Selective *Pneumocystis carinii* Dihydrofolate Reductase Inhibitors: Design, Synthesis, and Biological Evaluation of New 2,4-Diamino-5-substituted-furo[2,3-*d*]pyrimidines[1]

Aleem Gangjee,*,† Xin Guo,† Sherry F. Queener,‡ Vivian Cody,§ Nikolai Galitsky,§ Joe R. Luft,§ and Walter Pangborn§

Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282, Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202, and Hauptman-Woodward Medical Research Institute, Inc., 73 High Street, Buffalo, New York 14203

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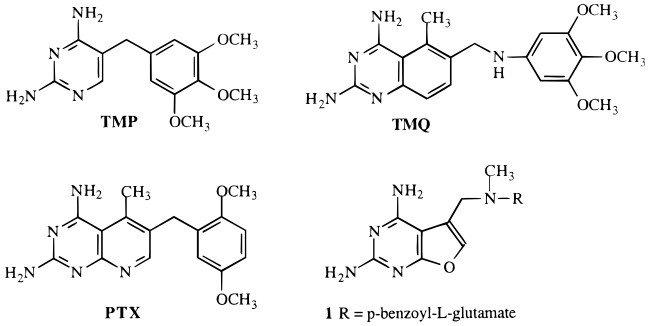
Nonclassical antifolates, 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines **3**−**12** with bridge region variations of C8−S9, C8−N9, and C8−O9 and 1-naphthyl, 2-naphthyl, 2-phenoxyphenyl, 4-phenoxyphenyl, and 2-biphenyl side chains were synthesized as phenyl ring appended analogues of previously reported 2,4-diamino-5-(anilinomethyl)furo[2,3-*d*]pyrimidines. The phenyl ring appended analogues were designed to specifically interact with Phe69 of dihydrofolate reductase (DHFR) from *Pneumocystis carinii* (pc) to afford selective inhibitors of pcDHFR. Additional substituted phenyl side chains which include 2,5-dichloro, 3,4-dichloro, 3,4,5-trichloro, 3-methoxy, and 2,5-dimethoxy analogues **13**−**17** were also synthesized. The compounds were prepared by nucleophilic displacement of 2,4-diamino-5-(chloromethyl)furo-[2,3-*d*]pyrimidine(**2**) with the appropriate thiol, amine, or naphthol. Compound **2** was obtained from 2,4-diamino-6-hydroxypyrimidine and 1,3-dichloroacetone. The compounds were evaluated as inhibitors against DHFR from *P. carinii*, *Toxoplasma gondii*, and rat liver. Two analogues, 2,4-diamino-5-[(2′-naphthylthio)methyl]furo[2,3-*d*]pyrimidine (**5**) and 2,4-diamino-5-[(2′-phenylanilino)methyl]furo[2,3-*d*]pyrimidine (**11**) showed significant selectivity and potency for pcDHFR compared to trimethoprim. The X-ray crystal structure of **5** with pcDHFR was also carried out, which corroborated the design rationale and indicated a hydrophobic interaction of the naphthalene ring of **5** and Phe69 of pcDHFR which is responsible, in part, for the more than 18-fold selectivity of **5** for pcDHFR as compared with rat liver DHFR.

Introduction

Pneumocystis carinii (pc) and *Toxoplasma gondii* (tg) are opportunistic organisms which are responsible for significant morbidity and mortality in immunocompromised patients such as those with AIDS.[2] Selectivity of inhibitors for dihydrofolate reductase (DHFR) from *P. carinii* and/or *T. gondii* over mammalian DHFR, such as rat liver (rl) DHFR along with high potency against these pathogenic DHFR, is a desirable goal. Currently used DHFR inhibitors against *P. carinii* and *T. gondii* include the weakly inhibitory monocyclic agents trimethoprim (TMP) and pyrimethamine, both of which need augmentation with sulfonamides for clinical utility, and the highly potent but nonselective bicyclic trimetrexate (TMQ), which is significantly toxic and must be used with leucovorin, a rescue agent for host cells.[3] The toxicities of these DHFR inhibitors or their combination with sulfonamides are severe enough in many cases to force discontinuation of therapy.[4]

Gangjee et al.[5,6] recently reported the inhibitory effects of a series of novel nonclassical and classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine[5] antifolates and nonclassical 2,4-diamino-5-substituted-pyrrolo[2,3-*d*]pyrimidine[6] antifolates against DHFR from *P. carinii* and *T. gondii*. It was found that the furo[2,3-*d*]pyrimidines were in general more selective for pcDHFR while pyrrolo[2,3-*d*]pyrimidines were more selective for tgDHFR. Though agents with selectivity for tgDHFR along with potency have been reported in the 6−6 and 6−5 fused bicyclic antifolates, selectivity for pcDHFR has been elusive in these series of antifolates. Several 6−6 fused analogues which include pteridines, quinazolines, and pyridopyrimidines and 6−5 fused systems which include thienopyrimidines and pyrrolopyrimidines show reasonable selectivity for tgDHFR vs rlDHFR but significantly lower or no selectivity for pcDHFR.[7] Thus the selectivity for pcDHFR vs rlDHFR of the series of 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines reported by Gangjee et al.[5] along with a complete lack of selectivity of these analogues for tgDHFR indicated a



† Duquesne University.
‡ Indiana University.
§ Hauptman-Woodward Medical Research Institute, Inc.

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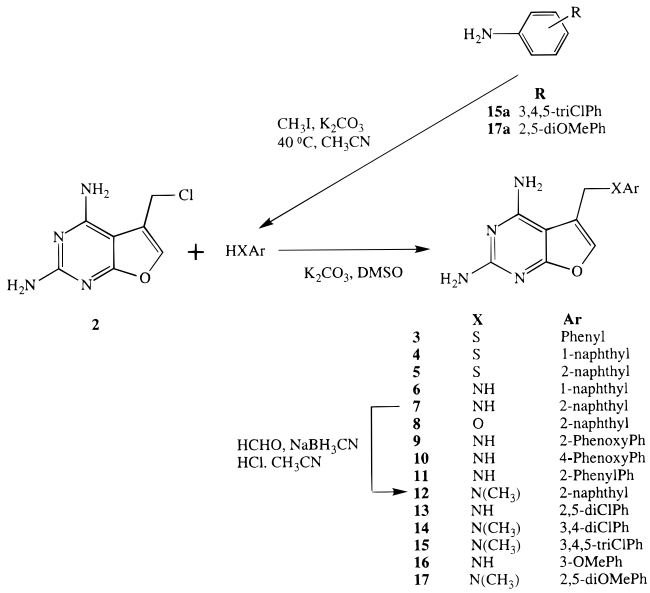
reversal in the general trend observed in the previously reported bicyclic 6−6 and 6−5 ring fused antifolates. It was possible that the furo[2,3-*d*]pyrimidine ring of the analogues reported was responsible, in part, for the pcDHFR selectivity. In the reported series of furo[2,3-*d*]pyrimidines, the most selective pcDHFR analogue was the classical N9-methyl analogue, **1**, with a selectivity ratio of 12.3 for pcDHFR. The most selective bicyclic pcDHFR inhibitor reported to date is 2,4-diamino-6-[(thiophenyl)methyl]pteridine which has a selectivity ratio of 25.9 (vs rlDHFR).[8] Since *P. carinii* lacks the active transport systems required for folates and reduced folate uptake, compound **1** would not be taken up by *P. carinii* cells, and hence was not a viable anti *P. carinii* agent. The lipophilic, nonclassical 2,4-diaminofuro[2,3-*d*]pyrimidine antifolates reported in the same study had selectivity ratios of 1.4 to 3.1. It was therefore of interest to explore further the 2,4-diaminofuro-[2,3-*d*]pyrimidine ring system with different side chain substituents to improve the selectivity for pcDHFR.

