2,4-Diaminothieno[2,3-*d*]pyrimidine Analogues of Trimetrexate and Piritrexim as Potential Inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* Dihydrofolate Reductase

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A series of eight previously undescribed 2,4-diaminothieno[2,3-*d*]pyrimidine analogues of the potent dihydrofolate reductase (DHFR) inhibitors trimetrexate (TMQ) and piritrexim (PTX) were synthesized as potential drugs against *Pneumocystis carinii* and *Toxoplasma gondii*, which are major causes of severe opportunistic infections in AIDS patients. 2,4-Diamino-5-methyl-6-(aryl/aralkyl)thieno[2,3-*d*]pyrimidines with 3,4,5-trimethoxy or 2,5-dimethoxy substitution in the aryl/aralkyl moiety and 2,4-diamino-5-(aryl/aralkyl)thieno[2,3-*d*]pyrimidines with 2,5-dimethoxy substitution in the aryl/aralkyl moiety were obtained by reaction of the corresponding 2-amino-3-cyanothiophenes with chloroformamidine hydrochloride. The aryl group in the 5,6-disubstituted analogues was either attached directly to the hetero ring or was separated from it by one or two carbons, whereas the aryl group in the 5-monosubstituted analogues was separated from the hetero ring by two or three carbons. 2-Amino-3-cyano-5-methyl-6-(aryl/alkyl)thiophene intermediates for the preparation of the 5,6-disubstituted analogues were prepared from ω-aryl-2-alkylidenemalononitriles and sulfur in the presence of a secondary amine, and 2-amino-3-cyano-4-(aryl/aralkyl)thiophene intermediates for the preparation of the 5-monosubstituted analogues were obtained from ω-aryl-1-chloro-2-alkylidenemalononitriles and sodium hydrosulfide. Synthetic routes to the heretofore unknown ylidenemalononitriles, and the ketone precursors thereof, were developed. The final products were tested *in vitro* as inhibitors of DHFR from *Pneumocystis carinii*, *Toxoplasma gondii*, rat liver, beef liver, and *Lactobacillus casei*. A selected number of previously known 2,4-diaminothieno[2,3-*d*]pyrimidines lacking the 3,4,5-trimethoxyphenyl and 2,5-dimethoxyphenyl substitution pattern of TMQ and PTX, respectively, were also tested for comparison. None of the compounds was as potent as TMQ or PTX, and while some of them showed some selectivity in their binding to *Pneumocystis cariniii* and *Toxoplasma gondii* versus rat liver DHFR, this effect was not deemed large enough to warrant further preclinical evaluation.

Opportunistic infections associated with loss of cell-mediated immunity due to progressive destruction of $CD4^+$ lymphocytes is well known as a major cause of morbidity and mortality in patients with acquired immune deficiency syndrome (AIDS).[1] Common examples of such infections which are not normally seen in immunocompetent individuals are *Pneumocystis carinii* pneumonia[2,3] and toxoplasmosis.[4,5] The latter typically occurs via reactivation of a previously acquired benign *Toxoplasma gondii* infection, which may occur in up to 50% of the U.S. population at some time and can exist in latent form throughout life. Toxoplasmosis is especially troublesome in AIDS patients because it can produce blindness, as well as dementia and painful and disabling encephalitis during the late stages of their disease.[6] Standard treatment or prophylaxis of *P. carinii* pneumonia utilizes various combinations of pentamidine, sulfonamides, and diaminopyrimidine antifolates.[7] Combinations of primaquine and clindamycin have likewise been found to be clinically useful.[8,9] Standard therapy for *T. gondii* infections relies mainly on the diaminopyrimidine antifolates and sulfonamides,[4,5] though the combination of a diaminopyrimidine antifolate and clindamycin has recently also met with some success.[10] However, all the standard agents currently used to treat *P. carinii* and *T. gondii* infections in AIDS patients and other immunocompromised individuals may cause side effects that are sometimes severe enough to require cessation of therapy. In addition, the emergence of drug resistance often becomes dose-limiting. Thus, there has been a vigorous effort to develop more effective drugs for the treatment of these AIDS-associated disorders. For example, a new hydroxynaphthoquinone has recently shown remarkable promise and may gain an important role in the treatment of patients with *P. carinii* pneumonia or *T. gondii* infections who cannot tolerate, or do not respond to, other drugs.[11-13]

An innovative recent approach to the treatment of *P. carinii* and *T. gondii* infections with antifolates takes advantage of the fact that these organisms are permeable to lipophilic nonclassical antifolates but, unlike mammalian cells, lack a carrier-mediated active transport mechanism for the uptake of folates with a polar glutamate side chain.[14] Thus, one can in principle selectively protect sensitive host issues from the toxic effects of lipophilic antifolates by coadministration of a reduced folate, typi-

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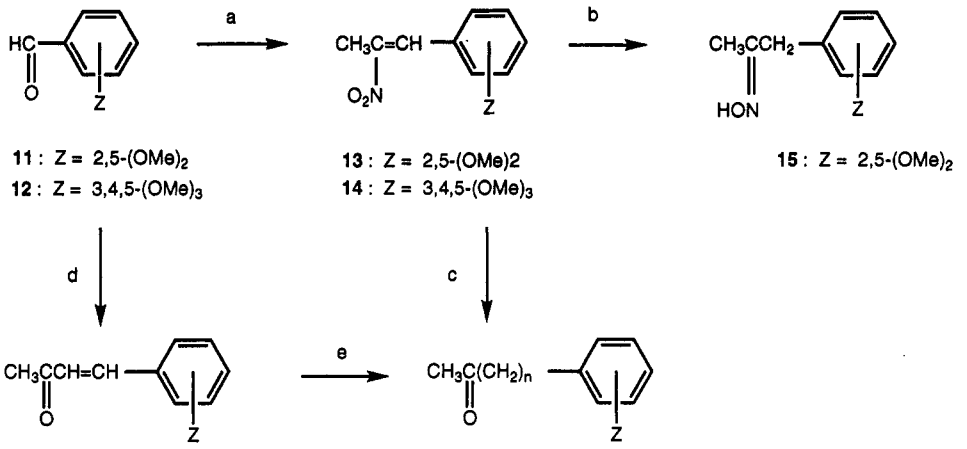
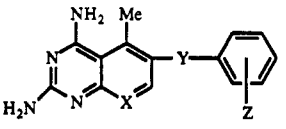
Scheme I[a]



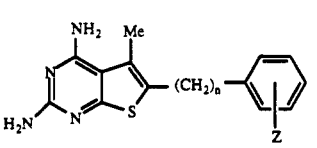
[a] Key: (a) $CH_3CH_2NO_2/NH_4OAc$; (b) H_2/Pd–C/AcOH/HCl; (c) H_2/RaNi, (d) $Ph_3P{=}CHCOCH_3$; (e) H_2/Pd–C/AcOH.

cally leucovorin [(6*R*,6*S*)-5-formyl-5,6,7,8-tetrahydropteroyl-L-gluatamate]. Two lipophilic antifolates which have been utilized in this way in the laboratory[14–19] and the clinic[20] are the potent dihydrofolate reductase (DHFR) inhibitors trimetrexate (TMQ, 1)[21] and piritrexim (PTX, 2).[22] While therapy of *P. carinii* and *T. gondii* opportunistic infections with TMQ or PTX and leucovorin is a conceptually attractive strategy, it unfortunately suffers from some disadvantages, including the fact that leucovorin is expensive and, more importantly in terms of therapeutic outcome, that these organisms could potentially become resistant by producing a mutant enzyme with a low affinity for diaminopyrimidine antifolates, as has been observed, for example, in pyrimethamine-resistant plasmodia.[22] Another possibility, theoretically, could be the appearance of a multidrug resistance phenotype of the kind already known to arise in cancer cells after selection with TMQ or PTX.[23,24]

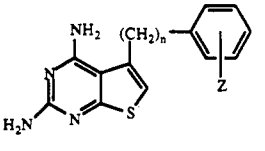


1 (TMQ): X = CH; Y = CH_2NH; Z = 3',4',5'-$(OMe)_3$
2 (PTX): X = N; Y = CH_2; Z = 2',5'-$(OMe)_2$

To circumvent the need for leucovorin as an antidote against myelosuppression, mucositis, and other life-threatening side effects during therapy with lipophilic DHFR inhibitors, it would be desirable to develop new analogues that (i) have a higher degree of selectivity for *P. carinii* or *T. gondii* DHFR than is shown by TMQ or PTX, (ii) are active against organisms with acquired resistance to TMQ and PTX, and (iii) are themselves slow to engender resistance. As part of a larger study of lipophilic antifolates, an area in which this laboratory has been involved for a number of years,[25] we have synthesized and tested the heretofore unknown 2,4-diaminothieno-[2,3-*d*]pyrimidines **3–10**, which may be viewed as analogues of **1** and **2**. The rationale behind our choice of these structures was that the sulfur atom is approximately isosteric with C^7–C^8 in **1** and C^7–N^8 in **2**. At the same time it was reasoned that if enough difference was introduced in other parts of the molecule, for example, in the bridge region, some selectivity for *P. carinii* or *T. gondii* DHFR might be produced. Selectivity could arise, for example, if protonation of the weakly basic diaminopyrimidine ring of this heterocyclic system[26] were to occur in the microenvironment of the active site of *P. carinii* or *T. gondii*, but not mammalian or bacterial, DHFR. It should be noted that 2,4-diaminothieno[2,3-*d*]pyrimidines have been examined previously for antibacterial[27] and antimalarial activity[28–30] but none of the reported compounds contained a di- or trimethoxyphenyl substituent. In addition to the synthesis of **3–10** and the results of assays of their inhibitory activity against *P. carinii*, *T. gondii*, and rat liver DHFR, we report enzyme and cell growth inhibition data for a selected group of compounds lacking the di- or trimethoxyphenyl substitution pattern of TMQ and PTX.



