

# Potent Dual Thymidylate Synthase and Dihydrofolate Reductase Inhibitors: Classical and Nonclassical 2-Amino-4-oxo-5-arylthio-substituted-6-methylthieno[2,3-*d*]pyrimidine Antifolates

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*N*-{4-[(2-Amino-6-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoyl}-L-glutamic acid (**4**) and nine nonclassical analogues **5–13** were synthesized as potential dual thymidylate synthase (TS) and dihydrofolate reductase (DHFR) inhibitors. The key intermediate in the synthesis was 2-amino-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (**16**), which was converted to the 5-bromo-substituted compound **17** followed by an Ullmann reaction to afford **5–13**. The classical analogue **4** was synthesized by coupling the benzoic acid derivative **19** with diethyl L-glutamate and saponification. Compound **4** is the most potent dual inhibitor of human TS (IC<sub>50</sub> = 40 nM) and human DHFR (IC<sub>50</sub> = 20 nM) known to date. The nonclassical analogues **5–13** were moderately potent against human TS with IC<sub>50</sub> values ranging from 0.11 to 4.6 μM. The 4-nitrophenyl analogue **7** was the most potent compound in the nonclassical series, demonstrating potent dual inhibitory activities against human TS and DHFR. This study indicated that the 5-substituted 2-amino-4-oxo-6-methylthieno[2,3-*d*]pyrimidine scaffold is highly conducive to dual human TS-DHFR inhibitory activity.

## Introduction

Most eukaryotic organisms synthesize the essential metabolite thymidylate via the thymidylate cycle which consists of three enzymes, serine hydroxymethyltransferase (SHMT<sup>a</sup>), thymidylate synthase (TS), and dihydrofolate reductase (DHFR). SHMT catalyzes the interaction of serine and tetrahydrofolate to form 5,10-methylenetetrahydrofolate (5,10-MTHF). TS then catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5' monophosphate (dTMP), with 5,10-MTHF as the cofactor, and the formation of dihydrofolate. DHFR then completes the cycle by catalyzing the conversion of dihydrofolate to tetrahydrofolate using NADPH as the reductant.<sup>1–5</sup> Inhibition of TS or of DHFR leads to "thymineless death" in the absence of salvage,<sup>6,7</sup> and inhibition of these enzymes has found clinical utility as antitumor, antimicrobial, and antiprotozoal agents.<sup>8,9</sup>

As shown in Figure 1, folate analogues that inhibit TS generally contain a 2-amino-4-oxo or 2-methyl-4-oxo substitution in the pyrimidine ring, for example, the clinically used pemetrexed (PMX)<sup>10</sup> and raltitrexed (RTX).<sup>11</sup> In contrast, folate analogues that inhibit DHFR generally contain 2,4-diamino substitution in the pyrimidine ring, typified by methotrexate (MTX),<sup>12</sup> a DHFR inhibitor that has been a mainstay in cancer chemotherapy. Raltitrexed (Figure 1) is a quinazoline based analogue that has been approved as a first-line agent for advanced colorectal cancer in several European countries, Australia, Canada, and Japan. PMX, RTX, and MTX are

transported into cells via the reduced folate carrier (RFC) and undergo rapid polyglutamylation by the enzyme folylpolyglutamate synthetase (FPGS).<sup>13–17</sup>

Pemetrexed, a 6–5 pyrrolo[2,3-*d*]pyrimidine represents an important example of a clinically used classical antifolate that has reported dual TS and DHFR inhibitory activity. Pemetrexed and its polyglutamylated metabolites are reported to be inhibitors of several important folate-dependent enzymes including TS, DHFR, glycinamide ribonucleotide formyltransferase (GARFT), and aminoimidazole carboxamideribonucleotide formyltransferase (AICARFT). Pemetrexed is designated as a multitargeted antifolate (MTA).<sup>18,19</sup> In combination with cisplatin, pemetrexed has been approved for malignant pleural mesothelioma and for non-small-cell lung cancer. The clinical success of pemetrexed has generated renewed interest in the design of single agents that function as dual inhibitors against TS and DHFR.<sup>20,21</sup> Such inhibitors could circumvent pharmacokinetic, drug–drug interactions, and/or toxicity disadvantages of administering two separate agents in combination chemotherapy protocols. In addition, the cost of a single agent might be less than two separate agents and might also improve patient compliance.

It has been our long-standing goal to design and synthesize single agents that are potent dual inhibitors against TS and DHFR. As part of a continuing effort to develop dual TS and DHFR inhibitors, Gangjee et al.<sup>20</sup> reported the synthesis of a classical antifolate *N*-{4-[(2-amino-6-methyl-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]benzoyl}-L-glutamic acid, **1** (Figure 2). Compound **1** is a potent inhibitor of human TS (IC<sub>50</sub> = 54 nM) and a moderate inhibitor of human DHFR (IC<sub>50</sub> = 2.2 μM), in its monoglutamate form, thus providing dual inhibition of human TS and human DHFR. More importantly, compound **1** was not a substrate for human FPGS at concentrations up to 1045 μM, indicating that **1**, unlike pemetrexed or raltitrexed does not require polyglutamylation for its potent inhibition of human TS. Molecular modeling using (SYBYL 6.91)<sup>22</sup> indicated that the 6-methyl group in **1** makes important hydrophobic contacts with Trp109 in human TS and also

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<sup>a</sup> Abbreviations: SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; DHFR, dihydrofolate reductase; dUMP, deoxyuridylate; dTMP, deoxythymidylate; 7,8-DHF, 7,8-dihydrofolate; MTX, methotrexate; RFC, reduced folate carrier; FPGS, folylpolyglutamate synthetase; GARFT, glycinamide ribonucleotide formyltransferase; AICARFT, aminoimidazole carboxamideribonucleotide formyltransferase; AIDS, acquired immunodeficiency syndrome; *P. carinii*, *Pneumocystis carinii*; *T. gondii*, *Toxoplasma gondii*; *E. coli*, *Escherichia coli*; PTX, piritrexim.

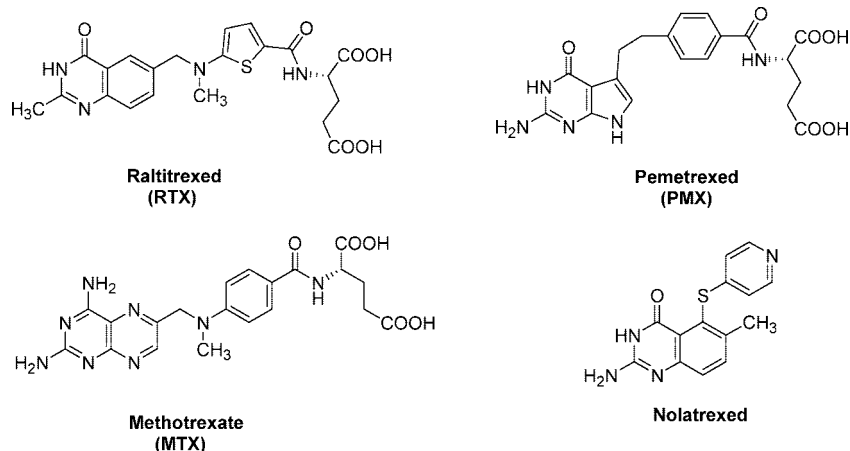


Figure 1

sterically restricts the rotation of the 5-position side chain so that it could adopt a favorable conformation for binding to human TS. In addition, molecular modeling of **1** suggested that the 6-methyl group makes hydrophobic contact with Val115 in human DHFR, which is absent in pemetrexed modeled in human DHFR. Thus, **1** is a promising lead compound that can be further structurally modified to optimize dual human TS and human DHFR inhibitory activities.