At the time the previous study had been completed, the crystal structure of pcDHFR was not available. The binding of the 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine was expected to parallel that of other 6−6 bicyclic compounds such as TMQ and the 2,4-diamino-6-substituted-pyrido[2,3-*d*]pyrimidine analogue piritrexim (PTX). The principal active site residues of pcDHFR were known to be the same as in human (h) DHFR or only conservatively different.[9] For example, the Glu30 of hDHFR, which forms the salt bridge with the N1 and the 2-amino group of the 2,4-diaminopyrimidine moiety of the antifolate, is replaced by Glu32 in pcDHFR. The Phe31 is replaced by Ile33 (pcDHFR) and the Phe34 is maintained as Phe36 in pcDHFR. Other residues which interact with the bicyclic system are all maintained or conserved in pcDHFR as evident from the crystal structures of TMP and PTX.[10] Replacement of Asn64 in hDHFR by Phe69 in pcDHFR is of particular interest because this residue lies just outside the diaminopyrimidine binding site.[9] Appropriate mutations of hDHFR to provide a pseudo pcDHFR were carried out by using SYBYL 5.5[11] and its BIOPOLYMER option. On the basis of this pseudo pcDHFR and molecular modeling with SYBYL, it was reasoned that by designing an additional phenyl ring into the side chain phenyl of the lipophilic nonclassical 2,4-diamino-5-[(substituted anilino)methyl]furo[2,3-*d*]pyrimidine antifolates reported previously[5] it may be possible to reach the Phe69 in pcDHFR in a hydrophobic "edge on" interaction of the added phenyl ring with Phe69. Such an interaction could provide selectivity for pcDHFR vs mammalian DHFR where an Asn64[9] resides to which the added phenyl ring was not expected to provide conducive binding.

Thus we designed compounds **4**−**12** (Scheme 1) which contain an additional phenyl ring either fused to or attached to the normal phenyl side chain. This took the form of a 1- or 2-naphthyl, biphenyl, or diphenyl ether substituent. Compounds **3** and **13**−**17** were also included as new examples of compounds with a single phenyl ring side chain with N9-CH_3 and ring substituents which had not been reported previously.[5]

To validate our hypothesis concerning the role of Phe69 and pcDHFR selectivity, we have also determined the crystal structure of compound **5** in a ternary complex with pcDHFR and NADPH.

Scheme 1



Chemistry

To develop a structure−activity/selectivity relationship for the 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines, it was necessary to use a versatile intermediate from which a series of nonclassical analogues **3**−**17** with variations in both the bridge and the 5-substituted side chain aromatic moiety could be obtained in a minimum of steps. The key intermediate chosen for this purpose was 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (**2**), reported by Secrist and Liu in 1978.[12] The chloromethyl moiety of **2** could be suitably transformed by nucleophilic displacements to afford the target molecules in a convergent manner similar to the previous report by Gangjee et al.[5]

Compound **2** was prepared from 2,6-diamino-4-hydroxypyrimidine and 1,3-dichloroacetone in anhydrous DMF at room temperature using an improved modification of the published method.[12] Analysis of the mixture by TLC after 24 h showed complete disappearance of the pyrimidine and the formation of a mobile product spot along with byproducts which remained at the origin. However, workup of the reaction proved difficult because **2** was unstable at room temperature and decomposed on prolonged exposure to air. It was reported[12] that filtration of the reaction mixture afforded the product as a precipitate and that chromatography of the filtrate afford some additional product (total yield 78%). In our hands, improved results were obtained by rapidly purifying compound **2** before condensing it with the appropriate nucleophiles. This was achieved by the addition of silica gel directly to the reaction mixture along with additional DMF once the reaction was over. The mixture was then sonicated and rapidly evaporated in vacuo at 50−55 °C. The resultant plug was kept in the dark and dried over P_2O_5 in vacuo overnight at room temperature. The plug was then ground into a fine powder and poured on top of a short, broad column of silica gel (15 w/w) which was eluted with methanol/chloroform, 1:7. The pure, dried compound **2** was immediately sealed and stored at low temperature (<−20 °C).

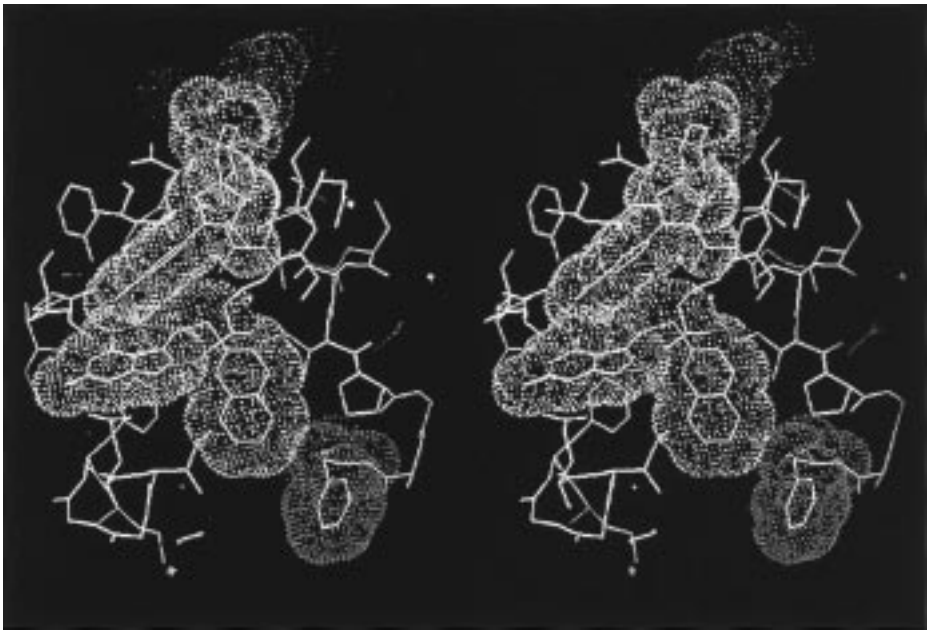


Figure 1. Stereoview of active site of pcDHFR complex with NADPH and compound **5** highlighting the intermolecular contacts of the 2-naphthyl group (yellow) with Phe-69 (green). The van der Waals surface is shown for NADPH, **5**, and Phe-69.