3: n = 0; Z = 2',5'-$(OMe)_2$
4: n = 1; Z = 2',5'-$(OMe)_2$
5: n = 2; Z = 2',5'-$(OMe)_2$
6: n = 0; Z = 3',4',5'-$(OMe)_3$
7: n = 1; Z = 3',4',5'-$(OMe)_3$
8: n = 2; Z = 3',4',5'-$(OMe)_3$

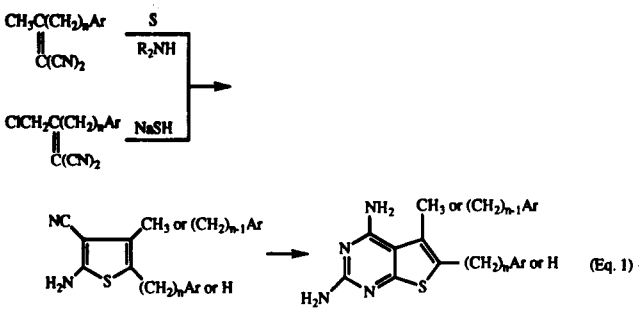


9 : n = 2; Z = 2',5'-$(OMe)_2$
10 : n = 3; Z = 2',5'-$(OMe)_2$

Chemistry

Convenient access to the diaminothienopyrimidine analogues **3–10** was gained by the previously described method[28–30] involving thermal fusion of chloroformamidine hydrochloride with appropriately substituted 2-amino-3-cyanothiophenes. The latter were prepared from ylidenemalononitriles by the general route shown below (eq 1).

The method used to obtain ylidenemalononitriles was dictated by the length of the bridge in the final product and the location of the (di- or trimethoxyphenyl)alkyl substituent. As shown in Scheme I, condensation of 2,5-dimethoxybenzaldehyde (**11**) and 3,4,5-trimethoxybenzaldehyde (**12**), with nitroethane afforded the nitro olefins



(Eq. 1)

13 (90%) and 14 (87%). Initial attempts to convert 13 directly to a ketone by hydrogenation under acidic conditions in the presence of palladium-on-charcoal unexpectedly led to oxime 15 (39%) as the only identified product, but when reduction was performed with Raney nickel the product was the desired ketone 16 (52%), presumably formed via the corresponding acid-labile enamine. Similar reduction of nitro olefin 14 yielded ketone 17 (65%). For the preparation of the next higher bridge homologues, 11 and 12 were condensed with 1-(triphenylphosphoranylidene)-2-propanone to form the unsaturated ketones 18 (95%) and 19 (94%), which on catalytic reduction afforded the saturated ketones 20 (91%) and 21 (94%).

Synthetic routes to the ketone intermediates needed for the preparation of TMQ and PTX analogues with a two-carbon bridge are given in Scheme II. Condensation of 11 with triphenyl(methoxymethylene)phosphorane afforded the enol ether 22 (64%) as a mixture of geometrical isomers. Although they were not separated, the isomers were easily visible in the ^{1}H NMR spectrum, which showed well-resolved doublets at δ 5.6 (J = 8 Hz) and δ 6.1 (J = 8 Hz) for the E-isomer and at δ 5.9 (J = 8 Hz) and δ 7.1 (J = 13 Hz) for the Z-isomer. Acidolysis of the mixture with perchloric acid in ether yielded the unstable aldehyde 23, which was condensed directly with 1-(triphenylphosphoranylidene)-2-propanone to form ketone 24 (57%). Catalytic reduction then gave 25 (47%). For the synthesis of the corresponding trimethoxy analogue, the sodium salt of ethyl acetoacetate was alkylated with 3,4,5-trimethoxyphenethyl bromide and the resulting keto ester 27 (52%) hydrolyzed and decarboxylated in base to obtain 28 (80%).

Condensation of 16, 17, 20, 21, 25, and 28 with malononitrile in refluxing benzene containing acetic acid and ammonium acetate afforded the ylidenemalononitriles 29–34 in yields ranging from 67% to 94% (Scheme III). Further reaction with elemental sulfur in the presence of diisopropylamine[31] produced the thiophene amino nitriles 35–40 in 30–60% yields. Fusion with chloroformamidine hydrochloride at 120 °C then gave the thienopyrimidines 3–8. The yields in these fusion reactions can be quite variable and need to be individually optimized. Generally, the components were simply heated in an oil bath until a melt was obtained and HCl gas evolution was evident. The reaction was exothermic, and the depth of the flask in the oil bath had to be adjusted continuously so as to maintain a steady *internal temperature* of 120 °C for 30 min. In the reactions of 37 and 40, it was noted that the filtrate remaining after isolation of the main product contained a second compound which was not the starting material. We assumed this to be a partially cyclized adduct[30] and therefore heated it under reduced pressure for an another 1.5 h in the presence of diisopropylamine to complete the ring closure. However, the second heating step did not seem to improve the results in all cases; the yield of product one can reasonably expect from this reaction appears to be in the 60–75% range, but the fusion conditions have to be optimized individually.

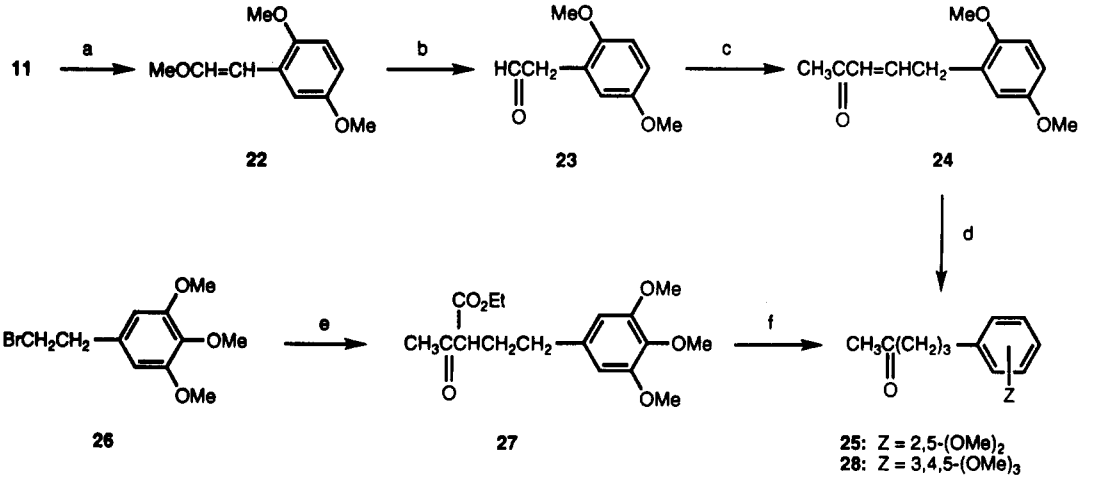
Treatment of the enolates of ketones 20 and 25 with trimethylchlorosilane and triethylamine in DMF[32] afforded the trimethylsilyl enol ethers 41 and 42, which on direct halogenation with N-chlorosuccinimide[33] were converted to chloro ketones 43 and 44 in ca. 35% overall yield (Scheme IV). Treatment of 43 and 44 with malononitrile as in the synthesis of 29–34 afforded ylidenemalononitriles 45 (71%) and 46 (69%), respectively. Further reaction with sodium hydrosulfide in refluxing ethanol[31] then converted 45 and 46 into the thiophene amino nitriles 47 (84%) and 48 (52%), which on fusion with chloroformamidine hydrochloride as in the synthesis of 3–8 gave 9 (29%) and 10 (36%).

As expected from their structures, compounds 3–10 were very lipophilic and could be easily purified on silica gel columns by elution with chloroform containing 10% methanol. Compound 9 was lipophilic enough to be recrystallized from a mixture of chloroform and hexanes. Ultraviolet absorption spectra of the 5-methyl-6-(arylalkyl) analogues 4, 5, 7, and 8 in ethanol solution showed maxima at 233 and 279 nm, whereas in the 5-methyl-6-aryl analogues 3 and 6 the long-wavelength peak was shifted to 310 nm, indicating conjugation between the phenyl and thienopyrimidine rings. Spectra of the 5-(arylalkyl) analogues 9 and 10 showed absorption maxima at 227 and 278 nm. Thus, the short-wavelength maximum, which was assigned to the thienopyrimidine chromophore, underwent a small blue shift in the 6-unsubstituted compounds in comparison with the 5,6-disubstituted compounds.