In the course of our structure-based drug design program, we investigated the thieno[2,3-*d*]pyrimidine scaffold, which could be considered an isostere of the pyrrolo[2,3-*d*]pyrimidine system, to develop potential dual TS and DHFR inhibitors. Thus, compound **4** (Figure 2) was designed as an isostere of **1** to determine the importance of the pyrrole 7-NH in **1** for binding to human TS and DHFR. The replacement of the NH of a pyrrolo[2,3-*d*]pyrimidine with a S to afford the thieno[2,3-*d*]pyrimidine was also anticipated to evaluate the importance of a hydrogen bond donor (NH) versus a hydrogen bond acceptor (S). In addition, the larger size of the sulfur atom compared to the nitrogen allows the thieno[2,3-*d*]pyrimidines to more closely mimic the size of the 6–6 pteridine system of folates. Molecular modeling using SYBYL 7.1 indicated that compound **4** should bind to human TS in the “normal” mode (Figure 3) and bind to human DHFR in the “flipped” mode similar to that proposed for pemetrexed,<sup>20</sup> thereby also allowing for human DHFR inhibition. We<sup>23,24</sup> and others<sup>25,26</sup> have shown, via X-ray crystal structures, that both the “normal” and “flipped” modes are viable binding modes for DHFR of similar analogues. Figure 4 shows the superimposition of **4** on pemetrexed in the X-ray crystal structure with human TS (PDB code 1JU6). Like compound **1**, the 6-methyl moiety of compound **4** makes important hydro-

phobic contacts with Trp109 in human TS and also serves to lock the 5-position side chain into favorable, low-energy conformations for TS binding. Both these aspects were anticipated to contribute to potent inhibitory activity of **4** against human TS in its monoglutamate form.

Molecular modeling revealed that compound **4** could also bind to human DHFR in either the “normal” folate binding mode to DHFR (Figure 5) or the “flipped” folate binding mode to DHFR (Figure 6). In the “normal” binding mode (Figure 5), compound **4** binds just like folic acid (PDB 1DRF). Superimposition onto folate (not shown) shows the 2-NH<sub>2</sub> and N3 moieties form hydrogen bonds with Glu30 (2.53 and 2.70 Å, respectively). The α-carboxyl group of **4** interacts with Arg70 in an ionic bond, and the thieno[2,3-*d*]pyrimidine and the phenyl ring make hydrophobic contacts with Phe31, Phe34, and Ile60 (Figure 5) in the binding pocket.

Additionally, molecular modeling indicated that compound **4** could also bind to the human DHFR in the “flipped” mode (Figure 6) compared to folic acid, in which the sulfur atom of the thieno ring is superimposed onto the 4-oxo moiety of folate. This is the “flipped” mode with respect to folate and is the binding mode for MTX to human DHFR, in its X-ray crystal structure (PDB 1U72). Superimposition of **4** onto MTX (not shown) in human DHFR (PDB 1U72) with the sulfur atom superimposed onto the 4-NH<sub>2</sub> group of MTX is shown in Figure 6. In this binding mode, the 4-oxo moiety of compound **4** makes hydrogen bonding with the backbone of Val115, Ile7, and Tyr121. The Glu30 residue also interacts with 2-NH<sub>2</sub> and N1 moieties of **4**. The *p*-aminobenzoyl ring along with thieno[2,3-*d*]pyrimidine ring makes hydrophobic interactions with Phe31,

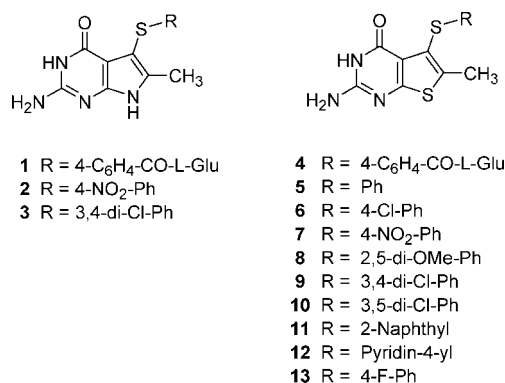
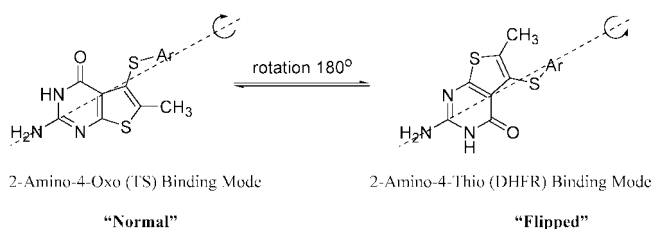
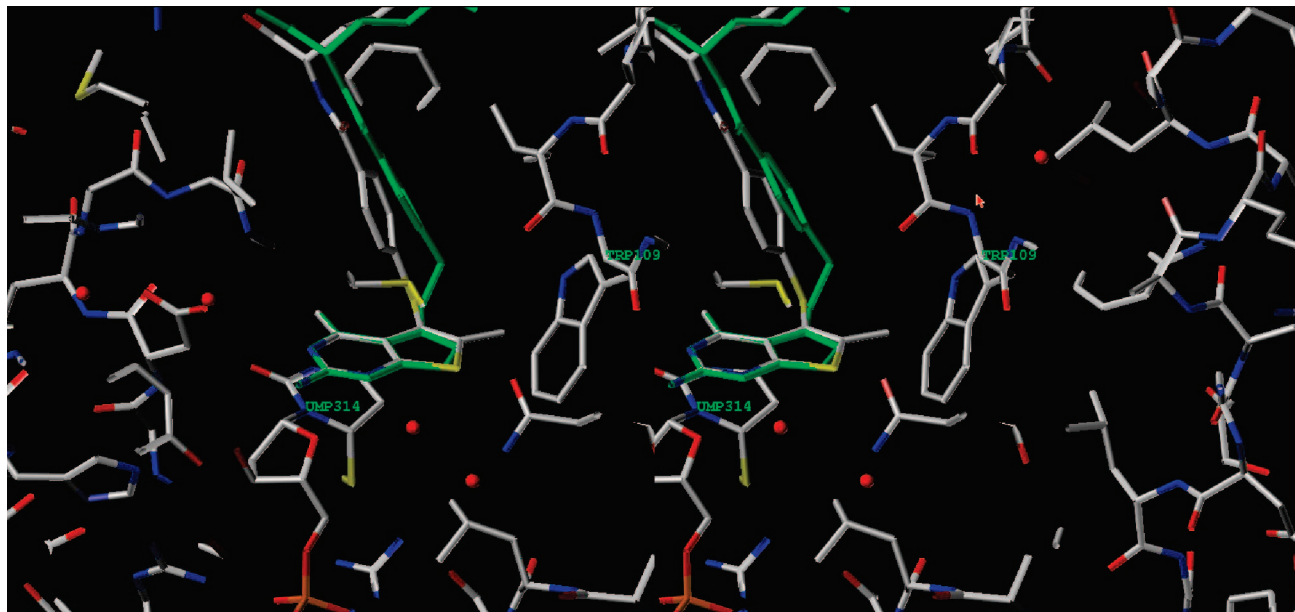


