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

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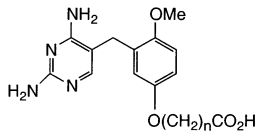
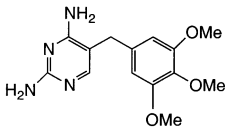
As part of an ongoing effort to discover novel small-molecule antifolates combining the enzyme-binding species selectivity of trimethoprim (TMP) with the potency of piritrexim (PTX), 10 previously unreported 2,4-diamino-5-(2′-methoxy-5′-substituted)benzylpyrimidines (**2**−**11**) containing a carboxyl group at the distal end of the 5′-substituent were synthesized and tested as inhibitors of dihydrofolate reductase (DHFR) from *Pneumocystis carinii* (Pc), *Toxoplasma gondii* (Tg), and *Mycobacterium avium* (Ma), three of the opportunistic pathogens frequently responsible for life-threatening illness in people with impaired immune systems as a result of HIV infection or immunosuppressive chemotherapy. The selectivity index of DHFR inhibition was evaluated by comparing the potency of each compound against the parasite enzymes with its potency against rat liver DHFR. 2,4-Diamino-5-[5′-(5-carboxy-1-pentynyl)-2′-methoxybenzyl]-pyrimidine (**3**) inhibited Pc DHFR with a selectivity index of 79 and was 430 times more potent than TMP. 2,4-Diamino-5-[5′-(4-carboxy-1-butynyl)-2′-methoxybenzyl]pyrimidine (**2**), with one less carbon than **3** in the side chain, had a selectivity index of 910 against Ma DHFR and was 43 times more potent than TMP. 2,4-Diamino-5-[5′-(5-carboxypentyl)-2′-methoxybenzyl]pyrimidine (**6**) had a selectivity index of 490 against Tg DHFR and was 320 times more potent than TMP. 2,4-Diamino-5-[5′-(6-carboxy-1-hexynyl)-2′-methoxybenzyl]pyrimidine (**4**), with one more carbon than **3**, was less potent against all three of the parasite enzymes than either **3** or **6** and also had a lower selectivity index than **3** against the Pc enzyme. However, **4** was the only member of the series with a selectivity index of >300 against both Tg and Ma DHFR. Given that PTX is at least 10 times more potent against rat DHFR than against *P. carinii* or *T. gondii* DHFR and that the selectivity index of several of the compounds matches or exceeds that of TMP as well as PTX, our results suggest that it may be possible to develop clinically useful nonclassical antifolates that are *both potent and selective* against the major opportunistic pathogens of AIDS.

Introduction

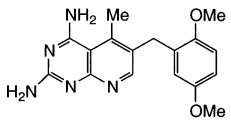
As part of a longstanding interest in the design and synthesis of small-molecule inhibitors of the dihydrofolate reductase (DHFR) enzymes from *Pneumocystis carinii* (Pc), *Toxoplasma gondii* (Tg), and *Mycobacterium avium* (Ma), three of the pathogenic organisms most often responsible for opportunistic morbidity and mortality in people with AIDS and other immune disorders,[1] we recently reported[2,3] that the 2,4-diamino-5-[2′-methoxy-5′-(4-carboxyalkyloxy)benzyl]pyrimidines **1a** and **1b** selectively inhibit DHFR from these three organisms compared with the enzyme from rat liver and that their potency is much higher than that of trimethoprim (TMP), the 2,4-diamino-5-(substituted benzyl)pyrimidine most widely used for the treatment and prophylaxis of pneumocystis pneumonia and toxoplasmosis. The most prominent feature of **1b** as a Pc DHFR inhibitor was that it surpassed TMP in terms of both potency and selectivity, a combination that has proved to be quite



1a: n = 3
1b: n = 4



Trimethoprim



Piritrexim

rare among the hundreds of diaminopyrimidine derivatives studied to date (reviewed in ref 2). Interestingly, the 2′,5′-substitution pattern of **1a** and **1b** was reminiscent of piritrexim (PTX),[4] which is more potent than TMP but is strongly selective for mammalian versus Pc DHFR. Thus, we had discovered in these compounds two rare examples in which the potency of PTX is combined with the desirable species selectivity of trimethoprim, a feature that was of considerable practical interest because PTX can only be given to patients in

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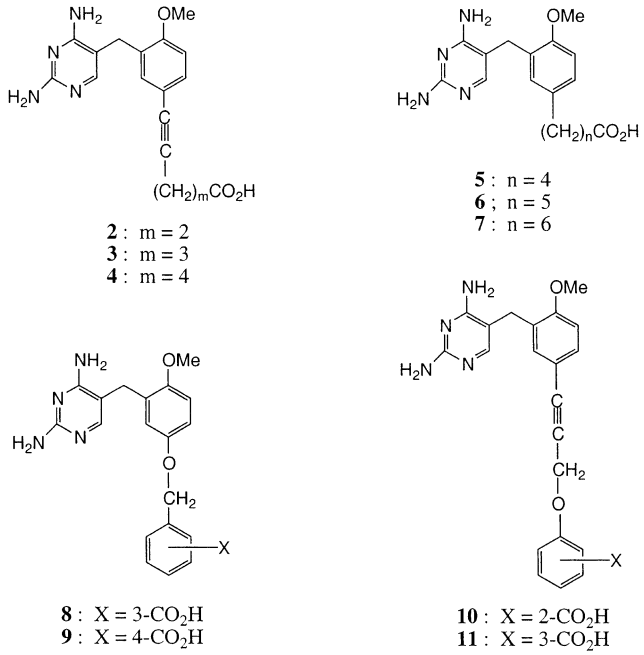


Figure 1. Structures of 2,4-diamino-5-(substituted benzyl)-pyrimidines **2**−**11**.

combination with a second drug, leucovorin (5-formyl 5,6,7,8-tetrahydrofolate), in order to prevent unacceptable side effects.[5] Computer modeling of the predicted binding of **1b** to Pc DHFR,[6] together with the ideas elegantly put forward by Kuyper and co-workers[7] as a rationale for the synthesis of 3′- and 4′-*O*-(*ω*-carboxyalkyl) analogues of TMP as selective inhibitors of *E. coli* versus rat DHFR, led us to hypothesize that selective binding of **1b** to Pc versus rat or human DHFR may be due to the ability of the terminal COOH group to interact with strongly basic residues, Arg 75 and Lys 37, in the active site of the Pc enzyme.[8,9] In the case of human DHFR, this interaction would not be possible; indeed, when **1b** was docked into the active site of human DHFR,[6] only a weak interaction with a nonbasic Gln35 residue seemed possible.

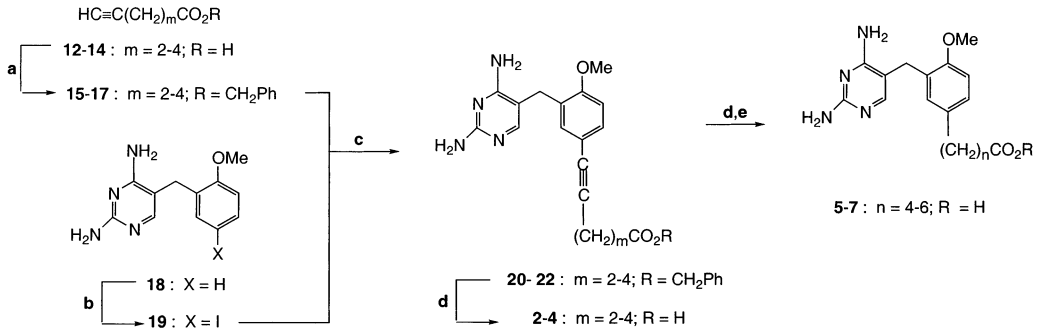
To further explore the scope of this finding, we have now synthesized a series of 10 analogues of **1a** and **1b** in which the nature of the linker between the COOH group and the 5′-position of the benzyl ring was altered as depicted in Figure 1. Three of the new analogues were of particular interest in terms of structure−activity correlation. One compound (**3**), in which the oxygen atom at the 5′-position was replaced by a carbon−carbon triple bond, was found to be twice as potent as **1b** against both Pc and rat DHFR, demonstrating that (i) a polar atom at the 5′-position is not necessary and (ii) a rigid linear bond between the first two atoms of the side chain is well tolerated. A second analogue containing this feature, compound **2**, was weaker and less selective than **3** against the Pc enzyme but was nonetheless a very potent and selective inhibitor of Ma DHFR despite its shorter side chain. The third analogue, compound **6**, in which the triple bond of **3** was reduced to a saturated carbon−carbon bond, was a weaker inhibitor of Pc and Ma DHFR than either **1b** or **3** but proved to be among the most selective inhibitors of the Tg enzyme reported to date.

