New 2,4-Diamino-5-(2‘,5‘-substituted benzyl)pyrimidines as Potential Drugs against Opportunistic Infections of AIDS and Other Immune Disorders. Synthesis and Species-Dependent Anti...

ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · APRIL 2004

Impact Factor: 5.45 · DOI: 10.1021/jm030438k · Source: PubMed

5 AUTHORS, INCLUDING:

Andre Rosowsky
Dana-Farber Cancer Institute
292 PUBLICATIONS **5,469** CITATIONS
SEE PROFILE

Sherry F Queener
Indiana University-Purdue University Indiana...
226 PUBLICATIONS **4,794** CITATIONS
SEE PROFILE

Additions and Corrections

2004, Volume 47

Andrea Guiotto, Mirta Canevari, Piero Orsolini, Olivier Lavanchy, Christine Deuschel, Norimasa Kaneda, Akinobu Kurita, Takeshi Matsuzaki, Takeshi Yaegashi, Seigo Sawada, and Francesco M. Veronese*: : Synthesis, Characterization, and Preliminary in Vivo Tests of New Poly(ethylene glycol) Conjugates of the Antitumor Agent 10-Amino-7-ethylcamptothecin.

Page 1281. Structure **4** in Figure 3 should contain the tripeptide -Gly-Leu-Gly- instead of the tripeptide -Gly-Phe-Gly-. The correct Figure 3 is the following:

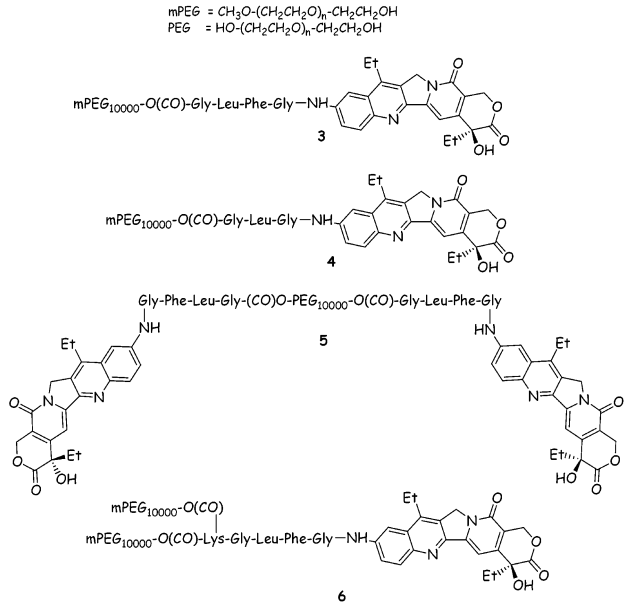


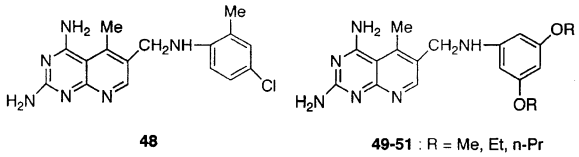
Figure 3. Synthesized PEG derivatives of 10-amino-7-ethylcamptothecin.

JM0408060

10.1021/jm0408060
Published on Web 05/28/2004

Andre Rosowsky,* Ronald A. Forsch, Carol Hopkins Sibley, Clark B. Inderlied, and Sherry F. Queener: : New 2,4-Diamino-5-(2′,5′-substituted benzyl)pyrimidines as Potential Drugs against Opportunistic Infections of AIDS and Other Immune Disorders. Synthesis and Species-Dependent Antifolate Activity.

Page 1479. In the middle of the right-hand column, compounds **48**−**51** were identified as benzyl rather than anilinomethyl derivatives and as [4,3-*d*l rather than [2,3-*d*] ring systems. Moreover, compounds **50** and **51** should have been referred to as 3′,5′- rather than 2′,5′-dialkoxy derivatives, and the structures at the end of that paragraph should be revised as shown below.



JM040098I

10.1021/jm040098i
Published on Web 06/08/2004

New 2,4-Diamino-5-(2′,5′-substituted benzyl)pyrimidines as Potential Drugs against Opportunistic Infections of AIDS and Other Immune Disorders. Synthesis and Species-Dependent Antifolate Activity

Andre Rosowsky,*[,†] Ronald A. Forsch,[†] Carol Hopkins Sibley,[‡] Clark B. Inderlied,[#] and Sherry F. Queener[§]

Dana-Farber Cancer Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, Department of Genome Sciences, University of Washington, Seattle, Washington 98195, Children's Hospital of Los Angeles, University of Southern California, Los Angeles, California 90027, and Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Received September 4, 2003

In a continuing effort to design small-molecule inhibitors of dihydrofolate reductase (DHFR) that combine the enzyme-binding selectivity of 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)-pyrimidine (trimethoprim, TMP) with the potency of 2,4-diamino-5-methyl-6-(2′,5′-dimethoxybenzyl)pyrido[2,3-*d*]pyrimidine (piritrexim, PTX), seven previously undescribed 2,4-diamino-5-[2′-methoxy-5′-(substituted benzyl)]pyrimidines were synthesized in which the substituent at the 5′-position was a carboxyphenyl group linked to the benzyl moiety by a bridge of two or four atoms in length. The new analogues were all obtained from 2,4-diamino-5-(5′-iodo-2′-methoxybenzyl)pyrimidine via a Sonogashira reaction, followed, where appropriate, by catalytic hydrogenation. The new analogues were tested as inhibitors of DHFR from *Pneumocystis carinii* (Pc), *Toxoplasma gondii* (Tg), and *Mycobacterium avium* (Ma), three life-threatening pathogens often found in AIDS patients and individuals whose immune system is impaired as a result of treatment with immunosuppressive chemotherapy or radiation. The selectivity index (SI) of each compound was obtained by dividing its 50% inhibitory concentration (IC_{50}) against Pc, Tg, or Ma DHFR by its IC_{50} against rat DHFR. 2,4-Diamino-[2′-methoxy-5′-(3-carboxyphenyl)-ethynylbenzyl]pyrimidine (**28**), with an IC_{50} of 23 nM and an SI of 28 in the Pc DHFR assay, had about the same potency as PTX and was 520 times more potent than TMP. As an inhibitor of Tg DHFR, **28** had an IC_{50} of 5.5 nM (510-fold lower than that of TMP and similar to that of PTX) and an SI value of 120 (2-fold better than TMP and vastly superior to PTX). Against Ma DHFR, **28** had IC_{50} and SI values of 1.5 nM and 430, respectively, compared with 300 nM and 610 for TMP. Although it had 2.5-fold lower potency than **28** against Ma DHFR (IC_{50} = 3.7 nM) and was substantially weaker against Pc and Tg DHFR, 2,4-diamino-[2′-methoxy-5′-(4-carboxyphenyl)ethynylbenzyl]pyrimidine (**29**), with the carboxy group at the para rather than the meta position, displayed 2200-fold selectivity against the Ma enzyme and was the most selective 2,4-diamino-5-(5′-substituted benzyl)pyrimidine inhibitor of this enzyme we have encountered to date. Additional bioassay data for these compounds are also reported.

Introduction

As part of an ongoing effort to design new lipophilic antifolates as drugs against the potentially life-threatening opportunistic parasites often found in patients with AIDS and other immune disorders, such as *Pneumocystis carinii* (Pc), *Toxoplasma gondii* (Tg), *Cryptosporidium parvum* (Cp), and *Mycobacterium avium* (Ma),[1,2] we recently described 10 novel compounds that could be viewed as structural hybrids of the well-known lipophilic dihydrofolate reductase (DHFR) inhibitors trimethoprim (TMP, **1**) and piritrexim (PTX, **2**).[3,4] When used alone, trimethoprim is not potent enough to achieve significant reduction of parasitemia and therefore requires coadministration of a sulfa drug to enhance efficacy. Piritrexim is not selective for microbial versus mammalian enzymes and thus requires coadministration of leucovorin to selectively protect the mammalian host from life-threatening hematopoietic toxicity. Therefore, the underlying goal of this program has been to discover new lipophilic DHFR inhibitors that will combine the binding species selectivity of trimethoprim with the potency of piritrexim. A compound with these properties should, in principle, obviate the need to coadminister either a sulfa drug or leucovorin.

Like trimethoprim, the compounds that provided the impetus for the present work were 2,4-diamino-5-(substituted benzyl)pyrimidines; however, as with piritrexim but in contrast to trimethoprim, the benzyl group was 2′,5′-disubstituted rather than 3′,4′,5′-trisubstituted. Furthermore, while the 2′-methoxy group of piritrexim was retained, the 5′-substituent consisted of either a hydrophobic long-chain alkoxy group (**3**−**7**) or various water-solubilizing *ω*-carboxyalkoxy (**8**−**13**), *ω*-carboxyalkynyl (**14**−**16**), *ω*-carboxyalkyl (**17**−**19**), carboxybenzyloxy (**20**, **21**), or carboxyphenoxypropynyl (**22**, **23**)

* To whom correspondence should be addressed. Phone: 617-632-3117. Fax: 617-632-2410. E-mail: andre_rosowsky@dfci.harvard.edu.
† Dana-Farber Cancer Institute.
‡ University of Washington.
# University of Southern California.
§ Indiana University School of Medicine.

10.1021/jm030438k CCC: $27.50
Published on Web 02/05/2004

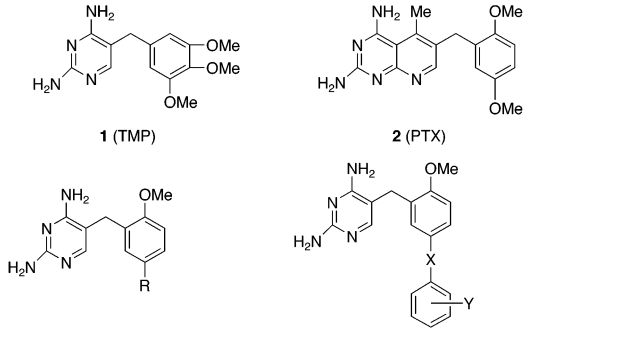


Figure 1. Structures of trimethoprim (**1**, TMP), piritrexim (**2**, PTX), and 2,4-diamino-5-[2′-methoxy-5′-(substituted benzyl)]pyrimidines **3**−**30**.

groups. The structures of trimethoprim and 2,4-diamino-5-(2′,5′-substituted benzyl)pyrimidines **2**−**23** are shown in Figure 1. As already noted earlier,[3,4] the rationale for these studies was based on the elegant work of Kuyper and co-workers[5] on 3′- and 4′-*O*-(*ω*-carboxyalkyl analogues of trimethoprim as selective inhibitors of *E. coli* DHFR. Four of the analogues (**14**−**16**, **18**), all containing a carboxyalkynyl or carboxyalkyl side chain, stood out from the rest in terms of potency and selectivity against Pc, Tg, and/or Ma DHFR relative to rat DHFR. A key finding was that the oxygen atom at the 5′-position of the benzyl group could be replaced by an alkyl or alkynyl carbon, in some cases with remarkable results. The four compounds (**20**−**23**) with a carboxyphenyl ring in the side chain were judged initially to be of less interest.[4] However, in the present paper we describe seven new analogues (**24**−**30**), one of which (**29**) does contain a carboxyphenyl ring in the side chain and yet to our satisfaction had *low nanomolar potency combined with 2200-fold selectivity* against Ma DHFR. A second analogue (**28**) was slightly more potent than **29** against the Ma enzyme but was only 430-fold selective.

