH), 11.50 (s, 1 H, NH). Anal. ($C_{29}H_{38}N_6O_7$) C, H, N.

***N*-{2-Pivaloylamino-4-oxo-6-methyl[(pyrrolo[2,3-*d*]-pyrimidin-5-yl)methylaminomethylene]benzoyl}-L-glut-**

amic Acid Diethyl Ester (12). To a solution of **7** (0.50 g, 1.64 mmol) in DMF (10 mL) was added CH_3I (0.06 mL, 1.48 mmol), and the mixture was stirred at room temperature for 2 h until the solution turned turbid. The glutamate ester **9** (0.498 g, 1.48 mmol) was added to this mixture which was ultrasonicated for 48 h until no more starting material could be detected on TLC. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in MeOH, and 1.00 g of silica gel was added to this solution and evaporated to dryness to form a plug which was loaded onto a dry silica gel column (2.4 cm × 20 cm) and eluted with a 0−5% MeOH:$CHCl_3$ gradient. Fractions containing the product eluted in 5% MeOH:$CHCl_3$ and were pooled together and evaporated to yield 0.17 g (17%) of **12** as a light brown powder: mp >190 °C dec; TLC R_f = 0.58 ($CHCl_3$/MeOH/NH_4OH, 9:2:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.17 (m, 6 H, CH_2CH_3), 1.21 (s, 9 H, $C(CH_3)_3$), 2.03 (m, 2 H, Glu β-CH_2), 2.27 (s, 3 H, 6-CH_3), 2.43 (t, 2 H, Glu γ-CH_2), 4.08 (m, 4 H, $OOCCH_2CH_3$), 4.24 (d, 4 H, CH_2−N−CH_2), 4.44 (m, 1 H, Glu α-CH), 7.59 (d, 2 H, 3′,5′-CH), 7.94 (d, 2 H, 2′,6′-CH), 8.80 (d, 1 H, CONH), 9.36 (bm, 1 H, C−NH−C), 10.97 (s, 1 H, NH), 11.80 (s, 1 H, NH), 12.22 (s, 1 H, NH).

N-{**2-Pivaloylamino-4-oxo-6-methyl[(pyrrolo[2,3-*d*]-pyrimidin-5-yl) aminomethylene]benzoyl**}-L-**glutamic Acid (13).** A suspension of **10** (0.20 g, 0.34 mmol) in 1 N NaOH (5 mL) was ultrasonicated for 15 min until a brown solution had formed which was stirred for an additional 2 h at room temperature and then neutralized with glacial AcOH. The resulting suspension was refrigerated for 2 h and filtered to yield 0.15 g (88%) of **13** as a bright yellow powder: TLC R_f = 0.14 ($CHCl_3$/MeOH, 10:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.24 (s, 9 H, $C(CH_3)_3$), 1.91 (m, 2 H, Glu β-CH_2), 2.08 (t, 2 H, Glu γ-CH_2), 2.28 (s, 3 H, 6-CH_3), 4.31 (d, 2 H, N−CH_2), 4.85 (m, 1 H, Glu α-CH), 6.45 (t, 1 H, 9-NH), 6.66 (d, 2 H, 3′,5′-CH), 7.64 (d, 2 H, 2′,6′-CH), 8.07 (d, 1 H, CONH), 10.78 (s, 1 H, NH), 11.34 (s, 1 H, NH), 11.86 (s, 1 H, NH). Anal. ($C_{25}H_{30}N_6O_7$·1.0 H_2O) C, H, N.