Chemistry

As shown in Scheme 1, the well-known Pd-catalyzed Sonogashira reaction between an aryl halide and a terminal acetylene[10] provided convenient access to the analogues of **1a** and **1b** with a 5′-(*ω*-carboxy-1-alkynyl), and ultimately a 5′-(*ω*-carboxyalkyl), side chain. The key intermediates needed for the coupling reaction were the terminal acetylenic esters **15**−**17** and the iodide **19**, the latter of which was obtained from 2,4-diamino-5-(2′-methoxybenzyl)pyrimidine (**18**) by reaction with iodine monochloride as described by Calas and co-workers.[11] The esters were readily prepared from the Cs salts of the commercially available acids **12**−**14** with benzyl bromide in dry DMF. Without purification, **15**−**17** were condensed with **19** in the presence of Et_3N and catalytic amounts of $(Ph_3P)_2PdCl_2$ and CuI in dry DMF at 60 °C under N_2. Because of their limited solubility in nonpolar solvents, the resulting acetylenic esters **20**−**22** did not lend themselves easily to column chromatography on silica gel and therefore were converted directly to the acids **2**−**4** by brief treatment with NaOH in DMSO at room temperature. The yield of **2** and **4** after purification by preparative HPLC on C_{18} silica gel using an isocratic mixture of MeCN and 0.1 M NH_4OAc, pH 7.4, as the eluent was 32% and 36%, respectively. In the case of **3**, we did not use HPLC but instead attempted to recrystallize the compound from MeOH. This proved to be a bad choice, inasmuch as heating the acetylenic acid in MeOH led to a sharp drop in final purified yield because of extensive degradation to a resinous, base-insoluble product of unknown composition (the crude yield had been comparable to that of **2** and **4**).

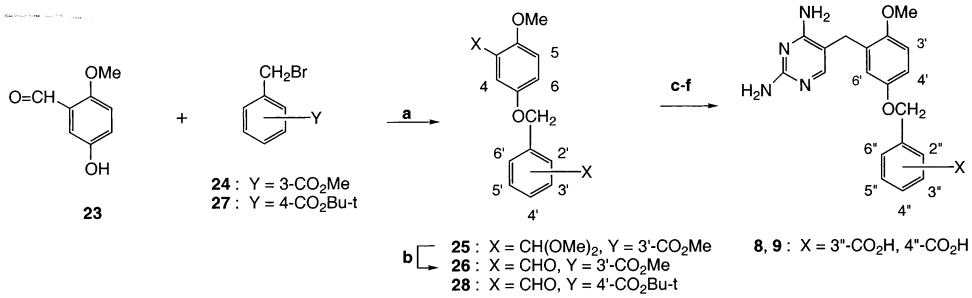
We had originally chosen benzyl esters in Scheme 1 with the intent of using catalytic hydrogenation (H_2/Pd−C) to reductively cleave the benzyl group and simultaneously reduce the triple bond to form the saturated acids **5**−**7**. However, when hydrogenation was attempted with **21** in DMF solution, the triple bond was reduced but the ester group survived. When reduction was performed in a 1:1 mixture of DMF and glacial AcOH with a view to protonating the diaminopyrimidine moiety, which we suspected might be deactivating the Pd−C catalyst enough to prevent debenzylation, the product proved to be totally insoluble in base and thus could not be a carboxylic acid. Fortunately, conversion of the acetylenic esters to the desired saturated acids was achieved satisfactorily by first cleaving the ester group with NaOH and hydrogenating the Na salt in situ in aqueous solution or, in the case of **7**, by isolating the free acid and carrying out the reduction in DMF solution. In contrast to **3** (see above), purification of **5** was achieved satisfactorily by recrystallization from MeOH. Preparative HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4) was used in the case of **6**; however, analytically pure **7** could be obtained by simply dissolving the product of the hydrogenation reaction in dilute NaOH and acidifying the solution with AcOH. The overall two-step yields of the purified acids **5**, **6**, and **7** was 35%, 46%, and 77%, respectively. The ^1H NMR spectra of all three compounds were consistent with reduction of the C≡C bond in that there were four additional CH_2 hydrogens and a slight upfield shift for the aromatic protons ortho to the *ω*-carboxyalkyl side chain.

Scheme 1[a]



[a] Reactants: (a) $PhCH_2Br/Cs_2CO_3$/DMF; (b) ICl/AcOH; (c) $(Ph_3P)_2PdCl_2$, CuI, Et_3N, DMF; (d) NaOH, DMSO; (e) H_2/10% Pd−C, DMF.

Scheme 2[a]

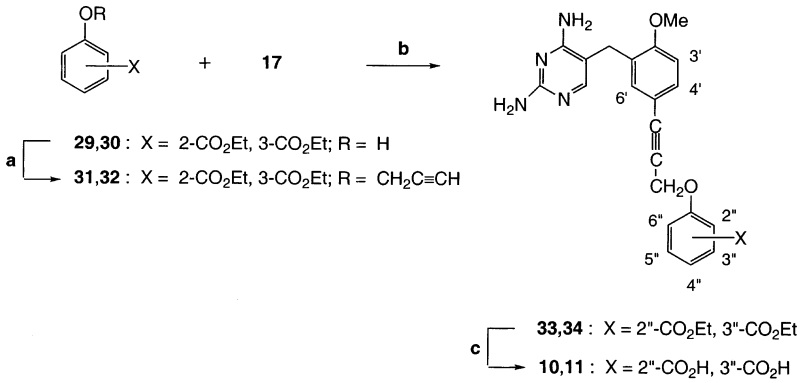


[a] Reactants: (a) K_2CO_3, 18-crown-6; (b) HCl; (c) 3-morpholinopropionitrile, NaOEt; (d) $PhNH_2{\bigcirc}HCl$; (e) $H_2NC(=NH)NH_2$; (f) NaOH.

Although we had originally planned to prepare compounds **8** and **9** from the Na salt of 2,4-diamino-5-(2′-methoxy-4′-hydroxybenzyl)pyrimidine, as we had done earlier to obtain **1a** and other 5′-(ω-carboxyalkoxy) analogues from ω-bromoalkanoic acids,[2] efforts to apply this method using methyl 4-(bromomethyl)benzoate were thwarted by several side reactions. To circumvent this problem, we chose to reverse the synthetic sequence so that formation of the diaminopyrimidine ring would be the last step. Thus, we carried out the synthesis of analogues **8** and **9** according to Scheme 2. Condensation of 3-hydroxy-6-methoxybenzaldehyde (**23**)[12] with methyl 3-(bromomethyl)benzoate (**24**) in the presence of K_2CO_3 and a catalytic amount of a crown ether as described in our earlier paper[2] unexpectedly gave a 79% yield of the previously unknown acetal **25** after recrystallization from MeOH; however, the latter was readily converted to the desired aldehyde **26** by treatment with dilute HCl. The structure of **25** was deduced from its elemental analysis, the absence of absorbance at 1685 cm^{-1} in the infrared spectrum, and the presence, in the ^1H NMR spectrum, of characteristic signals at δ 3.35 and δ 5.64 for the $CH(OMe)_2$ group. That O-alkylation had occurred in the expected manner was also evident from the presence of a peak at δ 5.07 corresponding to the OCH_2 protons. The overall two-step yield of **26** from **24** was 69%. Interestingly, when **23** was condensed with *tert*-butyl 4-(bromomethyl)benzoate (**27**), which we had used earlier in another synthesis,[13] only the expected aldehyde **28** was obtained even though the product was recrystallized from MeOH. The probable reason for this difference in behavior between the meta and para esters is that dilute citric acid was used during the workup of the reaction of **24** but not **27**. Thus, it is possible that some residual citric acid was present during the recrystallization from MeOH. The remaining steps were performed according to one of the standard routes for TMP analogues, in which the aldehyde is treated sequentially with 3-morpholinopropionitrile, aniline hydrochloride, guanidine,[14] and in this case NaOH to cleave the ester groups to acids. Although none of the intermediates in the four-step sequence from **26** and **28** to the final products **8** and **9**, respectively, were isolated in the pure state before proceeding to the next step, the final acids were purified by preparative HPLC on C_{18} silica gel using 18% MeCN in 0.l M NH_4OAc, pH 7.4, as the eluent. Large amounts of nonpolar impurities were easily removed in this manner, and the identity and purity of the products were confirmed by microchemical and ^1H NMR analyses. As expected, **8** and **9** were soluble in dilute aqueous base and precipitated at a weakly acidic pH.

The final two compounds in this series of analogues, **10** and **11**, were synthesized according to Scheme 3, starting from ethyl salicylate (**29**) and ethyl 3-hydroxybenzoate (**30**), respectively. Condensation of the phenols with propargyl bromide in the presence of K_2CO_3 and a crown ether catalyst in DMF afforded the *O*-propargyl ethers **31** and **32**. Further reaction of the ethers with iodide **19** in the presence of $(Ph_3P)_2PdCl_2$, CuI, and Et_3N, followed by saponification, afforded the desired acids in overall two-step yields of 21% and 24%. Neither **31** and **32** nor the ester intermediates **33** and **34** from the Sonogashira reaction were chromatographed, but the final products were carefully purified by preparative HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4) and characterized by their ^1H NMR spectra in DMSO-d_6 solution, which displayed the expected resonance signals at δ 3.50 (benzylic CH_2), 3.82 (OMe), 5.04 (CH_2O), and 7.40 (pyrimidine 6-H) in addition to appropriate peaks for the various protons of the phenyl rings.