Bioassays

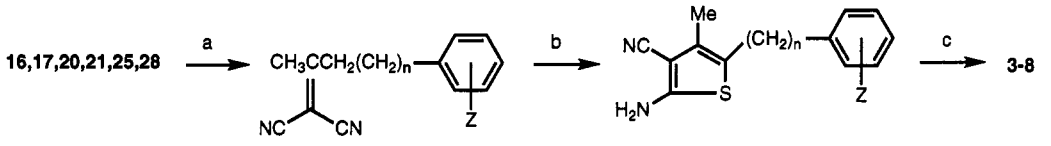
Comparative assays of the ability of 3–10 as inhibitors of *P. carinii*, *T. gondii*, and rat liver DHFR were carried out with a view to determining whether these thienopyrimidine analogues of PTX and TMQ would be more active or more selective toward the *P. carinii* and *T. gondii* enzymes. As shown in Table I, the IC_{50} values of 3–5 against *P. carinii* DHFR were in the 1.2–14 μM range, those of 6–9 showed no significant inhibition at 8 μM (the highest concentration that could tested for reasons of solubility), and those of 9 and 10 were 28 and 55 μM, respectively. The IC_{50} values of 3–5 against rat liver DHFR were in the 0.37–5.9 μM range, those of 6–9 were in the 1.8–51 μM range, and those of 9 and 10 were 3.1 and 26 μM, respectively. Thus, the 3,4,5-trimethoxyphenyl compounds were all less active than the 2,5-dimethoxyphenyl compounds, the compounds in which the aralkyl substituent was moved from the 6-position to the 5-position were even less active, and all of them were substantially less active than PTX and TMQ. The 3,4,5-trimethoxy compounds were also less active than the 2,5-dimethoxy compounds against human DHFR. On the other hand, it was of interest to note that, while the activity of the 2,5-dimethoxy analogues against *P. carinii* DHFR increased 4-fold as the bridge was lengthened from zero to two CH_2 groups, the same change produced a 15-fold decrease in activity against rat liver DHFR. As a result, 5 had a modest degree of selectivity against the *P. carinii* enzyme, whereas 3 and 4 did not. These results suggest that it may be possible to increase species selectivity among this group

Scheme II[a]



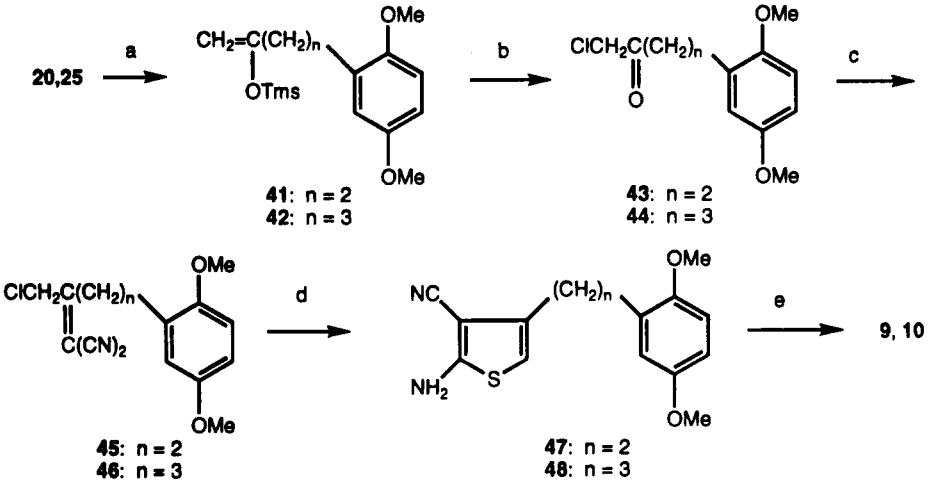
[a] Key: (a) $Ph_3P{=}CHOMe$; (b) $HClO_4/Et_2O$; (c) $Ph_3P{=}CHCOCH_3$; (d) H_2/Pd–C/EtOH; (e) $CH_3COCHCO_2Et$/NaH; (f) KOH, heat.

Scheme III[a]



[a] Key: (a) $CH_2(CN)_2/NH_4OAc$/HOAc; (b) S/i-Pr_2NH; (c) $ClC(=NH{\cdot}HCl)NH_2$.

Scheme IV[a]



[a] Key: (a) LDA/THF, then TMSCl/Et_3N/DMF; (b) NCS/CH_2Cl_2; (c) $CH_2(CN)_2/NH_4OAc$/HOAc; (d) NaSH; (e) $ClC(=NH{\cdot}HCl)NH_2$.

of DHFR inhibitors by increasing bridge length, perhaps because this subtle change allows the alkoxyaryl group to better adjust to small differences in the active site of one enzyme versus another.

A selected group of previously described diaminothienopyrimidines[28-30] were also tested as inhibitors of *P. carinii*, *T. gondii*, and rat liver DHFR with a view to assessing how electron-donating methoxy groups on the phenyl ring compare with hydrogen or electron-withdrawing chlorine atoms in terms of potency and selectivity. As shown in Table II, all the 5,6-disubstituted compounds without methoxy groups had IC_{50} values >10 μM against *P. carinii* DHFR, and moving the aryl substituent from the 6-position to the 5-position was just as detrimental when the phenyl ring was unsubstituted or chlorinated as it was when methoxy groups were present (cf. Table I). The 5-methyl-6-phenyl and 5-methyl-6-(3,4-dichlorophenyl) analogues were less active than the 5-methyl-6-(2,5-dimethoxyphenyl) analogue. However, while the 3,4-dichlorophenyl analogue was a better inhibitor of rat liver DHFR than of *P. carinii* or *T. gondii* DHFR, the phenyl analogue showed a modest amount of selectivity for both the *P. carinii* and *T. gondii* enzyme. Thus, it seemed once again (cf. Table I) that diminished potency was accompanied by enhanced selectivity of binding to nonmammalian DHFR. The 15-fold selectivity of the 5-methyl-6-phenyl analogue for the *T. gondii* enzyme is the best we have seen thus far in this series but falls short of what one would like to see in a therapeutically effective antitoxoplasmosis drug.

A shown in Table II, the activity of several 4,5-bridged analogues against *P. carinii*, *T. gondii*, and rat liver DHFR was also examined. IC_{50} values in the 1–10 μM range were obtained for the 5,6-tetramethylene (X = CH_2), 5,6-

Table I. Dihydrofolate Reductase Inhibition by 2,4-Diaminothieno[2,3-*d*]pyrimidine Analogues of Piritrexim (1) and Trimetrexate (2)

	DHFR species, IC_{50}[a] (μM)			
compd	*P. carinii*	*T. gondii*	rat liver	human
1 (TMQ)	0.042[b]	0.010	0.003[b]	0.0014[c]
2 (PTX)	0.038	0.011	0.0015	ND
3	4.8	0.13	0.37	0.75
4	14	0.07	0.40	0.73
5	1.2	3.3	5.9	>10
6	>8	0.32	1.8	>10
7	>8	0.63	51	>10
8	>8	18	25	>10
9	28	5.8	3.1	ND
10	55	24	26	ND

[a] The concentration of dihydrofolate substrate used in the *P. carinii*, *T. gondii*, and rat liver assays was 92 μM, whereas the concentrations used in the assays with beef liver (ref 35) and human enzyme (ref 36) were 66 and 50 μM, respectively. [b] Data from ref 34. [c] Data from ref 20.

pentamethylene (X = CH_2CH_2), and 5,6-hexamethylene (X = $CH_2CH_2CH_2$) derivatives against all three enzymes. However, activity against the *P. carinii* enzyme dropped off sharply with X = $(CH_2)_7$. The most active member of the tricyclic series was the one with a fused *N*-benzyltetrahydropyridine ring at the 5,6-position, whose IC_{50} was in the 0.5–1.0 μM range against both the *T. gondii* and rat liver enzyme. The higher binding affinity of this compound probably reflects the presence of the basic nitrogen at the 7-position, which would be expected to bring the pK_a of the molecule closer to the pH of the microenvironment of the DHFR active site. However, selectivity was either minimal or directed toward the wrong enzyme, reinforcing our impression that, at least in the 2,4-diaminothieno[2,3-*c*]pyrimidine series, the desired combination of high potency and high selectivity is difficult to achieve.

When the ability of the same two groups of compounds to inhibit DHFR from beef liver and *Lactobacillus casei* was compared (Table II), the beef liver enzyme proved to be somewhat more sensitive than the other enzymes, with several of the analogues giving IC_{50} values in the 0.1–1.0 μM range. However, all the compounds were weak inhibitors of human recombinant DHFR (IC_{50} >8 μM; J. H. Freisheim, personal comunication). The *L. casei* enzyme resembled the other enzymes in showing reduced sensitivity to compounds with an aryl or arylmethyl group at the 6-position. Five of the compounds were also tested as inhibitors of the growth of human lymphoblasts (CCRF-CEM cells) in culture (Table II), but their potency proved quite low and consistent with their weak binding to DHFR.

In summary, our bioassay results indicate that 2,4-diaminothieno[2,3-*d*]pyrimidine analogues of TMQ and PTX in which the phenyl and heterocyclic moieties are joined directly, or are separated by one to three carbons, are less potent than the parent compounds and show only a marginal increase in selective binding to either the *P. carinii* or *T. gondii* enzyme. Moreover, 2,5-dimethoxy or 3,4,5-trimethoxy substitution on the phenyl ring does not increase potency or selectivity relative to compounds in which the phenyl ring is unsubstituted or contains a 4-chloro or 3,4-dichloro substituent. It appears that the low basicity of 2,4-diaminothieno[2,3-*d*]pyrimidines as compared with 2,4-diaminoquinazolines and 2,4-diaminopyrido[2,3-*d*]pyrimidines impedes tight binding to *P. carinii* and *T. gondii* DHFR, just as it does to bacterial and mammalian DHFR. We conclude that, whatever subtle differences may ultimately be found at the molecular level between the DHFR of these organisms and that of their mammalian hosts, the simple 2,4-diaminothieno[2,3-*d*]pyrimidine analogues of PTX and TMQ reported in this paper do not exploit these differences well enough to warrant further study.

Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer; only peaks with wavenumbers greater than 1400 cm^{-1} are reported. Quantitative UV absorbance spectra were measured on a Varian Model 210 instrument. ^1H NMR spectra were recorded on a Varian Model EM360 or in some instances a Varian Model VXR500 instrument, using Me_4Si as the reference. TLC analyses were done on Baker Si250F silica gel plates, with spots being visualized under 254-nm illumination. Column chromatography was on Baker 3405 silica gel (60–200 mesh) or Baker 7024 flash silica gel (40-μm particle size). Solvents for moisture-sensitive reactions were dried over molecular sieves. Melting points are not corrected. Microanalyses were by Robertson Laboratory, Madison, NJ. Except where indicated, elemental analyses for C, N, S, and Cl were within ±0.4% of calculated values.