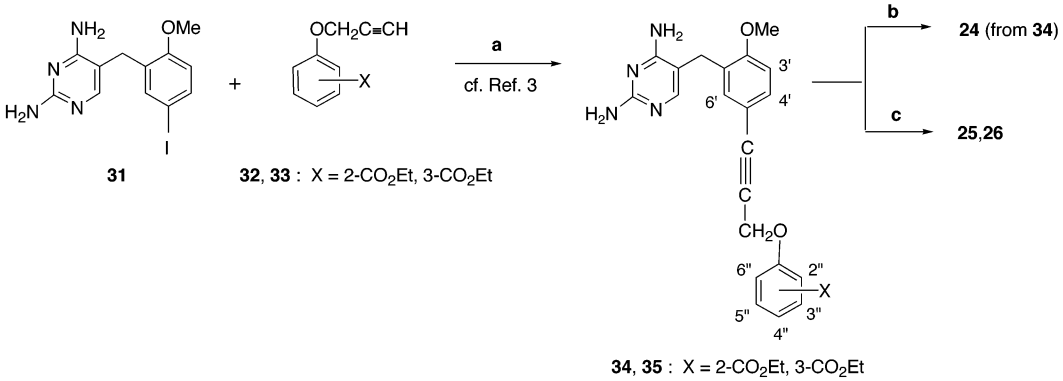
Chemistry

The synthesis of **24**−**30** made use of the key intermediate 2,4-diamino-5-(5′-iodo-2′-methoxybenzyl)pyrimidine (**31**)[6] and was adapted from our earlier work on **14**−**19**, **22**, and **23**, in which the 5′-position was likewise joined directly to a carbon rather than to an oxygen atom.[3] Condensation of **31** with ethyl 2-(propargyloxy)-benzoate (**32**) and ethyl 3-(propargyloxy)benzoate (**33**) via a Sonogashira reaction afforded the alkyne esters **34** and **35**, respectively (Scheme 1). Ester **34** was saponified with aqueous ethanolic $Ba(OH)_2$, the resulting insoluble barium salt was converted to a water-soluble ammonium salt, and an aqueous solution of the latter was subjected to catalytic hydrogenation in the presence of 5% Pd−C for 18 h. Somewhat surprisingly, the product under these conditions proved to be **24** rather than **25**. The presence of a cis double bond in **24** was deduced from the ^{1}H NMR spectrum, which featured a clean doublet at δ 6.59 (J = 12 Hz). This doublet was absent in **22**[4] and was consistent with a vinyl proton adjacent to an aromatic ring. A less deshielded signal at δ 5.87, partially obscured by a broad NH_2 peak, was assigned to the other vinyl proton next to CH_2.

Formation of the fully reduced analogue **25** from **34** occurred satisfactorily when catalytic hydrogenation preceded saponification and the reduction was performed with 10% Pd−C for 3 days rather than with 5% Pd−C for 18 h. Saponification was effected cleanly with NaOH in DMSO. As expected, the ^{1}H NMR spectrum of **25** lacked vinyl signals, and the CH_2O protons gave rise to a triplet at δ 3.93 instead of the singlet at δ 4.79 and the doublet at δ 4.80 seen in the spectra of **22**[4] and **24**, respectively. Catalytic hydrogenation of **34** under the same conditions as were used with **33**, followed by saponification with NaOH−DMSO again afforded a product (**26**) whose ^{1}H NMR spectrum and microanalysis were consistent with complete reduction of the triple bond.

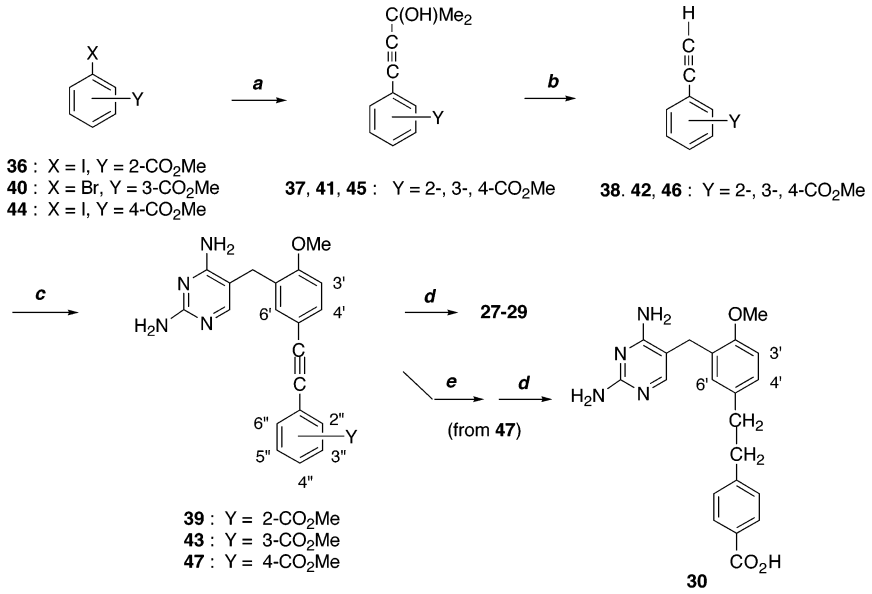
As shown in Scheme 2, condensation of methyl 2-iodobenzoate (**36**) with 2-methyl-3-butyn-2-ol under Sonogashira conditions followed by heating of the crude coupling product **37** directly in the presence of sodium hydride in toluene as described in the literature[7] afforded methyl 4-ethynylbenzoate (**38**) in nearly quantitative yield. A second Sonogashira condensation between **38** and **31** then yielded ester **39**, which was saponified directly with NaOH in DMSO to obtain the desired acid **27**. Purification of **27** was accomplished by preparative HPLC on C_{18} silica gel using 20% MeCN in 0.1 M NH_4OAc, pH 8.5, as the eluent. Appropriately pooled fractions of the eluate were freeze-dried, and the

Scheme 1[a]



[a] Reactants: (a) $(Ph_3P)_2PdCl_2$, CuI or $(Ph_3P)_3CuBr$, Et_3N, DMF, 65 °C, 3 h; (b) (i) $Ba(OH)_2 \cdot 8H_2O$, 50% EtOH−H_2O, room temp, 20 h, then $(NH_4)_2CO_3$; (ii) H_2, 5% Pd−C, 18 h; (c) (i) H_2/10% Pd−C, DMF, room temp, 3 days, then NaOH/DMSO.

Scheme 2[a]



[a] Reactants: (a) 2-methyl-3-butyn-2-ol, Ph_3P, $(Ph_3P)_2PdCl_2$, CuI, Et_3N; (b) NaH, toluene, heat; (c) **31**, $(Ph_3P)_2PdCl_2$, $(Ph_3P)_3CuBr$, Et_3N, DMF; (d) NaOH/DMSO; (e) H_2, 5% Pd−C, DMF, room temp, 18 h.

product was worked up in the usual way.[3,4] For the synthesis of meta and ortho acids **28** and **29**, the same sequence was followed, starting from methyl 3-bromobenzoate (**40**) and methyl 4-iodobenzoate (**44**). Elaboration of **40** and **44** to the ethynyl derivatives **42** and **46** via the acetylenic carbinols **41** and **45** was carried out in identical fashion, as was the subsequent preparation of esters **43** and **47** via **42** and **46** and their saponification and HPLC purification. The intermediate esters **39** and **43** were saponified directly, whereas ester **47** precipitated spontaneously from the reaction and was saponified separately.

Compound **30** was straightforwardly obtained from **47** by catalytic hydrogenation in DMF solution (5% Pd−C, 3 h) and was purified by preparative HPLC on C_{18} silica gel with 20% MeCN in 0.1 M NH_4OAc, pH 8.5, as the eluent. That reduction of the triple bond was complete was confirmed from the ^1H NMR spectrum, which showed no visible sign of a vinyl signal. Interestingly, the CH_2 groups in the CH_2CH_2 bridge could not be differentiated from each other because they coalesced into a broad singlet at δ 3.46.

Enzyme Inhibition Assays

Compounds **24**−**30** were tested for the ability to inhibit Pc, Tg, and Ma DHFR as described,[3,4] and their potencies (IC_{50} values) and selectivities (SI values) were compared with those of other analogues with a carboxyphenyl or carboxyphenoxy group in the side chain.[3] The results are presented in Table 1 and discussed individually for Pc, Tg, and Ma DHFR in comparison with rat DHFR in the following sections.

***Pneumocystis carinii* DHFR.** As shown in Table 1, the most potent of the new aromatic carboxylic acids **24**−**30** against Pc DHFR was the 2-(3-carboxyphenyl)-ethynyl analogue **28**, with an IC_{50} of 23 nM. The potency of this compound was substantially greater than that of the other 3-carboxyphenyl analogues **20** and **23**,[3] whose IC_{50} values against Pc DHFR were 890 and 5600 nM, respectively. However, the potency of **28** was also higher against rat DHFR, resulting in essentially no selectivity improvement for the Pc enzyme. Furthermore, while **28** was equipotent with **15**, the best of our previously reported simple 5′-(carboxyalkynyl) analogues,[3] it was less selective. Nevertheless, we were gratified to note that the oxygen atom at the 5′-position of **16** could be replaced by an sp carbon, at least where potency was concerned, and that shortening of the bridge in **23** by removal of the CH_2O moiety was likewise tolerated.

With regard to the effect of the location of the aromatic COOH group (Table 1), we observed that the ortho isomer **25** was less potent than the meta isomer **26** against both Pc and rat DHFR. This was likewise the case for both **27** (ortho) and **29** (para) relative to **28** (meta), suggesting that optimal binding of these compounds to the active site of both enzymes has a marked preference for meta substitution. The results were also consistent with an earlier modeling study in which it had been predicted that the COOH group in compounds such as these would be likely to interact favorably with a basic residue in the active site of Pc DHFR.[8] With regard to the spacer between the benzyl and phenyl rings, our results with **30** versus **29** indicate that a C≡C spacer favors Pc DHFR binding whereas a CH_2-CH_2 spacer favors rat DHFR binding, resulting in an overall selectivity improvement in the case of the alkyne analogue.