Scheme 3[a]



[a] Reactants: (a) $HC{\equiv}CCH_2Br$, K_2CO_3, 18-crown-6; (b) $(Ph_3P)_2PdCl_2$, CuI, Et_3N; (c) NaOH.

Table 1. Inhibition of *P. carinii*, *T. gondii*, *M. avium*, and Rat Dihydrofolate Reductase by 2,4-Diamino-5-(2′-methoxy-5′-substituted benzyl)pyrimidines

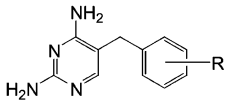
	IC_{50} [a] (μM)				selectivity index[b]		
compd	*P. carinii*	*T. gondii*	*M. avium*	rat	*P. carinii*	*T. gondii*	*M. avium*
1a	0.25 (0.21−0.30)	0.18 (0.15−0.20)	0.0048 0.0040−0.0059)	2.7 (2.5−2.9)	11 (8.5−13)	16 (12−19)	560 (430−730)
1b	0.054[c] (0.045−0.068)	0.11 (0.066−0.17)	0.058[c,d] (0.02−0.16)	4.6[e] (3.2−6.0)	85 (79−97)	42 (19−72)	79 (19−230)
2	0.13 (0.12−0.15)	0.097 (0.093−0.10)	0.0044 (0.0040−0.0049)	4.0 (3.5−4.6)	31 (24−39)	41 (34−39)	910 (700−1100)
3	0.028 (0.025−0.03)	0.032 (0.027−0.037)	0.0078 (0.0064−0.0095)	2.2 (1.9−2.5)	79 (63−100)	69 (52−91)	280 (2000−380)
4	0.87 (0.77−0.98)	0.072 (0.064−0.081)	0.041 (0.31−0.56)	25 (19−32)	28 (19−42)	340 (230−510)	590 (330−1100)
5	0.15 (0.13−0.18)	0.058 (0.052−0.065)	0.035 (0.025−0.048)	3.2 (2.5−4.0)	21 (14−31)	54 (39−77)	91 (51−160)
6	0.15 (0.13−0.17)	0.0084 0.075−0.009)	0.016 (0.011−0.022)	4.1 (3.1−5.3)	27 (18−41)	490 (340−700)	260 (140−460)
7	0.53 (0.43−0.65)	0.030 (0.026−0.034)	0.089 (0.071−0.11)	4.6 (4.2−5.0)	8.6 (6.4−11)	150 (120−190)	52 (38−70)
8	0.89 (0.73−1.1)	0.60 (0.53−0.69)	0.12 (0.1−0.14)	19 (14−24)	21 (13−33)	31 (21−46)	150 (100−230)
9	1.2 (0.098−1.5)	2.0 (1.7−2.2)	0.060 (0.053−0.069)	21 (13−29)	17 (9.6−30)	11 (6.4−18)	340 (210−560)
10	2.3 (2.1−2.5)	0.50 (0.44−0.56)	0.036 (0.032−0.041)	6.2 (5.2−7.4)	2.7 (2.1−3.5)	13 (9.3−17)	170 (130−230)
11	5.6[e] (4.4−7.2)	1.6 (1.1−2.4)	0.057 0.050−0.065)	14 (11−16)	2.4 (1.6−3.7)	8.3 (4.6−15)	240 (170−250)
TMP[f]	13 (10−16)	2.8 (2.4−3.3)	0.30 (0.26−0.35)	180 (160−210)	14 (10−20)	65 (48−87)	610 (460−810)
PTX[g]	0.013 (0.009−0.017)	0.0043 (0.0040−0.0046)	0.00061 (0.0053−0.0007)	0.0033 (0.0029−0.0039)	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 limits. The difference in IC_{50} between rat liver DHFR and each of the parasite enzymes was determined to be statistically significant at $P < 0.01$. [b] SI = IC_{50}(rat liver)/IC_{50}(Pc, Tg, or Ma). Numbers in parentheses are 95% confidence intervals, and represent a range calculated by dividing the lower end of the 95% confidence interval for rat liver DHFR by the high end of the 95% confidence interval for Pc, Tg, or Ma DHFR. [c] Mean of three experiments on different days. [d] The IC_{50} of **1b** against Ma DHFR was inadvertently recorded as 0.0058 μM in ref 2. As a result, the SI calculated therein for this compound was off by a factor of 10. [e] Mean of two experiments on different days. [f] TMP = trimethoprim, 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine. Data are taken from ref 2. [g] PTX = piritrexim, 2,4-diamino-5-methyl-6-(2′,5′-dimethoxybenzyl)pyrido[2,3-*d*]pyrimidine. Historical IC_{50} values for PTX, obtained with an older sample and cited previously for comparison purposes (e.g., see ref 2), were the following: Pc DHFR, 0.031 μM; Tg DHFR, 0.017 μM; rat liver, 0.0015 μM. The data in Table 1 were obtained with a newer sample of PTX kindly provided by Dr. Gary Smith, Glaxo-Wellcome Co., Research Triangle Park, NC. The IC_{50} of PTX against Ma DHFR had not been determined prior to this work.

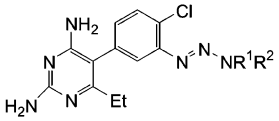
Enzyme Inhibition Assays

Compounds **2**−**11** were tested for the ability to inhibit Pc, Tg, and Ma DHFR according to the standardized spectrophotometric assays used in the Queener laboratory,[15,16] and their potencies and selectivities were compared with updated values for **1a**, **1b**, TMP, and PTX. The results are presented in Table 1 and discussed for each of enzyme in the following sections.

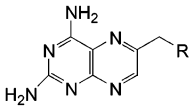
***Pneumocystis carinii* DHFR.** As shown in Table 1, the most potent inhibitor of Pc DHFR in this group was the 5′-(5-carboxy-1-pentynyl) analogue **3**, with an IC_{50} of 0.028 μM. Thus, against the enzyme from this organism, **3** was slightly more potent than **1b**. However, because potency increased by about the same extent against rat DHFR, the selectivity index (SI) as defined in Table 1, footnote b, was not changed significantly. Although there was no substantial improvement in potency or selectivity, this finding was nonetheless of interest because it demonstrated that the oxygen atom at the 5′-position of **1b** can be safely replaced by a carbon−carbon triple bond where Pc DHFR binding is concerned. It may be noted that the $C{\equiv}C(CH_2)_3CO_2H$ side chain in **3** contains the same number of atoms as the $O(CH_2)_4CO_2H$ side chain in **1b** but is less flexible



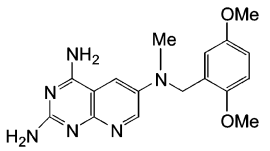
6 : R = 2'-OMe-5'-[$(CH_2)_5CO_2H$ (IC_{50} = 0.0084 μM, SI = 490)
35 : R = 2'-OMe-5'-$O(CH_2)_6CO_2H$ (IC_{50} = 0.18 μM, SI = 65)[2]
36 : R = 3',5'-$(OMe)_2$, 4'-(1-pyrrolyl) (IC_{50} = 0.47 μM, SI = 160)[17]



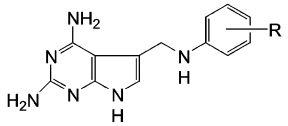
37 : R^1R^2 = morpholino (IC_{50} = 0.19 μM, SI = 140)[18]
38 : $R^1 = R^2$ = Me (IC_{50} = 0.31 μM, SI = 61)[18]



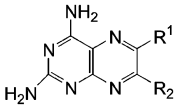
39 : R = $CH_2SC_6H_5$ (IC_{50} = 0.77 μM, SI = 319)[19]
40 : R = *N*-Dibenz[*b*,*f*]azepinyl (IC_{50} = 0.043 μM, SI = 102)[3g]



41 (IC_{50} = 0.028 μM, SI = 304)[17]



42 : R = 2',5'-$(OMe)_2$ (IC_{50} = 1.7 μM, SI = 92)[20]
43 : R = 2',3'-$(CH_2)_4$ (IC_{50} = 1.1 μM, SI = 54)[20]



44 : $R^1 = R^2 = C_6H_5$ (IC_{50} = 0.24 μM, SI = 58)[16]
45 : R^1 = 3-MeC_6H_4, $R^2 = NH_2$ (IC_{50} = 0.018 μM, SI = 47)[16]
46 : $R^1 = COC_6H_5$, $R^2 = C_6H_5$ (IC_{50} = 0.86 μM, SI = 66)[16]

Figure 2. Structures of *T. gondii* DHFR inhibitors previously reported to be more potent as well as more selective than trimethoprim.[2] Data for compound **5** are taken from Table 1. The calculated SI value given for **40** is based on titrations performed with human rather than rat DHFR and on assay conditions different from those used for the other analogues. When **40** was tested against Tg and rat liver DHFR according to the standardized method used by the Queener laboratory,[15] its selectivity was much lower.