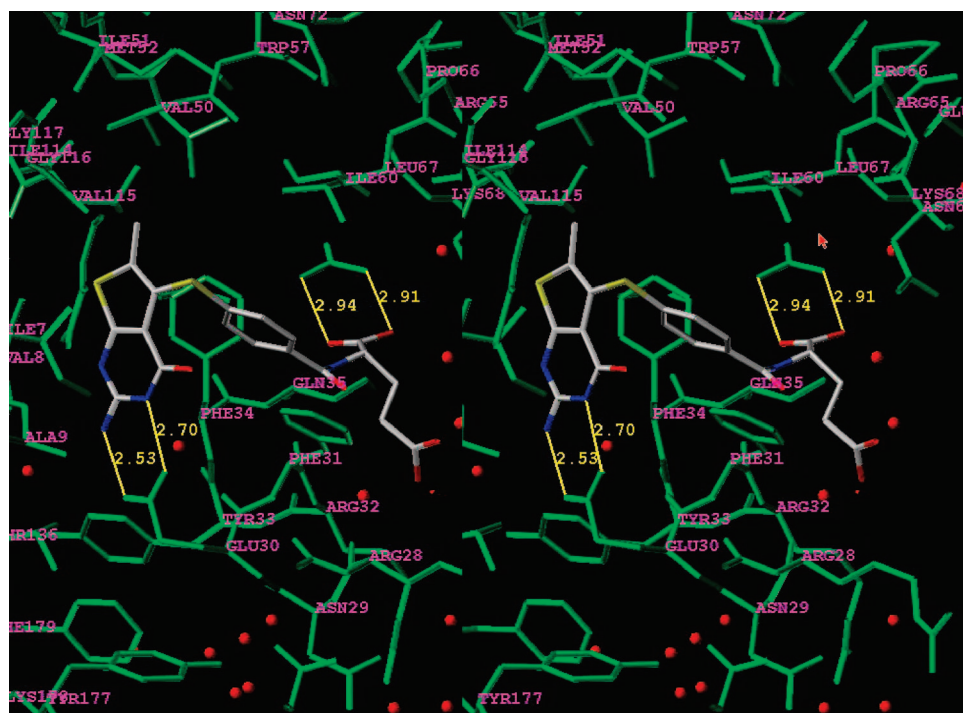
Figure 2



**Figure 3.** Proposed binding modes of 2-amino-4-oxo-5-substituted-6-methylthieno[2,3-*d*]pyrimidines. The “normal” mode is defined as the binding mode of pemetrexed and raltitrexed to human TS and folic acid (a 2-amino-4-oxopyrimidine system) to human DHFR. The “flipped” mode is defined as the binding mode when pemetrexed, raltitrexed, or folic acid is rotated about the C<sub>2</sub>–NH<sub>2</sub> bond by 180°.



**Figure 4.** Stereoview of compound **4** superimposed on pemetrexed (in green) in human TS (PDB code 1JU6).



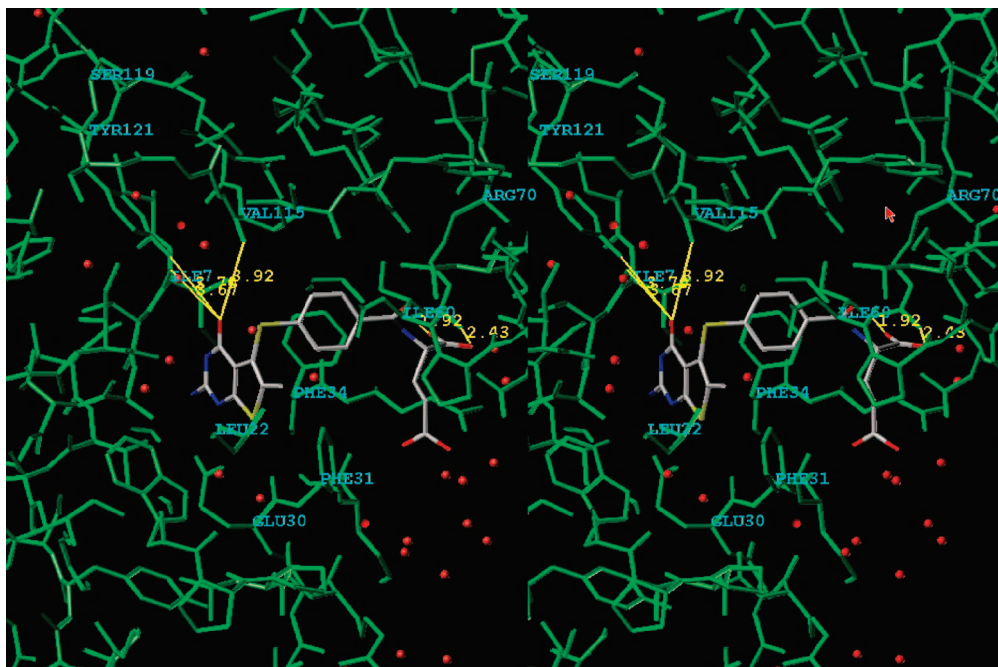
**Figure 5.** Stereoview: compound **4** bound to human DHFR in "normal" mode (PDB code 1DRF), superimposed on folic acid (not shown).

Phe34, and Ile60, and the  $\alpha$ -COOH forms an ionic bond with Arg70 just as MTX does with human DHFR.

On the basis of molecular modeling, the thieno[2,3-*d*]pyrimidine classical antifolate **4** can bind to human DHFR in either mode. Thus, *N*-{4-[(2-amino-6-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoyl}-L-glutamic acid, **4** (Figure 2), was designed and synthesized as a potential dual TS–DHFR inhibitor and an antitumor agent.

Tumor cells develop resistance to classical antifolates such as pemetrexed and raltitrexed, which depend on polyglutamylation for their antitumor effects by producing low or defective folylpoly- $\gamma$ -glutamate synthetase (FPGS).<sup>27,28</sup> Thus, the synthesis of classical antifolates that are potent inhibitors of TS and DHFR that do not require FPGS for their antitumor activity

is a desirable goal to overcome tumor resistance because of low or deficient FPGS. A further disadvantage of some classical antifolates as antitumor agents is that they require an active transport mechanism to enter tumor cells, which, when impaired, causes tumor resistance.<sup>29–32</sup> In addition, cells that lack these transport mechanisms, including several bacterial and protozoan cells, are not susceptible to the action of classical antifolates. In an attempt to overcome these potential drawbacks associated with classical antifolates, lipophilic nonclassical antifolates have also been designed and synthesized.<sup>3,33–35</sup> These lipophilic nonclassical antifolates such as nolatrexed (Figure 1) lack the polar glutamate moiety and hence do not depend on FPGS for their inhibitory activity.<sup>36,37</sup> In addition, nonclassical antifolates do not require the RFC system for active uptake into the cell,



**Figure 6.** Stereoview of compound **4** bound to human DHFR in the “flipped” mode (PDB code 1U72).

since they are lipophilic and are passively transported into cells. Gangjee et al.<sup>38</sup> described the design and synthesis of nonclassical antifolate analogues **2** ( $IC_{50} = 0.15 \mu M$ ) and **3** ( $IC_{50} = 0.13 \mu M$ ) as potent inhibitors of human TS, which were more potent against human TS than the clinically used pemetrexed. Thus, in addition to the classical analogue **4**, it was also of interest to synthesize nonclassical analogues **5–13** (Figure 2) with the thieno[2,3-*d*]pyrimidine scaffold as potential antitumor agents.