The effect of varying the hybridization state of the first two side chain carbons at the 5′-position of the benzyl ring on Pc versus DHFR binding can also be assessed from the IC_{50} values for the ortho carboxylic acids **22** (sp), **24** (sp^2), and **25** (sp^3) and the meta carboxylic acids **23** (sp) and **26** (sp^3). In the case of the 2-COOH derivatives, the alkyne **22** was a slightly better inhibitor of Pc DHFR than either **24** or **25**, whereas **24** and **25** had similar potency. The alkyne **22** was also a better inhibitor of rat DHFR than **25** but was not a better inhibitor than **24**. Interestingly, in the case of the meta carboxylic acids, **23** proved to be a *weaker*

Table 1. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, and Rat DHFR by 2,4-Diamino-5-[(2′-methoxy-5′-substituted)benzyl]pyrimidines Containing an Aromatic Carboxylic Acid Group at the End of the Side Chain

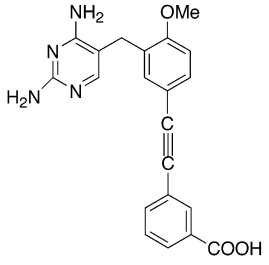
	IC_{50} (nM)[a]				selectivity index (SI)[b]		
compd	*P. carinii*	*T. gondii*	*M. avium*	rat liver	*P. carinii*	*T. gondii*	*M. avium*
19[c]	530 (430−650)	30 (26−34)	89 (72−110)	4600 (4.2−5.0)	8.6 (6.4−11)	150 (120−190)	52 (38−70)
20[c]	890 (730−1100)	600 (530−690)	120 (100−140)	19 000 (14 000−25 000)	21 (13−33)	31 (21−46)	150 (100−230)
21[c]	1200 (0.098−1.5)	2000 (1.7−2.2)	60 (0.053−0.069)	21 000 (14−29)	17 (9.6−30)	11 (6.4−18)	340 (210−560)
22[c]	2300 (2100−2500)	500 (440−560)	36 (32−41)	6200 (5200−7400)	2.7 (2.1−3.5)	13 (9.3−17)	170 (130−230)
23[c]	5600 (4400−7200)	1600 (1100−2400)	57 (50−65)	14 000 (11 000−16 000)	2.4 (1.5−3.6)	8.3 (6.7−15)	240 (170−320)
24	8600[d] (7000−11 000)	2500 (2200−2900)	380 340−420)	6400 (5700−7200)	0.74 (0.52−1.0)	2.6 (2.0−3.3)	17 (14−21)
25	7800 (6300−9700)	710 (640−800)	310 (270−350)	25 000 (21 000−30 000)	3.2 (2.2−4.8)	35 (26−47)	81 (60−110)
26	1100 (910−1200)	210 (170−270)	25 (23−26)	4000 (3600−4600)	3.6 (3.0−5.1)	19 (13−27)	160 (140−200)
27	4100 (3600−4800)	1900 (1100−3400)	100 (67−160)	25 000 (17 000−35 000)	6.0 (3.5−10)	13 (5−32)	250 (110−520)
28	23 (19−28)	5.5 (4.5−6.7)	1.5 (1.2−1.9)	650 (490−870)	28 (18−46)	120 (73−190)	430 (260−730)
29	1300 (650−2500)	340 (200−550)	3.7 (2.9−4.6)	8200 (7100−9300)	6.3 (2.8−14)	24(13−47)	2200 (1500−3200)
30	7100 (5200−9700)	1500 (820−2600)	25 (20−32)	13 000 (11 000−15 000)	1.8 (1.1−2.9)	8.7 (4.2−18)	520 (340−750)
TMP[e]	12 000 (10−16)	2800 (2.4−3.3)	300 (0.26−0.35)	180 000 (160 000−210 000)	14 (10−20)	65 (48−87)	610 (460−810)
PTX[f]	13 (9.0−17)	4.3 (4.0−4.6)	0.61 (0.53−0.7)	3.3 (2.9−3.9)	0.26 (0.17−0.42)	0.76 (0.63−0.97)	5.4 (4.1−7.2)

[a] Numbers in parentheses are 95% confidence intervals rounded off to two significant figures and based on IC_{50} values likewise rounded off to two significant figures. The difference in IC_{50} between rat liver DHFR and each of the parasite enzymes was determined to be statistically significant at $P < 0.01$ (Welch's *t*-test) for all compounds except **24**. [b] SI = IC_{50}(rat liver DHFR)/IC_{50}(*P. avium*, *T. gondii*, or *M. avium* DHFR). Numbers in parentheses are 95% confidence intervals rounded off to two figures and represent a range calculated by dividing the lower end of the 95% confidence interval for the IC_{50} against rat liver DHFR by the upper end of the 95% confidence interval for the IC_{50} against *P. carinii*, *T. gondii*, or *M. avium* DHFR. This calculated range affords values very close to those obtained by other statistical methods (ref 27) but is more conservative. [c] Data for comparison purposes are taken from ref 3. [d] Not statistically different from the IC_{50} against rat DHFR. [e] TMP = trimethoprim, 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine; data from ref 4. [f] PTX = piritrexim, 2,4-diamino-5-methyl-6-(2′,5′-dimethoxybenzyl)pyrido[2,3-*d*]pyrimidine; data from ref 3.

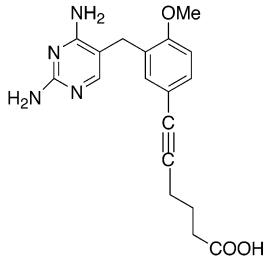
inhibitor of both enzymes than **26**, suggesting that the influence of sp hybridization on binding may be favorable only when the COOH group is at the ortho position. However, because the differences in binding to Pc versus rat DHFR among these five analogues were quite small, their selectivity was only marginal.

In summary, the compound in Table 1 with the best combination of potency and selectivity against Pc DHFR was the 5′-(3-carboxyphenyl)ethynyl analogue **28**. In terms of the distance separating the COOH group from the phenyl ring, **28** was intuitively viewed as an analogue of 2,4-diamino-5-[2′-methoxy-5′-(4-carboxy-1-pentynyl)benzyl]pyrimidine (**15**), whose IC_{50} against Pc and rat DHFR was previously reported to be 28 and 2200 nM, respectively.[3] It thus appears that replacement of the three CH_2 groups in the side chain of this compound with a phenyl ring was favorable for binding to both enzymes but unfortunately was more favorable for binding to the rat enzyme, resulting in lower selectivity. It is worth noting, however, that even though the Pc DHFR selectivity of **28** and **15** was not substantially different from that of trimethoprim, their potency was approximately 500-fold higher. By contrast, while the potency of **28** for Pc DHFR was not substantially different from that of piritrexim, only **28** was selective.[9]

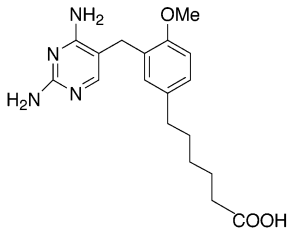
***Toxoplasma gondii* DHFR.** As in the case of Pc DHFR, the most potent and selective Tg DHFR inhibitor among the new compounds in Table 1 was **28**, with an



28



15 (ref. 4)



18 (ref. 4)

IC_{50} of 5.5 nM and a selectivity index of 120. We previously observed the corresponding values for **15** against this enzyme to be 32 nM and 69, respectively. Thus, replacement of three CH_2 groups by a phenyl ring was more favorable for Tg DHFR binding than for Pc DHFR binding and also led to a small improvement in selectivity.

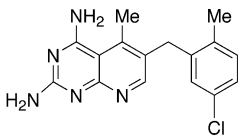
Whereas the pentynyl derivative **15** had been found previously[3] to have the best selectivity for Pc versus rat DHFR among the analogues without a phenyl ring in the side chain, the most potent and selective inhibitor with a simple spacer (i.e., with no phenyl ring inserted) in the case of the Tg enzyme was 2,4-diamino-5-[2′-methoxy-5′-(carboxypentyl)benzyl]pyrimidine (**18**), with an IC_{50} of 8.4 nM and a selectivity index of 490. It thus appeared that **28** was similar in potency to **18** in this assay but was not as selective. Nonetheless, we were pleased to see that **28** was considerably more selective than trimethoprim in this respect and clearly superior to piritrexim. The finding that a $(CH_2)_5$ spacer is optimal for Tg DHFR potency and selectivity, whereas the less flexible $C{\equiv}C(CH_2)_3$ spacer is optimal for Pc DHFR binding and selectivity, presumably reflects subtle differences in 3D structure between the active sites of the two proteins. A clearer picture of these differences awaits solution of the 3D structure of Tg DHFR (or preferably the bifunctional DHFR-TS protein) with NADPH also bound to the active site.[10]

With regard to other features in the new compounds **24**−**30** contributing to the structure−activity correlation, the most salient points to be made are that (i) potency was somewhat influenced by the hybridization state of the first two carbons in the side chain, although the effect is far from dramatic (compare **29** with **30** or **22** with **24** and **25**), (ii) a 3-carboxyphenyl group was superior to a 4-carboxyphenyl group when the spacer was a OCH_2 group (compare **20** and **21**) or C≡C group (compare **28** and **29**), and (iii) a 3-carboxyphenyl group was superior to a 2-carboxyphenyl group when the spacer was a $C{\equiv}CCH_2O$ group (compare **22** and **23**) or C≡C group (compare **27** and **28**). Taken together, these structure−activity correlations appear to be quite similar to those deduced above for the Pc enzyme, suggesting that the two proteins probably have a number of key features in common where the binding of these diaminopyrimidine inhibitors is concerned.

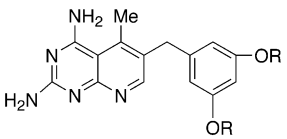
***Mycobacterium avium* DHFR.** As in the case of Pc DHFR and Tg DHFR, the most potent of the new compounds in Table 1 against Ma DHFR was **28**, with an IC_{50} of 1.5 nM. However, **28** was not the most selective analogue; rather, this honor belonged to the para isomer **29**, which was 2-fold less potent than **28** and yet had a remarkable selectivity index of 2200. This dramatic improvement in selectivity was the result of a 13-fold increase in the IC_{50} against rat DHFR, from 650 nM for **28** to 8200 nM for **29**. By comparison, the most potent and selective among the previously described carboxyphenyl analogues was **22**, with an IC_{50} of 36 nM (one-tenth the potency of **29**) and a selectivity index of only 340.[3] Two major structural differences characterize **29** relative to **22** and presumably account for its improved properties: (i) the spacer in the side chain of **29** is shorter (C≡C versus $C{\equiv}CCH_2O$) and (ii) the COOH group in **29** is at the para rather than the meta position.

Other structure−activity correlations that can be deduced for Ma DHFR from the data in Table 1, some of which are similar to those noted above for the other enzymes, are that (i) replacement of the triple bond in the ortho carboxylic acid **22** by a double (**24**) or single bond (**25**) leads to both diminished potency and diminished selectivity, (ii) replacement of the triple bond in the meta carboxylic acid **23** by a single bond (**26**) has a less pronounced effect than it does in the case of the ortho isomer **25**, (iii) replacement of oxygen at the 5′-position by a triple-bonded carbon leads to improved selectivity as well as potency (compare **29** with **21**), and (iv) moving the COOH group from the meta or para position to the sterically more hindered ortho position is not well tolerated (compare **23** versus **22**, **26** versus **25**, and **28** versus **27**). The unfavorable effect of ortho substitution suggests that this structural change is not optimal for binding and that it may be important for the COOH group to be able to lie in a coplanar orientation relative to the phenyl ring when the inhibitor binds to the active site.