because of the geometric constraints imposed by the triple bond. From the enzyme binding data, it appears that the terminal carboxyl group may nonetheless be adequately positioned in the active site to interact with Arg75. Of interest in this regard is that the shorter analogue **2** was a slightly weaker inhibitor of both Pc and rat DHFR than **3**, a pattern also observed in the previous series of 5′-(ω-carboxyalkoxy) analogues.[2] However, compound **4** displayed a more striking decrease in potency than either **2** or **3** against both the Pc and rat enzymes. Finally, with regard to the potency and selectivity of the alkynes **5**−**7** versus the alkanes **2**−**4**, a dramatic pattern of structure−activity relationships did not emerge, and we were not able to determine whether a carboxyalkyne or carboxyalkane side chain was superior.With respect to compounds **8**−**11** as inhibitors of Pc versus rat DHFR, it can be seen from Table 1 that they are all considerably weaker than **1b** or **3** against the Pc enzyme and that the potency difference is greater against this enzyme than against the rat enzyme, resulting in an overall decrease in selectivity. At least with these examples, therefore, it appears that the introduction of a bulky second phenyl ring in the side chain, together with replacement of the aliphatic COOH group by an aromatic one, was unfavorable. It may be noted that when the total number of side chain atoms is added, the number of atoms separating the carboxyl group from the 2′-methoxyphenyl ring is five in **8**, six in **9** and **10**, and seven in **11**. The corresponding number of atoms is four in **2** and **5**, five in **3** and **6**, and six in **4** and **7**. Thus, among the analogues with an extra phenyl ring, **8**−**10** most closely approximate **1b** and **2**−**7** in terms of the total number of atoms (aromatic and aliphatic) separating the carboxyl group from the rest of the molecule, whereas in **11** there is one additional atom.

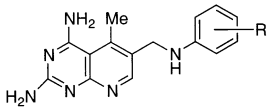
***Toxoplasma gondii* DHFR.** In contrast to Pc DHFR, the most potent inhibitor of the Tg enzyme was **6**, with an IC_{50} of 0.0084 μM (Table 1). Whereas this corresponded to a roughly 1-log increase in binding relative to **1b**, a comparable effect was not observed with rat DHFR. As a result, the selectivity index of **6** was 490 compared with only 42 in the case of **1b**. Thus, replacement of the oxygen atom at the 5′-position of **1b** by a saturated carbon resulted in a considerable improvement in both potency and selectivity. Interestingly, this was in contrast to the decreased potency and selectivity of **6** relative to **1b** against Pc DHFR and of **6** relative to **1a** against Ma DHFR.

The published literature on Tg DHFR inhibitors contains a large number of mono-, di-, and tricyclic 2,4-diaminopyrimidines that are much more potent than TMP but only a handful that are *both more potent and more selective* against Tg DHFR versus rat DHFR under our standardized assay conditions. As summarized in Figure 2, these compounds include **35**,[2] a two-carbon homologue of **1a**, the TMP analogue **36** (epiroprim),[17] the 2,4-diamino-5-aryl-6-ethylpyrimidines **37** and **38**,[18] the 2,4-diamino-6-substituted pteridines **39**[19] and **40**,[3g] the 2,4-diamino-6-benzylaminopyrido[2,3-*d*]pyrimidine **41** (albeit only when tested against Tg versus human instead of rat DHF and under assay conditions different from those typically used with the other compounds reported),[17] and the 2,4-diamino-5-substituted pyrrolo-[2,3-*d*]pyrimidines **42** and **43**.[20] Very surprisingly, three 2,4-diamino-6,7-disubstituted pteridines (**44**−**46**) have also been reported to be among this select group.[16] Bulky substitution at the 7-position of 2,4-diamino-

pteridines is generally considered very unfavorable for DHFR binding. However, it is possible that in the case of the Tg enzyme the selectivity of these compounds relative to rat DHFR reflects the fact that the Tg enzyme is a larger difunctional protein containing both a thymidylate synthase (TS) and DHFR domain.[21] This question will presumably remain unanswered until the 3D structure of the Tg enzyme is fully solved. On the basis of its 330-fold increase in potency and 7.5-fold increase in selectivity relative to TMP against Tg DHFR and in light of its very good profile when compared to the inhibitors shown in Figure 2, compound **6** may be viewed as a most interesting lead for further structure−activity optimization.

***Mycobacterium avium* DHFR.** As indicated in Table 1, compound **2** was a much better inhibitor of Ma DHFR than TMP and was similar to the previously reported 5′-*O*-(5-carboxybutyloxy) analogue **1a**. Indeed, with its nearly 3-log selectivity for Ma DHFR relative to the rat enzyme and its approximately 70-fold superiority over TMP in terms of potency, **2** ranks among the best inhibitors of Ma DHFR described to date. Although it stands out among the other compounds tested, there were also several analogues with respectable SI values in the 200−600 range. However, it should be noted that high SI values were not always accompanied by high potency. Thus, while the calculated SI of 590 listed for compound **4** had a range (330−1100) that overlapped that of **2** (700−1100), its potency against both Ma and rat DHFR was considerably lower.

Suling and co-workers[22] recently published data on a library of almost 80 2,4-diaminopyrido[2,3-*d*]pyrimidines with either arylamino or arylthiomethyl groups at the 6-position as inhibitors of Ma versus human DHFR. The most potent member of their series against Ma DHFR was 2,4-diamino-6-(2′-methyl-4′-chloroanilino)methyl-5-methylpyrido[2,3-*d*]pyrimidine (**47**), with an IC_{50} of 0.000 19 μM. However, because **47** was also a potent inhibitor of a mammalian DHFR (human in this case), its SI value was only 15. A second compound, 2,4-diamino-6-(2′,5′-diethoxyanilino)methyl-5-methylpyrido[2,3-*d*]pyrimidine (**48**), on the other hand, had IC_{50} values of 0.000 84 and 2.3 μM against Ma and human DHFR, respectively. Unfortunately an IC_{50} value



47: R = 2′-Me-4′-Cl
48: R = 2′,5′-$(OEt)_2$

for **48** against rat DHFR was not reported, and the assay conditions differed in some respects from those used routinely by the Queener laboratory.[16] The binding of inhibitors to DHFR can vary substantially depending on the pH, the temperature, and other variables.[23] Thus, it remains to be determined how **2** and **48** would compare if they were tested under identical assay conditions in the same laboratory. Irrespective of the outcome of such a comparison, the excellent combination of potency reported here over a range of 2,4-diamino-5-(substituted benzyl)pyrimidines, including some that are not particularly potent or selective against Pc or Tg DHFR, demonstrates that potency and selectivity against Ma DHFR do not require the inhibitor to be a fused-ring 2,4-diaminopyrimidine.

The 3D structure of the ternary complex of Ma DHFR with NADPH and TMP has not been reported. However, it is noteworthy that in a recent study of the closely related enzyme from *M. tuberculosis* by X-ray crystallography Li and co-workers[24] suggested that TMP analogues with extended hydrophobic substitution on the benzyl ring might be designed to selectively fit into a binding pocket that does not appear to be available in the active site of mammalian DHFR enzymes. Although they are 2′,5- rather than 3′,4′,5-trisubstituted benzyl derivatives, it would be of interest to determine whether **2**−**4** can bind to the Ma enzyme in such a way as to allow the hydrocarbon portion of the 5′-(ω-carboxy-1-alkynyl) side chain to make van der Waals contact with this hydrophobic domain.

Summary

Four new examples of small-molecule antifolates of the 2,4-diamino-5-(substituted benzyl)pyrimidine family, each more potent and/or more selective than TMP against at least one of three different opportunistic pathogens of AIDS, were discovered in this work. One of them, 2,4-diamino-5-[5′-(4-carboxy-1-pentynyl)-2′-methoxybenzyl]pyrimidine (**3**) inhibited Pc DHFR with a selectivity index of 79 relative to the rat liver enzyme and was 430 times more potent than TMP. A second compound, 5-[5′-(5-carboxy-1-butynyl)-2′-methoxybenzyl]pyrimidine (**2**), with one less carbon than **3** in the side chain, had a selectivity index of 910 against Ma DHFR and was 43 times more potent than TMP. The third compound, 2,4-diamino-5-[5′-(5-carboxypentyl)-2′-methoxybenzyl]pyrimidine (**6**) had a selectivity index of 490 against Tg DHFR and was 320 times more potent than TMP. The fourth compound, 2,4-diamino-5-[5′-(6-carboxy-1-hexynyl)-2′-methoxybenzyl]pyrimidine (**4**), was less potent against all three of the parasite enzymes than either **3** or **6** and also had a lower selectivity index than **3** against the Pc enzyme. However, it was the only member of the series with a selectivity index of >300 against both Tg and Ma DHFR. Attesting to the likelihood that subtle differences exist in the 3D structure of the active site of these three enzymes, each inhibitor was optimally active against only one of the enzymes despite the fact that there were only minor differences in molecular structure among the three compounds.

While the compounds described in this and our preceding paper[2] on 2,4-diamino-5-[2′-methoxy-5-(ω-carboxyalkoxy)benzyl]pyrimidines (e.g., **1a** and **1b**) show promise in terms of in vitro potency and selectivity, it has not been shown that they are efficiently taken up by *P. carinii*, *T. gondii*, or *M. avium*. Thus, before any practical clinical utility for such compounds can be established, it must be shown that they are able to penetrate these organisms in at least one stage of the life cycle or, if they cannot, that a suitable prodrug or other mode of cellular drug delivery can be developed. Although they were outside the scope of the work reported here, future studies of this nature would be of potential therapeutic interest.