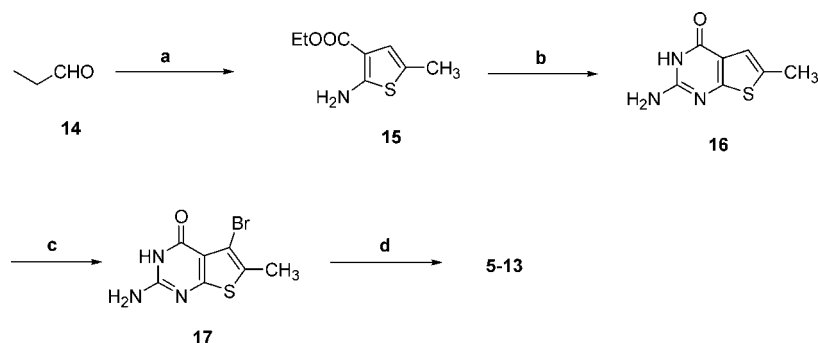
An additional aspect of our interest in nonclassical dual TS–DHFR inhibitors lies in the treatment of opportunistic infections in immunocompromised patients such as those with acquired immunodeficiency syndrome (AIDS).<sup>39</sup> The principal cause of death in patients with AIDS is opportunistic infections caused by *Pneumocystis carinii* (*P. carinii*)<sup>40</sup> and *Toxoplasma gondii* (*T. gondii*).<sup>41</sup> It should be noted that the thieno[2,3-*d*]pyrimidine scaffold has been examined previously for antibacterial and antimalarial activity.<sup>42–46</sup> Several nonclassical thieno[2,3-*d*]pyrimidine analogues have also been evaluated as inhibitors of *P. carinii* DHFR and *T. gondii* DHFR.<sup>43–46</sup> Some of these analogues have been found to be selective for DHFR from these pathogens.<sup>44,46</sup> However, none of these studies addressed the dual inhibitory effects of thieno[2,3-*d*]pyrimidines on TS and DHFR from *T. gondii*. Thus, we were also interested in evaluating the nonclassical analogues **5–13** as dual inhibitors of *T. gondii* DHFR and *T. gondii* TS.

## Chemistry

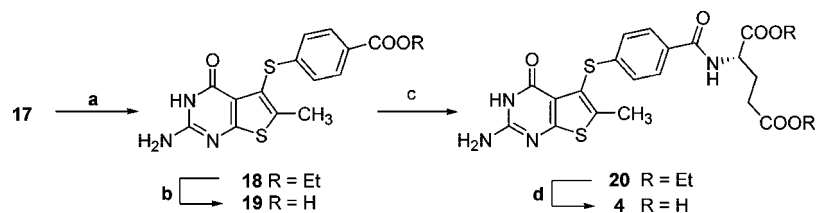
The synthetic method for the classical compound **4** and the nonclassical compounds **5–13** are outlined in Schemes 1 and 2. The key intermediate in the synthesis is 2-amino-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one, **16** (Scheme 1), which could be converted to the 5-bromo substituted compound **17** by bromination. Subsequent Ullmann coupling reaction with appropriate thiophenols would afford the target compounds **5–13** and the intermediate **18** for the classical target compound **4**. A literature search revealed that **16** has not been previously reported. Gangjee et al.<sup>46</sup> had reported that ethyl 2-amino-5-

alkyl-substituted-thiophene-3-carboxylates on cyclization with chlorformamidinium hydrochloride (generated from aminonitrile and HCl in ethyl ether as previously described<sup>46</sup>) afforded the corresponding 2-amino-6-alkyl-substituted-thieno[2,3-*d*]pyrimidin-4(3*H*)-ones in reasonably good yield. Thus, we envisioned that compound **16** could be obtained in a single step from chlorformamidinium hydrochloride and ethyl 2-amino-5-methylthiophene-3-carboxylate **15** using similar reaction conditions as reported previously. Treatment of commercially available propionaldehyde, **14** (Scheme 1), with ethylcyanoacetate, sulfur, and triethylamine in DMF under nitrogen for 2 h afforded **15** in 56% yield via a Gewald<sup>47</sup> reaction. Heating a mixture of **15**, chlorformamidinium hydrochloride and DMSO<sub>2</sub> at 125 °C for 30 min gave **16** in 87% yield.

With the key intermediate **16** in hand, it was initially anticipated that different arylthiols could be easily attached to the 5-position of **16** via an oxidative addition reaction using iodine in ethanol/water at reflux as reported by Gangjee et al.<sup>20,21,38</sup> Unfortunately, all attempts at this oxidative addition using a variety of reaction conditions of time and temperature variations were without success. Failure of the above method led us to explore a new alternative strategy that involved the bromination of the 5-position of intermediate **16** followed by displacement with arylthiols using the Ullmann coupling reaction. The first approach for the synthesis of 2-amino-5-bromo-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one, **17**, was to utilize NBS in a variety of solvents under different temperature conditions. When DMF or chloroform was used as solvent at room temperature or under reflux for 24 h, no new spot was detected on TLC, indicating that no reaction had occurred. However, when acetic acid was used as the solvent at reflux for 24 h, the brominated product **17** was obtained in a yield of 14%. The <sup>1</sup>H NMR of **17** in deuterated dimethyl sulfoxide indicated the absence of the 5-proton at 6.77 ppm. This along with elemental analysis indicated that bromination had occurred. In an attempt to circumvent the harsh reaction conditions (reflux 24 h) and to improve the overall yield, bromine instead of NBS was utilized as the brominating reagent. When DMF was used as the solvent at 50 °C for 24 h, no product was found (TLC).

Scheme 1<sup>a</sup>

<sup>a</sup> Reagents and conditions: (a) ethylcyanoacetate, TEA, sulfur, DMF, 50 °C to room temp, 2 h; (b) chloroformamidinium hydrochloride, DMSO<sub>2</sub>, 125 °C, 30 min; (c) Br<sub>2</sub>, AcOH, microwave 150 °C, 30 min; (d) thiols, Cu<sub>2</sub>O, K<sub>2</sub>CO<sub>3</sub>, DMF, microwave 180 °C, 30 min.

Scheme 2<sup>a</sup>

<sup>a</sup> Reagents and conditions: (a) ethyl 4-mercaptobenzoate, Cu<sub>2</sub>O, K<sub>2</sub>CO<sub>3</sub>, DMF, microwave 180 °C, 30 min; (b) 1 N NaOH, EtOH, room temp, 18 h; (c) L-glutamic acid diethyl ester hydrochloride, 2-chloro-4,6-dimethoxy-1,3,5-triazine, *N*-methylmorpholine, DMF, room temp, 5 h; (d) 1 N NaOH, EtOH, room temp, 24 h.