Nucleophilic displacement reactions of the chloride **2** with appropriate anilines, phenol, or thiophenols were accomplished in DMSO in the presence of potassium carbonate. The product was precipitated by quenching the reaction mixture with water and was purified by column chromatography. Analogues **3**, **5**, and **13**−**16** were synthesized from crude **2**, and the yields were very low (3−16% for two steps). However, when purified **2** was used, the reactions were much cleaner and the yields improved significantly (16−48%).

The N9-methyl analogues of this series of furo[2,3-*d*]-pyrimidines were prepared by the following two methods. For compounds **15** and **17**, the appropriate commercially available primary anilines were transformed into their N-methylated analogues, by alkylation with methyl iodide at 40 °C in CH_3CN in the presence of potassium carbonate. Reaction of the N-methylated anilines with **2** afforded **15** and **17.** In the second approach, reductive methylation using formaldehyde and sodium borohydride in an acidic medium was used to synthesize **12** directly from **7**. This method is well documented for the synthesis of N10-methyl bicyclic antifolates.[13] Since the furo[2,3-*d*]pyrimidines are not very stable under acidic conditions, previous attempts using acetic acid as both the acidic catalyst and the solvent resulted in slow reaction and yielded multiple products. However, when 2,4-diamino-5-(2-naphthylaminomethyl)furo[2,3-*d*]pyrimidine **7**, formaldehyde, and sodium cyanoborohydride were suspended in acetonitrile and concentrated HCl was added dropwise until a clear solution formed, the reaction was fast and completed within 1 h, which precluded significant degradation or dimethylation. The reaction afforded the desired monomethylated analogue **12** in 64% yield.

X-ray Crystal Structure

The overall characteristics of the pcDHFR ternary complex with NADPH and **5** are similar to those reported for the TMP complex[10] and for the classical furo[2,3-*d*]pyrimidine analogue **1**.[14] The active site region is homologous to those of the mammalian and bacterial enzymes, reemphasizing the conserved nature of the active site despite changes in enzyme size.

The binding orientation of **5** is similar to that observed for the furo[2,3-*d*]pyrimidine classical analogue, **1**.[14] The carboxylate oxygens of Glu32 protonate the furopyrimidine ring forming hydrogen bonds to N1 by OE2 and to the N2-amino nitrogen by OE1. In addition, OE1 and OE2 form hydrogen bonds to a conserved threonine and a structural water. The 2-amino group also hydrogen bonds to a structurally conserved water. In contrast, no structural water was observed near O8, as is seen near N8 of the pteridine antifolates.[15,16]

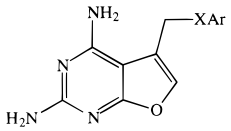
Because the geometry of the furo[2,3-*d*]pyrimidine differs significantly from that of pteridine, the 5-substituent of the furo[2,3-*d*]pyrimidine is oriented differently in the active site.[15,16] This change places the C8−N9 bridge of **5** in a different orientation from that observed for the C9−N10 bridge of MTX.

Analysis of the intermolecular interactions involving the 2-naphthyl group shows that it makes hydrophobic contacts (<4.0 Å) to Phe69 (Figure 1) and Pro66. Similar hydrophobic interactions with Phe69 and the glutamate methylene carbons of the classical furopyrimidine **1** have been observed in its ternary complex with pcDHFR and NADPH.[14]

Biological Results and Discussion

Compounds **3**−**17** were evaluated as inhibitors of DHFRs from *P. carinii*, *T. gondii*, and rat liver which served as the mammalian reference.[8,17] The inhibitory concentration (IC_{50}) values and the selectivity ratios (IC_{50} rlDHFR/IC_{50} pcDHFR or tgDHFR) for compounds **3**−**17** are listed in Table 1 along with epiroprim, TMP, and TMQ as reference standards. The low inhibitory activity with marginal pcDHFR selectivity previously observed[5] with the di- and tri-OCH_3Ph and di- and tri-ClPh nonclassical furo[2,3-*d*]pyrimidine analogues was again evident in most instances in the present study. Significantly, however, the 2-naphthylthio analogue **5**

Table 1. Inhibitory Concentrations (IC_{50}, μM) and Selectivity Ratios of 5-Substituted Furo[2,3-*d*]pyrimidines against pcDHFR, tgDHFR, and rlDHFR



			DHFR				
no.	X	Ar	pc	rl	rl/pc	tg	rl/tg
3	S	phenyl	>26	252	ND	>26	ND
4	S	1-naphthyl	19	23	1.2	19	1.2
5	S	2-naphthyl	0.65	12.3	18.9	11.6	1.1
6	NH	1-naphthyl	13.5	12	0.89	37	0.32
7	NH	2-naphthyl	41	36.5	0.89	38	0.96
8	O	2-naphthyl	14	60.3	4.31	>42	ND
9	NH	2-phenoxyPh	>12	>12	ND	>12	ND
10	NH	4-phenoxyPh	8.1	16.2	2.00	32.4	0.50
11	NH	2-phenylPh	7.7	137	17.79	45.4	3.02
12	$N(CH_3)$	2-naphthyl	14.8	14.6	0.99	23.6	0.62
13	NH	2,5-diClPh	50.9	71.9	1.4	>47	ND
14	$N(CH_3)$	3,4-diClPh	44.8	>27	ND	>27	ND
15	$N(CH_3)$	3,4,5-triClPh	284	34.3	0.1	21.5	1.6
16	NH	3-OMePh	>31.3	>31.3	ND	>31.3	ND
17	$N(CH_3)$	2,5-diOMePh	>27	>27	ND	>27	ND
	TMQ		0.042	0.003	0.07	0.010	0.3
	TMP		12	133	11.1	2.7	49
	Epiroprim		2.6	33.2	12.8	0.48	70.6

and the biphenyl-2-ylamino analogue **11** gave selectivity ratios of 18.9 and 17.8, respectively, against pcDHFR. These selectivity ratios rank compounds **5** and **11** as the second and third most selective bicyclic pcDHFR inhibitors (vs rlDHFR) reported to date.[8,18] Compound **5**, in addition to its considerable selectivity, was also reasonably potent against pcDHFR with an IC_{50} of 0.65 μM.

The most selective bicyclic pcDHFR inhibitor (vs rlDHFR) 2,4-diamino-6-[(thiophenyl)methyl]pteridine has a pcDHFR selectivity ratio of 25.9 with an IC_{50} of 9.5 μM.[8] Compound **5** is 15-fold more potent and is only slightly less selective with a selectivity ratio of 18.9. Compared to epiroprim and TMP, compound **5** is significantly more potent and more selective against pcDHFR in our assay, which is performed in the presence of saturating concentrations of the substrate and cofactor with a 37 °C incubating temperature.[8]

Comparison of the thio bridged analogues with the corresponding NH-bridged analogues (i.e., **4** and **5** with **6** and **7**) in the 1- and 2-naphthyl series indicates that for selectivity and potency against pcDHFR the *S*-2-naphthyl moiety was essential. All three of the other analogues, **4**, **6**, and **7**, not only lacked appreciable selectivity but also were poorly inhibitory. Compound **8** in which the heteroatom in the bridge was isosterically substituted by an oxygen has similar potency as the NH analogue with slightly improved selectivity for pcDHFR. These results indicate that both potency and selectivity are determined by the heterocycle as well as the side chain substituent including the bridge. On the basis of the significant potency and selectivity of **5**, three analogues, **9**−**11**, with an appended phenyl ring in the side chain were synthesized in the NH-bridged series. Compound **11**, with 17.8-fold selectivity and an IC_{50} of 7.7 μM, was comparable to epiroprim and TMP in its potency but had better selectivity against pcDHFR. The other analogues were neither potent nor selective for pcDHFR. The results in the CH_2NH-bridged series again underscore the previous conclusion that all three features, i.e., the heterocyclic ring, the bridge, and the side chain aryl moiety, combine to provide pcDHFR selectivity in this series.