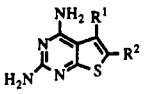
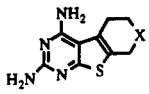
1-(2,5-Dimethoxyphenyl)-2-nitro-1-propene (13). To a solution of 11 (16.6 g, 0.1 mol) in nitroethane (100 mL) was added NH_4OAc (3 g), and the mixture was heated under reflux (105 °C) for 7 h. The solution was stirred for 15 min with anhydrous Na_2SO_4 (15 g), filtered, and evaporated to dryness under reduced pressure at 70 °C. The residue was recrystallized from a 1:8 mixture of CH_2Cl_2 and hexanes (450 mL), and the crystals were washed with hexanes (50 mL). The mother liquor and combined washings were concentrated to a volume of 30 mL and chilled over dry ice (90 min) to obtain a second crop, which was filtered and washed with hexanes (10 mL). The combined crops were dried in vacuo for 48 h, giving yellow crystals (20.7 g, 90%): mp 72–74 °C; R_f 0.68 (silica gel, CH_2Cl_2); IR (KBr) ν 3460, 3080, 3020, 2850, 1650, 1520, 1500, 1460, 1425 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.42 (s, 3H, $=CCH_3$), 3.77 (s, 3H, 2-OMe), 3.81 (s, 3H, 3-OMe), 6.8–6.9 (m, 3H, aromatic), 8.2 (s, 1H, $=CNO_2$).

1-(3,4,5-Trimethoxyphenyl)-2-nitro-1-propene (14). Starting with 12 (19.6 g, 0.1 mol), the procedure used for the synthesis of 13 afforded yellow crystals (21.2 g, 87%): mp 88–91 °C; R_f 0.35 (silica gel, CH_2Cl_2); IR (KBr) ν 3440, 2990, 2950, 2840, 1650, 1590, 1520, 1460, 1430 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.49 (s, 3H, $=CCH_3$), 3.87 (ps, 9H, 3-, 4-, and 5-OMe), 6.65 (s, 2H, aromatic), 8.0 (s, 1H, $=CNO_2$).

3-(2,5-Dimethoxyphenyl)-2-oximinopropane (15). A solution of 13 (2.33 g, 0.01 mol) in a mixture of glacial AcOH (50 mL) and 1 N HCl (10 mL) was hydrogenated in a Parr apparatus for 1 h over 10% Pd–C (0.2 g). After filtration, the colorless solution was evaporated under reduced pressure at 50 °C and the residue was partitioned between 1 M $NaHCO_3$ (50 mL) and $CHCl_3$ (50 mL). The aqueous phase was extracted with $CHCl_3$ (2 × 50 mL), and the pooled organic phases were concentrated to dryness on the rotary evaporator. The residue was redissolved in a 1:2 mixture of CH_2Cl_2 and hexanes and the solution kept at −20 °C overnight. The precipitate was filtered, washed with hexanes, and dried in vacuo to obtain white crystals (0.81 g, 39%); mp 91–93 °C; IR (KBr) ν 3260, 2980, 2920, 2850, 1610, 1520, 1480, 1430 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.88 (s, 3H, $CH_3C=N$), 3.55 (s, 2H, $CH_2C=N$), 3.75 (s, 3H, 2-OMe), 3.80 (s, 3H, 5-OMe), 6.73 (m, 3H, aromatic), 8.52 (s, 1H, N=OH).

3-(2,5-Dimethoxyphenyl)-2-propanone (16). A solution of 13 (4.46 g, 0.0196 mol) in glacial AcOH (100 mL) containing 2.5 mL of washed Raney Ni suspension (50% v/v) was was hydrogenated in a Parr apparatus at 25 °C for 2 h. The mixture was filtered, the filtrate evaporated under reduced pressure, and the blue-green residue partitioned between $CHCl_3$ (50 mL) an 1 M $NaHCO_3$ (250 mL). The aqueous phase was extracted with $CHCl_3$ (3 × 50 mL), and the combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure. The residue was distilled under high vacuum, and a fraction was collected at 102–105 °C/7–8 Torr; yield 2.0 g (52%); IR (thin film) ν 3000, 2950,

Table II. DHFR Inhibition and Cell Growth Inhibition by Miscellaneous 2,4-Diaminothienol[2,3-*d*]pyrimidines

compd		IC_{50}[a] (μM)[a]					
		DHFR species					
		P. carinii	*T. gondii*	rat liver	beef liver	*L. casei*	CCRF–CEM cells
							
R^1	R^2						
CH_3	C_6H_5[b]	26 (2.2)	3.9 (15)	57	0.65	1.5	14
CH_3	3,4-$Cl_2C_6H_3$[b]	16 (0.3)	>28 (<0.2)	4.6	4.6	2.0	ND
CH_3	$CH_2C_6H_5$[b,c]	35 (0.4)	6.2 (2.2)	14	0.64	1.3	15
4-ClC_6H_4	CH_3[b]	>100	>70	>100	7.7	>50	ND
$CH_2C_6H_5$	CH_3[b]	>100	>100	>37	19	>50	ND
							
X							
CH_2[d,e]		2.1 (1.9)	2.8 (1.4)	3.9	ND	ND	ND
$(CH_2)_2$[d]		1.4 (0.9)	0.86 (1.5)	1.3	ND	ND	ND
$(CH_2)_3$[d]		6.5 (0.3)	ND	1.9	0.4	45	12
$(CH_2)_7$[d]		>50 (<0.2)	ND	9.3	0.2	12	3.7
$N(CH_2C_6H_5)$[d,e]		5.2 (0.1)	0.85 (0.7)	0.57	ND	ND	ND

[a] Numbers in parentheses are approximate potency ratios relative to DHFR from rat liver. ND = not determined. [b] See ref 29. [c] See ref 27. [d] See ref 28. [e] See ref 30.

2910, 2840, 1725, 1715, 1610, 1590, 1500, 1470 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.09 (s, 3H, CH_3CO), 3.60 (s, 2H, CH_2CO), 3.75 (two s, 6H, 2- and 5-OMe), 6.68–6.87 (m, 3H, aromatic).

3-(3,4,5-Trimethoxyphenyl)-2-propanone (17). A solution of 14 (10.1 g, 0.04 mol) in glacial AcOH (100 mL) containing 5 mL of Raney Ni suspension (50% v/v) was hydrogenated in a Parr apparatus at 25 °C for 15 h. After filtration and evaporation, the residue was recrystallized from 3:1 MeOH–H_2O (30 mL). The white crystals were collected, the filtrate was evaporated to dryness, and the residue was redissolved in $CHCl_3$ (30 mL). The solution was dried ($MgSO_4$) and filtered through a bed of silica gel which was washed with another 60 mL of $CHCl_3$. The combined filtrates were evaporated, and the residue was combined with the first crop and recrystallized from 3:1 MeOH–H_2O (40 mL). The product was filtered, washed with H_2O (30 mL), and dried overnight in vacuo to obtain a waxy white solid (5.8 g, 65%): mp 64–65 °C; R_f 0.19 (silica gel, 3:7 EtOAc–hexanes); IR (KBr) ν 3660–3200, 3000, 2960, 2940, 2840, 1720, 1595, 1510, 1470, 1455, 1430 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.15 (s, 3H, CH_3CO), 3.61 (s, 2H, CH_2), 3.81 (ps, 9H, 3-, 4-, and 5-OMe), 6.40 (s, 2H, aromatic).

4-(2,5-Dimethoxyphenyl)-3-buten-2-one (18). A solution of 11 (16.6 g, 0.1 mol) and 1-(triphenylphosphoranylidene)-2-propanone (35 g, 0.11 mol) in CH_2Cl_2 (400 mL) was heated under reflux for 2.5 days and then evaporated to dryness under reduced pressure. The residue was stirred with Et_2O (500 mL), and the triphenylphosphine oxide was removed by passing the solution through a column of silica gel (60 g). After elution with an additional portion of Et_2O (300 mL), the volume of the combined eluates was reduced to 100 mL. Final traces of triphenylphosphine oxide were removed by applying the solution onto a fresh silica gel column and eluting with another portion of Et_2O (300 mL). The volume of the final eluate was concentrated to dryness and the residue kept at −80 °C overnight. The resultant solid was triturated with hexanes (200 mL), filtered, and dried in vacuo overnight at 25 °C to obtain white crystals (19.5 g, 95%): mp 48–50 °C; IR (KBr) ν 3000, 2950, 2840, 1670, 1620, 1605, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.40 (s, 3H, CH_3CO), 3.77 (s, 3H, 2-OMe), 3.83 (s, 3H, 5-OMe), 6.74 (d, J = 16.6 Hz, 1H, CH=C*H*-CO), 6.85–7.63 (m, 3H, aromatic), 7.86 (d, J = 16.6 Hz, 1H, C*H*=CHCO).

4-(3,4,5-Trimethoxyphenyl)-3-buten-2-one (19). A solution of 12 (19.6 g, 0.1 mol) and 1-(triphenylphosphoranylidene)-2-propanone (35 g, 0.11 mol) in $CHCl_3$ (400 mL) was heated under reflux for 5 h and evaporated to dryness under reduced pressure. Final traces of solvent were removed by raising the bath temperature to 80 °C for 0.5 h, and the residue was redissolved in a 1:2 Et_2O–CH_2Cl_2 (150 mL). The solution was kept for 48 h at 20–25 °C in a stoppered flask until large crystals of triphenylphosphine oxide formed, which were filtered off. The filtrate was then applied onto a column of silica gel (4.5-cm i.d. × 30 cm) which was eluted with $CHCl_3$. Fractions of 100 mL were collected, fractions 5–8 were pooled and evaporated, and the residue was recrystallized from Et_2O to obtain white crystals (22.3 g, 94%): mp 92–93 °C; IR (KBr) ν 3000, 2940, 2820, 1670, 1645, 1625, 1580, 1505, 1470, 1420 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.32 (s, 3H, CH_3CO), 3.86 (ps, 9H, 3-, 4-, and 5-OMe), 6.56 (d, J = 16.0 Hz, 1H, CH=C*H*CO), 6.69 (s, 2H, aromatic), 7.41 (d, J = 16.0 Hz, 1 H, C*H*=CHCO).