However, with acetic acid as solvent at reflux for 12 h a new spot was obtained on TLC with the disappearance of **16**, indicating that the reactant had been consumed. The isolated yield for **17** was 35%. Inspired by these initial results, the bromination of **16** was also attempted under microwave irradiation with bromine in acetic acid at 150 °C for 30 min, which afforded **17** in a yield of 80%. At this stage, with the intermediate **17** in hand, attention was turned to the synthesis of target compounds **5–13** (Scheme 1) and intermediate **18** (Scheme 2). The Ullmann coupling with **17** and appropriate arylthiols in the presence of Cu<sub>2</sub>O and K<sub>2</sub>CO<sub>3</sub> in DMF under microwave irradiation at 180 °C for 30 min afforded **5–13** in yields of 20–68%.

For the synthesis of the classical compound **4**, the required intermediate 4-(2-amino-6-methyl-4-oxo-3,4-dihydro-thieno[2,3-*d*]pyrimidine-5-ylsulfanyl)benzoic acid ethyl ester, **18** (Scheme 2), was prepared, using the same synthetic strategy as shown for **5–13** (Scheme 1) using ethyl 4-sulfanylbenzoate to afford **18** in a yield of 34%. Ester hydrolysis of **18** with 1 N NaOH at room temperature for 18 h afforded the corresponding acid **19** in 99% yield. Coupling of the acid **19** (Scheme 2) using conventional peptide coupling methods with L-glutamic acid diethyl ester hydrochloride and 2-chloro-4,6-dimethoxy-1,3,5-triazine as the activating agent followed by chromatographic purification afforded **20** in 65% yield.<sup>20,48</sup> The <sup>1</sup>H NMR of **20** revealed the newly formed peptide NH proton at 8.61 ppm as a doublet, which exchanged on addition of D<sub>2</sub>O. Hydrolysis of **20** with aqueous NaOH at room temperature, followed by acidification with 3 N HCl under ice cold conditions, afforded compound **4** in 60% yield.

## Biological Evaluation and Discussion

The classical and nonclassical analogues **4–13** were evaluated as inhibitors of human, *Escherichia coli* (*E. coli*), and *T. gondii* DHFR<sup>49</sup> and TS.<sup>50</sup> The inhibitory potencies (IC<sub>50</sub>) are listed in Table 1 and compared with pemetrexed, PDDF, MTX, and

trimethoprim. The classical analogue **4** was an excellent dual inhibitor of human TS (IC<sub>50</sub> = 40 nM) and human DHFR (IC<sub>50</sub> = 20 nM). Thus, compound **4** is a novel dual TS–DHFR inhibitor. To our knowledge this is the first example of a classical 2-amino-4-oxo-thieno[2,3-*d*]pyrimidine antifolate that possesses dual TS–DHFR inhibitory activity.

Against human TS, compound **4** was similar in potency to the previously reported **1** and about 2-fold more potent than PDDF and 238-fold more potent than pemetrexed. Against human DHFR, compound **4** was similar in potency to clinically used MTX (Table 1) and was 330-fold more potent than pemetrexed. These results indicate that isosteric structural modification of the pyrrolo[2,3-*d*]pyrimidine scaffold to a thieno[2,3-*d*]pyrimidine maintains high potency against human TS and remarkably increases human DHFR inhibition by 105-fold for **4** compared with compound **1**. Thus, the principal goal to increase the human DHFR inhibitory activity of **1** without compromising the potent human TS inhibitory activity of **1** was accomplished. Thus, on the basis of our results, the replacement of the smaller hydrogen bond donor pyrrolo NH with larger hydrogen bond acceptor S has little or no effect on human TS inhibition but increases human DHFR inhibitory potency by 105-fold for classical antifolates. The thieno[2,3-*d*]pyrimidine scaffold is the most conducive classical scaffold known to date for potent dual human TS/DHFR inhibitory activity.

On the basis of our molecular modeling, we have suggested the possibility of different binding modes of **4** to human DHFR. The actual mode of binding awaits the X-ray crystal structure that is currently underway. In addition to these molecular modeling studies there are three possible reasons for the better activities of thieno[2,3-*d*]pyrimidines than pyrrolo[2,3-*d*]pyrimidines against human DHFR. The first reason is that the larger 6–5 thieno[2,3-*d*]pyrimidine ring system more closely approximates the size of 6–6 bicyclic pteridine ring and 5-deaza folate systems of DHFR inhibitors MTX and 5-deaza folic acid than the pyrrolo[2,3-*d*]pyrimidine of **1**. Second, thieno[2,3-*d*]

**Table 1.** Inhibitory Concentrations (IC<sub>50</sub> in  $\mu$ M) against TS and DHFR<sup>a</sup>

compd	TS ( $\mu$ M)			DHFR ( $\mu$ M)			
	human <sup>b</sup>	<i>E. coli</i> <sup>b</sup>	<i>T. gondii</i> <sup>c</sup>	human <sup>d</sup>	<i>E. coli</i> <sup>e</sup>	<i>T. gondii</i> <sup>c</sup>	rh/tg <sup>f</sup>
<b>1</b> <sup>g</sup>	0.042	nd <sup>i</sup>	nd <sup>i</sup>	2.2	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>
<b>2</b> <sup>h</sup>	0.15	13	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>
<b>3</b> <sup>h</sup>	0.13	45	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>
<b>4</b>	0.04	0.04	0.036	0.02	0.2	0.008	2.5
<b>5</b>	1.2	29	8.4	3.5	>35 (18) <sup>i</sup>	0.12	29
<b>6</b>	0.26	5.2	2.6	1.5	28	0.031	48
<b>7</b>	0.11	1.2	1.2	0.56	1.2	0.056	10
<b>8</b>	4.6	>23 (36) <sup>i</sup>	>23 (0) <sup>i</sup>	22	>28 (0) <sup>i</sup>	0.056	393
<b>9</b>	0.11	2.3	1.2	2.8	>2.8 (0) <sup>i</sup>	0.028	100
<b>10</b>	1.0	23	2.3	5.6	>2.8 (0) <sup>i</sup>	0.064	88
<b>11</b>	0.12	2.4	1.2	2.9	58	0.044	66
<b>12</b>	0.28	1.1	1.4	2.5	35	0.034	74
<b>13</b>	1.1	10	4.0	2.6	32	0.048	54
pemetrexed <sup>j</sup>	9.5	76	2.8	6.6	230	0.43	15.4
PDDF <sup>k</sup>	0.085	0.019	0.43	1.9	23	0.22	8.6
MTX	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>	0.02	0.0088	0.033	0.6
trimethoprim	nd <sup>i</sup>	nd <sup>i</sup>	nd <sup>i</sup>	>340 (22) <sup>i</sup>	0.01	6.8	>50

<sup>a</sup> 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  $\pm 10\%$  of the value given. <sup>b</sup> Kindly provided by Dr. Frank Maley, New York State Department of Health. <sup>c</sup> Kindly provided by Dr. Karen Anderson, Yale University, New Haven, CT. <sup>d</sup> Kindly provided by Dr. J. H. Freisheim, Medical College of Ohio, Toledo, OH. <sup>e</sup> Kindly provided by Dr. R. L. Blakley, St. Jude Children's hospital, Memphis, TN. <sup>f</sup> rh/tg is selectivity ratio for *T. gondii* DHFR and is (IC<sub>50</sub> against rhDHFR)/(IC<sub>50</sub> against *T. gondii* DHFR). <sup>g</sup> Data derived from ref 20. <sup>h</sup> Data derived from ref 38. <sup>i</sup> Numbers in parentheses indicate the % inhibition at the stated concentration. <sup>j</sup> Kindly provided by Dr. Chuan Shih, Eli Lilly and Co. <sup>k</sup> Kindly provided by Dr. M. G. Nair, University of South Alabama. <sup>l</sup> nd = not determined.

d]pyrimidines are more aromatic like MTX and 5-deaza folic acid than pyrrolo[2,3-*d*]pyrimidines. Lastly, like the pteridine ring of MTX and 5-deaza folic acid, the thieno[2,3-*d*]pyrimidine has a hydrogen bond acceptor in the five-member heterocyclic ring rather than a hydrogen bond donor as in the pyrrolo[2,3-*d*]pyrimidine ring. Thus, the increased similarity of the thieno[2,3-*d*]pyrimidine ring system to the pteridine ring may explain the higher potency of **4** compared to **1** against human DHFR.