N-{**2-Amino-4-oxo-6-methyl[(pyrrolo[2,3-*d*]pyrimidin-5-yl)aminomethylene]benzoyl**}-L-**glutamic Acid (2).** A suspension of **10** (0.20 g, 0.34 mmol) in 1 N NaOH (5 mL) was ultrasonicated for 15 min until a brown solution had formed. MeOH (10 mL) was added to this solution which was stirred at 50 °C for 1 h. The solution was then concentrated under reduced pressure, cooled to 0 °C, and neutralized with glacial AcOH. The resulting bright yellow suspension was refrigerated for 2 h and filtered to yield 0.13 g (87%) of **2**: mp > 200 °C; TLC R_f = 0.52 (30% NH_4HCO_3, cellulose); ^{1}H NMR (Me_2SO-d_6) δ 1.86 (m, 2 H, Glu β-CH_2), 2.08 (s, 3 H, 6-CH_3), 2.23 (m, 2 H, Glu γ-CH_2), 4.13 (m, 2 H, N−CH_2), 4.24 (m, 1 H, Glu α-CH), 5.94 (s, 2 H, NH2), 6.45 (t, 1 H, 9-NH), 6.54 (d, 2 H, 3′,5′-CH), 7.53 (d, 2 H, 2′,6′-CH), 8.02 (d, 1 H, CONH), 10.20 (s, 1 H, NH), 10.67 (s, 1 H, NH). Anal. ($C_{20}H_{22}N_6O_6$·1.5 H_2O) C, H, N.

N-{**2-Amino-4-oxo-6-methyl[(pyrrolo[2,3-*d*]pyrimidin-5-yl)propargylaminomethylene]benzoyl**}-L-**glutamic Acid (3).** To a solution of **7** (0.50 g, 1.64 mmol) in DMF (15 mL) was added CH_3I (0.06 mL, 1.48 mmol), and the mixture stirred at room temperature for 2 h until the solution turned turbid. The propargyl glutamate ester (**8b**) (0.50 g, 1.48 mmol) was added to this mixture which was heated at 100 °C for 48 h. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in MeOH, and 1.00 g of silica gel was added to this solution and evaporated to dryness to form a plug which was loaded onto a dry silica gel column (2.4 cm × 20 cm) and eluted with a 0−5% MeOH:$CHCl_3$ gradient. Fractions containing the product eluted in 5% MeOH:$CHCl_3$ and were pooled together and evaporated to afford the pivaloylated ester **11** which was dissolved in MeOH (5 mL) containing 0.1 N NaOH. The mixture was heated at 50 °C for 1 h. The MeOH was evaporated, and the aqueous solution was acidified with glacial AcOH to afford a brown precipitate. This was filtered, washed with water, and dried over P_2O_5 to yield 0.030 g (4%) of **2** as a light brown powder: mp >200 °C dec; TLC R_f = 0.68 ($CHCl_3$/MeOH/NH_4OH, 9:2:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.01 (m, 2 H, CH_2), 2.22 (m, 2 H, CH_2), 2.33 (s, 3 H, CH_3), 3.13 (t, 1 H, C≡CH), 3.99 (s, 2 H, CH_2), 4.42 (s, 2 H, CH_2), 5.40 (m, 1 H, CH), 6.05 (bs, 2 H, NH_2), 6.67 (d, 2 H, C_6H_5), 7.10 (d, 2 H, C_6H_5), 7.75 (d, 1 H, CONH), 10.38 (bs, 1 H, NH), 10.8 (bs, 1 H, NH). Anal. ($C_{23}H_{24}N_6O_6$·0.5 H_2O) C, H, N.

N-{**2-Amino-4-oxo-6-methyl[(pyrrolo[2,3-*d*]pyrimidin-5-yl)methylamino methylene]benzoyl**}-L-**glutamic Acid (4).** A suspension of **12** (0.12 g, 0.20 mmol) in 1 N NaOH (0.5 mL) was ultrasonicated for 15 min until it formed a clear brown solution. MeOH (5 mL) was added to this solution which was stirred at 50 °C, for 1 h. The solution was then concentrated under reduced pressure, cooled to 0 °C and acidified (pH 3.5) with glacial AcOH. The resulting suspension was filtered, washed with water, and dried to yield 0.04 g (44%) of **4**: mp > 200 °C; TLC R_f = 0.15 (30% $CHCl_3$/MeOH/NH_4OH 9:2:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.03 (m, 2 H, Glu β-CH_2), 2.18 (s, 3 H, 6-CH_3), 2.37 (t, 2 H, Glu γ-CH_2), 4.07 (d, 2 H, N−CH_2), 4.17 (d, 2 H, N−CH_2), 4.37 (m, 1 H, Glu α-CH), 6.53 (s, 2 H, NH_2), 7.57 (d, 2 H, 3′, 5′−CH), 7.93 (d, 3 H, 2′, 6′−CH, C−NH−C), 8.47 (d, 1 H, CONH), 11.16 (s, 1 H, NH). Anal. ($C_{21}H_{24}N_6O_6$·1.5 CH_3COOH) C, H, N.