Experimental Section

IR spectra were obtained on a Perkin-Elmer model 781 double-beam recording spectrophotometer. Only peaks with wavenumbers above 1200 cm^{-1} are reported. ^1H NMR spectra

were recorded 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), triplet (t), doublet of triplets (dt), or pentet (p). Integrated peak areas are not listed when the resonance signal was partly obscured by water or DMSO or in the case of NH_2 groups on the pyrimidine ring. Signals for the aromatic protons in compounds with two phenyl rings are identified according to the numbering in Schemes 2 and 3. 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 were performed on C_{18} radial compression cartridges (Millipore, Milford, MA; analytical, 5 μm particle size, 5 mm × 100 mm; preparative, 15 μm particle size, 25 mm × 100 mm). Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Fisher, Pittsburgh, PA) and are not corrected. 3-Morpholinopropionitrile was prepared by adding acrylonitrile dropwise to an equimolar amount of morpholine in an ice bath and stirring the mixture at room temperature for 1 h. The resulting light-yellow oil did not require purification and was used directly. 2,4-Diamino-5-(2′-methoxybenzyl)pyrimidine (**18**) and 2,4-diamino-5-(5′-iodo-2′-methoxybenzyl)pyrimidine (**19**) were prepared according to the literature: **18**, 42% yield, mp 160−161 °C (lit.[11] 160 °C); **19**, 66% yield, mp 207−208 °C (lit.[11] 205 °C). 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. Where microanalytical results were consistent with residual acetic acid, its presence in the sample was confirmed by the finding of a methyl signal at δ 1.9 in the ^{1}H NMR spectrum.

2,4-Diamino-5-[5′-(4-carboxy-1-butynyl)-2′-methoxy)-benzyl]pyrimidine (2). **Step 1.** Benzyl 4-pentynoate (**15**) was prepared by stirring a solution of 4-pentynoic acid (**12**) (1.96 g, 0.02 mol) in dry DMF (25 mL) with Cs_2CO_3 (3.25 g, 0.01 mol) for 10 min, followed by addition of benzyl bromide (2.38 mL, 3.42 g, 0.02 mol). After 18 h of stirring at room temperature, the solvent was removed by rotary evaporation (vacuum pump) and the residue was partitioned between EtOAc and H_2O. Evaporation of the organic layer gave **15** as an oil whose NMR spectrum showed that it still contained some DMF and a trace of benzyl bromide but was suitable for use in the next step.

Step 2. A stirred mixture of **15** (1.3 g, estimated by NMR to contain ca. 6.0 mmol), iodide **19** (1.42 g, 4.0 mmol), $(Ph_3P)_2PdCl_2$ (15 mg), CuI (1 mg), and Et_3N (5 mL) in dry DMF (5 mL) was heated at 60 °C for 20 h. A clear solution formed after ca. 1 h. The solvent was removed by rotary evaporation, and the residue was triturated successively with isooctane and H_2O. Recrystallization from EtOH afforded **20** as a light-yellow powder (1.59 g, mp 208 °C), which was used in the next step without additional purification.

Step 3. A solution of **20** (416 mg, 1.0 mmol) in dry DMSO (4 mL) was treated dropwise with 1 N NaOH with swirling. The mixture was diluted immediately with H_2O (40 mL), acidified with 10% AcOH, and chilled in ice. The precipitate was collected and dried to obtain a beige solid: crude yield 239 mg. Analytically pure **2** for bioassay was obtained by preparative HPLC (C_{18} silica gel, 13% MeCN in 0.1 M NH_4OAc, pH 7.4). Appropriately pooled fractions were concentrated and freeze-dried: yield 123 mg (32% based on the amount of **19** used); mp 162−163 °C (softening without giving a true melt); IR (KBr) ν 3500−2800 (broad), 3350, 3210, 2930, 1665, 1560−1530, 1505, 1460, 1405, 1295, 1115 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.50 (m, 4H, CH_2CH_2, partly overlapped by DMSO peak), 3.49 (s, 2H, bridge CH_2), 3.87 (s, 3H, OMe), 5.84 (br s, NH_2), 6.16 (br s, NH_2), 7.00 (m, 2H, aryl 3′- and 6′-H), 7.22 (d, J = 9 Hz, 1H, aryl 4′-H), 7.39 (s, 1H, pyrimidine 6-H). Anal. ($C_{19}H_{22}N_4O_5$·0.2AcOH·2.5H_2O) C, H, N.

2,4-Diamino-5-[5′-(4-carboxy-1-pentynyl)-2′-methoxy)-benzyl]pyrimidine (3). **Step 1.** Benzyl 5-hexynoate (**16**) was prepared by stirring a solution of 5-hexynoic acid (**13**) (1.12 g, 0.01 mol) in dry DMF (25 mL) with Cs_2CO_3 (1.63 g, 0.005 mol) for 10 min, followed by addition of benzyl bromide (1.19 mL, 1.71 g, 0.01 mol). After 18 h of stirring at room temperature, the solvent was removed by rotary evaporation (vacuum pump) and the residue was partitioned between EtOAc and H_2O. Evaporation of the organic layer gave **16** as an oil suitable for use directly in the next step.

Step 2. A stirred mixture of **16** (0.65 g, estimated by NMR to contain ca. 36.0 mmol), iodide **19** (1.42 g, 4.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), CuI (1 mg), and Et_3N (3 mL) in dry DMF (3 mL) was heated at 60 °C for 72 h. The solvent was removed by rotary evaporation, and the residue was triturated successively with isooctane and H_2O. Attempted recrystallization from EtOH gave only a gum. Therefore, the solvent was evaporated, and the residue was redissolved in hot THF (40 mL). A small amount of insoluble material was filtered off, the filtrate was concentrated to dryness, and the residue, containing ester **21**, was taken up directly in dry DMSO (6 mL). The solution was treated dropwise with 1 N NaOH (6 mL) and then diluted with H_2O (100 mL) and chilled. A small amount of residual nonsaponifiable material was removed by filtration, and the filtrate was acidified with 10% AcOH. The precipitate was collected and dried in a lyophilization apparatus to obtain a beige solid. Attempted recrystallization from hot MeOH led to separation of a brown insoluble gum, suggesting that decomposition was occurring. The methanolic solution was immediately decanted and left to cool passively in the hood at room temperature, producing the first crop of analytically pure **3** as white crystals weighing 32 mg (5%): mp 121−124 °C (softening without giving a true melt); IR (KBr) ν 3500−2800 (broad), 3350, 3210, 2930, 1665, 1560−1530, 1505, 1460, 1405, 1295, 1115 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.72 (p, J = 7 Hz, 2H, C≡CCH$_2$*CH$_2$*CH$_2$), 2.37 (two overlapping t, 4H, C≡C*CH$_2$*CH$_2$*CH$_2$*), 3.50 (s, 2H, bridge CH_2), 3.80 (s, 3H, OMe partly overlapped by H_2O), 5.86 (br s, NH_2), 6.20 (br s, NH_2), 6.95 (m, 2H, aryl 3′- and 6′-H), 7.24 (dd, J = 8 Hz, J = 2 Hz, 1H, aryl 4′-H), 7.39 (s, 1H, pyrimidine 6-H). Another 153 mg (24%) of less pure **3** was obtained from the mother liquor and used for hydrogenation as described below. Anal. ($C_{18}H_{20}N_4O_3$·0.5AcOH·0.2 H_2O) C, H, N.

2,4-Diamino-5-[5′-(6-carboxy-1-hexynyl)-2′-methoxy)-benzyl]pyrimidine (4). Step 1. Benzyl 6-heptynoate (**17**) was prepared by stirring a solution of 6-heptynoic acid (**14**) (1.26 g, 0.01 mol) in dry DMF (15 mL) with Cs_2CO_3 (1.63 g, 0.005 mol) for 10 min, followed by addition of benzyl bromide (1.19 mL, 1.71 g, 0.01 mol). After 18 h of stirring at room temperature, the solvent was removed by rotary evaporation (vacuum pump) and the residue was partitioned between EtOAc and H_2O. Evaporation of the organic layer gave **17** as an oil suitable for use directly in the next step.