Analogues **3** and **13**−**17**, with a single phenyl ring in the side chain, generally showed low potency and poor selectivity for pcDHFR compared to the most potent and selective analogue **5**. Methylation of the bridge nitrogen in bicyclic 6−6 and 6−5 ring systems usually leads to an increase in DHFR potency.[7,11] In the furo[2,3-*d*]-pyrimidine series, the N9-CH_3 analogues **12**, **14**, and **17** had IC_{50} values similar to those of the corresponding N9-H analogues, and in the case of compound **15**, N9-methylation actually produced a 34-fold decrease in potency against pcDHFR.[5]

In contrast to the selectivity obtained for pcDHFR, these analogues were devoid of any selectivity for tgDHFR with the exception of **11** and **15,** which were marginally selective. The potency of these analogues against tgDHFR was also low, with IC_{50} values greater than 11 μM.

The idea that an added phenyl ring to the side chain phenyl of the previously reported 2,4-diaminofuro[2,3-*d*]pyrimidines would result in increased selectivity for pcDHFR was indeed realized in compounds **5** and **11**. It therefore became of interest to determine if this increased selectivity could be attributed to a favorable "edge on" interaction of the appended phenyl ring with the phenyl ring of Phe69 of pcDHFR as was originally proposed in our design of compounds **5** and **11**.

As illustrated in Figure 1, the X-ray crystal structure of **5** as a ternary complex with pcDHFR and NADPH verifies the close hydrophobic interactions of the second fused phenyl ring of the 2-naphthyl substituent of **5** with the Phe69 of pcDHFR. This interaction may be responsible, in part, for the selectivity of compound **5** for pcDHFR since such an interaction is not possible for the human enzyme as there is a sequence change to Asn64 at this position in human DHFR.

Compound **5**, on the basis of its potency and selectivity for pcDHFR, was evaluated in inhibitory studies of the growth of *P. carinii* cells in culture. Initial studies indicate that the IC_{50} against the growth of *P. carinii* cells in culture is between 1 and 10 μM and the compound is not cytotoxic at 10 μM. Compound **5** is currently undergoing animal studies in the *P. carinii* pneumonia mouse model.

In summary, compounds **4**−**11** were designed as "extended" phenyl ring analogues of previously reported pcDHFR selective 2,4-diaminofuro[2,3-*d*]pyrimidines, with the hypothesis that this extended phenyl ring would favorably interact with Phe69 on pcDHFR which is different from Asn64 in mammalian DHFR and perhaps provide selectivity for pcDHFR. Of the synthesized analogues, compounds **5** and **11** showed significant selectivity for pcDHFR, and the X-ray crystal structure of compound **5** with pcDHFR indicates that the B ring of the naphthyl moiety of **5** does indeed interact in an "edge on" hydrophobic binding with Phe69 of pcDHFR which translates into selectivity. Compound **5** is the second most selective analogue in the bicyclic series against pcDHFR with an inhibitory potency and selectivity better than both epiroprim and TMP for pcDHFR. Compound **5** is currently undergoing studies in animal models of *P. carinii* pneumonia, the results of which will be reported in a future communication.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus or a Mel-Temp II and are uncorrected. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz). The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard: s = singlet, d = doublet, t = triplet, m = multiplet, and dd = doublet of doublet. Thin-layer chromatography (TLC) was performed on silica gel plates with fluorescent indicator that was visualized with light at 254 or 366 nm. Proportions of solvents used for TLC and column chromatography are by volume. Column chromatography was performed on 230−400 mesh silica gel purchased from Aldrich, Milwaukee, WI. Solvents routinely used for reactions and purification were purchased from Aldrich, Milwaukee, WI, or Fisher Scientific, Pittsburgh, PA and were used as obtained. Samples for microanalysis were dried in vacuo over phosphorus pentoxide at room temperature. 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 were not prevented despite drying in vacuo and were confirmed by their presence in the ^{1}H NMR spectrum.

2,4-Diamino-5-[(phenylthio)methyl]furo[2,3-*d*]pyrimidine (3). To a 100 mL three-neck round-bottom flask were added sequentially anhydrous DMSO (15 mL), thiophenol (0.85 g, 7.50 mmol), crude **2** (0.75 g, 3.75 mmol), and anhydrous potassium carbonate (1.03 g, 7.50 mmol). The reaction mixture was stirred at room temperature for 2 h and then quenched with water (150 mL). The resultant suspension was stirred at room temperature for 10 h and filtered. The solid was extracted twice with MeOH (150 mL + 100 mL), and the extracts were mixed with silica gel (2 g). The mixture was evaporated under reduced pressure and dried in vacuo. The residue was powdered and poured on top of a dry column of silica gel (40 g). The column was washed with MeOH in $CHCl_3$: 200 mL (0.5%), 100 mL (1%), 100 mL (2%). The column was then eluted with 3% MeOH in $CHCl_3$. Fractions corresponding to a single spot (TLC) of the product were pooled and evaporated. The residue was washed with ether and air-dried to afford 0.17 g (16% for two steps) of **3** as a light pinkish solid: mp 227 °C (dec); TLC R_f 0.48 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.27 (s, 2 H, 5-CH_2), 6.03 (s, 2 H, 4-NH_2), 6.56 (s, 2 H, 2-NH_2), 7.14 (s, 1 H, 6-CH), 7.18 (m, 1 H, 4′-CH), 7.23−7.40 (m, 4 H, 2′,3′,5′,6′-CH). Anal. ($C_{13}H_{12}N_4OS\cdot0.10CH_3OH\cdot0.22H_2O$) C, H, N, S.

2,4-Diamino-5-[(1-naphthylthio)methyl]furo[2,3-*d*]pyrimidine (4). To a 50 mL three-neck round-bottom flask were added sequentially anhydrous DMSO (8 mL), 1-naphthalenethiol (0.89 g, 5.55 mmol), purified **2** (0.63 g, 4.63 mmol), and anhydrous potassium carbonate (0.92 g, 6.67 mmol). The reaction mixture was stirred at room temperature for 2 h and then quenched with distilled water (16 mL). The resultant suspension was stirred at room temperature for 4 h, cooled to 5 °C, and filtered. The solid was washed with cold water (2 × 5 mL) and then stirred in ether (20 mL) for 8 h. The suspension obtained was filtered and the residue dissolved in acetic acid (40 mL). The solution was mixed with charcoal (0.1 g) and stirred at 40 °C for 15 min. The mixture was filtered and the filtrate evaporated to dryness. The residue was washed thoroughly with MeOH to yield 0.44 g (29%) of **4** as a light yellow solid which was homogeneous on TLC: mp 215−218 °C (dec); TLC R_f 0.53 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.36 (s, 2H, 8-CH_2), 6.04 (s, 2H, 4-NH_2), 6.63 (s, 2H, 2-NH_2), 7.02 (s, 1H, 6-CH), 7.50−8.30 (m, 7H, $C_{10}H_7$). Anal. ($C_{17}H_{14}N_4OS\cdot CH_3$-COOH) C, H, N, S.