4-(2,5-Dimethoxyphenyl)-2-butanone (20). A solution of 18 (10.3 g, 0.05 mol) in absolute EtOH (100 mL) was hydrogenated over 10% Pd–C (0.5 g) in a Parr apparatus at 25 °C for 9 h. The mixture was filtered, the filtrate was evaporated under reduced pressure at room temperature, and the residue was chromatographed on a column of silica gel (2.8-cm i.d. × 40 cm) with 4:1 hexanes–EtOAc as the eluent. Fractions homogeneous by TLC were pooled and evaporated, and the residue was dried in vacuo overnight at 25 °C to obtain an oil which failed to crystallize (9.5 g, 91%): R_f 0.49 (silica gel, 3:17 EtOAc–hexanes); IR (KBr) ν 3000, 2980–2890, 2820, 1730, 1590, 1505, 1475–1410 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.13 (s, 3H, CH_3CO), 2.63–2.97 (m, 4H, CH_2CH_2), 3.73 (ps, 6H, 2- and 5-OMe), 6.70 (ps, 3H, aromatic).

4-(3,4,5-Trimethoxyphenyl)-2-butanone (21). A solution of 19 (11.8 g, 0.05 mol) in absolute EtOH (100 mL) was hydrogenated over 10% Pd–C (0.5 g) in a Parr apparatus for 16 h. After filtration and rotary evaporation at room temperature, with heating at 70 °C to remove the last traces of solvent, the oily residue was kept at −20 °C until it formed a waxy solid precipitate (11.2 g, 94%): mp 21–25 °C; R_f 0.26 (silica gel, 7:3 hexanes–EtOAc); IR (KBr) ν 3010–2870, 2840, 1715, 1590, 1510, 1460, 1425 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.15 (s, 3H, CH_3CO), 2.78 (ps, 4H, CH_2CH_2), 3.79 (s, 3H, 4-OMe), 3.81 (s, 6H, 3- and 5-OMe), 6.37 (s, 2H, aromatic).

1-(2,5-Dimethoxyphenyl)-2-methoxyethylene (22, 1:1 *E*/*Z* Mixture). To a stirred suspension of (methoxymethyl)triphenylphosphonium chloride (17.1 g, 0.05 mol) in THF (250 mL) at 0 °C under argon was added a 2.5 M solution of n-BuLi (22.0 mL, 0.055 mol) in hexanes. The resulting deep-red solution was stirred at 0 °C for 5 min, and to it was quickly added a solution of 11 (8.0 g, 0.048 mol) in THF (100 mL). The temperature was slowly raised to 25 °C, and stirring was continued for 1 h. The solvents were evaporated under reduced pressure, the residue was triturated with hot hexanes, and the precipitated triphe-

nylphosphine oxide was filtered off. The filtrate was evaporated, the residue redissolved in 9:1 hexanes–EtOAc, and the solution passed through a bed of silica gel and evaporated to a yellow oil (6.3 g, 67%): IR (thin film) ν 3000, 2940, 2440, 1640, 1490 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.6–3.8 (m, 9H, 2-OMe, 5-OMe, and CH=CHO*Me*), 5.6 (d, J = 8 Hz, 0.5H, C*H*=CHOMe, *E*-isomer), 5.90 (d, J = 13 Hz, 0.5H, C*H*=CHOMe, *Z*-isomer), 6.1 (d, J = 8 Hz, 0.5H, CH=C*H*OMe, *E*-isomer), 7.1 (d, J = 13 Hz, 0.5H, CH=C*H*OMe, *Z*-isomer), 6.6–7.6 (complex m, 3H, aromatic).

5-(2,5-Dimethoxyphenyl)-3-penten-2-one (24). A 40% aqueous solution of $HClO_4$ (3 mL) was stirred in Et_2O (15 mL) at room temperature until homogeneous, and to this mixture was added a solution of **22** (2.5 g, 0.0128 mol) in Et_2O (5 mL). As soon as the reaction was complete (45 min), the mixture was diluted with H_2O (30 mL) and Et_2O (30 mL), the excess acid was neutralized with solid $NaHCO_3$ (caution: gas evolution), and the layers were separated. The aqueous layer was extracted with Et_2O (2 × 30 mL), and the combined organic layers were washed with a half-saturated $NaHCO_3$ solution (10 mL), dried, and evaporated to dryness under reduced pressure. Column chromatography on silica gel with 4:1 hexanes–EtOAc as the eluent yielded 2,5-dimethoxyphenylacetaldehyde (**23**) as a yellow oil which was sufficiently pure for the next step: IR (thin film) ν 2900–3000, 2840, 1730 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.6 (d, 2H, CH_2), 3.7 (s, 6H, 2- and 5-OMe), 6.7–6.8 (m, 3H, aromatic), 9.61 (t, 1H, CH=O).

A solution of **23** (1.56 g, 8.7 mmol) and 1-(triphenylphosphoranylidene)-2-propanone (2.75 g, 8.7 mol) in $CHCl_3$ (40 mL) was heated under reflux for 4 h. The mixture was cooled to room temperature, the solvent was removed under reduced pressure, and the residue was stirred in hot hexanes (100 mL) for 10 min. The triphenylphosphine oxide precipitate was filtered off, the filtrate concentrated under reduced pressure, and the residue chromatographed on silica gel with 4:1 hexanes–EtOAc as the eluent to obtain a yellow oil (1.1 g, 57%): IR (thin film) ν 2900–3000, 2840, 1670 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3H, CH_3CO), 3.4–3.6 (m, 2H, CH_2), 3.7 (s, 6H, 2- and 5-OMe), 5.8–6.2 (m, 1H, CH=), 6.6–6.8 (m, 3H, aromatic).

5-(2,5-Dimethoxyphenyl)-2-pentanone (25). A solution of **24** (1.0 g, 4.6 mmol) in EtOH (25 mL) was hydrogenated over 10% Pd–C (50 mg) in a Parr apparatus for 2 h, the catalyst removed, and the filtrate concentrated to a colorless oil by rotary evaporation: yield 0.99 g (47%); IR (thin film) ν 2900–3000, 2840, 1720 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.5.1.8 (m, 2H, $CH_2CH_2CH_2CH_2$), 1.9 (s, 3H, $COCH_3$), 2.1–1.6 (m, 4H, $CH_2CH_2CH_2CH_2$), 3.5 (s, 6H, 2- and 5-OMe), 6.5 (m, 3H, aromatic).

Ethyl 5-(3,4,5-trimethoxyphenyl)-2-oxopentane-3-carboxylate (27). A heat-dried flask under argon was charged with NaH (0.105 g, 4.4 mmol; prewashed with hexanes) and THF (4 mL). A solution of ethyl acetoacetate (0.71 mL, 4.0 mmol) in THF (2 mL) was added dropwise at room temperature, followed by DMSO (2 mL), and the resulting homogeneous mixture was stirred for 30 min. A solution of bromide **26** (1.0 g, 3.6 mol) in THF (2 mL) and DMSO (2 mL) was then added, followed by solid NaI (5 mg). The reaction mixture was heated under reflux for 24 h, cooled, and quenched with saturated NH_4Cl (20 mL). The layers were separated, the aqueous layer was extracted with Et_2O (3 × 30 mL), and the combined organic extracts were washed with brine (2 × 20 mL), dried ($MgSO_4$), and evaporated under reduced pressure. Flash chromatography on silica gel with 7:3 hexanes–EtOAc as the eluent yielded a colorless oil (612 mg, 52%): IR (thin film) ν 2960, 2840, 1745, 1720, 1600, 1460 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.3 (t, 3H, OCH_2CH_3), 1.9–2.7 (m, 4H, CH_2CH_2), 2.2 (s, 3H, $COCH_3$), 3.4 (t, 1H, CH_2CHCO), 3.8 (broad s, 9H, 3-, 4-, and 5-OMe), 4.2 (q, 2H, OCH_2CH_3), 6.3 (s, 2H, aromatic).

5-(3,4,5-Trimethoxyphenyl)-2-pentanone (28). To a rapidly stirred suspension of **27** (612 mg, 1.88 mmol) in H_2O (30 mL) was added solid KOH (400 mg) in a single portion (exothermic reaction). The mixture was heated to 90 °C, stirred for 3 h, and then cooled to room temperature and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic layers were washed with 1 N KOH (2 × 20 mL), dried ($MgSO_4$), and concentrated by rotary evaporation. The residual oil was filtered through a plug of silica gel with a 5:1 mixture of hexanes and EtOAc as the eluent, and the filtrate was evaporated to a yellow oil under reduced pressure: yield 379 mg (80%); IR (thin film) ν 2960, 2880, 1720, 1600, 1470, cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.6–1.9 (m, 2H, $CH_2CH_2CH_2$), 2.1 (s, 3H, $COCH_3$), 2.2–2.7 (m, 4H, $CH_2CH_2CH_2CH_2$), 3.8 (broad s, 9H, 3-, 4-, and 5-OMe), 6.4 (s, 2H, aromatic).

3-(2,5-Dimethoxyphenyl)-2-propylidenemalononitrile (29). Glacial AcOH (0.24 mL) and NH_4OAc (80 mg) were added to a stirred solution of **16** (1.94 g, 0.01 mol) and malononitrile (0.661 g, 0.1 mol) in benzene (4 mL), and the mixture was heated at the reflux temperature for 3.5 h. After evaporation under reduced pressure, the residue was chromatographed on a column of silica gel (2.4 cm × 40 cm) with CH_2Cl_2 as the eluent. Fractions of 50 mL each were monitored by TLC (silica gel, CH_2Cl_2), and those containing a single spot with R_f 0.46 were pooled and evaporated. The solid was dried in vacuo at room temperature overnight to obtain yellow crystals, 2.36 g (97%): mp 42–43 °C; IR (KBr) ν 3200–3600, 2890–2990, 2840, 2240, 1600, 1590, 1500, 1460, 1425 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.12 (s, 3H, CH_3), 3.75 (s, 2H, CH_2), 3.79 (s, 3H, 2-OMe), 3.81 (s, 3H, 5-OMe), 6.67–6.95 (m, 3H, aromatic).