It is of interest to compare the potency and selectivity against Ma DHFR of the two best compounds in this paper with those of a large series of 2,4-diamino-5-methyl-(6-substituted benzyl)pyrido[2,3-*d*]pyrimidine inhibitors recently described by Suling and co-workers at the Southern Research Institute.[11] Among 78 compounds synthesized and tested by this group, nearly three-quarters had IC_{50} values of <10 nM and the two most potent were 2,4-diamino-5-methyl-6-(4′-chloro-2′-methylbenzyl)pyrido[4,3-*d*]pyrimidine (**48**) and 2,4-diamino-5-methyl-6-(3′,5′-dimethoxybenzyl)pyrido[4,3-*d*]-pyrimidine (**49**), with IC_{50} values of 0.19 and 0.64 nM. However, because their IC_{50} against human DHFR (the enzyme chosen for comparison in this case) was 2.8 and 1.5 nM, the selectivity of **48** and **49** was only 15- and 23-fold. The two most selective compounds in the series, on the other hand, were the 2′,5′-diethoxy analogue **50** and the 2′,5′-dipropyloxy analogue **51**, with selectivities of ca. 2700- and 7300-fold. The greatly increased selectivity of **50** and **51** relative to **48** and **49** was due to a striking 3 log reduction in binding to the human enzyme. It should be noted, however, that these assays were done at pH 7.0 in the presence of 1.0 mM EDTA and no added KCl, whereas the compounds in this and our earlier papers[3,4] were assayed at pH 7.4 in the presence of 150 mM KCl but no EDTA. Thus, a direct comparison of these compounds with **29** under the same assay conditions would be of interest.



48



49- **51** : R = Me, Et, n-Pr

In a recent comparison of the binding of several diaminopyrimidine inhibitors to rat DHFR (both wild-type and recombinant) and recombinant human DHFR enzymes,[12] the selectivity of trimethoprim was found to be about 50% higher when the reference enzyme was recombinant human DHFR than when it was rat liver DHFR. Several other standard antifolates such as methotrexate and pyrimethamine were similarly found to be more potent against the rat enzyme. Although this type of species-based difference for rat versus human DHFR is not reported with respect to Ma DHFR inhibition, it is certainly possible that the selectivity index of **29** relative to *human* as opposed to rat DHFR would in fact be greater than our observed value of 2200.

Table 2. Comparison of 2,4-Diamino-5-[2′,5′-disubstituted benzyl)pyrimidines as Inhibitors of the Growth of *Mycobacterium avium* Isolates from Human Patients[a]

	MIC (μg/mL)							
compd	MAC100	MAC101	MAC108	MAC109	MAC16	MIC50% (μg/mL)	MIC90% (μg/mL)	range (μg/mL)
4	32	32	32	32	32			
5	8	4	8	8	8	8	8	2−16
8	16	16	16	32	32			
9	16	16	>32	16	16	16	32	4−32
10	16	8	32	8	8	4	32	4−32
11	16	8	>32	8	16	4	32	2−32
12	8	4	32	2	2	4	8	2−32
13	8	2	>32	4	2	4	8	2−16
14	8	8	16	16	16	8	16	4−32
15	8	4	32	16	8	8	16	4−32
16	4	2	>32	4	2	8	16	2−32
18	32	16	>32	32	32			
19	>32	32	>32	32	16			

[a] MIC refers to the minimum inhibitory concentration, whereas MIC50% and MIC90% refer to MIC values against 50% and 90%, respectively, of 30 strains tested, with the range for all strains being in the last column. Assays were done according to the published method.[13] Compounds **17** and **20**−**23** gave MIC values of >32 μg/mL against an initial test panel consisting of MAC100, MAC101, MAC108l, MAC109, and MAC116, whereas the MIC values of **27**−**29** against this panel were >64 μg/mL. Trimethoprim at concentrations of up to 64 μg/mL was likewise not inhibitory, in agreement with an earlier study in which 10 human isolates of *M. avium* were found to be insensitive to trimethoprim at concentration of 0.5−1.5 μg/mL.[29] Because of their low potency against the initial test panel, **4**, **8**, **17**−**19**, and **20**−**23** were not tested further. Assays with **24**−**26** were not done. In molar terms, a concentration of 2 μg/mL in the cases of **5**, **11**−**13**, and **16** is equivalent to ca. 5000 nM.

In a follow-up to the cell-free assays against Ma DHFR reported here, many of the compounds synthesized previously and in the present work were tested in an in vitro assay using isolates of *M. avium* from AIDS patients.[13] Three of the compounds (**12**, **13**, **16**) were active against two out of five isolates at concentrations as low as 2 μg/mL (ca. 5000 nM) and against four isolates at concentrations of 8 μg/mL.[14] It thus appears that *M. avium* is permeable to at least some 2,4-diamino-5-(2′,5′-disubstituted benzyl)pyrimidines despite the presence of a negatively charged COOH group. Since 2 μg/mL is generally considered a physiologically achievable concentration, these compounds could have therapeutic utility in the treatment of disseminated *M. avium* infection. Interestingly, trimethoprim by itself is reported to be ineffective against human isolates of *M. avium* even at a concentration of up to 1.5 μg/mL,[15] although there is at least one report that it is clinically effective against *M. avium* infection when combined with sulfamethoxazole.[16] It may be noted that, while their in vitro molar equivalent potency is considerably lower than that of several popular macrolide and quinolone antibiotics,[13,17,18] the antifolate activity of **12**, **13**, and **16** would probably not be affected by macrolide resistance, an emergent problem in the clinical management of *M. avium* infection.[19] Thus, when used as a two-drug combination, or even as a three-drug combination with a sulfa drug like dapsone,[20,21] these compounds may be able to extend the usefulness of macrolides in the treatment of this disease. On the other hand, as indicated by the results in Table 2, cellular uptake of some of the compounds by *M. avium* in culture may be a problem, as suggested for example by the fact that the highly potent DHFR inhibitors **28** (IC_{50} = 1.5 nM) and **29** (IC_{50} = 3.7 nM) gave an MIC50% of >64 μg/mL whereas the carboxyalkynyl analogues **14** and **15** had an 8-fold lower MIC50% even though their reported IC_{50} values against MaDHFR were likewise in the 1−10 nM range. Indeed the MIC50% values of all the tested compounds with a bulky and hydrophobic phenyl ring in the side chain (**20**−**23**, **27**−**29**) were either >32 or >64 μg/mL (Table 2). We believe the lack of correlation between Ma DHFR inhibition and *M. avium* growth inhibition may be due to inefficient cellular uptake or to fact that some of the phenyl analogues (e.g., **29**) do not remain well dissolved when stock solutions in DMSO are added into the cell culture medium. Thus, in order for a highly selective inhibitor like **28** or **29** to be pharmaceutically useful, steps would have to be taken to improve solubility. Further complicating the correlation of cell culture data with DHFR binding data in this case is the fact that many serological variants (serovars) of *M. avium* exist in humans[21] and may differ in ways other than merely their surface polysaccharide composition. Therefore, it is conceivable that the DHFR in our enzyme assays, which came from a single source ("serovar 4"),[22] and the DHFR in the *M. avium* organisms used in the growth assays would not bind all inhibitors equally well. Moreover, as the data in Table 3 illustrates, there can be as much as a 16-fold range in the MIC when as many as 30 strains are tested. This variability may reflect differences in endogenous folate pools sizes and the rate of de novo folate biosynthesis. Because high MIC50% values for compounds **20**−**23** and **27**−**29** were only observed against five strains, we cannot exclude the possibility that lower MIC50% values would have been observed against at least some of the other 25. Expanded assays addressing this possibility are planned. However, a gratifying conclusion that can already be drawn from the preliminary data in Table 2 is that, despite initial fears to the contrary, these molecules can penetrate the cells at micromolar external concentrations despite the presence of a free COOH group and, more importantly in terms of the overall goal of this work, can inhibit growth without coaddition of a sulfa drug.

Other Potential DHFR Targets. A rapid and cost-effective assay was recently described in which the ability of a compound to inhibit DHFR from several different species of origin can be compared simultaneously in *Saccharomyces cerevisiae* specifically modified to express only a nonyeast enzyme (e.g., Pc DHFR,

Tg DHFR, human DHFR, etc.).[24,25] Briefly, this assay is based on the use of a dhfr$^-$ mutant with an appropriately designed plasmid encoding the target DHFR, or in some cases the DHFR-TS bifunctional enzyme, of choice. Appropriate dilutions of a test compound in a stock solution in DMSO are spotted at the center of a series of agar-coated Petri plates. A wild-type yeast control and up to eight different engineered strains expressing different nonyeast DHFRs, all in log phase, are then streaked onto each plate in a wheel-like pattern of "spokes". Sulfanilamide (1 mM) is also spread on the surface of the plate in order to take advantage of the synergy between the sulfa drug and the DHFR inhibitor, lowering the relative amount of test drug required to inhibit yeast growth; more importantly, sulfanilamide needs to be present in the medium because without it the yeast cells grow so aggressively that a difference between wild-type and mutant DHFR constructs in the presence of a DHFR inhibitor cannot be discerned. As the test compound diffuses outward from the center of the plate, individual spokes show varying degrees of growth in proportion to how well the compound inhibits the enzyme in that particular spoke. High selectivity against microbial versus human DHFR is indicated when growth of the construct encoding the microbial enzyme is visible only near the outer rim of the plate, whereas growth of the construct encoding the human enzyme extends all the way from the center to the edge. It should be noted that the various constructs are genetically identical except for the DHFR enzyme they express and that drug penetration is assumed to be the same in all cases. Plates are ranked according to whether compounds are (i) inactive, (ii) weak and nonselective, (iii) potent but nonselective, (iv) weak but selective, (v) potent and selective, and (vi) "reverse-selective" (i.e., more active against human than nonhuman DHFRs). It may be noted that because some sulfanilamide is present to prevent overgrowth of the yeast, the effect actually measured is *potentiation of de novo folate biosynthesis by the DHFR inhibitor*, in much the same way that trimethoprim is used in combination with sulfa drugs to treat opportunistic infections in patients. Additionally, it should be emphasized that comparisons of relative growth in this type of assay are made only between spokes on a given plate (i.e., comparing the effect of a particular compound against different DHFR species) and not among different plates (i.e., comparing the effect of different compounds against a particular DHFR). A representative set of plates illustrating the effect of prototypical examples of the six categories of inhibition defined above is shown in Figure 2. The concentration of each antifolate drug spotted at the center of its plate was 10 mM in a 10 μL volume, and the plates were incubated for 72 h. An example of selective inhibition of *C. parvum* versus human DHFR after 72 h of incubation is illustrated by the middle plate of the second row, in which the *C. parvum* spoke (C) is clearly shorter than the human spoke (E), which is nearly as long as the control spoke for wild-type yeast (I). A plate showing the effect of trimethoprim is given in Figure 3 (top row, far left). Because its effect was highly reproducible, trimethoprim could be used with a high degree of confidence as a positive control.