2,4-Diamino-5-[(2-naphthylthio)methyl]furo[2,3-*d*]pyrimidine (5). To a 50 mL three-neck round-bottom flask were added sequentially anhydrous DMSO (5 mL), 2-naphthalenethiol (1.12 g, 7.0 mmol), crude **2** (1.39 g, 6.98 mmol), and anhydrous potassium carbonate (0.97 g, 7.0 mmol). The reaction mixture was stirred at room temperature for 2 h and then quenched with distilled water (50 mL). The resultant suspension was stirred at room temperature for 8 h, cooled to 5 °C, and filtered. The solid was washed with cold water (2 × 5 mL) and then extracted several times with MeOH (100 mL). Extracts with a major spot of the product on TLC were pooled and mixed with silica gel (2 g). The mixture was evaporated under reduced pressure and then dried in vacuo. The residue was ground into a fine powder and poured on top of a dry column of silica gel (40 g). The column was washed with MeOH in $CHCl_3$: 200 mL (0.5%), 100 mL (1%), 100 mL (2%). The column was then eluted with 3% MeOH in $CHCl_3$. Fractions corresponding to a single spot of the product (TLC) were pooled and evaporated to afford 0.11 g of the pure product. Fractions with a major spot of the product (TLC) and a faint spot just above the product spot were also pooled and evaporated. The residue was washed thoroughly with ether to afford an additional 0.05 g of the pure product. Fractions with a major spot of the product and a faint spot just below the product spot were also pooled and evaporated. The residue was washed thoroughly with MeOH to afford an additional 0.06 g of the pure product (combined yield 10% for two steps) **5** as a light pinkish solid: mp 233−235 °C (dec); TLC, R_f 0.49 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.41 (s, 2H, 8-CH_2), 6.05 (s, 2H, 4-NH_2), 6.62 (s, 2H, 2-NH_2), 7.21 (s, 1H, 6-CH), 7.40−7.55 (m, 3H, $C_{10}H_7$), 7.70−7.90 (m, 4H, $C_{10}H_7$). Anal. ($C_{17}H_{14}N_4OS\cdot0.25H_2O$) C, H, N, S.

2,4-Diamino-5-[(1-naphthylamino)methyl]furo[2,3-*d*]-pyrimidine (6). To a 50 mL three-neck round-bottom flask were added sequentially anhydrous DMSO (5 mL), 1-aminonaphthalene (0.66 g, 4.64 mmol), purified **2** (0.50 g, 2.51 mmol), and anhydrous potassium carbonate (0.69 g, 5.00 mmol). The reaction mixture was stirred at 50 °C for 8 h under nitrogen in the dark. The reaction was then quenched with distilled water (15 mL), and the resultant suspension was stirred at room temperature for 4 h, cooled to 5 °C, and filtered. The residue was washed with cold water (2 × 5 mL) and then dissolved in hot MeOH, and the solution was mixed with silica gel (2 g). The mixture was evaporated under reduced pressure and then dried in vacuo. The residue was ground into a fine powder and poured on top of a dry column of silica gel (40 g). The column was washed with MeOH in $CHCl_3$: 200 mL (0.5%),

100 mL (1%), 100 mL (2%). The column was then eluted with 3% MeOH in $CHCl_3$. Fractions corresponding to a single spot of the product (TLC) were pooled and evaporated. The residue was washed with ether and air-dried to afford 0.25 g (33%) of **6** as a light yellow solid: mp 230 °C (dec); TLC R_f 0.48 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.41 (d, 2H, 8-CH_2), 6.00 (s, 2H, 4-NH_2), 6.60−6.74 (m, 3H, 2-NH_2 and 9-NH), 6.75 (d, 1H, 4′-CH, J = 7.4 Hz), 7.20 (d, 1H, 2′-CH), 7.28 (m, 1H, 3′-CH), 7.40−7.46 (m, 3H, 6, 6′,7′-CH), 7.78 (d, 1H, 5′-CH) 8.17 (d, 1H, 8′-CH, J = 9.2 Hz). Anal. ($C_{17}H_{15}N_5O\cdot 0.20H_2O$) C, H, N.

2,4-Diamino-5-[(2-naphthylamino)methyl]furo[2,3-*d*]pyrimidine (7). Compound **7** was prepared from anhydrous DMSO (4 mL), 2-aminonaphthalene (0.54 g, 3.76 mmol), purified **2** (0.61 g, 3.09 mmol), and anhydrous potassium carbonate (0.55 g, 4.00 mmol) as described for compound **6** to afford 0.27 g (29%) of **7** as a white solid: mp 260−265 °C (dec); TLC R_f 0.52 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.32 (d, 2H, 8-CH_2, J = 4.9 Hz), 6.04 (s, 2H, 4-NH_2), 6.38 (t, 1H, 9-NH), 6.55 (s, 2H, 2-NH_2), 6.98 (d, 1H, 1′-CH, J = 1.5 Hz), 7.08 (dd, 1H, 3′-CH, J = 8.8, 2.1 Hz), 7.16 (m, 1H, 6′- or 7′-CH), 7.33 (m, 1H, 6′- or 7′-CH), 7.43 (s, 1H, 6-CH), 7.55−7.70 (m, 3H, 4′,5′,8′-CH). Anal. ($C_{17}H_{15}N_5O\cdot 0.33H_2O$) C, H, N.

2,4-Diamino-5-[(2-naphthoxy)methyl]furo[2,3-*d*]pyrimidine (8). To a 50 mL three-neck round-bottom flask were added sequentially anhydrous DMSO (4 mL), 2-naphthol (0.27 g, 1.89 mmol), purified **2** (0.25 g, 1.26 mmol), and anhydrous potassium carbonate (0.26 g, 1.89 mmol). The reaction mixture was stirred at 45 °C for 24 h under nitrogen in the dark and then quenched with distilled water (12 mL). The resultant suspension was stirred at room temperature for 4 h, cooled to 5 °C, and filtered. The solid was washed with cold water (2 × 5 mL) and then dissolved in MeOH. The solution was mixed with silica gel (1 g), and the mixture was evaporated under reduced pressure and dried in vacuo. The residue was ground into a fine powder and poured on top of a wet column of silica gel (30 g) and eluted with 3% MeOH in $CHCl_3$. Fractions corresponding to a single spot of the product (TLC) were pooled and evaporated to afford 0.09 g of **8** (23%) as a white solid: mp 245−247 °C; TLC R_f 0.62 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 5.30 (s, 2H, 8-CH_2), 6.11 (s, 2H, 4-NH_2), 6.47 (s, 2H, 2-NH_2), 7.23 (dd, 1H, 3′-CH), 7.36 (m, 1H, 6′- or 7′-CH), 7.48 (t, 1H, 6′- or 7′-CH), 7.51 (s, 1H, 6- or 1′-CH), 7.55 (s, 1H, 6- or 1′-CH), 7.70−7.90 (m, 3H, 4′,5′,8′-CH). Anal. ($C_{17}H_{14}N_4O_2\cdot 0.20H_2O$) C, H, N.