The nonclassical analogues **5–13** were also evaluated as inhibitors of TS and DHFR (Table 1). All of the nonclassical analogues were reasonably potent inhibitors of human TS with IC<sub>50</sub> values ranging from 0.11 to 4.6  $\mu$ M. The electronic nature of the substituent on the side chain phenyl was an important factor in determining inhibitory potency. Analogues with electron withdrawing substitutions on the phenyl ring were more potent than analogues with electron donating substitutions or the unsubstituted phenyl. Electron withdrawing 4-chloro, 4-nitro, 3,4-dichloro, and 4-fluoro substituents in analogues **6**, **7**, **9**, and **13**, respectively, showed the most potent inhibition against isolated human TS. Compounds **7** and **9** were comparable in potency to the pyrrolo[2,3-*d*]pyrimidine analogues **2** and **3**, respectively. These data are consistent with SAR studies previously reported for the pyrrolo[2,3-*d*]pyrimidine series.<sup>38</sup> Like the classical analogue **4**, all of the nonclassical analogues were also more potent than pemetrexed as inhibitors of human TS. This result indicates that isosteric structural modification of the pyrrolo[2,3-*d*]pyrimidine to a thieno[2,3-*d*]pyrimidine maintains potent human TS inhibitory activity. Compounds **5–13** are also moderately potent inhibitors of human DHFR with IC<sub>50</sub> values ranging from 0.56 to 5.6  $\mu$ M. Analogue **7** (IC<sub>50</sub> = 0.56  $\mu$ M) was the most potent compound in this series against human DHFR. Compound **7** was 28-fold less potent against human DHFR than MTX but was more than 12-fold more potent than pemetrexed. In addition, all the nonclassical analogues were more potent human DHFR inhibitors than pemetrexed. These results demonstrate that the nonclassical analogues follow the same trend of dual inhibition against human TS and DHFR as the classical analogue **4** albeit with lower potency. Interestingly, all the nonclassical compounds were potent inhibitors of *T. gondii* DHFR with IC<sub>50</sub> values ranging from 0.028 to 0.12  $\mu$ M.

The IC<sub>50</sub> values of compounds **6–13** against *T. gondii* DHFR were similar in potency to MTX and were about 243-fold more potent than the clinically used trimethoprim (Table 1). In addition, all the nonclassical compounds showed good to excellent selectivity against *T. gondii* DHFR compared to human DHFR. Compound **8** with a 2,5-dimethoxy substitution on the phenyl ring was marginally active against human DHFR (IC<sub>50</sub> = 22  $\mu$ M) but very potent against *T. gondii* DHFR (IC<sub>50</sub> = 56 nM) exhibiting 393-fold selectivity, which indicated a distinct species difference in DHFR from different sources. This 2,5-dimethoxyphenyl substitution occurs in several other potent DHFR inhibitors that usually lack selectivity such as piritrexim (PTX). In this series of compounds, potency and selectivity were also found with the unsubstituted phenyl analogue and analogues with electron withdrawing substitutions. These result parallel the structure–activity relationship (SAR) we recently reported for the nonclassical N5-substituted 2-amino-4-oxo-6-methylpyrrolo[3,2-*d*]pyrimidines against DHFR.<sup>48</sup> The excellent selectivity of compound **8** against *T. gondii* DHFR attests to the fact that differences in mammalian and pathogen DHFR can be exploited with nonclassical DHFR inhibitors. We are in the process of developing other nonclassical TS inhibitors with potential selectivity toward nonmammalian DHFR and TS and other analogues as antitumor agents.

In summary, the 5-substituted 2-amino-4-oxo-6-methylthieno[2,3-*d*]pyrimidine classical antifolate **4** and nine nonclassical analogues **5–13** were designed and synthesized as potential dual TS–DHFR inhibitors. Compound **4** maintained the potent human TS inhibitory activity of **1** and significantly increased the human DHFR inhibition of **1** by 105-fold. Compound **4**, to our knowledge, is the most potent dual TS–DHFR inhibitor known to date. Compound **7** was the most potent compound in the nonclassical series, also demonstrating potent dual inhibitory activities against human TS (IC<sub>50</sub> = 0.11  $\mu$ M) and human DHFR (IC<sub>50</sub> = 0.56  $\mu$ M). In addition, excellent potency and high selectivity for *T. gondii* DHFR compared to human DHFR were observed for all the analogues (except **4** and **7**). This study indicated that the 5-substituted 2-amino-4-oxo-6-methylthieno-

[2,3-*d*]pyrimidine scaffold is most conducive to dual human TS–DHFR inhibitory activity.

## Experimental Section

Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P<sub>2</sub>O<sub>5</sub> at 80 °C. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (<sup>1</sup>H NMR) were recorded on a Bruker WH-300 (300 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Mass spectra were recorded on a VG-7070 double-focusing mass spectrometer or in a LKB-9000 instrument in the electron ionization (EI) mode. Chemical names follow IUPAC nomenclature. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230–400 mesh silica gel (Fisher, Somerville, NJ) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within 0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates could not be prevented in spite of 24–48 h of drying in vacuo and were confirmed where possible by their presence in the <sup>1</sup>H NMR spectra. Microwave-assisted synthesis was performed utilizing an Emrys Liberator microwave synthesizer (Biotage) utilizing capped reaction vials. All microwave reactions were performed with temperature control. All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received.

**Ethyl 2-Amino-5-methylthiophene-3-carboxylate (15).** In a 250 mL round-bottom flask, under nitrogen, were placed triethylamine (4.7 g, 51 mmol), ethylcyanoacetate (9.7 g, 90 mmol), sulfur (2.8 g, 90 mmol), and dry DMF (30 mL). The reaction mixture was stirred for 30 min at room temperature, and then propionaldehyde (5.0 g, 90 mmol) was added dropwise to this suspension while maintaining the temperature at 50 °C. When the addition was complete, the reaction mixture was allowed to cool to room temperature and then stirred for another 2 h. The reaction mixture was diluted with EtOAc (120 mL) and washed sequentially with H<sub>2</sub>O (30 mL) and brine (30 mL). The organic layer was separated and dried over MgSO<sub>4</sub>, filtered, and concentrated under reduced pressure to afford yellow oil. The crude product was purified by flash chromatography on silica gel (gradient, 5% EtOAc/hexane to 10% EtOAc/hexane) to afford 8.9 g (56%) of **15** as a yellow solid: *R*<sub>f</sub> = 0.6 (hexane/EtOAc, 3:1); mp 45–47 °C, (lit.<sup>47</sup> mp 46 °C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 1.22 (t, 3 H, *J* = 7.2 Hz), 2.17 (s, 3 H), 4.14 (q, 2 H, *J* = 7.2 Hz), 6.47 (s, 1 H), 7.06 (s, 2 H).