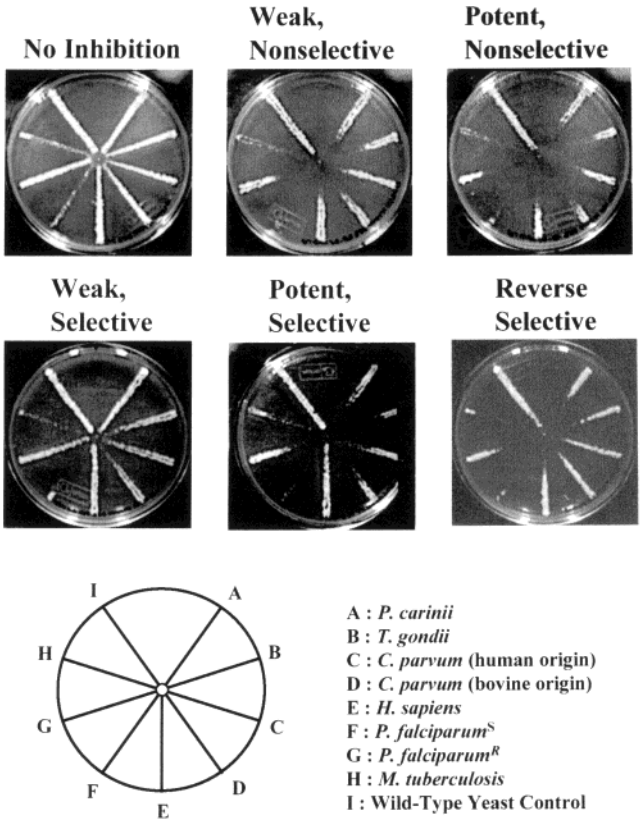


Figure 2. Semiquantitative classification of species-selective potency and selectivity of DHFR inhibitors into six types based on the results of assays with a panel of *S. cerevisiae* mutants engineered to express *P. carinii*, *T. gondii*, *C. parvum*, human, *P. vivax*, *P. falciparum*, or *M. tuberculosis* DHFR instead of wild-type yeast DHFR. For a detailed description of the method and a discussion of its scope and limitations, see refs 21 and 22. All compounds were spotted on the plate as 10 mM stock solutions in DMSO (10 μL), with 1 mM sulfanilamide previously spread over the agar in order to prevent overgrowth. The pyrimethamine-resistant *P. falciparum* construct in spoke F expressed a DHFR double-mutant (N51, S108N).[25]

According to the criteria defined in Figure 2, it would be classified as weak but selective (type iv).

Plates showing the effects of 14 of the analogues with a COOH side chain reported in this and in our two preceding papers in this series[3,4] are presented in Figure 3. The 5′-*O*-(4-carboxybutyl) analogue **9** appeared to be slightly more effective against both Cp DHFR constructs (spokes C and D) than against the human construct (spoke E) on the same plate. Some ability to discriminate between the Cp and human constructs on the same plate was also apparent in the case of **3**, **10**, **14**−**16**, and **18**. In contrast, **24**−**30** were less selective. Selective growth suppression was also observed with **9** against the Pc and Tg DHFR constructs (spokes A and B) and with **3**, **9**, and **10** against the *Mycobacterium tuberculosis* construct (spoke H), but the other compounds did not appear to be selective. Interestingly, almost all the analogues inhibited a wild-type *P. falciparum* DHFR construct (spoke G) but not a pyrimethamine-resistant construct with a double mutation (N51I, S108N) (spoke F).[25,26] The best inhibitor of the wild-type *P. falciparum* construct was the 5′-*O*-(5-carboxypentyl) analogue **18**, which produced nearly complete growth suppression. The fact that all the compounds were more active against the pyrimethamine-sensitive construct than the

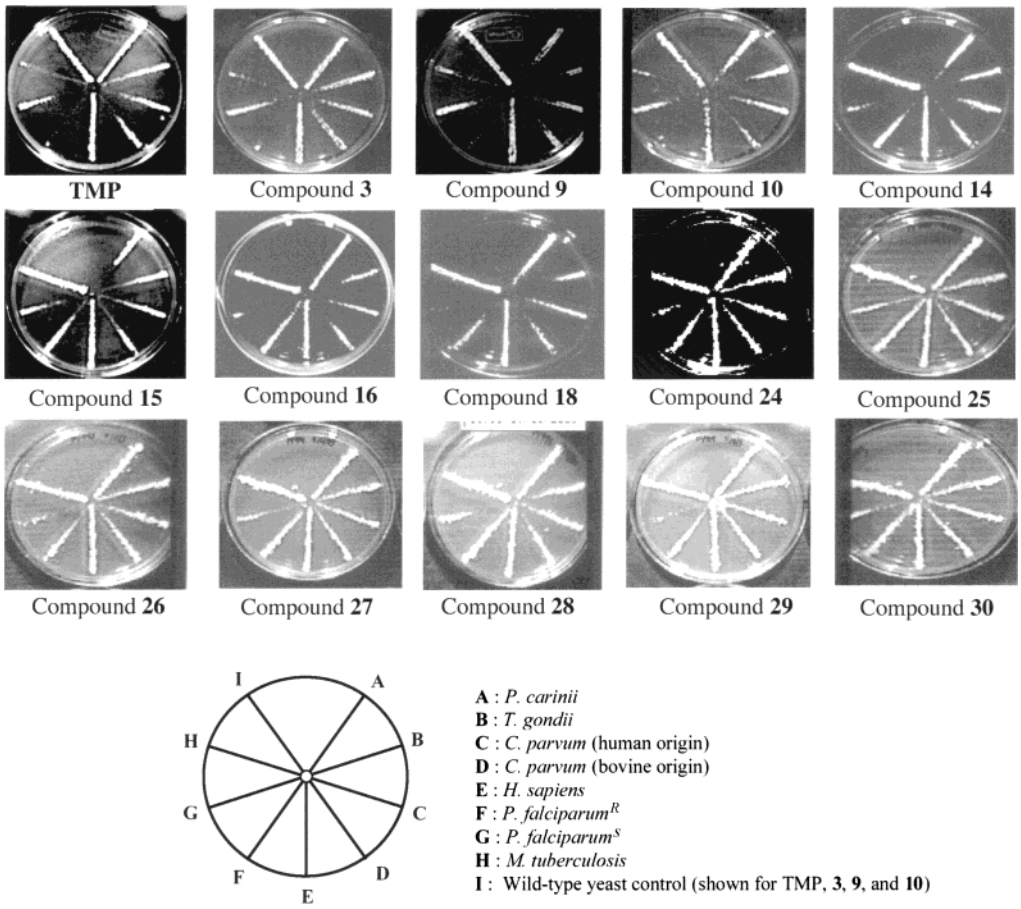


Figure 3. Results of yeast spoke assays using compounds **24**−**30** and selected examples of 2,4-diamino-5-[2′-methoxy-5′-(substituted benzyl)]pyrimidines synthesized earlier.[3,4] All the compounds except **26** and **27** were spotted as 10 mM stock solutions in DMSO (10 μL), with 1 mM sulfanilamide (1 mM) previously spread over the agar to prevent overgrowth. Because of solubility limitations, **26** could only be applied to the plate as a 5 mM solution and **27** as a 1.25 mM solution. A pyrimethamine-resistant *P. vivax* construct expressing DHFR with a single mutation (S117N)[26] gave similar results (not shown). Please note that the assignment of spokes F and G in Figure 3 to *P. falciparum*^{R} and *P. falciparum*^{S}, respectively, is the opposite of the assignment of spokes F and G in Figure 2 (cf. figure legends).

resistant construct provided indirect evidence that their site of action is indeed DHFR; trimethoprim was likewise more effective against the wild-type construct in this assay, in agreement with our earlier results.[24] In selected cases (results not shown), the identity of the pharmacologic target of these drugs was confirmed to be DHFR by including thymidylic acid (dTMP) in the medium, which allows the cells to escape the need to recycle tetrahydrofolate.

In parallel with the spoke assays, the ability of **9** and **14**−**18** to inhibit dihydrofolate reduction by NADPH in the presence of the recombinant Cp and human DHFR enzymes was determined spectrophotometrically (Table 3). Please note that the assignment of spokes F and G in Figure 3 to *P. facliparum*^{R} and *P. falciparum*^{S}, respectively, is the opposite of the assignment of spokes F and G in Figure 2 (cf. figure legends). The potency of the carboxylic acid analogues against hCp and bCp DHFR was not greatly different from that previously reported for trimethoprim itself.[24] Moreover, while there was a 60-fold difference in binding to hCp and human DHFR in the case of trimethoprim, the IC_{50} of **9** against the two enzymes was the same, indicating that this (and presumably other DHFR inhibitors of the 2,4-diamino-5-[2′-methoxy-5′-(substituted benzyl)pyrimidine type) would probably not be effective as single agents for the treatment of cryptosporidial infections unless leucovorin was used for selective protection in the same way that it is used with trimetrexate or piritrexim.

Table 3. Inhibition of *C. parvum* and Human DHFR by 2,4-Diamino-5-[2′-methoxy-5′-(substituted benzyl)pyrimidines **9** and **14**[a]

	IC_{50} (nM)			
compd	hCp	bCp	human	selectivity (human/hCp)
9	8000	5000	8000	1
14	30000	5000	90000	3
TMP	5000	2000	300000	60

[a] The assay mixture contained 20 μM dihydrofolate and 100 μM NADPH in 50 mM Tris buffer, pH 7.0, 1 mM EDTA, 75 mM 2-mercaptoethanol, and 1% bovine serum albumin to stabilize the enzyme.[23] Numbers are rounded off to one significant figure and are the mean values of two independent determinations. The IC_{50} values for TMP are taken from ref 21. Compounds **8**, **10**, and **15**−**18** were also tested, but because in most cases an IC_{50} was not reached against the human enzyme at the range of drug concentrations (1000−100 000 nM) used against the hCp and bCp enzymes, the results are not shown. Because the spoke assays indicated **24**−**30** to be nonselective, spectrophotometric assays of DHFR inhibition by these compounds were not carried out.

Interestingly, the selectivity of **9** and **14** for the microbial enzyme was lower than would be predicted from the spoke assay. This can be seen for example from the data in Table 3 for compound **9**, for which there was no difference in IC_{50} against the hCp and human enzyme despite a clear difference in growth between the

corresponding yeast constructs (cf. Figure 2). This may be explained if one assumes that in order for DHFR inhibition to be fully effective at preventing the growth of *C. parvum*, a sulfa drug also needs to be present. If this is correct, then the design of DHFR inhibitors for the treatment of cryptosporidiosis without coadministration of a sulfa drug may prove to be much more difficult than the design of such inhibitors targeted against Pc, Tg, or Ma.

Conclusion

Seven new examples of 2,4-diamino-5-(2′,5′-substituted benzyl)pyrimidine DHFR inhibitors with a carboxyphenyl substituent at the 5′-position of the benzyl moiety are described in this paper. One example (**28**) was ca. 500-fold more potent than trimethoprim against both Pc DHFR and Tg but showed only a small increase in selectivity relative to the rat enzyme. This compound also retained most of the potency of piritrexim against both enzymes but, unlike piritrexim, showed excellent selectivity. Although the other six compounds still compared favorably with trimethoprim, they were less potent and less selective than **28**. A second compound, **29**, had a low IC_{50} of 3.7 nM against Ma DHFR and a selectivity index of 2200. This compound was the most selective 2,4-diamino-5-[2′-methoxy-5′-(substituted benzyl)pyrimidine we have tested to date against this enzyme. Its activity against human isolates of intact *M. avium* in culture, as well as that of the other new compounds reported in this paper, was lower than that of the previously synthesized analogues with a simple ω-carboxyalkyloxy, ω-carboxyalkynyl, or ω-carboxyalkyl substituent at the 5′-position, suggesting less efficient cell penetration by compounds with bulkier 5′-substituents containing an extra phenyl ring. However, two of the previously synthesized 5′-(ω-carboxyalkyloxy) and 5′-(ω-carboxyalkynyl) analogues (**12**, **13**, **16**) were active at a physiologically realistic concentration of 2 μg/mL (ca. 5000 nM). In a rapid and cost-effective complementation assay utilizing *S. cerivisiae* constructs engineered to express Pc, Tg, Ma, Cp, and other DHFR proteins as opposed to the wild-type yeast enzyme, several of the analogues were found to inhibit the growth of the Cp constructs in the presence of the sulfanilamide, which is required in the assay to prevent overgrowth. However, their selectivity for the hCp construct relative to the human construct appeared to be only marginally greater than that of trimethoprim. Furthermore, in contrast to trimethoprim, direct spectrophotometric assays comparing the activity of the carboxylic acid analogues **9** and **14** against purified hCp and human DHFR failed to show a significant species difference in binding, suggesting that differences in the effect of these analogues on the growth of Cp constructs in the complementation assays may not have been due to DHFR inhibition alone but instead may have been the result of the combined action of the DHFR inhibitor and the sulfa drug. Despite the lack of success thus far in our effort to develop a better Cp DHFR inhibitor than trimethoprim, structure−activity correlations uncovered during this work with respect to Pc, Tg, and Ma DHFR inhibition offer potentially helpful guidelines for the future design of additional nonclassical DHFR inhibitors of these enzymes in which the potency of piritrexim and the selectivity of trimethoprim are combined into a single molecule to circumvent the need for coadministration of a sulfa drug or leucovorin.