2-Pivaloylamino-6-methyl-5-[(4′-pyridine)mercaptomethylene]-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidine (17). To a solution of 4-mercaptopyridine **16** (1.00 g, 9.04 mmol) in DMF (5 mL) was added NaH (0.10 g, 4.52 mmol), and the mixture was stirred for 1 h at room temperature. The Mannich base **7** (1.15 g, 3.16 mmol) was then added, and the reaction mixture was warmed to 50 °C for 48 h until no **7** was detected on TLC. The reaction mixture was then cooled to room temperature, and the solvent evaporated under reduced pressure. Water (150 mL) was added to the residue which was then filtered and dried to yield 0.50 g (42%) of **22**: mp > 150 °C dec; TLC R_f = 0.65 ($CHCl_3$/MeOH, 9:3, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.24 (s, 9 H, $C(CH_3)_3$), 2.26 (s, 3 H, 6-CH_3), 4.47 (d, 2 H, CH_2−S), 7.40 (d, 2 H, C_5H_4), 8.37 (d, 2 H, C_5H_4), 10.78 (s, 1 H, NH), 11.46 (s, 1 H, NH), 11.83 (s, 1 H, NH). Anal. ($C_{18}H_{21}N_5SO_2$·0.5 H_2O) C, H, N, S.

2-Amino-6-methyl-5-[(4′-pyridine)mercaptomethylene]-4-oxo-7 *H*-pyrrolo-[2,3-*d*]pyrimidine (5). A solution of **17** (0.5 g, 1.3 mmol) in MeOH (45 mL) containing 1 N NaOH (4.5 mL) was stirred at 45−50 °C for 1 h until no more **17** was detected on TLC. The solvents were evaporated under reduced pressure and water (50 mL) was added to the residue which was then neutralized with 50% HCl. The bright yellow suspension was filtered, and the residue was recrystallized from a mixture of ethyl acetate:glacial AcOH (9:1). The resulting light yellow solid was filtered and dried to yield 0.56 g (51%) of **5**: mp > 175 °C dec; TLC R_f = 0.56 ($CHCl_3$/MeOH 9:3, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.14 (s, 3 H, 6-CH_3), 4.38 (s, 2 H, CH_2−S), 6.02 (s, 2 H, NH_2), 7.31 (d, 2 H, C_5H_4), 8.33 (d, 2 H, C_5H_4), 10.28 (s, 1 H, NH), 10.91 (s, 1 H, NH). Anal. ($C_{13}H_{13}N_5SO$·0.1 H_2O·0.3$CH_3COOC_2H_5$) C, H, N, S.

Thymidylate Synthase Assay. Thymidylate synthase was assayed spectrophotometrically at 30° and pH 7.4 in a mixture containing 0.1 M 2-mercaptoethanol, 0.0003 M (6*R*,*S*)-tetrahydrofolate, 0.012 M formaldehyde, 0.02 M $MgCl_2$, 0.001 M dUMP, 0.04 M trisHCI, and 0.00075 M naEDTA.

This was the assay described by A. J. Wahba and M. Friedkin,[40a] except that the dUMP concentration was increased 25-fold as per V. J. Davisson, W. Sirawaraporn, and D. V. Santi.[40b] The reaction was initiated by the addition of an amount of enzyme yielding a change in absorbance at 340 nm of 0.016/min in the absence of inhibitor. The percent inhibition was determined at a minimum of four inhibitor concentrations within 20% of the 50% point. The standard deviations for determination of the 50% points were within ±10% of the values given.