Step 2. A stirred mixture of **17** (0.65 g, estimated by NMR to contain ca. 3.0 mmol), iodide **19** (0.71 g, 2.0 mmol), $(Ph_3P)_2PdCl_2$ (20 mg), CuI (2 mg), and Et_3N (5 mL) in dry DMF (5 mL) was heated at 65 °C for 2.5 h. Additional amounts of $(Ph_3P)_2PdCl_2$ (10 mg) and CuI (1 mg) were added, and heating was continued for 18 h. The solvent was evaporated under reduced pressure, and the residue was triturated successively with isooctane and H_2O. The solid was taken up in DMSO (5 mL) and treated with a solution of NaOH (220 mg, 4.4 mmol) in H_2O (1 mL). The solution was diluted to 80 mL with H_2O, then chilled, adjusted to ca. pH 8 with 10% AcOH, and filtered to remove a trace of insoluble material. The filtrate was subjected to preparative HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4, 10 mL/min) and fractions eluting in the major peak (ca. 12 min on the analytical column at 1.0 mL/min) were pooled and freeze-dried to obtain **4** as a white solid: yield 292 mg (36%); mp 187−189 °C (softening without giving a true melt); IR (KBr) ν 3370, 3220, 2950, 2880, 2850br, 1690sh, 1670, 1565, 1540, 1505, 1465, 1450sh, 1430, 1405, 1375s, 1340, 1320, 1295, 1350, 1300 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.63 (m, 4H, *CH$_2$*CH$_2$*CH$_2$CO$_2$H), 2.18 (t, J = 7 Hz, 2H, *CH$_2$*CO$_2$H), 2.39 (t, J = 7 Hz, C≡C*CH$_2$*), 3.80 (s, OMe, partly overlapped by broad H_2O peak), 5.74 (br s, NH_2), 6.09 (br s, NH_2), 6.93 (d, J = 8 Hz, 1H, 3′-H), 7.02 (d, J = 2 Hz, 1H, 6′-

H), 7.22 (dd, J = 8 Hz, J = 2 Hz, 1H, 4′-H), 7.41 (s, 1H, pyrimidine 6-H). The signal for the CH_2 bridge was completely obscured by a strong H_2O peak. Anal. ($C_{19}H_{22}N_4O_3 \cdot 2.75H_2O$) C, H, N.

2,4-Diamino-5-[5′-(4-carboxybutyl)-2′-methoxy)benzyl]-pyrimidine (5). A solution of recrystallized **20** (416 mg, 1.0 mmol) in dry DMF (20 mL) containing 10%% Pd−C (100 mg) was shaken under H_2 (50 psi initial pressure) for 18 h. After filtration to remove the catalyst, the solution was evaporated to a solid that was not soluble in NaOH, indicating that the ester group had survived. The solid, assumed to be the benzyl ester of **5**, was dissolved directly in hot EtOH (20 mL) and treated with 1 N NaOH (2 mL). The solvent was evaporated under reduced pressure, and the residue was taken up in H_2O. A trace of cloudiness, indicating that a trace of ester was still present, was discharged by adding another small portion of 1 N NaOH and some EtOH. The volume was reduced by rotary evaporation, and 10% AcOH was added dropwise until a solid formed, which was filtered and dried on a lyophilizer to obtain **5** as a white powder (117 mg, 35%): mp 134−137 °C (softening without giving a true melt); IR (KBr) ν 3330, 3180, 2930, 2850, 1660, 1560, 1505, 1455, 1400, 1290, 1250 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.46 (m, 4H, $CH_2CH_2CH_2CO_2H$), 1.77 (m, 4H, $CH_2CH_2CH_2CH_2CO_2H$), 3.7 (s, 2H, bridge CH_2), 3.75 (s, OMe, partly overlapped by broad H_2O peak), 5.66 (br s, NH_2), 6.03 (br s, NH_2), 6.89 (m, 3H, 3′-, 4′-, and 6′-H), 7.34 (s, 1H, pyrimidine 6-H). Anal. ($C_{17}H_{22}N_4O_3 \cdot 0.5$AcOH) C, H, N.

2,4-Diamino-5-[5′-(5-carboxypentyl)-2′-methoxy)benzyl]-pyrimidine (6). Benzyl 5-hexynoate (**16**) (0.65 g of nonpurified ester, ca. 3.0 mmol, prepared from **11** as described above) was heated with iodide **19** (0.71 g, 2.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), CuI (1 mg), and Et_3N (3 mL) in dry DMF (3 mL) under N_2 at 60 °C for 3.5 h. Solution occurred after ca. 1 h. The solvent was evaporated under reduced pressure, the residue was taken up in warm 95% EtOH (60 mL), and the solution, containing ester **21**, was treated with 1 M NaOH (6 mL). The EtOH was evaporated and replaced with H_2O (50 mL). The mixture was chilled and filtered, and the filtrate was transferred directly to a Parr apparatus and subjected to catalytic hydrogenation (42 psi initial pressure) in the presence of 10% Pd−C (85 mg). The catalyst was filtered off, and the filtrate was acidified with 10% AcOH. The precipitated solid was collected and dried on a lyophilizer: yield ca. 0.4 g. Analytical HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4, 1 mL/min) showed a major peak eluting at 12 min, along with several unidentified impurities. Preparative HPLC using the same eluent system, pooling of appropriate fractions, and freeze-drying afforded **6** as a white powder (334 mg, 46%): mp 96−98 °C (softening without giving a true melt); IR (KBr) ν 3350, 3200, 2930, 2860, 1660, 1560, 1505, 1460, 1405, 1290, 1250 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.26 (m, 2H, $CH_2CH_2CH_2CO_2H$), 1.45 (p, J = 7 Hz, 4H, $CH_2CH_2CH_2CH_2CO_2H$), 2.13 (t, J = 7 Hz, 2H, benzylic CH_2), 2.45 (t, CH_2CO_2H, partly overlapped by DMSO peak), 3.48 (s, bridge CH_2, partly overlapped by broad H_2O peak), 3.76 (s, OMe, partly overlapped by broad H_2O peak), 5.66 (br s, NH_2), 6.04 (br s, NH_2), 6.90 (m, 2H, aryl 3′- and 6′-H), 7.00 (dd, J = 8 Hz, J = 2 Hz, 1H, aryl 4′-H), 7.35 (s, 1H, pyrimidine 6-H). Anal. ($C_{18}H_{24}N_4O_2 \cdot 1.25H_2O$) C, H, N.

2,4-Diamino-5-[5′-(6-carboxyhexyl)-2′-methoxy)benzyl]-pyrimidine (7). A solution of **4** (120 mg, 0.3 mmol) in DMF (10 mL) was shaken under H_2 (initial pressure 3 atm) in the presence of 5% Pd−C in a Parr apparatus for 20 h. The catalyst was removed, and the solvent wasevaporated under reduced pressure. The residue, consisting of the benzyl ester of **7**, was treated with a small volume of dilute NaOH, and a trace of insoluble material was filtered off. The filtrate was acidified with 10% AcOH and chilled, and the precipitate was collected and dried on a lyophilizer to obtain **7** as a white solid: yield 115 mg (77%); mp 101−105 °C (softening without giving a true melt); IR (KBr) ν 2860, 3210, 2950, 2870, 1670, 1565, 1510, 1470, 1410, 1295, 1260 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.23 (poorly resolved m, 4H, $CH_2CH_2CH_2CH_2CO_2H$), 1.45 (m, 4H, $CH_2CH_2CH_2CH_2CH_2CO_2H$), 2.14 (t, J = 7 Hz, 2H, CH_2CO_2H), 2.43 (t, J = 7 Hz, benzylic CH_2, partly overlapped by DMSO), 3.74 (s, OMe, partly overlapped by broad H_2O peak), 5.65 (br s, NH_2), 6.03 (br s, NH_2), 6.88 (m, 2H, 3′- and 6′-H), 6.98 (d, J = 8 Hz, 1H, 4′-H), 7.32 (s, 1H, pyrimidine 6-H). The signal for the CH_2 bridge was obscured by a strong H_2O peak. Anal. ($C_{19}H_{26}N_4O_3 \cdot$AcOH$\cdot 1.3H_2O$) C, H, N.

Methyl 3-(3-Formyl-4-methoxyphenoxymethyl)benzoate (26). Step 1. A stirred mixture of 3-hydroxy-6-methoxybenzaldehyde (**23**) (456 mg, 3.0 mmol),[2] methyl 3-bromomethylbenzoate (**24**) (687 mg, 3.0 mmol), K_2CO_3 (1.04 g, 7.5 mmol), and 18-crown-6 (79 mg, 0.3 mmol) in dry DMF (10 mL) was heated at 70 °C for 20 h. The mixture was cooled to room temperature, the salts were filtered off, and the solvent was removed by rotary evaporation using a vacuum pump. The residue was partitioned between EtOAc and dilute aqueous citric acid. Evaporation of the organic layer gave a tan solid (1.03 g), which on recrystallization from MeOH proved unexpectedly to be the dimethyl acetal **25**: yield 816 mg (79%); mp 65−66 °C; IR (KBr) ν 3010, 2970, 2920, 2850, 1730, 1690w, 1615, 1595, 1505, 1470, 1455, 1440, 1405, 1380, 1370, 1290, 1235, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.35 (s, 6H, two OMe groups of acetal), 3.81 (s, 3H, ether OMe), 3.93 (s, 3H, ester OMe), 5.07 (s, 2H, benzylic CH_2), 5.64 (s, 1H, acetal CH), 6.82 (d, J = 9H, 1H, aryl 5-H), 6.91 (dd, J = 9 Hz, J = 3 Hz, 1H, aryl 6-H), 7.21 (d, J = 3 Hz, 1H, aryl 4-H), 7.45 (t, J = 8 Hz, 1H, aryl 5′-H), 7.64 (d, J = 7 Hz, 1H, aryl 6′-H), 7.99 (d, J = 7 Hz, 1H, aryl 4′-H), 8.11 (s, 1H, aryl 2′-H). Anal. ($C_{19}H_{22}O_6$) C, H.