Experimental Section

IR spectra were recorded on a Perkin-Elmer model 781 double-beam spectrophotometer. Only peaks with wavenumbers above 1200 cm^{-1} are reported. ^1H NMR spectra were obtained in DMSO-d_6 solution at 200 MHz on a Varian VX200 instrument. Each peak is denoted as a singlet (s), broad singlet (br s), doublet (d), doublet of doublets (dd), triple (t), doublet of triplets (dt), quartet (q), or multiplet (m). Integrated peak areas are not reported when the signal was partly obscured by water or DMSO or corresponded to NH_2 groups. Signals for aryl protons are identified according to the numbering in Schemes 1 and 2. The term "bridge CH_2" refers to the benzylic carbon between the pyrimidine and methoxyphenyl rings. TLC analyses were on Whatman MK6F silica gel plates with UV illumination at 254 nm. Column chromatography was on Baker 7024 "Flash" silica gel (40 μm particle size). HPLC separations at pH 7.4 were performed on Waters C_{18} silica gel radial compression cartridges (Millipore, Milford, MA; analytical, 5 μm particle size, 5 mm × 100 mm; preparative, 15 μm particle size, 25 mm × 100 mm), whereas those at pH 8.5 were performed on a stainless steel C_{18} silica gel column more suitable for work at this higher pH (Phenomenex, Torrance, CA; 15 μm particle size, 21.2 mm × 100 mm). The synthesis of 2,4-diamino-5-(5′-iodo-2′-methoxybenzyl)pyrimidine (**31**)[6] and the alkyne esters **32** and **33** was carried out as described.[4] $(Ph_3P)_3CuBr$ (97% yield, mp 170−171 °C, lit.[29] 164 °C) used in the preparation of alkyne esters **33**, **39**, **43**, and **47** was synthesized according to the literature[29] except that CuBr was used instead of $CuBr_2$, the P/Cu ratio was decreased from 4.0 to 3.2, and the solvent was MeCN rather than MeOH. Other chemicals were purchased from Sigma Aldrich (St. Louis, MO), Acros Organics (Pittsburgh, PA), and Lancaster Synthesis (Windham, NH). Elemental analyses were performed by Quantitative Technologies, Inc. (Whitehouse, NJ) and were within ±0.4% of theoretical values unless otherwise noted. Where the microchemical data indicated the presence of residual acetic acid in the analytical sample, its presence in the sample was confirmed by the finding of a methyl signal at δ 1.9 in the ^1H NMR spectrum.

2,4-Diamino-5-[2′-(3-(2-carboxyphenoxy)propen-1-yl)-5′-methoxybenzyl]pyrimidine (24). A mixture of iodide **31** (356 mg, 1.0 mmol), the alkyne ester **32** (306 mg, 1.5 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), CuI (1 mg), Et_3N (3 mL), and DMF (3 mL) was heated at 65 °C under an atmosphere of N_2 for 3 h. The volatiles were evaporated at 50 °C (water bath) under reduced pressure, and the residue was triturated successively with isooctane and H_2O, the triturate being decanted each time. The residue containing ester **34** was dissolved in 50% EtOH−H_2O (200 mL) at 50 °C, and after addition of $Ba(OH)_2 \cdot 8H_2O$ (947 mg, 3.0 mmol) and stirring at room temperature for 20 h, the mixture was treated with a solution of $(NH_4)_2CO_3$ (500 mg, 5 mmol) in H_2O (10 mL). The mixture was stirred vigorously for 10 min, the precipitated $BaCO_3$ was filtered off, and the filtrate was concentrated by rotary evaporation to remove the EtOH and cause a solid to form. Dilute ammonia was added until most of the solid dissolved; a trace of remaining insoluble material was filtered off. The volume of the filtrate was decreased by rotary evaporation, and the solution was hydrogenated in the presence of 5% Pd−C (100 mg) in a Parr apparatus (3 Torr) for 18 h. The catalyst was filtered off, and the filtrate was again concentrated by rotary evaporation (caution: frothing occurs because of gas evolution). A solid gradually formed as the volume was reduced and was redissolved by adding dilute NaOH. Analytical HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4, 1.0 mL/min) showed a major peak (13 min) corresponding to the product **24**, along with a faster-moving impurity (4 min) and additional minor peaks. The pH was adjusted to 9.0 with 10% AcOH, a small amount of grayish precipitate was removed by filtration, and the product was purified by preparative HPLC on C_{18} silica gel using the aforementioned system. Appropriately pooled

fractions were freeze-dried to obtain **24** as a white solid (142 mg, 33%): mp 125−129 °C (softening without giving a true melt); IR (KBr) ν 3350, 3200, 2950, 2850 (broad), 1670, 1610, 1595, 1560, 1535, 1510, 1495, 1455, 1395, 1300 sh, 1260 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.54 (bridge CH_2 partly obscured by H_2O), 3.82 (s, 3H, OMe), 4.80 (d, J = 7 Hz, 2H, CH_2O), 5.87 (m, 1 H, $CH_2CH{\equiv}C$), 6.21 (br s, NH_2), 6.59 (d, J = 12 Hz, $CH_2C{\equiv}CH$), 7.03 (m, 3H, 3′-, 4′-, 6′-H), 7.21 (dd, J = 8 Hz, J = 2 Hz, 1H, 6″-H), 7.42 (m, 3H, 4″- and 5″-H, pyrimidine 6-H), 7.60 (dd, J = 8 Hz, J = 2 Hz, 1H, 3″-H). Anal. ($C_{22}H_{22}N_4O_4 \cdot 0.2AcOH \cdot H_2O$) C, H, N.

2,4-Diamino-5-[2′-(3-(2-carboxyphenoxy)propyl)-5′-methoxybenzyl]pyrimidine (25). A solution of iodide **31** (178 mg, 0.5 mmol), the alkyne ester **32** (153 mg, 0.75 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), $(Ph_3P)_3CuBr$ (10 mg), Et_3N (3 mL), and DMF (3 mL) was heated at 65 °C for 3 days. Most of the volatiles were removed by rotary evaporation, the mixture was diluted with 10 volumes of H_2O, and the precipitate was collected and freeze-dried. The residue containing ester **34** was redissolved in DMF (15 mL), and the solution was shaken with 10% Pd−C (100 mg) in a Parr hydrogenation apparatus (3 atm pressure) for 3 days. After filtration of the catalyst and evaporation of the DMF, the residue was taken up in DMSO (4 mL) and the solution was stirred, treated dropwise with 2 N NaOH (1 mL), diluted with H_2O (40 mL), chilled, and filtered to remove a trace of undissolved solid. Analytical HPLC as described above revealed a major peak at 16 min (easily distinguished from a co-injected sample of **24**). For preparative HPLC the MeCN concentration in the buffer was initially decreased to 18% to remove faster-moving impurities, then increased to 20% as soon as the elution of **25** began. Appropriately pooled fractions were concentrated on the rotary evaporator and finally freeze-dried to a solid, and the latter was added to dilute NaOH. A trace of material that did not dissolve was filtered off, the filtrate was chilled and acidified with 10% AcOH, and the precipitate was collected and freeze-dried to obtain **25** as a colorless solid (38 mg, 9% overall yield for two steps): mp 133−140 °C (softening without giving a true melt); IR (KBr) ν 3330, 3170, 2920, 1655, 1595, 1550, 1495, 1445, 1380, 1385, 1245 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.89 (m, $CH_2CH_2CH_2O$, partly obscured by AcOH), 2.64 (t, J = 7 Hz, 2H, $CH_2CH_2CH_2O$), 3.45 (s, 2H, bridge CH_2), 3.73 (s, 3H, OMe), 3.93 (t, 2H, J = 6 Hz, $CH_2CH_2CH_2O$), 5.81 (br s, NH_2), 6.13 (br s, NH_2), 6.99 (m, 5H, 3′-, 4′-, 6′-, 6″-H, pyrimidine 6-H), 7.31 (m, 2H, 4″-, 5″-H), 7.51 (d, J = 6 Hz, 3″-H). For analysis, a 6 mg sample of the product was stirred overnight in 1 mL of 5% AcOH. Approximately two-thirds of the solid dissolved, and the rest was collected and freeze-dried. Anal. ($C_{22}H_{24}N_4O_4 \cdot AcOH$) C, H, N.

2,4-Diamino-5-[2′-(3-(3-carboxyphenoxy)propyl)-5′-methoxybenzyl]pyrimidine (26). A solution of the iodide **31** (356 mg, 1.0 mmol), the alkyne ester **33** (306 mg, 1.5 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), CuI (1 mg), Et_3N (3 mL), and DMF (3 mL) was heated at 60 °C for 2 h. The volatiles were removed in vacuo, and the residue was triturated successively with isooctane and H_2O, with decantation of the triturate each time. The residue containing ester **35** was taken up in 50% EtOH−H_2O (200 mL) at 50 °C, and the solution was treated with $Ba(OH)_2 \cdot 8H_2O$ (947 mg, 3.0 mmol) and stirred at room temperature for 3 days, whereupon a solution of $(NH_4)_2CO_3$ (500 mg, 5 mmol) in H_2O (10 mL) was added and the mixture was stirred vigorously for 10 min and filtered. The filtrate was concentrated under reduced pressure until the EtOH was removed, resulting in the formation of a solid. The solid was redissolved by adding diluted ammonia, and the solution was shaken with H_2 and 5% Pd−C (100 mg) in a Parr apparatus at 3 atm pressure for 20 h. The catalyst was filtered off and washed with a small volume of 0.1 N NaOH to dissolve a small amount of white solid that had deposited on the catalyst. The pooled filtrate and wash solution were concentrated under reduced pressure (caution: frothing due to ammonia evolution) while periodically adding dilute NaOH to keep the product in solution. After most of the H_2O had been removed, the pH was adjusted to 8.0 with 10% AcOH, the cloudy mixture was left to stand overnight, and a trace of solid was filtered off. HPLC analysis on C_{18} silica gel using an 85:15 mixture of 20% MeCN in 0.05 M NH_4OAc, pH 7.4, and 50% MeCN in H_2O as the eluent gave a major peak (15.5 min) corresponding to **26** along with a slower-moving peak (18.5 min) and several minor fast-moving impurities. Purification of the major peak by preparative HPLC as described in the preceding experiment afforded the product as a white powder (160 mg, 37% overall yield for two steps): mp 267−269 °C dec; IR (KBr) ν 3360, 3240, 2950 br, 1665, 1635, 1560, 1505, 1465, 1445, 1385, 1315, 1290, 1255 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.96 (m, 2H, $CH_2CH_2CH_2O$), 2.64 (t, J = 7 Hz, 2H, $CH_2CH_2CH_2O$), 3.50 (s, bridge CH_2, overlapping and partly obscured by H_2O), 3.76 (s, 3H, OMe), 3.97 (t, J = 6 Hz, 2H, $CH_2CH_2CH_2O$), 6.05 (br s, NH_2), 6.31 (br s, NH_2), 6.98 (m, 4H, 3′-, 4′-, 6′-H, pyrimidine 6-H), 7.32 (m, 3H, 2″-, 5″-, 6″-H), 7.50 (d, J = 7 Hz, 1H, 4″-H). Anal. ($C_{22}H_{24}N_4O_4 \cdot 0.15AcOH \cdot 0.85H_2O$) C, H, N.