2,4-Diamino-5-[(2′-phenoxyanilino)methyl]furo[2,3-*d*]pyrimidine (9). Compound **9** was prepared from anhydrous DMSO (4 mL), 2-phenoxyaniline (0.35 g, 1.89 mmol), purified **2** (0.25 g, 1.26 mmol), and anhydrous potassium carbonate (0.21 g, 1.51 mmol) as described for compound **8**, and fractions corresponding to a single spot (TLC) of the product were pooled and evaporated. The residue was washed with ether and air-dried to afford 0.16 g (37%) of **9** as a white solid: mp 245−250 °C (dec); TLC R_f 0.58 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.30 (d, 2H, 8-CH_2, J = 5.3 Hz), 5.79 (t, 1H, 9-NH, J = 5.3 Hz), 5.98 (s, 2H, 4-NH_2), 6.52 (s, 2H, 2-NH_2), 6.61 (m, 1H, 4′-CH), 6.75 (dd, 1H, 6′-CH, J = 7.9, 1.2 Hz), 6.85−7.00 (m, 4H, 3′,5′,2″,6″-CH), 7.08 (t, 1H, 4″-CH, J = 7.2 Hz), 7.31 (s, 1H, 6-CH), 7.34 (m, 2H, 3″,5″-CH). Anal. ($C_{19}H_{17}N_5O_2\cdot 0.25H_2O$) C, H, N.

2,4-Diamino-5-[(4′-phenoxyanilino)methyl]furo[2,3-*d*]pyrimidine (10). Using a method similar to that of **9**, 0.06 g of chromatographically pure **10** was obtained as a white solid. Fractions corresponding to a major spot of the product (TLC) and a spot of some unknown impurity just below were pooled and evaporated. The residue was washed with MeOH to afford an additional 0.12 g of the product which was homogeneous on TLC (total yield 41%): mp 245−250 °C (dec); TLC R_f 0.50 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.18 (d, 2H, 8-CH_2, J = 5.5 Hz), 6.04 (s, 2H, 4-NH_2), 6.06 (t, 1H, 9-NH, J = 5.5 Hz), 6.64 (s, 2H, 2-NH_2), 6.80 (d, 2H, 2′,6′-CH, J = 8.9 Hz), 6.85−6.95 (m, 4H, 3′,5′,2″,6″-CH), 7.01 (t, 1H, 4″-CH, J = 7.3 Hz), 7.31 (m, 2H, 3″,5″-CH), 7.38 (s, 1H, 6-CH). Anal. ($C_{19}H_{17}N_5O_2\cdot 0.20H_2O$) C, H, N.

2,4-Diamino-5-[(2′-phenylanilino)methyl]furo[2,3-*d*]pyrimidine (11). To a 50 mL three-neck round-bottom flask containing purified **2** (0.30 g, 1.51 mmol), 2-aminobiphenyl (0.38 g, 2.26 mmol), and K_2CO_3 (0.25 g, 1.812 mmol) was added anhydrous DMSO (3 mL), and the reaction mixture was stirred for 8 h at 45 °C under nitrogen in the dark. Distilled water (10 mL) was added to precipitate the product, and the suspension was stirred for 2 h, cooled to 5 °C, and filtered. The solid was washed with cold water (2 × 2 mL) and suspended in MeOH (200 mL). Silica gel (1 g) was added, and the suspension was sonicated and heated to 50 °C for 15 min, evaporated under reduced pressure, and dried in vacuo for 10 h. The residue was powdered and poured on top of a wet column of silica gel (30 g) and eluted with 3% of MeOH in $CHCl_3$. Fractions corresponding to the product spot (TLC) were pooled and evaporated. The residue was washed with ether and air-dried to afford 0.24 g (48%) of **11** as a light yellow solid. Recrystallization from MeOH afforded the analytically pure compound **11**: mp 250−260 °C (dec); TLC R_f 0.55 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.25 (d, 2 H, 8-CH_2), 5.10 (t, 1H, 9-NH), 5.59 (s, 2H, 4-NH_2), 6.61 (s, 2H, 2-NH_2), 6.72 (m, 1H, 4′-CH), 6.87 (d, 1H, 6′-CH), 6.98 (d, 1H, 3′-CH), 7.15 (m, 1H, 5′-CH), 7.30−7.50 (m, 6H, 2′-phenyl and 6-CH). Anal. ($C_{19}H_{17}N_5O$) C, H, N.

2,4-Diamino-5-[(*N*-methyl-2-naphthylamino)methyl]furo[2,3-*d*]pyrimidine (12). To a suspension of **7** (0.10 g, 0.32 mmol), 30% (w/w) aqueous solution of formaldehyde (0.1 mL), and $NaCNBH_3$ (0.063 g, 1 mmol) in acetonitrile (10 mL) was added dropwise concentrated hydrochloric acid until the suspension dissolved. The solution was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and the residue dissolved in a minimum amount of distilled water and the pH of the solution adjusted to 7 with concentrated NH_4OH. The resulting suspension was sonicated, cooled to 5 °C, and filtered. The residue was washed with cold water (2 × 3 mL), stirred in MeOH (5 mL) for 12 h, and filtered. The residue was washed with MeOH (2 × 3 mL) to afford 0.065 g (64%) of **12** as a light yellow solid: mp 234−240 °C (dec); TLC R_f 0.50 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 2.95 (s, 3H, N−CH_3), 4.54 (s, 2H, 8-CH_2), 6.10 (s, 2H, 2-NH_2), 6.60 (s, 2H, 4-NH_2), 7.39 (s, 1H, 6-CH), 7.20−7.80 (m, 7H, $C_{10}H_7$). Anal. ($C_{18}H_{17}N_5O\cdot 0.20H_2O$) C, H, N.

2,4-Diamino-5-[(2′,5′-dichloroanilino)methyl]furo[2,3-*d*]pyrimidine (13). To a 100 mL three-neck round-bottom flask was added in sequence anhydrous DMSO (30 mL), 2,5-dichloroaniline (2.43 g, 15 mmol), crude **2** (1.49 g, 7.5 mmol), and anhydrous potassium carbonate (2.07 g, 15 mmol). The reaction mixture was stirred at 45 °C for 8 h and then quenched with distilled water (150 mL). The resultant suspension was stirred at room temperature for 8 h and filtered. The solid was extracted with MeOH (100 mL) several times, the extracts which gave a major spot of the product on TLC were pooled and mixed with silica gel (2 g), and the mixture was evaporated under reduced pressure and then dried in vacuo. The residue was ground into a fine powder and poured on top of a dry column of silica gel (40 g). The column was washed with MeOH in $CHCl_3$: 200 mL (0.5%), 100 mL (1%), 100 mL (2%). The column was then eluted with 3% MeOH in $CHCl_3$. Fractions corresponding to a single spot of the product (TLC) were pooled and evaporated. The residue obtained was washed with ether and air-dried to afford 0.067 g (3% for two steps) of **13** as a light yellow solid: mp 258 °C (dec); TLC R_f 0.49 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.39 (d, 2H, 8-CH_2, J = 5.2 Hz), 6.02 (s, 2H, 4-NH_2), 6.27 (t, 1H, 9-NH), 6.62 (s, 2H, 2-NH_2), 6.64 (dd, 1H, 4′-CH, J = 8.4 Hz, 2.0 Hz), 6.84 (d, 1H, 6′-CH, J = 2.0 Hz), 7.27 (d, 1H, 3′-CH, J = 8.4 Hz), 7.39 (s, 1H, 6-CH). Anal. ($C_{13}H_{11}N_5OCl_2\cdot 0.25H_2O$) C, H, N, Cl.