3-(2,5-Dimethoxyphenyl)-2-butylidenemalononitrile (30). Solid NH_4OAc (0.5 g) was added to a solution of **20** (5.21 g, 0.0025 mol) and malononitrile (1.65 g, 2.5 mmol) in benzene (12.5 mL) and glacial AcOH (0.75 mL). The mixture was heated under reflux for 4 h, cooled to room temperature, left to stand for 18 h, and evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (30 mL), and the solution washed with H_2O (25 mL) followed by aqueous $NaHCO_3$ (25 mL). The organic layer was dried ($MgSO_4$) and evaporated to dryness and the residue recrystallized from hexanes containing ca. 5% Et_2O (v/v) to obtain white crystals (5.86 g, 92%): mp 74–75 °C; R_f 0.40 (silica gel, CH_2Cl_2); IR (KBr) ν 3200–3600, 2950, 2840, 2240, 1600, 1500, 1465 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, CH_3), 2.85 (ps, 4H, CH_2CH_2), 3.72 (s, 3H, 2-OMe), 3.77 (s, 3H, 5-OMe), 6.65–6.90 (m, 3H, aromatic).

5-(2,5-Dimethoxyphenyl)-2-pentylidenemalononitrile (31). Malononitrile (0.302 g, 4.59 mol), glacial AcOH (0.15 mL), and solid NH_4OAc (100 mg) were added to a solution of **25** (1.01 g, 4.59 mmol) in benzene (4 mL). The reaction mixture was heated under reflux for 2 h and then cooled and diluted with H_2O (20 mL) and EtOAc (80 mL). The layers were separated, the aqueous layer was extracted with EtOAc (2 × 20 mL), and the combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The residue was chromatographed on silica gel with 4:1 hexanes–EtOAc as the eluent to obtain a white solid (1.03 g, 88%). The analytical sample was prepared by recrystallization from 1:9 Et_2O–hexanes: mp 54–55 °C; IR (KBr) ν 2960, 2840, 2220 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.5–1.9 (m, 2H, $CH_2CH_2CH_2$), 2.1 (m, 3H, CH_3), 2.3–2.7 (m, 4H, $CH_2CH_2CH_2CH_2$), 2.6 (s, 6H, 2- and 5-OMe), 6.6 (s, 3H, aromatic).

3-(3,4,5-Trimethoxyphenyl)-2-propylidenemalononitrile (32). Starting with **17** (4.48 g, 2 mmol), the method used to prepare **30** afforded **32** as white crystals (3.87 g, 71%): mp 78–79 °C; R_f 0.13 (silica gel, CH_2Cl_2); IR (KBr) ν 3640–3160, 2980, 2940, 2840, 2240, 1590, 1510, 1460, 1420 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3), 3.79 (s, 2H, CH_2), 3.82 (s, 3H, 4-OMe), 3.86 (s, 6H, 3- and 5-OMe), 6.41 (s, 2H, aromatic).

4-(3,4,5-Trimethoxyphenyl)-2-butylidenemalononitrile (33). Starting with **21** (5.96 g, 2.5 mmol), the method used to prepare **30** afforded **33** as white crystals (6.66 g, 93%): mp 79–81 °C; TLC R_f 0.09 (silica gel, CH_2Cl_2); IR (KBr) ν 3600–3200, 2940, 2840, 2240, 1590, 1510, 1460, 1430 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.25 (s, 3H, CH_3), 2.85 (ps, 4H, CH_2CH_2), 3.80 (s, 3H, 4-OMe), 3.85 (s, 6H, 3- and 5-OMe), 6.39 (s, 2H, aromatic).

5-(3,4,5-Trimethoxyphenyl)-2-pentylidenemalononitrile (34). Starting with **28** (0.62 g, 2.46 mmol), the method used to prepare **31** afforded **34** as white crystals (0.5 g, 67%): mp 72–73 °C; IR (KBr) ν 29940, 2840, 2240, 1590, 1460 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.7–2.0 (m, 2H, $CH_2CH_2CH_2$), 2.2 (s, 3H, CH_3), 2.4–2.8 (m, 4H, $CH_2CH_2CH_2CH_2$), 3.8 (broad s, 9H, 3-, 4-, and 5-OMe), 6.3 (s, 2H, aromatic).

2-Amino-3-cyano-5-(2′,5′-dimethoxyphenyl)-4-methylthiophene (35). i-Pr_2NH (0.701 mL, 5 mmol) was added to a stirred suspension of **29** (1.21 g, 5 mmol) and sulfur (0.16 g, 5 mmol) in absolute EtOH (25 mL) at 55 °C under argon. The temperature was kept at 55 °C for 45 min, the resulting pale-amber solution was poured into 0.2 N HCl (25 mL), and the mixture was extracted with EtOAc (2 × 50 mL). The extracts

were dried ($MgSO_4$) and evaporated, and the residue was chromatographed on a silica gel column (2.8-cm i.d. × 35 cm) with CH_2Cl_2 as the eluent. TLC-homogeneous fractions were pooled and evaporated, and the residue was recrystallized from a mixture of EtOAc and hexanes to obtain pale-tan crystals (0.42 g, 31%): mp 107–108 °C; R_f 0.50 (silica gel, 24:1 CH_2Cl_2–EtOH); IR (KBr) ν 3410, 3320, 2940, 2200, 1630, 1520, 1500, 1400 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.09 (s, 3H, 4-CH_3), 3.73 (ps, 6H, 2′- and 5′-OMe), 4.81 (broad s, 2H, NH_2), 6.72–6.97 (m, 3H, aromatic).

2-Amino-3-cyano-5-(2′,5′-dimethoxybenzyl)-4-methylthiophene (36). Starting with **30** (1.54 g, 0.006 mol), the general method used to prepare **35** was followed except that in the workup the EtOAc extracts were evaporated and the residue was recrystallized from Et_2O: yield 0.52 g (30%); mp 134–136 °C; R_f 0.60 (silica gel, 24:1 CH_2Cl_2–EtOH); IR (KBr) ν 3420, 3305, 3205, 3000, 2940, 2835, 2205, 1630, 1525, 1495 cm^{-1}; ^1H NMR (10:1 $CDCl_3$-CD_3OD) δ 2.10 (s, 3H, 4-CH_3), 3.72 (s, 3H, 4′-OMe), 3.76 (s, 6H, 3′- and 5′-OMe), 3.80 (s, 2H, 5-CH_2), 4.51 (broad s, 2H, NH_2), 6.56–6.90 (m, 3H, aromatic).

2-Amino-3-cyano-5-[2-(2′,5′-dimethoxyphenyl)ethyl]-4-methylthiophene (37). Starting with **31** (0.510 g, 1.88 mmol), the general method used to prepare **35** afforded white crystals (0.376 g, 66%): mp 130–132 °C; IR (KBr) ν 3420, 3320, 2960–2920, 2200, 1580 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.0 (s, 3H, 4-CH_3), 2.7 (s, 4H, CH_2CH_2), 3.7–3.8 (m, 8H, 2′-OMe, 5′-OMe, NH_2), 6.7 (broad s, 2H, 3′- and 4′-H), 7.2 (s, 1H, 6′-H).

2-Amino-3-cyano-5-(3′,4′,5′-trimethoxyphenyl)-4-methylthiophene (38). Starting with **32** (2.72 g, 0.01 mol) and equimolar amounts of S and diisopropylamine, **38** was prepared by the same method as **35** except that a 2:3 mixture of EtOAc and hexanes was used instead of CH_2Cl_2 for column chromatography. Recrystallization from a mixture of Et_2O and hexanes afforded plate-tan crytsals (1.01 g, 33%): mp 157–158 °C; R_f 0.40 (silica gel, 24:1 CH_2Cl_2–EtOH); IR (KBr) ν 3460, 3320, 2200, 1585, 1515, 1410 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.24 (s, 3H, 4-CH_3), 3.93 (ps, 9H, 3′-, 4′-, 5′-OMe), 4.82 (broad s, NH_2), 6.50 (s, 2H, aromatic).

2-Amino-3-cyano-5-(3′,4′,5′-trimethoxybenzyl)-4-methylthiophene (39). Starting with **33** (2.86 g, 0.01 mol) and equimolar amounts of S and diisopropylamine, **39** was prepared by the same method as **35** except that a 3:7 mixture of EtOAc and hexanes was used for column chromatography: yield 1.52 g (48%); mp 169–170 °C; R_f 0.42 (silica gel, 24:1 CH_2Cl_2–EtOH); IR (KBr) ν 3420, 3335, 3205, 2200, 1630, 1590, 1505, 1460, 1420 cm^{-1}; ^1H NMR (10:1 $CDCl_3$–CD_3OD) δ 2.15 (s, 3H, 4-CH_3), 3.80 (s, 3H, 4′-OMe), 3.84 (s, 6H, 3′-OMe, 5′-OMe), 4.11 (s, 2H, 5-CH_2), 6.41 (s, 2H, aromatic).