Step 2. The acetal **25** from the preceding step was dissolved in THF (10 mL), and the solution was cooled in an ice bath and stirred while cold 1 N HCl (10 mL) was added dropwise, After 25 min at 0 °C, the mixture was diluted with isooctane. Partial precipitation occurred, but when TLC showed that both the solid and the solution contained an identical single spot (R_f = 0.5, silica gel, 1:1 EtOAc−isooctane), they were recombined in EtOAc and the solution was washed with 5% $NaHCO_3$. Evaporation of the organic layer afforded a solid (0.7 g crude yield), which on recrystallization from MeOH with three drops of added Et_3N afforded aldehyde **26** as off-white flakes (617 mg, 69%): mp 102−103 °C; IR (KBr) ν 2960w, 2880w, 1730, 1685, 1615, 1640w, 1500, 1475, 1455, 1430, 1405, 1385, 1320, 1295, 1280, 1265, 1225, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.91 (s, 3H, ether OMe), 3.93 (s, 3H, ester OMe), 5.09 (s, 2H, CH_2O), 6.96 (d, J = 9 Hz, 1H, aryl 5-H), 7.21 (dd, J = 9 Hz, J = 3 Hz, 1H, aryl 6-H), 7.43 (d, J = 3 Hz, 1H, aryl 4-H), 7.48 (d, J = 7 Hz, 1H, aryl 6′-H), 7.63 (d, J = 8 Hz, 1H, aryl 5′-H), 8.01 (d, J = 8 Hz, 1H, aryl 4′-H), 8.11 (s, 1H, aryl 2′-H), 10.45 (s, 1H, CH═O). Anal. ($C_{17}H_{16}O_5 \cdot 0.1H_2O$) C, H.

2,4-Diamino-5-[2′-methoxy-5′-(3″-carboxybenzyloxy)-benzylpyrimidine (8). **Step 1.** Metallic Na (23 mg, 1.0 mmol) was dissolved in absolute EtOH (30 mL). The solvent was removed by rotary evaporation, and the residue was redissolved in dry DMSO (2 mL). 3-Morpholinopropionitrile (280 mg, 2.0 mmol) was added, and the reaction mixture was placed in an oil bath preheated to 100 °C. A solution of **26** (600 mg, 2.0 mmol) in DMSO (3 mL, with slight warming as needed) was added all at once, and heating was continued for 20 min. A second portion of NaOMe (1.0 mmol in DMSO) was added, and heating was resumed for another 20 min. The reaction mixture was cooled and partitioned between EtOAc and dilute aqueous citric acid. The EtOAc layer was evaporated, the residue was dissolved in absolute EtOH (20 mL), aniline hydrochloride (389 mg, 3.0 mmol) was added, and the mixture was heated under reflux for 30 min and set aside until the next step.

Step 2. Metallic Na (184 mg, 8.0 mmol) was dissolved in absolute EtOH (25 mL), and guanidine hydrochloride (382 mg, 4.0 mmol) was added. The resulting mixture, containing some precipitated NaCl, was combined with the ethanolic solution from the preceding step. The reaction mixture was heated under reflux for 18 h and then was chilled and filtered. The filter cake was taken up in H_2O, and the pH was neutralized with 10% AcOH. A trace of solid precipitate was collected and redissolved in a small volume of 1 N NaOH. This solution was added back to the filtrate, which was then basified with 1 N

NaOH (5 mL) and concentrated to dryness by rotary evaporation. The residue was taken up in H_2O (40 mL), the solution was acidified with 10% AcOH and chilled, and the precipitate was collected, dried on a lyophilizer, and purified for elemental analysis and bioassay by preparative HPLC (C_{18} silica gel, 18% MeCN in 0.1 M NH_4OAc, pH 7.4). Appropriately pooled fractions were freeze-dried, and the solid was redissolved in 1 N NaOH. A trace of solid was filtered off, the filtrate was reacidified with 10% AcOH and chilled, and the precipitate was collected and dried in a lyophilizer to obtain **8** as a white solid (66 mg, 9% overall yield from **26**): mp ca. 140 °C (softening without giving a true melt); IR (KBr) ν 3325−3380, 2920w, 1655, 1535br, 1495, 1380, 1275 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.50 (s, bridge benzylic CH_2, partially overlapped by broad H_2O peak), 3.74 (s, OMe, partially overlapped by broad H_2O peak), 5.06 (s, 2H, CH_2O), 6.18 (br s, NH_2), 6.37(br s, NH_2), 6.83 (m, 3H, aryl 3′-, 4′-, and 6′-H), 7.32 (s, 1H pyrimidine 6-H), 7.47(t, J = 8 Hz, 1H, aryl 5″-H), 7.61 (d, J = 7 Hz, aryl 4″-H), 7.87 (d, J = 7 Hz, 1H, aryl 6″-H), 7.99 (s, 1H, aryl 2″-H). Anal. ($C_{20}H_{20}N_4O_4$·0.1AcOH·2H_2O) C, H, N.

***tert*-Butyl 4-(3-Formyl-4-methoxyphenoxymethyl)benzoate (28).** To a 4:1 THF−DMF mixture (10 mL) were sequentially added *tert*-butyl 4-bromomethylbenzoate (**27**) (1.08 g, 4.0 mmol),[13] 3-hydroxy-6-methoxybenzaldehyde (**23**) (496 mg, 3.25 mmol),[2] K_2CO_3 (1.38 g, 4.0 mmol), and 18-crown-6 (106 mg, 0.4 mmol). The mixture was heated at 70 °C for 40 min, after which the THF was blown off with a stream of air. TLC (silica gel, 1:1 EtOAc−isooctane) showed some unchanged bromide (R_f = 0.6) and phenol (R_f = 0.3), along with a small new spot at R_f = 0.5 (blue-fluorescent) corresponding to the desired product. Replacement of the THF and heating for another 66 h led to complete disappearance of the phenol (R_f = 0.3). The reaction mixture was diluted with EtOAc, and the combined organic solvents were decanted from the salts, washed with H_2O, and evaporated to dryness under reduced pressure to obtain a solid (1.3 g). Recrystallization from MeOH afforded **28** as a light-yellow powder (0.85 g, 76%): mp 91−92 °C; IR (KBr) ν 3000, 2980, 1860, 1700, 1675, 1615, 1585, 1575, 1495, 1470, 1455, 1435, 1425, 1410, 1395, 1365, 1315, 1300, 1280, 1255, 1220, 1200 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.60 (s, 9H, t-Bu), 3.90 (s, 3H, OMe), 5.11 (s, 2H benzylic CH_2), 6.94 (d, J = 9 Hz, 1H, 5-H), 7.21 (dd, J = 9 Hz, J = 3 Hz, 6-H), 7.41 (d, J = 3 Hz, 1H, 4-H), 7.46 (d, J = 8 Hz, 2H, 2′- and 6′-H), 8.00 (d, J = 8 Hz, 2H, 3′- and 5′-H). Anal. ($C_{20}H_{22}O_5$) C, H.

2,4-Diamino-5-(2′-methoxy-5′-(4″-carboxybenzyloxy)-benzylpyrimidine (9). Metallic Na (12 mg, 0.5 mmol) was dissolved in absolute EtOH (3 mL), and the solvent was evaporated under reduced pressure and replaced by dry DMSO (2 mL). 3-Morpholinopropionitrile (280 mg, 2.0 mmol) was added, and the mixture was placed in a bath preheated to 100 °C and was slowly treated with a solution of **28** (684 mg, 2.0 mmol) in dry DMSO (3 mL). After another 20 min at 100 °C, the reaction mixture was cooled and partitioned between EtOAc and dilute citric acid. The EtOAc layer was concentrated to dryness by rotary evaporation, and the residue was taken up in absolute EtOH (10 mL). Aniline hydrochloride (260 mg, 2.0 mmol) was added, and the reaction mixture was refluxed for 30 min. Separately, guanidine hydrochloride (478 mg, 5.0 mmol) was added to a solution of metallic Na (161 g, 7.0 mmol) in absolute EtOH (20 mL), and the mixture was swirled for 5 min and added to the foregoing ethanolic solution containing the anilino nitrile adduct. The mixture was refluxed for 18 h and then was chilled and filtered. The filter cake, which was completely soluble in H_2O, was discarded, and the filtrate was evaporated under reduced pressure. The residue was taken up in trifluoroacetic acid (5 mL), the solution was evaporated to dryness, and the residue was suspended in H_2O (30 mL) and dissolved by adding 1 N NaOH and stirring for several minutes until the pH was 10. Reacidification with 10% AcOH produced a solid, which was collected, dried in a lyophilization apparatus (crude yield 720 mg), and purified for elemental analysis and bioassay by preparative HPLC (C_{18} silica gel, 18% MeCN in 0.1 M NH_4OAc, pH 7.4). Appropriately pooled fractions were concentrated and freeze-dried, and the solid was redissolved in dilute NH_4OH and filtered. The filtrate was acidified with 10% AcOH and the precipitate was collected and freeze-dried to obtain **9** as a white solid (60 mg, 8% overall yield from **28**): mp 250−251 °C; IR (KBr) ν 3330, 3170, 1660, 1615sh, 1595sh, 1535, 1500, 1460, 1380, 1295, 1220 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.49 (s, bridge CH_2 partially overlapped by broad H_2O peak), 3.74 (s, OMe, partially overlapped by broad H_2O peak), 5.08 (s, 2H, benzylic CH_2), 5.90 (br s, NH_2), 6.20 (br s, NH_2), 6.83 (m, 3H, 3′-, 4′-, and 6′-H), 7.37 (s, 1H, pyrimidine 6-H), 7.50 (d, J = 8 Hz, 2H, 2″- and 6″-H), 7.93 (d, J = 8 Hz, 3″- and 5″-H). Anal. ($C_{20}H_{20}N_4O_2$·0.7AcOH) C, H, N.