Uracil Incorporation by Cultured *T. gondii*. Uracil is incorporated into nucleic acid by *T. gondii* grown in culture.[44] *T. gondii* was originally isolated from an AIDS patient at Indiana University School of Medicine. The organism was isolated by growth in a BALB/c mouse and subsequently amplifed by passage in mice. Organisms were taken from the peritoneal fluid of infected mice and preserved by freezing.

Frozen stocks were used to inoculate cultures for drug testing. The organism may be passed for several generations in culture without loss of viability. Because mammalian cells do not avidly incorporate uracil, the incorporation of uracil can be used as an index of growth of *T. gondii* on host cells. For these experiments *T. gondii* was grown on HEL (human embryonic lung) cells with minimum essential medium (MEM) supplemented with glutamine (2 mM), penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively), and fetal bovine serum (10%). HEL cells were grown to confluency in 24-well tissue culture plates using MEM as described above. To inoculate, old medium was removed and replaced with 0.5 mL of fresh medium containing 5×10^4 *T. gondii* tachyzoites obtained from mouse peritoneal fluid or from tissue culture. Four hours after addition of the inoculum, an additional 0.5 mL of medium containing drug or control diluent was added to the wells. Twenty-four hours after inoculation, 1 μCi of tritiated uracil (New England Nuclear) was added to each well, and incubation continued for an additional 24 h. At the end of this period, the medium was sampled to determine remaining counts in the supernatant. The cells were dislodged, suspended by agitation, and filtered through glass fiber filters (Whatman) using a well washing apparatus with a 24-place filter manifold. Each well was washed with cold isotonic saline and added to the filter. The filters were washed with approximately 20 mis of cold isotonic saline, dried overnight, removed to scintillation vials, and counted with Ultima Gold scintillant (Packard).

Culture of *P. carinii* for Drug Testing. Compounds were evaluated in short term culture using inocula from *P. carinii*-infected rat lung and cell cultures of human embryonic lung fibroblasts (HEL cells) as described.[45−48] *P. carinii* was orginally obtained from a spontaneous infection in an immunosuppressed Sprague−Dawley rat. The lung of that rat was used to make homogenates that contained viable *P. carinii* which were then transtracheally inoculated into immunosuppressed Sprague−Dawley rats that were initially free of any *P. carinii*. The organism has been passaged in this way for over 10 years, and frozen stocks are maintained to allow restart or expansion of the colony for drug studies. The chromosome pattern determined by PFGE has remained stable over time. This strain of *P. carinii* is available through the AIDS Research and Reference Reagent Program. Briefly, tissue cultures were prepared using 24-well plates in which HEL cells had been grown to confluency in MEM containing 10% fetal calf serum. They were inoculated with 7×10^5 viable *P. carinii* trophozoites per milliliter. Inoculum was prepared by grinding *P. carinii*-infected rat lung in MEM, centrifuging at 250*g* to remove fragments of tissue, and counting numbers of organisms in 10 μL samples of supernate with Giemsa staining and fluorescein diacetate/ethidium bromide viability stain. On the basis of these counts, the number of organisms per milliliter was adjusted by adding MEM to achieve the desired concentration.

Each drug concentration to be tested was incorporated into the medium of four wells on each of four plates. Each plate also contained four wells without drug that were inoculated with *P. carinii*; these wells served as positive growth controls. Experiments were discarded if numbers of organisms in these wells failed to increase more than 3-fold over 7 days. Plates were incubated in a gaseous mixture of 5% O_2, 10% CO_2, and balance N_2 at 35 °C.

Evaluation by Morphology. For determination of numbers of organisms by morphology, cultures were sampled by washing medium over cells with Pasteur pipets and removing 10 μL samples of the supernate to 1 cm^2 areas that had been etched on slides.[47,49] Slides were air-dried, fixed in methanol, and stained with Giemsa. Two individuals counted numbers of trophozoites, cysts, and cells in 10 oil immersion fields of each slide, and mean values were plotted.

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