2-Amino-3-cyano-5-[2-(2′,5′-dimethoxyphenyl)ethyl]-4-methylthiophene (40). i-Pr_2NH (0.14 mL, 0.001 mol) was added dropwise to a mixture of **34** (0.3 g, 0.001 mol) and S (0.045 g, 0.014 mol) in EtOH (2 mL) while stirring under reflux. The amber-colored solution was stirred for 1.5 h and then poured into 0.2 N HCl (2 mL). The solution was diluted with EtOAc (30 mL) and H_2O (20 mL), and the layers were separated. The aqueous phase was extracted with EtOAc (2 × 30 mL), and the combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure. The resulting brown solid was purified by chromatography on silica gel (3:2 hexanes–EtOAc) and recrystallization from hot Et_2O to obtain yellow needles (0.127 g, 38%): mp 153–154 °C; R_f 0.31 (silica gel, 3:2 hexanes–EtOAc); IR (KBr) ν 3420, 3320, 3210, 2940, 2840, 2200, 1590, 1530 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.9 (s, 3H, 4-CH_3), 2.7–2.8 (m, 4H, 5-CH_2CH_2), 3.8 (s, 9H, 3′-, 4′-, 5′-OMe), 4.1–4.6 (broad s, 2H, NH_2), 6.3 (s, 2H, aromatic).

1-Chloro-4-(2,5-dimethoxyphenyl)-2-butanone (43). i-Pr_2-NH (2.42 mL, 17.3 mmol) in dry THF (12 mL) was added to a heat-dried flask under an argon atmosphere, and the stirred solution was cooled to 0 °C and treated dropwise with n-BuLi (6.9 mL of 2.5 M solution, 17.3 mmol) over 15 min. The temperature was then lowered to −78 °C, followed by dropwise addition of ketone **20** (3.0 g, 14.4 mmol) in dry THF (4 mL). After being stirred at −78 °C for 40 min, the dark-yellow reaction mixture was treated with Me_3SiCl (1.99 mL, 15.8 mmol) in a single portion. The mixture was stirred at −78 °C for 20 min and then warmed slowly to room temperature, stirred for 1 h, and poured into half-saturated aqueous $NaHCO_3$ (30 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with aqueous $NaHCO_3$ (40 mL) and brine (40 mL), dried (Na_2SO_4), and concentrated by rotary evaporation. The oily residue (enol ether **41**) was redissolved in CH_2Cl_2 under gentle reflux, and the solution treated dropwise with *N*-chlorosuccinimide (1.92 g, 14.4 mmol) in CH_2Cl_2 (40 mL). The reaction mixture was refluxed for 1 h and then cooled and evaporated under reduced pressure. The residue was taken up in hot hexanes (60 mL). Upon cooling, a precipitate formed, which was collected and washed with warm hexanes. The filtrate was evaporated, the residue was dissolved in MeOH (40 mL), citric acid (1.5 g) was added, and the mixture was stirred at room temperature for 1.5 h. The yellow oil remaining after solvent evaporation was taken up in EtOAc (50 mL), the solution was carefully washed with half-saturated aqueous $NaHCO_3$, the basic aqueous layer was extracted with EtOAc (2 × 30 mL), and the combined organic layers were washed with brine (30 mL), dried (Na_2SO_4), and evaporated. Chromatography of the residue on silica gel using a 10:1 mixture of hexanes and EtOAc as the eluent afforded **43** as a yellow oil (1.31 g, 32% overall): R_f 0.30 (silica gel, 4:1 hexanes–EtOAc); IR (neat) ν 2990, 2930, 2830, 1735, 1720, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.9 (br s, 4H, CH_2CH_2), 3.9–3.8 (two s, 6H, 2- and 5-OMe), 4.05 (s, 2H, CH_2Cl), 6.75 (s, 3H, aromatic).

1-Chloro-5-(2,5-dimethoxyphenyl)-2-pentanone (44). Starting from ketone **25** (2.22 g, 0.01 mol), the method used to prepare **43** gave **44** as a yellow oil (0.307 g, 35% overall): R_f 0.29 (silica gel, 4:1 hexanes–EtOAc); IR (neat) ν 2990, 2930, 2820, 1730, 1715, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.8–2.4 (m, 2H, $CH_2CH_2CH_2$), 2.5–2.8 (m, 4H, $CH_2CH_2CH_2$), 3.8 and 3.9 (two s, 6H, 2- and 5-OMe), 4.1 (s, 2H, CH_2Cl), 6.8 (s, 3H, aromatic).

1-Chloro-4-(2,5-dimethoxyphenyl)-2-butylidenemalononitrile (45). Malononitrile (0.231 g, 3.5 mmol), glacial AcOH (70 mL), and NH_4OAc (10 mg) were added to a solution of **43** (0.85 g, 3.5 mmol) in benzene (30 mL). The reaction mixture was heated to reflux for 4 h, cooled, and then poured into a mixture of EtOAc (320 mL) and half-saturated $NaHCO_3$ solution (30 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2 × 20 mL). The combined organic layers were washed with $NaHCO_3$ (120 mL) and brine (20 mL), dried (Na_2SO_4), and then evaporated to dryness under reduced pressure. The resulting brown residue was chromatographed on silica gel with 4:1 hexanes–EtOAc to obtain a yellow oil. Upon being stored at −10 °C for 3 days, the oil solidified. Recrystallization from Et_2O–hexanes gave yellow needles (0.72 g, 71% yield): mp 77–79 °C; R_f 0.30 (silica gel, 4:1 hexanes–EtOAc); IR (KBr) ν 3020, 2950, 2840, 2240, 1590, 1500, 1460 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.8–3.0 (m, 2H, CH_2CH_2), 3.75 (s, 6H, 2- and 5-OMe), 4.3 (s, 2H, CH_2Cl), 6.6–6.8 (m, 3H, aromatic).

1-Chloro-5-(2,5-dimethoxyphenyl)-2-pentylidenemalononitrile (46). Starting with 0.78 g (0.3 mmol) of **44**, the method used to prepare **45** gave **46** as an oil (0.64 g, 69%): R_f 0.29 (silica gel, 4:1 hexanes–EtOAc); IR (neat) ν 3000, 2940, 2840, 2220, 1590, 1500, 1460 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.9–2.2 (m, 2H, $CH_2CH_2CH_2$), 2.6–3.2 (m, 4H, $CH_2CH_2CH_2$), 3.8 (s, 6H, 2- and 5-OMe), 4.4 (s, 2H, CH_2Cl), 6.8 (m, 3H, aromatic). Anal. Calcd: Cl, 11.63. Found: Cl, 11.00.

2-Amino-3-cyano-4-[2-(2′,5′-dimethoxyphenyl)ethyl]-thiophene (47). A solution of chloro ketone **45** (0.75 g, 2.5 mmol) in EtOH (10 mL) was added dropwise to a solution of NaSH (0.15 g, 2.6 mmol) in EtOH (20 mL) cooled to −42 °C (dry ice/MeCN bath). The reaction mixture was stirred at −42 °C for 1 h, slowly warmed to 0 °C over 20 min, and stirred at 0 °C for 2 h. The solvent was evaporated under reduced pressure and the residue dissolved in Et_2O (30 mL). The solution was washed with brine (40 mL) and the aqueous wash extracted with Et_2O (3 × 40 mL), and the combined Et_2O layers were dried ($MgSO_4$) and evaporated to dryness under reduced pressure. The dark red residue was chromatographed on silica gel with 3:1 hexanes–EtOAc, and the resultant orange solid was recrystallized from a mixture of Et_2O and hexanes to obtain white crystals (0.61 g, 84%): mp 80–81 °C; R_f 0.15 (silica gel, 3:1 hexanes–EtOAc); IR (KBr) ν 3400, 3300, 3210, 2993, 2980, 2820, 2205, 1630, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.7–2.8 (m, 4H, CH_2CH_2), 3.75 (s, 3H, 2′-OMe), 3.81 (s, 3H, 5′-OMe), 4.5–4.8 (broad m, 2H, NH_2), 5.95 (s, 1H, thiophene H_5), 6.75 (s, 3H, aromatic).

2-Amino-3-cyano-4-[3-(2',3-dimethoxyphenyl)propyl]-thiophene (48). Starting with chloro ketone **46** (0.20 g, 0.65 mmol) the method used to prepare **47** gave **48** (0.103 g, 52%): mp 95–96 °C; R_f 0.15 (silica gel, 4:1 hexanes–EtOAc); IR (KBr) ν 3420, 3320, 3240, 2995, 2930, 2840, 2205, 1630, 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.8–2.1 (m, 2H, $CH_2CH_2CH_2$), 2.4–2.8 (m, 4H, $CH_2CH_2CH_2$), 3.75 (s, 6H, 2'- and 5'-OMe), 4.6–4.8 (br m, 2H, NH_2), 5.95 (s, 1H, thiophene H_5), 6.7 (s, 3H, aromatic).

2,4-Diamino-6-(2',5'-dimethoxyphenyl)-5-methylthieno-[2,3-*d*]pyrimidine (3). Aminonitrile **35** (91.8 mg, 0.335 mmol) and chloroformamidine hydrochloride (147 mg, 1.28 mmol) were crushed to a fine powder, mixed thoroughly, and placed in a 25-mL pear-shaped flask. The mixture was triturated continuously with a glass rod while being heated to 120 °C (internal temperature) in an oil bath under an argon atmosphere. After 30 min of heating, the reaction mixture was cooled to room temperature and the resulting glassy solid triturated with a mixture of $CHCl_3$ (5 mL) and i-Pr_2NH (0.24 mL). The solid was filtered, the filtrate evaporated under reduced pressure, and the residue chromatographed on a silica gel column (2.8 cm × 40 cm) with 9:1 $CHCl_3$–MeOH as the eluent. Fractions homogeneous by TLC (silica gel, 9:1 $CHCl_3$–MeOH, R_f 0.49) were pooled and concentrated under reduced pressure to a volume of 0.5 mL. Dropwise addition of Et_2O, filtration of the precipitated solid, recrystallization from H_2O, and overnight drying in vacuo at 75 °C afforded white crystals (33.4 mg, 24%): mp 227–228 °C; IR (KBr) ν 3500, 3310, 3155, 1650, 1620, 1565, 1530, 1450 cm^{-1}; UV (EtOH) λ_{max} 233 nm (ϵ 40 350), 310 (19 900); ^{1}H NMR (500 MHz, CD_3OD) δ 2.21 (s, 3H, 5-CH_3), 3.73 (s, 3H, 2'-OMe), 3.76 (s, 3H, 5'-OMe), 4.64 (s, 2H, NH_2), 6.82 (d, $J_{6',4'}$ = 3 Hz, 6'-H), 6.92 (dd, $J_{4',3'}$ = 3 Hz, 1H, 4'-H), 6.98 (d, $J_{3',4'}$ = 9 Hz, 1H, 3'-H).