**2-Amino-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (16).** In a 100 mL round-bottom flask, under nitrogen, were added **15** (0.67 g, 3 mmol), chlorformamidinium hydrochloride (0.35 g, 4.5 mmol), and DMSO (10 g, 102 mmol). The resulting mixture was heated to 120–125 °C for 30 min. The reaction mixture was quenched with water (15 mL). The resulting solution was cooled in an ice bath, and the pH was adjusted to 8–9 with dropwise addition of NH<sub>4</sub>OH. This suspension was left at 5 °C for 1 h. The precipitate obtained was collected by filtration, washed with brine, dried in vacuo, and purified by flash chromatography on silica gel (gradient, 2% MeOH/CHCl<sub>3</sub> to 5% MeOH/CHCl<sub>3</sub>) to afford 0.56 g (87%) of **16** as a light-yellow solid: *R*<sub>f</sub> = 0.54 (MeOH/CHCl<sub>3</sub>, 1:7); mp 370–372 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.35 (s, 3 H), 6.43 (s, 2 H), 6.77 (s, 1 H), 10.82 (s, 1 H). Anal. (C<sub>7</sub>H<sub>7</sub>N<sub>3</sub>SO) C, H, N, S.

**2-Amino-5-bromo-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (17).** To a microwave reaction vial was added **16** (0.5 g, 3 mmol), bromine (1 g, 333 μL), and AcOH (10 mL). The reaction mixture was irradiated in a microwave apparatus at 150 °C for 30 min. After the reaction mixture was cooled to ambient temperature, the mixture was filtered and the filtrate was concentrated under reduced

pressure. The crude product was purified by flash chromatography on silica gel (1% MeOH/CHCl<sub>3</sub>) to afford 0.57 g (80%) of **17** as a yellow solid: *R*<sub>f</sub> = 0.60 (MeOH/CHCl<sub>3</sub>, 1:7); mp 254–256 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.33 (s, 3 H), 6.60 (s, 2 H), 10.97 (s, 1 H). Anal. (C<sub>7</sub>H<sub>6</sub>BrN<sub>3</sub>SO) C, H, N, Br, S.

**2-Amino-4-oxo-6-methyl-5-phenylsulfanylthieno[2,3-*d*]pyrimidine (5).** To a microwave reaction vial was added **17** (0.15 g, 0.6 mmol), K<sub>2</sub>CO<sub>3</sub> (0.25 g, 1.8 mmol), and dry DMF (15 mL). The mixture was evacuated and back-filled with nitrogen (three cycles). Cu<sub>2</sub>O (89 mg, 0.6 mmol) and benzenethiol (0.27 g, 2.4 mmol) were added, and the reaction mixture was degassed twice. The reaction mixture was irradiated in a microwave apparatus at 180 °C for 30 min. After the reaction mixture was cooled to ambient temperature, the mixture was filtered; the filtrate was concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel using 2% MeOH in CHCl<sub>3</sub> as the eluent. Fractions containing the product (TLC) were combined and evaporated to afford 0.12 g (68%) of **5** as a white solid: *R*<sub>f</sub> = 0.60 (MeOH/CHCl<sub>3</sub>, 1:7); mp 291–294 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.38 (s, 3 H), 6.56 (s, 2 H), 7.00–7.22 (m, 5 H), 10.77 (s, 1 H); HRMS (EI) calcd for C<sub>13</sub>H<sub>11</sub>N<sub>3</sub>OS<sub>2</sub> *m/z* = 289.0343, found *m/z* = 289.0351.

**2-Amino-5-[(4-chlorophenyl)sulfanyl]-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (6).** Compound **6** was synthesized as described for **5** with 4-chlorobenzenethiol and was obtained as a white solid (yield 49%); *R*<sub>f</sub> = 0.70 (MeOH/CHCl<sub>3</sub>, 1:7); mp >330 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.40 (s, 3 H), 6.58 (s, 2 H), 7.02 (d, 2 H, *J* = 7.2 Hz), 7.27 (d, 2 H, *J* = 7.2 Hz), 10.77 (s, 1 H); HRMS (EI) calcd for C<sub>13</sub>H<sub>10</sub>N<sub>3</sub>OS<sub>2</sub>Cl *m/z* = 322.9953, found *m/z* = 322.9944.

**2-Amino-6-methyl-5-[(4-nitrophenyl)sulfanyl]thieno[2,3-*d*]pyrimidin-4(3*H*)-one (7).** Compound **7** was synthesized as described for **5** with 4-nitrobenzenethiol and was obtained as a yellow solid (yield 20%); *R*<sub>f</sub> = 0.70 (MeOH/CHCl<sub>3</sub>, 1:7); mp >300 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.41 (s, 3 H), 6.63 (s, 2 H), 7.16–7.19 (d, 2 H, *J* = 7.8 Hz), 8.06–8.09 (d, 2 H, *J* = 7.8 Hz), 10.83 (s, 1 H). Anal. (C<sub>13</sub>H<sub>10</sub>N<sub>4</sub>O<sub>3</sub>S<sub>2</sub>·H<sub>2</sub>O) C, H, N, S.

**2-Amino-5-[(2,5-dimethoxyphenyl)sulfanyl]-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (8).** Compound **8** was synthesized as described for **5** with 2,5-dimethoxybenzenethiol and was obtained as a white solid (yield 26%); *R*<sub>f</sub> = 0.70 (MeOH/CHCl<sub>3</sub>, 1:7); mp >300 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.34 (s, 3 H), 3.52 (s, 3 H), 3.79 (s, 3 H), 5.93 (s, 1 H), 6.55 (s, 2 H), 6.58 (d, 1 H, *J* = 8.7 Hz), 6.86 (d, 1 H, *J* = 8.7 Hz), 10.77 (s, 1 H). Anal. (C<sub>13</sub>H<sub>10</sub>N<sub>4</sub>O<sub>3</sub>S<sub>2</sub>·0.7H<sub>2</sub>O) C, H, N, S.

**2-Amino-5-[(3,4-dichlorophenyl)sulfanyl]-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (9).** Compound **9** was synthesized as described for **5** with 3,4-dichlorobenzenethiol and was obtained as a white solid (yield 48%); *R*<sub>f</sub> = 0.64 (MeOH/CHCl<sub>3</sub>, 1:7); mp 297–300 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.41 (s, 3 H), 6.59 (s, 2 H), 6.95 (dd, 1 H, *J* = 1.5 Hz, *J* = 6.3 Hz), 7.23 (d, 1 H, *J* = 1.5 Hz), 7.45 (d, 1 H, *J* = 6.3 Hz), 10.79 (s, 1 H); HRMS (EI) calcd for C<sub>13</sub>H<sub>9</sub>N<sub>3</sub>OS<sub>2</sub>Cl<sub>2</sub> *m/z* = 356.9564, found *m/z* = 356.9567.

**2-Amino-5-[(3,5-dichlorophenyl)sulfanyl]-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (10).** Compound **10** was synthesized as described for **5** with 3,5-dichlorobenzenethiol and was obtained as a white solid (yield 54%); *R*<sub>f</sub> = 0.70 (MeOH/CHCl<sub>3</sub>, 1:7); mp >300 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.42 (s, 3 H), 6.64 (s, 2 H), 6.98 (s, 2 H), 7.33 (s, 1 H), 10.84 (s, 1 H). Anal. (C<sub>13</sub>H<sub>9</sub>N<sub>3</sub>OS<sub>2</sub>Cl<sub>2</sub>) C, H, N, S, Cl.