2,4-Diamino-5-[(*N*-methyl-3′,4′-dichloroanilino)methyl]furo[2,3-*d*]pyrimidine (14). Compound **14** was prepared

from anhydrous DMSO (30 mL), *N*-methyl-3,4-dichloroaniline (2.64 g, 15.00 mmol), crude **2** (1.49 g, 7.50 mmol), and anhydrous potassium carbonate (2.07 g,15.00 mmol), as described for compound **13** to afford 0.08 g (3% for two steps) of **14** as a white solid: mp 185−190 °C (dec); TLC R_f 0.49 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 2.93 (s, 3H, 9-NCH_3), 4.56 (s, 2H, 8-CH_2), 6.07 (s, 2H, 4-NH_2), 6.42 (s, 2H, 2-NH_2), 6.82 (dd, 1H, 6′-CH, J = 9.0 Hz, 2.9 Hz), 7.02 (d, 1H, 2′-CH, J = 2.9 Hz), 7.06 (s, 1H, 6-CH), 7.38 (d, 1H, 5′-CH, J = 9.0 Hz). Anal. ($C_{14}H_{13}N_5OCl_2$) C, H, N, Cl.

2,4-Diamino-5-[(*N*-methyl-3′,4′,5′-trichloroanilino) methyl]furo[2,3-*d*]-pyrimidine (15). To a 100 mL three-neck round-bottom flask was added 3,4,5-trichloroaniline (1.96 g, 10.00 mmol), potassium carbonate (1.66 g, 12.00 mmol), acetonitrile (25 mL), and iodomethane (1.42 g, 10.00 mmol). The reaction mixture was stirred at 40 °C for 3 days under nitrogen in the dark. The reaction mixture was then quenched with ethyl acetate (50 mL) and filtered. The residue was washed with ethyl acetate (3 × 20 mL). The filtrate and the washings were combined and washed with water (3 × 30 mL). The organic layer was dried (anhydrous $MgSO_4$) and evaporated under reduced pressure. The residue was dissolved in a minimum amount of ethyl acetate/hexane, 1:12, and the solution was placed on a wet column of silica gel (80 g) and eluted with ethyl acetate/hexane (300 mL (1:12), 500 mL (1:8)) to afford 0.88 g of *N*-methyl-3,4,5-trichloroaniline **15a** (42%) as a white solid. The product was used directly for the condensation without characterization. To a 100 mL three-neck round-bottom flask was added anhydrous DMSO (5 mL), *N*-methyl-3,4,5-trichloroaniline (0.88 g, 4.20 mmol), crude **2** (0.20 g, 1.00 mmol), and anhydrous potassium carbonate (0.56 g, 4.09 mmol). The reaction mixture was stirred at 45 °C for 24 h and then quenched with distilled water (15 mL). The resulting suspension was stirred at room temperature for 4 h and filtered. The residue was extracted with MeOH (100 mL) several times, and the extracts which showed a major spot of the product on TLC were pooled and mixed with silica gel (2 g). The mixture was evaporated under reduced pressure and dried in vacuo. The residue was ground into a fine powder and poured on top of a dry column of silica gel (40 g). The column was eluted with MeOH in $CHCl_3$: 200 mL (0.5%), 100 mL (1%), 100 mL (2%). The column was then eluted with 3% MeOH in $CHCl_3$. Fractions corresponding to a single spot of the product were pooled and evaporated. The residue was washed with ether and air-dried to afford 0.06 g (16% for two steps) of **15** as a light yellow solid: mp 280 °C (dec); TLC R_f 0.55 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 2.97 (s, 3H, 9-NCH_3), 4.64 (s, 2H, 8-CH_2), 6.07 (s, 2H, 4-NH_2), 6.39 (s, 2H, 2-NH_2), 7.00 (s, 2H, 2′, 6′-CH), 7.04 (s, 1H, 6-CH). Anal. ($C_{14}H_{12}N_5OCl_3 \cdot 0.50H_2O$) C, H, N, Cl.

2,4-Diamino-5-[(3′-methoxyanilino)methyl]furo[2,3-*d*]-pyrimidine (16). Compound **16** was prepared from anhydrous DMSO (15 mL), 3-methoxyaniline (0.93 g, 7.50 mmol), crude **2** (0.75 g, 3.75 mmol), and anhydrous potassium carbonate (1.04 g, 7.50 mmol) as described for compound **13** to afford 0.12 g (11% for the two steps) of **16** as a white solid: mp 225 °C (dec); TLC R_f 0.46 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.18 (d, 2H, 8-CH_2, J = 4.8 Hz), 6.04 (s, 2H, 4-NH_2), 6.09 (t, 1H, 9-NH, J = 4.8 Hz), 6.22 (d, 1H, 6′-CH), 6.31 (s, 1H, 2′-CH), 6.34 (d, 1H, 4′-CH), 6.56 (s, 2H, 2-NH_2), 7.00 (m, 1H, 5′-CH), 7.36 (s, 1H, 6-CH). Anal. ($C_{14}H_{15}N_5O_2$) C, H, N.