2,4-Diamino-6-(2',5'-dimethoxybenzyl)-5-methylthieno-[2,3-*d*]pyrimidine (4). This compound was prepared from aminonitrile **36** (288 mg, 1 mmol) and chloroformamidine hydrochloride (458 mg, 4 mmol) by the same method as **3** except that 9:1 $CHCl_3$–MeOH was used as the column chromatography eluent: yield 250 mg (76%); mp 175–176 °C; R_f 0.43 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3520, 3420, 3180, 1610, 1570, 1530, 1470, 1440 cm^{-1}; UV (EtOH) λ_{max} 233 nm (ϵ 27 050), 279 (14 000); ^{1}H NMR (500 MHz, CD_3OD) δ, 2.39 (s, 3H, 5-CH_3), 3.62 (s, 3H, 2'-OMe), 3.71 (s, 5H, 5'-OMe), 3.91 (s, 2H, 6-CH_2), 4.78 (s, 2H, NH_2), 6.60 (d, $J_{6',4'}$ = 3.5 Hz, 1H, 6'-H), 6.69 (dd, $J_{4',3'}$ = 9.5 Hz, 1H, 4'-H), 6. 79 (d, $J_{3',4'}$ = 9.5 Hz, 1H, 3'-H).

2,4-Diamino-6-[2-(2',5'-dimethoxyphenyl)ethyl]-5-methylthieno[2,3-*d*]pyrimidine (5). Aminonitrile **37** (210 mg, 0.69 mmol) and chloroformamidine hydrochloride (314 mg, 2.76 mmol) were copulverized thoroughly under argon, and the mixture was heated at 120 °C (internal) for 15 min. After being allowed to cool to room temperature, the mixture was dissolved in MeOH (1 mL) and the solution diluted with $CHCl_3$ (15 mL). The precipitate was filtered and washed with $CHCl_3$. i-Pr_2NH (0.5 mL) was added to the filtrate, the solution concentrated under reduced pressure, and the residual solid then heated for 1.5 h at 75–80 °C under reduced pressure (0.1 Torr) to complete the ring closure. Chromatography of the resulting product on silica gel (9:1 $CHCl_3$–MeOH) followed by recrystallization (9:1 i-PrOH–MeOH) afforded a white powder (144 mg, 61%): mp 185–187 °C; R_f 0.43 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3420, 3180, 2980–2860, 1660, 1630, 1510 cm^{-1}; UV (EtOH) λ_{max} 233 nm (ϵ 26 717), 279 (14 740); ^{1}H NMR ($CDCl_3$) δ 2.1 (broad s, 3H, 5-CH_3), 2.8–2.9 (m, 4H, CH_2CH_2), 3.61 (s, 3H, 2'-OMe), 3.70 (s, 3H, 5'-OMe), 6.6–6.7 (m, 3H, aromatic).

2,4-Diamino-5-methyl-6-(3',4',5'-trimethoxyphenyl)thieno-[2,3-*d*]pyrimidine (6). Starting with aminonitrile **38** (304 mg, 1 mmol) and chloroformamidine hydrochloride (458 mg, 4 mmol), **6** was prepared by the same method as **3** except that 98:2 $CHCl_3$–MeOH was used as the column chromatography eluent; white crystals (226 mg, 66%); mp 249–251 °C; R_f 0.46 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3500, 3480, 3150, 1650, 1620, 1560, 1520, 1490, 1435 cm^{-1}; UV (EtOH) λ_{max} 233 nm (ϵ 32 900), 310 (20 000); ^{1}H NMR (500 MHz, CD_3OD) δ 2.20 (s, 3H, 5-CH_3), 3.77 (s, 3H, 4'-OMe), 3.84 (s, 6H, 3'-OMe, 5'-OMe), 4.85 (s, 2H, NH_2), 6.60 (s, 2H, 2'-H, 6'-H).

2,4-Diamino-5-methyl-6-(3',4',5'-trimethoxybenzyl)thieno-[2,3-*d*]pyrimidine (7). Starting from aminonitrile **39** (159 mg, 0.5 mmol) and chloroformamidine hydrochloride (230 mg, 2 mmol), **7** was prepared by the same method as **3** except that 9:1 $CHCl_3$–MeOH was used as the column chromatography eluent, affording white crystals (100 mg, 60%): mp 241–242 °C; R_f 0.48 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3650–3100, 1590, 1565, 1520, 1460, 1450 cm^{-1}; UV (EtOH) λ_{max} 233 nm (ϵ 31 600), 279 (16 150); ^{1}H NMR (500 MHz, CD_3OD) δ 2.43 (s, 3H, 5-CH_3), 3.76 (s, 3H, 4'-OMe), 3.78 (s, 6H, 3'-OMe, 5'-OMe), 3.99 (s, 2H, 6-CH_2), 4.51 (s, 2H, NH_2), 6.45 (s, 2H, 2'-H, 6'-H).

2,4-Diamino-5-methyl-6-[2-(3',4',5'-trimethoxyphenyl)ethyl]thieno[2,3-*d*]pyrimidine (8). Starting from aminonitrile **40** (100 mg, 0.3 mmol) and chloroformamidine hydrochloride (137 mg, 1.2 mmol), **8** was prepared by the same method as **5** except that the reaction mixture was heated at 120 °C for 20 min and that the final product after chromatography was recrystallized from 9:1 $CHCl_3$–MeOH: yellow crystals (67 mg, 59%); mp 197–199 °C; R_f 0.51 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3500, 3440, 3300, 3180, 2940, 1640, 1590, 1560, 1460 cm^{-1}; UV (EtOH) λ_{max} 233 nm (ϵ 32 100), 278 (15 830); ^{1}H NMR ($CDCl_3$) δ 2.2 (s, 3H, 5-CH_3), 2.8–3.0 (m, 4H, CH_2CH_2), 3.8 (broad s, 9H, 3'-, 4', 5'-OMe), 4.8–5.4 (m, 4H, 2-NH_2, 4-NH_2), 6.3 (s, 3H, 2'-H, 6'-H).

2,4-Diamino-5-[2-(2',5'-dimethoxyphenyl)ethyl]thieno-[2,3-*d*]pyrimidine (9). A mixture of aminonitrile **47** (250 mg, 0.86 mmol) and chloroformamidine hydrochloride (396 mg, 0.347 mmol) was heated as described above. A dark brown oil emitting HCl gas formed at 80 °C (internal), and the temperature was increased to 120 °C (internal) and maintained with stirring for 20 min. The reaction mixture was cooled, the solid dissolved in MeOH (1.5 mL), $CHCl_3$ (15 mL) added, the solution chilled at 0 °C for 1 h, and the precipitate filtered. i-Pr_2NH (0.5 mL) was added to the filtrate and the solution concentrated to dryness under reduced pressure while maintaining the rotary evaporator bath at 65 °C. The resulting red solid was chromatographed on silica gel with 9:1 $CHCl_3$–MeOH to obtain a yellow solid. Recrystallization from $CHCl_3$–hexanes yielded pale-yellow crystals (84 mg, 29%): mp 183–184 °C; R_f 0.36 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3460, 3350, 3190, 2990, 1620, 1550, 1500 cm^{-1}; UV (EtOH) λ_{max} 277 nm (ϵ 35 500), 278 (12 700); ^{1}H NMR ($CDCl_3$) δ 2.8 (s, 4H, CH_2CH_2), 3.75 (s, 3H, 2'-OMe), 3.8 (s, 3-H, 5'-OMe), 4.6–4.8 (br s, 2H, NH_2), 6.5 (s, 1H, thiophene H_5), 5.6–5.8 (broad m, 2H, NH_2), 6.8 (s, 3H, aromatic).

2,4-Diamino-5-[3-(2',3'-dimethoxyphenyl)propyl]thieno-[2,3-*d*]pyrimidine (10). Starting from aminonitrile **48** (100 mg, 0.33 mmol), the method used to prepare **9** gave **10** as pale-yellow crystals (42 mg, 36%): mp 171–174 °C; R_f 0.36 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) ν 3490, 3310, 3190, 2940, 1675, 1550, 1500 cm^{-1}; UV (EtOH) λ_{max} 227 nm (ϵ 38 300), 278 (10 900); ^{1}H NMR ($CDCl_3$ + 10% CD_3OD) δ 1.8–2.2 (m, 2H, $CH_2CH_2CH_2$), 2.5–2.85 (m, 4H, $CH_2CH_2CH_2$), 3.75 (s, 6H, 2'- and 5'-OMe), 6.5 (s, 1H, thiophene H_5), 6.7 (s, 3H, aromatic).

Bioassays. Methods described in previous papers[15,34] were used to test compounds as inhibitors of *P. carinii*, *T. gondii*, and rat liver DHFR activity. Inhibition assays using *L. casei* and beef liver DHFR,[35] recombinant human DHFR,[36] and cultured CCRF-CEM lymphoblasts[37] were also done as reported earlier. The concentration of dihydrofolate substrate in the DHFR inhibition assays was 92 μM with the *P. carinii*, *T. gondii*, and rat enzymes, 66 μM with the beef enzyme, and 50 μM with the human enzyme.

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