2,4-Diamino-5-[2′-methoxy-5′-[3-(2″-carboxyphenoxy)-propyn-1-yl]benzyl]pyrimidine (10). Step 1. A mixture of ethyl salicylate (**29**) (1.66 g, 0.01 mol), K_2CO_3 (2.07 g, 0.015 mol), a catalytic amount of 18-crown-6 (26 mg), and propargyl bromide (1.12 mL, of 80% solution in toluene, calculated to contain 1.19 g, 0.01 mol) in dry DMF (10 mL) was stirred at room temperature for 20 h. The salts were filtered off, and the solvent was evaporated under reduced pressure in a bath maintained at 50 °C. The residue was partitioned between EtOAc and H_2O, and the organic layer evaporated to obtain the propargyl ether **31** (2.1 g) as an oil suitable for use directly in the next step: ^1H NMR ($CDCl_3$) δ 1.38 (t, J = 7 Hz, 3H, CH_2CH_3), 2.53 (t, J = 2 Hz), 1H, C≡CH), 4.36 (q, J = 7 Hz, 2H, CH_2CH_3), 4.79 (d, J = 2 Hz, 2H, ≡CCH_2O), 7.05 (dt, J = 8 Hz, J = 2 Hz, 1H, aryl 4-H), 7.12 (d, J = 8 Hz, 1H, aryl 3-H), 7.47 (dt, J = 8 Hz, J = 2 Hz, 1H, aryl 5-H), 7.80 (dd, J = 8 Hz, J = 2 Hz, 1H, aryl 6-H).

Step 2. A mixture of propargyl ether **31** (306 mg, 1.5 mmol), iodide **19** (356 mg, 1.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), CuI (1 mg), and Et_3N (3 mL) in dry DMF (3 mL) was heated under N_2 at 65 °C for 3 h. The solvent was evaporated under reduced pressure, and the residue was triturated with alternating portions of isooctane and H_2O. The residue, consisting of ester **33**, was dissolved in 50% EtOH (200 mL) at 50 °C and treated with $Ba(OH)_2$·8H_2O (947 mg, 3.0 mmol). The mixture was stirred at room temperature for 20 h, whereupon a solution of $(NH_4)_2CO_3$ (500 mg, 5 mmol) in H_2O (10 mL) was added. After 10 min of vigorous stirring, the solid was removed by filtration and the filtrate was concentrated to a small volume under reduced pressure. Pure product for elemental analysis and bioassay was isolated by preparative HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4OAc, pH 7.4, 280 nm). The major peak (eluting at 13 min on an analytical C_{18} column using the same elution system) was concentrated and freeze-dried, and the residue was dissolved in small volume of dilute NH_4OH. A small amount of insoluble material was filtered off, and the filtrate was chilled and acidified with 10% AcOH. The precipitate was collected and freeze-dried to obtain pure **10** as a white solid (104 mg, 24%): mp 147−153 °C (softening rather than giving a true melt); IR (KBr) ν 3360, 3210, 2960br, 2230, 1665, 1605, 1595, 1560, 1505, 1355, 1385, 1295, 1275sh, 1250 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.51 (s, bridge CH_2, partly overlapped by broad H_2O peak), 3.82 (s, OMe, partly overlapped by broad H_2O peak), 5.05 (s, 2H, CH_2O), 5.94 (br s, NH_2), 6.23 (br s, NH_2), 6.95−7.55 (complex m, 7H, aryl and pyrimidine protons), 7.62 (d, J = 7 Hz, 1H, 3″-H). Anal. ($C_{22}H_{20}N_4O_4$·1.5H_2O) C, H, N.

2,4-Diamino-5-[2′-methoxy-5′-[3-(3″-carboxyphenoxy)-1-propynyl]benzyl]pyrimidine (11). Step 1. A mixture of ethyl 3-hydroxybenzoate (**30**) (1.66 g, 0.01 mol), K_2CO_3 (2.07 g, 0.015 mol), a catalytic amount of 18-crown-6 (26 mg), and propargyl bromide (1.12 mL, 1.49 g of 80% solution in toluene, calculated to contain 0.01 mol) in dry DMF (10 mL) was stirred at room temperature for 20 h. The salts were filtered off, and the solvent was evaporated under reduced pressure in a bath maintained at 50 °C. The residue was partitioned between EtOAc and H_2O, and the organic layer was evaporated to obtain the propargyl ether **32** (2.3 g) as an oil suitable for use directly in the next step: ^1H NMR ($CDCl_3$) δ 1.39 (t, J = 7 Hz, 3H, CH_3CH_2), 2.53 (d, J = 1 Hz, 1H, C≡CH), 4.38 (q, J = 7 Hz, 2H, CH_3CH_2), 4.74 (s, 2H, ≡CCH_2O), 7.17 (d, J = 8 Hz,

1H, aryl 4-H), 7.36 (t, $J = 8$ Hz, 1H, aryl 5-H), 7.65 (m, 2H, aryl 2- and 6-H).

Step 2. A mixture of propargyl ether **32** (306 mg, 1.5 mmol), iodide **19** (356 mg, 1.0 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), CuI (1 mg), and Et_3N (3 mL) in dry DMF (3 mL) was heated under N_2 at 65 °C for 3.5 h. The solvent was evaporated under reduced pressure, and the residue was triturated with alternating portions of isooctane and H_2O. The solid, consisting of ester **34**, was collected and dissolved in DMSO (5 mL) with slight warming as needed. This solution was then swirled and treated dropwise with a solution of NaOH (120 mg, 3.0 mmol) in H_2O (1 mL). The mixture was diluted to 90 mL with H_2O and then brought to approximately pH 8 with 10% AcOH. Pure **9** for microchemical analysis and bioassay was isolated by preparative HPLC (C_{18} silica gel, 20% MeCN in 0.1 M NH_4-OAc, pH 7.4, 280 nm). Appropriately pooled eluates were concentrated and freeze-dried, the residue was redissolved in 0.1 N NaOH, and the solution was filtered. Acidification with 10% AcOH, followed by freeze-drying, yielded pure **9** as a white powder (100 mg, 21%): mp 149−153 °C (softening without giving a true melt); IR (KBr) ν 3360, 3200, 2960 (broad), 1670, 1610, 1560, 1505, 1460, 1445, 1390, 1275, 1245 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.51 (s, benzylic CH_2, partly overlapped by broad H_2O peak), 3.82 (s, OMe, partly overlapped by broad H_2O peak), 5.04 (s, 2H, CH_2O), 5.94 (br s, NH_2), 6.21 (br s, NH_2), 6.99 (d, $J = 8$ Hz, 1H, 3′-H), 7.11 (d, $J = 2$ Hz, 1H, 6′-H), 7.21 (dd, $J = 8$ Hz, $J = 2$ Hz, 1H, 4′-H), 7.28−7.45 (m, 3H, 5″- and 6″-H, pyrimidine 6-H), 7.54 (m, 2H, 2″ and 4″-H). Anal. ($C_{22}H_{20}N_4O_4$·AcOH·1.25H_2O) C, H, N.

Enzyme Assays and Data Analysis. Standardized spectrophotometric determination of Pc, Tg, Ma, and rat liver DHFR inhibition was performed as described earlier.[15,16] IC_{50} values were obtained with the aid of the curve-fitting program Prism 3.0. The data were transformed to percentage of uninhibited control for enzyme activity (y-axis) and the log of the molar drug concentration (x-axis). The analyses for individual curves had degrees of freedom ranging from 6 (2 curves) to 42 (1 curve); the mode for degrees of freedom was 12 (14 curves). The r^2 value for individual curves, determined using the program InStat 2.03, ranged from 0.71 (1 curve) to >0.99 (24 curves); all but two curves had r^2 values of >0.93. The range of SI values for each compound was calculated from the 95% confidence intervals for the individual IC_{50} values for that compound; all were referenced to rat liver DHFR. As an example of the reproducibility of these methods, when **1b** was assayed against Pc DHFR on three independent occasions, the mean ± SEM (standard error of the mean) was found to be 0.054 ± 0.0023 μM (i.e., the SEM was 4.3% of the mean). In quality control assays using TMP against the same enzyme, the SEM was 6.3% of the mean.

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