2,4-Diamino-5-[2′-(2-carboxyphenyl)ethynyl)-5′-methoxybenzyl]pyrimidine (27). Step 1. To a solution of **36** (3.02 mL, 5.24 g, 0.02 mol) and 2-methyl-3-butyn-2-ol (2.35 mL, 2.02 g, 0.024 mol) in Et_3N (60 mL) under N_2 were added Ph_3P (40 mg), $(Ph_3P)_2PdCl_2$ (20 mg), and CuI (20 mg). The reaction mixture was stirred under reflux for 18 h, then cooled and partitioned between EtOAc and H_2O. The organic layer containing ester **37** was separated and evaporated to dryness, the residue was taken up directly in dry toluene (40 mL), NaH (0.80 g of 60% dispersion in mineral oil, calculated to contain 0.02 mol) was added, and the toluene was slowly distilled off until 10 mL had been collected (caution: since residual NaH may still be present, evaporation to dryness at this stage must be avoided). The mixture was then quenched with 5% $NaHCO_3$ and the rest of the toluene was evaporated under reduced pressure. Column chromatography of the residue on a "Flash" silica gel column (50 g, 3 cm × 20 cm) with 2:1 isooctane−EtOAc as the eluent afforded ester **38** as an oil (0.74 g, 23%) that was used directly in the next step.

Step 2. A mixture of **38** (240 mg, 1.5 mmol), iodide **31** (356 mg, 1.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), $(Ph_3P)_3CuBr$ (10 mg), Et_3N (3 mL), and DMF (3 mL) was heated at 60 °C for 18 h. After evaporation of the volatiles under reduced pressure, the residue was swirled successively with isooctane and H_2O, each of which was decanted in turn. The remaining material containing ester **39** was taken up in DMSO (3 mL), and the solution was swirled while being treated dropwise with 2 mL of a 1:1 mixture of DMSO and 2 N NaOH. The saponification mixture was diluted with H_2O (50 mL), a solid that remained undissolved was filtered off, and the filtrate was purified by HPLC on C_{18} silica gel using 18% MeCN in 0.1 M NH_4OAc, pH 8.5, as the eluent. Appropriately pooled fractions were concentrated and freeze-dried, and the residue was partially redissolved in dilute NaOH. A small amount of undissolved solid was removed by filtration, and 10% AcOH was added dropwise to the filtrate until a precipitate formed, which was collected and freeze-dried to obtain **27** as a white solid (114 mg, 28% combined yield for the Sonogashira reaction and saponification): IR (KBr) ν 3340, 3190, 2930, 2850, 2210, 1765w, 1665, 1585, 1555, 1505, 1460, 1380, 1290, 1250 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.53 (s, 2H, bridge CH_2), 3.83 (s, 3H, OMe), 6.24 (br s, NH_2), 6.41 (br s, NH_2), 7.02 (d, J = 8 Hz, 1H, 3′-H), 7.14 (d, J = 2H, 6′-H), 7.39 (m, 4H, 4′-H, 5″-H, 6″-H, pyrimidine 6-H), 7.55 (dt, J = 8 Hz, J = 2 Hz, 1H, 5″-H), 7.81 (dd, J = 8 Hz, J = 1 Hz, 1H, 3″-H). For analysis, a sample of the HPLC-purified product was stirred with 5% AcOH for 18 h, then filtered and freeze-dried. Anal. ($C_{21}H_{18}N_4O_3 \cdot AcOH$) C, H, N.

2,4-Diamino-5-[2′-(3-carboxyphenyl)ethynyl)-5′-methoxybenzyl]pyrimidine (28). Step 1. To a solution of **40** (2.15 g, 0.01 mol) and 2-methyl-3-butyn-2-ol (1.17 mL, 1.01 g, 0.012 mol) in Et_3N (15 mL) under N_2 were added $(Ph_3P)_3CuBr$ (20 mg) and $(Ph_3P)_2PdCl_2$ (10 mg). The mixture was stirred under reflux for 18 h and then partitioned between EtOAc and H_2O. Evaporation of the organic layer gave **41** as an oil (R_f = 0.3, silica gel, 2:1 isooctane−EtOAc) that was taken up directly in dry toluene (50 mL) and treated with NaH (0.40 g of 60% dispersion in mineral oil, calculated to contain 0.24 g, 0.01

mol). Toluene was slowly distilled off until 20 mL (40% of the original volume) had been collected. The reaction was then quenched with 5% $NaHCO_3$, the rest of the toluene was removed by rotary evaporation, and the product was purified by chromatography ("Flash" silica gel, 20 g, 2 cm × 17 cm, 2:1 isooctane−EtOAc) to obtain alkyne ester **42** (421 mg, 29%) as an oil that was used directly in the next step.

Step 2. A mixture of **42** (240 mg, 1.5 mmol), iodide **31** (356 mg, 1.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), $(Ph_3P)_3CuBr$ (10 mg), and Et_3N (10 mL) in DMF (3 mL) was heated at 65 °C for 2 days. The solvent was evaporated, and the residue was swirled successively with isooctane and H_2O, each of which was decanted in turn. The residue left after trituration with H_2O, consisting of ester **43**, was collected, freeze-dried, and taken up in DMSO (4 mL). The solution was swirled and treated dropwise with 1 N NaOH (1.5 mL), then diluted with 10 volumes of H_2O and adjusted to pH 8−9 with 10% AcOH. A small amount of precipitate was filtered off, and the filtrate was purified by preparative HPLC on a C_{18} silica gel using a 98:2 mixture of 20% MeCN in 0.1 M NH_4OAc, pH 8.5, and 50% MeCN in H_2O as the eluent. Appropriately pooled fractions were reduced in volume by rotary evaporation and then freeze-dried. The residue was redissolved in dilute NaOH, the solution was acidified with 10% AcOH, and the precipitate was collected and dried on a lyophilizer to obtain **28** as a white solid (54 mg, 13% combined yield for the Sonogashira reaction and saponification): mp >250 °C dec; IR (KBr) ν 3420, 3330, 3110, 1675, 1615, 1585, 1560, 1505, 1465, 1425, 1375, 1320, 1300, 1285, 1250, 1210 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.57 (s, 2H, bridge CH_2), 3.86 (s, 3H, OMe), 6.36 (br s, NH_2), 6.52 (br s, NH_2), 7.07 (d, J = 8 Hz, 1H, 3′-H), 7.25−7.55 (m, 4H, 4′-H, 6′-H, 5″-H, pyrimidine 6-H), 7.70 (d, J = 8 Hz, 1H, 6″-H), 7.92 (d, J = 8 Hz, 1H, 4″-H), 8.02 (s, 1H, 2″-H). Anal. ($C_{21}H_{18}N_4O_3$·0.9AcOH) C, H, N.

2,4-Diamino-5-[2′-(4-carboxyphenyl)ethynyl)-5′-methoxybenzyl]pyrimidine (29). Step 1. To a solution of **44** (4.97 g, 0.019 mol) and 2-methyl-3-butyn-2-ol (2.24 mL, 1.93 g, 0.023 mol) in Et_3N (50 mL) under N_2 were added CuI (20 mg), Ph_3P (40 mg), and $(Ph_3)_2PdCl_2$ (20 mg). The mixture was stirred under reflux for 68 h, then cooled to room temperature and partitioned between EtOAc and H_2O. Evaporation of the organic layer and recrystallization of the solid from hexane afforded the alkyne ester **45** as a light-brown solid (4.14 g, ca. 100%): mp 74−76 °C (lit.[7] 83.5−84.5 °C after recrystallization from EtOH−H_2O instead of hexane). Anal. ($C_{13}H_{14}O_3$) C, H.

Step 2. A stirred solution of **45** (2.18 g, 0.01 mol) in dry toluene (50 mL) was treated with NaH (0.4 g of 60% dispersion in mineral oil, calculated to contain 0.24 g, 0.01 mol) was slowly heated in a distillation apparatus until the head temperature reached 110 °C and toluene began to collect in the receiver. After 15 mL had been removed, the reaction mixture was cooled and quenched with 5% $NaHCO_3$ (caution: gas evolution). This resulted in an emulsion that was difficult to separate into two layers. Two phases formed readily upon addition of EtOAc and 10% citric acid, the organic layer was separated and evaporated to a brown solid, and the latter was purified by chromatography ("Flash" silica gel, 20 g, 2 cm × 18 cm, 2:1 isooctane−EtOAc) to obtain **46** (1.43 g, 98%): mp 87−88 °C (lit.[7] 91−93 °C, purification by sublimation instead of chromatography).

Step 3. A stirred mixture of **46** (240 mg, 1.5 mmol), iodide **31** (356 mg, 1.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), $(Ph_3P)_3CuBr$ (10 mg), and Et_3N (3 mL) in DMF (3 mL) was heated at 60 °C for 18 h. A homogeneous solution formed within 10 min, followed a few minutes later by the appearance of a solid. The reaction mixture was chilled, and the solid was collected and washed with Et_2O to obtain the methyl ester **47** (292 mg, 78%): mp 239−240 °C; IR (KBr) ν 3480, 3370, 3160, 2950 w, 2830 w, 2200, 1700 (C═O), 1675, 1615, 1595, 1565, 1510, 1485, 1455, 1430, 1400, 1310, 1305, 1290, 1280, 1245 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.52 (s, 2H, bridge CH_2), 3.84 (s, 6H, two OMe), 5.71 (br s, NH_2), 6.09 (br s, NH_2), 7.04 (d, J = 8 Hz, 1H, 3′-H), 7.20 (s, 1H, 6′-H), 7.42 (m, 2H, 4′-H, pyrimidine 6-H), 7.62 (d, J = 8 Hz, 2H, 2″-H, 6″-H), 7.93 (d, J = 8 Hz, 2H, 3″-H, 5″-H). Anal. ($C_{22}H_{20}N_4O_3$) C, H, N.