**2-Amino-6-methyl-5-(2-naphthylsulfanyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one (11).** Compound **11** was synthesized as described for **5** with naphthalene-2-thiol and was obtained as a white solid (yield 31%); *R*<sub>f</sub> = 0.70 (MeOH/CHCl<sub>3</sub>, 1:7); mp 300–303 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.43 (s, 3 H), 6.57 (s, 2 H), 7.16 (d, 1 H, *J* = 7.2 Hz), 7.38–7.48 (m, 3 H), 7.72–7.83 (m, 3 H), 10.75 (s, 1 H); HRMS (EI) calcd for C<sub>17</sub>H<sub>13</sub>N<sub>3</sub>OS<sub>2</sub> *m/z* = 339.0466, found *m/z* = 339.0504.

**2-Amino-6-methyl-5-(pyridin-4-ylsulfanyl)thieno[2,3-*d*]pyrimidin-4(3*H*)-one (12).** Compound **12** was synthesized as described for **5** with pyridine-4-thiol and was obtained as a yellow solid (yield 56%); *R*<sub>f</sub> = 0.69 (MeOH/CHCl<sub>3</sub>, 1:7); mp >300 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ 2.32 (s, 3 H), 6.62 (s, 2 H), 6.92 (d, 2 H, *J* = 6.9

Hz), 8.29 (d, 2 H,  $J = 6.9$  Hz), 10.83 (s, 1 H); HRMS (EI) calcd for  $C_{12}H_{10}N_4OS_2$   $m/z = 290.0296$ , found  $m/z = 290.0302$ .

**2-Amino-5-[(4-fluorophenyl)sulfanyl]-6-methylthieno[2,3-*d*]pyrimidin-4(3*H*)-one (13).** Compound **13** was synthesized as described for **5** with 4-fluorobenzenethiol and was obtained as a white solid (yield 59%):  $R_f = 0.70$  (MeOH/ $CHCl_3$ , 1:7); mp  $>300$  °C;  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  2.41 (s, 3 H), 6.56 (s, 2 H), 7.07–7.09 (m, 4 H), 10.76 (s, 1 H). Anal. ( $C_{13}H_{10}N_3OS_2F \cdot 0.3H_2O$ ) C, H, N, S, F.

**4-(2-Amino-6-methyl-4-oxo-3,4-dihydro-thieno[2,3-*d*]pyrimidine-5-ylsulfanyl)benzoic Acid Ethyl Ester (18).** Compound **18** was synthesized as described for **5** with ethyl 4-sulfanylbenzoate and was obtained as a yellow solid (yield 34%):  $R_f = 0.60$  (MeOH/ $CHCl_3$ , 1:7); mp 282–284 °C;  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  1.26 (t, 3 H,  $J = 6.8$  Hz), 2.39 (s, 3 H), 4.23 (q, 2 H,  $J = 6.8$  Hz), 6.59 (s, 2 H), 7.05 (d, 2 H,  $J = 8.1$  Hz), 7.77 (d, 2 H,  $J = 8.1$  Hz), 10.79 (s, 1 H); HRMS (EI) calcd for  $C_{16}H_{15}N_3O_3S_2$   $m/z = 361.0554$ , found  $m/z = 361.0558$ .

**4-[(2-Amino-6-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoic Acid (19).** To a solution of **18** (0.25 g, 0.7 mmol) in ethanol (10 mL) was added aqueous 1 N NaOH (10 mL), and the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was evaporated to dryness under reduced pressure. The residue obtained was dissolved in water (5 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3–4 with dropwise addition of 3 N HCl. This suspension was left at 5 °C for 24 h. The precipitated solid was collected by filtration, washed with brine, and dried in vacuo to afford 0.23 g (99%) of **19** as a light, white solid: mp  $>300$  °C;  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  2.51 (s, 3 H), 6.61 (s, 2 H), 7.47–7.79 (m, 4 H), 10.82 (s, 1 H); HRMS (EI) calcd for  $C_{14}H_{11}N_3O_3S_2$   $m/z = 333.0241$ , found  $m/z = 333.0227$ .

**Diethyl-*N*-[4-[(2-amino-6-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-5-yl)thio]benzoyl]-L-glutamate (20).** To a solution of **19** (142 mg, 0.43 mmol) in DMF (8 mL) were added 6-chloro-2,4-dimethoxy-1,3,5-triazine (80 mg, 0.45 mmol) and *N*-methylmorpholine (52 mg, 0.52 mmol) at 0 °C. After the mixture was stirred at 0 °C for 2 h, *N*-methylmorpholine (52 mg, 0.52 mmol) and dimethyl-L-glutamate hydrochloride (95 mg, 0.45 mmol) were added together. The mixture was stirred at 0 °C for 2 h and at room temperature for 12 h. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel with 5% MeOH/ $CHCl_3$  as the eluent to afford 140 mg (65%) of **20** as a light-yellow solid:  $R_f = 0.50$  (MeOH/ $CHCl_3$ , 1:7); mp 211–212 °C;  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  1.01–1.20 (m, 6 H), 1.98–2.12 (m, 2 H), 2.27–2.32 (m, 2 H), 2.42 (s, 3 H), 4.00–4.13 (m, 4 H), 4.35–4.40 (m, 1 H), 6.60 (s, 2 H), 7.03 (d, 2 H,  $J = 7.8$  Hz), 7.70 (d, 2 H,  $J = 8.1$  Hz), 8.61 (d, 1 H,  $J = 7.5$  Hz), 10.79 (s, 1 H). HRMS (EI) calcd for  $C_{23}H_{26}N_4O_6S_2$   $m/z = 518.1293$ , found  $m/z = 518.1316$ .

***N*-[4-[(2-Amino-6-methyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoyl]-L-glutamic Acid (4).** To a solution of **20** (0.1 g, 0.19 mmol) in ethanol (15 mL) was added 1 N NaOH (12 mL), and the solution was stirred at room temperature for 24 h. The solution was evaporated under reduced pressure, and the residue was dissolved in water (10 mL) and stirred for a further 24 h. The solution was then cooled in an ice bath and acidified carefully to pH 4.0 with dropwise addition of 3 N HCl. This suspension was left at 0–5 °C for 24 h and filtered. The residue was washed well with water and dried over  $P_2O_5$ /vacuum to afford 71 mg (93%) of **4** as a yellow solid: mp 188–190 °C;  $^1H$  NMR (DMSO- $d_6$ )  $\delta$  1.88–2.10 (m, 2 H), 2.27–2.33 (t, 2 H,  $J = 7.2$  Hz), 2.39 (s, 3 H), 4.36 (m, 1 H), 6.59 (s, 2 H), 7.02 (d, 2 H,  $J = 8.4$  Hz), 7.70 (d, 2 H,  $J = 8.4$  Hz), 8.49 (d, 1 H,  $J = 7.2$  Hz), 10.79 (s, 1 H), 12.38 (s, 2 H). Anal. ( $C_{19}H_{18}N_4O_6S_2 \cdot 1.5H_2O$ ) C, H, N, S.

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**Supporting Information Available:** Results from elemental analysis and high resolution mass spectrometry. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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