2,4-Diamino-5-[(*N*-methyl-2′,5′-dimethoxyanilino) methyl]furo[2,3-*d*]pyrimidine (17). To a 100 mL three-neck round-bottom flask were added 2,5-dimethoxyaniline (1.53 g, 10.00 mmol), anhydrous potassium carbonate (1.66 g, 12.00 mmol), acetonitrile (25 mL), and iodomethane (1.42 g, 10.00 mmol). The reaction mixture was stirred at 40 °C for 3 days under nitrogen in the dark. The reaction mixture was quenched with ethyl acetate (50 mL) and filtered. The residue was washed with ethyl acetate (3 × 20 mL). The filtrate and the washings were combined and washed with water (3 × 30 mL). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The residue was dissolved in a minimum amount of ethyl acetate/hexane, 1:8. The solution was placed on a wet column of silica gel (80 g) and eluted with ethyl acetate/hexane (300 mL (1:8), 500 mL (1:5)) to afford 0.51 g of *N*-methyl-2,5-dimethoxyaniline **17a** (31%): TLC R_f 0.44 (hexanes/EtOAc, 3:1, silica gel); ^{1}H NMR ($CDCl_3$) δ 2.67 (d, 3H, N−CH_3, J = 5.2 Hz), 3.65 (s, 3H, 2-OCH_3), 3.69 (s, 3H, 5-OCH_3), 5.04 (q, 1H, NH, J = 5.2 Hz), 6.02 (s, 1H, 6-CH), 6.03 (m, 1H, 4-CH), 6.65 (d, 1H, 3-CH). To a 50 mL three-neck round-bottom flask with **2** (0.32 g, 1.61 mmol), *N*-methyl-2,5-dimethoxyaniline **17a** (0.40 g, 2.42 mmol), and anhydrous potassium carbonate (0.27 g, 1.93 mmol) was added anhydrous DMSO (3 mL), and the reaction mixture was stirred at 45 °C for 8 h under nitrogen in the dark. Distilled water (10 mL) was added to precipitate the product, and the suspension was stirred for 2 h, then cooled to 5 °C, and filtered. The solid was washed with cold water (2 × 2 mL) and suspended in MeOH (200 mL). Silica gel (1 g) was added, and the suspension was sonicated and then heated to 50 °C for 15 min. The suspension was then evaporated under reduced pressure and dried in vacuo at room temperature for 10 h. The residue was powdered, poured on top of a wet column of silica gel (40 g), and eluted with 5% of MeOH in $CHCl_3$. Fractions corresponding to the product spot (TLC) were pooled and evaporated. The residue was washed with ether and air-dried to afford 0.15 g (28%) of **17** as a white solid: mp 238−241 °C (dec); TLC R_f 0.54 ($CHCl_3$/MeOH, 7:1, with one drop of NH_4OH, silica gel); ^{1}H NMR (DMSO-d_6) δ 2.49 (s, 3H, N−CH_3), 3.70 (s, 3H, 2- or 5-OCH_3), 3.78 (s, 3H, 2- or 5-OCH_3), 3.95 (s, 2H, 8-CH_2), 6.01 (s, 2H, 4-NH_2), 6.61 (dd, 1H, 4′-CH, J = 2.9, 8.8 Hz), 6.72 (d, 1H, 6′-CH, J = 2.9 Hz), 6.49 (d, 1H, 3′-CH, J = 8.8 Hz), 7.20 (bs, 2H, 2-NH_2), 7.36 (s, 1H, 6-CH). Anal. ($C_{16}H_{19}N_5O_3$) C, H, N.

Biological Evaluation. The *P. carinii* DHFR used for these studies was recombinant enzyme produced from a clone with a sequence identical to that reported by Edman et al.[9] The properties of this enzyme were identical to those reported for the native enzyme.[8] Purification of the recombinant enzyme was with a folte-Sepharose affinity column, which yielded pure, active enzyme without refolding. DHFR assays were carried out at 37 °C in the presence of 90 μM dihydrofolic acid as the substrate and 119 μM NADPH as the cofactor. These concentrations saturated the enzyme and gave maximal activity. Assays for rlDHFR and tgDHFR also contained 150 mM KCl. Testing of DHFR inhibitors against *P.carinii* in culture was in a model that has been predictive of the activity of standard agents such as trimethoprim, pyrimethamine, and trimetrexate.[19]

Structure Determination and Refinement. Crystals were grown using the sitting drop vapor diffusion method. The protein solution of pcDHFR was prepared in 0.1 M imidazole HCl buffer, pH 7.5, incubated overnight at 4 °C with NADPH, followed by addition of the inhibitor.[5] Protein droplets (5 mL) containing 3.6 mg/mL pcDHFR in 24% (w/v) PEG 8K, 0.1 M imidazole HCl buffer, pH 7.5, were utilized for crystal growth. Crystals grew in 3 weeks time. Crystals of recombinant pcDHFR ternary complexes are monoclinic, space group $P2_1$, and diffract to 2.5 Å resolution. The lattice constants for the **5**−NADPH−pcDHFR ternary complex are a = 37.552 Å, b = 43.256 Å, c = 61.389 Å, and β = 94.97°. The data for the complex refined to R = 16.1% for data to 2.5 Å resolution (Table 2).

The structure was solved by molecular replacement methods using the coordinates of pcDHFR from the trimethoprim complex.[10] Inspection of the resulting difference electron density map, using the program CHAIN[20] running on a Silicon Graphics Elan workstation, revealed density for a ternary complex. However, there was an unusually broad electron density profile near the diaminopyrimidine ring of compound **5** and Glu32 of pcDHFR which suggested the presence of more than one mode of binding for the furopyrimidine ring of compound **5** by rotation of the furopyrimidine ring, or the presence of a second molecule. This disorder did *not* extend

Table 2. Refinement Statistics for MTOS−NADPH−pcDHFR Complex

lattice constants, Å	37.55, 43.26, 61.39, $\beta = 94.9$
space group	$P2_1$
Resolution range, Å	8.0−2.5
reflections collected	7117
reflections used (8−2.5 Å; 2σ)	3220
R factor, %	16.1
protein atoms	1678
water molecules	66
B factor (protein average) Å^2	19.34

	target σ	root mean square σ
distances, Å		
bonds	0.020	0.014
angles	0.040	0.051
planar 1−4	0.050	0.052
planar groups	0.020	0.010
chiral volume	0.150	0.173
nonbonded distances		
single torsion	0.500	0.225
multiple torsion	0.500	0.325
possible hydrogen bonds	0.500	0.275
torsion angles, deg		
planar	3.0	1.8
staggered	15.0	23.9
orthonormal	15.0	23.7

to the naphthyl group of **5** which had a clearly defined electron density. After several models were tested for the orientation of the diaminofuropyrimidine ring, which left the position of the 2-naphthyl group constant, it was concluded that the extra residual density could be the result of a folate-like molecule remaining in the original pcDHFR sample. It was noted that this particular sample had a yellow color which was not removed on repeated washing. Such contaminants have been observed in other protocols for pcDHFR purification.[21] In our hands, this is the first crystal structure that has such a contaminant, as it was not observed in the other crystals grown samples purified from the same protocols. The final cycles of refinement were carried out with the inhibitor **5** and folate present at half occupancy, and cofactor included using the program PROLSQ.[22] The Ramachandran conformational parameters from the last cycle of refinement, generated by PROCHECK,[23] show that 84.3% of the residues have the most favored conformation and none in the disallowed regions.

Further restrained refinement was continued for the ternary complex, including the cofactor and inhibitor which were modeled using the builder function of InsightII.[24] Between least-squares minimizations, the structures were manually adjusted to fit its electron density values and verified by a series of OMIT maps calculated from the current model with deleted fragments. The highest thermal parameters (30−50 Å^2) of the protein residues are near the binding site entrance and in flexible loop regions. The remaining side chain thermal parameters range from 10 to 25 Å^2. In the final refinement stages, 66 water molecules were added in accord with the criteria of good electron density and acceptable hydrogen bonds to other atoms. The final refinement statistics are summarized in Table 2.

The broad electron density profile near Glu32 suggested that the density represented a composite of more than one model and further analysis of this profile suggested the presence of folate which filled this density. After several possible orientations of the naphthalene inhibitor and folate were tested, the final model was that of folate and the inhibitor, **5**, at half occupancy. However, the resolution and extent of the current diffraction data preclude further discrimination of these models.

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