Step 4. A stirred solution of **47** (146 mg, 0.376 mmol) in slightly warm DMSO (4 mL) was treated dropwise with 2 N NaOH (0.5 mL), then diluted with H_2O (40 mL) and acidified with 10% AcOH. The mixture was chilled and the solid was collected and dried in a lyophilizer to obtain **29** as a white powder (118 mg, 84%): mp >300 °C dec; HPLC, 17 min (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4); IR (KBr) ν 3400, 3120, 1680, 1620, 1600, 1580, 1505, 1445, 1405, 1365, 1285, 1250, 1215 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.52 (s, 2H, bridge CH_2), 3.82 (s, 3H, OMe), 5.91 (br s, NH_2), 6.21 (br s, NH_2), 7.03 (d, J = 8 Hz, 1H, 3′-H), 7.20 (s, 1H, 6′-H), 7.46 (m, 2H, 4′-H, pyrimidine 6-H), 7.56 (d, J = 8 Hz, 2H, 2″-H, 6″-H), 7.89 (d, J = 8 Hz, 2H, 3″-H, 5″-H). Anal. ($C_{21}H_{18}N_4O_3$·0.8H_2O) C, H, N.

2,4-Diamino-5-[2′-(2-(4-carboxyphenyl)ethyl)-5′-methoxybenzyl]pyrimidine (30). A solution of ester **47** (140 mg, 0.361 mmol) in DMF (20 mL) was shaken with H_2 and 5% Pd−C (25 mg) in a Parr apparatus at 3 atm pressure for 18 h. The catalyst was filtered off, the filtrate was concentrated to dryness by rotary evaporation, the residue was taken up in DMSO (3 mL), and the solution was swirled and treated dropwise with 2 N NaOH (0.5 mL). The solution was then diluted with H_2O (30 mL), acidified with 10% AcOH, chilled in ice, and filtered. The collected solid was purified by preparative HPLC on C_{18} silica gel (20% MeCN in 0.1 M NH_4-OAc, pH 8.5), and appropriately pooled fractions were evaporated to dryness. The residue was taken up in dilute NaOH, the solution was acidified with 10% AcOH and chilled in ice, and the precipitate was collected and dried in a lyophilizer to obtain **30** as a white powder (51 mg, 35%): mp >250 °C dec; IR (KBr) ν 3330, 2930, 1655, 1610, 1505, 1460, 1390, 1250 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.79 (br s, 4H, CH_2CH_2), 3.46 (s, 2H, bridge CH_2), 3.73 (s, 3H, OMe), 5.87 (br s, NH_2), 6.16 (br s, NH_2), 6.84 (d, J = 8 Hz, 1H, 3′-H), 6.97 (m, 2H, 4′-H, 6′-H), 7.16 (d, J = 8 Hz, 2H, 2″-H, 6″-H), 7.28 (s, 1H, pyrimidine 6-H), 7.77 (d, J = 8 Hz, 2H, 3″-H, 5″-H). Anal. ($C_{21}H_{22}N_4O_3$·1.7H_2O) C, H, N.

Acknowledgment. This work was supported by Research Grants RO1-AI29904 (A.R.) and RO1-AI55064 (C.H.S.) and Research Contract NO1-AI25140 (C.B.I.) from the National Institute of Allergy and Infectious Diseases (NIAID), U.S. Department of Health and Human Services. The help and advice of Dr. Christopher Lambros of the AIDS Opportunistic Infections Program, NIAID, Bethesda, MD, are likewise acknowledged.

References

(1) Klepser, M. W.; Klepser, T. B. Drug treatment of HIV-related opportunistic infections. *Drugs* **1997**, *3*, 40−73.
(2) See ref 3 for a selected list of our publications in this area since 1993.
(3) Rosowsky, A.; Forsch, R. A.; Queener, S. F. Further studies on 2,4-diamino-5-(2′,5′-disubstituted benzyl)pyrimidines as potent and selective inhibitors of dihydrofolate reductases from three major opportunistic pathogens of AIDS. *J. Med. Chem.* **2003**, *46*, 1726−1736.
(4) Rosowsky, A.; Forsch, R. A.; Queener, S. F. Inhibition of *Pneumocystis carinii*, *Toxoplasma gondii*, and *Mycobacterium avium* dihydrofolate reductases by 2,4-diamino-[2-methoxy-5-(ω-carboxyalkyloxy)benzyl]pyrimidines: Marked improvement in potency relative to trimethoprim and species selectivity relative to piritrexim. *J. Med. Chem.* **2002**, *45*, 233−241.
(5) Kuyper, L. F.; Roth, B.; Baccanari, D. P.; Ferone, R.; Beddell, C. R.; Champness, J. N.; Stammers, D. K.; Dann, J. G.; Norrington, F. E.; Baker, D. J.; Goodford, P. J. Receptor-based design of dihydrofolate reductase inhibitors: comparison of crystallographically determined enzyme binding with enzyme affinity in a series of carboxy-substituted trimethoprim analogues. *J. Med. Chem.* **1985**, *28*, 303−311.
(6) Calas, M.; Barbieri, A.; Giral, L.; Balmayer, B.; Despaux, E. Synthesis of new trimethoprim analogs. Antibacterial structure−activity relationship. *Eur. J. Med. Chem.* **1982**, *17*, 407−504.
(7) Havens, S. J.; Hergenrother, P. M. Synthesis of arylacetylenes by the sodium hydride catalyzed cleavage of 4-aryl-2-methyl-3-butyn-2-ols. *J. Org. Chem.* **1985**, *50*, 1763−1765.

(8) Cody, V.; Rosowsky, A. Crystal structure determination of human DHFR binary complexes with TMP and modeling studies of TMP analogues. *Proc. Am. Assoc. Cancer Res.* **2002**, *43*, 1021.
(9) Structural studies are planned on the ternary complexes of **15** and **28** with PcDHFR and NADPH in order to determine the 3D orientation of the COOH group in relation to the benzyl moiety (V. Cody, personal communication).
(10) Work on the structure of Tg DHFR ternary complexes with several of the compounds in this paper is currently in progress in the laboratory of Dr. Amy Anderson at Dartmouth College and will be reported in due course.
(11) Suling, W. J.; Seitz, L. E.; Pathak, V.; Westbrook, L.; Barrow, E. W.; Zywno-van Ginkel, S.; Reynolds, R. C.; Piper, J. R.; Barrow, W. R. Antimycobacterial activities of 2,4-diamino-5-deazapteridine derivatives and effects on mycobacterial dihydrofolate reductase. *Antimicrob. Agents Chemother.* **2000**, *44*, 2784−2793.
(12) Wang, Y.; Bruenn, J. A.; Queener, S. F.; Cody, V. Isolation of rat dihydrofolate reductase gene and characterization of recombinant enzyme. *Antimicrob. Agents Chemother.* **2001**, *45*, 2517−2523.
(13) Bermudez, L. E.; Inderlied, C. B.; Kolonoski, P.; Petrofsky, M.; Aralar, P.; Wu, M.; Young, L. S. Activity of moxifloxacin by itself and in combination with ethambutol, rifabutin, and azithromycin in vitro and in vivo against *Mycobacterium avium*. *Antimicrob. Agents Chemother.* **2001**, *45*, 217−222.
(14) We are indebted to Dr. Chris Lambros of the AIDS Opportunistic Infections Program of the NIAID, Bethesda, MD, for kindly arranging to have these assays performed.
(15) Raszka, W. V., Jr.; Skillman, L. P.; McEvoy, P. L. In vitro susceptibility of clinical isolates of *Mycobacterium avium* and *M. intracellulare* to folate antagonists. *Diagn. Microbiol. Infect. Dis.* **1994**, *18*, 201−204.
(16) Chang, W. J.; Goetz, M. B. Response to treatment of infection due to *Mycobacterium avium* complex with trimethoprim-sulfamethoxazole. *Clin. Infect. Dis.* **1992**, *14*, 1267−1268.
(17) Ji, B.; Lounis, N.; Truffot-Pernot, C.; Grosset, J. Effectiveness of various antimicrobial agents against *Mycobacterium avium* complex in the beige mouse model. *Antimicrob. Agents Chemother.* **1994**, *38*, 2521−2529.
(18) Bermudez, L. L.; Kolonoski, P.; Wu, M.; Aralar, P. A.; Inderlied, C. B.; Young, L. S. Mefloquine is active in vitro and in vivo against *Mycobacterium avium* complex. *Antimicrob. Agents Chemother.* **1999**, *43*, 1870−1874.
(19) Bermudez, L. E.; Petrofsky, M.; Kolonoski, P.; Young, L. S. Emergence of *Mycobacterium avium* populations resistant to macrolides during experimental chemotherapy. *Antimicrob. Agents Chemother.* **1998**, *42*, 180−183.
(20) Bermudez, L. E.; Inderlied, C. B.; Kolonoski, P.; Petrofsky, M.; Young, L. S. Clarithromycin, dapsone, and a combination of both used to treat of prevent disseminated *Mycobacterium avium* infection in beige mice. *Antimicrob. Agents Chemother.* **1994**, *38*, 2717−2721.
(21) Rastogi, N.; Labrousse, V.; Bryskier, A. Intracellular activities of roxithromycin used alone and in association with other drugs against *Mycobacterium avium* complex in human macrophages. *Antimicrob. Agents Chemother.* **1995**, *39*, 976−978.
(22) Meissner, G.; Anz, W. Sources of *Mycobacterium avium* complex infection resulting in human diseases. *Am. Rev. Respir. Dis.* **1977**, *116*, 1057−1064.
(23) Chio, L. C.; Bolyard, L. A.; Nasr, M.; Queener, S. F. Identification of a class of sulfonamides highly active against dihydropteroate synthase from *Toxoplasma gondii*, *Pneumocystis carinii*, and *Mycobacterium avium*. *Antimicrob. Agents Chemother.* **1996**, *40*, 727−733.
(24) Brophy, V. H.; Vasquez, J.; Nelson, R. G.; Forney, J. R.; Rosowsky, A.; Sibley, C. H. Identification of *Cryptosporidium parvum* dihydrofolate reductase inhibitors by complementation in *Saccharomyces cerivisiae*. *Antimicrob. Agents Chemother.* **2000**, *44*, 1019−1028.
(25) Lau, H.; Ferlan, J. T.; Brophy, V. H.; Rosowsky, A.; Sibley, C. H. Efficacies of lipophilic inhibitors of dihydrofolate reductase against parasitic protozoa. *Antimicrob. Agents Chemother.* **2001**, *45*, 187−195.
(26) Hastings, M. D.; Sibley, C. H. Pyrimethamine and WR99210 exert opposing selection on dihydrofolate reductase from *Plasmodium vivax*. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13137−13141.
(27) For a recent critical review of the serious problem of controlling pyrimethamine-resistant malaria in areas of the world where it is endemic, such as sub-Saharan Africa, see the following. Sibley, C. H.; Hyde, J. E.; Sims, P. F. G.; Plowe, C. V.; Kublin, J. G.; Mberu, E. K.; Cowman, A. F.; Winstanley, P. A.; Watkins, W. M.; Nzila, A. M. *Trends Parasitol.* **2001**, *17*, 582−588.
(28) Motulsky, H. *Intuitive Biostatistics*; Oxford University Press: New York, 1995; p 286.
(29) Gujadhur, R.; Venkataraman, D. Synthesis of diaryl ethers using an easy-to-prepare, air-stable, soluble copper(I) catalyst. *Synth. Commun.* **2001**, *31*, 2865−2879.

JM030438K